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VIEWS

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Determination of Process Variables Using Factorial Design on Entrapment Efficiency of Levofloxacin Nanoparticles

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ABSTRACT

The Levofloxacin nanoparticles have been developed to controlled drug delivery systems to improve the bioavailability, reduce dose frequency, toxicity and patient compliance. Many processing variables can influence the characteristics of the resulting Levofloxacin nanoparticles. The objective of the present study was to study the effect of some selected process variables, drug/chitosan and concentration of tripolyphosphate on the encapsulation efficiency, by applying 3-level full factorial design analysis, accounting for percent drug entrapment as dependant variable. The general factorial design with an R^2 value of 0.9643 was selected as the suitable model for further analysis. The model F-value of 27.03 with probability P > F of 0.0037 implies that this model is significant with only a 0.37% chance that this F value could have occurred due to noise. Precision is a measure of the signal-to-noise ratio, and a value greater than 4 is required. The probability P-value is used to quantify this probability and is a very good indicator of significance. The SEM image of the nanoparticles revealed that they are spherical, non porous and uniform with smooth surface. The drug entrapment efficiency influenced by mainly two basic formulation factors which consist of drug/polymer ratio and concentration of tripolyphosphate. Findings established the role of the statistical design in predicting the values of independent variables for preparation of nanoparticles having predetermined percent drug entrapment.

Key words: Levofloxacin, Nanoparticles, Factorial design, Ionic gelation.

INTRODUCTION

Significant efforts have been done in recent years in the application of biodegradable nanoparticles as it offers a suitable means of site-specific and/or time-controlled delivery of small or large molecular weight drugs, improving efficacy of the drugs, improving bioavailability, extending drug or gene effect in target tissue and improving the stability of therapeutic agents against chemical/enzymatic degradation [1]. The nanoparticles protects the drug from premature degradation, interaction with biological environment, thereby it enhances absorption in the tissues and improves the intracellular penetration [2].

Oral drug delivery is the most favored manner of drug delivery for achieving systemic and local effects. The problem related with the conventional oral dosage form is frequent administration of drug to retain the concentration within the therapeutically effective range, which results in a fluctuated drug level and consequently undesirable toxicity and poor efficiency. So to overcome such problems associated with conventional oral dosage form, the idea of controlled drug delivery systems was introduced [3]. The real challenge in the development of a controlled drug delivery system is not just to control the drug release, also to extend the existence of the dosage form in the absorption site until the entire drug is completely released in the preferred period of time [4-6].

Levofloxacin (Biopharmaceutical Classification System I) is a broad spectrum antiinfective agent, under the third generation fluroquinolone derivative mainly used for the treatment of chronic obstructive pulmonary diseases (COPD), community acquired pneumonia (CAP), pyelonephritis and urinary tract infections. Levofloxacin is

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rapidly and completely absorbed after oral administration. Peak plasma concentrations are usually attained one to two hours after oral dosing. The mean terminal plasma elimination half-life of Levofloxacin ranges from approximately 6 to 8 hours following single or multiple doses of Levofloxacin given orally or intravenously [7].

Chitosan, a natural polysaccharide obtained from Crustaceans, insects, fungi etc and it has more properties such as bioadhesiveness, film-forming ability, gelation characteristics and as penetration enhancer. It has a favourable effect on tight junction opening epithelial cells. Due to its polymeric cationic characterization, it interacts with negatively charged molecules/polymers. The chitosan nanoparticles were formulated by ionic interaction between the cationic chitosan and anionic counter ions (tripolyphosphate) thereby the polymer linkage occurs [8]. The chitosan degradation depends on molecular weight and deacetylation degree. The absorption and distribution also depends upon molecular weight i.e. larger molecular weight excretes faster without absorption.

The present study focused on the preparation of Levofloxacin loaded nanoparticles by statistical optimization technique. The characteristics of the resultant Levofloxacin nanoparticles influence many processing variables. It is difficult to assess the effect of the variables individually or in combination. The objective of the present study was to study the effect of selected process variables, Levofloxacin/ chitosan (A) and amount of tripolyphosphate (B), on the entrapment efficiency by applying 3-level factorial design analysis.

Factorial design is a statistical tool that allows experimentation on several factors simultaneously. A technique of 3^2 factorial design taking two prime selected variables at three different levels, affecting the entrapment efficiency was used to design the experimental batches for the preparation of Levofloxacin loaded chitosan nanoparticles by ionic gelation method. The model is a mathematical equation used to predict a given response. The software is used to fit linear, quadratic, or cubic polynomials to the response. The aim of this investigation was to derive a mathematical model suitable for establishing a quantitative relationship among the variables and predicting the quantitative values of selected independent variables to prepare Levofloxacin loaded chitosan nanoparticles having predetermined encapsulation efficiency [9].

MATERIALS AND METHODS

Materials

Levofloxacin was obtained as a gift sample from Orchid chemicals, Chennai, India. All other reagents used were of analytical grade. Chitosan was purchased from Central fisheries department, Cochin, India. Tripolyphosphate (TPP) was purchased from Sigma Aldrich, Bangalore, India. Sodium hydroxide and acetic acid were purchased from Merck India Ltd. Mumbai. All other reagents used were of analytical grade.

Methods

Formulation of LEV - chitosan nanoparticles

Nanoparticles of Levofloxacin were prepared by the ionic gelation method. The compositions of different formulations of Levofloxacin nanoparticles are shown in **Table 1.** Chitosan gel solution (0.1, 0.2 and 0.3 %) was prepared using glacial acetic acid and stabilized overnight to obtain clear solution [10]. Tripolyphosphate solution (0.1, 0.3 and 0.5 %) were prepared in distilled water and added drop wise with a syringe to the above different concentration of gel solution (drug and chitosan solution) with constant stirring and further sonicated for 1 h. Then the resulting suspension was subsequently centrifuged at 10000 rpm for 10 min, further the pellets obtained were resuspended in deionised water by sonication, then centrifuged and dried at room temperature. Finally the resulting suspensions were centrifuged for 4 times (15 min) at 10000 rpm and washed with distilled water and dried [11]. The compositions of different Levofloxacin nanoparticles are shown in **Table. 1.**

Table 1. 3^2Factorial design for preparation of Levofloxacin loaded chitosan nanoparticles

Variables	Levels		
	Low	Medium	High
Levofloxacin/ chitosan (A)	1:1	1:2	1:3
Amount of tripoly-phosphate (B)	0.1	0.3	0.5

Drug entrapment efficiency

'The direct and indirect methods were adapted to estimate the encapsulation efficiency of Levofloxacin loaded in the nanoparticles. The direct method was performed by dissolving the samples (equivalent to 770 mg of Levofloxacin), in 100 mL of phosphate buffer (pH 6.8) at 37 ± 5 °C and which was stirred at 100 rpm [12]. The samples are centrifuged at10000 rpm for 30 min. Finally the supernatant solution was assayed by UV Spectrophotometer at 287 nm (Shimadzu 1800, Japan).

In indirect method, samples (equivalent to 770 mg of Levofloxacin) were dissolved in 100 mL of ethanol under occasional shaking for 1 h. Then, the aqueous medium was ultracentrifuged at 10000 rpm for 30 min to separate the samples. Further nanoparticles were separated from the suspending medium by filtration using 0.1 µm membrane filter. The amount of free Levofloxacin in the supernatant medium was assayed by UV spectrophotometer at 287 nm (Shimadzu 1800, Japan). The comparative studies of indirect

and direct method results showed almost similar results. The direct method was selected for the estimation of drug in nanoparticles and for further studies also

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total amount of drug loaded-free drug in supernatant}}{\text{Total amount of drug loaded}} \times 100$$

Surface morphology

Shape and surface morphology of Levofloxacin nanoparticles were studied using scanning electron microscopy (SEM). For shape and surface morphology the nanoparticles were mounted on metal stubs and the stub was then coated with conductive gold with sputter coater attached to the instrument. The photographs were taken using a Jeol scanning electron microscope (JEOL-JSM-AS430, Japan).

Statistical Design

Design-Expert software (Stat-Ease, Minnea-polis, MN) was used. A 3-level full factorial design with axial and center points was used. Nine runs were performed. Actual fitting of the model was computed using the statistical software. The design matrix is presented (Table 2).

Table 2
Experimental design matrix with entrapment efficiency

Stan-dard	Run	Factor A	Factor B	Entrapment Efficiency (%)
7	1	0.5	0.5	67.79
1	2	0.5	0.1	60.06
6	3	0.25	0.3	71.33
5	4	0.33	0.3	69.79
3	5	0.25	0.1	70.31
9	6	0.25	0.5	74.29
2	7	0.33	0.1	66.51
4	8	0.5	0.3	62.43
8	9	0.33	0.5	71.33

The model is validated using ANOVA calculation and then the estimation pure measurement error is done. The variance of these observations pooled over all to get an estimate of pure error of variance. The F-test on regression and lack of fit will be useful for judging descriptive properties of a model and the significance of model terms. Once a model is selected and validated, the brute force method is applied for the prediction of response. With the help of 3D-response surface or a 2D contour diagram, the prediction is done using these graphs either by grid search or feasibility search methods [9].

RESULTS

The general factorial design with an R^2 value of 0.9643 was selected as the suitable model for further analysis. ANOVA results are summarized inTable3.

ANOVA results indicated that drug/polymer ratio was the most significant factor influencing entrapment efficiency.

Final Equation in Terms of Coded Factors:

Entrapment efficiency (%) = 68.20-4.77 *A [1] + 1.005 *A [2] -2.57 *B [1] -0.35 *B [2]

The model F-value of 27.03 with probability P > F of 0.0037 implies that this model is significant with only a 0.37% chance that this F value could have occurred due to noise. The correlation co-efficient $R^2 = 0.9643$. Precision is a measure of the signal-to-noise ratio, and a value greater than 4 is required. The probability P-value is used to quantify this probability and is a very good indicator of significance [13].

Normal plot of residuals clearly showed the variables influencing entrapment efficiency. Both main and interactive effects lie far away from the centre (Figure 1) and the effect of chitosan and tripolyphosphate for entrapment efficiency is shown in Figure 2.

Scanning electron microscopy (SEM) is a helpful tool to examine shape of nanoparticles and surface characteristics. The SEM image of the nanoparticles revealed that they are spherical, non porous and uniform with smooth surface (Figure 3).

Table 3
ANOVA for selected factorial model

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	160.3093778	4	40.07734	27.03707	0.0037
A-A	114.2038889	2	57.10194	38.52224	0.0024
B-B	46.10548889	2	23.05274	15.55189	0.0130
Residual	5.929244444	4	1.482311	–	–
Cor Total	166.2386222	8	–	–	–

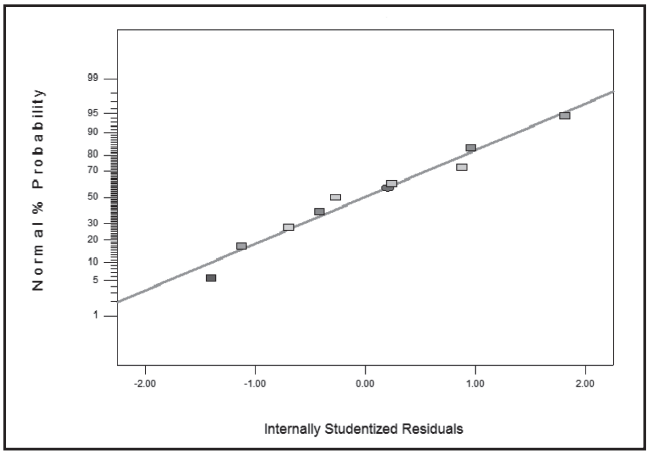

Fig.1: Normal plot of residuals for the selected variables

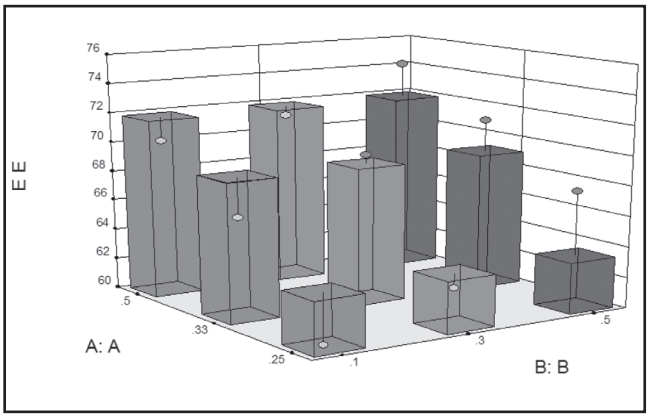

Fig.2: The effect of chitosan and tripolyphosphate for entrapment efficiency

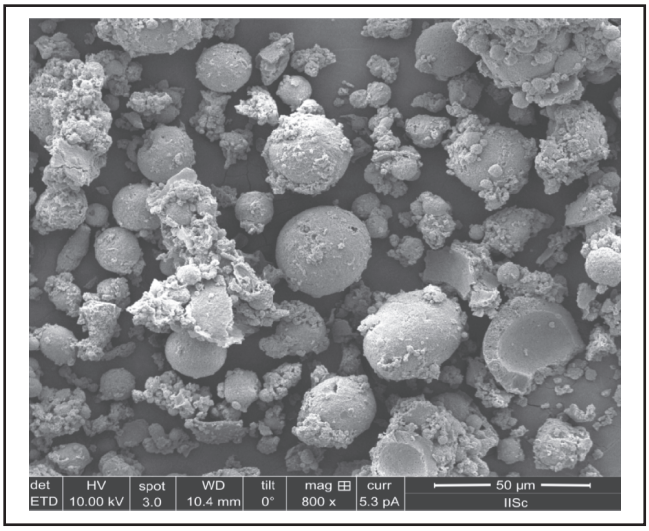

Fig.3: Surface morphology of Levofloxacin nanoparticles

DISCUSSION

The drug entrapment efficiency influenced by mainly two basic formulation factors which consist of drug/polymer ratio and concentration of tripolyphosphate. In the present study a factorial method was used as a replacement for traditionally evaluating one factor at a time. A factorial design provides a dissimilarity of averages and thus statistically proving the effects. One of the grand advantages of factorial design is that it reveals interaction between factors thus helps to recognize how different factors influence the drug entrapment. Moreover, various other inferences concerning the product can be made using factorial analysis. Therefore our main objective was to exploit this technique to comprehense the effects of the above mentioned factors and interactions on drug entrapment. The information obtained would then be used to gain a formulation with desired drug entrapment and analysis of statistical design results revealed a linear model. The response surface method used in this study approved us to produce maps of various responses in 3D plots and therefore able to assess the effects of different combinations of drug/polymer ratio and concentration of tripolyphosphate on various responses, so long as the factors evaluated remained within the ranges of the factorial design points.

Intermittently, due to the variations in molecular weight and other properties of the polymer, the nanoparticles obtained are not completely spherical. But the obtained nanoparticles were smooth and without any deformed or rough surfaces.

Additional, a checkpoint analysis was performed in order to evaluate the predictive potential of our statistical models for formulations that do not represent actual data points in the study. This approach enabled us to fully understand the effects of drug/polymer ratio, concentration of tripolyphosphate while performing statistically fewer experiments than if we had evaluated one factor at a time. Thus using a statistical design approach we obtained accurate models and understand the influence of various factors on drug entrapment.

The resulting response surface plot for entrapment efficiency shows highest entrapment at high concentration of chitosan and tripolyphosphate (crosslinking agent) and vice versa. The response surface plot shows how drug entrapment changes as the concentration of chitosan and tripolyphosphate varies. The data revealed that the drug entrapment efficiency dependent on polymer concentrations.

CONCLUSION

Findings established the role of the statistical design in predicting the values of independent variables for preparation of nanoparticles having predetermined percent drug entrapment.

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In Vitro Anthelmintic Activity of Stems of *Cuscuta Reflexa*

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ABSTRACT

Crude extracts of *Cuscuta Reflexa* (*Cuscutaceae Convolvulaceae*) were evaluated for in-vitro anthelmintic activity on the Indian adult earthworms *Pheritima posthuma*. The extracts of *Cuscuta Reflexa* have shown a dose dependant inhibition of spontaneous motility (Paralysis) of earthworms. It has been observed that all pet. Ether, chloroform, and methanolic extracts have shown anthelmintic activity, which was compared with albendazole as reference drug. The exact mode of action and the constituents responsible for the anthelmintic activity needs to be investigated.

Key words Anthelmintic, *Cuscuta reflexa*, Albendazole, *Pheritima posthuma*

Introduction

Infections caused by various species of parasitic *helminths* (worms) are among the most widespread of all chronic infections. It is estimated that over half the world's population may be infected with gastrointestinal helminths; children often become infected with one or more species almost as soon as they are born and may remain infected throughout their lives. In some cases (e.g. *threadworms*), these infections result mainly in discomfort and do not cause substantial ill health, but others, such as *schistosomiasis* (*bilharzia*) and *hookworm* disease, can produce very serious morbidity.

The current antihelminthic therapies act by incapacitating the parasite by paralysis, damaging the worm such that the immune system can eliminate it, or by altering its metabolic processes. Because the metabolic requirements of these parasites vary greatly from one species to another, drugs that are highly effective against one type of worm are ineffective against others and because of the prevalence of helminth infections, treatment of *helminthiasis* is one of very great practical therapeutic importance.[5]

Cuscuta reflexa, (syn. Dodder, Akashwel, amarwel), belonging to family *Cuscutaceae* a division of *Convolvulaceae is a* leafless parasite plants having yellow or orange thread like stem.[2,3] The stem has been used as purgative, in protracted fever, bilious disorders, induration of liver, abortifacient and in melancholia. Externally it has been used for itch and washing sores. Knowing such extensive use in the past, we decided to explore the anthelmintic activity of various extracts of the plant. [7, 8,9,10]

Materials And Methods

Plant material

The plant material *Cuscuta Reflexa* was collected from Taked,Nandi hills region (Dist. Nashik, Maharashtra) in Feb. 2010. The plant material were identified and authenticated by Dr.P.G. Diwakar from Botanical Survey of India, Pune (Ref no. BSI/WC/Tech/2010/374). The plant material was dried in sunlight, pulverized, passed through sieve no. 40 and stored in air tight container and used for further extraction.

Extraction

The shade dried and course powdered plant material of *Cuscuta reflexa* was subjected to extraction subsequently with Petroleum ether, Chloroform, and methanol.

Preliminary Phytochemical Screening

Petroleum ether, chloroform, ethanol, and aqueous extracts of *Cuscuta reflexa* were subjected to preliminary qualitative phytochemical investigations. All the extracts were screened for the presence of secondary metabolites such as steroids, alkaloids, flavonoids and tannins using standard methods

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Animals

Indian adult earthworms (*Pheretima posthuma),* collected near the swampy water in farms were used to evaluate anthelmintic activity. The average size of earthworm selected was 6-8 cm.

Drugs and Chemicals

Albendazole was purchased and used during the experimental protocol.

Anthelmintic Activity

Anthelmintic activity was carried as per the method reported by Ajaiyeoba *et al* with minor modifications. Intestinal round worms have a close resemblance with Indian earth worms which are readily available; hence the *in vitro* Anthelmintic activity of Cuscuta *reflexa* was performed on adult Indian earth worm *Pheritima posthuma.*[1]

Indian earthworms of desired size were selected for the activity, 42 worms were randomly devided in 7 groups of six worms each and added to a 50 ml solution containing different concentrations, of Pet. Ether, chloroform and methanolic extracts (20, 50 mg/ ml in normal saline). This was done in duplicate for all extracts. Albendazole 20 mg/ ml in 50 ml normal saline was used as a standard. All the extracts and the standard drug solution were freshly prepared before starting the experiments. Mean time for paralysis (in min) was noted when no movement of any sort could be observed except when the worm was shaken vigorously; time for death of worms (in min) was recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50°C).

Statistical Analysis

One way ANOVA was followed by Dunnet'smultiple comparision test

Results

Treat-ment	Conc. µg/ml	Time of Paralysis (Min)	Time of Death (Min.)
Alb.	20	3.348±0.12*	9.19±0.145*
PECR	20	9.143±0.134*	15.04±0.169*
PECR	50	8.137±0.206*	12.05±0.148*
CECR	20	7.256±0.036*	12.56±0.151*
CECR	50	5.236±0.112*	11.05±0.208*
MECR	20	5.216±0.063*	11.59±0.189*
MECR	50	4.282±0.049*	10.01±0.156*

Results of the anthelmintic activity of extracts of stems of Cuscuta reflexa are presented in table 1, the pet. Ether extract of Cuscuta reflexa (PECR), Chloroform extract of Cuscuta reflexa (CECR), and methanolic extract of Cuscuta reflexa (MECR) show dose dependent anthelmintic activity as compared with the satandard anthelmintic drug, Albendazole.

Table 1.-Anthelmintic activity of extracts of Cuscuta reflexa.Alb.-Albendazole, PECR- Pet. Ether extract of Cuscuta reflexa, CECR- Chloroform extract of Cuscuta reflexa, and MECR- Methanolic extract of Cucuta reflexa. Values expressed are mean±SEM. * indicated $p< 0.01$ when compared with control.

Discussion

In this study, anthelmintic assay of various extracts of *Cuscuta Reflexa* was performed on adult Indian earthworm, *Pheretima posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings and ease of availability, earthworms have been used widely for the initial evaluation of anthelmintic compounds in vitro. Various medicinal plants have been successfully screened for in vitro anthelmintic activity using this model. The results of the present study indicates that all the three extracts tested for the anthelmintic activity possess significant activity as compared to the Albendazole, a benzimidazole(BZA) derivative. The BZAs are versatile anthelmintic agents, particularly against GI nematodes, where their action is mediated locally in the intestinal lumen. Albendazole acts as anthelmintic agent by inhibiting microtubule polymerization by binding to b-tubulin, Immobilization i.e. paralysis and death of susceptible GI parasites occur slowly, and their clearance from the GI tract may not be complete until several days after treatment[10]. The anthelmintic effect shown by the extracts are dose dependent and may be attributed to the presence of tannins in the extracts, Tannins were shown to produce anthelmintic activities. Chemically tannins are polyphenolic compounds. Some synthetic phenolic anthelmintic (eg) niclosamide, oxyclozanide and bithionol are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation[11]. It is possible that tannins contained in the extracts of *Cuscuta Reflexa* produced similar effects. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastro intestinal tract of host animal or glycoprotein on the cuticle of the parasite and cause death[11].

Preliminary phytochemical studies on *Cuscuta Reflexa* revealed the presence of flavanoid s, steroids, carbohydrates, triterpenoids, tannins, proteins and flavanols. Some of these phytoconstituents may be responsible to show a potent anthelmintic activity. The efficacy of plant materials has been judged on the basis of the loss of spontaneous movement and/or complete destruction of the worm in *in-vitro* studies (Goto et al. 1990; Robinson et al. 1990; Togo et al.1992).

In conclusion, the traditional claim of *Cuscuta Reflexa* as an anthelmintic has been confirmed as the extracts shown activity against *Pheritima postuma*. Further studies are

necessary to isolate and reveal the active compound contained in the crude extracts of *Cuscuta Reflexa* responsible for activity and to establish the mechanism of action are required.

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Development and Validation of Bioanalytical Method for Simultaneous Quantification of Veratric Acid, Mebeverine Acid and Desmethyl Mebeverine Acid in Human Edta Plasma by Using LC-MS/MS

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ABSTRACT

Mebeverine undergoes facile hydrolysis and is metabolized completely after oral administration. The first step in metabolism is hydrolysis leading to Mebeverine alcohol (MAL) and Veratric acid (VA), mebeverine alcohol further gets converted to mebeverine acid (MAC) by oxidation. HPLC methods have been reported for estimation of Mebeverine, Mebeverine acid and mebeverine alcohol using HPLC and coulometric detection[1]. A simple, sensitive and selective method for the simultaneous determination of Veratric acid, Mebeverine acid and Desmethyl Mebeverine acid by using rapid high-performance liquid chromatography / negative heated electrospray ionization tandem mass spectroscopy has been developed for the first time. The metabolites Mebeverine acid (MAC), Desmethyl mebeverine acid (DMAC), Veratric acid (VA) and internal standard (IS) salicylic acid were extracted from human plasma by solid phase extraction (SPE) and separated on Alltima, 50×4.6 mm, 5µm within 8.5 min. Quantitation was performed on a triple quadrapole mass spectrometer employing heated electro spray ionization technique in selective reaction monitoring (SRM) and negative ion mode. The precursor to product transitions monitored for MAC, DMAC, VA and IS were m/z 278.302/130.100, 264.135/130.100, 181.038/137.100, 137.037/93.000 respectively. The assay method was validated with the linear range of 77.005-6129.762 ng/mL for MAC, 100.571-8005.616 ng/mL for DMAC, 322.666-25684.882 ng/mL for VA.

KEY WORDS: LC-MS\MS, Human Plasma, Bioanalytical, Veratric acid, Mebeverine acid Desmethyl mebeverine acid, Validation.

Objective

The objective of the present study was to develop and validate a simple and sensitive method for simultaneous determination of Veratric acid, Mebeverine acid and Desmethyl Mebeverine acid in human plasma using Liquid Chromatography- Tandem Mass Spectrometry

Introduction

Mebeverine is a musculotropic antispasmodic drug without atropic side effects whose major thearapeutic role in the treatment of irritable bowel syndrome. It is also indicated for treatment of gastrointestinal spasm secondary to organic disorder. Both in vivo and in vitro (in plasma/ blood samples), mebeverine is rapidly metabolized and yield at least 12-13 metabolites. The main ones have been identified as Veratric acid (VA), mebeverine alcohol (MAL), mebeverine acid (MAC) and O-desmethyl derivatives of MAL and MAC. Mebeverine is a Veratric acid ester of a substituted ethylamphetamine derivative. The first step in mebeverine degradation is hydrolysis (by esterases) to Veratric acid and mebeverine alcohol. In vivo hydrolysis of orally administered mebeverine starts already in the intestine and continuous in the blood. In fact, it has been suggested that orally administered mebeverine is completely hydrolyzed already pre-systemically and that no parent molecule reaches the systemic circulation, although sporadic

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reports on detection of mebeverine in blood/plasma samples (in living subjects or post-mortem) have been published.

In this report, we describe for the first time a simple and sensitive method for the simultaneous estimation of Veratric acid, Mebeverine acid and Desmethyl mebeverine acid in human plasma by using liquid chromatography-tandem mass spectrometry. Chemical structure of all the three metabolites are as below.

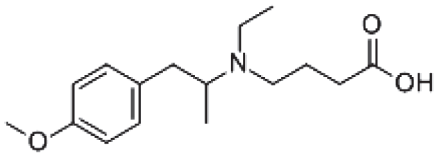

Fig.1 (a) Mebeverine acid

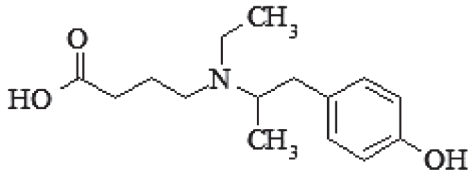

Fig.1 (b) Desmethyl mebeverine acid

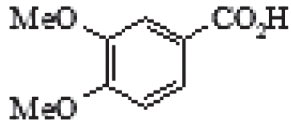

Fig.1 (c) Veratric acid

Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for all the analyte and IS, as well as a short run time. It was found that a mixture of 90:10 MeOH : 2mM Ammonium formate could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analytes and the IS at retention times of Veratric acid: 4.54 ±.0.3 min, Mebeverine acid: 2.58 ±.0.3 min, Desmethylmebeverine acid: 2.35 ±.0.3 min, ISTD: 5.62 ± 0.3 min. A flow rate of 0.5 mL/min produced good peak shapes and permitted a run time of 5.0 min in a single run. Solid phase extraction technique (Extraction method) was used for the sample preparation in this work. SPE can be helpful in producing a clean sample essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses.

Experimental

Standards, Solvents and Chemicals

- Veratric acid (RA Chem Pharma Ltd, Hyderabad)
- Mebeverine acid (RA Chem Pharma Ltd, Hyderabad)
- Desmethyl mebeverine acid (RA Chem Pharma Ltd, Hyderabad)
- Salicylic acid (as internal standard, RA Chem Pharma Ltd, Hyderabad)
- Methanol (HPLC Grade from JT Baker)
- Acetonitrile (HPLC Grade from JT Baker)
- Ammonium Acetate (Merck)
- Water (HPLC Grade)
- Human Plasma (Seha Blood bank, Hyderabad)

Instrumentation

HPLC	Shimadzu Prominence HPLC consisting of SIL-10 AD VP temperature controlled auot sampler and LC20-AD VP binary pump, a degasser and column oven
MS/MS	Thermo TSQ Quantum Ultra Tandem mass spectrometer equipped with HESI probe
Solid Phase Extraction unit	Orochem Technologies EZYPRESS™ 48 positive pressure processor

Sample preparation

Standard stock solutions of Veratric acid, Mebeverine acid, Desmethyl mebeverine acid and IS were separately prepared in methanol. Working solutions for calibration and quality controls were prepared by appropriate dilution in Methanol:Water (70:30). The IS working solution (500 ng/mL) was prepared by diluting its stock solution with a mixture of Methanol: Water (70:30). Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain concentration 306.465-9927.596 ng/mL for VA, 23.380-757.357 ng/mL for MAC, 35.360-1145.441 ng/mL for DMAC. The calibration and control bulk samples were divided into aliquots in polypropylene vials and stored in a freezer at below -80^{0}C along with STD Blank.

On each day of analysis STD Blank, STD Zero, CC, QC samples were retrieved from the deep freezer and allowed to thaw in water bath and mixed on a vortex mixture. 300 µL of STD Blank, STD Zero, CC and QC samples were transferred into pre-labeled polypropylene tubes. 50 µL of ISTD dilution (about 500 ng/mL) was added and mixed. 100 µL of 2% ortho phosphoric acid was added, samples were mixed on a vortex mixture and processed using 30 mg 1CC solid phase extraction cartridges as per the procedure given below.

Condition	: 1 mL of methanol
Equilibrate	: 1mL of Water
Sample Loading	: Load the prepared sample
Wash	: 1 mL of HPLC grade water
Wash	: 1 mL of 5 % Methanol

Drying : Dry the cartridge for approximately 2 minutes

Elution : Elute with 2×0.5 mL of Methanol

The eluent was evaporated to dryness under nitrogen at 40°C. Samples were reconstituted with 100 µL of methanol: water (70:30) and transferred to pre labeled HPLC vials.

Chromatographic conditions

Column : Alltima C_{18} (50 x4.6 mm), 5µ
Mobile Phase : 90:10 MeOH : 2mM Ammonium formate
Flow rate : 0.5 mL/min
Injection Volume: 10 µL
Column Oven Temperature : 35 ± 5°C

Method Validation

Method validation was carried out as per current regulatory guidelines for USFDA[2] and EU[3] including recommendations in the white papers and consisted of the following main parameters

- Linearity
- Precision and Accuracy
- Recovery
- Bench top stability
- Freeze-Thaw Stability
- Autosampler stability

A calibration curve was constructed from a blank sample (blank plasma processed without the IS), a zero sample (blank plasma processed with the IS) and eight non-zero samples covering the total range 322.666 - 25684.882 ng/mL for VA, 77.005 - 6129.762 ng/mL for MAC, 100.571 - 8005.616 ng/mL for DMAC including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by using ($1/x^2$) weighted least-squares linear regression on consecutive days. The acceptance criteria was

- Correlation coefficient (r^2) should be e" 0.99
- Back-calculated standard concentration must be within ±15% from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at ±20%.
- At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing six sets of QC samples on three different batches.

Mass Spectrometry Conditions

Parameters	**Value**				
Ion Source	Turbo ion heated source				
Ionization	Heated Electrospray Ionization				
Spray voltage	2500				
Vaporizer temperature	300				
Sheath gas pressure (Nitrogen)	30				
Auxiliary gas pressure (Nitrogen)	20				
Collision pressure	1.0				
Collision gas	Argon				
Capillary temperature	350				
Polarity	Negative				
Mode of Analysis	Selected Reaction Monitoring (SRM)				
Analyte	**Tube Lens**	**Skimmer offset**	**Collision Energy**	**Q1(m/z)**	**Q3(m/z)**
Veratric acid	66	10	14	181.038	137.100
Mebeverine acid	105	10	19	278.302	130.100
Desmethyl Mebeverine acid	86	16	14	264.135	130.100
Salicylic acid (IS)	39	10	15	137.037	93.000

The acceptance criteria for within- and between-batch precision were

- ±20% for LLOQ and ±15% for the other concentrations
- Accuracy was 100 ± 20% for LLOQ and 100 ± 15% for other concentrations.

Recovery of Veratric acid, Mebeverine acid, and Desmethyl Mebeverine acid, from the extraction procedure was determined by comparison of the peak area of Veratric acid, Mebeverine acid and Desmethyl Mebeverine acid in spiked plasma samples (six each of low, medium and high QCs) with the peak area of Veratric acid, Mebeverine acid and Desmethyl Mebeverine acid in samples prepared by spiking extracted drug-free plasma samples with the same concentration of Veratric acid, Mebeverine acid and Desmethyl Mebeverine acid, at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 6) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-80^{0}C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (low 210.843 ng/mL for MAC, 275.366 ng/mL for DMAC and 883.471 for VA; high 4016.051 ng/mL for MAC, 5245.059 ng/mL for DMAC and 16828.062 ng/mL for VA with six determinations for each.

Representative Spectrum (Full scan), Chromatograms and Calibration Curves for each analyte

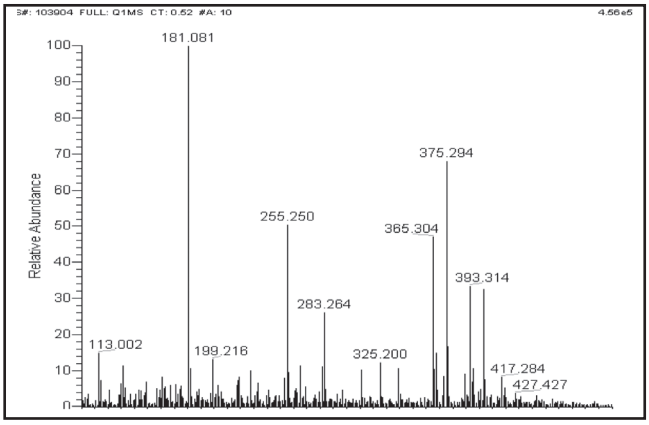

Fig.2(a). Veratric acid full scan mass spectrum

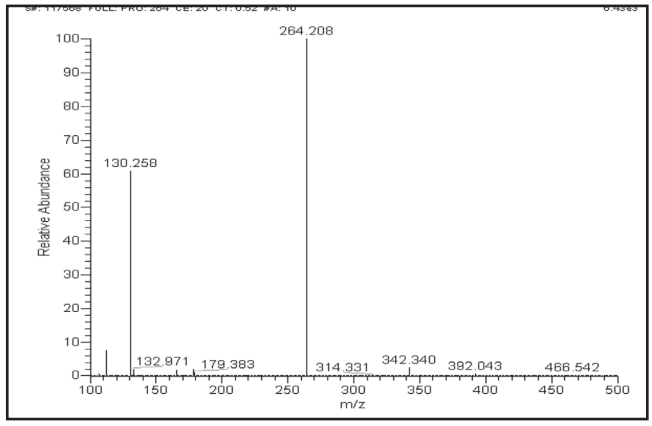

Fig.2(b). Desmethyl mebeverine acid full scan mass spectrum

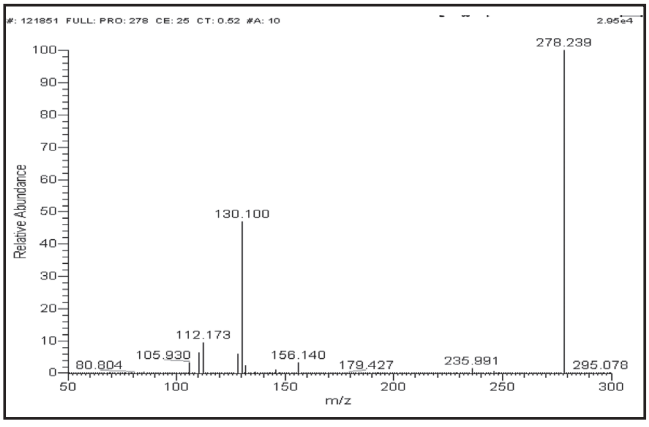

Fig.2(c). Mebeverine acid full scan mass spectrum

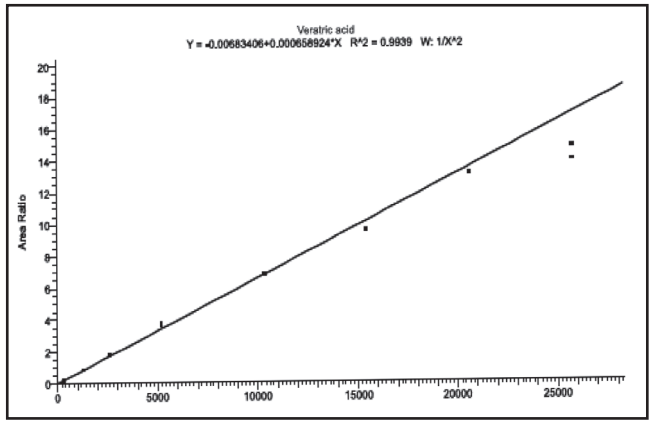

Fig.3(a). Calibration curve for Veratric acid

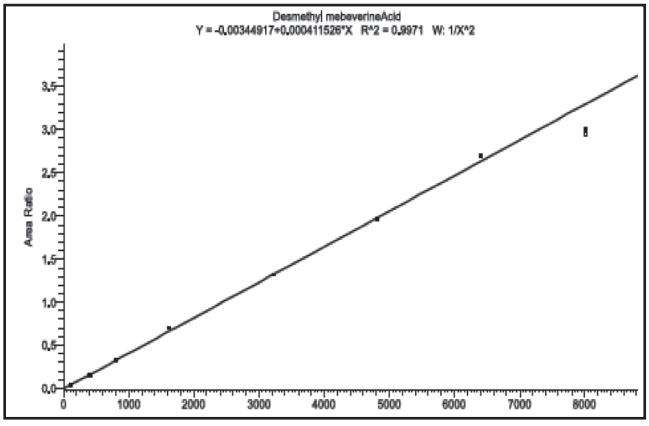

Fig.3(b) : Calibration curve for Desmethyl mebeverine acid

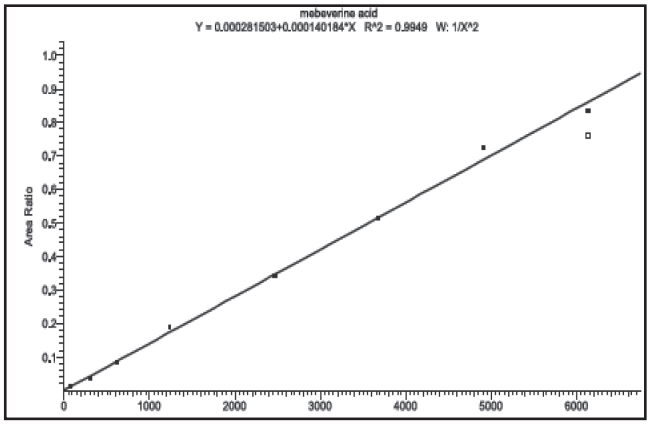

Fig.3(c) : Desmethyl mebeverine acid

The selectivity of the method was examined by analyzing six blank human plasma extract, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Excellent sensitivity was observed for a 10 µL injection volume. The SRM chromatograms obtained for an extracted LOQ plasma sample are depicted in Fig. 4(a), 4(b) and 4(c).

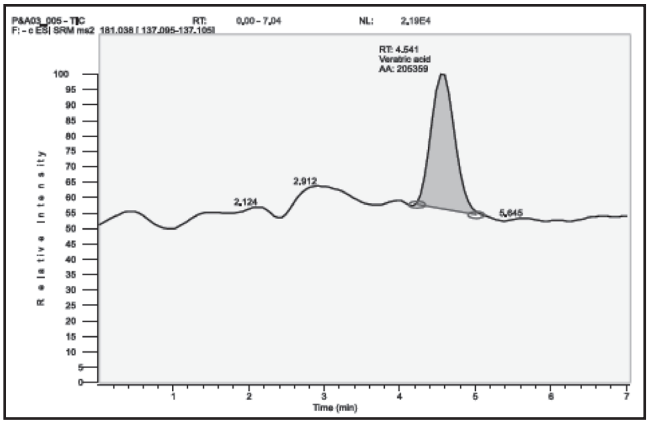

Fig.4(a).Chromatogram of Veratric acid

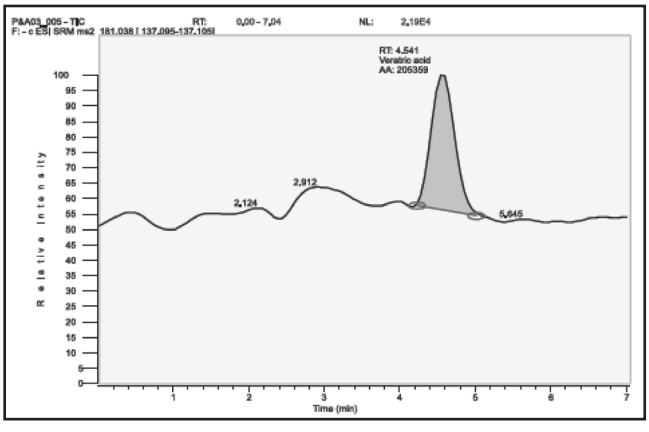

Fig.4(b).Chromatogram of Desmethyl mebeverine acid

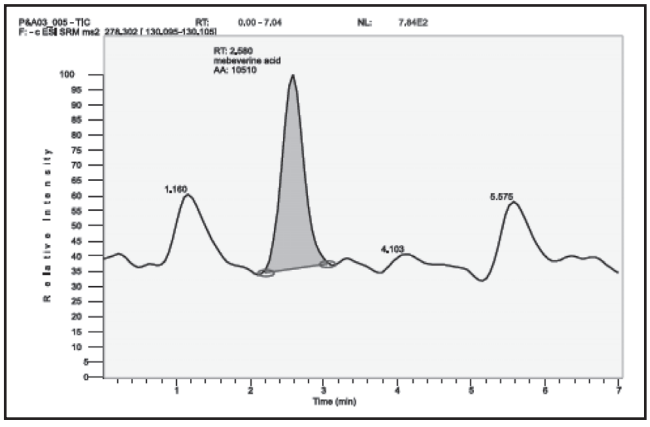

Fig. 4(c).Chromatogram of Mebeverine acid

Results and Discussions

Precision and accuracy data of back-calculated concentrations of calibration samples in human plasma.

Table: 1(a)
Veratric Acid

STD ID	Nominal Conc.	Mean	± S.D.	Precision (% C.V.)	% Accuracy
STD 1	322.666	318.1473	2.4723	0.8	99.0
STD 2	1290.665	1298.897	36.986	2.8	100.8
STD 3	2581.331	2739.516	42.2092	1.5	103.8
STD 4	5162.661	5733.493	67.4524	1.2	97.4
STD 5	10325.32	10625.56	169.1868	1.6	99.8
STD 6	15410.93	14394.81	223.9488	1.6	102.7
STD 7	20547.91	19962.72	145.2393	0.7	95.0
STD 8	25684.88	23144.96	549.1634	2.4	101.6

Table:1(b)
Mebeverine Acid

STD ID	Nominal Conc.	Mean	± S.D.	Precision (% C.V.)	% Accuracy
STD 1	77.005	79.071	0.217	0.3	102.5
STD 2	308.021	267.872	5.462	2.0	87.0
STD 3	616.041	625.4797	15.0624	2.4	101.5
STD 4	1232.082	1328.724	15.7654	1.2	107.8
STD 5	2464.164	2506.895	54.4195	2.2	101.7
STD 6	3677.857	3572.181	94.6441	2.6	97.1
STD 7	4903.809	5075.981	107.7379	2.1	103.5
STD 8	6129.762	6052.734	117.6615	1.9	98.7

Table:1 (C)
Desmethyl Mebeverine Acid

STD ID	Nominal Conc.	Mean	± S.D.	Precision (% C.V.)	% Accuracy
STD 1	100.571	101.849	0.7068	0.7	101.3
STD 2	402.282	370.737	10.7614	2.9	92.2
STD 3	804.564	821.751	5.5264	0.7	102.1
STD 4	1609.129	1721.238	12.1184	0.7	107.0
STD 5	3218.258	3280.912	64.9975	2.0	101.9
STD 6	4803.370	4655.556	155.1687	3.3	96.9
STD 7	6404.493	6581.000	60.3095	0.9	102.8
STD 8	8005.616	7672.766	327.389	4.3	95.8

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 323.351 ng/mL for Veratric acid, 77.168 ng/mL for Mebeverine acid, 100.784 ng/mL for Desmethyl mebeverine acid in human plasma. The mean response for the analyte peak at the assay sensitivity limit was ten-fold greater than the mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The precision and accuracy for within-batch and between-batch experiments at the High, middle, low and LOQ QC quantification levels were summarized in Table 2(a), 2(b) and 2(c). For the within and between batch experiments the precision and accuracy for the analyte met the acceptance criteria (d”15%). These results suggest that samples with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Table: 2(a). Precision and accuracy of Veratric acid

Within Batch (n=6)				Between Batch(n=6)		
Nominal Concentration	Back Calculated Concentration (mean ± SD)	Precision	Accuracy	Back Calculated Concentration (mean ± SD)	Precision	Accuracy
16828.02	16719.74 ± 585.23	3.5	99.4	16783.49 ± 87.91	0.5	99.7
11779.61	11871.87 ± 286.44	2.4	100.8	11937.09± 83.49	0.7	101.3
883.47	924.80 ± 49.64	5.4	104.7	908.42 ± 19.41	0.9	102.8
323.35	360.47 ± 10.90	3.5	111	329.09± 35.62	2.1	101.8

Table: 2(b). Precision and accuracy of Mebeverine acid

Within Batch (n=6)				Between Batch(n=6)		
Nominal Concentration	Back Calculated Concentration (mean ± SD)	Precision	Accuracy	Back Calculated Concentration (mean ± SD)	Precision	Accuracy
4016.05	4137.91±103.46	2.5	103.0	3984.77± 157.86	4.0	99.2
2811.23	2837.35± 143.65	5.1	100.9	2769.35 ± 69.32	2.5	98.5
210.84	195.45± 17.42	8.9	92.7	194.84 ± 3.63	1.9	92.4
77.16	82.15 ± 7.52	9.2	106.5	82.04± 3.52	4.3	106.3

Table:2(c). Precision and accuracy of Desmethyl Mebeverine acid

Within Batch (n=6)				Between Batch(n=6)		
Nominal Concentration	**Back Calculated Concentration (mean ± SD)**	**Precision**	**Accuracy**	**Back Calculated Concentration (mean ± SD)**	**Precision**	**Accuracy**
5245.05	5099.80 ± 90.51	1.8	97.2	5056.82 ± 40.75	0.8	96.4
3671.54	3546.20 ± 149.56	4.2	96.6	3542.35 ± 43.08	1.2	96.5
275.36	254.43 ± 18.05	7.1	92.4	246.59 ± 6.79	2.8	89.6
100.78	102.03 ± 2.97	2.9	101.2	96.15 ± 5.10	5.3	95.4

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 13hrs). Samples were extracted and analyzed as described above, and the results are given in Table 3(a), 3(b) and 3(c). These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the auto sampler for 2 days was also assessed. The results indicate that solutions of the three analytes and the IS can remain in the auto sampler for at least 2 days without showing significant loss in the quantified values, indicating that samples should be processed within this period of time. The data representing the stability of the three analytes in plasma at two QC levels over three freeze/thaw cycles are given in Table 4(a), 4(b) and 4(c). These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below -80^{0}C and thawed to room temperature. The stability study of the three analytes in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of the three analytes in plasma samples

Table: 3(a).Stability of Veratric acid in human plasma

Nominal Concentration (ng/mL)	Back Calculated Concentration (ng/mL)	Precision (%)	Stability(%)
	Bench Top Stability – 13 hours		
16828.026	16719.74	1.7	96.3
883.471	924.80	3.4	100.8
	Freeze Thaw Stability – 4 cycles		
16828.026	16746.94	1.6	92.2
883.471	886.97	4.9	92.0
	Auto sampler stability for 2 days		
16828.026	16883.78	2.0	102.3
883.471	913.50	6.8	105.6

Table: 3(b).Stability of Desmethyl mebeverine acid in human plasma

Nominal Concentration (ng/mL)	Back Calculated Concentration (ng/mL)	Precision (%)	Stability(%)
	Bench Top Stability – 13 hours		
5245.059	5099.80	1.3	98.5
275.366	254.43	2.6	89.7
	Freeze Thaw Stability – 4 cycles		
5245.059	5051.94	1.3	98.5
275.366	242.60	2.6	89.7
	Auto sampler stability for 2 days		
5245.059	5018.73	1.9	97.1
275.366	242.73	7.8	102.6

Table:3(c).Stability of Mebeverine acid in human plasma

Nominal Concentration (ng/mL)	Back Calculated Concentration (ng/mL)	Precision (%)	Stability(%)
	Bench Top Stability – 13 hours		
4016.051	4137.91	3.8	99.1
210.843	195.45	2.7	105.2
	Freeze Thaw Stability – 4 cycles		
4016.051	3993.84	8.9	96.5
210.843	198.14	9.6	89.2
	Auto sampler stability for 2 days		
4016.051	3822.57	1.7	101.5
210.843	190.94	6.8	99.2

at below -80^{0}C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 1 day and for 9 days under refrigeration (2-8ºC). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

The average absolute recoveries of analytes from spiked plasma samples was 74 % for MAC, 80.2 % for DMAC, 76 % for VA and 74.6% for IS. Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible.

Conclusions

In summary, a simple, rapid and reproducible method is developed and validated for the simultaneous determination of Veratric acid, Mebeverine acid, Desmethyl mebeverine acid in human plasma by LC/MS/MS in negative HESI mode using SRM. Several methods[4,5,6] have been reported for the estimation of mebeverine metabolites but none for simultaneous estimation of Veratric acid, Mebeverine acid and Desmethyl mebeverine acid has been reported . The method has shown acceptable precision and adequate sensitivity for the quantification of the three analytes/metabolites in human plasma and can be applied to a BA/BE studies. The desired sensitivity of the three analytes was achieved with an LLOQ of 323.351 ng/mL for Veratric acid, 77.168 ng/mL for Mebeverine acid and 100.784 ng/mL for Desmethyl mebeverine acid in human plasma with an acceptable within and between batch accuracy and precision. The simplicity of the assay and use of SPE and sample turnover rate of 5.0 min per sample make it a sensitive, reproducible and high-throughput method for bioanalysis of Veratric acid, Mebeverine acid, Desmethyl mebeverine acid.

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Design and Synthesis of 1, 3, 4-oxadiazole Derivatives as Antimicrobial Agents

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ABSTRACT

A series of novel 1-{2-[(5-benzyl-1, 3, 4-oxadiazol-2-yl) methoxy] phenyl}ethanone (IVa-j) were prepared via reaction of 2-(2-acetyl phenoxy)-*N*'-benzylideneacetohydrazide with chloramine-T. Further, compounds thus obtained were identified by IR, ^{1}H NMR and Mass spectral data and have been screened for their anti-microbial activity. All the synthesized compounds showed significant anti-bacterial activity and anti-fungal activity than those of standard antibacterial and antifungal agents used.

Key words: 2-hydroxy acetophenone, schiff's base, 1,3,4- oxadiazole and antimicrobial activity.

Introduction

Oxadiazole, a heterocyclic nucleus has attracted a wide attention of chemist in search for the new therapeutic molecules. Among them the derivatives of oxadiazoles have been playing an important role in the medicinal chemistry [1].Oxadiazole moiety and its various derivatives were studied frequently in the past few decades and found potent in various pharmacological and pathological conditions [2]. Literature reveals that 1, 3, 4-Oxadiazole is a highly privileged structure the derivatives of which exhibit a wide range of biological activities including antibacterial [3], antitubercular [4], antifungal [5], cytotoxic [6], anti-inflammatory & analgesic [7,8], hypolipidemic [9], anticancer [10] and ulcerogenic [11] activities. Oxadiazole derivatives have been found to possess broad spectrum antimicrobial activity and therefore are useful substructures for further molecular exploration.

The present work deals with the reaction of 1-(2-hydroxyphenyl) ethanone with ethyl chloroacetate to get ethyl (2-acetylphenoxy) acetate (1).When (1) react with hydrazine hydrate yields 2-(2-acetylphenoxy) acetohydrazide (2) which was made to react with various aromatic aldehydes to yeild 2-(2-acetylphenoxy)-*N*'-benzylideneacetohydrazide (3). Further (3) cyclised by reacting with a strong oxidizing agent chloramine-T to get final targeted 1-{2-[(5-benzyl-1,3,4-oxadiazol-2-yl)methoxy] phenyl} ethanone (4) derivatives. The structures of all the various synthesized compounds were assigned on the basis of IR ^{1}H NMR and Mass spectral data. Finally these compounds were screened for their antimicrobial activity.

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Materials and Equipments

Chemicals were purchased from Qualigens and S.D. fine chemicals as synthetic grade and used without further purification. Melting points were determined with open capillary and are uncorrected. The purity of compounds were checked by TLC using ethyl-acetate & n-hexane (2:8) as solvent system and the spots were located under either ultra violet or through exposure to Iodine vapours. I.R spectra were recorded on a Shimadzu FTIR model 8010 spectrophotometer, ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker supercon FT-NMR instrument using TMS as internal standard. Mass spectra were recorded on GCMS in dimethyl sulphoxide (University Science Instrument Center, Dharwad, India).

Methodology

Synthesis of ethyl (2-acetylphenoxy) acetate (1)

To an equimolar mixture of *o*-hydroxyacetophenone (0.05M) and ethyl chloroacetate (0.05M), solution of sodium hydroxide (0.45M) was added drop wise with stirring for half an hour. To the residue 150ml of water was added,

acidified with hydrochloric acid (5M) and product was filtered ,dried and recrystallized from ethanol.

Synthesis of 2-(2-acetylphenoxy) acetohydrazide (2)

To a suspension of (**1**) (0.01 mol) in absolute ethanol (200 ml), hydrazine hydrate (99%, 0.015 mol) was added and the reaction mixture was refluxed for 15hr on a water bath. The solution was concentrated and allowed to cool overnight. The resulting solid obtained was filtered, washed with cold ethanol, dried and recrystallized from ethanol. The compound was separated as brown crystals.

Synthesis of 2-(2-acetylphenoxy)-*N*'-benzylideneacetohydrazide (3a-j)

Equimolar amount of the hydrazide compound (0.002mol) and various aromatic aldehydes (0.002mol) in DMF (20 ml) were refluxed for 4-8 hrs. The resulting Schiffs bases (**3a-j)** were cooled and poured into crushed ice. The precipitate thus obtained was filtered, washed with cold water and recrystallized from ethanol.

Synthesis of 1-{2-[(5-benzyl-1,3,4-oxadiazol-2-yl) methoxy]phenyl}ethanone (4a-j)

An equimolar mixture of compound (0.001 mol.) **3a-j** and chloramine-T (0.001)was added in 50ml of ethanol and the reaction mixture was refluxed on a water bath for 5 hrs. The reaction mixture was cooled to room temperature and poured into crushed ice. The solid thus obtained was filtered, washed with water and recrystallized from ethanol. The physicochemical properties of the compounds **4a-j** is described in table **2**.

Characterization Data

1-{2-[(5-benzyl-1,3,4-oxadiazol-2-yl) methoxy]phenyl}ethanone (4a)

IR (KBr) cm^{-1} 3082.5 (Ar-CH), 1611.8 (C=O), 1543. 2 (C=N), 1119.8(C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.87-6.21 (m, 9H, Ar), 4.66 (2H, s, OCH_2), 3.12-2.77 (5H, m, $-CH_2$, $-CH_3$). Mass m/z: 309 M^+.

1-(2-{[5-(4-hydroxybenzyl)-1,3,4-oxadiazol-2-yl] methoxy} phenyl)ethanone(4b)

IR (KBr) cm^{-1} 3425.7 (OH), 3011(Ar-CH), 1724.3 (C=O), 1560.5 (C=N), 1160.4(C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.33-6.82 (m, 8H, Ar), 4.80 (s, 2H, OCH_2), 3.89 (s, 1H, OH), 2.87-2.34 (5H, m, CH_2CH_3).Mass m/z: 322 $M^{-1.}$

1-(2-{[5-(4-chlorobenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4c)

IR (KBr) cm^{-1} 3018.6 (Ar-CH), 1713.3 (C=O), 1582.3 (C=N), 1156.0 (C-O-C), 652 (C-Cl). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.52-6.35 (m, 8H, Ar), 4.02 (s, 2H, OCH_2), 3.26-2.65 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 343 M^{+1}

1-(2-{[5-(4-methoxybenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4d)

IR (KBr) cm^{-1} 2960.6 (Ar-CH), 1675.3 (C=O), 1576.5 (C=N), 1148.2 (C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.70- 6.54 (m, 8H, Ar), 4. 10 (s, 2H, OCH_2), 3.20 -2.78 (m, 8H, $-CH_2$, $-CH_3$, $-CH_3$). Mass m/z: 339 M^{+1}

1-(2-{[5-(2-bromobenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4e)

IR (KBr) cm^{-1} 2978.3 (Ar-CH), 1681.7 (C=O), 1575. 6 (C=N), 1168.6 (C-O-C), 617.2 (C-Br). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 8.12- 7.54 (m, 8H, Ar), 4. 52 (s, 2H, OCH_2), 2.95-2.28 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 388 M^{+1}

1-(2-{[5-(2-chloro-4-nitrobenzyl)-1,3,4-oxadiazol-2-yl] methoxy}phenyl)ethanone (4f)

IR (KBr) cm^{-1} 3043.9 (Ar-CH), 1765.2 (C=O), 1610.5 (C=N), 1554.7 (N=O), 1185.2 (C-O-C), 685.6 (C-Cl). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.29- 6.28 (m, 7H, Ar), 4. 24 (s, 2H, OCH_2), 2.54-2.18 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 388 M^{+1}

1-(2-{[5-(2,4-dichlorobenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4g)

IR (KBr) cm^{-1} 3019.2 (Ar-CH), 1632.6 (C=O), 1584.2 (C=N), 1163.8 (C-O-C), 658.3 (C-Cl). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.57- 6.14 (m, 7H, Ar), 4. 69 (s, 2H, OCH_2), 3.19 -2.61 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 378 M^{+1}

1-(2-{[5-(3,5-dinitrobenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4h)

IR (KBr) cm^{-1} 2987.1 (Ar-CH), 1683.5(C=O), 1597.6 (C=N), 1512.4 (N=O), 1172.4 (C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.92- 6.11 (m, 7H, Ar), 4. 37 (s, 2H, OCH_2), 3.10 -2-31 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 399 M^{+1}

1-(2-{[5-(3,5-dimethoxybenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4i)

IR (KBr) cm^{-1} 3096.6 (Ar-CH), 1687.9 (C=O), 1565. 7 (C=N), 1182. 4 (C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.85- 6.34 (m, 7H, Ar), 4. 54 (s, 2H, OCH_2), 3.26 -2-78 (m, 11H, $-CH_2$, $-CH_3$, $-CH_3$,$-CH_3$). Mass m/z: 369 M^{+1}

1-(2-{[5-(2,4-dihydroxybenzyl)-1,3,4-oxadiazol-2-yl]methoxy} phenyl)ethanone (4j)

IR (KBr) cm^{-1} 3342.5 (OH), 2966.0 (Ar-CH), 1643.9 (C=O), 1578. 3 (C=N), 1139.2 (C-O-C). ^{1}H NMR (DMSO D_6, 400 MHz) δ ppm 7.49- 6.22 (m, 7H, Ar), 4. 86 (s, 2H, OCH_2), 4.01(d, 2H, OH), 3.26 -2-78 (m, 5H, $-CH_2$, $-CH_3$). Mass m/z: 340 M^{+1}

Antimicrobial Activity

The newly synthesized compounds were screened for antibacterial activity studies at a concentration of 50μg/ml

and 100µg/ml using dimethylsulfoxide as a control against gram-positive (*Staphylococcus aureus and Bacillus subtilis*) and gram-negative (*Salmonella typhi and Escherichia coli*) bacteria. The antibacterial activity of the test compounds was compared with Ampicillin.

Two fold serial dilutions of the test compounds and reference drugs were prepared in Muller-Hinton agar. Test compounds, standard drug Ampicillin (6.4 mg) were dissolved in dimethylsulfoxide (1 ml) and the solution was diluted with distilled water (9 ml). Further progressive serial dilutions with melted Muller-Hinton agar were performed to obtain the required concentrations from 50-100 ìg/ml. The petridishes were inoculated with 1-5 x 10^4 colonies forming units (cfu/ml) and incubated at 37 ÚC for 24 h. The minimum inhibitory concentration (MIC) was the lowest concentration of the tested compound that yields no visible growth on the plate. To ensure that the solvent had no effect on the bacterial growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments. Similar procedure was carried out for the evaluation of antifungal activity by using Muller-Hinton agar using clotrimazole as standard drug against two fungi *Candida albicans* and *Aspergillus niger*. The plates were incubated at 37^0 C for 48 h.

Results and Discussion

Compounds like 4b and 4g showed significant antibacterial activity against *Staphylococcus aureus* and 4e & 4c also showed potent activity against *Bacillus subtilis* organisms when compared to standard drug. Compounds 4d & 4h showed significant antibacterial activity against *Salmonella typhi* and 4i showed potent activity against *Escherichia coli* organisms when compared to standard drug. Compounds like 4c, 4g & 4h showed potent activity against *Candida albicans* and 4d, 4e, 4h & 4i good activity against *Aspergillus niger* as compared to standard drug.

Conclusion

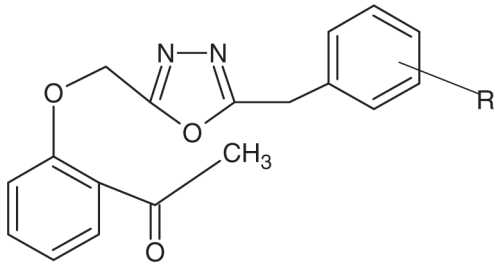
In view of all these observations we conclude that electron withdrawing groups present at ortho & para position shows good activity on gram positive organisms and fungi. Whereas electron releasing groups shows significant activity on gram negative organisms and mild activity on fungi. However, further structural evaluation is required to identify the potent molecule among the series.

Acknowledgement

The authors are thankful to Chemi Labs for providing laboratory facilities authors are also thankful to the head RSIC, SAIF, Chandigarh for spectral data.

Table 1:

Physicochemical Characterization Of 1-{2-[(5-benzyl-1,3,4-oxadiazol-2-yl)methoxy]phenyl}ethanone



Sl No	*R*	Mol. formula	R_f value	% Yield	m.p.ÚC
4a	H	$C_{18}H_{16}N_2O_3$	0.56	63	160-62
4b	4-OH	$C_{18}H_{16}N_2O_4$	0.74	87	175-77
4c	4-Cl	$C_{18}H_{15}ClN_2O_3$	0.68	72	152-54
4d	4-OCH_3	$C_{19}H_{18}N_2O_4$	0.42	66	75-77
4e	2-Br	$C_{18}H_{15}BrN_2O_3$	0.58	74	142-45
4f	2-Cl,4-NO_2	$C_{18}H_{14}ClN_3O_5$	0.82	70	179-82
4g	2,4-Cl	$C_{18}H_{14}Cl_2N_2O_3$	0.64	65	110-13
4h	3,5- $(NO_2)_2$	$C_{18}H_{14}N_4O_7$	0.72	74	114-16
4i	3,5-$(OCH_3)_2$	$C_{20}H_{20}N_2O_5$	0.50	76	108-10
4j	2,4-$(OH)_2$	$C_{18}H_{16}N_2O_5$	0.76	68	205-07

Scheme

Scheme

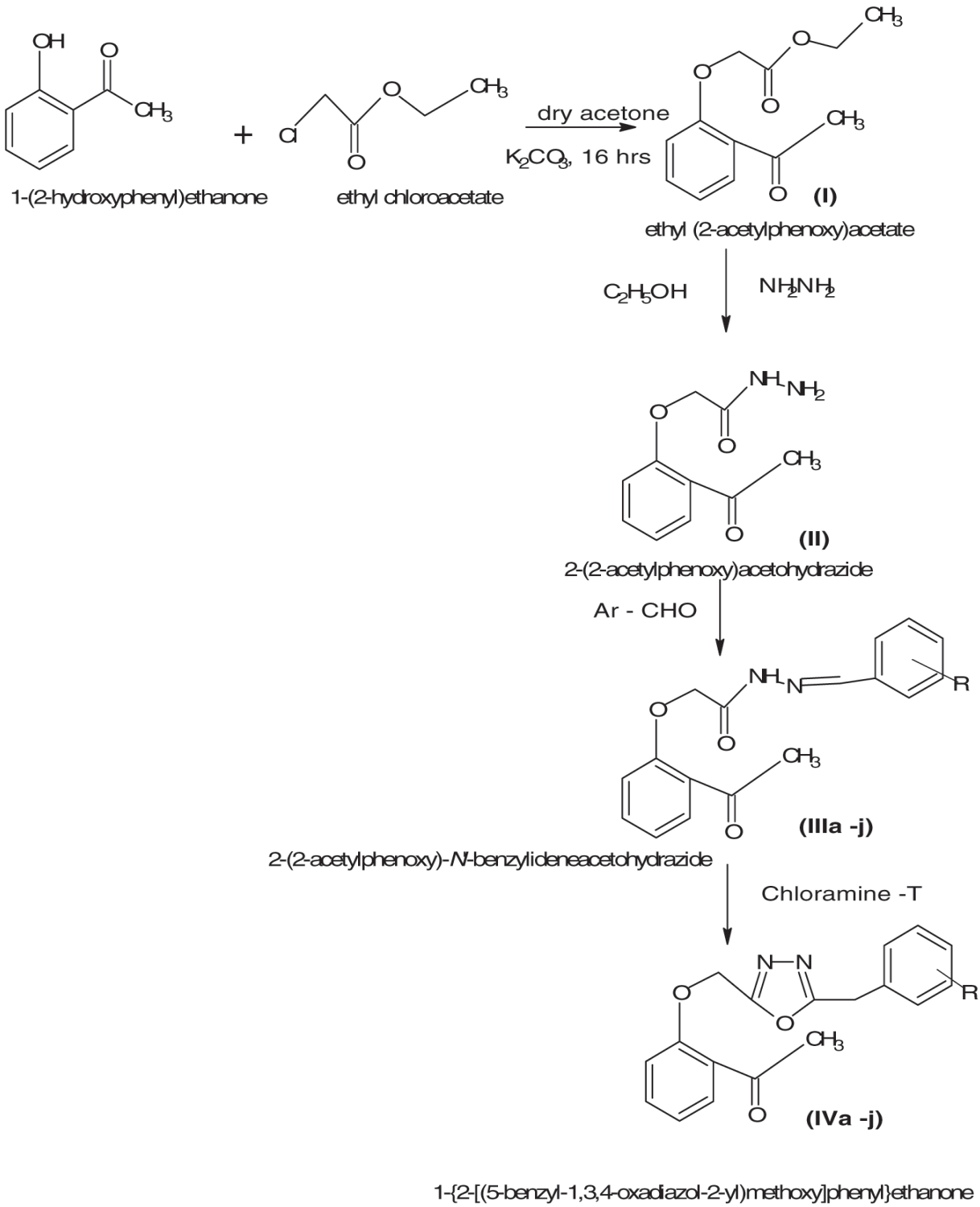
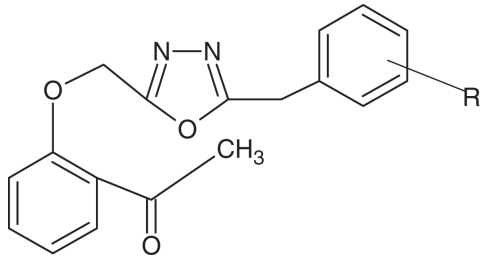


Table 2:

In vitro Anti-bacterial and Anti-fungal activity data of 1-{2-[(5-benzyl-1,3,4-oxadiazol-2-yl)methoxy]phenyl} ethanone Minimum inhibitory concentrations (MICs) µg/ml



Sl.No.	R	*S. aureus*	*B.Subtilis*	*E.Coli*	*S.typhi*	*C.Albicans*	*A.niger*
4a	H	50.0	50.0	100.0	50.0	100.0	50.0
4b	4-OH	12.5	25.0	50.0	50.0	50.0	50.0
4c	4-Cl	25. 0	50.0	50.0	50.0	25.0	50.0
4d	4-OCH_3	25.0	25.0	25.0	12.5	100.0	25.0
4e	2-Br	50.0	12.5	50.0	25.0	50.0	25.0
4f	2-Cl-4-NO_2	50.0	50.0	25.0	25.0	50.0	50.0
4g	2,4-$(Cl)_2$	12.5	50.0	50.0	50.0	25.0	50.0
4h	3,5-$(NO_2)_2$	25.0	50.0	25.0	12.5	25.0	25.0
4i	3,5-$(OCH_3)_2$	25.0	50.0	12.5	25.0	50.0	25.0
4j	2,4-$(OH)_2$	50.0	25.0	50.0	50.0	50.0	50.0
	Ampicillin	>12.5	>12.5	>12.5	>12.5	——-	——-
	Clotrimazole	——	——	——	——-	>25	>25

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Phytochemical and Antioxidant Activities of Different Solvent Extracts of Plant *Aerva Tomentosa*

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ABSTRACT

*Aervatomentosa*Linn. (Amaranthaceae) is is a rigid perennial much branched shrub, found as weed throughout India.The present study was designed for the investigation of phytochemical, antioxidantextracts of whole plant of *Aervatomentosa.*which is widely used in ayurveda to cure many remedies. Phytochemical investigation showed the presence of tannins, carbohydrate, glycosides. Antioxidant screening was studied by using H_2O_2 scavenging, reducing power and phosphor molybdenum method. Antioxidant studies reveal that both extracts of *Aervatomentosa Linn.*showed highest antioxidant activity.

Keywords: *Aervatomentosa. Linn. ,* Phytochemical, Antioxidant.

Introduction

Nature is and will still serve as the man's primary source for the cure of his ailments. However, the potential of higher plants as sources for new drugs is still largely unexplored. It is widely accepted that antioxidants are radical scavengers, which protect the human body against free radicals. *Aervatomentosa.Linn.*is a perennial wild plant which grows in pasture lands and hills. Traditionally it is used in the treatment of Yoke gall is a common problem in bullock sand is treated with extracts of a perennial plant locally called 'safedbuvariyo' (Aervatomentosa), it is also been reported by traditional healers for the treatment of laxative, anti helmintics, constipation, skin diseases, liver and antiviral. Studies on chemical and pharmacological activity [1], diuretic activity on ethanolic extract of *Aervatomentosa.Linn* [2].

Antioxidants are free radical quenching agents and used for the prevention of many diseases. Polyphenolic compounds are commonly found in both edible and non-edible plants and reported to have multiple biological effects due to their antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents [3]. Free radicals are fundamentals to any biochemical process, which represent an essential part of aerobic life and metabolism. They are continuously produced by the body during respiration and some cell–mediated immune functions. These free radicals are also generated from environmental pollutants. Cigarette smoke automobile exhaust fumes, radiation and pesticides. Free radicals when accumulated in cells cause cumulative damage of proteins, lipids, DNA, carbohydrates and membranes, resulting in oxidative stress. Oxidative stress causes food deterioration, aging and a wide range of human diseases including Alzheimer's disease, Parkinson's disease, dispatches, cancer diseases etc. Recently; interest has considerably increased in identifying naturally occurring antioxidants to replace synthetic antioxidants are cause toxin side effects such as cancers, many antioxidant compounds derived from plants have been identified as free radical (or) active oxygen scavengers. Therefore, plant –derived antioxidants are now receiving a special attention. The present study is designed to investigate phytochemical and antioxidant activity of the different solvent extracts of whole plant of *Aervatomentosa. Linn.*

Materials and Methods

Plant material

The aerial parts of *Aervatomentosa*(L) was collected from local supplier and was identified and authenticated by

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Dr.Reddy Raju Venkatapathi Raju, Botanist, SK University, Anatapur,Andhra Pradesh India.. A voucher specimen has been preserved in out laboratory for future reference. The aerial parts were dried under shade, powdered by a mechanical grinder and were passed through 40-mesh sieve and stored in airtight container for further use.

Preparation of Extract

About 1kg of the powdered plant material was exhaustively extracted using Hexane, Chloroform, Acetone and Aqueous Successive Solvents in a Soxhlet extractor. The Different Solent extracts were concentrated and the traces of the solvent were completely removed under reduced pressure and were stored in vacuum desiccators for further use.

Preliminary Phytochemical screening

The crude Hexane, Chloroform, Acetone and Aqueous extracts is dissolved in distilled water and subjected for preliminary phytochemical screening. The study was carried out by using standard procedure [4], [5]. The phytochemical reports are tabulated in Table.1 respectively.

Scavenging of hydrogen peroxide

A solution of H_2O_2 (20mm) was prepared in phosphate buffer saline (PBS, PH 7.4). Various concentration (10µg-100µg) of standard and extracts was prepared, 1ml of the extract and standard was dissolved in methanol in a separate volumetric flask and to this solution 2ml of H_2O_2 solution in PBS was added, the absorbance was measured at 230nm, after 10min against blank solution. The % inhibition of OD was calculated by the formula.

The percentage inhibition was calculated by using the formula.

$$\%\text{inhibition} = A_{control} - A_{sample}/A_{control} \times 100\%$$

Determination of Reducing Power

Method based on the principle of increase in the absorbance of the reaction mixture. Increase in the absorbance indicates increase in anti-oxidant activity [6]. Different concentration of extracts (20µg-100µg) in 1ml of distilled water were mixed with 2.5ml of phosphate buffer (0.2m;P--H6.6) & 2.5ml of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%), the resulting mixture was incubated at 50°C for half an hour. Then, 2.5ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000rpm for 10min. Finally 2.5ml of upper layer solution was mixed with 2.5ml of distilled water and 0.5ml of $FeCl_3$ (0.1%) were added. The absorbance was measured at 700nm in UV-Vis spectrophotometer against blank. Increasing of the reaction mixture indicates increasing reducing power [7]. The % inhibition of OD was calculated by the formula.

The percentage inhibition was calculated by using the formula.

$$\%\text{inhibition} = A_{control} - A_{sample}/A_{control} \times 100\%$$

Estimation of Phosphomolybdenum:

In this method quantitative determination of anti-oxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic PH. An aliquot of 0.3ml of sample solution containing a reducing species in DMSO was combined in a test tube with 3ml of reagent solution (0.6m H_2SO_4, 28mm sodium phosphate and 4mm ammonium molybdate) then the tubes were covered with aluminium foil and kept in a water bath at 95°c for 90min. Then the samples were cooled to room temperature, absorbance of each solution was measured at 695nm against blank. The total anti-oxidant was expressed as mm equivalent to DMSO [8]. The % inhibition of OD was calculated by the formula. The results are tabulated in (Table 2, Table 3, Table 4) and (Figure 1, Figure 2, Figure 3) respectively.

The percentage inhibition was calculated by using the formula.

$$\%\text{inhibition} = A_{control} - A_{sample}/A_{control} \times 100\%$$

Results and Discussions

Phytochemical screening of Hexane, Chloroform, Acetone and Aqueous extracts of *Aerva tomentosa. Linn* reveals the presence of tannins, carbohydrates, glycosides, flavonoids as majorly compounds tabulated in Table: 1. Antioxidant studies prove to show potent antioxidant activity for Hexane, Chloroform, Acetone and aqueous extracts of Aerva *tomentosa. Linn*. Presence of the tannins, flavonoids in past reported to possess antioxidant properties [9]. Hydrogen peroxide scavenging Hexane, Chloroform, Acetone and aqueous extract showed high activity, the reducing anti-oxidant activity shows the reducing property of the plant extracts on potassium ferricyanide. The absorbance is directly proportional to the reduction of ferric ions to ferrous ions, thus an increase in the absorbance denotes the reducing property of the plant extracts, Hexane, Chloroform, Acetone and aqueous extracts show higher anti-oxidant activity. In Phosphomolybdenum method the all extracts show potent anti-oxidant activity. All these extracts are Show Significant activity. The results are shown in Table: 2, Table: 3, Table: 4 and Figure: 1, Figure: 2, Figure: 3.

Table: 1

Preliminary Phytochemical Screening Test

Phytochemical Tests	Hexane	Chloroform	Acetone	Water
Test for Flavonoids				
Sinoda	+ve	-ve	-ve	-ve
Lead acetate	+ve	-ve	+ve	-ve
Sodium Hydroxide (NaoH)	+ve	-ve	+ve	-ve
Test for Carbohydrates				
Fehilings	+ve	-ve	+ve	+ve
Molishes	+ve	+ve	+ve	
Resorcinol	-ve	-ve	-ve	-ve
Benedict	+ve	+ve	+ve	+ve
Barfoeds	+ve	+ve	-ve	+ve
Test for Proteins				
Biuret	+ve	-ve	-ve	-ve
Millons	+ve	+ve	-ve	-ve
Ninhydrin		-ve	+ve	+ve
Nitropruside	+ve	+ve	-ve	-ve
Leadsulphide	-ve	-ve	-ve	-ve
Xanthoprotic	+ve	-ve	+ve	-ve
Heatcoagulation	-ve	-ve	-ve	-ve
Test for Fixed oils				
Boudouinis	-ve	+ve	-ve	-ve
Persic oil	+ve	+ve	-ve	-ve
Test for Tannins				
Vanilin-Hcl	+ve	+ve	+ve	+ve
Gelatin	+ve	-ve	+ve	-ve
Fecl3	+ve	+ve	-ve	-ve
Test for Resins				
Dis.water	+ve	-ve	+ve	-ve
Pet.ether	-ve	+ve	-ve	-ve
$(H_3CCO)_2O+H_2SO_4$	+ve	-ve	-ve	-ve
Test for Glycosides				
Born-tragors	+ve	-ve	-ve	-ve
Foam	+ve	-ve	-ve	-ve
Libermannbuchard	+ve	+ve	-ve	-ve
Baljets	-ve	+ve	-ve	+ve
Hydroxyanthroquinons	-ve	-ve	-ve	-ve
Legal	+ve	+ve	+ve	-ve
Bromine	-ve	-ve	-ve	-ve
Reymonds	-ve	-ve	-ve	-ve
Modified borntagers	+ve	-ve	-ve	-ve
Klungersisobarabuloin	-ve	-ve	-ve	-ve
Flavanoidglycosides	+ve	+ve	+ve	-ve
Saponinglycoside	+ve	-ve	-ve	+ve
Test for Alkaloids				
Mayers	+ve	-ve	-ve	-ve
Wagners	+ve	-ve	+ve	-ve
Murexide	-ve	-ve	-ve	-ve
Thalleoquin	+ve	-ve	-ve	-ve
Hagers	+ve	-ve	-ve	-ve
Dragondroffs	+ve	-ve	-ve	-ve
Br_2,H_2O,dil.HNO_3	-ve	-ve	-ve	-ve
Test for Terpenoids				
TCA	-ve	-ve	+ve	+ve
Test for Other groups				
Insulin	+ve	-ve	-ve	-ve
Waxes	+ve	+ve	-ve	-ve
Mucilase	-ve	-ve	+ve	-ve

Table: 2

Anti-oxidant activities of Different solvent extracts of *Aerovatomentosa Linn.*

Using H_2O_2-Scavenging Profile Method

Conc.	CONTROL	ATH	ATC	ATA	ATW
	Absorbance	Absorbance	Absorbance	Absorbance	Absorbance
10 µg	0.780±0.03	0.482±0.02(38.20)	0.475±0.04(39.10)	0.489±0.06(37.30)	0.643±0.04(17.5)
25 µg	0.836±0.02	0.480±0.02(42.58)	0.483±0.03(42.22)	0.385±0.07(53.94)	0.504±0.05(39.71)
50 µg	0.968±0.01	0.476±0.03(50.82)	0.397±0.02(58.98)	0.514±0.05(46.90)	0.418±0.06(56.81)
75 µg	1.016±0.01	0.349±0.01(65.64)	0.365±0.01(64.07)	0.554±0.05(45.47)	0.359±0.08(64.66)
100 µg	1.232±0.01	0.329±0.01(73.29)	0.348±0.01(71.75)	0.633±0.04(48.62)	0.323±0.09(73.78)

Table: 3

Anti-oxidant activities of Different solvent extracts of *Aerovatomentosa Linn.* Using Reducing Power Method

Conc.	CONTROL	ATH	ATC	ATA	ATW
	Absorbance	Absorbance	Absorbance	Absorbance	Absorbance
20 µg	0.530±0.05	0.376±0.02(29.05)	0.398±0.05(24.90)	0.387±0.08(26.98)	0.398±0.07(24.90)
40 µg	1.055±0.02	0.384±0.01(63.60)	0.492±0.02(53.36)	0.478±0.08(54.69)	0.383±0.08(63.69)
60 µg	1.107±0.02	0.388±0.01(64.95)	0.496±0.02(55.19)	0.454±0.09(58.98)	0.474±0.08(57.18)
80 µg	1.242±0.01	0.391±0.01(68.51)	0.499±0.02(59.82)	0.543±0.01(56.28)	0.565±0.09(54.50)
100 µg	1.433±0.01	0.398±0.01(72.22)	0.502±0.01(64.96)	0.548±0.01(61.75)	0.552±0.09(61.47)

Table: 4

Anti-oxidant activities of Different solvent extracts of *Aerovatomentosa Linn.*

Using Phosphomolybdenum Method

Conc.	CONTROL	ATH	ATC	ATA	ATW
	Absorbance	**Absorbance**	**Absorbance**	**Absorbance**	**Absorbance**
20 µg	0.580±0.05	0.355±0.05(38.79)	0.375±0.08(35.34)	0.371±0.07(36.03)	0.393±0.05(32.24)
40 µg	0.995±0.02	0.386±0.04(61.20)	0.363±0.09(63.51)	0.363±0.08(63.51)	0.389±0.06(60.90)
60 µg	1.027±0.02	0.392±0.04(61.83)	0.354±0.09(65.53)	0.372±0.07(63.77)	0.387±0.06(62.31)
80 µg	1.224±0.02	0.421±0.05(65.60)	0.461±0.09(62.33)	0.358±0.09(70.75)	0.373±0.08(69.52)
100 µg	1.483±0.01	0.463±0.06(68.77)	0.458±0.09(69.11)	0.449±0.09(69.72)	0.465±0.09(68.64)

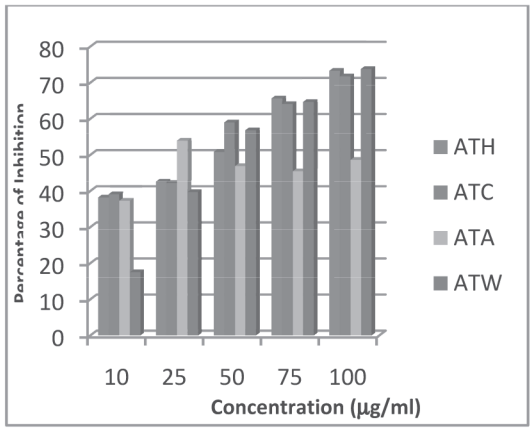

Fig. 1 : H_2o_2 Scavenging Method

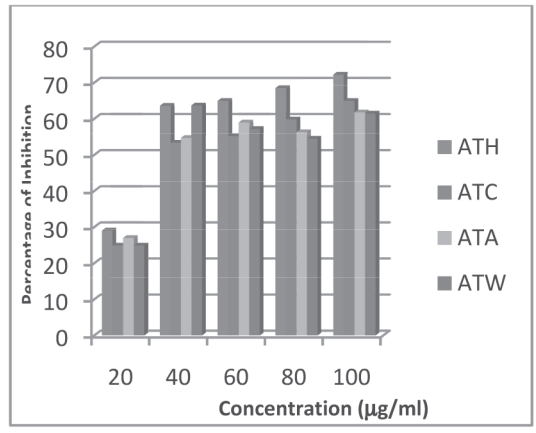

Fig. 2 : Reducing Power Method

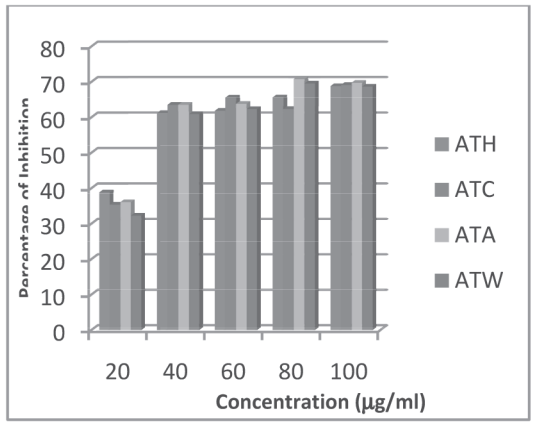

Fig. 3 : Phospomolybdenum Method

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Synthesis, Characterization and Evaluation of Analgesic Activity of Some New Thiazine Derivatives

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ABSTRACT

A series of some new thiazines were synthesized and evaluated for analgesic activity. Thiazines are reported to possess a wide spectrum of biological properties such as antibacterial, antifungal, antihelmenthic, anti-inflammatory and anticonvulsant activities. Regular clinical usage of non steroidal anti-inflammatory drugs is allied with major side effects such as gastrointestinal lesions, bleeding and nephrotoxicity. Hence, the discovery of new safer analgesic drugs represents a demanding target for this research area. The reaction of 4, 4- dimethylcyclohexanone with different aromatic aldehydes yield chalcones. The title compounds were synthesized by treating chalcones with thiourea in presence of potassium hudroxide. Their structures were confirmed by UV, IR, ^{1}H-NMR and mass spectra. Analgesic activity was evaluated for the synthesized compounds.

Key Words: Synthesis, thiazines, chalcones, Analgesic activity.

Introduction

The ability of thiazine to exhibit antibacterial [1], antifungal [2], antihelminth [3], anti-tumour [4], Antiproliferative [5], anti-inflammatory [6], analgesic[7] anticonvulsant [8], antitubercular[9], and which inactivate HIV in biological fluids[10] and used as cannabinoid receptor agonists[11]. In the light of these interesting biological activities, it appeared interest to synthesize some new thiazines derivatives. Analgesics are the drugs which decrease the pain sensation. NSAIDs are mainly used to treat integumental pain. The main physiological peripheral receptors are sensitized by pro-inflammatory autacoids like prostaglandin, 5-HT, histamine, bradykinin, interleukin etc. These drugs are most effective against pain associated with inflammation. When a tissue is injured, prostaglandin synthesis increases in that tissue which sensitizes the pain receptors at the nerve endings by lowering the threshold of response to painful stimuli. Thus, a drug that prevents synthesis of prostaglandins will be effective in treating pain due to inflammation. The mechanism of action involves the inhibition of cyclooxygenases enzymes in the arachidonic acid cascade for synthesis of prostaglandins. By considering all these facts it was planned to synthesize title compounds with an objective to get new potent analgesic agents. In the present study various benzylidene-5, 6, 7, 8-tetrahydro-6, 6-dimethyl-4-phenyl-1H-benzo[d][1, 3]thiazin-2(4H)-imines (Scheme-1) have been synthesized from 2, 6-dibenzylidene-4, 4-dimethylcyclohexanone (1a-h) and evaluated for their analgesic activity by hot plate method in mice. The compounds were characterized by UV, IR and ^{1}H NMR and mass spectroscopy.

Materials And Methods

Melting points of all synthesized compounds were determined in open capillary tubes on an electro thermal apparatus and are uncorrected. The progress of the reaction and purity of the compounds was checked by TLC on silica gel coated aluminium plates (Merck) as adsorbent and UV light as visualizing agent. UV Spectra were obtained on ELICO SL 244 UV Double Beam Spectrophotometer.IR spectra (KBr ν_{max} cm^{-1}) were recorded on a BRUKER FTIR spectrophotometer in the range of 4000-400 cm^{-1} .^{1}HNMR spectra were recorded on a INOVA (400 MHz) NMR

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spectrometer using $CDCl_3$ as solvent and TMS as an internal standard (chemical shifts in δ ppm).Mass spectra were recorded on a VG Autospec MS using ESI mode positive ion trap detector.

The drugs and fine chemicals were purchased from Sigma-Aldrich, India. All other chemicals and solvents were obtained from local firms (India) and were of highest pure and analytical grade. Polypropylene cages with paddy husk, Eddy's hot plate, diclofenac sodium as standard, test compounds, normal saline and double distilled water

Syntheis Procedures

General procedure for the synthesis of 2, 6-dibenzylidene-4, 4-dimethylcyclohexanone [12] (1a-i): A mixture of 10% NaOH, ethyl alcohol, 4, 4-dimethylcyclohexanone (0.01 mol), and aromatic aldehyde (0.02mol) was stirred at 20-25ÚC for 2h.Later the reaction mixture was kept in an ice chest over night. The product was filtered washed with ice cold water followed by ice cold ethanol, Dried and recrystallized by DMF.

General procedure for the synthesis of benzylidene-5,6,7,8-tetrahydro-6,6-dimethyl-4-phenyl-1H-benzo[d][1,3]thiazin-2(4H)-imine [13] (2a-i) : A mixture of 2,6-diarylidene cyclohexanone derivative (0.01mol), thiourea (0.015mol) and potassium hydroxide (0.01mol)dissolved in 10ml of water ,was refluxed in isopropyl alcohol for 16-18h.Later the solvent was removed under reduced pressure and the residue obtained was treated with ice cold water, filtered dried and recrystallized from ethanol (Fig1.Scheme) .The physical data of the compounds were given in the table 1.

Pharmacological Evaluation

The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) of Balaji College of Pharmacy, Anantapur, Andhrapradesh with CPCSEA Registration No: 1563/PO/a/11/CPCSEA.

Animals

Albino mice of either sex weighing 20-25g were used for performing acute toxicity studies and analgesic activity. Animals were housed individually in polypropylene cages, maintained under standard conditions of alternating 12hr light dark cycles at a constant temperature (25±2°C) and 40-60% room humidity).Animals were fed with standard rat pellet and water and libitum.

Acute Toxicity Studies[14]:

The acute toxicity test was carried out according to OECD guidelines11 to establish the effective dose of test compounds after obtaining ethical clearance from animal ethics committee. Healthy and adult albino mice of either sex weighing between 20-25g were used in this investigation. Animals were fasted for 24 hours and divided into groups of six animals each. The test compounds, suspended in sodium carboxymethyl cellulose (CMC) solution (0.1%) were administered intraperitoneally in doses of 5mg to 1000mg per kg (b.w.). The control groups of animals received only the vehicle (0.1% sodium CMC). The animals were observed for 48 hours from the time of administration of test compound to record the mortality.

Analgesic activity [15]

Analgesic activity was evaluated by hot plate method[16].Each group of six mice were selected for the study. One group served as control and received the vehicle, and one group received the standard drug diclofenac sodium (30 mg/kg, i.p.). The drug concentration of 50 mg/kg suspended in acacia was administered orally to other groups. The mice were placed on Eddy's hot plate kept at a temperature of 55 ± 0.5ºC for a maximum time of 15 sec. Reaction time was recorded when the animals licked their fore-and hind paws and jumped, at before 0 and 15, 30, 45, and 60 min after administration of test drugs. In Statistical Analysis all the results were expressed as mean ± standard error (SEM). Data was analyzed using one-way ANOVA followed by Dunnett's t-test. P-values <0.05 were considered as statistically significant. The results of analgesic activity of title compounds are presented in Table 2.

Scheme

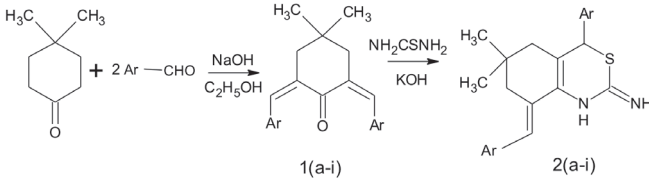

Fig.1: Schematic representation of synthesized compounds 2(a-i)

Results and Discussion

The structures of new compounds prepared during the present investigation have been authentically established by their UV, IR, NMR and mass spectral studies. In the following section the spectral studies of some selected compounds were dealt.

Spectral data of (8E)-8-benzylidene-5, 6, 7, 8-tetrahydro-6, 6-dimethyl-4-phenyl-1H-benzo[d][1,3]thiazin-2(4H)-imine (2a):

IR- imineNH-3262.4 cm^{-1}, Cyclic NH 3160.8cm^{-1}, C-N-1025 cm^{-1}, Aromatic-1543.9 &1462.7 cm^{-1},C=N – 1602 cm^{-1} , ^{1}HNMR - δ0.9 (s, $(CH_3)_2$, 6H), δ1.7 (d, CH_2, 2H), δ1.9 (d, CH_2, 2H), δ4.9 (s, -CH-S, 1H), δ8.2 (s, imine, 1H), δ6.8 (s, cyclic NH, 1H), δ7.2-7.5 (m, ArH, 10H), δ8.0 (s, bezylic-H, 1H), MS-m/z 361(M+).

Spectral data of (8*E*) – 8 - (4-chlorobenzylidene) -4- (4 - chlorophenyl) - 6, 6-dimethyl-1,4,5,6,7,8-hexahydro-2*H*-3,1-benzothiazin-2-imine (2b):

IR- imine NH 3259.6,Cyclic NH3154.8 cm^{-1}, C-N-

Table-1:

Physical data of the Compounds (1a-i) and (2a-i)

Compound	Ar	M.F	M.W	M.P (ÚC)	Yield (%)
1a	Phenyl	$C_{22}H_{22}O$	302.4	92-94	66
1b	*p*-chlorophenyl	$C_{22}H_{20}Cl_2O$	371.2	146-148	94
1c	*p*-methoxyphenyl	$C_{24}H_{26}O_3$	362.4	124-126	92
1d	*m*-nitrophenyl	$C_{22}H_{20}N_2O_5$	392.4	140-142	77
1e	2,5-Dimethoxyphenyl	$C_{26}H_{30}O_5$	422.5	112-113	69
1f	3,4,5-Trimethoxyphenyl	$C_{28}H_{34}O_7$	482.5	180-182	73
1g	*p*-Flurophenyl	$C_{22}H_{20}F_2O$	338.3	115-117	95
1h	*p*-Ethylphenyl	$C_{26}H_{30}O$	358.5	175-177	79
1i	*p*-Isopropylphenyl	$C_{28}H_{34}O$	386.5	193-195	90
2a	Phenyl	$C_{23}H_{24}N_2S$	360.5	148-150	70
2b	*p*-chlorophenyl	$C_{23}H_{22}Cl_2N_2S$	429.4	284-286	84
2c	*p*-methoxyphenyl	$C_{25}H_{28}N_2O_2S$	420.5	240-242	88
2d	*m*-nitrophenyl	$C_{23}H_{22}N_4O_4S$	450.5	210-212	78
2e	2,5-Dimethoxyphenyl	$C_{27}H_{32}N_2O_4S$	480.6	242-246	60
2f	3,4,5-Trimethoxyphenyl	$C_{29}H_{36}N_2O_6S$	482.5	286-288	75
2g	*p*-Flurophenyl	$C_{23}H_{22}F_2N_2S$	396.4	280-281	90
2h	*p*-Ethylphenyl	$C_{27}H_{32}N_2S$	416.6	270-272	86
2i	*p*-Isopropylphenyl	$C_{29}H_{36}N_2S$	444.6	260-262	83

1007cm^{-1}, Aromatic-1571.5&1480.2cm^{-1},C=N –1614.7cm^{-1}, ^{1}HNMR - δ0.9 (s, $(CH_3)_2$, 6H), δ1.6 (d, CH_2, 2H), δ1.8 (d, CH_2, 2H), δ4.9 (s,-CH-S, 1H), δ6.6 (s, cyclic NH, 1H), δ7.1-7.4 (m, ArH, 8H, benzylic H, 1H), δ7.7 (s, imine H, 1H), MS-m/z 429(M+).

Spectral data of (8E) -8- (4-methoxybenzylidene)-5, 6, 7, 8-tetrahydro – 4 - (4-methoxyphenyl) -6, 6- dimethyl-1H-benzo[d][1,3]thiazin-2(4H)-imine (2c):

IR- imineNH- 3498.3 cm^{-1}, Cyclic NH 3149.0cm^{-1}, C-N- 1023 cm^{-1}, Aromatic-1563.1 &1504.1 cm^{-1},C=N – 1601.2 cm^{-1}, ^{1}HNMR - δ0.9 (s, $(CH_3)_2$, 6H), δ 1.7 (d, CH_2, 2H), δ1.8 (d, CH_2, 2H), δ4.8 (s,-CH-S, 1H), δ 8.2 (s, imine, 1H), δ6.7 (s, cyclic NH, 1H), δ6.9-7.3 (m, ArH, 8H), δ7.9 (s, bezylic, 1H). MS-m/z 421(M+).

Spectral data of (8*E*) -8- (4-fluorobenzylidene) -4- (4-fluorophenyl) -6, 6-dimethyl-1,4,5,6,7,8-hexahydro -2*H*-3,1- benzothiazin -2- imine 2(g):

IR- imineNH-3371.9cm^{-1}, Cyclic NH3160 cm^{-1}, C-N-1015 cm^{-1}, Aromatic-1546.&1494. cm^{-1},C=N –1597.4cm^{-1}, ^{1}HNMR - δ0.9 (s, $(CH_3)_2$, 6H), δ1.7 (d, CH_2, 2H), δ1.9 (d, CH_2, 2H), δ4.9 (s,-CH-S, 1H), δ7.7 (s, imine, 1H), δ6.6 (s, cyclic NH, 1H), δ7.2-7.6 (m, ArH, 8H, benzylic H, 1H), MS-m/z 397(M+).

Spectral data of (8*E*)-8-(4-ethylbenzylidene)-4-(4-ethylphenyl)-6,6-dimethyl-1,4,5,6,7,8-hexahydro-2*H*-3,1-benzothiazin-2-imine (2h):

IR- imine NH- 3589.8 cm^{-1}, Cyclic NH 3176.1 cm^{-1}, C-N- 1022 cm^{-1}, Aromatic- 1544&1471 cm^{-1},C=N – 1611.6cm^{-1},^{1}HNMR - δ0.9(s,$(CH_3)_2$,6H), δ1.3(d,CH_3)$_2$,6H), δ2.3(d,CH_2,2H), δ2.5(d,CH_2,2H), δ2.9 (q,$(CH_2)_2$,4H), δ4.9 (s,-CH-S, 1H), δ6.8 (s, imine, 1H), δ6.6 (s, cyclic NH, 1H), δ7.2-7.5 (m, ArH, 10H), δ7.6 (s, bezylic, 1H), MS-m/z 417(M+).

Spectral data of (8*E*) -8- (4-propylbenzylidene) -4- (4-propylphenyl) -6, 6-dimethyl-1,4,5,6,7,8- hexahydro- 2*H*-3,1-benzothiazin -2- imine (2i):

IR- imineNH-3445.6 cm^{-1}, Cyclic NH 3189.3 cm^{-1}, C-N- 1017 cm^{-1}, Aromatic-1544 &1465 cm^{-1},C=N – 1606.0cm^{-1},^{1}HNMR -δ 0.9 (s, $(CH_3)_2$, 6H), δ 1.2 (d,$(CH_3)_4$,12H), δ 2.3-2.5(m,$(CH)_2$,2H), δ 1.7 (d, CH_2, 2H), δ 1.9 (d, CH_2, 2H), δ 4.9 (s,-CH-S, 1H), δ 6.8 (s, imine, 1H), δ 6.6 (s, cyclic NH, 1H), δ 7.2-7.5 (m, ArH, 8.0H), d7.7 (s, bezylic, 1H), MS-m/z 445(M+).The reactant and product melting points were different from each other. It clearly indicates the formation of new chemical entities (Table.1). All synthesized compounds were observed in a single spot whose Rf values are 0.45, 0.35, 0.54, 0.36, 0.46, 0.23, 0.49, 0.45 and 0.50, respectively for compounds 2 (a-i). It ultimately confirms the purity and completion of reaction.The above spectral values confirm the structure of the synthesized compounds and the mass spectral values also corresponds to their calculated molecular weight.

Analgesic Activity

All the compounds have been evaluated for their analgesic activity by Eddy's hot plate method. The results of analgesic activity are presented in Table 2**.** The data represents that none of the tested compounds shown analgesic activity as good as the standard drug. The results showed that the compounds 2b, 2g and 2h significantly increased the pain threshold to hot plate in mice, which suggested that the test compounds displayed peripheral analgesic effect and acted like non-steroidal anti-inflammatory drugs. Among the all tested compounds 2g, 2b, 2h, showed good analgesic activity and the remaining compounds showed very less to moderate analgesic activity when compared to the standard drug diclofenac [20 mg/kg (i.p.)].

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Table 2:

Analgesic activity of new 2 benzylidene-5, 6, 7, 8-tetrahydro-6, 6-dimethyl-4-phenyl-1H-benzo[d][1, 3]thiazin-2(4H)-imines 2(a-i)

Compound Mean ±SD	30min		60min		120min	
	Mean ± SD Time (minutes)	% Protection	Mean ± SD	% Protection	Mean ± SD	% Protection
Control	4.27 ± 0.16	NA	4.42± 0.19	NA	4.53 ± 0.11	NA
Standard	12.1 ± 0.89*	145.25	13.2 ± 0.05*	178.37	14.3 ± 0.34*	210.89
2a	5.11 ± 0.43	15.6	5.83 ± 0.17	36.53	6.37 ± 0.51	47.11
2b	8.56 ± 0.09*	93.66	9.42 ± 0.22*	120.60	10.65 ± 0.43*	145.40
2c	4.95 ± 0.22	12	5.7 ± 0.45	33.48	6.15 ± 0.82	42.03
2d	7.23 ± 0.25	63.5	8.2 ± 0.23	92.03	9.12 ± 0.22*	110.62
2e	5.13 ± 0.12	16	6.3 ± 0.53	47.54	7.2 ± 0.61	66.28
2f	5.38 ± 0.14	21.7	7.2 ± 0.12	68.61	8.72 ± 0.46*	101.38
2g	9.00 ± 0.22*	103.61	10.02 ± 0.15*	134.6	11.50 ± 0.44*	165.58
2h	9.15 ± 0.19*	107.01	10.18 ± 0.14*	138.90	12.08 ± 0.23*	178.98
2i	7.83 ± 0.41*	74.0	5.13 ± 0.75	14.0	4.8 ± 0.8	6.6

*** p<0.0001, ** p<0.001, * p<0.05 compared to control at respective Time period,

NA = Not Applicable, Standard = Diclofenac sodium

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Molecular Properties Prediction, Synthesis, and Docking Studies of 3- Benzimidazol-1-yl-1-(4-phenylpiperizin-1-yl) propan-1-one and their Derivatives

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ABSTRACT

In the present study a series of 3-benzimidazol-1-yl-1-(4-phenylpiperizin-1-yl) propan-1-one and their derivatives **(8a-8g)** were subjected to molecular properties prediction, drug likeness by mol inspiration and Molsoft software's, lipophilicity and solubility parameters calculated using ALOPGPS 2.1 program. The compounds followed the Lipinski 'Rule of five' were synthesized. All the synthesized compounds were characterized by IR, NMR, and mass spectral analysis. Furthermore, the binding conformations of these compounds **(8a-8g)** for anti inflammatory activities were determined in silico docking studies carried out in Mastro V 2011 in the active site of the cyclooxygenase-2 (COX-2) enzyme.

Key words: Benzimidazole, phenylpiperizine, Molecular properties, docking studies.

Introduction

In the development of drugs intended for oral use, good drug absorption and appropriate drug delivery are crucial. Nearly 30% of oral drugs fail in the process of development due to poor pharmacokinetics. Therefore the bioavailability related properties such as solubility, lipophilicity are important before actual synthesis to reduce the chemical expenses and valuable time. An *in Silico* model for predicting oral bioavailability is very essential prior to synthesis. It can be achieved with an appropriate balance between solubility and partitioning properties.

The molecular properties of phenylpiperizine analogues were calculated using Mol inspiration, Osiris,and Mol Soft Softwares. Lipophilicity and solubility parameters were calculated using ALOGPS 2.1 program to filter the compounds for further synthesis. [**1, 2**]

Benzimidazole and its derivatives are an important class of bioactive molecules. Their importance is due to their versatile application in the field of drugs and pharmaceuticals. [**3**] Benzimidazole structures are associated with a wide range of activities including anti-cancer, antiviral, anti-inflammatory, anti-microbial, anti- oxidant and proton pump inhibitor, anticoagulant properties [**4**].

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In recent years there is an enhanced tendency for drug regulatory authorities to treat racemic drugs as containing 50% impurities [**5, 6**] accordingly to encourage the development of chiral drugs containing only one enantiomer[**7**]. However, in some cases a drug containing a mixture of enantiomers is preferable to a pure chiral drug [**8**]. To develop an optically active chiral drug or a racemic drug should take into account the relative potency of the two drug enantiomers, both in the principal therapeutic activity and in side effects [**9**].

Well-known classes of 5-HT_{1A} receptor ligands are the "long chain" arylpiperazine derivatives. Among these Buspirone a partial agonist at 5-HT_{1A} receptors is an effective antianxiety and was the first arylpiperizine approved for clinical use.

Stereo controlled synthesis of the enantiomers of 1-[2-hydroxy-3-(4-phenyl-1-piperazinyl)-propyl]-pyrrolidin-2-one[**10**] .it **is** in a class of antiarrhythmic and also showed hypertensive effects and displayed $á_1$ and $á_2$ adrenergic blocking activities.Later Barbara reported the synthesis of 1-[2-hydroxy-3-(4-phenyl-1-piperazinyl)-propyl]-pyrrolidine[**11,12**]. Even though a variety of methods for the synthesis of phenyl piperizine derivatives have been developed there is no report dealing with 4-arylpiperizine, Predicted bioavailability and related properties Filtered drug synthesis, In silico docking studies were carried out for

drug discovery to select the most promising compounds for further clinical development.

Materials and Methods

Melting points of the compounds were determined on a Melter FP-51 melting point apparatus and are uncorrected. Analytical TLC was performed on Merck pre-coated silica gel-60 F_{254} plates. Visualization was done by exposing to iodine vapour,UV light. IR spectra (KBr pellet) are recorded on a Perkin Elmer model 283B and Nicolet-740 FT-IR spectrometer. ^{1}H NMR spectra were recorded on a Varion Gemini 200, Varian unity-400 and advance MHZ, BrukerUx-NMR spectrometer. Chemical shift values are given in ppm (δ) with tetra methyl silane as an internal standard. Mass spectra are recorded on VG micro mass 7070H (EI and CI) VG auto spec (FAB) using CS^+ ion gun.

Molecular properties prediction

Molecules which contain functional groups have properties consistent with most of the known drugs with the intention to achieve good oral drugs[**13**]. Molecular properties were calculated to a series of Benzimidazole linked to phenylpiperizins.

Drug likeness is a qualitative concept used to predict[**14**] molecular properties, mainly hydrophobicity, electronic distribution, hydrogen bonding characteristics, molecule size and flexibility of various pharmacophoric features which influence the behavior of molecules in a living organism, including bioavailability, transport properties, affinity to proteins, reactivity, toxicity, metabolic stability[**15**]. logP, molecular volume was calculated using Mol inspiration–cheminformatics online service[**16**].Since Molecular properties are fast and reliable estimation and very important process of drug discovery and development [**17**].

Molecular Polar surface area (PSA) was determined by the fragment-based method. PSA is sum of the surfaces of polar atoms (O, N, and attached Hydrogen) in a molecule. Hydrogen bonding capacity is an important parameter for describing drug permeability. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, and blood–brain barrier penetration. Number of rotatable bonds are important for conformational changes of molecules, logP is useful in rational drug design as a measurement of molecular hydrophobicity, and used in QSAR studies. Hydrophobicity affects drug absorption, bioavailability, metabolism of molecules, toxicity, hydrophobic drug receptor interactions. Molecular volume is used in QSAR studies to determine transport characteristics of molecules such as intestinal absorption or blood-brain barrier penetration [**16**].

Besides the above properties, Balance between solubility and partitioning properties are essential for good bioavailability. The ALOGPS 2.1 program used to predict lipophilicity calculations and aqueous solubility of compounds. The logKow (Kow-WIN) program estimates the log octanol/water partition coefficient (log*P*) of organic chemicals and drugs using an atom/fragment contribution method. The XLOGP2 is an atom-additive applying corrections. Both the XLOGP2 method and the logKow (Kow-WIN) are the best supported for the most of the compounds on the basis of lipophilicity (d"5) to consider an oral drug /leads [**18**].

High oral bioavailability is an important factor for the development of bioactive molecules as therapeutic agents. Molecular properties such as membrane permeability and bioavailability are always associated with some basic molecular descriptors such as logP value, molecular weight, hydrogen bond acceptors and donors in a molecule. Lipinski 'Rule five' states that most molecules with good membrane permeability have log*P* d"5, molecular weight d"500, number of hydrogen bond acceptorsd"10, and number of hydrogen bond donarsd"5. This rule is widely used as a filter for drug- like properties[**19**].

After successful prediction of molecular properties a series **(8a-g)** were selected for the next steps of synthesis

Chemistry:

Preparation of 3-benzoimidazole-1-yl-propionic acid methyl ester (2):

Benzimidazole **1** (10 g, 80 mmol) and methyl acrylate were dissolved in THF (4.54g, 52.8 mmol) Followed by the addition of CAN (2.89 g, 8 mmol) and the mixture is refluxed at 60 ^{o}C for 24 h under air atmosphere. Then the solvent was concentrated under reduced pressure and purified by silica gel (60-120 mesh) column chromatography using ethyl acetate, hexane as eluent. The compound was obtained as a syrupy liquid.

Yield: 62.03%. IR (KBr): υ_{max}1734 (C=O), 1497 (C=N), 1207 (C-O) cm^{-1}. ^{1}H NMR(300 MHz, $CDCl_3$): δ 2.85 (t, 2H, *J*=6.592Hz, CH_2-CO), 3.66 (s, 3H, -OCH_3), 4.49 (t, 2H, *J*=6.592Hz, N-CH_2), 7.22-7.28 (m, 2H, Ar-H), 7.33 (m, 1H, Ar-H), 7.76 (m, 1H, Ar-H), 7.91 (s, 1H, N=CH). Mass: ESI-MS m/z 205 $[M+H]^+$

Preparation of 3-benzoimidazol-1-yl-propionic acid (3):

The compound 3-benzoimidazol-1-yl-propionicacid-methylester (**2**) was taken in 20 mL of THF: Water system (2:1). To this lithium hydroxide (1.23 g, 29.41mmol) was added at O^{O}C. Then the mixture was stirred at RT for 1 hr and the reaction was monitored by TLC. After completion of reaction solvents were distilled under reduced pressure. Organic compound was dissolved in methanol, filtered, concentrated, and purified by silica gel column chromatography using ethyl acetate, hexane (80:20). A colorless crystalline solid was obtained.

Yield: 82.16%; m.p: 285-287 ^{o}C. IR (KBr): υ_{max} 3415

(-OH), 1588 (C=O) cm^{-1}. ^1H NMR(300 MHz, DMSO-d_6): δ 2.81 (t, 2H, *J*=6.267Hz, CH_2-CO-), 4.49 (t, 2H,*J*=6.267Hz, N-CH_2), 7.17-7.27 (m, 2H, Ar-H), 7.44 (d, 1H, Ar-H), 7.66-7.70 (m, 1H, Ar-H), 8.02 (s, 1H, N=CH). Mass: EI-MS m/z 191 $[M+H]^+$

Preparation of Benzoimidazol-1-yl-1-amine propanones 8a-8g:

General Procedure: To a solution of compound **3** (100 mg, 0.28 mmol), EDC (53 mg, 0.28 mmol), HOBt (38 mg, 0.28 mmol), DIPEA (72 mg, 0.56 mmol) and amine **7a-g** (45 mg, 0.28 mmol) in CH_2Cl_2 (5 ml) was stirred for overnight and solvents was evaporated under vacuum. The residue obtained was dissolved in water and extracted with ethyl acetate (3 × 25 ml). The organic phases were washed with sat. Sodium bicarbonate solution dried over sodium sulphate and evaporated under vacuum. The residue obtained was purified by column chromatography over silica gel using ethyl acetate: hexane (80:20), to afford following compounds:

Preparation of (1H-benzo[d]imidazol-1-yl)-N-methyl-N-phenethylpropanamide (8a)

The general synthetic method described above afforded **8a** as off semi solid mass

Yield 86%. IR (KBr): $\acute{\nu}_{max}$ 2924. ^1H NMR ($CDCl_3$): δ 2.60 (t, 2H, -CH_2-Ar), 2.70 (t, 2H, -CH_2-CO), 3.38 (t, 2H, -N-CH_2), 3.50 (s, 3H, -N-CH_3), 3.60 (t, 2H, -N-CH_2), 7.00-7.85 (m, 10H, Ar-H). ES-MS: m/z 330 [M+Na]. Anal. Cald for $C_{19}H_{21}N_3O$: C, 74.24; H, 6.89; N,13.67. Found: C, 74.14; H, 6.52;N,13.57.

Preparation of (1H-benzo[d]imidazol-1-yl)-N-(2-pheneylpropyl) propanamide (8b)

The general synthetic method described above afforded **8b** as off semi solid mass.

Yield 82%. IR (KBr): $\acute{\nu}_{max}$ 2922, ^1H NMR ($CDCl_3$): δ 1.00 (d, 3H, -CH_3), 2.55 (t, 2H, -CH_2-CO), 2.75 (t, 1H, -Ar-CH), 3.38 (t, 2H, -N-CH_2), 3.50 (s, 3H, -N-CH_3), 3.60 (t, 2H, -N-CH_2), 7.00-7.85 (m, 10H, Ar-H). Mass: ESI- MS m/z 308[M+Na]. Anal. Cald for $C_{19}H_{21}N_3O$: C, 74.24; H, 6.89;N, 13.65.Found: C, 74.14; H, 6.54.N,13.67.

Preparation of (1H-benzo[d]imidazol-1-yl)-1-(pyrolidin-1yl) propan-1-one (8c)

The general synthetic method described above afforded **8c** as off semi solid mass

Yield 80%. IR (KBr): $\acute{\nu}_{max.}$ 2970. ^1H NMR ($CDCl_3$): δ 1.80-1.98 (m, 4H, 2CH_2), 2.80 (t, 2H, -CH_2-CO), 3.18 (t, 2H, -NCH_2), 3.45 (t, 2H, -NCH_2), 4.60 (t, 2H-, -NCH_2-Ar), 6.40 (s, 1H, Ar-H), 7.25-8.18 (m, 4H, Ar-H). Mass:ESI-MS m/z 244[M+H]. Anal. Cald for $C_{14}H_{17}N_3O$: C, 69.11; H, 7.04; N,17.27. Found: C, 69.10; H, 7.06.N,17.37

Preparation of 1H-benzo[d]imidazol-1-yl)-1-(pyrolidin-1yl) propan-1-one (8d)

The general synthetic method described above afforded **8d** as off semi solid mass.

Yield 78%. IR (KBr): $\acute{\nu}_{max}$.2925. ^1H NMR ($CDCl_3$): δ 1.70-1.95 (m, 6H, 3CH_2), 2.70 (t, 2H, -CH_2-CO), 3.20 (t, 2H, -NCH_2), 3.30 (t, 2H, -NCH_2), 4.6 0(t, 2H-, -NCH_2-Ar), 6.39 (s, 1H, Ar-H), 7.45-8.12 (m, 4H, Ar-H). Mass: ESI-MS m/z 258[M+H]. Anal. Cald for $C_{15}H_{19}N_3O$: C, 70.01; H, 7.44; N,16.33 Found: C, 70.11; H, 7.34;N,16.23

Preparation of (1H-benzo[d]imidazol-1-yl)-1-(pyrolidin-1yl) propan-1-one (8e)

The general synthetic method described above afforded **8e** as off semi solid mass.

Yield 80%. IR (KBr): $\acute{\nu}_{max}$.2964. ^1H NMR ($CDCl_3$): δ 2.80 (t, 2H, -CH_2-CO), 3.25 (t, 4H, -2CH_2O), 3.45 (t, 4H, 2-NCH_2), 4.60 (t, 2H,-NCH_2-Ar), 6.39 (s, 1H, Ar-H), 7.30-8.10 (m, 4H, Ar-H). Mass:ESI-MS m/z 260[M+H]. Anal. Cald for $C_{14}H_{17}N_3O_2$: C, 64.85; H, 6.61. N,16.20. Found: C, 64.35; H, 6.52;N,16.10

Preparation of 3-benzoimidazol-1-yl-1-(4-phenyl-piperizin-1-yl)- propan-1- one (8f):

To a solution of compound **3** (100 mg, 0.28 mmol), EDC (53 mg, 0.28 mmol), HOBt (38 mg, 0.28 mmol), DIPEA (72 mg, 0.56 mmol) and 4-phenyl -1-piparazine **5a**, (45 mg, 0.28 mmol) in CH_2Cl_2 (5 mL) was stirred for overnight and solvents was evaporated under vacuum. The residue obtained was dissolved in water and extracted with ethyl acetate (3 × 25 mL). The organic phases were washed with sat. Sodium bicarbonate solution dried over sodium sulphate and evaporated under vacuum. The residue obtained was purified by column chromatography over silica gel using ethyl acetate: hexane (80:20), to afford 3-benzoimidazol-1-yl-1-(4-phenyl-piperizin-1-yl) - propan-1-one (**8a**) as a brown viscous mass. Yield: 85%. IR (KBr): υ_{max} 2924 (C-H), 1645 (C=O) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.90 (t, 2H, *J*=6.421Hz, CH_2-CO-), 2.95 (t, 2H, -NCH_2), 3.10 (t, 2H, -NCH_2) 3.40 (t, 4H, *J*=5.099Hz, N-CH_2), 3.75 (t, 4H, *J* =5.099Hz, N-CH_2), 4.60 (t, 2H, *J*=6.241Hz, N-CH_2 Ar), 6.81 (d, 2H, Ar-H), 7.14-7.32 (m, 4H, Ar-H), 7.51-5.54 (m, 2H, Ar-H), 7.69 (s, 1H, Ar-H), 8.10 (s, 1H, Ar-H), 8.28 (brs, 1H, N-CH). Mass:ESI- MS m/z 335 $[M+H]^+$ Anal. Cald for $C_{20}H_{22}N_4O$: C, 71.83, H, 6.63; N,16.75. Found: C, 71.76, H, 6.45; N,16.65

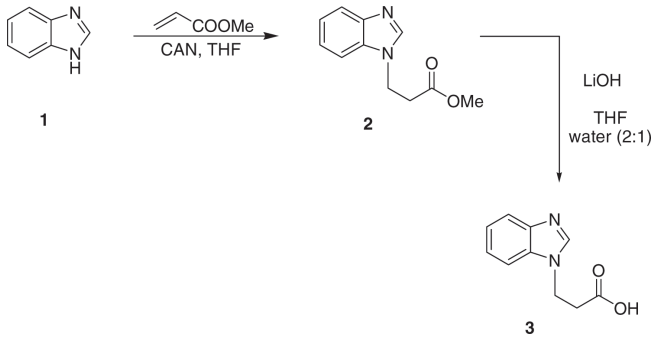
Preparation of 3-(1H-benzo[d]imidazol-1-yl)-1-(4-nitrophenyl-piperizin-1-yl)-propan-1-one (8g):

The general synthetic method described above afforded **8f** as a brown viscous mass.

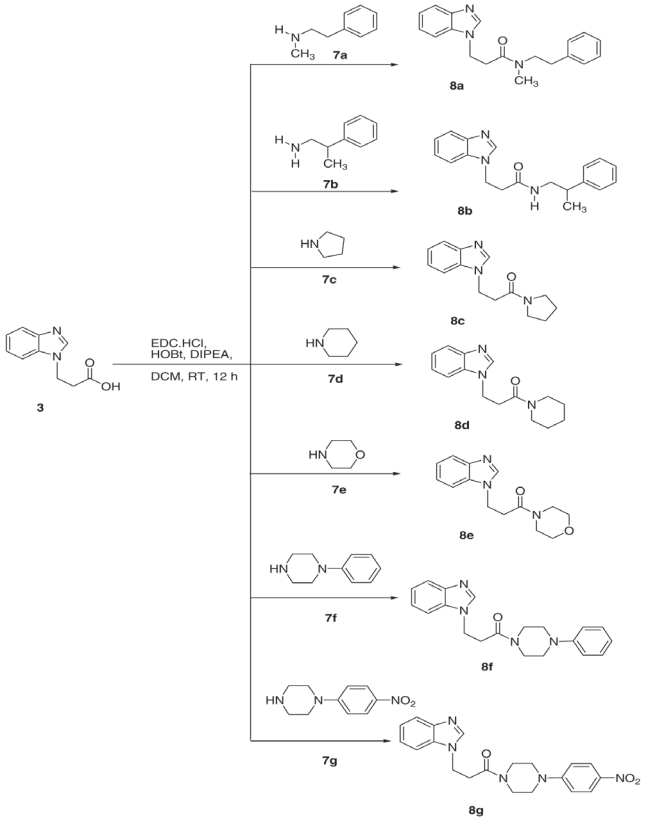
Yield: 85%. IR (KBr): υ_{max} 2925 (C-H), 1642 (C=O) cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 2.90 (t, 2H, *J*=6.421Hz, CH_2-CO-), 3.20 (t, 2H, -NCH_2), 3.35 (t, 2H, *J*=5.099Hz, N-CH_2), 3.45 (t, 2H, *J* =5.099Hz, N-CH_2), 3.75

(t, 2H, N-CH_2), 4.65 (t, 2H, *J*=6.241Hz, N-CH_2 Ar), 6.75 (d, 2H, Ar-H), 7.26-7.90 (m, 5H, Ar-H),8.15(brs, 1H, N-CH). Mass:ESI- MS m/z 379 $[M+H]^+$ Anal. Cald for $C_{20}H_{21}N_5O_3$: C, 63.31; H, 5.58; N,18.46. Found C, 63.41, H, 5.55;N,18.45.

Scheme-1



Scheme-2



Molecular Docking

Docking in a true sense is the formation of non-covalent protein-ligand complexes in silico.

The main aim of ligand-protein docking is to predict the predominant binding mode(s) of a ligand with a receptor to understand the mechanism. In this study, we have used flexible docking to study the binding orientations and predict binding affinities of title compounds with COX-2. A docking method estimates the forces involved in the protein-ligand recognition viz.eleectrostatic, van der walls and hydrogen bonding and place the ligand appropriately in the active site [**20, 21**].

The literature survey reveals that majority of nonsteroidal anti –inflammatory drugs (NSAIDs) are used as therapeutic agents for the treatment of pain and inflammation. cyclooxygenase (COX), an essential enzyme, [**22**]The molecular structures were further prepared along with the proteins by the docking engine. The structure of COX-2 bound to different drugs. Docking procedures were performed on COX-2 as a receptor, downloading its structure from the Protein Data Bank (PDB). Different files of COX-2 complexed with different ligands are available from that web site. The crystal structure of COX-2 (pdb code: 4COX) with 2.9 Å resolution was used in the study. Amino acid residues within 10 A radius of the ligand, Indomethacin were considered as active site residues. All the crystallographic waters outside the active site were removed and the protein was prepared for docking using the protein preparation tool implemented in the Schrodinger suite 2011 (Schrodinger LLC). The resulted structure was minimized using the OPLS-2005 force field with normal Batch Min cutoffs - 7.0 Å van der Waals [VDW]; 12.0 Å electrostatic [ELE]. The Generalized Born/Solvent Accessible (GB/SA) water solvation model was used in the minimization.

All the Docking studies were carried out using the Glide docking program in Schrodinger suite. The Docking grid was generated using the co-ordinates of the X-ray ligand (Indomethacin) with the standard settings.

Ligand Preparation

All the ligands were drawn in Mastro and converted to 3D conformations using ligand prep. All the possible tautomers and stereo isomers were generated using EPIK. The geometry optimization for all the molecules was carried out using the OPLS-2005 force field with Steepest Descent followed by truncated Newton conjugated gradient minimization methods. Partial atomic charges were computed using OPLS-2005 force field.

Results and Discussion

After successful prediction of molecular properties, values are within the limit, following Lipinski rule, fulfill the requirements of solubility, low polar surface area, total hydrogen bond count are important predictors of good oral bioavailability. Both XLOGP2 and KoW-WIN are the best supporters on the basis of lipophilicity to consider as oral

drug /lead. The title compounds were synthesized on the basis of molecular properties,Molecular bioactivity scores, partition coefficients and solubilities, values were recorded in Tables 1,2&3.

All the compounds are flexibly docked with Cox-2 using Glide (Schrodinger LLC). The docking scores obtained from both the SP and XP methods for the best docking conformation of each molecule was summarized in Table 4.and Characterization data is in Table 5.

In order to get deeper insight into the nature of binding and also the type of interactions involved in the binding, the complexes between each compound and COX-2 receptor was visualized in Maestro. Most of the compounds (**Molecules 8a, 8f& 8g**) show a bidentate H-bond interactions with Tyr 355 and Arg 120 similar to the side chain carboxylate group of indomethacin in the crystal structure (4COX). However, the most active molecule of the series, **Molecule 8e** shows this interaction with the side chain morpholin O-atom instead of the carbonyl O-atom. These interactions show the key role to attain the bioactive conformations. Apart from this, the ring nitrogens of the most active compounds (**Molecules 8e & 8a**) form a H-bond interaction with the hydroxyl group of Ser 530, similar to the benzoyl O-atom in the indomethacin.

Acknowledgements

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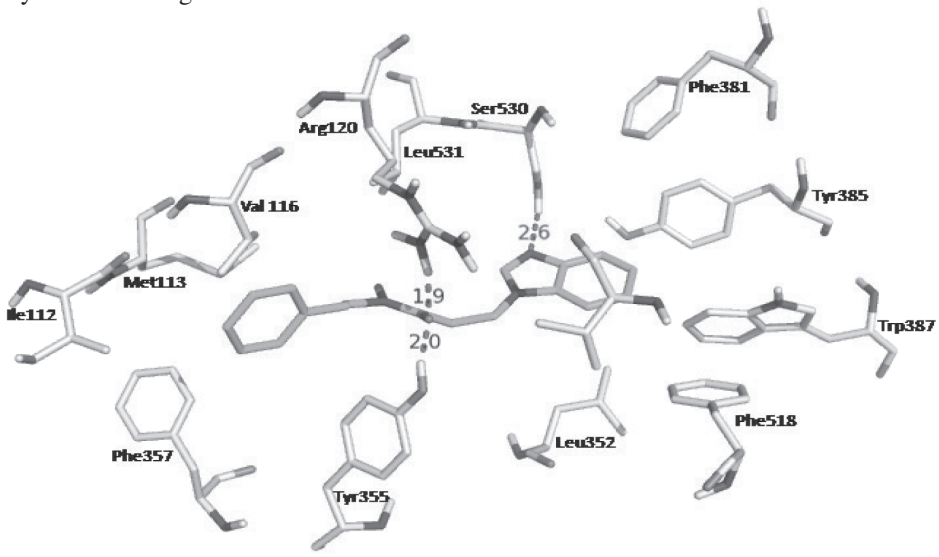

Fig.1: Docking conformation of Molecule8a in the active site of 4COX. The docked molecule is shown in green while the active site residues in grey. The H-bonds are shown as pink dotted lines while mentioning distances of the H-bonds.

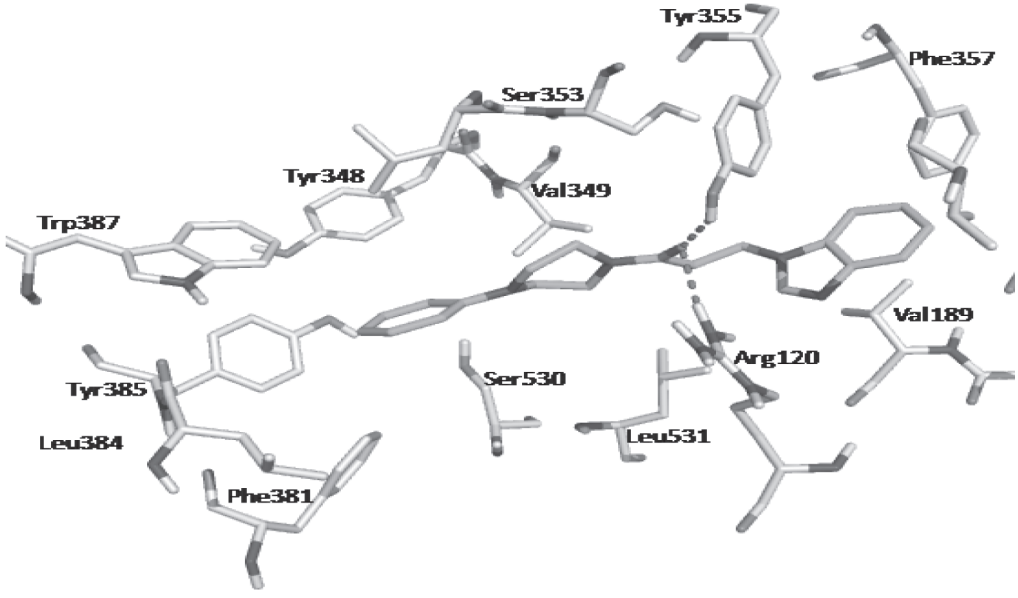

Fig.2: Docking conformation of Molecule 8f in the active site of 4COX.

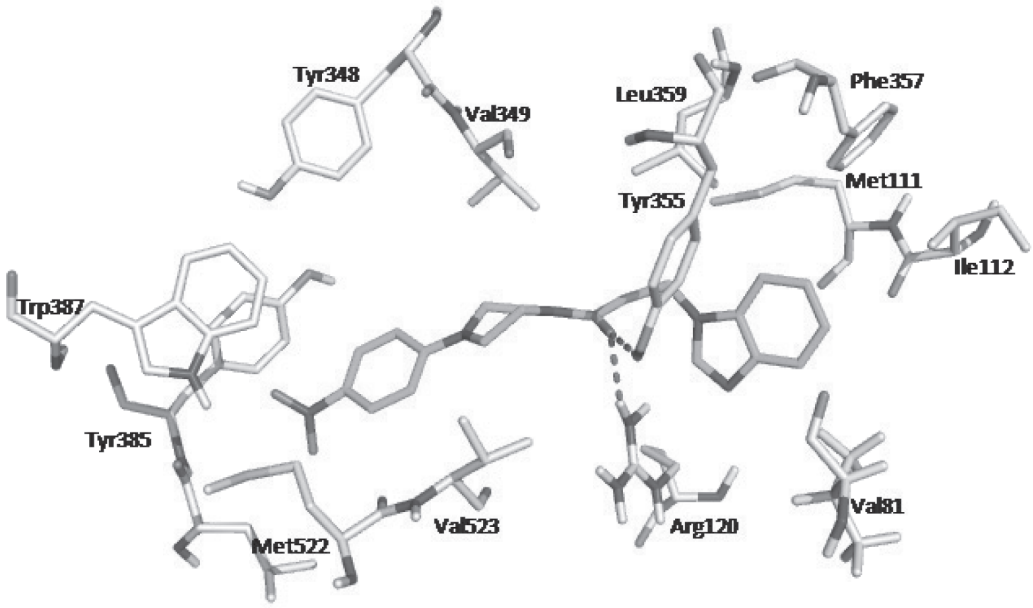

Fig. 2: Docking conformation of Molecule 8g in the active site of 4COX.

Table-1
Calculated partition coefficients and solubilities of the synthesized compounds

Compounds	ALOGPS	KoW-WIN	XLOGP2
8a	-3.82(46.87mg/l)	3.15	2.96
8b	-4.22(18.58mg/l)	3.36	3.11
8c	-2.17(1.64g/l)	1.83	1.60
8d	-2.43(0.96g/l)	2.32	1.96
8e	-1.96(2.86g/l)	0.57	0.69
8f	-2.60(0.85g/l)	2.67	2.90
8g	-2.98(0.40g/l)	2.49	2.80

Table 2:

Molecular properties

Drug likeness									
Compound	LogP	TPSA (A^2)	HBA nONatom	natoms	HBD nOHNH	lipinskirule nviolations	RB	Mol.wt	Mol. Volume
8a	2.923	38.135	4	23	0	0	6	307.397	296.19
8b	3.476	46.924	4	23	1	0	6	307.397	295.835
8c	1.522	38.135	4	18	0	0	3	243.31	230.982
8d	2.027	38.135	4	19	0	0	3	257.337	247.784
8e	0.965	47.369	5	19	0	0	3	259.309	239.967
8f	2.708	41.373	5	25	0	0	4	334.423	315.175
8g	2.667	87.197	8	28	0	0	5	379.42	338.509

Table-3

Molecular bioactivity score

Compounds	GPCR	ICM	KI	NRL	PI	EI
6a	0.16	-0.06	0.17	-0.26	-0.01	0.01
6b	0.01	-0.1	0.01	-0.32	-0.14	-0.08
8a	0.23	-0.03	0.14	-0.18	0.07	0.1
8b	0.17	-0.02	0.1	-0.35	-0.06	0.03
8c	0.01	-0.06	0.02	-0.46	-0.11	0.06
8d	0.08	-0.03	0.05	-0.41	-0.11	0.11
8e	-0.02	-0.15	0.08	-0.45	-0.15	0.04

Table 4

Docking score obtained for the molecules

Sl. No.	Title	docking score	glide gscore	glide evdw	glide ecoul	glide emodel	glide energy	glide einternal	glide ligand efficiency	XP GScore
1	str-8a	-7.57	-7.57	-24.04	-6.79	-50.42	-30.84	0.40	-0.40	-7.57
2	str-8b	-5.86	-5.86	-26.97	-6.31	-48.08	-33.28	7.28	-0.25	-5.86
3	str-8c	-4.46	-4.46	-35.62	-1.07	-52.59	-36.69	0.32	-0.25	-4.46
4	str-8d	-8.83	-8.83	-33.92	-6.53	-52.08	-40.45	3.95	-0.46	-8.83
5	str-8e	-8.40	-8.40	-33.59	-6.06	-55.72	-39.65	2.89	-0.44	-8.40
6	str-8f	-7.33	-7.33	-32.50	-5.51	-40.38	-38.00	3.84	-0.29	-7.33
7	str-8g	-8.57	-8.57	-41.10	0.25	-51.62	-40.85	7.32	-0.31	-8.57

Table-5:

Characterization data of synthesized compounds

Compound Code	Molecular Formula	Molecular Weight	% Yield	Found (Calcd)%		
				C	H	N
8a	$C_{19}H_{21}N_3O$	307	86	74.14	6.52	13.57
8b	$C_{19}H_{21}N_3O$	307	82	74.14	6.54	13.67
8c	$C_{14}H_{17}N_3O$	243	80	69.10	7.06	17.37
8d	$C_{15}H_{19}N_3$	257	78	70.11	7.34	16.23
8e	$C_{14}H_{17}N_3O_2$	259	80	64.35	6.52	16.10
8f	$C_{20}H_{22}N4O$	334	85	71.76	6.45	16.65
8g	$C_{20}H_{21}N_5O_3$	379	85	63.41	5.55	18.45

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