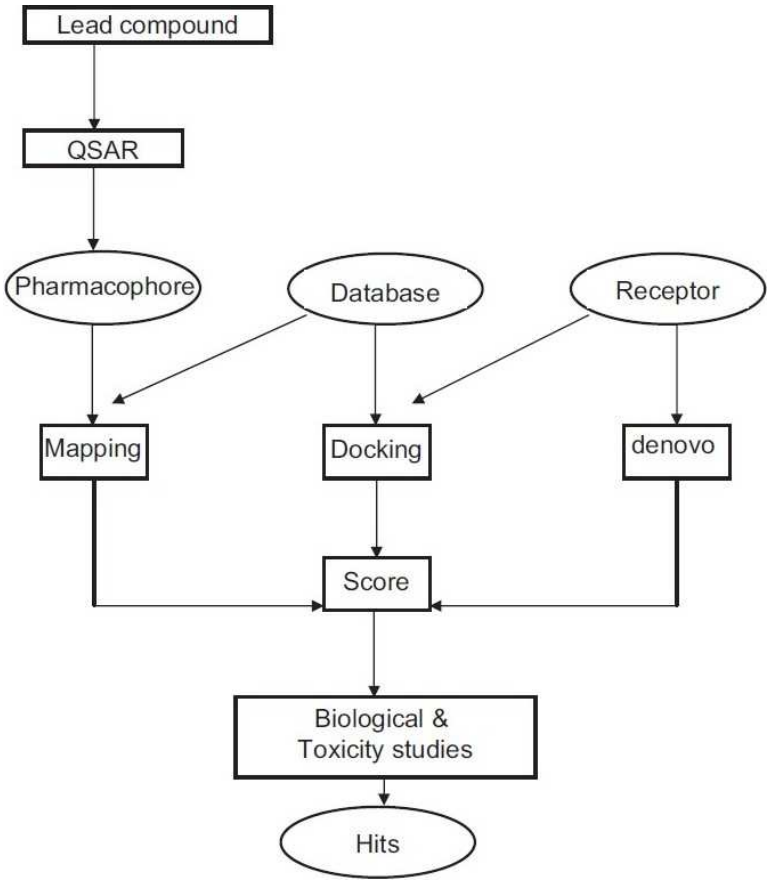
Design, Synthesis and Biological Screening of Heterocycles with Azetidinone, Thiazolidinone and Related Rings

1. INTRODUCTION

Medicinal chemistry involves the invention of new chemical entities for the treatment of diseases and the systematic study of the Structure Activity Relationships of these compounds. Such studies give the basis for development of better medicinal agents from lead compounds found through random screening, systematic screening and rational design. The most difficult aspect of drug discovery is that of lead discovery. Once a lead compound has been discovered for a particular therapeutic use, the next step is to determine the pharmacophore for this compound.[1]



Schematic representation of drug design with the help of computer tools

Once a pharmacophore hypothesis has been developed, structural databases of 3D structures can be searched rapidly for “hits” which are existing compounds with required functional groups and permissible spatial orientations as defined by the search query. Future

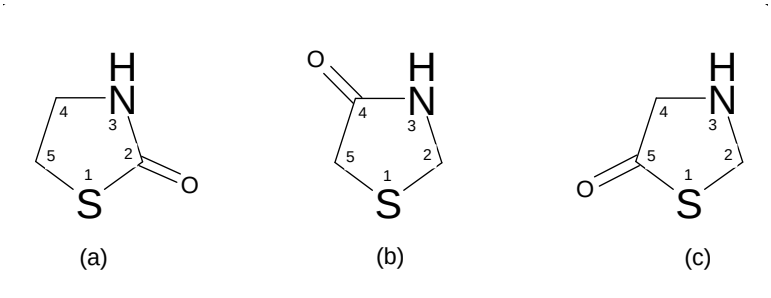
development can be done by conducting *in silico* screening of drug-receptor candidate interactions, known as *virtual* high-throughput screening (*v*HTS).This helps to identify potential lead compounds. The drug-receptor fit, predicted physicochemical properties (molecular weight, no. of hydrogen bonds, hydrophobicity etc.) and ADMET (absorption, distribution, metabolism, elimination and toxicity) are determined in the early design stage. Even though drug design is mainly based on modern computational chemical techniques, it also uses sophisticated knowledge of disease mechanisms and receptor properties.[2]

LEAD COMPOUND

Heterocyclic compounds have a wide range of applications as therapeutic agents. Among large number of heterocycles found in nature, nitrogen heterocycles are the most abundant specially those containing oxygen or sulphur due to their wide distribution in nucleic acid illustration and their involvement in almost every physiological process of plants and animals. Almost 80% of the drugs in clinical use are based on heterocyclic constitution because they have specific chemical reactivity.

The main objective of drug discovery phase is to synthesize lead compounds. A lead compound in drug discovery is a chemical compound that has pharmacological or biological activity and whose chemical structure is used as a starting point for chemical modifications in order to improve potency, selectivity, or pharmacokinetic parameters.[3]

THIAZOLIDIN-4-ONES AS LEADS



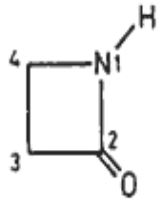
(a) thiazolidin-2-one; (b) thiazolidin-4-one; (c) thiazolidin-5-one

Thiazolidinones are the derivatives of thiazolidine which belong to an important group of heterocyclic compounds containing sulphur and nitrogen in a five member ring, and a carbonyl group at position 2, 4, or 5. 4-Thiazolidinone, a saturated form of thiazole with carbonyl group on fourth carbon, has been considered as a magic moiety which possesses almost all types of biological activities. This diversity in the biological response profile has

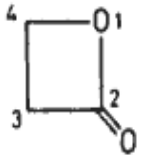
attracted the attention of many researchers to explore this skeleton to its multiple potential against several activities.[4]

The Mannich reaction is an organic reaction which consists of an amino alkylation of an acidic proton placed next to a carbonyl functional group by formaldehyde and a primary or secondary amine or ammonia. Mannich reaction provides a suitable method to introduce amino alkyl substituent into a molecule. In several instances, the Mannich derivatives exhibit better activity than the corresponding parent analogues. Moreover, the presence of Mannich side chain increases the solubility and hence the bioavailability of the drug molecule. Modification of thiazolidinones by replacing the active hydrogen atom at C-5 by an amino alkyl group via Mannich reaction to improve biological activity is also reported.[5]

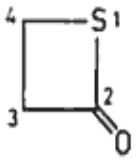
AZETIDIN-2-ONES AS LEADS



2-azetidinone



2-oxetanone



2-thietanone

The 2-carbonyl derivatives of the 4-membered heterocyclic compounds containing either nitrogen or oxygen or sulphur hetero atom in the ring are known as 2-azetidinone, 2-oxetanone, 2-thietanone respectively. The β-lactams are 4-membered cyclic amides derived from 3-amino propanoic acids. The 2-azetidinone ring is a common structural feature of a number of broad spectrum β-lactam antibiotics including penicillin, cephalosporins, carbapenams, nocardin and monobactams which have been widely used as chemotherapeutic agents to treat bacterial infection and microbial diseases.

The azetidinone core-structure offers a unique approach to the design and synthesis of new derivatives with unique biological properties. During the last two decades researches convincingly demonstrated that the prospect of structural modifications of monocyclic β-lactams with specific substituent is an effective procedure for the detection and improvement of important pharmacological effects different from antibacterial activity. As a matter of fact, new β-lactam compounds demonstrated biological activity as inhibitors of a wide range of enzymes. [6]

Most of the researches up to early 1990s focused on synthesis of 2- azetidinones and their antibacterial property. In recent years, renewed interest has been focused on the

synthesis and modification of β-lactam ring to obtain compounds with diverse pharmacological activities like cholesterol absorption inhibitory activity, human tryptase, thrombin and chymase inhibitory activity, vasopressin V1a antagonist activity, antidiabetic, anti-inflammatory, antiparkinsonian and anti-HIV activity.

Modification of azetidinone rings by combining other active rings is also tried. Various amino derivatives could be introduced by nucleophilic substitution at the C-3 position of 2-azetidinone ring.

TUBERCULOSIS

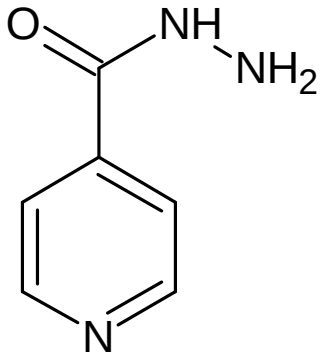
Tuberculosis (TB) is a chronic necrotizing bacterial infection with a wide variety of manifestations caused by bacteria called *Mycobacterium tuberculosis* and seven very closely related mycobacterial species together comprise what is known as the *M. tuberculosis* complex. TB, one of the most common diseases, has been a scourage of humanity for thousands of years and remains to be one of the major infectious diseases in the world.

Despite the availability of highly efficacious treatment for decades, TB remains a major global health problem. According to the WHO Global Tuberculosis Report 2013, there were 8.6 million new TB cases in 2012 and1.3 million TB deaths.[7]

Drug-resistant TB (DR-TB) threatens global TB control and is a major public health concern in several countries. The increasing emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) in the era of human immunodeficiency virus (HIV) infection presents a major threat to effective control of TB. [8]

Design of new substances based on privileged scaffolds is one of the successful directions in drug discovery. Drug discovery based on the existing lead molecules by modification of functional groups is a common strategy. A number of already existing drugs have been structurally modified for improving the activity, to reduce the side effects or in some cases to make the compound devoid of any unwanted effect. A large number of derivatives of already existing drugs have been synthesized and screened for various activities. Moreover, introducing two or more established rings in a single molecule for a combined effect is also tried.[9]

ISONIAZID (INH)



Isoniazid (Isonicotinic acid hydrazide) is the most commonly used drug for active infection and prophylaxis since its introduction for treatment of TB in 1952. Hearn et al (2009) synthesized isoniazid derivatives by lipophilic modification at N2 that combined low toxicity with serum concentration levels as much as three orders of magnitude above minimum inhibitory concentration (MIC) .[10]

Isoniazid is a prodrug that is activated by Kat G, the mycobacterial catalase-peroxidase. The activated form of isoniazid forms a covalent complex with an acyl carrier protein (AcpM) and Kas A, a beta-ketoacyl carrier protein synthetase, which blocks mycolic acid synthesis.

The bacterial type II fatty acid biosynthesis (FASII) pathway is an essential target for drug discovery. Enoyl-ACP reductases participate in fatty acid biosynthesis by utilizing NADH to reduce the trans double bond between positions C-2 and C-3 of a fatty acyl chain linked to the acyl carrier protein.[11] The enoyl-ACP rerductase from *M. tuberculosis,* known as InhA, is a member of an unusual FAS-II system that prefers longer chain fatty acyl substrates for the purpose of synthesizing mycolic acids, a major component of mycobacterial cell walls. [12]

Enzymatic acetylation of the antitubercular isoniazid (INH) by N-acetyltransferase represents a major metabolic pathway for INH in human beings. Acetylation greatly reduces the therapeutic activity of the drug, resulting in under dosing, decreased bioavailability and acquired INH resistance. Chemical modification of INH with a functional group that blocks acetylation, while maintaining strong antimycobacterial action, may improve clinical outcomes and help to reduce the rise of INH resistance. Structurally, the modifications engendering these desirable drug properties are optimally made at N-2 of the INH

framework. Such modifications block the resulting molecule against the action of N-arylaminoacetyl transferases (NATs).

Isoniazid in the treatment of tuberculosis is associated with mild to moderate elevation of liver enzyme activity in plasma, & serum hepatotoxicity in 1-2% patients Isoniazid induced hepatitis is the major toxic side effect. Acetyl hydrazine, a metabolite of INH, causes hepatic damage in adults.

Drug discovery and development consists of a series of processes starting with the demonstration of pharmacological effects in experimental cell and animal models and ending with drug safety and efficacy studies in patients. A main limitation is often the unacceptable level of toxicity with the liver as the primary target organ. Therefore, approaches to study hepatic toxicity in the early phase of drug discovery represent an important step towards rational drug development.

2. OBJECTIVE OF THE STUDY

In view of all the above aspects regarding drug designing, various heterocyclic rings and reactions, their biological activities, it was planned to conduct study on the following aspects to develop a pharmacologically effective drug especially for TB.

1. *In silico* modeling

In silico modeling of the molecules using softwares such as Chemsketch, to select compounds having optimum drug likeness, molecular descriptors resembling those of standard molecules and without violating the 'Lipinski's Rule of Five'.

2. Computer Aided Drug Design

i. To select and prepare targets of interest and carry out the docking studies of analogues using Schrodinger software to obtain the docking scores.

ii. *In silico* ADME property prediction: - To predict the ADME profile of the designed molecules using the application QikProp in Maestro Molecular modeling environment.

3. Synthetic Aspects

i. To synthesize hydrazones from isoniazid by the reaction with various aromatic aldehydes.

ii. To prepare 4-thiazolidinones from the above hydrazones.

iii. To obtain Mannich bases from these thiazolidinones.

iv. Conversion of hydrazones to 2-azetidinones is also planned.

v. To combine azetidinones with other biologically active ring systems.

4. Biological screening

i. To screen the synthesized compounds for anti tubercular activity using alamar blue assay method.

ii. Since both the rings are reported to have antimicrobial activity, to perform anti bacterial and antifungal activity using disc diffusion method.

iii. To screen for anti inflammatory activity by carrageenan induced paw edema method.

iv. To compare the *in vitro* hepatotoxicity effect of isoniazid derivative with the same effect produced by the parent molecule.

3. MATERIALS & METHODS

1. Materials

Chemicals used were purchased from Sigma-Aldrich, Hi media, LobaChem etc. and are of analytical grade. All the solvents were distilled and dried using standard procedure. Silica gel G was used as the stationary phase for TLC. Iodine chamber was used for developing spots.

2. Computational Methodology

2.1. Methodology of Docking

- Retrieving the crystal structure of protein from protein data bank (PDB)

The 3-D structure of the protein was obtained from PDB using their specific PDB ID. For the antitubercular study, crystal structure of *Mycobacterium tuberculosis* Enoyl- [acyl-carrier-protein] reductase (PDB ID 4DRE) in complex with NADH has been determined to a resolution of a 2.40A^0. The protein cyclooxygenase2 (PDB ID ICX2) was selected for anti-inflammatory study.[13]

- Protein Preparation

This was done using the protein preparation wizard in the Schrodinger software graphical user interface maestro v9.3.

- Ligand Preparation

  All the possible compounds belonging to both groups of derivatives were drawn using the workspace of Maestro and were converted into 3D form for the docking studies. All the ligands were built using Maestro build panel and preparation was done using 'Lig Prep'. [15]

- ADME Studies

  This is to analyze the pharmacokinetics and pharmacodynamics of the ligands by accessing the drug likeness properties using QikProp.

- Docking

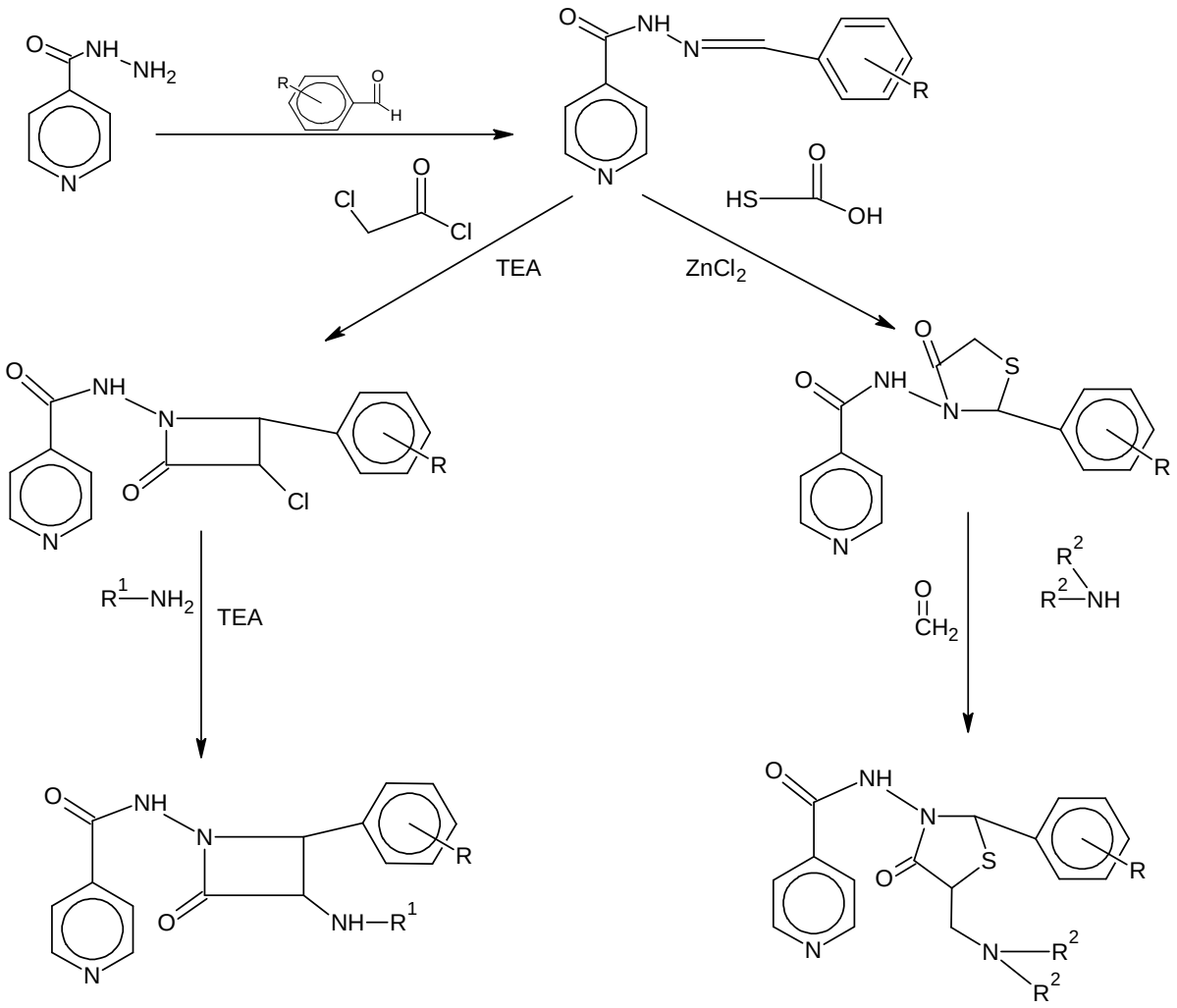
  It involves various steps like receptor grid generation, Glide standard precision (SP) ligand docking and Induced Fit Docking (IFD) Extra Precision (XP). Each ligand was rigorously docked into the induced fit receptor structure and the best docked pose with lowest Glide score was recorded for each ligand.

2.2. *In Silico* Molecular Studies (QSAR)

QSAR studies are used as a powerful drug design tool for the optimization of promising drug candidates. The various properties of a drug that appear to influence its activity like lipophilicity, electronic effects, steric effect, etc. are determined as molecular descriptors. Lipinski's rule of Five is determined using 'MOLINSPIRATION' software.

3. Synthetic Methodology

In the first step, isoniazid was combined with aromatic aldehydes and Schiff bases/ hydrazones were obtained. Thiazolidinones and azetidinones were obtained from Schiff bases by treatment with thioglycollic acid and chloroacetyl chloride respectively using conventional, microwave and ultrasonic methods. In the next step, azetidinone was converted to its derivatives using amines and thiazolidinone to Mannich bases using formaldehyde and secondary amines.



During each step, progress of the reaction was monitored by performing TLC. The final product was confirmed by single spot TLC and melting point determination. The products obtained were characterized by various spectroscopic analyses like IR spectroscopy, ^{1}HNMR, ^{13} CNMR and Mass spectroscopy.

4. BIOLOGICAL SCREENING

4.1. Antitubercular Screening by Alamar Blue Assay Method

Alamar blue is a dye used for screening of antitubercular activity. Alamar blue (oxidized form) turns pink in color upon reduction. Since Mycobacterium is an aerobic organism, presence of growth turns alamar blue pink. This principle has been used to predict the presence or absence of growth of *Mycobacterium tuberculosis* for testing antitubercular agents. Blue color shows absence of growth of *Mycobacterium tuberculosis* (inhibitory activity of agents tested).

Alamar blue gives a measure of cell proliferation, by detecting the level of oxidation during respiration. The reducing environment of the cells in the alamar blue assay is measured through the conversion of resazurin (oxidized form) to resorufin (reduced form).

Test organism: *Mycobacterium tuberculosis* H37Rv maintained in Lowenstein Jensen medium was used as the test organism for antimycobacterial screening studies.

4.2. Antimicrobial Screening

Antibacterial and antifungal study of selected compounds from each group was done by Disc Diffusion method. For antibacterial study both Gram positive (*Staphylococcus aureus*) and Gram negative (*E. coli*) were selected. Antifungal study was conducted against *Aspergillus niger* and *Candida albicans*. Gentamycin and ketoconazole were used as standards for antibacterial and antifungal respectively.

4.3. Acute Toxicity Study

The Institutional Animals Ethics Committee approved the use of animals for the study. (Ethical Clearance number: 002/PHD/UCP/CVR/13 dtd 14/5/13}.The acute toxicity study for the test drugs was carried out in mice according to OECD guidelines (OECD-423, 2004). The test drugs were administered as single dose of 2000 mg/kg, p.o. and animals were monitored individually and continuously for 30 min, 2 h and up to 24 h. The animals were then monitored for any mortality for the following 14 days.

4.4. Anti-inflammatory Activity by Carrageenan Induced Paw Edema

Procedure

The paw volume was measured at 1, 2, 3, 6 and 24 hr after carrageenan injection using a Plethysmograph. The difference between initial and subsequent reading gave the actual edema volume. The anti-inflammatory activity in animals that received the test compounds (100mg/kg) and Diclofenac (25mg/kg) was compared with that of vehicle control groups. The percentage inhibition of edema was calculated as follows:

$$\text{Percentage inhibition of edema} = 1 - Vt / Vc \times 100$$

Carrageenan induced paw edema method, Using Plethysmograph. Wistar albino rats were used and divided into 5 groups each containing 6 animals.[16]

4.5. *In vitro* Hepatotoxicity Study

In early stages of drug discovery, *in vitro* toxicity evaluation is very important to obtain quick evaluation with small amount of test compounds and reduce animal use.[17] Chang liver cells were used and sample solutions (INH and derivative) were added to grown cells at 10µg/ml, 50µg/mland100µg/ml concentrations. The % dfference in viability was measured using standard MTT assay method after 24hrs. Optical density was read at 540nm using DMSO as blank.

% viability = (OD of Test/ OD of Control) X 100

5. RESULT & DISCUSSION

5.1. *In Silico* Molecular Modeling

In silico molecular modeling studies were carried out on novel analogues of isoniazid containing azetidinone and thiazolidinone rings using various softwares like Schrodinger, ACDLABS Chemsketch and Molinspiration. The molecular descriptors for lipophilicity, steric and electronic parameters were determined by using ACDLABS Chemsketch software.

Drug likeness, a complex balance of various molecular properties and structural features which determines whether the particular molecule is similar to known drugs, was calculated using Molinspiration software. The drug likeness score of the proposed novel analogues were determined and compared with those of existing drugs.

The log P value, which is a measure of lipophilicity, for the various derivatives was also calculated and also their compliance with Lipinski's rule of five was determined using Molinspiration software.

5.2. Molecular Docking

ADMET studies were done with these derivatives using QikProp application of Schrodinger and most of the molecules passed ADMET studies. Then these molecules were docked with enoyl-[ACP]-reductase (PDB ID 4DRE) for antitubercular activity and for anti-inflammatory activity cyclooxygenase-2 (PDB ID 1CX2). Docking scores obtained according to molecule's binding affinity to the receptor were compared and only those with minimum scores were taken for synthesis.

5.3. Synthesis

Five different analogues of isoniazid from each category say azetidin-2-ones and their derivatives; thiazolidin-4 ones and their derivative Mannich bases were synthesized, with the standard chemicals and well established procedures by conventional, microwave irradiation and ultrasonication methods. The purification was done using column chromatography (Toluene: methanol solvent system). The purity of the synthesized compounds was further established by single spot TLC and melting point determination. All synthesized compounds were then characterized by IR, NMR and Mass spectroscopy.

5.4. Antitubercular Screening

The antitubercular activities of the tested derivatives are comparable with that of isoniazid and with other standard drugs. Most of the Schiff base derivatives are showing antitubercular activity at 50µg/ml. In the chlorine substituted derivatives, the para substitution is showing better activity than ortho and meta derivatives. Mannich bases and azetidinone derivatives are producing better antitubercular activity.

5.5. Antimicrobial Screening

All the compounds showed promising activity as antibacterial and antifungal agents in comparison with corresponding standards.

5.6. Acute Toxicity Study

As there was no toxic reaction or mortality observed, the test drugs were found to be devoid of any toxicity up to a maximum dose of 2000 mg/kg body weight. Hence the dose selected for animal study is 100mg/Kg.

5.7. Anti-Inflammatory Activity

One representative compound from each group of derivative was taken for anti-inflammatory study using male Wistar rat by carrageenan induced paw edema method. The data were statistically analyzed by one way ANOVA followed by Dunnet's test and compared with toxic control. Dose: 100mg/Kg. **P<0.01 against toxic control. Results are expressed as mean ± SEM for n=6 rats in each group. The results obtained for each group is comparable to those of standard diclofenac. The decrease in paw volume (percentage inhibition) produced at 1hr is low in comparison with diclofenac. But at 2hr, 3hr and 6hr, there is significant change. Among the various derivatives used, at 6hr, MB and AAZ

derivatives are producing percentage inhibition near to diclofenac, which in turn is more than TZ derivatives.

5.8. *In vitro* Hepatotoxicity Study

The results obtained show that at higher concentration the derivative produced comparatively less damage to the hepatic cells than INH.

6. CONCLUSION

In silico molecular modeling studies were carried out on various novel Mannich bases of 4-thiazolidinone derivatives of isoniazid and 2-azetidinone derivatives of isoniazid using various softwares like Schrodinger, ACDLABS Chemsketch, Molinspiration and Pass online etc.

The protein enoyl ACP reductase (inhA) involved in the synthesis of mycolic acid from fatty acid, the target protein for antitubercular activity of INH is selected as the target for antitubercular docking. In each group, the derivatives with minimum docking score and which satisfied all physicochemical parameters including Lipinski's rule were taken for synthesis using well established procedures.

The enzyme cyclooxygenase-2 (COX-2) which plays a major role in prostaglandin biosynthesis in the inflammatory cells was selected as the target for anti inflammatory docking studies.

The synthesized compounds were characterized by single spot TLC, melting point and are characterized by spectroscopic techniques like IR, NMR and Mass spectroscopy.

Selected compounds from each group were subjected to antitubercular screening by alamar blue assay method and antimicrobial study was conducted by disc diffusion method. The antitubercular activities of the derivatives are comparable with that of isoniazid. Most of the derivatives produced significant antibacterial and antifungal activity.

Acute toxicity study was performed and based on that the dose was determined as 100mg/Kg for anti-inflammatory study. Anti- inflammatory study was performed by carrageenan induced paw edema method and the activities produced by the derivatives are significant in comparison with standard diclofenac.

In vitro hepatotoxicity study was conducted in chang liver cells. The damage exhibited by derivative was less compared to INH.