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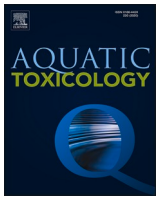

Application of QSAR for the identification of key molecular fragments and reliable predictions of effects of textile dyes on growth rate and biomass values of *Raphidocelis subcapitata*

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ABSTRACT

The current quantitative structure-activity relationship (QSAR) study seeks to explore the underlying causes of fluctuations in growth rate and biomass of microalgae mainly due to textile dyes. The derived QSAR models cover two endpoints: E_rC_{50} (growth rate) and E_bC_{50} (biomass) of *Raphidocelis subcapitata*. In order to extract the structural features involved, multiple PLS (partial least squares) models have been developed with easy to interpret and uncomplicated 2D descriptors having proper physico-chemical meaning. These descriptors were calculated from Dragon, SiRMS, and PaDEL-descriptor software. Then, the models were developed initially using stepwise regression followed by partial least squares (PLS) regression, and the model development procedure for both the endpoints (E_rC_{50} and E_bC_{50}) followed the stringent Organization for Economic Cooperation and Development (OECD) rules. Later on, the model validation was carried out with statistically significant and internationally accepted metrics (both internally and externally) in both the cases. Next, we have used the "Intelligent Consensus Predictor" tool (available from http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab/) to test the prediction quality with an "intelligent" approach to select multiple models. The estimated prediction quality for the appropriate test sets reveals that the consensus models (CM) surpass the quality shown by individual models (IM) for both the endpoints (E_rC_{50} and E_bC_{50}). Finally, the developed models were able to identify the major contributing features (hydrophobic units, unsaturation, saturation, electronegativity, branched atoms and charged fragments) related to aquatic toxicity of textile dyes.

1. Introduction

Nowadays, aquatic toxicity is a matter of great concern to the mankind. The society should be more cautious about the problems arising from the toxicants present in the water bodies. Increasing industrialization is the potential cause for aquatic toxicity as it introduces harmful effluents to the river or sea or other fresh water systems. Among these, textile dye industry is the one causing pollution of water bodies (Ali et al., 2019). Textile dyes provide color to the textile/fabric, and they differ from each other in terms of the chemical structures, process of dyeing, solubility and field of application. But all of them contain chromophores which are responsible for different dye colors. The most widespread chromophores are the azo, anthraquinone, nitro, aryl methane, carbonyl functional groups (Chequer et al., 2013). Textile dyes are used in dyeing and printing of the many kinds of textile materials (natural synthetic, man-made and mixed) like silk, polyester nylon and polyacetate, poly-urethane materials. Basically, textile dye industries deal with many kinds of chemicals in the textile dying process like acids, alkalis, surface active substances, and salts. In the process of textile production, the stages like dyeing, washing etc. require large volume of water because there is a need to handle with the large quantities of the dyes and chemicals. As a result, lot of polluted waste water is produced (Akbari et al., 2007; Joshi et al., 2004; Verma et al., 2012; Poon et al., 1999). Later, the waste water from the plant or industry is released into the flowing waters with the internal sewage system of the industry (**Fig. 1**). Textile dyes are classified in many ways, with reference to the production methods or chemical structure or applications (Kulkarni et al., 1985). Based on the chemical classification of

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textile dyes, there are 11 classes such as acid, azoic, basic, direct, disperse, fiber reactive, mordant, solvent, sulphur and vat dyes and optical/fluorescent brighteners (Carmen et al., 2012; Kulkarni et al., 1985). It is predicted that over 10,000 different textile dye varieties are used globally and over 7×10^5 tons of synthetic dyes are annually produced worldwide (Rajamohan et al., 2013; Sahoo et al., 2018; Xu et al., 2005).

The textile dyes such as acid orange 7 and reactive black 5 and derivatives of azo, and Vat Green 3 of anthraquinone dyes are commonly used for cotton and polymeric fiber dyeing. Food yellow 3 and food red 17 in addition to food yellow 4 are used in food, cosmetic and pharmaceutical industry (Kobylewski and Jacobson, 2010). Washing of the printed textile fabrics or materials may produce large quantity of waste water effluents which are heavily loaded with 10-60% of dye materials. These kind of dye effluents are responsible for the waste of 280,000 tons of dyes/year (Chequer et al., 2013). This sewage dye effluents mainly contain chemical substances and auxiliaries like nitrates and phosphates. Normally, phosphates and nitrates are useful nutrients, but when they become much concentrated in the water environments, they can create problems. The concentrated levels of phosphate and nitrate from textile dyes may cause "eutrophication" which is an issue when there is too much nutrient in a water body (e.g., rivers and lakes, fresh water sources). This may cause excessive growth of algae and other plants, which affects the water quality, damages plants and animals and makes the water unusable (Royer et al., 2009a; Royer et al., 2010b; Fazal et al., 2018; Rahman et al., 2016; Sahoo et al., 2017; Berradi et al., 2019; (Lima et al., 2007); (Mohan et al., 2002); (Umbuzeiro et al., 2005)). Normally, these groups (nitrates and phosphates) are eliminated from the effluent of a sewage treatment plant over a long-term operation in bioreactors packed with different combinations of wood and iron, with a trickling filter packed with foam ceramics. In order to find out the degree of pollution from textile dye effluents (waste water effluents from textile dyes), various physicochemical and biological parameters like coloring intensity, BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), TDS (Total dissolved solids), TN (Total Nitrogen), TP (Total Phosphorus) and non-biodegradable organic compounds (**Fig. 2**) are used. On the other hand, these effluents also contain heavy metals, metalliferous and phthalocyanine dyes, such as chromium (Cr), arsenic (Ar), copper (Cu) and zinc (Zn)). These substances are not biodegradable; hence, they accumulate in primary organs in the body and over time, begin to fester, leading to various diseases (Oh et al., 2009; Eh-sahbany et al., 2019; Mantzevinos et al., 2004). In the dying process, 10–15% proportion of the textile dyes are released into the aquatic environment, and in case of direct and reactive dyes, it is almost 30% final effluent released to the aquatic environment. Usually, biological treatment is used to treat such effluents; however, it has proved to be inefficient for complete dye removal (Pearce et al., 2003). When dyes reach to the aquatic environment, even they are at low concentrations; they may interfere in light penetration, inhibiting photosynthesis, and cause other deleterious effects to the aquatic organisms. However, there are still few studies that addressed the ecotoxicity of dyes (Oh et al., 2009; Eh-sahbany et al., 2019; Mantzevinos et al., 2004, Pearce et al., 2003).

To deal with above consequences, one should have knowledge about the physical, chemical and application properties of textile dyes, and information about the environmental release and impacts of dyes. Therefore, to know the properties of the chemicals or textile dyes, assays are required. The biological assays or toxicological assays are carried out for identifying the intensity and their effect of the chemical or dye. The assay always produces some qualitative results, and it is used to quantify effects of the untested chemicals. At the same time, it is useful for the risk assessment of existing chemicals or dyes by using the popular method namely quantitative structure-activity relationship (QSAR). Basically, the structure of a chemical or dye is correlated by QSAR with an activity/toxicity using several statistical approaches. QSAR models are used for the prediction of *in vivo* and *in vitro* activities of molecules which are not being tested experimentally (**Fig. 3**).

Till date, many researchers have reported QSAR of textile dyes. In 2014, Örücü et al. calculated isotherm constants and developed Langmuir and Freundlich isotherm models for experimental data of 33 anthraquinone and azo dyes. After qualitative analysis of experimental results, multiple linear regression (MLR), support vector regression (SVR) and back propagation neural network (BPNN) methods were used to develop quantitative structure–property relationship (QSPR) models with the novel adsorption data (Örücü et al., 2014). Sahoo and Berradi developed QSAR models to analyze the affinity of 27 azo dyes for cellulose fibers and the specific dye–fiber interactions of heterocyclic azo dyes (Oprea et al., 1997; Funar Timofei et al., 2012). Oh et al. reported a QSPR model to predict the maximum absorption wave-length (λ_{max}) of 191 azobenzene dyes. Recently, QSPR has been used to determine the structural features that influence the adsorption process of 22 commercial dyes on carbon cloths (Metivier-Pignon et al., 2007)

However, there is no satisfactory evidence about the characterization of textile dyes, and very few of them have experimental toxicological/

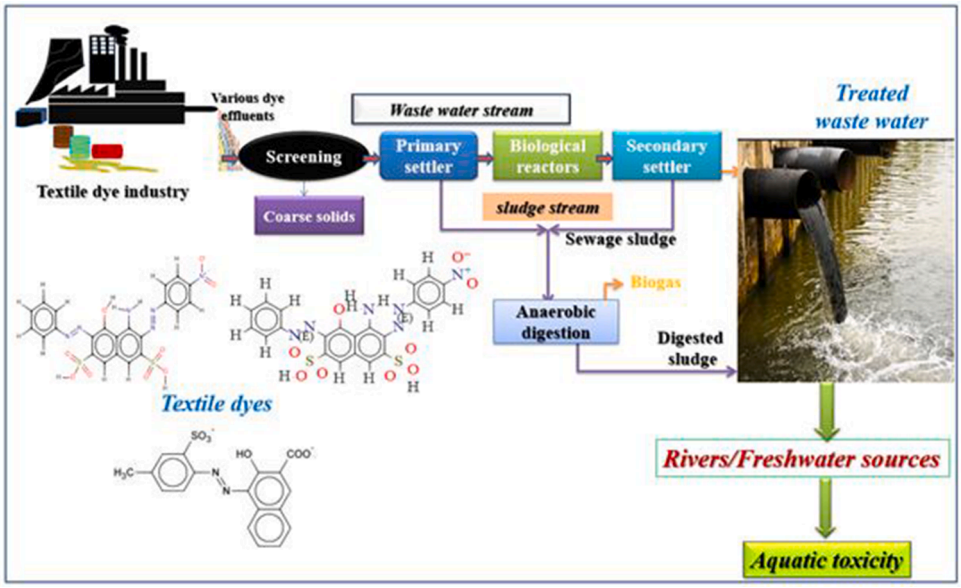

Fig 1. Flow of waste water stream from textile dye industry.

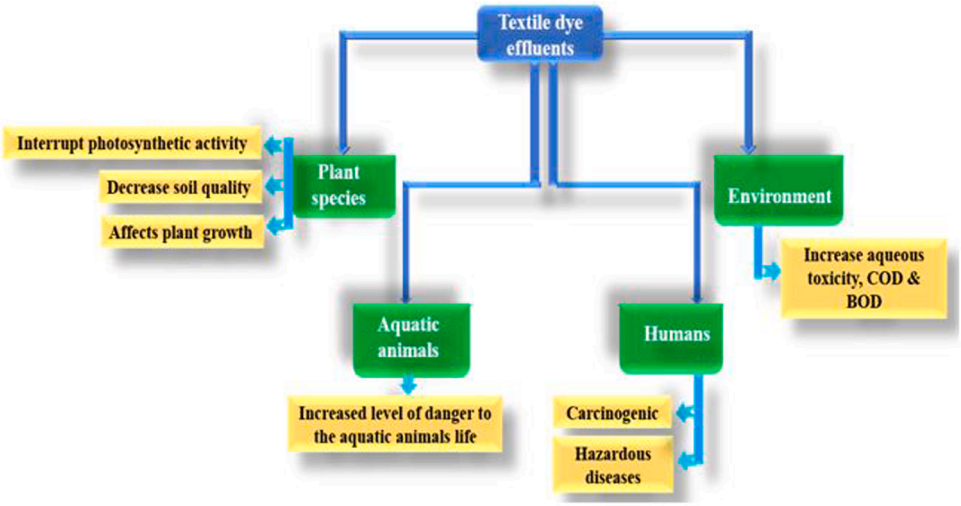

Fig 2. Effects of textile dye effluents.

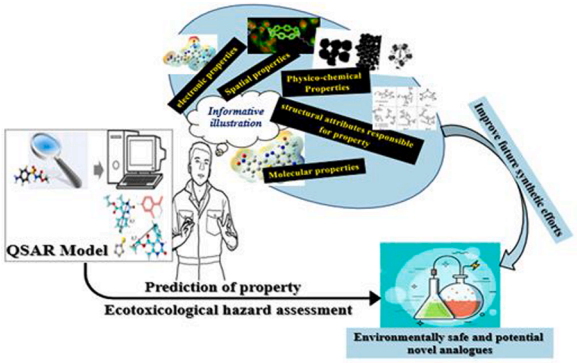

Fig 3. QSAR: informative illustration catalogue.

ecotoxicological data. In this direction, if we can predict new data for untested chemicals, it will be very much useful for risk assessment of new dyes. The data used in this study was taken from ecotoxicological bioassays of the algae named *Raphidocelis subcapitata.* It is one of the species recommended for ecotoxicological bioassays by the Organization for Economic Cooperation and Development (OECD) (OECD, 2011). *Raphidocelis subcapitata* is also known as *Pseudokirchneriella subcapitata*. It is a unicellular alga, often more sensitive to the various substances/chemicals/dyes compared to the other species. These algae are commonly found in fresh water. It has a quick generation time and higher growth rates. At present, most of the toxicity bioassays with microalgae uses integrative endpoints such as growth and cell viability. The population-based parameters used here are ecologically relevant, providing general information on the consequences of the presence of contaminants at the cellular and population level that can potentially affect higher trophic levels. However, these tests do not provide any mechanistic information on the toxic mode of action by which contaminants can disturb biological processes in algal cells (ISO 8692 (1989)).

In the present work, we have developed two-dimensional quantitative structure-activity relationship (2D-QSAR) models with partial least squares (PLS)-regression by using two different endpoints namely, growth rate (E_rC_{50}) and biomass (E_bC_{50}) values of the *Raphidocelis subcapitata*. Later, for both the datasets, we have applied an approach "multi-layered variable selection" for extracting the important features, to develop the final PLS-QSAR models. The model development procedure follows the strict OECD guidelines (https://www.oecd.org/env/ehs/risk-assessment/validationofqsarmodels.htm). The derived models can focus the attention on the molecular requisites, which are essential to design safer dyes which are eco-friendly. The study has recognized the structural characteristics in textile dyes, responsible for growth rate (E_rC_{50}) and biomass (E_bC_{50}) and their correlation to aquatic toxicity for further development of novel and environmentally safer textile dyes.

2. Methodology

2.1. Data set

Authenticated experimental data is a key requirement to build a statistically robust QSAR model. To comply with this criterion, we have collected a data set of 42 textile dye formulations which covers six different chemical classes (acid, direct, disperse, mordant, reactive and vat dyes) and different color index, so as to achieve the diversity of commercially available dyes. 2D-QSAR models were developed by using two different endpoints (Croce et al., 2017), i.e., growth rate (E_rC_{50}) and biomass (E_bC_{50}) concentrations of *Raphidocelis subcapitata* (commonly known as *Pseudokirchneriella subcapitata*) which were derived from homogenous experimental conditions, procedures and protocols (Croce et al., 2017). The structures were drawn by using MarvinSketch software (version 14.10.27) (Marvin Sketch software) (https://www.chemaxon.com) with appropriate aromatization and adding explicit hydrogens . Further, the structures were saved as MDL .mol files, which is required for descriptor calculation. In the current modeling procedure, growth rate (E_rC_{50}) and biomass (E_bC_{50}) values for the 42 textile dye formulations were expressed in negative logarithm of milligrams per liter and used for QSAR modeling. In the preliminary analysis of 42 textile dye formulations, one compound (**14**) was recognized as a stereoisomer having disparity in values, thus removed.

2.2. Descriptor calculation and data splitting

The dye structures were used for molecular descriptor computation using several software tools like PaDEL-Descriptor calculation software (Yap, 2011), Dragon (Mauri et al., 2006) and SiRMS (Kuz'min et al., 2005). For the ease of interpretation of models, we have computed 2D descriptors only, because of their definite physico-chemical meaning and absence of conformational complexity. The computed descriptors are 43 ETA descriptors (Roy and Ghosh, 2010) calculated from PaDEL-descriptor calculation software (Yap, 2011) and 590 descriptors covering eight different classes named as constitutional indices (e.g., MW, H%, N% etc), E-state indices (e.g., NssN, NdsN), 2D atom pairs (e.g., B10 [N-N], F10[N-N]), molecular property descriptors (e.g., LogP,

AMR), connectivity indices (e.g., X1, X1A), functional group counts (e.g., nPyrimidines, nPyridines and nArX), ring indices (e.g., nCIC, nCIR) and atom-centered (e.g., C-001, C-040) fragments calculated from the Dragon software (7.0). Additionally, 12,000 molecular fragments have been calculated using simplex representation of molecular structure software (SiRMS). Next, to avoid the overfitting complication of the final QSAR models, we have removed the constant (variance <0.0001) and intercorrelated descriptors (r >0.9). Both the endpoints are separately used to develop models with the combined descriptor pool and also using SiRMS descriptors with the aim of ascertaining the most contributing features to the response variables.

The prime objective of the present work was to develop robust and statistically reliable 2D QSAR models. The datasets of both the endpoints of the textile dye formulations were split into training (N_{train}= 31 and 32 dyes for E_rC_{50} and E_bC_{50} respectively) and test (N_{test}=10 and 8 dyes for E_rC_{50} and E_bC_{50} respectively) sets (the training set was meant for model development, while the test set was used for validation of the developed models) using approximately 75:25 ratio employing various data division tools available at http://dtclab.webs.com/software-tools. However, the statistically better models are obtained using the *k*-Medoid clustering technique in case of E_rC_{50}, and activity-sorted response method in case of E_bC_{50}. Other details like experimental response values and the predicted values along with the numerical values of modeled descriptors were mentioned in **SI-1** of Supplementary Material section.

2.3. QSAR modeling and validation

Prior to the development of QSAR models, it is very important to select important descriptors which are essential for the response from the large pool of descriptors. There are several feature selection approaches used to extract important descriptors like genetic algorithm (GA), stepwise selection, all possible subset selection, factor analysis, (Roy et al., 2015) etc. In the present work, we have employed stepwise regression with stepping criteria (also known as "Fisher criteria") F = 4.0 for inclusion and F = 3.9 for exclusion (Krishna et al., 2020). To identify the most important descriptors, the above procedure was repeated multiple times in a manner where each time selected descriptors were removed from previous runs and kept aside (Jillella et al., 2020). At the end of final stepwise run for each of the endpoints, we have selected 25 descriptors and those were subjected to best subset selection. We have selected best five models based on Mean Absolute Error (MAE) based criteria (Roy et al., 2016) for both the endpoints (six descriptor models were developed in case of E_rC_{50} and five descriptors models were developed in case of E_bC_{50}). Finally, the selected MLR models were subjected to partial least squares (PLS) analysis to avoid intercorrelation among the modeled descriptors and reduce the noise (since the PLS model stores the information of descriptors in the form of latent variables (LVs)). The partial least squares models were developed using MINITAB (Version 14.13) (http://www.minitab.com/en-US/default.aspx), and the PLS plots were generated by using SIMCA-P (SIMCA-P 2002) (Version 10.0.2.0) software, individually.

The robustness of the developed models was established by using different types of validation criteria. For all the developed models, we have employed determination coefficient (R^2), leave-one-out (LOO) cross-validation R^2 (Q^2) and $r^2_{m(LOO)}$ for the training set, R^2_{pred} or Q^2_{ext} (Q^2_{F1}) and Q^2F_2 and r^2_m (test) values for the test set. Finally, the predictions were subjected to the intelligent consensus predictor (ICP) tool for enhancing their prediction quality.

The "IntelligentConsensusPredictor" software tool assists us to find and understand the execution of consensus predictions from individual PLS models based on the MAE values, as a single model cannot give better prediction accuracies for each and every test set compound. The stages required for the PLS model development are represented below in Fig. 4.

2.4. Applicability domain (AD)

The applicability domain study was carried out by employing the DModX (distance to model in X-space) approach at 99 percent confidence level available in the SIMCA-P software (Umetrics, 2013) (https://umetrics.com/) to determine whether the test set compounds used in QSAR models are present in the chemical space of the training set compounds or not (Wold et al., 2001), i.e., to identify potential outliers.

2.5. Modeling Growth rate (E_rC_{50}) and Biomass (E_bC_{50}) using molecular fragments

Finally, QSAR modeling with fragment-based variables was implemented in the study to scrutinize the important structural features present in molecular structures of textile dyes. To accomplish this, we have computed a sequence of 2D structural features with SiRMS (simplex representations of molecular structure) software (Version 4.1.2.270). Later on, the calculated structural features were examined to identify the likely cause for producing aquatic toxicity (the notion is to identify the fragments which are responsible for reduction of both growth rate and biomass in *P. subcapitata*). The remaining steps such as splitting of the data, selection of the important variables and validation of the model were employed as stated by the above procedure. We have tried to develop the models using conventional descriptors only as well as combination of both conventional descriptors with SiRMS descriptors. In case of growth rate (E_rC_{50}), the models developed with the conventional

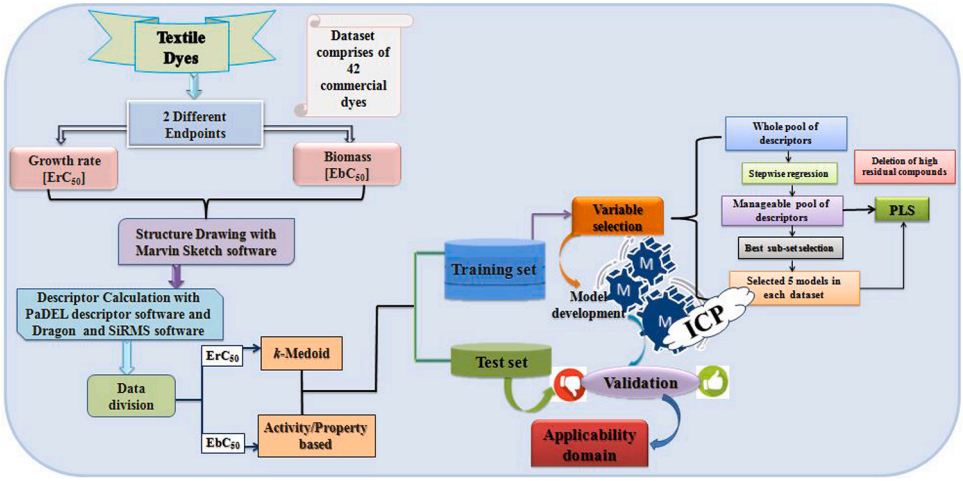

Fig. 4. The detailed methodology followed in the current work.

descriptors outperformed the SiRMS descriptors, while in case of the biomass (E_bC_{50}) endpoint, the models developed from combined set of descriptors are better.

2.6. Software used

For drawing the chemical structures, we have used Marvin sketch (version 14.10.27) software (https://www.chemaxon.com). Then, the descriptor computation performed with three different software tools, i.e., SiRMS (Version 4.1.2.270), Dragon version 7 (Mauri et al., 2006) and PaDEL-Descriptor (Yap, 2011). For splitting of the data set, we have employed freely accessible *k*-Medoids and DatasetDivisionGUI v1.2_9 tools (http://dtclab.webs.com/software-tools). In modeling, we have employed stepwise regression analysis in MINITAB Software (version 14.13) (http://www.minitab.com/en-US/default.aspx). Another freely accessible tool was employed i.e., "best subset selection" for pooling the best models (http://dtclab.webs.com/software-tools). Note that these free tools were developed in our laboratory. The PLS plots were generated with Simca-P (Version 10.0) software (Wold et al., 2001).

3. Results and Discussion

The numeric values of the attributes related to the calibration (training) and validation (test) sets are included in **SI-1** of the Supplementary Materials section. For modeling, a great care was taken for the time of collection of the data, data curation and validation of QSAR models to pass the stringent criteria i.e., OECD guidelines. The qualified models for the growth rate (E_rC_{50}) consist of 31 textile dye molecules in the calibration (training) set (N_{train}) and 10 textile dye molecules in the validation (test) set (N_{test}), and the data splitting was attained with the *k*-Medoids algorithm. In contrast, biomass (E_bC_{50}) models are built with 32 textile dye molecules in the calibration (training) set (N_{train}) and 8 textile dye molecules in validation (test) set (N_{test}); here, the activity/property-based dataset division was employed for the dataset splitting. After developing the models, the derived models were meticulously validated employing several internal validation and external validation metrics to substantiate the robustness and predictivity of derived models. The individual equations along with their validation metric values are given below in **Table S1** (Supplementary Materials SI-2) and elaborated thoroughly.

3.1. QSTR modeling of Growth rate (E_rC_{50}) against P. subcapitata

The final QSAR models for growth rate (E_rC_{50}) of *P. subcapitata* are derived from a set of 31 training dye molecules and subsequently validated with a set of 10 test dye molecules. The selected five models are straight-forward, uncomplicated and can be easily interpreted. Moreover, the models have statistically significant robustness and show the good quality predictions. The models can describe, approximately, 80% of variance of the training set and 67% in terms of leave-one-out (LOO) predictions; while explained 65% of the test set variance, i.e. the prediction quality was 65%. **Table S1**(Supplementary Materials SI-2) represents the summary of internal along with external validation metrics derived for both the sets (training and test) of the QSAR models. The equations derived from five partial least squares models are specified in **Box-1**. Table S2 in Supplementary Materials SI-2 deals with the frequency of descriptors appearing in growth rate (E_rC_{50}) models.

Box – I

IM-1

$$-\log(ErC50) = 0.514 + 0.403 \times B08[N-O] - 0.166 \times C-040 - 0.381 \times B08[N-N] + 0.552 \times B10[O-O] + 0.502 \times B08[C-N] - 0.157 \times nCp$$

IM-2

$$-\log(ErC50) = 0.514 + 0.403 \times B08[N-O] + 0.502 \times B07[C-N] - 0.166 \times C-040 - 0.381 \times B08[N-N] + 0.552 \times B10[O-O] - 0.157 \times nCp$$

IM-3

$$-\log(ErC50) = 0.512 + 0.475 \times B08[N-O] + 0.539 \times B09[O-S] - 0.119 \times C-040 - 0.420 \times B08[N-N] + 0.451 \times B08[C-N] - 0.127 \times nCp$$

IM-4

$$-\log(ErC50) = 0.530 + 0.412 \times B08[N-O] - 0.147 \times C-001 - 0.158 \times C-040 - 0.363 \times B08[N-N] + 0.564 \times B10[O-O] + 0.500 \times B08[C-N]$$

IM-5

$$-\log(ErC50) = 0.530 + 0.413 \times B08[N-O] - 0.147 \times C-001 + 0.453 \times B07[C-N] - 0.164 \times C-040 - 0.375 \times B08[N-N] + 0.562 \times B10[O-O]$$

Each of the selected five models comprise a unique combination of six descriptors, with a total of about nine descriptors in all models. The variable importance plot (VIP) analysis was performed to determine the relative importance of the descriptors appearing in each of the final models. According to the VIP plots (Figures S1-S5 in Supplementary Materials SI-2), the maximum contributing descriptors appearing in all individual models include B08[N-O] (presence/absence of N - O at topological distance 8), B10[O-O] (presence/absence of O - O at topological distance 10) and B09[O-S] (presence/absence of O - S at topological distance 9) with VIP scores greater than one in all models. In contrast, among the least contributing descriptors, C-040(R-C(=X)-X / R-C#X / X=C=X) and B08[N-N] (presence/absence of "2" nitrogen atoms at the topological distance 8) have VIP values less than one in all the models. Other descriptors like nCp (C(Sp3)), C-001(CH_3R / CH_4) and B08[C-N] (presence/absence of C - N at topological distance 8) are the least contributing in 2 to 3 models.

According to the loading plots of five individual models (Figures S1-S5 in Supplementary Materials SI-2), B08[N-O] and B10[O-O] have the greatest influence in regulating the growth rate (E_rC_{50}) of *P. subcapitata*, because they are far from the origin and close to the response in the loading plot, as ascertained by the VIP scores. On the other hand, the descriptors with minimum effect on growth rate (E_rC_{50}) of *P.subcapitata* were nCp (C(Sp3)), C-001(CH_3R / CH_4) and C-040(R-C(=X)-X / R-C#X / X=C=X). However, nine descriptors appeared repeatedly in five growth rate (E_rC_{50}) PLS models, and their frequency is listed in Table S1 in Supplementary Materials SI-2.

As shown in the above equations (BOX-1), all of the growth rate models have six descriptors; however, three descriptors were shared in all cases, namely B08[N-O], C-040, and B08[N-N], for a total of nine descriptors.

The descriptor B08[N-O] (2D-atom pair descriptor) is an important parameter in growth rate models of *P. subcapitata.* This descriptor shows the presence or absence of nitrogen and oxygen at the topological distance 8. The positive regression coefficient of the descriptor B08[N-O] suggests that the presence of this feature increases the growth rate of *P. subcapitata.*. It is observed that the textile dyes **7** (Acid Blue 324) and **8** (Acid Brown 235) show increased growth rate due to the presence of this feature, while textile dyes **33** (Mordant Yellow 10) and **42** (Vat Green 1) without the feature show lower growth rate.

The second important parameter having a major influence on the growth rate is the atom centered fragment (ACF) C-040 (R-C(=X)-X / R-C#X / X=C=X), which represents the number of carbon atoms attached to the heteroatom by single or multiple bonding and one valence is satisfied by an alkyl group (this feature lies in the bromoprop-2-enamide fragment of dye structure) (Tugcu et al., 2012). This descriptor's negative regression coefficient signifies that an increase in the number of such fragments reduces the growth rate of *P. subcapitata* as evidenced from the dyes **20** (Acid Yellow 42) and **36** (Reactive Red 83)**.** A lower value of this descriptor, on the other hand, accelerates growth rate of *P. subcapitata*, as evidenced by textile dyes **23** (Direct Blue 71) and **39** (Reactive Yellow 15).

Another ACF variable is C-001, which represents the 'C' atom in CH4 and CH3R, in which 'R' can be any group (most notably the presence of terminal methyl groups) and indicates the proportion of saturation when compared to the entire molecule. This characteristic has a negative relationship with the growth rate. As a result, we can assume that an increase in the saturation content of the entire molecule decreases the

growth rate significantly. The same is true for textile dyes **5** (Acid Blue 80) and **30** (Disperse Blue [Cl]-79), and vice versa in textile dyes like 17 (Acid Red 266) and 34 (NET (Mordent Black11).

The descriptor, B08[N-N] (2D atom pairs) indicated by the presence/absence of two nitrogen atoms at the topological distance 8. According to the regression coefficient plot, the above descriptor has a detrimental effect on the growth rate of *P. subcapitata.*. Therefore, the dyes containing two nitrogen atoms at the topological distance 8 has less growth rate (E_rC_{50}) as observed from the textile dyes **24** (Direct Blue 293) and **38** (Reactive Red 195) (Fig. 5). Again, in the dyes with no such fragments, there is an increase in the growth rate (E_rC_{50}), i.e., the dye no**.22** (Acid Yellow 61) and **31** (Disperse Orange 30).

Other descriptor B10[O-O] (2D atom pairs) (presence/absence of O-O atoms at the topological distance 10) exhibited a positive influence in enhancing the growth rate (E_rC_{50}) of *P. subcapitata*. The presence of this descriptor corresponds to an increase in lipophilicity of the molecule. Thus, the growth rate of the *P. subcapitata* may increase with high lipophilicity of the textile dyes as observed for the compounds **25** (Direct Green 26) and **39** (Reactive Yellow 15) (the presence of a greater number of aromatic hydrocarbons in the structure increases lipophilicity). On the other hand, the opposite may happen with the reduction in aromatic hydrocarbon content, size and surface area of the molecules as observed in compounds **21** (Acid Yellow 49) and **32** (Disperse Orange 61) (with simpler structures, small size, and low molecular bulk).

Other 2D atom pair based descriptors such as B08[C-N] (presence/absence of C-N at the topological distance 8) and B07[C-N] (presence or absence of carbon and nitrogen atoms at the topological distance 7) show positive contributions (as indicated by the regression coefficient plot) suggesting that both the descriptor values are directly proportional to the growth rate of *P. subcapitata*. Compounds having these descriptors indicate presence of hydrogen bonding groups like –OH, -NH_2, etc. at the terminal carbon atom of the compounds (Khan et al., 2019). The presence of increased number of hydrogen bond donors enhances the hydrophilicity of the molecules, which makes the compound less membrane permeable, thus increasing the growth rate of the *P. subcapitata*. The same can be observed in compounds **11** (Acid Green 111), **23** (Direct Blue 71) (for B08[C-N]) and **3** (Acid Black 26), **10** (Acid Green 68) (presence of B07[C-N]), whereas an opposite phenomenon takes place in the textile dyes **33** (Mordent Yellow 10), **42** (Vat Green 1) (B08[C-N]) and **12** (Acid Orange 7), **41** (Vat Blue 20) (B07[C-N] (with the absence of both (B08[C-N], B07[C-N]) features, the growth rate values decrease).

The descriptor B09[O-S] (2D atom pairs) describes the presence or absence of oxygen and sulfur atoms at the topological distance 9. According to the regression coefficient plot, the descriptor positively influenced the growth rate (E_rC_{50}) of *P. subcapitata.*. Thus, evidence from this descriptor suggests that molecules containing the oxygen and sulfur atoms at the topological distance 9 have a higher growth rate (E_rC_{50}), as evidenced by the textile dyes **10** (Acid Green 68) and **34** (NET (Mordent Black 11)). Again, the dyes like **16** (Acid Red 131) and **43** (Vat Green 9) have low growth rate (E_rC_{50}) due to the absence of this fragment (Fig. 6).

The number of total primary carbons (C(Sp3) present in the molecules is denoted by the functional group descriptor nCp. The negative regression coefficient of this descriptor indicates that a higher number of primary carbons in an aromatic ring is not favorable for growth rate (E_rC_{50}), as observed in textile dyes **24** (Direct Blue 293), **30** (Disperse Blue 79 [Cl]). The opposite behaviour is observed with the textile dyes **8** (Acid Brown 235) and **34** (NET (Mordent Black 11)). Fig. 7 depicts a scatter plot of observed versus calculated/predicted training/test set compounds.

3.2. QSTR modeling of Biomass (E_bC_{50}) against P. subcapitata

For the development of biomass (E_bC_{50}) models of *P. subcapitata,* we have employed the data set of 42 textile dyes. In this modeling procedure, SiRMS descriptors are included along with regular Dragon and PaDEL descriptors. The reason is that solely regular molecular descriptors failed to provide an acceptable model with required features for the desired property. SiRMS descriptors may help to identify the key fragments of the considered structures contributing to the biomass (E_bC_{50}) values of *P. subcapitata*. Only 2D descriptors are employed in the modeling for ease of understanding and to avoid the conformational complexity with 3D and 4D descriptors. The models after incorporation of SiRMS descriptors performed better than the previous conventional

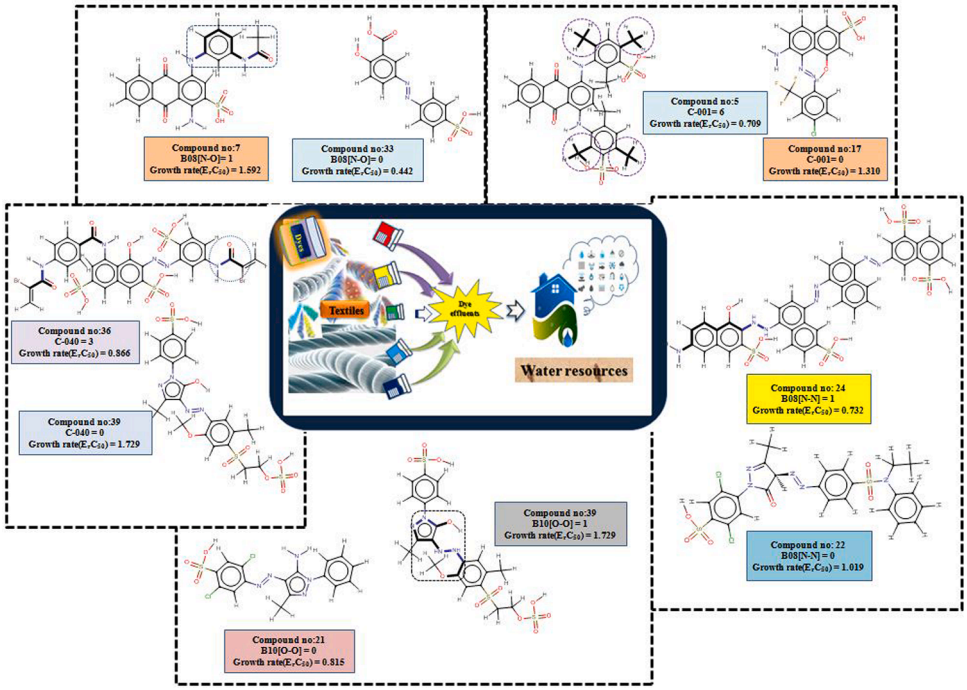

Fig 5. Importance of various descriptors involved in the growth rate (E_rC_{50}) QSAR modeling (Part-1).

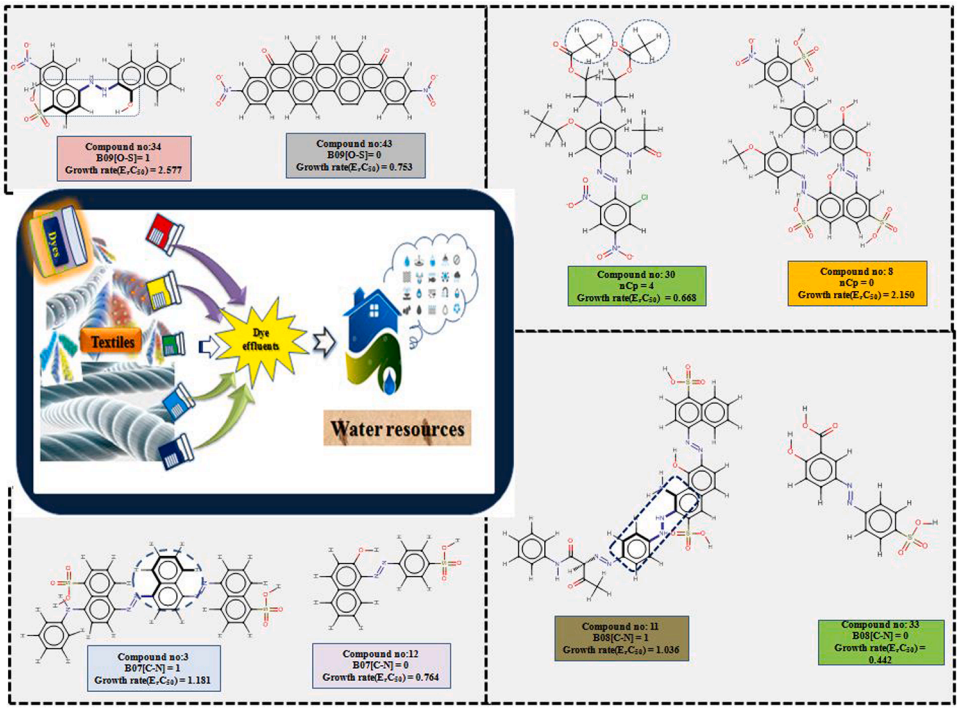

Fig 6. Importance of various descriptors involved in the growth rate (E_rC_{50}) QSAR modeling (Part-2).

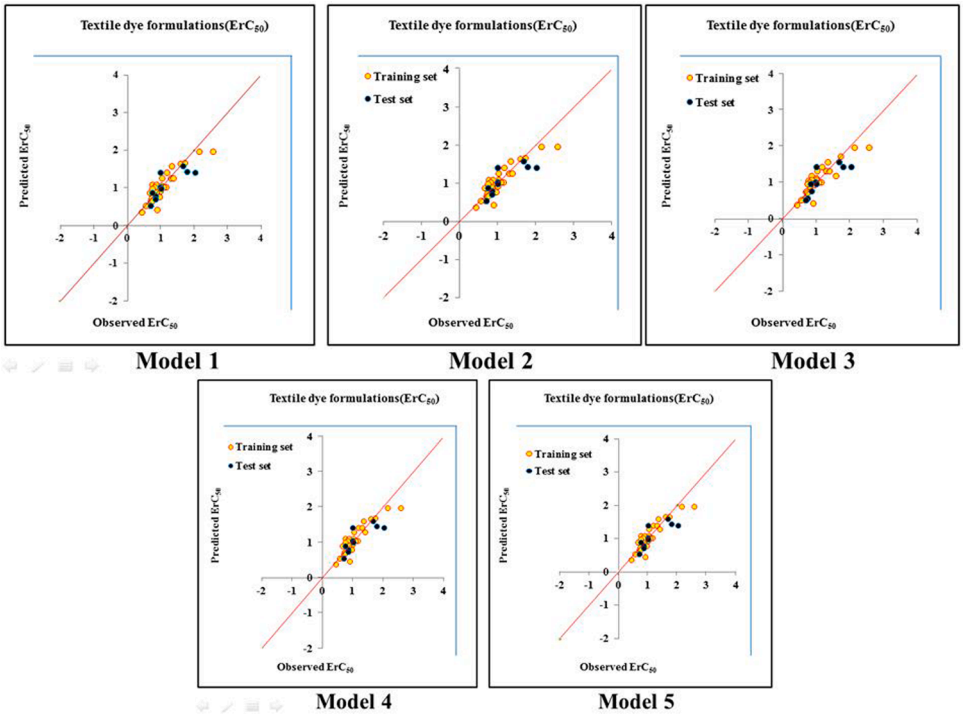

Fig 7. Scatter plots of the observed and the predicted growth rate values (E_rC_{50}) of the developed PLS models (Models 1-5).

models (using only regular descriptors) for the biomass (E_bC_{50}). The final models used 32 dyes in the training set for model generation and 8 dyes as the test set used for model validation purpose. After the model development, top five models were chosen based on their statistical significance, robustness and good prediction ability. The models can explain approximately 81 percent of the variance in the training set (average 67 percent in terms of LOO variance) and 76 percent of the variance in the test set (prediction quality). Table S1 (Supplementary Materials SI-2) shows a detailed summary of the statistical properties of the generated models. Each chosen model equation consists of a combination of five descriptors, with a total of nine variables in the final models.

According to the VIP plots (Figures S6-S10 in Supplementary Materials SI-2), the major contributing descriptors throughout all models are SiRMS (Simplex Representation of Molecular Structure) fragments such as chg1013 (Fr5(chg)/B_C_C_C_D/1_3a,1_4a,2_4a,2_5s/), type3018 (S_A

(type)/H_N.2_O.2_O.3/1_4s,2_3d/3), type3020 (S_A(type)/H_N.2_O.3_O.3/1_3s,2_4s/3) showing the VIP scores greater than or equal to "1". Again, the variables with low contributions are B10[N-N], C-040, F10 [N-N] (Todeschini and Consonni, 2009), chg340 (Fr5(chg)/A_B_B_C_D/1_5s,2_4a,3_4a,3_5a/), and chg529 (Fr5(chg)/A_B_D_D_D/1_3s, 1_4s,2_5a,4_5a/). According to the loading plot (Figures S6-S10 in Supplementary Materials SI-2), it is also evident that chg1013 (Fr5 (chg)/B_C_C_C_D/1_3a,1_4a,2_4a,2_5s/), type3018 (S_A(type)/H_N.2_O.2_O.3/1_4s,2_3d/3), type3020 (S_A(type)/H_N.2_O.3_O.3/1_3s,2_4s /3), SdssC variables are located far from the origin and close to the response value and have a great impact on the biomass values of *P. subcapitata*, whereas chg340 (Fr5(chg)/A_B_B_C_D/1_5s,2_4a,3_4a, 3_5a/), B10[N-N] (presence/absence of N-N at topological distance 10), F10[N-N] (frequency of N-N at topological distance 10), C-040 (R-C (=X)-X / R-C#X / X=C=X) and chg529 (Fr5(chg)/A_B_D_D_D/1_3s,1_4s, 2_5a,4_5a/) variables have a smaller impact on the biomass (E_rC_{50}) prediction because they are located closer to the origin and at a greater distance from the response.

BOX-2 contains the significant descriptors and fragments as well as the mathematical equations for five PLS models of biomass (E_bC_{50}) .

BOX-2

IM-1

$$-\log(EbC50) = 1.35 - 0.164 \times C-040 + 0.396 \times B10[N-N] + 0.494 \times Fr5(chg)/A\_B\_B\_C\_D/1\_5s,2\_4a,3\_4a,3\_5a/ + 1.43 \times Fr5(chg)/B\_C\_C\_C\_D/1\_3a,1\_4a,2\_4a,2\_5s/ + 0.093 \times S\_A(type)/H\_N.2\_O.2\_O.3/1\_4s,2\_3d\ /3$$

IM-2

$$-\log(EbC50) = 1.35 - 0.164 \times C-040 + 0.396 \times B10[N-N] + 0.494 \times Fr5(chg)/A\_B\_B\_C\_D/1\_5s,2\_4a,3\_4a,3\_5a/ + 1.43 \times Fr5(chg)/B\_C\_C\_C\_D/1\_3a,1\_4a,2\_4a,2\_5s/ + 0.093 \times S\_A(type)/H\_N.2\_O.3\_O.3/1\_3s,2\_4s\ /3$$

IM-3

$$-\log(EbC50) = 1.39 + 0.284 \times SdssC + 0.338 \times B10[N-N] + 0.502 \times Fr5(chg)/A\_B\_B\_C\_D/1\_5s,2\_4a,3\_4a,3\_5a/ + 1.38 \times Fr5(chg)/B\_C\_C\_C\_D/1\_3a,1\_4a,2\_4a,2\_5s/ + 0.103 \times S\_A(type)/H\_N.2\_O.2\_O.3/1\_4s,2\_3d\ /3$$

IM-4

$$-\log(EbC50) = 1.39 + 0.285 \times SdssC + 0.338 \times B10[N-N] + 0.501 \times Fr5(chg)/A\_B\_B\_C\_D/1\_5s,2\_4a,3\_4a,3\_5a/ + 1.38 \times Fr5(chg)/B\_C\_C\_C\_D/1\_3a,1\_4a,2\_4a,2\_5s/ + 0.102 \times S\_A(type)/H\_N.2\_O.3\_O.3/1\_3s,2\_4s\ /3$$

IM-5

$$-\log(EbC50) = 1.37 + 0.243 \times SdssC + 0.137 \times F10[N-N] + 0.588 \times Fr5(chg)/A\_B\_B\_C\_D/1\_5s,2\_4a,3\_4a,3\_5a/ + 1.30 \times Fr5(chg)/B\_C\_C\_C\_D/1\_3a,1\_4a,2\_4a,2\_5s/ + 0.377 \times Fr5(chg)/A\_B\_D\_D\_D/1\_3s,1\_4s,2\_5a,4\_5a/$$

(*continued on next column*)

(*continued*)

The biomass models with fragmental variables showed a slightly better robustness having five additional variables in addition to the previous descriptors. The fragmental QSAR models identified all the five features contributing positively to the biomass (E_bC_{50}) (see Box 1-5 of **Fig. 8**). Another major notable point here is that the positively correlating features were rich in aromatic bonds along with nitrogen of the amide group and carboxy groups. The SiRMS fragments of the biomass (E_bC_{50}) models mainly suggested the lipophilic ('type' fragments appearing in the models) and charged species ('Chg' fragments appearing in the models). Both fragment categories are enhancing the biomass values in *P. subcapitata*. Table S3 in Supplementary Materials SI-2 deals with the frequency of descriptors appearing in biomass (E_bC_{50}) models.

The descriptors and the fragments present in the models are elaborated below with the physico–chemical meaning along with the mechanistic influence on the biomass (E_bC_{50}) models of *P. subcapitata.* These descriptors are divided into two classes: the positively correlated ones include B10[N-N], F10[N-N], SdssC, Fr5(chg)/A_B_B_C_D/1_5s,2_4a, 3_4a,3_5a/, Fr5(chg)/B_C_C_C_D/1_3a,1_4a,2_4a,2_5s/, S_A(type)/H_N. 2_O.2_O.3/1_4s,2_3d/3, S_A(type)/H_N.2_O.3_O.3/1_3s,2_4s/3, Fr5 (chg)/A_B_D_D_D/1_3s,1_4s,2_5a,4_5a/)) and there is only one negatively correlated descriptor, i.e., C-040.

The descriptor B10[N-N] (2D atom pairs) denotes the presence or absence of two nitrogen atoms at the topological distance 10, whereas the other 2D atom pair descriptor, F10[N-N], denotes the frequency of two nitrogen atoms at the topological distance 10. According to the regression coefficient plot, these descriptors have a positive effect on the biomass values of *P. subcapitata.* As a result, the dyes with two nitrogen atoms at the topological distance 10 increase the biomass value, as confirmed by the textile dyes **11** (Acid Green 111), **28** (Direct Red 227) (For B10 [N-N]) and **35** (Reactive Black 5) and **37** (Reactive Red 120) (For F10[N-N]). Again, the compounds with no such fragments observed

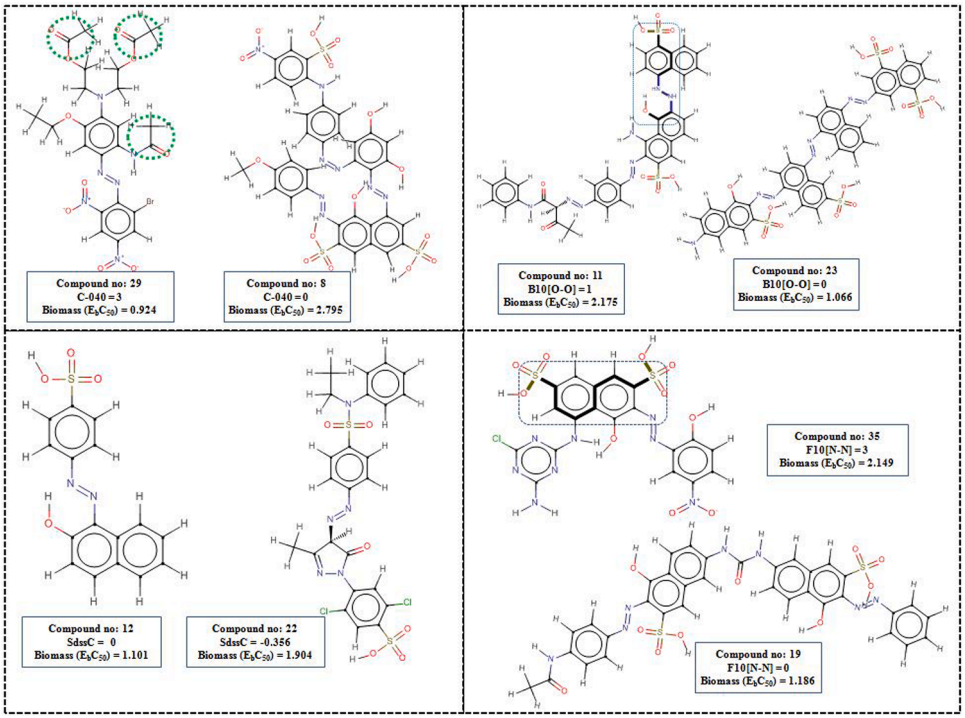

Fig 8. Importance of various descriptors appearing in biomass (E_rC_{50}) models.

less biomass values as shown in dyes **23** (Direct Blue 71), **33** (Mordent Yellow 10) (For B10 [N-N]) and **19** (Active Violet 48), **26** (Direct Red 23) (For F10[N-N]).

SdssC is a simple atom-type descriptor that clearly states the sum of double, single, and single bonded carbon E-states (=C<). According to the positive regression coefficient, this descriptor makes a positive contribution to biomass (E_bC_{50}) values. Consequently, the textile dyes which have higher values of the SdssC descriptor (dyes **12** (Acid Orange 7) and **42** (Vat Green 1)) have higher biomass values against *P. subcapitata* as presented in Fig. 9. In contrary, the dyes **5** (Acid Blue 80 (SdssC; -0.898)) and **22** (Acid Blue 80 (SdssC; -0.356)) having lower values of this descriptor showed less biomass values. Based on this observation, it can be asserted that the sum of the double, single, and single bonded carbon E-states (=C<) must be higher for improving the biomass (E_bC_{50}) values, as they contribute to a molecule's hydrophobicity, which is the most influential feature for toxicity prediction, as previously reported in other articles (Roy et al., 2014; Khan and Roy, 2017).

The following atom centered fragment C-040 (R-C(=X)-X / R-C#X / X=C=X) refers to the number of fragments comprising C(sp2) atoms attached by one electronegative atom (O, N, S, Se and halogens) with double/triple bonds and another single bond (in case of any other group) or double bond (in case the fragment has another electronegative atom) (Todeschini and Consonni, 2009). The negative regression coefficient indicates that the feature having C(sp2)/ C(sp) atoms directly attached with one or two electronegative atoms has a negative impact on biomass (E_bC_{50}) values. It can be seen in the dyes **16** (Acid Red 131) and **29** (Disperse Blue 79) (descriptor value "1" and "3" respectively), and vice versa in textile dyes **8** (Acid Brown 235) and **34** (Mordent Black 11).

The SiRMS descriptor, Fr5(chg)/A_B_B_C_D/1_5s,2_4a,3_4a,3_5a/, denotes the partial charge of a five atom fragments as shown in Box 1. The presence of the above fragment (i.e., '3' atoms inside aromatic ring and '1' is outside the ring) was intended to increase the biomass values due to the positive influence of the descriptor. The textile dyes such as **5, 8, 15, 17** and **39** have higher partial charges, due to the presence of the aforementioned fragment, which increases the biomass values. In dyes that do not contain such fragments (like **16, 19,** and **33**), the impact of this charge is not noticed, and there is no increase in biomass values.

Another SiRMS descriptor that shows the highest significance in all the models is Fr5(chg)/B_C_C_C_D/1_3a,1_4a,2_4a,2_5s/ which signifies the partial charge of any of the five atom descriptor as shown in Box 2 of Fig. 9. Here, this fragment can be found in the bicyclic region of the dye structure (naphthalene). Any such descriptor has a positive effect on biomass (E_bC_{50}); consequently, these groups enhance the biomass (E_bC_{50}) value. The textile dyes in the dataset with the same group as **6** (Acid Blue 113) and **34** (NET (Mordent Black 11)) have maximum biomass values. As a result, the significance of this fragment has led to the conclusion that the presence of electron delocalized aromatic rings such as naphthalene indicates higher biomass values.

The next descriptor is Fr5(chg)/A_B_D_D_D/1_3s,1_4s,2_5a,4_5a/, where it characterizes the partial charge of a four atom fragment as shown in Box 3. The existence of the specified group (i.e., two aromatic bonds present in the aromatic rings and two single bonds outside the aromatic ring) might enhance the biomass values due to its positive regression coefficients in the modeled equation. Textile dyes such as **11, 16, 34, 35,** and **40** have higher partial charges caused by the presence of the indicated fragments, resulting in higher biomass values. Again, other textile dyes lacking such fragments (such as **2, 26, 32,** and **43,** for example), do not have such charges, resulting in lower biomass (E_bC_{50}) value.

Another descriptor S_A(type)/H_N.2_O.2_O.3/1_4s,2_3d/3 is a four atomic fragment which signifies the given formula: H-N(sp^2)-C=O(sp^2). The possible fragment's structure could be similar to the amide group, as shown in Box 4. According to the positive regression coefficients of the descriptor values, the presence of such fragments increases biomass (E_bC_{50}) value in *P. subcapitata*. This is well observed in textile dyes **1, 8, 11, 34, 35** and **40**. In contrast, absence of such fragments lowers the biomass values as observed in textile dyes **29, 33** and **36** etc.

The least significant descriptor is S_A(type)/H_N.2_O.3_O.3/1_3s,2_4s/3 which indicates the compound's electronegativity through the four atomic fragment as in Box 5 of Fig. 9. The positive regression coefficient of this descriptor suggested that the presence of this kind of fragment has an impact on dye molecule's electronegativity, thus increasing the biomass value. The textile dyes **1, 8, 11, 34, 35, 40** contain such fragments, increasing the biomass (E_bC_{50}) values of *P. subcapitata*.

The scatter plots for the biomass (E_bC_{50}) models are shown in Fig. 10.

3.3. Assessment of Applicability domain (AD) and Y-randomization of the developed models

We used the DModX approach to verify the applicability domain for all of the individual models (IM1-IM5) developed from growth rate (E_rC_{50}) and biomass (E_bC_{50}) values of *P. subcapitata*. From the AD study, we found that all of the training set dyes are within the required D-critical value at 99 percent confidence level for growth rate (2.135–3.911) and Biomass (3.821-4.07) datasets. On the other hand, in test set dyes, for both the endpoints, all of the test set dyes also fall within the AD zone. Despite the structural diversity of textile dyes, 100% of them pass the AD test, and their predictions are totally reliable. We also performed the Y-randomization analysis to see if the models were generated by chance or not. The Y-randomization results ($R^2_{int} < 0.4$ and $Q^2_{int} < 0.05$) indicated that the models were not derived by chance, and

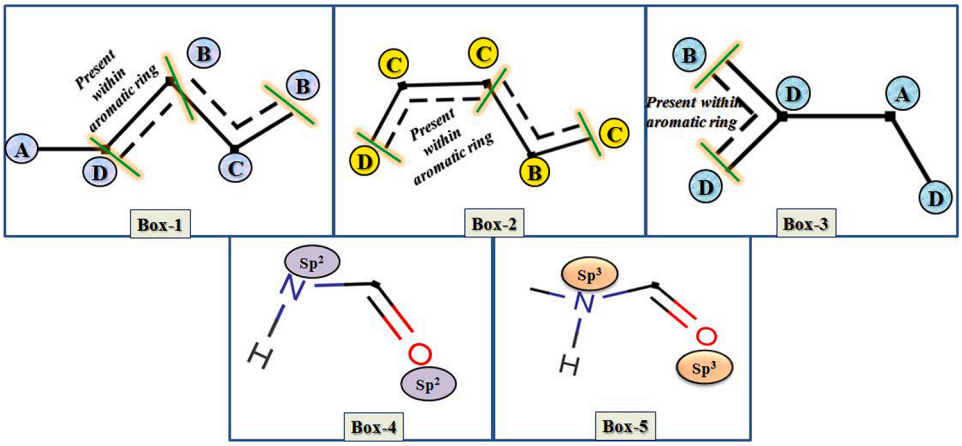

Fig 9. Simplex representation of molecular structures (SiRMS) fragments in biomass (E_bC_{50}) models.

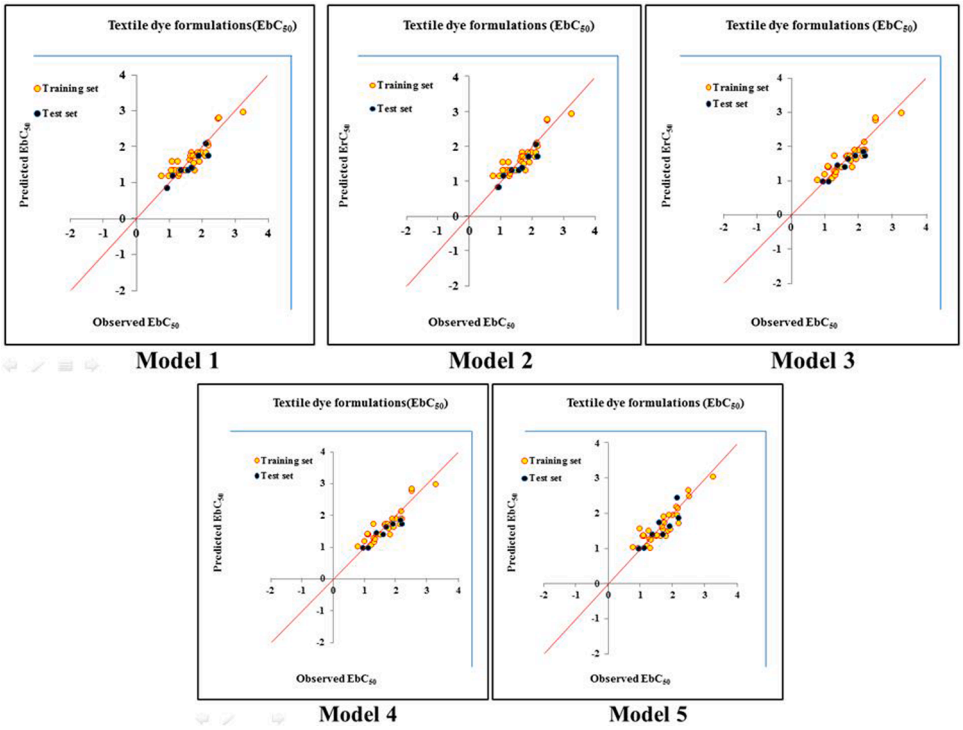

Fig 10. Scatter plots of observed v/s predicted responses against models Biomass (E_bC_{50}) Fragment models.

the results were given in Figures **S11-S20** which can be found in **SI-2** in the Supplementary Materials section.

3.4. Predictions of developed QSAR models on commercial textile dyes

To determine the quality of predictions and utility of the developed QSAR models, we have used 36 commercial textile dyes with unknown observed values of growth rate (E_rC_{50}) and biomass (E_bC_{50}) values of *P. subcapitata*. The individual five PLS models (in both cases i.e., E_rC_{50} (IM1-5) and E_bC_{50} (IM1-5)) were used to extract the predicted values for 36 commercial textile dyes; the quality of model derived predictions was checked using the "Prediction Reliability Indicator" (PRI) tool (freely accessible from https://dtclab.webs.com/software-tools) (Roy et al., 2018). The detailed information related to the predicted values of growth rate and biomass values of 36 commercial textile dyes are given in **SI-3** of Supplementary Materials section.

3.5. Comparison with previous reports

In a previous work (Croce et al., 2017), the authors used an in silico approach to assess the chemical similarity of selected textile dyes using their inbuilt similarity tool (istSimilarity v.1.0.5). They mentioned the similarity index (S.I), which ranges from 0 (not similar) to 1 (extremely similar). This similarity index calculation was executed based on VEGA platform of QSARs (www.vegahub.eu) (Floris et al., 2014). However, in the current study, we have developed a number of models (2D-QSAR) to predict the growth rate (E_rC_{50}) and biomass (E_bC_{50}) values of *P. subcapitata* against various textile dyes. Furthermore, we have used consensus modelling to reduce the error in selected response values. Finally, 36 commercial textile dyes were employed as an external set to assess the prediction quality of the QSAR models that were developed with our in house "Prediction Reliability Indicator" (PRI) tool (Roy et al., 2018). All 36 commercial dyes are within the model's domain in both cases, i.e. growth rate and biomass. The results of this study will aid in the rapid identification of key molecular fragments responsible for the effects of textile dyes on the growth rate and biomass values of *P. subcapitata*. However, these models contain no information about the 3D-pharmacophoric features required for *P. subcapitata* growth rate and biomass.

4. Conclusion

The textile industry is spread globally, generating around 1 trillion dollars, and contributing with 7% of the total world exports and employing around 35 million workers around the world. The ever-increasing use of textile dyes might give serious threats to the environment in the near future. Now, researchers are focusing on regulating the use of the textile dyes by knowing their risk benefit ratio towards the aquatic environment. At present, many regulatory agencies all over the world are thinking about the rationale to control the usage of textile dyes by applying conventional knowledge about them along with their physicochemical properties and fate of transformation, etc. For making safer textile dyes to the environment, initially we should minimize the toxicity data gap of the dyes because they are poorly characterized and their toxicological/ecotoxicological data is limited (available only for few dyes). In this work, we have developed 2D-QSAR models using Dragon and PaDEL-descriptor against the growth rate (E_rC_{50}). Additionally, simplex fragmental variables are employed in case of biomass (E_bC_{50}) values of *P. subcapitata* (*Raphidocelis subcapitata*). Following the stringent guidelines of QSAR model validation, we have performed the data set division, model development and AD estimation. Mainly, this work focuses on identifying molecular features responsible for growth rate (E_rC_{50}) and biomass (E_bC_{50}) values and quantitative predictions of toxicity for new or hypothetical structures. Consequently, these models might show the direction for the chemists to predict the toxicity of untested textile dyes according to their predefined threshold theoretically, and they may help for regulatory decisions and for proposing the mechanism for the safer design of textile dyes; thus, the resources and the time duration required for experimental work can be reduced.

CRediT authorship contribution statement

Gopala Krishna Jillella: Data curation, Methodology, Validation, Investigation, Writing – original draft. **Probir Kumar Ojha:** Validation,

Writing – review & editing. **Kunal Roy:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105925.

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Artificial Neural Network (ANN) Modeling of Odor Threshold Property of Diverse Chemical Constituents of Black Tea and Coffee

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ABSTRACT

The authors have developed an artificial neural network model using odor threshold (OT) property data for diverse odorant components present in black tea (76 components) and coffee (46 components). The models were validated in terms of both internal and external validation criteria signifying acceptable results. The authors found the significant features controlling the OT property using Mean Absolute Error (MAE)-based criteria in a backward elimination of descriptors, one in each turn. The present results well-corroborated the previously published PLS-regression based chemometric model results.

KEYWORDS

ANN, Black Tea, Coffee, MAE, Odor Threshold

1. INTRODUCTION

Tea is the most commonly consumed nonalcoholic beverage after water. The consumption of tea is a very ancient habit. In 1978, an Archeological research reported by Jelinek suggested that the infusion of leaves from the tea tree was probably practiced more than 500 000 years ago (Gutman, & Ryu, 1996). Black tea is originated from the two varieties such as *Camellia assamica* and *Camellia sinensis*. Black tea and green tea are produced from the same plant *Camellia sinensis* but the name varies due to how the plant's leaves are processed (Hara, Luo, Wickremashinghe, & Yamanishi, 1995). Black tea is grown and processed all over the world in varying geographies and climates. Though, China is the largest producer of tea, but India, Sri Lanka and Africa are the top three producers of black tea today (Mary, & Robert, 2011). Among the different varieties of tea, black tea is most widely used worldwide due to its flavour. In case of black tea preparation, before the heat processing and drying, the leaves are allowed to oxidize fully which turn the leaves from the rich dark brown to black colour for which black tea leaves are famous for. The change of colour occurs due to the interactions between the tea plant cell walls and oxygen. This oxidation process alters the flavor profile of black tea. The enzyme, catechol oxidase, acts as a catalyst in the oxidation process leading to the formation of theaflavins and thearubigins from flavanols which are responsible for the characteristic colour and flavor of fermented tea (Robertson, 1992; Borse, Rao, Nagalakshmi, & Krishnamurthy, 2002; Bhattacharyya et al., 2008). Thus, the characteristics flavour is the key element for identification or evaluation of tea. Flavour of black tea is due to the presence of taste and aroma active components. Volatile components (around 600 volatile compounds have been reported in tea

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leaves) like aldehydes, alcohols, ketones, furans, and aromatic compounds control the aroma of black tea while the non-volatile components like organic acids, polyphenols, sugars, caffeine, catechin, theaflavins, thearubigins and free amino acids, among others are responsible for taste of black tea (Wang et al., 2011; Rawat et al., 2007; Bondarovich et al., 1967). Though, the volatile components are present in minute amount, but these have high impact to regulate the flavour of the black tea due to their low threshold values (Rawat et al., 2007).

Like tea, coffee, though young, is also an important beverage used worldwide due to its flavour as well as its potential health benefit (Balentine, Wiseman, & Bouwens, 1997; Halder et al., 2005). Coffee is prepared from roasted coffee beans, the seeds of berries from certain *Coffea* species. This is one of the major sources of income for many countries like Brazil, Vietnam, Colombia, Indonesia, Ethiopia, India, Honduras, Uganda, Mexico, Guatemala, Peru, etc. As of 2018, Brazil is producing one-third of the world total coffee (Coffee Annual Brazil - USDA GAIN reports, 2018). The worldwide popularity of this beverage is due to some factors, among which, flavour is the main reason. Thus, distinctive characteristics of smell can help in the identification of different food and beverages. This is also very helpful in case of perfume and beverage industries for masking the obnoxious odor of chemicals used in different food, pharmaceuticals and cosmetic products. An odor is the impression in the brain obtained by the recognition of a volatile compound at a very low concentration by odorant receptors (ORs) which is perceived by the sense of olfaction of human or other animals.

1.1. Odor Threshold and Evaluation

The odor threshold (OT) is the minimum concentration at which all panelists have been able to recognize the odor sensitivity (Leonardos, Kendall, & Barnard, 1969). A trained Panel of four staff members of the Food and Flavor Section of Arthur D. Little, Inc. was used for determining the odor threshold of each chemical. The Panel members were selected from a pool of approximately fifteen observers with more than one year of analytical odor work. Only one chemical was observed per day by the panel. Before the observations of a chemical, the Panel examined the odor at diverse dilutions to familiarize with the odor type. Each chemical was examined with the five different concentrations. The first odor observation of the day was the background level of test room. A concentration range was selected for inclusion of the odor threshold. The Panel members were not informed of the concentration of the chemical in the test room. The results obtained from each panelist were examined separately. Each Panel member was required to be present for all the odor examinations scheduled for the day, as a different concentration was evaluated at each session. The Panel members were not allowed to make continuous observations, every observation was separated within 25 minutes. Afterwards, range and concentration were decided by the Panel members. A positive response is indicated for each concentration at which the Panelist described the odor of the chemical. The concentration ranges were changed on a random basis, the threshold concentration for each subject was determined by recording positive responses as a function of the concentration. The threshold is the least concentration, i.e., when the Panelist could define the odor and it would be constantly observed in higher concentrations. Chemical odor is compiled in this manner for each Panel member. The odor threshold reported is the concentration at which all four panel members could positively recognize the odor of the chemical (Leonardos, Kendall, & Barnard, 1974). This property is typical attribute for individual component and has been reliable in their response at all higher test concentrations. Since there is no such modern technology which can mimic the efficiency of human nose and can characterize different types of odor with the similar sensitivity.

1.2. Why Machine Learning Approach?

In this regard, *in silico* tool can be applied for the prediction of OT property of odorants. Quantitative structure-activity relationships (QSARs) have been used for prediction of biological activity/property/toxicity to understand of the mechanism(s) of action (Hansch, Kurup, Garg, & Gao, 2001). Sometimes, it is very difficult to explain all the sources of variability due to the complexity between the molecular

structure and activity/property/toxicity. In this complex situation, machine learning approach like artificial neural networks (ANNs) may be used for the predictions (Haykin, 1994; Agatonovic-Kustrin, & Beresford, 2000). However, most of the common drawback of machine learning approaches is failure to interpret the relationships between the independent variables and the response variables (Polishchuk, 2017).

In the present work, we have developed ANN models separately using OT property data for diverse chemical classes of odor active components present in black tea and coffee and tried to found out the significant features controlling OT property using Mean Absolute Error (MAE) based criteria. The ANN models were developed in this study keeping in mind the principles of Organization for Economic Co-operation and Development (OECD) (http://www.oecd.org/dataoecd/33/37/37849783.pdf).

2. METHODS AND MATERIALS

2.1. Dataset

The present work deals with modeling of the OT property data for diverse chemical classes (aldehydes, acids, esters, furans, sulfur containing compounds, thiols, thiophenes, thiazoles, furanones, ketones, norisoprenoids, phenolic compounds, pyrazines, pyridines, terpenes, etc.) of compounds present in black tea and coffee collected from the published literature (Yeretzian, 2017; Magagna et al., 2017). The whole datasets of black tea (76 compounds) and coffee (46 compounds) are presented in Tables 6 and 7 respectively in Supplementary Section. Here, we have developed two multi-layer perceptron (MLP) based ANN models separately using the odor active components present in black tea and coffee. The odor threshold (OT) of compounds present in black tea and coffee are expressed in mmol/kg and in μmol/kg respectively. The OT values are taken in the negative logarithmic scale [log(1/OT)] leading to Y values ranging from -0.935 to 7.677 (in case of black tea) and -1.736 to 5.532 (in case of the coffee). The initial modelling analysis identified one compound as potential outlier (based on a high residual value) in case of the tea dataset. Thus, the final ANN model was developed using 76 components present in black tea.

2.2. Descriptor Calculation

We have drawn individually all compounds in the data sets using Marvin Sketch software (http://www.chemaxon.com). Descriptors are "numerical values associated with chemical constitution for correlation of chemical structure with various physical properties, chemical reactivity or biological activity". Using the drawn structures, we have calculated the descriptors using three software tools namely Dragon software version 6 (http://www.talete.mi.it/products/dragondescription.htm), PaDEL-Descriptor (http://www.yapcwsoft.com/dd/padeldescriptor) software and Cerius 2 version 4.10 software (http://www.accelrys.com). Dragon software was used to calculate constitutional indices, ring descriptors, connectivity indices, functional group counts, atom centered fragments, atom type E-state indices and 2D atom pairs descriptors while PaDEL-descriptor software was used to calculate extended topochemical atom (ETA) indices (Das, & Roy, 2016). Before computation of 3D descriptors, all structures were optimized using the "optimal search method" available in Cerius2 software version4.10 (http://www.accelrys.com). All the 3D descriptors were calculated using geometry optimized molecules. Thus, the models were developed in this work using a pool of both 2D and 3D descriptors. From the initial pool of descriptors, we have removed those descriptors having constant and near constant values (standard deviation less than 0.0001), variables with at least one missing value, descriptors with all missing values and descriptors with (absolute) pair correlation larger than or equal to 0.95.

2.3. Division of the Dataset: Selection of Training and Test Sets

We have employed a clustering technique, "Modified *k*-medoids" (Park,& Jun, 2009), using a tool developed in our laboratory (http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab) for division of the datasets. Seven clusters were generated in case of components present in black tea, and four clusters were generated in case of components present in coffee based on the properties available for the respective dataset components. Based on the clusters, we have taken approximately 75% compounds randomly from each cluster for the training set (57 compounds and 36 compounds in case of the tea and coffee datasets respectively) and remaining 25% compounds for the test set (19 compounds in case of the tea dataset and 10 compounds for the coffee dataset). The ANN model was developed using the training set compounds, and the test set compounds were used to validate the ANN models. Note that we have used same division pattern of the data sets for development of final ANN models as reported in the paper published previously by our group (Ojha, & Roy, 2018).

2.4. Descriptor Selection

Using the whole pool of descriptors, first we have performed stepwise regression for selection of the descriptors. We have removed the selected descriptors after the first run of stepwise regression, and rerun stepwise regression using remaining pool of descriptors. In this way, we have selected 48 descriptors in case of the tea and 40 descriptors in case of the coffee dataset as also discussed in our previous paper (Ojha, & Roy, 2018). This reduced pool of descriptors was used for the development of the final ANN models.

2.5. Optimization of Parameters and Development of ANN Models

Artificial Neural Network (ANN) is a multilayered architecture made up of one or more hidden layers placed between the input and output layers (Agatonovic-Kustrin, & Beresford, 2000). In the present work, a multilayer perceptron (MLP) neural network with the Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm (Head, & Zerner, 1985) was used to construct ANN models. BFGS is also known as variant of Gradient descent method which overcomes the limitations of plain gradient descent by seeking the second derivative (a stationary point) of the cost function (Liu, & Nocedal, 1989). Hence, this domain is created to predict odorant property of black tea and coffee. The network requires iterative training phases, which may be slow, but the networks are quite compact, execute quickly once trained and in most cases yield better results than the other types of networks (Zuvela, David, & Wong, 2018). In this study, the same methodology of ANN was used to generate separately the predictive models correlating a set of reduced pool molecular descriptors and odorant properties of the components present in black tea and coffee using STATISTICA software (version 13.4) (Statistica, 2016). The process starts with splitting of the (training) datasets with subset variable sampling method. The training process is a feed forward network. The input layer contains all the relevant descriptors obtained after reducing the initial pool of descriptors in order to investigate the prediction capability of the proposed ANN methods. The next one is a hidden layer. The performance of neural network depends on the number of hidden layers and number of neurons in the hidden layers. In this study, the neural network architecture is trained with different combinations of hidden layers and single output layer with 5 types of activation functions/Transfer functions namely Identity, Logistic, Tanh, Exponential and Sine which are available for hidden and output layers. The final ANN model was developed using 3 nodes with Logistic activation function in case of the black tea dataset and 2 nodes with identity activation function in case of the coffee dataset. Basically, Identity function is the operation of artificial neural network which sum up the product of the associated weight and the input signal and produce an output (Godfrey, & Gashler, 2015). The second one is sigmoid or logistic activation function which normalizes the data between0to1. Therefore, it is especially used for models to predict the probability as an output. Sigmoid is the right choice because it is differentiable, that means, we can find the slope of the sigmoid curve at any two points (Godfrey, & Gashler, 2015). The third one

in the process is Tanh activation function, also known as hyperbolic tangent activation function. It is like logistic or sigmoid function, but better predictions are possible. The range of Tanh functions is from -1 to 1. The next activation function is the exponential activation function, which speeds up learning in neural networks and leads to higher classification accuracies. Here, α is an exponential hyper parameter, which controls the value to which an Exponential activation saturates for negative net inputs. This function diminishes the vanishing gradient effect like rectified linear units (ReLUs) and leaky ReLUs (LReLUs). The vanishing gradient problem is alleviated because the positive part of these functions is the identity. Therefore, their derivative is one and it is not contractive. In contrast, the exponential function has negative values which push the mean of the activations closer to zero. Mean activations that are closer to zero enable faster learning as they bring the gradient closer to the natural gradient. The exponential activation function saturates to a negative value when the argument gets smaller. Saturation means a small derivative which decreases the variation and the information that is propagated to the next layer (Clevert, Unterthiner, & Hochreiter, 2015). The final activation function of the process is *Sine*. It is a periodic function, and unlike other activation functions it is non-quasiconvex and non-monotonic. This means that for a periodic activation function, as the correlation with the input increases, the activation will oscillate between stronger and weaker activations. This apparently undesirable behavior might suggest that periodic functions might be just as undesirable as activation functions in a typical learning task (Parascandolo, Huttunen, & Virtanen, 2016). After selection of activation function, another pivotal parameter is the Weights of neural network. For the optimized predictive model, always less weight is preferable. An increase in weights may cause over fitting problem. Weights in the hidden layers and the output layer maintained uniformly throughout the process. Here, for the development of the final ANN models, we have selected the network weight decay in hidden and output layer is 0.0001-0.001. The training process is continued to develop the model until error reaches performance goal (minimum error and maximum accuracy) that will be decided by Initialization. It is an iterative process where the user defined the seed number to decide how many times the process need to be cycled. In this work, we have used 1000 iterations for the development of ANN model in case of the tea dataset and 120 iterations in case of the coffee dataset. During the process of training, the software provides summary details about the networks being built including the type of network, the activation functions utilized, the training cycle configuration and the appropriate error terms. Once the training process of neural network is completed, the results are displayed. The active neural networks grid will show the top five networks generated during the training in which only one predictive model will be selected based on the training set and test set performances.

2.6. Strategy Used to Find Out the Significant Descriptors

After selection of the optimized model, we wanted to see the relative importance of different descriptors used in model development employing MAE based criteria (Roy et al., 2016). To achieve that, every time one descriptor is removed and the model is rebuilt with the rest of the descriptors without interrupting the network architecture used to develop the parent model. In this way,48 individual descriptor omission models are constructed in case of the black tea dataset and 40 models are constructed in case of the coffee dataset. After that, we have calculated Mean Absolute Error (MAE) for models obtained from individual descriptor omissions in case of both the black tea and coffee datasets and checked the MAE variations in each case after comparing with the MAE of the parent models. Furthermore, based on the MAE variation, all the input descriptors were divided into three types, category-I (significant descriptors: MAE changes>+0.01), category-II (redundant descriptors: MAE varies between +0.01 to -0.01) and category-III (noisy descriptors: MAE changes<-0.01).

2.7. Statistical Validation Parameters and Applicability Domain (AD)

The developed ANN model was validated in terms of both internal (R^2_{train}) and external (Q^2_{F1}) validation parameters (Ojha, & Roy, 2011; Roy, Ambure, Kar, & Ojha, 2018; Ojha, & Roy, 2018;

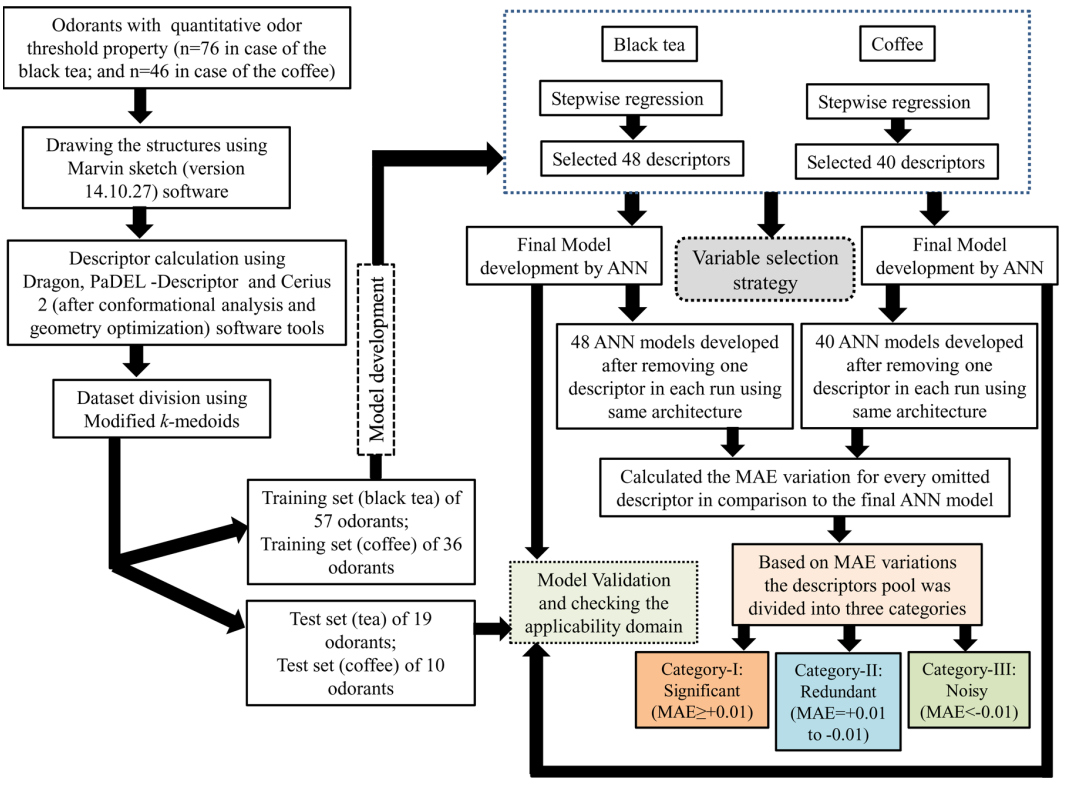
Roy, Ghosh, Ojha, & Roy, 2018; Ojha, Kar, Roy, & Leszczynski, 2018). The parameters obtained for the final ANN models are depicted in Table 1. The AD of a QSAR model is represented by the chemical space which is provided by the molecular properties of the training set compounds. The AD criteria help to check whether a query compound lies inside the domain of chemical space of the training set compounds or not. In this work, we have checked the applicability domain of test set compounds of the developed ANN models employing the standardization approach using the software developed in our laboratory (Roy, Kar, & Ambure, 2015). The reliability of prediction of a QSPR model is considered good if the molecules are present within the region of the chemical space of the training set molecules.

The methods employed for the development of ANN models are represented graphically in Figure 1.

2.8. Software Used

Marvin sketch (version 14.10.27) software (http://www.chemaxon.com/) was used to draw the chemical structures. Dragon version 6 (http://www.talete.mi.it/products/dragondescription.htm), Cerius 2 (version 4.0) (http://www.accelrys.com) and PaDEL-Descriptor (http://www.yapcwsoft.com/dd/padeldescriptor) software tools were used to calculate the molecular descriptors. Modified *k*-medoid (http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab) cluster analysis was performed by employing the software developed in our laboratory. The stepwise regression analysis was performed by using MINITAB software (version 14.13) (http://www.minitab.com/en-US/default.aspx). STATISTICA software (version 13.4) was used to develop the ANN models.

Figure 1. Schematic representation of the steps involved in the development of final ANN models



3. RESULTS AND DISCUSSION

To model the relationships between the molecular structure and the odor threshold (OT) property of black tea and coffee separately, we have employed *a-priori* selected reduced pool of molecular descriptors. The reduced pool descriptors were selected using various strategies as discussed in our previously published work (Ojha, & Roy, 2018) as well as here in Materials and methods section. In the present work, we have developed the multilayer perceptron (MLP) ANN models using the reduced pool descriptors only to reduce the noise. The ANN as a powerful non-linear mapping technique simulates biological neural networks. Although, it was proved mathematically that ANNs have an inherent capability of universal approximation of any function (Hornik, 1991), they are very susceptible to overfitting or underfitting. Prior to the development of final ANN models, we have applied different conditions to find out the optimum ANN parameters as discussed in Materials and Method section. The optimized parameters used to development of final ANN models and the results are depicted in Table 1.

3.1. ANN Model Developed from Odorants Present in Black Tea

The optimized parameters such as Logistic activation function, BFGS algorithm, one hidden layer with three nodes, 0.0001-0.001 weight decay and 1000 initialization are used to develop the final MLP type ANN model for odor active compounds. The statistical quality in terms of both internal (R^2_{train}=0.823) and external (Q^2_{F1} = 0.900) validation parameters suggested that the model was significantly robust. The scatter plot of the observed and predicted odorant properties is shown in Figure 2. This plot showed a minimal scattering of all the training set and test set compounds from the diagonal line depicting an acceptable fitting and prediction results. Note that the results obtained from the non-linear model is far better than the previously published PLS based regression model (Ojha, & Roy, 2018) (Table 1). The ANN model for the odor active compounds present in black tea was developed using the priori selected 48 descriptors as mentioned in the Table 2. However, interpretation of the model is not straightforward as in case of regression equations. In this work, we have tried to explore the significant descriptors from the descriptor pool used to develop the ANN model by employing the MAE based criteria. To find out the significant descriptors, we have removed one descriptor from the initial pool of descriptors in each turn, and generated the ANN model using the same architecture as used in the development of the final ANN model and calculated the variation of MAE for removing that particular descriptor. In this way, we have generated ANN models one by one for every omitted descriptor and calculated the MAE variation in each case. After removing the particular descriptor, if the MAE value is found to be higher as compared to that of the parent model then we can say that the impact of that particular descriptor is high. Based on the variation of MAE, we have divided the descriptors pool in three categories namely category-I, category-II and category-III (Table 2). The

Table 1. The statistical results obtained from ANN-model of TEA and Coffee

MLP		Activation function	Weight. decay		Network	Initialization	Q2F1	$\overline{r^2_{m(test)}}$	$\overline{r^2_{m(test)}}$	RMSEP	Training set		Test set	
(2-3 Hidden layers)						(seedcontrol)								
			Hidden layer	Output layer										
											MAE	MAE	MAE	MAE
											(100%)	(95%)	(100%)	(95%)
Tea		Logistic	0.0001-0.001	0.0001-0.001	48-3-1	1000	0.781	0.716	0.14	0.596	0.801	0.707	0.401	0.351
Coffee		Identity	0.0001-0.001	0.0001-0.001	40-2-1	120	0.947	0.911	0.021	0.4	0.708	0.646	0.299	0.23

Table 2. Categorical list of descriptors obtained from the ANN model based on MAE (95%) in case of black tea dataset

Category	Sl. No	Descriptor removed	Definition	Type	Architecture	MAE (95%)	Changes in MAE
Category-I: Significant descriptors	1	H-051	H attached to alpha-C	Atom centred fragments	47-3-1	0.591	0.113
	2	F10[C-O]	Frequency of C-O at topological distance 10	2DAtomPairs	47-3-1	0.584	0.106
	3	Jurs-RPSA	Relative polar surface area: total polar surface area divided by the total molecular solvent-accessible surface area	Spatial descriptor	47-3-1	0.582	0.104
	4	ETA_dAlpha_B	DaB	ETA descriptor	47-3-1	0.57	0.092
	5	nRCHO	number of aldehydes (aliphatic)	Functional group counts	47-3-1	0.568	0.09
	6	H-047	H attached to C1(sp3)/C0(sp2)	Atom centered fragments	47-3-1	0.566	0.088
	7	SssssC	Sum of ssssC E-states	Atom-type E-state indices	47-3-1	0.562	0.084
	8	Hbond donor	Represents number of hydrogen bond donor	Structural descriptor	47-3-1	0.56	0.082
	9	Jurs-RASA	Relative hydrophobic surface area: total hydrophobic surface area divided by the total molecular solvent-accessible surface area	Spatial descriptor	47-3-1	0.557	0.079
	10	F04[C-C]	Frequency of C-C at topological distance 4	2DAtomPairs	47-3-1	0.557	0.079
	11	nCq	number of total quaternary C(sp3)	Functional group counts	47-3-1	0.555	0.077
	12	AlogP	Ghose-Crippenoctanol-water partition coeff. (logP)	Molecular properties	47-3-1	0.552	0.074
	13	Jurs-RPCG	Relative positive charge: charge of most positive atom divided by the total positive charge		47-3-1	0.55	0.072
	14	ETA_ dEpsilon_C	DeC	Extended Topological Atom	47-3-1	0.545	0.067
	15	Jurs-RNCG	Relative negative charge: charge of most negative atom divided by the total negative charge	Spatial descriptor	47-3-1	0.54	0.062
	16	B03[C-O]	Presence/ absence of C-O at topological distance 3	2DAtomPairs	47-3-1	0.538	0.06
	17	ETA_dPsi_A	DyA	Extended Topological Atom	47-3-1	0.533	0.055

continued on following page

Table 2. Continued

Category	Sl. No	Descriptor removed	Definition	Type	Architecture	MAE (95%)	Changes in MAE
	18	ETA_Eta_F	hF	Extended Topological Atom	47-3-1	0.528	0.05
	19	ETA_Shape_X	(Sa)X /Sa	Extended Topological Atom	47-3-1	0.528	0.05
	20	ETA_BetaP_ns	Sbns	Extended Topological Atom	47-3-1	0.524	0.046
	21	Jurs-WNSA-3	Surface-weighted charged partial surface areas	Spatial descriptor	47-3-1	0.516	0.038
	22	LUMO	Lowest unoccupied molecular orbitals	Electronic descriptors	47-3-1	0.512	0.034
	23	SssO	Sum of ssO E-states	Atom-type E-state indices	47-3-1	0.505	0.027
	24	nCconj	Number of non-aromatic conjugated C(sp2)	Functional group counts	47-3-1	0.504	0.026
	25	MR	Molar refractivity	Fragment constants descriptor	47-3-1	0.496	0.018
	26	nROH	number of hydroxyl groups	Functional group counts	47-3-1	0.491	0.013
Category-II: Redundant descriptors	27	X5Av	average valence connectivity index of order 5	Connectivity indices	47-3-1	0.486	0.008
	28	nRCOOH	number of carboxylic acids (aliphatic)	Functional group counts	47-3-1	0.479	0.001
	29	C-001	CH3R / CH4	Atom-centred fragments	47-3-1	0.475	-0.003
	30	H-049	"H" attached to C3(sp3)/ C2(sp2)/ C3(sp2)/C3(sp)	Atom-centredfragments	47-3-1	0.474	-0.004
	31	X3A	average connectivity index of order 3	Connectivity indices	47-3-1	0.473	-0.005
	32	B07[C-C]	Presence/ absence of C-C at topological distance 7	2DAtomPairs	47-3-1	0.473	-0.005
	33	ETA_Psi_1	Y1	Extended Topological Atom	47-3-1	0.472	-0.006
	34	Jurs-RPCS	Relative positive charge surface area: solvent-accessible surface area of the most positive atom divided by descriptor	Spatial descriptor	47-3-1	0.469	-0.009
Category-III: Noisy descriptors	35	NdssC	Number of atoms of type dssC	Atom-type E-state indices	47-3-1	0.465	-0.013
	36	H-052	H attached to C0(sp3) with 1X attached to next C	Atom-centred fragments	47-3-1	0.461	-0.017
	37	O-060	Al-O-Ar / Ar-O-Ar / R..O..R / R-O-C=X	Atom-centred fragments	47-3-1	0.459	-0.019
	38	F04[C-O]	Frequency of C-O at topological distance 4	2DAtomPairs	47-3-1	0.454	-0.024

continued on following page

Table 2. Continued

Category	Sl. No	Descriptor removed	Definition	Type	Architecture	MAE (95%)	Changes in MAE
	39	X5A	average connectivity index of order 5	Connectivity indices	47-3-1	0.452	-0.026
	40	ETA_Epsilon_2	e2	Extended Topological Atom	47-3-1	0.446	-0.032
	41	ETA_AlphaP	Sa/NV	Extended Topological Atom	47-3-1	0.445	-0.033
	42	NssssC	Number of atoms of type ssssC	Atom-type E-state indices	47-3-1	0.427	-0.051
	43	nDB	number of double bonds	Constitutional indices	47-3-1	0.422	-0.056
	44	F03[C-O]	Frequency of C-O at topological distance 3	2DAtomPairs	47-3-1	0.42	-0.058
	45	Jurs-PNSA-1	Partial negative surface area: sum of the solvent-accessible surface areas of all negatively charged atoms	Spatial descriptor	47-3-1	0.416	-0.062
	46	nR=Cs	number of aliphatic secondary C(sp2)	Functional group counts	47-3-1	0.412	-0.066
	47	B06[C-C]	Presence/ absence of C-C at topological distance 6	2D Atom Pairs	47-3-1	0.412	-0.066
	48	B04[C-C]	Presence/ absence of C-C at topological distance 4	2D Atom Pairs	47-3-1	0.391	-0.087

*MAE (95%): determined after removing 5% test or training sets chemicals with high residual values in order to obviate the possibility of any outlier predictions; MAE (100%): Determined using all training and test sets chemicals.

Table 3. Comparison of numerical values of the descriptors with odorant property of five most potent and five less potent odorants present in black tea

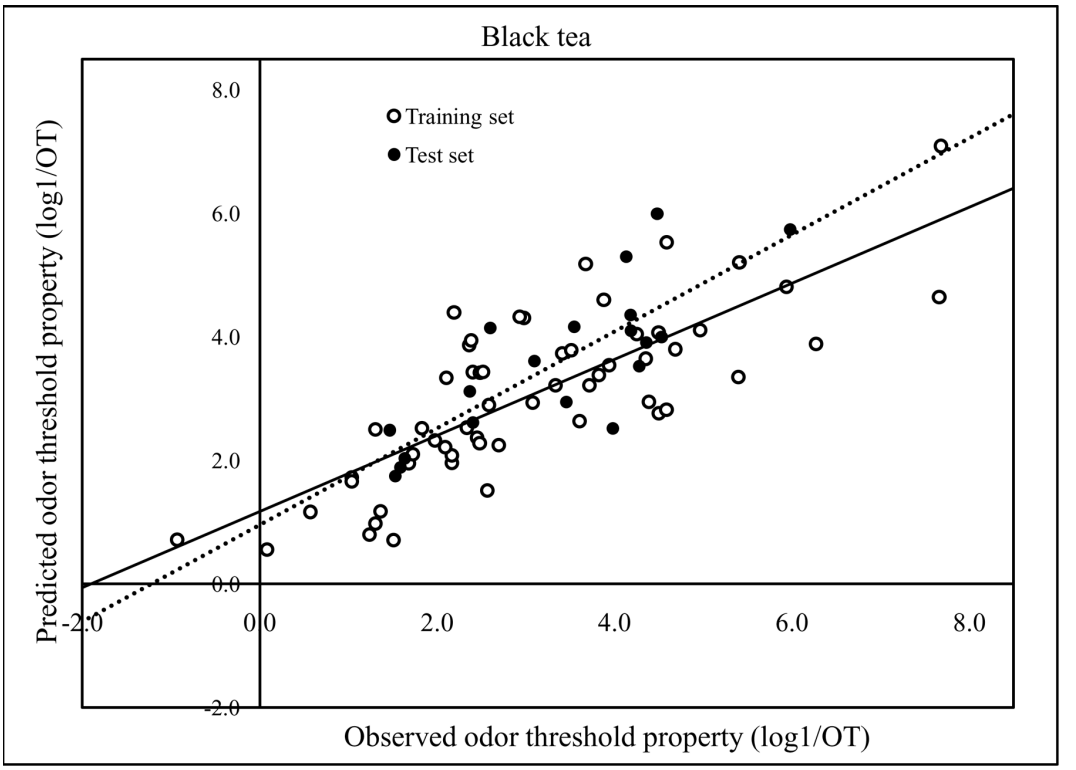
No.	NAME	log(1/OT)	H-051	Jurs-RPSA	ETA_ dAlpha_B	nRCHO	H-047	SssssC	Hbond donor	Jurs-RASA	F04[C-C]	AlogP
Five most potent odorants present in black tea												
68	β-Damascenone	7.677	0	0.072	0.012	0	4	-0.018	0	0.928	14	3.934
51	(E,Z)-2,6-Nonadienal	7.663	0	0.134	0.017	1	4	0	0	0.866	5	2.370
26	(Z)-4-heptenal	6.272	2	0.152	0.021	1	2	0	0	0.848	3	1.374
56	E,E)-2,4-Nonadienal	5.936	0	0.121	0.017	1	4	0	0	0.879	5	2.370
47	Linalool	5.410	0	0.076	0.015	0	4	-0.702	1	0.924	6	2.517
Five leastpotent odorants present in black tea												
12	2-Methyl propanoic acid	1.037	1	0.377	0.056	0	0	0	1	0.623	0	1.023
41	Benzyl alcohol	1.034	0	0.168	0.021	0	7	0	1	0.832	1	1.634
11	Propanoic acid	0.569	2	0.450	0.067	0	0	0	1	0.550	0	0.460
8	Acetic acid	0.080	3	0.561	0.083	0	0	0	1	0.439	0	-0.168
2	Acetone	-0.935	6	0.211	0.042	0	0	0	0	0.789	0	0.379

category-I (MAE varies +0.01 and above) descriptors are the most significant descriptors, category-II (MAE varies between +0.01 to -0.01) depicts redundant descriptors and the third category descriptors i.e., category-III (MAE varies -0.01 and lesser) are defined as noisy descriptors. In the black tea dataset, out of 48 descriptors, we have found out 26 descriptors as significant, 8 descriptors as redundant and 14 descriptors as noisy descriptors. Our results also well corroborated the previously published work by our group as out of six descriptors (obtained from the PLS regression-based model published previously by our group), five descriptors were identified as significant descriptors in the present work. According to the MAE variation as depicted in Table 2, it is clear that the descriptors like H-051, F10[C-O], Jurs-RPSA, ETA_dAlpha_B, nRCHO, H-047,SssssC, Hbond donor, Jurs-RASA, F04[C-C], AlogP,Jurs-RPCG, ETA_dEpsilon_C, Jurs-RNCG, B03[C-O], ETA_dPsi_A, ETA_Eta_F, ETA_Shape_X, ETA_BetaP_ns, Jurs-WNSA-3, LUMO, SssO, nCconj, MR and nROH play dominant role to regulate the odorant property of black tea. To find out the relationship between the odorant property and the molecular properties, we have selected five higher odorant property containing compounds and five lower odorant property containing compounds and checked the trend of changes of the numerical values of significant descriptors (here we have mentioned only 10 descriptors in Table 3) and odorant property of the constituents present in black tea. It has been found that the trend is very similar, i.e., for the higher active compounds, the numerical values of the descriptors are either in higher range or in lower range based on the descriptors and vice versa (Table 3). As for example, in case of H-051 descriptor (Table 3), only one compound out of the five most potent odorant molecules have a nonzero descriptor value (compound number 26) while four compounds (compounds 12, 11, 8 and 2) have nonzero descriptor values out of the five least potent odorant molecules. Thus, the trend is like that potent odorants have lower descriptor values for H-051 and less potent odorants bear higher descriptor values.A similar trend is also observed in case of JURS-RPSA, ETA_dAlpha_B and Hbond donor descriptors. As shown in Table 3, in case of nRCHO, H-047, SssssC, Jurs-RASA, F04[C-C] and AlogP descriptors, the trend or pattern of changes of the numerical values of these descriptors is similar i.e., the potent odorants contain higher range of descriptor values and less potent odorants bear a lower range of numerical values. We have checked the applicability domain of the ANN model using the standardization approach. We found that all the test set compounds are within the applicability domain of the developed model.

3.2. ANN Model Developed from Odorants Present in Coffee

The optimized parameters used to model the odorant properties of the components present in coffee are Identity as activation function, BFGS algorithm, 2 hidden layers, 0.0001-0.001 weight decay and 120 initialization. The model quality in terms of both internal ($R^2_{train} = 0.908$) and external ($Q^2_{F1} = 0.980$) validation parameters confirmed that the model was statistically robust (Table 1).The scatter plot of the observed and predicted odorant properties of the components present in coffee are shown in Figure 3, which showed limited deviations of the fitted values or predictions from the experimental values for the model confirming the acceptability of the model. The results obtained from the ANN models are better than those obtained from the previously published PLS based regression models (Ojha, & Roy, 2018). The ANN model for the odor active compounds present in coffee was developed by using the priori selected 40 descriptors as mentioned in the Table 4. We have found out the significant descriptors among the 40 descriptors used initially as input employing the same protocol as used in case of development of ANN model of odorant components present in black tea. Here also, we have divided the descriptors in three categories based on MAE variation in the same way as discussed earlier in case of the tea dataset. The MAE variation after removing each descriptor from the parent model is summarized in Table 4. Based on MAE variation, out of 40 descriptors as input, we have found out 24 descriptors as significant (category-I), 8 descriptors as redundant (category-II) and 8 descriptors as noisy descriptors (category-III). This results also well corroborated with the previously published work by our group as out of six descriptors (obtained from the PLS regression-based model published previously by our group), five descriptors were

Figure 2. The scatter plot of the observed and the predicted odorant properties [log(1/OT)] for the final ANN model developed from the components present in black tea. The dashed line indicates the best fit line based on test set compounds and the solid line indicates the best fit line based on the training set compounds.



identified as significant descriptors here. According to the MAE variation as depicted in Table 4, it can be suggested that the descriptors like F01[C-S], C-024, B07[C-C], nS, B04[C-C], F05[C-C], B07[C-O], S-106, nRCO, Jurs-WPSA-2, ETA_Shape_P, C-040, Apol, nHDon, H-049, B02[C-S], nH, nRCOOH, B01[C-O], Jurs-RNCS, ETA_dAlpha_B, F04[C-N], ETA_Eta and B01[C-S] play a crucial role to control the odorant property of the components present in coffee. In this case, we have also selected five most potent odorants and five least potent odorants present in coffee and compared the trend of changes in the descriptors values with the odorant property (Table 5). We observed that the descriptor values are either in higher range or in lower range in case of potent odor active molecule based on the descriptors, and a similar pattern is also shown in case of least potent odor active molecules. As for example, in case of F01[C-S] descriptor (Table 5), two compounds out of the five most potent odorant molecules having non-zero descriptor value (compound numbers 19 and 16) while none of the compounds having non-zero descriptor values in case of five least potent odorant molecules. This pattern (i.e., higher descriptors value in the potent odor active molecules) is also shown in case of B07[C-C], nS, B04[C-C], F05[C-C], S-106, nRCO, ETA_Shape_P and Apol descriptors. The opposite pattern (i.e., lower or less number of descriptors value in the potent odor active molecules) is shown in case of C-024 descriptor. To check the domain of applicability, we have used standardization approach and found only one compound (compound number 14) as outlier.

4. CONCLUSION

The MLP based ANN model for the odor active components present in black tea and coffee were developed using a priori selected descriptors. We have used a variable selection strategy to extract the significant descriptors prior to development of the final models thus reducing the noise. The ANN models were developed keeping in mind the OECD principles. The statistical results obtained from the ANN models justify the reliability and robustness of the developed models. The statistical results in terms of both internal and external validation parameters obtained from the ANN models are far better than the previously published PLS-regression based QSPR model. However, interpretation of the non-linear model is not straightforward as in case of regression equations. In this work, we have tried to explore the significant descriptors from the descriptor pool used to develop the ANN model by employing the MAE based criteria in a backward elimination of descriptors one in each turn. In case of the black tea dataset, we have found out 28 significant descriptors while in case of the coffee dataset we have found out 24 significant descriptors. As compared with the previously published PLS-regression based models, out of six descriptors as in case of both tea and coffee models, five common descriptors are found to be significant descriptors based on the respective ANN models. The present ANN models corroborate the findings of the previously published PLS-regression based chemometric models (Ojha, & Roy, 2018). Thus, the present approach can be used to find out the significant descriptors used for development of ANN models.

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Figure 3. The scatter plot of the observed and the predicted odorant properties [log(1/OT)] for the final ANN model developed from the components present in coffee. The dashed line indicates the best fit line based on test set compounds and the solid line indicates the best fit line based on the training set compounds.

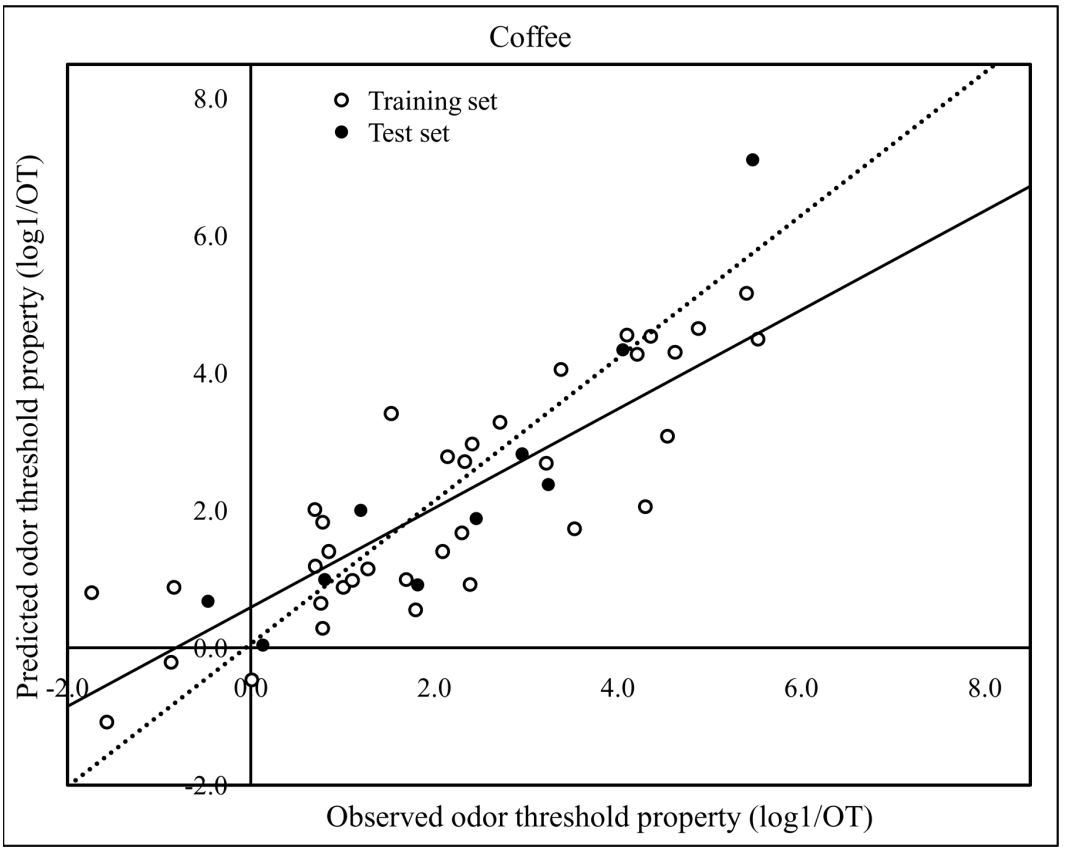

Table 4. Categorical list of descriptors obtained from the ANN model based on MAE (95%) in case of coffee dataset

Category	Sl. No.	Descriptor removed	Definition	Type	Architecture	MAE (95%)	Change in MAE
Category-I: Significant descriptors	1	F01[C-S]	Frequency of C-S at topological distance 1	2D Atom Pairs	39-2-1	0.444	0.191
	2	C-024	R--CH--R	Atom-centred fragments	39-2-1	0.404	0.151
	3	B07[C-C]	Presence/absence of C-C at topological distance 7	2DAtom Pairs	39-2-1	0.399	0.146
	4	nS	number of Sulfur atoms	Constitutional indices	39-2-1	0.399	0.146
	5	B04[C-C]	Presence/absence of C-C at topological distance 4	2DAtom Pairs	39-2-1	0.339	0.086
	6	F05[C-C]	Frequency of C-C at topological distance 5	2D Atom Pairs	39-2-1	0.326	0.073
	7	B07[C-O]	Presence/absence of C-O at topological distance 7	2DAtom Pairs	39-2-1	0.326	0.073
	8	S-106	R-SH	Atom-centred fragments	39-2-1	0.318	0.065
	9	nRCO	number of ketones(aliphatic)	Functional group counts	39-2-1	0.317	0.064
	10	Jurs-WPSA-2	Surface-weighted charged partial surface areas	Spatial descriptor	39-2-1	0.315	0.062
	11	ETA_Shape_P	$(\Sigma\alpha)_P / \Sigma\alpha$	Extended Topological Atom	39-2-1	0.312	0.059
	12	C-040	R-C(=X)-X / R-C#X / X=C=X	Atom-centred fragments	39-2-1	0.308	0.055
	13	Apol	Sum of atomic polarizabilities.	Electronic descriptors	39-2-1	0.303	0.050
	14	nHDon	number of donor atoms for H-bonds (N and O)	Functional group counts	39-2-1	0.302	0.049
	15	H-049	H attached to (sp3)/C2(sp2)/C3(sp2)/ C3(sp)	Atom-centred fragments	39-2-1	0.294	0.041
	16	B02[C-S]	Presence/absence of C - S at topological distance 2	2DAtom Pairs	39-2-1	0.294	0.041
	17	nH	number of Hydrogen atoms	Constitutional indices	39-2-1	0.292	0.039
	18	nRCOOH	number of carboxylic acids (aliphatic)	Functional group counts	39-2-1	0.289	0.036
	19	B01[C-O]	Presence/absence of C - O at topological distance 1	2DAtom Pairs	39-2-1	0.289	0.036
	20	Jurs-RNCS	Relative negative charge surface area: solvent-accessible surface area of most negative atom divided by descriptor	Spatial descriptor	39-2-1	0.284	0.031
	21	ETA_dAlpha_B	$\Delta\alpha_B$	Extended Topological Atom	39-2-1	0.275	0.022
	22	F04[C-N]	Frequency of C - N at topological distance 4	2DAtom Pairs	39-2-1	0.266	0.013
	23	ETA_Eta	η	Extended Topological Atom	39-2-1	0.266	0.013
	24	B01[C-S]	Presence/absence of C - S at topological distance 1	2DAtom Pairs	39-2-1	0.263	0.010
Category-II: Redundant descriptors	25	X1v	valence connectivity index of order 1	Connectivity indices	39-2-1	0.260	0.007
	26	B03[O-S]	Presence/absence of O - S at topological distance 3	2DAtom Pairs	39-2-1	0.257	0.004
	27	C-006	CH2RX	Atom-centred fragments	39-2-1	0.256	0.003
	28	nR=Ct	number of aliphatic tertiary C(sp2)	Functional group counts	39-2-1	0.252	-0.001
	29	F04[C-C]	Frequency of C - C at topological distance 4	2DAtom pairs	39-2-1	0.251	-0.002
	30	S-107	R2S / RS-SR	Atom-centred fragments	39-2-1	0.250	-0.003
	31	ETA_EtaP	η/N_V	Extended Topological Atom	39-2-1	0.248	-0.005
	32	C-029	R--CX--X	Atom centred fragments	39-2-1	0.246	-0.007
Category-III: Noisy descriptors	33	nCsp3	number of sp3 hybridized Carbon atoms	Constitutional indices	39-2-1	0.241	-0.012
	34	Jurs-RNCG	Relative negative charge: charge of most negative atom divided by the total negative charge	Spatial descriptor	39-2-1	0.240	-0.013
	35	C-027	R--CH--X	Atom centred fragments	39-2-1	0.237	-0.016
	36	nROH	number of hydroxyl groups	Functional group counts	39-2-1	0.233	-0.020
	37	SssO	Sum of ssO E-states	Atom-type E-state indices	39-2-1	0.233	-0.020
	38	nArOR	number of ethers (aromatic)	Atom centred fragment	39-2-1	0.232	-0.021
	39	H-050	H attached to heteroatom	Atom centred fragment	39-2-1	0.227	-0.026
	40	nArCHO	number of aldehydes (aromatic)	Functional group counts	39-2-1	0.212	-0.041

Table 5. Comparison of numerical values of the descriptors with odorant property of five most potent and five less potent odorants present in coffee

Compound no.	NAME	F01[C-S]	C-024	B07[C-C]	nS	B04[C-C]	F05[C-C]	S-106	nRCO	ETA_ Shape_P	Apol
Five most potent odorants present in black tea											
19	3-Methyl-2-butene-1-thiol	1	0	0	1	0	0	1	0	0.550	4706.700
30	(E)-Beta-damascenone	0	0	1	0	1	8	0	1	0.341	8070.440
41	2-Methoxy-3-isopropylpyrazine	0	0	0	0	1	5	0	0	0.292	5317.440
16	3-Mercapto-3-methylbutyl formate	1	0	0	1	1	2	1	0	0.481	5704.260
27	1-Octen-3-one	0	0	1	0	1	3	0	1	0.308	5015.620
Five least potent odorants present in black tea											
43	Pyridine	0	3	0	0	0	0	0	0	0.000	2999.580
8	3-Methylbutyric acid	0	0	0	0	0	0	0	0	0.526	3506.000
35	2,3-Dimethylpyrazine	0	0	0	0	1	0	0	0	0.263	3946.120
42	Ethyl pyrazine	0	0	0	0	1	1	0	0	0.132	3946.120
12	5-Methyl-2-furancarboxyaldehyde	0	2	0	0	1	0	0	0	0.227	3878.500

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APPENDIX A: SUPLEMENTRY TABLES

Table 6. List of aroma components present in black tea with observed and model derived odor threshold property

Compound no.	Structure Name	Observed value (log(1/OT))	Predicted value
1	2-Methyl propanal	4.496	3.770
2	Acetone	-0.935	0.595
3	Butanal	3.603	3.514
4	Ethyl acetate	1.972	1.830
5	1-Butanol	2.694	3.013
6	3-Methyl butanal	3.821	3.825
7	2-Methyl butanal	3.935	4.021
8	Acetic acid	0.080	1.078
9	1-Penten-3-ol	2.333	2.488
10	Pentanal	3.333	2.900
11	Propanoic acid	0.569	1.191
12	2-Methyl propanoic acid	1.037	1.320
13	(E)-2-Pentenal	2.448	3.102
14*	1-Pentanol	1.468	1.538
15	Butanoic acid	2.565	1.233
16*	Hexanal	3.098	3.041
17	Furfural	1.506	1.345
18	3-Methyl butanoic acid	2.164	1.335
19	2-Methyl butanoic acid	2.164	1.437
20*	(E)-2-Hexenal	2.369	3.269
21	(Z)-3-hexen-1-ol	1.825	1.948
22	(E)-2-hexen-1-ol	1.302	1.661
23*	1-Hexanol	2.407	1.841
24*	Pentanoic acid	1.532	0.945
25	2-Heptanone	2.581	2.385
26	(Z)-4-heptenal	6.272	3.617
27	Heptanal	2.359	3.326
28*	(E,E)-2,4-Hexadienal	3.983	3.465
29	(E)-2-Heptenal	4.351	3.656
30	Benzaldehyde	2.482	2.160
31	1-Heptanol	4.588	3.159
32	1-Octen-3-ol	3.409	4.149
33	6-Methyl-5-hepten-2-one	2.101	2.780
34	2-Octanone	2.400	3.061
35*	Hexanoic acid	1.588	0.922
36*	2-Pentyl furan	4.362	3.893
37	(Z)-3-Hexen-1-ol acetate	4.250	4.492
38*	Octanal	2.603	3.587
39	(E,E) 2,4-Heptadienal	2.486	4.013
40*	Limonene	4.134	3.861
41	Benzyl alcohol	1.034	1.432
42*	Phenyl acetaldehyde	4.280	3.164
43	(E)-2-Octenal	4.499	4.088
44	(E,E)-3,5-Octadien-2-one	5.395	3.955
45	1-Octanol	3.073	3.761
46*	Heptanoic acid	1.638	0.977
47	Linalool	5.410	6.018
48	6-Methyl-3,5-heptadien-2-one	2.514	3.298

continued on following page

Table 6. Continued

Compound no.	Structure Name	Observed value (log(1/OT))	Predicted value
49	Nonanal	2.977	3.643
50	2-Phenyl ethanol	2.087	1.843
51	(E,Z)-2,6-Nonadienal	7.663	4.752
52	(E)-2-Nonenal	2.193	4.136
53*	1-Nonanol	3.460	3.858
54	Octanoic acid	1.682	1.364
55	Decanal	2.381	3.533
56	(E,E)-2,4-Nonadienal	5.936	4.721
57*	β-Cyclocitral	4.484	4.885
58	Geraniol	4.683	4.543
59*	(E)-2-Decenal	4.188	3.948
60	Geranial	3.677	4.606
61	Nonanoic acid	1.722	1.573
62	2-Undecanone	4.386	3.089
63*	(E,Z)-2,4-Decadienal	4.183	4.446
64*	Undecanal	4.532	3.566
65	(E,E)-2,4-Decadienal	2.927	4.457
66	Dihydro-5-pentyl-2(3H)-Furanone	3.717	4.257
67	Decanoic acid	1.236	1.091
68	β-Damascenone	7.677	6.142
69	Vanillin	3.881	3.851
70	Dodecanal	4.965	3.610
71	α-Ionone	4.585	5.698
72	Geranyl acetone	3.510	3.507
73*	β-Ionone	5.983	5.507
74	Dodecanoic acid	1.302	1.321
75*	Tetradecanal	3.549	3.667
76	Tetradecanoic acid	1.359	1.581

*denotes test set compounds

Table 7. List of aroma components present in coffee with observed and model derived odor threshold property

Compound No.	Structure name	Observed value ((log(1/OT))	Predicted value
1*	2-Methylbutanal	1.821	1.853
2	3-Methylbutanal	2.391	1.859
3*	(E)-2-Nonenal	3.244	3.096
4	Acetaldehyde	1.799	0.456
5	4-Methoxybenzaldehyde	0.703	0.541
6	Propanal	0.764	0.920
7	2-Methylbutyric acid	1.009	-0.356
8	3-Methylbutyric acid	-0.836	-0.357
9	Ethyl-2-methylbutyrate	2.412	2.028
10	Ethyl-3-methylbutyrate	2.333	1.959
11*	Furfural	-0.464	-0.834
12	5-Methyl-2-furancarboxyaldehyde	-1.736	-1.086
13	Dimethyl trisulfide	4.101	3.497
14*	Bis(2-methyl-3-furyl)disulfide	5.474	5.461
15	Methional	2.717	3.609
16	3-Mercapto-3-methylbutyl formate	4.627	5.767
17*	2-Furfuryl thiol	4.058	4.528
18	2-Methyl-3-furanthiol	4.212	4.163
19	3-Methyl-2-butene-1-thiol	5.532	4.973
20	Methanethiol	3.381	3.003
21	Dihydro-2-methyl-3(2H)-furanone	4.302	3.477
22	2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone	0.852	1.309
23*	3-Hydroxy-4,5-dimethyl-2(5H)-furanone	0.807	0.859
24	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	1.108	0.711
25	5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone	1.278	0.701
26	5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone	2.092	1.325
27	1-Octen-3-one	4.545	3.333
28*	2,3-Butanedione	2.458	1.603
29	2,3-Pentanedione	0.700	1.310
30	(E)-Beta-damascenone	5.404	5.387
31	Guaiacol	1.696	1.015
32	4-Ethyl Guaiacol	0.785	2.447
33	4-Vinyl Guaiacol	2.302	1.971
34	Vanillin	0.784	0.245
35	2,3-Dimethylpyrazine	-0.869	-0.411
36*	2,5-Dimethylpyrazine	0.131	-0.337
37	2,3-Diethyl-5-methylpyrazine	3.223	2.519
38	2-Ethyl-3,5-dimethylpyrazine	3.532	1.537
39*	2-Ethyl-3,6-dimethyl-pyrazine	1.200	1.613
40	2-Methoxy-3,5-dimethylpyrazine	4.362	4.062
41	2-Methoxy-3-isopropylpyrazine	4.881	4.795
42	Ethylpyrazine	-1.568	-0.644
43	Pyridine	0.012	-1.038
44*	Linalool	2.958	2.642
45	Limonene	1.532	2.780
46	Geraniol	2.147	2.907

*denotes test set compounds

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COMPREHENSIVE REVIEW

Therapeutics for COVID-19: from computation to practices—where we are, where we are heading to

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Abstract

After the 1918 Spanish Flu pandemic caused by the H1N1 virus, the recent coronavirus disease 2019 (COVID-19) brought us to the time of serious global health catastrophe. Although no proven therapies are identified yet which can offer a definitive treatment of the COVID-19, a series of antiviral, antibacterial, antiparasitic, immunosuppressant drugs have shown clinical benefits based on repurposing theory. However, these studies are made on small number of patients, and, in majority of the cases, have been carried out as nonrandomized trials. As society is running against the time to combat the COVID-19, we present here a comprehensive review dealing with up-to-date information of therapeutics or drug regimens being utilized by physicians to treat COVID-19 patients along with in-depth discussion of mechanism of action of these drugs and their targets. Ongoing vaccine trials, monoclonal antibodies therapy and convalescent plasma treatment are also discussed. Keeping in mind that computational approaches can offer a significant insight to repurposing based drug discovery, an exhaustive discussion of computational modeling studies is performed which can assist target-specific drug discovery.

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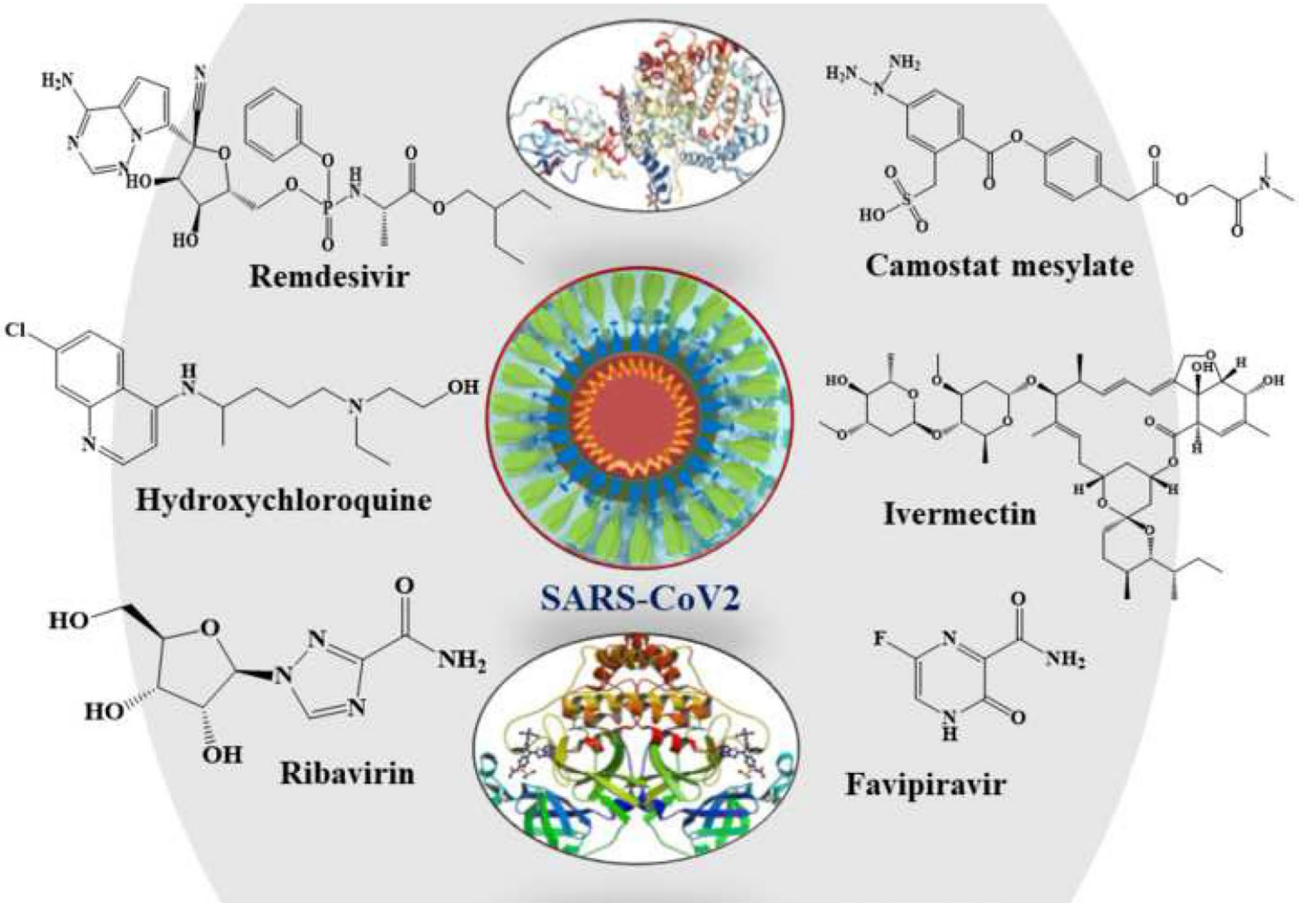
Graphic abstract



Keywords Computational · Coronavirus · COVID-19 · Drug · SARS-CoV-2 · Vaccine

Introduction

A form of pulmonary disease was first reported in China from a city called Wuhan in the Hubei Province on December 31, 2019 [1]. The deadly disease was later termed as COVID-19 by the World Health Organization (WHO) on February 11, 2020. The identified causative novel coronavirus (2019-nCoV) is termed as severe acute respiratory syndrome-related coronavirus SARS-CoV-2 as it shares around 79.6% of genome similarity with SARS-CoV which also previously emerged in China during 2002–2003 [2]. With the announcement of COVID-19 as 'Global Pandemic' by WHO on March 11, 2020, SARS-CoV-2 has eventually affected 212 countries and territories around the world and 2 international conveyances. As of August 13, 2020, 20,881,635 cases have been confirmed with 748,503 deaths and 13,771,549 recovery cases, while among the active cases, 6,297,028 cases are in mild condition and 64,555 cases in a serious or critical condition [3] (Fig. 1a).

The literature reported seven coronaviruses (CoVs) that are known to cause human disease where the strains 229E (α-CoV), HKU1 (β-CoV), OC43 (β-CoV) and NL63 (α-CoV) caused mild infections of the upper respiratory tract in humans [4]. On the contrary, other two strains SARS-CoV (occurring in 2002–2003) and MERS-CoV (Middle East respiratory syndrome occurring in 2012) and the newly identified SARS-CoV-2 belonging to β-CoV have caused serious health threat and fatality [5]. The present scenario and available pathophysiology specify that SARS-CoV-2 is highly transmittable and contagious than its progenitor affecting not only the respiratory system but also the gastrointestinal system, central nervous system, kidney, heart and liver leading to multiple organ failure [6]. The SARS-CoV-2 spike S glycoprotein has 72% identical sequence with human SARS with a unique furin-like cleavage site, which is absent in other SARS-like CoVs [7]. The Cryo-EM structural evidence has revealed that SARS-CoV-2 has 10–20 times higher binding affinity to the ACE2 receptor

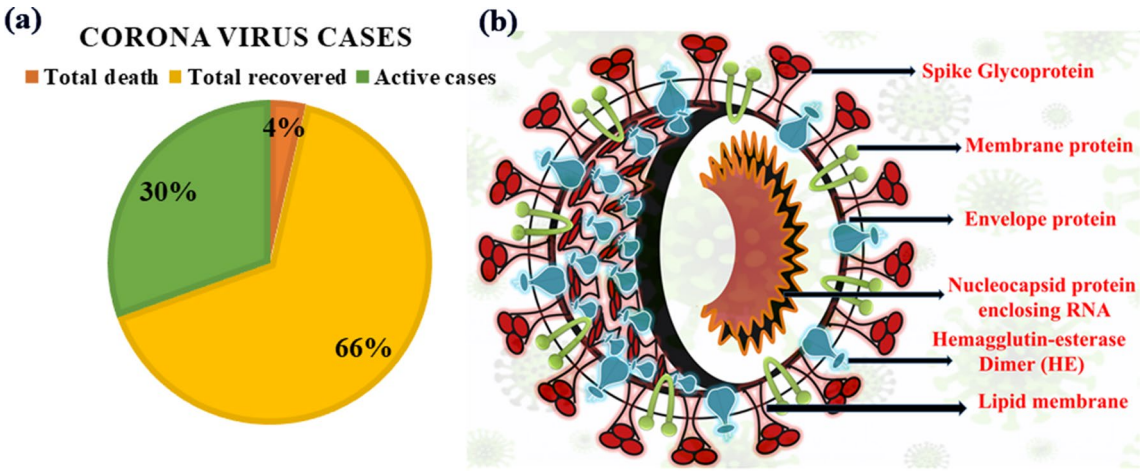


Fig. 1 **a** The global trend of COVID-19 reported death, recovered and active cases till August 13, 2020; **b** structure of SARS-CoV-2

than SARS-CoV which may lead to higher transmission and contagiousness [8]. Therefore, blocking of the isolated viral S protein at its host receptor region and/or binding within the S protein-ACE2 interface are the two most important strategies to design probable drugs for COVID-19 (Fig. 1b) [9]. The SARS-CoV-2 virus replicates via multiple processes after entering into the host cell, and the proteins associated with these replication steps are the principal targets to treat the infected patients by blocking the viral replication. The replication-associated proteins are [10]:

a. Translation of genomic RNA,
b. Proteolysis of the translated polyprotein with viral 3C-like proteinase,
c. Replication of genomic RNA with the viral replication complex which comprises 3′-to-5′ exonuclease, RNA-dependent RNA polymerase (RdRp), endoRNAse and helicase, 2′-*O*-ribose methyltransferase,
d. Assembly of viral components.

Along with the above-mentioned targets, the most commonly employed drug targets and drug discovery strategies employed all over the world right now are illustrated in Fig. 2.

Presently, there is no specific treatment or approved drugs available to treat COVID-19. In most of the active cases, physicians are relying on symptom-based treatment for mild cases and primarily oxygen therapy (if required, with ventilator support) for critically ill patients. A set of approved marketed drugs like hydroxychloroquine (HCQ) [11, 12], chloroquine (CLQ) [12], combination of HCQ and azithromycin [13], remdesivir [14], lopinavir [15] and ritonavir [15] are being evaluated for the infection treatment; their clinical trials are also going on in different pharmaceutical industries. A series of new vaccines are also under clinical trial such as mRNA-1273 [16], Ad5-nCoV [17] and ChAdOx1 nCoV19 [18] along with existing Bacillus Calmette–Guerin (BCG) vaccine [19, 20] to check its efficiency in COVID-19. Due to severity and contagious nature of the SARS-CoV-2, researchers are exploring multiple in silico approaches and artificial intelligence [21–26] with the aim of identifying target-specific and potent therapeutic agents to speed up the discovery process. The RCSB protein data bank (PDB) (www.rcsb.org) has already deposited around 110 protein crystal structures associated with SARS-CoV-2 and COVID-19 to allow understanding important structural binding sites which can be explored in rational designing of small molecules.

The current review discusses the most updated and probable drug candidates which are being experimentally used to treat patients in different parts of the world. Also, their possible targets and pharmacological mechanisms of action which might not be clear in many cases and their pathophysiology along with the details about the status of convalescent plasma treatment and ongoing vaccine trials are discussed. We have compiled up-to-date in silico studies providing information related to computational tools, employed protein crystal structure used in the study followed by probable future drug candidates evolved from the repurposed virtual screening (VS) study employing docking, molecular dynamics (MD) and homology modeling. Therefore, the details related to SARS-CoV-2 transmission, protein structures, epidemiology, disease spectrum, diagnosis and testing are not discussed here at all as they have already been discussed in multiple literatures and separate reviews [1, 2, 4–7, 27–29]. The present review is significantly different from the other recently published ones on a similar topic in that it covers and gives emphasis on the in silico modeling studies in

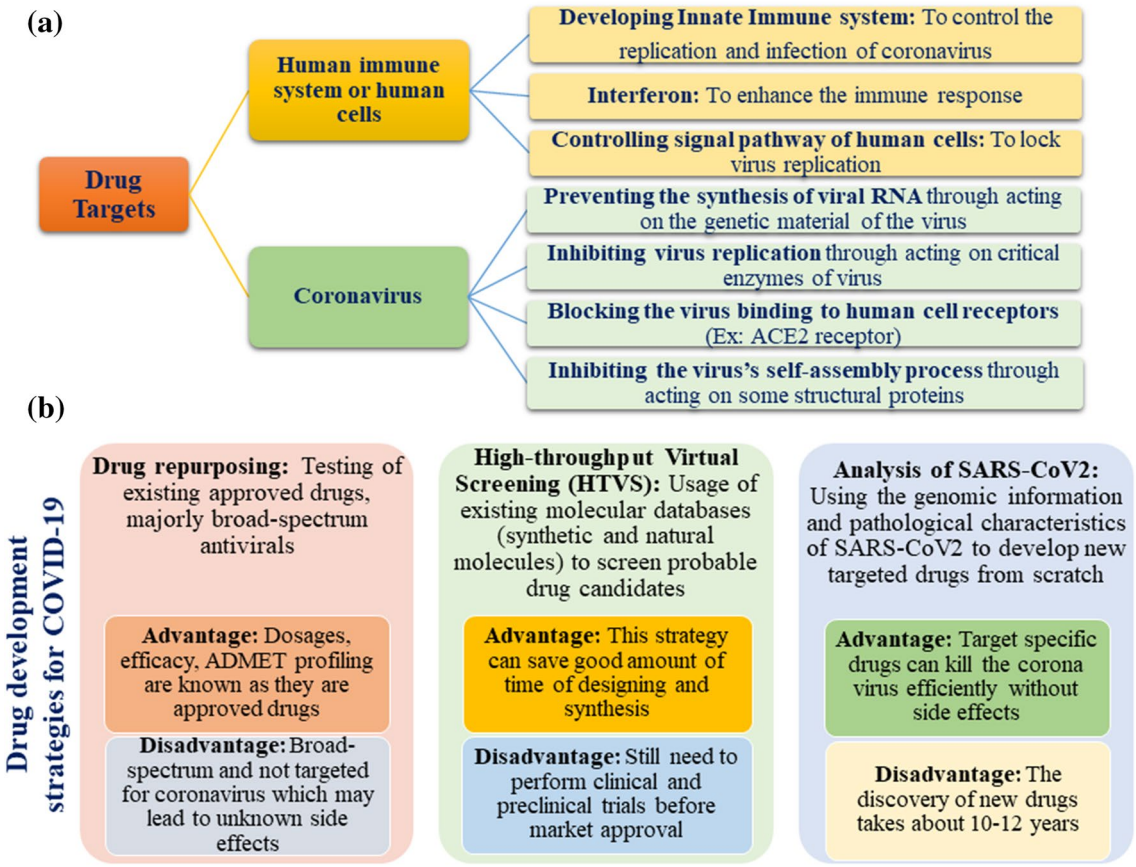


Fig. 2 **a** Possible drug targets and **b** drug development strategies to fight COVID-19

search of drugs against COVID-19. Thus, this paper provides an important source for the knowledge about possible drug candidates and vaccines along with their targets and pathophysiology information.

Investigational drugs or combination of drugs against COVID-19 up-to-date

At the time of writing this article, there are no clinically approved drugs or vaccine available for the treatment of COVID-19 [30]. However, there are many drugs under trial for the treatment of COVID-19 (chemical structures **1**–**19** in Figs. 3, 4, 5) including angiotensin II type I receptor (AT1R) blockers, antiviral drugs, antimalarial drugs, interferon, IL-6 inhibitors, corticosteroids, ascorbic acid, some antibacterial antibiotics, etc. An up-to-date list of drugs under trials against COVID-19 with their targets, mechanisms of action, the developer companies or institutions, uses and recent status are tabulated in Table 1. Several industries and research institute are also trying to develop miscellaneous drugs and/or therapeutics agents, followed by investigation of the effectiveness of combination of drugs listed under Box 1. Among these tabulated drugs, most effective ones are discussed here. Mechanisms of different categories of drugs used in COVID-19 patients on various stages of SARS-CoV-2 life cycle are schematically depicted in Fig. 6.

The entry of SARS-CoV-2 to the host cell can occur in two ways, i.e., either via plasma membrane fusion or via endosomes (endocytosis blockers: CLQ and HCQ) (Fig. 6). In both ways, spike proteins (S1, S2) of SARS-CoV-2 mediate attachment to the membrane of a host cell and engage angiotensin-converting enzyme 2 (ACE2) as the entry receptor. Inhibitors like convalescent plasma, monoclonal antibodies bind to the spike glycoprotein, thus preventing the viral entry. When virions are taken up into endosomes, the spike protein can be activated by the cellular serine protease TMPRSS2 in close proximity to the ACE2 receptor, which initiates fusion of the viral membrane with the plasma membrane. Camostat mesylate inhibits the TMPRSS2 receptor. The plasma membrane fusion entry is less likely to trigger host cell antiviral immunity and therefore more efficient for viral replication. After the viral RNA is released into the host cell, polyproteins are translated. The coronavirus genomic RNA encodes non-structural proteins that have a critical role in the synthesis of viral RNA and structural

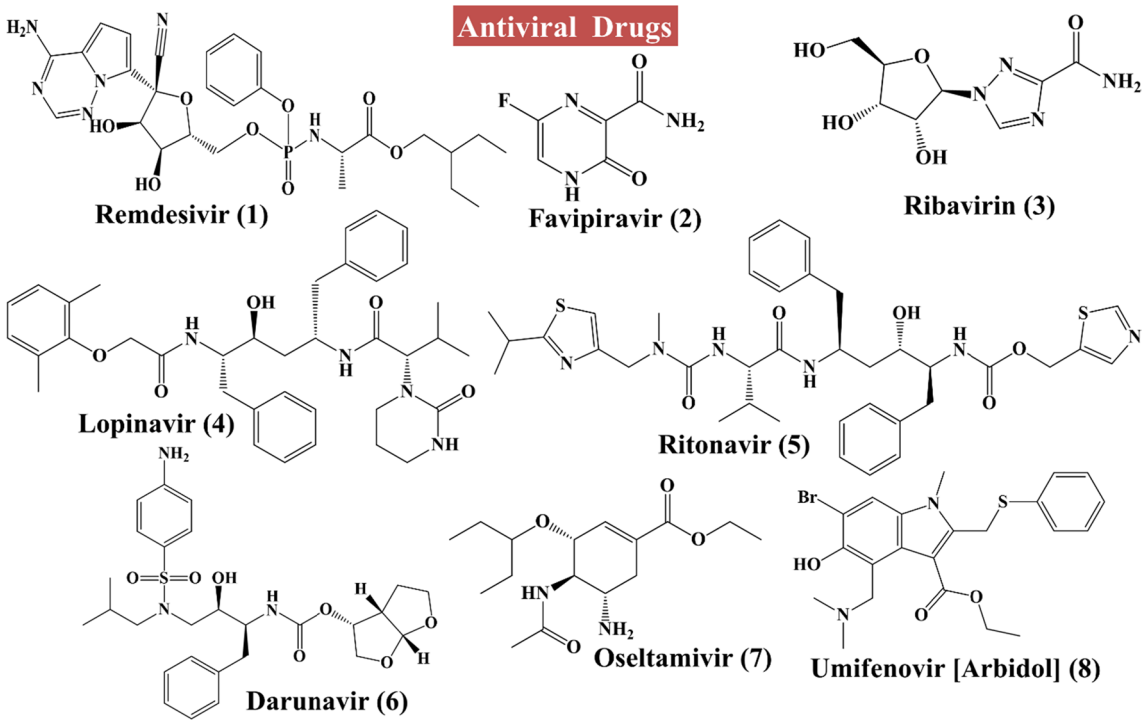


Fig. 3 Structures of antivirals to combat COVID-19 (**Compounds 1–8**)

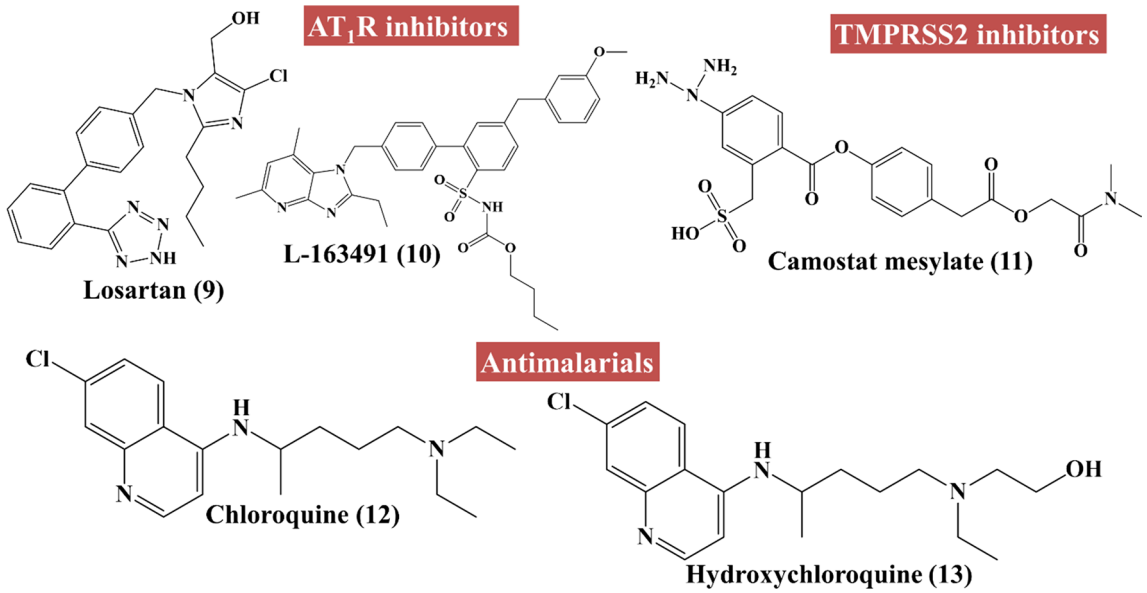


Fig. 4 Structures of angiotensin II type I receptor (AT1R) inhibitors (**Compounds 9–10**), type 2 transmembrane serine protease (TMPRSS2) inhibitors (**Compound 11**) and antimalarials (**Compounds 12–13**) to combat COVID-19

proteins which are important for virion assembly. First, polyproteins are translated and cleaved by some of proteases like $3CL^{pro}$, PL^{pro}, etc. (lopinavir, ritonavir and darunavir act as inhibitors of this step) to form RNA replicase-transcriptase complex. The non-structural protein RdRp is responsible for replication of structural protein RNA. (Remdesivir, favilavir and ribavirin act as inhibitors of this enzyme). Structural proteins S1, S2, envelope and membrane are translated by ribosomes that are bound to the endoplasmic reticulum (ER) and presented on its surface as preparation of virion assembly. The nucleocapsids (N) remain in cytoplasm and are assembled from genomic RNA. They fuse with the virion

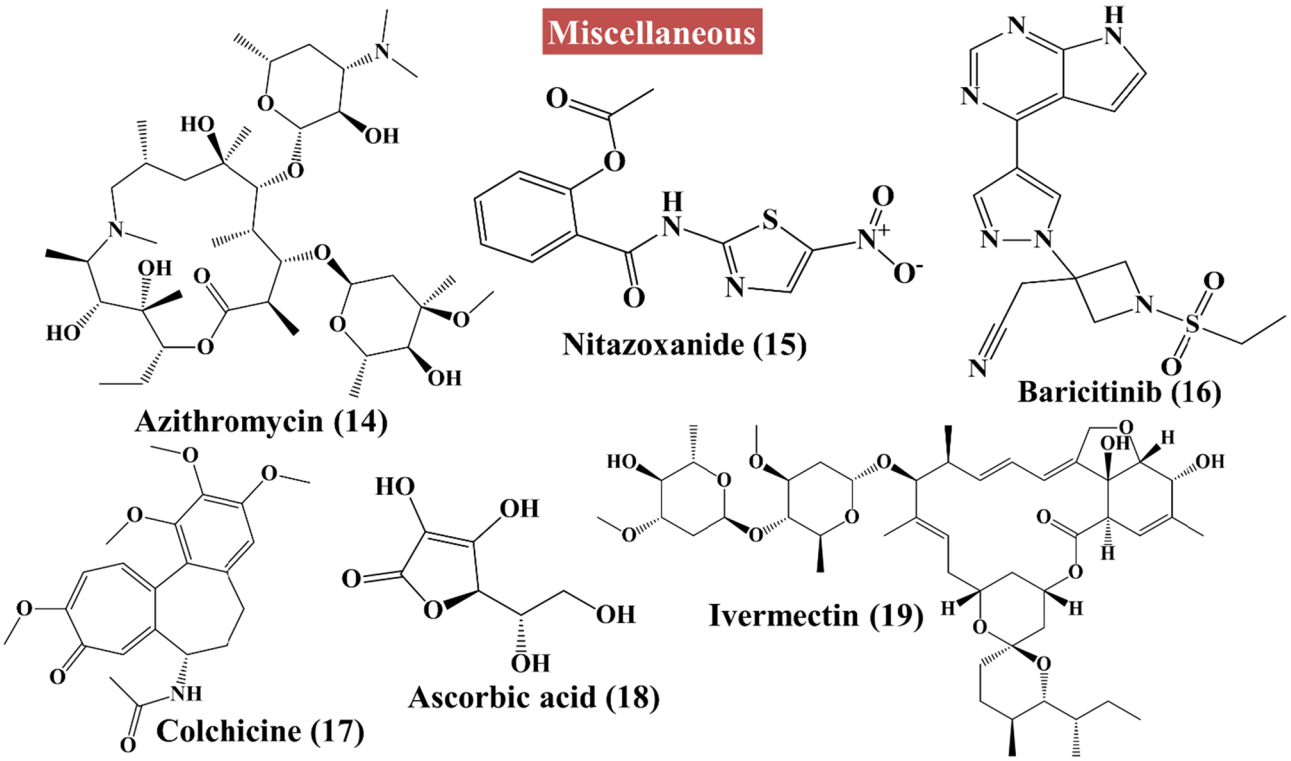


Fig. 5 Structures of miscellaneous drugs to combat COVID-19 (**Compounds 14–19**)

precursor which is then transported from the ER through the Golgi apparatus to the cell surface via small vesicles. The mature virions are then released from the infected cell through exocytosis, and then, they search another host cell (Fig. 6).

Angiotensin II type I receptor (AT1R) blockers

SARS-CoV-2 has transmembrane spikes (S protein). The spikes attached to the lipid membrane of the coronavirus recognize a host cell to attach and infect it with its viral RNA. The attachment of the coronavirus S protein to angiotensin converting enzyme 2 (ACE2) at its cellular binding site promotes the entry into human cells. The S protein contains two subunits such as an N-terminal S1 subunit which is responsible for receptor-virus binding and a C-terminal S2 subunit which is responsible for the fusion of virus with cell membrane [31, 32]. The S1 subunit has two domains such as a receptor-binding domain (RBD) and an N-terminal domain (NTD). At the time of infection, at first coronavirus binds to the human cell through interaction between the cell ACE2 receptor and S1-RBD of coronavirus. As a result, conformational changes in the S2 subunit are triggered followed by virus-cell fusion and entry into the target cell. ACE2 is a natural protein present in the lungs and the intestine (epithelial cells), and in the heart and the kidneys (endothelial cells). ACE2 regulates the blood pressure by converting angiotensin molecules. It was found that the coronavirus which caused the SARS outbreak in 2002 also binds to the same ACE2 molecule, but in case of SARS-CoV-2, the binding affinity is 10 to 20 times more on human cells than the spike from the SARS virus of 2002, which makes it a suitable target for COVID-19. Due to the high affinity of this virus to human cells, it is spread without any difficulty from one person to another than the earlier virus [33].

The entry of SARS-CoV-2 to the host cells via binding with ACE2 enzyme leads to ACE2 down-regulation. As a result, angiotensin II is produced excessively by the correlated enzyme ACE1, while a lower amount of ACE2 is not capable of transforming it to the vasodilator heptapeptide angiotensin 1–7. Thus, expression of higher ACE2 resulting from frequently medicating COVID-19 patients with AT1R blockers may resist them against acute lung injury. This can be described by following two complementary mechanisms: (1) blocking the excessive angiotensin II-mediated AT1R activation caused by the viral infection, (2) upregulation of ACE2 decreasing angiotensin II production by ACE and enhancing the production of the vasodilator angiotensin 1–7 [34]. The role of ACE2 to enter the coronavirus into the host cell and mechanism of action of ACE2 inhibitors to control the COVID-19 are depicted in Fig. 7.

Table 1 Drug candidates under trial against COVID-19 with probable targets, mechanism of action, pathophysiology, application and current status [11, 12, 14, 30, 40, 43, 45, 53, 55, 57–88]

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
Remdesivir [14, 57–59] (antiviral: nucleoside analog)	Gilead Sciences	RdRp	(1) Inhibit the RdRps (2) Premature termination of viral RNA transcription	(1) Broad-spectrum antiviral (2) In vitro activity against coronaviruses (under trial) (3) Reduces pulmonary pathology (4) Tried in Ebola virus too	Phase-III clinical trials are underway in both China and the USA
Favipiravir [favilavir or Avigan] [60, 61] (antiviral)	Fujifilm Toyama Chemical	RdRp	(1) Purine nucleoside leading to erroneous viral RNA synthesis (2) Inhibiting the RdRp	Viral infection	Yet to be approved by the US FDA for COVID-19
Ribavirin [58, 62, 63] (antiviral: guanine derivative)		RdRp	Inhibit RNA polymerization	(1) RSV infection, hepatitis C, some viral hemorrhagic fevers (2) In case of COVID-19, this drug is tried in combination with IFN-α or lopinavir/ritonavir	Performed two trials: NCT04254874, 2/5/20; ChiCTR2000029308, 1/23/20)
Lopinavir/ritonavir [Kaletra] [45, 57, 64] (antiviral: HIV protease inhibitor)	AbbVie	(1) Coronavirus main protease $3CL^{pro}$ (2) Papain-like protease PL^{pro}	(1) Inhibits $3CL^{pro}$ and PL^{pro} (viral protease). Bind to M^{pro}, a key enzyme for coronavirus replication	(1) Approved drug for HIV infection (2) WHO has mentioned it as an agent that can be tried for COVID-19 (3) It can also be used in combination with Interferon alpha or Ribavirin (4) Powerful CYP3A4 inhibitor (monitor for drug interactions)	(1) At least three trials are going on (e.g., ChiCTR2000029603, 2/6/20) (2) European Society of Intensive Care Medicine (ESICM) and Critical Care Medicine (SCCM) Surviving Sepsis Campaign recommended the routine use of lopinavir; ritonavir in critically ill adults
Darunavir/darunavir + cobicistat [Prezcobix] [65] (antiviral)	Shanghai public health clinical center	HIV-1 Protease	(1) HIV-1 Protease inhibitor (2) CYP3A inhibitor	(1) HIV infection (2) Studied as a possible treatment for SARS-CoV-2	Clinical trials are underway
Oseltamivir [66] (antiviral)		Neuraminidase enzyme	Inhibits neuraminidase enzyme in influenza	Used in influenza patient. This drug is used for repurposing only in combination with other drugs like ASC09F and ritonavir	No trials on COVID-19 yet. Due to lack of suitable control group in the studies, definite evidence of efficacy is questionable
Umifenovir [Arbidol] [38, 39] (antiviral)	Pharmstandard	(1) ACE 2 (2) Viral spike glycoprotein	(1) Interrupts the attachment of viral envelope protein to host cells thus prevents viral entry to the target cell (2) Inhibits viral-host cell fusion	(1) An antiviral treatment for influenza infection used in Russia and China (2) Proposed reduction of cytokine storm	At present, there are no clinical data to support either starting or discontinuing ACEi/ARBs on any patients with COVID-19

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
L-163491 [67]		Angiotensin AT1/AT2 receptor	Partial antagonist of AT1 receptor and partial agonist of AT2 receptor	To treat coronavirus-induced lung injury	Yet to be determined
Losartan [34]	University of Minnesota	Angiotensin II type 1 receptor (AT1R)	Inhibit AT1 receptor	Reduce organ failure	Phase-I clinical trials.
Camostat mesylate [40]		Serine protease TMPRSS2	(1) Inhibit TMPRSS2 (2) Able to block SARS-CoV-2 entry to the host cells	Treats SARS-CoV-2 infection of lung cells	A clinically approved TMPRSS2 inhibitor, was able to block SARS-CoV-2 infection of lung cells
Chloroquine [Aralen] [11, 12, 14, 43, 45] (antimalarial)	Sanofi (Aralen)	Endosome/ACE2	(1) Inhibits viral DNA and RNA polymerase, viral protein glycosylation, virus assembly, new virus particle transport and virus release (2) Inhibits fusion of the virus to the receptor, thus prevent the entry into the host cell (3) Also acts by preventing the sialic acid containing glycoprotein and gangliosides (key binding factors along the respiratory tract) intermediated attachment to the S protein	(1) CLQ has been proven effective in treating coronavirus in China (2) Treating pneumonia patients with SARS-CoV-2 infection	Approved for clinical trial against COVID-19

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
Hydroxychloroquine [Plaquenil] [11, 12, 44] (antimalarial)	Sanofi (Plaquenil); Mylan, Teva, Novartis, Bayer, Rising Pharmaceuticals (generics)	Endosome/ACE2	(1) Hampers viral replication (2) Inhibits viral DNA and RNA polymerase, Viral protein glycosylation, virus assembly, new virus particle transport, and virus release (3) May also involve ACE2 cellular receptor inhibition (4) Acts by inhibiting fusion of the virus to the host cells thus prevents the entry to the host cells (5) Immunomodulation of cytokine release (6) Also acts by inhibiting the sialic acid containing glycoprotein and gangliosides (key binding factors along the respiratory tract) that intermediate binding to the S protein	Used to treat COVID-19 disease. This drug has also immunomodulating properties	At present, one of the most highlighted drug against COVID-19
Baricitinib [68]	Concert Pharmaceuticals, Inc., USA	Janus kinase inhibitor (JAK)	Affects the inflammatory processes	(1) Approved drug for rheumatoid arthritis (2) Used to treat acute respiratory disease in COVID-19 patients	Eli Lilly has announced plans to conduct a clinical trial of baricitinib (Olumiant)
Azithromycin [13] (Macrolide antibacterial)	Pfizer	50S subunit of the bacterial ribosome	(1) Their direct effects on viral clearance are not clear (2) Downregulates inflammatory responses and decreases the excessive cytokine release related with respiratory viral infections (3) Immunomodulatory properties in pulmonary inflammatory disorders	(1) Prevents bacterial superinfection (2) Used as adjunct therapy due to immunomodulatory properties	Combination with HCQ showed promising activity against COVID-19 Pfizer has announced positive clinical trial data performed in France for this drug along with HCQ
Nitazoxanide [58]	Materno-Perinatal Hospital of the State of Mexico	Pyruvate:ferredoxin oxidoreductase (PFOR) enzyme	Inhibits viral protein expression	Used to treat various infections caused by helminth, protozoa and virus	Phase-IV clinical trial (NCT04341493) is going on using Nitazoxanide and combination of Nitazoxanide and HCQ

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
Tocilizumab [atlizumab] [69, 70] (immunosuppressant drug)	Roche (as Actemra)	Interleukin-6 (IL-6) receptor	Acts by inhibiting IL-6-mediated signaling	Used to treat severe disease in COVID-19 patients Initial analysis suggests that tocilizumab may have a clinical advantage as an adjunctive therapy	Roche launched a Phase-III trial (COVACTA) to assess tocilizumab's efficiency
Ivermectin [53] (antiparasitic agent)	Biomedicine Discovery Institute, Australia	HIV-1 integrase protein (IN) and the importin (IMP) α/β1 heterodimer	Inhibits nuclear transport	Broad-spectrum antiviral	Phase-III clinical trial in dengue patients Need to design further study to check the worthiness in SARS-CoV-2 treatment
Mavrilimumab [71, 72] (monoclonal antibody)	Kiniksa Pharmaceuticals	Granulocyte macrophage colony stimulating factor (GM-CSF) receptor alpha	Showed antagonistic activity of GM-CSF signaling by binding to the α-subunit of the GM-CSF receptor (GM-CSFRα)	Kiniksa reported that as of 31st March 2020 the patients treated with this drug experienced early resolution of fever and enhanced oxygenation within 1–3 days and also reported that none of the patients required mechanical ventilation as of that time	A consortium of US academic sites plans to initiate parallel prospective, interventional studies with mavrilimumab in patients with pneumonia and hyper-inflammation caused by SARS-Cov-2. Kiniksa also reported that they are engaging with FDA about the path forward for potential
Lenzilumab [73, 74] (monoclonal antibody)	Humanigen	GM-CSF	This drug neutralizes GM-CSF which is a crucial cytokine in the initiation of cytokine storm	Have a defensive consequence against cytokine release syndrome (CRS) associated with CAR-T therapy. It can aid cytokine-mediated immunopathology of lung injury and acute respiratory distress syndrome (ARDS)	On 2 April 2020, FDA authorized the use of lenzilumab in COVID-19 patients under an eIND application. Humanigen is planning a Phase-III clinical trial with lenzilumab for the prevention of ARDS in patients with pneumonia caused by SARS-CoV-2
Leronlimab [75] (monoclonal antibody)	CytoDyn (as PRO 140)	Humanized IgG4 monoclonal antibody	Blocks the CCR5 co-receptor on the surface of immune cells like CD4 cells	(1) Leronlimab in combination with carboplatin can be used for the treatment of CCR5-positive metastatic triple-negative breast cancer. It is also used in antiretroviral therapy (HAART) in HIV (2) This drug can increase the immune response in patients with CRS from respiratory distress	FDA authorized the use of leronlimab in COVID-19 patients under an eIND. It has been showed that patients treated under the eIND have a lower level of cytokine storm and lower levels of IL-6 and TNF-alpha

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
Gimsilumab [76] monoclonal antibody	Roivant	GM-CSF	Inhibits GM-CSF	Used as anti-inflammatory drug	Phase-I clinical trial
Sarilumab [77] (anti-rheumatic drug) (monoclonal antibody)	Sanofi and Regeneron (as Kevzara)	IL-6 receptor	IL-6 receptor antagonist	Used to reduce the inflammatory response in the lungs associated with the COVID-19 patients who develop ARDS	A Phase-II/III trial (NCT04327388) of 400 patients sponsored by Sanofi. Regeneron is currently underway in the USA. A second, Phase-II/III trial is being conducted in Italy, Spain, Germany, France, Canada and Russia also
Aviptadil [78] (analog of vasoactive intestinal polypeptide)	NeuroRx and Relief Therapeutics	IL-6	(1) IL-6 inhibitor (2) Reduction of inflammatory cytokines	(1) Used for the treatment of inflammation produced by cytokines (2) Used in ARDS (3) Erectile dysfunction	Phase-II clinical trials
Siltuximab [Sylvant] [79] (monoclonal antibody)	EUSA Pharma	IL-6	IL-6 inhibitor	To treat Acute Respiratory Distress Syndrome as a result of Covid-19	EUSA Pharma initiates the study of siltuximab to treat Covid-19 patients Based on the clinical data, the company reported that 16 patients using siltuximab were stable or had improved disease at the interim analysis
Camrelizumab [AiRuiKa] [80] (monoclonal antibody)	South East University, China	Programmed cell death 1 (PD-1)	Programmed cell death 1 (PD-1) inhibitor	(1) Classical Hodgkin lymphoma (2) Treat pneumonia (3) Sepsis	Phase-II clinical trials (NCT04268537)
Eculizumab [Soliris] [81] (monoclonal antibody)	Alexion	Binds terminal complement protein C5	Modulates the activity of the distal complement preventing the formation of the membrane attack complex	(1) In severe Pneumonia (2) In ARDS	Plan for Phase-II clinical trials
Bevacizumab [Avastin] [82]	Qilu Hospital of Shandong University	Vascular endothelial growth factor (VEGF)	Inhibit vascular endothelial growth factor (VEGF)	May be used as a promising drug for acute lung injury (ALI) and/or ARDS in COVID-19 through suppression of pulmonary edema	Under clinical trial (NCT04275414) Bevacizumab, an anti-VEGF drug, approved by the FDA on February 26, 2004 and widely used in clinical oncotherapy

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
CD24Fc [83]	OncoImmune	Recombinant fusion protein	(1) It targets a novel immune pathway checkpoint and modulates immune response through binding to Danger-Associated Molecular Patterns (DAMPS) and sialic acid-binding immunoglobulin-type lectins (Siglecs) (2) It also acts by reducing multiple inflammatory cytokines	Used for the treatment of graft-versus-host disease (GVHD) in leukemia patients receiving hematopoietic stem cell transplantation	OncoImmune is planning a Phase-III clinical trial against COVID-19 patients with absolute lymphocyte counts ≤ 800/mm in peripheral blood (NCT04317040)
Colchicine [84]	Montreal Heart Institute	Tubulin	Tubulin disruption	Used as anti-inflammatory agents	Phase-III clinical trials for CIVID-19
SNG001 [85]	Synairgen Plc	Antiviral protein interferon-beta 1a (IFN-β), a natural antiviral produced in lungs during viral lung infections	Delivers extra IFN-β direct to the lungs, correcting this deficiency and counteracting viral strategies to evade the host's immune defenses by inhibiting natural IFN-β production	Used to treat severe lower respiratory tract illness caused by cold and flu infections when they spread to the lungs	Phase-II clinical trials
COVID-19 convalescent plasma [55] (Immunoglobulin)	Mount Sinai	Passive antibody therapy; possible sources of antibody for SARS-CoV-2 are human convalescent sera	Plasma collected from recovered COVID-19 patients that may contain antibodies to SARS-CoV-2	In China, a case series of 5 patients suffering from SARS-CoV-2 and ARDS treated with convalescent plasma showed better clinical status	Clinical trials are going on to assess the use of COVID-19 convalescent plasma to treat patients with COVID-19 infections
Tissue plasminogen activator (tPA) [86] [alteplase] (anti-clotting drug)	Researchers at Beth Israel Deaconess Medical Center (BIDMC)	Serine protease	It catalyzes the conversion of plasminogen to plasmin	Used to treat COVID-19 induced ARDS. Used for heart attacks or stroke	Under clinical trial. A recent report suggested that the use of this drug could reduce deaths among the COVID-19 patients with ARDS
Corticosteroids [87]	Beijing Chao Yang Hospital, China	Cytokines	Modulating a variety of cytokines involved in the inflammatory response	May be used to treat ARDS	Not indicated in treating SARS-CoV-2 as per available evidence. Might prolong viral shedding

Table 1 (continued)

Drug candidates under trial	Company	Targets	Mechanism	Application	Current status against COVID-19
Ascorbic acid [88]	University of Palermo	T-lymphocytes	(1) Recent studies showed that ascorbic acid (vitamin C) clearly affects the development and maturation of T-lymphocytes, natural Killer cells (NK cells) involved in the immune response to viral agents. It also contributes to the remodulation of cytokine network responsible for systemic inflammatory syndrome by inhibiting ROS production (2) It blocks the expression of intercellular adhesion molecule 1 (ICAM-1) and activation of NF Kappa B that are involved in inflammatory, neoplastic, and apoptotic processes by the inhibition of TNF alpha	Effective against COVID-19 pneumonia	Under clinical trials (NCT04323514)

Box 1 Miscellaneous therapeutics under investigation for COVID-19

TAK-888 (antibodies from recovered COVID-19 patients developed by Takeda; preclinical stage), **REGN3048-3051** (antibodies from mice developed by Regeneron; Phase-I preclinical stage), **Thymosin** (PD-1 blocking antibody developed by Southeast University, China, which is under Phase-II clinical trial), **antibodies from recovered COVID-19 patients** (companies like Celltrion, Kamada, Vir Biotech/WuXi Biologics/Biogen, Lilly/Ab-Cellera, Swiftscale Biologics, Erasmus MC/Utrecht University, and AstraZeneca are trying to develop), **Galidesivir** (developed by BioCryst Pharmaceuticals; preclinical stage), **Combination of ebastine, lopinavir and interferon alpha** (developed by Wuhan Red Cross Hospital; clinical stage), **Combination of Ganovo and danoprevir** (hepatitis C virus NS3 protease inhibitor), **Ritonavir and interferon** (approved in China to treat hepatitis C, developed by Ascletis; clinical stage), **ASC09** (HIV protease inhibitor developed by Ascletis Pharma; clinical stage), **Truvada combination of Emtricitabine and Tenofovir** (both are HIV-1 nucleoside analog reverse transcriptase inhibitors developed by Gilead/Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital; clinical stage), **Xofluza** (polymerase acidic endonuclease inhibitor developed by Roche; clinical stage), **Azvudine** (reverse transcriptase inhibitor developed by Henan Provincial People's Hospital; clinical stage), **Washed microbiota transplantation** (The Second Hospital of Nanjing Medical University; clinical stage), **Jakafi/Jakavi (Ruxolitinib in combination with mesenchymal stem cells)** (Department of Hematology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology/Incyte Corp; clinical stage), **Peginterferon alfa-2b** (PegIntron, Sylatron, IntronA; clinical stage), **Novaferon** (Zhejiang University Medical School), **Interferon** (Zhejiang University Medical School), **Ifenprodil (NP-120)** (an NDMA receptor glutamate receptor antagonist targeting Glu2NB developed by Algernon Pharmaceuticals: preclinical stage), **APN01** (a physiological formulation of recombinant soluble human ACE2 developed by University of British Columbia/Apeiron Biologics; clinical stage), **Brilacidin** (a defensin mimetic developed by Innovation Pharmaceuticals; preclinical stage), **BXT-25** (a glycoprotein developed by Bioxytran; preclinical stage), **Peptides** (developed by CEL-SCI; preclinical stage), **Gilenya (fingolimod)** (developed by Fujian Medical University/Novartis; clinical stage), **A number of synthesized nanoviricide drug candidates** (developed by different industries like Novartis; preclinical stage), **Scanning compounds to repurpose** (Janssen Pharmaceutical Companies, Novartis, Merck, Pfizer, Materia Medica/Cyclica, Enanta Pharmaceuticals, Southwest Research Institute, Takeda), **RNA-based treatment like RNAi–testing 150 RNAis** (Sirnaomics; preclinical), **siRNA candidates** (Vir Biotech/Alnylam Pharmaceuticals; preclinical stage), **Ampligen** (AIM ImmunoTech/National Institute of Infectious Diseases in Japan; preclinical stage), **OT-101** (a TGF-Beta antisense drug candidate developed by Mateon Therapeutics; preclinical stage), **Cell-based therapies like PLX cell product** (placenta-based cell therapy developed by Pluristem Therapeutics/BIH Center for Regenerative Therapy/Berlin Center for Advanced Therapies; preclinical stage), **Mesenchymal stem cells** (numerous trials with Chinese research sponsors; clinical stage), **Ryoncil (Remestemcel-L)** (allogenic mesenchymal stem cells developed by Mesoblast; preclinical stage). **Dexamethasone (Dextenza, Ozurdex, others)** (University of Oxford; Phase-II/III)

Under parenthesis companies and research institutes along with stage of development are mentioned

The SARS-CoV-2 uses the S protein to facilitate viral entry into the host cells. The pathogen S protein consists of two subunits S1 and S2, of which "S1" allows entry of pathogen and binding of S protein to ACE2 (cellular receptor) (Fig. 7). In addition, the entry requires S protein priming by cellular proteases, which is responsible for cleavage of S protein. After this, "S2" subunit employs fusion of viral and cellular membranes. SARS-CoV-2 engages ACE2 as the entry receptor that can be blocked by ACE2 inhibitors and Arbidol, and it employs the cellular serine protease TMPRSS2 for S protein priming which is inhibited by camostat mesylate (Fig. 7). Conclusively, SARS-CoV-2/ACE2 interface occurs in the molecular level, and the efficiency of ACE2 usage is a key determinant of SARS-CoV-2 transmissibility.

Although it is widely accepted that coronavirus enters into the host cell through the ACE2 receptor, due to limited number of studies, it is yet to establish how ACE2, AT1 and AT2 receptors exert their activities in coronavirus-induced diseases [35, 36]. Thus, ACE2 inhibitors and AT1 receptor antagonists [e.g., L-163491 as a partial antagonist of AT1 receptor and partial agonists of AT2 receptor; losartan, valsartan, irbesartan, candesartan cilexetil, telmisartan, and eprosartan (FDA-approved AT1 receptor blockers)] may be used as important drug candidates to control lung injury of COVID-19 patients [34]. The binding of viral S protein with its receptor ACE2 on host cells followed by viral endocytosis into the cells may also be a possible drug target. For example, the broad-spectrum antiviral drug Arbidol recently entered the clinical trial for the treatment of SARS-CoV-2 which may act by inhibiting virus-host cell fusion, thus preventing the viral entry into host cells against influenza virus [37–39].

Camostat mesylate

The serine protease TMPRSS2 produced by the host cells plays a key role for cell entry of coronaviruses by S protein priming to the receptor ACE2 binding in human cells (Fig. 7). A recent study shows that camostat mesylate, a clinically approved inhibitor of TMPRSS2 (responsible for S protein priming), has been able to block SARS-CoV-2 infection of lung cells. Thus, this drug may be a potential drug candidate for COVID-19 [40].

Remdesivir

Remdesivir, a nucleoside analog and a monophosphoramidate prodrug of remdesivir-triphosphate (RDV-TP) developed by Gilead Sciences Inc. (USA), was previously

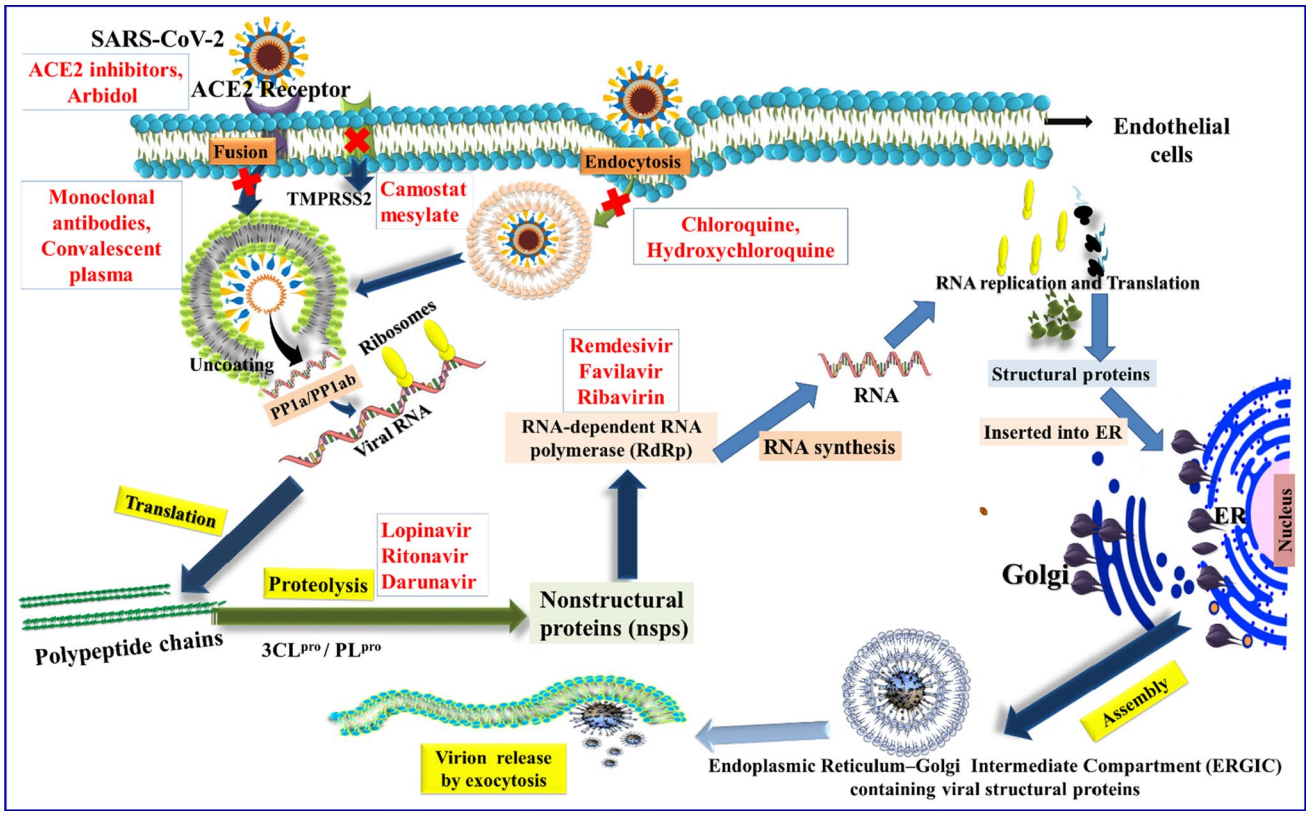


Fig. 6 Mechanisms of action of different categories of drugs used in COVID-19 patients acting on various stages of the SARS-CoV-2 life cycle

tried for the Ebola virus disease, and it showed promising effects in MERS and SARS. It acts by inhibiting RNA-dependent RNA polymerases (RdRp). Incorporation of this drug into nascent viral RNA chain causes premature termination. For incorporation of remdesivir-TP into nascent viral RNA chains, it competes with adenosine-triphosphate. After incorporation into the viral RNA at position *i*, remdesivir-triphosphate arrests RNA synthesis at position $i+3$. Due to the incorporation of 3 additional nucleotides after RDV-TP, it does not cause instant chain termination because these three additional nucleotides may protect the inhibitor from excision by the viral 3′–5′ exoribonuclease activity (Fig. 6). Recent reports showed that the EC_{90} value of remdesivir against COVID-19 in VeroE6 cells was 1.76 µM, half-cytotoxic concentration (CC_{50}) was greater than 100 µM, and the selective index (SI) was greater than 129.87, suggesting that its working concentrations are likely to be achieved in nonhuman primate (NHP) [14, 41]. This drug is also able to inhibit virus infection proficiently in human liver cancer Huh-7 cells sensitive to COVID-19. A recent case study revealed that treatment with remdesivir improved the clinical condition of the first patient infected by SARS-CoV-2 in the USA [42]. A recent in vitro data showed that remdesivir and chloroquine (CQ) phosphate are capable of inhibiting SARS-CoV-2 infection [14]. Remdesivir is currently being studied in Phase-III clinical trials against SARS-CoV-2 in Wuhan, China, as on February 4, 2020, and in the USA.

Chloroquine/hydroxychloroquine

Chloroquine (CLQ) and hydroxychloroquine (HCQ) have received deep attention because of positive results from some small studies. An antimalarial drug, CLQ, has recently been reported to have potential in vitro activity against SARS-CoV-2. CLQ protects from viral infection by enhancing endosomal pH (making the environment unfavorable) which is required for virus-cell fusion. This drug may also block viral infection by inhibiting viral enzymes or processes like viral DNA and RNA polymerases, virus assembly, new virus particle transport, immunomodulation of cytokine release and virus release. CLQ also affects the glycosylation process of ACE2 (as discussed earlier that to enter the host cell, the viral S protein binds with this receptor) [11, 14, 43, 44]. Besides this mechanism, a recent report [12] showed that this drug also acts by inhibiting the sialic acid containing glycoprotein and gangliosides (act as primary attachment factors along the respiratory tract) mediated attachment to the S protein which is the first step for viral replication. In the NTD of the S protein of SARS-CoV-2, a

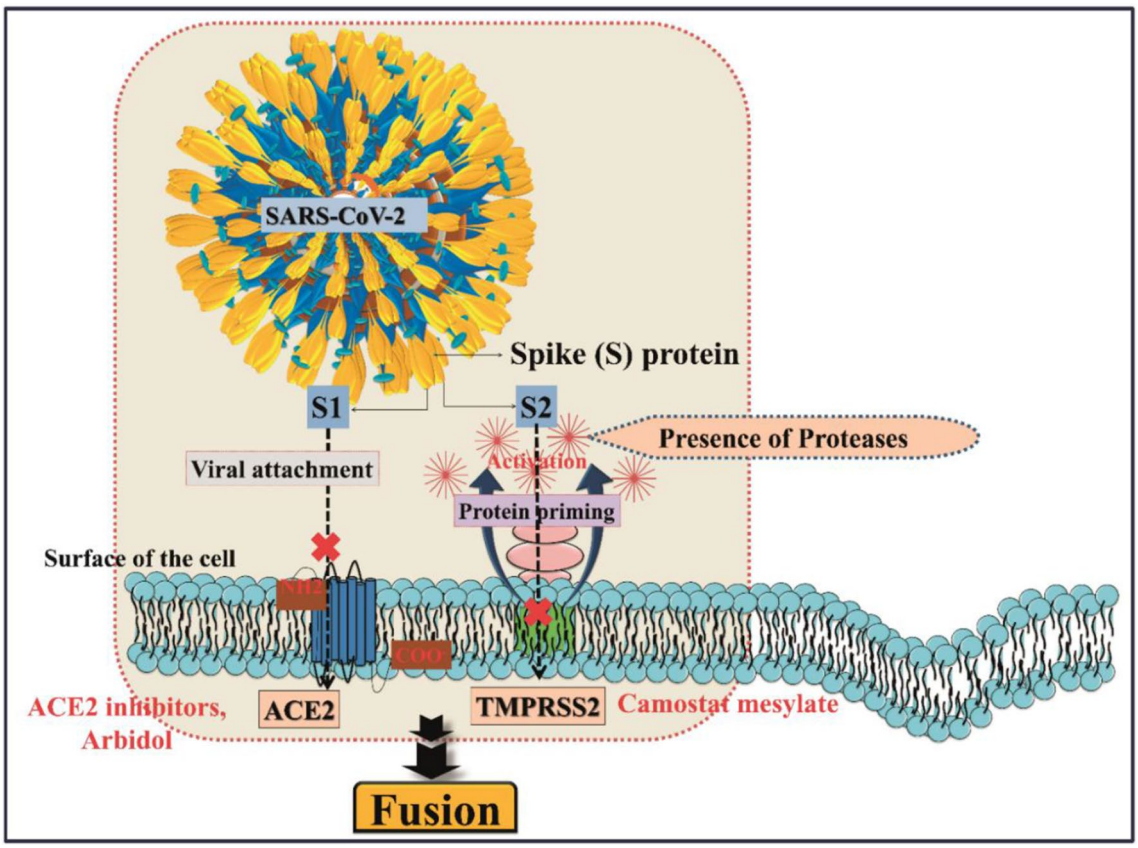


Fig. 7 The role of ACE2 receptor for the entry of coronavirus into the host cell, and mechanism of action of ACE2 inhibitors and TMPRSS2 inhibitors to control COVID-19

ganglioside-binding site was recognized. The antimalarial drug CLQ was found to be a probable blocker of the S–ganglioside interaction. Thus, this drug may be used to fight pathogenic coronaviruses especially SARS-CoV-2 which is responsible for COVID-19. A detailed mechanism of action of CLQ and HCQ against SARS-CoV-2 is illustrated in Fig. 8. A recent report showed that the EC_{90} value of CLQ against the SARS-CoV-2 in VeroE6 cells was 6.9 µM, which may be clinically achievable. Although specific data are not available, this drug is able to inhibit the exacerbation of pneumonia patients with SARS-CoV-2 infection.

Figure 8 explains two possible mechanisms of CLQ and HCQ against SARS-CoV-2. The mechanism 1 is that CLQ and its derivative HCQ are weak bases, which can raise the pH of acidic intracellular organelles, such as endosomes/lysosomes, essential for membrane fusion. On the other hand, mechanism-2 explains the entry of SARS-CoV-2 into the host cells also depends upon sialic acid (Neu5Ac) containing glycoproteins and gangliosides that act as the key binding factors along the respiratory tract. A ganglioside-binding site at the N-terminal domain (NTD) of the S glycoprotein of SARS-CoV-2 was recognized, and CLQ was found to be a possible blocker of the S–ganglioside interaction which occurs in the first step of the viral replication cycle (i.e., attachment to the surface of respiratory cells, intermediated by the S protein) [12]. The interaction was augmented by placing the negative charge of the carboxylate anion of Neu5Ac and one of the two positive charges of CLQ. SARS-CoV-2 especially interacted with 9-*O*-acetyl-*N*-acetylneuraminic acid (9-*O*-SIA). In this case, the carboxylic acid group of the sialic acid interacted with the cationic group of the nitrogen-containing ring of CLQ. The formed complex of CLQ and 9-*O*-SIA was further stabilized by OH-π and van der Waals interactions. Next, the complex developed from HCQ was very close to that obtained from CLQ, although numerous conformational adjustments happened for the period of the simulations. Interestingly, the –OH group of HCQ reinforced the binding of CLQ to Neu5Ac via formation of a hydrogen bond. The formed complex of CLQ-OH and 9-*O*-SIA will be stabilized again like CLQ to form a protective layer against fusion of the SARS–CoV-2.

HCQ is a hydroxy derivative of CLQ, which can block the viral infection by a similar mechanism as chloroquine; thus, this drug may also be a potential candidate against SARS-CoV-2. This drug is less toxic (~40%) than

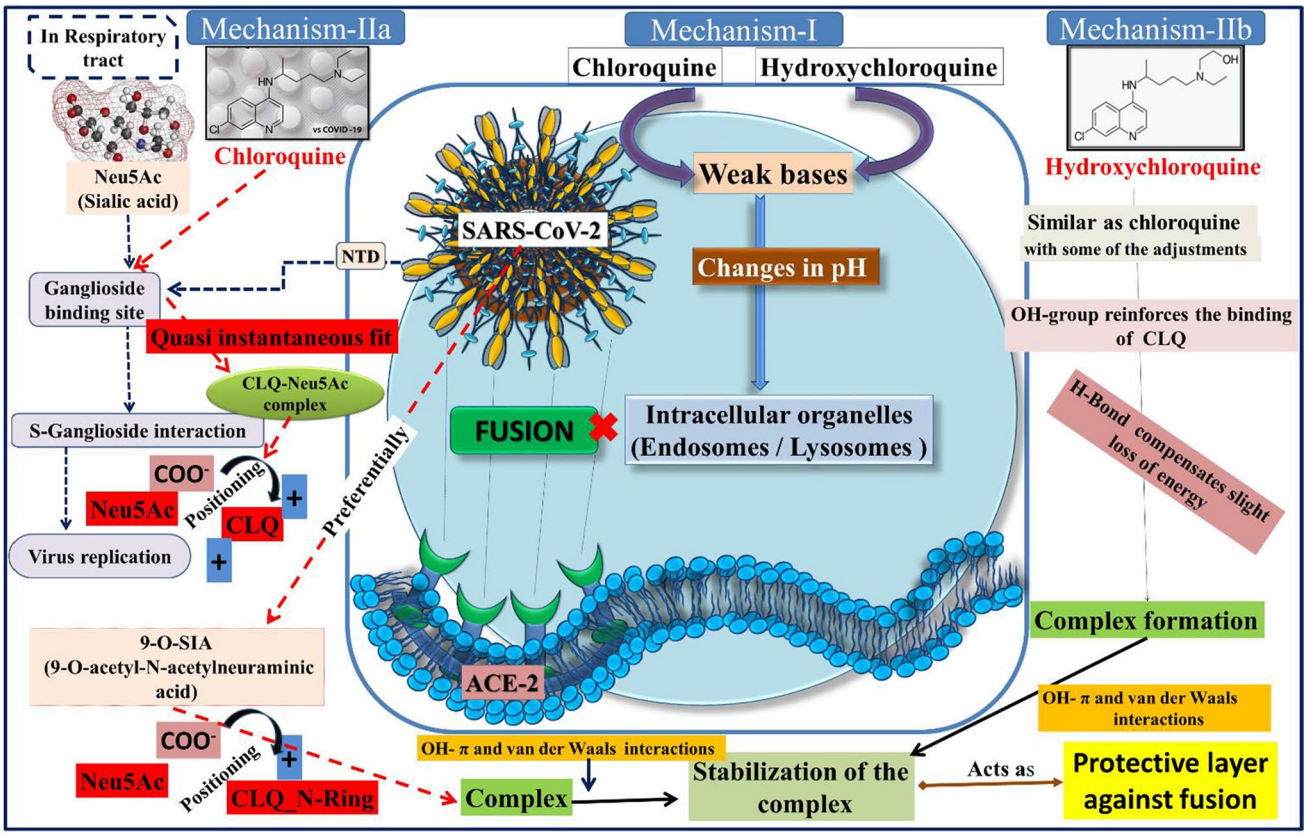


Fig. 8 Schematic representation of different mechanistic pathways of CLQ and HCQ against SARS-CoV-2

chloroquine in animals. It was found that there were seven clinical trials registered as on February 23, 2020, in the Chinese Clinical Trial Registry (http://www.chictr.org.cn), for using HCQ to treat COVID-19. The in vitro results suggested that this drug can efficiently inhibit SARS-CoV-2 infection. Based on an in vitro report, it was suggested that HCQ may be more potent than CLQ to treat COVID-19 [11, 44]. (EC_{50} values for CLQ > 100 µM at 24 h and 18.01 µM at 48 h; EC_{50} values for HCQ were 6.25 µM at 24 h and 5.85 µM at 48 h). Due to the relatively low selectivity index (SI) of HCQ, it requires cautious designing and conducting of clinical trials to attain resourceful and safe control of the SARS-CoV-2 infection.

Favipiravir or Favilavir (Avigan)

A purine nucleoside presently labeled as Avigan, developed by Fujifilm Toyama Chemical of Japan, has recently been approved for Phase-III clinical trial (March 31, 2020) for the COVID-19 patients. This drug is approved for manufacturing and sale in Japan for the treatment of influenza as an antiviral. In case of Influenza virus, it selectively inhibits its RNA polymerase which is essential for viral replication when human cells are infected. It is believed that this drug may be effective for the treatment of COVID-19 as SARS-CoV-2 uses same enzyme (RNA polymerase) for replication and classification into the same type of single-stranded RNA virus like influenza. Thus, this drug acts by inhibiting the RdRp leading to inaccurate viral RNA synthesis (Fig. 6). This drug is recommended by the director of the China National Center for Biotechnology Development under the Ministry of Science and Technology to treat COVID-19. Italy has also approved the drug to treat COVID-19 cases. Due to the effectiveness of this drug against COVID-19, it is being mass-produced as generic version in China [45, 46].

Ritonavir and lopinavir (Kaletra)

These drugs are approved HIV-1 protease inhibitors, used in combination with other anti-retroviral drugs to treat HIV-1 infection in both adults and pediatric patients who is older than 14 days. Coronaviruses encode either two or three protease enzymes like papain-like proteases (PL^{pro}), a serine-type protease, the main protease, or M^{pro} which cleave the polyproteins into non-structural polyproteins (nsps). These nsps are essential for viral RNA synthesis. Ritonavir and

lopinavir act by inhibiting these protease enzymes. The mechanisms of action of these drugs suppressing coronavirus activity [47] are depicted in Fig. 6. A combination of these drugs is recommended in Italy to treat COVID-19 patients. Several trials are going on worldwide using these drugs or in combination of other drugs. A collaborative research from China and the UK conducted a clinical trial to examine the effectiveness of a combination of these two drugs against COVID-19 which was published in the New England Journal of Medicine (NEJM) [15]. The output of their trial did not provide any significant benefit in the patients with COVID-19. They suggest that "future trials in patients with severe illness may help to confirm or exclude the possibility of a treatment benefit." Among those trials, two trials are investigating against pneumonia caused by COVID-19. One trial is conducted in the Tongji Hospital, Wuhan, China, using lopinavir–ritonavir against Arbidol hydrochloride (influenza drugs) and oseltamivir (NCT04255017). In South Korea, a comparative study of lopinavir–ritonavir against HCQ in patients with mild cases of COVID-19 (NCT04307693) was made. The two arms of the WHO SOLIDARITY trial are lopinavir–ritonavir alone and in combination with interferon-beta [48].

Ivermectin

Ivermectin is an FDA-approved broad-spectrum antiparasitic drug, which recently showed in vitro antiviral activity against SARS-CoV-2. It acts by inhibiting the interaction between HIV-1 integrase protein (IN) and the importin (IMP) α/β1 heterodimer which is responsible for IN nuclear import [49, 50]. Therefore, (IMP) α/β1 is unable to bind to the viral protein and preventing it from entering the nucleus, thus inhibiting HIV-1 replication [49, 50]. As a result, inhibition of the antiviral responses is reduced leading to a normal, more efficient antiviral response.

Monoclonal antibodies

The trial of potential monoclonal antibody-based therapy against COVID-19 is going on by using the previous knowledge on the neutralizing monoclonal antibodies (nMAb) against similar coronaviruses such as SARS-CoV and MERS-CoV. Monoclonal antibodies targeting the vulnerable sites of trimeric spike (S) glycoproteins on the viral surface which are responsible for the entry to the host cell

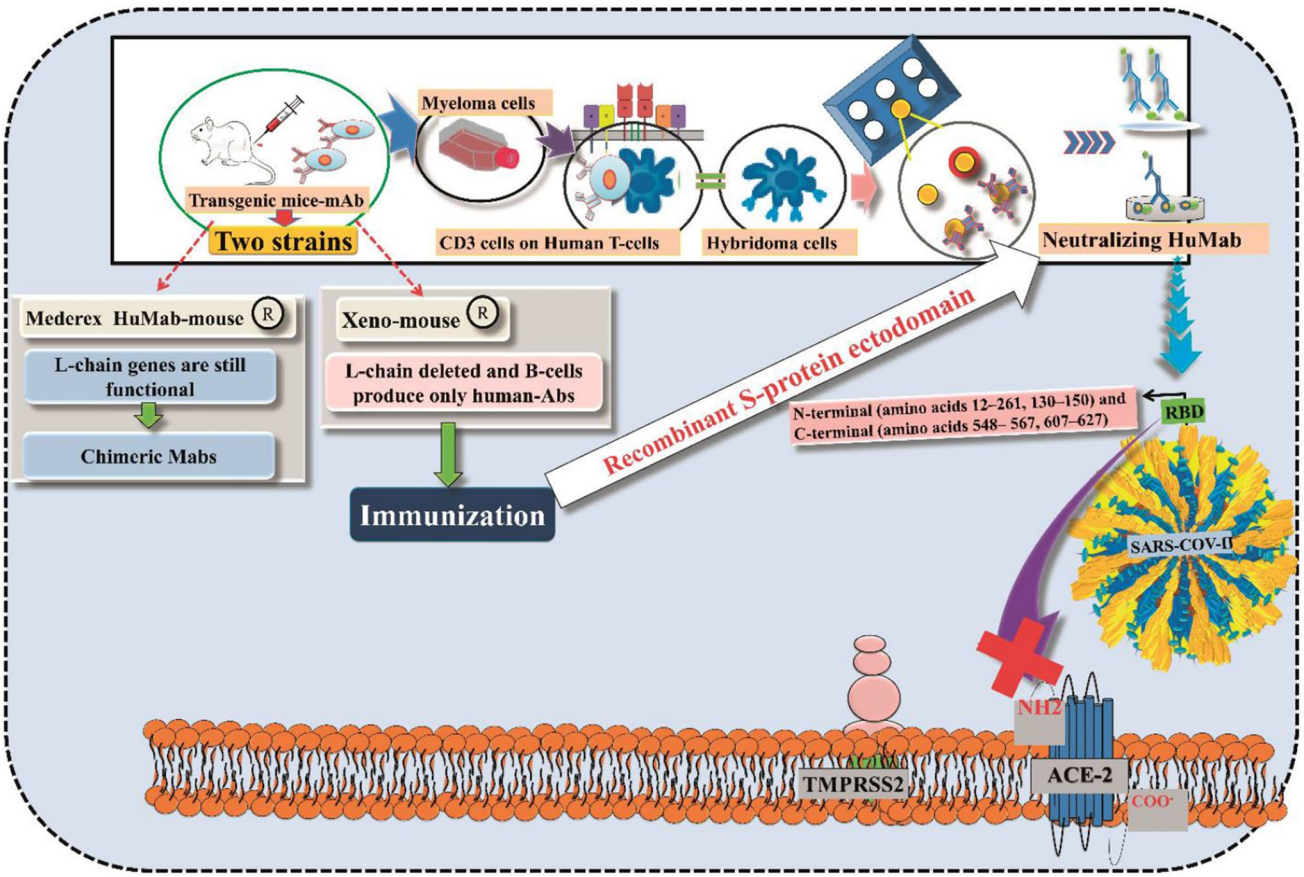


Fig. 9 Schematic diagram of the role of human monoclonal antibodies to block SARS-CoV-2

are increasingly being recognized as a promising class of drugs against COVID-19. Potential neutralizing monoclonal antibodies mainly targeting the receptor-interaction domain at S1 subunit ultimately disabled cell–receptor interactions [51–53]. The detailed mechanism of action of this class of drugs is depicted in Fig. 9. Recently, several monoclonal antibodies, namely tocilizumab (atlizumab), mavrilimumab, lenzilumab, leronlimab, gimsilumab, sarilumab, siltuximab (Sylvant), camrelizumab (AiRuiKa), eculizumab (Soliris), etc., are being tried to investigate their potency against COVID-19 disease, and these are tabulated in Table 1.

The human monoclonal antibodies (hmAbs) are developed by using several strategies against SARS-CoV-2 including preparation of hybridomas (using a transgenic mouse), phage display technologies and the immortalization of convalescent B cells. At present, the hmAbs production has been carried out with two strains of transgenic mice, i.e., Medarex HuMab-Mouse and Xeno-mouse [54]. The difference between these two strains of mice is that mouse L chain genes are still functional in the Medarex HuMAb-Mouse; thus, these mice can produce chimeric mAbs. The Xeno-Mouse from Amgen has all the mouse L chain genes deleted, and B cells produce only human Abs. One of these mice monoclonal antibodies [chimeric (Medarex HuMAb) (or) hybridomas (Xeno-mouse)] was immunized to produce neutralizing antibodies and further evaluated. The developed neutralizing antibodies bind to a specific portion to the RBD (N-terminal (amino acids 12–261, 130–150), and C-terminal of the RBD (amino acids 548–567, 607–627)) to prevent fusion of SARS-CoV-2 with the target cells.

COVID-19 convalescent plasma

Due to emergency of COVID-19 patients, recently, FDA has recommended to healthcare providers and investigators the use of convalescent plasma collected from individuals who have recovered from COVID-19 that may contain antibodies to SARS-CoV-2. However, till now, this therapy has not yet been shown to be effective and safe against this disease. Thus, it is very essential to investigate the safety and effectiveness of COVID-19 convalescent plasma in clinical trials. For this therapy, FDA has recommended some guidelines as follows [55]: (1) the pathways for use of investigational COVID-19 convalescent plasma; (2) patient eligibility; (3) collection of COVID-19 convalescent plasma, including donor eligibility and donor qualifications; (4) labeling, and (5) record keeping.

The pharmacological safety data including dose, drug–drug interactions and toxicities of selected potential drug candidates are provided in Table 2.

Vaccines

Due to the worldwide outbreak of the COVID-19, the general public are keenly watching the progress of development of COVID-19 vaccines [56]. Dr. Anthony S. Fauci, Director of National Institute of Allergy and Infectious Diseases (NIAID), said that "finding a safe and effective vaccine to prevent infection with SARS-CoV-2 is an urgent public health priority." But development of a new vaccine against a new disease is not an easy task. Various research institutes and industries are giving their full efforts to develop a vaccine against this pandemic disease with its earliest. Thankfully, the progress is rapid due to various reasons such as: (1) sharing the efforts to sequence the genetic material of SARS-CoV-2 by China throughout the world, (2) coronaviruses were already on the radar of health science researchers, (3) the knowledge from SARS and MERS caused by corona viruses and (4) also learnings from the vaccines against SARS and MERS which were stopped or postponed when those outbreaks were controlled may still be used to defeat COVID-19. The progress of most promising vaccines against the pandemic disease COVID-19 being made by various industries and research institutes is tabulated in Table 3. Several miscellaneous vaccines under investigation for COVID-19 are listed under Box 2.

In silico modeling applied to search the future drug candidates

To combat the COVID-19 pandemic, researchers must fight with the time to save as many lives as possible. To save the time and speed up the drug discovery process, in silico modeling provides one the best possible options. Till now, in most of the cases, researchers are trying the computational repurposing theory of existing approved drugs (synthetic as well as from natural origin) for SARS-CoV-2 employing docking, homology modeling and molecular dynamics (MD) studies to identify probable magic drugs for COVID-19. Huang et al. [21] computationally designed a short protein fragment or peptide which may block the coronaviruses' ability to enter human cells by binding to the viral protein which is one of the first kinds of peptide treatments routing for experimental efforts.

Smith and Smith [22] analyzed 8000 small drug molecules and natural products (SWEETLEAD library database) employing restrained temperature replica-exchange MD simulations combining virtual screening through the ensemble docking to identify the effective drug for COVID-19 which might stop the virus by two ways: (a) disrupting S protein and ACE2 receptor interface stability; or (b) by troubling the capability of the S protein to recognize

Table 2 Pharmacological safety data of selected potential drug candidates [11, 12, 14, 34, 38, 39, 43–45, 57–59, 64, 69, 70, 89]

Drug	Dose	Drug–drug interaction	Toxicity
Chloroquine phosphate (Aralen) [11, 12, 14, 43, 89]	500 mg by mouth every 12–24 h × 5–10 days	CYP2D6 and CYP3A4 substrate	Mild: Abdominal cramps and gastrointestinal intolerance Serious: Cardiovascular effects with QTc prolongation, hematologic effects, central nervous system effects, hypoglycemia, retinal toxicity and neuropsychiatric effect
Hydroxychloroquine sulfate (Plaquenil) [11, 12, 44, 89]	400 mg by mouth daily × 5 days **OR** 400 mg by mouth every 12 h × 1 day, then 200 mg by mouth every 12 h × 4 days **OR** 200 mg by mouth 3 times/day for 10 days	CYP2D6, CYP3A4, CYP3A5 and CYP2C8 substrate	Mild: Gastrointestinal disorders Serious: Blood and lymphatic system disorders, Cardiac disorders (cardiomyopathy, QT interval prolongation, and ventricular arrhythmias), Ear and labyrinth disorders, Eye disorders (Irreversible retinopathy with retinal pigmentation changes), maculopathies (macular degeneration), decreased dark adaptation
Favipiravir [60, 61, 89]	1600 mg doses on the first day and two doses of 600 mg for the next 13 days	CYP2C8 and aldehyde oxidase inhibitor	Mild: Diarrhea, Serious: Hyperuricemia, elevated transaminases, reduction in neutrophil count, teratogenicity
Lopinavir/ritonavir (Kaletra) [45, 57, 64, 89]	400 mg/100 mg by mouth every 12 h for up to 14 days **OR** lopinavir/ritonavir 400-mg/100-mg per 5-mL oral solution	P-gp substrate; CYP3A4 inhibitor and substrate; CYP2D6 substrate; CYP1A2, CYP2B6, CYP2C9, CYP2C19 Inducer; UGT1A1 inducer	Mild: Gastrointestinal disorders Serious: Hyperlipidemia, cardiac conduction disorders, hepatotoxicity, pancreatitis
Losartan [34]	Initial dose: 50 mg orally once a day Maximum dose: 100 mg orally once a day	Interacts with drugs that may increase the level of potassium in the blood such as ACE inhibitors including benazepril/lisinopril, birth control pills containing drospirenone	Mild: Dizziness, chest pain, hypoglycemia Serious: Hyperkalemia, hypotension, and orthostatic hypotension
Remdesivir [14, 57–59, 89]	200 mg × 1, 100 mg every 24 h IV infusion	Not a significant inducer/inhibitor of CYP enzymes	Reversible elevated transaminases, kidney injury, liver toxicity
Tocilizumab (Actemra) [69, 70, 89]	400 mg IV or 8 mg/kg × 1–2 doses. If required, then second dose after 8–12 h of first dose	IL-6 reduces mRNA expression for several CYP450 isoenzymes, including CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4	Mild: Upper respiratory tract infections (chances of tuberculosis), headache, nasopharyngitis, hypertension Serious: Gastrointestinal perforations, hematologic effects, hepatotoxicity, hypersensitivity reactions
Umifenovir (Arbidol) [38, 39, 89]	200 mg every 8 h by mouth 7–14 days	Metabolized by CYP3A4, flavin-containing monooxygenase (FMO) family, and UDP-glucuronosyltransferase (UGT) family	Mild: Gastrointestinal upset Serious: Allergic reaction, elevated transaminases

Table 3 The progress in development of vaccines by different companies and institutes throughout the world [16–20, 90, 91]

Vaccine under trial	Name of the company	Platform	Discussion	Current status
mRNA-1273 [16]	Moderna	RNA	This is a novel lipid nanoparticle (LNP)-encapsulated mRNA-based vaccine that encodes for a full-length, prefusion stabilized spike (S) protein of SARS-CoV-2. The protein S complex is necessary for membrane fusion and infection of host cells. Thus, the stabilized prefusion coronavirus spike protein responsible for Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS) can be used as a vaccine antigen to produce robust neutralizing antibody responses. The vaccine is packed with mRNA. The genetic material originates from DNA and makes proteins. Moderna carries its vaccine with mRNA that encodes the correct coronavirus proteins, which are injected into the body. The immune cells present in the lymph nodes can process this mRNA and start making the protein in the right way for new immune cells to identify it and mark it for damage	Phase-III clinical trial (NCT04470427)
Ad5-nCoV [17]	CanSino Bio and Institute of Biotechnology of the Academy of Military Medical Sciences	Non-Replicating Viral Vector	In China, Ad5-nCoV is the first novel coronavirus vaccine for COVID-19. This is a genetically engineered vaccine candidate with the replication-defective adenovirus type 5 as the vector to express SARS-CoV-2 spike protein. Preclinical animal studies of this vaccine candidate showed that it can persuade a strong immune response in animal models. Preclinical animal safety studies also exhibited a good safety profile	A Phase-III trial in Saudi Arabia is currently underway

Table 3 (continued)

Vaccine under trial	Name of the company	Platform	Discussion	Current status
AZD1222 (previously called ChAdOx1) [18]	Jenner Institute-University of Oxford	Non-Replicating Viral Vector	This vaccine candidate was developed by Jenner Institute based on a chimp adenovirus vector. For the clinical trials, recruiting 510 healthy volunteers, aged between 18 and 55 years, in a single-blinded, randomized, placebo-controlled, multi-center study to determine efficacy, safety, and immunogenicity of this vaccine	Phase-II/III clinical trial
LV-SMENP-DC [90]	Shenzhen Geno-Immune Medical Institute	Lentiviral	A synthetic minigene has been engineered based on conserved domains of the viral structural proteins and a polyprotein protease. The entry of SARS-CoV-2 is intermediated through attachment of the S protein to the ACE2 receptor and the viral replication depends on molecular mechanisms of all of these viral proteins. In this vaccine, a competent lentiviral vector system (NHP/TYF) was used to convey COVID-19 minigenes to express viral proteins and immune modulatory genes to modify dendritic cells (DCs) and to activate T cells	Phase-I/II clinical trial (NCT04276896)
Bacillus Calmette–Guérin (BCG) Vaccine [19]	Research group, Netherlands	Live Attenuated Virus	The Research group will recruit 1000 healthcare workers in eight Dutch hospitals who will either receive the BCG vaccine, or a placebo	Phase-III clinical trial
BCG Vaccine [20]	Murdoch Children's Research Institute	Live Attenuated Virus	This is one of the oldest vaccines available in the market. It is made from live, attenuated bovine tuberculosis bacillus, *Mycobacterium bovis* which induces an adaptive immune response against tuberculosis bacterium. Due to availability in the market, they utilize this vaccine directly in Phase-III clinical trials. This is an open-label, two-group, Phase-III randomized controlled trial in up to 4170 healthcare workers across Australia	Phase-III clinical trial (NCT04327206)

Table 3 (continued)

Vaccine under trial	Name of the company	Platform	Discussion	Current status
Oral bacTRL-Spike [91]	Symvivo Corporation	Oral vaccine for COVID-19	bacTRL-Spike-1 will be the first-in-human study of bacTRL-Spike, and the first-in-human use of orally delivered bacTRL. It is a genetically modified, probiotic-based oral vaccine for COVID-19. The study design is a phase 1, randomized, observer-blind, placebo-controlled trial in 84 healthy adults [63 receiving active vaccine in bacterial medium and 21 receiving placebo (bacterial medium only)]	Phase-I clinical trial (NCT04334980)

the ACE2 receptor. The simulation was performed in the IBM's supercomputer SUMMIT which is recognized as the world's most powerful computer with 200 petaflops speed. The authors reported the predicted binding affinities of all studied molecules for isolated SARS-CoV-2 S protein and the S protein human ACE2 receptor interface, followed by proposed 77 small molecules by docking studies (47 ligands employing interface docking and 30 ligands identified by the isolated S protein docking). Among the identified 77 molecules, the authors finally reported top 7 regulatory approved small molecules [Pemirolast (Zinc ID: 5783214), Isoniazid pyruvate (Zinc ID: 4974291), Eriodictyol (Zinc ID: 58117) and Nitrofurantoin (Zinc ID: 3875368) binding within the S protein-ACE2 interface; Ergoloid (Zinc ID: 3995616), Cepharanthine (Zinc ID: 30726863), and Hypericin (Zinc ID: 3780340) binding with the S protein receptor] which may serve as possible drug candidates for COVID-19 (Fig. 10a).

Ton et al. [23] identified 1000 noncovalent inhibitors for SARS-CoV-2 main protease (M^{pro}) using 1.36 billion compounds from the ZINC15 library employing deep docking platform using Glide SP module which utilizes QSAR models trained on docking scores. The 4MDS protein with 1.6 Å resolution bound to a noncovalent inhibitor was used for the docking study, and among the identified 1000 noncovalent inhibitors, ZINC000541677852 is the top hit drug candidate (Fig. 10b).

α-Ketoamide inhibitors are proposed as new drug candidates by designing, synthesis, followed by a docking study on the main protease (M^{pro}, $3CL^{pro}$) which has a crucial role in processing the polyproteins that are translated from the viral RNA [24]. Three different PDB structures were employed for the study; they are 6Y2E, 6Y2F and 6Y2G. The authors modified previously designed inhibitor (1) (earlier used for beta-, alpha coronaviruses and 3C proteases of enteroviruses) by incorporating the P3-P2 amide bond into a pyridone ring to enhance the half-life of the newly designed (2) compound in plasma followed by replacing the hydrophobic cinnamoyl moiety with less hydrophobic Boc group (Fig. 11a). Although the inhibitory concentration decreased from 0.18 ± 0.02 μM (1) to 2.39 ± 0.63 μM (2), but molecule 2 reported three times higher plasma binding and 19 times better plasma solubility compared to molecule 1. Further, to improve the antiviral activity against betacoronaviruses of clade b, the authors replaced the P2 cyclohexyl moiety of molecule 1 by the smaller cyclopropyl fragment to produce compound 3 which showed IC_{50} value of 0.67 ± 0.18 μM for the purified recombinant SARS-CoV-2 M^{pro}.

Zhou et al. [25] reported an integrative antiviral drug repurposing analysis employing pharmacology-based network medicine platform by a two-step process: (a) quantifying the relationship between the human coronaviruses

Box 2 Miscellaneous vaccines under investigation for COVID-19 [92, 93]

Live attenuated vaccines (AJVaccines, Altimmune, Arcturus Therapeutics and Duke-NUS Medical School, Baylor College of Medicine, BIOCAD, Biological Research Institute, Codagenix)
Molecular clamp vaccine (CSL and The University of Queensland)
Synthetic mRNA that stimulates the immune system to produce antibodies (CureVac)
Two Ii-Key peptide vaccine candidates (EpiVax)
Ii-Key peptide vaccine (ExpreS^{2}ion Biotechnologies, Generex Biotechnology)
Ankara virus-like particles (MVA-VLP) (Geovax and Bravovax)
COVID-19 S-Trimer (GlaxoSmithKline)
Adenovirus-based vector vaccine (Greffex)
gp96-based vaccine (Heat Biologics)
INO-4800; currently in preclinical stage (Inovio Pharmaceuticals)
Plant-based vaccine (Institute Pasteur, Themis Bioscience, University of Pittsburgh, Johnson & Johnson, Medicago)
Modified avian coronavirus vaccine (MIGAL Galilee Research Institute)
BNT162 (Merck, Novavax, Peter Doherty Institute for Infection and Immunity, Pfizer and BioNTech)
A chimera that combines DNA from the SARS-CoV-2 with a harmless virus that can stimulate the immune system (Sanofi)
Formalin-inactivated and alum-adjuvanted candidate vaccine (Sinovac)
Gene-encoded antibody vaccine (Sorrento Therapeutics, Inc. and SmartPharm Therapeutics Inc.)
DNA-based vaccine (Takis Biotech)
Vaccine candidate based on Tonix's horsepox vaccine, TNX-1800 (Tonix Pharmaceuticals and Southern Research)
CoronaVac (Sinovac; Phase-III), **Adjuvant recombinant vaccine candidate** (Anhui Zhifei Longcom Biopharmaceutical, Institute of Microbiology of the Chinese Academy of Sciences; Phase-II), **ZyCoV-D** (Zydus Cadila; Phase-II), **Covaxin** (Bharat Biotech, National Institute of Virology; Phase-II), **BBIBP-CorV** (Beijing Institute of Biological Products, China National Pharmaceutical Group (Sinopharm); Phase-I/II), **GX-19** (Genexine; Phase-I/II), **Sputnik V** (Gamaleya Research Institute, Acellena Contract Drug Research and Development; Phase-I/II), **Self-amplifying RNA vaccine** (Imperial College London; Phase-I/II), **LUNAR-COV19** (Arcturus Therapeutics and Duke-NUS Medical School; Phase-I/II), **mRNA-based vaccine** (CureVac; Phase-I/II), **SCB-2019** (GlaxoSmithKline, Sanofi, Clover Biopharmaceuticals, Dynavax and Xiamen Innovax; Phase-I), **COVAX-19** (Vaxine Pty Ltd.; Phase-I), **NVX-CoV2373** (Novavax; Phase-I), **V590** (Merck, IAVI; Phase-I), **GRAd-COV2** (ReiThera, Leukocare, Univercells, Phase-I)

Under parenthesis companies and research institutes are mentioned

(HCoV) and host interactome and (b) identifying the drug targets in the human protein–protein interaction network. As per phylogenetic analyses, SARS-CoV-2 has the highest nucleotide sequence identity (79.7%), followed by the envelope and nucleocapsid protein sequence identities of 96% and 89.6%, respectively, with SARS-CoV. Employing the network proximity analyses between drug targets and HCoV-associated proteins followed by gene set enrichment analysis (GSEA), it was possible to identify 16 probable anti-HCoV drugs (e.g., melatonin, mercaptopurine, and sirolimus, etc.). Later, the drugs were validated through HCoV-induced transcriptomics data in human cell lines and enrichment analyses of drug-gene signatures. The authors also reported three effective drug combinations among the probable hits identified through 'complimentary exposure' pattern (Fig. 11b).

Grifoni et al. [26] employed the Immune Epitope Database and Analysis Resource (IEDB) to characterize the sequence similarity between SARS-CoV and SARS-CoV-2 through homology modeling. The epitope prediction identified a priori potential B and T cell epitopes for SARS-CoV-2 which are the promising targets for immune recognition, followed by the discovery of diagnostics and future vaccines.

Within a short period of time, a huge number of in silico results were deposited in the preprint servers from all over the world and at the same time a few are already published. As in most of the cases, multiple in silico drug designing and virtual screening tools were employed for repurposing theory of approved existing drugs [94], thus, major information is gathered in Table 4 to avoid similar discussion several times [95–119].

An interesting aspect is that most of the studies are based on repurposing theory of existing approved drug employing docking and MD supported VS. Majorly the authors relied on approved antiviral drugs along with DrugBank database, Zinc database, Natural compounds' databases along with one of the most discussed molecules of recent time, HCQ. Thus, the basic ideas of implication of in silico tools and repurposing of approved drugs are same, but the only difference is selection of the target protein in different studies. Without any doubt, the crystal structure of COVID-19 main protease (M^{pro}) in complex with an inhibitor N3 (PDB: 6LU7) is the most accessed protein for the drug discovery. But based on the target type, the choice of proteins can be different. Thus, to assist researchers, we have classified the target proteins into 14 types covering 110 PDB crystal ID from PDB (https://www.rcsb.org/) available until April 12, 2020, and enlisted in Table 5.

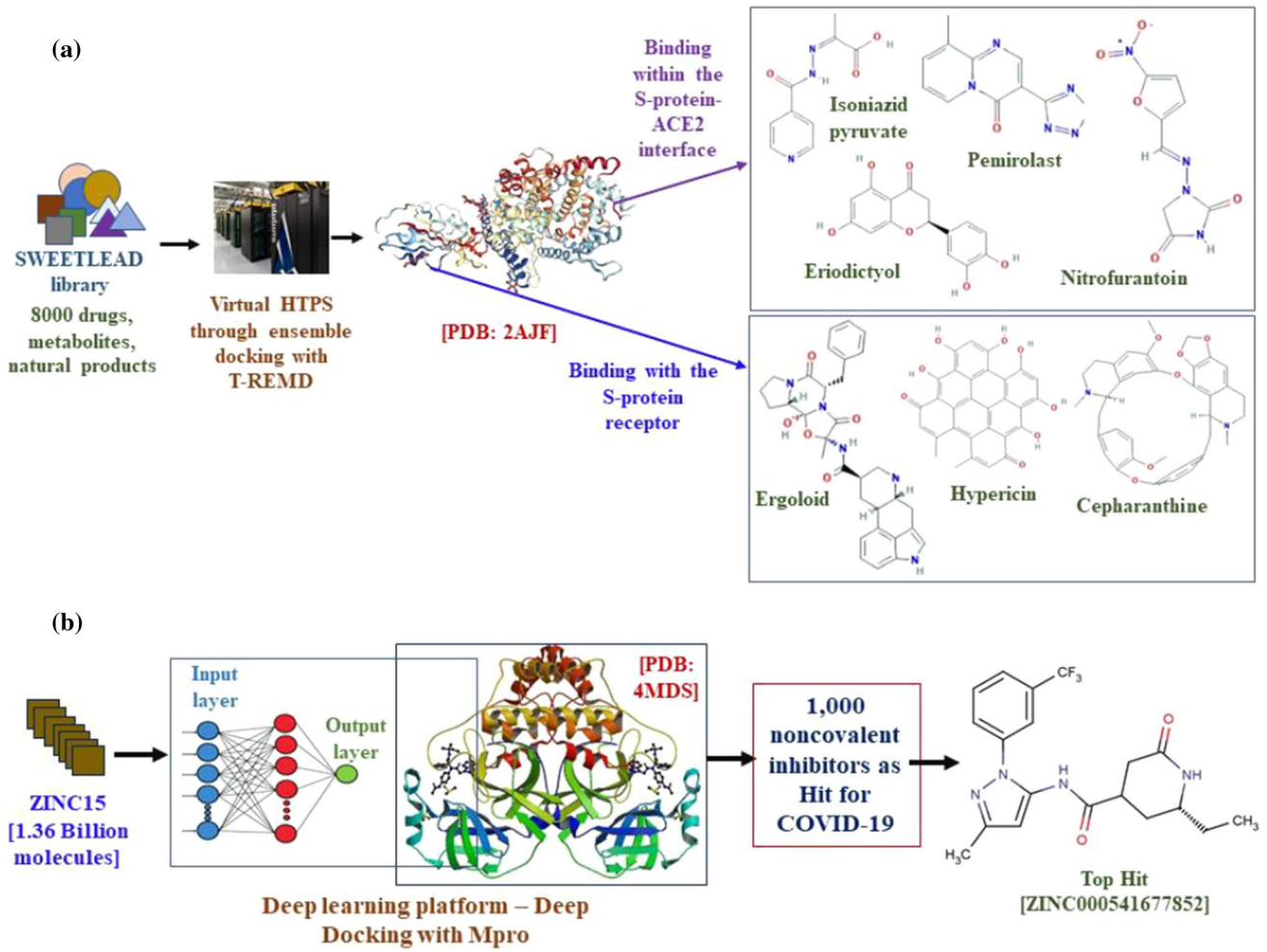


Fig. 10 Schematic workflows performed by Smith and Smith [22] (**a**) and by Ton et al. [23] (**b**)

Is interspecies modeling the future of COVID-19 drug discovery? An unexplored concept under in silico tools

Multiple incidents illustrated the outbreak of corona virus in animals, especially in pets [120, 121]. Animals from dogs, cats, tigers, lions to minks are already tested positive and showed mild to severe symptoms of corona virus, followed by death of few instances. Although these events are scattered and not enough to study in the middle of human crisis, we cannot ignore the fact of human to animal transmission. If human to animal transmission is true, then there is a possibility of mutations, insertions and deletions in the genome sequence of this deadly virus in these animals in future with probabilities of zoonotic transfer of a stronger form of present SARS-CoV-2 virus from them to human in the near future. Thus, these small incidents need to be checked very carefully to avoid any future transmission.

The above-mentioned facts help to build an interspecies analysis by Kar and Leszczynski [122] for animals to human transmission or vice versa to aid in the drug discovery process of COVID-19 in upcoming days. The in silico interspecies-quantitative structure–activity relationship (i-QSAR) modeling correlates and then extrapolates the response (activity/toxicity) data from animal source to human which will be helpful for drug discovery. Due to genome sequence similarity of SARS-CoV-2 with the pangolins and bat, experimental data of drug candidates to pangolins/bat along with structural and physicochemical properties of drugs can be correlated with human response endpoint which is the first step of modeling. In the next step, extrapolation of animal data to human data can lead to no human testing through developed i-QSAR model. Once the acceptable predictive i-QSAR model is ready, there might be no need of future animal testing. As bat and pangolins are endangered species, thus future introspection can be performed in dogs and cats due to their better accessibility as well as considering them possible carriers of SARS-CoV-2 from the recent incidents.

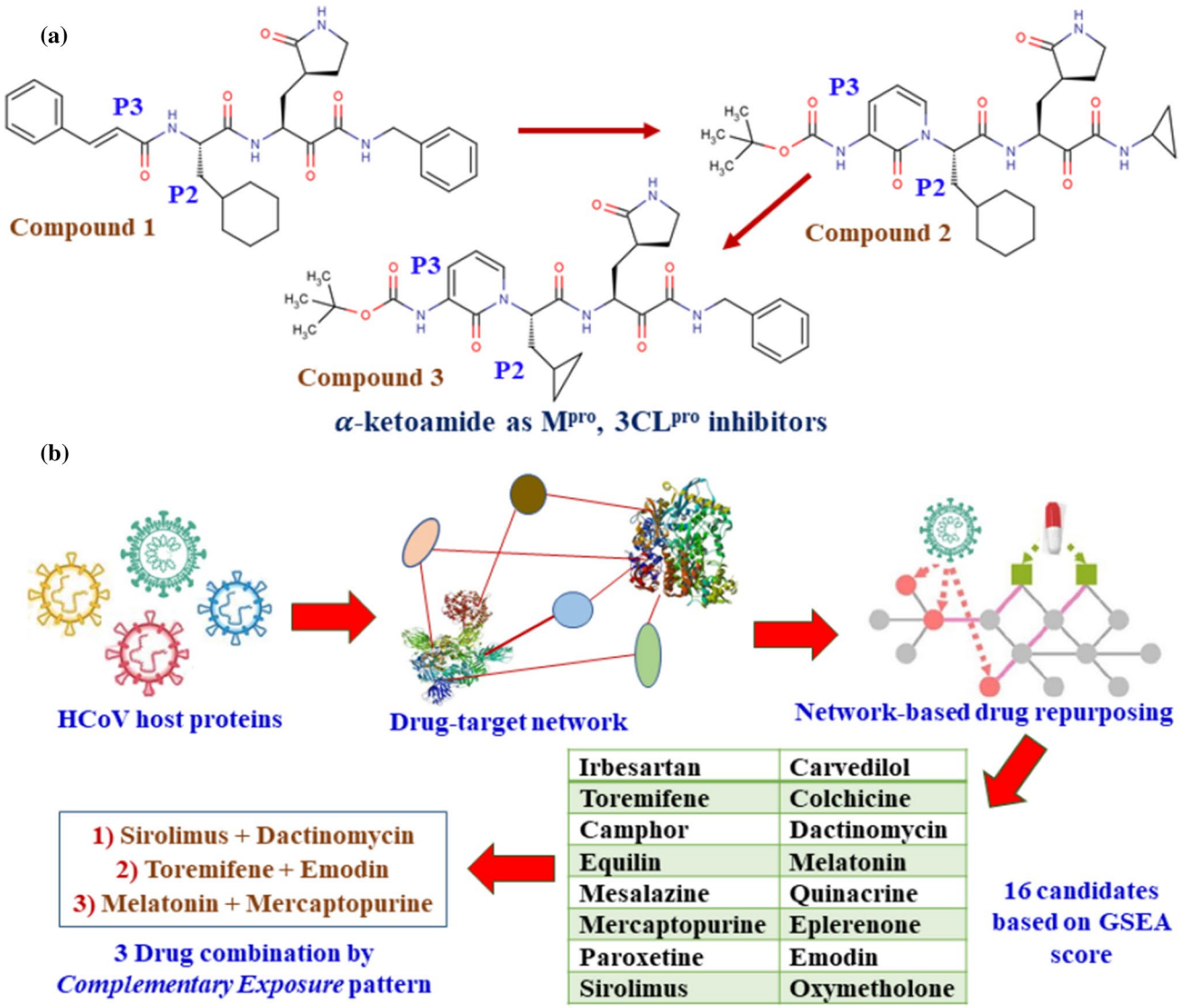


Fig. 11 Schematic workflows performed by Zhang et al. [24] (**a**) and by Zhou et al. [25] (**b**)

Conclusion

Researchers from all over the world are trying to find the medicines which will help to stop the transmission of the virulent SARS-CoV-2 virus, mitigate the symptoms of the infected patients and help to lower the death toll throughout the world. Unfortunately, till now there is no silver gunshot which can solve this pandemic COVID-19. We have reported here a comprehensive and updated discussion on drug or drug candidates under investigation with their probable targets and mechanisms of action which might not be clear for many cases earlier, along with the effort of ongoing vaccine trials, monoclonal antibodies therapy and convalescent plasma treatment. We have presented here the ongoing computational efforts related to in silico tools to explore the probable drug candidates

Table 4 A comprehensive list of in silico studies in search of drug molecule to fight COVID-19 [95–119]

Implied in silico methods and tools	Database	PDB ID	Pharmacological target and mechanism of action of study	Number and name of leads/hits
Classical MD (NAMD and the CHARMM36 protein force field)	Peptides	2AJF	Peptide inhibitors are formed by two sequential self-supporting α-helices extracted from the protease domain of ACE2 which provide a stable binding and are extremely specific to the SARS-CoV-2 receptor-binding domains	3 (Inhibitor 2, Inhibitor 3 and Inhibitor 4) [95]
Docking followed by VS (AutoDock Vina, SMINA)	SuperDRUG2 (3639 approved drugs)	6LU7, 5R82, 6YB7	Main protease (M^{pro}) was utilized in MD and VS of approved drugs followed by ranking of drug candidates based on predicted binding energy	2 (Saquinavir and Beclabuvir) [96]
VS through docking (PLANTS) followed by MD (AMBER, MOE2019)	Selleckchem and TargetMol (3118 FDA-approved drug)	6LU7, 4MDS	VS of two databases on two targets (M^{pro} in complex with peptidomimetic inhibitor and SARS-CoV 3C-Like proteinase $3CL^{pro}$)	4 (Indinavir, lopinavir, atazanavir, cobicistat) [97]
VS employing docking study (AutoDock 4.2)	ZINC (1615 FDA-approved drug)	6LU7	Repurposing of FDA-approved drugs as inhibitors for M^{pro} protein of SARS-CoV-2	1 (Simeprevir) [98]
Hierarchical VS through docking (GLIDE), MD (AMBER), Binding Free Energy Calculations (MM-PBSA-WSAS)	DrugBank (Approved, investigational, experimental drugs)	6LU7	Repurposing of DrugBank molecules as inhibitors for M^{pro} protein of SARS-CoV-2	7 (Carfilzomib, Eravacycline, Valrubicin, Lopinavir, Elbasvir, Streptomycin, PubChem 23727975) [99]
Docking (Molegro Virtual Docker)	Terpenoids (9)	6LU7	Terpenoids of plant origin were checked as inhibitors of M^{pro} of SARS-CoV-2	1 (Ginkgolide A) [100]
Docking (AutoDock Vina)	DrugBank 5.1.5	6Y84, 6Y2F, 6Y2G, 6W63	Drug repurposing against SARS-CoV-2 M^{pro} or $3CL^{pro}$	29 (Remdesivir, Asunaprevir, Ciluprevir, Simeprevir, Danoprevir, Glecaprevir, etc.) [101]
Docking (DOCK6), MD (SOMD, GROMACS)	ZINC (1615 FDA-approved drug)	6LU7	Combination of docking and MD simulations of FDA-approved drugs to identify inhibitors that could potentially bind to the active site of SARS-CoV-2 M^{pro}	11 (Dronedarone, Aliskiren, cobicistat, Isavuconazonium, Capreomycin, Tessalon, Pradaxa, Saquinavir, Ceftolozane, Naloxegol Carfilzomib) [102]
Docking (Autodock 4.2)	Traditional Chinese Medicine Systems Pharmacology Database (TCMSP)	5E6J, 1UJ1, 6CAD	Screening of Chinese medicinal herbs that are frequently used in treating viral respiratory infections through docking and network pharmacology analysis targeting $3CL^{pro}$, papain-like protease (PL^{pro}) and spike protein	13 (betulinic acid, coumaroyltyramine, tanshinone IIa cryptotanshinone, sugiol, desmethoxyreserpine, dihomo-γ-linolenic acid, lignan, dihydrotanshinone, kaempferol, quercetin, moupinamide, *N-cis*-feruloyltyramine) [103]

Table 4 (continued)

Implied in silico methods and tools	Database	PDB ID	Pharmacological target and mechanism of action of study	Number and name of leads/hits
Homology Modeling (ICM 3.7.3), Docking (ICM)	ZINC (2924), Natural products (1066), Anti-viral drugs (78)	2K87,2FKV,2W2G, 3E9S, 2K87, 2AW0, 2AHM, 3EE7,1Z8A 5NFY,5NUR, 6JYT, 5NFY, 2H85, 3R24, 1YO4, 3SCI, 1SSK, 2CJR, 5X29	19 SARS-CoV-2 targets and 1 human target were prepared by homology modeling. Along with these targets and human ACE2 target, selected databases were screened for repurposing of drug candidates and targets	42 PL^{pro} inhibitors, 53 $3CL^{pro}$ inhibitors, 40 RdRp inhibitors [104]
Molecule Transformer-Drug Target Interaction (MT-DTI), Docking (AutoDock Vina)	Drug Target Common (DTC) database, BindingDB database, FDA-approved antiviral drug	$3CL^{pro}$, RdRp, 2′-*O*-ribose methyltransferase, 3′-to-5′ exonuclease, endoRNAse	Pre-trained deep learning-based drug–target interaction model named MT-DTI was used to identify commercially available antiviral for SARS-CoV-2	6 (Atazanavir, remdesivir, efavirenz, ritonavir, dolutegravir, lopinavir) [105]
Homology modeling (Blastp, Modeller 9.12), Docking (Maestro, Schrodinger), MD (DESMOND)	DrugBank (2300 approved drug), Super Natural II (3,00,000)	2HSX	Modeled non-structural protein NSP1 employing homology modeling, docking and followed by MD simulation-based VS to identify new drug candidates through drugs repurposing theory	32 (Remdesivir, Edoxudine, Esculin, Acarbose, Shogaol, Glycyrrhizic acid, Gingeronone) [106]
Homology modeling (SWISS-MODEL), Docking (AutoDock Vina), MD (GROMACS)	LOPAC	2AJF, 6VSB	High-throughput VS was employed to investigate FDA-approved drugs against both ACE2 host cell receptor and the S protein to identify drug candidate through repurposing theory	5 inhibitors of ACE2 receptor (TNP, Eptifibatide acetate, GNF5, RS504393, GR 127935 hydrochloride hydrate); 5 inhibitors of viral S protein (KT185, KT203 GSK1838705A, BMS195614, RS504393) [107]
Docking (AutoDock Vina), VS (MTiOpenScreen)	Drugs-lib (7173)	2DUC	Molecular modeling of the $3CL^{pro}$ of the SARS-CoV-2 followed by VS performed for already approved drug	16 (Diosmin, Hesperidin, MK-3207, Venetoclax, Dihydroergocristine, Bolazine, R428, Ditercalinium, Etoposide, Ledipasvir UK-432097, Teniposide, Irinotecan, Velpatasvir, Eluxadoline, Lumacaftor) [108]
Docking (AutoDock Vina), VS (MTiOpenScreen), MD (AMBER 16)	Approved drugs (28)	6LU7, 1UK4, 6M0J, 6NUR	Searching of antagonists which will inhibit the M^{pro} of the SARS-CoV-2 virus, modulate the ACE2 receptors and reduce viral replication by inhibiting NSP12 RNA Polymerase	4 (Simeprevir, baricitinib, paritaprevir, remdesivir) [109]
Docking, MD (SYBYL-X 1.1)	Essential oils in garlic including organosulfur compounds (18)	6LU7	Inhibitory effect of essential oils from garlic is established and found interactions with the amino acids of the human ACE2 protein and the M^{pro} of SARS-CoV-2	2 (Allyl disulfide, Allyl trisulfide) [110]

Table 4 (continued)

Implied in silico methods and tools	Database	PDB ID	Pharmacological target and mechanism of action of study	Number and name of leads/hits
Docking (AutoDock Vina)	Artemisinin and derived compounds (12)	6LZG	Explained how HCQ interferes in the prevention of Lys353 in human ACE2 from interacting with the corresponding binding hotspot exist on the Spike protein through docking. This was followed by screening of artemisinin derived compounds to show better docking score (two mode of interactions with Lys31 and Lys353 binding hotspots of the Spike protein) of them compare to HCQ	3 (Artesunate, Artenimol Artemisinin) [111]
MD (CHARMM36)	CLQ, HCQ	6VSB	Structural and molecular modeling reported that CLQ and HCQ bind to sialic acids and gangliosides with high affinity (S protein uses sialic acids linked to host cell surface gangliosides binding domain at the tip of the N-terminal domain (amino acids 111-158) of the SARS- CoV2 for entry to host along with ACE2)	2 (CLQ-OH[preferably], CLQ) [112]
Docking (PatchDock, FireDock)	HCQ, Curcumin (2)	6Y84, 2GHV	Molecular modeling indicated that combination of HCQ with curcumin disrupt the stability of SARS-CoV-2 receptor proteins effectively by binding with main protease and S1 RBD. The combination therapy has higher efficacy than single dose of HCQ	Combination therapy of HCQ and Curcumin [113]
Docking (GLIDE- Schrodinger), Binding free energy calculations (MM-GBSA)	SELLEKCHEM, DrugBank, Repurposing hub	6LU7, 6M03	M^{pro} target was utilized in molecular docking and VS of approved drugs followed by binding energy calculation to identify potential drug candidate	6 (Pepstatin A, Leupeptin Hemisulfate, Nelfinavir, Birinapant, Lypression, Octeotide) [114]
2D-QSAR, Docking (Autodock tool 1.5.6) followed by VS	Binding DB (69 molecules as $3CL^{pro}$ for QSAR model), ZINC15 database and Chemical Abstracts Service (CAS) reported as COVID-19 antiviral candidates for VS	6LU7	MLR based 2D-QSAR model was developed using $3CL^{pro}$ inhibitor followed by docking study was also explored. Further, inhibitory activity of a total of 50,437 compounds was computed. Top 100 compounds were reported with inhibitory activity in mM.	100 lead compounds from ZINC15 and CAS database [115]

Table 4 (continued)

Implied in silico methods and tools	Database	PDB ID	Pharmacological target and mechanism of action of study	Number and name of leads/hits
Pharmacophore (vROCS (OpenEye)) and Docking study followed by VS (AutoDock 4.2)	Four pharmacophores (OEW, remdesivir, hydroxychloroquine and N3) and 50,000 natural compounds from ZINC database for VS	6LU7 and 6Y7M	Pharmacophore and docking based virtual screening performed employing natural compounds of ZINC database targeting SARS-CoV-2M protease	11 ligands as (ZINC1845382, ZINC1875405, ZINC2092396, ZINC2104424, ZINC44018332, ZINC2101723, ZINC2094526, ZINC2094304, ZINC2104482, ZINC3984030, and ZINC1531664) [116]
Sequence analysis and homology modeling followed by VS employed docking (AutoDock VINA) and later using machine learning technique CNN (BindScope)	MCULE database (44,704,142)	6VSB	44 million compounds were screened to find potential inhibitor able to inhibit the surface glycoprotein responsible for virus entry and binding	3 molecules (benzylfuran-2(5H)-one); ((2,5-difluorophenyl)thio)-2,2-difluoroacetic acid) and (2-methylfuran-3-yl)methanesulfonyl fluoride) [117]
Similarity searching and Docking (Autodock Vina, version 1.1.2 and MOE v.2019) and ADMET profiling (SwissADME)	FooDB (22,880), Dark Chemical Matter (DCM) (139,329), ZINC (top 10 hit from literature), Actives (11)	6LU7, 5N5O	Consensus VS of DCM and Food Chemicals as potential inhibitors of SARS-CoV-2 M^{Pro}	105 hits of which, three are commercially available [118]
Docking (GLIDE-Schrodinger), MM-GBSA, ADMET profiling (pkCSM and ProTox-II), MD (DESMOND- Schrodinger)	MolPort database (10,305)	6LU7, 6Y2E, 6Y2F, 6Y2G, 6M03, 6Y84, 5RF8, 5RG0, 5R8T	Protein reliability analysis among 9 M^{pro} proteins followed by VS through docking, MM-GBSA calculation, ADMET profiling and MD to find natural M^{pro} inhibitors for SAS-COV-2 which is already commercially available for further testing	3 hits (two natural compounds MolPort-005-944-636 and MolPort-005-945-924, as well as a Noricaritin (MolPort-039-052-338) derived from *Epimedium brevicornu* Maxim) [119]

Table 5 Type of target protein to identify the efficient drug molecule of SARS-CoV-2

Type of target protein	PDB crystal ID
Structure of the 2019-nCoV HR2 Domain	6LVN
Nsp9 RNA-binding protein of SARS-CoV-2	6W4B, 6W9Q
N-terminal RNA-binding domain of the SARS-CoV-2 nucleocapsid phosphoprotein	6YI3, 6M3M, 6VYO
SARS-CoV-2 main protease (M^{pro}/$3CL^{pro}$)	5R84, 5R83, 5R7Y, 5R80, 5R82, 5R81, 5R8T, 5R7Z, 5REA, 5REC, 5REB, 5REE, 5RED, 5REG, 5REF, 5RE9, 5RE8, 5RE5, 5RE4, 5RE7, 5RE6, 5RFB, 5RFA, 5RFD, 5RFC, 5RFF, 5RFE, 5RFH, 5RFG, 5REY, 5REX, 5RF9, 5REZ, 5RF2, 5REP, 5RF1, 5RES, 5RF4, 5RER, 5RF3, 5REU, 5RF6, 5RET, 5RF5, 5REW, 5REV, 5RF7, 5REI, 5REH, 5REK, 5REJ, 5REM, 5REL, 5REO, 5RF0, 5REN, 5RFZ, 5RFY, 5RFR, 5RFQ, 5RFT, 5RFS, 5RFV, 5RFU, 5RFX, 5RFW, 5RFJ, 5RFI, 5RFL, 5RFK, 5RFN, 5RFM, 5RFP, 5RFO, 5RG0, 6Y2E, 6Y84, 6W63, 6YB7, 5RF8, 6Y2G, 6Y2F, 6LU7
ADP ribose phosphatase of NSP3 from SARS-CoV-2	6VXS, 6W02. 6W6Y
papain-like protease (PL^{pro}) of SARS-CoV-2	6W9C
SARS-CoV-2 RNA-dependent RNA polymerase	7BTF, 6M71
post fusion core of 2019-nCoV S2 subunit	6LXT
NSP16–NSP10 Complex from SARS-CoV-2	6W4H, 6W61, 6W75
NSP15 Endoribonuclease from SARS-CoV-2	6VWW, 6W01
SARS-CoV-2 receptor-binding domain in complex with human antibody CR3022	6W41
Spike glycoprotein with a single receptor-binding domain (RBD) [plus bound to ACE2]	6VSB, 6VXX, 6M0J, 6VYB, 6LZG
Chimeric receptor-binding domain complexed with its receptor human ACE2	6VW1
RBD/ACE2-B0AT1 complex	6M17

against COVID-19 along with the up-to-date target protein information. The information provided from in silico approaches may work as a magic bullet for the medicinal chemists to accelerate the drug discovery and development process. Overall, this review provides a strong intellectual foundation to support progress of ongoing research related to thorough knowledge of drugs or investigational drugs, vaccines and in silico approaches which can be helpful for the development of new drug candidates for the treatment of COVID-19.

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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Full paper

Chemometric modeling of power conversion efficiency of organic dyes in dye sensitized solar cells for the future renewable energy

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ABSTRACT

The present study reports chemometric modeling of power conversion efficiency (PCE) of dye sensitized solar cells (DSSCs) using the biggest available data set till date which comprises around 1200 dyes covering 7 chemical classes. To extract the best structural features required for higher PCE, we have developed multiple partial least squares (PLS) quantitative structure-property relationship (QSPR) models for the Triphenylamine, Phenothiazine, Indoline, Porphyrin, Coumarin, Carbazole and Diphenylamine chemical classes using descriptors derived from the best subset selection method followed by selection of best five models in each dataset based on the Mean Absolute Error (MAE) values. The models were validated both internally and externally followed by the consensus predictions employing "Intelligent Consensus Predictor" tool to examine whether the quality of predictions can be improved with the "intelligent" selection of multiple PLS models. The quality of predictions for the respective external sets showed that the consensus models (CM) are better than the individual models (IM) in most of the cases. From the insights of the developed models, we concluded that attributes like a packed structure toward higher conductivity of electrons, auxiliary donor fragment of aromatic tertiary amines, number of thiophenes inducing the bathochromic shift and augmenting the absorption, presence of additional electron donors, enhancement of electron-donating abilities, number of non-aromatic conjugated C(sp^2) which helps as conjugation extension units to broaden the absorption and highly conjugated π-systems exert positive contributions to the PCE. On the contrary, features negatively contributing to PCE are the followings: fragments which lower the tendency of localized π–π* transition, fragments related to larger volume and surface area of dyes along with hydrophobicity resulting in poor adhesion, fragment RC = N causing dye hydrolysis, steric hindrance for π electronic mobility, fragments enhancing polarity, *etc*. The identified features from the best QSPR model of the coumarin dataset was employed in designing of ten more efficient coumarin dyes (predicted %PCE ranging from 8.93 to 10.62) than the existing ones.

1. Introduction

A dye-sensitized solar cell (DSSC) is a molecular photovoltaic (PV) system that mimics nature's photosynthesis principle employing a dye to absorb solar radiant energy to generate charge carriers which are then separated, transported and collected as harnessed solar electricity [1]. In the last decade, the DSSCs have attracted considerable attention as an alternative renewable energy source, and there is an extensive ongoing effort towards the design of organic dyes for DSSCs with high power conversion efficiency (PCE) to surpass some of the disadvantages of the previous inorganic solar cell systems like limiting weight, reducing cost, improving resources, and performing in an environment friendly manner [2]. In DSSCs, the dye acts as a photoactive component which converts photon to electricity through a series of stages where dye is

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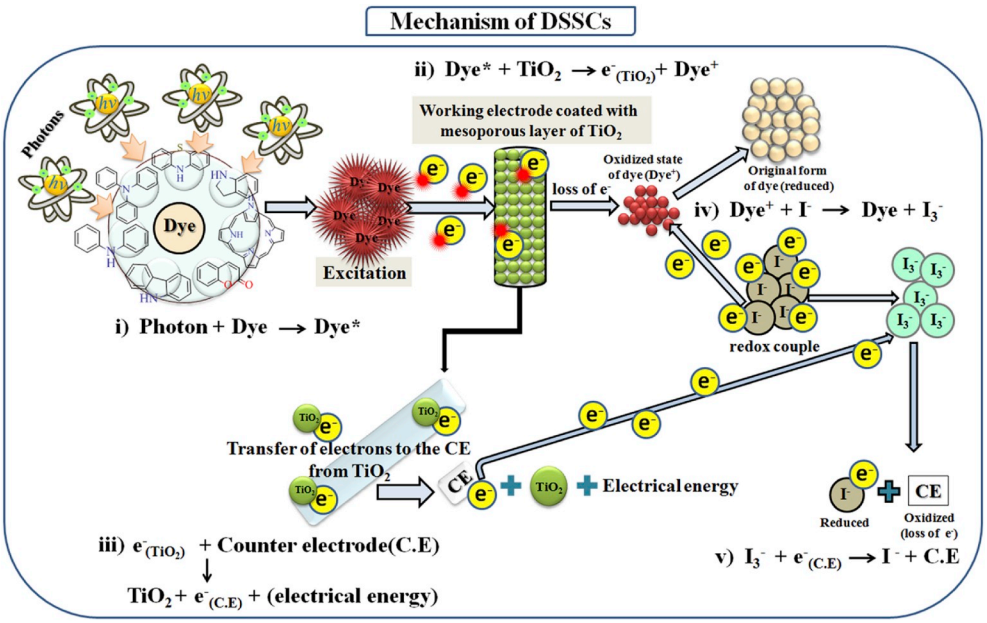


Fig. 1. General mechanism of Dye sensitized solar cells (DSSCs).

coated to the wide band gap semiconductor (in most of the cases TiO_2) known as working electrode (Fig. 1). The DSSC is prepared with a mixture of high-performance electrolytes like iodide/triiodide (I^-/I_3^-) redox shuttle and additives [3]. The sensitizer is the key element as it controls the photon harvesting and electron transport into the nano-structured semiconductor surface and can offer a mean to govern the interfacial behavior of electron transfer at the TiO_2/dye/electrolyte interface [4]. Most of the organic dyes in DSSCs are prepared based on the donor-acceptor (D-A)-like structure linked through a conjugated π spacer such as polyene and oligothiophene (D–π–A) and usually have a rod like configuration. The electron donor units are composed of moieties like indoline, triarylamine, coumarin, *etc.*, while carboxylic acid, cyanoacrylic acid, and rhodamine units are used as electron acceptors to fulfill the requirement [4]. The performance of DSSCs depends on important quantum properties like the highest occupied molecular orbital (HOMO), the lowest unoccupied molecular orbital (LUMO), and their distributions in the photosensitizers. Thus, dye's molecular structure and its orientation in the form of well-established high-performance D−π−A structure framework [5] and/or D−A−π−A structure [6] (where D and A stand for donor and acceptor fragments and π is the conjugated linker between D and A), which combines an auxiliary acceptor, improves their intramolecular charge transfers (ICT) and diminishes the optical band gap, are very important areas to study. Therefore, keeping constant all other components of DSSCs and just by changing the chemical structure of dyes, one can develop highly power conversion efficient solar system.

Dye-sensitizers can majorly be of two types: inorganic metal-based dye sensitizers and organic-metal free dye-sensitizers. The organic dyes are more environment friendly as well as easy to be modified structurally. Thus, a good number of organic dyes are explored for DSSCs such as triphenylamines [7,8], indolines [6], diketopyrrolopyrroles [9], anthocyanin dyes [10], perylenes [11], carbazole dyes [12], *etc*. In the current situation, ruthenium [13] and Zn-based DSSCs achieve PCE of 13% [14] experimentally, while organic-metal free dyes (triazatruxene (TAT) based D−π–A dye) achieve PCE of 13.6% (as reported by Zhang et al. [15], which is the experimentally reported highest value at the present time). Interestingly, according to National Renewable Energy Laboratory solar cell efficiency chart, the DSSCs are the least performers among all existing solar cell types which shows the importance and requirement of more efficient and practical outcome-oriented research in this specific field. As optimum performance of DSSCs depends on multi-layered aspects and components, thus a proper scheme needs to be followed for the design of dyes where all possible parameters of an efficient dye should be checked initially even before its synthesis. Over the last few years, low cost computational method like quantitative structure-property relationship (QSPR) is a recognized *in silico* tool in designing of potential dyes for DSSCs [16]. Instead of designing sensitizer dyes blindly spending a considerable amount of time and money, QSPR models can be a fruitful and reasonable approach to explore multiple chemical classes in search of the best possible dyes for DSSCs with higher PCE values than the existing ones in the market [17].

Multiple QSPR models have previously been explored for the designing of solar cell systems [18–25]. Fullerene-based polymer solar cell systems have been designed by our group for the very first time [18,19] employing QSPR models and virtual screening strategically. Venkatraman et al. [20,21], Li et al. [22] and our group [23–25] are actively working in the designing of organic dyes for DSSCs from the least explored chemical classes like phenothiazines, indolines, tetrahydroquinolines, N,N′-dialkylanilines *etc*. We have already designed and proposed potentially efficient 'lead dyes' theoretically, employing QSPR models employing for 273 dyes followed by electrochemical and optoelectronic parameter evaluation which reported better (predicted) PCE values than the existing dyes, *i.e.* 18.88, 19.24, and 13.87 for tetrahydroquinolines, N,N′-dialkylanilines and indolines, respectively [23–25], inspiring synthesis and experimental studies of two efficient lead dyes from the N,N′-dialkylanilines family in future studies [17]. Venkatraman et al. [20] developed QSPR models based on eigenvalue (EVA) descriptors generated from vibrational frequencies which suggested the best goodness-of-fit as well as prediction capability. Venkatraman et al. [21] proposed effective de novo design employing QSPR analysis and projected five lead phenothiazine dyes where all designed compounds had predicted PCE values ranging from 9.2 to 9.52. Li et al. [22] developed cascaded QSPR models for the overall PCE using quantum chemical descriptors which successfully predicted the PCE for 354 organic dyes, offering a valuable tool for the design of future dye sensitizers with efficient PCE. It is obvious that the quantitative models not only predict the PCE of newly designed dyes, but also explore the structural as well as physicochemical features responsible for changes in PCE values which can be employed for future designing as well as alteration of structure scaffolds by experimentalists.

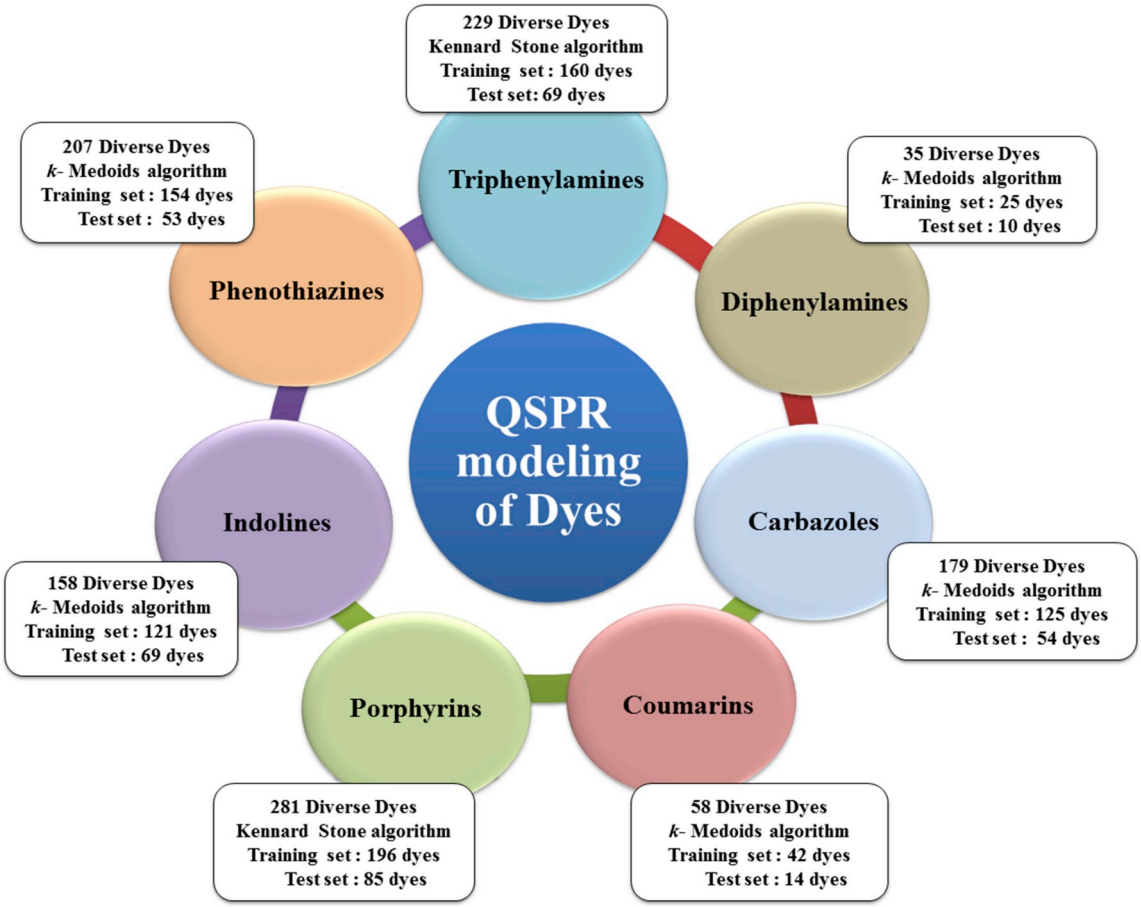


Fig. 2. Representation of various dye data sets used in QSPR modeling of dyes.

In this background, we have developed here multiple QSPR models for one of the biggest dye datasets for DSSCs consisting of around 1200 dyes covering seven important chemical classes, *i.e.* triphenylamines, phenothiazines, indolines, porphyrins, coumarins, carbazoles, and diphenylamines following all five principles of Organisation for Economic Co-operation and Development (OECD) for QSPR model development. The developed models should be significant tools for the prediction and screening of new and untested dye datasets as well as rich resources for the designing criteria of individual chemical classes, as each model has explored necessary structural fragments and molecular prerequisites for efficient dyes for DSSCs.

2. Materials and methods

2.1. Datasets

The experimental PCE values used for modeling of various chemical classes of dyes are obtained from the Dye Sensitized Solar Cell Database (DSSCDB) (https://www.dyedb.com/) [26]. Currently, the database is holding over 4000 experimental results for a diverse set of chemical classes. In a preliminary analysis of data, we have screened the dyes based on solar simulator (AM 1.5G 100 mW/cm2) and TiO_2 electrode. After that, we have divided the database into individual chemical classes of dyes; in this process, the mixture of dyes were discarded, the standalone chemical classes of dyes were separated for QSPR modeling. Since the response variable (PCE) refers to the energy terms, the modeling was performed without logarithmic conversion of the response (as customary in biological QSPR). Finally, around 1200 dyes were considered and classified into seven chemical classes and their experimental power conversion efficiency data were employed for QSPR modeling. The seven datasets consist of 244 Triphenylamines (%PCE range: 0.053–10.1), 215 Phenothiazines (%PCE range: 0.12–8.18), 170 Indolines (%PCE range: 0.046–9.2), 300 Porphyrins (%PCE range: 0.0013–12.5), 56 Coumarins (%PCE range: 0.33–7.4), 179 Carbazoles (%PCE range: 0.038–12.5), and 35 diphenylamine (%PCE range: 0.4–8) dyes. The details of the data sets are provided in a Supplementary Information excel file.

2.2. Descriptor calculation

"*The molecular descriptor is the ultimate result of a logical and mathematical operation which transfigures the chemical information encrypted within a symbolic representation of a molecule into the result of some regularized (standardized) experiments*" [27]. The dye structures were drawn by using the Marvin Sketch 5.10.0 software [28]. Dragon software version 7 [29] and PaDEL-descriptor 2.21 software [30] were employed for the computation of 2D descriptors covering constitutional, ring descriptors, connectivity index, functional group counts, atom centered fragments, atom type E-states, 2D atom pairs, molecular properties (Dragon Software) and extended topochemical atom (ETA) indices descriptors (PaDEL-Descriptor software). To identify and interpret the structural fragments and physicochemical properties with ease and to avoid conformational complexity, we have employed only 2D descriptors.

2.3. Data set division

Individual datasets were divided into a training and a test set using Kennard-Stone (Triphenylamine, Porphyrin datasets) and "Modified k-medoid" [31] (Phenothiazine, Indoline, Coumarin, Carbazole, and

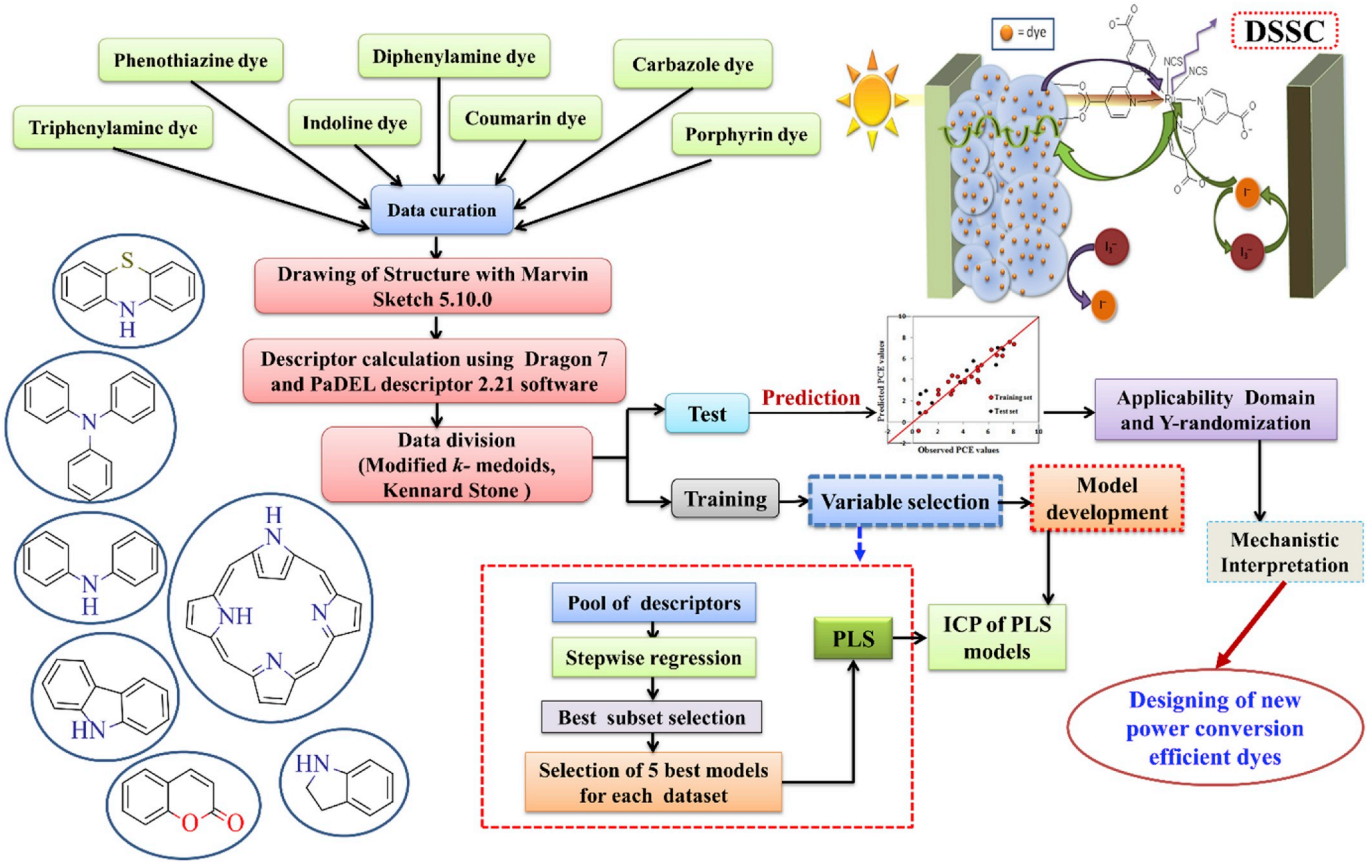


Fig. 3. Schematic representation of the steps involved in the development of QSPR models.

Diphenylamine datasets) algorithms. We have selected around 30% of dyes from each data set for the test set and the remaining 70% of compounds for the training set. The training set was used to develop the models, and the respective test set was used to validate the models for prediction purposes. The composition of training and test sets for the individual dataset is illustrated in Fig. 2.

2.4. Variable selection and model development

The descriptor matrices of the training sets were pre-treated to eliminate intercorrelated descriptors from all the data sets followed by "multistage" stepwise regression analysis was performed to remove less important descriptors from the initial large pool of descriptors [32–36]. In this way, we have selected some manageable number of descriptors and made a reduced pool of descriptors for further processing. After that, we have run best features selection separately for all datasets using the reduced pool of descriptors employing 'Best Subset selection v2.1 software' [37]. We have developed multiple 13 descriptor models for Triphenylamines, 14 descriptor models for Phenothiazines, 13 descriptors models for Indolines, 12 descriptor models for Porphyrins, 5 descriptors models for Coumarins, 11 descriptor models for Carbazoles and 4 descriptor models for Diphenylamines datasets. Among all models obtained from the best subset selection, we have selected the best five models based on Mean Absolute Error (MAE) [38] values in case of each dataset. Descriptors selected in all five multiple linear regression (MLR) models for individual datasets were further subjected to partial least squares (PLS) modeling separately to refine the models in terms of predictivity and robustness. The MLR and PLS models were developed using MINITAB [39] and SIMCA-P software [40], respectively.

To explore whether the quality of predictions of external (test) compounds could be enhanced through an "intelligent" selection of multiple models (here, five models), we have further used our in house Intelligent consensus predictor (ICP) tool [31,41]. The ICP software tool helps for finding and understanding the performance of consensus predictions in comparison to the quality obtained from the individual PLS models based on the MAE values (95%), as any single model may not be good for predictions for all test set compounds. The use of ICP is quite a rational approach for the prediction of test compounds considering multiple QSPR models developed from the same the training set. The steps involved in the development of PLS models are represented schematically in Fig. 3.

2.5. Statistical validation metrics, AD study and Y-randomization test

For judging goodness-of-fit and predictive ability of the developed QSPR models, we have checked the statistical quality employing both internal and external validation metrics. Statistical parameters like determination coefficient (R^2), explained variance (R^2_a), leave-one-out cross-validated correlation coefficient (Q^2_{LOO}), variance ratio (F), and standard error of estimate(s) were used to check the quality of training set fitting [42]. For external or test set validation, R^2_{pred} or Q^2F_1 and Q^2F_2 parameters were implemented [43]. We have also employed stringent validation metrics like r^2_m [43] and the mean absolute error (MAE) [38] values for both internal and external validation. The error based metrics were used to determine the true indication of the prediction quality in terms of prediction errors since they do not evaluate the performance of the model in comparison with the mean response [38]. Model Y-randomization test was performed using SIMCA-P software [40] to check whether the models are obtained by any chance or not. The applicability domain (AD) study was performed for each model using the DModX (distance to model X) approach at 99% confidence level using SIMCA-P software [40].

Table 1
Statistical quality and validation parameters obtained from the developed PLS models.

Dataset	Type of model	Training set				Test set					AD criteria	Dixon Q-test	Euclidian distance
		R^2	$Q^2_{(LOO)}$	$\overline{r^2_{m(LOO)}}$	$\Delta r^2_{m(LOO)}$	R^2_{pred} or Q^2F_1	Q^2F_2	$\overline{r^2_{m(test)}}$	$\Delta r^2_{m(test)}$	MAE (95%)			
Tri-phenyl amine	IM1 (LV:7)	0.67	0.60	0.47	0.19	0.60	0.60	0.47	0.20	1.06			
	IM2 (LV:6)	0.68	0.61	0.48	0.20	0.59	0.59	0.46	0.24	1.03			
	IM3 (LV:5)	0.67	0.61	0.48	0.20	0.59	0.59	0.47	0.18	1.04			
	IM4 (LV:6)	0.65	0.59	0.46	0.22	0.58	0.58	0.44	0.24	1.05			
	IM5 (LV:7)	0.67	0.60	0.47	0.20	0.60	0.60	0.47	0.19	1.06			
	CM1	–	–	–	–	0.61	0.61	0.49	0.25	1.04	NO	NO	0.4
	CM2	–	–	–	–	0.62	0.61	0.49	0.20	1.05			
	CM3	–	–	–	–	0.61	0.61	0.48	0.23	1.01			
Phenothiazine	IM1 (LV:6)	0.70	0.64	0.51	0.20	0.69	0.69	0.58	0.18	0.91			
	IM2 (LV:6)	0.70	0.64	0.51	0.20	0.69	0.69	0.58	0.18	0.91			
	IM3 (LV:6)	0.70	0.63	0.51	0.20	0.70	0.70	0.60	0.13	0.85			
	IM4 (LV:6)	0.70	0.63	0.51	0.20	0.70	0.70	0.60	0.13	0.85			
	IM5 (LV:6)	0.70	0.63	0.51	0.20	0.70	0.70	0.60	0.15	0.86			
	CM0	–	–	–	–	0.70	0.70	0.60	0.16	0.88	NO	NO	0.4
	CM1	–	–	–	–	0.71	0.71	0.60	0.18	0.87			
	CM2	–	–	–	–	0.71	0.71	0.61	0.18	0.87			
	CM3	–	–	–	–	0.73	0.73	0.63	0.18	0.83			
Indoline	IM1 (LV:7)	0.74	0.66	0.55	0.16	0.72	0.72	0.67	0.12	0.72			
	IM2 (LV:7)	0.74	0.66	0.54	0.17	0.68	0.68	0.63	0.14	0.74			
	IM3 (LV:6)	0.73	0.66	0.55	0.17	0.67	0.67	0.64	0.18	0.68			
	IM4 (LV:7)	0.73	0.65	0.53	0.17	0.71	0.71	0.66	0.12	0.73			
	IM5 (LV:7)	0.75	0.67	0.56	0.16	0.68	0.68	0.64	0.13	0.76			
	CM0	–	–	–	–	0.71	0.71	0.66	0.13	0.71	NO	NO	0.4
	CM1	–	–	–	–	0.71	0.71	0.66	0.12	0.70			
	CM2	–	–	–	–	0.71	0.71	0.66	0.13	0.69			
	CM3	–	–	–	–	0.74	0.74	0.69	0.15	0.66			
Porphyrin	IM1 (LV:6)	0.70	0.66	0.54	0.19	0.66	0.65	0.57	0.16	0.97			
	IM2 (LV:6)	0.70	0.66	0.54	0.19	0.66	0.66	0.57	0.16	0.96			
	IM3 (LV:6)	0.70	0.65	0.54	0.19	0.66	0.66	0.58	0.13	0.97			
	IM4 (LV:6)	0.70	0.65	0.54	0.19	0.67	0.66	0.58	0.13	0.96			
	IM5 (LV:5)	0.68	0.64	0.52	0.20	0.67	0.67	0.56	0.20	1.01			
	CM0	–	–	–	–	0.68	0.67	0.58	0.17	0.96			
	CM1	–	–	–	–	0.68	0.68	0.59	0.16	0.94	YES	YES	NO
	CM2	–	–	–	–	0.68	0.68	0.59	0.16	0.94			
	CM3	–	–	–	–	0.69	0.69	0.60	0.15	0.93			
Coumarin	IM1 (LV:2)	0.78	0.71	0.61	0.15	0.60	0.58	0.43	0.29	0.95			
	IM2 (LV:2)	0.74	0.67	0.56	0.17	0.63	0.61	0.41	0.48	0.85			
	IM3 (LV:3)	0.75	0.67	0.57	0.15	0.61	0.59	0.36	0.33	0.89			
	IM4 (LV:2)	0.75	0.67	0.56	0.16	0.62	0.60	0.36	0.34	0.88			
	IM5 (LV:3)	0.71	0.65	0.54	0.19	0.68	0.66	0.53	0.24	0.84			
	CM0	–	–	–	–	0.65	0.63	0.42	0.30	0.88	0.4	NO	NO
	CM1	–	–	–	–	0.63	0.61	0.40	0.31	0.92			
	CM2	–	–	–	–	0.63	0.61	0.40	0.31	0.92			
	CM3	–	–	–	–	0.61	0.63	0.37	0.34	0.89			
Carbazole	IM1 (LV:5)	0.75	0.71	0.98	0.19	0.75	0.74	0.61	0.20	0.64			
	IM2 (LV:4)	0.75	0.70	0.99	0.20	0.73	0.71	0.58	0.21	0.65			
	IM3 (LV:5)	0.75	0.71	0.99	0.18	0.73	0.71	0.58	0.21	0.66			
	IM4 (LV:4)	0.74	0.70	1.01	0.19	0.74	0.72	0.57	0.21	0.64			
	IM5 (LV:4)	0.74	0.69	1.01	0.20	0.73	0.71	0.55	0.22	0.67			
	CM0	–	–	–	–	0.75	0.73	0.58	0.21	0.63	NO	NO	0.4
	CM1	–	–	–	–	0.75	0.73	0.58	0.20	0.63			
	CM2	–	–	–	–	0.75	0.73	0.58	0.20	0.63			
	CM3	–	–	–	–	0.75	0.73	0.58	0.21	0.63			
Di-phenyl amine	IM1 (LV:3)	0.88	0.81	0.74	0.01	0.83	0.83	0.62	0.15	0.65			
	IM2 (LV:2)	0.86	0.81	0.74	0.03	0.74	0.73	0.49	0.24	0.73			
	IM3 (LV:2)	0.87	0.82	0.74	0.12	0.83	0.82	0.70	0.14	0.77			
	IM4 (LV:2)	0.87	0.81	0.74	0.09	0.81	0.80	0.65	0.16	0.92			
	IM5 (LV:2)	0.88	0.82	0.76	0.04	0.80	0.79	0.57	0.19	0.65			
	CM0	–	–	–	–	0.84	0.83	0.62	0.16	0.61	NO	NO	NO
	CM1	–	–	–	–	0.84	0.83	0.62	0.16	0.61			
	CM2	–	–	–	–	0.84	0.83	0.63	0.15	0.61			
	CM3	-	-	-	-	0.85	0.84	0.74	0.12	0.65			

LV: Latent variable for PLS models; CM0: Ordinary consensus predictions; CM1: Average of predictions from 'qualified' Individual models; CM2: Weighted average predictions from 'qualified' Individual models; CM3: Best selection of predictions compound wise from 'qualified Individual models'; Best model for individual dataset is marked in bold.

3. Results and discussion

Statistically acceptable and robust individual (IM), as well as consensus models (CM), were developed as depicted in Table 1. Analyzing the obtained results, we found that in most of the cases, consensus predictions of multiple PLS models were better than the results obtained from the individual PLS models. From among all validation metrics, we have selected the best models based on $MAE_{(95\%)}$ to give more importance on the prediction error of test or external compounds. The CM3 model which signifies 'the best selection of compound wise

Box 1

IM1

$$PCE = 2.971 - 48.538 \times GD - 0.599 \times F06[N-O] + 1.587 \times B09[C-S] - 0.298 \times SdssC + 3.545 \times B06[C-O] - 2.415 \times nN(CO)2 - 2.992 \times NdsN - 2.805 \times (C-038) + 4.578 \times (nRC=N) - 0.685 \times F05[N-N] - 2.257 \times B07[O-S] - 2.597 \times (C-043) + 1.465 \times B06[O-S]$$

IM2

$$PCE = -1.208 - 56.313 \times GD - 0.310 \times SdssC - 0.703 \times F06[N-O] + 2.597 \times B06[C-O] + 5.304 \times (nRC=N) - 2.849 \times NdsN - 3.098 \times (C-038) - 2.201 \times nN(CO)2 + 85.425 \times X4Av - 0.654 \times F05[N-N] - 2.032 \times B07[O-S] - 2.817 \times (C-043) + 11.530 \times ETA\_Shape\_Y$$

IM3

$$PCE = 1.495 - 55.860 \times GD - 0.329 \times SdssC - 0.622 \times F06[N-O] + 3.116 \times B06[C-O] - 2.996 \times NdsN + 2.403 \times nN(CO)2 + 88.416 \times X4Av - 3.061 \times (C-038) + 4.744 \times (nRC=N) - 2.487 \times B07[O-S] - 0.556 \times F05[N-N] - 2.786 \times (C-043) + 1.632 \times B06[O-S]$$

IM4

$$PCE = 1.424 - 54.789 \times GD - 0.749 \times B02[N-O] - 0.511 \times F06[N-O] - 1.574 \times nN(CO)2 - 0.271 \times SdssC + 3.385 \times B06[C-O] + 83.242 \times X4Av - 3.152 \times NdsN + 4.815 \times (nRC=N) - 1.894 \times B07[O-S] - 2.657 \times (C-038) - 0.595 \times F05[N-N] - 2.570 \times (C-043)$$

IM5

$$PCE = 2.971 - 48.538 \times GD - 0.599 \times F06[N-O] + 1.587 \times B09[C-S] - 0.298 \times SdssC + 3.545 \times B06[C-O] - 2.415 \times nN(CO)2 - 2.992 \times NdsN - 2.805 \times (C-038) + 4.578 \times (nRC=N) - 0.685 \times F05[N-N] - 2.257 \times B07[O-S] - 2.597 \times (C-043) + 1.465 \times B06[O-S]$$

predictions from the selected individual models' is the winner model for following datasets: tri-phenylamines, phenothiazines, indolines, and porphyrins. However, all four consensus models evolved as the winner model in case of the carabazole dataset, whereas CM0 (ordinary consensus predictions), CM1 (average of predictions from 'qualified' Individual models) and CM2 (weighted average predictions from 'qualified' Individual models) models are winner for the diphenylamine dataset. In contrast, in case of the coumarin dataset, individual model 5 (IM5) is the best model. It is quite evident from the outcome that predictability of consensus models is much better than the individual models; the former not only nullifies the error of predictions from an individual model but also enhances the reliability of the predictions for the true external dataset. All the individual models are mechanistically interpreted based on the modeled descriptors. In case of all the datasets, we have selected five PLS models in each case based on MAE values followed by development of consensus models. The selected models contain 13, 14, 13, 12, 5, 11 and 4 descriptors for the triphenylamine, phenothiazine, indoline, porphyrin, coumarin, carbazole, and diphenylamine datasets, respectively. To understand the order of significance of the modeled descriptors or variables in a descending order, we have prepared Variable Importance in Projection (VIP) plot for individual models of each dataset which can be found in Figs. S1–S7 in the Supplementary materials file.

3.1. Dataset 1: modeling of PCE property of triphenylamine dyes

The significant descriptors obtained from the five PLS models are indicated in Box 1. The mechanistic interpretation of all the descriptors is discussed below.

i) The atom type E-state descriptor NdsN represents number of nitrogen atoms with double and single bonds (=N-) contributing negatively to the PCE indicating that presence of this feature in the dye may decrease the PCE as reflected for following examples: **40** (NdsN = 1; PCE = 0.44), **161** (NdsN = 1; PCE = 0.45) and **144** (NdsN = 1; PCE = 0.18) and *vice versa* in case of dyes like **97** (NdsN = 0; PCE = 7.83), **204** (NdsN = 0; PCE = 8.06) and **238** (NdsN = 0; PCE = 10.1). This fragment lowers the tendency of localized π–π* transition due to intramolecular charge transfer transition (ICT) from the triphenylamine donor. As a result, the absorption maxima will decrease and thereby the PCE values may decrease [44].

ii) The 2D atom pair descriptor B06[C–O] denotes presence/absence of carbon and oxygen atoms at the topological distance 6, which contributed positively towards the PCE due to its positive regression coefficients. Thus, presence of this fragment in the dye molecules may increase the PCE property as shown in dyes **162** (B06[C–O] = 1; PCE = 7.78), **178** (B06[C–O] = 1; PCE = 6.95) and **189** (B06[C–O] = 1; PCE = 6.82) and *vice versa* in case of dyes **166** (B06[C–O] = 0; PCE = 0.087), **169** (B06[C–O] = 0; PCE = 0.058) and **171** (B06[C–O] = 0; PCE = 0.093). Presence of this group in the dye molecules leads to bathochromic shift of the absorption spectrum and enhancement of the molar extinction coefficient of the dye which directly causes higher PCE [45].

iii) The 2D atom pair descriptor B07[O–S] describes the presence/absence of oxygen and sulfur atoms at the topological distance 7, contributing negatively towards the PCE. Due to the presence of this fragment in the donor groups, there is a narrowing of the absorption range of dyes which causes latency decrease of rapid π-conjugation [46]. Eventually, the PCE values decrease as shown in the dyes **63** (B07[O–S] = 1, PCE = 0.77), **67** (B07[O–S] = 1; PCE = 0.45) and **144** (B07[O–S] = 1; PCE = 0.18). In contrast, the dyes having no such fragments may experience an enhancement in the PCE property as shown in dyes **14** (B07[O–S] = 0, PCE = 6.22), **95** (B07[O–S] = 0, PCE = 6.51) and **107** (B07[O–S] = 0, PCE = 7.67).

iv) Another 2D atom pair descriptor B09[C–S] denotes the presence/absence of carbon and sulfur atoms at the topological distance 9 which signifies longer chain in a molecule where sulfur atom is a part of thiophene ring resulting in a slight hypsochromic and a hypochromic effect in the ICT band. This may be explained by the steric hindrance induced by the branched-chain that increases the torsion angle between the triphenylamine moiety and the thiophene unit. This torsion impedes good delocalization of the π electrons and blue-shifts the position of the ICT band and augment the absorption [47]. Thus, the dyes having such fragment may have an enhanced PCE property as shown in dyes **14** (B09[C–S] = 1, PCE = 6.22), **94** (B09[C–S] = 1, PCE = 7.03) and **95** (B09[C–S] = 1, PCE = 6.51) and *vice versa* in case of dyes **8**

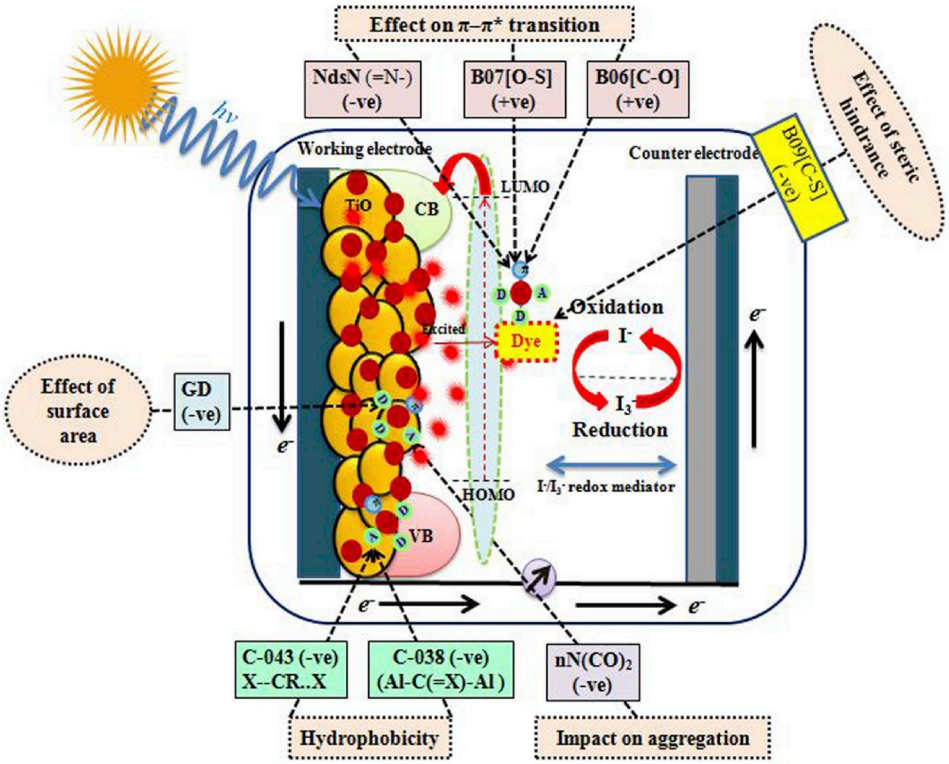


Fig. 4. Contribution of features on controlling PCE values of the Triphenylamine dyes.

(B09[C–S] = 0, PCE = 0.08), **9** (B09[C–S] = 0, PCE = 0.83) and **40** (B09[C–S] = 0, PCE = 0.44).

v) The atom-centered fragment descriptor C-038 represents the Al–C(=X)-Al fragment (where, Al:aliphatic groups and X: any electronegative atoms like O, N, S, P, Se, halogens) while another descriptor C-043 represents X–CR..X (R: any group linked through carbon) [48,49]. Both descriptors contributed negatively towards the PCE property by contributing to hydrophobicity and acting as a buffer between the semiconductor and the electrolyte, thus effectively preventing back-transfer of electrons from the semiconductor's conduction band to the redox couple. Therefore, the charge recombination is reduced [50]. Thus, the PCE values may decrease in the dyes containing these fragments as shown in dyes like **68** (C-038 = 2, PCE = 0.95), **69** (C-038 = 2; PCE = 1.1) and **217** (C-038 = 1; PCE = 2.03) for C-038 descriptor); and **49** (C-043 = 2; PCE = 2.98), **72** (C-043 = 1, PCE = 3.34) and **91** (C-043 = 1, PCE = 1.02) for C-043 descriptor. On the other hand, the dyes having no such fragments may have enhanced PCE property as shown in dyes **97** (C-038 = 0 & C-043 = 0; PCE = 7.83), **204** (C-038 = 0 & C-043 = 0; PCE = 8.06) and **238** (C-038 = 0 & C-043 = 0; PCE = 10.1).

vi) The functional group count descriptor nN(CO) represents the number of imides(thio) in the dye structures. The negative regression coefficient of this descriptor indicates that presence of this fragment in the dye molecules may decrease the PCE property as shown in dyes **148** ($nN(CO)_2$ = 1; PCE = 0.18) and **170** ($nN(CO)_2$ = 2; PCE = 0.053) and *vice versa* in case of dyes **113** ($nN(CO)_2$ = 0; PCE = 7.21), **175** ($nN(CO)_2$ = 0; PCE = 7.28) and **234** ($nN(CO)_2$ = 0; PCE = 7.25**).** Presence of this feature favours dye hydrolysis which improves the aggregation property of the dye over the TiO_2 surface and improves the recombination reaction between redox electrolyte and electrons in the TiO_2 nanolayer. As a result, the linkage will be distorted and thereby the PCE values may decrease [51].

vii) The ETA_Shape_Y descriptor deals with size and branching in the molecular structure. This descriptor contributes positively towards the PCE property as indicated by its positive regression coefficient. The higher numerical value of this descriptor may enhance the bulk of dyes resulting in sensitized wide-bandgap in the nanostructured photoelectrode [52]. The PCE values may increase with an increase of this descriptor value as shown in the dyes **48** (ETA_Shape_Y = 0.366; PCE = 6.01), **120** (ETA_Shape_Y = 0.342; PCE = 7.66) and **179** (ETA_Shape_Y = 0.360; PCE = 7.58). On the other hand, the lower numerical value of this descriptor may decrease the PCE property as shown in dyes **40** (ETA_Shape_Y = 0.235; PCE = 0.44), **115** (ETA_Shape_Y = 0.241; PCE = 0.6) and **134** (ETA_Shape_Y = 0.170; PCE = 1.7).

viii) Graph density (GD) is derived from the H-depleted molecular graph and calculated from the following formula:

$$GD = \frac{2.nBo}{nSK.(nSK-1)}$$

Here, nBO is the number of graph edges (i.e., non-H bonds) and nSK is the number of vertices in the graph (i.e., non-H atoms). This descriptor indicates the surface area of the dye which leads to prolongation of the electron injection into the nano-structured TiO_2 [53]. Thus, higher surface area may decrease the PCE property of dyes in DSSC as evident from the negative contribution. The higher numerical values of this descriptor may decrease the PCE property as shown in dyes **8** (GD = 0.104; PCE = 0.08), **9** (GD = 0.098; PCE = 0.83) and **115** (GD = 0.133; PCE = 0.6) and *vice versa* in case of dyes **143** (GD = 0.019; PCE = 6.6), **204** (GD = 0.017; PCE = 8.06) and **243** (GD = 0.0189; PCE = 6.69).

ix) The positive regression coefficients of 2D atom pair descriptor B06[O–S] (presence or absence of oxygen and sulfur atoms at the topological distance 6) and the connectivity index X4Av (average valence connectivity index of order 4) as well as the functional group count descriptor nRC = N (number of aliphatic imines) indicate that presence of these fragments in the triphenylamine

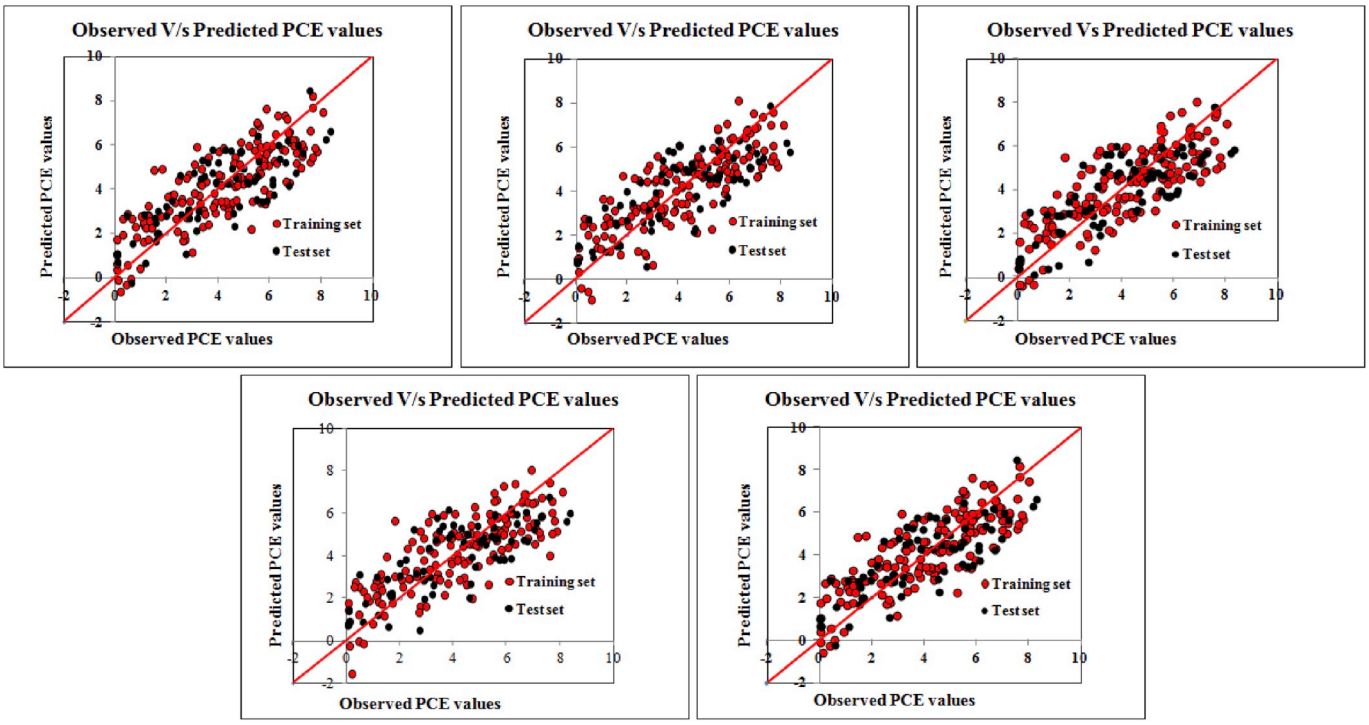


Fig. 5. Scatter plots of the observed and the predicted PCE values of the developed PLS models (IM1-IM5).

dye molecules may enhance the PCE property as shown in dyes **107** (B06[O–S] = 1; PCE = 7.67), **179** (B06[O–S] = 1; PCE = 7.58) and **122** (B06[O–S] = 1; PCE = 5.5) in case of B06[O–S] descriptor; **88** (X4Av = 0.072; PCE = 6.65), **89** (X4Av = 0.067; PCE = 7.6) and **239** (X4Av = 0.062; PCE = 6.91) for X4Av descriptor; and **70** (nRC = N = 2; PCE = 3.3) and **71**(nRC = N = 2; PCE = 3.9) for nRC = N descriptor and *vice versa* in case of dyes **7** (B06[O–S] = 0; PCE = 1.78), **39** (B06[O–S] = 0; PCE = 1.49) and **91** (B06[O–S] = 0; PCE = 1.02); **115** (X4Av = 0.028; PCE = 0.6), **119** (X4Av = 0.029; PCE = 1.1) and **159** (X4Av = 0.028; PCE = 0.3); **144** (nRC = N = 0; PCE = 0.18) and **166** (nRC = N = 0; PCE = 0.087), respectively.

x) The negative regression coefficient of 2D atom pair descriptors like F05[N–N] (frequency of two nitrogen atoms at the topological distance 5), F06[N–O] (frequency of nitrogen and oxygen atoms at topological distance 6), B02[N–O] (presence or absence of nitrogen and oxygen atoms at the topological distance 2) and the atom type E-state descriptor SdssC (sum of double, single and single bonded carbon E-states) (=C<) indicate that presence of these fragments in triphenylamine dye molecules may decrease the PCE property as shown in dyes **149** (F05[N–N] = 1; PCE = 2.6), **156** (F05[N–N] = 1; PCE = 2.2) and **159** (F05[N–N] = 1; PCE = 0.3) for F05[N–N]; **63** (PCE = 0.77), **166** (PCE = 0.087) and **171** (PCE = 0.093) for F06[N–O], **67** (PCE = 0.45), **87** (PCE

Box 2

IM1

$$PCE = 61.755 + 2.021 \times B04[N-O] - 2.604 \times B04[O-S] - 0.719 \times (H-052) + 1.727 \times B08[C-O] + 1.552 \times B07[N-S] + 0.438 \times O\% - 103.322 \times Mi + 59.547 \times X0A + 3.926 \times totalcharge - 1.520 \times F09[S-S] + 1.348 \times B10[C-S] - 0.127 \times F05[C-O] + 0.002 \times D/Dtr05 + 0.370 \times (C-022)$$

IM2

$$PCE = 61.755 + 2.021 \times B04[N-O] - 2.604 \times B04[O-S] - 0.719 \times (H-052) + 1.727 \times B08[C-O] + 1.552 \times B07[N-S] + 0.438 \times O\% - 103.322 \times Mi + 59.547 \times X0A + 3.926 \times totalcharge - 1.520 \times F09[S-S] + 1.348 \times B10[C-S] - 0.127 \times F05[C-O] + 0.002 \times D/Dtr05 + 0.370 \times nR\#C -$$

IM 3

$$PCE = 64.630 + 2.348 \times B04[N-O] - 2.711 \times B04[O-S] + 1.632 \times B08[C-O] + 1.561 \times B07[N-S] - 102.274 \times Mi + 0.418 \times O\% + 0.978 \times F04[O-O] + 3.789 \times totalcharge - 0.133 \times F05[C-O] + 53.834 \times X0A - 1.530 \times F09[S-S] + 0.002 \times D/Dtr05 + 0.424 \times nR\#C - + 1.243 \times B10[C-S]$$

IM4

$$PCE = 64.630 + 2.348 \times B04[N-O] - 2.711 \times B04[O-S] + 1.632 \times B08[C-O] + 1.561 \times B07[N-S] - 102.274 \times Mi + 0.418 \times O\% + 0.978 \times F04[O-O] + 3.789 \times totalcharge - 0.133 \times F05[C-O] + 53.834 \times X0A - 1.530 \times F09[S-S] + 0.002 \times D/Dtr05 + 0.424 \times (C-022) + 1.243 \times B10[C-S]$$

IM5

$$PCE = 63.969 + 2.333 \times B04[N-O] - 2.582 \times B04[O-S] + 1.568 \times B08[C-O] + 1.537 \times B07[N-S] - 104.701 \times Mi + 0.427 \times O\% + 4.242 \times totalcharge + 58.245 \times X0A - 1.897 \times F09[S-S] + 0.002 \times D/Dtr05 + 0.424 \times (C-022) - 0.117 \times F05[C-O] + 1.202 \times B10[C-S] + 0.824 \times B07[S-S]$$

= 0.63) and **103** (PCE = 1.12) for B02[N–O] descriptor; and **66** (SdssC = 4.65, PCE = 0.45) and **67** (SdssC = 3.08; PCE = 0.27) for SdssC descriptor. On other hand, absence of these fragments may be important for the PCE property of dyes as shown in the dyes like **162** (PCE = 7.78), **172** (PCE = 7.02) and **175** (PCE = 7.28) for F05[N–N] descriptor; **107** (PCE = 7.67), **113** (PCE = 7.21) and **177** (PCE = 6.95) for F06[N–O] descriptor; **97** (PCE = 7.83), **189** (PCE = 6.82) and **204** (PCE = 8.06) for B02[N–O] descriptor, **195** (PCE = 6.78), **205** (PCE = 6.57) and **231** (PCE = 7.67) for SdssC descriptor.

The mechanistic interpretation of the triphenylamine dyes from all models is schematically portrayed in Fig. 4. The scatter plots of observed vs. predicted PCE property related to the triphenylamine dyes of DSSCs for all the PLS models are shown in Fig. 5.

3.2. Dataset 2: modeling of PCE property of phenothiazine dyes

The significant descriptors along with the mathematical equations of the five PLS models are illustrated in Box 2. The modeled descriptors are discussed below in detail with their meaning along with how they influence the PCE values.

The constitutional descriptor total charge is defined as sum of the charges of the individual atoms which contributes positively towards the PCE property as indicated by its positive regression coefficient. The dyes with a higher value of the descriptor may push forward the π–π* transitions leading to the efficient ICT in the donor groups of dyes resulting in an increase in the PCE values [54]. Thus, the dyes bearing higher charges atoms may have higher PCE property as shown in the dyes **191** (Total charge = 1; PCE = 7.1) and **192** (Total charge = 1; PCE = 6.9). On the other hand, the dyes having lower charges may have low PCE property as evidenced from the dyes **62** (Total charge = 0; PCE = 0.4), **66** (Total charge = 0; PCE = 1.8) and **70** (Total charge = 0; PCE = 0.6).

The 2D atom pair descriptor B07[N–S] indicates the presence or absence of the nitrogen and sulfur atoms at the topological distance 7. The positive regression coefficient of this descriptor indicates presence of these two atoms at the topological distance 7 in the dye may increase the PCE as evidenced by dyes **51** (B07[N–S] = 1; PCE = 7.7), **141** (B07[N–S] = 1; PCE = 7.87) and **180** (B07[N–S] = 1; PCE = 6.64) and *vice versa* in case of dyes **114** (B07[N–S] = 0; PCE = 1.12), **155** (B07[N–S] = 0; PCE = 0.93) and **157** (B07[N–S] = 0; PCE = 0.12). Presence of nitrogen and sulfur atoms at the topological distance 7 improves the photo-excitation by increasing the localized π–π* transition of the dye [46]. The photo excitation of the dye increases the PCE property.

The constitutional descriptor O% denotes the percentage of oxygen atoms and has a positive contribution to the PCE property as evident from the dyes **78** (O% = 11.764; PCE = 6.72), **197** (O% = 11.764; PCE = 6.09) and **199** (O% = 10; PCE = 6.58). On the other hand, the dyes containing low number of oxygen atoms may decrease the PCE property as shown in dyes **111** (O% = 0; PCE = 0.7) and **112** (O% = 0; PCE = 1.06). Oxygen atoms are involved in the conduction of electrons (towards the excitation state) and they have a natural tendency to form a closely packed structure (https://neutronsources.org/news/scientific-highlights/neutron-power-finding-useful-oxygen-atoms-and-ions.html) [55]. As a result, the higher conductivity carriers in the dyes enhance the PCE property.

The 2D atom pair descriptor B08[C–O] indicates the presence/absence of carbon and oxygen atoms at the topological distance 8 with positive effects to the PCE property as shown in the dyes **5** (B08[C–O] = 1; PCE = 6.98), **122** (B08[C–O] = 1; PCE = 6.82), **142** (B08[C–O] = 1; PCE = 7.98) and **158** (B08[C–O] = 1; PCE = 7.33) and *vice versa* in case of dyes **27** (B08[C–O] = 0; PCE = 1.88), **98** (B08[C–O] = 0; PCE = 2.66) and **112** (B08[C–O] = 0; PCE = 1.06). Presence of carbon and oxygen atoms at the topological distance 8 signifies the effect of donor and additional donor through a linkage in the dye system. This specific structural fragment helps to achieve absorption band broadening which inflences the PCE property of dye molecules [54]. Thus, the broadening of the absorption band increases the PCE property of dye molecules in the solar cell system.

Another 2D atom pair descriptor B04[N–O] is defined as the presence or absence of nitrogen and oxygen atoms at the topological distance 4 which positively contributes to the PCE property as evident from the dyes **12** (B04[N–O] = 1; PCE = 6.29), **127** (B04[N–O] = 1; PCE = 6.87) and **129** (B04[N–O] = 1; PCE = 8.08). On the contrary, dyes like **56** (B04[N–O] = 0; PCE = 0.73), **57** (B04[N–O] = 0; PCE = 0.33) and **70** (B04[N–O] = 0; PCE = 0.6) have low PCE values. This descriptor signifies the distance between the nitrogen and the oxygen atoms which is referred to a strong cyano acceptor and a chelating anchoring mode of the carboxylation which plays a crucial role to regulate the PCE property of dyes [56]. Thus, the presence of strong acceptor and chelating anchors in dye leads to higher PCE values.

The connectivity descriptor X0A denotes average connectivity index of order 0 and the ring descriptor, D/Dtr05, states distance/detour ring index of order 5. These descriptors have an impact on the surface area of the dye molecules. The positive regression coefficients of these descriptors indicated that the dyes having large surface areas may have higher PCE property as shown in the dyes **12** (X0A = 0.713; PCE = 6.29), **63** (X0A = 0.728; PCE = 6.8) and **126** (X0A = 0.708; PCE = 7.44) (in case of X0A descriptor) and other dyes like **10** (D/Dtr05 = 807; PCE = 6.67), **75** (D/Dtr05 = 674; PCE = 7.3) and **186** (D/Dtr05 = 782; PCE = 7.94) **(**in case of D/Dtr05 descriptor**)**. On the contrary, lower numerical values of these descriptors may reduce the PCE value of dye molecules as shown in dyes like **27** (X0A = 0.696, D/Dtr05 = 0; PCE = 1.83), **56** (X0A = 0.701, D/Dtr05 = 0; PCE = 0.99) and **57** (X0A = 0.697, D/Dtr05 = 0; PCE = 0.73). Large surface area of the dyes may affect the photon capturing ability due to the sensitized wide band gap in the photo electrode [52]. As a result of the sensitized wide band gap in the nano-structured photo electrode, the PCE values may be enhanced.

The atom centered fragment descriptor H-052 denotes H^e attached to $C0(sp^3)$ with 1X attached to next carbon (where, X: any electronegative atom O, N, S, P, Se, halogens; the superscript ^{e} represents the formal oxidation number) which has a negative contribution to the PCE. Thus, the dyes bearing this fragment may have lower PCE values as shown in the dyes **56** (H-052 = 2; PCE = 0.73), **57** (H-052 = 2; PCE = 0.99) and **65** (H-052 = 1; PCE = 1.3), whereas the dyes **141** (H-052 = 0; PCE = 7.87), **142** (H-052 = 0; PCE = 7.98) and **143** (H-052 = 0; PCE = 8.06) showed higher PCE values as these dyes are devoid of this fragment. The presence of this fragment favors the lipophilicity of the dyes which causes alterations in the energy cascade as a result of the physicochemical changes occurring in the dyes [50]. Thus, the physicochemical alterations such as poor solubility on semiconductors' porous layer decreases the PCE values.

The constitutional descriptor Mi represents the mean first ionization potential (scaled on a carbon atom) which shows a negative contribution to the PCE. It was found in case of dyes **41** (Mi = 1.049; PCE = 2.14), **61** (Mi = 1.041; PCE = 1.3) and **200** (Mi = 1.037; PCE = 2) that with an increase in the value of the mean first ionization potential, there is a significant decrease in the PCE and *vice versa* in case of dyes **44** (Mi = 1.012; PCE = 7.48), **45** (Mi = 1.011; PCE = 6.56) and **100** (Mi = 1.010; PCE = 6.59). The mean first ionization potential is related to polarity of the molecules which plays an essential role to regulate the PCE property of dyes in solar cell. Due to presence of small electronegative atoms, dyes behave like polar molecules. It is known that polarity tends to attain the aggregation of dye molecules on the semiconductor [51]. Thus, for enhancement of the PCE property of dye molecules, the mean first ionization potential of dye molecules should be low.

The positive regression coefficient of the 2D atom pair descriptors like B10[C–S] (presence or absence of carbon and sulfur atoms at the topological distance 10), B07[S–S] (presence or absence of 2 sulfur atoms at the topological distance 7) and F04[O–O] (the frequency of the two oxygen atoms at the topological distance 4) as well as C-022 (which accounts for #CR/R = C = R; where R: any group linked through carbon;

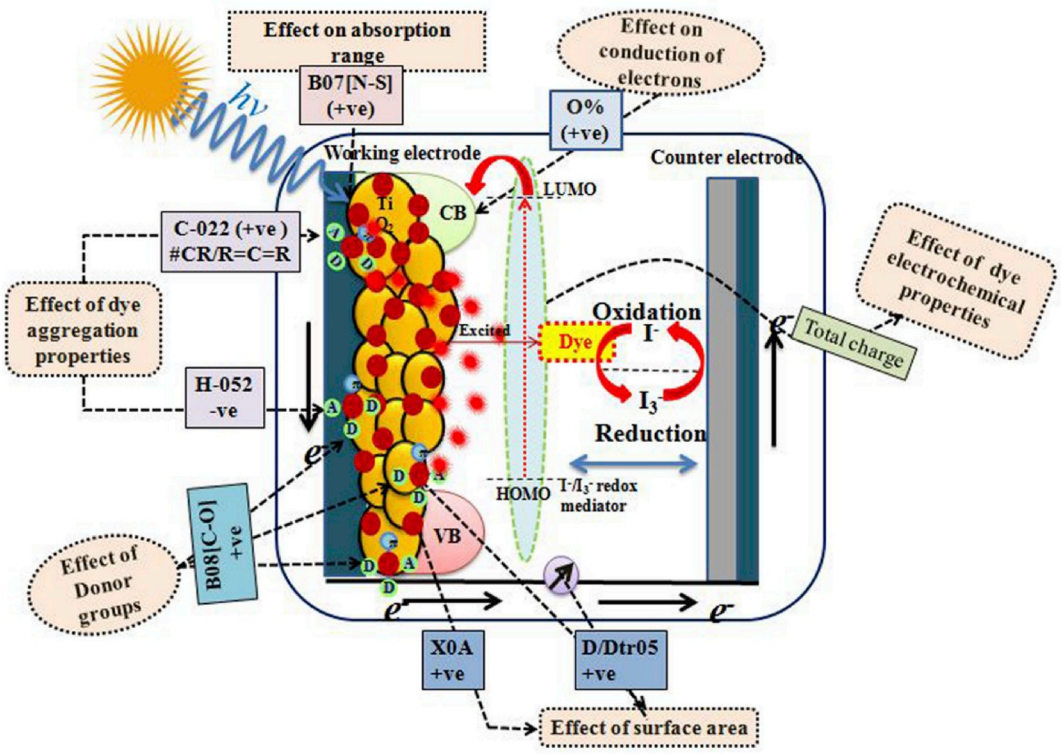


Fig. 6. Contribution of modeled descriptors on controlling PCE values of the Phenothiazine dyes.

X: any electronegative atom O, N, S, P, Se, halogens; = : a double bond; #: a triple bond) indicate that presence of such fragments in the dye molecules may enhance the PCE property in DSSCs as evidenced by the dyes **2** (PCE = 7.5), **3** (PCE = 6.87), **4** (PCE = 7.78) where the numerical value of B10[C–S] descriptor in all three cases is 1; **4** (PCE = 7.78), **78** (PCE = 6.78), **126** (PCE = 7.44), where the numerical value of B07[S–S] descriptor in all these cases is 1; **2** (PCE = 7.5), **51** (PCE = 7.7) and **191** (PCE = 7.1) for which the numerical value of F04[O–O] descriptor in all these cases is 1; and **179** (PCE = 7.44), **180** (PCE = 6.64), **182** (PCE = 6.06) where the numerical value of C-022 in all these examples is 4. On the other hand, the absence of these fragments may reduce the PCE property as shown in the dyes **1** (PCE = 2.71), **114** (PCE = 1.12) and **189** (PCE = 2.82) for B10[C–S] fragment; **61** (PCE = 1.3), **65** (PCE = 1.3) and **68** (PCE = 1.2) for B07[S–S] descriptor; **27** (PCE = 1.83) and **67** (PCE = 1.9) for F04[O–O] descriptor; and **56** (PCE = 0.73), **57** (PCE = 0.99) and **70** (PCE = 0.6) for C-022 fragment.

On the contrary, 2D atom pair descriptors like B04[O–S] (presence or absence of oxygen and sulfur atoms at topological distance 4), F09[S–S] (frequency of 2 sulfur atoms at topological distance 9) and F05[C–O] (frequency of carbon and oxygen atoms at topological distance 5)

Box 3

IM1

$$PCE = 2.982 + 0.907 \times nCrq + 0.992 \times B07[N-N] - 1.520 \times B04[S-S] - 0.733 \times NsssN + 0.272 \times F04[C-N] - 2.060 \times B06[N-N] + 1.382 \times B05[O-S] - 6.394 \times SaaaC + 0.089 \times F10[C-N] - 0.825 \times B05[S-S] + 0.930 \times F07[N-S] + 0.649 \times B09[O-S] + 0.642 \times F07[N-O]$$

IM2

$$PCE = 3.004 + 1.042 \times nCrq + 1.224 \times B07[N-N] + 0.268 \times F04[C-N] - 1.206 \times F04[S-S] - 2.200 \times B06[N-N] + 1.327 \times B05[O-S] - 6.174 \times SaaaC + -0.097 \times F10[C-N] - 0.949 \times B05[S-S] + 0.639 \times F07[N-O] + 0.997 \times F07[N-S] + 0.482 \times B09[O-S]$$

IM3

$$PCE = 3.119 + 1.045 \times nCrq + 0.866 \times F07[N-N] - 0.291 \times B04[S-S] + 0.278 \times F04[C-N] - 6.600 \times SaaaC - 0.758 \times NsssN - 1.162 \times F04[S-S] + 1.404 \times B05[O-S] - 1.968 \times B06[N-N] - 0.111 \times F10[C-N] + 1.006 \times F07[N-S] - 1.014 \times B05[S-S] + 0.734 \times F07[N-O]$$

IM4

$$PCE = 3.161 + 1.123 \times B07[N-N] - 0.950 \times nCrq - 1.549 \times B04[S-S] - 0.039 \times nCconj + 0.276 \times F04[C-N] - 0.697 \times NsssN - 2.059 \times B06[N-N] + 1.288 \times B05[O-S] - 6.516 \times SaaaC - 0.095 \times F10[C-N] + 1.018 \times F07[N-S] + 0.644 \times F07[N-O] - 0.720 \times B05[S-S]$$

IM5

$$PCE = 3.119 + 0.922 \times nCrq + 1.123 \times B07[N-N] - 1.101 \times B02[N-O] - 0.849 \times B04[S-S] + 0.287 \times F04[C-N] - 0.576 \times NsssN + 1.119 \times B05[O-S] - 6.387 \times SaaaC - 2.143 \times B06[N-N] - 0.707 \times F10[O-S] - 0.124 \times F10[C-N] + 0.665 \times F07[N-O] + 1.056 \times F07[N-S]$$

contribute negatively towards the PCE property of dyes in DSSC. The negative regression coefficients of these descriptors indicate that presence of such features in the structures of dye molecules may reduce the PCE property as observed in the dyes **65** (PCE = 1.3), **68** (PCE = 1.2), **71** (PCE = 1.3) for which the numerical value of B04[O–S] is 1 in all cases; dye **206** (PCE = 1.5) for which the F09[S–S] value is 2; **155** (PCE = 0.12) and **157** (PCE = 0.93) with F05[C–O] descriptor values of 20 and 19, respectively. On the other hand, absence of such features in the dyes may enhance the PCE property in DSSCs as shown in the dyes like **51** (PCE = 7.7), **132** (PCE = 6.13), **180** (PCE = 6.64) for B04[O–S] and **141** (PCE = 7.87) for F09[S–S]. Dyes **126** (PCE = 6.9) and **192** (PCE = 7.44) showed quite higher PCE due to much lower descriptor values for F05 [C–O] than other dyes *i.e.*2.

The mechanistic interpretation of the phenothiazine dyes from all the models is schematically portrayed in Fig. 6. The scatter plots of observed vs. predicted PCE property related to the Phenothiazine dyes for all the PLS models are depicted in Fig. S8 in Supplementary material.

3.3. Dataset 3: modeling of PCE property of indoline dyes

Significant five PLS models are reported in Box 3. The descriptors appearing in the models are explained below with the most feasible mechanistic interpretation towards the PCE property of dyes in DSSCs.

The positive regression coefficients of the 2D atom pair descriptors like F04[C–N] (frequency of carbon and nitrogen atoms at topological distance 4), F07[N–S] (frequency of nitrogen and sulfur atoms at topological distance 7) and F07[N–O] (frequency of nitrogen and oxygen atoms at topological distance 7) indicate that presence of these fragments in the indoline dyes may enhance the PCE as shown in dyes **13** (F04[C–N] = 21; PCE = 8.43), **18** (F04[C–N] = 18; PCE = 7.28) and **24** (F04[C–N] = 18; PCE = 9.2) for F04[C–N]; **8** (F07[N–S] = 1; PCE = 7.12), **107** (F07[N–S] = 2; PCE = 7.63) and **146** (F07[N–S] = 4; PCE = 6.71) for F07[N–S]; and **43** (F07[N–O] = 4; PCE = 5.4), **112** (F07[N–O] = 3; PCE = 6.51) and **115** (F07[N–O] = 4; PCE = 5.93) for F07[N–O] and *vice versa* in case of dyes **147** (F04[C–N] = 5, F07[N–S] = 0, F04 [N–O] = 0; PCE = 1.8), **149** (F04[C–N] = 4, F07[N–S] = 0, F04[N–O] = 0; PCE = 1.92) and **164** (F04[C–N] = 3, F07[N–S] = 0, F04[N–O] = 0; PCE = 2.08). The dyes containing donor group and groups with non-planar structure are very important for the PCE property. It was already reported that the above mentioned descriptors are present as a part of dye donors with non-planar structures of the Indoline dyes [54, 57].

The other 2D atom pair descriptors F10[O–S] (frequency of oxygen and sulfur atoms at the topological distance 10) and F10[C–N] (frequency of carbon and nitrogen atoms at the topological distance 10) contributed negatively towards the PCE of indoline dyes. These descriptors actually represent bulk of dye molecules which may weaken the interactions between the semiconductor and the dye molecules due to steric hindrance followed by restriction of the transfer of electrons from the dye molecules to the semiconductor [58]. Therefore, the dyes bearing such fragments may reduce the PCE property of dyes in DSSC as shown in the dyes **14** (F10[O–S] = 2; PCE = 3.85), **53** (F10[O–S] = 2; PCE = 2.96), and **150** (F10[O–S] = 2; PCE = 2.1), for F10[O–S]; and **34** (F10[C–N] = 6; PCE = 2.7), **35** (F10[C–N] = 5; PCE = 1.2) and **55** (F10 [C–N] = 7; PCE = 3.9) for F10[C–N] and *vice versa* in case of dyes **44** (F10[O–S] = 0, F10[C–N] = 3; PCE = 6.96), **59** (F10[O–S] = 0, F10 [C–N] = 2; PCE = 8.34) and **94** (F10[O–S] = 0, F10[C–N] = 2; PCE = 8.42).

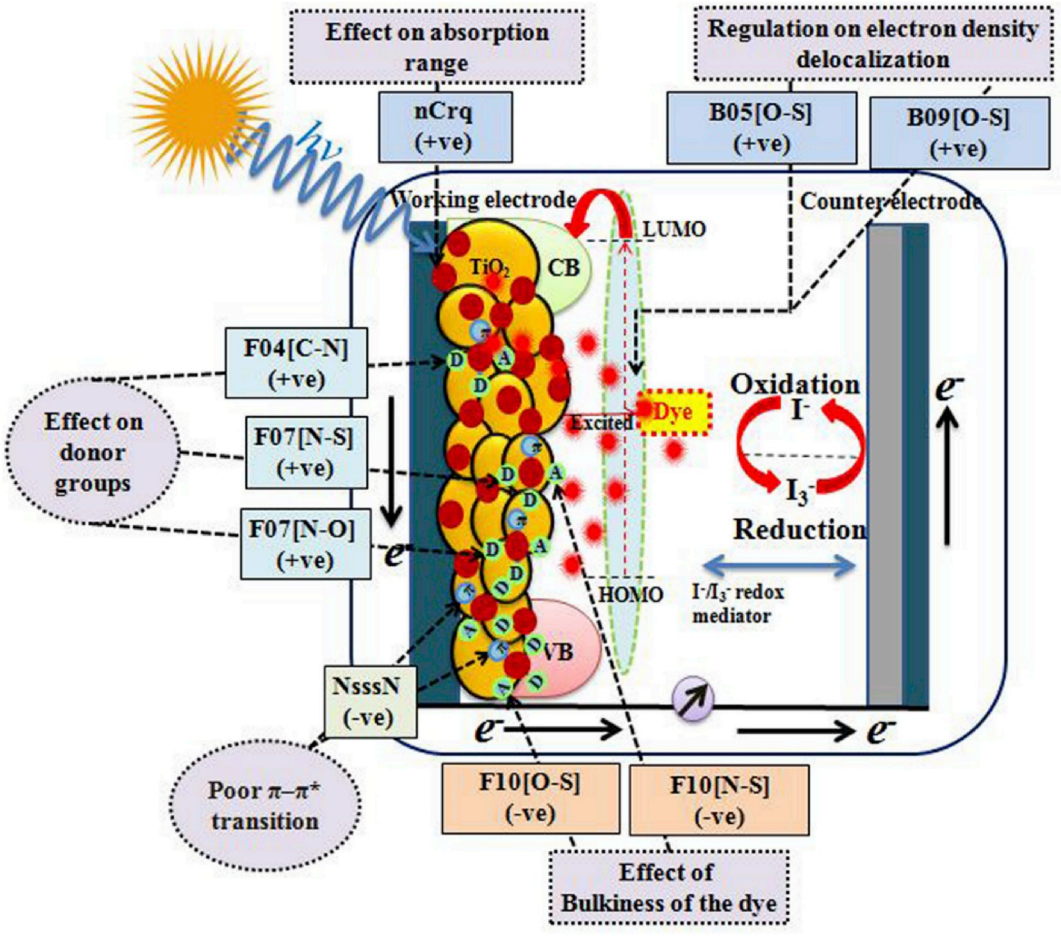


Fig. 7. Contribution of features on controlling PCE values of the Indoline dyes.

Box 4

IM1
PCE = − 16.058 + 0.560 × F06[O − X] + 0.296 × F07[C − X] − 0.421 × O% + 224.846 × X4A − 0.067 × F08[C − O] − 2.395 × B04[N − N] + 0.359 × F10[N − O] − 2.179 × (N − 072) − 1.155 × F03[N − X] + 2.267 × B05[N − S] − 0.191 × SdsCH + 0.887 × F03[N − O]
IM2
PCE = − 16.139 + 0.294 × F07[C − X] + 0.556 × F06[O − X] − 0.407 × O% + 224.846 × X4A − 0.071 × F08[C − O] − 2.434 × B04[N − N] + 0.347 × F10[N − O] − 2.130 × (N − 072) − 1.126 × F03[N − X] + 2.228 × B05[N − S] − 0.189 × SdsCH + 1.810 × B03[N − O]
IM3
PCE = − 14.909 − 0.429 × O% + 0.291 × F07[C − X] + 0.574 × F06[O − X] + 191.057 × X4A − 0.066 × F08[C − O] − 2.434 × B04[N − N] + 0.403 × F10[N − O] − 2.079 × (N − 071) + 2.651 × B05[N − S] − 2.079 × (N − 072) − 0.216 − × SdsCH − 1.196 × F03[N − X] + 0.988 × F03[N − O]
IM4
PCE = − 15.101 − 0.413 × O% + 0.288 × F07[C − X] + 0.571 × F06[O − X] + 193.418 × X4A − 0.070 × F08[C − O] + 0.390 × F10[N − O] − 1.157 × (N − 071) + 2.619 × B05[N − S] − 0.215 × SdsCH − 2.045 × (N − 072) − 1.173 × F03[N − X] + 1.946 × B03[N − O]
IM5
PCE = − 14.102 + 0.362 × F07[C − X] + 202.714 × X4A − 0.483 × O% + 1.237 × (N − 071) − 2.755 × B04[N − N] + 2.322 × B05[N − S] − 1.109 × F03[N − X] + 0.287 × F10[N − O] − 0.177 × SdsCH − 1.987 × (N − 072) + 0.802 × F03[N − O] − 1.101 × nR08

The functional group count descriptor nCrq indicates the number of ring quaternary carbons with sp^3 hybridization, and it has a positive contribution towards the PCE. The presence of ring quaternary carbon with sp^3 hybridization is essential for tunable absorption properties and it produces high molar extinction coefficients leads to improve the energy level reactions in the solar cell [59]. Thus, presence of higher number of sp^3 hybridized quaternary carbon atom in dyes may enhance the PCE as observed in dyes like **126** (nCrq = 1; PCE = 6.9), **144** (nCrq = 3; PCE = 8.78) and **157** (nCrq = 3; PCE = 7.08). In contrary, the absence of such type of fragment in the dyes may reduce the PCE as evidenced by the dyes **32** (nCrq = 0; PCE = 1.48), **93** (nCrq = 0; PCE = 0.35), and **129** (nCrq = 0; PCE = 1.48).

The count of atom-type E-State descriptor NsssN states the number of atoms of type sssN (), which has a negative contribution towards the PCE. Due to the presence of such fragments, the dyes experience poor π–π* transition (the fragment is less reactive than imines) which results in slow energy cascade mechanism [22]. Thus, the dyes containing such fragments may have lower PCE property in DSSCs as evidenced by dyes **27** (NsssN = 4; PCE = 2.12), **99** (NsssN = 3; PCE = 1.42) and **169** (NsssN = 3; PCE = 1.71) and *vice versa* in case of dyes **77** (NsssN = 0; PCE = 6.86), **78** (NsssN = 0; PCE = 7.99) and **146** (NsssN = 0; PCE = 6.71).

The positive coefficient of other 2D atom pair descriptors B05[O–S] (presence or absence of oxygen and sulfur atoms at topological distance 5) and B09[O–S] (presence or absence of oxygen and sulfur atoms at topological distance 9) indicated that presence of these fragments in dyes may increase the PCE property in DSSCs. In the dye system, oxygen and sulfur atoms regulate the electron density delocalization which is favorable for the π-bond conjugation. As a result, the molar extinction coefficient of the dye is enhanced which leads to the bathochromic shift of the absorption spectrum [45]. Therefore, the dyes containing these fragments may show good PCE property in DSSCs as evident by the dyes **143** (B05[O–S] = 1; PCE = 7.25), **144** (B05[O–S] = 1; PCE = 8.78) and **145** (B05[O–S] = 1; PCE = 7.4) for B05[O–S]; and **21** (B09[O–S] = 1; PCE = 6.12), **78** (B09[O–S] = 1; PCE = 7.99) and **131** (B09[O–S] = 1; PCE = 6.11) for B09[O–S] and *vice versa* in case of dyes **30** (B05[O–S], B09[O–S] = 0; PCE = 0.77), **32** (B05[O–S], B09[O–S] = 0; PCE = 0.63) and **35** (B05[O–S], B09[O–S] = 0; PCE = 1.2).

The 2D atom pair descriptors F07[N–N] (frequency of 2 nitrogen atoms at the topological distance 7), B07[N–N] (presence or absence of 2 nitrogen atoms at topological distance 7) and the functional group count descriptor nCconj (the number of non-aromatic conjugated carbon with sp^2 hybridization) contributed positively towards the PCE which indicates that the PCE values increase with an increase in the numerical value of these descriptors as shown in dyes **8** (B07[N–N] = 2; PCE = 7.12), **115** (B07[N–N] = 2; PCE = 5.93) for F07[N–N]; **135** (B07[N–N] = 1; PCE = 8.38), **141** (B07[N–N] = 1; PCE = 8.61) for B07[N–N]; and **13** (nCconj = 13; PCE = 8.43), **18** (nCconj = 13; PCE = 7.28) and *vice versa* in case of dyes **32** (F07[N–N] = 0, B07[N–N] = 0, nCconj = 1; PCE = 0.63), **93** (F07[N–N] = 0, B07[N–N] = 0, nCconj = 3; PCE = 0.35) and **108** (F07[N–N] = 0, B07[N–N] = 0, nCconj = 3; PCE = 0.046).

The 2D atom pair descriptor B06[N–N] represents the presence or absence of 2 nitrogen atoms at the topological distance 6. B04[S–S] means presence or absence of 2 sulfur atoms at the topological distance 4 and F04[S–S] stands for frequency of 2 sulfur atoms at the topological distance 4, B02[N–O] states presence or absence of nitrogen and oxygen atoms at the topological distance 2 and the atom centered fragment SaaaC represents sum of aromatic carbons (–C(–)–) (–represents an aromatic bond). The negative regression coefficients of these descriptors indicate that an increase in the numerical values of the descriptors may reduce the PCE values as observed in the dyes **93** (B06[N–N] = 1, B04[S–S] = 1 & F04[S–S] = 1, B02[N–O] = 1; PCE = 0.35), **108** (B06[N–N] = 1, B04[S–S] = 1 & F04[S–S] = 1, B02[N–O] = 1; PCE = 0.046), **30** (SaaaC = 0.697; PCE = 0.77) and **32** (SaaaC = 0.857; PCE = 0.63). In contrary, compounds having no such fragments may show higher PCE values as observed in case of dyes **18** (B06[N–N] = 0, B04[S–S] = 0, F04[S–S] = 0, B02[N–O] = 0; PCE = 7.08), **157** (B06[N–N] = 0, B04[S–S] = 0, F04[S–S] = 0, B02[N–O] = 0; PCE = 7.79), **13** (PCE = 8.43) and **144** (PCE = 8.78).

The mechanistic interpretation from all models is schematically portrayed in Fig. 7 for Indoline dyes. The scatter plots of observed vs. predicted PCE property related to the indoline dyes for all the PLS models are depicted in Fig. S9 in Supplementary material.

3.4. Dataset 4: modeling of PCE property of porphyrin dyes

The modeled descriptors for porphyrin dyes obtained from the five PLS models are illustrated in Box 4. The best possible mechanistic interpretation of the descriptors is discussed below with the examples of studied dyes.

The 2D atom pair descriptors F10[N–O] (frequency of N–O at

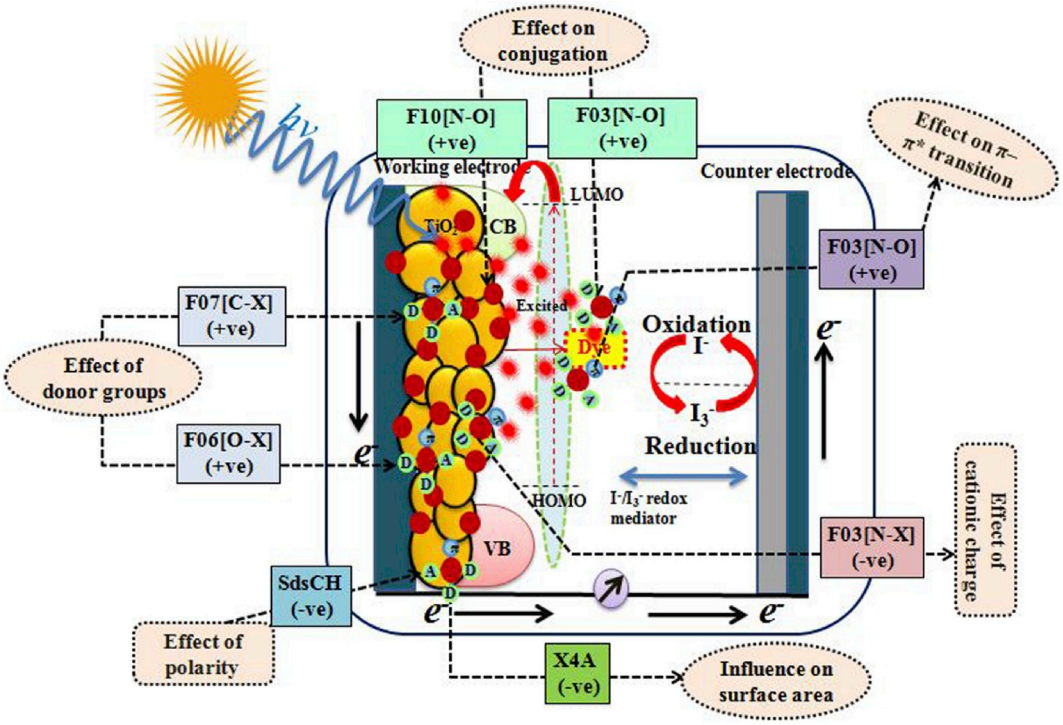


Fig. 8. Contribution of significant features on controlling PCE values of the porphyrin dyes.

topological distance 10) and F03[N–O] (frequency of N–O at topological distance 3) contributed positively towards the PCE. Thus, presence of such fragments in the dyes may enhance the PCE as showed in dyes **37** (F10[N–O] = 6; PCE = 9.1), **38** (F10[N–O] = 8; PCE = 7.2), **39** (F10[N–O] = 8; PCE = 6.9) for F10[N–O]; **34** (F03[N–O] = 2; PCE = 8.3), **208** (F03[N–O] = 1; PCE = 7.94), **213** (F03[N–O] = 1; PCE = 8.6) for F03[N–O], while absence of these fragments may reduce the PCE property as verified by the dyes **113** (PCE = 2.46), **265** (PCE = 0.63) and **271** (PCE = 0.38). Presence of nitrogen and oxygen atoms has a contribution to the special stability (conjugation) and allows the smaller HOMO-LUMO gap, followed by the red shift of the absorption spectrum [60]. Thus, the conduction valence edge level increases, and in result the PCE values of DSSCs increases.

The 2D atom pair descriptors F06[O-X] and F07[C-X] denote the frequency of carbon and heavy metal (X) at topological distance 6 and 7, respectively. They contribute positively towards the PCE. The descriptor F07[C-X] signifies the carbon is a part of meso aryl substituted portion of Zinc porphyrin which will act as a donor in the dye [61]. On the other hand, in case of F06[O-X], the oxygen atom is a part of the long-chain alkoxy group which impairs interfacial back electron transfer reaction [62]. Thus, electron donating ability and presence of electron back transfer groups may increase the PCE property as shown in the dye molecules **40** (F07[C-X] = 12, F06[O-X] = 4; PCE = 8.6), **48** (F07[C-X] = 12, F06[O-X] = 4; PCE = 8.42) and **99** (F07[C-X] = 10, F06[O-X] = 4; PCE = 9.19) and *vice versa* in case of dyes **63** (F07[C-X] = 0, F06[O-X] = 0; PCE = 0.42), **102** (F07[C-X] = 0, F06[O-X] = 0; PCE = 0.3) and **123** (F07[C-X] = 0, F06[O-X] = 0; PCE = 0.11).

The atom type E-state index SdsCH defines the E-state atom index of C atom of the fragment dsCH (=CH-) which contributes negatively to the PCE property. Therefore, presence of this fragment in the dyes decreases the PCE property as observed in dyes **96** (SdsCH = 25.43; PCE = 2.3), **263** (SdsCH = 18.19; PCE = 1.64) and **268** (SdsCH = 19.21; PCE = 2.37) and *vice versa* in case of dyes **141** (SdsCH = 1.41; PCE = 8), **217** (SdsCH = 1.39; PCE = 7.88) and **218** (SdsCH = 1.39; PCE = 8.14). Presence of this non-polar group makes the negative shift in the solvatochromic properties (ability of chemical substance to change color due to a change in polarity) of the dye. Thus, the dyes cannot adhere properly to the semiconductor which results in a negative effect on absorption and stability of the dye [63].

Another significant descriptor X4A indicates average connectivity index of order 4; it encodes the 'χ' value across four bonds which can be calculated on the basis of Kier and Hall's connectivity index [64]. It contributes positively towards the PCE. This indicates that the PCE property of dyes increases with an increase in the numerical value of this descriptor as shown in dyes **81** (X4A = 0.1123; PCE = 10.17), **196** (X4A = 0.1123; PCE = 9.25) and **201** (X4A = 0.1145; PCE = 10.24) and *vice versa* in case of dyes **42** (X4A = 0.097; PCE = 0.6), **45** (X4A = 0.096; PCE = 0.02) and **105** (X4A = 0.096; PCE = 1.1). This descriptor is related to surface area of dyes which is directly related to light-harvesting capability which could be achieved maximum when the surface area of the dyes is large [53].

The 2D atom pair descriptor B03[N–O] indicates the presence or absence of nitrogen and oxygen atoms at the topological distance 3 which offers a positive effect to the PCE property as evidenced by dyes **34** (PCE = 8.3), **213** (PCE = 8.6) and **214** (PCE = 8.7) due to their descriptor values being equal to 1 in all cases and *vice versa* (descriptor value zero) for dyes **44** (PCE = 0.0013), **45** (PCE = 0.02) and **286** (PCE = 0.03) in absence of this fragment. This fragment may represent the conjugation units to 'π' system of the dye, which engenders a lower internal resistance to the transport of positive charges. Thus, the conjugated π-system may enhance the PCE property of dye molecules in DSSC [65].

The 2D atom pair descriptor F03[N-X] denotes frequency of the nitrogens and heavy metal atoms (Zn) at the topological distance 3 which contributes negatively towards the PCE property as indicated by bearing dyes like **252** (F03[N-X] = 4; PCE = 0.73), **253** (F03[N-X] = 4; PCE = 1.54) and **292** (F03[N-X] = 4; PCE = 0.92) which have lower PCE values. Again, dyes not having this feature showed higher range of PCE as evidenced by the dyes **213** (F03[N-X] = 0; PCE = 8.6), **214** (F03[N-X] = 0; PCE = 8.7) and **216** (F03[N-X] = 0; PCE = 9.5). The presence of the nitrogen atom at topological distance 3 from Zinc in the porphyrin moiety has a positive influence on the cationic charge of the dye endowing the aggregation resulting in low PCE values [66].

The positive regression coefficients of 2D atom pair descriptor B05

Box 5

IM1
$PCE = -1.672 + 1.383 \times nRCN + 0.979 \times F08[N-S] + 2.615 \times nArNR2 - 3.358 \times B09[S-S] + 0.261 \times nCconj$
IM2
$PCE = -1.628 + 1.455 \times nRCN + 2.647 \times nArNR2 - 3.508 \times B09[S-S] + 1.115 \times B08[N-S] + 0.249 \times nCconj$
IM3
$PCE = -1.953 + 1.265 \times nRCN + 0.323 \times (C-034) + 2.658 \times nArNR2 - 3.354 \times B09[S-S] + 0.300 \times nCconj$
IM4
$PCE = -1.574 + 1.398 \times nRCN + 0.627 \times nThiophenes + 2.649 \times nArNR2 - 3.229 \times B09[S-S] + 0.230 \times nCconj$
IM5
$PCE = 0.082 + 1.151 \times nR=Ct + 0.539 \times (C-034) - 0.098 \times T(S..S) - 0.946 \times nR\#C - + 0.539 \times (C-040)$

[N–S] which signifies the presence or absence of the nitrogen and sulfur atoms at topological distance 5 and the atom centered fragment descriptor N-071 (Ar-NAl2; where, Al and Ar: aliphatic and aromatic groups, respectively) indicate that the presence of these fragments is influential for the PCE property of dyes in DSSC as observed for the dyes **217** (PCE = 7.88) and **218** (PCE = 8.14) for which B05[N–S] value is 1 for both cases; and **178** (PCE = 9.73) and **180** (PCE = 9.51) for which N-071 descriptor value is 1 for both cases. In contrast, the dyes having no such fragments showed poor PCE property in DSSC as observed in case of dyes like **92** (PCE = 0.65), **252** (PCE = 0.73) and **292** (PCE = 0.92).

The 2D atom pair descriptors B04[N–N] (presence or absence of 2 nitrogens at the topological distance 4), F08[C–O] (frequency of carbon and oxygen atoms at the topological distance 8) and the atom centered fragment N-072(RCO-N</>N-X = X), the constitutional descriptor O% (percentage of oxygen atoms) as well as the ring descriptor nR08 (number of 8 membered rings) contribute negatively towards the PCE property as suggested by their negative regression coefficients. As the numerical values of these descriptors increase, the PCE property of the dyes will decrease. For example, the dyes **292** (PCE = 0.92) and **294** (PCE = 0.133) for B04[N–N] descriptor where the numerical value is 1 for both cases; **252** (PCE = 0.73) and **120** (PCE = 2.48) for which the numerical values of F08[C–O] are 60 and 46, respectively; **120** (N-072 = 2, nR08 = 1; PCE = 2.48) and **121** (N-072 = 2, nR08 = 1; PCE = 2.58) for N-072 and nR08 descriptors; **44** (PCE = 0.6) and **45** (PCE = 0.02) for O% descriptor with the numerical value of 9.41 in both cases have lower PCE values and *vice versa* in case of dyes **217** (PCE = 7.88) and **218** (PCE = 8.14) where B04[N–N] is absent; **188** (PCE = 8.1) and **198** (PCE = 8.77) with descriptor value being 2 for F08[C–O] in both cases; **214** (PCE = 8.7) and **216** (PCE = 9.5) in absence of both features N-072 and nR08; **198** (PCE = 8.77) and **205** (PCE = 8.26) for O% where the descriptor values are 1.09 and 1.38, respectively.

The mechanistic interpretation of the models for porphyrin dyes is schematically portrayed in Fig. 8. The scatter plots of observed vs. predicted PCE property related to the Porphyrin dyes for all the PLS

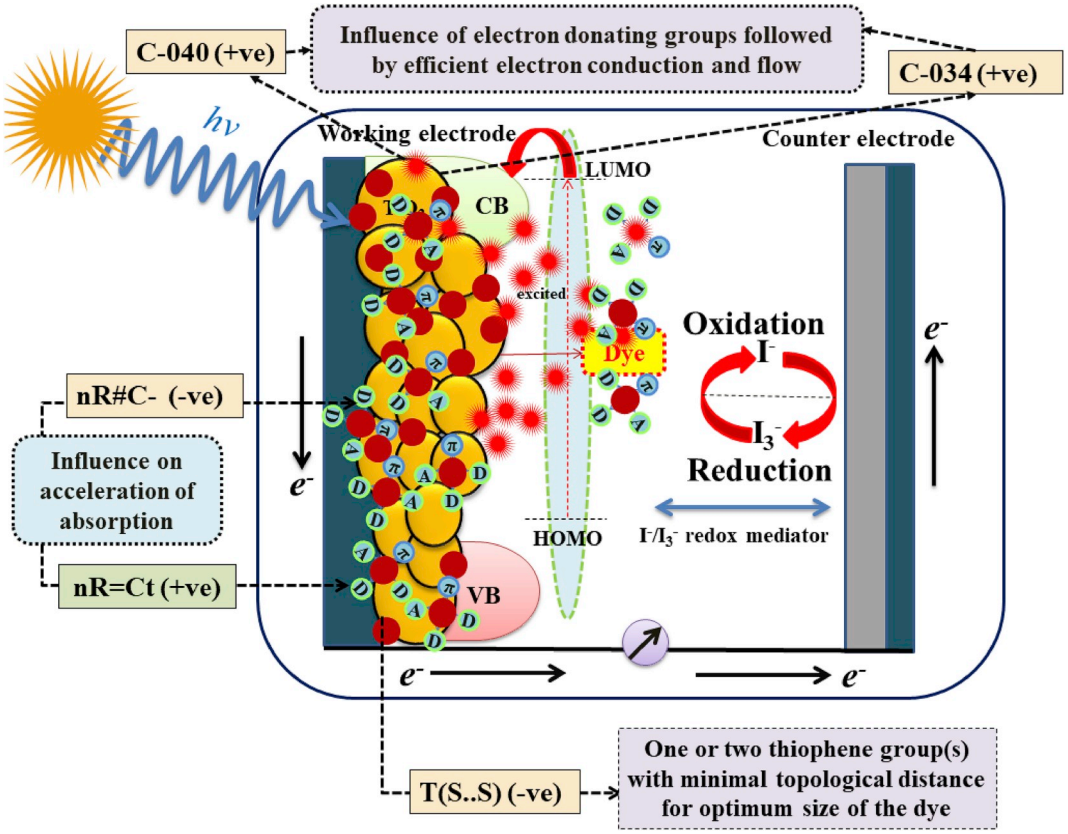


Fig. 9. Contribution of modeled descriptors on controlling PCE values of the Coumarin dyes.

Box 6

IM 1
$$PCE = 0.541 + 0.019 \times F06[C - C] - 0.154 \times NaasC + 1.808 \times B04[N - O] + 1.409 \times F08[O - O] + 1.339 \times B02[C - S] + 1.177 \times F06[N - O] + 0.918 \times B08[O - S] - 0.266 \times N\% + 0.143 \times F04[C - N] + 0.601 \times nR10 - 1.445 \times F06[N - N]$$
IM2
$$PCE = 0.177 + 0.019 \times F06[C - C] - 0.125 \times NaasC + 1.933 \times B04[N - O] + 1.263 \times F08[O - O] + 1.261 \times B10[C - S] + 1.072 \times F06[N - O] + 0.949 \times B08[O - S] - 0.202 \times N\% + 0.129 \times F04[C - N] + 0.714 \times nR10 - 1.444 \times F06[N - N]$$
IM3
$$PCE = -0.415 + 0.021 \times F06[C - C] - 0.141 \times NaasC + 1.734 \times B04[N - O] + 1.377 \times F08[O - O] + 1.009 \times B02[C - S] + 1.108 \times F06[N - O] + 0.638 \times B06[N - S] + 1.007 \times B08[O - S] + 0.732 \times nR10 + 0.114 \times F04[C - N] - 1.588 \times F06[N - N]$$
IM4
$$PCE = 0.576 + 0.016 \times F06[C - C] - 0.098 \times NaasC + 2.044 \times B04[N - O] + 1.652 \times B02[C - S] + 1.614 \times F08[O - O] + 0.933 \times F06[N - O] - 0.295 \times N\% + 0.108 \times F04[C - N] + 0.694 \times nR10 - 0.029 \times F06[O - S] - 1.346 \times F06[N - N]$$
IM5
$$PCE = 0.307 + 0.020 \times F06[C - C] - 0.130 \times NaasC + 1.804 \times B04[N - O] + 2.442 \times F08[O - O] + 1.206 \times B10[C - S] + 0.853 \times B08[O - S] - 0.189 \times N\% + 0.133 \times F04[C - N] + + 0.670 \times nR10 - 1.547 \times F06[N - N] - 0.546 \times B04[O - S]$$

models are depicted in Fig. S10 in Supplementary Materials.

3.5. Data set 5: modeling of PCE property of coumarin dyes

The identified and extracted vital features obtained from the five PLS models for coumarin dyes are reported in Box 5. The mechanistic interpretation of all the descriptors with suitable examples of the studied dyes is discussed below to demonstrate how the features are affecting the PCE property.

Unlike other chemical classes, here we are not discussing descriptor interpretations for consensus models. IM5 is the best model for coumarin dyes, and the modeled descriptors only are discussed below. The functional group count descriptors nR#C- (number of non-terminal carbon with "sp" hybridization) and nR = Ct (number of aliphatic tertiary carbon with "sp^2" hybridization) had negative and positive contributions respectively towards PCE property of coumarin dyes. Absence of non-terminal "sp" hybridized C atom and presence of tertiary aliphatic "sp^2" hybridized C atoms in the dyes enhance the frequency of the S-character (S means strong absorption) which is important to the enhancement of PCE values [67].

The atom centered fragments C-034 (R–CR..X) and C-040 (R–C(=X)-X/R–C#X/X = C = X) (where, R: any group linked through carbon; X: any electronegative atom O, N, S, P, Se,halogens; #: a triple bond; –: an aromatic bond as in benzene or delocalized bonds such as the N–O bond in a nitro group; ..: aromatic single bonds as the C–N bond in pyrrole) contribute positively to the PCE. Thus, the presence of such fragments in the dyes may enhance the PCE property in DSSC as observed in case of dyes **19** (F08[N–S] = 2, C-034 = 3, C-040 = 4; PCE = 7.4) and **32** (F08 [N–S] = 1, C-034 = 4, C-040 = 4; PCE = 6.5) and *vice versa* in case of dyes **7** (F08[N–S] = 0, C-034 = 2, C-040 = 1; PCE = 1.1) and **24** (F08 [N–S] = 0, C-034 = 2, C-040 = 0; PCE = 1.04).

The 2D atom pair descriptor T(S..S) means the sum of topological distance between two sulfur atoms (where, .. signifies aromatic single bonds), and it shows a negative contribution to the PCE of Coumarin dyes. It has been found that with the an increase in the numerical value of this descriptor, the PCE property of dyes decreases, as clearly observed in case of dyes **1** (T(S..S) = 52; PCE = 1.39) and **3** (T(S..S) = 28; PCE = 1.77), while in case of dyes **29** (T(S..S) = 3; PCE = 6.07) and **35** (T (S..S) = 0; PCE = 6.20), the PCE values are increased due to absence of such fragment.

The mechanistic interpretation of the descriptors for coumarin dyes from all the models is schematically portrayed in Fig. 9. The scatter plots of observed vs. predicted PCE property related to the coumarin dyes for all the PLS models are depicted in Fig. S11 in Supplementary Material.

3.6. Data set 6: modeling of PCE property of carbazole dyes

The modeled descriptors obtained from the five PLS models for carbazole dyes are reported in Box 6. The mechanistic interpretation of all the descriptors is discussed below with suitable examples.

The 2D atom pair descriptor F06[C–C] denotes the frequency of two carbon atoms at the topological distance 6 with a positive effect towards the PCE. Thus, dyes bearing this fragment like **99** (F06[C–C] = 149; PCE = 7.58), **101** (F06[C–C] = 139; PCE = 8.09) and **130** (F06[C–C] = 207; PCE = 9.8) showed higher range of PCE property in DSSC. Again, dyes having lower frequency of this fragment showed lower range of PCE value as evidenced by the dyes **47** (F06[C–C] = 14; PCE = 2.43), **97** (F06 [C–C] = 0; PCE = 0.0538) and **98** (F06[C–C] = 0; PCE = 0.0387). Presence of this descriptor signifies the importance of alkyl linear chains in the dyes which may enhance the electron transfer (these chains improve the surface protection which will facilitate electron injection [68] from donor to the acceptor resulting in generation of the charge separated species followed by an increase in the PCE value [69]).

Another 2D atom pair descriptor F08 [O–O] represents the frequency of two oxygen atoms at topological distance 8, which contributes positively towards the PCE. We can see in the dyes **132** (F08[O–O] = 3; PCE = 12.5) and **133** (F08[O–O] = 2; PCE = 9.32), the PCE property is high due to the higher numerical value of this descriptor, and the opposite is observed in case of dyes **62** (PCE = 1.88), **66** (PCE = 1.44) and **96** (PCE = 1.36) with the absence of F08[O–O] feature. This descriptor signifies the oxygen in the enamine of the Carbazole moiety and the anchoring functional groups (such as carboxylate, alkoxysilanes *etc.)* which may strengthen the π–π interactions of the dye system to help in an increase in PCE [50].

The 2D atom pair descriptor F04[C–N] states that the frequency of carbon and nitrogen atoms at the topological distance 4 affects PCE positively. Thus, presence of this fragment in the dye may increase the PCE property as reported in dyes **50** (F04[C–N] = 22; PCE = 7.52), **101** (F04[C–N] = 17; PCE = 8.09) and **130** (F04[C–N] = 15; PCE = 9.8) and *vice versa* in case of **97** (PCE = 0.0538), **98** (PCE = 0.0837) in absence of F04[C–N] fragment. The presence of carbon and nitrogen atoms in the carbazole moiety may enrich the electron donating capability in the dyes in DSSCs which may increase the PCE values [54].

The ring descriptor nR10 denotes the number of 10-membered rings in the carbazole dyes with a positive contribution to the PCE. The presence of 10 membered ring N-annulated indenoperylene (electron donor) in the photo-chemically inactive segments of the carbazole dye can be conjugated *via* triple bond with an electron-acceptor for a metal-free donor or acceptor dye without the use of any co-adsorbate, which might be responsible for high PCE value of DSSCs [70]. Presence of 10

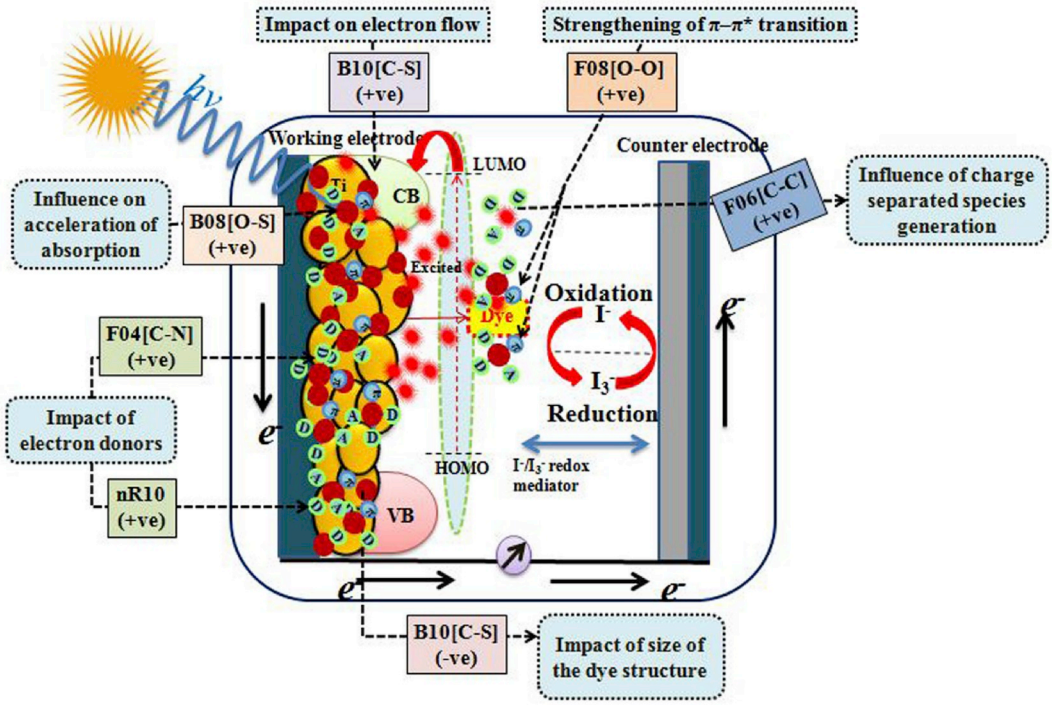


Fig. 10. Contribution of different descriptors on controlling PCE values of the carbazole dyes.

membered ring in the dyes correlate to higher PCE as observed in dyes **103** (nR10 = 1; PCE = 7.54), **130** (nR10 = 6; PCE = 9.8) and **131** (nR10 = 6; PCE = 7.6).

Another 2D atom pair descriptor B08[O–S] defines presence or absence of oxygen and sulfur atoms at the topological distance 8 bearing a positive effect to PCE. From the analysis of structures, we have found that these two atoms are present in methoxy and thiophene moieties of dyes where oxygen atom acts as linker for donor or acceptor whereas sulfur in thiophene moiety accelerates absorption by inducing bathochromic shift [47]. Thus, presence of such fragment in dye molecules may enhance the PCE property of dyes in DSSC as shown in dyes **50** (PCE = 7.52), **94** (PCE = 7.33) and **178** (PCE = 7.43) (descriptor value is 1 in all cases), while the absence of such fragments may detrimental to the PCE property as shown in compounds **91** (PCE = 0.19), **157** (PCE = 0.21) and **159** (PCE = 0.31).

The negative regression coefficient of 2D atom pair descriptors such as B04[O–S] (which means presence or absence of oxygen and sulfur atoms at topological distance 4) and F06[O–S] (indicates frequency of oxygen atom and sulfur at topological distance 6) indicate that presence of these fragments may reduce the PCE property of dyes in DSSC as evidenced by the dyes **12** (PCE = 1.79), **13** (PCE = 0.55) and **92** (PCE = 0.24) for B04[O–S] descriptor where the value of descriptor is 1 in all cases); **66** (PCE = 1.44), **154** (PCE = 0.07) and **156** (PCE = 0.06) for F06 [O–S] descriptor where the numerical value is 1 in all cases and *vice versa* in case of dyes **94** (PCE = 7.33), **101** (PCE = 8.09) and **133** (PCE = 9.32), where both features are absent. These fragments may cause weak interaction between the semiconductor and carbazole dyes due to steric hindrance which make difficulty in the electron flow to the semiconductor.

The 2D atom pair descriptor B10[C–S] states the presence or absence of carbon and sulfur atoms at topological distance 10 offers a positive effect on the PCE property. Presence of this fragment favors the bulkiness of the dyes which may enhance the sensitized wide bandgap in the nano-structured photoelectrode [53]. As a result, the PCE values may increase in the presence of this fragment as shown in the dyes **50** (PCE = 7.52), **103** (PCE = 7.54) and **178** (PCE = 7.43). On the other hand, the absence of this feature may decrease the PCE property as shown in dyes **32** (PCE = 0.695), **53** (PCE = 0.99) and **112** (PCE = 0.96).

The 2D atom pair descriptors B06[N–S] (presence or absence of nitrogen and sulfur atoms at the topological distance 6), B04[N–O] (presence or absence of nitrogen and oxygen atoms at topological distance 4), F06[N–O] (frequency of nitrogen and oxygen atoms at topological distance 6) and B02[C–S] (presence or absence of carbon and sulfur atoms at topological distance 2) contribute positively towards the PCE property of dyes in DSSC. The positive regression coefficients of these parameters suggest that the presence of such fragments in the Carbazole dyes enhances the PCE property as shown in the dyes **94** (PCE = 7.33), **99** (PCE = 7.58), **100** (PCE = 6.98) (for B06[N–S] descriptor, the descriptor value is "1" in all cases), **50** (PCE = 7.52), **103** (7.54), **178** (7.43) (for B04[N–O] descriptor, the numerical value of the descriptor is "1" in all cases), **104** (PCE = 6.93), **134** (PCE = 6.16), **135** (PCE = 6.33) (for F06[N–O], the descriptor value is "1" in all cases), **130** (PCE = 9.8), **131**(PCE = 7.6) (for B02[C–S], descriptor value is "1" in all cases). On the other hand, the dyes **124** (PCE = 1.04), **126** (PCE = 0.91) (the numerical value of these descriptors is "0" in all cases) showed lower PCE values for the Carbazole dyes due to absence of such fragments.

The negative regression coefficients of the 2D atom pair descriptor F06[N–N] (indicating the frequency of 2 nitrogen atoms at the topological distance 6), the atom type E-state descriptor NaasC (representing the number of atoms of aasC (–C(–)-)) and the constitutional descriptor N% (indicates the percentage of nitrogen atoms) suggested that the presence of these specific features might reduce the PCE values as shown in case of dyes **138** (PCE = 2.17), **150** (PCE = 2.49) for F06[N–N], where the descriptor values are 2 and 1, respectively; **62** (PCE = 1.88), **116** (PCE = 1.78) for NaasC, where the descriptor values are 15 and 18, respectively); **91** (PCE = 0.89), **176** (PCE = 0.07) for N% with values of 6.5 and 6.2, respectively, and *vice versa* in case of dyes **132** (PCE = 12.5) and **133** (PCE = 9.32), where F06[N–N] fragment is absent; **99** (PCE = 7.58) and **101** (PCE = 8.09), where NaasC descriptor value is 9 for both dyes; **133** (PCE = 9.32) and **99** (PCE = 7.58) for which N% values are 2 and 1.8, respectively.

The mechanistic interpretation of the PCE property of carbazole dyes from all models is schematically portrayed in Fig. 10. The scatter plots of observed vs. predicted PCE property related to the carbazole dyes for all

Box 7

IM1

$$PCE = 6.155 + 0.278 \times F08[C-N] + 3.681 \times nPyrimidines - 0.661 \times F01[C-N] - 0.170 \times StsC$$

IM2

$$PCE = 5.567 + 0.294 \times F08[C-N] + 3.354 \times nPyrimidines - 0.622 \times F01[C-N] - 0.143 \times nCsp$$

IM3

$$PCE = 2.857 - 2.974 \times B08[N-N] - 1.99 \times (C-041) + 0.498 \times nHAcc - 0.267 \times StsC$$

IM4

$$PCE = 4.471 - 3.186 \times B08[N-N] + 2.919 \times nPyrimidines - 1.278 \times F04[N-S] - 1.016 \times nR\#C -$$

IM5

$$PCE = 5.714 + 0.241 \times F08[C-N] - 0.068 \times ETA\_dBeta - 0.634 \times F01[C-N] + 3.729 \times nPyrimidines$$

the PLS models are depicted in Fig. S12 in Supplementary material.

3.7. Dataset 7: modeling of the PCE property of diphenylamine dyes

The identified significant descriptors obtained from the five PLS models are illustrated in Box 7 with their respective contributions to the PCE property. The mechanistic interpretation of all the descriptors provided below with reasonable examples.

The 2D atom pair descriptor F08[C–N] defines the frequency of carbon and nitrogen atoms at the topological distance 8 which contribute positively towards the PCE. This fragment is a part of many electron donating groups (EDGs) in this dye system (seen in more than 50 structures in the form of hexyloxy amines and ethylhexyloxy amino groups, dithiadiazole groups) [71]. Therefore, presence of this fragment in the dyes may enhance the PCE as observed in the dyes **15** (F08[C–N] = 16; PCE = 6.66), **26** (F08[C–N] = 16; PCE = 7.1) and **27** (F08[C–N] = 20; PCE = 8) and *vice versa* in dyes **14** (F08[C–N] = 2; PCE = 3), **33** (F08[C–N] = 1; PCE = 0.44) and **35** (F08[C–N] = 1; PCE = 0.4).

The negative regression coefficient of B08[N–N] (representing presence/absence of the two nitrogen atoms at the topological distance 8) and F01[C–N] (designating the frequency of carbon and nitrogen atoms at topological distance 1) indicate that presence of these fragments in the dyes may reduce the PCE of DSSCs as shown in dyes **29** (B08[N–N] = 1 & F01[C–N] = 8; PCE = 1.99), **34** (B08[N–N] = 1 & F01[C–N] = 10; PCE = 1) and **35** (B08[N–N] = 1 & F01[C–N] = 11; PCE = 0.44). In contrast, the dyes having no such fragments may have enhanced PCE property as shown in dyes **15** (B08[N–N] = 0 & F01[C–N] = 6; PCE = 6.66), **17** (B08[N–N] = 0 & F01[C–N] = 6; PCE = 6.19) and **26** (B08[N–N] = 0 & F01[C–N] = 6; PCE = 7.05). Due to the presence of these groups, there is an effect on the polarity of the dye molecules which may cause dye aggregation on semiconductors mesoporous layer followed by minimization of photon absorption [72].

The functional group count descriptor nPyrimidines represents the number of pyrimidines present in the structure of a dye. The positive regression coefficient of the descriptor indicates the presence of pyrimidine ring favors the PCE property as shown in dyes **7** (nPyrimidines = 1; PCE = 7.05) and **8** (nPyrimidines = 1; PCE = 7.64), while dyes **14** (nPyrimidines = 0; PCE = 3), **28** (nPyrimidines = 0; PCE = 2.8) and **34** (nPyrimidines = 0; PCE = 1) show lower range of PCE values due to absence of the pyrimidine ring. Due to presence of pyrimidine in the dye,

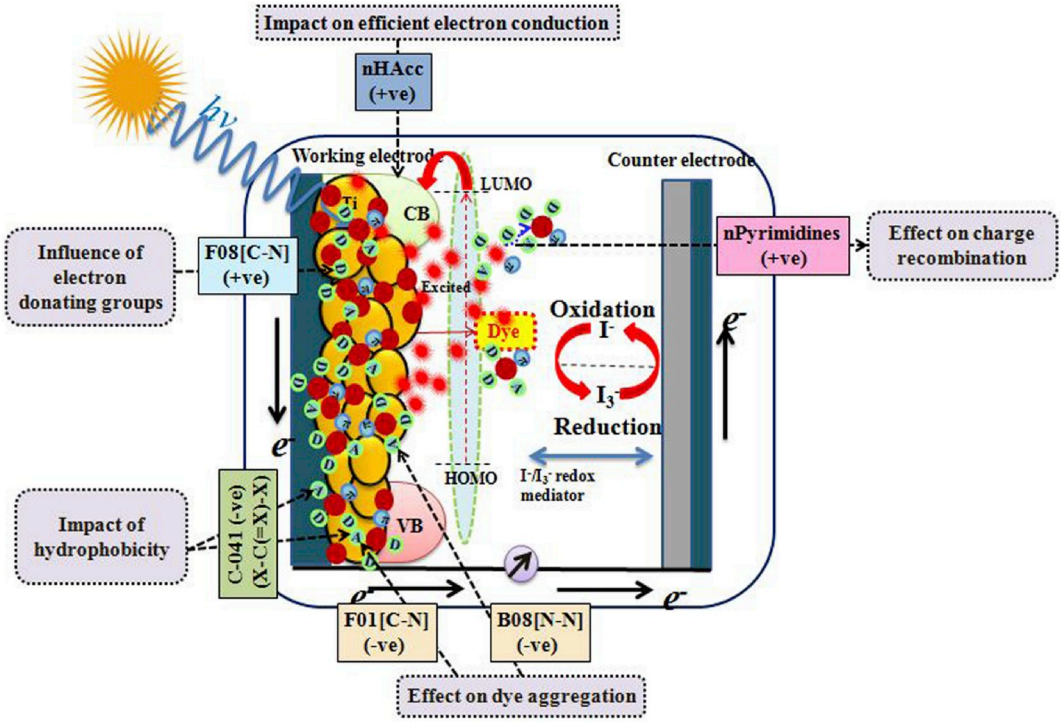


Fig. 11. Contribution of different descriptors on controlling PCE values of the Diphenylamine dyes.

the LUMOs lay over the conduction band edge of TiO_2, while their HOMOs are under the reduction potential energy of the electrolytes (I^-/I^-_3); thus, the ability of electron transfer from the dye (excited state) to TiO_2 mesoporous layer increases, and charge regeneration occurs effectively. Thus, the effective charge regeneration may enhance the PCE property of dye molecules in DSSC [73].

The atom centered fragment C-041 [48,49] corresponds to X–C (=X)-X, where, X can be any electro negative atom O, N, S, P, Se and halogens connected with the carbon atom; it has a negative impact on the PCE property of dyes as found in dyes **33** (C-041 = 1; PCE = 0.44), **34** (C-041 = 2; PCE = 0.4) and **35** (C-041 = 2; PCE = 1). On the other hand, the dyes with lower numerical values of the descriptor show higher PCE values as observed in case of dyes **3** (C-041 = 0; PCE = 5.4), **7** (C-041 = 0; PCE = 7.05) and **27** (C-041 = 0; PCE = 8). This fragment favours the hydrophobicity of dyes by acting as a buffer between semiconductor and the electrolyte which leads to an effect on charge recombination followed by reduction of PCE property.

Another functional group count descriptor nHAcc stands for the number of acceptor atoms for H-bonds (N, O and F) which contributes positively to the PCE property of diphenylamine dyes. Thus, the presence of higher number of acceptor atoms in the dyes for hydrogen bonding favors forming a closely packed structure that increases its conductivity [74] as well as the PCE values as shown in the dyes **27** (nHAcc = 10; PCE = 8), **8** (nHAcc = 8; PCE = 7.64) and **7** (nHAcc = 8; PCE = 7.05). In contrast, the PCE property decreases when there is a decrease in the numerical value of this descriptor as shown in case of dyes **10** (nHAcc = 4; PCE = 1.99) and **33** (nHAcc = 5; PCE = 0.44).

The atom type E-state descriptor StsC (Sum of tsC E-states ≡C—), the constitutional descriptor nCsp (number of sp hybridized Carbon atoms) and the extended topochemical atom descriptor, ETA_dBeta (measuring the relative unsaturation content (Δβ)) contribute negatively towards the PCE property of diphenylamine dyes as indicated by negative regression coefficient of these descriptors. This means that with an increase in the numerical values of the mentioned descriptors, the PCE value decreases as observed in case of dyes **10** (StsC = 8.29 & nCsp = 3, ETA_dBeta = 7; PCE = 1.99), **14** (StsC = 8.31 & nCsp = 3, ETA_dBeta = 8; PCE = 3) and *vice versa* in case of dyes **7** (StsC = 1.64 & nCsp = 1, ETA_dBeta = 1.5; PCE = 7.05), **8** (StsC = 1.67 & nCsp = 1, ETA_dBeta = −5; PCE = 7.64).

The mechanistic interpretation of the diphenylamine dyes from all models is schematically portrayed in Fig. 11. The scatter plots of observed vs. predicted PCE property related to the Phenothiazine dyes for all the PLS models are depicted in Fig. S13 in Supplementary material.

We have also checked the applicability domain of all the individual models (IM1-IM5) developed from the seven datasets using the DModX approach. Based on the AD study, we have found that all the training set dyes are within the stipulated D-critical value under 99% confidence limit for the Triphenylamine (1.7622–1.879), Phenothiazine (1.768), Indoline (1.598–1.916), Porphyrin (1.584–1.853), Coumarin (2.536–2.912), Carbazole (1.638–1.904) and Diphenylamine (2.135–3.911) datasets, respectively. In case of test set dyes, **132** (for model IM-1), **167** (for model IM-2), **165** (for model IM-3) and **160** (for model IM-5) for Triphenylamine dataset (Figs. S14–S18); **181**, **28** and **160** (IM1-IM5) for Phenothiazine dataset (Figs. S19–S23); **151** (IM1-IM5) for Indoline dataset (Figs. S24–S28); **270** (IM1-IM5) for Porphyrin dataset (Figs. S29–S33); **129** (IM1-IM-5) for Carbazole dataset (Figs. S34–S38) are identified as out of AD. In case of Coumarin (Figs. S39–S43) and Diphenylamine (Figs. S44–S48) datasets, all the test set compounds are within the AD zone. Considering the huge number of dyes, almost 99% of dyes pass the AD test, and their predictions are completely reliable.

We have performed Y-scrambling or Y-randomization analysis to check whether we got our model accidently. But interestingly all scrambled models failed to achieve R^2 and Q^2 values of more than or equal to 0.5 which suggest that our developed models had not generated by chance. The quality of these randomized models is so bad that no single R^2 value is over 0.1 and all Q^2 values are in negative range. The complete results can be found in Table S1 and the figures can be found in Figs. S49–S55 in the Supplementary materials file.

3.8. Overview of the obtained interpretation from QSPR models

We have developed multiple QSPR models for around 1200 organic dyes classified under seven chemical classes. The developed individual and consensus models helped us to identify the essential structural fragments and physicochemical features of the studied dyes which are responsible for variation of the PCE values in DSSCs. The models offer a series of mechanistic interpretation of the variation of PCE values with molecular structures of a large number of dyes which can be employed by the investigators to reduce the experimental testing, time, and money by several folds. Moreover, the exploratory information may help to design new, improved lead dyes for all seven classes. The quantitative structural analysis lead to the following interpretations for efficient PCE of individual chemical classes:

3.8.1. Triphenylamine dyes

The fragment = N- lowers the tendency of localized π–π* transition due to ICT transition from the triphenylamine donor to the anchor group and lowers the absorption maxima followed by a decrease of PCE values. The fragments Al–C(=X)-Al (Al: aliphatic, X: O, N, S, P, Se, halogens) and X–CR..X contribute to the hydrophobicity and act as a buffer between the semiconductor and the electrolyte which prevent the back-transfer of electrons from the semiconductor's conduction band to the redox couple and ultimately lower the PCE. Presence of imides (thio) favours dye hydrolysis which helps the aggregation of the dye over the TiO_2 surface and improves the recombination reaction between redox electrolyte and electrons in the TiO_2 nanolayer. On the other hand, presence of O and S atoms in the donor groups narrows the absorption range of dyes which causes latency decrease of rapid π-conjugation. Thus, all these fragments need to be avoided during the design of triphenylamine dyes.

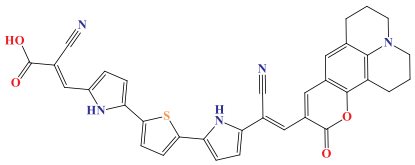
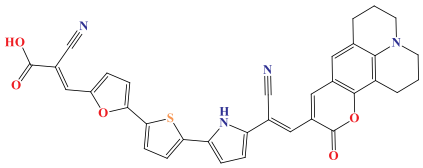
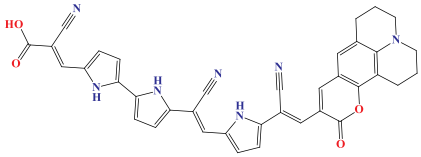
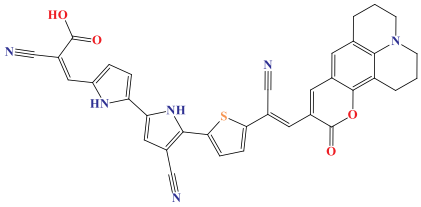
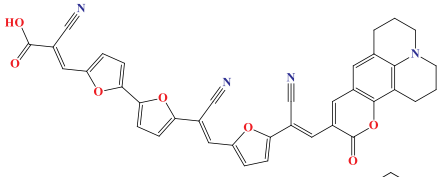
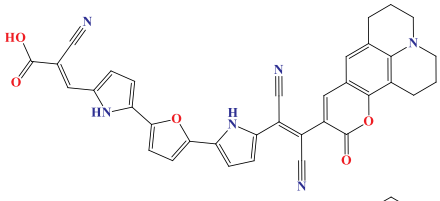
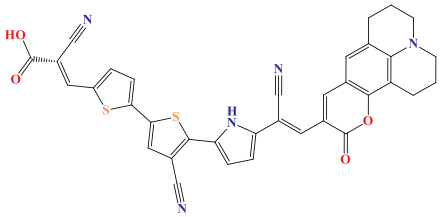
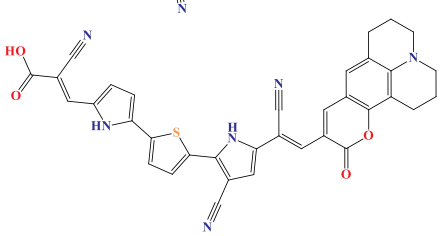
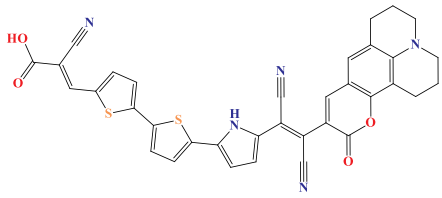
On the contrary, long branching in thiophene ring results in a slight hypsochromic and hypochromic effect in the ICT band where the steric hindrance is induced by the branched-chain increases the torsion angle between the triphenylamine moiety and the thiophene unit. This torsion impedes good delocalization of the π electrons and blue-shifts the position of the ICT band and augment the absorption. Aliphatic imines (nRC = N) also help in the mobilization of free electrons to offer better PCE.

3.8.2. Phenothiazine dyes

H attached to C0(sp^3) with 1X attached to next C fragment favors the lipophilicity or hydrophobicity of the dyes which causes alterations in the energy cascade as a result of the physicochemical alterations such as poor solubility on semiconductors porous layer decreases the PCE values. Again, the mean first ionization potential on a scaled C atom is related to polarity of the molecules due to presence of small electronegative atoms which results in the aggregation of dyes on the semiconductor and ultimately lowers the PCE.

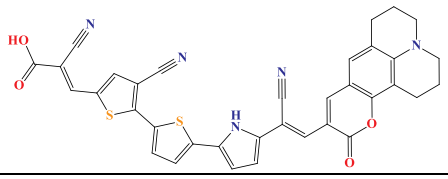
Higher number of O atoms is good as they are involved in the conduction of electrons towards the excitation state due to their natural tendency to form a closely packed structure. As a result, the higher conductivity carriers in the dyes enhance the PCE. Fragment like #CR/R = C = R (= double bond; # a triple bond) is good for mobilization of free electrons and ultimately flow of current in the solar system. Again, large surface area of the dyes may affect the photon capturing ability due to the sensitized wide band gap in the photo electrode which is one of the reasons for high PCE values. The presence of C and O atoms at topological distance 8 signifies the effect of donor and an additional donor through a linkage in the dye system which helps to achieve absorption band broadening followed by an increase in the PCE. The N and O atoms

Table 2
Calculated descriptors and predicted % PCE of the designed coumarin dyes (NCM1 to NCM10).

Dye	Structure	Computed Descriptor					Predicted (%) PCE
		nR = Ct	C-034	T(S..S)	nR#C-	C-040	
NCM1		3	6	0	0	4	8.93
NCM2		3	6	0	0	4	8.93
NCM3		4	6	0	0	5	10.62
NCM4		3	6	0	0	5	9.46
NCM5		4	6	0	0	5	10.62
NCM6		4	6	0	0	5	10.62
NCM7		3	6	3	0	5	9.17
NCM8		3	6	0	0	5	9.46
NCM9		4	6	3	0	5	10.32

(*continued on next page*)

Table 2 (*continued*)

Dye	Structure	Computed Descriptor					Predicted (%) PCE
		nR = Ct	C-034	T(S..S)	nR#C-	C-040	
NCM10		3	6	3	0	5	9.17

at the topological distance 4 signifies to a strong cyano acceptor and a chelating anchoring mode of the carboxylate ion which plays a crucial role to regulate the PCE property of dyes.

3.8.3. Indoline dyes

Dyes with nitro group,  and  fragments experience poor π–π* transition (less reactive than imines) which results in a slow energy cascade mechanism followed by lower PCE values. O–S and C–N atoms at the topological distance 10 represent bulkiness of dyes which may weaken the interactions between the semiconductor and the dye due to steric hindrance followed by restriction of the transfer of electrons from the dye to the semiconductor. Therefore, smaller fragments with lower branching is expected to increase PCE.

Ring quaternary carbon with sp^3 hybridization and non-aromatic conjugated carbon with sp^2 hybridization are good for PCE as this feature is essential for tunable absorption properties, and they produce high molar extinction coefficients leading to improved energy level reactions in the solar cell. Indoline dyes containing donor groups and groups with non-planar structure are very important for the PCE property. Fragments with O and S atoms at the topological distances 5 and 9 regulate the electron density delocalization which is favorable for the π-bond conjugation. As a result, the molar extinction coefficient of the dye is enhanced which leads to the bathochromic shift of the absorption spectrum followed by better PCE.

3.8.4. Porphyrin dyes

Presence of methine bridges (=CH-), a non-polar group makes the negative shift in the solvatochromic properties of the dye which cannot adhere properly to the semiconductor which results in a negative effect on the absorption and stability of the dye. Additionally, fragments like RCO-N< or >N-X = X and 8 membered rings could be avoided for better PCE values.

Aliphatic and aromatic amines, the N–O at topological distances 3 and 10 fragments have a contribution to the special stability (conjugation) and allow a smaller HOMO-LUMO gap, followed by the red shift of the absorption spectrum. Thus, the conduction valence edge level increases, and as a result, the PCE values of DSSCs increases. Again, C and O at the topological distances 6 and 7 with heavy metal (X) suggest the long-chain alkoxy group which impair interfacial back electron transfer reaction help in electron donating ability, and the C in meso aryl substituted portion of Zinc porphyrin acts as a donor in the dye, and these may increase the PCE property. The average connectivity index of order 4 related to surface area of dyes is directly related to light-harvesting capability which could be achieved maximum when the surface area of the dyes is large.

3.8.5. Coumarin dyes

The topological distance between two sulfur atoms needs to be lower in case of coumarin dyes. One or two thiophene group(s) would be optimal, preferably one. Again, if two thiophene groups are present in a coumarin dye, they should be connected with a single bond to have the lowest topological distance between the S atoms. Absence of a non-terminal carbon with "sp" hybridization is also good for enhancement of PCE.

The aliphatic nitriles increase the electron withdrawing ability which is meant to interact with the electron-donating groups present in the dye. Thus, this kind of dipolar fragment provides directionality of the electronic orbitals in the excited state which leads to photo-induced charge-transfer excitation of the dye-to TiO_2 bands. The non-aromatic conjugated sp^2 hybridized C atoms and aliphatic tertiary carbon with "sp^2" hybridization help in the conjugation extension units increase which helps in broadening the absorption and photosensitization properties of the coumarin dyes followed by better PCE. Fragments like R–CR..X, R–C(=X)-X or R–C#X or X = C = X (#: triple bond; –: aromatic bond as in benzene or delocalized bonds such as the N–O bond in a nitro group; ..: aromatic single bonds as the C–N bond in pyrrole) are good for PCE.

3.8.6. Carbazole dyes

Fragments with O and S atoms at the topological distances 4 and 6 may cause weak interaction between the semiconductor and carbazole dyes due to steric hindrance which causes difficulty in the electron flow to the semiconductor. Presence of  fragment and 2 N atoms at the topological distance 6 results in poor π–π* transition followed by lower PCE values.

In contrast, 10-membered ring N-annulated indenoperylene (electron donor) in the photo-chemically inactive segments of the carbazole dye can be conjugated *via* triple bond with an electron-acceptor for a metal-free donor or acceptor dye without use of any co-adsorbate which might be responsible for high PCE values. Fragments with two O atoms at the topological distance 8 signify the oxygen in the enamine of the carbazole moiety and the anchoring functional groups (such as carboxylate, alkoxysilanes *etc.),* which may strengthen the π–π interactions of the dye system to help in an increase of PCE values. Again, a fragment with C and N atoms at the topological distance 4 moiety may enrich the electron donating capability in the dyes which may increase the PCE values. The fragment with the O and S atoms at the topological distance 8 present in methoxy and thiophene moieties of dyes where the O atom acts as linker for donor or acceptor and S in thiophene moiety accelerates absorption by inducing bathochromic shift.

3.8.7. Diphenylamine dyes

Fragments like X–C(=X)-X and  favour the hydrophobicity of dyes by acting as a buffer between semiconductor and the electrolyte which have an effect on charge recombination followed by reduction of PCE property. Again, number of sp hybridized C atoms and higher number of unsaturation content in the dye result in the reduction of PCE values. Again, fragments like 2 N atoms at the topological distance 8 and C and N atoms at the topological distance 1 have an effect on the polarity of the dye which may cause dye aggregation on semiconductor's mesoporous layer followed by minimization of photon absorption.

The pyrimidine scaffold has the ability of electron transfer from the dye (excited state) to TiO_2 mesoporous layer, and charge regeneration is done effectively for higher PCE. Again, presence of more acceptor atoms for H-bonds (N, O and F) in the dyes for hydrogen bonding favour to form a closely packed structure that increases its conductivity followed

by PCE. The electron donating groups like hexyloxy amines, ethylhexyloxy amino groups and dithiadiazole groups help in an increase of PCE values.

4. Design of new dyes

The success of a QSPR model is in its implementation to design new dyes with an enhanced response (here %PCE). Therefore, we have tried to design power conversion efficient dyes based on our generated best models. In our previous studies, we have already designed indoline, diphenylamine and tetrahydroquinoline dyes [17,24,25] employing QSPR analysis and quantum chemical studies. In the present study, we have considered the coumarin chemical class for designing purpose due to its least %PCE value compared to all other studied chemical classes. Among the studied 58 coumarin dyes, the highest experimental %PCE value is 7.4 and only 5 dyes has %PCE value more than 6. So, undoubtedly coumarin dye is the least explored and developed classes of dyes among the studied ones. Thus, model IM5 is considered for the design of coumarin dyes as it is the best developed model as discussed in the Results and Discussion section. Based on the interpretation of the modeled descriptors of equation IM5 of Box 5, we have designed 10 coumarin dyes (NCM1-NCM10) (See Table 2) for which theoretical predictions showed % PCE ranging from 8.93 to 10.62. Compared to the highest reported experimental %PCE of 7.4 in the studied dataset, the designed dyes showed a 20.68–43.51% increase in PCE values. In order to check the AD of the designed dyes, we have applied the DModX approach under 99% confidence limit and found that all 10 compounds reside under the modeled D-critical value of 2.912. The AD plot of the designed coumarin dyes is illustrated in Fig. S56 in Supplementary materials.

5. Conclusion

In the overall conclusion, the identified requisite fragments are important for photophysical properties and optimal balance of short-circuit current (JSC) and the open-current voltage (VOC). The present manuscript has largely explored the 2D structural fragments features, as the quantum and electrochemical analyses for large number of dyes are time consuming. Aliphatic or aromatic amines/imines, thiophenes, pyrimidines, and pyrrole ring systems with an overall requirement in terms of π electrons or aromatic system for the mobilization of free electrons in the form of current, more acceptor atoms for H-bonds in the form of D−A−π−A structure framework, polarity, optimum chain length to avoid hydrophobicity of dyes are the overall features for most of the chemical classes with some specific exceptions to individual chemical classes. The interpreted features from the QSPR models helped us in designing of power conversion efficient ten coumarin dyes. The potentially best designed dye showed predicted %PCE value of 10.62 which is a 43.5% increase compared to the existing coumarin dye with the highest PCE value in the modeled dataset. Our suggested exploratory features of dyes from other chemical classes may also help to design more efficient dyes similar to the case of coumarins saving time and money.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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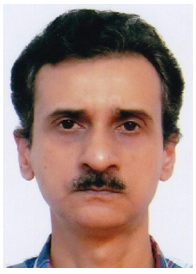

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QSPR modeling of absorption maxima of dyes used in dye sensitized solar cells (DSSCs)

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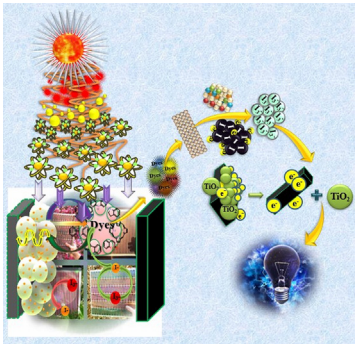
HIGHLIGHTS

- Predictive models for absorption maxima of dyes for usage in dye sensitive solar cells are developed.
- One of the largest accessible dye data sets, including five chemical classes, is used.
- The models were constructed and verified in compliance with the OECD standards.
- Only 2D descriptors are used to obtain the models to serve as fast query tools for novel or untested dyes.
- The models should aid in the development of novel dyes with enhanced absorption maxima.

GRAPHICAL ABSTRACT



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ABSTRACT

Dye-sensitized solar cells (DSSCs) have recently received a significant attention as possible sources of renewable energy. As a result, a significant effort is being made to develop organic dyes for highly power conversion efficient DSSCs, in order to overcome the disadvantages of previous solar cell systems, such as cost reduction, weight reduction, and production methods that minimize environmental pollution. As shown by multiple recent research publications, computational techniques such as quantitative structure–property relationship (QSPR) modeling may aid in the development of suitable dyes for DSSCs satisfying many fundamental desired characteristics. The current report provides robust, externally verified QSPR models for five chemical classes of organic dyes (Triphenylamines, Phenothiazines, Indolines, Porphyrins and Coumarins) based on experimentally determined absorption maxima values. The size of the dye data points utilized to develop the models is the largest known to date. The QSPR models were constructed using only two-dimensional descriptors with clear physicochemical meaning. Using the best subset selection approach, we built 5, 3, 4, 3 and 2 descriptor models for the Triphenylamine, Phenothiazine, Indoline, Porphyrin and Coumarin classes, respectively. The models were validated both internally and externally, and then consensus predictions were made for specific categories of dyes using the developed partial least squares (PLS) models, and the "Intelligent consensus predictor" tool (http://teqip.jdvu.ac.in/QSAR_Tools/) was used to determine whether the quality of test set compound predictions can be improved through the "intelligent" selection of multiple PLS models. We identified from the insights gained from the developed models several chemical attributes that are important in enhancing the absorption maxima. Thus, our study may be utilized to predict the λ_{max} values of novel or

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untested organic dyes and to give insights that will aid in the development of new dyes for use in solar cells with increased λ_{max} values and enhanced power conversion efficiency.

1. Introduction

The growing global demand for energy, combined with the draining of easily accessible, thus inexpensive, fossil fuel reserves, produce a serious warning to the human global economy in near future [1]. After considering the negative environmental impact of conventional energy sources, it is evident that to develop a clean alternative energy source is a prominent necessity [2]. Solar energy is only the source of external energy for harnessing clean, non-hazardous, and inexhaustible energy to fulfil the primary goals of all alternative energy strategies [3]. In this regard, dye-sensitized solar cells (DSSCs) have been studied as one of the most promising methods for future renewable energy sources to convert solar energy to electrical energy [4]. The use of DSSCs over silicon-based solar cells is advantageous due to recent advancements in producing high conversion efficiency, limiting weight, improving resources, low production cost, eco-friendly performance and short-term energy payback [5–18]. The DSSC architecture mainly consists of a mechanical support of transparent conductive oxide, semiconductor (TiO_2 or ZnO) mesoporous layer, redox mediator (electrolyte), sensitizer (dye), and counter electrode. In dye sensitized solar cells, the dye molecule plays the role of a "sensitizer" since it is a photoactive molecule, and it is coated on the surface of nano-crystalline semiconductor (typically TiO_2) for converting photons to electric energy through a series of energy changes. The DSSC is made up of a combination of high-performance electrolytes such as the iodide/triiodide (I^-/I_3^-) redox mediator and additives [7].

"Dye" is a key element in the DSSC architecture; since it is a photoactive component, it regulates the photon harvesting. Later the photo-excited dye injects the electrons into the nano-structured surface of the semiconductor (most of the cases TiO_2). Thus, the electrons are directed through the semiconductor's mesoporous layer to reach the external circuit (interfacial behavior), then collected and stored as solar electricity. Finally, oxidized dye is regenerated with liquid electrolyte, specifically an iodide/triiodide (I^-/I^{3-}) redox shuttle. The performance of "dye sensitized solar cell" is measured by their key quantum properties such as HOMO-LUMO energies, and its frequencies in photo-active molecules. Hence, the photoactive molecule's structure and its configuration in the direction of authentic high-quality D − π − A or D − A − π − A structure framework (D: donor, A: acceptor, π: donor/acceptor connector with conjugation), which couples with an auxiliary acceptor, enhances the ICT (intramolecular charge transfers) and lowers a threshold for photons to be absorbed (optical band gap of semiconductor), are crucial research fields to explore. Diverse classes of materials are used in the construction of DSSCs. Among them, "organic dye molecule" plays a critical role in the entire working mechanism like managing photon capture, charge carrier generation and recombination of TiO_2 electrons with electrolyte species. Thus, by maintaining most other elements of dye sensitized solar cells unchanged and modifying the chemical compositions of dye molecules, one may create a highly effective dye solar cell system. This inference of dye's role in DSSCs also creates a greater desire to investigate the electronic properties of various organic dyes. To achieve the goal of high-power conversion efficiency, organic dyes must have high molar extinction coefficients and absorbance across the entire visible/near-IR spectrum. It has been investigated and demonstrated that a sensitizer (dye molecule) with longer π -conjugation has higher molar extinction coefficients and better light harvesting ability, resulting in higher DSSC efficiency and stability [19,20]. Considering the observations related to the sensitizer, it is important to study the spectroscopic properties of various dye molecules used in DSSCs for improving the photoconversion efficiency (PCE) values of organic dyes. In this context, there is a necessity to explore the absorption maxima (λ_{max}) of various organic dyes, because λ_{max} is one of the most significant spectroscopic properties, mostly defined by the composition of the dyes. The use of experimental approaches to determine absorption maxima (λ_{max}) of dyes is the most apparent and reliable method; nevertheless, there are certain disadvantages, such as the need for testing equipment and the massive workload. Furthermore, the techniques are not readily applicable to poisonous, reactive, hazardous, or radio-active compounds, and they cannot be utilized if the dye has not yet been synthesized. For instance, several types of dyes are suspected to be carcinogens or mutagens; dyes' high coloring strength causes aesthetic damage: dye concentrations less than 1 mg/L can cause noticeable coloration and hence public complaint [21]. As a result, analytical techniques must be developed to compensate for the scarcity of experimental methods. Methods for quantitatively estimating the absorption maxima (λ_{max}) of dyes based on their molecular configurations can be extremely useful not only for application of dyes, but also in the molecular design of new dyes. Until now, theoretical attempts to estimate the absorption maxima (λ_{max}) have relied primarily on quantum-chemistry calculations such as density functional theory (DFT) and *ab initio* approaches. However, these absorption profile measurements are comparatively time-consuming and complicated, precluding the use of such approaches to estimate thousands of dyes in a quick and accurate manner. Furthermore, it was discovered that the absorption maxima (λ_{max}) values of certain dyes determined by DFT generated bad performance [22–26]. Alternatively, the quantitative structure–property relationship (QSPR) offers a successful approach for predicting λ_{max} by fitting experimental results with descriptors extracted solely from molecular structures. The QSPR approach is focused on the principle that variations in compound nature, as demonstrated by some measured physicochemical properties, may be compared with numerical differences in structural features of all compounds, referred to as "molecular descriptors."[27–29]. The benefit of this approach is that it only involves understanding of the chemical structure and is not based on any experimental properties. If a hypothesis of correlation is constructed, it may be used to predict the properties of new substances that have not yet been synthesized or discovered. As a result, the QSPR approach will speed up the synthesis of new molecules and materials with desirable properties.

The QSPR approach has been used successfully to study the absorption maxima (λ_{max}) in relation to the structures of different compounds. Chen et.al., (2018) recently suggested a QSPR approach with non-linear ensemble methods to study the fluorescence absorption wavelengths and emission wavelengths of 413 fluorescent dyes [30]. Baboli et.al., (2013) [31] developed QSPR models for the prediction of the absorption maxima (λ_{max}) of organic dyes used in solar cells with MARS and ANFIS combined with Monte Carlo methods. Fayet et al. (2011) [32] suggested statistically robust QSPR models using quantum chemical descriptors for predicting the absorption maxima (λ_{max}) of 22 azobenzenes and

24 anthraquinones. Xu et al. (2010) developed artificial neural network (ANN) based QSPR models to predict absorption maxima of azobenzene dyes used in DSSCs. Liu et al. (2008) [33] used a stepwise multiple linear regression (MLR) and a radial base structure neural network (RBFNN) to investigate the absorption maxima (λ_{max}) of flavones. Buttingsrud et al. (2007) [34] developed empirical models that relate molecular electronic structure of azobenzene dyes and critical points in the electron density distribution. Several other studies were performed to estimate absorption maxima of various organic dyes [21,35] and 2nd order non-linear optical chromophores [36]. The present authors' group has recently modeled absorption maxima of carbazole dyes used in DSSCs [37]. Such predictive models not only forecast the absorption maxima (λ_{max}) of new customized dyes, they can also be used to investigate the constitutional and electrochemical properties responsible for improvements in absorption maxima (λ_{max}) of dyes that can be exploited by experimentalists for possible design and modification of dye structural scaffolds.

The current research aims to provide robust consensus QSPR models that can be used to calculate the absorption maxima values of various organic dyes. It entails creating QSPR models for five different dye classes using sufficiently large datasets in each case i.e., triphenylamines, phenothiazines, indolines, porphyrins and coumarins. To avoid the complications that arise with 3D descriptors (like conformational complexity and energy minimization), only 2D theoretical descriptors were used to develop simple and interpretable models. The descriptors used in the modelling process were carefully chosen so that they could produce meaningful models with appropriate physicochemical meaning in order to identify chemical features responsible for organic dye absorption maxima (λ_{max}). This level of comprehension could aid in the development of efficient dyes for use in DSSCs. As customary in QSPR analysis, the models were rigorously validated using internationally accepted validation metrics. The study was conducted to help chemists design efficient dyes by demonstrating the impact of absorption maxima values of organic dyes used in dye sensitized solar cells. Finally, this in silico approach highlights the importance of absorption maxima in the development of new potential sensitizers that can directly improve device performance.

2. Materials and methods

2.1. Dataset collection

The dye sensitized solar cell database (DSSCDB) (https://www.diyedb.com/) was used to extract the absorption maxima values for various chemical classes of dyes [38]. Currently, the collection contains over 4,000 experimental findings for a variety of chemical groups. In the preliminary analysis, we screened the available data for uniform experimental conditions such as a Solar simulator (AM 1.5 G 100 mW/cm2) and TiO2 electrodes which were subsequently used in this report. Following that, we partitioned the database into distinct chemical dye groups, excluding dye mixtures, and the stand-alone chemical dye classes were used for QSPR modeling. **SI-1** of supplementary materials section provides the information of various dye data sets with corresponding absorption maximum values. The response variable (λ_{max}) refers to the power (energy) requirements, which is the customary way in non-biological QSPR to model the response without a logarithmic conversion. Ultimately, 600 dyes were evaluated and grouped into five chemical classes. The absorption maxima data were used as the response variable for QSPR modelling. The five datasets are composed of 159 triphenylamines: 357–760 (λ_{max} range), 97 phenothiazines: 471–775 (λ_{max} range), 69 indolines: 470–766(λ_{max} range), 228 porphyrins: 597–766 (λ_{max} range) and 44 coumarins: 464–720 (λ_{max} range).

2.2. Descriptor calculation

Descriptors are fundamental features that provide knowledge about the correlation between structures and their physical, chemical, and bio-active properties. For descriptor calculation, PaDEL-Descriptor and Dragon software tools were used. The constitutional, ring descriptor, connectivity index, functional group counts, atom-centered, atom-type fragments, 2D atom pairs, molecular properties (using Dragon software version 6), and extended atomic topochemical indices (ETA) were calculated (using PaDEL-Descriptor software). Descriptors with a correlation exceeding 0.95 were not included in the descriptor pool.

2.3. Data division and model development

In the current work, the primary datasets for five endpoints were divided by using several defined algorithms into training and validation sets. Using various data division techniques (Kennard-Stone process, Euclidean distance, and Sorted response), approximately 75% of organic dye molecules were assigned to the training group, while the remaining 25% were assigned to the test set. The training set was used solely for feature selection and model creation, while the test set was used to evaluate the established models' predictability. Stepwise regression was used to determine features using stepping parameters, also known as "Fisher criteria," with an inclusion threshold of F = 4.0 and an exclusion threshold of F = 3.9. [39]. The procedure was repeated multiple times in a fashion where in each cycle, selected descriptors were removed from previous runs and kept aside.

Consequently, at the end of multistage stepwise analysis, a list of the best 25 variables was compiled and subjected to best subset selection. The top five models for each of the five endpoints were then subjected to partial least squares (PLS) regression to minimize the model noise (PLS regression measures latent variables (LVs) from the selected variables and also controls the noisy descriptors). During PLS model construction, the input data scaling (standardization) is done followed by computation of latent variable scores (the real regressing variables); however, the final regression coefficients are expressed in terms of the original un-scaled variables (similar to MLR equations). Unlike MLR models, where the standard errors of regression coefficients are determined, the PLS models show the relative importance of different descriptors in terms of VIP (variable importance plot) **(SI-2 in Supplementary Materials). (**See Fig. 1**)**

2.4. Statistical validation and "intelligent" selection of multiple models

For validation, various quantitative validation metrics were used for defining the quality of the developed models which were evaluated in terms of stability, robustness, fitness and predictivity. The coefficient of determination (R^2), internal predictivity metrics like leave-one-out cross-validated R^2 or Q^2_{LOO} and external predictivity metrics like R^2_{pred} or Q^2_{ext} and Q^2F_2 were calculated [40]. We have also checked the MAE (mean absolute error) based criteria for both internal and external validation sets. Additionally, highly stringent r^2_m parameters for validations were also calculated to check for model robustness [41]. Among all the models obtained from the "best subset selection", we have selected the best five models based on MAE (Mean Absolute Error) [42] values for each of the endpoints. To enhance the prediction quality of the individual models for external compounds, "intelligent" consensus predictions of multiple models were attempted. For that, we have used our in-house intelligent consensus predictor (ICP) tool (http://teqip.jdvu.ac.in/QSAR_Tools/).

Fig. 1. Various dye chemical classes of dyes used for absorption maxima (λ_{max}) QSPRmodeling.

The ICP tool assists in finding and understanding the performance of consensus predictions in comparison to the quality of the individual PLS models based on the MAE values, as single model may not be effective for predictions for all test set compounds. The steps involved in the development of the PLS models are represented schematically in Fig. 2.

2.5. Applicability domain and Y-Randomization

According to the OECD principle 3 [43], a QSAR model should have a specific applicability domain. The applicability domain is a specific region or chemical space identified by the molecular properties or structural details of the chemicals used in model development. The established QSAR model can predict the compounds that lie within the region of chemical space of the internal set compounds. We tested the applicability domain of the final PLS model using the standardization approach [44] as well as the distance to model X (DModX) approach at the 95% confidence level using SIMCA-P software [45] to see whether all of the test set compounds are within or beyond the applicability domain. Model Y-randomization test was performed with the SIMCA-P software [45] to assure the models were not developed by any chance.

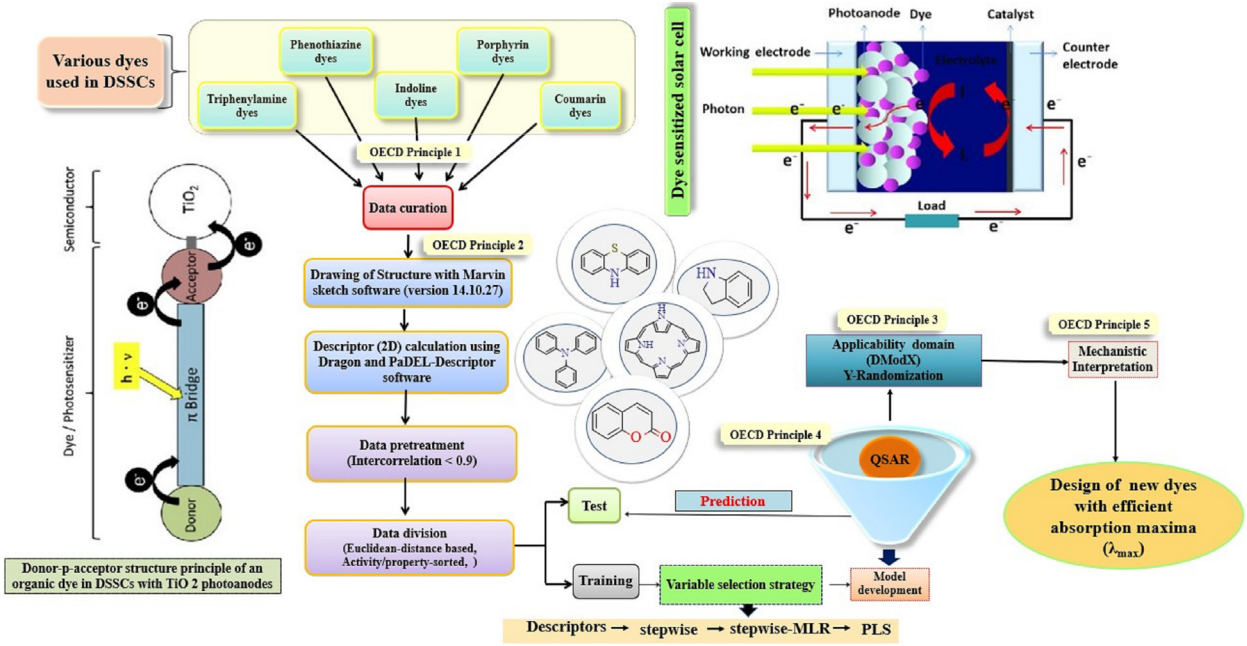


Fig. 2. Schematic overflow of methodology followed in the current work.

2.6. Software used

MarvinSketch version 5.5.0.1 software [46] was used for drawing various chemical structures of dyes. Dragon [47] and PaDEL descriptor software tools [48] were used for descriptor calculation. "Dataset Division GUI 1.2" tool [49] was used for dataset division. MINITAB software version 13.14 [50] was employed for stepwise regression and PLS analysis. Best subset selection and "IntelligentConsensusPrediction" (ICP) tool [49] was used for model development and evaluation of external statistical parameters. The applicability domain of the test set compounds was checked by using SIMCA-P software version 10.0 [51]. Additionally, a sample Y-randomization test was conducted using the SIMCA-P program [45] to ensure that the models were not created by chance.

3. Result and discussion

On the basis of the MAE criterion, we have selected five PLS models for each endpoint and developed corresponding consensus models for each dataset. The details of statistically precise and valid individual (IM) and consensus models (CM) are shown in Table 1. Upon examination of the obtained results, we have found that in majority of the cases the consensus predictions of several PLS models outperform the predictions of individual PLS models. The CM3 model, which reflects the best range of compound wise predictions from the chosen individual models, was chosen as the winner for the datasets tri-phenyl amines, phenothiazines, and porphyrins. However, individual model 5 (IM5) emerged as the winner in the class of indolines, while individual model 3 (IM3) is the finest model in the coumarin dataset. The findings demonstrate that consensus models are significantly more predictive than individual models in the most of the cases; consensus modeling not only reduces the estimation errors associated with individual models but also increases predictability for actual external datasets. Many of the individual models are mechanistically represented using the modeled descriptors. Each of the models for triphenylamine, phenothiazine, indoline, porphyrin, and coumarin datasets have seven, eight, five, seven, and four descriptors, respectively.

3.1. Dataset 1: Modeling of absorption maxima (λ_{max}) values of triphenylamine dyes

Each of the five PLS models included seven descriptors. These models adequately explain the absorption maxima of the triphenylamine dyes used in DSSCs as indicated in Box 1. The following section discusses the mechanistic interpretation of all the descriptors (Fig. 3).

The functional group count descriptor nCrq denotes the number of ring quaternary carbons with sp3 hybridization, and it contributes negatively to the values of the absorption maxima (max). The inclusion of ring quaternary carbon with 'sp^{3}' hybridization shortens the optical band difference and results in a lower molar extinction coefficient, which reduces the energy level reactions in the solar cell [52]. Hence, the existence of a greater number of sp^3 hybridized quaternary carbon atoms in dyes may result in a reduction of the absorption maxima, as observed in dyes such as **70** (nCrq = 2; λ_{max} = 569), **71** (nCrq = 2; λ_{max} = 584) and **101** (nCrq = 2; λ_{max} = 470), and conversely in dyes such as **83** (nCrq = 0; λ_{max} = 745), **217** (nCrq = 0; λ_{max} = 715) and **218** (nCrq = 0; λ_{max} = 677).

The fact that the constitutional descriptor X% (percentage of halogen atoms) has a positive coefficient means that presence of these features in dyes can increase the absorption maxima (max) values. The halogen (Fluorine (F), Chlorine (Cl), Bromine (Br), Iodine (I), and astatine (At)) atoms in the dye system control electron density delocalization, which is advantageous for π-bond conjugation. As a consequence, the dye's molar extinction coefficient increases, resulting in a redshift and modification of the abso+rption bands in the spectra [53]. As a result, dyes containing halogen fragments can exhibit greater absorption maxima (max) values in DSSCs, as shown by the dyes 23(X%= 4.88; λ_{max} = 706), 29(X% = 5.5555; λ_{max} = 640) and 53 (X%= 5.5555; λ_{max} = 620). From the other end, dyes lacking these fragments may exhibit low absorption maxima, as shown by the dyes 7 (X%= 0; λ_{max} = 479), 9 (X% = 0; λ_{max} = 425) and 35 (X%= 0; λ_{max} = 458).

The descriptor, F05[C-N] denotes the frequency of carbon and nitrogen at the topological distance 5, while F06[C-N] indicates the frequency of carbon and nitrogen at the topological distance 6. Both descriptors are showing negative contributions to the absorption maxima (λmax) values. It was found that dyes such as 74 (F05[C-N] = 14; λ_{max} = 585;), 76 (F05[C-N] = 14; λ_{max} = 547) and 77 (F05[C-N] = 21; λ_{max} = 554) and 44 (F06[C-N] = 16; λ_{max} = 565), 45 (F06[C-N] = 16; λ_{max} = 583) and 48 (F06[C-N] = 13; λ_{max} = 584) have an increased frequency of carbon and nitrogen at the topological distances 5 and 6 resulting in a substantial decrease in absorption maxima (λ_{max}) values for dyes, and the opposite occurs in the dyes like 175 (F05[C-N] = 4; λ_{max} = 654), 218 (F05[C-N] = 4; λ_{max} = 677) and 219 (F05[C-N] = 4; λ_{max} = 678) and 18 (F06[C-N] = 3; λ_{max} = 700), 19 (F06[C-N] = 3; λ_{max} = 708) and 20 (F06[C-N] = 3; λ_{max} = 709). Presence of these fragments leads to the transitions in dye structures (mainly due to the nitrogen atom). Carbon and nitrogen atoms form the emission bands with various levels of vibrational transitions of the ground state and excited state [54]. From that vibrational electrical discharge of the nitrogen atom, further radiative transition is highly forbidden. Therefore, the dyes having this fragment has low range of absorption maxima values [55].

Box-1

IM- 1

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 690.87 - 1919.61 \times \text{GD} + 49.18 \times \text{B06[C-S]} + 12.19 \times \text{X\%} - 7.97 \times \text{F06[C-N]} + 4.67 \times \text{F07[C-O]} + 97.27 \times \text{B01[N-S]} - 94.03 \times \text{nCrq}$$

IM- 2

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 661.83 - 88.81 \times \text{nCq} + 42.13 \times \text{F01[N-S]} - 1577.31 \times \text{GD} + 51.29 \times \text{B06[C-S]} + 11.47 \times \text{X\%} + 3.73 \times \text{F07[C-O]} - 6.104 \times \text{F05[C-N]}$$

IM −3

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 661.83 - 88.81 \times \text{nCq} - 1577.31 \times \text{GD} + 51.29 \times \text{B06[C-S]} + 11.47 \times \text{X\%} + 3.73 \times \text{F07[C-O]} + 84.27 \times \text{B01[N-S]} - 6.10 \times \text{F05[C-N]}$$

IM − 4

$$\text{AbsorptionMaxima}(\lambda\,\text{max}) = 661.83 - 88.81 \times \text{nCq} - 1577.31 \times \text{GD} + 51.29 \times \text{B06[C-S]} + 11.47 \times \text{X\%} + 3.73 \times \text{F07[C-O]} + 84.27 \times \text{B01[N-S]} - 6.10 \times \text{F05[C-N]}$$

IM – 5

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 690.87 - 94.03 \times \text{nCq} - 1919.61 \times \text{GD} + 49.18 \times \text{B06[C-S]} + 12.19 \times \text{X\%} - 7.97 \times \text{F06[C-N]} + 4.67 \times \text{F07[C-O]} + 97.27 \times \text{B01[N-S]}$$

Table 1
Statistical results obtained from QSPR models of absorption maxima.

Dataset	Type of model	Training set statistics				Test set statistics							cRp^2 values	AD criteria	Dixon Q-test	Euclidian distance
		Model R^2	Model $Q^2_{(LOO)}$	$r^2_{m(LOO)}$	$\Delta r^2_{m(LOO)}$	R^2_{pred} or Q^2F_1	Q^2F_2	CCC	$r^2_{m(test)}$	$\Delta r^2_{m(test)}$	MAE $_{(100\%)}$	MAE $_{(95\%)}$				
Triphenylamine Dyes	IM1 (LV:5)	0.684	0.635	0.501	0.230	0.606	0.606	0.723	0.343	0.378	35.604	31.578	0.648			
	IM2 (LV:4)	0.668	0.620	0.482	0.243	0.620	0.620	0.730	0.338	0.389	33.247	28.929	0.629			
	IM3 (LV:4)	0.668	0.620	0.482	0.243	0.620	0.620	0.730	0.338	0.389	33.247	28.929	0.646			
	IM4 (LV:5)	0.684	0.635	0.501	0.243	0.606	0.606	0.723	0.343	0.378	35.604	31.578	0.654			
	IM5 (LV:5)	0.684	0.635	0.501	0.230	0.606	0.606	0.723	0.343	0.378	35.604	31.578	0.654			
	CM0	-	-	-	-	0.621	0.621	0.731	0.340	0.386	34.272	30.145	**-**	**Yes**	**Yes**	**0.4**
	CM1	-	-	-	-	0.620	0.620	0.730	0.338	0.388	34.510	30.398				
	CM2	-	-	-	-	0.622	0.622	0.731	0.338	0.389	34.378	30.256				
	CM3	**-**	**-**	**-**	**-**	**0.629**	**0.629**	**0.736**	**0.343**	**0.386**	**33.152**	**28.910**				
Phenothiazine Dyes	IM1(LV:3)	0.690	0.602	0.473	0.185	0.624	0.609	0.801	0.563	0.104	26.655	22.212	0.631			
	IM2(LV:3)	0.687	0.601	0.470	0.194	0.620	0.605	0.799	0.556	0.094	27.161	22.708	0.637			
	IM3(LV:3)	0.711	0.621	0.496	0.175	0.610	0.594	0.820	0.576	0.189	27.210	22.714	0.649			
	IM4(LV:3)	0.699	0.605	0.477	0.177	0.590	0.573	0.816	0.567	0.193	28.706	24.067	0.629			
	IM5(LV:3)	0.682	0.600	0.469	0.186	0.601	0.585	0.789	0.548	0.121	28.709	24.407	0.608			
	IM6(LV:3)	0.682	0.600	0.469	0.186	0.601	0.585	0.789	0.548	0.121	28.709	24.407	0.618			
	CM0	-	-	-	-	0.639	0.625	0.816	0.591	0.146	27.557	23.274	**-**	**Yes**	**Yes**	**0.4**
	CM1	-	-	-	-	0.648	0.633	0.814	0.591	0.119	27.045	23.274				
	CM2	-	-	-	-	0.658	0.644	0.818	0.599	0.111	26.507	22.778				
	CM3	-	-	-	-	**0.643**	**0.628**	**0.809**	**0.564**	**0.042**	**25.757**	**21.904**				
Indoline Dyes	IM1 (LV:4)	0.759	0.713	0.604	0.177	0.759	0.757	0.861	0.703	0.129	25.679	23.880	0.720			
	IM2 (LV:4)	0.765	0.720	0.612	0.182	0.739	0.736	0.853	0.661	0.094	27.314	25.257	0.713			
	IM3 (LV:4)	0.765	0.720	0.612	0.182	0.739	0.736	0.853	0.661	0.094	27.314	25.257	0.717			
	IM4(LV:4)	0.765	0.720	0.612	0.182	0.739	0.736	0.853	0.661	0.094	27.314	25.257	0.714			
	IM5(LV:4)	**0.803**	**0.750**	**0.651**	**0.133**	**0.780**	**0.778**	**0.878**	**0.710**	**0.083**	**24.447**	**22.417**	**0.761**			
	CM0	-	-	-	-	0.756	0.754	0.862	0.685	0.103	26.116	24.112	**-**	**Yes**	**Yes**	**0.3**
	CM1	-	-	-	-	0.756	0.754	0.862	0.685	0.103	26.116	24.112				
	CM2	-	-	-	-	0.759	0.757	0.863	0.687	0.107	25.931	23.932				
	CM3	-	-	-	-	0.771	0.769	0.870	0.711	0.094	24.943	23.172				
Porphyrin Dyes	IM1(LV:1)	0.633	0.612	0.470	0.279	0.634	0.624	0.763	0.538	0.223	16.322	14.082	0.633			
	IM2(LV:1)	0.633	0.612	0.470	0.279	0.653	0.644	0.780	0.554	0.200	15.714	13.436	0.629			
	IM3(LV:2)	0.655	0.624	0.491	0.250	0.621	0.611	0.754	0.501	0.235	17.195	15.252	0.635			
	IM4(LV:2)	0.647	0.611	0.474	0.255	0.641	0.632	0.785	0.540	0.114	16.383	14.243	0.629			
	IM5(LV:2)	0.642	0.614	0.478	0.259	0.629	0.619	0.754	0.505	0.256	16.728	14.627	0.617			
	CM0	-	-	-	-	0.665	0.656	0.782	0.558	0.233	15.502	13.315	**-**	**Yes**	**Yes**	**0.4**
	CM1	-	-	-	-	0.660	0.651	0.781	0.572	0.227	15.686	13.510				
	CM2	-	-	-	-	0.660	0.651	0.781	0.573	0.224	15.682	13.495				
	CM3	-	-	-	-	**0.668**	**0.660**	**0.789**	**0.585**	**0.208**	**15.379**	**13.136**				
Coumarin Dyes	IM1(LV:1)	0.777	0.751	0.656	0.178	0.868	0.867	0.910	0.674	0.104	21.613	19.119	0.755			
	IM2(LV:3)	0.800	0.720	0.633	0.089	0.934	0.933	0.964	0.874	0.049	15.309	13.247	0.715			
	IM3(LV:3)	**0.796**	**0.700**	**0.607**	**0.108**	**0.952**	**0.951**	**0.976**	**0.920**	**0.030**	**11.482**	**9.363**	**0.762**			
	IM4(LV:3)	0.781	0.700	0.598	0.196	0.928	0.927	0.957	0.809	0.057	15.413	13.203	0.733			
	IM5(LV:3)	0.786	0.698	0.609	0.101	0.935	0.935	0.968	0.915	0.007	14.839	12.487	0.727			
	CM0	-	-	-	-	0.957	0.956	0.975	0.864	0.033	12.656	10.980	**-**	**Yes**	**Yes**	**0.3**
	CM1	-	-	-	-	0.918	0.917	0.950	0.793	0.060	16.725	13.742				
	CM2	-	-	-	-	0.922	0.921	0.952	0.811	0.055	16.096	13.050				
	CM3	-	-	-	-	0.915	0.914	0.951	0.866	0.056	16.107	13.063				

LV: For PLS models, latent variable; CM0: Ordinary consensus predictions; CM1: Average of predictions from 'qualified' Individual models; CM2: Weighted average of predictions from 'qualified' Individual models; CM3: Best set of compound-wise predictions from 'qualified Individual models'; The best model for each dataset is highlighted in bold.

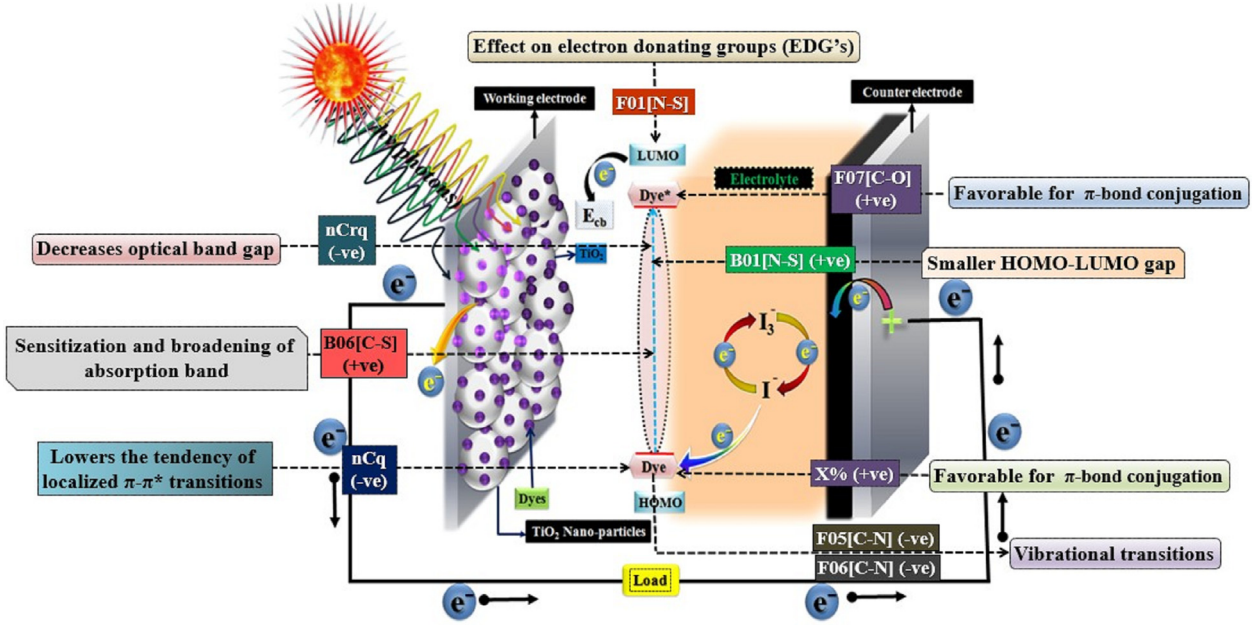


Fig. 3. Contribution of various properties to the regulation of triphenylamine dyes' absorption maxima (λ_{max}) values.

The two-dimensional (2D) atom pair descriptor B01[N–S] denotes the presence/absence of nitrogen and sulfur atoms at the topological distance 1, implying a shorter chain length in a thiadiazole ring-containing molecule. Basically, thiadiazole moieties are electron-deficient units, and they are considered here to show the lower LUMO levels of dyes while at the same time maintaining the HOMO levels, thereby leading to a smaller HOMO-LUMO gap and a more red-shifted UV/Vis spectrum [56–62]. Thereby, the dyes having such fragments may have an enhanced absorption maxima (λ_{max}) values as demonstrated by the dyes 61 (B01[N-S] = 1; λ_{max} = 729), 83 (B01[N-S] = 1; λ_{max} = 745) and 86 (B01[N-S] = 1; λ_{max} = 760). Oppositely, 90 (B01[N-S] = 0; λ_{max} = 480), 101 (B01[N-S] = 0; λ_{max} = 470) and 102 (B01[N-S] = 0; λ_{max} = 452) do not have this fragment; hence, their the absorption maxima values are low.

Another two-dimensional atom pair descriptor, F07[C–O], indicates the existence or absence of carbon and oxygen atoms at the topological distance 7, which correlates favorably to absorption maxima (λ_{max}) values due to its positive regression coefficients. Thus, the inclusion of this fragment in dye molecules can result in an increase in absorption maxima (λ_{max}) values, as shown by dyes 42 (F07[C-O] = 16; λ_{max} = 641), **230** (F07[C-O] = 16; λ_{max} = 612) and 72 (F07[C-O] = 12; λ_{max} = 608) and vice versa in case of dyes 6 (F07[C-O] = 2; λ_{max} = 489), 7 (F07[C-O] = 2; λ_{max} = 478) and 9 (F07[C-O] = 0; λ_{max} = 425). The inclusion of this feature in dye molecules results in a bathochromic shift in the absorption spectrum and an increase in the dye's molar extinction coefficient, which has a direct effect on the λ_{max} values [63].

Other two-dimensional atom pair descriptor, B06[C-S], describes the presence/absence of carbon and sulfur atoms in the dye molecular structure at the topological distance 6. As shown by its positive regression coefficient, this descriptor relates favorably to the absorption maxima (λ_{max}) values. The existence of this descriptor can enhance the bulk of dyes, sensitizing and broadening the band in the photo-electrode, resulting in an increase in the dyes' absorption maxima [64]. The absorption maxima (λ_{max}) might enhance with the existence of B06[C-S], as shown in dyes - such as 15 (B06[C-S] = 1; λ_{max} = 631), 41 (B06[C-S] = 1; λ_{max} = 623) and 189 (B06[C-S] = 1; λ_{max} = 630). On the other hand, in absence of the B06[C-S] feature, there is a reduction in the absorption maxima (λ_{max}) values as shown in the dyes 109 (B06[C-S] = 0; λ_{max} = 357) and 110 (B06[C-S] = 0; λ_{max} = 358).

The descriptor graph density (GD) is determined using the following formula from the H-depleted molecular graph:

$$Graphdensity(GD) = \frac{2.nBo}{nSK.(nSK-1)}$$

In this formula, nBO denotes the number of graph edges (i.e., non-H bonds) and nSK denotes the number of graph vertices (i.e., non-H atoms). This descriptor GD influences the dye surface region, which contributes to the electron injection continuance into the nanostructured TiO_2 [65]. The negative contribution of larger surface area may lead to a decrease in the absorption maxima (λ_{max}) values of dyes. Therefore, the increased numerical values for GD descriptor can result in a decrease of absorption maxima (λ_{max}) values, as demonstrated in the dyes. 9 (GD = 0.098814229; λ_{max} = 425), 102 (GD = 0.0942; λ_{max} = 452) and 117 (GD = 0.0942; λ_{max} = 502) and opposite observed in the dyes 94 (GD = 0.0375; λ_{max} = 666), 42 (GD = 0.0309; λ_{max} = 641) and 218 (GD = 0.0298; λ_{max} = 677).

The functional group count descriptor nCq denotes the total number of quaternary sp^3 hybridized carbons that contributes negatively to the absorption maxima (max) values, implying that the existence of this attribute in dyes may result in a decrease in the λ_{max} values, as shown in the following examples: 101 (nCq = 2; λ_{max} = 470), **70** (nCq = 2; λ_{max} = 569) and **206** (nCq = 1; λ_{max} = 512) and vice versa in case of dyes like 1 (nCq = 0; λ_{max} = 658), 18 (nCq = 0; λ_{max} = 700) and 219 (nCq = 0; λ_{max} = 658). This fragment inhibits local π–π* transitions and raises the wavelength owing to intramolecular charge transfer (ICT) from the triphenylamine donor. As a consequence of this, the dye's absorption maxima would decrease [66].

F01[N-S] is a two-dimensional atom pair descriptor that determines the frequency of nitrogen and sulphur atoms at the topological distance 1, which has a positive contribution to the absorption maxima (λ_{max}). This fragment is a component of a large number of electron donation groups (EDGs) in this dye system (found in over 50 structures as hexyloxy amines and ethylhexyloxy amino groups, as well as dithiadiazole groups) [67]. As a result, the existence of this fragment in dyes can increase their absorption maxima (λ_{max}) values, as observed in dyes 83 (F01[N-S] = 2; λ_{max} = 745), 84 (F01[N-S] = 2; λ_{max} = 760) and 86 (F01[N-S] = 2; λ_{max} = 740), and conversely in dyes 31 (F01[N-S] = 0; λ_{max} = 605), 40 (F01[N-S] = 0; λ_{max} = 575) and 52 (F01[N-S] = 0; λ_{max} = 582).

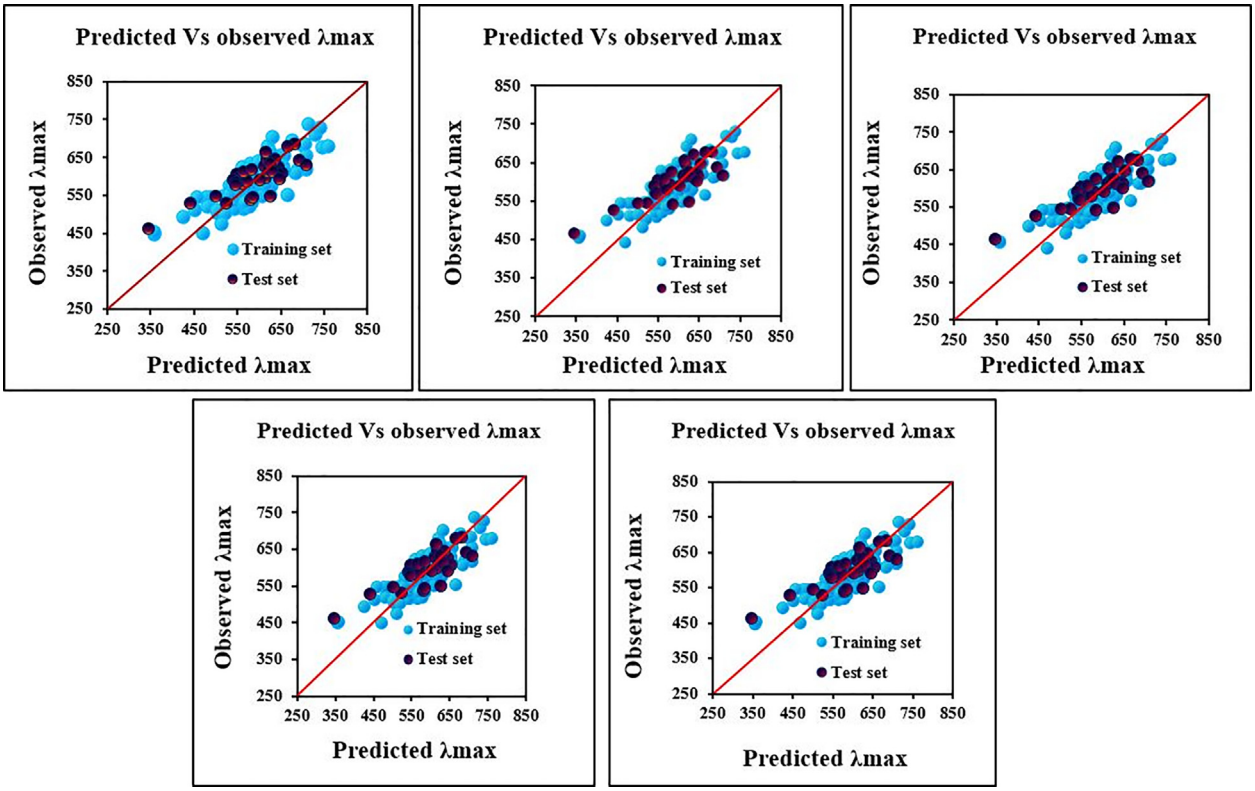


Fig. 4. Scatter plots of the established PLS models observed and predicted absorption maxima (λ_{max}) values (IM1-IM5) (triphenylamine dyes).

Fig. 4 shows scatter plots of the observed and expected absorption maxima (λ_{max}) values for the evolved PLS models (IM1-IM5) (triphenylamine dyes).

3.2. Dataset 2: Modeling of absorption maxima (λ_{max}) values of phenothiazine dyes

Box-2 illustrates the major descriptors and mathematical equations for the final PLS models (Fig. 5). The modeled descriptors are described in depth below, along with their significance and effect on the values of the absorption maxima (λ_{max}).

Box-2

IM-1

$$\text{AbsorptionMaxima}(\lambda\text{max}) == 468.38 + 62.33 \times \text{B07[C}-\text{S]} + 14.31 \times \text{F08[N}-\text{S]} - 7.91 \times \text{nCconj} - 6.95 \times \text{F10[N}-\text{S]} + 3444.69 \times \text{ETA\_EtaP\_B} - 7.26 \times \text{nCar} + 43.19 \times \text{NsssCH} + 62.47 \times \text{B03[N}-\text{S]}$$

IM – 2

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 512.90 + 77.56 \times \text{B07[C}-\text{S]} - 12.58 \times \text{nCconj} - 13.51 \times \text{F10[N}-\text{S]} + 32.41 \times \text{B08[C}-\text{S]} + 2744.80 \times \text{ETA\_EtaP\_B} - 6.48 \times \text{nCar} + 38.98 \times \text{NsssCH} - 13.87 \times \text{nArOR}$$

IM – 3

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 506.74 + 66.35 \times \text{B07[C}-\text{S]} + 14.50 \times \text{F08[N}-\text{S]} - 11.11 \times \text{nCconj} - 12.77 \times \text{F10[N}-\text{S]} + 26.82 \times \text{B08[C}-\text{S]} + 3891.01 \times \text{ETA\_EtaP\_B} - 39.62 \times \text{SsssCH} - 7.84 \times \text{nCar}$$

IM – 4

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 506.74 + 66.35 \times \text{B07[C}-\text{S]} + 14.50 \times \text{F08[N}-\text{S]} - 11.11 \times \text{nCconj} - 12.77 \times \text{F10[N}-\text{S]} + 26.82 \times \text{B08[C}-\text{S]} + 3891.01 \times \text{ETA\_EtaP\_B} - 39.62 \times \text{SsssCH} - 7.84 \times \text{nCar}$$

IM – 5

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 477.98 + 63.36 \times \text{B07[C}-\text{S]} + 14.90 \times \text{F08[N}-\text{S]} - 11.47 \times \text{nCconj} + 3398.01 \times \text{ETA\_EtaP\_B} - 5.03 \times \text{nCar} + 38.69 \times \text{NsssCH} - 11.34 \times \text{nArOR} + 54.84 \times \text{B03[N}-\text{S]}$$

IM – 6

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 477.98 + 63.36 \times \text{B07[C}-\text{S]} + 14.90 \times \text{F08[N}-\text{S]} - 11.47 \times \text{nCconj} + 3398.01 \times \text{ETA\_EtaP\_B} - 5.03 \times \text{nCar} + 38.69 \times \text{NsssCH} + 54.84 \times \text{B03[N}-\text{S]} - 11.34 \times \text{O} - 060$$

B07[C–S] is a two-dimensional atom pair descriptor that shows the existence or absence of carbon (C) and sulfur (S) atoms at the topological distance 7. The positive correlation coefficient suggests that the presence of C-S atoms at the topological distance 7 in the dye molecules increases the absorption maxima (λ_{max}). This can be observed in the dyes 10 (B07[C–S] = 1; λ_{max} = 743), 163 (B07[C–S] = 1; λ_{max} = 677) and 210 (B07[C–S] = 1; λ_{max} = 704). On the other hand, dyes 25 (B07[C–S] = 0; λ_{max} = 471), 189 (B07[C–S] = 0; λ_{max} = 509) and 211 (B07[C–S] = 0; λ_{max} = 558), which do not possess this fragment, show lower absorption maxima values. Therefore, it is confirmed the existence of C-S atoms at the topological distance 7 enhances the photoexcitation by growing the dye's localized π–π* transformation. Therefore, the dye's absorption maxima values will be increased by the photoexcitation [66].

The two-dimensional atom pair descriptor F08[N-S] denotes the frequency of N-S atoms at the topological distance 8, which has a positive influence on the absorption maxima of dyes 3 (F08[N–S] = 4; λ_{max} = 661), 7 (F08[N–S] = 2; λ_{max} = 676) and 104 (F08[N–S] =

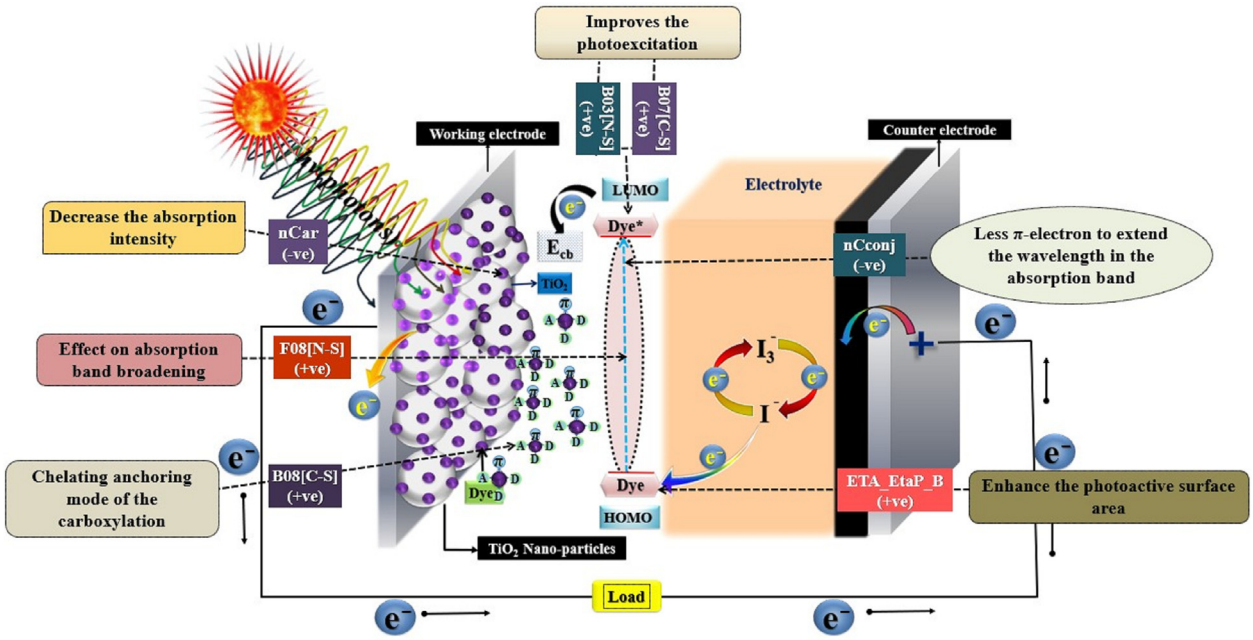


Fig. 5. Contribution of different features in regulating Absorption maxima (λ_{max}) values of phenothiazine dyes.

3; λ_{max} = 642), and inversely for dyes 146 (F08[N–S] = 0; λ_{max} = 550), 205 (F08[N–S] = 0; λ_{max} = 550) and 206 (F08[N–S] = 0; λ_{max} = 549). The existence of nitrogen (N) and sulfur (S) atoms at the topological distance 8 indicates the influence of a donor and an additional donor through the linkage of a dye system. This particular structural fragment contributes to the broadening of the absorption band, which affects the absorption maxima of dye molecules [68]. Consequently, the broadening of the absorption band raises the absorption maxima of dye molecules in the solar cell system.

The descriptor nCconj is a functional group count descriptor indicating the quantity of non-aromatic conjugated carbon of sp^2 hybridization that correlates negatively to the absorption maxima values. This means that as the numerical value of the nCconj descriptor increases, the absorption maxima values decrease, as demonstrated for dyes **177** (nCconj = 10; λ_{max} = 556), **157** (nCconj = 7; λ_{max} = 577) and **205** (nCconj = 6; λ_{max} = 550) and vice versa in case of dyes **11**(nCconj = 2; λ_{max} = 648), **12** (nCconj = 2; λ_{max} = 655) and **63** (nCconj = 2; λ_{max} = 657). Owing to the inclusion of non-aromatic conjugated carbon with sp^2 hybridization, molecules are unable to achieve strong absorption maxima due to a lack of π - electron to expand the wavelength in the absorption band [69].

The descriptor F10[N-S] is a two-dimensional atom pair descriptor denoting the frequency of N-S at the topological distance 10, which relates negatively to the absorption maxima. Therefore, the dyes containing this fragment should have lower absorption maxima values, as shown by the dyes **8** (F10[N-S] = 2; λ_{max} = 577), **85** (F10[N-S] = 2; λ_{max} = 539) and **87** (F10[N-S] = 2; λ_{max} = 572), whereas in the dyes **4** (F10[N-S] = 0; λ_{max} = 662), **126** (F10[N-S] = 0; =665) and **215** (F10[N-S] = 0; λ_{max} = 666), the existence of cyano-propane and phenothiazine sulfur atoms is likely to form the F10[N-S] fragment [70]; as a result, an increase in the numerical value of this descriptor results in a reduction in the values of the absorption maxima.

Another two-dimensional atom pair descriptor, B08[C–S], is described as the existence or absence of carbon and sulfur atoms at the topological distance 8, as demonstrated by the dyes 62 (B08[C-S] = 1; λ_{max} = 659), 63 (B08[C-S] = 1; λ_{max} = 657) and 167 (B08[C-S] = 1; λ_{max} = 659). On the contrary, dyes like 121 (B08[C-S] = 0; λ_{max} = 572), 146 (B08[C-S] = 0; λ_{max} = 550) and 211 (B08[C-S] = 0; λ_{max} = 558) have very low absorption maxima. B08[C–S] descriptor refers to the gap between a strong cyano acceptor and a chelating anchoring mode of carboxylation, which is crucial in regulating the absorption maxima property of dyes [39].

nCar is a descriptor denoting the number of aromatic carbons with sp^2 hybridization. The negative regression coefficient of this descriptor suggests that the inclusion of this fragment in dye molecules will result in a decrease in the absorption maxima, as demonstrated in the dyes **83** (nCar = 8; λ_{max} = 562), **137** (nCar = 8; λ_{max} = 538) and **150** (nCar = 6; λ_{max} = 565), and the opposite is observed in the dyes like **7**(nCar = 0; λ_{max} = 676), **210**(nCar = 0; λ_{max} = 704) and **215**(nCar = 0; λ_{max} = 666). This property favors a decrease in absorption strength caused by geometry distortion, which illustrates the hypochromic influence. As a result, the dye's absorption maxima decreases [71].

The descriptor NsssCH belongs to atom-type E-state indices that specifies the number of atoms of type sssCH. As shown by its positive regression coefficient, this descriptor contributes positively to the absorption maxima property. For example, in the dyes **13** (NsssCH = 1; λ_{max} = 656), **12** (NsssCH = 1; λ_{max} = 655) and **66** (NsssCH = 1; λ_{max} = 655), the absorption maxima values increase as the descriptor values are increased. On the other side, a low number of this descriptor might result in a reduction in the absorption maxima property, as shown by dyes **51** (NsssCH = 0; λ_{max} = 598), **189** (NsssCH = 0; λ_{max} = 509) and **197** (NsssCH = 0; λ_{max} = 546).

B03[N-S] is a two-dimensional atom pair descriptor that denotes the existence or absence of N-S atoms at the topological distance 3. The positive correlation coefficient of this descriptor suggests that the inclusion of N-S atoms (topological distance 3) in dye molecule increases the absorption maxima (λ_{max}), as shown by the dyes such as **3** (B03[N-S] = 1; λ_{max} = 661), **4** (B03[N-S] = 1; λ_{max} = 662) and **5** (B03[N-S] = 1; λ_{max} = 655), and inverse happen in the dyes like **25** (B03[N-S] = 0; λ_{max} = 471), **189** (B03[N-S] = 0; λ_{max} = 509) and **190** (B03[N-S] = 0; λ_{max} = 599). The presence of

N-S atoms (at topological distance 3) enhances the photoexcitation by increasing the dye's localized $\pi-\pi^*$ transformations [72]. The dye's absorption maxima are increased by photoexcitation of dye molecules.

B03[N-S] is a two-dimensional atom pair descriptor that denotes the presence or absence of nitrogen (N) and sulfur (S) atoms at topological distance 3. The positive correlation coefficient of this descriptor shows that the presence of these two atoms at the topological distance 3 in the dye may result in an increase in the absorption maxima (λ_{max}), as shown by the dyes **3** (B03[N-S] = 1; λ_{max} = 661), **4** (B03[N-S] = 1; λ_{max} = 662) and **5** (B03[N-S] = 1; λ_{max} = 655), and vice versa for dyes **25** (B03[N-S] = 0; λ_{max} = 471), **189** (B03[N-S] = 0; λ_{max} = 509) and **190** (B03[N-S] = 0; λ_{max} = 599). Photoexcitation increases with the localized $\pi-\pi^*$ transitions of N-S atoms at the topological distance 3 [72]. Therefore, the dye molecules' absorption maxima will be enhanced with the photoexcitation.

The ETA_EtaP_B descriptor describes the relationship between the branching index EtaB and the molecular size. As indicated by its positive regression coefficient, this descriptor contributes positively to the absorption maxima values. The greater value of this descriptor may result in an increase in the dye's photoactive surface area [64]. As demonstrated by the dyes **63** (ETA_EtaP_B = 0.0 1698; λ_{max} = 657), **162** (ETA_EtaP_B = 0.02364; λ_{max} = 626) and **163** (ETA_EtaP_B = 0.02273; λ_{max} = 677), the absorption maxima values may increase with an increase in this descriptor value. On the other side, a relatively low value for this descriptor can result in a decrease in the max property, as demonstrated by dyes **86** (ETA_EtaP_B = 0.00827; λ_{max} = 538), **136** (ETA_EtaP_B = 0.00687; λ_{max} = 537) and **137** (ETA_EtaP_B = 0.0066; λ_{max} = 538).

nArOR is a functional group count descriptor that denotes the number of aromatic ethers and contributes negatively to the absorption maxima. The presence of aromatic ethers in the molecular dye system without a spacer reduces the absorption maxima [73]. Thus, the presence of a greater number of this fragment in dyes may result in a reduction of the absorption maxima, as seen in dyes such as **151** (nArOR = 2; λ_{max} = 559), **153** (nArOR = 2; λ_{max} = 546) and **197** (nArOR = 2; λ_{max} = 546). On the other hand, the absence of such groups in dye molecules can result in an increase in the absorption maxima values, as demonstrated by the dyes **3** (nArOR = 0; λ_{max} = 661), **4** (nArOR = 0; λ_{max} = 662), and **7** (nArOR = 0; λ_{max} = 676).

SsssCH, is another atom type descriptor that refers to the sum of sssCH E-states that contributes negatively to the absorption maxima. As a result, the presence of this fragment in dyes **9** (SsssCH = 0; λ_{max} = 589), **51** (SsssCH = 0; λ_{max} = 598) and **122** (SsssCH = 0; λ_{max} = 568) decreases the absorption maxima, and vice versa in dyes **62** (SsssCH = -0.878; λ_{max} = 659), **66** (SsssCH = -0.981; λ_{max} = 655) and **69** (SsssCH = -0.949; λ_{max} = 651).

The positive regression coefficients for the two-dimensional atom pair descriptor F07[S-S] (frequency of two sulfurs at a topological distance of 7) showed that the proportion of such group in phenothiazine dye molecule enhances the absorption maxima, as demonstrated for dyes **3** (F07[S-S] = 2; λ_{max} = 661), **7** (F07[S-S] = 1; λ_{max} = 676) and **104** (F07[S-S] = 2; λ_{max} = 642). Consequently, dyes lacking this characteristic exhibit a narrower range of absorption maxima, as demonstrated by the dyes **146** (F07[S-S] = 0; λ_{max} = 550), **177** (F07[S-S] = 0; λ_{max} = 556) and **197** (F07 [S-S] = 0; λ_{max} = 546).

The atom-centered fragment O-060 denotes Al-O-Ar / Ar-O-Ar / R..O..R / R-O-C = X and contributes negatively to the absorption maxima of phenothiazine dyes. This descriptor shows that when the numerical value of this descriptor increases, the absorption maxima of phenothiazine dyes become low. Similarly, the dyes **150** (O-060 = 2; λ_{max} = 565) and **151** (O-060 = 2; λ_{max} = 559) exhibit the same behavior. Inversely, the absorption maxima values of dyes **11** (O-060 = 0; λ_{max} = 648) and **12** (O-060 = 0; λ_{max} = 655) are higher because of absence of such group.

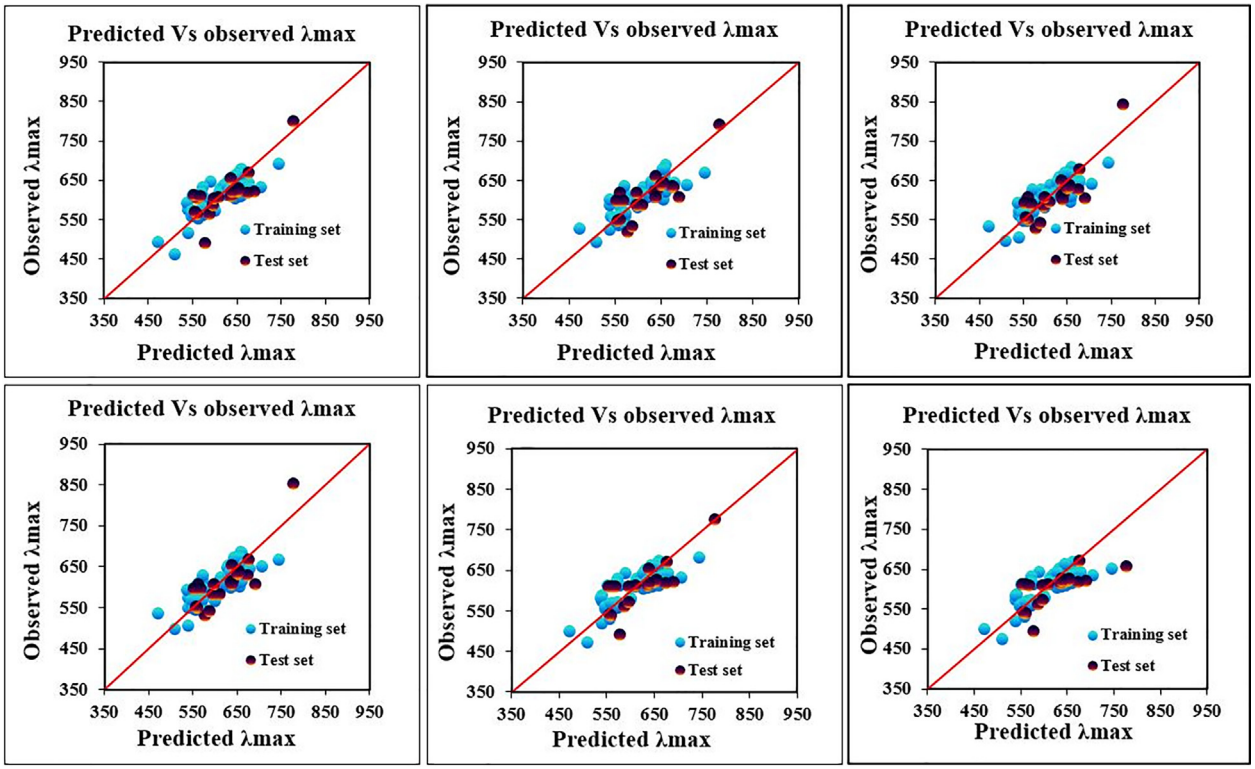


Fig. 6. Observed vs predicted scatter plots of the phenothiazines absorption maxima (λ_{max}) models (IM1-IM6) (phenothiazine dyes).

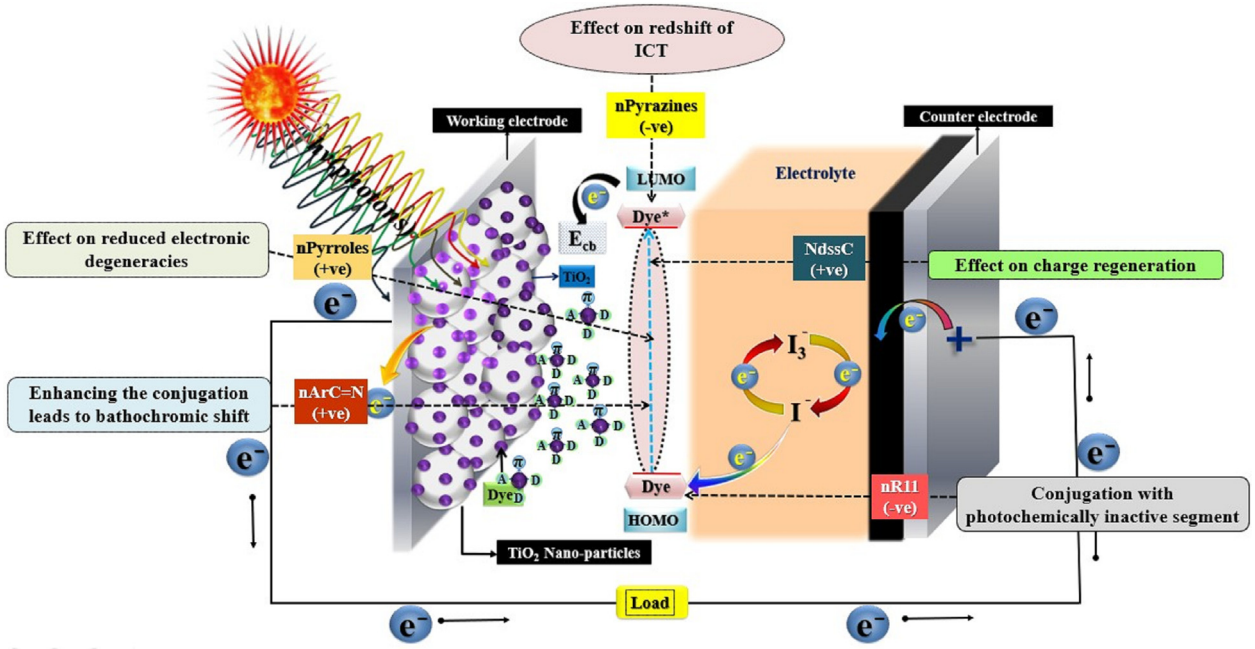


Fig. 7. Contribution of different features in regulating Absorption maxima (λ_{max}) values of indoline dyes.

Fig. 6 shows scatter plots of the observed and predicted absorption maxima (max) values for the developed PLS models (IM1-IM5) (phenothiazine dyes).

3.3. Dataset 3: Modeling of absorption maxima (λ_{max}) values of indoline dyes

Box-3 summarizes five significant PLS models. The descriptors used in the models are discussed below, along with the most plausible mechanistic explanation (Fig. 7) for the dyes' absorption maxima (λ_{max}) property.

Box-3

Model − 1.

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 464.659 + 149.657 \times \text{nArC} = \text{N} + 61.957 \times \text{nRNR2} + 10.46 \times \text{NdssC} - 4.473 \times \text{F07[C} - \text{N]} + 8.367 \times \text{B03[N} - \text{N]}$$

Model − 2.

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 514.526 - 75.361 \times \text{nR11} + 160.308 \times \text{nArC} = \text{N} - 5.031 \times \text{F07[C} - \text{N]} + 10.594 \times \text{nCsp2} - 96.485 \times \text{nPyrazines}$$

Model − 3.

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 514.526 - 75.361 \times \text{nR11} + 160.308 \times \text{nArC} = \text{N} - 5.031 \times \text{F07[C} - \text{N]} + 10.594 \times \text{nCsp2} - 96.485 \times \text{C} - 025$$

Model − 4.

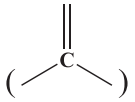
$$\text{AbsorptionMaxima}(\lambda\text{max}) = 514.526 - 75.361 \times \text{nR11} + 160.308 \times \text{nArC} = \text{N} - 5.031 \times \text{F07[C} - \text{N]} + 10.594 \times \text{nCsp2} - 96.485 \times \text{nPyrroles}$$

Model − 5.

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 468.917 - 69.284 \times \text{nR11} + 192.057 \times \text{nArC} = \text{N} + 58.762 \times \text{nRNR2} + 10.987 \times \text{NdssC} - 5.084 \times \text{F07[C} - \text{N]}$$

nArC = N (=: double bond), a functional group count descriptor, denotes the number of aromatic imines present in the dye molecules. This descriptor's positive regression coefficient indicates that the absorption maxima is directly proportional to the numerical value of nArC = N. Thus, the compounds having a higher number of aromatic imines may have an enhanced absorption maxima property. Compounds like **150** (λ_{max} = 752) and **151** (λ_{max} = 766) have their corresponding descriptor values "1". In contrast, compounds like **31** (λ_{max} = 506) and **32** (λ_{max} = 500) have lower absorption maxima because these compounds have no such fragment. In the nArC = N fragment, the lone pair of electrons on nitrogen might be delocalized into the aromatic ring; thus, the conjugation is enhanced, leading to the bathochromic shift [74]. From these observations, we have concluded that aromatic imines are important for absorption maxima of dyes used in DSSCs.

Another functional group count descriptor, nRNR2, indicates the number of aliphatic tertiary amines. The positive impact of this descriptor recommends that an increase in the numerical value of this descriptor leads to higher absorption maxima values of dyes as evidenced by the compounds **26** (nRNR2 = 2; λ_{max} = 712), **27** (nRNR2 = 2; λ_{max} = 700) and **50** (nRNR2 = 1; λ_{max} = 670). On the contrary, the dye molecules without this group displays lower absorption maxima values as seen in the dyes **30** (nRNR2 = 0; λ_{max} = 460), **31**(nRNR2 = 0; λ_{max} = 506) and **83**(nRNR2 = 0; λ_{max} = 545).

NdssC, is an atom type descriptor that indicates the quantity of dssC  atoms. This descriptor favors the absorption maxima values of a dye as it has a positive correlation coefficient.

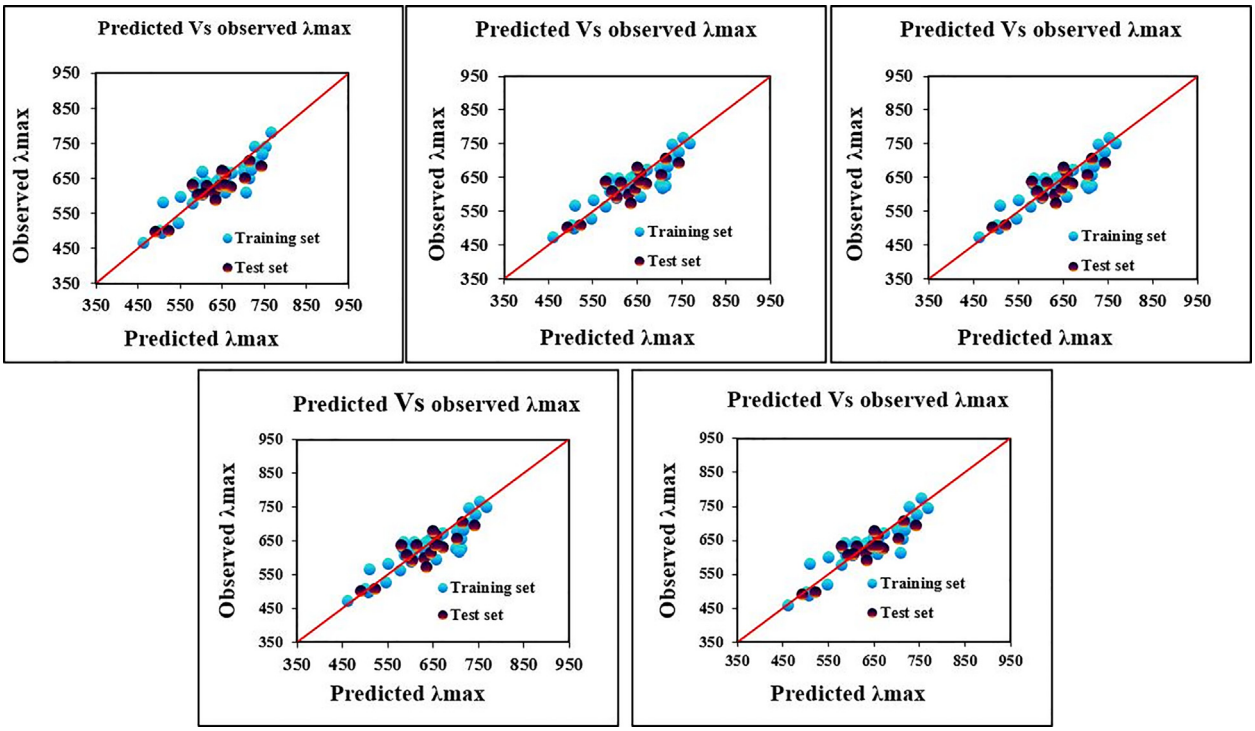


Fig. 8. Observed vs predicted absorption maxima (λ_{max}) values of the developed indoline PLS models (IM1-IM5) (indoline dyes).

The same can be observed in the dyes **63** (NdssC = 20 ; λ_{max} = 656), **170** (NdssC = 24 ; λ_{max} = 727) and **169** (NdssC = 22 ; λ_{max} = 743), while dyes **47**(NdssC = 10 ; λ_{max} = 587), **93** (NdssC = 10; λ_{max} = 549) and **100** (NdssC = 8 ; λ_{max} = 577) showing lower values of absorption maxima due to disappearance of the dssC feature. The presence of dssC in the dyes leads to charge regeneration, which improves the absorption maxima of dye molecules in DSSCs [39].

Another 2D atom pair descriptor, F07 [C–N], indicates two nitrogen atoms at the topological distance of 7. The negative regression coefficient of this descriptor suggests that the presence of carbon and nitrogen atoms at the topological distance 7 has an inversely proportional effect to the dyes' absorption maxima values. This is noticed in case of the dye molecules **9** (F07[C-N] = 16; λ_{max} = 641), **62** (F07[C-N] = 15; λ_{max} = 652), and **65** (F07 [C-N] = 14; λ_{max} = 662). The opposite is observed in the compounds **26**(F07[C-N] = 9; λ_{max} = 712), **169** (F07[C-N] = 8; λ_{max} = 743) and **170** (F07[C-N] = 8; λ_{max} = 727).

Another 2D atom pair descriptor, B03 [N–N], denotes the presence/absence of two nitrogen atoms at the topological distance 3. This descriptor positively influences the absorption maxima of dyes as suggested by its positive regression coefficient. Thus, the compounds containing a higher number of N–N fragments at the topological distance 3 may have high absorption maxima as evidenced by the compounds **150** (λ_{max} = 752) and **151**(λ_{max} = 766) (their corresponding descriptors values are "1"). On the other hand, the molecules which do not contain such a feature may have lower absorption maxima, as shown in compounds **93** (λ_{max} = 508) and **102** (λ_{max} = 549) (Fig.).

nR11 is a ring descriptor indicating how many number of 11-membered rings are present in the indoline dyes. This specific feature has a negative contribution to the absorption maxima property. The N-annulated indenoperylene is a 11 membered ring in the indoline dyes that acts as a photo-chemically inactive segment, thus reducing the absorption maxima [75]. The presence of 11 membered ring in the dyes correlates to low λ_{max} values as observed in dyes **30** (λ_{max} = 460), **31** (λ_{max} = 506), and **32** (λ_{max} = 500). On the contrary, the dyes in absence of 11-membered rings have high absorption maxima values as shown in the dyes **94** (nR11 = 1; λ_{max} = 602) and **95** (nR11 = 1; λ_{max} = 602).

The constitutional descriptor nCsp2 represents a number of sp^2 hybridized carbon atoms, which contributes positively to indoline dyes' absorption maxima property. Thus, the presence of more sp^2 hybridized carbon atoms in indoline dyes favors the higher absorption maxima values. The same can be observed in case of dyes **151** (nCsp2 = 23; λ_{max} = 766), **169** (nCsp2 = 24; λ_{max} = 743) and **170** (nCsp2 = 26; λ_{max} = 727). In contrary, λ_{max} value diminishes as the numerical value of this descriptor decreases as can be observed in the dye molecules **30** (nCsp2 = 9; λ_{max} = 460), **100** (nCsp2 = 8; λ_{max} = 577), and **102** (nCsp2 = 7; λ_{max} = 508).

nPyrazines is a functional count descriptor that specifies the number of pyrazine rings which are present in the dye molecules. The negative regression coefficients of this descriptor indicates that the presence of pyrazine rings in dye molecules reduces the absorption maxima as found in dyes **30**(λ_{max} = 460) and **83** (λ_{max} = 545) (containing one pyrazine ring in each case). Simultaneously, the absence of such fragment in the dyes shows higher absorption maxima, as observed in the case of compounds **1** (λ_{max} = 651) and **3** (λ_{max} = 654). The presence of this group has an effect on the redshift of ICT, thereby reducing the absorption maxima [76].

C-025 is an atom-centered fragment type descriptor representing the fragment R--CR--R (R: any group linked through carbon; --: an aromatic bond as in benzene or delocalized bonds such as the N_O bond in a nitro group). This feature negatively influences the absorption maxima of dyes as suggested by the developed models, which indicates that this feature does not enhance the absorption maxima of dye molecules as found in compounds **31** (λ_{max} = 506), **32** (λ_{max} = 500) (with descriptor values one for both). On the other

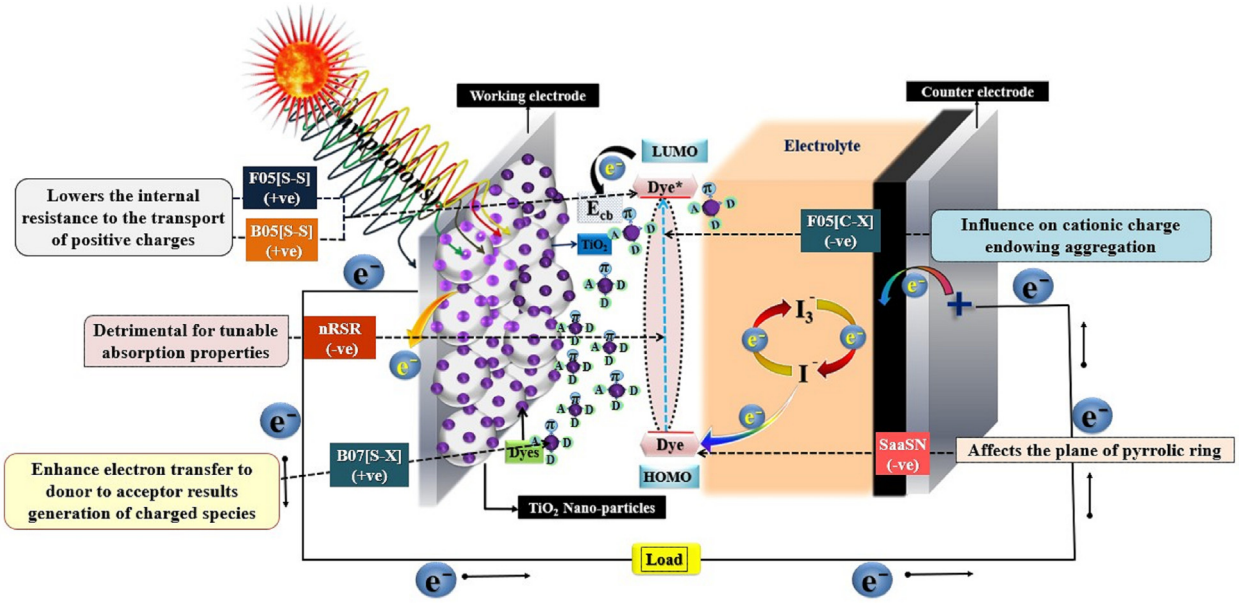


Fig. 9. Contribution of different features in regulating Absorption maxima (λ_{max}) values of porphyrin dyes.

hand, the dyes which do not contain such feature have higher absorption maxima values as found in compounds **6** (λ_{max} = 662) and **26** (λ_{max} = 712).

Another functional group count descriptor, nPyrroles, describes the number of pyrrole rings present in the dyes. This descriptor contributes negatively towards the absorption maxima of the dyes. Thus, the dye molecules bearing a higher number of pyrrole rings may have lower absorption maxima as shown in dye molecules such as **30** (λ_{max} = 460) and **32** (λ_{max} = 500) (both compounds have the descriptor value is '1′), whereas in the contrary, the dye molecules **160** (λ_{max} = 706), **163** (λ_{max} = 712) and **164**(λ_{max} = 701) which do not contain any such fragment show higher absorption maxima values. From this observation, it can be concluded that the number of pyrrole rings should be low in dye structures for better absorption maxima values of dyes used in DSSCs because this fragment has an effect on reduced electronic degeneracies.

Fig. 8 shows scatter plots of the observed and predicted absorption maxima (max) values for the developed PLS models (IM1-IM5) (indoline dyes).

3.4. Dataset 4: Modeling of absorption maxima (λ_{max}) values of porphyrin dyes

Box-4 summarizes the descriptors used in the five PLS models for porphyrin dyes. The mechanistic interpretation of all descriptors (Fig. 9) is discussed in a great detail below, along with appropriate examples.

The descriptor nCIR denotes the number of circuits (bigger loops through two or even more rings) inside a molecule, where a circuit is a self-returning route, i.e., a walk with no repeated vertices except for the beginning and last. The positive coefficient value of this descriptor implies that a surge in the proportion of circuits (which is primarily due to the presence of more fused aromatic rings such as pentacyclic or hexacyclo aromatics) increases the dyes' absorption maxima, as demonstrated by the compounds **104** (nCIR = 162; λ_{max} = 766), **105** (nCIR = 146; λmax = 759) and **4**(nCIR = 77; λ_{max} = 686) [77]. But at the other end, a lower value of this descriptor (corresponding to a reduction in the number of fused rings) results in a reduction in the absorption maxima, as shown by compounds **57** (nCIR = 44; λ_{max} = 616), **94** (nCIR = 39; λ_{max} = 612) and **259** (nCIR = 45; λ_{max} = 612).

nRSR, is a functional group count descriptor, which describes the presence of a lot of sulfides and contributes negatively to the dyes' absorption maximum values. Sulfides have an adverse effect on tunable absorption characteristics, and their presence results in low molar extinction coefficients, which leads to improved energy level responses in the solar cells [78]. Thus, the inclusion of sulfides in the dye structure may result in a reduction of the absorption maxima, as shown in dyes such as **258** (nRSR = 1; λmax = 602), **259**(nRSR = 1; λ_{max} = 612) and **269**(nRSR = 1; λ_{max} = 597). On the other hand, the lack of such fragments in dyes results in increased absorption maxima, as shown by the dyes **81** (nRSR = 0; λ_{max} = 748), **105** (nRSR = 0; λ_{max} = 759) and **184** (nRSR = 0; λ_{max} = 750).

Box-4

Model-1

$$\text{AbsorptionMaxima}(\lambda\text{max}) == 646.245 + 0.65 \times \text{nCIR} - 58.704 \times \text{nRSR} - 23.183 \times \text{NaasN} + 30.992 \times \text{B05[S-S]} + 0.919 \times \text{F07[C-N]} + 8.367 \times \text{nRC\#} - 22.106 \times \text{B07[S-X]}$$

Model-2

$$\text{AbsorptionMaxima}(\lambda\text{max}) == 646.245 + 0.65 \times \text{nCIR} - 58.704 \times \text{nRSR} - 23.183 \times \text{NaasN} + 0.919 \times \text{F07[C-N]} + 8.367 \times \text{nRC\#} - 22.106 \times \text{B07[S-X]} + 30.992 \times \text{F05[S-S]}$$

Model-3

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 676.562 + 0.834 \times \text{nCIR} - 17.621 \times \text{NaasN} + 56.274 \times \text{B05[S-S]} - 4.287 \times \text{F05[C-X]} + 0.805 \times \text{F05[C-N]} + 8.607 \times \text{nRC\#} - 33.404 \times \text{B07[S-X]}$$

Model-4

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 633.647 + 0.677 \times \text{nCIR} - 61.128 \times \text{nRSR} + 0.957 \times \text{F07[C-N]} - 23.019 \times \text{B07[S-X]} + 2.386 \times \text{StsC} - 8.236 \times \text{SaasN} + 32.272 \times \text{F05[S-S]}$$

Model-5

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 671.061 + 0.811 \times \text{nCIR} - 62.983 \times \text{nRSR} + 43.912 \times \text{B05[S-S]} - 4.343 \times \text{F05[C-X]} + 0.911 \times \text{F07[C-N]} + 2.154 \times \text{StsC} - 7.157 \times \text{SaasN}$$

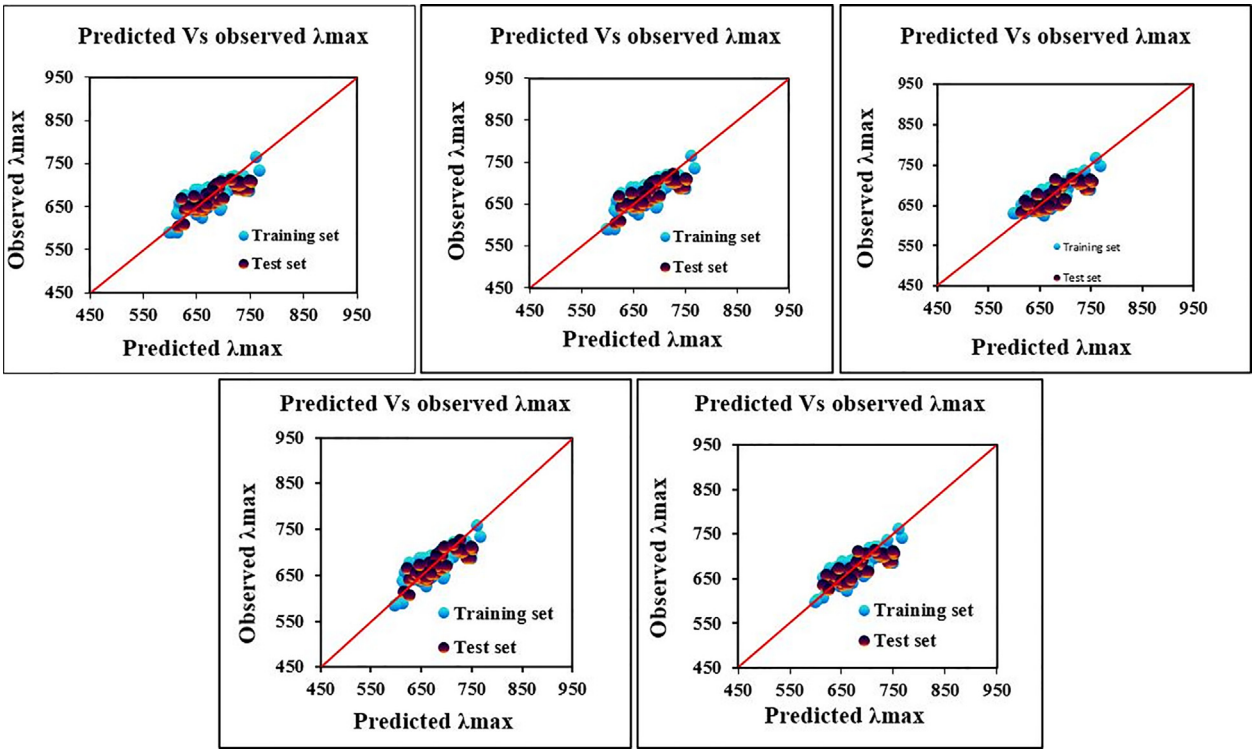
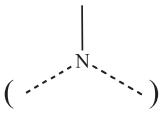


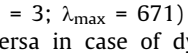
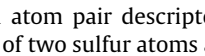
Fig. 10. Observed vs predicted scatter plots of absorption maxima (λ_{max}) values of the developed PLS models (IM1-IM5) (porphyrin dyes).

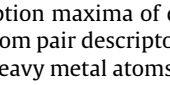
NaasN is an atom type E-state descriptor that denotes the number of atoms of type aasN  that contributes negatively to the absorption maxima. The existence of such features results the lower reactivity because poor π–π* transition causes delayed energy cascade process of the dye molecules [77]. Consequently, dyes comprising these fragments might exhibit reduced absorption maxima in DSSCs, as shown by dyes **85** (NaasN = 3; λ_{max} = 671), **207** (NaasN = 3; λ_{max} = 671) and **209** (NaasN = 3; λ_{max} = 673) and vice versa in case of dyes **105** (NaasN = 0; λ_{max} = 759), **265** (NaasN = 0; λ_{max} = 736) and **283** (NaasN = 1; λ_{max} = 715).

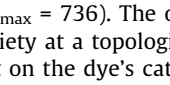
The two-dimensional atom pair descriptor B05[S-S] identifies the existence or absence of two sulfur atoms at the topological distance 5. It has a beneficial effect mostly on absorption maxima values as suggested by dyes **67** (λ_{max} = 703), **262** (λ_{max} = 705), and **263** (λ_{max} = 712) due to their descriptor values being equal to one in all cases, and zero for dyes **127** (λ_{max} = 624), **150** (λ_{max} = 623) and **164** (λ_{max} = 622). This segment may represent the conjugation units in the dyes' "π" system, which results in a decreased internal barrier to positive charge transfer [67]. Thus, the conjugated π--system may exhibit greater absorption maxima of dyes in DSSCs.

The two-dimensional atom pair descriptor F05[C-X] denotes the frequency of carbons and heavy metal atoms (Zn) at the topological distance 5, that also corresponds negatively to the absorption maxima, as suggested by dyes such as **1** (F05[C-X] = 12; λ_{max} = 644), **2** (F05[C-X] = 12; λ_{max} = 648) and **5** (F05[C-X] = 14; λ_{max} = 645) having lower absorption maxima. Similarly, the dyes without this property exhibit larger absorption maxima, as seen by the dyes **80** (F05[C-X] = 10; λ_{max} = 747), **81** (F05[C-X] = 10; λ_{max} = 748) and **220** (F05[C-X] = 10; λ_{max} = 736). The occurrence of a carbon atom in the porphyrin moiety at a topological distance of 5 from zinc has a beneficial effect on the dye's cationic charge, resulting in low aggregation values [80].

The positive correlation coefficient for the two-dimensional atom pair descriptor F07[C-N] indicates that the frequency of C-N atoms at the topological distance 7 has a positive effect on the absorption maxima of dyes used in DSSCs, as observed for dyes **14** (F07[C-N] = 59; λ_{max} = 697), **183** (F07[C-N] = 63; λ_{max} = 697) and **270** (F07[C-N] = 58; λ_{max} = 700). In contrast, the dye molecules without such fragments have suboptimal absorption maxima in DSSCs, as shown by dyes such as **26** (F07[C-N] = 15; λ_{max} = 621), **94** (F07[C-N] = 13; λ_{max} = 622) and **95** (F07[C-N] = 16; λ_{max} = 612).

StsC is an atom type E-state descriptor, which describes the sum of tsC E-states  , contributes positively towards the absorption maxima of porphyrin dye molecules as evident from the positive regression coefficient of the descriptor. Existence of numerous "  " fragments increase the absorption maxima values as shown by the dye molecules **179** (StsC = 22.815; λ_{max} = 720), **181**(StsC = 22.874; λ_{max} = 713) and **220** (StsC = 23.223; λ_{max} = 736). Again, the dye molecules **57** (StsC = 0; λ_{max} = 616), **26** (StsC = 0; λ_{max} = 621) and **55** (StsC = 0; λ_{max} = 621) have no such fragments and thus they have lower λ_{max} values.

The descriptor nR#C- belongs to the family of functional group counts, which signifies the number of non-terminal "C (sp)" (sp-hybridized carbon)  . It has a positive impact on the absorption maxima (λ_{max}) of dyes as discovered in the dyes such as **179** (nR#C- = 6; λ_{max} = 720), 180 (nR#C- = 6; λ_{max} = 710) and 181 (nR#C- = 6; λ_{max} = 713). In contrary, the dyes lacking this feature display minimum absorption maxima values (λ_{max}) as found in the dyes **27** (nR#C- =0; λ_{max} = 632), **72** (nR#C- =0; λ_{max} = 629) and **77** (nR#C- =0; λ_{max} = 630).

SaasN, is an atom type E-state descriptor denoting the sum of aasN E-states  which has a negative regression coefficient denoting that an increase in the number of aromatic rings

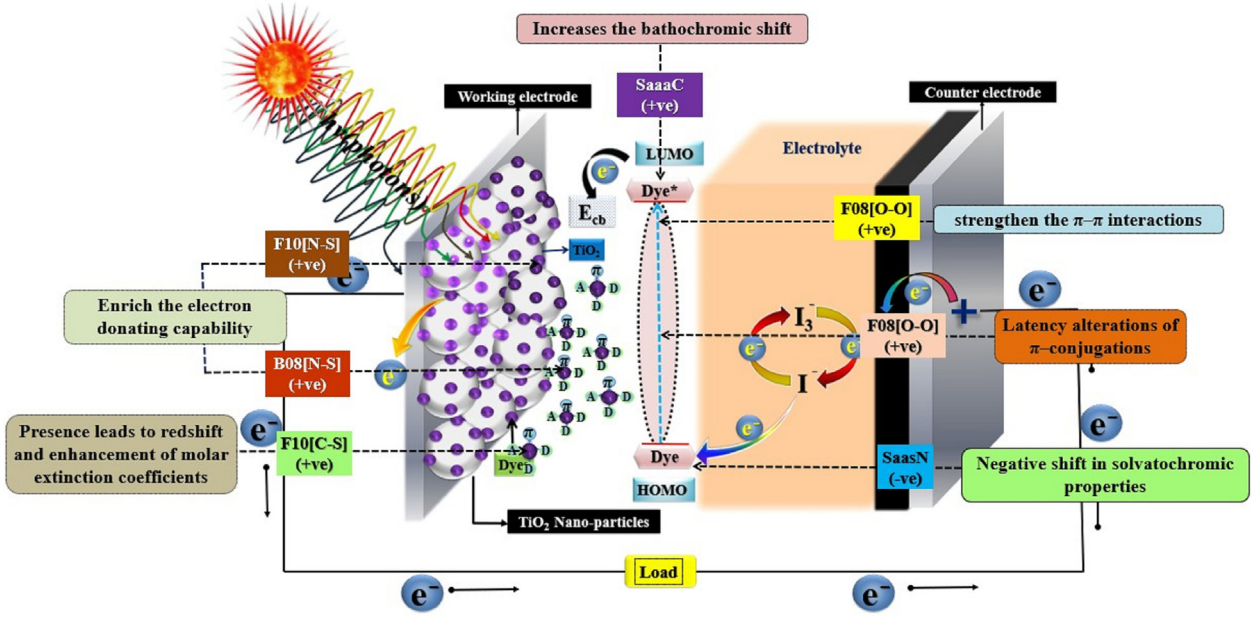


Fig. 11. Significance of various features on regulating Absorption maxima (λ_{max}) values of coumarin dyes.

accompanied by the "N (nitrogen)" groups distorts the plane of porphyrin dye's pyrrole ring resulting in blue shift and lowering of the absorption maxima as found in case of the dyes **168** (SaasN = 7.060; λ_{max} = 657), **169** (SaasN = 7.093; λ_{max} = 648) and **170** (SaasN = 7.101; λ_{max} = 617). In contrast, a reduction in the number of aromatic rings with fused "N (nitrogen)" atom enhances the dyes' absorption maxima, as shown by compounds **232** (SaasN = 4.475; λ_{max} = 703), **100** (SaasN = 4.503; λ_{max} = 703) and **190** (SaasN = 4.506; λ_{max} = 725) [79].

B07[S-X] is a two-dimensional atom pair descriptor that indicates the existence or absence of sulfur and heavy metal atoms (Zn) with a positive influence on the absorption maxima (λ_{max}). Thus, dyes with this fragment, such as **67** (B07[S-X] = 1; λ_{max} = 703), **51** (B07[S-X] = 1; λ_{max} = 684) and **50** (B07[S-X] = 1; λ_{max} = 675), exhibit a wider range of absorption maxima in DSSCs. On the other hand, dyes with a lower frequency of this fragment exhibit a narrower range of maximum values, as shown by the dyes **57** (B07[S-X] = 0; λ_{max} = 616), **160** (B07[S-X] = 0; λ_{max} = 617) and **169** (B07[S-X] = 0; λ_{max} = 617). This descriptor indicates the significance of the thiophene ring connected to the porphine ring in porphyrin dyes, which may facilitate electron transfer from donor to acceptor, resulting in the creation of charge-separated species and a rise in the λ_{max} value [70,81].

One more two-dimensional atom pair descriptor, F05[S-S], indicates the presence or absence of two sulfur atoms at the topological distance 5, which has a positive effect on the absorption maxima values, as demonstrated by the dyes **67** (F05[S-S] = 1; λ_{max} = 703), **262** (F05[S-S] = 1; λ_{max} = 705) and **263** (F05[S-S] = 1; λ_{max} = 712), which have this fragment resulting in an increase in absorption maxima (λ_{max}) values. On the other hand, the dyes **74** (λ_{max} = 632), **75** (λ_{max} = 633) and **76** (λ_{max} = 632) do not have this group resulting in a decrease in the λ_{max} value of dyes. Therefore, it is concluded that the presence of this fragment in the dye molecules may represent the conjugation units in the dye's 'π' system, which results in a decreased internal barrier to positive charge transfer. Thus, the conjugated π -system may exhibit greater absorption maxima of dye molecules used in DSSCs [67].

Fig. 10 shows scatter plots of the observed vs predicted absorption maxima (λ_{max}) values for the proposed PLS models (IM1-IM5) (porphyrin dyes).

3.5. Dataset 5: Modeling of the absorption maxima (λ_{max}) of coumarin dyes

The five PLS models revealed important descriptors, which are displayed in Box-5 along with their corresponding absorption maxima (λ_{max}) property contributions. The mechanical explanation of each descriptor (Fig. 11) is presented below, along with some suitable instances.

Box-5

Model-1

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 512.619 + 52.679 \times \text{B08[N}-\text{S]} + 8.522 \times \text{F08[O}-\text{O]} + 80.413 \times \text{F10[N}-\text{S]} + 61.628 \times \text{SaasN}$$

Model-2

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 503.126 + 15.133 \times \text{F08[O}-\text{O]} + 16.426 \times \text{F09[C}-\text{N]} + 11.195 \times \text{F10[C}-\text{S]} - 0.661 \times \text{T(N..O)}$$

Model-3

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 475.892 + 65.687 \times \text{B08[N}-\text{S]} + 13.12 \times \text{F08[O}-\text{O]} + 68.133 \times \text{F10[O}-\text{O]} + 10.233 \times \text{F10[C}-\text{N]}$$

Model-4

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 507.512 + 60.855 \times \text{B08[N}-\text{S]} + 11.44 \times \text{F08[O}-\text{O]} + 87.475 \times \text{F10[N}-\text{S]} + 20.228 \times \text{SaaaC}$$

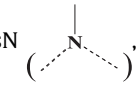
Model-5

$$\text{AbsorptionMaxima}(\lambda\text{max}) = 472.924 + 13.652 \times \text{F08[O}-\text{O]} + 69.486 \times \text{F10[N}-\text{S]} + 45.541 \times \text{F08[N}-\text{S]} + 11.554 \times \text{F10[C}-\text{N]}$$

B08[N-S] is a two-dimensional atom pair descriptor that indicates that the presence or absence of nitrogen and sulfur atoms at the topological distance 8, It has a favorable effect on the absorption maxima values. Thus, the inclusion of this fragment in the dyes may result in a rise in the absorption maxima property, as observed for dyes **37** (B08[N-S] = 1; λ_{max} = 714) and **44** (B08[N-S] = 1; λ_{max} = 619). Conversely, the dyes **17** (B08[N-S] = 0; λ_{max} = 470) and **56** (B08[N-S] = 0; λ_{max} = 464) in absence of the B08[N-S] fragment show lower absorption maxima. The inclusion of nitrogen and sulfur atoms in the coumarin moiety may enhance the dyes' capacity to donate electrons, hence the absorption maxima values of the dyes will be enhanced [68].

F08 [O-O], another two-dimensional atom pair descriptor, denotes the frequency of two oxygen atoms at the topological distance 8, which impacts favorably to the absorption maxima values. This can be seen from dyes **10** (F08 [O-O] = 8; λ_{max} = 610) and **39** (F08 [O-O] = 4; λ_{max} = 617); the λ_{max} attribute is set to a high value owing to the descriptor's larger numerical value in such cases. In the absence of the F08[O-O] characteristic, the dye molecules **6** (F08[O-O] = 0; λ_{max} = 510) and **16** (F08[O-O] = 0; λ_{max} = 505) exhibit the opposite behavior. This descriptor refers to the oxygen in the coumarin moiety's enamine and the functional anchoring groups (such as carboxylate, alkoxysilanes, etc.) that may assist to enhance the dye system's π–π interactions, resulting in an increase in the absorption maxima [83].

Another two-dimensional atom pair descriptor, F10[N-S], claims that the frequency of nitrogen and sulfur atoms at the topological distance 10 has a positive effect on absorption maxima (λ_{max}). Hence, the inclusion of this fragment in the dyes may result in a rise in the absorption maxima values recorded for dyes **7** (F10 [N-S] = 1; λ_{max} = 673) and **27** (F10 [N-S] = 2; λ_{max} = 720); conversely for dyes **56** (F10 [N-S] = 0; λ_{max} = 464), **57** (F10 [N-S] = 0; λ_{max} = 489), the absence of the F10 [N-S] fragment decreases the absorption maxima. The inclusion of nitrogen and sulfur atoms at the topological distance 10 in the coumarin moiety may enhance the dyes' capacity to donate electrons (electron donating groups (EDGs)); hence, it will be helpful to increase their absorption maxima values [68].

The atom type E-state index SaasN specifies the E-state atom index of the 'N' atom in the fragment aasN  , favorably affecting the absorption maxima feature. As a result, the inclusion of this fragment in dyes enhances their absorption maxima, as shown by the dyes **37** (SaasN = 2.381; λ_{max} = 714) and **7** (SaasN = 1.765; λ_{max} = 673), and oppositely by the dyes **16** (SaasN = 0; λ_{max} = 505) and **17** (SaasN = 0; λ_{max} = 470). The presence of this non-polar group has a detrimental effect on the dyes' solvatochromic characteristics (the chemical substance's capacity to change color in response to a change in polarity). As a result, the dyes are unable to stick adequately to the semiconductor, impairing the dye's absorption and stability causes less absorption maxima [84].

F09[C-N] is a two-dimensional atom pair descriptor which specifies the frequency of carbon and nitrogen atoms at the topological distance 9, and it influences the absorption maxima positively. Furthermore, the dyes having this fragment like **27** (F09 [C-N] = 15; λ_{max} = 720) and **37** (F09 [C-N] = 10; λ_{max} = 714) show increased values of absorption maxima in DSSCs. Conversely, dyes such as **4** (F09 [C-N] = 4; λ_{max} = 490) and **5** (F09 [C-N] = 4; λ_{max} = 494) without this fragment show minimum absorption maxima values.

F10[C-S] is a two-dimensional atom pair descriptor that specifies the frequency of carbon and sulfur atoms at the topological distance 6, which contributes favorably to absorption maxima owing to their positive correlation coefficients. Therefore, the inclusion of this group in dye molecules may enhance the absorption maxima property, as shown by dyes **22** (F10[C-S] = 8;

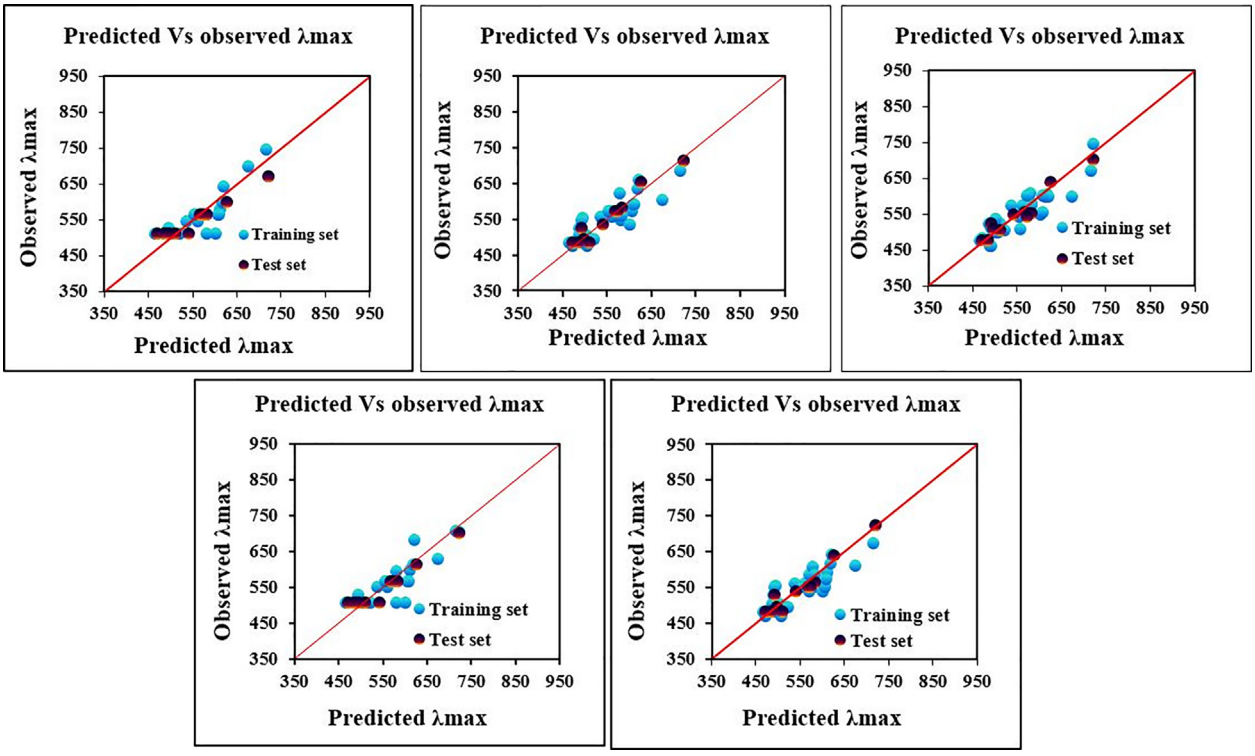


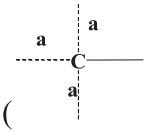
Fig. 12. Observed vs predicted values of scatter plots of absorption maxima (λ_{max}) values of the coumarin dyes (IM1-IM5) (coumarin dyes).

λ_{max} = 607) and **54** (F10[C-S] = 10; λ_{max} = 570), and the opposite happens with the dyes like **17** (F10[C-S] = 0; λ_{max} = 470) and **56** (F10[C-S] = 0; λ_{max} = 464) because of absence of this fragment. This group adds an alkyl substitution at the third position of the thiophene ring, which results in redshift of the absorption spectrum and an increase in the dyes' molar extinction coefficient, which results in greater absorption maximum values [85].

T(N..O) is a two-dimensional atom pair descriptor that reflects the sum of topological distances between nitrogen and oxygen atoms. This feature has a detrimental effect on the absorption maxima of dyes used in DSSCs. Thus, we can infer that the single aromatic bonds of nitrogen and oxygen atoms tend to have a lower potential to improve absorption maxima. It is evidenced from the dye molecules **3** (T(N..O) = 260; λ_{max} = 487) and **5** (T(N..O) = 214; λ_{max} = 494). On the other hand, the reverse was seen with dye molecules **39** (T(N..O) = 58; λ_{max} = 617) and **10** (T(N..O) = 58; λ_{max} = 610).

The two-dimensional atom pair descriptor F10[C-N] denotes the frequency of carbon and nitrogen atoms at the topological distance 10, and it influences positively to the coumarin dyes' absorption maxima. Therefore, the presence of such groups enhances the absorption maxima values, as seen in dyes 22 (F10[C-N] = 5; λ_{max} = 607) and 44 (F10[C-N] = 5; λ_{max} = 619), whereas dyes 16 (F10[C-N] = 0; λ_{max} = 505) and 17 (F10[C-N] = 0; λ_{max} = 470) exhibit decreased absorption maxima values because of the non-existence of this descriptor.

SaaaC is an atom type E-state index descriptor that describes the total of three aromatic bonds formed by carbon atoms



in dye molecules. This descriptor shows a positive ( ) effect on the absorption maxima values of dyes employed in dye-sensitized solar cells. Basically, this descriptor determines the delocalization of electrons, and thereby, it enhances the bathochromic shift of the dyes [86]. As a result, dyes containing this group may exhibit higher absorption maxima, as shown by the dyes **7** (SaaaC = 1.855; λ_{max} = 673), and **44** (SaaaC = 1.407; λ_{max} = 619). Conversely, the dyes **17** (SaaaC = 0; λ_{max} = 470), and **24** (SaaaC = 0; λ_{max} = 485) which do not have this fragment display lower absorption maxima values.

F08[N-S] is a two-dimensional atom pair descriptor that defines the frequency of N(nitrogen) and S (sulfur) atoms at the topological distance 8, which contributes positively to the absorption maxima. The presence of this fragment in the donor groups influences the dye molecules' absorption range, resulting in π-conjugation latency changes [87]. Consequently, in dyes 40 (F08[N-S] = 2; λ_{max} = 569) and 51 (F08[N-S] = 2; λ_{max} = 581), the absorption maxima values increase with the presence of this fragment. In contrary, dyes without such groups may exhibit a decrease in absorption maxima, as can be seen in the dye molecules like **8** (λ_{max} = 490), 17 (λ_{max} = 470), and 56 (λ_{max} = 464).

The scatter plots obtained by observed vs predicted absorption maxima (λ_{max}) values for the developed coumarin PLS models (IM1-IM5) are depicted in Fig. 12**.**

Equations and various statistical metrics derived from mixed models (i.e., PLS models derived from the combination of descriptors appearing in different models) have been mentioned in Supplementary Materials SI-2. Additionally, we have used the DModX approach to assess the applicability domain of each of the individual models (all of the models IM1 to IM5) derived from the five datasets. According to the applicability domain analysis, all of the training datasets of organic dyes are within the specified D-critical values at the 99% confidence level for the Triphenylamine (2.227–2.498), Phenothiazine (2.024), Indoline (3.408), Porphyrin (1.585–1.911) and Coumarin (2.5–3.696) datasets. In the case of test set dyes, all compounds except **28, 54** (for models IM-2, IM3) for the Triphenylamine dataset; all compounds except **6, 200** (IM1-IM5) for the Phenothiazine dataset; all compounds except **136, 227, 260** and **270** (IM1-IM5) for the Porphyrin and all of the test set chemicals in the indoline and coumarin dataset are inside the applicability domain. Given the large number of dyes, over 99 % compounds passed the AD test, indicating that their predictions are totally trustworthy and reliable.

4. Overview

In this study, several QSPR models have been constructed for 700 organic dyes divided into five chemical types. Individual and consensus models were created to assist us exploring the critical structural components and physicochemical properties of the dyes that contribute to the variety of absorption maxima (λ_{max}) values in DSSCs. The models provide a series of mechanistic explanations for the fluctuation of absorption maxima (λ_{max}) values with molecular structures for a large number of dyes that investigators/researchers may use to significantly minimize experimental testing, time, and money. Additionally, the exploratory data may aid in the development of novel lead dyes for all five chemical classes. The chemometric modeling study results in the following findings on the effective absorption maxima (λ_{max}) of the specific chemical classes:

4.1. Triphenylamine dyes

The sp^3 hybridized ring quaternary carbons lower the optical band gap, and thereby shorten the wavelength, which produces a lower molar extinction coefficient leading to a reduction in the solar cell reaction energies [52]. Again, a greater number of halogen atoms are favorable for π- bond conjugation and consequent changes in absorption bands of the spectra followed by increased absorption maxima (λ_{max}) values [53]. Furthermore, the triphenylamine dyes with vibrational electrical discharge of the nitrogen atom exhibit a low range of absorption maxima values [54,55]. The thiadiazole ring being an electron-deficient unit may lower the LUMO levels at the same time maintaining the HOMO levels and thus lead to a smaller HOMO-LUMO gap and a more red-shifted UV/Vis spectrum [56–62]. Benzothiadiazoles are used as the additional acceptors as they are electron withdrawing groups and expected to function as an electron trap where charge separation occurs, thus facilitating the electron migration direction to the anchor (cyanoacetic acid) unit. These groups incorporated adjacently to the dye donors show several favourable characteristics in the beneficial light harvesting and efficiency [88,89]:

i) an optimization in energy levels resulting in the long responsive wavelength to NIR region;

ii) exhibiting a very small blue-shift in absorption peak on thin TiO2 film with respect to that in solution;

iii) an improvement in the electron distribution of donor unit to distinctly increase the photo-stability of synthetic intermediates and final sensitizers. Hence, the HOMO energy level is maintained, resulting in a smaller HOMO-LUMO energy gap.

According to the studies conducted by various groups [88,89] and observations from the NMR data, during the synthetic process, reasonably, nitrogen-containing electron donors are very sensitive under irradiation due to their low oxidation potential. Specifically, for the indoline unit, the saturated five-member ring cannot disperse the lone pair electrons as the phenylene in triphenylamine unit, resulting in less photo-stability. In contrast, the intermediate becomes completely photo-stable when attaching a benzothiadiazole unit. Clearly, the incorporated strong electron-withdrawing

unit of benzothiadiazole can distinctly increase the photo-stability of the intermediate due to the favourable electron distribution of donor section (showed red-shift of absorption band in UV/visible spectrum because of long π- conjugation and narrow band gap). Such a stable intermediate is beneficial to the synthetic process. Consequently, we expect the insertion of benzothiadiazole moiety as an additional acceptor to construct D-A- π -A configuration organic dyes specifically improves their photostability, which is always critical to the commercialization of solar cells. Chemical reactivity theory of DFT might be more suitable for explaining the reason for the red shift of the UV/visible spectrum.

Again, triphenylamines with higher surface area causes a larger charger transfer (electron injection) time into the mesoporous semi-conductor layer (TiO_2) resulting in reduced λ_{max} values [66]. Hence, these groups should not be considered while designing new dyes. In contrary, sp^3 hybridized carbons tends to reduce the regional $\pi-\pi^*$ transformations and increase the wavelength owing to the ICT (intramolecular charge transfer transition) from the donor moiety of triphenylamine dye, thereby it enhances the λ_{max} values [66]. Hexyloxy amines and ethylhexyloxy amino groups help as electron donating groups to offer better absorption maxima (λ_{max}) [67]. Thus, these fragments would be useful in developing new triphenylamine dyes.

4.2. Phenothiazine dyes

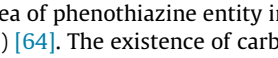
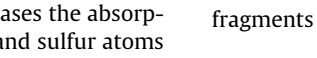
Dye molecules having the sp^2 hybridized non-aromatic conjugated carbons produce less π-electrons to extend the wavelength in the absorption band; thus, such molecules fail to attain high absorption maxima [66]. On the other hand, the enhanced photoactive surface area of phenothiazine entity increases the absorption maxima (λ_{max}) [64]. The existence of carbon and sulfur atoms of the donor fragment of phenothiazine dye encounters chelating anchoring mode and powerful cyano acceptor for carboxylation, hence controlling the λ_{max} values of phenothiazines [66]. In phenothiazines, D-π-A's increased photoactive surface area enhances the maintenance of bathochromic shift [68]. Again, presence of N and S atoms improves the photoexcitation by increasing the localized $\pi-\pi^*$ transition of the dyes [72]. In case of phenothiazines, the sulfur atom likely forms a link with cyano-propane and decreases the λ_{max} value [70]. Furthermore, regional $\pi-\pi^*$ transformation of the dyes improves the photoexcitation due to the existence of C-S atoms at the topological distance 7[39].

Higher number of N and S atoms at the topological distance 8 denotes the donor and additional donor's effect through a dye system coupling [68]. This particular descriptor influences the broadening of absorption band, that effects the λ_{max} value. Again, the carbons with aromatic nature and sp^2 hybridization favor a decrease in absorption intensity due to the geometry distortion, which reflects the hypochromic effect [69]. Thus, the absorption maxima of the dye decreases. Therefore, these fragments should be avoided while designing phenothiazine dyes.

4.3. Indoline dyes

Presence of aromatic imines improves π- conjugation leading to delocalization in the aromatic ring of indolines and resulting in the bathochromic shift [74]. Similarly, nitro group of dye molecules,  and  structural features have an influence on the effective charge regeneration leading to enhanced absorption maxima [39]. Simultaneously, inclusion of 11-membered ring (N-annulated indenoperylene) acts as an electron donor in indoline dyes enabling the conjugation with an electron acceptor through a triple bond, resulting in a metal-free donor or acceptor dye without the usage of any co-adsorbate, which may account for the high absorption maximum values [75]. Again, the presence of pyrazine rings and sp^2 hybridized carbon atoms has an effect on redshift of ICT, thereby it reduces the absorption maxima. The fragment R--CR--R having an aromatic bond as in benzene or delocalized bonds such as N_O bond in a nitro group has an influence on the hypsochromic effect. This feature negatively influences the absorption maxima of dyes. Again, pyrrole and pyrazine rings should be low in number in the dye structures for better absorption maxima, because this fragment has an effect on reduced electronic degeneracies [76].

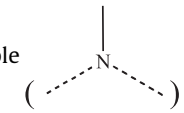
4.4. Porphyrin dyes

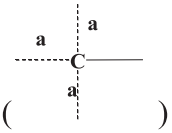
Dyes having merged ring systems (fused aromatic rings) such as pentacyclic or hexacyclo aromatics show enhanced absorption maxima. Again, a higher sulfide content is detrimental to the adjustable absorption capabilities of dyes, since they have low molar extinction coefficients, resulting in lower absorption maxima [78]. Similarly, dyes with the  fragment with two aromatic bonds cause a slow energy cascade mechanism, hence the molecules experience the poor $\pi-\pi^*$ transition as they have less absorption maxima [79]. Furthermore, distance of carbon to Zn at the topological distance 5 in the porphyrin moiety has an influence on the cationic charge of the whole dye molecular structures. Thus, it endows the aggregation resulting low λ_{max} values [80]. Other fragments like  and  have an influence towards bathochromic shift, improving the λ_{max} of the dyes, thus these fragments can be included in efficient molecular design of dye molecule.

In contrary, dyes with the fragment SaasN with an increase number of electron delocalized (aromatic) rings surrounded by nitrogens affect the plane of the pyrrolic ring of the porphyrin dye leading to the distortions within the dye structures, resulting in blue shift, thereby decreasing the absorption maxima values. Therefore, this feature should be avoided in molecular design of novel dyes [81,82]. Moreover, the inclusion of thiophene ring within porphyrin dyes might facilitate electron transport from donor to acceptor, leading to the creation of charge-separated species and a rise in the absorption maxima values. The feature B05[S-S] could constitute the alternative double bonds (conjugation units) for the dye's conjugated π-system, which results in a decreased internal barrier to positive charge transfer [67]. Thus, the conjugated π -system may exhibit greater absorption maxima of dye molecules in DSSCs.

4.5. Coumarin dyes

Presence of N and S atoms in coumarin dyes influences the electron donating groups (EDGs) and thereby it increases the capability of electron donation in the dyes used in DSSCs [68], which may increase the absorption maxima values. In addition, the groups that represent the oxygen in the coumarin moiety's enamine group and the functional anchoring groups (such as carboxylate, alkoxysilanes, etc.) reinforce the π–π interaction, raising the absorption maxima [83]. Again, the nitrogen and sulfur (N-S) atoms act as electron donating groups (EDG), since these atoms are electron rich units associated with vibrational frequencies and improve the blueshift of organic dyes [68,90]. Simultaneously,

with another variable  (among three bonds, two are within the aromatic ring), solvatochromic characteristics undergo a negative change in coumarin dyes [84]. As a result, the dyes are unable to stick adequately to the semiconductor, impairing their absorption and stability. Furthermore, C and S atoms at the topological distance 6 introduce an alkyl substitution at the 3rd position of the thiophene ring leading to the redshift of the absorption spectrum resulting in the enhancement of absorption maxima [85]. In addition to this, single aromatic bonds (like C_N bonds in pyrrole) enhance the absorption maxima. Thus, these moieties can be included in the design of novel dyes.

Again, the fragment  improves the electron delocalization and then increases the bathochromic shift within coumarin dyes. The presence of nitrogen and sulfur atoms in the donor groups at the topological distance 8 causes latency alterations of π-conjugation and influences the absorption range of dyes [86]. Thus, these fragments augment the absorption maxima and they are helpful for the design of efficient dyes.

5. Conclusion

In summary, the meaningful attributes or descriptors indicated as above are crucial for photocatalytic characteristics, cell short-circuit current (Isc) and open-circuit voltage (V_{OC}) points, as well as the maximum power point (Vmp, Imp). This work has focused mostly on characteristics of 2D structural fragments, since quantum and electrochemical examination of specific organic dye molecules is time demanding and requires extensive computational resources. The current work employs a simple methodology based on 2D descriptors to investigate critical chemical characteristics associated with greater absorption maxima. Incorporation of aliphatic or aromatic amines/imines, thiophene, thiadiazole, pyrazine, or pyrrole rings into the molecular structures of dyes improves the flow of electrons to acceptor atoms for H-bonds through the D-A-π-A structural framework. Additionally, all dye chemical classes have the adhesion characteristic (solvatochromism) and the requirement for a chain length free of hydrophobic attribute.

CRediT authorship contribution statement

Jillella Gopala Krishna: Data curation, Methodology, Validation, Investigation, Writing – original draft. **Kunal Roy:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2021.120387.

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QSAR modelling of organic dyes for their acute toxicity in *Daphnia magna* using 2D-descriptors

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ABSTRACT

The present study reports quantitative structure-activity relationship (QSAR) models for 22 organic dyes spanning a broad chemical domain to predict their toxicity in *Daphnia magna* [log ($1/EC_{50}$)]. Only two-dimensional descriptors with clear physicochemical meaning were used to construct the QSAR models. The process of development, validation, and interpretation of models adheres to the stringent recommendations of the Organization for Economic Cooperation and Development (OECD) guidelines. In this study, the multi-layered stepwise regression method and linear discriminant analysis (LDA) method were employed for the deployment of regression – and classification-based models respectively; however, the final regression-based QSAR models were obtained through the partial least squares (PLS) regression. Additionally, the applicability domain of the developed models was verified. The constructed models should be applicable in the absence of toxicity data of new or untested dye structures, particularly when the compounds fall within the developed models' scope, and also implementable to develop more environmentally friendly alternatives.

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Introduction

The usage of dyes has become more common in our day-to-day life because many industries are depending on dyes for their commercial activities. These dyes are the medium to impart colour to pharmaceuticals, food materials, textiles, solar cells, etc.; thus, this colouring is not readily altered by heat, light, wash, and other factors to which it gets exposed. For these reasons, it is more important to know their characteristics in various aspects. All of the dyes can make some wastewater during the dye preparation and their use or other applications with the dyes; this dye wastage is released to several aquatic ecosystems such as rivers, oceans, or even other bodies of fresh water. Another category of dyes is food dyes, which are promising colourants used to colour a wide range of foods. These food dyes not only colour food but also increase appetite and visual appeal to consumers [1]. 1–2% of dyestuffs are lost in manufacture, while 10–15% of dyes are discharged as effluent [2]. The discharge of dye effluents from dye manufacturing or

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consumption units into water bodies poses major health risks to humans, plants, animals, and aquatic biota. Even trace levels of dyes in wastewater (less than 1 mg/dm^3 for some dyes) are unpleasant and undesirable [3]. Organic dyes like azo dyes have aromatic centres in their molecular structures, and they are well-established carcinogens, mutagens, and DNA adducts [4]. Furthermore, dyes can colour water, limiting transparency (sunlight penetration) and aeration, reducing photosynthetic efficiency, and reducing dissolved oxygen (DO) levels in the water. Dye effluents have direct and indirect effects on aquatic ecosystems. Dissolved oxygen loss, reduced reoxygenation potential, dye leaching from soil into groundwater, reduced light penetration into water inhibiting photosynthesis (red signal to aquatic flora and fauna) are some of the direct impacts [5,6]. Indirect impacts of dye effluents include aquatic organism death, genotoxicity and micro-toxicity induced by coloured allergens, immune system depression, allergic reactions, and hyperactivity in children (ADHD), bladder cancer in humans, and the lethal process of eutrophication [7].

Coming to the application of dyes, both natural and synthetic dyes have altered the modern world, transforming both the household and industrial sectors. These days, dyes are essential because they are used in a wide variety of applications including electronic goods, paper, and pulp, photography, textiles, rubber, food, leather, cosmetics, specifically dye-sensitized solar panels, pharmaceuticals, pigment, agriculture research, paints, printing inks, cosmetics, biomaterials and colouring of plastics products [7,8,9]. But there is still a quest for potential approaches, notably in the health care field. The quick creation of novel dye-based pharmaceutical formulations would benefit from the ability to search vast databases comprising a diverse range of molecular configurations. Furthermore, dyes can be used to determine the effectiveness of sewage and wastewater treatment plants, as well as agricultural research. Textile industries consume more than 70% of all dyes produced on a global scale [10,11].

The removal of dye matter from the aquatic ecosystems is more important for water purification and the recycling of water. This process might be helpful for the survival of flora and fauna present in those water sources. Dye pollution has drawn the attention of environmental chemists worldwide, particularly in industrialized countries like the USA, UK, Germany, China, Japan, Scandinavian countries, etc. Global environmental standards have become stricter, requiring more accurate technological solutions, attracting the attention of environmental experts worldwide for the last two decades. Physicochemical procedures, enzymatic degradation, microbiological treatment, chemical approaches, and sophisticated oxidation methods have all been developed [12–14]. However, because of their large/complex size and inert nature, most organic dyes are non-biodegradable, thermally stable, and resistant to biochemical oxidation. These properties make organic dyes resistant to decolorization by standard wastewater treatment processes such as filtration, coagulation, biochemical and physicochemical methods. Thus, for the last two decades, environmental scientists all over the world have been researching more efficient and advanced dye removal procedures from wastewater to reduce or eradicate water contamination [15]. The dye abatement techniques in water are divided into two categories such as segregation (adsorption, nanofiltration, or coagulation) and degradation (chemical

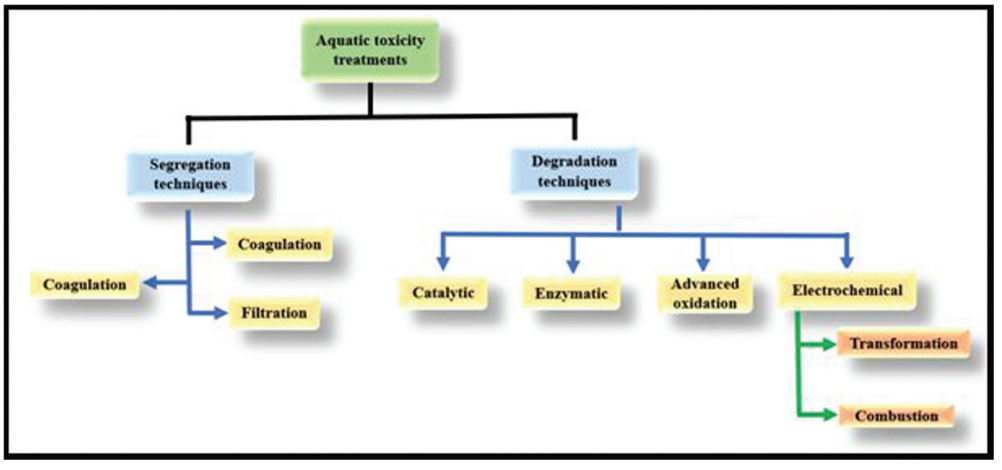


Figure 1. Methods for removing dyes from wastewater.

degradation, AOPs or advanced oxidation process, microbiological and enzymatic degradation techniques, catalytic degradation, electrochemical techniques) techniques (Figure 1).

Industries that use dyes or colours are a significant source of water contamination [16]. Humans have developed a strong aversion to prevention of water pollution. The public should be made more aware of the dangers associated with hazardous materials found in aquatic environments. Rapid industrialization may contaminate rivers, seas, and other sources of fresh water, resulting in aquatic toxicity.

Numerous publications discuss quantitative structure-activity relationship (QSAR) models for the acute toxicity of chemicals in general to *Daphnia magna*, since it is frequently used as a test organism in aquatic toxicology. But very few numbers of studies have been reported based on dyes. In 2019, Umbuzeiro et al. verified that the recently developed ant colony optimization-support vector machine (ACO-SVM) QSAR models would be a good tool to correctly predict the acute ecotoxicity available from existing experimental data as well as for a freshly tested subset of dyes from the Max A Weaver Dye Library (MWDL) [17]. Some are intended to represent specific chemical classes, while others are intended to represent chemical substances in general. Several evaluations have assessed the effectiveness of several QSAR models for *D. magna* acute toxicity [18,19]. Moosus and Maran [20] discussed a number of these models in detail, while Toropova et al. [21] explored several others more recently. Although dyes are employed more frequently in numerous fields such as food, pharmaceuticals, textiles, diagnostic agents, and so on, the existing QSAR models are insufficient to cope with the manifestations associated with organic dyes because these models are primarily focused on organic compounds in general. For example, the Toxicity Estimation Software Tool (TEST) (https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test) allows users to easily estimate the toxicity of chemicals using QSARs. However, this software was not specifically designed to predict toxicity of dyes and it also not known to us whether the QSAR models used by TEST employed any dye compound in their training set. Thus, it is always advisable to develop local QSAR models for better accuracy of predictions. Therefore, in our modelling work, we exclusively employed organic dyes.

The development of models that explicitly explain the physicochemical features of organic dyes is critical. It is important to understand the physical, chemical, and application qualities of different categories of dyes as well as their environmental impacts. Assays are necessary to determine the characteristics of chemicals or dyes and pigments. Biological or toxicological assays are used to determine the dye's concentration and effects. The assay always yields qualitative data and is used to quantify unproven chemical or dye effects. It is also useful for assessing the risk of existing chemicals or dyes utilizing QSAR, which correlates a chemical or dye's structure with activity/toxicity using statistical methods. These QSAR models predict in vivo and in vitro activities of compounds that have not been investigated experimentally.

Considering limited experimental data for toxicity of dyes and pigments being available, developing prediction models appears to be of timely interest. We have built here 2D-QSAR models using *D. magna* acute aquatic toxicity (log ($1/EC_{50}$)) data of dyes. We have employed a 'multi-layered variable selection' technique to select descriptors for the regression-based QSAR models and the modelling process follows strict/stringent OECD guidelines [22]. We have also developed a classification – based model using linear discriminant analysis (LDA). The produced models can shine a spotlight on the molecular requisites for eco-friendly dye design.

Methodology

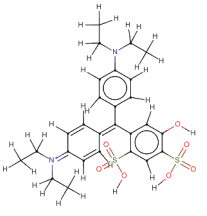
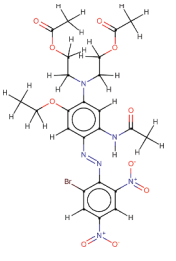
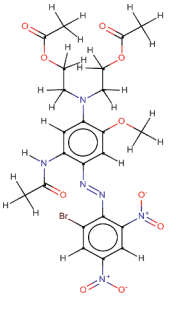
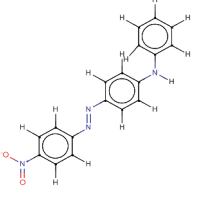
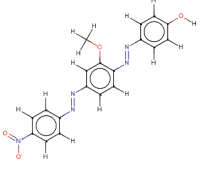
Dataset preparation

The acute toxicity data of 22 commercial dyes and pigments (Table 1) were carefully collected from the literature [23–29] as per OECD guideline 201 (1987) [30]. The dataset comprises three water-insoluble organic pigments, thirteen sparsely water-soluble disperse dyes, and six water-soluble dyes (2 FD&C and 4 acid dyes). The majority of organic dyes available at the Max Weaver Dye Laboratory (MWDL) are disperse dyes. We also included dyes that are commonly used in detergents and for which ECHA and REACH registration dossiers provide experimental toxicity data [31]. 2D-QSAR models were developed using acute aquatic toxicity concentrations of *Daphnia magna*, which have been produced from identical experimental circumstances, techniques, and procedures. The acute toxicity tests were carried out in accordance with the guidelines in the Organization for Economic Cooperation and Development's (OECD) Test No. 202: *Daphnia* species acute immobilization test with the selected organic dyes. The structures of dyes and pigments were drawn using MarvinSketch (version 14.10.27) software (https://www.chemaxon.com) [32], with suitable aromatization and explicit hydrogen addition. Moreover, MDL. mol files of the structures were saved, which are essential for descriptor calculation. The present modelling approach used the negative logarithm of molar concentration to express the experimental EC_{50} values of *Daphnia magna* [31].

Molecular descriptors

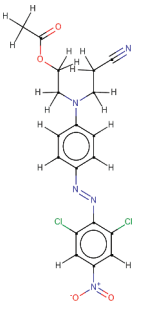
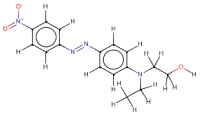
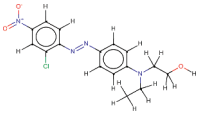
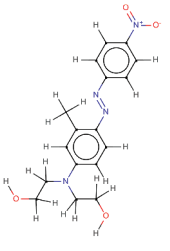
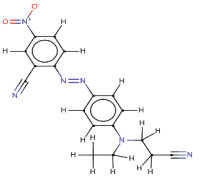
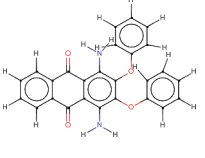
The molecular descriptors are the fundamental characteristics, which reveal the relationship between structures and their physical, chemical, and biological qualities. We developed our models in this work by utilizing only 2D descriptors that are quite efficient for

Table 1. Dataset of dye compounds used for modelling study.

S. No	*Comp. No*	*Name of the dye*	*Structure*	*Experimental EC_{50}*	*log ($1/EC_{50}$)*
1	4	C.I. Acid Blue 3 [23]		42,900	1.117094
2	12	C.I Disperse Blue 79 [25]		4.5	5.152608
3	13	C.I Disperse blue 79:1 [25]		4.5	5.142973
4	14	C.I Disperse Orange 1 [26]		10	4.502918
5	15	C.I Disperse Orange 29 [25]		70	3.731692

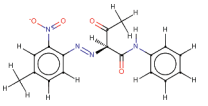
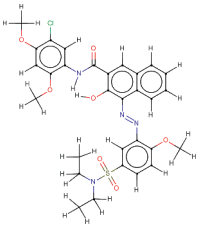
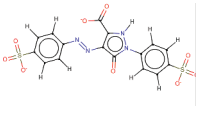
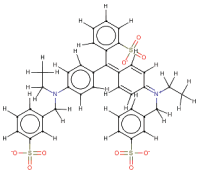
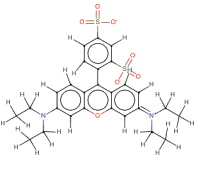
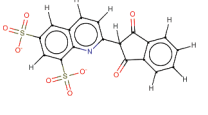
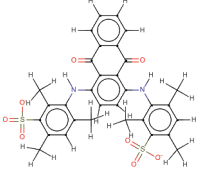
(*Continued*)

Table 1. (Continued).

S. No	*Comp. No*	*Name of the dye*	*Structure*	*Experimental EC_{50}*	*log ($1/EC_{50}$)*
6	16	C.I Disperse Orange 30 [27]		0.03	7.17639
7	17	C.I Disperse Red 1 [28]		0.18	6.242182
8	18	C.I Disperse Red 13 [29]		0.0187	7.27076
9	19	C.I Disperse Red 17 [25]		98	3.54585
10	20	C.I Disperse Red 73 [25]		110	3.500685
11	21	C.I Disperse Violet 31 [27]		177.9	3.37561

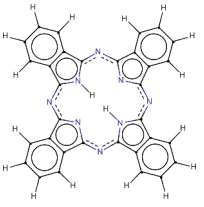
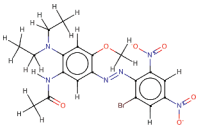
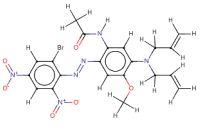
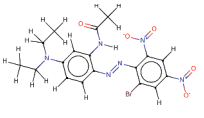
(*Continued*)

Table 1. (Continued).

S. No	*Comp. No*	*Name of the dye*	*Structure*	*Experimental EC_{50}*	*log ($1/EC_{50}$)*
12	1*	C.I Pigment Yellow 1 [23]		>100	>0.531951
13	2*	C.I Pigment Red 5 [23]		>100	>0.797385
14	3*	FD&C Yellow 5 [23]		>125	>0.570944
15	5*	FD&C Blue no. 1 [23]		>100	>0.873286
16	6*	C.I Acid Red 52 [23]		>120	>0.667235
17	7*	C.I Acid Yellow 3 [23]		>100	>0.6349
18	8*	C.I Acid Blue 80 [23]		>67	>0.975164

(*Continued*)

Table 1. (Continued).

S. No	Comp. No	Name of the dye	Structure	Experimental EC_{50}	log $(1/EC_{50})$
19	9*	C.I Pigment Blue 16 [23]		>500	>0.012483
20	10*	C.I Disperse Blue 291 [24]		>0.02	>4.405995
21	11*	C.I Disperse blue 373 [24]		>0.005	>5.028067
22	22*	C.I Disperse violet 93 [24]		>0.02	>4.379605

*test set compounds in the regression models

predictions, avoiding the computational complexity of conformational analysis, energy minimization, and alignment problems. PaDEL-Descriptor and Dragon software tools were used to calculate the 2D descriptors. The constitutional, ring descriptors, 2D-matrix based, connectivity indices, functional group counts, atom-centred, atom-type fragments, 2D-atom pairs, and molecular properties (using Dragon software version 7) and extended topochemical atom (ETA) descriptors were calculated (using PaDEL-Descriptor software). Before developing the model, we deleted intercorrelated ($|r| > 0.95$), constant (variance 0.0001), and other defective and extraneous data using the data pre-treatment tool V-WSP v1.2 (available from http://dtclab.webs.com/software-tools) and the salts were removed by using Data treatment KNIME workflow available from https://sites.google.com/site/dtclabdc/ [33]. Finally, we used a pool of 461 two-dimensional descriptors.

Data set partitioning and model development

The current work divided 22 commercial dye datasets into training and test sets. Among the collected dye toxicity data, the responses with definite values were chosen for model development as a training set, while responses reported in the range were used to

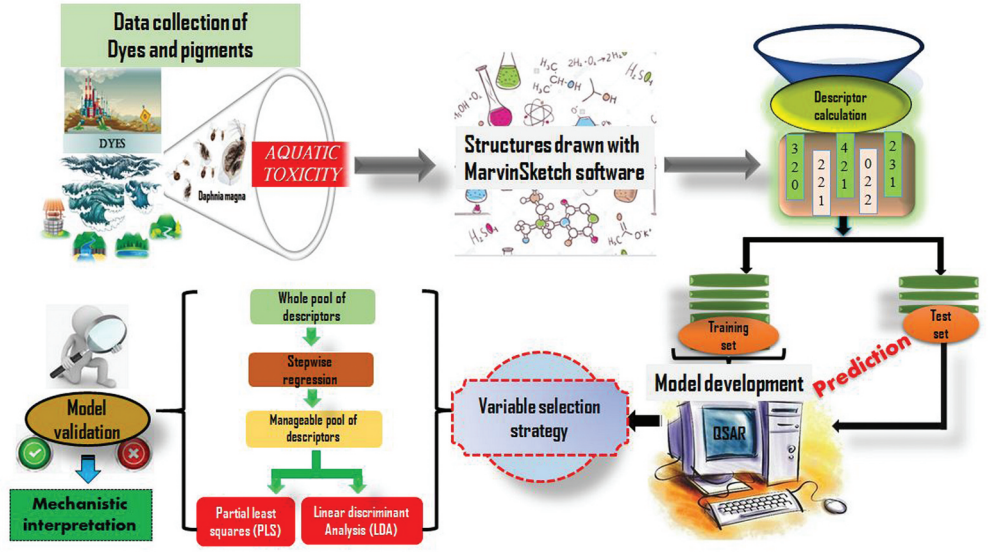


Figure 2. The stages involved in the development of PLS models against *D. magna*.

validate the regression-based models as a test set. The training set was utilized primarily for feature selection and model development, while the test set was used to evaluate the developed models' predictability. For feature selection, stepwise regression was employed with a predefined threshold of $F = 4.0$ for inclusion and $F = 3.9$ for exclusion [34–37]. The technique was applied several times, with selected descriptors from previous runs being set aside each time. After that, the selected descriptors in the top models for the specified endpoint were subjected to partial least squares (PLS) regression analysis [38,39] to reduce model noise (PLS regression produces latent variables (LVs) from the original variables and also controls for noisy descriptors). Within the scope of PLS model construction, the software performs input data scaling (standardization) and then measures latent variable scores (the real regressing variables), although the final regression coefficients are expressed in terms of the initial un-scaled variables (similar to multiple linear regression or MLR equations) [40]. In this case, we have developed four models of organic dyes acute toxicity against *D. magna* (Model I, Model II, Model III, and a pooled descriptor model from Models I, II, III). Unlike MLR models, where standard errors of regression coefficients are determined, PLS models depict the relative importance of distinct descriptors in terms of VIPs (variable importance plots). The stages involved in developing the PLS models are schematically depicted in Figure 2.

A linear discriminant analysis model was also developed to achieve the goal of developing classifier models with appropriate chemical characteristics capable of classifying compounds according to their acute aquatic toxicity potential (log ($1/EC_{50}$)). The discriminant model may be used as a preliminary filter to classify (or categorize) the chemical into 'highly toxic' or 'less toxic' groups. The discrimination model was created using the LDA methodology with a stepwise approach, incorporating *F*-criterion for variable inclusion (F to enter = 4.0) and exclusion (F to remove = 3.9), using the STATISTICA program [41].

Setting a threshold value for classification analysis

In the classification analysis study, we have used the models to categorize 22 organic dyes into 'very harmful' and 'less toxic' classes. The categorization threshold was established using the median value (4.502 M in log unit) of the reported response values. The entire dataset was partitioned around 50:50 between high and low toxicity compounds, resulting in 11 substances in each class. The training set of the final models contains 6 (54.55%), 5 (45.45%), 7 (63.64%) highly hazardous chemicals for Model I, Model II, and Model III respectively, whereas the remaining 5 (45.45%), 7 (63.64%) and 4 (36.36%) substances are less dangerous to *Daphnia magna*.

Statistical validation

Several quantitative validation measures were employed to define the quality of developed PLS models, which were assessed in terms of stability, robustness, fitness, and predictivity. We estimated the coefficient of determination (r^2), internal predictivity metrics such as leave-one-out cross-validated r^2 or Q^2_{LOO} for assessing the quality and internal stability of the regression models, and for the external validation, we used metrics such as sensitivity, specificity, accuracy, precision, and recall, F1-score, and MCC (Matthews' correlation coefficient) due to non-availability of quantitative observed values for test compounds. Similarly, in the case of the LDA model, different validation measures were employed such as Wilks' statistic, Canonical index (Rc), squared Mahalanobis distance, chi-squared, probability level, F-measure, G-means, Cohen's kappa, Guner Henry score, and Matthews' correlation coefficient (MCC). The model's discriminating capacity was further evaluated by plotting a receiver operating characteristic (ROC) curve for both the training and test sets [42].

Software used

The chemical structures were drawn using the Marvin Sketch version 5.5.0.1 software (http://www.chemaxon.com/) [32]. Dragon (http://www.talete.mi.it/products/dragon description.htm) [43] and PaDEL-Descriptor software tools (http://www.yapcwsoft.com/dd/padeldescriptor) [44] were used to calculate the descriptors. The models were developed by using stepwise regression and PLS analysis using MINITAB software version 13.14 (http://www.minitab.com/en-US/default.aspx) [45, 46] and SIMCA-P software (www.umetrics.com) [47], respectively. The LDA model was built using the STATISTICA program (version 13.4) [41], while SPSS 9.0 was used to plot the ROC curves.

Results and discussion

Development of regression-based 2D- QSAR models to assess *D. magna* acute toxicity

The developed models were robust, internally stable, and predictive as found with different classification-based metrics using test compounds (with only graded observed toxicity values) that were not employed during model building. In this work, we have sought to provide a mechanistic interpretation of the modelled descriptors and to identify structural elements associated with dyes' aquatic toxicity.

The resulting models and their associated values for various validation measures are listed below. Additional analyses were conducted using the SIMCA-P software (Simca-P, 2002) for Variable Importance Plot (VIP) and Y-Randomization; the VIP plot indicated the relative quantitative importance and contribution of individual variables to the corresponding response, whereas the Y-randomization test established the robustness of the developed models. The detailed results are discussed below.

Model I

Model I (Eq. 1) shows the first regression equation developed using the PLS method implemented in MINITAB software. The model consists of three 2D predictor variables namely, X%, F05[C-C], and F09[C-C].

$$\log(1/\mathrm{EC50}) = 6.63489 + 0.54993 \times \mathrm{X\%} - 0.08218 \times \mathrm{F05[C-C]} - 0.04983 \times \mathrm{F09[C-C]} \quad (1)$$

$$\mathrm{LV} = 2, n_{\mathrm{training}} = 11,\ r^2 = 0.71, Q^2 = 0.558,\ s\ = 67.2, F = 22.27, {}^{c}r^2{}_{\mathrm{p}} = 0.756$$

$$\mathrm{PRESS} = 14.9268$$

The developed regression model predictions were used to assess the external set of 11 compounds (with graded experimental values only) using the classification-based validation metrics such as sensitivity, specificity, precision, accuracy, F1_score, and MCC by keeping the threshold limit 4.502918 (Threshold: Median of the experimental log ($1/EC_{50}$)). The details of validation metrics are depicted in Table 2.

The number of true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN) present in the training and test sets were given below in Table 3.

Interpretation of the descriptors. The most significant descriptor is F09[C-C], which is a member of the 2D atom pair block and is defined as the frequency of two carbon atoms at the topological distance 9. It contributes negatively to the response in the reported model, implying that increasing the amount of C-C at the topological distance 9 reduces the log ($1/EC_{50}$) as well the toxicity of the modelled dyes and vice versa. For example, compounds such as Disperse violet 31 (F09[C-C] = 30; log ($1/EC_{50}$) = 3.37) and Acid Blue 3 (F09[C-C] = 32; log ($1/EC_{50}$) = 1.117) have high values of the descriptor, indicating low toxicity, whereas

Table 2. Statistical validation parameters for the training and test sets for Model I developed against *D. magna*.

Sets	*Data*	*Sensitivity*	*Specificity*	*Accuracy*	*Precision*	*F1-Score*	*MCC*
Training	11	0.83	0.40	0.64	0.63	0.72	0.26
Test	11	1.00	0.60	0.64	0.20	0.33	0.35

Table 3. Confusion matrix for Model I.

Class	*Training set*	*Test set*
True positives (TP)	5	1
False positives (FP)	3	4
True negatives (TN)	2	6
False negatives (FN)	1	0

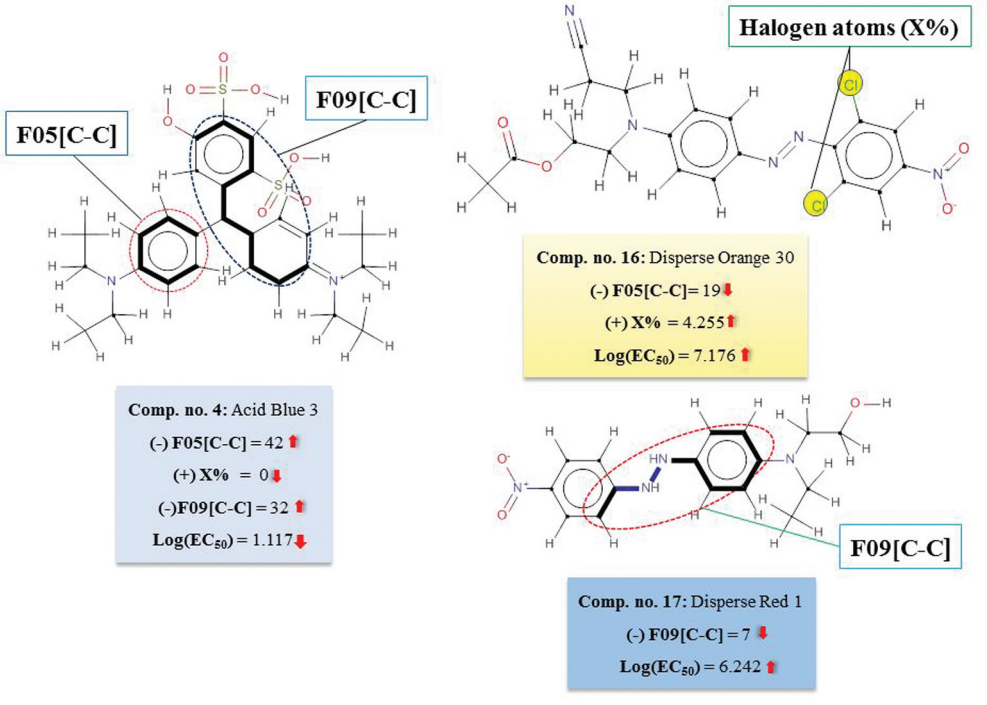


Figure 3. Significance of various descriptors involved in the aquatic toxicity of *D. magna* (Model I).

compounds lacking/with less C-C atoms at topological distance 9 in their chemical structures, such as Disperse red 13 (F09[C-C] = 7; log ($1/EC_{50}$) = 7.270), and Disperse red 1 (F09 [C-C] = 7; log ($1/EC_{50}$) = 6.242) have high log ($1/EC_{50}$) values and thereby increase the aquatic toxicity (Figure 3). These non-conjugated carbons with sp^2 hybridization in the dye structures do not involve in the electrophilic/proto-electrophilic specific toxicity mechanism, Since the reactive species generation is not possible with the 'nCconj' groups, they are thus useful to be part in the design of the environmentally safer chemical/dyes.

The second most significant variable is another 2D atom pair descriptor, i.e. F05[C-C], and it refers to the frequency of two carbon atoms at the topological distance 5 in the chemical structures of dyes. The fact that this descriptor has a negative contribution (as seen by the regression coefficient plot) indicates that it is inversely connected to the log ($1/EC_{50}$). The same phenomenon was observed with the dyes Acid Blue 3 (F05[C-C] = 42; log ($1/EC_{50}$) = 1.117) and Disperse Violet 31 (F05[C-C] = 33; log ($1/EC_{50}$) = 3.37) (lower log ($1/EC_{50}$) due to their corresponding numerical descriptor values being in the higher range), whereas the opposite is found in dyes Disperse Orange 30 (F05[C-C] = 19; log ($1/EC_{50}$) = 7.176), and Disperse Red 13 (F05[C-C] = 14; log ($1/EC_{50}$) = 7.270) (increase in the log ($1/EC_{50}$) as their corresponding numerical descriptor values are in the lower range) (Figure 3).

Finally, the least contributing descriptor, i.e. X% belongs to the block of constitutional indices, and it refers to the percentage of halogen atoms in the chemical structures of dyes. This descriptor has a positive coefficient; a rise in the percentage of this group increases the log ($1/EC_{50}$) values, which means that this descriptor increases the toxicity of *D. magna*. The halogen (Fluorine (F), Chlorine (Cl), Bromine (Br), Iodine (I), and astatine (At)) atoms in the dye system are reactive electrophilic toxicants and make covalent interaction with the biological

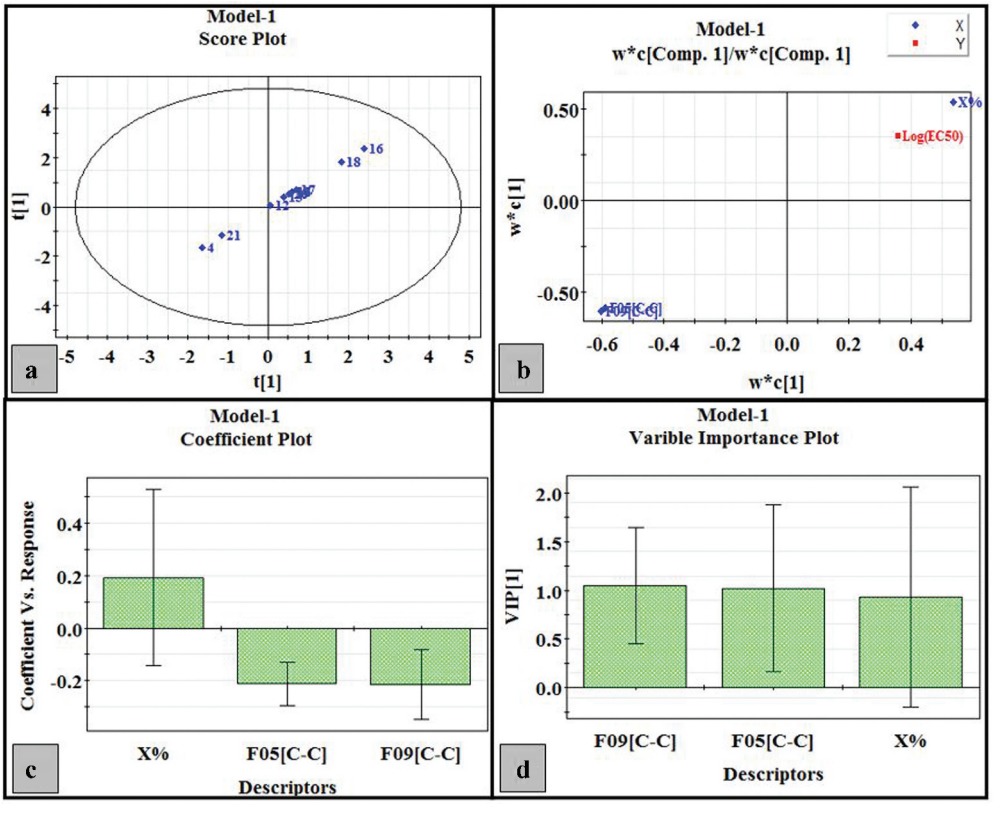


Figure 4. Various SIMCA plots developed for Model I.

system (i.e. *D. magna*) [32]. As a result, dyes containing halogen fragments can exhibit greater toxicity values in *Daphnia magna*, as shown by the dyes Disperse Orange 30 having 2 aqueous toxicophores namely, nitrile and 4-nitrobenzene (X% = 4.255; log ($1/EC_{50}$) = 7.176) and Disperse Blue 79:1 (X% = 1.539; log ($1/EC_{50}$) = 7.270). And from the other end, dyes lacking these fragments may exhibit low log ($1/EC_{50}$) values, thereby reducing the toxicity, which can be seen from the dyes Disperse Orange 29 (X% = 0; log ($1/EC_{50}$) = 3.731), Disperse Red 73 (X % = 0; log ($1/EC_{50}$) = 3.50) (Figure 3).

Interpretation of various plots obtained in the modelling study. The score plot (Figure 4(a)) illustrates the distribution of compounds in the latent variable space specified by the scores. The first two components' scores, t1 and t2, have been plotted. The ellipse denotes the model's applicability domain, as described by Hotelling's t^2. The t^2 statistic is a multivariate generalization of the student's *t*-test defined by Hotelling. The approach can be used to determine whether a compound adheres to multivariate normality [48–50]. Compounds that are close in proximity to one another on the plot have similar properties, whereas compounds that are far apart have dissimilar properties in terms of their log ($1/EC_{50}$). Consider compounds 14, 17, 19, and 20, which are clustered together on the plot space and might be deemed to have comparable features. On the other hand,

compounds 18, 16, 4, and 21 are entirely separated from one another and exhibit variability in their properties. Due to the absence of compounds outside the ellipse, we may conclude that this approach does not produce outliers.

The loading plot demonstrates the link between the X–variables (i.e. descriptors) and the Y-variable (i.e. response) [34]. Three X–variables and one Y-variable are depicted in Figure 4(b). The plot is typically constructed using the first and second components. A loading graph reveals how much each variable contributes to the model and which variable has the highest contribution. The distance from the origin is taken into account during interpretation. Correlations and groupings are used to bring together descriptors that are similar and contribute similarly. Descriptors located further away from the plot's origin should have a bigger effect on the Y-response. The loading plot demonstrates that the descriptors F09[C-C] and F05[C-C] are located further from the plot origin, implying a greater influence, which is also described by the variable importance plot (VIP). A PLS plot also takes into account the positive or negative algebraic symbol. Due to their proximity to log ($1/EC_{50}$) in the plot, the feature explained by the descriptor X% is conducive for log ($1/EC_{50}$). On the other hand, descriptors which are present in the opposite side of the plot origin are considered to contribute negatively to the response variable.

The regression coefficient plot (Figure 4(c)) [34] reveals the positive or negative contribution of the descriptor to the activity (log ($1/EC_{50}$)) of the compounds. A positive regression coefficient suggests that as the descriptor values increases, the log ($1/EC_{50}$) value increases as well. A negative regression coefficient, on the other hand, shows that as the descriptor value increases, the log ($1/EC_{50}$) value decreases. The VIP plot is shown in Figure 4(d).

Y-Randomization. Model randomization provides an insight into the model's relevance and assures that it is not the result of accidental correlation [49,50]. A randomized model is developed by shifting or rearranging different combinations of X- or Y-variables (in this case, just the Y-variable) and evaluating the fit of the reordered model. We used 100 permutations in this study, which can be changed according to the user's preference. A randomized model's statistics should be extremely poor. The r^2 and Q^2 coefficients for the random models are shown against the correlation coefficient between the original and permuted Y values (X-axis); the r^2y intercept should not exceed 0.3, and the Q^2y intercept should not exceed 0.05. Figure 5 plots the relationship between the original Y-vector and the permuted Y-vector against the cumulative r^2y and cumulative Q^2y intercepts of 0.0665 and −0.0914, respectively, demonstrating that the model is resilient and non-random.

Model II

Model II (Eq. 2) represents the model developed by PLS. The model consists of three 2D descriptors out of which two belong to the class of 2D-atom pairs (B08[N-O] and B06 [O-O]) and the remaining one is a 2D-matrix based descriptor (Chi_Dt). A detailed interpretation of the three descriptors is given below.

$$\log(\mathrm{EC50}) = 8.1891 + 3.4841 \times \mathrm{B08[N-O]} - 2.1709 \times \mathrm{B06[O-O]} - 34.7307 \times \mathrm{Chi\_Dt}$$
$$\mathrm{LV} = 2,\ n_{\mathrm{training}} = 11,\ r^2 = 0.918,\ Q^2 = 0.774, s = 42.9, F = 45.32, {}^{c}r^2{}_{\mathrm{p}} = 0.829$$
$$\mathrm{PRESS} = 2.586 \tag{2}$$

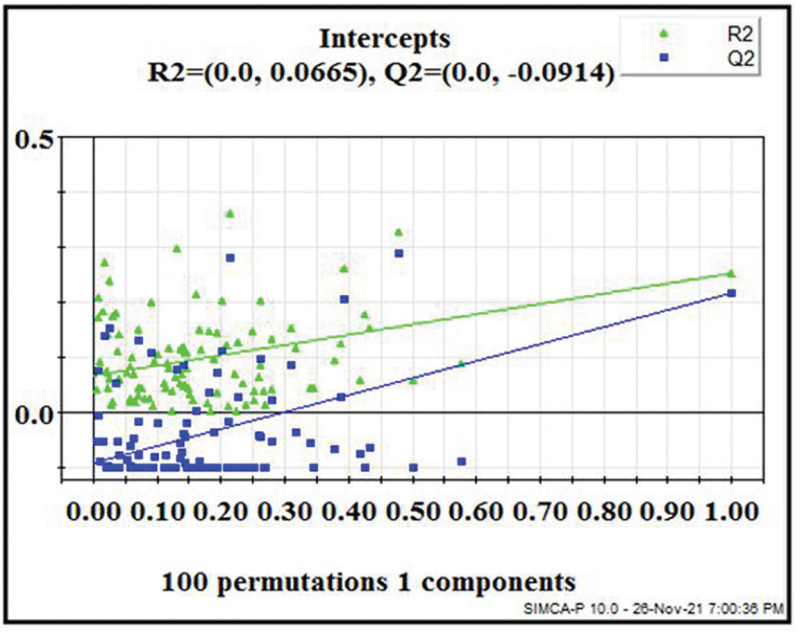


Figure 5. Y-Randomization plot for Model I.

Here also, we have used the median value of the experimental EC_{50} of training compounds as the threshold for computation of various validation metrics for the training and test sets, and the results are shown in Table 4.

The number of true positives, false positives, true negatives and false negatives found in training and test sets of Model II is mentioned below in Table 5.

Model II: descriptor interpretation. The most important descriptor among the modelled ones is B08 [N-O], which belongs to the 2D atom pairs descriptor representing the presence or absence of the nitrogen and oxygen atoms at the topological distance 8. This descriptor contributing positively to the log ($1/EC_{50}$) values indicates that the presence of this feature in the dye molecular system might increase the toxicity of *D. magna*. The same can be reflected in the following examples: Disperse Orange 30 has 2 aqueous toxicophores namely, nitrile and 4-nitrobenzene (B08 [N-O] = 1; log

Table 4. Different statistical validation parameters of the Model II developed against *D. magna*.

Sets	*Data*	*Sensitivity*	*Specificity*	*Accuracy*	*Precision*	*F1-Score*	*MCC*
Training	11	0.83	1.00	0.91	1.00	0.91	0.83
Test	11	1.00	0.40	0.45	0.14	0.25	0.23

Table 5. Confusion matrix for Model II.

Class	*Training set*	*Test set*
True positives (TP)	5	1
False positives (FP)	0	6
True negatives (TN)	5	4
False negatives (FN)	1	0

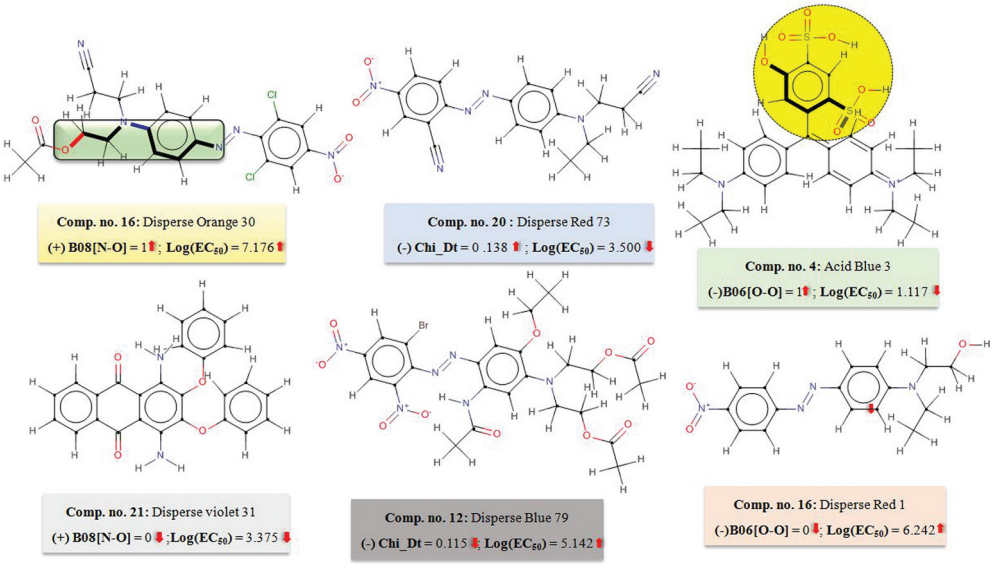


Figure 6. Significance of various descriptors involved in the aquatic toxicity of *D. magna* (Model II).

($1/EC_{50}$) = 7.176) and Disperse blue 79 (B08[N-O] = 1; log ($1/EC_{50}$) = 5.152) and vice versa in case of dyes like Disperse Red 17 (B08[N-O] = 0; log ($1/EC_{50}$) = 3.545) and Disperse Violet 31 (B08[N-O] = 0; log ($1/EC_{50}$) = 3.500) (Figure 6). As per the reports [51], the electronegative groups help in electrophilic or protoelectrophilic substitution which may lead to reactive species generation, resulting in photosensitization and highly toxic effects to *Daphnia magna* [52].

Another important descriptor is Chi_Dt, which represents the Randic-like index from the Detour matrix. The negative regression coefficient of this descriptor indicates that the presence of such features in the structure of a dye molecule contributes negatively towards log ($1/EC_{50}$) as observed in the dyes Acid Blue 3 (Chi_Dt = 0.131; log ($1/EC_{50}$) = 1.117) and Disperse red 73(Chi_Dt = 0.138; log ($1/EC_{50}$) = 3.500) the log ($1/EC_{50}$). On the other hand, decrease of occurrence of such features in the dyes like Disperse Blue 79 (Chi_Dt = 0.115; log ($1/EC_{50}$) = 5.142), Disperse Blue79:1 (Chi_Dt = 0.116; log (EC_{50}) = 5.152) the log ($1/EC_{50}$) increases the aquatic toxicity (Figure 6).

The 2D-atom pair descriptor B06 [O-O] refers to the presence or absence of two oxygen atoms at the topological distance 6. This descriptor contributes the least to the model as per the variable importance plot, and it has a negative impact on log ($1/EC_{50}$). As a result, we can assume that an increase in the oxygen atoms at the topological distance 6 decreases the log ($1/EC_{50}$) significantly. The same is true for the dyes Disperse Red 17 (B06 [O-O] = 1; log ($1/EC_{50}$) = 3.545) and Acid Blue 3 (B06 [O-O] = 1; log ($1/EC_{50}$) = 1.117), and vice versa in dyes like Disperse Orange 30 (which contains aqueous toxicophores namely, nitrile and 4-nitrobenzene) (B06 [O-O] = 0; log ($1/EC_{50}$) = 7.176) and Disperse Red 1 (B06 [O-O] = 0; log ($1/EC_{50}$) = 6.242) (Figure 6).

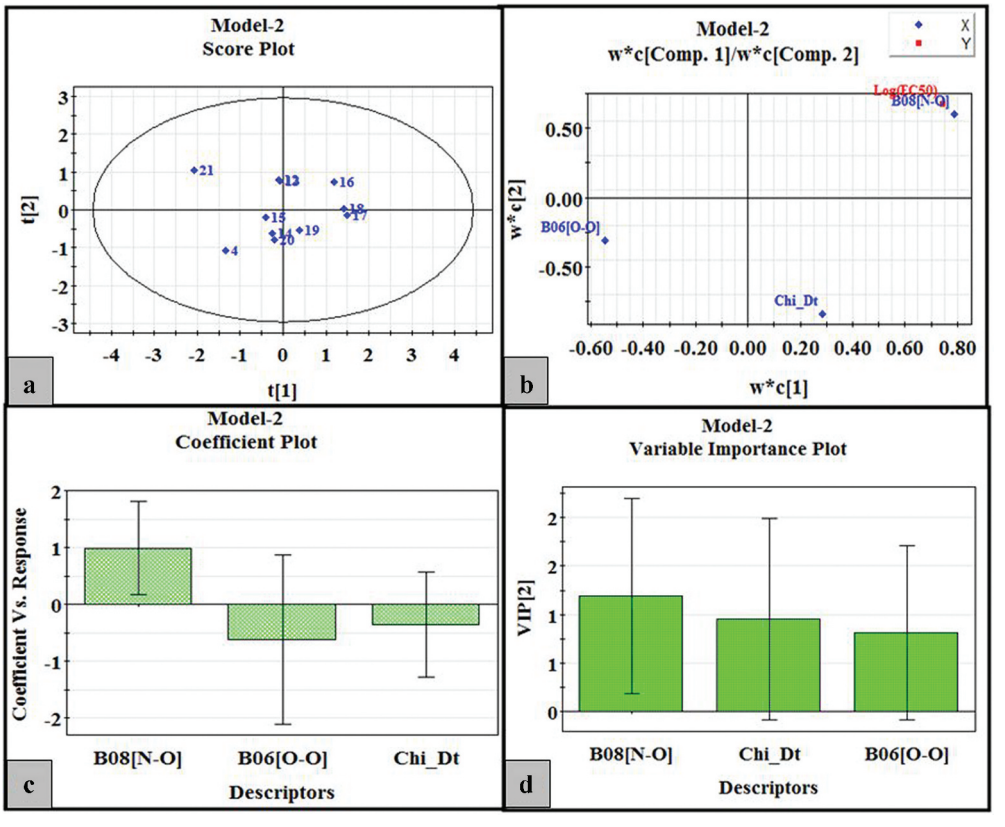


Figure 7. Various SIMCA plots developed for Model II.

Plot interpretation. According to the score plot (Figure 7(a)), dyes 14, 20, and 17, 18 are crowded together around the plot space and might even be considered to share similar properties. Compounds 4, 13, 14, and 16 are completely distinct from one another and display a wide range of features.

The loading plot (Figure 7(b)) for Model II demonstrates that the descriptor B08[N-O] is conducive to the log ($1/EC_{50}$). On the other hand, B06 [O-O], and Chi_Dt values located on close proximity to the plot origin were deemed to be less influential for the dependent variable (log ($1/EC_{50}$).

According to with regression coefficient plot (Figure 7(c)) for Model II, the modelled descriptors have a positive or negative effect on the response variable, i.e. log ($1/EC_{50}$) of the dyes. Figure 7(d) shows the VIP plot for Model II.

As per the Y-Randomization plot (Figure 8), the relationship between the original and permuted Y-vectors is plotted with the cumulative r^2y and Q^2y intercepts being 0.0814 and −0.382, respectively, proving that the model is non-random.

Model III

Model III (Eq. 3) represents the third PLS model, and it consists of two 2D descriptors, of which one belongs to the block of constitutional (nCconj) and the other one is a 2D matrix-based descriptor (B01[C-Cl]). A detailed interpretation of the three descriptors is given below.

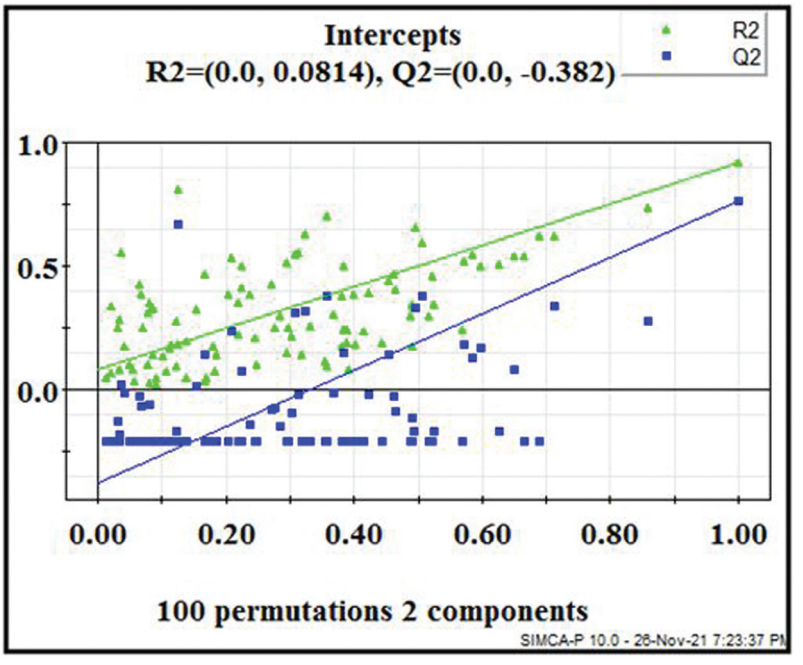


Figure 8. Y-Randomization plot for the Model II.

$$\log(1/\mathrm{EC50}) = 4.53331 + 2.68134 \times \mathrm{B01[C-Cl]} - 2.0337 \times \mathrm{nCconj} \tag{3}$$

$$\mathrm{LV} = 1, n_{\mathrm{training}} = 11, r^2 = 0.810,\ Q^2 = 0.640, s = 89.40, F = 38.43, {}^{c}r^2_{\mathrm{p}} = 0.714$$

$$\mathrm{PRESS} = 8.977$$

A similar threshold was set for the validation (same as Model I and Model II), i.e. after converting into molar scale, the median value of the experimental log ($1/EC_{50}$) was taken as the threshold. Later, we have calculated various validation metrics for the training and test sets. The results can be found in Table 6.

The number of true positives, false positives, true negatives and false negatives found in training and test sets for Model III are mentioned below in Table 7.

Table 6. Different statistical validation parameters of the Model III developed against *D. magna*.

Sets	*Data*	*Sensitivity*	*Specificity*	*Accuracy*	*Precision*	*F1-Score*	*MCC*
Training	11	1.00	0.40	0.73	0.67	0.80	0.52
Test	11	1.00	0.50	0.54	0.50	0.29	0.29

Table 7. Confusion matrix for Model III.

Class	*Training set*	*Test set*
True positives (TP)	6	1
False positives (FP)	3	5
True negatives (TN)	2	5
False negatives (FN)	0	0

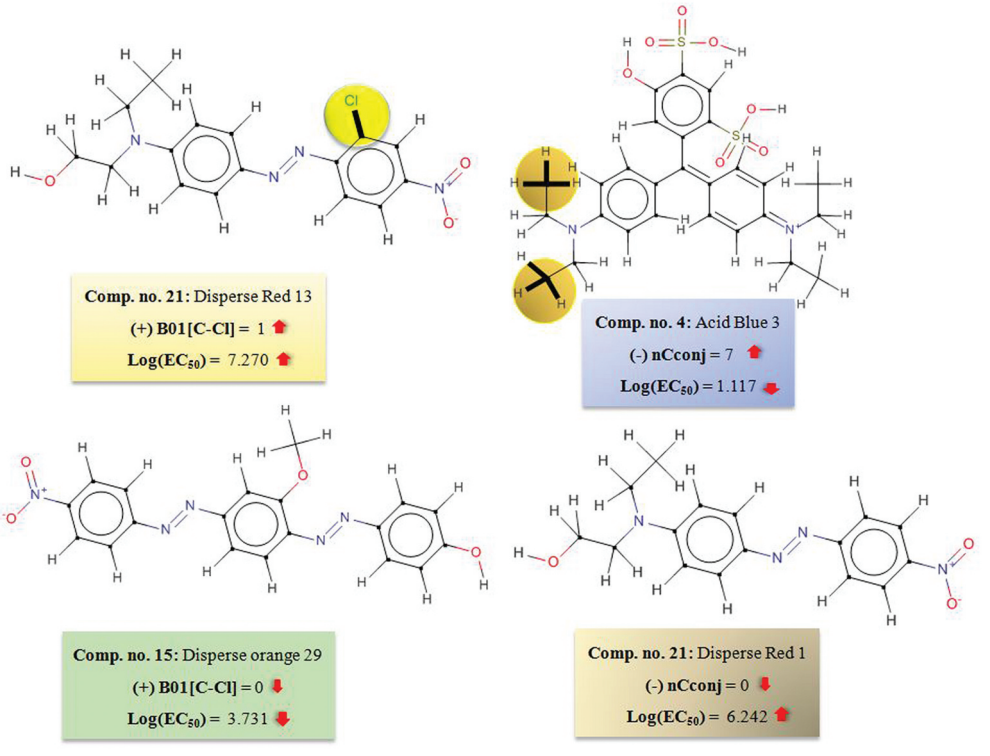


Figure 9. Significance of various descriptors involved in the aquatic toxicity of *D. magna* (Model III).

Model III: descriptor interpretation. According to the VIP, the most significant variable, B01[C-Cl] is a 2D atom pair descriptor, which represents the presence/absence of carbon and chlorine atoms at the topological distance 1. This descriptor shows a positive contribution to the log ($1/EC_{50}$) values as indicated by its positive regression coefficient, which means that an increase in the B01[C-Cl] descriptor values enhance the log ($1/EC_{50}$) values. It was found in case of dyes Disperse Orange 30 (B01[C-Cl] = 1; log ($1/EC_{50}$) = 7.176), Disperse Red 13 (B01[C-Cl] = 1; log ($1/EC_{50}$) = 7.270) that there is a significant decrease in the log (EC_{50}) values and vice versa in case of dyes Disperse Orange 29 (B01[C-Cl] = 0; log ($1/EC_{50}$) = 3.731), Disperse Violet 31 (B01[C-Cl] = 0; log ($1/EC_{50}$) = 3.375) (Figure 9). The existence of chlorine (halogen) atoms promotes lipophilicity of the molecules, and hence diffusion across the cell membrane grows stronger. In this manner, chlorine-containing compounds may result in greater toxicity [53]. In case of C.I Pigment red 5 (number 2), the descriptor B01[C-Cl] has a positive contribution to the aquatic toxicity of *D. magna* while other features like F05[C-C], F05[C-C], B06[O-O] and nCconj groups contribute negatively to the acute aquatic toxicity. Therefore, overall, this dye compound shows a low toxicity.

Another descriptor belongs to the block of functional group counts i.e. nCconj, which is a member of the functional group counts and is defined as the number of non-aromatic conjugated C (sp^2). It contributes negatively to the response in the reported model, implying that an increase in the number of non-aromatic conjugated C (sp^2) reduces the log ($1/EC_{50}$) as well as the toxicity of the modelled dyes and vice versa. For example,

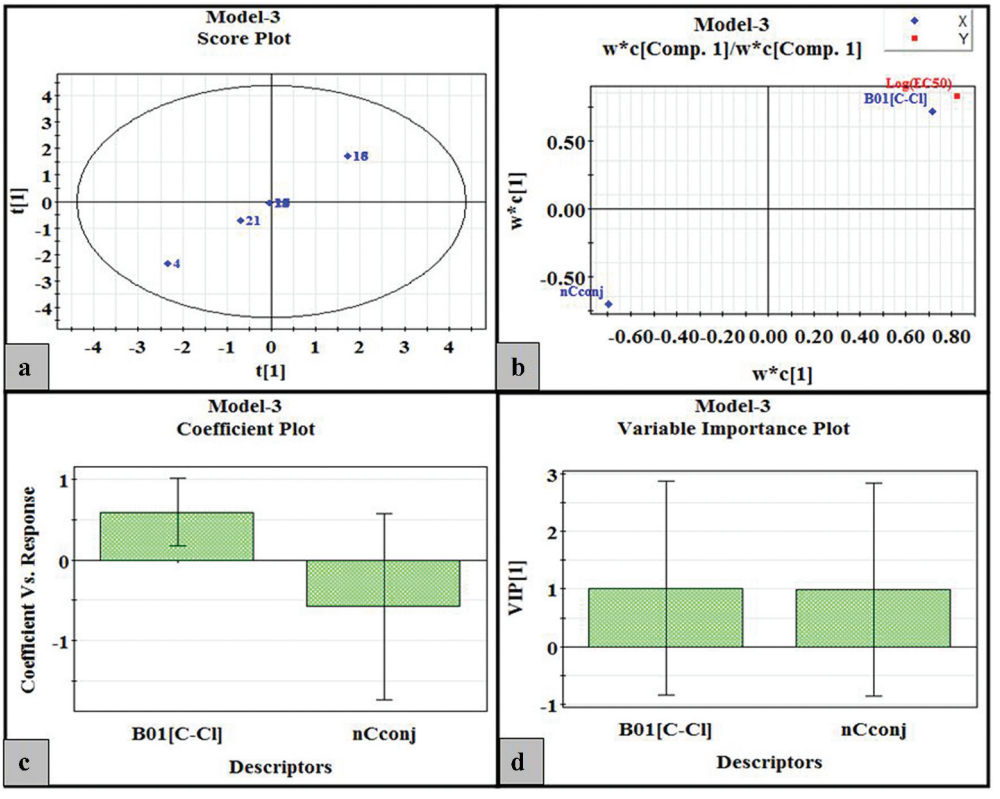


Figure 10. Various SIMCA plots obtained for Model III.

compounds such as Disperse Violet 31 (nCconj = 2; log (1/EC_{50}) = 3.37) and Acid Blue 3 (nCconj = 7; log (1/EC_{50}) = 1.117) have high values of the descriptor, indicating low toxicity, whereas compounds lacking non-aromatic conjugated C (sp^2) in their chemical structure, such as Disperse Red 13 (nCconj = 0; log (1/EC_{50}) = 7.270), and Disperse Red 1 (nCconj = 0; log (1/EC_{50}) = 6.242) have highest log (1/EC_{50}) values (Figure 9). These non-conjugated carbons with sp^2 hybridization in the dye structure do not involve the electrophilic/protoelectrophilic specific toxicity mechanism [54].

Plot interpretation. According to the score plot (Figure 10(a)), compounds 4, 18, and 21 are wholly unique and exhibit a wide variety of properties. The regression coefficient plot (Figure 9(d)) for Model III indicates that the modelled descriptors have a positive or negative effect on the log (1/EC_{50}) values of the dyes.

The loading plot (Figure 10(b)) for Model III shows that the descriptor 'B01[C-Cl]' is conducive to log (1/EC_{50}), while nCconj lying near to the plot origin has less influence on the response (log (1/EC_{50})). Figure 10(c) and 10(d) show regression coefficient plot and VIP plot, respectively, for Model III.

For the Y-Randomization plot (Figure 11) for Model III, the original and permuted Y-vectors generate the cumulative r^2y and Q^2y intercepts of 0.018 and –0.208, respectively, demonstrating that the model is non-random.

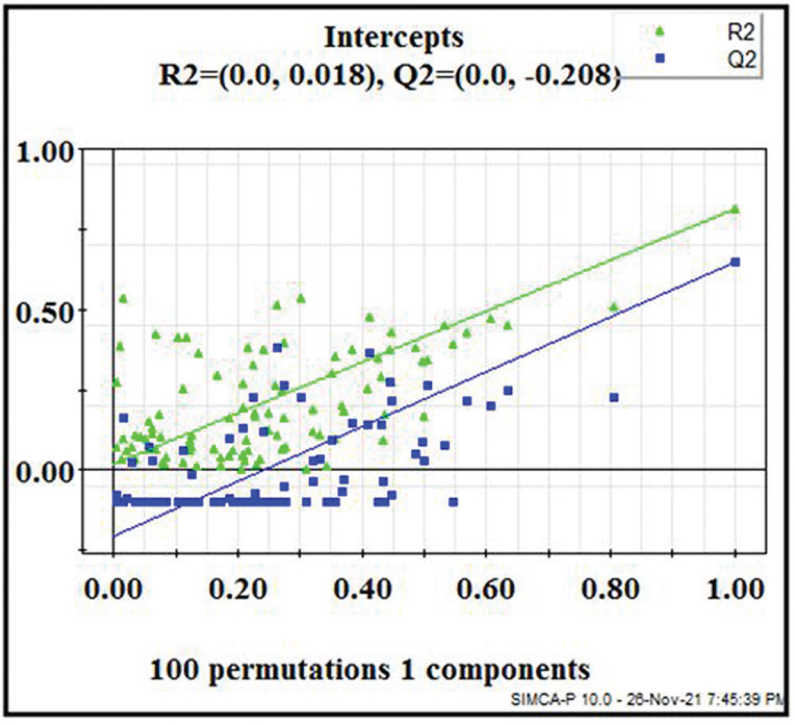


Figure 11. Y-Randomization plot for the Model III.

Finally, we have developed a model by pooling all the descriptors in the aquatic toxicity regression models (Model I, Model II, Model III) of *D. magna* in a PLS model and the quality as shown below.

$$\log(1/\mathrm{EC50}) = 4.48914 + 0.25238 \times \mathrm{X\%} - 0.03771 \times \mathrm{F05[C-C]} - 0.02287 \times \mathrm{F09[C-C]} + 0.66809 \times \mathrm{B0[N-O]} - 0.4625 \times \mathrm{B06[O-O]} + 6.7527 \times \mathrm{Chi_{Dt}} + 0.84896 \times \mathrm{B01[C-Cl]} - 0.15727 \times \mathrm{nCconj} \quad (4)$$

$$\mathrm{LV} = 1,\ n_{\mathrm{Training}} = 11,\ r^2 = 0.801\ ,\ Q^2 = 0.700, s = 67.20, F = 36.36, {}^{c}r^2{}_{\mathrm{p}} = 0.472$$

$$\mathrm{PRESS} = 10.1443$$

The corresponding qualitative validation parameters of the model developed after pooling all the descriptors from Model I, Model II, and Model III were given in Table 8.

Table 8. Different statistical validation parameters of the pooled descriptor model developed against *D. magna*.

Sets	Data	*Sensitivity*	*Specificity*	*Accuracy*	*Precision*	*F1-Score*	*MCC*
Training	11	0.83	0.40	0.64	0.63	0.72	0.26
Test	11	1.00	0.80	0.82	0.33	0.50	0.52

Table 9. Confusion matrix for pooled descriptor model from Model I, Model II and Model III.

Class	*Training set*	*Test set*
True positives (TP)	5	1
False positives (FP)	3	2
True negatives (TN)	2	8
False negatives (FN)	1	0

The number of true positives, false positives, true negatives, and false negatives found in training and test sets were mentioned in Table 9.

Classification-based model: linear discriminant analysis (LDA)

Model V (Eq. 5) displays the discriminant function equation computed using the LDA technique available within STATISTICA software [41]. The model comprises two 2D predictors namely nRCOOR and VE1_B(v). The developed LDA model is defined by encouraging Wilks' lambda value of 0.214 where zero stands for ideal distribution and >0.5 relates to random distribution. The model also generated pretty noticeable Fisher-distribution (F-value) and chi-square distribution (χ^2) parameter values which suggest an acceptable classification of data. Lastly, the model also demonstrated high values of squared Mahalanobis distance (dM^2) and canonical correlation coefficient (Rc) complementing the considerable degree of discrimination between the assigned extremely hazardous and less dangerous class of dyes. The details of various validation measures are presented below.

$$DF = -125.201 - 15.980 \times nRCOOR + 67.540 \times VE1\_B(v) \tag{5}$$

$$\begin{aligned} n_{training} = 11, n_{test} = 11, p - value = 0.002, Wilks\ \lambda = 0.214, \chi^2 = 177, R_c \\ = 0.886, AURO_{training} = 0.91, AURO_{test} = 0.84, Eige_{value} = 3.652, F(2,8) \\ = 14.63, dM^2 = 12.934 \end{aligned}$$

The qualitative validation parameters for the linear discriminant analysis model were given below in Table 10.

Based on the threshold value of 4.502918, the LDA model correctly classified 4 (100%) of 4 highly toxic organic chemicals and 7 (100%) of 7 fewer toxic chemicals in the training set, while 2 (66.6%) of 3 compounds were correctly classified as highly toxic chemicals and 7 (88%) of 8 compounds were correctly classified as less toxic chemicals in the test set. Qualitative classification measures were extremely encouraging and demonstrated the generated LDA models' capacity to categorize. Additionally, we computed G-means for both the training and test sets (training = 0%, test = 76%), which indicated that the model is capable of distinguishing effectively between extremely dangerous and less harmful organic compounds. The ROC curves (AUROC) were generated to evaluate the

Table 10. Qualitative validation parameters for the training and test sets for LDA model.

Sets	*Data*	*Sensitivity*	*Specificity*	*Accuracy*	*Precision*	*F-Measure*	*G-Means*
Training	11	1.00	0.00	1.00	1.00	1.00	0.00
Test	11	0.87	0.67	0.82	0.88	0.88	0.76

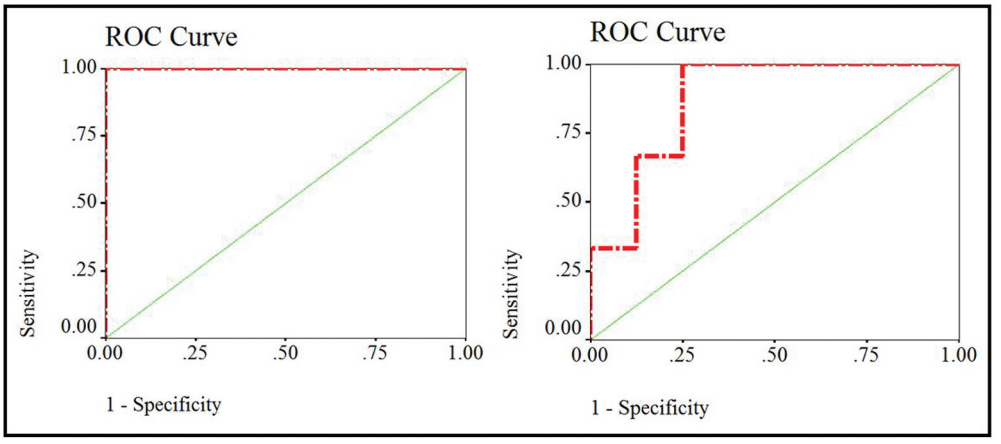


Figure 12. ROC curves for the training and test sets.

performance of the model on training and test sets; surprisingly, both the training and test sets produced promising values (Figure 12), guaranteeing the proposed model's dependability and acceptability.

We have attempted to illustrate the interpretations of the descriptors in the model by demonstrating their ability to categorize compounds into more and less toxic commercial dyes. The classifier model is composed of two 2D-descriptors and both the descriptors signify lipophilic or hydrophilic properties.

The relationship between toxicity and non-polar characteristics has been examined extensively and established in several papers [22,36]. The variables in the model may be classified into two groups: those in the first group result in less toxic molecules, while those in the second group result in highly toxic compounds.

The descriptor nRCOOR indicates the number of esters in the dye chemical structure. This variable has a negative regression coefficient, indicating that increasing the value of this attribute results in a decrease in DF scores. When the ester count of the dye structure increases, it shows a negative effect on the log ($1/EC_{50}$) values of *D. magna*. Some compounds like Disperse Blue 79 (Comp no 12) (nRCOOR = 2) and Disperse Blue 79:1 (Comp no 13) (nRCOOR = 2) follow this pattern.

The next descriptor is VE1_B(v), which is the coefficient sum of the last eigenvector from Burden matrix weighted by van der Waals volume. The presence of this variable results in an increased DF score and highly toxic compounds. This variable indicates the lipophilic content of the molecule as represented by the majority of the highly toxic compounds in the training set; for example, comp no 17 (Disperse Orange 30) has 2 aqueous toxicophores namely, nitrile and 4-nitrobenzene and comp no 18 (Disperse Red 13) have higher values of this descriptor, i.e. a higher lipophilic content increases the DF score and also makes the dye more toxic to the water bodies.

The Contribution plot in Figure 13 illustrates the degree of discriminating power of specific descriptors for classifying dyes into extremely hazardous and less toxic compounds based on the chosen threshold. The contribution plot illustrates the discriminating ability of model descriptors, with VE1_B(v) having the most positive contribution to highly toxic dye compounds (standardized coefficient of 67.54).

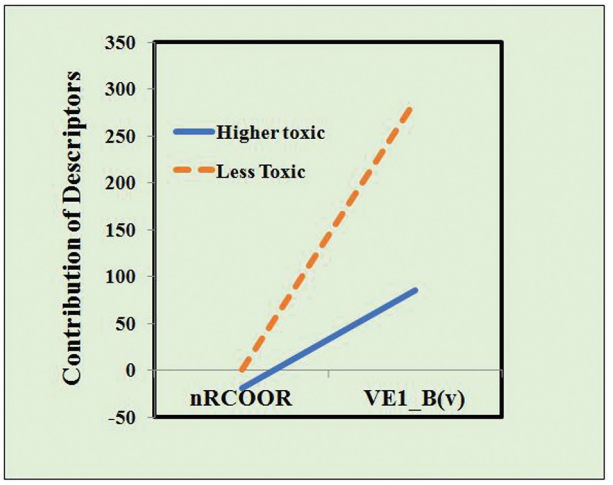


Figure 13. Contribution plot illustrating the discriminating potential of different variables contained in the LDA model.

Finally, we have conducted an analysis of the applicability domain using a standardized approach and found no outliers found in the dataset [42].

Comparison with previous studies

Table 11 compares the statistical data produced from the current QSAR model to the previously published models. Based on both internal and external validation criteria, the model described in this work is statistically significant and robust enough in comparison to the previously published models (Table 11). For model construction, only 2D descriptors were used in the present study. Previously, multiple researchers presented QSAR models for predicting acute toxicity of various organic chemicals against *Daphnia magna* using a variety of approaches, including ACO-SVM, ACO-MLR, CORAL Models, employing Multiple Linear Regression (MLR) analysis and Partial least squares (PLS) regression. However, this is the first attempt to develop models on acute aquatic toxicity of chemical dyes using simple, interpretable 2D descriptors against *D. magna*. Dyes are commonly mischaracterized, and it is vital to comprehend both their physicochemical properties and their environmental implications. We employed a variable selection strategy based on multilayered stepwise regression before building the final models in this work, followed by the best subset selection method. In this study, we built regression and classification models to assess the acute toxicity of chemical dyes against *D. magna*. The PLS regression technique was used to build the final regression models, which had 3, 3, 2 descriptors and 2, 2, 1 LVs. The chosen descriptors reflect the underlying structural features of compounds, which are important in modelling the acute toxicity of organic dyes against *D. magna*. In terms of statistical quality, equation length, LVs, and so on, the models created in this work outperform earlier models. According to Aalizadeh et al. [17] in Table 11, the ACO-SVM and ACO-MLR models were built against *D. magna* using 1353 organic compounds (including dyes), but the model quality was subtle and the models' mechanistic interpretation was ignored.

Table 11. A quick assessment of existing conventional models on the acute aquatic toxicity of selected organic compounds against *Daphnia magna* with our models.

Sr No.	*Working group*	*Type of chemicals*	*Model*	*Dataset size*	r^2	Q^2	*Remarks**
1	Current manuscript	Only organic dyes	Model I(PLS)	22	**0.71**	**0.56**	Models with mechanistic interpretation and possible validation parameters are produced
			Model II(PLS)		**0.91**	**0.77**	
			Model III(PLS)		**0.81**	**0.64**	
			Model I, II, III(PLS)		**0.80**	**0.70**	
2	Marzo et al. [55]	Biocides	LR	133	0.83	—	Complicated descriptors used and no PLS models developed
			RF		0.97		
			CORAL Model		0.76		
3	Aalizadeh et al. [17]	Organic compounds	ACO-SVM	1353	0.92	0.695	No mechanistic interpretation
			ACO-MLR		0.60	0.60	

In this study, we only employed solely organic dyes, because dyes are commonly mischaracterized and their ecotoxicological impacts are critical for aquatic life as well as humans. So, we used 22 chemical dyes with acute toxicity data against *D. magna* and used PLS and LDA to build regression and classification – based models. Marzo et al. [55] also employed organic molecules to construct a linear regression (LR) model, a random forest (RF) model, and a CORAL model (133 compounds). Table 11 shows information about several validation parameters obtained from our model and previously reported models.

Conclusion

Globally, several sectors are utilizing dyes and pigments and generating some trillion dollars revenue, which accounts for a large portion of total world exports employing approximately 40 million people. The growing usage of dyes and pigments may pose severe environmental problems shortly. Researchers are currently concentrating their efforts on controlling the use of dyes by determining their risk-benefit ratio for the aquatic environment. Numerous regulatory authorities throughout the world are currently considering the justification for regulating the use of dyes and pigments by utilizing available information about them, as well as their physicochemical qualities and transformation fate, among other things. To make these dyes (food, diagnostic and textile, etc.) more eco-friendly, we must first bridge the toxicity data gap for the dyes, which are poorly defined and have scanty toxicological/ecotoxicological data (available only for a few dyes). In this work, we have created two-dimensional QSTR models against the log ($1/EC_{50}$) utilizing 2D descriptors. We have developed both PLS and LDA models with the corresponding data obtained from the literature and estimated the different validation criteria following the strict standards for QSAR model validation. This work is primarily concerned with elucidating the molecular mechanisms (such as impact of increase in the bulk, addition of halogens and electronegative groups, and electrophilic/proto-electrophilic specific toxicity mechanism, etc.) underlying acute aquatic toxicity (log ($1/EC_{50}$)) towards water flea *Daphnia manga*, as well as making precise predictions of toxic effects for novel or speculative dye compounds. As a result, these models may show the way for chemists to theoretically predict the toxicity of untested dyes and pigments based on their predetermined threshold, and they may aid in decision making and suggesting a framework for relatively safe food, diagnostic and textile dye design; thus, the time and resources required for experimental work can be minimized.

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Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Application of QSARs in identification of mutagenicity mechanisms of nitro and amino aromatic compounds against *Salmonella typhimurium* species

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ABSTRACT

In an attempt to describe the underlying causes of mutagenicity mainly due to organic chemicals, quantitative structure-activity relationship (QSAR) models have been developed using two different *Salmonella typhimurium* mutagenicity endpoints with or without presence of liver metabolic microsomal enzymes (S9) namely TA98-S9 and TA98 + S9. The models were developed using simple 2D variables having definite physicochemical meaning calculated from Dragon, SiRMS, and PaDEL-descriptor software tools. Stepwise regression followed by partial least squares (PLS) regression was used in model development following the strict OECD guidelines for QSAR model development and validation. The models were validated using coefficient of determination R^2, cross-validation coefficient Q^2_{LOO} (leave one out) while the test set predictions were analyzed using Q^2F_1 (coefficient of determination for the test set). Several other internationally accepted validation metrics like $MAE_{95\%train}$, average $r_{m(LOO)}{}^2$ and $\Delta r_{m(LOO)}{}^2$ (for the training set) were used to check model robustness while predictive efficiency was evaluated using $MAE_{95\%test}$, average $r_m{}^2$ and $\Delta r_m{}^2$ (for the test set). The scope of predictions was defined by applicability domain analysis using the DModX approach, a recommended tool for PLS models. The major contributing features related to mutagenicity include lipophilicity, electronegativity, branching and unsaturation, etc. The present manuscript is the first attempt to undertake modeling of two different endpoints (TA98-S9 and TA98 + S9) in order to explore major contributing molecular features linked directly or indirectly to mutagenicity.

1. Introduction

Organic chemicals (OCs) constitute a large collection of chemicals employed in several spheres of life including dyes, polymers, pesticides, textiles, explosives and several food substances, etc. With the gradual rise of chemical consumption in diaspora, there arises a need to check for adversity that follows. The exponential rise in the consumption of OCs has compelled scientists to explore the probable toxicity caused to the flora and fauna at the genetic level starting from the building block of life, i.e., deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (Moretti et al., 2002; Tsuboy et al., 2007). OCs have long been identified as carcinogenic or mutagenic materials in several ecologically important species including humans along with various bacterial cell lines such as *Salmonella typhimurium* (Chung and Cerniglia, 1992; Chung et al., 1997; Rashid et al., 1987; Sabbioni, 1994; Tomita et al., 1982). Mutagens are distinct from the carcinogens where the former leads to one or the other types of cancer due to mutation while carcinogens need not cause mutation in genetic material to develop cancer (Maisanaba et al., 2015). Among the various functionalities present in the OCs, nitroaromatics and aromatic amines have proven to have greater mutagenic potential compared to other classes of chemicals.

For many decades, scientists have relied upon the Ames test (Stead et al., 1981) for the mutagenicity study. It is estimated from the Ames test that the TA98 strain consists of a full complement of nitroreductases required to activate the reduction reaction of nitro aromatics, whereas aromatic and hetero aromatic amines demand the presence of an exogenous metabolic activation system, i.e. S9, to initiate the oxidation reaction, suggesting that mutagenicity can only take place at the nitro moiety in nitroaromatic amines in *S. typhimurium* strain TA98 without S9 mix (Fu, 1990; Fu and Herreno-Saenz, 1999). Table 1 and Fig. 1 depict the experimentally proved mechanisms leading to mutagenicity.

The major drawbacks of using the Ames test in mutagenicity

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Table 1
Some of the reported mechanisms leading to mutagenicity.

Sr. no.	Modes of mutagenicity	Mechanism
1	Alkylation	Most of the Alkylating agents damage the DNA with the formation of N2-alkylG (where G stands for guanine) and other lesions; for example formaldehyde reacts with the exocyclic amino group of deoxyguanosine to produce N2-methylG (Yasui et al., 2001). Some other examples of alkylating agents include ethanol which enzymatically get oxidized to acetaldehyde and thus forms N2-ethylG found in liver DNA and urine of alcoholic patients (Cheng et al., 2008). Other alkylating agents having sufficient mutagenic potential include polycyclic aromatic hydrocarbons (PAH-DNA adducts), nitrosamines (formation of the O6-methylG (O6-MeG)) and bis-electrophilic agents like 1, 3-Butadiene (BD can be oxidized to 1, 2, 3, 4-diepoxybutane (DEB), a prominent bis-electrophilic carcinogenic metabolite).
2	Oxidation	Oxidizing agents can produce 7, 8-dihydro-8-oxo-2′-oxodeoxyguanosine (8-oxodG) lesions. 8-oxodG is a ubiquitous lesion arising from the oxidation of the C8 atom of G to form a hydroxyl group by free radical intermediates of oxygen that are produced by chemical oxidation, ionizing radiation, or UV irradiation (Degan et al., 1991; Fraga et al., 1990). The enol (a lactim) at the C8-N7 position of G is converted to the more stable 8-oxodG lactam form.
3	Amination	Several aminating agents like aryl amines and *N*-acetyl aryl amines possess higher propensity to act like potential mutagenic substances. This group is extensively studied for their mutagenic activity and also implemented in several in silico studies due to their presence in various occupational settings like tobacco smoke, chemical dyes etc. These chemicals go on to form adducts like 2-aminofluorene (AF-dG) and *N*-acetyl-2-aminofluorene (AAF-dG) through amination of the C8 atom of guanine (via an initial N7 reaction, linking the amine group of the aryl amine) (Vrtis et al., 2013).
4	Co-ordination	Heavy metal ions also produce mutagenicity with the formation of DNA-DNA intra-strand and inter-strand cross-links via coordination bonds. For example, chromium (VI) complex permeates the cell membranes and gets reduced to form chromium (III) complex, following which it then coordinates with oxygen atoms of phosphate backbone of two adjacent nucleotides within one DNA strand or between two DNA strands, yielding chromium (III)-DNA intra-strand thus yielding an inter-strand cross-links (O'Brien et al., 2002).
5	Photo-addition	The ultra violet (UV) radiation leads to the formation of photoproducts (e.g., CPD) by cycloaddition of the C5-C6 double bonds with adjacent pyrimidine bases; thus it behaves like a non-chemical mutagenic agent. Six diastereomers are generated, depending on the position of pyrimidine moieties with respect to the cyclobutane ring (cis/trans stereochemistry) and on the relative orientation of the two C5-C6 bonds (syn/anti regiochemistry) (Cadet et al., 1985). The cis-syn form is formed preferentially to the trans-syn diastereomers within double-stranded DNA. The trans-anti and trans-syn photoproducts are only present within single-strand or denatured DNA (Ravanat et al., 2001).
6	Hydrolysis	The final proposed mechanism for mutagenesis is by hydrolysis, where AP (apurinic/apyrimidinic) sites are generated by spontaneous reactions, chemical induction or by enzyme-catalyzed hydrolysis of the N-glycosyl bond (Wilson Iii and Barsky, 2001) resulting in the loss of genetic information. In mammalian cells, it has been estimated that approximately 12,000 purines are lost spontaneously per genome per cell generation (20*h*) in the absence of any protective effects of chromatin packaging. It was subsequently shown that depyrimidination occurs at a rate about 100 times more slowly than depurination (Wilson Iii and Barsky, 2001). Damaging chemicals, e.g., free radicals and alkylating agents, promote base release, mostly by generating base structures that destabilize the N-glycosyl linkage due to positively-charged leaving groups (Wilson Iii and Barsky, 2001).

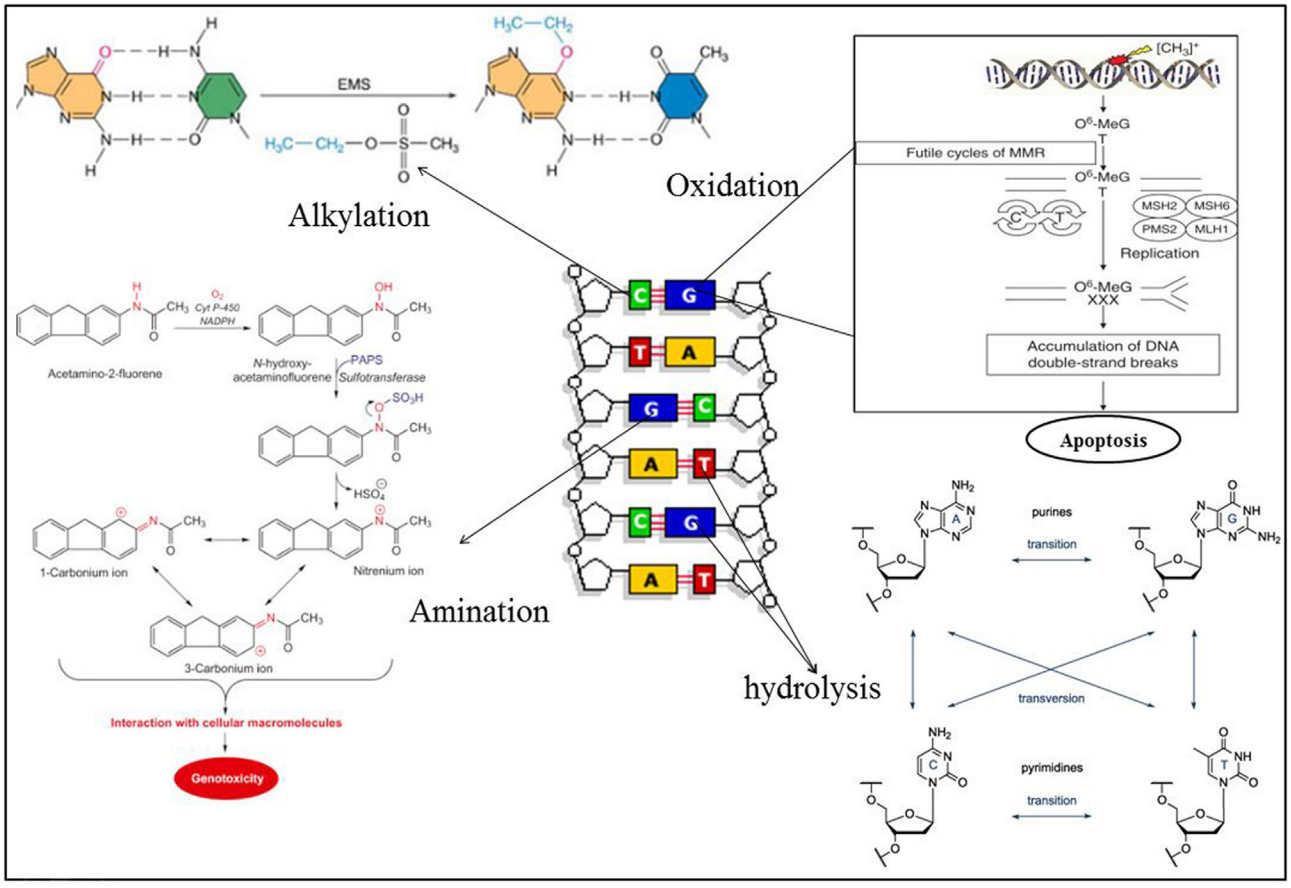

Fig. 1. Known mechanisms of mutagenicity proposed by several experts (Cadet et al., 1985; Cheng et al., 2008; Degan et al., 1991; Fraga et al., 1990; O'Brien et al., 2002; Ravanat et al., 2001; Vrtis et al., 2013; Wilson Iii and Barsky, 2001; Yasui et al., 2001).

estimation lies in its associated time, cost and man power. Finally, a number of existing organic chemicals (more than several millions) make it impossible to undertake experimental evaluation of all chemicals through the Ames test against enormous number of endpoints. Several computational (in silico) techniques like QSAR (quantitative structure-activity relationship) and pharmacophore modeling can help to fill the data gap. QSAR offers several advantages over other techniques as it utilizes limited experimental resources, cost and offers time efficient outcome (Dearden, 2016). Due to these encouraging features, QSAR is recommended for use in early detection of various toxic chemicals (Khan et al., 2019b; Khan et al., 2019c).

A number of scientific groups have attempted to identify the mutagenic features present in the various subclasses of organic compounds, some of them are discussed here. Garg et al. developed a QSAR model correlating the experimental mutagenicity of 43 aminoazobenzenes using several molecular descriptors calculated from quantum-chemical semi empirical approach (Garg et al., 2002). Gramatica and colleagues (Gramatica et al., 2007) used a dataset of 48 nitro-PAHs (polycyclic aromatic hydrocarbons) mutagenicity data against TA100 (*S. typhimurium*) strain to analyze the possible toxicophores using QSAR study, while Wang et al. (2005) performed comparative molecular field analysis (CoMFA) and molecular orbital theory based classic structure-activity relationship analysis in order to explore the structural fragments responsible for mutagenicity using 219 nitro aromatics compounds (Wang et al., 2005).

The current manuscript is the first report giving in silico QSAR models correlating the mutagenicity of nitro aromatics and aromatic/hetero aromatic amines against *Salmonella typhimurium* species (TA98) with or without presence of liver metabolic microsomal enzymes (S9). The previous reports on this topic were solely consisting of either TA98 + S9 or TA98-S9 toxicity endpoints. Only 2D descriptors with definite physicochemical meaning were employed here in model development in order to derive models of relatively less complexity from the interpretation perspective. The models were validated using some of the very stringent validation metrics. The applicability domain study was checked in order to give models a definite zone for reliable prediction for unknown or untested chemicals.

2. Methods and materials

2.1. Dataset

A reliable QSAR model can only be obtained from trusted sources of experimental data. To achieve this, the authors have compiled two sets of mutagenicity data against *Salmonella typhimurium* (TA98) bacterial species with (TA98 + S9) or without (TA98-S9) implementing microsomal activating enzyme named S9, solely collected from literatures (Bhat et al., 2005; Ding et al., 2017; Leong et al., 2010). For the ease of acceptability, the collected data were selectively filtered for uniform experimental procedures, conditions and protocol. The mutagenic endpoint TA98-S9 consists of 295 nitro aromatic compounds along with their derivatives, while the TA98 + S9 endpoint data was derived of 309 chemicals with reported acute mutagenicity against *S. typhimurium* and composed of aromatic amines and hetero aromatic amines along with their derivatives (See Sheet 2-3 in SI-1). The mutagenicity in all cases was expressed as the logarithm of the number of revertants per nmol and was used as such. Out of 295 TA98-S9 endpoints, four compounds (**191**, **192** and **197**, **198**) were identified as stereoisomers having contrast mutagenicity values, thus excluded from the initial analysis. The dataset chemicals in both the endpoints are commercially employed to make several important products or product mixtures such as dyes, personal care products, organic reactants, product intermediate, resins and also for research purposes, thus making them indispensible from day to day life and necessitating their evaluation for mutagenic potential. All the structures were manually drawn in MarvinSketch version 4.0 (available on https://chemaxon.com/products/marvin) and cross verified from the source paper in order to avoid any miscalculation of molecular descriptors at the later stage. Finally, the structures were cleaned in 2D, aromatic bonds and explicit hydrogens added and saved as MDL.mol format, a recommended input format for Dragon (Mauri et al., 2006), SiRMS (Kuz'min et al., 2005) and PaDEL-descriptor (Yap, 2011) softwares.

2.2. Descriptor calculations

For the ease of interpretation, simple 2D descriptors with definite physicochemical meaning were used during the QSAR modeling. A total of 43 ETA indices (extended topochemical indices) (Khan and Roy, 2019) were calculated from PaDEL-descriptor (version 2.21) (Yap, 2011) software while from Dragon (version 7.0) (Mauri et al., 2006) eight different classes of descriptors were generated including constitutional indices, *E*-state indices, 2D atom pairs, molecular property descriptors, connectivity indices, functional group counts, ring indices and atom-centered fragments giving a total of 467 different variables (Mauri et al., 2006). Additionally, nearly 15,000 molecular fragments as 2D descriptors were calculated from simplex representation of molecular structure (SiRMS) software (Kuz'min et al., 2005). The descriptors with correlation more than > 0.9 (R^2) were excluded from the analysis in order to avoid problems of over fitting (Khan et al., 2019a). The endpoints are separately modeled with SiRMS descriptors in order to identify the most contributing features present in the respective datasets.

2.3. QSAR modeling and validation

The initial datasets for the both endpoints were split into training and validation set using defined algorithms. Various data division techniques (Sorted response, Euclidean distance and Kennard-Stone method) partitioned approximately 75% molecules into the training set while remaining 25% were placed in the test set. However, the best division which gave the most reliable models was obtained by the Euclidean distance based partitioning (Golmohammadi et al., 2012) using a software tool (available at http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab/). The training set was solely employed for feature selection and model development while the test set was used to analyze predictivity of the developed models. For feature selection, stepwise regression with stepping criteria also known as “Fisher criteria” was used with specified threshold of $F = 4.0$ for the inclusion and $F = 3.9$ for exclusion (Hossain and Roy, 2018). The process was repeated several times (after removing selected descriptors in the previous runs) in order to identify the significant descriptors. Finally, a set of best 25 variables were collected at the end of stepwise analysis and were subjected to best subset selection. The best two models for both the endpoints were then subjected for partial least squares (PLS) analysis in order to reduce noise form the models, as PLS regression calculates latent variables (LVs) from the original variables and can also handle a lot of noisy descriptors. As a part of the PLS model development, the software internally performs input data scaling (standardization) followed by computation of latent variables scores (the actual regressing variables), although the final regression coefficients are presented in terms of the original un-scaled variables (similar to multiple linear regression or MLR equations). Unlike MLR models, determination of standard errors of regression coefficients for PLS models is not straight-forward; however, the relative importance of different variables can be presented in terms variable importance plot (VIP). For validation, various quantitative validation metrics were used for defining the quality of the developed models which are evaluated in terms of stability, robustness, fitness and predictivity. The coefficient of determination (R^2), internal predictivity metrics like leave-one-out cross-validated R^2 or Q^2_{LOO} and external predictivity metrics like R^2_{pred} or Q^2_{ext} and Q^2F_2 were calculated (Roy and Mitra, 2011). We have also checked the MAE (mean absolute error) based criteria for both internal and external validation sets (Roy

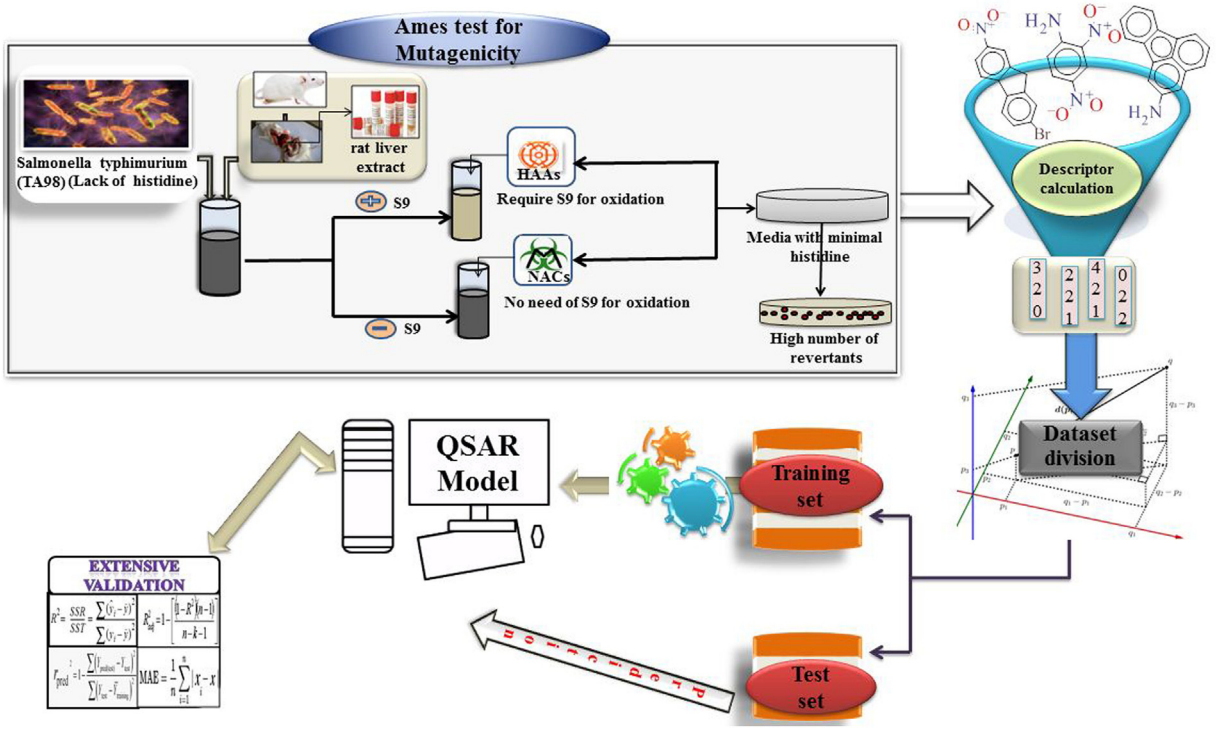

Fig. 2. The detailed methodology followed in the present work.

et al., 2016). Additionally, highly stringent rm^2 parameters for validations were also calculated to check for model robustness (Roy et al., 2012). The prediction error of the developed QSTR model was evaluated by the statistical parameters of the model for the external set which should be within the response and chemical domains of the internal or training set. Model randomization was performed to substantiate the robustness of developed PLS model. Additionally, a variable importance plot was constructed for each model depicting individual contributions of the modeled descriptors. The performed methodology is summarized in Fig. 2.

2.4. Applicability domain (AD)

The applicability domain is a concept of chemical space explained by the model descriptors and the modeled response. In the present study, we have implemented DModX (distance to model in X-space) approach using SIMCA-P software (Umetrics, 2013) to scrutinize the applicability domain of the developed QSTR model. The DModX approach was implemented at 99% confidence level to examine whether the test compounds used in modeling study lie in the chemical space or outside the chemical space of the training compounds used for developing the model (Wold et al., 2001).

2.5. Modeling mutagenicity using molecular fragments

Lastly, QSAR modeling with fragmental variables was performed in order to explore major contributing features embedded in the structures of organic molecules. To achieve this, a series of 2D molecular fragments were computed using simplex representations of molecular structure (SiRMS) software (Version 4.1.2.270). The molecular fragments were then analyzed for their probable cause leading to mutagenicity in *S. typhimurium* as per the recommended OECD guidelines for model interpretations. The remaining procedures like data division, features selection and model validation were performed as per the protocol specified above.

2.6. Software used

Marvin sketch (version 14.10.27) software (http://www.chemaxon.com/) was used to draw the chemical structures. SiRMS (Version 4.1.2.270), Dragon version 7 (http://www.talete.mi.it/products/dragondescription.htm) and PaDEL-Descriptor (http://www.yapcwsoft.com/dd/padeldescriptor) software tools were used to calculate the molecular descriptors. For data division, freely available DatasetDivisionGUIv1.2_9May2017 tool at http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab was used. The stepwise regression analysis was performed by using MINITAB Software (version 14.13) (http://www.minitab.com/en-US/default.aspx). Best subset selection was performed using freely available QSAR tools at http://teqip.jdvu.ac.in/QSAR_Tools/DTCLab. Simca-p (Version 10.0) was used for the generation of various PLS plots.

3. Result and discussion

The current work reports QSAR modeling of mutagenicity potential of selected organic chemicals mainly against *Salmonella typhimurium* species (TA98) employing two different strategies. In the first approach, the data comprise mutagenicity of 291 nitroaromatic compounds measured without using microsomal activation (Rat S9) while the second dataset consists of 309 aromatic and hetero-aromatic amines having mutagenicity data with the employment of microsomal activation. In order to get reliable models, a great care was taken in data collection, curation, and validation of QSAR models in order to obey the strict OECD guidelines for QSAR model development as described in the methods and materials section (Khan et al., 2018). The developed models were rigorously validated using various internal and external validation parameters in order to prove their robustness. Additionally, the MAE based criteria for the test set were checked to enhance acceptance of the final models (Roy et al., 2016). The models developed with employing S9 passed the MAE criteria (Roy et al., 2016). The individual equations depicting their validation metrics are given below with descriptors elaborated thoroughly.

3.1. Modeling nitro-aromatic compounds against Salmonella typhimurium strain (TA98) without microsomal activation (S9)

From the total of 291 nitro-aromatic compounds, 219 molecules were utilized for the model development while the remaining 72 molecules were used to validate the final models. Initially, a number of QSAR models were generated using the best subset selection; however the best two models were finally selected from the large pool of generated models based on the most interpretable chemical features or toxicophores. Both the models gave a moderate level of robustness with coefficient of determination showing values ranging from 0.71–0.73 (0.68–0.71 for leave-one-out cross validation), while the predictivity of the test set was measured using predictive R^2 covering 74–76% variance of the test set molecules. Eqs. (1)–(2) detail the validation metrics obtained for both the models along with regression coefficients of individual variables arranged in a descending order of contribution to the mutagenicity as per the VIP plots. The VIP values rank model descriptors based on their significance to the response and are calculated using classical regression coefficient, weight vector and t-statistic, etc. (Akarachantachote et al., 2014). A VIP score of > 1 corresponds to significant descriptors whereas a score < 1 is considered as insignificant. Model 1 consists of three highly significant variables namely RDCHI (reciprocal distance sum Randic-like index), ETA_dBeta (a measure of relative unsaturation content in a molecule) and nCIR (number of circuits or loops) with VIP scores of 1.45, 1.4 and 1.2 respectively. Model 2 for TA98-S9 endpoint contained relatively more significant variables such as ETA_Beta_ns (a measure of electron-richness of the molecule), X5A (average connectivity index of order 5), ETA_dBeta, RCI (ring complexity index or size) and RDCHI with VIP scores of 1.25, 1.25, 1.10, 1.10 and 1.05 respectively. The remaining variables in both the cases were less significant. The loading plot in both models hinted towards a greater positive contribution from ETA indices and RDCHI due to their closeness to the dependent variable, i.e., mutagenicity. Finally, Y-randomization implemented in SIMCA-P was performed at 100 permutations to check for the non-randomness of the obtained models. Interestingly both the QSAR models on TA98-S9 gave intercepts for determinant coefficients of 0.003 and 0.004 and those for cross validated determinant coefficients of −0.28 and −0.17, which are far below then expected cut off; for a good model, the intercept for R^2 should be < 0.4 and that for Q^2 should be < 0.05. Additionally a stringent correlation (r) cut off of 0.9 was implemented at every stage of modeling to avoid problems of overfitting. The obtained modeled descriptors with highest influence on mutagenicity are grouped in four sub classes such as descriptors depicting unsaturation in molecules like RDCHI, ETA_dBeta and ETA_Beta_ns, descriptors designating presence of various rings like NRS, nCIR, and RCI, descriptors with more hydrophobic influence which include X5Av, X5A, F07[C−C], and finally, descriptors with more electronegative element content such as F09[N−N], B08[Cl−Cl] and B09[N−N]. Various qualitative plots generated by SIMCA tool are given in Figs. S1-S2 in Supplementary Materials (SI-2).

3.1.1. Mechanistic interpretation of the TA98-S9 models

$$\begin{aligned} Log(TA98 - S9)rev/nmol &= -17.487 + 4.310 \times \text{RDCHI} + 0.326 \times \text{ETA\_dBeta} - 0.040 \times \text{nCIR} + 24.010 \times \text{X5Av} - 0.206 \times \text{F07[C - C]} - 1.892 \times \text{NRS} + 1.390 \times \text{F09[N - N]} - 2.220 \times \text{B08[Cl - Cl]} \end{aligned} \quad (1)$$

$n_{training} = 219$, LV = 6, $R^2 = 0.731$, $Q^2 = 0.710$, $r^2_{m(LOO)} = 0.603$, $\Delta r^2_{m(LOO)} = 0.196$, MAE = 0.852

$n_{test} = 72$, $R^2_{pred} = 0.756$, $Q^2_{F2} = 0.751$, $r^2_{m(test)} = 0.649$, $\Delta r^2_{m(test)} = 0.194$, MAE = 0.728

$$\begin{aligned} Log(TA98 - S9)rev/nmol &= -17.4872 - 0.11793 \times \text{ETA\_Beta\_ns} + 37.18461 \times \text{X5A} + 0.35196 \times \text{ETA\_dBeta} + 2.77124 \times \text{RCI} + 4.866 \times \text{RDCHI} - 0.25617 \times \text{F07[C - C]} + 1.48697 \times \text{B09[N - N]} - 1.06744 \times \text{NRS} \end{aligned} \quad (2)$$

$n_{training} = 219$, LV = 6, $R^2 = 0.707$, $Q^2 = 0.679$, $r^2_{m(LOO)} = 0.564$, $\Delta r^2_{m(LOO)} = 0.202$, MAE = 0.883

$n_{test} = 72$, $R^2_{pred} = 0.736$, $Q^2_{F2} = 0.727$, $r^2_{m(test)} = 0.628$, $\Delta r^2_{m(test)} = 0.145$, MAE = 0.781

Both the models for TA98-S9 consist of 8 descriptors as shown in above equations; however, 4 descriptors were common in both cases; i.e. RDCHI, ETA_dBeta, NRS and F07[C−C] thus making a total of 12 descriptors. The descriptor RDCHI is defined by analogy with Randic connectivity index (X1), where the simple vertex degrees are replaced by the row sums of the reciprocal distance matrix as depicted in Eq. (3).

$$RDCHI = \sum_{i=1}^{A-1} \sum_{j=i+1}^{A} a_{ij}.\,(RDS_i.\,RDS_j)^{-1/2} \quad (3)$$

Here, A is the number of vertices and a_{ij} is equal to 1 only for pairs of adjacent vertices and zero; R is the topological radius, D is the topological diameter and 'S' means sum. The RDCHI descriptor values increases with the size of the molecules whereas it decreases with branching. The positive regression coefficient of this parameter suggests that the presence of this fragment in nitro-aromatics enhances mutagenic potency of a molecule. This effect can be seen in compounds **17** (presence of pyrene or polycyclic aromatic hydrocarbon rings and four nitro groups), **176** (consists of 6 aromatic rings (benzene) and 2 nitro groups) and **212** (presence of a nitro-phenol ring) where a higher magnitude of this descriptor produces more toxic, potentially more mutagenic nitro-aromatic compounds. In contrast, molecules with lower RDCHI values were relatively less toxic and less mutagenic as seen in compounds **43** (having only one naphthalene ring), **80** and **177** (a single benzene ring).

The second most influential descriptor enhancing mutagenicity value was ETA_dBeta which gives the relative unsaturation content (Δβ) in the studied compounds. The index of unsaturation can be calculated by the following Eq. (4):

$$\Delta\beta = \sum \beta_{ns} - \sum \beta_{s} \quad (4)$$

Here, Σβs is the summed β values for all the sigma bonds (VEM (Valence Electron Mobile environment) sigma contribution) and Σβns is the summed β values for all the non-sigma bonds including lone electron pairs capable of resonance if any (VEM non-sigma contribution) (Roy and Das, 2017). This descriptor contributes positively towards the mutagenicity as indicated by its positive regression coefficient. This indicates that the mutagenicity of the nitro-aromatic compounds increases with an increase in the numerical value of unsaturation in the form of double bonds ("=") as can be seen in compounds **99, 111** and **271,** while a low level of unsaturation in the form of double bonds will lead to less mutagenic chemicals mainly seen in case of **154, 182** and **228**. Presence of more unsaturation prevailed in most of the highly mutagenic nitro-aromatic compounds. Again, ETA_Beta_ns, which is a measure of electron richness in a molecule, is an index of non-sigma electrons including lone pair of electrons. The index is calculated from all the π bonds and electron lone pairs present on heteroatoms, carbonyls and the atoms which are capable of resonance like N, O, S and halogens present in aromatic compounds. In contrast to ETA_dBeta, this index exerted a negative effect towards mutagenicity possibly due to an

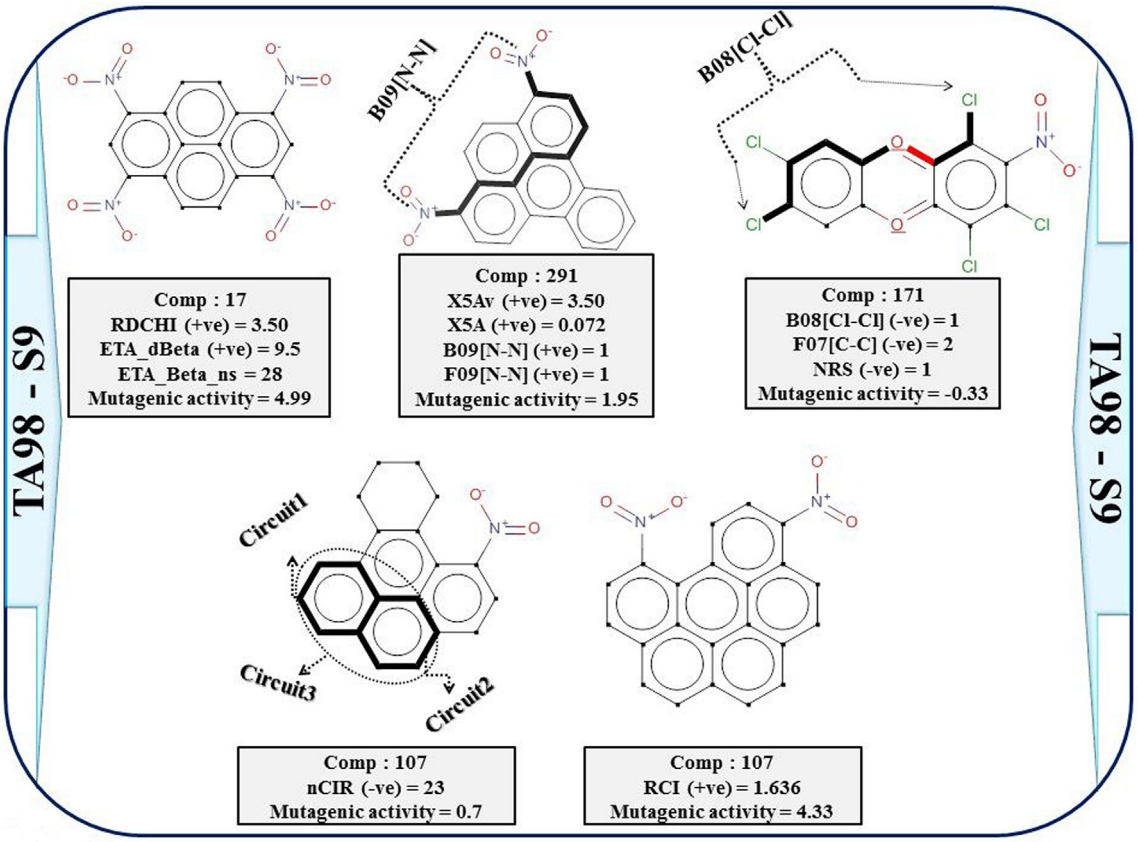

Fig. 3. Molecular features enhancing or reducing mutagenicity of TA98-S9.

increase in polar bulk in the molecule mainly due to sum effects of polar atoms such as N, O and S. Some of the low mutagenic chemicals with higher electron richness include compounds **4**, **141** and **171**.

The second major sub-class having a major influence on mutagenicity is the presence of various ring systems. The descriptor nCIR represents the count of the circuits (larger loop around two or more rings) in a molecule, the circuit being a self-returning path, i.e., a walk with no repeated vertices other than its first and last ones (Mauri et al., 2006). The negative regression coefficient of this descriptor indicates that an increase in the number of circuits (which are mostly due to presence of more fused aromatic rings such as pentacyclo or hexacyclo aromatics) reduces the potential of the mutagenicity within nitro-aromatics as evidenced from the compounds **88** and **284**. On the other hand, a lower value of this descriptor (with a decrease in the count of fused rings) enhances mutagenicity of nitro-aromatics as evident from compounds **68** and **264**. Another variable NRS representing the number of ring system, which gives the proportion of cyclic content when compared with the whole molecule, had a negative correlation with mutagenicity of bacterial species as seen in Eq. (5)

$$NRS = (nBO - B_R) - (nSK - A_R) + 1 \quad (5)$$

Here, nBO and nSK are the total numbers of bonds and atoms in the H-depleted molecular graph, respectively; B_R and A_R are the number of atoms and bonds belonging to rings, respectively. Thus, we can infer that with an increase in the ring proportion against the entire molecule, there will be a considerable decrease in the mutagenicity value as seen in the compounds **220, 224** and **233** and vice versa in compounds **54, 183** and **238**. The third variable RCI denoting ring complexity index gives the ratio of summed ring size of all the single cycles, over the total number of atoms in the ring systems, and it is calculated from Eq. (6).

$$C_R = \frac{R}{A_R} \quad (6)$$

Here, R and A_R are the total ring size and total number of atoms belonging to any ring system, respectively. Due to the positive regression coefficient, the high ring complexity causes higher mutagenicity in nitro-aromatics. Some examples with higher ring complexity values showing higher mutagenic characteristics include compounds **111, 176** and **179** (presence of many monocyclic rings), whereas opposite was observed in case of compounds **216**, **223** and **226.**

The presence of higher lipophilic bulk mainly due to carbon skeleton as represented by X5A and X5AV (average valence connectivity index of order 5) exhibited a positive influence in enhancing mutagenicity. The higher values of these descriptors correspond to an increase in size and non-polar surface area of the molecule. Thus, the mutagenicity of the compounds may increase with an increase in the surface area and size of the molecules as shown by the compounds **48**, **280** and **290** (presence of bromine and chlorine atoms as well as polycyclic hydrocarbon in the structure tends to increase the lipophilicity). On the other hand, the opposite may happen with the reduction of size, surface area and lipophilicity of the molecules as can be seen from compounds **16, 74** and **181** (with simple structures like nitrobenzene, flourobenzene having a small size and low molecular bulk). Another variable F07[C−C] contributing negatively towards mutagenicity was found to be less significant in controlling toxicity of nitro-aromatics owing to its lower VIP score.

The last group of variables denotes the presence of more electronegative elements in a molecule such as nitrogen and chlorine where the former contributes positively towards mutagenicity while the latter has a negative correlation coefficient. The descriptor F09[N−N] stands for frequency of two nitrogen atoms at the topological distance 9 contributing to the electronegativity in the nitro-aromatics, capable of undergoing alkylation (Rosenkranz and Klopman, 1995). As per Eq. (1), the descriptor F09[N−N] is positively correlated with the mutagenicity of nitro-aromatics. The presence of more electronegative atoms in the

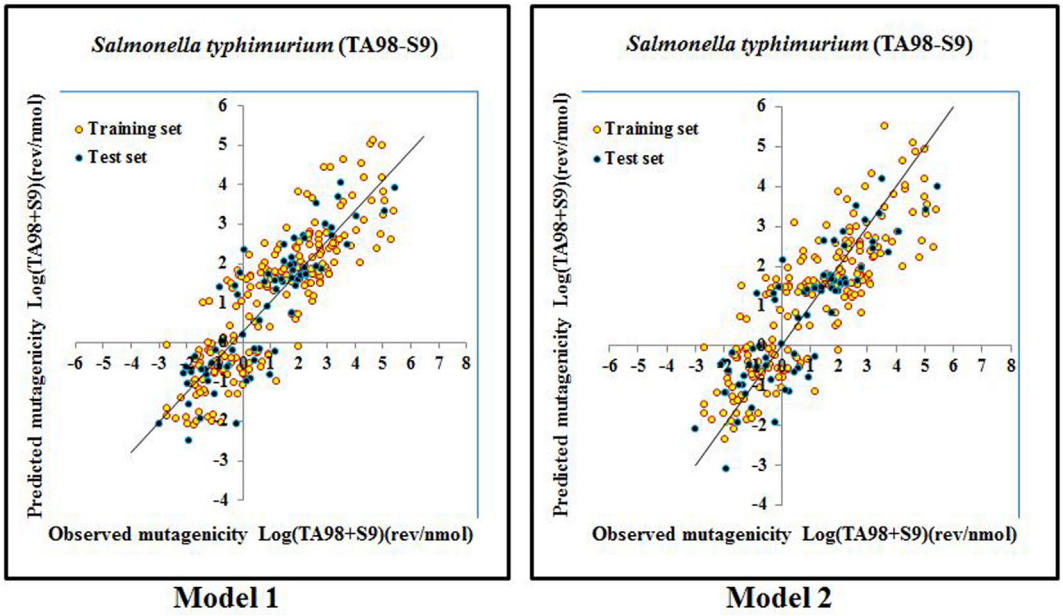

Fig. 4. Scatter plots of observed v/s predicted responses against model 1 and 2 of TA98-S9.

form of nitrogen increases the mutagenicity of nitro-aromatics as evident from several examples like **133**, **160** and **244** being more mutagenic than other molecules such as **138**, **142** and **143** which are devoid of this fragment. Another closely related variable B09[N—N] stands for the presence or absence of two nitrogen atoms at topological distance 9 also has a positive influence on mutagenicity of nitro-aromatics. The last variable appearing in the TA98-S9 model is B08[Cl—Cl] representing the presence or absence of two chlorine atoms located at the topological distance 8. Although this descriptor has a negative regression coefficient in the equation, the descriptor was found to be relatively insignificant as suggested by very low VIP score of 0.2 (found in three molecules i.e. **132**, **141** and **171**). The coefficient plot as shown in Fig. S1–S2 depicts (SI-2) the contribution of individual variables with reference to the algebraic sign. The chemical features enhancing or reducing mutagenicity of TA98-S9 endpoints are summarized in Fig. 3. The goodness of fit for the training set and corresponding predictive ability (test set) in the form of scatter plots are represented in Fig. 4.

3.2. Modeling of Salmonella typhimurium strain (TA98) with microsomal activation (S9) using 309 aromatic and hetero aromatic compounds

The dataset of 309 compounds was further split into a training set containing 232 compounds for model building and a test set of 77 molecules in order to validate the models. The final two models for TA98 + S9 also exhibited moderate robustness as both the models could explain nearly 70% of the training set variance (68% in terms of LOO) while for the test set 68–70% variance was predicted. For details of the metric values, one can see Eqs. (7)–(8) as given below. Both the final models were derived of two highly significant variables namely ETA_Epsilon_3 (a measure of electronegative atom count), a sub-type of extended topochemical indices and an *E*-state index namely SaaaC (sum of atom-type E-State of::C:) having VIP scores of 1.55–1.77 and 1.25–1.45, respectively. Apart from the descriptor B06[C—C], the remaining variables were considered relatively less significant as suggested by their respective low VIP scores. The loading plot of TA98 + S9 models highlights more towards impact of mutagenicity enhancing factor which are placed in close proximity to each other on co-ordinate 1. The Y-randomization plots gave intercepts for determinant coefficients of 0.0002 to −0.0009 and those for cross-validated determinant coefficients of −0.25 and −0.21 proving robustness and non-random nature of the models. Like the TA98-S9 models, intercorrelation among the descriptors were kept below 0.9 in order to avoid problems of over fitting. The variables appearing in the TA98 + S9 models were grouped into three subcategories based on their features. The first group consists of descriptors showing presence of more electronegative elements include ETA_Epsilon_3, B02[N—N], F02[N—N], nPyridines, nImidazoles and SaaNH, while the second group represented hydrophobic moieties such as C-034, C-027, B06[C—C], SaaaC and D/Dtr09. The remaining variable sssCH was grouped in the third group influencing branching in a molecule. The various qualitative plots generated by SIMCA tool are given in Fig. S3–S4 in Supplementary Materials (SI-2).

3.2.1. Mechanistic interpretation of TA98 + S9 models

$$Log(TA98 + S9)rev/nmol = -54.056 + 120.748 \times \text{ETA\_Epsilon\_3} + 0.224 \times \text{SaaaC} + 0.614 \times \text{B06[C - C]} + 0.010 \times \text{D/Dtr09} - 1.473 \times \text{C - 034} + 1.585 \times \text{F02[N - N]} - 0.743 \times \text{SsssCH} - 1.658 \times \text{nPyridines} \quad (7)$$

$n_{training} = 232$, LV = 4, $R^2 = 0.701$, $Q^2 = 0.680$, $r^2_{m(LOO)} = 0.566$, $\Delta r^2_{m(LOO)} = 0.200$, MAE = 0.767

$n_{test} = 77$, $R^2_{pred} = 0.696$, $Q^2_{F2} = 0.695$, $r^2_{m(test)} = 0.588$, $\Delta r^2_{m(test)} = 0.162$, MAE = 0.747

$$Log(TA98 + S9)rev/nmol = -98.228 + 222.822 \times \text{ETA\_Epsilon\_3} + 0.09347 \times \text{SaaaC} + 3.535 \times \text{B02[N - N]} + 1.43394 \times \text{nImidazoles} - 0.753 \times \text{SaaNH} - 1.364 \times \text{C - 027} - 1.270 \times \text{nPyridines} - 0.775 \times \text{SsssCH} \quad (8)$$

$n_{training} = 232$, LV = 5, $R^2 = 0.700$, $Q^2 = 0.683$, $r^2_{m(LOO)} = 0.567$, $\Delta r^2_{m(LOO)} = 0.220$, MAE = 0.766

$n_{test} = 77$, $R^2_{pred} = 0.677$, $Q^2_{F2} = 0.676$, $r^2_{m(test)} = 0.568$, $\Delta r^2_{m(test)} = 0.144$, MAE = 0.768

The presence of electronegative atom count due to ETA_Epsilon_3 was found to be the most crucial descriptor enhancing mutagenicity.

The descriptor ETA_Epsilon_3 is a second generation extended topochemical variable (see Eq. (9)); it gives the summation of epsilon (ε) values relative to the total number of atoms including hydrogen in the connected molecular graph of the reference alkane.

$$\varepsilon_3 = \frac{[\sum \varepsilon]_R}{N_R} \tag{9}$$

Here, $\Sigma\varepsilon$ and N are summation of electronegative atoms, heteroatoms and multiple (double or triple) bonds replaced by carbon and single bonds and total number of atoms including hydrogen respectively in the molecular graph of the original structure. Here, R denotes the parent reference alkane having no functional moiety present within the molecule. The positive regression coefficient of ETA_Epsilon_3 suggests that with an increase in the electronegative element content (mainly nitrogen), the tendency of molecules to behave as potent mutagenic entity is enhanced in aromatics and hetero-aromatics. Other variables denoting the presence of high electronegative element content in the aromatic or hetero-aromatic amine datasets include a functional group count descriptor nImidazoles (number of imidazoles), 2D atom pair descriptors F02[N−N] (frequency of two nitrogens at topological distance 2) and B02[N−N] (presence or absence of the 2 nitrogen atoms at topological distance 2); all three of them were positively correlated with the mutagenicity of the studied chemicals. Some examples with higher electronegative element content (mainly nitrogen) include **115S, 116S** and **118S** whereas an inverse effect was seen in compounds having lower nitrogen content, for example **105S** and **288S**. The last variable denoting the electronegative count in a molecule was SaaNH (−-NH—moiety, where (−-) denotes aromatic bonds). The last fragment (aromatic bond-NH-aromatic bond) exerted a very little contribution to enhance the mutagenicity of aromatic and hetero aromatic compounds (low VIP score). A lot of such fragments were the parts of more toxic imidazole ring, and thus it can be inferred that the imidazole moiety plays a crucial role in regulating the mutagenicity of aromatic and hetero aromatic compounds. Some examples of compounds with higher electronegative content and enhanced mutagenicity include **115S**, **118S** and **119S,** whereas the reverse was seen in compounds **81S, 150S** and **265S**. With imidazole enhancing mutagenic potency of aromatic and hetero-aromatic compounds, a reverse was observed with the presence of pyridines. Despite it showing a negative regression coefficient, we cannot confidently comment on the mutagenic potency of pyridines as the level of significance of this variable is very low with VIP score of < 0.5.

The second subgroup represents the lipophilic bulk of the organic chemicals. Lipophilicity being an important criterion having a positive influence on mutagenicity, it is represented by several attributes in the models, the most important being an atom centered fragment SaaaC which represents sum of aromatic carbons ((−-C(−-)–), where "–"represents an aromatic bond). The positive coefficient of the SaaaC denotes that with an increase in the number of aromatic rings surrounded by carbons enhances the hydrophobicity thereby enhancing the mutagenicity values, as observed in the compounds **88S**, **177S** and **205S** having higher mutagenicity values of **3.8, 3.23** and **3.5**, respectively. Conversely, with a decrease in the number of aromatic rings surrounding by carbons decreases the mutagenic potential of the molecule as seen in compounds **15S**, **21S** and **298S** with mutagenicity values of −3, −3 and −2.7 respectively. The other lipophilicity enhancing variables appearing in the model was B06[C−C] (presence or absence of two carbons at topological distance 6) and D/Dtr09 (distance/detour ring index of order 9), both of these variables represent a larger chain length in the carbon skeleton. Higher values of these descriptors tend to increase the non-polar surface area and bulkiness of the molecule. It was observed that the mutagenicity of the compounds increases with an increase in non-polar surface area as evident from the molecules **174S**, **177S** and **220S** where most of them contained a flouranthane moiety having a larger non-polar surface. Similarly with a reduced non-polar surface area as seen in compounds **28S, 48S** and **304S** mutagenicity decreases. The remaining two variables enhancing lipophilicity include C-034 (R–CR..X) and C-027 (R–CH—X) (where R is any group linked through carbon; X is any electronegative atom (O, N, S, P, Se, halogens); − is an aromatic bond as in benzene or delocalized bonds such as the N,O bond in a nitro group; .. denotes aromatic single bonds). These two variables represent very less number of molecules in spite of having a close relation with ETA_epsilon_3 descriptor where the latter has a larger influence in controlling mutagenicity of aromatic and hetero-aromatics chemicals for the same compounds as seen in **115S** and **118S**.

The last variable SsssCH represents the presence of tertiary carbon atoms and denotes branching in the molecule. The descriptor is an atom type *E*-state index calculated from the sum of E-states of > CH– fragment. This descriptor contributed negatively towards the mutagenicity of the aromatic and hetero aromatic compounds. Thus, we can infer that the highly branched organic chemicals tend to have lower potential to cause mutagenicity in *Salmonella typhimurium* when compared to the less branched molecules. Some of the more branched and less mutagenic compounds include **10S**, **84S** and **290S**, while the reverse was seen with compounds **88S**, **90S** and **102S**. The chemical features enhancing or reducing mutagenicity of TA98-S9 endpoints are summarized in Fig. 5**.** The scatter plots showed that the points were close to the line of fit for both TA98 + S9 models (see Fig. 6).

3.3. Modeling mutagens with molecular fragments derived from SiRMS software

In many cases, the conventional molecular descriptors fail to provide the definite features actually responsible for the desired response. To obviate such deficiency, the authors have additionally incorporated simplex molecular variables in order to identify the actual fragments present within the molecules with contribution to bacterial mutagenicity. The SiRMS variables constitute a group of 1D-4D tetratomic fragments; however, to avoid the complications of energy minimizations needed for 3D and 4D fragment computation, only 2D descriptors were used in the present study. The final selected models with fragments were slightly better in predictivity for the test sets when compared to the previous models for the respective endpoints (See Eqs. (10)–(13)).

3.3.1. Fragmental QSAR models against TA98-S9

$$\begin{aligned} Log(TA98-S9)rev/nmol = {} & -8.50 + 3.28 \times RDCHI \\ & + 0.0543 \times Fr5(d\_a)/I\_I\_I\_I\_I \\ & /1\_2s,\ 2\_4a,\ 3\_5a,\ 4\_5a/(Box2) \\ & + 0.0337 \times S\_A(lip)/B\_C\_C\_C/1\_2a,\ 3\_4a \\ & /3(Box1) + 0.264 \times Fr5(att)/E\_E\_E\_E\_E \\ & /1\_2s,\ 1\_3s,\ 2\_4a,\ 3\_5a/(Box3) \\ & + 1.15 \times F09[N-N] - 0.841 \times NRS \\ & - 0.127 \times F07[C-C] \\ & - 0.0126 \times S\_A(rep)/A\_B\_B\_B/1\_2s,\ 3\_4a \\ & /3(Box4) \end{aligned}$$

$n_{training} = 219$, LV = 5, $R^2 = 0.738$, $Q^2 = 0.710$, $r^2_{m(LOO)} = 0.604$, $\Delta r^2_{m(LOO)} = 0.187$, MAE = 0.847

$n_{test} = 72$, $R^2_{pred} = 0.747$, $Q^2_{F2} = 0.739$, $r^2_{m(test)} = 0.640$, $\Delta r^2_{m(test)} = 0.176$, MAE = 0.753 (10)

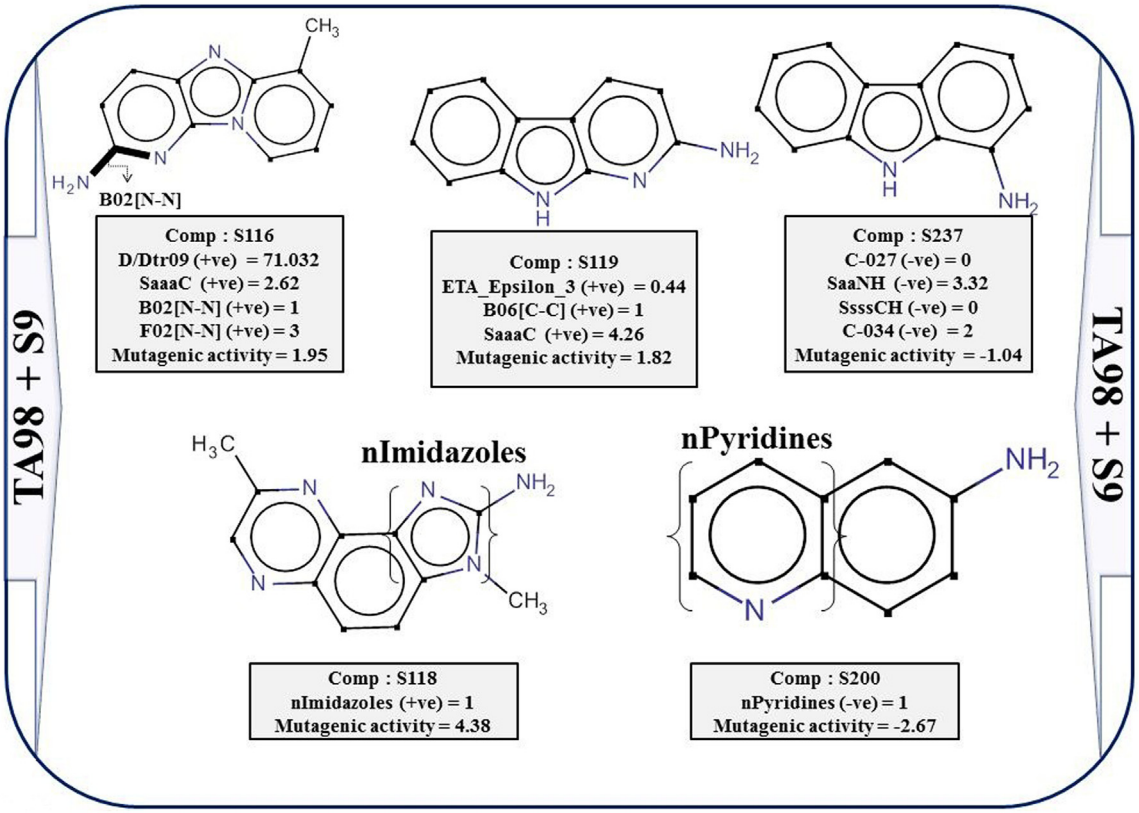

Fig. 5. Molecular features enhancing or reducing mutagenicity of TA98 + S9.

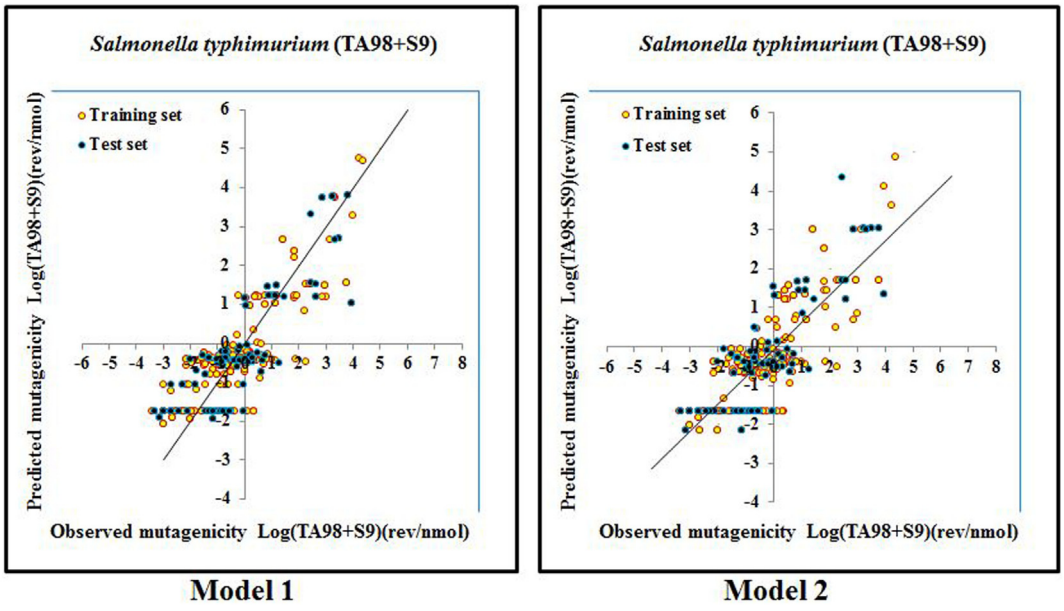

Fig. 6. Scatter plots of observed v/s predicted responses against model 1 and 2 of TA98 + S9.

$$Log(TA98 - S9)rev/nmol = -7.66 + 2.97 \times RDCHI + 1.33 \times F09[N - N] + 0.51 \times S\_A(chg)/B\_B\_B\_C /1\_2s, 2\_3a, 3\_4a/6(Box5) + 0.0821 \times Fr5(d\_a)/I\_I\_I\_I\_I /1\_2s, 2\_4a, 3\_5a, 4\_5a/(Box2) + 0.0319 \times S\_A(lip)/B\_C\_C\_C/1\_2a, 3\_4a /3(Box1) - 0.0204 \times S\_A(rep)/A\_B\_B\_B /1\_2s, 3\_4a/3 - 0.787 \times Fr5(d\_a)/A\_I\_I\_I\_I /1\_3d, 2\_4s, 3\_5s, 4\_5d/(Box6) - 1.061 \times NRS$$

$n_{training} = 219$, LV = 6, $R^2 = 0.739$, $Q^2 = 0.709$, $r^2_{m(LOO)} = 0.603$, $\Delta r^2_{m(LOO)} = 0.187$, MAE = 0.825

$n_{test} = 72$, $R^2_{pred} = 0.757$, $Q^2_{F2} = 0.749$, $r^2_{m(test)} = 0.650$, $\Delta r^2_{m(test)} = 0.195$, MAE = 0.750 (11)

The TA98-S9 models with fragmental variables showed a slightly better robustness having six additional variables in addition to the previous descriptors. The fragmental QSAR models identified four features correlated positively with the bacterial mutagenicity (see Box 1–3 and 5 of Fig. 7) while the remaining two fragments (see Box 4, 6 of Fig. 7) exerted a negative influence in controlling mutagenicity of nitro aromatics. Another major notable point here is that the positively correlated features were rich in aromatic bonds along with nitrogen of the nitro group. The fragments of TA98-S9 models mainly hinted towards lipophilic and electronegative group dependent mutagenicity of nitro aromatic chemicals. The scatter plots for the TA98-S9 SiRMS models are given in Fig. 8.

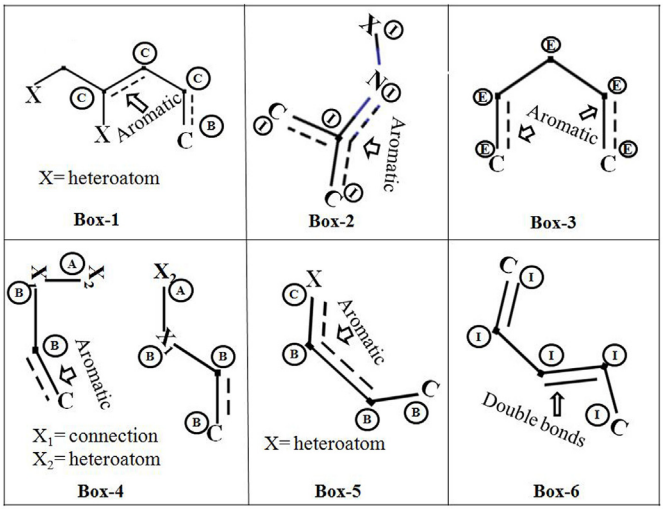

Fig. 7. Simplex representation of molecular structures (SiRMS) fragments appearing in both of the TA98-S9 model.

3.3.2. Fragmental QSAR models against TA98 + S9

$$Log(TA98 + S9)rev/nmol = =-3.035 + 0.229 \times Fr3(lip)/C\_C\_C /1\_3a, 2\_3a/(Box5) + 0.32516 \times Fr3(lip)/B\_C\_C/1\_3a, 2\_3s /(Box1) + 0.908 \times Fr5(elm)/C\_C\_C\_C\_C /1\_2s, 2\_3a, 3\_4s, 4\_5a/(Box7) + 0.46272 \times S\_A(chg)/A\_B\_D\_D /1\_3a, 2\_4a/3(Box3) - 1.659 \times Fr5(chg)/B\_B\_C\_C\_D /1\_4a, 2\_3a, 2\_4a, 4\_5a/(Box8) + 1.24174 \times Fr3(elm)/C\_N\_N/1\_2s, 1\_3a /(Box2) + 0.816 \times Fr5(lip)/B\_B\_B\_B\_C /1\_2s, 2\_5a, 3\_4a, 4\_5a/(Box6) - 0.052 \times Fr3(chg)/B\_C\_C/1\_2s, 1\_3s /(Box4)$$

$n_{training} = 232$, LV = 4, $R^2 = 0.726$, $Q^2 = 0.704$, $r^2_{m(LOO)} = 0.596$, $\Delta r^2_{m(LOO)} = 0.197$, MAE = 0.733

$n_{test} = 77$, $R^2_{pred} = 0.760$, $Q^2_{F2} = 0.754$, $r^2_{m(test)} = 0.663$, $\Delta r^2_{m(test)} = 0.147$, MAE = 0.485 (12)

$$Log(TA98 + S9)rev/nmol = -3.170 + 0.240 \times Fr3(lip)/C\_C\_C /1\_3a, 2\_3a/(Box5) + 0.328 \times Fr3(lip)/B\_C\_C/1\_3a, 2\_3s /(Box1) + 0.458 \times S\_A(chg)/A\_B\_D\_D /1\_3a, 2\_4a/3(Box3) + 0.822 \times Fr5(elm)/C\_C\_C\_C\_C /1\_2s, 2\_3a, 3\_4s, 4\_5a/(Box7) - 1.824 \times Fr5(chg)/B\_B\_C\_C\_D /1\_4a, 2\_3a, 2\_4a, 4\_5a/(Box8) + 1.379 \times Fr3(elm)/C\_N\_N/1\_2s, 1\_3a /(Box2) + 0.853 \times Fr5(lip)/B\_B\_B\_B\_C /1\_2s, 2\_5a, 3\_4a, 4\_5a/(Box6) - 0.509 \times Fr5(d\_a)/D\_I\_I\_I\_I /1\_2s, 2\_5a, 3\_5a, 4\_5a/(Box9)$$

$n_{training} = 232$, LV = 6, $R^2 = 0.725$, $Q^2 = 0.702$, $r^2_{m(LOO)} = 0.594$, $\Delta r^2_{m(LOO)} = 0.194$, MAE = 0.731

$n_{test} = 77$, $R^2_{pred} = 0.760$, $Q^2_{F2} = 0.760$, $r^2_{m(test)} = 0.663$, $\Delta r^2_{m(test)} = 0.157$, MAE = 0.496 (13)

The performance of TA98 + S9 fragmental QSAR models were superior when compared to the previous two models with conventional descriptors providing better robustness as well as predictivity. Both the models solely consist of fragmental variables with nine fragments in total. Six (see Box 2–7 of Fig. 9) out of nine variables exerted positive contributions towards mutagenicity while the remaining three variables (see Box 1, 8 and 9 of Fig. 9) exerted a negative influence on bacterial mutagenicity. Like the TA98-S9 models, lipophilicity proved to be a major contributing feature for mutagenicity as evident from its repetition in several positively correlated fragments in the TA98 + S9 models. Among the negatively correlated features was branching, as seen in Box 9 of Fig. 9. Additionally, plots of observed against predicted response for theTA98 + S9 fragment models are shown in Fig. 10.

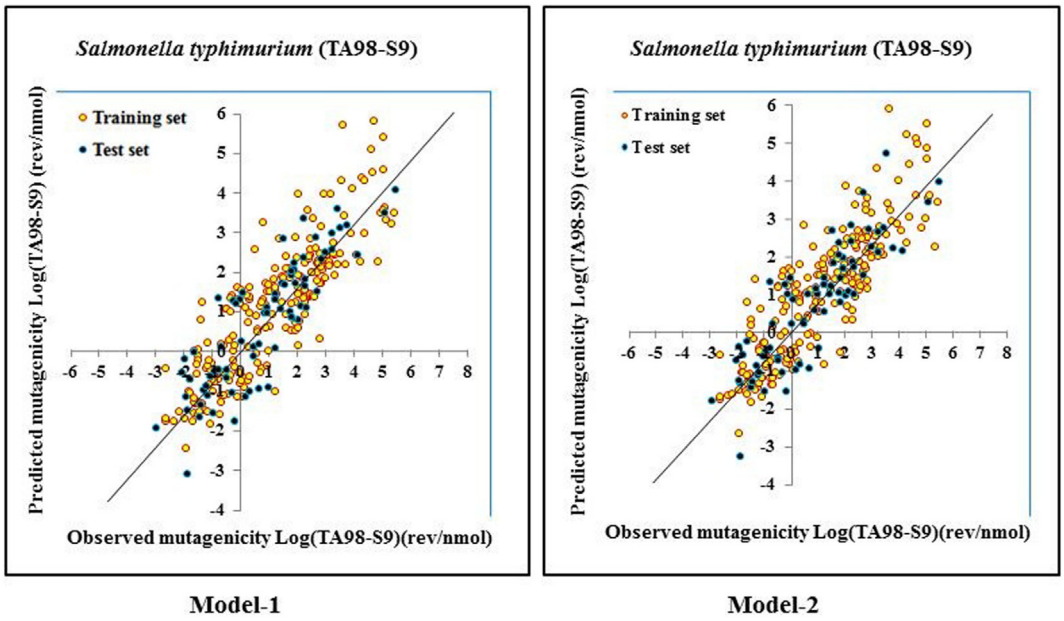

Fig. 8. Scatter plots of observed v/s predicted responses against models 1 and 2 of TA98-S9 fragment models.

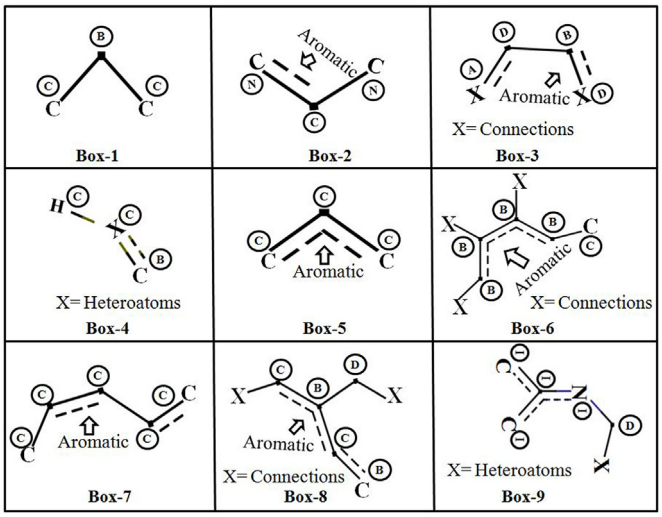

Fig. 9. Simplex representation of molecular structures (SiRMS) fragments appearing in both of the TA98 + S9 model.

3.4. Summary of the overall finding of individual QSAR models: A mechanistic view

To summarize, the authors have assembled various model features into four different groups based on their physicochemical attributes responsible for enhancing or reducing mutagenicity. All the chemical features obtained in the process can simply be grouped into four classes: (1) effect of lipophilicity on mutagenicity, (2) mutagenicity due to electronegative atoms such as nitrogen, (3) mutagenicity due to unsaturation, and finally (4) effect of branching on mutagenicity.

3.4.1. Effect of non-polar moieties on mutagenicity

The lipophilicity of polycyclic, aromatic and planar chemical structures enables them to readily penetrate cellular membranes (Yu et al., 2016). Furthermore, these moieties undergo metabolism (phase-I and II), hence, these molecules are converted into more water-soluble entities in order to be removed from the body easily. However, these chemical structures can also be converted to more mutagenic or carcinogenic metabolites because of their heavy lipophilic nature. Some of the highly contributing lipophilicity variables such as higher connectivity indices (X5Av, X5A), atom pair indices (B08[Cl—Cl] and F07[C—C]) and fragmental variables (Box 1 and 4 of Fig. 7 and Box 4–9 in Fig. 9) contributed to the mutagenicity of the compounds of the studied dataset. Since these molecules are highly lipophilic in nature, they tend to promote fluidization of phospholipid bilayer of cell membrane in order to facilitate more and more accumulation within the cell. This, in turn, it can lead to the formation of several intermediates. Finally, there is a high possibility of these intermediates entering into redox cycle thereby causing oxidative stress and inducing the production of reactive radical cations inside the cell (Yu et al., 2016). These radical cations have potential to form covalent bonds with the exocyclic amino group of the phosphodiester bonds, a leading cause for mutations leading to genotoxicity. Fig. 11 schematically demonstrates the probable lipophilicity induced mutagenicity.

3.4.2. Effect of electronegativity on mutagenicity

Electronegative features present in the planar polycyclic aromatic molecules intercalate into DNA in the space between two adjacent base pairs via nucleophilic aromatic substitution. This might induce the changes in DNA structure like double helix unwinding and elongation of DNA strands. Several such features were predominant in all the developed QSAR models against both the endpoints. Some of the most predominant descriptors correlating electronegativity against mutagenicity include ETA indices like ETA_epsilon_3 and ETA_Beta_ns, atom pair variables (F02[N—N, F09[N—N] and B02[N—N]), presence of imidazole ring and several simplex fragments (see Box 2, 3 and 5 in Fig. 7 and Box 2 and 3 of Fig. 9**).** These structural modifications may lead to mutagenicity because of the sequential alterations in the DNA strands; furthermore, the DNA gyrase may not distinguish the actual DNA and mutated DNA leading to more lethal conditions. Besides mutations, these features can also lead to retardation or inhibition of transcription and replication. The process is summarized in Fig. 12**.**

3.4.3. Effect of unsaturation on mutagenicity

There is also a sufficient number of features present within the QSAR models possibly hinting towards unsaturation leading to mutations. Some of the important descriptors include ETA indices like ETA_Beta_ns and ETA_dBeta, connectivity indices like RDCHI and several molecular fragments such as Box 2 and 6 of Fig. 7 and Box 3 of Fig. 9**.** Like lipophilicity, unsaturation is also capable of augmenting

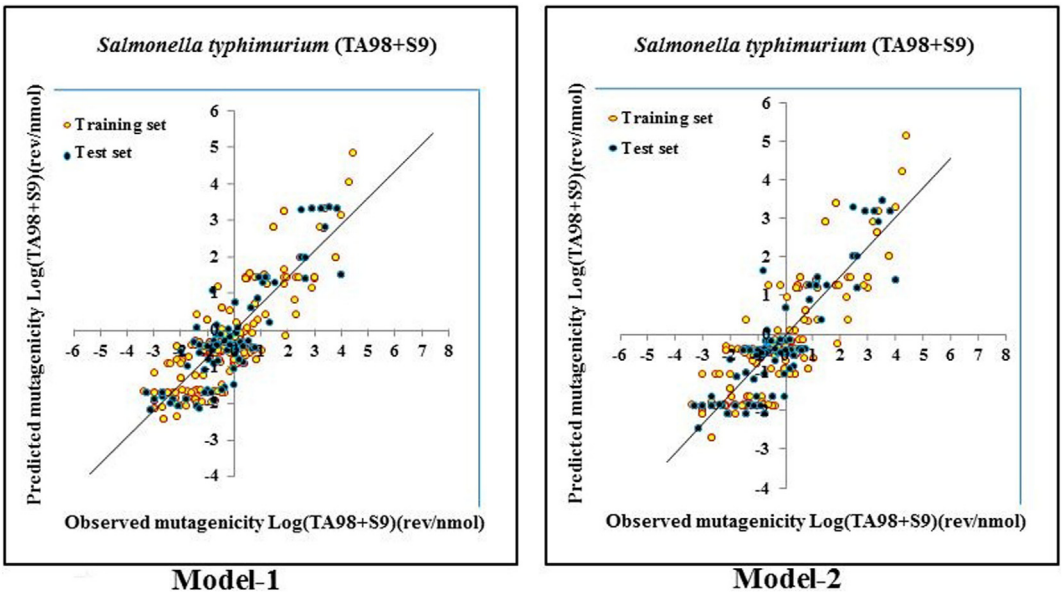

Fig. 10. Scatter plots of observed v/s predicted responses against models 1 and 2 of TA98 + S9 fragment models.

membrane fluidization which results in more invasions of the organic chemicals within the cell. The process is followed by metabolism giving reactive intermediates leading to adduct formation as shown in Fig. 13. Two types of adducts are reported to form with these reactive intermediates named "Bay region adducts" and "Fjord region adducts" where the former is less reactive than the other (Munoz and Albores, 2011; Yu et al., 2016). These adducts go on to form the covalent bonds with the exocyclic amino group of the adenine and guanine as shown in Fig. 13. To be specific, "Bay region adducts" form covalent bonds with guanine, whereas "Fjord region adducts" form covalent bonds with adenine (Munoz and Albores, 2011; Yu et al., 2016).

3.4.4. *Effect of branching on mutagenicity*

The last effective attribute having a negative influence on mutagenicity was found to be enhanced branching within the molecules. Some of variables influencing more branching in the molecules include SsssCH and several fragments as shown in Box 1, 2, 8 and 9 of Fig. 9**.** In general, highly branched organic chemicals show lower toxicity. The reason of their less toxic behaviour could lie in their physical properties which alters the lipophilic bulk and increase of the hydrophilic nature. This is followed by the cascade of membrane fluidization which is sufficiently hampered due to more hydrophilic nature of the entity thus ending the mutagenic pathway as explained in lipophilic section. For ease of understanding, please see Fig. 14.

3.5. *Applicability domain analysis*

The applicability domains (ADs) of individual models were checked using DModX approach embedded in Simca-P (version10.0), a recommended method for PLS models. The AD was checked at 99% confidence level with D-critical limit of 0.01. All the developed QSAR models could cover a minimum of 95% hypothetical AD space with

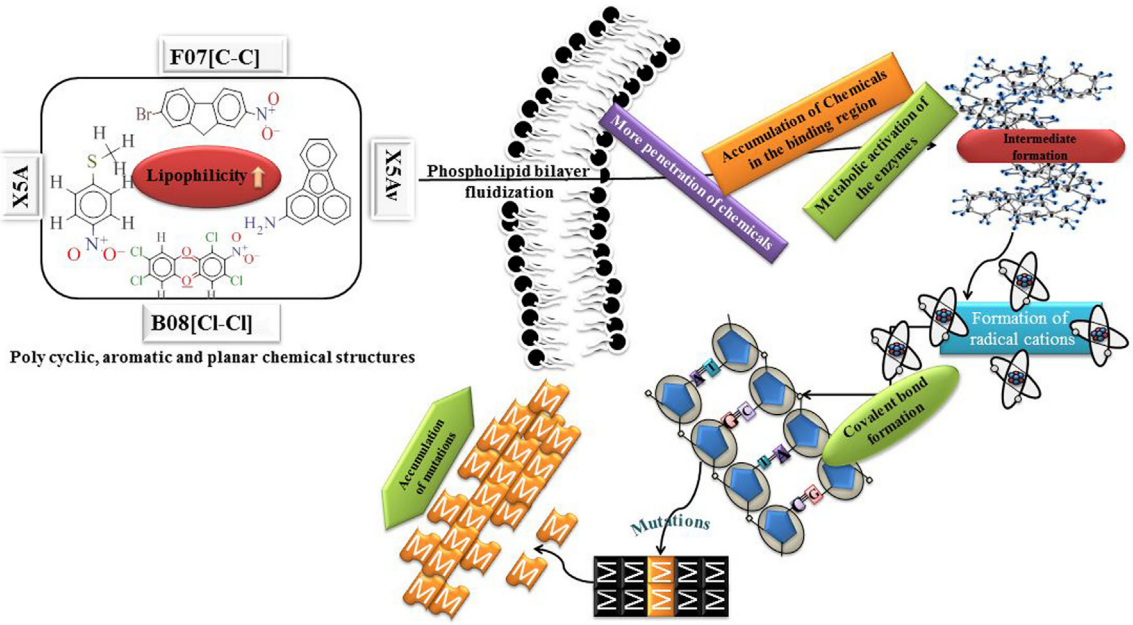

Fig. 11. Effect of lipophilic moieties on mutagenicity present in the organic chemicals.

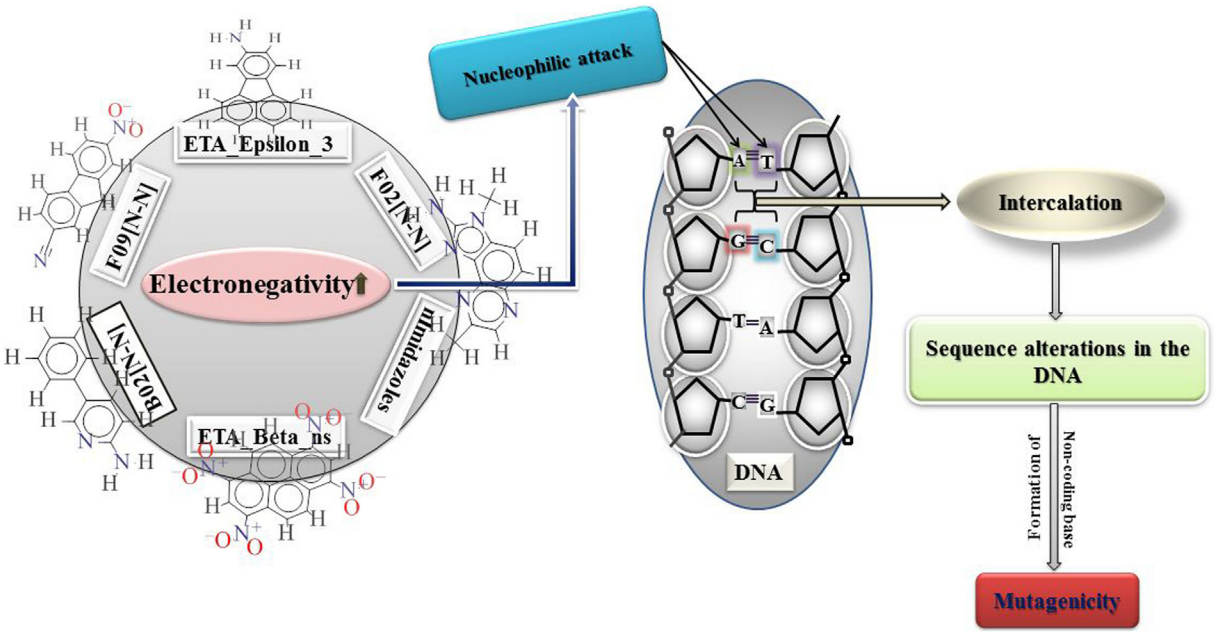

Fig. 12. Effect of electronegative moieties on mutagenicity present in the organic chemicals.

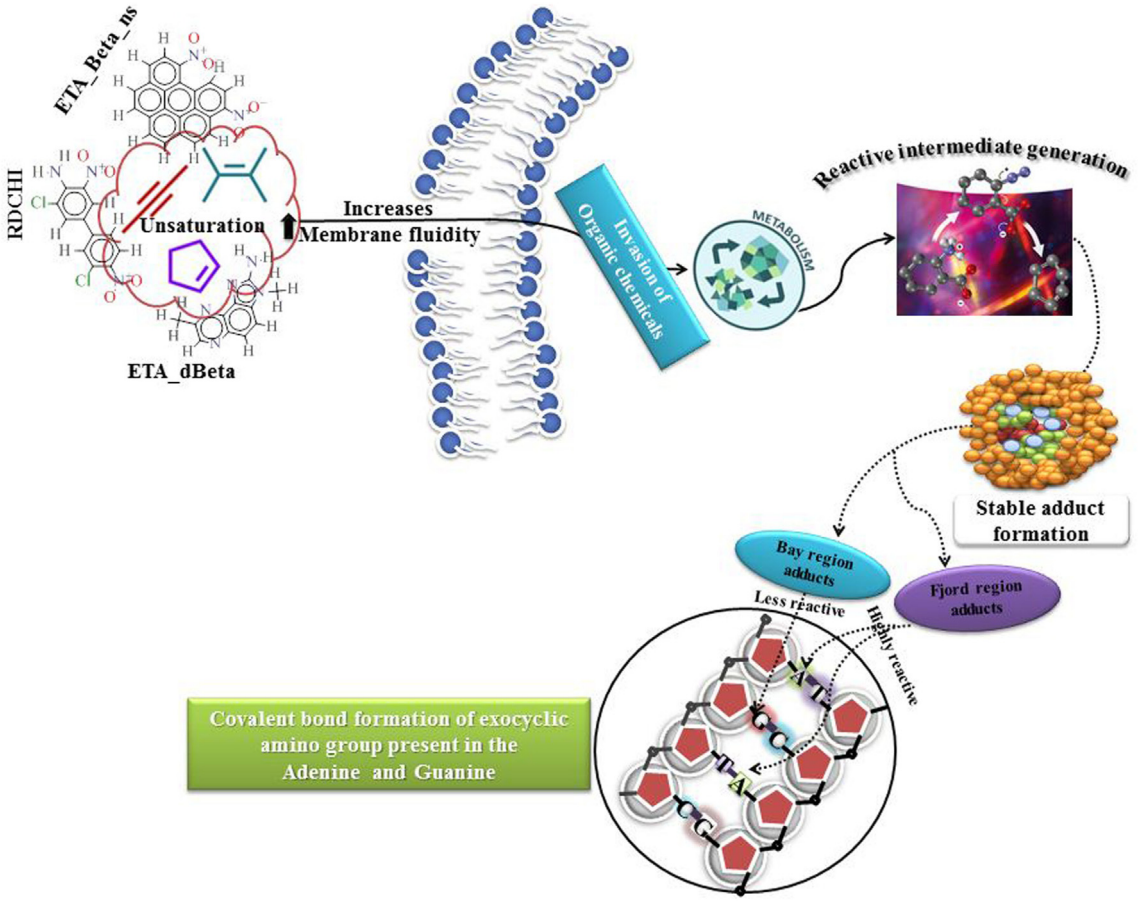

Fig. 13. Effect of unsaturation on mutagenicity present in the organic chemicals.

TA98-S9 models covering maximum of 96–99% of chemical space. Although a number of outliers were identified in the training set and a number of chemicals were outside the domain in the test set, the outliers were retained in the final models since the developed models could predict their mutagenicity with absolute error of less than two (< 2) log units. For the details of AD analysis, please see Fig. S5–8 in SI-2.

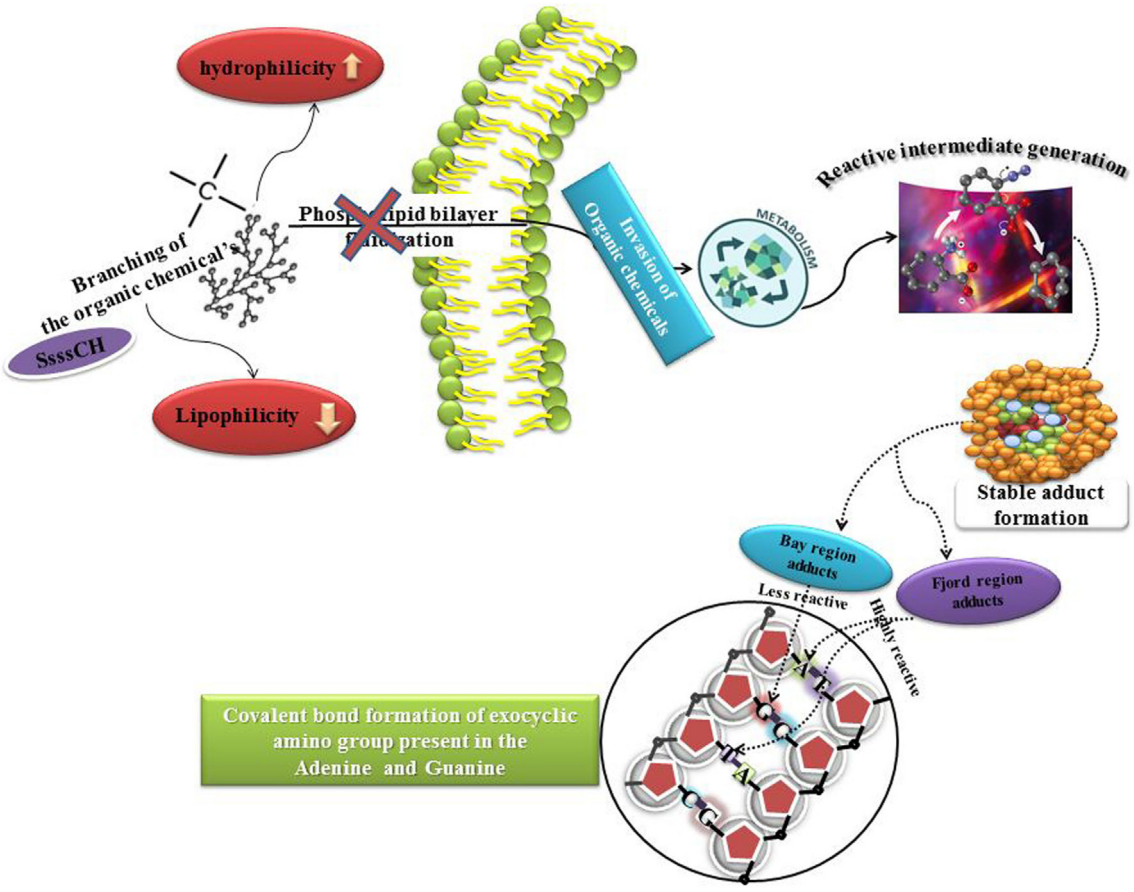

Fig. 14. Effect of unsaturation on mutagenicity present in the organic chemicals.

3.6. Comparison of present models with existing QSAR models on mutagenicity

The application of novel QSAR models cannot be justified unless compared with practicing standards. Thus, we have tried to give a brief comparison with some of the known models already developed in order to study the probable mode of mutagenicity of selective organic chemicals against *Salmonella typhimurium*. The authors make a point here that the present manuscript outperforms all the previous linear QSAR models in terms of both statistical quality as well as domain of applicability. For a detailed comparison, please see Table 2.

4. Conclusion

The present paper proposes sufficiently robust QSAR models employing simple 2D variables generated from Dragon and PaDEL-descriptor softwares against the TA98-S9 and TA98 + S9 endpoints. Additionally, more predictive QSAR models consisting of simplex fragmental variables were also proposed. The data division, model development and AD estimation were performed following the strict guidelines for QSAR model validation. The study focuses mainly on the mechanistic approach of QSAR application by providing a detailed analysis of probable cause of mutagenicity by simply taking into

Table 2
The brief comparison of already existing linear 2D QSAR models with our models on mutagenicity of selected organic chemicals against *S. typhimurium*.

Sr no.	Working group	Type of chemicals	Endpoint(S)	Dataset size	R^2	Q^2	Q^2F_1	Remarks[a]
1	Current manuscript	Nitro aromatics	TA98-S9	291	0.73	0.71	0.76	Model without fragments
		Nitro aromatics	**TA98-S9**	**291**	**0.74**	**0.71**	**0.76**	**Model with fragments**
		Amino aromatics	TA98 + S9	309	0.70	0.68	0.70	Model without fragments
		Amino aromatics	**TA98 + S9**	**309**	**0.73**	**0.70**	**0.76**	**Model with fragments**
2	Ding et al. (2017)	Nitro aromatics	TA98-S9	282	0.72	0.69	0.70	Complicated descriptors used
3	Bhat et al. (2005)	Amino aromatics	TA98 + S9	181	0.67	0.64	0.65	Multiple linear regression model
4	Leong et al. (2010)	Amino aromatics	TA98 + S9	122	0.35	–	0.77	No valid PLS models
5	Garg et al. (2002)	Amino aromatics	TA98 + S9	43	0.85	–	–	Very small dataset, no validation set
6	Gadaleta et al. (2017)	Azoaromatics	Non specific	354	–	–	–	Classification model
7	Pasha et al. (2008)	Azoaromatics	TA98 + S9	43	0.95	0.51	0.65	3D model, non-robust
8	Ren (2003)	Phenols	*Tetrahymena pyriformis*	200	0.65	–	–	No validation set, use of more complex descriptors
9	Gramatica et al. (2007)	nitro-PAHs[b]	TA100-S9	48	0.88	0.86	0.75	Very small dataset and used of more complex descriptors
10	Abbasitabar and Zare-Shahabadi (2017)	Phenols	*Tetrahymena pyriformis*	206	0.72	0.69	0.69	Use of more complex descriptors

[a] The best models are highlighted with **bold.**
[b] Poly aromatic hydrocarbons.

account QSAR equation (OECD principle 5 for model interpretations). From the statistical point of view, the models were validated using some stringent metrics such as r_m^2 and MAE. Finally, the established models were compared with many already existing QSAR models on mutagenicity against *S. typhimurium* and related species. The obtained QSAR model outperforms almost all the existing linear QSAR models for at least some of the validation parameters. The authors strongly believe that the performed methodology will greatly help various groups of researchers working in the field of mutagenicity of synthetic organic chemicals. Lastly, the developed models can also be used to screen untested or unknown or not yet synthesized chemicals based on their acute mutagenic potential.

Author contributions

All the coauthors have equally contributed to this work. The authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2020.104768.

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Original Research Article

Evalution Of Nootropic Activity Of Methanolic Extract Of Grangea Maderaspatna In Wister Albino Rats.

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ABSTRACT:
The term "dementia" refers to a group of symptoms that severely impair memory, reasoning, and social functioning. While many illnesses can cause dementia, Alzheimer's disease is the most common cause of progressive dementia.Over 55 million individuals worldwide already have dementia, and there are almost 10 million new cases diagnosed each year. Beta amyloid deposition and neuro-fibrillary tangles, which induce the loss of synapses and neurons and result in gross atrophy of the affected parts of the brain, usually starting in the mesial temporal lobe, are the underlying pathophysiological causes. In the current investigation the efficacy of the methanolic extract of Grangea maderaspatana [GM] was tested in wistar albino rats against scopolamine (4mg/kg) induced Alzheimer's disease. A dose of 200 mg/kg of piracetam is used as a standard.The doses for GM were 250 mg/kg and 500 mg/kg. The nootropic activity was evaluated using the elevated plus maze model, novel object recognition test, morris water maze, and Y-Maze, as well as biochemical tests such as Acetylcholinesterase activity, brain reduced glutathione levels, melanoldehyde, and catalase activity were measured in order to assess the level of oxidative stress. During behavioural studies, Scopolamine administration reduces learning and memory enhancement. A significant reduction in time spent in the preferred arm of the Y-maze, escape latency, time spent exploring the novel object, and discrimination index of the familiar object was also observed. Acetylcholinesterase activity increased in rats, indicating a significant impairment of the central cholinergic system. Grangea maderaspatana (250 and 500 mg/kg orally administered) significantly improved rat memory in the y-maze, elevated plus maze, and novel object recognition tests, and also improved mouse locomotion in the open field. Treatment with a methanolic extract of Grangea maderaspatana also reduces brain oxidative stress and AChE activity. We find that treatment of Grangea maderaspatana [GM] methanolic extract improves rat memory by lowering AChE activity and exhibiting antioxidant effects. The presence of phenolic chemicals and flavonoids was confirmed by the phythochemical screening of the GM, making it appear to be an effective source for improving memory and learning.

KEYWORDS: Alzheimer's disease, Dementia, Grangea maderaspatana, Scopolamine, Melonaldehyde, Novel object recognition test.

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INTRODUCTION :

Neurodegenerative disorders cause nerve cell degeneration and impair the nervous system's normal functioning. and may have an impact on a person's ability to move about, speak, perceive, think, and remember during their lifetime depending on the regions affected[1]. Alzheimer's disease is a type of dementia that worsens over time and has an impact on memory, thinking, and behaviour. Clinically, it manifests as memory loss, an inability to learn new things and recollect the past, difficulty thinking, etc. According to WHO, According to brain histology, Alzheimer's disease is the sixth leading cause of death in the United States, and approximately 450 million people worldwide suffer from a mental or behavioural problem.[2]. Neuronal loss is one of the fundamental neuropathological factors underlying Alzheimer's symptoms, and when the disease is examined under a microscope, senile plaques and neurofibrillary tangles (NFTs) are the most visible features.. A number of hypothesised processes have been proposed to better understand the pathophysiology of Alzheimer's disease, including cholinergic dysfunction, oxidative damage, beta amyloid toxicity, tau protein hyperphosphorylation, and senile plaque inflammation[3][4]. The loss of cholinergic neurons, along with a decline in their production, causes the learning and memory failure that is characteristic of Alzheimer's disease. The cholinergic system, which is made up of cholinergic neurotransmitters, is crucial for memory processing[5].Nootropic drugs, which are used to improve memory, work by specifically improving the integrative function of the central nervous system, which has an impact on cognition, learning, and memory[6].

The Indian medical system places a strong emphasis on using herbs, nutraceuticals, and lifestyle modifications to treat age-related neurodegenerative illnesses[7].Traditional medicinal plants' pharmacological and therapeutic effects have been linked to a variety of Chemical components isolated from crude extracts; in particular, active compounds with antioxidant activity have been linked to a significant role in a number of neurodegenerative diseases[8].

The current treatment strategy focuses on inhibiting the acetylcholinesterase (AChE) enzyme with drugs such as donepezil, gallantamine, rivastigmine, and memantine [9].Although these medications have a number of limitations that prevent them from being viable pharmacological candidates for the treatment of AD, including low efficacy, poor bioavailability, unfavourable peripheral cholinergic side effects, restricted therapeutic ranges, and hepatotoxicity [10].

In Indian traditional medicine, the medicinal plant Granger Maderaspatna Pior is frequently used to cure a variety of illnesses. GM, also known as madras carpet, is a weed that thrives in sandy wastelands and subtropical regions of Africa, Asia, and Baluchistan. Other important chemical elements of plants include steroidal compounds, hardwickic acid, auranamide, penta and haxamethoxyflavones. The root is diuretic, anthelmentic, astringent to the intestines, and an appetiser. The herb is said to have astringent and anti-implantation properties..[11],[12],[13].

MATERIALS AND METHODS

Drugs and chemicals:

Piracetam **(**sun pharamaceuticals), Scopolamine, Methanol, Carboxy methyl cellulose.

Animals:

Wistar albino rats of either sex, weighing 150–200g, were used for the screening. They were obtained from the animal house of the Dr. K. V. Subba Reddy Institute of Pharmacy, Kurnool, and were maintained under standard laboratory conditions (temperature 23–20 c, relative humidity 55–10%, and 12-hour light–dark cycle). Throughout the research period, animals were fed with the regular laboratory food and water at their discretion. The experiments were carried out after the IAEC of Dr.K.V.Subba Reddy of the Institute of Pharmacy approved the experimental protocol.

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Plant material:
G. Maderaspatana plant materials (whole plant) were collected from a field near Nandanapalle village in Kurnool district, Andhra Pradesh. And authenticated by Dr.K.V.Madhusudhan, Dept of Botany, Silver Jubilee Government Degree College for Men, Kurnool. The plant was collected in December and shade dried at room temperature before being subjected to extraction procedures.

Preparation of extract:
Methanol was utilised in the extraction procedure since almost all of the Grangea maderaspatana whole plant's constituents are soluble in it. A free-flowing powder was created by electrically grinding the entire Grangea maderaspatana plant after it had been shade-dried. This powder was prepared by extracting dehydrated alcohol at room temperature. Using soxhlet, the extracted was dried at 40–50°C for 24 hours after being filtered through Whatman filter paper.

Acute toxicity study:
An acute toxicity study was carried out in accordance with OECD guideline 423. The test solution was administered to six swiss albino rats weighing 150-200g at a dose of 2000mg/kg. Rats were observed for clinical signs, gross behavioural changes, and mortality after receiving the test formulation at intervals of 30 minutes, 1 hour, 2 hours, 4 hours, 24 hours, 48 hours, and 74 hours for a total of 14 days**.**

Experimental Protocol for Nootropic activity:
Scopolamine-induced dementia model:
Scopolamine-induced acute dementia treatment protocol.
Group-I : Normal control ; Orally adminstered by CMC (0.5%w/v)
Group-II : Disease Control ; Treated with scopolamine (0.4mg/kg I.P) + CMC (0.5%w/v)in 0.5% CMC.
Group-lll : Standard: Treated with scopolamine (0.4mg/kg I.p) + piracetam (200mg/kg)
Group-IV : Low dose test ; Animals are orally adminstered by extract 250mg/kg dissloved in 0.5% CMC and + scopolamine (0.4mg/kg I.P)
Group-V : High dose test ; 500mg/kg extract orally +scopolamine (0.4mg/kg I.p) Animlas were randomly divided into 5 groups each group contains six animals. Scopolamine, used for inducing acute dementia was given 30min. Prior to the recommended treatment.

Y Maze Test:
The Y maze had three arms, each 40 cm long, 12 cm tall, 3 cm wide at the bottom, and 10 cm wide at the top, and they all converged in an equilateral triangular central area. During an 8-minute session, each rat was placed at the end of one arm and allowed to freely roam the maze. To be able to alternate, the rat must be aware of which arm they have already visited. The sequences of arm entries, including potential returns into the same arm, were visually recorded. The performance of instant working memory was measured by recording spontaneous alternation behaviour. When the rat's hind paws had completely entered the arm, entry was considered complete. On overlapping triplet sets, alternation was defined as successive entries into the three different arms (A, B, and C). % Alteration was calculated by the formula.
% Alteration = (Number of arm alteration/total arm entry-2) *100.

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Elevated Plus-Maze Test :

The elevated plus maze was constructed of wood and featured two open arms (35 6 cm) and two enclosed arms (35 6 15 cm). The maze had been raised to a 40 cm height. Each rat should be positioned at the end of one arm of the elevated plus maze, facing away from the centre. The transfer latency (TL)—the length of time it took the rat to move from the open arm to one of the closed arms—was then measured. The first day the rats were allowed to explore the plus maze for 20 seconds. Rats were returned to their original cages after the first experiment's measurement of TL. Twenty-four hours later, the rats were again individually positioned on the elevated plus-maze as before, and TL was once more recorded. The first (L1) and second (L0) days' TL measurements served as the acquisition and retrieval parameters, respectively. Using the following, the inflexion ratio (IR) was calculated from these:

formula: **IR=L1/L0.**

Novel Object Recognition (NOR) Test :

The open field apparatus was made of white plywood (70x60x30 cm) with a grid floor that could be cleaned with hydrogen peroxide after each trial. The box's diagonally opposite corners were where the objects to be distinguished were placed. On the test day for the first trial (T1), two identical objects were placed in two corners of the box that were in opposition to one another, and the time it took each rat to complete a 20-second exploration was recorded. Exploration was defined as aiming the nose at an object from less than 2 cm away and/or touching it16. The time spent examining new (N) and familiar (F) objects was recorded during the second trial (T2, 90 minutes following T1). One of the objects presented during T1 was replaced with a new object during T2. (N-F)/(N+F) is the formula used to determine the discrimination index (DI). Thirty minutes before to the first trial, the animals were given the vehicle or medicines.

Morris Water Maze:

The apparatus consists of a circular pool with a smooth inner surface that is 100 cm in diameter and 45 cm high. The pool was divided into four equal quadrants, each with an equal area, and filled with opaque water (kept at 22 2 °C) to a height of 30 cm. At the centre of one of the four quadrants, there was a platform (29 cm 6 cm) that was positioned one centimetre below the water's surface (the target quadrant). Throughout the whole experiment, the platform's position remained constant. On day 10 of the treatment period, the test was started, and the rats were given time to acclimate by swimming for 120 seconds without the platform. Each animal got four 120-second learning trials separated by 60-second intertrial intervals throughout the course of the following four days. The rat was submerged for each learning trial, its back to the pool wall and diagonally across from the quadrant holding the platform. The escape latency time for each trial was calculated based on how long it took the animal to find the submerged platform.In this scenario, the escape latency time was 120 s because the animal was unable to find the platform within 120 s, it was pointed in the direction of the platform, where it was allowed to rest for 60 s. The hidden platform trials or acquisition tests took place during these sessions, which were recorded. The platform was removed from the water on day 15 (24 hours following the last learning trial), and the rats were put through a probe trial session to gauge memory recall. Each rat was dropped into the water across from the target quadrant, and given 60 seconds to swim and locate the quadrant where the platform had previously been installed. We kept track of how much time the animal spent in the target area.

Statislical Analysis :

Invivo study data were presented as the mean SEM. One-way analysis of variance was used to determine how the control and treated groups differed from one another (ANOVA). Statistics were considered to be significant at P-values under 0.05. Multiple comparisons were conducted using

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Dunnet's post hoc test. The software programme graphpad prisom version No. 5.0 was used for the statistical analysis.

Estimation of biochemical parameters:

Preparation of brain sample:

Rats from each group were subjected to euthanasia using a carbon dioxide chamber after the learning and memory paradigm in scopolamine induced amnesia was evaluated. The brains were rapidly removed and stored in ice-cold saline.

Quick dissections of the frontal cortex, hippocampus, and septum were performed on a petridish with eyes crushed (this part of the brain cannot be identified in a small rat brain ;therefore, the whole brain was taken).

In 0.1M phosphate buffer, the tissues were weighed and homogenised (P^H8). The homogenates of the rat brain were collected in different test tubes and examined for various enzymes, such as acetylcholinesterase, MDA, reduced glutathione protein thioles, and catalase. Enzymatic tests employed the supernatant.

RESULTS

Acute toxicity study:

According to OECD guideline 423, the methanolic extract of dried leaves of G. maderaspatana was tested for acute toxicity. The animals were observed for signs of toxicity at differed time intervals 0,30min,1,2,4,6,8,12h and then daily for a peroid of 14 days.No signs of toxicity were obsereved in tested animals.

Nootropic activity

Y-**MAZE :** When compared to the negative control group (scopolamine), the G.M. treatment groups with methanolic extract demonstrated dose-dependent increases in percentage alteration.

S.NO	GROUP	TREATMENT	ALTERATION(%)
1.	Normal control	(CMC,P.O)	64.31 ± 2.45
2.	Negative control	Scopolamine(1mg/kg.p.o)	33.81 ± 3.19***
3.	Standard treatment	Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	78.30 ± 3.61*
4.	Low dose extract	(250mg/kg p.o)+scopolamine(1mg/kg i. p)	44.25 ± 2.51**
5.	High dose extract	(500 mg/kg p.o) + scopolamine(1mg/kg i.p)	68.23 ± 2.19**

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

Elevated plus maze :

The effects of elevated plusmaze are shown in table .Low dose of G.M (250mg/kg) and high dose of G.M (500mg/kg) were anticipated for 7 consecutive days.Orally treated revealed significant reduction in TL.High dose produced highly significant effect (P<0.01) and low dose produced low significance effect (P<0.05) after associated through scopolamine - induced amnesia as well as normal control group, on 6^{th}-7th days.The animals were administred with small to high dose showed a significant decline in TL of the 6^{th}-7th days as part of learning and memory. Scopolamine injected prior training considerably increased TL on the 6^{th}-7th days indicating impairment of learning and memory. Piracetam at a dose of 100mg/kg additionally revealed considerable.

GROUP	TREATMENT	TL ON 6TH DAY	TL ON 7TH DAY
I	Normal Control (CMC,P.O)	25 ± 0.89	26 ± 0.72
II	Negative Control Scopolamine(1mg/kg.p.o)	56 ± 2.6	57 ± 2.7***
III	Standard Treatment Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	20 ± 0.9	21 ± 0.9**
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	18 ± 0.54	19 ± 0.65***
V	High dose extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	5.8 ± 0.9	5.9 ± 0.71*

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

Morris Water Maze:

There is a rise in escape latency in scopolamine evoked animals when compared with the standard of each times [4,25= 66.05] (p < 0.001).

Methanolic extract of GM low dose (250mg/kg) dose not show any significance on the 6th ,7th days. Higher dose of MEGM (500mg /kg) indicates slight significance on the 6th day and high significance on 7th day.

The animals administered with the high dose MEGM showed remarkable decrease in escape latency of 6th-7th as part of learning and memory.

IMPACT OF MEGM ON ESCAPE LATENCY OF RAT USING MORRIS WATER MAZE.

GROUP	TREATMENT	ESCAPE LATENCY TIME (SECS) DAY 1	ESCAPE LATENCY TIME (SECS) DAY 2
I	Normal Control(CMC,P.O)	84.431 ± 4.0212	75.213 ± 0.5165
II	Negative Control Scopolamine(1mg/kg.p.o)	97.5 ± 2.396***	83.313 ± 0.9821***
III	Standard Treatment Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	79.091 ± 3.2235*	53 ± 0.5306**
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	89.205 ± 0.7102***	72.320 ± 1.003l3***
V	High Dose Extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	90.506 ± 0.7724**	67.53 ± 1.1757*

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

Novel Object Recognition Test:

When compared to the scopolamine-treated group, methanolic extract of GM related groups showed a dose-dependent increase in discrimination index.

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Table: Effect of methanolic extract of GM on descrimination index in scopolamine induced ammesia in albino wistar rats.

GROUP	TREATMENT	DESCRIMATION INDEX (DI)
I	Normal Control (CMC,P.O)	51.20 ± 1.062
II	Disease Control	40.16 ± 1.306*
III	Standard Treatment Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	58.75 ± 1.051*
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	54.015 ± 1.021**
V	High Dose Extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	57.12 ± 2.013**

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

Biochemical Test :

1. Brain Acetylcholine Esterase Levels:

Comparing the methanolic extract of GM to the disease control group, the acetylcholin- esterase levels decreased in a dose-dependent manner.

Table: Effect of MEGM on acetylcholine level in scopolamine induced amnesia in albino wistar rats.

GROUP	TREATMENT	ACETYLCHOLINE LEVEL
I	Normal Control (CMC,P.O)	11.09 ± 1.34
II	Negative Control Scopolamine(1mg/kg.p.o)	29.21 ± 2.16***
III	Standard Treatment Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	11.07 ± 1.06*
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	22.89 ± 1.08**
V	High Dose Extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	24.12 ± 2.013**

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

2. Brain Melanaldelyde Levels :

When compared to the disease control group, which received scopolamine alone, the co-administration of the methanolic extract of GM at a dose of 500 mg/kg and scopolamine significantly decreased the amount of brain melanaldehyde (p0.0001). Piracetam similarly decreased the level of MDA..

GROUP	TEARTMENT	MELANALDEHYDE
I	Normal Control (CMC,P.O)	0.14 ± 0.006
II	Disease Control	0.62 ± 0.0010**
III	Standard Treatment Piracetam(200mg/kg I.p)+scopolamine (1mg/kg I.p)	0.43 ± 0.0024*
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	0.20 ± 0.0010***
V	High Dose Extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	0.18 ± 0.004**

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

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3. Brain Reduced Glutathione Levels:

Administration of the GM methanolic extract at doses of 250 mg/kg and 500 mg/kg significantly (P 0.0001) reduced the amount of GSH induced by scopolamine.

GROUP	TREATMENT	GSH
I	Normal Control (CMC,P.O)	21 ± 0.22
II	Disease Control	12.18 ± 1.22**
III	Standard Control (200mg/kg I.p)+scopolamine (1mg/kg I.p)	16.06 ± 0.12*
IV	Low Dose Extract (250mg/kg p.o)+scopolamine(1mg/kg i. p)	20.02 ± 0.41***
V	High Dose Extract (500 mg/kg p.o) + scopolamine(1mg/kg i.p)	20.62 ± 0.24***

n=6, Values are Mean ±SEM, *p<0.05, **p<0.01, ***p<0.001, as compared with control group(ANOVA followed by dunnet's test)

Histopathology:

In figure and table, the findings of the histological analysis are presented. While the histopathological analysis of the groups receiving standard treatment drugs (figure 1B) revealed vascular degeneration, neuronal degeneration, and gliosis that were found to be lower as compared with the negative control group, the histopathological analysis of the normal control rat revealed neuronal degeneration without vascular degeneration and gliosis. The most significant pathological alterations were seen in the scopolamine-treated groups. When compared to the normal control, the negative control, and the standard treatment, the methanolic extract of GM in both low and high doses demonstrated good regeneration scores.

DISCUSSION:

The number of AD patients is progressively increasing worldwide every day [38]. Degenerative changes in the brain cause memory loss in Alzheimer's disease [37]. Because scopolamine is an antimuscarinic agent that causes memory deficits after administration, the primary cause of AD is the loss of cholinergic neurons in the basal forebrain area.

The objective of the current investigation was to evaluate whether Grangea maderaspatana may enhance cholinergic pathways and alleviate memory impairment. Memory loss with Alzheimer's disease is managed in a substantial way by medicinal plants. In this work, we used the Y-maze, Elevated Plus Maze, NOR, and Morris Water Maze tests to assess the impact of Geangea Maderaspatana on the memory function of amnesia rat. As was previously described [26], scopolamine caused amnesia in rats by impairing memory and blocking the brain's muscarinic cholinergic receptors. In the present investigation, prolonged scopolamine treatment to rats increased the latency time to enter the preferred arm, resulting in a reduction in the amount of time spent in the preferred arm of the y-maze. Chronic administration of GM methanolic extract, on the other hand, caused a reduction in latency time, which increased the amount of time spent in the preferred arm. According to earlier reports [27], the reduction in latency time indicated an improvement in memory. The considerable increase in entries and the length of time spent in the preferred arm reflex indicate that the memory is working well [28,29].

Neuroscience and memory studies of rat and mouse behaviour and brain functioning have been extensively exploited in an open field [30,31]. According to the results of the novel object recognition test, scopolamine increases the time it takes to find a familiar object when compared to a new one (NOR). It also reduced the amount of time spent exploring an object in relation to the

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frequency of investigation.The descrimination index dropped dramatically in the scopolamine-treated rats, indicating that the learning and recognition processes were impaired. Scopolamine is thus an anticholinergic drug that blocks muscarinic receptors, which have been linked to impaired learning and memory in both humans and animals.[32].

The animals' learning capacity is indicated by how long it takes them to go to the centre platform of the raised plus-maze. If the time it takes to go to the central platform is shortened, the animal is said to have learned.

The morris water maze (MWM) task was used as a behavioural task to demonstrate the mechanism by which G.M extracts show neuroprotective activity.
The findings show that, like piracetam, G.M significantly reduced the learning and retension deficits caused by repeated scopolamine doses. The time spent in the invisible platform during the retension phase was reduced by using a methanolic extract of G.M. During the retension phase, G.M also increased the amount of time spent in the target quadrant significantly. Our findings with the Morris water maze indicate that pre-treatment with G.M extract prevented scopolamine-induced learning and memory deficits, implying that G.M is neuroprotective. [33].
Memory disorders caused by scopolamine are linked to oxidative stress in the brain [34,35], as evidenced by an increase in MDA levels, a negative effect of reactive oxygen species [36]. Because the brain is composed of lipids, the action of ROS causing lipid peroxidation may directly cause brain death. The current study confirmed previous findings by Lee et al. [37] that the brain's antioxidant defence mechanism had collapsed, as evidenced by higher MDA levels and lower catalase and glutathione activity in the scopolamine-treated group versus the control group. Catalase activity, glutathione level, and MDA level in the rat brain all improved significantly after treatment with G.M. methanolic extract.

CONCLUSION

The purpose of this study was to assess whether grangea maderaspatana methanolic extract could protect against Scopolamine-induced memory loss. Scopolamine is a muscarinic acetylcholine receptor antagonist. Scopolamine-induced memory and learning deficits in Y-maze and object recognition tests resulted in an increase in oxidative stress as well as an increase in acetylcholinesterase activity.

In conclusion, Grangea maderaspatana possesses antiamnesic activity in a scopolamine rat model and enhances learning and memory in normal rats. It had a positive impact on brain levels of SOD, CAT, and MDA, which helped to reverse the neurotoxicity and learning and memory deficits caused by scopolamine. These findings at least partially support the widespread use of Grangea maderaspatana as a neuroprotective and antiamnesic herb in traditional medicine. To fully comprehend the methods by which Grangea maderaspatana exerts its effects, however, more research is required.

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CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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Antioxidant And Acetylcholinesterase Inhibitory Activities Of Methanolic Extract Of Grangea Maderaspatana

Aruna B[1]*, V Santhosh Kumar[2]

Abstract

Background: Grangea maderaspatana has traditionally been used to treat neurological disorders. The purpose of this study was to look into the phenolic content, antioxidant activity, and acetylcholinesterase enzyme (AChE) inhibitory activities of various fractions of Grangea maderaspatana grown in local areas of kurnool.

Methods: The whole plant of Grangea maderaspatana was extracted with methanol and total phenolic content was estimated by folin-catechu method. DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay was used to measure antioxidant activity, while Ellman's colorimetric method was used to measure AChE inhibitory activity.

Results: Our findings indicated that the methanolic fraction had the highest total phenol concentration, which is comparable to 25.21.4 mg quercetin/g of fraction. Our findings also showed that the methanolic fraction had the highest antioxidant activity and AChE inhibitory activity when compared with the standard in a dose-dependent manner.

Conclusions:Our findings suggest that grangea maderaspatana methanolic extract could be a promising source of AChEinhibitors for Alzheimer's disease.

Keywords: Alzeimer›s disease, DPPH radical, IC 50, HPTLC fingerprinting.

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INTRODUCTION

Plant-based medicine has been practised throughout the world since time immemorial. According to the WHO, approximately 80% of people rely on traditional and herbal medicine systems for primary health care.[1]. Phyto-chemicals found in medicinal plants are the secondary metabolites that serve specific biological functions in the plant host and have potential disease-inhibiting abilities in animals [2]. Indian medicine systems like Ayurveda, Siddha and Unani use traditional herbs and plants against various diseases. Recently, there is a thrust in the research and development of traditional plant based drugs in modern medicine due to their remarkable versatility and proven efficacy. It is estimated that 25% of drugs are plant based, suggesting the significant role of plants as a medicine source [3].

Grangea maderaspatana, also known as Madras carpet, is one such weed that grows in sandy lands and waste areas. It contains flavonoids, diterepenes, sesquiterpenoids, ste-roid, and essential oil, according to reports.It is a medicinal plant that is widely used in Indian traditional medicine to treat a variety of

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ailments. The herb is useful for eye and ear pain.The root is an appetiser, diuretic, anthelmintic, emmenogogue, galactogogue, stimulant, beneficial in griping, chest and lung problems, headache, paralysis, rheumatism in the knee joint, piles, muscle pain, spleen and liver diseases, and reduces sweating. The plant has stomachic and uterine stimulant properties.[4].

According to Hegde et al., each plant has a distinct nature in terms of botany, chemistry, and therapeutic potency, and it is critical to study a medicinal plant's pharmacognostic characteristics not only for proper identification, but also to understand its structure and biology [5]. According to the literature, only a few studies on Grangea maderaspatana have been conducted. As a result, the dried plant material of Grangea maderaspatana was studied for its pharmacognostic properties in this study.

MATERIALS AND METHODS

Collection and identification of plant material

G. maderaspatana plant materials (whole plant) were collected from a field near Nandinapalle village in the Kurnool district. AP. The plant was identified and authenticated by the taxonomist Prof. P. Jayaraman of Plant Anatomy Research Centre, Chennai. The plant was dried under shade. Air dried plant material was ground to #10 powder and the plant material was then used for further investigations.

Chemicals, reagents, and solvents

All of the chemicals, reagents, and solvents utilised in the experiment were of analytical quality.

Physicochemical screening

Physicochemical parameters such as moisture content, ash value, , and extractive values were determined in accordance with WHO quality control methods for herbal materials. [6-8].

The methanolic extract was subjected to preliminary phytochemical screening to determine the presence of alkaloids,glycolsides, flavonoids, phytosterols, tannins/ phenolic compounds, carbohydrates, proteins, and fats.The extracts were filtered after being treated with a few drops of diluted HCl. The filtrates were then treated with Wagner's, Hager's, and Dragendroff's reagents, In order to detect the presence of alkaloids. one gram of the extract was dissolved in a few drops of dry acetic acid, 3 ml of acetic anhydride, and then a few drops of strong sulfuric acid in order to qualitatively analyse the presence of phytosterol.Three independent assays were carried out for tannins and phenolics using 5% diluted ferric chloride solution, 1% solution of gelatin with 10% NaCl, and 10% lead acetate solution. Shinoda's test was run for flavonoids.The extracts were dissolved in alcohol, to which a piece of magnesium was added after conc.Hcl was poured drop by drop. The mixture was then heated to obtain a magenta colour.The extracts were diluted in 5 ml of distilled water, filtered, and then tested for carbohydrates using Molisch's, Fehling's, and Barfoed's methods using the filtrate.In order to conduct Biuret and Millon's test to identify proteins, the extracts were diluted in water. The extracts were individually pressed bet-ween two filters papers to get the presence of fixed oils. It is clear that solidified oil was present from the oil spots on the paper. Additionally, a test known as a saponification was conducted to see if fixed oils and fats developed as a result of adding 0.5N alcoholic potassium hydroxide and phenolphthalein to the mixture and heating it in a water bath.[9, 10].

HPLTC fingerprinting

HPTLC fingerprinting for oleanolic acid and ursolic acid was done on G. maderaspatanawa extracts in chloroform and methanol. 20 mg of each extract were precisely weighed into a volumetric flask, and 10 mL of methanol were then added. Whatman Filter Paper No. 1 was used to dissolve it, filter it, and use the results for HPTLC profiling.The HPTLC chromategraphic condition is given in Table 1.

Quantification of Quercetin-3-rutinoside by HPLC

Estimation of Quercetin-3-rutinoside in methanol extracts of G. maderaspatana was performed by HPLC [13]. *G. maderaspatana* powder (50 mg) was dispersed in 50 ml of methanol. Solution was sonicated (for 15 minutes) and then vortexed (for <1 minutes). Obtained methanolic extract had concentration of 1 mg/ml this solution was used for the HPLC analysis. The HPLC chromatographic condition is given in Table 2.

Acetylcholinesterase (AChE) inhibition assay

Using a spectrophotometric technique, AChE inhibitory activity was evaluated [14–16]. When acetylthiocholine is hydrolyzed by enzyme and then combines with DNTB (5, 5'-dithiobis-2-nitrobenzoic acid) ion, thiocholine produces a yellow colour, which was used to measure the enzyme activity. At 405 nm, this is detectable. Tris-HCl buffer 50 mM, pH 8, 0.1 percent BSA as an enzyme blank, and galantamine as a reference standard were employed as the positive controls (enzyme activity without extract), respectively. The enzyme (0.2 U/mL) was dissolved in 50 mM of Tris-HCl buffer, pH 8 and 15 mM of the substrate ATCI (Acethylthiocholine Iodide). kinetic reaction was seen for three minutes.

Following equation was used to calculate the amount of enzyme inhibition (I percent) of the enzymatic reaction:

$$I\% = \frac{(E - S)}{E} \times 100$$

Where,

E: The enzyme's substrate hydrolysis kinetics without the test substance

S: The enzyme's substrate hydrolysis kinetics witH the test substance

DPPH assay for measuring antioxidant activity

Preparation of DPPH solution

In a conical flask, DPPH (5 mg) was accurately weighed and dissolved in ethanol (10 ml). Further, DPPH solution (2 ml) was diluted with ethanol (38 ml) in such way that the OD of DPPH solution was 0.7 at 517 nm, to get DPPH working solution [17, 18].

Preparation of sample solution

In a micro centrifuge tube, *G. maderaspatana* powder (10mg) was accurately weighed and methanol (2 ml) was added. It was vortexed for 4min and centrifuged at 8000 rpm for 5 min. Supernatant (5 mg/ml) was collected. 200 l of the supernatant was collected in a micro centrifuge tube and diluted with methanol to make a solution containing 1 mg/ml. 200 l of the aforementioned solution was taken in a micro centrifuge tube and diluted to a volume of 2 ml with methanol to produce a solution containing (0.1 mg/ml).This solution was used for DPPH assay. Ethanol (1 ml) was added to the DPPH working solution (0.5 ml), and absorbance at 517 nm was measured. The test sample was made according to Table 3's instructions. Samples were incubated for 20 minutes in a dark atmosphere after reactants were added. At 517 nm, absorbance was measured. For each concentration, the test was run in triplicate.

Calculation

The following formula was used to calculate The percent inhibition (% IC) of DPPH radical,%IC= [(ODDPPH control-ODtest)/OD DPPHcontrol] X 100

Calculation of 50% inhibition conc. (IC_{50}):

The concentration which scavenges 50% of the radical (IC_{50}) is calculated by plotting % IC in Y-axis and sample doses in X-axis.

Statistical Analysis

According to regression analysis of the relationship between scavenging activities (in percent) and different concentrations of the extract, the amount of effective concentration of the extract required to inhibit free radicals by 50%, or Inhibitory Concentration (IC50), was calculated. The antioxidant assay was run in triplicate, and the results were shown as mean + SD.

RESULTS & DISCUSSION

Phytochemical screening

Table 4 contains a compilation of the findings from the various analyses. An indication of the earthy matter, inorganic composition, and other contaminants present with the medicine was provided by the ash values of the drug. The primary function of the extractive values is to identify medicine that has been exhausted or that has been adulterated with.

Grangea maderaspatana powdered portions were extracted using several solvents in succession (except water extract which was prepared by decoction). Table 5 lists the various extracts that were collected along with their yield percentage, colour, and consistency.

qualitative analysis

The results of the various qualitative chemical tests performed on the extracts obtained through successive solvent extraction processes to identify the presence of various phyto-

constituents, such as steroids, carbohydrates, alkaloids, glycosides, phenolics, and tannins, among others, are shown in Table 6.

HPTLC fingerprinting

Utilizing thin layer chromatography, quick screening of oleanolic acid and ursolic acid was investigated (TLC). Toluene:Ethyl acetate:Formic acid (8:2:0.1) led to a better separation. The visualizer recorded the TLC run data, and Figure 1 shows a picture of the chromate-graphic plate along with the results. After the anisaldehyde-sulphuric acid reagent was sprayed, all four bands were recognised (Figure 1). In Table 7, Rf values and the AUC for each of the four bands were listed. Figure 2-5 shows the HPTLC chromatogram of the standard oleanolic acid, standard ursolic acid, and the methanol and chloroform extract of G. maderaspatana. Oleanolic acid and ursolic acid are present in 4.0 percent and 9.5 percent, respectively, of the chloroform extract of Grangea maderaspatana, whereas the methanol extract containsEstimation of Quercetin-3-rutinoside in methanol extracts of G. maderaspatana by HPLC method3.0% and 6.5% of oleanolic acid and ursolic acid respectively.

HPLC programming for mobile phase is given in Table 8.To assign the peaks in chromatogram, standard compounds (Quercetin-3-rutinoside) was injected in same HPLC gradient system. The corresponding retention time for the standard compound Quercetin-3-rutinoside was 22.22 min. The HPLC chromategram of *G. maderaspatana* methanol extract and Quercetin-3-rutinoside were given in figure 6 and 7.

DPPH radical scavanging of methanolic extract of G. maderasapatna

The DPPH radical inhibition percentage for the methanolic extract of G. maderaspatna was determined. The average IC50 was determined, and Table 9 shows the results. The methanolic extract of G. maderasapatna used in in vitro assays to determine its antioxidant capacity demonstrated DPPH activity. Antioxidant-DPPH interaction results in 1, 1-diphenyl-2-picryl hydrazine, a persistent free radical. The decrease in absorbance at 517 nm was used to gauge the capacity to neutralise the stable free radical DPPH. The IC50 value of the extract is the amount of extract required to block the DPPH free radical 50% of the time. A lower IC50 value indicates that the sample is more effective in scavenging. Figure 8 depicts the percentage inhibition.

AchE Inhibition of methanolic extract of G. maderasapatna

Table 10 and Figure 9 both show the methanolic extract of G. maderaspatna's inhibitory efficacy against AChE at concentra-tions of 50 to 250 g/mL. The percentage of inhibition of G. maderaspatna's methanolic extract ranged from 27.080.12 to 41.220.26. The presence of phenolic acids, flavonoids, and other antioxidant substances may be the cause of the inhibition. Antioxidant substances might be responsible for inhibiting AChE.

CONCLUSION

The phytochemical studies described in the current study require additional scientific research to determine their identity down to the component level. To support the use of G. madarasapatna by traditional healers, research is required on a variety of biological processes that are similar to those of G. madarasapatna. The standardisation of G. madarasapatna's quantitative and qualitative properties will be helped by the pharmacognostic characteristics that were researched. For G. madarasapatna, comprehensive differential studies utilising molecular and chemical markers are needed, particularly for their authentication in their medication form.

ACKNOWLEDGMENT

The authors are thankful to the principal, Dr.KV Subbareddy Institute Of Pharmacyfor providing the necessary facilities.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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Table 1: HPTLC Chromatographic condition

System	CAMAG Linomat 5
Method	Quercetin-3-rutinoside
Column	Merk –TLC/HPTLC silica gel 60 F254 on Aluminum sheets (10×10 cm)
Injection volume	10µL
Mobile phase	Toluene:Ethyl acetate:Formic acid (8:2:0.1)
Spray agent	p-anisaldehyde reagent
Absorbance	510nm

Table 2: HPLC Chromatographic condition

System	Alliance-Water 9PDA 2996)
Method	Quercetin-3-rutinoside
Column	Xterra RP column (250×4 mm, 5 µm merk)
Solven system	Gradient; (A) 0.1% Aqueous Formic acid (B) Methanol
Detection	270 nm (PDA detector)
Flow rate	0.7 ml/min
Run time	35 min

Table 3: Preparation of test sample for DPPH assay

Quantity of sample	Volume taken (in µl)			Total volume
	G.maderaspatna powder (0.1mg/ml)	Ethanol	DPPH working solution	
1µg	10	990	500	1.5ml
2µg	20	980	500	
3µg	30	970	500	
4µg	40	960	500	
5µg	50	950	500	
6µg	60	940	500	
8µg	80	920	500	
10µg	100	900	500	
12µg	120	880	500	

Table 4: Physico-Chemical Parameters of powder of *G. maderaspatana*

S. No	Parameters	Values (% w/w)
1	Loss on drying	8
2	Ash value	
	Total ash	11.60
	Acid insoluble ash	1.80
	Water soluble ash	4.50
3	Extractive value	
	Water soluble extractive	10
	Alcohol soluble extractive	10

The values given here are expressed as percentage of air dried material. Each value is averageof three determinations.

Table 5: Preliminary phytoprofile fo G. maderaspatana

S. No	Solvent	Colour	Consistency	% yield w/w
1	Petroleum ether	Light green	Slight sticky	4.82
2	Chloroform	Darak green	Slight sticky	1.37
3	Ethyl acetate	Brownish green	Sticky	2.50
4	Methanol	Brown	Slight sticky	6.20
5	Water	Dark brown	Sight sticky	5.34

Table 6: Phytochemical screening of extracts of G. maderaspatana

Chemical constitutents	Pertroleum ether extract	Chloroform extract	Ethylacetate extract	Methanol extract	Water extract
Carbohydrate	-	-	-	+	+
Protein	-	-	-	-	-
Phenolics & Tannins	-	-	-	+	+
Saponins	-	-	-	+	+
Flavanoids	-	-	-	+	+
Terpenes	+	+	+	+	-
Steriods	+	+	-	-	-
Alkaloids	-	-	-	-	-

+ positive, - negative

Table 7: Result of HPTLC analysis of extracts of *G. maderaspatana*

	Sample			
	Methanoloic extract	Oleanolic acid	Ursolic acid	Chloroform extract
Rf value	0.50	0.48	0.48	0.49
AUC	1517.5	24373.6	11179.7	2142.7

Table 8: Gradient system of solvent-A and solvent-B

Time (Min.)	Solvent A (%)	Solvent B (%)
0	95.0	5.0
12.0	60.0	40.0
13	95.0	5.0
15	95.0	5.0

Table 9. Percent inhibition of DPPH radical by test samples at different concentrations

Quantity of sample (µg)	Percentage inhibition of DPPH radical
1	12.56 (1.35)
2	26.41 (1.95)
4	46.83 (1.30)
5	55.35 (0.87)
6	63.47 (0.42)
8	76.79 (1.18)
10	84.48 (0.23)
12	88.95 (0.55)

Values are expressed in Mean (SD) (n=3)

Table 10:% AchE Inhibition of methanolic extract of G. maderasapatna

S. No	Conc. (µg/ml)	% Inhibition AchE
1	50	27.08±0.12
2	100	29.24±0.26
3	150	32.36±0.29
4	200	40.18±0.18
5	250	41.22±0.26

All values in the table represent mean ±SD (n=3)

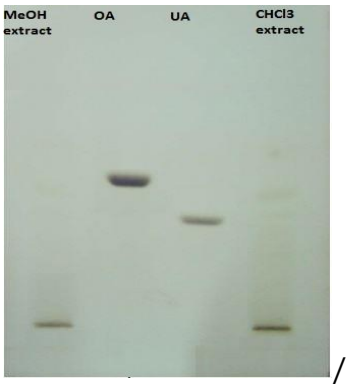


Figure 1: HPTLC plate of G. maderaspatana extracts after derivatization (MeoH extract - G. maderaspatana-methanolic extract, OA- Oleanolic acid (standard 1), UA - Ursolic acid (standard 2), CHCl3- G. maderaspatana chloroform extract)

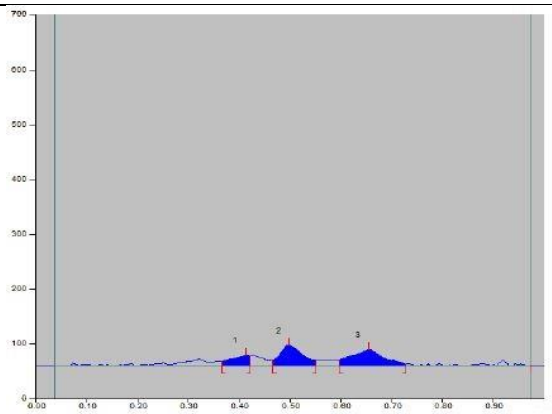


Figure 2: Chromatogram of methanol extract of G. maderaspatana

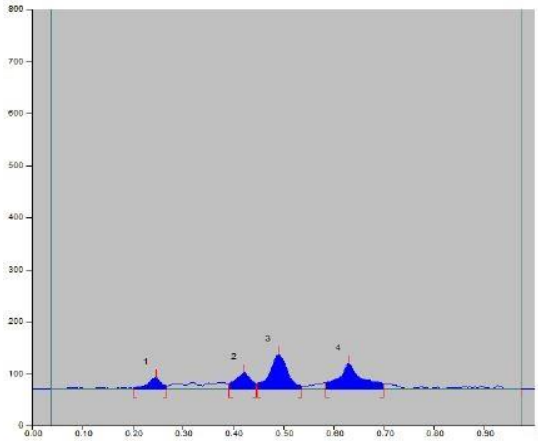


Figure 3: Chromatogram of chloroform extract of *G. maderaspatana*

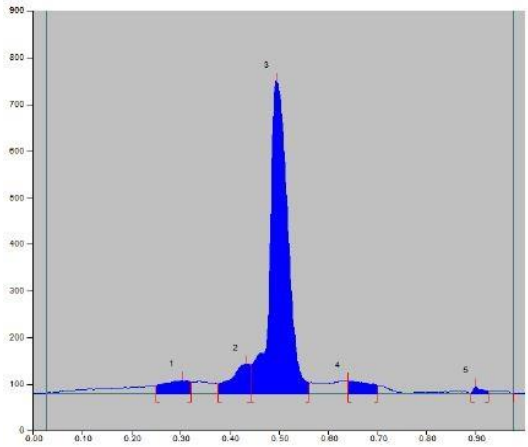
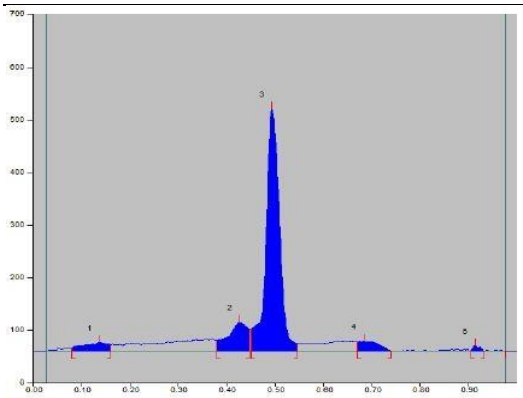


Figure 4: Chromatogram of standard Oleanolic acid



Fgure 5: Chromatogram of standard Ursolic acid

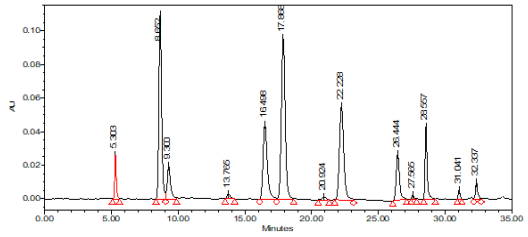


Figure 6: HPLC chromatogram of *G. maderaspatana* methanol extract

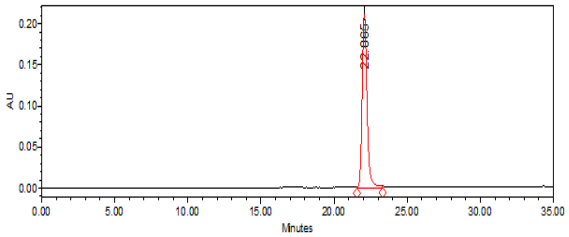


Figure 7: HPLC chromatogram of Quercetin-3-rutinoside

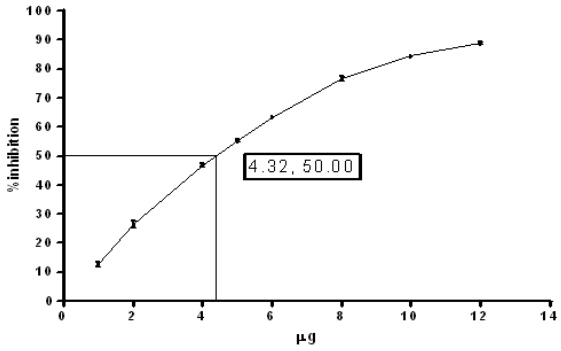


Figure 8. Percent inhibition of DPPH scavenging by methanolic extract of G. maderaspatna at different concentrations

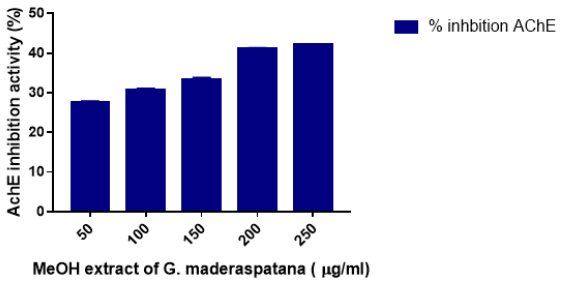


Figure 9: MeOH extract of G. maderaspatana inhibits acetylcholinesterase. The extract was tested for its acetylcholinesterase inhibitory activity. Each bar represents the mean percentage of inhibitory activity ±SD. (*P<0.001; n=3)

Neuropharmacological exploration of grangea maderaspatana(L.) poir an inclusive review.

Aruna B[1]*, V Santhosh Kumar[2]

Abstract

Grangea maderaspatana(L.)poir is a popular indian medicinal plant belongs to the family asteraceae. This plant is commonly known as madras carpet. This species is utilized in many countries for medicinal purposes. The leaves are used as stomachic, sedative, an emenogogue, and an anti -flatulent, a carminative. The roots are used as an appetizer, astringent to the bowels, galactogogue etc. in many countries the whole plant is used. Phytochemical composition of this plant has marked effect on brain cholinergic, dopaminergic and serotonergic systems. Usefulness of this plant in various neurobehavioral disorders is explained in this review.

Keywords:G.maderaspatana, neuropharmacology, anxiety, depression, parkinsonism, autism.
DOI Number: 10.48047/ nq.2021.19.12.NQ21220 **NeuroQuantology2021;19(12): 297-302**

Introduction

Herbal medicines play an important role in preventing and treating diseases in human beings. In recent years there has been a resurgence of interest rediscover medicinal plants as a source of potential drug candidate. The asteraceae family is one of the largest plant families with more than one thousand generes and twenty thousand species. Many of the plants of this family are used in traditional medicine. Since ancient times, plants have been the used in traditional medicine systems such as Ayurvedic, Chinese and African traditional medicines. The interest of modern medicine for medicinal plants significantly increased due to the recently found enormous therapeutic potential of these plants[1]. Thus, many plants of the Asteraceae family are used for symptomatic relief of several neuropsychiatric disorders. More than that, animal and cellular modelling experiments were performed to present scientific proof for their proper correlations between traditional use and scientific research on Asteraceae family plants for treatments of neuropsychiatric disorders such as AD, PD, schizophrenia, autism, depression and. In this way, this review aims to therapy. These plants are widely used in alternative medicine due to their effects on the nervous system, being studied both on cellular and animal models. In this way, medicinal plants are preferred in drug therapy research due to the wide population interest. The plants phytochemical composition is mainly consisted in cholinergic, dopaminergic or serotonergic molecules which are involved in the pathophysiology of the neuropsychiatric disorders likeschizophrenia, autism, anxiety or depression. Therefore, this review aims to comprise all the available information regarding this family plants in order to find further correlations between or to explain their neuropsychiatric use. Thus, many plants of this family are used for symptomatic relief of several neuropsychiatric disorders [9]. More than that, animal and cellular modeling experiments were performed to present scientific proof for their properties [10-11]. Correlations between traditional use and scientific research on family plants for treatments of neuropsychiatric disorders such as AD, PD, schizophrenia, autism, depression

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of

and anxiety are necessary for neuropsychiatric potential molecules discovery. Unlike the potentially addictive and forceful action of widely used psychostimulants, chronic and moderate administration of g.maderaspatana, appears to nourish rather than deplete neurons. In this way, this review aims to assemble traditional and Asteraceae family plants.

G.maderaspatana-Plant profile

Kingdom: Plantae
Subkingdom: Planta Tracheophyta
Subdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida (Dicotyledons)
Subclass: Asteriidae
Order: Asterals
Family: Asteraceae
Synonyms
Grangea maderaspatana (L.) Poir., G. adansonia, Artemesiamaderaspatana

Vernacular names

Gujarati: Jhinkimundi, Nahanigora, Khamundi
Hindi: Mukhatari, Mustaru
Malayalam: Nelampala;
Marathi: Mashipatri
Tamil: Mashipatri
Telugu: Machi-Patri
Urdu: Afsantin

Phytoconstituents

The chemical constituents reported in different parts of the G.maderaspatana L. plant are mentioned below.

Steroidal constituents, hardwickiic acid, the corresponding 1, 2- dehydro-derivative, acetylenic compounds, eight new clerodane diterpenes including five clerodane, a nor clerodane, a secoclerodane and a norseco-clerodane derivatives along with auranamide, grangolide and eudesmanolides have been reported from various parts of G. maderas-patana[3]. A clerodane derivative, 15-hydroxy-16- oxo-15,16H-hardwickiic acid (15-hydroxycleroda – 3, 13-dien-15, 16-olide-18-oic acid) has been isolated from the aerial parts of G. maderaspatana[4]. Three components viz., eudesmanolide, (-) frullanolide, (-) -7-alpha-hydroxyfrullanolide and a new eudesmanolide (+) -4 alpha, 13-dihydroxyfrullanolide have been isolated from the whole plant of G. maderaspatana. A new eudesmanolide was named (+) – Grangolide[5]. Penta and hexamethoxy flavones have been isolated as 3‟5- dihydroxy- 3,4‟,5‟,6,7-pentamethoxy flavone, 4‟,5-dihydroxy-3,3‟,5‟,6,7-pentamethoxy flavone (murrayanol) and 5-hydroxy-3,3‟,4‟,5‟6,7-hexamethoxy flavone in addition to previously reported clerodane ditepenes from the Diethyl ether – Petrol – Methanol (1:1:1) extract of the aerial parts of Grangea maderaspatana[6]. Two new 5-deoxyflavones, 6-hydroxy-2′,4′,5′-trimethoxyflavone (1), 6-hydroxy-3′,4′,5′-trimethoxyflavone (2) and a known flavone, 7,2′,4′-trimethoxyflavone (3) have been isolated from the whole plant of Grangea maderaspatana[7]. The plant contains diterpenoid compounds of labdane and clerodatetrean type, such as the analgesic constituentent-15, 16-epoxy-7-hydroxy-3, 13, 14-clerodatrien-18-oic acid; steroids, chondrillasterone and chondrillasterol; diterpene, strictic acid, a phenylalanine derivative, auranamide and the allergenic compounds, eudesmanolides, (-)-frullanolide, (-)-hydroxyfrullanolide and (+)-grangolide[8]. A new diterpenoid has been isolated as 8-hydroxy- 13E-labdane-15yl-acetate from the acetone extract of Grangea maderaspatana[9] 1 Singh et al. (2013)[14] were identified 21 constituents constituting 91.5 % of the oil from aerial parts of Grangea maderaspatana (L.) Poir. It was characterized by the dominant presence of sesquiterpenoids (sesquiterpenoid hydrocarbons 36.1 % and oxygenated sesquiterpenoids (28.4 %). Most abundant compounds are γ-gurjunene (26.5%), terpinyl acetate (20.8%) and hinesol (11.7%).

Pharmacological activities:

Jain et al., 1993 assessed a mixture of flavonoids extracted from the Grangea maderaspatana plant for oestrogenicity and antiimplantational activities, in the mouse. In the 3 dayuterotrophic bioassay, administration of the drug at a dose of 20 mg/kg body weight per day, intramuscularly to ovariectomized females, resulted in a highly significant (pof the oil obtained by steam distillation of extract of aerial parts of Grangea maderaspatana (L.) Poir., using, DPPH radical scavenging, metal chelating and reducing power assays. The oil showed antioxidant potential with significant reducing power (ASE/mL 2.01 ± 0.00), chelating activity (IC50 1.80 ± 0.15) and DPPH

radical scavenging activity (IC50 2.90 ± 0.96)[11].

Omhare et al., 2012 identified that the aqueous and ethanol extract (250 mg/kg, 500 mg/kg, p.o.) of Grangea maderaspatana Poir., effectively inhibited CCl4 and paracetamol induced changes in the serum marker enzymes (SGOT, SGPT and ALP) in a dose-dependent manner as compared to the normal and the standard drug silymarin treated groups. Hepatic steatosis, hydropic degeneration and necrosis observed in CCl4 and paracetamol treated groups were completely absent in histology of the liver sections of the animals treated with the extracts. The results suggest that the ethanol extract of G. maderaspatana possess significant hepatoprotective activity[12].

Singh et al., 2013 demonstrated an Antimicrobial activity of the oil obtained by steam distillation of aerial parts of Grangea maderaspatana (L.) Poir., against gram positive bacteria, gram negative bacteria and fungi using agar well diffusion method. The zone of inhibition (ZOI) values of the oil was in the range of 2.67 ± 0.58 to 11.00 ± 0.00 mm and minimum inhibitory concentration (MIC) of the oil was ranged from 5 to 30 µL/ mL for tested microorganisms. The activity was more pronounced against Candida albicans (ZOI = 11.00 ± 0.00 mm, MIC = 5 µL/mL) followed by Streptomyces candidus (ZOI = 9.33 ± 0.58 mm, MIC = 5 µL/mL), while the oil was least effective against Aeromonas hydrophila and Klebsiella pneumonia [13].

Rachchh et al., 2013 evaluated Anti-inflammatory activity of methanol extract of G. maderaspatana (1000 mg/kg, p.o.) using acute model of carrageenan induced rat paw edema. Indomethacin was used as standard in this model. The extract showed significant protection against carrageenan induced rat paw edema indicating its anti-inflammatory activity[14].

Rachchh et al., 2013 also evaluated Antiarthritic activity of methanol extract of G. maderaspatana (1000 mg/kg, p.o.) using Complete Freund's Adjuvant (CFA) induced arthritis in rats. Dexamethasone was used as a standard in this model. The degree of arthritis was evaluated by hind paw swelling, body weight changes, erythrocyte sedimentation rate, rheumatoid factor, Creactive protein and arthritic index supported by histopathology of ankle joints. The extract treatment declined CFA induced rise of erythrocyte sedimentation rate, rheumatoid factor, Creactive protein significantly in rats. Histopathological study of ankle joint revealed that extract inhibited edema formation and cellular infiltration induced by CFA[15]

Ahmed et al., 2001 assessed diuretic activity of Grangea maderaspatana (L.) Poir[16]

Acute toxicity studies

Omhare et al., 2012 evaluated Acute oral toxicity by following Organization of Economic Co-operation and Development (OECD) guidelines 420- Fixed Dose Procedure (FDP). Results indicated that the aqueous and alcohol extract of G. maderaspatana up to a dose of 2000 mg/kg; p.o. did not produced any mortality

Neuropharmacological activity

G.maderaspatana has been studied extensively in animal models and invitro. While long term effects ofg.maderaspatana on humans is unknown, but animal models suggest considerable protection against age-related neurodegeneration, anxiety and depression.

Putative mechanism of action

In this review we discussed about the possible mechanisms responsible for antioxidant/neuroprotection, acetylcholine esterase inhibition, choline acetyltransferase activation, increased cerebral blood flow, and monoamine potentiation and modulation.

Anti-oxidant/neuroprotection. Oxidative stress (OS) occurs when free radicals (chemical species with unpaired electrons, produced during normal metabolism) overcome the cell's homeostatic defense mechanisms[36]. Protective, free radical–quenching enzymes include superoxide dismutase, catalase, glutathione peroxidase (GPx), glutathione reductase (GSR), and others. Anti-oxidant compounds also play a key protective role, including vitamins A, C, E, and myriad phytonutrients (particularly phenols)[37]. OS plays a role in many diseases, even aging itself, [38] by degrading ligands, peroxidizing lipids, disrupting metabolic pathways, denaturing proteins, and breaking DNA strands[39].The brain is especially susceptible to OS because it

is metabolically active, possesses high levels of pro-oxidant iron, and is composed of unsaturated lipids (prone to lipid peroxidation)[40]. Furthermore, the blood–brain barrier prevents many exogenous anti-oxidants from quenching reactive oxygen species (ROS) in the brain[41].

Darshan singh at al assessed the extract of aerial parts of Grangeamaderaspatana (L.) Poir., obtained by steam distillation was analyzed by gas chromatography and gas chromatography coupled with mass spectroscopy. Twenty one constituents were identified, constituting 91.5 % of the oil. It was characterized by the dominant presence of sesquiterpenoids (sesquiterpenoid hydrocarbons 36.1 % and oxygenated sesquiterpenoids 28.4 %). Most abundant compounds are γ-gurjunene (26.5%), terpinyl acetate (20.8%) and hinesol (11.7%). The in vitro antioxidant potential of the oil was evaluated using, DPPH radical scavenging, metal chelating and reducing power assays. The oil showed antioxidant potential with significant reducing power (ASE/mL 2.01 ± 0.00), chelating activity (IC50 1.80 ± 0.15) and DPPH radical scavenging activity (IC50 2.90 ± 0.96).

Galani at al evaluated the antioxidant activity of the extract was evaluated using five in vitro assays and was compared to standard antioxidant (Ascorbic acid). Further, Total phenolic contents of the extract were determined by using FolinCiocalteu method in order to evaluate a relationship between the antioxidant activity and the phytochemical constituents. The total phenolic content was found to be 121.45 ± 2.56 µg Gallic acid equivalent of phenol. The extract and ascorbic acid were found to have different levels of antioxidant activity in the systems tested. Methanolic extract of Grangea maderaspatana (GMME) exhibited significant anti oxidant activity.

Actylcholine-esterase inhibitory property

Bhubanendrasingh at al characterizedthe volatile components (non-polar compounds) of g. maderaspatana and evaluated its acetylcholinesterase inhibition potential. The essential oils (yield 0.06% v/w) were obtained from fresh aerial part of G. maderaspatana. The characterization of volatile components (non-polar compounds) was performed by GC-MS data and with those of reference compounds compiled in the spectral library of in-house database. The in vitro acetylcholinesterase (AChE) inhibition of the volatile organic constituents (VOC's) of G. maderaspatana aerial part was evaluated in varying concentration ranges (0.70-44.75 µg/mL) with Ellman's method. The major components were α-humulene (46.3%), β-caryophyllene (9.3%), α-copaene (8.2%), β-myrcene (4.3%), Z(E)-α-farnesene (3.7%), and calarene (3.5%). Chemical variability among other Artemisia spp. from different climatic regions of India and countries namely Iran and France was observed. The experimental results showed that diverse volatile organic constituents of A. maderaspatana have significant acetylcholi-nesterase inhibitory activity (an IC50 value of 31.33 ± 1.03 µg/mL). This is the first report on the inhibition of acetylcholinesterase properties of essential oil of G. maderaspatana obtained from fresh aerial part. The present results indicate that essential oil of G. maderaspatana isolated from the northern region of India could inhibit AChE moderately. Therefore, the possibility of novel AChE inhibitors might exist in VOCs of this plant.

Cerebral blood flow and vasodilation.

Adequate perfusion of blood to capillary beds within the brain is of utmost importance. Otherwise, deficits of oxygen and nutrients will ensue alongside the buildup of cytotoxic waste. Diminished cerebral blood flow is implicated in various pathologies, including dementia. Kamkaew et al. compared the effect of daily oral BM (40 mg/kg oral) and Gingko biloba (60 mg/kg oral) on cerebral blood flow (CBF) in rats. In their 8-week trial, rats treated with BM saw a significant 25% increase in CBF, although Gingko biloba increased CBF by 29% (albeit at a 20-mg higher dosage). Chronic oral BM administration had no effect on blood pressure, whereas intravenous infusion decreased diastolic blood pressure *31 mmHg with 40 mg/kg of either extract, correspondingly decreasing CBF by 15%. BM appears to act as a vasodilator by releasing NO from the endothelium and inhibiting calcium fluctuations in and out of the sarcoplasmic reticulum. More research on this property of g.maderaspatana need to be explored.

Neurotransmitter potentiation. Adaptogens enable the body to better cope with the deleterious mental and physical consequences of stress. Eleutherococcussenticosus (Siberian ginseng), Rhodiola rosea, and Panax ginseng are classic adaptogens. Others include Ocimum sanctum (Sweet Holy Basil or Tulsi), Withaniasomnifera (Ashwaghanda), Astragalus propinquus, Ganoderma lucidum (Reishi mushroom), and many others.98 BM also exhibits adaptogenic qualities. One putative action of the adaptogen is modulation of neurotransmitter production, release, and synaptic concentration.

Dementia and cognitive dysfunction. Dementia is a global loss of cognitive ability. Aging is a major risk factor for dementia, which includes various types, such as vascular dementia, frontotemporal degenerative dementia, Lewy body dementia, and Alzheimer disease. Dementia results secondarily from many neurodegenerative disorders. The exact etiology of Alzheimer dementia is uncertain and controversial, but there is a general consensus about some of the factors that may be involved. Free radical-induced OS is one such

Conclusion

World wide, the neurodegenerative disorders like alzeimers, dementia, parkinsonism, huntington's chorea, remain a challenge to researchers. Plants are an invaluable source of chemical compounds which could cure many diseases. Our review showed that g.maderaspatana, belongs to the family asteracaceae, an important family of medicinal plants, is used in search for medicinal plants.this plant contains phytochemicals that could affectbrain dopaminergic, cholinergic and serotonergic systems, which are involved in the pathogenesis of alzeimers, parkinsonism, autism and depression or anxiety. The conventional and therapeutic application of g.maderaspatana(L).poir, with its phytochemical profile need to be explored further based on its different activities.

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R E S E A R C H A R T I C L E

Method Development and Validation for the Simultaneous estimation of Velpatasvir and Sofosbuvir in tablet dosage form by using RP – HPLC

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A B S T R A C T

The present research work mainly focused on method development and validation for the simultaneous estimation of velpatasvir and sofosbuvir in tablet dosage form by using RP-HPLC. Chromatographic separation was carried on YMC C_{18} column (4.6*150mm, 5µ) and it maintained flow rate was 1.0 ml/min. The mobile phase consist of phosphate buffer pH 3.0 was adjusted with ortho phosphoric acid and acetonitrile with the ratio of 50:50%v/v, then UV spectrum was monitored at 255 nm. The retention time was found to be 3.472 min for velpatasvir and 5.505 min for sofosbuvir. The method shows linear response in the range of concentration was 50-250µg/ml for velpatasvir and 200-1000 µg/ml for sofosbuvir respectively. The developed method was stastically validated according to ICH guidelines. The validation parameters are present in with in the limit. So, the developed method was simple, precise, accurate and robust. It can be suggest that routine quality control analysis of analytical laboratories.

Keywords: Velpatasvir, Sofosbuvir, Mobile phase, Validation, ICH guidelines

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C O N T E N T S

1. Introduction

Valpatasvir and Sofosbuvir is a pangenotypic NS5A-NS5B inhibitor single pill combination regimen potent activity against hepatitis C virus (HCV) genotypes 1,2,3,4,5 and 6. It provides a much needed option for patients with HCV genotype 3 infection4,5. Treatment with combined sofosbuvir and velpatasvir has resulted in high sustained virological response rates in patients chronically infected with hepatitis C virus (HCV) with genotypes 1–6 in clinical trials and real-world settings, but its efficacy and safety has not been assessed in Asia, a region with diverse HCV genotypes.with compensated cirrhosis [4,5].

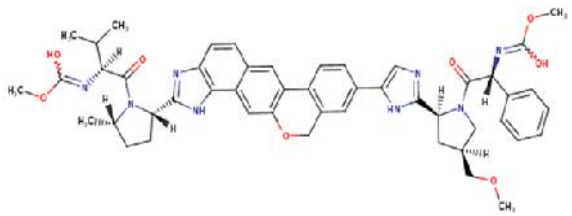

Fig 1: Structure of Valpatasvir

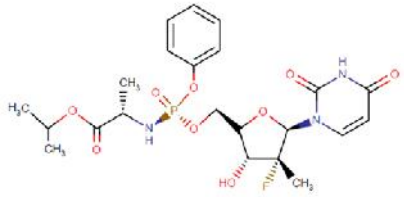

Fig 2: Structure of Sofosbuvir

The earlier literature reveals the analytical methods like UV and HPLC were reproted for determination of Velpatasvir and Sofosbuvir individually and other combinations[8,9,10]. Therefore the present study has been undertaken in order to develop new, simple, rapid, efficient and reproducible method for the analysis of Velpatasvir and Sofosbuvir.

2. Materials and Methods

Instruments used:

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemical used:

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Velpatasvir	PHARMATRAIN
2	Sofosbuvir	PHARMATRAIN
3	KH_2PO_4	FINER chemical LTD
4	Water and Methanol for HPLC	LICHROSOLV (MERCK)
5	Acetonitrile for HPLC	MOLYCHEM
6	Ortho phosphoric Acid	MERCK

Method development

Preparation of mobile phase:

Accurately measured 500 ml (50%) of above buffer and 500 ml of Acetonitrile HPLC (50%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 100 mg of Velpatasvir and 400 mg of Sofosbuvir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer 100 mg of Velpatasvir and 400 mg of Sofosbuvir working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Wave length selection:

UV spectrum of 10µg/ml Velpatasvir and 10 µg/ml Sofosbuvir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 255 nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. YMC (4.6 x 150mm, 5 m) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimized chromatographic conditions:

Instrument used **:** Waters HPLC with auto sampler and UV detector
Temperature **:** Ambient (25^{0}C)
Mode of separation**:**Isocratic mode
Column **:** YMC column (4.6*150mm, 5µ)
Mobile phase **:** 50% phosphate buffer (pH 3.0) 50% Acetonitrile
Flow rate **:**1.0 ml /min
Wavelength **:**255 nm
Injection volume**:** 20 µl
Run time **:** 10 min.

System Suitability:

Tailing factor for the peaks due to Velpatasvir and Sofosbuvir in Standard solution should not be more than 2.0. Theoretical plates for the Velpatasvir and Sofosbuvir peaks in Standard solution should not be less than 2000. Resolution for the Velpatasvire and Sofosbuvir in Standard solution should not be less than 2.0.

Method validation

Method validation parameters like specificity, linearity, accuracy, precision and robustness. The validation was done for the according to ICH guidelines Q2 (R1)[11,12].

Linearity:

From the standard stock solution five different aliquot's are prepared and obtained concentration range from 50-250µg/ml for velpatasvir and 200-1000 µg/ml for sofosbuvir. Inject the each aliquot into the chromatographic system and measure the peak area. Then Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision:

Intraday and intermediate precision was performed for the estimation of valpatasvir and Sofosbuvir. The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

For accuracy determination, three different spiked concentrations were prepared separately i.e. 50%, 100% and 150% standard solutions were injected into the chromatographic system. Calculate the Amount found and Amount added for Velpatasvir & Sofosbuvir and calculate the individual recovery and mean recovery values.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 0.9 ml/min to 1.1ml/min:

Standard solution 150 ppm of Velpatasvir & 600 ppm of Sofosbuvir was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The Organic composition in the Mobile phase was varied from ±10%:

Standard solution 150 ppm of Velpatasvir & 600 ppm of Sofosbuvir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10.

3. Results and Discussions

System suitability:

All the system suitability parameters for developed method were within the limit. The results were shown in table 3 and fig 3.

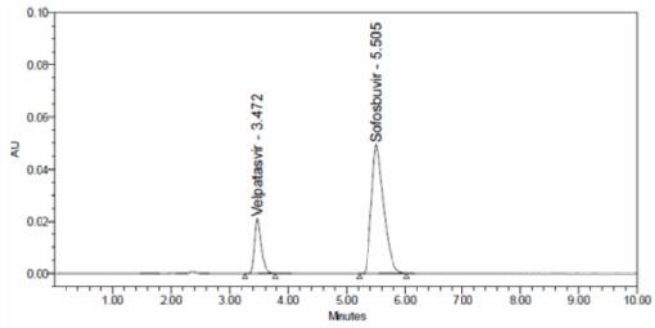

Fig 3: Chromatogram for System suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

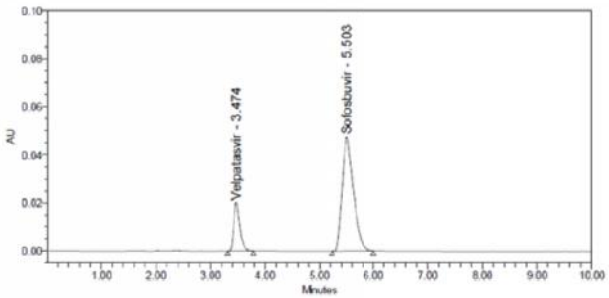

Fig 4: Chromatogram for Standard

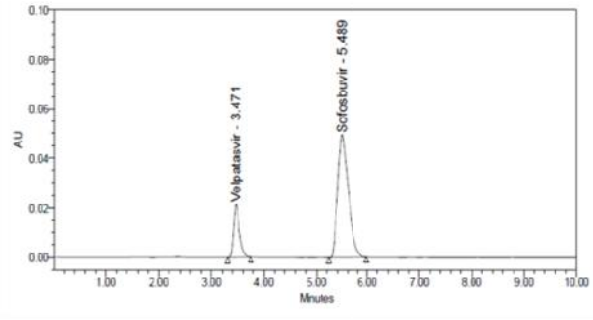

Fig 5: Chromatogram for Sample

Linearity:

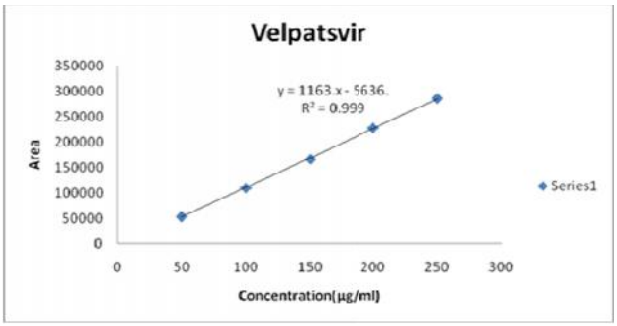

Fig 6: Calibration graph for Velpatasvir

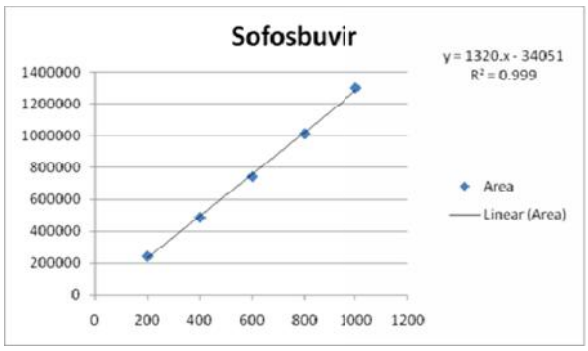

Fig 7: Calibration graph for Sofosbuvir

Precision:

Precision of the method was carried out for both sample solutions as described under experimental work. The results were reported in table 6 &7.

Accuracy:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. Results are shown in table 8 & 9.

Robustness:

The standard and samples of Velpatasvir and Sofosbuvir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The results were shown in table 11 & 12.

Table 3: Results of system suitability parameters

S. No	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Velpatasvir	3.472	161234	21536	2.81	1.36	4822.40
2	Sofosbuvir	5.505	747339	36534		1.26	4722.40

Table 4: Results of Assay for Velpatasvir and Sofosbuvir

Drug	Label Claim (mg)	% Assay
Velpatasvir	100	100.08
Sofosbuvir	400	99.97

Table 5: Linearity results for Velpatasvir and Sofobusvir

		Valpatasvir		Sofosbuvir	
S. No	Linearity Level	Concentration	Area	Concentration	Area
1	I	50	53953	200	243401
2	II	100	110011	400	488042
3	III	150	166601	600	744612
4	IV	200	227887	800	1013904
5	V	250	285840	1000	1300811
Correlation Coefficient			0.999	0.999	

Table 6: Method precision results of Velpatasvir and Sofosbuvir

Injection	Area for Velpatasvir	Area for Sofosbuvir
Injection-1	161345	747339
Injection-2	161232	746432
Injection-3	161671	747131
Injection-4	161999	747399
Injection-5	162898	747018
Injection-6	164679	747649
Average	162304.0	747161.3
Standard Deviation	1308.1	419.3
%RSD	0.8	0.1

Table 7: Intermediate Precision for Velpatasvir and Sofosbuvir

Injection	Area for Velpatasvir	Area for Sofosbuvir
Injection-1	162345	744533
Injection-2	162432	747232
Injection-3	162971	744531
Injection-4	162899	744399
Injection-5	162898	744018
Injection-6	162333	744689
Average	162646.3	744900.3
Standard Deviation	305.8	1164.7
%RSD	0.2	0.2

Table 8: The accuracy results for Velpatasvir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	80505	50	49.72	99.43	100.08%
100%	161649	100	99.82	99.82	
150%	245309	150	151.49	100.99	

Table 9: The accuracy results for Sofosbuvir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	373486	200	199.69	99.84	100.02%
100%	746639	400	398.74	99.68	
150%	1129342	600	603.12	100.52	

Table 10: Results of LOD & LOQ

Parameter	Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
LOD	Velpatasvir	58	174	3.00
	Sofosbuvir	58	173	2.98
LOQ	Velpatasvir	58	579	9.98
	Sofosbuvir	58	580	10.00

Table 11: Results for variation in flow for Velpatasvir and Sofosbuvir

Drug	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
Velpatasvir	0.9	4353.29	1.30
Sofosbuvir		3433.94	1.42
Velpatasvir	1	4822.40	1.36
Sofosbuvir		4722.40	1.26
Velpatasvir	1.1	4543.29	1.40
Sofosbuvir		3863.94	1.44

Table 12: Results for variation in mobile phase composition for Velpatasvir and Sofosbuvir

Drug	Change in Organic Composition in the Mobile Phase	System Suitability Results		USP Resolution
		USP Plate Count	USP Tailing	
Velpatasvir	10% less	4543.29	1.40	9.50
Sofosbuvir		3863.94	1.44	
Velpatasvir	*Actual	4822.40	1.36	9.50
Sofosbuvir		3115.92	1.12	
Velpatasvir	10% more	4543.29	1.40	9.50
Sofosbuvir		3863.94	1.44	

***** Results for actual Mobile phase composition have been consideredfrom Accuracy standard.

4. Conclusion

The estimation of Velpatasvir and Sofosbuvir was done by RP-HPLC. The assay of Velpatasvir and Sofosbuvir was performed with tablets and the % assay was found to be 100.08 and 99.97 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.1for Velpatasvir and Sofosbuvir which shows that the method is precise. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.08% and 100.02% for Velpatasvir and Sofosbuvir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The LOD and LOQ for Velpatasvir was found to be 3.0 and 9.98 and LOD and LOQ for Sofosbuvir was found to be 2.98 and10.00. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

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RESEARCH ARTICLE

Simultaneous estimation and validation of Lumacaftor and Ivacaftor in the tablet dosage form using RP-HPLC method

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ABSTRACT

The aim present research work to development and validation of RP-HPLC method for the simultaneous estimation of Lumacaftor and Ivacaftor. Chromatographic separation was evaluated by Phenomenex C18 column (250 X 4.6 mm, 5 µ) using the mobile phase consisting of Phosphate buffer and Acetonitrile in the ratio of 40:60%v/v (pH was adjusted to 4.5 with O-phosphoric acid). The mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 255 nm. The retention time of Lumacaftor and Ivacaftor were found to be 2.857min and 6.329 min. The linearity was obtained in the range of 100-500µg/ml for lumacaftor and 62.5-312.5 µg/ml for Ivacaftor with correlation coefficient was 0.999. The proposed method was found to be simple, accurate, precise, robust and cost effective. It can be applied for routine quality control analysis for simultaneous estimation of Lumacaftor and Ivacaftor in pharmaceutical dosage forms.

Keywords: Lumacaftor, Ivacaftor, RP-HPLC, Mobile phase, Acetonitrile, Retention time

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CONTENTS

1. Introduction

Ivacaftor is a drug used to treat cystic fibrosis in people with certain mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, who account for 4–5% cases of cystic fibrosis. Cystic fibrosis is caused by any one of several defects in a protein, cystic fibrosis. Trans membrane conductance regulator, which regulates fluid flow within cells and affects the components of sweat, digestive fluids, and mucus. It is a potentiator of the CFTR protein. The CFTR protein is a chloride channel present at the surface of epithelial cells in multiple organs. Ivacaftor

facilitates increased chloride transport by potentiating the channel-open probability (or gating) of the G551D-CFTR protein[1,2].

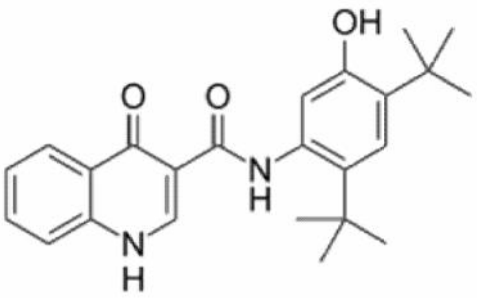

Fig 1: Chemical Structure of Ivacaftor

Lumacaftor/ivacaftor (brand name Orkambi) is a combination drug available a single pill that is used for the treatment of cystic fibrosis in people who have the F508del mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. It is a combination drug that consists of lumacaftor and ivacaftor. Ivacaftor increases the activity of the CFTR protein at the surface of epithelial cell, while lumacaftor acts as a chaperone during protein folding and increases the number of CFTR proteins that are trafficked to the cell surface[1,2]. It was approved by the US FDA in July 2015.

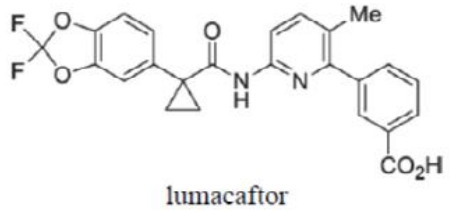

Fig 2: Chemical Structure of Lumacaftor

Literature reveals different methods for their analysis in their formulations. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated[3,4,5,6].

2. Materials and Methods

Instruments used:

The following instruments are used to determination of Lumacaftor and Ivacaftor.

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

The following chemicals are used to determination of Lumacaftor and Ivacaftor.

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Lumacaftor	PHARMATRAIN
2	Ivacaftor	PHARMATRAIN
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	MOLYCHEM
5	Ortho phosphoric Acid	MERCK

HPLC Method development

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to orthophosphoric acid with buffer (pH 4.5), Acetonitrile in proportion 40: 60 v/v respectively.

Wave length selection:

UV spectrum of 10 µg/ml Lumacaftor and Ivacaftor in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 255nm. At this wavelength both the drugs show good absorbance.

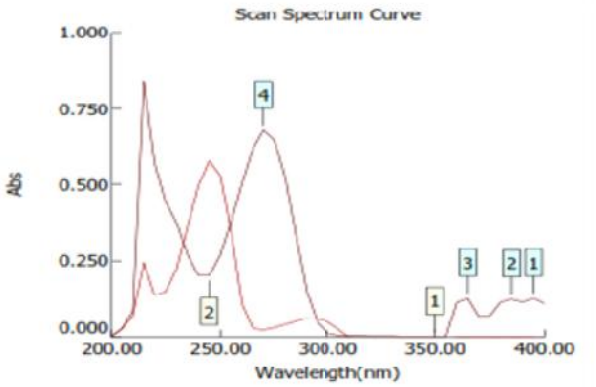

Fig 3: UV Spectra of Lumacaftor and Ivacaftor

Optimized Chromatographic Conditions:

Instrument used **:**Waters HPLC with auto sampler and 2487 UV detector.
Temperature **:**Ambient
Column **:** Phenominex (4.6 x 250mm, 5µm)
Buffer **:**1ml of orthophosphoric acid in 1000ml water, pH adjusted with NaOH.
pH **:** 4.5
Mobile phase **:**40% buffer 60% Acetonitrile
Flow rate **:**1.0 ml/min
Wavelength **:**255 nm
Injection volume **:**10 µl
Run time **:** 10 min
.

Preparation of mobile phase:

Accurately measured 400 ml (40%) of above buffer and 600 ml of Acetonitrile HPLC (60%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 20 mg of Lumacaftor and 12.5 mg of Ivacaftor working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 1000 mg of Lumacaftor and 10mg Ivacaftor (marketed formulation=1250.08 mg of tablet Powder) sample into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.44 micron Injection filter (Stock solution). Further pipette 1.5 ml of Lumacaftor and Ivacaftor from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability Parameter:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established.

Acceptance criteria:

- Resolution between two drugs must be not less than 2.
- Theoretical plates must be not less than 2000.
- Tailing factor must be not more than 2.

Method Validation

The developed method was statically validated according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision, LOD & LOQ and robustness[10,11].

Linearity:

The linearity was determined for Lumacaftor and Ivacaftor six different concentrations were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity concentrations are made from in the range of 100-500µg/ml for Lumacaftor and 62.5-312.5µg/ml for Ivacaftor.

Precision:

The standard solution was injected for six times and measured the area for all six Injections in HPLC. It was done for the within the day and between the days with same chromatographic conditions.The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

Percentage mean recovery was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels such as 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

LOD & LOQ:

The sensitivity of the proposed method for measurement of Lumacaftor and Ivacaftor were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

a) The flow rate was varied at 0.9 ml/min to 1.1ml/min:

Standard solution 300 ppm of Lumacaftor & 187.5 ppm of Ivacaftor was prepared and analysed using the varied flow rates along with method flow rate.

b) The Organic composition in the Mobile phase was varied from 50% to 50%:

Standard solution 300 ppm of Lumacaftor & 187.5 ppm of Ivacaftor was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Forced degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. In present research work the standard solutions of Lumacaftor and Ivacaftor were placed in different stress conditions like acid, base, peroxide, thermal and photolytic condtions. Then observe the solutions in some period of time and calculate the percentage amount of drug degraded in above stress conditions.

3. Results and discussion

System suitability: The specificity of this method was determined by complete separation of Lumacaftor and Ivacaftor. The tailing factor was less than 2% and resolution was satisfactory. The peaks obtained for sharp and have clear baseline separation. The system suitability parameters are given in Table 3.

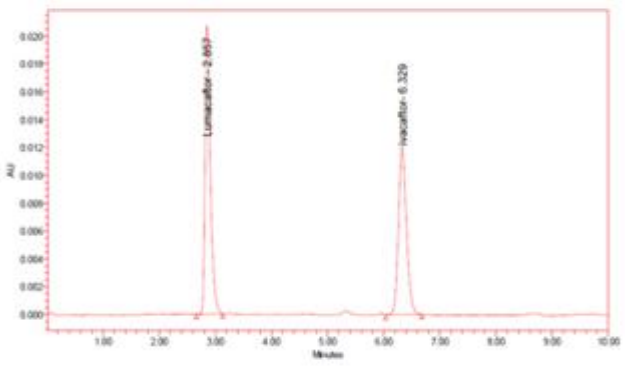

Fig 4: Chromatogram for system suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity:

The linearity range was found to lie from 100µg/ml to 500µg/ml of Lumacaftor, 62.5µg/ml to 312.5µg/ml of Ivacaftor and then plotting the graph concentration Vs peak area. The correlation coefficient was found to be 0.999 for both. The results were reported in table 5 and fig 5&6.

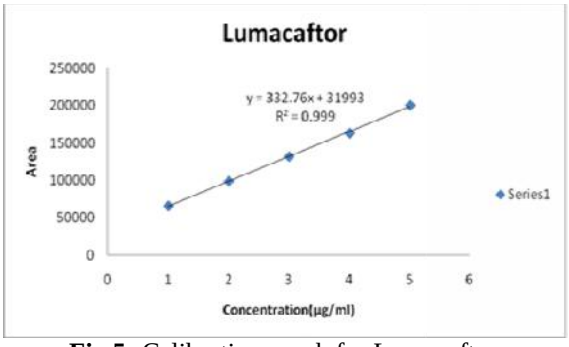

Fig 5: Calibration graph for Lumacaftor

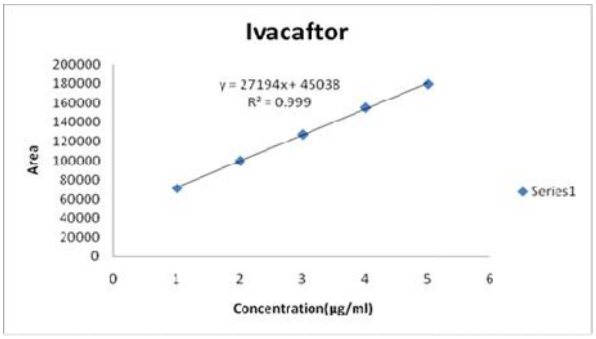

Fig 6: Calibration graph for Ivacaftor

Precision: Precision of the method was carried out for both sample solutions as described under experimental work. The % RSD was found to be less than 2%. The data was shown in table 6.

Accuracy:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The mean percentage recovery was found to be 100.53 for lumacaftor and 100.13 for ivacaftor. It was present in within the limit. The data was shown in table 7&8.

Robustness: The standard and samples of Lumacaftor and Ivacaftor were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. All the paremeters are present in between the limit. The results were shown in table 10&11.

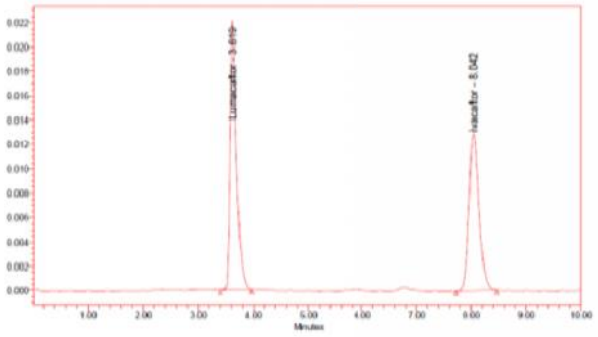

Fig 7: Chromatogram showing less flow

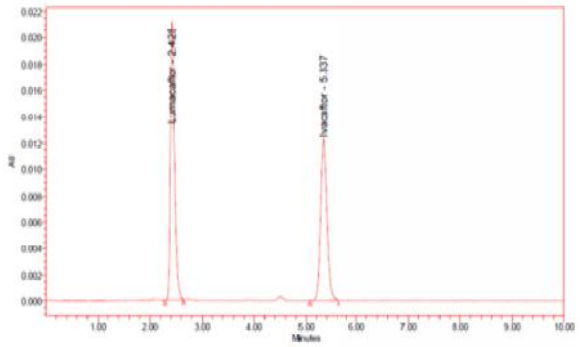

Fig 8: Chromatogram showing more flow

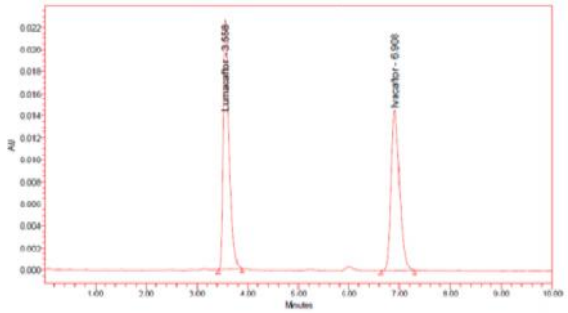

Fig 9: Chromatogram showing less organic composition

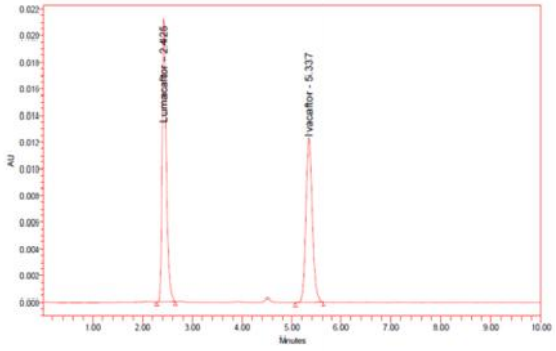

Fig 10: Chromatogram showing more organic composition

Table 3: Results of system suitability parameters

S.No	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Lumacaftor	2.857	134796	20824		1.47	4509.57
2	Ivacaftor	6.329	120104	12053	16.02	1.15	9239.89

Table 4: Results of Assay for Lumacaftor and Ivacaftor

Drug	Label Claim (mg)	% Assay
Lumacaftor	200	100.39
Ivacaftor	125	100.17

Table 5: Area of different concentration of Lumacaftor and Ivacaftor

S. No	Lumacaftor		Ivacaftor	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	100	65792	62.5	71267
2	200	98696	125	99725
3	300	131638	187.5	127369
4	400	162911	250	155275
5	500	200063	312.5	179461

Table 6: Results for intraday and inter day precision

Injection	Intraday precision		Intermediate precision	
	Peak area of Lumacaftor	Peak area of Ivacaftor	Peak area of Lumacaftor	Peak area of Ivacaftor
Injection-1	141368	128876	139453	122535
Injection-2	140717	127224	137162	121224
Injection-3	142655	129055	139458	122915
Injection-4	143939	128739	138377	123391
Injection-5	143013	126699	138482	123108
Injection-6	142282	129220	139771	122959
Average	142329.0	128302.2	138783.8	122688.7
Std Dev	1156.8	1064.1	976.1	769.7
%RSD	0.8	0.8	0.7	0.6

Table 7: Accuracy results for Lumacaftor

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	67838.3	10	10.00	100.02	100.53%
100%	136568.7	20	20.13	100.67	
150%	205309.3	30	30.27	100.90	

*Average of three determinations

Table 8: Accuracy results for Ivacafttor

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	60620.7	6.25	6.27	100.37	100.13%
100%	121845	12.5	12.6	100.87	
150%	179676.0	18.75	18.59	99.16	

*Average of three determinations

Table 9: Results for LOD & LOQ

Parameter	Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
LOD	Lumacaftor	66	198	3.00
	Ivacaftor	66	199	3.02
LOQ	Lumacaftor	66	659	9.98
	Ivacaftor	66	660	10.00

Table 10: Results for variation in flow for Lumacaftor and Ivacaftor

S. No	Flow Rate (ml/min)	System Suitability Results			
		Lumacaftor		Ivacaftor	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing

1	0.9	4685.09	1.12	4731.46	1.21
2	1.0	4509.7	1.47	4509.7	1.47
3	1.1	4065.51	1.40	4549.3	1.12

***** Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 11: Results for variation in mobile phase composition

S. No	Variation in mobile phase ratio	System Suitability Results			
		Lumacaftor		Ivacaftor	
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing
1	10% less	4382.7	1.12	4643.64	1.26
2	*Actual	4509.7	1.47	4509.7	1.47
3	10% more	4982.7	1.17	4987.28	0.95

***** Results for actual Mobile phase composition have been consideredfrom Accuracy standard.

Table 12: Degradation results for Lumacaftor and Ivacaftor

Sample Name	Lumacaftor		Ivacaftor	
	Area	% Degraded	Area	% Degraded
Standard	135383.3	-	121004.3	-
Acid	125453	7.33	115289	4.72
Base	127849	5.57	117420	2.96
Peroxide	125131	7.57	113076	6.55
Thermal	128347	5.20	113704	6.03
Photo	129359	4.45	116820	3.46

4. Conclusions

The proposed method was simple, specific, precise and accurate can be used for simultaneous analysis Lumacaftor and Ivacaftor in bulk samples and its dosage form. The result of the study follows the protocol of ICH guidelines and it can be successfully applied for the simultaneous estimation of the marketed products of Lumacaftor and Ivacaftor in bulk samples and its combined dosage form.

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R E S E A R C H A R T I C L E

Development of new simultaneous RP-HPLC method for the estimation of Dapagliflozin and Saxagliptin in tablet dosage form

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A B S T R A C T

The aim of present research work made to develop and validate RP-HPLC method for the simultaneous estimation of Dapagliflozin and Saxagliptin in bulk and combined dosage form. The optimized mobile phase was consists of 0.1% OPA: Methanol: Acetonitrile (30: 60: 10) and chromatographic separation was carried on Xterra C 18, column (4.6*150mm, 5µ). The detection of absorption maxima was monitored at 225nm. The flow rate was maintained at 1.0 ml/min. The linear concentration Dapagliflozin and Saxagliptin were found to be from 20-100 µg/ml and 10-50 µg/ml with regression coefficient was 0.999 for both drugs. The values of % RSD are less than 2% indicating accuracy and precision of the method. The mean percentage recovery was found to be 100.64% of Dapagliflozin and 100.36% of Saxagliptin. The proposed method is precise, simple and accurate to determine the amount of Dapagliflozin and Saxagliptinin formulation. So the method can be useful in the routine quality control of these drugs.

Keywords: Dapagliflozin and Saxagliptin, RP-HPLC, Mobile phase, Accuracy, Regression coefficient

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CONTENTS

1. Introduction

Dapagliflozin is indicated for the management of diabetes mellitus type 2, and functions to improve glycemic control in adults when combined with diet and exercise. Dapagliflozin is a sodium-glucose co transporter 2 inhibitor, which prevents glucose reabsorption in the kidney. Using dapagliflozin leads to heavy glycosuria (glucose excretion in the urine), which can lead to weight loss and tiredness. Dapagliflozin was approved by the FDA on Jan 08, 2014. Dapagliflozin is not recommended for patients with type 1 diabetes mellitus or for the treatment of diabetic ketoacidosis[1].

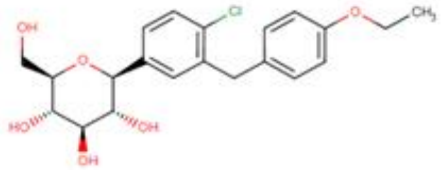

Fig 1: Chemical structure of Dapagliflozin

Saxagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor antidiabetic for the treatment of type 2 diabetes. DPP-4 inhibitors are a class of compounds that work by affecting the action of natural hormones in the body called incretins. Incretins decrease blood sugar by increasing consumption of sugar by the body, mainly through increasing insulin production in the pancreas, and by reducing production of sugar by the liver. DPP-4 is a membrane associated peptidase which is found in many tissues, lymphocytes and plasma. DPP-4 has two main mechanisms of action, an enzymatic function and another mechanism where DPP-4 binds adenosine deaminase, which conveys intracellular signals via dimerization when activated. The inhibition of DPP-4 increases levels active of glucagon like peptide 1 (GLP-1), which inhibits glucagon production from pancreatic alpha cells and increases production of insulin from pancreatic beta cells[2].

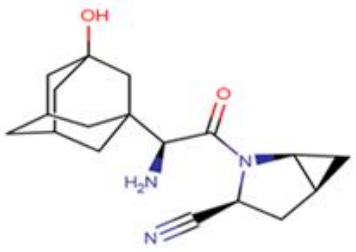

Fig 2: Chemical structure of Saxagliptin

Dapagliflozin and Saxagliptin existing drugs. Literature reveals different methods for their analysis in their formulations3,4,5. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used:

The following instruments are used to determination of Dapagliflozin and Saxagliptin.

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

The following chemicals are used to determination of Dapagliflozin and Saxagliptin.

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Saxagliptin	PHARMATRAIN
2	Dapagliflozin	PHARMATRAIN
3	KH_2PO_4	FINER chemical LTD
4	Water and Methanol for HPLC	LICHROSOLV (MERCK)
5	Acetonitrile for HPLC	MOLYCHEM
6	Ortho phosphoric Acid	MERCK

Wave length selection:

UV spectrum of 10 µg/ml Saxagliptin and 10 µg/ml Dapagliflozin in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 225 nm. At this wavelength both the drugs show good absorbance.

HPLC Method Development

Preparation of mobile phase:

Accurately measured 300 ml (30%) of 0.1% OPA Buffer, 600 ml (60%) of Methanol and 100 ml (10%) of Acetonitrile were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 10 mg of Saxagliptin and 20 mg of Dapagliflozin working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 10 mg of Saxagliptin and 20 mg of Dapagliflozin sample into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Saxagliptin and Dapagliflozin.

Optimized Chromatographic Conditions:

Instrument used **:** HPLC with auto sampler and UV detector.
Temperature **:**Ambient (25°C)
Mode of separation **:**Isocratic mode
Column **:** Xterra C 18, column (4.6*150mm, 5µ)
Buffer **:**0.1% OPA
Mobile phase **:**0.1% OPA: Methanol: Acetonitrile (30: 60: 10)
Flow rate **:** 1 ml per min
Wavelength **:**225 nm
Injection volume **:** 20 µl
Run time **:** 15 min.

System Suitability:

System suitability is an integral part of many analytical procedures. The system suitability parameters such as theoretical plates, tailing factor and resolution. Tailing factor for the peaks due to Saxagliptin and Dapagliflozin in Standard solution should not be more than 2.0. Theoretical plates should not be less than 2000. Resolution should not be less than 2.

Method Validation

Method validation was done for the according ICH guidelines Q2 (R1). The validation parameters like linearity, specificity, accuracy, precision, LOD & LOQ and robustness[11,12].

Linearity:

For determination of linearity five different concentrations i.e. 25%, 50%, 100%, 125%, 150% were prepared and injected in triplicate. Then plotting the graph concentration Vs peak area and measure the correlation coefficient. It should not more than 0.999.

Precision:

The standard and sample solutions were injected into the five times in intraday and inter day, the peak areas were recorded. The mean and percentage relative standard deviation were calculated from the peak area.

Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same. Each solution was injected three times under optimized conditions and then calculates the mean percentage recovery.

LOD & LOQ:

The sensitivity of the proposed method for measurement of dapagliflozin and saxagliptin were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 0.9 ml/min to 1.1ml/min:

Standard solution 30 ppm of Saxagliptin & 60 ppm of Dapagliflozin was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The Organic composition in the Mobile phase was varied from ±10%:

Standard solution 30 ppm of Saxagliptin & 60 ppm of Dapagliflozin was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10.

3. Results and discussion

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in fig 3 & 4.

Table 3: Assay Results

Drug	Label Claim (mg)	% Assay
Dapagliflozin	10	100.53
Saxagliptin	5	100.10

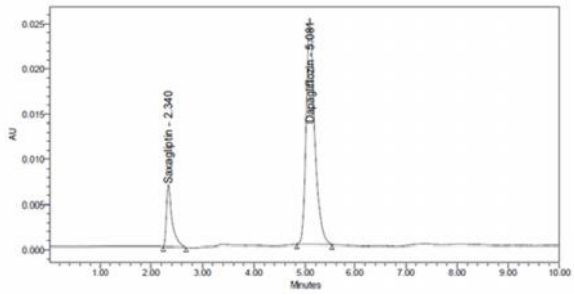

Fig 3: Chromatogram for Standard

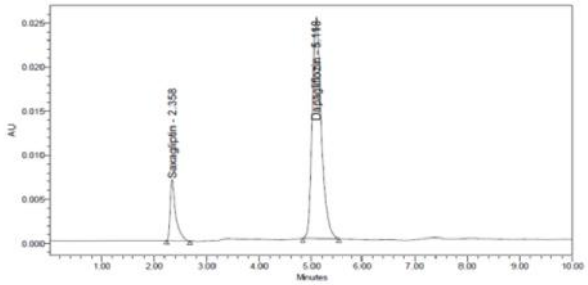

Fig 4: Chromatogram for Sample

System Suitability:

The system suitability of the method was checked by injecting five different preparations of the Dapagliflozin and Saxagliptin standard. The parameters of system suitability were checked. It was found from above data that all the system suitability parameters for developed method were within the limit. The results were shown in table 4.

Linearity:

The linearity range was found to lie from 20µg/ml to 100µg/ml of Dapagliflozin, 10µg/ml to 50µg/ml of Saxagliptin and chromatograms are shown in table 5.

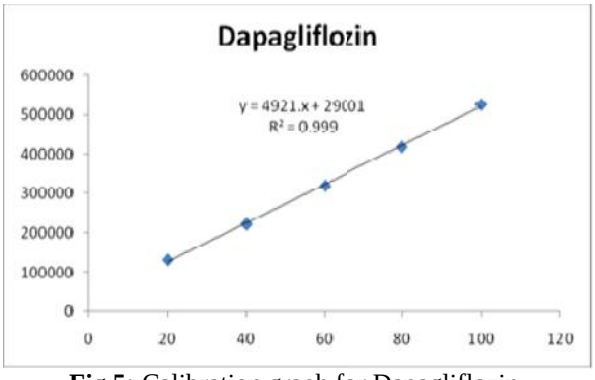

Fig 5: Calibration graph for Dapagliflozin

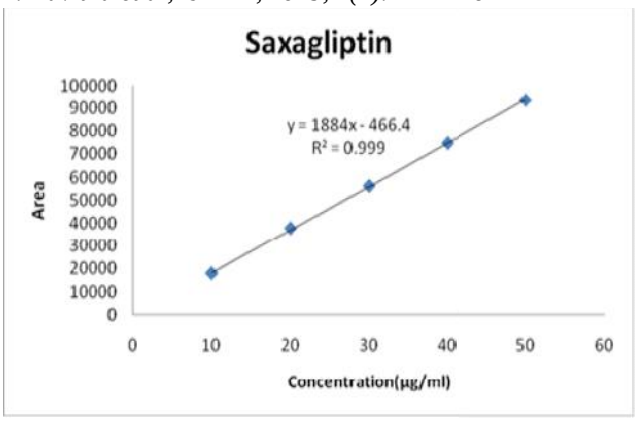

Fig 6: Calibration graph for Saxagliptin

Precision:

Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results were reported in table 6.

Accuracy: Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results are shown in table 7 & 8.

Robustness:

The standard and samples of Dapagliflozin and Saxagliptin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The results are shown in table 10 &11.

Table 4: Results of system suitability parameters

S. No.	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Saxagliptin	2.347	56445	6857	11.53	1.41	2593.29
2	Dapagliflozin	5.086	320903	25250		1.18	4843.11

Table 5: Area of different concentration of Dapagliflozin and Saxagliptin

S. No	Dapagliflozin		Saxagliptin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	20	132359	10	17896
2	40	223105	20	37780
3	60	320315	30	56233
4	80	419173	40	74754
5	100	526461	50	93611
Slope (m)		4921	-	1884
Intercept (c)		29001	-	466.4
Correlation coefficient (R^2)		0.999	-	0.999

Table 6: Results of Precision for Dapagliflozin and Saxagliptin

Injection	Intraday precision		Inter day precision	
	Area for Dapagliflozin	Area for Saxagliptin	Area for Dapagliflozin	Area for Saxagliptin
Injection-1	318752	56407	316450	56082
Injection-2	316862	56050	318607	56734
Injection-3	320903	56444	316347	56133
Injection-4	315150	56445	319509	56124
Injection-5	320979	56203	319175	56948
Injection-6	316258	56139	317693	56919
Average	318150.7	56281.3	317963.5	56490.0
Std Dev	2457.0	172.6	1359.9	419.8
%RSD	0.8	0.3	0.4	0.7

Table 7: Accuracy (recovery) data for Dapagliflozin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	161058.3	10	10.04	100.39	100.64%
100%	323719.3	20	20.18	100.89	
150%	484374.0	30	30.19	100.64	

*Average of three determinations

Table 8: Accuracy (recovery) data for Saxagliptin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	28244.7	5	5.01	100.26	100.36%
100%	56457.3	10	10.02	100.20	
150%	85035.3	15	15.09	100.61	

Table 9: Results of LOD & LOQ

Parameter	Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
LOD	Dapagliflozin	43	130	3.02
	Saxagliptin	43	129	3.00
LOQ	Dapagliflozin	43	433	10.07
	Saxagliptin	43	431	10.02

Table 10: Results for variation in flow for Saxagliptin and Dapagliflozin

Drug	Flow Rate (ml/min)	System Suitability Results		USP Resolution
		USP Plate Count	USP Tailing	
Saxagliptin	0.9	3067.03	1.29	12.44
Dapagliflozin		5361.12	1.16	
Saxagliptin	1.0	2589.12	1.44	11.46
Dapagliflozin		4825.77	1.19	
Saxagliptin	1.1	2526.15	1.27	11.48
Dapagliflozin		4766.36	1.13	

Table 11: Results for variation in mobile phase composition for Saxagliptin and Dapagliflozin

Drug	Change in Organic Composition in the Mobile Phase	System Suitability Results		USP Resolution
		USP Plate Count	USP Tailing	
Saxagliptin	10% less	3078.29	1.19	12.97
Dapagliflozin		12.97	4573.25	
Saxagliptin	*Actual	2589.12	1.44	11.46
Dapagliflozin		11.46	4825.77	
Saxagliptin	10% more	2521.39	1.19	9.37
Dapagliflozin		9.37	4756.36	

*****Results for actual Mobile phase composition have been consideredfrom Accuracy standard.

Table 12: Results for Stability of Dapagliflozin and Saxagliptin

Sample Name	Dapagliflozin		Saxagliptin	
	Area	% Degraded	Area	% Degraded
Standard	320211.3		56232.7	
Acid	295636	7.67	54275	3.48
Base	302783	5.44	52453	6.72
Peroxide	289767	9.51	53967	4.03
Thermal	316254	1.24	51867	7.76
Photo	286735	10.45	50162	10.80

4. Conclusions

The simultaneous estimation of Dapagliflozin and Saxagliptin was done by RP-HPLC.The assay of Dapagliflozin and Saxagliptin was performed with tablets and the % assay was found to be 100.53 and 100.10 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The method show precision 0.8 and 0.3 and intermediate precision 0.4 and 0.7 for Dapagliflozin and Saxagliptin which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.64% and 100.36% for Dapagliflozin and Saxagliptin. The validation of developed

method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criterion for LOD and LOQ is 3 and 10.The LOD and LOQ for Dapagliflozin was found to be 3.02 and 10.07 and LOD and LOQ for Saxagliptin was found to be 3.00 and 10.02. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits which shows that the method is having good system suitability and precision under given set of conditions.

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R E S E A R C H A R T I C L E

Estimation of Netupitant and Palonosetron and in combination with other drugs using RP-HPLC method

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A B S T R A C T

A simple precise and accurate reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of Netupitant and Palonosetron. Chromatographic separation was achieved on YMC C18 column (4.6 x 150mm, 5µ) using the mobile phase consisting of Phospahte buffer and Methanol in the ratio of 70:30. The mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 210 nm. The retention times were 2.254 min for Netupitant and 3.048 min for Palonosetron. The linearity ranges for Netupitant and Palonosetron were 300-1500µg/ml and 0.5-2.5µg/ml respectively with correlation coefficient 0.999. The proposed method was found to be simple, accurate, precise and reproducible and could be applied for routine quality control analysis for simultaneous estimation of Netupitant and Palonosetron in pharmaceutical dosage forms.

Key words: Netupitant, Palonosetron , RP-HPLC, Mobile phase, Retention time

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C O N T E N T S

1. Introduction

Netupitant is a neurokinin 1 receptor antagonist. Delayed emesis (vomiting) has been largely associated with the activation of tachykinin family neurokinin 1 (NK1) receptors (broadly distributed in the central and peripheral nervous systems) by substance P. As shown in in vitro and in vivo studies, netupitant inhibits substance P mediated responses. Once absorbed, netupitant is extensively metabolized to form three major metabolites: desmethyl derivative, M1; N-oxide derivative, M2; and OH-methyl derivative, M3. Metabolism is mediated primarily by CYP3A4 and to a lesser extent by CYP2C9 and CYP2D6.

Metabolites M1, M2 and M3 were shown to bind to the substance P/neurokinin 1 (NK1) receptor[1].

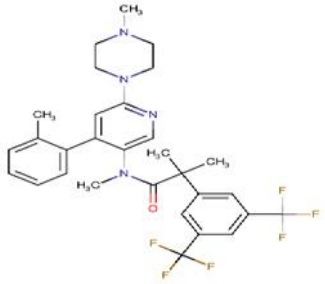

Fig 1: Chemical structure of Netupitant

Palonosetron is a selective serotonin 5-HT3 receptor antagonist. The antiemetic activity of the drug is brought about through the inhibition of 5-HT3 receptors present both centrally (medullary chemoreceptor zone) and peripherally (GI tract). This inhibition of 5-HT3 receptors in turn inhibits the visceral afferent stimulation of the vomiting center, likely indirectly at the level of the area postrema, as well as through direct inhibition of serotonin activity within the area postrema and the chemoreceptor trigger zone. Alternative mechanisms appear to be primarily responsible for delayed nausea and vomiting induced by emetogenic chemotherapy, since similar temporal relationships between between serotonin and emesis beyond the first day after a dose have not been established, and 5-HT3 receptor antagonists generally have not appeared to be effective alone in preventing or ameliorating delayed effects[2].

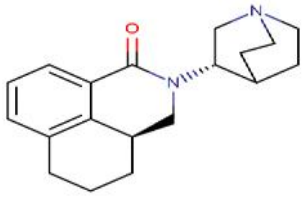

Fig 2: Chemical structure Palonosetron

The literature review reveals that few HPLC methods for the estimation of Netupitant and Palonosetron alone and in combination with other drugs[3,4,5]. Very few methods are reported for estimation of both drugs from formulation. We intend to develop RP-HPLC method by simultaneous determination with simple, rapid, greater sensitivity and faster elution.

2. Materials and Methods

Instruments used:

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module, UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Netupitant	PHARMATRAIN
2	Palonosetron	PHARMATRAIN
3	KH_2PO_4	FINER chemical LTD
4	Water and Methanol for HPLC	LICHROSOLV (MERCK)
5	Acetonitrile for HPLC	MOLYCHEM
6	Ortho phosphoric Acid	MERCK

HPLC Method Development

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 70: 30 v/v respectively.

Wave length selection:

UV spectrum of 10µg/ml Netupitant and Palonosetron in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 210nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. YMC column (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used**:** Waters HPLC with auto sampler and UV or detector.

Temperature **:** Ambient
Column **:**YMC 4.6*150mm 5µ
Buffer **:** 3.4g of KH2PO4 is taken in 1000 ml water pH adjusted with NaOH.
pH **:**3.0
Mobile phase **:** 70% buffer and 30% methanol
Flow rate **:**1.0 ml per min
Wavelength **:**210 nm
Injection volume **:**20 µl
Run time **:**8 min.

Preparation of mobile phase:

Accurately measured 700 ml (70%) of above buffer and 300 ml of Methanol HPLC (30%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 300 mg of Netupitant and 0.5 mg of Palonosetron working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer 300 mg of Netupitant and 0.5 mg of Palonosetron working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability:

Tailing factor for the peaks due to Netupitant and Palonosetron in Standard solution should not be more than 2.0. Theoretical plates for the Netupitant and Palonosetron peaks in Standard solution should not be less than 2000.Resolution for the Netupitant and Palonosetron peaks in standard solution should not be less than 2.

Method validation

The developed method was validated stastically according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision and robustness[11,12].

Specificity:

For Specificity Blank and Standard are injected into system. There is no any interference of any peak in blank with the retention time of the analytical peaks.

Linearity:

The linearity was determined for Netupitant and Palonosetron five different aliquiots were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity in the concentration ranges of Netupitant for 300- 1500 µg/mL and Netupitant for 0.5-2.5 µg/mL respectively.

Precision:

The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

Variation of flow rate:

Standard solution 900ppm of Netupitant & 1.5 ppm of Palonosetron was prepared and analysed using the varied flow rates along with method flow rate.On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

Change the organic composition of Mobile phase: Standard solution 900 ppm of Netupitant & 1.5 ppm of Palonosetron was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10.

Degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The standard solutions are subjected in various stress conditions like acid, base, thermal, oxidative and photolytic conditions. Then observe the percentage of drug degraded in given stress conditions.

3. Results and discussion

System suitability:

The specificity of this method was determined by complete separation of Netupitant and Palonosetron. The tailing factor for peaks of Netupitant and Palonosetron was less than 2% and resolution was satisfactory. The average retention time for Netupitant and Palonosetron were found to be 2.254 and 3.048 min respectively, for six replicates. The peaks obtained for Netupitant and Palonosetron were sharp and have clear baseline separation. The system suitability parameters are given in Table 3 and fig 3.

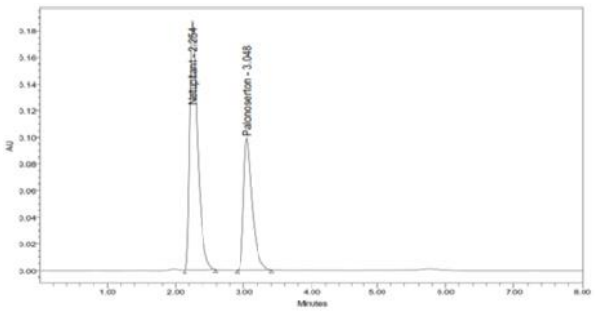

Fig 3: Chromatogram for System suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity: The linearity range was found to lie from 300µg/ml to 1500µg/ml of Netupitant, 0.5µg/ml to 2.5µg/ml of Palonosetron. The results are shown in table 5 and fig 4 & 5.

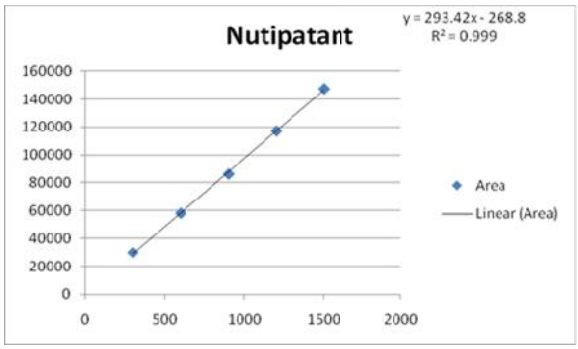

Fig 4: Calibration graph for Netupitant

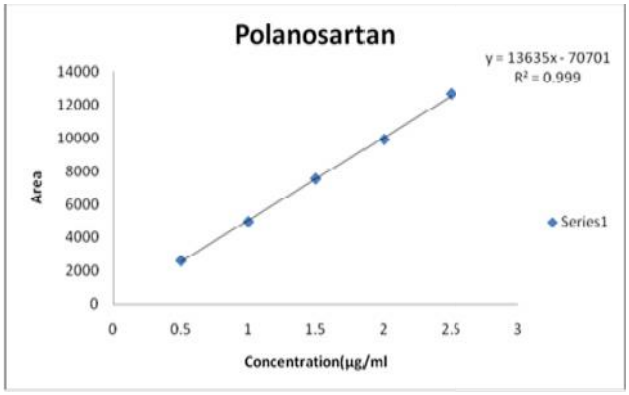

Fig 5: Calibration graph for Palonosetron

Precision:

Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are shown table 6.

Accuracy:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results were reported in table 7 & 8.

Robustness:

The standard and samples of Netupitant and Palonosetron were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was shown in table 10 & 11.

4. Conclusion

The estimation of Netupitant and Palonosetron was done by RP-HPLC. The assay of Netupitant and Palonosetron was performed with tablets and the % assay was found to be 100.08 and 100.04 which shows that the method is useful for routine analysis. The linearity of Netupitant and Palonosetron was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.3 for Netupitant and Palonosetron which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.4 for Netupitant and Palonosetron which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 100.43% and 100.50% for Netupitant and Palonosetron. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10.The LOD and LOQ for Netupitant was found to be 3.02 and 9.98 and LOD and LOQ for Palonosetron was found to be 3.00 and 10.00. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

Table 3: Results of system suitability parameters

S. No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Netupitant	2.254	86345	187425		1.14	3930.1
2	Palonosetron	3.048	7573	103066	3.72	1.11	2910.11

Table 4: Assay results

Drug	Label Claim (mg)	% Assay
Netupitant	300	100.08
Palonosetron	0.5	100.04

Table 5: Linearity results

S. No	Netupitant		Palonosetron	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	300	30018	0.5	2613
2	600	58216	1	4969
3	900	86174	1.5	7547
4	1200	117088	2	9909
5	1500	147293	2.5	12640
Slope (m)	293.42		13635	
Intercept (c)	268.8		70701	
Correlation coefficient (R^2)	0.999		0.999	

Table 6: Results for precision

	Intraday precision		Inter day precision	
Injection	Area for	Area for	Area for	Area for

	Netupatant	Palonosetron	Netupatant	Palonosetron
Injection-1	87799	7524	86017	7508
Injection-2	86973	7519	86172	7587
Injection-3	86232	7524	86652	7576
Injection-4	87604	7581	86680	7534
Injection-5	85975	7558	86818	7558
Injection-6	87018	7565	86585	7517
Average	86933.8	7545.2	86933.8	7546.7
Std Dev	723.5	26.2	723.5	32.1
%RSD	0.8	0.3	0.8	0.4

Table 7: Accuracy (recovery) data for Netupitant

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	43148.6	10	10.01	100.08	100.43
100%	86625.0	20	20.09	100.46	
150%	130313.3	30	30.23	100.75	

Table 8: Accuracy (recovery) data for Palonosetron

%Concentration (at specification Level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	3818.7	5	5.04	100.75	100.50
100%	7587	10	10.01	100.08	
150%	11447	15	15.10	100.67	

Table 9: Results of LOD & LOQ

Parameter	Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
LOD	Netupitant	58	175	3.02
	Palonosetron	58	174	3.00
LOQ	Netupitant	58	579	9.98
	Palonosetron	58	580	10.00

Table 10: Results for variation in flow for Netupitant and Palonosetron

Drug	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
Netupitant	0.9	3962	1.17	3.60
Palonosetron		3110	1.13	
Netupitant	1	3914.29	1.17	3.69
Palonosetron		3017.92	1.13	
Netupitant	1.1	3199.71	1.14	2.66
Palonosetron		2675.77	1.12	

Table 11: Results for variation in mobile phase composition for Netupitant and Palonosetron

Drug	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
Netupitant	10% less	3591	1.42	4.01
Palonosetron		2410	1.34	
Netupitant	*Actual	3914.29	1.17	3.69
Palonosetron		3017.92	1.13	
Netupitant	10% more	3340.78	1.17	2.17
Palonosetron		3341.82	1.18	

* Results for actual Mobile phase composition have been considered from Accuracy standard

Table 12: Showing degradation results

Sample Name	Netupitant		Palonosetrone	
	Area	**% Degraded**	**Area**	**% Degraded**
Standard	86056.0	-	7565.7	-
Acid	81872	4.86	7239	4.32
Base	81285	5.54	7298	3.54
Peroxide	82049	4.66	7267	3.95
Thermal	82411	4.24	7245	4.24
Photo	82185	4.50	7264	3.99

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R E S E A R C H A R T I C L E

Treatment of Asthma by Chronotherapeutic Drug Delivery System using Salbutamol Sulphate

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A B S T R A C T

The objective of the present investigation was to formulate and evaluate Salbutamol sulphate by chronotherapeutic drug delivery system. In chronopharmacotherapy (timed drug therapy) drug administration is synchronized with biological rhythms to produce maximal therapeutic effect and minimum harm for the patient. Salbutamol is a short-acting, selective beta2-adrenergic receptor agonist used in the treatment of asthma and COPD. Seven formulations of extended release tablets were prepared using 7 mm concave punch with single station tablet compression machine with polymer such as HPMC K100, HPMC K15M, sodium CMC, methyl carboxy cellulose and magnesium stearate in different ratios. The evaluation results revealed that all formulations comply with the specification of official pharmacopoeias and/or standard reference with respect to general appearance, content uniformity, hardness, friability and dissolution. Out of all the formulation developed, formulation F2 containing of HPMCK15 showed better Pulstile drug release of Salbutamol sulphate. Formulated tablets showed satisfactory results for various Post compression evaluation parameters like: tablet thickness, hardness, weight variation, floating lag time, total floating time, content uniformity and in vitro drug release.

Keywords: Salbutamol sulphate, Chronopharmacotherapy, HPMC K100, Extended release, Formulation, Pulsatile drug release

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C O N T E N T S

1. Introduction

Oral controlled drug delivery systems represent the most popular form of controlled drug delivery system for the more obivous advantage of the oral routes of the administration. Such systems release the drug with constant or variable release rates1. These dosage forms offer many advantages, such as nearly constant drug level

at the site of action, prevention of peak-valley fluctuations, reduction in dose of drug, reduced dosage frequency, avoidance of side effects, and improved patient compliance. In other words, it is required that the drug should not be released at all during the initial phase of dosage form administration. Such a release pattern is known as pulsatile release. The principle rationale for the use of pulsatile release is for the drugs where a constant drug release, i.e., a zero-order release is not desired. The release of the drug as a pulse after a lag time (an interval of no drug release) has to be designed in such a way that a complete and rapid drug release follows the lag time. In chronopharmacotherapy (timed drug therapy) drug administration is synchronized with biological rhythms to produce maximal therapeutic effect and minimum harm for the patient. By basing drug delivery on circadian patterns of Diseases, drug effect can be optimized and side effects can be reduced[2].

Salbutamol is a short-acting, selective beta2-adrenergic receptor agonist used in the treatment of asthma and COPD. It is 29 times more selective for beta2 receptors than beta1 receptors giving it higher specificity for pulmonary beta receptors versus beta1-adrenergic receptors located in the heart. Salbutamol is formulated as a racemic mixture of the R- and S-isomers. The R-isomer has 150 times greater affinity for the beta2-receptor than the S-isomer and the S-isomer has been associated with toxicity[3]. The main objective of present research work to design and characterize an oral, pulsatile drug delivery system of Salbutamol Sulphate intended to approximate the chronobiology of asthma, proposed for colonic targeting. It is a chronopharmaceutical approach for the better treatment of nocturnal asthma. Salbutamol administered intravenously has a half-life of 2 to 4 hours and is cleared partly renally and partly by metabolism to the inactive 4'-0- sulphate (phenolic sulphate) which is also excreted primarily in the urine.

2. Materials and Methods

Materials:

Table 1: List of Materials

S.No	Ingredients	Supplier
1	Salbutamol	Pharma Train
2	HPMC	Colorcon
3	Sodium CMC	Colorcon
4	Avicel Ph102(MCC)	Fmc Bio Polymer, Mumbai
5	Lactose Mono Hydrate	Sd Fine Chemicals, Mumbai
6	Magnesium Stearate	Evonik, India
7	Sodium Starch Glycolate	Sd Fine Chemicals, Mumbai
8	Aerosil	Sd Fine Chemicals

Table 2: List of Equipment's

S.No	Name of the Equipment	Model
1	Electronic weighing balance	Scale-Tec
2	Friabilator	Roche Friabilator Electrolab, Mumbai
3	Laboratory oven	Dtc-00r
4	Compression machine	Cmd(Cadmach)
5	Tablet hardness tester	Pfizer Hardness Tester, Mumbai
6	UV	Labindia Uv 3000+
7	Dissolution apparatus	Electrolab TDT-08L
8	Vernier callipers	Cd-6"Cs

Methods

Analytical method development:

Standard solution of Salbutamol sulphate by using 0.1 N Hcl: 100mg of drug is dissolved in 100ml of methanol. This is first stock solution.10ml of 1st stock solution is diluted with 100ml of 0.1N Hydrochloric acid buffer. This is 2nd stock solution. Now from 2nd stock, various concentrations of 2ug/ml, 4ug/ml, 6ug/ml, 8ug/ml, and 10ug/ml were prepared by using same 0.1 N Hydrochloric acid buffer. Blank was also prepared with same buffer composition except the drug. All the samples were analyzed at 276 lambda max with respect to the blank.

Standard solution of Salbutamol sulphate by using 6.8 Buffer Solution: 100mg of drug is dissolved in 100ml of methanol. This is first stock solution.10ml of 1st stock solution is diluted with 100ml of 6.8 buffer. This is 2nd stock solution. Now from 2nd stock, various concentrations of 10ug/ml, 20ug/ml, 30ug/ml, 40ug/ml, and 50ug/ml were prepared by using same 6.8 buffers. Blank was also prepared with same buffer composition except the drug. All the samples were analyzed at 276 lambda max with respect to the blank.

Formulation of Salbutamol ER Tablets by Direct Compression Method

Preparation of ER Tablets: All the excipients except Mg stearate were cosifted through # 40 ASTM & blended in a poly bag for 10 min. The above mixture # 60 ASTM passed Mg stearate were added & lubricated by blending in a poly bag for 5 min. ER tablets of 100 mg weight were prepared by direct compression method using 7 mm concave punch with single station tablet compression machine.

Evaluation of Tablets

The formulated tablets were evaluated for the following Pre, post compression quality control studies & In vitro Buoyancy studies and dissolution studies.

Pre Compression studies: Angle of Repose: Angle of Repose of granules was determined by the funnel method. Accurately weighed powder blend was taken in the funnel. Height of the funnel was adjusted in such a way the tip of the funnel just touched the apex of the powder blend. Powder blend was allowed to flow through the funnel freely on to the surface. The angle of repose has been used to characterize the flow properties of solids.

$$^{-} = \tan^{-1}(h/r)$$

Where:

θ = angle of repose; h = height in cms; r = radius in cms

Bulk density (BD): It is the ratio of total mass of powder to the bulk volume of powder Weigh accurately 25 g of

granules, which was previously passed through 22 # sieve and transferred in 100 ml graduated cylinder.

Bulk density = weight of powder / Bulk volume

Tapped density (TD): It is the ratio of total mass of powder to the tapped volume of powder. Weigh accurately 25 g of granules, which was previously passed through 22# sieve and transferred in 100 ml graduated cylinder of tap density tester which was operated for fixed number of taps until the powder bed volume has reached a minimum, thus was calculated by formula.

Tapped density = Weigh of powder / Tapped volume

Carr's Index: Compressibility index of the powder blend was determined by Carr's compressibility index. It is a simple test to evaluate the BD and TD of a powder and the rate at which it packed down[19]. The formula for Carr's index is as below:

$$\textbf{Compressibility index} = 100x \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}}$$

Hausner's Ratio: Hausner's Ratio is a number that is correlated to the flow ability of a powder.

$$\textbf{Hausner's Ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}$$

Post compression studies

General appearance:

The formulated tablets were assessed for its general appearance and observations were made for shape, colour, texture and odour.

Average weight/Weight Variation:

20 tablets were selected and weighed collectively and individually. From the collective weight, average weight was calculated. Each tablet weight was then compared with average weight to assure whether it was within permissible limits or not. Not more than two of the individual weights deviated from the average weight by more than 7.5% for 300 mg tablets and none by more than double that percentage

%weight variation =

$$\frac{\text{Average weight} - \text{weight of each tablet}}{\text{Average weight}} \times 10$$

Thickness:

Thickness of the tablets (n=3) was determined using a Vernier calipers.

Hardness test:

Hardness of the tablet was determined by using the Monsanto hardness tester (n=3) the lower plunger was placed in contact with the tablet and a zero reading was taken. The plunger was then forced against a spring by turning a threaded bolt until the tablet fractured. As the spring was compressed a pointer rides along a gauge in the barrel to indicate the force.

Friability test:

This test is performed to evaluate the ability of tablets to withstand abrasion in packing, handling and transporting. Initial weight of 20 tablets is taken and these are placed in the Friabilator, rotating at 25rpm for 4min. The difference in the weight is noted and expressed as percentage. It should be preferably between 0.5 to 1.0%.

$$\textbf{\%Friability} = [(W_1 - W_2)/W_1] \times 100$$

Assay Procedure:

Weigh and finely powder not less than 20 tablets. Transfer an accurately weighed portion of the powder equivalent to about 10mg Of model drug a 10 ml volumetric flask. Add approximately 6ml of 0.1N HCl and shake and sonicate for 10 min to complete the extraction. Dilute the methanol to volume and mix. Pipette 1ml aliquot into a 10ml volumetric flask, dilute with mobile phase to volume, mix and filter. From it withdraw take 1ml aliquot and make up to mark with buffer. Calculate the quantity in mg of model drug hydrochloride in the portion taken by the formula

Assay = test absorbance/standard absorbance*standard concentration/sample concentration*purity of drug/100*100

In vitro Dissolution Study:

900 ml of 0.1N HCl was placed in the vessel and the USP-II apparatus (Paddle method) was assembled. The medium was allowed to equilibrate to temperature of $37^0C \pm 0.5^0C$. A tablet was placed in the vessel and was covered; the apparatus was operated up to 12 hrs at 50 rpm. At definite time intervals, 5 ml of dissolution medium was withdrawn; filtered and again replaced with 5 ml of fresh medium to maintain sink conditions. Suitable dilutions were done with dissolution medium and were analyzed spectrophotometrically at λ_{max} =238 nm using a UV-spectrophotometer (Lab India).

In vitro Release Kinetics Studies: The analysis of drug release mechanism from a pharmaceutical dosage form is important but complicated process and is practically evident in the case of matrix systems. The order of drug release from FDDS was described by using zero order kinetics or first order kinetics. The mechanism of drug release from FDDS was studied by using Higuchi equation and the Peppa's-Korsemeyer equation.

3. Results and Discussion

Standard calibration curve of Salbutamol sulphate in 0.1N HCl: The absorbance of the solution was measured at 276nm, using UV spectrometer with 0.1N HCl as blank. The values are shown in table no 4. A graph of absorbance Vs Concentration was plotted which indicated in compliance to Beer's law in the concentration range 10to 30 µg/ml.

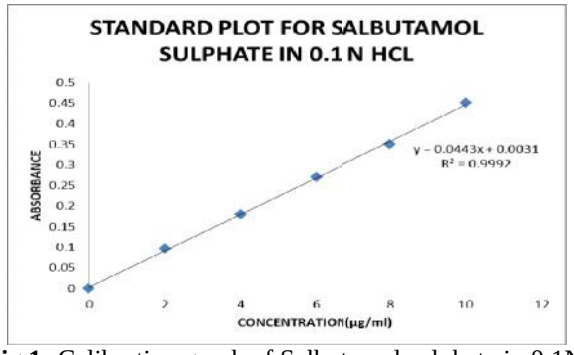

Fig 1: Calibration graph of Salbutamol sulphate in 0.1N HCl

Standard calibration curve of Salbutamol sulphate in 6.8 phosphate buffer: The absorbance of the solution was measured at 276nm, using UV spectrometer with 6.8 phosphate buffer as blank. The values are shown in table no 5. A graph of absorbance Vs Concentration was plotted which indicated in compliance to Beer's law in the concentration range 10 to 50 µg/ml.

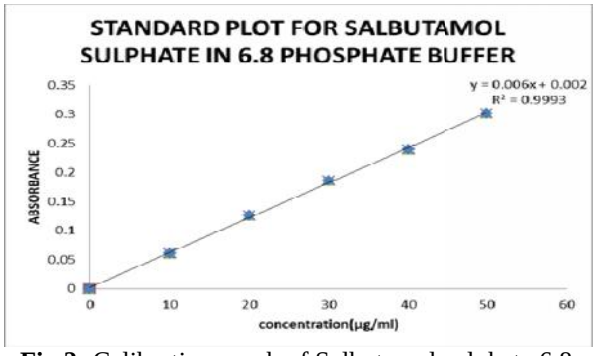

Fig 2: Calibration graph of Salbutamol sulphate 6.8 phosphate buffer

Evaluation of Tablets

Pre Compression studies:

The blends prepared for Dry granulation of tablets were evaluated for their flow properties; the results for the blends of compression tablets were shown in Table 6. The bulk density and the tapped density for all formulations were found to be almost similar. The Carr's index and Hausner's ratio were found to be in the range of ≤ 18 and 1.0 to 1.23 respectively, indicating good flow and compressibility of the blends. The angle of repose for all the formulations was found to be in the range of 9.92-12.73˚ which indicating passable flow (i.e. incorporation of glidant will enhance its flow).

Post compression studies:

The variation in weight was within the range of ±7.5% complying with pharmacopoeia specifications of USP. The thickness of tablets was found to be between 4.9-5.2 mm. The hardness for different formulations was found to be between 4.2 to 5.0 kg/cm2, indicating satisfactory mechanical strength. The friability was < 1.0% W/W for all the formulations, which is an indication of good mechanical resistance of the tablet. The drug content was found to be within limits 98 to 102 %.

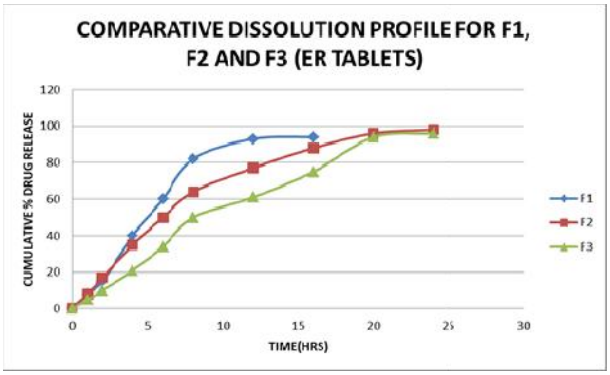

Fig 3: Comparative disso profile for F1, F2 and F3 ER Tablets

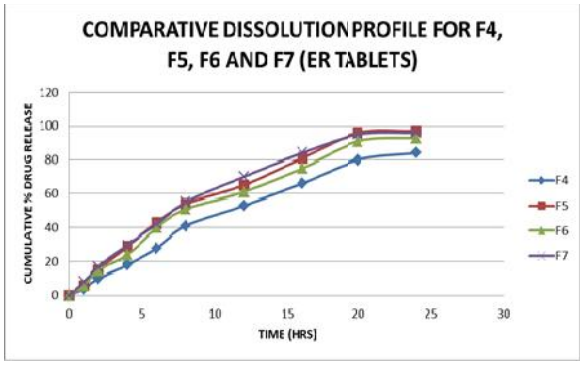

Fig 4: Comparitive disso profile for F4, F5, F6 and F7 ER Tablets

In-vitro drug release kinetics:

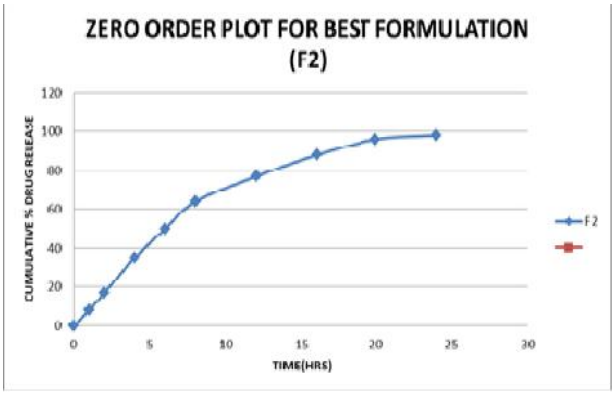

Fig 5: Zero order plot for best formulations F2 for ER Tablets

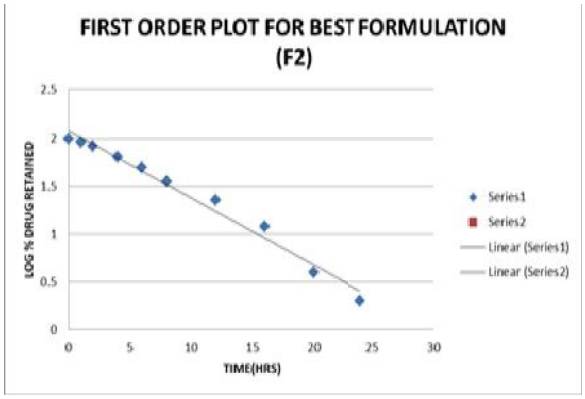

Fig 6: First order plot for best formulation F2 for ER tablets

Among the different control release polymers HPMC k15m was showing highest drug release retarding capacity. F2 was showing the satisfactory results and having better sustainability. When we plot the release rate kinetics for best formulation f2 was following first order because correlation coefficient value of first order is more than zero order 2 value. F2 formulation diffusion exponent n value is 0.45 < n >0.89 so they are following anomalous (Non-Fickian) diffusion. Higuchi plots F2 formulation is having good correlation values so the drug is releasing diffusion mechanism.

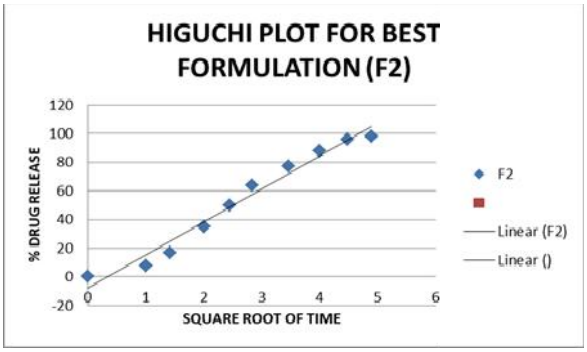

Fig 7: Higuchi plot for best formulation F2 for ER tablets

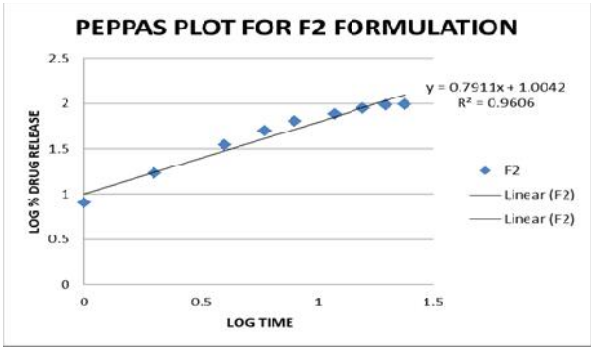

Fig 8: Kors mayers pepas plot for best formulation F2 for ER tablets

Table -3: Formulation of Salbutamol sulphate ER tablets by direct compression Method

Ingredients	F1 mg/tab	F2 mg/tab	F3 mg/tab	F4 mg/tab	F5 mg/tab	F6 mg/tab	F7 mg/tab
Salbutamol	6	6	6	6	6	6	6
HPMC K100m	-	-	50	75	50	60	37.5
HPMC K15	50	75	-	-	-	-	-
Sodium CMC	25	-	25	-	-		37.5
MCC	18	18	18	18	43	33	18
Magnesium Stereate	1	1	1	1	1	1	1
Total Weight	100	100	100	100	100	100	100

Table -4: Calibration graph values of Salbutamol sulphate in 0.1N HCl

Conc.(µg / ml)	Absorbance at λ_{Max} = 276 nm
0	0
10	0.097
15	0.18
20	0.27
25	0.35
30	0.45

Table -5: Calibration graph values of Salbutamol sulphate 6.8 phosphate buffer

Conc.(µg / ml)	Absorbance at λ_{Max} = 276 nm
0	0
10	0.061
20	0.126
30	0.186
40	0.239
50	0.302

Table -6: Pre compression studies of Salbutamol sulphate ER tablets *n=3

Formulation Code	Bulk density (Kg/cm^3)	Tapped density (Kg/cm^3)	Cars index	Hausners ratio	Angle of repose (˚)
F1	0.40	0.48	16	1.2	12.73
F2	0.39	0.48	18	1.23	11.96
F3	0.50	0.58	13	1.16	11.58
F4	0.44	0.50	12	1.1	9.92
F5	0.37	0.41	9.75	1.1	11.14
F6	0.36	0.39	7.6	1.0	11.03
F7	0.41	0.45	8.8	1.0	11.85

Table -7: Post compression studies of Salbutamol sulphate floating tablets

Formulation Code	% weight vaiation	Thickness± SD n=3 (mm)	%* friability	%Drug Content± SD n=3	Hardness (Kg/cm^2) Avg wt hardness ± SD n=3
F1	Pass	5.03±0.05	0.132	99.6±1.5	4.63 ±0.057

F2	Pass	5.03±0.15	0.143	98.9 ±2.3	4.2 ±0.057
F3	Pass	4.93±0.05	0.110	100.2± 1.7	4.7 ±0.1
F4	Pass	5.1±0.1	0.133	100.5± 1.4	4.53 ±0.057
F5	Pass	5.06±0.11	0.142	101.3 ±1.2	4.56 ±0.057
F6	Pass	5.06±0.15	0.151	102.3 ±1.7	5.03 ±0.115
F7	Pass	5.03±0.057	0.62	100.1 ±1.2	5 ±0.1
F8	Pass	5.1±0.1	0.154	100.7 ±1.1	4.63 ±0.057

Table -8: In-vitro Dissolution Studies for an Salbutamol sulphate ER Tablet

TIME (hrs)	%DRUG RELEASED IN 6.8 PH BUFFER, USP II,50 rpm						
	F1	F2	F3	F4	F5	F6	F7
0	0	0	0	0	0	0	0
1	8	8	5	4	6	6	8
2	15	17	10	10	15	15	17
4	40	35	21	18	29	24	30
6	60	50	34	28	43	40	42
8	82	64	50	41	54	51	55
12	93	77	61	53	65	61	70
16	94	88	75	66	81	75	84
20	-	96	94	80	96	91	95
24	-	98	96	84	97	93	96

Table -9: Results for in vitro drug release kinetics

Formulation code	R square value				n value
	Zero order	First order	Higuchi plot	Pepas plot	
F2	0.948	0.99	0.986	0.98	0.791

4. Conclusion

The present research work can be concluded that HPMC K15M was respectively showed better Pulstile drug release of Salbutamol sulphate. When drug: polymer concentration increases the release rate decreases this is because of reason when the concentration of polymer increases the diffusion path length increases. Formulated tablets showed satisfactory results for various Post compression evaluation parameters like: tablet thickness, hardness, weight variation, floating lag time, total floating time, content uniformity and in vitro drug release. Formulation F2 gave better-controlled drug release and in comparison to the other formulations. The release pattern of the F2 formulation was best fitted to Korsmeyer-Peppas model, Higuchi and first-order model. The most probable mechanism for the drug release pattern from the formulation was Anomalous (Non-Fickian) diffusion.

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R E S E A R C H A R T I C L E

Development of new simultaneous RP-HPLC method for the estimation of Glecapravir and Pibrentasvir in tablet dosage form

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A B S T R A C T

The aim present research work to development and validation of RP-HPLC method for the simultaneous estimation of Glecapravir and Piberentasvir. Chromatographic separation was evaluated by Xterra C18 column (250 X 4.6 mm, 5 µm) using the mobile phase consisting of 50% Water: 50% Acetonitrile and the mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 255 nm. The retention time of Glecapravir and Piberentasvir were found to be 2.205 min and 4.996 min. The linearity was obtained in the range of 100-500µg/ml for Glecapravir and 40-200 µg/ml for Piberentasvir with correlation coefficient was 0.999. The proposed method was found to be simple, accurate, precise, robust and cost effective. It can be applied for routine quality control analysis for simultaneous estimation of Glecapravir and Piberentasvir in pharmaceutical dosage forms.

Keywords: Glecapravir, Piberentasvir, RP-HPLC, Mobile phase, Acetonitrile, Retention time

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CONTENTS

1. Introduction

Glecaprevir is a direct acting antiviral agent and Hepatitis C virus (HCV) NS3/4A protease inhibitor that targets the the viral RNA replication. In combination with Pibrentasvir, glecaprevir is a useful therapy for patients who experienced therapeutic failure from other NS3/4A protease inhibitors. It demonstrates a high genetic barrier against resistance mutations of the virus. In cell cultures, the emergence of amino acid substitutions at NS3 resistance-associated positions A156 or D/Q168 in HCV genotype 1a, 2a or 3a replicons led to reduced susceptibility to glecaprevir. The combinations of amino acid substitutions at NS3 position Y65H and D/Q168 also results in greater reductions in

glecaprevir susceptibility, and NS3 Q80R in genotype 3a patients also leads to glecaprevir resistance[5].

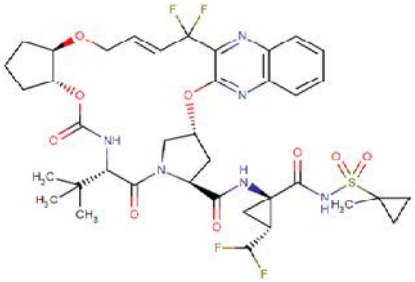

Fig 1: Chemical structure of Glecaprevir

Pibrentasvir is a direct acting antiviral agent and Hepatitis C virus (HCV) NS5A inhibitor that targets the the viral RNA replication and viron assembly. In combination with Glecaprevir, pibrentastiv is a useful therapy for patients who experienced therapeutic failure from other NS5A inhibitors. In cell cultures, the emergence of amino acid substitutions at known NS5A inhibitor resistance-associated positions in HCV genotype 1a, 2a or 3a replicons led to reduced susceptibility and resistance to pibrentasvir. Individual NS5A amino acid substitutions that reduced susceptibility to pibrentasvir include M28G or Q30D in a genotype 1a replicon and P32-deletion in a genotype 1b replicon[5].

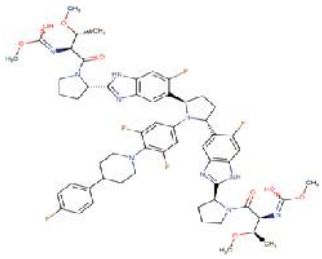

Fig 2: Chemical structure of Pibrentasvir

Glecapravir and Pibrentasvir are existing drugs. Literature reveals different methods for their analysis in their formulations6. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used: The following instruments are used to determination of Glecapravir and Piberentasvir.

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

The following chemicals are used to determination of Glecapravir and Piberentasvir.

Table 2: List of chemicals

S. No	Chemical	Brand
1	Glecaprevir	Supplied by Pharmatrain
2	Pibrentasvir	Supplied by Pharmatrain
3	KH_2PO_4	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
6	WaterHPLC	MERCK
7	Ortho phosphoric acid	MERCK

HPLC Method Development

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to 0.1% OPA: Methanol in proportion 30: 70 v/v respectively.

Wave length selection:

UV spectrum of 10µg/ml Glecaprevir and 10 µg/ml Pibrentasvir in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 251 nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Xterra C18 column (4.6 x 150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used **:**Waters HPLC with auto sampler and PDA detector.
Temperature **:** Ambient (25° C)
Mode of separation **:** Isocratic mode
Column **:** Xterra C18 column (4.6 x 150mm, 5µm)
Buffer **:** 0.1% OPA
Mobile phase **:** 30% buffer 70% Methanol
Flow rate **:** 1ml per min
Wavelength **:** 251 nm
Injection volume **:** 20 µl
Run time **:** 15 min.

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 25 mg of Glecaprevir and 10 mg of Pibrentasvir working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml

of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation: Accurately weigh and transfer equivalent to 25 mg of Glecaprevir and 10 mg of Pibrentasvir working standard into a 25 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability:

System suitability defined as integral part of many analytical procedures. The measured system suitability parameters like theoretical plates, tailing factor and resolution. Tailing factor for the peaks due to Glecaprevir and Pibrentasvir in Standard solution should not be more than 2.0 Theoretical plates for the Glecaprevir and Pibrentasvir peaks in Standard solution should not be less than 2000.Resolution for the Glecaprevir and Pibrentasvir peaks in standard solution should not be less than 2.

Method Validation

The developed method was statically validated according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision, LOD & LOQ and robustness[10,11].

Specificity:

For Specificity Blank and Standard are injected into system. there is no any inteferece of any peak in blank with the retiontime of the analytical peaks.

Linearity:

The linearity was determined for Glecaprevir and Pibrentasvir five different concentrations were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity concentrations are made from in the range of 100-500µg/ml for Glecaprevir and 40-200 µg/ml for Pibrentasvir.

Precision:

The standard solution was injected for six times into the within the day and between the days and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

Percentage mean recovery was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels such as 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

LOD & LOQ:

The sensitivity of the proposed method for measurement of Glecaprevir and Pibrentasvir were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 0.9 ml/min to 1.1ml/min: Standard solution 300 ppm of Glecaprevir & 120 ppm of Pibrentasvir was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The Organic composition in the Mobile phase was varied from ±10%: Standard solution 300 ppm of Glecaprevir & 120 ppm of Pibrentasvir was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Glecaprevir and Pibrentasvir using the proposed method. The standard solutions were subjected stress conditions like acid, base, thermal, photolytic and oxidative conditions. After some period of time observe the amount of drug degraded in selected stress conditions.

3. Results and discussion

System suitability:

The specificity of this method was determined by complete separation of Glecaprevir and Pibrentasvir. The tailing factor was less than 2% and resolution was satisfactory. The peaks obtained for sharp and have clear baseline separation. The system suitability parameters are given in Table 3 and fig 3.

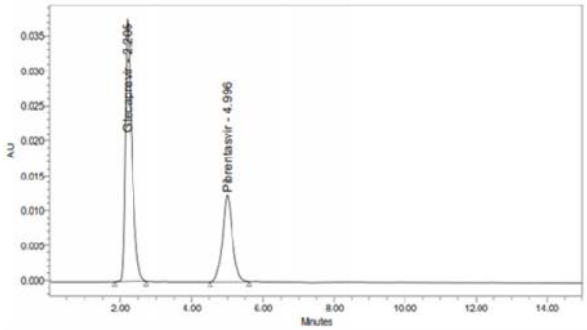

Fig 3: Chromatogram for system suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity:

The linearity range was found to lie from 100µg/ml to 500µg/ml of Glecaprevir, 40µg/ml to 200µg/ml of Pibrentasvir and chromatograms are shown in table 5 and fig 4 & 5.

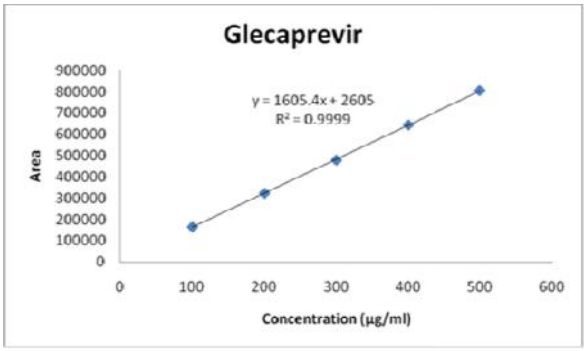

Fig 4: Calibration graph for Glecaprevir

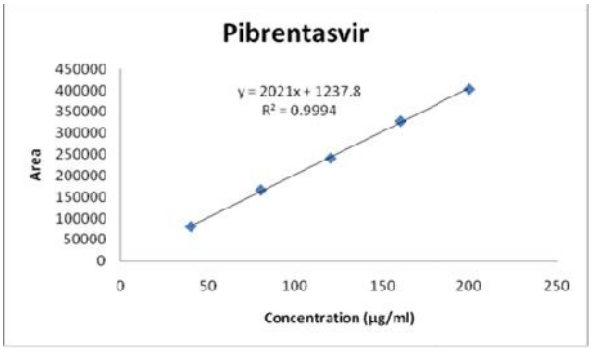

Fig 5: Calibration graph for Pibrentasvir

Precision: Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are given in table 6.

Accuracy:

Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results were reported in table 7 & 8.

Robustness:

The standard and samples of Glecaprevir and Pibrentasvir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was given in table 10 & 11.

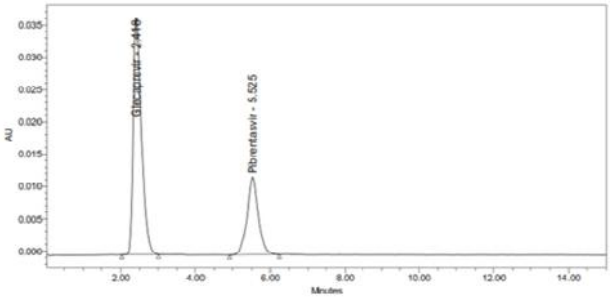

Fig 6: Chromatogram showing less flow

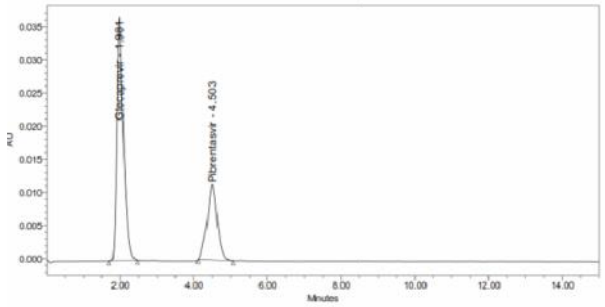

Fig 7: Chromatogram showing more flow

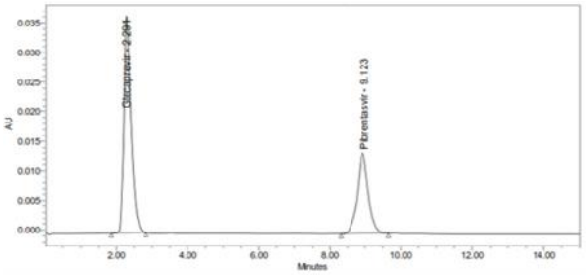

Fig 8: Chromatogram showing less organic composition

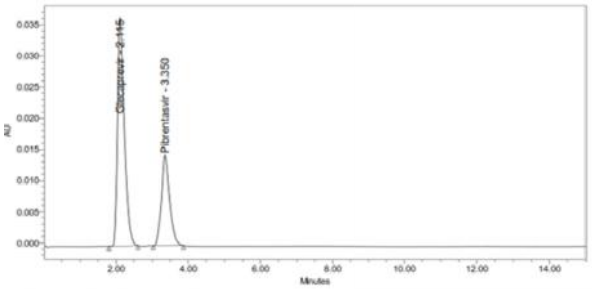

Fig 9: Chromatogram showing more organic composition

Table 3: Results of system suitability parameters

S.No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Glecaprevir	2.205	478222	36550	6.76	1.58	3677.56
2	Pibrentasvir	4.996	239609	12483		1.04	4683.62

Table 2: Results of Assay

Drug	Label Claim (mg)	% Assay
Glecaprevir	100	100.83
Pibrentasvir	40	100.23

Table 3: Area of different concentration of Glecaprevir and Pibrentasvir

S. No	Glecaprevir		Pibrentasvir	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	100	165076	40	80057
2	200	323694	80	166200
3	300	480198	120	241067
4	400	645116	160	328200
5	500	807077	200	403253
Slope (m)	1605.4		2021	
Intercept (c)	2605		1237.8	
Correlation coefficient (R^2)	0.999		0.999	

Table 4: Results for intraday and inter day precision

Injection	Intraday precision		Intermediate precision	
	Peak area of Glecaprevir	Peak area of Pibrentasvir	Peak area of Glecaprevir	Peak area of Pibrentasvir
Injection-1	483912	242261	482579	241793
Injection-2	479899	241331	489171	241873
Injection-3	487806	244327	482292	241291
Injection-4	486352	243371	483377	241423
Injection-5	482426	242500	483324	241328
Injection-6	484893	241079	480775	242453
Average	484214.7	242478.2	483586.3	241693.5
Std Dev	2822.8	1227.7	2894.6	444.5
%RSD	0.6	0.5	0.6	0.2

Table 7: Accuracy (recovery) data for Glecaprevir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	242024.3	12.5	12.57	100.54	100.40
100%	484977.0	25	25.18	100.73	
150%	721772.3	37.5	37.48	99.94	

*****Average of three determinations

Table 8: Accuracy (recovery) data for Pibrentasvir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	120660.7	5	5.01	100.23	100.25
100%	241976.0	10	10.05	100.50	
150%	361205.0	15	15.00	100.02	

*Average of three determinations

Table 9: Results for LOD & LOQ

Parameter	Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
LOD	Glecaprevir	58	173	2.98
	Pibrentasvir	58	174	3.00
LOQ	Glecaprevir	58	580	10.00
	Pibrentasvir	58	579	9.98

Table 10: Results for variation in flow for Glecaprevir and Pibrentasvir

S. No	Flow Rate (ml/min)	System Suitability Results				
		Glecaprevir		Pibrentasvir		USP Resolution
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing	
1	0.9	3672.96	1.59	4701.86	1.04	6.84

2	1.0	3678.77	1.57	4652.35	1.04	6.71
3	1.1	3574.36	1.46	4388.51	1.01	6.20

* Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 11: Results for variation in mobile phase composition for Glecaprevir and Pibrentasvir

S. No	Variation in mobile phase	System Suitability Results				
		Glecaprevir		Pibrentasvir		USP Resolution
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing	
1	10% less	3668.63	1.45	4446.54	0.83	12.51
2	Actual *	3678.77	1.57	4652.35	1.04	6.71
3	10% more	3575.02	1.51	4051.10	1.20	3.23

* Results for actual Mobile phase composition have been consideredfrom Accuracy standard.

Table 12: Results for Stability of Glecaprevir andPibrentasvir

Sample Name	Glecaprevir		Pibrentasvir	
	Area	% Degraded	Area	% Degraded
Standard	480497	-	240280	-
Acid	446911	6.99	230160	4.21
Base	453105	5.70	222491	7.40
Peroxide	427335	11.06	213084	11.32
Thermal	421312	12.32	207446	13.66

4. Conclusions

The estimation of Glecaprevir and Pibrentasvir was done by RP-HPLC. The assay of Glecaprevir and Pibrentasvir was performed with tablets and the % assay was found to be 100.83 and 100.23 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The method show precision 0.6 and 0.5 for Glecaprevir and Pibrentasvir which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.6 and 0.2 for Glecaprevir and Pibrentasvir which shows that the method is repeatable when performed in different days also. The total recovery was found to be 100.40% and 100.25% for Glecaprevir and Pibrentasvir. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits. Which shows that the method is having good system suitability and precision under given set of conditions.

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RESEARCH ARTICLE

Solubility Enhancement of Nifedipine Using Liquisolid Compact Technique

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A B S T R A C T

The objective of the present investigation was to formulate and evaluate conventional tablets of Nefidipine by solubility enhancement liquisolid technique. Liquisolid technique is a new and promising method that can change the dissolution rate of drugs. Nifedipine as a long- and short-acting 1,4-dihydropyridine calcium channel blocker. Twelve formulations of conventional tablets were prepared using liquisolid technique with nonvolatile solvent systems (Tween 80, Propylene glycol and PEG 400) termed as liquid vehicles with different ratio. The evaluation results revealed that all formulations comply with the specification of official pharmacopoeias and/or standard reference with respect to general appearance, content uniformity, hardness, friability and buoyancy. Out of all the formulation developed, Formulation 12 containing tween 80 shows rapid rate of disintegration when compared with other formulations.

Key words: Nefidipine, Liquisolid technique, Nonvolatile solvent, Formulation, Tween 80

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C O N T E N T S

1. Introduction

Liquisolid system is a novel concept of drug delivery via oral route. This technique is applied to water insoluble drugs and lipophilic drugs to sustain their release. Formulation and manufacture of the liquisolid tablets is quite simple method according to new mathematical model described by Spire's et al. It involves dissolving the drug in suitable non-volatile solvent and then adding this liquid medication to the mixture of carrier and coating materials. Mixing of this will lead to liquisolid system which is subjected to tabletting by direct compression. Increase in dissolution rate and in turn improvement in bioavailability is observed in case of poorly water soluble drugs[1].

Drug profile: Nifedipine has been formulated as both a long- and short-acting 1,4-dihydropyridine calcium channel blocker. It acts primarily on vascular smooth muscle cells by stabilizing voltage-gated L-type calcium channels in their inactive conformation. By inhibiting the influx of calcium in smooth muscle cells, nifedipine prevents calcium-dependent myocyte contraction and vasoconstriction. A second proposed mechanism for the drug's vasodilatory effects involves pH-dependent inhibition of calcium influx via inhibition of smooth muscle carbonic anhydrase. Nifedipine is used to treat hypertension and chronic stable angina.

The aim of present work is to enhance the dissolution rate of Nifedipine drug using liquisolid compacts. Liquisolid technique is a new and promising method that can change the dissolution rate of drugs. The technique of liquisolid preparation is used to formulate drug solution in solid dosage forms. Drug solution is generally, prepared by dissolving the drug in non-volatile water-miscible solvent. The prepared liquisolid formulation contains the drug held in solution2. Accordingly, the dissolution step, a pre-requisite for drug absorption, may be by-passed and better bioavailability of poorly soluble drugs is achieved. Liquisolid system refers to formulations formed by conversion of liquid drugs, drug suspension or drug solution in non-volatile solvents into dry, non adherent, free flowing and compressible powder mixtures by blending the suspension or solution with selected carriers and coating materials[3].

2. Materials and Methods

Materials:

Table 1: List of Materials

S.No	Ingredients	Supplier
1.	Nifedipine	Supplied By Pharma Train
	PEG 400	Colorcon
2.	Propylene Glycol	Colorcon
3.	Tween 80	Colorcon
4.	Avicel Ph102(Mcc)	Fmc Bio Polymer, Mumbai
5.	Silica Gel Powder	Sd Fine Chemicals, Mumbai
6.	Sodium Starch Glycolate	Sd Fine Chemicals, Mumbai
7.	Talc	Evonik, India

Table 2: List of Equipment's

S.No	Name of the Equipment	Model
1	Electronic weighing balance	Scale-Tec
2	Friabilator	Roche Friabilator Electrolab, Mumbai
3	Compression machine	Cmd(Cadmach)
4	Tablet hardness tester	Pfizer Hardness Tester, Mumbai
5	UV	Labindia Uv 3000+
6	Dissolution apparatus	Electrolab TDT-08L
7	Vernier calipers	Cd-6"Cs

Methods

Analytical method development:

Preparation of 7.2 pH phosphate buffer solution: 6.8g of monobasic potassium phosphate was weighed and diluted up to 1000 ml to get stock solution of monobasic potassium phosphate. 0.2M Sodium hydroxide solution was prepared and add to the monobasic potassium phosphate drop by drop to adjust the 7.2 pH.

Standard solution of Nifedipine by using 7.2 Buffer Solution:

100mg of drug is dissolved in 100ml of methanol. This is first stock solution.10ml of 1st stock solution is diluted with 100ml of 7.2 buffer. This is 2nd stock solution. Now from 2nd stock, various concentrations of 2ug/ml, 4ug/ml, 6ug/ml, 8ug/ml, and 10ug/ml were prepared by using same 7.2 buffer. Blank was also prepared with same buffer composition except the drug. All the samples were analyzed at 470 lambda max with respect to the blank.

Formulation of liquisolid compacts:

Nifedipine drug was initially dispersed in the nonvolatile solvent systems (Tween 80, Propylene glycol and PEG 400) termed as liquid vehicles with different drug : vehicle ratio. Then a mixture of carrier (Avicel pH 102) was added to the above liquid by continuous mixing for a period of 10 to 20 minutes in a mortar. Then to the above mixture coating material (silica gel powder) was added and mixed thoroughly. The amount of carrier and coating materials added were based on the 'R' value. To the above binary mixture disintegrant like sodium starch glycolate and other remaining additives such as Glidant (Talc) are added according to their application and mixed in a mortar.The final mixture was punched into Tablet.

Preparation of Conventional tablet of Nifedipine:

Conventional Tablet of Nifedipine was prepared by mixing 5mg of drug with 100mg of micro crystalline cellulose (Avicel pH 102), 5mg of silica gel powder and sodium starch glycolate 5% (w/w) as a disintegrant and mixed for 10 minutes. Talc is added and then Punched into Tablet.

Evaluation of Tablets

The formulated tablets were evaluated for the following Pre, post compression quality control studies & In vitro Buoyancy studies and dissolution studies

Pre Compression studies:

Angle of Repose: Angle of Repose of granules was determined by the funnel method. Accurately weighed powder blend was taken in the funnel. Height of the funnel was adjusted in such a way the tip of the funnel just touched the apex of the powder blend. Powder blend was allowed to flow through the funnel freely on to the surface. The angle of repose has been used to characterize the flow properties of solids.

$$^{-} = \tan^{-1}(h/r)$$

Where:

θ = angle of repose; h = height in cms; r = radius in cms

Bulk density (BD): It is the ratio of total mass of powder to the bulk volume of powder Weigh accurately 25 g of granules, which was previously passed through 22 # sieve and transferred in 100 ml graduated cylinder.

Bulk density = weight of powder / Bulk volume

Tapped density (TD): It is the ratio of total mass of powder to the tapped volume of powder. Weigh accurately 25 g of granules, which was previously passed through 22# sieve and transferred in 100 ml graduated cylinder of tap density tester which was operated for fixed number of taps until the powder bed volume has reached a minimum, thus was calculated by formula.

Tapped density = Weigh of powder / Tapped volume

Carr's Index: Compressibility index of the powder blend was determined by Carr's compressibility index. It is a simple test to evaluate the BD and TD of a powder and the rate at which it packed down[19]. The formula for Carr's index is as below:

$$\textbf{Compressibility index} = 100 \times \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}}$$

Hausner's Ratio: Hausner's Ratio is a number that is correlated to the flow ability of a powder.

$$\textbf{Hausner's Ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}$$

Post compression studies

General appearance:

The formulated tablets were assessed for its general appearance and observations were made for shape, colour, texture and odour.

Average weight/Weight Variation:

20 tablets were selected and weighed collectively and individually. From the collective weight, average weight was calculated. Each tablet weight was then compared with average weight to assure whether it was within permissible limits or not. Not more than two of the individual weights deviated from the average weight by more than 7.5% for 300 mg tablets and none by more than double that percentage.

$$\%\text{weight variation} = \frac{\text{average weight} - \text{weight of each tablet}}{\text{Average weight}} \times 10$$

Thickness:

Thickness of the tablets (n=3) was determined using a Vernier calipers.

Hardness test:

Hardness of the tablet was determined by using the Monsanto hardness tester (n=3) the lower plunger was placed in contact with the tablet and a zero reading was taken. The plunger was then forced against a spring by turning a threaded bolt until the tablet fractured. As the spring was compressed a pointer rides along a gauge in the barrel to indicate the force.

Friability test: This test is performed to evaluate the ability of tablets to withstand abrasion in packing, handling and transporting.Initial weight of 20 tablets is taken and these are placed in the Friabilator, rotating at 25rpm for 4min. The difference in the weight is noted and expressed as percentage. It should be preferably between 0.5 to 1.0%.

$$\%\text{Friability} = [(W_1 - W_2)/W_1] \times 100$$

Assay Procedure: Weigh and finely powder not less than 20 tablets. Transfer an accurately weighed portion of the powder equivalent to about 10mg Of model drug a 10 ml volumetric flask. Add approximately 6ml of 7.2 phosphate buffer and shake and sonicate for 10 min to complete the extraction. Dilute the methanol to volume and mix. Pipette 1ml aliquot into a 10ml volumetric flask, dilute with mobile phase to volume, mix and filter. From it withdraw take 1ml aliquot and make up to mark with buffer. Calculate the quantity in mg of model drug hydrochloride in the portion taken by the formula

Assay = test absorbance/standard absorbance*standard concentration/sample concentration*purity of drug/100*100

In vitro Dissolution Study:

900 ml of 7.2 phosphate buffer was placed in the vessel and the USP-II apparatus (Paddle method) was assembled. The medium was allowed to equilibrate to temperature of 370C±0.50C. A tablet was placed in the vessel and was covered; the apparatus was operated up to 60 minutes at 50 rpm. At definite time intervals, 5 ml of dissolution medium was withdrawn; filtered and again replaced with 5 ml of fresh medium to maintain sink conditions. Suitable dilutions were done with dissolution medium and were analyzed spectrophotometrically at λ_{max} =338 nm using a UV-spectrophotometer (Lab India).

3. Results and Discussion

Standard calibration curve of nifedipine in 7.2 phosphate buffer: The absorbance of the solution was measured at 338nm, using UV spectrometer with 7.2 phosphate buffer as blank. The values are shown in table no 20. A graph of absorbance Vs Concentration was plotted which indicated in compliance to Beer's law in the concentration range 2 to 10 µg/ml. Standard plot of Nefidipine plotted by taking absorbance on Y – axis and concentration (µg/ml) on X – axis, the plot is shown fig No.1.

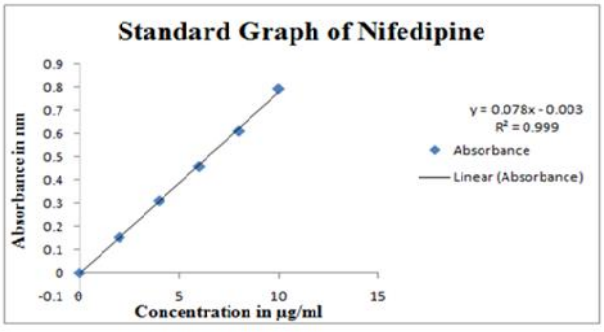

Fig 1: Calibration curve for nifedipine in 7.2 phosphate buffer

Pre Compression studies:

The prepared tablets were evaluated for their flow properties; the results for the blends of compression tablets were shown in Table 5. The bulk density and the tapped density for all formulations were found to be almost similar. The Carr's index and Hausner's ratio were found to be in the range of ≤ 18 and 1.0 respectively, indicating good flow

and compressibility of the blends. The angle of repose for all the formulations was found to be 11.14 which indicating passable flow (i.e. incorporation of glidant will enhance its flow).

Post compression studies:

The variation in weight was within the range of ±7.5% complying with pharmacopoeia specifications of USP. The thickness of tablets was found to be between 4.9-5.2 mm. The hardness for different formulations was found to be between 3 to 4.12 kg/cm2, indicating satisfactory mechanical strength. The friability was < 1.0% W/W for all the formulations, which is an indication of good mechanical resistance of the tablet. The drug content was found to be within limits 98 to 102 %.

In vitro dissolution study:

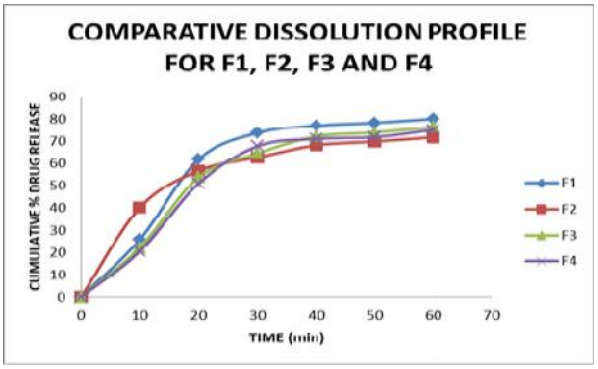

Fig 2: Dissolution profiles of Nefidipine Tablets for F1, F2, F3 and F4 formulations

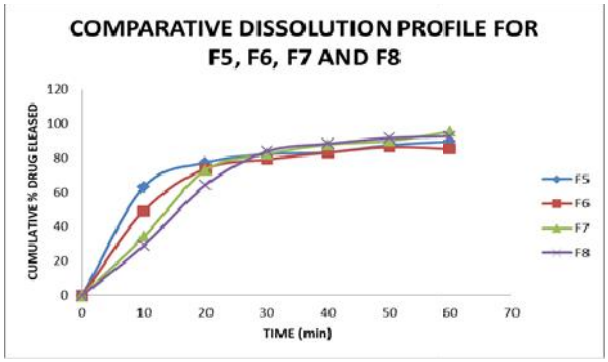

Fig 3: Dissolution profiles of Nefidipine Tablets for F5, F6, F7 and F8 formulation

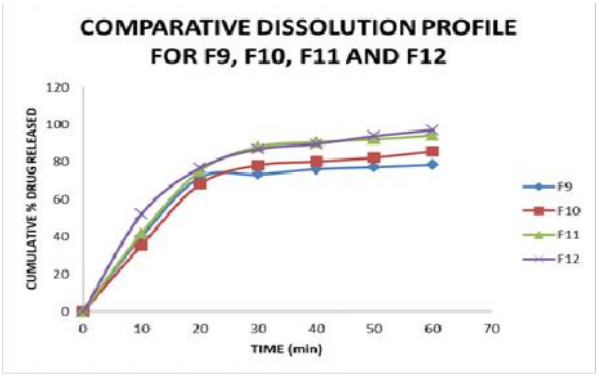

Fig 4: Dissolution profiles of Nefidipine Tablets for F9, F10, F11 and F12 formulations

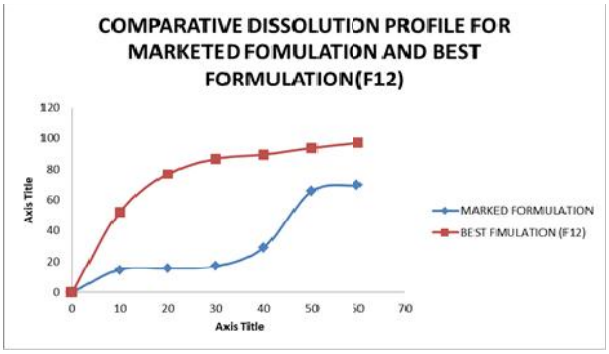

Fig 5: Dissolution profiles of Nefidipine marketed formulation and best formulation (F12)

4. Conclusion

Nefidipine tablets were formulated by using liquid solid compaction method using Tween 80, Propylene glycol, PEG 400 as liquid vehicle, Avicel pH 102 as carrier, silica gel powder as coating material, sodium starch glycolate as disintegrating agent and talc as glidant. Compatibility studies were carried out for the physical mixture and the drug was found to be compatible with all excipients used in different formulations. The blend was compressed into tablets and were analysed for the parameters such as average weight, disintegration, friability, thickness and hardness. Concluded that Formulation 12 containing tween 80 shows rapid rate of disintegration when compared with other formulations.

Table -3: Formulation of Nefidipine conventional tablets

Liqui solid systems	Non-volatile liquid vehicle	Drug concentration in liquid vehicle (%w/w)	Carrier: Coating material (R)	Liquid load factor (L_f)	Nifedipine (in mg)	Liquid vehicle (in mg)	Carrier (in mg)	Coating material (in mg)	Super disintegrant (in mg)	Glidant (in mg)	Unit dose (in mg)
F1	PEG 400	66.66	20:1	0.075	5	2.5	100	5	5.625	0.590	118.715
F2	PEG 400	50	20:1	0.1	5	5	100	5	5.75	0.603	121.353
F3	PEG 400	33.33	20:1	0.15	5	10	100	5	6	0.63	126.63
F4	PEG 400	25	20:1	0.2	5	15	100	5	6.25	0.656	131.906
F5	Propylene glycol	66.66	20:1	0.075	5	2.5	100	5	5.625	0.590	118.715
F6	Propylene glycol	50	20:1	0.1	5	5	100	5	5.75	0.603	121.353

F7	Propylene glycol	33.33	20:1	0.15	5	10	100	5	6	0.63	126.63
F8	Propylene glycol	25	20:1	0.2	5	15	100	5	6.25	0.656	131.90
F9	Tween 80	66.66	20:1	0.075	5	2.5	100	5	5.625	0.590	118.715
F10	Tween 80	50	20:1	0.1	5	5	100	5	5.75	0.603	121.35
F11	Tween 80	33.33	20:1	0.15	5	10	100	5	6	0.63	126.63
F12	Tween 80	25	20:1	0.2	5	15	100	5	6.25	0.656	131.90
Conventional	–	–	20:1	–	5	–	100	5	5.5	0.5775	116.077

Table -4: Calibration graph values of nifedipine in 7.2 phosphate buffer

Concentration (µg/Ml)	Absorbance
0	0
2	0.153
4	0.310
6	0.457
8	0.609
10	0.790

Table -5: Pre compression studies of Nefidipine tablets *n=3

Formulations	Bulk density (Kg/cm^3)	Tapped density (Kg/cm^3)	Cars index	Hausner's ratio	Angle of repose (˚)
F1	0.37	0.41	9.75	1.1	21.61
F2	0.43	0.52	17.3	1.41	22.62
F3	0.40	0.46	13.0	1.50	22.29
F4	0.44	0.51	13.7	1.25	20.29
F5	0.39	0.47	17.0	1.56	28.23
F6	0.42	0.52	19.2	1.45	23.24
F7	0.41	0.50	18.0	1.50	27.4
F8	0.41	0.51	19.6	1.53	22.26
F9	0.44	0.52	15.3	1.40	23.62
F10	0.42	0.49	14.2	1.35	25.24
F11	0.41	0.51	19.6	1.53	21.26
F12	0.44	0.51	13.7	1.25	20.29

Table -6: Post compression studies of Nefidipine tablets

Formulation Code	% weight vaiation	Thickness± SD n=3 (mm)	% *Friability	%Drug Content± SD n=3	Hardness (Kg/cm^2) Avg wt hardness ± SD n=3
F1	Pass	5.03±0.15	0.14	98.9 ±2.3	3.2 ±0.057
F2	Pass	4.93±0.05	0.11	100.2± 1.7	3.7 ±0.1
F3	Pass	5.06±0.11	0.14	101.3 ±1.2	3.56 ±0.057
F4	Pass	5.06±0.15	0.15	102.3 ±1.7	4.03 ±0.115
F5	Pass	5.03±0.057	0.62	100.1 ±1.2	3 ±0.1
F6	Pass	5.1±0.1	0.15	100.7 ±1.1	3.63 ±0.057
F7	Pass	4.99±0.03	0.23	99.3 ±2.2	3.37 ±0.14
F8	Pass	5.15±0.12	0.19	100.2± 1.4	4.23 ±0.11
F9	Pass	5.04±0.11	0.17	99.7 ±1.3	3.98 ±0.12
F10	Pass	4.83±0.06	0.53	99.6 ±1.2	4.12 ±0.12
F11	Pass	5.02±0.07	0.23	101.2± 0.9	3.22 ±0.16
F12	Pass	5.04±0.14	0.12	99.8 ±1.2	3.32 ±0.14

*Test for Friability was performed on single batch of 20 tablets

Table -7: Dissolution profile of liquisolid compacts with PEG 400

Time (in minutes)	% Cumulative drug releaseof F1 (1:0.5 ratio)	% Cumulative drug release of F2 (1:1 ratio)	% Cumulative drug releaseof F3 (1:2 ratio)	% Cumulative drug release of F4 (1:3 ratio)
0	0	0	0	0
10	25.84	40.15	22.15	0.53
20	61.90	56.75	53.78	51.05
30	73.66	62.68	64.53	67.76
40	77.01	68.21	72.16	71.05
50	78.30	69.87	73.87	71.82
60	80.07	71.78	76.51	75.13

Table -8: Dissolution profile of liquisolid compacts with propylene glycol

Time (in minutes)	% cumulative drug release of F5 (1:0.5 ratio)	% cumulative drug release of F6 (1:1 ratio)	% cumulative drug release of F7 (1:2 ratio)	% cumulative drug release of F8 (1:3 ratio)
0	0	0	0	0
10	63	49.15	34.15	29.07
20	77.31	73.69	72.84	64.01
30	82.08	79.12	82.18	83.64
40	83.21	82.99	87.69	88.25
50	87.42	86.27	89.80	91.52
60	89.50	85.12	95.38	92.50

Table -9: Dissolution profile of liquisolid compacts with tween 80

Time (in minutes)	% Cumulative drug release of F9 (1:0.5 ratio)	% Cumulative drug release of F10 (1:1 ratio)	% Cumulative drug release of F11 (1:2 ratio)	% Cumulative drug release of F12 (1:3 ratio)
0	0	0	0	0
10	40.15	35.53	42	52.15
20	71.75	68.24	75.23	76.73
30	73.23	78.22	88.06	86.81
40	76.11	80.23	90.64	89.60
50	77.17	82.25	92.08	93.80
60	78.45	85.67	94.23	97.11

Table-10: Dissolution profile of marketed & optimised formulation

Time (in minutes)	% Cumulative drug release of marketed formulation	% Cumulative drug release of optimised formulation (F12)
0	0	0
10	14.53	52.15
20	15.39	76.73
30	16.94	86.81
40	29.13	89.60
50	65.44	93.80
60	69.39	97.11

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R E S E A R C H A R T I C L E

Development of New simultaneous RP-HPLC method for the estimation of Pentazocine HCl and Naloxone HCl in tablet dosage form

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A B S T R A C T

The aim of present research work made to develop and validate RP-HPLC method for the simultaneous estimation of Pentazocaine and Naloxone in bulk and combined dosage form. The optimized mobile phase was consists of 30% OPA buffer: 70%Methanol, the pH was maintained at 3.0. The chromatographic separation was carried on Inertsil ODS C 18, column (4.6*150mm, 5µ). The detection of absorption maxima was monitored at 239 nm. The flow rate was maintained at 1.0 ml/min. The retention time of Pentazocaine and Naloxone were found to be from 2.401min & 3.374 min respectively. The values of % RSD are less than 2% indicating accuracy and precision of the method. The mean percentage recovery was found to be 100.43% of Pentazocaine and 100.50% of Naloxone. The proposed method is precise, simple and accurate to determine the amount of Pentazocaine and Naloxone in formulation. So the method can be useful in the routine quality control of these drugs.

Keywords: Pentazocaine and Naloxone, RP-HPLC, Mobile phase, Accuracy, Regression coefficient

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CONTENTS

1. Introduction

Naloxone is a specific opiate antagonist that has no agonist activity. It is a competitive antagonist at mu, delta, and kappa opioid receptors. It naloxone antagonizes the opioid effects by competing for the same receptor sites, especially the opioid mu receptor. Recently, naloxone has been shown to bind all three opioid receptors (mu, kappa and gamma) but the strongest binding is to the mu receptor. Naloxone has been shown to block the action of pain-lowering endorphins which the body produces naturally. These endorphins likely operate on the same opioid receptors that

naloxone blocks. It is capable of blocking a placebo pain-lowering response, both in clinical and experimental pain, if the placebo is administered together with a hidden or blind injection of naloxone. Pentazocine antagonizes the opioid effects by competing for the same receptor sites, especially the opioid mu receptor.

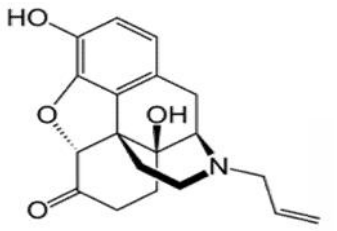

Fig 1: Structure of Naloxone

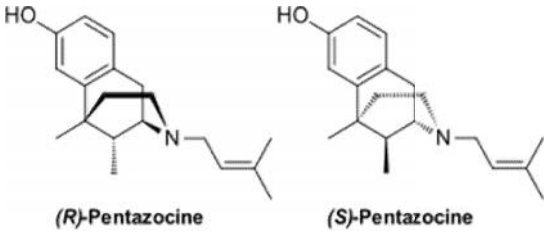

Fig 2: Structure of Pentazocaine

Pentazocine HCl and Naloxone HCl are existing drugs. Literature reveals different methods for their analysis in their formulations2,3. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used:

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector
2	UV/VIS spectrophotometer	LABINDIA UV 3000^{+}
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

Table 2: List of Chemicals

S .No	Chemical	Brand
1	Pentazocine HCl	Supplied by Pharmatrain
2	Naloxone HCl	Supplied by Pharmatrain
3	Tri ethyl amine	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
6	HCl, H_2O_2, NaOH	MERCK

HPLC Method Development

Wave length selection:

UV spectrum of 10 µg/ml Pentazocine HCl and Naloxone HCl in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 239nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Inertsil ODS (4.6 x 150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used **:**Waters HPLC with auto sampler and uv detector.
Temperature **:**Ambient
Column **:**Inertsil ODS (4.6 x 150mm, 5µm)
Buffer **:**OPA buffer
pH **:**3.0
Mobile phase **:**30% OPA buffer: 70%Methanol
Flow rate **:**1 ml per min
Wavelength **:**239 nm
Injection volume **:** 20 µl
Run time **:** 10 min

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 500 mg of Pentazocine HCl and 5 mg of Naloxone HCl working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 500 mg of Pentazocine HCl and 5 mg of Naloxone HCl working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability: System suitability is an integral part of many analytical procedures. The system suitability parameters such as theoretical plates, tailing factor and resolution. Tailing factor for the peaks due to Pentazocaine and Naloxane in Standard solution should not be more than 2.0. Theoretical plates should not be less than 2000. Resolution should not be less than 2.

Method Validation

Method validation was done for the according ICH guidelines Q2 (R1). The validation parameters like linearity, specificity, accuracy, precision, LOD & LOQ and robustness[11,12].

Linearity:

For determination of linearity five different concentrations were prepared from the standard stock solution and injected in triplicate. Then plotting the graph concentration Vs peak area and measure the correlation coefficient. It should not more than 0.999.

Precision:

The standard and sample solutions were injected into the five times in intraday and inter day, the peak areas were recorded. The mean and percentage relative standard deviation were calculated from the peak area.

Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same. Each solution was injected three times under optimized conditions and then calculates the mean percentage recovery.

LOD & LOQ:

The sensitivity of the proposed method for measurement of Pentazocaine and Naloxone were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 0.9 ml/min to 1.1ml/min:

Standard solution 1500 & 15 µg/ml of Pentazocine HCl & Naloxone HCl prepared and analysed using the varied flow rates along with method flow rate.

The Organic composition in the Mobile phase was varied from ±10%:

Standard solution 1500 & 15 µg/ml of Pentazocine HCl & Naloxone HCl was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Pentazocine HCl and Naloxone HCl using the proposed method. The standard solutions are placed in various stress conditions like acid, base, peroxide, thermal and photolytic conditions and calculate the amount of drug degraded in given stress conditions.

3. Results and discussion

System Suitability:

The system suitability of the method was checked by injecting five different preparations of the Pentazocine HCl and Naloxone HCl standard. The parameters of system suitability were checked. It was found from above data that all the system suitability parameters for developed method were within the limit. The results were shown in table 3.

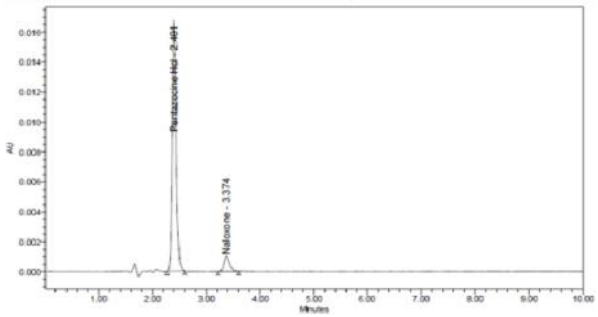

Fig 3: Chromatogram for System suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity: The linearity range was found to lie from 500µg/ml to 2500µg/ml of Pentazocine HCl, 5µg/ml to 25µg/ml 0f Naloxone HCl and chromatograms are shown in table 5 and fig 4 & 5.

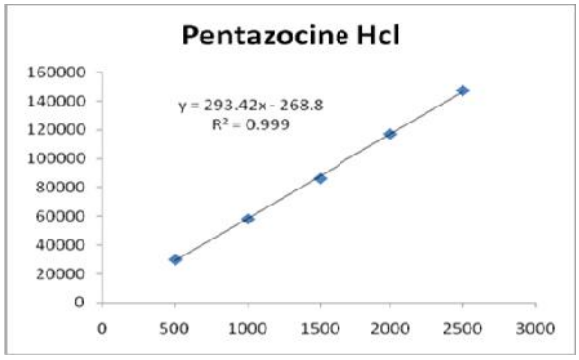

Fig 4: Calibration graph for Pentazocine HCl

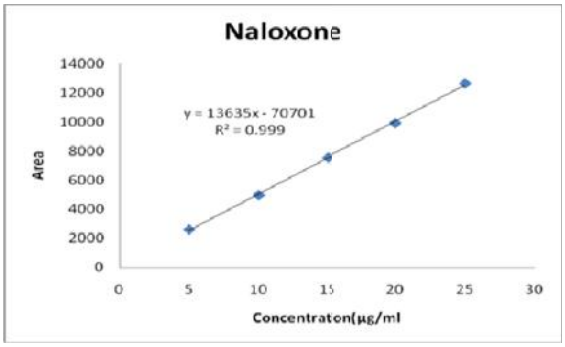

Fig 5: Calibration graph for Naloxone HCl

Precision: Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are given in table 6.

Accuracy: Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The data was given in table 7 & 8.

Robustness: The standard and samples of Pentazocine HCl and Naloxone HCl were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The results were reported in table 10 & 11.

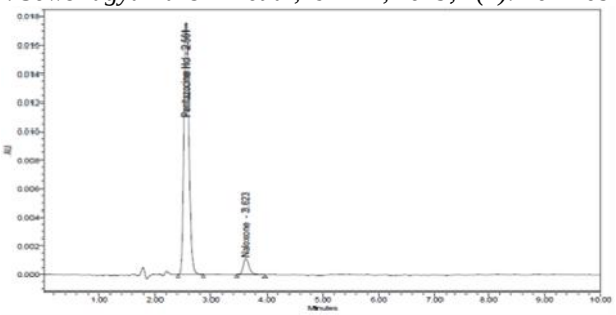

Fig 6: Chromatogram showing less flow

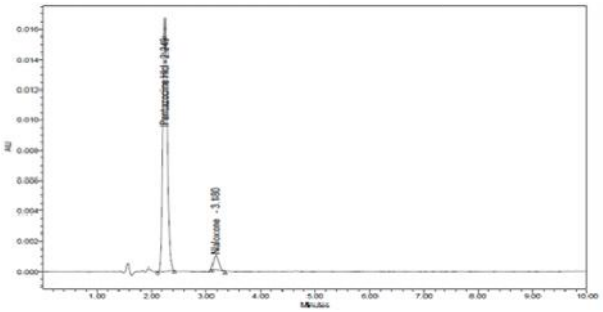

Fig 7: Chromatogram showing more flow

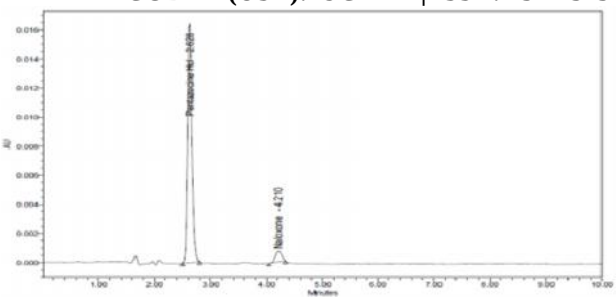

Fig 8: Chromatogram showing less organic composition

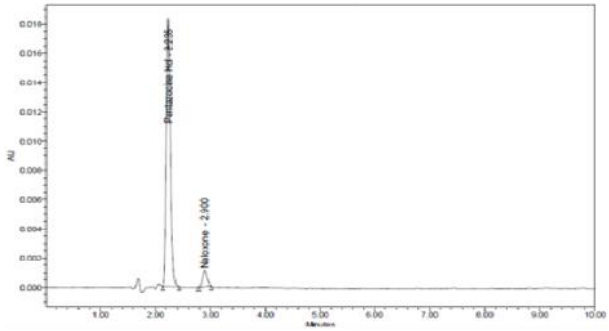

Fig 9: Chromatogram showing less organic composition

Table 3: Results of system suitability parameters

S. No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Pentazocine HCl	2.401	86345	16547		1.18	4682.77
2	Naloxone HCl	3.374	7556	1033	5.75	1.20	4633.60

Table 4: Results of Assay for Pentazocine HCl and Naloxone HCl

Drug	Label Claim (mg)	% Assay
Pentazocine HCl	50	100.08
Naloxone HCl	0.5	100.04

Table 5: Area of different concentration of Pentazocine HCl and Naloxone HCl

S. No	Pentazocine HCl		Naloxone HCl	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	500	30018	5	2613
2	1000	58216	10	4969
3	1500	86174	15	7547
4	2000	117088	20	9909
5	2500	147293	25	12640

Table 6: Results of Precision for Pentazocine HCl and Naloxone HCl

Injection	Intraday precision		Inter day precision	
	Area for Pentazocaine	Area for Naloxone	Area for Pentazocaine	Area for Naloxone
Injection-1	87799	7524	86017	7508
Injection-2	86973	7519	86172	7587
Injection-3	86232	7524	86652	7576
Injection-4	87604	7581	86680	7534
Injection-5	85975	7558	86818	7558
Injection-6	87018	7565	86585	7517
Average	86933.8	7545.2	86933.8	7546.7
Std Dev	723.5	26.2	723.5	32.1
%RSD	0.8	0.3	0.8	0.4

Table 7: Accuracy (recovery) data for Pentazocine HCl

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	43148.6	250	250.20	100.08	100.43%
100%	86625.0	500	502.30	100.46	
150%	130313.3	750	755.63	100.75	

*Average of three determinations

Table 8: Accuracy (recovery) data for Naloxone HCl

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	3818.7	2.5	2.52	100.75	100.50%
100%	7587	5	5	100.08	
150%	11447	7.5	7.55	100.67	

*Average of three determinations

Table 9: Results of LOD & LOQ

Parameter	Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
LOD	Pentazocaine	58	175	3.02
	Naloxone	58	174	3.00
LOQ	Pentazocaine	58	579	9.98
	Naloxone	58	580	10.00

Table 10: Results for variation in flow for Pentazocine HCl and Naloxone HCl

Drug	Flow Rate (ml/min)	System Suitability Results		USP Resolution
		USP Plate Count	USP Tailing	
Pentazocaine	0.9	4531.39	1.20	5.90
Naloxone		4857.7	1.27	
Pentazocaine	1.0	4529.07	1.18	5.75
Naloxone		4633.60	1.20	
Pentazocaine	1.1	4072.7	1.15	5.97
Naloxone		5791.3	1.35	

* Results for actual flow (1.0ml/min) have been considered from Assay standard.

Table 11: Results for variation in mobile phase for Pentazocine HCl and Naloxone HCl

Drug	Variation in mobile phase	System Suitability Results		USP Resolution
		USP Plate Count	USP Tailing	
Pentazocaine	10 % less	4683	1.21	5.97
Naloxone		5278. 62	1.20	
Pentazocaine	Actual*	4529.07	1.18	5.75
Naloxone		4633.60	1.20	
Pentazocaine	10% more	4383	1.21	5.97
Naloxone		5201.62	1.20	

* Results for actual Mobile phase composition have been considered from Accuracy

Table 12: Degradation results for Pentazocine HCl and Naloxone HCl

Sample Name	Pentazocine HCl		Naloxone HCl	
	Area	% Degraded	Area	% Degraded
Standard	86056.0	-	7565.7	-
Acid	81872	4.86	7239	4.32
Base	81285	5.54	7298	3.54
Peroxide	82049	4.66	7267	3.95
Thermal	82411	4.24	7245	4.24
Photo	82185	4.50	7264	3.99

4. Conclusions

The estimation of Pentazocine HCl and Naloxone HCl was done by RP-HPLC. The assay of Pentazocine HCl and Naloxone HCl was performed with tablets and the % assay was found to be 100.08 and 100.04 which shows that the method is useful for routine analysis. The linearity of Pentazocine HCl and Naloxone HCl was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.8 and 0.3 for Pentazocine HCl and Naloxone HCl which shows that the method is precise. The accuracy limit is the percentage recovery should be in the range of 98.0% - 102.0%. The total recovery was found to be 100.43% and 100.50% for Pentazocine HCl and Naloxone HCl. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

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RESEARCH ARTICLE

Development of new simultaneous RP-HPLC method for the estimation of Allopurinol and Lesinurad in tablet dosage form

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ABSTRACT

A simple precise, accurate and robust reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for simultaneous estimation of Allopurinol and Lesinurad. Chromatographic separation was achieved Inertsil ODS C18 column (4.6 x 150mm, 5µ) using the mobile phase consisting of Phospahte buffer 3.5 pH and Acetonitrile ratio of 70:30. The mobile phase was pumped at a flow rate of 1.5 mL/min and detection was done by UV detector at 255 nm. The retention times were 4.974 min for Allopurinol and 6.006 min for Lesinurad. The linearity ranges for Allopurinol and Lesinurad were 75-375 µg/ml and 50-250 µg/ml respectively with correlation coefficient 0.999. The proposed method was stastically validated according to ICH guidelines. All the validation parameters are present in within the limit. The developed could be applied for routine quality control analysis for simultaneous estimation of Allopurinol and Lesinurad in pharmaceutical dosage forms.

Keywords: Allopurinol, Lesinurad, RP-HPLC, Mobile phase, Retention time

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CONTENTS

1. Introduction

Allopurinol inhibits the enzyme xanthine oxidase, blocking the conversion of the oxypurines hypoxanthine and xanthine to uric acid. Elevated concentrations of oxypurine and oxypurine inhibition of xanthine oxidase through negative feedback results in a decrease in the concentrations of uric acid in the serum and urine. Allopurinol also facilitates the incorporation of hypoxanthine and xanthine into DNA and RNA, Leading to a feedback inhibition of de

novo purin synthesis and a decrease in serum uric acid concentrations as a result of an increase in nucleotide concentration[1].

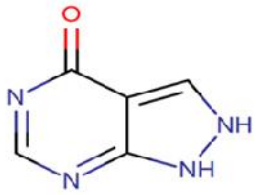

Fig 1: Chemical structure of Allopurinol

Lesinurad inhibits the activity of uric acid transporter 1 (urat1) and organic anion transporter 4 (oat4). Urat1 is a major transporter enzyme responsible for reuptake of uric acid from the renal tubules; inhibition of urat1 function thereby increases excretion of uric acid. Lesinurad is an oral uric acid transporter 1 (urat1) inhibitor indicated for the treatment of hyperuricemia associated with gout[2].

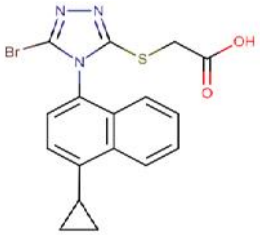

Fig 2: Chemical structure of Lesinurad

Allopurinol and Lesinurad are existing drugs. Literature reveals different methods for their analysis in their formulations[3,4]. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used:

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Table 2: List of Chemicals

S. No	Chemical	Manufacturer
1	Allopurinol	Supplied by Pharmatrain
2	Lesinurad	Supplied by Pharmatrain
3	KH_2PO_4	FINAR chemical LTD
4	Water and Methanol for HPLC	Standard solutions Ltd
5	Acetonitrile for HPLC	Standard solutions Ltd
6	WaterHPLC	MERCK
7	Ortho phosphoric acid	MERCK

Wave length selection:

UV spectrum of 10µg/ml Allopurinol and 10 µg/ml Lesinurad in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 255 nm. At this wavelength both the drugs show good absorbance.

HPLC Method Development

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: methanol with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to Phosphate buffer pH 3.0: Methanol in proportion 70: 30 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Inertsil ODS (150 x 4.6, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.5 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used **:**Waters HPLC with auto sampler and PDA detector.
Temperature **:**Ambient (25° C)
Mode of separation**:**Isocratic mode
Column **:** Inertsil ODS (150 x 4.6, 5µm)
Buffer **:**Phosphate buffer pH 3
Mobile phase **:**Phosphate buffer pH 3: Acetonitrile (70: 30)
Flow rate **:**1.5ml per min
Wavelength **:**255 nm
Injection volume **:** 20 µl
Run time **:** 15 min.

Preparation of mobile phase:

Accurately measured 700 ml (70%) of above Buffer and 300 ml (30%) of Acetonitrile were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 300 mg of Allopurinol and 200 mg of Lesinurad working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 300 mg of Allopurinol and 200 mg of Lesinurad sample into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.75 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability:
Tailing factor for the peaks due to Allopurinol and Lesinurad in Standard solution should not be more than 2.0. Theoretical plates for the Allopurinol and Lesinurad peaks in Standard solution should not be less than 2000. Resolution for the Allopurinol and Lesinurad peaks in standard solution should not be less than 2.

Method validation
The developed method was validated stastically according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision and robustness[11,12].

Specificity:
For Specificity Blank and Standard are injected into system. There is no any interference of any peak in blank with the retention time of the analytical peaks.

Linearity:
The linearity was determined for Allopurinol and Lesinurad five different aliquiots were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity in the concentration ranges of Allopurinol for 75-375 µg/mL and Lesinurad for 50-250 µg/mL respectively.

Accuracy:
Recovery assessment was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels in 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

Precision:
Precision studies were evaluated by intraday and inter day. The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Robustness:
As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 1.35 ml/min to 1.65ml/min:
Standard solution 225 ppm of Allopurinol & 150 ppm of Lesinurad was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The Organic composition in the Mobile phase was varied from ±10%:
Standard solution 225 ppm of Allopurinol & 150 ppm of Lesinurad was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method.On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10.

Degradation studies:
The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The standard solutions of Allopurinol and Lesinurad are subjected in various stress conditions like acid, base, thermal, oxidative and photolytic conditions. Then observe the percentage of drug degraded in given stress conditions.

3. Results and Discussions

Assay:
Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 3 and fig 3 & 4.

Table 3: Results of Assay for Allopurinol and Lesinurad

Drug	Label Claim (mg)	% Assay
Allopurinol	300	99.93
Lesinurad	200	99.95

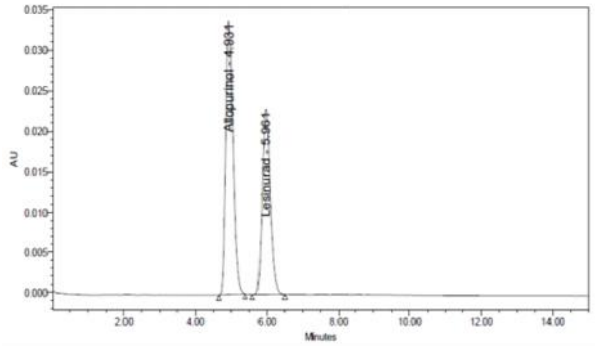

Fig 3: Chromatogram for Standard

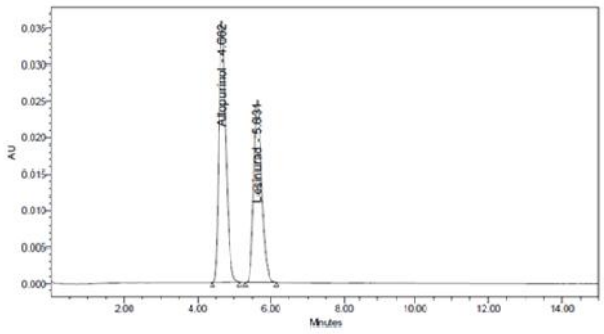

Fig 4: Chromatogram for Sample

System suitability:
The specificity of this method was determined by complete separation of Allopurinol and Lesinurad. The tailing factor for peaks of Allopurinol and Lesinurad was less than 2% and resolution was satisfactory. The average retention time for Allopurinol and Lesinurad were found to be 4.974 and 6.006 min respectively, for six replicates. The peaks obtained for Darunavir and Ritonavir were sharp and have clear baseline separation. The system suitability parameters are given in Table 4.

Linearity:
The linearity range was found to be 75 µg/ml to 375 µg/ml of Allopurinol, 50µg/ml to 250µg/ml of Lesinurad. The results are shown in table 5.

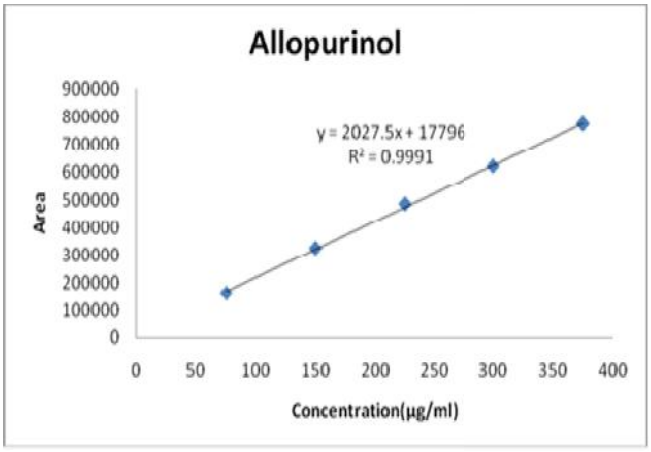

Fig 5: Calibration graph for Allopurinol

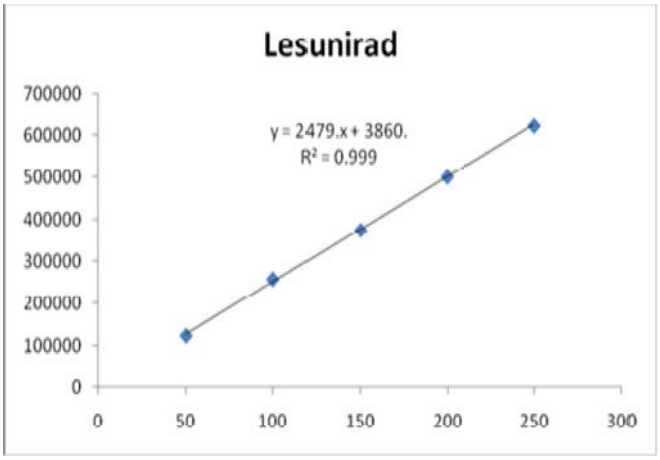

Fig 6: Calibration graph for Lesinurad

Precision: Precision of the method was carried out for both sample solutions as described under experimental work. The results are given in table 6.

Accuracy: Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results are reported in table 7 & 8.

Robustness:
The standard and samples of Allopurinol and Lesinurad were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was shown in table 10 &11.

4. Conclusion

The present work concluded that estimation of Allopurinol and Lesinurad was done by RP-HPLC. The assay of Allopurinol and Lesinurad was performed with tablets and the % assay was found to be 99.93% and 99.95&% which shows that the method is useful for routine analysis. The linearity of Allopurinol and Lesinurad was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The method show precision 0.6 and 0.8 for Allopurinol and Lesinurad which shows that the method is precise. The total recovery was found to be 99.60% and 100.15% for Allopurinol and Lesinurad. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criterion for LOD and LOQ is 3 and 10.The LOD and LOQ for Allopurinol was found to be 3.07 and 10.09 and LOD and LOQ for Lesinurad was found to be 2.95 and 9.93. The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits which shows that the method is having good system suitability and precision under given set of conditions.

Table 4: Results of system suitability parameters

S.No	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Allopurinol	4.974	463731	34151	2.69	1.29	3122.36
2	Lesinurad	6.006	373273	23654		1.22	3422.48

Table 5: Area of different concentration of Allopurinol and Lesinurad

S. No	Allopurinol		Lesinurad	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	75	163126	50	123687
2	150	324879	100	258151
3	225	484999	150	374272
4	300	622089	200	500737
5	375	774838	250	622363
Slope (m)	2027.5		2479.9	
Intercept (c)	17796		3860.6	
Correlation coefficient	R^2=0.999		R^2 =0.999	

Table 6: Results of for Allopurinol and Lesinurad

Injection	Intraday precision		Inter day precision	
	Allopurinol	Lesinurad	Allopurinol	Lesinurad

Injection-1	469199	378542	466111	372909
Injection-2	466480	370422	463354	378218
Injection-3	463505	377395	467721	375833
Injection-4	465113	375692	463219	376144
Injection-5	463129	375700	469297	379868
Injection-6	460972	372893	462378	377714
Average	464733.0	375107.3	465346.7	376781.0
Std Dev	2876.4	2985.9	2797.8	2398.4
%RSD	0.6	0.8	0.6	0.6

Table 7: Accuracy (recovery) data for Allopurinol

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	233775.3	150	150.42	100.28	99.60
100%	462242.7	300	297.42	99.14	
150%	695121.3	450	447.25	99.39	

*Average of three determinations

Table 8: Accuracy (recovery) data for Lesinurad

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	188250.7	100	100.19	100.19	100.15
100%	374491	200	199.32	99.66	
150%	567073.3	300	301.81	100.60	

*Average of three determinations

Table 9: Results of LOD & LOQ

Parameter	Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio
LOD	Allopurinol	43	132	3.07
	Lesinurad	43	127	2.95
LOQ	Allopurinol	43	434	10.09
	Lesinurad	43	427	9.93

Table 10: Results for variation in flow for Allopurinol and Lesinurad

Drug	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
Allopurinol	1.35	3095.23	1.30	2.68
Lesinurad		3407.85	1.23	
Allopurinol	1.5	3022.90	1.30	2.66
Lesinurad		3302.43	1.23	
Allopurinol	1.65	3088.28	1.28	2.66
Lesinurad		3345.08	1.20	

* Results for actual flow (1.5ml/min) have been considered from Assay standard

Table 11: Results for variation in mobile phase for Allopurinol and Lesinurad

Drug	Change in Organic Composition in the Mobile Phase	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
Allopurinol	10% less	6196.70	1.12	3.16
Lesinurad		6196.70	1.12	
Allopurinol	*Actual	3022.90	1.30	2.66
Lesinurad		3302.43	1.23	
Allopurinol	10% more	4608.01	1.49	2.11
Lesinurad		5906.60	1.46	

*Results for actual Mobile phase composition have been consideredfrom Accuracy standard

Table 12: Results for Stability of Allopurinol and Lesinurad

Sample Name	Allopurinol		Lesinurad	
	Area	% Degraded	Area	% Degraded
Standard	465326.7	-	375025.0	-
Acid	446578	4.03	359788	4.06
Base	453567	2.53	362545	3.33
Peroxide	439786	5.49	343876	8.31
Thermal	448788	3.55	349675	6.76
Photo	437675	5.94	351989	6.14

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R E S E A R C H A R T I C L E

Development of New RP-HPLC method for the estimation of Glycopyrrolate and Formoterol fumarate in the tablet dosage form

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A B S T R A C T

The present research work mainly focused on method development and validation for the simultaneous estimation of Glycopyrrolate and Formeterol fumarate in tablet dosage form by using RP-HPLC. Chromatographic separation was achieved on Xterra C18 column (4.6*150mm, 5µ) and it maintained flow rate was 1.0 ml/min. The mobile phase consist of 0.1% OPA buffer pH 3.0 was adjusted with sodium hydroxide and methanol with the ratio of 40:60%v/v, then UV spectrum was monitored at 220nm. The retention time was found to be 4.268 min for Glycopyrrolate and 3.129 min for Formeterol fumarate. The method shows linear response in the range of concentration was 4.8-24 µg/ml for Glycopyrrolate and 9.45µg/ml for Formeterol fumarate respectively. The developed method was stastically validated according to ICH guidelines. The validation parameters are present in with in the limit. So, the developed method was simple, precise, accurate and robust. It can be suggest that routine quality control analysis of analytical laboratories.

Keywords: Glycopyrrolate, Formeterol fumarate, Mobile phase, Validation, ICH guidelines

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C O N T E N T S

1. Introduction

Glycopyrrolate binds competitively to the muscarinic acetylcholine receptor. Like other anticholinergic (antimuscarinic) agents, it inhibits the action of acetylcholine on structures innervated by postganglionic cholinergic nerves and on smooth muscles that respond to acetylcholine but lack cholinergic innervation. These peripheral cholinergic receptors are present in the autonomic effector cells of smooth muscle, cardiac muscle,

the sinoatrial node, the atrioventricular node, exocrine glands and, to a limited degree, in the autonomic ganglia. Thus, it diminishes the volume and free acidity of gastric secretions and controls excessive pharyngeal, tracheal, and bronchial secretions.

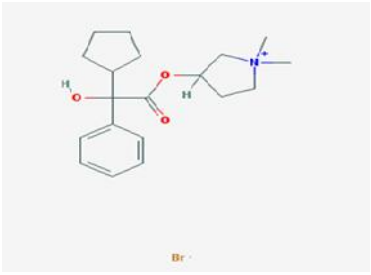

Fig 1: Chemical structure of Glycopyrrolate

Formoterol is attributable to stimulation of intracellular adenyl cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3', 5'-adenosine monophosphate (cyclic AMP). Increased cyclic AMP levels cause relaxation of bronchial smooth muscle and inhibits the release of pro-inflammatory mast-cell mediators such as histamine and leukotrienes. Formoterol also inhibits histamine-induced plasma albumin extravasation in anesthetized guinea pigs and inhibits allergen-induced eosinophil influx in dogs with airway hyper-responsiveness. The relevance of these in vitro and animal findings to humans is unknown.

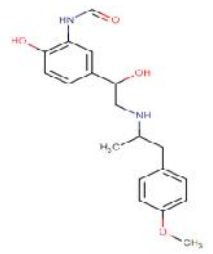

Fig 2: Chemical structure of Formoterol

Literature reveals different methods for their analysis in their formulations[8,9]. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used: The following instruments are used to determination of Glycopyrrolate and Formoterol.

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Chemicals used:

The following chemicals are used to determination of Glycopyrrolate and Formoterol.

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Glycopyrrolate	PHARMATRAIN
2	Formoterol fumarate	PHARMATRAIN
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	MOLYCHEM
5	Ortho phosphoric Acid	MERCK

HPLC Method Development

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to orthophosphoric acid with buffer (pH 3), methanol in proportion 60: 40 v/v respectively.

Wave length selection:

UV spectrum of 10 µg/ml Glycopyrrolate and Formoterol fumarate in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 220nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Xterra (4.6 x 150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used :Waters HPLC with auto sampler and 2487 UV detector.
Temperature :Ambient
Column :Xterrra 4.6*150mm, 5µm
Buffer :1ml of orthophosphoric acid in 1000ml water, pH adjusted with NaOH.
pH : 3
Mobile phase :40% buffer 60% methanol
Flow rate : 1 ml per min
Wavelength :220 nm
Injection volume :10 µl
Run time :10 min

Preparation of mobile phase:

Accurately measured 400 ml (40%) of above buffer and 600 ml of Acetonitrile HPLC (60%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further

pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer 9 mg of Glycopyrrolate and 4.8 mg of Formoterol fumarate working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

System Suitability:

Tailing factor for the peaks due to Glycopyrrolate and Formoterol fumarate in Standard solution should not be more than 2.0. Theoretical plates for the Glycopyrrolate and Formoterol fumarate peaks in Standard solution should not be less than 2000. Resolution for the Glycopyrrolatee and Formoterol fumarate peaks in standard solution should not be less than 2.

Method validation

Method validation parameters like specificity, linearity, accuracy, precision and robustness. The validation was done for the according to ICH guidelines Q2 (R1)[14,15].

Linearity:

From the standard stock solution five different aliquot's are prepared and obtained concentration range from 4.8-24 µg/ml for Glycopyrrolate and 9-45 µg/ml for Formoterol. Inject the each aliquot into the chromatographic system and measure the peak area. Then Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision:

The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate precision:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory. The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits Chromatograms were recorded.

Accuracy:

For accuracy determination, three different spiked concentrations were prepared separately i.e. 50%, 100% and 150% standard solutions were injected into the chromatographic system. Calculate the Amount found and Amount added for Glycopyrrolate and Formoterol and calculate the individual recovery and mean recovery values.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

Variation in flow rate: Standard solution 14.4 ppm of Glycopyrrolate & 27 ppm of Formoterol fumarate was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

Variation in the Mobile phase composition: Standard solution 27 ppm of Glycopyrrolate & 14.4 ppm of Formoterol fumarate was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10%.

Degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Glycopyrrolate and Formoterol fumarate using the proposed method. The standard solutions were subjected in different stress conditions like acid, base, peroxide, thermal and photolytic conditions and calculate the amount of drug degraded in selected stress conditions.

3. Results and Discussions

System suitability: All the system suitability parameters for developed method were within the limit. The results were shown in table 3 and fig 3.

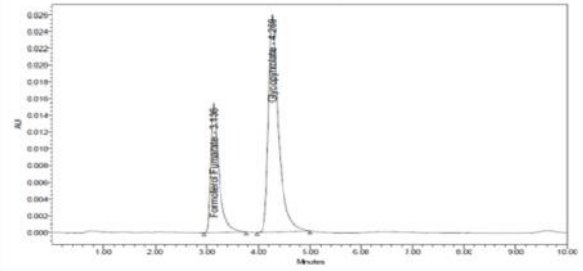

Fig 3: Chromatogram for system suitability

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 4.

Linearity:

To plotting the graph concentration Vs peak area, then calculate the correlation coefficient. It should not more than 0.999. The data was shown in table 5 and fig 4& 5.

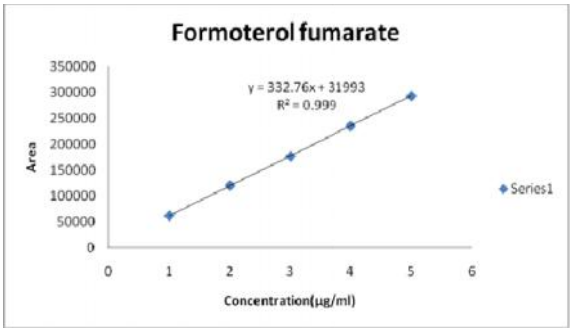

Fig 4: Calibration graph for Formoterol fumerate

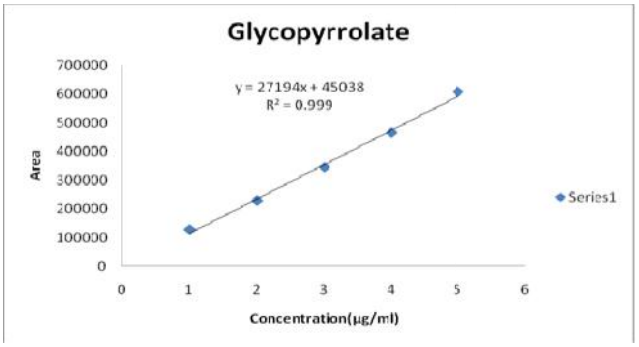

Fig 5: Calibration graph for Glycopyrrolate

Precision: Precision of the method was carried out for both sample solutions as described under experimental work. The corresponding chromatograms and results are shown in table 6.

Intermediate Precision (ruggedness): There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation. The results are given in table 7.

Accuracy: Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results are given in table 8 & 9.

Robustness:

The standard and samples of Formoterol fumerate and Glycopyrrolate were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was given in table 11 & 12.

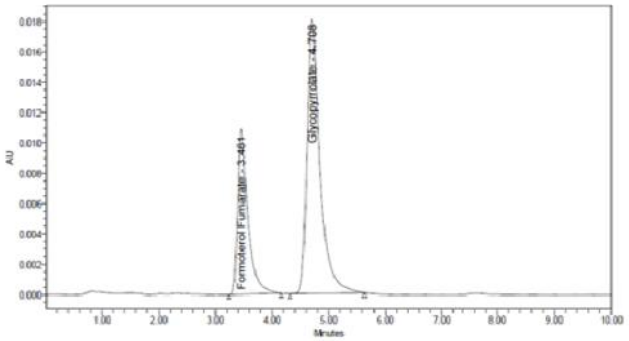

Fig 6: Chromatogram showing less flow

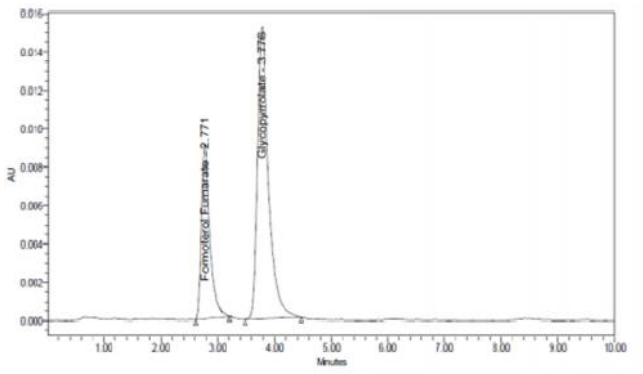

Fig 7: Chromatogram showing more flow

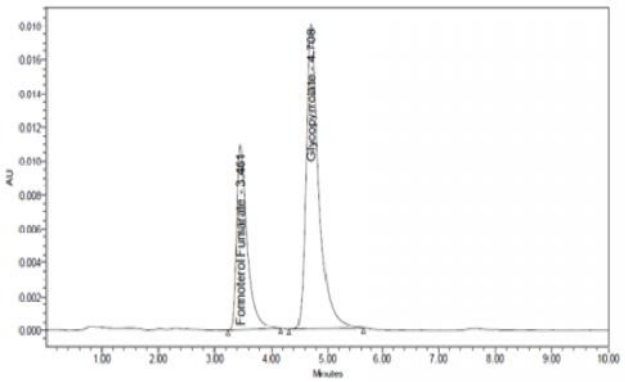

Fig 8: Chromatogram showing less organic composition

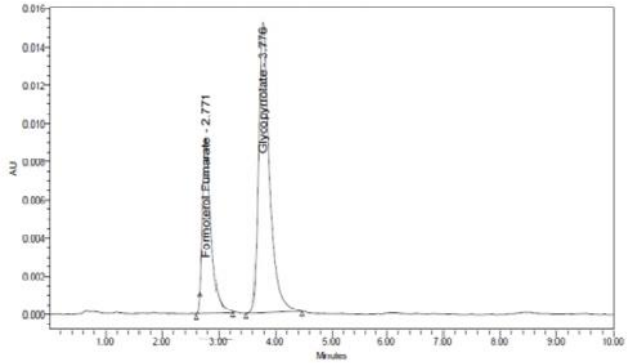

Fig 9: Chromatogram showing more organic composition

Table 3: Results of system suitability parameters

S.No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Formoterol	3.129	170698	14786	3.78	1.12	2137.25
2	Glycopyrrolate	4.268	346853	26148		1.14	2657.72

Table 4: Results of Assay for Formoterol fumerate and Glycopyrrolate

Drug	Label Claim (mg)	% Assay
Formoterol fumerate	9	99.80
Glycopyrrolate	4.8	99.72

Table 5: Linearity results for Glycopyrrolate and Fomoterol

		Glycopyrrolate		Fomoterol	
S. No	Linearity Level	Concentration	Area	Concentration	Area

1	I	4.8	127774	9	61241
2	II	9.6	228918	18	119943
3	III	14.4	345340	27	176636
4	IV	19.2	465502	36	235363
5	V	24	607979	45	293580
Correlation Coefficient			0.999	0.999	

Table 6: Results of Precision for Formoterol fumerate and glycopyrrolate

Injection	Area for Glycopyrrolate	Area for Formoterol fumarate
Injection-1	341368	178876
Injection-2	340717	177224
Injection-3	342655	179055
Injection-4	343939	178739
Injection-5	343013	176699
Injection-6	342282	179220
Average	342329.0	178302.2
STD	1156.8	1064.1
%RSD	0.3	0.6

Table 7: Results of Intermediate precision for Formoterol fumerate & Glycopyrrolate

Injection	Area for Glycopyrrolate	Area for Formoterol fumarate
Injection-1	349453	172535
Injection-2	347162	171224
Injection-3	349458	172915
Injection-4	348377	173391
Injection-5	348482	173108
Injection-6	349771	172959
Average	348783.8	172688.7
STD	976.1	769.7
%RSD	0.3	0.4

Table 8: The accuracy results for Glycopyrrolate

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	172505.0	4.5	4.47	99.38	100.01%
100%	346412	9	8.98	99.78	
150%	525309.0	13.5	13.62	100.88	

Table 9: The accuracy results for Formoterol fumarate

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	85620	2.4	2.40	99.85	100.34%
100%	171845	4.8	4.81	100.21	
150%	259676.0	7.2	7.27	100.95	

*Average of three determinations

Table 10: Results of LOD & LOQ

Parameter	Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
LOD	Glycopyrrolate	66	197	3.02
	Fomoterol	66	198	3.0
LOQ	Glycopyrrolate	66	660	10.00
	Fomoterol	66	659	9.98

Table 11: Results for variation in flow for Glycopyrrolate and Formoterol fumarate

Drug	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
Glycopyrrolate	0.9	2452	1.12
Fomoterol		2025.5	1.18
Glycopyrrolate	1	2718.66	1.64
Fomoterol		3961.26	1.15
Glycopyrrolate	1.1	2255	1.22
Fomoterol		2644.17	1.13

* Results for actual flow (1.0ml/min) have been considered from Assay standard

Table 12: Results for variation in mobile phase composition for Glycopyrrolate and Formoterol fumarate

Drug	Variation in mobile phase	System Suitability Results	
		USP Plate Count	USP Tailing
Glycopyrrolate	10% less	2452	1.10
Fomoterol		2025	1.18
Glycopyrrolate	Actual*	2718.66	1.64
Fomoterol		3961.26	1.15
Glycopyrrolate	10% more	2055.73	1.13
Fomoterol		3644	1.10

* Results for actual Mobile phase composition have been considered from Accuracy standard

Table 13: Degradation results for Glycopyrrolate and Fometorol

Sample Name	Glycopyrrolate		Formoterol fumarate	
	Area	% Degraded	Area	% Degraded
Standard	346468.0	100	171146.0	100
Acid	325453	93.93	155289	90.73
Base	327849	94.63	157420	91.98
Peroxide	325131	93.84	163076	95.28
Thermal	328347	94.77	163704	95.65
Photo	329359	95.06	156820	91.63

4. Conclusion

The estimation of Glycopyrrolate and Formoterol fumarate was done by RP-HPLC. The assay of Glycopyrrolate and Formoterol fumarate was performed with tablets and the % assay was found to be 99.80 and 99.72 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.6 for Glycopyrrolate and Formoterol fumarate which shows that the method is precise. The total recovery was found to be 100.01% and 101.34% for Glycopyrrolate and Formoterol fumarate. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of conditions.

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RESEARCH ARTICLE

Development of new simultaneous RP-HPLC method for the estimation of Dolutegravir and Rilpivirin in tablet dosage form

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ABSTRACT

The aim present research work to development and validation of RP-HPLC method for the simultaneous estimation of Dolutegravir and Rilpivirin. Chromatographic separation was carried on Inertsil ODS C18 column (200 X 4.6 mm, 5µm) using the mobile phase consisting of 0.1% OPA: Acetonitrile (80: 20). The mobile phase was pumped at a flow rate of 1.5 mL/min and detection was done by UV detector at 230 nm. The retention time of Dolutegravir and Rilpivirin were found to be 3.417min and 4.392 min. The linearity was obtained in the range of 50-250 µg/ml for Dolutegravir and 25-125 µg/ml for Rilpivirin with correlation coefficient was 0.999. The proposed method was found to be simple, accurate, precise, robust and cost effective. It can be applied for routine quality control analysis for simultaneous estimation of Dolutegravir and Rilpivirin in pharmaceutical dosage forms.

Keywords: Dolutegravir, Rilpivirin, RP-HPLC, Mobile phase, Acetonitrile, Retention time

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CONTENTS

1. Introduction

Rilpivirine is non-nucleoside reverse transcriptase inhibitor (NNRTI) which is used for the treatment of HIV-1 infections in treatment-naive patients. It is a non-competitive NNRTI that binds to reverse transcriptase. Its binding results in the blockage of RNA and DNA-dependent DNA polymerase activities, like HIV-1 replication. It does not present activity against human DNA polymerases α, β and γ. Rilpivirine binds to the HIV-1 reverse transcriptase (RT) and its flexible structure around the aromatic rings allows the adaptation to changes in the non-nucleoside RT binding pocket.

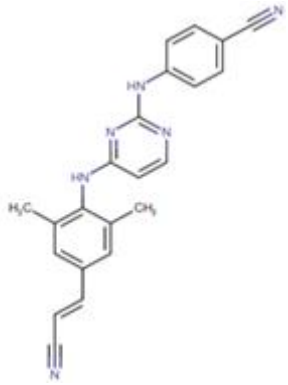

Fig 1: Structure of Rilpivirine

Dolutegravir is a HIV-1 intergrase inhibitor that blocks the strand transfer step of the integration of the viral genome into the host cell (INSTI). It inhibits HIV integrase by binding to the active site and blocking the strand transfer step of retroviral DNA integration in the host cell. The strand transfer step is essential in the HIV replication cycle and results in the inhibition of viral activity.

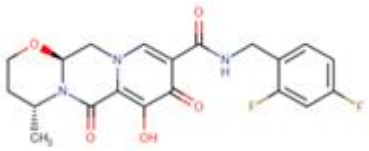

Fig 2: Structure of Dolutegravir

Dolutegravir and Rilpivirin are existing drugs. Literature reveals different methods for their analysis in their formulations1-5. But our present plan is to develop a new, simple, precise& accurate method for its analysis in formulation after a detailed study a new RP-HPLC method was decided to be developed and validated.

2. Materials and Methods

Instruments used:

The following instruments are used to determination of Dolutegravir and Rilpivirin.

Table 1: List of Instruments

S. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A

Table 2: List of Chemicals

S. No	Chemical	Company Name
1	Dolutegravir	PHARMATRAIN
2	Rilpivirine	PHARMATRAIN
3	KH_2PO_4	FINER chemical LTD
4	Water and Methanol for HPLC	LICHROSOLV (MERCK)
5	Acetonitrile for HPLC	MOLYCHEM

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ortho phosphoric acid buffer and Methanol: phosphate buffer, Acetonitrile: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to 0.1% OPA: Acetonitrile in proportion 80: 20 v/v respectively.

Wave length selection:

UV spectrum of 10µg/ml Dolutegravir and 10 µg/ml Rilpivirine in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 230 nm. At this wavelength both the drugs show good absorbance.

Optimization of Column:

The method was performed with various columns like C18 column Phenomenex column, YMC, and Inertsil ODS column. Inertsil ODS (200 x 4.6mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.5 ml/min flow.

Optimized Chromatographic Conditions:

Instrument used **:**Waters HPLC with auto sampler and UV detector.
Temperature **:**Ambient (25° C)
Mode of separation **:**Isocratic mode
Column **:** Inertsil ODS, column (200 x 4.6mm, 5µm)
Buffer **:**Water
Mobile phase **:**0.1% OPA: Acetonitrile (80: 20)
Flow rate **:** 1.5 ml per min
Wavelength **:**230 nm
Injection volume **:**20 µl
Run time **:** 10 min.

Preparation of mobile phase:

Accurately measured 800 ml (80%) of above Buffer and 200 ml (20%) of Acetonitrile were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Standard Solution Preparation:

Accurately weigh and transfer 50 mg of Dolutegravir and 25 mg of Rilpivirine working standard into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3.0 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 50 mg of Dolutegravir and 25 mg of Rilpivirine sample into a 100 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

System Suitability:

Tailing factor for the peaks due to Dolutegravir and Rilpivirine in Standard solution should not be more than 2.0.Theoretical plates for the Dolutegravir and Rilpivirine peaks in Standard solution should not be less than 2000.Resolution for the Dolutegravir and Rilpivirine peaks in standard solution should not be less than 2.

Method Validation

The developed method was statically validated according to ICH guidelines Q2(R1). The validation parameters like specificity, linearity, accuracy, precision, LOD & LOQ and robustness[10,11].

Linearity:

The linearity was determined for Dolutegravir and Rilpivirine five different concentrations were analyzed and calibration curve was constructed by plotting mean response factor against the respective concentration. The method was evaluated by determination of the correlation coefficient and intercept value. Linearity concentrations are made from in the range of 50-250µg/ml for Dolutegravir and 25-125 µg/ml for Rilpivirine.

Precision:

The standard solution was injected for six times and measured the area for all six injections in HPLC. It was done for the within the day and between the days with same chromatographic conditions. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

Percentage mean recovery was obtained by using standard addition technique which was by adding known quantities of pure standards at three different levels such as 50%, 100% and 150% to the pre analysed sample formulation. From the amount of drug found, amount of drug recovered and percentage recovery were calculated which sense to conformation that the proposed method was accurate.

LOD & LOQ:

The sensitivity of the proposed method for measurement of Dolutegravir and Rilpivirine were estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ).The LOD and LOQ were calculated by using the slope and SD of response (intercept).The mean slope value and SD of response were obtained after plotting six calibration curves.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 1.35 ml/min to 1.65ml/min: Standard solution 150 ppm of Dolutegravir & 75 ppm of Rilpivirine was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

The Organic composition in the Mobile phase was varied from ±10%: Standard solution 150 ppm of Dolutegravir & 75 ppm of Rilpivirine was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10.

Forced degradation studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. In present research work the standard solutions of Dolutegravir and Rilpivirine were placed in different stress conditions like acid, base, peroxide, thermal and photolytic condtions. Then observe the solutions in some period of time and calculate the percentage amount of drug degraded in above stress conditions.

3. Results and Discussions

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown in table 3 and fig 3&4.

Table 3: Results of Assay for Dolutegravir and Rilpivirin

Drug	Label Claim (mg)	% Assay
Dolutegravir	50	100.08
Rilpivirin	25	99.94

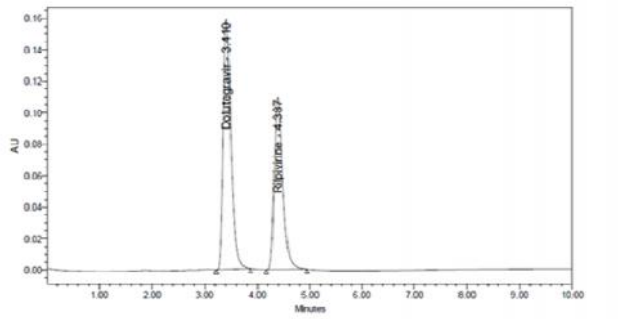

Fig 3: Chromatogram for Standard

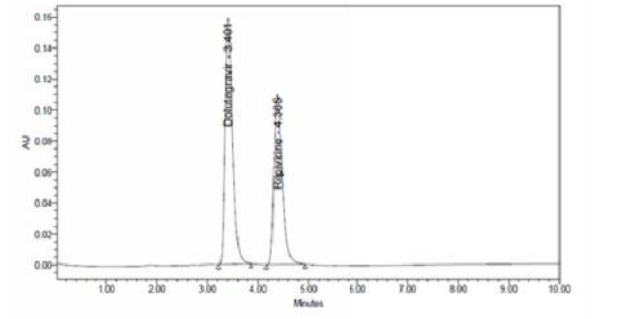

Fig 4: Chromatogram for Sample

System suitability:

The specificity of this method was determined by complete separation of Dolutegravir and Rilpivirin. The tailing factor was less than 2% and resolution was satisfactory. The peaks obtained for sharp and have clear baseline separation. The system suitability parameters are given in Table 4.

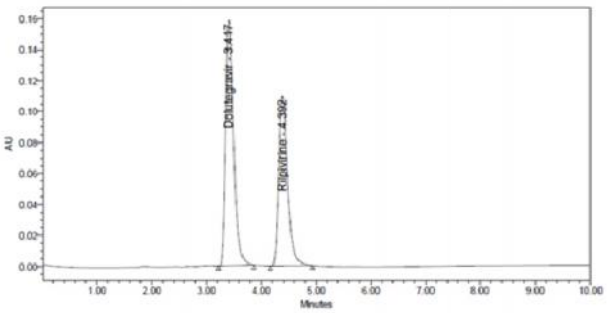

Fig 5: Chromatogram for system suitability

Linearity: The linearity range was found to lie from 50µg/ml to 250µg/ml of Dolutegravir, 25µg/ml to 125µg/ml of Rilpivirin and chromatograms are shown in table 5 and Fig 6 & 7.

Precision:
Precision of the method was carried out for both sample solutions as described under experimental work. The % RSD was found to be less than 2% for intraday precision and inter day precision. The results are explained in table 6.

Accuracy:
Sample solutions at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated. The results were shown in table 7& 8.

Robustness:
The standard and samples of Dolutegravir and Rilpivirin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count. The data was shown in table 10 & 11.

Table 4: Results of system suitability parameters

S.No	Name	RT (min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Dolutegravir	3.417	1593654	157646	3.49	1.45	2603.72
2	Rilpivirin	4.392	1218284	109089		1.41	3596.31

Table 5: Area of different concentration of Dolutegravir and Rilpivirin

S. No	Dolutegravir		Rilpivirin	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	50	524876	25	380761
2	100	1059982	50	782401
3	150	1574201	75	1164038
4	200	2068062	100	1549472
5	250	2604868	125	1965315
Slope (m)	10336		15745	
Intercept (c)	15979		12456	
Correlation coefficient (R^2)	0.999		0.999	

Table 6: Results for intraday and inter day precision

Injection	Intraday precision		Intermediate precision	
	Peak area of Dolutegravir	Peak area of Rilpivirin	Peak area of Dolutegravir	Peak area of Rilpivirin
Injection-1	1610934	1228406	1604507	1214125
Injection-2	1609985	1223300	1594158	1210517
Injection-3	1619309	1213803	1591505	1212127
Injection-4	1608645	1201667	1601953	1211539
Injection-5	1610885	1228897	1598025	1219177
Injection-6	1618951	1220372	1604821	1203992
Average	1613118.2	1219407.5	1599161.5	1211912.8
Std Dev	4731.4	10327.1	5538.0	4950.5
%RSD	0.3	0.8	0.3	0.4

Table 7: Accuracy (recovery) data for Dolutegravir

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	809552.3	25	25.21	100.82	100.39
100%	1611682	50	50.18	99.36	
150%	2408440.7	75	74.99	99.98	

*****Average of three determinations

Table 8: Accuracy (recovery) data for Rilpivirin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	617877.7	12.5	12.59	100.75	100.04
100%	1224225.3	25	24.95	99.81	
150%	1831657.7	37.5	37.33	99.55	

*Average of three determinations

Table 9: Results for LOD & LOQ

Parameter	Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
LOD	Dolutegravir	52	160	3.08
	Rilpivirin	52	156	3.00
LOQ	Dolutegravir	52	525	10.10
	Rilpivirin	51	521	10.02

Table 10: Results for variation in flow for Dolutegravir and Rilpivirin

S. No	Flow Rate (ml/min)	System Suitability Results				USP Resolution
		Dolutegravir		Rilpivirin		
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing	
1	1.35	2630.90	1.45	3726.83	1.45	3.49
2	1.5	2657.20	1.42	3669.74	1.40	3.52
3	1.65	2611.73	1.35	3385.64	1.30	3.44

Results for actual flow (1.5ml/min) have been considered from Assay standard

Table 11: Results for variation in mobile phase for Dolutegravir and Rilpivirin

S. No	Variation in mobile phase	System Suitability Results				USP Resolution
		Dolutegravir		Rilpivirin		
		USP Plate Count	USP Tailing	USP Plate Count	USP Tailing	
1	10% less	2569.17	1.39	3485.60	1.30	3.48
2	*Actual	2657.20	1.42	3669.74	1.40	3.52
3	10% more	2526.40	1.38	3416.12	1.34	3.44

Table 13: Results for Stability of Dolutegravir and Rilpivirin

Sample Name	Dolutegravir		Rilpivirin	
	Area	% Degraded	Area	% Degraded
Standard	1602702		1224118	
Acid	1583722	1.18	1207822	1.33
Base	1528333	4.64	1173832	4.11
Peroxide	1558673	2.75	1146223	6.36
Thermal	1492533	6.87	1196732	2.24
Photo	1509356	5.82	1127897	7.86

4. Conclusion

The estimation of Dolutegravir and Rilpivirin was done by RP-HPLC. The assay of Dolutegravir and Rilpivirin was performed with tablets and the % assay was found to be 100.08 and 99.94 which shows that the method is useful for routine analysis. The linearity was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.8 for Dolutegravir and Rilpivirin which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.3 and 0.4 for Dolutegravir and Rilpivirin which shows that the method is repeatable when performed in different days also. The total recovery was found to be 100.39% and 100.04% for Dolutegravir and Rilpivirin. The

validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criterion for LOD and LOQ is 3 and 10.The LOD and LOQ for Dolutegravir was found to be 3.08 and 10.10 and LOD and LOQ for Rilpivirin was found to be 3 and 10.02 The robustness limit for mobile phase variation and flow rate variation are well within the limit, the % degradation results are in limits which shows that the method is having good system suitability and precision under given set of conditions.

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Research

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Silver-catalysed azide–alkyne cycloaddition (AgAAC): assessing the mechanism by density functional theory calculations

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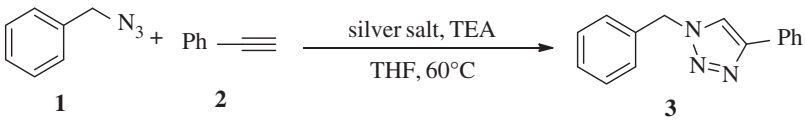
'Click reactions' are the copper catalysed dipolar cycloaddition reaction of azides and alkynes to incorporate nitrogens into a cyclic hydrocarbon scaffold forming a triazole ring. Owing to its efficiency and versatility, this reaction and the products, triazole-containing heterocycles, have immense importance in medicinal chemistry. Copper is the only known catalyst to carry out this reaction, the mechanism of which remains unclear. We report here that the 'click reactions' can also be catalysed by silver halides in non-aqueous medium. It constitutes an alternative to the well-known CuAAC click reaction. The yield of the reaction varies on the type of counter ion present in the silver salt. This reaction exhibits significant features, such as high regioselectivity, mild reaction conditions, easy availability of substrates and reasonably good yields. In this communication, the findings of a new catalyst along with the effect of solvent and counter ions will help to decipher the still obscure mechanism of this important reaction.

1. Introduction

Reactions to produce small building blocks from selective components are synthetically highly demanding [1–3]. Copper



1 2 3

Scheme 1. Silver catalysed click reaction.

Table 1. Effects of different silver salts in triazole formation. All reactions are carried out with 0.5 mmol of **1** and 0.60 mmol of **2** in 4 ml THF.

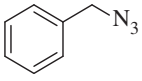
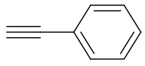
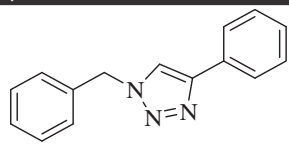
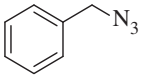
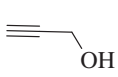
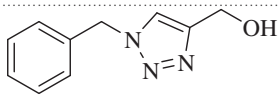
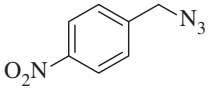
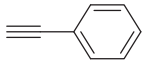
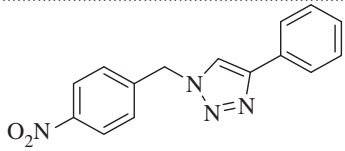
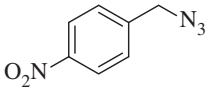
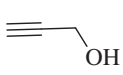
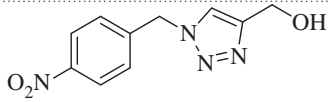
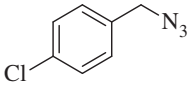
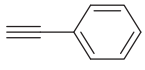
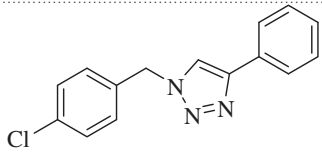
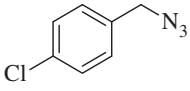
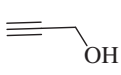
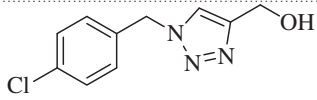
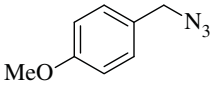
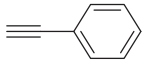
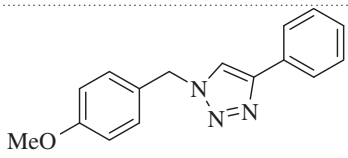
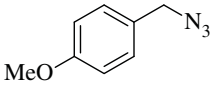
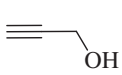
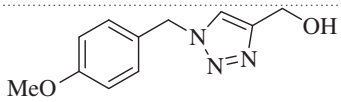
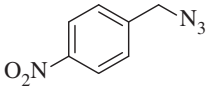
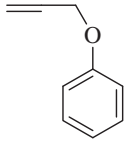
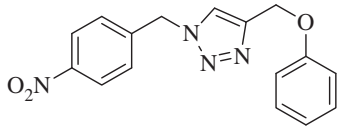
entry	silver salt (20 mol%)	base (equiv.)	yield (%)
1	AgOAc	TEA (5)	44
2	Ag_2O	TEA (5)	32
3	$AgNO_3$	TEA (5)	13
4	Ag_2CO_3	TEA (5)	61
5	AgCl	TEA (5)	87
6	AgI	TEA (5)	45
7	AgCl	—	trace

(I)-catalysed Huisgen dipolar cycloaddition reaction of terminal alkynes with azides yields 1,4 and 1,5 triazoles [4,5]. It is the most convenient method for the synthesis of triazoles, which are widely used in chemistry, biology and materials science [6–9]. In such cycloaddition reactions (also known as click reactions), the products are obtained in very high yields with little or no by-product [10–12]. Click reactions can be performed under many conditions and are least affected by the nature of the other functional groups [13]. For these reasons, click chemistry has made a great impact in the pharmaceutical and synthetic world [14]. In the past years, considerable efforts have been made to enhance the efficiency and general applicability of this reaction [15,16]. All kinds of copper catalyst systems including the Cu/Cu_2O nanoparticle catalyst systems have been developed to facilitate click chemistry and also to expand the substrate scope [17–20]. Nevertheless, the current transformation catalysed by silver salts and its mechanism have remained largely unexplored. Recently, Erick Cuevas [21] has described a process for the synthesis of 1,2,3-triazoles by using silver chloride and silver N-heterocyclic carbene complex. Abdul Aziz *et al*. [22] synthesized 1,4-disubstituted-1,2,3 triazoles by using $AgN(CN)_2$ catalyst at room temperature. The use of silver (I) oxide nanoparticles and different silver (I) complexes was also reported for the synthesis of 1,4-disubstituted-1,2,3 triazoles [23–25]. In this work, we have demonstrated the catalytic activity of silver (I) in the Huisgen cycloaddition reaction of azides and alkynes and also a general computational investigation has been carried out to study the mechanisms of the silver-catalysed triazole formation reaction. It is noteworthy to mention here that the catalytic activity of Ag(I) species is remarkably controlled by its conjugate anion. This may be the reason for the better chemical yield over Cu(I) salt-catalysed reactions. Silver chloride salt in this reaction produces clean products with high yield. We have also explored the mechanism of this important transformation using quantum mechanical computations.

2. Results and discussion

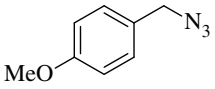
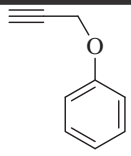
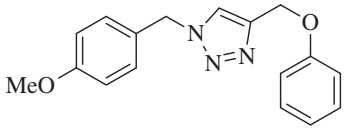
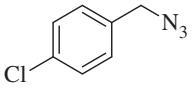
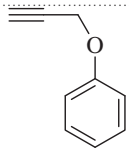
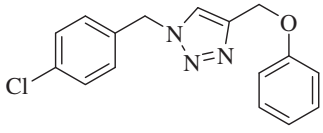
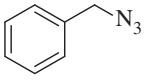
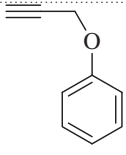
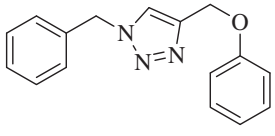
In this study of the synthesis of silver-catalysed triazole compounds (**3a–l**), we screened several silver salts as catalysts for the click reaction (table 1). Here, we reported our findings on the synthesis of various silver-catalysed various triazole rings both in intermolecular and intramolecular fashion. The reaction was investigated in a series of control experiments. Accordingly, different acetylene compounds (**2**) were reacted with different azides (**1**), (1:1.2) in THF solvent, in the presence of different Ag(I) salts and the reaction mixture was heated at 60°C in the presence of approximately 5 equiv. of triethylamine, to get the desired triazole product, (**3**) Scheme 1. Among all the silver catalysts screened for this reaction (AgOAc, Ag_2O, $AgNO_3$, Ag_2CO_3, AgI and AgCl), AgCl produced the highest yield, 87% (table 1, entry 5). Yield

Table 2. The list of different triazole compounds (**3a–l**) synthesized with the optimized condition.

s. no	substrate (1)	substrate (2)	product	yield (%)	time (h)
1	 **1a**	 **2a**	 **3a**	87	4
2	 **1b**	 **2b**	 **3b**	81	4
3	 **1c**	 **2c**	 **3c**	85	4
4	 **1d**	 **2d**	 **3d**	75	4
5	 **1e**	 **2e**	 **3e**	77	6
6	 **1f**	 **2f**	 **3f**	68	6
7	 **1g**	 **2g**	 **3g**	79	6
8	 **1h**	 **2h**	 **3h**	72	6
9	 **1i**	 **2i**	 **3i**	70	6

(*Continued*.)

Table 2. (*Continued*.)

s. no	substrate (1)	substrate (2)	product	yield (%)	time (h)
10	 **1j**	 **2j**	 **3j**	82	6
11	 **1k**	 **2k**	 **3k**	74	6
12	 **1l**	 **2l**	 **3l**	75	6

in all the other cases was substantially lower, as shown in table 1. Without triethylamine, the reaction may proceed but it was extremely sluggish. Under the optimized reaction conditions (AgCl and TEA, table 1, entry 5), full conversion to the triazole product was achieved within 4–6 h at 60°C. With this optimized synthetic protocol, we further synthesized a small library of triazole compounds with different substituents as shown in table 2.

The structures of all the synthesized triazole compounds (**3a–l**) were established spectroscopically by FTIR, ^{1}H NMR, ^{13}C NMR and HRMS (electronic supplementary material). After having the optimized condition in hand, we turned our attention to the one pot intramolecular triazole synthesis reaction. The four step reaction was initiated by converting the amine to the corresponding diazo compound followed by *in situ* displacement of the diazo group by azide resulting in compound **5**, scheme 2.

Thus, 1-azido-2-prop-2-ynyloxymethyl-benzene (**4**) undergoes intramolecular reaction to form 4H,6H-[1,2,3]triazolo[1,5-*a*][4,1]-benzoxazepine (**5**) in 75% yield under the same condition (scheme 2) [21].

3. Density functional theory calculation

GAUSSIAN 09 Revision C.01 software was used for the quantum mechanical calculations. All the geometry optimizations were performed *in vaccuo* at density functional theory (DFT) level of theory using B3LYP/3–21G basis set for all the atoms. Molecules were drawn in GAUSSVIEW 5. For theoretical calculations, the silver-catalysed 1,3 dipolar cycloaddition of methyl azide with propyne was studied. As proposed recently, for calculations we consider the involvement of the silver-centred acetylides, and the charge of the complexes was neutralized by adding chloride ions [26]. The initial guess of the transition state (TS) was obtained by scanning the N3–C4 and N1–C5 distances on a stable pre-reaction complex. All the other coordinates were relaxed during the scan. The TS was optimized using Berny algorithm (opt = ts) at the same level of theory. Molecular orbitals were calculated on the geometry optimized structures at the same level of theory. Coordinates for the optimized geometries are given in the electronic supplementary material. Relative energies were calculated with respect to the most stable pre-reaction complex. The energy values were converted to kilocalories per mole from Hartree per particle using the conversion factor of 627.509467.

The Ag-catalysed reaction process has been modelled using quantum mechanical calculations. Figure 1*a* shows the potential energy landscape for the 1,4 disubstituted cycloaddition reaction. From

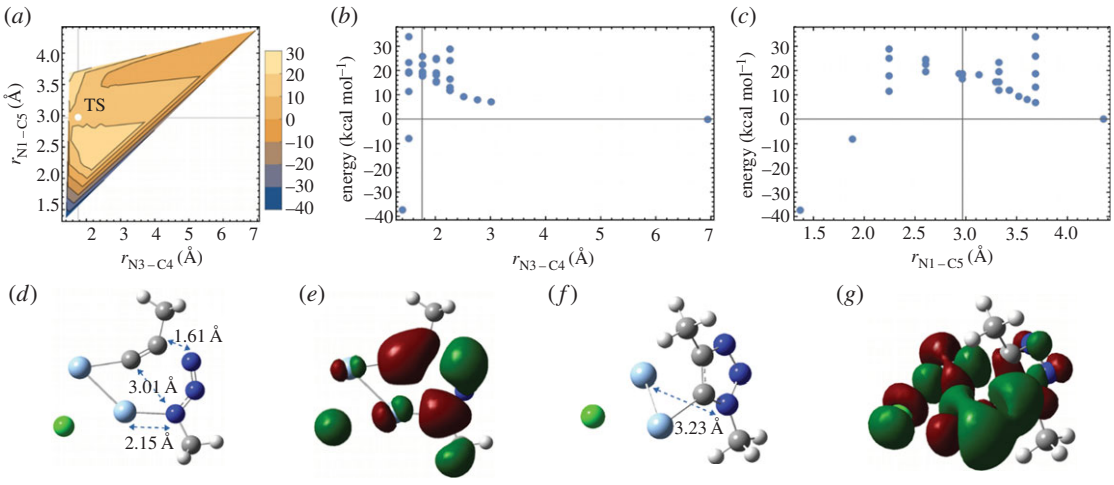
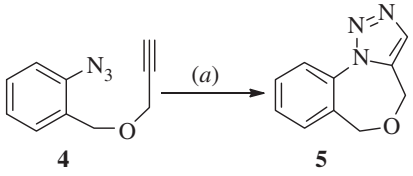


Figure 1. Quantum mechanical analysis of the silver-catalysed azide–alkyne cycloaddition. (*a*) Contour plot of the potential energy landscape for cycloaddition. Transition state (TS) is marked by a white dot. (*b*) Potential energy diagram for the N3–C4 bond formation. The vertical grid line indicates the TS bond length. (*c*) Potential energy diagram for N1–C5 bond formation. The vertical grid line indicates the TS bond length. (*d*) Transition state of the 1,4-disubstituted cycloaddition reaction involving a dinuclear silveracetylide. Colour key: H, white; C, grey; N, blue; Cl, green and Ag, cyan. Reaction coordinates are shown. (*e*) Molecular orbital (HOMO) of the transition state. (*f*) Reaction product 1,4-dimethyl-triazole attached to the metal centre. (*g*) HOMO of the product. A silver chloride is readily released from the product leaving (1,4-dimethyl-1H-1,2,3-triazol-5-yl) silver, the optimized geometry and molecular orbitals for which is given in the electronic supplementary material.



Scheme 2. (*a*) AgCl (20 mol %), TEA (5 equiv.), THF, 60°C, 4 h.

the energy landscape, it appears that N3–C4 bond formation occurs at first, which then facilitates the N1–C5 bond formation. The saddle point in this potential energy landscape, which indicates the TS, is also highlighted in figure 1*a*. Figure 1*b*,*c* shows the reaction coordinates for N3–C4 and N1–C5 bond formations, respectively. The saddle point coordinates were used as initial guess for the TS optimization. Figure 1*d* shows the optimized geometry of the TS structure. Electron densities in the highest occupied molecular orbital (HOMO) at the TS are depicted in figure 1*e*. From this TS, the activation energy for the Ag-catalysed 1,4 disubstituted cycloaddition was computed to be 18.52 kcal mol^{-1}. Optimized geometry of the product, i.e. after the N3–C4 and N1–C5 bond formation, is given in figure 1*f*. The Gibbs free energy for this two bond formation was found to be −37.51 kcal mol^{-1}. Figure 1*g* shows HOMO of the reaction product.

It has been established that the click reactions are catalysed by dinuclear metal centres [16,27]. The detailed DFT analysis of the of copper-catalysed click reactions has also been reported previously in the literature [26]. According to the detailed DFT analysis by Cantillo *et al*. [26], the energy barriers for the uncatalysed azide–alkyne coupling in the absence of copper (I) species was approximately 36 kcal mol^{-1}, whereas in the presence of a dinuclear copper centre the barrier leading to the 1,4-disubstituted triazole formation becomes approximately 16.0 kcal mol^{-1} which is comparable to the silver-catalysed reaction. However, the opposite regioisomer formation (1,5-approach) proceeds with a higher barrier, thus accounting for the observed regioselectivity [26]. Further, the comparison of the TS in silver-catalysed click reaction shows a very similar structure to that reported in the presence of the copper. In the presence of silver the N3–C4 and N1–C5 distances were calculated to be 1.61 Å and 3.01 Å, respectively (figure 1*d*), whereas in the presence of copper the N3–C4 and N1–C5 distances were

1.74 Å and 2.87 Å, respectively. N1–Cu distance was 2.01 Å, whereas the N1–Ag distance was computed to be 2.15 Å. In both the cases, the TS structures were planar in geometry.

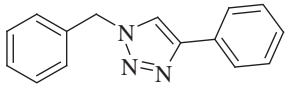
4. Conclusion

We have reported a silver catalyst for click reactions illustrating the transformations which are experimentally simple, robust and reliable. We have successfully developed an AgAAC catalytic reaction method for the cycloaddition of different acetylenes with azide compounds. This reaction exhibited good general applicability and regioselectivity with a variety of acetylenes and azide compounds under mild conditions. We have also explored the mechanism of this reaction using DFT, which suggested the involvement of a dinuclear silver centre, which is also reported in copper-catalysed click reactions.

5. General procedure and characterization data

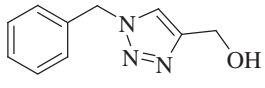
To an alkyne substrate (10 mmol) in THF (10 ml) was added AgCl (2 mmol), TEA (50 mmol) followed by azide substrate (12 mmol), and the reaction mixture was stirred vigorously at 60°C for 4 h. The reaction mixture was extracted with ethyl acetate, and washed with brine solution. After that the organic layer was separated, dried over sodium sulfate, filtered and evaporated under reduced pressure. The residue was finally purified by column chromatography (silica gel 100–200, ethyl acetate–hexane) to obtain the corresponding triazole compounds in 68–87% yield.

1-Benzyl-4-phenyl-1H-1,2,3-triazole (compound **3a**):



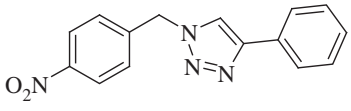
White solid; yield 87%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 5.59 (2 H, s), 7.32–7.345 (3 H, m), 7.38–7.434 (5 H, m), 7.68 (1 H, s), 7.80–7.82 (2 H, m); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 147.79, 134.23, 130.07, 128.71, 128.36, 127.62, 127.61, 125.25, 119.04, 53.79; mass: [EI-HRMS] ($C_{15}H_{13}N_3$) calc. 235.1109 Da, found: 235.1089 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3500, 3139, 3039, 1608, 1458, 1353, 1215, 1070, 972, 808, 764, 720, 695.

(1-Benzyl-1H-1,2,3-triazol-4-yl)methanol (compound **3b**):



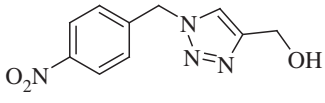
White solid; yield 81%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 2.43 (1 H, s), 4.78 (2 H, d, $J = 6$), 5.53 (2 H, s), 7.28–7.302 (2 H, m), 7.37–7.41 (3 H, m), 7.46 (1 H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 147.54, 134.01, 128.71, 128.39, 127.69, 121.09, 56.19, 53.77; mass: [EI-HRMS] ($C_{10}H_{11}N_3O$) calc. 189.0902 Da, found: 189.0903 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3352, 3141, 2927, 2861, 1607, 1553, 1496, 1335, 1221, 1127, 1046, 797, 722.

1-(4-Nitrobenzyl)-4-phenyl-1H-1,2,3-triazole (compound **3c**):



White solid; yield 85%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 5.71 (2 H, s), 7.345–7.375 (1 H, m), 7.42–7.47 (4 H, m), 7.77 (1 H, s), 7.81–7.84 (2 H, m), 8.24–8.26 (2 H, m); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 148.28, 147.64, 141.30, 129.63, 128.47, 128.10, 128.06, 125.29, 123.9, 119.24, 52.73; mass: [EI-HRMS] ($C_{15}H_{12}N_4O_2$) calc. 280.0960 Da, found: 280.0967 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3124, 3084, 1706, 1606, 1517, 1348, 1215, 1071, 1044, 866, 762, 692.

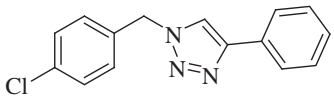
(1-(4-Nitrobenzyl)-1H-1,2,3-triazol-4-yl)methanol (compound **3d**):



White solid; yield 75%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 2.43 (1 H, s), 4.83 (2 H, s), 5.66 (2 H, s), 7.425 (2 H, d, $J = 6$), 7.56 (1 H, s), 8.245 (2 H, d, $J = 6$); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm)

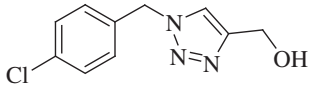
147.66, 141.05, 128.16, 123.90, 121.44, 56.13, 52.72; mass: [EI-HRMS] ($C_{10}H_{10}N_4O_3$) calc. 234.0753 Da, found: 234.0744 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3263, 3113, 1608, 1536, 1468, 1349, 1227, 1126, 1012, 854, 797, 729, 678.

1-(4-Chlorobenzyl)-4-phenyl-1H-1,2,3-triazole (compound **3e**):



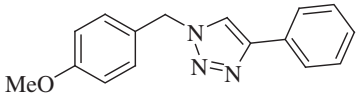
White solid; yield 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (in ppm) 5.54 (2 H, s), 7.21–7.26 (2 H, m), 7.3–7.45 (5 H, m), 7.67 (1 H, s,), 7.77–7.82 (2 H, m); ^{13}C NMR (75 MHz, $CDCl_3$): δ (in ppm) 134.77, 133.15, 130.30, 129.69, 129.32, 128.80, 128.24, 126.04, 125.65, 119.44, 53.41; mass: [EI-HRMS] ($C_{15}H_{12}ClN_3$) calc. 269.0720 Da, found: 271.0702 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3447, 3082, 1488, 1462, 1351, 1217, 1083, 1015, 808, 764.

(1-(4-Chlorobenzyl)-1H-1,2,3-triazol-4-yl)methanol (compound **3f**):



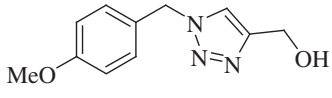
White solid; yield 68%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 4.75 (2 H, s), 5.5 (2 H, s), 7.21 (2 H, d, $J = 6$), 7.34 (2 H, $J = 12$), 7.47 (1 H, s,); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 147.84, 134.38, 132.51, 128.97, 128.87, 128.64, 128.61, 121.25, 55.78, 52.97; mass: [EI-HRMS] ($C_{10}H_{10}ClN_3O$) calc. 223.0512 Da, found: 223.0491 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3263, 3110, 2923, 2853, 1596, 1489, 1439, 1291, 1230, 1086, 1022, 858, 777, 661.

1-(4-Methoxybenzyl)-4-phenyl-1H-1,2,3-triazole (compound **3g**):



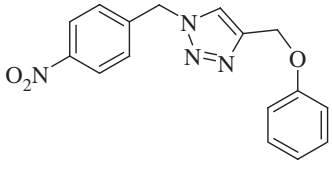
White solid; yield 79%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 3.82 (3 H, s), 5.52 (2 H, s), 6.91–6.94 (2 H, m), 7.27–7.29 (2 H, m), 7.30–7.33 (1 H, m), 7.40 (2 H, t, $J = 6$), 7.63 (1 H, s), 7.78–7.80 (2 H, m); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 159.50, 147.68, 130.07, 129.23, 128.34, 127.69,126.13, 125.22, 118.83, 114.06, 54.90, 53.35; mass: [EI-HRMS] ($C_{16}H_{15}N_3O$) calc. 265.1215 Da, found: 265.1223 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3449, 3123, 2932, 2839, 1610, 1513, 1459, 1301, 1247, 1073, 1026, 828, 763, 694.

(1-(4-Methoxybenzyl)-1H-1,2,3-triazol-4-yl)methanol (compound-**3 h**):



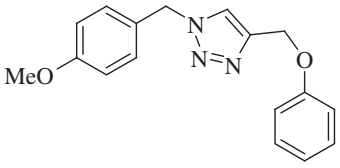
White solid; yield 72%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (in ppm) 3.04 (1 H, s), 3.79 (3 H, s), 4.73 (2 H, s), 5.43 (2 H, s,), 6.88 (2 H, d, $J = 6$), 7.22 (2 H, d, $J = 9$), 7.41 (1 H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ (in ppm) 158.87, 148.09, 129.70, 126.43, 121.52, 114.52, 56.14, 55.32, 53.68; mass: [ESI-HRMS] ($C_{11}H_{13}N_3O_2$) ($M + Na^+$) calc. 242.0905 Da, found: 242.0819 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3288, 3115, 2691, 2838, 1609,1513, 1460, 1247, 1034, 846, 785, 644.

1-(4-Nitrobenzyl)-4-(phenoxymethyl)-1H-1,2,3-triazole (compound **3i**):



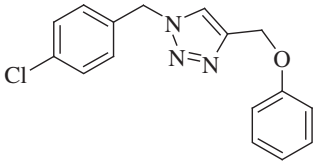
White solid; yield 70%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 5.24 (2 H, s), 5.67 (2 H, s), 6.96–7.00 (3 H, m), 7.31 (2 H, t, $J = 6$), 7.42 (2 H, d, $J = 12$), 7.63 (1 H, s), 8.25 (2 H, t, $J = 6$); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 157.57, 147.66, 144.92, 141.02, 129.14, 128.14, 128.04, 127.81, 123.90, 123.64, 123.54, 122.34, 120.96, 114.27, 61.49, 52.74; mass: [EI-HRMS] ($C_{16}H_{14}N_4O_3$) calc. 310.1066 Da, found: 310.1055 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3291, 2925, 1646, 1599, 1515, 1346, 1240, 1008, 829, 733.

1-(4-Methoxybenzyl)-4-(phenoxymethyl)-1H-1,2,3-triazole (compound **3j**):



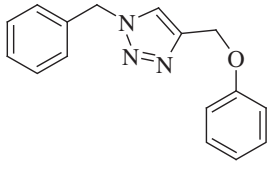
White solid; yield 82%; ^{1}H NMR (300 MHz, $CDCl_3$): δ (in ppm) 3.81 (3 H, s), 5.18 (2 H, s), 5.46 (2 H, s), 6.88 (1 H, s), 6.91 (1 H, s), 6.94–6.99 (3 H, m), 7.22 (1 H, s), 7.26 (2 H, d, $J = 6$), 7.31 (1 H, s), 7.49 (1 H, s); ^{13}C NMR (75 MHz, $CDCl_3$): δ (in ppm) 159.91, 158.15, 144.52, 129.70, 129.48, 126.36, 122.32, 121.18, 114.69, 114.45, 61.97, 55.30, 53.76; mass: [EI-HRMS] ($C_{17}H_{17}N_3O_2$) calc. 295.1321 Da, found: 295.1326 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3075, 1605, 1513, 1245, 1176, 1033, 841, 754.

1-(4-Chlorobenzyl)-4-(phenoxymethyl)-1H-1,2,3-triazole (compound **3k**):



White solid; yield 74%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 5.21 (2 H, s), 5.51 (2 H, s), 6.98 (3 H, d, $J = 12$), 7.22 (2 H, d, $J = 6$), 7.285–7.315 (2 H, m), 7.36 (2 H, d, $J = 6$), 7.55 (1 H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 157.67, 144.49, 134.44, 132.51, 129.10, 128.97, 128.92, 122.06, 120.86, 114.30, 61.55, 53.04; mass: [EI-HRMS] ($C_{16}H_{14}ClN_3O$) calc. 299.0825 Da, found: 299.0823 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3138, 3100, 2928, 1593, 1492, 1293, 1224, 1087, 1008, 851, 751.

1-Benzyl-4-(phenoxymethyl)-1H-1,2,3-triazole (compound **3l**):



White solid; yield 75%; ^{1}H NMR (600 MHz, $CDCl_3$): δ (in ppm) 5.21 (2 H, s), 5.55 (2 H, s), 6.96–7 (3 H, m), 7.28–7.31 (4 H, m), 7.39 (3 H, t, $J = 6$), 7.54 (1 H, s); ^{13}C NMR (150 MHz, $CDCl_3$): δ (in ppm) 157.71, 144.26, 133.98, 129.06, 128.69, 128.36, 127.67, 122.08, 120.79, 114.29, 61.59, 53.81; mass: [EI-HRMS] ($C_{16}H_{15}N_3O$) calc. 265.1215 Da, found: 265.1206 Da; FTIR (KBr, ν_{max}, cm^{-1}): 3131, 2923, 2855, 1738, 1593, 1493, 1221, 756.

Data accessibility. The detailed DFT calculations and NMR spectra of the final compounds are provided in the electronic supplementary material.

Authors' contributions. S.K.P., U.P., B.B. and N.C.M. conceived and designed the experiments. S.K.P., U.P., K.C. and S.K.K. performed the experiments. All the authors analysed the data. S.K.P. and U.P. drafted the manuscript. All authors read and approved the final manuscript.

Competing interests. The authors declare that they have no competing interests.

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Synthesis of Triazole-Substituted Quinazoline Hybrids for Anticancer Activity and a Lead Compound as the EGFR Blocker and ROS Inducer Agent

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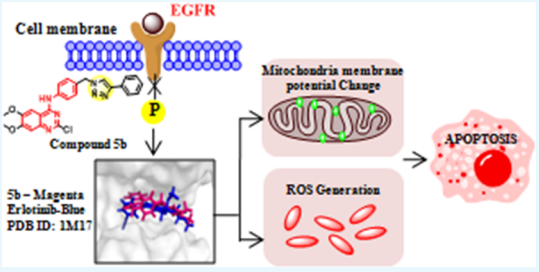
Supporting Information

ABSTRACT: A series of triazole-substituted quinazoline hybrid compounds were designed and synthesized for anticancer activity targeting epidermal growth factor receptor (EGFR) tyrosine kinase. Most of the compounds showed moderate to good antiproliferative activity against four cancer cell lines (HepG2, HCT116, MCF-7, and PC-3). Compound **5b** showed good antiproliferative activity (IC_{50} = 20.71 μM) against MCF-7 cell lines. Molecular docking results showed that compound **5b** formed hydrogen bond with Met 769 and Lys 721 and π−sulfur interaction with Met 742 of EGFR tyrosine kinase (PDB ID: 1M17). Compound **5b** decreases the expression of EGFR and p-EGFR. It also induces apoptosis through reactive oxygen species generation, followed by the change in mitochondrial membrane potential.



INTRODUCTION

Cancer is one of the most devastating diseases in the developing countries.[1] The epidermal growth factor receptor (EGFR) plays an important role in cell survival, growth, differentiation, and tumorigenesis. Dysregulation of EGFR is a common mechanism in cancer progression especially in nonsmall cell lung cancer (NSCLC). Also, overexpression of EGFR has been observed in different types of cancers such as breast, ovarian, head and neck, colon, and so forth.[2] Some FDA-approved drugs, EGFR inhibitors such as erlotinib[3] (i), gefitinib[4] (ii), icotinib[5] (iii), lapatinib[6] (iv), and afatinib[7] (v), are used for the treatment of the above-mentioned cancers (Figure 1). The interplay of reactive oxygen species (ROS) and the EGFR plays an important role in cancer progression. Excessive ROS can induce negative responses such as growth inhibition or death of cancer cells. Mitochondrial dysfunction is also the major mechanism inducing oxidative stress. Higher ROS levels can trigger overoxidation of the Met residue of EGFR T790M and shut down the EGFR downstream survival pathway.[8] Therefore, direct EGFR inhibition or inhibition of EGFR function via excessive ROS generation or both may be a feasible therapeutic approach for cancer treatment.

Side effects are major problems with the current EGFR inhibiting anticancer drugs. For example, erlotinib significantly reduced the levels of white blood cells, red blood cells (RBCs), and hemoglobin. It increased liver function markers, aspartate aminotransferase and alanine aminotransferase levels, and damaged the internal organs in an experimental rat model.[9] Similarly, unusual hematologic complications were detected after erlotinib was administered in patients with advanced NSCLC.[10] Therefore, it is important to design new EGFR inhibitors as anticancer agents with low toxicity on normal organs and blood cells.

Quinazoline is an important heterocyclic moiety used in drug discovery because of its diverse biological activities.[11] Especially, 4-aminoquinazoline moiety showed good efficacy against various cancers. The structure−activity relationship (SAR) of EGFR inhibitors such as erlotinib and lapatinib revealed a quinazoline moiety to play an important role in antitumor activity, especially 4-aminoquinazoline moiety. 4-Aminoquinazoline moiety seemed particularly very important for activity and showed diverse biological activities such as anticancer,[12] antitubercular,[13] antimalarial,[14] antileishmanial,[15] and antibacterial and antifungal activities.[16]

1,2,3-Triazole is another important pharmacophore in medicinal chemistry and it can form hydrogen bonding with biological targets,[17] which will be useful for the activity. Also, triazole moiety-containing molecules (vi−ix, Figure 1) are known to show various pharmacological activities such as anticancer,[18] anti-human immunodeficiency virus,[19] antituber-

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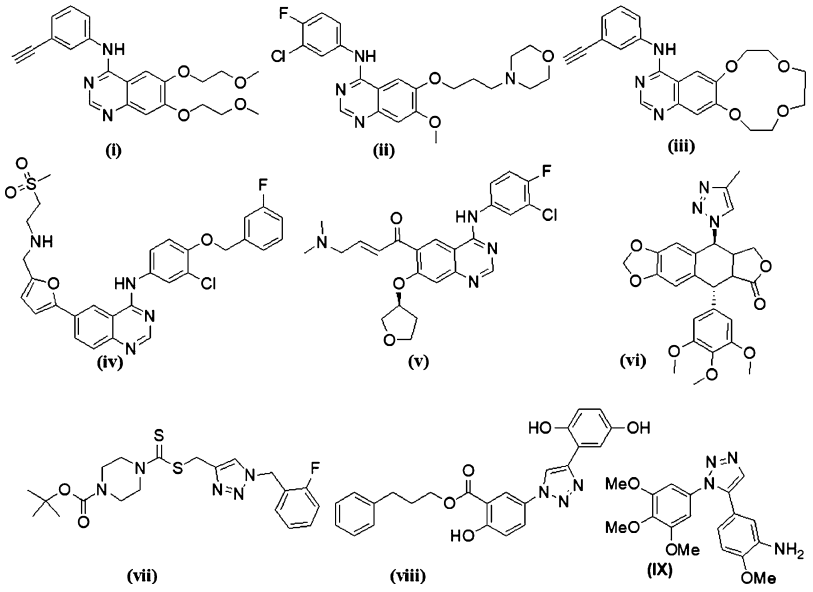


Figure 1. Chemical structure of some reported anticancer agents.

cular,[20] and anti-inflammatory[21] activities. These structural features and importance in various biological activities have made this moiety very important in drug discovery. The fight against cancer and hence the research to cure the disease are continuing since last many years. Many novel therapeutics were tried, but most of them suffer from severe toxicities. In an ongoing project in our laboratory on the discovery of new anticancer agents,[22] we were interested to make EGFR inhibitors. Recently, molecular hybridization approach has been widely used for the design and synthesis of small hybrid compounds for the treatment of cancer. The approach mainly involves combining two or more different pharmacophore moieties in a single molecule having a common scaffold. These hybrid molecules have many advantages over the conventional drugs such as toxicity,[23] solubility, multidrug resistance, and so forth. In the present study, we are using the molecular hybridization strategy to combine the biologically important two scaffolds, quinazoline and 1,2,3-triazole, to get a small set of new hybrid compounds (Figure 2). As discussed earlier, both quinazoline and 1,2,3-triazole moieties are very important for the anticancer activity, thus we synthesized 20 triazole-containing quinazoline hybrid compounds and performed cytotoxicity studies and the molecular docking studies thereafter. A lead compound was used to study EGFR inhibition, ROS generation, and toxicity in normal cells as well as in blood cells.

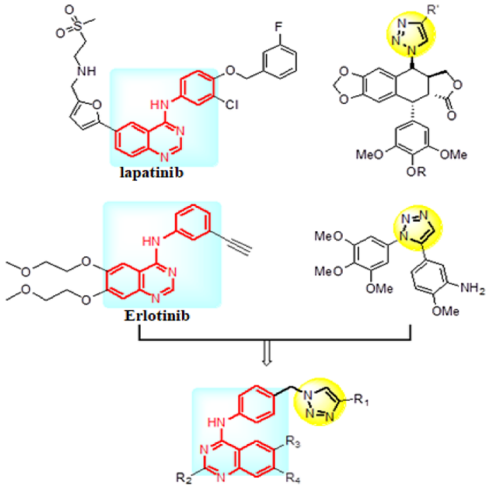
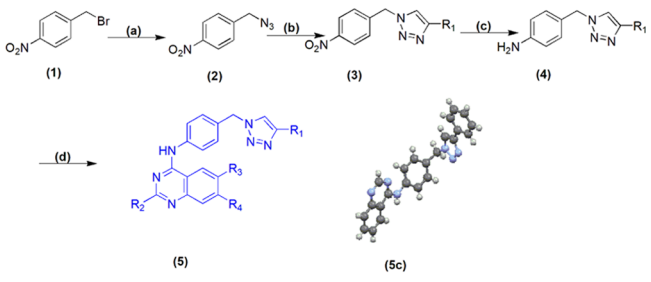


Figure 2. Design strategy of the target molecule.

RESULTS AND DISCUSSION

The synthesis was started by converting 4-nitro-benzylbromide **1** to the corresponding azide **2** in the presence of sodium azide in tetrahydrofuran (THF)−water in 95% yield. Compound **2** was reacted with different mono-substituted alkynes under classical "click" condition to produce different triazole compounds **3** in good yields. Finally, nitro group in **3** was reduced (Fe/NH_4Cl in ethanol and water) to the corresponding amine **4**, which was further coupled with different 4-chloro quinazolines to give the desired target compounds (**5a**−**5t**) in 73−88% yield (Scheme 1). All compounds were fully characterized by ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, Fourier transform infrared (FT-IR), and high-resolution mass spectrometry (HRMS) data.

Scheme 1. Synthetic Route of the Target Molecule and Crystal Structure of Compound 5c (ccdc: 1854806)[a]



[a]Reagents and conditions: (a) NaN_3, THF/H_2O (4:1), RT, 1 h, 95%; (b) CuI, THF, different alkynes, reflux, 80 °C, 3 h, 80−85%; (c) Fe, NH_4Cl, EtOH/H_2O (1:1), reflux, 80 °C, 2 h, 76−80%; (d) NaOAc, THF/H_2O (4:1), different quinazolines, reflux, 80 °C, 6 h, 73−88%.

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Table 1. Cytotoxicity Activities of Compounds 5a−5t against HepG2, MCF-7, HCT116, and PC-3 in Micromolar

S.No	Name	R_1	R_2	R_3	R_4	HepG2	MCF-7	HCT116	PC-3
1)	5a		H	OMe	OMe	52.57±4.13	39.67±1.55	72.01±3.45	89.43±2.56
2)	5b		Cl	OMe	OMe	42.46±1.96	20.71±1.2	67.96±1.96	59.33±3.89
3)	5c		H	H	H	74.52±3.27	48.07±3.47	87.09±3.52	70.52±3.78
4)	5d		H	OMe	OMe	76.19±4.86	34.48±3.99	79.35±5.19	88.52±2.23
5)	5e		Cl	OMe	OMe	63.06±3.95	34.23±2.07	82.53±2.76	67.67±1.74
6)	5f		H	H	H	58.75±6.78	36.94±4.07	86.65±3.98	76.54±3.33
7)	5g		H	OMe	OMe	49.01±4.13	35.92±2.67	61.42±3.25	86.02±3.78
8)	5h		Cl	OMe	OMe	102.25±5.12	33.53±2.02	78.32±1.34	70.52±3.78
9)	5i		H	H	H	76.41±3.45	41.66±1.98	81.33±2.71	75.63±4.56
10)	5j		H	OMe	OMe	60.87±2.97	33.36±1.8	72.03±3.56	70.90±3.06
11)	5k		Cl	OMe	OMe	46.18±3.06	25.67±0.8	65.66±4.13	44.65±2.89
12)	5l		H	H	H	52.51±4.13	42.88±1.89	64.80±3.45	67.43±1.77
13)	5m		H	OMe	OMe	64.79±4.23	38.97±3.35	67.87±3.27	88.77±4.44
14)	5n		Cl	OMe	OMe	63.11±4.97	30.84±3.22	71.32±4.67	65.92±3.56
15)	5o		H	H	H	63.17±3.86	41.05±2.33	70.08±3.65	52.87±1.78
16)	5p		Cl	H	H	67.45±3.1	33.49±2.8	62.34±3.9	39.98±2.56
17)	5q		Cl	H	H	51.22±2.6	39.92±2.1	59.96±2.3	59.21±1.22
18)	5r		Cl	H	H	56.39±1.9	37.35±1.3	69.96±1.67	66.89±3.4
19)	5s		Cl	H	H	52.44±2.7	28.24±1.56	68.84±3.33	53.61±1.9
20)	5t		Cl	H	H	68.89±3.03	36.65±1.11	72.96±2.2	58.67±2.5
	Erlotinib					17.68±3.29	11.57±1.79	20.46±4.25	26.87±2.25

The average 50% inhibitory concentration (IC_{50}) values (concentration needed to inhibit cancer cell line proliferation by 50%) of the compounds (**5a−5t**) against four human cancer cell lines that include HCT116 (human colorectal cancer cell line), HEPG2 (human liver cancer cell line), MCF-7 (human breast cancer cell line), and PC-3 (human prostate cancer cell line) were determined using the cytotoxicity assay method. The IC_{50} values are listed in Table 1, and the marketed anticancer drug erlotinib was used as positive control. On the basis of the cytotoxicity assay results, further efforts were made to elucidate the SAR. All of the synthesized compounds were screened against different cancer cell lines (HepG2, MCF-7, HCT116, and PC-3). Results suggested that most of the compounds showed moderate to good efficacy toward MCF-7 compared to other cell lines. Among all, compound **5b** showed the best activity with an IC_{50} value of 20.71 μM in MCF-7 cell lines. Moreover, it was observed that compounds bearing the −Cl atom at the second position of quinazoline (**5b**, **5e**, **5h**, **5k**, and **5n**) were better compared to other substitutions. Surprisingly, all of the above-mentioned active compounds containing −OMe group at the 6, 7 position of the quinazoline moiety are resulted, strongly suggesting that the chlorine atom at the 2nd position and −OMe group at the 6, 7 position of quinazoline are necessary for the activity.

The best compound **5b** has an IC_{50} value of 20.71 μM, which is higher than the standard compound erlotinib (11.57 μM). Interestingly, **5b** also shows lower toxicity than erlotinib on a normal human epithelial kidney cell line (40.32 ± 4.43 and 29.48 ± 3.32 μM), in human blood RBC (45.6 ± 2.65 and 16.23 ± 3.23 μM), and in human peripheral blood mononuclear cell (37.38 ± 3.55 and 20.46 ± 4.1 μM, respectively). Therefore, though compound **5b** shows relatively low toxicity than erlotinib in cancer cells, it has low toxicity in normal cell lines and normal blood cells. Thus, compound **5b** is expected to be a better drug candidate than erlotinib. Gratifyingly, compound **5b** also has 4-amino quinazoline moiety as the pharmacophore similar to that of erlotinib and gefitinib, and we thought that compound **5b**

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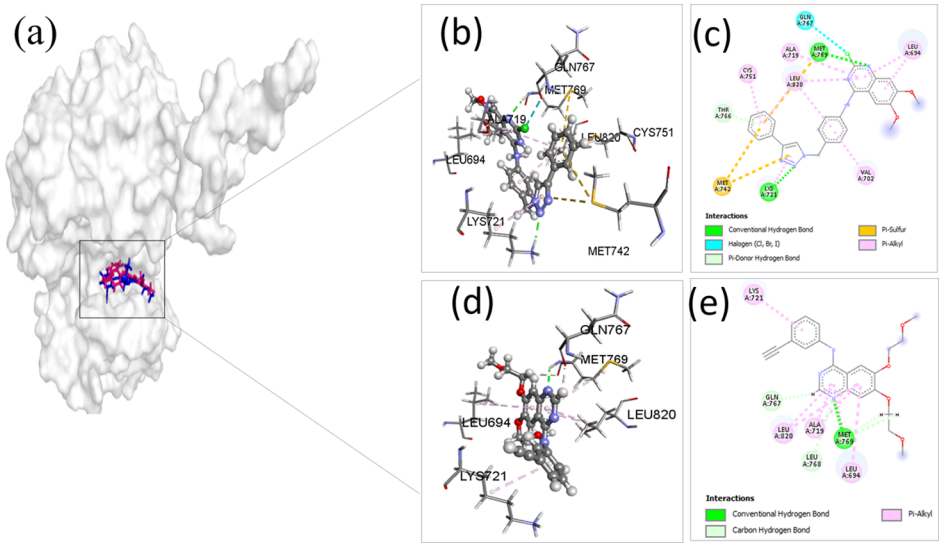

Figure 3. (a) Surface representation of the EGFR protein (PDB ID 1M17) along with compound **5b** (magenta) and erlotinib (blue). (b,c) Three-dimensional (3D) and two-dimensional (2D) representation of molecular docking interactions of compound **5b** in the ATP-binding pocket of EGFR tyrosine kinase (1M17). (d,e) 3D and 2D molecular docking interactions of erlotinib in the ATP-binding pocket of EGFR tyrosine kinase.

shows anticancer activity through the EGFR-mediated pathway. For this study, we selected the crystal structure (PDB ID: 1M17)[24] of EGFR tyrosine kinase for the molecular modeling study. To study the interaction between EGFR tyrosine kinase and triazole-substituted quinazoline hybrid derivatives, molecular docking of the EGFR protein (PDB ID: 1M17) and compound **5b** using discovery studio software was performed. Discovery studio was used for the visualization of interaction of the target molecule with EGFR tyrosine kinase. Docking results showed that compound **5b** goes and binds in the adenosine 5′-triphosphate (ATP)-binding pocket of 1M17 and showed two hydrogen bonding interactions with N1 of quinazoline with the main-chain NH group of Met 769 at a distance of 3.13 Å and triazole N2 with Lys 721 in the ATP-binding pocket at a distance of 3.04 Å. Both erlotinib and compound **5b** go and bind in the same ATP-binding pocket of EGFR tyrosine kinase (1M17, Figure 3).

Compound **5b** also showed other interactions such as halogen interaction with Gln 767, π−alkyl interaction with Leu 694, Leu 820, and Cys 751, and π−sulfur interaction with Met 742. Erlotinib was used as the reference compound, which also formed a similar H-bond with Met 769 at a distance of 2.85 Å.

EGFR is an important signaling network in the case of cell proliferation, adhesion, migration, and survival. In order to study the mechanism of triazole-substituted quinazoline hybrids, we investigated the effect of compound **5b** on the EGFR signaling pathway in MCF-7 cell lines using western blot analysis.[25] After treatment with compound **5b** in MCF-7 cell lines, the level of EGFR and p-EGFR decreases with different time intervals of 12 and 24 h (Figure 4). In this experiment, erlotinib was used as positive control. These results confirmed that the antiproliferative effect of compound **5b** in MCF-7 cell lines is mainly due to the decrease of EGFR and phosphorylation of EGFR and its downstream process. EGFR inhibition leads to the activation of ROS generation, which leads to DNA damage and cell death. Cellular generation of ROS is an important factor of apoptotic cell death. We have examined here the status of ROS generation by compound **5b**. The maximum ROS generation was observed at 24 h after the treatment of MCF-7 cells with compound **5b**.

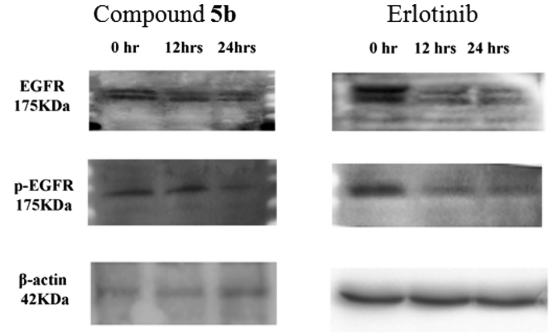

Figure 4. Western blot image of expression of EGFR and p-EGFR upon treatment with compound **5b** and erlotinib (positive control).

Also, flow cytometric analysis revealed that the fluorescein isothiocyanate (FITC) mean intensity was 765 in control cells but 1683 in compound **5b** (20.71 μM) treated cells after 24 h, indicating a shift in FITC mean intensity from the control cells to the cells treated with compound **5b**. These results demonstrated that compound **5b** (20.71 μM) inducing apoptosis in MCF-7 cells at 12 and 24 h might proceed via the ROS-mediated pathway.[26]

Apoptosis is the desired way of cancer cell death. To determine the effect of compound **5b** on apoptosis, fluorescence-activated cell sorting (FACS) was performed on MCF-7 cell lines treated with compound **5b** at different time intervals.[27] At the initial stage of apoptosis, phosphatidyl serine is exposed from inside cell membrane to outside and this can bind with annexin V. After treatment of MCF-7 cells with compound **5b** (20.71 μM) at time points (0, 12, and 24 h), MCF-7 cells were stained with annexin V-FITC and propidium iodide (PI) and monitored by flow cytometry. It was observed that early apoptosis rates increased from 5.9 to 24.6% and the late apoptosis rates increased from 0.7 to 14.2%. The results showed that compound **5b** increased cellular apoptosis in a time-dependent manner (Figure 5). Flow cytometric analysis of control cells revealed that 84.4% of the cell population exhibited fluorescence at the PE-Texas Red A channel, indicating a higher level of cells having a healthy $\Delta\Psi_m$,

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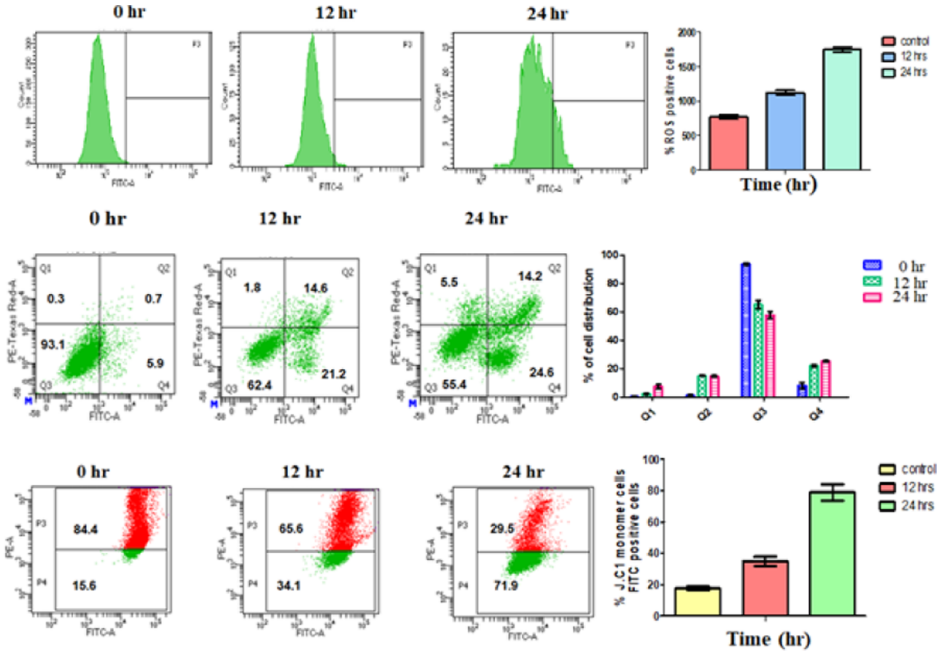

Figure 5. Effect of compound **5b** in (i) apoptosis induction through ROS generation, (ii) analysis of apoptosis, and (iii) apoptosis induction through the mitochondrial membrane potential assay in MCF-7 cells at different time points (0−24) h.

whereas compound **5b** (20.71 μM) treated cells at 24 h revealed that 29.5% of the cell population exhibited fluorescence at the PE-Texas Red A channel, which showed a loss of $\Delta\Psi_m$ in 54.9% of cell population after 24 h.[28] These results indicate that compound **5b** might induce apoptosis by generation of ROS via the mitochondrial pathway as it leads to lowering of mitochondrial membrane potential.

CONCLUSIONS

In conclusion, a series of triazole-substituted quinazoline hybrid molecules were designed and synthesized as anticancer agents. The results showed that most of the compounds had moderate to good antiproliferative effects against four different cell lines HCT116, HepG2, PC-3, and MCF-7. Among them, compound **5b** showed good antiproliferative effects against MCF-7 cell lines. Molecular docking studies showed that compound **5b** formed hydrogen bond with Met 769 of the EGFR protein, which regulates the conformation of EGFR which is responsible for antiproliferative activity. Compound **5b** decreased the expression of EGFR and p-EGFR in MCF-7 cell lines, from which we can conclude that compound **5b** exhibits antiproliferative effects in MCF-7 cell lines through the EGFR signaling pathway. Compound **5b** also caused change in mitochondrial membrane potential and ROS-mediated apoptosis in MCF-7 cell lines. From the above results, it may be concluded that compound **5b** acts as the EGFR inhibitor and can be used in the treatment of cancer. Further optimization of the structure to show improved EGFR inhibitory and antiproliferative activity is ongoing in the laboratory.

EXPERIMENTAL SECTION

General Remarks. All chemicals and reagents were purchased from Sigma-Aldrich. Column chromatography purifications were performed using silica gel grade 9385, 100−200 mesh, neutral alumina (Sigma). Thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates with 0.20 mm thickness (Merck, Germany), which was visualized under an ultraviolet light chamber (254 and 365 nm). NMR experiments were run on Bruker Avance III 600 (600 MHz for ^{1}H and 150 MHz for ^{13}C). ^{1}H NMR spectra were recorded for solution in $CDCl_3$ or dimethyl sulfoxide (DMSO) with tetramethylsilane as the standard. Chemical shifts for ^{1}H and ^{13}C spectra were recorded in parts per million (ppm). Data were reported as follows: chemical shift (ppm), integrated intensity, multiplicity (indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), and coupling constants (J) in hertz (Hz). HRMS spectra were obtained on a JEOL MS station 700 (JEOL Ltd., Akishima-Shi, Japan). The FT-IR spectra of the samples were recorded on a JASCO FT-IR 4200 spectrometer (JASCO, Easton, MD, USA) using a KBr disk technique. The spectra were recorded from 400 to 4000 cm^{-1}. JASCO software was used for data processing. Apoptosis, ROS, and JC1 assay were performed by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Synthetic Procedure. *General Procedure To Synthesize Compound 2.* To the solution of compound **1** (1 equiv) in THF was added sodium azide (1.5 equiv) which was dissolved in water dropwise at 0 °C. The reaction mixture was then stirred for 1 h (0 °C to rt) and was monitored through TLC. After the complete consumption of the starting material, the solvent was evaporated. The crude mixture was then dissolved in EtOAc and washed with water and brine (3 × 10 mL). Thereafter, the organic layer was collected and dried over Na_2SO_4 and concentrated under reduced pressure to get yellow liquid in 95% yield.

General Procedure To Synthesize Compound 3. To the solution of compound **2** (1 equiv) in THF were added different acetylene (1 equiv) and copper iodide (0.2 equiv), which was heated to reflux temperature (80 °C) for 3 h. After the total consumption of the starting material (monitored through TLC), the solvent was evaporated. The reaction mixture was then diluted with ethyl acetate, filtered through a Celite bed, and washed with water and brine (3 × 10 mL) in a

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separating funnel. Thereafter, the organic layer was collected and dried over Na_2SO_4 and then evaporated under reduced pressure to get a crude solid, which was purified by column chromatography (silica gel 100−200, per ether/ethyl acetate 4:1) to afford the desired product as yellow solid with 80−85% yield.

General Procedure To Synthesize Compound ***4***. To the solution of compound **3** (1 equiv) in ethanol−water (1:1) were added iron powder (4 equiv) and ammonium chloride (10 equiv), which was heated to reflux temperature (80 °C) for 2 h. The reaction was monitored through TLC until total consumption of the starting material. After completion, ethanol was evaporated, and the crude mixture was diluted with ethyl acetate and passed through a Celite bed. Then, it was washed with water and brine (3 × 10 mL) in a separating funnel. Thereafter, the organic layer was collected and dried over Na_2SO_4 and evaporated under reduced pressure to get the crude solid. It was then purified by column chromatography (silica gel 100−200, per ether/ethyl acetate 1.5:1) to afford the desired product as yellow solid with 76−80% yield.

General Procedure To Synthesize Compound ***5***. To the solution of compound **4** (1 equiv) in THF/water (4:1), different substituted 4-chloro quinazoline (0.9 equiv) and anhydrous sodium acetate (3 equiv) were added. The reaction mixture was heated to reflux temperature (80 °C) for 6 h and monitored through TLC. After total consumption of the starting material, the solvent was evaporated. The residue was then dissolved in EtOAc and washed with water and brine (3 × 10 mL). The organic layer was thereafter collected and dried over Na_2SO_4. It was then purified by column chromatography (neutral aluminum oxide, dichloromethane/methanol 49:1) to afford the desired product in 73−88% yield as a white powder.

In similar manner, all other triazole-containing quinazoline hybrid compounds (**5a**−**5t**) were prepared.

Characterization Data. *6,7-Dimethoxy-N-(4-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl)phenyl)quinazolin-4-amine (**5a**).* Yield 87% (32 mg); pale yellow solid; mp: 220−221 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.53 (s, 1H), 8.65 (s, 1H), 8.44 (s, 1H), 7.86 (d, J = 7.8 Hz, 2H), 7.82 (m, 3H), 7.44 (t, J = 6 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.33 (t, J = 7.2 Hz, 1H), 7.18 (s, 1H), 5.63 (s, 2H), 3.95 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ (ppm) 156.7, 154.7, 153.2, 149.3, 147.4, 147.1, 139.9, 131.1, 129.3, 128.7, 128.3, 125.6, 122.9, 121.8, 109.3, 107.6, 100.3, 56.6, 56.2, 53.2. IR (KBr) [cm^{-1}] ν: 3386, 3314, 3201, 3138, 3007, 1624, 1577, 1517, 1467, 1426, 1351, 1245, 1146, 1070.41, 993, 923, 855, 763, 691, 657. HRMS (ESI-m/z): calcd for $C_{25}H_{22}N_6O_2$, [M + H]$^+$ 439.1822; found, 439.1892.

*2-Chloro-6,7-dimethoxy-N-(4-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl)phenyl)quinazolin-4-amine (**5b**).* Yield 82% (37 mg); white solid; mp: 292−293 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.90 (s, 1H), 8.68 (s, 1H), 7.86 (d, J = 7.2 Hz, 3H), 7.74 (d, J = 8.4 Hz, 2H), 7.43 (m, 4H), 7.33 (t, J = 7.2 Hz, 1H), 7.17 (s, 1H), 5.66 (s, 2H), 3.94 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 157.9, 155.0, 154.2, 149.0, 148.2, 146.6, 138.4, 131.7, 130.7, 128.9, 128.3, 127.9, 125.2, 122.9, 121.5, 107.2, 106.6, 102.2, 56.3, 56.0, 52.7. IR (KBr) [cm^{-1}] ν: 3361, 3138, 2988, 2948, 1621, 1572, 1515, 1458, 1426, 1240, 1150, 1001, 963, 842, 763, 697. HRMS [EI-m/z]: calcd for $C_{25}H_{21}N_6O_2Cl$, [M]$^+$ 472.1415; found, 472.14136.

*N-(4-((4-Phenyl-1H-1,2,3-triazol-1-yl)methyl)phenyl)-quinazolin-4-amine (**5c**).* Yield 85% (30 mg); white solid; mp: 270−271 °C; ^{1}H NMR (600 MHz,, DMSO-d_6): δ 9.85 (s, 1H), 8.66 (s, 1H), 8.58 (s, 1H), 8.55 (d, J = 8.4 Hz, 1H), 7.87 (m, 5H), 7.79 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 7.42 (m, 4H), 7.33 (t, J = 7.2 Hz, 1H), 5.64 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.1, 154.8, 150.1, 147.1, 139.6, 133.5, 131.5, 131.1, 129.3, 128.7, 128.3, 128.2, 126.7, 125.6, 123.4, 123.0, 121.8, 115.6, 53.2. IR (KBr) [cm^{-1}] ν: 3258, 3135.49, 3087, 1611, 1570, 1524, 1501, 1415, 1357, 1224, 1074, 1043, 922.80, 766, 683, 511. HRMS [EI-m/z]: calcd for $C_{23}H_{18}N_6$, [M]$^+$ 378.1593; found, 378.15329.

*N-(4-((4-(4-Ethylphenyl)-1H-1,2,3-triazol-1-yl)methyl)-phenyl)-6,7-dimethoxyquinazolin-4-amine (**5d**).* Yield 88% (39 mg); white solid; mp: 230−231 °C; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.68 (s, 1H), 8.06 (d, J = 12 Hz, 1H) 7.71 (s, 1H), 7.68 (m, 4H), 7.43 (d, J = 6 Hz, 1H), 7.27 (s, 1H), 7.22 (m, 4H), 5.55 (s, 2H), 4.03 (s, 3H), 3.92 (d, J = 1.8 Hz, 3H), 2.65 (q, J = 7.8 Hz, 2H), 1.23 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 156.4, 154.7, 153.4, 149.6, 148.5, 147.5, 144.6, 139.3, 138.9, 129.9, 128.5, 128.4, 127.5, 125.6, 122.3, 119.6, 109.3, 107.7, 99.9, 56.3, 56.2, 53.8, 28.6, 15.4. IR (KBr) [cm^{-1}] ν: 3379, 2956, 1623, 1577, 1516, 1462, 1423, 1394, 1240, 1140, 1061, 996, 840, 798, 540. HRMS (ESI-m/z): calcd for $C_{27}H_{26}N_6O_2$, [M + H]$^+$ 466.2195; found, 467.2189.

*2-Chloro-N-(4-((4-(4-ethylphenyl)-1H-1,2,3-triazol-1-yl)-methyl)phenyl)-6,7-dimethoxyquinazolin-4-amine (**5e**).* Yield 84% (34 mg); white solid; mp: 277−278 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.89 (s, 1H), 8.61 (s, 1H), 7.85 (s, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.73 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8.4 Hz, 2H), 7.16 (s, 1H), 5.64 (s, 2H), 3.94 (s, 3H), 3.91 (s, 3H), 2.61 (q, J = 7.8, 2H), 1.19 (t, J = 7.8, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.3, 155.4, 154.6, 149.5, 148.6, 147.2, 143.9, 138.9, 132.2, 128.7, 125.6, 123.4, 121.6, 107.7, 107.1, 102.6, 56.7, 56.4, 53.1, 28.3, 15.9. IR (KBr) [cm^{-1}] ν: 3356, 2965, 1621, 1573, 1515, 1455, 1427, 1343, 1294, 1240, 1150, 1002, 962, 839, 800, 580, 530. HRMS [EI-m/z] calcd for $C_{27}H_{25}N_6O_2Cl$, [M]$^+$ 500.1728; found, 500.17178.

*N-(4-((4-(4-Ethylphenyl)-1H-1,2,3-triazol-1-yl)methyl)-phenyl)quinazolin-4-amine (**5f**).* Yield 87% (28 mg); white solid; mp: 250−251 °C; ^{1}H NMR (600 MHz,, DMSO-d_6): δ 9.86 (s, 1H), 8.59 (d, J = 6 Hz, 2H), 8.54 (d, J = 12 Hz, 1H), 7.88 (d, J = 6 Hz, 2H), 7.85 (d, J = 6 Hz, 1H), 7.79 (d, J = 12 Hz, 1H), 7.76 (d, J = 12 Hz, 2H), 7.64 (t, J = 12 Hz, 1H), 7.41 (d, J = 6 Hz, 2H), 7.27 (d, J = 7.8 Hz, 2H), 5.62 (s, 2H), 2.61 (q, J = 7.8 Hz, 2H), 1.19 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.2, 154.8, 150.1, 147.2, 143.9, 139.5, 133.5, 131.6, 128.7, 128.7, 128.6, 128.2, 126.7, 125.6, 123.4, 123.0, 121.4, 115.6, 53.2, 28.3, 15.9. IR (KBr) [cm^{-1}] ν: 3422, 3290, 3103, 2967, 2927, 1616, 1571, 1526, 1498, 1422, 1358, 1319, 1224, 1049, 924, 830, 770, 677. HRMS [EI-m/z] calcd for $C_{25}H_{22}N_6$, [M]$^+$ 406.1915; found, 406.19156.

*(1-(4-(6,7-Dimethoxyquinazolin-4-ylamino)benzyl)-1H-1,2,3-triazol-4-yl)methanol (**5g**).* Yield 77% (29 mg); off-white solid; mp: 295−296 °C; ^{1}H NMR (600 MHz,, DMSO-d_6): δ 9.52 (s, 1H), 8.43 (s, 1H), 8.02 (s, 1H), 7.82 (s, 1H), 7.77 (d, J = 7.8 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.18 (s, 1H), 5.55 (s, 2H), 5.18 (t, J = 5.4 Hz, 1H), 4.51 (d, J = 6 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 156.7, 154.7, 153.2, 149.3, 148.7, 147.4, 131.3, 128.7, 123.1, 122.9, 109.3, 107.6, 102.3, 56.6, 56.2, 55.5, 52.9. IR (KBr) [cm^{-1}] ν: 3315, 3146, 3004.33, 2928, 2834, 1623, 1579, 1517, 1466, 1425, 1246, 1140, 1050, 994, 924, 854, 779, 657, 557, 515. HRMS (ESI-m/z) calcd for $C_{20}H_{20}N_6O_3$, [M + H]$^+$ 392.1675; found, 393.1675.

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*(1-(4-(2-Chloro-6,7-dimethoxyquinazolin-4-ylamino)-benzyl)-1H-1,2,3-triazol-4-yl)methanol (**5h**).* Yield 74% (24 mg); light yellow solid; mp: 265−266 °C; ^{1}H NMR (600 MHz,, DMSO-d_6): δ 9.89 (s, 1H), 8.04 (s, 1H), 7.85 (s, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 7.17 (s, 1H), 5.57 (s, 2H), 5.20 (s, 1H), 4.51 (s, 2H), 3.94 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.4, 155.4, 154.6, 149.5, 148.8, 148.6, 138.7, 132.4, 128.8, 123.4, 107.06, 107.04, 102.6, 56.7, 56.4, 55.5, 52.8. IR (KBr) [cm^{-1}] ν: 3390, 3282, 3037, 3007, 2930, 1604, 1576, 1511, 1465, 1420, 1309, 1241, 1136, 1057, 992, 849, 781, 751, 726, 695. HRMS [EI-m/z] calcd for $C_{20}H_{19}N_6O_3Cl$, $[M]^+$ 426.1207; found, 426.1216.

*(1-(4-(Quinazolin-4-ylamino)benzyl)-1H-1,2,3-triazol-4-yl)methanol (**5i**).* Yield 73% (21 mg); white solid; mp: 268−269 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.84 (s, 1H), 8.58 (s, 1H), 8.54 (d, J = 8.4 Hz, 1H), 8.02 (s, 1H), 7.85 (d, J = 8.4 Hz, 3H), 7.79 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 8.4 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 5.56 (s, 2H), 5.16 (t, J = 6 Hz, 1H), 4.51 (d, J = 6.6 Hz, 2 H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.2, 154.8, 150.1, 148.7, 139.4, 133.5, 131.79, 128.7, 128.2, 126.7, 123.4, 123.1, 123, 115.5, 55.5, 52.8. IR (KBr) [cm^{-1}] ν: 3433, 3308, 3129, 2923, 1604, 1576, 1532, 1426, 1402, 1364, 1325, 1229, 1128, 1049, 93, 770. HRMS [EI-m/z] calcd for $C_{18}H_{16}N_6O$, $[M]^+$ 332.1386; found, 332.13748.

*6,7-Dimethoxy-N-(4-((4-(phenoxymethyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)quinazolin-4-amine (**5j**).* Yield 87% (33 mg); white solid; mp: 224−225 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.52 (s, 1H), 8.44 (s, 1H), 8.29 (s, 1H), 7.83 (s, 1H), 7.79 (d, J = 7.8 Hz, 2H), 7.36 (d, J = 7.8 Hz, 2H), 7.29 (t, J = 7.8 Hz, 2H), 7.18 (s, 1H), 7.02 (d, J = 8.4 Hz, 2H), 6.94 (t, J = 7.2 Hz, 1H), 5.59 (s, 2H), 5.13 (s, 2H), 3.95 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.4, 156.7, 154.7, 153.2, 149.3, 147.4, 143.5, 139.9, 131.1, 129.9, 128.7, 124.9, 122.8, 121.2, 115.1, 109.3, 107.6, 102.2, 61.4, 56.6, 56.2, 53.0. IR (KBr) [cm^{-1}] ν: 3375, 1621, 1575, 1514, 1461, 1421, 1237, 1137, 997, 853, 758. HRMS [EI-m/z] calcd for $C_{26}H_{24}N_6O_3$, $[M]^+$ 468.1910; found, 468.1891.

*2-Chloro-6,7-dimethoxy-N-(4-((4-(phenoxymethyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)quinazolin-4-amine (**5k**).* Yield 84% (29 mg); light yellow solid; mp: 260−261 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.88 (s, 1H), 8.32 (s, 1H), 7.86 (s, 1H), 7.72 (d, J = 8.4 Hz, 2H), 7.39 (d, J = 8.4 Hz, 2H), 7.29 (t, J = 7.2 Hz, 2H), 7.17 (s, 1H), 7.03 (d, J = 7.8 Hz, 1H), 6.95 (t, J = 7.2 Hz, 2H), 5.62 (s, 2H), 5.14 (s, 2H), 3.95 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.4, 158.3, 155.4, 154.6, 149.4, 148.6, 143.5, 138.8, 132.1, 129.9, 128.8, 125, 123.3, 121.2, 115.1, 107.6, 107.1, 102.6, 61.4, 56.7, 56.4, 52.9. IR (KBr) [cm^{-1}] ν: 3364, 2922, 2853, 1601, 1573, 1515, 1458, 1427, 1293, 1240, 1151, 1004, 961, 840, 758. HRMS (ESI-m/z) calcd for $C_{26}H_{23}N_6O_3Cl$, [M + H]$^+$ 503.1598; found, 503.1599.

*N-(4-((4-(Phenoxymethyl)-1H-1,2,3-triazol-1-yl)methyl)-phenyl)quinazolin-4-amine (**5l**).* Yield 85% (32 mg); white solid; mp: 208−210 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.85 (s, 1H), 8.59 (s, 1H), 8.55 (d, J = 8.4 Hz, 1H), 8.29 (s, 1H), 7.87 (m, J = 6, 3H), 7.79 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 13.2 Hz, 1H), 7.37 (d, J = 8.4 Hz, 2H), 7.28 (t, J = 7.2 Hz, 2H), 7.03 (d, J = 8.4 Hz, 1H), 6.94 (t, J = 7.8 Hz, 2H), 5.60 (s, 2H), 5.13 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.4, 158.1, 154.8, 150.1, 143.5, 139.5, 133.52, 131.53, 129.9, 128.8, 128.2, 126.7, 124.9, 123.4, 123, 121.2, 115.5, 115.1, 61.2, 53. IR (KBr) [cm^{-1}] ν: 3355, 3126, 2922, 1606, 1572, 1531, 1498, 1416, 1311, 1218, 1122, 1041, 828, 776, 683, 506. HRMS [EI-m/z] calcd for $C_{24}H_{20}N_6O$, $[M]^+$ 408.1703; found, 408.1703.

*(6,7-Dimethoxy-quinazolin-4-yl)-[4-(4-phenylaminomethyl-[1,2,3]triazol-1-ylmethyl)-phenyl]-amine (**5m**).* Yield 85% (31 mg); light yellow solid; mp: 261−262 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.51 (s, 1H), 8.43 (s, 1H), 8.01 (s, 1H), 7.82 (s, 1H), 7.77 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 7.18 (s, 1H), 7.06 (t, J = 7.8 Hz, 2H), 6.62 (t, J = 7.8 Hz, 2H), 6.53 (t, J = 12 Hz, 1H), 6.02 (t, J = 6 Hz, 1H), 5.54 (s, 2H), 4.27 (d, J = 6 Hz, 2H), 3.95 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 156.7, 154.7, 153.2, 149.3, 148.8, 147.4, 146.5, 139.7, 131.2, 129.2, 128.6, 123.1, 122.8, 116.4, 112.7, 109.3, 107.6, 102.2, 56.6, 56.2, 55.3, 52.8, 39. IR (KBr) [cm^{-1}] ν: 3390, 3282, 3037, 3007, 2930, 1604, 1576, 1511, 1465, 1420, 1309, 1241, 1136, 1057, 992, 849, 781, 751, 726, 695. HRMS (ESI-m/z) calcd for $C_{26}H_{25}N_7O_2$, [M + H]$^+$ 468.2148; found, 468.2150.

*(2-Chloro-6,7-dimethoxy-quinazolin-4-yl)-[4-(4-phenylaminomethyl-[1,2,3]triazol-1-ylmethyl)-phenyl]-amine (**5n**).* Yield 82% (29 mg); light yellow solid; mp: 280−281 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.87 (s, 1H), 8.03 (s, 1H), 7.85 (s, 1H), 7.69 (d, J = 6 Hz, 2H), 7.35 (d, J = 9 Hz, 2H), 7.17 (s, 1H), 7.06 (t, J = 8.4 Hz, 2H), 6.62 (d, J = 9.6 Hz, 2H), 6.53 (t, J = 7.2 Hz, 1H), 6.03 (t, J = 6 Hz, 1H), 5.57 (s, 2 H), 4.28 (d, J = 6.6 Hz, 2H), 3.94 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.3, 155.5, 154.6, 149.4, 148.8, 148.6, 146.5, 138.7, 132.4, 129.2, 128.7, 123.4, 123.2, 116.4, 112.7, 107.6, 107.1, 102.6, 56.7, 56.4, 52.7, 39. IR (KBr) [cm^{-1}] ν: 3406, 1603, 1572, 1513, 1426, 1241, 1150, 961, 844, 755. HRMS (ESI-m/z) calcd for $C_{26}H_{24}N_7O_2Cl$, [M + Na]$^+$ 524.1578; found, 524.1586.

*[4-(4-Phenylaminomethyl-[1,2,3]triazol-1-ylmethyl)-phenyl]-quinazolin-4-yl-amine (**5o**).* Yield 81% (29 mg); light yellow solid; mp: 250−251 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 9.83 (s, 1H), 8.58 (s, 1H), 8.54 (d, J = 8.4 Hz, 1H), 8.01 (s, 1H), 7.86 (m, 3H), 7.79 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 8.4 Hz, 1H), 7.34 (d, J = 8.4 Hz, 2H), 7.06 (t, J = 8.4 Hz, 2H), 6.62 (d, J = 7.8 Hz, 2H), 6.53 (t, J = 7.2 Hz, 1H), 6.02 (t, J = 6 Hz, 1H), 5.55 (s, 2H), 4.28 (d, J = 6 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 158.1, 154.8, 150.1, 148.8, 146.5, 139.4, 133.5131.7, 129.2, 128.7, 128.2, 126.7, 123.4, 123.1, 123, 116.4, 115.5, 112.7, 52.8, 39. IR (KBr) [cm^{-1}] ν: 3422, 3289, 2923, 1604, 1571, 1524, 1498, 1414, 1357, 1316, 1256, 1123, 1051, 924, 773, 687, 510. HRMS [EI-m/z] calcd for $C_{24}H_{21}N_7$, $[M]^+$ 407.1858; found, 407.1855.

*(2-Chloro-quinazolin-4-yl)-[4-(4-phenyl-[1,2,3]triazol-1-ylmethyl)-phenyl]-amine (**5p**).* Yield 86% (39 mg); white solid; mp: 204−206 °C; ^{1}H NMR: (600 MHz, DMSO-d_6): δ 10.25 (s, 1H), 8.68 (s, 1H), 8.55 (d, J = 8.4 Hz, 1H), 7.86 (m, 3H), 7.79 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 7.2 Hz, 1H), 7.43 (m, 4 H), 7.32 (t, J = 7.8 Hz, 1H), 5.65 (s, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.8, 156.6, 151.3, 147.1, 138.6, 134.6, 132.6, 131.1, 129.4, 128.8, 128.4, 127.4, 127.2, 125.6, 123.9, 123.6, 122, 114.2, 53.1. IR (KBr) [cm^{-1}] ν: 3311, 2922, 2854, 1620, 1564, 1516, 1423, 1342, 1222, 1187, 1077, 1049, 948, 753. HRMS [ESI-m/z] calcd for $C_{23}H_{17}N_6Cl$, [M + H]$^+$ 413.1281; found, 472.1286.

*(2-Chloro-quinazolin-4-yl)-{4-[4-(4-ethyl-phenyl)-[1,2,3]-triazol-1-ylmethyl]-phenyl}-amine (**5q**).* Yield 85% (36 mg); white solid; mp: 205−207 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.24 (s, 1H), 8.61 (s, 1H), 8.55 (d, J = 8.4 Hz, 1H), 7.88 (t, J = 8.4 Hz, 1H), 7.79 (d, J = 8.4 Hz, 2H), 7.76 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 7.8 Hz, 1H), 7.66 (t, J = 8.4 Hz, 1H),

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7.42 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 7.8 Hz, 2H), 5.64 (s, 2H), 2.61 (q, J = 7.8 Hz, 2H), 1.18 (t, J = 7.8 Hz, 3H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.8, 156.6, 151.3, 147.2, 143.9, 138.5, 134.7, 132.7, 128.8, 128.7, 128.6, 127.4, 127.1, 125.7, 123.9, 123.6, 121.6, 114.2, 53.1, 28.4, 16. IR (KBr) [cm^{-1}] ν: 3379, 2964, 2924, 1623, 1605, 1562, 1528, 1497, 1424, 1345, 1290, 1220, 1195, 1078, 951, 839, 763. HRMS [ESI-m/z] calcd for $C_{25}H_{21}N_6Cl$, $[M + H]^+$ 441.1594; found, 441.1627.

*{1-[4-(2-Chloro-quinazolin-4-ylamino)-benzyl]-1H-[1,2,3]-triazol-4-yl}-methanol (**5r**).* Yield 83% (34 mg); white solid; mp: 224−226 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.24 (s, 1H), 8.54 (d, J = 8.4 Hz, 1H), 8.04 (s, 1H), 7.88 (t, J = 7.2 Hz, 1H), 7.76 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 1H) 7.64 (t, J = 7.2 Hz, 1H), 7.38 (d, J = 8.4 Hz, 2H), 5.57 (s, 2H), 5.17 (t, J = 6 Hz, 1H), 4.5 (d, J = 4.8 Hz, 2 H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.8, 156.6, 151.3, 148.8, 138.5, 134.6, 132.9, 128.8, 127.4, 127.2, 123.9, 123.6, 123.3, 114.2, 55.5, 52.8. IR (KBr) [cm^{-1}] ν: 3334, 2925, 1607, 1566, 1527, 1424, 1410, 1288, 1200, 1126, 1057, 953, 859, 766. HRMS [ESI-m/z] calcd for $C_{28}H_{15}N_6OCl$, $[M + H]^+$ 367.1074; found, 367.1162.

*(2-Chloro-quinazolin-4-yl)-[4-(4-phenoxymethyl-[1,2,3]-triazol-1-ylmethyl)-phenyl]-amine (**5s**).* Yield 85% (36 mg); white solid; mp: 200−202 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.25 (s, 1H), 8.56 (d, J = 8.4 Hz, 1H), 8.31 (s, 1H), 7.88 (t, J = 7.2 Hz, 1H), 7.77 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 1H), 7.65 (t, J = 8.4 Hz, 1H), 7.38 (d, J = 8.4 Hz, 2H), 7.29 (t, J = 8.4 Hz, 2H), 7.02 (d, J = 7.8 Hz, 2H), 6.93 (t, J = 7.2 Hz, 1H), 5.62 (s, 2H), 5.12 (s, 2 H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.8, 158.5, 156.6, 151.3, 143.5, 138.5, 134.7, 132.7, 129.98, 128.9, 127.4, 127.2, 125.1, 123.9, 123.6, 121.3, 115.1, 114.2, 61.4, 52.9. IR (KBr) [cm^{-1}] ν: 3336, 2922, 2853, 1621, 1565, 1529, 1516, 1426, 1410, 1366, 1237, 1216, 1187, 1058, 1006, 853, 760. HRMS [ESI-m/z] calcd for $C_{24}H_{19}N_6OCl$, $[M + H]^+$ 443.1387; found, 443.1450.

*(2-Chloro-quinazolin-4-yl)-[4-(4-phenylaminomethyl-[1,2,3]triazol-1-ylmethyl)-phenyl]-amine (**5t**).* Yield 85% (36 mg); white solid; mp: 212−214 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.23 (s, 1H), 8.54 (d, J = 8.1 Hz, 1H), 8.03 (s, 1H), 7.89 (t, J = 7.2 Hz, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 1H) 7.64 (t, J = 8.4 Hz, 1H), 7.35 (d, J = 9 Hz, 2H), 7.05 (t, J = 7.2 Hz, 2H), 6.61 (d, J = 7.8 Hz, 2H), 6.52 (t, J = 7.2 Hz, 1H), 6.04 (t, J = 6 Hz, 1H), 5.56 (s, 2H), 4.27 (d, J = 6 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6): δ 159.8, 156.6, 151.3, 148.8, 146.5, 138.4, 134.7, 132.9, 129.3, 128.8, 127.4, 127.2, 123.9, 123.6, 123.3, 116.5, 114.2, 112.8, 52.8, 31.2. IR (KBr) [cm^{-1}] ν: 3366, 2922, 2850, 1621, 1604, 1561, 1451, 1409, 1366, 1287, 1195, 1079, 1056, 957, 839, 765. HRMS [ESI-m/z] calcd for $C_{24}H_{20}N_7Cl$, $[M + H]^+$ 442.1547; found, 441.1564.

General Experimental Procedures for Biological Studies. *Cytotoxicity Assay.* Exponentially growing cells were seeded into 96-well plates at a concentration of 1 × 10^5 cells per well. After 24 h incubation at 37 °C, the cells were treated with all compounds in different concentrations in triplicates. The cells were incubated for another 24 h. A 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL) was added to all wells and incubated for 4 h at 37 °C. The solution was discarded, and the insoluble dark blue crystals (formazan) were dissolved in 40 μL DMSO. After 15 min, the absorbance was measured using an ELISA reader at a wavelength of 595 nm. The IC_{50} of all compounds is the concentration (μg/mL) of the compound at which there was 50% growth inhibition with respect to the control culture in different cell lines.

Analysis of Cellular apoptosis. Apoptosis was assayed by using an annexin-V FITC apoptosis detection kit (Calbiochem, CA, USA). Briefly, cells were treated with or without the compounds in a time-dependent manner (0, 12, and 24 h) and then washed and stained with PI and annexin-V-FITC in accordance with the manufacturer's instructions. The percentages of live, apoptotic, and necrotic cells were determined by the flow cytometric method (Becton Dickinson, San Jose, CA, USA). Data from 1 × 10^6 cells were analyzed for each sample.

Measurement of ROS. ROS generation was measured by dichlorofluorescein diacetate (DCF-DA). After treatment with the compounds for the time periods (0, 12, and 24 h), the cells were incubated with 10 μM DCF-DA at 37 °C for 20 min. Then cell pellets were suspended in 1 mL phosphate-buffered saline (PBS). Samples were analyzed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Measurement of Mitochondrial Membrane Potential (JC1 Assay). MCF-7 cells were seeded for 24 h and then treated with and without compounds in a time-dependent manner (0, 12, and 24 h). Cells were washed twice with ice-cold PBS and then incubated with JC-1 dye (5 μg/mL) in darkness for 20 min at room temperature (37 °C). Emission was determined by the flow cytometric method (Becton Dickinson, San Jose, CA, USA) at 525 nm.

Western Blot. Treated or untreated cells were collected and lysed. Lysates (30 μg of protein per well) were separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to poly(vinylidene difluoride) membranes using a Trans-Blot system (Trans Blot wet transfer; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin in TBST (tris buffered saline containing 0.1% Tween-20, pH 7.6) for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After washing with TBST, membranes were incubated with horseradish peroxidase conjugated secondary antibody for 1 h. Immunoreactive bands were visualized by chemiluminescence using tetramethylbenzidine as the substrate. β-Actin was used as the loading control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01960.

^{1}H NMR and ^{13}C NMR spectra of all compounds and crystal data of compound **5c** (PDF)

Crystallographic data of compound **5c** (CIF)

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Notes

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Communication

Decarboxylative Acylation

Rh^{III}-Catalyzed Decarboxylative *o*-Acylation of Arenes Bearing an Oxidizing Directing Group

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Abstract: Here in we report, rhodium(III)-catalyzed decarboxylative acylation of arenes using α-oxocarboxylic acids as acyl surrogate. In this strategy, O–NHAc group act as an autocleavable oxidizing directing group (ODG^{auto}), thus giving rise to *ortho*-acylated phenols in moderate to good yields. Mechanistic studies provided strong support for a kinetically relevant C–H bond activation. According to the best of our knowledge, this is the first report of Rhodium catalyzed decarboxylative acylation.

Introduction

Transition-metal-catalyzed chemical transformation of C–H bond has emerged as a highly focused area of research to the synthetic community since last few decades. Diverse types of C–H bond functionalization have been explored intensely. Acyl functionalization to C–H bond always draw considerable attention due to the large abundance of acyl group in the family of organic molecular systems. In the recent years, α-oxocarboxylic acids have been explored as efficient acyl surrogates for acylation to C–H bond of aryl systems containing diverse types of directing groups such as pyridines,[1] acetanilides,[2] azoarene,[3] *O*-methyl ketoximes,[4] amides,[5] *N*-nitrosoanilines[6] etc. These methods produce CO_2 as the waste instead of often toxic species. Goossen was the pioneer person to develop a synthetic route to achieve aryl ketones via Pd-catalyzed decarboxylative cross coupling using aryl halides with potassium α-oxocarboxylates in the presence of a Cu^{I} source.[7] After this pioneering work, several groups have reported C–H acylation through Pd-catalyzed decarboxylation of α-oxocarboxylic acids. But there is no report of Rhodium catalyzed decarboxylative acylation. Now, the catalytic activity changes altering the transition metal. Specific transition metal is required for a particular type of C–H bond transformation. Thus any kind of new catalytic activity of any transition metal always draw considerable attention. In past few years, Rhodium or Iridium-catalyzed various kinds of aryl C–H functionalization have been reported using auto cleavable oxidizing directing group (ODG^{auto}) such as CONHOMe,[8] N–NHR,[9] O–NHAc.[10] These types of directing groups, become traceless in the products to generate valuable functional groups. In case of traditional directing groups(DG) mediated C–H functionalization, generally very harsh conditions are required for removal of the DG after C–H functionalization to achieve the target molecule, whereas use of auto cleavable DG's circumvent the harsh conditions.

In accordance with the above information and inspired by the recent advances in transition-metal-catalyzed C–H functionalization to access phenol,[10e,10g,11] we aimed to synthesize *ortho*-acylated phenols via Rh^{III}-catalyzed direct C–H acylation of arenes bearing O–NHAc group as ODG^{auto}. Phenols containing acyl group are considered as significant structural motifs for their great abundance in diverse type of bio-active molecules including natural products[12] (Figure 1). Among the family of acyl group assisted phenols, *o*-acyl phenols are most important for their wide range of application. They serve as the prime building blocks for the synthesis of various drugs such as celiprolol, acebutolol, warfarin, propafenone etc.[13] In addition to this, *o*-acylated phenols are effecttive synthetic intermediates to access various types of oxygen-containing heterocyclic scaffolds such as benzoxazole, benzofuranone, dibenzoxazepine and chromanone etc. Therefore, development of a method for the preparation of *ortho*-acyl phenols is always appreciated.

The traditional methods involved in synthesis of *o*-acylated phenols (like Friedel–Craft acylation, Fries Rearrangement)[14,15] suffer from various kinds of drawbacks such as lack of regioselectivity or harsh reaction condition etc. Since, last few decades direct C–H functionalization technique has become complementary to the classical protocols. So, accessing *o*-acyl phenol through C–H functionalization is in focus now a days to overcome the problems of classical methods. Recently transition metal catalyzed direct *ortho*-hydroxylation of aromatic ketone has been explored for the preparation of *o*-acyl phenols but this approach also suffers from site selectivity in few cases of bi-aryl ketones.[16] Therefore synthesis of *o*-acyl phenols via

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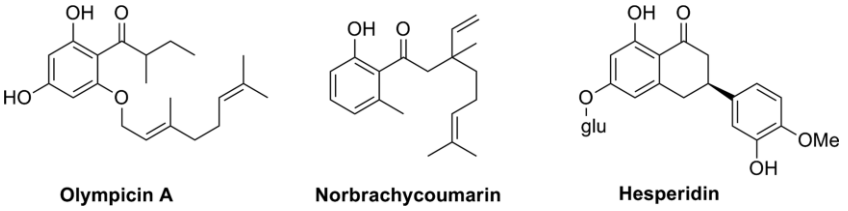


Figure 1. Some bio-active molecules containing *o*-acyl phenol.

direct C–H transformation is expected to have the potential to draw considerable attention.

Results and Discussion

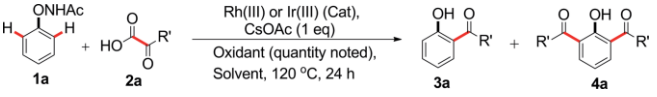
However, our investigation started with exploring the coupling of *N*-phenoxyacetamide, **1a** (1 equivalent) with phenylglyoxylic acid, **2a** (1.2 equivalent) in the presence of 4 mol-% $[Rh(Cp^*Cl_2)]_2$ and 2 equivalent $Cu(OAc)_2$ as an oxidant along with 1 equivalent of CsOAc as an additive in acetonitrile solvent with constant heating at 120 °C for 24 hours. This condition provided 7 % combined yield of mono (**3a**) and bis-*ortho*-acylated phenol (**4a**) in 5:1 ratio (Table 1, entry 1). Next, we screened the solvent system where only in DMF, the combined yield increased to a very little extent (12 %) with 4:1 ratio of mono & bis-*ortho*-acylated product. Then we optimized the reaction condition with different oxidizing agents. Oxidants like TBHP, CAN, $K_2S_2O_8$ did not give the desired product. Gratifyingly, we found Ag_2CO_3 (20 mol-%) with stoichiometric amount of $K_2S_2O_8$ (1.5–2 equivalent) to be the superior oxidizing system over other oxidants producing the highest yield (Table 1, entry 10–11). The combination of Ag_2CO_3 with other oxidants like TBHP, CAN, Oxone did not satisfied the reaction condition. However, 20 mol-% Ag_2CO_3 with 2 equiv. $K_2S_2O_8$ produced 84 % combined yield of mono- and bis-*ortho*-acylated phenol in 2.8:1 ratio.

To improve the selectivity towards mono-acylation the amount of $K_2S_2O_8$ was reduced to 1.5 equivalent from 2 equivalent and as a result the ratio of mono-and-bis product was turned to almost 3.2:1 with 78 % combined yield. Using DMSO as solvent, the yield was not improved and Both of $[IrCp^*Cl_2]_2$ and $[Rh(COD)Cl]_2$ as the catalyst instead of $[RhCp^*Cl_2]_2$ were unable to promote the reaction. We also made an attempt to achieve the bis-*ortho*-acylated product in major. To serve this issue, a reaction was carried out using *N*-phenoxyacetamide, **1a** (1 equivalent) with phenylglyoxylic acid, **2a** (3 equivalent) in the presence of 5 mol-% $[Rh(Cp^*Cl_2)]_2$ and 100 mol-% Ag_2CO_3 with 2 equiv. $K_2S_2O_8$ as the combined oxidant along with 1 equivalent of CsOAc as additive in DMF solvent at 120 °C for 24 hours.

Remarkably, this reaction condition provided the ratio of mono- and bis-*ortho*-acylated phenol nearly to 1:20 with 83 % combined yield.

Thereafter, the substrate scope was explored using the optimized reaction condition. It is interesting to note that the *N*-phenoxyacetamides bearing strong electron withdrawing groups (**1g**, **1h**: CF_3, CO_2Me) at the *para*-position afforded exclusively mono-acylated compounds **3g**, **3h** in satisfactory yield

Table 1. Optimization of reaction conditions.[a]

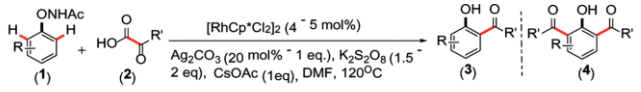


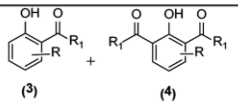
Entry	Catalyst	Oxidant	Solvent	Yield [%][c]	Ratio (**3a**/**4a**)[d]
1	$[RhCp^*Cl_2]_2$	$Cu(OAc)_2$ (2 equiv.)	MeCN	7 %	5:1
2	$[RhCp^*Cl_2]_2$	$Cu(OAc)_2$ (2 equiv.)	MeOH	0	–
3	$[RhCp^*Cl_2]_2$	$Cu(OAc)_2$ (2 equiv.)	DMF	12 %	4:1
4	$[RhCp^*Cl_2]_2$	TBHP (2 equiv.)	DMF	0	–
5	$[RhCp^*Cl_2]_2$	CAN (2 equiv.)	DMF	0	–
6	$[RhCp^*Cl_2]_2$	$K_2S_2O_8$ (2 equiv.)	DMF	0	–
7	$[RhCp^*Cl_2]_2$	AgOAc (20 mol-%) $K_2S_2O_8$ (2 equiv.)	DMF	27 %	3:1
8	$[RhCp^*Cl_2]_2$	$AgNO_3$ (20 mol-%) $K_2S_2O_8$(2 equiv.)	DMF	55 %	2.7:1
9	$[RhCp^*Cl_2]_2$	Ag_2O (20 mol-%) $K_2S_2O_8$(2 equiv.)	DMF	75 %	2.8:1
10	.$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) $K_2S_2O_8$ (2 equiv.)	DMF	84 %	2.8:1
11	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) $K_2S_2O_8$ (1.5 equiv.)	DMF	78 %	3.2:1
12	$[RhCp^*Cl_2]_2$	Ag_2CO_3(20 mol-%) $(NH_4)_2S_2O_8$ (1.5eq)	DMF	72 %	3:1
13	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) $K_2S_2O_8$ (1.5 equiv.)	DMSO	43 %	2.8:1
14	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) TBHP (1.5 equiv.)	DMF	0	–
15	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) CAN (1.5 equiv.)	DMF	0	–
16	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) Oxone (1.5 equiv.)	DMF	0	–
17	$[IrCp^*Cl_2]_2$	Ag_2CO_3 (20 mol-%) $K_2S_2O_8$ (1.5 equiv.)	DMF	0	–
18	$[Rh(COD)Cl]_2$	Ag_2CO_3 (20 mol-%) $K_2S_2O_8$ (1.5 equiv.)	DMF	0	–
19	$[RhCp^*Cl_2]_2$	Ag_2CO_3 (1 equiv.) $K_2S_2O_8$ (2 equiv.)	DMF	83 %	1:20

[a] Reaction Conditions: **1a** (0.2 mmol), **2a** (0.24 mmol), $[RhCp^*Cl_2]_2$ (4 mol-%), oxidant (quantity noted), CsOAc (1 equiv.), solvent (2 mL), 120 °C, 24 h in sealed tubes. [b] Condition: **1a** (0.2 mmol), **2a** (0.6 mmol), $[RhCp^*Cl_2]_2$ (5 mol-%), oxidant (quantity noted), CsOAc (1 equiv.), solvent (2 mL), 120 °C, 24 h in sealed tube. [c] Combined Yields of **3a** and **4a**. [d] Ratio of **3a** and **4a** was determined by ^{1}HNMR analysis of crude reaction mixture.

(Table 2). In addition, the reaction of *meta*-substituted *N*-aryloxyacetamides exclusively occurred at the more sterically accessible position to afford the corresponding mono-acylated phenol as a single product. These data suggest that steric effects of the substrate strongly interfere with either the formation of the cyclometalated intermediate or the proximity of α-oxocarboxylic acid to the cyclometalated intermediate. The

Table 2. Substrate scope for *o*-acyl phenol preparation.



Entry	N-Aryloxy acetamide	α-Oxocarboxylic acid	Products	Yield% (3:4)
	 (1)	 (2)	 (3) + (4)	
1	**1a** R = H	**2a** R_1 = Ph	**3a + 4a**	78 % (3.2 : 1)
	1b R = *o*-Me	**2a**	**3b**	73 %
	1c R = *m*-Me	**2a**	**3c**	79 %
	1d R = *m*-Ome	**2a**	**3d**	82 %
	1e R = *m*-Ph	**2a**	**3e**	75 %
	1f R = *m*-CF_3	**2a**	**3f**	78 %
	1g R = *p*-CF_3	**2a**	**3g**	76 %
	1h R = *p*-CO_2Me	**2a**	**3h**	62 %
2	**1f**	**2b** R_1 = *p*-Tolyl	**3i**	83 %
	1f	**2c** R_1 = *p*-Fluorophenyl	**3j**	61 %
	1f	**2d** R_1 = *p*- Chlorophenyl	**3k**	65 %
	1f	**2e** R_1 = *p*- Bromophenyl	**3l**	78 %
	1f	**2f** R_1 = 2-Furyl	**3m**	67 %
3	**1d**	**2g** R_1 = Isobutyl	**3n**	51 %
4	**1a** R = H	**2a** R_1 = Ph	**4a**	83 %
	1i R = *p*-Me	**2a** R_1 = Ph	**4b**	88 %
	1j R = *p*-^{t}Bu	**2a** R_1 = Ph	**4c**	85 %
	1k R = *p*-Ph	**2a** R_1 = Ph	**4d**	79 %

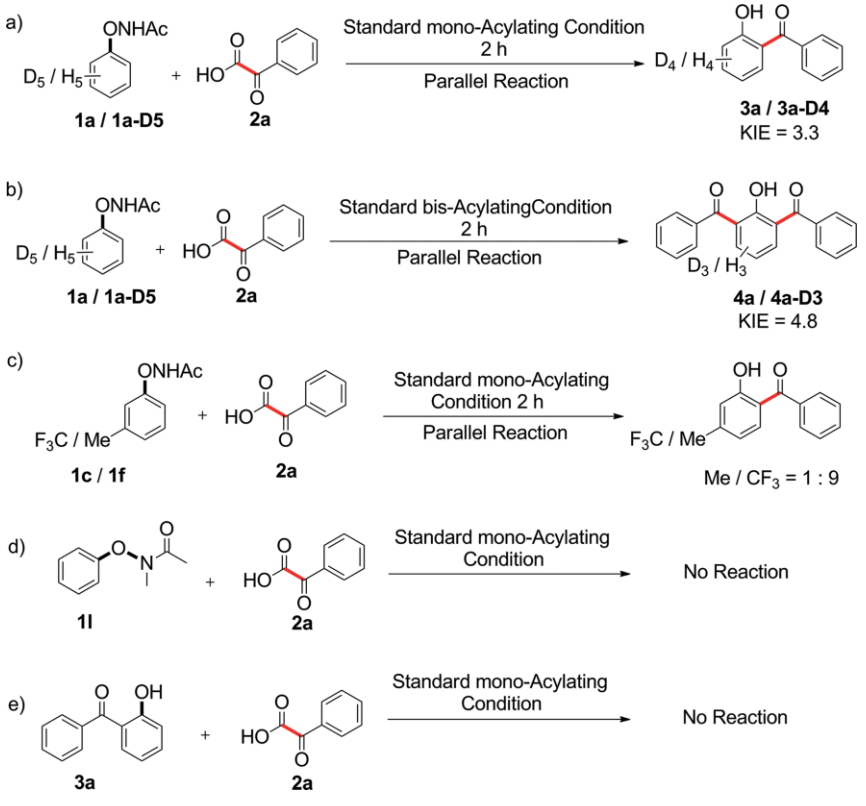
[a] Reaction Conditions for mono-Acylation (from **3a** to **3n**): *N*-phenoxyacetamide (0.2 mmol), α-oxocarboxylic acid (0.24 mmol), $[RhCp^*Cl_2]_2$ (4 mol-%), Ag_2CO_3 (20 mol-%) + $K_2S_2O_8$(1.5 equiv.), CsOAc (1 equiv.), DMF (2 mL), 120 °C, 24 h in sealed tubes. [b] Reaction Conditions for bis-Acylation (from **4a** to **4d**): *N*-phenoxyacetamide (0.2 mmol), α-oxocarboxylic acid (0.6 mmol), $[RhCp^*Cl_2]_2$ (5 mol-%), Ag_2CO_3 (1 equiv.) + $K_2S_2O_8$ (2 equiv.), CsOAc (1 equiv.), DMF (2 mL), 120 °C, 24 h. [c] Yield isolated by column chromatography.

ortho-substituted *N*-aryloxyacetamides are also compatible to the reaction condition.

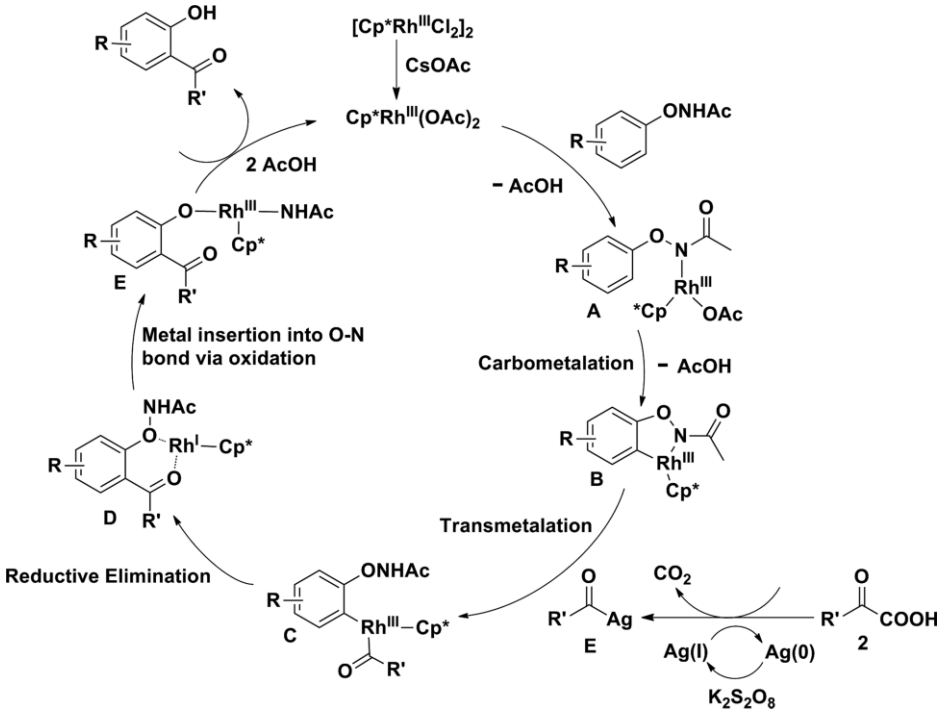
Next, we aimed to achieve bis-*ortho*-acylated phenols for the *N*-aryloxyacetamides bearing electron donating groups at *para*-position using the reaction condition for bis-acylation(Table 1, entry 19). Symmetrical *N*-phenoxyacetamides (1 equivalent) with methyl, *tert*-butyl and phenyl group at the *para*-position (**1i, 1j**, **1k**) underwent smooth bis-acylation reaction producing the corresponding bis-acylated products **4b**, **4c**, **4d** with high yield (Table 2). But surprisingly, this bis-acylating condition was unable to generate bis-acylated product for the *N*-phenoxyacetamides bearing strong electron withdrawing groups like CF_3, CO_2Me (**1g, 1h**) at the *para*-position. The scope of α-oxocarboxylic acids was also explored. Arylglyoxylic acid with electron donating group was favourable in comparison to arylglyoxylic acid substituted with electron withdrawing group. Hetero-arylglyoxylic acids and aliphatic-glyoxylic acids are also suitable for this methodology. Different types of arylglyoxylic acids were prepared by well known Riley oxidation method: oxidation of corresponding acetophenone derivatives using selenium dioxide as oxidant.

Next, a set of experimental investigations were conducted to explore the deep insight of the reaction mechanism. First, the isotope-labeling experiment was carried out with deuterium-labeled *N*-phenoxyacetamide $[D_5]$-**1a**.

Treatment of **2a** with the equimolar amount of both 1a and $[D_5]$-1a for 2 hours under standard mono-acylating condition



Scheme 1. Mechanistic studies. (a) kinetic isotope study for mono-acylation. (b) kinetic isotope study for bis-acylation. (c) Competitive experiment. (d) Replacing ONHAc group by ONMeAc. (e) Necessity of ONHAc group for bis-acylation.



Scheme 2. Plausible mechanism for this transformation.

gave a relatively large KIE value (k_H/k_D = 3.3). The result indicated that the cleavage of C–H bond might be involved in the rate-determining step (Scheme 1a). Thereafter, same amount of both 1a and [D_5]-1a were treated with **2a** for 2 hours under bis-acylating condition and this experiment resulted greater KIE value (k_H/k_D = 4.8) than earlier experiment which supports the fact considering the C–H bond cleavage to be involved in the rate-limiting step.[10g]

Next, to delineate the electronic preference of the reaction a competitive coupling between an equimolar amount of **1c** and **1f** with **2a** was conducted (Scheme 1c). The ratio of products showed that the electron-deficient 1f was preferentially transformed (**3c**/**3f** = 1:9), suggesting that the C–H activation process might follow a concerted metalation–deprotonation (CMD) mechanism.[8f,8l,17]

Subsequently, the coupling reaction between *N*-methyl-substituted phenoxyacetamide **1l** and phenylglyoxylic acid **2a** was carried out under standard mono-acylating condition (Scheme 1d) but this reaction did not produce the desired product. This fact indicates that the N–H bond of O–NHAc is indispensable for this transformation which is consistent with the previous reports of Lu[10e] and Yi.[10g]

Then, **3a** was subjected to couple with phenylglyoxylic acid **2a** to evaluate the necessity of O–NHAc group for the second *o*-acylation during bis-acylation and this time also no transformation of **3a** happened (Scheme 1e). This indicates that the O-N bond of the oxidizing directing group is cleaved after acyl functionalization i.e. these two processes are not concerted.

It is believed that this transformation begins with the *ortho*-metallation of *N*-aryloxyacetamide with Rh^{III} to provide the five membered Rhodacycle **B** which undergoes a transmetalation step with acyl-silver species formed by the silver mediated decarboxylation of α-oxocarboxylic acids to afford complex **C**. Thereafter, reductive elimination of **C** provides the aryl-acylated intermediate **D** and Rh^{I}. Finally, Rh^{I} get reoxidized to Rh^{III} by the oxidizing directing group itself and consequently the directing group is being cleaved to generate the hydroxyl functional group (Scheme 2).

It may be considered that bis-acylated phenols are formed when the mono acylated *N*-aryloxyacetamides become involved again in carbometalation process with remaining Rhodium(III) at another *ortho*-position and go through the same catalytic pathway.

Conclusions

In summary, we have developed a new Rhodium(III)-catalyzed decarboxylative strategy for *ortho*-C-H acylation of arenes, associated with autocleavable oxidizing directing group (ODG^{auto}) to achieve *o*-acylated phenols. As per our knowledge, this is the first report for Rh^{III}-catalyzed decarboxylative acylation. Our ongoing studies need to focus over the unexplored area based on redox neutral C-H acylation.

Conflict of Interest

The authors declare no competing financial interest.

Acknowledgments

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Keywords: C–H activation · C–C coupling · Acylation · Homogeneous catalysis

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Detection of Lysosome by a Fluorescent Heterocycle: Development of Fused Pyrido-Imidazo-Indole framework via Cu-Catalyzed Tandem N-Arylation

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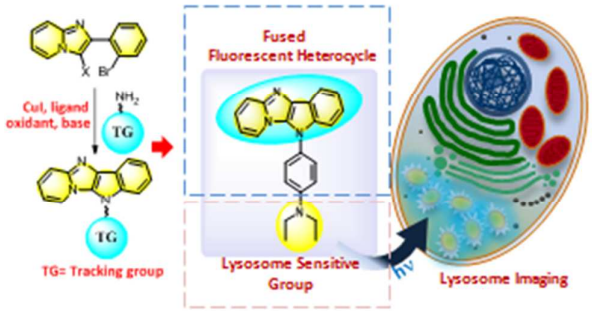


Abstract:

Fluorescent active small molecules for organelle-specific bio-imaging are in great demand. We have synthesized twenty different pyrido-imidazo-indole fused heterocycles (6-5-5-6 ring) via copper catalyzed tandem N-arylation reaction in moderate-good yields. Due to decent fluorescent property, lysosome-directing moieties were attached on two of these heterocycles. Delightfully, those molecules tracked lysosome with bright blue fluorescence and co-localised with a known lysosome marker (Lysotracker Red) in human/murine cells. Therefore, it may be considered as a rapid (10 minutes) lysosome staining probe.

Introduction:

In living systems, cells are the most important core unit, inside which all the biochemical reactions take place continuously. These various chemical reactions simultaneously transduct into various important signals though out the body responsible for life sustainabilities.[1] 'Organelle-Targetable Fluorescent Probes' (OTFPs) are namely those (Figure **1**) which can directly anchor to a specific organelle inside the cell and make the target organelle visible by the principle of fluorescence.[2] In recent times, nitrogen containing heterocycles have gained high importance due to their impactful and versatile applications in various

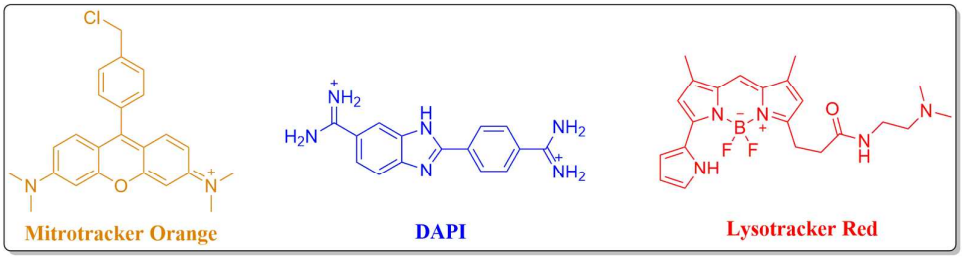


Figure 1: Some examples of Organelle Targeting Fluorescent Probes (OTFPs).

fields.[3-10] Thus, researchers are interested in synthesis of these kinds of fused heterocycles. These fused heterocycles due to their extensive conjugations are also highly fluorescent

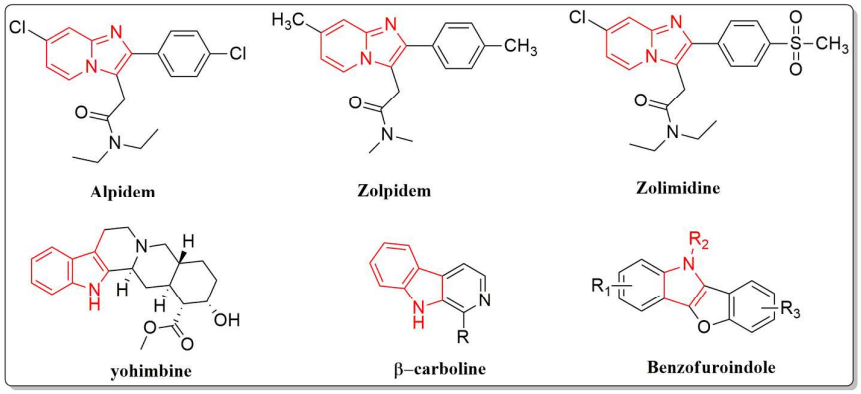


Figure 2: Some famous imidazo[1,2-a]pyridine & indole containing compounds and natural products.

active and can be used in bio-imaging.[11-13] Imidazo[1,2-a]pyridine, one of the most important example of nitrogen bearing heterocycles was found to be major constituent of different pharmaceuticals (Figure **2)**. Some of the reported anti-cancer,[14] antiparasitic,[15] anti-inflammatory,[16] anti-viral,[17] and anti-bacterial[18] drugs are consist of imidazo[1,2-a]pyridine motif. As a result, efforts were made for the preparation and derivation of imidazo[1,2-a]pyridine for a long period of time. On the other hand, indole and its derivatives

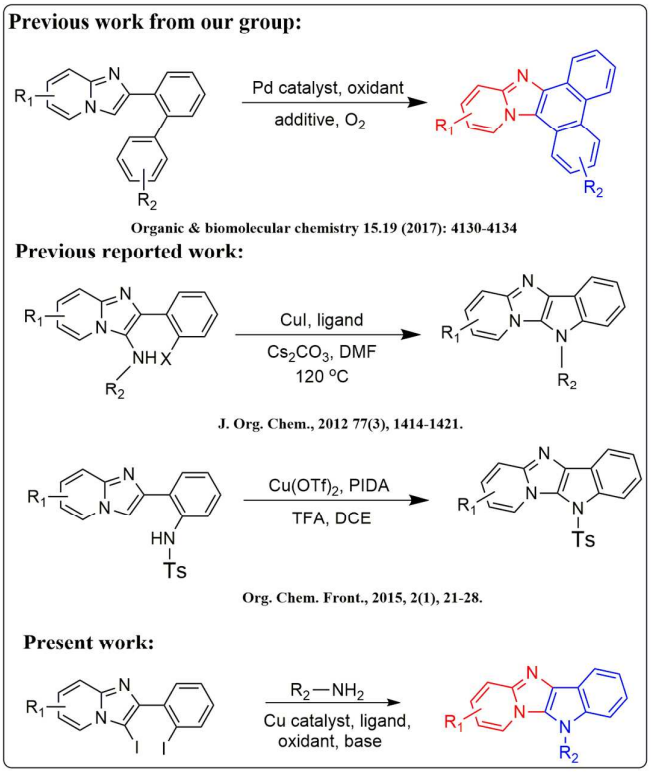
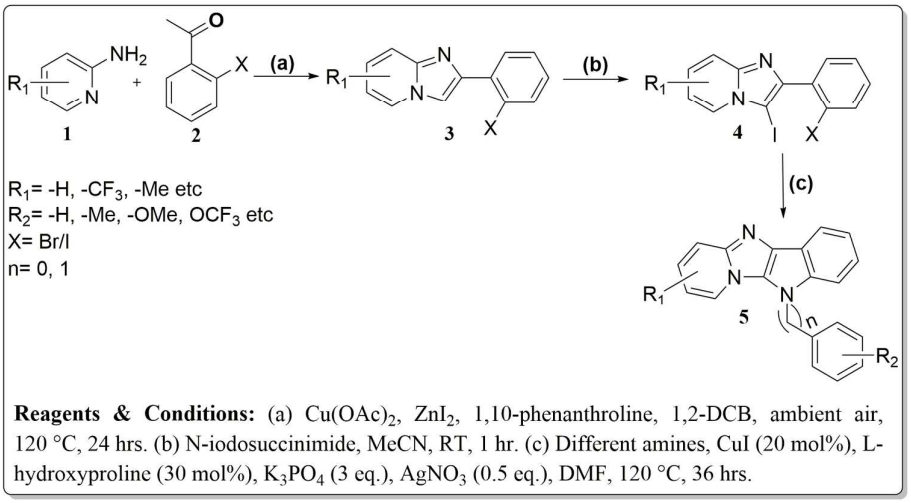


Figure 3: Some previously reported work along with the present work.

were prevalent in a wide class of natural products with enormous biomedical activities (Figure **2**). [19-24] In an ongoing project in our laboratory, we were interested to build up a new fused heterocycle consisting of pyrido-imidazo-indole framework. Previously our group had reported the synthetic methodology of fused pyrido-imidazo-phenthridine scaffold (Figure **3**).[11] In the present study, we have developed a new synthetic route to prepare imidazo[1,2-a]pyridine-indole fused heterocycles (**5**) using Ullmann type tandem N-arylation reaction of various amines with the corresponding dihalo-biaryl substrates (**4**). Unlike previous reported methods (Figure **3)**,[25-28] this methodology avoids the uses of toxic chemicals like, isocyanide and is not dependant to any specific structural orientation thus, resulting a wide functional group tolerance with a variety of substrates. Due to the decent luminescence properties of the synthesized molecules, their photo physical characteristics were further explored and extended to live cell imaging. Two of these new hetreocycles were pre-installed with lysosome directing groups at one end, precisely to examine their cellular localization behaviour. Gratifyingly, we observed that those molecules can selectively go into lysosome inside the cell and gave bright blue fluorescence. Being acidic in nature, lysosome plays key role in cellular defence, antigen processing, apoptosis and autophagic pathways and also in various other physiological processes.[29,30] Moreover, lysosome is involved in receptors cycling and trafficking. Thus, by tracking lysosome at the cellular level, these fused heterocycles can be used as new potent fluorescent probes.

Results & Discussion:



Scheme 1: Synthetic scheme for the preparation of fused heterocycle, **5**.

The synthesis started with a well-established C-H amination strategy by reacting compound **1** & **2** to get compound **3**.[31] The dihalo-biaryl precursor, **4** was synthesized from compound **3**

using *N*-iodosuccinimide (NIS) in MeCN at room temperature. Compound **4** was then subjected to copper catalysed (CuI) Ullmann type consecutive C-N arylation reaction in DMF at 120 ^{o}C for 36 hrs using L-hydroxyproline as a ligand, K_3PO_4 as a base and $AgNO_3$ as a SET oxidant (Scheme **1**). After completion, the reaction mixture was subjected to column chromatography to purify compound **5** as a 6-5-5-6 fused heterocycle in moderate to good yields (52-91%) (Table **2**). In search of the best condition for this reaction, optimisation studies were performed. Various copper catalysts along with different ligands and environments were thoroughly examined. Our optimisation study was started with the coupling partner $Cu(OAc)_2$ & 1,10-phenanthroline as catalyst and ligand respectively. After 24 hrs, the yield of the reaction was found to be 45%, which did not improve much even after 48 hrs. Changing the ligand from 1,10-phen to bipyridine did not give any better result.

Entry	Catalyst	Ligand	Base	Oxidant	yield (%)
1	$Cu(OAc)_2$	1,10-phen	K_3PO_4	-	45
2	$Cu(OAc)_2$	1,10-phen	Cs_2CO_3	-	30
3	$Cu(OAc)_2$	Bipy	K_3PO_4	-	42
4	$Cu(OAc)_2$	Xantphos	K_3PO_4	-	23
5	CuI	1,10-phen	K_3PO_4	-	60
6	CuI	L-hydroxyproline	K_3PO_4	-	72
7	**CuI**	**L-hydroxyproline**	**K_3PO_4**	**$AgNO_3$**	**89**
8	CuI	-	K_3PO_4	$AgNO_3$	-
9	CuI	1,10-phen	K_3PO_4	$AgNO_3$	74
10	CuI	Xantphos	K_3PO_4	$AgNO_3$	51
11	CuI	L-hydroxyproline	Cs_2CO_3	$AgNO_3$	65
12	CuI	L-hydroxyproline	NaO^tBu	$AgNO_3$	63

Table 1: Optimization of the reaction condition along with the yield obtained.

Thereafter, we checked other various ligands but still there was no satisfactory result. However, changing the catalyst from $Cu(OAc)_2$ to CuI produced a better yield of the product (from 45% to 60%) (Table **1**, entry **5)**. In search of the best ligand as a partner with CuI, we found L-hydroxyproline as the best one. We have also performed the reaction under N_2 atmosphere but ended up with no desired product formation. However, introducing $AgNO_3$ (0.5 eq., twice in stoichiometry with respect to CuI) as a SET oxidant satisfactorily yielded (**89%**) the desired product (Table **1**, entry **7**), which therefore infers that the redox couple Cu(I)/Cu(II), formed during single electron transfer[32] is playing the key role in this reaction. Among different bases screened, K_3PO_4 was found to be the best in this reaction. Literature reports show that performing an Ullmann reaction is favourable under inert atmosphere with the help of perfectly degassed solvents.[33] To our delight, this methodology produced decent yields under non-nitrogenous environment and in non-degassed DMF. It is noteworthy to mention here that elimination of the ligand from the reaction medium ended up with no desired product formation (Table **1**, entry **8**). Therefore, having the optimum reaction condition in hand, we applied this methodology to make a series of well decorated derivatives

to generalize the scope of the reaction. We have used a number of substituted anilines and benzyl amines to make the series. Here, we observed that substitution at different positions of anilines and benzylamines have no effect on the yield of the final product, however the electronic effect played a vital role in this case. Presence of electron donating groups [-OMe, -Me, -$C(Me)_3$] produced satisfactory yields as compare to electron withdrawing groups (CF_3, -OCF_3, -F, etc.) (see Table **2**). Heterocyclic amines on the other hand reacted efficiently

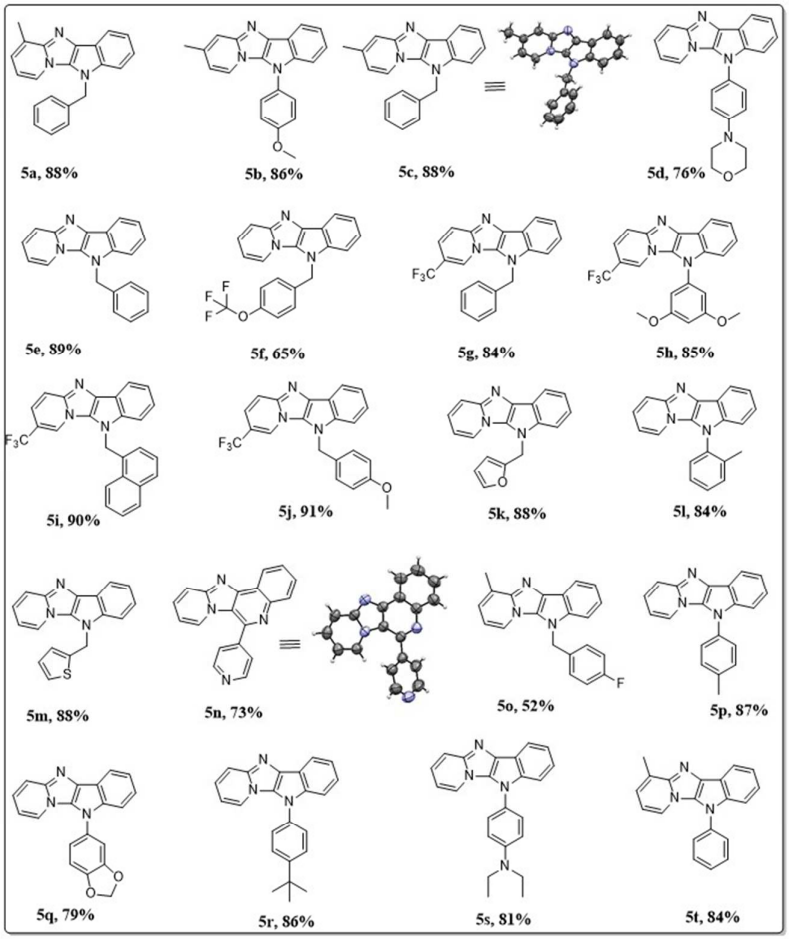


Table 2: List of the synthesized compounds with the crystal structure (CCDC 1844829 & CCDC 1844830) and isolated yields respectively.

under the optimized condition, producing good yields except the example of picolyl amine. In case of 4-picolylamine, we found a very interesting result. Single crystal data confirmed the formation of a fused 6-5-6-6 ring system instead of the 6-5-5-6 ring system, which was not expected under this reaction condition (Table **2**; **5n**). Therefore, a new product, i.e., pyrido-imidazo-quinoline fused heterocycle instead of desired pyrido-imidazo-indole fused heterocycle was formed under the same reaction condition. This is may be due to the strong electron withdrawing nature of the pyridine ring which made the methylene proton *para* to it so acidic[34] that it took part in the cyclisation. Literature study showed only a few reports [35-37] to synthesize this kind of fused heterocycles.

From the mechanistic point of view, the reaction goes via Ullmann type consecutive intermolecular and intramolecular amination reactions as shown in Figure **4**. As per as the Ullmann reaction is concerned, the reactivity of the halides generally decreases from ArI to ArBr.[38] Therefore, the mechanism would likely to start with the intermolecular N-arylation on the imidazo[1,2-a]pyridine ring due the presence of more electronegative atom (i.e., Nitrogen) *ortho* to iodo group (intermediate **II**), followed by the second N-arylation leading to the final cyclised product (**IV**).

The synthesized compounds showed decent fluorescent property (blue to greenish blue, Figure **5A**) in solution. Thus, we studied their photo-physical properties in detail. For that, we selected eight different compounds from the series with different substituents. Both absorbance and fluorescence spectra were taken in CH_2Cl_2 at 5μM concentration.

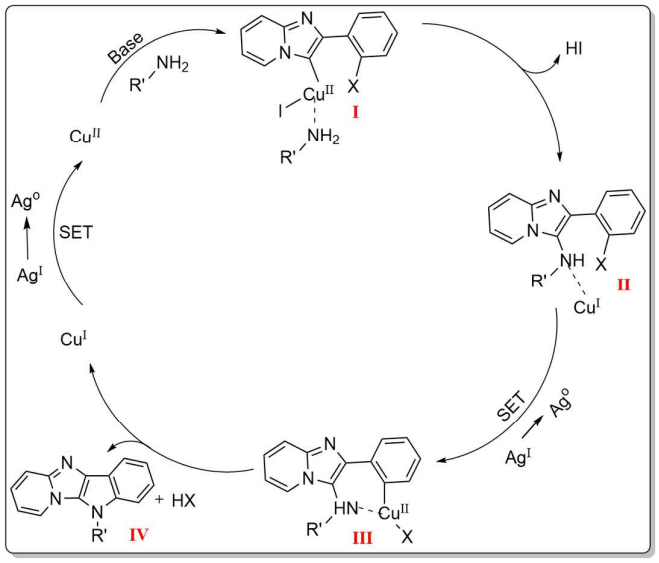


Figure 4: Mechanistic pathway of the Ullmann type tandem N-arylation reaction.

The absorbances of all the compounds have fallen almost in the same region (between 266-273 nm, Table **3** & ESI Fig. **S1**) but, the emission bands showed some interesting result. It was observed that among all, compounds having $-CF_3$ substituent (Table **2**, entries **5g-5j**) in the imidazo[1,2-a]pyridine ring and compound **5s** showed different colour (i.e., bluish green,

Entry	Compound	λ_{max} (nm)	[ε ($M^{-1}cm^{-1}$)][a]	λ_{em}[a](nm)	Φ_F[b]
1	5q	266	39333	436	0.56
2	5r	267	24834	436	0.57
3	5h	273	35000	464	0.58
4	5l	266	44833	436	0.55
5	5k	266	36167	436	0.45
6	5i	272	30500	469	0.47
7	5d	267	22166	439	0.36
8	5s	270	51000	448	0.59

Table 3: Photo-physical properties of the fused compounds. [a]Concentration: 5 μM in CH_2Cl_2. [b]Quantum yield was determined using phenanthrene ($\Phi_F = 0.125$, $\lambda_{ex} = 253$ nm) as a standard.

Figure **5A**) and emitted at around 12-30 nm longer wavelength (Figure **5B**) than others. Notably, the Stoke shifts of the compounds were found to be high, ranging 170-197 nm in CH_2Cl_2. The fluorescence quantum yield was also calculated and remained in the range 0.36-0.59 for the series.

To further explore the fluorescent property of the synthesized molecules, we extended our study in a more focused way. We have used this fluorescence property to distinguish the localisation of one of the most vital organelle of the eukaryotic cells i.e., lysosome. In course of that, we used compound **5d** & **5s** with preinstalled lysosome directing groups along with

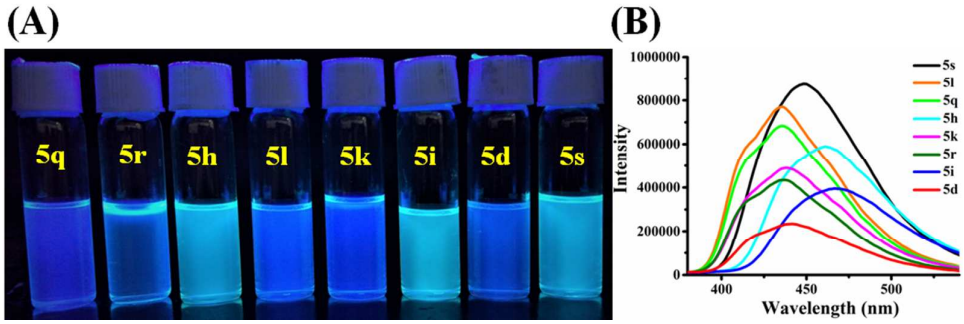


Figure 5: (A) Fluorescence (under long UV, 365 nm) of a few selected compounds of the series in CH_2Cl_2 along with (B) their respective spectra (5 μM in CH_2Cl_2, λ_{ex}= 275 nm).

compound **5i** having no lysosome sensitive group. Human pancreatic adenocarcinoma (AsPC-1) and murine macrophage (J774A.1) cells were stained with these compounds to visualize the cellular localization via confocal imaging. Compounds **5d**, **5s** & **5i** (100 nM) were stained for 30 mins at 37 °C and found to be membrane permeant. A commercially available lysosome specific stain, Lysotracker Red DND-99 dye (75 nM) was also used to

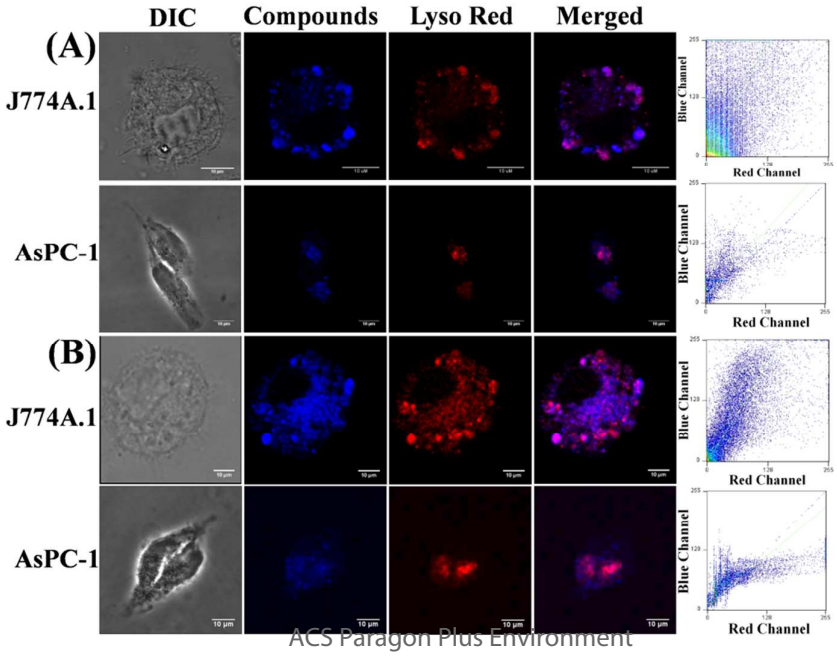


Figure 6: Confocal microscopic images of lysosome co-localisation of compound **5d (A)** and **5s (B)** **(**using DAPI filter) with Lysotracker Red in J774A.1 & AsPC-1 cell lines along with correlation plot of Lysotracker red and compound **5d**, **5s** intensities.

compare the subcellular localisation. Confocal imaging revealed that compounds **5d** & **5s** were successfully localised in the same compartment as like Lysotracker Red (Figure **6**). Pearson correlation coefficient values of greater than 0.5 indicates that both the compounds colocalized with Lysotracker Red and are successfully targeted to label lysosome. Interestingly, compound **5s** rapidly localised just after 10 minutes. However, imaging with compound **5i** was not limited and ended up with whole cytosol localization (see ESI, Fig. **S4**).

Conclusion:

To conclude, we have developed a new, efficient methodology to synthesize a series of imidazo[1,2-a]pyridine-indole fused heterocycles in good yields. Due to the strong fluorescence emissions, their photo-physical properties were thoroughly studied and were successfully applied to cell organelle imaging. Two such molecules **5d** & **5s** were specifically tagged with lysosome sensitive groups and were found to be co-localized with Lysotracker Red having excellent Pearson coefficient values rapidly. These molecules are therefore a new addition to OTFPs.

Experimental Section:

General Information:

All the reagents and solvents used in this study were purchased from Sigma Aldrich and Thermo Fischer Scientific respectively. Melting points were measured using one side open capillary tubes. IR spectrum of the solid sample was recorded in the range 400 to 4000 cm^{-1} in a Bruker FT-IR spectrometer. All the ^{1}H and ^{13}C NMR spectra were recorded in a Bruker 600 MHz spectrometer. ESI mass spectral analysis was done using LCQ-ORBITRAP-XL instrument. All the UV & Fluorescence data were collected using Jasco & Cary Eclipse spectrophotometer respectively. Bruker Kappa Apex II X-ray crystallography machine was used to solve the crystal structure. Singlet (s), doublet (d), double doublet (dd) triplet (t) & multiplet (m) were designated as ^{1}H NMR multiplicity patterns. Silica gel (100-200 mesh) and (230-400 mesh) were used for column chromatographic separations.

Synthetic Procedures:

General Procedure for the synthesis compound 3:

To a solution of **1** (1 g, 1.0 eq.) and **2** (2.61 g, 1.0 eq.) in 1,2-DCB (4 mL), was added $Cu(OAc)_2.H_2O$ (212 mg, 0.1 eq.), ZnI_2 (340 mg, 0.1 eq.) & 1,10-Phenanthroline (192 mg, 0.1 eq.) and heated in a 100 mL round bottom flask under ambient air for 24 hours. After completion of the reaction, monitored by TLC, the reaction mixture was diluted with dichloromethane and filtered through celite bed. The organic layer was then concentrated

under reduced pressure and subjected to column chromatography (silica gel 60-120 mesh size, ethyl acetate: pet ether) for further purification to get the desired compound, **3** in 85% yield as a yellow solid.

General Procedure for the synthesis compound 4:

To the solution of **3** (1 g, 1.0 eq) in acetonitrile, was added NIS (703 mg, 1.0 eq.) and stirred at room temperature for 1 hr. After completion of the reaction, monitored by TLC, the reaction mixture was diluted with ethyl acetate, washed with cold water (2×10 mL) and brine (1×10 mL). Then, the organic layer was filtered, dried over Na_2SO_4 and concentrated under reduced pressure to get the solid product, **4** in 90% yield.

General Procedure for the synthesis of fused pyrido-imidazo-indoles, 5:

To a solution of compound **4** (1.0 eq.) and different amines (1.5 eq.) in dry DMF (1 mL), was added CuI (0.2 eq.) as catalyst, L-hydroxyproline (0.3 eq.) as ligand, $AgNO_3$ (0.5 eq.) as oxidant and K_3PO_4 (3 eq.) as base and stirred while heating at 120 ^oC in a sealed tube. After completion of the reaction, monitored by TLC, the reaction mixture was diluted with ethyl acetate, filtered through celite bed and then washed with cold water (4× 10 mL) and brine (2×10 mL). The organic layer was then collected and dried over Na_2SO_4. After that, the organic extract was evaporated under reduced pressure to get crude solid. It was then subjected to column chromatography (silica gel 230-400 mesh size, ethyl acetate: pet ether) for further purification to get the desired compound, **5 (5a-5t)** in 52-91% yield.

5-benzyl-10-methyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5a):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 88% (123.2 mg) ; yellowish solid, m.p= 135-137 ^oC; ^1H NMR (600 MHz, Chloroform-*d*) δ 8.21 (d, *J* = 6.0 Hz, 1H), 7.81 (d, *J* = 6.0 Hz, 1H), 7.46 (d, *J* = 12.0 Hz, 1H), 7.37 (t, *J* = 6.0 Hz, 1H), 7.32 – 7.27 (m, 4H), 7.14 (d, *J* = 6.0 Hz, 2H), 6.87 (d, *J* = 6.0 Hz, 1H), 6.58 (t, *J* = 12.0 Hz, 1H), 5.70 (s, 2H), 2.71 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.9, 141.4, 137.1, 130.8, 130.6, 129.2, 128.7, 128.6, 128.2, 128.0, 126.0, 123.3, 120.9, 120.1, 119.8, 119.6, 118.3, 111.0, 109.7, 47.8, 17.8; FTIR (ν_{max}, cm^{-1}): 3491, 3383, 3282, 3243, 3152, 3050, 2842, 2358, 1562, 1450, 1336, 1187, 1137, 1069, 1032, 839, 718, 667; **HRMS (ESI-TOF) m/z:** $[M+H]^+$calculated for $C_{21}H_{18}N_3$ 312.1500 found 312.1501

5-(4-methoxybenzyl)-9-methyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5b):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 86% (120.4 mg); yellowish solid, m.p= 189-191 ^oC; ^1H NMR (600 MHz, Chloroform-*d*) δ 8.12 (t, *J* = 12.0 Hz, 1H), 7.60 (d, *J* = 12.0 Hz, 1H), 7.46 (t, *J* = 12.0 Hz, 3H), 7.40 – 7.38 (m, 1H), 7.31-7.29 (m, 2H), 7.14 (d, *J* = 6.0 Hz, 2H), 6.32 (d, *J* = 6.0 Hz, 1H), 3.94 (s, 3H), 2.42 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.1, 145.9, 142.0, 132.9, 131.1, 129.6, 129.3, 128.0, 123.3, 121.4, 120.7, 119.4, 118.6, 116.7, 115.1, 113.6, 110.7, 55.7, 21.6; FTIR (ν_{max}, cm^{-1}): 3168, 3114, 3051, 2963.31, 2913, 2843, 2357, 1649, 1558, 1512, 1455, 1402, 1299, 1254, 1210, 1173, 1029, 848, 757, 722; **HRMS (ESI-TOF) m/z:** $[M+H]^+$calculated for $C_{21}H_{18}N_3O$ 328.1449 found 328.1457

5-benzyl-9-methyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5c):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 88% (114.4 mg); yellowish solid, m.p= 144-146 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.12 (d, *J* = 6.0 Hz, 1H), 7.74 (d, *J* = 6.0 Hz, 1H), 7.44 (t, *J* = 12.0 Hz, 2H), 7.35 (t, *J* = 12.0 Hz, 1H), 7.30 – 7.28 (m, 4H), 7.14 (d, *J* = 6.0 Hz, 2H), 6.47 (d, *J* = 6.0 Hz, 1H), 5.67 (s, 2H), 2.37 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.1, 137.1, 132.9, 132.0, 130.8, 129.9, 129.2, 128.7, 128.1, 127.3, 126.0, 123.2, 120.9, 120.1, 119.6, 118.2, 116.6, 113.8, 109.7, 47.9, 21.5; FTIR (ν_{max}, cm^{-1}): 3058, 3028, 1704, 1649, 1609, 1560, 1530, 1494, 1442, 1336, 1308, 1187, 1163, 1135, 1041, 735, 702, 650; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{21}H_{18}N_3$ 312.1500 found 312.1501

4-(4-(5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indol-5-yl)phenyl)morpholine (5d):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 76% (106.4 mg); brownish solid, m.p>300 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.19 (d, *J* = 6.0 Hz, 1H), 7.93 (d, *J* = 12.0 Hz, 1H), 7.74 (d, *J* = 6.0 Hz, 1H), 7.44 (d, *J* = 12.0 Hz, 2H), 7.39 (d, *J* = 6.0 Hz, 1H), 7.34 – 7.28 (m, 2H), 7.16 – 7.12 (m, 3H), 6.73 (t, *J* = 12.0 Hz, 1H), 3.95 (t, *J* = 6.0 Hz, 4H), 3.32 (t, *J* = 6.0 Hz, 4H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 150.9, 145.2, 142.3, 130.6, 130.4, 129.5, 127.9, 127.8, 123.9, 122.5, 122.2, 120.8, 120.1, 118.4, 117.9, 116.1, 111.4, 110.8, 66.8, 48.8; FTIR (ν_{max}, cm^{-1}): 3142, 3035, 2998, 2969, 2866, 2830, 1706, 1561, 1510, 1449, 1405, 1357, 1235, 1209, 1120, 924, 825, 720, 673; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{23}H_{21}N_4O$ 369.1715 found 369.1723

5-benzyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5e):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 89% (115.7 mg); yellowish solid, m.p= 140-142 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.16 (d, *J* = 12.0 Hz, 1H), 7.90 (d, *J* = 12.0 Hz, 1H), 7.70 (d, *J* = 12.0 Hz, 1H), 7.49 (d, *J* = 6.0 Hz, 1H), 7.39 (t, *J* = 12.0 Hz, 1H), 7.34 – 7.29 (m, 4H), 7.17 (d, *J* = 6.0 Hz, 2H), 7.07 (t, *J* = 12.0 Hz, 1H), 6.66 (t, *J* = 12.0 Hz, 1H), 5.72 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.5, 137.1, 129.3, 128.1, 126.8, 126.5, 126.0, 124.4, 124.2, 123.6, 121.9, 121.6, 120.3, 119.8, 119.6, 118.5, 118.4, 111.2, 109.8, 47.9; FTIR (ν_{max}, cm^{-1}): 3160, 3063, 3024, 3003, 2964, 1882, 1560, 1494, 1450, 1432, 1337, 1299, 1234, 1187, 1131, 1074, 793, 716; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{20}H_{16}N_3$ 298.1344 found 298.1345

5-(4-(trifluoromethoxy)benzyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5f):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 65% (91 mg); yellowish solid, m.p= 180-182 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.15 (d, *J* = 12.0 Hz, 1H), 7.89 (d, *J* = 6.0 Hz, 1H), 7.70 (d, *J* = 6.0 Hz, 1H), 7.43 (d, *J* = 6.0 Hz, 1H), 7.38 (t, *J* = 12.0 Hz, 1H), 7.33 (t, *J* = 12.0 Hz, 1H), 7.18 – 7.14 (m, 4H), 7.10 – 7.06 (m, 1H), 6.69 (t, *J* = 12.0 Hz, 1H), 5.70 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 148.9, 145.5, 141.3, 135.7, 131.5, 129.8, 128.4 (q, $^{1}J_{C-F}$ = 283.5 Hz), 123.7, 121.9, 121.8, 121.4, 121.2, 120.7, 120.3, 119.9, 119.6 (q, $^{1}J_{C-F}$ = 286.5 Hz), 119.5, 118.3, 111.4, 109.7, 47.3; FTIR (ν_{max}, cm^{-1}): 3533, 3357, 3063, 2355, 1910, 1492, 1251, 1218, 1158, 1012, 920, 827, 813, 729; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{21}H_{15}F_3N_3O$ 382.1167 found 382.1174

5-benzyl-8-(trifluoromethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5g):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 84% (117.6 mg); yellowish solid, m.p= 190-192 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.17 (d, *J* = 6.0 Hz, 1H), 8.03 (s, 1H), 7.74 (d, *J* = 12.0 Hz, 1H), 7.54 (d, *J* = 12.0 Hz, 1H), 7.44 (t, *J* = 12.0 Hz, 1H), 7.37 – 7.34 (m, 4H), 7.22 (d, *J* = 6.0 Hz, 2H), 7.15 (d, *J* = 12.0 Hz, 1H), 5.69 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.6, 141.9, 139.9, 136.5, 133.0 (q, $^{4}J_{C-F}$ = 4.5 Hz), 130.3, 129.6, 129.5 (q, $^{1}J_{C-F}$ = 268.5 Hz), 129.2, 128.8, 124.5 (q, $^{1}J_{C-F}$ = 268.5 Hz), 121.0 (q, $^{3}J_{C-F}$ = 6.0 Hz), 120.7, 120.00, 118.7, 117.8 (q, $^{2}J_{C-F}$ = 30.0 Hz), 117.3 (q, $^{4}J_{C-F}$ = 3.0 Hz), 115.2 (q, $^{2}J_{C-F}$ = 33.0 Hz), 111.8, 109.9, 48.2; FTIR (ν_{max}, cm^{-1}): 3038, 2923, 2849, 1745, 1649, 1553, 1432, 1403, 1269, 1133, 1068, 939, 792, 741, 679, 632; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{21}H_{15}F_3N_3$ 366.1218 found 366.1223.

5-(3,5-dimethoxyphenyl)-8-(trifluoromethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5h):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 85% (119 mg); yellowish solid, m.p= 224-226 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.32 (s, 1H), 8.16 (d, *J* = 6.0 Hz, 1H), 7.84 (d, *J* = 12.0 Hz, 1H), 7.63 (d, *J* = 12.0 Hz, 1H), 7.42 – 7.36 (m, 2H), 6.69 (s, 2H), 6.63 (s, 1H), 5.93 – 5.87 (m, 1H), 3.87 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 161.8 (q, $^{2}J_{C-F}$ = 40.5 Hz), 148.3, 144.8, 141.9, 137.5, 133.9, 129.2, 124.7, 123.6 (q, $^{1}J_{C-F}$ = 268.5 Hz), 121.5, 121.3 (q, $^{3}J_{C-F}$ = 6.0 Hz), 120.0, 118.9, 118.5, 117.4, 115.2 (q, $^{2}J_{C-F}$ = 34.5 Hz), 111.3, 104.3, 100.2, 93.7, 90.9, 55.7; FTIR (ν_{max}, cm^{-1}): 3282, 3067, 3011, 2971, 2847, 2349, 1597, 1454, 1300, 1203, 1158, 1122, 1064, 1030, 946, 835, 741; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{22}H_{17}F_3N_3O_2$ 412.1272 found 412.1279.

5-(naphthalen-1-ylmethyl)-8-(trifluoromethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5i):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 90% (117 mg); yellowish solid, m.p= 194-196 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.22 (d, *J* = 6.0 Hz, 1H), 8.09 (d, *J* = 6.0 Hz, 1H), 7.97 (d, *J* = 6.0 Hz, 1H), 7.86 (d, *J* = 6.0 Hz, 1H), 7.81 (s, 1H), 7.73 (d, *J* = 9.6 Hz, 1H), 7.66 – 7.59 (m, 2H), 7.52 (d, *J* = 12.0 Hz, 1H), 7.44 (t, *J* = 12.0 Hz, 1H), 7.39 (t, *J* = 12.0 Hz, 1H), 7.30 (t, *J* = 12.0 Hz, 1H), 7.11 (d, *J* = 6.0 Hz, 1H), 6.94 (d, *J* = 12.0 Hz, 1H), 6.13 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 144.7, 141.8, 133.9, 133.1, 131.4, 130.6, 130.2, 129.6 (q, $^{1}J_{C-F}$ = 262.5 Hz) 129.3 (q, $^{3}J_{C-F}$ = 6.0 Hz), 127.2, 126.5, 125.6, 124.5, 123.2 (q, $^{1}J_{C-F}$ = 268.5 Hz), 120.9 (q, $^{3}J_{C-F}$ = 6.0 Hz), 120.8, 120.0, 118.8, 118.0, 117.7, 117.2, 115.2 (q, $^{2}J_{C-F}$ = 33.0 Hz), 111.8, 110.1, 46.1; FTIR (ν_{max}, cm^{-1}): 3266, 3223, 3063, 2898, 1650, 1573, 1454, 1329, 1302, 1256, 1151, 1117, 1070, 1024, 944, 837, 794, 724; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{25}H_{17}F_3N_3$ 416.1374 found 416.1379.

5-(4-methoxybenzyl)-8-(trifluoromethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5j):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 91% (118.3 mg); yellowish solid, m.p= 192-194 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.16 (d, *J* = 12.0 Hz, 1H), 8.01 (s, 1H),

7.73 (d, *J* = 12.0 Hz, 1H), 7.56 (d, *J* = 6.0 Hz, 1H), 7.45 (t, *J* = 12.0 Hz, 1H), 7.35 (t, *J* = 12.0 Hz, 1H), 7.15 (d, *J* = 6.0 Hz, 3H), 6.88 (d, *J* = 12.0 Hz, 2H), 5.62 (s, 2H), 3.78 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.8, 144.6, 141.9, 133.1, 130.4, 128.3, 127.7, 124.3, 122.6 (q, $^{1}J_{C-F}$ = 268.5 Hz), 121.2 (q, $^{3}J_{C-F}$ = 6.0 Hz), 120.6, 119.9, 118.7, 117.9, 117.2 (q, $^{4}J_{C-F}$ = 3.0 Hz), 115.1 (q, $^{2}J_{C-F}$ = 33.0 Hz), 114.9, 110.0, 99.9, 55.3, 47.8; FTIR (ν_{max}, cm^{-1}): 2938, 2840, 1650, 1612, 1568, 1509, 1454, 1403, 1358, 1324, 1301, 1239, 1150, 1119, 1069, 1024, 830, 738; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{22}H_{17}F_3N_3O$ 396.1323 found 396.1331

5-(furan-2-ylmethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5k):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 88% (132 mg); grey solid, m.p= 194-196 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.27 (d, *J* = 6.0 Hz, 1H), 8.10 (d, *J* = 6.0 Hz, 1H), 7.70 (d, *J* = 6.0 Hz, 1H), 7.53 (d, *J* = 6.0 Hz, 1H), 7.39 (t, *J* = 12.0 Hz, 1H), 7.33 – 7.29 (m, 2H), 7.14 – 7.08 (m, 1H), 6.80 (t, *J* = 12.0 Hz, 1H), 6.26 (dd, *J* = 6.0, 6.0 Hz, 2H), 5.60 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 149.7, 145.4, 142.9, 141.1, 131.5, 130.0, 123.5, 121.9, 121.7, 120.4, 119.7, 118.5, 111.1, 110.7, 109.8, 108.1, 41.5; FTIR (ν_{max}, cm^{-1}): 3094, 3043, 2964, 1567, 1526, 1454, 1344, 1326, 1304, 1181,1140, 1010, 915,818., 792, 677; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{18}H_{14}N_3O$ 288.1136 found 288.1138.

5-(o-tolyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5l):

Eluent: petroleum ether/ethyl acetate4:1; yield: 84% (109.2 mg); yellowish solid, m.p= 139-141 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.19 – 8.16 (m, 1H), 7.72 (d, *J* = 12.0 Hz, 1H), 7.53 – 7.49 (m, 2H), 7.45 (d, *J* = 12.0 Hz, 3H), 7.35 – 7.31 (m, 2H), 7.15 – 7.12 (m, 1H), 7.08 (t, *J* = 12.0 Hz, 1H), 6.64 (t, *J* = 12.0 Hz, 1H), 2.07 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.3, 141.8, 137.1, 135.2, 131.7, 131.3, 130.1, 129.3, 128.9, 127.4, 123.6, 121.9, 121.4, 120.6, 119.6, 118.4, 111.0, 110.9, 77.2, 17.6; FTIR (ν_{max}, cm^{-1}): 3256, 3054, 2826, 1653, 1561, 1490, 1426, 1398, 1347, 1321, 1250, 1212, 1130, 1090, 785, 711, 667; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{20}H_{16}N_3$ 298.1344 found 298.1347.

5-(thiophen-2-ylmethyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5m):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 88% (105.6 mg); yellowish solid, m.p= 191-193 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.12 (d, *J* = 6.0 Hz, 1H), 8.05 (d, *J* = 12.0 Hz, 1H), 7.70 (d, *J* = 12.0 Hz, 1H), 7.54 (d, *J* = 12.0 Hz, 1H), 7.40 (t, *J* = 12.0 Hz, 1H), 7.32 (t, *J* = 12.0 Hz, 1H), 7.22 – 7.19 (m, 1H), 7.09 (t, *J* = 12.0 Hz, 1H), 6.92 (d, *J* = 6.0 Hz, 2H), 6.75 (t, *J* = 12.0 Hz, 1H), 5.81 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 145.5, 141.4, 139.7, 131.8, 129.8, 127.3, 125.7, 125.3, 123.6, 121.9, 121.7, 120.6, 119.8, 118.7, 118.5, 111.3, 109.9, 43.7; FTIR (ν_{max}, cm^{-1}): 3053, 2921, 2852, 1567, 1524, 1453, 1343, 1301, 1273, 1246, 1226, 1185, 1139, 1033, 844, 718; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{18}H_{14}N_3S$ 304.0908 found 304.0909

6-phenylpyrido[2',1':2,3]imidazo[4,5-*c*]quinoline (5n):

Eluent: petroleum ether/ethyl acetate (1:3); yield: 73% (102.2 mg); white solid, m.p= 259-261 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.95 (d, *J* = 6.0 Hz, 2H), 8.82 (d, *J* = 12.0 Hz, 2H),

8.30 (d, *J* = 6.0 Hz, 1H), 8.10 (d, *J* = 6.0 Hz, 1H), 7.97 (d, *J* = 12.0 Hz, 1H), 7.84 (t, *J* = 12.0 Hz, 1H), 7.78 (t, *J* = 12.0 Hz, 1H), 7.71 (d, *J* = 6.0 Hz, 2H), 7.59 (t, *J* = 12.0 Hz, 1H), 6.88 (t, *J* = 12.0 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 150.9, 149.8, 147.8, 146.1, 145.1, 144.9, 130.3, 129.7, 129.3, 127.2, 126.8, 123.5, 122.7, 121.7, 119.9, 118.4, 112.5; FTIR (ν_{max}, cm^{-1}): 3113, 3092, 3020, 2355, 1639, 1603, 1567, 1517, 1484, 1429, 1360, 1324, 1250, 1218, 1175, 1130, 1105, 956, 816, 751, 733, 685; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{19}H_{13}N_4$ 297.1140 found 297.1145

5-(4-fluorobenzyl)-10-methyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5o):

Eluent: petroleum ether/ethyl acetate (3:1); yield: 52% (72.8 mg); brownish solid, m.p= 202-204 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.21 (d, *J* = 12.0 Hz, 1H), 7.82 (d, *J* = 6.0 Hz, 1H), 7.45 (d, *J* = 6.0 Hz, 1H), 7.37 (t, *J* = 12.0 Hz, 1H), 7.31 (t, *J* = 12.0 Hz, 1H), 7.12 – 7.08 (m, 2H), 6.98 (t, *J* = 12.0 Hz, 2H), 6.90 (d, *J* = 6.0 Hz, 1H), 6.62 (t, *J* = 12.0 Hz, 1H), 5.67 (s, 2H), 2.72 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 162.4 (d, $^1J_{C-F}$ = 246.0 Hz), 141.5, 141.3, 132.8 (d, $^4J_{C-F}$ = 3.0 Hz), 130.4, 128.9, 128.4, 127.7 (d, $^3J_{C-F}$ = 7.5 Hz), 123.4, 121.0, 120.3, 119.9, 119.4, 118.4, 116.2 (d, $^2J_{C-F}$ = 21.0 Hz), 111.2, 110.9, 109.6, 47.2, 17.7; FTIR (ν_{max}, cm^{-1}): 3489, 3344, 2926, 2850, 1558, 1471, 1448, 1404, 1353, 1297, 1210, 1136, 1030, 834, 693; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{21}H_{17}FN_3$ 330.1406 found 330.1412.

5-(p-tolyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5p):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 87% (113 mg); yellowish solid, m.p= 142-144 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.42 (d, *J* = 6.0 Hz, 1H), 8.31 (d, *J* = 12.0 Hz, 1H), 7.90 (d, *J* = 6.0 Hz, 1H), 7.60 (t, *J* = 12.0 Hz, 1H), 7.51 (d, *J* = 6.0 Hz, 2H), 7.49 – 7.45 (m, 3H), 7.42 (d, *J* = 12.0 Hz, 2H), 7.14 (t, *J* = 12.0 Hz, 1H), 2.55 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 142.1, 141.3, 132.1, 131.1, 130.3, 130.0, 127.7, 126.9, 126.4, 124.9, 124.7, 122.8, 122.5, 121.4, 115.7, 111.2, 110.4, 21.3; FTIR (ν_{max}, cm^{-1}): 3489, 3344, 2926, 2850, 1558, 1471, 1448, 1404, 1353, 1297, 1210, 1136, 1030, 834, 693; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{20}H_{16}N_3$298.1344 found 298.1347

5-(benzo[d][1,3]dioxol-5-yl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5q):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 79% (110.6 mg); yellowish solid, m.p= 243-245 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.16 – 8.13 (m, 1H), 7.79 (d, *J* = 6.0 Hz, 1H), 7.74 (d, *J* = 6.0 Hz, 1H), 7.46 – 7.43 (m, 1H), 7.35 – 7.31 (m, 2H), 7.15 – 7.09 (m, 1H), 7.06 – 6.99 (m, 3H), 6.72 (t, *J* = 12.0 Hz, 1H), 6.15 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 148.7, 147.5, 145.5, 142.4, 131.8, 130.3, 129.6, 123.8, 122.1, 121.9, 121.0, 120.3, 119.6, 118.7, 118.5, 110.9, 110.8, 108.8, 108.0, 102.1; FTIR (ν_{max}, cm^{-1}): 3031, 2899, 1558, 1485, 1402, 1231, 1205, 1131, 1036, 936, 808, 735; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{20}H_{14}N_3O_2$ 328.1086 found 328.1088.

5-(4-(tert-butyl)phenyl)-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5r):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 86% (103.2 mg); yellowish solid, m.p= 243-245 °C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.19 – 8.13 (m, 1H), 7.78 (d, *J* = 6.0 Hz,

1H), 7.74 (d, *J* = 12.0 Hz, 1H), 7.64 (d, *J* = 12.0 Hz, 2H), 7.52 (dd, *J* = 6.6, 2.6 Hz, 1H), 7.50 – 7.45 (m, 2H), 7.36 – 7.31 (m, 2H), 7.15 – 7.08 (m, 1H), 6.72 (t, *J* = 12.0 Hz, 1H), 1.45 (s, 9H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 150.9, 145.5, 142.0, 133.8, 132.1, 129.5, 126.9, 126.0, 123.7, 122.5, 121.8, 120.9, 119.6, 118.8, 118.4, 110.9, 110.8, 34.8, 31.4; FTIR (ν_{max}, cm^{-1}): 3049, 2959, 1635, 1603, 1573, 1561, 1448, 1351, 1244, 1213, 1132, 729; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{23}H_{22}N_3$ 340.1813 found 340.1816.

N,N-diethyl-4-(5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indol-5-yl)aniline (5s):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 81% (105.3 mg); yellowish solid, m.p= 244-246 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.14 (dd, *J* = 6.0, 6.0 Hz, 1H), 7.79 (d, *J* = 6.0 Hz, 1H), 7.71 (d, *J* = 6.0 Hz, 1H), 7.43 – 7.40 (m, 1H), 7.36 (d, *J* = 6.0 Hz, 2H), 7.33 – 7.29 (m, 2H), 7.09 – 7.05 (m, 1H), 6.84 (d, *J* = 6.0 Hz, 2H), 6.67 (t, *J* = 12.0 Hz, 1H), 3.47 (q, *J* = 18.0 Hz, 4H), 1.28 (t, *J* = 18.0 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 147.5, 145.2, 142.5, 131.1, 130.2, 128.2, 123.7, 123.3, 122.3, 121.6, 120.3, 119.4, 118.4, 118.2, 111.9, 110.9, 110.5, 44.5, 12.6; FTIR (ν_{max}, cm^{-1}): 3285, 2975, 2926, 2872, 1878, 1612, 1519, 1449, 1398, 1347, 1264, 1203, 1141, 1083, 1012, 877, 814, 738, 713, 661, 524; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{23}H_{23}N_4$ 355.1923 found 355.1944.

10-methyl-5-phenyl-5*H*-pyrido[2',1':2,3]imidazo[4,5-*b*]indole (5t):

Eluent: petroleum ether/ethyl acetate (4:1); yield: 84% (100.8 mg); yellowish solid, m.p= 191-193 ^{o}C; ^{1}H NMR (600 MHz, Chloroform-*d*) δ 8.25 – 8.21 (m, 1H), 7.64 (q, *J* = 12.0, 6.0 Hz, 3H), 7.55 (d, *J* = 6.0 Hz, 2H), 7.53 – 7.48 (m, 2H), 7.34 – 7.31 (m, 2H), 6.94 (d, *J* = 6.0 Hz, 1H), 6.63 (t, *J* = 12.0 Hz, 1H), 2.76 (s, 3H).; ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.1, 141.9, 136.6, 131.7, 130.0, 129.9, 128.2, 127.7, 126.6, 123.6, 121.0, 120.4, 119.8, 119.0, 110.8, 110.7, 17.7; FTIR (ν_{max}, cm^{-1}): 3031, 2920, 1706, 1592, 1551, 1486, 1442, 1397, 1341, 1206, 1079, 1020, 927, 853, 712, 556, 455; **HRMS (ESI-TOF) m/z:** $[M+H]^+$ calculated for $C_{20}H_{16}N_3$ 298.1344 found 298.1347.

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SUPPORTING INFORMATION

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^{1}H and ^{13}CNMR spectra of all the final compounds, X-ray crystal data of compound **5c** and **5n**, UV-Vis spectra of some selected compounds as well as the details of biological study.

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Cellular Detection of Hydrazine as Isoniazid Metabolite by a New Turn-On Fluorescent Probe: Synthesis, Live Cell Imaging and In Vitro Toxicity Studies

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A new turn-on fluorescent probe 2-((pyren-3-yl) methyl) isoindoline-1,3-dione was synthesized in a single step, high yielding reaction to detect hydrazine in solution. This ligand is highly sensitive to detect hydrazine in micromolar (1.1 μM) concentration in solution. It has high selectivity towards hydrazine compared to other analytes. Different biophysical experiments like hydrazine screening from both tap water and distilled water, vapour phase detection, anti-interference studies with different analytes also corroborate the phenomenon of selective detection of hydrazine by our ligand. NMR titration and mass spectrum established the mechanism of hydrazine sensing. In addition, the probe was successfully used to trap exogenously added hydrazine inside the cell, which was further utilized to study insitu hydrazine released from an anti-tubercular drug Isoniazid (INH), after its metabolism.

Introduction

Hydrazine is most widely used chemical in various industries worldwide.[1] It is used as a blowing agent[2] for the production of plastics, gas-forming agent in air bags, agricultural chemicals and preservatives. Because of its flammable characteristic, hydrazine is used as propellant in rockets and missiles.[3] It is also used as an antioxidant in nuclear power plant[4] and as anti-corrosion agent in electrical power plants. Some nitrogen fixing bacteria releases hydrazine as a by-product.[5] Even though there is no endogenous hydrazine in our body, hydrazine is easily absorbed by skin, orally through inhalation while usage, manufacturing, transport and disposal. Hydrazine causes environmental pollution and health related problems. Despite of all these, it is potentially harmful to organs like kidney, liver and central nervous systems (CNS). It induces hepatotoxicity, neurotoxicity[6] and mutagenicity.[7] Hydrazine also causes lipid peroxidation,[8] reactive oxygen species (ROS) formation and increase oxidative stress[9] and finally leads to nonspecific damage to DNA.[10] Isoniazid (INH), which is a highly used antibiotic drug for the treatment and prevention of tuberculosis, releases toxic hydrazine metabolites in the liver.[11] During the treatment of tuberculosis, INH mediated hepatotoxicity is a common problem. Interestingly this toxicity is caused due to the formation of hydrazine as metabolite. Hence, selective and sensitive detection of even trace amount of hydrazine at the cellular level is quite important. For such reasons, U.S. Environmental Protection Agency (EPA) marked hydrazine as human carcinogen with a low threshold limit value (TLV) of 10 ppb.[12] Thus design and synthesis of small molecular probes which can selectively detect hydrazine is a challenging job. Different analytical methods including titrometry,[13] electrochemical,[14] chromatographic,[15] colorimetric and UV-Visible spectral changes were demonstrated[16] for the detection of hydrazine. Recently fluorescent spectroscopic technique for analyte detection gained special attention due to their high sensitivity, low detection limit, real time, in-vivo detection[17] and enzymatic quantification.[18,19] Consequently, development of new fluorescent chemodosimeter for the detection of hydrazine both in solution and vapour phase[20] as well as in live cell would be highly beneficial.

In the present study we chose pyrene moiety as the fluorophore and phthalimide as the sensing unit. In order to sense hydrazine, an electrophilic centre should be present in the ligand and in our case phthalimide was supposed to serve the purpose.[21] Accordingly, we have designed probe **3**, as a hybrid of phthalimide (recognition site for hydrazine) and methyl-pyrene (signal transducer unit). Thus, we report an easy one step synthesis of 2-((pyren-3-yl) methyl) isoindoline-1, 3-dione **(3)** with the aim to detect hydrazine with high specificity. Large stoke shift value rendered pyrene to be selected as fluorescent signalling unit.[22]Apart from an active sensing unit for hydrazine, phthalimide also acted as a masking agent[20a,23] to turn-off the fluorescence property of methyl-pyrene. Therefore, sensing of hydrazine with probe **3** accounted for a

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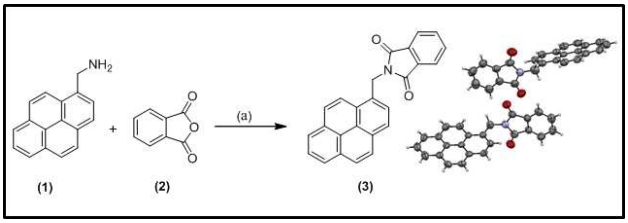
nucleophilic attack on phthalimide ring, generating 2, 3-dihydrophthalazine-1,4-dione (side product) and free methyl-pyrene, which attributed to fluorescence turn-on.

Results and Discussion

Synthetic procedure for the probe 2-((pyren-3-yl) methyl) isoindoline-1,3-dione

To a solution of pyrene-methyl amine 1, (1.0 eq) in 2:1 DMF: Acetic acid (3 ml) were added phthalic anhydride 2 (1.2 eq) and the mixture was refluxed under Nitrogen atmosphere at 120 °C for 2 hrs. It was then cooled to room temperature, the reaction mixture was evaporated and the residue was extracted with ethyl acetate, filtered and the organic part was dried over Na_2SO_4. It was concentrated under reduced pressure and purified by column chromatography (silica gel 100–200, Ethyl acetate and Petroleum ether) to get the desired compound (3) as light yellow solid in 80% yield (Scheme. 1). The structure of probe 3 was fully characterised using NMR and mass spectroscopic techniques. The single X-ray crystal (CCDC no 1581719) structure also confirmed the structure of probe 3 (inset, Scheme 1).



Scheme 1. Reagent and Conditions: Synthesis of probe (**3**), (a) DMF and Acetic acid (2:1), refluxed at 120 °C, 2 hr, inset: single unit cell of the crystal structure of **3**.

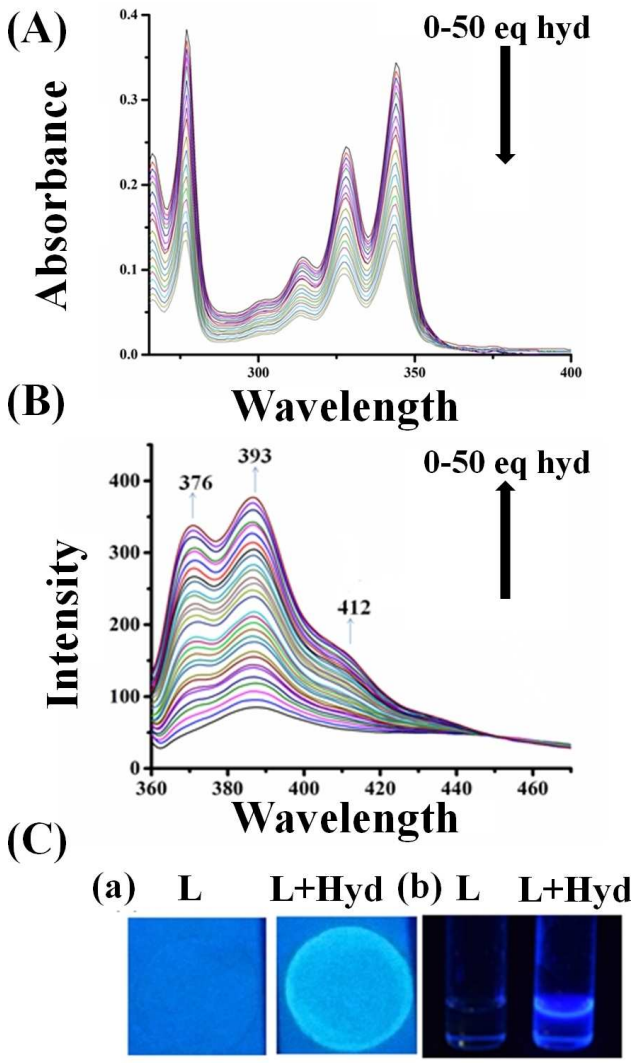


Figure 1. UV (A) and Fluorescence (B) Titration of Probe **3**, (10 μM) dissolved in 3:7, DMSO: H_2O upon addition of various amount of hydrazine; (C) (a) Snapshot of visually detectable changes in fluorescence of silica surface modified with Probe **3**, (1 mM) in absence and presence of hydrazine (b) Visible fluorescence colour change on hydrazine sensing

UV-Vis and Fluorescence titration studies of probe 3 with hydrazine

Recognition property of the synthesized probe **3** towards different analytes was investigated by UV-Vis and fluorescence titration. In the UV-Vis absorption spectrum, solution of probe **3** dissolved in H_2O–DMSO (7:3, v/v) showed three absorption maxima at 277 nm, 328 nm and 344 nm respectively accounting for π-π* transition. Upon gradual addition of Hydrazine (0 to 50 eq), a steady decrease in absorbance of the probe **3** at each maxima were observed (Figure 1A). With this UV profile in hand, we performed fluorescence titration experiments with probe **3** in presence of hydrazine and other analytes with excitation at 344 nm. Emission spectrum of the probe **3** dissolved in H_2O–DMSO (7:3, v/v) showed one broad emission peak at 393 nm along with a hump at 376 nm. To our delight, here a steady increment of the fluorescent intensity of the probe **3** was observed in presence of hydrazine with increasing concentration (Figure 1B). Upon gradual addition of hydrazine to the probe **3**, enhancement of the intensity of the emission peak at 376 nm, and 393 nm along with the appearance of a new peak at 412 nm were observed in contrast to free probe **3**. Appearance of the new peak at 412 nm along with the other two sharp peaks at 376 nm and 393 nm indicated generation of new molecule resulted from the reaction between hydrazine and probe **3**. Fluorescence spectrum of free pyrenemethyl amine (Figure **S5**) indicated the generation of pyrene methyl amine accounted for the reaction of hydrazine with probe **3**. Thus, both UV & fluorescent titrations supported hydrazine recognition phenomenon, with the limit of detection 1.1 μM (Figure **S2**). Furthermore, fluorescence intensity varies linearly with the hydrazine concentration added to the probe solution with coefficient $R^2 = 0.99$, indicated that probe **3** will be effective for the quantitative determination of hydrazine (Fig-

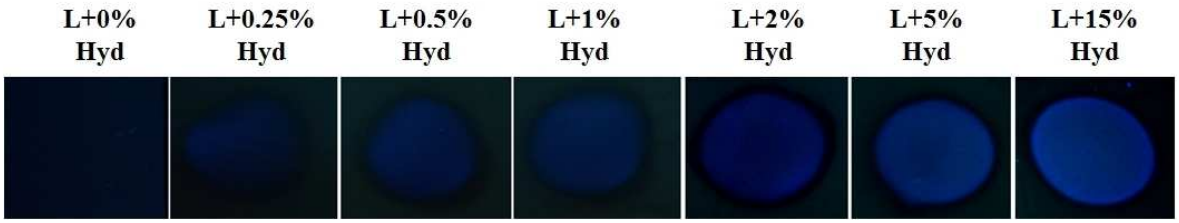


Figure 2. Fluorescence colour changes of the probe **3** coated TLC plates treated with different concentrations of hydrazine in DCM solution. Fluorescence colour changes were observed using a hand-held UV lamp with excitation at 365 nm

ure **S2**). It was observed from the time dependent fluorescence spectra of the probe **3** in presence of 2 eq hydrazine that the fluorescence intensity of the probe **3** was increased up to 12 minutes and then became saturated accounting for the completion of the reaction between hydrazine and the probe **3** (Figure **S4**). We have calculated photo physical properties of compound **1** and **3** (Table S**1**). The visually detectable fluorescence of probe **3** (1 mM) coated silica surface in the absence/presence of hydrazine was checked with hand UV lamp with excitation of 365 nm and appearance of bluish fluorescence further suggested the detection of hydrazine by probe **3** (Figure **1 C-a**).

Fluorescence colour change of probe 3 coated on TLC plate at different concentration of hydrazine

Report of severe health problems including liver damage due to very low vapour pressure of hydrazine rendered us to check the applicability of this probe towards the detection of hydrazine in the vapour phase. For this purpose, silica gel TLC plates were immersed into a THF solution of the probe **3** (c = 1 mM) and-then it was dried. The probe-loaded TLC plates were subjected to exposure (15 minutes) of the hydrazine vapour generated from the hydrazine solution with various concentrations (blank, 0.25%, 0.5%, 1%, 2%, 5%, 15% hydrazine in Dichloromethane) prepared in airtight containers at 25 °C. Gradual increment of fluorescent intensity (excited at 365 nm) of the probe loaded TLC plates was observed with increasing concentration of hydrazine vapour associated with hydrazine solution in Figure 2.

Selectivity and anti-interference study of probe 3 with different analytes

The affinity of probe **3** towards other analytes was also evaluated by fluorescence spectroscopy. However, fluorescence profile of the probe **3** was not changed significantly in presence of large excess (50 eq) of different amines including NH_2OH, urea, aniline, histidine (His), glutathione (GSH), cysteine, (Cys), homocysteine (HCY) and other anions like NO_3^-, N_3^-, I^-, SO_4^{-2}, HCO_3^- as well as cations including Hg^{+2}, Zn^{+2}, K^+, NH_4^+, Na^+ (Figure 3).

So, probe **3** was inert towards others analytes showing selectivity towards hydrazine only. In order to check this selectivity of the probe **3** towards hydrazine in the presence of other ions, we carried out fluorescence interference experiment with 50 equiv. of amines and other ions as stated above. No noticeable perturbation of the fluorescence response of the probe **3** in presence of hydrazine (25 eq), was observed by the competitive anions and cations.

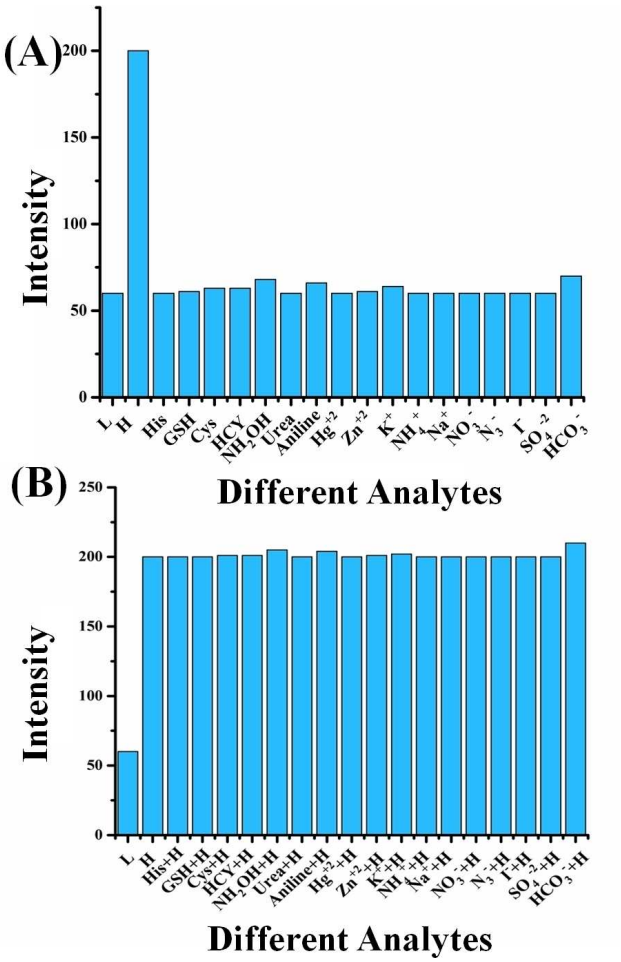


Figure 3. Fluorescence intensity at 393 nm of probe **3** (10.0 μM) upon addition of various analytes (50 equiv.) along with hydrazine (25 equiv). **(B)** Fluorescence intensity at 393 nm of probe **3** (10.0 μM) upon addition of various analytes (50 equiv.) in presence of hydrazine (25 equiv). Where L is Probe **3** and H is hydrazine.

Therefore, probe **3** is able to selectively recognize hydrazine even in the presence of other different analytes. Consequently, it can be concluded that a turn-.

On fluorescence response in presence of hydrazine suggested high selectivity and specificity of probe **3** towards hydrazine over the other stated analytes.

^{1}H NMR and EI-Mass study of probe 3 with hydrazine

Next, we monitored the reaction of hydrazine and probe **3**, by ^{1}H NMR titration experiments in DMSO-d_6. It was observed that after addition of 2 eq. of hydrazine in the DMSO-d_6 solution of probe **3**, a new peak (c; in triplet multiplicity) at δ 9.1 ppm appeared which was presumably due to the free amine-NH_2, (**1**), released by hydrazine after reaction. While, before addition of hydrazine, pthalimide peak appeared at δ 7.8 ppm and $-CH_2$ peak came at δ 5.5 ppm in probe **3**. Hence a shift in their peak positions (a, b) were observed after addition of hydrazine (Figure 4). This indeed confirmed a very fast reaction of

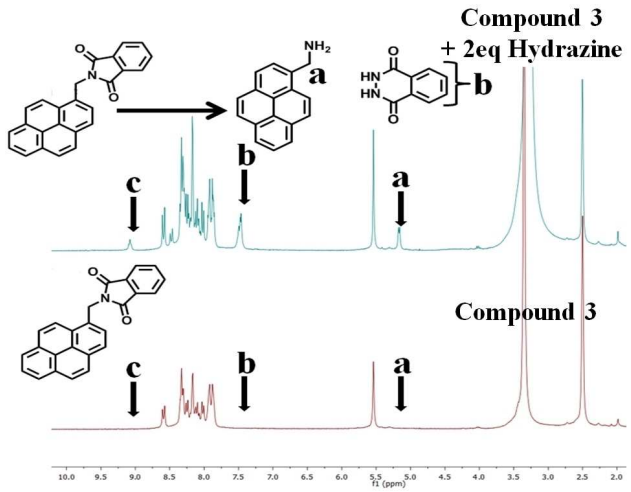


Figure 4. NMR-stack plot comparison of blank probe **3** with probe **3** + 2 eq. Hydrazine.

hydrazine with probe **3**. Mechanistically, this reaction was simultaneous substitution-elimination type with a very low detection limit (1.1 μM) of hydrazine. The reaction mixture was subjected to EI Mass spectrometry to further confirm the underlying mechanism of action. The mass spectrum showed a characteristic peak at m/z = 361 along with two new peaks at m/z = 231 and 162 respectively. The peak at 361 corresponded to the original probe **3**, while 231 was due to pyrene methyl amine **1** and 162 was due to the appearance of 2,3-dihydrophthalazine-1,4-dione, **4** (side product) after the reaction (Figure 5). The other peaks at 202 and 215 are due to the further fragmentation of compound **3**. This also unambiguously proved the presence of three different species as suggested by the mechanism. Our hypothesis therefore concurred with the experimental data. In order to show biological application, the probe should also function in physiological pH. For this

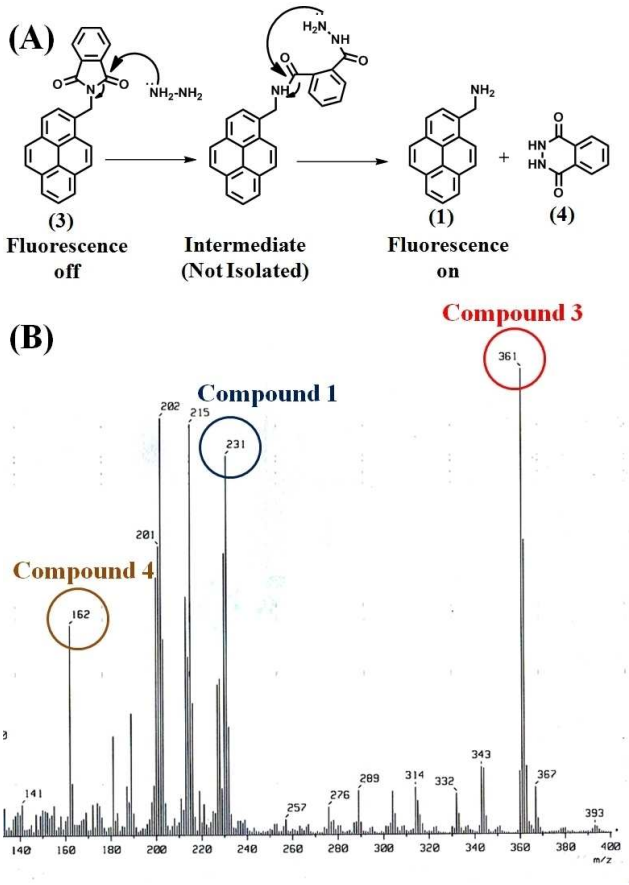


Figure 5. (A) Mechanism of Hydrazine sensing (B) EI – Mass spectra of probe **3** up on addition of 2 eq of hydrazine.

purpose, the emission responses of probe **3** in different pH were investigated. Gratifyingly, it was found that the probe **3** was stable in pH range of 4.5 to 8.5; (ESI; Figure **S1**). Accordingly, it may be concluded that probe **3** is capable of sensing hydrazine at physiological pH.

Application of probe 3 in tap water and distilled water hydrazine detection

In order to establish the versatility of this probe, we checked hydrazine sensing capacity of the probe in distilled water and tap water as well. Hydrazine possess carcinogenic property and widely used in various industrial processes, there by detection of hydrazine in aqueous samples is of special interest. For this purpose, an aliquot of hydrazine was added to both distilled and tap water and their fluorescence was measured (Figure 6). The analysis of hydrazine from both the aqueous systems agreed well at hydrazine concentrations up to 100 μM. Therefore, it may be concluded that probe **3** is also quite sensitive towards different water samples.

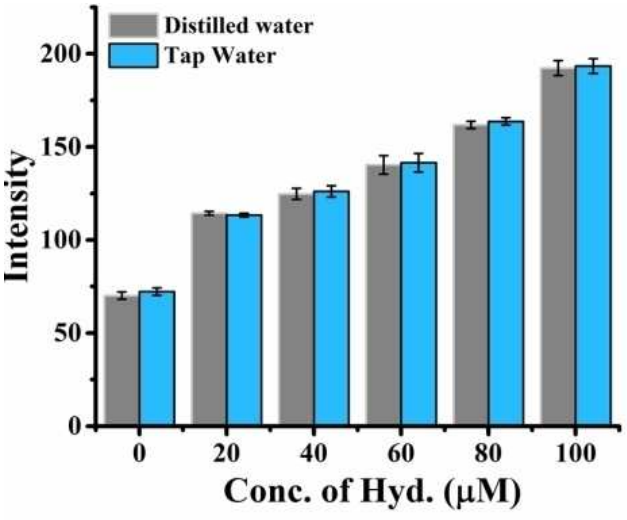


Figure 6. Fluorescence detection of hydrazine in distilled water and tap water by probe **3** (10 μM) in 3:7 DMSO: H_2O mixture.

Application of probe 3 in Cell imaging studies

Next we were interested to check the presence of hydrazine in cell via cell imaging studies. In course of that, HepG2 cells were cultured in DMEM medium & then incubated with hydrazine (6 μM) in phosphate buffer saline (PBS, 10 mM, pH = 7.2) at 37 °C for 1hr. Next the cells were further incubated with probe **3** (10 μM) for 1 hr. After the incubation time for 30-min-1hr, considerable amount of compound **3**, hydrazine and Isoniazid [24] will be present in intracellular region by diffusion, while washing with PBS mostly removes the extracellular molecules. Control cells did not show any fluorescence with probe **3** alone under fluorescence microscope (Figure 7). Gratifyingly, a strong blue intracellular fluorescence was observed in the presence of hydrazine and probe **3** under fluorescence microscopy, indicating the reaction (sensing) of the probe **3** with exogenously added hydrazine.

Fluorescence microscopy images of HepG2 cells incubated with hydrazine (6 μM) for 15 min and added probe **3** at

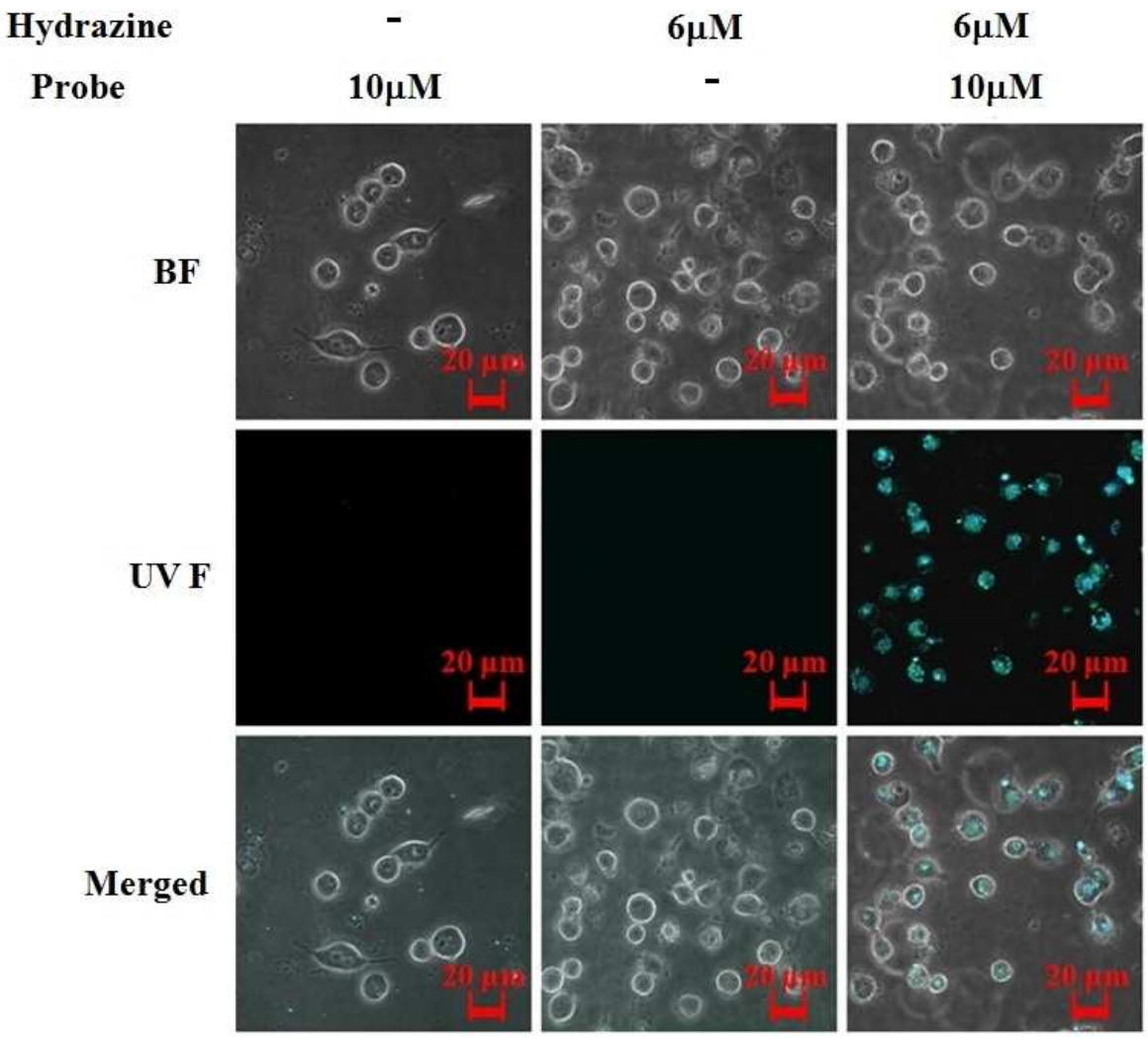


Figure 7. Fluorescence microscopic images of HepG2 cancer cells bright field (BF); UV filter (UV F, 330–385 nm); Merged fluorescence microscopic images of HepG2 cells incubated with Hydrazine (6 μM) for 1 h and then further exposed to probe **3** (10 μM) for 1 h at 37 °C.

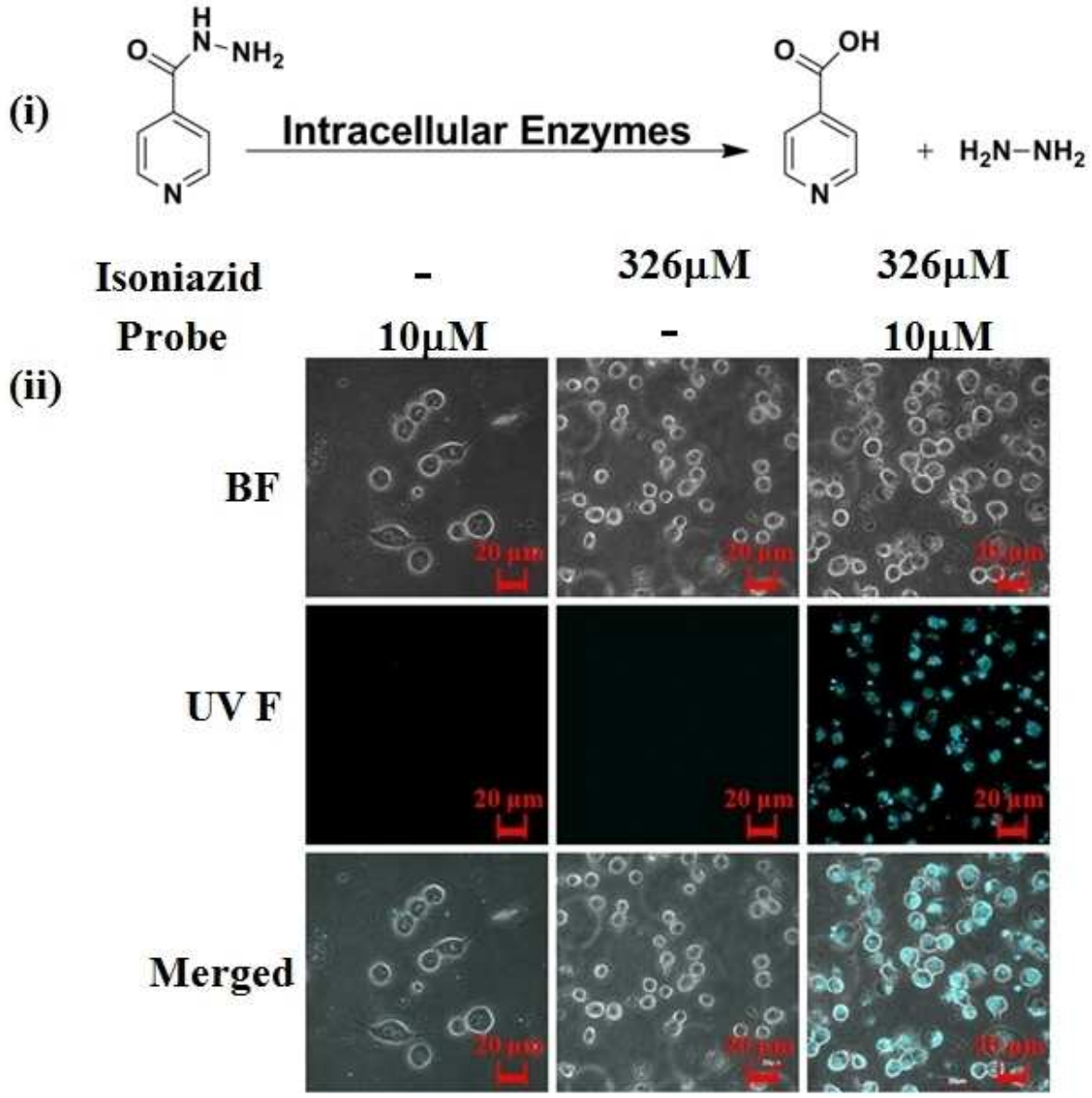


Figure 8. (i) Biotransformation of Isoniazid, (ii) fluorescence microscopic images of HepG2 cancer cells: bright field (BF); uv filter (UV F, 330–385 nm); Merged fluorescence microscopic images of HepG2 cells incubated with INH (326 μM) for 1 h and then further incubated with probe **3** (10 μM) for 1 h at 37 °C.

different time intervals (5 min, 10 min and 20 min)) were recorded (Figure **S6**). With cell imaging experiments performed, we extended our studies to detect hydrazine released through Isoniazid (INH) metabolism inside the cell. Isoniazid is a well known antibiotic, most pronounced for the treatment of tuberculosis. During its biotransformation process, INH was metabolized to release free hydrazine metabolite (Figure 8) in the liver. Among many other reasons, this hydrazine imparts to hepatotoxicity. To find out the ability of probe **3** to detect the hydrazine metabolite of INH, HepG2 cells were incubated with INH for 1hr. After that, cells were exposed to probe **3** (10 μM) for 1hr. To our delight, strong blue fluorescence confirmed the presence of hydrazine as the metabolic product from INH.

The result suggested that probe **3** indeed was also sensitive towards cellular hydrazine metabolized through INH metabolic pathway (Figure 8). Fluorescence microscopy images of HepG2 cells incubated with Isoniazid at different time intervals (30 min, 1 h, 1.5 h and 3 h) with and without probe **3** (10 μM) were recorded (Figure 9). Increase in the fluorescence intensity with time suggested more production of metabolic hydrazine from INH, which was detected by probe **3**. Here it is worthy to mention that INH half lifetime is 3 hrs only.

MTT Assay

The cell viability studies suggested that probe **3** had no significant toxicity upto 70 μM (Figure 10A). Next, we wanted

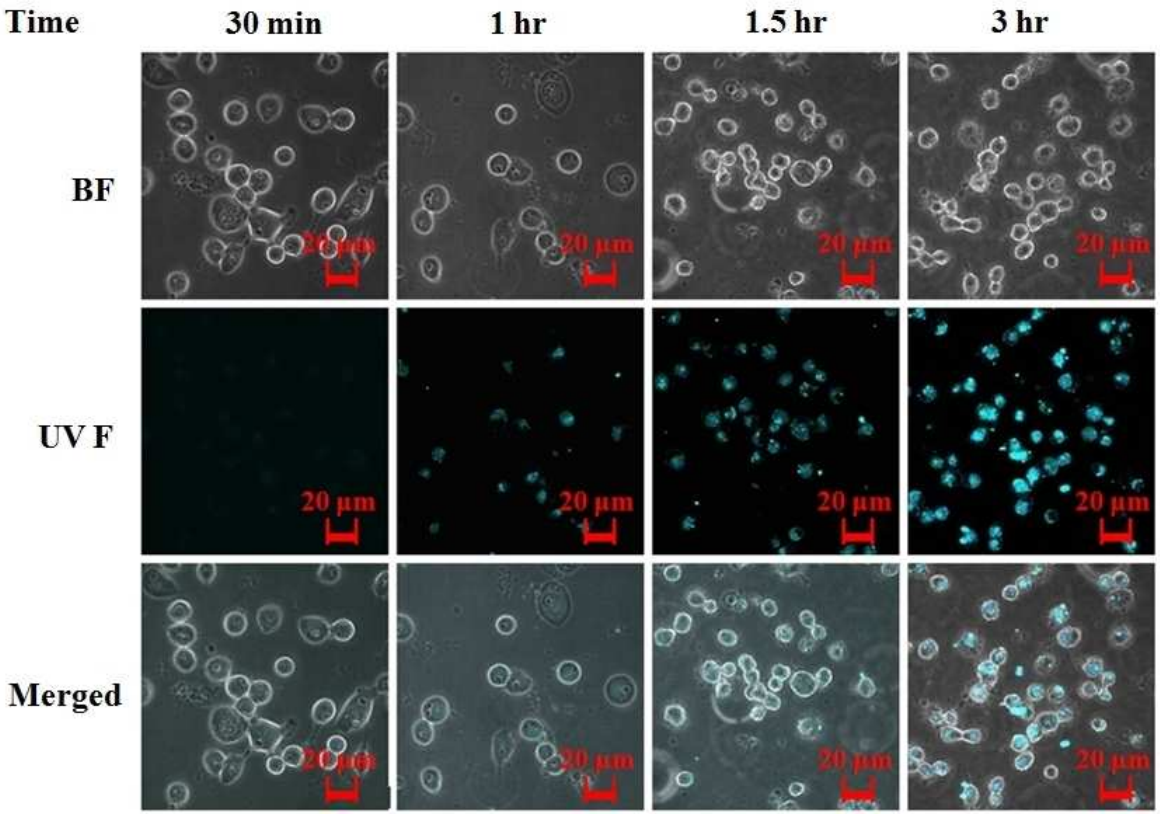


Figure 9. Time dependent fluorescence microscopic images of HepG2 cells treated with Isoniazid (326 μM) ανδ probe **3** (10 μM): Cells were incubated with the Isoniazid for different time intervals: 30 min, 1 h, 1.5 h and 3 h. Then probe **3** (10 μM) was added and the cells were incubated for another 1 h. Bright field (BF); uv filter (UV F, 330–385 nm); Merged images.

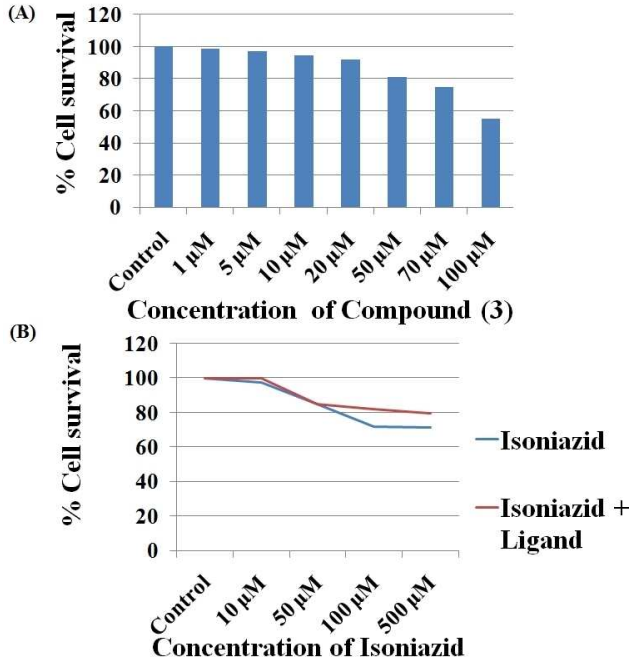


Figure 10. A) MTT assay of probe **3** B) MTT assay of Isoniazid in the presence and absence of probe **3** (10 μM) in HepG2 cells.

to check if probe **3** could effectively reduce the hydrazine toxicity in cell produced by INH metabolism. We extended our studies to check if the probe **3** was involved in the effective scavenging of intracellular toxic, hydrazine released by enzymatic action on Isoniazid. The MTT assay results suggested that in the presence of probe **3**, the toxicity of the Isoniazid was reduced which confirmed that probe **3** effectively lowered (by the reaction mechanism shown earlier) the intracellular hydrazine concentration produced through INH metabolism (Figure 10B).

Conclusion

To conclude, we successfully designed and synthesized a new probe for sensing hydrazine in aqueous medium by an easy single step reaction in good yield. This probe selectively detected hydrazine in vapour and solution phase. The preliminary spectroscopic experimental results suggested that the designed probe was able to detect hydrazine by a turn-on fluorescence mechanism with a detection limit of 1.1 μM. NMR and mass spectroscopic studies had clearly established the mechanism of hydrazine sensing by our probe. In addition, the probe was effective to distinguish the fluorescence colour change at different concentration of hydrazine vapour associated with hydrazine solution (0.25-15%) within 15 min at 25 °C.

Interestingly, our probe is able to detect hydrazine in real water samples quantitatively. Cell imaging studies were performed and the results clearly suggested that probe can easily detect the exogenously added hydrazine in the cell as well as hydrazine metabolite released by the anti-tubercular drug, Isoniazid during its biotransformation. Here the major advantage of this probe **3** is the rapid detection time. Moreover this hybrid probe can reduce the cytotoxicity of Isoniazid; thereby it may be used as a combination along with Isoniazid for the treatment of tuberculosis to reduce the side effects of Isoniazid.

Supporting Information summary

The related supplementary data and experimental section can be found in supplementary material.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Fluorescence · Hydrazine · Isoniazid · Pthalimide · Pyrene

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ARTICLE

Expedient Synthesis of Phenanthro-Imidazo-Pyridine Fused Heteropolynuclear Framework via CDC coupling: A New Class of Luminophores

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We here in report, design and synthesis of a group of fused phenthro-imidazo[1,2-a]pyridine derivatives, as a new class of luminescent materials through Pd(II) catalyzed intramolecular CDC (cross dehydrogenative coupling) reaction. This method thus unlocked aconvenient & expedient way for the synthesis of a new molecular framework containing π– extended fused heteropolycycle. The heteropolycycles showed very good fluorescent property both in solid and solution phase which was further utilized in live cells imaging. These kind of molecules have potential to be used as therapeutic probes and also its solid state luminescence property can be further utilized in making optoelectronic devices.

Introduction

In recent years, different fused aromatic motifs received considerable interest due to their enormous applications in different fields. Among different fused motifs, *N*-fused heterocycles dominates due to their significant biological importance[1-2] and presence in wide class of natural products and functional materials.[3-6] Construction of new heteropolycycles through C-H bond functionalization is highly challenging. It has been always a challenge to synthesize versatile fused heteropolycycles in a simple, convenient, and high yielding method. Serious research efforts are going on to construct new class of fused heteropolycycles using transition metal catalyzed C-H bond functionalization procedure[7-8] which is atom economical and cost efficient. C-C bond formation through direct dehydrogenative cross coupling is one of the most used methods to construct fused heteropolycycles.[9-12] Recently, arylation of different heterocyles like, pyrroles,[13] azoles,[14-15] phenanthroimidazoles,[16-17] polyfluoroarenes[18-19] benzo[*h*]quinolines[20-21] etc. through direct dehydrogenative cross coupling reaction using various transition metals as catalyst has been reported. It was observed that among different transition metals, palladium is widely used due to its catalytic and synthetic efficiency. Different functionalized Imidazo[1,2-a]pyridines[22] are recently under the limelight due to their appealing and vast applications. Imidazo[1,2-a]pyridine is an important pharmacophore found in various natural products as well as some marketed drugs like, zolimidine (antiulcer), alpidem (anxiolytic) , zolpidem (hypnotic) etc.[23-28] Some of this classes of molecules have also great contribution in the field material science (Figure **1**).[29]

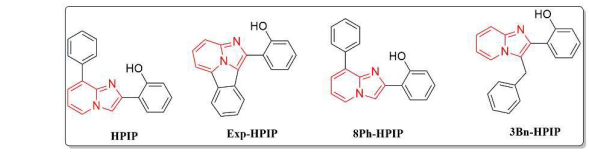

Figure 1: Some reported imidazo[1,2-*a*]pyridine derivatives[29] having very distinct optical properties, exhibiting excited state intramolecular proton transfer (ESIPT).

In the present study, we have synthesized a new class of imidazo[1,2-a]pyridine heteropolycycles using dehydrogenative cross coupling as a key step. An optimized reaction condition was established for synthesizing these new heteropolycycles and thereafter their photo physical properties were examined thoroughly.

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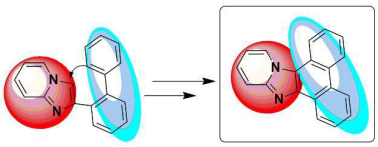


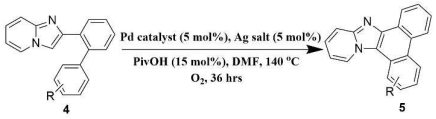
Figure 2: Dehydrogenative cross coupling strategy to form fused heteropolycycle

Due to the extended conjugation through π–expansion, the synthesized molecules have shown interesting fluorescence properties both in the solution and solid phase which was further applied in the live cell imaging studies. Our studies therefore unlocked a new class of luminophores with immense potential to be explored in the emerging field of optoelectronics or could be very useful as therapeutic tools.

Result & Discussion

The synthesis of the fused heteropolycycle (**5**) was started by synthesizing the Imidazo[1,2-a]pyridine ring (**3**) from 2-amino pyridine,(**1**) & 2'-iodo acetophenone, (**2**) using an established C-H amination protocol (in presence of $Cu(OAc)_2$, ZnI_2, 1,10-phenanthroline),[30] scheme **1**. This was then subjected to routine Suzuki coupling reaction in Dry DMF to get compound, **4** in good yield. Compound **4** was further subjected to a palladium (II) catalysed intramolecular oxidative CDC reaction in dry DMF using Ag_2CO_3 as an oxidant and PivOH as an additive at 140 ^{o}C under positive pressure of molecular oxygen for 36 hrs. The reaction mixture was purified afterwards through column chromatography to get compound **5** in moderate to good yield (52-85%). Furthermore, optimisation studies were carried out and the detail of the same was summarized in table **1**. The effect of different solvent, co-oxidant and additive used were thoroughly studied.

It was observed that among all the co-oxidant used, Ag_2CO_3 gives the best result with a combination of $Pd(OAc)_2$ as a catalyst, PivOH as an additive in dry DMF under ambient pressure of molecular oxygen (Table **1**, entry **7**). The other co-oxidants tested (such as BQ, $FeCl_3$ etc.) were found to be not so effective to produce better yield. It was also observed that the presence of molecular oxygen was necessary to get the desired heterocycle in good yield. Without molecular oxygen, very less or no conversion was occurred (see table **1,** entry **9**



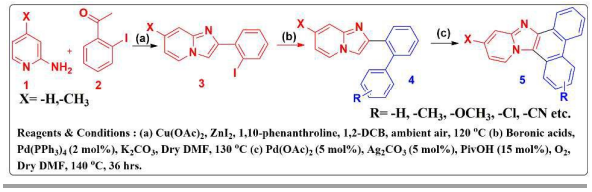
Entry	Catalyst	Oxidant	Co-oxidant	Solvent	Additive	Yield (%)
1	$PdCl_2$	O_2	$Cu(OAc)_2$	Toluene	HOAc	15
2	$Pd(OAc)_2$	O_2	$Cu(OAc)_2$	Toluene	HOAc	25
3	$Pd(OAc)_2$	O_2	$Cu(OAc)_2$	Toluene	PivOH	32
4	$Pd(OAc)_2$	O_2	$Cu(OAc)_2$	DMSO	PivOH	44
5	$Pd(OAc)_2$	O_2	Ag_2O	DMF	PivOH	10
6	$Pd(OAc)_2$	O_2	AgOAc	DMF	PivOH	56
7	**$Pd(OAc)_2$**	**O_2**	**Ag_2CO_3**	**DMF**	**PivOH**	**85**
8	$Pd(OAc)_2$	O_2	Ag_2CO_3	DMSO	PivOH	70
9	$Pd(OAc)_2$	O_2	-	DMF	PivOH	Trace
10	$Pd(OAc)_2$	Air	Ag_2CO_3	DMF	PivOH	28
11	$Pd(OAc)_2$	O_2	BQ	DMF	PivOH	-
12	$Pd(OAc)_2$	O_2	$FeCl_3$	DMF	PivOH	-
13	$Pd(OAc)_2$	-	-	DMF	PivOH	-
14	$PdCl_2(PPh_3)_2$	O_2	Ag_2CO_3	DMF	PivOH	20
15	$Pd(acac)_2$	O_2	Ag_2CO_3	DMF	PivOH	17
16	$Pd(PPh_3)_4$	O_2	Ag_2CO_3	DMF	PivOH	26

Table 1: Optimization of the Reaction condition of Pd catalyzed dehydrogenative cross coupling.

&**12**). Pivalic acid was found to be better additive as compare to acetic acid.

Thus it was clear from the result that both the oxidant and co-oxidant play an important role during cyclization. Different palladium catalysts were also examined in search of optimal catalyst for the cyclization. We found $Pd(OAc)_2$ as a worthy catalyst in this reaction. The solvent screening resulted DMF as an efficient solvent. Thus, having an optimal reaction condition we explored this synthetic methodology to prepare a group of Imidazo[1,2-a]pyridine/Phenanthrene hybrid scaffolds to explore its substrate scope. Different substituted boronic acids were used to do Suzuki coupling reaction.

There were some problems in the Suzuki cross coupling reactions with some boronic acid derivatives (like pyridine-2-boronicacid, 4-fluorophenylboronicacid, 2-methoxyphenylboronicacid). For each case, either reaction did not proceed or very low yield was observed. Thus it was not possible to proceed further with these substrates. Moreover in



Scheme 1: Synthesis of the fused heteropolycycle, **5.**

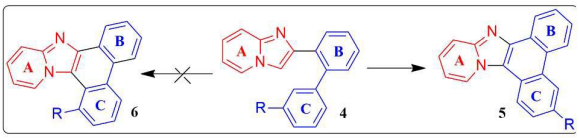


Figure 3: Regioselectivity in the **C** ring during final Intramolecular C-C coupling

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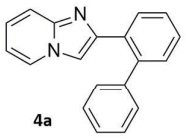
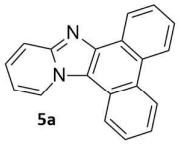
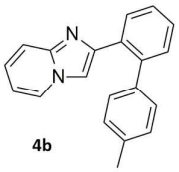
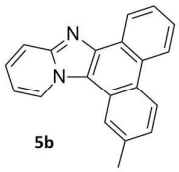
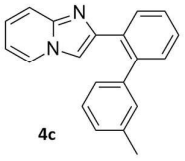
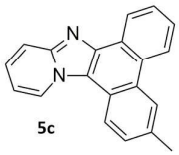
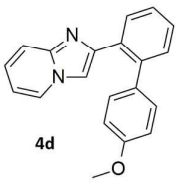
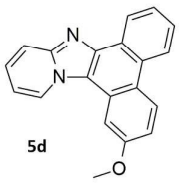
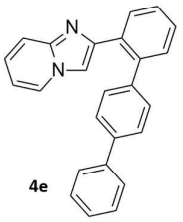
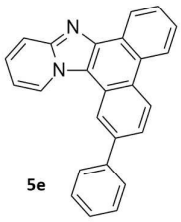
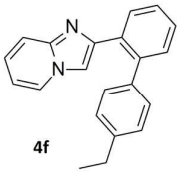
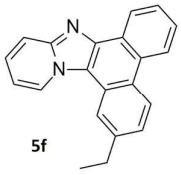
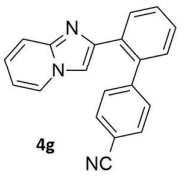
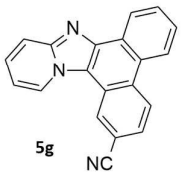
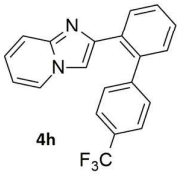
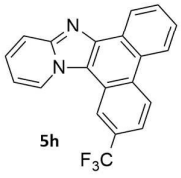
Entry	Precursor	Product	Yield[a]
1	 4a	 5a	85%
2	 4b	 5b	83%
3	 4c	 5c	81%
4	 4d	 5d	84%
5	 4e	 5e	82%
6	 4f	 5f	80%
7	 4g	 5g	56%
8	 4h	 5h	52%

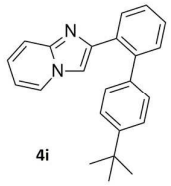
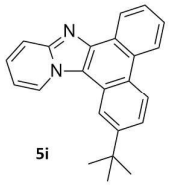
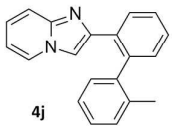
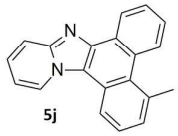
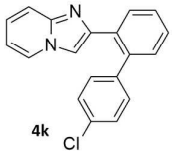
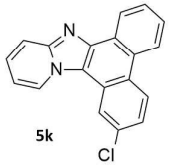
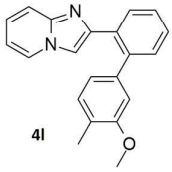
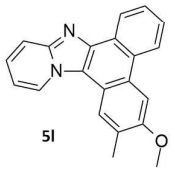
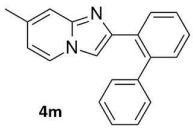
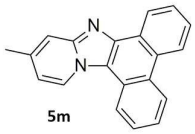
Entry	Precursor	Product	Yield[a]
9	 4i	 5i	78%
10	 4j	 5j	81%
11	 4k	 5k	75%
12	 4l	 5l	72%
13	 4m	 5m	83%

Table 2: Reactions were carried out by using precursors **4 (a-m),** $Pd(OAc)_2$ (5 mol%), Ag_2CO_3 (5 mol%), PivOH (15 mol%) under O_2 atm in Dry DMF at 140 ^oC for 36 hours. [a]Yield of the isolated product.

case of some precursors (**4**), made from 3-nitrophenylboronicacid, 3-chlorophenylboronicacid and 2-napthylphenylboronicacid, the yield of the final product (**5**) was extremely poor (≤ 5% yield) and the reactions were very sluggish.Keeping aside those substrates, a number of 13 substituted and well decorated Imidazo[1,2-a]pyridine/Phenanthrene hybrid polyheterocycles (**5a-m**) were

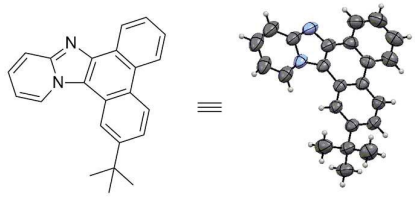


Figure 4: Crystal structure of compound **5i** (CCDC 1515438)

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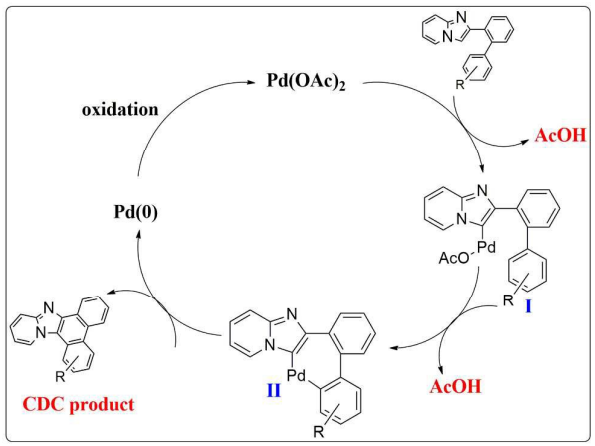

Figure 5: Proposed mechanism of the oxidative CDC coupling

synthesized by this methodology in moderate to good yields. This is noteworthy to mention here that the presence of electron donating groups (like methyl or methoxy) in the **C**-ring produced better result in case of final construction of the fused ring (Table **2** entry **5b** & **5d**). The presence of electron withdrawing groups (like trifluoromethyl or cyano) however resulted in poor yield (Table **2** entry **5g** & **5h)**. The question of regioselectivity in case of substrate (**4**), having meta substituted aromatic **C** ring was also examined and gratifyingly, only one regioisomer having minimal steric hindrance (**5c**) was selectively formed (see Figure **3**) and no trace of the other product (**6**) was observed. The general structure of the polyheterocycles was further confirmed from the crystal structure of compound **5i** (Figure **4**).

On the basis of the above results, we propose a plausible reaction mechanism (Figure **5**). In the first step, intermediate **I** was generated by $Pd(OAc)_2$ through electrophilic substitution of the C-H proton of Imidazolium ring, the most acidic one. The next step was the key step in which the intramolecular deprotonation takes place, forming the intermediate **II** followed by the formation of the desired product through CDC Coupling. Finally the catalytic cycle was closed by the regeneration of more active Pd(II) from Pd(0) via oxidation.

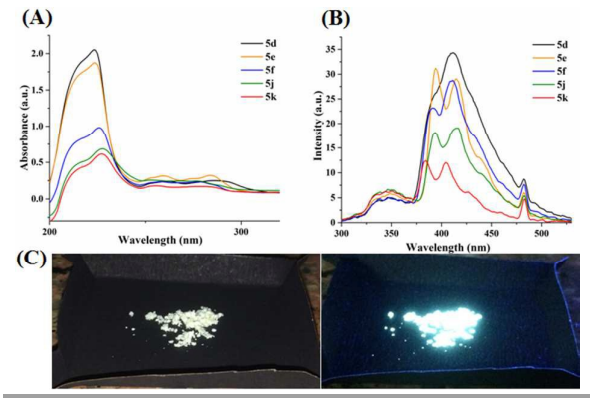

Figure 6: (A) Absorbance & **(B)** Emission spectra of some selected compounds (5 μM in CH_2Cl_2, λ_{ex}= 230 nm) along with **(C)** Normal light & solid state fluorescence of compound **5e** (hand held UV lamp at 365 nm)

Compounds	λ_{max} [a] (nm)	λ_{em} [b] (nm)	Φ_F [c]	Ref
5d	223	413	0.61	-
5e	224	416	0.44	-
5f	226	410	0.47	-
5j	228	414	0.35	-
5k	227	405	0.22	-
HPIP	345	573	0.005	29
4'MeO-HPIP	334	572	0.01	29
5'Br-HPIP	347	370	0.013	29
4'Br-HPIP	347	541	0.055	29
4'HO-HPIP	351	560	0.019	29

Table 3: [a]Absorption maximum and [b]Emission maximum of the synthesized compounds (**5d-5k** in 5 μM in CH_2Cl_2) along with some reported molecules. [c]Fluorescence quantum efficiencies were determined using Phenanthrene (Φ_F=0.125, λ_{ex} = 255 nm) as a standard for compound **5d-5k.**

The synthesized compounds (**5**) showed significant fluorescence response (blue) when irradiated at 365 nm. Therefore the photo physical properties of these molecules were thoroughly investigated. UV/Vis and fluorescence spectra of some selected compounds were recorded in CH_2Cl_2 (Figure **6**). It was observed that the presence of electron donating group in C-ring enhances the fluorescence intensity (especially in para position, **5d**) due to extended conjugation. Emission maxima of all the compounds appeared in the blue light region. Fluorescence quantum efficiencies of these compounds were calculated to be in the range of 0.22-0.61 using phenanthrene (Φ_F= 0.125) as a standard (Table **3**). A comparison chart of quantum efficiencies with some previously reported Imidazo[1,2-a]pyridine derivatives were tabulated (Table **3**). The synthesized compounds also show a very interesting solid state fluorescence (white emitting, Figure **6c**) when irradiated at 365 nm (Figure **6**). Compound **5e** and **5k** showed best solid state fluorescence activity among all. These types of compounds with such properties have high potential to be used in the emerging field of optoelectronics such as OLEDs (Organic Light Emitting Diodes).[31-34]

Due to decent fluorescence properties of the synthesized compounds, further attempt to explore the fluorescence activity of the compounds in live cells were taken forward. We planned to perform cell imaging study with **5d** (Φ_F= 0.61) & **5e** (Φ_F= 0.44). Initially the cytotoxic effect of **5d** & **5e** were

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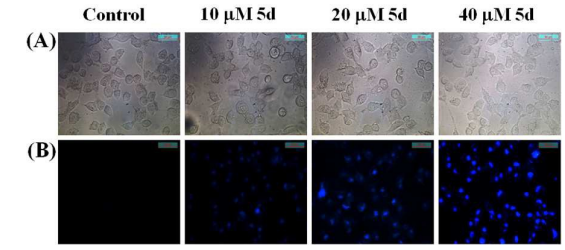

Figure 7: (A) Bright field & **(B)** Fluorescence images of HepG2 cells at 40X magnification after preincubation with 10 µM, 20µM and 40µMof **5d** for 60 min at 37 °C followed by washing twice with 1X PBS.

examined using a cell viability assay in HepG2 cells. Reaching up to 70 µM concentration, no significant reduction in the tetrazolium salt was found, reflecting a decrease in formazan production. More than 80% of cells were viable up to a dose of 40 µM (see SI, Figure **S2**). Therefore, no significant cytotoxicity was observed up to 40 µM concentration of both the compounds **5d** & **5e**. Now, taking into consideration the fluorescence efficiency of the compounds, live cell fluorescence imaging was recorded for both the compounds in vitro in different concentrations (Figure **7** for compound **5d**). In case of HepG2 cells, incubated with 10 µM of **5d** and **5e** for 60 min at 37 °C, we observed a distinct blue fluorescence emission. Increasing concentration of **5d** & **5e** (from 10 µM to 20 µM to 40 µM) resulted in concentration-dependent increase in the intracellular blue fluorescence. The fluorescence signal also indicated a distinct localization of the compounds in the cytoplasmic region. Hence, the present compounds, **5d** & **5e** have low cytotoxic effects and are biocompatible for fluorescence imaging of the live cells. Fluorescence images of compound **5e** were shown in the ESI (Figure **S4**).

In summary, we have developed a new strategy to construct fused Phenanthro-Imidazo-Pyridine heterocypolycles through intramolecular CDC reaction. We showed that these fused heteropolycycles are efficient luminophores. Cell imaging studies were performed to show its efficiency. In our laboratory, currenty we are trying to utilise the synthesized compounds as fluorophores in different sensing techniques. Due to its very good solid state fluorescence, it can also be useful for developing optoelectronic devices, like solid state lighting in OLEDs.

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Arylation

Palladium-Catalyzed Direct Synthesis of Phenanthridones from Benzamides through Tandem N–H/C–H Arylation

Biswadip Banerji,*[a,b] Satadru Chatterjee,[a] K. Chandrasekhar,[a] Chinmay Nayan,[a] and Sunil Kumar Killi[a]

Abstract: We report a palladium-catalyzed method for the direct synthesis of phenanthridones from benzamides in a single step. Unlike previous reports, the current protocol does not need any directing groups or any harsh conditions. This methodology has a wide functional group tolerance therefore a series of phenanthridones were synthesized with a yield up to 87 %. The efficacy of this protocol was further explored by synthesizing some important naturally occurring amaryllidaceae alkaloids in a single step with very good yields.

Introduction

Synthesis of new heterocycles continues to be a major part of pharmaceutical research, as most bioactive compounds or their congeners belong to a direct heterocycle or a hybrid scaffold.[1] Phenanthridones are one such heterocyclic motif, and they are present in a wide class of bioactive alkaloids (such as amaryllidaceae alkaloids).[2] Different anticancer and antiviral drugs,[3] Poly (ADP-ribose) polymerase (PARP) inhibitors,[4] and acetylcholine esterase inhibitors[5] contain phenanthridones as a core motif (Figure 1). Due to their significant pharmaceutical importance, phenanthridones are always under the limelight in the therapeutic domain. Serious research work is going on to enrich the chemistry of phenanthridones by evolving new efficient synthetic routes.[6] Though a number of methods are available for the synthesis of phenanthridone and its derivatives (Figure 2), these also have some limitations.[6b,7] In each case, either a directing group dictates the course of the reaction, or

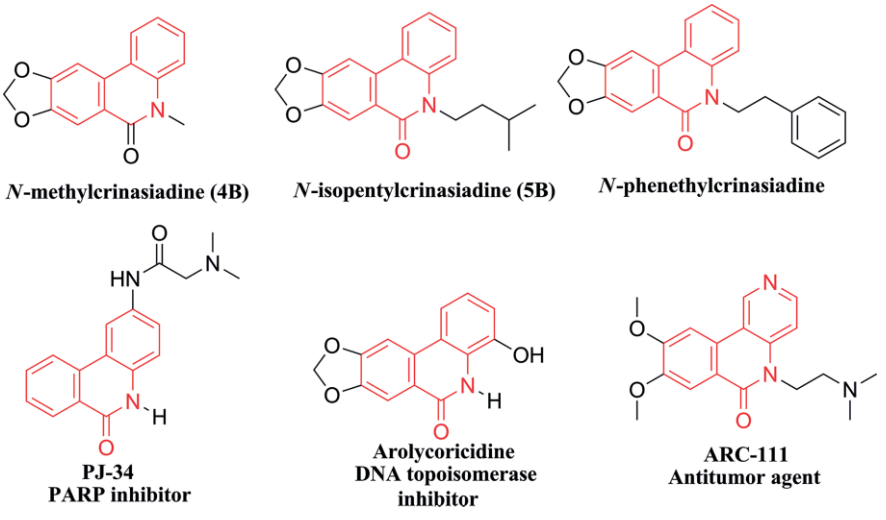

Figure 1. Some natural products along with some popular drugs having phenanthridone as a core motif.

the ligand used plays a pivotal role. Moreover, in most of the cases, the conditions used are too harsh, and the substrate scopes were limited. Therefore a quick and easy access to a variety of phenanthridones is still a synthetic challenge.

In recent years, transition-metal-catalyzed construction of different heterocycles and carbocycles has become common practice.[8] Palladium catalysts are the most frequently used

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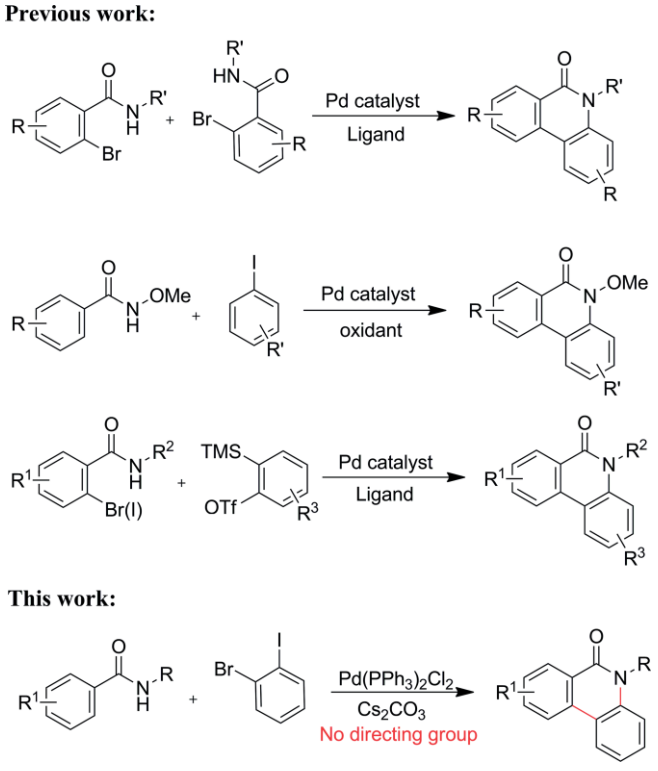


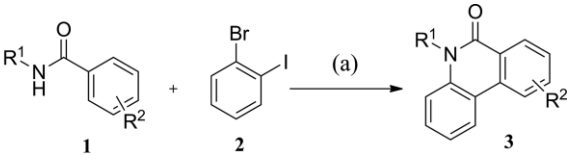
Figure 2. Previously reported synthetic protocols of phenanthridones with respect to our current work.

transition metal catalysts due to their efficiency and compatibility with a large number of functional groups. Most of the previously reported phenanthridone derivatives were synthesized using palladium catalysts.[6d,e,9]

In the present study, we developed a new synthetic method to construct a group of phenanthridone derivatives with ease from different simple amides in moderate to good yields. No directing group or external ligand was required for the reaction to proceed. The reaction conditions are relatively mild, and clean products were achieved in decent yields. The reaction is intermolecular and simultaneously forms a C–C and a C–N bond to generate the desired phenanthridone moiety. As the reaction needs no directing groups or ligands to proceed, a large number of target heterocycles can be synthesized by this simple method in a shorter time. Our study therefore unveiled a new way to access a wide variety of phenanthridones quickly and efficiently.

Results and Discussion

The synthetic study started by preparing different simple and substituted benzamides **1**. These were then subjected to reaction with 1-bromo-2-iodobenzene (**2**) in a sealed tube in the presence of $Pd(PPh_3)_2Cl_2$ as catalyst and Cs_2CO_3 as base in dry DMF at 140 °C under a N_2 atmosphere for 16 h to form different phenanthridone derivatives **3** via simultaneous formation of an intermolecular C–C and C–N bond in good to excellent yields (Scheme 1).

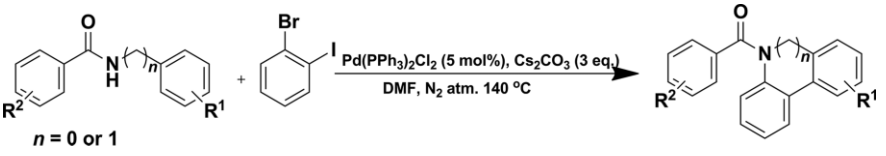


R^1 = Ph, Bn, propyl etc.
R^2 = Me, CN, CF_3 etc.

Scheme 1. One pot synthesis of phenanthridones. (a) Reaction conditions: $Pd(PPh_3)_2Cl_2$, Cs_2CO_3, dry DMF, 140 °C, N_2 atmosphere, 16 h.

Optimization studies were performed in search of the best conditions to carry the reaction forward and to get higher yields. At first, oxidative conditions, using molecular oxygen as

Table 1. Optimization of the reaction conditions.



Entry	Catalyst	Ligand	Base	Solvent	Yield [%]
1	$Pd(OAc)_2$	X-Phos	Cs_2CO_3	DMF	18
2	$Pd(OAc)_2$	Xantphos	Cs_2CO_3	DMF	16
3	$PdCl_2$	X-Phos	Cs_2CO_3	DMF	Trace
4	$Pd(acac)_2$	X-Phos	Cs_2CO_3	DMF	Trace
5	$Pd(dba)_3$	X-Phos	Cs_2CO_3	DMF	Trace
6	$Pd(PPh_3)_4$	X-Phos	Cs_2CO_3	DMF	59
7	$Pd(PPh_3)_4$	Xantphos	Cs_2CO_3	DMF	57
8	$Pd(PPh_3)_4$	X-Phos	K_2CO_3	DMF	49
9	$Pd(PPh_3)_2Cl_2$	X-Phos	Cs_2CO_3	DMF	88
10	**$Pd(PPh_3)_2Cl_2$**	**–**	**Cs_2CO_3**	**DMF**	**87**
11	$Pd(PPh_3)_2Cl_2$	–	K_2CO_3	DMF	76
12	$Pd(PPh_3)_2Cl_2$	–	Cs_2CO_3	1,4-dioxane	67
13	$Pd(PPh_3)_2Cl_2$	–	Cs_2CO_3	Toluene	58
14	$Pd(PPh_3)_2Cl_2$	–	Cs_2CO_3	DMSO	79

oxidant, $Pd(OAc)_2$ as catalyst, X-Phos as ligand, and Cs_2CO_3 as base in dry DMF at 140 °C were tried; but, to our disappointment, no desired product was formed. Then we tried various other ligands (like dppf, Xantphos etc.), but still ended up with the same result. Subsequently, we varied the catalyst system as well as using different ligands, but still no satisfactory results were obtained. Then we changed from an O_2 to a N_2 atmosphere, and, to our surprise, the desired compound was formed in poor yield using $Pd(OAc)_2$ as catalyst, X-Phos as ligand, and Cs_2CO_3 as base in dry DMF at 140 °C after 16 h. The yield was further increased by changing the catalyst system to $Pd(PPh_3)_4$ and finally to $Pd(PPh_3)_2Cl_2$. Thereafter, we observed that the presence or absence of ligand in the reaction medium does not affect the yield of the final product (Table 1, entry 10).

It was also observed that lowering the reaction temperature ended up in lower yield. Cs_2CO_3 was found to be superior to K_2CO_3. Among the different solvents tested, DMF was found to be the best. Thus, having the best reaction conditions in hand, we explored this methodology to prepare a wide variety of phenanthridones in good yields (Figure 3). Both *N*-substituted benzyl and phenyl groups react equally well in this case. Different simple and heteronuclear amides afforded the correspond-

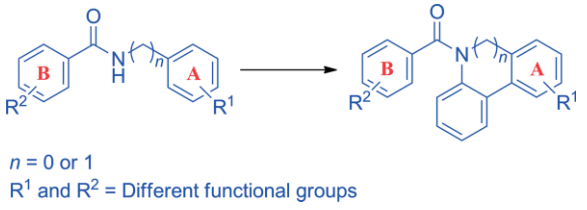


Figure 3. Schematic representation of the cyclization.

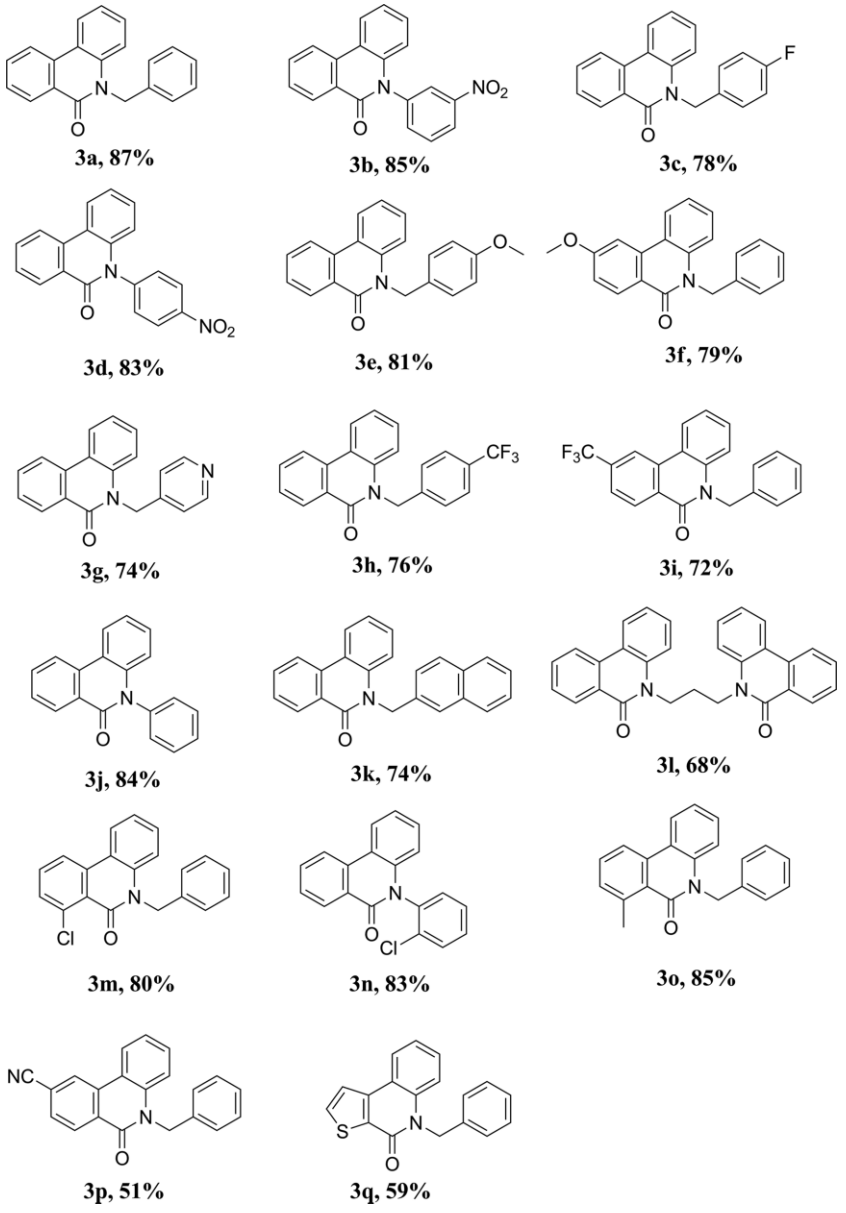


Figure 4. Substrate scope of the reaction with various kinds of amides along with percent isolated yields.

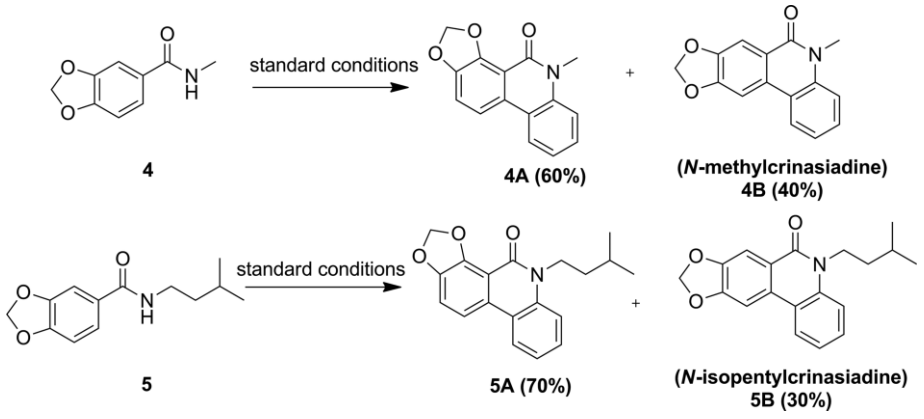


Figure 5. Application of Pd-catalyzed synthesis of phenanthridone.

ing products in good yields. The effects of substitution at different positions as well as the electronic nature of the aromatic rings were thoroughly studied. It was observed that the presence of electron-withdrawing or electron-donating groups did not affect the yield of the final product much except for $-NO_2$ groups in the B ring, which resulted in a sluggish reaction and –CN groups, for which the reaction yield was poor (Figure 4). A dimer product was obtained when using aliphatic diaminopropane (**3l** in Figure 4). In case of amides with bulky aliphatic groups (such as tertiary butyl or isopropyl), the reactions did not proceed at all. The positions of the different functional groups were also equally examined, and it was gratifying to observe that substitution at different positions of rings A or B did not actually affect the yield of the final product. Surprisingly, we got only a single isomer for compound **3m** instead of a mixture of two, which might be due to the negative inductive effect (–I) of chlorine at the *ortho* position, which facilitated the coupling at that position.

The success of any good methodology lies in its fruitful application. Thus, we tried to utilize this new method by synthesizing two important naturally occurring amaryllidaceae alkaloids, *N*-methylcrinasiadine (**4B**) and *N*-isopentylcrinasiadine (**5B**), in a single step. Most of the previously reported procedures were multistep and proceeded under very harsh conditions. Surprisingly, in the course of this reaction, two regioisomers (**4A**, **4B** and **5A**, **5B** in Figure 5) were formed in ratios of 6:4 (**4A**/**4B**) and 7:3 (**5A**/**5B**). To the best of our knowledge, the new regioisomeric compounds **4A** and **5A** (with yields greater than or equal to 60 %) belong to a new class of phenanthridones.

On the basis of the aforementioned results, we propose a tentative mechanism for phenanthridone synthesis (Figure 6).

The reaction cycle starts with the formation of intermediate **I** through oxidative insertion of palladium(0) to the C–I bond. Intermediate **I** is then converted to intermediate **II** via a C–N bond formation, eliminating HI. Again oxidative insertion of palladium(0) to C–Br bond results in intermediate **III**, which is further transformed to the palladacycle (intermediate **IV**), eliminating HBr. Finally, the desired phenanthridones are generated by reductive elimination, ending the catalytic cycle.

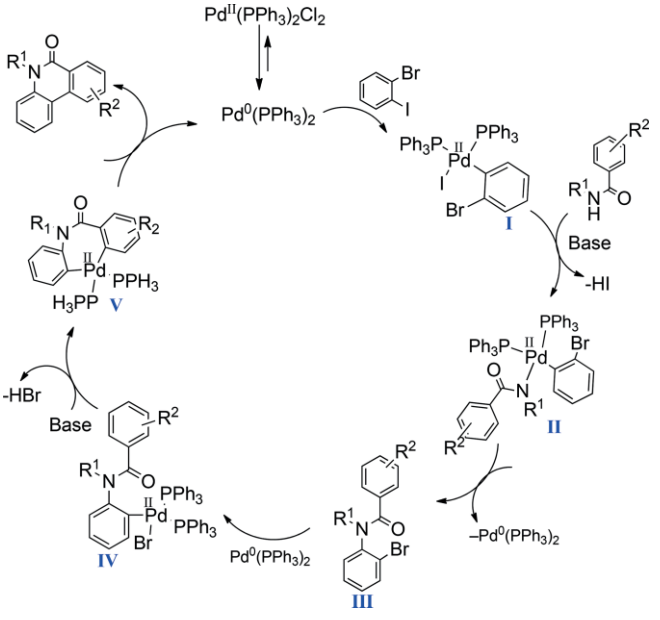


Figure 6. Proposed reaction mechanism.

Conclusion

An efficient method was developed to synthesize phenanthridone derivatives from easily available synthons. This new methodology opens up new avenues to successfully synthesize a variety of different substituted phenanthridones in moderate to good yields. This method was further employed to synthesize two natural products along with their regioisomers in single steps. Owing to the therapeutic importance of this particular heterocycle, the present methodology will be a valuable addition towards synthetic endeavors.

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Keywords: Synthesis design · Heterocycles · Homogeneous catalysis · Tandem processes

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Available online at: http://www.iajps.com *A Case Report*

DRUG INDUCED DYSELECTROLYTEMIA –A CASE REPORT

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Abstract:

Spiranolactone and Eplerenone were belongs to the class of potassium sparing diuretics where as Torsemide belongs to the loop diuretic class, they mainly acts by blocking the function of aldosterone hormone to retain the sodium and excrete potassium. By irrational use of these drugs induced dyselectrolytemia like hyperkalemia and hyponatremia. A 55 years female patient was admitted in cardiology department with the chief complaints of drowsiness and slow response to commands by using of the diuretics (Spiranolactone , Eplerenone and Torsemide) causes the electrolytes induced hyperkalemia and hyponatremia. These electrolytes abnormalities will causes the cardiac arrhythmias , muscle paralysis and sometimes death also. Whereas both rechallenge and dechallenge was done and the ADR assessment scales like Naranjo and WHO gives certain ADR and it can be managed by stopping of the above drugs. So, clinical pharmacist plays a major role in detecting, monitoring and managing of ADRs.

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INTRODUCTION:

Spironolactone and Eplerenone belongs to potassium sparing diuretic class it's mainly bind to mineralocorticoid receptor blocking the functions of aldosterone hormone to retain sodium and excrete potassium. Torasemide or torsemide is a pyridine-sulfonylurea type loop diuretic. Torasemide inhibits the $Na^+/K^+/2Cl^-$-carrier system (via interference of the chloride binding site) in the lumen of the thick ascending portion of the loop of Henle, resulting in a decrease in reabsorption of sodium and chloride.

Spironolactone, eplerenone and torasemide are associated with electrolytic imbalances like hyperkalemia and hyponatremia**.** Hyperkalemia is life threating metabolic disorder occurs when serum potassium levels is greater than 5 meq/l [1,2]. Symptoms of hyperkalemia is divided into mild, moderate and severe in mild to moderate includes generalized weakness fatigue, nausea vomiting and diarrhea whereas severe hyperkalemia causes cardiac arrhythmias and muscle paralysis. Causes of hyperkalemia medication induced hyperkalemia. insufficiency of aldosterone hormone and distribution of potassium between intracellular and extracellular was impaired [1]. Treatment for hyperkalemia includes intravenous administration of calcium lowers the risk of cardiac arrhythmias and stabilizing the causing of myocardial infraction. Metabolic acidosis is reduced by giving sodium bicarbonate that lowers the potassium where as insulin and beta 2 agonist are helpful for shifting of potassium intracellularly. The use of loop diuretics along with polystyrene resins reduces risk of volume overload because of sodium exchange for that potassium by resins. Long terms treatment includes restriction the potassium diet [(1)].

Whereas hyponatremia occurs when the sodium in serum is less than 136 meq/l [2]. Symptoms of hyponatremia is divided into acute symptoms and chronic symptoms in acute symptoms occurs less than in 48 hrs it includes seizures and impaired mental status where as chronic symptoms occurs in greater than 48 hrs it includes GIT symptoms like nausea ,vomiting and loss of appetite and second symptom is neurological abnormality.

Causes of hyponatremia are medication induced dehydration and liver, heart and kidney problems [5].

Treatment for hyponatremia: To correct the hyponatremia, tolvaptan and conivaptan was administered intravenously or orally [2].

Incidence: The incidence of hyperkalemia is about 1% to 8% due to its life threating arrhythmias. Whereas for hyponatremia incidence is 4% in hospitalized patients [2].

CASE REPORT:

A 55 years female patient was admitted in cardiology department with chief complaints of drowsiness since 3 days followed by slow response to commands. She was a known case of diabetes mellitus since 3 years, hypertension since 2 years, hypothyroidism since 1 year and coronary artery disease since 1 month. She was under regular medication for Diabetes mellitus Glimipiride,HypertensionTelmisartan,Hypothyroidism- thyroxine and Coronary artery disease spironolactone, eplerenone, torsemide, clonazepam , metalozone , Rabeprazole ,ambroxol, buclizine, sodium picosulphate, desloratidine + montelukast. She was using the following drugs for her disease condition they are metolazone (used as diuretic), eplerenone + spironolactone (used for heart failure), telmisartan (used for hypertension), Rabeprazole(for gastric discomfort), thyroxine (hypothyroidism), clonazepam (used for sleep), ambroxol (used for cough), buclizine(used as antiemetic), torasemide (used as diuretic), glimepiride(used for diabetes mellitus), desloratidine+montelukast (used for allergy).

Investigations: On examination vitals shows that BP: 100/60 mm of hg, PR: 77 Bpm, T: 98.6 ^{0}f where as systemic examination includes CVS: S1, S2+ and RS: Clear. Her renal functions shows increased **BUN (146 mg/dl↑)** and **serum creatinine values (1.8 mg/dl↑)** Where as electrolytes indicates increased in **potassium (5.8 mmol/l↑) and chloride (140mmol/l↑) levels with low serum sodium concentration (107 mmol/l↓).** Based on subjective and objective evidence the patient is newly diagnosed with spironolactone , eplerenone and torsemide induced dyselectolytemia. The following figure shows abnormalities in ECG like Long QT interval, abnormal QRS-T angle and marked ST Depression, possible subendocardialinjury(V4,V5).

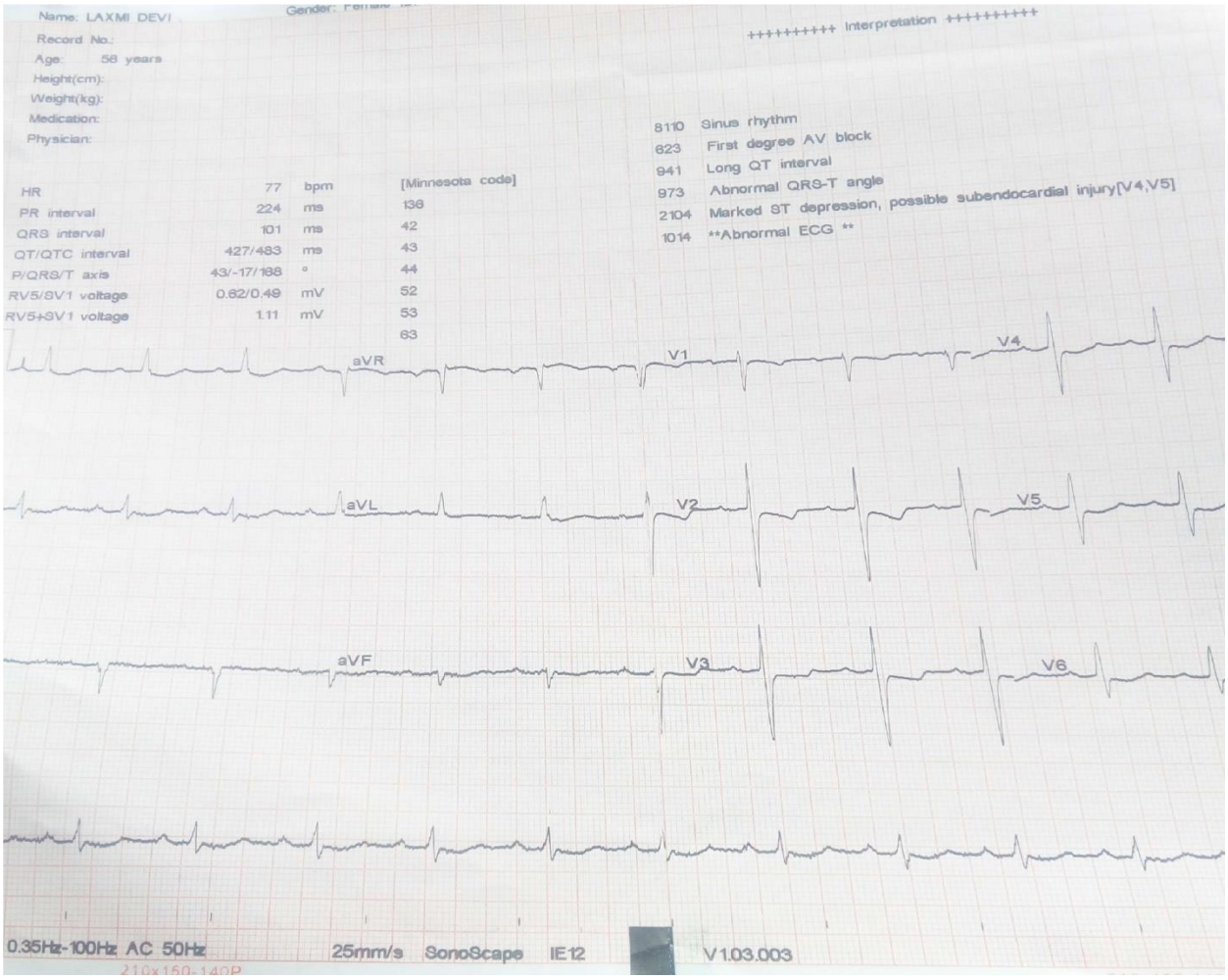

Fig 1: shows the following abnormalities in ECG

DISCUSSION:

Spironolactone and eplerenone are both are androgen receptor antagonist the pharmacology of spironolactone is structurally similar to the progesterone molecule and produces anti androgenic effects where as eplerenone is a derivative of spironolactone does not produce much anti androgenic effects compared to that of spironolactone. The major pharmacokinetic effects of eplerenone inhibit aldosterone binding by 50% where as only half by that spironolactone undergoing with rapid metabolism of 3 active metabolites of prolonged half lives (13.8-16.5 hrs) similarly the eplerenone under goes with extensive metabolism with short elimination half life (4-6hrs) [3]. Spironolactone induced hyperkalemia is happened by blocking the potassium excretion by binding directly with the mineralocorticoid antagonist is seen in patients treated with potassium sparing diuretics within 10 days[4]. Torasemide Rapidly absorbed following oral administration. Absolute bioavailability is 80%. Volume of distribution 12 to 15 L. Metabolized via the hepatic CYP2C8 to 5 metabolites. The major metabolite, M5, is pharmacologically inactive. There are 2 minor metabolites, M1, possessing one-tenth the activity of torasemide, and M3, equal in activity to torasemide. Overall, torasemide appears to account for 80% of the total diuretic activity, while metabolites M1 and M3 account for 9% and 11%, respectively.half life 3.5 hours. Symptoms of overdose include dehydration, hypovolemia, hypotension, hyponatremia, hyperkalemia, hyperchloremic alkalosis, and hemoconcentration[6].

ADR ASSESMENT: CASUALITY ASSESMENT OF SUSPECTED ADR

Both dechallenge and rechallenge was done

S.NO	SUSPECTED DRUG	SUSPECTED ADR	NARANJO SCALE	WHO-UMC
1	Spironolactone, Eplerenore and Torsemide	Dyselectrolytemia	Certain	Certain

ADR management: Attempts are made to treat spironolactone , eplerenone and torsemide induced dyselectrolytemia by stopping of these drugs and correcting the electrolyte imbalance of sodium by supplying normal saline.

CONCLUSION:

The irrational use of diuretics like Spiranolactone , Eplerenone and Torsemide causes electrolytic imbalances like Hyperkalemia and Hyponatremia and in severe conditions it may leads to cardiac arrythmiasis , muscle paralysis and death. So clinical pharmacist plays a major role in detecting, monitoring and managing of ADRs.

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Research Article

POLYPHARMACY INDUCED DRUG-DRUG INTERACTIONS- AN OBSERVATIONAL AND INTERVENTIONAL STUDY

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A R T I C L E I N F O

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Key words:

Polypharmacy, drug-drug interactions (DDIs), drug related problems, Cardiology, General medicine.

A B S T R A C T

Aims and Objectives:

- To study the incidence and pattern of drug-drug interactions
- To identify and minimise preventable drug-drug interactions as far as possible.
- To minimize health care cost burden on patients and health care system.
- To improve the patients quality of life
- To improve the rationality of drug therapy
- To decrease the hospitalization of patients

Methodology: An observational and interventional study was conducted for a period of 6 months in the various departments of VishwaBharathi super specialty hospital, Kurnool. A total of 60 patients/ prescriptions were included in this study. A total of 102 DDIS were identified from 27 prescriptions.

Results: Our study concluded that a high percentage of 54(52.94%) DDIs were found in patients of age group >60 years and whereas distribution of drug-drug interactions based on severity showed many major drug-drug interactions (59.80%). Further the study concluded that a high number of DDIs were found in cardiology department (32 DDIS) followed by General Medicinedepartment (28 DDIs).

Conclusion: Hence, monitoring of prescriptions with polypharmacy is highly necessary in order to reduce the occurrence of DDI's and other drug related problems. In this aspect, Clinical pharmacist play an important role in healthcare system by assisting the physicians in dosage and duration adjustments, in discontinuation of unnecessary and inappropriate medications, in establishing a balance between risks and benefits of multiple drug therapies, thereby preventing the occurrence of DDIs, and thus improving the rationality of drug therapy, patients quality of life, decreasing the hospitalization of patients and health care cost burden to the patient and society.

INTRODUCTION

Polypharmacy is the main issue of patient safety in all healthcare settings in world wide.[1] Polypharmacy can be defined as the use of multiple drugs or increase in number of drugs that are medically necessary[2] or sometimes Polypharmacy, means the use of 7 or more than 7 drugs per day. The word Polypharmacy is derived from two Greek words 'Poly' meaning 'more than one' and Pharmacy meaning 'drug'. Polypharmacy is more common in older ambulatory care, hospital inpatients department, and nursing home patients. [2] Unfortunately, this increased use of multiple medications or Polypharmacy is associated with the increase of negative consequences such as increased risk of adverse drug reactions, Drug interactions, reduced functional capacity, poor quality of life and multiple geriatric syndromes. As a result of this negative consequences, Polypharmacy sets up a barrier to treatment adherence by creating a complex therapeutic regimens. Polypharmacy increases the risk of numerous negative consequences mainly in the elderly patients due to changes in biochemical composition of tissue, progressive decrease in physiological capacity, reduced ability to adopt to stimuli, increased susceptibility and vulnerability to disease and increased risk of death. [3, 2]

Polypharmacy is the main cause of many potential drug interactions in prescriptions in day to day practice. [4] A drug-druginteraction (DDI) occurs when one substance (usually another drug) alters the pharmacological effect of other drug when both administered simultaneously. The pharmacological effect of both the drugs may be increased (synergistic action) or decreased (antagonistic action) or a new or unanticipated adverse or toxic effect may be produced. Usually, in hospital

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or in clinical settings drug interactions not only exist with the drugs (drug-drug interactions) but also with the food products (drug food interactions)**.** Drugs interact in two major ways. Pharmacokinetically and pharmacodynamically. A pharmacokinetic drug-drug interaction occurs when one drug causes a change in other drugs serum concentration by altering its absorption, distribution, metabolism or elimination. A pharmacodynamics drug-drug interaction (interaction at drug receptors) occurs when the clinical effect is altered by administering the two drugs concomitantly such as those yielding synergistic or antagonistic therapeutic effect and those yielding overlapping or additive toxicities.[5]

The incidence of drug-drug interactions increases exponentially as the number of drugs co-prescribed increases for a individual patient.[4,6] Critically ill or chronically ill patients are mainly at increased risk of drug-drug interactions due to polypharmacy as well as impaired homeostatic mechanisms. [6]

By using Micromedex 2.0® software, drug.com and STOCKLEY's book, Potential drug-drug interactions were categorized into different levels as follows:

Onset

- Rapid: The effect of interaction occurs within 24 hours of administration.
- Delayed: The effect occurs if the interacting combination is administered for more than 24 hours i.e., days to week(s)
- Unspecified: The occurrence of effect of interaction is not specified.

Severity

- Contraindicated: The drug-combination is contraindicated for concurrent use.
- Major: There is risk of death and/or medical intervention is required to prevent or minimize serious negative outcomes.
- Moderate: The effect of interaction can deteriorate patient's condition and may require alteration of therapy.
- Minor: Little effects are produced that don't impair therapeutic outcome and there is no need of any major change in therapy. [4]

Polypharmacy and its negative consequences like drug interactions imposes a healthcare cost burden on both the patient and the health care system. [2]

Incidence of DDIs is estimated to vary from 6%-30% in hospitalized patients which leads to an increase on risk to the patients health outcomes and an increase in health care cost burden to patient and healthcare system.[7]

The probability of DDIs increases to 50% in patients taking 5-9 medications where as the risk/probability increases to 100% in patients taking 20 or more medications.[2]Therefore, based on the above incidence and prevelance of DDIs ,we hypothesis the need for careful monitoring of prescriptions with polypharmacy.

Prescriptions containing polypharmacy can be accepted as long as they are clinically appropriate or meet the criteria for the need of the patient condition, but monitoring is required for the occurrence of drug related problems.[4] .This creates the need of adopting some successful interventions to optimize prescribing multiple drugs or polypharmacy.[3] One of the most best intervention for improving/optimizing polypharmacy is involving an interprofessional approach which often includes a clinical pharmacist.[2]

Therefore, the clinical pharmacist have a potential role in the healthcare setting in assisting medical practitioner / physician in altering or reducing the number of medicines taken, in reducing the frequency of doses to be taken, reducing the no: of ADRs, DDI's, improving health related quality of life and decreasing the health care cost burden on the patient and health care system.[4]

Thus, the purpose of the present study was to conduct a broader integrated review aimed at identifying and summarizing studies examining drug-drug interactions in patients who are polymedicated.

Aims and Objectives

- To study the incidence and pattern of drug-drug interactions in our hospital and to identify whether the drug interactions are associated with polypharmacy
- To identify and minimise preventable drug-drug interactions as far as possible.
- To minimise health care cost burden on patients and health care system.
- To improve the patients quality of life
- To improve the rationality of drug therapy
- To decrease the hospitalization of patients.

MATERIALS AND METHODS

Place of the study

The study was conducted in the various departments (General medicine, Psychiatry, Neurology, Diabetology, Cardiology) of a territory hospital, Vishwa Bharathi superspeciality hospital, Kurnool, after obtaining the ethical clearance.

Duration of the study

The study was conducted for a period of 6 months during September 2016 to Feb 2017

Study population

Patients prescribed with seven or more than seven drugs from all the departments of hospital

Sample size

60 Patients

Study design

An Interventional and observational study

Patients Eligibility criteria

Patients are enrolled in the study based on inclusion and exclusion criteria.

Inclusion Criteria

- Patients of both gender from all the departments (General medicine, psychiatry, neurology, diabetology, and cardiology) of hospital receiving seven or more than seven drugs were included.
- Both new and old patients who are on polypharmacy.
- Patients from both inpatient and outpatient departments.

- Patients who are willing to participate in the study

Exclusion Criteria

- The patients from departments other than those mentioned in inclusion criteria.
- Pregnant and lactating females, Paediatrics.
- Cases of therapeutic failure: intentional or accidental poisoning, with a history drug abuse.
- Patients taking oncological drugs, ointments (topical medications) eye drops, vitamin supplements, mineral supplements, homeopathic medications and herbal treatments.
- Patients who are not willing to participate in the study.

METHOD OF STUDY

Sources of Data

A separate data entry format was specially designed including the drug chart and Pharmacist intervention column. The data was collected during routine ward rounds from various sources such as patient's case sheets, treatment charts and patient interviews and/or care takers interviews and transferred to specially designed data entry format for evaluation

Methods of detecting drug interactions

Patient who met the study criteria were included in the study.The first step in the detection of Drug interactions is collection of data. The data to be collected include patient's demographic data; presenting complaints; past medication history; drug therapy details including current medications and medication on admission; and lab data. All the cases were reviewed retrospectively and monitored extensively for the pattern of drug uses like their category, indication, and rationality of the prescription, concurrent drugs prescribed and/or number of drugs in prescriptions. Drug-drug interactions were identified and documented by using MICROMEDEX 2.0® software. Potential drug-drug interactions were categorized on the basis of gender, age, severity, department, number of DDIs per prescription.

RESULTS AND DISCUSSION

As per the patient demographic data obtained, a total of 60 patients prescriptions (35males (58.33%) and 25(41.77%) females) were included in this study. Out of 60 patients, 27 patients (9 females (33.33%) and 18(66.66%) males) were found to have drug-drug interactions in their prescriptions. A total of 102 DDIs were identified from 27 prescriptions.

In this study, a maximum of 19 patients belongs to an age group of above 60 years followed by 12 patients belongs to an age group of 51-60 years .This data shows that , geriatric patients are more prone to diseases, for which multiple drugs will be prescribed.

Age wise distribution of DDIs

Table 1 Age wise distribution of DDIs

Age group	No of prescriptions	No of prescriptions with DDIs	Total number of DDIs	% of DDIs
11-20	3	1	1	0.9803
21-30	6	4	10	9.803
31-40	10	0	0	0
41-50	10	4	25	24.50
51-60	12	5	12	11.76
>60 yrs.	19	13	54	52.94
Total	60	27	102	100

A maximum of 54(52.94%) DDIs were found in patients belonging to age group of above 60 years followed by 25(24.50%) drug- drug interactions among patients belonging to age group of 41-50 years and then 12(11.76%) drug interactions among patients belonging to age group of 51-60 years. The results of our study are in correlation with results of the study conducted by Kumar Swamy R.C., Jignesh U Ramani and Bushipaka Ramesh, *et al*. in which a high incidence of DDIs (175) were found in patients belonging to age group of >60 years followed by 160 DDIs in patients of 41-60years age group. [7]

This data suggests that geriatric patients are more prone to diseases and may be having concomitant disease conditions for which multiple drugs will be prescribed and thus making the patient more prone to DDIs.

Gender wise distribution of Drug-drug interactions

Table 2 Gender wise distribution of Drug-drug interactions

Gender	No of patients prescriptions	Number of prescriptions with DDIs	No of DDIs	% of DDIs
Female	25	9	28	27.45
Male	35	18	74	72.54
Total	60	27	102	100

Among 102 Drug interactions, a high incidence of 74(72.54%) Drug-drug interactions were found in male population and 28(27.45) drug-drug interactions were found in female population.

Severity of Drug-drug interactions

Based on severity, Drug-drug interactions were classified into minor, moderate and major. Minor drug interactions will be having very little clinical effect and hence not considered in this study. Only major and moderate drug interactions were considered in this study.

Table 3 Severity of Drug-drug interactions

Severity	Number of drug-drug interactions	% of drug interactions
Major	61	59.80%
Moderate	41	40.19%
Total	102	100

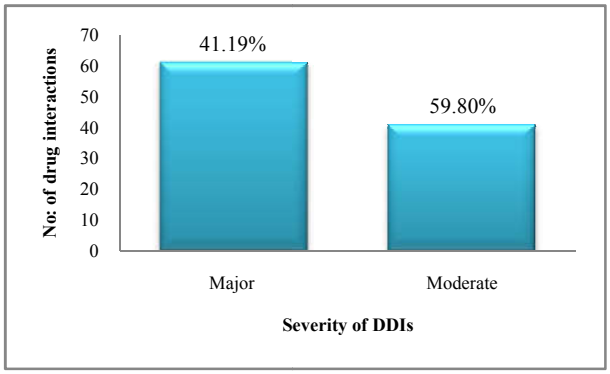

Figure 1 Severity of Drug-drug interactions

In this study we found that most of the patient prescriptions were having major drug-druginteractions. A total of 61(59.80%) major DDIs were found followed by 41(41.19%) moderate DDIs. Thus this discrepancy in prevalence may be due to patient's expectation and demand of quick relief, incorrect diagnosis and the influence of the attractive

promotional programs by the pharmaceutical companies. A study by Kumar Swamy R.C., Jignesh U Ramani, Bushipaka Ramesh, etal.Showed 155 moderate DDIs, 123 major DDIs and 122 minor DDIs whose results are in contrast to our study[7]. A study by Nimmy N. John, R.H. Udupi and K.M.Binu on Incidence of Polypharmacy induced DDIs in a tertiary care hospital showed a high number of moderate DDIs (66.2%) followed by 20.1% of minor DDIs and 13.63% of major DDIs. [6]A study by Ashok Kumar Malpani, RiyazMiya showed a high number of 37 moderate DDIs, 5 major DDIs and 23 minor DDIs. [8]

Department wise distribution of drug-drug interactions

Table 4 Department wise distribution of drug-drug interactions

Department	No of drug-drug interactions	% of drug-drug interactions
Cardiology	32	31.37
Neurology	27	26.47
General medicine	28	27.45
Pulmonology	4	3.92
Nephrology	7	6.86
Gynecology	4	3.92
Total	102	100

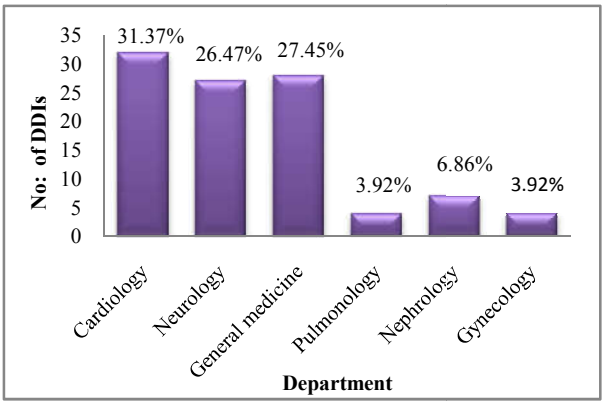

Figure 2 Department wise distribution of drug-drug interactions

Out of 102 drug-drug interactions, a majority of 32(31.37%) drug-drug interactions were from cardiology department followed by 28 drug interactions from general medicine and 27 drug-drug interactions were from neurology department.
This data confirms that patients admitted in cardiology department have high exposure to polypharmacy and thus are more prone to drug interactions.

The results of our study are in correlation with the results of the study conducted by Nimmy N. John, R.H. Udupi, and K.M. Binu who reported a high incidence of 38 DDIs from Pulmonology department followed by 34 DDIs from Gastroenterology department, 29 DDIS from Cardiology department and 14 DDIs from Neurology department. [6]

Effect of drug-drug interactions on P^K and P^D parameters

Table 5 Effect of drug-drug interactions on $P^{K \text{ and}}$ P^D parameters

Effect of DDIs	No of DDIs	% of DDIs
On Pharmacokinetics	2	1.96
On Pharmacodynamics		
• Drug action increased	68	66.66
• Drug action decreased	30	29.41
• Either increased or decreased	2	1.96
Total	102	100

Drug-drug interactions can alter or have effect on P^K and P^D of interacting drugs. In this study, 2(1.96%) drug-drug interactions were found to alter the pharmacokinetics of interacting drugs. 100 (99.4%) DDIs were found to alter (either increase or decrease the action of drugs) pharmacodynamics of interacting drugs.

Distribution of drug-drug interactions based on management

Management of drug-drug interactions is very important in order to improve the patient's quality of life, to decrease the adverse effect of drug-drug interactions on patients, to decrease cost burden due to drug interactions to the patients to society, to improve rationality of treatment

Table 6 Distribution of drug-drug interactions based on management

Management of drug-drug interaction	No of drug-drug interactions	% of drug-drug interactions
By close monitoring	52	50.98
By changing duration of drug administration	44	43.13
By drug discontinuation	1	0.98039
By using alternative drug	5	4.901
Total	102	100

A majority of 52(50.98%) drug-drug interactions were managed by close monitoring of either vitals, electrolytes, ECG, laboratory parameters of patients. About 44(43.13%) drug-drug interactions were managed by changing the duration of administration of interacting drugs. only 1 drug-drug interaction was managed by discontinuation of drug due to serious issue of major bleeding by concomitant administration of enoxaparin and NSAID (diclofenac)

Number of drug-drug interactions per individual patient

Around 6 patients were found to have at least 1 potential to drug-drug interaction, followed by 3 patients with 2 drug-drug interactions, 5 patients with 3 drug-drug interactions , 6 patients with 4 drug-drug interactions , 2 patients with 6 drug-drug interactions , 2 patients with 7 drug-drug interactions and 2 patients with 10 drug-drug interactions.

Drug-drug interactions encountered in the study

Clopidogrel+ Aspirin (8 DDIs), Clopidogrel+ Rabeprazole (5 DDIs), Amlodipine+ Clopidogrel (7 DDIs) are most commonly encountered major DDIs in this study. Atorvastatin+ Clopidogrel (9 DDIs) are the more commonly encountered moderate DDIs in the study.A study by Kumar Swamy R.C., Jignesh U Ramani, Bushipaka Ramesh, et al. showed Ofloxacin+ On dansetron (31DDIs), Metformin+Ofloxacin (19 DDIs) as the most common major DDIs and Azithromycin+Ondansetron (43 DDIs), Metronidazolr+Theophylline (37 DDIs) as the most common moderate DDIs. [7]

Aspirin (Antiplatelet), Clopidogrel (Antiplatelet), Atarvostatin (Dyslipidemic), Rabeprazole (Antacid), Ondensetron (5 -HT receptor antagonist), Ofloxacin (antibiotic) are most common drugs involved in drug drug interactions.

Severity of DDIs	Interacting Drugs	Class of Interacting Drugs	Mechanism of DDI	Effect of DDI		Management of DDIs	Frequency of DDIs	% of DDIs
				On Pharmacokinetics(P^K)	On Pharmacodynamics(P^D)			
Major	Oflaxacin +Ondensetron	Quinolones+ 5 -HT receptor antagonist	Increased Q-T interval prolongation	-	Increased	Closely monitor of ECG	3	2.94%
Major	Aceclofenac +Furosemide	NSAIDs +Diuretic	Decreased diuretic effect and possible nephrotoxicity	–	Decreased	Avoid concomitant use or change duration of drugs administration.	1	0.98%
Major	Aceclofenac+ Spironolactone	NSAIDs +Diuretic	Decreased diuretic effect and possible nephrotoxicity	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Aceclofenac+ Aspirin	NSAIDs+ Antiplatelet	Increased risk of bleeding	–	Increased	Monitor blood count	1	0.98%
Major	Aceclofenac+Clopidogrel	NSAIDs+Antiplatelet	Increased risk of bleeding	–	Increased	Monitor blood count	1	0.98%
Major	Amlodipine+Clopidogrel	Antihypertensive+Antiplatelet	Increased risk of thrombotic events and decreased anti platelet effects	–	Increased	Avoid concomitant use or change duration of drugs administration	7	6.86%
Major	Aspirin+Furosemide	Antiplatelet +Diuretic	Reduced diuretic effect and possible nephrotoxicity	–	Decreased	Monitor renal function test	2	1.96%
Major	Artemether/Lumefentrine +Ondansetron	Anti malarial +5 HT receptor antagonist	Increased Q-T interval prolongation	–	Increased	Closely monitor ECG	1	0.98%
Major	Aspirin+ Enoxaparin	Antiplatelet +Anticoagulant	Decrease the risk of major bleeding events	–	Increased	Discontinuation of an NSAID prior to initiation of LMWH therapy	1	0.98%
Major	Aspirin+Eplerenone	Antiplatelet+Diuretic	Decreased diuretic effect and possible nephrotoxicity	–	Decreased	Monitor renal function test	1	0.98%
Major	Aspirin+Piracetam	Antiplatelet +Cognitive Enhancers	Increased risk of bleeding	–	Increased	Monitor blood count	1	0.98%
Major	Aspirin+Spironolactone	Antiplatelet +Diuretic	Reduced diuretic effect and possible nephrotoxicity	–	Decreased	Monitor renal function test	1	0.98%
Major	Aspirin+Telmisartan/ Hydrochlorothiazide	Antiplatelet +Antihypertensive	Increased risk of bleeding	–	Increased	Monitor blood count	1	0.98%
Major	Atarvostatin +Diltiazem	Antiplatelet +Calcium channel blocker	Increased risk of rhabdomylosis	–	Increased.	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Ciprofloxacin+Insulin	Quinolones + Antidiabetic	Increased risk of thrombosis	–	Increased.	Avoid concomitant use or change duration of drugs administration	2	1.96%
Major	Ciprofloxacin+ Ondansetron	Quinolones + 5 HT receptor antagonist	Increased risk of QT interval prolongation	–	Increased.	Closely monitor ECG	1	0.98%
Major	Clopidogrel +Aspirin	Antiplatelet+Antiplatelet	increased risk of bleeding	–	Increased.	Monitor blood count	8	7.84%
Major	Clopidogrel+Dilitiazem	Antiplatelet +Calcium channel blocker	Decreased anti platelet effect and increased risk of thrombotic events	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Clopidogrel+Enoxaparin	Antiplatelet +Anticoagulant	Increase the risk of bleeding	–	Increased	Monitor patients closely for signs or symptoms of bleeding	1	0.98%
Major	Clopidogrel+ Rabeprazole	Antiplatelet +H2 receptor blocker	Increase the risk of thrombosis	–	Increased	Use of H2 blockers	5	4.90%
Major	Clopidogrel+Piracetam	Antiplatelet +Cognitive Enhancers	Increased risk of bleeding	–	Increased	Monitor blood count	1	0.98%
Major	Aspirin+Heparin	Antiplatelet +Anticoagulant	Increased risk of bleeding		Increased	Monitor blood count	2	1.96%
Major	Dexemethasone+ Diclofenac	Corticosteroids+NSAIDs	Increased risk of GIT ulcer bleeding		Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Diclofenac +Naproxen	NSAID+ NSAID	Increased risk of bleeding		Increased	Monitor blood count	1	0.98%

Major	Domperidone+ Ondensetron	Antiemetics+5 -HT receptor antagonist	Increased Q-T interval prolongation		Increased	Closely monitor ECG	2	1.96%
Major	Eplerenone+Metoprolol	Diuretic +Beta blocker	Result in hyperkalemia		Increased	Monitor electrolytes	1	0.98%
Major	Escitalopram+Ofloxacin	Antidepressant +Quinolones	Increased Q-T interval prolongation		Increased	Closely monitor ECG	1	0.98%
Major	Furosemide+Aspirin	Diuretic +Antiplatelet	Decreased diuretic effect and possible nephrotoxicity		Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Heparin+Clopidogrel	Antiplatelet +Anticoagulant	Increased risk of bleeding	–	Increased	Monitor blood count	3	2.94%
Major	Heparin+Nitroglycerin	Antiplatelet +Antianginal	Decreased in partial thromboplastintime	–	Decreased	Careful monitoring of partial thromboplastin time	1	0.98%
Major	Insulin +Oflaxacin	Antidiabetic +Quinolones	Increased risk of hypo or hyperglycemic effect	–	Increased	Monitor blood glucose levels	1	0.98%
Major	Labetalol+Diltiazem	Anti hypertensive +Calcium channel blocker	Increased risk of hypotension ,bardycardiaand conduction disturbances	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Metaprolol+ Diltiazem	Beta blocker + Calcium channel blocker	Increased risk of hypotension ,bardycardiaand conduction disturbances	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Major	Metronidazole +Ofloxacin	Nitroimidazole+ quinolones	Increased Q-T interval prolongation	–	Increased	Closely monitor ECG	2	1.96%
Major	Metronidazole +Ondensetron	Nitroimidazoles +5 - HT receptor antagonist	Increased Q-T interval prolongation	–	Increased	Closely monitor ECG	1	0.98%
Moderate	Aspirin+ Sodium bicarbonate	Antiplatelet +Gastric acid neutralizers	Decreased aspirin effectiveness	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Aspirin+ Insulin	Antiplatelet +Antidiabetic	Increased risk of hypoglycemia	-	Increased	Monitor blood glucose levels	3	2.94%
Moderate	Aspirin+Metoprolol	Antiplatelet +Beta blocker	Increased Blood pressure	–	Increased	Monitor Blood pressure	2	1.96%
Moderate	Aspirin+Nitroglycerin	Antiplatelet +Antianginal	Increase in nitroglycerin concentration and additive platelet function depression	–	Increased	Avoid concomitant use or change duration of drugs administration	2	1.96%
Moderate	Aspirin+Ramipril	Antiplatelet +Antihypertensive	Decreased ramipril effectiveness	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Aspirin+Sorbitrate	Antiplatelet +Antianginal	Increased nitroglycerin concentration and additive platelet function	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Atenolol +Glimepiride	Beta blocker + Anti diabetic	Result in hypoglycemia or hyperglycemia	–	Increased or Decreased	Monitor blood glucose levels	1	0.98%
Moderate	Atenolol +Metformin	Beta blocker + Anti diabetic	Result in hypoglycemia or hyperglycemia	–	Increased or Decreased	Monitor blood glucose levels	1	0.98%
Moderate	Atarvostatin + Clopidogrel	Hypolipidaemic+ Antiplatelet	Decreased formation of Clopidogrel active metabolite resulting high on treatment platelet reactivity	Decreased	–	Avoid concomitant use or change duration of drugs administration	9	8.823
Moderate	Dexemethasone+ Phenytoin	Corticosteroids+ Antiepileptic	Decreased Dexomethasone effectiveness		Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Hydrocortisone +Phenytoin	Corticosteroid +Antiepileptic	Decreased hydrocortisone effectiveness		Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%

Moderate	Insulin+Telmisartan/Hydrochlorothiazide	Antidiabetic +Antihypertensive	Increased risk of hypoglycemia		Increased	Monitor blood glucose levels	2	1.96%
Moderate	Labetalol+Aspirin	Antihypertensive +Antiplatelet	Increased blood pressure		Increased	Avoid concomitant use or change duration of drugs administration	2	1.96%
Moderate	Lorazepam+Telmisartan	General Anaesthetic +Antihypertensive	Decreased BP	–	Decreased	Monitor BP	1	0.98%
Moderate	MemantineHcl+Sulfamethaxazole	Cognition Enhancers +Sulfonamide	Increased memantine effect	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Metronidazole +Phenytoin	Nitroimidazole+ Anti-epileptic	Increased risk of phenytoin toxicity	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Midazolam+Ranitidine	General Anaesthetic +H2 receptor blocker	Increased midazolam bioavailability	Increased	–	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Midazolam+ Theophylline	General Anaesthetic +Bronchodilator	Decreased benzodiazepine formation	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Ondensetron+ Magnesium Hydroxide	5 -HT receptor antagonist +Antacids	Increased heart rhythm	–	Increased	Closely monitor ECG	1	0.98%
Moderate	Phenytoin+ Sulfamethaxazole	Anti epileptic +Sulfonamide	Increased phenytoin exposure	–	Increased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Quinine-+Magnesium Hydroxide	Antiarrhythmic +Antacid	Increased the risk of irregular heart rhythm	–	Increased	Closely monitor ECG	1	0.98%
Moderate	Quinine+Ondansetron	Antiarrhythmic + 5 -HT receptor antagonist	Increased the risk of irregular heart rhythm	–	Increased	Closely monitor ECG	1	0.98%
Moderate	Ramipril+Aspirin	Ramipril+Aspirin	Decreased ramipril effectiveness	–	Decreased	Avoid concomitant use or change duration of drugs administration	1	0.98%
Moderate	Tramadol+Ondansetron	CNS Analgesic+ Opioid Analgesics	Decreased the effect of Tramadol	–	Decreased	Avoid concomitant use or change duration of drugs administration	3	2.94%
			Total				102	100%

CONCLUSION

Prescription of more drugs i.e., polypharmacy to treat multiple comorbid conditions (or) to gain quick patient satisfactorial response and prescription of additional drugs to treat side effects increases the risk of drug- drug interactions. Our study concluded that a high percentage of DDI were found in patients of age group >60 years and whereas distribution of drug-drug interactions based on severity showed many major drug-drug interactions (59.80%). Further the study concluded that cardiology department followed by General medicine department are more prone to polypharmacy, so more drug-drug interactions were noted in those departments. Hence, monitoring of prescriptions with polypharmacy is highly necessary in order to reduce the occurrence of DDI's and other drug related problems. In this aspect, Clinical pharmacist play an important role in healthcare system by assisting the physicians in dosage adjustments, in discontinuation of unnecessary and inappropriate medications, in establishing a balance between risks and benefits of multiple drug therapies, thereby preventing the occurrence of DDIs, ADRs and thus improving the rationality of drug therapy, patients quality of life, decreasing the hospitalization of patients and health care cost burden to the patient and society.

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A CASE REPORT OF NEUROLOGIC MANIFESTATIONS AFTER MULTIPLE BEE STINGS

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ABSTRACT

Honeybees are very common flying insects, encountered in daily life and accidents associated with them are also equally frequent. The sting venom mostly causes local anaphylactic reactions, and rarely shows neurological symptoms. We report a case of such rare reaction in a 48 yr old diabetic male patient with complaints of aphasia, lower limb weakness and cough, after 12 hours of honey bee attack. In the CT-scan acute multi focal infarcts were observed, confirming the condition to be ischemic stroke. The treatment included mannitol, aspirin, atorvastatin for ischemic stroke along with symptomatic therapy for respiratory problem. The patient was discharged from the hospital after his limb monoparesis gradually improved and advised to visit after 2 months for further follow up. The systemic immune mediated reactions, along with bee venom toxins like mellitin & phospholipase mediated intravascular coagulation effect might be the possible mechanism contributing to cerebral ischemia.

KEYWORDS: ***Bee sting, Infarcts, neurological symptoms, stroke***

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INTRODUCTION

Bee's envenomation is mostly seen as occupational hazards in farmers, tree dwellers and honey collectors.[1] The most common symptoms include signs of anaphylactic reaction like local and generalized allergic reactions like oedema, erythematic reaction, burn-like sensation, pruritus, urticaria, along with hypotension, vomiting, diarrhoea. Bee stings also produce severe manifestations like anaphylactic shock, arrhythmias like atrial fibrillation, myocardial infarction, renal failure, and pulmonary haemorrhage.[2]This emphasizes the need for reporting such neurological complications, which in turn would lead to a better therapeutic planning.

CASE REPORT

A 48 yr old diabetic male patient was presented in the neurology ward with the chief complaints of aphasia, lower limb weakness, along with cough and fever. He had a history of honey bee sting bite all over the body 12 hours prior the admission. He was treated as an outpatient with sting extraction, intravenous antihistamines and steroid at the local hospital. Patient was having a medical history of type 2 diabetes mellitus since 5years and was under tablet Glimepride 2mg + Metformine 500mg dose twice daily. His personal history and habits include alcoholism (occasionally) and mixed type of diet. On examination he was conscious and oriented but his blood pressure showed hypotension signs as noted in table 1.

Table 1
Patient's vital signs

Vital signs (units)	Day-1	Day-2	Day-3
Temperature (^{0}f)	100.6	99.6	98.6
BP (mm of hg)	90/60	110/80	110/60
PR(bpm)	82	84	84
RR(/min)	24	24	20

BP= Blood Pressure; PR= Pulse rate; bpm= Beats per minute; RR= Respiratory rate.

All his lab investigations showed normal reports except his elevated postprandial sugar (165 mg/dl) and elevated direct serum bilirubin levels (0.3 mg/dl). ECG and Echo cardiography was observed to be within normal range. Fundus examination revealed a normal study in both the eyes. CT brain showed acute multiple focal infarct in left fronto-parietal and adjacent sub cortical deep phernic white matter, along with left hemorrhagic nucleus & focal hemorrhagic transformations noted at left pre central and post central gyri. The Doppler study gives the conclusion of right common carotid artery atherosclerotic plaque and left soft plaque. The treatment was given to the patient for five days, with drugs like mannitol 20% three times daily dose for his cerebral oedema,tablet aspirin (300mg once daily dose) and tablet atorvastatin (40mg three times daily dose) as a drug of choice for ischemic stroke patients. Both oral hypoglycaemic and parenteral insulin was administered for his elevated sugar levels. Symptomatic therapy was given for fever and cough including *Deletus D* Syrup, composed of the following active ingredients like Phenylephrine(5 MG), Dextromethorphan(10 MG), Triprolidine(1.25 MG)at three times daily dose. An antibiotic Gemtrex, containing ceftriaxone 1 gm twice daily dosing was also administered. Over this five days of therapy the patients showed gradual improvement in his left limb monopheresis and advised to visit after 2 months for further follow up.

DISCUSSION

Honey bees are common insects in rural India and farmers regularly encounter accidents associated with them.[2] A single bee sting can inject around 0.33mg of venom, which is a mixture of amines, peptides and enzymes that can trigger cell injury instantaneously.[2-3] In usual cases a local anaphylactic reaction and its associated signs & symptoms are commonly seen with bee sting attack. But along with local symptoms, systemic complications are also observed in several cases especially cough, diarrhoea, vomiting, dysphagia, angina, hypotension, syncope, respiratory failure, convulsions, unconsciousness and even death. Loss of vision is also observed in few cases where a direct sting attack to eye was encountered.[2-5]Along with the above mentioned consequences, cerebral infarctions or neurological manifestations are rare phenomenon, which are mostly associated with multiple bee sting bites. Literatures supporting these symptoms are very few, emphasizing the need to report our case for better approach to treat the future complications. In our case the patient was observed with neurological symptoms like aphasia and monoparesis along with systemic symptoms like hypotension and cough. Seven case comparison done by Rajendiran.C et.al, also reported similar results .[4] The incidence of symptoms are also varying from <1 hour to one day later, and the patient observed for the present study was admitted 24 hrs later with neurological signs.[4] Complete eye checkup is one parameter missed in many studies, but in our case a full fundus examination was done and found to be normal. Seven case reports were mentioned in literature presenting neurological manifestations along with renal failures, optic neuropathy, rhabdomyolysis and other.[1,4] A case report by rajendiran C et al., also represented similar report,where infarcts in right fronto parietal and occipital regions were evident in his CT report along with hemorrhagic transformation.[4] In our case also patient's CT presented multiple focal infarct in left fronto-parietal and adjacent sub cortical deep phernic white matter, along with left hemorrhagic nucleus & focal hemorrhagic transformations. Different mechanisms were postulated in previous case reports for the underlying cause, but in this patient the reason can be due to the following

a) Hypotension caused by anaphylaxis may certainly induce cerebral and optic nerve ischemia [1,2,4]

b) The systemic immune mediated reactions was postulated as one of the cause for occurrence of stroke and the mechanism involved in this was toxins present in bees venom like mellitin & phospholipase, along with inflammatory mediators resulted in intravascular coagulation leading to vasoconstriction. The details are depicted in figure 1.

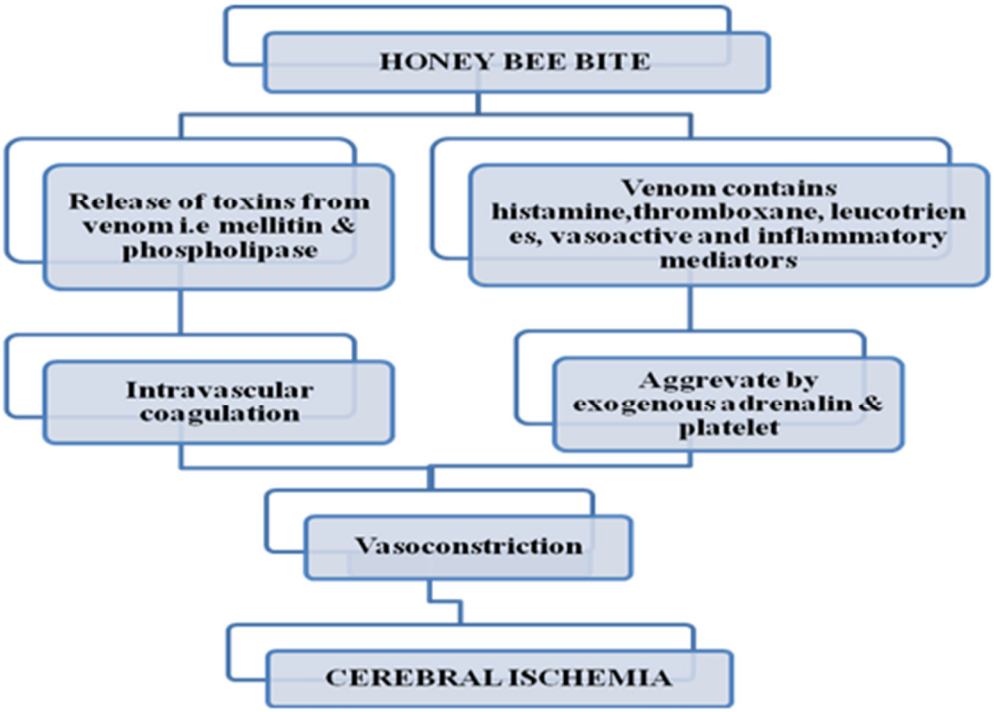

Figure 1
Illustration of systemic immune mediated mechanism of bee sting leading to ischemic stroke [1,2 4 6-10]

CONCLUSION

Thus a patient presented with severe multiple bee stings, should be carefully assessed for any onset of neurological signs and symptoms, especially in elderly patients or in those with risk for cerebrovascular accidents so as to improve the therapeutic outcome.

CONFLICT OF INTEREST

Conflict of interest declared none.

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Spironolactone Induced Gynecomastia: A Case Report

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Abstract: *Gynaecomastia is generally caused by increased ratio of free circulating oestrogens/androgens or altered effects of these hormones on their correspondent intracellular receptors in the mammary tissue. The pathologies influencing the levels of circulating sexual hormones (i.e. testicular or adrenal neoplasias, hepatic cirrhosis, hyperthyroidism hypogonadism obesity, refeeding syndrome. The active principles known for most frequently causing gynecomastia are exogenous oestrogens, antiandrogens, 5 alpha reductase inhibitors, spironolactone and cimetidine. Medical history plays a fundamental role in the diagnosis of drug induced gynecomastia. A large variety of drugs have been implicated in its pathogenesis and they may induce gynecomastia by decreasing testosterone production ,increasing peripheral conversion of testosterone to estradiol and displacing estradiol from sex hormone binding globulin. We present a case report of 41 old male patient affected by spironolactone induced gynecomastia and discuss its pathogenetic mechanism.*

Key Words: *Gynaecomastia, Spironolactone, Decreased Testosterone Production, Conversion of Testosterone to estradiol, Spironolactone induced Gynaecomastia, Drug induced Gynaecomastia*

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I. Introduction

Gynaecomastia is clinically defined as benign enlargement of male breast due to proliferation of glandular component with deposition of fat. Gynaecomastia is a well described adverse effect of spironolactone and is related to dose and duration of treatment.

Spironolactone induces gynecomastia by decreasing testosterone production, increasing peripheral conversion of testosterone to estradiol and displacing estradiol from sex hormone binding globulin.

A Large variety of drugs are known to cause gynecomastia among them Spironolactone are rarely reported. Here in we report a case of 41 years old male patient with Spironolactone induced Gynaecomastia.

II. Case Report

A 41 Year old male patient was referred to General Medicine Department Government General Hospital Kurnool with the chief complaints of fever, fatigue, black colored stools , swelling around umbilicus . He has similar complaints in the past since 1 yr known case of HBV related Decompensated cirrhosis of liver disease with portal hypertension with esophageal varices grade 3-2 column, Acute kidney injury shortness of breath recovered , known case of pulmonary TB one yr back. On physical examination He showed enlargement of male breast was present(GYNECOMASTIA) Medical History revealed that the patient had received Spironolactone 50mg/ day from 30 may 2018 for Decompensated cirrhosis of liver with portal hypertension and from 6 months there was slowly enlargement of breast was observed but no pain was appeared .

The patient had been taking spironolactone 50mg/day for 2yrs as a part of his medication regimen for Decompensated Cirrhosis of Liver Disease with Portal Hypertension.

The patient reported the chief complaints of fever, fatigue, black colored stools, swelling around umbilicus from 1 yr.

There was enlargement of male breast was also reported.

Based on the physical examination and on the relationship between the drug and onset of gynecomastia a diagnosis of drug induced Gynaecomastia was made.
Withdrawal of the culprit drug and short term tablet Inderol 40 mg/ day was given led to complete and permanent remission of the disease. Rechallenge was done to avoid unnecessary risk to the patient.

TABLE 1: DRUGS MOST FREQUENTLY INVOLVED IN DRUG INDUCED GYNECOMASTIA

1. P0TASSIUM SPARING DIURETICS : Spironolactone
2. CALCIUM CHANNEL BLOCKERS : Nifedipine
   Amlodipine
   Diltiazem
   Verapamil
3. ANGIOTENSIN CONVERTING ENZYME INHIBITORS : Captopril
   Enalapril
4. ALPHA RECEPTOR BLOCKERS : Doxazosin
   Prazosin
5. CENTRALLY ACTING AGENTS : Clonidine
   Methyldopa
   Reserpine
6. ANTIANDROGENS : Bicalutamide
   Flutamide
7. 5 ALPHA REDUCTASE INHIBITORS : Finasteride
   Dutasteride
8. H2 HISTAMINE RECEPTOR BLOCKER : Cimetidine
9. PROTEASE INHIBITORS OF ANTIRETRO VIRAL THERAPY : Saquinavir
   Lopinavir
10. ANTIPYSCHOTIC DRUG : Haloperidol
11. SEVERAL CHEMOTHERAPY DRUGS : Methotrexate
    Carmustine
    Etoposide
    Cytarabine
12. ANTIRETRO VIRAL DRUGS REVERSE TRANSCRIPTAS INHIBITORS: Stavudine
    Zidovudine
    Lamivudine
13. ENVIRONMENTAL EXPOSURE : Phenothrin
    anti parasitical
14. EXOGENOUS HORMONES : Oestrogens
    Androgens
15. ANTIFUNGAL DRUG : Ketoconazole
16. PROTON PUMP INHIBITORS : Omeprazole
17. 17. CARDIOVASCULAR DRUGS: Phytoestrogens
18. DRUGS RARELY CAUSING GYNECOMASTIA :

Amiod arone, Amphetamine, Aripiprazole, Atorvastatin, Captopril, Cetrizine, Clonidine ,Dasatinib Diazepam, Diethyl stilbestrol, Digoxin, Domperidone, Entecavir, Ethanol , Fenofibrate, Fluoxetine,Gabapentin, Heroin, Imatinib, Lisinopril, Loratadine, Marijuana ,Methadone, Metronidazole, Misoprostol, Paroxetine, Penicillamine, Pravastatin , Pregabalin, Ranitidine, Rosuvastatin, Sulindac, Sulpiride, Sunitinib,Theophylline, Venlafaxine .

III. Discussion

Gynecomastia is clinically defined as benign enlargement of male breast due to proliferation of glandular component with deposition of fat. Normally estrogen stimulates the proliferation of breast epithelial cells ,and androgens have an inhibitory. Gynecomastia usually results due to imbalance between actions of estrogens and androgen on the breast tissue .

The causes for gynecomastia can be either physiological (neonatal , pubertal or involutional) or pathological conditions (drug induced endocrine disorders such as testicular , adrenocortical or pituitary tumors , hyperthyroidism , and non-endocrine causes such as cirrhosis ,starvation , stress and renal failure .

Drugs associated with gynecomastia are bicalutamide , flutamide , nilutamide , leuprolide ,metronidazole ,ketoconazole ,isoniazide, minocycline, digoxin , spironolactone, amlodipine , nifedipine, verapamil, captopril, enalapril, amiodarone, methyldopa, minoxidil, methotrexate , vincristine, diazepam,

phenytoin, androgens, anabolic steroids, estrogen, theophylline, d- penicillamine, cimetidine , and metoclopramide.

Spironolactone does alter the peripheral metabolism of testosterone resulting in changes in the ratio of testosterone to estradiol which could contribute to the production of gynecomastia.

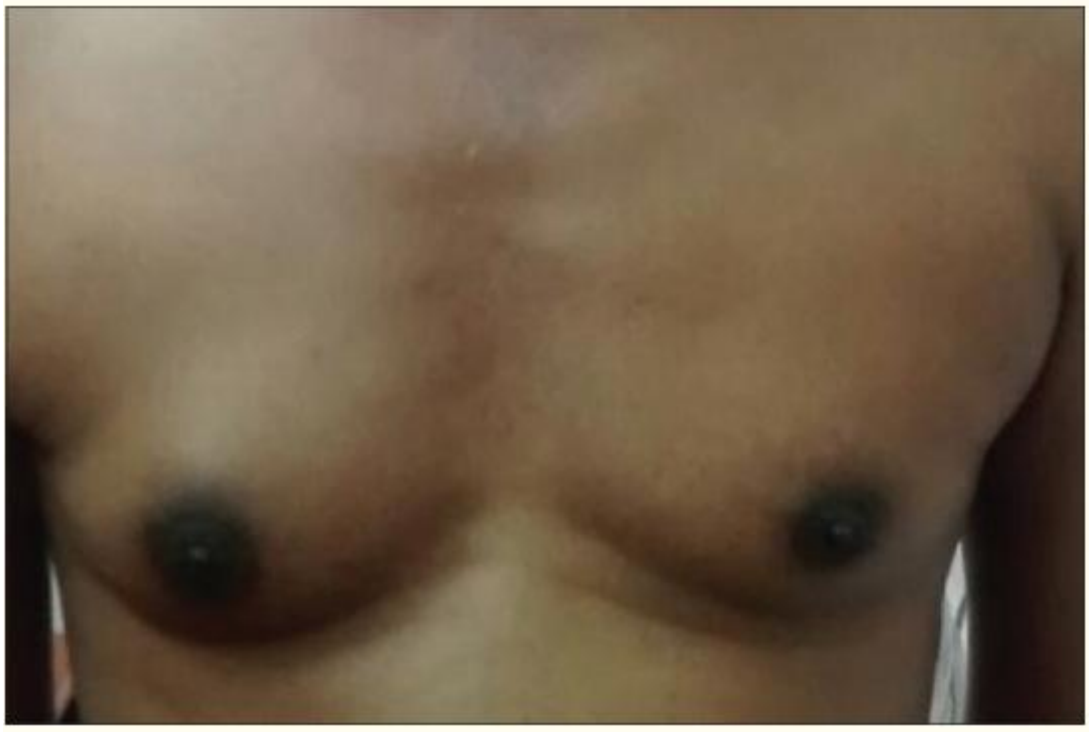
Spironolactone is a well known cause of gynecomastia and may act by displacing androgen from the androgen receptor and sexual hormone binding globulin and by causing increased metabolic clearance of testosterone and higher estradiol production.

The patients spironolactone was replaced with inderol that lowers the incidence of gynecomastia.

Spironolactone induces gynecomastia by blocking androgen production, by blocking androgens from binding to their receptors and by increasing both total and free estrogen levels.

Production of testosterone is decreased by inhibiting 17 alpha hydroxylase and 17, 20 –desmolase, which are enzymes in the testosterone synthesis pathway.

Oestrogens levels are increased by enhancing the peripheral conversion of testosterone to estradiol and by displacing estradiol from sex hormone binding globulin.



IV. Conclusion

Spironolactone causing bilateral gynecomastia is well established. Eliciting proper history and performing examination can result in correct diagnosis.

Stopping the offending drug resolves the problem and thereby can save the patient from embarrassment, anxiety, physical discomfort of investigations.

Patients should be informed about this side effect while prescribing this drug, and alternatively inderol can be used.

Physician should discuss about serious adverse drug reactions while prescribing a medication, if he get any adverse drug reaction he will discontinue the drug and consult the physician.

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Research Article

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FORMULATION AND EVALUATION OF TOPICAL GEL OF *RUBIA CORDIFOLIA* (MANJISTHA)

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ABSTRACT

The objective of the present study was to formulate and evaluate Topical gel containing Manjistha as a drug. A total of six gel formulations were prepared by homogenization technique using stearic acid, cholesterol, tween 80 and chloroform as excipients. Preformulation studies like UV spectrophotometry and FTIR were performed for the drug. Carbopol was used as a gelling agent in the preparation of these gels. Evaluation studies like pH, spreadability, viscosity, homogeneity and *in vitro* drug release studies were carried out. All the results were obtained to state that prepared gels have shown optimum results. Also, a gel containing stearic acid as a lipid (1:2) has shown maximum drug release compared to other gels.

Keywords: Manjistha, Cholesterol, Stearic acid, Topical gel.

INTRODUCTION

Rubia cordifolia (Rubiaceae) is also known as, Manjishtha, Indian madder, distributed throughout India[1]. It is found throughout the hilly districts of India from the northwest Himalayas eastwards, ascending to 8000 feet and southwards to Ceylon. The roots of this plant are of high medicinal value and are recognized as official[2]. This perennial herbaceous prickly creeper or climber is up to 10m long, found throughout the country ascending to 3750 m and grows well in light (sandy), medium (loamy) and heavy (clay) soils[3].

Rubiaceae comprises about 450 genera and 6500 species and includes trees, shrubs and infrequently herbs. *Rubia cordifolia* L. (Rubiaceae), also known as 'manjistha', it is a perennial, herbaceous climbing plant, with long roots, cylindrical, flexuous, and thin red bark. Stems often have a long, rough, grooved, woody base. Plants belonging to this the family contains substantial amounts of anthraquinones, especially in the roots [4].

The traditional therapeutic use of the plant has been for skin disorders and anticancer activity. Furthermore, the anthraquinones of the Rubiaceae family exhibit some interesting in vivo biological activities, such as anti-tumour, anti-inflammatory, urinary disorders, antistress antimicrobial, hepatoprotective, radioprotective, and anticancer, antimicrobial, antifungal, hypotensive, analgesic, antimalarial, antioxidant, antileukemic and mutagenic functions, immunomodulatory, anti-inflammatory and antioxidant[5].

Apart from its medicinal value, this plant has also been used as natural food colourants and natural hair dyes. The interest in the isolation of natural dyes and colouring, matters are increasing due to their applications in food, drugs and other human consumptions[6]. This plant has also been listed officially as herbal medicine in the Chinese Pharmacopeia for the treatment of arthritis, dysmenorrhea, hematuria and hemostasis, which are free radical related diseases. It has a variety of uses, such as a blood purifier. It is helpful in treating skin diseases, in blood purification, increasing appetite and in stimulation and contraction of the uterus [7].

Antimicrobial action can be characterized as an aggregate term for all active principles that inhibit the development of microscopic organisms, forestall the arrangement of microbial states, and may destroy microorganisms [7].

The primary classes of antimicrobial specialists are disinfectants, which kill a broad scope of organisms on non-living surfaces to forestall the spread of sickness, germicides (which are applied to living tissue and assist with decreasing contamination during a medical procedure), and anti-infection agents (which annihilate microorganisms inside the body). The expression "anti-microbial" initially portrayed just those details from living microorganisms yet is presently additionally applied to engineered specialists, like sulfonamides or fluoroquinolones [7].

Most, as of late, found antimicrobial specialists are altered regular mixtures, and this adjustment is made through substance mode, for instance: b-lactams (penicillin's), carbapenems, or cephalosporin. Unadulterated everyday items, for example, aminoglycosides, and other manufactured anti-toxins, for instance: sulfonamides, are likewise often utilized. The antimicrobial specialists could be named the specialists that can either be bactericidal, which kills microscopic organisms, or bacteriostatic, which delays the development of microorganisms. Antibacterial specialists are the main in battling irresistible sicknesses. However, with their wide use and misuse, bacterial obstruction toward antibacterial specialists has become a significant issue for the present drug industry. Opposition is most regularly founded on formative cycles, for instance, an anti-infection treatment that prompts inheritable resistance [8].

The topical delivery of drugs is an attractive method for local and systemic treatment and is commonly used in treating inflammatory conditions like musculoskeletal injuries and dermatological diseases. There are many advantages to the topical application compared to conventional dosage forms. Especially some systemic severe and adverse effects are avoided [8].

When the drug is delivered topically, it can penetrate deeper into the skin and give better absorption. Topical preparation can be used to prevent the metabolism of the drug in the liver. It can be used to avoid gastrointestinal disorders, risks and inconvenience of intravenous therapy etc. Furthermore, the bioavailability of the drug is increased, and targeted action can be achieved.

The topical delivery with gels can increase the time of presence of the drug on the skin and improve the delivery and release of the substance.

In the present study, an attempt has been made to prepare gels of Manjista using stearic acid and cholesterol as lipids separately.

MATERIALS AND METHODS

Manjistha power was procured from Chaitanya Agro Herbals, Mysuru, as a gift sample. Stearic acid, Cholesterol, Carbopol 934, Triethanolamine, Propylene glycol, and Tween 80 from various sources were of analytical grade.

Analytical method for Manjistha

Calibration curve in pH 6.8 phosphate buffer

A stock solution was prepared from the standard solution to give a 100 µg/ml concentration in ethanol. Aliquots of 0.5, 1.0, 1.5, 2.0 and 2.5 ml from the stock solution were pipetted into 10 ml volumetric flasks. These dilutions gave 5, 10, 15, 20 and 25 µg/ml concentrations of Manjistha. The absorbance of prepared solutions was measured at 540 nm spectrophotometrically against an ethanol blank. The Results and discussion chapter reported standard plot data of Manjistha in ethanol.

Phyto chemical Analysis

Qualitative analysis of phytochemicals

Preliminary phytochemical screening was performed for herbal extract. The presence of various phytochemicals was tested. Different phytochemicals present in both extracts were identified. Procedures and results obtained are given in Table 2. The results state that the extract contained amino acids, alkaloids, flavonoids, tannins, carbohydrates, glycosides, saponins, and triterpenoids. [9-11]

Fourier transform infrared (FT-IR) spectroscopic analysis

Fourier transform infrared analysis was conducted to verify the interaction between the drug and polymer. The sample powder was dispersed in KBr powder, and pellets were made by applying 4 kg/cm^2 pressure. FT-IR spectra were obtained by powder diffuse reflectance on an FT-IR spectrophotometer type 8400S Shimadzu. [12]

Gel Preparation

An appropriate quantity of carbopol 934 was soaked in water (around 5 ml) for 2 hours. Carbopol was then neutralized with triethanolamine (TEA) with stirring. Then specified drugs and lipids were dissolved in an appropriate and pre-weighed amount of propylene glycol. The solvent blend was transferred to a carbopol container and agitated for an additional 20 min. The dispersion was then allowed to hydrate and swell for 60 min; finally, the pH was adjusted with 98% TEA until the desired pH value was approximately reached (6.8-7). During pH adjustment, the mixture was stirred gently with a spatula until a homogeneous gel was formed. All the samples were allowed to equilibrate for at least 24 hours at room temperature before performing rheological measurements. The formulation chart of the gel is given in Table 1. The prepared gel formulation is shown in Figure 1. [12-14]

Evaluation studies

pH determination: The pH of the gels was determined using a digital pH meter by placing the glass electrode completely into the gel system. The readings were taken an average of 3 times. [15]

Homogeneity: All developed gels were tested for homogeneity by visual inspection after the gels had been set in the container. They were tested for the appearance and presence of any aggregates.

Spreadability test: Place 0.5 gm gel in a premarked circle (1cm diameter) on a glass plate. Another glass plate was placed over the gel, and a weight of 500 gm was placed over this upper glass plate for 5 min. The experiment was carried out in triplicate, and spreadability was expressed in gm.cm/sec. Spreadability can be calculated by using the formula.

$$S = M.L/T$$

Where S = Spreadability, M = Weight tied to upper slide, L = Length of glass slide, T = Time taken to separate the glass slide from each other.

Rheological studies: The rheological measurements were performed on the Brook field viscometer. All measurements were carried out using parallel plate measuring systems having 50 mm diameter and 1 mm gap at 25 ^{0}C. The rheological properties of the formulated gels were studied at different shear rates (rpm), and the viscosity was measured in cP

***In vitro* drug release studies:** The gel was permeated through the dialysis bag. An optimized formulation was selected for these studies. 0.5 gm of gel was placed in the bag in a beaker containing 150 ml phosphate buffer of pH 7.4 and constantly stirred with a small magnetic bead. During the experiment, the temperature was maintained at 37± 0.5 ^{o}C to simulate the human skin condition. 5 ml samples were withdrawn at 0.5, 1, 2, 6 and 12 h and replaced with fresh receptor solution. The samples drawn were analyzed spectrophotometrically at 540 nm. The amount of drugs released was calculated, and the percentage of drugs released was plotted against time.

Table 1: Formulation chart of various gel formulations

Formulation	Drug (mg)	Stearic acid (mg)	Cholesterol (mg)	Tween 80 (%)	Tri ethanolamine (TEA)	Propylene glycol	Carbopol 934	Distilled water
F1	50	50	_	1	1%	0.5 ml	0.5 gm	Q.S
F2	50	100	_	1	1%	0.5 ml	0.5 gm	Q.S
F3	50	150	_	1	1%	0.5 ml	0.5 gm	Q.S
F4	50	_	50	1	1%	0.5 ml	0.5 gm	Q.S
F5	50	_	100	1	1%	0.5 ml	0.5 gm	Q.S
F6	50	_	150	1	1%	0.5 ml	0.5 gm	Q.S

Table 2: Phytochemicals present in Ethanolic extract of Manjistha

Constituents	Test	Observation	Results
Alkaloids	Mayer's test	Formation of the creamy precipitate.	Present
Flavonoids	Lead acetate test	Formation of yellow precipitate	Present
Carbohydrates	Molisch's test	Formation of the violet ring at the junction.	Present
Triterpenoids and steroids	Salkowski test	The presence of steroids is confirmed if the lower layer turns red and that of triterpenoids by the Golden yellow layer at the bottom	Present
Deoxy sugars	Killer killani's test	Formation of blue colour in the acetic acid layer	Absent
Glycosides	Legal's test	Formation of pink to blood red colour	Present
Reducing sugars	Benedict's test	Depending on the amount of reducing sugar present in the test solution appears green or yellow, or red	Absent
Amino acids	Ninhydrin's test	Formation of blue colour	Present

Table 3: Evaluation studies of Formulations F1-F6

Formulation	pH	Spreadability	Viscosity
F1	5.6	0.494	15230
F2	6.2	0.370	14870
F3	5.4	0.492	14400
F4	5.8	0.395	15900
F5	6.0	0.370	15188
F6	5.8	0.518	14203

Table 4: *In vitro* drug diffusion studies (F1-F6)

Time	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0
0.5	3.55	6.75	3.46	0.57	8.09	3.99
1	10.68	17.31	13.80	7.35	19.83	11.62
2	22.42	35.21	32.59	20.57	35.95	27.07
4	45.72	56.91	52.64	42.83	56.73	48.21
6	71.52	81.46	75.43	69.27	79.07	73.14

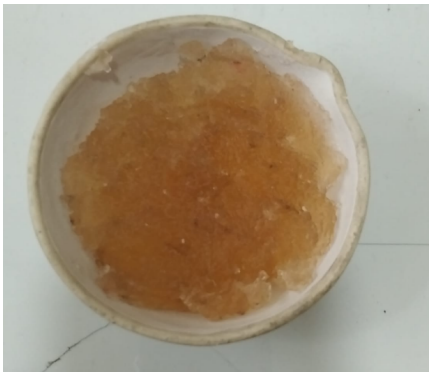

Figure 1: Topical Gel formulation

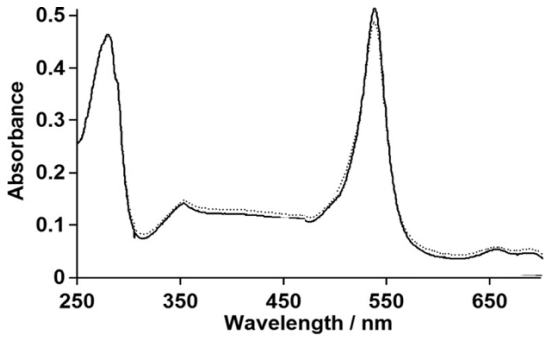

Figure 2: UV-Spectra of Manjistha in 6.8 pH buffer

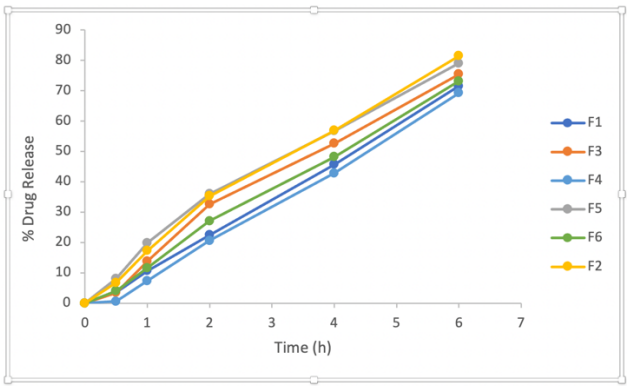

Figure 3: *In vitro* drug diffusion studies (F1-F6)

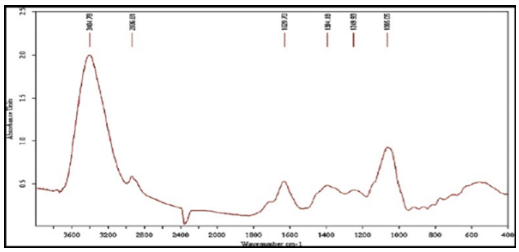

Figure 4: FTIR Spectrum of Pure Drug (Manjistha)

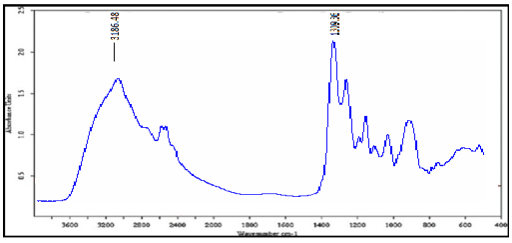

Figure 5: FTIR Spectrum of Formulation (F2)

RESULTS AND DISCUSSION

Calibration curve in 6.8 pH buffer: Standard plot data of Manjishtin in 6.8 pH buffer is reported in Figure 2.

Phytochemical Analysis

Qualitative analysis of phytochemicals: The extract contained amino acids, alkaloids, flavonoids, tannins, carbohydrates, glycosides, saponins, and triterpenoids. Results are given in Table 2.

Evaluation of gels

pH determination: The pH of the obtained gel was found to be between 5.8 to 6.9, which is near neutral pH. This shows prepared gel does not cause any skin irritation.

Homogeneity: All the prepared gels are clear and transparent. It shows that no aggregates were present.

Spreadability: The spreadability of all prepared formulations was calculated and found to be between 0.3 to 0.5 gm.cm/sec. From the results obtained, it was confirmed that all the prepared gels were spreading easily on the skin.

Rheological studies: Viscosities for prepared gels were between 14000-16000 cps approx. And the viscosities of all formulations were found feasible for topical drug delivery. All the viscosities obtained were almost near since no change in Carbopol quantity was done. The results are shown in Table 3.

***In vitro* drug diffusion studies**: *In vitro,* drug release studies were performed for all formulations. The results obtained in *vitro* release studies were plotted per cent cumulative drug release Vs time and shown in Figure 3. It was found that in the case of F1-F3 formulations where stearic acid was taken as lipid, the F2 formulation has shown maximum *in vitro* drug release and in the case of F4-F6 where cholesterol was the lipid used, F5 has full release. This could be due to the presence of the optimum amount of lipid in both cases. *In vitro* drug release studies of all the formulations are given in Table 4.

Drug compatibility study: Fourier- transform infrared spectroscopy (FTIR) spectra of pure drug and F2 formulation are presented in Figures 4 and 5, respectively.

FTIR spectroscopy was used to identify the functional groups of the active components present in Manjistha based on the peak values in the IR region. FTIR analysis of Manjistha has confirmed the presence of alcohol, phenols, amines, and carboxylic acids. The major IR stretching frequency at 3404 cm^{-1} was due to primary amines. The frequency at 2936 cm^{-1} was due to hydroxyl and aromatic C-H stretching frequency. The band at 1249 cm^{-1} and 1384 cm^{-1} were due to the >C=C< and CH_2 groups, respectively. This together indicates the presence of a carboxylic acid group. The absorbance at 1065 cm^{-1} was due to OH stretching, which suggests the presence of alcohols and phenols functional groups. This result shows that RC does not contain any fatal toxic substances.

FTIR spectrum of the pure drug in F2 Formulation spectra was intact without any significant deviations. Hence there is no interaction between drugs and excipients.

CONCLUSION

the pH of prepared gels was found to be between 5.8 to 6.9, and this indicates the gel causes no skin irritation. The viscosity and spreadability studies were performed, and the results obtained were within limits. *In vitro* drug diffusion studies were performed for prepared gels. Results stated that formulation F2 containing Drug: Lipid in 1:2 ratio has shown maximum *in vitro* drug diffusion compared to other formulations. The FTIR spectra observed that similar characteristic peaks appeared with minor differences in drug and formulation. This indicated no chemical interaction between the drug and the polymers used.

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Research Article

FORMULATION AND EVALUATION OF SOLID LIPID NANOPARTICLES CONTAINING CAFFEINE TO TREAT CLINICAL MASTITIS

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ABSTRACT

Objective: The objective of the present study was to formulate and evaluate caffeine loaded solid lipid nanoparticles (SLNs) in the treatment of clinical mastitis.

Methodology: These were prepared by homogenization technique using cholesterol, tween 80, and chloroform as excipients. Preformulation studies such as ultraviolet spectrophotometry, Fourier transform infrared (FTIR), and differential scanning calorimetry (DSC) were performed for the drug. Entrapment efficiency and *in vitro* dissolution studies were carried out for prepared SLN's and the optimum formulation (F2) was taken for further studies such as FTIR, DSC, scanning electron microscopy, particle size, and zeta potential analysis.

Results: Obtained results stated that prepared SLNs are roughly spherical in nature and are in nanorange. These were incorporated in Carbopol gel and further evaluation studies such as pH, spreadability, viscosity, homogeneity, and *in vitro* drug diffusion studies were carried out. All the results obtained state that prepared nanogel has shown sustained release of drug. The antimicrobial study was carried out using *Staphylococcus aureus* and it was confirmed by appearance of the zone of inhibition.

Conclusion: Nanogel that contains Caffeine SLNs with 1:2 ratio drug:lipid has shown good *in vitro* release. Sustained release of Caffeine drug till 12 h was achieved by delivering it in the form of nanogel.

Keywords: Caffeine, Cholesterol, Clinical mastitis, Solid lipid nanoparticles, Antimicrobial activity.

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INTRODUCTION

Clinical mastitis is an inflammatory condition of the breast that may occur in the breastfeeding women during the puerperium and is reported in women who continue to breast feed up to 1 year after delivery [1].

Recently, a condition called subclinical mastitis has been described. Subclinical mastitis is diagnosed from the finding of a raised sodium-potassium ratio in the milk and an increased concentration of interleukin-8 (IL-8) when there is no clinical mastitis. Increased sodium and IL-8 levels are thought to indicate that an inflammatory response is occurring despite the absence of clinical signs. Due to this disease milk production falls below 400 ml per day.

Two principle causes of mastitis are milk stasis and infection. Milk stasis is usually the primary cause which may or may not be accompanied by progress to infection. Intra mammary infections caused by *Escherichia coli* are commonly considered to be limited in duration. Sometimes, microorganisms may even be eliminated before or shortly after the onset of clinical symptoms. Therefore, the host defense system appears to eliminate *E. coli* efficiently especially when the infection occurs late in lactation. *Staphylococcus aureus* is also causative organism for clinical mastitis [2].

In treating and managing clinical mastitis, culture based therapy and severity levels play ey role. The antibiotic therapy is strongly recommended for clinical mastitis [3].

The veterinary in clinical practice is often confronted with cases of mastitis that require systemic antibacterial treatment in addition to local treatment. Furthermore, nonsteroidal anti-inflammatory drug's are most commonly used in the treatment. Few examples of drugs used are penicillin's, oxytetracycline, chloramphenicol, sulfonamides, and trimethoprim [4].

Caffeine is a natural alkaloid found in coffee beans, tea leaves, cocoa beans, cola nuts, and other plants. It is probably the most frequently ingested pharmacologically active substance in the world. It is found in common beverages (coffee, tea, and soft drinks), products containing coca, chocolate, and over counter stimulants. Caffeine is used to treat tiredness and drowsiness and used along with other pain relievers to improve their effect [5]. Caffeine is a methyl xanthine moiety capable to hinder the phosphodiesterase (PDE) enzyme which helps in hydrolysis of cyclic nucleotides resulting in elevated concentration of intracellular cyclic adenosine monophosphate (cAMP). Cell surface receptors inhibition for adenosine is another proposed mechanism. Reduced intracellular cAMP levels are seen in cutaneous leukocytes of patients with psoriasis. Many researchers proposed that as a PDE inhibitor and methyl xanthine, caffeine increases intracellular cAMP levels, which consequently suppress inflammatory pathways [6].

Solid lipid nanoparticles (SLNs) are submicrons colloidal carrier ranging from 50 to 100 nm which are composed of a physiological lipid dispersed in water or in aqueous surface solutions. They consist of macromolecular material in which the active compound is dissolved, entrapped, or to which the active compound is dissolved or attached. SLNs are generally spherical in shape and diameter from 10 to 100 nm. Advantages are it controls or target drug release, improve stability of pharmaceutics, and feasibilities of carrying both lipophilic and hydrophilic drugs more affordable [7,8].

Various lipids used in the preparation of SLNs are triglycerides (tri-stearin), partial glycerides (Imwitor), fatty acids (stearic acid,

and palmitate acid), and steroids (cholesterol), and waxes (acetyl palmitate). Various emulsifiers and their combination (pluronic F 68, F 127) have been used to stabilize the lipid dispersion. Among them, cholesterol is widely used lipid because of its GRAS status lipids and thus know to be safe and used widely in pharmaceutical and cosmeceutical industries. No skin irritations are reported using these lipids from the reports and sources obtained. Hence, this lipid is used in the present study [9,10].

The topical delivery of drugs is an attractive method for local and systemic treatment and commonly used in the treatment of inflammatory conditions such as musculoskeletal injuries and dermatological diseases. There are many advantages in topical application compared to conventional dosage forms. Especially, some serious systemic and adverse effects are avoided.

When the drug is delivered topically, it can penetrate deeper into skin and hence give better absorption. Topical preparation can be used to prevent the metabolism of drug in the liver. It can be used to avoid the gastrointestinal disorders, risks and inconvenience of intravenous therapy, etc. Furthermore, bioavailability of the drug is increased and targeted action can be achieved.

The topical delivery with gels can increase the time of the presence of drug on the skin and improve the delivery and release of the substance [11].

In the present study, an attempt has made to prepare SLN's of Caffeine using cholesterol as lipid. The novelty of this work is though caffeine SLN's were prepared earlier by others using them in the treatment of clinical mastitis was not reported till date. These SLN's help in increasing bioavailability of drug and also provide sustained release of the drug.

MATERIALS AND METHODOLOGY

Caffeine was obtained from Loba Chemie, Mumbai, India. Cholesterol and other excipients were obtained from Pallav Chemicals & Solvents Pvt, Ltd., Boisar, India. All the reagents used were of analytical grade.

Methodology

Analytical method for caffeine

Calibration curve in pH 7.4 phosphate buffer

From the standard solution, a stock solution was prepared to give a concentration of 100 µg/ml in 7.4 buffer. Aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 ml from the stock solution were pipetted out into 10 ml volumetric flasks. The volume was made up to the mark with 7.4 buffer. These dilutions gave 5, 10, 15, 20, and 25 µg/ml concentration of Caffeine, respectively. The absorbance of prepared solutions of Caffeine in 7.4 buffer was measured at 275 nm spectrophotometrically against 7.4 buffer blank. Standard plot data of Caffeine in 7.4 pH buffer are reported in Fig. 1.

Preparation of SLN

Homogenization technique

Cholesterol and Caffeine were dissolved in 10 mL chloroform in a glass flask (organic phase). Tween 80 was added in 30 ml distilled water and heated up to 75°C using water bath (aqueous phase). Then, the organic phase was added to the aqueous phase under continuous stirring at 1000 rpm using a homogenizer. About 2.5 h later, the flask was removed from the water bath, 10 mL of ice-cold distilled water was added, and stirring of the mixture at 1000 rpm was continued for 2 h. Then, the resulting suspension was washed twice with distilled water by centrifuging at 10,000 rpm for 30 min to remove the supernatant, which contained the free (unreacted) drug. It was followed by freeze-drying that converted it to powder form [12,13]. Three SLN formulations were prepared by varying the drug: lipid ratio. They are given in Table 1.

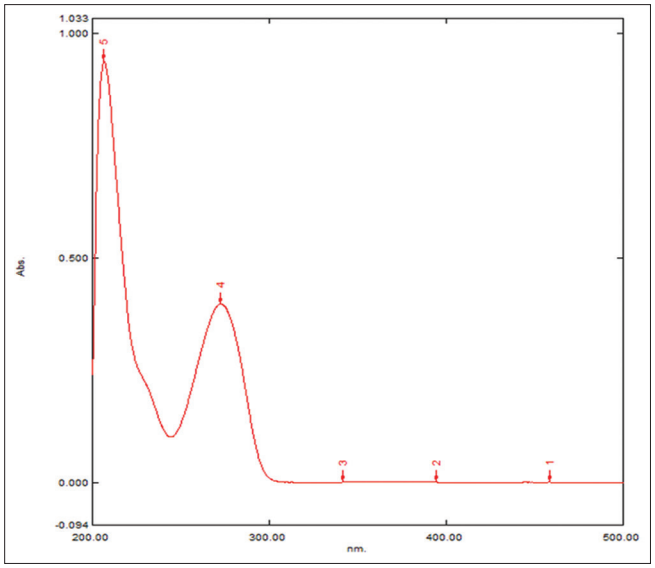

Fig. 1: Ultraviolet-spectra of Caffeine in pH 7.4 phosphate buffer

Table 1: Formulation chart of various SLN formulations

Formulation	Drug (mg)	Cholesterol (mg)	Chloroform (ml)	Tween 80 (%)
F1	50	50	10	1
F2	50	100	10	1
F3	50	150	10	1

SLN: Solid Lipid Nanoparticle

Characterization and evaluation of SLN's [14-16]

Entrapment efficiency (EE)

To calculate the EE, accurately weighed quantity of 100 mg SLN was taken and dissolved in 7.4 pH buffer. It was stirred for 10 min to break the complex. Then, the solution was filtered and 2 ml was taken from above solution and diluted up to 10 ml with 7.4 pH buffer [14,15]. It was kept aside for few minutes and absorbance was measured by ultraviolet (UV)-spectrophotometer at 421 nm. It can be calculated using the formula.

In vitro drug diffusion studies

In vitro studies were carried out using cellophane membrane soaked in pH 7.4 buffer overnight. For this study, Franz diffusion cell was taken and in donor compartment pH 7.4 buffer was taken. In between donor and receiver compartments, cellophane membrane was placed and tightly held using rubber band. On the membrane gel was applied. The buffer which passes from donor to receiver compartments through the membrane was collected in receiver compartment using a syringe tube. Sampling was done at regular intervals of 15, 30 min, 1 h, 2 h ,3 h, 4 h, 5 h, and 6 h (each time 2 ml of sample was collected and replaced with similar amount of buffer). The obtained samples were analyzed using UV spectrophotometer [14-16]. 7.4 pH buffer is used because human breast milk pH is around 7.4

Fourier transform infrared (FTIR) spectroscopic analysis

FTIR analysis was conducted to verify the interaction between drug and polymer. The sample powder was dispersed in KBr powder and pellets were made by applying 4 kg/cm^2 pressure. FTIR spectra were obtained by powder diffuse reflectance on a FTIR spectrophotometer type 8400S Shimadzu.

Differential scanning calorimetry (DSC)

DSC was performed on pure drug and its formulations using DSC-60 instrument. Calorimetric measurements were made with empty cell (high purity alpha alumina disks) as the reference. The dynamic scans were taken in nitrogen atmosphere at the heating rate of 10°C min-1. The energy was measured as J/Kcal.

Scanning electron microscopy (SEM)

The surface morphology of formulations was determined using a scanning electron microscope. Samples were mounted on aluminum mount, using double-sided adhesive tape and sputtered by gold under vacuum and were scanned at an accelerating voltage of 15 KV before observation.

Particle size and zeta potential analysis

The average particle size distribution and charge of the resulting nanoparticles were determined by dynamic light scattering using C:\ Microtrac\FLEX 11.0.0.2 Instruments, United Kingdom. The experiment was performed using clear disposable zeta cell, water as a dispersant which has refractive index – 1.330 and viscosity (cP) – 0.898 and the temperature was kept constant at 25°C. The optimized SLN formulation was further incorporated into topical nanogel prepared.

Nanogel preparation

Appropriate quantity of Carbopol 934 was soaked in water (around 5 ml) for a period of 2 h. Carbopol was then neutralized with triethanolamine (TEA) with stirring. Then, specified amount of SLN's was dissolved in appropriate and pre-weighed amount of propylene glycol. Solvent blend was transferred to Carbopol container and agitated for additional 20 min. The dispersion was then allowed to hydrate and swell for 60 min; finally, the pH was adjusted with 98% TEA until the desired pH value was approximately reached (6.8–7). During pH adjustment, the mixture was stirred gently with a spatula until homogeneous gel was formed. All the samples were allowed to equilibrate for at least 24 h at room temperature before performing rheological measurements [17,18]. Formulation chart of nanogel is given in Table 2.

Evaluation studies

pH determination

The pH of the gels was determined using digital pH meter by placing the glass electrode completely into the gel system. The readings were taken for average of 3 times [17,18].

Homogeneity

All developed gels were tested for homogeneity by visual inspection after gels have been set in the container. They were tested for the appearance and the presence of any aggregates [17,18].

Spreadability test

Place 0.5 g gel in a premarked circle (1 cm diameter) on a glass plate. Another glass plate was then placed over the gel and weight of 500 g was placed over this upper glass plate for 5 min. The experiment was carried out in triplicate and spreadability expressed in gm.cm/sec [17,18]. Spreadability can be calculated using the formula.

- S = M.L/T
- Where, S = Spreadability
- M = Weight tied to upper slide
- L = Length of glass slide
- T = Time taken to separate the glass slide completely from each other.

Table 2: Formulation of nanogel

S No.	Ingredients	Amount
1.	Drug (F2)	100 mg
2.	TEA	1%
3.	Propylene glycol	0.5 ml
4.	Carbopol 934	0.5 g
5.	Distilled water	Q.S

TEA: Tri ethanolamine

Rheological studies

The rheological measurements were performed on the Brookfield viscometer. All measurements were carried out using parallel plates measuring systems having 50 mm diameter and 1 mm gap at 25°C. The rheological properties of the formulated gels and nanogel were studied at different shear rates (rpm) and the viscosity was measured in cP [17,18].

In vitro drug diffusion studies

The caffeine SLN loaded nanogel was permeated through dialysis bag. Optimized formulation was selected for these studies. 0.5 g of nanogel was placed in the bag and is placed in a beaker containing 150 ml of phosphate buffer of pH 7.4 and constantly stirred with a small magnetic bead. During the experiment, temperature was maintained at 37±0.5°C to simulate the human skin condition. 5 ml of samples were withdrawn at 0.5, 1, 2, 6, and 12 h and replaced with fresh receptor solution. The samples withdrawn were analyzed spectrophotometrically at 235 nm. The amount of drug released was calculated and the percentage drug released was plotted against time [17,18].

Similarly, it was done for marketed topical antibiotic gel.

Antimicrobial test

Organisms used
S. aureus (Gram-positive bacteria).

Procedure
Nutrient agar medium was prepared in aseptic condition (2.8 g of agar powder in 100 ml distilled water). Prepared agar medium was poured in sterilized Petri plates and allowed to solidify. Above mentioned organism was collected from the culture tube with a prior sterilized metal loop and streaked on solidified agar plates. Then, bores or cups were made in the agar Petri plates with a sterilized metal borer and the sample was placed in the cups. The Petri plates were placed in the incubator for 36 h [19].

RESULTS AND DISCUSSION

EE

EE studies were carried out for F1-F3 formulations. All the entrapment efficiencies were between 55% and 69%. When cholesterol was used as lipid, F2 has highest EE (69.12%). It is because, in case of F1 (1:1 ratio) equal amount of drug and lipid was present for interaction. Hence, the release rate was reduced due to no extra drug molecules present. In case of F3 (1:3 ratio) formulations excess lipid molecules were present in the formulation and these molecules formed a thick sheet around the drug leading to delay in drug release. In case of F2 (1:2) formulations drug and lipid ratio were satisfactory. In spite of excess lipid molecules, high % release was observed in F2 formulation, since only a thin sheet of lipid layer has formed around the drug which does not hinder the release of drug.

The drug entrapment efficiencies noted for different formulation are given in Table 3.

***In vitro* drug diffusion studies**

The SLN formulations were subjected to *in vitro* release studies. The results obtained in *in vitro* release studies were plotted in percent

Table 3: Entrapment efficiencies of F1-F3 formulations

S. No.	Formulation	EE (%)
1.	F1	55.79
2.	F2	69.12
3.	F3	60.22

EE: Entrapment efficiency

cumulative drug release versus time and shown in Fig. 2. It was found that formulation containing drug:lipid in 1:2 ratio has shown maximum *in vitro* drug release, as compared to other formulations. This could be due to poor loading seen in F1 due to insufficient lipid and incase of F3 it could be able to inability of drug to enter into the larger network of lipid. *In vitro* drug diffusion studies of all the formulation are given in Table 4.

SEM

From the results obtained that SLNs were roughly spherical. It shows formation of nanoparticles resembling spheres. SEM image of F2 formulation is given in Fig. 3.

Particle size analysis and zeta potential and measurement

Particle size of solid lipid nanoparticles was found between 100 and 2500 nm. Average particle size was 190.5 nm. Obtained results state that prepared nanoparticles were in nanosize which is one of the objectives of the study. The particle size distribution of F2 formulation is given in Fig. 4.

Zeta potential is the major function which determines the interaction of formulation with biological system. It determines the charge type present on the nanoparticle surface. Zeta potential of the prepared SLN's was found as -18.9 mv. It shows that decrease in particle size has led to increase in surface area that resulted in higher zeta potential.

Table 4: *In vitro* drug diffusion studies (F1-F3)

Time (h)	F1	F2	F3
0	0	0	0
0.5	17.15	25.13	16.18
1	31.25	39.24	29.47
2	37.26	49.60	37.99
4	43.32	56.39	46.95
6	47.68	60.75	52.76
8	53.22	67.74	58.30
12	63.01	72.48	66.64

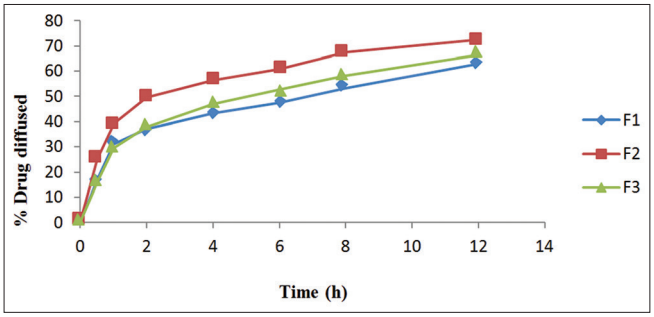

Fig. 2: *In vitro* drug diffusion studies (F1-F3)

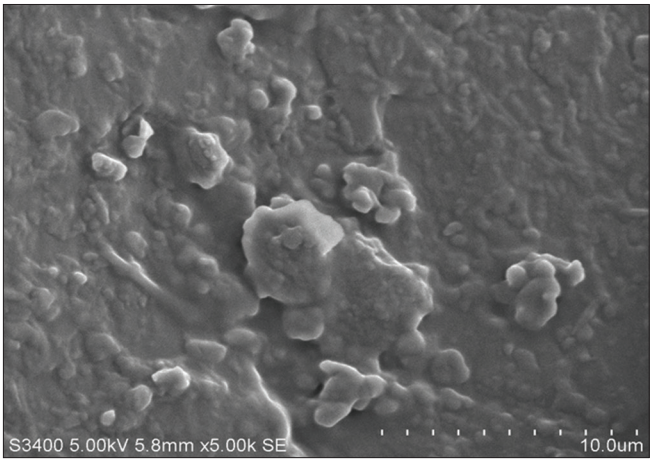

Fig. 3: Scanning electron microscopy photograph of F2 formulation

FTIR spectroscopy

Major peaks of Caffeine, i.e., amines, amides, and carboxylic groups with bond frequency of 3300–3000, 3500–3000, and 1710–1650, respectively, are seen in pure caffeine at 3112, 1599 and 3510, and 1661 and 1698, respectively. FTIR spectrum of pure caffeine is shown in Fig. 5.

Similar peaks were observed at 1552, 1655, and 1701 frequencies in F2 formulation along with other characteristics peaks pertaining to Caffeine. This indicates the presence of drug in SLNs. Some of the major peaks pertaining to amine and amides, i.e., 3112 and 3510 cm^{-1} disappeared in final formulation.

This shows that though pure drug is present, it is entrapped in the lipid system. This is also confirmed with blunt peaks obtained in FTIR spectrum. FTIR spectra of F2 formulation are shown in Fig. 6.

DSC

DSC endograms of pure drug and F2 formulations are given in Figs. 7 and 8, respectively. Melting point of pure caffeine was observed at 242°C. In case of F2 formulation, peak pertaining to cholesterol was observed at 145°C. Drug peak was observed at around 235°C but was not sharp. The absence of sharp peak pertaining to drug indicates the presence of drug but embedded in lipid system showing drug-lipid compatibility.

Evaluation of nanogels

pH determination

pH of obtained gel was found to be 7.4 which is near to neutral pH. This shows that prepared gel does not cause any skin irritation as it is near to skin pH.

Homogeneity

All the gels prepared clear and transparent. It shows that no aggregates were present.

Spreadability

Prepared gel was spreaded on skin and was found to spread easily. It shows that prepared gel has good viscosity.

Rheological studies

Viscosity for prepared gel was found to be good. It shows that obtained results were optimum which helped in good spreadability.

All the above results are given in Table 5.

In vitro drug diffusion studies

The nanogel containing F2 formulation and normal antibiotic gel was subjected to *in vitro* diffusion studies. The results obtained were

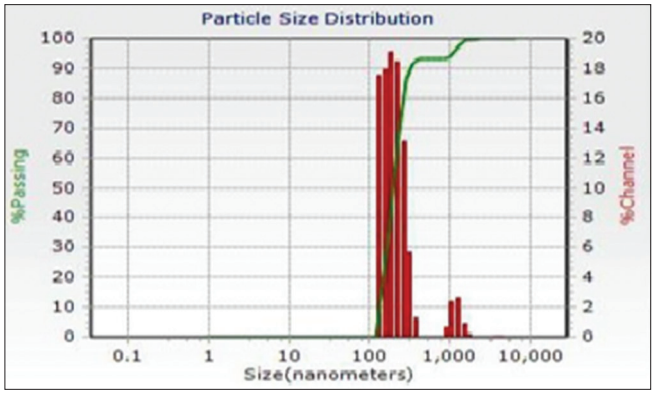

Fig. 4: Particle size distribution of formulation F2

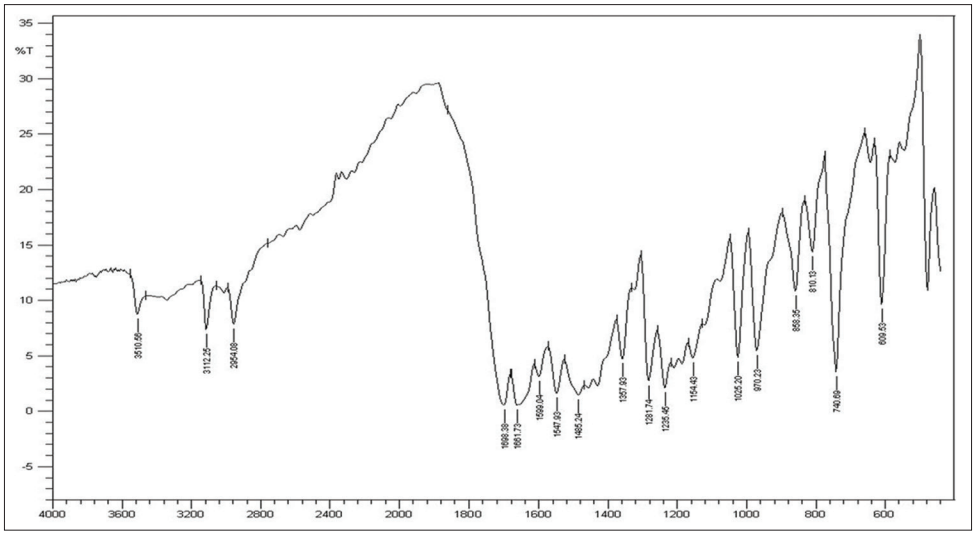

Fig. 5: Fourier transform infrared spectrum of pure caffeine

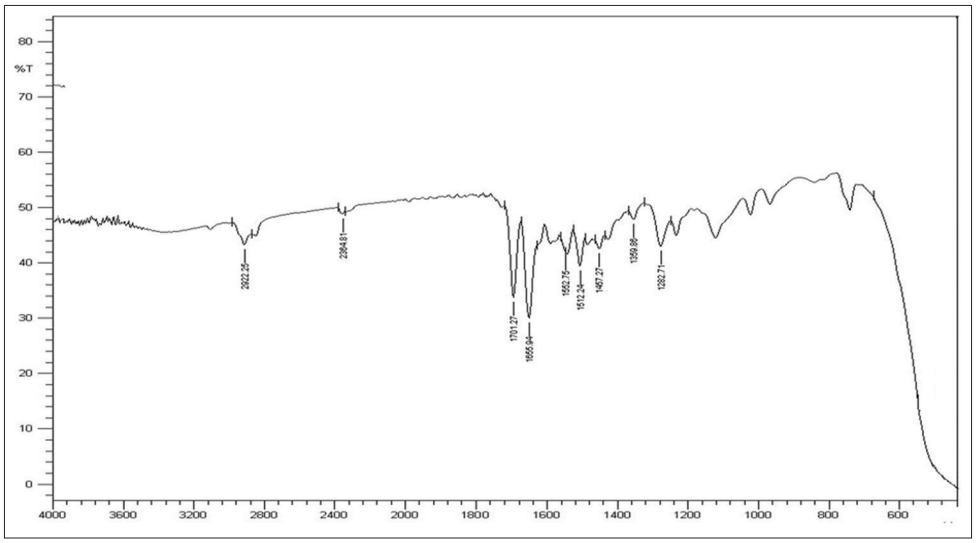

Fig. 6: Fourier transform infrared spectra of formulation F2

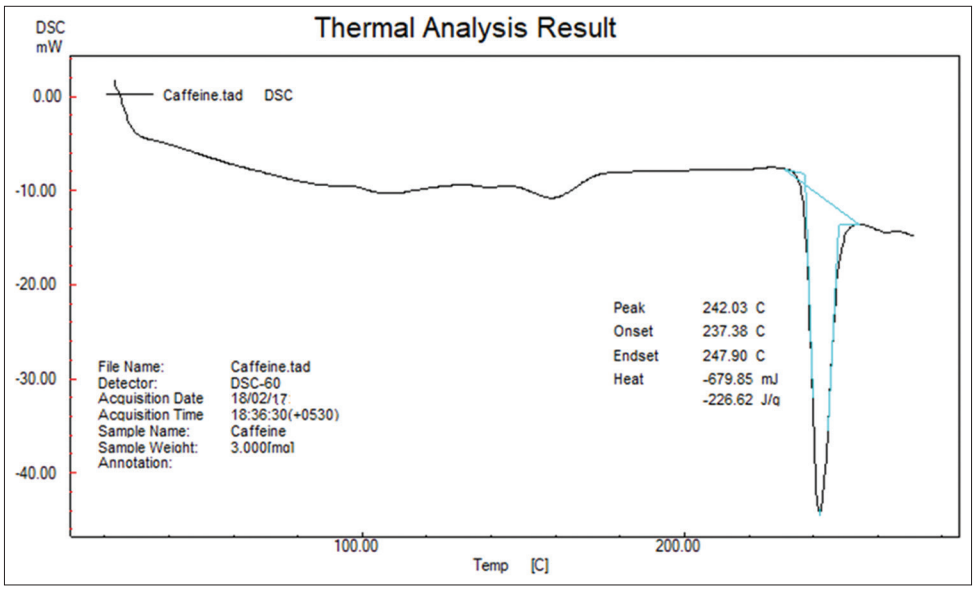

Fig. 7: Differential scanning calorimetry thermogram of caffeine

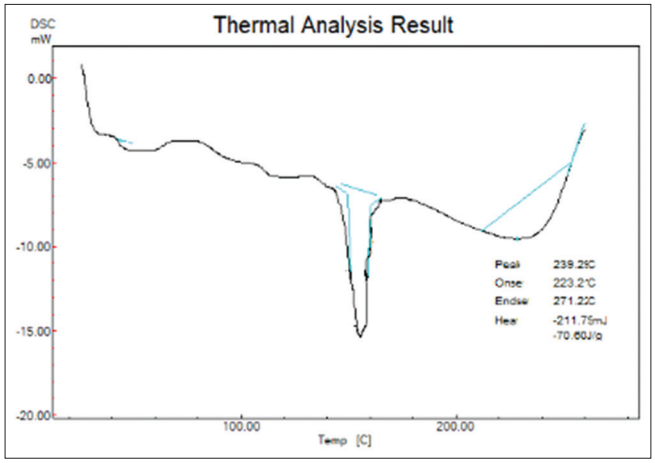

Fig. 8: Differential scanning calorimetry thermogram Formulation F2

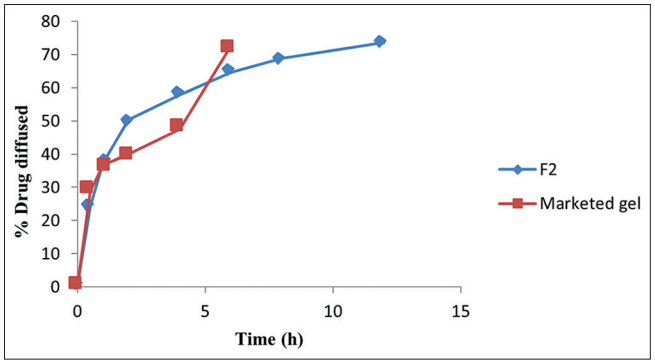

Fig. 9: *In vitro* % drug diffusion studies

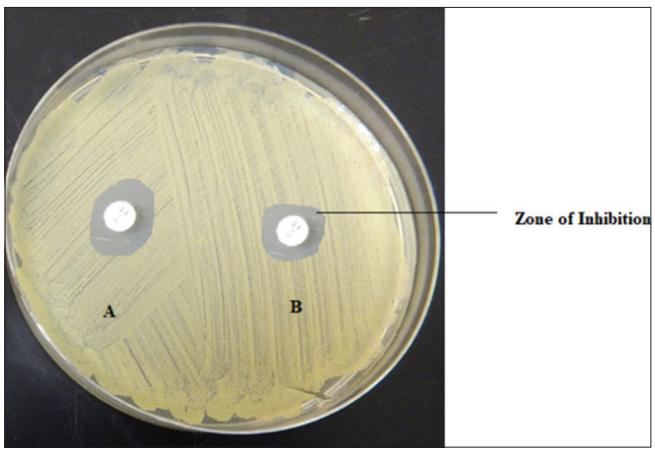

Fig. 10: Antimicrobial activity

plotted in percent cumulative drug release versus time in Fig. 9. It was found that F2 nanogel has shown maximum *in vitro* drug diffusion of 73.6% and ordinary antibiotic gel release was 72.68%. Furthermore, sustained release up to 12 h was found in former but it was limited to 6 h in later. This could be due to entrapment and slow release of drug from polymer complex in nanogels whereas due to availability of free drug quick release was seen in marketed formulation. *In vitro* % drug diffusion results of F5 and marketed gel are given in Table 6.

Antimicrobial test

The zone of inhibition was observed and is shown in Fig. 10. From the results, it can be stated that prepared SLN formulation has antimicrobial activity.

Table 5: Evaluation parameters of F2 nanogel

S. No.	Evaluation parameter	Results
1.	pH	7.4
2.	Homogeneity	Clear and Transparent
3.	Spreadability	Good
4.	Rheological studies (cp)	9872

Table 6: *In vitro* % drug diffusion studies

S. No.	Time (h)	F2	Marketed
1.	0	0	0
2.	0.5	24.3	28.84
3.	1	37.5	36.77
4.	2	50.3	39.97
5.	4	57.9	47.55
6.	6	64.6	72.68
7.	8	68.9	-
8.	12	73.6	-

CONCLUSION

Nanogel that contains Caffeine SLNs with 1:2 ratio drug:lipid has shown good *in vitro* release. Sustained release of Caffeine drug till 12 h was achieved by delivering it in the form of nanogel.

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AUTHOR'S CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTEREST STATEMENT

Declared none.

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Research Article

FORMULATION AND EVALUATION OF CAFFEINE-LOADED SOLID LIPID NANOPARTICLES TO TREAT CLINICAL MASTITIS

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ABSTRACT

Objective: The objective of the present study was to formulate and evaluate caffeine-loaded solid lipid nanoparticles (SLNs) in the treatment of clinical mastitis.

Methods: These were prepared by homogenization technique using stearic acid, Tween 80, and chloroform as excipients. Pre-formulation studies such as UV spectrophotometry, Fourier transform infrared (FTIR), and differential scanning calorimetry (DSC) were performed for the drug. Entrapment efficiency and *in vitro* dissolution studies were carried out for prepared SLNs and the optimum formulation (F2) was taken for further studies such as FTIR, DSC, SEM, particle size, and zeta potential analysis.

Results: Obtained results stated that prepared SLNs are roughly spherical in nature and are in nano range. These were incorporated in Carbopol gel and further evaluation studies such as pH, spreadability, viscosity, homogenicity, and *in vitro* drug diffusion studies were carried out. All the results stated that prepared nanogel has shown sustained release of drug. Antimicrobial study was carried out using *Staphylococcus aureus* and it was confirmed by the appearance of zone of inhibition.

Conclusion: Nanogel that contains caffeine SLNs with 1:2 ratio drug:lipid has shown good *in vitro* release. Sustained release of caffeine drug till 12 h was achieved by delivering it in the form of nanogel.

Keywords: Caffeine, Stearic acid, Clinical mastitis, Solid lipid nanoparticles, Antimicrobial activity.

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INTRODUCTION

Clinical mastitis is an inflammatory condition of the breast that may occur in the breastfeeding women during the puerperium and is reported in women who continue to breast feed up to 1 year after delivery [1].

Recently, a condition called subclinical mastitis has been described. Subclinical mastitis is diagnosed from the finding of a raised sodium-potassium ratio in the milk and an increased concentration of interleukin-8 (IL-8) when there is no clinical mastitis. Increased sodium and IL-8 levels are thought to indicate that an inflammatory response is occurring despite the absence of clinical signs. Due to this disease, milk production falls below 400 ml/day.

Two principle causes of mastitis are milk stasis and infection. Milk stasis is usually the primary cause which may or may not be accompanied by progress to infection. Intramammary infections caused by *Escherichia coli* and *Staphylococcus aureus* (*E. coli*) are commonly considered to be limited in duration. Sometimes, microorganisms may even be eliminated before or shortly after the onset of clinical symptoms. Therefore, the host defense system appears to eliminate microorganisms efficiently especially when the infection occurs late in lactation [2].

In treating and managing clinical mastitis, culture-based therapy and severity levels play a key role. Antibiotic therapy is strongly recommended for clinical mastitis [3].

The veterinary in clinical practice is often confronted with cases of mastitis that requires systemic antibacterial treatment in addition to local treatment. Furthermore, NSAIDs are most commonly used in treatment. Few examples of drugs used are penicillin's, oxytetracycline, chloramphenicol, sulfonamides, trimethoprim, etc. [4].

Caffeine is a natural alkaloid found in coffee beans, tea leaves, cocoa beans, cola nuts, and other plants. It is probably the most frequently ingested pharmacologically active substance in the world. It is found in common beverages (coffee, tea, and soft drinks), products containing coca, chocolate, and over counter stimulants. Caffeine is used to treat tiredness and drowsiness and used along with other pain relievers to improve their effect [5]. Caffeine is a methyl xanthine moiety capable to hinder the phosphodiesterase (PDE) enzyme which helps in hydrolysis of cyclic nucleotides resulting in elevated concentration of intracellular cyclic adenosine monophosphate (cAMP). Cell surface receptors inhibition for adenosine is another proposed mechanism. Reduced intracellular cAMP levels are seen in cutaneous leucocytes of patients with psoriasis. Many researchers proposed that as a PDE inhibitor and methyl xanthine, caffeine increases intracellular cAMP levels, which consequently suppress inflammatory pathways [6].

Solid lipid nanoparticles (SLNs) are submicrons colloidal carrier ranging from 50 to 100 nm which are composed of a physiological lipid dispersed in water or in aqueous surface solutions. They consist of macromolecular material, in which the active compound is dissolved, entrapped or to which the active compound is dissolved or attached. SLNs are generally spherical in shape and diameter from 10 to 100 nm. Advantages are it controls or target drug release, improve stability of pharmaceutics, feasibilities of carrying both lipophilic and hydrophilic drugs more affordable [7,8].

Various lipids used in the preparation of SLNs are triglycerides (tristearin), partial glycerides (Imwitor), fatty acids (stearic acid and palmitate acid), steroids (cholesterol), and waxes (acetyl palmitate). Various emulsifiers and their combination (Pluronic F 68, F 127) have been used to stabilize the lipid dispersion. Among them, stearic acid is widely used because of its GRAS status and thus known to be safe and

used widely in pharmaceutical and cosmeceutical industries. No skin irritations are reported using this lipid from the reports and sources obtained. Hence, it is used in the present study [9,10].

The topical delivery of drugs is an attractive method for local and systemic treatment and commonly used in the treatment of inflammatory conditions such as musculoskeletal injuries and dermatological diseases. There are many advantages in topical application compared to conventional dosage forms. Especially, some serious systemic and adverse effects are avoided.

When the drug is delivered topically, it can penetrate deeper into skin and hence give better absorption. Topical preparation can be used to prevent the metabolism of drug in the liver. It can be used to avoid the gastrointestinal disorders, risks, inconvenience of intravenous therapy, etc. Furthermore, bioavailability of the drug is increased and targeted action can be achieved.

The topical delivery with gels can increase the time of presence of drug on the skin and improve the delivery and release of the substance [11].

In the present study, an attempt has made to prepare SLNs of caffeine using stearic acid as lipid. The novelty of this work is though caffeine SLNs were prepared earlier by others using them in the treatment of clinical mastitis which was not reported till date. These SLNs help in increasing bioavailability of drug and also provide sustained release of the drug.

MATERIALS AND METHODOLOGY

Caffeine was obtained from Loba Chemie, Mumbai, India. Stearic acid was obtained from Qualikems Pvt. Ltd., Vadodara, India. All other excipients were obtained from Pallav Chemicals & Solvents Pvt., Ltd., Boisar, India. All the reagents used were of analytical grade.

METHODOLOGY

Analytical method for caffeine

Calibration curve in pH 7.4 phosphate buffer

From the standard solution, a stock solution was prepared to give a concentration of 100 µg/ml in 7.4 buffer. Aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 ml from the stock solution were pipetted out into 10 ml volumetric flasks. The volume was made up to the mark with 7.4 buffer. These dilutions gave 5, 10, 15, 20, and 25 µg/ml concentration of caffeine, respectively. The absorbance of prepared solutions of caffeine in 7.4 buffer was measured at 275 nm spectrophotometrically against 7.4 buffer blank. Standard plot data of caffeine in 7.4 pH buffer are reported in Fig. 1.

Preparation of SLNs

Homogenization technique

Stearic acid and caffeine were dissolved in 10 ml chloroform in a glass flask (organic phase). Tween 80 was added in 30 ml distilled water and heated up to 75°C using water bath (aqueous phase). Then, the organic phase was added to the aqueous phase under continuous stirring at 1000 rpm using a homogenizer. About 2.5 h later, the flask was removed from the water bath, 10 mL of ice-cold distilled water was added, and stirring of the mixture at 1000 rpm was continued for 2 h. Then, the resulting suspension was washed twice with distilled water by centrifuging at 10,000 rpm for 30 min to remove the supernatant, which contained the free (unreacted) drug. It was followed by freeze-drying that converted it to powder form [12,13]. Three SLN formulations were prepared by varying the drug:lipid ratio. They are given in Table 1.

Characterization and evaluation of SLNs

Entrapment efficiency (EE)

To calculate the EE, accurately weighed quantity of 100 mg SLN was taken and dissolved in 7.4 pH buffer. It was stirred for 10 min to break the complex. Then, the solution was filtered and 2 ml was taken from

Table 1: Formulation chart of various solid lipid nanoparticle formulations

Formulation	Drug (mg)	Stearic acid (mg)	Chloroform (ml)	Tween 80 (%)
F1	50	50	10	1
F2	50	100	10	1
F3	50	150	10	1

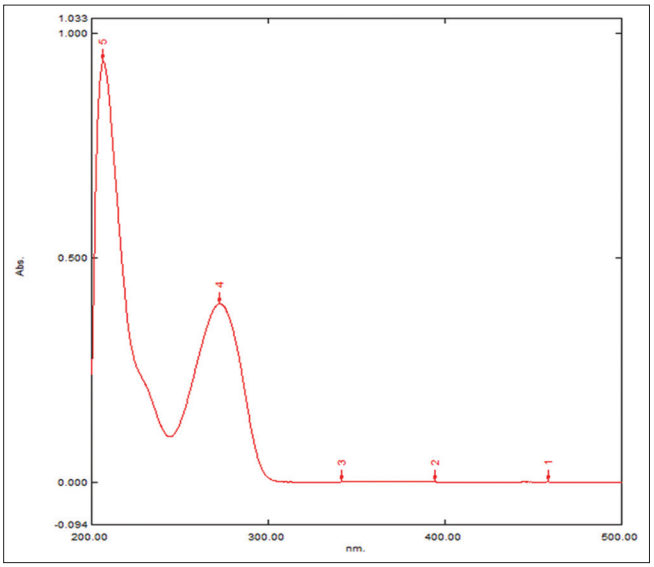

Fig. 1: Ultraviolet spectra of caffeine in pH 7.4 phosphate buffer

above solution and diluted up to 10 ml with 7.4 pH buffer [14-16]. It was kept aside for few minutes and absorbance was measured by UV spectrophotometer at 421 nm. It can be calculated using the formula.

$$EE = \frac{\text{Actual drug content in NS}}{\text{Theoretical drug content}} \times 100$$

In vitro drug diffusion studies

In vitro studies were carried out using cellophane membrane soaked in pH 7.4 buffer overnight. For this studies, Franz diffusion cell was taken and in donor compartment, pH 7.4 buffer was taken. In between donor and receiver compartments, cellophane membrane was placed and tightly held using rubber band. On the membrane, gel was applied. The buffer which passes from donor to receiver compartments through the membrane was collected in receiver compartment using a syringe tube. Sampling was done at regular intervals of 15 and 30 min, 1 h, 2, 3, 4, 5, and 6 h (each time 2 ml of sample was collected and replaced with similar amount of buffer) [14-16]. The obtained samples were analyzed using UV spectrophotometer.

pH buffer is used because human breast milk pH is around 7.4.

Fourier transform infrared (FT-IR) spectroscopic analysis

FT-IR analysis was conducted to verify the interaction between drug and polymer. The sample powder was dispersed in KBr powder and pellets were made by applying 4 kg/cm^2 pressure. FT-IR spectra were obtained by powder diffuse reflectance on a FT-IR spectrophotometer type 8400S Shimadzu.

Differential scanning calorimetry (DSC)

DSC was performed on pure drug and its formulations using DSC-60 instrument. Calorimetric measurements were made with empty cell (high purity alpha alumina discs) as the reference. The dynamic scans were taken in nitrogen atmosphere at the heating rate of 10°C min^{-1}. The energy was measured as J/Kcal.

Scanning electron microscopy (SEM)

The surface morphology of formulations was determined using a SEM. Samples were mounted on aluminum mount, using double-sided adhesive tape and sputtered by gold under vacuum and were scanned at an accelerating voltage of 15 KV before observation.

Particle size and zeta potential analysis

The average particle size distribution and charge of the resulting nanoparticles were determined by dynamic light scattering using C:\ Microtrac\FLEX 11.0.0.2 Instruments, United Kingdom. The experiment was performed using clear disposable zeta cell, water as a dispersant which has refractive index – 1.330 and viscosity (cP) – 0.898, and the temperature was kept constant at 25°C. The optimized SLN formulation was further incorporated into topical nanogel prepared.

Nanogel preparation

Appropriate quantity of Carbopol 934 was soaked in water (around 5 ml) for a period of 2 h. Carbopol was then neutralized with triethanolamine (TEA) with stirring. Then, specified amount of SLNs was dissolved in appropriate and pre-weighed amount of propylene glycol. Solvent blend was transferred to Carbopol container and agitated for additional 20 min. The dispersion was then allowed to hydrate and swell for 60 min; finally, the pH was adjusted with 98% TEA until the desired pH value was approximately reached (6.8–7). During pH adjustment, the mixture was stirred gently with a spatula until homogeneous gel was formed. All the samples were allowed to equilibrate for at least 24 h at room temperature before performing rheological measurements [17,18]. Formulation chart of nanogel is given in Table 2.

Evaluation studies

pH determination

The pH of the gels was determined using digital pH meter by placing the glass electrode completely into the gel system. The readings were taken for average of 3 times [17,18].

Homogenicity

All developed gels were tested for homogenicity by visual inspection after gels have been set in the container. They were tested for the appearance and presence of any aggregates [17,18].

Spreadability test

Place 0.5 g gel in a pre-marked circle (1 cm diameter) on a glass plate. Another glass plate was then placed over the gel and weight of 500 g was placed over this upper glass plate for 5 min [17,18]. The experiment was carried out in triplicate and spreadability expressed in gm.cm/sec. Spreadability can be calculated using the formula.

S=M.L/T

Where, S=Spreadability
M=Weight tied to upper slide
L=Length of glass slide
T=Time taken to separate the glass slide completely from each other.

Rheological studies

The rheological measurements were performed on the Brookfield viscometer. All measurements were carried out using parallel plates measuring systems having 50 mm diameter and 1 mm gap at 25°C. The rheological properties of the nanogel were studied at different shear rates (rpm) and the viscosity was measured in cP [17,18].

In vitro drug diffusion studies

The caffeine SLN-loaded nanogel was permeated through dialysis bag. Optimized formulation was selected for these studies. A 0.5 g of nanogel was placed in the bag and is placed in a beaker containing 150 ml of phosphate buffer of pH 7.4 and constantly stirred with a small magnetic bead. During the experiment, temperature was maintained at 37± 0.5°C to simulate the human skin condition. A 5 ml of samples were withdrawn at 0.5, 1, 2, 6, and 12 h and replaced with fresh receptor solution [17,18]. The samples withdrawn were analyzed spectrophotometrically at 235 nm. The amount of drug released was calculated and the percentage drug released was plotted against time.

Similarly, it was done for marketed topical antibiotic gel.

Antimicrobial test

Organisms used: *S. aureus* (Gram-positive bacteria).

Procedure

Nutrient agar medium was prepared in aseptic condition (2.8 g of agar powder in 100 ml distilled water). Prepared agar medium was poured in sterilized Petri plates and allowed to solidify. The above-mentioned organism was collected from the culture tube with a prior sterilized metal loop and streaked on solidified agar plates. Then, bores or cups were made in the agar Petri plates with a sterilized metal borer and the sample was placed in the cups. The Petri plates were placed in the incubator for 36 h [19].

RESULTS AND DISCUSSION

- EE

EE studies were carried out for F1-F3 formulations. All the EEs were between 55% and 69%. For F2, EE was 68.24% which is higher than F1 and F3. It is because, in case of F1 (1:1 ratio), equal amount of drug and lipid was present for interaction. Hence, the release rate was reduced due to no extra drug molecules present. In case of F3 (1:3 ratio) formulations, excess lipid molecules were present in the formulation and these molecules formed a thick sheet around the drug leading to delay in drug release. In case of F2 (1:2) formulation, drug and lipid ratio was satisfactory. Although lipid molecule was in excess, they only formed a thin sheet around the drug and hence high % release was observed.

The drug EEs noted for different F1 formulation are given in Table 3.

- *In vitro* drug diffusion studies

The SLN formulations were subjected to *in vitro* release studies. The results obtained in *in vitro* release studies were plotted in percentage cumulative drug release versus time and shown in Fig. 2. It was found that formulation containing drug:lipid in 1:2 ratio has shown maximum *in vitro* drug release, as compared to other formulations. This could be due to poor loading seen in F1 due to insufficient lipid and in case of F3, it could be able to inability of drug to enter into the larger network of lipid. *In vitro* drug diffusion studies of all the formulation are given in Table 4.

- SEM

From the results, obtained SLNs were roughly spherical. It shows formation of nanoparticles resembling spheres. SEM image of F2 formulation is given in Fig. 3.

Table 2: Formulation of nanogel

S. No.	Ingredients	Amount
1.	Drug (F2)	100 mg
2.	Triethanolamine	1%
3.	Propylene glycol	0.5 ml
4.	Carbopol 934	0.5 g
5.	Distilled water	Q.S

Table 3: Entrapment efficiencies of F1-F6 formulations

S. No.	Formulation	Entrapment efficiency (%)
1.	F1	56.78
2.	F2	68.24
3.	F3	61.53

- Particle size analysis and zeta potential and measurement

Particle size of solid lipid nanoparticles was found between 100 and 1000 nm (steric acid). Average particle size was 181.5 nm in stearic acid. Obtained results state that prepared nanoparticles were in nanosize which is one of the objectives of the study. Particle size distribution of F2 formulation is given in Fig. 4.

Zeta potential is the major function which determines the interaction of formulation with biological system. It determines the charge type present on the nanoparticle surface. Zeta potential of the prepared SLNs was found as −15.2 mv. It shows decrease in particle size has led to increase in surface area that resulted in higher zeta potential.

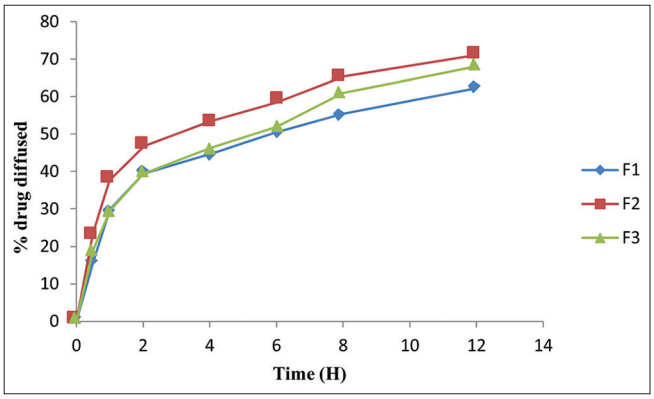

Fig. 2: *In vitro* drug diffusion studies (F1-F3)

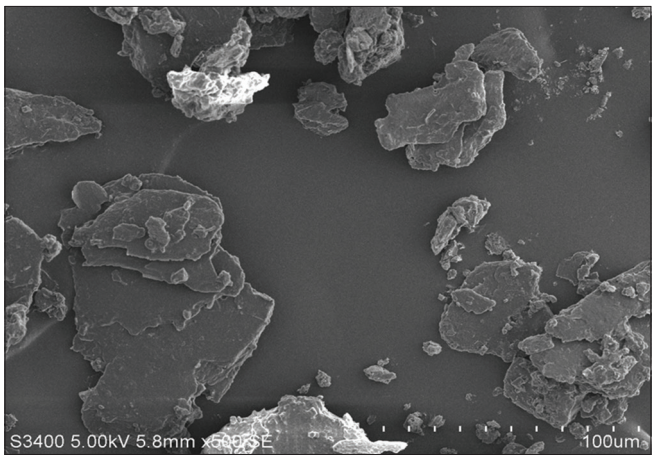

Fig. 3: Scanning electron microscopy photograph of F2 formulation

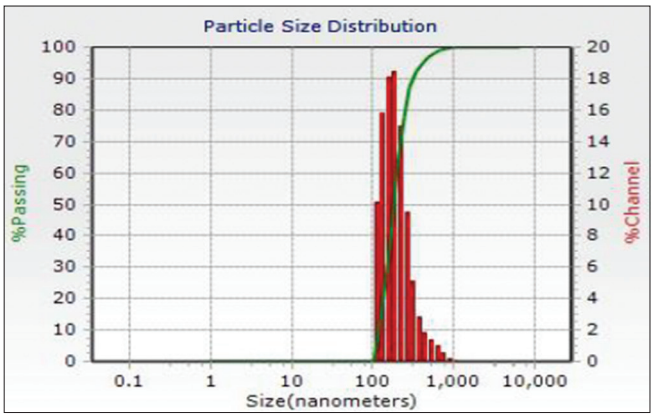

Fig. 4: Particle size distribution of formulation F2

- FT-IR spectroscopy (FTIR)

Major peaks of caffeine, i.e., amines, amides, and carboxylic groups with bond frequency of 3300–3000, 3500–3000, and 1710–1650, respectively, are seen in pure caffeine at 3112, 1599 and 3510, and 1661 and 1698, respectively. FTIR spectrum of pure caffeine is shown in Fig. 5.

Similarly, in case of F2, where stearic acid has been taken as lipid, peaks pertaining to amines, amides were observed at 1625 and 2917, respectively. Major peaks such as amines, amides, and carboxylic groups disappeared.

This shows that though pure drug is present, it is entrapped in the lipid system. This is also confirmed with blunt peaks obtained in FTIR spectrum. FTIR spectra of F2 formulation are shown in Fig. 6, respectively.

- DSC

DSC endograms of pure drug and F2 formulation are given in Figs. 7 and 8, respectively. Melting point of pure caffeine was observed at 242°C. In case of F2 formulation, an endothermic peak was seen at 58°C that resembles melting point of stearic acid. Drug peak was observed at around 230°C but was not sharp. The absence of sharp peak pertaining to drug indicates the presence of drug but embedded in lipid system showing drug-lipid compatibility.

Evaluation of nanogels

- pH determination

  pH of obtained gel was found to be 7.4 which is near to neutral pH. This shows that prepared gel does not cause any skin irritation as it is near to skin pH.
- Homogenicity

  All the gels prepared clear and transparent. It shows that no aggregates were present.
- Spreadability

  Prepared gel was spread on skin and was found to spread easily. It shows that prepared gel has good viscosity.
- Rheological studies:

  Viscosity for prepared gel was found to be good. It shows that obtained results were optimum which helped in good spreadability. All the above results are given in Table 5.
- *In vitro* drug diffusion studies

  The nanogel containing F2 formulation and normal antibiotic gel was subjected to *in vitro* diffusion studies. The results obtained were plotted in percent cumulative drug release versus time in Fig. 9. It was found that F2 nanogel has shown maximum *in vitro* drug diffusion of 72.4% and ordinary antibiotic gel release was 72.68%. Furthermore, sustained release up to 12 h was found in former, but it was limited to 6 h in later. This could be due to entrapment and slow release of drug from polymer complex in nanogels, whereas due to availability of free drug, quick release was seen in marketed formulation. *In vitro* % drug diffusion results of F2 and marketed gel are given in Table 6.

Antimicrobial test

Zone of inhibition was observed and is shown in Fig. 10. From the results, it can be stated that prepared SLN formulation has antimicrobial activity.

Table 4: *In vitro* drug diffusion studies (F1-F3)

Time (h)	F1	F2	F3
0	0	0	0
0.5	15.70	23.68	19.09
1	29.80	37.79	29.47
2	39.44	46.70	39.44
4	44.78	53.49	46.23
6	50.58	58.57	52.03
8	55.40	65.56	61.20
12	62.28	71.20	68.09

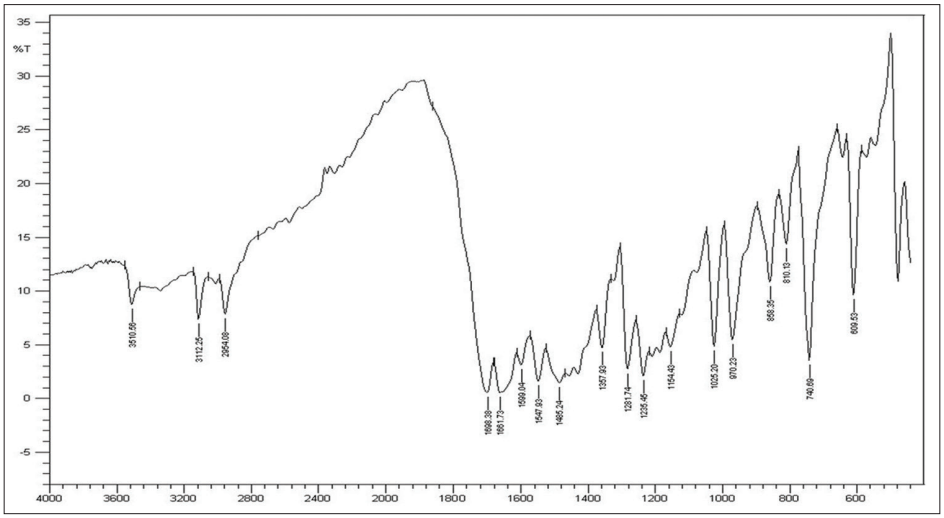

Fig. 5: Fourier transform infrared spectrum of pure caffeine

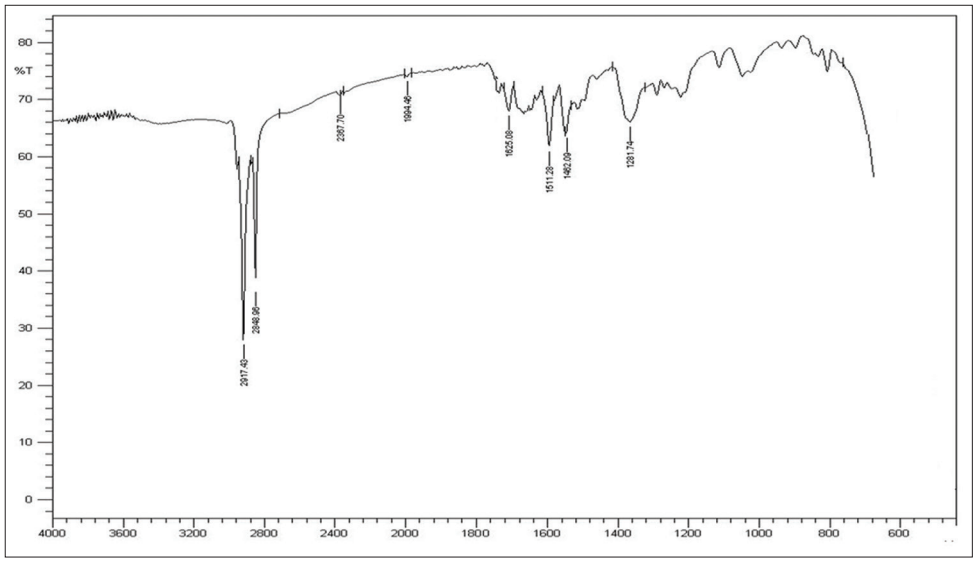

Fig. 6: Fourier transform infrared spectra of formulation F2

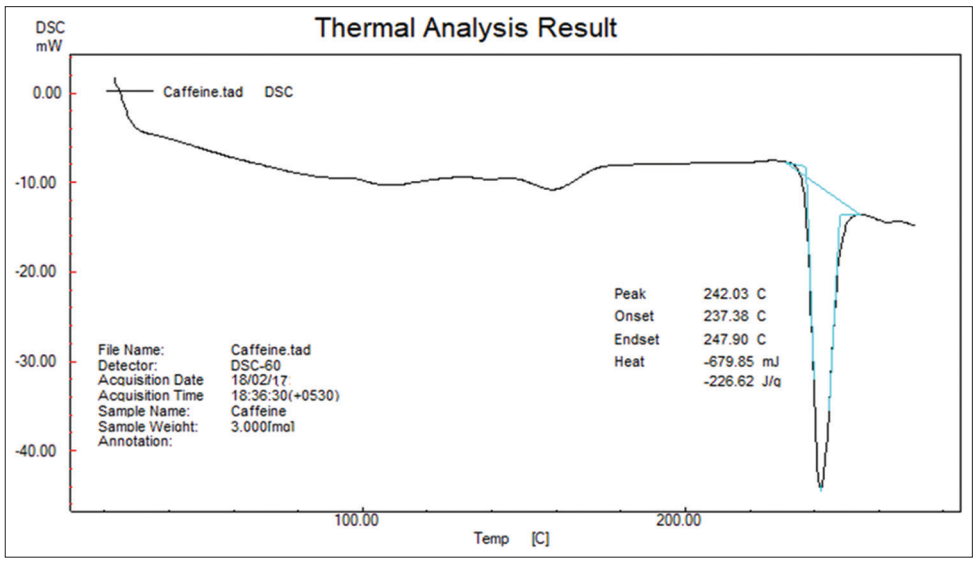

Fig. 7: Differential scanning calorimetry thermogram of caffeine

Table 5: Evaluation parameters of F2 nanogel

S. No.	Evaluation parameter	Results
1.	pH	7.4
2.	Homogenicity	Clear and transparent
3.	Spreadability	Good
4.	Rheological studies (cp)	10,282

Table 6: *In vitro* % drug diffusion studies

S. No.	Time (h)	F2	Marketed
1	0	0	0
2	0.5	25.1	28.84
3	1	39.2	36.77
4	2	49.6	39.97
5	4	56.3	47.55
6	6	60.7	72.68
7	8	67.7	-
8	12	72.4	-

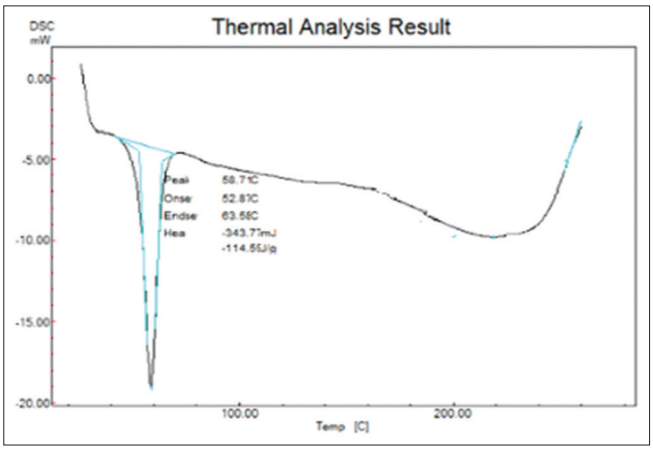

Fig. 8: Differential scanning calorimetry thermogram formulation F2

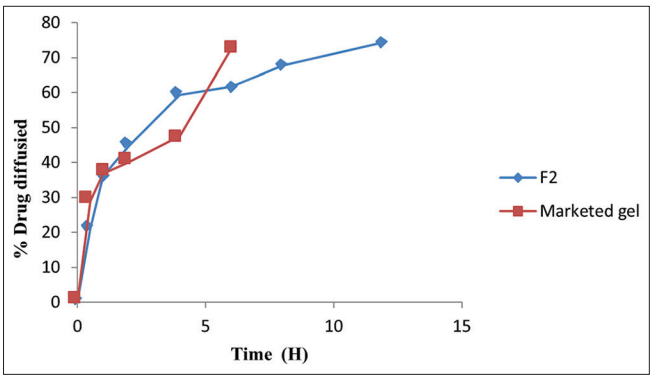

Fig. 9: *In vitro* % drug diffusion studies

CONCLUSION

Nanogel that contains caffeine SLNs with 1:2 ratio drug:lipid has shown good *in vitro* release. Sustained release of caffeine drug till 12 h was achieved by delivering it in the form of nanogel.

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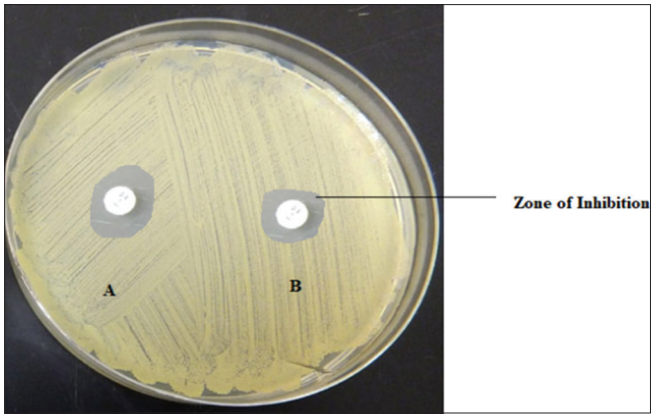

Fig. 10: Antimicrobial activity

AUTHORS' CONTRIBUTIONS

All the authors contributed equally in preparation of manuscript.

CONFLICTS OF INTEREST

Authors have none to declare.

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Research Article

FORMULATION AND EVALUATION OF CREAMS CONTAINING CELECOXIB INCLUSIONS FOR TREATING PSORIATIC ARTHRITIS

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ABSTRACT

Objective: The main objective of the present work was to prepare and evaluate creams of celecoxib inclusions which are nonsteroidal anti-inflammatory drug to treat psoriatic arthritis.

Methods: Celecoxib inclusions were prepared using β-cyclodextrin to increase the solubility. These inclusions were incorporated in creams. Cocoa butter, triethanolamine, stearic acid, methylparaben, gum acacia, and coconut oil were used in cream preparation. Fourier transform infrared and differential scanning calorimetry studies were carried out for pure celecoxib and inclusions. Viscosity, pH, homogeneity, and type of emulsion under dye test were evaluation parameters done for creams.

Results: All the results obtained were within the limits and confirmed increased solubility of celecoxib inclusions.

Conclusion: From the results obtained, increase in solubility of celecoxib drug was confirmed by forming inclusions using β-CD as polymer.

Keywords: β-cyclodextrin, Psoriatic Arthritis, Creams, Celecoxib, Inclusions.

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INTRODUCTION

Psoriatic arthritis (PsA) is a chronic, systemic, and inflammatory disease that affects peripheral joints, connective tissues, and axial skeleton and is associated with psoriasis of skin and nails [1,2]. PsA is a seronegative inflammatory arthropathy that occurs in 5–7% of people with psoriasis [3,4]. It is thought that environmental triggers which are infectious in nature, triggers the disease in genetically susceptible individuals [5]. Currently available therapies for PsA are surgery, light therapy, narrowband ultraviolet (UV) B phototherapy, excimer laser, and psoralen and ultraviolet A (PUVA) [6]. Available drugs are corticosteroids, tacrolimus, tumor necrosis factor inhibitors, methotrexate, and sulfasalazine [7]. The disadvantages of these drugs are the radiation can dry out the skin and cause itching and redness, burning, blistering of the skin, etc. [8]. Celecoxib is a cyclooxygenase-2 (COX-2)-specific inhibiting agent that inhibits the conversion of arachidonic acid to the prostaglandins that mediate pain and inflammation [9]. In theory, a drug such as celecoxib that selectively inhibits COX-2 might block inflammation, pain, and fever [10]. Creams refer to disperse systems, in which one insoluble phase is dispersed as droplets in a second liquid phase. Creams are often preferred over other topical preparations because they are less irritating and easy to apply [11]. β-cyclodextrins are widely used in the pharmaceutical field for their ability of improving the solubility and the stability of drugs by complex formation at the solid state [12]. The use of cyclodextrins and their derivatives can encapsulate the bioactive compounds and protect the compounds from environmental conditions and improves the aqueous solubility and increases their capacity to function [13]. In the present study, an attempt has been made to develop inclusions (complexes) of celecoxib using β-CD. These inclusions are prepared to increase the solubility of the drug which is poorly water soluble. Prepared inclusions are incorporated into creams to obtain formulations for which further evaluation studies have been conducted.

METHODS

Celecoxib is a gift sample obtained from Wexford Laboratories Pvt., Ltd., Bengaluru. β-cyclodextrin was obtained from HiMedia, India. Cocoa butter was procured Rolex Chem Industries, India. Stearic acid and methylparaben were obtained from Qualikems Fine Chemicals Pvt. Ltd., Vadodara. Gum acacia was obtained from SD Fine-Chem Limited, Mumbai, triethanolamine from Pallav Chemicals and Solvents Pvt., Ltd., Boisar, India, and coconut oil from Herbs Nutri Products Pvt., Ltd., Mumbai.

Methodology

Solubility studies of celecoxib in water and in pH 7.4 buffer

Drug solubility was determined by adding excess amount of pure celecoxib in water and pH 7.4 phosphate buffer at 37±0.5°C in vials, respectively. These vials were kept in orbital shaker for 24 h at 100 rpm. Final solution was filtered through membrane filter 0.45 μm. The concentration of the samples was measured using UV–visible spectrophotometer (UV 1800, Shimadzu, Japan). Each sample was analyzed in triplicate.

Analytical method for celecoxib

Calibration curve in pH 7.4 phosphate buffer

From the standard solution, a stock solution was prepared to give a concentration of 100 μg/ml in 7.4 buffer. Aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 ml from the stock solution were pipetted out into 10 ml volumetric flasks. The volume was made up to the mark with 7.4 buffer. These dilutions gave 5, 10, 15, 20, and 25 μg/ml concentration of celecoxib, respectively. The absorbance of prepared solutions of celecoxib in 7.4 buffer was measured at 252 nm spectrophotometrically against 7.4 buffer blank. Standard plot data of celecoxib in 7.4 pH buffer are reported in Fig. 1.

Fourier-transform infrared (FT-IR) spectroscopy analysis

FT-IR analysis was conducted to verify the interaction between drug and polymer. The sample powder was dispersed in KBr powder and

pellets were made by applying 4 kg/cm^2 pressure. FT-IR spectra were obtained by powder diffuse reflectance on a FT-IR spectrophotometer type 8400S Shimadzu. It was performed for pure celecoxib drug and F2 formulation.

Differential scanning calorimetry (DSC)
DSC was performed on pure drug and its formulations using DSC-60 instrument. Calorimetric measurements were made with empty cell (high purity alpha alumina discs) as the reference. The dynamic scans were taken in nitrogen atmosphere at the heating rate of 10°C min^{-1}. The energy was measured as J/Kcal. It was performed for pure celecoxib drug and F2 formulation.

Preparation of celecoxib cream
Oil-in-water (O/W) emulsion-based cream (semisolid formulation) was formulated. The emulsifier (stearic acid) and other oil-soluble components (cocoa butter, acetyl alcohol, and coconut oil) were dissolved in the oil phase (Part A) and heated to 75°C. The preservatives and other water-soluble components (methylparaben, triethanolamine, propylene glycol, and drug celecoxib inclusions) were dissolved in the aqueous phase (Part B) and heated to 75°C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring and perfume was added [14]. The formulations of different creams prepared are given in Table 1.

Evaluation of creams
Type of emulsion under dye test
The scarlet red dye is mixed with the cream. A drop of the cream was placed on a microscopic slide and then it was covered with a cover slip and examined under a microscope. If the disperse globules appear red and the ground is colorless, the cream is O/W type. The reverse condition occurs in W/O type cream, i.e., the disperse globules appear colorless in the red ground [15].

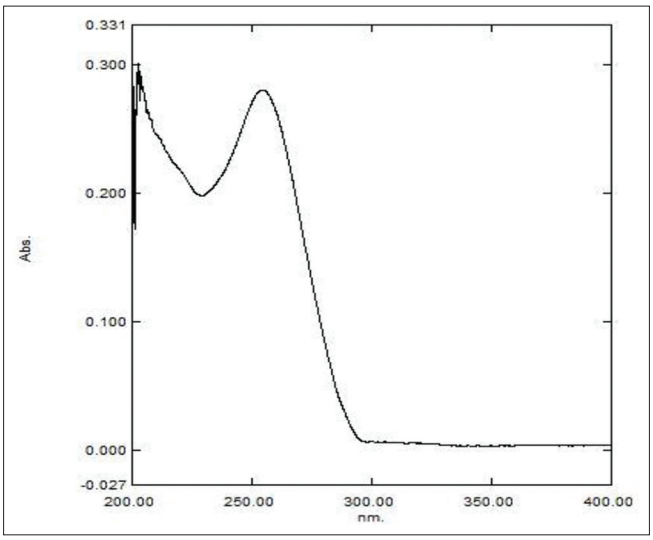

Fig. 1: Ultraviolet spectra of celecoxib in pH 7.4 phosphate buffer

Table 1: Formulation chart of creams (F1-F3)

S. No.	Ingredients	F1	F2	F3
1.	Celecoxib inclusions (mg)	50	50	50
2.	Cocoa butter (g)	1	2	3
3.	Stearic acid (g)	1	2	3
4.	Gum Acacia (%)	0.5	1	1.5
5.	Coconut oil (ml)	2	2	2
6.	Methylparaben (%)	1	1	1
7.	TEA (ml)	0.5	0.5	0.5

Appearance
The appearance of the cream was judged by its color, opalescence, and roughness and graded [15].

Homogeneity
The formulations were tested for the homogeneity by visual appearance and by touch [15].

pH
The pH meter was calibrated using standard buffer solution. About 0.5 g of the cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured [15].

Viscosity
Viscosity of the formulation was determined by Brookfield Viscometer at 100 rpm, using spindle no. 7 [15].

In vitro drug diffusion studies
The F2 cream was permeated through a dialysis bag. A 0.5 g of cream was taken in the bag and is placed in a beaker containing phosphate buffer of pH 7.4 and constantly stirred with a small magnetic bead. During the experiment, temperature was maintained at 37±0.5°C to simulate the human skin condition. A 5 ml of samples were withdrawn at 0.5, 1, 2, 6, and 12 h and replaced with fresh receptor solution. The samples withdrawn were analyzed spectrophotometrically at 235 nm. The amount of drug released was calculated and the percentage drug released was plotted against time.

Similarly, it was done for pure drug.

Statistical analysis (Analysis of variance [ANOVA])
Both the study and control groups were compared. ANOVA is performed for cream F2 and pure drug to determine the differences among the products tested.

RESULTS AND DISCUSSION

Solubility studies of celecoxib in water and in pH 7.4 buffer
From the results obtained, it was confirmed that solubility of celecoxib is more in pH 7.4 buffer. Hence, further studies were carried out using pH 7.4 buffer. The results are given in Table 2.

Evaluation studies
Type of emulsion
Prepared cream was found to be O/W emulsion. It shows that the cream has good spreadability and penetration on applying on the skin. Furthermore, it can be washed of easily.

Appearance
Prepared creams were light cream in color, opaque.

Homogeneity
Prepared creams are homogenous. No aggregates are present in the formulation.

pH
pH of the prepared creams was in the range of 6–7. It shows that obtained pH is near to skin pH and it does not cause any skin irritation.

Table 2: Solubility studies for celecoxib

Concentration of celecoxib in water (µg/ml)	Concentration of celecoxib in pH 7.4 (µg/ml)
6.87 (0.01)	13.55±0.0057

*n=3 [16]

Viscosity

It was carried out by Brookfield Viscometer. Obtained viscosity was in the range of 2020–2060. It shows that F3 has higher viscosity (2060) and F1 has lowest (2020).

All the obtained results are given in Table 3.

From the above results, it was found that F2 cream has high viscosity. Hence, it is considered for further studies such as FTIR and DSC. F2 cream formulation is shown in Fig. 2.

FT-IR studies

The characteristic peaks of pure celecoxib and F2 formulation are shown in Fig. 3 and 4. From the data, it was observed that characteristic peaks of drug appeared almost similar in F2 spectrum with disappearance of some peaks. Hence, it can be inferred that there is no chemical interaction between drug and polymer and it can be concluded that the characteristic bands of pure drug were not affected by β-cyclodextrin and method used for preparation.

DSC

Thermogram of pure drug has shown a sharp endothermic peak at 163.10°C, which corresponds to its melting point and is represented in Fig. 5. Formulation F2 has shown endothermic peak at 160.10°C which corresponds to the melting point of the drug and it is represented in Fig. 6. The DSC thermograms revealed that there was significant difference between the drug and the excipients. From the thermograms, it was evident that melting point of celecoxib was changed when it was formulated as complex. This was due to thermal transition behavior. Decrease in the melting point of the drug was due to decrease in the crystallinity of the compound and also might be due displaced peak of drug.

Fig. 2: Prepared cream formulation (F2)

***In vitro* drug release studies**

In vitro drug release studies were carried out for both F2 formulation and pure drug. Pure drug has shown drug release for 8 h and after that no release was noticed, whereas F2 formulation has shown release till 24 h (Max 91.78%). In case of F2 formulation, drug release occurred in prolonged manner. In the beginning, drug which is not encapsulated in the inclusions got released. This is the reason for increased % drug release during initial hours. Then, after attaining saturation state, drug release from inclusions occurred. This was seen till 24 h. However, in case of pure drug, no encapsulation of drug is seen hence release occurred till 8 h and also since celecoxib is poorly water soluble, drug release was also less. Drug release of both F2 formulation and pure drug is given in Table 4 and Fig. 7.

Statistical analysis (ANOVA)

ANOVA shows that *in vitro* results obtained by comparison between F5 and pure drug formulations have shown statistically significant differences ($p < 0.05$), as shown in Table 5.

Table 3: Evaluation results of creams

S. No.	Evaluation studies	F1	F2	F3
1.	Appearance	Light cream	Light cream	Light cream
2.	Homogeneity	No aggregates	No aggregates	No aggregates
3.	Viscosity	2020	2040	2060
4.	pH	6	7	7

Table 4: *In vitro* % drug release

S. No.	Time	F5	Pure drug
1.	0	0.00	0.00
2.	0.5	89.72	14.23
3.	1	76.98	28.45
4.	2	63.42	41.67
5.	4	59.43	54.82
6.	8	71.12	67.75
7.	12	82.43	-
8.	24	91.78	-

Table 5: Statistical analysis (ANOVA)

Newman–Keuls multiple comparison test	Mean difference	q	Significant ($p<0.05$)
F2 versus pure drug	−20.16	37.42	Yes

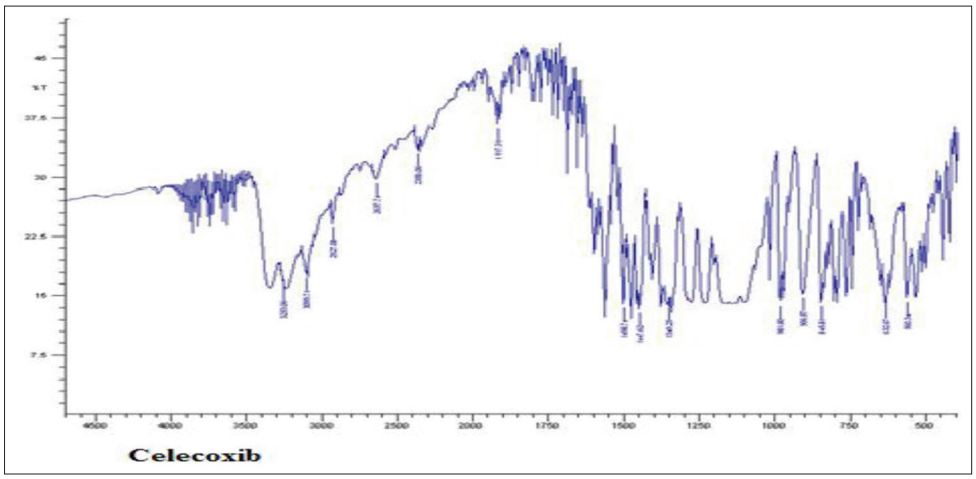

Fig. 3: Fourier transform infrared spectral data of pure celecoxib

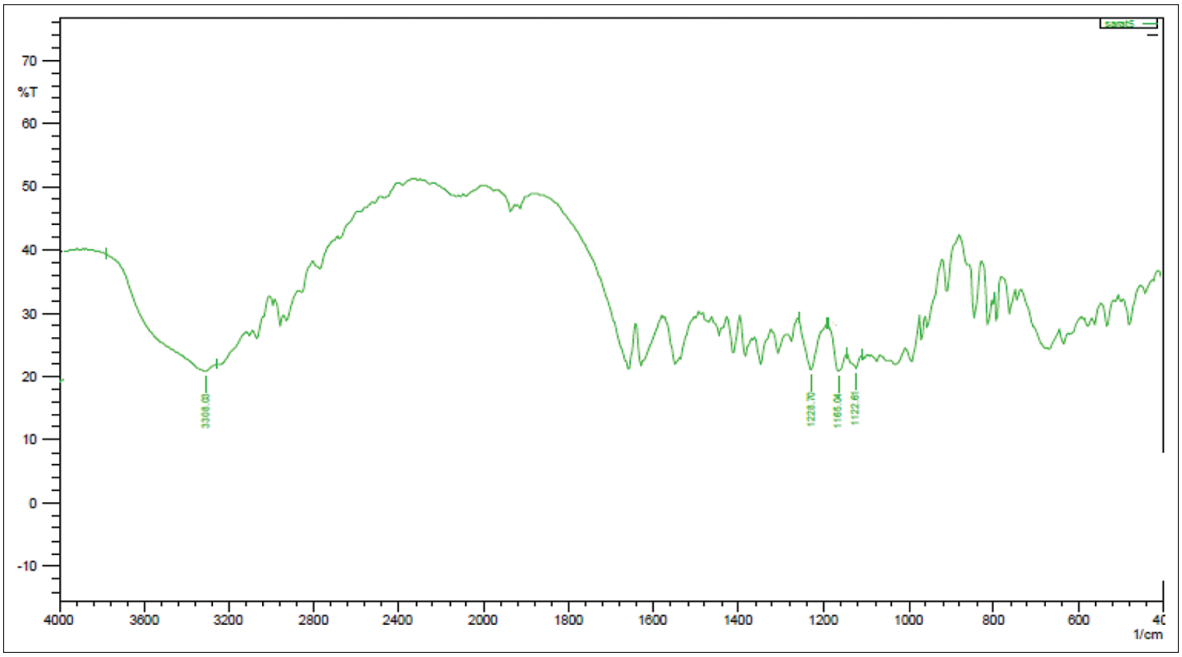

Fig. 4: Fourier transform infrared spectra of F2 formulation

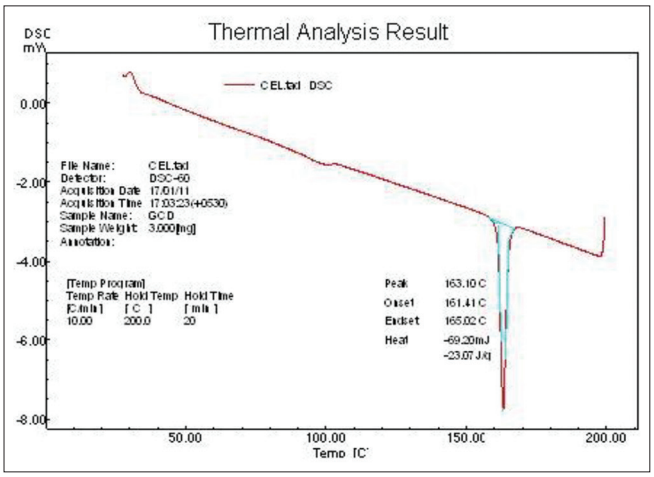

Fig. 5: Differential scanning calorimetry thermogram of pure celecoxib

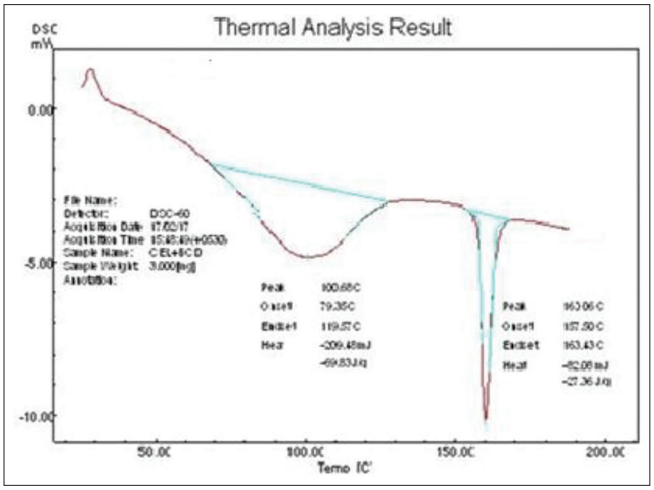

Fig. 6: Differential scanning calorimetry thermogram of F2 formulation

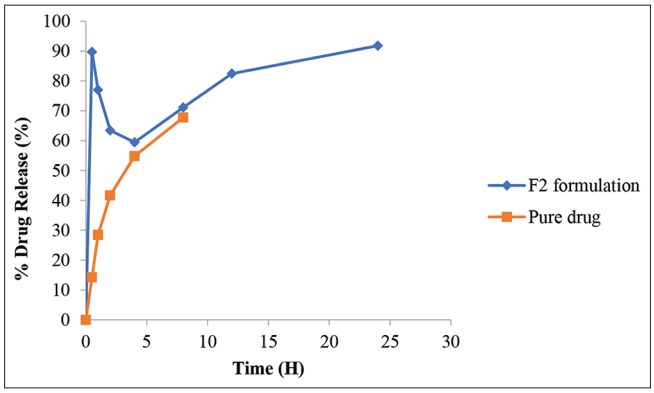

Fig. 7: Comparison of drug release between F2 formulation and pure drug

CONCLUSION

Solubility of celecoxib drug was increased by forming inclusions using β-CD as polymer. This further leads to increased bioavailability. From FT-IR and DSC studies, compatibility between drug and polymer was confirmed. All the results obtained from evaluation parameters found to be within the limits and state that prepared creams were having good spreadability and no skin irritation on topical use. Prolonged release of celecoxib drug from 8 h to 24 h was achieved. This helps in decreasing the dose frequency of drug thus increases patient compliance.

ACKNOWLEDGMENTS

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AUTHORS' CONTRIBUTIONS

All the authors contributed equally in preparation of manuscript.

CONFLICTS OF INTEREST

Authors have none to declare.

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Research Article

FORMULATION AND EVALUATION OF TRANSDERMAL FILMS CONTAINING CELECOXIB INCLUSIONS FOR TREATING PSORIATIC ARTHRITIS

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ABSTRACT

Objective: The main objective of the present work was to prepare and evaluate transdermal films and creams of celecoxib inclusions which are nonsteroidal anti-inflammatory drug to treat psoriatic arthritis.

Methods: Celecoxib inclusions were prepared using β-cyclodextrin to increase the solubility. These inclusions were incorporated in transdermal films. Hydroxy Propyl Methyl Cellulose (HPMC), dibutyl phthalate, propylene glycol, and glycerin were used to prepare the film. Fourier transform infrared and differential scanning calorimetry studies were carried out for pure celecoxib and inclusions. Morphological properties, weight variation, surface pH, percentage elongation, folding endurance, *in vitro* disintegration, and *in vitro* diffusion studies were carried out for films.

Results: All the results obtained were within the limits and confirmed the prolonged drug release and increased solubility of celecoxib inclusions.

Conclusion: From the results obtained, increase in solubility of celecoxib drug was confirmed by forming inclusions using β-CD as polymer.

Keywords: β-cyclodextrin, Psoriatic Arthritis, Transdermal films, Inclusions, Celecoxib.

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INTRODUCTION

Psoriatic arthritis (PsA) is a chronic, systemic, and inflammatory disease that affects peripheral joints, connective tissues, and axial skeleton and is associated with psoriasis of skin and nails [1,2]. It is a seronegative inflammatory arthropathy that occurs in 5–7% of people with psoriasis [3,4]. Genetic factors have an important role in PsA. It is thought that environmental triggers which are infectious in nature trigger the disease in genetically susceptible individuals [5]. Currently available therapies for PsA are surgery, light therapy, narrowband ultraviolet (UV) B phototherapy, excimer laser, and Psoralen and Ultra Violet A (PUVA) [6]. Available drugs are corticosteroids, tacrolimus, tumor necrosis factor inhibitors, methotrexate, and sulfasalazine [7]. The disadvantages of these drugs are that the radiation can dry out the skin and cause itching and redness, burning, blistering of the skin, etc. [8]. Celecoxib is a cyclooxygenase (COX)-2-specific inhibiting agent that inhibits the conversion of arachidonic acid to the prostaglandins (PGs) that mediate pain and inflammation [9]. The mechanism of action of celecoxib is conversion of arachidonate to the PGs which is important in homeostatic function; COX-2 is present in immune cells, blood vessel endothelial cells, and synovial fibroblasts. Classic nonsteroidal anti-inflammatory drugs inhibit both COX isoenzymes by occupying the COX active site, preventing access by arachidonic acid. In theory, a drug such as celecoxib that selectively inhibits COX-2 might block inflammation, pain, and fever [10]. Transdermal patch generally refers to topical application and delivers agents to healthy intact skin either for localized treatment of tissues underlying the skin or for systemic therapy. Transdermal patch offers many advantages over the conventional dosage forms or controlled release oral systems. Transdermal patch maintains constant blood levels, avoids first pass metabolism, increases patient compliance, and avoids dose dumping [11,12].

METHODS

Celecoxib is a gift sample obtained from Wexford Laboratories Pvt., Ltd., Bengaluru. β-cyclodextrin is a polymer obtained from HiMedia, Mumbai, India. HPMC is a polymer obtained from Rolex Chemical Industries Pvt., Ltd. Dibutyl phthalate is a plasticizer obtained from Pallav Chemicals and Solvents Pvt. Ltd., Biosar, India. Propylene glycol is a solubilizer obtained from Qualikems Fine Chem Pvt., Ltd., Vadodara. Glycerine is a humectants obtained from Simson, Mumbai, India.

Solubility studies of celecoxib in water and in pH 7.4 buffer

Drug solubility was determined by adding excess amount of pure celecoxib in water and pH 7.4 phosphate buffer at 37 ± 0.5°C in vials, respectively. These vials were kept in orbital shaker for 24 h at 100 rpm. Final solution was filtered through membrane filter 0.45 µm. The concentration of the samples was measured using UV–visible spectrophotometer (UV 1800, Shimadzu, Japan). Each sample was analyzed in triplicate.

Analytical method for celecoxib

Calibration curve in pH 7.4 phosphate buffer

From the standard solution, a stock solution was prepared to give a concentration of 100 µg/ml in 7.4 buffer. Aliquots of 0.5, 1.0, 1.5, 2.0, and 2.5 ml from the stock solution were pipetted out into 10 ml volumetric flasks. The volume was made up to the mark with 7.4 buffer. These dilutions gave 5, 10, 15, 20, and 25 µg/ml concentration of celecoxib, respectively. The absorbance of prepared solutions of celecoxib in 7.4 buffer was measured at 252 nm spectrophotometrically against 7.4 buffer blank. Standard plot data of celecoxib in 7.4 pH buffer are reported in Fig. 1.

Fourier-transform infrared (FT-IR) spectroscopy analysis

Fourier transform infrared analysis was conducted to verify the interaction between drug and polymer. The sample powder was dispersed in KBr powder and pellets were made by applying 4 kg/cm^2 pressure. FT-IR spectra were obtained by powder diffuse reflectance on a FT-IR spectrophotometer type 8400S Shimadzu. It was performed for pure celecoxib drug and F2 formulation.

Differential scanning calorimetry (DSC)

DSC was performed on pure drug and its formulations using DSC-60 instrument. Calorimetric measurements were made with empty cell (high purity alpha alumina discs) as the reference. The dynamic scans

Table 1: Formulation chart of transdermal films (F1-F5)

Formulation	Drug complex (mg)	HPMC (mg)	Dibutyl phthalate (ml)	Propylene glycol (%)	Glycerine (ml)
F1	50	50	0.5	0.5	0.5
F2	50	75	0.5	0.5	0.5
F3	50	100	0.5	0.5	0.5
F4	50	125	0.5	0.5	0.5
F5	50	150	0.5	0.5	0.5

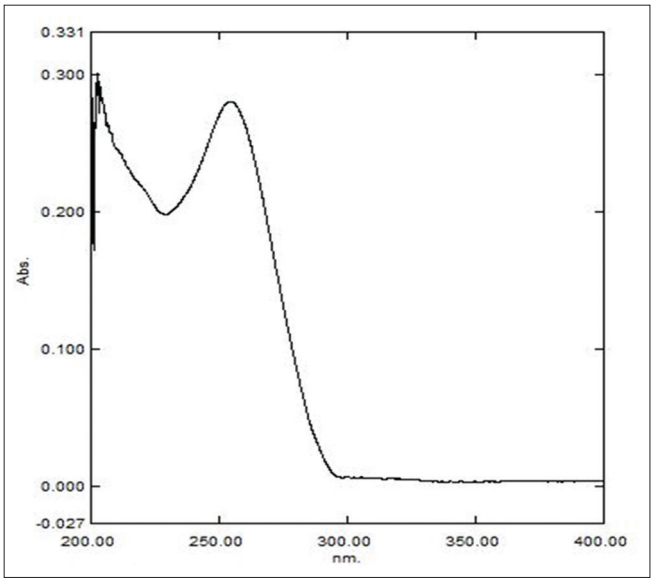

Fig. 1: Ultraviolet spectra of celecoxib in pH 7.4 phosphate buffer

were taken in nitrogen atmosphere at the heating rate of 10°C min^{-1}. The energy was measured as J/Kcal. It was performed for pure celecoxib drug and F2 formulation.

Procedure for preparation of transdermal films

The transdermal films of celecoxib were prepared by solvent casting method. HPMC polymer was soaked in distilled water for 1 h. Calculated amount of celecoxib was added to polymeric solution, followed by propylene glycol, dibutyl phthalate, and glycerin. The mixture was stirred well for uniformity. The resultant mixture was poured into a Petri plate and dried for 24 h. Obtained films were stored in dry conditions for further evaluation studies [13]. Formulation chart of prepared transdermal films (F1-F5) is given in Table 1.

Evaluation of celecoxib films [14]

Morphological properties

Homogeneity, color, and transparency of films were tested visually [14].

Weight variation

Weight of 2 × 2 cm^2 film from different batches of the formulations was noted on electronic balance [14].

Surface pH study

A 2 × 2 cm^2 prepared films were dissolved in a Petri plate, until the film dissolves and the pH of the solution was checked using pH stripes [14].

Percentage elongation (%E)

Percentage elongation was calculated by measuring the increase in length of the film after tensile strength measurement using the following formula [14].

$$\text{Percentage elongation} = [L\text{-}L_0] \times 100/L_0$$

Where, L = Final length, L_0= initial length

Table 2: Solubility studies for celecoxib

Concentration of celecoxib in water (µg/ml)	Concentration of celecoxib in pH 7.4 (µg/ml)
6.87 ± 0.01	13.54±0.0057

*n=3 [15]

Folding endurance

Folding endurance indicates brittleness of the film and was determined by repeated folding of the film at the same place till the film breaks [14].

***In vitro* disintegration time**

The film strip was placed in a glass Petri plate containing 25 ml of distilled water at 37°C with swirling at every 10 s. Disintegration time was recorded as the time at which the film starts to break or disintegrate [14].

***In vitro* drug release method**

Beaker stirring method

These studies were conducted using 150 ml glass beaker with 125 ml of 7.4 pH buffer as dissolution medium. Film was placed on one side of the beaker using double-sided tape. Medium was stirred at a speed of 200 rpm using magnetic stirrer. A 5 ml samples were withdrawn at 0, 1, 2, 4, 8, 12, and 24 h time intervals and every time replaced with 5 ml of fresh medium solution. Samples were analyzed by UV at 252 nm [14].

Similar procedure was used for pure drug also. Drug release from prepared film (F5) was compared with pure drug. All the absorbance values at different time intervals are noted. From that, % drug release was calculated.

Statistical analysis (analysis of variance [ANOVA])

Both the study and control groups were compared. ANOVA is performed for film F5 and pure drug to determine the differences among the products tested.

RESULTS AND DISCUSSION

Solubility studies of celecoxib in water and in pH 7.4 buffer

From the results obtained, it was confirmed that solubility of celecoxib is more in pH 7.4 buffer. Hence, further studies were carried out using pH 7.4 buffer. Results are given in Table 2.

Evaluation parameters

Appearance

Prepared films were transparent. It shows that they were spread equally without any aggregates.

Weight variation

Weight variation of transdermal films of different formulations was between the limits of 0.03 and 0.07 mg.

Percentage elongation

It is carried out using the following equation:

$$\% \text{ elongation} = [L\text{-}L_0] \times 100/L_0$$

Percentage elongation of different formulations was between 5% and 30%. As the percentage increases, the rate of drug release increases. The maximum percentage elongation was seen in F5 formulation.

***In vitro* disintegration time**

The disintegration times for different formulations are between 10 and 65 s. It shows that the results are optimum.

Fig. 2: Prepared transdermal films

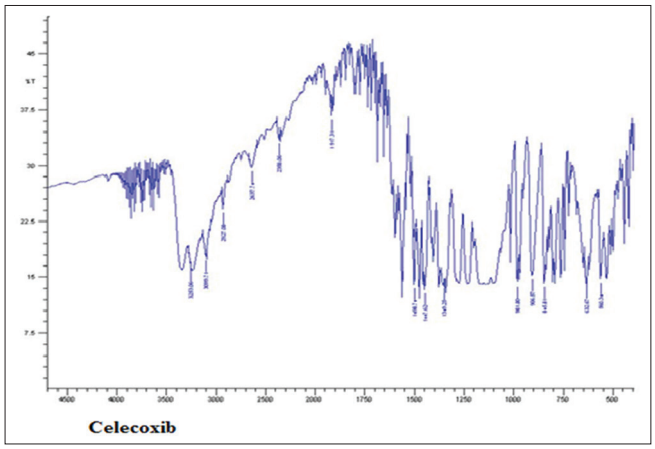

Fig. 3: Fourier transform infrared spectral data of pure celecoxib

Folding endurance

The folding endurance of different formulations was found between 59 and 65. It shows that all the films have optimum elasticity.

Surface pH

The pH of the prepared film was found to between 6 and 7 which is nearer to the pH of the skin; hence, it does not show skin irritation.

All the obtained results are given in Table 3.

From the above results, it was found that F5 film has high folding endurance, good % elongation, and *in vitro* disintegration time. Hence, it is considered for further studies such as FT-IR, DSC, and *in vitro* drug release. F5 formulation film is shown in Fig. 2.

FT-IR studies

The characteristic peaks of pure celecoxib and F2 formulation are shown in Figs. 3 and 4. From the data, it was observed that characteristic peaks of drug appeared almost similar in F2 spectrum with disappearance of some peaks. Hence, it can be inferred that there is no chemical interaction between drug and polymer and it can be concluded that the characteristic bands of pure drug were not affected by β-cyclodextrin and method used for preparation.

DSC

Thermogram of pure drug has shown a sharp endothermic peak at 163.10°C, which corresponds to its melting point and is represented in Fig. 5. Formulation F2 has shown endothermic peak at 160.10°C which corresponds to the melting point of the drug and it is represented in Fig. 6. The DSC thermograms revealed that there was significant difference between the drug and the excipients. From the thermograms, it was evident that melting point of celecoxib was changed when it was formulated as complex. This was due to thermal transition behavior. Decrease in the melting point of the drug was due to decrease in the crystallinity of the compound and also might be due displaced peak of drug.

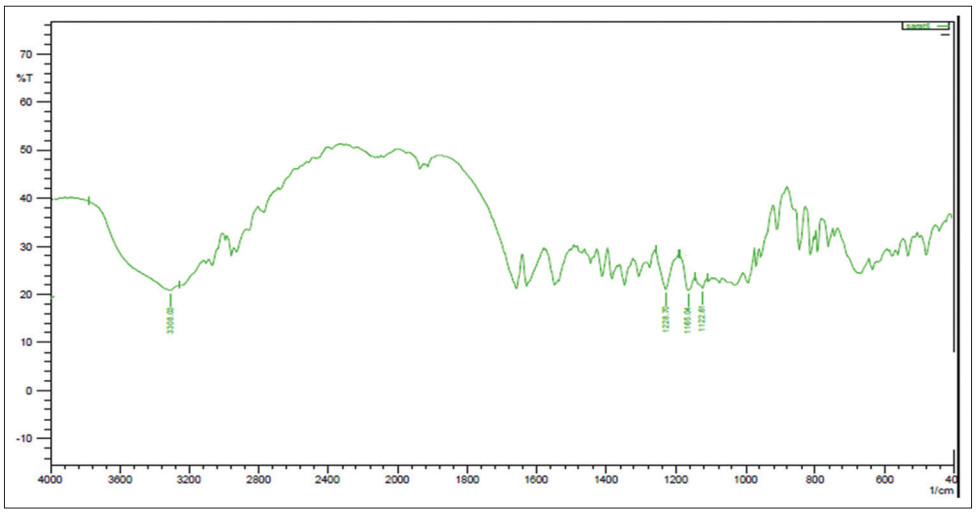

Fig. 4: Fourier transform infrared spectra of F2 formulation

Table 3: Evaluation results of transdermal films

S. No.	Evaluation parameter	F1	F2	F3	F4	F5
1.	Appearance	H and T*	H and T*	H and T*	H and T*	H and T*
2.	Weight variation (mg)	0.03	0.05	0.05	0.06	0.07
3.	Folding endurance	59	61	57	63	65
4.	*In vitro* disintegration time (s)	11	22	42	51	60
5.	% elongation	5	10	15	20	30
6	pH	4	5	4.5	5	6

*H and T: Homogenous and transparent

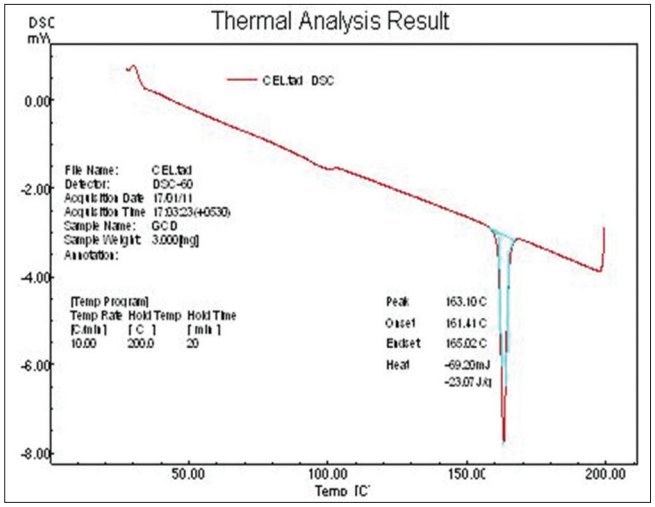

Fig. 5: Differential scanning calorimetry thermogram of pure celecoxib

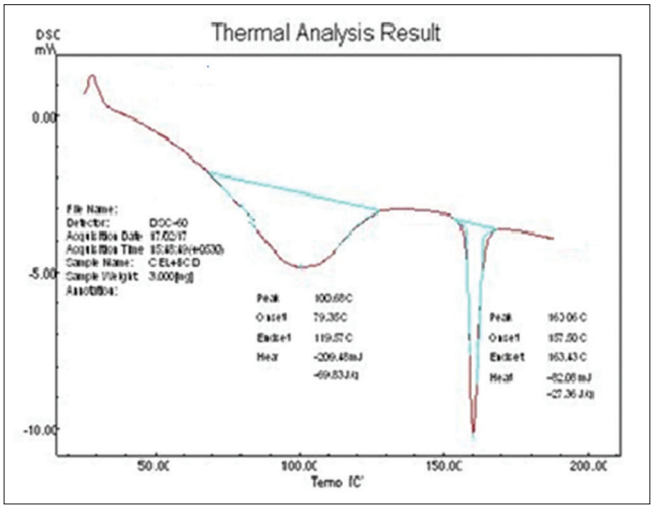

Fig. 6: Differential scanning calorimetry thermogram of F2 formulation

In vitro drug diffusion studies

In vitro drug diffusion studies were carried out for both F5 formulation and pure drug. Formulation containing pure drug has shown drug release for 8 h and after that, no release was noticed, whereas F5 formulation has shown release till 24 h (Ma×93.05%). In case of F5 formulation, drug release occurred in prolonged manner. At the beginning, drug which is not encapsulated inside the inclusions released that has led to increased % release of drug. Then, after attaining saturation state, drug release from inclusions occurred. This was seen till 24 h. However, in case of pure drug, no encapsulation of drug is seen; hence, release occurred till 8 h and also since celecoxib is poorly water soluble, drug release was also less. Drug release of both F5 film formulation and pure drug is given in Table 4 and Fig. 7.

From the obtained diffusion studies, it was confirmed that drug which is not entrapped inside the cyclodextrin cup is released in the beginning 4 h and then release from cyclodextrin cup occurred. This release was prolonged till 24 h.

Statistical analysis (ANOVA)

ANOVA shows that *in vitro* results obtained by comparison between F5 and pure drug formulations have shown statistically significant differences ($P < 0.05$), as shown in Table 5.

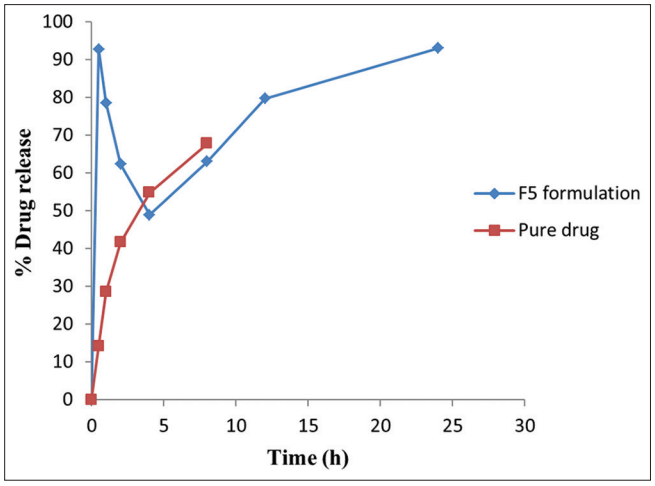

Fig. 7: Comparison of drug release between F5 formulation and pure drug

Table 4: *In vitro* % drug release

S. No.	Time	F5	Pure drug
1.	0	0.00	0.00
2.	0.5	92.81	14.23
3.	1	78.58	28.45
4.	2	62.34	41.67
5.	4	48.95	54.82
6.	8	63.02	67.75
7.	12	79.71	-
8.	24	93.05	-

Table 5: Statistical analysis (analysis of variance)

Newman–Keuls multiple comparison test	Mean difference	q	Significant ($p<0.05$)
F5 versus pure drug	−21.16	39.42	Yes

CONCLUSION

Solubility of celecoxib drug was increased by forming inclusions using β-CD as polymer. This further leads to increased bioavailability. From FTIR and DSC studies, compatibility between drug and polymer was confirmed. All the results obtained from evaluation parameters found to be within the limits and state that no skin irritation and prepared films were ideal for topical use. Prolonged release of celecoxib drug from 8 h to 24 h was achieved. This helps in decreasing the dose frequency of drug thus increases patient compliance.

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AUTHORS' CONTRIBUTIONS

All the authors contributed equally in preparation of manuscript.

CONFLICTS OF INTEREST

Authors have none to declare.

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Original Article

DEVELOPMENT AND EVALUATION OF NANOSPONGE LOADED TOPICAL HERBAL GEL OF WRIGHTIA TINCTORIA

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ABSTRACT

Objective: Psoriasis is the most common chronic autoimmune disease. The main objective of the present study was to develop Nanosponge (NS) based Topical gels of *Wrightia tinctoria* extract using cross-linker and polymer by melting method. It is used in treating Psoriasis.

Methods: Phytoconstituents present in the herbal extract were identified by Liquid Chromatography-Mass Spectroscopy (LC-MS) studies. *In vitro* drug release and Entrapment Efficiency of all NS were determined. The optimized NS were incorporated in the gel to formulate nano topical gel. Evaluation studies like homogenicity, viscosity, spreadability, pH and *In vitro* studies were carried out for all gel formulations.

Results: The prepared gels were transparent, had good viscosity and spreadability. SEM photographs confirmed that the prepared formulation were roughly spherical and porous in nature. *In vitro* diffusion studies showed drug release of 92.15% in 24 h.

Conclusion: From this study it can be concluded that constituents responsible for treating psoriasis are present in the obtained extract and prepared NS based topical gel has significant effect in providing sustained drug release.

Keywords: LC-MS, Nanosponges, Olibanum gum, Psoriasis, Topical gel, Wrightia tinctoria

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INTRODUCTION

Psoriasis is a skin disease which is distinguished by massive proliferation, thick inflammatory cell infiltrates, generation of new blood vessels, modifications in lymphatic structure and impaired differentiation of epidermis. It is an autoimmune disorder where environment and genetic components have a Dimethylformamide function. The immune system releases proinflammatory cytokines and growth factors that accelerate the growth of skin cells which accumulate and form thick red patches of skin on various parts of the body [1-3].

Nanoparticles are the colloidal systems with particle size varying from 10 nm to 1000 nm. These are nano or sub-nano-sized structures which are made of synthetic or semi-synthetic polymers. Nanoparticles of plant medicine are gaining a lot of attention currently. Different types of nanoparticles are Polymeric nanoparticles, Solid lipid nanoparticles, Liposomes, Proliposomes, Niosomes, Liquid crystalline systems, Quantum dots etc. But all these nanoparticles have certain disadvantages like drug loading, toxicity etc [2].

Nanosponges (NS) can conquer these problems. They solubilize poorly water-soluble drug and provide prolonged release as well as improves drugs bioavailability. They can load both hydrophilic and hydrophobic drug molecules because of their inner hydrophobic cavities and external hydrophilic branching. They can move in the body until they come across the exact target site and attach on the surface and start releasing the drug in a controlled manner [5, 6].

In preparation of NS cyclodextrins are the most preferable polymer because these are having more capacity to increase the solubility of poorly soluble drugs when compared to other polymers. Cyclodextrins are nanometric biomaterials with a close relationship between molecular status and supramolecular properties. They are a class of cyclic glucopyranose oligomers and are synthesised by enzymatic action on hydrolysed starch [7].

For delivery of NS, topical gels are very attractive drug delivery systems. In-gel preparation usually synthetic and semi-synthetic polymers are used which are expensive and less biocompatible. Plant products serves as alternative to synthetic products because of local accessibility, environmentally friendly nature, lower prices and non-toxic compared to imported synthetic products. Olibanum gum which can be used as a natural polymer in gel preparation, is a dried, gummy exudation obtained from *Boswellia serrate* (family: *Burseraceae*). Gum Olibanum is used as an anti-inflammatory remedy. It controls the drug release rate from the dosage form and thus acts as a sustained release polymer [8].

In the present study, NS of *Wrightia tinctoria* are prepared which are delivered by incorporating into gel. *Wrightia tinctoria* (Pale indigo) belongs to the family Apocynaceae. Methanolic extract of seeds is used. The most important bioactive principles in seeds are Quercitin, Lupeol, α-amyrin, β-amyrin, wrightial and β-sitosterol. Different activities exhibited by this plant are wound healing, anti-bacterial, antifungal, anti-inflammatory, antiviral, anti-cancer, anti-diabetic, anti-ulcer, antipsoriatic [9, 10].

This research study states that Wrightia extract along with Olibanum gum as a polymer in the form of NS gives sustained release of drugs and exhibits promising antipsoriatic activity. The novelty of this work is this combination of drug and polymer has not been reported till date.

MATERIALS AND METHODS

Wrightia tinctoria extract was obtained from Sri Nidhi Industries, Mysuru, India. Olibanum gum was bought from Govinda Raj Shetty stores, Mysuru, India. β-CD was obtained from Himedia, Mumbai, India. Dimethyl carbonate and carbopol were taken from Loba Chemie Mumbai. All other reagents used were of analytical grade.

Phytochemical analysis

Qualitative analysis of phytochemicals

Preliminary phytochemical screening was performed for herbal extract. Presence of various phytochemicals was tested [9-11].

Identification of phytoconstituents in extracts using LC-MS studies

The herbal extract was analyzed by LC-MS method in order to identify different constituents present in them. Specifications of the instrument used in the study were as below: [8, 11, 12].

Specifications

LC column: ACQUITY UPLC BEH C18 1.7 µm**,** Solvent selection A: 0.1%Formic acid in water, B: Acetonitrile**,** Mobile Phase: Water: Methanol**,** Ionization Mode: ES+**,** Mode: Positive, Injection Volume: 2 microlitre**,** Column Dimension: 25 cm×2.5 mm**,** Mass range: 50, 1500Software: 1.40.2532.

Experimental methods

Synthesis of drug-loaded NS by melting method

Three formulations of NS containing β-cyclodextrin and Dimethyl carbonate (Cross linker) in 1:2, 1:4, and 1:8 ratios were prepared as shown in table 1. Calculated amount of β-cyclodextrin was dissolved completely in Dimethyl formamide (DMF) in a round bottom flask. Dimethyl carbonate (DMC) was added and the solution was allowed react for 4 h at 100 °C. This mixture was ground in a mortar and extracted with water and ethanol in order to remove impurities and unreacted DMC. After purification, 100 mg of prepared NS were mixed with water and sonicated for 1 h in order to avoid aggregates. The sample was dried thoroughly. Into this aqueous suspension of NS 100 mg drug was dispersed. This suspension was stirred constantly at 2000 rpm for 24 h and complexes were obtained. After complexation, the undissolved drug was separated by centrifugation [13-15].

Table 1: Preparation of NS in different ratio

S. No.	Formulation	Drug (g)	Polymer: cross linker
1.	NS1	1	1:2
2.	NS2	1	1:4
3.	NS3	1	1:8

Preparation of NS loaded the topical gel

Measured amount of carbopol 934 was soaked in water (around 5 ml) for 2 h. It was then neutralized with triethanolamine (TEA) with stirring. Olibanum gum and drug loaded NS of particular amount were dissolved in pre weighed and an appropriate amount of propylene glycol. This was transferred to carbopol container and mixed further for 20 min. The dispersion was kept aside for 60 min for hydrating and swelling. All the samples were allowed to equilibrate for at least 24 h at room temperature prior to performing viscosity studies. Table 2 represents various formulations prepared [16].

Table 2: Composition of different formulations

Formulation	Drug (g)	Carbapol-934 (%)	Olibanum gum (%)	Propylene glycol (ml)	TEA(ml)	Propyl paraben (ml)	Water (ml)
F1	1	0.2	0.5	3	0.5	0.2	Q. S
F2	1	0.3	0.5	3	0.5	0.2	Q. S
F3	1	0.4	0.5	3	0.5	0.2	Q. S
F4	1	0.2	1	3	0.5	0.2	Q. S
F5	1	0.3	1	3	0.5	0.2	Q. S
F6	1	0.4	1	3	0.5	0.2	Q. S

Evaluation studies of NS

Entrapment efficiency (EE)

Accurately weighed quantity of 100 mg NS was dissolved in 7.4 pH buffer and stirred up to 10 min to break the complex. The solution was filtered and 2 ml was taken from it and diluted up to 10 ml with 7.4 pH buffer. It was kept aside for a few minutes and absorbance was measured by UV-spectrophotometer at 550 nm [15].

$$EE = \text{Actual drug content in NS} / \text{Theoretical drug content} \times 100$$

***In vitro* drug diffusion studies (For NS)**

In vitro drug release studies were carried out for all formulations using the dialysis membrane method. The membrane was soaked for 24 h in 7.4 pH buffer and receptor compartment filled with buffer and kept for stirring on a magnetic stirrer. NS powder equivalent to 100 mg was loaded in membrane. The speed of stirring was kept constant (600rpm) for 12 h. 5 ml of drug sample was taken at 1 hr interval and replaced with equal amount of freshly prepared buffer. The drug analysis was done using UV-Spectrophotometer at 550 nm. Drug release amount was calculated [15, 17-19].

Based on the above evaluation results, optimized formulation was chosen and SEM study was performed. This formulation was incorporated into gel and further evaluation studies for nanogel were carried out.

Scanning electron microscopy (SEM)

The surface morphology of formulations was determined using a scanning electron microscope. Samples were mounted on aluminum mount, using the double-sided adhesive tape and sputtered by gold under vacuum and were scanned at an accelerating voltage of 15 KV before observation [15].

Particle size and zeta potential analysis

The average particle size distribution and charge of the resulting nanoparticles were determined by dynamic light scattering using C: \Microtrac\FLEX 11.0.0.2 Instruments, United Kingdom. The experiment was performed using clear disposable zeta cell, water as a dispersant which has a refractive index (RI)-1.330 and viscosity (cP)-0.898 and the temperature was kept constant at 25 °C. The optimized NS formulation was further incorporated into topical nanogel prepared.

Evaluation of nanogel

Determination of pH

Digital pH meter was used to determine the pH of the gels. Glass electrode was completely placed into the gel system [15-17].

Homogenicity

After placing the gels in the container, all formulations were tested for homogenicity (appearance and presence of any aggregates) by inspecting visually [15-17].

Spreadability test

0.5 gm gel was kept on a glass plate in a 1 cm diameter circle. On this, another glass plate was placed and 500 gm of weight was placed for 5 min above upper glass plate. Spreadability was calculated. Below formula was used to calculate spreadability [15-17].

$$S = M.L/T$$

Where, S = Spreadability, M = Weight tide to upper slide, L= Length of the glass slide, T = Time taken to separate the glass slide completely from each other.

Rheological studies

All measurements were carried out by using parallel plates measuring systems having 50 mm diameter and 1 mm gap at 25 °C. The rheological properties of the formulated gels and nanogel were studied at different rpm and the viscosity was calculated in Pa S [15-17].

***In vitro* drug diffusion studies (For gel)**

Nanogels were permeated through an artificial cellophane membrane. 0.5 gm of nanogel was placed in the donor compartment. The receptor medium consists of pH 7.4 phosphate buffer. The temperature was maintained at 37±0.5 °C to simulate the human skin condition during the experiment. 5 ml of sample was withdrawn at 0.5, 1, 2, 6, 12 and 24 h and replaced with fresh receptor solution. Collected samples were spectrophotometrically analyzed at 550 nm. The drug release amount was calculated [18].

***Ex vivo* permeation study of NS loaded the topical gel**

Animals were purchased from Adita Biosys Private Limited, Tumkur and housed at the institution animal facility. Study was conducted after the approval from the institutional animal's ethics committee, (Proposal No. P12-282/2018). The skin of BALB/c mice was collected and used for *ex vivo* permeation study.

Nanogel containing pure *Wrightia tinctoria* extract and nanogel with optimized NS were prepared and permeated through the dorsal skin of mouse. A procedure similar to that in *in vitro* drug diffusion studies was followed [19].

RESULTS AND DISCUSSION

Phytochemical analysis

Qualitative analysis of phytochemicals

Different phytochemicals present in both the extracts were identified. Results obtained were also given in table 3. From the results, it can be concluded amino acids, alkaloids, flavonoids, tannins, carbohydrates, glycosides, saponins, triterpenoids are present in the extract.

Table 3: Phytochemicals present in methanolic extracts of wrightia

S. No.	Constituents	Test	Obesrvation	Results
1.	Alkaloids	Mayer's test	Formation of creamy precipitate.	Positive
2.	Flavanoids	Lead acetate test	Formation of yellow precipitate	Positive
3.	Carbohydrates	Molish's test	Formation of violet ring at the junction.	Positive
4.	Triterpenoids and steroids	Salwonski test	Presence of steroids is confirmed if the lower layer turns red and that of triterpenoids by Golden yellow layer at bottom	Positive
5.	Deoxy sugars	Killer kiliani's test	Formation of blue color in the acetic acid layer	Positive
6.	Glycosides	Legal's test	Formation of pink to a blood-red color	Positive
7.	Reducing sugars	Benedict's test	Depending on the amount of reducing sugar present in the test solution appears green or yellow or red	Positive
8.	Amino acids	Ninhydrin's test	Formation of blue color	Positive

Identification of phytoconstituents in extracts using LC-MS studies

Base peak Ionization (BPI) Chromatogram of methanolic Wrightia extract was obtained as shown in fig. 1, which states that retention time of constituent β-amyrin (Molecular weight-426.787 g/mol) present in the methanolic extract was around 2.75. Fig. 2 shows the Mass spectrum related to this RT. Similarly, RT of another constituent Quercetin Hexaacetate (Molecular weight-720.721g/mol) was around 4.42. Its Mass spectrum is shown in fig. 3.

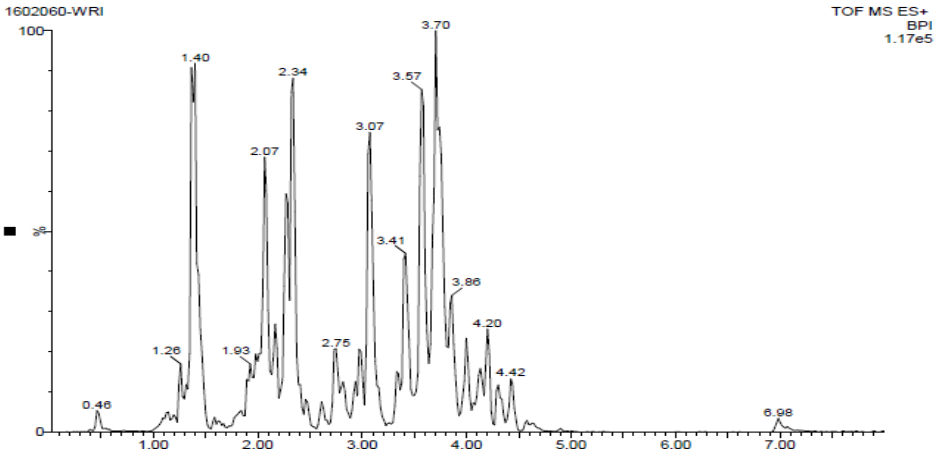

Fig. 1: Base peak ionization (BPI) chromatogram of wrightia

Table 4: Evaluation parameters for nanosponges

S. No.	Formulation	Entrapment efficiency	*In vitro* diffusion studies
1.	NS1	44.14±0.050330	83.34±0.13
2.	NS2	52.28±0.020816	92.67±0.24
3.	NS3	47.31±0.015275	86.21±0.18

*mean±SD = Standard Deviation from the mean, n = 6

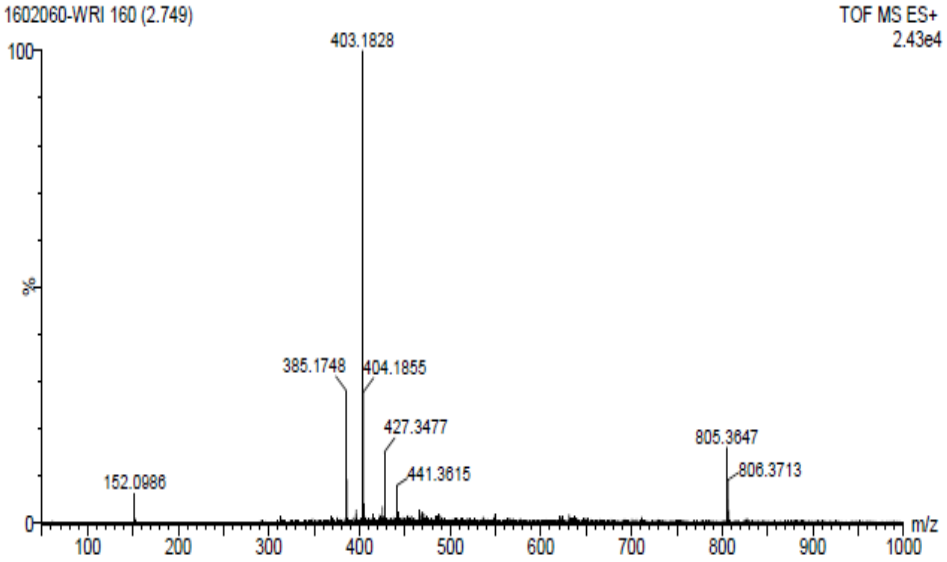

Fig. 2: Mass spectrum of β-amyrin

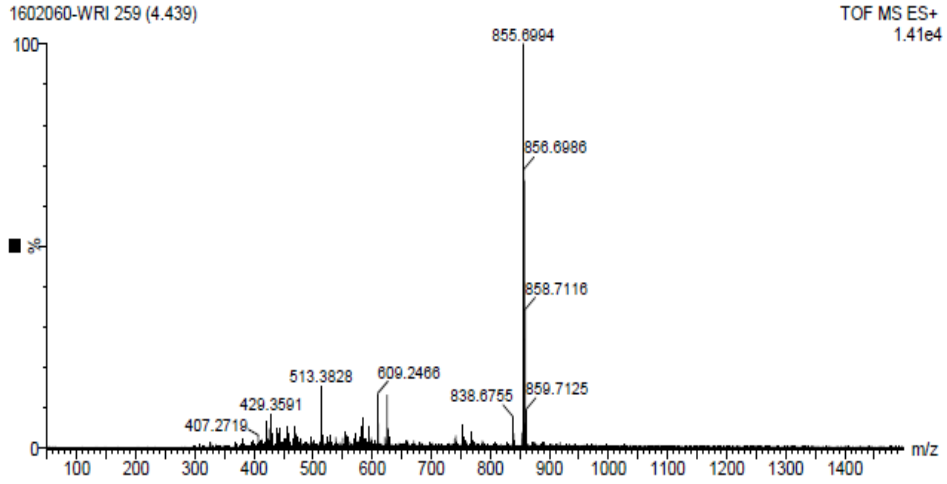

Fig. 3: Mass spectrum of quercetin hexa-acetate

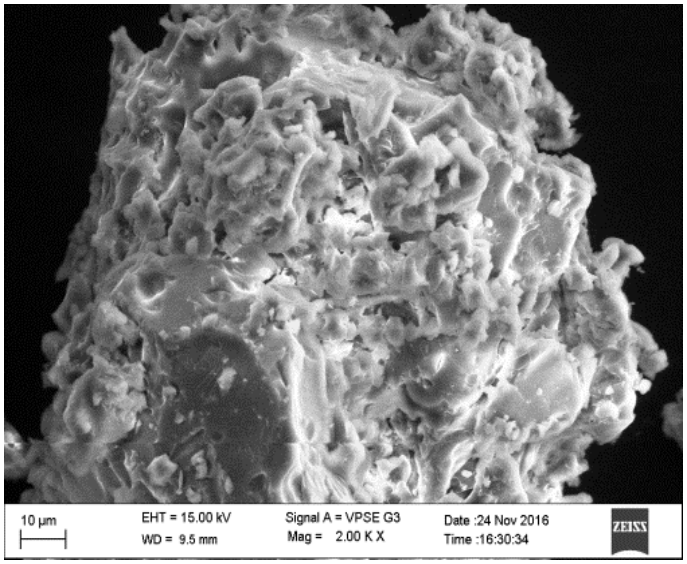

Fig. 4: Scanning electron microscopy (SEM) of NS formulation (NS2)

Evaluation studies of NS

Entrapment Efficiency and *In vitro* dissolution studies were conducted for all formulations prepared by varying the drug: polymer ratio. Results are given in table 4.

Entrapment efficiency shows the amount of drug entrapped in β-cyclodextrin. It was calculated for all NS and ranged from 83.34 % to 92.67%. It changes according to the variation in drug: polymer ratio and depends upon the cross-linker used in the formulation. The highest loading efficiency was found for NS2 formulation, where a greater amount of drug was encapsulated. From the encapsulation studies, it was confirmed that the dimethyl carbonate was considered as an efficient cross-linker for the formulation of NS.

Scanning electron microscopy (SEM)

Scanning electron microscopy for the optimized formulation was performed to check the morphology of nanosponges. The prepared optimized NS (NS2) formulation was roughly irregular in shape and porous in nature. The SEM photograph is shown in fig. 4.

Particle size and zeta potential analysis

The particle size was measured for optimized formulation NS2 by light scattering method using Zeta sizer. Size of a particle is a very important parameter in NLC performance because drug release rate, extend and drug absorption are majorly affected by it. As the particle size decreases, the interfacial area available for drug diffusion increases and thus improve in drug release can be seen. The Ostwald ripening probability was conquered because of narrow size distribution [20].

The mean diameter of formulation NS2 was around 192.5 nm. Particle distribution was given in fig. 5.

In the interaction of formulation with the biological system, zeta potential plays a significant role and it has been reported in various studies [21]. Zeta potential gives the type of charge present on the surface of the NS. This gives the stability of the prepared formulation. The dividing line between the stable and unstable suspensions was generally taken at+30 mV or-30 mV, which were considered as the limits for the zeta potential. The zeta potential for the prepared formulation was found to be 21.5 mv. This indicates that particles are moderately stable.

Evaluation of nanogel

Nanogels prepared were evaluated for different parameters and the values were given in table 5. *In vitro* drug release graph was shown in fig. 6.

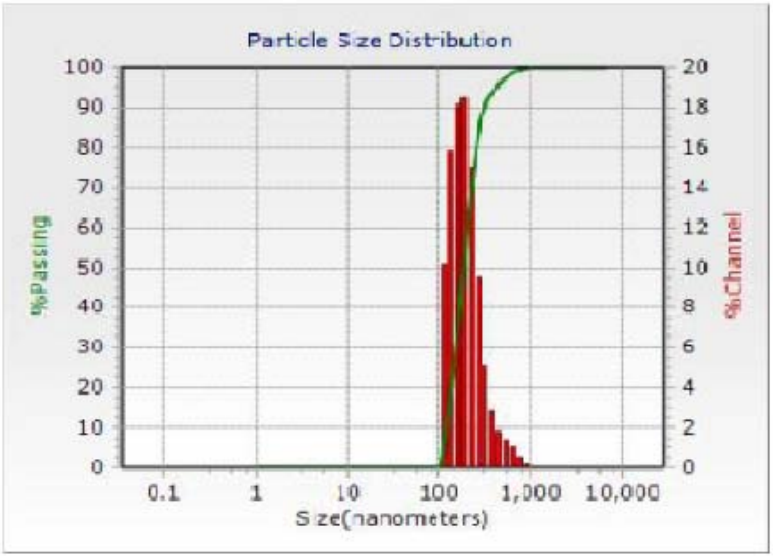

Fig. 5: Particle size distribution of formulation NS2

pH determination

pH values of all gels formulations were found to be between 5.2-7.5, which states that the values were within the range near to that of skin pH. An increase in pH values may lead to skin irritation.

Homogenicity

All the prepared gels were homogenous and clear without any aggregates.

Spreadability

Spreadability is inversely proportional to the viscosity of prepared gel. Spreadability decreased as the polmer and carbopol amounts increased. The spreadability of the prepared carbopol gel formulation was in the range of 3.8 to10.5 gm/cm. These values were represented in table 5. All the formulations showed good spreadability.

***In vitro* drug diffusion studies**

The NS formulations were subjected to *in vitro* release studies. Obtained results shows that F5 formulation containing 0.3% of carbopol-934 and 1% of olibanum gum has shown the drug release of 16.18 % in initial 0.5 h and upto 91.64 % in 24 h. Carbopol 934, when taken in medium amount, provides good viscosity to the gel which further shows good spreadability. An increase or decrease in carbopol percent (0.2% and 0.4%) leads to decreased drug release. Similarly olibanum gum amount when taken in 0.5% amount gives less drug release. For immediate control of disease symptoms, optimum concentration is provided from initial burst release and concentration required for overall treatment is provided by prolonged drug release. The conclusions drawn from the study were supported by the findings of Joshi *et al.* [22]. The results obtained in *in vitro* release studies were plotted as percent cumulative drug release Vs time and shown in fig. 6.

***Ex vivo* permeation study of NS loaded the topical gel**

The formulation containing pure drug extract has shown drug release for 12 h and after that no release was noticed; whereas NS formulation gel has shown release till 24 h (Max 87.15%).

The results obtained in *ex vivo* release studies were plotted as percent cumulative drug release Vs time and shown in fig. 7. Since NS gel is a nano formulation, sustained release of drug is noticed and the release time of this formulation was prolonged.

Table 5: Evaluation parameters for nanogels

Formulation	pH	Homogenicity	Spreadability (gm/cm²)	Viscosity (Pa. S)	*In vitro* diffusion studies (%)
F1	6.2	Clear, transparent	3.82±0.251	0.581	70.23±1.12
F2	5.9	Clear, transparent	4.55±0.035	1.482	78.19±0.98
F3	5.3	Clear, transparent	10.51±0.036	2.852	73.23±0.93
F4	6.1	Clear, transparent	8.64±0.026	0.984	85.22±1.03
F5	7.5	Clear, transparent	5.2±0.041	1.913	91.44±0.76
F6	5. 7	Clear, transparent	9.53±0.025	2.975	82.16±0.94

*mean±SD = Standard Deviation from the mean, n = 6

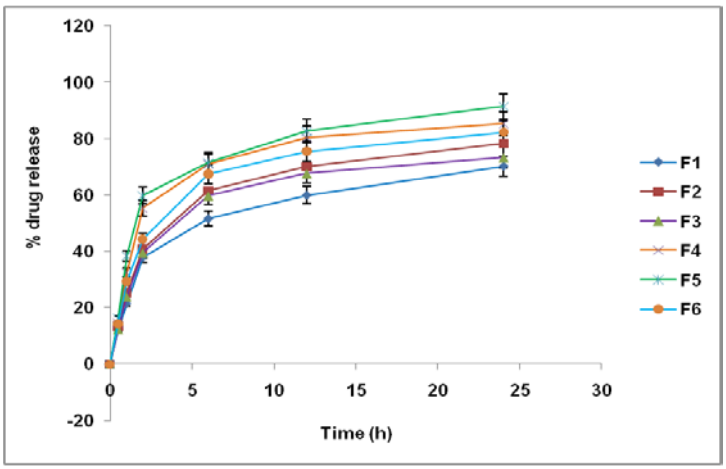

Fig. 6: *In vitro* drug release studies

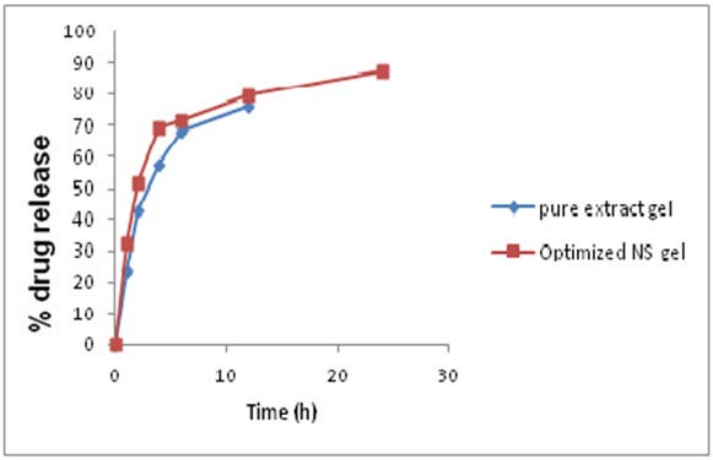

Fig. 7: *Ex vivo* drug permeation studies

CONCLUSION

NS were effective vehicles for delivery of *Wrightia* extract. Olibanum gum, a natural polymer, when used in 1% amount, has proved to show sustained release activity. From all the above results it was concluded that NS with 1:2 ratio polymer and crosslinker has shown good *in vitro* release. Olibanum gum and Carbapol-934 in 1% and 0.3% respectively exhibited good release activity. Sustained release of *Wrightia* extract was achieved till 24 h by delivering in the form of topical nanogel.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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RESEARCH ARTICLE

Formulation and Evaluation of Novel Oral System (Chewing Gum) of Domperidone

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Abstract

Oral Delivery Systems have proven through time as best kind of approach for delivering various drugs. But certain drugs given by oral route undergo extensive First pass metabolism which leads to low bioavailability, making them less effective. To overcome this Novel Oral Drug Delivery Systems, i.e., Chewing Gums were developed as an alternate for conventional oral systems. These were used for both systemic and local delivery of drugs. These systems have advantages like increased patient compliance, increased Bioavailability and self administration. Domperidone, an anti emetic drug was given in the form of chewing gums in this study. As this drug is poorly water soluble, to enhance its solubility, solubilizers were used in various ratios. For the obtained formulations, evaluation studies were carried out. Weight variation, Drug content values were found to be within standard limits prescribed. *In vitro* studies were carried out using modified disintegration apparatus and all the formulations showed release between 70-90%. *Ex vivo* studies were carried out using porcein buccal mucosa and up to 60% of drug release was found. Drug excipient compatibility was studied by FTIR studies. ANOVA was performed to determine significance between formulations.

INTRODUCTION

Oral route has been the most commonly adopted and most convenient route for the drug delivery. Conventional Oral Drug Delivery Systems are Tablets and Capsules. But they have certain disadvantages like some drugs cause Gastric irritation, some undergo first pass metabolism and difficulty in swallowing for those suffering from Dysphagia.

To avoid these disadvantages an alternate system for conventional dosage forms which can be given through this route are developed like Lozenges, chewing gums, chewable tablets. Among these, chewing gums has been very well received by the patients especially in children.

Chewing gums are defined as " Solid, single- dose preparations with a base consisting mainly of gum that are intended to be chewed but not swallowed, providing a slow, steady release of the medicine contained" (European pharmacopoeia 2004**).** Drug release from dosage form is induced by masticatory process. During chewing the drug contained in the gum product is released from the mass into saliva and is absorbed through the oral mucosa.

Merits of Chewing Gums

Chewing gum has advantages like it avoids first pass metabolism and increases Bioavailability. Does not require water to swallow so can be easily taken during journey. It is easy for patients having difficulty in swallowing. Fast onset of action is seen due to absorption into systemic circulation. Useful in overcoming conditions like Xerostamia

(Dry mouth syndrome) caused by some diseases. Treatment can be terminated at any time, if required. (Conway, B et al., 2003 and Morjaria, Y et al., 2004).

Anti emetics are drugs that are effective against Nausea and Vomiting. These drugs are typically used in treating motion sickness, side effects caused by opoid analgesics, general anesthetics and chemotherapy directed against cancer. Anti emetic drugs are available in the form of tablets, capsules, injections, transdermal patches, oral solutions etc.

Most of the anti emetics have side effect called Dry mouth syndrome (Xerostamia).This is common with anti emetics because these drugs decrease the salivary secretion. To overcome this problem a sugar free chewing gum is prescribed along with anti emetic drugs by the physician. Also when drugs are given in tablets or capsules form, they may undergo First pass metabolism and thus reduction in bioavailability is seen.

1. MATERIAL AND METHODS

1.1. Drug and Excipients

Domperidone was obtained from SL Drugs and pharmaceuticals, Hyderabad. Gum base was purchased from Kamco Chew Food Pvt Ltd., Madhya Pradesh. Other excipients such as β-cyclo dextrin and Poly Ethylene Glycol-6000 (PEG-6000) were obtained from SD Fine Chem. Ltd., India. Dibutyl phthalate and Saccharine Sodium from Loba Chemie, Mumbai and Peppermint flavor from Hi-media, Mumbai

1.2. Analytical method for Domperidone

1.2.1. Preparation of 6.8 pH & 7.4 pH phosphate buffers:

Phosphate buffers of 6.8 pH & 7.4 pH were prepared (Indian Pharmacopoeia, 2007)

1.2.2. Calibration curve in 6.8 pH & 7.4 pH phosphate buffers:

100 mg of drug was dissolved in 100 ml of methanol. From this stock solutions of various concentrations, i.e., 1000 µg/ml, 100 µg/ml and 10 µg/ml were prepared. Aliquots of 0.5, 1.5, 2.0, 2.5, and 3.0 were transferred into series of 10 ml volumetric flasks and the volume was made up to 10 ml using 6.8 pH phosphate buffer. The absorbance of these solutions was measured at 284 nm by UV spectrophotometer.

Similar method was followed to prepare 7.4 pH phosphate buffer but volume was made up using 7.4 pH phosphate buffer. (Indian Pharmacopoeia, 2007)

1.3. Saturation Solubility Studies:

These studies were carried out in water, Simulated Gastric Fluid (SGF), Simulated Intestinal Fluid (SIF), 6.8 pH & 7.4 pH phosphate buffers (Indian Pharmacopoeia, 2007).

1.4. Preparation of Chewing Gums:

Gum base was melted at 50-60° C on steam bath and to this plasticizer (Di butyl phthalate) and mannitol were added. Then physical mixtures of Domperidone and solubilizers (β-cyclo dextrin and PEG-6000) in different ratios, talc, saccharine and sodium chloride and flavor were added and mixed evenly. (Cherukuri et al., 1988) Composition of 7 different formulations were explained in Table 1.

1.4.1. Preparation of Domperidone and β cyclo dextrin physical mixtures:

Physical mixtures of domperidone and β cyclo dextrin of various ratios i.e., 1:1, 1:2, 1:3 were prepared. (Gaurav, S et al., 2010)

1.4.2. Preparation of Domperidone and PEG-6000 physical mixtures:

Physical mixtures of Domperidone and PEG-6000 of various ratios i.e., 1:1, 1:2, 1:3 were prepared. (Patel, K et al., 2010)

1.5. Evaluation

1.5.1. Weight variation:

All the obtained formulations were weighed on a digital balance and weight of each gum was noted.

1.5.2. Drug content:

Drug content of the formulation was determined using 6.8 pH phosphate buffer. For 1 gm of formulation 50 ml of buffer was added and shaken well for about one hour on a mechanical

shaker at 100rpm. The obtained filtrate is made up to the mark and drug cobncentrations of dilutions was determined by measuring the absorbance at 284 nm using UV Visible Spectrophotometer. (Klaus, F et al., 2005)

1.5.3. *In vitro* Dissolution studies:

In vitro drug release for the prepared formulations was carried out using Modified Disintegration apparatus. These studies are carried out in 6.8 pH phosphate buffer.

Procedure:

1 g of formulation was adhered on to the wooden block which was placed in the glass beaker containing 200 ml of 6.8pH phosphate buffer. Samples were withdrawn at regular intervals. Collected samples were estimated by UV Visible Spectrophotometer at 284 nm. The modified disintegration apparatus has been shown in Figure 1.

1.5.4. *Ex vivo* Studies:

Ex vivo studies were carried out for the best formulations using porcine buccal mucosa. (Shivang, C et al., 2012). The sample was collected from the receiver compartment and analyzed by UV spectrophotometer at 284 nm.

1.5.5. FTIR:

The Infrared spectra of pure drug and formulation were recorded at a scanning range of 400 to 4000 cm^{-1} using FTIR Spectrophotometer. This study was done to rule out any possible interactions between Domperidone and the excipients.

1.5.6. Stability studies:

The objective of stability studies was to predict the shelf life of a product by accelerating the rate of decomposition, preferably by altering the temperature. The best formulation F4 was subjected to stability studies by storing at 4°C and room temperature for 30 days. These samples were analyzed and checked for changes in physical appearance and drug content at regular intervals.

1.6. Statistics (ANOVA)

Both the study and control groups were compared. Analysis of Variance (ANOVA) is performed for formulations F1, F4 & F7 to determine the differences among the products tested.

2. RESULTS & DISCUSSION:

2.1. Calibration curves in 6.8 pH & 7.4 pH phosphate buffers:

Calibration curves of Domperidone were plotted in 6.8 pH & 7.4 pH phosphate buffers and exhibited linearity as per beer lamberts law. (Figures 2 & 3)

2.2. Saturation Solubility Studies:

It was observed that the maximum solubility was seen in phosphate buffer of pH 6.8 (Figure 4).

2.3. Weight variation:

The weight variation test for all the formulations compiles within I.P. limits, i.e. ± 7.5% (Indian Pharmacopoeia, 2007), as shown in Table 2.

2.4. Drug content:

It was observed that all the formulations were found to contain percentage of drug content between 80-90% as per content uniformity studies (Figure 5).

2.5. *In vitro* Dissolution studies:

The *in vitro* release data for domperidone chewing gum was represented in Figure.6. From all the formulations, it was noticed that F4 (90.59%) and F7 (88.60%) formulations showed good drug release within 30 minutes. Release of drug was

mainly dependent on polymer used and its ratio. The drug-polymer ratio was found to affect the drug release characteristics of the prepared chewing gums. At higher drug-polymer (1:3) ratio drug release from chewing gum was faster compared to lower drug-polymer ratio.

2.6. *Ex vivo* studies:

F1 acted as a control, F4 showed good *in vitro* release among formulations formulated using β cyclodextrin and F7 showed good *in vitro* release among formlations formulated using PEG 6000 as solubilizer. *Ex vivo* studies were carried out for these three formulations. Among the three formulations F4 showed maximum release (64.23%) within 10 minutes compared to other two formulations. Higher polymer ratios enhances drug solubility, which can lead to increase in the amount of drug absorption (Gaurav, S *et al.*, 2010). It showed that solubility enhancement of β-cyclodextrin with domperidone was more compared to PEG-6000 which led to increased permeation through mucosa. (Figure 7).

2.7. Stability studies:

The best formulation F4 was subjected to stability studies (Table 3) and it was observed that there was no marked change in the physical property and the drug content during the study period.

2.8. FTIR studies:

The characteristic peaks of the pure drug were compared with the peaks obtained for Formulations F4. From the data it was observed that characteristic peaks of Domperidone and formulation F4 were similar. Hence it can be inferred that there is no chemical interaction between drug and polymer and it can be concluded that the characteristics bands of pure drug were not affected by the method used to prepare chewing gum.(Figures 8 and 9)

Range of wave lengths, bond nature and bond attribute of functional groups obtained were mentioned in Table 4.(Elementary Organic Spectroscopy; Principles And Chemical Applications by Y.R. Sharma)

2.9. Analysis of Variance (ANOVA):

Analysis of Variance shows that *in vitro* results obtained by comparison between F4 and F7 formulations showed statistically insignificant difference and those obtained by comparison between F1, F4 and F1, F7 formulations show statistically significant differences ($p < 0.05$) as shown in Table.5.

Ex vivo results obtained by comparison between F1vsF4, F1vsF7 and F4vsF7 showed statistically significant differences ($p < 0.05$) as shown in Table 6.

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Tables and Figures:

Ingredients	F1	F2	F3	F4	F5	F6	F7
Domperidone (mg)	10	10	10	10	10	10	10
β-cyclodextrin(mg)	-	10	20	30	-	-	-
PEG- 6000(mg)	-	-	-	-	10	20	30
Gum base(mg)	900	900	900	900	900	900	900
Di butyl phthalate (mg)	5	5	5	5	5	5	5
Saccharine Na(mg)	4	4	4	4	4	4	4
Sodium chloride (mg)	1	1	1	1	1	1	1
Mannitol(mg)	50	40	30	20	40	30	20
Talc(mg)	20	20	20	20	20	20	20
Peppermint flavor(mg)	10	10	10	10	10	10	10

Table 1: Composition of different Formulations

No	**Formulation**	**Weight variation (g)**
1	F1	1.080 ± 0.05
2	F2	0.995 ± 0.06
3	F3	1.012 ± 0.06
4	F4	1.098 ± 0.01
5	F5	1.294 ± 0.05
6	F6	0.981 ± 0.01
7	F7	1.071 ± 0.01

Table 2: Results of Weight Variation (%/wt)

Stability conditions	**Days**	**Drug Content (mg) Mean ± SD***
4°C	0	90.59 ± 0.13
	15	90.76 ±0.23
	30	90.64 ±0.12
25°C	0	90.89 ±0.29
	15	90.89 ±0.36
	30	90.45 ±0.25

Table 3: Stability Studies of F4 formulation

S No	Range	Drug (A)	Formulation (B)	Bond nature and Bond attribute
1.	3500-3300	3025.66	3278.33	N-H stretching vibrations in amines
2.	1900-1600	1684.98	1730.77	C=O stretching
3.	2960-2850	2937.64	2916.23	C-H stretching in alkane
4.	1620-1590	1686.12	1609.62	N-H deformation in amines

Table 4: FTIR Spectral Data of pure Domperidone (A) and Formulation F4 (B)

Newman-Keuls Multiple Comparison Test	Mean difference	q	Significant (p<0.05)
F1vsF4	-17.83	19.04	Yes
F1vsF7	-15.76	16.83	Yes
F4vsF7	-2.065	2.206	No

Table 5: ANOVA Results for Formulations (*In vitro* results)

Newman-Keuls Multiple Comparison Test	Mean difference	q	Significant (p<0.05)
F1vsF4	-23.53	43.56	Yes
F1vsF7	-20.72	38.37	Yes
F4vsF7	-2.805	5.194	Yes

Table 6: ANOVA Results for Formulations (*Ex vivo* results)

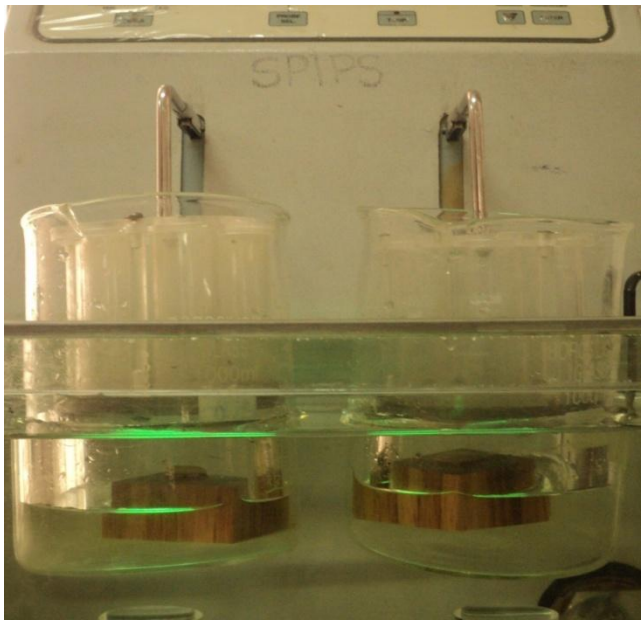

Figure 1: Modified Disintegration Apparatus for *In vitro* Dissolution studies

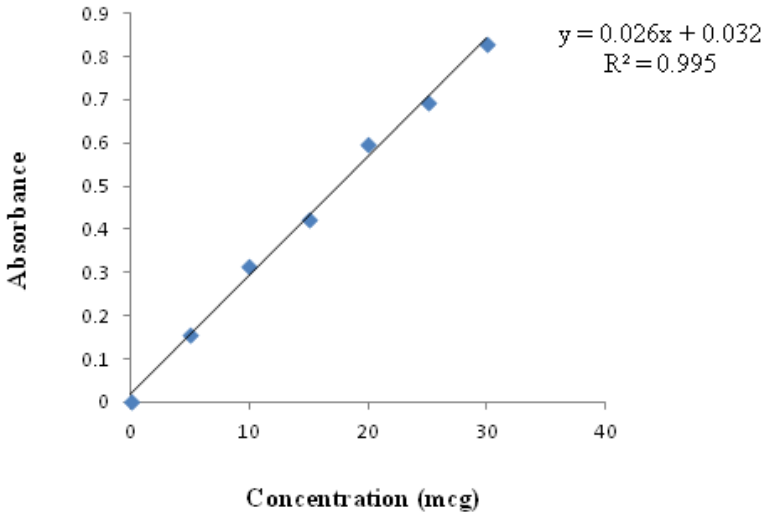

Figure 2: Calibration curve in 6.8 pH phosphate buffer

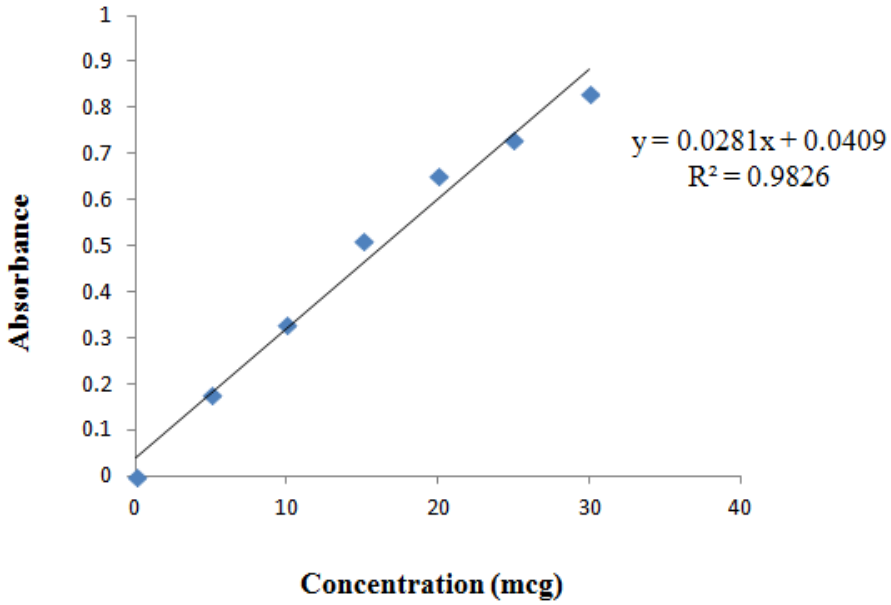

Figure 3: Calibration curve in 7.4 pH phosphate buffer

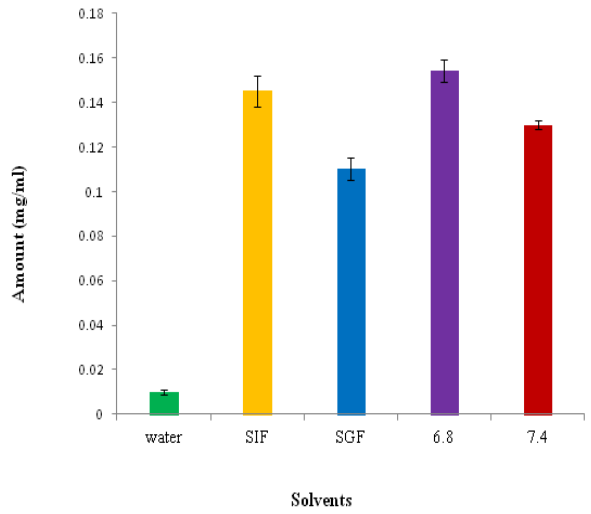

Figure 4: Saturation Solubility Studies

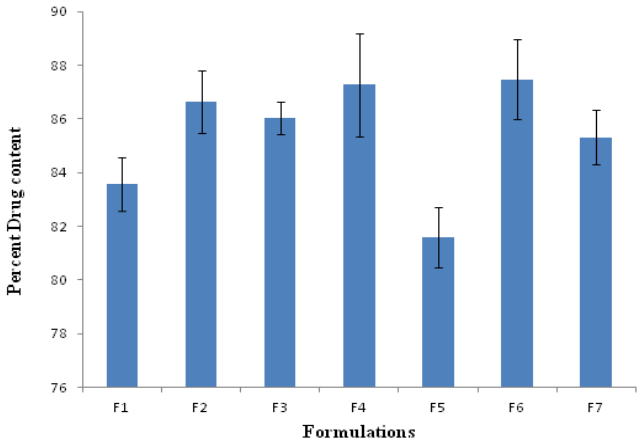

Figure 5: Comparison studies of Drug Content

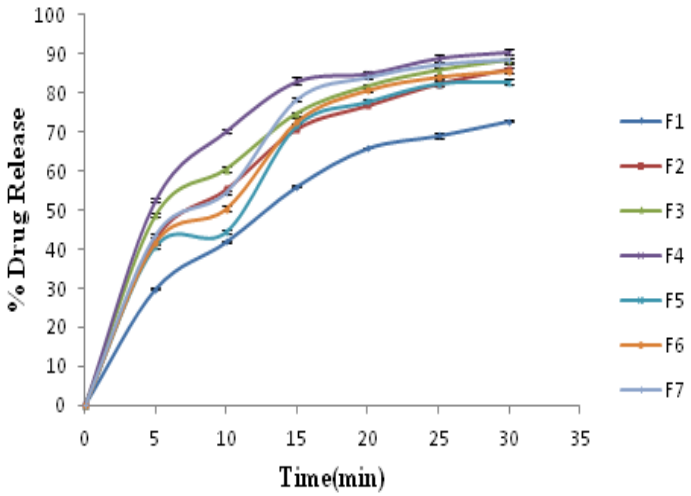

Figure 6: *In vitro* Dissolution profile

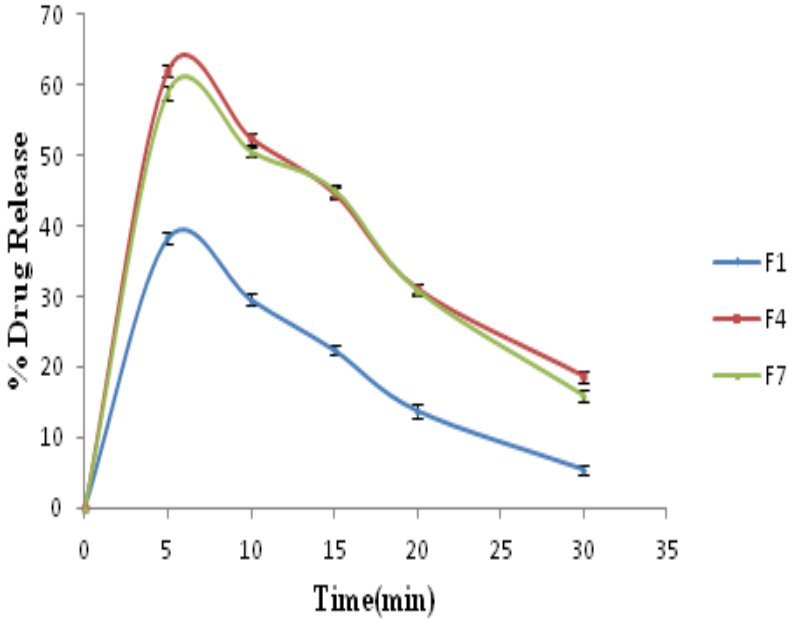

Figure 7: *Ex vivo* permeation studies through porcine buccal mucosa

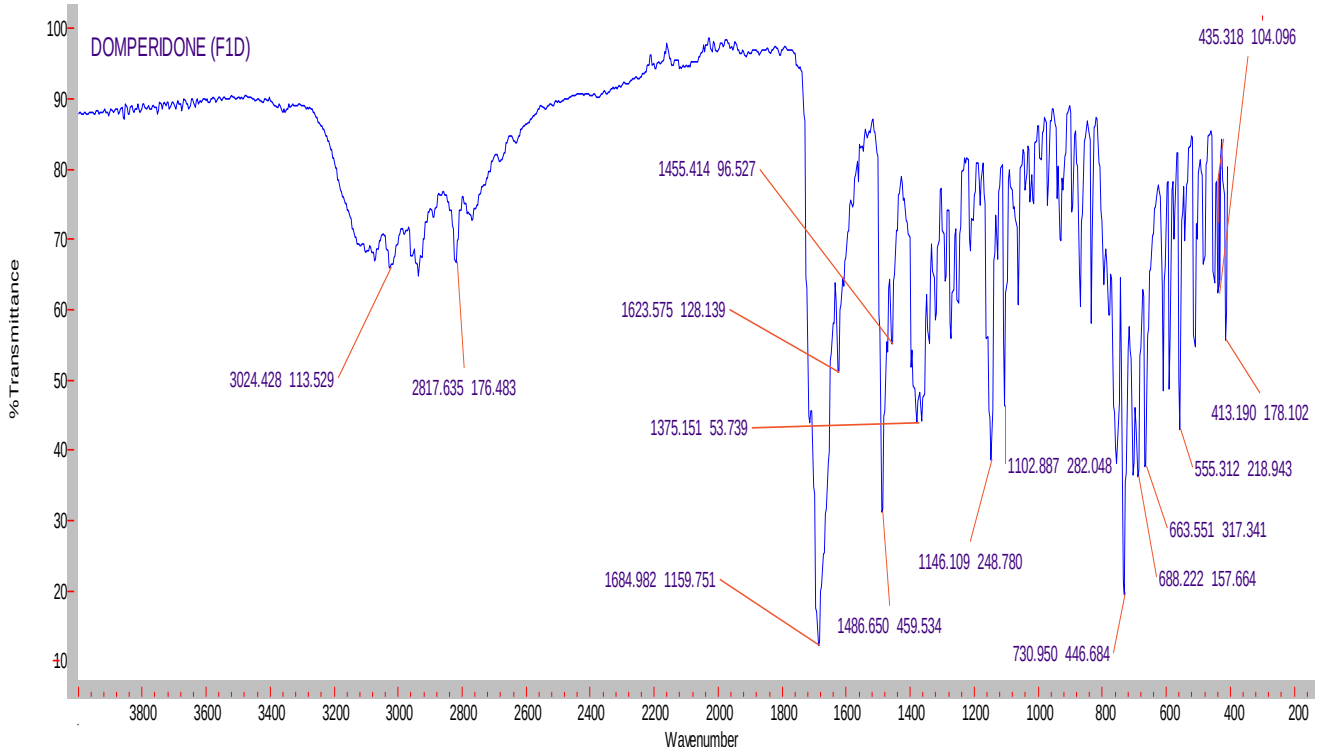

Figure 8: FTIR spectra of pure drug Domperidone (A)

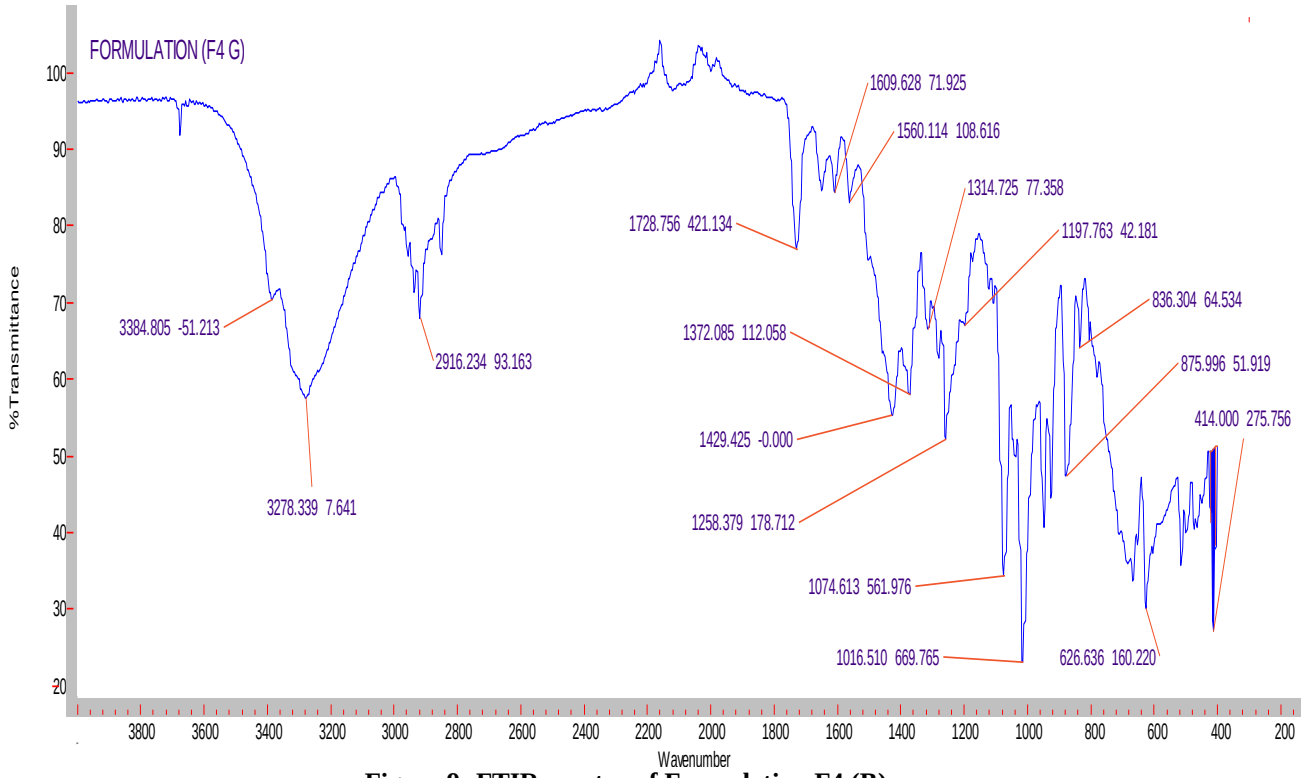

Figure 9: FTIR spectra of Formulation F4 (B)

Conclusion:

From this study it was concluded that chewing gum that contains highest amount of β-Cyclodextrin showed good release in both *in vitro* and *ex vivo* studies. It indicates that β-Cyclodextrin acts as a good solubilizer which solubilizes the drug Domperidone.Higher polymer ratios enhances drug solubility, which leads to increase in the amount of drug absorption. By delivering Domperidone in the form of chewing gum, it directly enters into systemic circulation thus bypasses First Pass Metabolism and hence Bioavailability of drug increases.

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NANOTECHNOLOGY IN MANAGEMENT OF PSORIASIS: A FOCUS ON HERBAL THERAPY

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ABSTRACT

Psoriasis is a hyper proliferative, autoimmune skin disorder. Despite the fact that many therapies are there in treating psoriasis, no single treatment gives complete and satisfactory cure. There are several therapeutic agents used topically and systemically but they have adverse effects. As an alternative for these drugs, herbal medicine have been widely used. These have better therapeutic value and have less side effects. Nano herbal drug delivery systems have a great future for increasing the activity and succeeding in dealing with the problems related with medicinal plants. Various marketed herbal formulations are available.

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INTRODUCTION: [1]

Psoriasis is a skin disease which is distinguished by massive proliferation, thick inflammatory cell infiltrates, generation of new blood vessels, modifications in lymphatic structure and impaired differentiation of epidermis .It is an autoimmune disorder where environment and genetic components have a major function.

Historical perspective and epidemiological studies: [1, 2]

Psoriasis is an inflammatory disease which has been existing from ancient times and is cosmopolitan. It was initially described in the Corpus Hippocraticum. The term psora was used by Hippocrates (460-377 BC), that means, 'to itch'. In simple terms Psoriasis is a non communicable regular skin disorder that leads to instant skin cell reproduction leading in red, dry patches of thickened skin. The dry flakes and skin scales are the result of the instant and sudden formation of skin cells. Psoriasis commonly affects the skin of the elbows, knees, and scalp. Skin sores can not only be itchy and defaced, in 10-30% of patients there can also be nail dystrophy associated with psoriatic arthritis. The swelling in the joints is alike in few forms to Rheumatic arthritis (RA), although in psoriasis it is a seronegative arthritis (no rheumatoid factor is present in the blood). Thus the various clinical evidences of psoriasis make it more than a skin disorder, as it hampers certain usual daily actions, like using hands, sleeping, walking etc. The Crohn disease which is an inflammatory bowel disorder, is one of the most serious problem related with psoriasis other than arthritis.

Both the sex are equally affected by this disorder and is seen at all ages, especially first time between the ages 15-25 years. It is a lifelong inflammatory disease.

Pathophysiology of Psoriasis:

Very rapid multiplication of keratinocytes occur in people having psoriasis and their movement from the stratum basale (basal layer) to the upper layer of epidermis occurs in 4 days. Thick dry patches or plaques form, as the skin does not shed the cells quickly[3]. In some people very mild psoriasis exists which cannot be even suspected as a skin disorder. Others have very severe psoriasis where almost whole body is covered with scaly, thick and red skin. Despite the fact that psoriasis occurs in population of all age groups, i.e., paediatrics to geriatrics, generally it is diagnosed in adolescence of a person.

The other causative factors for psoriasis are genetics, sudden changes in genes (mutations), climate, mental or emotional strain, contagion, and wounds [4, 5].

The major pathological phennomenon associated with psoriasis are [6]

1. T cell activation
2. Activated T cells migration into the skin
3. Reactivation of T cells in dermis and epidermis

Types of psoriasis:

Plaque psoriasis: [6, **7]**

It is also known as psoriasis vulgaris, which generally occurs (almost 80-85%). It is usually seen on elbows, knees, scalp and lower back. Symptoms include,

- Spherical lesions which ranges about 0.99 cm to several cms in width, and may further grow into patches.
- Red colored lesions which are encrusted with silvery, loose and shining skin.
- Lesions that are generally seen on the elbows, knees, and trunk.

Causes due to which this type occurs are generally infections, skin abrasion, medications, sunlight, strain, smoking and drinking.

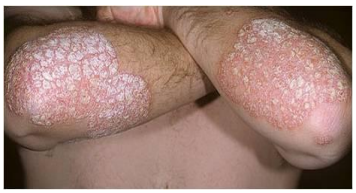

Fig 1: Plaque psoriasis [8]

Guttate psoriasis: [6]

It is also known as rain drop psoriasis and is the second general form (around10%) seen in population with psoriasis.Symptoms are

- Several minute rain drops sized lesions
- Lesions grow instantly, generally on the trunk, arms, legs, and scalp
- Eruption of lesions which may occur along with any upper respiratory infection.

Causes are streptococcal infection, viral or bacterial contagions, skin wounds and burns, insect bites etc, sunlight, medicine etc.

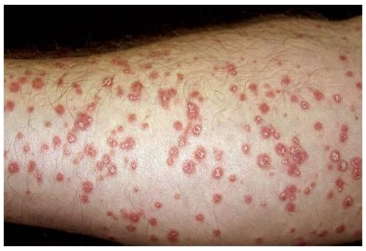

Fig 2: Guttate psoriasis [8].

Psoriatic arthritis: [6, 9]

It is a condition in which swelling occurs which shows impact on the joints. Generally appears in 6% to 40% of population having this skin disorder.

Symptoms of psoriatic arthritis include:

- Inflated, sore, thick, and painful joints,
- Above signs may be seen earlier, along with, or following the development of symptoms of the skin.
- In the hands and feet, joint symptoms are seen.

Causes are shock or wounds on skin, medications, agents that cause irritation of skin, smoking and drinking etc

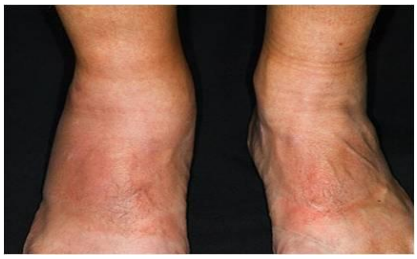

Fig 3: Psoriatic arthritis [8].

Pustular psoriasis: [6]

It can occur as patches which are small or wide spread on areas of feet, hands or fingertips. Seen in 5% or less population having psoriasis. Symptoms are,

- Fluid-filled lesions which are seen on soles and palms. Very scaly skin.
- Alterations in nail.
- Eruptions which are seen after discontinuation of certain medications and creams.

Causes are pregnancy, overexposure to UV light, systemic steroids, contagions, mental and emotional strain, and sudden with drawal of certain medications.

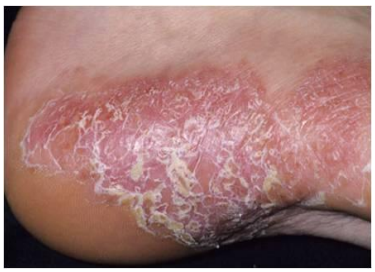

Fig 4: Pustular psoriasis [8].

Erythroderma, or exfoliative psoriasis: [6]

It is a very uncommon type that may be damaging or lethal. In population with this type, along with skin, symptoms are seen on whole body like

- Inflation and soreness which occurs on entire body skin. The skin may slough off and is generally itchy and tender.
- Incapable to monitor temperature of the body and chills.

Causes are use of steroid, extreme sun burn, strain, drinking alcohol, contagions, sensitivity etc.

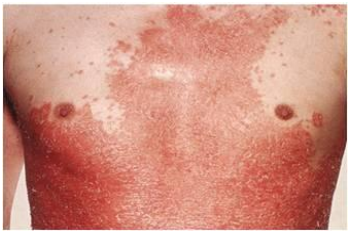

Fig 5: Erythroderma [8].

Nail psoriasis: [6]

Along with building up of skin cells under epidermis, in half of the psoriasis population they even develop under nails, which becomes impenetrable. They mostly split and in extreme cases, they fall down or collapse. Yellow or brownish red spots are seen below the nail.

Causes of this type is not known exactly, but generally it is considered having a genetic factor. [10]

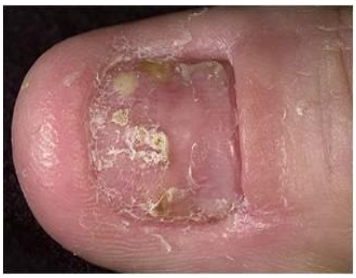

Fig 6: Nail psoriasis [8].

Genetic factors

Human genome scans show almost nine various loci which are susceptable to psoriasis (PSORS1-9).PSORS-1, is the main genetic factor of this disorder, which results up to 50% of genetic sensitivity to psoriasis. The risk of psoriasis may be increased due to certain variations and changes, which are related with four auto immune diseases: type 1diabetes, Celiac disease, Grave's disease and rheumatoid arthritis describing that all above disorders have the same genetic factors. [11]

Trigger Factors

Climate, strain, wounds, medicines are indirect causes and infections like HIV, one of the uncommon forms of Human Papiloma viruses (HPV) like EV-HPV, Streptococcal infections in upper respiratory tract lie sinusitis, tonsillitis are mostly important in triggering the disease process that commences and intenses psoriasis. [12]

Diagnosis:

Based on clinical studies such as skin rash, alterations in nails, joint involvement etc psoriasis is diagonised. Occasionally patients show uncommon skin sores that have to be distinguished from mycosis fungicides, seborrhoiec dermatitis, tinea, discoid lupus or undesignated skin syptoms like very little scaling of the scalp, isolated flexural erythema, or genital lesions. Thorough inspection of the body sites must be done to reveal unrecognised, clinically useful characteristics, and occasionally a skin biopsy may be required. Fusions of the various types may develop occasionally along with or later in the same patient. The silvery white scales, which are filled with dark pink or red lesions having prominent edges can be separated easily for diagnosis of psoriasis. After scraping off the moist skin, tiny blood droplets appear under the scales which are seen under pinkish moist tender skin. Scraping or skin biopsy and blood analysis may be necessary sometimes, to approve the diagnosis. [4]

Severity of disease: [13]

The extremity of this disease occurs from one or two small flaky swollen patches to extensive pustular psoriasis which in atypical cases can be lethal. To help in determining the suitable treatment for a patient, physicians generally classify psoriasis as mild to severe which is usually done based on percentage of skin impacted.

- Mild: Affected body area is < 3% affected. Less than 2% of the skin is affected in most cases of psoriasis.
- Moderate: 3-10% skin is impacted.
- Severe: >10% of the body is influenced.

Treating severe psoriasis is harder. However, though not categorized under severe psoriasis, few types of psoriasis are unaffected to treatment which include,

- Hand and Foot psoriasis (Any psoriasis that occur on palms and soles)
- Psoriasis that is seen in the fold of the skin (Inverse)
- Psoriatic arthritis
- Scalp psoriasis

Therapies to treat psoriasis:

There are various therapies for treating psoriasis. Any therapy should be considered on long term basis. General therapies present for treating psoriasis are systemic agents (medicines within the body), topical agents (medicines applied to the skin) and phototherapy. All these treatments may be used single or in combination with one another. Best treatment for the individual is determined by the physician situated on the kind and extremity of psoriasis.

Various approaches are:

Topical Therapy:

These are the medications that are directly applied on the skin and are the first line of therapy options. Topical creams, sprays and lotions are very effective for mild psoriasis and is also safe to use. Corticosteroids, vitamin D-3 derivatives [14], retinoids, coal tar or anthralin [15] are the main topical treatments. Combination of these preparations can be given sometimes. For example, keratolytics are often added to topical preparations.

Because of interference with each other some preparations should never be mixed together. For example,when salicylic acid is added to calcipotriene cream or ointment (a form of vitamin D-3) it inactivates the substance, while salicylic acid is required to be to medicines like anthralin to work effectively.

Photo therapy: [13]

Skin would be exposed to ultraviolet (UV) radiation in case of failure of topical treatments in achieving desired goal. This type of treatment is called phototherapy. This therapy includes subjection to UV radiations with the help of special apparatus utilizing fluorescent light source discharging specific wavelength of radiation. Psoriasis responds to ultraviolet rays. Symptoms get reduced on frequent subjection to sun or artificial ultraviolet rays. Methods are:

- Subjection to ultraviolet B light,i.e., UVB(290-320nm)
- Subjection to UV rays (320-400nm) combined with the drug psoralen,i.e.,PUVA, which expands the skin's light sensitivity.

There are other new methods like lasers, that emphasize the useful results of light particularly on psoriatic sores. UV light therapy is the basic treatment with ultimate outcomes for treating psoriasis.

Systemic Therapy:

Systemic Therapy is majorly used in moderate to severe cases or when no response for,topical treatment and photo therapy is seen [6]. Those patients who undergo systemic treatment must have frequent blood and liver function tests since the medicines used are toxic [13]. Generally three main drugs are used in systemic treatment.

Methotrexate:

This is a popular anti-metabolite which is very effective agent used in treating severe psoriasis [13]. Irrespective of its benefits it has limitations as it is related with extreme acute and chronic adverse reactions that involve acute hematologic toxicity and acute and chronic hepatotoxicity. This treatment should be restricted to patients with disabling, refractory psoriasis. [17]

Cyclosporine:

It is a cyclic polypeptide and mostly used in organ transplantation as an immunosuppressant. It shows inhibitory effects on T-cells and acts on psoriasis. Cyclosporine should be limited for patients with severe psoriasis. Major side effects associated with this drug are nephrotoxocity and hypertension. Risk of malignancies may be increased. [13]

Acitretin:

Actretin is an oral retinoid which act by its anti-inflammatory action. It shows good effect on combining with topical agents or light therapy in the generalized pustular and erythrodermic types of psoriasis [5] Though this drug has related teratogenic risk which exists for two to three years after terminating therapy it is the safest systemic treatment for psoriasis. [13]

Treatment using Biologics:

Biologics are means that inhibit molecular steps vital in the pathogenesis of this disease. They have grown in past few years as a good substitute therapeutic choices in treating psoriasis. They are of 2 types: agents that target the cytokine tumour necrosis factor α (example, adalimumab, etanercept) and agents that target T cells or antigen presenting cells (example, efalizumab). Though they have to be administered parentally, they are widely accepted by patients due to dissatisfaction with other treatments [18] and have led to high demand.

Combination Therapy

Combining various therapies like topical, photo, and systemic often results in reduction of doses of each therapy and can result in increased effectiveness. Therefore, physicians are focusing more on combination therapy. [27]

Herbal Therapy:

Plants and its constituents are used in herbal therapy which has less side effects than synthetic drugs. These days, herbal resources play an important role in the management of the skin and inflammatory diseases. [13]

Advantages:

- Increases therapeutic value by reducing toxicity and side effects
- Easily available
- More effectiveness

Disadvantages:

- High cost
- Difficulty in scaling up
- Have toxicity and stability issues

There are many herbal drugs which show anti psoriatic effect.

Wrightia tinctoria:

It is commonly known as Dyer's oleander, Indrajau and belongs to the family Apocynaceae. Leaves has astringent, anti inflammatory and anti bacterial properties and is used in treating various skin disorders [19]. From leaves of this plant a hydro alcoholic extract was obtained which shows antipsoriatic effect. This extract has a good antioxidant activity. These leaves contain beta amyrin and glucoside, which are reasonable for the skin healing properties of plant. [22]

Silybum marianum:

Its common name is Milk Thistle and belongs to the family Asteraceae. Liver neutralizes certain toxins associated with psoriasis. This herb increases liver function and prevents outbreak of psoriasis. It may prevent human T-cell activation that is normally seen in this disorder[13]. Active ingredient which protects liver is Silymarin, a chemical constituent extracted from the seeds. Silymarin consists of a group of flavonoids (Silibinin, Silidianin and Silicristin) that repairs hepatic cells [23].

Aloe vera:

It belongs to the family Asphodelaceae. The gel and the rind of the aloe vera leaf contains pharmacologically active components like anthraquinone and acemannan which have properties like analgesic , anti inflammatory, anti pruritic, wound healing etc. and hence can be used in treating psoriasis [13, 19].

Aloe vera moisturizes skin by forming a protective layer which prevents bacterial growth. Saliclic acid is a proven antiseptic agent used in psoriasis treatment which is present in Aloe vera [24, 25].

Work done on aloe vera states that 0.5% Aloe vera extract in a hydrophilic cream was well tolerated and found to be very effective [26].

Angelica sinensis: [19]

It is commonly known as Dong quay and belongs to the family Apiaceae. Extracts of this herb contains furocoumarin,i.e, Psoralen which acts as a potent photosensitizer in existance of ultraviolet A. Exposure to UVA along with Psoralen consumption leads to cross linking of epidermal DNA which reduces epidermal DNA synthesis rate.

Capsicum annuum:

It is commonly known as Cayenne and belongs to the family Solanaceae. Major component is capsaicin. Substance- P (SP) mediates a neurogenic inflammatory condition which occurs in psoriasis [19].

Capsaicin decreases neurotransmitters from the sensory nerves and thus reduces pain and itching associated with psoriasis [27].

Smilax china:

It is commonly known as China root, which belongs to the family Smilacaceae. Rhizome part of the plant is used and this contains flavonoid quercitin which can be isolated from the methanolic extract. This flavonoid shows antipsoriatic activity by reducing epidermal thickness, which is due to reduction of leucocyte migration [19, 20, 28].

Ulmus rubra: [20]

It is commonly known as slippery elm and belongs to the family Ulmaceae. It contains a mucilage component which is derived from the inner bark of the elm.

Mahonia aquifolium:

It belongs the family Berberidaceae and is commonly called as Oregon grape. Bark extract of this drug is used which consists of berberine, berbamine, oxycanthine as major constituents. It inhibits keratinocytes growth [19, 21].

Alkaloids like berberin, protoberberine, berbamine and oxycanthine that are present in the root and bark extract of this plant have very good antimicrobial and antifungal activity. These alkaloids arrest the proliferation of various tumour cells as well as incorporate powerful antioxidant activity which hinders keratinocytes and reduces inflammation. [29]

Nigella sativa:

It is commonly known as black cumin and belongs to the family Ranunculaceae . 95% of ethanol extract of seeds of this herb shows significant epidermal differentiation and reduces epidermal thickness [19].

A study conducted on seed extract of this plant on an animal model of psoriasis, in which the histological effects were examined has shown that Nigella sativa has anti-psoriatic activity [30].

Thespesia populnea:

It is commonly known as ortia tree and belongs to the family Malvaceae. It has been traditionally used in treatment of psoriasis along with other skin disorders like ring worm, eczema, scabies etc. Oil which is obtained by boiling the bark in coconut oil is applied externally in scabies psoriasis [31].

In one study the screening for anti psoriatic activity was carried out by topically applying various extracts and isolated compounds like TpF-1, TpF-2 and TpS-2 [32].

Apart from the above mentioned drugs many other herbal drugs are used in treatment of psoriasis like Matricaria recutita, Curcuma longa, Alpinia galangal, Indigo naturalis, Gaultheria procumbens, Cassia tora, Momordica charantia etc.

All these drugs show significant anti psoriatic effect by following different mechanism pathways.

Nutritional Therapy:

To reduce severity of psoriasis, more water should be consumed. Lots of green leafy vegetables should be taken which will reduce the intensity of this condition [13] . Few foods trigger the disease and they should be avoided. Eg: Coke, Vit-C,junk food, oily food, red meat, acidic food etc. Psoriasis worsens in those people who follow poor diet. Consumption of alcohol is proved to be a probability component for this disorder in men. Avoiding gluten (found in wheat, barley) is also good. Fumaric acid, fish oil, triglcerides, Vit-D, folic acid are found to be effective against psoriasis [33, 34].

Hydro therapy: [27]

Hydro or Water therapy is used as alternative therapy for psoriasis in which the skin is supplied with moisture, heat and certain minerals. The use of warm water for bathing improves blood circulation.Water rich in sulphur and other minerals should be used for bathing which will tend the skin.

To prove water therapy works in treating psoriasis, the sufferers were made to bathe in the Dead sea which is rich in salt and minerals and due to presence of ultraviolet light radiation the location was perfect for sunbathing.

Role of Nanotechnology in treating Psoriasis:

Nano particles are the colloidal systems with particle size varying from 10 nm to 1000nm. These are nano or sub nano sized structures which are made of synthetic or semi synthetic polymers. Nanoparticles of plant medicine is gaining a lot of attention currently [35].

Herbal drug delivery has certain problems due to poor solubility, low bioavailability, instability in biological medium and high first pass metabolism and poor permeability. Nanotechnology is useful to overcome all these problems [36, 37].

Merits of plant nanoparticle delivery system: [38, 39]

- Delivery of formulation to the targeted site
- Drug solubility and Pharmacokinetics can be improved by encapsulating the drugs within nanoparticles
- Bioavailability of drugs can be increased
- Degradation of drugs can be avoided
- Delivering the drug in small particle size increases the surface area of the drugs which allocates quicker dissolution in the blood
- Decreases side effects
- Decreases the dose of the formulation

Approaches of NanoTechnology:

Polymeris nanoparticles, Solid lipid nanoparticles, Liposomes, Proliposomes, Niosomes, Liquid crystalline systems, Quantum dots etc are the various approaches of nanotechnology.

Herbal constituents :

Various constituents that possess anti psoriatic activity are isolated from many herbal plants.

- Artesunate is an active constituent which is derivative of Artemisinin that is processed from plant Artemisia annua L. of family Asteraceae. It acts by controling the expression of CXCR2 and increases the secretion of TGFβ, in vitro [40].
- Capsaicin is derived from Capsicum annuum of family Solanaceae [41].
- Colchicine, an active constituent Colchicum autumnale of family Colchicaceae [42].
- Curcumin is derived from Curcumin longa belonging to the family Zingiberaceae shows its activity as selective phosphorylse kinase inhibitor, thereby decreasing inflammation through inhibition of NF_KB [43].
- Psoralen, is derived from Psoralea corylifolia of family Fabaceae. It inhibits epidermal DNA synthesis and thereby cell division [44].
- Koumine is derived from plant Gelsemium elegans belonging to the family Loganiaceae. It acts by inhibiting epidermal cell proliferation, promoting formation of granular cells, decreasing serum IL-2 levels [45].

Some other constituents derived from various plants are Iso-Camptothecin Hypericin, Podophyllotoxin, Isoquinoline etc [46, 47, 48, 49].

Herbal anti psoriatic formulations:

Several anti psoriatic herbal formulations are available and few of them are listed in Table 1.

Table 1: Anti psoriatic Herbal formulations.

Formulation	Mode of action
Ginkgo and megranate (Chinese in vitro medicine)	Downregulates the expression of VEGF and PlGF [50]
Composite Shendi decoction (CSDD) and Diyin tablet (DYT) (Chinese formulation)	Serum IL-8 and plasma endothelin level reduced [51]
Chunghyuldan (Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus, Rhei Rhizoma) (Chinese formulation)	Regulation of COX-II produced by macrophage cells, and IFN-γ and IL-4 produced by T cells [52]
Lixue xiaoyin decoction (Chinese formulation)	Improves microcirculation and inhibits division of epidermal cells [53]
Qingdai compound capsule (Chinese formulation)	Decrease in expression of c-myc in keratinocytes [54]
Quyin granule (Chinese formulation)	Inhibition of faster epidermal cell proliferation and improvement of parakeratotic epidermal cells [55]
Radix Sophorae Flavescentis (Sophora flavescens Ait.)	Inhibition of hyperplasia of epidermis cells and the promotion to the formation of granular layer [56]

Other herbal formulations which are used in treating psoriasis are Yinxie capsules, Tuhuai extract, Relieva, Vitamin B12 cream containing Avocado oil etc [57, 58, 59, 60].

Patents on antipsoriatic herbal products:

List of patents on anti psoriatic herbal products are given in table 2.

Table 2: Patents on anti psoriatic herbal formulations.

Composition	Formulation
Carthami tinctorii Flores, Guaiacum officinale, Pardanthi dichotomy Radices, Parmeliae herba of moss, Pyrus baecate	Chinese medicinal composition [61]
Herba Chelidonii (Chelidonium majus)	Topical emulsion or ointment [62]
Et Hg thiosalicylate, plantain extract, rose extract, orange extract	Topical composition [63]
Arnica Herb, marigold, Fructus Rhodomyrti, Radix Hamamelis Mollis, Avocado	Medicinal ointment [64]
Turmeric extract, α-hydroxy acids,α1-antitrypsin	Topical formulation [65]
Moisturizing cream, berberine, oleuropein, glucosamine	Cream [66]
Mussel, Carnis Rapanae Thomasianae, Concha Ostreae, Concha Meretricis Seu Cyclinae, Liushugu, Scolopendra	Topical or oral composition [67]
Psorberine (M. aquifolium alcohol-water extract)	Topical formulation [68]
Mimosine and idebenone	Occlusive patch, cream, gel, emulsion, spray [69]
Wrightia tinctoria oil extract	Hydrophobic topical formulation (cream, ointment) [70]

Other than those listed in above table, many more patents are present which are claimed on antipsoriatic herbal formulations.

Future scope:

Use of herbal drugs in treatment of psoriasis is gaining importance gradually. So, dosage forms which can overcome the present existing disadvantages of herbal formulations can be prepared. Among them nanoparticles occupy the first place. Researchers and scientists are currently working to develop nanoparticulate drug delivery systems that can treat psoriasis with minimal side effects.

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Formulation and Evaluation of Nano Copper Gel For Treatment of Clinical Mastitis

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ABSTRACT

Clinical mastitis is considered to be a sizeable clinical problem in dairy industry. Apart from cows it is also reported in sheep and even in breast feeding mothers. *Staphylococcus aureus* bacteria accounts for infections to a great extent and its control is a vital part in curing programs. Metal nano copper has gathered much attention in antifungal/antimicrobial applications. In the present work Carbapol-934 gel, infused with nano copper was formulated and evaluated, which can be a remarkable breakthrough in the field. The antimicrobial test on nano copper against Staphylococcus aureus and Escherichia coli proved the antimicrobial property of the former. The prepared gel was evaluated for PH, homogeneity, spreadability, drug content, *in-vitro* drug release and *ex-vivo* permeation. The accelerated stability studies and rheological studies were also conducted. The results of above tests were encouraging for the evolution of the above gel to cure clinical mastitis.

KEY WORDS: Mastitis, Carbopol Gel, In Vitro, Ex Vivo

1. INTRODUCTION

These days copper nano particles have gathered much attention especially in the field of antifungal and anti microbial activities [1,2]. The metal nano particles especially copper were found to have sound anti microbial activity [3]. The anti microbial properties of copper nano particles find several applications but much attention has not yet been gathered in treating Clinical Mastitis. Since 1990 several studies were reported on economics of Mastitis. Mastitis can be defined as an endemic disease which is one of the most costly and frequent diseases in dairy industry. There are several economic consequences due to this disease and among them the associated costs can be classified among 10 vital factors[4]. For successful implementation of any Mastitis control program it is vital to identify Staphylococcus aureus Mastitis – infected cows quickly and reduce the opportunity of pathogen spread in the herd[5].

Mastitis is also found in breast feeding mothers and about 20 to 30 percent of them were reported to affect by it [6 - 9]. Recent studies have showed that Mastitis, usually indicated through elevated sodium concentrations in breast milk, is mapped with higher HIV load in the milk along with higher risks of mother-to-child HIV transmission [10].

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Few researchers have studied the elevated concentrations of immune proteins in Mastitis breast milk [11]. The complete and innate consequences of infections in Mastitis is yet to be know but in cows, Mastitis can permanently damage lactational performance and mild yield[12]. Among the pathogens of Mastitis, Staphylococcus aureus is believed to be an agent vital canorn due to low cure rate of infections by antibiotics and due to its ability to persist in the form of innate, subclinical infection [13]. Hence external gel application to treat Mastitis can be remarkable insight. This is one of the cogent attempts made in this study. It was found that recurring mastitis episodes were often influenced by Escherchia coli [14].

The present study aims at synthesizing nano copper gel in treatment of clinical mastitis, which is novelty of the work. Topical gel applications at pathological sites has remarkable advantage of faster drug release to the action zone.The as synthesized gel was undergone into studies like *invitro*, *exvivo*, spreadability and viscosity etc.

2. MATERIALS AND METHODS

2.1. Synthesis of Nano Copper Gel

Carbopol 934 (Merck Ltd), Propylene glycol, Triethanolamine & Proyl Paraben (Loba Chem, India) were purchased from the local market. Nano copper, which was used as a drug in this gel, was synthesized biogenically, and details pertaining to its morphology are represented

in the **Figure-1 (a& b)**.The formulation chart of the gel is presented below in **Table-1** and its preparation procedure is mentioned as follows.

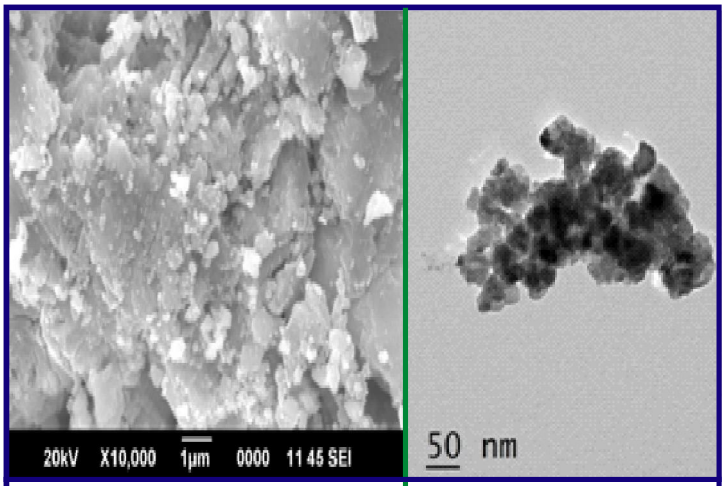

Fig. 1a SEM image of the drug | **Fig. 1b. TEM image of the drug**

Table. 1: Showing Preparation details for 1 gram of gel.

Ingredients	Use	Amount
Copper powder	Drug	2 mg
Carbopol- 934	Gelling agent and Polymer	500 mg
Propylene Glycol	Stabilizer and solvent	3 ml
Triethanolamine	Emulsifier and pH neutralizer	0.5 ml
Propyl paraben	Preservative	0.2 ml (2 drops)
Distilled water		Q.S

Appropriate quantity of carbopol 934 was soaked in water (around 5 ml) for a period of 2 hours. It was neutralized with triethanolamine (TEA) with stirring. Specified amount of drug was dissolved in appropriate and pre weighed amount of propylene glycol . Solvent blend was transferred to carbopol container and agitated for additional 20 min. The dispersion was then allowed to hydrate and swell for 60 min. pH was adjusted with 98% TEA with gentle stirring until the desired pH value was approximately reached (6.8-7) and homogeneous gel was formed. All the samples were allowed to equilibrate for at least 24 hours at room temperature prior to performing rheological measurements.

2.2. Evaluation studies:

Before the gel was formulated, the nanocopper was tested for its antimicrobial properties. For this two bacterial species,viz *.staphylococcus auresus* and *escherichia Coli* were obtained and maintained on Luria – Bertani agar. Both were gram positive, and gram negative species respectively. Prior to their incubation with the powder, they were cultured overnight in 5 ml of Luria – Bertani broth (Fluka) in a certomat BS-T incubation shaker (Sartorius Stediun Biotech, Aubagne, France) at an rpm of 370 and 150 until the culture reached on OD 600 of 1.0, corresponding to 8 x 108 colony forming units per mL. This was determined using an ultrospec ultraviolet – visible 3000 spectrophotometer. Further zone of inhibition method was used to determine their anti-microbal activity.

As depicted in Fig. 2a and 2b. Gram-positive and Gram-negative bacteria were cultivated on Nutrient agar medium. Approximately 2.8 gms of agar powder was mixed in 100 ml of distilled water. The medium was powered in sterilized petriplates and it was allowed to solidify. After their surfaces solidify, they were allowed to dry in an incubator former to the streaking of organisms onto the surface of the agar plates. The above organisms were collected from the culture tube using a prior sterilized metal loop and streaked on solidified agar plates. A sterilized metal borer was used to make bores/cups in the agar plates, and the sample was located in the cup. The petriplates were kept in the incubator for 36 hours.

The pH of the gel was determined using digital pH meter by placing the glass electrode completely into the gel system. The readings were taken for average of 3 times.All developed gels was tested for homogeneity by visual inspection after gels have been set in the container. They were tested for the appearance and presence of any aggregates. A sample of 0.5 g of gel was placed between two slides and left for about 5 minutes where no more spreading was expected. Diameters of spreaded circles were measured in cm and were taken as a comparative value of spreadability. The experiment was carried out in triplicate. Spreadability was expressed in cm. The rheological measurements were performed on the Viscolab 3000 Rheometer. The rheological properties of the formulated gel were studied at different shear rates (rpm) and the viscosity was measured in centipoise. To study the drug content,100 mg of nano gel formulation was taken in a 100ml volumetric flask. The volume was made up to 100 ml using 7.4 pH buffers. Then drug concentration was determined by measuring the absorbance using UV- Spectrophotometer.

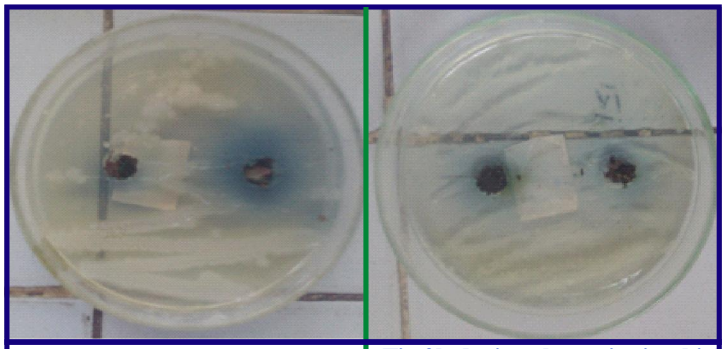

Fig 2a shows the anti microbial activity of Gram Positive bacteria | **Fig 2b depicts the anti microbia activity of Gram Negative bacteria**

2.3. In vitro drug release studies:

In vitro studies were carried out using a franze diffusion cell and cellophane membrane (soaked in pH 7.4 buffer overnight). In donar compartment of diffusion cell pH 7.4 buffer was taken. On the

membrane, gel was applied. Sampling was done at regular intervals of 15, 30 mins, 1 h, 2 ,3, 4, 5 and 6 hours (each time 2 ml of sample was collected and replaced with similar amount of buffer). The obtained samples were analyzed using UV spectrophotometer.

2.4. Ex vivo drug permeation studies:

Ex vivo studies were carried out for the formulation using rat abdominal skin using franze diffusion method. 7.4 pH phosphate buffer was placed in donor compartment. Sampling was done at regular intervals of 15, 30 mins, 1 hour, 2 ,3, 4, 5 and 6 hours (each time 2 ml of sample was collected and replaced with similar amount of buffer). The obtained samples were analyzed using UV spectrophotometer. Detailed comparison of in vitro and ex vivo studies are shown in **Fig. 3**.

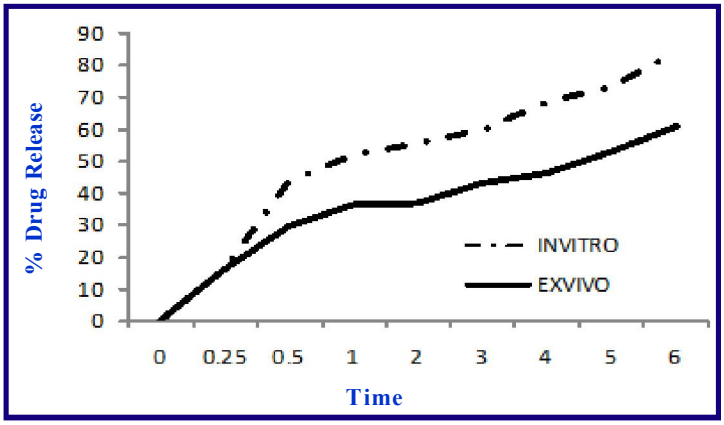

Fig 3 :Graph Representing In vitro and Ex vivo drug release

2.5. Accelerated Stability Studies:

Stability studies are conducted to predict the shelf life of a product by accelerating the rate of decomposition, preferably by increasing the temperature and relative humidity (RH) conditions. Formulation was packed in a screw capped bottle and studies were carried out for 30 days by keeping in stability chambers at 25 ± 2 °C & 60 ± 5 % RH and 40 ± 2 °C & 75 ± 5 % RH.

Samples were withdrawn on 0th, 15th and 30th days and were analyzed for Physical appearance and drug content spectrophotometrically at 252 nm as per ICH Q1A (R2) guidelines.

3. RESULTS AND DISCUSSIONS

3.1. pH determination

pH values of gel formulation was found to be 5.53. This shows that the pH value was near to that of skin pH which is quite important for topical formulations. Increase in the pH values may cause skin irritation.

3.2. Homogeneity

The prepared gel was visually evaluated for homogeneity. The formulation was clear without any aggregates.

3.3. Spreadability studies

Spreadability is the important factor to be considered in formulation of gels. The spreadability of the prepared carbopol gel formulation was 5.74 ± 0.33 cm. Amount of carbapol and polymer shows effect on spreadability, i.e, more their amount, less is the spreadbility and vice versa.

3.4. Rheological studies:

Rheology testing performed on the gel showed that the designed formulation had a non-Newtonian, pseudoplastic, thixotropic behaviour at temperatures 25ºC. This type of rheological behaviour indicates an appropriate spreadability in case of topical administration of the tested gels. Viscosity of the developed formulation was 16,844 centi poise.

3.5. Drug content

The prepared topical gel was analyzed for drug content. The percentage drug content in topical gel formulation was 91.758 %. It was observed that the drug content in the prepared topical gel was satisfactory and the drug was uniformly distributed in the formulation.

3.6. Anti microbial activity:

It was carried out for 36 hours. After that the petri plates were removed from the incubator and examined. Zone of inhibition occurred around the cups, which indicates that the sample has anti microbial activity.

3.7. In vitro drug release studies:

Samples were collected at different time intervals and drug release was calculated. At the end of 6th hour drug release was found to be 83.99 %. This shows that sustained release was obtained. The details are furnished in **Table 2**.

Table 2:In Vitro calculations

Time	abs	df	conc	amt	Ca	%dr
0	0	0	0	0	0	0
0.25	0.391	1	22.8655	19.05458	8.163781	16.32756
0.5	0.447	1	26.14035	21.78363	21.80649	43.61298
1	0.532	1	31.11111	25.92593	25.97493	51.94986
2	0.569	1	33.27485	27.72904	27.80916	55.61832
3	0.611	1	35.73099	29.77583	29.88922	59.77844
4	0.698	1	40.81871	34.01559	34.16472	68.32943
5	0.747	1	43.68421	36.40351	36.63713	73.27427
6	0.856	1	50.05848	41.7154	41.99908	83.99817

3.8. Ex vivo drug permeation studies:

For these studies drug permeation through porcein bucca mucosa was measured and drug release was calculated. At the end of 6th hour 60.85 % drug release was obtained as represented in detail in **Table 3**.

Table 3: Ex Vivo Calculations

Time	abs	df	conc	amt	ca	%dr
0	0	0	0	0	0	0
0.25	0.311	1	13.4632	11.21934	8.163781	16.32756
0.5	0.412	1	17.8355	14.86291	14.87638	29.75276
1	0.504	1	21.81818	18.18182	18.21312	36.42623
2	0.513	1	22.20779	18.50649	18.55961	37.11922
3	0.598	1	25.88745	21.57287	21.6482	43.29639
4	0.637	1	27.57576	22.9798	23.08101	46.16202
5	0.732	1	31.68831	26.40693	26.5674	53.13481
6	0.838	1	36.27706	30.23088	30.42763	60.85527

3.9. Accelerated Stability Studies:

The optimized formulation was subjected to stability Studies according to ICH guidelines by storing at 25 ºC/60 % RH and 40 ºC/ 75 % RH for 30 days. Sample was analyzed and checked for changes in physical appearance and drug content at regular intervals. The results are given in **Table 4**.

Table 4 : Stability studies of optimized gel

Stability condition	Sampling interval (days)	Physical appearance	% Drug content Gel (mean ± S.D*)
25º±2ºC/60±5% RH	0	No change	91.25 ± 0.01
	15	No change	90.63 ± 0.01
	30	No change	89.98 ± 0.02
40º±2ºC/75±5% RH	0	No change	91.62 ± 0.02
	15	No change	90.01 ± 0.01
	30	No change	89.54 ± 0.01

**SD=Standard deviation, n=3*

4. CONCLUSION

Nano copper gel was formulated and evaluated. It has been inferred from the results that the formulated gel was good in appearance, homogeneity, possess appreciable spreadability, exhibited anti microbial activity. In vitro and ex vivo studies has shown 83.99% and 60.85 % respectively in 6 h. This shows that the formulated gel has sustained release activity. However, further investigations on these lines have to be done which can lead towards the evolution of a topical gel against clinical mastitis.

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Oral dissolving films of Celecoxib inclusion complexes using *Musa paradisiaca* as super disintegrant in treatment of Psoriatic arthritis

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ABSTRACT

Celecoxib is a poorly water soluble drug which is a selective COX-2 inhibitor used in treating Psoriatic arthritis. It shows poor dissolution and flow properties. So physical mixures of the drug using solubilizers like β CD were prepared in order to enhance its solubility and dissolution rate. Super disintegrant plays a vital role in increasing drug release. Musa paradisiaca, a natural excepient is used in this study as a super disintegrant. The aim of present study was to formulate oral dissolving films of Celecoxib using solubilizer and evaluate them for *in vitro* disintegration and dissolution rates. It was found that β CD had shown good impact on increasing solubility and dissolution rate. Drug excepient compatibility was studied by FTIR studies.

KEY WORDS: Celecoxib; Psoriatic arthritis; Musa paradisiaca; Oral dissolving film.

1. INTRODUCTION

Celecoxib is generally used in treatment of Rheumatoid arthritis (RA), Osteoarthritis (OA) and Psoriatic Arthritis (PsA). Psoriatic Arthritis therapy should target both rash and joint disease. Joint damage that occurs in PsA patients have been shown to be comparable with that in patients with RA. Therefore NSAID's that are generally used in treatment of RA, OA are also widely used in treating PsA, but it is more based on clinical experience than that on clinical trials.[1, 6, 7]

Celecoxib, 4-[5-4(4-methyl phenyl-3-(trifluoromethyl)-1H-Pyrazol-1-yl] benzene sulphonamide, belongs to a novel class of agents that selectively inhibit cyclogenase-2 (cox-2) enzymes. According to the biopharmaceutical classification system (BCS), Celecoxib is a class II compound that means it has low solubility and high permeability. Numerous efforts have been made in order to improve drug dissolution rates like, [2]

- using water soluble carriers to form inclusion complexes
- reducing particle size to increase surface area
- using prodrugs and drug derivatization
- solubilization in surfactant systems etc

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Oral dissolving films are being used as an alternative to tablets and capsules for pediatric and geriatric patients suffering from Dysphagia. These are thin films which are placed on tongue or mucosal tissue of patients and instantly get wet by saliva and dissolves rapidly. This disintegrates and dissolves to release the medication for oral mucosal absorption. [3,4]

Since Celecoxib is a poorly water soluble drug, to enhance its solubility solubilzers like β-CD were used. Cyclodextrins are hydrophilic cyclic oligosaccharides with a lipophilic central cavity. In aqueous solutions cyclodextrins were able to solubilize many hydrophobic drugs by taking up some lipophilic moiety of the drugs into the cavity, that is, through formation of water-soluble inclusion complexes.[8,9]

Now a day's natural excepients have gained more importance due to their safety, less side effects, low cost, biocompatibility, bioacceptance etc. In this study Musa paradisiacal mucilage is used as a natural superdisintegrant which helps in rapid disintegration of film on tongue.[5]

So in present study an attempt has been made to develop physical mixtures of Celecoxib with solubilizer β CD to increase solubility and dissolution rate of the drug. This physical mixture formulated was used in formulating ODF's of Celecoxib drug.

2. MATERIALS AND METHODS:

Celecoxib was received as a gift sample from Microlabs, Bengaluru, India. β-CD was obtained from SD Fine chem.. LTD., India. Musa paradisiaca powder was purchased from Srinidhi Pharmaceuticals, Mysore. HPMC E15, Tween 80 were obtained from Loba Chemicals, Mumbai, India. Aspertame and Mannitol from Signet Chemical Corporation, Mumbai, India. Glycerine, Cirtic acid from Fisher Scientific Chemicals, Mumbai, India and Methanol from Merck pvt. LTD., Mumbai, India.

2.1. UV Analytical Method:

The UV Calibration curve of Celecoxib was constructed in methanol. 6.8 pH buffer was used for making up volumes. 10 mg of drug was dissolved in 100 ml of methanol (100µg/ml). From this 1 ml was pippeted out and made up to 10 ml (10µg/ml). Similarly stocks of 5, 15, 20, 25 and 30 µg/ml were prepared. Absorbance of these solutions was measured at 252 nm by UV Spectrophotometer.[10]

2.2. Solubility studies of Celecoxib in different buffers:[11]

Solubility studies of Celecoxib were carried in water and in different phosphate buffer solutions of pH, 6.4, 6.8 and 7.4 as per the IP specification. Excess amount of drug was added to each 10ml of buffer solutions in the conical flasks and kept on the mechanical shaker at100 rpm for 2 hrs. Then the conical flasks were removed from mechanical shaker and kept aside over night. Next day samples were filtered. A volume of filtrate was taken and appropriate dilutions were made, filtered and absorbance was measured at 252 nm using UV Spetrophotometer. Using the standard calibration curve the quantity of drug dissolved was calculated. The results were presented in the **Table 1**.

Table 1: Solubility studies in water and different buffers

pH	Solubility (mg/ml) ± SD
Water	0.00744 ± **0.01**
6.4	0.1224 ± 0.01
6.8	0.1416 ± 0.03
7.4	0.1470 ± 0.02

2.3. Preparation of Inclusion complexes of Celecoxib and β-CD:

Appropriate amounts (1:3) of drug and solubilizer were mixed in a mortar by geometrical dilution technique.[12] The resulting mixtures were sieved through sieve no: 60 collected and stored. The ratios were mentioned in **Table 2**.

Table 2: Ratio of drug: Solubilizer for inclusion complexes

Drug (mg)	β-CD (mg)
50	50
50	100
50	150
50	-
50	-
50	-

2.4. Preparation of Celecoxib Oral Dissolving Films:[4,11]

Polymer was soaked in distilled water (5ml) for 1h. Sweetener, plasticizer, mannitol and other ingredients were dissolved in 95% ethanol (around 2ml). Then drug and solubilizer complex equivalent to 100 mg and superdisintegrant were taken and added to the polymer solution. The entire mixture was stirred for 1h at 1000 rpm and sonicated for 10 mins to remove the entrapped air. This mixture was poured on an ointment slab and allowed to air dry. Then films of 2×2 cm^2 were cut and were wrapped in a butter paper followed by aluminium foils and kept in dessicator for further studies. The compositions of the different drug loaded films were given in Table 3. Obtained film was shown in **Figure 1**.

Table 3: Composition of different formulations

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9
Drug β-CD complex (mg)	100	100	100	100	100	100	100	100	100
HPMC E15 (mg)	200	300	400	200	300	400	200	300	400
Musa paradisiaca gum (mg)	50	50	50	100	100	100	150	150	150
Glycerine (%)	2	2	2	3	3	3	4	4	4
Aspartame (mg)	3	3	3	3	3	3	3	3	3
Tween 80 (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mannitol (mg)	10	10	10	10	10	10	10	10	10
Citric acid (mg)	1	1	1	1	1	1	1	1	1
Methyl paraben (mg)	1	1	1	1	1	1	1	1	1

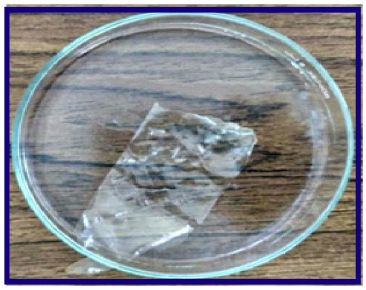

Figure 1. Prepared oral film

2.5. Evaluation of Celecoxib films:

2.5.1. Morphological properties:[13]

Homogenicity, color and Transparency of films were tested visually. The results were given in **Table 4**.

Table 4: Evaluation parameters for oral dissolving films

Formulations	Appearance	Weight variation	Folding Endurance	*In vitro* Disintegration Time
F1	Homogenous and Transparent	119.3 ± 0.89	59±0.19	38 ± 1.2
F2	Homogenous and Transparent	123.5 ± 0.61	61±0.42	42 ± 2.3
F3	Homogenous and Transparent	125.4± 0.54	57±0.35	36 ± 1.8
F4	Homogenous and Transparent	118.6±0.22	63±0.52	44 ± 1.1
F5	Homogenous and Transparent	121.9± 0.72	65±0.11	41 ± 1.6
F6	Homogenous and Transparent	124.7±0.36	59±0.34	46 ± 2.2
F7	Homogenous and Transparent	125.9±0.48	62±0.28	32 ± 1.5
F8	Homogenous and Transparent	120.5±0.63	67±0.16	43 ± 1.6
F9	Homogenous and Transparent	118.2±0.31	58±0.33	39 ± 1.7

2.5.2. Weight variation: [13]

Weight of 2×2 cm^2 film from different batches of the formulations was noted on electronic balance. The results were given in Table 4.

2.5.3. Surface pH Study

The surface pH of oral film was determined in order to investigate the possibility of any side effects *in vivo*. It should be kept close to neutral since acidic or basic surface pH may cause oral mucosal irritation. A combined pH electrode was used for this purpose. Oral film was slightly wet with the help of water. The pH was measured by bringing the electrode in contact with the surface of the oral film. The experiments were performed in triplicate.[4]

2.5.4. Tensile Strength:

It is the maximum stress applied to a point at which the film breaks. It is calculated by the load at rupture divided by the cross-sectional area of the film as given below:

Tensile strength = Load Failure ×100/ Film thickness × film width

It was measured using Shimadzu AG-100kNG (Winsoft tensile and compression testing). The film of size 2×2 cm^2 was placed between two clamps held 10 mm apart. The film was pulled by clamp at a rate of 5mm/min. Whole experiment was carried out in triplicate. [14,15]

2.5.5. Percent Elongation (%E):[14,16]

Strain occurs when the film sample stretches due to applied stress. Strain is basically the deformation of the film divided by the original dimension of the film. Percentage elongation was calculated by measuring the increase in length of the film after tensile strength measurement by using the following formula.

Percentage Elongation = $[L-L_0]$ X 100 / L_0

Where, L = Final length, L_0 = initial length

2.5.6. Folding endurance:[11]

Folding endurance indicates brittleness of the film and was determined by repeated folding of the film at the same place till the film breaks. The results are given in Table 4.

2.5.7. In vitro Disintegration Time:[14]

The film strip was placed in a glass petri plate containing 25 ml of distilled water at 37p C with swirling at every 10 secs. Disintegration time was recorded as the time at which the film starts to break or disintegrate.

2.5.8. Drug content: [14]

2×2 cm^2 film was taken into a 10 ml volumetric flask and dissolved in methanol (10 ml). Then it was kept aside for 2 hrs and then filtered through 0.45 µm membrane filter and absorbance as checked at 252nm.

2.6. *In vitro* dissolution method:[14]

2.6.1. Beaker stirring method:

These studies were conducted using 150 ml glass beaker with 125 ml of 6.8 pH buffer as dissolution medium. Film was placed on one side of the beaker using double sided tape. Medium was stirred at a speed of 200 rpm using magnetic stirrer. 5 ml samples were withdrawn at 5, 10, 15, 20, 25, 30, 60 secs time intervals and every time replaced with 5 ml of fresh medium solution. Samples were analyzed by UV at 252 nm.

2.6.2. Ex vivo drug permeation studies: [17]

Drug permeation studies were carried out using porcine cheek pouch as permeation barrier on a standard two chambered Franz diffusion cell, to determine the rate and extent of mucosal permeation of Celecoxib. The water jacket was maintained at 37 ± 1°C. The receptor compartment was filled with PBS (pH 7.4). Film of size 2 × 2 cm^2 was cut and weighed. The film was mounted in donor compartment which was filled with 7 ml of dissolution medium. The dissolution media was stirred at 50 rpm making use of a magnetic bead. Samples were withdrawn at predetermined time intervals from the receptor compartment, suitably diluted, and analyzed using UV spectrophotometer at λ_{max} 252 nm against a blank (UV 1800, Shimadzu, Japan).

2.7. Fourier Transforms Infrared Spectroscopy (FTIR) analysis:

The Infrared spectra of pure drug and optimized formulation were recorded at a scanning range of 400 to 4000 cm^{-1} using FTIR Spectrophotometer. This study was done to rule out any possible interactions between Celecoxib and the excipients.

2.8. Stability studies:

The objective of stability studies was to predict the shelf life of a product by accelerating the rate of decomposition, preferably by altering the temperature. The optimized formulation F7 was subjected to stability studies by storing at 4°C and room temperature for 30 days. These samples were analyzed and checked for changes in physical appearance and drug content at regular intervals.

3. RESULTS AND DISCUSSION:

3.1. Solubility studies of Celecoxib in water and different buffers:

The pH of saliva varies in individuals based on the diet, health condition etc. The pH of the normal individual is in the range of pH 6.2 to 7.4. The solubility studies of Celecoxib were conducted in water and in different phosphate buffers within the salivary pH range. The results of solubility studies at different pH indicated that Celecoxib was more soluble in alkaline pH than in acidic pH . This is due to the acidic nature of Celecoxib by virtue of its sulphonamide group. Hence pH 7.4 was used as dissolution medium.

3.2. Surface pH Study:

The surface pH of the oral films was ranging from 6.7 and 7.2. Since surface pH of films was found to be around neutral pH, any kind irritation to the mucosal lining of the oral cavity was not seen.

3.3. Tensile Strength and % elongation:

ODFs should possess moderate tensile strength, and high % elongation (% E). As the plasticizer amount increases, % elongation increases. Tensile strength is high if polymer concentration is less and superdisintegrant is high **(Table 5)**.

Table 5: Evaluation parameters for oral dissolving films

Formulations	Tensile strength	% Elongation	Drug content	*In vitro* drug release
F1	1.25 ± 0.21	20.24 ± 1.20	98.57 ± 1.02	90.15 ± 1.12
F2	1.12 ±0.23	23.52 ± 0.66	99.36 ± 0.21	86.19 ± 0.98
F3	1.24 ± 0.34	22.51 ± 0.11	99.20 ± 1.63	75.23 ± 0.93
F4	2.54 ± 0.26	34.32 ± 0.68	97.85 ± 1.44	91.22 ± 1.03
F5	2.62 ± 0.17	31.44 ± 1.53	95.00 ± 0.92	87.44 ± 0.76
F6	2.93 ± 0.28	36.53 ± 0.30	99.40 ± 0.86	79.16 ± 0.94
F7	1.95 ± 0.14	42.29 ± 0.88	98.53 ± 0.37	94.38 ± 1.17
F8	1.71 ± 0.29	45.68 ± 0.75	99.20 ± 0.56	87.93 ± 0.55
F9	1.85 ± 0.22	41.52 ± 0.50	97.34 ± 0.93	80.14 ± 1.23

3.4. In vitro Disintegration time:

All the batches of oral dissolving strips (F1– F9) were found to disintegrate in less than 60 sec. *In vitro* disintegration time was found to increase with increase in the amount of HPMC used in the formulations F7 was found to give fastest *in vitro* disintegration time (32.00 secs) as compared to other formulas. The results were given in **Table 4**.

3.5. Drug content:

The drug content in all the formulations varied between 95 to 99 %. The results were given in **Table 5**. These results indicated a good uniformity of Celecoxib within films and overall, good solubilization of drug in the formulations.

3.6. In vitro Dissolution studies:

The drug release study was carried out for 5 mins at time interval of 30 secs. The drug release profile for all batches was shown Figures 2. As the concentration of the superdisintegrant increased there was considerable increase in the drug release. But with the increase in the polymer concentration the inverse results were observed. The F7 batch containing 200 mg of HPMC and 150 mg of superdisintegrant has shown the drug release of 58.71% in initial 30 seconds and upto 99.64% in 5 mins.

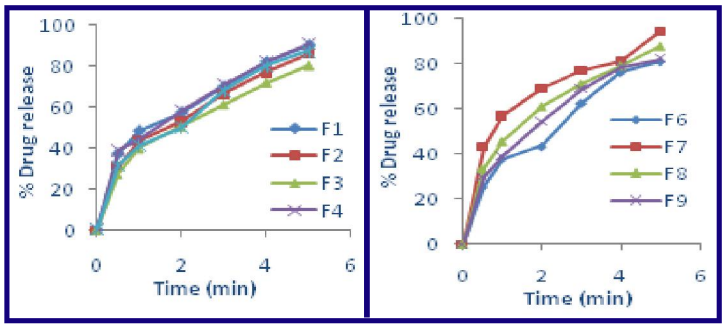

Figure 2. *In vitro* dissolution profile of formulations

3.7. Ex vivo drug permeation studies:

The *ex vivo* permeation of Celecoxib from the film had shown that the drug permeated well across porcine buccal mucosa over a period of 5 min. The *ex vivo* permeation from F7 was found to be 74.56 ± 1.2% in 5 min. In case of formulation F4, nearly 69% drug permeated in 5 min Film F1 had shown minimum *ex vivo* drug permeation in 5 min, i.e, 57% **(Figure 3)**.

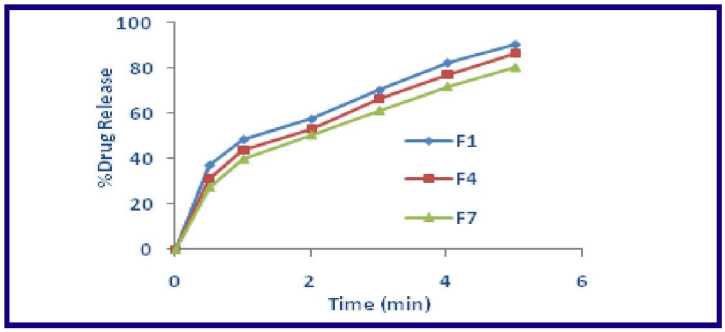

Figure 3. *Ex vivo* permeation studies through porcine buccal mucosa

3.8. Fourier Transforms Infrared Spectroscopy (FTIR) analysis:

The characteristic peaks of the pure drug were compared with the peaks obtained for Formulations F7. From the data it was observed that characteristic peaks of Celecoxib and formulation F7 were similar. Hence it can be inferred that there is no chemical interaction between drug and polymer and it can be concluded that the characteristics bands of pure drug were not affected by the method used to prepare oral films **(Figure 4)**. Range of wave lengths, bond nature and bond attribute of functional groups obtained were mentioned in **Table 6**.

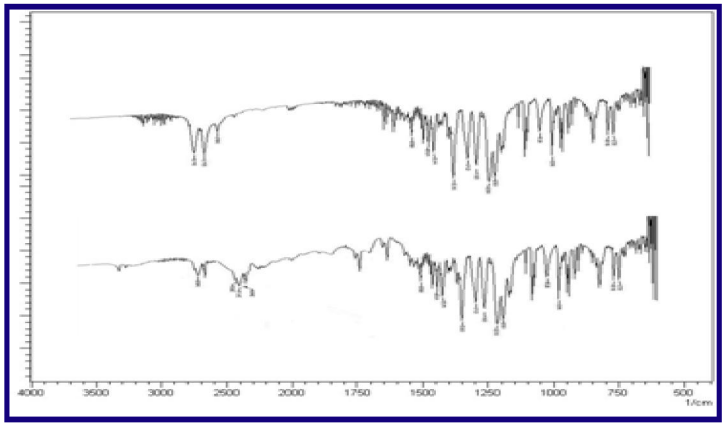

Figure 4: FTIR spectra of pure drug celecoxib (A) and formulation F7 (B)

Table 6: FTIR spectral data of pure celecoxib (A) and formulation F7 (B)

S. No	Range	Drug (A)	Formulation (B)	Bond nature and Bond attribute
1.	3500-3300	3341	3428	N-H Stretching vibrations in amines
2.	1370-1335	1347	1359	S=O asymmetric
3.	1170-1155	1165	1161	S=O symmetric
4.	1400-1000	1230	1238	C-F Stretching

3.9. Stability studies:

The stability studies for the optimized formulation were carried out and the results were given in **Table 7**. No considerable variations in the physical property and the drug content were observed during the study period.

Table 7: Stability studies of F7 formulation

S. No	Time period (days)	Storage conditions	Percent drug content (w/v) ±S.D.
1.	0	-	93.78 ± 3.24
2.	30	30°C and 60% RH	92.24 ± 3.36
3.	30	40°C and 75% RH	93.10 ± 4.12
4.	90	30°C and 60% RH	92.36 ± 4.49
5.	90	40°C and 75% RH	92.57 ± 3.65

4. CONCLUSION:

From this study it was concluded that β-CD that has been used as a solubilizer, enhances the solubility of Celecoxib which is a class II drug. *Musa paradisiaca* acted as a good super disintegrant. The films prepared using these excepients has shown good *in vitro* and *ex vivo* release. Higher the super disintegrant and plasticizer amounts, higher is the drug release.

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Research Article

A Prospective Study on Clinical Profile with Special Reference to Etiology, Management and Drug Utilisation Review on Cerebrovascular Accident with Ischemic and Hemorrhagic Stroke

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ABSTRACT

Background: Stroke is one of the leading causes of death and long-term disability in world. Stroke is an important cause of premature death and disability in low-income and middle-income countries like India, largely driven by demographic changes and enhanced by the increasing prevalence of key modifiable risk factors. Main aim of our study was to assess the clinical profile with special reference to the etiology of the condition, the management and drug utilization review. **Methods:** A Prospective observational study of 6 months was performed at general medicine department of the tertiary care government general hospital, Kurnool. Total of Hundred Patients aged above 20 years with Cerebrovascular accident with ischemic and hemorrhagic stroke were included following inclusion and exclusion criteria to observe their demographic profile and drug use. The data was analyzed summarized as frequency and percentage by using Microsoft Excel. **Results:** In our prospective study of hundred patients on cerebrovascular acccident with ischemic and hemorrhagic stroke. We observed males (70%) were more than females (30%). Stratification based on age showed patients admitting to general ward were frequently within the age group of 50-60 and 60-70. Among 100 patients studied, Hypertension (74%), Diabetes mellitus (23%), Smoking (34%) and alcohol (34%) were the risk factors respectively. **Conclusion:** We observed age, hypertension, diabetes mellitus, smoking and alcohol were the major risk factors. We conclude that proper risk factor management and following the guidelines in the treatment reduces the severity, thereby the prognostic factors will be good.

Key words: Ischemic Stroke, Hemorrhagic Stroke, Precipitating Factors, WHO Prescribing Indicators, Drug Utilisation Review.

INTRODUCTION

Stroke is an acute neurologic injury occurring as a result of vascular pathologic processes which manifest either as brain infarction or hemorrhage. Stroke is one of the leading causes of death and long-term disability in India. Stroke is an important cause of premature death and disability in low-income and middle-income countries like India, largely driven by demographic changes and enhanced by the increasing prevalence of key modifiable risk factors. Among all the neurological diseases of adult life, cerebrovascular accidents clearly first in the frequency of importance. Almost fifty percent of neurological diseases in general hospital are due to stroke. Cerebrovascular accident includes ischemic stroke, hemorrhagic stroke and cerebrovascular anomalies such as an intracranial aneurysm, AV malformation and cortical venous thrombosis. Stroke after heart disease is the second most common cause of death among non-communicable disease.[1] With the introduction of effective treatment for hypertension, there has been a marked reduction in the frequency of stroke. Diabetes mellitus by virtue of its association with microvascular and macrovascular disease is an important risk factor in the genesis of stroke. Most of the diabetic patients with stroke have raised glycosylated

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hemoglobin indicating that most of them have uncontrolled diabetes.[2] Diabetes and stress hyperglycemia have severe strokes resulting in a poor outcome. Stroke is twice more common in diabetes than in non-diabetics.[3] Hypertension is common in diabetes and accelerates atherosclerosis which promotes intracranial small vessel disease and heart disease leading to lacunar and embolic infarction respectively. There are several risk factors that determine the outcome of a stroke.[4] The studies related to drug utilization in neurology is limited in India. Drug Utilization Research (DUR) was defined by 'World Health Organization' (WHO) in 1977 as 'Marketing, distribution, prescription and use of drugs in society with special emphasis on resulting medical, social and economic implication. Such studies have to be carried out from time to time. Drug utilization study, especially at the neurology department in India, is very less. Keeping these things in mind we conducted a single centered prospective study in general medicine inpatient department at a tertiary care hospital with objectives to study the profile of patients admitting to the general medicine department, to study the prescriptions based on WHO prescribing indicator and to identify utilization pattern of drugs. A Drug is a complex process. Uncertainties in diagnosis, treatment and medication adherence contribute to wide variations in the way drugs are used for any given condition Drug use evaluation or drug utilization review is an ongoing, authorized and systematic quality improvement process, which is designed to[5]

- Review drug use and/or prescribing patterns.
- Provide feedback of results to clinicians and other relevant groups.
- Develop criteria and standards which describe optimal drug use.
- Promote appropriate drug use through education and other intervention.

MATERIALS AND METHODS

Study Site: Government General Hospital, Kurnool.

Study Period: The study was carried out for 6 months i.e., from August 2018 to January 2019.

Study Size: 100 patients fit into inclusion criteria from both male and female General Medicine Departments.

Study Design: A Prospective observational study.

Inclusion Criteria

Patients should be above the age of twenty, have been admitted within twenty-four hours of onset of symptoms, must be diagnosed as a cerebrovascular accident with ischemic or hemorrhagic stroke.

Exclusion criteria

Pediatric patients and patients presented with illness having stroke-like symptoms from the hospital were excluded.

IRB Approval

The research protocol was duly approved by IRB of Dr. K. V. Subba Reddy Institute of Pharmacy vide approval number KVSP/IRB 2018-2019/Pharm.D/PROJ/002.

Method of Study

Selection of the patient was based upon their inclusion criteria and final diagnosis. All the data collected in a patient profile collecting proforma for the study. For drug-drug interactions drug information resources like "Medscape Drug Interaction Checker", "Stockley's Drug Interactions" were reviewed. Only major and moderate interactions were mentioned which have more probability to appear in patients. The data was analyzed by using Microsoft Office Excel, synthesized the results and interpreted the data in simple frequency and percentages

RESULTS

Age and Sex incidence

Our study means that as age advances there is a frequent chance of having a neurological illness which is in conjuncture with other result findings.

Gender Distribution

In our study of a hundred patients, the majority of them belonged to male sex showing a male preponderance which is commonly seen in most studies which are represented in Table 1 and Figure 1.

Age wise distribution of patients

The majority (38%) of the patients were in the age group of 50 – 60 years. as represented in the Table 2 and Figure 2.

Age wise and sex wise distribution of patients

In our study majority of the patients were among the age group of 50-60 years and 60-70 years with male preponderance than female as such 26 (37.1%) males were of 50-60 years and 16 (22.9%) were of 60-70 years age group and 12 (40%) females were of 50-60 years and 9 (30%) were of 60-70 years age group as represented in the Table 3.

Incidence of risk factors

Hypertension

In our study among the hundred patients, we observed 74 (74%) patients were with hypertension and 26 (26%) were without hypertension which was shown in the Table 4. Among the hypertensive patients we had observed 51 were males and 23 were females.

Diabetes Mellitus

In our study out of a hundred patients, 23 (23%) patients were with diabetes mellitus and 77 (77%) were without diabetes mellitus which was shown in the Table 5. Among the diabetic patients 17 were males and 6 were females.

Smoking

Excluding females, 34 (34%) males were smokers and 66 (66%) were nonsmokers was shown in the Table 6.

Alcoholic

In our study, excluding females 34 (34%) patients were alcoholic and 66 (66%) were nonalcoholic among all the 100 patients was shown in the Table 7.

Table 1: Gender distribution.

Sex	Frequency (*N*)	Percentage (%)
Male	70	70.0
Female	30	30.0
Total	100	100.0

Table 2: Age wise distribution of the patients.

Age group	Frequency (*N*)	Percentage (%)
20 – 30	4	4.0
30 – 40	10	10.0
40 – 50	18	18.0
50 – 60	38	38.0
60 – 70	25	25.0
70 – 80	3	3.0
80 – 90	2	2.0

Table 3: Age wise and sex wise distribution.

Age groups	Male	Female
20 – 30	01 (1.4%)	03 (10%)
30 – 40	09 (12.8%)	01 (3.33%)
40 – 50	14 (20%)	04 (13.33%)
50 – 60	26 (37.1%)	12 (40%)
60 – 70	16 (22.9%)	09 (30%)
70 – 80	02 (2.9%)	01 (3.33%)
80 – 90	02 (2.9%)	0
Total	70 (100%)	30(100%)

Types of Stroke

In our study 66 (66%) patients had ischemic stroke among which 45 were male and 21 were female and 32 (32%) had hemorrhagic stroke among which 21 were males and 11 were females and 2 (2%) were having both ischemic and hemorrhagic stroke which was seen only in males as shown in the Table 8 and Figure 3.

Risk Factors

In our study of hundred stroke patients, the major etiology was found to be hypertension (74%) followed by smoking (34%), alcohol (34%), diabetes mellitus(23%), recurrent attacks (17%), cardiac diseases (7%) and drug noncompliance (4%) respectively as shown in the Table 9.

In our study of hundred stroke patients, based on sex wise distribution the frequency of the risk factors were observed as 51 males and 23 females were hypertensive, 17 males and 6 females were diabetic, excluding females, the frequency of both smoking and alcoholics were same i.e., 34, both the sexes with the same frequency 4 were presented with cardiac diseases, 12 males and 5 females had recurrent attacks and 4 males were presented with drug noncompliance as shown in the Table 10 and Figure 4.

Table 4: Percentage of Hypertensive patients.

	Frequency (*N*)	Percentage (%)
Yes	74	74.0
No	26	26.0
Total	100	100.0

Table 5: Percentage of Diabetic patients.

	Frequency (*N*)	Percentage (%)
Yes	23	23.0
No	77	77.0
Total	100	100.0

Table 6: Percentage of smoking.

	Frequency (*N*)	Percentage (%)
Yes	34	34.0
No	66	66.0
Total	100	100.0

Table 7: Percentage of alcoholic.

	Frequency (*N*)	Percentage (%)
Yes	34	34.0
No	66	66.0
Total	100	100.0

Table 8: Types of stroke.

Types of stroke	Male	Female	Frequency (*N*)	Percentage (%)
Ischemic Stroke	45	21	66	66.0
Hemorrhagic Stroke	21	11	32	32.0
I.S+ H.S	2	0	2	2.0
Total	68	32	100	100.0

Table 9: Percentage of risk factors.

Etiology	Frequency (*N*)	Percentage (%)
Hypertension	74	74.0
Diabetes Mellitus	23	23.0
Smoking	34	34.0
Alcohol	34	34.0
Cardiac diseases	7	7.0
Recurrent attacks	17	17.0
Drug noncompliance	4	4.0

Table 10: Risk factors based on sex wise distribution.

Etiology	Male	Female
Hypertension	51	23
Diabetes	17	6
Smoking	34	0
Alcohol	34	0
Cardiac diseases	4	4
Recurrent attacks	12	5
Drug noncompliance	4	0

Table 11: WHO Prescribing indicators.

WHO Prescribing indicators	Total
Number of cases reviewed	100
Ischemic stroke cases	67
Hemorrhagic stroke cases	33
Number of drugs utilized	782
Percentage of injections utilized	41.9%
Number of antibiotics	8.95%
Average number of drugs per prescription	7.82

Assessment of WHO Prescribing Indicators

We observed 782 medicines were prescribed in 100 prescriptions. An average number of drugs in prescription was 7.82, Percentage of injections utilized was found to be 41.9% (328), Percentage of patients with antibiotics was found to be 8.95% (70) as represented in Table 11.

Utilization Pattern of Drugs: Classification

The utilization of various drugs and its classification in both ischemic and hemorrhagic stroke was presented. Total 22 different drug classes were used with different drugs. Eleven different classes i.e., only half of the classes that were utilised had been accounted for 95% of drugs utilisation in both which included Gastrointestinal agents (*N*=105, 13.4%), Hypolipidemics (*N*=100, 12.7%), Supplements (*N*=98, 12.53%), Antihypertensives (*N*=96, 12.27%), Osmotic diuretics (*N*=83, 10.6%), Antiplatelet (*N*=70, 8.95%), Antibiotics (*N*=70, 8.95%), Nootropics (*N*=28, 3.5%), Antipyretics and Analgesics (*N*=25, 3.1), Antidiabetics (*N*=23, 2.94%) respectively. Very infrequently used a class of drugs included Antidiarrheal (Purgatives), Antiemetics, Antiviral, Laxatives, Bronchodilators, Skeletal muscle relaxants, Corticosteroids, Mucolytics, Hepatoprotectives Table 12.

Utilisation Pattern of Drugs: Nature and Extent of Drug Use

73 Different drugs were utilised in both ischemic and hemorrhagic stroke of total 100 patients which was as shown in table. Most frequently Hypolipidemic Atorvastatin (12.7%) was used followed by Osmotic diuretic Mannitol (10.3%), Aspirin (8.2%), Pantoprazole (8.2%), Amlodipine (5.7%), B-complex (5.1%), Calcium+VitaminD3 (4.5%), Ranitidine (4.5%), Citicholine (2.8%), Acetaminophen (2.8%), Phenytoin (2.3%), Telmisartan (2.2%), Insulin (2.0%), Iron Supplements (1.6%), Metronidazole (1.5%), Labetalol (1.5%), Lorazepam (1.2%), Thiamine (1.1%), Nimodipine (0.76%), Duoiln+Budecort (0.6%), Multivitamins (0.6%), Cefotaxim (0.5%), Atenolol (0.5%), Cefixime (0.5%), Nootropil (0.5%), Clopidogrel (0.5%), Ondansetron (0.5%), Piperacillin+Tazobactam (0.35%), Amoxicillin Clavulanate (0.3%), Enoxaparin (0.3%), Theophylline (0.3%), Acenocoumarol (0.2%), Nifedipine (0.2%), Divalproexsodium+Valproic acid (0.2%), Metformin+Glimepride (0.2%), Metoprolol (0.2%), Metformin (0.2%), Baclofen (0.2%), Dexamethasone (0.2%), Levitriacetam (0.2%), Artesunate (0.2%), Doxyxcyline(0.2%), Ambroxol (0.2%), Midazolam (0.2%), Atenolol+Amlodipine (0.1%), Prazosin (0.1%), Metformin+Glibenclamide (0.1%), Losartan (0.1%), Escitalopram+Clonazepam (0.1%), Ursodeoxycholic Acid (0.1%), Salbutamol (0.1%), Sandostatin(0.1%), Rifampin+Ethambutol+Isoniazid+Pyrizinamide (0.1%), Glibenclamide (0.1%), Enalapril (0.15), Pyridoxine+Hydrochloride (0.1%), Ciproflaxacin (0.1%), Streptomycin (0.1%), Hydrocortisone (0.1%), Dopamine (0.1%), Acyclovir (0.1%), Racecadotril (0.1%), Vitamin C (0.1%), Potassium Chloride (0.1%), Tramadol (0.1%), Methyl Prednisoolone (0.1%), Pregabalin (0.1%), Spiranolactone+Torsemide (0.1%), Lactulose (0.1%) and Gabapentin (0.1%) Table 13.

Table 12: Utilization pattern of drugs: Classification.

S. No	Class of drug	I.S	H.S	Frequency (N)	Percentage (%)
1	Antiplatelets	70	0	70	8.95
2	Antihypertensives	42	54	96	12.27
3	Anticoagulants	5	0	11	1.4
4	Supplements	71	27	98	12.53
5	Antiepileptics	22	19	41	5.24
6	Gastrointestinal agents	66	39	105	13.4
7	Antibiotics	45	25	70	8.95
8	Nootropics	18	10	28	3.5
9	Hypolipidemic	68	32	100	12.7
10	Antipyretics and analgesics	18	7	25	3.1
11	Antidiabetics	17	6	23	2.94
12	Osmotic diuretics	50	33	83	10.6
13	Laxatives	2	0	2	0.25
14	Bronchodilators	9	0	9	1.15
15	Skeletal muscle relaxants	2	0	2	0.25
16	Corticosteroids	3	1	4	0.51
17	Ionotropic	1	0	1	0.12
18	Antiviral	1	0	1	0.12
19	Antidiarrheal	8	4	12	1.5
20	Mucolytics	2	0	2	0.25
21	Antiemetics	1	3	4	0.51
22	Hepatoprotectives	0	1	1	0.12
	Total	521	261	782	100.36

Table 13: Utilization pattern of drugs: Nature and extent of drug use.

S.No.	Name of utilized drugs	IS	HS	Frequency	Percent
1	Mannitol	48	33	81	10.3
2	Aspirin	65	0	65	8.2
3	Atorvastatin	68	32	100	12.7
4	Pantoprazole	42	23	65	8.2
5	Amlodipine	26	20	46	5.7
6	Calcium+Vitamin D3	26	10	36	4.5
7	Thiamine	6	3	9	1.1
8	Librium	2	2	4	0.5
9	B-Complex	28	12	40	5.1
10	Telmisartan	4	14	18	2.2
11	Human Insulin	12	4	16	2
12	Phenytoin	8	11	19	2.3
13	Iron supplements	12	2	14	1.6
14	P+G+M	1	0	1	0.1
15	Ranitidine	23	14	37	4.5
16	Acetaminophen	17	6	23	2.8
17	Gabapentine	1	0	1	0.1
18	Citicholine	13	9	22	2.8
19	Labetalol	1	11	12	1.5
20	Prazosin	0	1	1	0.1
21	Clopidogrel	4	0	4	0.5
22	Ceftriaxone	28	20	48	19.7
23	Theophylline	3	0	3	0.3
24	Atenolol+Amlodipine	1	0	1	0.1

25	Acenocoumarol	2	0	2	0.2
26	Enoxaparin	3	0	3	0.3
27	Metronidazole	8	4	12	1.5
28	Cefotaxime	2	2	4	0.5
29	Atenolol	2	2	4	0.5
30	Metformin+Glibenclamide	1	0	1	0.1
31	Cefixime	3	1	4	0.5
32	Duolin+Budecart	5	0	5	0.6
33	Amoxicilline+Clavulanate	3	0	3	0.3
34	Nootropil	3	1	4	0.5
35	Lorazepam	6	4	10	1.2
36	Nifedipine	0	2	2	0.2
37	Losartan	1	0	1	0.1
38	Escitalopram+Clonazepam	1	0	1	0.1
39	Divalproex sod.+Valproic acid	2	0	2	0.2
40	Multivitamins	5	0	5	0.6
41	Metformin+Glimepiride	2	0	2	0.2
42	Metoprolol	1	1	2	0.2
43	Ursodeoxycholic acid	0	1	1	0.1
44	Metformin	1	1	2	0.2
45	Salbutamol	1	0	1	0.1
46	Sandostatin	1	0	1	0.1
47	Enalapril	1	0	1	0.1
48	Glibenclamide	0	1	1	0.1
49	Baclofen	2	0	2	0.2
50	R+I+P+E	1	0	1	0.1
51	Pyridoxine+Hydrochloride	1	0	1	0.1
52	Dexamethasone	1	1	2	0.2
53	Piperacillin+Tazobactum	1	2	3	0.3
54	Ciprofloxacin	1	0	1	0.1
55	Streptomycin	1	0	1	0.1
56	Hydrocortisone	1	0	1	0.1
57	Levetiracetam	1	1	2	0.2
58	Ondansetron	1	3	4	0.5
59	Dopamine	1	0	1	0.1
60	Artesunate	2	0	2	0.2
61	Doxycycllin	2	0	2	0.2
62	Acyclovir	1	0	1	0.1
63	Racecadotril	1	0	1	0.1
64	Nimodipine	1	5	6	0.76
65	Vitamin-C	0	1	1	0.1
66	Potassium chloride	1	0	1	0.1
67	Tramadol	1	0	1	0.1
68	Methylprednisalone	1	0	1	0.1
69	Ambroxol	2	0	2	0.2
70	Pregabiline	0	1	1	0.1
71	Midazolam	2	0	2	0.2
72	Spiranolactone+Torsemide	1	0	1	0.1
73	Lactulose	1	0	1	0.1
	Total	521	261	782	100.03

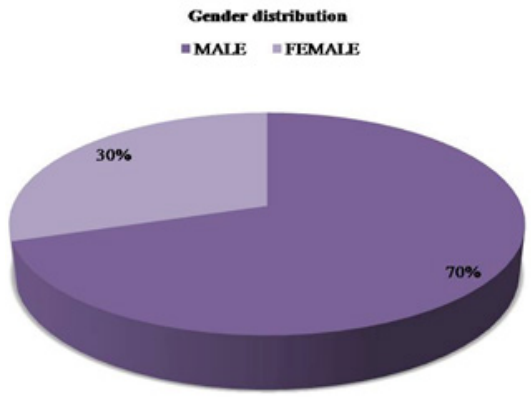

Figure 1: Graphical representation of gender distribution.

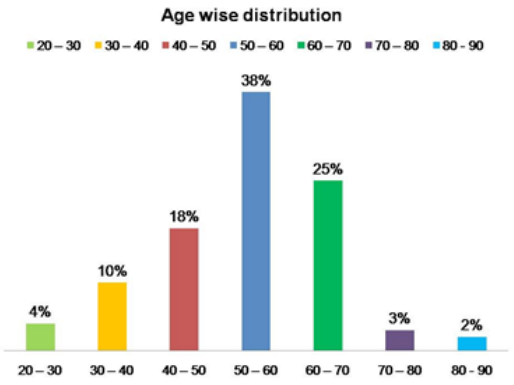

Figure 2: Graphical representation of age wise distribution.

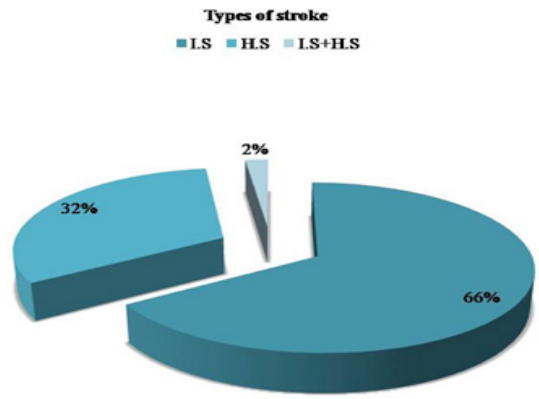

Figure 3: Graphical representation of the percentage of the types of stroke.

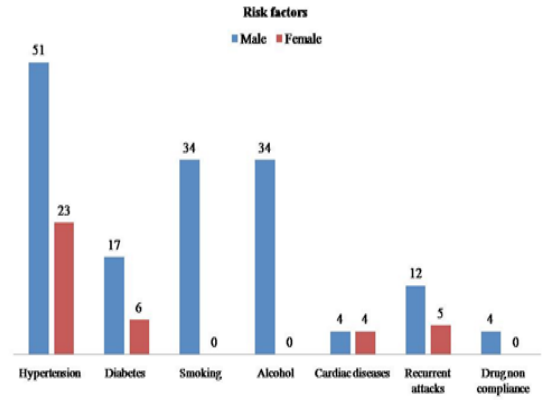

Figure 4: Graphical representation of risk factors based on sex wise distribution.

DISCUSSION

The stroke is considered as an emergency medical condition which is to be treated as early as possible. Other than ischemic and hemorrhagic stroke, there are other conditions like transient ischemic attack and silent ischemic stroke. Our study observed that stroke is being a major burden to neurological illness. In our hospital, the Cerebrovascular accident was a most common diagnosis of stroke but it can vary from region to region and hospitals. Most people with stroke were between 50-60y and 60-70y in age group. Our study also shows that being a male has more chances of stroke. One reason may be due to different-sex steroid hormones, notably estrogens and testosterone. Estrogens have terribly potent effects on endothelial that promote dilation and blood flow whereas testosterone has opposing effect indicating women are protecting with endogenous estrogens.[6] We already knew that as the number of risk factors increases, the progression and complications of the disease increases. Even in the present study, we found that there were more patients who have involvement of multiple risk factors than single or no risk factors. Proper intervention is to be done assessing risk factors. Measures are to be taken in order to avoid progression of certain risk factors. Because improper control over stroke leads to disability. The major risk factor in stroke patients in the present study was found to be hypertension (74%) followed by diabetes (23%) and age and the least risk was seen with a past history of coronary artery disease (4%). The reasons for the progression with these risk factors to the stroke might be due to the change in capillary tone and advances glycation product formation. Drug utilization pattern provides the rationality in drug prescribing and analysis. Prescribing indicators assess the drug use problems in prescriptions from rationality standpoint.[7] Among different indicators drugs per prescription is high (7.82). This number is high in relation to get drug-drug interactions. In fact, we observed prescription which can theoretically cause effects of serious drug interactions like Ceftriaxone + Enoxaparin, Phenytoin + Nifedipine and Aspirin + Enalapril and moderate drug interactions like Aspirin + Insulin aspartate, Aspirin + Telmisartan, Telmisartan + Atorvastatin, Aspirin + Clopidogrel, Amlodipine + Atenolol, Pantoprazole + Clopidogrel, Phenytoin+ Atorvastatin, Phenytoin + Pantoprazole, Calcium + Amlodipine, Metronidazole + Atorvastatin, Amlodipine + Metformin. Close and careful monitoring

was done to these patients to optimize therapy.

We observed utilization of drugs like Mannitol, Aspirin, Atorvastatin, Multivitamins, Amlodipine, Ceftriaxone, Pantoprazole, Phenytoin, Citicoline, Clopidogrel, Acetaminophen, Metronidazole. Apart from these drugs, we observed the use of a combination of drugs like Atenolol + Amlodipine, Metformin + Glibenclamide, Metformin + Glimepiride, Calcium+ Vitamin D3, Spironolactone + Torsemide, Clopidogrel + Aspirin. Antihypertensives like β-blockers, Diuretics, Calcium channel blockers, α+β blockers, Angiotensin II receptor blockers were used to reduce target BP for these patients. It shows the fact that antihypertensive's were frequently used agents for stroke patients, along with Antihypertensives, Antiplatelets, Anticoagulants, Dyslipidemics, Nootropics, Multivitamins, Antidiabetics were also prescribed. Drugs to be administered for conditions occurring after the initial event (epilepsy, depression, anxiety and dementia) like Antiepileptics Antipsychotics, CNS stimulants were also prescribed to the patients.[8-10] Our results are in conjuncture to these findings. In our Study, we have observed that Clopidogrel was given only to the patients with cardiac disorders and to the patients who are allergic to Aspirin. Primary management of stroke includes Antiplatelet therapy with Aspirin, together with Clopidogrel used in the management of stroke and in patients with past history of cardiac disorders and Clopidogrel, Statin therapy and blood pressure management. Primary prevention interventions are expected to target behavioral modifications such as to reduced smoking, alcohol, salt consumption patterns and increased physical activity. Increasing fruit and vegetable consumption is considered to reduce the risk of stroke by 6 %.[11] Secondary prevention is through pharmacological therapy. Tertiary prevention is attributed to maintaining their ability to carry out daily activities if they receive rehabilitation services at home. Stroke rehabilitation is expected to begin as soon as possible after the person has a stroke and continue for as long as it is clinically appropriate.[8] Treatment pattern of illness other than stroke involved specific pharmacological agents like Antiepileptics like Levetiracetam, Gabapentin, Phenytoin, Lorazepam and Antipsychotics like Escitalopram and Clonazepam for Seizures related to Stroke or Epilepsy. Steroids like Dexamethasone and Methyl Prednisolone were used primarily. Other agents were used for symptomatic treatment like Antibiotics, Alkaliser's, Supplements, Antipyretics, Laxatives, Sedative-Hypnotics, Antidepressants, Antidiabetics.

CONCLUSION

In most of the cases, drug utilization review was done effectively, an average number of drugs per prescription with different types of drug classes used in both ischemic and hemorrhagic stroke were found accordingly. Most of the patients attending the department of general medicine with ischemic and hemorrhagic stroke were above the age of fifty and geriatric patients. Stroke occupies topmost burdens. We observed high drugs per prescription for stroke cases mostly generic drugs were used based on the socioeconomic status of the patient. In our study, we observed age, Hypertension (74%), Diabetes mellitus (23%), Smoking (34%) and Alcohol (34%) were the major risk factors. Various type of drugs were used depending on the diseased condition illness of the patient. In our study most commonly Antiplatelets, Statins, Osmotic Diuretics like Aspirin, Atorvastatin and Mannitol were respectively utilized based on the types of stroke.

A primary prevention strategy to reduce highly prevalent neurological cases like stroke is needed. Finally, we conclude that proper risk factor management and following the guidelines in the treatment reduces the severity, thereby the prognostic factors will be good. Early identifications of risk factors and pattern of therapy play a crucial role in qualitative patient care such that can reduce the recurrent attacks of the stroke.

ACKNOWLEDGEMENT

It is a great pleasance to convey our gratification and sincere thanks to Dr. K. Narasimhulu, M.D. (Gen.Med.), DTCD., Professor and HOD, Department of General Medicine, Govt. General Hospital and Dr. M. Ramanjulu, M.S., M.Ch, (Neurosurgery), HOD, Department of Neurosurgery, Govt. General Hospital for their guidance, supervision and support during the study. We take this opportunity to acknowledge the management and principal of Dr. K. V. Subba Reddy Institute of Pharmacy, Kurnool for providing necessary facilities and timely help to complete our work.

ABBREVATIONS

BP: Blood Pressure; **DUR:** Drug Utilization Review; **H.S:** Hemorrhagic Stroke; **IH:** Ischemic Stroke; **WHO:** World Health Organization.

SUMMARY

- The study was carried out in an attempt to find out the major risk factors and to evaluate the quality of drugs prescribing at Tertiary care Hospital. The study showed an active involvement of clinical pharmacists in different wards of General Medicine Department.
- The major risk factor found in our study was hypertension followed by diabetes mellitusin order

to control high blood pressure patient have to follow regular physical activity, maintaining a healthy body weight, reducing salt (sodium chloride) intake, consuming a diet that emphasizes fruits, vegetables and low-fat dairy products and that is reduced in fat and cholesterol. Male patients were more prone to be affected with the condition due to smoking and consumption of alcohol which have to be avoided.

- Stroke is a time-sensitive condition regardless of whether it is ischemic or hemorrhagic. although Pharmacotherapeutic treatment options are limited, they have been shown to reduce the complications associated with stroke. Pharmacotherapeutic treatment options also reduce the risk of recurrent stroke

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Evaluation of the activity of trans-Resveratrol alone and in combination with Amlodipine and Pioglitazone against Fructose induced metabolic syndrome rats

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Abstract

Metabolic syndrome (MS) is a cluster of conditions that cause an increase in the risk of diabetes, heart disorders, and stroke.

The present research was completed in Wistar rats, in which Metabolic Syndrome (MS) was induced with a High Fructose Diet. Animals were randomly divided into 7 gatherings and the test group animals received Resveratrol (RSVT), Amlodipine (AML), Pioglitazone (PIO), Resveratrol+Amoldipine, and Resveratrol+Pioglitazone at different doses for 5 weeks. Various behavioral, biochemical, and histopathological parameters were estimated.

AML alone and along with RSVT was found to reduce diastolic and systolic pressures, there was the reduction in BP in the remaining groups. There was a significant reduction in serum insulin and Fasting glucose level (FGL) in all the treatment groups. And there was a noticeable reduction in the levels of total glycerides (TG), total cholesterol (TC) along with LDL, HDL, and VLDL when compared to the control group and HFD group. Histopathological study revealed that there was a reduction in the deposition of lipids in liver cells and aorta as compared to HFD group.

The outcomes showed the defensive mechanism of Resveratrol against fructose-induced Metabolic Syndrome. The mechanism of protection may be due to an escalation of cellular antioxidants. The activity was found to increase in combination with amlodipine and pioglitazone.

Keywords: Amlodipine, Fructose, Metabolic syndrome, Pioglitazone, Resveratrol.

INTRODUCTION

International Diabetes Federation (IDF), National Cholesterol Expert Program Adult Treatment Program III (NCEP ATP III), World Health Organization (WHO)1, and harmonized criteria were used to define the term "metabolic syndrome", these criteria include central obesity, elevated triglycerides, reduced high-density lipoprotein (HDL), raised blood pressure (BP), and fasting plasma glucose (FPG) or fasting glucose levels (FGL)2. Diagnostic criteria for metabolic syndrome often include central obesity and any two of the risk variables3.

Reaven initially coined the term metabolic syndrome (MS), also known as syndrome X, in 1988 to refer to the presence of atherogenic risk factors and underlying insulin resistance. The World Health Organization (WHO) improved the definition in 1997 to refer to a particular grouping of risk factors for type 2 diabetes and cardiovascular diseases, including abdominal obesity, high blood pressure, atherogenic dyslipidemia, stroke, cardiovascular disease, hyperglycemia, insulin resistance, hyperuricemia, and proinflammatory state4,5,6.

The WHO definition stipulates the presence of three or more of the following factors: fasting blood glucose >110 mg/dl, blood pressure >130/85 mm Hg, triglycerides >150 mg/dl plasma, high-density lipoproteins (HDL) 40 mg/dl plasma, and waist circumference >102 cm for men and >88 cm for women. In the world, metabolic syndrome affects more than 25% of the population7,8. The change in dietary habits, particularly the increased intake of simple sugars, primarily fructose, which are frequently used in sugar-sweetened drinks and the food industry, is one of the most significant factors contributing to the rising prevalence of MS, obesity, and type 2 diabetes mellitus around the world9,10. Rats have frequently been used as models for high fructose intake. High fructose feeding causes hypertension, hypertriglyceridemia, insulin resistance, and hyperinsulinemia in these models11.

Environmental and genetic variables are both involved in the multifactorial etiology of metabolic syndrome. The combination of a modern sedentary lifestyle with a diet high in fat and low in dietary fiber, bioactive compounds, and micronutrients accelerates the development of metabolic syndrome. Therefore, preventing metabolic syndrome with weight loss, a good diet, exercise, medication, and bariatric surgery is crucial. However, most drugs on the market are ineffective and linked to negative drug reactions3.

A stilbenoid, or natural phenol, called resveratrol (3,5,4′-trihydroxy-trans-stilbene), is a phytoalexin produced by several plants in reaction to injury or when the plant is being attacked by pathogens like bacteria or fungi12,13. The skin of grapes, blueberries, raspberries, mulberries, and peanuts are among the food sources of resveratrol14,15. There is no solid proof that resveratrol lengthens lifespan or significantly affects any human disease, even though it is frequently used as a dietary supplement and investigated in lab models of human diseases16.

There is insufficient proof that resveratrol affects human metabolic syndrome. There isn't much support for using resveratrol to treat diabetes, according to an analysis17. A meta-analysis found scant support for resveratrol's potential impact on diabetes biomarkers18.

In one review, there was insufficient proof that resveratrol helped diabetics with fasting plasma glucose levels. According to two reviews, resveratrol administration may lower body weight and body mass index, but not fat mass or total blood cholesterol19. Resveratrol supplementation may lessen the indicators of inflammation of TNF- and C-reactive protein, according to a 2018 review20.

The body may respond to resveratrol in a variety of ways, including by widening blood arteries and decreasing blood clotting. Additionally, it might lessen pain and swelling, lower blood sugar levels, and support the body's immune system. The most typical illnesses for which resveratrol is suggested are high cholesterol, cancer, heart disease, and many more.

MATERIALS:

Sources of fine chemicals:

Fructose & casein were purchased from Hi-media Ltd., Baroda, coconut oil was purchased from National Chemicals, Baroda. Trans-resveratrol & Amlodipine were obtained from Zydus Research Centre, Ahmedabad. All other reagents and chemicals obtained were of analytical grade.

Drugs:

Trans-resveratrol (RSVT) was dissolved in DMSO (dimethyl sulphoxide) & suspended in saline so that the final concentration of DMSO would not exceed 5%. Amlodipine (AML) and Pioglitazone (PIO) were dissolved in DMSO & suspended in saline so that the final concentration of DMSO would not exceed 5%. They were given orally.

METHODS:

Animals:

All experiments and protocols described in the present study were approved by the Institutional Animal Ethics committee (IAEC) of M. S. University, Baroda and with the permission from Committee for Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Healthy adult rats of either sex (200-250 gms) were used for the study. Rats were housed in small cages with free access to food and water ad libitum. During the period of the experiment, the animals were fed the standard laboratory diet, unless specified. Healthy rats of either sex (200-250gm) were reused. Animals were placed in small cages and maintained under standardized conditions (12-hr light/dark cycle, 24oC, 35 to 60% humidity) and provided free access ta o palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt. Ltd., Pune) and purified drinking water ad libitim unless specified.

EXPERIMENTAL DESIGN:

Rats (n=30) were randomized into the following groups.

1. Control: They were administered DMSO (DMSO in saline, final conc. of DMSO was 5%) p.o for 5 weeks.

2. High Fructose Diet (HFD): They were administered DMSO (DMSO in saline, final conc. of DMSO was 5%) along with HFD (60% fructose, 20% casein, 5%coconut oil, 15% std chow diet) & water ad libitium for 5 weeks.

3. HFD +Resveratrol (RSVT): They were administered RSVT (20mg/kg/day) p.o along with HFD & water ad libitium for 5 weeks.

4. HFD + Amlodipine (AML): They were administered Amlodipine (10mg/kg/day) p.o along with HFD & water ad libitium for 5 weeks.

5. HFD + RSVT + AML: They were administered RSVT (20 mg/kg/day) p.o & amlodipine (10mg/kg/day) p.o along with HFD & water ad libitium for 5 weeks.

6. HFD + Pioglitazone (PIO): They were administered pioglitazone (20 mg/kg/day) p.o along with HFD & water ad libitium for 5 weeks.

7. HFD + RSVT + PIO: They were fed administered RSVT (20 mg/kg/day) p.o& pioglitazone (20mg/kg/day) p.o along with HFD & water ad libitium for 5 weeks.

Estimation of hemodynamic parameters:

At the end of the study, blood pressures were monitored indirectly in conscious, prewarmed, slightly restrained rats by the tail-cuff method. All the animals were acclimatized in PANLAB Non-Invasive Tail Cuff Pressure Recorder for blood pressure measurement for 7 days during the end of the treatment. At the time of measurement, five individual readings were obtained in a rapid sequence. The highest and the lowest readings were not counted, and the average of the remaining three readings was accepted as the measurement.

Serum parameters:

The blood samples were withdrawn from the retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept at 4ºC until used. The separated serum was used to estimate various biochemical parameters such as uric acid (Reckon Diagnostics Pvt. Ltd, India.), glucose (Beacon Diagnostics Pvt. Limited), serum triglycerides (TG), total cholesterol (TC), low-density lipids (LDL), and High-density lipids (HDL) were estimated by a kit obtained from Span Diagnostics Ltd. Total lipids (TL) (lipids react with vanillin in the presence of sulphuric and phosphoric acid to form a pink-colored complex). Estimation of very low-density lipids (VLDL)-cholesterol was done using the Fried Ewald formula: VLDL cholesterol = triglycerides / 5.

Estimation of serum insulin:

The amount of insulin in rat serum was estimated by ELISA kit as per the instruction of Mercodia ELISA kits.

Antioxidant Parameter Study:

The liver, kept in cold conditions (precooled in an inverted Petri dish on ice) was removed. It was cross-chopped with a surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight Teflon pestle of glass homogenizer at a speed of 2500 rpm. The prolonged homogenizer under hypotonic conditions was designed to disrupt as far as possible the ventricular structure of the cells to release soluble protein and leave only membrane and nonvascular matter in a sedimental form. The clear supernatant was used for other enzyme assays.

Superoxide Dismutase (SOD):

SOD was estimated by the method of Mishra and Vidovich, 1972. To 0.5ml of dilute brain homogenate, added 0.5ml of ethanol followed by the addition of 0.15ml of chloroform. Shake for 1min, then Centrifuge at 2000rpm, and separate supernatant. Take 0.5ml of supernatant, add 1.5ml of carbonate buffer, and 0.5ml 0f Ethylenediamine tetraacetic acid (EDTA).

The reaction was initiated by the addition of epinephrine and the change in optical.

Density/min was measured at 480 nm, taken for 3 min at 30-second intervals and results are reported in Units/ mg of protein.

Catalase:

Catalase was estimated by the method of Hugo E. Abe, 1987. To 2ml of diluted homogenate added 1ml of phosphate buffer pH 7 followed by the addition of 1ml of Hydrogen Peroxide (H_2O_2). Add H_2O_2 just before taking absorbance at 240 nm and take the reading for 3 min with a 15-second interval and report the results in units µ mol of H_2O_2 evolved/ mg of protein.

Reduced glutathione:

Reduced glutathione (GSH) was estimated by the method of Moran et. al, 1979. To 1ml of homogenate added 1ml of Tri Chloro acetic acid (TCA) (10%). Cool for 10 min and centrifuged at 2000 rpm take 0.5 ml of supernatant. Take 0.5ml of supernatant, and add 4 ml 5-5¹-dithiobis-2-nitrobenzoic acid (DTNB)+1.5 ml Phosphate buffer. Mix well and keep at room temperature. Read the absorbance against blank at 412 nm using a spectrophotometer and report the results in µg of GSH / mg of protein.

Lipid peroxidation:

Malondialdehyde formation (MDA) was estimated by the method of Slater and Sawyer, 1979. To 2ml of homogenate added 2 ml of TCA. Cool for 15 min and centrifuged, take the supernatant. Take 2ml of supernatant and, add 2ml of Thiobarbituric acid (TBA).

Keep in boiling water bath for 10 min. Read the absorbance of the test against blank at 535 nm using a spectrophotometer. The results are reported in nm of MDA / gm of tissue.

Histopathology:

Thoracic aortas & liver were collected after the rats were sacrificed. After blotting free of blood and tissue fluids. Paraffin-fixed tissues were made into 5-15µm thick sections on a Leitz microtome in a horizontal plane, stained by eosin and hematoxylin. Later, sections were mounted on a glass slide. The sections were observed and desired areas were photographed in an Olympus photomicroscope for morphometric studies of different cells. The sections were viewed under 40X and 100X magnification.

RESULTS:

Hemodynamic study:

There was a significant ($P<0.05$) increase in diastolic B.P of fructose-treated animals as compared to control animals. Trans-Resveratrol (RSVT) was found to reduce diastolic B.P significantly. Amlodipine (AML) significantly ($p<0.01$) reduced diastolic B.P. as compared to fructose treated group. RSVT combined with AML was also found to reduce diastolic B.P significantly ($p<0.01$). Pioglitazone (PIO) alone & PIO in combination with RSVT were reducing diastolic B.P significantly as compared to fructose-treated animals but not as AML group (fig 1).

There was a significant ($P<0.001$) increase in systolic B.P of fructose-treated animals as compared to control group animals. RSVT was found to reduce systolic B.P significantly. However, AML significantly ($p<0.001$) reduced systolic B.P. as compared to fructose treated group. RSVT combined with AML also was found to reduce systolic B.P significantly ($p<0.01$). PIO alone & PIO in combination with RSVT were reducing systolic B.P significantly as compared to fructose-treated animals (fig 1).

Serum Parameters:

There was a significant increase ($p<0.001$) in serum fasting glucose level (FGL) level in HFD group as compared to the control group. RSVT was found to decrease serum FGL significantly ($p<0.01$) as compared to HFD group. AML alone & in combination with RSVT were found to reduce serum FGL significantly ($p<0.001$ & $p<0.01$, respectively) as compared to HFD group. PIO alone & in combination with RSVT were found to reduce serum FGL significantly ($p<0.001$ in both groups) as compared to HFD group.

There was a significant increase ($p<0.001$) in serum insulin level of HFD group as compared to the control group. RSVT was found to decrease serum insulin significantly ($p<0.001$) as compared to HFD group. AML alone & in combination with RSVT were found to reduce serum insulin significantly ($p<0.001$ in both groups) as compared to HFD group. PIO alone & in combination with RSVT were found to reduce serum insulin significantly ($p<0.001$ in both groups) as compared to HFD group (fig 1).

In the case of serum uric acid, no significant increase was found in HFD group as compared to the control group (fig 1).

There was a significant increase ($p<0.001$) in serum TG level in HFD group as compared to the control group. RSVT was found to decrease serum TG significantly ($p<0.01$) as compared to HFD group. AML alone & in combination with RSVT were also found to reduce serum TG significantly ($p<0.05$ & $p<0.001$ respectively) as compared to HFD group. PIO alone & in combination with RSVT were found to reduce serum TG significantly ($p<0.001$ in both groups) as compared to HFD group (fig 1).

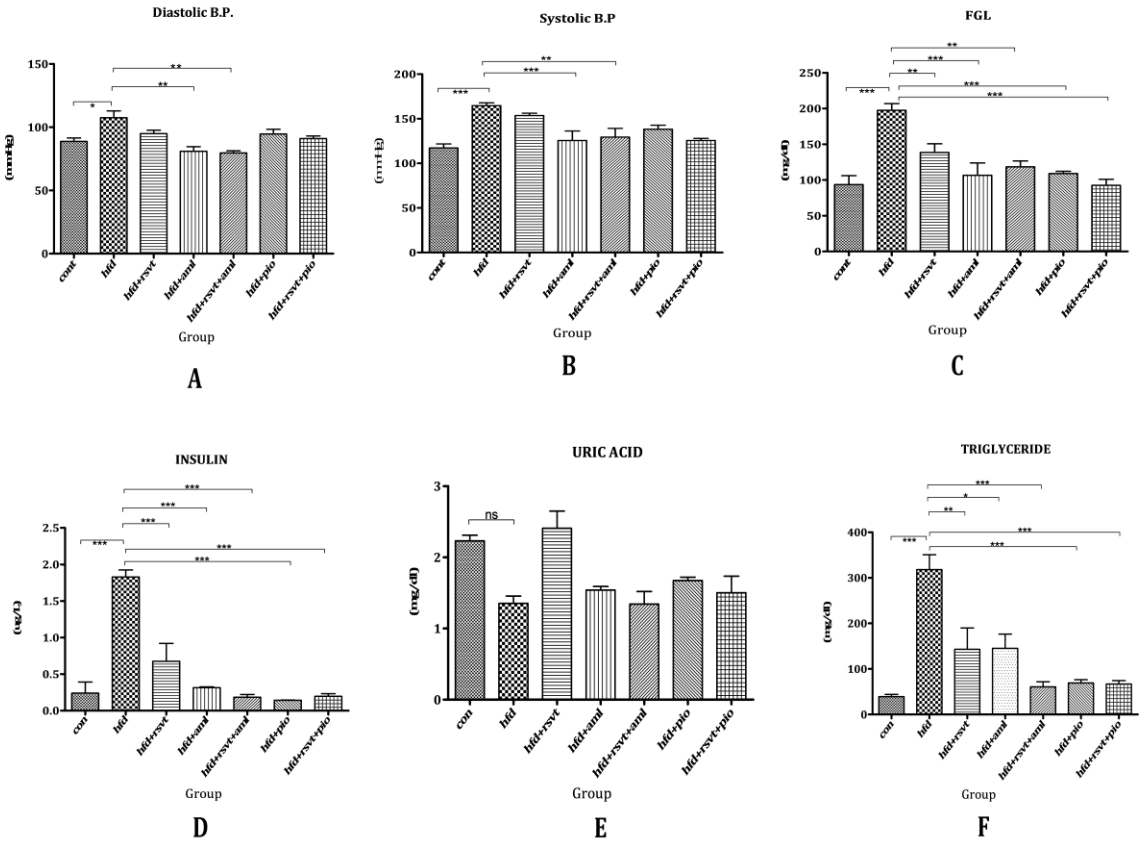

Fig. 1: Estimation of Diastolic and Systolic B.P and biochemical estimations like FGL, insulin, uric acid, Triglycerides.

There was a significant increase ($p<0.05$) in serum TC level in HFD group as compared to the control group. In this, case all of the treated groups were found to decrease serum TC significantly as compared to HFD group (fig 2).

There was a significant increase ($p<0.001$) in serum VLDL and LDL levels in HFD group as compared to the control group. RSVT was found to decrease serum VLDL significantly ($p<0.01$) as compared to HFD group. AML alone & in combination with RSVT were also found to reduce serum VLDL and LDL significantly ($p<0.05$ & $p<0.001$ respectively) as compared to HFD group. PIO alone & in combination with RSVT were found to reduce serum VLDL and LDL significantly ($p<0.001$ in both groups) as compared to HFD group (fig 2).

In the case of serum HDL, no significant increase was found in HFD group as compared to the control group.

There was a significant increase ($p<0.001$) in serum total lipid level in HFD group as compared to the control group. RSVT was found to decrease serum total lipid significantly ($p<0.05$) as compared to the HFD group. AML alone & in combination with RSVT were also found to reduce serum total lipid significantly ($p<0.001$ in both groups) as compared to HFD group. PIO alone & in combination with RSVT were found to reduce serum total lipid significantly ($p<0.001$ in both groups) as compared to HFD group (fig 2).

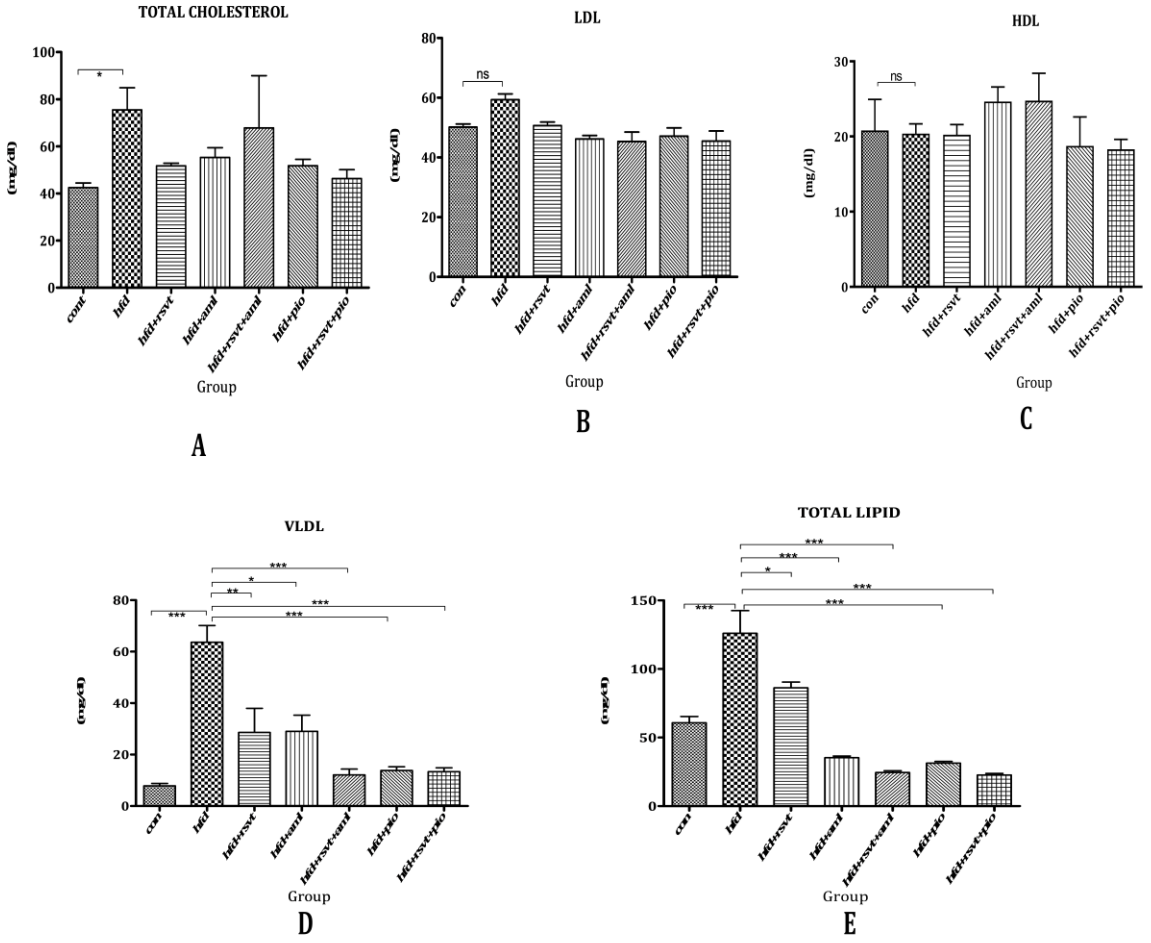

fig. 2: Biochemical estimations including total cholesterol, LDL, HDL, VLDL, total lipid.

Antioxidant study:

Liver parameters showed that fructose induces oxidative stress so, SOD, catalase (CAT), and reduced glutathione (GSH) were found to be decreasing ($p<0.01$ in all the cases), and lipid peroxidation was found to be increasing significantly ($p<0.01$) in HFD group. RSVT (20mg/kg) was found to increase SOD ($p<0.05$), catalase & reduced glutathione significantly as compared to HFD rats, whereas decrease in lipid peroxidation ($p<0.05$) significantly (fig 3).

Effect of RSVT (20 mg/kg) on change in liver antioxidant enzyme as well as lipid peroxidation in HFD-induced MS in rats

(Values are expressed as mean ± SEM, P value * <0.05; ** <0.01, *** <0.001)

Group	**Lipid Peroxidation** (nm of MDA /gm of tissue)	**Antioxidant Enzymes**		
		GSH (mg of GSH / gm of tissue)	**Catalase** (µ mol of H_2O_2 evolved/ mg of protein)	**SOD** (U/gm of tissue)
Control	1.110 ± 0.00	54.83 ± 2.598	24.87 ± 1.278	49.33 ± 0.6667

HFD	2.040 ± 0.13**	27.33 ± 1.139***	15.45 ± 1.776**	14.67 ± 0.6677**
HFG+RSVT	1.377 ± 0.1333*	36.03 ± 1.560*	19.44 ± 0.5336^{ns}	34.67 ± 7.265 *

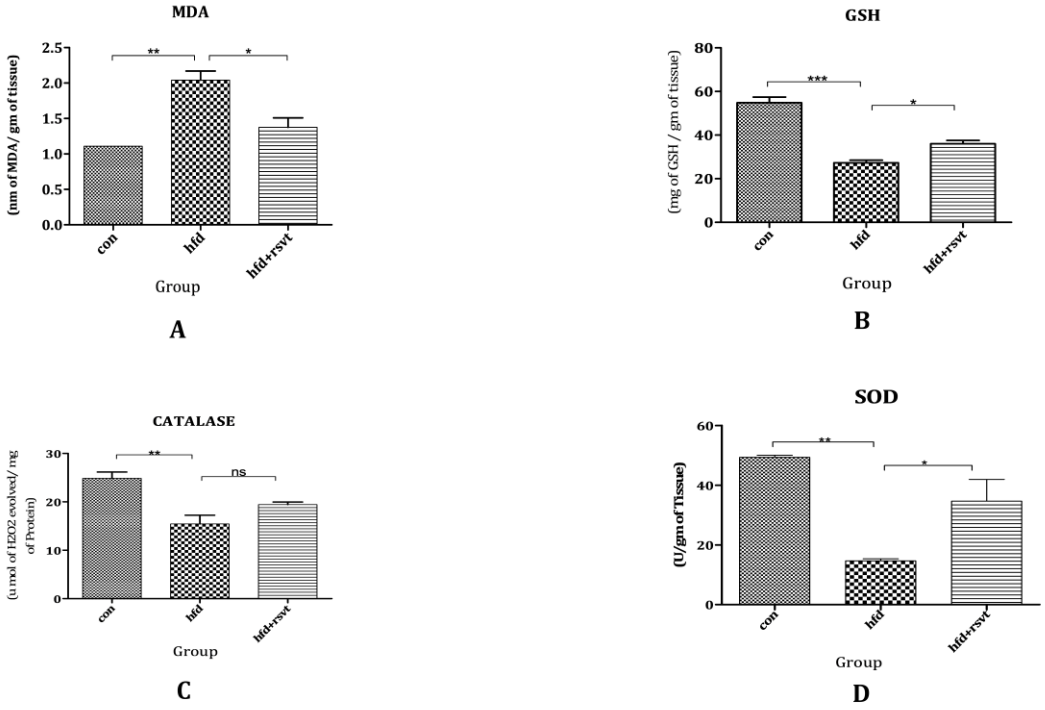

Fig. 3: Bar diagrams indicating antioxidant activity of control group, high fructose diet group and high fructose diet + RSVT group.

Histopathological study:

HFD showed an increase in lipid deposition and endothelial dysfunction as compared to the normal animal aorta. RSVT, AML, and PIO as well as a combination of AML & PIO with RSVT showed decreased lipid deposition as well as endothelial dysfunction after 35 days of treatment (fig 4.- fig 7.).

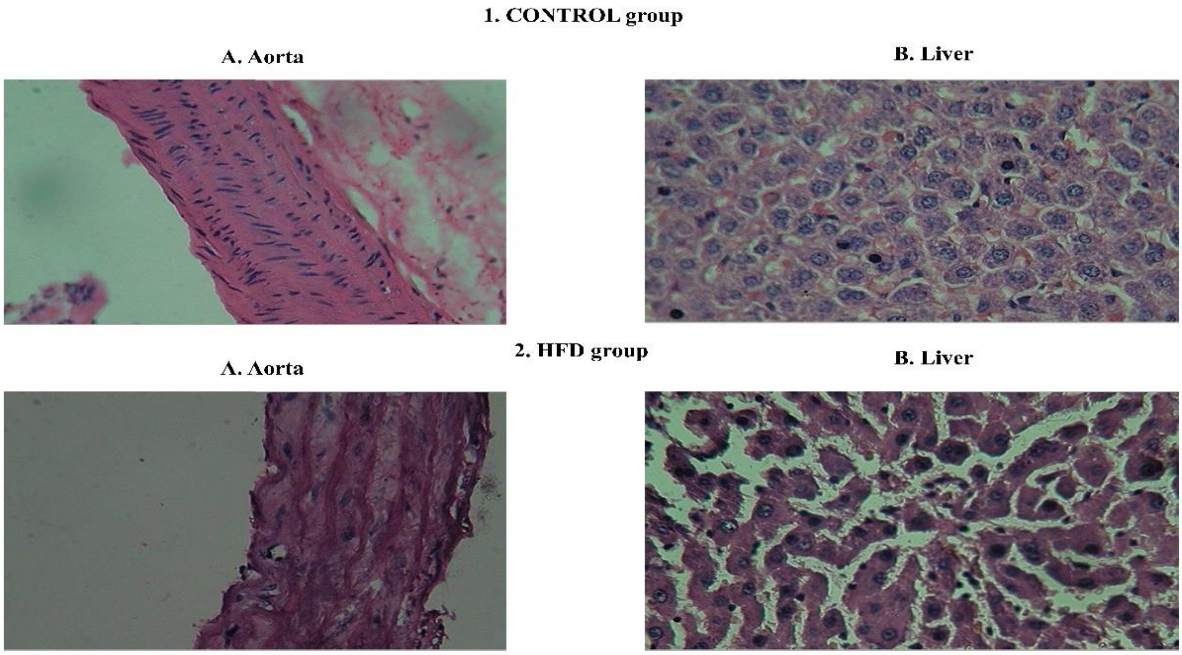

Fig 4: (1. Control group) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with Standard laboratory diet (Control group) and HFD group respectively. (2. HFD group) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD.

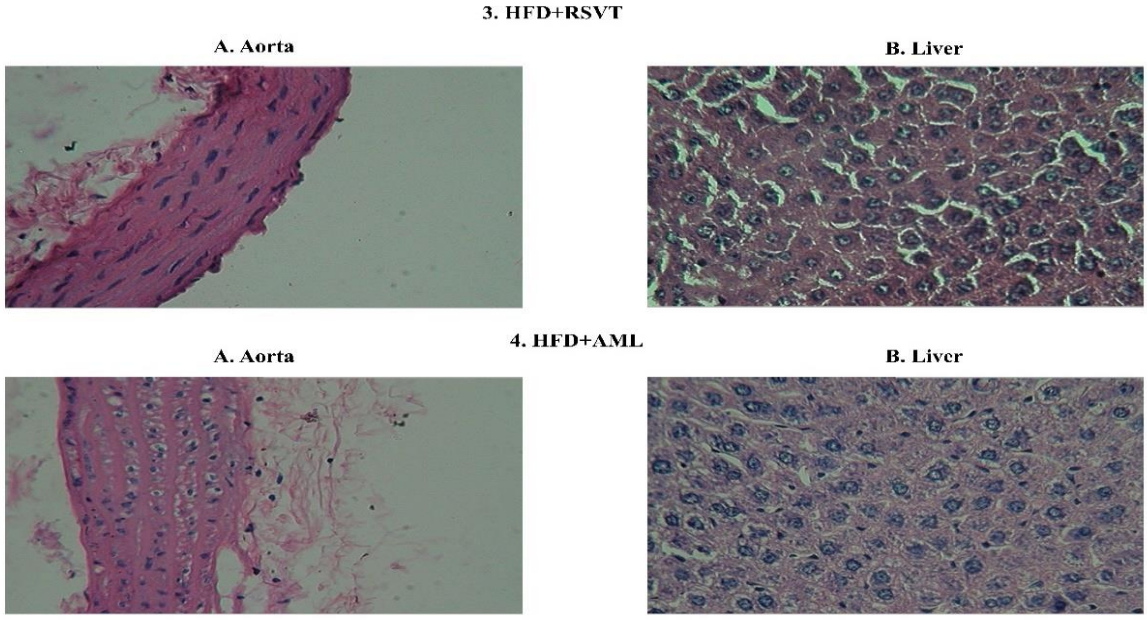

Fig 5. (3. HFD+RSVT) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD + RSVT (20 mg/kg). (4. HFD+AML) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD + AML (10 mg/kg).

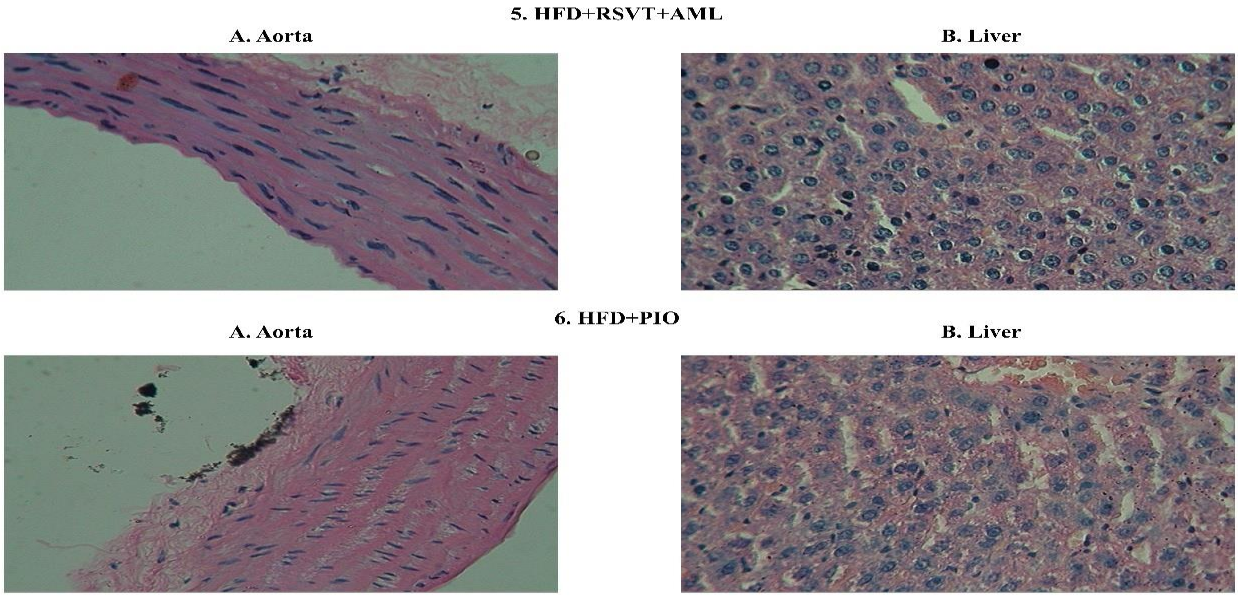

Fig 6. (5. HFD+RSVT+AML) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD + AML (10 mg/kg) + RSVT (20mg/kg). 6. HFD+PIO - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD + PIO (20mg/kg).

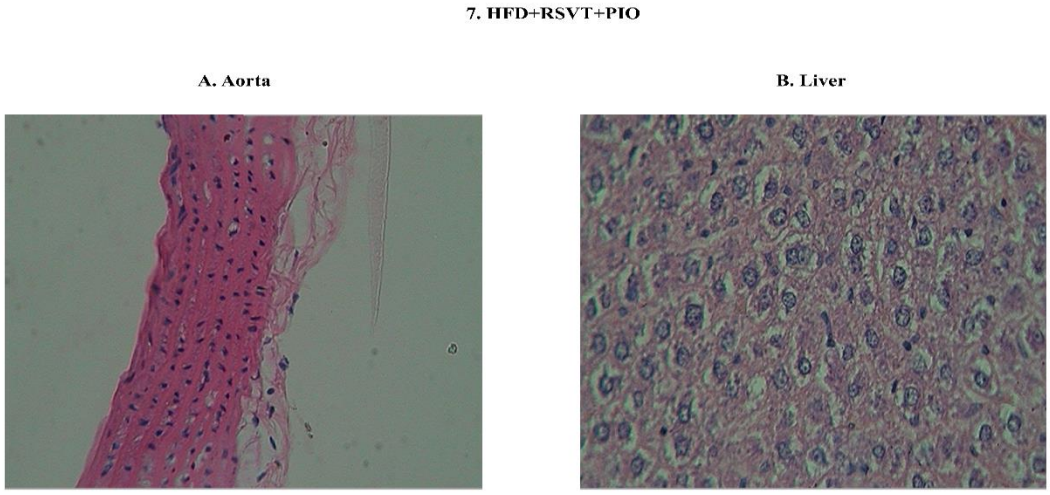

Fig 7. (7. HFD+RSVT+PIO) - Histopathological sections of Aorta and Liver (stained with Haematoxylin & Eosin) of rat fed with HFD + PIO (20 mg/kg) + RSVT (20mg/kg)

Statistical Analysis: All the data were expressed as mean ± SEM (standard error of the mean). Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test as appropriate using a computer-based fitting program (Prism, GraphPad). Differences were considered to be statistically significant when $p < 0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, non-significant(ns) $p>0.05$).

DISCUSSION:

Metabolic syndrome (MS) has gained attention as a global public health issue. Since most patients find it challenging to adhere to exercise or dietary plans, it has become crucial to look into potential treatments for the harmful effects of metabolic syndrome. A constellation of abnormalities, including abdominal obesity, dyslipidemia, and insulin resistance, make up metabolic syndrome and lead to hypertension in the end. The patient is at a greater risk of acquiring type 2 diabetes mellitus and cardiovascular morbidities as a result of these abnormalities21. Over the past few decades, there has been a correlation between high fructose consumption and an increase in MS cases22. In studies, fructose has received widespread acceptance as an animal model for metabolic syndrome (MS) that replicates the symptoms affecting human participants23.

The current study showed that keeping rats on HFD for 5 weeks caused the classic symptoms of MS, which were manifested by a significant rise in systolic BP, diastolic BP, a significant rise in blood glucose and serum insulin levels, a significant rise in total cholesterol, triglycerides, and LDL-C, and a significant decline in HDL-C compared to control rats.

RSVT decreased the serum glucose level maybe by SIRT1 activation can also encourage GLUT4 (glucose transporter type-4) translocation into cell membranes; resveratrol binding to estrogen receptors can facilitate GLUT4 translocation which leads to a reduction in serum glucose levels24. The observed lipid-lowering effect by RSVT may be due to increased plasma lipid uptake by the liver and adipose tissue or by decreased hepatic fatty acid synthesis. Also, RSVT is a strong antioxidant. RSVT acts by changing the oxidative stress of the diabetic tissues and thus improves the functional states of the metabolic machinery of the cells & the improved cellular redox status helps maintain the normal function of the mediators involved in insulin signaling and promotes vasodilation by enhancing nitric oxide production or improved nitric oxide bioavailability by the reduction of hydrogen peroxide RSVT decreased BP.

AML increases the hormone-sensitive triglyceride lipase to cause rapid breakdown of triglycerides and mobilization of free fatty acids, thereby leading to a fall in levels of serum triglycerides. AML acts by relaxing the smooth muscle in the arterial wall decreasing total peripheral resistance and hence reducing B.P.

PIO showed significant change in systolic and diastolic BP. PIO selectively stimulates the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-Y). PIO reduces insulin resistance in the liver and peripheral tissues.

CONCLUSION:

Resveratrol has (20mg/kg) reduced serum TG, insulin, FGL & total lipids significantly. AML & PIO also reduced all the above serum parameters. There was a significant change of action of drugs with a combination of RSVT was found. In case of BP, RSVT decreased significantly. AML & AML+RSVT were found to decrease both systolic as well as diastolic B.P. In the case of the antioxidant enzyme of the liver, RSVT significantly increased liver SOD, catalase, and GSH as compared to HFD animals. It also reduced liver lipid peroxidation significantly. Histopathological study of liver & aorta showed that RSVT, PIO & AML alone & their combination with RSVT prevented damage to tissue from HFD to animals.

RSVT possesses antioxidant activity. Also, it has anti-hyperglycemic anti hyper triglyceridemic & anti hyperinsulinemic effects but we haven't found any drastic increase in the efficacy when given with combinatios.

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Pfizer's inventive Nirmatrelvir/Ritonavir (PaxlovidTM): Another arrow in the quiver against SARS-CoV-2

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Pfizer's inventive Nirmatrelvir/Ritonavir (PaxlovidTM): Another arrow in the quiver against SARS-CoV-2.

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Abstract

SARS-CoV-2 was a devastating global pandemic that swept the globe in late 2019, claiming the lives of an estimated 4 million people. Amidst challenging times, we are remembering Martin Luther King Jr.'s remark, "Mankind must put an end to war or war will put an end to mankind." In that sense, scientists are repurposing drugs meticulously to curb the nCOVID-19. New antiviral drugs on the other hand are being developed at unparallel rates. Among those Pfizer's inventive Nirmatrelvir/Ritonavir (PaxlovidTM), which inhibits the main protease (Mpro) of SARS-CoV-2, 3CL protease, will be another arrow in the quiver to mount resistance towards SARS-CoV-2. In patients treated with nirmatrelvir/ritonavir within five days of symptom onset, COVID-19-related hospitalizations and mortality were dramatically reduced. Paxlovid's high oral availability, allows it to be used in both hospitalized and outpatient patients. In this brief review, we presented pharmacokinetic, preclinical, and clinical evidence on Paxlovids for the treatment of moderate nCOVID-19.

Keywords: SARS-CoV-2, Paxlovid.

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Introduction

Coronavirus illness 2019 (COVID-19) has created a worldwide outbreak spread via respiratory infection caused by coronavirus 2 (severe acute respiratory syndrome) (SARS-CoV-2).[1,2,3]COVID-19 can produce a wide range of symptoms, often from no symptoms to severe hypoxia, multiorgan or respiratory failure, and even death.Out of 272 million confirmed cases, the SARS-CoV-2 pandemic has claimed the lives of 5.5 million individuals by the end of December 2021[4].SARS-structural CoV-2 proteins include spike glycoproteins (S), tiny envelope glycoproteins

(E), glycoprotein membranes (M), nucleocapsids (N), and various auxiliary proteins.[5]In the fight against the COVID-19 pandemic, vaccines are regarded to be the most significant and effective strategy. Remdesivir, molnupiravir, and nirmatrelvir/ritonavir are only a few of the new medications being studied or authorised to address COVID-19.Remdesivir is the sole FDA-approved antiviral medication for the treatment of SARS-CoV-2 infection (RDV; GS-5734). RDV, on either hand, must be administered intravenously, restricting its clinical application to patients with severe conditions who need to be admitted to the hospital.[6] In a phase III study enrolling non-hospitalized adults with mild to moderate COVID-19 and expelling at least one risk factor for severe disease within five days of symptom onset, the RNA-dependent RNA polymerase inhibitor Molnupiravir showed 30% efficacy in reducing hospitalisation or death compared to the placebo group[7]. The most hopeful of these new medications is the oral version of nirmatrelvir/ritonavir, which decreases the risk of hospitalisation or death by 89 percent.[8]It is projected that nirmatrelvir/ritonavir will alter the path of the COVID-19 pandemic.[9]

Emergency Use Authorization(EUA)

AEUA is a US Food and Drug Administration authorization for the emergency use of an unapproved product or the unapproved use of an approved product (i.e., drug, biological product, or device) in the United States under certain conditions, such as when the Secretary of Health and Human Services (HHS) declares a public health emergency that threatens national security or the health and security of US citizens living abroad (s).

On December 22, 2021, the U.S. Food and Drug Administration approved Pfizer's Paxlovid (nirmatrelvir tablets and ritonavir tablets, co-packaged for oral use) as an emergency use authorization (EUA) for the treatment of mild-to-moderate coronavirus illness (COVID-19) in adults and paediatric patients (12 years of age and older weighing at least 40 kilos or approximately 88 pounds) with positive results of direct SARS-CoV-2. Paxlovid is only accessible by prescription and should be started as soon as possible following a COVID-19 diagnosis and within five days of symptom onset.

Paxlovid is made up of nirmatrelvir, which suppresses a SARS-CoV-2 protein and prevents the virus from reproducing, and ritonavir, which reduces nirmatrelvir's breakdown and allows it to stay in the body for longer at greater concentrations. Paxlovid is given in the form of three pills (two nirmatrelvir tablets and one ritonavir tablet) taken orally twice daily for five days, for a total of 30 tablets. Paxlovid should not be used for more than five days in a row.

Paxlovids' Efficacy in Preventing Severe CoVID-19 Death

According to Pfizer's experimental treatment, using paxlovid (nirmatrelvir [PF-07321332] tablets and ritonavir tablets) reduces the risk of hospitalisation or death by 89 percent (onset of symptoms within 3 days) and 88 percent (5 days of symptoms) as compared to the placebo group.[14]The EPIC-HR study enrolled 2246 participants and discovered that 0.7 percent of people who underwent (nirmatrelvir [PF-07321332] tablets and ritonavir tablets) were hospitalised through Day 28 following randomization (5/697 hospitalised, no deaths), compared to 6.5 percent of patients who received placebo and were hospitalised or died (44/682 hospitalised, 9 deaths).As per the company, the comparative risk reduction in patients 65 and older was 94 percent; 1.1 percent of people who underwent (nirmatrelvir [PF-07321332] tablets and ritonavir tablets) were hospitalised through Day 28 (1/94 hospitalised with no deaths), compared to 16.3 percent of patients who received placebo (16/98 hospitalised with 6 deaths), with high statistical significance ($p \leq 0.01$). There were no fatalities recorded in the whole trial group of Paxlovid patients until Day 28, compared to 12 (1.2 percent) deaths among placebo participants. [15]

The issuing of an EUA is not the same as FDA approval.When deciding whether to issue an EUA, the FDA considers all available scientific information and carefully weighs any known

or possible dangers against any known or potential benefits of the product.Based on an evaluation of the available scientific information, the FDA decided that it is reasonable to assume Paxlovid may be helpful in the treatment of mild-to-moderate COVID-19 in permitted patients.The agency has also assessed that the current and prospective advantages of Paxlovid exceed the known and possible hazards of the medication when used under the terms and conditions of the authorization. Paxlovid has no appropriate, authorised, or available alternatives for the treatment ofCOVID-19[10].https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19

Ritonavir Enhanced Nirmatrelvir Preclinical Aspects

According to in vitro research, nirmatrelvir has stronger anti-SARS-COV-2 action. SARS-CoV-2 viral proliferation was suppressed in differentiated normal human bronchial epithelial cells treated for three days with varied doses of nirmatrelvir with no evident cytotoxicity. Nirmatrelvir has minimal in vitro cytotoxicity, demonstrating that it is a safe medication. In vitro investigations revealed that CYP3A4 was important in the breakdown of nirmatrelvir, implying that co-treatment with the powerful CYP3A4 inactivator, ritonavir, might increase blood concentrations of nirmatrelvir.[8] Ritonavir improves the pharmacokinetic action of the majority of HIV inhibitors (e.g., darunavir and lopinavir) that are metabolised by CYP3A4.[11] Thus, ritonavir and nirmatrelvir were combined to boost treatment effectiveness.Leist et al. developed a widely used mouse model of SARS-CoV-2 infection. In a summary, 10-week-old BALB/c mice were intranasally infected with SARS-CoV-2 MA10, which concluded in a 10% decrease in body weight. This model mimics the illness spectrum and host immune responses of COVID-19, such as elevated T-helper (Th)-1 cytokines and failure of surfactant expression and pulmonary function in connection with acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), demonstrated a dose-dependent upsurge in mortality and morbidity over the course of 14 days with SARS-CoV-2 MA10 (ARDS).[12]

Mice were protected from weight loss after infection with SARS-CoV-2 MA10 when given nirmatrelvir at 300 and 1000 mg/kg dosages twice daily.Furthermore, nirmatrelvir significantly decreased SARS-CoV-2 MA10 pulmonary virus levels in mice.Furthermore, mice given nirmatrelvir showed decreased inflammation in their perivascular tissues, and also their bronchial and bronchiolar epithelia.The good antiviral action of nirmatrelvir in these animals makes it a suitable option for additional clinical studies to test its potential for treating SARS-CoV-2 infection. [13]

Drug Information

PAXLOVID is a combination of nirmatrelvir and ritonavir pills.Nirmatrelvir is the main protease (Mpro) inhibitor for SARS-CoV-2, while ritonavir is an HIV-1 protease inhibitor and CYP3A inhibitor.

Nirmatrelvir

The nirmatrelvir chemically designated as (1R,2S,5S)-N-((1S)-1-Cyano-2-((3S)-2-oxopyrrolidin-3-yl)ethyl)-3-((2S)-3,3-dimethyl-2-(2,2,2-trifluoroacetamido)butanoyl)-6,6dimethyl-3-azabicyclohexane-2-carboxamide]. It has thechemical formula of $C_{23}H_{32}F_3N_5O_4$ and a atomic weight of 499.54.Nirmatrelvir is a peptidomimetic blocker of SARS-CoV-2 Mpro, also known as 3C-like protease (3CLpro) or nsp5 protease.SARS-CoV-2 Mpro inhibition makes it incapable of digesting polyprotein precursors, halting viral replication.

Nirmatrelvir is marketed as a film-coated tablet for immediate release. Each tablet includes 150 mg nirmatrelvir along with colloidal silicon dioxide, croscarmellose sodium, lactose monohydrate, microcrystalline cellulose, and sodium stearyl fumarate as inactive components.The film coating contains the following ingredients: iron oxide red, hydroxy propyl methylcellulose, titanium dioxide and polyethylene glycol.

Ritonavir

Ritonavir molecular name is 10-Hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1 methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, [5S-(5R*,8R*,10R*,11R*)]. Its chemical formula is $C_{37}H_{48}N_6O_5S_2$, and its atomic weight is 720.95.Ritonavir is an HIV-1 protease inhibitor, however, it does not affect SARS-CoV-2 Mpro.Ritonavir blocks the CYP3A-mediated metabolism of nirmatrelvir, leading to higher nirmatrelvir plasma concentrations.

Ritonavir is available in the form of film-coated tablets. Each pill includes 100 mg ritonavir as well as the inactive components listed below:sorbitan monolaurate, anhydrous dibasic calcium phosphate, colloidal silicon dioxide, sodium stearyl fumarate, andcopovidone. The following components may be present in the film coating:polyethylene glycol, colloidal silicon dioxide, titanium dioxide, colloidal anhydrous silica, hydroxypropyl cellulose, hypromellose, polysorbate 80, and talc.

Paxlovid's potential side effects[18]

Symptoms include a loss of taste, diarrhoea, elevated blood pressure, and muscular pains. Taking Paxlovid alongside certain other medications may result in potentially serious drug interactions. Paxlovid use in persons with uncontrolled or undetected HIV-1 infection may result in HIV-1 treatment resistance. Because ritonavir can cause liver damage, Paxlovid should be used with caution in individuals who have a history of liver illness, abnormal liver enzymes, or liver inflammation[19].

Allergic reactions-Hives, difficulty swallowing or breathing, puffiness of the mouth, lips, or face, throat discomfort, hoarseness, pruritus.

Liver Problems-Appetite loss, yellowing of the skin and whites of the eyes, black urine, pale faeces, itchy skin, stomach ache.

Resistance to HIV medicines-If a person has an untreated HIV infection, PAXALOVID may cause some HIV medications to fail in the future.

Drug Interactions

Lurasidone, simvastatin, ergotamine, Alfuzosin, midazolam, phenobarbital, St. John's Wort (hypericum perforatum), propoxyphene, Ranolazine, methylergonovine, sildenafil (Revatio) for pulmonary arterial hypertension (PAH), Lovastatin, propafenone, Pethidine, Triazolam, flecainide, Carbamazepine, phenytoin, Amiodarone, quinidine, Dihydroergotamine, Colchicine, clozapine, pimozide, Apalutamide, Rifampin, dronedarone.

Contraindications

Paxlovid is contraindicated with certain drugs that are highly dependent on those enzymes for metabolism and for which elevated concentrations of certain drugs are associated with serious and/or life-threatening reactions because it works in part by inhibiting a group of enzymes that break down certain drugs. Paxlovid is also contraindicated in combination with drugs that strongly induce those same enzymes, resulting in the faster breakdown of nirmatrelvir or ritonavir, because lower concentrations of nirmatrelvir or ritonavir may be associated with potentially losing virologic response and developing viral resistance. Because the effects of such drugs persist after withdrawal, Paxlovid cannot be begun immediately after discontinuation.

Paxlovid should not be used in people who have significant renal or liver damage. A lower Paxlovid dosage is required in patients with significant renal impairment. Patients with renal or liver issues should talk to their doctor about if Paxlovid is good for them.[15]

Authorized use Restrictions

1. PAXLOVID is not approved for use in individuals who require hospitalisation due to severe or critical illness due to COVID-19
2. PAXLOVID is not approved for use as a pre- or post-exposure treatment for COVID-19 prevention.
3. PAXLOVID should not be used for more than 5 days in a row.

PAXLOVID may only be given to a single patient by doctors, advanced practice registered nurses, and medical specialists who are licenced or allowed by state law to prescribe medications in the therapeutic class to which PAXLOVID belongs (i.e., anti-

infectives).PAXLOVID has not been licenced for any application, including the treatment of COVID-19.

PAXLOVID is permitted only for the period of the declaration that circumstances exist requiring the emergency use of PAXLOVID under section 564(b)(1) of the Act, 21 U.S.C.§ 360bbb-3(b)(1) unless the authorisation is terminated or withdrawn sooner.

Information on Available Alternatives for EUA Authorized Use

Veklury (remdesivir) is an FDA-approved treatment for COVID-19 in adults and paediatric patients (12 years of age or older weighing at least 40 kg) with positive direct SARS-CoV-2 viral testing, who are not hospitalised and have mild-to-moderate COVID-19, and who are at high risk of progression to severe COVID-19, including hospitalisation or death.Veklury is given as an intravenous infusion over the course of three days.Although Veklury is an approved alternative treatment for mild-to-moderate COVID-19 in adults and paediatric patients, the FDA does not consider Veklury to be an appropriate alternative to PAXLOVID for this authorised use because it may not be feasible or practical for certain patients (e.g., it requires an intravenous infusion daily for 3 days).https://www.fda.gov/emergency-preparedness-and-response/mcm-legalregulatory-and-policy-framework/emergency-use-authorization.

Effectiveness of PF-07321332 against SARS-CoV-2 Variants

Nirmatrelvir possesses antiviral activity against all known human coronaviruses, including beta- and alpha-coronaviruses (SARS-CoV-2, SARS-CoV-1, MERS-CoV, HKU1, and OC43).It also demonstrates the effect on mutant SARS-COV-2.[16]The level of nirmatrelvir inhibition against these five primary protease variations was comparable, with 5 nM inhibiting activity by 50%, 20 nM inhibiting activity by >50%, and 100 nM inhibiting the activity of protease versions of five SARS-CoV-2 lineages.The P132H mutation in nsp5 (Mpro) in the new Omicron version was detected, however, structural research reveals that it has no effect on the active site and so may not alter nirmatrelvir's antiviral effectiveness.[17]

Pharmacokinetic profile

In healthy subjects, the time to maximum drug concentration (Tmax) following a single dosage of 300 mg nirmatrelvir (2 ×150 mg tablet formulation) provided combined with a 100 mg ritonavir tablet was 3.00 h and 3.98 h, respectively, and the mean half-life (t1/2) was 6.05 h and 6.15 h, respectively.Nirmatrelvir is primarily excreted by the kidneys, whereas ritonaviris through the liver.

The prescribing information states that the individual should consult with a healthcare provider if he or she has any allergies, liver or kidney disease, are pregnant or plans to become pregnant, are breastfeeding a child, or has any serious illnesses, as well as about all the medications he or she takes, including prescription and over-the-counter medications, vitamins, and herbal supplements.Some medications may interact with PAXLOVID, resulting in significant adverse effects. Maintain a list of the medicines to show the healthcare provider and pharmacist when getting a new medicine[10]

Summary and Outlook

Paxlovid appears to be substantially more effective than any similar anti-virals in reducing the risk of hospitalization and death and shows 89% efficacy compared to the placebo group, the ongoing trials on the drug will help prevent disease transmission and plays a vital role in the future. The use of the effective oral agent nirmatrelvir/ritonavir is a ray of hope in the covid 19 pandemic.

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PHARMACOLOGICAL EVALUATION OF ANTIDEPRESSANT AND ANTIANXIETY ACTIVITY OF BUPLEURUM FALCATUM IN ANIMAL MODELS

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ABSTRACT

Bupleurum falcatum, belongs to the family Apiaceae. Anxiety and Depression are widespread psychiatric disorders affecting around 5% of the population. Furthermore, it is difficult to predict which patient will respond to any given treatment. In the traditional systems of medicine, many plants have been used to treat anxiety and depression for thousands of years. The present study was designed to evaluate the antianxiety and antidepressant activity of the alcoholic and aqueous extracts of *Bupleurum falcatum* leaves in rodents. Antianxiety activity was tested by exposing rats to unfamiliar aversion in different methods like elevated plus maze model and actophotometer. The results infer that reduced aversion fear elicits antianxiety activity. The antidepressant activity was tested by using forced swim test and tail suspension test. The results infer that reduced immobility time elicits antidepressant activity. It was concluded that alcoholic and aqueous extracts of *Bupleurum falcatum* leaves having antianxiety and antidepressant activity. Alcoholic extract of *Bupleurum falcatum* leaves showing more significant activity over the aqueous extract.

Keywords: *Bupleurum falcatum*, Antianxiety activity, Antidepressant activity, Elevated plus maze, Actophotometer, Despair swim test, Tail Suspension Test.

INTRODUCTION

Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science or approved by regulatory agencies such as the United States Food and Drug Administration or European Food Safety Authority to have medicinal effects. World Health Organization (WHO) has provided a definition of medicinal plants, that is "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drugs."[1]

World Health Organization (WHO) reported that 80% of the world's population depends on medicinal plants for their primary health care. In the Plant Kingdom, Medicinal plants form the largest single grouping of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are trees [2] Plants are known to be the source of many chemical compounds. Medicinal plants were used by people of ancient cultures without knowledge of their active ingredients. The common practice of taking crude extract orally is laden with hazards as the extracts may contain some toxic constituents. There is an ever increasing need to limit toxic clinical drugs.In modern times, the active ingredients and curative actions of medicinal plants were first investigated through the use of European Scientific methods[3]. The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steriods, phenols glycosides and tannins[2].

The information obtained from extracts of medicinal plants makes pharmacological studies possible. The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized. Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens. Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity[4].

There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants [5]. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural products play a vital role in modern drug development in the pharmaceutical industry[6].

History of plants in medicine [7]

The earliest known medical document is a 4000-year-old Sumerian clay tablet that recorded plant remedies for various illnesses. The ancient Egyptian Ebers papyrus from 3500 year ago lists hundreds of remedies. The Pun-tsao contains thousands of herbal cures attributed to Shennung, China's legendary emperor who lived 4500 years ago. In India, herbal medicine dates back several thousand years to the Rig-Veda, the collection of Hindu sacred verses. The Badianus Manuscript is an illustrated document that reports the traditional medical knowledge of the Aztecs. Western medicine can be traced back to the Greek physician Hippocrates, who believed that disease had natural causes and used various herbal remedies in his treatments. Early Roman writings also influenced the development of western medicine, especially the works of Dioscorides, who compiled information on more than 600 species of plants with medicinal value in De Materia Medica. Many of the herbal remedies used by the Greeks and Romans were effective treatments that have become incorporated into modern medicine (e.g., willow bark tea, the precursor to aspirin). Dioscorides' work remained the standard medical reference in most of Europe for the next 1500 years.

The beginning of the Renaissance saw a revival of herbalism, the identification of medicinally useful plants. This coupled with the invention of the printing press in 1450 ushered in the Age of Herbals. Many of the herbals were richly illustrated; all of them focused on the medicinal uses of plants, but also included much misinformation and superstition. The Doctrine of Signatures, for example, held that the medicinal use of plants could be ascertained by recognizing features of the plant that corresponded to human anatomy. For example, the red juice of bloodwort suggests that it should be used for blood disorders; the lobed appearance of liverworts suggests that it should be used to treat liver complaints; the "humanoid" form of mandrake root suggests that is should be used to promote male virility and ensure conception.

Many of the remedies employed by the herbalists provided effective treatments. Studies of foxglove for the treatment of dropsy (congestive heart failure) set the standard for pharmaceutical chemistry. In the 19th century, scientists began purifying the active extracts from medicinal plants (e.g. the isolation of morphine from the opium poppy). Advances in the field of pharmacology led to the formulation of the first purely synthetic drugs based on natural products in the middle of the 19th century. In 1839, for example, salicylic acid was identified as the active ingredient in a number of plants known for their pain-relieving qualities; salicylic acid was synthesized in 1853, eventually leading to the development of aspirin. It is estimated that 25% of prescriptions written in the U.S. contain plant derived ingredients (close to 50% if fungal products are included); an even greater percentage are based on semisynthetic or wholly synthetic ingredients originally isolated from plants.

While Western medicine strayed away from herbalism, 75% to 90% of the rural population of the rest world still relies on herbal medicine as their only health care. In many village marketplaces, medicinal herbs are sold alongside vegetables and other Wares. The People's Republic of China is the leading country for incorporating traditional herbal medicine into a modern health care system; the result is a blend of herbal medicine, acupuncture, and Western medicine. Plantations exist in China for the cultivation of medicinal plants, and thousands of species are thus available for the Chinese herbalist; prescriptions are filled with measured amounts of specific herbs rather than with pills or ointments. In India, traditional systems have remained quite separate from Western medicine. In addition to Ayurvedic medicine, which has a Hindu origin, Unani medicine, with its Muslim and Greek roots, is another widely practiced herbal tradition in India. The renewed interest in medicinal plants has focused on herbal cures among indigenous populations around the world, especially those in the tropical rain forests. It is hoped that these investigations will add new medicinal plants to the world's pharmacopoeia before they are lost forever. In addition to the destruction of the forests, the erosion of tribal cultures is also a threat to herbal practices.

MATERIALS AND METHODS

Drugs and Chemicals

Drugs and Chemicals used in this study were of analytical grade and of highest purity procured from standard commercial sources in India.

ACUTE ORAL TOXICITY:

The acute oral toxicity of aqueous and alcoholic extracts of *Bupleurum falcatum* was determined by using rats and mice which were maintained under standard conditions. The animals were fasted 12 hour prior to the experiment, up and down procedure OECD guideline no. 425 were adopted for toxicity studies. Animals were administered with single dose of individual extract up to 2000mg/kg and observed for its mortality during 2days and 7days study period (short term) toxicity and observed up to 7days for their mortality, behavioral and neurological profiles.

SCREENING FOR ANTIANXIETY AND ANTIDEPRESSANT ACTIVITY

The aqueous and alcoholic extracts of *Bupleurum falcatum* leaves were tested for antianxiety activity using elevated plus maze and actophotometer and antidepressant activity using despair swim test and tail suspension test.

Treatment

Animals were divided into four (I-IV) groups.

Group I - Control group received distilled water (1ml, p.o).

Group II - Standard group received Diazepam (10mg/kg i.p).

Group III - Test group received aqueous extract of *Bupleurum falcatum* (200mg/kg p.o).

Group IV - Test group received alcoholic extract of *Bupleurum falcatum* (200mg/kg p.o).

Procedure for Ant anxiety Activity

- **Elevated plus maze (EPM) model**

The apparatus comprises of two open arms (35x5cm) and two closed arms (30x5x15cm) that extend from a common central platform (5x5cm). The floor and walls of the closed arms are made of wood and painted black. The entire maze is elevated to a height of 50 cm above the ground level. Rats weighing (150 – 200gms) were housed in a pair of 10 days prior to the test in the apparatus. During this time the rats were handled by the investigator on alternate days to reduce stress. 30 min and 60min after oral administration of the drug treatment, each rat was placed in the center of the maze facing one of the enclosed arms. During five minutes session, number of entries into open arm and time spent in the open arm were noted14,15. The procedure was conducted preferably in a sound attenuated environment.

- **Locomotor activity**

The locomotor activity can be easily studied with the help of actophotometer, the rats were grouped and treated with drugs. Turn on the equipment (check & make sure that all the photocells are working for accurate recording) and placed individually each rat in the activity cage for 10 minutes. Note the basal activity score of all the animals. Inject the drug diazepam (Dose: 5 mg/kg, ip; make a stock solution containing 0.5 mg/ml of the drug & inject 1 ml/100 g body wt of mouse), and after 30 mins re-test each mouse for activity scores for 10 mins[16]. Note the difference in the activity, before & after chlorpromazine. Calculate percent decrease in motor activity.

Procedure for Antidepressant Activity

- **Despair Swim Test Apparatus**

For the determination of antidepressant activity, forced swim test (FST) protocol was employed. During the test, animals were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with water up to a height of 10cm, at 25 ± 2°C. All animals were forced to swim for 5 min and the duration of immobility was observed and measured during the 5 min interval of the test. Immobility period was regarded as the time spent by the rats to float in water with no struggle and making only those movements necessary to keep its head above the water. In order to check the fitness level of each test animal, a pre-test was carried out 24 h before the FST by subjecting each test animal to a session of 15 min swimming.

- **Tail suspension test**

Tail suspension test was performed based on the method prescribed. The mice were suspended 58cm above the floor by means of an adhesive tape, placed approximately 1cm from the tip of the tail. The total duration of immobility was quantified during a test period of 5min. Mice were considered immobile when they were completely remain motionless

Statistical analysis

The values were expressed as mean ± SEM data was analyzed using one-way ANOVA followed by T-test. Two sets of comparision had made. i.e. Normal control Vs All treated groups. Differences between groups were considered significant at $P<0.001$ and $P < 0.05$ levels.

RESULTS

ANTIANXIETY ACTIVITY OF BUPLEURUM FALCATUM

➢ ELEVATED PLUS MAZE TEST

Anxiolytic property of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Elevated plus maze experiment.

In elevated plus-maze test (EPM), the ethanolic and aqueous extracts of *Bupleurum falcatum* leaves at a dose of 200 mg/kg p.o. significantly increased the number of entries and time spent into the open arm. The magnitude of the anti anxiety effects 200mg/kg p.o. of alcoholic and aqueous extracts of *Bupleurum falcatum* was comparable to that of diazepam 10 mg/kg i.p. (Figure 1 and 2).

Table: - Data obtained from Elevated Plus Maze experiment

S.No	Groups	Dose	% Preference	Open arm and enclosed arm			
				No.of entries in open arm(M±SEM)		Average time spent (sec±SEM)	
				(O)	(C)	(O)	(C)
1.	Control	-	Open	1	2	34	268
			Closed	1	1	12	282
			Open	2	1	42	258
2.	standard	10	Open	2	1	11	284
			Open	1	1	24	274
			Open	1	1	35	264
3.	AQEBF	200	Open	3	2	42	126
			Open	2	1	32	145
			Open	1	1	28	142
4.	ALEBF	200	Open	3	2	47	130
			Open	1	1	29	151
			Open	2	1	33	147

From the experiment it was observed that mice taken aqueous and alcohol soluble fraction at dose of 200 mg/kg body weight, stayed more time in open arm of Elevated plus Maze apparatus in comparison to standard and negative control group. Moreover they were also stayed less time in closed arm of Elevated plus Maze apparatus in comparison to standard and negative control group. The value obtained from these fraction were statistically significant ($p<0.05$).

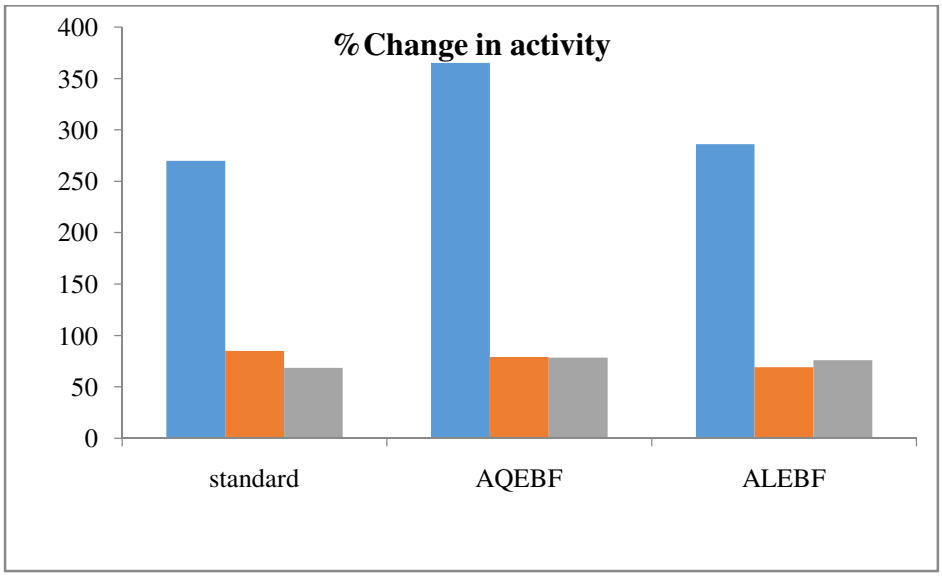
ACTOPHOTOMETER TEST:

Anxiolytic property of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Actophotometer experiment.

The percentage of reduction in locomotor activity with diazepam (10 mg/kg i.p) after 1 hour is 91.0 % i.e. there is highly significant ($P<0.000$) decrease in locomotor activity compare to control, where as dose of AQEBF and ALEBF (200mg/kg i.p) showed dose dependent decrease in locomotor activity that is 78.3% and75.8% respectively when compared to standard. The values are highly significant ($P<0.000$) (Table No:----).

Table No: --. Effect of extracts of *Bupleurum falcatum* on Locomotor activity.

S.No	Groups	Dose (mg/kg)	Locomotor activity (scores) in 10 min		
			Before	After	%change in activity
1.	control	-	245	--	---
2.	standard	30	270	85	68.5
3.	AQEBF	200	365	79	78.3
4.	ALEBF	200	286	69	75.8



The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

ANTIDEPRESSANT ACTIVITY OF BUPLEURUM FALCATUM

- **FORCED SWIM TEST**

Antidepressant activity of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Forced Swim Test experiment.

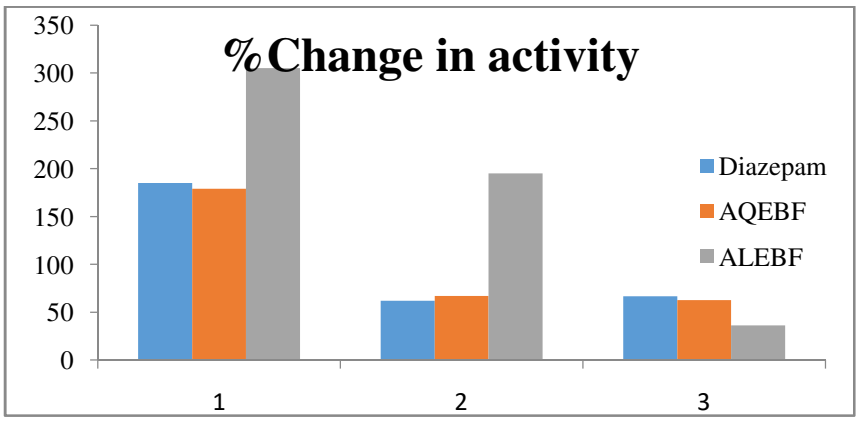
The anti-depressant activity of AQEBF and ALEBF was assessed using Forced Swimming Test in Swiss albino rats were illustrated in Table No:---. It was observed that AQEBF and ALEDB at a dose of 200mg/kg exhibited significant reduction in immobility time when compared to control in dose dependent manner. Similarly the animals treated with diazepam (10mg/kg) as expected showed significant decrease in immobility time.

Table No: ---. Effect of extracts of *Bupleurum falcatum* on Anti-depressant activity.

S.No	Group	Dose(i.p; mg/kg)	Immobility period		% change in activity
			Before	After	
1	Control	5ml/kg	134	--	---
2	Diazepam	10mg/kg	185	62	66.48%
3	AQEBF	200mg/kg	179	67	62.6%

4	**ALEBF**	**200mg/kg**	305	195	36.06%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.



Graph-1: Effect of extracts of *Bupleurum falcatum* on Anti-depressant activity

- **TAIL SUSPENSION TEST**

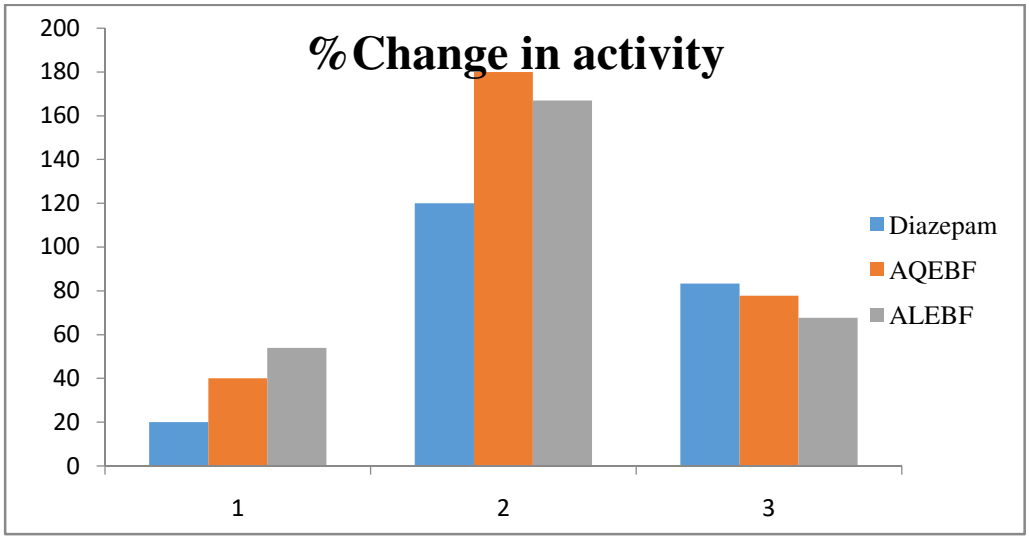
Antidepressant activity of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Forced Swim Test experiment.

In tail suspension test, the alcoholic and aqueous extracts of leaves of *Bupleurum falcatum* at a dose of 200 mg/kg i.p. significantly decreased the immobility time. The magnitude of the antidepressant effects of 200 mg/kg i.p. of alcoholic and aqueous leaves of *Bupleurum falcatum* was comparable to that of Diazepam 10 mg/kg i.p. (Table ---).

Effect of Ethanolic and Aqueous Extracts of *Bupleurum falcatum* Leaves on Tail Suspension Test in Mice at Different Time Intervals

S.No	**Treatment**	**Dose (mg/kg)**	**Duration of immobility**		**%change in activity**
			Before	**After**	
1.	Control	---	40	-----	------
2.	Standard	10	20	120	83.33%
3.	AQEDB	200	40	180	77.8%

4.	ALEDB	200	54	167	67.7%



DISCUSSION:

PHYTOCHEMICAL ANALYSIS:

The phytoconstituents are known to play an important role in bioactivity of medicinal plants. In qualitative phytochemical analysis reveals the presence of alkaloids, flavonoids, tannins, terpenoids and saponins have associated with various degree of anti-microbial, anti-bacterial, anti-fungal, anti-oxidant and anti-termites. Therefore, the anti-diabetic, hypoglycemic, anti-depressant, anti-anxiety, skeletal muscle relaxant property, locomatar activity, anti-inflammatory, analgesic and diuretic activities were observed in this study may be due to the presence of chemical constituents in both aqueous and alcoholic extracts of *Bupleurum falcatum.*

BEHAVIOURAL ACTIVITIES

ANTI-DEPRESSANT ACTIVITY

OPEN FIELD TEST

Open field behavioral model was used to study exploratory and locomotor activity in this investigation. Reported studies have shown that stress factors account for the decreases in mobility and functional responses against novel environment. The purpose of including this test was to assess the general activity of the animals after performing FST. The results observed in the open field test showed that i.p administration of aqueous and alcoholic extracts of *Bupleurum falcatum* (200 mg/kg) did not significantly increase the locomotor activity in unstressed groups

of rats as compared with their control groups. However, aqueous and alcoholic *Bupleurum falcatum* administered rats following the exposure to repeated restraint stress showed significant ($p<0.01$) increases in locomotor / exploratory activity on an open field arena. It is therefore, suggested that the extract has the ability to reverse or normalize the locomotor suppressant behavior in laboratory animals and hence may help to cope with immobility factor associated with depression in humans. In the present study that administration of aqueous and alcoholic *Bupleurum falcatum* at the dose of 200 mg/kg significantly altered the behavioral deficits induced by injections of atypical neuroleptic, haloperidol and increased brain serotonin metabolism in mice. The results are in general agreement with our previous studies in continuation to this plant and indicating its antidepressant-like activity in behavioral models of depression.

FORCED SWIM TEST

Mood disorders are one of the most common mental illnesses, with a lifetime risk of 10% in general population. Prevalence of depression alone in general population is estimated to be around 5% with suicide being one of the most common outcomes. Commonly used Antidepressants often cause adverse effects, and difficulty in tolerating these drugs is the most common reason for discontinuing an effective medication, for example the side -effects of Selective Serotonin Reuptake Inhibitor (SSRIs) include: nausea, diarrhea, agitation, headaches. Sexual side-effects are also common with SSRI's. The Food and Drug Administration requires Black Box warnings on all SSRIs, which state that they double suicidal rates (from 2 in 1,000 to 4 in 1,000) in children and adolescents. Side effects of Tricyclic Antidepressants (TCA's) include drowsiness, anxiety, emotional blunting (apathy/anhedonia), confusion, restlessness, dizziness, akathisia, hypersensitivity, changes in appetite and weight, sweating, sexual dysfunction, muscle twitches, weakness, nausea and vomiting, hypotension, tachycardia, and rarely, irregular heart rhythms.

In the present study we have evaluated the antidepressant activity of *Bupleurum falcatum* of both aqueous and alcoholic extracts in FST. The development of immobility when rodents are placed in an inescapable cylinder of water during FST reflects the cessation of their persistent escape-directed behavior. Conventional drugs reliably decrease the duration of immobility in animals during this test. This decrease in duration of immobility is considered to have a good predictive value in the evaluation of potential antidepressant agents. Exact mechanisms

underlying the antidepressant action cannot be concluded at the moment due to the presence of large number of Phytochemical in the *Bupleurum falcatum*. However, the antidepressant activity may be attributed to the presence of saponins, flavonoids and tannins in the extract. It is possible that the mechanism of anxiolytic action of AQEEU and ALEEU could be due to the binding of any of these phytochemical to the $GABA_A$ -BZD_S complex.

ANTI-ANXIETY ACTIVITY

ELEVATED PLUS MAZE METHOD:

Anti-anxiety activity of *Bupleurum falcatum* was evaluated by employing a widely used model, i.e. elevated plus-maze. The mean number of entries and time spent by mice in open arms Amongst aqueous and alcoholic extracts of *Bupleurum falcatum* significantly increased mean number of entries and mean time spent by mice in open arms of elevated plus maze apparatus at the dose of 200 mg/kg with respect to control, thereby producing anti-anxiety activity. Phytochemical screening of aqueous and alcoholic extracts showed presence of flavonoids and tannins. Flavonoids have shown anti-anxiety activity in various studies. Further, the anxiolytic effect of flavonoids has been attributed to its effect on central nervous system and benzodiazepine receptors. Therefore, flavonoids of aqueous and alcoholic extracts of *Bupleurum falcatum* may be responsible for the anti-anxiety activity. It may possible that the mechanism of anxiolytic action of AQEBF and ALEBF could be due to the binding of any of these phytochemicals to the GABAA-BZD complex. In support of this, it has been found that flavones bind with high affinity BZD site of the $GABA_A$ receptor. The plant *Bupleurum falcatum* also contains flavones which may responsible for its anxiolytic activity. So the anxiolytic activity of AQEBF and ALEBF might involve an action on GABAergic transmission or effects on serotonergic transmission or due to its mixed aminergic potentiating effect.

ACTOPHOTOMETER TEST:

Spontaneous locomotor activity is considered as an index of alertness and can be helpful to confirm the general depressive activity of any drug. The decrease in motor activity gives an indication of the level of excitability of the CNS and this decrease may be related to sedation resulting from depression of CNS. However, in the present study the AQEBF and ALEBF was found to have decreased effect on the locomomotor activity in actophotometer.

It is reported that GABA, an inhibitory neurotransmitter is involved in the pathophysiology of depression. Moreover, neurochemical research has revealed that the

monoamines (5-HT, NA, and dopamine) have a crucial role in the development of the depression syndrome. It has been found that flavonoids isolated from plant species such as *Bupleurum falcatum* showed antidepressant activity. Thus, it is likely that flavonoids present in AQEBF and ALEBF may be responsible for the observed antidepressant effect. The antidepressant effect shown by AQEBF and ALEBF suggests etiological similarity in the development depression. Several hypotheses have been proposed to explain this aspect. The serotonergic theory postulates excessive functioning of the serotonergic neurotransmission for the prevention of the cause of depression. It can be hypothesised that AQEBF and ALEBF may have acted by modulating one or more of the above-mentioned neurotransmitters. Moreover, cholinergic transmission also plays the promising role in CNS. Based on its irregular distribution within the CNS and the observation that peripheral cholinergic drugs could produce marked behavioral effects after central administration. Therefore, it can be predicted that the higher level of choline in EU leaves may be responsible to act on cholinergic transmission in CNS and may be helpful to prevent depression.

Moreover triterpepenoids (steroidal compounds) are present in the leaves, those are able to cross blood brain barrier (BBB) due to their lipophilic nature and so it can be assumed that such steroidal compounds might also be responsible to elicit antidepressant and other neuropharmacological activities at molecular level in CNS (brain).

SUMMARY

1. The fresh leaves of *Bupleurum falcatum* used for this project work .
2. The dried leaves of *Bupleurum falcatum* were successively extracted with alcohol and water. Percentage yield was calculated.
3. Therapeutic dose of the extracts were calculated after carrying acute oral toxicity studies in both rats and mice.
4. Extracts were tested for their anti-depressant activity in mice.

- By Forced Swim Test.
  - Both aqueous and alcoholic extracts (200 mg/kg) showed significant decrease in duration of immobility time.
- By OPEN FIELD TEST.
  - Both aqueous and alcoholic extracts (200 mg/kg) showed significant decrease in duration of immobility time.

5. Extracts were tested for their anti-anxiety by elevated plus maze in rats.

- By Elevated Plus Maze Test.
  - Both aqueous and alcoholic extracts (200mg/kg) showed significant increased in mean number of entries and time spent in open arms.
- By Actophotometer Test
  - Both aqueous and alcoholic extracts (200 mg/kg) was showed decreased effect on the locomotar activity in rats.

CONCLUSION

The results obtained in this study indicate that the n-hexane, ethyl acetate and methanol fractions of the leaves of *Bupleurum falcatum* have significant CNS Depressant and Anxiolytic activities in animal model systems. The medicinal values of the plant leaves may be related to their constituent phytochemicals. So, further detailed investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be done. It will help in the development of novel and safe drugs for the treatment of different types of CNS disorders.

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Covid-19: A Formidable Challenge to the Healthcare System

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Covid-19: A Formidable Challenge to the Healthcare System

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ABSTRACT

Coronaviruses are important disease causing animal pathogen and recently with the mutations has resulted into the infection in humans. Beta type of corona virus a subgenus cause severe acute respiratory syndrome. Older patients with comorbid conditions including pulmonary disease, cardiac disease, kidney disease, diabetes, and hypertension have been associated with fatality. More than 2.06 million cases have been reported in 212 countries/regions, resulting in more than 137,108 fatalities across the world. The COVID-19 infection is characterized by respiratory symptoms like cough, pneumonia symptoms, gastrointestinal symptoms, high fever etc. At present there is no treatment or drug available for the disease and its changing structure and infection symptoms is posing a challenge for pharmaceutical and biomedical industry. The present review article highlights the on-going researches on pathogenesis, clinical characteristics of COVID 19.

Keywords-- Comorbid conditions, Corona virus, COVID-19, Pneumonia

INTRODUCTION

Corona viruses square measure a family of viruses noted for holding strains that cause doubtless deadly diseases in mammals and birds. First delineate very well within the 60s, the coronavirus gets its name from a particular corona or 'crown' of sugary-proteins that comes from the envelope encompassing the particle. Secret writing the virus's make-up is that the longest ordering of any RNA-based virus - one strand of macromolecule roughly measures 6,000 to 32,000 bases long. At present corona disease is one of the big problem which is difficult to control infection and to treat the disease. This coronavirus was initially named as the 2019-novel coronavirus (2019-nCoV) on 12 January 2020 by World Health Organization (WHO). WHO officially named the disease as coronavirus disease 2019 (COVID-19) on 11 February 2020. The subgroups of coronaviruses are alpha (α), beta (β), gamma (γ) and delta (δ) coronavirus [1].

HISTORY

In 1960 Corona viruses was discovered and 8098 individuals were infected all over 26 countries with mortality rate of 9%. A couple of Saudi Arabian nationals in 2012 were infected with other corona virus. The virus is named as the Middle East Respiratory Syndrome Corona virus (MERS-CoV), as a member of corona virus. It infected more than 2428 people and 838 deaths. The infection starts from upper respiratory tract and progresses to severe respiratory diseases. Patients infected with MERS suffer pneumonia, ARDS and renal failure. Geriatric patients with diseased conditions are highly infected with the virus [2, 4].

EPIDEMIOLOGY

The Mortality rate till date is 5.1% across 213 countries [3]. By novel corona virus and it's transmission rate is higher than SARS- CoV. The genetic transmission in the RBD region at S-Protein might be the reason for its enhanced transmission ability.

A number of pneumonia cases were emerged at Wuhan, China during early December 2019. Majority of cases were observed at the Huanan Seafood Wholesale Market area, those were associated with selling many species of live animals. It was rapidly spread to various parts of China and many countries across six continents. Chinese Centre for Disease Control and Prevention (China CDC) was initially identified a novel member of enveloped RNA corona virus at branch alveolar lavage fluid from affected person on 3rd January 2020. WHO named as COVID-19 – those illness associated with nCoV as the 2019 corona virus disease on 11th February 2020. WHO identified the emergence of nCOV19 and declared a public health emergency of international concern (PHEIC) in Guangdong, strong public health response, high-level political commitment and sufficient funding is required to the outbreak. As of 26 June 2020, 4,72,804 Covid-19 cases are confirmed in India, as per the health ministry. 14,894 are dead and 271696 have recovered [3]. The details are given below

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Table 1: *The states which have significant morbidity of COVID-19 are depicted below*

State/Union Territory	Total number of cases
Maharashtra	1,42,900
Delhi	70,390
Tamilnadu	67,468
Gujarat	28,943
Uttar Pradesh	19557
Rajasthan	16,009
West Bengal	15,173
Madhya Pradesh	12,448
Haryana	12,010
Telanagana	10,444
Andhra Pradesh	10331
Karntaka	10118

Worldwide, the number of Covid-19 cases is over 96, 08,814 lakhs; the death toll is 4, 89,405 and 48, 08,236 have been recovered. The death toll in USA has crossed 1, 26,218- at 24, 65,127 the highest in the world have been confirmed [3].

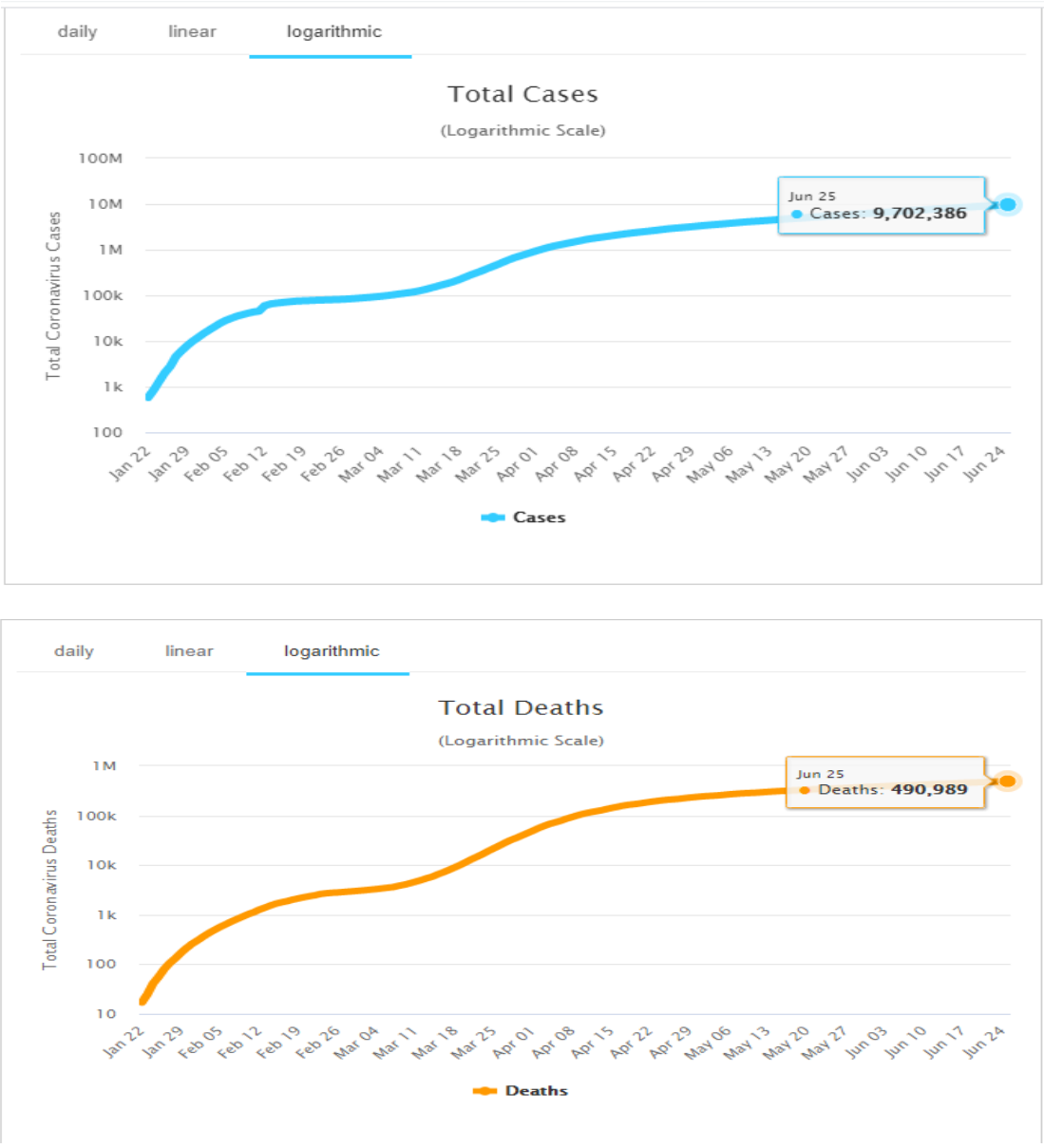

Figure 1: *Total number of corona virus cases and deaths.*

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Nature and Type

Corona viruses belong to family Coronaviridae, order Nidovirales. Outer surface of corona virus contains crown-like spikes having dimensions (65-125 nm in diameter). The nucleo-capsid, created from a super molecule shell referred to as a capsid and containing the infectious agent nucleic acids, is whorled or cannular and it contains a single-stranded positive sense Subtypes of corona virus are Alpha (α), beta (β), gamma (γ) and delta (δ) . The SARS and MERS virus virus causes acute respiratory distress syndrome (ARDS) and lung injury resulting in pulmonary failure and fatality.

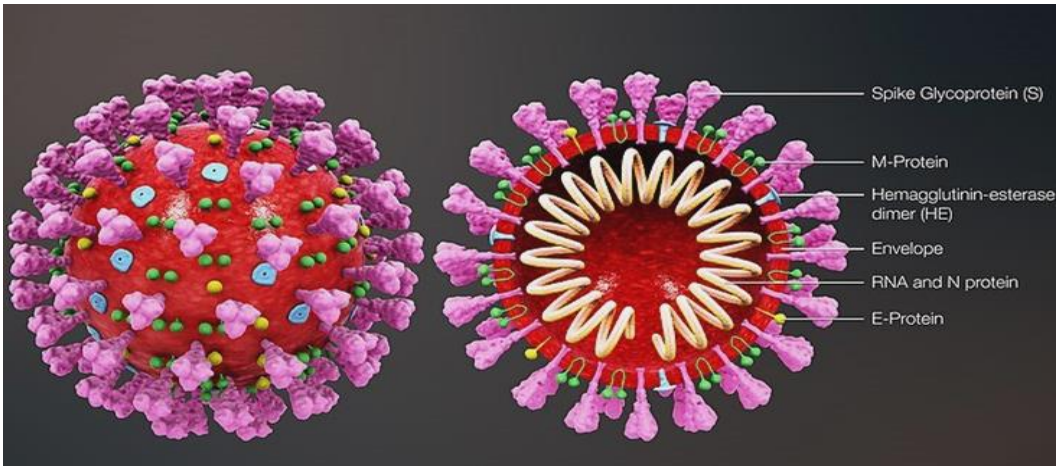

Figure 2: *Structure of Corona Virus.*

TRANSFORMATION OF VIRUS IN THE BODY

Mammals are the link between viruses and humans as it shows 88% genomic sequence, identify with SARS like virus. Lose contact with humans infected droplets, sneezing, coughing. They penetrate via inhalation from nose or mouth. The key reservoirs are only α and β corona viruses infect humans. Close contact with infected person and consumption of infected animals are main reasons for transmission. People who are contacted with incubation carriers are the transmitters [4]. Dotted black arrow - possibility of viral transfer, solid black arrow - confirmed transfer.

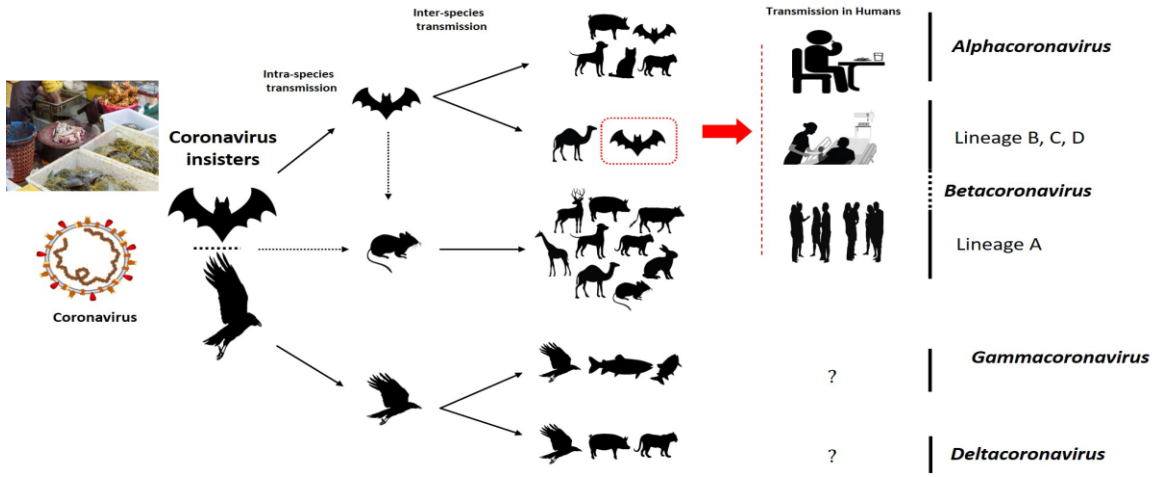

Figure 3: *The key reservoirs and mode of transmission of corona viruses [1].*

Entry Mechanism

The common shaped spikes of glycoprotein on the outer side of corona virus are responsible for attachment of virus to host cells. These are specific genes in ORF1 encode proteins for spikes formation and replication. The virus can infect multiple hosts the Receptor – binding Domine (RBD) is loosely attached to virus. The virus enters by binding to ACE -2 which is important factor of infection. SARS CoV-2 possesses structure, with protein spikes and other expressed poly nucleon and membrane proteins.

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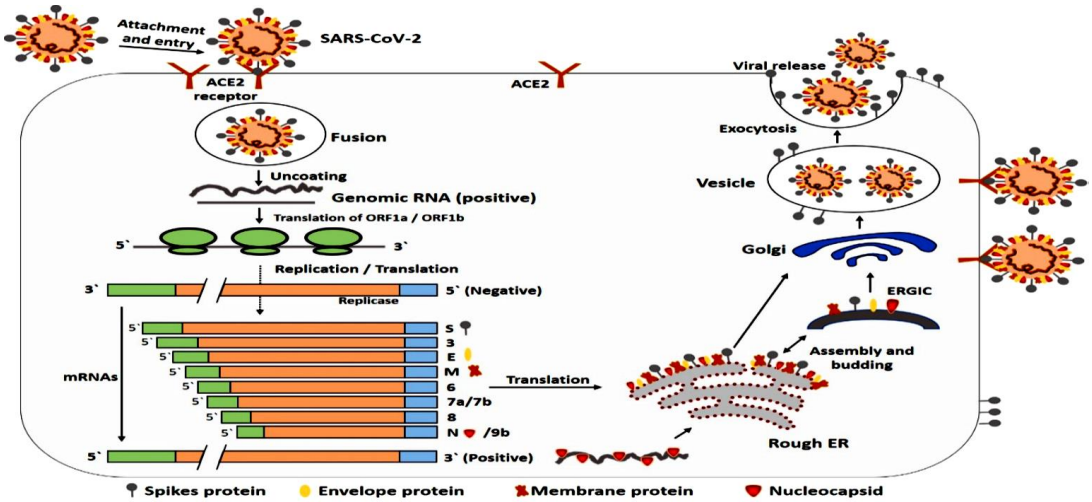

Figure 4: *Life cycle of SARS-CoV-2 in host cells [1].*

ACE2, angiotensin-converting enzyme 2; ER, endoplasmic reticulum; ERGIC, ER–Golgi Intermediate Compartment

The life cycle of virus starts when cellular receptor ACE-2 gets linked with S-Protein. Viral envelope fusion is done by endosomal pathway after conformation change in S protein with receptor. Then viral RNA released into the host cell and translated in presence of viral replicase polyproteins pp1a and 1ab, which are then cleaved into small products by viral proteinases. Relevant vial protein formed by a series of subgenomic mRNAs by discontinuous transcription in presence of polymerase. Viral proteins and genome RNA are subsequently assembled into virions in the ER and Golgi and then transported via vesicles and released out of the cell. The disease may worsen in Patients with metabolic syndrome, obesity and would make any sick person because it can produce the "cytokine storm" that generates damage to lung tissue. Case reports showed that increase in number of leucocytes, pro inflammatory cytokines in plasma were observed in COVID -19 patients [5].

Symptoms

The symptoms of COVID-19 may appear after an incubation period of approximately 5.2 days. Onset of symptoms was observed from 6th day and its lethal effects approximately 41 days. This period depends on the status of patient immunity and age. This period is shorter in patients with more than 70 years age and they affected rapidly compare to under 70 years age. It showed some unique clinical features that include the targeting of the lower airway as evident by upper respiratory tract symptoms like rhinorrhoea, sneezing, sore throat, anosmia, detection of RNAaemia and acute respiratory distress syndrome. Other than these, it affects cardio vascular system and may cause acute cardiac injury, developed intestinal symptoms like diarrhoea [6] only a low percentage of MERS-CoV or SARS-CoV patients exhibited diarrhoea. 44-60% Asian patients were experienced with myalgia, fatigue also [6]. Myoclinic reported that during emergency the patient may experience with following signs and symptoms include trouble in breathing, persistent chest pain, inability to stay awake, blue lips or face [7].

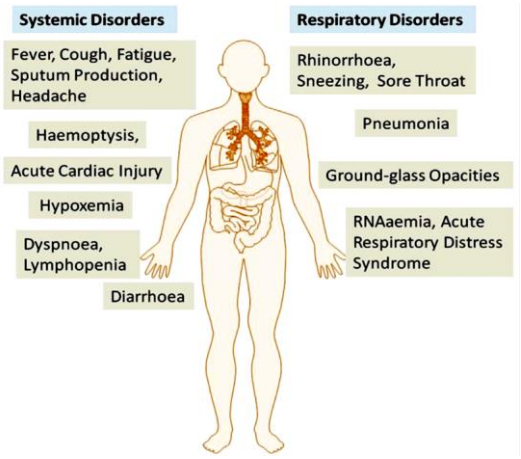

Figure 5: *Symptoms caused by COVID-19 infection.*

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More than 40,000 persons with COVID-19 from china showed illness of severity Mild to moderate is characterized by fever, fatigue, myalgia, cough, sore throat, runny nose, and sneezing. Severe symptoms characterized by dyspnea, hypoxia, image shows >50% lung involvement and critical condition can be characterized by respiratory failure, shock or multi organ dysfunction [8].

RISK FACTOR FOR SEVERE ILLNESS

The patients admitted to the intensive care unit had number of comorbidities than those not admitted in ICU. Comorbid conditions of patients with COVID-19 shown more severe illness and mortality which include:

- Cardiovascular disease
- Diabetes mellitus
- Hypertension
- Chronic lung disease
- Cancer
- Chronic kidney disease
- Immunocompromising conditions
- Severe obesity
- Liver disorders

Majority of patients who died with COVID -19 were suffering with pre-existing co morbidities [9].

Diagnosis

In most cases of infection, diagnosing of coronaviruses is not sensible, because the illness can naturally run its course. However, it should be vital in bound clinical associated veterinary settings or in medical specialty studies to spot an etiological agent. Patients infected with COVID -19 shows 83% of increase in leucocytes, elevation in pro inflammatory cytokines and C- reactive protein, lymphopenia, neutripenia from complete blood picture[7]. Elevated levels of serum alanine aminotransferase and aspartate aminotransferase levels, lactate dehydrogenase are observed. Multiple patchy ground glass opacities in bilateral subpleural areas were observed by High resolution computed tomography (HRCT). Swab test from Branchoalveolar lavage fluid (BALF) is more accurate than oropharengeal or nasopharengeal swab test [8]. SARS-CoV-2 RNA can be detected in patient sputum, blood and stool specimens by RT-PCR assay. In some cases live virus has been cultured from stool. Chest radiography (CXR) is best method to view the extension and typology of lung parenchyma involvement [11]. The hallmarks of COVID-19 infection on imaging were bilateral and peripheral ground-glass and consolidative pulmonary opacities and consolidation with or without vascular enlargement, interlobular septal thickening, and air bronchogram sign are common CT features of COVID-19 [12].

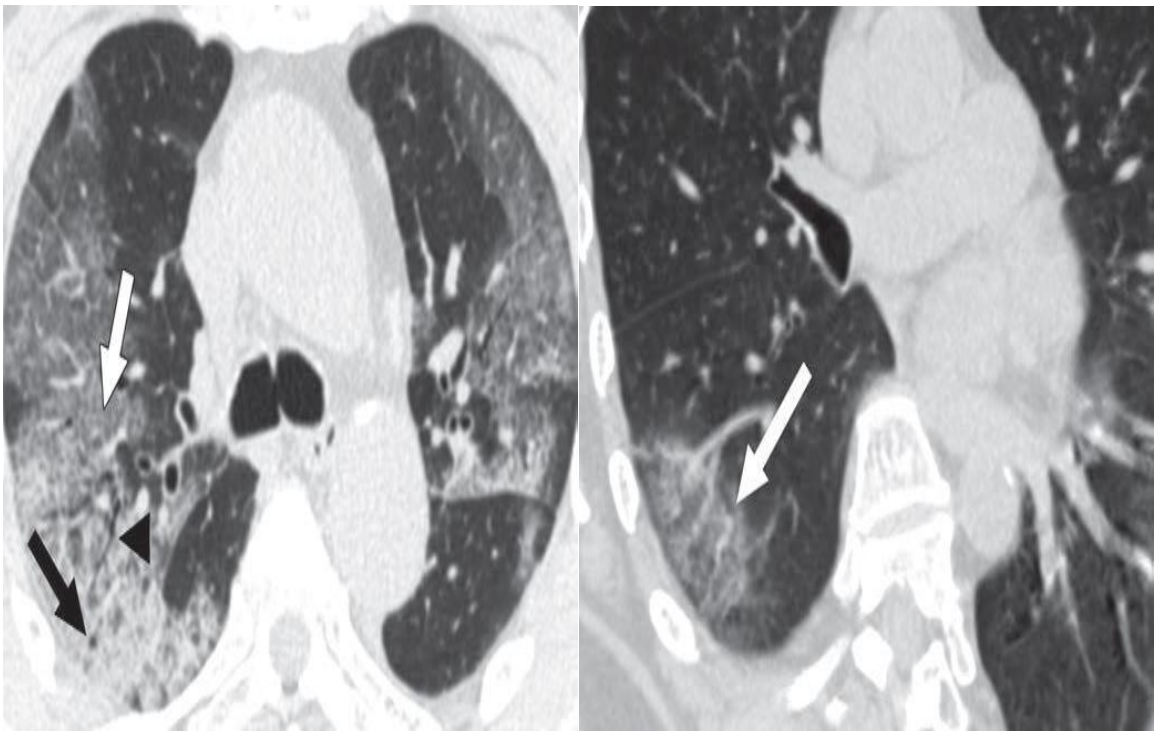

Figure 6: *(a) CT scan shows multiple ground-glass opacities and with consolidation with thickened intralobular and interlobular septum (white arrow).*
(b) Oblique transverse CT image shows ground-glass opacities vascular enlargement (arrow).

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The duration of viral load is variable and which depends on severity of illness. In one study of 21 patients, some patients with mild illness showed negative to nasopharyngeal swabs by 10 days after onset of symptoms. Whereas tests were positive for longer in patients with severe symptoms. One study found that PCR confirmed cases of SARS-CoV infection in children who were asymptomatic. RT-PCR is also used to measure viral RNA shedding. Medical science assays are vital in cases wherever RNA is also troublesome to isolate, isn't any longer gift, and for medical specialty studies. CSIR lab developed India's first paper-strip test which uses Crispr-Cas9 to target and identify the genomic sequences of the novel corona virus. India-based Mylab Discovery has become the first company in the country to receive approval for its coronavirus (Covid-19) diagnostic test kits, called reverse transcription polymerase chain reaction (RT-PCR) tests. Abbott Company launched its Abbott m2000 Real Time SARS-CoV-2 EUA test, which provides results just in 5 minutes.

Preventive measures [13]

Avoid physical contact while greeting. Follow safe greeting include a wave or a bow or a node. Maintain at least 1 meter (3 feet) distance between yourself and anyone who is coughing or sneezing. Regularly and thoroughly washing your hands with soap and water or using alcohol-based hand rub kills viruses that may be on your hands. Avoid touching eyes, mouth and nose to prevent entry of virus in to body. During coughing and sneezing cover your mouth and nose with napkin or tissues or with your bent elbow to prevent droplet infection. Wear a mask if you are sick. Consult the physician if patient fell unwell like fever, cough, and difficulty in breathing

CELLULAR VACCINE BY SORRENTO THERAPEUTICS FOR COVID-19 [12]

An I-Cell cellular vaccine, STI-6991 comprises replication-deficient human erythroleukemia K562 cells. It express membrane-bound S1 protein of the SARS-CoV-2. T and B-cell immunities are expected to induce by this vaccine against the novel coronavirus. Sorrento Therapeutics intends to achieve this immune response by using a replicating cell line (human erythroleukemia, K562) to integrate SARS-CoV-2's S1 domain onto the cellular membrane and present the viral antigen on a decoy cell surface. At present, the company is testing I-Cells' ability to elicit T and B-cell immunities in an animal model. Furthermore, the company has already started process development, validation and scaled up manufacturing testing kits in its cell therapy facilities in San Diego, US to support registration trials and commercialisation. Human trials should begin in mid of this year.

POSSIBLE TREATMENT OPTIONS [14-16]

There is no specific standard treatment for covid-19. But treatment with anti-viral agents like (remdesivir, ribavirin, chloroquine), glucocorticoid therapy, extracorporeal support like artificial liver system (ALS) and extracorporeal membrane oxygenation (ECMO) depends on requirement.
Combination of lopinavir and ritonavir: they are protease inhibitors. They mainly bind to M^{pro} enzyme and interfere with corona viral replication.

Remdesivir

It approved in India which speeds the recovery by shutting down the replication, Used in moderate stage. It is broad spectrum anti-viral agent effective against MERS and SERS. It mainly inhibits the EBOV RNA dependent RNA polymerase complex in remdesivir triphosphate form.

Ribavirin

It is also broad spectrum nucleoside anti-viral agent. The phosphorylated ribavirin inhibit viral synthease and prevents viral transcription there by it inhibits necessary proteins require for synthesis of new virions. Insufficient clinical effects were observed in SARS infected patients. Used in human respiratory fusion viral infection. Chinese used ribavirin in COVID 19 treatment in combination with interferons. According to cellular trails in SARS and MERS showed that ribavirin can be reduced when used with interferon due to synergistic effect.

Fapirair

This is also antiviral activity and approved in india. It alters the immune mediated lung injury and slowdown the progression of respiratory failure. It reduce the risk of death in ICU patients

Azithromycin

It is a macrolide antibiotic. It may prevent bacterial super infection and immunomodulatory property in COVID-19 treatment. It mainly down regulate inflammatory responses thereby reduce cytokinin release in respiratory infections. It may also reduce mucosal hyper secretion, reduce the chemotaxis of neutrophils to lungs by inhibiting cytokine species, block the nuclear transcription

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factors, accelerating neutrophil apoptosis and decrease the levels of reactive oxygen species. The major drawback with azithromycin is Q-T prolongation, drug interactions.

Toclizumab

IL-6 receptor inhibiting monoclonal antibody. Majorly it bind to IL-6 receptor there by inhibits interleukin actions. There by it stops the release of pro inflammatory mediators like cytokines. It induces the activation of T cells , immunoglobulins, T and B-cells, lymphocytes, monocytes and fibroblasts. Risk of GI perforation, hepatotoxicity, infusion related reactions and throbocytopenea and neotropenia are major drawbacks.

COVID-19 Convalescence Plasma

Here plasma which is collected form recovered COVID-19 person that may contain anti-bodies to SARS CoV-2. Clinical trials showed that convalescence plasma is preferred in severe and immediate life threatening patients infected with COVID -19 (Plasma therapy).

Chloroquine

It is an antimalarial agent. It mainly interfere with viral replication by altering the endosomal pH during viral cell fusion and elevates immunity throughout body. *In vitro* studies shown that it is powerful inhibitor of SARS-CoV cell culture. It also interfere with binding of s-protien to ACE_2 (angiotensin converting enzyme 2) receptor. There by it prevent the SARS-CoV infection. The use of chloroquine is limited because the cellular and animal studies showed cardiac toxicity, irreversible eye damage and its pharmacokinetic parameters like its longer plasma half-life 2.5 – 10 days, metabolized in liver its metabolites are toxic to liver during hepatotoxic condition. There is risk of cardiac arrhythmias (Q-T prolongation), renal damage upon long term use.

Corticosteroids

It is used in corona viral infections such as SARS and MERS. Some studies shown that the use of corticosteroids are not recommended because of the unwanted effects like avascular necrosis, diabetes and alteration in viral clearance. It showed arrhythmia and acute cardiac injury in patients with COVID-19. Other than this it showed multiple organ dysfunctions (MOD) like shock, acute respiratory failure and also it may cause death.

Ivermectin

It is an inhibitor of the COVID-19 causative virus (SARS-CoV-2) *in vitro*. A single treatment able to effect ~5000 fold reduction in virus at 48h in cell culture. It is an inhibitor of interaction between the human immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the importin (IMP) α/β1 heterodimer responsible for IN nuclear import, Ivermectin has since been confirmed to inhibit IN nuclear import and HIV-1 replication. It is FDA-approved for parasitic infections, and therefore has a potential for repurposing.

ROLE OF RAAS IN COVID -19 [17]

ACE2 receptors have strong association for SARS-CoV-2 viral entry. ACE2 has been expressed in various parts of the human body viz., gastrointestinal system, heart, and kidney. Recent studies showed the expression of ACE2 in type II alveolar cells of lungs. Various animal models showed that administration of angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) affect the severity and mortality of COVID-19. At the same time they elevate ACE2 levels and that increase ACE2 mediated viral penetration in to lung tissues. These findings suggested that administration of these drugs increase severity of infections.

JAMA CLINICAL GUIDELINES SUGGESTED FOLLOWING RECOMMENDATIONS OF CRITICALLY ILL ADULTS WITH COVID -19 [3]

Infection Control and Testing

Health care workers were provided with N95 respirators instead of surgical masks to avoid infection.

Hemodynamic Support

1. For acute resuscitation adults with shock: using conservative fluid administration strategy using balanced crystalloids rather than colloids by measuring dynamic parameters.
2. Adults with shock norepinephrine are recommended as first line vasoactive, use of vasopressin with epinephrine if norepinephrine is not valuable.

Ventillatory Support

1. If Spo_2 is less than 90% supplemental oxygen is strongly recommended and it should maintained not more than 96%.

2. Use of high flow nasal cannula (HFNC) is suggested during hypotoxic respiratory failure. If HFNC is not available non-invasive positive pressure ventilation is recommended.
3. Use of low tidal volume ventilation (in range of 4-8 mL/kg) recommended in patients with Acute respiratory distress syndrome (ARDS). Plateau pressure maintained <30 cmH_2O is recommended. Using higher positive end expiratory pressure (PEEP) strategy is suggested over lower PEEP.
4. 12-16 hours of mechanical ventilation is suggested in patients with moderate to severe ARDS. Hemodynamic support: Conservative fluid administration strategy using balanced crystalloids were preferred in adult patients with acute shock condition.

CONCLUSION [18]

We have to be ready for further outbreaks and must focus our attention to the prompt diagnosis of the cases and infection control procedures with isolation of suspected cases, and to anticipate those that may be the complications and evolutions in the course of the COVID-19 epidemic. Now, promising clinical treatments or prevention strategies have not developed against corona viruses. However, the researchers are working to develop efficient therapeutic strategies to cope with the novel coronaviruses.

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ANTIDIABETIC ACTIVITY AND PHYTOCHEMICAL SCREENING OF LEAVES EXTRACT OF DIOSPYROS PEREGRINE IN ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

Diabetes mellitus is a most common endocrine disorder, affecting more than 300 million people worldwide. For these therapies developed along the principles of allopathic are often limited in efficacy, Carry the risk of adverse effects, and are often too costly, especially for the developing world. In order to identify complementary or alternative approaches to existing medications, we studied the anti-diabetic potential of leaves of *Diospyros peregrine*. The acute oral toxicity studies of the extracts revealed no toxic effects up to the levels of 2000mg/kg b.wt. The aqueous and alcoholic extracts of 20 and 30mg/kg body weight of *Diospyros peregrine* was screened for the presence of hypoglycemic and antidiabetic activity. In this study diabetes was induced by a single IP dose Alloxan monohydrate in 72hrs fasted rats. The FBGL was carried on 7th, 14th and 21st day and OGTT was measured on 8th, 15th and 22nd day. Glibeclamide was taken as the standard and the results are quite comparable with it. The studies were indicated that the leaves of *Diospyros peregrine* are effective in regeneration of insulin secreting β-cells and thus possess antidiabetic activity. The aqueous and alcoholic extracts showed significant effect in decreasing the Fasting blood Glucose level and oral glucose tolerance test of rats and it's also showed good hypoglycemic activity in normal glycemic rats. The preliminary phytochemical analysis of the extracts of *Diospyros peregrine* revealed the presence of Alkaloids, Tannins,

Anthraquinones, Flavonoids, Saponins and Terpenoids as the possible biologically active principles.

Keywords: *Diospyros peregrine*, Alloxan monohydrate, Glibenclamide, FBGL and OGTT.

INTRODUCTION

Diabetes Mellitus (DM):

Diabetes is one of the most common non-communicable diseases and a serious life-long condition appearing worldwide. The etiology of diabetes is a complex interaction of genetic and environmental factors. It is a heterogeneous group of metabolic disorders characterized physiologically by dysfunction of pancreatic beta cells and deficiency in insulin secretion or insulin activity and clinically by hyperglycemia or impaired glucose tolerance and other manifestable disorders. It is an endocrinological syndrome abnormally having high levels of sugar in the blood. This may be either due to insulin not being produced at all, is not made at sufficient levels, or is not as effective as it should be.

Diabetes is still a serious health problem all over the world since it is associated with increased morbidity and mortality rate. When compared with the general population, mortality and morbidity increase in diabetes is mainly due to the associated chronic complications both specific (microvascular) and nonspecific (macrovascular). Since the disease prevails in both genders and in all age groups, the general public has a concern about its control and treatment[1].

Classification of DM

Diabetes is classified by underlying cause. The most common forms of diabetes are categorized as

Type 1, or insulin-dependent diabetes mellitus (IDDM) - an autoimmune disease in which the body's own immune system attacks the pancreatic beta cells, rendering it unable to produce insulin and

Type 2 or non - insulin - dependent diabetes mellitus (NIDDM) - in which there is resistance to the effects of insulin or a defect in insulin secretion.

Type 2 diabetes commonly occurs in adults associated with obesity. There are many underlying factors that contribute to the high blood glucose levels in these individuals. An

important factor is the resistance to insulin in the body essentially ignoring its insulin secretions. A second factor is the decreased production of insulin by the cells of the pancreas. Therefore, an individual with Type 2 diabetes may have a combination of deficient secretion and deficient action of insulin. In contrast to Type 2 diabetes, Type 1 diabetes most commonly occurs in children and is a result of the body's immune system attacking and destroying the beta cells. The trigger for this autoimmune attack is not clear, but the result is the end of insulin production[2].

Multiple risk factors for the development of Type 2 diabetes mellitus[3]:

- Family history (parents with diabetes).
- Obesity (i.e., ≥20% over ideal body weight or body mass index ≥25kg/m^2).
- Habitual physical inactivity.
- Impaired glucose tolerance.
- Hypertension (≥140/90mm Hg in adults).
- High density lipoprotein (HDL) cholesterol ≤ 35mg/dl and/or triglyceride level ≥ 250mg/dl.

History

The term “Diabetes” was first used around 250 B.C. It is a Greek word meaning “to syphon”, reflecting how diabetes seemed to rapidly drain fluid from the affected individual. The Greek physician Aretaeus noted that affected individuals passed increasing amounts of urine as if there was “liquefaction of flesh and bones into urine”. The complete term “diabetes mellitus” was coined in 1674 by Thomas Willis. Mellitus is Latin for honey, which is how Willis described the urine of diabetics[5].

Historical accounts reveal that as early as 700-200 BC, diabetes mellitus was a well recognized disease in India and was even distinguished as two types, a genetically based disorder and other one resulting from dietary indiscretion. Ancient Hindu writings document how black ants and flies were attracted to the urine of diabetics. The Indian physician Sushruta in 400 B.C. described the sweet taste of urine from affected individuals, and for many centuries to come, the sweet taste of urine was a key to the diagnosis.

Physicians have observed the effects of diabetes for thousands of years. One of the effects of diabetes is the presence of glucose in the urine (glucosuria). For much of the time, little was known about this fatal disease that caused weight loss of body, extreme thirst, and

frequent urination. It was in 1922 that the first patient was successfully treated with insulin. Till the mid-1800s, the treatments offered for diabetes varied tremendously. A breakthrough in the puzzle of diabetes came in 1889. German physicians Joseph von Mering and Oskar Minkowski surgically removed the pancreas from dogs. The dogs immediately developed diabetes. Now that a link was established between the pancreas and diabetes, research focused on isolating the pancreatic extract that could treat diabetes. Dr. Frederick Banting succeeded in his experiments of isolating a pancreatic extract. The diabetic dog was kept alive for eight days by regular injections until supplies of the extract, at that time called "isletin", was exhausted. Experiments on dogs showed that extracts from the pancreas caused a drop in blood sugar, caused glucose in the urine to disappear, and produced a marked improvement in clinical condition.

A young boy, Leonard Thompson, was the first patient to receive insulin treatment in the year 1922 and lived for thirteen years. Over the next 70 years, insulin was further refined and purified. A revolution came with the production of recombinant human DNA insulin in 1978. Instead of collecting insulin from animals, new human insulin could be synthesized. In 1923, Banting and Macloed were awarded the Nobel Prize for the discovery of insulin. In his Nobel Lecture, Banting concluded the following about their discovery: “Insulin is not a cure for diabetes; it is a treatment.”

MATERIALS AND METHODS

Alloxan from Quali Kems Fine Chem Pvt, Ltd, Vadodara and provided by **Suralabs, Dilsuknagar** Methanol from Merck, India.Alcohol from Merck, India.Glibenclamide from Orchid Pharma Ltd, Chennai, provided by **Suralabs, Dilsuknagar.**

Plant Material Collection

The leaf of Diospyros peregrine was collected from the local market in Hyderabad in the month of January and was identified and authenticated from Department of Pharmacognosy. The plant material was cleaned, reduced to small fragments, air dried under shade at room temperature and coarsely powdered in a mixer. The powdered material was stored or taken up for extraction process.

Preparation of plant extracts:

Preparation of Aqueous Extract:

Dried leaves of *Diospyros peregrine* were taken about 20gms into 250ml beaker containing 200ml of water. The contents were mixed well and then the mixture was boiled upto 80-90^{0}C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Preparation of Alcoholic Extract:

Dried leaves of *Diospyros peregrine* were taken about 20gms into 250ml beaker containing 200ml of Alcohol. The contents were mixed well and then the mixture was boiled up to 50-60^{0}C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Preliminary phytochemical analysis of the extracts

The extracts so obtained were subjected to preliminary phytochemical screening. Phytochemical studies were performed to identify the presence of various phytoconstituents as follows:

RESULTS

Phytochemical screening of *Diospyros peregrine*.

The present investigation concluded that the isolated compounds from the plant *Diospyros peregrine* shows the various Pharmacological effects was determined due to the presence of different phytochemical compounds. Further study is needed for the isolation of the constituents present in the plant and its individual pharmacological activity should need to consider and ultimately it should be implemented for the benefit to human beings.

Table 1: Phytochemical screening of *Diospyros peregrine*.

S.No.	Phytoconstituents	Aqueous	Alcoholic
1.	Alkaloids	-	-
2.	Flavonoids	+	+
3.	Steroids	+	-
4.	Tannins	+	+
5.	Anthraquinones	-	-
6.	Terpenoids	+	+
7.	Cardiac glycoside	+	+
8	Saponins	+	-

- **Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats**

Table No:2- Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats.

Treatment	Dose (mg/kg)	Blood glucose level(mg/dl)		
		7th day	14th day	21st day
Normal control	-	85.31±1.05	78.14±0.23	68.19±3.93
Glibenclamide	10	82.87±2.78	75.93±2.12	65.3±2.05
AQDP1	20	86.51±2.91	80.17±3.32	72.97±5.20
AQDP2	30	81.93±2.49	73.96±1.57	68.36±2.93
ALDP1	20	78.2±0.19	62.11±0.49	57.65±5.73
ALDP2	30	83.0±2.24	72.36±4.89	67.30±1.56

Values are expressed as mean± S.E.M. n=6. Significant values were compared with p<0.005, normal control Vs all groups. Parent thesis indicates % reduction in BGL.

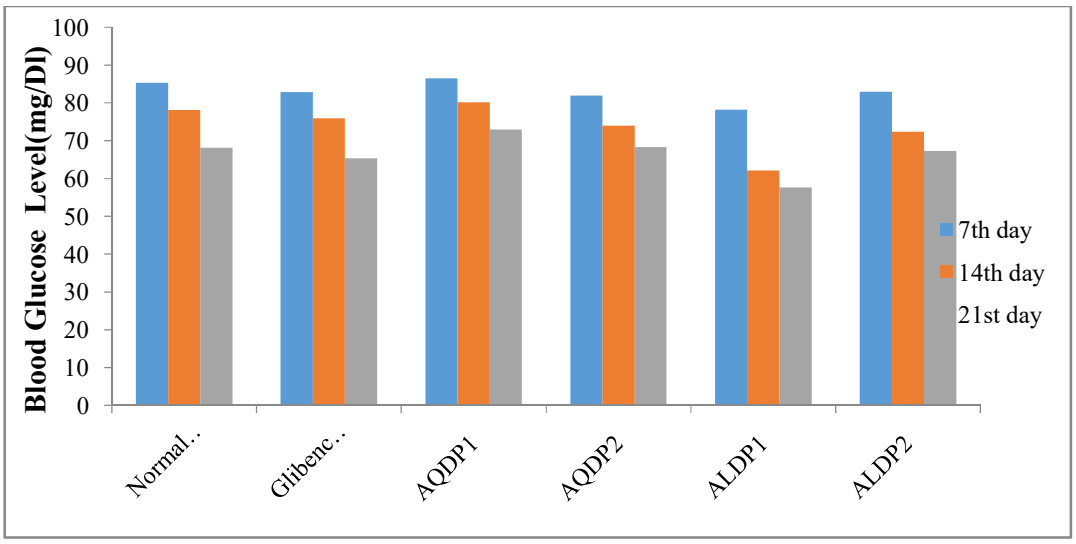

Figure 1: - Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats.

Table No: 3- Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats on 7th day.

Treatment	Dose (mg/kg)	Blood glucose level(mg/dl) (7th day)			
		1st hr	2nd hr	4th hr	8th hr
Normal control	-	42.26±2.08	51.89±0.15	76.32±1.89	85.31±1.05
Glibenclamide	10	50.14±1.39	61.21±1.20	71.5±0.05	82.87±2.78
AQDP1	20	53.52±0.76	65.31±1.03	70.14±8.52	86.51±2.91
AQDP2	30	47.72±1.25	60.59±2.87	75.83±1.06	81.93±2.49
ALDP1	20	45.1±0.54	58.35±1.50	62.15±3.28	78.2±0.19
ALDP2	30	53.1±0.08	65.28±6.19	78.20±2.75	83.0±2.24

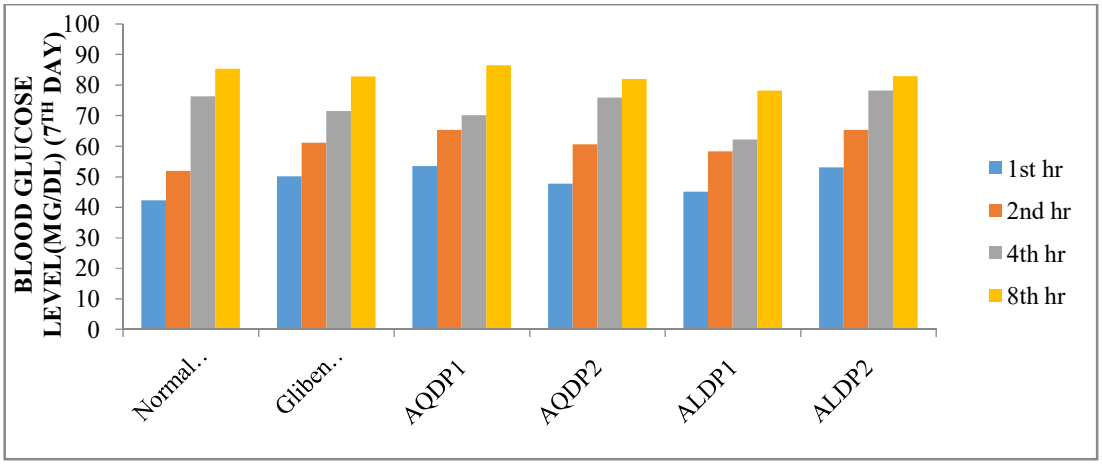

Figure: 2- Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats on 7th day.

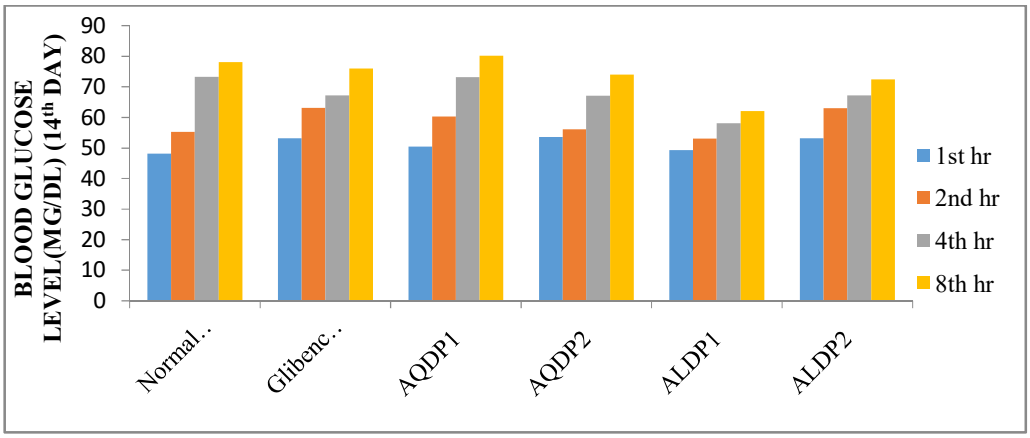

Figure No: 3- Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats on 14th day

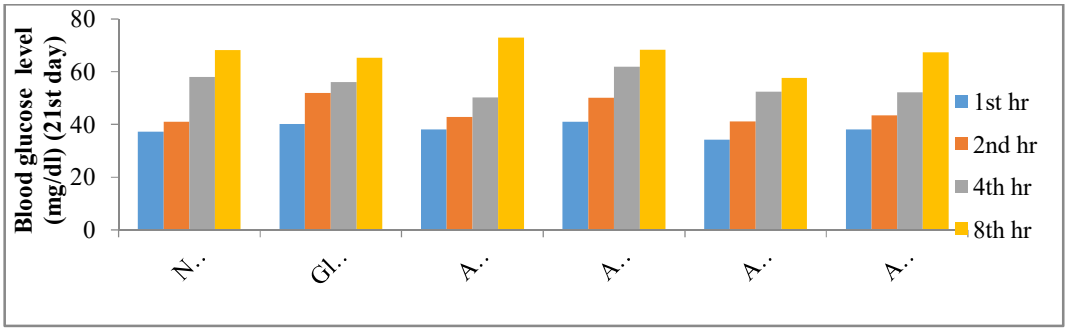

Figure: 4- Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in normal rats on 21st day

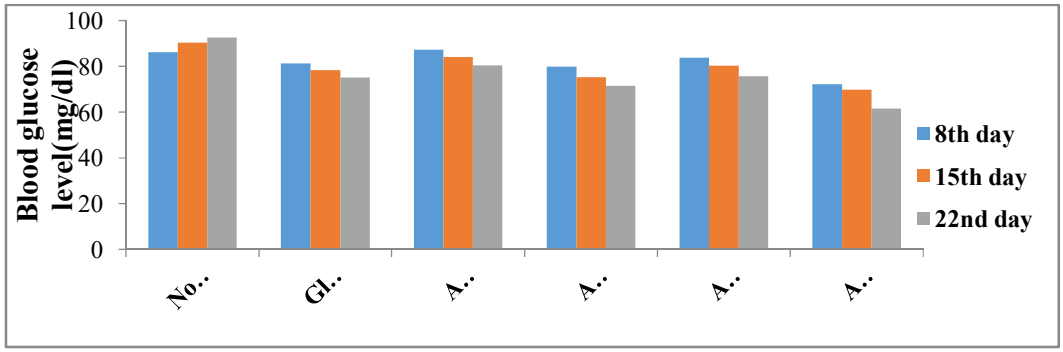

Figure: 5- Effect of extracts of *Diospyros peregrine* on 8th, 15th and 22nd day in normal rats.

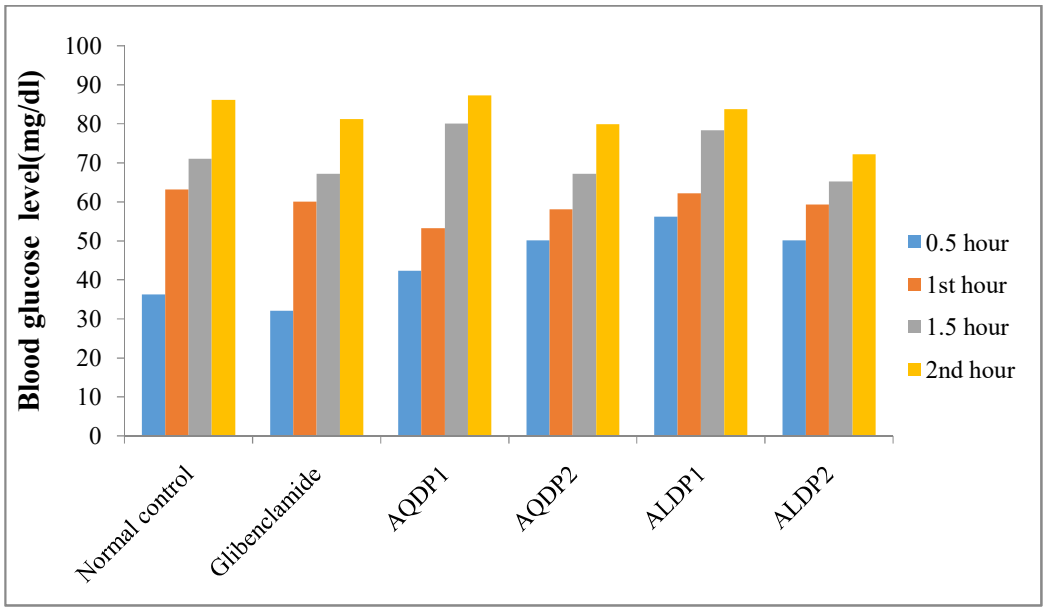

Figure: 6- Effect of extracts of *Diospyros peregrine* on 8th day in normal rats.

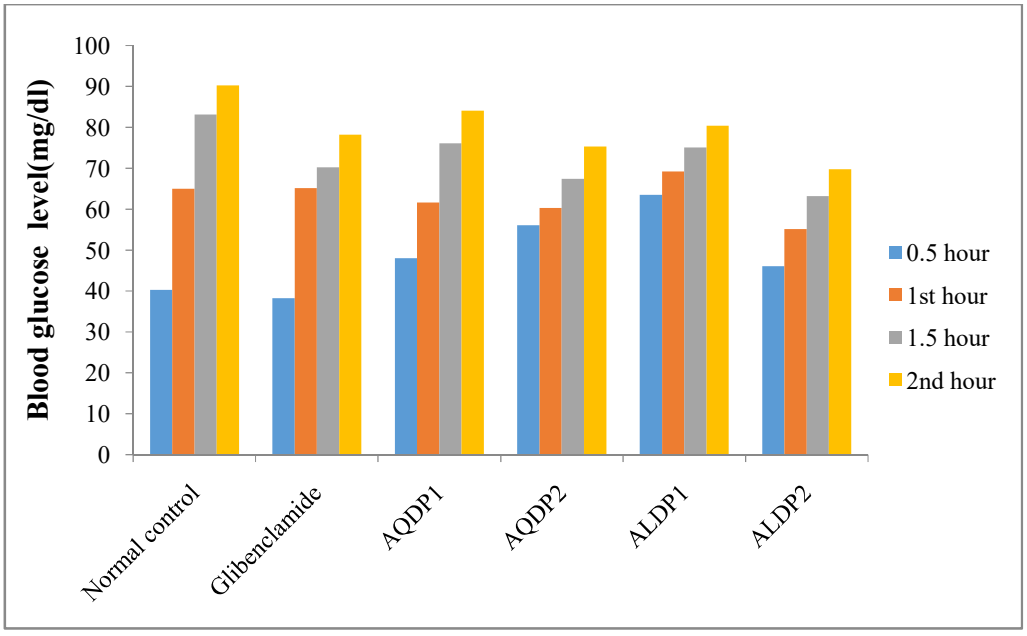

Figure 7- Effect of extracts of *Diospyros peregrine* on 15th day in normal rats

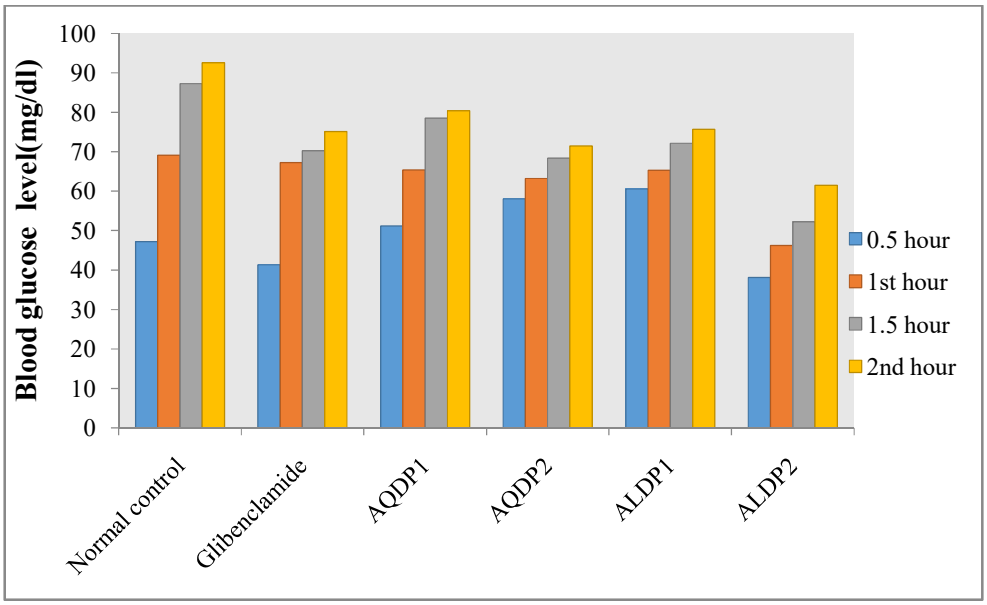

Figure 8:- Effect of extracts of *Diospyros peregrine* on 22nd day in normal rats

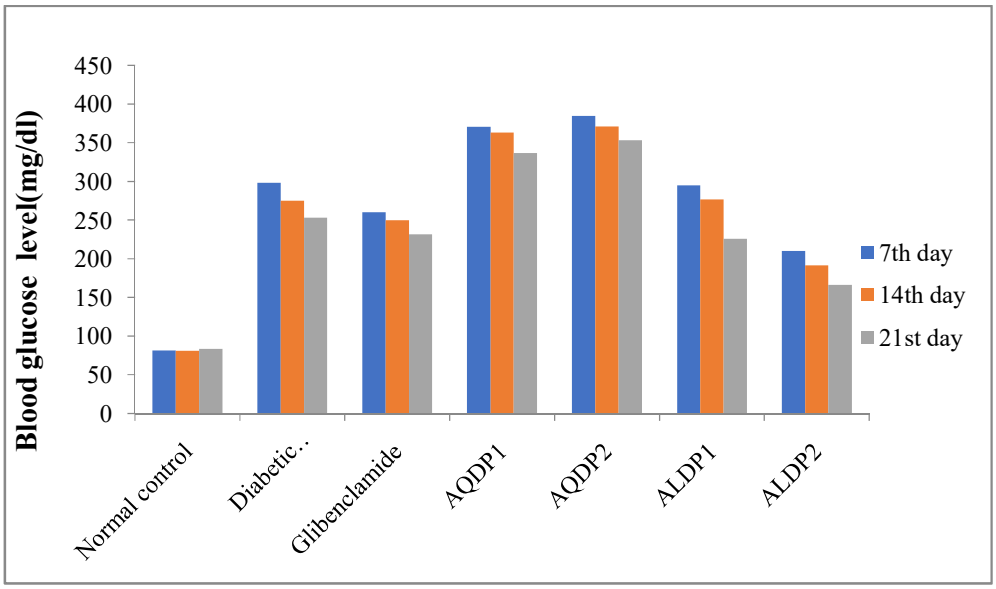

Figure 9: Effect of extracts of *Diospyros peregrine* on fasting blood glucose level (FBGL) in Alloxan induced diabetic rats.

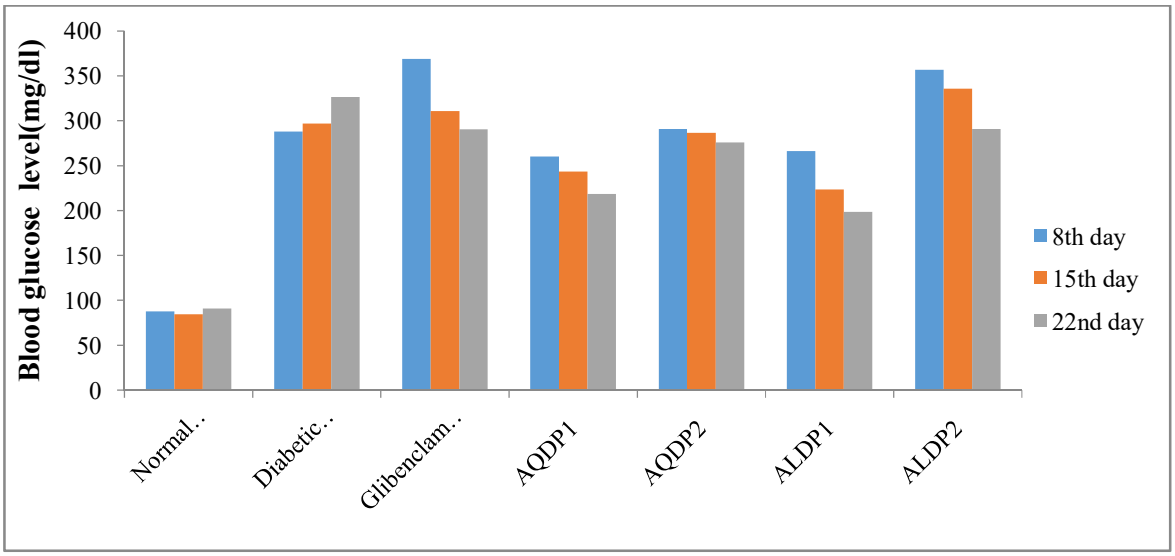

Figure: 10- Effect of extracts of *Diospyros peregrine* on 8th, 15th and 22nd day in Diabetic rats.

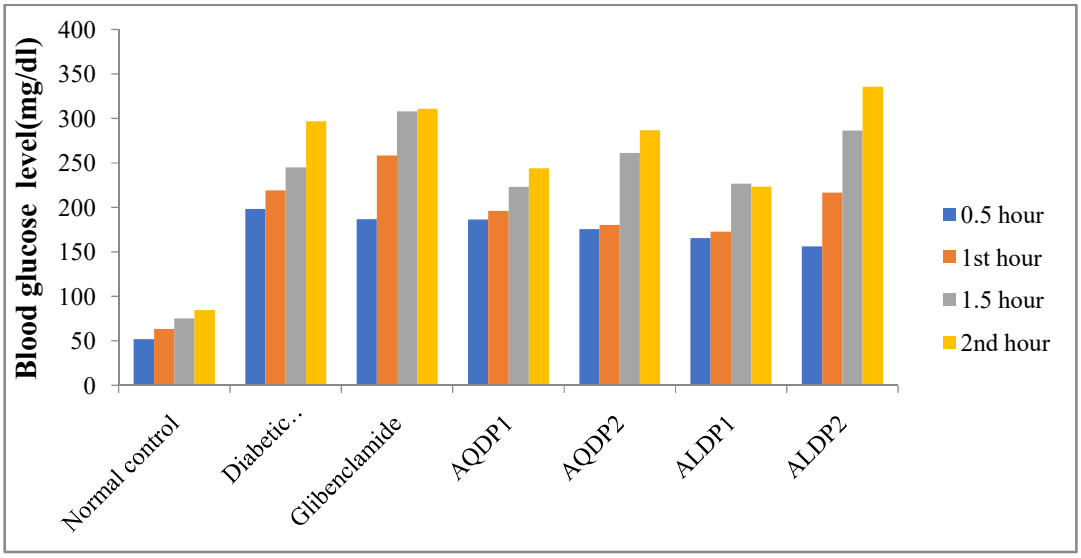

Figure: 11- Effect of extracts of *Diospyros peregrine* on 8th day in Diabetic rats

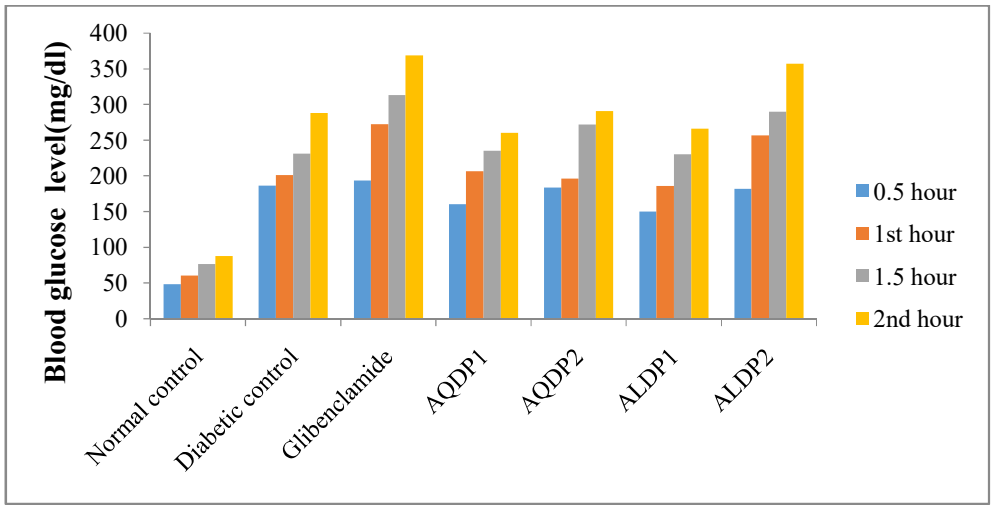

Figure: 12- Effect of extracts of *Diospyros peregrine* on 15th day in Diabetic rats.

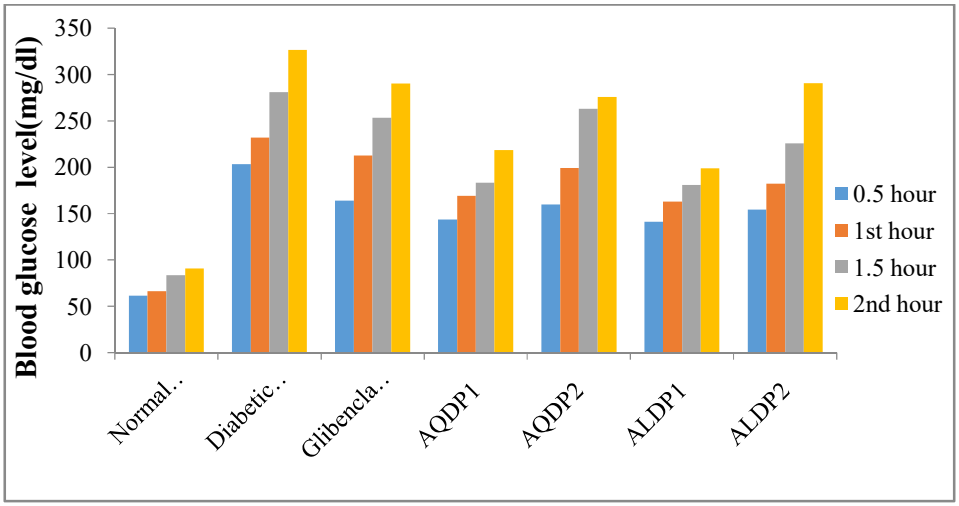

Figure: 13- Effect of extracts of *Dios*

DISCUSSION

Despite the fact that diabetes has high prevalence, morbidity and mortality globally, it is regarded as non curable but controllable disease. Different synthetic drugs, plant remedies and dietary modification play an effective role in the reduction of the suffering that it causes. The potential role of medicinal plants as antidiabetic agents has been reviewed by several authors. In order to identify the plants with antidiabetic properties various plants have been tested *in-vivo* using animal models, for example rats, against the complications caused by inducers of diabetes, and it has been established that many plants possesses the potential to lower the fasting blood glucose levels and besides help in improving other diabetic

complications. The sustained reduction in hyperglycemia automatically decreases the risk of other major complications of diabetes. Effective glucose control is the key for preventing or reversing the diabetic complications and improving the quality of life of the diabetics.

Many natural active compounds have been isolated from plants of different species. These active principles are complex Alkaloids, Tannins, Anthraquinones, Flavonoids, Saponins, Terpenoids, Sterols and others. These compounds have been shown to produce potent hypoglycemic, anti-hyperglycemic and glucose suppressive activities. These effects might be achieved by facilitating insulin release from pancreatic ß-cells, inhibiting glucose absorption in gut, stimulating glycogenesis in liver and/ or increasing glucose utilization by the body. These compounds may also exhibit Anthelmintic, Antibacterial, Antitumor and antioxidant activities, and restore enzymatic functions, repair and regeneration of pancreatic islets and the alleviation of liver and renal damage.

Crude aqueous and alcoholic extracts of leaves of *Diospyros peregrine*at a dose of 20 and 30mg/kg showed significant effect on the glucose tolerance of rats and it also showed reduction in the fasting blood glucose levels of the normoglycaemic rats, thus revealing the hypoglycaemic nature of the extracts. The effect was more pronounced for both extracts. These findings indicate that the extracts might be producing hypoglycaemic effect by a mechanism independent from the insulin secretion e.g. by the inhibition of endogenous glucose production or by the inhibition of intestinal glucose absorption.

Alloxan monohydrate is one of the chemical agents used to induce diabetes mellitus in animals. It induces diabetes by dose dependent destruction of β -cells of islets of langerhans. It is a generator of free radicals of oxygen which cause extensive DNA damage. It was observed that single intravenous dose of alloxan exhibited significant hyperglycemia. Excessive hepatic glycogenolysis and gluconeogenesis associated with decreased utilization of glucose by tissues is the fundamental mechanism underlying hyperglycemia in the diabetic state. As the hyperglycemia induced by alloxan falls under category of mild diabetes and may reverse after a few weeks, the hypoglycemic effect of the plant in hyperglycemic rats was studied during 22 days treatment. The difference observed between the initial and final fasting serum glucose levels of extract treated hyperglycemic rat's revealed antihyperglycemic effect of leaves of *Diospyros peregrine* throughout the period of study. The effect of the extracts was compared to that of reference standard, Glibenclamide and was found to be significant.

Phytochemical analysis of extracts of leaves of *Diospyros peregrine* revealed the presence of secondary metabolites that have been shown to possess antidiabetic effect in

other plants. Flavonoids, alkaloids and Steroids which were responsible for the antidiabetic effect in other plants were also detected in the extracts of this plant. The presence of phenols in the plant could also be responsible for the antidiabetic effect have been shown to prevent the destruction of β-cells by inhibiting the peroxidation chain reaction and thus they may provide protection against the development of diabetes. Extracts of leaves of *Diospyros peregrine* appear to be attractive materials for further studies leading to possible drug development for diabetes. Development of phytomedicines is relatively inexpensive and less time consuming; it is more suited to our economic conditions than allopathic drug development which is more expensive and spread over several years.

SUMMARY

1. The dried leaves of *Diospyros peregrine* for this project work were procured locally.
2. The dried leaves of *Diospyros peregrine* were successively extracted with water and alcohol.
3. Therapeutic dose of the extracts were calculated after carrying acute oral toxicity studies in rats.
4. Extracts were tested for their anti-diabetic activity in normal and alloxan induced diabetic rats.
5. The following parameters were assessed:
   - fasting blood glucose levels
     At 7^{th}, 14^{th} and 21^{st} day in normal and alloxan induced rats.
   - Oral Glucose Tolerance Test
     At 8^{th}, 15^{th} and 22^{nd} day in normal and alloxan induced rats.
6. Aqueous and Alcoholic extracts of *Diospyros peregrine* (20 and 30 mg/kg) showed significant effect in blood glucose lowering activity and improved oral glucose tolerance test (OGTT) in short term (7^{th} day) and long term (14^{th} and 21^{st} day) repeated administration in normal and alloxan induced diabetic rats.
7. The above studies showed that Aqueous and Alcoholic extracts of *Diospyros peregrine* had potent anti-diabetic activity on repeated administration.

CONCLUSION

The study was performed to find out the beneficial effects of two different extracts of leaves of *Diospyros peregrine* in normoglycaemic rats and alloxan induced diabetic rats and the results reveal that the plant has beneficial effects on blood glucose levels.

In current scenario, herbs are the potent sources of medicines used in the treatment of various disease and disorders. Since, plants are used as medicine there is prompt need of evaluation of plant species, therefore, the present work was conceived to evaluate the phytochemical and pharmacological screening of leaves of *Diospyros peregrine*. Alkaloids, Tannins, Anthraquinones, Flavonoids, Saponins, Terpenoids.

The aqueous and alcoholic extracts had hypoglycemic activity because the presence of flavonoids which are rich in treatment of hypoglycaemia with less side effects. Flavonoids might be producing hypoglycaemic effect by a mechanism independent from insulin secretion e.g. by the inhibition of endogenous glucose production or by the inhibition of intestinal glucose absorption. The present study *Diospyros peregrine* of both aqueous and alcoholic extracts was showed significant effect on glucose tolerance and also showed reduction in fasting blood glucose levels in normal diabetic rats.

The data of the blood glucose level of rats treated with Alloxan (150mg/kg body weight) produced diabetes within 72 hours. After 72 hours of Alloxan administered the blood glucose levels of rats were observed. It was observed that significant lowering of sugar in aqueous and alcoholic extract. The administration of different extracts at a dose of 20 and 30 mg/kg showed significant anti-hyperglycaemic effect at 22^{nd} day which was evident from the 7^{th} day on wards as compared to standard. The aqueous and alcoholic extract of *Diospyros peregrine* has showed better anti-hyperglycaemic effect of the extract on the fasting blood sugar levels on diabetic rats are shown in table. The decreasing blood glucose levels are comparable with that of 10 mg/kg of Glibenclamide. The Glibenclamide (10 mg/kg body weight) shows significant effect on compare to the initial and more significant effect on the 22^{nd} Day compare to the initial. The aqueous and alcoholic extracts of 20 and 30mg/kg body weight shows significant ($P^*<0.05$), effect.

Results of anti-diabetic activity in normal and alloxan induced rats the extracts established the scientific basis for the utility of these plants in the treatment of diabetes. The extracts have shown significant reduction in blood glucose levels in normal and alloxan induced diabetic rats and produced maximum anti-diabetic activity and are higher than the

hypoglycaemic activity of Glibenclamide in the diabetic rats. In glucose loaded animals, the drug has reduced the blood glucose to the normal levels. It is possible that the drug may be acting by potentiating the pancreatic secretion or increasing the glucose uptake. In conclusion, these extract showed significant anti-diabetic effect in normal and diabetic rats after administration. Thus the claim made by the traditional Indian systems of medicine regarding the use of these plants in the treatment of diabetes stands confirms.

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EVALUATION OF ANTIDIABETIC ACTIVITY OF *SWERTIA CHIRAYITA* AND *PANAX GINSENG*

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ABSTRACT

Diabetes mellitus, one of the most common endocrine disorders has caused significant morbidity and mortality due to macro vascular and micro vascular complications. Currently available therapies for diabetes include insulin and various oral anti diabetic drugs have number of serious adverse effect; therefore the search for more effective and safer hypoglycemic agents is one of the important areas of investigation. Some medicinal plants have been reported to be useful in diabetes worldwide. The herbs like *swertia chirayata* shown to protect the liver. It contains xanthones which is reputedly effective against Malaria, Tuberculosis. It also cures constipation and used for treating dyspepsia with all other properties the *swertia chirayita* shows good anti diabetic activity. The other herb which was used to carry out the experiment *panax ginseng* is well effective in case of anti-sterility in men, it prevents cancer and fight chemical dependency (anti proliferative). The study was conducted to examine the possible antidiabetic activity of *swertia chirayata and panax ginseng* leaf extraction on male wistar rats. Gold thio glucose method was used to induce diabetes in rats. Initially blood glucose levels were increased abruptly after induction. After giving the oral administration of ethanolic extract of *swertia chirayat* (100mg/ Kg, 200mg/kg) and *panax gingseng* (250mg/kg, 100mg/kg). Finding of this research showed that ethanolic extract of a plant *swertia* possess phytochemicals like steroids, alkaloids, tannins, flavonoids and *panax ginseng* possess alkaloids, carbohydrates, flavonoids and tannins significant ($P < 0.05$) anti diabetic activity. The results were compared with standard drug metformin (400mg/kg).

KEYWORDS: *Swertia chirayita*, *panax ginseng*, Antidiabetics.

INTRODUCTION

DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose concentration (hyperglycemia) caused by insulin deficiency often combined with insulin resistance (Rang and Dale, 2008). Diabetes mellitus refers to the group of diseases that leads to high blood glucose level due to defect in either insulin secretion or insulin action in the body (Rother, 2007).

Hyperglycemia occurs because of uncontrolled hepatic glucose output and reduced uptake of glucose by skeletal muscle with reduced glycogen synthesis. When the renal threshold for glucose reabsorption is exceeded, glucose spills over into the urine (glycosuria) and causes an osmotic diuresis (polyuria), which in turn results in dehydration, thirst and increased drinking of water (polydipsia).

The characteristic symptoms of diabetes mellitus are polyuria, polydipsia, polyphagia (increased hunger), blurred vision, these symptoms may be absent if the blood sugar is only mildly elevated.

IMPORTANT TYPES OF DIABETES MELLITUS

A. TYPE I DIABETES MELLITUS

Type I diabetes mellitus is characterized by loss of the insulin producing beta cells of the islets of Langerhans in the pancreas leading to insulin deficiency. Type I diabetes can be further classified as immune mediated or idiopathic. Type I diabetes is majorly of the immune mediated variety, where beta cell loss is a T-cell mediated auto immune attack (Rother, 2007). Type I diabetes is also called as juvenile diabetes (childhood) or insulin dependent diabetes mellitus (IDDM).

There is no preventive measure that can be taken against this type I diabetes. Diet and exercise cannot reverse or prevent type I diabetes. Sensitivity and responsiveness to insulin are usually normal especially in early stages.

B. TYPE II DIABETES MELLITUS

Type II diabetes mellitus is characterized differently and it is due to insulin resistance or reduced insulin sensitivity and it may be absolutely due to reduced insulin secretion in some of the cases. Insulin receptor sensitivity decreases on insulin receptors.

Type II diabetes is also called as adult onset diabetes mellitus, maturity onset diabetes mellitus or no insulin dependent diabetes mellitus (NIDDM) Type II diabetes mellitus is characterized by insulin resistance, impaired glucose induced insulin secretion and inappropriately regulated glucagon secretion which in combination eventually results in hyperglycaemia and in the longer term micro vascular and macro vascular diabetic complications. There are numerous theories as to the exact cause and mechanism in type II diabetes. Central obesity (fat concentrated around the waist in relation to abdominal organs, but not subcutaneous fat) is known to predispose individuals to insulin resistance. Abdominal fat is especially active hormonally, secreting a group of hormones called adipokines that may possibly impair glucose tolerance. Obesity was found to be the reason in approximately 55% of patients diagnosed with type II diabetes.

C. GESTATIONAL DIABETES MELLITUS

Gestational diabetes develops during pregnancy and it may persists or disappear after delivery. Gestational diabetes may damage the health of foetus or mother, and about 20%-50% of women with gestational diabetes develop type II diabetes later in life. Gestational diabetes mellitus (GDM) occurs in about 2%-5% of all pregnancies, including high birth weight (Macrosomia), foetal malformation and congenital heart disease. It requires careful medical supervision during the pregnancy (Lawurence *et al*., 2008).

CAUSES OF DIABETES MELLITUS: (Arlal Rosen bloom *et al*., 2003)

- Shortage or defective imperfect insulin produced by the body.
- Stress, fear and tension
- Hereditary either parent suffering from diabetes
- Frequent administration of steroids
- Insomnia
- Alcoholic habituation
- Addiction to sweets
- Swollen pancreas
- Smoking
- Malnutrition

COMPLICATIONS OF DIABETES MELLITUS

- Kidney (Diabetic nephropathy)
- Nerves (Diabetic neuropathy)
- Retina (Diabetic retinopathy)
- Testes (Infertility in males)
- Coronary thrombosis
- Cerebral thrombosis
- Hemorrhage

DIAGNOSIS OF DIABETES MELLITUS

The following tests performed when the patient complaint symptoms suggesting diabetes.

- Urine test for glucose and ketones
- Measurement of random blood glucose, plasma electrolytes
- Measurement of fasting blood glucose levels
- Glucose tolerance test(GTT)
- Glycosylated hemoglobin test (HbA1c)

Materials and source

Sodium citrate-Virat labs, Hyd, India
Diethyl ether-Finar chemicals limited, Ahmadabad.
Methanol -E-Merk, Mumbai, India.
Normal saline-Claris life sciences, Ahmadabad, India.
Formaldehyde- Finar chemicals limited, Ahmadabad, India
Chloroform- Molychem, Mumbai, India.
Gold thio glucose -Sigma, St Louis, U.S.A.
Metformin-MSN Formulations, HYD, India.

EQUIPMENTS USED

Centrifuge –Remiequipments Pvt, Ltd, Hyd, India.
Shimadzu electronic balance- Toshvin Analytical Pvt. Ltd, India
Shimadzu UV-spectrophotometer- Toshvin Analytical Pvt. Ltd, Mumbai.
Inverted microscope- Boeckl + co, Hamburg.

GOLD THIOGLUCOSE

Gold thio glucose is diabetogenic compound, which is induced hyperphagia and severe obesity induced Type -2 diabetes.

Chemical Properties

• It is derivative of sugar glucose.
• Gold thio glucose is precipitated with methanol and recrystallized with water and methanol.

Mechanism of Action

Gold thio glucose developed obesity induces diabetes in genetically normal mouse strains. Gold thio glucose treated DBA/2 (Dilute Brown Non- Agouti), C57BLKs, and BDF1 Rats gained weight rapidly and significantly increase non fasting plasma glucose level within 8-12 weeks. These Rats showed impaired insulin secretion, mainly in early phase after glucose load and reduced insulin content in pancreatic islets.

METHODS

Collection and authentification of Plant Material

The whole plant of swertia chirayita and Panax ginseng are collected and authenticated by Dr k madhava chetty, department of botany, Sri Venkateswara University, Tirupathy.

Extraction of Plant to a coarse powder with the help of suitable grinder.

Cold Extraction (Methanol Extraction)

In this work the cold extraction process was done with the help of methanol. About 200gms of powdered material was taken in a clean, flat bottomed glass container and soaked in 750 ml of methanol. The

container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

Evaporation of Solvent

The filtrates (methanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vacuum desiccator for 7 days.

PHYTOCHEMICAL SCREENING

GROUPSP	TEST PERFORMED	OBSEVATIONS
Alkaloids	Mayer's test	Yellow colored precipitate
Carbohydrates	Molisch's test	Violet ring at the junction
Saponin	Froth test	1 cm layer of foam
Steroids	Salkowaski's test	Golden yellow color
Phenols	Ferric chloride test	Bluish black color
Tanins	Gelatin test	White precipitate
Flavonoid	Leaad acetate test	Yellow color precipitate

Animals

Healthy Swiss Rats (Wistar strain) weight about 40-60 g were kept in individual polyethylene cages and maintained standard condition (12 h dark and 12 h light circle; 25 ± 5°C; 40-60% humidity), and the animals were fed ad libitum with normal laboratory chow standard pellet diet, purchased from the Sanzyme pvt. Limited, Hyderabad, India. The animals were allowed to acclimatize for 5 days before commencing the experiments. All the studies were conducted in accordance with the Animal Ethical Committee.

Acute toxicity studies

The Acute oral toxicity test of the extracts was determined prior to the experimentation on animals according to the OECD (Organization for Economic Co-operation and Development) guidelines no 423. Female Albino Swiss Rats (40-60 g) were taken for the study and dosed once with 2000 mg/kg (*swertia chirayita*) 5000mg/kg (*panax ginseng*). The treated animals were monitored for 14 days to observe general clinical signs and symptoms as well as mortality. No mortality was observed till the end of the study revealing the 2000 mg/kg (*swertia*) and 5000mg/kg (*panax)* dose to be safe. Thus, 1/10 and 1/20 doses of 2000 mg/kg i.e. 200 mg/kg and 100 mg/kg (*swertia*) and 500mg/kg and 250mg/kg (*panax)* were chosen for subsequent experimentation.

Experimental procedure of diabetes induction

After 1 week of acclimatization, Rats of each strain were divided into control or GTG groups. Because the expected rates of GTG-induced obesity ranged from 40 to 80% depending on the mouse strain, a large number of Rats (12–20 Rats) were assigned to GTG groups with the aim of obtaining six to eight obese Rats per group. The Rats received an intra peritoneal administration of saline or GTG (Cat. No. 1045508; USP, Rockville, MD) at the following optimal doses: 0.8 g/kg for Rats groups, respectively. After 4 or 6 weeks, Rats that developed obesity (GTG-obese Rats) were selected for further studies on showing a greater weight gain compared with the average weight gain measured in the control Rats.

The development of obesity-induced diabetes was investigated in control and GTG-obese Rats of each strain (n = 6–8 per group) for 12–14 weeks. Body weights were measured every 2 or 4 weeks and the development of diabetes was monitored by measuring non fasting plasma glucose levels between 9:00 and 10:30 am.

Experimental Study Design of *swertia chirayita* for Diabetic screening

Diabetic rats were divided in to five groups with each group four animals.

- Group-I: Rats served as normal control group.
- Group-II: served as diabetic/disease control.
- Group-III: Diabetic rats treated with *swertia chirayita* at a dose 100 mg/kg.
- Group-IV: Diabetic rats treated with *swertia chirayita* at a dose of 200 mg/kg
- Group V: Diabetic rats treated with Metformin (standard drug) at 450mg/kg.

The treatment was given for 14days and blood samples were collected at different intervals.

Experimental Study Design of *Panax ginseng* for Diabetic screening

Diabetic rats were divided in to five groups with each group four animals.

- Group-I: Rats served as normal control group.
- Group-II: served as diabetic/disease control.
- Group-III: Diabetic rats treated with *Panax ginseng* at a dose 250 mg/kg.
- Group-IV: Diabetic rats treated with *Panax ginseng* at a dose of 500 mg/kg
- Group V: Diabetic rats treated with Metformin (standard drug) at 450mg/kg.

The treatment was given for 14days and blood samples were collected at different intervals.

Experimental Study Design of *swertia chirayat* and *Panax ginseng* for Diabetic screening

- Diabetic rats were divided in to five groups with each group four animals.
- Group-I: Rats served as normal control group.
- Group-II: served as diabetic/disease control.
- Group-III: Diabetic rats treated with *ME-SCPG* at a dose 250 mg/kg.
- Group-IV: Diabetic rats treated with *ME-SCPG* at a dose of 500 mg/kg
- Group V: Diabetic rats treated with Metformin (standard drug) at 450mg/kg

Collection of blood samples

Blood samples were collected from all the groups of animals at 0, 7, 15th day intervals.

EVALUTION PARAMETER

Glucose Method[23]: GOD/POD method

Principle

D-glucose + H_2O + O_2 glucose oxidase ⟶ (GOD) gluconic acid + H_2O_2

H_2O_2 + 4-AAP + Phenol peroxidase ⟶ (POD) Quinoneimine dye + H_2O

RESULTS

Swertia chirayita

%Yield value of Methanolic Extract from *swertia chirayita* was found to be **12.27%.**

Preliminary Phytochemical Screening

Investigation revealed the presence of steroid, Alkaloid, Tannins & Flavonoid in Methanolic Extract of *swertia chirayita.*

Table: Phytochemical screening.

Phytochemical	Results
Steroid	+
Alkaloid	+
Tannin	+
Carbohydrate	-
Phenol	-
Flavonoid	+
Saponin	-

(+) Present **(-)** Absent

Procedure

- Wavelength/filter : 505 nm (Hg 546 nm) / Green
- Temperature : 37^0 C / R.T.
- Light path : 1 cm
- Pipette into clean dry test tubes labeled as Blank (B), Standard (S) and Test (T)

Mix well and incubate at 37^{0}C for 10 min or at room temperature (25^{0}C) for 30 mins. Measure absorbances of the Standard (Abs.S) and Test Sample (Abs.T) compare these against the Blank within 60 mins.

Additional sequence	B (ml)	S (ml)	T (ml)
Glucose reagent L1	1.0	1.0	1.0
Distilled water	0.01	-	-
Glucose standard (s)	-	0.01	-
Sample	-	-	0.01

STATISTICAL ANALYSIS

All the values will be expressed as mean ±standard deviation (S.D). Statistical comparisons between different groups will be done by using one way analysis of variance. P value <0.05 will be considered as statistically significant.

Table: Effect of *swertia chirayita* extract on serum glucose levels (mg/dl) in diabetic rat.

Groups/Interval	0th Day	7th Day	15th Day
Normal	83.3±4.23	79.1±5.36	77.7±5.62
Diabetic control	283.8±5.01	286.4±12.4	300.3±8.64
MESC(100mg/kg)	293.1±9.83	152.9±6.91**	110.1±17.1**
MESC(200mg/kg)	280.5±42.4	85.5±7.20***	64.7±20.7***
Metformin(450mg/kg)	211.0±34.7	79.7±10.2***	68.3±2.4**

MESC-METHANOLIC EXTRACT OF *SWERTIA CHIRAYITA*

*All the values of mean ± SD; n=6; ** indicates p<0.01, *** indicates [a]p<0.001 vs diabetic control.*

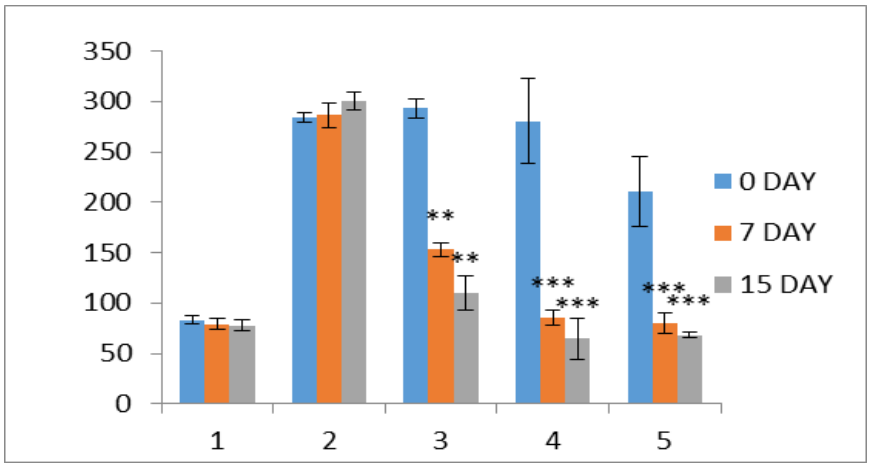

Fig. 3: Effect of *swertia chirayita* methanolic extract on serum glucose levels (mg/dl) in diabetic rats.
*All the values of mean ± SD; n=6; ** indicates p<0.01, *** indicates [a]p<0.001 vs. diabetic control.*

Panax ginseng

%Yield of Ethanolic Extract from Aerial Parts of *panax ginseng* was found to be **12.43%.**

Table: 7 Preliminary Phytochemical Screening
Glucose

Phytochemical	Results
Steroid	-
Alkaloid	+++
Tannin	+
Carbohydrate	++
Phenol	-
Flavonoid	+
Saponin	-

(+) Present. **(-)** Absent

Table: Effect of *Panax ginseng* (EPG) on serum glucose levels (mg/dl) in diabetic rats.

Groups/Interval	0th Day	7th Day	15th Day
Normal	65.48±0.35	67.89±0.18	69.96±0.78
Diabetic control	185.65±0.64	188.64±0.65	189.67±0.95
EEPG (250mg/kg)	164.48±0.48	159.74±0.74	145.86±0.64
EEPG (500mg/kg)	142.65±0.46	138.65±0.71	135.75±0.48
Metformin (450mg/kg)	120.34±0.75	115.75±0.48	111.34±0.69

All the values of mean ± SD; n=3.

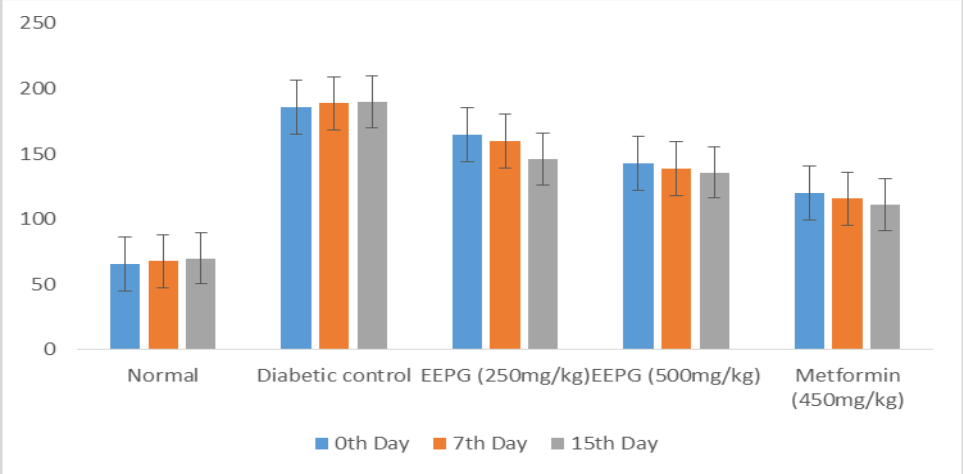

FIG: Effect of *Panax ginseng (EEPG)* on serum glucose levels (mg/dl) in diabetic rats.

MIXED EXTRACTS OF *SWERTIA CHIRAYITA* AND *PANAX GINSENG*

Table: Effect of *mixed* extract on serum glucose levels (mg/dl) in diabetic rats.

Groups/Interval	0th Day	7th Day	15th Day
Normal	81.3±4.1	72.0±5.6	74.7±7.9
Diabetic control	269.0±12.6	277.5±12.7	306.0±10.2
ME-SCPG (250mg/kg)	260.1±14.1	152.2±15.2**	110.0±11.2**
ME-SCPG (500mg/kg)	270.7±14.2	97.8±11.1***	72.6±4.1***
Metformin(450mg/kg)	260.1±16.8	90.2±9.5***	71.2±7.1***

*All the values of mean±SD; n=6; ** indicates p<0.01, *** indicates [a]p<0.001 vs diabetic control.*

ME-SCPG=MIXED EXTRACTS OF *SWERTIA CHIRAYITA* AND *PANAX GINSENG*

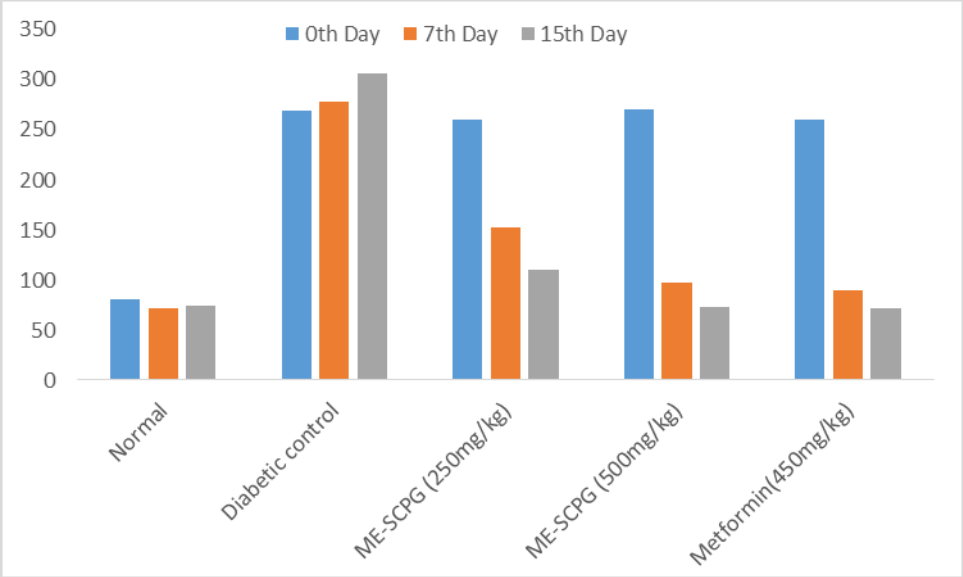

FIG:. Effect of *mixed* extract on serum glucose levels (mg/dl) in diabetic rats.

DISCUSSION

This study aimed to evaluate the synergestic antidiabetic activity of *sertia chirayata* and *panax ginseng.* Leaves were used to prepare the extract of *swertia chirayata* and *panax ginseng*. The physical appearance of the extract was dark greenish in color, non-sticky in nature and odourless.

Phytochemical screening of methanolic extract revealed the presence of phytochemicals in *swertia chirayata* such as steroids, alkaloids, tannins and flavonoids, and in *panax ginseng* alkaloids, carbohydrates, flavonoids and tannins.

Gold thioglucose method (GTG) was used to induce diabetes in rats. The rats received an intraperitonial administration of GTG at the optimal dose of 0.8gm/kg. After 2 weeks rats develop diabetes. Gold thioglucose causes hypertrophy of pancreatic islets associated with degranulation of β-cells. It causes hyperinsulinemia, hyperglycemia, glucosuria and depressed insulin sensitivity.

Therefore the development of diabetes was monitored by measuring non fasting plasma glucose levels between 9-10:30 am. Which was defined as diabetes, because non fasting plasma glucose levels values were higher than 300mg/dl.

The extract of the plants *swertia chirayata* and *panax ginseng* was administered orally at the dose of 100, 200 mg/kg (*swertia chiarayata)*, 250, 500 mg/kg (*panax ginseng*) and mixed extract the dose of 500mg/kg for 14 days.

In disease treated animals diabetes was reduced by α-amylase inhibition, decrease in cholesterol, triglycerides and blood glucose levels in *swertia chirayata*. And in *panax ginseng* it worked by protecting pancreatic islets and inhibition of β-cells. It also modulates the production or secretion of insulin, glucose metabolism and uptake and obesity reduction.

Gold thioglucose induced diabetes treated at the dose of 200mg/kg when compared to 100mg/kg of *swertia chirayata* shows more anti diabetic activity and in case of *panax ginseng* the higher dose 500mg/kg shows more antidiabetic activity than the lower dose 250 mg/kg.

Mixed extract of *swertia chirayata* and *panax ginseng* showed synergistic effect at the higher dose 500mg/kg when compared to lower dose 250mg/kg with the reference drug metformin 450mg/kg.

CONCLUSION

- *Swertia chirayita* have many medicinal properties useful to cure anorexia, ulcers etc. *swertia chirayita*

have different medicinal properties due to its active phytochemical constituents and able to treat diabetes & diabetics complications.

- In the literature review the acute toxicity studies (OECD guidelines no. 423) shows methanolic extract of *swertia chirayita* is safe to use up to the dose of 2000mg/kg.
- The methanolic extract of *swertia chirayita* was found to be in dose dependent way against GTG induced diabetes in rats. The reduction of the elevated blood glucose levels in diabetic rats on treatment with the extract at two different concentrations confirmed that methanolic extract of *swertia chirayita* possess antidiabetic activity & has shown significant effect when compared to GTG administration.
- The acute toxicity studies revealed that the ethanolic extracts were safe for oral administration is safe upto 5000mg/kg. and the results for anti diabetic activity of *Panax ginseng* against GTG induction is found to be dose dependent and gave positive results.
- It needs comprehensive investigations for developing a safe and effective herbal drug. Further research is required to isolate the biomolecules responsible for the antidiabetic and antidiabetic complications.

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EVALUATION OF ANTI INFLAMMATORY AND ANALGESIC ACTIVITIES OF THE EXTRACT PREPARED FROM *ALOYSIA POLYSTACHYA* IN EXPERIMENTAL ANIM ALS

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ABSTRACT

Aloysia polystachya used as an appetite suppressant herb for millennia. It also has antioxidant, ant diabetic, and nootropic actions. It is proved that it is a natural anti obesogenic agent and is widely consumed in India. Its actions like anti-atherosclerotic is of high medicinal value. The phytochemical screening of extract shows the presence of alkaloids, phytosterols, phenolic compunds and tannins using various methods. In the present work an attempt has been made to evaluate the anti inflammatory, analgesic activities of ethanolic extract of *aloysia polystachya* (100mg/kg, 200mg/kg) and the results were found to be positive. The results were compared with the standard drug indomethacin (10mg/kg), pentazocin (10mg/kg) and aspirin (10mg/kg). Hence, *aloysia polystachya* contains anti inflammatory and analgesic activity. The present work was done to demonstrate the anti inflammatory and analgesic activity of the ethnolic extract obtained from the leaves of *aloysia polystachya* (verbenaceae). Inflammation was induced by carrageenan induced paw edema and pain was induced by eddy's hot plate and tail flick method. Thermal and radiant heat is used in hot plate and tail flick method respectively.

KEYWORDS: *Aloysia polystachya*, analgesic, anti inflammatory activity.

1. INTRODUCTION

Pain is the most common reason for physician consultation. It is a major symptom in many medical conditions. It can significantly interfere with a person's quality of life and general functioning.[1] It is a part of the body's defence system, producing are flexiveretraction from the painful stimulus, and tendencies to protect the affected body part while it heals, and avoid that harmful situation in the future.[2,3] Pain is the most common reason for using complementary and alternative medicine.[4,5] Pain is primarily managed with analgesics. Opioid analgesics are commonly used for treatment of pain. Although opioids are strong analgesics, there are other drugs used for the treatment of pain.

Inflammation is the body's immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury.[6] It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Inflammation can be classified as either acute or chronic status depending on onset time. Acute inflammation is the primary response of the body to injurious stimuli and it involves the local vascular and immune response. On the other hand, chronic inflammation is a pathological condition characterized by progressive destruction and recovery of the injured tissue from the inflammatory response.[7]

Though a variety of chemical mediators or signalling molecules such as histamine, serotonin, leukotrienes, prostaglandins are involved in the inflammatory response the mechanism of inflammation injury is attributed to release of ROS (reactive oxygen species) from activated neutrophils and macrophages. The over production of ROS by macrophages causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins.[8] In addition, ROS propagate inflammation by stimulating release of cytokines such as interleukin-1, tumor necrosis factor and interferon which stimulate recruitment of additional neutrophils and macrophages. Further ROS activates Nuclear Factor ƙ-β(NF-ƙ-β) which regulates various cellular genes involved in immune and acute phase inflammatory responses and in cell survival. Thus free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation.

2. MATERIAL AND METHODS

2.1 Material

Drugs and chemicals

1. Pentazocine inj. (Ranbaxy laboratories limited).

2. Indomethacin capsules (Ranbaxy laboratories limited).
3. Aspirin (Ranbaxy laboratories limited).

All Other chemicals used for this investigation were of analytical grade from S.D Fine chemicals, Mumbai, India.

Plant sample collection
The whole plant (leaves) of *aloysia polystachya* will be collected and will be authenticated by dr k madhava chetty, department of botany, Sri Venkateswara University, tirupathy.

Extraction of Plant Material
The plant leaves are sun dried for 2 weeks then grinded in to a coarse powder with the help of a mixer (remi motor grinder-rm-200).

2.2 Hot Continuous Extraction (Soxhlet)
In this method, the finely ground crude drug is placed in a porous bag or "thimble" made of strong filter paper, which is placed in chamber E of the soxhlet apparatus. The extracting solvent in flask A is heated, and its vapours condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A.

This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated.

The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

Evaporation of Solvent
The filtrates (ethanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vaccum dissecator for 7 days.

2.3 ACUTE TOXICITY STUDIES:
The acute oral toxicity test of the extract was determined prior to the experimentation on animals according to the OECD (Organisation for Economic Co-operation and Development) guidelines no. 423. Albino mice (25-30g) were taken for the study and dosed once with 2000 mg/kg. The treated animals were monitored for 14 days to observe general clinical signs and symptoms as well as mortality. No mortality was observed till the end of the study revealing LD50 dose as 2000 mg/kg. Therefore therapeutic dose was calculated as $1/10^{th}$ and $1/20^{th}$ie; 100 mg/kg and 200 mg/kg of the dose were chosen for subsequent experimentation.

2.4 PHYTOCHEMICAL SCREENING
The extract where subjected to preliminary phytochemical screening for possible presence of bioactive anti inflammatory and analgesic compounds.

Table1: Phytochemical screening method.

Test	EEAP
Alkaloids	+
Carbohydrates and Glycosides	-
phytosterols	+
Fixed oils and fats	-
Phenolic componds and tannins	+
Proteins and amino acids	-

2.5 Experimental Animals and Housing of Animals
Albino mice weighing 25-30 g of either sex were used for the study in different models. The animals were procured from National institute of Nutrition (Hyderabad) at least 2 weeks prior to the study, so that animals could acclimatize to the new environment.

Animals were kept in well-maintained room under standard hygienic conditions. Commercial pellet diet and water were made available *ad libitum*. They were housed in propylene cages (32 x 24 x 16 cm) with stainless steel grill top, bedded with rice husk.

2.6 Screenings of anti-inflammatory activity
Carageenan induced hind paw edema in mice: Albino mice weighing between 25-30gms were divided into 5 groups of 6 mice each; three animals being housed in labeled cage each. Animals were given a period of time to adjust to the new environment provided with food & water ad libitum.

Grouping
Group 1: Animals were administered 0.1ml saline p.o.
Group 2: Animals served as disease control (carrageenan induced).
Group 3: Animals were administered standard (Indomethacin 10 mg/kg) p.o.
Group 4: Animals were administered *Aloysia polystachya*(100 mg/kg) p.o.
Group 5: Animals were administered *Aloysia polystachya*(100 mg/kg dose) p.o.

Procedure: All mice of II, III, IV & V (except I group) groups were injected with 0.1ml of carageenan (1%) in normal saline into sub planter area of right hind paw. All the drugs were given orally 1hr prior to carageenan injection.

Paw volume was measured by mercury plethysmograph at 0, 1, 2, 3, 6, hrs after the carageenan injection.

2.7 Screening of analgesic activity

A. Eddy's hot plate method

Albino mice weighing between 25-30gms were divided into 4 groups of 6 mice each; three animals being housed in labeled cage each. Animals were given a period of time to adjust to the new environment provided with food & water ad libitum.

Grouping

Group 1: Animals were administered 0.1ml saline p.o.

Group 2: Animals were administered standard reference Pentazocine (10 mg/kg)i.p.

Group 3: Animals were administered *Aloysia polystachya*(100 mg/kg) p.o.

Group 4: Animals were administered *Aloysia polystachya*(200 mg/kg) p.o.

Procedure: The time for licking paws or jumping in hot plate was recorded as response, prior and 0, 30, 60, 90,120 min after administration of respective drugs.

B.Tail flick method

Grouping

Group 1: Animals were administered 0.1ml saline p.o.

Group 2: Animals were administered standard reference aspirin (10 mg/kg)i.p.

Group 3: Animals were administered *Aloysia polystachya*(100 mg/kg) p.o.

Group 4: Animals were administered *Aloysia polystachya*(200 mg/kg) p.o.

Procedure

The tail flick latency was assessed by analgesiometer (INCO, INDIA).The strength of the current passing through the naked nichrome wire was kept constant at 6 amperes. The distance between the heat source and tail skin was 1.5 cm. The site of application of the radiant heat in the tail was maintained at 2.5c.m. measured from the root of tail. The cutoff reaction time was fixed at 10 seconds to avoid tissue damage.

3. RESULTS AND DISCUSSION

3.1 STATISTICAL ANALYSIS

Results were expressed as mean±S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA). If the overall *P*-value was found statistically significant ($P < 0.05$).

3.2 Carageenan induced paw edema in mice

In carageenan induced paw edema *aloysia polystachya* significantly inhibited the edema in a dose dependent manner as shown in Table.3. The paw volume in normal control group rats on 2ndhr was found to be 0.2148 ± 0.0122ml. The paw volume in mice pretreated with lower dose of *aloysia polystachya* (100 mg/kg/day), higher dose of *aloysia polystachya* (200 mg/kg/day) and indomethacin (10 mg/kg/day) at 2ndhr were found to be 0.191 ± 0.0061 ml, 0.158 ± 0.0042** ml and 0.1369 ± 0.0054** ml.

Table 2: Anti-inflammatory effect of *Aloysia polystachya* on carageenan induced paw edema in rats.

Treatment	Paw volume in ml at different Hrs (Mean + S.E.M.)				
	0	1	2	3	6
Normal Control	0.101± 0.0058	0.101± 0.0058	0.101± 0.0058	0.101± 0.0058	0.101± 0.0058
Inflammatory control	0.1225 ± 0.0079^{+++}	0.1876 ± 0.007^{+++}	0.2148 ± 0.0122^{+++}	0.2083 ± 0.0094^{+++}	0.165 ± 0.0076^{+++}
Indomethacin 10mg/kg, p.o.	0.1249 ± 0.0061	0.1427 ± 0.0071**	0.1369 ± 0.0054**	0.1442 ± 0.007**	0.1449 ± 0.0060
Aloysia polystachya (100mg/kg)	0.1210 ± 0.0186	0.152 ± 0.008	0.191 ± 0.0061	0.196 ± 0.006	0.159 ± 0.009*
Aloysia polystachya (200mg/kg)	0.1016 ± 0.0070	0.132± 0.0057**	0.158 ± 0.0042**	0.1542 ± 0.0071**	0.1542 ± 0.0136

Values are expressed as (Mean±S.E.M) n=6; One way ANOVA followed by Dunnet's test.

+++ P<0.001 Vs Normal control &** P< 0.01 Vs Inflammatory Control.

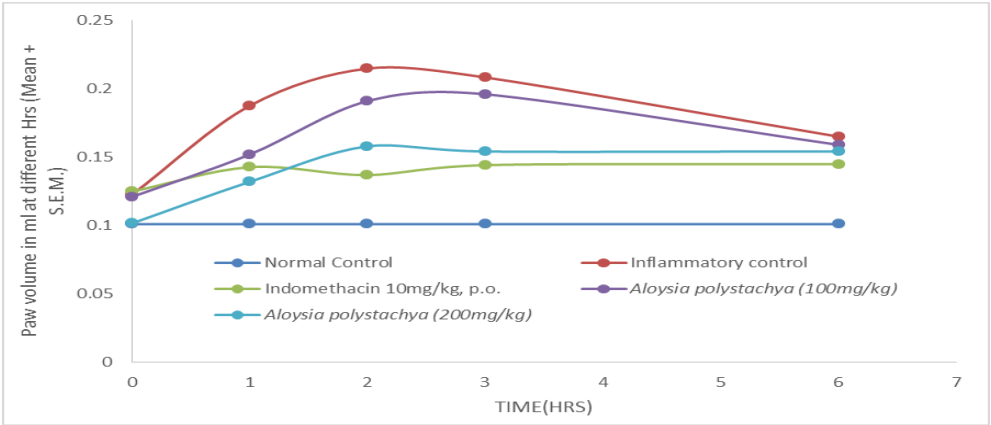

Figure 1: Anti-inflammatory effect of *Aloysiapolystachya* on carrageenan induced paw edema in mice.

3.3 Analgesic activity

Eddy's hot plate

Aloysia polystachya showed maximum analgesic activity at 60, 90 min for 100 and 200mg/kg dose. The reaction time in normal control group at 60, 90 min was found to be 3.52±0.002, 4.08±0.161 sec. The reaction time (paw licking / jumping response) in mice pretreated with lower dose of *Aloysia polystachya* (100mg/kg), higher dose of *Aloysia polystachya* (200mg/kg/day) and Pentazocine(10 mg/kg) at 60, 90 min were found to be 9.26±0.851, 7.16 ± 0.193, 9.82 ± 0.894 and 8.60 ± 0.992, 9.12 ±0.372, 14.12±3.182 respectively when compared to control group mice.

The duration of analgesic effect was more in 200 mg/kg compared to 100 mg/kg and reference drug pentazocine at 10 mg/kg dose significantly increased the reaction time at 90 minutes as shown in Table 2.

Table 3: Effect of *Aloysia polystachya* on reaction time (sec) in Eddy's hot plate.

Treatment	Reaction time in seconds				
	0	30	60	90	120
Control	3.51 ± 0.277	3.80 ± 0.343	3.52 ± 0.455	4.08 ± 0.161	3.93± 0.067
Pentazocine (10mg/kg)	4.11 ± 0.238	6.64 ± 0.430**	9.82 ± 0.894**	14.12 ± 3.182**	9.41± 0.650**
Aloysia polystachya (100mg/kg)	4.02 ± 0.194	5.01 ± 0.332	9.26 ± 0.851**	8.60 ± 0.992	6.30± 0.259**
Aloysia polystachya (200mg/kg)	3.81 ± 0.230	7.09 ± 0.523**	7.16 ± 0.193**	9.12 ± 0.372	8.21± 0.671**

Values are expressed as (Mean±S.E.M) n=6; One way ANOVA followed by Dunnet's test.
** P< 0.001 Vs control, * P< 0.05 Vs control.

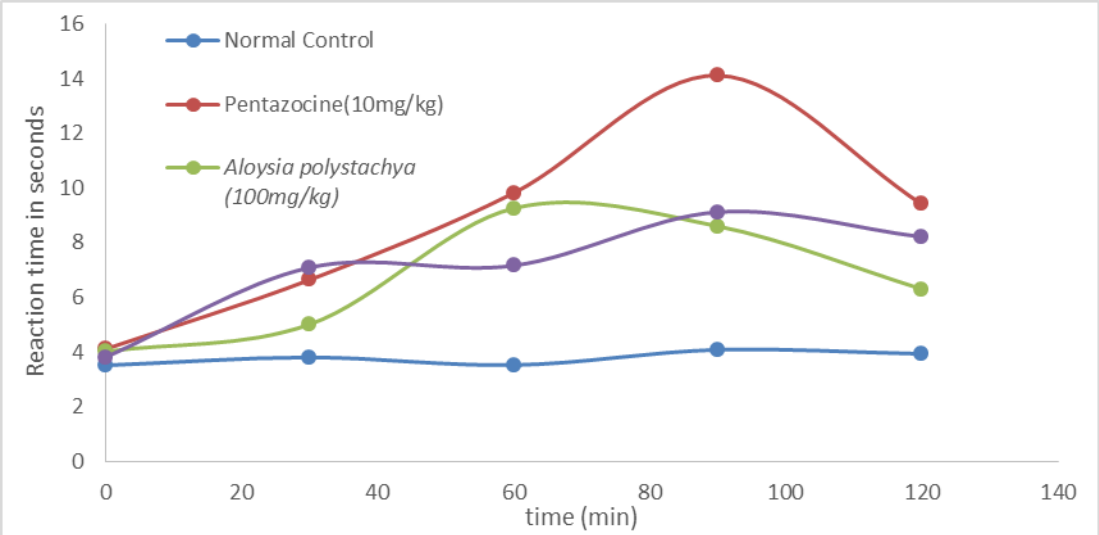

Figure 2: Effect of*Aloysia polystachya* on reaction time (sec) in Eddy's hot plate method.

Tail flick method

Table 3: Mean TFL of Tail flick method at Various Time Interval.

Treatment	DOSE Mg/kg	MEANBASAL TIME (Seconds)	MEAN RESPONSE OF TAIL FLICK METHOD AT VARIOUS TIME INTERVAL(in minutes)					
			30	60	90	120	180	240
Distilled water	0.5ml	4.19±0.029	3.76±0.041	4.68±0.046	4.77±0.041	4.77±0.035	4.23±0.047	4.07±0.037
Asprin	10	4.29±0.045	7.88±0.042	8.41±0.042	9.47±0.046	9.47±0.036	10.68±0.037	9.94±0.041
Aloysia polystachya (100mg/kg)	100	4.52±0.057	4.41±0.010	4.52±0.034	5.32±0.013	5.77±0.020	5.52±0.022	5.38±0.041
Aloysia polystachya (200mg/kg)	200	4.21±0.016	4.79±0.016	4.89±0.005	5.48±0.025	6.82±0.012	6.16±0.010	0.07±0.035

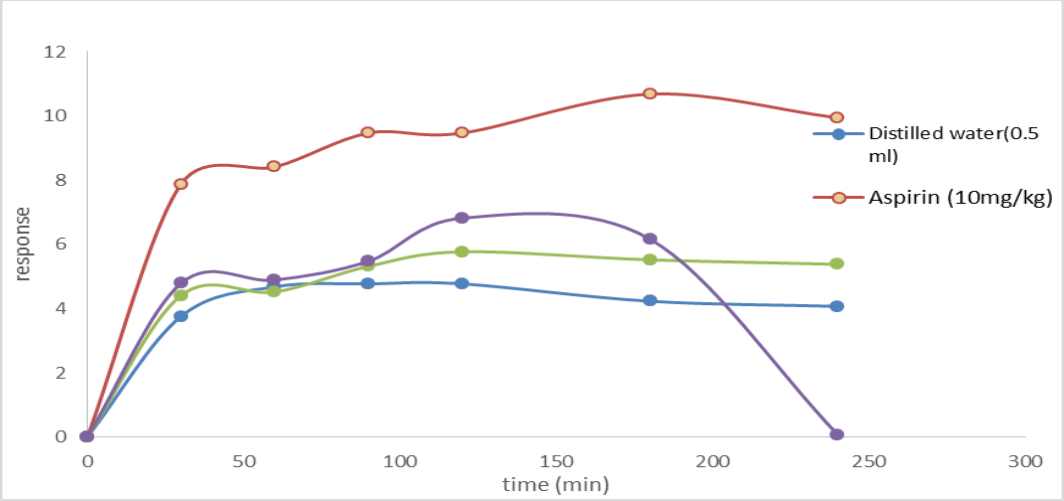

Figure 3: Mean TFL of Tail flick method at Various Time Interval.

3.4 DISCUSSIONS

The present study was done about anti inflammatory and analgesic activity of ethanolic extract of *aloysia polystachya*. Inflammation was induced by carragenan induced paw edema and pain was induced by eddy's hot plate and tail flick method.

The physical appearance of the ethanolic extract of *aloysia polystachya* was greenish black with semisolid and non sticky in nature.

The phytochemical investigation revealed the presence of significant phytoconstituents in the extract such asalkaloids, phytosterols, phenolic compunds and tannins for ethanolic extract of *aloysia polystachya*.

Carragenan paw edema method was used to determine anti inflammatory activity of the extract by using 0.1 ml of 1 % w/v carrageenan was injected into the sub plantar tissue of right hind paw of each mice.

Carragenan inuced paw edema is a commonly used primary test for the screening of new anti inflammatory agents and is believed to be biphasic. The first phase (1-2h) is due to the release of histamine or serotonin,bradykinin, and to a less extent prostaglandins produced by cyclooxygenase enzymes (COX) and the second phase of edema is due to the release of prostaglandins.[9] Release of the neutrophil-derived free radicals, nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), and interleukin-1 β (IL-1 β) also involved in the delayed phase of carrageenan-induced acute inflammation.[10]

The results of this study indicate that the extract of *aloysia polystachya* (100mg/kg,200mg/kg) significantly reduces carragenan induced paw edema in mice. Therefore, the mechanism of action by inhibition of histamine, serotonin or prostaglandins synthesis.The results were compared with the standard drug indomethacin (10mg/kg).

This study also involves the investigation of the anti nociceptive property of the extract of *aloysia polystachya* by thermal models of nociception- eddys hot plate method and tail flick method. These models are based on polysynaptic reflexes intiated at the spinal level and modulated from supraspinal centers. Pro nociceptive mediators activate primary afferent neurons directly or indirectly to enchance nociceptive signal transmission to the CNS excitation of primary afferent by peripherally originating mediators so called "peripheral sensitization".

Peripheral sensitization is the synthesis of ATP, glutamate, kinins, cytokinins and tropic factors. These reflexes are generated due to the application of heat, cold, mechanical and electrical stimulus.

Thermal and radiant heat are used in hot plate and tail flick method respectively. In eddy hot plate the duration of analgesic effect was more in 200 mg/kg compared to 100 mg/kg and reference drug pentazocine at 10 mg/kg dose significantly increased the reaction time at 90 minute and in tail flick method analgesic effect was observed at 100mg/kg when compared with the standard drug asprin (10mg/kg).

CONCLUSION

The findings in this study suggest that the *aloysia polystachya* possess anti- inflammatory and analgesic activity. The results have been obtained in carefully controlled experiments with laboratory animals where psychological factors can presumably be ruled out. In all the tests the responses have been assessed by actual measurement and not by subjective comparisons which may be influenced by the observer. Therefore the statistical validity of the findings has been proved and they provide a scientific foundation for the use of the biologically active ingredients of *aloysia polystachya* in inflammatory and pain conditions and explain the clinical effectiveness of the *aloysia polystachya*.

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Amelioration of CCl_4 Induced Liver Injury in Albino Rats by Antioxidant Rich Leaf Extract of *Biophytum sensitivum.* L

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Abstract
Objective: The present study was aimed to evaluate the antioxidant and hepatoprotective activity of *Biophytum sensitivum* L. plant extracts against CCl_4 induced liver damage in albino rats. Materials and methods: Hepatotoxicity was induced by using CCl_4.

Keywords
Antioxidant, Hepatoprotective activity, *Biophytum sensitivum* L

*****

INTRODUCTION:
For thousands of year's human beings are using plant sources to cure illness[1]. The active chemical compounds are synthesized from the plants. Various plant materials are exclusively used in pharmaceuticals, cosmetics, and food industry, and are recommended as efficient antioxidants[2]. Plants are the chemical factories of nature, producing many secondary metabolites, some of which have medicinal and insecticidal properties *Biophytum sensitivum* (L.) is an herbaceous plant belonging to oxalidaceae family[3]. The phytochemical constituents of the plant shown that amentoflavone[4], 3,8 – biepigenin[5], proanthocynidines[6] and Phenolic compounds[7]. It has several medicinal properties like antiseptic, the plant parts are used in the treatment of asthma, inflammatory diseases and diabetes[8-10]. The plant shows hypoglycemic[11], hypocholesterolemic, immunomodulatory[12], anti-inflammatory, chemoprotective[13], antitumor, cell-mediated immune response[14], and antibacterial activity.

MATERIALS AND METHODS:
Collection of plant material
The Leaves of *Biophytum sensitivum* L. (*Oxalidaceae*) plant was collected during the month of November 2013 from Tirumala Hills, Tirumal, Chittoore District, India. The plant was authenticated by Dr. Madhava Setty, Department of Botany, S.V University, Tirupathi.

Preparation of the extracts
After collection of leaves of *B.Sensitivum* the leaves were shade dried and powdered. The powder was subjected to successive solvent extraction by using methanol and water. Then the dried extract was obtained by evaporation of the solvent using a rotatory vacuum evaporator at 50 ºC and kept in

desiccator. The extract is further studied for phytochemical investigations and animal studies.

Pharmacological activities:

Experimental animals:

Wistar albino rats weighing 150-200g were used for experimental purpose they acclimatized for one week in experimental room. After acclimatization the animals were selected for final allotment of the study. Feed and water were given *ad libitum* throughout the study. All the animal experiments were conducted according to the ethical norms approved by the Institutional ethical committee of CPCSEA, New Delhi (Reg. No.: 1722/Ro/Ere/S/13CPCSEA).

Determination of Acute toxicity (LD_{50}):

Plant extracts of *B.sensitivum* L*.,* up to a higher dose of 2 g/kg were administered orally to normal rats. During the first four hours after the drug administration, the animals were observed for gross behavioral changes. The parameter such as hyperactivity, grooming, convulsions, sedation, hypothermia, body weight and mortality was observed up to 14 days. No mortality was observed with oral administration of all the extracts even at the highest dose of 2 g/kg, p.o. according to OECD guidelines.

Experimental design:

To evaluate the antioxident and hepatoprotective potential of *B sensitivum* L., in Carbon tetrachloride (CCl_4) - induced hepatic damage, 42 rats was randomly divided into seven equal groups. Group I served as a control. Group II received CCl_4 (1ml/kg b.w., p.o, in olive oil 1:1 ratio) once daily for 21 days Group III received daily oral dose of Silymarin (50mg/kg b.w.) along with CCl_4, Group IV and V received once daily oral dose of 200, 400mg/kg b.w. of MEBS respectively along with CCl_4, Group VI and VII received once daily oral dose of 200, 400mg/kg b.w. of AQEBS, respectively along with CCl_4, All groups were administered with their respective drugs for 20 days and blood was collected by retro orbital plexus and transferred to sterilized non-heparinized syringes to separate serum for biochemical analysis and liver was collected and preserved for histopathological examinations. The serum was stored at -10 ^{o}C until biochemical analysis which was carried out within 24 hrs.

Determination of serum biochemical parameters: The biochemical parameters includes serum enzymes SGPT, SGOT by (uv kinetic method)[15], ALP by by p-NPP method[16] by Schlebusch *et al.*, 1974., Total Bilurubin by taylor RLS *et al*, 1996[17] and Total Protein by Lowry's method by Dunn *et al*, 1992[18] were estimated.

Determination of anti-oxidant enzymes from liver homogenate: anti-oxidant parameters includes SOD by method of pyrogallol[19], CAT by the method of Aebi (1974)[20], GSH by the method of Ellman, (1959) [21]and lipid peroxidation by ohkawa *et al,* 1979 [22]by using liver homogenate.

Histopathological examination The sections of liver were processed for histopathological examination involving tissue fixation and were then mounted using DPX for microscopic examinations[23].

Statistical analysis Experiments were performed in triplicate and data analyzed are Mean ± SEM subjected to one-way ANOVA by using Graph Pad Prism 7. Means were separated by the Tukey's multiple range test when analysis of variance (ANOVA) was significant ($p<0.05$). Pearson correlation test was used to assess correlations between means.

RESULTS AND DISCUSSION:

Acute toxicity studies[1]

Acute toxicity studies for dried methanolic and aqueous extracts of *B. sensitivum* L., *was* carried out in different groups of mice (10 mice/group) with one group served as control group, at different graded doses (oral) of the dried plant extract of *B. Sensitivum* L., (200 – 4000 mg/kg, orally), diluted with 1% w/v of CMC suspension, showed no gross evidence of any toxicity and abnormalities in the mice up to 72 hr of the observation period. As no mortality signs had been observed even after administration of dose up to 4000 mg/kg of body wt, the extract might have LD50 value beyond 4000 mg/kg. Hence, further pharmacological investigation was carried at dose levels equivalent to 1/10th of LD50 (here maximum therapeutically safe dose) eqvalent to 400mg/kg and below. Acute toxicity study was done as per OECD Guidelines 423.

Rats treated with CCl_4 developed a significant hepatic damage observed as elevated serum levels of biochemical parameters like SGOT, SGPT, ALP, Total Protein and Total Bilrubin when compared to normal control. Pretreatment with Silymarin, and extracts of *Biophytum Sensitivum* L. leaves had showed good protection against CCl_4 induced toxicity to liver. Silymarin and extract treated animals showed significant reduction in elevated levels of biochemical parameters compare to toxic control animals which is evident in table no: 2

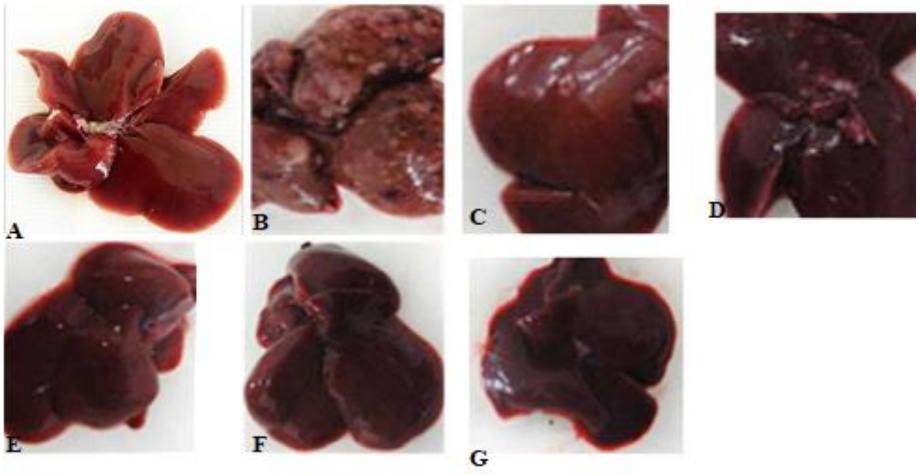
Antioxidant activity:

Effect of *B.sensitivum.L* leaf extracts on antioxidant parameters in CCl_4 induced hepatotoxic rats.

Rats treated with CCl_4 developed a significant hepatic damage observed by marked decreased in levels of antioxidant parameters like Superoxide dismutase, Catalase, Reduced Glutathione and increase in levels of lipid peroxidation when compared to normal control. Pretreatment with silymarin, and extracts of *B. Sensitivum.L* leaves had showed good protection against CCl_4 induced toxicity to liver. Silymarin and extract treated animals showed significant increase in levels of antioxidant enzymes and decrease in levels of lipid peroxidation compare to toxic control animals which is evident in table no: 3

Fig:1 Extraction by Roary Flask Evoparator

Photographs of isolated rat livers:



Effect of *Biophytum Sensitivum. L* on CCL_4 induced hepatotoxicity in Wister rats

A. Normal B. Disease control c. Standard D.Low dose of methanol extract E. High dose of methanol extract F. Low dose of aqouse extract G. High dose of aqouse extract

Fig: 2 Effect of *Biophytum sensitivum* L. on isolated rat livers

Histopathological studies:

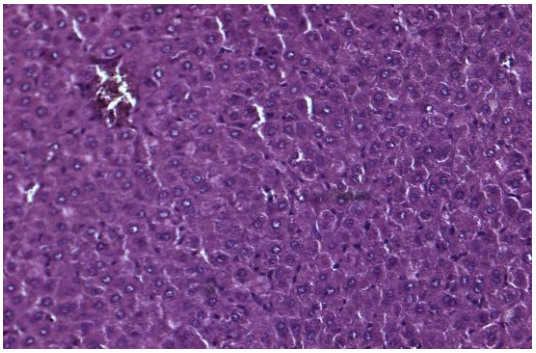


Fig: 3 Group: I Normal control

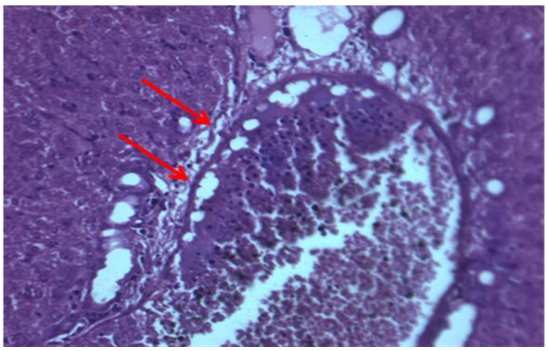


Fig: 4 Group: II Disease Control (CCL_4)

Fig.3 control shows hepatocytes appeared normal, no degeneration of necrosis and inflammation.
Fig.4 moderate to severe periportal fibrosis in which proliferation of connective tissue is noticed with red arrow

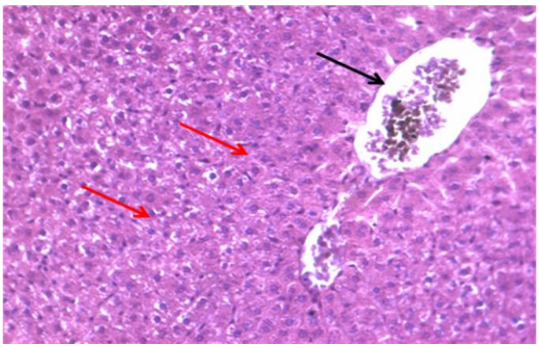


Fig: 5 Group: III Standard (Silymarin)

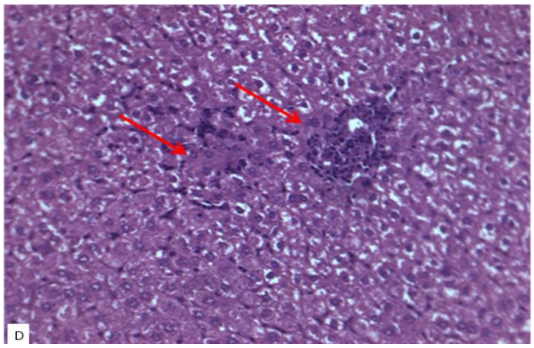


Fig: 6 Group: IV Methanol extract(200 mg/kg

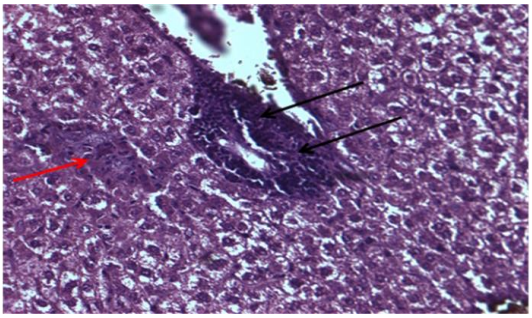


Fig: 7Group: V Methanol extract (400 mg/kg)

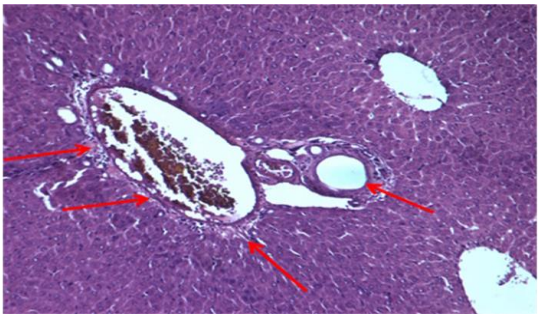


Fig: 8 Group: VI Aqueous extract e**xtract (200 mg/kg)**

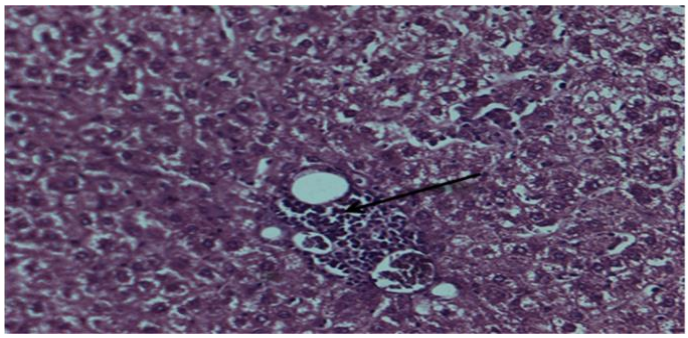


Fig: 9 Group: VII Aqueous extract (400 mg/kg)

Fig.5 sliymarin treated shows Central vein [portal vein], peri portal region appeared normal – black hepatocytes appeared normal – Red arrow Fig.6 200 mg/kg methanol extract treated shows multiple foci of inflammation surrounding central vein in liver – Red Fig: 7 400mg/kg methanol extract treated shows Mild periportal inflammation in which infiltration inflammatory cells particularly lymphocytes noticed in the liver – Black arrow

also, mild foci of necrosis in centri lobular region of liver – Red arrow. Fig.8 200 mg/kg aqueous extract treated shows Mild to moderate peri portal inflammation and fibrosis noticed in the liver – Red arrow.Fig.9 400 mg/kg aqueous extract treated shows Mild foci of inflammation in which infiltration inflammatory cells particularly lymphocytes noticed surrounding the central vein in liver – Black arrow

Table: 1The % yield of both methanol and aqueous extracts are as follows

S.NO	Extracts	Colour	Consistency	%Yield (w/w)
I	Methanol	Greenish black	Semisolid and non- sticky	17.4
II	Aqueous	Light dark green	Semisolid and nonsticky	21.1

Table no: 2 Effect of *Biophytum sensitivum* L. Extracts on biochemical parameters in CCL_4 induced hepatotoxic rats.

Groups	Dose (mg/kg)	AST(U/L)	ALT (U/L)	ALP (U/L)	TBL (mg/dl)	TP (U/L)	Liv.wt (g/100g)	liv.vol (ml/100g)
Normal control (1ml of CMC)	1ml	50.94±1.61	80.23±1.62	129.30±2.07	0.31±0.01	7.8±0.14	3.72±0.08	4.32±0.09
Disease control (CCL_4)	1ml	231.10±4.1$^{###}$	324.88±4.75$^{###}$	373.32±5.41$^{###}$	6.75±0.24$^{###}$	4.11±0.14$^{###}$	6.75±0.15$^{###}$	7.43±0.12$^{###}$
Standard (silymarin)	50	73.75±2.06	129.54±3.57	174.±2.12	0.71±0.02	6.89±0.12	4.28±0.03	4.82±0.03
MEBS low dose	200	170.45±3.87	185.94±3.13	177.07±3.57	2.9±0.15	4.47±0.12	4.27±0.03	5.23±0.02
MEBS high dose	400	121.26±3.96**	131.35±2.74**	121.97±5.28**	1.43±0.09**	5.37±0.06***	3.87±0.05***	4.85±0.06**
AEBS low dose	200	159.76±3.53	152.76±3.53	167.32±3.72	1.81±0.05	5.30±0.04	4.11±0.04	4.95±0.08
AEBS high dose	400	100.55±3.67***	110.60±4.31***	106.59±5.04***	1.14±0.05***	5.95±0.04***	3.72±0.06***	4.15±0.08***

Values are expressed in Mean ± SEM (n=6) Tukey's multiple range test when analysis of variance (ANOVA) was significant (p<0.05).

Graph: 1 Graphical representation of biochemical parameters of *Biophytum sensitivum* L. in CCL_4 induced hepatotoxicity rats.

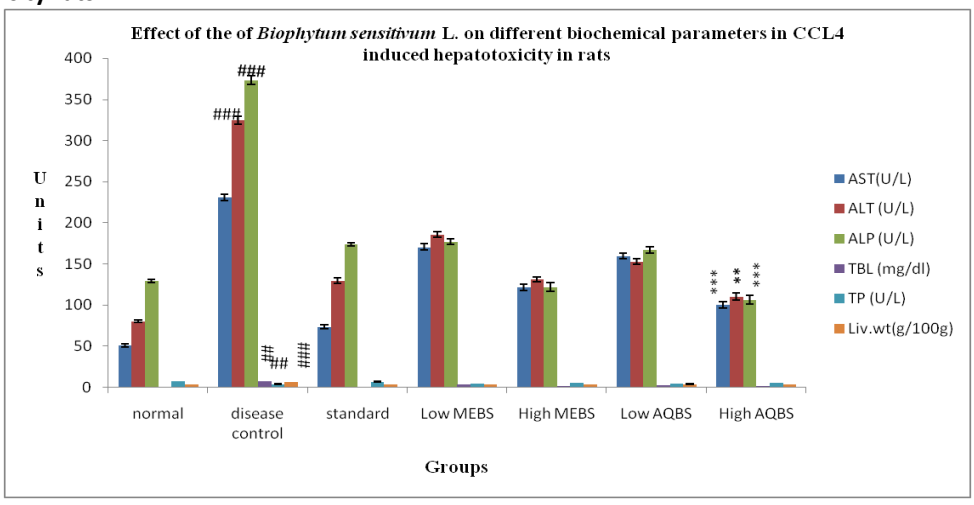
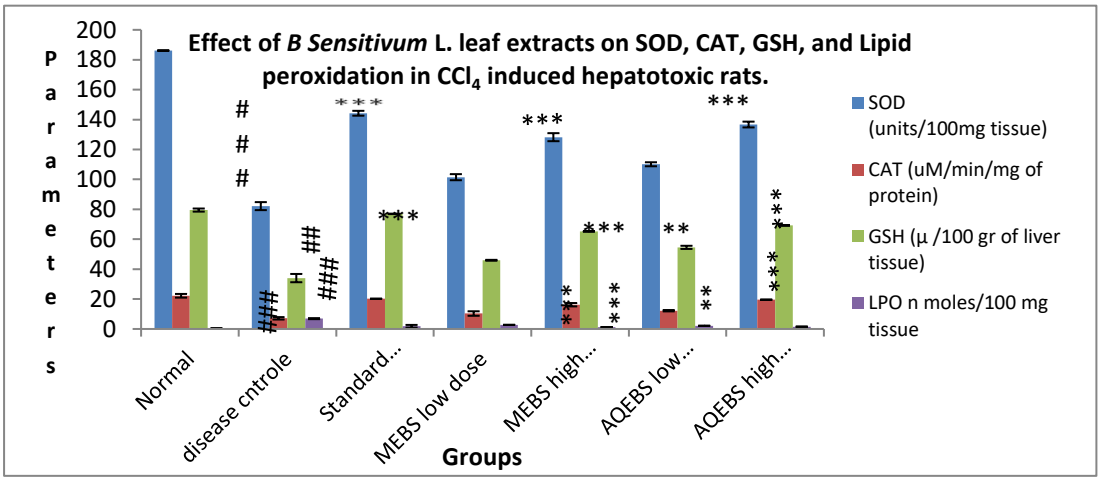


Table no. 3: Effect of *B Sensitivum* L. leaf extracts on SOD, CAT, GSH, and Lipid peroxidation in CCl_4 induced hepatotoxic rats.

Groups	Treatment	Dose (mg/kg)	SOD (units/100mg tissue)	CAT (uM/min/mg of protein)	GSH (µ moles/100 gr of liver tissue)	LPO n moles/100 mg tissue
I	0.9% Saline	1ml	186.15±0.11	22.19±1.23	79.5±1.04	0.29±0.2
II	CCL_4	1ml	82.14±2.68 ##	7.12±.81 ##	34.01±2.77 ##	6.9±0.34 ##
III	Standard (silymarin)	50	144.23±1.62***	20.19±0.1***	76.96±0.15***	1.8±0.9
IV	MEBS low dose	200	101.49±2.03	10.32±1.51	45.92±0.3	2.74±0.07
V	MEBS high dose	400	128.24±2.68	16.21±1.12	65.23±0.06	1.24±0.08
VI	AQEBS low dose	200	110.12±1.34**	12.21±0.46**	54.56±1.05**	2.12±0.06**
VII	AQEBS high dose	400	136.69±1.94***	19.56±0.2***	69.23±0.09***	1.52±0.21***

Values are expressed in Mean ± SD (n=6)
##indicate significance from the control group at P<0.01 probability level.
*****indicate significance from the CCl4 group at P<0.01 probability level.**

Graph: 2 Graphical representation of effect of *B.Sensitivum* L. leaf extracts on SOD, CAT, GSH and Lipid Peroxidation in CCL_4 induced hepatotoxicity rats



DISCUSSION

In the Present research work the MEBS and AQBS of dose (400 mg/kg) have shown better results of hepatoprotection against CCL_4 induced hepatic damage. The trichloromethyl radicals that are metabolites of CCL_4 binds to organs and cell membranes covalently and causes peroxidation of lipids that leads to hepatic damage by altering the functional integrity of mitochondria of liver. The phytoconstituents of extract induces enzymatic activity resulting in inhibited lipid peroxidation or accelerated excretion of CCL_4. The following phytoconstituents such as (biflavones, three flavonoids luteolin-7-methyl ether, isoorientin, 3'-methoxyluteolin 7-O-glucoside, as well as two acids, 4-caffeoylquinic acid and 5-caffeoylquinic acid) have been detected in plant extracts which were already proven for hepatoprotective activity.

The parameters referred as markers for liver damage were serum SGOT, SGPT, ALP and Total Bilurubin. There was significant change or decrease in the levels of above markers upon administration of high doses of MEBS and AQBS. Whereas no change was observed for low doses of extracts. Among the two extracts AEBS as shown dose dependent and high hepatoprotective activity and hence was selected to compare with positive control group (Silymarin).

Carcinogensis of tissue is a result of oxidative stress induced by generation of free radicals and decreased antioxidant levels in target cells and tissues. Leak

down of enzymes to the circulatory fluid and their assessment serves as markers in cell membrane damage. Lipid peroxidation can be inhibited by free radical scavenging. The groups pretreated with standard drug and high dose of extract have shown decreased lipid peroxidation by decrease in MDA levels. Elevation of GSH, SOD and CAT activities was found. In present study the rats treated with standard and high doses of extracts shown increase in catalase, superoxide dismutase and GSH and decrease in MDA levels indicating that they possess antioxidant and hepatoprotective activity.

The histopathology studies conducted shows that low doses of extracts have shown multiple foci of necrosis or mild to moderate inflammation in liver. Whereas the high doses of extracts show mild inflammatory changes by compared to disease control (CCL_4 induced).

CONCLUSION:

Based on above findings it can be concluded that antioxidant and hepatoprotective activities reported for high dose of aqueous extract shows significant protection against CCL_4 induced hepatic damage.

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PHARMACOLOGICAL EVALUATION OF ANTIDEPRESSANT AND ANTIANXIETY ACTIVITY OF BUPLEURUM FALCATUM IN ANIMAL MODELS

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PHARMACOLOGICAL EVALUATION OF ANTIDEPRESSANT AND ANTIANXIETY ACTIVITY OF BUPLEURUM FALCATUM IN ANIMAL MODELS

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ABSTRACT

Bupleurum falcatum, belongs to the family Apiaceae. Anxiety and Depression are widespread psychiatric disorders affecting around 5% of the population. Furthermore, it is difficult to predict which patient will respond to any given treatment. In the traditional systems of medicine, many plants have been used to treat anxiety and depression for thousands of years. The present study was designed to evaluate the antianxiety and antidepressant activity of the alcoholic and aqueous extracts of *Bupleurum falcatum* leaves in rodents. Antianxiety activity was tested by exposing rats to unfamiliar aversion in different methods like elevated plus maze model and actophotometer. The results infer that reduced aversion fear elicits antianxiety activity. The antidepressant activity was tested by using forced swim test and tail suspension test. The results infer that reduced immobility time elicits antidepressant activity. It was concluded that alcoholic and aqueous extracts of *Bupleurum falcatum* leaves having antianxiety and antidepressant activity. Alcoholic extract of *Bupleurum falcatum* leaves showing more significant activity over the aqueous extract.

Keywords: *Bupleurum falcatum*, Antianxiety activity, Antidepressant activity, Elevated plus maze, Actophotometer, Despair swim test, Tail Suspension Test.

INTRODUCTION

Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science or approved by regulatory agencies such as the United States Food and Drug Administration or European Food Safety Authority to have medicinal effects. World Health Organization (WHO) has provided a definition of medicinal plants, that is "A medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drugs."[1]

World Health Organization (WHO) reported that 80% of the world's population depends on medicinal plants for their primary health care. In the Plant Kingdom, Medicinal plants form the largest single grouping of plants. It is estimated that 30,000 species worldwide fall in this group, of which around 33% are trees [2] Plants are known to be the source of many chemical compounds. Medicinal plants were used by people of ancient cultures without knowledge of their active ingredients. The common practice of taking crude extract orally is laden with hazards as the extracts may contain some toxic constituents. There is an ever increasing need to limit toxic clinical drugs.In modern times, the active ingredients and curative actions of medicinal plants were first investigated through the use of European Scientific methods[3]. The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steriods, phenols glycosides and tannins[2].

The information obtained from extracts of medicinal plants makes pharmacological studies possible. The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized. Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens. Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity[4].

There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants [5]. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural products play a vital role in modern drug development in the pharmaceutical industry[6].

History of plants in medicine [7]

The earliest known medical document is a 4000-year-old Sumerian clay tablet that recorded plant remedies for various illnesses. The ancient Egyptian Ebers papyrus from 3500 year ago lists hundreds of remedies. The Pun-tsao contains thousands of herbal cures attributed to Shennung, China's legendary emperor who lived 4500 years ago. In India, herbal medicine dates back several thousand years to the Rig-Veda, the collection of Hindu sacred verses. The Badianus Manuscript is an illustrated document that reports the traditional medical knowledge of the Aztecs. Western medicine can be traced back to the Greek physician Hippocrates, who believed that disease had natural causes and used various herbal remedies in his treatments. Early Roman writings also influenced the development of western medicine, especially the works of Dioscorides, who compiled information on more than 600 species of plants with medicinal value in De Materia Medica. Many of the herbal remedies used by the Greeks and Romans were effective treatments that have become incorporated into modern medicine (e.g., willow bark tea, the precursor to aspirin). Dioscorides' work remained the standard medical reference in most of Europe for the next 1500 years.

The beginning of the Renaissance saw a revival of herbalism, the identification of medicinally useful plants. This coupled with the invention of the printing press in 1450 ushered in the Age of Herbals. Many of the herbals were richly illustrated; all of them focused on the medicinal uses of plants, but also included much misinformation and superstition. The Doctrine of Signatures, for example, held that the medicinal use of plants could be ascertained by recognizing features of the plant that corresponded to human anatomy. For example, the red juice of bloodwort suggests that it should be used for blood disorders; the lobed appearance of liverworts suggests that it should be used to treat liver complaints; the "humanoid" form of mandrake root suggests that is should be used to promote male virility and ensure conception.

Many of the remedies employed by the herbalists provided effective treatments. Studies of foxglove for the treatment of dropsy (congestive heart failure) set the standard for pharmaceutical chemistry. In the 19th century, scientists began purifying the active extracts from medicinal plants (e.g. the isolation of morphine from the opium poppy). Advances in the field of pharmacology led to the formulation of the first purely synthetic drugs based on natural products in the middle of the 19th century. In 1839, for example, salicylic acid was identified as the active ingredient in a number of plants known for their pain-relieving qualities; salicylic acid was synthesized in 1853, eventually leading to the development of aspirin. It is estimated that 25% of prescriptions written in the U.S. contain plant derived ingredients (close to 50% if fungal products are included); an even greater percentage are based on semisynthetic or wholly synthetic ingredients originally isolated from plants.

While Western medicine strayed away from herbalism, 75% to 90% of the rural population of the rest world still relies on herbal medicine as their only health care. In many village marketplaces, medicinal herbs are sold alongside vegetables and other Wares. The People's Republic of China is the leading country for incorporating traditional herbal medicine into a modern health care system; the result is a blend of herbal medicine, acupuncture, and Western medicine. Plantations exist in China for the cultivation of medicinal plants, and thousands of species are thus available for the Chinese herbalist; prescriptions are filled with measured amounts of specific herbs rather than with pills or ointments. In India, traditional systems have remained quite separate from Western medicine. In addition to Ayurvedic medicine, which has a Hindu origin, Unani medicine, with its Muslim and Greek roots, is another widely practiced herbal tradition in India. The renewed interest in medicinal plants has focused on herbal cures among indigenous populations around the world, especially those in the tropical rain forests. It is hoped that these investigations will add new medicinal plants to the world's pharmacopoeia before they are lost forever. In addition to the destruction of the forests, the erosion of tribal cultures is also a threat to herbal practices.

MATERIALS AND METHODS

Drugs and Chemicals

Drugs and Chemicals used in this study were of analytical grade and of highest purity procured from standard commercial sources in India.

ACUTE ORAL TOXICITY:

The acute oral toxicity of aqueous and alcoholic extracts of *Bupleurum falcatum* was determined by using rats and mice which were maintained under standard conditions. The animals were fasted 12 hour prior to the experiment, up and down procedure OECD guideline no. 425 were adopted for toxicity studies. Animals were administered with single dose of individual extract up to 2000mg/kg and observed for its mortality during 2days and 7days study period (short term) toxicity and observed up to 7days for their mortality, behavioral and neurological profiles.

SCREENING FOR ANTIANXIETY AND ANTIDEPRESSANT ACTIVITY

The aqueous and alcoholic extracts of *Bupleurum falcatum* leaves were tested for antianxiety activity using elevated plus maze and actophotometer and antidepressant activity using despair swim test and tail suspension test.

Treatment

Animals were divided into four (I-IV) groups.

Group I - Control group received distilled water (1ml, p.o).

Group II - Standard group received Diazepam (10mg/kg i.p).

Group III - Test group received aqueous extract of *Bupleurum falcatum* (200mg/kg p.o).

Group IV - Test group received alcoholic extract of *Bupleurum falcatum* (200mg/kg p.o).

Procedure for Ant anxiety Activity

- **Elevated plus maze (EPM) model**

The apparatus comprises of two open arms (35x5cm) and two closed arms (30x5x15cm) that extend from a common central platform (5x5cm). The floor and walls of the closed arms are made of wood and painted black. The entire maze is elevated to a height of 50 cm above the ground level. Rats weighing (150 – 200gms) were housed in a pair of 10 days prior to the test in the apparatus. During this time the rats were handled by the investigator on alternate days to reduce stress. 30 min and 60min after oral administration of the drug treatment, each rat was placed in the center of the maze facing one of the enclosed arms. During five minutes session, number of entries into open arm and time spent in the open arm were noted14,15. The procedure was conducted preferably in a sound attenuated environment.

- **Locomotor activity**

The locomotor activity can be easily studied with the help of actophotometer, the rats were grouped and treated with drugs. Turn on the equipment (check & make sure that all the photocells are working for accurate recording) and placed individually each rat in the activity cage for 10 minutes. Note the basal activity score of all the animals. Inject the drug diazepam (Dose: 5 mg/kg, ip; make a stock solution containing 0.5 mg/ml of the drug & inject 1 ml/100 g body wt of mouse), and after 30 mins re-test each mouse for activity scores for 10 mins[16]. Note the difference in the activity, before & after chlorpromazine. Calculate percent decrease in motor activity.

Procedure for Antidepressant Activity

- **Despair Swim Test Apparatus**

For the determination of antidepressant activity, forced swim test (FST) protocol was employed. During the test, animals were individually placed in a glass cylinder (20 cm in height, 14 cm in diameter) filled with water up to a height of 10cm, at 25 ± 2°C. All animals were forced to swim for 5 min and the duration of immobility was observed and measured during the 5 min interval of the test. Immobility period was regarded as the time spent by the rats to float in water with no struggle and making only those movements necessary to keep its head above the water. In order to check the fitness level of each test animal, a pre-test was carried out 24 h before the FST by subjecting each test animal to a session of 15 min swimming.

- **Tail suspension test**

Tail suspension test was performed based on the method prescribed. The mice were suspended 58cm above the floor by means of an adhesive tape, placed approximately 1cm from the tip of the tail. The total duration of immobility was quantified during a test period of 5min. Mice were considered immobile when they were completely remain motionless

Statistical analysis

The values were expressed as mean ± SEM data was analyzed using one-way ANOVA followed by T-test. Two sets of comparision had made. i.e. Normal control Vs All treated groups. Differences between groups were considered significant at $P<0.001$ and $P < 0.05$ levels.

RESULTS

ANTIANXIETY ACTIVITY OF BUPLEURUM FALCATUM

➢ ELEVATED PLUS MAZE TEST

Anxiolytic property of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Elevated plus maze experiment.

In elevated plus-maze test (EPM), the ethanolic and aqueous extracts of *Bupleurum falcatum* leaves at a dose of 200 mg/kg p.o. significantly increased the number of entries and time spent into the open arm. The magnitude of the anti anxiety effects 200mg/kg p.o. of alcoholic and aqueous extracts of *Bupleurum falcatum* was comparable to that of diazepam 10 mg/kg i.p. (Figure 1 and 2).

Table: - Data obtained from Elevated Plus Maze experiment

S.No	Groups	Dose	% Preference	Open arm and enclosed arm			
				No.of entries in open arm(M±SEM)		Average time spent (sec±SEM)	
				(O)	(C)	(O)	(C)
1.	Control	-	Open	1	2	34	268
			Closed	1	1	12	282
			Open	2	1	42	258
2.	standard	10	Open	2	1	11	284
			Open	1	1	24	274
			Open	1	1	35	264
3.	AQEBF	200	Open	3	2	42	126
			Open	2	1	32	145
			Open	1	1	28	142
4.	ALEBF	200	Open	3	2	47	130
			Open	1	1	29	151
			Open	2	1	33	147

From the experiment it was observed that mice taken aqueous and alcohol soluble fraction at dose of 200 mg/kg body weight, stayed more time in open arm of Elevated plus Maze apparatus in comparison to standard and negative control group. Moreover they were also stayed less time in closed arm of Elevated plus Maze apparatus in comparison to standard and negative control group. The value obtained from these fraction were statistically significant ($p<0.05$).

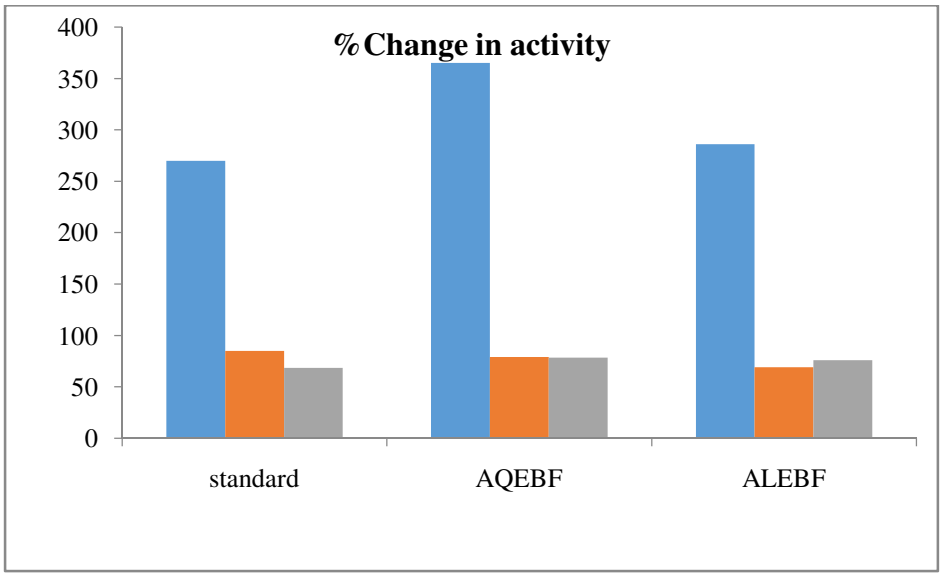
ACTOPHOTOMETER TEST:

Anxiolytic property of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Actophotometer experiment.

The percentage of reduction in locomotor activity with diazepam (10 mg/kg i.p) after 1 hour is 91.0 % i.e. there is highly significant ($P<0.000$) decrease in locomotor activity compare to control, where as dose of AQEBF and ALEBF (200mg/kg i.p) showed dose dependent decrease in locomotor activity that is 78.3% and75.8% respectively when compared to standard. The values are highly significant ($P<0.000$) (Table No:----).

Table No: --. Effect of extracts of *Bupleurum falcatum* on Locomotor activity.

S.No	Groups	Dose (mg/kg)	Locomotor activity (scores) in 10 min		
			Before	After	%change in activity
1.	control	-	245	--	---
2.	standard	30	270	85	68.5
3.	AQEBF	200	365	79	78.3
4.	ALEBF	200	286	69	75.8



The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.

ANTIDEPRESSANT ACTIVITY OF BUPLEURUM FALCATUM

- **FORCED SWIM TEST**

Antidepressant activity of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Forced Swim Test experiment.

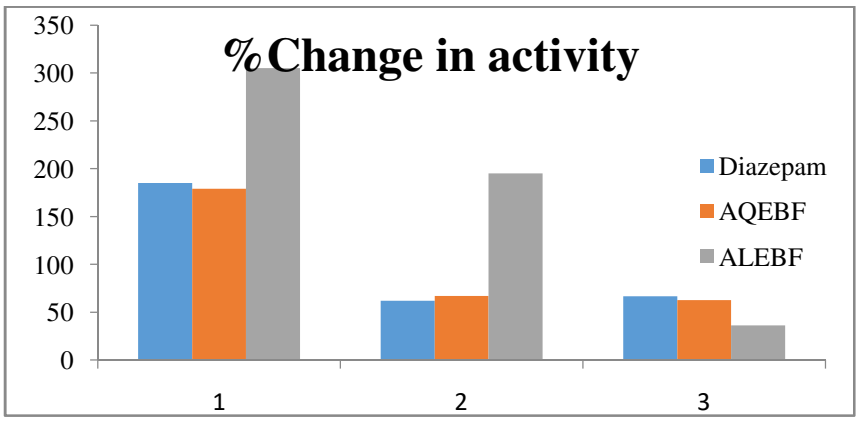
The anti-depressant activity of AQEBF and ALEBF was assessed using Forced Swimming Test in Swiss albino rats were illustrated in Table No:---. It was observed that AQEBF and ALEDB at a dose of 200mg/kg exhibited significant reduction in immobility time when compared to control in dose dependent manner. Similarly the animals treated with diazepam (10mg/kg) as expected showed significant decrease in immobility time.

Table No: ---. Effect of extracts of *Bupleurum falcatum* on Anti-depressant activity.

S.No	Group	Dose(i.p; mg/kg)	Immobility period		% change in activity
			Before	After	
1	Control	5ml/kg	134	--	---
2	Diazepam	10mg/kg	185	62	66.48%
3	AQEBF	200mg/kg	179	67	62.6%

4	**ALEBF**	**200mg/kg**	305	195	36.06%

The results are expressed as means ± S.E.M Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA). Statistical significance was assessed as $p < 0.05$.



Graph-1: Effect of extracts of *Bupleurum falcatum* on Anti-depressant activity

- **TAIL SUSPENSION TEST**

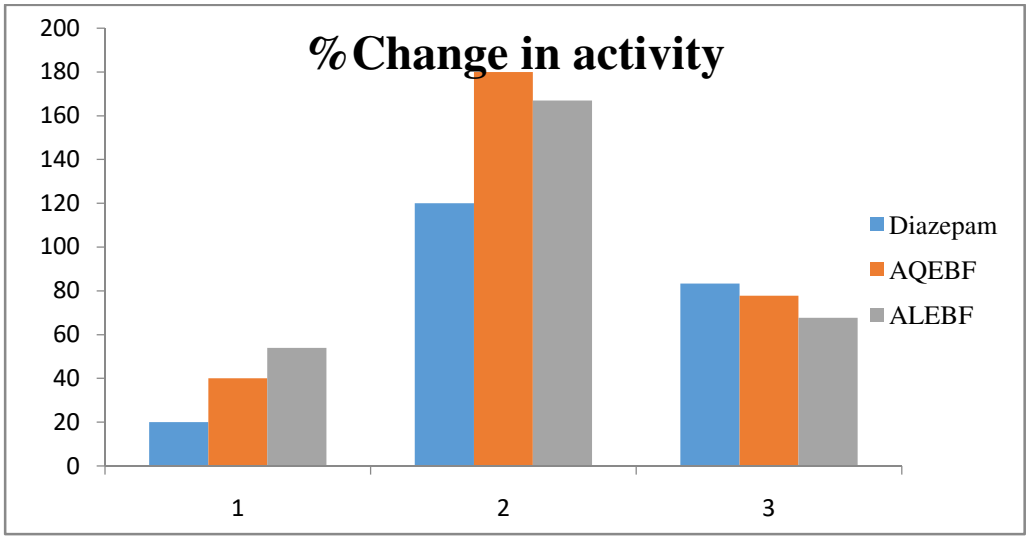
Antidepressant activity of aqueous and alcohol solvent soluble fraction of the leaves of *Bupleurum falcatum* studied at a dose of 200 mg/Kg, using Forced Swim Test experiment.

In tail suspension test, the alcoholic and aqueous extracts of leaves of *Bupleurum falcatum* at a dose of 200 mg/kg i.p. significantly decreased the immobility time. The magnitude of the antidepressant effects of 200 mg/kg i.p. of alcoholic and aqueous leaves of *Bupleurum falcatum* was comparable to that of Diazepam 10 mg/kg i.p. (Table ---).

Effect of Ethanolic and Aqueous Extracts of *Bupleurum falcatum* Leaves on Tail Suspension Test in Mice at Different Time Intervals

S.No	**Treatment**	**Dose (mg/kg)**	**Duration of immobility**		**%change in activity**
			Before	**After**	
1.	Control	---	40	-----	------
2.	Standard	10	20	120	83.33%
3.	AQEDB	200	40	180	77.8%

4.	ALEDB	200	54	167	67.7%



DISCUSSION:

PHYTOCHEMICAL ANALYSIS:

The phytoconstituents are known to play an important role in bioactivity of medicinal plants. In qualitative phytochemical analysis reveals the presence of alkaloids, flavonoids, tannins, terpenoids and saponins have associated with various degree of anti-microbial, anti-bacterial, anti-fungal, anti-oxidant and anti-termites. Therefore, the anti-diabetic, hypoglycemic, anti-depressant, anti-anxiety, skeletal muscle relaxant property, locomatar activity, anti-inflammatory, analgesic and diuretic activities were observed in this study may be due to the presence of chemical constituents in both aqueous and alcoholic extracts of *Bupleurum falcatum.*

BEHAVIOURAL ACTIVITIES

ANTI-DEPRESSANT ACTIVITY

OPEN FIELD TEST

Open field behavioral model was used to study exploratory and locomotor activity in this investigation. Reported studies have shown that stress factors account for the decreases in mobility and functional responses against novel environment. The purpose of including this test was to assess the general activity of the animals after performing FST. The results observed in the open field test showed that i.p administration of aqueous and alcoholic extracts of *Bupleurum falcatum* (200 mg/kg) did not significantly increase the locomotor activity in unstressed groups

of rats as compared with their control groups. However, aqueous and alcoholic *Bupleurum falcatum* administered rats following the exposure to repeated restraint stress showed significant ($p<0.01$) increases in locomotor / exploratory activity on an open field arena. It is therefore, suggested that the extract has the ability to reverse or normalize the locomotor suppressant behavior in laboratory animals and hence may help to cope with immobility factor associated with depression in humans. In the present study that administration of aqueous and alcoholic *Bupleurum falcatum* at the dose of 200 mg/kg significantly altered the behavioral deficits induced by injections of atypical neuroleptic, haloperidol and increased brain serotonin metabolism in mice. The results are in general agreement with our previous studies in continuation to this plant and indicating its antidepressant-like activity in behavioral models of depression.

FORCED SWIM TEST

Mood disorders are one of the most common mental illnesses, with a lifetime risk of 10% in general population. Prevalence of depression alone in general population is estimated to be around 5% with suicide being one of the most common outcomes. Commonly used Antidepressants often cause adverse effects, and difficulty in tolerating these drugs is the most common reason for discontinuing an effective medication, for example the side -effects of Selective Serotonin Reuptake Inhibitor (SSRIs) include: nausea, diarrhea, agitation, headaches. Sexual side-effects are also common with SSRI's. The Food and Drug Administration requires Black Box warnings on all SSRIs, which state that they double suicidal rates (from 2 in 1,000 to 4 in 1,000) in children and adolescents. Side effects of Tricyclic Antidepressants (TCA's) include drowsiness, anxiety, emotional blunting (apathy/anhedonia), confusion, restlessness, dizziness, akathisia, hypersensitivity, changes in appetite and weight, sweating, sexual dysfunction, muscle twitches, weakness, nausea and vomiting, hypotension, tachycardia, and rarely, irregular heart rhythms.

In the present study we have evaluated the antidepressant activity of *Bupleurum falcatum* of both aqueous and alcoholic extracts in FST. The development of immobility when rodents are placed in an inescapable cylinder of water during FST reflects the cessation of their persistent escape-directed behavior. Conventional drugs reliably decrease the duration of immobility in animals during this test. This decrease in duration of immobility is considered to have a good predictive value in the evaluation of potential antidepressant agents. Exact mechanisms

underlying the antidepressant action cannot be concluded at the moment due to the presence of large number of Phytochemical in the *Bupleurum falcatum*. However, the antidepressant activity may be attributed to the presence of saponins, flavonoids and tannins in the extract. It is possible that the mechanism of anxiolytic action of AQEEU and ALEEU could be due to the binding of any of these phytochemical to the $GABA_A$ -BZD_S complex.

ANTI-ANXIETY ACTIVITY

ELEVATED PLUS MAZE METHOD:

Anti-anxiety activity of *Bupleurum falcatum* was evaluated by employing a widely used model, i.e. elevated plus-maze. The mean number of entries and time spent by mice in open arms Amongst aqueous and alcoholic extracts of *Bupleurum falcatum* significantly increased mean number of entries and mean time spent by mice in open arms of elevated plus maze apparatus at the dose of 200 mg/kg with respect to control, thereby producing anti-anxiety activity. Phytochemical screening of aqueous and alcoholic extracts showed presence of flavonoids and tannins. Flavonoids have shown anti-anxiety activity in various studies. Further, the anxiolytic effect of flavonoids has been attributed to its effect on central nervous system and benzodiazepine receptors. Therefore, flavonoids of aqueous and alcoholic extracts of *Bupleurum falcatum* may be responsible for the anti-anxiety activity. It may possible that the mechanism of anxiolytic action of AQEBF and ALEBF could be due to the binding of any of these phytochemicals to the GABAA-BZD complex. In support of this, it has been found that flavones bind with high affinity BZD site of the $GABA_A$ receptor. The plant *Bupleurum falcatum* also contains flavones which may responsible for its anxiolytic activity. So the anxiolytic activity of AQEBF and ALEBF might involve an action on GABAergic transmission or effects on serotonergic transmission or due to its mixed aminergic potentiating effect.

ACTOPHOTOMETER TEST:

Spontaneous locomotor activity is considered as an index of alertness and can be helpful to confirm the general depressive activity of any drug. The decrease in motor activity gives an indication of the level of excitability of the CNS and this decrease may be related to sedation resulting from depression of CNS. However, in the present study the AQEBF and ALEBF was found to have decreased effect on the locomomotor activity in actophotometer.

It is reported that GABA, an inhibitory neurotransmitter is involved in the pathophysiology of depression. Moreover, neurochemical research has revealed that the

monoamines (5-HT, NA, and dopamine) have a crucial role in the development of the depression syndrome. It has been found that flavonoids isolated from plant species such as *Bupleurum falcatum* showed antidepressant activity. Thus, it is likely that flavonoids present in AQEBF and ALEBF may be responsible for the observed antidepressant effect. The antidepressant effect shown by AQEBF and ALEBF suggests etiological similarity in the development depression. Several hypotheses have been proposed to explain this aspect. The serotonergic theory postulates excessive functioning of the serotonergic neurotransmission for the prevention of the cause of depression. It can be hypothesised that AQEBF and ALEBF may have acted by modulating one or more of the above-mentioned neurotransmitters. Moreover, cholinergic transmission also plays the promising role in CNS. Based on its irregular distribution within the CNS and the observation that peripheral cholinergic drugs could produce marked behavioral effects after central administration. Therefore, it can be predicted that the higher level of choline in EU leaves may be responsible to act on cholinergic transmission in CNS and may be helpful to prevent depression.

Moreover triterpepenoids (steroidal compounds) are present in the leaves, those are able to cross blood brain barrier (BBB) due to their lipophilic nature and so it can be assumed that such steroidal compounds might also be responsible to elicit antidepressant and other neuropharmacological activities at molecular level in CNS (brain).

SUMMARY

1. The fresh leaves of *Bupleurum falcatum* used for this project work .
2. The dried leaves of *Bupleurum falcatum* were successively extracted with alcohol and water. Percentage yield was calculated.
3. Therapeutic dose of the extracts were calculated after carrying acute oral toxicity studies in both rats and mice.
4. Extracts were tested for their anti-depressant activity in mice.

- By Forced Swim Test.
  - Both aqueous and alcoholic extracts (200 mg/kg) showed significant decrease in duration of immobility time.
- By OPEN FIELD TEST.
  - Both aqueous and alcoholic extracts (200 mg/kg) showed significant decrease in duration of immobility time.

5. Extracts were tested for their anti-anxiety by elevated plus maze in rats.

- By Elevated Plus Maze Test.
  - Both aqueous and alcoholic extracts (200mg/kg) showed significant increased in mean number of entries and time spent in open arms.
- By Actophotometer Test
  - Both aqueous and alcoholic extracts (200 mg/kg) was showed decreased effect on the locomotar activity in rats.

CONCLUSION

The results obtained in this study indicate that the n-hexane, ethyl acetate and methanol fractions of the leaves of *Bupleurum falcatum* have significant CNS Depressant and Anxiolytic activities in animal model systems. The medicinal values of the plant leaves may be related to their constituent phytochemicals. So, further detailed investigations are needed to isolate and identify the active compounds present in the plant extract and its various fractions and their efficacy need to be done. It will help in the development of novel and safe drugs for the treatment of different types of CNS disorders.

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AQUATIC EXTRACT OF ACACIA CONCINA LINN HAS ANTIBACTERIAL AND ANTHELMINTIC PROPERTIES.

S MD. Abdulla, G. Swetha, Dr. B.V. Ramana

Abstract:

Herbal shampoo made from shikakai fruit is used to heal skin conditions including jaundice and constipation. Research quality is low, as seen by this plant's literature review, making it difficult to find novel therapeutics. Acacia concinna leaf aqueous extract showed considerable antibacterial activity against both gram-positive and gram-negative bacteria in an antibacterial screening. Greatest inhibition was shown with the gram-positive bacterium Micrococcus luteus, while maximum antibacterial activity was shown against E. coli. When compared to piperazine citrate at a dosage of 5mg/ml, the aqueous extract of Acacia concinna demonstrated considerable anthelimentic action. The plant extract, in addition to having inert chemical elements, also has multiple of active therapeutically active ingredients accountable for more therapeutic action than the single active constituent of synthetic medication in the event of resistant microbial strains.

Keywords: *Acacia concinna, Shikakai, Effectiveness as an anthelmintic and as a bactericidal*

INTRODUCTION:

Current efforts are being made to combat the rising issue of antibiotic-resistant bacterial strains and the development of resistance in gastro-intestinal helminthes to anthelmintic medications. This has resulted in a worldwide appeal for the development of novel antimicrobial medications, especially those derived from nature. Since their biological origin means they are less likely to cause harm, medications produced from natural sources tend to have less adverse effects than their synthetic counterparts. New antibacterial and anthelmintic drugs derived from plants were the inspiration for this study.

Shikakai (Acacia concinna, Fabaceae) (Acacia concinna, Fabaceae)

Saponins, which are found in abundance in the plant's fruit, are what cause lather to form when the fruit is used to wash hair. The shikakai shrub might be mistaken for a little tree. South India's dry, hot climate is ideal for these trees and shrubs with tree-like leaves. The leaves taste sour, like Tamarind pulp[1]. A widespread shrub in India's forests. The naturally gentle pH of shikakai fruit cleans hair without stripping it of its natural oils, earning shikakai the nickname "fruit for the hair" [2, 3]. As an anti-dandruff treatment, shikakai also speeds up hair development and fortifies the follicles at their base.

To clean hair, use either the powder or an extract made from the bark, leaves, or pods. Cleaning oil and grime from hair is a breeze with this. When using Shikakai, it is not necessary to follow up with a rinse or conditioner since it also aids in detangling the hair.

• Oxalic acid, tartaric acid, citric acid, succinic acid, ascorbic acid, and two alkaloids (calyctomine and nicotine) give the leaves their acidic flavor, making them ideal for use in chutneys. Other than that, an infusion of the leaves is utilized in anti-dandruff products.

The roasted and pulverized pods have been used to treat a number of skin disorders.

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Traditional medicine uses an extract of Shikakai leaves to treat malarial fever, while a decoction of the pods alleviates biliousness and functions as a purgative. Additionally, a saponin found in the bark has been shown to have spermicidal effect against human semen. The astringent properties of the leaves and pods make them an effective first aid remedy for minor cuts, wounds, and dental issues [4].

MATERIALS AND METHODS:

Herbarium specimen validated by Prof B. Sujatha, College of Science and Technology, Andhra University, Visakhapatnam; voucher number BS-00125; collection location: Yeleswaram (at 17.2833°N 82.1000°E), East Godavari district, Andhra Pradesh, India; collection of Acacia concinna leaves.

Plant material was extracted by placing 100 grams of dried powdered leaf material in a water bath with distilled water as a solvent for 6 hours, and then filtering the extract through a vacuum filter. Repeated extraction with hot water for 3 hours was used to remove the residual stain. The same filtering process was used for the second extract. This extraction process was carried out three times and then merged. The combined extract filtrate was heated to 60 degrees Celsius in a water bath until it became a semisolid dark brown color. A desiccator was used to preserve the extract. Chemical analyses on the dried extract revealed that it was high in carbs, tannins, and flavonoids.

Antibacterial activity The medication Ciprofloxacin, supplied by Suvarna Scientific Chemicals and Equipments, was utilized as the reference standard drug in this investigation.

Usually, pharmaceuticals are administered at a concentration of 20 g/ml.

Test organisms: The total of six bacteria was chosen for this investigation. To name a few, gram-positive bacteria include Bacillus subtilis MCC 2049, Staphylococcus aureus MCC2043, Micrococcus luteus MCC 2155, and Streptococcus aeruginosa MCC 2081. Escherichia coli MCC 2079 and Proteus vulgaris MCC 2543 are examples of gram-negative bacteria.

Three test extract solutions, T1, T2, and T3, were prepared at concentrations of 100mg/l, 50mg/l, and 25mg/l, respectively. Control vehicle: Sterile water for injection Procedure:

Under aseptic circumstances in the laminar air flow chamber, 30 ml of sterile nutrient agar medium was transferred to test tubes and inoculated with the fresh stock cultures of test organisms; the inoculated medium was then put onto sterile Petri plates and allowed to harden.

Antibacterial activity was tested using the cup plate agar diffusion technique against six different bacterial strains. A sterile aluminum borer was used to create five wells (cups/holes) of the same diameter (6mm) in the solidified agar medium. After that, in a laminar air flow cabinet, 30 l of each test extract concentration, standard drug concentration, and vehicle control (sterile water for injection) were pipetted and placed directly into the appropriate wells.

A place where fluid may move easily and quickly. It took 15 minutes of careful refrigeration of the Petri plates for the solution in the wells to diffuse into the medium. After that, we placed all of the Petri plates in an incubator at 37°1°C for 24 hours. Next, we looked for the existence of growth inhibition zones in the petri dishes and assessed their widths around each disc.

measures in millimeters [Including the well's diameter, which is 6mm]. The diameter of every inhibitory zone was measured twice in opposite directions to get an average value. Three separate runs of the experiment were conducted, with the average results shown in table-1[7, 8

RESULTS:

Table 1: **Antibacterial activity of Aqueous extracts of fruits of the *Acacia concinna***

S.No.	Bacterial strains used	Diameter of inhibition zone (mm)				
		Aqueous extract (mg/ml)				Positive control
		100	50	25	Control	20 µg/ml
1.	Staphylococcus aureus	18.05 ± 2.11	13.09 ±2.45	9.50 ±4.10	---	30.10 ±1.59
2.	Bacillus subtilis	14.50 ±1.28	11.50 ±3.43	9.50 ±3.63	---	29.21 ±2.15
3.	Escherichia coli	23.50 ± 3.15	18.05 ±1.85	15.05 ±3.81	---	36.50 ±3.18
4.	Proteus vulgaris	20.01 ±1.89	16.50 ±3.51	11.50 ±2.56	---	26.10 ±2.13
5.	Micrococcus luteus	23.12 ±2.65	18.15 ±2.89	14.10 ±2.89	---	27.50 ±3.43
6.	Streptococcus aeruginosa	21.50 ±3.41	19.11 ±1.62	13.20 ±3.56	---	25.50 ±2.15

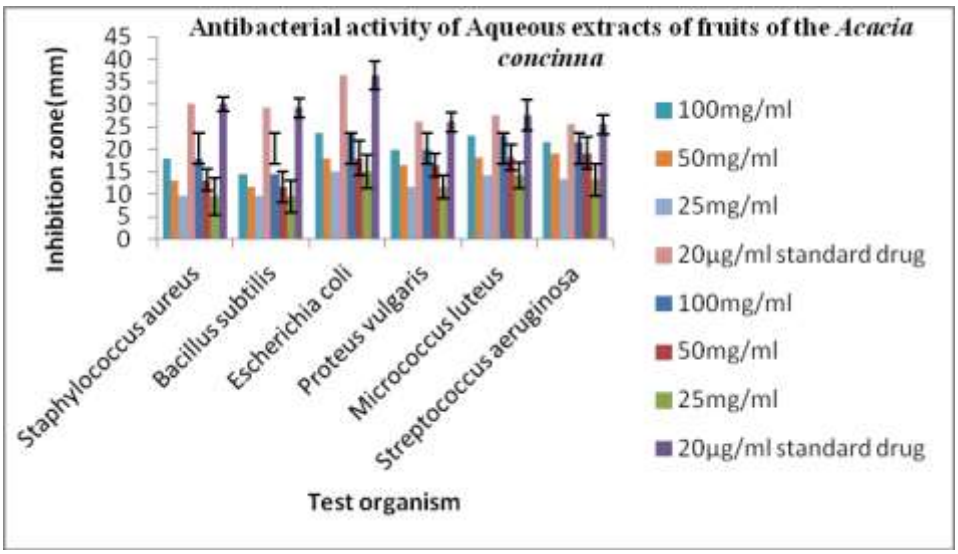
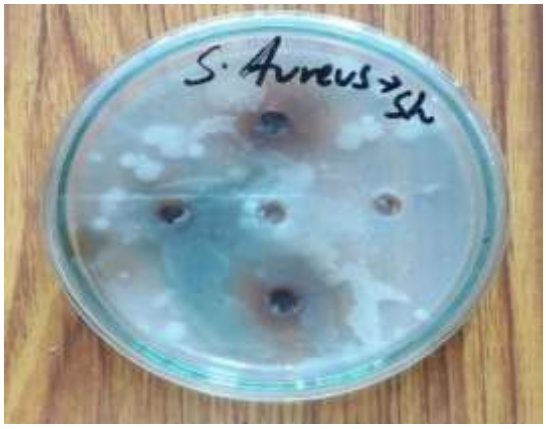


Fig 1: Graphical presentation of antibacterial activity of aqueous extract of leaves of *Acacia concinna* Linn.


E.coli- Sh


M.L- Sh


S. Aureus Sh


B.S - Sh


P.V - Sh
S.Aerogin - Sh

Pictures of antibacterial activity of aqueous extract of *Acacia concinna* Linn.

DISCUSSION:

Acacia concinna aqueous extract showed considerable antibacterial activity against both gram-positive and gram-negative bacterial strains in an antibacterial screening. Increasing the concentration also boosted the antibacterial effect. Aqueous extract showed the most antibacterial action against Escherichia coli, followed by the greatest suppression against Micrococcus luteus and Streptococcus aeruginosa (all gram-positive organisms). Repetition of the experiments showed that the examined organisms' zone of inhibition findings were within the range of -5 to +5 standard deviations. The aqueous extract of Acacia concinna showed more activity against gram-negative bacteria than it did against gram-positive bacteria. The uninhibited group showed no signs of slowing down.

Antihelmintic activity: most currently available anthelmintics have unwanted side effects as nausea, vomiting, dizziness, and constipation. Natural anthelmintics show promise as a potential therapy for parasite infestations. Common Medicine: valfred Pharmaceuticals Limited's Piperazine Citrate Syrup, USP, 30ml.

Indian adult earthworms (Pheretima posthuma) were gathered from damp soil close to the Aditya College campus, washed with normal saline to remove soil particles, and then stored in normal saline. The 0.2-0.3-centimeter-thick earthworms may grow to a length of 6-8 centimeters. thickness were used for the analysis. Because of their physiological and anatomical similarities to human intestinal roundworm parasites, Pheretima posthuma were utilized in the current work.

Generally, a drug's concentration is set at 5mg/l.

T1, 100mg/ml; T2, 50mg/ml; T3, 25mg/ml; these are the test extract concentrations.

The adult Pheritima posthuma was subjected to several quantities of test extract and standard medication to determine its anthelmintic activity [9-12]. In two different 10-milliliter Petri plates, we tested both the test extract and the standard concentration. The standard of care was normal saline. The earthworms used in all of the Petri dishes were carefully chosen to be around the same size. Each Petri plate was stored at ambient temperature.

The duration of paralysis and death of individual worms was tracked. If the worms were still alive, they would have been subjected to external stimuli designed to get them moving. Paralysis set in when no worm activity could be seen, even when violently shook. Worms were considered dead if they stopped responding to a strong shaking or a dip in warm water (50°C). The test was repeated three times, and the averages were recorded. In table-2 below, you'll see the results of the anthelmintic activity.

RESULTS:

Table 2: Antihelmintic activity of aqueous extract of the fruits of the *Acacia concinna*

Substance tested	Concentration(mg/ml)	Time taken for paralysis (min)	Time taken for death (min)
Control (normal saline)	**…**	**…**	**…**
Piperazine citrate (standard)	**5 mg/ml**	**2.50 ± 1.05**	**5.25 ± 1.56**
Aqueous Extract (Acacia concinna)	**100 mg/ml**	**8.05 ± 0.98**	**13.45 ± 1.85**
	50 mg/ml	**11.30 ± 1.76**	**18.05 ± 1.95**
	25 mg/ml	**17.5 ± 2.10**	**23.40 ± 1.85**

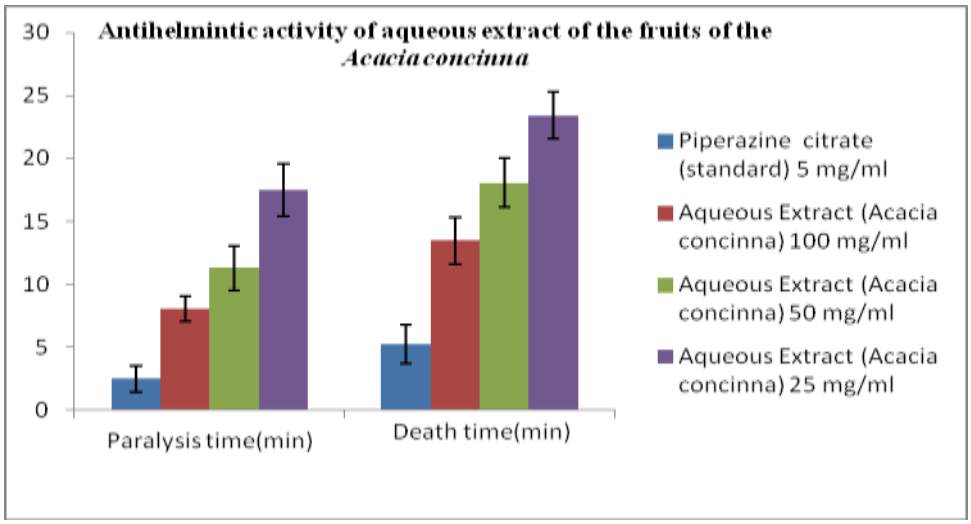
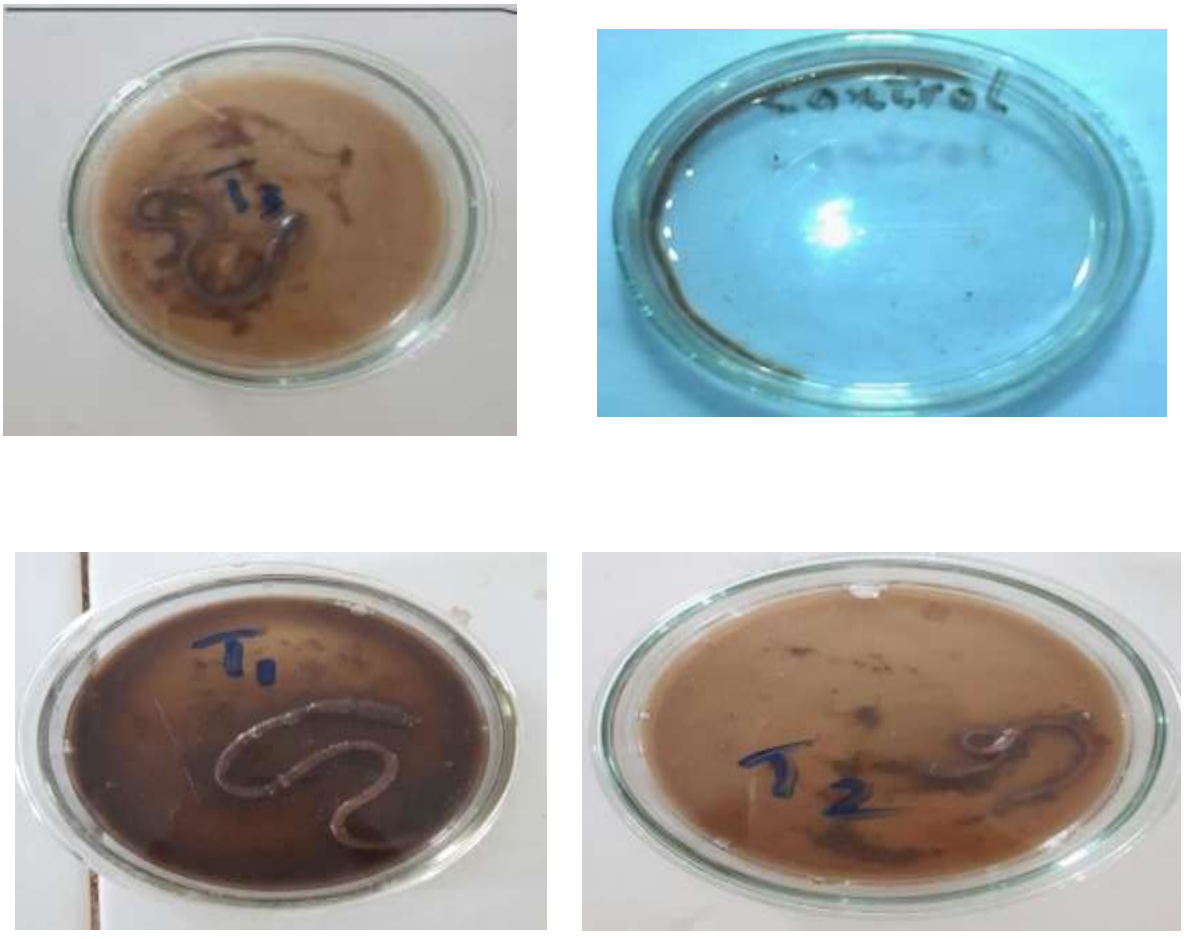


Fig 2: Graphical representation of anthelmintic activity of aqueous extract of the leaves of *Acacia concinna*



Pictures of anthelmintic activity of aqueous extract of the *Acacia concinna.*

DISCUSSION:

When compared to piperazine citrate at a dosage of 5mg/ml, the aqueous extract of Acacia concinna demonstrated considerable anthelimentic action. The anthelmintic activity of Acacia concinna aqueous extract increased with increasing concentration. The plant extract, in addition to having inert chemical elements, also has multiple of active therapeutically active ingredients accountable for more therapeutic action than the single active constituent of synthetic medication in the event of resistant microbial strains. Repeated experiments showed that the examined organism had anthelmintic activity, with values within 5 standard deviations of the mean.

CONCLUSION:

At a concentration of 5mg/ml, the aqueous extract of Acacia concinna leaves exhibited much higher anthelimentic activity than piperazine citrate. We need to extract the active ingredients in shikakai leaves so that we may use them to create new therapies to treat infections caused by bacteria, viruses, and fungi that have developed resistance to existing treatments.

ACKNOWLEDGEMENT:

I'd want to take this opportunity to thank the administrations of Aditya Pharmacy College in Surampalem, East Godavari district and A.U. College of Pharmaceutical Sciences in Visakhapatnam, Affiliated to Andhra University Recognized by PCI for making the resources I needed for my study possible.

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Emerging guidelines for the regulation of plant-based drugs are being put into effect by the Pharma-Planta initiative.

B. Lavanya, B. Mayuri, Dr. C. H. Naveen Kumar

Abstract; Rapid progress in recent years has brought plant-made pharmaceuticals (PMPs) out of the early stages of research and into clinical trials; the first commercial medications for human use are projected to hit the market in 2009. It is yet to be seen if PMP technology will be commercialized in Europe, although at least one product has already entered phase II clinical testing. These new goods pose a threat to the existing, convoluted regulations that control the creation of both genetically modified (GM) plants and "conventional" medicines. Specific rules for the regulation of PMPs are now being drafted, and the areas of responsibility amongst the several EU regulatory bodies are being mapped out. In this post, we'll go through some of the challenges that have come up during the process of developing rigorous risk assessment and risk management methods focused on health and environmental effect, all while cooperating with EU regulatory agencies to provide adequate regulatory monitoring..

Keywords; Medical plants as medicines; PMP; biosafety; regulations

Introduction

The next big commercial breakthrough in biotechnology will probably be the harnessing of plants to create medicinal and industrial proteins. Some advantages of plant-based cell culture systems have been identified over more traditional mammalian and bacterial cell culture methods (Twyman et al. 2005). There are several advantages to mass production, including as inexpensive initial investment, scalability, and storage options, and the possibility of producing large quantities of products at low unit prices. Plant manufacture may be the sole alternative for certain high-demand medicinal goods, especially in underdeveloped nations where they are critically required.

The USDA Centre for Veteri- nary Biologics registered Dow AgroSciences' first plant-derived vaccination in January 2006 (http://www.dowagro.com/animal- health/). The item was a vaccination for chickens.

tobacco cells in sterile, airtight containers to combat Newcastle disease. In validating the safe use of genetically modified plant cells to create therapeutic proteins, this was a major step forward for the industry. Tobacco plant production of the monoclonal antibody CB-Hep.1 was approved by Cuba's Med- ication Quality Control Agency in April 2006. The Cuban Institute of Biotechnology and Genetic Engineering (CIGB) (www.cigb.edu.cu). The production of hepatitis B vaccines requires this specific monoclonal antibody. The contemporary pharmaceutical business and authorities may be more accustomed with biore- cycling settings, but this product is the first commercial use of complete plants as a production vehicle for reagents utilized in a clinical manufacturing procedure.

Although some devices have progressed to the clinical trials stage, PMP technology has not yet been used commercially in Europe. Cobento Biotech produces human intrinsic factor in Arabidopsis thaliana, while Meristem Therapeutics produces gastric lipase and lactoferrin in maize (www.meristem-therapeutics.com) (www.cobento.dk). Several organizations are now responsible for regulating these crops; which organizations are involved will vary on factors such as the host plant used, the location of cultivation, the product selected, the final formulation employed, and the population for whom the crop is intended.

Pharma-Planta

In 2004, the European Union (EU) established an academic research group called Pharma-Planta (www.pharma-planta.org) to address many issues related to the use of plants as manufacturing platforms for medicines. One of the consortium's goals is to establish a plant-based manufacturing platform for medicines suitable for the European market, and another is to aid in the creation of suitable regulatory control in the EU.

The second goal is to create a system for manufacturing transgenic plants that can make recombinant antibodies in accordance with established guidelines for doing so, known as Good Manufactur-

Human Phase I Clinical Trials, Pre-Clinical Toxicology Testing, and Good Manufacturing Practice (GMP) in Europe.
Third, to show concrete support for the humanitarian use of PMPs and the related industrial technologies in low-income nations. A unique consortium-wide Statement of Intent on the use of Pharma- Planta intellectual property for humanitarian purposes in underdeveloped

stringent regulatory restrictions pertaining to other GM crops, such as the 2001/18 EU laws and the USDA/APHIS permit application criteria for the USA (for field grown plants), but also the regulations posed by authorities that regulate the manufacturing of pharmaceuticals. In 2002, both the FDA (US Food and Drug Administration, URL: http://www.fda.gov/cber/gdlns/bioplant.pdf) and the EMEA (The European Agency for the

countries and the establishment of a PMP licensing approach for humanitarian reasons have enabled this.
To define the most up-to-date ideas on appropriate international regulatory oversight and the benefits and drawbacks of using various plant species for the production of recombinant pharmaceuticals, a Pharma-Planta sub-group responsible for the analysis of biosafety issues conducted a consultation exercise. Recent years have seen a proliferation of reviews devoted to this same subject (Commandeur et al. 2003; Mascia and Flavell 2004; Petersen and Arntzen 2004; Ma et al. 2005a, b). The purpose of this study is not to repeat the information found in these other works, but rather to examine the more general implications involved in the development of recombinant pharmaceuticals in plants. We talk about picking products, production hosts, and ideal growing conditions. Choosing the proper product is crucial for the industry to assure the success of this new technology and its adoption by regulators.

Where do we go from here?

Many technological and regulatory considerations must be addressed for PMP production to be effective (Horn et al. 2004; Ma et al. 2003). How well the target product can be produced, constructed, and stored in the host plants, as well as how effectively it can be removed, all play a role (Gomord 2004; Tekoah 2004). The pharmaceutical product yield per hectare that can be achieved, the cost of inputs, harvesting, transportation, and processing, and the cost of marketing are all factors to consider while deciding on a production host (Fischer et al. 2004; Stoger et al. 2005; Giddings et al. 2000). Regulatory agencies have a number of requirements that the production system must meet.

Evalua- tion of Medicinal Products, URL: http://www.
emea.eu.int/pdfs/human/bwp/076402en.pdf)
published draft documents addressing quality aspects of the production of medicinal products in GM plants.
Ability to induce enough expression and accumulation of the recombinant protein in plants is a critical factor in determining economic

viability. In order to fully take use of plants' scalability in agriculture, this is an essential first step (Ma et al. 2003; Twyman et al. 2003; Hood et al. 2002). Although the plant species utilized in production has some bearing on absolute yields, there are many other variables that have a role in determining which crop is selected for cultivation. What are the pros and downsides of various crop production systems? What are the most pressing biosafety and regulatory concerns? These were some of the primary topics discussed throughout the consultation.

Production Method Selection

The production host's biological characteristics must be evaluated from several angles, including productivity, environmental impact, food safety, and human health. There is probably not a single plant species that can meet all the needs. In order to choose the optimum species for a certain application, it is sometimes necessary to strike a compromise between competing factors. There are three possible "classes" of plant species to utilize: wild species, crops that are not consumed by humans, and crops that are consumed by humans.

Species that aren't bred in captivity

The term "non-cultivated species" is used to describe the wide variety of plant life that is not cultivated by humans. Like

The main benefit of non-food crops is that they are not included in the human food chain. Conversely, little is understood about the genetics and biology of such organisms, including whether or not they generate poisons and whether or not they are capable of outcrossing. To make field farming more possible, little to no effort will have been made to domesticate such species. Due to a lack of domestication, seed yields are frequently poor, making leaf material the most probable harvestable target tissue.

The cultivation of non-domesticated plant species in bioreactors or other forms of confinement is theoretically viable. According to Biolex (http://www.biolex.com/), who bought Lemnagene (http://www.lemnagene.com) in 2005, duckweed has a high potential for scalability (Lemna minor). One may also argue that raising this species in captivity has its benefits.

It's quite improbable that we'll be able to successfully domesticate a creature that isn't already part of human culture anytime soon. Although it may be possible to create new species of "pharmaceutical crop" in the long run, the appropriate methods should be assessed in tandem with studies to use already domesticated species rather than in place of them.

(i) Non-food crops

The main advantage of non-food crops is that, although they have been developed and bred as crops, they are not used for food or feed. Consequently, it should be relatively easy to keep them separate from crop products used in the human or animal food chain. The main species being considered in the non-food category are tobacco and falseflax. Tobacco is a strong candidate for the commercial production of recombinant proteins since it already has a track record in PMP research (Stoger et al. 2002) and has recently been used in Cuba for the commercial production of a recombinant anti- body against hepatitis B (Ramirez et al. 2003; Valdés et al. 2003; Pujol et al. 2005Principal tobacco benefits infrastructure set up for massive processing that works properly. Unless it is produced in rotation with a food crop, it is quite improbable that tobacco material will accidentally mix with material intended for the human or animal food chain (cf. the ProdiGene maize incident, discussed later). These problems should be resolved when Good Agricultural Practice becomes more well-developed.

Toxic alkaloids are produced in high concentrations by many tobacco cultivars and must be eliminated during processing, however there are low-alkaloid types that might be used to create medicines (Fischer and Emans 2000; Ma et al. 2003). Alternatively these alka- loids are allegedly not present in cell suspension cultures, which might also be utilized to create recombinant proteins (Doran, 2000; Hellwig et al. 2004), but not on the scale necessary for the antibodies selected as target molecules in the Pharma-Planta initiative. Alternatively, proteins might be sent along the secretory route and then secreted by the plant at the root or leaf level (Drake et al. 2003; Kormarnytsky et al. 2000; Borisjuk et al. 1999). There are phenolic compounds in tobacco that are produced during grinding and protein extraction that may impede further processing steps. However, developments in downstream processing, such as the use of smart membranes during the clarifica- tion and capture stages, will

potentially enable manufacturers to target and eliminate these undesirable molecules, making them no more a problem than the removal of any other protein in the purification process.

The Finnish Biotech Company UniCrop (www.unicrop.fi) is developing falseflax (Camelina sativa) to manufacture recombinant proteins for the pharmaceutical business. Protein is recovered from the soft sprout material generated from transgenic seedlings in fully contained air-lift bioreactors, eliminating the need to separate the fibres and oil later in the processing chain.

(ii) Food crops

Cultivating food crops and undergoing transformation operations are accompanied by a wealth of information. also clear cut for a few key agricultural species. The regulatory benefits of using GRAS (Generally Recognized as Safe) plants are not to be overlooked. EMEA draft guidance paper on PMPs, www.emea.eu.int/pdfs/ human/bwp/076402en.pdf, emphasizes that GRAS status only considers oral administration and does not apply to topical or injectable forms.
Seed crops (a), vegetable crops (b), and fruit/green leaf crops (c) are the three main categories of food crops. The primary distinction between the three classes is the amount of time that passes after harvest before the plant tissue holding the medicinal component must be preserved in some other way, such as by desiccation or freezing

Regulation of plants for pharmaceutical production (US and EU)

Most of our expertise in regulating the field release of pharmaceutical plants has been acquired in North America. European Union officials are making an effort to treat pharmaceutical crops the same way they treat other agricultural crops—on a case-by-case basis. However, most regulatory systems do not conveniently accept pharmaceutical crops since the laws have mostly been devel- Developed for use in food and feed crops, with consideration given to any possible environmental consequences. Although some effort has been made to modify these rules to accommodate pharmaceutical crops, the European Union regulatory process currently provides no "natural home" for conducting such an evaluation. Currently, under 2001/18/EC, the responsible authority in the country of release must be notified of every field-grown pharmaceutical crop growing inside the EU. Both food and non-food crops have their release plans governed by these rules and regulations. In accordance with regulation 1829/2003/EU, the European Food Safety Authority (EFSA) would evaluate a request for commercial distribution of a food crop. EFSA would also have the determining responsibility if a non-food crop was proposed, but would normally only intervene in instances where Member States were not in agreement. Currently, EFSA is working on a set of guidelines geared specifically for PMPs. Note that the field release restrictions would not apply to a pharmaceutical crop cultivated in containment; instead, the confined regulations (Directive 90/219/EEC as revised by Directive 98/81/EC) would govern. Whether cultivated in confinement or not, medicinal products from plants would also need to conform to the 2309/93/EU standards. During the early stages of clinical trials, the relevant national authority is in charge of these rules, but at the point of commercial application, the European Medicines Evaluation Agency (EMEA), which is roughly comparable to the FDA in the US, takes control. In 2002, the EMEA released some preliminary recommendations that are now being revised (http://www.emea.eu.int/pdfs/hu-man/bwp/076402en.pdf) (publication due by the end of 2006). The biological and semantic distinctions between plant-based manufacturing and traditional systems based on cells grown in bioreactors have contributed to the delay in finalizing these guideline notes. Concepts like working and master bank stocks, batch-to-batch consistency, standard operating procedures, and so on all need to be specified precisely for plants (inputs, downstream processing, QA etc). It is still being determined at what point in the process each regulatory authority gets engaged and the scope of their power. Field-release A permit from APHIS is needed for PMP crops planted in the US (the Animal and Plant Health Inspection Service of the USDA). A containment strategy for growing, harvesting, and transporting plants from the field is required. Seed production, pollination

schedules, harvest dates, crop destruction, shipping, quarantine, and storage and usage of equipment are all subject to APHIS scrutiny. Up to five inspections of the fields may be carried out during the growing season, each time coinciding with a crucial stage of harvest. Field test permits are issued by APHIS to organizations like companies and universities, who then may subcontract with individuals like farmers. Training on the necessary permits and their implementation is mandatory for subcontractors (Elbehri 2005).

There are no plans for APHIS (USDA) to deregulate any medicinal crops at this time. That's why it's probable that commercial and research crops alike will continue to need an experimental permit and the extra scrutiny that comes with it. The possibility for accidental cross-pollination between pharmaceutical and food crops is of special concern to the FDA. Attitudes on the use of food crops to create "drugs" have been profoundly impacted by the Star Link maize issue (while not a PMP crop), in which GM maize intended for animal feed, invaded the food chain. The USDA has the right to mandate environmental impact statements for any permit applications they deem necessary (EA). Such EAs might be triggered by factors like the proposed site's location or the projected cultivation conditions. The United States Department of Agriculture (USDA) has published two separate Environmental Assessments (EAs) for Prodi- Gene's maize field experiments (Permits 04-121- 01r and 04-114-01r, available at http://www.aphis.usda.gov/brs/ ea pubs.html). These materials were made available for public review, and the deadline for submission of comments was extended (from the standard 30 days). Subsequently, we decided to stop considering the applications.

International developments in regulation

Canada's Food Inspection Agency (CFIA) held a technical conference for researchers from across the world in 2004.
shop on how PMP products and by-products should be separated and handled in commercial settings. Experts in grain handling and identity preservation were among the participants, along with members from the PMP sector, federal government organizations, agriculture and agribusiness organisations, and more. Since many of the plants being researched for PMP production are also used as food and feed (such as safflower and alfalfa), the first step in formulating a regulatory framework was to investigate whether PMP products and by-products could be adequately segregated from other commodities, and more specifically from commodities intended for the food and feed chains. The workshop's findings are available online at www.inspection.gc.ca/english/plaveg/bio/mf/ segrege.shtml on the CFIA site.

In 2004, APHIS held a similar international workshop titled "Confinement of Genetically Engineered Crops During Field Testing." The primary objective of this session was to analyze data from existing crop plants that have been planted with APHIS field trial permits to create PMPs and plant produced industrials (PMIs). Summaries of the many environmental consequences and confinement concerns discussed during the workshop may be accessed on the APHIS website (www.aphis.usda.gov/brs/confine workshop 2004. html).

The European Food Safety Authority (EFSA) and the European Medicines Agency (EMEA) are both working on guidance notes for the regulation of PMPs in Europe. As items are put through the new regulatory procedures, these rules are certain to change further..

There is an unexpected occurrence of pharmaceutical crops in food crops.

Gens encoding pharmaceuticals may be difficult to contain. Both the spread of genes and the appearance of volunteer plants in successive harvests pose serious risks to both ecosystem health and human wellbeing. The attention and care given to this problem is reflected in the activities taken by regulatory bodies and the biotechnology industry in the past. Prod- iGene Inc. was at the epicenter of a 2002 controversy about how to keep pharmaceutical crops grown outdoors from spreading (Hoag 2003). Volunteer transgenic maize plants appeared in the soybean crop the year after the pharmaceutical maize crop, and the incident received widespread media attention. Soybean in the storage silo was seized and destroyed because of the presence of maize plant debris.

As part of the subsequent settlement, Prodigene agreed to pay $250,000 in civil penalties, the expense of cleaning the premises and equipment, and the price of 500,000 bushels of soybeans. While this instance reveals that mechanisms were in place to prevent tainted foods from entering the food supply, it also highlights that the biology of the production host crop and subsequent crops in the cycle must be taken into consideration for effective regulation and containment. While this should help lessen the possibility of contamination, it may be unrealistic to promise that it will be eliminated entirely. According to Elbehri (2005), a coalition of food companies favored the inclusion of a food-safety review per event prior to obtaining a permit. In reality, this might shift the focus of agricultural research and development away from staple crops like maize and toward non-food crops (tobacco).

Transparency

Many forms of regulation need openness to the public. Generally speaking, releasing GMOs into the environment needs authorization from the appropriate authorities in nations with biosafety legislation. In nations that are not yet regulated for biosafety but are signatories to the Cartagena Protocol on Biosafety (CPB) (http://www.biodiv.org/biosafety/default.aspx), notice is required before any genetically modified organisms (GMOs) are moved across borders for release into the environment. When a product is released into the environment in the European Union (EU), either as an experimental release (2001/18/EC part B) or as a commercial release (2001/18EU part C) (http://gmoinfo.jrc.it/), the EU system requires that a summary of the notifier's application and the assessment report be made available to the public. When it comes to government agencies in the United States, some (like the USDA) make their applications and notices available to the public, while others (like the FDA) only do so upon request. According to South Africa's GMO Act, information on the GMO's description, the release's intended purpose and location, the release's monitoring, and the evaluation of its environmental effects "must not be kept hidden."

Strategies for reducing the impact

Low-tech methods, such as careful planning and execution of each operation, are primarily what is needed to prevent pharmaceutical crops from entering the food chain. To prevent the introduction of the industrial characteristic into traditional breeding stock, the crop must be cultivated in isolation from breeding materials. Given the difficulty in detecting such mixture in reality, proper rules for handling and labeling are crucial. Similarly, traditional agricultural crop experiments must be conducted separately from both local and large-scale field trials. To prevent accidental pollination from occurring, commercially produced parent seed and commercial crops must be cultivated in isolation from other plants of the same species or wild relatives.

For plants that rely on either wind or insects for pollination, achieving an adequate amount of isolation may be a significant challenge. In all likelihood, the new crop shouldn't be produced in areas where it may come into contact with food crops or related wild weeds. places where the species is often cultivated for human consumption, or where it is abundant in the wild.

The qualities of the chemical, the biology of the crop, and the nature of the environment in which it is cultivated are only a few examples of the variables that will determine the most effective mitigation methods for a certain pharmaceutical crop. Here are a few of the recommended preventative measures: (reviewed by Commandeur et al. 2003; Dunwell 2005).

• Using marker genes to make the crop or its products (such as seeds) physically distinguishable from food and feed crops, such as DsRed (Disco- soma sp. red fluorescent protein; www.clon- tech.com/).

Injecting the crop with a bitter or unpleasant flavor to make it unattractive; Expressing the crop after harvest; Keeping the crop at a safe distance from sexually compatible crops, weeds, and feral species; Using barrier crops to reduce cross pollination

• Geographical and temporal separation to prevent genetic mixing of crops. The risk of cross-pollination may be reduced by sowing crops at intervals of time other than when they are being harvested.

Pollination may be avoided by physically removing flowers from a garden. In order to prevent pollen from spreading from the female transgenic parent, tassel removal is a common isolation method in maize.

Agricultural crops and their products should not come into contact with PMP crops, hence precautions should be taken to prevent this. Measures to prevent the negative consequences of volunteer plants growing in subsequent years; partial processing of the pharmaceutical product at the production site; secure land with security fencing; dedicated agricultural machinery; dedicated storage facilities; secure methods of transporting seeds for establishing the crop; and secure methods of transporting the pharmaceutical containing crop product (such as seeds) and crop residues.

Conclusions

While there are certainly advantages to using maize in pharmaceutical manufacture, numerous biotechnology firms have looked at the viability of using a variety of crops, and thus far, no one crop has stood out as the obvious winner. The preferences of one company may differ from another's based on the specifics of its business strategy. The adoption of a food crop is likely to have the greatest impact on the social and political factors that drive crop selection. It would seem that maize's numerous benefits in the short-term include its familiarity, infrastructure, scalability, product stability, and processing simplicity. The introduction of alternative crops has the potential to streamline production and improve public image over the long run. If genetic modification (GM) technology is to reach its full potential, it is imperative that it get widespread public support. Thus, it is important to thoroughly examine both food crops and non-food crops.

The anti-HIV monoclonal antibody production under the Pharma-Planta initiative will center on maize. The product (a topical cream) has entered phase 1 clinical testing, and the crop will be cultivated in a controlled environment. The potential of tobacco as a supplementary crop is being studied. By addressing many of the issues raised in this article, it is intended that this program would serve as a beta test for the emerging regulatory requirements for PMPs.

There is a substantial possibility of a demonstrable public benefit from pharmaceutical crops. Any mistakes made with pharmaceutical crops might represent a big setback for their future implementation, especially in light of the existing bad perceptions surrounding GM crops. Any manufacturing system that has the potential to provide universal access to medications, especially in countries with substantial poverty, deserves careful consideration for the value it might provide to mankind.

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The IPWG discusses the challenges and opportunities facing the pharmacogenomics industry.

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Abstract

Genomic data has been increasingly used by the pharmaceutical industry in the identification of therapeutic targets and the development of precision medicine strategies. Large pharmaceutical corporations often acquire DNA samples from trial participants and undertake pharmacogenomic (PGx) investigations as part of their standard operating procedure. However, there are several obstacles to implementing PGx trials throughout clinical development. Among these obstacles include the need to respond to a globally regulatory climate that is in a perpetual state of flux, difficulties in research design and clinical execution, and rising worries about patient privacy. The availability of massive genetic databases connected to patient health information, the rising usage of polygenic risk scores, and the direct sequencing of participants in clinical trials are all examples of how advances in genomics are creating new possibilities for pharmaceutical firms. Companies in the pharmaceutical industry that are involved in pharmacogenomics work together as part of the Industry Pharmacogenomics Working Group (I-PWG). Here, the I-PWG offers a bird's-eye view of the initiatives being undertaken by the pharmaceutical industry to meet these difficulties and seize new scientific openings.

Introduction

It is now common practice for pharmaceutical firms to gather DNA samples from trial participants, particularly in preliminary stages of clinical studies. However, there are a number of obstacles that businesses may encounter while adopting PGx research, ranging from the ever-changing global regulatory framework to rising concerns about patient privacy and data access. Meanwhile, recent breakthroughs in genetics have opened up exciting new avenues for PGx study. Due to the dramatic drop in price of high-throughput sequencing and genotyping over the last several years, businesses frequently do complete genetic profiling of clini- cal trial participants. An increasing amount of patient health and genetic data is being stored in large databases, making these records invaluable tools for the pharmaceutical industry. These databases may be used for both the discovery of new targets and a more in-depth analysis of already discovered ones. An organization of pharmaceutical industry professionals, the Industry Pharmacogenomics Working Group (I-PWG) company that is now engaged in the area of pharmacog-enomics. Each year, the 26 member companies that make up the I-PWG conduct hundreds of clinical studies that need DNA collection as part of their procedures. This I-PWG viewpoint aims to provide an overview of the opportunities and threats that pharmaceutical firms encounter in the area of clinical pharmacogenomics (PGx). Future clinical trials may benefit from industry-sponsored PGx investigations if researchers and regulators collaborate to resolve the problems mentioned in this viewpoint while also taking advantage of new scientific prospects.

Pharmaceutics

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Recent breakthroughs in science: sequencing participants in PGx clinical trials

Companies commonly do extensive genetic characteriza- tion of individuals in clinical trials due to the declining costs of high-throughput sequencing and genotyping in recent years. According to an unpublished 2017 survey of I-PWG members, almost 80% of member businesses reported employing next-generation sequencing (NGS) technologies for internal PGx investigations (in at least one study), and more than a third of member companies were using these technologies extensively. There were 53 percent of firms that said they used NGS for whole-genome sequencing and 71 percent that said they used it for whole-exome sequencing in clinical trials. Companies claimed that NGS technologies were being employed for PGx investigations in a wide variety of non-oncology therapeutic areas, including as cardiology, neurology, immunology, and rare disorders, however cancer was by far the most common use. Incorporating next-generation sequencing (NGS) into clinical trial samples enables a more in-depth genomic examination of trial participants and, perhaps, a more expansive study for PGx analysis that takes into account both common and unusual genetic variation (Schwarz et al. 2019).

Problems with Global Regulations for Clinical PGx Studies

The European Medicines Agency (EMA) and the Food and Drug Administration (FDA) both recommend collecting DNA samples for PGx evaluation throughout all phases of clinical development (EMA 2018; FDA 2013), but this is not always possible due to global laws and regulations or the opinions of individual Investigational Review Boards and Independent Ethics Committees (IRB/IEC). Companies conducting worldwide clinical trials must comply with a complicated set of standards governing the collection and use of DNA and generated data for PGx research. Completely Extra! The laws, rules, and recommendations that have an effect on PGx studies are included in Table S1. This chart is not comprehensive, but it does include a substantial portion of the nations and regulations that are most often faced by sponsors.

Obtaining DNA samples or conducting genetic research is prohibited by law in several nations. The collection, maintenance, use, or provision of China's human genetic resources to foreign organizations, for instance, are all regulated under the country's Regulation of Human Genetic Resources. This rule was modified in 2019 to increase the severity of punishments for noncompliance and to further clarify the expanded scope of actions that fall within the purview of monitoring. In actuality,

Multinational corporations have been asked to provide information on the assay, vendor, and contract for carrying out genetic testing. These rules might also necessitate conducting tests on Chinese subjects inside China, which could increase the assay variability of global research. When combined, these rules might make it difficult for businesses to export samples or even gather them in the first place for future use. In addition, the law mandates that the Chinese partner get ownership of any IP developed throughout the course of the research collaboration (i.e., the clinical site in China). In addition, in October of this year, China enacted a new Biosecurity Law that aims to further strengthen the standards established in the control of genetic resources. Companies are still trying to figure out how this new rule will affect them, but in the meantime, it's making it harder than ever to gather biospecimens and evaluate people's DNA, which might slow the progress of pharmacogenomic research in China.

Resolution 340/2004 (NHC 2004) pertaining to genetic research and Resolution 2201/2001 on biorepository and biobank requirements have implications for the conduct of genetic research and storage of genetic specimens, including the requirements to share any biobanked samples with investigators in Brazil. Specific duties for the conduct of clinical trials with genetic research components are outlined in the Israeli government's Guideline for Clinical Trials in Human Subjects (2006). This may include an extra approval procedure dedicated only to the genetic research component. Last but not least, several nations have biobanking regulations that affect a company's capacity to biobank genetic material for research (e.g., Taiwan (MoHW 2019), Sweden (Regerings- kansliet 2002), and Finland (MoSAH 2012)).

Further complicating matters is the fact that different laws and regulations in various parts of the world address the issue of an individual's right to access their own genetic information that is derived from these samples. Research participants in Brazil, for instance, are guaranteed access to their genetic data, informed consent, and genetic counseling upon request according to Resolution 340/2004. Access to genetic information is also guaranteed under the Biomedical Research Law 14/2007 of Spain and by Italy's General Authorization No.8/2014 (IDPA 2014) for the processing of genetic data. Data privacy laws exist in some countries that grant citizens access to their own personal information, which may include genetic research results; examples of such countries include Norway (2000) and Argentina (2000). More generally, the EU General Data Protection

Regulations (GDPR) also grants citizens access rights to personal data (GDPR 2016). In addition, several regional and national ethical bodies have taken stands on the repatriation of inci- dental results. Research ethics regulations in Denmark have been established by the National Committee on Health Research Ethics (NVK). that uses whole-genome sequencing and requires therapeutically relevant data to be sent to participants proactively if they wish to have it returned to them (NVK 2020). Similarly, the Ethics Committee for Clinical Research (CEIC) in Portugal has established guidelines for dealing with unexpected results from genetic testing in the course of clinical studies.

Returning genetic information to people who participated in clinical trials is a complicated process, the details of which have been covered at length elsewhere (Downey et al. 2018; MRCT-Center 2017; Prucka et al. 2015). It is important to remember that providing individual genetic data to participants and their healthcare practitioners may be unethical and illegal in PGx research that is being undertaken for exploratory reasons (Thorogood et al. 2019). Analytical and clinical validity standards for diagnostic testing are not often met by the research-grade assays typically used in PGx studies. Quality standards for the testing of human specimens in laboratories for the purposes of disease diagnosis, prevention, and treatment were set, for instance, in the United States by the Clinical Laboratory Improvement Amendments (CLIA) (CLIA 2003). No clinical decision should be made based on genetic research data that were not generated in a CLIA-certified lab and did not fulfill proper analytical criteria. These results should be seen as exploratory in nature (MRCT-Center 2017). Since most clinical studies rely on underpowered exploratory research, it is important to think about how to interpret the data. While progress has been made, sponsors still face a minefield when trying to decide if, when, and how genetic data from worldwide trials should be returned to participants.

Disparities in the use of con-

Challenges for using PGx techniques in global research include the duct of PGx studies and even local variances in IRB/ EC requirements and preferences. Because of these variations, it may be difficult to manage and keep track of things like informed consent agreements, data needs, and constraints on sample usage. Although the significance of these regional requirements is recognized, the administrative effort required to handle them may discourage the collection and use of PGx samples from certain locations, thereby compromising analytical rigor and the transferability of results to other areas. We think the scientific and regulatory community can overcome some of the difficulties caused by this complexity and help forward vital genetic research.

Clinical development pitfalls for PGx analysis

Clinical trial data genetic analysis may serve as a foundation for better decisions across the clinical development life cycle, opening up new avenues for patient classification and commercialization of therapeutic value propositions (Nelson et al. 2016). However, doing genetic analysis during clinical development presents a number of obstacles and constraints, including as small research sizes, lack of worldwide representation, and issues in validating results.

To begin, the majority of clinical trials do not prioritize testing a genetic and/or PGx hypothesis. The primary goal of a study is to test a treatment hypothesis, and its power is calculated so that any differences in safety and effectiveness may be detected. Except when data from numerous trials are combined, phase I studies seldom have enough participants to perform even candidate variant analysis (Guo et al. 2019; Kobie et al. 2019). It is common for genome-wide association studies to be underpowered, especially in bigger phase II and phase III research.

One such difficulty is the general lack of variety in clinical trial populations. Most people who take part in clinical trials are of European descent (FDA 2017). The existing corpus of research in genetics is limited by this imbalance, and it is not specific to genetic analysis performed in clinical trials (Popejoy and Fullerton 2016). However, key signals may be overlooked in clinical practice if global genetic diversity is not effectively captured in PGx investigations. In fact, non-European groups either lack or have much greater frequencies of numerous recognized clinically important PGx indicators. Certain East Asian and South Asian populations have the HLA-B*15:02 allele, which is linked to skin responses to carbamazepine and oxcarbazepine (Phillips et al. 2018). Another case in point: Asians seem to have a greater prevalence of the CYP2C19 poor metabolizer phenotype, which is linked to varying degrees of medication toxicity and ineffectiveness (Scott et al. 2012). The inability to identify such relationships depends on the inclusion of a sufficiently broad sample of the population. Furthermore, when the number of participants from whom to draw conclusions is small, assessing the applicability of results from a genetic association study of drug response done in a dataset dominated by people of European ancestry to other (non-European) groups may be difficult.

Lastly, the information gathered from early clinical development programs is probably the first and only information available for new chemical entities and/or medications with unique mechanisms.

Therefore, it is difficult to validate or deny fresh genetic discoveries until further clinical trials have been undertaken. However, it may be difficult to interpret PGx data from subsequent clinical trials because to changes in clinical trial design, population heterogeneity, and a lack of statistical power for replication (Hopewell et al. 2019; Shen et al. 2020). The danger of an uninterpretable, unconfirmable exploratory discovery may exceed the upside potential, and this ambiguity in interpreting therapeutic usefulness for genetic analysis during drug development is a general barrier for commencing exploratory research.

Studying the ADME of PGx is not without its difficulties.

The majority of the known PGx correlations may be attributed to genetic variations that cause changes in drug metabolizing enzymes and drug transporters (FDA 2015; Tremaine et al. 2015). Such variations might affect medication safety or effectiveness because they modify enzyme or transporter function, resulting in inter-individual variability in exposure that can go beyond the therapeutic window for small molecules. There are a number of obstacles that are particular to PGx research in early phase studies, in addition to the problems with small clinical trial sizes that we've already covered. Emergence of novel variations with clinical importance, and the possibility of ambiguity in defining metabolic routes for new medicines in early clinical development, are two examples. To maximize the likelihood of success when conducting analyses in very small trial datasets, PGx studies in early phase studies should be conducted in a targeted manner, giving higher priority to variants in genes that have been shown via preclinical work to be important for the disposition of the compound. However, major and minor metabolic pathway investigations in vitro are generally not finished prior to phase II or even pivotal trials in humans. Therefore, it is generally necessary to combine as much PK data from early phase clinical trials as feasible to increase statistical power and evaluate a larger group of ADME genes. The population PK modeling estimates of PK parameters from bigger phase II/III studies may also be utilized to evaluate the possible effect of variations in ADME genes (Guo et al. 2019; Kobie et al. 2019). However, it's possible that these data sets don't have enough statistical power to discover genetic connections. In instance, individuals may have more than one functional mutation in a set of metabolizing enzymes, which makes it difficult to find uncommon variations that potentially alter safety exposure. Drugs that are primarily metabolized by highly polymorphic Cytochrome P450 enzymes (CYPs) like CYP2D6 have been largely phased out of use in recent decades as rational drug design has shifted focus to maximizing the distribution of drug metabolism across many CYPs and other enzyme families. Even yet, the possibility is not completely nullified possibility that a patient's exposure will vary due to the presence of a poor metabolizer phenotype in two of those enzymes. Studies of PGx have also shown promise in illuminating the potential importance of other metabolic clearance pathways, such as glucuronidation and the function of membrane transporters (Desai et al. 2003; Guillemette 2003; Yee et al. 2018). PGx investigations may be necessary to understand the possible influence of genetic polymorphisms in these other groups of metabolic enzymes or in membrane transporters on PK and pharmacodynamics, despite the typically weaker previous clinical data supporting their functional impact.

Genotyping for ADME genes should be performed in both early and late stage clinical trials, and it is advised that the metabolic pathways of all incoming clinical candidates be thoroughly evaluated. It is reasonable to investigate the possible impact during NCE development if the medication is metabolized via pathways with known polymorphism variation and there is substantial evidence suggesting clinically important effects for other authorized agents in the same class. When there is unexpected PK variability that cannot be explained by standard PGx genotyping, the European Medicines Agency (EMA) has issued PGx recommendations that suggest the possible use of wider, whole-exome or whole-genome sequencing to investigate potential new vari- ants (EMA 2018). Additional difficulties arise with more comprehensive genome sequencing, such as the potential requirement for regulatory authorities to demand phenotypic confirmation of new variations.

Clinical implementation challenges

The number of clinically relevant indicators that might be used to enhance patient care is rising (FDA 2015; Relling et al. 2020), however despite the commitment of resources by business and academia in developing PGx biomarkers, such information is still not frequently employed in clinical practice. Many factors contribute to this; these have been reviewed at length elsewhere (Chenoweth et al. 2020; Klein et al. 2017) and include, but are not limited to, challenges with ordering, reimbursement, and interpretation of genetic tests; a lack of education for both patients and clinicians; and limited evidence supporting the clinical utility and health economic value of many PGx bio-markers. Further, it has been found that different regulatory agencies for the same drug have different recommendations for PGx testing included in drug labels (Koutsilieri et al., 2020; Shekhani et al., 2020), suggesting that the lack of consensus guidelines for genetic testing and implementation may be an additional barrier for clinicians attempting to incorporate PGx information into clinical practice.

The obstacles to clinical implementation have been studied and attempts made to remove them. Organizations

such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) and

For clinical use of PGx data, the Dutch Pharmacogenomics Working Group (DWPG) has developed recommendations (Bank et al. 2018). Some hospitals and healthcare networks have begun doing pre-emptive PGx testing (Cecchin et al. 2017, Dunnenberger et al. 2015) so that doctors don't have to wait for test results before giving medicine. For certain commonly prescribed medications, studies have been conducted to establish the clinical validity, utility, and economic worth of PGx biomarkers (Anderson et al. 2007; Claassens et al. 2019; Pereira et al. 2020; Wadelius et al. 2009; Zhu et al. 2020). However, genetic testing are still not widely used in clinical practice, and the lack of a PGx companion diagnosis is often seen as a major roadblock in the pharmaceutical industry. Industry's emphasis on and investment in PGx research is projected to increase as PGx data gradually finds its way into clinical practice.

It's important to remember that environmental, anthropometric, and genetic variables, as well as biological subsystems impacted by the illness, may all interact to produce a medication response that is very complicated in many circumstances (Armstrong 2008). This means that the use of genetic markers, biomarkers, and other single stratifying factors is likely to be constrained by the fact that no one factor is likely to capture the entire extent of the complexity involved and provide sufficiently accurate predictions for therapeutic use. This motivates researchers to look into novel avenues, such as polygenic risk scores (discussed further below) and machine learning techniques, for further progress in the area. Increases in processing capacity and the development of machine learning algorithms have made it possible to combine different forms of data for a more complete picture of a patient's reaction to a treatment, leading to greater accuracy in predictions and easier clinical translation.

Polygenic risk ratings in PGx research: a new frontier

The use of polygenic risk scores to PGx research is a hot new topic in the field. The use of polygenic risk scores for coronary artery disease (CAD) precision medicine has been recommended by a number of research. Patients with higher CAD polygenic risk scores have been shown to benefit more from statin therapy, according to two meta-analyses (Mega et al. 2015; Natarajan et al. 2017). Patients with high polygenic risk scores for coronary artery disease had more clinical benefit from therapy with PCSK9 inhibitors in two large, independent retrospective investigations (Damask et al. 2020; Levin and Rader 2020; Marston et al. 2020). Likewise, polygenic risk scores have been investigated in the prevention of atherothrombotic events. In a retrospec- tive PGx study of clopidogrel, Lewis et al. identified a poly- genic risk score that was associated with increased platelet reactivity, risk of developing major adverse cardiovascular events, and risk of cardiovascular death (Lewis et al. 2020). Finally, in the field of oncology a recent study found that high vitiligo, high psoriasis, and low atopic dermatitis polygenic risk scores were associated with longer overall survival after treatment with atezolizumab (anti-PD-L1) monotherapy compared to treatment with chemotherapy in bladder cancer patients (Khan et al. 2020).

Patient enrolment in clinical trials may be improved with the use of polygenic risk ratings. It is possible for people with high polygenic risk scores to have illness risk equivalent to that seen in those with monogenic disorders (Khera et al. 2018). There is a possibility that clinical trial sizes may be reduced or the length of event-driven studies could be shortened if individuals with high polygenic risk scores were included selectively. Although the association between polygenic risk scores and treatment response is a relatively new topic of inquiry in drug development, it is expected to garner increasing scientific attention across a wide variety of illnesses and therapeutic domains in the future.

Applying methods like polygenic risk scores and machine learning to patient selection tactics in clinical research has regulatory ramifications, including potential effects on medication labeling and the necessity for a companion diagnosis. As a result, once a medicine is authorized, regulatory agencies want to know that there is a reliable way to identify the patients who would get the most benefits from it and that the label appropriately represents the enrichment tactics used to choose them. Regular communication with regulatory agencies throughout medication development is essential, as is consideration of the impact of enrichment tactics on labeling and the path to approval of any test intended as a companion diagnostic (FDA 2019).

Patients with common cancers and their families in the NHS, as well as those with rare diseases and their families substances currently available on the market (Diogo et al. 2018; McInnes et al. 2020). The success rates of drug development projects are higher when the medication target has genetic proof to back it up

(Nelson et al. 2015). It's possible that various types of genetic variation might provide light on potential therapeutic targets. In particular, LOF variations have attracted a lot of attention because of their potential as therapeutic targets. When this kind of genetic polymorphism is protective against illness risk, it may analagize to the actions of therapeutic antagonists (like PCSK9) (Cohen et al. 2006). Phenomenon-wide association studies (PheWAS) inside these massive datasets may characterize pharmacological targets to find new indications, related indications, or even possible safety flags (Diogo et al. 2018; Jerome et al. 2020). Additional evidence in favor of variations found by PGx analysis of current clinical trials for medications in development may be gathered from the material included in these huge databases. In conclusion, unique genetic patient subpopulations may be discovered for precision medicine clinical development programs or for call back studies to do further in-depth patient phenotyping.

Summary

Pharmaceutical corporations have already invested extensively in genomic technology, databases, and PGx research, and this trend will only accelerate. From initial target identification through late-stage clinical development, genomics is now an essential aspect of the drug development process. Although the difficulties highlighted here are substantial, they will be overcome as pharmaceutical firms increasingly use precision medicine tactics across the drug development process. One of the biggest obstacles to doing international research is the ever-evolving legal and regulatory framework in which such studies must be conducted. Worries, while

While worries about patient privacy and the exploitation of patient data are warranted, too restrictive laws would stifle progress in PGx discoveries and, by extension, precision medicine for the world's populations as a whole. Positively, developments in genomic technology are accelerating, and pharmaceutical firms are adapting to the new landscape. In the next years, clinical trial PGx investigations will likely benefit from the addition of patient-level sequencing, polygenic risk scores, and data from massive electronic health record (EHR)/genomic databases to seed their results.

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The evolving nature of pharmacy practice and the consequent requirement for a revised educational framework

C. S. Parameswari, S. Afreen Jaha, Dr. G. Nagarajan

A B S T R A C T

Pharmacy The practice of pharmacy has gone from an early concentration on drugs to a more recent shift toward a greater emphasis on the needs of individual patients. Although pharmacists formerly had a larger role in the development and production of pharmaceuticals, this function has been greatly diminished during the last century. With this newfound responsibility, pharmacists must collaborate with other members of the healthcare team to improve patient care and ultimately help the world move closer to reaching the Millennium Development Goals. To keep up, modern pharmacists need to broaden their responsibilities to encompass pharmaceutical care, transforming them from a commodity medication vendor into a critical member of the healthcare team. As a result, it is incumbent upon pharmacy colleges to provide a curriculum that is up to date with the evolving responsibilities of the modern pharmacist. Learning to think critically, solve problems, and make educated decisions about drug treatment are all abilities that should emerge from this training. The student should learn to work with other health professionals and improve the quality of life for people locally and globally via better health, as well as to generate, disseminate, and apply new information based on cutting-edge research in the pharmacological, social, and clinical sciences..

Keywords: Healthcare from a Pharmacist Moderate medical treatment Rational pharmacotherapy in the pharmacy curriculum

Introduction

Pharmacy profession

Pharmaceutical compounding was the primary focus of pharmacists in the previous century. The compounding functions were drastically cut down during the last decade, and the dispensing side became the primary focus. However, dispensing alone isn't enough to meet demand, thus a new function for the profession has had to emerge in recent years. 1,2 A modern pharmacist has to be more than just a drug salesperson; they need to be trained in phar- maceutical care principles that elevate them to the level of a health care professional. 3 Helping individuals and communities maximize the benefits gained from pharmaceuticals and other health care options is central to the profession of pharmacy. 4 Problems with drugs are identified, prevented, and resolved, and healthy medication and lifestyle habits are promoted.

patient-centered therapies centered on promotion and education, leading to improved therapeutic outcomes.

As stated in the previous paragraph, it is important to evaluate the standards for applicability when there is a gap between how they are used in various environments. In addition, with the introduction of new duties comes the need for pharmacists to rethink traditional workflow models and the distribution of responsibilities. 6 The drug information practice model, the self-care practice model, the clinical pharmacy practice model, the pharmaceutical care practice model, and the distributive practice model are all examples of different types of practice models. 7 Depending on the pharmaceutical demands of a given region, the available resources, and the level of respect shown to pharmacists, one or more of these models may be implemented.

Pharmaceutics

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1. The pharmacist's role in promoting safe and effective drug usage

When it comes to making choices about medications, the pharmacist's input is crucial for encouraging safe and effective usage. Promoting rational medication use requires pharmacists to adapt to their new roles as patient counselors and educators in outpatient settings (RUD). 8 The procedure The quality of medication usage is governed by the information provided by pharmacists and other healthcare professionals throughout the drug's acquisition, storage, distribution, and dispensing processes.

To have access to "medicines suited to their clinical needs, in dosages that satisfy their own specific requirements, for an acceptable period of time, and at the lowest cost to them and their community," as stated by the World Health Organization, constitutes a 9 RUD (WHO). 10 The pharmacist's job is to make that the correct dose of the correct drug is given to the correct patient at the correct time through the correct channel. 11 Academic training of pharmacists and related health professionals is one method of achieving this goal. Countries like Pakistan have already taken this step, with all Doctor of Pharmacy students required to study the WHO's rational drug module.

Pharmacy ethics

Ethical standards can only be derived from broad, overarching ideas that are grounded in widely held Western ideals. Unequal access to the health care system seems to be the ethical issue. The role of the pharmacist may be better defined by an examination of pharmacological treatment from an ethical perspective. To a large extent, the field of pharmacy is intertwined with the notion of non-maleficence. There has to be greater attention paid by pharmacists to the potential side effects of drug treatment. Therefore, the pharmacy profession places a premium on monitoring the patient's medication, identifying and preventing probable bad effects, conducting good communication, and providing appropriate information on the use of medicines. This may be made easier by the identification of patient subsets requiring medicinal attention. 12 The ethical standards for pharmacists should be followed.

1. **Pharmaceutical care**

As the idea of pharmaceutical care develops, a more systemic and complete approach is needed to ensure that patients are protected against adverse drug reactions and that existing issues with medications are addressed. 13,14 It was in the 1990s when Hepler and Strand, two American researchers, first presented the idea of pharmacological care. 15 In contrast to conventional pharmacies, where the emphasis is on the order (prescription/OTC), which is fulfilled on demand, and the pharmacist, who is oriented to the drug product, obeys the order that he or she receives, the focus of the process is on the patient, and it is continuous in its delivery with the strategy to anticipate and improve the patient outcome of the drug therapy. The care model places a premium on the pharmacist-patient connection and the patient's active participation in the treatment process, both of which improve the likelihood of a positive clinical result. Pharmacists use a standardized process called the pharmacist's workup of drug therapy (PWDT) to provide pharmaceutical care to patients. This involves gathering patient history, creating a CORE pharmacotherapy plan, identifying the patient's PRIME pharmacotherapy problems, and writing FARM (FINDING, ASSESSMENT, REGIME, and MANAGER) progress notes. Academics and training programs have responded to the birth of this notion by emphasizing the philosophy of pharmacological care in all facets of the pharmacy profession. 16 In order to provide effective pharmaceutical care, pharmacists need to acquire knowledge and practice skills in areas such as patient assessment, education, and counseling; the formulation of individualized care plans; the implementation of treatment protocols; the titration of medications; the choice of therapeutic alternatives and preventive therapies; and the formulation of dosing schedules. But many locals are unfamiliar with the idea.

pharmacists working in many of the developing countries.[17] Moreover, there are many barriers to adoption/adaptation of pharmaceutical care idea in reality, including as experts' inadequate knowledge of the topic, a dearth of available training opportunities, limited financial resources, and, most importantly, a lack of political will to see the plan through. The curriculum is sometimes insensitive to changes in the pharmacy practice industry. In order for pharmacists to aid their countries in achieving their Millennium Development Goals and commitments, each region and country will need to develop its own model of practice by taking into account its unique context and outlining the specific steps and actions necessary for getting started.

2 Drug treatment based on solid scientific evidence

If pharmacists want to provide the highest standard of pharmaceutical treatment, they must shift their focus from opinion to evidence. 4,18 The adoption and application of clinical guidelines in everyday practice is a significant difficulty in most of the contexts, including the pharmacy, and has only recently gained notice despite having been there since the early 1990s. 19 There is evidence to show that patients often get treatment that is both ineffective and perhaps hazardous, yet is outside of the doctors' purview. 2,20e22 With the constant emergence of new medical sub-disciplines, it may be difficult to stay up with the state of the art. Evidence-based practice is predicated on the idea that all choices on actual practice should be founded on research papers chosen in accordance with strict criteria for the quality of quantitative, qualitative, and theoretical research. 23 Some argue that it is challenging to put evidence-based medicine into practice; one approach to do so is to improve information systems in order to give decision support; this will aid in reducing the likelihood of mistakes being made while making treatment recommendations. 24 A curriculum mindful of the maturation of expertise in evidence-based pharmacy is urgently required.

Teaching in the Pharmaceutical Sciences

For the purposes of the Taskforce, "pharmacy education" shall mean the educational structure and capacity to train personnel for a wide range of service provision and competence (e.g., technical support staff, pharmacists, and pharmaceutical scientists) in a variety of settings (e.g., community, hospital, research and development, academia) (e.g., under- graduate, postgraduate, lifelong learning).

Education for pharmacists varies in both time and scope from one nation to the next.

27,28 The fundamentals of pharmacy education are universal, although the pharmaceutical care philosophy varies by area and nation. 29e35 It has recently been shown that simulation centers for health professional schools may provide a fresh approach to teaching and assessing health

care procedures at the individual level.

Pharmacy faculty pedagogical interventions

Improving pharmacotherapy education is necessary because of the growing need for competent pharmacy services. Pharmacologists usually speak during pharmacotherapy classes at universities that provide both medical and pharmacy degrees. However, putting their theoretical pharmacotherapy knowledge into reality is typically a challenge for pharmacy students. As a result, new approaches (like the Groningen model) are being developed for pharmacotherapy education at medical schools. Recently, the 'Turkish Pharmacological Society'37 established a unique model based on dispensing ratings, and Turkish and Northern Cyprus universities provide courses in "problem-solving methods"–based rational pharmacotherapy.

Four Point Strategy for Pharmacy Education

Pharmacists are well positioned for job shifting in health care due to their familiarity with both medications and clinical therapeutics; they may also be taught to do other activities, such as clinical management and laboratory diagnostics. Although research shows that pharmacists are excellent resources for patient care and public health initiatives, they are underutilized globally. To ensure that there is a sufficient supply of competent pharmacists available for such positions, there must be a concerted and comprehensive push to improve workforce planning, training, and education.

However, health care requirements and practice standards, as well as educational opportunities, differ significantly among nations. Consequently, the World Health Organization; the United Nations Educational, Scientific, and Cultural Organization; and the International Pharmaceutical Federation formulated the Global Pharmacy and the Education Action Plan 2008–2010. To achieve/ensure the competence, it is necessary to define a vision, frameworks, guidelines, and case studies; gather evidence and advocate for change; speed up action in individual countries; and provide a worldwide forum for dialogue. Quality assurance, academic and institutional capacity, competency, and vision for pharmacy education are the four pillars around which the Action Plan is built. The framework was created and refined in the course of two FIP-sponsored international workshops on pharmacy education (Inter- national Pharmaceutical Federation). The Taskforce will keep an eye on it to see how far along it is in its larger mission of "disseminating evidence-based guidance and frameworks that facilitate the development of pharmacy education (and higher education capacity) to enable sustainability of a pharmacy workforce appropriately skilled to provide pharma- ceutical services."

2. Conclusion

Pharmacy colleges need to adapt their curricula to reflect the new realities of pharmacy practice and prepare their graduates for the expanding responsibilities of the pharmacy profession. Ability to think critically, solve problems, and make decisions during medication are all qualities that should be fostered by this training. The student should be prepared to collaborate with other health professionals, improve the quality of life for people in our society and the global community through better health, and create, transmit, and apply new knowledge based on cutting-edge research in the pharmaceutical, social, and clinical sciences.Conflicts of interest

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How Maltese citizens see their neighborhood drugstores and pharmacists

C. Anusha, S. Neelofer Sulthana, Dr. B. V. Ramana

Abstract

Our goal was to get an idea of how regular Maltese citizens feel about their neighborhood pharmacy and the services it offers. Psychometric evaluation was used to look at the trustworthiness and validity of a self-reported survey. Five hundred clients were surveyed, or 10 from each of fifty community pharmacies. They conducted a few descriptive analyses.

Standout outcomes Ninety-five percent of respondents said they were extremely or very satisfied with their pharmacists in terms of their responsiveness to their requests, while ninety-one percent said they were satisfied with their pharmacists' ability to maintain confidentiality when discussing their patients' medical histories, and ninety percent said they were satisfied with the professional nature of their interactions with their pharmacists. The pharmacy had the lowest satisfaction rate (69%) for personal space. More than eighty-seven percent of respondents said they would use a diagnostic test performed by a pharmacist, and eighty-three percent said they would use the pharmacist for longer hours.

Conclusions Consumers in Malta generally have a favorable impression of community pharmacies and the services they provide. They supported expanding existing professional services to accommodate more clients..

Keywords neighborhood druggist; neighborhood pharmacy; neighborhood patient; neighborhood patient satisfaction

Introduction

Quality of treatment may be gauged by looking at how satisfied patients are with their primary care providers. The pharmacist's role may be assessed in terms of both quality and patient satisfaction, allowing for adjustments to be made in response to feedback about the pharmacist's emphasis on care. Public education regarding the community pharmacist's role in health care delivery may raise awareness of how pharmacists may utilize their pharmacological and illness expertise to enhance patient outcomes (as stated in [1,2]). Primary care community pharmacists have challenging decisions as they juggle commercial and professional responsibilities. Since most governments don't finance community pharmacies [4], they need to make a profit. How effectively to balance a community pharmacist's commercial and professional responsibilities is a matter of ongoing debate. Community pharmacists who place profit maximization ahead of customer happiness will see advising patients on appropriate medication use as a lost opportunity and will spend less time doing it. A community pharmacist's responsibilities include, but are not limited to, consulting with patients to learn about their symptoms and other pertinent information, making product recommendations based on that data, and coordinating treatment with the patient's physician and other healthcare professionals. [6–8] A pharmacist has an ethical obligation to refuse to dispense a product if he or she has reasonable suspicion that doing so may endanger a patient. Professionals have succeeded when they are able to refuse service to a consumer while still giving them a reasonable, personable justification.

prescription drugs instead of worrying about the bottom line.

Community pharmacists' promotion of a service focused on individual patients is expected to boost public opinion and, in turn, revenue.

A Maltese community pharmacy

Independent and a part of the European Union, Malta is a sovereign nation. Malta has one of the greatest pharmacy to population ratios in the European Union, with 209 community pharmacies serving a population of around 414 000. [9] The only place to get any kind of medication, prescription or otherwise, is in a community pharmacy. In Malta, community pharmacies are independently owned and operated for profit without government assistance. According to a schedule set by the Malta Medicines Authority, community pharmacies in Malta are open from 9:00 am to 12:00 pm and from 16:00 pm to 19:00 pm on weekdays, and from 9:00 am to 12:00 pm on weekends and public holidays.

According to findings from a 2009 research conducted in the country, 30% of Malta's pharmacists are employed in community pharmacies. There is a high need for pharmacists in the healthcare consulting industry (16%), as well as in hospital and clinical pharmacy (9%), industrial pharmacy (8%), regulatory affairs (4%) and pharmacy management (3%). Not practicing, teaching, retiring, or living abroad account for the remaining 5-10% of pharmacists.

Pharmaceutics

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MOU with Government General Hospital &KMC, K urnool

prescription drug store .[10]

The purpose of this research was to learn how Maltese customers see their local pharmacists and how satisfied they are with the pharmaceutical services now available in Maltese pharmacies.

Method

Sample Selection

Every community pharmacy in Malta was included in the sample period. These were then tabulated alphabetically in a Microsoft Excel spreadsheet, separated into the five districts specified by the Maltese National Statistics Office. Starting with pharmacy number one in each district, the Excel file lists the pharmacies in numerical order. Using a random number table and a stratified random sample method, ten community pharmacies were chosen from each district. Using convenience sampling, we were able to collect data from 500 customers at 50 different pharmacies.

Statistics from the Questionnaire

A questionnaire for self-completion was prepared. The questionnaire was developed after a thorough examination of the relevant literature. [1,5,11–17] The survey asked respondents about their experiences with pharmacies, the quality of care they received, their impressions of the pharmacist, how they felt about the pharmacist's expanded role in the community, and how they felt about having to pay extra for the convenience of having a local pharmacy treat minor ailments.

The questionnaire has 14 well-defined items and was offered in both English and Maltese. Questions on the questionnaire were closed and did not allow for free-form responses. including a comment box and demographic data collection questions. Attitudes and views of customers were gauged by presenting respondents with statements and asking them to indicate the degree to which they agreed or disagreed on a five-point Likert-type scale. When answering questions about the services provided by a community pharmacy, customers have the option of selecting several answers.

Evaluating psychological factors

The questionnaire underwent testing for its content validity, face validity, reliability, adaptability, and practicability. Ten participants (two hospital pharmacists, two community pharmacists, two primary care physicians, two pharmacy students, and two consumers) participated in a focus group to validate the questionnaire. Participants in the validation panel were given the survey and asked for feedback. After that, the investigator convened a roundtable discussion (FW). This was done to find out whether any crucial details were left out, if the structure made sense, and if the questions posed were easily grasped or if they needed to be reworded. Instructions were studied carefully.

Ten consumers were selected at random and given a questionnaire to fill out at time 0 (Test 1) and again a week later (Test 2) for the purposes of test/retest reliability testing (Test 2). Cronbach's alpha for the questionnaire was 0.90, which is rather high. It took respondents an average of 6 minutes to complete the survey (range 3–12 min).

Data collection

The investigator went to each of the 50 randomly chosen community pharmacies to collect signed permission forms. After that, the researcher met with the subjects for a total of fifty 3-hour meetings during which they filled out the questionnaire. These visits were spread out throughout the week, however most occurred between the hours of 16:00 and 19:00. This approach took some time, but it was straightforward, inexpensive, and yielded an impressively high rate of responses. Ten randomly chosen customers were given the questionnaire. Customers who came into the drugstore to make purchases or see a doctor at the clinic were approached, given information about the research, and given the option to take part. Patients were informed that their participation in the study was entirely optional, that their care would continue unaffected by their decision not to take part, and that their responses would be kept anonymous. Customers who were unable to complete the survey at the time were given a questionnaire and a self-addressed, stamped envelope to send it back later.

Results

Statistics on the composition of the consumer market

The whole research population consisted of 500 customers. It was a very even split between the sexes, with 52% women and 48% men. Nearly half of the market share (41%) had advanced degrees. The majority of buyers were between the ages of 36 and 45 (24%), while the average and median shopper ages were 40 and 38. (range 18–84 years). The majority of buyers were either managers, administrators, or executives (29%), followed by professionals (21%; think teachers, attorneys, and accountants) and technicians (21%), clerks (16%), and finally housewives (16%).

Consumer behavior about trips to the local pharmacy

Forty-eight percent of customers only go to their local pharmacy once a month or less, 32 percent go there twice a month, 14 percent go once a week, and just 6 percent go more than once a week. Most customers (67%) usually visited the same community pharmacy, 22% always visited the same community pharmacy and 11% seldom visited the same community pharmacy.

Ninety percent of customers at community pharmacies were there to fill a prescription and sixty-five percent were there to stock up on over-the-counter medications. Cosmetics and toiletries accounted for 40%, followed by advice from the pharmacist at 25% and infant items at 5%.

Eighty percent of customers who frequented a specific community pharmacy did so because it was conveniently located near their residence or place of employment.

Afterwards, 44% said they looked for a sympathetic and approachable pharmacist before deciding on a community pharmacy. The devotion of customers to their pharmacy (18%), the store's layout and appearance (17%), and the availability of a broad variety of items (38%).

Customer contentment with pharmacist traits

The majority of customers had a positive impression of their neighborhood pharmacy and were either very or somewhat satisfied with a variety of aspects of their service, including the pharmacist's efficiency in responding to their requests (95%), the pharmacist's clarity in explaining how to take medications (94%), the pharmacist's use of language (93%), the pharmacist's discretion (91%), the pharmacist's ability to maintain a professional relationship with each customer (90%), the pharmacist's ability to explain the mechanism of action of medications (86%), and the pharmacist' Both the level of privacy in the pharmacy (69%) and the interaction time between the customer and pharmacist (73%), received the lowest ratings from customers (Figure 1).

Aspects of business vs those of a professional nature

Overall, 56% of customers saw pharmacists as having a foot in both the

business and healthcare worlds, whereas 35% saw them more as healthcare specialists and 9% saw them more as entrepreneurs. Most customers who were polled regarding whether or not they would pay for services rendered by their local pharmacy's community pharmacists said they did not want to pay for any kind of service, even getting advice on whether over-the-counter medications were safe and effective for their specific

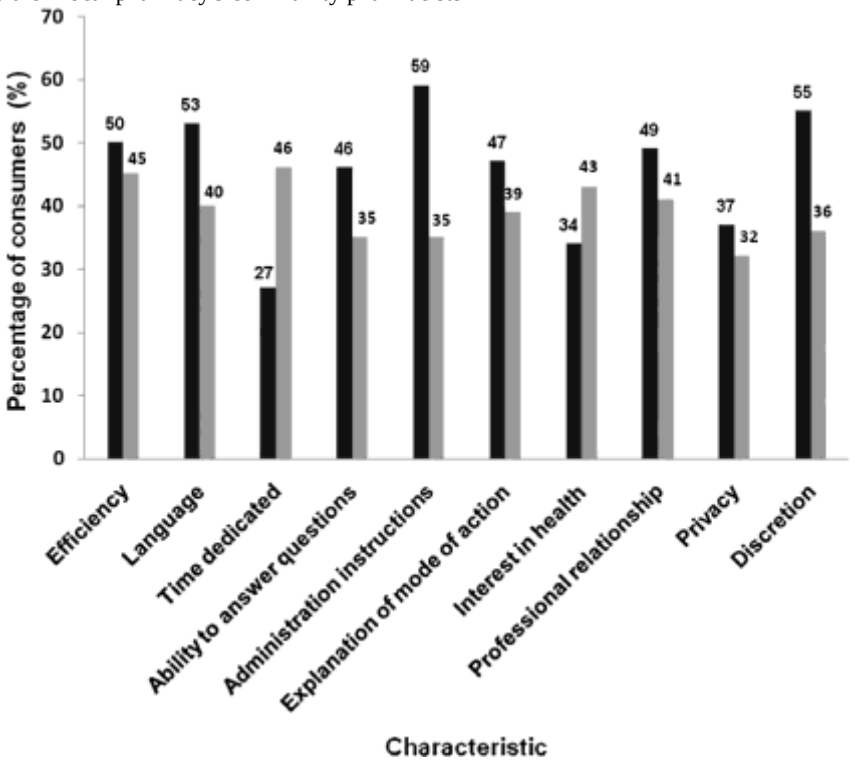

Figure 1 Satisfaction with pharmacist characteristics (n = 500). (■ Very satisfied; (▒ Fairly satisfied.

counsel offered while buying a prescription medicine (83%), and general advise given regarding any problems expressed (72%).

Purchasing non-prescription medication andhealth advice

Seventy-five percent of respondents said they would trust their neighborhood pharmacist's recommendation when buying an OTC drug. The majority of people (76%) would go to their family doctor before visiting their local pharmacy (11%). Family and friends (10%) and the internet (4% of all respondents) also provided helpful guidance.

Eighty percent of consumers would go to a community pharmacist for advice if their problem wasn't serious enough to warrant a trip to the doctor, fifteen percent would go to the com- munity pharmacist if they didn't have time to wait for a doctor's appointment, thirteen percent would rather speak with a pharmacist, and six percent would rather get free advice from a pharmacist. Consumers are more likely to seek guidance from their neighborhood pharmacy than from any other professional (13% vs. 86%).

Health care for minor injuries and illnesses

Consumers were given a list of 13 common diseases and asked to decide whether they would see a local pharmacy, doctor, or treat the condition themselves. Consumers are more likely to see a community pharmacist for a cough (44%) or gastrointestinal issue (38%). Acne, spots, rashes, colds, flu, dyspepsia, and diarrhea are all common reasons why customers visit their neighborhood pharmacy (Figure 2).

Consequences of a wider view of the community pharmacist's responsibilities

The majority of customers (91%) support the idea of a community pharmacist coordinating treatment with other doctors (87%), diagnostic tests (87%), longer pharmacy hours (83%), and the availability of a private consultation place in the drugstore (80%). Less than two-thirds of respondents supported the idea that community pharmacists and doctors should work together to treat patients with chronic diseases, and just a third thought that community pharmacists should be available to patients outside of the drugstore.

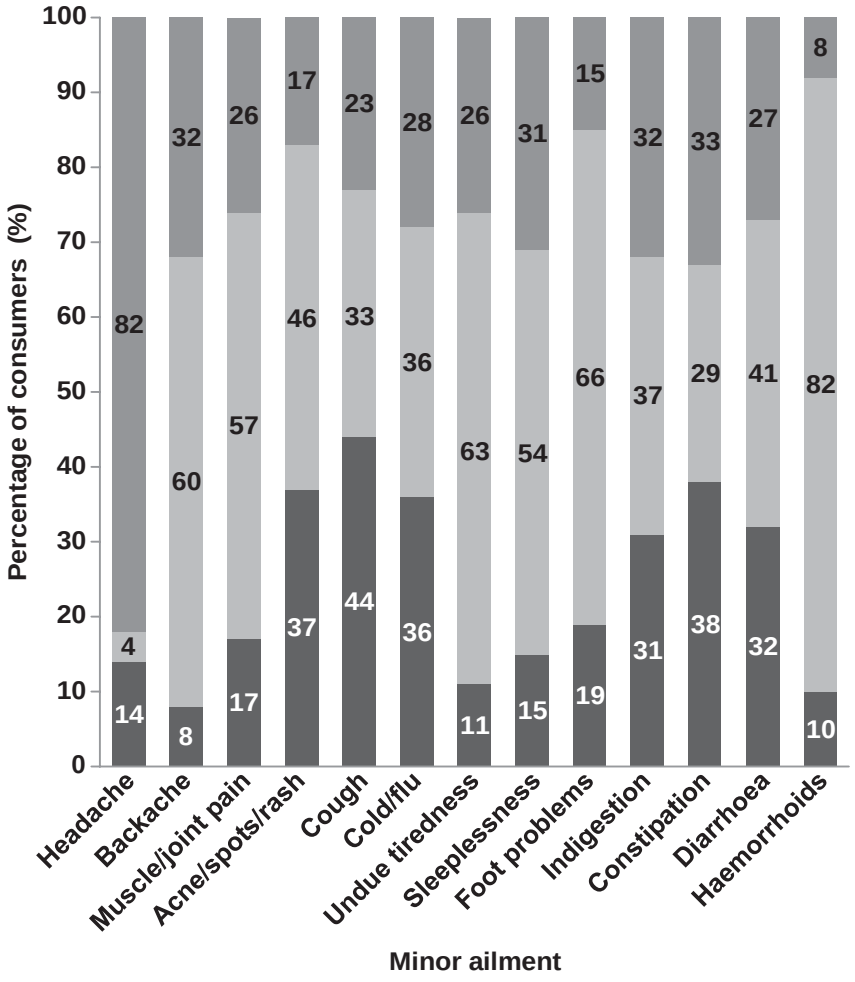

Figure 2 Treatment of minor ailments (n = 500). ( ) Self-treat; ( ); Doctor; ( ) Pharmacist.

Table 1 Community pharmacist extended roles (n = 500)

	Very important	Fairly important	Neither important nor unimportant	Not very important	Not important
Longer pharmacy opening hours	56%	27%	10%	4%	3%
Pharmacist accessibility outside pharmacy opening hours	39%	28%	18%	9%	6%
Private area for consultation	46%	34%	10%	7%	3%
Diagnostic testing (urinalysis, blood pressure, blood glucose monitoring)	57%	30%	9%	2%	2%
Liaison with primary and secondary care-based physicians	62%	29%	7%	2%	0%
Management of chronic conditions (asthma, hypertension, diabetes)	33%	35%	23%	7%	2%
Pharmacist prescribing	15%	32%	21%	22%	10%

opening hours (67%) and community pharmacist prescribing(47%) (Table 1).

Discussion

Consumers in Malta have a favorable impression of community pharmacists and the services they provide, according to this survey.

When evaluating the quality of care delivered, patient happiness is crucial.

[18] Many customers reported being either extremely pleased or somewhat satisfied with various aspects of their local pharmacist. Results reported in the United States[1,19], the United Kingdom[15], and the Netherlands were quite similar. [17] High levels of pharmacy patronage were found in this research, with the majority of customers visiting the same community pharmacy on a regular or even daily basis. This is promising information since it suggests that customers' high level of loyalty to their preferred community pharmacy may be indicative of their contentment with the services received there. Both British[14,15] and Canadian[16,17] research supports this result. [20]

Similar to the findings of this study, research conducted in the United Kingdom[21,22] and South Africa[23] reports that customers frequent a specific community pharmacy for reasons like proximity to home or work and the warmth and compassion of the pharmacist. Similar to findings by Bell et al.[15] in the UK, the most prevalent reason for visiting a community pharmacy reported in the present investigation was to acquire drugs recommended by a physician.

Similarly to a research conducted by Hargie et al.[14] in the UK, the majority of customers in the present study saw pharmacists as businesspeople as well as healthcare professionals. Most Maltese customers in this research, however, were unwilling to pay for the pharmacist's assistance. In contrast, a survey of Canadian customers found that both willingness to pay and reimbursement levels were increasing. [20]

Most Maltese customers said they would see a community pharmacy for assistance on a variety of minor diseases and when their condition wasn't critical enough to see a doctor, suggesting that they accept the pharmacist's advising function in respect to minor ailments. Consumers in Malta also said that they would trust their local pharmacist's recommendation when buying an over-the-counter medication.

For similar reasons, the local pharmacy in the United Kingdom was traditionally considered the go-to spot for the treatment of less serious medical issues.

[24] However, in two different investigations conducted out by the According to surveys conducted by the Welsh School of Pharmacy[25,26], only a tiny minority of customers said that they would seek a pharmacist for guidance on treating minor diseases because they think that pharmacists do not know enough about their specific health. In Scotland once again, it was found that fewer than 10% of the general people saw the pharmacist as the 'first person for guidance on health concerns. [27] In Canada, both pharmacists and doctors are regarded as "go-to" experts on patient health. [20]

In this survey, customers rated pharmacy privacy as low on a scale from 1 to 10, with a private consultation space ranking highest in importance. It has been claimed that people in the Netherlands[17] and the United Kingdom have had the same issues with privacy at community pharmacies. [28] Private consultation rooms in community pharmacies are highly valued in the United Kingdom and should be a priority for any new or remodeled pharmacies in the country. [14,16,29] Longer pharmacy hours and community pharmacist availability outside of pharmacy hours were also seen as a positive service enhancement among research participants. A research done in the United Kingdom came to the conclusion that extending the professional part of community pharmacy might benefit by offering telephone assistance lines, pharmacist domiciliary visits, and longer or more variable

operating hours to better meet the requirements of patients. [30]

Consumers in Malta also noted improvements in physician cooperation for the treatment of chronic illnesses and diagnostic tests as examples of professional services provided by community pharmacies.

[16]Limitations

One drawback of this research is that because customers were recruited from within a pharmacy context, it is probable that only those consumers who frequently frequented pharmacies and had a favorable overall view of the pharmacist were included in the study, leading to potential bias. The surveys might be given out to people scouted during public and social gatherings. The research might be strengthened by recording the specific reasons why people who filled out the surveys were going to the pharmacy, such as to buy medicine or to see doctors at the pharmacy clinic.

Conclusion

Customers in Malta generally have a favorable impression of community pharmacies and the services they provide.
nity pharmacies is consistent with the findings of the vast majority of research conducted in Europe and the United States. The results of the survey show that customers want community pharmacists to invest more heavily in collaborative care practice, diagnostic testing, and longer store hours.

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BIOMOLECULES IN GLYCOSMIS PENTAPHYLLA L. FRUIT-SEEDS MAY SERVE AS A HEPATOPROTECTIVE AGENT.

K. Sara Sirisha, Nd. Nizamuddin, P. T. Naga Raju

Abstract:

*The primary purpose of this study was to determine the in vitro and Research Lab, GIET School of Pharmacy, NH-16, Chaitanya Knowledge City, Rajahmundry-533296. Research Lab, GIET School of Pharmacy, NH-16, Chaitanya Knowledge City, Rajahmundry-533296. Research Lab, GIET School of Pharmacy, NH-16, Chaitanya Knowledge City, Rajahmundry-533296.in vivo Hepatoprotective activity of biomolecules that could be extracted from Glycosmis pentaphylla L fruit seeds. The DPPH test was used to determine the Hepatoprotective activity in vitro. The EEFSGP was measured to have an IC50 value of 212.96 g/ml (50% inhibition). For the in-vivo Hepatoprotective activity, albino rats were used. Rats treated with an ethanolic extract of Glycosmis pentaphylla fruit seeds showed a substantial (*P0.05) decrease in their CCl4-induced increased levels of SGOT, SGPT, ALP, and serum bilirubin (EEFSGP). The levels of SGOT, SGPT, ALP, and serum bilirubin were all reduced by 6.23% ns(non significantly), 28.96% ns(non significantly), 8.81% ns(non significantly), and 11.11 % ns(non significantly), respectively, after treatment with EEFSGP at a dose of 250 mg/kg b. wt. A higher dose of 500 mg/kg b. wt. was A conventional dose of silymarin revealed inhibition by CCl4 of 55.09, 68.98, 57.46, and 35.04%, respectively. Based on these results, it can be concluded that the biochemical parameters of the ethanolic extract-treated group were considerably lower than those of the CCl4-treated group. The elevated levels of AST, ALT, ALP, and bilirubin in hepatotoxic rats were also dramatically decreased after treatment with the extract. Histopathological analysis demonstrated that the EEFSGP exhibited moderate to good hepatoprotective activity at both dosages (250 mg/kg b.w. and 500 mg/kg b.w.), but at 500 mg/kg b.w. performed exceptional hepatoprotective efficacy against CCl4-induced injured hepatocytes.*

Key words :*: Biomolecules; Intoxication; Hepatocytes; SGOT; SGPT; SALP; DPPH test;*

INTRODUCTION:

The Glycosmis pentaphylla, often called orangeberry and gin berry, is a flowering plant species in the Rutaceae (citrus) family. Its natural range includes India, northern Australia, and Southeast Asia. Its pink fruits are harvested for human consumption. Those who live in more temperate climates may grow it as an indoor houseplant [1].

Word shape [2]

Correa is a shrub or a small tree, and its scientific name is Glycosmis pentaphylla (Retz.) There are anywhere from three to five folioles on each leaf, and the leaflets may be anywhere from entirely obtuse to sharply crenate. There are also anything from two to twelve pairs of lateral nerves. 5-merous flowers that bloom in the axils and are very, very long tight racemes/cymes Globose to ellipsoid with a glandular pericarp characterizes the fruit.

Elements [2]:

In the leaves, you'll find the quinolone alkaloid glycolone. The alkaloids and amide (benzamide-2-methylamino) found in flowers include arborine, arborinine, skimmianine, glycorine, and glycosmicine. The carbazole alkaloid mupamine is also present. Dictamine, -fagarine, skimmianine, -sitosterol, coumarin, stigmasterol, myricylalcohol, base glyborine, triterpenes arborinolA, arborinolB, arborine, arborinine, carbazolealkaloid glycosinine, glycozolidol, and 3-formylcarbazole are all found in the roots. Glycophymine, glucosolone, glucocolone, and amide-glycomide are all alkaloids found in root bark, along with the acridine alkaloids noracronycine, de-methylacronycine, and e-N-methylnoracronycine.

This herb has been utilized for centuries in traditional medicine to treat a variety of conditions, including cough, rheumatism, anemia, and jaundice (see also [3]). The bitter leaf juice has medicinal uses for treating fever, easing liver issues, and as a vermifuge. Eczema and other skin problems are treated using a paste made from the leaves and ginger. Inflammations of the face are treated with a decoction made from the root.

Substances and Procedures: Drugs and Chemicals

All of the chemicals employed in the extraction process and the screening for phytochemicals were classified as LR or AR. Silymarin, the reference medicine, was acquired from a neighborhood drugstore, while the solvents and other chemicals used were all of a "AR grade" and sourced from a university or hospital chemistry warehouse.

Hepatotoxin

A rat model of hepatotoxicity caused by CCl4 was used to assess hepatoprotective efficacy in this investigation.

Animals used in experiments

Two hundred and fifty to two hundred and fifty gram male albino rats were utilized. They came from the animal facility of the C.L. Baid Metha College of Pharmacy in Chennai (Reference Number IAEC-CPCSEA).

IAEC/XXIX/10/2010. The participants were closely monitored for around 7 days before to the start of the trial to rule out the possibility of a concomitant infection, and they were given unrestricted access to their regular meals and drinking water during this time. The animals were kept in clean, well-ventilated plastic cages at room temperature (255 °C) with a standard 12-hour light/dark cycle.

Method of Extraction (Soxhlet Extraction)Introduction

To isolate an organic compound from a solid, we use an organic solvent that does not dissolve the unwanted byproducts. Extraction from solids is a time-consuming process that often requires prolonged contact and heating with the solvent. To do this, the Soxhlet Extractor is used. It's a glass cylinder with a siphon and a tube on the side. Water condenser is located at the top of the cylinder, and the whole thing is placed into the neck of a boiling round bottom flask [4].

Methodology

Before the equipment can be assembled, the dried fruits and seeds must be ground into a fine powder in a food processor. Suitable solvent, such as ethanol, is heated in a flask placed in a water bath or on a heating mental. When the solvent is heated to a boil, the vapors escape through the side tube and condense on the top of the water tank. The powdered material in the thimble is exposed to the condensed liquid, which dissolves any organic compounds present and then filters out into the gap between the thimble and the glass cylinder. The solution drains back into the boiling flask via the siphon as the liquid level in this container increases. Once again, the solvent is evaporated, and the extracted material is left behind in the flask. A steady stream of pure solvent is dripped over the solid, where it dissolves the substance of interest and then flows back into the original flask. The organic material is separated from the solvent by distillation at the conclusion of the procedure [4]. After concentrating the ethanolic extract in a water bath, you may chill it and store it in the freezer in a clean, dry beaker. The first phytochemical analysis must be performed on the ethanolic extract of Glycosmis pentaphylla fruit seeds (EEFSGP).

Preliminary Phytochemical Screening [5, 6, 7, 8] The preliminary phytochemical screening of EEFSGP revealed the presence of a wide variety of biologically active molecules, including carbohydrates, amino acids and peptides, phytosterols,

carotenoids, alkaloidsds (higher concentration), terpenoids especially diterpenoids, tri and tetra terpinoids di, and aromatic acids and alcohols, etc.

Evaluation of Acute Toxicity [9]

The current investigation assessed the EEFSGP for acute oral toxicity using the acute toxic class technique. Three Wister rats were to be used at each stage of this technique to determine the extract's toxicity. Prior to treatment, the rats were fasted for three to four hours (food but not drink should be withheld). After the fasting period was over, the animals were weighed again, and an oral dosage of 2000 mg/Kg b.w. of the extract was given to them. After administration of the dose, each animal was watched at least once within the first 30 minutes, then at regular intervals over the next 24 hours (with extra care provided during the first 4 hours), and then every day for 14 days.

Evaluation of In vitro Antioxidant Activity by DPPH Assay (Free Radical Scavenging Activity) [10, 11]

Principle

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test measures the reduction in color intensity that happens when a free radical scavenger contributes a hydrogen atom to quench a DPPH radical.

Method

The DPPH free radical scavenging activity of the ethanolic plant extract was determined using a modified version of the technique described by Pan et al. [10]. We mixed 8 milliliters of a 0.004% (w/v) stock solution of DPPH in 95% ethanol with 0.2 milliliters of extract solutions at varying concentrations. Absorbance at 517 nm was measured using a UV-Visible spectrophotometer until the reaction reached a steady state, at which point the DPPH radical scavenging activity could be calculated. Gallic acid, a synthetic antioxidant, was utilized as a positive control. All counts were done three times for accuracy. This equation was used to determine the percent scavenging activity against DPPH radicals:

S% = [(Acontrol-Asample) /Acontrol].

×100 Asample = absorbance of test sample; Acontrol = absorbance of blank control (containing all reagents except the extract solution).

Study Design for Evaluating In Vivo Hepatoprotetive Activity [12]

Thirty rats in total were used, with each group consisting of six animals. Group I received just vehicle (1 mL/kg/day of 1% CMC; p.o.)

Class II: The Negative Control 1 milliliter of CCl4 per kilogram intramuscularly or intraperitoneally (1:1 CCl4 in olive oil).

Positive Control/Standard Group (Group III) Standard Silymarin 100 mg/kg p.o. for 7 days Plus CCl4 1 mL/kg i.p. (1:1 of CCl4 in olive oil)

Organizations that Provide Different Forms of Therapy

High Dose Group [CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p + EEFSGP (500 mg/ kg b. w., p.o.]

Low Dose Group [CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p + EEFSGP (250 mg/ kg b. w., p.o.] Over the course of seven days, the patients received an oral medication.

Blood Donation: After an overnight fast of 8 hours, blood was drawn from the retro orbital abyss under light ether anesthesia on day 8. In order to separate the blood components, the samples were centrifuged at 3000 rpm for 20 minutes. Before undergoing biochemical assessments, serum was isolated and frozen at -200 C for later use.

The Use of Biochemical Methods

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and bilirubin were all tested on the serum samples taken.

Histopathological Analysis

Hepatic tissue was removed by dissection and then preserved in 10% formalin solution. Ethanol (between 50% and 100%) was used to remove moisture, then xylene was used to clean it, and finally paraffin wax was used to embed it. Following this, thick slices (5-6 mm) were cut and stained with hematoxylin and eosin dye for photo microscopic examination. V.H.S. Hospital in Chennai was responsible for all of the biochemical and histological testing.

RESULT AND DISCUSSION:

Table 1: Results of DPPH Scavenging Activity

Sl. No.	Concentration (ug/mL)	Absorbance (A)	S% = [(A_0-A)÷A_0] X 100	IC_{50} (µg/ml)
Control (DPPH Sol.)				
1.	0.1mM in ethanol	1.174 (A_0)		-
Std (Ascorbic Acid)				
1	4	0.983	16.26	
2	6	0.954	18.73	
3	8	0.917	21.89	
4	10	0.870	25.89	39.87

5		25	0.565	51.87	
6		50	0.037	96.84	
EEFSGP					
1		10	1.044	11.07	
2		25	0.936	20.01	
3		50	0.850	27.59	212.96
4		75	0.786	33.04	
5		100	0.702	40.20	
6		250	0.568	51.61	

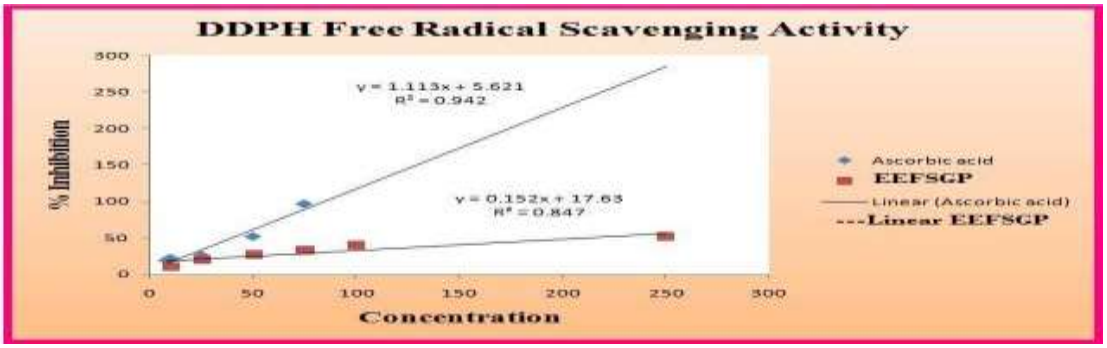

Fig 1: DDPH scavenging Activity of Ascorbic Acid and EEFSGP

Table 2: Results of Hepatoprotective Activity

Group	Treatment	AST(SGOT)	ALT(SGPT)	ALP(SALP)	Serum bilirubin
		IU/L	IU/L	IU/L	mg/dL
1	Normal Control Group (only the vehicle, 1% CMC; p.o.)	53.00±8.672***	46.60±11.95***	139.2±6.914***	0.58±0.08***
2	Negative Control (1:1 of CCl_4 in olive oil; i.p.)	202.2±30.45	204.4±47.74	399.2±16.18	1.17±0.16
3	Low dose [(1:1 of CCl_4 in olive oil) i.p + EEFSGP (250 mg/ kg b. w., p.o.)]	189.6±14.48ns	145.2±39.75*	364.0±16.52*	1.04± 0.15ns
4	High dose [(1:1 of CCl_4 in olive oil) i.p + EEFSGP (500 mg/ kg b. w., p.o.)]	151.6±13.52***	107.0±19.47***	303.0±38.78***	0.85±0.20**
5	Positive Control/Standard				
	Group[(1:1 of CCl_4 in olive oil)i.p.+ Silymarin 100 mg/kg orally (p.o.)]	90.80±17.61***	63.40±15.73***	169.8±8.55***	0.76±0.14***

Data are expressed as mean±SD (n = 6). One-way ANOVA followed by Dunnett's Multiple Comparison Test (P< 0.05) compared with group 2 ;(ns=non significant.*

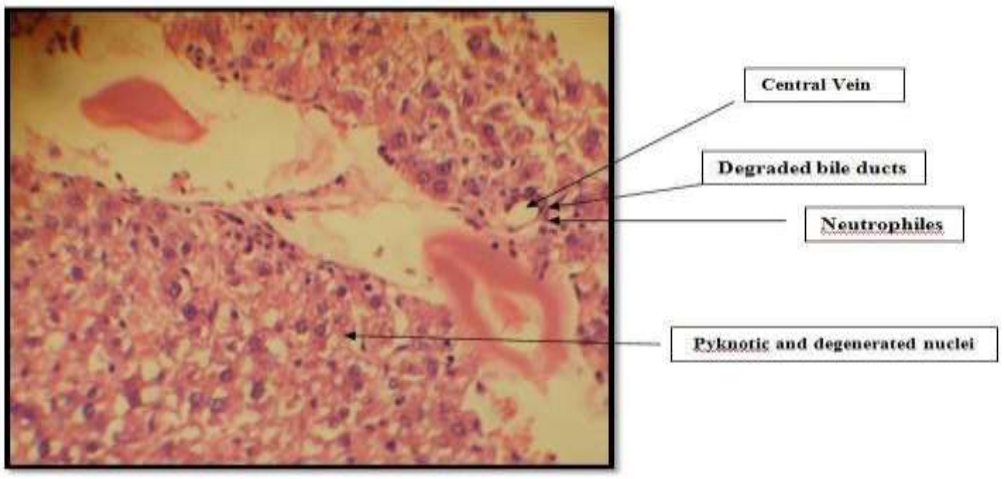

Fig 2: Liver Section of CCl4 Treated Rats.

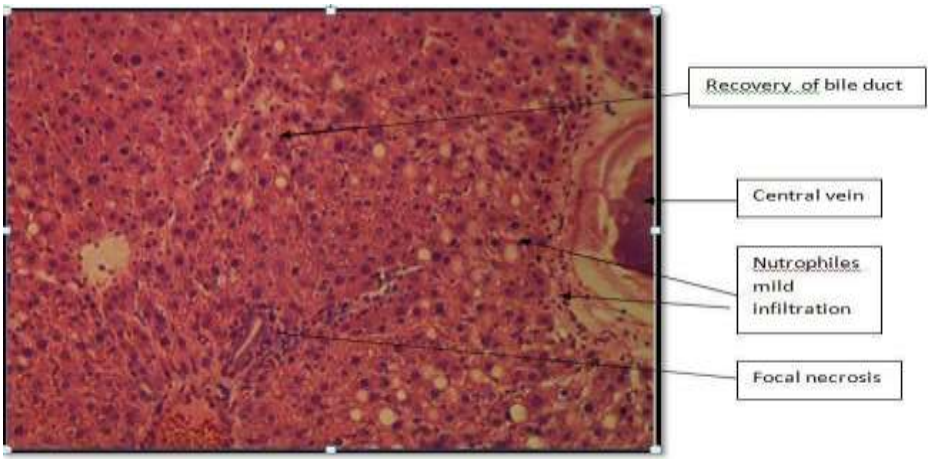

Fig 3: Liver Section of Rats Treated CCl_4 and 100 mg/kg of Silymarin.

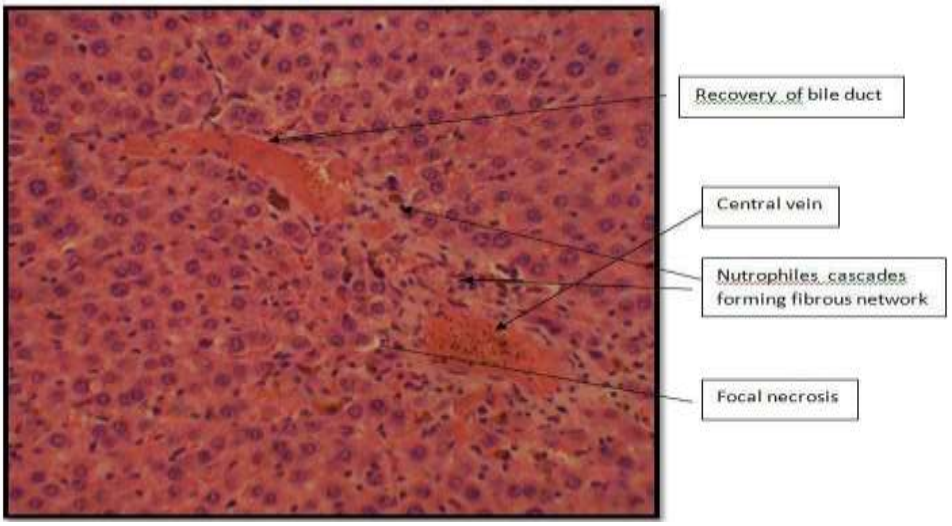

Fig 4: Liver Section of Rats Treated CCl_4 and 500 mg/kg of EEFSGP.

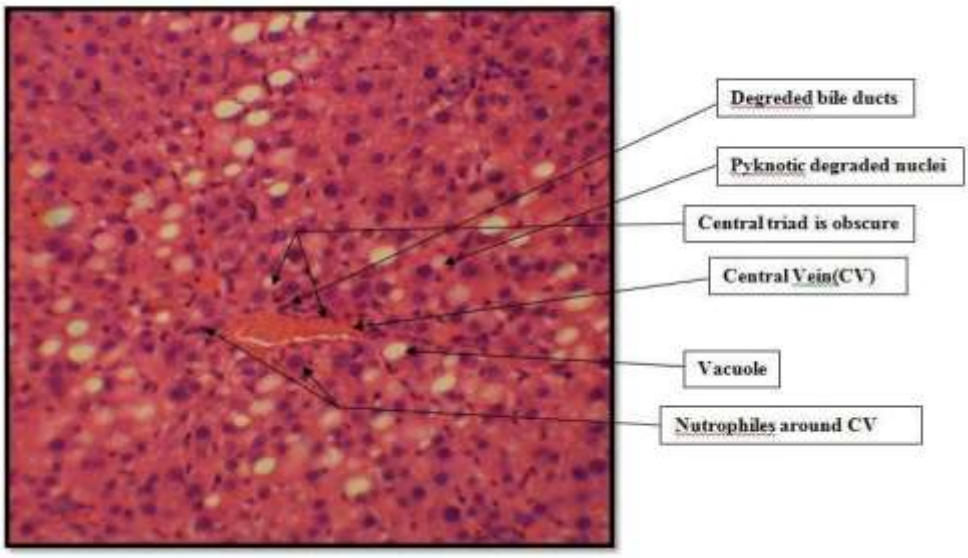

Fig 5: Liver Section of Rats Treated CCl_4 and 250 mg/kg of EEFSGP.

1. Phytochemical Screening

Carbohydrates, amino acids, peptides, phytosterols, carotenes, alkaloidsds (in greater concentration), terpenoids (particularly diterpenoids, triterpenoids, and tetraterpenoids di), aromatic acids, and alcohols were all found in EEFSGP during preliminary phytochemical screening..

2. Acute Oral Toxicity Studies

"Acute toxic class techniques (OECD guideline- 423)" were used to determine the acute oral toxicity in this research. The extract was given orally at a dosage of 2,000 milligrams per kilogram of body weight. Neither major toxicity nor any other significant changes in behavior were seen over the observation period. Significant dosages were determined to be 250 mg/kg body weight per oral (LD) and 500 mg/kg body weight per oral (ED) (HD) considerably.

3. Hepatoprotective Activity

(i) Statistical analysis

Methods from the "acute toxic class" (OECD guideline 423), which measure oral toxicity quickly, were employed in this study. The oral administration of the extract was at a rate of 2 milligrams per kilogram of body weight. During the course of the study, no substantial toxicity or behavioral alterations were detected. An oral LD (lowest effective dose) of 250 mg/kg and an ED (highest effective dose) of 500 mg/kg were shown to be significantly effective..

(ii) Analysis of DPPH free Radical Scavenging Activity

Antioxidant activity in the extract was supposed to be measured in vitro using DPPH free radical scavenging. Ascorbic acid, itself a natural antioxidant, was used as the reference antioxidant throughout the research. Antioxidant activity was measured using DPPH free radical scavenging, and the findings were reported as a percentage of suppression of produced free radicals at different doses. One may see a concentration-dependent impact, as greater concentrations were shown to show a greater percentage of inhibition. The X-axis of the graph represents the percentage of inhibition achieved, while the Y-axis represents the different doses used in the experiment (Fig 1). In every study, both the EEFSGP and the gold standard ascorbic acid were tested to find their respective IC50 values (50% inhibition).

Table 1 displays the findings of an experiment where an ethanolic extract of the fruit-seeds of Glycosmis pentaphylla (EEFSGP) was tested for its ability to scavenge DPPH radicals, in comparison to ascorbic acid. Figure 6.3 shows a Microsoft Office Excel 2007 plot of the percentage inhibition at several concentrations of ethanolic extract of fruit-seeds of Glycosmis pentaphylla (4-50 g/ml) and standard Ascorbic acid (10-250 g/ml). Graph analysis revealed that the IC50 values for Ascorbic acid and EEFSGP, respectively, are 39.87 g/ml and 212.96 g/ml.**)**.

(iii) Biochemical Analysis

Table 2 shows how EEFSGP affects liver marker enzymes and bilirubin levels in the blood. The results indicated that whereas the Normal Control Group had levels of AST, ALT, and bilirubin within the normal range, the CCl4-treated group showed raised levels of these markers, demonstrating that CCl4 caused hepatocellular degeneration at higher dosages. An sign of liver cell disruption and the subsequent release of enzymes is an increase in cytoplasmic AST and ALT. Chemically induced hepatic damage has been measured by measuring bilirubin levels.

Table 2 shows that in rats treated with EEFSGP, the raised levels of SGOT, SGPT, ALP, and Serum bilirubin caused by CCl4 intoxication were considerably (*P 0.05) decreased. The levels of SGOT, SGPT, ALP, and serum bilirubin were reduced by 6.23 percent (nonsignificant), 28.96 percent (nonsignificant), 8.81 percent (nonsignificant), and 11.1 percent (nonsignificant), respectively, after treatment with ethanolic extract at a dose of 250 milligrams per kilogram of body weight. A higher dose of 500 milligrams per kilogram of body weight was even more effective. A conventional dose of silymarin revealed inhibition by CCl4 of 55.09, 68.98, 57.46, and 35.04%, respectively. Data in Table 2 revealed that the EEFSGP-treated group had considerably lower biochemical parameters than the CCl4-treated group. Treatment with the EEFSGP also dramatically decreased the elevated levels of AST, ALT, ALP, and bilirubin seen in hepatotoxic rats.

Histopathological Analysis

Section of a CCl4-treated liver is shown in Fig. The loss of cellular boundaries, pyknotic, and degraded nuclei, as well as extensive infiltration of the lymphocytes surrounding the central vein, were all seen in the affected rats' tissues.

Liver sections from CCl4 and 100 mg/kg Silymarin-treated rats showed minor fatty change, necrosis, and localized necrosis in the central vein, as well as in other areas of the liver (Fig. B) (dilatation).

C. Figure 4: Liver sections from CCl4 and 500 mg/kg EEFSGP-treated rats showed normal liver architecture, with minimal inflammatory cellular infiltration around the central vein, no necrosis, and a fibrous network formed by neutrophil cascades providing substantial protection and large septa of connective tissue flowing together and penetrating the parenchyma.

D. Fig. 5: Liver sections from CCl4 and 250 mg/kg EEFSGP-treated rats showed very little recovery, with an indistinct central triad and infiltration of neutrophils surrounding the central vein, a deteriorated fatty change, necrosis, and localised necrosis (dilatation), and a loss of

cellular boundaries.

CONCLUSION:

Finally, we demonstrate that ethanolic extract of Glycosmis pentaphylla fruit-seeds (EEFSGP) showed the capacity to regenerate hepatocytes in vivo and had potential antiinflmmatory action, as validated by liver biopsy, with an IC50 value of 212.96 g/ml. When compared to the gold standard medicine silymarin, the hepatoprotective activity of EEFSGP may be regarded outstanding.

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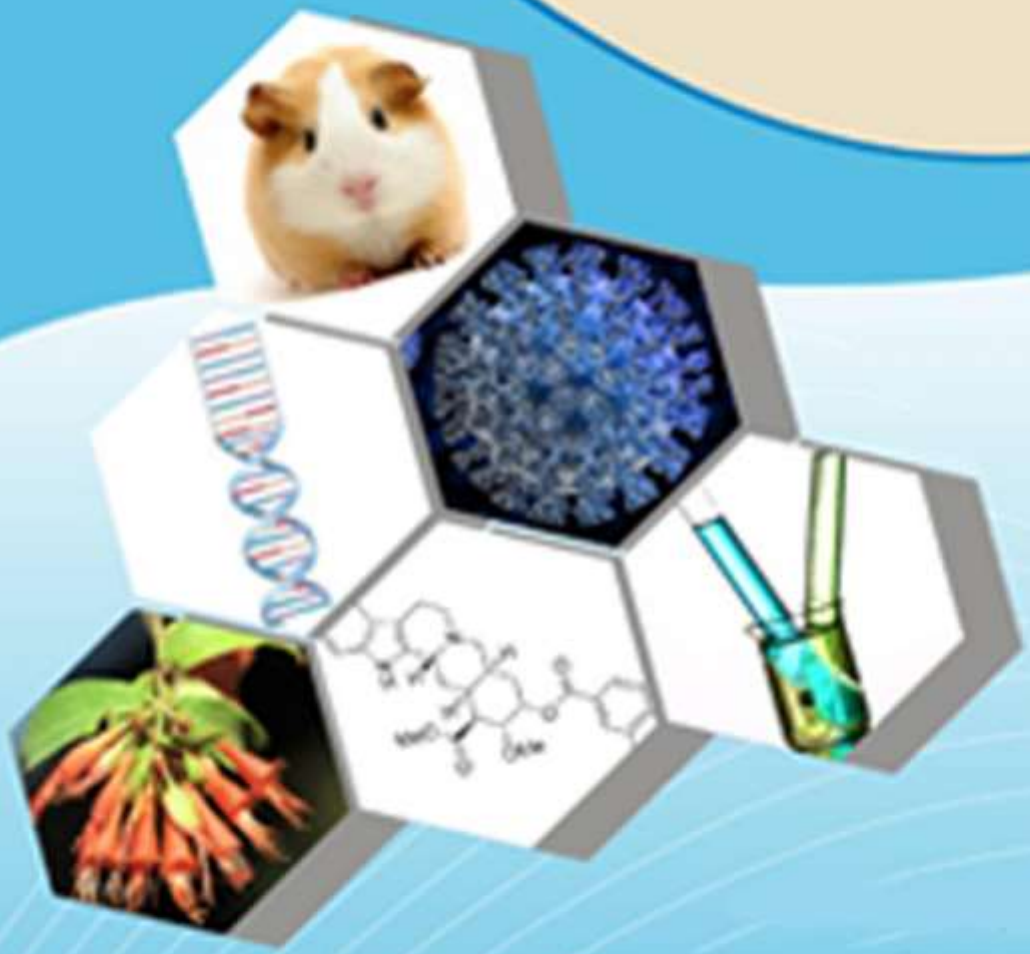

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A PHYTOCHEMICAL INQUIRY INTO THE DRIED LEAF OF AERVA LANATA AND AN EXAMINATION OF ITS IN-VITRO ANTIBACTERIAL ACTIVITY

G. Kamlesh Goud, P. Anil Kumar Yadav, Dr. C. H. Naveen Kumar

Abstract:
The study was carried out to ascertain the anti bacterial properties present in different extracts of dried scale leavesof Aerva lanata. The Anti bacterial testing of leaves extract Aerva lanata was evaluated by Agar well diffusion method using gram positive bacteria like Staphylococcus aureus, Bacillus subtilis, gram negative bacteria like Escherichia coli, Klebsiella pneumoniae. Amongst the test extracts, the results suggested that, Chloroform, Ethanol extracts of leaves showed significant antibacterial activity compared with standard drug.

Keywords: *Aerva lanata, Gentamycin, Flavonoids, Anthraquinons.*

INTRODUCTION:

Traditionally plants are used as drugs and have genuine utility because they contain some components which have healing and pain relieving properties. For the primary health care about 80% of rural population depends on these medicinal plants. Usage of plants for the treatment of diseases is as old as human species which produces various secondary metabolites like alkaloids, terpenoids, steroids, phenols, tannins, flavonoids, and other metabolites and which have antimicrobial and antioxidant types of properties. Plants are the main source of food and rich nutrients content. Traditional societies around the world had deep knowledge of various plants and their medicinal value, though they did not possess knowledge on components present and their mode of action. Medicinal properties attributed to various herbs have paved way to the discovery of new drugs, as they are the reservoirs of potential chemical compounds. For the benefit of mankind it is necessary to prefer herbal usages to avoid chronic stress and synthetic drugs.

Herb is an immeasurable wealth of nature not only from the global environmental perspective but also from the and lesser side effects. The chemical constituents present in them are a part of the physiological functions of living flora and hence they are believed to have better compatibility with thehuman body. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re emerging infectious diseases. Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections. *Aerva lanata* linn. belonging to the family Amaranthaceae. Herbs are perennial, 5–50 cm tall. Stem branched from base; branches ascending or stoloniferous, white lanose. Leaves opposite or nearly whorled, sessile, grayish green,subulate, linear, 1–2.5 cm × ca. 1 mm, abaxially white lanose, adaxially glabrous, base attenuate, sometimes vaginate. Spikes terminal, narrowly ovate or terete, 0.5–2.5 cm, 3–5 mm in diam., white lanose; rachis very short or absent. Bracts and bracteoles lanceolate, 1–2 mm, abaxially white lanose. The Phytoconstituents reported from stem are flavonoids, tannins and anthraquinones. However, from the above account, it is obvious that there is no information available about the anti bacterial activity of stem and leaves of *Aerva lanata.* The present investigation was to explore the anti bacterial activity of dried leaves of *Aerva lanata.*

Pharmaceutical Analysis
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MATERIALS AND METHODS:

Collection of plant material

The leaves of *Aerva lanata* were collected from surrounding places of Rangareddy Dist.

Phytochemical Evaluation

The different chemical tests were performed for establishing profile of the extract for its chemical composition; the following chemical tests for various phytoconstituents in the petroleum ether, chloroform, ethyl acetate, alcohol and water extracts were carried out as described below.

(A) Alkaloid detection test: I Dragendroff's reagent color development in 1ml extract in test tube after adding a few drops of Dragendroff's reagent. When alkaloids are present, they cause the color to become orange.

The presence of alkaloids was confirmed by adding 2 ml of Wagner's reagent to the extract, and the resulting reddish brown precipitate.

Extract was mixed with 2 ml of Mayer's reagent, and the presence of alkaloids was indicated by the formation of a dingy white precipitate.

iv) Hager's Test: 2 ml of Hager's reagent was added to the extract, and the presence of alkaloids was verified by the production of a yellow precipitate.

Salkowski test I 1 ml of extract was mixed with 1 ppm tin and 0.1 ml of thionyl chloride. Terpenoids are present when a pink hue is seen.

Hirshonn reaction (ii): The material turned from red to purple when heated with trichloroacetic acid.

As for the steroid test, which is option (C), here are the results:

"(D) Liebermann Burchard" For this test, we mixed 1 milliliter of extract with 1 milliliter of glacial acetic acid, 1 milliliter of acetic anhydride, and 2 drops of concentrated sulphuric acid. Steroids are present when the solution becomes red, then blue, and lastly bluish green. In order to detect coumarins, we mixed 1 milliliter of extract with 1 milliliter of 10% sodium hydroxide. The appearance of a bright yellow tint is a telltale sign that coumarins are present.

Tannins were detected using the following method (E): I after adding ferric chloride to a little amount (only a few mg) of extract, a dark blue or greenish black hue developed, indicating the presence of tannins.

When the extract was combined with a basic lead acetate solution, a white precipitate formed, proving the presence of tannins.

(F) Test for saponins: \sTo 1 ml of the extract, 5 ml of water was added and the tube was shaken briskly. The presence of saponins is shown by the production of a large amount of lather.

The flavone content is determined by the Shinoda The existence of flavones was determined by adding a few magnesium turnings and 2 drops of strong hydrochloric acid to the extract, which caused a crimson hue to appear.

ii) Ten percent sodium hydroxide or ammonia was added to the extract, which became a dark yellow hue due to the presence of flavones.

To conduct the quinones test (method H), 1 ml of the extract was mixed with 1 ml of concentrated sulphuric acid. The presence of quinones is indicated by the development of a red hue.

I. Sodium hydroxide test for flavanones: I adding 10% sodium hydroxide to the extract causes a change in color from yellow to orange, proving the presence of flavanones.

The presence of flavanones is indicated by a change in color from orange to blood red upon addition of concentrated sulphuric acid to the extract.

Anthocyanins may be detected using the following procedure (J): I Adding 10% sodium hydroxide to the extract causes a blue tint, which is indicative of the existence of anthocyanins.

The presence of anthocyanins in the extract was confirmed by adding concentrated sulphuric acid, which produced a yellowish orange tint.

The Borntrager test (K) for anthraquinones involves macerating the extract with ether and then adding aqueous ammonia or caustic soda after it has been filtered. In the presence of anthraquinones, the aqueous layer will become a pinkish red or violet hue following shaking.

Test for phenols (L): Ferric chloride test; a few drops of 10% aqueous ferric chloride were added to the extract. For phenols to be present, a blue or green hue must appear.

I added 1 ml of a 40% sodium hydroxide solution and 2 drops of a 1% copper sulphate solution to the extract to conduct the I Biuret Test for proteins. In the presence of proteins, a violet hue forms.

The Xanthoprotein ii) Test included adding 1 ml of strong nitric acid to the extract. There was a white precipitate that was cooked and chilled. Twenty percent ammonia or sodium hydroxide was then added. The presence of aromatic amino acids is shown by an orange hue.

iii) Tannic Acid Test: 10% tannic acid was added to the extract. When proteins are present, they tend to precipitate out into a white color.

For the (N) carbohydrate test, I Molisch's Test, 1 ml of alpha-naphthol solution and concentrated sulphuric acid were added to the extract through the test tube's sides. Carbohydrates were identified by the appearance of a purple or reddish violet tint at the interface of the two fluids.

After adding the same volume of fehling's solution A and B to the extract, we heated the mixture to see if any carbs would precipitate out, and sure enough, we got a nice brick red precipitate, so we know we had carbs.
Extract was added to 5 ml of Benedict's reagent, heated for 2 minutes, and then chilled for the iii) Benedict's Test. Carbohydrates were detected due to the formation of a crimson precipitate.
(O) Amino acid screening:
Amino acid content was confirmed by the ninhydrin test, in which two drops of ninhydrin solution were added to the extract to produce a distinctive purple hue.
Extraction Technique
A coarse powder was made by grinding dried Aerva lanata leaves. For the manufacture of various extracts, the powder was extracted with various solvents such Ethanol, Chloroform by soxhlation for 6 hours, and the resultant extracts were tested for antibacterial properties.

Microorganisms

The test organisms included for study were gram positive bacteria like *Staphylococcus aureus*, *Bacillussubtilis*, gram negative bacteria like *Escherichia coli*, *Klebsiella pneumoniae*. All the bacterial strains were

procured from Osmania University, Hyderabad, Telangana. The bacteria were grown in the nutrient broth at 37 ^{0}C and maintained on nutrient agar slants at 4 ^{0}C.

Bacterial media

Muller Hinton Media was mixed with distilled water and then sterilized in autoclave at 15 lb pressure for 15 minutes. The sterilized media were poured into Petri dishes and allowed for solidification. The solidified plates were bored with 5mm diameter cork borer. The plates with wells were used for the antibacterial studies.

RESULTS AND DISCUSSION:

Antibacterial activity of the plant extracts

Different leaves extracts of *Aerva lanata* at a concentration of 500µg/ml, 750µg/ml, 1000µg/ml were tested against the gram positive bacteria like*Staphylococcus aureus*, *Bacillus subtilis*, gramnegative bacteria like *Escherichia coli*, *Klebsiellapneumoniae* by Well Diffusion Method.

Well Diffusion Method

Antibacterial activity of the plant extract was tested using Well diffusion method. The prepared culture plates were inoculated with different selected strains of bacteria using streak plate method. Wells were made on the agar surface with 6mm cork borer. The dried extracts were dissolved in 95% of ethanol for preparation of different concentration ranges of extracts. The extracts were poured into the well using sterile syringe. The plates were incubated at 37 ^{0}C±2 ^{0}C for 24 hours for bacterial activity. The plates were observed for the zone clearance around the wells. The extracts of the dried scale leaves were used for the study. The extracts were dissolved in sterile distilled water to form dilution such as 500µg/ml, 750µg/ml and 1000µg/ml. Each concentration of the extract was tested against different bacterial pathogens. Gentamycin at a concentration of 5µg/ml and 10µg/ml was used as standard antibacterial drug. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all three replicates and the average values were tabulated.

Table 1: Preliminary phytochemical screening of *Aerva lanata* leaves

Constituents	Pet ether Extract	Chloroform extract	Ethyl acetate extract	Alcohol extract	Water extract
Terpenoids	-	-	-	-	-
Saponins	+	+	-	+	-
Steroids	-	+	+	-	-
Phenols	-	-	+	-	-
Flavonoids	-	-	-	+	-
Coumarins	-	-	+	+	+
Reducing sugars	-	+	-	-	-

Alkaloids	-	-	+	+	-
Quinones	-	+	+	+	+
Tannins	-	+	+	-	-
Proteins	-	-	-	-	-
Amino acids	-	-	-	-	-
Anthraquinones	-	+	+	+	-

+ Present, - Absent

Antibacterial assay of the Ethanol, Chloroform extracts of dried leaves of *Aerva lanata* exhibited dose dependent antibacterial activity against the tested microorganisms at three different concentrations of 500, 750 and 1000µg/ml. The potential sensitivity of the extracts was obtained against all the tested micro organisms and the zone ofinhibition was recorded and presented in the table given below (Table 2). From the above study the zone of inhibition obtained was dose dependent and the activity shown by the Chloroform, Ethanol extracts of leaves of *Aerva lanata* at a concentration of 1000µg/ml against gram positive bacteria like *Staphylococcus aureus, Bacillus subtilis,* and gram negative bacteria like *Escherichia coli, Klebsiella pneumoniae* strains involved in present study was more in comparison to Gentamycin at a concentration of 5µg/ml. The extracts prepared by solvents like water, isopropyl alcohol showed no zone of inhibition. The zone of inhibition shown by the water were tabulated in the below given below (Table 3). The antibacterial potential exhibited by leaves extracts may be contributed to the presence of tannins, flavonoids and anthraquinones in preliminary phytochemical investigations. Further study is needed to characterize the active principles.

Table 2: Zone of inhibition shown by the Gentamycin and the Ethanol, Chloroform extracts of dried leaves of *Aerva lanata*

Micro organism	Zone of inhibition (mm)			
	GENTAMYCIN		EXTRACTS (1000µg/ml)	
	5µg/ml	10µg/ml	Ethanol extract	Chloroform extract
Bacillus subtilis	7.5 mm	9 mm	8 mm	7 mm
Escherichia coli	7 mm	9 mm	6.5 mm	6 mm
Klebsiella pneumoniae	7 mm	9 mm	8 mm	7 mm
Staphylococcus aureus	7.5 mm	9 mm	8 mm	8 mm

Table 3: Zone of inhibition shown by the Gentamycin and the Water, Isopropyl alcohol extracts of leaves of *Aerva lanata*

Micro organism	Zone of inhibition (mm)			
	GENTAMYCIN		EXTRACTS (1000µg/ml)	
	5µg/ml	10µg/ml	Water extract	Isopropyl alcohol extract
Bacillus subtilis	7.5 mm	9 mm	--	--
Escherichia coli	7 mm	9 mm	--	--
Klebsiella pneumoniae	7 mm	9 mm	--	--
Staphylococcus aureus	7.5 mm	9 mm	--	--

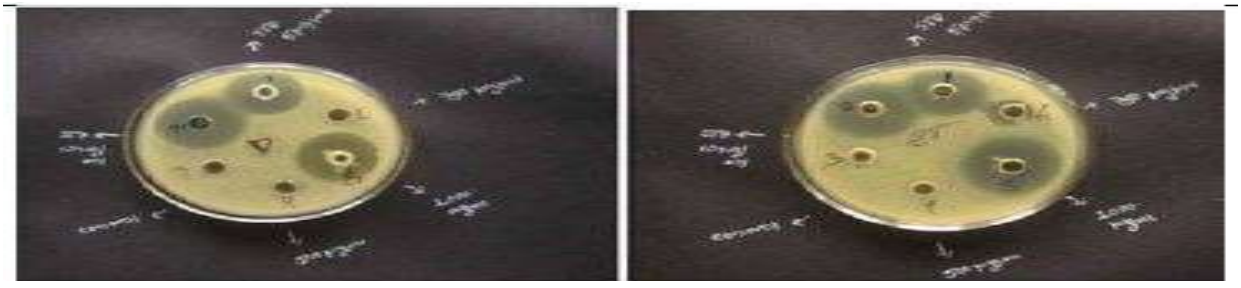

Fig 1: Zone of inhibition shown by the Ethanol and Chloroform extracts of leaves of *Aerva lanata* on *Bacillus subtilis* bacteria

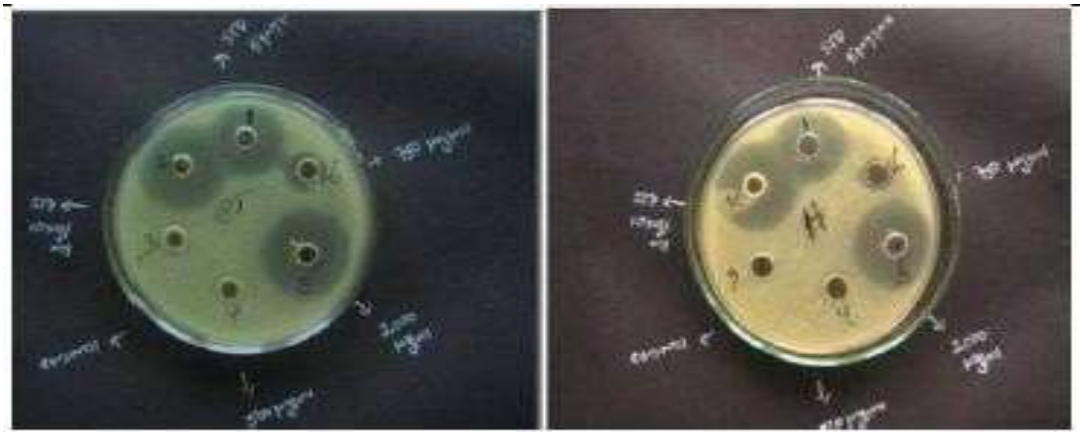

Fig 2: Zone of inhibition shown by the Ethanol and Chloroform extracts of leaves of *Aerva lanata* on *klebsiella pneumoniae* bacteria

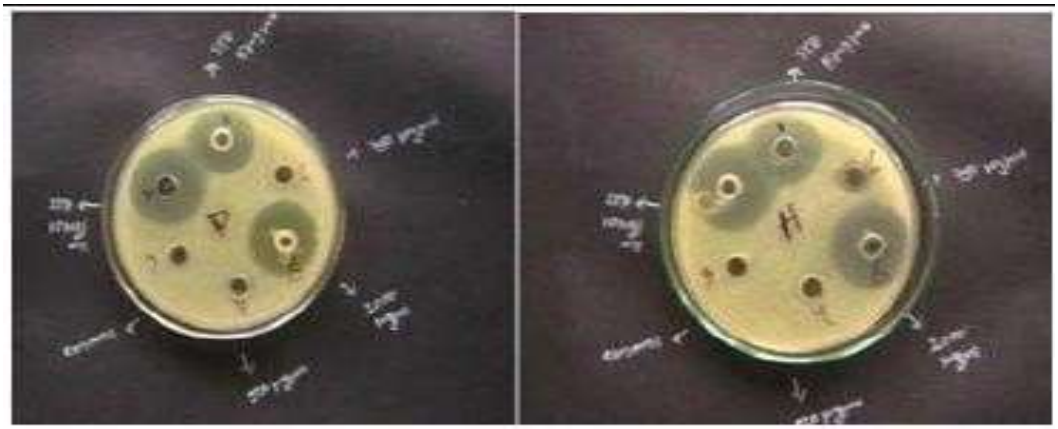

Fig 3: Zone of inhibition shown by the Ethanol and Chloroform extracts of leaves of *Aerva lanata* on *Staphylococcus* bacteria

Fig 4: Zone of inhibition shown by the Ethanol and chloroform extracts of leaves of *Aerva lanata* on *E. coli* Bacteria

CONCLUSION:

From the above study, it is concluded that the leaves of *Aerva lanata* may represent a new source of anti bacterial with stable, biologically active components that can establish a scientific base for the use of this in modern medicine. These local ethno medical preparations of plant sources should be scientifically evaluated and then disseminated properly. Thisknowledge about the medicinal plants usage can also be extended to other fields like field of pharmacology.

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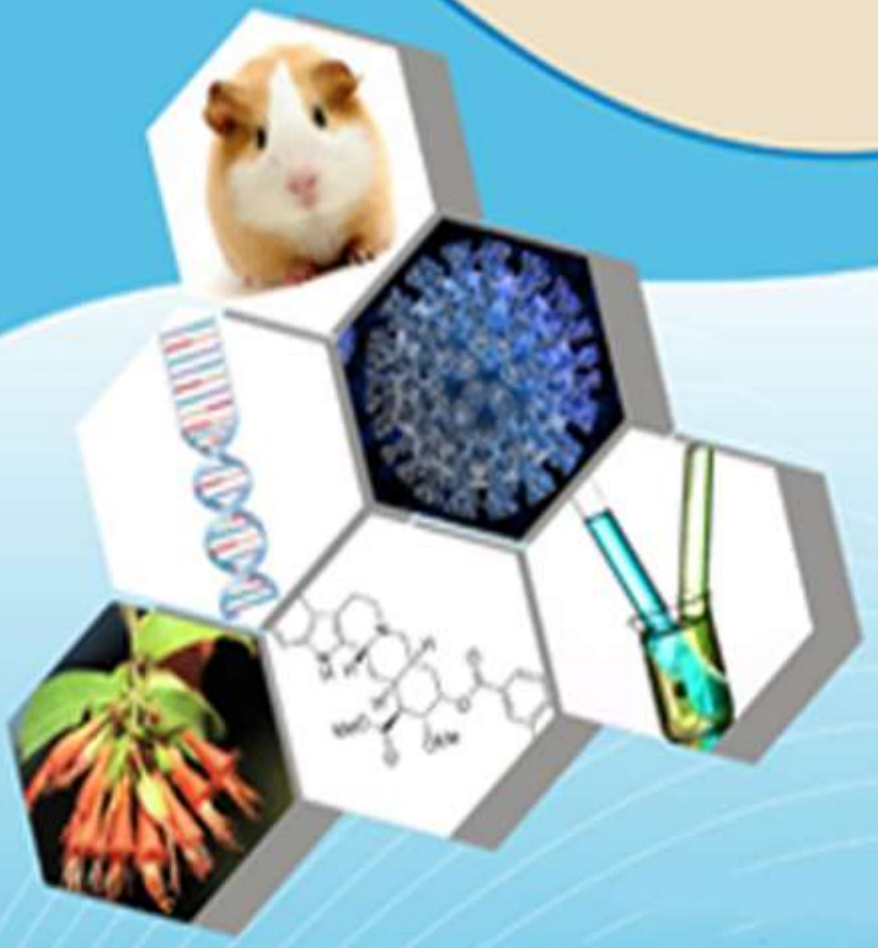

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Pharmacy serving the neighborhood

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Abstract

This article describes the evolution of community pharmacy in the Australian health system, and assesses its current and potential future contribu- tion to health care. A central theme is the unique extent and accessibility of community pharmacy to the public, with a vast and dispersed infrastructure that is funded by private enterprise. The viability of community pharmacy as a retail trade depends on a diversification of its service roles and retention of its product-supply roles. Initiatives by the phar- macy profession, the pharmacy industry and the Australian Government are likely to give commu- nity pharmacy an increasingly prominent place in health promotion and primary, secondary and tertiary prevention, especially in relation to the management of chronic diseases.

Introduction

Community pharmacies' roles in health care systems

Community pharmacists are unique in that they make up a large professional body of individuals who are vocationally trained at university level, fully accredited by state and territory registration boards, subject to Australian Government and state and territory government regulations, work in a retail environment handling a multiplicity of health care products, have extensive interactions with other health professionals (especially the medical profession), and balance the delivery of professional services with the supply of a wide range of products and the management of retail business.

Traditionally, pharmacists and pharmacies have been the main suppliers of medicines for the Australian population. Increasingly, how- ever, the pharmacy is becoming an important source of a wide range of health care services in the community. People perceive pharmacists as highly reliable advisers on many personal health matters, trustworthy independent pur- veyors of health care products, and steadfast partners of the medical profession. This has been clearly shown in national and interna- tional literature on consumers' views and expe-riences of community pharmacy services (Aslani, Benrimoj & Emerson 1999).

Community pharmacy practice in Australia is highly regulated through state and territory Pharmacy and Pharmacists Acts and through the *National Health Act 1953* (Cwlth). Regulation covers the registration of pharmacists, acceptable courses of study to become a pharmacist, and ownership and location of pharmacies.

Pharmaceutics

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As at 30 June 2002, there were 4926 approved community pharmacies (chemists' shops) in Australia (Pharmacy Guild of Australia 2004). Because of their wide distribution in cities and all major towns, pharmacies have become the most accessible points of contact for individuals with the health care system. People can enter a pharmacy without an appointment, can expectto receive professional attention almost immediately, and retain a high level of control over the extent of their engagement with the pharmacist. In contrast, people who consult a doctor often need to make an appointment in advance, often have to wait, and often surrender personal con- trol in the course of the doctor's history-taking and physical examination. While these two types of professional encounter usually differ in their scope and intent, pharmacy offers a con- venient encounter with the health system for many purposes.

The role of community pharmacy is becoming increasingly diversified, with a proliferation of professional services in addition to the tradi- tional supply role of dispensing medications. Six types of community pharmacy services have been identified (Emerson, Whitehead & Benri- moj 1998):

Provision of drug information. Pharmacists pro- vide drug information to patients when medi- cations are dispensed, either in written form (mainly using Consumer Medicines Informa- tion) or as spoken advice (Koo, Krass & Aslani 2002). This advice can improve patients' understanding of medications and awareness of adverse effects, and improve adherence to prescriptions, resulting in better health out- comes. Pharmacists can also provide drug information to doctors, which can result in improved prescribing.

- *Provision of 'pharmacist only' and 'pharmacy' medicines.* These are 'over-the-counter', non- prescription medications (for example, bron- chodilator sprays to treat asthma and certain stronger pain-killers such as paracetamol com- bined with codeine). Pharmacists both provide advice on the selection and use of these drugs, and supply them. In doing so, pharmacists make a major contribution to the provision of primary health care (Benrimoj & Gilbert 2002). For 'pharmacist only' medicines, pharmacists must ascertain a therapeutic need. For all intents and purposes, they must dispense these medicines as they would for 'prescription only' medicines, asking appropriate questions and providing advice and information.
- *Clinical interventions.* A clinical intervention occurs where a pharmacist identifies a medi- cation-related problem and intervenes to resolve the problem. Examples of problems managed through clinical interventions are inappropriate prescribing (incorrect agent, dose, dosing schedule, or interactions), adverse reactions, and prescription of drugs which are contra-indicated.
- *Medication management services.* These services include medication reviews, in which a medical practitioner refers a patient to a pharmacist for a review of medication use. The reviews, con- ducted in patients' homes or in a nursing home, are described below.
- *Preventive care services for patients with chronic conditions*. These services, which are provided in conjunction with patients' own doctors, include screening, health-promotion informa- tion, drug information, monitoring of adher- ence to prescriptions and of factors that affect adherence, and monitoring of intended drug- therapy outcomes and adverse effects.
- *Participating in therapeutic decisions.* Pharmacists may make an active contribution to therapeutic decisions, for example in providing advice on asthma management, weight management, and smoking cessation.

Through these six types of services, community pharmacy makes a major contribution to (a) the care of patients of medical practitioners, (b) the individuals who present direct to pharmacists to ask for advice, and (c) prevention and public health more broadly. A systematic review of

wellbeing and relief of symptoms for

pharmacist professional services has recently been published and is available through the Pharmacy Guild of Australia (Roughead, Semple & Vitry 2003). The development of these services has been discussed in Australia throughout most of the last decade (Carr & Benrimoj 1996).

Changes in the Workforce of Community Pharmacies

In 2001, the Australian Bureau of Statistics Cen-sus of Population and Housing identified 13 902 persons as 'pharmacy workers' (AIHW 2004). This figure is similar to the total number of individuals listed as pharmacists by state and territory registration boards. Census data com- bined with data on part-time participation in the pharmacy workforce (Health Care Intelligence Pty Ltd 2003) suggest that there were just under 12 000 full-time equivalent pharmacists Aus- tralia-wide. The pharmacist workforce is charac- terised by a high level of female participation (52% of the 'pharmacy workers' enumerated in the 2001 Census were women) and increasing part-time participation. Survey data indicate that just under 80% of registered pharmacists prac- tised in community pharmacy in 2002 (Health Care Intelligence Pty Ltd 2003). Thus there are about 11 000 practising community pharmacists in Australia, or about 9500 full-time equivalents. Two factors have influenced the development of the pharmacy profession. The first is the dichotomy of retailing versus professional activi- ties. Pharmacists in community practice are retail- ers, at the same time as undertaking a wide range of professional activities in the six types of serv- ices described above. The second is the shift from an apprenticeship system to a university-degree-
qualified workforce.

Before 1960, the training of pharmacists involved attendance at some university courses on a part-time basis, combined with on-the-job experience in an apprentice role. State and terri-tory pharmacy registration boards issued the qualification 'Pharmaceutical Chemist' (PhC) to those who fulfilled the requirements for practice. The PhC qualification was apt for the function of pharmacists at the time; they were primarily 'chemists', preparing and dispensing medicinal substances and products, with no other signifi- cant clinical responsibilities.

In 1960, the first Bachelor of Pharmacy (BPharm) degree program in Australia was intro- duced by The University of Sydney. Other uni- versities soon followed with bachelor's degree programs, initially of three years' duration. All pharmacists entering practice in Australia are now required to have at least a four-year Bach-elor of Pharmacy (BPharm) degree from a recog- nised university, and to have satisfactorily completed a period of supervised practice (of one year's duration, or equivalent) in a phar- macy. The BPharm degree and the practicum together confer eligibility for the competency- based registration examinations run by state and territory pharmacy registration boards. Thus a third party, representing the interests of the public, is responsible for registration examina- tions. Arrangements exist for registration boards to admit overseas-trained pharmacists.

When the BPharm degree was first introduced, the curriculum still concentrated on pharmaceu- tical chemistry, and pharmacists still saw them- selves as 'chemists' rather than as 'pharmacists'. The term 'pharmacist' implies a professional with a range of clinical skills and responsibilities and the training and capacity to interact with patients and others in the community.

Graduates responded in three different ways to the 'chemist' role. Some found it to be a satisfying profession which could be pursued in a fairly conservative manner. Some found it unsatisfying, and left. Others had an entrepreneurial bent, enjoyed business, and developed the retailing side of running a 'chemist's shop'. The develop-ment of professional services was constrained by legislation, which prevented

advertising of profes- sional services but allowed pharmacists as retail- ers to promote their businesses and products.

the supply and distribution of medicines, but inhibited professional services.

Several forces changed the balance between the product orientation and the service orientation in community pharmacy. The emergence of the sci- ence of pharmacokinetics in the 1960s and 1970s was a major factor. Pharmacokinetics deals with the interaction between pharmaceutical substances and normal or abnormal physiological systems in the body. The introduction of pharma- cokinetics led to an increasing engagement between the discipline of pharmacy and human biology, linking pharmacy with life-science fields such as physiology and biochemistry. Pharmacokinetics also came to occupy an increasing amount of curriculum time in pharmacy-degree programs which had previously concentrated on pharmaceutical chemistry. It strengthened the academic base of pharmacy training, linking pharmacy with the cause and mechanisms of disease. Indirectly, it started a process of re- positioning pharmacy practice in health-care sys- tems, enabling the profession to contribute more prominently to clinical aspects of health care.

By 1975, the pharmacy profession in the United States of America had begun to demand changes to training programs that would give graduates the skills and capacity for a broader range of clinical activities in both communitypractice and hospital practice. US educational institutions responded by adding courses in phar- macy practice to the existing basic sciences, phar- maceutical chemistry and pharmaceutics content of their programs. Institutions moved to offer higher degrees, such as the Doctor of Pharmacy (PharmD), in recognition of the new programs' scope and duration. Australian universities sim- ply expanded the content of the BPharm, and in 2000 the BPharm was lengthened from a three- year to a four-year full-time program. Indeed, Australian schools (led by Victoria in 1981) werethe first outside the USA to introduce psychoso- cial sciences

This led to the establishment of good systems for

into their curricula, in recognition of the fact that pharmacists spend much of their time interacting with people. More recently, there has been a move for the basic-science content of pharmacy-degree programs to have a strongerclinical orientation, and for more new areas to beadded to the pharmacy curriculum. These include herbal medicines, pharmacogenomics, pharmaceutical management, and the delivery of more advanced clinical services such as disease state management and home medication reviews. Vocational education programs are now produ- cing graduates who are much better equipped to deliver the professional and clinical services that pharmacists are expected to be able to provide, including public-health services such as health promotion and services for primary, secondary and tertiary prevention.

The Role of Community Pharmacies in the Federal Medicines Policy

Through both its traditional role in the supply of medicines and its evolving role in the provision of professional services, community pharmacy makes a critical contribution to the implementa- tion of the National Medicines Policy. The Policy has four elements:

- equitable access to necessary medicines;
- medicines of high quality, safety and efficacy;
- quality use of medicines; and
- a viable and responsible local pharmaceuticalindustry.

The development of a service orientation in community pharmacy is particularly important for the quality use of medicines (QUM), which isdefined in the Box .

The National Strategy for the Quality Use of Medicines places an onus on community pharma- cists, as health practitioners and educators, to:

- maintain their own knowledge and expertise;

- use objective information as the basis for deci- sions and advice;
- improve medication use by recognising and taking action to correct problems associatedwith medicines;
- enhancing understanding of the risks and ben- efits associated with medication use; and
- assisting people in making informed decisions about their treatment options.

In order to encourage community pharmacy to contribute to the implementation of QUM poli-cies, the Australian Government has introduced three new funded opportunities, as follows.

- Pharmacists can now claim a payment of 10 cents per prescription item from the Health Insurance Commission for ensuring that writ- ten drug information is included in medication packaging, or for providing such information separately. This is known as Consumer Medi- cine Information (CMI). CMI may be given to the patient as a leaflet produced by the manu- facturer (either included in medication packag- ing or handed to the patient separately) or as a computer printout produced by the pharmacist (Koo, Krass & Aslani 2002).
- Pharmacists who supply medications to the patients in a nursing home can claim $100 per bed per annum for reviewing patients' medica- tions, under the Residential Medication Man- agement program.
- Pharmacists can undertake Home Medicines Reviews (HMRs) for patients who are referred by their doctors. The referring doctor supplies clinical information to the pharmacist with the patient's consent. Each review may include an interview with the patient, an examination of medicines stored by the patient, an assessment

The Pharmaceutical Benefits Scheme and Local Pharmacies

The introduction of the funded opportunities to participate in QUM reflects a general trend to engage community pharmacy in the implementa- tion of health policy initiatives by providing financial incentives. They build on a long history of fee-for-service for the dispensing function of community pharmacy under the Pharmaceutical Benefits Scheme (PBS).

The PBS subsidises the cost of some 792 generic medications that are available on pre- scription or for medical practitioners to carry as doctors' bag supplies. Before any medicine is approved for listing on the PBS, it must be approved for use in Australia by the Therapeutic Goods Administration. Applications for PBS list- ing are usually made by pharmaceutical manu- facturers. The application process is very rigorous. The application is assessed by an expert committee (the Pharmaceutical Benefits Advisory Committee, or PBAC) which examines the clinical effectiveness, safety, and cost-effec- tiveness of the proposed new listing in relation to other treatments (see Sansom in this issue *page 194*). If the PBAC recommends listing, the medicine is considered by the Pharmaceutical Benefits Pricing Authority, and a price is negoti- ated between the manufacturer and the Depart- ment of Health and Ageing. The listing is then considered by the Australian Government, with final approval coming from the Minister for Health.

For example, the negotiated price plus pharma- cists' fees for the drug azathioprine (which in different doses has a wide range of indications, from the treatment of cancer to the treatment of inflammatory bowel disease) is $73.70 for the standard prescription quantity of 100 tablets, each containing 50 mg of the drug. A patient would pay $23.70, so the cost to the Australian Government (as the PBS subsidy) is $50.00 per prescription. Of the total of $73.70, the dispens- ing pharmacist receives $10.94, made up of a standard dispensing fee of $4.66 for a ready- prepared medicine plus 10% of the net cost of the drug. At present, most patients make a co-pay- ment of $23.70 for each PBS prescription item, and the remainder of the cost is covered by the PBS subsidy. Patients with low incomes

and patients who receive sickness benefits make a co- payment of $3.80. The co-payment is higher if a particular brand of a PBS item is specified in the prescription, and if the specified brand has a higher price than other brands of the same drug. A 'safety net' protects both patients and their families from the need for excessive expenditure on PBS medicines. From the beginning of 2005, PBS co-payments will increase by $4.90 for general patients and 85 cents for concession patients. The Australian Government's expenditure on the PBS has increased markedly. In 2002–03, there were 158.5 million community PBS pre- scriptions, an increase of 2.6% over the 2001–02 figure of 154.5 million and of 7.4% over the 2000–01 figure of 147.6 million. These figures do not include repatriation PBS prescriptions or doctors' bag prescriptions (15.4 million and 0.5 million respectively in 2002–03). The cost to the Government of PBS prescriptions (excluding repatriation PBS prescriptions) rose from $4.18 billion in 2000–01 to an estimated $4.57 billion in 2002–03 (a 9.4% increase). Total patient con- tributions rose from $744 million to $860 million (a 15.6% increase) (AIHW 2004).

Given these increases, it is not surprising that the Australian Government has been developing policies to contain the cost of the PBS. Changes to the PBS inevitably affect pharmacists' incomes, because pharmacists' mark-ups on PBS medica- tions is fixed at a maximum of 10% (the percent- age is lower for some high-cost drugs). An instance of a recent change is the introduction of the Therapeutic Group Premium Policy, whereby the PBS provides a subsidy up to the price of the lowest-priced drug in a group of similar drugs which have been shown to be of similar safety and produce similar health outcomes. Examples of such drugs are H_2-receptor antagonists (which suppress gastric acid secretion) and dihydropyri- dine derivative calcium-channel blockers (used in the treatment of hypertension, among other things) (Health Insurance Commission 2004). If a drug other than the lowest-priced drug is pre- scribed, the patient bears the additional cost. The pharmacist's 10% markup is based on the lowest- priced drug in the group. Another instance is the Brand Premium Policy. This applies where manu- facturers develop generic equivalents of drugs for which original patent restrictions have expired. Manufacturers can apply to have generic equiva- lents listed on the PBS, and these are usually cheaper than the originator drugs. The PBS pro- vides a subsidy up to the price of the lowest- priced brand, so the price is then set by a generic rather than the originator brand (Health Insur- ance Commission 2004).

Alongside the changes in the PBS, pharmacists in community pharmacies face competition from internet marketing of medicines. For some con- sumers, internet marketing can be an effective medium both for supply of and the provision of information about medicines. However, its role in the Australian medicines market has not yet been evaluated. As far as we are aware, no data are available on the volume or value of internet sales or trends.

Proposed Community Pharmacy Directions

Future directions for community pharmacy are influenced by economic factors such as changes in the PBS and the introduction of new incen- tives, and by professional factors. Every five years, an agreement is negotiated between the Austral- ian Government and the Pharmacy Guild cover- ing the directions for the forthcoming five years. The agreement is a blueprint for the development of professional services and other activities of community pharmacy. It sets out the remunerated roles for community pharmacy, and thus becomes the determinant of sustainable practice for the profession. The Fourth Community Pharmacy

Agreement is currently being negotiated; the Third Agreement covers the period from 1 July 2000 to 30 June 2005 (Department of Health and Ageing 2003).

Circumstances of the Economy

Pharmacy sales can be grouped as follows:

- Sales of prescribed medications
- Sales of non-prescription products which are sold only by pharmacists. These include Schedule 2 (S2) medicines (such as large packs of simple analgesics such as paraceta- mol — small packs are available from other retailers, such as supermarkets) and Schedule3 (S3) medicines, ie, products which cannot be obtained from other outlets in any form or quantity (eg, salbutamol inhalers for asthma). For the latter, the sale must be supervised directly by a pharmacist. Regulations for the sale of S2 and S3 medicines vary slightly among states and territories.
- Sales of other goods in pharmacies, ranging from wound dressings to photography goods. Most of these are obtainable from other types of retailers.

Sales of prescription drugs involve acting on the instructions of a medical practitioner. Sales of non-prescription (S2 and S3) drugs involve the pharmacist in primary care, or involve the patient in self care, or both. For over a decade there has been an international trend to for products toshift from prescription to non-prescription status (Blenkinsopp 2004).

Despite this trend, the proportion of revenue from sales of prescription drugs is going up relative to revenue from non-prescription drugs and other goods. Sales of non-prescription drugs are fairly constant, while sales of other goods are declining.

However, profits from prescription-drug sales are declining mainly because margins from the dispensing of medicines on the PBS are declin- ing. Only the large volume of sales prevents overall prescription-drugs profits from declining further. Profits from sales of non-prescription drugs are growing marginally (Pharmacy Guild of Australia 2003).

The reliance on the Australian Government as the single payer for PBS prescription medicines (apart from patient co-payments) is a major threat to pharmacy incomes. In the 1960s, the pharmacist's markup on a PBS prescription was 50% of the net medication cost. This decreased to 33%, and (as described above) it is now 10%, or less for high-cost drugs (Health Insurance Commission 2004).

These trends have caused the pharmacy indus- try to examine opportunities for a broader remu- neration base that is less reliant on the volume generated by the supply of PBS medicines. The industry has a strong interest in an expansion of remunerated service roles, but wishes to retain the product-supply role because the product and service roles are mutually reinforcing. A combina- tion of product-supply and service roles is there- fore likely to continue.

The owners of community pharmacies are rep- resented by the Pharmacy Guild of Australia, and the pharmacy industry negotiates with the Aus- tralian Government through the Guild. Thestrength of the Guild depends, in turn, on the strength of the network of community pharmacy owners. At insistence of the Guild, payments for services are made to pharmacy owners. The phar- macists who supply the services that attract the remuneration may be employees or contractors ofowners. Government policy is therefore effected through pharmacy owners, rather than the pro- fession as a whole. The only exception to this arrangement is payment for Residential Medica- tion Reviews, which is made direct to the phar- macists who provide the services.

On behalf of its members, the Guild is seeking opportunities to extend the range of services that community pharmacy can supply in imple- menting QUM policy. The Guild has linked recent initiatives to the quality-improvement aspects of QUM policy. To this end, it has supported the introduction of the Quality Care Pharmacy Program (QCPP). The QCPP is a mechanism for assuring Government and the public that individual pharmacies provide high- quality services. As negotiated between the Guild and the

Australian Government, pharmacies that register and attain accreditation with the QCPP attract incentive payments totalling about $15 000. The QCPP makes use of competency standards and professional practice standards developed by the Pharmaceutical Society of Australia (PSA 2004a; PSA 2004b).

In addition to drug information, Residential Medication Reviews, Home Medication Reviews, and QCPP, the Guild is exploring other possible avenues for strengthening the role and service-remuneration base of pharmacy in the healthsystem. These include increasing involvement in chronic-disease management, through more active participation in the management of conditions such as asthma and diabetes mellitus; more active participation in preventive services, such as weight reduction and smoking cessation; assessments of concordance with treatment recommendations; case conferencing with other health-care professionals; and quality-improvement activities. Overall, economic influences on community pharmacy are likely to promote integration of normal professional practice into a business orientation that is appropriate for a retail setting. They are also likely to cement an interdependence that has grown in the last 10–20 years between a product orientation and a service orientation.

Factors relevant to one's career

Two major professional service factors are likely to influence future directions for community pharmacy.

The first is an increase in cognitive pharmacy services, that is, professional services such as medication management and clinical interventions. Pharmacists' capacity to deliver these services is likely to be strengthened as cohorts of new graduates who have received appropriate vocational training gradually replace older practitioners who were educated as 'chemists'. Fulfillment of accreditation requirements will entitle practitioners to claim remuneration. This provides an incentive to assure the quality ofcognitive services.

The second is an increasing emphasis on quality-assurance systems. As part of the overall tightening of quality and safety in health systems, there is likely to be increasing pressure for pharmacy to have a formal service-quality framework.This will build on the pharmacy industry's substantial experience of competency standards and professional practice standards (PSA 2004a; PSA 2004b), with incentives and support through the QCPP and other programs. A recent National Competition Policy Review of Pharmacy has already recommended that state and territory pharmacy registration boards "should implement competency-based mechanisms as part of re-registration processes for all registered pharmacists" (Wilkinson 2000).

The development and uptake of quality-assurance systems is at the core of a debate about the deregulation of pharmacy, which has been continuing for at least two decades. The stimulus for the debate was an attempt by the British pharmacy chain, Boots the Chemist, to enter the Australian market with multiple retail outlets and non-pharmacist ownership. The Boots attempt was unsuccessful, but it prompted Australian supermarket chains to pursue the same perceived market opportunity. The pharmacy profession has repeatedly won the deregulation argument on public benefit grounds (Wilkinson 2000), with strong government and non-government support in the media (see, for example, Brooker 2004). The purported public benefit of independent professional ownership is the assurance of safety and quality; the Pharmacy Guild of Australia is encouraging community pharmacists to work together as a network of independent professionals who have a capacity to implement and sustain safety and quality standards (PSA 2004a; PSA 2004b).

Conclusion

Pharmacy education in Australia has already responded to changes in the health

system, and has led changes in the pharmacy profession to equip graduates for new professional roles. These changes are likely to be consolidated in the near future

Future agreements between the Pharmacy Guild of Australia and the Australian Government will provide both a policy framework and a remuneration framework for community phar- macy, anticipating an increasing recognition of the potential of community pharmacy to make a major contribution to the implementation of health policy through health promotion and pri- mary, secondary and tertiary prevention.

and to continue as vocational education adapts to the evolving roles of pharmacy in the health system.

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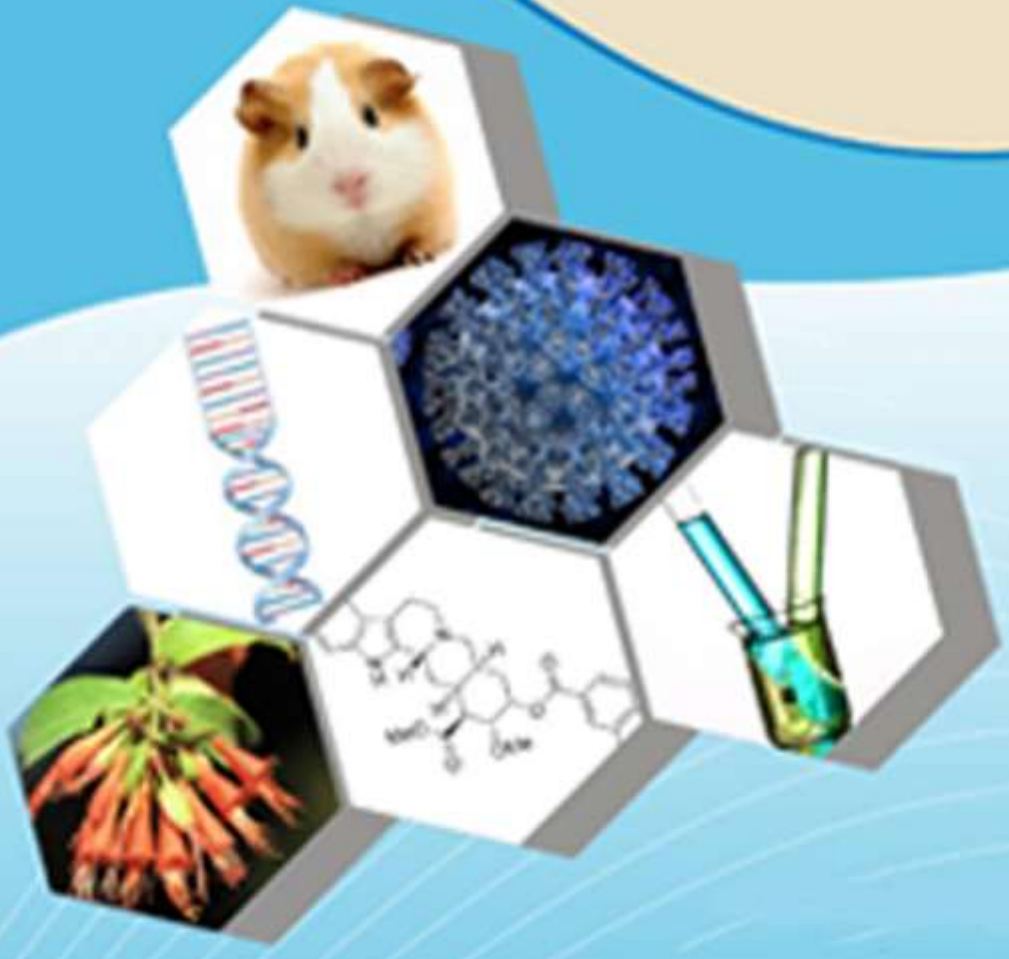

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Integrity and credibility in qualitative pharmaceutical studies

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A B S T R A C T

Researchers in the field of social pharmacy all over the world have been increasingly turning to qualitative research techniques, either on their own or as part of more comprehensive mixed methodologies investigations, thanks to the insights they may provide. Due of this growth, it is essential to equip researchers with guidelines for using these techniques effectively. The theoretical foundations and practical issues of ensuring quality in qualitative social pharmacy research are discussed in this discussion. Specifically, it describes the many methods used to demonstrate the credibility, transferability, dependability, and con- firmability that Lincoln and Guba offer as criteria for trustworthiness. In addition, a short introduction to authenticity is provided, which is a more contemporary and less often utilized set of criteria that requires proof of fairness, ontological authenticity, educational authenticity, catalytic authenticity, and tactical authenticity. The commentary defines each of these words, explains how it relates to social pharmacy research, and offers advice on when and how to utilize it. These are supplemented by illustrative cases from the pharmaceutical literature. At the conclusion of the discussion, we summarize several approaches to determining quality in the scholarly literature and ask you to consider how these criteria may be applied to your own qualitative study.

Introduction

Four characteristics distinguish science from non-science. Science is theory-based, uses systematic research techniques, is cumulative and is predictive.[1] All science is embedded in belief systems known as para- digms, which are frameworks based on assumptions about ontology (nature of being) and epistemology (nature of knowledge and ration- ality of belief). Four paradigms are used most commonly: positivism, post-positivism, interpretivism, and critical theory,[2] each with their own important differences.[2,3]

Research carried out within the positivist/post-positivist paradigm strives towards objectivity and neutrality and typically employs quan- titative studies with a focus on numbers, precision, and general- izability.[4] A positivist approach, however, may not be the best way to address some research questions. Hence, there has been a notable in-crease in interpretivist studies using qualitative methods in health

services research, including pharmacy research, to tackle questions that do not lend themselves to a positivist paradigm. Social pharmacy re- searchers have been using qualitative methods to understand, explain, discover, and explore patients' and health care practitioners' beliefs, perceptions, and feelings. With considerable variety in qualitative ap- proaches,[5] qualitative research provides considerable options for re- searchers to pick when approaching a research question.[6]

Qualitative research allows a researcher to provide an interpretation of observed experiences and actions of individuals and groups in dif- ferent contexts.[2] It lends itself to health services research in general and social pharmacy research in particular, where an in-depth under- standing of the participants' experiences is needed. As it pertains to more critical theoretical studies, qualitative methods can also be used for the "democratization" of research through carrying out studies that are more inclusive, collaborative, and involving partnerships and co- production.[7] Indeed, such methods can provide a framework that is not only "about" or "on" participants but, rather, with and by participants as co-creators.[8] This is particularly important, especially with the increasing calls for increased patient involvement in health servicesresearch.[9]

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Quality in qualitative research

A number of articles have addressed issues related to the rigor of qualitative health services research in recent years. Recently, an insightful study highlighted the importance of demonstrating rigor in qualitative pharmacy research and briefly defined strategies to ensure its rigor.[10] In that paper, Hadi and Closs[10] included a brief review of pharmacy research that uncovered gaps in demonstrating rigor. In the field of pharmacy education, guidance for publishing qualitative re- search with checklists were provided[11] along with best practices of steps of designing, conducting, and reporting qualitative research in a step-wise approach using attributes of quality.[12] Another important manuscript provided recommendations for key components for con- ducting qualitative research, articulating the process, presentation and contextualization of results in pharmacy education studies.[13]
More generally, Tong et al.[14] provide a consolidated set of criteria for reporting qualitative research (COREQ) comprising a 32-item checklist to help researchers and reviewers incorporate and assess how a qualitative research article describes aspects of the research team, methods, context, findings, analysis and interpretations. This and several critical appraisal instruments have been designed to facilitate an assessment of methodological quality of qualitative studies.[15] While helpful for ensuring details about a project and its methods are assessable, checklists do not necessarily ensure quality in a qualitative project. A reflection of quality criteria and their use in pharmacy would help the reader better discern a strategy for establishing quality according to the specific project context including the research question and method used.
This paper adds to earlier work by providing a more detailed and thorough discussion of what qualitative quality criteria are, when and how to use each standard, their pros and cons, and concrete examples in the pharmacy literature to further demonstrate them. Additionally, this paper provides an overview of the authenticity criteria and criteria is particularly worth noting since a common problem with qualitative pharmacy research has been spending inadequate time in a "culture" before researchers produced their understanding of a process. Quite often a researcher may conduct a number of interviews with pharmacy staff or patients and wrap the project hastily without al- lowing oneself the opportunity to get the full scope of issues under study. The reader may wonder on the amount of time that would be enough for a prolonged engagement. Lincoln and Guba[16] posit that, while this is relative, there can be signs that prolonged engagement has
been achieved. Those signs include being able to survive without a challenge in a culture and building trust with its members. As a technique, prolonged engagement involves researchers being cognizant of potential distortions coming from one's own prior formulations about an issue. Equally important for the researcher is to be cognizant of
potential intended and unintended distortions coming from participants who, for example, can have "situated motives" including the motive to say things that would be more appropriate or please the researcher or can misconstrue the researchers' questions.[16] One pitfall for prolonged engagement is described as "going native". In particular, Lincoln and
Guba[16] caution that a researcher may "lose detached wonder" and
become unable to discover something compared to a less involved researcher.
It is important for the researcher to consider this while reflecting on the epistemological perspective adopted. While these criteria were proposed with constructivism in mind, the notion that a researcher may "fail to discover a phenomenon" favors objectivism, embracing the belief that a researcher can come to one reality. Within a more con- structivist approach, motives of researchers and participants are ac- knowledged and addressed in a transparent manner while adjacent sub-criteria, which have not been addressed in the context of qualita-tive social pharmacy research. This is done with the aim of providing clear and comprehensive guidance for pharmacy researchers who are invested in demonstrating rigor in their research. It is important to alert the reader that the criteria discussed in this commentary are not meant to be treated like a checklist. Strategies addressing criteria that will be discussed here should be considered in combination with the under- lying problem and specific research question in each qualitative study. Thus, researchers are encouraged to reflect on those criteria and con- sider ways in which they could be of use in their qualitative or mixed methods research projects.
The paper begins by presenting techniques used for establishing *trustworthiness* subdivided into credibility, transferability, dependability and confirmability. These techniques are prolonged engagement and persistent observation, referential adequacy, member checking, trian- gulation, negative case analysis, thick contextual description, external audit/audit trail, and reflexivity and transparency. The second section of the paper provides a brief overview of the so-called authenticity criteria including fairness, ontological authenticity, educative authen- ticity, catalytic authenticity, and tactical authenticity and their value in social pharmacy research ().

Trustworthiness

Prolonged engagement is a technique that gets the researcher to spend adequate time to learn about the culture in which the research is conducted, build trust, and reflect on potential distortions introduced by the researcher and participants along with their impact.[16] This

discussing the choices that have been made (see the discussion of reflexivity and transparency below).
One example for prolonged engagement involved a study that addresses clinician patient communication and care in relation to Muslim patients considering fasting during Ramadan. Participants in this study included patients with chronic conditions representing different ethnic groups from two countries.[17] In the US, some participants were recruited from Fort Wayne, Indiana, which houses a large Burmese population. Before data collection, two of the researchers connected with the Burmese Muslim Education and Community Center. They had dis- cussions with members and leaders of the community about the topic, attended events organized by the community including an annual Iftar ceremony, and recruited translators from that community. This allowed for a strong relationship and trust between researchers and the com- munity.

Persistent observation

"If the purpose of prolonged engagement is to render the inquirer open to the multiple influences - the mutual shapers and contextual factors - that impinge upon the phenomenon being studied, the purpose of persistent ob- servation is to identify those characteristics and elements in the situation that are most relevant to the problem or issue being pursued and focusing on them in detail. If prolonged engagement provides scope, persistent observation provides depth" [13 (p. 304)].
Qualitative researchers would expectedly make analytical or at least contextual decisions from their data that are particularly salient to their research. Persistent observation helps a researcher become mindful of aspects that are particularly important to the research question and has them focus on those aspects in detail. As part of a constant process, the researcher should keep a record of what are believed to be salient factors and explore them in greater depth. Then, the researcher would ask oneself as to whether the analysis is

superficial or erroneous. The process would be repeated and temporary labels revised in enough
detail that the researcher would be able to describe the process. Thus, persistent observation provides "depth" as compared to prolonged engagement which provides "scope." Prolonged engagement compels the
researcher to reflect on multiple influences shaping context, while persistent observation requires that researchers screen irrelevancies and explore in detail issues that are truly important. Lincoln and Guba[16] caution against the so-called "premature closure" where a researcher hastily decides on what is determined to be salient for the study question. Ongoing healthy "skepticism" of participants' intentions should be practiced, particularly where concerns about deception are present. The reader may note that a researcher would need to exercise both prolonged engagement and persistent observation to allow for reflexivity and a thick description of data, which will be discussed further below.

A unique example of persistent observation and prolonged engagement is demonstrated in a study conducted by several and rather different researchers.[18] The overall purpose of the study was to conduct a Health Technology Assessment (HTA) of automated dose dispensing in the Danish primary health care sector. The HTA research group carefully made notes throughout the study period, which lasted for several years. The professional background of the project members was in diverse fields such as nursing, social science, health economics, and social pharmacy. All but one researcher had worked in their field for more than 20 years. Because the HTA was conducted on technical, economic, organizational, and patient-related aspects of dose-dispen- sing, a variety of research methods were applied, including focus group and individual interviews, surveys, register-based calculations, health economic analysis and documentary material analysis. About half-way through the study, the project group realized that the majority of the problems experienced stakeholders were related to the dose-dispensing system, but there were quite a few that were not due to the technology itself. Rather, the use of technology acted as a magnifying glass for problems that already existed in the system. Reaching this conclusion would have been much more difficult had the researchers prematurely closed the project. Instead, they took their time and kept records of their presumptions, ideas, and doubts throughout the process. Their extensive knowledge from carrying out other studies focusing on problems in the Danish health care sectors also contributed to this level of depth.

Member checking

Member checking is recommended by Lincoln and Guba[19] as a vital technique available for the qualitative researcher to strengthen the credibility of data. In the process of member checking the researcher returns data, analytic categories, data interpretations, and/or even conclusions to study participants. The argument is that by giving participants the opportunity to review research work, a researcher can claim that the work adequately presents 'own and multiple realities'. As suggested by Lincoln and Guba (1985), member checking can be per-
formed formally or informally.[16] Informal member checking is more immediate and involves recordings or transcripts involving a participant being played back to that participant or observations from a set of participants tested with others. Informal member checking helps a re- searcher in a number of ways including the opportunity to assess in- tentionality, meaning that the participant actually intended to provide such information to researchers. It also provides the opportunity for a participant to recall additional points/ideas, correct errors, and provide context. Formal other hand, is more struc- tured. It is important to note that the member checking team is asked as to examine whether constructions provided to them by the research team are viewed as fair even if they do not totally agree with them.[16] Here, the researcher provides the study report to the member check team in advance, a process that can take one or more days. This team would include individuals from different groups included in the study. Feedback from the member checking team can be obtained using different modes according to the nature of the study and participants.[19,20] The researcher might ask for written feedback or meet face-to-face to discuss any revisions and comments on the notes or analyzed data (i.e.

thematic summaries).[20]

As the reader would note, member checking could be particularly relevant and feasible for many pharmaceutical health services research projects, which usually involve patients, physicians, and of course, pharmacists. A recent example of the use of member checking is a study that aimed to better understand prescription opioid abuse related communication among prescribers and pharmacists.[21] That study de- scribed intraprofessional and interprofessional prescription opioid re- lated communication among and between opioid prescribers and community pharmacists. To enhance credibility of the reported themes, member checking interviews were conducted with one participant from each of the five focus groups in the study. According to the authors, while member checking interviews did not result in major changes to themes, some were expanded upon to improve clarity of the report. A more comprehensive example on the use of member checking comes from a study that used grounded theory to describe patients' perspec- tives of medication-taking tasks associated with long-term medication use.[22] It also contributed to an understanding of how these tasks relate to patients' medication information needs. In that study, the emergent framework was presented to participants who provided their input on how the emerging framework explained medication-taking practices and their education needs. Participants invited to participate in the member checking team came from groups who represented varied approaches to medication-taking practices. As part of the member checking process, members agreed that patients using medications for long intervals may undergo continuous change in how they think (lo-
gically) and feel (emotionally) about their medications because of changes in the patients' conditions and their situational context.[22] They also felt that the presented model helped them understand perplexing actions and behaviors they observe with their patients that they could not understand or tackle before.[22]

Participants in qualitative research are always part of the data generation in qualitative research. For example, in qualitative interviews, it is common to test preliminary hypothesis and analysis with the informants.[23] Despite this fact and the argument that member checking can be a key process in establishing the credibility of the collected data, there are some disputes about its use. Morse,[24] for example, does not recommend member checking as a strategy. She argues that since the analysis would usually involve a synthesis of different interviews, a participant would not easily recognize their own story in the presented text. The participant's ability to critique the methods and outcomes
used is at play here. The issue of favoring participants with better lit- eracy is not exclusive to qualitative research – a research participant may find it hard to check and comment on quantitative data, particu- larly those involving complex statistical analysis. This could partially be tackled through doing member checking in a more tailored manner that purposefully provides opportunities for underprivileged participants to contribute.

A researcher could show results to a participant and ask him/her to "find oneself" in the results. Instead of having participants point their own quotes, which could breach participants' confidentiality, partici- pants would be asked more nuanced questions: Do the results make sense? Does the approach itself make sense? Having lived the experi- ence, such as, for example, illness and medication use, a participant is in a position to provide a unique perspective on data that a researcher may not be able to bring no matter how trained and experienced in the topic he/she is. This could provide a more nuanced or even different
view of reality, which is particularly important to address in qualitative research. A researcher might face a tough decision when the participant does not feel the analysis is correct. Should changes be made even if the researcher "outranks the participant as a judge" of the conceptual fra- mework and research methods used in analysis? While there may not be a preferred way of integrating participants' input in the analysis, ide-ally, the perspective of participants should be given equal weight to allow for *fairness* as will be described below.

This is particularly im- portant if the interpretation of a participant varies with that of the researcher. As the practice of modern medicine moves towards patient- centered care, involving patients in research is becoming critical. In fact, the US Patient-Centered Outcomes Research Institute (PCORI) requires funded applicants to involve patients and relevant stakeholders in different phases of a study including helping to interpret findings.[9] While PCORI does not put a condition that involved patients are re- cruited from the ones who are being studied, some might argue that member checking would create a unique opportunity for patient in- volvement.

Another concern is that some study participants might refuse to be part of this process of member checking. Participants who agree to participate might concur with the researcher's interpretation of the analyzed data and might not oppose the researcher's interpretation due to politeness or other reasons. When carrying out this process, Lincoln and Guba[16] particularly caution researchers to be cognizant of this, which could lead to "reconstructing an average or typical position", defeating the purpose of a qualitative study.

Lastly, the kind of training that is provided to the formal member checking team is worth reflecting. While providing such training could help researchers provide a report that adheres to requirements by theresearcher, this training may inadvertently lead to the loss of authen- ticity in the feedback provided thus defeating a key value for member checking. Researchers should be cognizant of this issue when providing instructions or training to the member checking team. One approach ofaddressing this issue is to break the member checking team into two halves and train one of them while leaving the other with minimal instructions. Striking this balance, however, may be resource intensive and may not be feasible in smaller studies.

Triangulation

Triangulation is a general approach to check and establish the credibility of qualitative findings by analyzing a research question members who do not dispense antibiotics injudiciously allowed for a better understanding of factors driving the process. Data were also obtained, not just from pharmacists, but also from pharmacy assistants who provided valuable information on their role in the provision of antibiotics. This complemented the data generated from interviews with pharmacists.

Investigator triangulation: This type of triangulation applies to qualitative studies in which data output emerges from researchers' analytical work. To allow for credibility, several different researchers within the field study are involved. It is expected that team members, applying the same technique, arrive to the same results.[26,27] Using teams of re- searchers with different backgrounds (e.g. demographic and/or pro- fessional) may be used to check the process of data collection and/or interpretation. It should be noted, however, that an enhancement in credibility should not be viewed as the only goal of investigator trian- gulation. Qualitative researchers have used this technique to ensure that the analysis is rich, comprehensive, and inclusive of insights and interpretations by different researchers. The researcher is encouraged to apply this technique with these two applications in mind. The study addressing injudicious dispensing of antibiotics described above in- volved three authors with an interest in public health. The first author was a former practicing pharmacist with an interest in research exploring pharmacists' behavior. The second author was a non-practicing physician with an interest in public health and antimicrobials, while the third author was a primary care practitioner.[32] The presence of practitioners and non-practitioners, a pharmacist along with physicians, meant that a diversity of perspectives were used allowing for credibility and a richer interpretations when analyzing data. So, for example, re- search physicians and the research pharmacist recognized the role of patient pressure in providing antibiotics to patients, which improved credibility in that finding. On the other hand, the researcher with pharmacy background provided additional insights on the role of the ministry of health inspection mechanisms in relation to community pharmacists, which were related to his prior experience.

from more than one perspective.[25] Triangulation can be categorized into four classical types: methodological, data, investigator, and theoretical tri- angulation, along with a growingly important and prevalent fifth one called environmental triangulation.[26,27]

Methodological triangulation: With this frequently used approach to triangulation, the researcher uses more than one method or a combination of methods to compensate for the limitations of one approach with the strengths of another, aiming to improve the credibility of the findings when these are broadly convergent.[26,27] According to Bryman,[28] there is within-method triangulation and between-method triangulation. The first uses assortments of the same method; for example, conducting an open-ended, drawing-based interview as well as a semi-structured interview with the same asthmatic children.[29] The second uses contrasting methods, such as combining interviews with observation.[30] Pharmaceutical education has been a fertile field for methodological triangulation approaches. An interesting example comes from an introductory, team-based, interprofessional education course for first-year students that used small-group methods for health- professions students' learning of interprofessional collaboration.[31] A triangulation of assessment involved conjoint use of quantitative and qualitative methods. This enabled researchers to effectively assess various outcomes including students' self-ratings of attaining learning objectives, perceptions of other professions obtained from word cloud responses, and student satisfaction through end-of-course evaluations. *Data triangulation*: The researcher promotes data collection through different sampling strategies, such as different times and/or contexts for the same or varying participants. An example is demonstrated in a study that attempted to understand why antibiotics were dispensed injudiciously in community pharmacies.[32] In that study, 13 pharmacy staff members were recruited from community pharmacies that dis- pensed antibiotics, while two were recruited from pharmacies that did not dispense antibiotics as identified by simulated client visits in the quantitative phase of the study.[33] Perceptions of pharmacy staff

Theoretical triangulation: When using theoretical pluralism, the researcher combines multiple perspectives and frameworks to explore and make sense of a single data set. Contrasting with the data and investigator triangulation, this method usually involves using experts outside the study main area, from different disciplines or positions. When interpretations are convergent, research findings are assumed to be credible.[26,27] One example that demonstrates this, is a study where Kotter's model of change management was used simultaneously with the normalization process theory, a sociological tool, to explore the implementation and sustainability of medication reviews in older pa- tients by clinical pharmacists.[34] This combination provided a more rigorous support to explore events, actions as well as other factors in- volved in the investigated process.

Environmental triangulation: Somewhat similar in concept to data or methodological triangulation, this type of triangulation is seldom em- ployed.[35] This method uses a range of different settings, such as varying locations, times, days, seasons, and other relevant factors related to the 'ecosystem' in which the study takes place. Again, if the findings endure across varying environmental conditions, then the researcher can be more confident in the evidence found. One example is given by a pharmaceutical education intervention study, in which Introductory Pharmacy Practice Experience (IPPE) students and Advanced Pharmacy Practice Experience (APPE) students experienced a layered learning model (LLM) for developing ambulatory clinical skills.[36] Both IPPE and APPE students, who participated in the LLM completed a mixed methods course evaluation addressing their experiences in three tradi- tional semesters. Each semester comprised a different environment where the schedule varied with some IPPE students attending alone or in pairs, which provided a more comprehensive understanding of the impact of the LLM. As a note, environmental triangulation should not be understood as an ecological triangulation, with the latter being conceptualized as an approach to qualitative meta-synthesis.[37]

Benefits of triangulation is the increase in findings' credibility as well as a providing a richer and more elaborate understanding of

the phenomenon under investigation. If the researcher is looking for as- sembling findings on to the same conclusion, triangulation contributes to improved credibility by overlapping data sources, checking, and confirmation.[24] However, when interpretive, critical, or postmodern paradigms are predominant, reality is viewed as multiple, fractured, and/or socially constructed. In this case, study credibility should emerge from crystallization rather than triangulation, i.e. the practice of using multiple data sources, researchers and lenses contributes to a
more complex understanding where multiple truths are being constructed.[38] In Patton's words, "*data inconsistencies should not be seen as weaken evidence, but an opportunity to uncover deeper meanings*".[39]
So, for example, if a number of researchers are identifying transcribed data
where analysts agree on a certain interpretation, this would be a good indicator of reliability. However, when one of the analysts provides a view that is different from what the majority of the team comprehended, the "minority" view should not be ignored or shunned. Instead, this point of view should be closely discussed, examined and further explored to make sure it is equally presented in the final report. All researchers would discuss not just the interpretations they reached but also how they reached them allowing for transparency. This is parti- cularly important since qualitative researchers often approach their research questions with relativist ontology where that reality is to be constructed through the meanings and understandings that are devel-
oped socially and experientially. It also contributes to the authenticityof the study as will be discussed below.
Perhaps the main disadvantage of triangulation is that it can be resource consuming, by adding layers of time, effort, and expense. Furthermore, while valuable, contradictory findings from different sources pose additional challenges leading to analytical tension and latent conflict between research team members when reporting results.[28]

Peer debriefing

Peer debriefing is a technique used to establish a study's credibility.It involves the researcher exposing oneself to a disinterested peer who would examine aspects that may remain implicit in the researcher's mind.[16] By answering questions from the peer debriefer, a researcher can reflect on biases affecting different aspects of the research process including the research question formulation, methodological design and interpretation. Ideally, the peer debriefer should have considerable knowledge of the studied topic and the method used to investigate it. Also, the peer debriefer should be of equal status; so for example, having a committee member serve as a doctoral student's peer debriefer would not be recommended. While the peer debriefer should be playing the role of the devil's advocate, Lincoln and Guba[16] encourage a peer debriefer to be empathic when providing feedback to a fellow re- searcher. Peer debriefing sessions themselves should be documented to be a reference for the researcher and to help with the audit trail as willbe explained below.
While a peer debriefing may appear similar in some aspects to investigator triangulation in having multiple parties contribute to the research process, it is important to note the differences. Unlike in peer debriefing, investor triangulation does not require the fellow investigator be of equal status. So, with investigator triangulation, a more seasoned researcher can contribute to a research project that is led by a novice researcher with all their interpretations equally considered. Moreover, a peer reviewer is likely to take a broader look at the various details of the research project beyond the analysis. A peer debriefershould be a disinterested party, which is not the case of the fellow in- vestigator employed in investigator triangulation who would likely be a partner in the entire research process.
Peer debriefing has been mentioned in a study assessing perspectives of children and parents regarding pediatric patients' knowledge

and medication use experiences for chronic conditions and community pharmacist–provided counselling.[40] It was also used in a study examining stakeholders' experiences and views on the logistics of settingup and maintaining pharmacy services in general practice in the UK.[41]
The second study specifically used peer debriefing for feedback on interviews and in the development of understandings of the examined processes.

Negative case analysis

Negative case analysis, also called the analysis of deviant cases, involves deliberate sampling, searching for, and discussing of elementsin the data that do not support or appear to contradict the findings emerging from data analysis.[16] It is a process for refining the analysis until it is possible to explain or account for all known cases, actually broadening and confirming the patterns emerging from data analysis.[16] By actively seeking cases that contradict or conflict with the pre- dominant theory, the researcher develops an understanding about why these outliers exist, amplifying their understanding of the data strengths and weaknesses, and reinforcing the study findings trustworthiness.[42] Negative cases often provide the key to recognize the norm and are critical to understand the whole phenomenon.[24]
There are few examples of actual studies reporting overtly negative case analysis in pharmacy research. One example concerns how phar- macists' ethical attention, reasoning, intention, and action may con- tribute to decision-making; the predominant ethical inaction and pas- sivity emerged from negative opposite cases of ethical attention and action.[43] Two related papers, one describing pharmacists' isolation and subordination to physicians as explanatory factors of ethical inactivity and a previous paper exploring the nature of pharmacists' ethical di- lemmas, also made use of deviant cases, with these exceptions actually lending further support to data analysis.[44,45] Rieck and Pettigrew[46] assessed physicians' and community pharmacists' (CPs) perceptions of
the CPs' role in Australian primary care and how these perceptions may influence the quality of physician/CPs chronic disease management
programs. Here, the negative cases were recruited purposively by the interviewer being made aware during previous interviews, enabling the emergence of a more complete description of physician/CPs collaboration.[46]
The advantages described previously are mostly based on the gap between the expected outcome and the explanation sparked off by the deviant cases.[47] These draw the researcher's focus on to why the out- come predicted by the previous results or a theory did not occur, whichis often more useful to study credibility than a repeated examination ofpositive cases. The detailed empirical knowledge of a single case may be a fruitful mechanism to discern credibility, knowing that dissim- ilarity improves the understanding of new principles when compared to no variation underlying generalization. A second advantage relates to the easier distinction between important and irrelevant events, pro- cesses, structures, and patterns; in practice, these are much more dif- ficult to confirm when there are negative cases, since all cases actuallycontributed to the outcome.[47] The main disadvantage from negative case analysis is similar to that described in the triangulation section, especially additional resources consumption, mostly associated with the need to perform an efficient search for deviant cases, which may be hard for the researcher to locate.
One way to apply a negative case analysis begins with sampling, i.e. to purposively select participants that are known to provide opposing viewpoints. Their opinions are carefully analyzed, and additional cases are sought and compared, aiming to reach saturation.[24] Saturation means that no new or additional data are found by which a researcher can develop attributes of a category.[48] It has attained widespread acceptance as a methodological principle in qualitative research and is widely viewed in health services research. Worth noting, however, is that saturation's position as a gold standard in assessing quality has been questioned. Another debate is whether saturation should be
viewed as an event or a process. Saunders et al.[48] argue that considering saturation as a process would encourage authors to provide evidence for saturation rather simply stating the number of participants with whom saturation has been reached. In that sense, an

analysis would not unexpectedly become 'rich' or 'insightful' after that one particular in- terview but, instead, would become richer or more insightful. To en- courage the use of saturation in such a way that meets the aims of the research, Saunders et al.[48] described four models of saturation that vary
in the extent to which an inductive or a deductive logic is followed and the relative prominence of data collection, data analysis, and theo- rizing. When used, saturation models should be operationalized in a way that is reflecting of the research question, theoretical position, and analytic framework.[48] Additionally, pharmacy researchers, especially those with interest in grounded theory, might benefit from being fa- miliar with *abduction*, an inferential process that utilizes surprising re- search evidence in producing new hypotheses and theories.[49] This ap- proach fits with grounded theory, since it involvies moving back and forth between data and theory iteratively; it also fits within the con- structivist paradigm where anomalies would be investigated with great detail rather than shunned.

Thick contextual description

With qualitative research, transferring the results of a study is ul- timately the responsibility of the reader.[16] The reader, however, should not be alone in this endeavor, and this is where thick description comes into importance. The qualitative researcher has the challenging task of providing thick descriptions of the setting, subjects, and other persons involved, quotes, and other data compelling interpretation and synth-esis to position the reader to (if deemed applicable) transfer the findings to their own context.
Transferability in qualitative research, perhaps especially in an ap- plied field like social pharmacy, can be hindered by a superficial in- vestigation of the issue of interest. Qualitative studies in pharmacy may limit reporting to categories like barriers and facilitators that are yielding "time constraints" as a barrier with little depth to the meaning of the concept with regard to how it affects actors and their motiva- tions, changes over time, or how the interplay of different persons and systems contribute to an overall picture. A more in- depth exploration of a "time barrier" may yield that there are conflicting priorities at the site and that someone in power is aligning incentives for the pharmacist to engage in other activities, thus contributing to detachment among employees. Thin description presents itself in studies that simply list codes with little or no integration, as if topics voiced in interviews were independent and exist in a vacuum. Thin description may be an in- dication that the researchers needed more time in the field, more sub- jects, and more observations. On the other hand, as noted above, per- sistent observation and prolonged engagement serve for a thick description that advances our knowledge of a certain topic.
A potential misconception about thick description is that it is all about providing copious details about the setting, that it was a rainy day or that there was a large stack of manila folders in the interview room. Ample detail should be included, especially in the methods and communicated to the reader as a means to establish credibility. However, through persistent observation, researchers should carefully consider what detail is needed to so they can be privy to people's mo- tivations, intentions, or the social system being analyzed. While details should be provided to give the reader a vivid experience, this can be difficult due to the temptation to tell rather than show or demonstrate to the reader.[38]

External audit/audit trail

An audit is a unique technique for trustworthiness, in the fact that like reflexivity, it can be used to establish more than one of the trust- worthiness sub-criteria. Lincoln and Guba[16] encourage the reader to compare an audit for a qualitative study to a fiscal audit, which involves an official financial inspection of an organization's accounts by an in- dependent auditor. By examining the *process* by which the research has been carried out, the auditor can corroborate the study's *dependability*. While by examining the *product,* emerging data and interpretations, *confirmability* can be established. Ideally, the auditor should be disin- terested, experienced in the methods and the topic studied, and rea- sonably close in status to the auditee that no one dominates the other. A key part in the external audit process is the establishment of an audit trail, which in its own can contribute to a researcher being cognizant of decisions being made. An audit trail is a residue of records originating from a research endeavor.[53] The Halpern audit trail categories are 1) raw data, including recordings, field notes, and other documents; 2) data reduction and analysis products, including summaries; 3) data reconstruction and synthesis product, including themes, results, con- clusions, and reports; 4) process notes, including notes related to methods used and trustworthiness; 5) materials addressing intentions and dispositions, including reflexive notes; 6) instrument development information, including pilot forms and observation charts.[53]
The audit process itself consists of 1) *preentry*, where the auditor and the auditee meet and see if the audit would be useful and accordingly decide if they want to continue, continue conditionally or discontinue the audit; 2) *determination of auditability,* where the auditor familiarizes him/herself with the study and determines if the audit trail is com- prehensible, complete, and useful; 3) *formal agreement*, which should include a timeline for the audit, audit's specific goals, roles of auditor, and auditee; logistics; and format of the audit report; 4) *determination of trustworthiness criteria*, where confirmability, dependability, and pos- sibly, credibility are assessed; and 5) *closure,* where the auditor meets the auditee and provides feedback before writing a structured final report.
While the process may appear to be onerous to some researchers, Lincoln and Guba[16] argue that even in sophisticated projects, it can be done in seven to ten days. Other than effort and logistics, external audit presents a unique challenge. If the auditor is contacted at the end of a
project and finds issues with its trustworthiness, it would be too late to make changes to salvage the study. On the other hand, if the auditor is brought earlier, the auditor may be "coopted" into a role different from the one intended by the researcher calling disinterestedness into question.
While the authors have not been able to identify an example of a pharmacy study using external audit and audit trail, a few examples from other areas of health services research may be useful to the reader. Using grounded theory, researchers explored perceptions of prevention practitioners regarding their new role in the Building on Existing Tools to Improve Chronic Disease Prevention and Screening

pre- sented for a very specific context. Such articles may not contain the requisite thick description about the phenomena, motivations, and so- cial meaning of those involved to leave the specific pharmacy setting. Ideally, a study would add to a deep understanding within pharmacy settings and even transcend to other health professions and other helping professions like education or social work. While not every qualitative study in pharmacy has the potential to transcend fields, researchers must push themselves to dig further and seek deep under- standings (thick interpretations) of persons and their systems. Qualitative researchers aim to leverage the time spent carefully collecting, interpreting, and reporting data to lead to meaningful findings that are useful to stakeholders and others, add depth to how issues and phenomena are understood, and make theoretical insights about people and systems that can be applied, and thus transferred, beyond the original context. Transferability, sometimes referred to as applicability,[16,50] is the aspirational product of rigorous qualitative studies that contain thick description and thick interpretation.
Thick description is often used in describing qualitative research methods but too often without apparent substance. Denzin[51] described thick description as going beyond facts and surface appearances. He suggests thick description should illuminate social interactions and their meanings. Situations should be presented in a way that the sig- nificance of the background and context to communicate the sig- nificance of what is going on. Thick descriptions lend themselves to thick interpretations.[52] This is important for two main reasons. First, thick description is seen as a form of building trustworthiness and va- lidity. The reader can see for themselves the depth of the data and analyses. Second, the depth of the descriptions allows the reader to determine how the study findings may transfer to other situations.
One way to consider the concept of thick description is in contrast to thin description.[52] An example of thin description may be a study

in Family Practice (BETTER) Trial. This included the development of the practi- tioners' role, perceived barriers, facilitators, benefits, and disadvantages as well as exploring the feasibility and sustainability of this approach for chronic disease prevention and screening (CDPS).[54] In that study, researchers documented their activities through an audit trail, journals, field notes, and memos. The audit trail consisted of a "step-by-step chronological accounting of the project activities including interviews, discussions and decisions."[54] Another example comes from a study that aimed to provide an in-depth understating of "the conditions of engagement" necessary for a therapeutic relationship between physiotherapists and their patients.[55] In addition to main- taining an audit trial, researchers reported employing an external audit on completion of the project, which confirmed that the described re- search process was complete and the quality and nature of the results were in harmony with the described process.[55] Researchers properly acknowledged the contribution of the external auditor in their paper.

Reflexivity

Reflexivity is unique in the fact that it can be used for establishing all trustworthiness sub-criteria. It provides researchers with means to deal with the inherent influence that the researcher brings to this typeof investigation. It is both the positioning of the researcher[56] and a systematic approach for the researcher to be attentive to their role in the construction of knowledge during each step of the research process.[57] Qualitative research has struggled with how to deal with a potension for the researcher is the openness to criticism that may ensue with the vulnerability that reflexive writing and process may provide.[57] In the end, we collaborate with our research participants. As in anyrelationships, feelings may emerge that are important to account for, as they may provide insight into our understanding of the topic. On the flip side, we may have blind spots that compromise our ability to detect aspects influencing our understanding. Additionally, the disciplinary or theoretical perspectives chosen for the research may influence how the data are analyzed and presented, and, thus, influence outcomes.[56] Re- flexivity executed, and then communicated via the text, has the po- tential to provide a credible and trustworthy account of the research process and knowledge produced.

The practice of reflexivity is encouraged through the use of effective record keeping. Throughout the research process, all assumptions, decisions, interpretations, and reflections should be documented.[56] Several techniques may facilitate this process, including careful notetaking regarding choices available, decisions made and why, changing directions, and personal reactions. For example, one can look at the research question and how it is framed for insight, motivations for interest in a particular topic, the basis for selection of interviewees and choice of interview questions, and expectations about what the research may yield.[60] Carefully used, posing "why" questions such as "Why this?" and "Why not that?" may allow the researcher to delve more deeply into the underlying motivations for choosing a topic or engaging a particular aspect of research in order to better understand the effects this may have on the research process.[60] During the data collection phase, personal thoughts and feelings that may be prompted during this process should be collected to encompass the differing reactions that likely arise for the researcher with different participants. This will inform the analysis alongside the research collected data.[60] Throughout, one should ask difficult questions of oneself[60] and maintain these reflections as well as thoughts and experiences in a research diary or journal be- fore, during, and after data collection and analysis.[61] These insights may provide the basis for competing conclusions from the findings.[51] Alternatively, they may occur only once then can be justifiably ignored later if their relevance is lacking. These insights should, however, becaptured unedited and followed through.

A number of studies described the use of reflexivity in the social pharmacy literature. Witry and Doucette[62] acknowledge their position as pharmacists with an interest to advance the pharmacy profession. They employed contact summary forms, check coding, and regular meetings as strategies to help foster reflexivity, which helped assist in reflection and documentation of the process as well as assisted in being cognizant of and in managing inherent biases.[62] Garcia et al.[63] discuss the possible influence that the undergraduate master pharmacy student may have had on their study findings. The student's background as an inexperienced qualitative researcher who had positive perceptions about the program may lead to different findings than an experienced qualitative researcher, someone with negative perceptions about the program, or someone with a different professional background.[63] In another instance, Hanna et al.[64] discuss how the use of debrief meetings was used to accomplish reflexivity as a team. While it was encouraging to find a number of social pharmacy studies mentioning reflexivity as trustworthiness technique, discussions were often too brief and did not elaborate on the impact that reflexivity had on findings. To be mean- ingful for the reader, we suggest reflexivity be discussed in detail to shed light not just on how it was employed, but also on its possible influence and to help situate findings.

sitivist approach to knowledge construction. Accordingly, reflexivity is expected and logically argued to have become a standard for evincing rigor in qualitative investigations.[57] The situated nature of qualitative knowledge construction, the uncertainties and incompleteness, and the context and conditions under which knowledge is produced become more available for the reader to interpret for themselves when reflex- ivity is properly attended to in research.[56]

It may be argued that all research is reflexive, as awareness of one's own reality is the only avenue by which we may come to know, interpret, or understand any reality. Reflexivity accounts for these researcher preconceptions during each stage of the research process, including choice of research domain, question, methodology, data collection, data analysis, and in the writing and presentation of findings.[56] Bias would not eliminated, but it can be accounted for in this process. The issue of subjectivity emerges as an issue if the effect of the researcher is not taken into consideration.[57] This is particularly important since researchers do not enter the field naively.[58] They bring a background of experience, both personal and professional, to the investigation including beliefs about reality, what needs to be investigated, qualifications to do so, and theoretical and educational interests.[57] Any preconceptions of how things are perceived must be accounted for prior to and during the research process so that any influence can be transparently noted. These personal sources of interest can be strengths, as long as they are accounted for in the research. With proper attention to reflection and awareness, the researcher is co-con- structing knowledge, deeper insights and, thus, potentially richer un-derstanding of a given human experience.

As researchers, we must account for these reflections in our writing to frame limitations and strengths and transferability of findings.[59] The

Transparency

While not described separately as part of trustworthiness criteria, itis important for qualitative pharmacy researchers to reflect on transparency, the principle that researchers should make key components of their work visible to peers, as a key indicator of quality. In arguing for transparency as a precondition for quality, Moravcsik[65] cautions that without it, many key aspects of the research including perceptions, beliefs, interests, processes, and even choices would be assumed or implied than actually depicted in an empirical manner. He argues that transparency has three dimensions: data, analytic, and production transparency. *Data transparency* gives readers access to the evidence and data that a researcher has used when making research claims. Transparency with data allows readers to consider the richness and nuance of what sources say, assess how they relate to claims made, and evaluate whether data have been interpreted and analyzed in a sound manner. Second is *analytic transparency*, which involves the provision of information about data analysis-how the researcher addressed inter- pretation and inferred that the evidence supports a specific claim. Going through this process would allow the researcher to consider multiple interpretations and one's own motives. This would serve quality by having the research team weigh alternative

sources and in- terpretations and assess ambiguities, strains, contradictions, as well as synergies that exist among them. Finally, *production transparency* pro- vides readers with access to information about ways by which specific cited evidence, arguments, and methods were selected from among the full body of possible choices. Thus, production transparency requiresthat researchers would explain how choices of evidence, theory, and method were made. This would be particularly significant with someprojects where researchers report the use a certain method, such as focus groups for example, without providing any elaboration on such choice.

Authenticity

Lincoln and Guba[66] argue that trustworthiness criteria make an incomplete set because they deal only with issues that loom important from a positivist paradigm. The positivist standard criteria are primarily *methodological,* overlooking such issues as power, pluralism, multiple values, representation, empowerment, and accountability. To address these issues Guba and Lincoln[66] proposed the term authenticity. This addition takes the influence of context into consideration by addressing additional intrinsic naturalistic criteria. Certain initial conditions are prerequisite to achieve authenticity sub-criteria, including fairness, ontological authenticity, educative authenticity, catalytic authenticity and tactical authenticity. First, respondents ideally are to be drawn from all at-risk groups, fully informed consent is to be in place, caringand trusting relationships are to be nurtured, inquiry procedures are to be rendered transparent to all guaranteeing balance (since nothing can), but at least it would improve the likelihood that balance is well approximated.[66] Specific procedures/ techniques should be followed to fulfill a balanced view that presentsall possible constructions and the values that uphold them. All the previous constructions of participants and researchers are to be ob- tained, compared, and contrasted, with each enjoying similar privilege; respondents and inquirers are to negotiate the kind of data to be col- lected, methods that will be employed, interpretations to be made, modes of reporting, recommendations, and actions to be proposed. Additionally, introspective statements about changes experienced by participants and researchers are to be collected, and the extent of em- powerment felt by participants, such as patients, is to be assessed.[67]
Procedures would include a “negotiation”; that is, ideally open, carried
out from equal positions of power, and under circumstances that allowall sides to possess equally complete information and carried out by“bargainers” of equal skill.[66] The negotiation should focus on all re- levant matters with rules that are agreed upon. Techniques that serve fairness included member checks, thick description along with peer debriefers and auditors. Other steps include continuous fully informed consent with respect to an evaluation's procedures and constant member-checking as described earlier. Finally, *fairness* would require defining mechanisms that should be agreed upon if one party sensesthat agreed upon rules have not being observed by others.[66]
An attempt to incorporate *fairness* as a criterion in a pharmacy practice study might be the above described study by Herborg, Haugbølle and Lee[18] on dose-dispensing. The fairness criterion was addressed in the study in several ways. First, all interviews were guided by the same detailed interview guide -though adjusted depending on developing meanings and on whether the interview was performed at the user level, a practitioner level, or at decision maker level. This gave all participants the chance to make their opinion heard on different aspects of the issue. Second, the same social constructivist theoretical approach was used to analyze all interviews. Most importantly, the three discourse types identified in the study were not labelled as
“truths”, but as social constructions true for the specific relevant group
embedded in the discourses. Last but not least, the authors explicitly mention that they are also “voices” in the discourse system having se- lected, described, and discussed the most meaningful interpretations possible to establish encompassing the empirical material.[18]

Ontological authenticity

Ontological authenticity is defined as “the extent to which in- dividual respondents' (and the inquirer's) early *constructions are im- proved*, matured, expanded, and elaborated, so that all parties possess *more information*, become more sophisticated in its use, and get their consciousness raised”.[67] In some cases this will entail “realization of
contextual shaping”, while in others it will mean better appreciation of
complexities previously not accounted for adequately or at all.[66] Helpful procedures for fulfilling the ontological authenticity criterion include establishing clarity of participants' and researchers' a priori positions; comparison of participants' earlier and later constructions; solicitation of participants' and the researcher's introspective statements about their growth, as well as the testimony of selected participants regarding their changing constructions; and the establishment of an audit trail demonstrating changes made.[67] Examples of ontological authenticity applied to social pharmacy research can be found in phe- nomenologically and/or hermeneutically based studies. An interesting example addressed ontological understandings of consultant nurses and midwives in how they approach patient care delivery in difficult si- tuations.[68] The authors describe how participants came to an under- standing that they must develop new pathways to being clinically ef- fective. When they sensed that the effectiveness of patient care was jeopardized, they then chose to break through barriers and introduce changes for themselves and for the organization.

Educative authenticity

Educative authenticity is defined as “the extent to which individual respondents (and the inquirer) possess *enhanced understanding* of, ap- preciation for, and tolerance of the constructions of others outside their own stakeholding group”.[67] Helpful procedures include: having a peer debriefer and an external audit, comparison of participant's' and re- searcher's assessments of the constructions held, introspective state- ments about their understandings of others' constructions, participant testimony, and maintenance of an audit trail.[67] An example of a social pharmacy study in which educative authenticity was an explicit quality criterion is a pharmacy-based, action-oriented study on cognitive ser- vices conducted by pharmacy interns.[69] The basic tenet of the project was the *learning* for all parties that were involved. The study steering group considered pharmacy preceptors' practical knowledge and re- searchers' theoretical knowledge as equal prerequisites for being able to
carry out the study. When learning was investigated among different

participants and audiences, and partici- pant-inquirer collaboration has to be built into every step, with full agreement on the rules to govern the inquiry and with information fully shared. Besides, the inquiry report is to be available to all respondents and audiences. Finally, a clear mechanism is to be established in cases of conflict or disagreement.[66] What follows is a brief discussion of eachof the sub- criteria, together with recommended procedures/techniques to establish them as they are described by Guba,[67] followed by concrete examples of where the specific sub-criterion has been applied or might fruitfully be applied.

Fairness

Fairness is defined as “the extent to which all competing construc- tions of reality, as well as their underlying value structures, have been accessed, exposed, deconstructed, and taken into account in shaping the inquiry product, that is, the emergent reconstruction”. Of all criteria of authenticity, *fairness* is considered to be most important.[67] It is parti- cularly relevant in a qualitative study since, in a “value bound” inquiry, a researcher has to make every attempt to avoid a situation where some values are suppressed with their holders exploited and that others will be enhanced with their holders advantaged. Noting “value-pluralism”, a researcher attempts to clarify and honor constructions in a balanced, impartial way as attested by multiple parties.[66] One should ask about the way analysts proceeded with their tasks. This would not be

parties, it was found that not all of them had learned equally. Whereas researchers and pharmacy preceptors in the steering group entered into a lengthy learning process, the individual pharmacies tended to see the study as just one of many options offered to them, which discouraged them from wanting to take on further obligations. Similarly, the phar- macy interns found participating in the study during the duration oftheir internship to be appealing as long as it did not demand too muchof their time.

Catalytic authenticity

Catalytic authenticity is defined as "the extent to which *action* (clarifying the focus at issue, moving to eliminate or ameliorate the problem, and/or sharpening values) is stimulated and facilitated by the research being carried out".[67] The argument for catalytic authenticity is that knowledge in and of itself is insufficient to deal with the different issues that participants raise during the research process. Thus, for elucidation, deliberate action must also be described. Useful procedures include: developing a joint construction that aims at consensus when possible or an explication of conflicting values, including the assign- ment of responsibility and authority for upcoming action(s); plans for participant-researcher collaboration; accessibility of the produced re- port; and evidence of practical applications. A well-planned follow-up over time to assess the sustainability of outcomes and gathering testi- monies of a sample of participants would also be helpful techniques. The pharmacy practice member checking; and early agreements about power. As the reader would imagine, participants' and stakeholders' testimonials indicating they were empowered during the study and afterwards during follow- up activities would serve as the best indicator of tactical authenticity. Tactical authenticity bears resemblance with the design action re- search, described above in a study by Sørensen and Haugbølle.[65,66,71]

An action-oriented (or co-construction based) study which does not *empower* and/or raise collective awareness among specific, selected group (patients, vulnerable groups, selected group of health care practitioners, etc.) does not fulfill the tactical authenticity criteria.

Guba and Lincoln[67] argue that perhaps the most significant ac- complishment to date related to the authenticity criteria is simply its existence, a demonstration of the fact that it is possible to think outside in more creative and suitable ways about quality assessments.

Final thoughts

While this commentary provides guidance on promoting rigor in qualitative research, it is not meant to be a comprehensive manual on conducting qualitative research methods. The reader is encouraged to review some of the classic texts cited in this manuscript for guidance on how to design and conduct qualitative research.

Second, this commentary does not expound upon the importance of sound conceptual and/or theoretical underpinnings as critical to the success of qualitative research endeavors. That has been explained elsewhere, including within this journal[73]; so to that end, it will not be elaborated upon further in this commentary beyond stating that the authors herein and the journal, itself, firmly believe in the importance of a theoretical basis or in some cases the induction of theory from qualitative inquiries. In considering trustworthiness and the techniques used to discern it, the need for a solid and sound basis for the quali- tative study becomes apparent, as the various techniques discussed here, including triangulation, negative case analyses, reflexivity, and thick description will be more successful under the auspices of a solid theoretical foundation. It is difficult to imagine thick description and its resultant transferability in the absence of such a foundation, or, like- wise, how the results of a negative case analysis will not become muddled, even impossible to make sense of, without a sound basis for inquiry. Likewise, a strong foundation also assists with the establish- ment of authenticity, an emerging facet to establishing the rigor in a qualitative study.

Third, the reader should also be made aware that other ways of establishing rigor have been suggested by others. Some qualitative re- searchers share the view that no set of criteria should be used in evaluating qualitative research. Hammersley,[72] for example, argues that qualitative research should focus on political action rather than production of knowledge. Notwithstanding, he suggests that "certain" criteria, in the form of "guidelines," can contribute to improved rigor of qualitative research. Others propose an approach to qualitative re- search evaluation that further emphasizes pluralism through reflexive dialogue and agenda based on engagement, processing, interpretation, and (self-)critique while dealing in a reflexive manner with preconditions and consequences of research, critique, usefulness, relevance, and ethics.[74]

The reader is encouraged to examine those other guidelines and make a choice of the approach that would establish rigor in qualitative study he or she is planning. However, one way or other, the author is expected to demonstrate evidence of rigor in a manuscript describing qualitative research.

Finally, the authors would like to alert the reader that it is not ex- pected for one study to apply all the trustworthiness or authenticity criteria concomitantly. That is practically not feasible. According to Creswell,[75] researchers should utilize more than one in any qualitative study. As Morse[24] recommends, we encourage authors to study all those sub-criteria carefully and make decisions on why and how they have been used. While it was encouraging to see social pharmacy articles mentioning the use of the described quality criteria, quite often the description of how they were utilized and how they impacted the re- search inquiry was lacking. These details should be included in quali- tative manuscripts along with philosophical/paradigmatic standpoints of authors. The underlying point is that documenting the evidence of trustworthiness and authenticity provides the reader with greater confidence in the study results. In no one type of study can 100% confidence be inspired, regardless of methodological approach used ordiscipline/area in which the study was conducted. More [evidence] is better, but given the nature of science, all [complete evidence] is not possible.

Conclusion

This commentary provided an explanation of each of the criteria of trustworthiness proposed by Lincoln and Guba (*credibility, transfer- ability, dependability and confirmability*) and different techniques used in establishing them. It also provided an overview of authenticity, a more recent and less widely used set of criteria that has been specifically designed for qualitative research. Those included *fairness, ontological authenticity, educative authenticity, catalytic authenticity, and tactical au- thenticity*. For each of these terms, the commentary provided examples from the literature where the criteria have been

example of catalytic authenticity comes from the action study mentioned above, this time described in Haugbølle et al.[70] The paper specifically maps actions taken and decisions made in study pharmacies as to changing counselling practices towards angina pectoris patients. In 29 (85% of the participating) pharmacies, decisions were made following the study to develop and implement different activities for angina pectoris patients. Two main types of activities were suggested by the 29 pharmacies, namely different staff-oriented and patient-oriented activities. Staff-oriented activities were organizing and holding meetings for pharmacy staff, developing or adapting brief in- structions to staff on angina pectoris, and developing or changing po- licies and instructions on counter procedures were initiated in 23 pharmacies. Patient-oriented activities such as handing out pamphlets, improving over-the-counter counselling and holding open meetings for pharmacy customers were initiated in nine pharmacies.

Tactical authenticity

Tactical authenticity is defined as "the degree to which all partici- pants are *empowered* to take the action(s) that the inquiry implies or proposes."[67] Useful procedures/techniques applicable when striving towards tactical authenticity include confidentiality, negotiations ad- dressing the kind of data that would be collected, how these data will be interpreted and reported; using elaborate and clear consent forms;

used.

Research in Social and Administrative Pharmacy (RSAP) was founded as a vehicle to advance the discipline by publishing theory-driven and methods-intensive research. It is hoped that this commentary contributes to this message by providing guidance that assists authors, reviewers, editors, and even readers when considering the quality of qualitative research manuscripts addressing social pharmacy issues.

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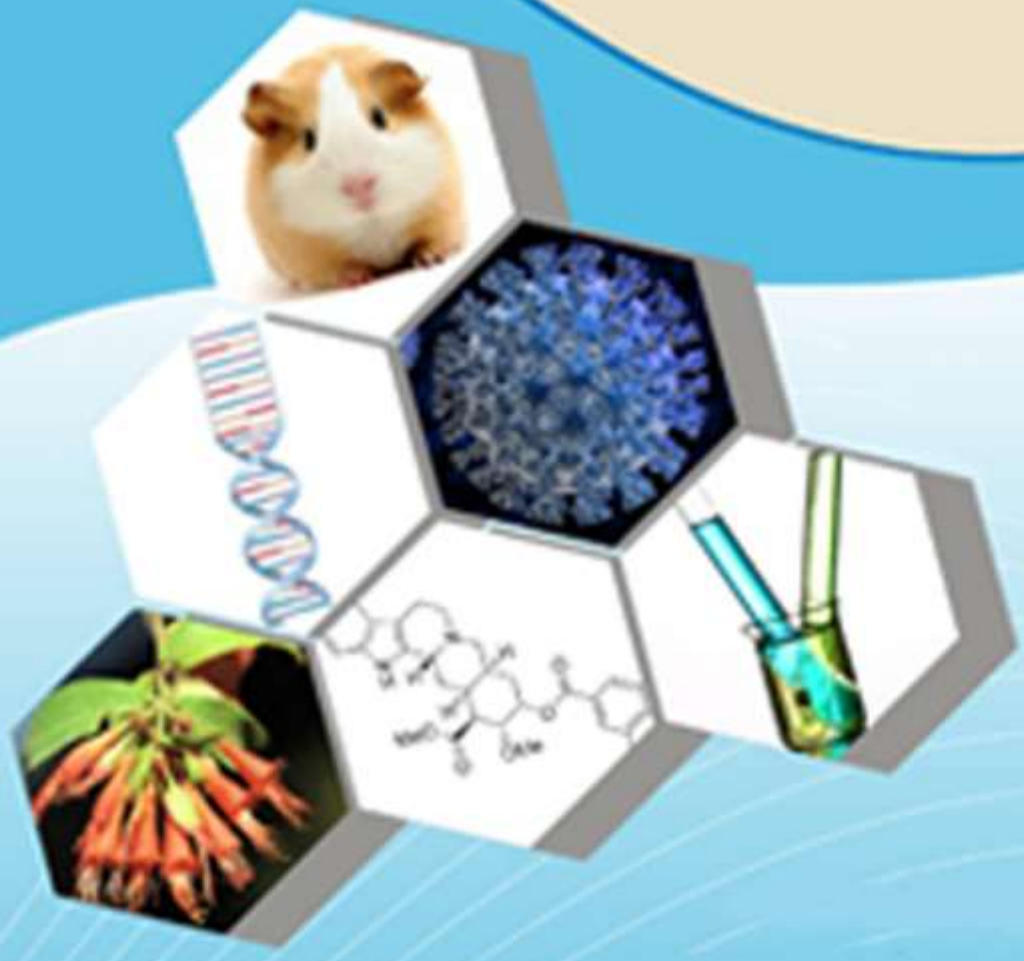

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Perspectives from various regions on pharmacogenomics EHR development and implementation

S. Kusuma Kumari, C. renuka Thejeswini, R. Jona Methusala

Abstract

There are few documented instructions to implementation, yet the design of electronic health records is vital to the effective introduction of novel genomic services.

In this study, we followed the four-year development process of a locally produced electronic health record that serves a major pharmacogenomics program at a tertiary-level academic medical facility.

Electronic health record (EHR) procedures for ordering a pharmacogenomics panel in anticipation of clinical need (preemptive genotyping) or in response to a particular therapeutic indication were developed and implemented by program personnel. As a result, panel-based genotyping results were kept separate from the EHR until clinically actionable evidence of drug-gene interactions was found. A

The drug-response phenotype prediction service supplied a summary of drug-gene interactions, prompted inpatient and outpatient clinical decision support, updated laboratory records, and produced gene results inside online personal health records.

Conclusion: Generalizability of a locally constructed electronic health record that incorporates pharmacogenomics into its design. Scalability of the model to larger collections of genomic data is examined, as is the difficulty of putting genomic data in a way that is both understandable and therapeutically useful.

Key words; electronic health records; implementation; pharmacogenomics

Introduction

As the price of genotyping reduces dramatically1 and new studies demonstrate the utility of testing, the use of diagnostic gene tests in clinical care has expanded significantly in the United States. 2 Since many commonly given medications now have increasingly well-validated connections to adverse events or lower efficacy when gene variations are present, pharmacogenomics is primed to see comparable expansion. 3–5 In addition, the cost of panel tests comprising hundreds of genes has decreased thanks to developments in genotyping technology, opening the door to the possibility of testing individuals once and utilizing their genetic data frequently for the rest of their lives. There is a high probability that a patient may be exposed to a medicine with published pharmacogenomic connections, given the labels of 119 US Food and Drug Administration-approved pharmaceuticals presently include germ- line or tumor pharmacogenomic information. Sixty-five percent of ambulatory-care patients tracked longitudinally at our institution were exposed to at least one drug with a documented pharmacogenomic connection during a 5-year time period, demonstrating the great potential for using variations from a pharmacogenomic panel test. 6 Communicating the importance of genetic data to practicing physicians and managing genomic data across a fragmented care-delivery system are crucial to realizing the promise of translating pharmacogenomics to clinical practice. 7 Electronic health records (EHRs) and clinical decision support (CDS) are only two examples of the health information technology (HIT) that has become essential in modern medicine. However, there is little documentation of successful clinical pharmacogenomics implementations of these technologies. 8,9 Fortunately, the voids are being filled by a number of NIH-funded consortiums. Knowledge management and clinical decision support (CDS) best practices have been created and published by the Clinical Pharmacogenetics Implementation Consortium.

Pharmacy Practice

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(Approved by AICTE,P.C.l New Delhi& Permanently Affiliated to JNTUA Anantapuramu
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The recommendations of the Clinical Pharmacogenetics Implementation Consortium are well annotated, backed by a hierarchy of evidence, and accessible for no cost. 10–14 To further facilitate translation, two multi-institute consortia, the Electronic Medical Records and Genomics Network (also known as the eMERGE Network) and the Translational Pharmacogenomics Project of the Pharmacogenomics Research Network, are actively piloting efforts to integrate genomic information with EHRs. SPECIAL ARTICLE of pharmacogenomics to the clinical setting and to capitalize on the wealth of clinical data contained in the EHR for research.

Vanderbilt University Medical Center (VUMC) has estab- lished a large pharmacogenomic program known as PREDICT (Pharmacogenomic Resource for Enhanced Decisions in Care and Treatment).[15] PREDICT is based on the principles that pharmacogenomic testing should be preemptive and harness HIT to facilitate ordering, storage, and timely dissemination of genetic results at the point of care. The design and implementa- tion model presented here arose out of a 4-year development process to adapt a largely locally developed EHR[16] to enable the maintenance, interpretation, and distribution of panel-based pharmacogenomic data to a broad base of providers and patients(**Figure 1**). For this article, the term EHR is inclusive of all clini- cal information systems that manage or manipulate genomic information while serving clinician information needs. In addi- tion, we include a brief description of the connection between the EHR and the personal health record (PHR). We believe ourexperiences can inform adaptations of both locally developedand commercial EHRs for pharmacogenomics.

EHR DESIGN PRINCIPLES FOR A PHARMACOGENOMICS IMPLEMENTATION

PREDICT was established as a quality-improvement program in 2010 to apply clinically significant gene variants designated by the US Food and Drug Administration as pertinent to deci- sions involving drug selection and dosing.[15] EHR features were developed with the expectation that panel-based pharmacoge- nomic testing will become pervasive, and genomic consider- ations will routinely influence prescribing. Accordingly, the design of supportive EHR functions has followed 10 objectives (**Table 1**), which seek to give universal, comprehensible, and timely access to clinically significant genetic variants. Displays of pharmacogenomics results were created to be highly visible,

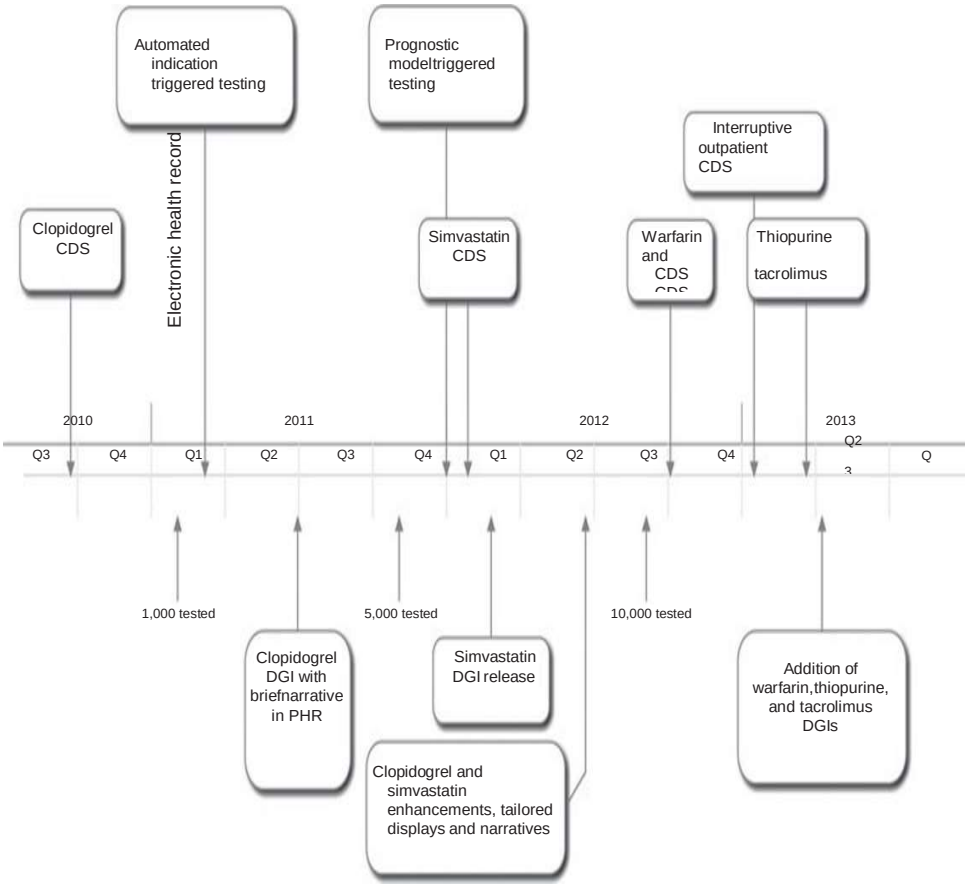

Figure 1 PREDICT EHR development timeline. PREDICT has undergone a 4-year process of design, implementation, and iterative refinement. Several milestones, including new drug–genome interaction implementation as well as high-impact EHR design features, are highlighted. CDS, clinical decision support; DGI, drug–genome interaction; EHR, electronic health record; PHR, personal health record; PREDICT, Pharmacogenomic Resource for Enhanced Decisions in Care and Treatment.

Table 1 Design objectives for a pharmacogenomics-enabled electronic health record

1. Display universally accessible and highly visible gene variant and phenotype information within EHR
2. Flag patients likely to benefit from knowledge of genomic variants in advance of clinical need (preemptive genotyping)
3. Facilitate genotyping among patients with an immediate clinical need (indication-based genotyping)
4. Sequester all variants with selective promotion of actionable variants to EHR upon institutional pharmacy and therapeutics approval
5. Create and maintain a centralized service to translate genotype to phenotype
6. Create a centralized knowledge base of therapeutic alternatives and dosing algorithms for clinical decision support
7. Rapidly distribute genetic results to laboratory, patient portal, inpatient and outpatient prescribing environments, and the associated clinical decision support subsystems
8. Implement surveillance and quality assurance interventions for post–prescription drug–genome “conflicts”
9. Create notification to patients of their genomic results with patient-friendly interpretations
10. Ensure systems are scalable to genomic variant data sets that are much larger than those currently in clinical use

The above objectives were prospectively addressed in the design and implementation of pharmacogenomics CDS within VUMC’s EHR.

CDS, clinical decision support; EHR, electronic health record; VUMC, Vanderbilt University Medical Center.

in an effort to prevent priority results from being “buried” among other laboratory data. Preemptive identification of patients who were expected (based on statistical prediction models) to benefit from panel-based gene variant data to tailor future therapies was incorporated into outpatient workflow. All gene variant data were stored long term, but selective, clinically actionable drug–gene combinations that met the burden of evidence for a significant drug–genome interaction (DGI) and attained institutional approval for release, and for which we had developed CDS logic to guide the physician, were promoted to the EHR. The design for disseminating results features a sin- gle source for both genetic variant data and genotype to drug phenotype interpretation, reinforcing the consistency and reli- ability of genotype reporting. Knowledge and data sources were constructed using service-based software architecture such that both genetic variant data and the DGI knowledge base could be easily updated and the updates would propagate to all linked systems. Finally, the EHR mechanisms for reporting the results and delivering CDS were initially designed to serve a small set of targeted DGIs but easily scale to support a large quantity of pharmacogenomic variants.

A LOCALLY DEVELOPED EHR PERSPECTIVE

The biomedical informatics and genomics fields foresaw the need to store, manage, analyze, display, and communicate genetic data early in the process of developing the translation of genomic medicine to clinical practice. 17 Locally designed clinical information systems are ideal for pharmacogenomics adoption since hospitals have more say over the structure and compatibility of their HIT. Historically, "homegrown" EHRs have been acknowledged for serving as a proving ground for novel HIT concepts, allowing for the evaluation of clinical efficacy, and offering proof-of-concept implementations for the broader informatics community. 18–21 Furthermore, locally designed EHRs benefit from a dedicated user base that developers can interact with face-to-face to get input and make iterative adjustments that enhance the software's usability and functionality. While many big academic medical facilities and integrated health systems have invested decades in technological infrastructure and programming, few have maintained this competence as clinical information demands have grown. The existing monolithic architecture of EHRs, in which huge HIT ecosystems from a single vendor or institution are interoperable internally but lack the capacity to connect outside, is another possible drawback. For instance, due to the difficulties in standardizing EHR systems, three of the eMERGE pharmacogenomics implementation sites are pursuing either partial or complete development separately. When it comes to information sharing and dissemination, eMERGE and the Translational Pharmacogenomics Project locations

standards for design and information management that may be used as a guide by other businesses. From prescription to clinical application, pharmacogenomics relies on electronic health records (EHRs), and this article will detail how these records and their associated features facilitate this process (Figure 2).

IMPLEMENTATION OF PREEMPTIVE AND INDICATION-BASED PHARMACOGENOMIC TESTORDERING

For most of the prescribing scenarios currently covered by PREDICT, including the drugs warfarin, simvastatin, clopidogrel, tacrolimus, and thiopurines, the genomic information contributes diminishing returns to clinical outcomes after the patient has achieved a stable dose or drug selection through experience or sequential drug trials. 3–5,22–25 The clinical impact of genetic data, such as VKORC1 and CYP2C9 variant status, is thought to wane considerably after a stable international normalized ratio is achieved, an event which generally occurs within the first 2 weeks of therapy. This is because warfarin dosing is sto- chastically adjusted in response to serial international normalized ratio measurements. Like CYP2C19 variant individuals, those taking clopidogrel had a greater chance of developing in-stent throm- bosis during the first 30 days after stent implantation. Thus, in order to optimize the

influence of the genotype data on clinical treatment, the program has emphasized testing before to or simultaneously with medication commencement.

Due to this, two pharmacogenomics ordering methodologies, preventative and indication-triggered testing, were developed. All patients with upcoming general care or cardiology appointments now get an alert in the EHR if a statistical risk score hits or exceeds the thresh- old, allowing for preventative genotyping. With a risk score of 40%, the molecular diagnostics lab would be fully used, predicting a patient's likelihood of obtaining simvastatin, warfarin, or clopidogrel over a 3-year time horizon. When a patient's record is marked, the outpatient order entry system generates a draft order for the PREDICT test, which the treating clinician must then approve. Indication-specific testing has been implemented by adding the PREDICT panel test to order sets or preprocedure planning prior to cardiac catheterization (to capture catheterization patients who receive intracoronary stents and antiplatelet therapy such as clopidogrel) and certain orthopedic procedures (e.g., joint replacements) for which warfarin-based anticoagulation is standard. Preemptive genotyping is advantageous because it removes the need to wait for the genotype, which typically takes between 2 and 5 days to get.

In light of the declining cost of genotyping, the potential exposure to multiple medications with pharmacogenomic indications, and the very high cost of severe adverse events, we propose significant cost savings using a preemptive panel- based genotyping strategy as compared to serial single-gene tests.

6,26,27 Over the course of a patient's lifetime, multiplexed gene testing will most likely be used.

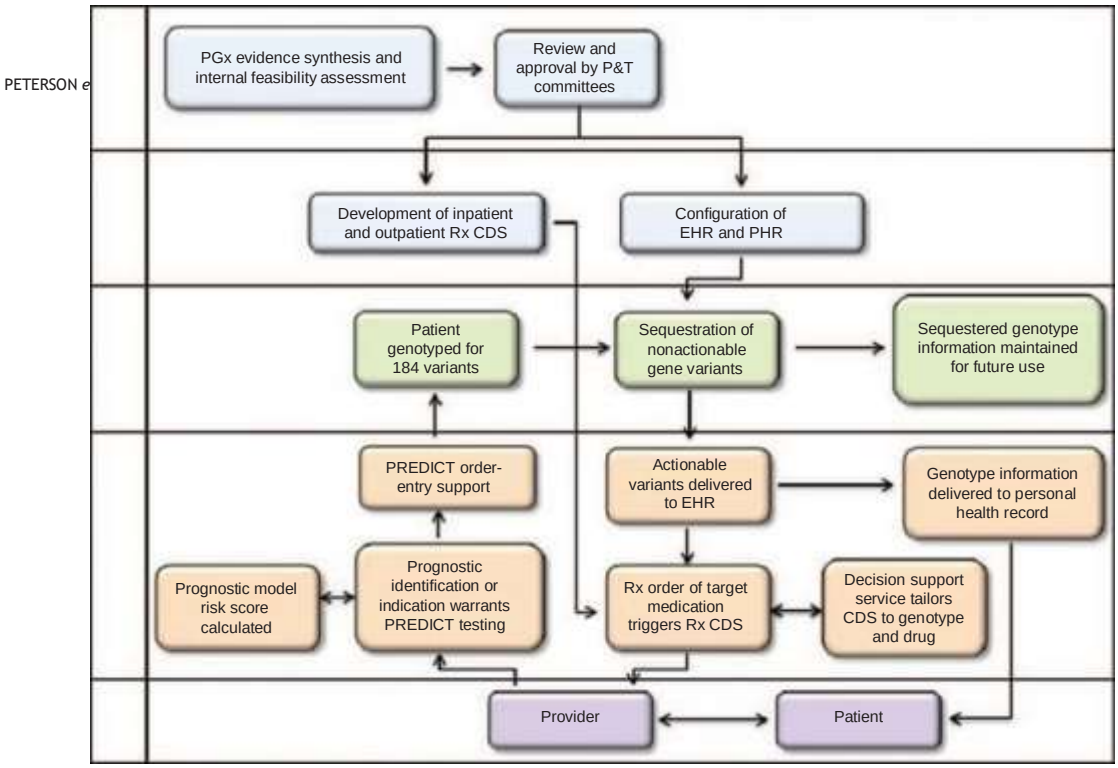

Figure 2 EHR development and operational processes. Pharmacogenomics implementation requires preimplementation research and assessment, technical development of informatics infrastructure, and integration with laboratory and clinical operations. Accessibility to users, both patients and providers, is integral. CDS, clinical decision support; EHR, electronic health record; P&T, pharmacy and therapeutics; PGx, pharmacogenomics; PHR, personal health record; PREDICT, Pharmacogenomic Resource for Enhanced Decisions in Care and Treatment; Rx, prescription.

to be less expensive relative to the potential benefit, particu- larly in patients with a common set of cardiovascular risk fac- tors likely to need associated therapies. However, no health economic studies have determined the value of panel-based genetic tests outside of oncology, and there is a paucity of evi- dence relating panel-based genetic tests to health-care spend- ing. VUMC has supported the PREDICT program costs with institutional funds, including assay costs, reagents, labor, instrumentation for processing, empiric research among patients and providers, development of patient informational materials, decision-support tools that provide point-of-care interventions and drug/dosing guidance based on test results, and education and training given the associated dearth of knowledge and familiarity among prescribers.[15] A key goal of this investment is to catalyze further pharmacoeconomic analyses of this approach.

EHR STORAGE MODEL FOR SEQUESTRATION AND REPOSITORY

National data standards for genetics are in early stages; a model to exchange genetic testing results is proposed by HealthLevel 7,[28] with contributions by Pharmacogenomics ResearchNetwork–affiliated academic groups[29,30] and EHR vendors.[31,32] In the absence of established standards in

2010, and to meet the immediate needs of the program, PREDICT developers created a coded storage model to meet local requirements for

CDS and distribution to multiple clinical information systems. Future adaptation to emerging standards such as Health Level7 is planned to support communication with external sys- tems. Genetic variant data produced by the Illumina VeraCode Absorption, Distribution, Metabolism, and Excretion Core Panel for PREDICT are provided either as a Portable Document Format or as plain text. As the former does not provide com- putable results, automatic parsing of the text format is required to extract the gene name, variant result in star nomenclature, and a call rate, which indicates the ability of the panel to yield a result at a specific variant. In the event of a call rate <98.7%, the test result is manually reviewed and generally retested by Molecular Diagnostics Laboratory staff; otherwise, it is released to an Oracle database, which initially sequesters all results from the main EHR storage.

The Oracle database is exposed to downstream systems through a filtered view limited to actionable approved variants. An automated script queries the filtered database view hourly to extract new or updated entries and, if discovered, creates a new or updated entry in the genotype section of the Patient Summary Service, a central Web service that is available to all components of the EHR and CDS (see **Supplementary Figure S1** online). Examples of four components of the EHR that use Patient Summary Service are shown in **Figure 3**. Patient Summary Service serves as a single source of patient-specific knowledge for medications, diagnoses, allergies, and othe

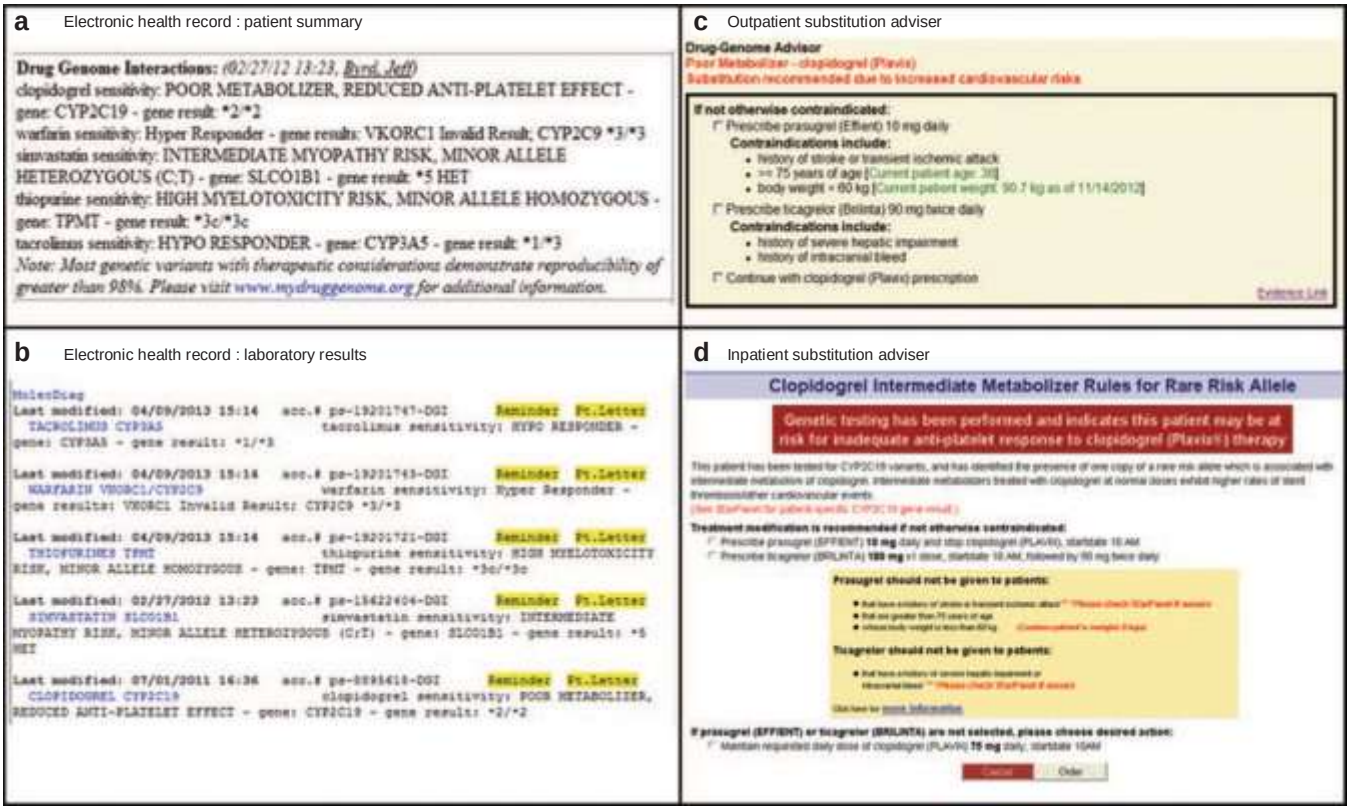

Figure 3 Task-specific views of genomic results present in the EHR. (**a**) The patient summary, which serves as the front page of each patient's record, includes a drug-genome interaction section detailing the patient's genotype in star allele nomenclature as well as phenotype and implications for prescribing. (**b**) Genomic results and phenotypes are also available in the lab results section of the EHR. When a drug is ordered for a patient with an actionable genotype, clinical decision support (CDS), such as the representative (**c**) outpatient substitution adviser, is presented to the ordering clinician. Similarly, parallel mechanisms offer CDS in the (**d**) inpatient setting.

significant family and social history, and this infrastructure was expanded to manage genomic variants and their interpretations.

GENOTYPE-TO-PHENOTYPE TRANSLATION

Although the advantages of multiplexed genetic testing are becoming increasingly apparent, there are clear challenges asso- ciated with managing panel-based genetic data. Raw genotype output is not typically delivered in a standardized format and does not include phenotypic interpretations, which may be drug and patient specific. In order for the genetic results to be useful for clinical implementation through PREDICT, results were individually categorized to create a translation layer, which assigns a coded phenotype category and generates the DGI text string used for display in the EHR and CDS, when triggered (see **Supplementary Figure S1** online). The assigned phenotypes are drawn from a translation table, which relates the raw genotype text string to drug and metabolism effect categories (**Table 2**). Translations are made based on actionable variants, defined as variants that have been reviewed and approved for clinical implementation by the VUMC Pharmacy and Therapeutics Committee; however, a large proportion of variants on the PREDICT platform are not actionable due

to insufficient evi- dence. For *CYP2C9*, for example, only 2 of the 13 variants tested on the platform have been approved for implementation. Genetic

variants that are not deemed actionable are sequestered withina separate database, outside of the EHR, and are not accessible to patients or providers. The genotype data will only be released into the EHR as new genotypes are deemed actionable and new DGIs are incorporated into clinical care.[15]

The model for the current genotype-to-phenotype transla- tion table is to assign a value to every result produced by the Absorption, Distribution, Metabolism, and Excretion plat- form, even if rare. For variants without sufficient evidence to be deemed actionable, a category labeled "indeterminate" was created (**Table 2**).

For purposes of CDS implementation, no change to usual care is recommended for indeterminate genotypes. Other pharmacogenomics implementation sites have used similar approaches,[33] and several consortia have been established to develop and maintain consistent guide- lines for translation of genotype test results, including the Clinical Pharmacogenetics Implementation Consortium and the Translational Pharmacogenomics Project.[34] The translated interpretations are viewable by providers via the EHR and incorporated into the EHR advisers; however, they are not tailored to background level of provider pharmacogenomic knowledge. Therefore, developing phenotype interpretations that are meaningful and clinically useful for providers presents its own set of challenges.

Table 2 Example genotype-to-phenotype translations

			Phenotype	
Gene name(s)	**Raw genotype result**	**Simplified genotype**	**Phenotype category**	**Phenotype detail**
CYP2C19	*17 VAR	*17/*17	Clopidogrel sensitivity	Rapid metabolizer
CYP2C19	*4 VAR	*4/*4[a]	Clopidogrel sensitivity	Poor metabolizer
SLCO1B1	*1A/*1A	*1/*1	Simvastatin sensitivity	Normal risk
SLCO1B1	*1B HET;*2 HET;*5 HET	*1/*5	Simvastatin sensitivity	Intermediate risk
VKORC/CYP2C9	*VKORC1* -1639G>A No Call, *CYP2C9* *1A/*1A	*VKORC1* indeterminate; *CYP2C9* *1/*1	Warfarin sensitivity	Normal responder
VKORC/CYP2C9	*VKORC1* -1639G>A No Call, *CYP2C9* *2 HET;*11 HET; *15 No Call	*VKORC1* indeterminate; *CYP2C9* *1/*2	Warfarin sensitivity	Hyper-responder
VKORC/CYP2C9	*VKORC1* c.-1639 VAR, *CYP2C9* *2 HET	*VKORC1* -1639 AA; *CYP2C9* *1/*2	Warfarin sensitivity	Hyper-responder
VKORC/CYP2C9	*VKORC1* NMD, *CYP2C9* *2 No Call	*VKORC1* -1639 GG; *CYP2C9* indeterminate	Warfarin sensitivity	Indeterminate

Translation entries exist for all encountered genotype combinations and phenotype categories shown in the table, which ultimately drive decision support. Currently, there are a total of 971 unique, observed diplotype genotype entries, mapping to 19 phenotypes.

[a]Denotes a rare variant.

EHR REPRESENTATIONS OF GENOTYPE ANDPHENOTYPE

The centralized service architecture of the genotype-to-phe- notype translation layer allows simultaneous population of multiple clinical information systems, supporting the clini- cian through EHR views and patients through their access toa PHR hosted on a patient portal (see **Supplementary Figure S2** online). For each client system, the service responds to requests for new or updated genomic results. Whenever a phenotype assignment is changed (such as when *CYP2C19**3 heterozygotes are added to an actionable "poor metabolizer" status for clopidogrel), the translation table within the service is updated manually, which triggers automatic revision of theresults displayed in the EHR and PHR. Following the principle of high visibility and universal access, four task-specific views of genomic results are supported in the EHR (**Figure 3a–d**). First, the program team created a space for genomic variants to be visible within the patient summary that serves as the "front page" of the electronic chart and adjacent to the medication list. Much like an "allergy" section, this space is intended to communicate significant genomic variant information whena target medication is contemplated and before initiating a prescription. During review of the design, clinicians and the Pharmacy and Therapeutics Committee required the display of any pharmacogenomic result whether indicating a variant or not, such that there was a quick method of determining if apatient had already been tested. This current presentation for- mat does not scale to many implemented DGIs; therefore, a redesign is in progress.

Second, the phenotype delivered by Patient Summary Service triggers CDS within the outpatient e-prescribing environment as well as the inpatient computerized physician order entry environment when a prescription or medication order con- flicts with the phenotype status (see **Supplementary Figure S1** online). For example, providers prescribing clopidogrel for a

patient with an intermediate-metabolizer or poor-metabolizer phenotype will receive therapeutic guidance to switch to an alternative antiplatelet therapy (see **Supplementary Table S1** online). Finally, new pharmacogenomic information is released from the laboratory. This mechanism (along with the patient

summary) supports reconsideration of patient therapy when- ever new DGIs are released. Among the challenges encoun- tered, EHR designers must decide how to represent risk; the potential impact of phenotype labeling and the utility of add- ing quantitative-risk measures to these brief interpretations are currently unknown.

DISPLAY OF GENOMIC RESULTS IN PHRs

PREDICT genetic results are released into the patient's EHR to guide therapy and clinical decision making. In addition, given the burgeoning body of literature suggesting the importance of empowering patients with health information and increased efforts surrounding the Health Information Technology for Economic and Clinical Health Act,[35] PREDICT genetic results have also been made available to patients through VUMC's patient portal, My Health at Vanderbilt, a resource that allows patients to view EHR data, message their health-care provid- ers, and read general health information tailored to their medi- cal history. Through PREDICT, we have added content in My Health at Vanderbilt related to a patient's genetic test results (see **Supplementary Figure S2** online). The first release of genomic results contained a simplified copy of what was displayed to pro- viders in the EHR: the genetic test result with a brief interpreta- tion, e.g., "CYP2C19, one copy of the variant, poor metabolizer of clopidogrel." Feedback from focus groups overwhelmingly indicated that patients preferred detailed, descriptive back- ground information related to drug side effects and how genet- ics may affect a patient's risk for adverse events. On the basis of this feedback, more comprehensive narratives with graphics are being developed and provided at a seventh-grade reading level.

EVOLUTION OF PREDICT SINCE LAUNCH

PREDICT was launched in September 2010 with genotype- tailored dosing guidance for clopidogrel.[15] The decision to focus on clopidogrel was made following the Food and Drug Administration black box warning alerting physicians and patients to the role of *CYP2C19* variants in medication response.[3] The Food and Drug Administration did not indicate how to incorporate *CYP2C19* variants into clinical decision making; however, an efficacious alternative, the antiplatelet drug prasugrel, was not affected by *CYP2C19* genotype.[36–39] Therefore, the initial clopidogrel adviser was designed to acti- vate when patients were homozygous for *CYP2C19**2 or *3 allele and displayed recommendations to increase clopidogrel maintenance dose to 150 mg daily or switch to prasugrel bar- ring any contraindications.

Since launching the program, over 75 articles have been pub- lished with the potential to influence genotype-to-phenotype mappings or the content of the clopidogrel CDS. Following publication of a large meta-analysis[3] and our internal analysis,[40] which both showed significant reduction in clopidogrel efficacy in individuals heterozygous for *CYP2C19* variants, we added such individuals to the program. Moreover, new, rare *CYP2C19* variants were determined to impair clopidogrel metabolism,[14] and new, effective alternatives to clopidogrel were released on the market. These advances warranted modifications to both the genotype–phenotype translations and the clopidogrel CDS recommendations. Updating the knowledge base and chang- ing the user interface for the CDS to add additional choices required comparatively less effort than the initial development, partially because of the separation of these components into Enterprise Services (see **Supplementary Figure S1** online). However, modifications to the phenotype map often changed the risk status of patients who were already genotyped, requir- ing providers to reconsider the initial drug selection or dosing. For each of these scenarios, we organized a communication plan, identifying affected patients and manually notifying pro- viders using secure electronic messaging within the EHR.[41]

The program continues to expand and incorporate CDS for additional DGIs into the EHR, including recommendations for warfarin, simvastatin, thiopurines, and tacrolimus. Two of the released DGIs are relevant to pediatric populations and required the development of guidelines applicable to both adult and pediatric populations, as well as DGI-specific suppression of genetic results and EHR advisers for those DGIs that were not applicable to a pediatric population (e.g., warfarin advis- ers). Infrastructure available at the time of these deployments allowed for a simple, alternative set of text for adult and pediat-ric patients. This required changes in both the database model and the presentation layer to determine, on the basis of the age of the patient, which text was appropriate for display.

DISCUSSION

The design and implementation of EHR features to support a large multi-DGI pharmacogenomics program required iterative refinements, in part because there is little published guidance on how to leverage HIT to translate genomic medi- cine to clinical practice. We described our initial design choices and subsequent changes in an effort to inform other institu- tions that are contemplating or have initiated a similar effort. One of the major successes in the past 5 years is the formation of cooperative efforts from pioneering institutions associated with the Pharmacogenomics Research Network to organize and curate the pharmacogenomics knowledge base relat- ing genomic variation to therapeutic decision making in the form of clear, accessible guidelines.[10–14] Similar efforts to share implementation practices among members of the Translational Pharmacogenomics Project and the eMERGE Network have made substantial progress.[34] Overall, the gap between the con- ceptual model of personalized medicine and actual clinical implementation is closing but remains wide for most health systems.[42] The PREDICT implementation approach is distinct because of the scope of drug–genome interactions that are tar- geted for adult and pediatric populations, the duration of the program, and the emphasis on preemptive testing. In addi- tion, the ability to leverage on-site developers familiar with the locally developed EHR allowed efficient implementation. Although the specific form of this implementation is institution specific, the abstracted challenges described in this article are generalizable.[33]

We found the major challenges for incorporating PREDICT relate to the complexity of raw genotype data and the lack of existing standards to store and transmit genomic data. Genotyping platforms do not output results in a coded refer- ence standard and are not accompanied by interpretations. Integrating with downstream EHR tasks required parsing of the gene result report and a translation layer able to contend with undefined variants. Manufacturers of genotyping instruments can improve the ease of implementation by adhering to coded standards (as they are developed) and providing more detailed documentation of potential genomic output. Second, we sought to preemptively map all variants but discovered rare variants that were undefined; an automated process within the EHR infrastructure to track and examine new, undefined variants would be valuable to ensure the timely updating of a trans- lation table and could eventually serve as a tool for discovery of potential variant function. Third, EHR integration of genomic data requires a process to manage the release of new or materi- ally updated drug–genome data as thousands of patient records are affected. Such releases also require significant communica- tion and education efforts to inform providers of emerging or changing evidence. Finally, the scalability of EHR integration is challenged by several technical factors, including limited screen "real estate" to display significant variants and inflexible models of displaying results that may not yet be pertinent to patient care.

LIMITATIONS OF THE PREDICT EHR MODEL

The application of pharmacogenomic testing to clinical care is complex and requires established and comprehensive infra- structures to support implementation. With quickly genotyping (and genome sequencing) technologies, emerg- ing evidence, and changes in therapies, these infrastructures must be prepared to accommodate rapid modifications and an explosion in genetic variants. Although PREDICT represents one viable model for implementation of pharmacogenomic information into the EHR, there are limitations and challenges that offer opportunity for improvement and fine-tuning of the program. Despite attention to the succinct and understandable interpretation of genomic results, the EHR displays may not be sufficient for providers without specific pharmacogenomics training. The brief interpretations provided presume a baseline knowledge of pharmacogenomics and are not intended to be educational. Furthermore, PREDICT affects providers in mul- tiple specialties, creating even greater provider education challenges. The provider EHR displays are not currently customiz- able by specialty, health-care role, or baseline knowledge, but such flexibility may be needed as the number of implemented DGIs increase. Moreover, results may be returned outside of the context of a clinical encounter, for example, when a DGI is released into the EHR many years after the patient's initial genetic testing. Similarly, although significant effort has been made to develop understandable and meaningful PHR dis- plays, further research is warranted to elucidate more effective methods of communicating complex genomic information to patients. In addition, there is currently no infrastructure in place to automatically and reliably deliver genetic results to pro- viders outside of Vanderbilt's EHR system; thus, some patients may be tested through PREDICT but not benefit from future decision support after they return to their primary providers outside of the Vanderbilt network. Although PREDICT rec- ommendations are based on the most up-to-date evidence and expert opinions, incorporating genomic information with clin- ically relevant nongenomic factors in CDS recommendations is currently outside of the scope of the program.

PHARMACOGENOMIC ADOPTION: THE WAY FORWARD

The challenges and lessons learned from PREDICT imple- mentation highlight the need for improved EHR integration and interoperability. For patients not receiving care exclusively at VUMC, improved communication and transfer of genetic results to external providers is the first step toward this inte- gration and is necessary to advance genotype-tailored decision making. Clinical notification of high-priority genetic results (e.g., those associated with life-threatening adverse events or with prolonged clinical utility) could be achieved by leveraging national electronic messaging infrastructures and will pave the way for full EHR integration. Pharmacogenomic adoption is limited by provider knowledge and usability of EHR-displayed genomic information. Maintaining awareness of evolving phar- macogenomic evidence and emerging therapies and incorpo- rating this information into clinical practice require procedures for systematic evidence review and an informatics infrastruc- ture that enables prompt modifications of genomic advisers within the EHR system.[15] Improved advisers and information displays that can be modified easily and incorporated within the EHR with very little informatics support will be vital as existing DGIs are updated and additional DGIs continue to be implemented. Moreover, portability of internally developed CDS across EHR systems will be critical for dissemination of clinical pharmacogenomics. We believe that use of Internet- based Web services to encapsulate genetic results and securely communicate relevant guideline-based recommendations and knowledge across institutional boundaries will compel efficient and widespread clinical adoption of pharmacogenomic evi- dence in real-world medical practice.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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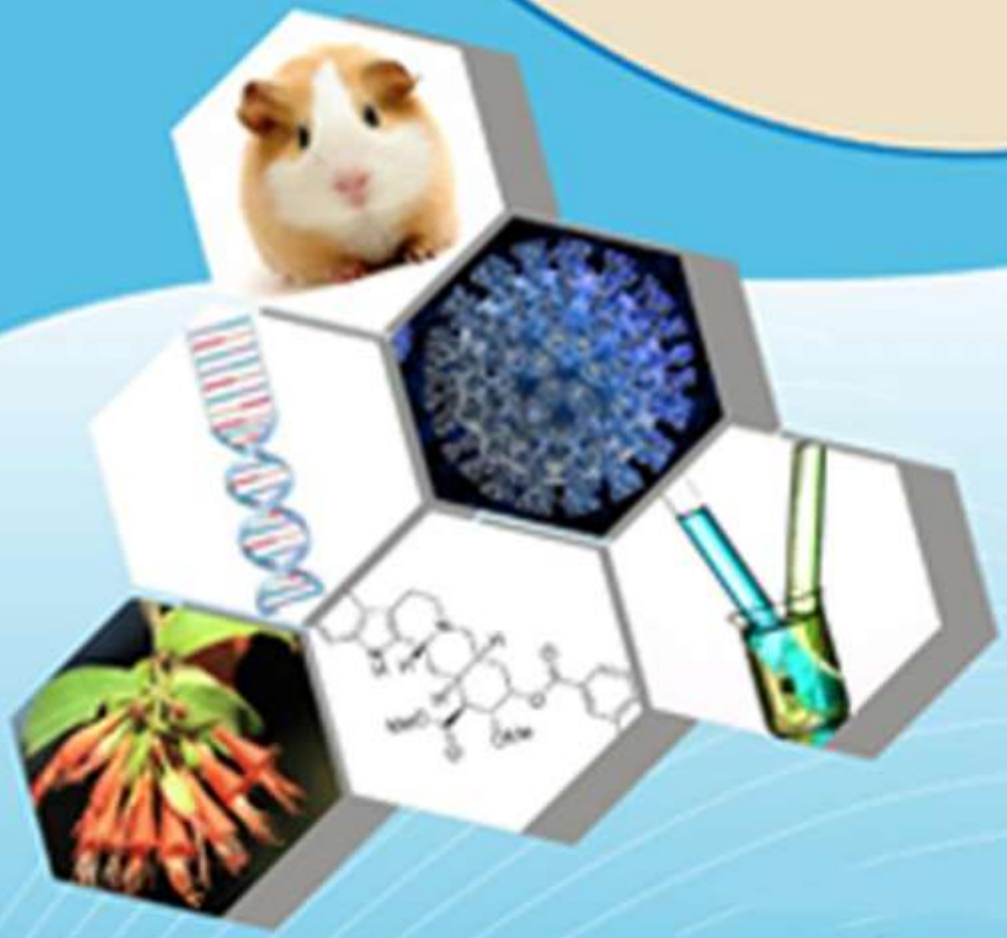

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EXTRACTION, CHARACTERIZATION AND ACTIVITY OF *CAPSICUM ANNUM* ON RHEUMATOID ARTHRITIS

M. Sree harini, R. Naganjaneyulu, Dr. B. V. Ramana

Abstract:

Background:

This study report was on plant of Capsicum annum, widely cultivated Capsicum annum *important as a vegetableand spice crop world-wide,it is one of the most diverse crops.*

The aim of this study was to investigate, extraction and characterization of Capsicum Annuum. The characterization by preliminary study, extraction of chemical constituents from the plant and find out the chemical constituents which help in treatment of rheumatoid arthritis. Rheumatoid arthritis (RA) is a long lasting inflammatory disorder characterized by joint swelling, joint compassion, and devastation of synovial joints, leading to severe disability and premature mortality it shows limited motion and function of joints.

Keywords: *Capsicum Annum, , Soxhelation Extraction, Characterization, Rheumatoid arthritis.*

INTRODUCTION:

Rheumatoid Arthritis:

Arthritis in Greek word means “arthr” meaning joint and “itis” meaning inflammation, in simply can say arthritis means inflammation on joints. And the term *Inflammation* means “*surface*” phenomenon produced by immunological and non-immunological stimuli, capable of initiating both humoral and cellular systems, normally present in the body in an inactive state and regulated by systemic inhibitors or Pain ,stiffness, redness and swelling [1].

Symptoms:

The stiffness seen in active RA is most frequent nastiest in the morning. It may precede one to two hours. Stiffness for an elongated time in the morning is a suspicion that you may have RA, while few otherarthritic disorders work this way.

Some of the common symptoms of RA are: Pain on joints, Swelling, Exhaustion, frustration and gloominess, Flu like symptoms feeling hot, ill and sweating, Anemia

The symptoms which are less common are: - Loss of weight, Swelling on eyes, Rheumatoid nodules (fleshy lumps below the elbows or on hands and feet)[2].

Pharmacognosy
Dr.K.V. Subba Reddy Institute of Pharmacy
(Approved by AICTE,P.C.l New Delhi& Permanently Affiliated to JNTUA Anantapuramu
MOU with Government General Hospital &KMC, K urnool

Causes:
As Rheumatoid arthritis is autoimmune disorder ; as we know that the immune system of our body have the defending mechanism against foreign bodies; but in case of RA, the immune system start damaging own tissues instead of attacking germs and viruses which causes swelling .Also different autoimmune disease attack different parts of body tissue but RA only affect joints.
Although physician are not confirm about the exact cause of rheumatoid arthritis, it's thought that it may result from a combination of genetics and environmental triggers (smoke ,eat a lot of red meat, drink, a lot of coffee)[3].

PLANT PROFILE:

Capsicum annuum:-

Scientific

Classification:

Kingdom	:	Plantae
Class	:	Eudicots
Subclass	:	Asterids
Family	:	Solanaceae
Genus	:	Capsicum
Species	:	C. annuum

Fig 1: Plant and fruit of Capsicum

annumSynonym:
chillies, bell peppers, green peppers, red peppers, umbrella chilli in Tamil & in India Shimla.

Biological Source:
Capsicum consists of dried ripe fruits of *Capsicum annu* Linn.var: *minimum*, belonging to family Solanaceae. It contains not less than 12% of non volatile ether soluble extractives [4].

Geographical Source:
Capsicum is cultivated in almost all the tropical countries. East Africa, West Africa and India are the regions producing the drug on commercial scale. In India, it is grown in Andhra Pradesh, Uttar Pradesh, Gujarat, Maharashtra, Assam and Tamil Nadu.

Macroscopical characters:

Colour	Dull orange red to brownish-red
Odour	Characteristic
Taste	Pungent
Size	About 12 to 25 cm in length and up to 7mm in width
Shape	It is conical or oblong

Table 1: Macroscopical characterization ofCapsicum annum

Phytochemical constituents:
Capsicum contains about 0.5 to 0.9% colourless, crystalline and pungent principle, known as capsaicin which is volatile above 65°C. Capsicum also contains fixed oil (4 to 16%),proteins and pigments, viz. capsanthin and carotene. Pigments are responsible for red colour. Thiamine and ascorbic acid are the other contents of the drug.

Uses:
1) It is used as a carminative, an appetizer and astomachic.
2) Externally, it is used as a counter irritant in thetreatment of rheumatism, lumbago and neuralgia.
3) It is also used in spices [5].

Article Review:
1) Maria de Lourdes Reyes-Escogido et.al; has showed “Capsaicin is a unique alkaloid found primarily in the fruit of the *Capsicum* genus and is what provides its spicy flavor. Generally extracted

directly from fruit, high demand has driven the use of established methods to increase production through extraction and characterization. Over time these methods have improved, usually be applying existing techniques in conjunction. An increasingly wide range of potential applications has increased interest in capsaicin" [6].

2) Enkelejda Goc, Entela Haloçi, et al; has showed "Capsicum (due to its capsaicin content) is currently used for various therapeutic purposes such as asthma, coughs, sore throats, to relieve toothaches, counter- irritant balm for external application, to alleviate pain in shingles, rheumatoidarthritis, diabetic neuropathy, etc. The pungent components responsible for the medical uses are the alkaloids called capsaicinoids. Isolation of capsaicin from Capsicum fruits was described by many authors with different methods of extraction. The most used methods are maceration, Soxhlet extraction, supercritical fluid extraction (CO2) and ultrasound the best solvents were ethanol and methanol."

3) Zeid Abdullah Al Othman, Et.al; has showed "Theaim of the present study was to determine the content of capsaicin and dihydrocapsaicin in *Capsicum* samples collected from city markets in Riyadh (Saudi Arabia), calculate their pungency in Scoville heat units (SHU) and evaluate the average daily intake of capsaicin for the population of Riyadh. The investigated samples consisted of hot chillies, red chillies, green chillies, green peppers, red peppers and yellow peppers. Extraction of capsaicinoids was done using ethanol as solvent, while high performance liquid chromatography (HPLC) was used for separation, identification and quantitation of the components. The limit of detection (LOD) of the method was 0.09 and 0.10 µg/g for capsaicin and dihydrocapsaicin, respectively, while the limit of quantification (LOQ) was 0.30 and 0.36 µg/g for capsaicin and dihydrocapsaicin, respectively" [7].

Fig 2: Exaction of Capsicum annum.

METHOD OF EXTRACTION:

1) Extraction method for *capsicum annum*: -

A. By Soxhelation method:

- Dry the fruits of capsicum annum and weighabout 100gm of dried fruits.
- Fill the dried fruit in the soxhelation apparatus with ethanol about 250-300ml in RBF and boil it at 50-60°C temperature for 5-6 hrs.
- Collect the crud syrup mass of extract at the bottom of flask.

B. By Maceration:
Take fresh fruits of *Capsicum annum* wash it properly & weight it in 2.5 grams and dissolve it in 25ml of ethanol, macerate it for 24 hours to get activeconstitute out of it.

Evaluation Of Preparations:-

1. Measurements of pH:
2.5gm Ointment sample was taken in 100 ml dry beaker, and then 50 ml water was added to it. Beaker was heated on water bath maintained at about 60ºC to 70ºC for 10 minutes, cooled to room temperature. Then pH measurements were done by using a digital type pH meter by dipping the glass electrode into the ointment formulation.

2. Determination of viscosity:
The measurement of viscosity of the formulated Ointment was done by using Brookfield Viscometer. Spindle No. S63 and S64 were used for the determination of viscosity of ointment. Spindle was rotated at different rpm for 30 second for each measurement. The results are shown in Table 2 and 3 below.

3. Determination of extrudability:
Extrudability test is the measure of the force required to extrude the material from a collapsible tube when

certain amount of force has been applied on it in the form of weight. In the present study the quantity in percentage of cream extruded from the tube on application of certain load was determined. More the quantity extruded, better was the extrudability of ointment.

4. Determination of spreadability:
One of the criteria for a cream, ointment or gel is that it should possess good spreadability. Spreadability is a term expressed to denote the extent of area to which the cream readily spreads on application to skin or affected part. The therapeutic efficacy of a formulation also depends on its spreading value. Hence determination of spreadability is very important in evaluating ointment characteristics. Special apparatus was designed to study the spreadability of ointment formulations. The spreadability is expressed in terms of time in seconds taken by two slides to slip off from ointment, placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, better the spreadability of ointment.

5. Patch test:
This is done by shaving the mice and applied Capsicum Ointment.

Before Application of Dosage form:	After Application of Dosage form:
	

RESULT & DISCUSSION:
Results and Discussion Determining the PH, Viscosity, Spreadibility and extrudability, and the results are shown in Table 2 and 3.

Preliminary Studies of Extract:

Chemical Constituents:

Table 2: Chemical test and inference of Capsicum annum.

S.N.	Phytochemicals test.	*Inference*
1.	**Alkaloids:**	
	Wagner's Test	+
	Hager's Test	+
	10% Tannic acid test	+
2.	**Phenolic compound**	+
3.	**Fixed Oil:** Saponification test	+
4.	**Steriods:** Salkowski test	+
5.	**Carbohydrate:**	
	Molish Test	+
	Solubility	+
	Interconversion Test	+
6.	**Protein:**	
	Ninhydrin test	–
	Million reagent test	–
7.	Volatile oil	–
8.	Gums	–

Evaluation of *Capsicum Annum* Ointment:

1. Measurements of pH:
pH was obtained as **5.84**.

2. Determination of viscosity:
The viscosity of ointment was determined by using spindle no 63 & 64.

Table 3: viscosity of ointment in spindle S63 and S64

S.N.	RPM	Spindle S63		Spindle S64.	
		Torque (%)	Viscosity(P)	Torque (%)	Viscosity(P)
1	0.3	42.4	848000	–	–
2	0.5	57.2	686000	–	–
3	0.6	54.6	545900	6.5	65000
4	1.0	68.3	409700	17.3	10380
5	1.5	47.4	189600	26.1	10440
6	2.0	59.2	177600	22.1	66300
7	2.5	63.1	151400	40.8	97900
8	3.0	54.0	108000	42.6	85200
9	4.0	67.3	100900	52.4	78600
10	5.0	93.9	117300	56.7	68000
11	6.0	82.5	82460	59.4	59300
12	10	81.5	51716	63.5	37450

3. Determination of extrudability:
The extrudability of ointment was obtained as **85%.**

4. Determination of spreadability:
The spreadability of ointment was found to be **4.5 gmcm/sec.**

5. Patch test on mice:
No change was found in mice.

CONCLUSION:

In this project ethanolic extract of *Capsicum annum* was obtained by soxhelation and preliminary work had done in which chemical constituents like alkaloids, fixed oil, phenolic compound, steroids and carbohydrates were found. Different parameters of phytochemical screening of herbs, their antioxidant properties and evaluated like PH, Viscosity, Speadibility, extrudability test were covered and result was as predetermined accept the patch test on mice that was negative.

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The Scope of pharmacy ethics—an evaluation of the international research literature,

C. Anusha, S. Md Abdulla , IVL. Padmini

Abstract

This paper attempts to provide a critical overview of international published discourse relating to ethical issues in pharmacy practice from 1990 to 2002. We found that there is little research literature specifically addressing ethics in pharmacy practice and almost none addressing fundamental philosophical issues or values for pharmacy ethics. There is no dedicated journal for pharmacy ethics. Most material relating to pharmacy ethics is articulated as codes or pronouncements from professional bodies, as opinion or reflection in textbooks and in debate such as letters and articles. However, this should not be taken to mean that pharmacy and ethics are strangers; simply that such matters are not frequently analysed in published pharmacy literature. The presumption is usually that most matters of pharmacy ethics are very familiar and require no exploration or explanation.

Where the research literature does target ethical issues, the most common method is to employ ‘‘the scenario approach’’. This term describes the technique of using a vignette or scenario from actual pharmacy practice and then exploring a variety of possible options to identify one or more defensible solutions. The vast majority of scenarios related to the delivery of healthcare per se; rather fewer derived from delivery of healthcare in a commercial environment. One notable exception to this approach is the body of work by Latif and colleagues on moral reasoning and community pharmacy practice.

Keywords: pharmacy ethics—an evaluation of the international

Introduction

Over the 20 years prior to 2002, pharmacists practising in the health systems of the developed world have expanded their activities from a predominant emphasis on the supply of medicines to an increasingly clinical and advisory role. Migration from compounding and provision of medicines has been a long-term process, (chronicled, for example, by Anderson, 2001) and is by no means complete. Policy directions, typified in England and Wales by the NHS Plan and Pharmacy in the Future (Department of Health 2000a, b), describe significant roles, principally for community pharmacists, in prescribing and the provision of prescribing advice, in assuming accountability for therapeutic outcomes and in contributing to patient care decisions within multi-faced by other clinical colleagues but will also encounter particular challenges of their own. Moreover, the position of community pharmacists straddles both the public and private sectors: in the UK, for example, the pharmacist is, or is employed by, a private employer contracted to the NHS to dispense prescriptions—a public service. The pharmacist is also a private sector retailer of other medicines and other products, health- related or otherwise. Pharmacists working in the community daily experience patients as customers as well as patients receiving complex and sophisticated therapeutic regimens. Practising pharmacists need there- fore to be fully engaged with and competent to deal with ethical issues arising from the increasing challenges of ‘‘hi-tech’’ healthcare and its delivery in a business environment. This literature review aims to establish the scope and extent of the literature documenting such engagement.

Scope

Healthcare ethics encompasses a reasonably well-established spectrum of issues typified by the UK core curriculum for medical ethics (Consensus group of teachers of medical ethics and law in UK medical schools, 1998) and supported by original work in America by Beauchamp and Childress (2001) and classic texts on ethics in healthcare (Mason, McCall Smith, & Laurie, 1999; Seedhouse, 1998). Many issues arise from the relationships between healthcare professionals and patients, relatives and the general public. Others arise from the research and development process that under- pins healthcare or the constraints of balancing demand and costs, especially within state-funded systems. Healthcare ethics has traditionally related to patients within a healthcare system rather than to situations where the ''patient'' is, in reality, a fully autonomous consumer in the private sector—for example, a customer selecting their own non-prescription medicines. How- ever, social science contributions, for example, from Denzin and Mettlin (1968), Dingwall and Wilson (1995) and Hibbert, Bissell, and Ward (2002) have explored commercial and business influences on professionalism in community pharmacy.

Pharmacy practice is usually understood to describe those activities of pharmacists that most closely and directly impact on or interact with the final consumer of medicines, be they patients or users. The main practice areas are therefore within hospitals (17%), within community (or retail) pharmacies (62%) and in associa- tion with GP practice (perhaps 3%) (Hassell, Fisher, Nichols, & Shann, 2002). Pharmacy practice has the potential to raise ethical challenges across the full spectrum of healthcare ethics, although not always to such an extent or to levels that may, for example, face medical or nursing practitioners, when negotiating the switching off of life support or terminating a pregnancy. Conversely, community pharmacists, being in the private sector, have daily to balance their obligations to make a living, nay a profit, with their professional duties as providers of advice and support to optimise the use of medicines. In addition, in Britain, national multiples operate roughly 40% of community pharma- cies so that the organisational values and targets adopted by these companies (and countless small local groups) to secure adequate profits for shareholders or owners may have a powerful influence on the ability of individual pharmacists to exercise independent profes- sional judgement and morality.

This literature review identified two additional bodies of published material that were excluded because they are not directly relevant to pharmacy practice. They were, firstly, accounts of work in the teaching of pharmacy ethics. Since much of this work is intended to inform and support the moral awareness and reasoning capacity of future pharmacists, further evaluation of this material may be appropriate else- where. The second body of work describes the moral dilemmas surrounding the pharmaceutical industry, third world poverty and availability of medicines. As such, it was decided that this was outside the scope of the present review.

This review therefore concentrates on two areas of literature in which ethical challenges arise for practising pharmacists: those that arise from

- Delivery of healthcare per se and.
- Delivery of healthcare in a business environment.

Method

Details of the search strategy with selection and exclusion criteria appear in Appendix 1. Electronic databases were searched from 1st January 1990 to 1st July 2002. This period was selected to overlap with and extend material in two major British reviews (Pharmacy Practice Research Resource Centre, 1994a & b), to identify published discourse consequent on a new British Code of Ethics in 1992 and to span a sufficient period for tangible changes in the role of pharmacists to be evident.

Part One: Healthcare practice related issues

Philosophy

In terms of philosophical discourse, very little material has been published in journals on the core values that might underpin pharmacy ethics. Instead, national and international professional organisations have largely defined the scope of pharmacy ethics through their official pronouncements and codes of ethics or conduct (for example, see website references for American Pharmaceutical Association, European Phar- macy Group, International Pharmacy Federation, Pharmaceutical Society of Ireland, Pharmacy Boards of Queensland or New South Wales, the Royal Pharmaceutical Society of Great Britain and the World Health Organisation).

Some US textbooks attempt discussion on ethical norms (Weinstein, 1996; Smith, Strauss, Baldwin, & Alberts, 1991); other UK textbooks (Appelbe & Wingfield, 2001; Mullan, 2000; Taylor & Harding, 2001), have sought to add brief commentary on the concepts of professional ethics and the operation of national codes of ethics. The current British Code of Ethics, (RPSGB, 2001), claims to be underpinned by

three key principles: beneficence, competence and integrity (Anon, 2001), although only the second of these terms actually appears in the text of the code. Also, at least in Britain, successive codes have included short preambles about duties or responsibilities of pharma- cists derived from discussions amongst leading practi- tioners or commentators in the profession, consultation with rank and file pharmacists and building from earlier versions of similar codes. That is not to say that these deliberations do not lead to principled statements of ethics; simply that the scope of the principles derive from practice rather than a philosophical analysis of core values in the profession.

Two American papers have both supported and criticised the effectiveness of pharmacy organisations in setting the core values of the pharmacy profession. A study by Garst and Berardo (2000) examined the role of the American Pharmaceutical Association in relation to changes in ethics and practice in pharmacy over the previous century and concluded that the Association had been a stabilizing social structure to stimulate debate and disseminate new ideas and standards. A much less comfortable view was taken by Redman (1995) who, whilst congratulating the Society of Health System Pharmacists on their innovative work in tackling ''drug misadventuring'', challenged pharmacy as a whole to demonstrate the ''ethics of leadership'' in coping with the ''hurricane of change'' surrounding the transformation of pharmacy into a fully clinical discipline. Citing work by Americans Pellegrino and Thomasma (1993) on medicine as a moral community, she questioned why there was virtually no collaboration between the three professions—nursing, medicine and pharmacy—to develop a cross-professional conception of ethics. International work in this direction did commence with the Tavistock Group, (Smith, Hiatt, & Berwick, 1999; Berwick, Davidoff, Hiatt, & Smith, 2001)but the outcomes were mostly concerned with economic and resource constraints in health systems and the balancing of interests of health professionals, organisa- tions, managers and the public rather than developing a universal code of ethics by which health professionals should organise their work. Nevertheless, the group made the telling point, as did Redman (above) that all healthcare providers should be brought together in a consistent moral framework. Such a philosophy may not recognise that core values such as equity and equality of access which underpin the British NHS or any publicly funded health service are not necessarily reflected in the private sector.

A paper by a Dutch pharmacist (Dessing, 2000) attempts a philosophical analysis of the application of ethics to pharmacy practice. Dessing postulates three fundamental principles—autonomy, democracy and solidarity—as being the basis for ethical pharmaceutical care. Drawing on the views of Rorty (1989), Dessing says that the latter two principles are necessary for the avoidance of anarchy that would result if personal autonomy were always unopposed. Dessing goes on to recognise that therapy, in this case medicinal treatment, should always be viewed as an ethical endeavour aimed at restoring the recipient to the maximum possible degree of autonomy. Dessing stresses the importance of developing a relationship with the patient as being the crucial precursor to an ethical obligation towards them. From this position there flows the imperative of non- maleficence (limiting harm) in therapy and the concepts of informed consent and participation (concordance) in drugs treatment. Dessing also criticises the FIP (Inter- national Pharmaceutical Federation) Code for stating lofty principles in places and providing very limited, specific practical guidance in others: e.g. ''the pharma- cist (shall show) respect for human life.'' and will ensure that ''when a pharmacy closes, the patients are informed of the pharmacy to which their records have been transferred''.

Published work examining pharmacy ethics from a philosophical perspective is relatively scarce; literature documenting the application of specific ethical concepts to pharmacy practice is more widespread and the following account gives examples only of typical papersin each area.

Application of ethical concepts to practice

By far the most common example of literature covering pharmacy ethics assumes that pharmacists have knowledge of ethical norms and moves straight to application of ethical (and legal) principles to scenarios from pharmacy practice. In the UK Nathan and Grimwade (1993) undertook an early form with eight scenarios ''to test y law and ethics knowledge''; in truth, all but one raised questions of law rather than ethics and even the eighth, on confidentiality of contraceptive prescribing, defended the ''answer'' principally by reference to data protection and other legislation. The Weinstein and Smith textbooks (see above) provide examples of ''practice scenarios'' (including business related dilemmas) where it is necessary first to identify the questions of ethics that arise in a given situation before being in a position to deal with them. In the UK, Wingfield, Taylor, and Lee (1997) and later Appelbe, Wingfield, and Taylor (2002) proposed the application of a systematic ''stepwise'' approach to decision making in practice situations where legal and ethical principles may conflict. (This can be summarised as: gathering facts, identifying issues, assigning priorities and interests, generating options for action and choos- ing an option as a reasoned decision.)

Two papers in the American Journal of Hospital Pharmacy appear at first sight to be simply scenario based but in fact include philosophical analysis of the schools of thought at work in the opposing ''solutions'' suggested by each of two commentators. In a case in which the pharmacist has to question the motives behind a change in physician prescribing,

Veatch (1990) advances deontological and utilitarian arguments in support of the two options, before concluding that duties such as the ''ethics of respect'' for the patient's rights should probably prevail. In 1993, referring to a patient who does not wish to disclose serious symptoms to his physician, Veatch (1993) also contrasts older paternalistic models of confidentiality with more mod- ern thinking on respect for autonomy.

The following sections exemplify illustrations of ethical principles as applied to pharmacy practice, often within papers that did not set out specifically to examine ethical questions.

Consent and confidentiality

The most frequent literature citation in pharmacy ethics is confidentiality, and the associated concept of consent to use and disclosure of patient information. A French study (Auguste, Guerin, & Hazebroucq, 1997) investigated the reactions of 15 hospital pharmacists to situations that might compromise confidentiality. All the pharmacists in the study contended that, in their undergraduate studies, they had received no training in biomedical ethics and insufficient exposure to practice to feel confident of their ability to deal competently with these situations. Resort to a guide to good practice was seen as the main solution. The same shortcomings, and solution, were features of a later UK study (Wills, Brown, & Astbury, 2002) into the dilemmas faced by medicines information pharmacists. Having tradition- ally confined their telephone service to other health professionals, such services are increasingly being accessed by the public. The study reported conflicts between those pharmacists who prefer to withhold sensitive information for more propitious disclosure than by phone and others who argue that as most of the information sought is in the public domain, it should be available on request. Again, of 151 centres for medicine information which responded, over half said the pharmacists providing the service had received no training in ''ethical issues'' despite the fact that over 70% of them had gained postgraduate clinical qualifications.

Other published material on consent and confidenti- ality uses the ''scenario and possible solutions'' ap- proach described above. In the US, Haddad (1993) discussed a situation in which a patient had disclosed information that suggested a serious threat to his health with a strict injunction not to share it with the patient's physician. She suggests that the duty of confidentiality rests on two fundamental ethical concepts: respect for the patient's autonomy to decide what happens to them, and fidelity, implicit in an unspoken pledge by the pharmacist to keep silent, if the patient wishes. In this case, the deciding factor is held to be that articulated wish. If the patient had said nothing, disclosure might have been an alternative option. In the UK, in a series of scenario discussions, Daly and Bower (1997a and b) included other examples of information provided by the patient to the pharmacist on a confidential basis, such as deliberate refusal to take the prescribed medication or withholding information that could compromise the effectiveness of medication. In a series analysing the process of ethical decision making, the present author (Wingfield et al., 1997, cited above) included a confidentiality scenario about disclosure of patient records after death to illustrate the overarching nature of ethical obligations beyond statutory law. Other articles have adopted a more descriptive approach by documenting the implications for confidentiality of changes in practice (Justice, 1997 USA) or a change in the Code of Ethics (Rodgers, 2000 UK). One further article (Sheu, 1998 USA) examines the practical ways in which both security and privacy can be assured with the use of a telephone link and a buffer zone to distance waiting patients from the patient consultation in progress.

The advent of electronic communication has gener- ated the largest amount of literature about pharmacy and confidentiality. On both sides of the Atlantic, there has been an explosion in statutory regulation to be described (Wingfield, 2000a, b; Tribble, 2001; Nahra & Ryland, 2001) and much comment on how to cope with the opportunities and threats that ''telepharmacy'' provides (Angaran, 1999). The acquisition of electronic patient medication records provides opportunities for ''data mining'' by pharmaceutical companies in an effort to target their drug promotion activities more efficiently and profitably. In the UK, the state took a hand in attempting to outlaw this activity claiming that it would drive up drug costs to be borne by the taxpayer (Anon, 1999). The state lost, principally because the data processor could show that the patient identity was effectively removed (Anon, 2000). In Canada, similar activity by the same data processor evoked a call for regulation by the Medical Association to protect physician confidentiality (Zoutman, Ford, & Bassili, 2000). Reference is made in this paper to the unilateral action by British Columbia to prohibit the participation of pharmacies in the collection of this data and substituted a province-wide, on-line pharmacy system instead. Ironically, the NHS in the UK already has such data within its state reimbursement systems but does not yet choose to use it in this way.

The capacity to create and transmit electronic prescriptions is in its infancy in the UK but early research (Porteous, Robertson, Bond, Hannaford, & Reiter, 2000) suggests that confidentiality and consent will be contentious issues. The ability to process personal health information within state systems such as the UK NHS or employment linked Patient Benefit Organisations in the US provides real and potential opportunities to manage drug costs or improve patient health outcomes. Such opportunities

also raise conflicts between organisational efficiency and patients' rights to withhold consent to such manipulation of their data.These rights are likely to be more about a right to be properly informed than a right to opt-out. In 1998, a media outcry followed a US scheme that used prescrip- tion information to promote a new drug to patients without their prior consent (O'Harrow, 1998 a and b). In an editorial discussing this development, Talley (1998) draws attention to earlier acknowledgement by Zellmer (1994) that the interests of pharmacy providers such as owners of businesses or Pharmacy Benefit Managers (PBMs) might be different from those of pharmacy practitioners. Pomerantz (1999) and Ohliger (1999) highlighted the threat to privacy inherent in collections of data being accessible to large numbers of employees in behavioural (mental) health PBMs. Lo and Alpers (2000), in a paper addressed to the Canadian medical profession, suggest that use of patient data in PBMs should be subject to clear evidence of patient benefit, independent scrutiny and patient authorisation, with such authorisation a necessary condition for continuing care.

Internet pharmacies continue to grow globally. Most comment focuses on the commercial implications for more traditional ''bricks and mortar'' pharmacies but the implications of privacy and confidentiality of email communications are described in a US news item by Landis (1999) and discussed by Spooner (1999). The formulation of operational and ethical standards for on- line supply of medicines has been undertaken by professional organisations in Britain and Europe (Anon, 1997; FIP, 2002). Electronic communications may help community pharmacists in particular to integrate with the wider healthcare team; conversely, the vast capacity and capability of electronic communication means that pharmacists must ensure they possess a clear and thorough understanding of privacy and confidentiality aspects of sensitive personal data and implement rigorous measures to preserve them.

Other consent issues

In the lexicon of health care ethics, consent has traditionally related to consent to treatment. For pharmacists however, the usual context is within clinical trials either at industry level or within a hospital setting (Kayne, 1996) although the present author (Wingfield &Gibb, 2001) has raised questions about the limitations of applying the traditional research governance mechan- isms to randomised controlled trials with autonomous customers in a community pharmacy setting. The extension of consent to participation in practice research, and the corresponding role of research ethics committees in the UK has been described by Jesson (1997) and Smith (1998). Raynor, Petty, Lowe, and Vail (2000) have published some early research on the reluctance of patients to consent to medication re- view—a key component of the pharmacist's expanding role. Once again, the scenario-solution approach has included an example where the pharmacist discovers an inadequately informed patient in a clinical trial being conducted by the district nurse at home (Daly & Bower, 1997c). Boggs and Daly (1998) also highlight the need for greater recognition of patient consent to treatment with unlicensed medication or ''off-licence or off-label'' indications. Conroy et al. (2000) found almost 50% of drug prescriptions in European countries were either unlicensed or off-label. Subsequent correspondence from Andrew, Riordan, Ruperto, and Martini (2000) and others pointed out that such use was by no means limited to hospital wards.

A thoughtful exposition of the concept of consent in a pharmacy context appears in a paper by Americans Wick and Zanni (2001). They argue that ''pharmacists have a professional obligation to counsel so that their patients are, in effect, informed co-managers of their drug therapy''. This approach (called ''concordance'' in the UK) is a logical extension of the theoretical approach taken by Dessing (above). The ethical rationale of medical treatment is to restore as far as possible patient autonomy. Partnership by patients in therapeutic decisions requires that they have both knowledge and understanding of their treatment and options; the pharmacist should accept accountability for ensuring that such informed consent to treatment is achieved, or at least offered. More work may be needed firstly to ensure that new and practising pharmacists are thoroughly instructed in the concepts of consent and confidentiality; secondly to research best practice in securing concordance.

The beginning and end of life

Pharmacists are rarely front line health practitioners at moments of birth or death but they may be the suppliers of medicines used in these circumstances. Reservations amongst UK pharmacists (and general practitioners) about the supply of emergency hormonal contraception (EHC) (Harper & Barrett, 1998; Barrett & Harper, 2000) diminished considerably when a proges- togen only form of EHC with a low risk profile was introduced as an over-the-counter pharmacy only medicine and legally sanctioned, locally agreed direc- tions were instituted allowing participating pharmacists to exercise to the full their professional judgement as to the suitability of supply outside the licence. Bissell and Anderson (2003) found that community pharmacists supplying EHC via a prescribing protocol had few ethical objections to this role.

Regarding ''hastened death'', an international series of papers published in the mid 1990s attempted to identify issues for pharmacists as the agents of supply for life-terminating medications. From the US, Rupp (1995) suggests a need for a ''conscience clause'' protection for those pharmacists who did not wish to be involved. McAuley, Smith, and Szeinbach (1996) report research on the views of American hospice pharmacists in

withholding or withdrawing life-sustain- ing medications. Marcus (1995) discusses the response of pharmacists to the Oregon Death with Dignity Act and highlights the lack of recognition of ethical challenges for pharmacists in the framing and passing of this law. In other American studies, Rupp and Isenhower (1994) undertook work on the views of pharmacists towards physician-assisted suicide (PAS) establishing that, although over 70% of pharmacists believed that patients were sometimes justified in wanting to end their own lives, fewer than half supported the concept of PAS. Commentators from Northern Ireland and the US (Mullan, Allen, & Brushwood, 1996) described the supervening ethical dilemma in this area: conflicts between personal convictions of pharmacists as employ- ees and the policies and customer expectations of their employing organisation. Monsanto, Fabregas, and Velez (1999) surveyed the adoption of a conscience clause by professional associations and boards across the United States. In a Canadian paper on terminal sedation, Tisdale and Woloschuk (1999) drew careful distinction between this and PAS. They distinguished between, in terminal sedation, the continuing obligation to monitor the patient's ''progress'' and ensure that suffering does not recur with PAS, is likely to be technically easy and brief. They argue that the ethical imperatives of beneficence and non-maleficence could
still be satisfied because terminal sedation is intended to maintain a satisfactory quality of life until the moment of death, not to hasten death.

A study in Britain (Hanlon, Weiss, & Rees, 2000) aimed to establish if British pharmacists had differing views from those in the Rupp studies. By and large they did not, with 38% not feeling the need to be informed about the purpose of the medication in these instances and 25% positively wanting to be kept in the dark. The present author drew attention to this rather disappoint- ing flight from involvement (Wingfield, 2000a, b) and (Hackett & Francis, 2001) noted that this reluctance seemed to be more marked in community pharmacists. They suggested that this might be due largely to their relative isolation and lack of opportunity to discuss issues with colleagues.

community pharmacy. They suggest that many of the economic pressures of solo practice identified lead directly to ethical dilemmas. Increased consumerism, the loss of resale price maintenance (price fixing on OTC medicines), pressure to ''violate ethical and professional norms'' to stay in business—all contribute to a feeling of ''ethical loneliness''. They found that there was a clear cultural gap between the thinking of NHS planners, civil servants and the entrepreneurial spirit of the solo professional, and that the policies of government and professional body policies ''simply fail to engage with the circumstances of many solo practitioners''.

Moral reasoning and professional independence

A prolific researcher in this field is Latif, who has undertaken many studies involving community pharma- cies in USA. In all, 18 citations of his work were identified; nine of these have been directed at

It might also reflect the diminu- tion of ethical reasoning skills in pharmacists working in retail settings as extensively researched in the USA by Latif (see below).

In the Netherlands, the state has already legalised euthanasia, albeit subject to a number of conditions. A paper by Lau, Riezbos, Abas, Porsius, and De Boer(2000) reported on a survey undertaken in 1994 on the views of pharmacists. Naafs (2001) comments further on the Dutch findings and argues that for pharmacists to assist in legalised euthanasia is a logical last step to alleviate suffering. Another perspective from Hirsch, Marriott, Wilson, and Faull (2001) highlighted the ethical dilemmas around clinical trials in palliative care and again reflects on the role of research ethicscommittees.

Part two: Business related issues

A fundamental dilemma, which goes to the heart ofthe position of community pharmacists, is whether they are health professionals or retailers in their community pharmacies or ''chemists' shops''. This conflict has long been identified (Quinney, 1964; Denzin & Mettlin, 1968; Ladinsky, 1971; Kronus, 1975) and may have implica- tions for the quality of care that can be delivered from community pharmacies and for the adoption of more clinically orientated roles in patient care, such as structured advice, management of repeat dispensing, supplementary prescribing and medicines management. The frequent, often daily, experience of such conflicts may affect the professional satisfaction and moral reasoning skills of pharmacists who work in this sector as well as the nature of their engagement with a broader ethics agenda than that solely associated with the provision of healthcare.

Dingwall and Watson (2002) have undertaken pre- liminary work in England to inform a full-scale investigation of the social and economic position of solo practitioners (usually owner-proprietors) in

progress in learning and teaching before qualification. The remain- ing nine are outlined below and represent a burgeoning body of empirical research into the moral reasoning skills of community-based pharmacists.

Latif's first identified paper (1997 with Berger) reported on work with pharmacy students in one School of Pharmacy and a random sample of community pharmacists in a South East US city. The aim was to examine their moral reasoning using Rest's DefiningIssues Test (DIT). In a comprehensive introduction, Latif sets out the genesis of this test, from its origins by Piaget (1932) and by Kohlberg (1969) leading to its ultimate formulation by Rest (1979, 1990). Briefly, the DIT is a self-administered questionnaire that measures the subjects' moral reasoning according to cognitive developmental theories. It consists of six hypothetical dilemmas (a short-form version comprises three dilem- mas). Each dilemma is followed by a series of 12 statements

about each dilemma. For each dilemma, subjects must select and rank order those issues that have, in their opinion, the most significant effect on the dilemma's resolution. The four highest ranked items are included in scoring the DIT. Of these four items, only those that represent principled thinking are included in the ''score''. These scores can then be compared across a range of subjects both within pharmacy and other professions. The DIT test can be administered in a range of settings controlled for organisational values and rewards systems.

Latif and Berger (1997) in their study have applied the DIT test to the work of nurses, physicians, physiothera- pists and surgeons. He found that the DIT scores of
42.47 in first year students are similar to the mean for college students (42.3) and for adults in general (40.0). However, it is less than the obtained means for other health profession student groups such as medical students (50.2) and dental students (47.6). Moreover,

the score for the community pharmacist group was 36.4 which compares unfavourably with other professional groups, such as practising physicians (49.2) and staff nurses (46.3). He concludes that, ''if the community pharmacy setting does in fact impede moral develop- ment, then pharmacy organisational climates and cultures must be changed if we expect to foster the ethical covenant between the patients and the pharma- cist that is required to provide pharmaceutical care''.

In subsequent years, using larger samples of data from the US, Latif has gone on to identify situational factors which affect the decision making behaviour of commu- nity pharmacists. Latif (1998a) published a survey of 450 independent and chain pharmacists in a large South Eastern USA city indicating that workload did not significantly affect clinical decision making but employ- er's approval or disapproval of the provision of pharmaceutical care were highly

confirm that pharmacists' moral reasoning accounted for a significant amount of the variance associated with their clinical performance. Moreover, that those phar- macists with higher levels of moral reasoning were significantly more likely to score low on social desir- ability—defined as ''a tendency to provide a socially desirable response that overstates actual behaviour''. In the same year, Latif (2000b) reported on further studies which found that the DIT scores for community pharmacy practitioners were lower than for practising physicians and for medical, dental and pharmacy students. He suggested three factors might be at work: the selection of lower ethical reasoners to the community pharmacy setting, the exodus to other pharmacy settings of higher reasoners and a possible retrogression in the ethical cognition of community practitioners. Further work has focused on the effect of age and tenure on moral reasoning scores Latif (2000c), differences be-tween moral reasoning scores in pharmacists working in chain and independent pharmacies (Latif, 2000d) and a model for community

significant. Based on this finding, he suggested that the effect of the organisational culture within the organisation employ- ing the pharmacist should be more widely researched. In the same year Latif et al. (1998) published results from the use of Rest's defining issues test (DIT) in the same population. These suggested that, after controlling for situational factors, pharmacists' moral reasoning ac- counted for a significant amount of the variation associated with their self-reported and actual clinical performance. In the third paper that year Latif (1998b) proposed that the study of patient care by community pharmacists would be incomplete without considering the effect of reward system within the employing pharmacy organisation and the ethical cognition level of the pharmacist. This paper set out a future research agenda of hypotheses to be tested, all referring to Kohlberg's six stage of morality:

- That most community pharmacists reason at con- ventional levels of cognitive moral reasoning (Kohl- berg's levels 3 and 4).
- That those pharmacists at higher levels (Kohlberg's levels 5 and 6) would deliver more consistent patient care judgement and behaviour.
- That systems which reward or emphasise prescription volume over patient care will reduce consistency amongst community pharmacists at lower (Kohl- berg's levels 1–4) levels.
- That community pharmacists at lower levels will be more influenced by significant others, and by the organisation's reward system, than those at the higher levels.
- That if excessive organisational pressure rewards volume over patient care, those pharmacists with higher levels will leave the organisation.

Latif (2000a) has since published findings (again with community pharmacists in a South East US city) that

pharmacists to adopt to cope with the impact of managed care and Pharmacy Benefit Management companies (Latif, 2000e).

Business conflicts: healthcare goals Versus business goals

Health services such as instalment supply, supervised supply, needle and syringe supply and disposal of ''used works'' for drug misusers, when provided from com- munity pharmacy premises, raise conflicts beyond whether they are ''right'' or not. Such activities can directly conflict with demands of the business environ- ment. In the UK, Harding, Smith, and Taylor (1992) studied the attitudes of (only) community pharmacists to these services. Whilst some objections reflected value judgements about expending valuable time on misusers, most reflected the constraints of healthcare in a retail environment—the potential to deter other customers, a perceived negative effect on other customers, the likelihood of

shoplifting, unpleasantness for counter staff and customers. Other objections reflected conflict with business imperatives—there was no long-term financial benefit to participation. A similar UK study with pharmacy students, at undergraduate and pre-registration stages (Sheridan & Barber, 1993) confirmed suggestions that younger pharmacists were likely to adopt the most non-judgemental approach to drug abusers, with support for the statement that ''pharma- cists should deal with all aspects of healthcare''. However, Tucker (1997), found that a significant objection to participating in these voluntary services remained the possibility of adverse effects on the regular customer base.

Rees, Harding, and Taylor (1997) identified a key business-related complication. Most pharmacists, other than those who are owner-proprietors do not have autonomy in making decisions about service provision. Thus the decision as to whether to participate is in fact taken by managers, often very senior, and sometimes not pharmacists themselves. Matheson and Bond (1999) undertook further qualitative analysis of Scottish com- munity pharmacists' views and identified a concern about the sheer numbers of supplies and exchanges for drug abusers as well as maintaining a welcoming environment for other shoppers.

These papers demonstrate that whilst the convenience and informality of a pharmacy ''shop'' may seem to be appropriate to the management of drug misusers in the community, success is limited by the need to remain attractive to other shoppers and to fit within corporate brand strategies and environments.

The same commercial conflicts arise with the optional supply of EHC outside its licence under local directions (see above). A British report (Anon, 2002) describes pressure from pro-life groups that led a national chain of supermarket pharmacies to abandon EHC supply via patient group directions which allow discretionary supply to females under 16 and some vilification in the pages of the pharmaceutical press (Gray and O'Brien, 2002; Atkinson, 2002; Bowyer, 2002). Whilst a decision to supply or not supply may be a matter of personal conscience and personal business interests for an independent owner-proprietor, participation by larger national pharmacy chains is a decision taken by senior executives, acutely aware of how boycotts and demon- strations threaten share prices and the prosperity of retailing activities wholly unrelated to healthcare.

Other business conflicts arise from the provision of what may be regarded by many as simple commodities from a healthcare setting. Resnik, Ranelli, and Resnik (2000) examined the impact of the USA business environment and the time available to meet a legal requirement to provide individual advice to each and every customer and patient. Two researchers from Iceland and Denmark (Almarsdottir & Morgall, 1999; Almarsdottir, Morgall, & Grimsson, 2001) examined the effect of removal of all price fixing for medicines and the introduction of licensing and quality audit for pharma- cies in Iceland since 1996. One unwanted outcome was that, at least in the cities, patients and customers were found to ''shop around'' avidly for discounts, making the proper use of patient medication records or any other monitoring of patient progress next to impossible. Prayle and Brazier (1998) published a UK paper examining ethical aspects of deregulating medicines from prescription control to OTC. They examined the ethical implications of enhancing access from the perspective of patients, the impact on the legal and ethical responsibilities of community pharmacists and considered whose interests really benefit from deregula- tion. They concluded that beneficence becomes difficult to deliver when pharmacists, or their assistants, simply

''supply the product that customers, aided by manufac- turers' advertising agents, have determined they desire''. Hibbert et al. (2002 see above) noted that pharmacists and their staff have sought to formalise their involve- ment in the surveillance of medicine sales through the use of questioning protocols; they also document how these strategies rapidly become ineffectual with ''smart consumers''.

Such considerations are germane when considering the misuse of OTC medicines such as codeine linctus, certain proprietary cough mixtures, laxatives and anti-histamines. In Britain, the Royal Pharmaceutical Society (2002) stated that pharmacists have a professional duty to intervene and few have challenged this obligation, only how it should be discharged. Work in Northern Ireland by Hughes, McElnay, Hughes, and McKenna (1999) has attempted to quantify the nature and frequency of OTC medicine abuse, and what measures pharmacists take to limit the incidence. By far the most common strategy is to remove the offending product from display, thus necessitating a special request for its supply and the opportunity to enquire about its use, refuse it or, as the author can testify, assert that ''it is out of stock''; a strategy still apparent in research by MacFadyen, Eadie, and McGowan (2001). Not a victory for truth-telling perhaps, but a pragmatic solution, well understood by regular misusers, which often avoids a potentially confrontational situation. The researchers found that pharmacists were nevertheless willing to consider several more professional options such as referral to the general practitioner, referral to a substance misuse treatment centre or enrolment in a harm-reduction programme for such misusers. The same researchers reported on a pilot study for such options several years on (Fleming, McElnay, & Hughes, 2001). Matheson, Bond, and Pitcairn (2002) also researched the incidence of OTC abuse in Scotland and found that the pharmacist's role still seemed to be one of professional policing of OTC medicines.

A recent Scottish study (Kennedy & Moody, 2000) elaborated on factors which affect selection of OTC medicines. Finding that proprietors are more likely to be influenced by economic factors than employees, they concluded that such pressures were not excessive and might only involve the selling of a branded

product rather than an unbranded product which was never- theless appropriate for the consumer. They did not point out, however, that an employee pharmacist generally has no control over the inventory of medicines being stocked in the first place. As we have seen with participation in services to drug misusers (above), this choice is usually exercised at senior management level and, in the case of stock inventory, rarely by pharmacists.

Brown and DiFranza (1992) surveyed the incidence and attitudes of pharmacists in the USA to selling cigarettes. They found that 95% stocked tobacco products and 81% were willing to illegally sell cigarettes to underage buyers. Some were also happy to stock ''candy tobacco'' such as sugar cigarettes and display advertisements which were said to foster tobacco use among teenagers. Bentley, Branahan, McCaffrey, Gar- ner, and Smith (1998) found that around half of the respondents were still selling tobacco despite clear statements from professional bodies that such sales were incompatible with the ethics of the profession. Once again, this study highlighted the role of ''higher management'' in sustaining this position although many independent pharmacies also continued to sell tobacco, presumably of their own volition.

Discussion

This review suggests that examination of published and refereed papers alone on pharmacy ethics may provide too narrow a view of its scope and too limited an impression of the extent to which ethical issues are encountered in practice. A more comprehensive picture appears using appropriate references to articles, news features, letters and other informal publications, as we have done in this review. Some citations in this review indicated work that may have been undertaken within associations or at conferences of special interest pharmacists—in academia, in hospital practice, in palliative care, in mental health or paediatric special- ties—but had not led to full peer-reviewed papers. None of this invalidates the classifications set out in the body of this review since the intention was only to identify the scope rather than undertake an exhaus- tive examination of published discourse. Nevertheless, some areas of ''classical'' healthcare ethics such as research governance, resource allocation, and the ethical aspects of advances in pharmacogenomics do not yet figure in the published deliberations of practising pharmacists.

There is a Journal of Medical Ethics and a Journal of Nursing Ethics, but no dedicated journal for pharmacy ethics. Since little research in pharmacy has specifically targeted ethical issues then it is necessary to trawl a wide range of generalist and specialist practice research journals to obtain a picture of the scope of pharmacy ethics. One interpretation of this finding might suggest that in pharmacy, ethics is so integrated and intrinsic to daily practice that there is no need to single ethical issues out for special attention. Certainly, many citations found in the search process flagged key words such as ''consent'' in pharmacy practice research papers but did not, in fact, explore consent at all. Consent was simply a precursor to research that involved patients. Whether or not such consent was properly valid—from individuals with full capacity, information and understanding of what they were actually consenting to—was not examined. This may imply that these matters are too commonplace to require elaboration.

A less positive interpretation may be that obtaining consent is not identified as an ethical issue. As was seen in the introduction, pharmacists have traditionally been told what their ethics should be in Codes and pronouncements from their professional bodies. They have not customarily been schooled in a principled approach to ethics but rather have been provided with detailed guidance on what behaviour will be regarded as ethical in a given set of circumstances. The limitations of this approach are obvious when new practice is developed. Cribb and Barber (1998), in a discussion paper, characterised this situation as a need for greater ''value literacy'' in pharmacy. They defined value literacy as ''an awareness of, interest in and capability in identifying, discussing and handling value and ethical issues in pharmacy''. Such value literacy, they assert, is necessary for pharmacists to play a full part in addressing dilemmas in health policy, in respecting users' cultural scepticism or religious beliefs, to deal with the growth of institutional and personal accountability for healthcare and the blurring of boundaries across differing health professional roles.

If the literature on traditional healthcare ethics and pharmacy is sparse and diffuse, that covering the impact of business imperatives on ethics in community practice is positively rare. An obligation to make a profit to stay in existence seems likely to have some effect on the capacity of community pharmacists to act as indepen- dent ethical practitioners. Taylor and Carter (2002) state that of active pharmacists working in community practice, around half now work as self-employed locums. No work appears to have been undertaken to explore the differing impact on ethical behaviour amongst pharmacists who own their own businesses, those who occupy managerial positions or junior employee positions in large multiple chain pharmacies (as predominate in the UK) or those who work as a ''hired gun'' for a wide variety of businesses large or

small. Nor indeed, is there research on the role and influence of non-pharmacists in the management of ethical positions adopted in corporate pharmacy such as participation in certain services or the choice of goods tostock. Latif's work does expose the pressures of organisational values and reward systems (which them- selves often reflect a consumer culture and remuneration within a state health system) on the consistency and quality of care in community pharmacies. Latif's work is however confined to the USA with a predominantly insurance based health system; would the same levels of moral reasoning obtain in community pharmacists working in other countries within other systems? The employment trends now apparent in Britain at least (Hassell et al., 2002, cited above) suggest that research in this area might assist employers and government alike in addressing those drivers which negatively affect the motivation and quality of care provided in community pharmacists.

Although deemed outside the scope of this review of ethics in pharmacy practice, papers cited from educa- tional journals were found that attempted to extrapolate backwards, as it were, from ethical challenges in practice to inform the content and delivery of undergraduate pharmacy courses. A greater willingness by practising pharmacists to publish accounts of actual dilemmas they encounter and to invite debate on how they could or should be resolved might facilitate the teaching of a principled approach to ethics. More effort could perhaps be made by pharmacy practice researchers to be alert to and aware of the ethical issues surrounding their work, and for them to consider the educational potential of their findings in raising such awareness amongst present and future pharmacy practitioners.

Conclusion

Pharmacists in practice do encounter a number of ethical challenges. Most of these may be dealt with a limited foundation in moral philosophy and exposure to dilemmas in the course of training. Whilst it may be argued that many pharmacists appear to tackle such situations pragmatically, using prior experience and common sense, more clinically oriented practice and an increasingly competitive retailing environment may mean that ethical challenges are likely to become more daunting and more likely to be disputed. There is a need for the knowledge base in pharmacy ethics to be systematised and integrated into the wider scheme of general healthcare ethics and for deeper and more open analysis of the conflicts that arise from the commercial context of practice in community pharmacy. These tensions will rise unless community based pharmacy services become part of the state health service or limits are set to define what activities in community pharmacies are purely retail transactions requiring no additionalprofessional input.

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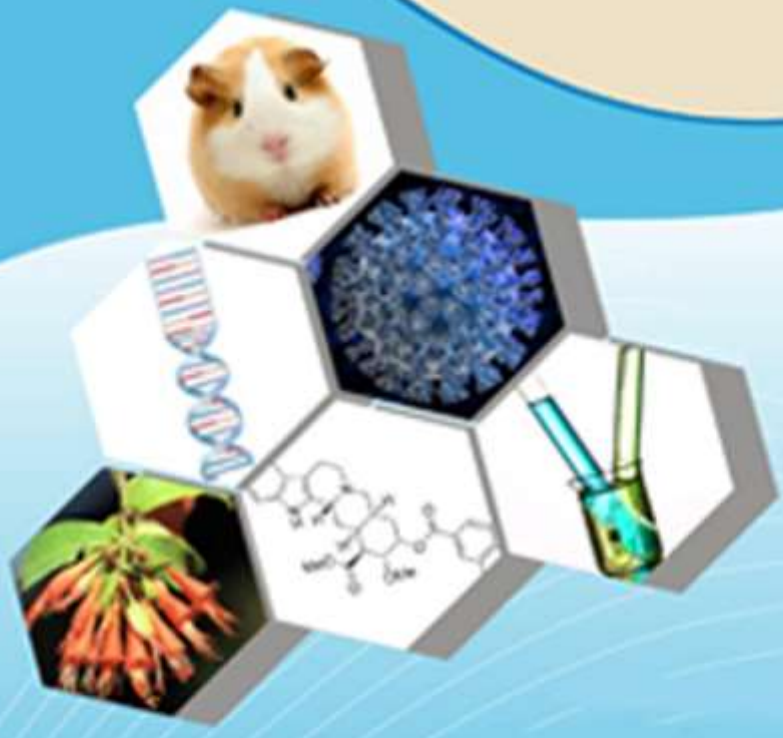

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Pharmaceutical sociology: recent developments and future directions

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Abstract

This paper takes a critical look at progress and prospects regarding the sociology of pharmaceuticals over the years. Key themes examined include: (i) medicalisation and pharmaceuticalisation; (ii) regulation; (iii) consumption and consumerism; (iv) expectations and innovation. Papers in the monograph are also introduced and discussed in relation to these themes. The paper concludes with some further comments and reflections on progress and prospects in this field, emphasising the continuing importance of sociological engagement with these personal and political issues in the 21st century.

Keywords:medicalization,pharmaceuticalization,pharmaceuticals,regulation, consumption, consumerism, expectations, innovation, pharmaceuticals

Introduction

The pharmaceutical business has become more powerful and influential over our lives as patients, customers, and citizens, and this has contributed to a recent uptick in interest in the intersection of drugs and society. With the pharmaceutical industry in the United Kingdom being the third most profitable after tourism and finance, it is no surprise that the National Health Service (NHS) in England spends over £7 billion annually on medicine, with the vast majority of that amount going toward branded (patented) products (House of Commons Health Committee 2005). In turn, these numbers are a part of the bigger picture of global pharmaceuticals sales, which are expected to grow by five to six per cent between 2007 and 2008 to more than US $735 billion a year (IMS MIDAS 2008 http:// www.imshealth.com), with sales in North America accounting for nearly half of this market and sales in North America and Europe together accounting for more than three-quarters of global pharmaceuticals sales. Additionally, pharmaceutical items and procedures are the subject of several news stories almost daily. New 'miracle medications' are constantly being heralded in the media. However, the media often repeats accounts of medication crises or controversies, increasing public anxiety while also piqueing their interest in the pharmaceutical sector. The pharmaceutical industry unquestionably contributes significantly to the reduction of human misery and the preservation of human life. However, they also spark heated debate and argument about everything from their creation and testing to their marketing and even their meaning and use.

With the aim of shedding further critical sociological light on these trends, this book serves as both a reflection of and a reaction to the recent increase of interest in the relationship between drugs and society. arguments and talks In this regard, there are several topics and concerns that, when considered as a whole, show the evolution of sociological study of medicines and its promising future.

The rise of medicine and pharmaceuticals; medical professionals, illnesses, and treatment

Pharmaceuticals' part in the increasing medicalization of society has been the first and, perhaps, longest-running sociological issue. Illich (1975), writing in the 1970s, criticized our 'over- reliance' or 'dependency' on pharmaceuticals and physicians when he discussed the iatrogenic implications of modern medicine and how the consumption of medical goods helped promote a'morbid society.' However, others who are more firmly established in medical sociology, especially in North American medical sociology, have also taken up similar topics, although in a less radical or libertarian vein than Illich. These writers have focused on the increasing scope of medical authority and control over our lives, justified by concerns about disease and wellness (Zola 1970, Freidson 1970, Conrad and Schneider 1980a,b). However, the pharmaceutical industry's position within these processes was a relatively subdued or disregarded subject in the medicalization literature from the 1970s to the 1990s, as sociological emphasis shifted to the role of medicine in the social construction of sickness and judgments about its treatment. However, new research has started to reevaluate these processes in light of recent advances in the field of medicalization of society. For instance, Conrad, in updating his earlier work in this area (Conrad 1992, Conrad and Schneider 1980a,b), has pointed to what he terms the "shifting engines" or "drivers" of medicalization over time; see also Clarke et al. (2003) for a somewhat different line or emphasis on transitions from medicalization to so-called "biomedicalization." While physicians remain at the center of the medicalization concept, Conrad contends that consumer demand, managed care markets, and advances in biotechnology (particularly the pharmaceutical sector) are the principal drivers of the medicalization process today.

Other, more critical writers (many of whom are not sociologists, which is crucial) have gone even farther, arguing that what was formerly considered medicalization is now better characterized as open "disease-mongering," in which the pharmaceutical business plays a big role. Through a series of case studies, critics like Moynihan (Moynihan 2002, Moynihan and Henry 2006, Moynihan et al. 2002) and Blech (2006) have shown that pharmaceutical companies, in collaboration or conjunction with doctors, pressure groups, and the media, are no longer just manufacturers of drugs but of diseases for these drugs to treat. – For more on "Big Pharma," read Law's (2006) study. For example, in a recent issue of the Public Library of Science - Medicine, an entire section was devoted to essays on this very topic, covering a wide variety of diseases and disorders, from attention deficit hyperactivity disorder (Phillips, 2006) to erectile dysfunction (Lexchin, 2006) and female sexual dysfunction (Tiefer, 2006) to bipolar disorder (Healy 2006). Without a doubt, it is vital to hear these comments. However, not all kinds of medicalization entail spreading false information about diseases. Similarly, not all medications or pharmaceutical procedures are required for medicalization. A value-neutral definition of medicalization would be one that only indicates the act of transforming anything into a medical concern, the efficacy of which may be investigated empirically case by case (Conrad 2007, 1992). As with many things, medicalization may have both good and bad outcomes, with some people benefiting while others suffer.

Whatever the merits of the argument against explicit illness mongering, direct-to-consumer advertising (DTCA) is a significant vehicle for the growth of pharmaceutical markets, a trend which is now only seen in nations like the United States and New Zealand. One way of looking at it is as a radical change or start again. Conversely, historical patent medication advertisements may provide as an illuminating comparison and antecedent. The study by Conrad and Leiter, for instance, provides helpful new insights on these questions. Using Lydia E. Pinkham's vegetable compounds for 'women's complaints' in the late 19th century and Levitra for 'erectile dysfunction' (ED) today as case studies, the authors draw illuminating similarities between the patent medicine period and the DTCA age. Conrad and Leiter point out that one of the ironies of DTCA is that it deepens the ties between pharmaceutical firms, doctors, and patients in ways that echo or bring us back to the situation that prevailed in Lydia Pinkham's time, when drug manufacturers had a direct and independent relationship with patients. Even though the law has reined in Pinkham's exaggerated claims, we still have to deal with the fact that advertising has evolved into a much more nuanced and sophisticated attempt to persuade or convince consumers that its products are the right ones in an increasingly competitive pharmaceutical marketplace. Conrad and Leiter argue that DTCA has made the pharmaceutical industry and consumers more central participants in medicalization.

Of course, as Conrad and Leiter's study on DTCA shows, the media also plays a significant role in these medicalizing processes. Sociological research has shown, for example, that media coverage of pharmaceuticals may range from celebratory to critical, depending on the medium, the specific medicine in issue, the degree to which the drug is novel on the market, and the significance of the story. When benzodiazepine tranquilizers were initially administered in the 1960s, for instance, they were largely hailed as starting a new therapeutic age and were met with widespread enthusiasm in the UK and US media. However, as their fame rose, the therapeutic benefits they provided were no longer noteworthy, and a more critical coverage emerged, feeding off the opinions of a small but expanding band of professional and lay critics. Users were portrayed in the local and national UK press as innocent victims, through no fault of their own, who then tried to withdraw and embark on a "return journey" to normalcy, a narrative that began in the 1970s with claims about their overuse as a "chemical crutch" for personal problems and shifted to claims about these drugs' "addictive" potential in the 1980s and 1990s (Gabe and Bury 1996a, 1996b) (Gabe et al. 1991). Furthermore, via various modes of mediation and marketing, drugs may begin to take on identities of their own, attaining a quasi-mythic or celebrity status in the public imagination, perceived or manufactured as the archetypal hero or villain (see for example Martin 2007, Nelkin 1995).

For instance, Williams and co-authors tackle some of these problems in their article on the press coverage of the wakefulness-inducing medication Modafinil (brand name Provigil). This article demonstrates how the print news media has constructed this drug in a variety of ways, from largely uncritical endorsement of its clinical applications as a 'breakthrough' or 'wonder drug' for a growing list of sleepiness-related conditions to somewhat more cautious or critical coverage of its wider (potential) uptake as a drug of choice for a lifestyle or in sporting or military contexts. In the form of this wakefulness-inducing drug, we once more witness the now-familiar if not-common rehearsal of concern over the blurring or shifting boundaries between "treatment" and "enhancement," as well as the broader articulation of cultural anxieties about a shift toward a 24/7 society in which sleep becomes

increasingly optional, if not obsolete. In this regard, the paper's authors' choice for the phrase

'pharmaceuticalisation' rather than'medicalisation' to capture these worries in the press; that is, concerns about the possible broad usage and adoption of pharmaceuticals for a wide variety of reasons that go much beyond medicine or the purely medical.

The Internet and cyberspace/culture are also prominent examples of medicinal mediation; for references, see authors like Miah and Rich (2008). It's not enough to only be able to look up information about drugs online; now people can actually buy drugs from e-pharmacies and talk to one another about their experiences in online support groups and chat rooms (Fox et al. 2005a,b). For better or worse, these and other pathways are emerging that may eliminate the need for the conventional doctor-patient interaction in the mediation of medications. Fox and Ward's study on the pharmaceuticalization of everyday life, for example, addresses some of these questions; as in Williams et alresearch, .'s the preference for pharmaceuticalization over medicalization is again striking. Using literature from social science, economics, and health services research, as well as their own research on pharmaceutical consumption, Fox and Ward (who are concerned with the new emphasis on lifestyle in the production, marketing, and consumption of pharmceuticals) identify two broad processes at work here. One trend is the increased use of computers to get and consume pharmaceuticals in the private sphere of the house, such as the bedroom and the kitchen. The second trend is the increasing reliance on medications for resolving a variety of mundane issues; for many people, drugs have become "magic bullets" in this regard. In this way, the biological effects of the drug on the body, the legitimacy of the problem or disorder at hand, the willingness of consumers to adopt the technology as a'solution' to a problem in their lives, and the corporate interests of the pharmaceutical industry all come together to form a complex mixture or heady brew that influences the domestication of pharmaceutical consumption and the pharmaceuticalization of life. The authors argue that the social interactions surrounding modern pharmaceutical manufacturing and consumption "bind the realm of business to the private world of people, creating new illnesses and therapies from the very fabric of everyday life."

Pharma industry, politics, and scientific regulation

While studies of medicalization and pharmaceuticalism have been important to the study of medicines and society throughout the years, studies of the science and politics of the pharmaceutical industry—including concerns of development, testing, and regulation—have also been fruitful. Abraham, for example, has been at the forefront of this research over the past 15–20 years, documenting through extensive empirical case study work and comparative analysis elements of controversy and corporate "bias" that, at the same time, demonstrate the inadequacy of existing regulatory practices and procedures, and the nee See also Busfield's (2007a) recent sociological analysis of scientific "fact making" in clinical trials of drugs and in post-approval drugs assessment and the subsequent Abraham (2007)-Busfield (2007b) debate for more information.

The House of Commons Health Committee (2005) Report on The Influence of the Pharmaceutical Industry addressed many of these concerns head-on. While it is true that pharmaceuticals have the potential to improve public health, the report is replete with criticisms of a "failing" regulatory system, "lax oversight," and "practices on the part of the pharamaceutical industry that act against the public interest," all of which are understandable given the industry's massive market share and pervasive marketing efforts. Greater transparency and independent assessment of evidence in the licensing process, enhanced mechanisms for restraints on medicines promotion, stricter restrictions and greater vigilance to guard against 'excessive' or 'inappropriate' prescribing, and a fundamental review of the Medicine and Health Care Products

Regulatory Agency (MHRA) are all on the list of recommendations.

Abraham's paper is timely because it reviews the past 20 years of sociological research on pharmaceutical development and regulation and because it reasserts the importance of a realist empirical research programme for the investigation of these issues, based on the notion of "objective interests" (i.e., the objective interests of pharma- ceutical companies in profit maximization and the objective interests of patients/public health in the optimization of drug safety and efficacy). Abraham uses worldwide comparisons of drug regulation to demonstrate how commercial interests have skewed the science of drug testing and review away from patients and the public and in favor of the corporation. He calls this phenomenon "neo-liberal corporate bias." In addition, these worldwide comparisons are helpful in showing that there is much room for improvement, rather than being a "inevitable by-product" of technological scientific advancement in medicines. In a similar vein, Abraham argues that the global spread of neo-liberal corporate bias in pharmaceutical regulation is not the inevitable price to be paid for faster development of therapeutically valuable medicines, but rather is more likely a consequence of a decline in technoscientific standards for drug safety in the EU, the US, and Japan.

Another crucial factor is the gender and sexual politics of drug research, testing, and regulation. Casper and Carpenter's work on the politics and controversy surrounding efforts to introduce the human papillomavirus (HPV) vaccination for cervical cancer in the United States of America is a prime illustration of this. This work has "animated longstanding worries about immunization... and young women's bodies and behavior," as the authors put it. In this regard, the HPV vaccine brings to mind both historical vaccination debates and present day political worries over sexual morality. The authors state that vaccines are a unique class of pharmaceuticals that draw on metaphors of transmission and confinement. In addition, pharmaceuticals generate their own histories, with political influences shaping their paths at every turn. Therefore, the most significant aspect of this narrative is not the public arguments over vaccination per se, but rather the fact that its aim is a sexually-transmitted illness, which brings into the debate much more sensitive questions of sex, gender, and women's bodies. Briefly put, these authors believe that the introduction of the HPV vaccination seems to have "inflamed" US health care politics, which has subsequently impacted marketing strategies for the medicine. Again, this highlights the very political nature of the pharmaceutical industry as a whole, as well as the battles sparked by the introduction of this new gendered technology. The global character and dynamics of the pharmaceutical sector as a whole also raise broader problems. Outsourcing or offshoring is becoming more common in the pharmaceutical sector, and there have been discussions over the 'globalization' of medication control (Abraham and Reed, 2003) and whether or not the pharmaceutical industry is genuinely 'globalised' in the first place.

sending clinical studies to countries with laxer or nonexistent regulations and a more affordable supply of human subjects, sometimes known as "pharming" (Shah 2007). The existing system of medicine production and distribution has created worldwide disparities and injustices, so it's important to examine questions such, "Who gets what, when, and where?" Especially noteworthy is the fact that essential life-saving treatments for persons in poorer regions of the globe, many of whom subsist on less than a dollar a day, are often overlooked in favor of pharmaceuticals for rich countries where chronic illnesses abound and lucrative markets beckon (Busfield 2007a, Petryna et al. 2006, Shah 2007).

Antiretroviral therapy (ART) treating HIV patients in Brazil is one apparent success story on this front, at least at first glance. Cataldo's paper, based on extensive ethnographic research in a Brazilian favela (shanty town), suggests that local definitions of illness, problems with adherence to treatment, structural violence, political alienation, and a lack of perspectives about the future all challenge or problematize the universal character of this public health programme. In addition, these shifts, which are echoed by other authors (Petryna 2002, Petryna et al. 2006, Biehl 2004, Rose 2007), indicate the emergence of fresh forms of socio-political

identification and involvement centered on the concept of "therapeutic" or "biological citizenship." In particular, they question the feasibility and long-term viability of public health policy in a "developing" or "middle income" nation, as well as the right to treatment and health care.

Medications on the market; consumerism and consumption.

Consumption and consumerism are the subject of a third body of sociological inquiry on the pharmaceutical industry. An first effort in this direction was to conduct a "social audit" of the community's prescription drug use (e.g. Dunnell and Cartwright 1972). Anti-hypertensives (Morgan 1996) and benzodi-azepine tranquilizers (Helman 1981, Gabe and Lipshitz-Phillips 1982, 1984, Gabe and Thorogood 1986) were the focus of research into the social meaning of medication in the 1980s and 1990s, with a particular emphasis on how users' ethnicity and gender shaped such meanings (Cooperstock and Lennard 1979, Gabe and Thorogood 1986, Ettorre and Riska 1995).

Users of drugs are increasingly being seen as knowledgeable and reflective actors, able to weigh the pros and drawbacks and make educated decisions in cooperation with medical experts, thanks to the rising sociological interest in consumption and consumerism (Rief, 2008). (Fox et al. 2007, Fox et al. 2005a). This kind of consumerism is then fostered by UK government policy that treats people like experts and urges doctors to have a "relationship" with them (Taylor and Bury 2007). In their study, Stevenson, Leontowitsch, and Duggan investigate these shifts by thinking about interactions between customers and pharmacists about OTC medications and how pharmacists strive to preserve their expertise in the face of rising health-care consumerism. They show that the pharmacists' efforts to involve customers in decisions about their treatment did not diminish the value of pharmaceutical expertise based on interviews with customers and pharmacists, focus groups with pharmacists, and tape recordings of consultations and observations in two pharmacies. Rather, the value of the pharmacist-customer knowledge gap was recognized by both parties. However, there were instances when clients did not value pharmacist knowledge and instead saw OTC drugs as a commodity and pharmacy transactions as no different from those at any other store. Pharmacy technicians and pharmacists

were cognizant of the fact that they were engaged in commercial activity and, as such, should be alert to the possibility of losing business if they refused to offer a product that a client had requested.

Individual consumers as informed and self-reflective participants in their own healthcare is not the exclusive focus of sociological research on consumerism. How users band together in self-help groups, patient-advocacy organizations, and health social movements has also received a lot of attention (Kelleher 2004, Brown et al. 2004). This has included examining the interactions between the pharmaceutical business and health consumer organizations, or non-profits that advocate for patients' rights. Jones's study centers on these findings. In light of the growing role of health consumer organizations in the UK policymaking process, she investigates how these organisations handle disclosure and management of ties to pharmaceutical corporations. Her research centers on assertions that businesses seek out organizations in an effort to influence or even "capture" their policymaking process. She finds that only about a quarter of organizations known to get money or in-kind assistance publicly acknowledge to this, based on evidence from group and industry websites and interviews with representatives of consumer groups, industry, and other health-care players. Jones, however, disagrees with the assumption that these organizations' policy agendas have been "captured" due to their lack of openness. Instead, she emphasizes the fact that industrial sponsorship often makes up just a tiny percentage of these organisations' revenue and that both sides have similar goals (both sides care that drugs be readily accessible). Nevertheless, she concedes that the unwillingness of policymakers to recognize consumer organizations as the genuine voice of patients in the policy process is a direct result of the lack of openness surrounding the disclosure of funds, which in turn supports criticisms of undue influence.

Expectations and innovation; pharmacogenomics, regenerative medicine and beyond . . .

Finally, cutting-edge advances in the fields of bioscience, biomedicine, and biotechnology—including pharmaceuticals—have captured the attention of sociologists as they together hint at or promise reimagined futures. For instance, Rose (2007), in his comprehensive assessment of this burgeoning area, emphasizes what he sees as the increasing "politicization" of all forms of life due to the quick speed of discoveries in bioscience, biotechnology, and biomedicine at the turn of the 21st century. Changes in the definition of what it means to be human, the emergence of new biosocial identities, groups, and forms of citizenship, and the redrawing of lines between health and sickness, treatment and augmentation are all part of this politicization of biomedicine. For example, the recent flurry of reports on cognitive enhancement agents by the Academy of Medical Sciences (2008), the British Medical Association (2007), and the Department of Health and Human Services (2008) all highlight the need for informed dialogue and debate across disciplinary boundaries and the wider public and policy-making arenas in light of the myriad of social, legal, ethical, political, and economic issues that have been raised by developments in neuroscience, including novel forms of psychopharmacological intervention or enhancement (2005). Thus, psychopharmaceuticals are rapidly assuming a more fundamental role in social regulation, compelling people to practice "constant risk management," or the "constant monitoring and evaluation of mood, emotion, and cognition, according to finer and more continuous processes of self-scrutiny" (Rose 2007: 223).

These shifts prompt us to consider the biopolitics of the future from a sociological perspective, with concerns like sociology's place in the co-construction or collaborative creation of contrasting bio-futures. As one example, recent studies in the sociology of expectations have highlighted I the dynamic role that expectations play in defining roles, attracting investors, and building mutually binding obligations; (ii) how expectations differ across different social groups (e.g. scientists, policy communities, industry, consumers, public); and (iii) how the futures they envision are 'contingent,' 'contested,' embraced, 'imagined,' including both the'retrospecting of p and

The creation of new vaccines is a glaring illustration of this phenomenon; it gives rise to expectations and apprehensions, as well as moral and political agendas that look backwards and forwards in time (for more reading, see the work by Casper and Carpenter cited above). These problems also become glaringly apparent when one considers the recent advances in the fields of pharmacogenetics and pharmacogenomics (the merging of pharmacological and genetic/genomic information to anticipate medication effects). There is a lot of hype and optimism in this area about the dawn of a new age of "personalized," "bespoke," or "tailor-made" medications, which are seen as the ideal response to the widespread occurrence of unwanted side effects and other problems with conventional, "one-size-fits-all" pharmaceuticals. While pharmacogenetics as a word of reference or organizing concept dates back to the late 1950s, significant pharmaceutical industry interest and investment in this field is considerably more recent. The proliferation of genetic testing, the "racial politics" of personalized medicine, and the potential problems of "over-segmented" (read "unprofitable") markets are just a few of the issues that have been raised in response to these developments (for helpful discussions of some of these issues, see Brown and Webster (2004) and Sneddon (2000); for an illuminating study of the politics of pharmcogenetics in relation to Alzheimer's disease, see Hedgecoe 2004). Also, it's not clear how

individualized these innovations may become in the long run. Hedgecoe (2004: 5) sarcastically compares the prospect of "personalized" medicine to buying a small, medium, or large T-shirt from GAP rather than being fitted for a sleek tailor-made Savile Row suit.

Conclusion

Taken together then, a variety of sociological agendas, both old and new, coalesce around pharmaceuticals and society, as this introduction clearly attests; issues, to repeat, which touch all our lives as patients, consumers and citizens. At a time of reinvigorated debate about the political and public faces of sociology (Burawoy 2005, Turner 2004), and the biopolitics of life itself (Rose 2007), sociological research on pharmaceuticals holds out the potential, promise or prospect of analyses which combine an appreciation of what Wright Mills (1959), in the *Sociological Imagination*, classically described as personal troubles and broader public issues of social structure. In potentially holding those in positions of power to account, moreover, and in engaging in informed dialogue and debate with its publics, sociological research on pharmaceuticals admirably demonstrates the continuing importance of the discipline to these developments, discourses and debates. The contribu- tions gathered together in this monograph, we believe, exemplify this promise and potential in an era where the power and force of pharmaceuticals to treat or enhance us, and the interests shaping their development and distribution, manufacture and marketing, look setto grow well into the 21st century.

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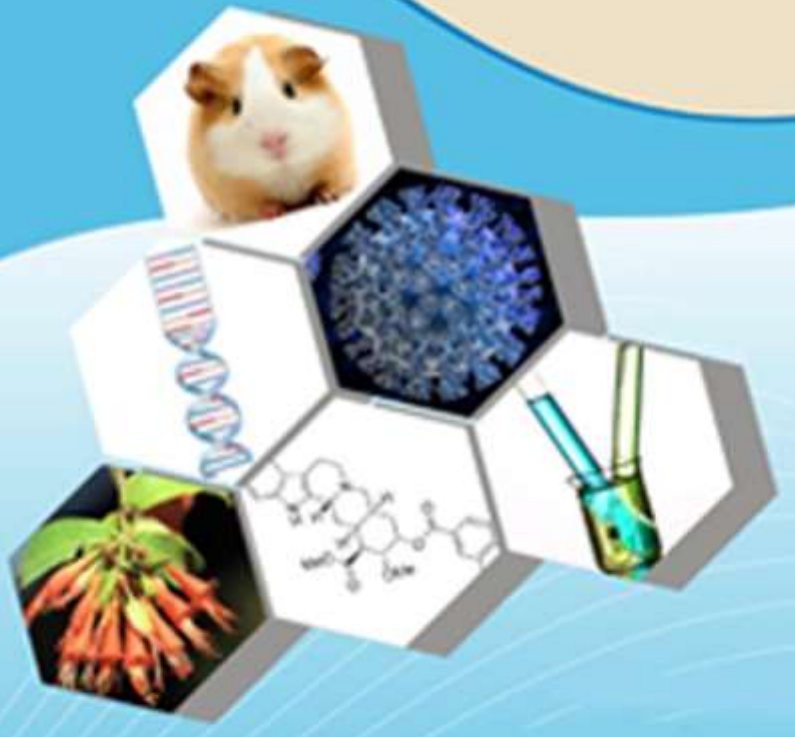

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Human medications are being studied for their potential to be transported via the environment.

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Abstract

The occurrence of 12 selected pharmaceutical compounds and pharmaceutical compound metabolites in sewage treatment works (STW) effluents and surface waters was investigated. The substances selected for the monitoring programme were identified by a risk ranking procedure to identify those substances with the greatest potential to pose a risk to the aquatic environment. STW final effluent and surface water samples were collected from Corby, Great Billing, East Hyde, Harpenden and Ryemeads STWs. Ten of the 12 pharmaceutical compounds were detected in the STW effluent samples: propranolol (100%, median = 76 ng/l), diclofenac (86%, median = 424 ng/l), ibuprofen (84%, median = 3086 ng/l), mefenamic acid (81%, median = 133 ng/l), dextropropoxyphene (74%, median = 195 ng/l), trimethoprim (65%, 70 ng/l), erythromycin (44%, < 10 ng/l), acetyl-sulfamethoxazole (33%, median = < 50 ng/l), sulfamethoxazole (9%, median = < 50 ng/l), tamoxifen (4%, median=< 10 ng/l). In the corresponding receiving streams, fewer compounds and lower concentrations were found: propranolol (87%, median = 29 ng/l), ibuprofen (69%, median = 826 ng/l), mefenamic acid (60%, median = 62 ng/l), dextropropoxyphene (53%, median = 58 ng/l), diclofenac (47%, median=< 20 ng/l), erythromycin (38%, median=< 10 ng/l), trimethoprim (38%, median = < 10 ng/l), acetyl sulfamethoxazole (38%, median=< 50 ng/l). Four human pharmaceutical compounds were detected in samples upstream of the STWs sampled: ibuprofen (57%, median = 181 ng/l), trimethoprim (36%, median < 10 ng/l), erythromycin (17%, median=< 10 ng/l), propranolol (14%, median=< 10 ng/l), suggesting that longer range stream transport of some compounds is possible.

Keywords: Pharmaceutical compounds; Environmental occurrence; Trimethoprim; Diclofenac; Sulfamethoxazole; Acetyl-sulfamethoxazole;Paracetamol; Mefenamic acid; Ibuprofen; Erythromycin; Dextropropoxyphene; Lofepramine; Tamoxifen; Propranolol

Introduction

Increased attention is currently being played to pharmaceutical substances as a class of environmen- tal contaminants (Daughton and Ternes, 1999; Küm- merer, 2001). Pharmaceutical substances are used in human and veterinary medicine and can enter the aquatic environment following manufacture, use or ingestion/excretion (see Halling Sørensen et al., 1998 for review). The majority of human pharma- ceutical compounds enter aquatic systems after ingestion and subsequent excretion in the form ofthe non-metabolised parent compounds or as metab- olites. Inputs of pharmaceutical substances into aquatic systems). Much of this work has been conducted in continental Europe and the United States with very little or no recent work being performed in the UK. The absence of data for the UK is significant since use patterns and volumes differ from country to country and occurrence data obtained in Europe may not be applicable to the UK. Occurrence data are therefore required for human pharmaceuticals in aquatic systems in the UK. In order to address this gap in knowledge the Environment Agency of England and Wales (EA) commissioned a study to conduct targeted monitor- ing of pharmaceutical compounds in STW effluents and receiving waters.

Approximately 2000 different human pharmaceu- tical substances are registered for use in the UK. Prior to conducting a targeted monitoring study it is there- fore necessary to rank substances on their relative risk, enabling those substances with the greatest potential to pose a risk to the aquatic environment to be identified. The Environment Agency conducted a ranking procedure using a combination of traditional risk assessment procedures, persistence, bioaccumu- lation and toxicity (P,B,T) data, occurrence data from other countries, whether suitable analytical methods were available and whether the compound was repre- sentative of different therapeutic classes (Thomas and Hilton, 2003). The approach adopted highlighted substances which are used in relatively large quanti- ties, but also identified substances which were either acutely toxic, persistent or bioaccumulative. The po- tential impact of substances that are not acutely toxic, but which exert other harmful impacts such as endo- crine disruption were not specifically addressed in this ranking procedure. In addition, substances which may only have a low tonnage usage, such as the contra- ceptive pill and certain anti-cancer drugs, will not have been captured by the ranking procedure adopted. As the contraceptive pill and natural female hormones are subject to detailed investigation by the Environ- ment Agency as part of an extensive research programme, it was decided therefore to focus on other general human pharmaceuticals.

pose minimal hazard to the environment. These in-cluded:

- Substances naturally occurring in the environment;
- Inorganics;
- Polymers;
- Gaseous compounds.

In addition, substances whose main use is in other sectors were removed from the list, these included:

- Veterinary medicines;
- Agricultural pesticides;
- General chemicals.

Risk characterisation ratios

Risk characterisation ratios were determined for the remaining substances on the list. Predicted Environmental Concentrations (PECs) were derived for the substances based on the approach detailed in the EU technical guidance on risk assessment (1996) (Eq. (1)). The calculation uses a simple equation based on usage, population and wastewater

1. Materials and methods

Risk prioritisation

Pharmaceutical sales data for the year 1999 for the top 500 substances (in terms of tonnage) were obtained from the British Pharmaceutical Industry- audit of purchases by retail pharmacies and dispensing doctors of registered pharmaceutical products. Sales to hospitals and sales of over-the-counter (OTC) products in to outlets such as supermarkets, garages, etc., are excluded. The data covered the whole of the UK, including Northern Ireland.

An initial screen of the substance list removed a number of classes whose compounds were believed to

production to generate the PEC_w and provides a likely 'worst case' concentration for the pharma- ceuticals in surface waters. It was assumed that there was no removal during sewage treatment and that the effluent is diluted by a factor of 10 in receiving waters.

to the lack of experimental data in the public domain on the ecotoxicity and environmental behaviour of substances, it was not possible to prioritise the sub- stances based on experimental data alone. Two approaches were therefore used to predict the no effect concentrations.

(1) A therapeutic dose approach. This method was used to provide an indication of relative potency of individual substances. This method used a simple equation using the maximum therapeutic dose/1000 to produce a $PNEC_D$.
(2) An approach using experimental ecotoxicology data and Quantitative Structure–Activity Rela-tionships (QSAR) predictions to generate a single acute ecotoxicity value for each com- pound, to which a safety factor of 100 was applied to produce a $PNEC_T$. The software used in this study was the Syracuse

Research Corporation's ECOSAR (Meylan and Howard,1998).

To enable the substances to be prioritised, a singleconcentration value was chosen to represent acutetoxicity. The value was selected from either

$$PEC_w = \frac{A \times (100 - R)}{365 \times P \times V \times D \times 100}$$

where PEC_w is the predicted concentration in sur- face water; A is the amount of substance used per year (mg year^{-1}); R is the removal rate in sewage treatment (set to 0); P is the population under consideration (i.e. England (55,000,000)); V is the volume of waste water produced per capita per day (assumed to be 150 l) and D is the dilution factor in

2.3. Prediction of no-effect concentrations (PNEC)

Toxicity data were collected from the open litera-ture on properties and effects of the substances. Due

Table 1
Risk characterisation ratios of the top 10 compounds

Substance		Therapeutic class
Lofepramine	1.99	Anti-depressant
Dextropropoxyphene	1.67	Analgesic
Procyclidine	1.60	Anti-depressant
Tramadol	0.97	Analgesic
Paracetamol	0.83	Analgesic
Clotrimazole	0.71	Antifungal
Thioridazine	0.60	Anti-depressant
Mebeverine	0.54	Gastrointestinal
Aminophylline	0.45	Respiratory
Tamoxifen	0.32	Anticancer

2.5. Results

A substance is considered to be "of potential concern" if its risk characterisation ratio is higher than 1. (EU, 1996). No substances fall into this category when using the PNECD method, while the PNECT method found that lofepramine (an antidepressant), dextropropoxyphene (an analgesic), and procyclidine (an antidepressant) all had ratios greater than one,

the predicted or experimental acute toxicity data. Thelowest value for fish, daphnids or green algae was used in the prioritisation. Very little chronic toxicity data was publicly available so for consistency acute toxicity data were used for the prioritisation of sub- stances in this study.

2.4. Risk characterisation

The potential risk of the substances to the environ-
ment was characterised by comparing the PEC_w valuewith the PNEC values (Eq. (2)).

the environment (default of 10).
Risk characterisation ratio $= \frac{PEC}{PNEC}$ or $PNEC_T$

Risk characterisation ratios were obtained for each type of PNEC ($PNEC_D$ and $PNEC_T$). The resulting ratios were then used to produce two priority lists, one based on dose, the other based on experimental data and/or QSAR predictions for ecotoxicity.

character-isation ratio of the substances; the higher of the two

$PNEC_D$) was used in the final ranking. The top 10

The final ranking was based on the risk values produced for each substance ($PNEC_T$ and

suggesting that they could be a problem in the aquatic environment.
To ensure that all relevant parameters (toxicity, use, and dosage) were included, a single ranking list was compiled using both PNEC methods.
products of this procedure are listed in Table 1).
The OSPAR dynamic selection and prioritisation mechanism for hazardous substances (DYNAMEC) (OSPAR Com- mission, 2002) criteria were used to evaluate the persistence, bioaccumulation, and toxicity (P,B,T) of the top 10 compounds and a selection of the additional substances at the top of the ranking. To be included in OSPAR DYNAMEC, a drug has to meet all three requirements. Even though none of the substances tested met the requirements for bioaccumulation, some were identified as hazardous, persistent, or both. It brought to light other drugs outside the top 10 compounds (such as fluoxetine, trimetho- prim, sulfamethoxazole, fenofibrate, and diclofenac) that might be cause for worry (Table 2).
After reviewing the available literature, we found that

few of the compounds at the top of the prioritising list had credible analytical procedures that could be employed in a monitoring plan. Surface water and/or sewage effluent samples may be collected in many circumstances by adapting procedures from other contexts.

Table 2
Summary data on P,B,T and analytical method availability

Substance	Risk characterisation ratio		Therapeutic class	Detected?	Method?	Method development possible?	OSPAR, p = persistence, t = toxicity
Lofepramine	1.99		Anti-depressant	×	×	U	
Dextropropoxyphene	1.67		Analgesic	×	×	U	U t
Procyclidine	1.60		Anti-depressant	×	×	No data	
Tramadol	0.97		Analgesic	×	×	Not easy	
Paracetamol	0.83		Analgesic	U	U		
Clotrimazole	0.71		Antifungal	×	×	No data	
Thioridazine	0.60		Anti-depressant	×	×	Not easy	U p,t
Mebeverine	0.54		Gastrointestinal	×	×	U	
Aminophylline	0.45	Antibiotic	Respiratory	723-46-6	×	No data	
Tamoxifen	0.32		Anticancer	×	×	U	
Fluoxetine	0.07		Anti-depressant	×	×	Not easy	U p,t
Trimethoprim	0.01	Analgesic	Antiinfective	1038-90-2	U		U t
Sulfamethoxazole	0.001		Antibiotic	U	U		U t
Fenofibrate1	0.04		Metabolism	U	U		U t
Diclofenac	0.01	Anti-inflammatory	Anti-inflammatory	61-68-7	U		U t
Mefenamic acid							
Ibuprofen		Analgesic		15687-27-1			
Erythromycin		Antibiotic		114-07-8			
Dextropropoxyphene		Analgesic		469-62-5			
Lofepramine		Anti-depressant		23047-25-8			
Tamoxifen		Anti-cancer		10540-29-1			
Propranolol		Antihypertensive		525-66-6			

analysed (Table 2). (Table 2). Although the top 10 chemicals (Table 1) may be priorities for a monitoring program, the lack of accessible techniques made it less likely that they would be included in the final list. Making a comprehensive list for follow-up.

The full list of compounds selected for the focused monitoring plan is shown in Table 3. After the preliminary evaluation, all of the compounds were placed towards the top of a prioritized list. Substances were selected from a wide variety of therapeutic classes; where the best option within a given class was unavailable due to a lack of an appropriate analytical technique, the second best option was selected. All of the chemicals, except for tamoxifen, dextropropoxyphene, and lofepramine, have been found in either sewerage effluent or surface waters using established analytical methods. Due to the very conservative nature of the underlying assumptions, this method should only be used to identify relative hazards; any inferences regarding the possible harm of particular drugs should be avoided.

It has been reported that ibuprofen, diclofenac, paracetamol, and proprano- lol either biodegrade or are eliminated during sewage treatment (Richardson and Bowron, 1985; Ternes, 1998; Stumpf et al., 1999). Variation in the rate of elimination is to be expected, and all substances on the list have been identified in monitoring studies. For the EA research, three substances—tamoxifen, dextropropoxyphene, and lofepramine—had new analytical procedures established (Hilton and Thomas, 2003).

After entering the body and undergoing metabolism, the vast majority of a medication is excreted as a conjugate, whereas just a small fraction is excreted unchanged. Researchers have shown that the glucoronide forms of these conjugates may quickly decompose back into the free molecules (Ternes et al., 1999). One of the first goals of the study was to attempt to measure all compounds known to metabolize the parent molecule (sulfamethoxazole, paraceta- mol, ibuprofen, diclofenac, propranolol, lofepramine, dextropropoxyphene and tamoxifen). Except for one metabolite, no reliable analytical technique could be established in time for use in the program.

Table 3
Details of the STWs chosen for this study

STW	Treatment process	Population equivalent (PE)	Trade PE	Mean flow during study (l s^{-1})
Corby	Inlets works: 6 mm screens, 4 mm drum screens, Kaldnes (Moving Fixed Film biological treatment process), intermediate settlement tanks, oxidation ditch (suspended biological treatment process), final settlement tanks and tertiary treatment (sand filter). [Ferric chloride is dosed in the ISTs and the oxidation ditch for phosphate removal. All return liquors are returned post screening]	150,000	105,000	284
Gt. Billing	Combination of filter and activated sludge plant with 40% of flows receiving biological P removal, flows are settled in conventional humus tanks, before being discharged to river. Secondary treatment only.	296,100	67,400	820
East Hyde	Oxidation ditch, final tanks then tertiary sand filters.	143,801	35,478	608
Harpenden	Primary sedimentation tanks then half of the plant is double biological filtration and the other half is alternate double filtration. Both followed by tertiary sand filters	31,905	324	138
Ryemeads	Conventional aeration, final tanks then tertiary lagoons	365,071	44,377	977

Monitoring sites

Corby, Northamptonshire, Great Billing, Northamptonshire, East Hyde, Bedfordshire, Harpenden, Hertfordshire, and Ryemeads, Hertfordshire were all selected as STWs for this investigation. Table 4 and Figure 1 offer specifics about these STWs. The pharmaceutical substances in the water samples were preserved to the greatest extent possible by collecting and transporting them back to the lab on the same day. Since the analyzing facility was positioned in the southeast of England, the study areas were limited to that region. Importantly, the local water companies in question provided their full assistance, and the locations selected had all been utilized in previous monitoring studies for trace organic chemicals. Last but not least, most of the chosen STWs were made entirely using domestic components. Online resources of this kind often include drug dosage information in the manner of widespread human consumption as opposed to concentrated industrial output.

Scheduling and data collecting examples

In 2002, samples were collected once a month in May, June, and July from each of the five locations. On each visit, three separate samples of the final effluent were collected at one-hour intervals for separate analysis. The operators of the STW submitted data showing that the first sample was collected 1 hour before peak flow, at peak flow, and 1 hour after peak flow. At each location, we also took a sample 1 kilometer upstream and 1 kilometer downstream from the discharge point. At the time of sampling, we gathered discharge flow rates to determine the total pharmaceutical load discharged by each STW. In addition to temperature and salinity, the pH of each sample was determined.

Fig. 1. Location of sewage treatment works sampled.

Additionally, water sample measurements were taken and treated as constant.

Surface water flows were expected to be low in May, June, and July of 2002, thus samples were collected at those times. Targeted dry spells were chosen because effluent dispersion via STWs would be minimal during those times, and river flows would be reduced as a result. The samples were taken during the moment of highest STW flow (information provided by the STW operators). Since dilution would be maintained to a minimum, it was assumed that the detected amounts of any substances would be a 'worst case' scenario. Heavy rain in July, however, prevented sample collection during dry times (particularly for Corby and Billing).

Depending on the effluent sam- pling sites available at each STW, samples (2.7 l) were collected immediately into a silanized, clean amber glass winchester using a stainless steel water sampler (Law et al., 1994).

Analysis

Liquid chromatography with either electrospray mass spectrometry or tandem mass spectrometry, as described by Hilton and Thomas, was used to analyze all substances (2003). After adding an internal standard, materials were extracted and concentrated using solid phase extraction (SPE), and then analyzed (13C-phenacetin). Table 5 presents an overview of the results obtained using various techniques. The techniques were unsuitable for environmental monitoring due to their poor recoveries of lofepramine and tamoxifen. Number crunching

Prior to analysis, concentration data were log-transformed, and their approximation to normality was validated visually using probability charting. For inputs that were more than lod, we substituted 0.5*lod. Due to the limited sample sizes and the sometimes high number of values lod, attempts were made to apply more sophisticated distributional approaches for gap-filling (as stated in Newman, 1995). Paracetamol and lofepramine were both tested for, and neither were found at concentrations over the detection limit.

Table 5
Performance data for analysis of selected pharmaceuticals

Compound	% Recoveries[a] (RSD)	LOD[b] (ng/l^{-1})
Sulfamethoxazole	120 (16)	50
Acetyl-sulfamethoxazole	56 (5.4)	50
Trimethoprim	123 (2.5)	10
Erythromycin	73 (30)	10
Paracetamol	75 (6.9)	50
Ibuprofen	117 (22)	20
Mefenamic acid	24 (7.9)	50
Diclofenac	62 (20)	20
Clofibric acid	83 (7.0)	50
Propranolol	45 (5.6)	10
Dextropropoxyphene	63 (3.9)	20
Lofepramine	4.2 (35)	10
Tamoxifen	42 (40)	10

[a] Calculated using: Recovery = 100(X_S — X_U)/K, where X_S = concentration measured in spiked sample, X_U = concentration measured in unspiked sample and K = known value of the spike inthe sample. n = 3. 100 ng spiked into each sample.

[b] Limit of detection (LOD) calculated using a signal to noise ratio of 10.

had not been subjected to statistical analysis. Tamoxifen and sulfame- thoxazole were also detected in very low concentrations and were thus removed from the study.
secondly, the analysis and commentary
Checking in on certain areas

Table 6 provides a summary of the incidence data collected for this investigation.

Ibuprofen
The amounts of ibuprofen found were by far the highest of any of the pharmaceutical compounds studied. median concentration of ibuprofen in STW effluents was f 3 Ag l— 1, the mean concentration was 4 Ag l— 1, and the quantities detected in receiving surface waters ranged from 0.02 to f 5 Ag l— 1. (Table 6). The highest concentration of silver found in Great Billing STW effluent was 27 g/l, but the highest concentration found downstream of the works was just 0.8 g/l (Fig. 2). A surface water sample obtained downstream of the Corby STW discharge had the highest concentration observed.

Table 6
Occurrence of selected pharmaceutical compounds in STW effluent and rivers

Compound	Sample type	Mean (ng/l^{-1})	Median	Max	Min	Frequency (%)[a]
Ibuprofen	Upstream	432	181	1555	< 20	57
	Final effluent	4201	3086	27256	< 20	84
	Downstream	1105	826	5044	< 20	69
Diclofenac	Upstream	< 20	< 20	< 20	< 20	0
	Final effluent	599	424	2349	< 20	86
	Downstream	154	0	568	< 20	47
Propranolol	Upstream	10	< 10	115	< 10	14
	Final effluent	93	76	284	16	100
	Downstream	41	29	215	< 10	87
Dextropropoxyphene	Upstream	< 20	< 20	< 20	< 20	0
	Final effluent	199	195	585	< 20	74
	Downstream	147	58	682	< 20	53
Mefenamic acid	Upstream	< 50	< 50	< 50	< 50	0
	Final effluent	273	133	1440	< 50	81
	Downstream	86	62	366	< 50	60
Erythromycin	Upstream	< 10	< 10	57	< 10	17
	Final effluent	109	< 10	1842	< 10	44
	Downstream	159	< 10	1022	< 10	38
Trimethoprim	Upstream	< 10	< 10	36	< 10	36
	Final effluent	128	70	1288	< 10	65
	Downstream	12	< 10	42	< 10	38
Acetyl-sulfamethoxazole	Upstream	< 50	< 50	< 50	< 50	0
	Final effluent	161	< 50	2235	< 50	33
	Downstream	70	< 50	239	< 50	38
Sulfamethoxazole	Upstream	< 50	< 50	< 50	< 50	0
	Final effluent	< 50	< 50	132	< 50	9
	Downstream	< 50	< 50	< 50	< 50	0
Tamoxifen	Upstream	< 10	< 10	< 10	< 10	0
	Final effluent	< 10	< 10	42	< 50	4
	Downstream	< 10	< 10	< 10	< 10	0

[a] Percentage of samples analysed where pharmaceutical was detected.

despite the fact that the ibuprofen concentration measured in Corby STW effluent was only 3 Ag l— 1, suggesting minimal degrees of dilution, a positive charge was detected. 84% of the collected wastewater samples had detectable levels of ibuprofen.

Since ibuprofen concentrations of up to 3.4 g l— 1 have been documented in STW effluents, its presence at such a high concentration is not unexpected (Ayscough et al., 2000; Ollers et al., 2001; La Farre et al., 2001; Kolpin et al., 2002). This is likely attributable to both the low degree of human metabolism and the large quantities of ibuprofen used as an anti-inflammatory and painkiller. According to research by Buser et al. (1999), the parent component of ibuprofen would be eliminated in the urine of 70% to 80% of a human therapeutic dosage.

or as metabolites; it is also believed to be very durable in aquatic systems (t1/2 = 50 days; Singer et al., 2002), however a half-life of 1 day has also been recorded (Richardson and Bowron, 1985). The STW technique has also been found to be compatible with the natural biodegradation of ibuprofen (Ayscough et al., 2000). Different sewage treatment systems have varying degrees of success, however. Activated sludge treatment was found to be more successful in removing ibuprofen (75%) than biological filtration (22%), according to data from a study of the removal of 11 pharmaceutical chemicals in a Brazilian sewage treatment works (Stumpf et al., 1999). Evidently high and detectable levels of ibuprofen were found in the STW effluents studied in this investigation.

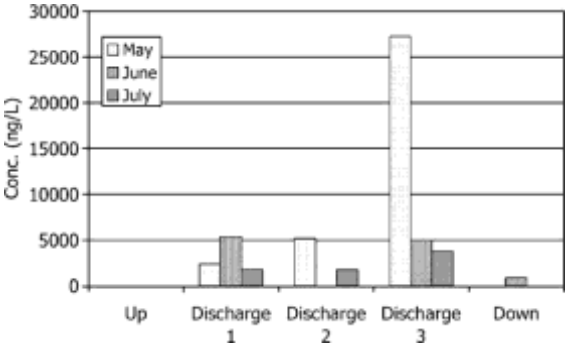


Fig. 2. Monthly concentration of ibuprofen in samples collected from Great Billing STW.

exist in surface waterways that get treated STW effluent. Diclofenac

The mean concentration of diclofenac in the collected effluents was found to be second highest (0.6 Ag l— 1; Table 6). There is a very high level of diclo-

fenac (2.3 Ag l— 1) in a sample taken from Great Billing STW; nevertheless, a low concentration of diclofenac was identified in receiving waters downstream, indicating substantial levels of dilution (as was the case with ibuprofen). Downstream of Harpenden STW, the maximum measured concentration of diclofenac in surface waters was 0.5 Ag l— 1. (Fig. 3).

The presence of diclofenac was confirmed in 86% of the samples analyzed from the collected wastewater.

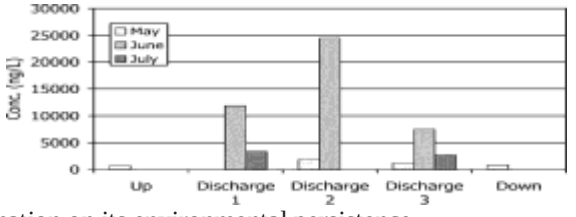
Although there was a lack of information on the overall diclo- fenac use in the UK, over a million prescription items were given in 1997. (Ayscough et al., 2000). This study's diclofenac concentrations are comparable to those found in other studies of sewage effluents (Ayscough et al., 2000; Ollers et al., 2001; La Farre et al., 2001; Ternes et al., 2003). It is well established that diclofenac sodium is removed by metabolism, with the glu- curonide and the sulfate conjugates of the metabolites being excreted intact in the urine and bile, respectively (1% of the parent drug) (EMC, 2002). It has been found that diclofenac is not persistent in the aquatic environment, has a half-life of less than one day, and is susceptible to photodegradation (t1/2 =4 h) (Buser et al., 1998; Ayscough et al., 2000).

It will deteriorate very quickly. Diclofenac concentrations are said to drop by 69% after being subjected to primary sedimentation, aeration, and phosphate removal (Ternes, 1998). Available environmental fate data imply that diclofenac is environmentally labile, which would explain why its concentration is low in samples collected upstream from STWs and why it was not detected in any samples collected downstream. Propranolol

Each and every STW effluent sample tested positive for propranolol. Effluent from Harpenden STW had an average value of 0.09 Ag l— 1 for propranolol and a maximum concentration of 0.28 Ag l— 1. (Table 6). Samples taken underneath the chosen works had propranolol concentrations between 0.01% and 0.2% Ag l— 1. Effluent propranolol concentrations were positively correlated with downstream concentrations, as measured by Pearson's Correlation (P 0.0001). Effluent samples from German sewage treatment works and surface waters show concentrations of propranolol that are consistent with these observations (Hirsch et al., 1996; Ternes et al., 2003). A total of around 12 metric tons of propranolol are used each year in the United Kingdom (Ayscough et al., 2000). Pro- pranolol is primarily metabolized in the liver and eliminated from humans through the

urine as metab- olites,

with very little unmodified propranolol (Martin- dale, 2002). There is a lack of widely accessible



information on its environmental persistence.

Fig. 3. Monthly concentration of diclofenac in samples collected from Harpenden STW.

Dextropropoxyphene

Seventy-four percent of the collected effluent samples had detectable levels of dextropropoxyphene, with a mean concentration of 0.2 Ag l— 1. (Table 6). The highest concentration of silver found in a sample was 0.59 mg Ag l— 1.

Samples taken downstream of STWs, with the maximum concentration found downstream of Corby STW, containing between 0.02 and 0.6 Ag l— 1. According to research conducted in the UK in the 1980s, dextropropoxyphene was found in several rivers at amounts of up to 1 g l— 1. (Richardson and Bowron, 1985). In this research, the average concentration of dextropropoxyphene downstream was 0.15 Ag l— 1. About 43 tons of dextro-propoxyphene are used each year in the United Kingdom (Ayscough et al., 2000). The majority of this drug is eliminated via the kidneys as metabolites (Martindale, 2002). Because of its long half-life of 1 year, any undegraded dextro-propoxyphene that makes it through the body is likely to stick around (Richardson and Bowron, 1985). Acid mefenamic

The anti-inflammatory drug mefenamic acid was found in 80% of the collected wastewater samples (Table 6). One sample of Great Billing STW's effluent reached a maximum value of 1.4 Ag l— 1, but the average concentration across all samples was just 0.27 Ag l— 1. Downstream of the STWs, the average concentration was 0.09 Ag l— 1, with a high value of 0.3 Ag l— 1 recorded from the Great Billing STW. All of the samples taken before the chosen STW discharges had concentrations of less than 0.01 g Ag l— 1. So far as we can tell, there is a lack of information on the prevalence of mefenamic acid in STW discharges. To put these numbers in context, mefenamic concentrations of f 0.01 Ag l— 1 have been observed in samples taken in Upper Austria (Ahrer et al., 2001). Little is known about the destiny of mefenamic acid in STWs and the environment, despite the fact that it is mostly eliminated in the urine as metabolites (EMC, 2002). Trimethoprim

At a mean concentration of 0.1 Ag l— 1 and a maximum con- centration of 0.1 mg l— 1, trimethoprim was the most often identified antibiotic in this study's effluent samples (65% of them).

concentration of 1,3 Ag l—1 (Table 6). Great Billing STW was the source of the highest concentration sample. Upstream of Great Billing STW, trimethoprim was found in surface waters at a maximum concentration of 0.04 Ag l— 1. However, the average concentration of silver at the water's surface was lower than the method's LOD of 0.01 Ag l— 1. Trimethoprim concentrations in effluent were positively correlated with concentrations in downstream environments (Pearson's Correlation, P 0.0001). There have been reports of trimethoprim at concentrations as high as 0.66 g l— 1 (median: 0.3 g l— 1) in German STW effluents, 0.2 g l— 1 (median: 0.02 g l— 1) in German surface waters, and 0.7 g l— 1 (median: 0.15 g l— 1) in US surface waters (Hirsch et al. These numbers are similar to what was found in this investigation. Unchanged trimethoprim is mostly eliminated in the urine (Martindale, 2002). Trimethoprim's destiny in STW is poorly understood, however its half-life in the environment has been estimated to be between 20 and 100 days (Zuccato et al., 2001). Erythromycin

Although it was discovered at higher amounts less often than trimethoprim (44%) in the samples, erythromycin was still the most common second choice (Table 6). It has been determined that the average concentration of silver in STW effluents is 0.1 mg/l, with a maximum concentration of silver in a sample taken at Rye Meads STW reaching 1.8 mg/l. Upstream and downstream surface water samples showed erythromycin concentrations as high as 1 Ag l— 1. There was a positive correlation between erythromycin concentrations in effluent and those in downstream environments (Pearson's Correlation, P 0.0001). Effluents from German STWs have been reported to contain up to 6 Ag l— 1, while surface waters in Germany and the United States have been found to have up to Ag l— 1. (Hirsch et al., 1999; Kolpin et al., 2002). According to Richardson and Bowron (1985), the average concentration of silver in UK rivers is 1 Ag l—1. The annual erythromycin consumption in the UK is 48 metric tons. Sulfamethoxazole

Acetyl-sulfa- methoxazole, a metabolite of sulfamethoxazole, was intermittently identified in the last plasma sample.

samples of wastewater collected (Table 6). There

were three times as many occurrences of acetyl-sulfamethoxazole as there were of sulfamethoxazole. The highest concentration of acetylsulfamethoxazole found in STW effluents was 2.2 micrograms per liter, whereas the highest quantity of sulfamethoxazole was just 0.1 micrograms per liter in samples taken from the Corby and East Hyde STWs. Acetylsulfamethoxazole concentrations upstream were positively correlated with those downstream (Pearson's Correlation, P 0.0001). Ternes et al. (2003) found sulfamethoxazole at a mean concentration of 0.62 Ag l— 1 in sewage discharge, and Hirsch et al. (1999) found it at a concentration of 0.03 Ag l— 1 in surface waters, while Kolpin et al. (2002) found sulfamethoxa- zole in 12.5% of surface water samples at a median concentration of 0.15 Ag l— 1. Sulfamethoxazole is mostly found as its chief metabolite acetyl-sulfame- thoxazole, and its content in UK STW effluents is lower than that seen in the US and Germany. Acetylsulfamethoxazole has not been studied in the past, thus there are no baseline statistics to compare to.

Contraceptive pill, pain reliever, and antidepressant

Tamoxifen, one of the remaining targeted pharmaceutical com- pounds, was found in STW effluent samples twice, with values of 0.02 and 0.04 Ag l— 1 in samples obtained from Corby and East Hyde STWs, respectively, but not in surface water samples. Both STW and surface water samples were negative for the presence of paracetamol and lofepramine. Tamoxifen and lofepramine have not been documented in the literature before.

Although paracetamol has been found in concentrations as high as 6 Ag l— 1, these findings should be interpreted with caution due to the fact that both the median and 90th percentile figures presented are less than 0.5 Ag l— 1. (Ternes, 1998). The findings of this investigation are supported by historical occurrence data. Further, it is well established that 95% of paracetamol is excreted as glu- curonide and sulphate conjugates, whereas the remaining 5% is excreted as the parent component (Martindale, 2002). It also breaks down quickly in nature (t1/2 = 1 day) (Richardson and Bowron, 1985). Distinct local variations

The presence and quantity of the desired medicinal ingredients showed substantial variation among the obtained samples. The detected concentrations of some, but not all chemicals, were affected by the kind of STW tested and the month in which the sample was taken. For instance, the quantities of mefenamic acid, propranolol, dextropropoxyphene, erythromycin, and trimeth- oprim in the effluent from the Great Billing STW were much greater than those found in other effluents. Although the month of the sample had a substantial influence on concentrations, no clear monthly pattern developed across all chemicals, and there was no overall significant effect of time of sample. Researchers have noticed that the concentration of chemicals in STW effluents varies from study to study (Williams et al., 2003). Studying what happens to steroid oestrogens proved fruitless since researchers were unable to link the hormones' varying concentrations to a specific direction of movement. Natural steroid inputs were thought to correspond to the (steady) number of people using the works' services, and changes in steroid concentrations were thought to reflect shifts in STW plant performance, which might play a significant role in determining the effluents' ultimate concentrations (Williams et al., 2003). Variability in the concentration of targeted pharmaceutical compounds may be under some control of plant performance, but it is anticipated that the input of pharmaceutical compounds and their metabolites into STW will be very variable. Use patterns, quantity utilized, the proximity to hospitals, and the proximity to pharmaceutical production facilities are only few of the aspects that might alter the input into a job. Thus, the finding that this was present in the samples used for the current investigation does not come as a huge surprise. Information on the usage of drugs in humans in the UK has been given by Sebastine and Wakeman (2003). Prescription data from the Department of Health was used to determine yearly drug intake on a weight basis (UK). Nine of the chemicals analyzed in this research had given tonnage information, with a significant correlation (Spearman Rank Correlation, P = 0.015) between the two data sets. However, the frequency data derived for this investigation did not show any statistically significant correlation with the total frequency data.

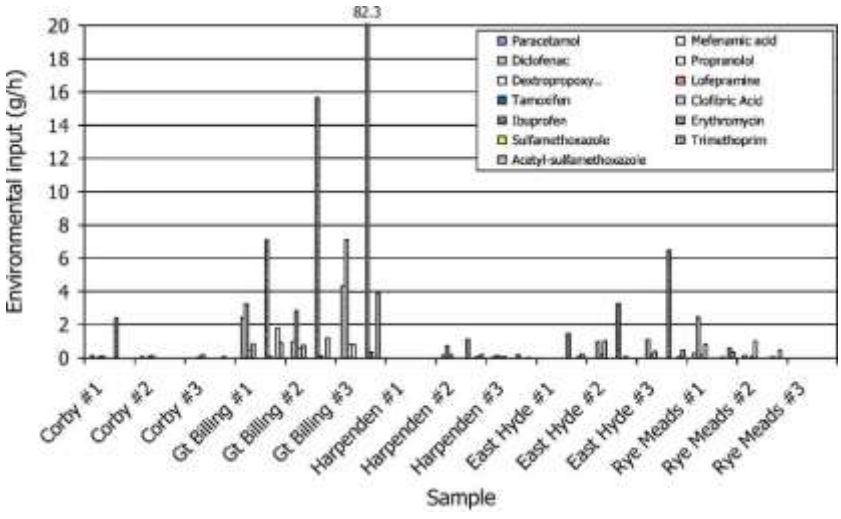


Fig. 4. Pharmaceutical compound inputs from (g h^{-1}) from five selected UK STW effluents during May 2002.

with which different substances were detected in effluent. However, there was some evidence to suggest that usage data are positively associated

with concentrations of pharmaceuticals in effluent and, particularly, with concentrations measured in surface waters below STWs.

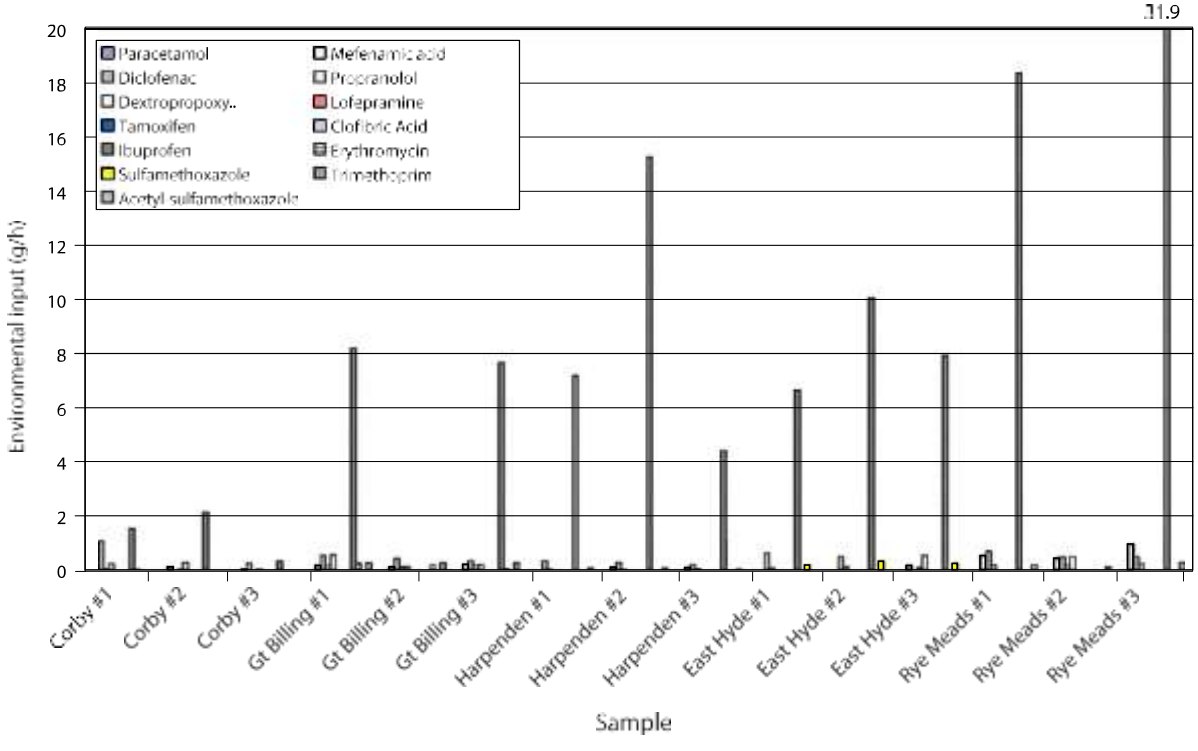


Fig. 5. Pharmaceutical compound inputs from (g h^{-1}) from five selected UK STW effluents during June 2002.

The coefficients of variation for substances ranged from 10.1% (propranolol) to 49.4% (erythromycin), showing that there is a difference in the precision of measured concentrations for different substances. In any future monitoring programme, the sampling strat- egy would need to take account of the variation reported between substances in this study. Composite sampling may have helped reduce the observed var- iability, but constraints in access to the sites and the financial implications involved did not allow for this and a pragmatic approach of using grab samples was

adopted.

Environmental load

The environmental input, in g h^{-1}, of each targeted pharmaceutical was calculated for each STW effluent sampling event using effluent flow rates supplied by the STW operators (Figs. 4–6). The data are dependent on the effluent flow rate and concentration of targeted pharmaceutical at the time of sampling. The time of sampling was around the time of peak flow of the STWs and therefore likely that they are among the highest for the sites monitored. Within the context of this study, Great Billing had both high final effluent flow rates and high concentrations of targeted pharmaceutical compounds. Therefore the highest loads were calculated to be from Great Billing STW. Similarly, the load data for Rye Meads STW are also high due to its high flow rates. Corby and Harpenden STWs provided the lowest load data, Corby STW due to the combination of low concentration of targeted pharmaceuticals determined in the final effluent and low flow rates. The loads of targeted pharmaceuticals from Harpenden STW were low due to correspondingly low flow rates.

Since the environmental load of each targeted pharmaceutical is dependent on its concentration, ibuprofen provides the most significant loads into the receiving waters. These loads are commonly in the low g h^{-1} range, while regularly reaching the order of tens of g h^{-1}. Within the context of this

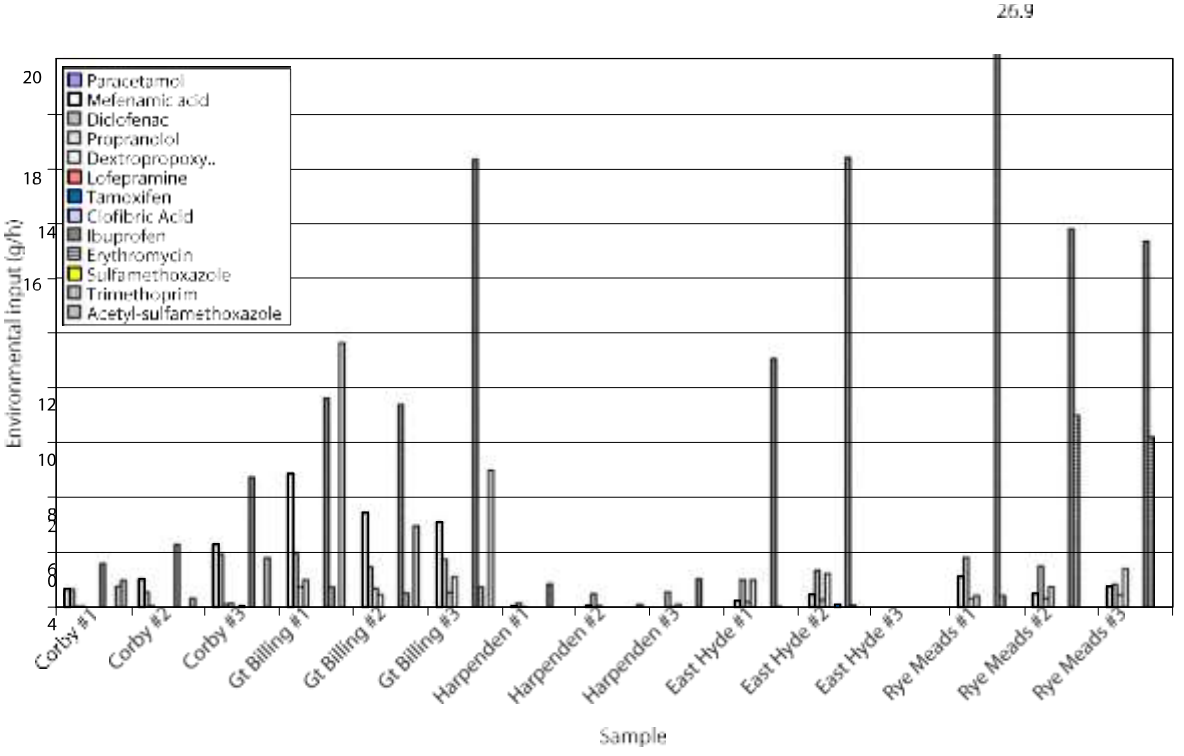


Fig. 6. Pharmaceutical compound inputs from (g h^{-1}) from five selected UK STW effluents during July 2002.

Table 7
Comparison of PECs and measured concentrations

Substance	Therapeutic class	Predicted environmentalconcentration (ng/l)	Actual concentration measured downstream(mean for five STW) (ng/l)	
			Mean	Maximum
Paracetamol	Analgesic	76400	N/d	N/d
Ibuprofen	Analgesic	10800	1105	5044
Mefenamic acid	Anti-inflammatory	677	86	366
Diclofenac	Anti-inflammatory	1090	154	568
Propranolol	Anti-hypertensive	365	41	215
Dextropropoxyphene	Analgesic	1332	147	682
Lofepramine	Anti-depressant	140	N/d	N/d
Tamoxifen	Anti-cancer	63	N/d	N/d
Erythromycin	Antibiotic	1594	159	1022
Trimethoprim	Antibiotic	289	12	42
Sulfamethoxazole	Antibiotic	40	N/d	N/d

targeted study, mefenamic acid, diclofenac and to a lesser extent dextropropoxyphene are all regularly providing a significant environmental input. However, the concentration of the target compound within the receiving watercourse, and therefore any associated biological effect, is very much dependent on the volume of the receiving waters, the degree of dilution that occurs and the environmental fate properties of the pharmaceutical. Although propranolol was detected in all of the discharge samples collected, an overall mean concentration of f 0.2 Ag l^{-1} suggests that overall input from individual effluent discharges is low in comparison to the other commonly detected target compounds. However, it would appear that propranolol is ubiquitous in the sewage discharges monitored.

PEC/PNEC comparisons

Predicted Environmental Concentrations were de- rived in the initial prioritisation exercise. In all cases the measured downstream concentrations, expressed as an average for all five sewage treatment works examined, are an order of magnitude below the PECs derived as part of the prioritisation process (Table 7). As described previously, the derivation of the PECs was precautionary, with no removal during sewage treatment or human metabolism being included in thecalculation. This could have led to the discrepancy between the PECs and the concentrations measured inthe field.

Table 8 outlines a comparison of the PNECs derived during the initial screening exercise and the

Table 8
Comparison of PNECs and measured concentrations

Substance	PNEC (ng/l)	Ratio (mean measured concentration/PNEC)	Actual concentration measured downstream (mean for five STW) (ng/l)	
			Mean	Maximum
Paracetamol	92,000	N/d	N/d	N/d
Ibuprofen	115,000	0.01	1105	5044
Mefenamic acid	15,000	0.006	86	366
Diclofenac	99,090	0.002	154	568
Propranolol	23,520	0.002	41	215
Dextropropoxyphene	800	0.18	147	682
Lofepramine	70	N/d	N/d	N/d
Tamoxifen	200	N/d	N/d	N/d
Erythromycin	78,000	0.002	159	1022
Trimethoprim	26,264	0.0005	12	42
Sulfamethoxazole	45,000	N/d	N/d	N/d

concentrations measured at the study sites. The ma- jority of concentrations reported are all significantly lower than the PNEC, often several orders of magni- tude lower. The maximum concentration reported downstream for dextropropoxyphene is close to the PNEC derived for this substance and the mean con- centration is also within the same order of magnitude. With the exception of paracetamol and ibuprofen, all the derived PNECs were based on predicted traditional toxicity end-point data, whereas the mode of action of these substances may be significantly different and the chronic effects have not been sufficiently taken into account. There is therefore some uncertainty associated with these values.

As the risk prioritisation approach used in this study was precautionary, it is likely that the substances with a risk characterisation ratio greater than
1 could be further reduced using additional information. Human metabolism of substances would provide information on the species of compound being excreted, i.e. parent compound, metabolite or bound conjugate. Improved usage data would pro- vide a better indication of the actual quantities of different compounds being used in the UK. At present, sales to hospitals and sales of over-the-counter (OTC) products in to outlets such as supermarkets, garages, etc., are excluded. These omissions could represent a significant input that at present is not being considered.

Removal/degradation data within STWs will pro- vide an indication of the substances likely to be in the final effluent and thus reaching receiving waters. Recently reported data indicate that many pharma- ceuticals are broken down during treatment and the efficiency of removal can be increased by using ozonation (Ternes et al., 2003). The fate of a range of pharmaceutical compounds in STWs has been modelled by Jones et al. (2002). This information will enable monitoring and management actions to be more effectively targeted at the media of concern,
i.e. waste water or sewage sludge.

With the exception of oestrogenic compounds, there is little peer-reviewed data on the aquatic toxicity of human pharmaceutical compounds. In addition, the data that is available rarely considers an end point with a relevant mode of action. An increasing number of studies are, however, being reported for a limited number of substances (Brooks
et al., 2003; Pascoe et al., 2003). This data will help to assess the significance of concentrations measured in monitoring programmes, but consideration of the endpoints utilised in such studies needs to be addressed to increase the relevance of the data reported.

2. Conclusions

- Environmental occurrence data has been obtained for 11 pharmaceutical (or pharmaceutical metabo- lite) compounds in samples collected from STW final effluent discharges and receiving waters at five UK STWs.
- The results indicate that a range of pharmaceutical
compounds from different therapeutic classes is present in both STW effluents and receiving waters in England. The values reported are within the same range as those reported in Europe and the US where more extensive monitoring has beenconducted.
- Ten pharmaceutical compounds, ibuprofen, mefe-
namic acid, diclofenac, propranolol, dextropropox- yphene, erythromycin, trimethoprim, tamoxifen, sulfamethoxazole and acetyl-sulfamethoxazole were detected in STW final effluent samples.
- Eight pharmaceutical compounds, ibuprofen, mefe-
namic acid, diclofenac, propranolol, dextropropox- yphene, erythromycin, trimethoprim, and acetyl- sulfamethoxazole were detected in receiving sur- face water samples.
- Paracetamol and lofepramine were not detected in
any of the effluent or receiving water samples collected.
- The anti-inflammatory pharmaceutical ibuprofen
was consistently found to be present in the effluent samples collected at the highest median concen- tration (1.3 $\mu g\ l^{-1}$).
- Environmental input data showed that significant
amounts of the targeted pharmaceutical compounds are entering UK surface waters from STW effluentdischarges.
- There was some evidence to suggest that usage
data are positively associated with concentrations of pharmaceuticals in effluent and, particularly, with concentrations measured in surface watersbelow STWs.

- The occurrence of some pharmaceuticals in upstream samples suggest that longer-range trans- port is possible for some compounds.
- The risk prioritisation procedure adopted in this
  study was precautionary and could be refined with additional data concerning human metabolism of substances, fate in STW and more inclusive usage data.
- The paucity of suitable effects data with which to
  put monitoring data into context prevents the risk of these substances to organisms exposed to low level concentrations over long periods of time to beassessed.

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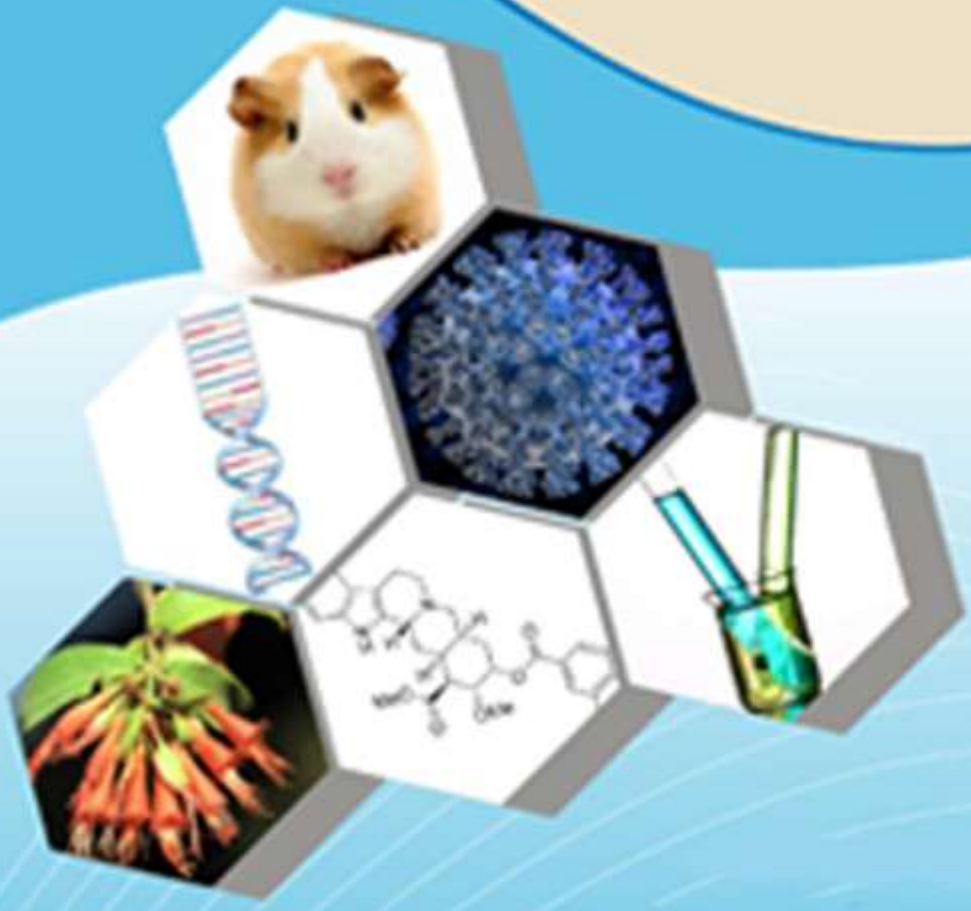

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Knowledge, attitudes, and practices of oncology health professionals on complementary medicines

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Abstract

Context: About 50% of cancer patients use some kind of CAM, raising worries about possible drug interactions with standard cancer therapy. This research aimed to investigate oncology staff members' CAM knowledge, attitudes, and behaviors in order to better understand how they may contribute to the safe use of these therapies.

The purpose of this research was to evaluate the CAM knowledge, attitudes, and behaviors among oncology professionals in Australia.

MethodsThree national oncology professional organizations' members were surveyed through online questionnaire to assess their familiarity with, and comfort with using, complementary and alternative medicine (CAM). Nine physicians, seventy nurses, and twenty pharmacists responded to the survey, for a total of 99 completed forms. Sixty-eight point four percent of those polled felt unprepared to answer patients' inquiries about CAMs because of a lack of expertise about the topic. Respondents, on the whole, agreed that CAMs play a supplementary function in oncology, however they voiced certain safety concerns. The respondents said that fewer than 40% of their patients would be open to discussing complementary and alternative medicine (CAM), with the absence of scientific evidence and guidelines for CAM usage being major hurdles to such talks. Our research reveals that a lack of awareness of CAMs among cancer health professionals may cause them to be less confident when advising patients and raises concerns about patient safety. This affects the way they talk about CAMs with patients and may explain why some people don't tell their doctors they use CAMs. Education on CAMs in oncology would help raise clinicians' confidence in addressing these treatments, leading to more patient disclosure of CAMs and safer treatment decision making for persons with cancer.

Keywords: knowledge, attitudes, practices, complementary and alternative medicine, oncology.

INTRODUCTION

The number of cancer patients who turn to CAM (complementary and alternative medicine) has increased during the last several decades. Twenty-five percent of persons with cancer undergoing treatment employed these methods prior to the 1990s. 1 Among the previous decade, the use of CAM has climbed to an average of 51% of patients2, with usage being more prevalent in people with cancer than the general population. 3

Given its prevalence, there is cause for worry over the safety of standard anticancer therapy. Antiplatelet activity observed in garlic and turmeric4 are examples of biologically based CAMs that might impact bleeding risk, and they may also modify the pharmacokinetics of chemotherapy. agents. In the second scenario, the therapeutic efficacy may be diminished, and the risk of adverse effects and toxicity may rise. 5,6 In addition, over half of cancer patients are not revealing CAM usage to their physicians due to the doctors' apparent lack of interest, understanding, or permission. 7

Considering their prevalence, it is important to learn how oncology doctors now view CAMs. Two systematic reviews of oncologists' and nurses' (or nurses') CAM knowledge, attitudes, and practices (KAP) have been published so far8 and 9, respectively. In most cases, doctors and nurses were found to be woefully uninformed about CAMs. Pharmacists were found to be more neutral, while nurses were found to be more encouraging of their patients' use of CAMs, while oncologists and other physicians were shown to be more likely to oppose CAM Process of integrating complementary and alternative medicine (CAM) into standard care.

8 Both evaluations, however, noted that heterogeneity in KAP study designs prevented them from drawing any firm conclusions. 8,9

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In addition, previous studies conducted over the last decade have either examined the KAPs of many professions collectively (as in study 10) or have zeroed in on the KAPs of a single profession.

8,11,12 Only one research was found that compared the KAPs of various health professionals; Stub et al.13 examined the KAPs of Norwegian doctors, nurses, and CAM practitioners with regards to CAM usage in cancer; this study was published in 2018. The results of this research indicate that medical professionals who have not had formal CAM training are wary of their patients using CAMs and are reluctant to have conversations with them about the topic. This contradicts the findings of complementary and alternative medicine (CAM) practitioners and health professionals with formal training in CAM treatments13, demonstrating that CAM expertise may influence CAM-related attitudes and behaviors.

This research seeks to be the first of its kind to examine the KAPs of oncology physicians, nurses, and pharmacists in Australia in regards to CAM use by cancer patients.

METHOD

Individuals in the Sample

In Australia, oncology professionals made up the bulk of this study's sample cohort. Three groups in particular were involved: the Clinical Oncology Society of Australia (COSA), the Cancer Nurses Society of Australia (CNSA), and the Oncology and Haematology Interest Group of the Society of Hospital Pharmacists of Australia (SHPA) (SHPA). There are a total of 2923 members of the COSA14 and CNSA15, as reported in their yearly reports and corroborated by SHPA. All active oncology physicians, nurses, and pharmacists who are members of relevant professional organizations were eligible to take part. No restrictions were placed on participants based on their level of oncology expertise or number of years in the field.

Study Tool Design

The definition of CAMs used in this study is taken from the National Centre for Complementary and Integrative Health (NCCIH). They classify CAMs into three

natural products, which includes herbs and vitamins, and mind and body prac- tices, which includes physical therapies and mindfulness techniques like meditation and yoga; and other com- plementary health approaches, which comprises tradi- tional health systems and those not in the other categories, such as Ayurveda, traditional Chinese medi- cine, and homeopathy. 16

An online questionnaire was used to gather information on people's beliefs, expectations, and experiences with CAM for cancer treatment. With their permission, we predominantly used the survey produced by Lee et al.17 in their 2014 work that studied the KAPs of American oncologists on herbal supplements in oncology. The first part of the questionnaire consisted of 10 multiple-choice questions designed to test respondents' familiarity with the topic of complementary and alternative medicine (CAM) interactions with cancer medicines and CAM indications in oncology.

The second part of the survey measured participants' opinions by having them rate how much they agreed with statements on the use of CAM in oncology on a five-point Likert scale. As part of the survey, participants were asked to rate how crucial it was to bring up patient and treatment-related aspects while discussing CAMs with patients.

Practitioners were asked what proportion of their patients they thought were using CAMs, what proportion of their patients they had discussed CAMs with, and what proportion of those conversations they had started in the final phase of the questionnaire analyzing practices. In addition, respondents were asked to share their experiences with supporting patients' use of CAMs and to name any obstacles they've encountered when bringing up the topic with their patients. While using a Likert scale, we choose the most common response to represent the population's norm when answering the question.

The respondents were questioned in Section 4 whether they had any CAM training throughout their undergraduate studies. After that, they were asked about their demographics, including their age, gender, greatest level of education, and current occupation. Twenty-one medical professionals at Townsville University Hospital were used in a pilot study to examine the questionnaire for clarity and accuracy. The results from the trial run were discarded before the full analysis was performed.

The use of SurveyMonkey for the dissemination of the questionnaires was a convenient and reliable method (Momentive, Waterford, NY, USA). A survey was sent out to members of the COSA and CNSA via their designated survey administrators, and members of the SHPA Oncology and Haematology Inter- est Group were able to participate in the survey using the group's online forum.

users are presented with a URL and encouraged to take part in the study. This was a link to a website that provided background on the study and survey. Participants who gave their informed consent were sent to a web-based survey. Two months later, we remembered everyone in each group by email. Between February 2021 and October 2021, you might fill out this survey.

Statistics and Data Collection

In order to conduct statistical analysis, data from the SurveyMonkey website were downloaded into a Microsoft Excel (Microsoft Corporation, Red- mond, WA, USA) spreadsheet and then imported into SPSS Version 25 (IBM Corp, Armonk, NY, USA). When comparing the overall replies to those of physicians, nurses, and pharmacists, we used chi-square tests and independent t-tests to establish statistical significance.

RESULTS

A total of 127 persons gave their permission to be studied during recruiting. Seventeen people didn't bother to answer any of the questions. The remaining 11 respondents either did not complete the questionnaire or did not provide their occupation. The final tally of questionnaires analyzed was 99. This would enable results to be reported with a 95% confidence level and a 9.68% margin of error, based on the total memberships of the professional organizations that were surveyed.

There were nine medical physicians, seventy nurses, and twenty pharmacists in this group. Table 1 displays the results of our demographic survey. The majority of respondents were women with advanced degrees in the medical field. Doctors had a more equitable gender split than other medical professionals (55.6% male, 44.4% female; p 0.001 vs. 2.9% male, 97.1% female in the nursing sector).

Knowledge

Ten multiple-choice questions were used to test students' familiarity with the concepts of CAM interactions with conventional cancer therapy and CAM indications in cancer care. The majority of responders had scores between 3 and 4. Separated by occupation, the average score for physicians was 4.6, the average score for nurses was 3.4, and the average score for pharmacists was 5.8. It was revealed that pharmacists had a significantly higher score than nurses (p 0.001, data not provided).

Attitudes

Responses to statements on the use of CAMs in oncology were used to gauge respondents' opinions, as shown in Figure 1. The majority of respondents (68.7%) did not think that their cultural or religious views influenced their perspective on CAMs, and the majority (74.4%), did not accept that CAMs had anticancer qualities. Nearly seventy-two percent of those polled didn't think they knew enough about CAMs to correctly answer questions regarding them. The majority of people (58.6%) agreed that CAMs might assist with side effects of cancer therapy, and the majority (71.8%) and the majority (63.6%) agreed that CAMs had favorable benefits on psychological (71.8%) and physical (63.6%) symptoms. Eighty-four percent of respondents said they would back a patient's use of CAMs if none better were available, but more than three-quarters (78.8%) agreed that patients spend too much money on CAMs. As a final point, 92.9% of those who took the survey expressed worry about possible negative interactions between CAMs and anticancer therapies.

A higher percentage of pharmacists (75% vs. 20.2% & 22.0%, respectively, p 0.001 & p = 0.004) reported feeling confident in their ability to answer patients' inquiries about CAMs. Nurses were more optimistic than pharmacists (71.4 percentage points) regarding the efficacy of CAMs in reducing adverse effects.

Table 1 Participant demographic data

	Frequency (%)	Doctors, frequency (%)	Nurses, frequency (%)	Pharmacists, frequency (%)
Gender				
Male	14 (14.1%)	5 (55.6%)	2 (2.9%)	7 (35%)
Female	84 (84.8%)	4 (44.4%)	68 (97.1%)	12 (60%)
Prefer not to say	1 (1%)	0	0	1 (5%)
Highest education level				
Health-related Postgraduate degree	48 (48.5%)	4 (44.4%)	34 (48.6%)	10 (50%)
Research-related postgraduate degree	8 (8.1%)	3 (33.3%)	4 (5.7%)	1 (5%)
Graduate diploma and graduate certificate	27 (27.3%)	0	24 (34.3%)	3 (15%)
Bachelor's degree	16 (16.2%)	2 (22.2%)	8 (11.4%)	6 (30%)

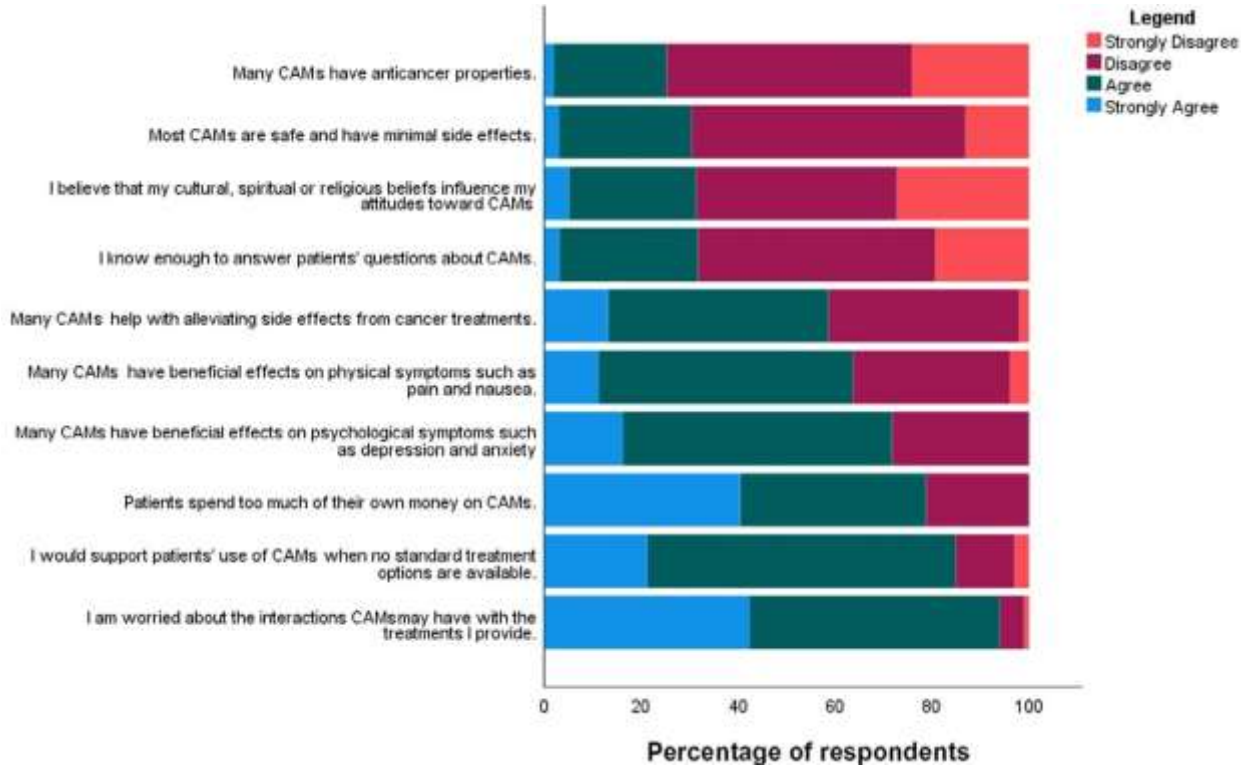

Figure 1 Responses to attitude-related statements.

nurses vs 20% of pharmacists, p 0.001), alleviating psychological symptoms (78.5% versus 40% of pharmacists, p 0.001), and relieving physical problems (76.3 versus 25% of pharmacists, p 0.001).

Respondents were then asked to indicate the relevance of several aspects when discussing CAMs with patients in the last half of this section. All respondents ranked the safety of CAMs as either "most essential" or "very important," making it clear that this is the issue that stands out most. Patients' preferences (93.4%), clinical experience (85.7%), and CAMs' efficacy (84.9%) followed in order of importance.

Table 2 Self-estimated practice patterns of respondents

	Mean	Standard deviation
In the past 12 months what is the percentage of your patients or customers with a diagnosis of cancer that currently use CAM? (98 respondents)	41.8	20.6
In the past 12 months, with approximately what percentage of your patients or customers with a diagnosis of cancer have you discussed the topic of CAM? (97 respondents)	40.6	26.8
Please estimate what percentage of these discussions about CAM were initiated by you (90 respondents)	35.9	31.0

Practices

First, we looked at how many patients respondents said they had discussed CAMs with and how many patients they estimated really used them (summarized in Table 2). There was a correlation between the number of patients that doctors thought were using CAMs and the number of patients that they discussed CAMs with. Only about a third of the time did medical professionals even start the conversation about complementary and alternative medicine.

Respondents stated they would reply as follows during CAM talks with patients:

would be most inclined to support their patients' CAM use (82.8% would often or sometimes support) than dis- courage (70.4%) or remain neutral (63.9%). Recommend- ing CAM was close

to evenly divided among all health professionals (52.5% would often or sometimes recom- mend,

KAP on CAM by oncology professionals

Table 3 Barriers to discussion of CAM use with people with cancer by all health professionals

	Frequency	Percentage
Do not believe in CAMs	14	14.3%
Limited time during consultation	32	32.7%
No interest in using CAMs	12	12.2%
Lack of scientific data on safety and efficacy	78	79.6%
Lack of professional/hospital guidelines	63	64.3%
Other	18	18.4%

As can be seen in Table 3, there are certain medical professionals that feel uncomfortable bringing up complementary and alternative medicine (CAM) with their patients. Lack of professional or hospital guidelines (64%) and a lack of scientific evidence on safety and efficacy (79.6%) were the two most common obstacles.

Education

Almost three quarters of participants (71.7% to be exact) said that they had not gotten any knowledge on CAM in their undergraduate degree. There was a statistically significant difference between pharmacists (65%) and nurses (18.6%, p 0.001, data not shown) in terms of the percentage of respondents who reported having CAM education.

DISCUSSION

This research surveyed oncology professionals in Australia to ascertain their familiarity with complementary and alternative medicine (CAM) and their perspectives on its role in cancer treatment. Overall, health care providers scored below 50% on the knowledge evaluation and were unprepared to address patients' inquiries concerning CAMs. The results of the survey indicated that respondents thought CAMs may complement conventional therapy. They did, however, express worries regarding the security of CAMs. Most health professionals claimed they were likely to support their patients' use of CAMs, but less than half said they had brought the topic up with their patients. Lack of scientific proof regarding safety and efficacy was seen as the greatest barrier to discussing these medicines with

in for cancer nurses to have conversations about CAMs with their patients. Our recommendations are supported by their observation that inexperience leads to a cautious approach to CAM usage because of the possibility of unintended consequences.

We also discovered that our respondents had conservative views on the prevalence of CAMs, with an

data not shown).

patients.

consistent with the study's other results, which showed that pharmacists were more likely to obtain training in CAMs as part of their undergraduate curriculum than nurses were. These findings point to the critical need of CAM training for practitioners.

In a survey of American oncologists, Lee et al.17 reported that when asked about herbal supplements' potential interactions with standard cancer therapy, doctors gave an average score of 1.8 out of 4 (45%). With our average of the physicians' responses at 46%, this is in line with their findings. In contrast to our finding of an average of 58% from pharmacists, Harnett et al.18, who surveyed Australian community and hospital pharmacists about their KAPs toward CAM and cancer, found an average score of 10 out of 16 (63%). The time difference in issue may explain this mismatch. The absence of standardization in questionnaires and the style of knowledge evaluation (self- assessment vs. testing) hinders the comparability of research, as was shown in the systematic KAP review of health professionals. 8

The Effect of Information on Opinions

More over two-thirds of respondents felt they did not know enough about CAMs to address their patients' inquiries, despite the knowledge ratings. This indicates that most medical professionals feel unprepared to discuss complementary and alternative medicine with their patients.

Respondents' concerns about the safety of CAM usage in cancer were evident, and this may have an effect on professionals' perspectives. Concern regarding interactions was shared by over 90% of respondents, and 69.7% of those polled did not feel that CAMs are safe. When asked about CAMs, most respondents agreed that safety comes first. Finally, the most often identified hurdles to CAM conversations were a lack of evidence on safety and efficacy. It was also found in the health professions evaluation that medical professionals were worried about the side effects and interactions that may occur while using CAM treatments alongside traditional medicine. 8 These results suggest that future training efforts should concentrate on ensuring that professionals in these fields are adequately educated about the safety of CAMs for persons with cancer. Furthermore, it implies causality with the knowledge section results. Most respondents felt unprepared to address CAMs with their patients, which may translate to a more cautious outlook on the topic of safety. The qualitative interviews with oncologists and patients that Broom et al.19 conducted

average estimate of CAM use among their patients of 41.8%. When comparing the three professions, this was greater than the estimated range of 25%-40% of patients by physicians. 8 A 2019 systematic analysis, however, indicated that CAMs were used by an average of 51% of cancer patients worldwide during the preceding decade. 2 This shows that medical practitioners often underestimate the prevalence of CAM use among their patients.

When comparing pharmacists and nurses on their perspectives on CAM's role in oncol- ogy, the former are more likely to agree that CAMs are useful in treating the side effects of cancer therapy and the physical and psychological symptoms of cancer. This is consistent with the findings from the review of health professionals, which found that nurses generally supported the use of CAMs in the treatment of symptoms and side effects, while half of doctors supported CAM use as a complementary to conventional treatment and pharmacists were neutral on the use of CAMs for symptomatic relief and improvement in quality of life. 8 A majority of our sample of oncologists had mixed feelings about CAMs' place in the field, although they were mostly favorable about their potential psychological benefits. These results are consistent with those found by a study of Italian hospital professionals conducted by Beretta et al.11. Half of the oncologists polled thought CAMs may be useful in cancer treatment. In contrast, a study conducted by Yang et al.12 among Chinese oncologists found that 95.3% of oncologists favored integrative oncology, or the use of CAMs in conjunction with traditional therapy. Note, however, that over half of the doctors who filled out the poll considered themselves integrative medicine practitioners; this might have skewed the results.

Broom and Adams19, based on interviews, corroborate our findings by suggesting that nurses are more likely to use a patient-centered, holistic approach to treatment when it comes to CAMs. Nurses were seen as more likely to support a patient's use of CAM than physicians were by respondents.

Influence on Practices

Responses to the practice section showed a general trend to be less inclined to engage with patients' CAM average of 40.6% of patients had a conversation about complementary and alternative medicine (CAM) usage. This finding is consistent with the findings of Powers-James et al.20, who surveyed American oncologists online and found that respondents discussed CAMs with 41% of patients on average. The Italian research by Berretta et al.11, on the other hand, indicated that oncologists typically discussed complementary and alternative medicine (CAM) with 49.2% of their patients. Since this was the first investigation of KAPs in Italian doctors, relating CAM usage by persons with cancer, the reason for the greater rate in the later study is not immediately evident.

When looking at all three occupations combined, an average of 36% of CAM conversations were started by respondents. This is a little more than the 25% of responding oncologists who initiated conversations about CAMs reported by Powers-James et al.20. This indicates that cancer healthcare providers may be hesitant to initiate conversations on CAMs, instead expecting patients to bring up the topic first. Moreover, this supports the findings of a comprehensive review of communication of complementary and alternative medicine (CAM) usage in cancer treatment, which found that a primary reason patients did not disclose CAMs to their health providers was because they had not been asked. 7

A majority of physicians (63%) and a large majority of nurses (93%), according to the assessment of health professionals, are willing to assist patients who desire to utilize CAMs.

8 Our findings are consistent with this range, with 82.8% of respondents expressing support for CAM usage on a regular or occasional basis. Nonetheless, it is worth noting that the second most common behavior among our questioned health professionals would be to discourage or stay neutral towards mixing CAMs with conventional treatment, suggesting that reactions made by health professionals might be extremely diverse. This is further shown by the almost even splintering of opinion across respondents about their own recommendation of CAM treatments. While 57.6% of oncologists would prescribe CAMs, the assessment of health professionals indicated that just a small percentage of MDs and RNs would make such a recommendation to their patients. 8 It's also worth noting that 84.8% of those who participated in our survey said they would support a patient's use of complementary and alternative medicine (CAM) if conventional therapies had failed. This wide range of responses shows that additional study is needed to characterize how medical professionals interact with cancer patients who use CAMs.

Weaknesses in the Research

The study had flaws, but then again, so does any research. There was a disproportionate lack of physicians and other medical professionals in this sample. This might have skewed the comparison between groups and affected the results for this occupation. Members were only reminded once to fill out the survey according to the policies set out by the COSA and CNSA. As a result, this may have discouraged prospective volunteers from taking part in our research..

CONCLUSION

To our knowledge, this is the first research to examine the similarities and differences between the perspectives of medical physicians, nurses, and pharmacists in regards to the use of complementary and alternative medicine (CAM) in oncology.

Our results imply that cancer health professionals' knowledge, attitudes, and behaviors toward CAMs are interconnected. Lack of confidence in addressing CAMs with patients might be related to poor knowledge or a perceived lack of awareness about CAMs. This would cause cautious attitudes toward these therapies, motivated by worries about their compatibility with standard medical care. As a consequence, practitioners may be hesitant to bring up CAMs with patients and may react differently when patients express interest in or commitment to CAMs. Filling up the gaps in CAMs'

knowledge might have a beneficial effect on people's perspectives and choices. Health practitioners' comfort level in discussing CAM usage with cancer patients might be boosted by the creation of easily available, high-quality, evidence-based material.

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The perspectives of pre-med, med, and pharm students on cross-disciplinary learning

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Abstract

Shared learning is becoming more important in health professional education throughout the world due to the widespread notion that increased cooperation and communication within and between health care teams will lead to better patient outcomes. Negative student attitudes may be the most difficult to alter, even more so than institutional roadblocks that prevent the implementation of interprofessional learning. The purpose of this research was to quantify first-year medical, nursing, and pharmacy students' pre-course sentiments regarding interprofessional education.

Desigm First-year medical, nursing, and pharmacy students at the University of Auckland were given the Readiness for Interprofessional Learning Scale (RIPLS) (University of Liverpool, Department of Health Care Education). The dissimilarities between the three categories were examined.

Settimg School of Health and Medical Sciences,

As a result, most students saw collaborative learning as a pleasant experience. Patient care and professional relationships were both considered to benefit from the development of cooperation abilities via collaborative learning. Students in the fields of nursing and pharmacy, on the other hand, were more confident that improved collaboration would result from their shared educational experiences. Although they were the least confident in their future careers, medical students believed they needed to learn more than their nursing and pharmacy counterparts.

Concluding; As the first year of medical school, students should concentrate on learning how to operate well in a team. When and how students should be exposed to information about various occupations remains an open question.

Keyrords Education, medical/*methods; education, pharmacy/*methods; education, nursing/*methods;

Introduction

More and more areas of expertise in the medical field are becoming redundant. As a result of these shifts, the traditional lines of demarcation between the different health professions have become more porous. Interprofessional learning (learning activities involving two professional groups) and multiprofessional learning are being offered as a means through which health professional education across the world is responding to this shift in health care delivery (learning activities invol- ving three or more professional groups). A quick recap from the available literature, it is generally accepted that health care providers, both in the community and in hospitals, need to work together and as a team to deliver the best care possible for their patients. For example, Parsell and Bligh state that the need to produce practitioners who are adaptable, Rexible, collaborative team workers with highly developed interpersonal skills is providing both the impetus and justification for the introduction of more shared learning opportunities, and l Davies describes the benefits of working "together" rather than "alongside" as energizing and resulting in new ways of tackling old problems. 3However, it is unclear from the data whether or not "learning together" during undergraduate education leads to better "working together" practices and/or greater patient outcomes. Even though Zwarenstein

While recent findings by & Reeves suggest that nurse-doctor teamwork may improve patient outcomes,4 it is evident that further study is required to draw firm conclusions. To yet, we have not succeeded in bridging the gap between dogma and evidence. ł Nonetheless, it is generally agreed that "shared learning" "should be introduced early in the undergraduate course and continue throughout the curriculum in sessions or topics where two or more distinct health curricula might contribute." 6

By contrasting it with "shared teaching," the phrase "shared learning" is used here. The term "shared teaching" is used to describe situations in which students from diverse academic disciplines attend the same lectures without a clearly defined educational method or predetermined set of results in mind. Economic rather than pedagogical considerations often drive the adoption of shared teaching. It's possible, however there's evidence to suggest it might backfire and fuel negative perceptions and feelings of anger. 6 Students may be mostly receptive, or "passive," in a shared-teaching environment, with little opportunity for active participation.

Interprofessional learning, on the other hand, is a method of teaching in which students from different disciplines work together to gain knowledge. The objective of this kind of education is to provide students access to information, practices, and perspectives in the workplace that they would not have access to in any other manner. This is how future health care workers are taught to navigate the challenges of a collaborative workplace. The necessity to train multidisciplinary health care teams comprised of individuals who can work well together and possess superior interpersonal skills and an appreciation for the unique contribution that each discipline provides to patient care and health outcomes is a major factor in this trend.

The University of Auckland's Faculty of Medical and Health Sciences made significant changes to the curriculum that prepares students for jobs in medicine in the year 2000, when it established two new programs: the Bachelor of Nursing and the Bachelor of Pharmacy. While there is a considerable population of students pursuing health science and scientific degrees at the University of Auckland, undergraduate education for other health professional groups is not offered there. In 2000, the Faculty had a total of around 770 first-year undergraduate students. There were 79 pre-meds, 49 aspiring nurses, and 2 hopeful pharmacists. The need to generate multiprofessional learning opportunities was recognized throughout the preparation of the revised medical curriculum and the two new programs (both of which were new to the University), but the means by which this would occur were less evident.

The various challenges of collaborative education are documented. These include issues with scheduling, disparities in student numbers among groups, varying learning and evaluation approaches, varying course durations, a lack of student commitment, and issues with planning and resources (such as a shortage of small-group space). These issues arose when we deliberated how to improve the three professions' access to multidisciplinary training. In the first semester, students take turns teaching required papers in the fundamental biophysical sciences as well as certain social science topics. Science and health majors also benefit from this kind of instruction. Since there might be as many as 800 students enrolled in a single course (taught in two sections), students are expected to participate actively in discussions and actively learn from one other. All three disciplines take a second-semester course on "Population Medicine." As a means of expanding our understanding and informing public health and healthcare policies, this course focuses on observations of communities and people. As a result of this course, participants will have the knowledge and abilities necessary to function productively and successfully in a team setting. The first-year students have not yet formed a professional identity, thus the learning goals were selected with that in mind. At this stage, students should be able to work together effectively, thus it's important for them to understand how to study as a team. 7 This is most effective in classrooms when students share and work together to attain academic and professional objectives. The students' progress will be linked with common learning goals, such as an understanding of the function of each profession.

Attitude issues have been cited as a major barrier to the development and implementation of shared learning.

8 According to Parsell and Bligh, the hardest part is getting people to change their minds, not overcoming obstacles like poor infrastructure or a lack of leadership. 9 The goal is to develop mutual understanding and knowledge of the many professions in an effort to lessen or eliminate biases that may exist between them. The first of these is concerned with the variation in attitudes between professional groups that may need exploration when considering shared learning. It is reported that a shift in attitudes is needed to make interprofessional learning effective.8 Accordingly, in order to design shared learning activities, assessing student attitudes to shared learning on entry to their courses and at other times during their programme might be useful. The Department of Health Care Education at the University of Liverpool has developed an instrument to measure students' attitudes to shared learning, the Readiness for Interprofessional Learning Scale (RIPLS).l0 At the University of Auckland it was decided to assess the readiness of medical, nursing and pharmacy students for multiprofessional shared learn- ing, prior to their undertaking shared learning activities and the Population medicine course. The assessment is to be repeated at the end of the second semester, fol- lowing the Population medicine course, and students' attitudes following their joint learning experiences will be compared. This paper reports on the attitudes of medical, nursing and pharmacy students to multipro- fessional learning at the University of Auckland as they commence their studies.

Readiness for Interprofessional LearningScale (RIPLS)

The RIPLS was developed to measure student attitudes to shared learning. It consists of l9 statements meas- uring the strengths of students' beliefs concerning shared learning. The statements in the questionnaire are based on the desired or intended positive outcomes of successful shared learning. Parsell & Bligh describe the development of the instrument, its piloting with students from eight professions, and a further study involving almost l000 students in five professions, which confirmed the content and construct validity of the scale.8,l0 They conclude that the l9-item scale, with three subscales named 'teamwork and collabor- ation', 'professional identity' and 'roles and responsi- bilities', appears to reveal a causal relationship between the variable 'readiness for shared learning' and some of the attributes needed for teamwork and collaboration, professional roles and practice, interpersonal relation- ships, personal growth and benefits to patients.l0 In the study reported here, the University of Liverpool RIPLS instrument was used to quantify attitudes towards interprofessional learning, prior to shared learning activities.

Methods

The RIPLS tool, entitled the Multiprofessiomal shared learmimg guestiommaire, was administered to first-year medical, nursing and pharmacy students within 4 weeks of the commencement of their studies at the University of Auckland. In order to make it appropriate to the University of Auckland context, and following a pilot study with second-year medical students, the definition of shared learning, included at the top of the questionnaire, was amended to read: 'Shared learning is learning interactively with other health professionalstudents'.

The data were analysed using chi-squared to test observed

differences between the groups, and the fre- quencies of responses were evaluated.

Response

Overall, l80 students returned the questionnaire. This represents a response rate of 90%, and includes 98% of first-year nursing students, 92% of first-year medical students and 83% of first year pharmacy students(Table l).

The majority of respondents were female (7l%), were studying for their first degree (8ł%) and spoke English as their first language (7l%) (Table l).

Results

Subscale 1: teamworking and collaboration

The items in the first subscale deal with the 'acquisition and effectiveness of teamworking skills and…the need for positive relationships between professionals and other health care students'.[8] The majority of students at the University of Auckland responded positively to the

There was strong agreement with the statements comprising this subscale. Over two-thirds of students agreed or strongly agreed with all three propositions,

Male	3ł	44	2	4	l6	3l
Female	44	ł6	47	96	36	69
Current study is first degree	70	89	36	73	47	90
Current study is not first degree	9	ll	l3	27	ł	l0
English first language	ł7	72	42	86	28	ł4
English not first language	2l	27	7	l4	24	46

qualification (m = l47, 82%); that 'Shared learning will help me think positively about other health care pro- fessionals' (m = l40, 78%), and that 'For small-group learning to work, students need to trust and respect each other (m = l64, 9l%).

Two significant differences between the responses were revealed: only medical students expressed disagreement with the proposition that shared learning would nine statements contained in this subscale (Table 2). The items in this subscale are clustered into two groups: 'effective teamworking' and 'relationships withother professionals'.

(a) Effective teamrorkimg

Overall, the responses to the items in this first subscale indicated that, for first-year medical, nursing and pharmacy students, shared learning is considered to enhance their effectiveness at work; and conveyed a recognition by the students of the need to share knowledge and skills as a way of understanding clinicalproblems in the workplace.

The majority of the l80 respondents agreed or strongly agreed that shared learning would make them more effective in the health care team (m = lł4, 8ł%) and that 'Patients would ultimately benefit if health care students worked together' (m = l6ł, 92%). Students indicated that the 'ability to under- stand clinical problems' would be enhanced by shared learning (m = l3ł, 7ł%). Respondents were also very positive about teamworking benefits, as the majority agreed or strongly agreed that 'Teamworking skills areessential for all health care students to learn' (m = l60, 89%), and that 'Communication skills should be learned with other health professionals' (m = l30, 72%). That 'Shared learning will help me understand my own professional limitations' was thought to be true by the majority of respondents (m = ll2, 62%)(Table 2).

However, there was a significant difference in the responses to two of the questions. Nursing students indicated more strongly than medical or pharmacy students that 'Learning with other students will help me to become a more effective member of a health care team, and medical students were least likely to consider that 'Shared learning with other health care students will increase my ability to understand clinical problems'(Table 2).

encourage them to think positively about other health care professionals, and a greater proportion of female students strongly agreed that 'For small-group learningto work, students need to trust and respect each other'.

Subscale 2: professional identity

The items in subscale 2 are based on ideas of negative and positive professional identity. These 'reRect the importance attached to the acquisition of professional identities by students as a means of defining their lives, and the power of individual professional cultures'.[8]

Negative professiomal idemtity

Respondents mostly disagreed with the three items in this section. The majority of respondents disagreed with the assertions 'I don't want to waste my time learning with other health care students' (m = l3ł, 7ł%) and 'It is not necessary for undergraduate healthcare students to learn together' (m = l03, ł7%). A smaller majority of students from all three health pro- fessional groups felt that climical problem-solvimg meed mot be learmt solely rith studemts from their orm professiom (m = 96, ł3%). There was one significant difference between the responses of the different health care professionals:

Nursing students disagreed most strongly with the statement that 'It is not necessary for undergraduate health care students to learn together'.

(a) Positive professiomal idemtity

Over two-thirds of respondents from each health profession group agreed or strongly agreed with the four items in this section. Respondents agreed that shared learning with other health care professionals would help them to communicate better with patients and other professionals (m = l4l, 78%), and that it would 'help to clarify the nature of patient problems' (m = l20, 67%). Most would 'welcome the opportunity to work on small

Tab1e 1 (Comtd.)

Subscales/statements	Student group	Strongly disagree	Disagree	Neutral	Agree	Strongly agree	Total	P
(b) Positive professiomal idemtity								
Shared learning other health care professionals will help me to communicate better with patients and other professionals	Medicine	1	1	11	44	18		
	Nursing	0	3	3	27	14		
	Pharmacy	0	2	11	28	10		
	Total	1	6	29	99	42	177	0·3
I would welcome the opportunity to work on small group projects with other health care students	Medicine	0	6	13	41	11		
	Nursing	0	0	10	28	9		
	Pharmacy	1	2	11	28	9		
	Total	1	8	34	101	33	177	0·44
Shared learning will help to clarify the nature of patient problems	Medicine	1	4	24	38	12		
	Nursing	0	0	12	26	8		
	Pharmacy	0	1	13	29	7		
	Total	1	1	49	93	27	171	0·47
Shared learning before qualification will help me to become a better team-worker	Medicine	2	3	11	41	22		
	Nursing	0	3	3	23	19		
	Pharmacy	0	1	8	29	14		
	Total	2	7	22	93	11	179	0·41
Subsca1e S: Ro1es and responsibi1ities								
The function of nurses and pharmacists is mainly to provide support for doctors	Medicine	9	21	17	17	11		
	Nursing	28	12	4	2	1		
	Pharmacy	14	22	12	3	0		
	Total	11	19	33	22	12	177	0·001
I'm not sure what my professional role will be	Medicine	13	24	17	19	6		
	Nursing	13	24	7	1	0		
	Pharmacy	7	21	13	1	2		
	Total	33	73	37	29	8	180	0·03
I have to acquire much more knowledge and skills than other health care students in the Faculty	Medicine	0	1	21	34	19		
	Nursing	3	21	19	4	0		
	Pharmacy	1	11	27	7	1		
	Total	4	41	67	41	20	177	0·001

group projects with other health care students (m = 134, 74%), and considered that 'Shared learning before qualification will help me to become a better teamworker' (m = 148, 82%).

Overall, all three groups of students are positive aboutthe benefits of shared learning.

The majority of vrecognise benefits of shared learning, that the acquisition of teamworking skills is useful for their future working lives, beneficial to the care of patients and likely to enhance professional working relationships. There is a consistency across the three subscales for all three groups. Communication is seen as an area where skills should be learned with other students. Implementation of this in our programmes is an area to be considered, where English is not the first language, in particular for pharmacy students (46%) compared with 27% of medical students and 14% of nursing students. Students acknowledge that teamworking skills are an essential component of their learning, and that learning together may improve working relationships after qualification.

In this study, the students were at the beginning of their careers and did not yet have a professional iden- tity, and the staging of multiprofessional learning and the particular learning outcomes to be achieved at dif- ferent levels are significant issues for consideration. The decision to focus on teamwork in the first year, without consideration of the different roles of each professional group, is appropriate. While the literature on teamwork and collaboration in health care has only tentative findings about the benefits which exist for health set- tings and patient outcomes, there are reports that bar- riers to teamwork include a lack of knowledge about the roles of different health professionals.ll Davies describes as important the recognition of what each professional brings that is different, which makes

Subscale 3: roles and responsibilities

The items in this scale are concerned with the idea that professional practice promotes some health professional roles, notably medical, over others. Medical students are least opposed to the idea that 'The function of nurses and pharmacists is mainly to provide support for doctors' and more medical students than nurses or pharmacists consider that they have more kmorledge amd skills to acguire im the course of their studies. Medical students are the least sure of what their professional role will be. Each of these differences was statistically sig- nificant (Table 2).

Analysis also revealed statistically significant differ- ences between the responses of male and female stu- dents: male students were less likely to disagree that the'function of nurses and pharmacists is mainly to provide support for doctors', and a greater proportion of femalerespondents than of male respondents were sure of theirprofessional role.

Discussion

The findings from this study have provided a focus for planning multiprofessional learning strategies within the Faculty of Medical and Health Sciences. While the findings allow only an exploration of attitudes and do not suggest important differences between the attitudes of the three groups, a number of issues are raised.

collaborative work more powerful than working sepa- rately: ‘It is the questions and challenges that arise from the differences that are vital’.2

The timing of learning about different professional roles is an issue to be resolved and determined for our programmes. The literature is not clear on when this should most usefully occur. Harden suggests that what matters most is that an approach is adopted which is appropriate for the phase or stage of the students’ learning.ł Other reports suggest that the timing of this aspect of multiprofessional learning should best be left to a post-basic level or when students can undertake clinical practice together.l,ll It is acknowledged that undergraduate multiprofessional learning is qualita- tively very different from that at a post-basic level; however, opportunities for small-group learning or problem-based multidisciplinary case studies can pro- vide a focus for undergraduate students to consider different professional roles.l While Pirrie et al. report that in general undergraduate students seek to develop a profession-specific knowledge and skills base,l the nursing and pharmacy students in this study were more certain about what their professional role would be than were the medical students. The situation where differ-ing roles of the professions should be considered may vary for each group, and issues are raised about how to place this when curriculum structures are quite different. Much of the literature on teamwork and collaboration focuses on relationships between doctors and nurses with little about other health professionals.ll Parsell & Bligh suggest that ‘the boundaries which delineate roles in professional practice and the role of academic training in supporting these divisions, are key issues’.8 The lit- erature supports the view of the medical students in this study: the tendency to view doctors as having pre- eminence over other health professionals. While multi-professional learning provides an opportunity for this attitude to alter, Davies suggests that nursing is no more conducive to collaborative working than medicine. ‘Both nursing and medicine need to change if a colla- borative model is to work’.2 Perceptions that the inRu- ence of ‘stereotypical attitudes’ affects collaborative working practices are identified by Pirrie et al., alongside a belief that these can be altered through multiprofes- sional learning.l The suggestion of Poldre that pro- gramme goals should include not only deliberate learning strategies and opportunities to understand different professional roles, but also encourage social interaction amongst students is a further area for consideration.ll The work of Terenzini & Pascerella supports this view.l2 They found student learning to be closely associated with non-classroom interactions with teaching staff, the nature of peer group interactions and extracurricular activities. Although the reported research and commen- tary focus in general on interprofessional and multipro- fessional learning opportunities, the value of social activities and non-classroom interaction is an area for research which should not be overlooked.

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Evaluating pharmacy high-needs criteria: a tool for identifying inpatients at risk of medication-related problems

K. Anjaneyulu, E. Honey, M Sriramachandra

Abstract

Due to the high cost of clinical pharmacy services, vulnerable patients should be given priority in healthcare systems with limited resources. For our healthcare system, this meant formulating high-needs pharmacy criteria to identify those patients who would benefit most from clinical pharmacy care. To assess the efficacy of the high-needs pharmacy criteria in identifying patients at elevated risk of medication-related poor clinical outcomes, a retrospective research was conducted on 761 patients admitted to four hospitals in metropolitan Melbourne. The medical histories of potential high-needs patients were examined. The computerized data were mined for information on patient stays, 30-day readmission rates, medication issues, and medication-related occurrences. Patients who met at least one high-needs criterion were in the hospital longer (mean 6.7 days vs 3.1 days, p 0.01), were more likely to be readmitted within 30 days (27% vs 16%, p 0.01), and had a greater incidence of medication-related issues (15% vs 7.6%, p 0.01). Patients with medication issues, medication events, or readmission within 30 days were detected with a sensitivity of over 80% using the high-needs criterion. Overall, the high-needs pharmacy criteria successfully identified older patients with longer lengths of stay who are at increased risk for 30-day readmission and medication-related issues.

Keywords: pharmacy practice, drug safety, drug evaluation, drug review, drug consultation, and pharmacy care.

Introduction

Clinical pharmacy services aim to minimise medication risks, improve patient safety, and optimise health out- comes.[1] Inpatient clinical pharmacist activities in Australia include medication reconciliation, medication clinical review, therapeutic drug monitoring, adverse drug event (ADE) management, providing medicine-related informa- tion to patients, and ensuring continuity of medication management at transitions between care settings.[1]

Medication-related problems (MRPs) refer to circum- stances which involve a patient's drug treatment that actually or potentially interferes with the achievement of an optimal outcome.[2] MRPs include medication errors, ADEs, and adverse drug reactions (ADRs). An ADE is defined as harm caused by appropriate or inappropriate use of a drug whereas an ADR is a subset of these events, where harm is directly caused by a drug under appropriate use.[3]

Clinical pharmacy services can be costly and in resource-constrained healthcare services should be priori- tised towards patients with the greatest potential risks.[2,4] Among healthcare organisations, prioritisation is com- monly achieved via organisational policies or individual clinical judgement.[5,6] Tools which have been developed to date frequently target specific patient groups and are often not validated against clinical outcomes.[2]

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The few tools which have been validated against clini- cal outcomes have been validated against the patients' risks of developing an ADE or MRP.[7–9] Less commonly, outcomes used to validate risk assessment tools include the 30-day readmission rate.[10] Previous studies have often been limited to specific clinical populations, including obstetrics,[11] geriatrics,[9–12] paediatrics,[13] or cardiology,[7] thus limiting the generalisability of these tools.

In order to make it easier for clinical pharmacists to use these criteria, they were revised to focus on the first assessment of hospitalized patients. Departmental and senior clinical pharmacists as well as the pharmacy management committee all gave their stamp of approval to the finalized HN criteria. The effectiveness of these HN criteria remains to be

At our health network, prioritisation of clinical phar- macy services occurs through the use of high-needs (HN) criteria (Table 1), a modification of the Society of Hospital Pharmacists of Australia (SHPA) *Fact Sheet: Risk factors for medication-related problems*.[14] The risk fac- tors identified by SHPA were considered too extensive for efficient use in daily practice, therefore the HN.

Table 1 High-needs clinical pharmacy criteria

Diagnosis or patient comorbidities
Cardiovascular and cerebrovascular events
Cognitive impairment (delirium, dementia, Alzheimer's disease)
Epilepsy
Liver disease – acute or chronic
Medication misadventure (including misuse and overdose)
Renal impairment (eGFR ≤ 30 mL/min or SCr > 200 1mol/L) – acute or chronic
Type I and II diabetes (excluding gestational and diet-controlled diabetes)
Transplant
Patient factors
Breastfeeding (excluding maternity patients unless fulfils another criteria)
Extreme BMI (cachexia or morbidly obese)
Pregnancy (excluding maternity patients unless fulfils another criteria)
Age < 12 years old
Medications
Antineoplastic drugs (cytotoxic and noncytotoxic, including "nibs" and "mabs")
Clozapine and depot antipsychotics
Continuous subcutaneous syringe drivers
Drugs requiring specialised monitoring (e.g. theophylline, lithium, phenytoin, digoxin, carbamazepine, valproate)
Epidural or blocks
Immunosuppressants
Insulin (excluding use in gestational diabetes)
Intravenous orders requiring manufacturing by pharmacy
Parkinson's medication
Pharmacotherapy (methadone or buprenorphine)
Restricted antimicrobials
Therapeutic anticoagulation
Non formulary medications
Inpatient circumstances
Intensive care unit transfer
Feeding tube, parenteral nutrition or medication modification as per speech pathologist
Pathology
• Patient with potassium <3 or >6 mmol/L
• Patient with sodium <125 or >155 mmol/L
• Patient with INR >2.5 and other abnormal coagulation pathology
• Patient with drug levels reported

BMI = body mass index; eGFR = estimated glomerular filtration rate; INR = international normalised ratio; SCr = serum creatinine.

Patients at risk of MRPs, readmission, or extended hospital stays are identified.

AIM

The purpose of this research was to examine whether locally designed HN pharmacy admission criteria can reliably identify patients at high risk for MRPs, medication-related events, and readmission across all hospital inpatients.

METHOD

Patients who were hospitalized to one of four public hospitals in the same healthcare network in metropolitan Melbourne, Australia, were the subjects of a retrospective cross-sectional observational research. For this reason, we limited our study to the four institutions with the most readily accessible computerized medical data. All patients aged 18 and above hospitalized between 10 and 16 February 2020 were considered for inclusion. Patients in the emergency room, those treated in an outpatient clinic, and those with LOSs of less than 24 hours were not included. In order to ascertain whether or not clinical pharmacy services were delivered and whether or not the patient satisfied the HN criteria, the researchers analyzed all relevant sections of each patient's medical record. The patients were classified as follows:
Patients with low needs (LN) did not fulfill any of the high-needs (HN) criteria.

(1) High Needs: Patient Meets HN Criteria but Receives No Clinical Pharmacy Services
Patient satisfied HN requirements and was provided clinical pharmacy services, therefore their needs were classified as "high."
The administrative database of the healthcare network was queried to calculate readmission rates after 30 days. The healthcare plan covered readmission to any of the seven affiliated hospitals. The Australian Commission on Safety and Quality in Health Care used the CHADx definition of a medication-related problem to establish MRPs. 15 When it comes to flagging adverse occurrences in hospitals, the Australian healthcare system often uses CHADx, a derivative of ICD-10 coding. 15 The Victoria Health Incident Management System provided data on medication-related occurrences.
In order to determine whether there was a statistically significant relationship between the various categories, we employed the chi-square test or Fisher's exact test. For continuous variables, we employed the Mann-Whitney U test (Wilcoxon rank-sum test). Every Statistical Formula are results of R (version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria). The study was approved by the health network's ethics and research council after being classified as a quality assurance project.

RESULTS

There were 761 participants from 4 hospitals who participated in this research (Table 2). Among those who satisfied HN requirements, 71% were treated by a clinical pharmacist, and 39% were given one or more clinical pharmacy services. Patients with longer LOS (mean days: 8.3 vs 3.6, p 0.01) and those who were hospitalized during the week were more likely to get clinical pharmacy services than those hospitalized on the weekend (42% vs 27%, p 0.01).

Patients with HN had a significantly higher mean age (67 vs 42, p 0.01) and a significantly longer LOS (6.7 vs 3.1 days, p 0.01). Compared to patients hospitalized to the mental health (65%), surgical (59%) and women's and children's (28%) units, those admitted to the geriatric (97%), general (92%), and speciality (86%) medical units were more likely to fulfill one or more HN criteria. For 17 individuals (HN 9, LN 8), we were unable to retrieve their ICD-10-coded data, hence they were not included in our MRP calculations. Patients who met the HN pharmacy criteria for MRP, medication-related event reporting, or readmission within 30 days had a sensitivity of over 80%. (Table 2). Patients who met one or more HN criteria were more likely to have an MRP during their hospitalization (15 percent vs. 7.6 percent, p 0.01) and to be readmitted within 30 days (27 percent vs. 16 percent, p 0.01). Those patients who met one or more of the HN pharmacy criteria had a higher incidence of reported medication-related events (3.9% vs. 1.4%, p = 0.07), although this difference did not achieve statistical significance.

There was a trend toward higher rates of readmission within 30 days (30% vs 24%, p > 0.05), MRPs (2.7% vs 0%, p > 0.05), and medication incidents (4.2% vs 2.7%, p > 0.05) in patients meeting one or more HN pharmacy criteria when excluding those who received clinical pharmacology services, but none of these differences were statistically significant..

DISCUSSION

Our research shows that older patients who are at higher risk for a prolonged LOS, an MRP, or readmission within 30 days after discharge may be reliably identified using the HN pharmacy criteria already in use at the health network. A large sample size and patients from a variety of therapeutic settings are two of the study's strengths. Moreover, other validated clinical pharmacy triage techniques have been research based but too difficult for application in everyday practice,7 need sophisticated electronic systems that may not be accessible at all institutions,7 or have been assessed in very particular patient groups. ¯ insignificant results from statistical analysis. A typical flaw in readmission-based assessment studies is their inability to identify readmission beyond the study hospital network. 10 Due to the size and scope of our healthcare network, we expect this possible constraint to have had a modest influence on the outcomes of our research. Most unexpected readmissions are likely to have presented to one of our three emergency departments.

Conclusion

Although we were able to apply the HN pharmacy criterion based on a comprehensive review of the patient's admission medical records, clinicians may not have access to all of this information during actual patient care. Further research is needed to determine how well our findings correlate with clinical evaluations in practice.

While the results of this research show that the HN pharmacy criteria may be used to identify individuals at risk for the outcomes of interest, the specificity of the criteria remains poor, and inter-rater reliability was not studied. If the HN tool can be simplified further to ensure consistent simplicity of use and/or increase its specificity, then further study into the individual criteria is warranted. Our health network is also exploring the possibility of developing more advanced and automated clinical pharmacy HN criteria and referral-based work-flows as a result of the implementation of electronic healthcare systems.

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Does splitting a tablet obtain an accurate dose? A systematic reviewand meta- analysis

B. Akhila, P. Anil Kumar Yadav, Dr. N. Padmaja

Abstract

The requirement to divide tablets into two or more doses in order to optimize medication for specific patients has led to widespread tablet splitting. Problems may develop if a tablet is divided in an uneven manner.

The goal of the study was to provide a comprehensive review of the research on how tablet splitting affects dosage accuracy.

Study inclusion criteria were studies published before January 2020 that examined the impact of tablet splitting on dosage accuracy and were located using a search of MEDLINE, EMBASE, CINAHL, and Cochrane. Any medication study in which the pill was broken might be considered. The screening and data extraction were done by two separate reviewers. There have been meta-analyses done to see whether pill splitting affects how well a dosage works. (Project number: CRD42018106252 at PROSPERO) Of the 25 studies considered, 16 looked at how tablet splitting affected tablet weight, 1 looked at how it affected drug concentration, and 8 looked at both. The weight and drug content of split tablets were found to vary by just a little amount in a meta-analysis (0.87% and 0.24%, respectively, 95% confidence range 0.63%-1.11% and 95% confidence interval 0.06%-0.43%). There was considerable discrepancy across trials with regards to weight, but not with regards to drug content fluctuation (I2 =.50, vs.1%). Tablet features and the technique used to divide the tablets both had a role in determining how well they worked.

This investigation reveals that the weight and drug content variance is modest independent of the technique and tablet properties, even if tablet splitting may affect dosage accuracy. To further understand the function of tablet splitting on dosage accuracy, more research is required to measure medication plasma concentrations as well as the effect on patient health outcomes including blood pressure and cholesterol levels.

Keywords: tablet, dosage, precision, adjustment, evaluation.

INTRODUCTION

Tablet splitting is a widespread practice resulting from the need to alter doses into two or more parts and opti- mise medicine usage in individual patients. Almost a quarter of all drugs administered in primary care are split.[1] Subdividing a tablet is a common part of modern-day drug therapy. Patients may need to over- come dysphagia to facilitate swallowing large tablets.[2–4] This practice can be relatively unproblematic since it can bring the required amount to the patient. 5 However, complications may occur if a pill is broken and then portion of it is abandoned or consumed at a different time.

Dosage manipulation by splitting a tablet may be difficult because of the potential for ingestion of uneven pieces or loss of tablet weight, both of which might result in an inaccurate dose.

6,7 Drugs with different amounts of active ingredients or other ingredients may have different effects on the body. 8 This is particularly crucial for medications with a narrow therapeutic index and a short half-life, since their effects are highly dose-dependent. 9 The quality of stored pills may also decline with time. In order to meet the criteria for content or mass homogeneity, split pieces must be consistent. 10,11 Product that is exclusive to a certain brand To the extent that appropriate credit is given to the author(s), the work may be used for noncommercial purposes and distributed in any media, according to the provisions of the Creative Commons Attribution-NonCommercial-NoDerivs License.

Pharmaceutical Analysis

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The medicine package inserts may include information on whether or not a certain pill is suitable for splitting. This advice, however, is often disregarded. 12 Many hospitals also have their own medication information and medicine formularies that address tablet splitting. However, there is frequently a discrepancy between the information obtained from these many sources. Patients, doctors, pharmacists, and nurses might all become confused because of this, so it's important to pay attention. We need standardized documentation and data on tablet splitting. Although changing the dosage by dividing pills is common practice, there is little literature that summarizes the information on whether or not this really works. Previous analyses have narrowed their attention to a single group, medicine, or illness. 13,14 Despite the fact that the 2012 assessment didn't zero in on any one group of people, any one medicine, or any one ailment, it's clear that we need to do some updating. 15 This review aims to summarize the research on how tablet splitting affects dosage precision.

METHODS

The results of this systematic review are presented following the guidelines of the PRISMA declaration for 2020. PROSPERO is aware of the review and has registered it (Registration Number: CRD42 018106252). The procedures used to conduct this review have been previously documented and will only be briefly described here. 16

Procedure for Seeking

Together with a medical research librarian, we formulated a search strategy and compiled a list of relevant keywords.

All studies published in MEDLINE, EMBASE, CINAHL, and the Cochrane Library between their inceptions between 1946 and 2020 were considered for inclusion in the review. A search for citations in pivotal publications and a study of references in included research led to the discovery of additional studies. The following is an outline of the included keywords:

1.Intervention, (tablet* split*, (tablet* break*, (tablet* cut*, (tablet* manipulate*)).

Title, abstract, original title, name of substance, topic heading, floating subheading, keyword heading, protocol supplementary concept, rare illness supplementary concept, unique identifier, and synonyms are all examples of mp.

The primary effect is to (pill split, pill shatter, pill cut, pill manipulate).

title, abstract, original title, substance name word, topic heading word, floating subheading word, [keywords for headings, a secondary concept for a procedure, a secondary concept for a rare condition, a unique identifier, and some synonyms].

Study Selection

The eligibility of abstracts and full texts was determined by two researchers (KC, MK) working separately and using predefined inclusion and exclusion criteria. The senior author was brought in to address the differences of opinion between the two reviewers.

Eligibility Criteria

Eligible studies might have been conducted with any medicine, and it was not necessary that the pill be broken. Participant characteristics were not limited in any way. Any procedure that required the patient to manipulate tablets (but not capsules) for oral administration was considered. Tablets might be manipulated by slicing, chopping, or breaking them into manageable pieces. In this case, we had an entire, uncracked tablet to use as a reference point.

Outcomes

The major result was the weight or percentage of drug content retained after splitting the tablet, indicating how accurately the dosage was preserved. Evaluation of the effect of physical tablet properties, the impact of tablet splitting on health outcomes, patient satisfaction, and other secondary outcomes were also considered.

Data Extraction

Two researchers (KC, MK) used data from three of the included studies to prototype and iteratively optimize the data extraction form. 16 The data from all included studies were retrieved by a single researcher separately. A secondary researcher checked all of the study data to ensure its accuracy.

Analytical Statistics

To account for within-study variability, we used fixed-effects and random-effects models based on a binomial distribution in our meta-analyses of proportions. Confidence intervals (CIs) for the mean were computed using the Wilson Score technique, with a 0.5 continuity correction applied when the numerator was zero, and the CIs were limited by zero and one. We did subgroup meta-analyses based on splitting technique (manual vs. mechanical vs. sharp tool) and tablet characteristics (size, shape, and score line), and we presented the results

I2 measures the fraction of total variability that can be attributed to real world causes as opposed to random variation. Stata 15 (StataCorp, College Station, TX, USA) was used for all statistical analyses, and the metaprop tool was used to determine overall prevalence rates across all groups.

Judgment of Possible Bias

Two researchers independently used suitable risk of bias techniques to evaluate the potential for bias in the included studies.

Discussions between reviewers and the senior author resulted in a resolution of 16 disagreements. If a study satisfied all of the other inclusion criteria, we did not eliminate it because of a problem with its methodology.

RESULTS

The literature search identified 1259 potentially eligible articles. Of these articles, 1188 were excluded on title

and abstract screening. The remaining 71 underwent fulltext screening, and an additional 46 articles were excluded due to failure to meet inclusion criteria. The final analysis included 25 studies. Figure 1 shows the process of study selection.

Characteristics of Included Studies

The search for relevant literature yielded 1259 possible items. To be specific, 1188 articles were disqualified due to title issues. and screening in the abstract The remaining 71 were subjected to a thorough text screening, after which another 46 were disqualified for not meeting inclusion requirements. Twenty-five studies were included in the final tally. The procedure for choosing studies is shown in Figure 1.

Inclusion Criteria for Studies

There were no randomized controlled trials that matched the inclusion criteria, hence all research that were included were conducted in a lab. Six thousand six hundred and seventy pills containing a total of eleventy-six distinct medications were lost throughout the included trials. More over half (n = 63, 54%) of these 116 medications were cardiovascular aids; the remainder were either analgesics, anti-inflammatories, or psychotropics.

medications for epilepsy (n = 7), diabetes (n = 6), and other conditions (n = 17) make up 15% of the total. About a third (n = 9, 36%) of the included research took place in the United States, while the remaining 14 took place in other countries. Perspectives of fragmenting groupings antiepileptic (*n* = 7, 6%); diabetic (*n* = 6, 5%); and other (*n* = 17, 15%) drugs. From the 25 studies include, approximately a third were conducted in the United States (*n* = 9, 36%), with the rest in various different countries. The experience of groups engaged in splitting

iPad users varied from healthcare professionals (n = 15, 60%) to the general community (n = 4, 16%), but few research reported on the demographics of the splitters or their degree of expertise. Sixteen (64% of the total) of the studies cited American pharmacopoeias, five (20%) cited European pharmacopoeias, one (4%) cited British, one (4%) cited Indian, and two (8%) cited none.

Outcomes

Accuracy of dosing as determined by weight or medication content was the main endpoint (Table 1). Most studies used halved pills. Very few studies have attempted to divide tablets into thirds or halves; only one has even tried to divide a tablet into halves.

Precision of Dose Determined by Drug Mass and Concentration

Using the drug's weight, twenty-four trials determined the efficacy of the doses. If you were to divide a pill in half, the average weight difference would be 0.87 percent (95% CI 0.63 to 1.11 percent, I2 50%, Figure 2a). Dose accuracy was evaluated in nine investigations based on the percentage of active ingredients in tablets. Figure 2b shows that the average drug content variation of a split tablet was 0.24% (95% CI: 0.06-0.43%).

Dosage Accuracy Using a Table-Splitting Method

Different dose-splitting techniques produced varying degrees of dose precision (Table 2). There were three main types identified: I dividing pills by hand, (ii) using a dedicated tablet splitter, and (iii) using a sharp instrument such a razor or knife. Tablet splitters were employed in eight out of the ten investigations.

Table 1 General characteristics of included studies

Reference	Method of splitting	Dose accuracy	Pharmacopeia referenced	Outcome
Boogie (2004)[18]	Tablet cutter, hand	Weight	USP	Splitting of unscored valdecoxib tablets resulted in low mean weight variation
Cook, Edwards[19]	Tablet cutter, knife	Weight	USP	Split tablets varied considerably in weight
Dosti, Malaj[20]	Tablet cutter	Weight	EP	Weight deviation depended on presence of score line
Elliott, Mayxay[3]	Not specified	Weight	EP	10% of tablets deviated by more than 25%; coated and unscored tablets resulted in greater weight variation
Habib, Alanizi[21]	Tablet cutter, hand	Weight and drug content	USP	There was greater variation in weight when tablets were split by hand. Drug content variation appeared to be attributable to weight variation that occurred during the splitting process
Helmy (2015)[22]	Knife	Weight and drug content	USP	Approximately 15% of half-tablets fell outside USP specification of weight and drug content. The subsequent mean percentage weight loss was less than 1.5% for all drugs. Drug content variation was attributable to weight variation that occurred during splitting process
Hill (2009)[23]	Tablet cutter	Weight and drug content	USP	Almost a quarter of split tablets fell outside USP specification of drug content. Drug content variation in half-tablets appeared to be attributable primarily to weight variation due to splitting process
Kadi (2016)[24]	Tablet cutter	Weight and drug content	USP	Following splitting, approximately three quarters of split tablets fell outside USP specification of drug content; recommend not to split unscored tablets
McDevitt (1988)[25]	Tablet cutter, hand	Weight	USP	Less than an eighth of tablets split deviated in weight by more than 20%
Nidanapu (2016)[26]	not specified	Weight and drug content	IP	Splitting tablets resulted in suboptimal dosage and plasma concentrations
Nolly (2005)[27]	Tablet cutter	Weight	USP	Tablet splitting was acceptable, resulting in acceptable weight variations
Madathilethiu (2018)[28]	Tablet cutter	Weight and drug content	USP	Quartering 10 mg hydrocortisone tablets produced unacceptable dose variations
Polli (2003)[29]	Tablet cutter	Weight	USP	Two thirds of drugs passed the weight uniformity test, with remaining third generally within 20% of target weight range

separating tablets by hand, and by using a blade, by the count of nine. Two research failed to detail their partitioning techniques (Table 1). Using a pill splitter resulted in less weight variation (0.58%, 95% CI 0.35-0.82%), while using a sharp instrument resulted in more weight variation (1.43%, 95% CI 0.74-2.11%), although manually dividing the medicine had the least weight variation (0.44%, 95% CI 0.00-1.00%). When compared to using a sharp instrument (0.24, 95% CI 0.01-0.58%) or manually breaking the tablet (1.56, 95% CI 0.01-66.82%), the tablet splitter resulted in the least variance in drug content (0.13%, 95% CI 0.00-0.36%).

Features of Tablets That Affect How Well They Reconstitute After Being Split for Dosage

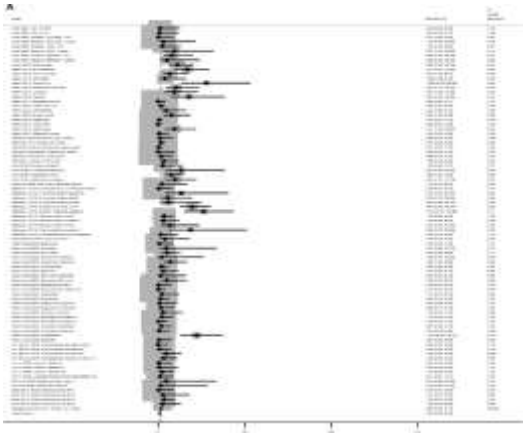
Scientists have reported on a variety of tablet features that might affect the split tablet's ability to deliver the correct amount (Table 2). From the total of 25 research, 23 (or 92%) mentioned whether or not a score line was used. Weight variation for scored tablets was 0.72 percent (95% confidence interval [CI] 0.45 percent to 0.98 percent) and drug content variation was 0.29 percent (CI 0.03 percent to 0.6 percent) lower than weight variation for unscored tablets (1.09 percent [CI] 1.09 to 1.09 percent).

Drug content fluctuation was 0.87 percent (95% confidence interval [CI]: 0.56 to 1.63 percent).

According to the studies (n = 16, 64%) that reported on tablet shape, the weight and drug content of oval-shaped tablets were more consistently distributed (weight variation: 0.54%, 95% CI: 0.25-0.83%; drug content variation: 0.48%, 95% CI: 0.51-1.48%) than those of round shape (weight variation: 0.99%, 95% CI: 0.45-1.52%; drug content variation: 2.36%, 95% CI: 0.66-4.06%).

Moreover, there was not much of a difference in the range of drug content variation between tablets with and without coating (1.61%, 95% CI 0.14% to 3.08%), as reported by 60% of the 25 investigations (n = 15). But there was more variance in weight among coated pills (1.15 percent, 95% CI 0.38 to 1.91 percent) than among uncoated tablets (0.65 percent, 95% CI 1.16 to 1.15 percent).

The Effects of Tablet Splitting on Health Outcomes and Patient Satisfaction



Almost three quarters of individuals in the research had plasma medication concentrations that were beyond the optimum range. 26 Patients' demographics were not discussed in any of the included studies.

whether taking the broken pill is a pleasant or bearable experience. No information was provided on tablet splitting's effect on taste or stickiness. But, according to one research

There was no mention of any health consequences from taking the broken pill in any of the studies we reviewed. Still, one

Figure 2 (Continued)

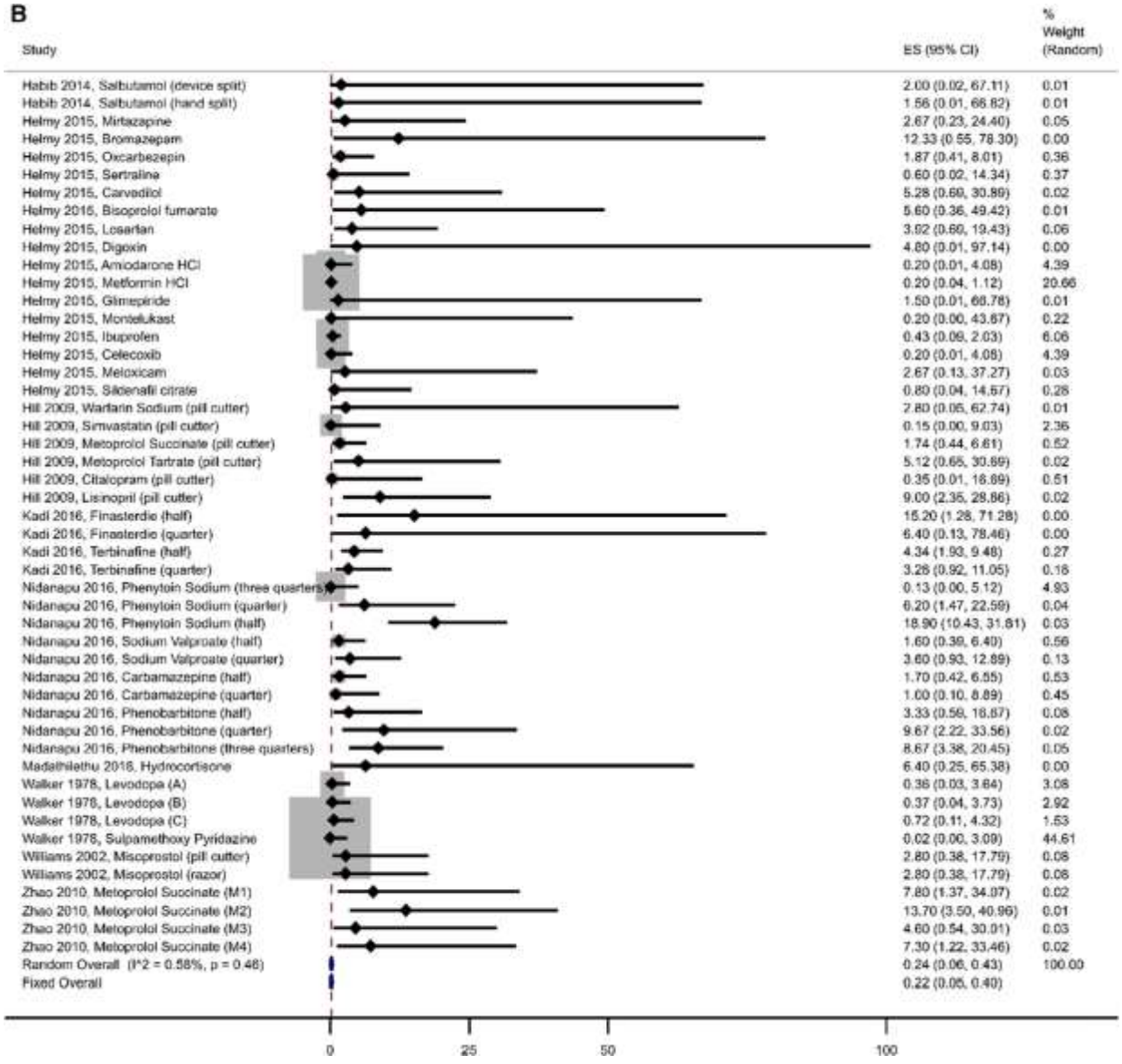


Figure 2 (a) Meta-analysis on mean weight variation on split tablet. (b) Meta-analysis on mean drug content variation on split tablet.

found that almost all participants (96.8%) would choose a lower dosage formulation over splitting a pill if given the option. Furthermore, over 80% of participants polled for this survey said they would pay more (with the median increase being 20%) for a lower dose strength. 26

Evaluation of the Quality of Studies

Figure 3 displays the results of the quality evaluation of the included studies. Studies used a combination of pills, and there were no randomized trials. Appropriate

Most research employed approaches intended to achieve their goals. More than half of the studies described their data collection methods and sample sizes for each medication. Studies also failed to account for the relative standard deviation of split tablets in their analyses. Many research attempted to accomplish their stated goals, but many failed to provide an adequate discussion of their findings.

DISCUSSION

Tablet splitting, according to previous studies,21 raises issues for drug and content variance.

Table 2 Weight and drug content variation of tablets within the studies

	Weight		Drug content	
Number of studies	24		9	
Variation overall	0.87%	(95%	0.24%	(95%
	CI	0.62–1.11%)	CI	0.06–0.43%)
Method of splitting				
Manual splitting	0.44%	(0.00–1.00%)	1.56%	(0.74–2.11%)
Tablet splitter	0.58%	(0.35–0.82%)	0.12%	(0.00–0.36%)
Sharp tool	1.43%	(0.74–2.11%)	0.24%	(0.01–0.58%)
Scoring				
Scored tablets	0.72%	(0.45–0.98%)	0.29%	(0.03–0.61%)
Unscored tablets	1.09%	(0.56–1.63%)	0.87%	(0.14–1.60%)
Shape				
Oval	0.54%	(0.25–0.83%)	0.48%	(0.51–1.48%)
Round	0.99%	(0.45–1.52%)	2.36%	(0.66–4.06%)
Coating				
Coated	1.15%	(0.38–1.91%)	1.61%	(0.14–3.08%)
Uncoated	0.65%	(1.16–1.15%)	1.61%	(0.20–3.03%)

and that this may have an effect on people's health. This meta-analysis is the first of its kind to systematically review the literature on the topic of pill splitting and its effect on dosage accuracy. Our objective was to learn more about how dividing pills affected dosage accuracy. Fortunately, this systematic review and meta-analysis indicated that the effects of dividing a tablet on its weight and pharmacological content were minimal.

The meta-analysis results indicated that the splitting procedure and tablet properties were

accurate dosing, albeit the variations were small. There was an improvement in accuracy while dividing scored pills compared to those that were not scored. The review indicated that the least amount of weight fluctuation occurred while breaking tablets manually, whereas the least amount of drug content variation occurred when using a pill splitter. Previous research has demonstrated that hefty tablets with deep score lines can be broken into pieces of the predicted weight. Alternatively, phenobarbital pills, which are tiny and lack a score line, produced subpar splitting results both in terms of precision and percentage content. 32 Small variations in dosage may result in sub- or supra-therapeutic levels, making such considerations clinically relevant for drugs like warfarin that have a narrow therapeutic index. 5 To ensure that patients are receiving consistent dosing of their medicine, it may be essential to reformulate such tablets into capsules providing the requisite therapeutic dosage.

Keep in mind that not all medications can be divided, notwithstanding the results of this article. Splitting extended-release pills, for instance, might cause toxicity owing to the fast and uncontrolled release of the drug's active component. Coated tablets were more consistent in their drug content after being split than uncoated tablets, however there was a significant weight difference between the two. Coated pills may attract water when broken, which might cause the medicine to become unstable and reduce its therapeutic benefits.

Different pill splitting techniques and their effects on dosage accuracy were evaluated in this overview.

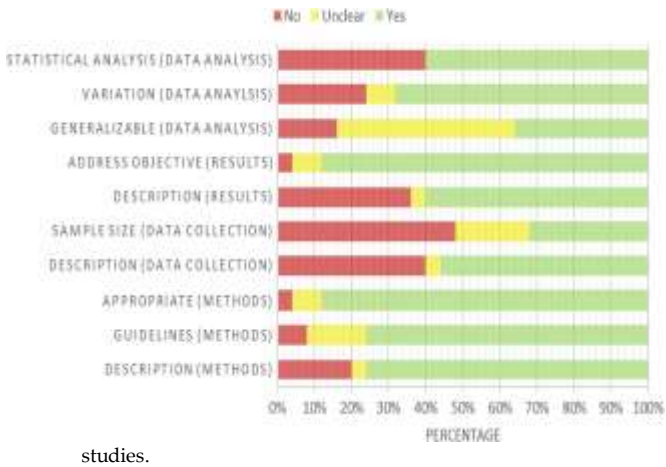


Figure 3 Review of authors' judgements about each risk of bias item, presented as percentages across all included studies.

a breakup. Confusion has arisen since there is no universally agreed-upon, standardized procedure for dividing pills or determining the efficacy of split dosages. Official sources do not provide any information on tablet splitting, as noted by Arnet and Hers- berger for the vast majority of scored tablets. 5

No research that were considered evaluated how patients felt after taking a split pill. Splitting a pill may be troublesome if it leads to people taking different amounts of the drug, and some research suggests that doing so may not affect health outcomes. 6 However, the split pills in this evaluation were so similar to one another in terms of weight and pharmacological content that serious clinical implications are very improbable. For certain medications, including warfarin, it is possible that little variations in drug content might affect results. In instances where extremely tiny dosage differences might have significant therapeutic implications, studies evaluating the dose accuracy of a split tablet and examining the impact of ingestion may be warranted.

In clinical settings, dividing tablets is routine. It is normal practice to divide a larger tablet into two smaller ones in order to save money. 1,6 Where the price per pill does not grow proportionately with increasing dosage strength, splitting has resulted in savings of up to 45 percent for medications used in primary care. 1 To meet the specific requirements of certain patients, tablets may be broken in half.

There were several caveats to our analysis. First, studies that might have affected secondary outcome findings may have been excluded since they did not provide the main outcome (dose accuracy by weight or drug content). Second, there may be unmeasured differences in dosage accuracy due to individual differences in splitting techniques and user experience. To reduce the potential for a systemic impact, subgroup analyses were performed. Thirdly, we were restricted by the low-quality of the available research; specifically, the absence of randomized trials, the failure to report on patient satisfaction results, and the small number of studies evaluating dosage accuracy based on drug content.

CONCLUSION

Based on the data presented here, it seems that splitting tablets may have an impact on dosage accuracy, but that the effects are modest in terms of differences in weight and drug content. Not all medications can be divided in half without risking serious side effects.

the indices, since the outcomes may be either beneficial or harmful. Splitting tablets with a slow-release coating may alter the formulation's underlying structure and therapeutic efficacy, therefore it's best to avoid doing so. There is a need for further research, particularly that which measures the correlation between medication plasma concentrations and clinical outcomes like blood pressure and cholesterol levels in patients.

— are necessary to fully comprehend the impact of tablet splitting on dosage precision. Therapeutic drug monitoring, pharmacokinetic and pharmacodynamic measurements, and specific stability studies are all necessary for medications having a limited therapeutic window.

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PHYTOCHEMICAL AND ANTHELMINTIC SCREENING OF *MORINGA OLEIFERA* STEMETHANOLIC EXTRACT

R. Mohana Priya, K. Sara Sirisha, Dr. G. Nagarajan

ABSTRACT

Helminthic infections are the most common infection in human beings affecting a large proportion of the world's population. Antihelminthic (Anthelmintic) drugs are the drugs which are used to kill or reduce the number of helminthic parasites in the intestinal tract or tissues of the body. *Moringa oleifera* belonging to the moringaceae family is a highly valued plant, distributed in many countries of the tropical and subtropical. The standard drugs such as piperazine citrate, albendazole, mebendazole and thiabendazole possess some side effects such as nausea, vomiting, stomach and abdominal pain, headache, dizziness, and temporary hair loss etc. But the herbal drug shows fewer side effects. The plant which shows muscle relaxant property may also shows antihelminthic activity. So we have selected *Moringa oleifera* which is an herbal drug and shows muscle relaxant property. Hence the present study was undertaken for phytochemical evaluation and to test the antihelminthic activity of ethanolic extract of *Moringa oleifera* stem. Preliminary phytochemical investigation indicatesthe presence of alkaloids, glycosides, flavonoids, steroids, carbohydrates and tannins. Indian adult earthworms (*Pheretima posthuma*) were used to study antihelminthic activity. The activity was checked in ethanolic extract with three different concentrations (100, 200, and 400mg/ml) and compared with the standard drug albendazole (40mg/ml) and control as distilled water. The result was expressed in the terms of paralysis time and death time of worms. Ethanolic extract of *Moringa oleifera* stem shows antihelminthic activity in dose dependent manner and maximum efficacy is seen at 400mg/ml concentration. Hence it was concluded that ethanolic extract of *Moringa oleifera* stem have antihelminthic activity

Keywords; *Moringa Oleifera*,Earthworms, Anthelmintic Activity,Albendazole, Paralysis, *Pheretima Posthuma*

INTRODUCTION

Anthelmintic or antihelminthics are the antiparasitic drugs that expel parasitic worms (helminths) and other internal parasites from the body by killing them and without causing significant damage to the host. They are also called as vermicides (those that kills) or vermifuges (those that stun). Antihelmintics are used to treat people who are infected by helminths, a condition called helminthiasis. These drugs are also used to treat infected animals. At the present time there are effective and broad spectrum of agents which cure or control infections caused by flukes and intestinal helminths. Due to the use of unsafe and repeated dose the parasite develop resistance towards the commercially available drugs.

Helminths are the parasitic worms which are most common infectious agents of humans in developing countries. It produces a global burden of diseases that exceeds conditions including malaria and tuberculosis. There are two major phyla of helminthis. They are nematodes and platyhelminths. Nematodes are also known as roundworms which includes the intestinal worms and filarial worms. The common roundworm infection is ascariasis. About 10 % of the developing world is infected with intestinal worms, according to world health organization (WHO). Ascariasis is an infection of the small intestine caused by a species of roundworm called *Ascaris lumbricoides*. The symptoms like nausea, vomiting, irregular stools, visible worms in the stool, stomach or abdominal pain and weight loss is seen in ascariasis. Some people with a large infection may have other symptoms such as fatigue and fever[1].

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The world health organization reveals that over two billion people suffering from parasitic worm infection[2]. It is estimated that by the year 2025, about 57% of the population in developing countries will be influenced by parasitic worm infection[3]. The prevalence of parasitic helminths typically displays a negative binomial distribution with in an infected population such that relatively few persons carry heavy parasite burdens. Without treatment, those individual are likely to become ill and to perpetuate infection within their community[4].

Antihelminthic are drugs that may act locally to expel worms from the GIT or systemically to eradicate adult helminths or development forms that effect organs and tissue of the body[5] This helminths infection also causes acute as well as chronic ill health among the various human being and cattle's. More than half of the population is suffering from the worm infection and mostly cattle'ssuffers from this worm infection[6]. In most of the developing countries the major health concern is helminths infection because they predispose humans to other infection such as fungal and bacterial infections[7]. Intestinal infections with worm can easily treated because it can easily be killed by the drug and this drug needs not to be absorbed when given by oral route[8].

This antihelminthic drug sometimes produces some side effects. This side effect includes abdominal pain, loss of appetite, nausea, vomiting, headache, diarrhoea[9]. Antihelminthic produces from the natural sources play key role in the treatment of this parasite infection[10]. In ethno medicine (study of the traditional medicine practice by various ethnic groups) at least 80% of the world population in developing countries uses plant material as there source of primary health care[11]. Due to this it increases problems of development of resistance in helminths against antihelminthic. So the medicinal plants are used for antihelminthic activity[12]. There is an increase in the antihelminthic resistance and the impact of conventional antihelminthic on the environment. So it is important to have alternative method against gastrointestinal nematode.

Moringa oleifera is the most widely cultivated spices belonging to the family moringaceae. It is the only genus in the family moringaceae. *Moringa oleifera* is the fast-growing, drough-resistant tree and it is widely cultivated

in tropical and subtropical area. It is native to Himalayas in north-western India. The leaves and young seeds pods of *Moringa oleifera* are used as vegetables. It is used in water purification and in herbal medicine. The leaf powder is used for washing hands. The plant which shows muscle relaxant property may also shows antihelminthic activity. So we have selected *Moringa oleifera* which is an herbal drug and shows muscle relaxant property.

Figure 1: *Moringa oleifera* tree.

Botanical classification:

Kingdom	**Plantae**
Class	Angiosperms
Order	Brassicales
Family	Moringaceae
Genus	*Moringa*
Species	*Oleifera*
Bionominal Name	*Moringa oleifera*

Table 1: Scientific classification of *Moringa oleifera.*

MATERIALS AND METHODS:

Plant collection:

The fresh stem of the plant was collected in the month of November and December from the local areas of Secunderabad,Telangana, India.

Preparation of extract:

- The stems were collected and washed thoroughly in water, chopped, air dried for a week and pulverized in electric grinder.
- 150 gm. of the powder subjected to soxhlet apparatus using ethanol as solvents for 8-12 hrs.
- The extract was stored at 4 ^{0}C until used.
- The prepared extract is used for the antihelminthic activity.

Phytochemical evaluation:

It is performed on plant extract or separated phytoconstituents to explore the possible primary and secondary activeconstituents present in the extract or phytochemicals. It includes:-

- Screening for primary metabolites
- Screening for secondary metabolites

Qualitative photochemical screening for primary metabolites:-

It is performed on plant extract/phytoconstituents to explore carbohydrates, proteins, amino acids, fats and fixed oils present in it.

1. Test for carbohydrates: -

About 50mg of the extract is dissolved in 5ml distilled water and filtered. The filtrate is tested for the presence of carbohydrates.

Molisch test:-

2ml of filtrate, 2drops of alcoholic solution of α – napthol is added. Mixture is shaken well and 1ml concentrated H_2SO_4 is added slowly along the sides of test tube and is observed for colour. The formation of violet ring at the junction of two liquids indicates the presence of carbohydrate.

Fehling's test:-

2ml of filtrate is neutralized with alkali. The mixture is heated with 1ml of Fehling's solution A and B each and observed for precipitate formation. Red precipitate formed indicates the presence of reducing sugars.

Barfoed's test:-

To 1ml of filtrate, 1ml of barfoed's reagent is added and heated on a boiling water bath for 2 minute and observed forprecipitate formation. Red precipitate formed indicates the presence of reducing sugars.

Benedict's test:-

To 0.5ml filtrate, 0.5ml benedicts reagent is added. The mixture is heated on a boiling water bath for 2 minutes and observed for precipitate formation. Formation of orange red precipitate indicates the presence of reducing sugars.

2. Test for proteins and amino acids:-

About 100mg of extract is dissolved in 10ml of distilled water, filtered through wattmann number 1 filtrate paper and filtrate subjectedto test for proteins and amino acids.

Millon's test:-

To 2 ml of filtrate, 2ml millons reagent is added heated to boil and observed for precipitate. The precipitate is again heatedand observed formation of white precipitate which turns to red upon heating indicates the presence of proteins and amino acids.

Biuret test:-

To 1ml of filtrate. 1ml 10% NaOH solution is added heated to boil. To this a drop of $CuSO_4$ is added and observed for colour.

Formation of purple violet colour indicates the presence of proteins.

Ninhydrin test:-

To 2ml of filtrate, few drops of 0.5N ninhydrin reagent is added and boiled for few minutes observed for colour. Formationof violet-blue colour indicates the presence of amino acids.Test of fixed oils and fats :- Saponification test:-

Treat the extract with few drops of 0.5N alcoholic KOH solution and a drop of phenopthalein solution. The resultant is heated on a water bath for about 1-2 hours. Formation of soap due to neutralization of alkali indicates the presence of fatty material.

Qualitative phytochemical screening for secondary metabolites:-

It is performed on plant extract/phytoconstituents to explore alkaloids, glycosides, steroids, terpenoids, phenolic compounds, tannins, flavonoids and saponins present in it.

3. Test for alkaloids: -

About 50mg of solvent free extract is dissolved in the same solvent used for extraction and filtered. The filtrate is tested for the presence of alkaloids.

Mayer's test: -

To 0.5ml of filtrate, 2drops of mayer's reagent (solution of potassium mercuric iodide) is added along the sides of the test tube and observed for precipitate. The formation of creamy precipitate indicates the presence of alkaloids.

Wagner's test: -

To 0.5ml of filtrate, 2 drops of wagner's reagent (solution of iodine in potassium iodide) is added along the sides of the test tube and observed for the precipitate. The formation of reddish brown precipitate indicates the presence of alkaloids.

Dragendroff's test: -

To 0.5ml of filtrate, 2drops of dragendroff's reagent is added and observed for precipitate. The formation of prominent reddish brown colour precipitate indicates the presence of alkaloids.

Hager's test: -

To 0.5ml of filtrate, 1ml of hager's reagent (saturated picric acid solution) is added and observed for precipitate. Formation ofprominent yellow colour precipitate indicates the presence of alkaloids.

4. Test for glycosides: -

For detection of glycosides about 50mg of extract is hydrolysed with concentrated HCl for 2hrs on a water bath and filtered.

The hydrolyzed is subjected to following test.

Borntragers test: -

To 2ml of hydrolysate, 3 ml of chloroform is added and shaken well. To the separated chloroform layer, 1ml of 10% ammoniasolution is added and observed for colour. Formation of pink colour indicates the presence of anthraquinone glycosides.

Keller-killiani test: -

about 50mg of the extract is dissolved in 2 ml of glacial acetic acid and 2drops of 5% ferric chloride solution is added and mixed to this 1 ml of H_2SO_4 is added, reddish brown colour appears at the junction of two liquid layers and the upper layer appears bluish green colour indicating the

presence of steroidal glycosides.Test for steroids and terpenoids:-Libermann-burchards test: -

To the 50 mg of extract dissolved in 2 ml of chloroform is treated with 2drops of acetic anhydride, 2 drops of concentrated H_2SO_4 is then added along the sides of the test tube and observed for colour. Red, pink or violet colour at the junction of the liquids indicates the presence of steroids and tritepenoids and their glycosides.

Salkowski test: -

50mg of extract in 2ml of $CHCl_3$ is treated with 2 drops of concentrated H_2SO_4, shaken well and allowed to stand and observed for colour. The formation of yellow coloured layer indicates the presence of triterpenes and formation of reddish brown coloured layer indicates the presence of steroids.

Test f_3 test: -

About 50mg of extract is dissolved in 2 ml of distilled water and then 2 drops of neutral 5% $FeCl_3$ solution is added and observed for colour. Formation of blur, green or black colour indicates the presence of phenolic compounds and tannins.

Lead acetate test: -

About 50mg of extract is dissolved in 2 ml of distilled water and to this 3ml of 10% lead acetate solution is added and observed for the precipitate. The formation of white precipitate indicates the presence of phenolic compounds and tannins.

Bromine water test: -

About 50 mg of the extract is dissolved in 2 ml of distilled water. 1 ml of bromine water is added and observed for the decolouration of bromine water. Discoloration of bromine water indicates the presence of phenolic compounds and tannins

Test forlavonoids :-Schinoda test: -

10 mg of extract is dissolved in 2 ml of alcohol, to these 2 fragments of magnesium turnings and 0.5 ml of concentrated HCl wereadded and observed for the colour. Formation of magenta colour/ crimson red colour indicates the presence of flavonoids.

Alkaline reagent test: -

10mg of extract is dissolved in 2 ml of water and treated with 1 ml of 10 % ammounium hydroxide solution and observed for the colouration 2 drops of dilute HCl is added and again observed for the discolouration. The formation of an intense yellow colour which turns to colourless on addition of dilute HCl indicates the presence of flavonoids.

5. Test for saponin glycosides:-

Saponin glycosides contain either steroids or triterpenoids aglycone and therefore they always give positive libermann burchard's test. Saponin glycosides give positive results with foam test and haemolysis test.

Foam test: -

Shake 2 ml solution of test sample in a test tube for half minute. Stable foam is formed indicating presence of saponin glycosides.

Haemolysis test: -

Treat 2 ml solution of test sample prepared in normal saline with 0.2 ml of blood in normal saline and mix well. Saponins cause complete haemolysis of the blood.

Worm collection:

Indian adult earthworms (*Pheretima posthuma*) collected from moist soil and washed with normal saline to remove all faecal matter which was used for the antihelminthic study. The earthworms of 3-5 cm in length and 0.1-0.2 cm in width were used for all the experimental protocol due to their anatomical and physiological resemblance with the intestinal roundworm parasites of human beings.

METHOD:

- Five groups of approximately equal sized Indian earthworms consisting of four earthworms in each group were released into 10 ml of desired formulation.
- Group first serve as control, receive only water (negative). Group second serve as standard receive standard drug albendazole (positive) of 400mg concentration. Group third serve as low dose of extract. Group forth serve as medium dose and Group fifth serve as high dose.
- Observations were made for the time taken to paralysis and death of individual worms.
- Maximum time is 120 minutes.
- Paralysis was said to occur when the worms did not revive even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colour.

RESULTS AND DISCUSSION

Preliminary phytochemical screening has shown the presence of carbohydrates, alkaloids, glycosides, steroids, terpenoids, tannins and phenolic compounds in ethanolic extracts of plants was illustrated in Table 2.

Table 2: Phytochemical screening for ethanolic extract of *Moringa oleifera* stem.

TEST
Test for carbohydrates
• Molish's test
• Fehling's test
• Benedict's test
• Barfoed's test
Test for proteins and amino acids
• Million's test
• Biuret test
• Ninhydrin test
Test for fixed oils and fats
• Saponification test

Test for alkaloids	
• Mayers test	+ve
• Wagners test	+ve
• Dragendroff's test	+ve
• Hagers test	+ve
Test for glycosides	
• Kellar killani test	+ve
• Borntragers test	-ve
Test for steroids and terpenoids	
• Liebermann-burchard test	+ve
• Salkowski test	+ve
Test for tannins and phenolic compounds	
• Ferric chloride test	+ve
• Lead acetate test	+ve
• Bromine water	+ve
Test for flavonoids	
• Shinoda test	-ve
• Alkaline reagent test	-ve
Test for saponin glycosides	
• Foam test	-ve
• Haemolysis test	-ve

Figure 2: photographs of antihelminthic activity of ethanolic extract of *Moringa oleifera* stem before experiment.

Figure 3: Photographs of antihelminthic activity of ethanolic extract of *Moringa oleifera* stem after experiment.

Table 3: Time of paralysis.

Group	Treatment	Concentration	Paralysis(min)
1	Standard (albendazole)	40mg	71.25±1.10 [a]
2	Low dose of extract	100mg	89.25±0.47 [c]
3	Medium dose of extract	200mg	84.75±1.70 [d]
4	High dose of extract	400mg	80.5±2.02 [be]
5	Control	-	-

All Values represents Mean± SEM; n=4 in each group
Significance of a is ****
Significance of b is ****
Significance of c is ****
Significance of d is ***
Significance of e is **

The values are expressed as mean ±SEM of 4 worms. Superscript letters represents the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests. [a] P<0.0001, indicates comparison of group 5 with group 1, [b] P<0.0001, indicates comparison of group 5 with group 4, [c] P<0.0001, indicates comparison of group 1 with group 2, [d] P<0.0001, indicates comparison with group 1 with group 3 and [e] P-0.0012, indicates comparison with group 1 with group 4.

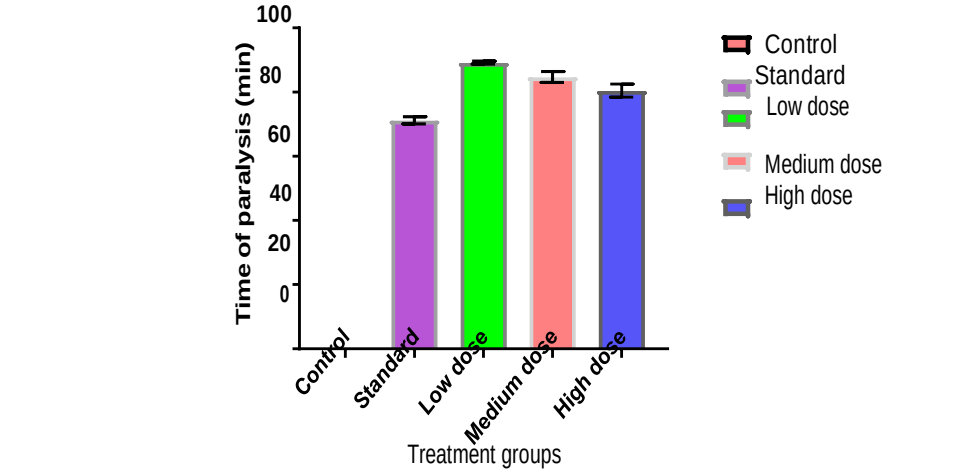

Figure 4: Paralysis time.

Table 4: Time of death.

Group	Treatment	Concentration	Death (min)
1	Standard (albendazole)	40mg	80.75±0.85 [a]
2	Low dose of extract	100mg	104.3±2.17 [b]
3	Medium dose of extract	200mg	91±0.70 [c]
4	High dose of extract	400mg	86.25±1.10 [de]
5	Control	-	-

All Values represents Mean± SEM; n=4 in each group
Significance of a is ****
Significance of b is ****
Significance of c is ***
Significance of d is *
Significance of e is ****

The values are expressed as mean ±SEM of 4 worms. Superscript letters represents the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests. [a] P<0.0001, indicates comparison of group 5 with group 1, [b] P<0.0001, indicates comparison of group 1 with group 2, [c] P-0.0002, indicates comparison of group 1 with group 3, [d] P-0.0364, indicates comparison with group 1 with group 4 and [e] P<0.001, indicates comparison with group 5 with group 4.

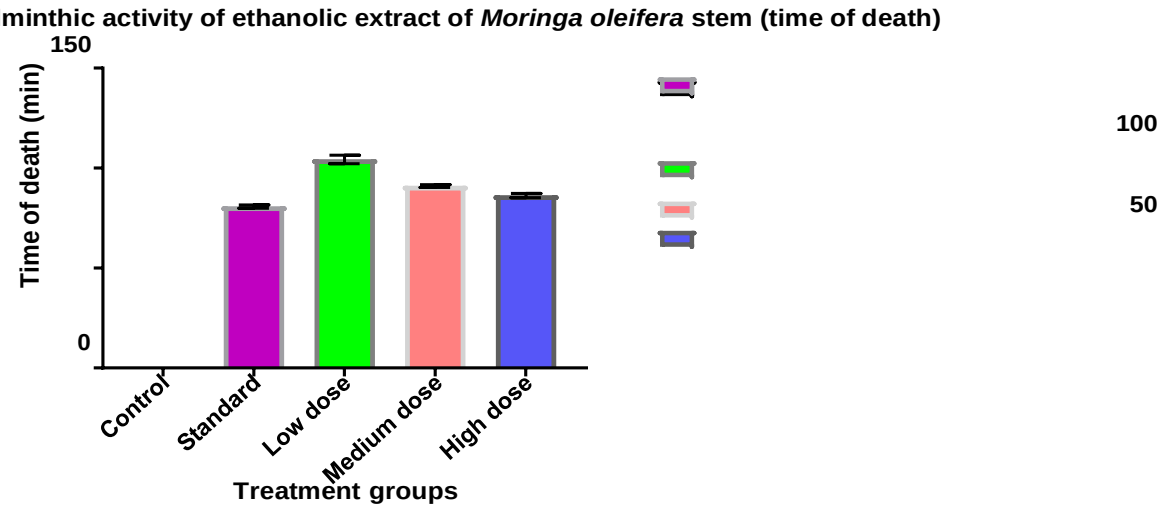

Figure 5: Death time.

Moringa oleifera was collected and dried for a week. The ethanolic extract was prepared by subjecting to soxhlet apparatus for 8-12 hours. The percentage yield of ethanolic extract was found to be 3.33% w/w. Phytochemical screening was performed on ethanolic extract of *Moringa oleifera*. It shows the presences of carbohydrates, alkaloids, glycosides, steroids and terpenoids, tannins and phenolic compounds (Table 2). The presence of some of these phytochemical constituents may produce antihelminthic activity. The evaluation of antihelminthic activity was done. Five groups were taken consists of 4 earthworms in each group. Two parameters were observed that is time of paralysis and time of death was illustrated in Table 3 and 4 respectively. When the three concentrations of extract were compared with standard drug it shows activity in a dose-dependent manner showing maximum efficacy at high dose than to the medium dose followed by low dose.

The statistical values for time of paralysis is 71.25±1.10 > 80.5±2.02 > 84.25±1.70 > 89.25±0.47 it represents standard drug, high dose, medium dose, low dose respectively. The statistical values for time of death is 80.75±0.85 > 86.25 ±1.10 > 91±0.70 > 104.3±2.17 it represents standard drug, high dose, medium dose, low dose respectively. Further studies should be done to identify the active constituents responsible for the antihelminthic activity.

CONCLUSION

From our observations, higher concentration of extract produced paralytic effects much earlier and the time taken for death was shorter when compared with other two concentrations. Ethanolic extract of *Moringa oleifera* shows antihelminthic activity in dose-dependent manner showing maximum efficacy at high dose (400mg/ml concentration). Antihelminthic activity of the extract was compared with the standard drug albendazole. From the above results, we can conclude that *Moringa oleifera* stem extract exhibited significant antihelminthic activity; therefore further study must be carried to know the active chemical constituents responsible for antihelminthic activity.

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Continuous manufacturing is used in the pharmaceutical business. Mental Reorientation

S. Shaheena Begum, S. Rajesh Raja, R. Jona Methusala

Abstract

The manufacture of pharmaceuticals is subject to strict guidelines known as good manufacturing practice. Though continuous processing might often result in the production of purer goods and thus have financial benefits, batch processing has traditionally dominated the industrial industry.

After the product and process have been licensed, the regulatory framework often stifles any efforts to make changes to the method. Because of this, many experts in the field now believe that batch operations are the only viable option. However, the regulatory authorities, especially the Food and Drug Administration in the United States, have realized that continuous processing has the potential to enhance product quality and are urging the sector to rethink its ideas. This study investigates how chemical engineers might take use of the newfound freedom afforded them by these revised regulations to reevaluate their own perspectives and inspire a paradigm shift in the field as a whole.

Keywords: *Continuous processing; GMPs; the pharmaceutical business; roadblocks to progress.*

INTRODUCTION

Many processes that would normally be continuous, such as in-line milling and spray drying, or semi-continuous, such as tablet compression, are converted to batch processes because of the pharmaceutical industry's preference for them. This has developed for historical causes, and it is reinforced by the regulatory atmosphere and the necessity to deliver new items to market as quickly as feasible. This is related to the belief that batch operations are necessary for any product development if the end result is to meet stringent quality standards imposed by government agencies.

Contrarily, there are a few issues plaguing batch operations, the most notable being the difficulty in scaling up and the challenge in producing uniform processing conditions. As a result, the quality of the produced goods is typically compromised. Unwanted effects may be caused by by-products, and it's possible that some products have been rejected during clinical testing in the past because they weren't pure enough.

There has been a shift in both the regulatory and commercial climates. Environmental, health, and safety law is pushing the pharmaceutical business toward more efficient procedures, and the licensing authorities recognize that continuous processing may play a role. It's becoming increasingly difficult and costly to test out brand-new items. time-consuming to find, develop, and bring to market, and generic producers have ramped up competition.

The time is ripe for technical experts in the field to further the efficient use of continuous processing. This paper looks at how this opportunity came to be and how to go forward with shifting the mindset of industry experts.

Pharmacology

Dr.K.V. Subba Reddy Institute of Pharmacy

(Approved by AICTE,P.C.l New Delhi& Permanently Affiliated to JNTUA Anantapuramu

MOU with Government General Hospital &KMC, K urnool

THE PHARMACEUTICAL MANUFACTURINGPROCESS

The Ingredients

A pharmaceutical product contains two groups of materials, active ingredients and excipients. The active ingredients are those materials that have a therapeutic effect, whilst the excipients have no therapeutic effect but are necessary to ensure the final dosage form acts as intended. Typical excipients include water, lactose, starch, sugar and colouring but in practice there are a very large number of excipients in common usage.

The Manufacturing Stages

The pharmaceutical manufacturing can be broken down into a number of stages, see Figure 1. Frequently it isbroken down into two major stages usually known as Pri- mary and Secondary. These stages are commonly carried

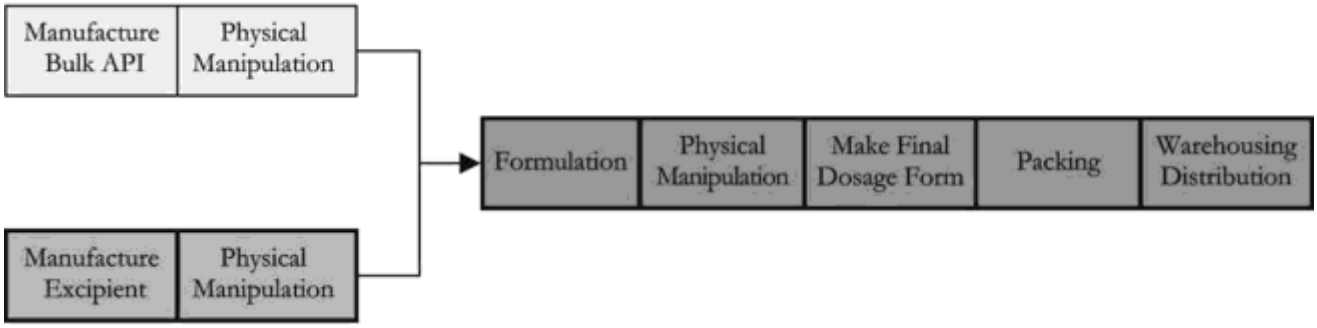


Figure 1. Overview of the pharmaceutical manufacturing process.

carried out at several locations, and by a variety of manufacturers. Production of APIs, which may include chemical synthesis or biological processing followed by physical manipulation steps including drying, purification, and size reduction, constitutes the Primary stage. Shipping the API in large quantities to a secondary production facility is standard practice.

Excipients are manufactured by mainstream companies that conform parts of their goods to the pharmaceutical requirements laid forth in the different pharmacopoeias, therefore technically speaking, excipient manufacturing is also part of the Primary stage. Except for sterile water used in injectable treatments, all other components in pharmaceuticals are typically manufactured off-site and subjected to the same rigorous quality controls as the drug's active ingredients.

Bulk formulation of the final product, including the combination of one or more APIs with a variety of excipients, often kicks off the Secondary stage. These might be subjected to further physical processing, including drying, sizing down, sizing up, filtering, and sterilizing. The final dosage form is created from the bulk substance. Although tablets account for well over 80% of all dosage forms, other options include capsules, liquids contained in ampoules, vials, or bottles, creams housed in tubes or jars, and aerosols housed in canisters or suppositories. Products are then placed into their final containers, such as bottles, blister packs, or foil wraps, which prominently display the product's information.

QUALITY REGULATION

Good Manufacturing Practice

The pharmaceutical industry is highly regulated with a number of regulatory systems. The technical requirements of Good Manufacturing Practice (GMP) are being harmo- nized but the regulations that oversee these are very different for the USA, the EU and Japan. However, all of these majormarkets require that products must be manufactured in linewith GMP if they are to be allowed into the market place. In the European Union the requirements for GMP are covered by Directive 2003/94/EC. Whereas in theUnited States the Federal Food, Drug and Cosmetic Act states that a pharmaceutical product may not be sold if it is adulterated. Section 501 (a)(2)(B) states that if a pharma- ceutical product is not made to GMP it is by definition

adulterated and cannot be sold.

GMP is defined by the EU Guide to Good Manufacturing Practice (European Regulations, Vol. 4, 2002) as follows:

Good Manufacturing Practice is that part of Quality Assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required be the Marketing Authorisation or product specification.

GMP must be applied to the manufacturing process after the ‘critical step’, which usually occurs when the final active molecule is being produced or an intermediate is being produced that has a major impact on the quality of the final product. This critical step usually occurs near the end of the API manufacturing process. Quality assur- ance measures also need to be in place for those parts of the process prior to the critical step. Since excipients donot include APIs they are not subject to the same level of scrutiny but quality assurance systems must be in placeand the products must meet the specifications given

in the appropriate pharmacopoeia.
The major thrust of GMP is to prevent the product becoming contaminated by particulates and micro-organisms (particularly from the human plant opera- tives) in the room environment, particulates generated by equipment, chemicals leached from the product contact parts and from other products being manufactured. It isthe need to avoid contamination and cross contamination from other products that dominates the design of pharma- ceutical manufacturing facilities and leads to the need for regular and extensive cleaning.
The guidelines to GMP, whilst not compulsory, form a detailed framework and working outside of them is only permissible if good scientific evidence for the alternative approach can be provided. The guidelines are fairly pre-

manufacture the product must have a manufacturing licence. Both of these licences are time consuming and expensive to obtain.
The product licence includes information about how the product is manufactured and therefore once the licence has been obtained it is to be expected that any change to process will require more time and money to be spent ona revised product licence.

Impact on Process Changes

It can be seen that the regulatory requirements to comply with GMP and the impact of including the method of man- ufacture in the product licence contribute to a mind set that is averse to moving outside the guidelines and/or changing the process after it has been licensed.

SAFETY, HEALTH AND ENVIRONMENTAL (SHE) LEGISLATION

There is a perception that the licensing and GMP regulations are more important within the pharmaceutical industry than SHE legislation. However, a recent incident (pers. comm., 2004) illustrates the fallacy of this view. A relatively minor incident relating to the cleaning equipment on some key manufacturing equipment in a tablet facility was report to the Health and Safety Executive as a result of the RIDDOR legislation within the UK. The resulting investigation nearly resulted in a Prohibition Order, if this had occurred the whole of the tablet facility would have shut down within a few days with the resultant loss of large amounts of money. This one safety issue could have stopped the production of a number of product ranges.

ATEX Directive

The European Union ATEX directive has had significant impact on pharmaceutical manufacturing within the union. This is because it has codified the requirements for poten- tial explosive atmospheres created by dust clouds. That is not to say that the industry has been ignoring the potential hazard but since there is now a legal requirement for manufacturers to classify the hazardous areas, equipment is being more rigorously specified and risk assessments have become more detailed. Also there is now a statutory requirement to reduce the potential risk by reducing the inventory of material within the manufacturing environment.

scriptive, the EU guidelines include 18 annexes that include guidelines on the manufacture of various groups of pro- ducts. The annex covering sterile products for example, indicates that sterile products shall be terminally sterilized using steam in an autoclave unless there a scientific reason why this is not possible.

Product and Manufacturing Licences

Before pharmaceutical products can be sold they must have a product licence from the relevant regulatory body, Medicines and Healthcare products Regulatory Agency in the UK, the Food and Drug Administration in America, and so on. Also, the facility that is used to

Control of Substances Hazardous to Health (COSHH/OSHA)

As products become ever more potent, the requirement to comply with COSHH/OSHA regulations becomes more complex and expensive. Product containment making use of special devices such as split butterfly valves or various forms of barrier isolators is becoming common. However, these add significantly to the capital cost of the equipment [up to 50% more (Cliff, 2004)] and make the facility more complex to operate. Continuous processes allow the manufacturing equipment to be contained without resorting to this sort expensive measure and are likely to lead to significant capital cost savings.

Integrated Pollution Prevention and Control (IPPC)and Other Environmental Legislation

The production processes for APIs tend to have low yields. This leads to the need to dispose of large quantities of waste. With multi-step complex processes there will be more waste product than saleable product even if the yield of each step is greater than 90%. The disposal of this material is becoming more expensive and more com- plex. There is an obvious advantage is increasing yields and reducing this problem.

NEW PRODUCT DEVELOPMENT AND THE PHARMACEUTICAL MARKET PLACE

The driving force behind the pharmaceutical industry has been and remains the discovery of new molecular entities that can be patented. Once patented, licensed and launched on to the market there is the potential to make large profits. However, in general the cost of the research efforts is increas- ing whilst its efficiency is falling. The major pharmaceutical companies are finding it difficult to find new products at the rate necessary to continue with the expected profit growth. This means the issue of the cost of manufacturing products is starting to become more important. Following on from the recent mergers and acquisitions activities there is a drive to reduce costs by reducing the number of research, development and manufacturing facilities.

Laboratory Scale versus Manufacturing Scale

New molecular entities are obviously developed at the laboratory scale. A large number of new molecules are pro- duced by chemical synthesis or by biological fermentation processes, these are screened for pharmacological activity to find new products of potential commercial value. New molecules that appear to have potential have to be sub- jected to three stages of

clinical trials. Each stage requires ever larger quantities of materials as the number of trials required increases at each stage. This means that in parallel with the clinical trials it is necessary to carry out scale-up trials to demonstrate that the molecule can be produced at a large enough scale to supply the third stage clinical trials. Much of this work is abortive because products frequently fail to move forward because they are not suffi- ciently efficacious or have too many side effects.

The scale required for Stage 3 clinical trials is usually large enough to produce sufficient quality for the initial launch of a product but if it successful it is not very long before further scale-up is required to allow the required tonnage of material to be made.

One way to increase the speed with which a product can be brought to the market is to reduce the development time. If the scale-up step could be avoided it may be possible to gain months/years of additional time covered by the patent. Continuous processing via micro-reactors offers the possi- bility to avoid this scale-up phase as discussed before.

Commercial Risk

The opportunity cost of failing to sell a successful pharmaceutical product can easily run into hundreds of millions of dollars. This means that pharmaceutical compa- nies take a cautious approach to business risk. Using novel processes and untried manufacturing techniques will increase the business risk and so high level management will always err toward the tried and tested.

One area of risk that particularly affects the batch proces- sing mind set is product recall. If a batch of material is found to out of specification or contaminated and needs to be recalled this is inevitably expensive and it reflects badly on the manufacturer. The commercial risk is reduced if the batch size is kept small so that any recalls require the minimum amount of material to be returned and destroyed. The use of continuous processes will have to address this issue, if company managers are to accept the associated commercial risks.

Product Pricing

The pharmaceutical market place falls into two major groups, the so called ethical products, prescription only patented products and generic products that have no patent protection. Generic products fall into two further categories, those that require a prescription and those that can be sold over the counter (OTC). The over the counter market differs in each country as the regulations covering the requirements for prescriptions are very different even across the EU. Antibiotics being OTC in Spain but pre- scription only in the UK, for example.

It would be expected that OTC products such as aspirin, paracetomol and ibuprofen would be under severe cost pressure, however this is not the case. A visit to any super- market will show that branded versions of these products still command a price premium of four or five times over the unbranded products. This is surprising when you consider that in a highly regulated market it unlikely that there will be any quality difference between the products. This seems to show the power of the 'reassuringly expensive' approach.

What is perhaps even more surprising that whilst these products are made in large volumes there are no

Speed to Market

The life of a patent is finite and varies around the world, in general more than half of the patent life of a new molecular entity is lost before the product can be launched. Once the patent protection has been lost it is possible forthe original product developer to lose up to 90% of their market share, to generic manufacturers, within 12 months. With large numbers of products coming off patent there are plenty of companies who can make good profits from entering the market as soon as a patent expires.This is a developing market place.

manufac- turers using a continuous process. However, this is one area where continuous processing could be a serious possibility, there are the production volumes to make use of the commercial available equipment and so scale down (see below) should not be a problem. Also, competition from supermarket own brands is likely to begin to have sufficient impact on the product cost to provide the driving force for a change.

THE NATURE OF BATCH PROCESS

Much of the resistance to change arises from the fear of the impact on product quality. However, batch processes are not actually very good for product quality. Although the product leaving a batch reactor, crystalliser, dryer or whatever may be homogeneous the heat, mass and momen- tum transfer environment that the molecules and crystals within the batch have experienced during the process will be significantly different. During the process, temperature, velocity and concentration profiles will vary with position within the equipment and with time leading to processing inefficiencies. Most of the practices within the industry have been developed to overcome these deficiencies but this is poorly recognized and many of the practices are seen as a prerequisite to good quality management.

Poor Understanding

Batch processes are poorly understood, time dependant and scale dependant operations. They are poorly under- stood at the micro-scale and have not until recently been studied in detail. In spite of this method of operation being used for thousands of years it still produces fluctua- ting and unpredictable results.

By comparison, most continuous processes are much simpler and far better understood. The mathematical tools to analyse these processes have been available for many years.

Poor Yield

The varying heat, mass and momentum transfer in a batch reaction generally mean that yields are sub-optimal. If a 10 stage process has a yield of 50% at each stage then the overall yield will be 0.510 ~ 100% i.e., 0.097% raising the stage yield to 80% raises the overall yield to over 10% thereby giving more than a hundred times greater throughput in the same equipment.

Continuous processes for making the relevant chemicals have been reported in the literature that give significant improvements in yield over the batch process mainly because of improvements in temperature control and mass transfer.

Intermediates

As a result of the low purity of materials made by batch processes and perhaps because of the approach taken by chemists at the laboratory scale, most APIs are isolated as a number of intermediates (usually solids). These arise as a result of the purification (often crystallization pro- cesses) that are required to isolate the desired chemical. Intermediates also allow the purity of the product to be checked at each stage and to identify problems before the final product is made. However, if reaction yields and con- versions were improved and classic stirred tank vessels. These processes are difficult to scale up and in general the regulatory authorities do not likeprocesses to be scaled up by more than $10-1$ at each stageof development. The scale-up problem can be illustrated byconsideration of the blend time in a stirred vessel with baf- fles and a pitched blade turbine.

Nagata (1975) proposed that $u \cdot N$ (where u is the blend time and N is the impeller speed), for a geometry similarsystem, is constant for a Renold's number greater than 10^4. Also the power absorbed by an impeller is given by Oldshue (1985) $P = N_p r N^3 D^5$ (where N_P is the power number of the impeller, r is the liquid density, N is the impeller speed and D is the impeller diameter) for a pitched

blade turbine a typical value of N_p is 1.5.

Consider the scale-up of a process from 100 l to 10 000 l with a vessel L/T = 1 and D/T = 0.33. Table 1 shows that scaling up to give a constant blend time requires impractical increase in power consumption. Using constant power per unit volume gives a more practical solution but the blend time will increase. Similarly scale-up using constant tip speed, also gives a practical power consump- tion but the blend time will increase nearly five times with a commensurate deterioration in yield and conversion resulting from a significant amount of time required toproduce a reaction mass approaching homogeneity.

As a further problem the velocity profile within the vessel varies, there will be a high velocity close to the tip of the impeller and lowering velocity as the distance from the impeller increases. Numerous studies have shown that they are frequently complete dead spots within a stirred tank. These variations in velocity lead to variations in reactions were more predic- table, less purification would be required. Also product quality could be monitored using on-line analysis.

A further major disadvantage of producing and analysing intermediates is cost of storing (working capital) and hand- ling the solid intermediates (labour costs).

Batch Processes Do Not Scale Up Well

Most pharmaceutical batch operations are carried out in agitated vessels, for liquids and slurries these will be the

heat, momentum and mass transfer causing variations in temp- erature and concentration within the stirred mass. These variations lead to sub-optimal reaction profiles and a reductions in product quality. In summary a stirred vessel is not good at producing a high quality consistent product.

The discussion above has been based on stirred tanks containing liquids or slurries but the above points also apply to more solid based processes such as crystallization, filtration and drying.

Table 1. Batch agitation scale up

Parameter		Units	Lab	Pilot	Production
Volume	V	l	100	1000	10 000
Vessel diameter	T	m	0.503	1.084	2.311
Height of liquid	L	m	0.503	1.084	2.311
Impeller diameter	D	m	0.168	0.361	0.771
Liquid density	r	kg/m^3	1000	1000	1000
Liquid viscosity	m	Pa·s	0.001	0.001	0.001
Scale-up on constant blend time i.e., constant speed					
Speed	N	s^{-1}	5.00	5.00	5.00
Renold's number	Re		137 807	639 643	2 968 962
Process power	P	kW	0.023	1.097	50.94
Scale-up on constant power per unit volume					
Speed	N	s^{-1}	5.00	2.99	1.80
Scale-up on constant tip speed ND					
Speed	N	s^{-1}	5.00	2.32	1.08
Renold's number	Re		137 807	296 896	639 645
Process power	P	kW	0.023	0.109	0.509

Flexibility

One major advantage of batch processing equipment is flexibility. A set of stirred tank reactors, batch filters, centrifuges and dryers can, with persuasion, make virtually any chemical providing the materials of construction are suitable. Since the annual requirements for the APIs can often be very low, production facilities need to be as flexible as possible. Continuous processing will need to match this level of flexibility.

CONTINUOUS PROCESSES

In contrast to batch processing, continuous processing is relatively well understood. At steady state, there are only three-dimensional problems instead of four dimensional problems because time is not relevant. Small scale conti- nuous processes are easy to control and a constant tempera-ture profile is easy to maintain.

Number Up Instead of Scale Up

The Institution of Chemical Engineers 2004 Conference 'Switching from Batch to Continuous' had a number of papers demonstrating that various forms of micro-reactors can be used for chemical synthesis with very promising improvements in conversion and product quality. Thesereactors have the advantage that a large number of themcan be used in parallel and so that basic scale is not altered. The production rate is increased by numbering up insteadof scaling up.

A major advantage of this approach is that there is no need to carry out the scale-up development work in parallel with the clinical trials and scale-up to full manufacturing is achieved by having sufficient micro-reactors in parallel to achieve the require throughput.

At present this approach has a major problem. The micro-reactors so far developed are suitable for liquid but not for solids due to their very narrow pathways. Since most pharmaceutical active ingredients are large

molecules they are also in general solids. This means that an alternative approach is required for crystallization, purifi- cation, drying, and other physical manipulation activities such as milling and granulation.

Solid Processing and the Scale Down Problem

By the very nature of the pharmaceutical industry the product quantities are usually small. Figure 2 shows the product portfolio of a major pharmaceutical company. The biggest output is only 1200 t.p.a and most products are in the 100 – 1000 t.p.a. range. However, as products become even more potent the trend is for lower production rates in the range 10 – 100 t.p.a. Suitable equipment isavailable to manufacture most stages of the secondary production by continuous means, however most of this equipment is too large to be of use. For example, the Hosokawa Bepex laboratory scale Extrud-o-mat2 that can be used for continuous granulation produces 10 – 25 kg h. If this were operated continuous for 6000 h per annum it would produce 60 to 150 t.p.a., i.e., greater than the full production scale for many of the new potent products.

Laboratory development trials either require even smal- ler machines than those currently available or alternatively short product runs have to be carried out with the associated start up and shut down problems. This scale down problem needs to be addressed if progress is to be made in this area.

Naturally Continuous Processes

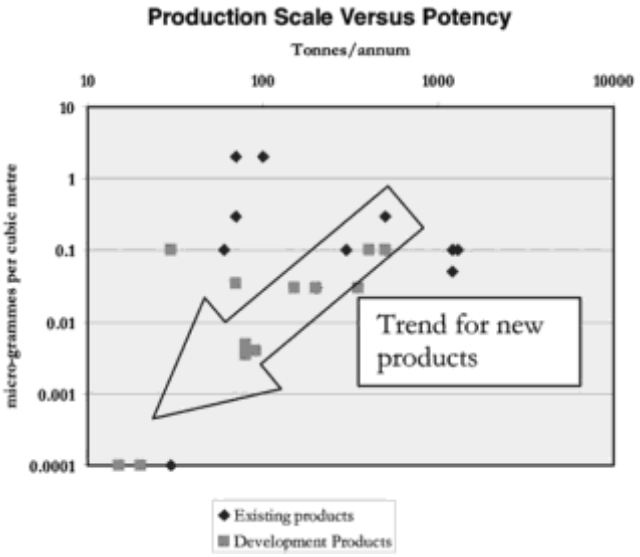
Pharmaceutical processes often contain naturally continuous processes such as in-line milling and spray drying and semi-continuous processes such as tablet compression. However, the desire to maintain the batch approach and to form intermediates for analytical testing is so great, that these processes are started and stopped to make them batch processes. This start up and shut down is likely to form out of specification material so the whole 'batch' is charged to a blender to ensure the resulting batch is homo- geneous. These naturally continuous processes are one of the obvious areas to start to change the mind set. Also, it will be easy to connect batch processes to these naturally continuous processes and run them semi-continuously and avoid the need to blend away the start up and shut down material.

Process Analytical Technology

The Food and Drug Administration (FDA) in the USA has launched an initiative under the umbrella of Process Analytical Technology (Guidance for Industry, 2004). The initiative is promoting the use of in-line analytical technology as a way of improving the development, manu- facture and quality assurance of pharmaceutical manufac- turing. Interestingly the guidance document says that the initiative is: 'Facilitating continuous processing to improve efficiency and manage variability

- using small-scale equipment (to eliminate certain scale-up issues) and dedicated manufacturing facilities;
- improving energy and material use and increasing capacity'.

Figure 2. Typical product portfolio (Cliff, 2004).



Process analytical technology has been successfully applied in a number of areas, laser diffraction for on-lineparticle size analysis during in-line milling, near infra-red (NIR) technology for determining blend homogeneity and NIR technology for the on-line determination of moisture content. These successes have demonstrated the potential for these methods to be used to control continuous pharma-ceutical processes in real time.

Reduced Waste

One of the major advantages of continuous processing should be the reduction in the amount waste produced asa result of higher yield under better control. With environ- mental legislation become ever more restrictive and the cost of waste disposal rising rapidly, this is likely to a major driver for the improvements to pharmaceutical processes.

Reduced Energy

Batch processes are inefficient users of energy. They require large fixed utility generation equipment that is subject to large variations in load. A recent study by Thomas (2004) showed that by converting a batch process to a continuous process many of the utility and energy requirements could be cut to as much as 95%. One of the major area of saving arises from the heating, ventilatingand air conditioning savings arising from a much smaller facility. Similarly, an actual conversion (Thomas and Ramsay, 2004) from batch to continuous realized signifi- cant savings by eliminating the need for 16 000 tonnes of solvent and improving equipment efficiency from 30% to greater than 81%.

Reduced Cost

With reduced waste and reduced energy, cost savings should accrue. Savings are also likely to occur in labour costs. The handling of batches of solids is nearly always labour intensive and close coupled continuous processes could overcome this problem.

Cost savings should also arise from smaller equipment (because of smaller utility needs as well as improved effi- ciency) but also form the space saved.

Since secondary pharmaceutical manufacturing space costs in the order of
$3000 per m^2 there is a lot of potential for capital cost saving in this area.

THE MIND SET

The Current Mind Set

The current mind set is perhaps typified by following words from Guidance for Industry (2004):

> Unfortunately the pharmaceutical industry has been hesitant to introduce innovative systems into the manufacturing sector for a number of reasons. One reason often cited is regulatory uncertainty, which may result from the perception that our existing regulatory system is rigid and unfavourable to the introduction of innovative systems. For example, many manu- facturing procedures are treated as being frozen and many process changes are managed through regulatory submissions.

This is also true in the European Union, Lennart Ernerot (2004) suggests that the EU directives and GMP guidelines emphasize the need to consider batches and homogeneity.

The ideas and approaches of the regulatory bodies have permeated through the minds of all of the professionals within the pharmaceutical industry so that they are reluctant to move away from the tried and tested approach. It is not possible to blame any one group since they have all become used to this environment.

Changing the Mind Set

There is now a window of opportunity to change the mind set of the professionals within the industry. The FDA have launched the Process Analytical Technology initiative that recognises that new approaches are required if product quality is to be improved. They have also recog- nized that continuous processing has a role to play.

The regulatory authorities have recognised that a time basis can be used instead of a batch basis for the release of finished products. They have also recognized the value of parametric release whereby a product is released by the Qualified Person on the basis that it has been manu- factured in compliance with a number of parameters. Actual final analytical testing does not take place untilafter the material has been released.

Importantly the quality assurance professionals are starting to change their views, Michael Warmuth (2004), Corporation Divisional VP, Corporate Quality Operation, Abbott Laboratories, said:

> Today the pharmaceutical industry focuses on R&D, with less emphasis on operations and maintenance and continuous improvement. Speed to market, no matter what production efficiency, was considered the key to success. But now the development of blockbuster drugs has slowed down the indus- try now needs to refocus its energies. The paradigm shift that the industry must make, to move forward, will be to gradually change from batch processes to continuous ones.

To make use of the window it will be necessary to have a strong group of people who will act as project champions and have the will to progress the necessary changes. In the author's opinion, Chemical Engineers are uniquely positioned to act as project champions because their education and experience provides them with the necessarytools and skills. Also, their drive will be enhanced by knowing that the reputation of the profession is likely tobe greatly enhanced by helping to make the changes. However, success will depend on much more than justtechnical skills.

To ensure that all the relevant professionals are engaged in this exercise it will be necessary to focus on the follo- wing aspects.

- The potential to increase product quality thus helping to diffuse the concerns of quality assurance professionals and regulatory bodies. Also the potential to increase the benefit to the users of medicines in term of increased efficaciousness and reduced side effects.
- The technical solutions required to allow pharmaceutical products to be produced continuously.
- The benefits to pharmaceutical producers in terms of the speed to market for new products and/or in reducedproduction costs.
- The benefits to everyone resulting from improved occu- pational health, safety and environmental performance.

As well on focusing on these aspects it will be beneficial to keep the focus as tight as possible and initially attack the batch processing mindset in two distinct areas. Firstly, there is one area where continuous processing should be accepted more readily than other areas, this is the primary productionof APIs by chemical synthesis. The second area is the sec- ondary production of tablets, change will be more difficult in this area but since more than 80% of pharmaceutical products are sold as tablets, it is very important to change the mind set in this area.

Example 1—API chemical synthesis

APIs manufactured by chemical synthesis will benefit significantly from continuous production and implemen- tation should be relatively straight forward particularly for those steps upstream of the critical step where GMP require- ments are less onerous. This is because simpler to understand and control continuous processes should improve product quality, reduce SHE issues and reduce waste. Perhaps most importantly of all, allow the time to market to be reduced due the elimination of the need to scale-up the process.

The papers presented at the 2004 Conference 'Switching from Batch to Continuous' demonstrated that there is significant interest in this area. Research within universities has demonstrated that the required chemical reactions can be carried out in continuous reactors that can be numbered up to the required production scale. There are however, a number of stumbling blocks. Firstly, in order to move into secondary processing the products will need to be separated as solids and this area does not appear to have had much attention. Secondly, it is difficult to change existing pro- cesses to continuous processes because of the embodiment of the process in manufacturing licences.

The author is in no doubt that the focus will move to the solid separation problem once continuous processes are developed for new products. It is the speed to market factor that is most likely to drive this change and once a continuous route is chosen it will be locked in by the man- ufacturing licence. Also, if it can be demonstrated that an API produced continuously is purer than one

produced by a batch process then the regulatory authorities will be push- ing for as many new APIs to be manufactured continuously as is possible.

Moving from batch to continuous processing in this area is currently being championed by a number of organization including the Institution of Chemical Engineers and the Crystal Faraday Partnership. Their annual conferences are providing a show case for the new technology and creating significant interest.

In summary, the change of mind set needs to take place in the development departs of the pharmaceutical manufac- turers where there is the opportunity to introduce conti- nuous process for new products, thereby author has already taken a lead in this area by setting a working group under the auspices of the Institution of Chemical Engineers Pharma Subject Group (PSG). This working group includes a wide cross section of engineers and the scientists from the pharmaceutical industry. The objectives of this group are:

To make use of the naturally continuous processes to overcome the conservatism of the industry by adapting the stages of the manufacturing process so that conti- nuous concepts can be developed with well known and trusted processes, thereby minimizing commercial risk.

To examine the product quality benefits that can arise from using continuous process and how the use of 'Process Analytical Technology' will be simpler with continuous processes. Also to quantify the impact of the poor control resulting batch processing on product quality.

To set up a precompetitive demonstrator project with the intention of demonstrating the practicality and flexibility of producing pharmaceutical products in tablet form using a continuous process.

To investigate the scale down problem with particular emphasis on allowing production lines to be dedicated and thereby reduce the need for extensive down time for between-product cleaning.

To fully quantify the benefits of continuous processing in terms of safety, occupational health, environmental, issues in particular, COSHH, ATEX and IPPC; including the impact of reduced inventories resulting for compact continuous processes.

To fully quantify the achievable reduction in product cost.

To investigate the impact of continuous processing on the whole supply chain where there is opportunities for significant simplification and cost saving.

To develop sources of funding that will allow the tech- nology to be moved forward independent of immediate commercial requirements.

It is the general view of those involved that the technical issues will be easier to overcome than the softer issues of the natural conservatism of a highly regulated industry. However, in spite of the attraction of making the full jump to a continuous process, taking small steps forward is likely to be more effective in overcoming conservatism and reducing commercial risk.

An example of this kind of step forward would be the use of the naturally continuous processes of milling wet granulate before drying and milling the dry granulate and replacing the batch drying with continuous drying. These three stages are at the heart of the traditional granulation process and the batch drying is very energy and space intensive and significant benefits could be achieved by just changing the drying process. If the rest of the process remains unaltered confidence can be built allowing this technology to permeate into the API manufacturing indus- try and overcoming the difficulty of the manufacturing process being embodied in the manufacturing licence.

Example 2—Tablet manufacture

Although the manufacturer of tablets is heavily focused on solids and materials handling, Chemical Engineers have built up a significant amount of expertise in this tech- nology and can also act as champions in this area. The

up in continuous processing and further continuous steps added over time.

What are the Chances of Success?

A number of companies and groups of professionals have tried to make this move in the past. In view of the poor per-formance of batch process illustrated above, it would have been expected that this move would have been easy to make. However, the conservatism of the regulators and

the industry has fixed batch processing into the mind set and attempts to move to continuous processes have notbeen a success.

However, recent changes have increase the likelihood of success.

The regulatory authorities, particularly the FDA have begun to realise that batch processes are not as easy to bring under control as continuous process.

Research projects are demonstrating that many of the required chemicals can be made to a higher purity with continuous processes than batch processes.

The pressure to bring new products to market is signifi- cantly greater than it used to be.

The cost of manufacture is becoming more important as established products reach the end of their patent life.

The pharmaceutical industry is coming under increasing pressure to improve their safety, occupational health and environmental performance.

Chemical engineers have become more and more involved in the pharmaceutical industry over time and they have now realised that they are in a lead position to drive forward the technologies to make this change.

In the discussions that the author has had with other tech- nical professionals there has been a strong recognitions the softer people orientate issues will be more difficult to over- come that the technical issues. This acceptance is causing the group set up by the PSG to look at ways of meetingthe challenge of the people issues.

With technical professions spending time thinking about how to get their ideas generally accepted and bearing in mind these significant changes listed above, along with amount interest that has been shown and the progress that has been to made to date; the chance of success appear tobe high.

We should also remember that there are significant benefits to moving to continuous processes. The benefits will affect all parts of the industry and have an impact on the speed to market, product quality, facility capital cost and product cost as well as leading to a marked improve- ment in safety, occupational health and environmental factors. These benefits will be more marked now than they would in the past because the climate of the pharma- ceutical industry has made all of them

significantly more important. Such as the strengths of these benefits that they should far outweigh the negative effects of conserva- tism and concerns about the commercial risk. As Michael Warmuth (2004) said, without the blockbusters to carry the industry forward a paradigm shift is required to embrace the benefits of continuous processing.

THE CHEMICAL ENGINEER'S CHALLENGE

As a profession, Chemical Engineers must now ensure that they are involved with pharmaceutical product of their efforts to improve efficiency, I believe that the Chemical Engineers working within the pharmaceutical industry are duty bound to set about changing their own mind set and helping to change that of others.

If this opportunity is missed, it is unlikely that the profession will have another opportunity to have such an impact on the industry and the initiative will pass to a different group of professionals. It is unlikely than any other group will have the required skill to reap all of the benefits to mankind that Chemical Engineers can and we will all be the poorer for their failure.

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develop- ment in the laboratory, so that they can use their skills to ensure that the most efficient process is used to manufacture pharmaceutical products. If they work together with the other professionals in the industry they have the opportunity to bring about the level of change that came about within the oil and gas industry during the 1950s and 1960s. Since the health mankind and the industry's impact on the natural environment of the world will be to a great extent dependent

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In-hospital de prescribing in the real world – a clinician-led approach to hyper poly pharmacy

Pulicherla Manju Bhargavi, M. Vasavi, Dr. B. V. Ramana

Abstract

Insufficient information on how to safely stop taking many medications has aided in the spread of polypharmacy. The chance to begin deprescribing is particularly useful during hospitalization. Pharmacists or multidisciplinary teams often lead deprescribing efforts, which are generally well-received by patients and have little risks. However, only a small number of research have looked into therapies that can really be used by doctors in the clinic.

The study's goal is to determine whether or not a deprescribing initiative guided by clinicians can be successfully implemented on an acute general medicine ward. Procedures Patients with hyperpolypharmacy (> 10 drugs) were subjected to a comprehensive intervention including (a) deprescribing education sessions and (b) a deprescribing alert in their bedside files. We used a historical cohort research design to compare the intervention cohort's data to those of a control group from the past. After the intervention was over, a sample of the group was queried to gauge sentiments about describing.

Conclusions Out of a total of 1333 patients enrolled, 1169 had full data sets analyzed (nintervention = 888, ncontrol = 281). Despite a drop in the prevalence of hyperpolyphar- macy from 28% to 26% in the intervention group, this difference was not statistically significant (net change = —1, IQR = —2-0; p = 0.26). Furthermore, the intervention did not lead to a statistically significant increase or decrease in medication use among any of the other categories. Many people who were interviewed about deprescribing agreed that they were taking too many drugs. In conclusion, we showed that it is possible to implement clinician-led deprescribing programs even in resource-poor, high-volume inpatient units, despite not finding a statistically significant impact of the intervention. Further research in large inpatient cohorts is needed to examine the long-term patient outcomes and harmful effects of medications after simple, creative deprescribing methods in hospitals.

Keywords: Deprescribing, Hyperpolypharmacy, Polypharmacy, Acute Medical Ward, and Clinician-Led.

INTRODUCTION

Many medicinal treatments have clear reasons for starting, but there are less guidelines for when to stop taking a prescription that has been given for lengthy periods of time. In other words, it is not normal practice to stop using a medicine for a chronic ailment after therapy has begun. Because of this, polypharmacy and hyperpolypharmacy have become more prevalent, especially among elderly patients. 1 The World Health Organization (WHO) defines polypharmacy as the use of five or more medications at once2, and the term hyperpolypharmacy might be used to describe the use of ten or more drugs at once. 3 Although statistics from other published publications reveal figures ranging from 36% to as high as 91%,4 the estimated incidence of polyphar- macy in older individuals on inpatient wards in Australia is 48%.

5–8 One negative aspect of polypharmacy is that it is often linked to the use of potentially inappropriate drugs (PIMs), in which the risks of adverse drug events are deemed to exceed the therapeutic benefits of the drug being taken. 9, 10 Polypharmacy is problematic since it has been linked to not just negative health outcomes but also functional decline, falls, disorientation, and increased healthcare expenditures.

Pharmaceutics

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treatment in a hospital, and a higher risk of dying. 11 When many medications are used, the risk increases because of the different ways they interact with one another and the different ways they could cause unwanted side effects. Sedatives, antipsychotics, antidepressants, anticoagulants, anti-cholinergics, and antihypertensives all fall within this category. 9, 10

Deprescribing, or the elimination of unnecessary medicine with the aim of enhancing clinical results, is one method that has shown promise in this regard.

12 While being hospitalized is a good chance to start deprescribing, there are several obstacles in the way. Among the difficulties doctors confront include limited deprescribing guidelines, prescriber confusion, a lack of time, and an absence of evidence-based counseling. 13 Furthermore, it might be challenging and counterproductive to closely adhere to deprescribing guidelines or tools since the process has to be individualized, and patient-specific methods are linked to better results. 14 Concerns about recurrence of symptoms, reluctance to change, and a lack of information about deprescribing are all examples of patient-related obstacles to deprescribing. 13 Both pharmacist- and MDT-led deprescribing treatments should be reviewed by the medical team before being implemented.

15 Various methods were employed in earlier research to help in deprescribing suggestions. Many PIMs have been found with the use of the Beers Criteria. 10 Another intervention designed to cut down on PIMs and spot any prescription omissions is the STOPP (Screening Tool of Older Persons' Prescriptions)/ START (Screening Tool to Alert to Right Treatment) cri- teria. 9 There have been other deprescribing experiments that have relied on implicit criteria, such as evaluative questionnaires and consultations with the patient's primary care physician (GP). 16 It has been shown in previous research that medication reduction is possible after a deprescribing intervention, with normal median reductions ranging from a drop of one to three medicines per patient. 17–19 Deprescribing has been demonstrated to be effective in reducing the amount of drugs a patient is on, and it has also been proved to be safe, which might result in fewer adverse drug reactions and fewer unscheduled hospitalizations. 16, 17, 19

Deprescribing has been shown to improve clinical outcomes including depression, mental health, function, and frailty.

The literature on the impact of deprescribing on quality of life, falls, and cognitive function is conflicting. 16, 17.\s16, 17, 19 Prescription medication overuse and PIMs may be reduced by the use of deprescribing techniques, as shown by the available data. Targeted interventions have been shown to be successful, with 72%-91% of deprescribing recommendations provided by pharmacists or MDTs being put into practice. 16–19 In addition, a Canadian research looking at the financial effects of deprescribing indicated that patients may save an average of CA$94.28.18 per year when their pharmacists used the STOPP criterion.

The lack of a dedicated pharmacist, large multidisciplinary teams (MDTs), and generally short durations of stay might make it difficult to adopt most existing techniques for deprescribing on an acute general medicine ward. Therefore, a concrete intervention that is simple for a hospital-based doctor to undertake at the bedside is required to enable deprescribing.

For this reason, we evaluated a real-world, clinician-led intervention to decrease polypharmacy in a busy, acute general care ward that lacked an electronic medical record system as part of a retrospective cohort research. The intervention's efficacy was the major focus of our investigation. The feasibility of such an intervention in this context was a secondary goal of this study. We postulated that a deprescribing strategy would be practically and clinically practicable in reducing overall drug loads, without impeding physicians' discretion.

METHODS

Intervention

To aid in the decline of polypharmacy, a multimodal intervention was developed to enhance doctors' knowledge and shift their perspective on deprescribing. This was accomplished in two stages: first, doctors were educated, affecting all patients in the intervention group; second, individuals with hyperpolypharmacy were educated. During the first stage, top medical professionals conducted weekly education sessions regarding deprescribing during morning clinical handover meetings. Sessions discussed the research supporting deprescribing, referencing a number of relevant standards and resources, including the Beers Criteria developed by the American Geriatrics Society. 10 Education sessions occurred continuously during the intervention period and took the form of brief oral presentations (often between two and three minutes) given to senior and junior medical personnel, senior nursing staff, pharmacists, allied health professionals, and medical students. The second step was to have all clinical and ward personnel engaged in the care of patients with hyper- polypharmacy fill out a deprescribing alert form (Figure 1) and put it on the front cover of the patient's bedside folder.

was bolstered by interdisciplinary team meetings held daily in the ward and educational seminars.

In addition, the investigator group (consisting of the hospital's Director of General Medicine, the ward's Nurse Unit Manager, and the hospital's Director of Pharmacy) kept their respective teams updated on the project through regular email correspondence and in-person meetings. The medical team during rounds, the ward pharmacist during admission medication reconciliation, the nursing staff during medication rounds, and the junior medical staff during charting of routine prescriptions are all instances when hyperpolypharmacy was found and the alert form was established. At the conclusion of each workday, an investigator checked the medication records of all admitted and discharged patients to see whether any of them had hyperpolypharmacy and, if so, if an alert form had been properly filed away. In the event that a patient had been forgotten, the ward personnel would get a reminder email. The notification form was developed.

It is highlighted in the patient's folder with purposeful use of color and font size to stand out during the daily morning ward round. Moreover, the form was laminated so that it could be used more than once, meaning that it was not thrown away when a patient was released. Forty alert forms were kept on hand in the ward at all times for quick use.

Simple instructions for the medical staff were included in the warning form, and six high-priority drug classes that should be considered for deprescribing were highlighted: statins, anti-hypertensives, proton pump inhibitors (PPIs), opioids, anticoagulants, and psychiatric drugs. These medications were regularly found in patients admitted to the ward and are included in the Beers and STOPP criteria as being acceptable for deprescribing9, 10. Otherwise, it was up to the treating medical teams to deprescribe on their own.

Study Cohort

Patients hospitalized to the General Medicine ward at Maroondah Hospital in Ringwood, Melbourne, Australia, were included in the research group. Patients who received the intervention between January and July 2018 (the study cohort) were followed and compared to a separate group of patients who did not get the intervention between September and October 2016 (the control group) (historical control group).

Collecting Information

Medication used during hospitalization and after release was documented (see the Supplementary Material for data col- lection methods). To simplify matters, just the active component of typical drugs was included in the tally. Regular drugs did not include those that were short-term in nature or had an established end date (such as antibiotics or brief courses of oral or topical corticosteroids). Also excluded were medications that required regular administration but were not included because of their minimal risk of drug interactions and harmful effects, such as topical skin moisturisers, ocular lubricants, vitamins, minerals, and plant or animal extracts. A drug was considered deprescribed only if it was a regular medicine at admission but no longer was upon discharge. However, dose decreases were not considered deprescribing. The confidentiality of the data was protected by keeping the treating physicians in the dark about the analytical criteria for the medications being used.

After discharging from the hospital, patients who had been exposed to the intervention and who were willing to be contacted again were polled about their feelings regarding their drug regimen and the concept of deprescribing. They were asked questions on a five-point Likert scale by an investigator who was unaware of whether or not the patient was in the intervention or control group. Patients' Attitudes to Deprescribing (PATD) questionnaire items were selected since they were shown to be most predictive of deprescribing intent. 22

Analytical Statistics

RStudio was used for the data analysis (Version 1.3.1093, Posit PBC, Boston, MA, USA). The Shapiro-Wilk test was used to check the normality of the 'net change' data (i.e. difference in number of drugs from admission to discharge) for each cohort. Due to the non-normal distribution of the data, the Mann-Whitney U test was used to evaluate the disparity in 'net change' between the control group and the experimental group. Influence size was determined by determining the Mann-Whitney U's r value. The statistical threshold for significance was set at a p value of less than 0.05. You may learn more about the techniques used in statistical analysis by checking out the supplementary materials.

RESULTS

There were a total of 1333 individuals in the cohort, albeit only 1169 had full drug dataa. Of the 1052 patients who were part of the intervention group, 888 had full medication dataa available. Some 281 patients were randomly selected to serve as the historical control group. The Supplemental Materials provide a recruiting process flowchart (Figure S1). From admission to discharge, there was a small rise in the rates of polypharmacy and hyperpolypharmacy in both the control and study groups. While hyperpolypharmacy was still somewhat common among the study population, its frequency fell from 28% to 26%. The characteristics of both cohorts are included in Table 1, as well as the rates of polypharmacy, hyperpolypharmacy, and high-risk prescription types at both admission and discharge.

In none of the overall cohorts did the number of medications alter (IQR -1-1) between admission and discharge. There was no discernible net change in either cohort, but when the data was narrowed down to only those patients who had

polypharmacy, a statistically significant difference in net change emerged between the two groups (p = 0.009). There was no net change in the control group (0, IQR —1-0) but there was in the research cohort (—1, IQR —2-0) after excluding patients with hyperpolypharmacy. However, there was no statistically significant difference between the two groups. There was no statistically significant difference between the control and study groups when individual high-risk medications were included. Table 2 provides a summary of the data, and figures are provided in the Appendices (Figure S2).

Patient Opinions on Drug Reduction

After being released from the hospital, 30 patients in the intervention group shared their thoughts on their drug regimens and their views on deprescribing. Two-thirds of patients felt they were taking too many prescriptions and wanted to stop taking at least one. While the majority of patients were pleased with their current medicine, approximately a third were not.

. These results are summarised inFigure 2.

Table 1 Characteristics and prevalence of medications in historical control and study cohorts

Group	Control cohort, *n* (%[a]) *n* = 281	Study cohort, *n* (%[a]) *n* = 888
On admission		
Patients with polypharmacy	159 (57%)	610 (69%)
Patients with hyperpolypharmacy	61 (22%)	253 (28%)
Any medication	214 (76%)	828 (93%)
Opioids	75 (27%)	251 (28%)
Antihypertensives	130 (46%)	518 (58%)
Anticoagulants	112 (40%)	408 (46%)
Proton pump inhibitors (PPIs)	104 (37%)	406 (46%)
Psychotropics	97 (35%)	375 (42%)
Statins	81 (29%)	316 (36%)
On discharge		
Patients with polypharmacy	162 (58%)	621 (70%)
Patients with hyperpolypharmacy	64 (23%)	230 (26%)
Any medication	217 (77%)	856 (96%)
Opioids	94 (33%)	354 (40%)
Antihypertensives	121 (43%)	443 (50%)
Anticoagulants	112 (40%)	387 (44%)
PPIs	111 (40%)	417 (47%)
Psychotropics	97 (35%)	391 (44%)
Statins	75 (27%)	288 (32%)

[a]Percentage of total, control, or study cohort.

DISCUSSION

To date, there hasn't been a lot of research done on deprescribing in hospital settings, so this study is a welcome addition. 16–21 However, its methodology is unusual in that it combines a first stage of education for physicians with an intervention based on visual alerts. Instead of relying on a more systematic, inflexible strategy to alert placement that would be impossible to execute in a busy, resource-poor situation, an opportunistic approach was used to assure sustainability of the intervention. The two parts of the intervention were simple to set up and keep going. While previous interventions have usually been pharmacist-led or directed by a multidisciplinary team (MDT), the intervention itself allowed for more clinician autonomy to give a more straightforward and easily accessible manner of deprescribing. 17–21 It also presented an original Australian viewpoint on dealing with polypharmacy in an emergency inpatient medical facility. The visual warning is similar to the warnings shown on electronic medical record systems when some medications are about to expire. The paper form, on the other hand, was expected to offer a more noticeable signal to the physician, since electronic notifications have a propensity to be disregarded and frequently do not improve patient care. 23

For either the "all patients" or the "polypharmacy" group, the intervention did not significantly improve deprescribing rates. Similarly, it seemed that none of the high-risk pharmaceutical categories were affected. The number of drugs taken by patients with hyperpolypharmacy was lower in the study cohort than in the control cohort at discharge (-1, IQR -2-0), albeit this difference was not statistically significant. Patients with hyperpolypharmacy are at the highest risk of medication-related adverse effects and have higher overall morbidity and mortality24; therefore, they are the group that will benefit the most from deprescribing, even though this shift was not replicated in other subgroups of the study cohort. However, it is debatable as to whether or not a decrease in drug count that is too modest to be statistically significant has any therapeutic significance at all. If this modification were to minimize medication-related side effects and increase patient quality of life, it would need to be connected to long-term patient outcomes. Patient-specific deprescribing treatments were linked with significant decreases in mortality in people with polypharmacy, according to a study published by Page et al.14, while general educational interventions did not have this effect. Given that patient-specific therapies are not always possible in resource- and time-poor settings, we think it is necessary to examine teaching techniques. Moreover, deprescribing benefits extend beyond this, and a decrease in unpleasant effects and drug interactions connected to medications may still be attainable via deprescribing educa- tion for physicians even if mortality benefits are not.

As was previously mentioned, the drop in drugs found in our trial was not statistically significant, and the decrease in the hyperpolypharmacy group was somewhat smaller than what has been produced by prior deprescribing interventions17-19. Due to time constraints, a lack of patient history, and the likelihood that patients will need drugs to manage acute illnesses, deprescribing in hospital settings is undeniably challenging. In outpatient or primary care settings, it is frequently easier to identify

and discontinue unneeded drugs. For patients who have experienced or are at high risk of medication-related side effects or interactions, we feel that an individualized approach to beginning deprescribing in an inpatient environment is useful and may offer benefit. Improved results have been linked to individualized deprescribing, according to recent meta-analyses. 14 Similar gains in clinical outcomes and patient satisfaction have been shown using the Garfinkel approach of deprescribing, which is guided by clinicians.

Table 2 Median net change in number of medications from admission to discharge, stratified by type of medication

Group	Control cohort[a] (n = 281)	Stu y cohort[a] (n = 888)	p value[b]
All patients			
All medication types	0 (IQR —1–1)	0 (IQR —1–1)	p = 0.58
Opioids	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.78
Antihypertensives	0 (IQR —1–0)	0 (IQR —1–0)	p = 0.90
Anticoagulants	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.29
Proton pump inhibitors Proton pump inhibitors (PPIs)	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.49
Psychotropics	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.49
Statins	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.82
Patients with polypharmacy on admission			
All medication types	0 (IQR —1–1)	0 (IQR —2–1)	p < 0.01*
Opioids	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.26
Antihypertensives	0 (IQR —1–0)	0 (IQR —1–0)	p = 0.59
Anticoagulants	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.18
Proton pump inhibitors (PPIs)	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.53
Psychotropics	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.75
Statins	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.58
Patients with hyperpolypharmacy on admission			
All medication types	0 (IQR —1–0)	—1 (IQR —2 to 0)	p = 0.26
Opioids	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.42
Antihypertensives	—1 (IQR —1–0)	—1 (IQR —1 to 0)	p = 0.57
Anticoagulants	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.73
Proton pump inhibitors (PPIs)	0 (IQR 0 to 0)	0 (IQR 0 to 0)	p = 0.86
Psychotropics	0 (IQR 0 to 0)	0 (IQR 0 to 0)	p = 0.17
Statins	0 (IQR 0–0)	0 (IQR 0–0)	p = 0.84

IQR = interquartile range.
[a]Data are presented as 'Median net change in number of medications from admission to discharge (quartile 1 – quartile 3)'.
[b]As per Mann–Whitney U test comparing median net change in medications in study cohort compared to median net change in medications in control cohort.
*p values < 0.05 were considered statistically significant.

contentment and signs and symptoms16 The burden of overtreatment is likely to be underestimated by several recommendations and generalized methodologies, such as Beer's Criteria10 and the START/STOPP criteria9, to tally each patient' PIMs. As a result, we believe that deprescribing should only be attempted after thorough review of each patient's unique circumstances by the treating team. Our interventions had a small impact on overtreatment rates, but they provide credence to the concept that a clinician-led strategy to deprescribing is doable and warrants further investigation as a form of individualized deprescribing.

While the deprescribing in this initiative was initiated by clinicians, we acknowledge the need of considering polypharmacy from a variety of angles. Deprescribing may be accomplished with the help of multidisciplinary teams (MDTs), and pharmacist participation in particular has been demonstrated to be beneficial. 17–19 Nevertheless, primary care physicians (PCPs) should not be overlooked in discussions about how to begin and maintain deprescribing. Considering primary care physicians' continuing interaction with their patients, sustained

Deprescribing is impossible without the help of primary care physicians. According to Nguyen et al.25, primary care physicians place a premium on receiving up-to-date information on their patients from hospital-based doctors while those patients are still in the facility. If GPs are to understand and continue deprescribing following a patient's release, it is crucial that discharge summaries be thorough and provide sufficient information regarding the reason for deprescribing.

Patients are a crucial group to consider as a stakeholder in deprescribing. Deprescribing is more likely to be successful and long-lasting if patients' drug preferences are taken into account. Based on our analysis of patient opinions towards deprescribing, it seems that the vast majority of patients want to lessen their drug load. This is particularly crucial to think about in low-resource countries where the combined expense of many prescriptions may be too much for a patient to bear. Several research, both in Australia and elsewhere, seem to corroborate this, showing that patients are ready to and willing to participate in deprescribing. 26–28 On the other hand, a major

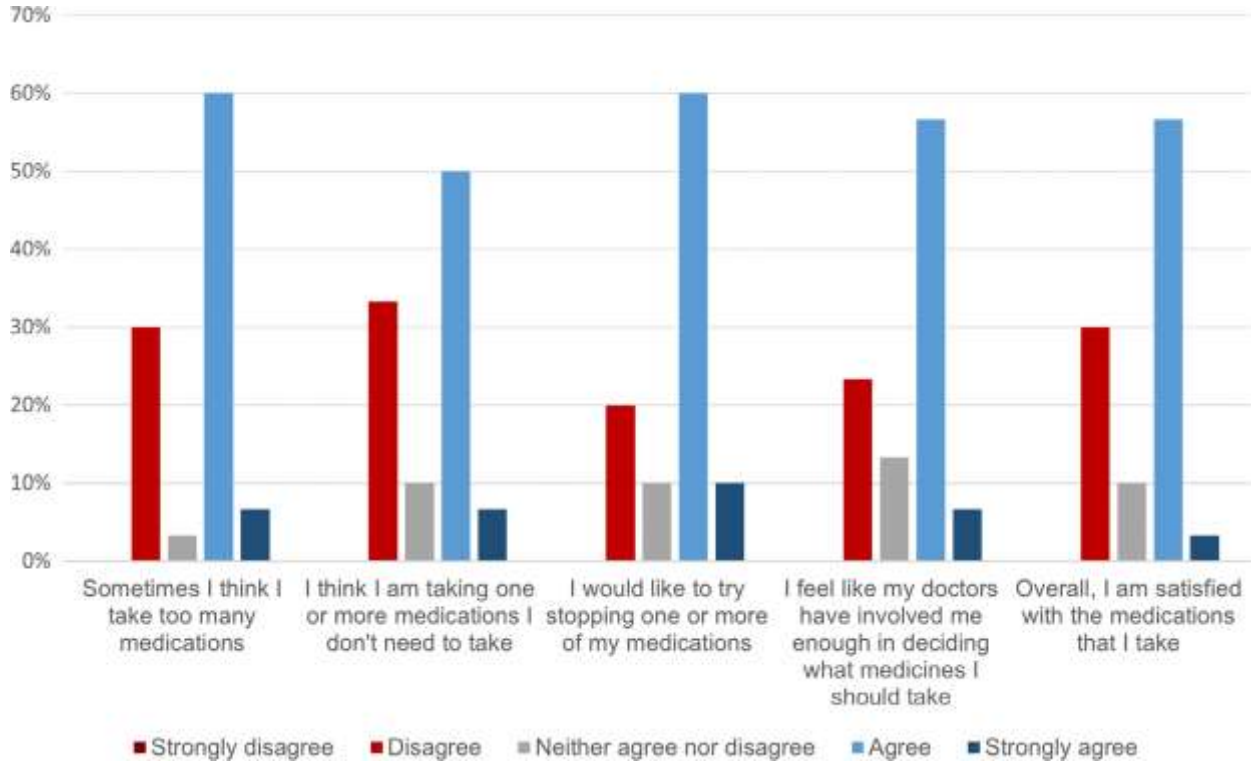

Figure 2 Patient attitudes toward their medication regimen and deprescribing as assessed by their responses to five questions recorded on a five-point Likert scale, adapted from the Patients' Attitudes Toward Deprescribing (PATD) questionnaire.

however a significant percentage of patients reported being happy with their treatment. Similar discrepancies in attitudes about deprescribing were identified by Hopper et al.29; many patients reported being satisfied with their prescriptions, but their treating doctors had better ideas of which drugs they would deprescribe. Because of this discrepancy, it is crucial that clinician-led deprescribing strategies take into account patient perspectives about their drugs. Before stopping any drugs, the patient and their doctor should have a conversation about which ones may be unneeded.

This study's merits include its large sample size (1333 total patients), which is greater than the majority of studies of this kind, and its careful documentation of different drug types, especially those used by high-risk populations. Another point in the positive direction is the inclusive nature of the process used to identify patients with hyper- polypharmacy on the ward. This'real-world,' opportunistic method demonstrated that diagnosing hyperpolypharmacy on a busy medical inpatient unit was feasible, even in resource-poor settings without electronic medical records (such as rural and regional hospitals) or the resources to produce electronic warnings. The supplementary

The use of hyperpolypharmacy warning actions may lead to unwarranted attempts at deprescribing. Determining the efficacy of alternative or complementary approaches to deprescribing in an emergency care context necessitates the exploration of novel approaches to deprescribing.

CONCLUSION

Clinicians learned not just when to deprescribe but also how and when to prescribe new drugs in these educational sessions.

Our method may have underestimated the frequency of hyperpolypharmacy since some patients with the condition may have been overlooked, particularly on weekends and during times of ward staff turnover. It's also important for the strategy to be constantly pushed at an institutional level, as it may otherwise lose steam as time goes on and people who work on the intervention leave and are replaced. Since we didn't evaluate whether or not deprescribing was warranted, we couldn't say whether or not the deprescribing that really occurred was clinically beneficial. Still, most deprescribing was probably clinically sound since it was only performed by experienced doctors who had been trained in deprescribing. Finally, the study's use of a historical control group rather than a parallel control group may have limited the ability to draw meaningful comparisons.

To more accurately monitor the long-term benefits of deprescribing and to ascertain whether or not an intervention has therapeutic significance, future studies should attempt to gather continuous clinical outcome and patient satisfaction data. Researchers might potentially learn whether comparable results were seen by linking their findings to clinical outcome data

It might be difficult for clinicians to practice deprescribing in an acute medical ward because of the urgency of patients' conditions and the limited time they spend in the hospital. Overall, individuals with hyper-polypharmacy did not show statistically significant reductions in prescriptions as a consequence of the intervention reported in this research. The majority of patients we surveyed expressed a desire to decrease their drug load, and we discovered that inpa- tient settings

provide excellent possibilities for depre- scribing to be addressed and begun. To further understand the therapeutic significance of deprescribing strategies led by clinicians, future research should focus on assessing their long-term efficacy and sustainability, as well as their connection to patient outcomes and medication-associated side effects.

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DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF VALSARTAN AND SACUBITRIL IN TABLET DOSAGE FORM

D. Reshma Bhanu, M. G. Maddilety, S. Neelofar Sulthana

ABSTRACT

Objective: A New method was established for simultaneous estimation of Sacubitril And Valsartan by RP-HPLC method. Methods: Chromatographic separations were carried using Spurcil C18 (4.6 x 250mm, 5µm) (Dikma column) column with a mobile phase composition of 0.1% OPA buffer and Acetonitrile(50:50) have been delivered at a flow rate of 1ml/min and the detection was carried out using waters HPLC auto sampler, separation module 2695 with PDA detector at wavelength 237 nm. Results: The retention time for Sacubitril and Valsartan were 3.119 and 6.851 minute respectively. The correlation coefficient values in linearity were found to be 0.999 and concentration range 12-60 µg/ml for Sacubitril and 13- 65µg/ml for Valsartan respectively. For accuracy the total recovery was found to be 99.98% and 100.28% for Sacubitril and Valsartan. The LOD and LOQ concentration for Sacubitril were found to be 0.02 µg/ml and 0.05 µg/ml and LOD and LOQ for Valsartan were found to be 0.01µg/ml and 0.04 µg/ml. The force degradation studies were performed and the results are within the limits. Conclusion: The results of study showed that the proposed RP-HPLC method is a simple, accurate, precise, rugged, robust, fast and reproducible, which may be useful for the routine estimation of Sacubitril and Valsartan in pharmaceutical dosage form.

Keywords ;Sacubitril,Valsartan, RP-HPLC, Validation.

INTRODUCTION

Sacubitril is an antihypertensive drug used in combination with valsartan[1].The combination drug valsartan/sacubitril, marketed under the brand name Entresto, is a treatment for heart failure[2]. Sacubitril is a prodrug that is activated to sacubitril at by de-ethylation via esterases[3]. Sacubitrilat inhibits the enzyme neprilysin. The most common adverse reactions with sacubitril plus valsartan included hypotension, hyperkalemia, cough, dizziness, and renal failure. Sacubitrilis chemically (S)-5-[(4-phenylphenyl)methyl] pyrrolidin-2-one. Structre shown in fig.1. Sacubitril Slightly soluble in water, sparingly soluble in dehydrated alcohol, freely soluble in methanol.

Valsartan is used to treat high blood pressure, congestive heart failure, and to reduce death for people with left ventricular dysfunction after having had a heart attack[4]. Valsartan blocks the actions of angiotensin II, which include constricting blood vessels and activating aldosterone, to reduce blood pressure[5]. The drug binds to angiotensin type I receptors (AT1), working as an antagonist. This mechanism of action is different than the ACE inhibitor drugs, which block the conversion of angiotensin I to angiotensin II. Since valsartan acts at the receptor, it can provide more complete angiotensin II antagonism since angiotensin II is generated by other enzymes as well as ACE.(B) Most common side effects include dizziness, low blood pressure, and diarrhea. Valsartan is chemically (2S)-3-methyl-2-(N-{[2'-(2H-1,2,3,4-tetrazole-5-yl)biphenyl-4- yl]methyl} pentanamido) butanoic acid. Structre shown in

Pharmaceutics

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(Approved by AICTE,P.C.l New Delhi& Permanently Affiliated to JNTUA Anantapuramu

MOU with Government General Hospital &KMC, K urnool

fig.2. Valsartan Soluble in Acetonitrile, practically insoluble in water also soluble in methanol

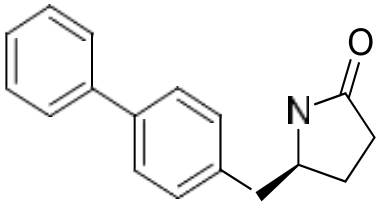

Figure 1: Structure of Sacubitril.

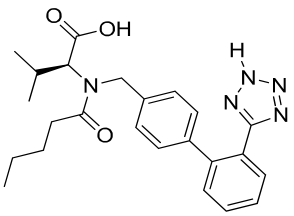

Figure 2: Structure of Valsartan.

Literature survey shows that a number of methods have been reported for estimation of Sacubitril And Valsartan individually or in combination with other drugs Those are HPLC Methods[6-16], LC-MS Methods[17], Ultraviolet spectrophotometry[18-19], However, there is only one HPLC method is reported for the simultaneous estimation of these drugs in combined dosage forms[20]. But it not explained force Degradation study.

The aim of the present study was A New Rp-Hplc Method For Simultaneous Estimation Of Sacubitril And Valsartan In Its Bulk And Tablet Dosage Form And Its Force Degadation Studies As Per Ich.

MATERIALS AND METHODS:

Chemicals and Reagents:

Sacubitril and Valsartan were obtained from Eisai pharmaceutical India pvt Ltd, Visakhapatnam. Orthophosphoric acid (Merck), Acetonitrile (Molychem, HPLC grade) and Water for HPLC (LICHROSOLV (MERCK).

Equipment and Chromatographic Conditions:

The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, PDA detector and Empower 2 software. Analysis was carried out at 237 nm with an Spurcil C18 (4.6 x 250mm, 5µm) (Dikma column) dimensions at ambient temperature. The optimized mobile phase consists of 0.1% OPA and Acetonitrile in the ratio of 50:50 v/v. Flow rate was maintained at 1 ml/min and run time for 16 min.

Preparation of solutions:

Preparation of buffer:

Take 1 ml of ortho phosphoric acid in 1000 ml volumetric flask and make up to the mark with HPLC water and sonicate for15minutes then then filter through 0.45µ filter under vacume filteration.

Preparation of mobile phase:

Mix a mixture of above buffer 500 mL (50%) and 500 mL of Acetonitrile HPLC (50%) degas in ultrasonic water bath for 5minutes. Filter through 0.45 µ filter under vacuum filtration

Preparation of diluent:

The Mobile phase was used as the diluent.

Preparation of standard stock solution:

Accurately weigh and transfer 12&13mg of Sacubitril & Valsartan working standard into a 10mL clean dry volumetric flaskadd Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 1.0 ml of Sacubitril & Valsartan of the above stock solution into a 10ml volumetric flask and dilute up to the mark withdiluent.

Further pipette 3.0ml of Sacubitril & Valsartan of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of Sample stock solution:

Accurately weigh and transfer equivalent to 12 &13mg of Sacubitril & Valsartan s (marketed formulation=130.9 mg of tablet Powder) sample into a 10m clean dry volumetric flask add about 7 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.44 micron Injection filter. (Stock solution)

Further pipette 1.0 ml of Sacubitril & Valsartan of the above stock solution into a 10ml volumetric flask and dilute up to themark with diluent.

Further pipette 3.0 ml of Sacubitril & Valsartan the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for the Sacubitril & Valsartanpeaks and calculate the %Assay by using the formulae.

METHOD:

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness androbustness as per ICH guidelines.

System suitability parameters:

To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 1ml/min for 16 minutes to equilibrate the

column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20µL of standard into Spercil C18 (4.6 x 250mm, 5µm) (Dikma column), the mobile phase of composition 0.1% OPA buffer and acetonitrile in the (50:50) was allowed to flow through the column at a flow rate of 1ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed methodare shown in table 1.

Assay of pharmaceutical formulation:

The proposed validated method was successfully applied to determine Sacubitril & Valsartan in their tablet dosage form. The result obtained for Sacubitril & Valsartan was comparable with the corresponding labeled amounts and they were shown in Table-2.

Validation of Analytical method:

Linearity and Range:

Stock solution was prepared by dissolving the appropriate amount of Sacubitril and Valsartan in 10 ml of diluent and further diluted to the required concentrations with diluent. The solution was prepared at five concentration levels ranging from 12µg/ml to 60µg/ml of Sacubitril and 13µg/ml to 65µg/ml of Valsartan. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The resulte are shown in table 3.

Accuracy studies:

The accuracy was determined by help of recovery study. The recovery method carried out at three level 50%, 100%, 150%. Inject the standard solutions into chromatographic system. Calculate the Amount found and Amount added for Sacubitril and Valsartan and calculate the individual recovery and mean recovery values. The resulte are shown in table 4.

Precision Studies:

Precision was caliculated from Coefficient of variance for six replicate injections of the standard. The standard solution was injected for six times and measured the area for all six Injections in HPLC. The %RSD for the area of six replicate injections was found. The resulte are shown in table 5.

Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day. The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found. The resulte are shown in table 6.

Method Precision:

To evaluate the method precision six individual samples solutions were prepared and calculate the % of Assay. The % RSD for the area of six standard injections results should not be more than 2%. The resulte are shown in table 7.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method. The flow rate was varied at 0.8 ml/min to 1.2ml/min. The Organic composition in the Mobile phase was varied from 45% to 55%.

LOD and LOQe sensitivity of RP-HPLC was determined from LOD and LOQ.For LOD S/N=3

For LOQ S/N=10

Where, S = Signal

N = Noise

Solution Stability:

The standard and sample solutions prepared under assay, has been kept in bench top for 24hours to perform solution stability. And inject standard and sample solutions after 24hours. The analysis performed solution stability with freshly prepared mobile phase. The % Area difference should not be more than 2%. The resulte are shown in table 8.

Force degradation Studies:

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Sacubitril and Valsartan using the proposed method. The resulte are shown in table 9.

Preparation of stock:

Accurately weigh and transfer 12.0 mg of Sacubitril and 13.0 mg of Valsatan working standards into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 1.0 ml of Sacubitril & Valsartan of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Hydrolytic degradation under acidic condition

Pipette 3 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Hydrolytic degradation under alkaline condition

Pipette 3 ml of above solution into a 10ml volumetric and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 24 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.44 microns syringe filters and place in vials.

Thermal induced degradation

Ten tablets were accurately weighed and triturated it to a fine powder form and transferred into a petri dish. The petridish contained sample was subjected to thermal stress at 70 ^{0}C for about one day .The Sample solutions were prepared as equivalent concentrations of 36 µg/mL, 39 µg/mL of VAL and SAC and transferred in vials and injected in to the HPLC system.

Oxidative degradation

Pipette 3 ml above stock solution into a 10ml volumetric flask and 1ml of 12.5% w/v of hydrogen peroxide added in 10 ml ofvolumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15min. Filter the solution with 0.45 microns syringe filters and place in vials.

Photo degradation:

Ten tablets were accurately weighed and triturated it to a fine powder form and transferred into a petri dish. The petridish contained sample was subjected to strees condition in the U.V chamber at 1.2 million lux for one week. The Sample solutions were prepared as equivalent concentrations of 36 µg/mL, 39 µg/mL of VAL and SAC and transferred in vials and injected in to the HPLC system.

RESULTS AND DISCUSSION

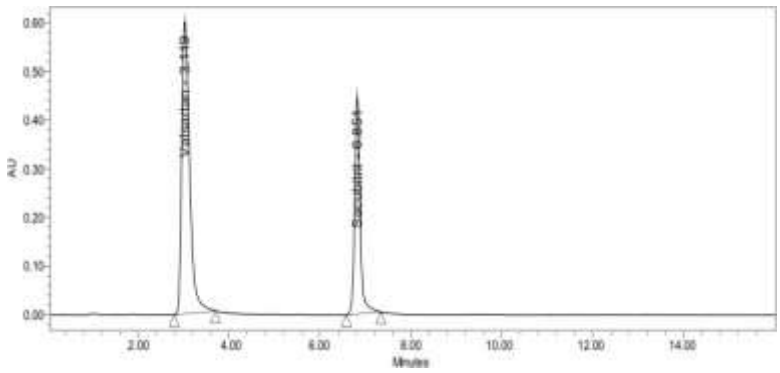

Figure 3: Standard chromatogram.

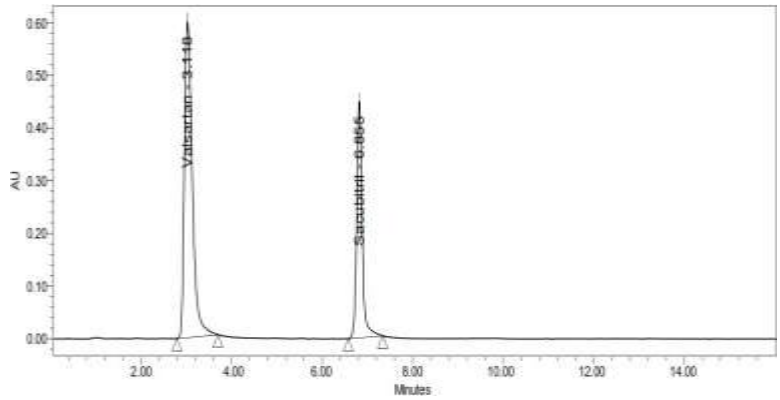

Figure 4: Sample chromatogram.

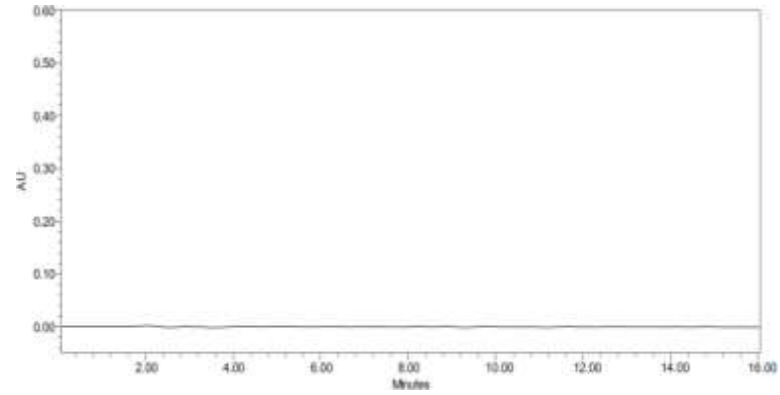

Figure 5: Blank chromatogram.

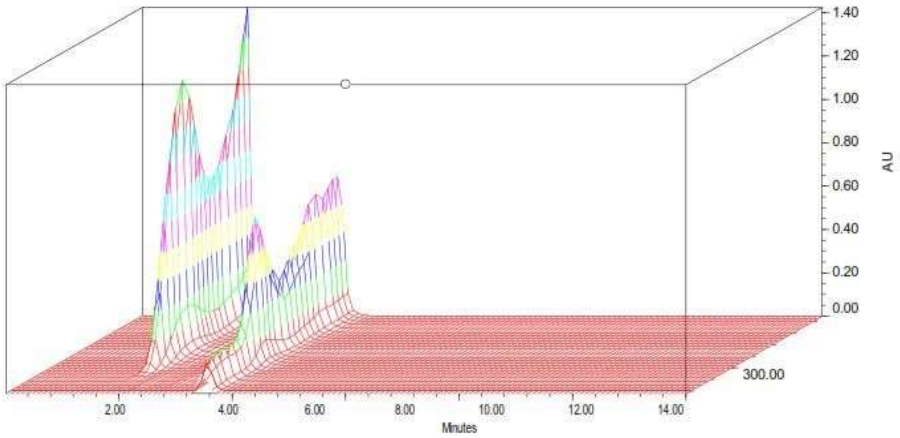

Figure 6: 3D chromatogram for standard.

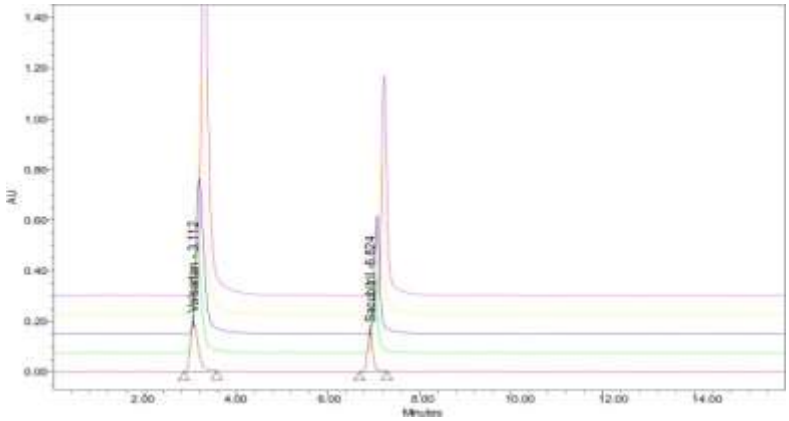

Figure 7: Linearity Overlay Chromotogram.

Table 1: System suitability parameters.

Parameters	Valsartan	Sacubitril
Retention time	3.119	6.851
USP Plate count	3558.28	17434.12
USP Tailing	1.50	1.52

Table 2: Assay results for Sacubitril and Valsartan.

	Label Claim (mg)	% Assay
Sacubitril	24	99.37
Valsartan	26	99.79

Table 3: Linearity results for Sacubitril and Valsartan.

Sacubitril		Valsartan	
Concentration(µg/ml)	Area	Concentration(µg/ml)	Area
12	307716	13	687517
24	619777	26	1203954
36	916722	39	1849001
48	1267369	52	2447459
60	1546648	65	3012348
Correlation coefficient	0.999	Correlation coefficient	0.999

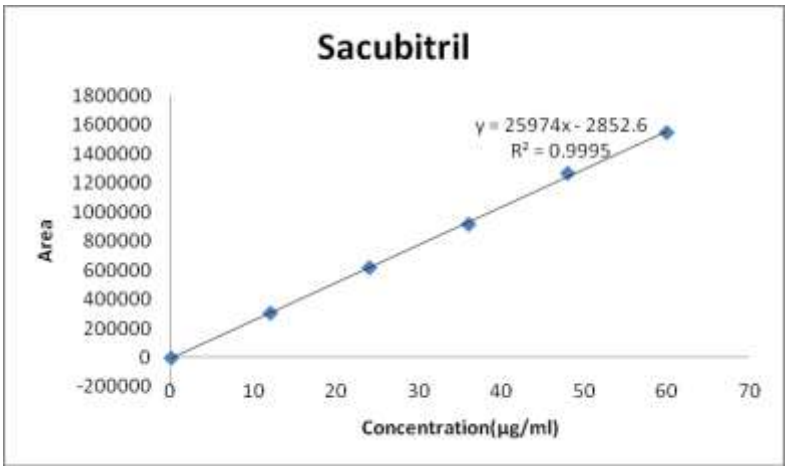

Figure 8: Linearity graph for Sacubitril.

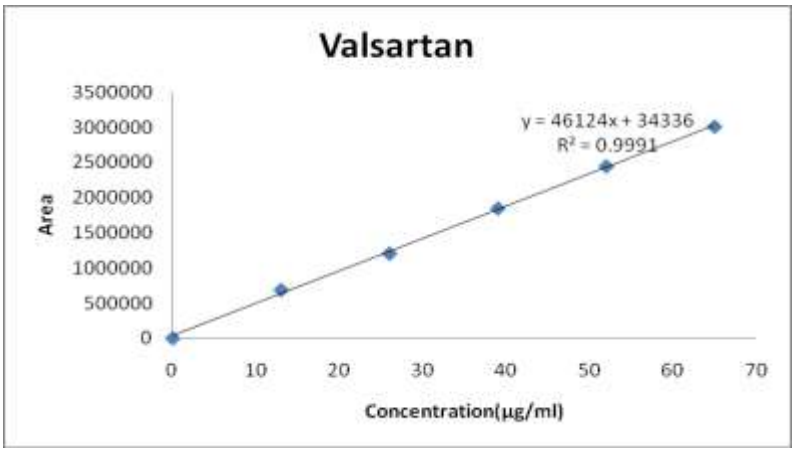

Figure 9: Linearity graph for Valsartan.

Table 4: Accuracy results for Sacubitril and Valsartan.

Drug Name	%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
Sacubitril	50%	465164	6	6.07	100.43	
	100%	913108	12	11.91	99.28	99.98
	150%	1382968	18	18.04	100.25	
Valsartan	50%	928676	6.5	6.50	100.03	
	100%	1858389	13	13.01	100.09	100.28
	150%	2804810	19.5	19.64	100.71	

Table 5: Precision results for Sacubitril and Valsartan.

Injection	Sacubutril Area	Valsartan Area
Injection-1	911197	1841184
Injection-2	910379	1845734
Injection-3	911492	1854819
Injection-4	911965	1865021
Injection-5	916979	1864411
Injection-6	916214	1868428
Average	913037.7	1866693
Standard Deviation	2814.9	11231.4
%RSD	0.3	0.6

Table 6: Ruggedness results for Sacubitril and Valsartan.

Injection	Sacubitril Area	Valsartan Area
Injection-1	915722	1862811
Injection-2	913937	1877531
Injection-3	919277	1870669
Injection-4	912006	1851835
Injection-5	900493	1872547
Injection-6	907901	1864770
Average	911556.0	1866693.8
Standard Deviation	6612.0	9023.0
%RSD	0.7	0.5

Table 7: Method precision results for Sacubitril and Valsartan.

Sample Name	% Assay for Sacubitril	% Assay for Valsartan
Method precision-1	99.74	100.63
Method precision-2	100.01	99.75
Method precision-3	99.55	99.27
Method precision-4	99.8	100.43
Method precision-5	99.9	100.31
Method precision-6	98.73	100.03
Average	99.62	100.07
Standard deviation	0.46	0.5
% RSD	0.46	0.5

Table 8: Solution stability results for Sacubitril and Valsartan.

S. No	Standard Area (Mean*3)	Standard area after 24hrs (Mean*3)	Sample area after 24hrs (Mean*3)	% Variation	% Assay
Sacubitril					
1	917883	915465	916168	0.26	99.39
Valsartan					
1	1853001	1845322	1846369	0.41	99.22

Table 9: Degradation results for Sacubitril and Valsartan.

Parameters	Sacubitril	% Degraded	Valsartan	% Degraded
Standard	917883.3		1853001	
Acid	896214	2.36	1738428	6.18
Base	861197	6.18	1711184	7.65
Peroxide	871965	5.00	1735021	6.37
Thermal	902006	1.73	1751835	5.46
Photo	899277	2.03	1770669	4.44

CONCLUSION

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Sacubitril and Valsartan in pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Sacubitril and Valsartan in pure and its pharmaceutical dosage forms.

ACKNOWLEDGEMENT

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Relationship of Serum Zinc, Insulin, and C-Peptide in Patients with Type 2 Diabetes Treated at a Tertiary Care Center

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ABSTRACT

Pancreatic beta cells have been shown to use zinc in the production and secretion of insulin and C-peptide. It is important to investigate the potential for a link between these factors, especially in respect to diabetes mellitus. Taking blood levels of Insulin, C-peptide, and zinc in people with type 2 diabetes, we hoped to analyze this connection in the Indian population. One hundred patients with type 2 diabetes were surveyed cross-sectionally in a tertiary hospital in North India (MMIMSR). Zinc levels were evaluated spectrophotometrically, whereas C-peptide and insulin levels in the serum were determined using chemiluminescence immunoassay. Serum C-peptide and insulin levels were both reported to be elevated (2.59 1.45 ng/ml and 11.24 10.57 IU/ml), whereas serum zinc levels were within reference ranges (97.82 11.27 g/dl). However, zinc levels were shown to have extremely significant inverse relationships with both serum C-peptide (r=0.649, p0.001) and insulin (r= -0.423, p0.001). Our findings suggest that zinc may have a role in the etiology and treatment of type 2 DM because of its high correlation with blood insulin and c-peptide levels in these individuals

Keywords Diabetes Mellitus,Zinc, Insulin,C-Peptide.

INTRODUCTION

Type 2 DM, which was until recently considered to be a disease of the rich countries, is now emerging in epidemic proportions in the developing countries [1]. 72.1 million people, which is about 1/6th of all the adults with diabetes in the world, live in India. The number is expected to rise to 134.3 million in 2045 [2].Significantly raised levels of insulin have been reported in type 2 diabetics in some studies [3,4]. Recent studies have also shown that C-peptide, a co product of insulin secretion, is a biologically active peptide with a host of physiological roles [5,6,7]. Raised C-peptide levels have been reported in diabetics in some studies [8,9]. Zinc, one of the most important trace elements in our body, is required for multiple steps in insulin synthesis, release and action [10,11]. Estimation of serum zinc levels in diabetic patients has yielded varying results with some authors like Basaki et al and Al- Maroof et al reporting decreased levels while Zargar et al reporting normal levels and Fujimoto et al reporting raised levels [12-15]. With the above background, the current study was planned. Serum insulin, c-peptide zinc levels were estimated in North Indian type 2 diabetic patients. The objective was to see if there was a relationship between the serum levels of these parameters which could then be explored for therapeutic benefit.

MATERIALS AND METHODS

The study was conducted in the Departments of Biochemistry and General Medicine, Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana, Ambala, Haryana on hundred type 2 diabetic patients admitted in the Department of General Medicine /attending the Medical OPD. Prediagnosed diabetic patients, >30 yrs of age, were chosen for the study. Subjects with Type 1 DM, patients receiving insulin, supplements or medications containing zinc (multi vitamins etc), pregnant and lactating women and patients with thyroid dysfunction were excluded from the study. Patients were selected randomly.

DM was defined according to the American Diabetic Association (ADA) guidelines [16], which are as under:

- HbA1C >6.5%. OR
- FPG >126 mg/dL (>7.0 mmol/L). (Fasting = no caloric intake for at least 8 h.)*OR
- 2-h plasma glucose>200mg/dL (>11.1mmol/L). OR
- In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose >200 mg/dl (>11.1mmol/L).

Clinical data

Demographic and clinical data were collected with the help of a self designed, pretested questionnaire.

Sample collection and transport

For accurate comparison to established normal values, fasting morning serum samples were obtained after an overnight fast of atleast 8 hrs. The blood was collected aseptically, preferably from anticubital vein in a plain red-top venipuncture tube containing clot activator (*BD™ Vacutainer™*) (without anticoagulants or gel barrier). Care was taken to ensure that no hemolysis took place during or after sample collection. The samples were allowed to clot for 30-60 minutes at room temperature. This was followed by centrifugation using *Remi™ R-23* research centrifuge. Serum was then separated and aliquots were made. Serum C-peptide, insulin, fasting blood glucose and zinc levels were then measured. Samples that required storage for upto 5 days were kept under refrigeration at 2-8°C. Samples that required storage for longer periods (upto 30 days) were stored at -40°C in deep freezer (*Remi™* Ultra Low Freezer).

Quantitative assays

Estimation of serum insulin and C-peptide levels was done by Chemiluminescence immunoassay technique on *Autoplex™ CLIA* (chemiluminescence immunoassay) *workstation* using *Acculite™* C-peptide and Insulin test kits (*Monobind ™ Inc.*(US)).

Estimation of zinc was done by Colorimetric method on *Pace plus* ™ semi auto analyser using *Coral™* zinc kit

Fasting blood glucose was estimated by Trinders method (GOD-POD) on *Pace plus* ™ semi auto analyser using *Erba glucose kit(Erba diagnostics Manheim, Germany*. The serum samples were analysed for glucose levels within 2 hrs of sample collection.

Statistical analysis

Statistical analysis was performed using IBM SPSS and Microsoft Excel. Descriptive analysis was performed, followed by graphical representation of different parameters in terms of mean / median and standard deviation / range. Patients were grouped on basis of age, BMI and duration of DM. Since many parameters like insulin, C-peptide, did not follow a Gaussian distribution, log transformation was performed on these parameters to normalize the distribution, as has been reported in several other studies [17-21]. Pearson's coefficient was used to analyse the different parameters for correlation and student t test was performed to analyse the hypertensive and normotensive patients for association. P value <0.05 was considered significant.

Ethical justification

Ethical approval was obtained from Institutional Ethics Committee (IEC) of the institute on the agreement that patient anonymity must be maintained, good laboratory practice, quality control ensured, and that every finding will be treated with utmost confidentiality and for the purpose of this research only.

RESULTS AND DISCUSSION

Descriptive statistics:

We found that serum insulin levels were raised in diabetic patients. Mean insulin levels were 11.24±10.57 µIU/ml (reference interval = 0-10 µIU/ml). Median, interquartile range and SEM were 7.1, 3.98-13.35 and 1.06µIU/ml respectively. Similar results were reported by Gayoso-diz et al (2013) and Islam et al (2013) [22, 23]. The raised serum insulin levels can be attributed to the fact that type 2 diabetes mellitus primarily involves insulin resistance (unlike type 1 where decreased insulin secretion is the main defect). To overcome this resistance, β cells of pancreas increase the synthesis and release of insulin, thereby leading to hyperinsulinemia.

In our study, we observed that serum C-peptide levels were also raised in diabetic patients 2.59 ± 1.45 ng/ml (reference interval = 0.78-1.89 ng/ml) Median, interquartile range and SEM were 2.00, 1.6-3.7 and 0.15 ng/ml respectively. Similar findings were reported by Kim et al (2011) and Chowta et al (2010) [8,9]. The elevation of C-peptide levels can be explained by the fact that it is produced in equimolar amounts by β cells of pancreas along with insulin by splitting of the common precursor, proinsulin. In patients with type 2 DM, the compensatory increase in C-peptide levels is attributed to the increased rate of synthesis and release of its precursor, proinsulin in an effort to reverse the hyperglycemia seen in the disease. Proinsulin, after release, splits into insulin and C-peptide.

In our study, we found that serum zinc levels were within reference intervals in diabetic patients. Mean values were 97.82 ± 11.27 µg/dl (reference interval = 80-120 µg/dl). Median, interquartile range and SEM were 96.9, 89.55-108 and 1.13 µg/dl respectively (Fig 1). Zargar et al (1998) reported slightly raised zinc levels of 112 ± 32.13 µg/dl (17.19 ±4.92µmol/L) [14]. In other studies, however, many different types of relationships have been reported between diabetes mellitus and serum zinc levels [24,15] . Decreased levels of serum zinc were reported in type 2 diabetic patients by Parham et al (2008) (76 ±16 µg/dl) [25], Ferdousi et al (2012) (72.70±8.43µg/dl) [26] and Al-Maroof et al (2006) (68.9 +/- 11.9 mg/dl) [13]. On the other hand, Fujimoto et al (1986) reported increased zinc levels have been found in patients with diabetes mellitus previously treated with insulin [15]. Similarly Rusu etal (2005) reported raised zinc levels in type 2 diabetics [27]. These differences could be at least partly due to heterogeneity in patient selection, study design and method of detection of serum zinc levels.

In our study we found no significant difference in the zinc levels in males and females (98.65 ± 11.15 in females and 97.30 ± 11.41 in males) (p=0.562). Saharia et al (2013) in their study on newly diagnosed type 2 DM cases observed that the mean serum zinc concentration was 80.83 ± 13.1 µg/dL in case of males and 77.56 ± 14.2 µg/dL in case of females. The difference was statistically insignificant (p > 0.05) [28]. Masood et al (2009) also reported similar insignificant gender difference in zinc levels (p=0.10) [24].

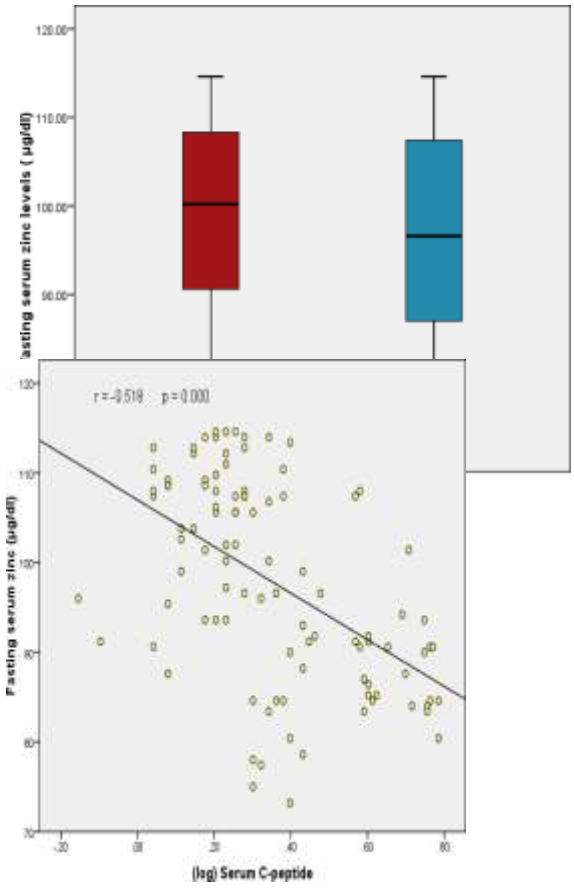

Figure 1: Median and interquartile range of fasting serum zinc levels.

Correlations:

A strong positive correlation was observed between (log) insulin and (log) C-peptide levels. The correlation was statistically highly significant (r=0.649, p=0.000). This strong correlation can in part be attributed to the fact that insulin and C-peptide are released in equimolar amounts from beta cells of pancreas. Because fasting C-peptide levels are 5 -10 % higher than those of insulin owing to a longer half life of C-Peptide, and because unlike insulin, C-peptide does not undergo first pass metabolism in liver and its concentration is not affected by interference from insulin antibodies often present in patients receiving insulin therapy, many authors have proposed the use of C-peptide as a surrogate marker insulin release and β cell function [7,29].

A strong negative correlation was found between serum zinc and (log) insulin levels. The correlation was highly significant (r= -0.423,p<0.001) (fig 2)

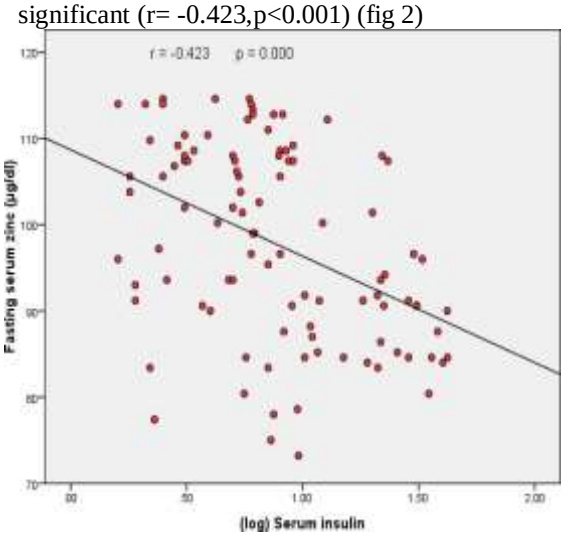

Figure 2: Correlation between fasting serum zinc levels (µg/dl) and (log) serum insulin.

A strong negative correlation was found between serum zinc and (log) C-peptide levels. The correlation was highly significant (r = -0.518, p=<0.001) (fig 3).These negative correlations can probably be explained by the biochemical role played byzinc in insulin release and action. Zinc is known to increase the sensitivity of insulin by several mechanisms. Zinc facilitates hexamerisation and crystal formation of insulin, processes which been suggested that crystal formation increases the degree of conversion of soluble pro-insulin to insoluble insulin [30] Several other modes of action have been described to explain the improved action of insulin by Zinc. It appears that Zinc can have direct insulin-like effects, which may be due to inhibition of the important glycogen-regulating enzyme GSK3. Other mechanisms include stimulation of the postreceptor proteins Akt and PI3-kinase. Zn can also reduce cytokines such as IL-1β as well as NFκB. Zn induces metallothionein synthesis, whereby Zn may have indirect efficacy. [30-32]. All these proposed mechanisms can lead to increased insulin sensitivity (and decreased insulin resistance). In view of the improved sensitivity of insulin, its release by β cells will be decreased. Thus, serum levels of insulin, C-peptide as well as β cell activity will be less if zinc levels are more and vice versa.

Figure 3: Correlation between fasting serum zinc levels (µg/dl) and (log) serum C-peptide.

SUMMARY AND CONCLUSIONS

In our study we found highly significant correlations between serum levels of zinc, C-peptide and insulin in patients of type 2DM. While Serum levels of zinc were within reference intervals, those of insulin and C-peptide were raised in patients of type 2 DM. Given the fact that insulin and c-peptide are fundamental to the origins of

type 2 DM, the strong negative correlation that zinc shares with these parameters points out towards a possible role of zinc in the pathogenesis of the disease. Large scale collaborated studies with a wider patient base are required to through further light on the subject. Further, therapeutic trials evaluating this role a need ofthe hour.

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AUTHOR STATEMENTS

Competing interests

The authors declare no conflict of interest.

Ethical issues

The study was done after obtaining proper ethical approval by the Institutional Ethics Committee of MMIMSR.

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Pharmaceuticals in wastewater and their treatment in Valencia,

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ABSTRACT

A survey on the presence of pharmaceuticals in urban wastewater of a Spanish Mediterranean area (Castellon province) was carried out. The scope of the study included a wide variety of pharmaceuticals belonging to different therapeutical classes. For this purpose, 112 samples, including influent and effluent wastewater, from different conventional wastewater treatment plants were collected. Similar median concentrations were found over the year and seasonal variation was not clearly observed. The removal efficiency of pharmaceuticals in the wastewater treatment plants was roughly evaluated. Our results indicated that elimination of most of the selected compounds occurred during the treatment process of influent wastewater, although it was incomplete.

Keywords

Pharmaceuticals; Liquid chromatography-tandem mass spectrometry; Effluent and influent wastewater; Waste water treatment plant; Occurrence; Removal efficiency.

1. Introduction

Pharmaceutical consumption is continuously increasing around the word. Only in Spain, about 729 millions of prescriptions were sold in 2004. Six years later, the consumption increased around 30% reaching 958 millions prescriptions (http://www.msps.es/profesionales/farmacia/datos/home.htm). This has lead to an increasing concern regarding possible ecological risks coming from pharmaceuticals released into the environment. Pharmaceuticals are used extensively in human and veterinary medicine to prevent illness and also as growth promoters in livestock and fish farming as well as in agriculture. After administration, pharmaceuticals can be transformed in the human body into more polar and soluble forms as metabolites or as conjugates of glucuronic and sulphuric acid (Heberer, 2002; Nikolaou et al., 2007). Pharmaceuticals and their metabolites are readily excreted with urine and faeces and enter into urban wastewater treatment plants (WWTPs).

Some of these compounds are eliminated by chemical or biological processes while others are degraded during sewage treatment processes or removed from the water phase by adsorption onto solid phase (e.g. sludge) (Jones et al., 2005). Data recently reported show that some pharmaceuticals are accumulated in sewage sludge. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent. In general, the elimination of most of the substances is incomplete and improvements of the wastewater treatment and subsequent treatments of the produced sludge are required to prevent the introduction of these micro- pollutants in the environment (Jelic et al., 2011). At present, urban wastewaters are considered the most important source of pharmaceutical compounds in the aquatic environment. WWTPs were designed to remove organic pollutants, mainly estimated as dissolved organic matter, solids and nutrients but not pharmaceutical compounds. Disposal of unused pharmaceuticals directly into domestic waste and application to livestock as veterinary drugs and feed additives can also contribute to their introduction in the environment (Heberer, 2002; Nikolaou et al., 2007).

Removal efficiencies in WWTPs depend on several factors such as compound physico- chemical properties, the climate conditions (e.g. temperature and sunlight intensity), the type of treatment process employed, the operational conditions of the treatment process (temperature of operation, redox conditions, solids retention time and hydraulic retention time) as well as the age of the activated sludge used in the plant (Castiglioni et al., 2006; Suárez et al., 2008; Le- Minh et al., 2010). Therefore, removal efficiencies can vary significantly from plant to plant and within a plant at different time periods (Vieno et al., 2007).

WWTPs typically employ conventional sewage treatment consisting on primary sedimentation followed by secondary treatment and final sedimentation. Organic pollutants can be transformed from the aqueous phase by hydrolysis, biotransformation or sorption to primary and secondary sludges (Le-Minh et al., 2010). However, the removal efficiency is variable as it is highly affected by the compound affinity to remain in the aqueous phase of the treated effluent (hydrophilic pharmaceuticals) or to be adsorbed to sludge (hydrophobic chemicals). In contrast, tertiary treatment or advanced treatment processes such as membrane filtration, activated carbon or oxidative processes (chlorination, ozonation and ultraviolet irradiation) seem to be more efficient when they work under optimum conditions. Nevertheless, their use is not widespread due to their high cost in terms of energy consumption.

Little is known about possible human and ecological adverse effects derived from the presence of pharmaceuticals in the aquatic

environment. Although the concentration levels detected after wastewater treatment processes seem not to cause toxic effects on human health and in the aquatic environment, there is a big concern on the long-term exposure of aquatic organisms to pharmaceuticals. Antibiotics are of special interest because they can promote bacterial resistance in the environment due to continuous exposure (Kümmerer, 2009a, 2009b; Zuccato et al., 2010). It is a problematic issue for flora and fauna as well as for humans, especially in those places where treated effluents are used to supplement drinking water supplies (Le-Minh et al., 2010). Consumption on antibiotics varies from country to country.Spain is one of the most consuming countries in terms of total amount. Broad spectrum antibiotics, which have the greatest impact on the development of resistance, are widely consumed according to the European Surveillance of Antimicrobial Consumption (ESAC) homepage (http://app.esac.ua.ac.be/public/index.php/en_eu/antibiotic/ antibiotic-consumption).

The aim of this paper is to investigate the occurrence and behavior of pharmaceuticals in wastewater treatment plants placed in the Castellon province (Spanish Mediterranean area) in order to have a realistic knowledge of the presence of pharmaceuticals in this region. A total of 112 samples (untreated and treated urban wastewater samples) from three WWTPs were analyzed by liquid chromatography coupled to tandem MS, along two monitoring programmes over the four seasons: summer (June), winter (January), spring (April), and autumn (October). Up to 47 pharmaceuticals were determined including a notable number of antibiotics. The occurrence and removal of these pharmaceuticals in different WWTPs and the effect of the seasonal variation on the elimination of pharmaceuticals was assessed.

2. Experimental

2.1. Reagents and chemicals

Reference standards were purchased from Sigma–Aldrich (St Louis, MO, USA), LGC Promochem (London, UK), Toronto Research Chemicals (Ontario, Canada), Across Organics (Geel, Belgium), Bayer Hispania (Barcelona, Spain), Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma (Madrid, Spain).

Isotopically labeled compounds used were omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3, from CDN Isotopes (Quebec, Canada); atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3, from Toronto Research Chemicals (Toronto, Canada); sarafloxacin-d_8 hydrochloride trihydrate, from Sigma–Aldrich; and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$, from Isotope Cambridge Laboratories (Andover, MA, USA).

HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from purification of demineralised water in a Milli-Q Gradient A10 (Millipore, Bedford, MA, USA). Formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide

(NaOH, >99%) were supplied by Scharlab (Barcelona, Spain).

Standards were dissolved in MeOH, except macrolides, sulfonamides and lincosamides that were prepared in ACN. The addition of NaOH was necessary for the proper dissolution of acidic analytes like quinolones. A mix of all compounds was prepared in MeOH and subsequently diluted with water to obtain working standard solutions. A mix of isotopically labeled internal standards (ILISs) was also prepared in MeOH and used as surrogate. All standard solutions and ILIS mix were stored in amber glass bottles at −20 °C in a freezer.

Cartridges used for SPE were Oasis HLB (60 mg) from Waters (Milford, MA, USA).

2.2. Instrumentation

Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC) analysis was carried out using an Acquity UPLC system (Waters, Milford, MS, USA), equipped with a binary solvent pumping. In the first monitoring, chromatographic separation of the 20 pharmaceuticals was achieved using an Acquity UPLC BEH column, 1.7 µm, 50 mm × 2.1 mm (i.d.) (Waters). Later, when the number of compounds increased up to 47, a longer column (Acquity UPLC HSS T3, 1.8 µm, 100 mm × 2.1 mm (i.d.)) was required for a satisfactory separation of all analytes but maintaining similar chromatographic runs. The LC system was interfaced to a TQD (triple quadrupole) mass spectrometer with an orthogonal electrospray ionization source Z-spray (Waters Corp.). MS/MS analysis was performed under selected reaction monitoring (SRM) mode, working in positive and negative ionization modes simultaneously. Chromatographic and mass spectrometry conditions can be found in detail in our previous papers (Gracia-Lor et al., 2010, 2011).

2.3. Analytical procedure

Water samples were extracted as described in Gracia-Lor et al. (2010, 2011). Briefly, the procedure was as follows: 100 mL water sample (100 mL effluent wastewater (EWW) or 20 mL influent wastewater (IWW) diluted with water to 100 mL) spiked with the ILIS mix working solution was passed through the Oasis HLB cartridge, previously conditioned. Analytes were eluted with 5 mL MeOH and the extract was evaporated and reconstructed with 1 mL MeOH–water (10:90, v/v). Finally, 20 µL of the final extract were injected in the UHPLC–MS/MS system. Quantification was made using calibration standards prepared in solvent, based on relative responses analyte/ILIS or on absolute analyte responses, depending on whether ILIS was used for correction or not. All methods applied were previously validated (Gracia-Lor et al., 2010, 2011).

2.4. Sampling

EWW and IWW samples were collected along 2008 and 2009. They were obtained from three WWTPs (Castellon de la Plana, Benicassim and Burriana) of the Castellon province (Spanish Mediterranean area). These WWTPs are designed to treat wastewaters (urban o mixed urban and industrial) operating with secondary treatment using

conventional activated sludge. At present, the Castellon de la Plana WWTP has a tertiary treatment operating with sand filtration and ultraviolet irradiation, but it was not operating when the monitoring was carried out. Castellon de la Plana WWTP has a population equivalent of 265,000 inhabitants, while Benicassim and Burriana WWTPs serve to a population around 18,000 and 35,000inhabitants. For each plant, 24-h composite untreated (influent) and treated wastewater samples (effluent) were obtained. Samples were frozen and stored at −18 °C until analysis.

Sampling was carried out in two campaigns. In the first monitoring, samples were collected along one complete week in June 2008 and in January 2009 and the occurrence of 20 pharmaceuticals was investigated (Gracia-Lor et al., 2010). In the second monitoring, in the light of the results obtained, the number of investigated compounds was increased up to 47 in order to have a wider knowledge of the presence of pharmaceuticals in wastewaters. Most of pharmaceuticals added in the second monitoring corresponded to antibiotics. In this case, only EWW and IWW samples from the Castellon de la Plana WWTP (the main town of the Castellon province) were analyzed as no significant differences between the three studied WWTPs were observed and this treatment plant serves a larger population. 24-h Composite samples (IWW and EWW) were collected during one complete week in April 2009 and October 2009.

3. Results and discussion

3.1. First monitoring

First of all, a group of 20 pharmaceuticals were selected including the most consumed active principles with medical prescription in Spain (Ministry of Health, 2008, 2009). Several compounds with low official sales volumes (in terms of medical prescription) but frequently detected in urban wastewater as reported by other authors (Ternes, 2001; Gros et al., 2006; Hernando et al., 2007; Pedrouzo et al., 2007) were also included (e.g. diclofenac, naproxen or bezafibrate). In addition, two metabolites were considered: salicylic acid, which is the main metabolite of acetylsalicylic acid, and 4-aminoantipyrine, which is a metabolite of dipyrone. These metabolites were selected because they had been frequently determined in the aquatic environment (surface water and wastewater) according to scientific literature (Ternes et al., 2001; Heberer, 2002; Metcalfe et al., 2003; Wiegel et al., 2004). Thus, 20 pharmaceuticals for human use were selected (Table 1). Target analytes represented a broad range of chemicals classes including analgesic and anti-inflammatory, cholesterol lowering statin drugs, lipid regulators, antidepressants, anti-ulcer agents, psychiatric drugs, ansiolitics and cardiovasculars.

In total, 84 wastewater samples were analyzed in this monitoring, and collected from three WWTPs of the Castellon province. Sample collection was performed in summer 2008 (June) and winter 2009 (January). Table 1 shows the percentage of positive findings of the selected compounds, as well as median concentrations in IWW and EWW analyzed during this period.

13 out of 20 compounds were detected in IWW. All 13 pharmaceuticals were identified in more than 95% of the samples, with the exception of salicylic acid and pravastatin, the latest only being present in 26% of IWW samples. Analgesics/anti-inflammatories and lipid regulators were the most commonly detected groups. Moreover, the highest values in this type of samples corresponded to salicylic acid, acetaminophen and ibuprofen (these three compounds belong to the anti-inflammatory therapeutic group) with maximum levels of 277, 201 and 40 μg L^{-1}, and median concentration of 35.1, 44.8 and 12.4 μg L^{-1}, respectively. Quantification of the samples with high analyte levels (typically above 100 μg L^{-1}) required an additional analysis with previous dilution of the sample before the SPE step.

When comparing the percentage of positive findings in IWW collected in summer and in winter, no relevant differences were found. However, when comparing the maximum levels found, higher concentrations were observed for some compounds in the winter samples. For example, in the case of acetaminophen, salicylic acid and ibuprofen, maximum concentrations increased from 84 to 201 μg L^{-1}, from 47 to 277 μg L^{-1}, and from 20 to 40 μg L^{-1}, respectively. For the rest of compounds, no relevant variations in concentrations were observed.

Table 1

Summary of the results obtained in the first monitoring programme of 20 pharmaceuticals (June 2008 and Janu ary 2009).

	Therapeutic group	Influent wastewater (n = 42)				Effluent wastewater (n = 42)			
		% Positive findings	Median concentration (µg/L)	Minimum-Maximum levels (µg/L)	LOQ (µg L-1)[a]	% Positive findings	Median concentration (µg/L)	Minimum-Maximum levels (µg/L)	LOQ (µg L-1)[a]
Acetaminophen	Analgesics and anti-inflamatories	100	44.8	1.13-201	0.11	0	n.d.	n.d.	0.09
4-Aminoantipyrine		100	2.26	0.53-6.45	0.03	100	0.69	0.42-1.68	0.04
Diclofenac		100	0.56	0.26-1.49	0.14	100	0.33	0.06-0.74	0.05
Ibuprofen		98	12.4	2.28-39.8	0.64	33	<LOQ	<LOQ	0.25
Ketoprofen		100	0.48	<LOQ-1.17	0.11	100	0.30	0.15-0.62	0.07
Naproxen		100	1.55	0.27-3.58	0.05	100	0.17	<LOQ-0.72	0.03
Salicylic acid		76	35.1	3.10-277	0.97	26	<LOQ	<LOQ-236	0.43
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.11	<LOQ-0.45	0.03	76	0.02	0.01-0.16	0.007
Pravastatin		26	0.20	0.14-0.24	0.12	30	0.10	0.07-0.17	0.02
Bezafibrate		100	0.16	0.02-0.46	0.02	100	0.07	0.02-0.39	0.01
Gemfibrozil		100	1.11	0.16-2.12	0.05	100	0.54	0.15-1.24	0.02
Paroxetine	Antidepressants	0	n.d.	n.d.	0.20	0	n.d.	n.d.	0.04
Venlafaxine		100	0.17	0.04-0.52	0.01	100	0.14	0.06-0.30	0.004
Omeprazole	Anti-ulcer agents	0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.01
Pantoprazole		0	n.d.	n.d.	0.07	65	0.13	0.05-0.18	0.03
Olanzapine	Psychiatric drugs	0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.01
Risperidone		0	n.d.	n.d.	0.009	0	n.d.	n.d.	0.006
Alprazolam	Ansiolitics	0	n.d.	n.d.	0.03	38	<LOQ	<LOQ	0.01
Lorazepam		0	n.d.	n.d.	0.05	55	0.04	0.03-0.06	0.03
Enalapril	Cardiovascular	96	0.14	0.02-0.29	0.02	0	n.d.	n.d.	0.007

n.d. (not detected)

[a] Data on LOQ taken from (Gracia-Lor et al., 2010).

Regarding EWW, up to 14 target compounds were detected. Analgesic and anti- inflamatories were frequently found (the exception was acetaminophen, which was never detected in the EWW samples in contrast to IWW where it was present in the 100% of samples). Cholesterol lowering statin drugs and lipid regulators were also found in a high number of samples but, with the exception of gemfibrozil, their median concentrations were below 0.10 μg L^{-1}. Other compounds frequently detected were venlafaxine, pantoprazole and lorazepam.

The removal of pharmaceuticals during wastewater treatment was estimated from concentration data in IWW and EWW. Considering that pharmaceuticals have rather different physico-chemical characteristics, their removal during treatment is expected to be diverse. In the literature, the removal efficiency is generally computed as the percentage of reduction between the dissolved aqueous phase concentration of the contaminant in the influent and the dissolved aqueous phase concentration of the contaminant in the effluent. Except for a few studies, pharmaceutical concentrations in sludge or suspended solid are generally not considered nor measured, probably because of the difficulty to sample and to analyze such complex matrices (Miège et al., 2009). However, the screening of sewage sludge showed that these micro-pollutants are very present in this medium. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent (Jelic et al., 2011). When comparing pharmaceutical concentrations in IWW and EWW, like in this work, lower levels in EWW would be interpreted as a removal of the compound in the WWTP. This fact might be due to different factors like chemical and physical transformations, biodegradation and sorption to the solid matter. Thus, the conversion of a given pharmaceutical to compounds other than the analyzed one would lead to lower pharmaceutical levels in EWW concluding that an “apparent” removal takes place.

In this work, acetaminophen, enalapril and ibuprofen were completely removed during the treatment processes (present in 100% and 96% of IWW samples, and never detected in the EWW samples), while the antidepressant venlafaxine, lipid regulator compounds, as well as analgesic and anti-inflammatory pharmaceuticals (with the exception of acetaminophen and ibuprofen) were detected in all EWW samples, although at concentrations lower than in IWW. On the other hand, some pharmaceuticals were not detected in IWW but they were present in EWW. This behavior was observed for pantoprazole and for the ansiolitic compounds alprazolam and lorazepam. This is in agreement with previous studies where some compounds were reported to be more abundant in effluents than in influents (Lacey et al., 2008; Gros et al., 2010; Jelic et al., 2011). In the case of the ansiolitic compounds, they were detected at very low concentrations in EWW (around or below the LOQ level). Maybe they were also present in the IWW samples but could not be detected due to the lower sensitivity of the method in this type of waters. The higher complexity of the influents leads to strong matrix effects (commonly

ionization suppression), which can hamper the detection of some analytes at very low levels.

The absence of ansiolitic compounds in the IWW might be also due to the enzymatic cleavage of the compound glucuronides and other conjugated metabolites and the subsequent release of the parent compound during the treatment process (Vieno et al., 2007; Lacey et al., 2008; Gros et al., 2010).

Predicting the removal efficiencies of compounds during treatment processes is quite difficult because they are significantly affected by the specific operating conditions of each WWTP. However, some information can be obtained from the data reported by others on the behavior of pharmaceuticals during the treatment processes. For instance, analgesics and anti- inflammatory pharmaceuticals have been detected in the aquatic environment in a broad number of studies. Within this group, our data showed that acetaminophen was removed by the three WWTPs. For salicylic acid, an efficient removal was also obtained in contrast to diclofenac, ketoprofen and naproxen that seemed to persist to the water treatment, although their levels in EWW were lower than in IWW. This behavior is consistent with scientific literature (Heberer, 2002; Gros et al., 2010).

In the case of lipid regulators and cholesterol lowering statin drugs, they showed a variety of removal rates between 30% and 100% which is in fairly good agreement with previous studies (Jelic et al., 2011). In our case, the highest levels and frequency of detection were found for lipid regulators, especially for gemfibrozil.

Comparing the three studied WWTPs, no significant differences in terms of removal efficiencies were observed for the analyzed compounds. This is because they work at similar operational conditions.

3.2. Second monitoring

A notable number of compounds (around 30 antibiotics and a cholesterol lowering statin drug) were added to the target list of our previous method in order to have a more realistic knowledge of the presence of pharmaceuticals in the environment. Many antibiotics were included due to the special concern on their potential negative effects on the aquatic environment, whereas simvastatin, a cholesterol statin drug, was added to the list due to its increased consumption with medical prescription.

As differences among the three WWTPs were hardly observed in the first survey, in the second monitoring only the Castellon de la Plana WWTP was monitored, in two different seasons: spring (April 2009) and autumn (October 2009). This treatment plant was selected because it serves the largest population of the Castellon province (Table 2). Moreover, data obtained in the first monitoring revealed that the samples from this treatment plant typically presented the highest pharmaceutical levels. In this second monitoring, 28 wastewater samples

(14 IWW and 14 EWW) were collected and analyzed (Table 3), corresponding to one wholeweek of April 2009 and one whole week of October 2009.

Table 2
Characteristics of the Castellon de la Plana treatment plant.

WWTP	Population(he)

Type of treatment

Type of wastewater

Designed treatment	Average flow (m3/day)	Minimum flow capacity (m^3d^{-1}) estimated (L/s)	Maximum flow estimated (L/s)	Sampling			
Castellon dela Plana				265,000	Secondary[a]	Urban and industrial	

42,000	36,000	139.06	752.31	Time-proportional composite (eve	ry 60 minutes)

[a] Secondary treatment was applied at the time of the monitoring was performed. At present, a tertiary treatment is applied.

In IWW, for those 20 pharmaceuticals also analyzed in the first monitoring, no relevant differences were observed except for diclofenac, which showed a lower frequency of detection. Similarly to the previous study, the highest concentrations in IWW were found for acetaminophen (134 µg L^{-1}), salicylic acid (64 µg L^{-1}) and ibuprofen (19 µg L^{-1}). As pointed out before, these compounds are frequently prescribed but they can also be acquired without medical prescription, the so-called "over the – counter" (OTC) drugs.

In the case of antibiotics, it is difficult to establish a general trend for each group. As shown in Table 3, 9 out of 26 selected antibiotics were detected in the influent samples. Among them, seven compounds (ciprofloxacin, clarithromycin, norfloxacin, ofloxacin, pipemidic acid, sulfamethoxazole and trimethoprim) were detected in all the samples. On the contrary, lincomycin, which can be used in both human and veterinary medicine, and sulfathiazole were detected in around 20% of IWW. Except for ciprofloxacin, antibiotic median concentrations in IWW did not exceed 1 µg L^{-1}.

Regarding EWW, our data suggest that elimination of most of the compounds analyzed is incomplete. Again, we may distinguish different behaviors. First of all, there is a group of compounds that were fully eliminated in the treatment plant (i.e. acetaminophen, enalapril, ibuprofen, salicylic acid). These results are in agreement with those reported by other authors (Gros et al., 2010) and supported the behavior observed in the first monitoring, with slight differences observed for salicylic acid. On the other hand, there are several compounds partly removed by the treatment processes. For these compounds, concentrations after treatment were normally lower than in IWW, but they were still present in the EWW analyzed. This is the case of most lipid regulators and anti-inflammatory drugs. In some particular cases, e.g. gemfibrozil, concentrations were slightly higher in the effluent. Another group of pharmaceuticals included those compounds that showed poor or non elimination in the treatment plant, as some macrolide antibiotics, ansiolitics and the anti-ulcer agent pantoprazole, which presented even higher percentages of positive findings in EWW than in IWW. This fact has been previously reported in other studies (Göbel et al., 2007; Gros et al., 2010). As pointed out before, this phenomenon might be explained by the higher LOQs in IWW compared to EWW, or by the release of the parent compound from glucuronides or other conjugated

metabolites during the treatment process. Finally, several target analytes were never found either in IWW or EWW. It was not expected for those compounds such as simvastatin, omeprazole or paroxetine that belong to the list of the most consumed pharmaceuticals in Spain with medical prescription. Their absence might be explained because their excretion was mainly as metabolites or due to the parent compound transformation/degradation in the sewer system. Thus, searching for metabolites and/or transformation products of these compounds seems necessary to evaluate their impact into the aquatic ecosystem.

Concerning sulfonamide antibiotics, only sulfamethoxazole was detected in EWW. In fact, it was present in 100% EWW analyzed although at very low levels, below 0.06 μg L^{-1}. Some contradiction exists about its removal (Le-Minh et al., 2010) as some studies have observed an effective removal (Choi et al., 2008) while others not (Brown et al., 2006). This fact might be explained by differences in operational conditions of each WWTP.

Table 3
Summary of the results obtained in the second monitoring programme of 47 pharmaceuticals (April 2009 and October 2009).

	Therapeutic group	Influent wastewater (n = 14)				Effluent wastewater (n = 14)			
		% Positive findings	Median concentration ($\mu g\ L^{-1}$)	Minimum-Maximum levels ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)[a]	% Positive findings	Median concentration ($\mu g\ L^{-1}$)	Minimum-Maximum levels ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)[a]
Acetaminophen	Analgesics and anti-inflamatories	100	55.1	18.2-134	0.19	0	n.d.	n.d.	0.09
4-Aminoantipyrine		100	2.30	0.90-3.20	0.03	100	0.74	0.56-0.95	0.02
Diclofenac		36	0.53	<LOQ-0.74	0.14	100	0.34	0.21-0.62	0.05
Ibuprofen		100	14.6	6.1-19.1	0.73	0	n.d.	n.d.	0.15
Ketoprofen		100	0.37	0.25-0.41	0.14	100	0.33	0.12-0.42	0.05
Naproxen		100	1.32	0.87-2.24	0.08	100	0.13	0.09-0.28	0.02
Salicylic acid		100	38.1	10.9-63.7	0.24	0	n.d.	n.d.	0.08
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.22	0.11-0.33	0.01	93	0.02	0.01-0.04	0.004
Pravastatin		50	<LOQ	<LOQ-0.25	0.20	0	n.d.	n.d.	0.03
Bezafibrate		100	0.08	<LOQ-0.10	0.03	100	0.06	0.04-0.08	0.01
Gemfibrozil		100	0.21	0.10-0.54	0.07	100	0.49	0.34-0.91	0.004
Simvastatin		0	n.d.	n.d.	0.13	0	n.d.	n.d.	0.02
Paroxetine	Antidepressants	0	n.d.	n.d.	0.27	0	n.d.	n.d.	0.17
Venlafaxine		100	0.87	0.78-0.98	0.05	100	0.29	0.20-0.55	0.007
Omeprazole	Anti-ulcer agents	0	n.d.	n.d.	0.06	0	n.d.	n.d.	0.02
Pantoprazole		0	n.d.	n.d.	0.06	100	0.01	0.01-0.02	0.004
Olanzapine	Psychiatric drugs	0	n.d.	n.d.	0.17	0	n.d.	n.d.	0.05
Risperidone		0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.003
Alprazolam	Ansiolitics	0	n.d.	n.d.	0.03	100	0.01	0.01-0.01	0.004
Lorazepam		0	n.d.	n.d.	0.15	100	0.14	0.10-0.16	0.05
Enalapril	Cardiovascular	100	0.15	0.09-0.20	0.02	0	n.d.	n.d.	0.006
Erithromycin	Macrolide antibiotics	0	n.d.	n.d.	0.02	100	0.08	0.05-0.12	0.008
Clarithromycin		100	0.23	0.13-0.62	0.01	100	0.02	0.01-0.06	0.003
Tylosin		0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.002
Roxithromycin		0	n.d.	n.d.	0.03	50	<LOQ	<LOQ	0.02
Moxifloxacin	Quinolone antibiotics	0	n.d.	n.d.	0.33	100	0.16	0.12-0.18	0.11
Norfloxacin		100	0.40	0.29-1.07	0.16	100	0.13	0.09-0.15	0.03
Pefloxacin		0	n.d.	n.d.	0.08	0	n.d.	n.d.	0.05
Ofloxacin		100	0.76	0.29-0.96	0.01	100	0.44	0.33-0.50	0.01
Marbofloxacin		0	n.d.	n.d.	0.12	0	n.d.	n.d.	0.11
Ciprofloxacin		100	2.45	1.21-3.85	0.32	100	0.70	0.52-1.08	0.05
Enrofloxacin		0	n.d.	n.d.	0.04	0	n.d.	n.d.	0.02
Sarafloxacin		0	n.d.	n.d.	0.04	0	n.d.	n.d.	0.03
Flumequine		0	n.d.	n.d.	0.06	0	n.d.	n.d.	0.01
Oxolinic acid		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.01
Nalidixic acid		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.006
Pipemidic acid		100	0.28	<LOQ-0.54	0.21	100	0.10	<LOQ-0.12	0.09
Sulfamethoxazole	Sulfonamide antibiotics	100	0.45	0.22-0.64	0.02	100	0.05	0.04-0.06	0.01
Sulfamethazine		0	n.d.	n.d.	0.003	0	n.d.	n.d.	0.001
Sulfadiazine		0	n.d.	n.d.	0.08	0	n.d.	n.d.	0.05
Sulfathiazole		29	0.06	0.06-0.07	0.04	0	n.d.	n.d.	0.01
Lincomycin	Lincosamide antibiotics	14	0.49	0.10-0.88	0.01	79	0.01	0.01-0.16	0.002
Clindamycin		0	n.d.	n.d.	0.04	100	0.02	0.01-0.02	0.006
Furaltadone	Other antibiotics	0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.005
Furazolidone		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.02
Trimethoprim		100	0.10	0.06-0.16	0.04	100	0.09	0.06-0.10	0.01
Chloramphenicol		0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.02

n.d. (not detected)

[a] Data on LOQ taken from (Gracia-Lor et al., 2011).

The presence of trimethoprim is usually related to the detection of sulfamethoxazole since these pharmaceuticals are often administered together. In agreement with other studies (Ternes, 2001; Gros et al., 2010; Jelic et al., 2011), the removal of trimethoprim during the wastewater treatment was incomplete.

In the case of macrolide antibiotics, all compounds belonging to this therapeutic group were detected in EWW, except for tylosin which was never found. Among them, the percentage of positive findings and concentrations may differ due to their different consumption pattern. The incomplete removal of macrolide antibiotics by WWTP is in agreement to previous works (Clara et al., 2005; Göbel et al., 2007). In our study, erithromycin and roxithromycin were present in the effluent samples, but absent in the corresponding influent. Some authors suggest that this might be due to the release of these compounds from faeces during the biological treatment (Göbel et al., 2007).

Regarding quinolone antibiotics, they have been frequently detected in wastewaters from several countries, especially norfloxacin and ciprofloxacin. In our case, 5 out of 12 compounds that belong to this group were found in EWW (see Table 3).

The results obtained in this monitoring work support the interest for including antibiotics when monitoring pharmaceuticals in wastewater, as they have been found rather frequently in the samples.

In all samplings carried out, samples were collected during one complete week. In general, concentration of pharmaceuticals did not significantly change along the week. This indicates that the consumption of the studied compounds is quite constant over the week in contrast to illicit drugs, which consumption clearly increases during the weekends and in special events (Bijlsma et al., 2009).

In this survey, a more complete seasonal variation analysis could be made for the Castellon de la Plana WWTP, which was the only one sampled in all monitoring programmes (four seasons, from summer 2008 to autumn 2009). Regarding the 20 most consumed pharmaceuticals, initially selected, they did not show big variations in median concentrations over the year (Fig. 1a and b). This is in accordance to their use, which is rather constant along the year. For a few compounds higher concentrations were found in winter for IWW (acetaminophen, salicylic acid, naproxen or diclofenac). These compounds are analgesic and anti-inflammatory pharmaceuticals, which are consumed along the whole year, but especially in winter.

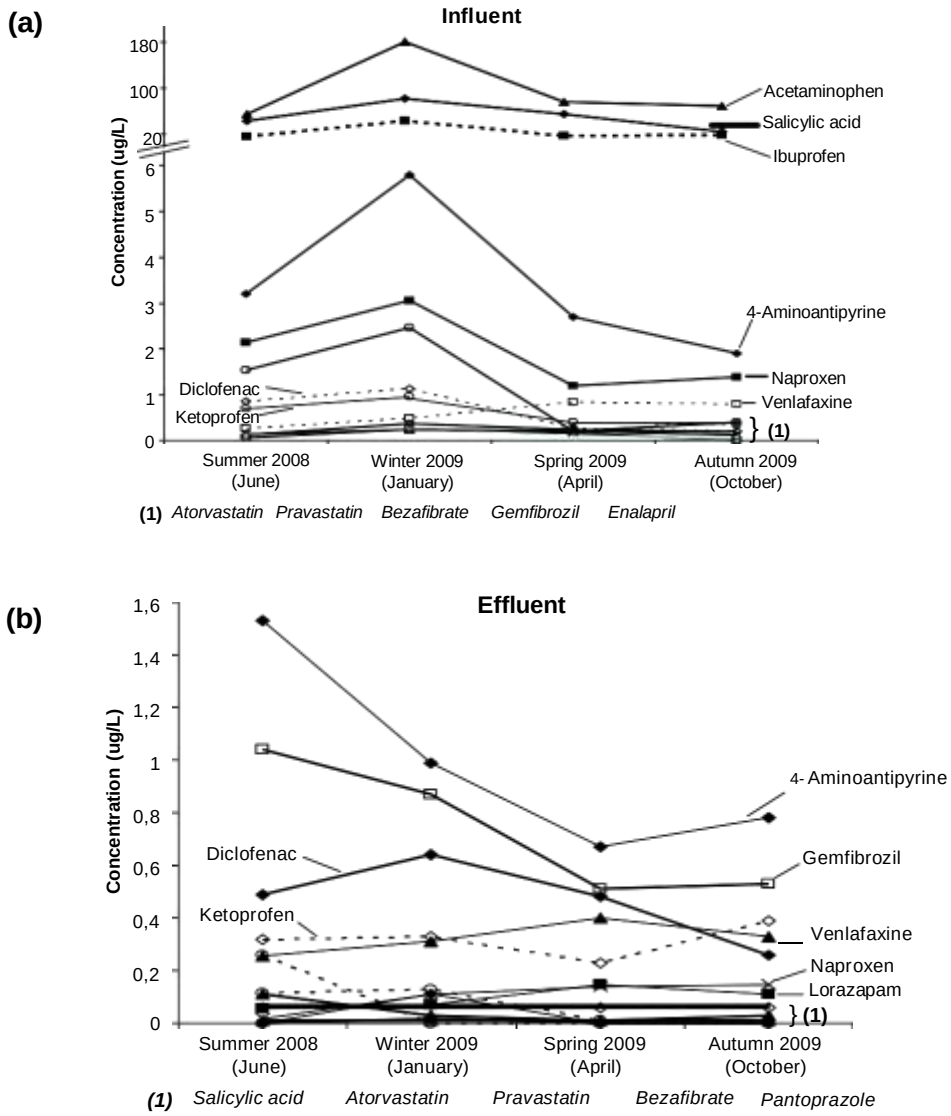

Figure 1. Median pharmaceutical concentration in the influent (a) and in the effluent (b) of the Castellon de la Plana WWTP monitored along four seasons.

Regarding antibiotics, a comparison between spring and autumn concentrations was made, as they were only determined in these two seasons (Fig. 2a and b). We did not observe relevant differences, as the same compounds were detected in both seasons at similar median concentrations. However, it is noteworthy that antibiotic concentrations were notable lower than for the rest of pharmaceuticals, probably because they are less consumed. The only exception was ciprofloxacin.

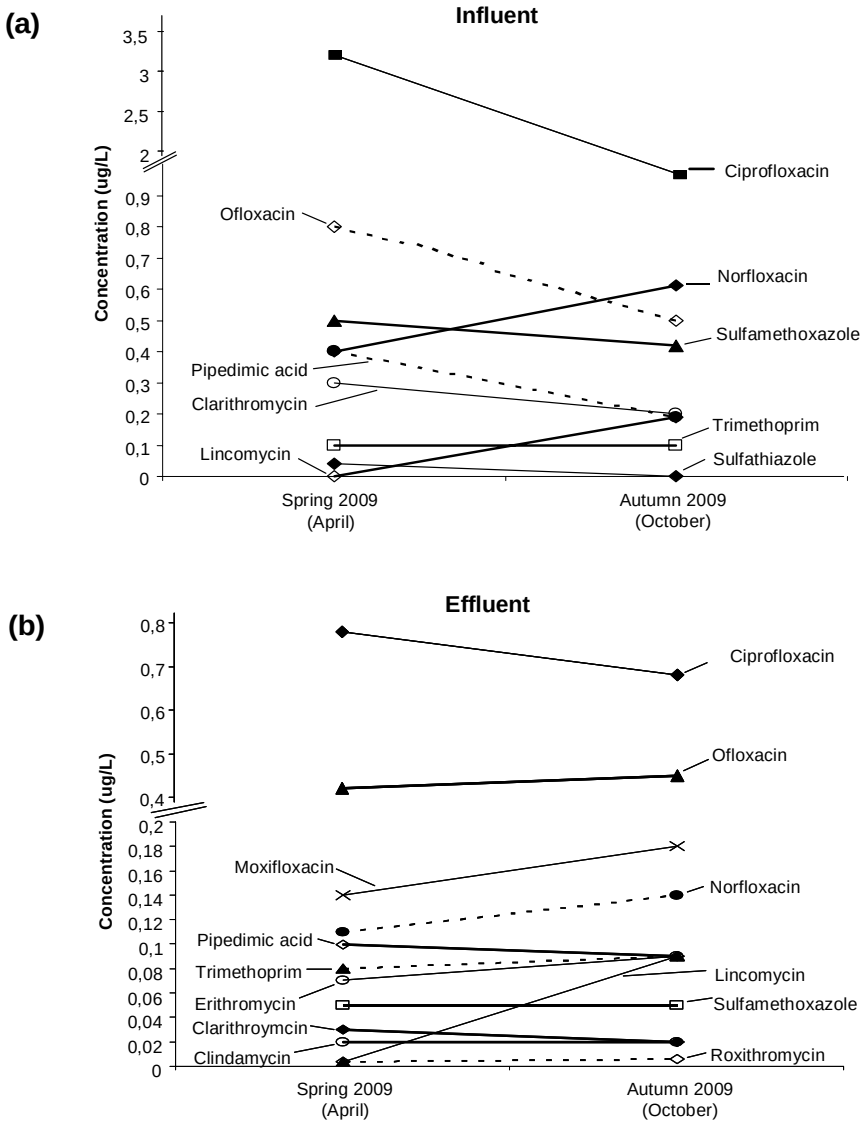

Figure 2. Median antibiotics concentration in the influent (a) and in the effluent (b) of the Castellon de la Plana treatment plant monitored in spring and autumn 2009.

The removal efficiency (RE) of the Castellon de la Plana WWTP is illustrated in Fig. 3. Those pharmaceuticals that were not detected in influent and in effluent wastewater samples (e.g. simvastatin, paroxetine, pefloxacin, etc.) have been omitted in this figure. RE values were calculated as the ratio between the median concentration levels of each pharmaceutical in influents and effluents. Data from samples collected along a whole week in April 2009 have been used in this figure. This WWTP seemed to have good removal efficiency for most analgesics/anti-inflammatories like acetaminophen, ibuprofen, naproxen or salicylic acid (RE around 100%). As regards the four cholesterol lowering statin drugs/lipid regulators detected in wastewater, two of them seemed to be efficiently removed (atorvastatin, pravastatin), while partial removal was suggested for bezafibrate (RE around 40%) and no removal was observed for gemfibrozil. The cardiovascular enalapril was also efficiently removed.

In relation to antibiotics, 6 out of 13 compounds detected in wastewater were rather efficiently removed in the WWTP, whereas ofloxacin and trimethoprim showed RE between 20% and 40%. However, negative RE were observed for 5 antibiotics because these pharmaceuticals were not detected in IWW samples but were present in the corresponding EWW samples. In this case, it was not possible to calculate the RE actually, and a reference value (−100%) was given in order to show their behavior in the figure. The same situation was observed for three more compounds (pantoprazole, alprazolam and lorazepam) that were not found in IWW although they were detected in EWW (all compounds marked as (*) in Fig. 3). As previously stated, this situation might be due to the non-detection in IWW as a consequence of the higher complexity of this matrix, with typically higher matrix suppression, and the higher LOQs resulting in IWW. It must be taken into account that concentration levels found in EWW were normally low for all those compounds. Thus, they might be present at low levels in the IWW as well, and might not have been detected. Therefore, this assigned arbitrary value of - 100% for al these 8 compounds might be questioned.

In the case of gemfibrozil and diclofenac, negative RE values were due to a slight increase of their concentration during the treatment process, i.e., they were detected at higher concentration levels in the effluent.

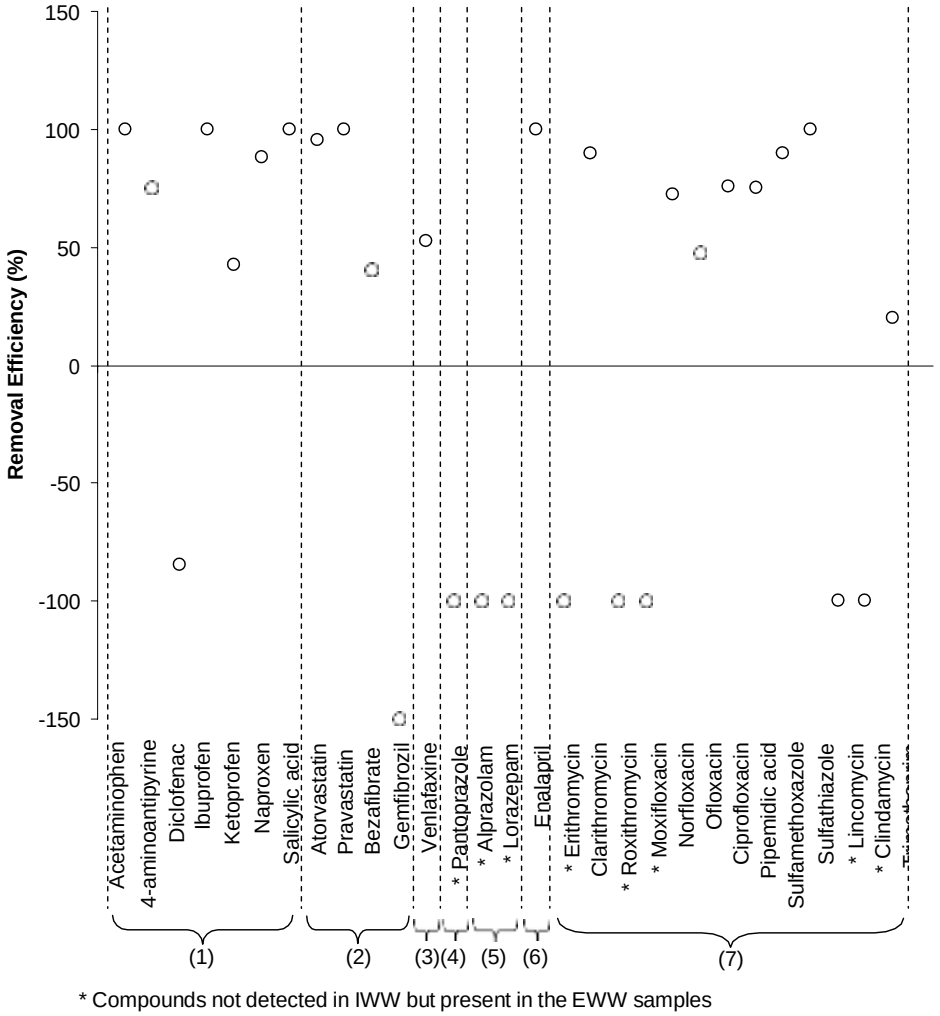

Figure 3. Removal efficiency of the Castellon de la Plana WWTP. (Data from April 2009). (1) Analgesics and anti-inflammatories, (2) cholesterol lowering statin drugs and lipid regulators, (3) antidepressants, (4) anti-ulcer agents, (5) ansiolitics, (6) cardiovasculars, and (7) antibiotics.

4. Conclusions

In this study, a monitoring of around 50 pharmaceuticals has been made in IWW and EWW from three different WWTPs. Up to 17 compounds were detected in both IWW and EWW indicating that conventional treatment processes do not completely remove these micro- pollutants. Among them, analgesics and anti-inflamatories, lipid regulators as well as quinolone and macrolide antibiotics were the major groups found.

Selected pharmaceuticals could be divided into four groups according to their behavior in WWTPs: a few compounds were completely removed during the treatment processes (e.g. acetaminophen, enalapril, ibuprofen); another group of analytes were not fully removed, although their concentrations after treatment were significantly lower than in influent (e.g. lipid regulators). A third group of compounds were not detected in IWW but were present in the

EWW samples (e.g. ansiolitics and macrolide antibiotics). Finally, some pharmaceuticals werenever detected in either IWW or EWW (e.g. simvastatin, paroxetine, sulfamethazine).

Searching for metabolites may offer valuable information (Tarcomnicu et al., 2011), especially for those analytes never found in wastewater despite they were frequently used. Future research will be directed towards the investigation of metabolites by using quadrupole time of flight (QTOF) mass spectrometry. In those particular cases where pharmaceuticals were not detected in IWW but detected in the corresponding EWW samples, QTOF would also be an ideal approach to identify glucuronide and conjugated metabolites, if present in IWW. Thus, the occurrence of metabolites and conjugated compounds could be studied by this technique.

Seasonal variation in terms of median concentration values was not clearly observed in IWW and EWW. However, when comparing the maximum levels reached, higher concentrationswere found in winter (January 2009), especially for analgesic and anti-inflamatory pharmaceuticals, possible due to a higher consumption during this period of the year to treat, forexample, seasonal flu.

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Drug Interactions: The Most Common, the Worst, and What to Do About Them in Long-Term Care

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ABSTRACT

The medical community is aware of the issue of medication interactions and has made significant attempts to track them, but they have been unsuccessful so far in predicting and preventing drug interactions. Unfortunately, there was no foolproof way to anticipate medication interactions; instead, people had to wait for them to show up in the scientific literature. Known drug interactions may not occur in all patients taking the medicine or even another drug in the same class, adding more complexity and unpredictability to the already difficult process of prescribing medications. Most medication interactions are unpredictable and happen with chronic drug usage. This article focused on the effects, cause, treatment, and prevention of potentially lethal medication interactions that may arise in long-term care settings. The possibility for adverse events and the processes behind them are best understood in the context of long-term care medication combinations.

Keywords;Genser 2008

INTRODUCTION

Medications can help people live healthy for a prolonged period. Although medicines are prescribed often, it is important to know than ever about the medicines administered and should be used with caution (Genser 2008). Medications, both prescription and over-the-counter, are used every day to treat acute and chronic illness. Taking several different medicines due to polypharmacy or having more than one health condition carry the risk of adverse interactions, such as drug interactions (Paul et al., 2000). Whenever two or more drugs are taken concurrently there is a chance of an interaction among the drugs that could manifest as an increase or decrease in their effectiveness or an adverse reaction or a totally new side effect that is not seen with either drug alone that can be severe enough to alter the clinical outcome and warrant hospital admissions (Ansari 2010). The likelihood of these interactions is increased if certain drugs in combination used for longer period. There is less emphasis of physicians on drug interaction on long term care. This article addressed dangerous drug interactions on long term care for commonly used drug combinations.

WARFARIN – NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs) (Chan 1995)

The most dangerous drug combinations in the nursing home population involve warfarin interactions with nonsteroidal anti- inflammatory drugs (NSAIDs).

Impact:

Potential for serious gastrointestinal bleeding and increased INR.

Mechanism of Interaction:

NSAIDs increase gastric irritation and erosion of the protective lining of the stomach causing gastrointestinal bleeding which is likely to be more severe if warfarin is also given. Additionally, NSAIDs decrease the cohesive properties of platelets and inhibits aggregation necessary in clot formation. Altogether leads to severe gastrointestinal bleeding.

Pharmaceutical Analysis
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MOU with Government General Hospital &KMC, K urnool

Prevention:

Avoid concomitant use of an NSAID with warfarin. Identify reason for NSAID therapy. If anti-pyretic effects are desired, then consider acetaminophen. Acetaminophen in doses less than 2g/day on a short-term basis does not appear to affect the INR. Long- term use of acetaminophen for anti-pyretic and analgesic effects is controversial. If anti-inflammatory effects are necessary, then consider cyclooxygenase-2 (COX-2) inhibitor therapy. These agents minimization of gastric irritation combined with the lack of anti- platelet action, support the cautious use of COX-2 inhibitors in anticoagulation patients. There are some case reports discussing the elevation of INRs with COX-2 inhibitors (Shaefer et al., 2003). If analgesic effects are desired, caution should also be exhibited with the use of tramadol; there are a few case reports describing an elevation of the INR with concomitant administration of tramadol with warfarin (Dumo et al., 2006).

Management:

Dosage reduction of 25-30% in warfarin followed by Prothrombin time and international normalized ratio (INR) should be monitored every week with co-administration of an NSAID. Signs and symptoms of an active bleed should be monitored with particular attention to the appearance and patterns of bruises. Signs of an active bleed include: coughing up blood in the form of coffeegrinds (hemoptysis), gum bleeding, nose bleeds, cola- or tea-colored urine (hematuria), or black, tarry stools (hemoccult positive).

WARFARIN - SULFA DRUGS (Cook et al., 1994; Ahmed et al., 2008)

Impact:

Increased effects of warfarin, with potential for bleeding

Mechanism of Interaction:

Clinicians hypothesize that warfarin's activity is prolonged due to inhibition of warfarin metabolism and decreased production of vitamin K by intestinal flora affected by systemic antibiotic administration. Sulfa drugs also cause the displacement of warfarin from protein binding sites and increases free drug concentration and increases prothrmbin time (Wen et al., 2002).

Prevention:

Avoid concomitant use of a sulfa drug with warfarin, particularly sulfamethoxazole and trimethoprim. Consider use of any other antibiotic alternative to sulfa drugs with warfarin. If use of a sulfa drug is imperative, then reduce warfarin dose by 50% during antibiotic administration and for one week following completion of the antibiotic. If sulfamethoxazole-trimethoprim therapy is required, then monitor INR every other day for elevating trends.

Management:

Prothrombin time and INR should be monitored every week during co-administration of warfarin with a sulfa drug. Also monitor for vitamin K levels at regular intervals. Signs and symptoms of an active bleed should be monitored daily with particular attention to the appearance and patterns of bruises. Signs of an active bleed include: coughing up blood in the form of coffee grinds (hemoptysis), gum bleeding, nose bleeds, hematuria, and black, tarry stools (hemoccult positive).

WARFARIN – MACROLIDES (Penti et al., 1992)

Impact:

Increased effects of warfarin, with potential for bleeding

Mechanism of Interaction:

Macrolides inhibits the metabolism and subsequent clearance of warfarin from the body. The activity of warfarin may also be prolonged due to alterations in the intestinal flora and its production of vitamin K for clotting factor production (Woldtvedt et al., 1998)

Prevention:

The interaction between warfarin and macrolide antibiotics is highly probable and often delayed. Concomitant use of a macrolide with warfarin should be avoided; switch to an alternative antibiotic. Microbial pathogen identification prior to antibiotic initiation will decrease the prevalence of unnecessary drug interaction risk. Consider culture sensitivity screening as research indicates cautious use of any antibiotic with warfarin.

Management:

If use of a macrolide is imperative, then monitor prothrombin time every other day and adjust warfarin dosing as necessary (Oberg 1998). Signs and symptoms of an active bleed should be monitored daily with particular attention to the appearance and patterns of bruises. Signs of an active bleed include: coughing up blood in the form of coffee grinds (hemoptysis), gum bleeding, nose bleeds, cola- or tea-colored urine (hematuria), and black, tarry stools (hemoccult positive)(WHO 2004).

WARFARIN- QUINOLONES (Carroll et al. 2008, Cade B Jones et al. 2002)

Impact:

Increased effects of warfarin, with potential for bleeding

Mechanism of interaction:

The exact mechanism for the warfarin-quinolone drug interaction is unknown. Reduction of intestinal flora responsible for vitamin K production by antibiotics is probable as well as decreased metabolism and clearance of warfarin. Displacement of warfarin from protein binding sites could be another possible reason for potentiation of warfarin effect (Carroll et., al. 2008)

Prevention:

Culture and identify microbial pathogen prior to initiation of antibiotic therapy. Consider culture sensitivity

screening. The metabolism of warfarin may be delayed in patients administered enoxacin, ciprofloxacin, norfloxacin, or ofloxacin; thus, quinolone selection should focus on one of the newer agents that has not demonstrated significant impairment of warfarin metabolism. Additionally, microbial pathogen identification and sensitivity prior to antibiotic initiation will decrease the prevalence of unnecessary drug interaction risk (Carroll et., al. 2008).

Management:

Prothrombin time and INR should be monitored during co-administration of warfarin with a quinolone. If use of ciprofloxacin is imperative, then monitor INR every other day and adjust warfarin dose as necessary. Signs and symptoms of an active bleed should be monitored daily with particular attention to the appearance and patterns of bruises. Signs of an active bleed include: coughing up blood in the form of coffee grinds (hemoptysis), gum bleeding, nose bleeds, cola- or tea-colored urine (hematuria), and black, tarry stools (hemoccult positive).

WARFARIN- PHENYTOIN (Panegyres and Rischbieth 1991)

Impact:

Increased effects of warfarin and/or phenytoin

Mechanism of Interaction:

Phenytoin may increase or decrease the anticoagulant effect of warfarin. A decrease in the effect has been attributed to increase in the metabolism of warfarin as phenytoin causes hepatic enzyme induction. Increase in the effect has been attributed to displacement of warfarin from plasma protein binding sites by phenytoin. In addition phenytoin itself can prolong prothrombin time (Levine and Sheppard 1984).

Prevention:

Obtain baseline phenytoin levels prior to initiation of warfarin. Monitor prothrmbin time and INR during coadministration.

Target INR should be towards the lower end of the therapeutic range.

Management:

Prothrombin time, INR , and phenytoin levels should be monitored during co-administration.Signs and symptoms of an active bleed should be monitored daily with particular attention to the appearance and patterns of bruises. Signs of an active bleed include: coughing up blood in the form of coffee grinds (hemoptysis), gum bleeding, nose bleeds, cola- or tea-colored urine (hematuria), and black, tarry stools (WHO 2004).

ACE INHIBITORS -POTASSIUM SUPPLEMENTS (Palmer et al., 2004)

Impact:

Elevated serum potassium

Mechanism of Interaction:

Inhibition of ACE results in decreased aldosterone production and potentially decreased potassium excretion.

Prevention:

Measure serum potassium level prior to initiation of ACE-inhibitor in a patient. Monitor potassium levels for 3 to 5 days after initiation of therapy and with each dose increment, followed by one week later.

Management:

Potassium levels greater than 5 should be monitored carefully due to risk of severe hyperkalemia and EKG changes. Watchrenal function (BUN, SCr) also. Adjust potassium supplementation if levels increase

ACE INHIBITORS – SPIRONOLACTONE (Bauersachs et al., 2000 ; Eike Wrenger et al., 2003)

Impact:

Elevated serum potassium levels

Mechanism of interaction:

Unknown, possibly an additive effect.

Prevention:

Evaluate need for additional drug therapy**.** Measure serum potassium level prior to initiation of spironolactone in a patient.

Monitor potassium levels for 3 to 5 days after initiation of therapy and with each dose increment, followed by one week later.

Management:

Potassium levels greater than 5 mmol/L should be monitored carefully due to risk of severe hyperkalemia and EKG changes. Watch renal function (BUN, SCr) also. Avoid potassium supplements in patients taking this combination of medications, unless the need is documented and the patient is monitored closely for hyperkalemia (Pitt et al., 1999).

DIGOXIN – AMIODARONE (Fenner et al., 2009)

Impact:

Digoxin toxicity

Mechanism of Interaction:

Multiple theories exist, but actual mechanism is unknown. Digoxin does not undergo any metabolism via the cytochrome P450 (CYP450) enzyme system but rather is a major substrate for the efflux pump known as multidrug resistance-associated protein (MDR) or more commonly called, P-glycoprotein (P-gp) (Cavet et al., 1996). Amiodarone is inhibitor of P-gp. Since digoxin is a major substrate for P-gp and amiodarone is a known inhibitor of P-gp, leads to decrease digoxin clearance, resulting in prolonged digoxin activity. There may also be an additive effect on the sinus node of the heart (Koonlawee et al., 1984).

Prevention:

Obtain digoxin level prior to initiation of amiodarone therapy. Then, Consider reducing digoxin dosing by 50% when givingit with amiodarone, and monitor digoxin levels once weekly for several weeks.

Management:

Maintain digoxin level between 1-2. Monitor for signs and symptoms of digoxin toxicity (abdominal pain, anorexia, bizarre mental symptoms in the elderly, blurred vision, bradycardia, confusion, delirium, depression, diarrhea, disorientation, drowsiness, fatigue, hallucinations, halos

around lights, reductionin visual acuity, mydriasis nausea, neuralgia, nightmares, personality changes, photophobia, restlessness,vertigo, vomiting, and weakness).

DIGOXIN – VERAPAMIL (Pedersen et al., 1981)
Impact:
Digoxin toxicity

Mechanism Of Interaction:

Synergistic effect of slowing impulse conduction and muscle contractility, leading to bradycardia and possible heart block. The mechanism of the digoxin-verapamil interaction consists of decreases in both renal and extrarenal clearance of digoxin by verapamil. Since the creatinine clearance does not change under the influence of verapamil, the decreased renal clearance of digoxin appears to be due to an inhibition of tubular secretion. The elevated plasma digoxin concentration induced by verapamil is associated with an inotropic effect as assessed by a measurement of systolic time intervals. When the serum digoxin levels are markedly elevated as a result of coadministration of verapamil, lethal cardiac toxicity may occur (Klein et al., 1982).

Prevention:

Monitor heart rate and EKG–PR interval. Evaluate selection of verapamil and digoxin. If patient has CHF, note that verapamil has no proven benefit in reducing mortality or morbidity; furthermore, digoxin offers no additional benefit in mortality, but does improve symptomatology (Zatuchni 1984).

Management:

Monitor heart rate and EKG–PR interval. Monitor for signs and symptoms of digoxin toxicity (abdominal pain, anorexia, bizarre mental symptoms in the elderly, blurred vision, bradycardia, confusion,delirium, depression, diarrhea, disorientation, drowsiness, fatigue, hallucinations, halos around lights, visualacuity, mydriasis, nausea, neuralgia, nightmares, personality changes, photophobia, restlessness, vertigo,vomiting, and weakness) (Matthew 2016).

THEOPHYLLINE – FLUOROQUINOLONES (Radandt et al., 1992**)**
Impact:
Theophylline toxicity

Mechanism of Interaction:

The fluoroquinolones have been shown to interact with the hepatic metabolism of theophylline and increase serum theophylline concentrations. The quinolone metabolite, 4-oxoquinolone, inhibits the N-demethylation of theophylline, leading to a decrease in the clearance of theophylline. The resultant rise in theophylline concentrations corresponds with the decrease in clearance and possible toxicity (Matuschka and Vissing 1995).

Prevention:

Obtain theophylline level prior to administartion of a quinolone and the dosage of theophylline may need to be reduced in order to avoid toxicity. Of the quinolones, enoxacin and ciprofloxacin reduce theophylline clearance by 30-84%. In contrast, ofloxacin and norfloxacin cause less inhibition of the metabolism of these compounds, and reduction of the theophylline dosage is not routinely required. Hence, switching to gatifloxacin, levofloxacin, moxifloxacin, or trovafloxacin; these agents appear not to inhibit theophylline metabolism can prevent theophyllin toxicity (Wijnands and Vree 1988).

Management:

Reduce the dose and monitor theophylline levels. Maintain level within targeted range of 5-15mcg/mL; however, theophylline toxicity may result even when the level is within the targeted range. Signs and symptoms of theophylline toxicity include nausea, vomiting, irritability with unrest, anxiety, trouble sleeping, seizures or a fast heartbeat (Sano et al., 1988).

CONCLUSION

It is desirable to understand the basic pharmacology of drugs so as to avoid giving drugs that are additive in nature or those acting on the same or multiple sites. Special care is needed while prescribing certain drugs with the greatest propensity for interactions. It would be easy to conclude from the above facts that it is extremely risky to give a patient more than one drug not only on long term even for short term. It is prudent to remember the subsets of populations like the elderly, critically ill, and those suffering from chronic disease as they are more susceptible. Recommended future research.

LIST OF ABBREVIATIONS

NSAIDs : Non Steroidal Anti-inflammatory DrugsCOX-2 : Cyclooxygenase-2INR : International Normalized Ratio SCr : Serum Creatinine
P-gp : P-glycoprotein

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