Development and evaluation of novel anti-tuberculosis drug molecules

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY INDORE
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A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
Of
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by

Shalini Shukla

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"Development and evaluation of novel anti-tuberculosis drug molecules** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore** is an authentic record of my own work carried out during the period from August 2021 to May 2022 under the supervision of Prof. Avinash Sonawane, Professor, Discipline of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree at this or any other institute.

Shalini

Signature of the student
SHALINI SHUKLA

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Signature of the Supervisor
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Date: 28/05/22

Shalini Shukla has successfully given his/her M.Sc. Oral Examination held on **5th May 2022**.

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Shalini Shukla

DEDICATION

My M. Sc. thesis is dedicated to all my loved ones, especially my mother, father, and sisters.

ABSTRACT

Bacterial infections have become a major problem for humanity. Among these, tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb) continues to be one of the leading causes of death around the world. Due to the evolution of Mtb, it has developed unique mechanisms to reside inside macrophages and induce persistent infections.

In this chemotherapy era, many anti-TB drugs have been discovered; however, with the rise in multidrug-resistant (MDR) strains the therapeutic efficacy of the drugs is minimized. Several new medications are currently being tested, however many of them fail in clinical trials due to non-targeted therapy, long regimen durations, and multiple adverse effects. As drug-resistant TB is a major public health concern, finding an effective way to deliver drugs to the target site is important.

Researchers are looking for a promising way to safely deliver the drugs to the infected site as well as to boost the treatment efficacy by combining current antibiotics with metal complexes such as nanoparticles (NP). Despite their benefits, such as immunomodulatory effects and strong antibacterial activity, certain NPs are hazardous, limiting their usefulness as a delivery platform. As a result, there is a great desire for an alternative that can operate as a vehicle for anti-TB drug delivery. Due to several promising qualities such as high porosity, wide surface area, high drug loading capacity, and higher

encapsulation efficiency, a novel class of material known as "metal-organic framework (MOF)" is receiving interest. Thus, it could act as a drug delivery vehicle to deliver anti-TB drugs.

To study more on the efficacy and biocompatibility of MOFs, in this study, the synthesis of Cu-MOFs and the encapsulation of one of the potent 1st line anti-TB drugs, isoniazid (INH) was carried out (i.e., INH@CuMOF). Characterization of INH@Cu-MOF confirmed the encapsulation of INH into Cu-MOFs and demonstrated the compound is thermally stable. *In vitro* experiments revealed that INH@Cu-MOF has anti-mycobacterial activity with no cytotoxicity for RAW 264.7 macrophages. INH@CuMOF also demonstrated less biofilm-forming and more biofilm-disrupting ability. Thus, our study suggests Cu-MOFs can be a potential drug delivery vehicle to sustainably deliver INH for the treatment of TB.

Further, as M.tb strains have developed several mechanisms to evade standard anti-TB treatment, it necessitates the development of novel anti-TB drugs. Drug repurposing is one of many other methods that can be tested to combat MDR TB infections. As the traditional drug development process is known to be costly as well as time-consuming thus, we performed the *in-silico* structural as well as functional characterization of mycobacterial cell wall protein to understand its functionality and determine any previously known drug that can be repurposed for the treatment of TB. It was followed by structure-based virtual screening to discover potentially novel or repurposed anti-TB drugs.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Background and Introduction

Chapter 2: Materials and Methods

Chapter 3: Results and Discussions

LIST OF TABLES

CHAPTER 2: Results and Discussions

LIST OF ABBREVIATIONS

TB…… Tuberculosis

MTB…… *Mycobacterium tuberculosis*

MS…… *Mycobacterium smegmatis*

MOFs…… Metallic Organic Framework

Cu-MOF…… Copper MOF

E-MOF…… Encapsulated MOFs

INH@Cu-MOF..... Encapsulated MOFs

RIF…… Rifampicin

INH…… Isoniazid

PZA …… Pyrazinamide

EMB…… Ethambutol

HIV…… Human Immunodeficiency Virus

Cu-MOF…… Copper Metallic Organic Framework

TNF α…… Tumour necrosis factor-alpha

NK……Natural killer cell

BCG…… Bacillus Calmette-Guerin

CTLs…… Cytotoxic T lymphocytes

INFγ…… Interferon

IL…… Interleukin

ER…… Endoplasmic reticulum

DOT…… Directly Observed Treatment

MHC…… Major histocompatibility complex

MDR TB…… Multidrug resistant TB

XDR TB…… Extensively drug-resistant TB

TDR TB……Totally drug-resistant TB

RR TB…… Rifampicin resistant TB

FQ…… Fluoroquinolones

MIC……. Minimum Inhibitory Concentration

SM…… Streptomycin

KM…… Kanamycin

AMK……. Amikacin

LFX ……. Levofloxacin

MFX…… Moxifloxacin

CIP……... Ciprofloxacin

AB…… Antibiotic

NP……. Nanoparticle

His ……Histidine

Asp …… Aspartic acid

Asn …… Asparagine

Val ……Valine

Ser …….Serine

Ala …….Alanine

ADME ……. Absorption, Distribution, Metabolism and Excretion

CHAPTER 1

1.1 Background and Introduction:

1.1.1 A historical look at TB:

Humans are susceptible to a wide range of bacterial infections, which can result in several health problems. To assist battle these disorders, many medications are currently available on the market. Mtb is responsible for TB, which is one of the leading causes of death globally [1]. Tuberculosis (TB) is the second most infectious disease worldwide after Human Immunodeficiency Virus (HIV) AIDS. The capacity of Mtb to stay in the latent phase for decades shows that the bacteria has persisted in the human host for a long time and may have evolved alongside [2]. According to studies, skeletal malformations with peculiar TB traits were discovered in Egyptian mummies circa 2400 BC. Interestingly, ancient scientists recognized the pulmonary form of TB and its infectious nature TB was present in Europe in the late VIII-XIX centuries, according to archaeological and pharmacological data [3]. Malnutrition, overcrowding, and poor sanitation were all risk factors for illness epidemics during the Industrial Revolution (during the 18th century). TB became widespread during this time as the mortality rate increased to 900 fatalities per 100,000 persons each year. Many researchers then attempted to learn more about the condition. In 1882, however, Robert Koch was able to isolate tubercle bacillus, inoculate it in animal serum, and effectively recreate the sickness in a susceptible host. He discovered tuberculin from tubercle bacteria while continuing his MTB research. He believed that administering tuberculin could stop MTB from multiplying. Finally, in 1905, Robert Koch received the Nobel Prize in Medicine for finding TB [4].

1.1.2. Epidemiology of TB:

The rising death rate is a severe concern to public health because MTB has been there for thousands of years [5]. Each year, over 1.7 million people die from TB, with 80 percent of infections occurring in developing nations. According to a WHO report, 2021, it is estimated that around 10 million people were infected with TB while a total of 1.5 million people died in the year 2020 [6].

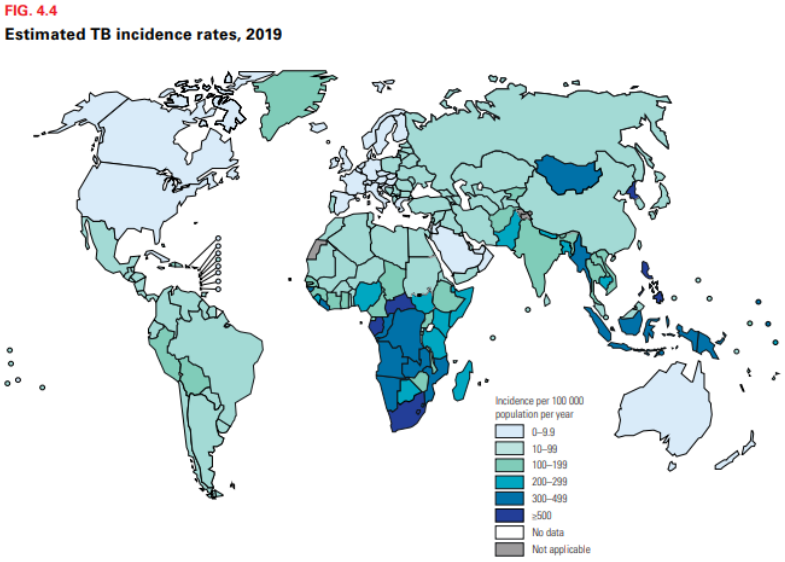


Fig 1.1. Epidemiology of TB: Global tuberculosis report 2021[6]

1.1.3. TB transmission and pathophysiology:

Mtb is spread through the air as droplet nuclei from an infected person to an uninfected person [7]. After being transmitted in small droplets, tubercle bacilli infect the lungs and reach the alveolar gaps. If the infection is not eradicated by the host's immune system, the bacilli grow inside alveolar macrophages and eventually kill the cells.

Infected macrophages produce chemokines and cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1B, IL-6, IL-12, IL-15, IL-18, and interferon (IFN)-γ, which attract additional phagocytic cells such as alveolar macrophages,

monocytes, and neutrophils to form the tubercle, a tubular granulomatous structure. Until a viable cell-mediated immune (CMI) response occurs, the bacilli will continue to grow [8] Antigen-presenting cells present antigen to CD4+ T-cells in a complex with the MHC class II complex, inducing cytotoxic T cell production to kill bacteria. Stress in the endoplasmic reticulum (ER) activates downstream signaling pathways, causing apoptosis of the infected host cells. Autophagy also plays an important role in naturally eliminating the pathogen. However, there are lesions formation- if bacterial replication cannot be controlled, thus bacteria remain in the dormancy stage [9]. Mtb has discovered a variety of ways for suppressing macrophage activation. Inhibition of phagocytic action, which can change the acidic environment of the lysosome, is one of the escape mechanisms. It also suppresses autophagy produced by the host cells and neutralizes the host oxidative stress response [9].

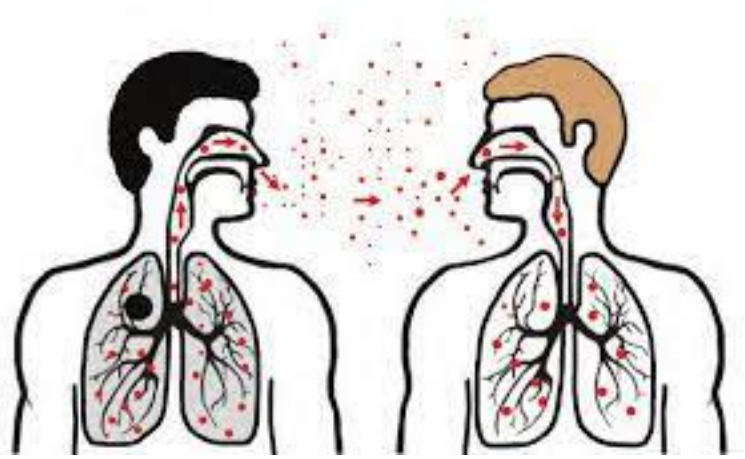


Figure 1.2 Schematic representation of transmission of MTB

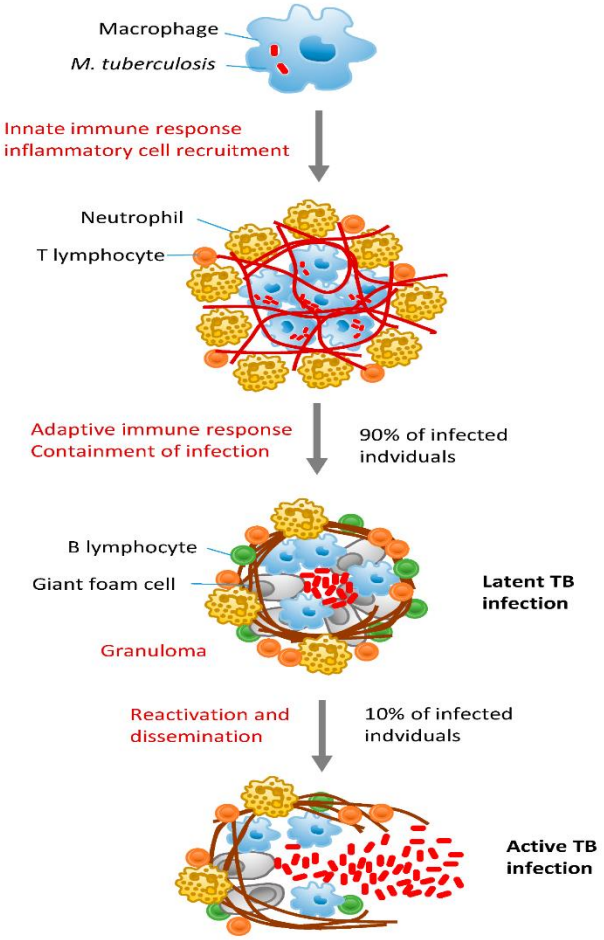


Figure 1.3 Schematic representation of pathogenesis of MTB [10]

1.2 Statement of the problem:

Even though TB has been around for over a century, the only vaccine created to combat it is Bacille Calmette-Guerin (BCG) [10]. The mortality rate of TB is still a major concern. Because of the number of antibiotics employed and the relatively long course of treatment, drug-related toxicity is an undesirable side effect of this TB treatment. Occasionally, the severity of a situation can be exaggerated when treatment is discontinued before completion of the medication course. In Mtb strains, this frequently results in resistance. To avoid being killed by anti-TB drugs, Mtb has been discovered to use a variety of molecular pathways. The development of drug efflux pumps, drug-resistant lipid-rich membranes, the emergence of

mutation in Mtb genes, etc. is some examples [11]. Several Mtb strains exhibit different type of drug resistance. They can be classified as:

1) Multidrug-resistant tuberculosis: Mtb strains that are resistant to at least isoniazid and rifampicin (most potent first-line anti-TB drugs) are known as MDR-TB [12].
2) Extensively drug-resistant tuberculosis: MDR-TB strains that are resistant to fluoroquinolones and one of the injectable second-line anti-TB drugs is called XDR-TB [12].
3) Totally drug-resistant tuberculosis: Mtb strains that are resistant to both the first and second line of anti-TB drugs are referred to as TDR-TB [12].

Due to the emergence of resistant strains of Mtb, the survival and persistence of these bacteria in a non-replicating dormant state for a long time in the host is established. The dormant bacteria become active years later after the encounter of initial infection. This relapse of TB is referred to as latent TB (LTBI) [13]. As a result, there is an urgent need for novel therapeutic strategies to be developed. As we live in the antibiotic era, many unique and repurposed medications are being investigated; unfortunately, some drugs fail in clinical trials or have negative consequences on the human body [14].

1.3 Existing treatment strategy to treat TB:

The first discovered antibiotic against TB was streptomycin which was discovered in the year of 1944 [15]. Over time, several other drugs have been discovered and approved by FDA which are categorized under first-line TB drugs, second-line TB drugs, etc. Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), and Ethambutol (EMB) is the first-line quadruple treatment medications that are metabolized mostly by the liver and hence have the potential to be hepatotoxic

[16]. This regimen consists of a mixture of four first-line TB medications (RIF, INH, EMB, and PZA for the first two months, followed by isoniazid and rifampicin for the next four months [17].

1.3.1 First-line TB Drugs:

1. Isoniazid:

Even though the INH molecule is simpler, the mechanism of action against MTB is fairly complicated. INH primarily targets the production of mycolic acid. With the help of catalase-peroxidase, the pro-drug INH is activated (KatG). Furthermore, the activated form establishes a strong interaction with the ACP reductase InhA, an enzyme implicated in the elongation of fatty acids in Mtb, restricting the synthesis of fatty acids. INH-resistant bacteria commonly produce mutations in the KatG and InhA genes. In the same way, as mutations in the InhA gene prevent INH from resisting mycolic acid synthesis, mutations in the KatG gene prevent INH from binding with pro-drug isoniazid [18].

2. Rifampicin:

The most potent antibiotic among all the 1st line drugs, rifampicin, was licensed by the FDA in 1971. It inhibits Mtb's DNA-dependent RNA synthesis by blocking DNA-dependent RNA polymerase. Rifampicin inhibits the transcription of RNA by binding to the pocket of RNA polymerase β subunit within the DNA/RNA channel, thus preventing the RNA synthesis by blocking RNA elongation and further preventing the synthesis of bacterial proteins [19].

3. Pyrazinamide:

PZA is employed for a short course of anti-TB chemotherapy. It becomes active when it is exposed to an acidic environment. inhibits the production of mycolic acid in semi-dormant

bacteria. The enzyme pyrazinamidase (PZase) converts PZA (pro-drug) to active pyrazinoic acid (POA) in the presence of an acidic environment. The enzymes FAS-I and FAS-II, which are involved in the synthesis of fatty acids, are inhibited by this active form [18].

4. Ethambutol:

The mechanism of action of EMB is to interfere with the biosynthesis of the cell wall component arabinogalactan. EMB hinders the transfer of mycolic acid to Mtb's cell wall and eventually limits arabinogalactan biosynthesis. When used in combination with other drugs, EMB is more effective. It's a bacteriostatic drug [19].

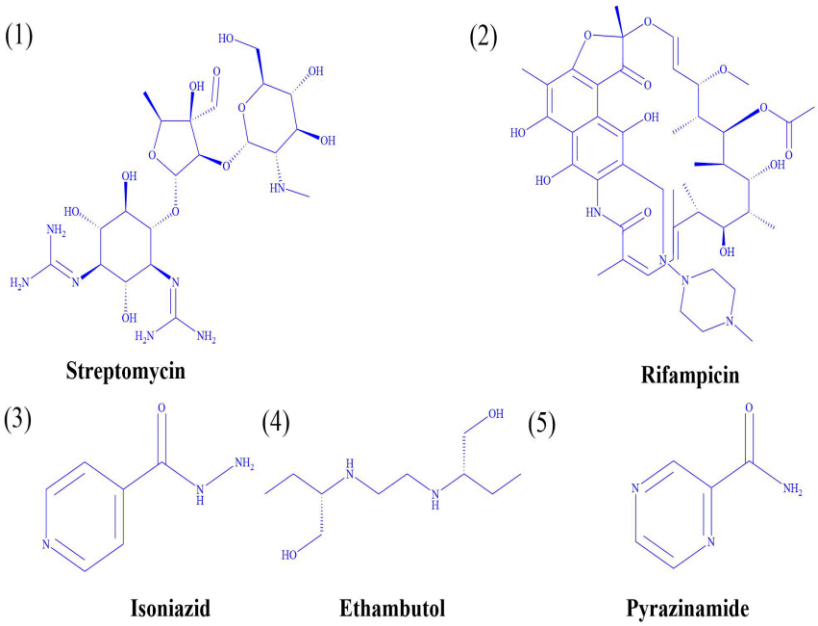


Fig 1.4 First line anti-TB drugs

1.3.2 Second-line TB Drugs:

One of the most critical factors contributing to slow treatment response and the development of drug resistance has been identified as pharmacokinetic (PK) variability [20]. Pharmacokinetics is the study of the relationship between a drug's plasma profile and its dose. Individual changes in a drug's absorption, distribution, and elimination pathways

cause pharmacokinetic (PK) variability. To treat drug-resistant TB, the second line of TB drugs has been developed [20].

1. Fluoroquinolones (FQ):

FQ prevents replication fork movement and transcription by blocking topoisomerase 2 (DNA gyrase). Ciprofloxacin, levofloxacin, cycloserine, clofazimine, moxifloxacin, and gatifloxacin are some of the antibiotics that fall in this category, available in the market. [17].

2. Injectable:

These drugs show an inhibitory effect on protein synthesis (translation). Capreomycin (CAP) and amikacin (AMK) are examples of second-line anti-TB injectables [21].

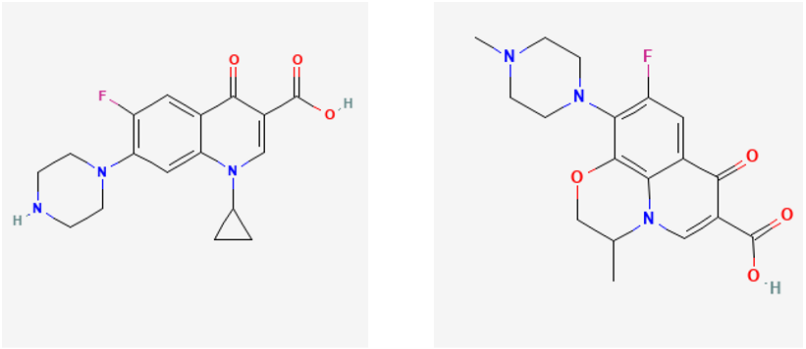


Fig 1.5 Second line anti-TB drugs

1.4 Need of the hour (New Drugs):

The major drawback with the current TB chemotherapy is that when a drug is administered orally or intravenously, it is dispersed throughout the body via the systemic blood circulation as a result majority of the drugs do not reach their target and remain in the body creating undesirable side effects [22]. As these drugs have a short plasma life, they eliminate rapidly from the system thereby limiting their effectiveness [23]. Hepatotoxicity is another drawback that arises due to the administration of conventional anti-TB drugs. The

pharmacokinetics study of the anti-TB drugs has also demonstrated Pharmacokinetics Variability that contributes to slow treatment response and development of drug resistance [24]. To address these challenges faces by anti-TB medications and restore the TB treatment success rate, we need new TB drug strategies that can overcome these obstacles [25]. Since there are few newly produced drugs, and several are still in clinical studies, to deal with the side effects produced by the conventional anti-TB drugs, the drug delivery mechanism must be modified. Targeted drug delivery and point-of-care diagnostics may aid in the control of TB [26], [27]. The nanoparticle-based drug-delivery technology is now being improved in the treatment of TB. The method is based on decreasing drug dosage, limiting adverse effects, and slowly and carefully releasing the drug [28]. Although some specific nanoparticle (carbon tubes, iron oxide NP) drug delivery has been demonstrated to be more effective, several *in-vitro* investigations have revealed detrimental effects on macrophages. Specifically, iron oxide NPs show observable toxicity against murine macrophage cells, human hepatocellular carcinoma cells, human macrophages, and rat mesenchymal stem cells [29]. Thus, the drug carrier must be safer, biocompatible, high efficacy, controlled, and targeted drug release phenomenon. MOFs are potential drug carrier candidates because they possess all of the desired structural characteristics. Several studies have also demonstrated the antibacterial properties of MOFs along with the targeted delivery of drugs [14].

Another major growing concern regarding TB treatment is the emergence of drug-resistant Mtb strains. The development of biofilm by the Mtb provides protection against the antibacterial drugs [30]. Several drug target proteins are mutated by introducing mutations in the cell wall when treatment is

discontinued before completion of the medication course [31]. This leads to the formation of drug resistance Mtb strains. For example, some Mtb strains cause a mutation in the rpoB gene (which codes for the β subunit of DNA-dependent RNA polymerase), leading to protein synthesis and bacterial survival [32]. Traditional drug discovery involves the determination of protein structure, its cloning, expression, and purification, which is a time-consuming process [33]. Similarly, crystallization procedures have methodological and technical challenges that might delay or obstruct crystal formation [34]. To overcome the hurdles of finding novel anti-TB drugs, predictive methods like homology modeling can be used. For decades, biochemical researchers have used computational approaches to predict protein structure and ligand-protein interactions [35]. First, homology modeling was used to create the mycobacterial cell wall proteins, which were then optimized using MD simulations. The stability and trustworthiness of the 3D structure were proved. Molecular docking was used to determine the ligand binding mechanisms of putative phytochemical binders based on the 3D structure [36].

1.5. Scope and Objective of my study:

Our research aims to synthesize and characterize isoniazid (INH) encapsulated metallic organic frameworks (MOFs), as well as investigate their biological properties. For this, first, we have synthesized Cu-MOFs followed by the encapsulation of one of the first-line anti-TB drugs, INH, into synthesized Cu-MOFs. In addition, the antibacterial activity of the INH@Cu-, MOF has been investigated along with the cellular toxicity. Our studies have demonstrated the synthesized MOFs as a potential drug delivery vehicle for INH.

We have also performed homology modeling to create the mycobacterial cell wall proteins, followed by predicting binding pockets. Further, we have used molecular docking to determine the ligand-binding mechanisms of putative phytochemical binders based on the 3D structure. Finally, we have determined the ADMET properties of potential drug molecules.

1.6. Research Questions:

What are MOFs?

Metal-organic frameworks (MOFs) are a type of porous crystalline material with an endless network structure made up of multitopic organic ligands and metal ions or metal clusters. These are also known as porous coordination polymers (PCPs) [37].

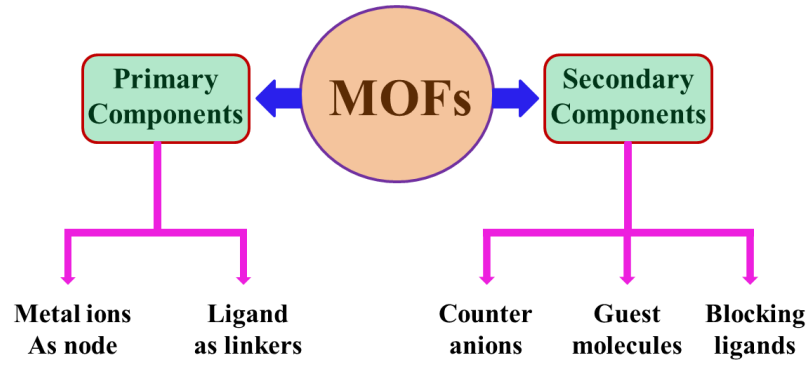


Fig 1.6 Components of MOFs

These frameworks are perfectly regular, highly porous have a large surface area, adjustable chemical composition, and are well designable [38]. Due to their plentiful active sites and biodegradability, they are frequently employed in a range of sectors, including chemical applications such as fluorescence-based and gas storage catalysis, as well as producing new nanomedicines for treating diseases and biosensors [39]. Even though many MOFs have been synthesized, only a few have been utilized in drug delivery because of their large drug loading capacity and long-term sustainable drug releasing

properties [40]. To create better-regulated release along with therapeutic impact, MOFs must be more stable in the physiological environment and contain a specific functional group or large pore aperture to coordinate and encapsulate drugs [41].

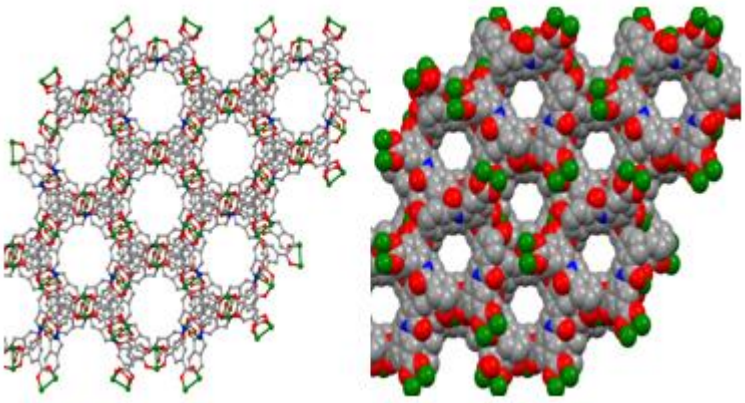


Fig 1.7 Representations of MOFs

Cu(II)/Cu(I)-based MOFs are biocompatible, have a strong SERS (Surface-Enhanced Raman Scattering) impact, possess high loading capacity, and have photothermal characteristics [41]. As a result of these characteristics, copper-based MOFs are the best candidate for nanomedicine. Copper-based MOFs also have good biological characteristics as the two-dimensional copper-based metal-organic frameworks were used for nanomedicine development in recent times [42].

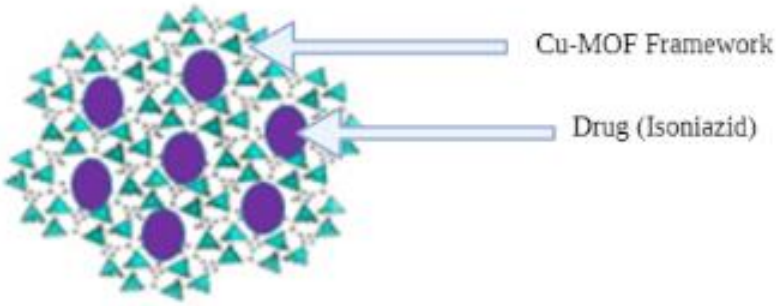


Fig 1.8 Representation of drug encapsulated Cu-MOF

What is Virtual screnining and molecular docking?

Structure-based virtual screening is a common computational tool for identifying novel and potential lead drugs that can be employed as inhibitors for a specific target [43]. The *in-silico* method of predicting the binding energy between a target and a molecule is known as molecular docking. The docking method entails predicting the conformation and orientation (or posing) of ligands within a certain binding site. In general, docking investigations have two goals: accurate structural modeling and precise activity prediction. It can also be utilized for performing virtual screening to discover novel potential anti-TB drugs [44].

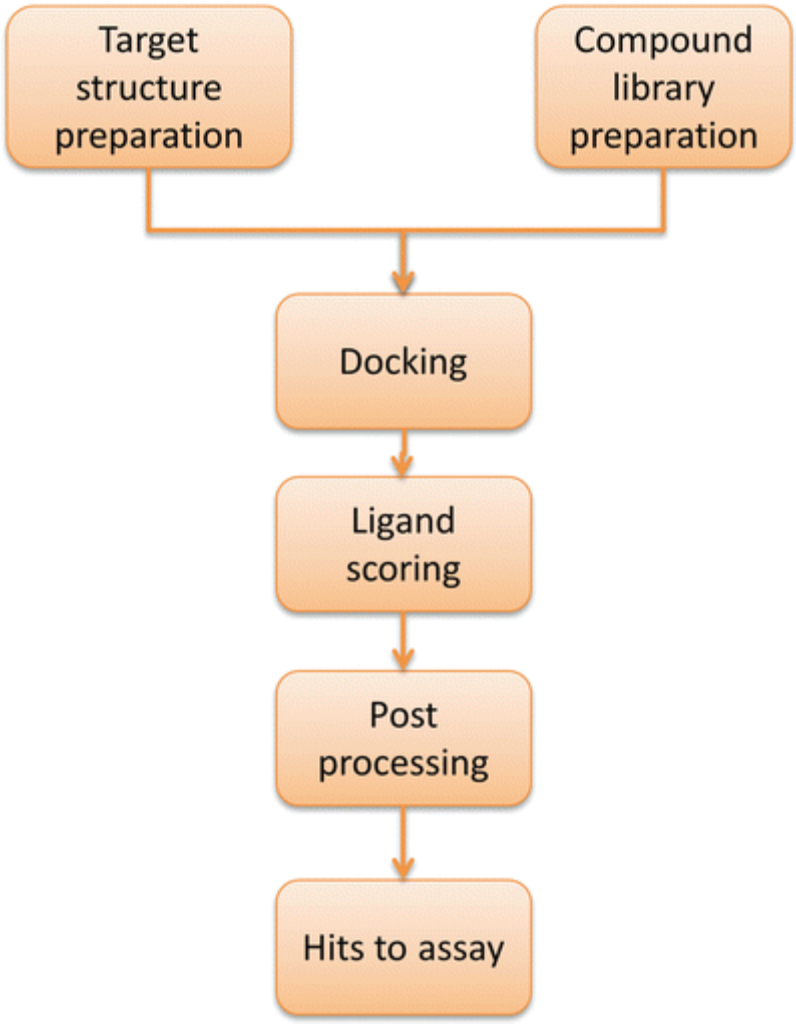


Fig 1.9 Workflow of virtual screening for drug discovery

1.7. Significance of our study:

A thermally stable porous 3D Cu-MOF.was synthesized in a collabaraitive work with Dr. Shaikh M Mobin in department of chemistry. The same Cu-MOF had been utilized for the encapsulation of INH. The antibacterial activity and the cellular toxicity of INH@Cu-MOF was further demonstarted. In the other part of our work, we were able to screen some of the potential anti-TB drugs from the given list of phytochemicals. The absorption distribution, metabolism and excretion (ADME) of the potential phytochemicals was also analyzed.

Low efficacy, non-targeted drug release, drug-resistant strains, and many other detrimental effects are all problems with traditional chemotherapy [22], [23], [24]. MOFs, on the other hand, exhibit targeted drug delivery, systemic drug release, and a large drug-loading capacity [16]. The chemical and structural characteristics of MOFs reveal that larger pores can load drugs at a higher dose. It releases more drugs at a lower pH, i.e., an acidic environment that makes it easier for a drug to reach its target locations in disease. Again, drug release at the target region may not harm neighboring cells, lowering the risk of cytotoxicity. These characteristics highlight the necessity of employing MOFs as a medication carrier in the treatment of TB.

Thus, with these unique properties of MOFs, the major problems for conventional chemotherapy can be overcome making them a promising class of drug delivery vehicle for the sustainable release of drugs along with effective antibacterial properties against intracellular pathogens. In this study, we have synthesized anti-Tb drug encapsulated Cu-MOF to fulfill the above-mentioned criteria for being a better anti-TB drug delivery system.

Further, with the increase in the emergence of novel resistant forms of Mtb, there is a constant need to discover novel antibacterial drugs. Traditional drug discovery has many drawbacks. We believe our

study may provide novel potential drug molecules that can be studied for use as anti-TB drugs.

Chapter 2

Materials and Methods:

2.1 Materials

2.1.1 Bacterial strains and reagents:

Mycobacterium smegmatis mc^2155 were grown in Middlebrook's 7H9 broth medium (Difco) containing 0.05% Tween80 at 37^0C at 120 r.p.m. *Mycobacterium smegmatis* biofilm was grown in Sauton's media (Himedia). Isoniazid, Diethyl-5-aminoisopthalate, and 2-Bromomethylbenzonitrile were purchased from Sigma Aldrich, and 2-Methyl imidazole, Crystal Violet from MP bio medicals. DMF, HCl, and acetone were purchased from Spectrochem. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from SRL.

2.2. Methods:

2.2.1. *In-vitro* growth curve of *M.smegmatis*:

A single colony of *M.smegmatis* was inoculated in 3 mL of 7H9 media. O.D. of the bacterial culture was set to 0.05 by making required dilutions. The culture was incubated at 37˚C in a shaker at 120 r.p.m for 36h. 1% of the grown bacterial culture was sub-cultured in 100mL of 7H9 broth. The optical density (OD_{600}) was measured every 12 hours to determine *in-vitro* growth [45].

2.2.2. *In-vitro* killing assay:

Mid-log phase grown culture of *M.smegmatis* was pelleted, washed with 1X PBS and the $O.D_{600}$ was adjusted to 0.1. Different concentrations of Cu-MOF, INH@Cu-MOF, and INH (1-25) µg/ml were incubated with 0.1 OD bacteria for 24h. Next, the incubated bacteria with different drug molecules are serially diluted up to 10^{-5} and plated for colonies to form. After 36h, bacterial colonies were counted and CFU plotting was done using GraphPad Prism [46].

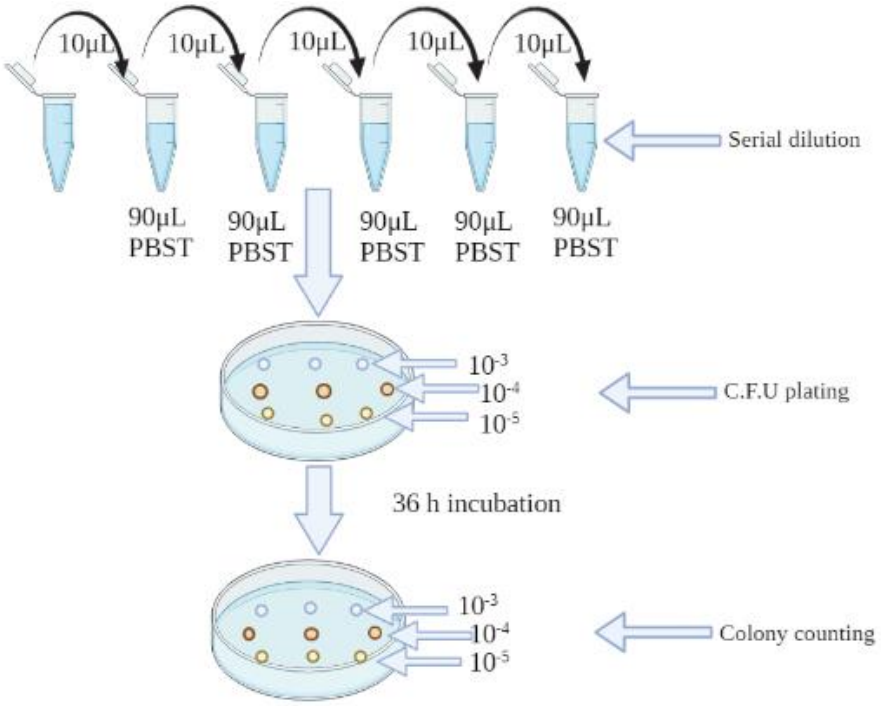


Fig 2.1 Representation of C.F.U counting

2.2.3 Agar diffusion assay

Mid-log phase grown culture of *M.smegmatis* was pelleted, washed with 1X PBS and the $O.D_{600}$ was adjusted to 0.5. The suspended culture (100 µL) was evenly spread on 7H9 plates and incubated for 30 minutes at 37°C. Different concentrations of Cu-MOF, INH@Cu-MOF, and INH (1-25) µg/ml were incubated with 0.5 OD bacteria for 24h. After 24 hours, the diameter of the zone of inhibition was measured to determine the zone of inhibition. [47].

2.2.4 Biofilm formation assay:

Mid-log phase grown culture of *M.smegmatis* was pelleted, washed with 1X PBS, and adjusted to OD_{600} 0.5. 10µg/ml concentration of Cu-MOF, INH@Cu-MOF, and INH (1-25) µg/ml was incubated with 0.5 OD bacteria in a 12-well microtitre plate for 5 days. After 5 days, the media was discarded carefully with the help of a 1 mL syringe without disrupting the biofilm formed. The unattached cells were removed by washing the wells with distilled water. 0.01% Crystal violet (MP bio medicals) was then added to the wells for staining the cells for 1 h. After 1h of incubation, crystal violet was discarded with the help of a 1 mL syringe. Stained cells were washed with the help of

distilled water to remove the unbound dye. Stained cells were resuspended in a dissolving reagent (Ethanol: Acetone:: 70:30) for 15 minutes to remove the remaining dye. The visualization of the stained biofilm was done under a light microscope [48].

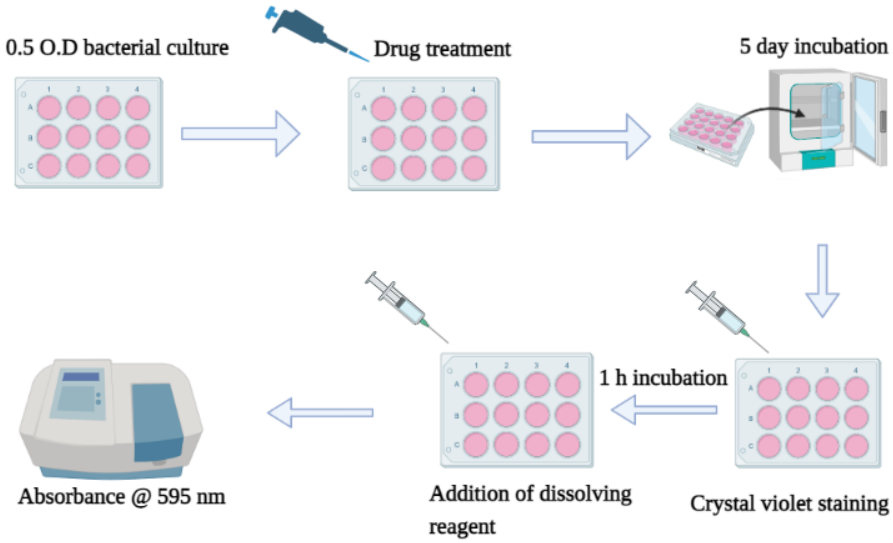


Fig 2.2 Schematic representation of biofilm formation assay

2.2.5 Biofilm disruption assay:

Mid-log phase grown culture of *M.smegmatis* was pelleted, washed with 1X PBS and the $O.D_{600}$ was adjusted to 0.5. Bacterial cultures were incubated in a 12-well microtitre plate for 5 days. After 5 days, the mature biofilm was formed. The media was discarded carefully with the help of a 1 mL syringe without disrupting the biofilm formed. The mature biofilms were treated with a 10µg/ml concentration of drugs and incubated for 24 h. After 24 h, the unattached cells were removed by washing the wells with distilled water. 0.01% Crystal violet (MP bio medicals) was then added to the wells for staining the cells for 1 h. After 1h of incubation, crystal violet was discarded with the help of a 1 mL syringe. Stained cells were washed with the help of distilled water to remove the unbound dye. Stained cells were resuspended in a dissolving reagent (Ethanol: Acetone:: 70:30) for 15 minutes to remove the remaining dye. The visualization of the stained biofilm was done under a light microscope [48].

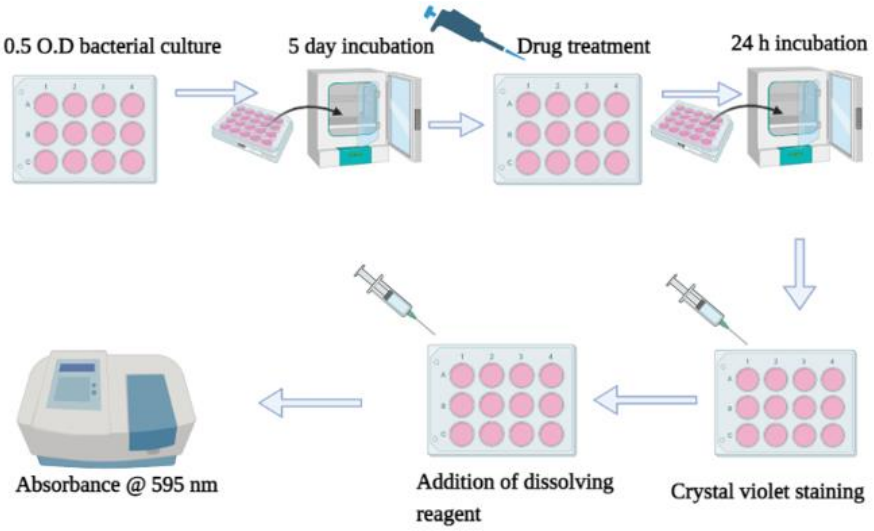


Fig 2.3 Schematic representation of biofilm disruption assay

2.2.6 Cytotoxicity assay:

To check the *in vitro* toxic effects of Cu-MOF, INH@Cu-MOF, and INH on mouse macrophages, RAW 264.7, MTT assay was performed. RAW 264.7 cells ($2x10^4$ cells/well) were seeded and grown in Dulbecco modified Eagle medium (DMEM) in a 96-well plate at 37℃, 5% CO_2 for 24 h. The cells were then treated for another 24 hours with various concentrations of Cu-MOF, INH@Cu-MOF, and INH. In order to measure cell viability, MTT (0.1 mg/mL) was then added to the wells and incubated for 4 hours in the dark. It was followed by dissolving these formazan crystals in a dissolving buffer (11 g SDS in 50 mL of isopropanol and 50 mL of 0.02 M HCl). It was followed by measuring the absorbance at 570 nm in the microplate reader [49]. The percentage of viable cells was calculated by comparing it with the untreated cells. The morphology of the cells was visualized under a light microscope 24 hours after treatment [50].

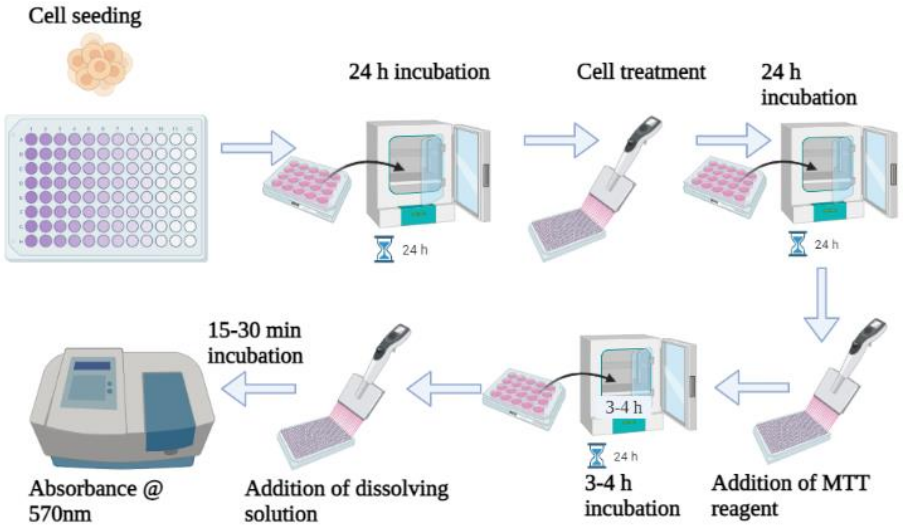


Fig 2.4 Schematic representation of MTT assay

2.2.7 *In silico*-based structure and function characterization:

The crystallographic structure of MTB cell wall protein polyketide synthase (PDB ID:1Q9J) was extracted from the protein data bank (PDB) database [51]. Using the PredictProtein tool, we analyzed the secondary structure of the mycobacterial cell wall protein polyketide synthase to determine the composition of sheets, helices, and random coils responsible for forming hydrogen bonds between the oxygen of the carboxyl group and hydrogen of the amine group [52].

2.2.8 Molecular docking study with the phytochemicals:

According to the previous studies, many phytochemicals have shown antibacterial activity in different classes of bacteria. Therefore, molecular docking studies were performed to check if any phytochemical binds to the cell wall protein of mycobacterium. For performing the molecular docking study, AutoDock Vina software was used [53], [54]. Molecular docking studies were performed in three steps:

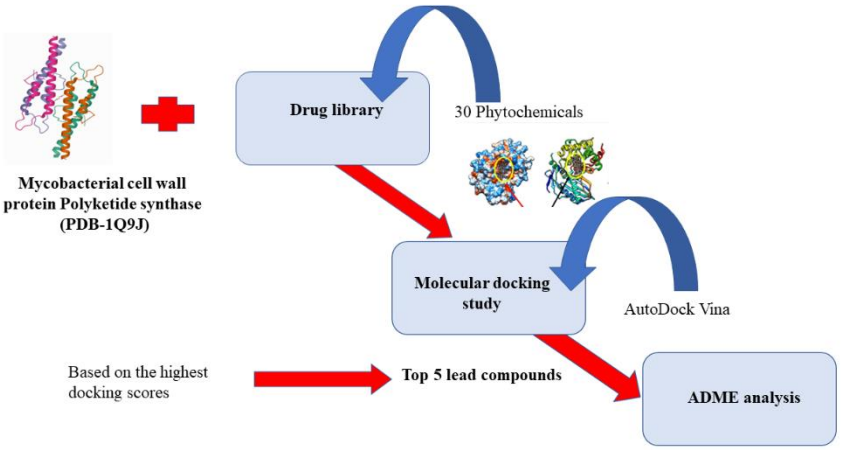


Fig 2.5 Schematic representation of virtual screening

2.2.9 Prediction of binding cavities:

The prediction of the ligand-binding site present in the mycobacterial cell wall protein was done using the CASTp web server [55], [56]. This tool provides information regarding the identification as well as the measurement of the surface accessible pockets and the inner inaccessible pockets. It gives the surface area and volume of each pocket, as well as the amino acid residues that line them. Firstly, the CASTp web server was opened. Then the PDB ID of the protein of interest was entered.

2.2.10 Ligand and protein file preparation:

Since, while performing molecular docking studies the ligand and protein files should be in PDB format, hence in this step both the protein and ligands were saved in PDB format. The two-dimensional structure of the protein was downloaded in PDB format from Protein Data Bank [51]. Polyketide synthase is a dimer protein. As a result, in the screening and selection of drug candidates, Pymol was used to analyze a single chain from the dimer protein [57]. This single-chain protein was then saved in a PDB format of the protein. Firstly, the PubChem was used to download the two-dimensional structure of the ligand [58]. The ligand file's .sdf format was converted to .pdb format using the Open Babel tool [59]. For this, 30 possible anti-TB

compounds were selected from the PubChem database and saved as PDB format file. Later to predict drug-like properties, ADMET properties of top-five lead compounds were assessed using the Molinspiration tool [60], [55].

2.2.11 Molecular docking:

Firstly, both the ligand as well as protein file weres converted to the PDBQT file. The AutoDock Tools interface was opened first, followed by the PDB format protein file. The protein file was prepared by adding polar hydrogen atoms and kollman charges and saved in PDBQT format. Then the ligand file was converted into the PDBQT format. For this, the PDB format of the ligand file was open and it was followed by detecting the root in the torsion tree tab. Next, the GPF (Grid Parameter File) was prepared. For this, the name of the protein molecule was selected, and then the necessary residues for docking were selected. Next, a grid box was constructed such that it covers the selected residues, and later the resulting file was saved in GPF format. Then, the protein file in PDBQT format was selected followed by the selection of ligand under the docking tab. The parameters of docking were accepted, and the resulting output file was saved in DPF format. This was followed by the running of vina file in Autodock Vina. Using the defined binding pockets, 30 phytochemicals were docked on the protein. AutoDock Vina was used for docking studies. Then the ligand file was converted to PDBQT format. This was done by opening the ligand file in PDB format and using the torsion tree tab to detect the root. After that, the ligand file was saved in PDBQT format.

2.2.12 Visualization of docked complexes:

Chimera was are used to determine probable binding orientations based on ligand and receptor molecular structures of docked complexes [39]. Firstly, Chimera was opened followed by opening the “Structure/Binding Analysis” tab. Then the “View Dock” tab was opened where the docked files were uploaded [61]. Discovery Studio

2.0 was used to analyze comparative docking acquired with Autodock Vina docking software. DS Visualizer was used for visualizing the docked complexes after docking [62]. Later the individual binding energies of the docked complexes were noted down for better analysis.

2.2.13 ADME analysis:

The analysis of the ADME properties was done to determine the pharmacokinetic profile of the lead compounds. The pharmacokinetic profile includes the absorption, distribution, metabolism and excretion of the compound inside the body. For determining the ADME properties of the lead compound, Molinspiration online tool was used [60], [63]. The " Calculation of Molecular Properties and Prediction of Bioactivity" tab of the Molinspiration server was opened, followed by entering the SMILES format of the drug. Then, the "Calculate Properties" tab was opened

CHAPTER 3

Results:

3.1 *In-vitro* growth kinetics of *M.smegmatis*:

The *in-vitro* growth pattern of *M.smegmatis*. As *M.smegmatis* is slow growing bacteria, hence a minimal growth in the initial time point was observed followed by rise in the growth after 36h onwards. However, a decrease in the absorbance was observed at 60h indicating the less growth of bacteria, followed by an increase in the absorbance showing an increase in the growth of bacteria.

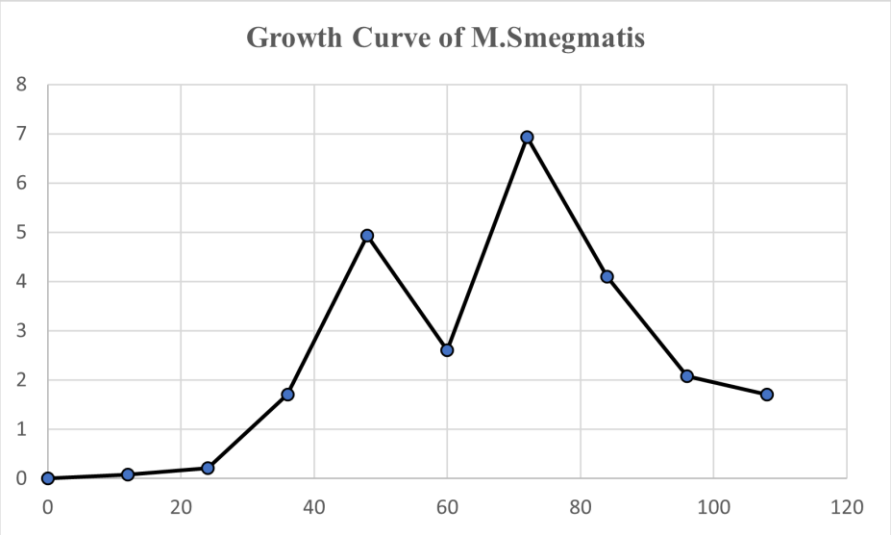


Fig 3.1 Growth Curve of *M.smegmatis*: The growth curve shows different phases such as lag, log, and death phases.

3.2 Antimycobacterial activity of Cu-MOF, INH@Cu-MOF, and INH:

The susceptibility of *M.smegmatis* was determined against Cu-MOF, INH@Cu-MOF, and INH. With the increase in concentrations of Cu-MOF as well as INH@Cu-MOF, a significant reduction in bacterial CFU was observed.(Figure 3.3-A, B) However, INH@Cu-MOF demonstrated effective killing of *M.smegmatis* as compared to the INH (10% loading content).

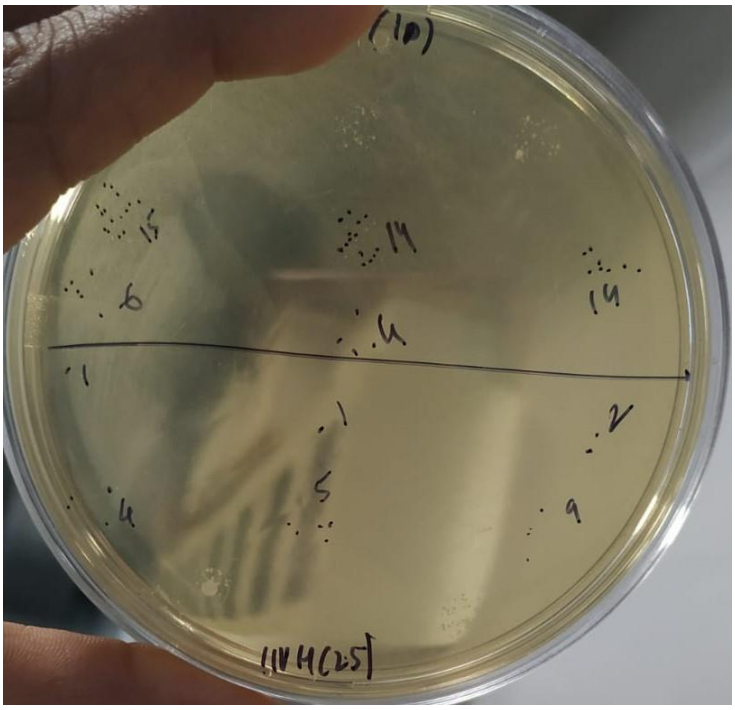


Fig 3.2 C.F.U counting plates.

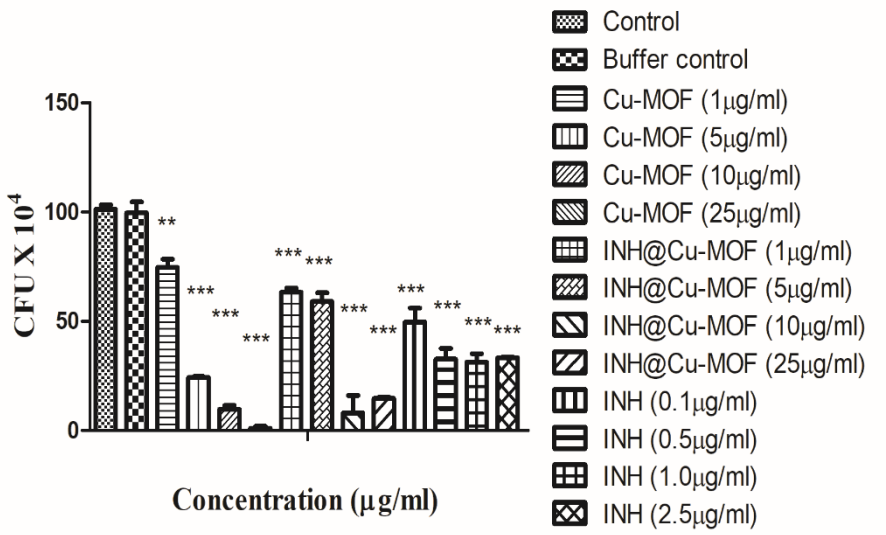


Fig 3.3 A Graph for *In-vitro* killing assay of *M.smegmatis*: Significant killing is observed in INH@Cu-MOF as compared to the INH.

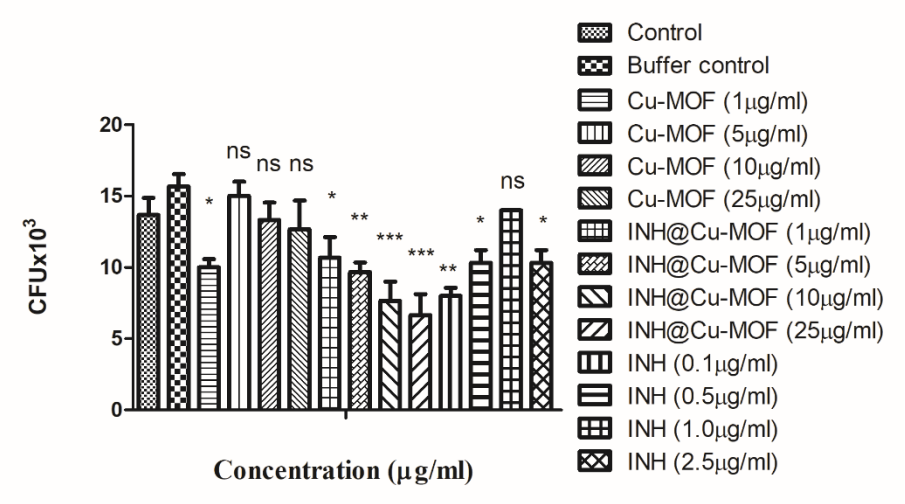


Fig 3.3 B Graph for *In-vitro* killing assay of BCG: Non-significant killing is observed in INH@Cu-MOF as compared to the INH

3.3 Antibacterial activity of Cu-MOF, INH@Cu-MOF, and INH through agar diffusion assay:

For confirming the antibacterial activity of Cu-MOF, INH@Cu-MOF, and INH, another qualitative method was used – the agar diffusion method. 20µL of different drugs were loaded in the well-formed 7H9 agar containing an equal number of bacteria. After 24h of the incubation, plates loaded with Cu-MOF, INH@Cu-MOF showed a clear zone of inhibition of different diameters but the plate loaded with buffer displayed a dense population of bacteria (Fig 3.4).

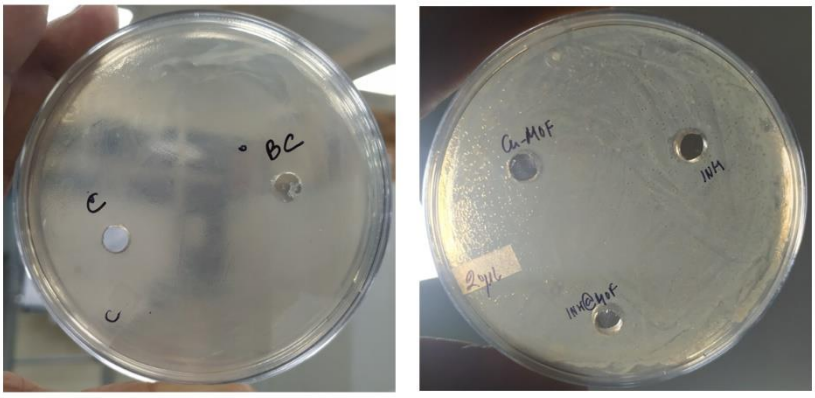


Fig 3.4 Antibacterial activity of Cu-MOF by agar diffusion assay: INH@Cu-MOF showed more diameter of zone of inhibition

Drug concentration	Control	Buffer control	Cu-MOF	INH@Cu-MOF	INH
20 µL	0 cm	0 cm	0.9 cm	1.3 cm	0.2 cm

Table 3.1 Diameter of zone of inhibition in agar diffusion assay.

The presence of a zone of inhibition near the wells of loaded drugs signifies the antibacterial action of respective drugs resulting in no bacterial growth.

3.4 Antibiofilm activity of Cu-MOF, INH@Cu-MOF, and INH during biofilm formation:

The formation of the biofilms by Mtb aids in the development of necrosis and cavity formation in the lung tissue. Also, the presence of biofilm somehow prevents the diffusion of the drug molecule, resulting in the progression of infection; hence, the inhibition of the biofilm formation is crucial to prevent bacterial colonization. The crystal violet dye binds to the adherent biofilm, representing the efficient biofilm formation. Hence it is used as a colorimetric assay to quantify the extent of the biofilm formation or disruption In this investigation, the intensity of crystal violet dye was found to decrease, indicating a considerable inhibition in biofilm formation in the drug-treated bacterial culture compared to the buffer control. Further, a significant antibiofilm activity was shown by INH@Cu-MOF (Fig 3.5)

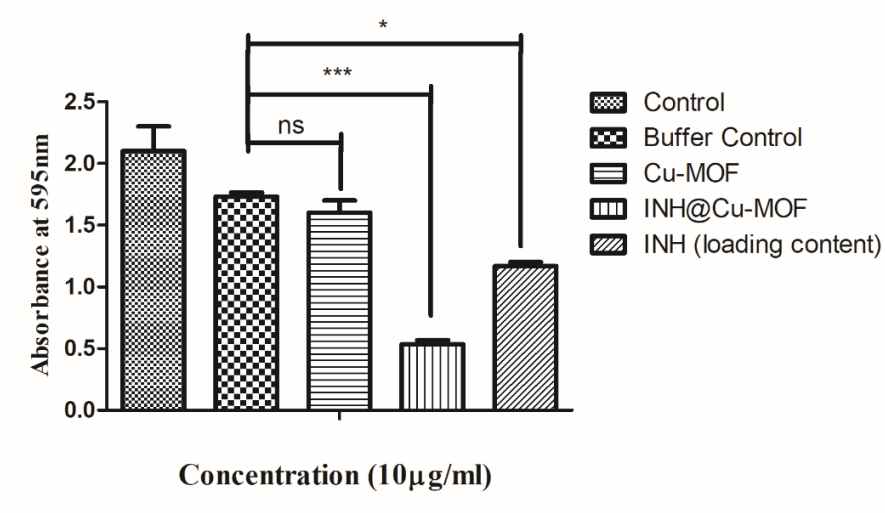


Fig 3.5 Graph of the effect of Cu-MOF, INH@Cu-MOF, and INH on the formation of biofilm: INH@Cu-MOF shows significant reduction in the biofilm formation as compared to INH.

3.5 Antibiofilm activity of Cu-MOF, INH@Cu-MOF, and INH during the disruption of biofilm formed:

In this study, INH@Cu-MOF did not show significant antibiofilm activity however, a comparative biofilm disruption in INH@Cu-MOF was observed as compared to INH treated *M.smegmatis* biofilm which was judtified by the decreased intensity of crystal violet (Fig 3.8).

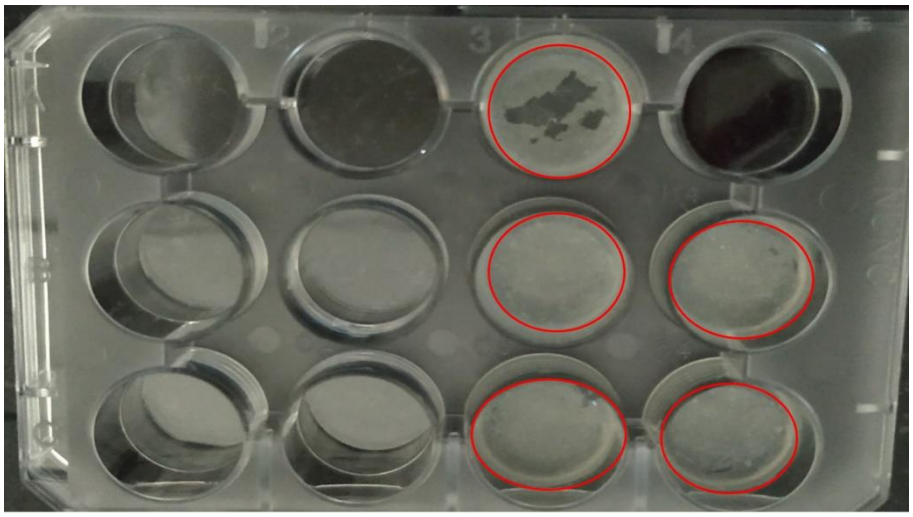


Fig 3.6 Biofilm formation after 5 days of incubation: the encircled (red) wells represent formed biofilms.

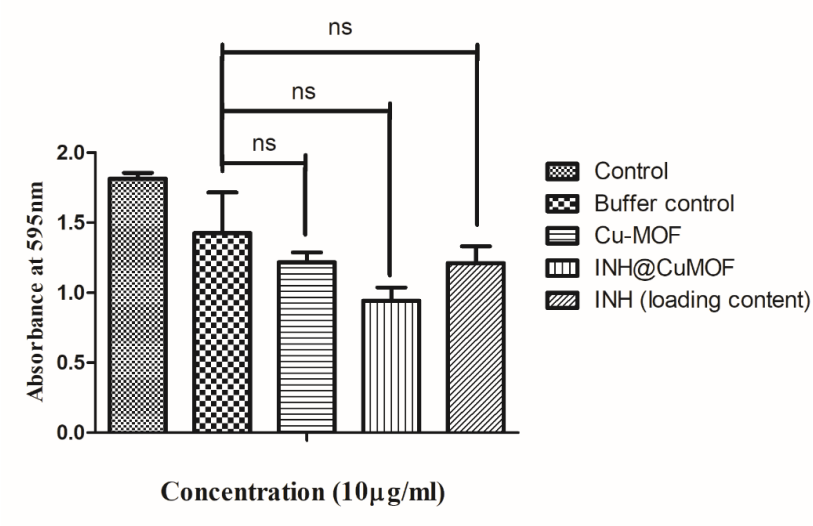


Fig 3.7 Graph of biofilm disruption after 5 days of incubation: INH@Cu-MOF did not show significant antibiofilm activity however, a comparative biofilm disruption in INH@Cu-MOF was observed as compared to INH treated *M.smegmatis* biofilm.

3.6 Less cellular toxicity of INH@Cu-MOF in treated RAW macrophages:

The utmost important property of a molecule to act as a therapeutic agent is that it should not produce a deleterious effect on cell viability. In the present study, our aim was to focus on the concentration of drug that can kill the bacteria without killing the macrophages. A MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide/ tetrazolium salt} assay was performed to determine the cytotoxic effect of Cu-MOF, INH@Cu-MOF, and INH against RAW 264.7 murine macrophages. In metabolically active cells, MTT reduces to an insoluble purple formazan {*N*'-anilino-*N*-[(4,5-dimethyl-1,3-thiazol-2-yl)imino]benzenecarboximidamide}.

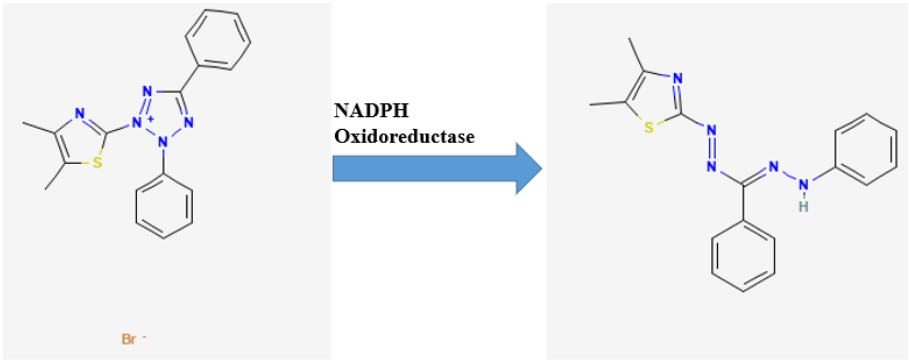
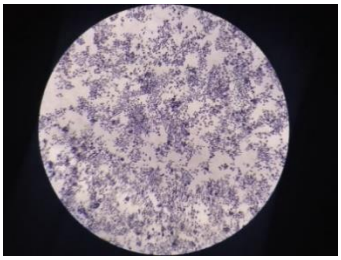
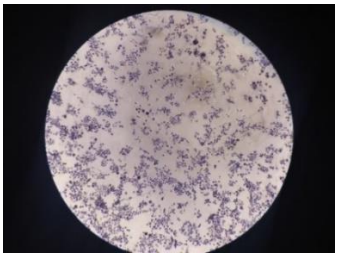


Fig 3.8 Chemical reaction showing reduction of MTT into a insoluble formazan salt by the viable cells.

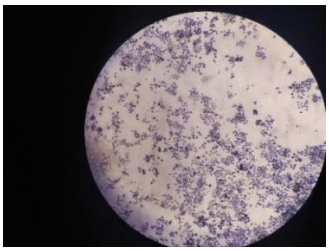
The intensity of the dye at 570 nm varies linearly with the number of viable cells. In other words more the intensity, the more will be the number of viable cells. A concentration-dependent cellular cytotoxicity was observed. As the concentration or the dose of the drug was increased, the intensity of the dye decreased, indicating more cellular toxicity.



3.9 A



3.9 B



3.9 C

Fig 3.9 Visualization of formazan crystals for different concentrations of the drug: with an increase in the concentration of the drug, the intensity of the purple color decreases showing that viability of cells decreases.

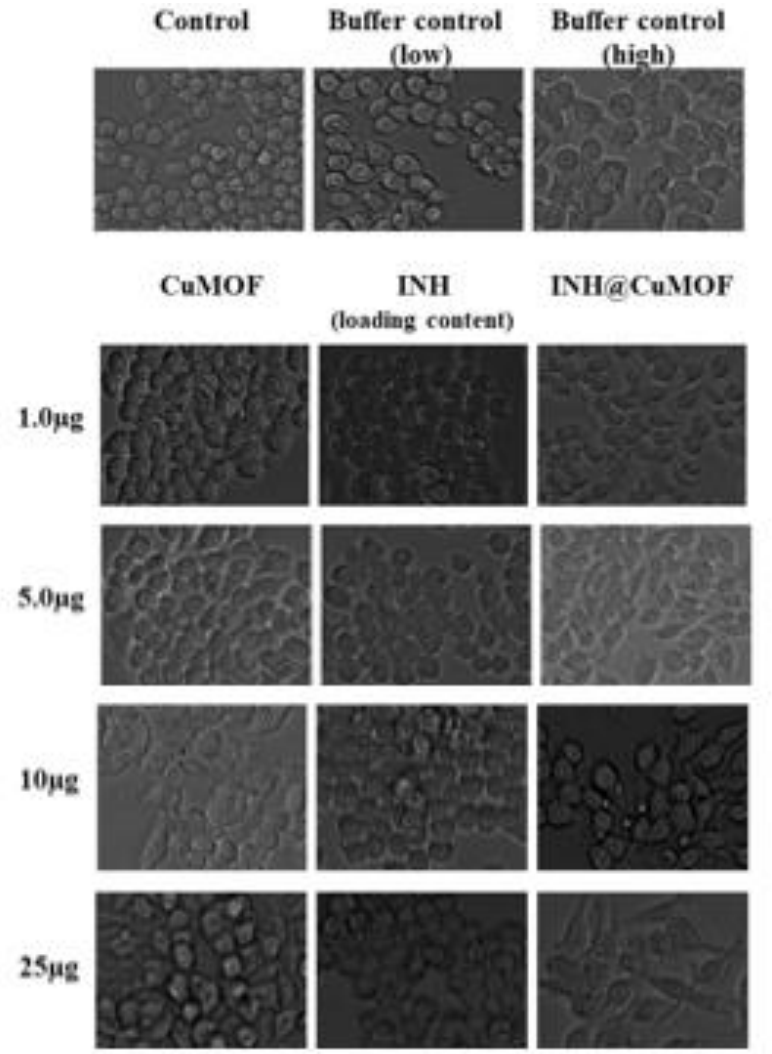


Fig 3.10 Bright-field microscopic images of RAW 264.7 showing comparative cell viability of Cu-MOF, INH@Cu-MOF, and INH.

Later % the viability of cells was calculated by making the control cell viability 100%. Accordingly, the percentage cell viability of the treated cells was determined using the following formula:

$$\% \text{ Cell viability for control} = \frac{\text{OD control}}{\text{Mean OD control}} \times 100$$

$$\% \text{ Cell viability for treatment} = \frac{\text{Mean OD treatment}}{\text{Mean OD control}} \times 100$$

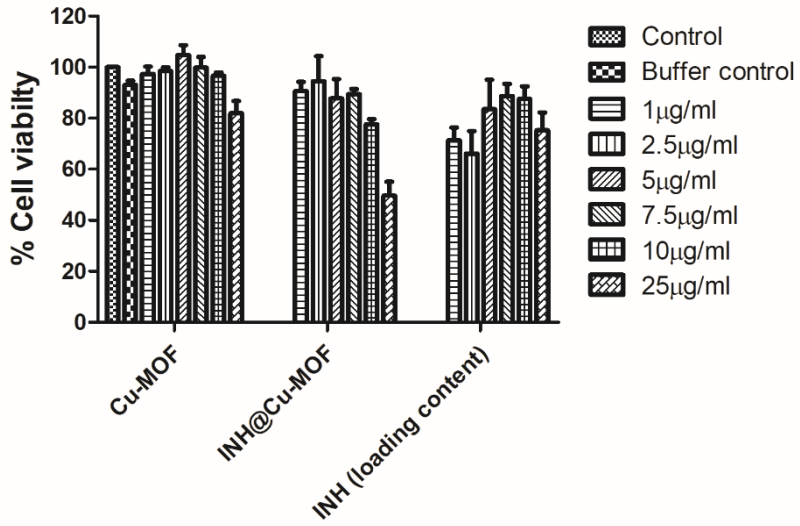


Fig 3.11 Cell viability assay of Cu-MOF, INH@Cu-MOF, and INH on mouse RAW264.7 macrophage cells; With the increase in drug concentration (upto 10µg/mL), no cytotoxicity was observed

A more than 50% cell viability was observed for the RAW cells when treated with all drugs even at a high concentration of 25 µg/mL. Hence, the results from the colorimetric assay suggests that Cu-MOF, as well as INH@Cu-MOF, might not be causing cellular toxicity.

3.7 Analysis of Secondary structure of Polyketide Synthase:

The Protein Data Bank (PDB) was used to obtain the 422 amino acid long sequence of mycobacterial cell wall protein polyketide synthase (PDB ID: 1Q9J). PredictProtein tool was utilized for determining the secondary structure of the protein (Figure 3.8). The analysis of its subcellular localization indicated that it was located in the cell wall. The prediction about the composition of its secondary structure

reveals that it consists of 22% helices, 20% strands, and 58% loops. A change in the direction of the protein chain has been observed as a result of the high content of loops, allowing the folding of protein back on itself. This results in a protein indicating a more compact structure. Due to the high percentage of hydrophobic amino acid residue within the 47% buried region, protein has compact and stable folding.

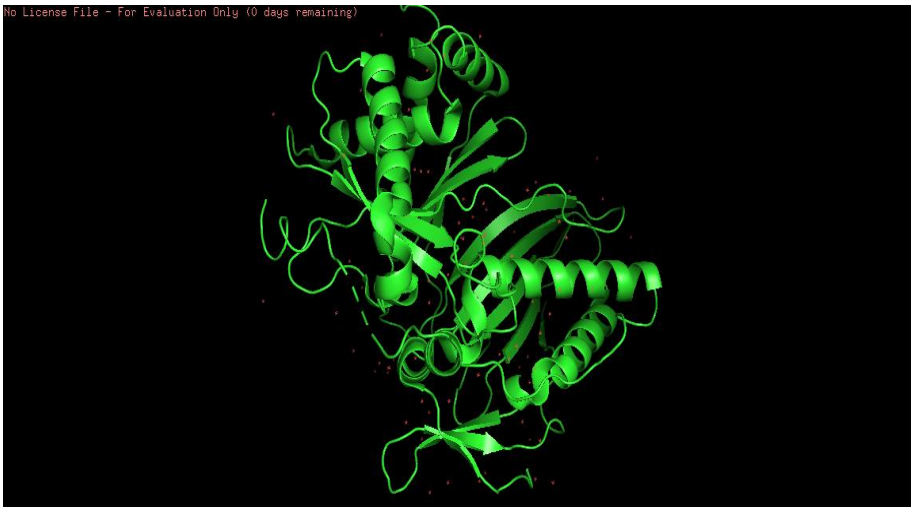
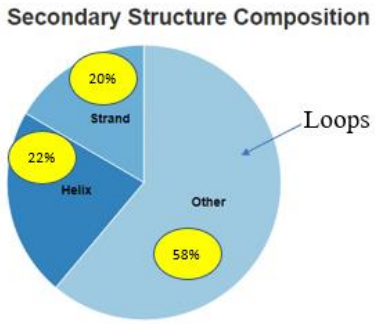


Figure 3.12 Side view of protein visualized by PyMol software

Figure 3.13 A



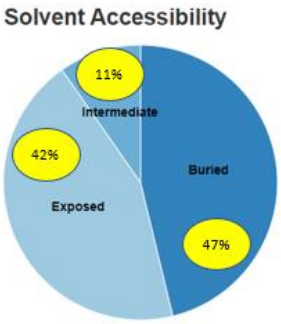


Figure 3.13 B **Figure 3.13 C**

Figure 3.13 Prediction of the secondary structure of *Mtb* cell wall protein polyketide synthase. A, Predicted components of the structure (Light Blue bar: helix, red bar: strand, light blue bar: buried, yellow bar: exposed region) of polyketide synthase has been shown here. Percentage chart of (B) loop, strand and helix and (C) buried, the intermediate and exposed region has been shown

3.8 Prediction of Binding cavities in Polyketide Synthase:

The binding pocket was predicted with the help of the CASTp server. The amino acid residues lining the binding cavity were noted. The docking studies were successfully performed with the help of AutoDock Vina. Then the amino acid residues lining the binding cavity with the highest binding energy were noted. Accordingly, the most common binding site was predicted. Next, the 2-D diagram of the interacting amino acid residues with different bonds were analyzed. The location of the binding cavity was observed at the junction of chains A and B of the protein (Fig 3.14). The binding cavity was found to be formed by 125 amino acid residues. The most common amino acid residues were found to be His, Asp, Ala , Ser and Asn(Fig 3.16).

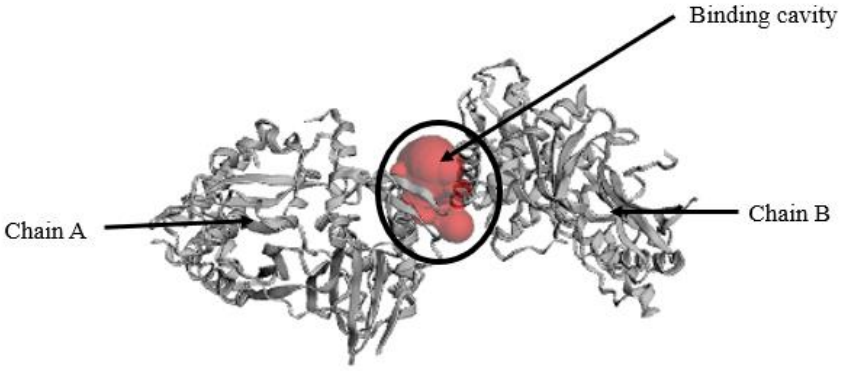


Figure 3.14 Prediction of the binding cavity in polyketide synthase: Binding pocket lies at the junction of chains A and B.

3.9 Potential lead phytochemicals to treat TB:

The interactions related to the relevant phytochemical and active binding site of polyketide synthase, as well as the binding energy, were noted (Table 3.1).

The top five lead compounds with strong binding energy were found to be Rutin, Silymarin, Naringin, Hesperidin, and chicoric acid (Table 3.2). These phytochemicals may act as a potential inhibitor against polyketide synthase. Rutin was found to have strong binding energy (-8.1 kcal/mol).

S.No	Name of ligand	Binding Energy (kcal/mol)	Interacting Amino acids
1.	Rutin	-8.1	Asn; Ser- (H-Bonding)
2.	Silymarin	-7.9	His,Asp,Asn-(H-Bonding)
3.	Naringin	-7.7	Asp,Asn,Ser-(H-Bonding)
4.	Hesperidin	-7.6	His,Ala,Asp-(H-Bonding)
5.	Chicoric acid	-7.3	Val, Asp, Ser, Ala-(H-Bonding)

Table 3.2 List of five lead compounds with their respective docking energy

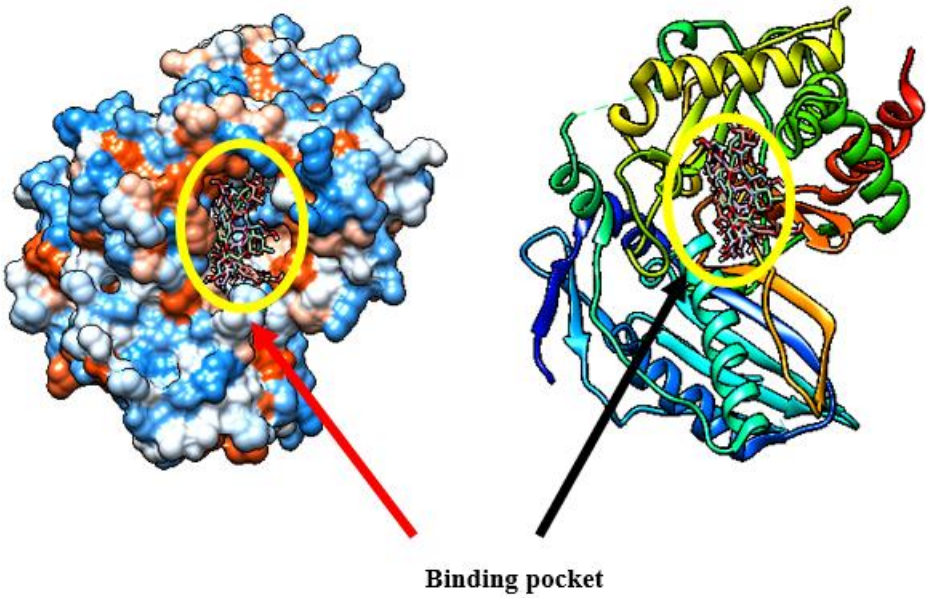


Fig 3.15 Molecular docking analysis of top 5 lead compounds: All the top five lead compounds bind to the binding pocket as indicated by the yellow highlighted region

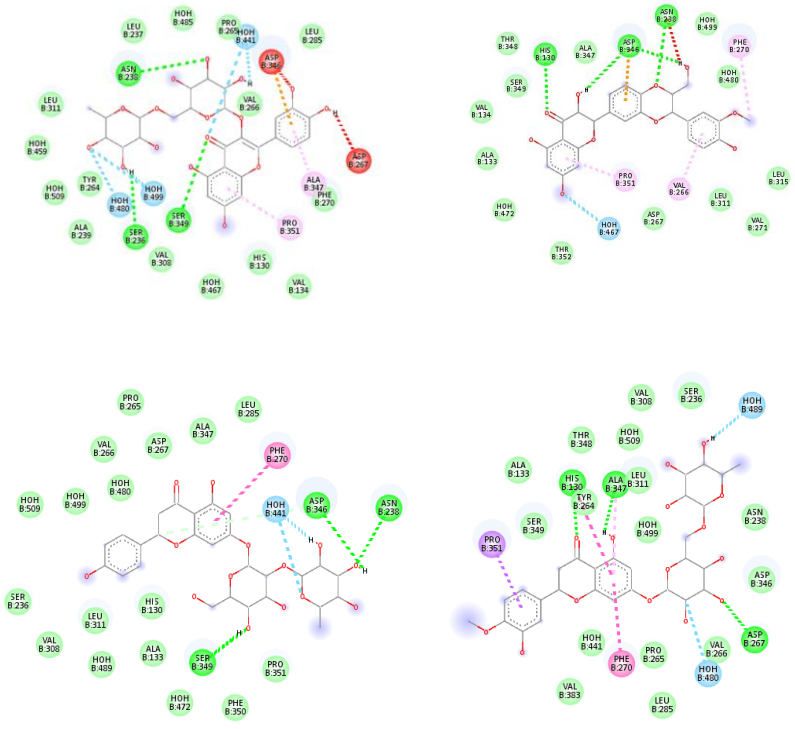


Fig 3.16 2-D diagram representing the interaction between the binding site residues and the ligand: His, Asp, Ala , Ser and Asn residues were observed to interact predominantly with the drug molecules.

3.10. ADME analysis for better drug-like properties of 5 lead compounds:

To study the drug-like properties of five lead compounds (Rutin, Silymarin, Naringin, Hesperidin, and chicoric acid), ADME analysis was performed. The pharmacokinetic properties of five lead compounds were compared using the Molinspiration tool, as shown in table 3.2. According to Lipinski's rule of five, a superior drug candidate should include 10 hydrogen bond acceptors, 5 hydrogen bond donors, miLogP ≤5, and a molecular weight of 500. The silymarin lead compound showed the maximum number of feasible pharmacokinetic properties in accordance of Lipinski's rule of 5. miLogP value determines the lipophilicity as well as the absorption efficiency of the compound. It was found that the miLogP value of (1.47), the polar topological surface area (TPSA) (155.15), molecular weight (482.44) of silymarin are in close agreement with the Lipinski's rule of 5. These pharmacokinetic properties suggest that the potential lead compound may show better cell permeability, bioavailability and absorption. Hence it can be a better drug candidate.

Properties	**Lead-1** (Rutin)	**Lead-2** (Silymarin)	**Lead-3** (Naringin)	**Lead-4** (Hesperidin)	**Lead-5** (Chicoric acid)
miLogP	-1.06	1.47	-0.37	-0.55	1.27
TPSA	269.43	155.15	225.06	234.30	208.12
MW	610.52	482.44	580.54	610.57	474.37
Volume	496.07	400.86	486.25	511.79	385.95

Table 3.3 Analysis of ADME properties of top five lead compounds

CHAPTER 4

Conclusion:

INH is a powerful first-line anti-TB drug; however, due to several side effects, longer treatment duration and the rise of MDR strains, conventional treatment using INH is less efficacious. There is an urgent need to improve TB therapy. Modifying and reengineering traditional chemotherapy can be an effective way to fight TB. MOFs have been found to exhibit antibacterial properties, which could be useful in the fight against TB. (MOFs) have the potential to be used as drug transporters. Cu-MOFs are a good option for acting as an antibacterial agent due to their structural qualities of substantial porosity, controlled drug release, large surface area, biocompatibility, and faster release rate.

In this study, a thermally stable porous 3D Cu-MOF was synthesized. This framework can maintain its structural integrity after guest (water) removal. We have used the synthesized Cu-MOF to encapsulate INH (INH@Cu-MOF). Characterization of the Cu-MOF shows the framework to be thermally stable. SEM analysis was performed to detetrmine the morphology of Cu-MOF which suggests that the morphology of Cu-MOF is similar to a shape of a microcapsule. PXRD data of INH@Cu-MOF indicates successful encapsulation of INH into the Cu-MOFs. A drug release study was performed to check the drug release kinetics of INH@Cu-MOF. The result suggests that the release of INH from INH@Cu-MOF increases with the increase in the pH.

Further to check the antibacterial activity of Cu-MOF, INH@Cu-MOF, and INH (10% loading content), *in-vitro* killing assay, agar diffusion assay as well as biofilm disruption and formation assays were performed on *M.smegmatis*. The *in-vitro* killing assay showed that Cu-MOF and INH@Cu-MOF have better antibacterial activity as compared to INH. Agar diffusion assay showed more diameter of

zone of inhibition indicating the efficient antibacterial activity of Cu-MOF and INH@Cu-MOF as compared to INH. Then to determine antibiofilm activity of INH@Cu-MOF, biofilm formation and disruption assays were performed. The biofilm formation results suggest that INH@Cu-MOF shows significant reduction in the biofilm formation as compared to INH. On the other hand, the biofilm disruption assay results suggests that INH@Cu-MOF did not show significant antibiofilm activity however, a comparative biofilm disruption in INH@Cu-MOF was observed as compared to INH treated *M.smegmatis* biofilm.

Further, along with the antibacterial activity, cytotoxicity assay was performed to check the cellular toxicity of Cu-MOF, INH@Cu-MOF, and INH against RAW 264.7 mouse macrophages which suggests that for low concentration of Cu-MOF and INH@Cu-MOF, the cellular toxicity is insignificant. The characterization of INH-Cu-MOF and biological assays were performed to determine the efficacy of the Cu-MOFs as a drug delivery vehicle and the sustained release of INH in macrophages.

In summary, in this study, the data and the results suggest that INH@Cu-MOF exhibits antibacterial activity. The efficacy of killing Cu-MOF as well as INH@Cu-MOF is more as compared to INH.

On the other hand, MOFs showed less cytotoxicity at the lower concentrations, hence suggesting that MOFs have no toxic effects on the cells.

Nowadays, the emergence of various types of Mtb-resistant strains is a matter of concern. In this study, we employed virtual screening of drugs against the Mtb polyketide synthase protein using molecular docking. Our result suggests that rutin binds most effectively to the binding pocket of Mtb polyketide synthase protein which is justified by the high binding energy. However Silymarin was observed to show better drug-like. Hence silymarin may act as a potent inhibitor of polyketide synthase.

Chapter 5

Future directions:

MOFs are emerging as a novel drug delivery system that enables targeted and sustained drug delivery. Many biological studies have been performed to check its efficacy as a drug delivery system. However, more studies are required to crosscheck its efficacy-

1.) Combination chemotherapy encapsulated Cu-MOFs: Many studies have been done to treat cancer with the help of combination chemotherapy. As EMB when combined with RIF shows better efficacy in treating TB, RIF can be encapsulated along with EMB Cu-MOFs to check whether the sustainable release of both EMB and RIF has a better effect.
2.) For better understanding of the antibacterial and antibiofilm activity of INH@Cu-MOF, biological assays can be done against Mtb H37Rv in a Biosafety Level 3 facility.
3.) *In-vivo* studies on the mouse: A novel drug/drug delivery vehicle discovery not only requires *in-vitro* studies, but it also requires studies on an animal model such as a mouse to confirm its effectiveness. Different *in-vivo* studies such as histopathological findings as well as DNA damage will provide more information about the efficacy of the encapsulated drug and its effectiveness on targeted drug delivery.
4.) More cytotoxic studies of Cu-MOFs: Apart from cell viability assay, certain other studies such as ROS production assay etc. can be done to determine the cytotoxic profile of Cu-MOFs.
5.) To check whether INH@Cu-MOF can kill all the good bacteria present in the gut flora.

Chapter 6

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