1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (Mtb). It is the most dangerous bacterial infection responsible for severe increase in death cases. The tubercle bacillus was discovered by Robert Koch in 1882. There are several reports indicating that tuberculosis (TB) is an age old dreadful disease even from ancient times.\(^1\) The disease was called "consumption" in the past because of the way it would consume from within anyone who became infected.

Tuberculosis is a chronic granulomatous infectious disease. Infection occurs via aerosol, and inhalation of a few droplets containing *M. tuberculosis* bacilli. After infection, *M. tuberculosis* pathogenesis occurs in two stages. The first stage is an asymptomatic state that can persist for many years in the host, called latent TB. When the immune system is weak, the bacteria begin replicating and cause characteristic symptoms such as cough, chest pain, fatigue and unexplained weight loss. If left untreated, the disease eventually culminates in death.

In the year 1993, World Health Organization (WHO) declared TB a global public health emergency. About one-third of the world’s population (> 2 billion), are infected with TB bacilli. 10% of the people infected with TB bacilli will become sick with active TB in their lifetime.\(^2,3\) According to WHO report, global population with burden of disease caused by TB from 1990-2011 was 6948 million and total number of Multi-Drug Resistant (MDR) cases from 2005-2011 were 61690.\(^3\) In 2011, there were an estimated 8.7 million incident cases of TB (range, 8.3 million–9.0 million) globally. Highest number of incidences were reported in Asia (59%) and Africa (26%). Estimates of the burden of TB disease among children have also been carried out. The figures are 4,90,000 cases and 64,000 deaths among HIV-negative children per year. TB is one of the leading causes of death among women. 0.5 million women succumbed to TB. This includes 3,00,000 (range, 2,50,000–3,50,000) TB deaths among HIV-negative women.

The burden of TB is highest in Asia and Africa. In 2011, largest number of cases was reported from India, China, South Africa, Indonesia and Pakistan. India and China alone accounted for 26% and 12% of global cases, respectively. Of the 8.7 million TB incident cases reported in 2011, about 1.2 million people are also suffering from HIV. In the African region, 39% of TB cases were estimated to be co-infected with HIV.\(^3\)

In 2011, approximately 1.4 million people died of TB, of whom 0.5 million were women. 1 million HIV-negative people died of TB and about 0.4 million people who were HIV-positive died of TB.
MDR-TB is defined as resistance to isoniazid and rifampicin, with or without resistance to other first-line drugs. XDR-TB is defined as resistance to at least isoniazid and rifampicin, and to any fluoroquinolone, and to any of the three second-line injectables (amikacin, capreomycin, and kanamycin). It has been estimated that 3.7% of new patients and 20% of previously treated patients are suffering from MDR-TB. India, China, the Russian Federation and South Africa have almost 60% of the world’s cases of MDR-TB. The highest proportions of TB patients with MDR-TB are in Eastern Europe and central Asia. Extensively drug-resistant TB (XDR-TB) has been identified in 84 countries globally. The proportion of MDR-TB cases with XDR-TB was 9.0%. It has been reported from India that there are patients who do not respond to any of the antiTB drugs and termed as “extremely drug resistant” (“XDR-TB”) and “totally drug-resistant TB” (“TDR-TB”).

Burden of disease in India is 1241 million. In 2011, 1.3 million new TB cases were reported in India. About 0.5 million people died of TB and 4237 MDR cases. Male and female population at the age of 25-34 is the maximum affected. The major factors which caused wide spread of TB include HIV infection and emergence of MDR strains. Irregular medication or patient noncompliance have contributed for the development of resistance and is often a corollary to HIV infection.

Directly Observed Treatment Short course (DOTS), introduced by the WHO consists of mainly standard short course regimen with first line drugs (isoniazid, pyrazinamide, rifampicin and streptomycin or ethambutol or both). The therapy has long course of duration (6 -12 months and 1-2 years for MDR TB), with severe drug side effects.

The urgent need for the discovery of newer antitubercular (antiTB) drugs has been felt worldwide. As a result, for the first time in 40 years, a coordinated effort has begun for the development of newer antiTB drugs. Currently 11 new or repurposed antiTB drugs are in clinical trials. Simultaneously efforts to develop new diagnostics and vaccines for TB have intensified. There has been increase in funding for TB research and development from US$ 363 million in 2005 to US$ 630 million in 2010 spurting the hope for early discovery of antiTB drugs.

Thus there is an urgent need for new drugs to treat tuberculosis with special emphasis on shortening the regimen than the current drugs and as well as novel pathway for mechanism of action to treat MDR-TB.
2. Review of Literature

The lipid rich cell wall of Mtb acts as a barrier to entry of molecules into the cell and is responsible for pathogenecity and resistance to attack by the host.\textsuperscript{11} The genomic sequence of Mtb has been studied by Cole \textit{et al.},\textsuperscript{12} It is found that the genome of \textit{Mycobacterium tuberculosis} encodes 20 different cytochrome P450 enzymes (P450s). This is an indication that their presence is having a crucial role in Mtb physiological functions. P450s are mono-oxygenases, which facilitate the use of carbon sources in prokaryotes. Venter \textit{et al.} proposed that the human genome (nearly 3000 Mb) encodes 57 P450s, whereas Mtb encodes 20 P450s (4.41 Mb).\textsuperscript{13} Greater CYP in Mtb indicates that the organism relies heavily on P450 enzymes for its survival. Odds \textit{et al.} reported that Azoles inhibit the fungal growth by having strong affinity at CYP51 active site. These drugs act as antifungals by inactivating the redox system by coordinating with heme iron through nitrogen atom of azole. As a result, the interaction of substrate with heme iron is hindered by azoles which indicates the possibility of targeting Mtb P450s as well.\textsuperscript{14} Gatfield \textit{et al.} reported that enzymes responsible for sterol-synthesis are absent in Mtb genome and thus incapable of de novo sterol synthesis. Cholesterol is necessary for Mtb infectivity in humans and thus sterol binding activity of CYPs is an important factor for Mtb survival in the host.\textsuperscript{15} Genomic analysis of Mtb P450 system by Waddle \textit{et al.} and Kendall \textit{et al.} has suggested the involvement of some P450s in cell viability and infectivity.\textsuperscript{16,17} Schnappinger \textit{et al.} and Sassetti \textit{et al.} have reported that Mtb gene Rv3545c encodes for protein CYP125 is induced in Mtb during its engulfment in macrophages and are essential for survival in infected mice.\textsuperscript{18,19} McLean \textit{et al.} reported antifungal azoles as potent inhibitors of cytochrome P450 and mycobacteria growth.\textsuperscript{20} Jackson \textit{et al.} reported the bactericidal activity of antifungal azoles against \textit{Mycobacterium smegmatis}.\textsuperscript{21} Ouellet \textit{et al.} and Podust \textit{et al.} have performed protein co-crystallization of CYP 130 and 51 with different antifungal azoles and found tight binding of the drugs to active site, thus confirming the new pathway and new azole agents as antitubercular agents.\textsuperscript{23,24} McLean \textit{et al.} have studied the binding mode of azoles to the active site of CYP 125.\textsuperscript{24} Boshoff \textit{et al.} have studied the transcriptional responses of Mtb in presence of metabolic inhibitors. The study has indicated that clotrimazole and econazole have non specific interactions with succinate dehydrogenase but no effect on NADPH system. Whereas new azoles like fluconazole and voriconazole are systemically tolerated and found to have good interaction with the CYPs of Mtb, indicating that these azoles can be used as templates for designing antitubercular agents.\textsuperscript{25} Murphy \textit{et al.} reported that CYP125A1 (Rv3545c) is a potential and
exploring target for new candidates as antitubercular agents. It has been experimentally proved that, among all the mutants of H37Rv strain studied for survival, only CYP125A1 is necessary for \textit{in vivo} growth. This importance of CYP125A1 has also been confirmed by Chang \textit{et al.} Capyk \textit{et al.} have shown that CYP125A1 in Mtb helps in terminal oxidation of cholesterol and C27 steroids, thus using host’s cholesterol for Mtb survival in macrophages. The binding of azoles like econazole and miconazole with CYP125A1 enzyme is with high affinity (<3 μM) through type-1, but not like other P450s which coordinate to heme iron. This fact was supported by co-crystal structure of CYP125 with econazole, which shows funnel shape entrance towards heme, thus stopping the heme coordination for ligands. Ouellet \textit{et al.} have also elaborated about CYP125A1 as a potential target for antitubercular drug designing. McLean \textit{et al.} have demonstrated the essentiality of CYP 121 for the viability of M.tb \textit{in vitro} by gene knock-out experiments. Leys \textit{et al.} have reported the role of CYP 121 in polyketide or polycyclics metabolism. McLean \textit{et al.} have reported the characterization of crystal structure of CYP 121. The crystal structure of Fluconazole-CYP121 complex was studied by Seward \textit{et al.} and suggested the role of azoles in tight binding to CYP 121 by direct contact with heme. Sundaramurthi \textit{et al.} have reported the docking studies of azoles with CYP 121 and suggested the potentiality of azoles as inhibitors of M.tb cytochromes. Hudson \textit{et al.} has reported the fragment screening of variousazole derivatives as inhibitors of CYP121 and suggested the role of CYP 121 as a potential drug target for designing antitubercular agents.

![Fig. 1. Structures (A) CYP125 Econazole complex\textsuperscript{29} (B) CYP121 Fluconazole complex\textsuperscript{34}](image-url)

Milano et al. recently reported that the CYP P450s are not involved in the mechanism of resistance.\textsuperscript{37} Thus we found that targeting these enzymes (CYP121 and CYP 125) for designing antitubercular agents is useful.

Triazole derivatives have been explored for anti-infective and other therapeutically important activities. Many researchers have reported that triazoles apart from having antitubercular activity, possess potential antimicrobial\textsuperscript{38,39}, antifungal\textsuperscript{40}, anti-inflammatory\textsuperscript{41}, anticancer activity.\textsuperscript{42-44}

![Fig. 2. Compounds 1 (antimicrobial)\textsuperscript{38}, 2 (antifungal)\textsuperscript{40}, 3 (anticancer)\textsuperscript{42}, 4 (anti-inflammatory)\textsuperscript{41}](image)

Bhat et al. have reported the antitubercular activity of 1,2,4-triazoles. presence of amide bond with the triazoles nucleus, prepared from pyrazinoic acid, thus making the structure rigid have shown antiTB activity < 1µg/mL.\textsuperscript{45}

1,2,4-triazole derivatives reported by Shiradkar et al. have been found to posses antitubercular activity ≤ 6.25 µg/mL (5).\textsuperscript{46}

![Fig. 3. Compound 5 (MIC ≤ 6.25 µg/mL)\textsuperscript{46}](image)

Patel et al. reported the synthesis of 1,2,4-triazoles (6) having moderate antiTB activity.\textsuperscript{47}
Triazolyl imidazoles synthesized by Jadhav et al., possessed MIC less than 6.25µg/ml against *Mycobacterium tuberculosis*.

S-derivatives of 1,2,4-triazoles-3-thiols synthesized by Shiradkar et al. (8) were found to possess antitubercular activity in the range 6.25 and 0.39µg/ml.

Foks et al. have synthesized triazole derivatives which were found to possess antitubercular activity in the range 25–100 µg/mL. N-substituted triazoles (9) were found to possess activity against Mtb lesser than the S-substituted triazoles. Thus indicating that substitution at sulphur is preferred than at ring nitrogen in 1,2,4-triazoles.

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**Fig. 4.** Compound 6

**Fig. 5.** Compound 7 (MIC ≤ 6.25 µg/mL)

**Fig. 6.** Compound 8 (MIC ≤ 6.25 µg/mL)

**Fig. 7.** Compound 9 (MIC 25 µg/mL)
Marrapu et al. have reported the synthesis of triazoles derivatives. Ten compounds have exhibited good antitubercular activity with MIC in the range of 3.12-0.78 µg/mL. Most of the compounds with potent in vitro activities were found non-toxic against VERO cell line and mouse derived macrophages. Two compounds showed good ex-vivo activity with 99% and 71% inhibition of growth of intracellular bacilli.

Fig. 8. Compound 10 (99% inhibition), 11 (71% inhibition).

N-(3-aryl-1,2,4-triazol-5-yl) cinnamamide derivatives (12) designed through topless tree approach by Kakwani et al., have shown antiTB activity in the range of 4-100 µM with good safety profile (13).

Fig. 9. Compound 12 (MIC 0.46 µM), 13 (MIC 4.8 µM).

Kini et al., have reported the synthesis and evaluation of antitubercular activity of 1,2,4-triazoles. Two of them (14, 15) possessed antitubercular activity at 1 µg/mL.

Fig. 10. Compound 14 (MIC 1 µg/mL), 15 (MIC 1 µg/mL).

Joshi et al. have reported synthesis of 5-substituted-4-amino-1,2,4-triazolin-3-thione with moderate antitubercular activity (16).

Fig. 11. Compound 16 (MIC 62.5 µg/mL).
Kumar et al. have reported the synthesis of 2-substituted-5-[isopropylthiazole] clubbed 1,2,4-triazole (17) which possessed moderate to good antitubercular activity.

![Fig. 12. Compound 17 (MIC 85 μg/mL)](image)

The study on clubbed triazoles with various alkyl and aryl substitution on thiol and 4-amino position of 1,2,4-triazoles (18, 19) has been reported by Shiradkar et al. The compounds were found to possess MIC in the range of 0.39 to 6.25 μg/mL against *Mycobacterium tuberculosis*. This study also claimed that better activity is achieved when highly electronegative substitution or hydrophobic group is at sulfhydryl group. This was also considered during our designing of 1,2,4-triazoles nucleus.

![Fig. 13. Compound 18 (MIC 6.25 μg/mL), 19 (MIC 3.13 μg/mL)](image)

A novel 3-cyclohexylpropanoic acid-containing triazole derivatives with aryl and alkyl substitution on the nitrogen at fourth position (20, 21) and having antiTB activity has been reported by Gobis et al.

![Fig. 14. Compound 20 (MIC 50 μg/mL), 21 (MIC 50 μg/mL)](image)

A series of 4-alkyl/aryl-2,4-dihydro-5-((6-(4-bromophenyl)imidazo[2,1-b]thiazol-3-yl)methyl)-3H-1,2,4-triazole-3-thiones (22) have been reported by Guzeldemirci et al. and are found to possess moderate antitubercular activity.
A series of galactose-linked nitro substituted 1,2,4-triazoles (22) have been designed by Mugunthan et al., which have shown a moderate antitubercular activity. These researchers have reported that the prepared triazoles were active under aerobic condition, whereas the standard metronidazole is inactive. This indicated that the triazoles are having good activity under aerobic conditions also.  

Kaplancikli et al. have reported the antitubercular activity of 3-alkylsulfanyl-1,2,4-triazoles (23).  

Fig. 15. Compound 22 (MIC 50 μg/mL).  

Fig. 16. Compound 23 (MIC 12.5 μg/mL).  

Fig. 17. Compound 24 (MIC 6.25 μg/mL).
3. Purpose of work

For over 40 years, Isoniazid (isonicotinic acid hydrazide, INH) has been utilized as a frontline agent in drug mixtures to treat *Mycobacterium tuberculosis*. Introduction of drugs like INH, Rifampicin, Pyrazinamide and Streptomycin resulted in rapid decline in TB cases worldwide. As a result, the research in the area of discovery of newer antitubercular drugs lost prominence. Drug development for tuberculosis and other neglected diseases has been at a virtual standstill for decades. Several factors lead to the emergence of resistant strains of *Mycobacterium tuberculosis*. HIV infection also contributed to the escalating burden of tuberculosis.

Nowadays, there are reports of MDR TB, XDR TB and TDR TB. There is an urgent need for the development of newer antitubercular agents. Due to the awareness created with regard to the disease burden, during the last decade, many industries have turned their attention towards the discovery of newer antitubercular drugs and many molecules are under investigation. However, the number of candidate compounds is still small compared to the drug pipelines for diseases such as cancer or cardiovascular diseases (and the number of companies engaged in the latter is also greater) which are major concern to the wealthy countries. Antitubercular drugs with novel mechanism of action are need of the hour.

Triazoles have received much attention during the last few decades as antimicrobial\(^{38,39}\), antifungal\(^{40}\), anti-inflammatory\(^{41}\), anticancer\(^{42-44}\) and antitubercular agents\(^{45}\). Among the triazole class of compounds, 1,2,4-Triazoles were reported as most promising heterocyclic compounds having antitubercular activity.\(^{45-59}\) Although several studies have been carried out on 1,2,4-triazoles, still there is a scope to explore the structure activity relationships, and to improve its druglikeness. Recent studies have shown thatazole antifungals act as antitubercular agents by inhibiting P-450 mono-oxygenases.\(^{10}\)

Recent studies have indicated that azole drugs exhibit antitubercular activity in mice. Newer generation azoles and triazoles have less severe interactions with human P450s.\(^{34,60}\) Econazole which possesses antitubercular activity has been reported to display strong affinity towards CYP 125 receptor.\(^{29}\) The interaction study of azoles with CYP 125 has shown that azoles have high affinity to the enzyme, thus inhibiting the interaction of cholesterol at the receptor site.\(^{28,29}\) Another enzyme CYP121 has also proved to be essential for the viability of *M. tuberculosis*.\(^{33,34}\) The *in vitro* and *ex vivo* antitubercular activity of azole drugs, which were reported to have binding affinities with CYP 121 and CYP 125 against *M. tuberculosis* has been studied.\(^{61-63}\) Based on above facts, it was decided to synthesize 1,2,4-triazole
derivatives and evaluate them for antitubercular activity. Since azoles have interactions with two important mycobacterial enzymes namely CYP 121 and CYP 125 enzyme, it was decided to study the interaction of synthesized 1,2,4-triazoles with both of these enzymes. The evaluation of the activity has been restricted to Mycobacterium tuberculosis H37Rv strain in silico. Other mycobacterial strains have not been included in the study. We made an attempt to identify stronger inhibitors of CYP121 and CYP 125 of M. tuberculosis using molecular docking studies.

Following were the specific objectives of the present study.

- To design and synthesize novel 1,2,4-triazole derivatives.
- To characterize the synthesized 1,2,4-triazole derivatives.
- To evaluate the antitubercular activity of synthesized compounds.
- To carry out docking studies to understand the interactions of synthesized molecules with CYP enzymes and correlate it to the antitubercular activity.
- To carry out cytotoxicity and hepatotoxicity evaluation of the synthesized molecules.
- To determine the antitubercular activity of compounds with selectivity index (SI) ≥ 10 against MDR strain.
- To quantify the physicochemical properties (Log P and pKa) of synthesized compounds.
- To study the microsomal stability of selected compounds.