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SIMILARITY BASED DESIGN, SYNTHESIS AND CHARACTERIZATION OF ISONIAZID ANALOGUES AND THEIR THERAPEUTIC POTENTIAL

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ABSTRACT

The alarming rise of Mycobacterium tuberculosis (Mtb) strains that are multi-drug resistant has prompted the scientific community to scramble for fresh, effective anti-tubercular treatments. Despite the different medications that are now being studied, isoniazid remains the most important and efficient part of all multi-therapeutic regimens that the WHO recommends. The computational design, synthesis, and in vitro anti-tubercular activity of a number of potent isoniazid derivatives against H37Rv are all covered in this study. In this study, docking tests were done to determine the biological activity of three isoniazid derivatives that were created. A simple and efficient method was developed for the synthesis of designed compounds and the structures of the compounds were characterized by means of their FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. The anti-tubercular activity of the compound with the relatively high binding affinity for the chosen target (2NSD) was evaluated. Microplate Alamar Blue Assay (MABA) was used to measure the anti-tubercular activity. According to the experimental findings, compound IBF1 demonstrated positive anti-tubercular activity with a MIC of 3.12 g/ml.

KEYWORDS: Isoniazid, Computational design, In-vitro anti-tubercular activity, Microplate Alamar Blue Assay.

1. INTRODUCTION

Tuberculosis (TB) is a major cause of ill health, one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent. A total of 1.6 million people died from TB in 2021 (including 187 000 people with HIV). Worldwide, TB is the 13th leading cause of death and the second leading infectious killer after COVID-19 (above HIV/AIDS). In 2021, an estimated 10.6 million people fell ill with tuberculosis (TB) worldwide.[1] Tuberculosis (TB) is defined as a disease caused by members of the *M. tuberculosis* complex, which includes the tubercle bacillus (*M. tuberculosis*), *M. bovis*, *M. africanum*, *M. microti*, *M. canetti, M. caprae* and *M. pinnipedi*. *M. tuberculosis* (Mtb) belongs to the genus Mycobacterium that includes more than 80 other species.[2] Tuberculous infection may be propagated by contiguous spread, via lymphatics or blood stream, by lymph-hematogenous spread (e.g., thoracic duct to left subclavian vein), through the bronchial tree (bronchogenic spread), or by intra-canalicular spread (e.g., bronchi to trachea to larynx to gastro intestinal tract).[3] Currently, there are 10 drugs that are approved by the FDA for the treatment of active TB of these approved drugs, the first-line pharmacological intervention that forms the core treatment regimen includes isoniazid, rifampin, ethambutol, and pyrazinamide.[4] Isoniazid (isonicotinic acid hydrazide, INH) is an essential drug used in the treatment of tuberculosis.[5] INH is a pro-drug, which is oxidatively activated in vivo by the katG-encoded mycobacterial catalase peroxidase to generate an isonicotinoyl radical.[6] This highly reactive species then reacts non-enzymatically with the cellular pyridine nucleotide coenzymes, NAD+ and NADP+, to generate 12 isonicotinoyl-NAD(P) adducts [INH-NAD(P)].[7] One of these, a nicotinoyl-NAD isomer inhibits the activation of enoyl acyl carrier protein reductase (InhA) and β-ketoacyl acyl carrier protein synthase (KasA). Inhibition of these enzymes inhibits synthesis of mycolic aid, an essential component of the mycobacterial cell wall, leading to bacterial cell death. Another adduct, a nicotinoyl-NADP isomer, potently inhibits mycobacterial dihydrofolate reductase, thereby interfering with nucleic acid synthesis.[8]

The drug resistant strains can then spread from person to person like drug susceptible bacteria. Strains of *M. tuberculosis* that are resistant against isoniazid and rifampicin, the most effective drugs against tuberculosis, are defined as multi drug resistant (MDR-TB).

In 2006 the WHO released the first data on extensively drug resistant strains (XDR-TB). These strains are resistant to any fluoroquinolones and at least to one of the injectable drugs kanamycin, capreomycin and amikacin, in addition to isoniazid and rifampicin and occur in every part of the world. Patients infected with XDR-TB are virtually untreatable with current drug.[1]

In this study three isoniazid derivatives were designed and docking studies were carried out for their biological activity. A simple and efficient method was developed for the synthesis of designed compounds and the structures of the compounds were characterized by means of their FT-IR, 1H NMR, 13C NMR and mass spectrometry. The compound with the comparatively high binding affinity toward the selected target (2NSD) was evaluated for anti-tubercular activity. The anti-tubercular activity was carried out by Microplate Alamar Blue Assay (MABA) method.

2. MATERIAL AND METHODS
Materials
2.1 Chemicals used
The chemicals and reagents were procured from Sigma Aldrich, Alchemy Lab Solutions, Edappally Kochi.

2.2 Instruments used
IR spectra were recorded on Bruker FT-IR (Shimadzu 8201 PC) spectrophotometers (Al Shifa College of Pharmacy, Perinthalmanna) and values are expressed in cm-1. 1HNMR and 13C-NMR spectra were recorded on Bruker Avance-500, FTNMR spectrometer (National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab) at 500MHz and the chemical shifts are reported in parts per million (δ value), taking TMS (δ 0 ppm for 1H NMR) as the internal standard. Mass spectra were recorded on ESI-MS Q-ToF Micro Waters Mass Spectrometer instrument (National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab) using LC-ESI Technique.

Methods
2.3 Insilico Study
All computational analysis was carried out on a Windows 10 Pro OS platform on a Desktop with an Intel (R)Pentium (R) CPU J3710@1.60GHz and 4GB RAM.

2.3.1 Physiochemical properties
Molinspiration® Molecular Viewer allows the visualization of molecule which is encoded as SMILES or SD file for the calculation of important molecular description (Log P, polar surface area , number of hydrogen bond donors, number of hydrogen bond accepters etc.) as well as prediction of bioactivity score of important drug targets.

2.3.2 Pharmacokinetic Study by SwissADME
SwissADME is a web tool giving free access into physiochemical properties (Molecular weight, Molar refractivity, Polar surface area) pharmacokinetics (substrate or non-substrate of P-gp, CYP inhibition), drug-likeness of potent molecule. It also produces predictive models such as BOILED-Egg allows the evaluation of passive gastrointestinal absorption (HIA) and brain penetration (BBB) of drug molecule. The white region is for high probability of passive absorption by the GI tract and yolk region is for high probability of brain penetration.

2.3.3 In-Silico Toxicity Prediction- OSIRIS
In silico toxicity prediction is done using OSIRIS® Property Explorer. It is a free software available for access in the Organic Chemistry Portal. Using this prediction tool, mutagenicity, tumerogenicity, skin irritation and reproductive effects can be calculated. The prediction properties rely on a precompiled set of structure fragment that gives rises to toxicity alerts in case they are encountered in the structure currently drawn. These fragment lists is created by rigorously shredding all compounds in the data base known to be active in a certain toxicity class. During the shredding any molecule is first cut at every rotatable bonds leading to a set of core fragments.

2.3.4 Molecular docking
Docking of small molecules and compounds into the binding site of receptor and estimating the binding affinity of complex is considered to be the important part of structure based drug design. Molecular docking is achieved by AutoDock Vina. The 3D crystallographic structures of proteins are downloaded from protein data bank (PDB ID:2NSD).

Auto Dock Vina is an open source program offering a complete molecular viewer and graphical support for all the steps inevitable for set up and docking analysis. Autodock® is a suite of software for predicting the optimal bound conformations of ligands to proteins. It relies on a number of approximations to predict the conformation and free energy of binding during a docking simulation. The ligand is treated as flexible and the target molecule is treated as rigid. During the docking simulation, a grid-based method is used for energy evaluation, where interaction energies are pre-calculated around the target structure and then used as look-up tablet to allow rapid evaluation of ligand-protein interaction.

2.4 Chemistry
2.4.1 General procedure for the synthesis of isoniazid derivatives
A mixture of isoniazid (0.5g, 0.0036 mol) and substituted aromatic aldehyde (0.0036 mol) was stirred in 10 ml water for prescribed time period at room temperature. In a few minutes the temperature of the reaction mixture was raised due to the heat evolved during the exothermic reaction, but it should not be allowed to exceed 20°C above the room temperature. The crystalline product was obtained was filtered, washed with water, dried, recrystallized from ethanol.

2.5 Characterization
2.5.1 Melting point determination
The melting point of the synthesized compound was determined by open capillary tube method. The temperature at which the compound starts losing its crystallinity and changes from solid to liquid form was found and recorded.

2.5.2 Thin layer chromatography
The reactants and products were dissolved in ethanol. It was spotted on the TLC plate. A single principal spot for the product and the absence of secondary spots for parent compounds and intermediates confirmed the purity of the product. Stationary phase: pre-coated silica gel GF using appropriate mobile phase was used. The spots were detected in a UV chamber.

2.5.3 IR spectroscopy
Infrared spectroscopy is one of most commonly used spectroscopic technique for identification of functional groups in molecules. IR spectroscopy is an important tool in the structural elucidation of organic compounds. In IR spectroscopy finger print region is used to compare the two compounds. Infrared spectrum shows percentage transmittance versus frequency expressed as wave numbers.

2.5.4 NMR spectroscopy
Nuclear Magnetic Resonance (NMR) spectroscopy is an important analytical technique used in the structural elucidation of organic molecules. It involves the interaction of the electromagnetic radiation and the proton of a nucleus of an atom when placed under an externally applied static magnetic field. NMR spectra provide the detailed information about a molecule's structure. The chemical shift is used to predict the number of protons with refers to TMS as standard. The NMR spectra are recorded on 300 MHZ BRUKER advance III NMR spectrometer. DMSO is used as a solvent.

2.5.5 LC-MS
LC-MS is a hyphenated technique, combining separation power of HPLC with the detection power of Mass Spectrometry. Mass spectra was recorded on Shimadzu LC-MS using Electron Spray Ionization Technique and was quantified using Lab Solutions Software 7.0.

2.6 Biological Evaluation
2.6.1 Microplate Alomar Blue Assay (MABA)
Principle
Microplate Alamar Blue Assay (MABA) is a non-toxic rapid, inexpensive and high throughput assay for anti-tubercular drug screening. The principle behind Microplate Alamar Blue assay is that, in the presence of cellular metabolism resazurin (oxidized for of Alamar blue), which is non fluorescent blue in colour is converted to resorufin (reduced form of Alamar blue) which is fluorescent pink in colour.
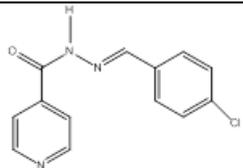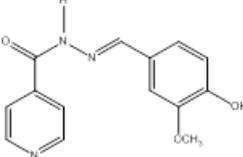
Procedure
The anti-mycobacterial activity of the compounds is to be assessed against M. tuberculosis using Microplate Alamar Blue Assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200 ml of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation. The 96 wells plate received 100μl of the Middle brook 7H9 broth and serial dilution of compounds are placed directly on plate. The final drug concentrations tested is made up to 100 to 0.2μg/ml) Plates were covered and sealed with para film and incubated at 37^{0}C for five days. After this time, 25μl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% Tween 80 was added to the plate and incubated for 24hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC is defined as lowest drug concentration which prevents the colour change from blue to pink.
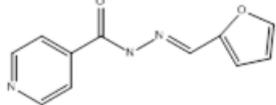**The percentage inhibition = Test well fluorescence/ mean fluorescence of triplicate wells**

3. RESULT AND DISCUSSION
3.1 *In silico* studies
In-silico Studies: Pharmacokinetics parameters of these derivatives were calculated using Molinspiration Online software. From all these parameters Table, the compounds obeying Lipinski's rule of five was selected for docking studies.

Compound Id	Structure	MW (g/mol)	HA	HD	Log P	nrotb	Violation
IBF 1		259.70	3	1	2.49	3	0
IBF 2		271.28	5	2	1.15	4	0

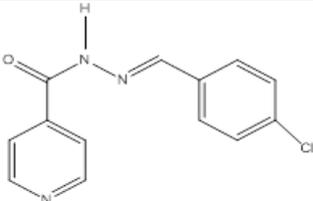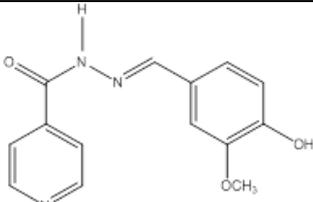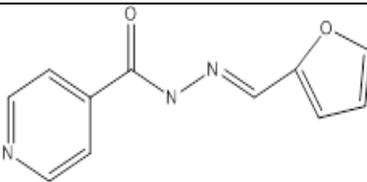|							
IBF 3		215.21	4	1	1.07	3	0

Analysis of Lipinski rule of five using Molinspiration

| Compound ID	GI Absorption (High/Low)	BBB Permeant (Yes/No)	P-gp substrate (Yes/No)	PAINS (alert)
IBF 1	High	Yes	No	0
IBF 2	High	No	No	1
IBF 3	High	Yes	No	0

Pharmacokinetic study by SwissADME

The designed molecules were docked against the selected *Mycobacterium tuberculosis* enoyl acyl carrier protein reductase (PDB ID:2NSD). And the compounds were filtered using docking score. The best and stable pose was selected based on the docking score and the basis of multiple interactions. Comparing the binding structure of all the three molecules, the hydrazide holds a key group for binding affinity in the binding pocket. Among the docked molecules, the compound IBF1 shows the highest binding affinity towards the protein, and IBF5 shows the least binding affinity.

| Sl. no	Compound	Structure	Docking score	Interacting residue
1	IBF 1		-10.3	ASP A:148 ALA A:191 PHE A:149 MET A:155 TRP A:222 LEU A:218 PRO A:193
2	IBF 2		-9.7	ALA A :191 MET A:147 PHE A:149 MET A:103 TYR A:158 ILE A:202 ALA A:157
3	IBF 3		-8.8	LEU A:218 ILE A:215 PRO A:193 TRP A:222 MET A:199 PHE A :149

Docking result
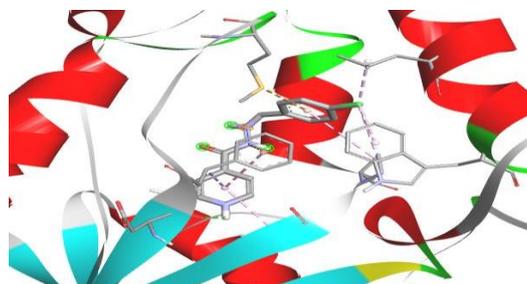
Crystal structure of IBF 1 bound to enoyl acyl carrier reductase protein (PDB ID: 2NSD)
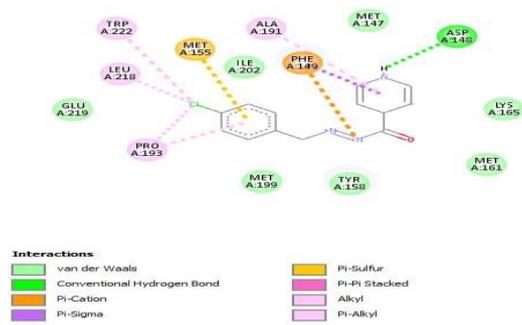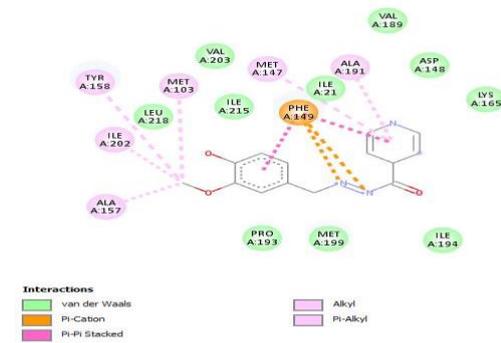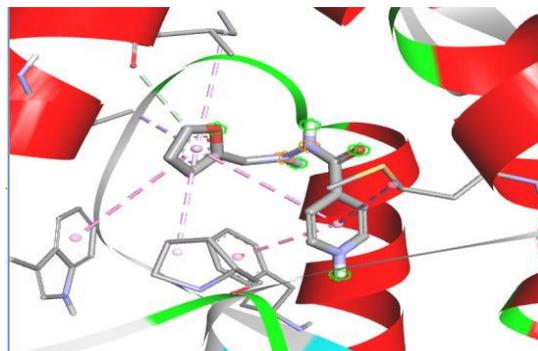
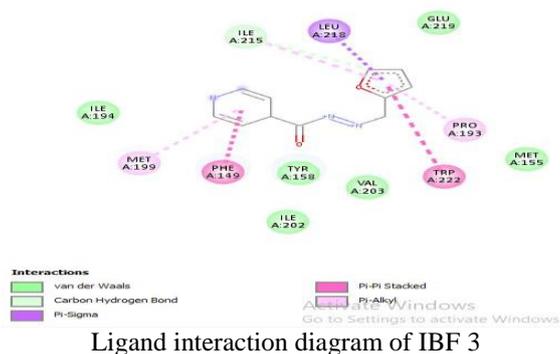**Ligand interaction diagram of IBF 1**
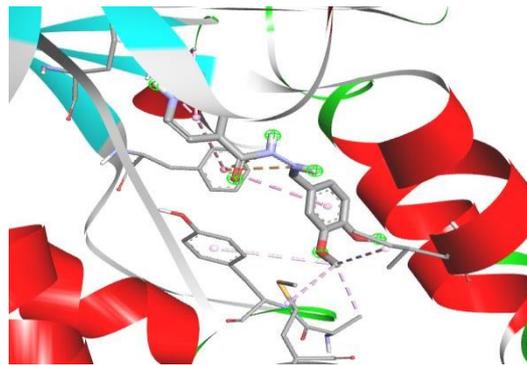
Crystal structure of IBF 2 bound to enoyl acyl carrier reductase protein (PDB ID: 2NSD)

Ligand interaction diagram of IBF 2

Crystal structure of IBF 3 bound to enoyl acyl carrier reductase protein (PDB ID: 2NSD)

Ligand interaction diagram of IBF 3

3.2 Chemistry

Three isoniazid derivatives were designed and synthesized by the reaction of equimolar amounts of isoniazid and different substituted aldehydes (4-chlorobenzaldehyde, Vanillin and Furfuraldehyde). The compound IBF1 took 1 hour to complete the reaction, whereas compounds IBF2 and IBF3 took 1 and 2 hours to complete the reaction respectively. The progress of the reaction was monitored by TLC. The yield of the compounds ranges from 60-72% with reasonable purity. All the synthesized derivatives were found to have a sharp melting point. The filtered products were recrystallized from ethanol to give crystalline compounds.

3.3 Characterization of the synthesized compounds

1. (E)-N'-(4-chlorobenzylidene) isonicotinohydrazide (IBF1)

White powder(EtOH), MP=192-202°C, IR(ZnSe) peaks 3555cm-1 (NH stretching) 3162cm-1 (Aromatic CH) 2991cm -1 (Aliphatic CH) 1658cm-1 (C=O stretching) 1592cm1 (C=N).1H NMR(500MHz,DMSO):δ ppm=7.55-8.80 (m,8H,ArH), 8.56 (s,1H,CH) 12.13 (br,1H,NH). 13C NMR (500MHz, DMSO): δ ppm=122.00, 123.55, 128.9, 129.56, 133.53, 135.33, 150.83, 158.13, 162.18, ESI-MS: 260.06 (M+1)

2. (E)-N'-(4-hydroxy-3-methoxybenzylidene) isonicotinohydrazide (IBF2)

Yellow crystal(EtOH), MP=194-210°C, IR(ZnSe) Peaks 3213 (NH Stretching) 3055 (aromatic CH) 1658(C=O stretching) 1599(C=N) 1H NMR(500mhz, DMSO): δ ppm= 6.85-8.77 (m,8H,ArH) 8.77 (s,1H,CH) 9.60 (br,1H,OH) 12.07 (br,1H,NH) 13C

NMR(500mhz,DMS):δ ppm= 56.06, 109.58, 115.93, 115.95, 121.96, 125.86, 151.17, 158.55, 159.90, 150.03, 150.76, 161.71, ESI-MS: 272.11(M+1).

3. (E)-N'-(furan-2-ylmethylene) isonicotinohydrazide (IBF3)

White powder(EtOH); MP=190-200°C; IR(ZnSe) peaks 3252cm-1 (NH stretching) 3052cm-1 (Aromatic CH) 1658cm-1 (C=O stretching) 1582cm-1 (C=N). 1H NMR(500mhz, DMSO):δ ppm= 6.66-8.79 (m,7H,ArH) 8.35 (s,1H,CH) 12.00 (br,1H,NH).13C NMR(500mhz,DMSO): δ ppm=112.77, 115.79, 121.95, 139.12, 150.87, 156.03, 159.62, 150.81, 161.98, ESI-MS: 216.07(M+1).

3.4 *In vitro* studies

All the newly synthesized compounds were investigated for their potential anti-tubercular activity. The anti-mycobacterial activity of compounds was assessed against Mycobacterium tuberculosis using Microplate Alamar Blue Assay (MABA). The organism used in the study is Mycobacterium tuberculosis H37Rv.Among the synthesized compounds, IBF1 showed anti-mycobacterial activity in varying degrees against the organism tested with an MIC value of 3.12µg/ml.

Standard Strain used: *Mycobacterium tuberculosis* (Vaccine strain, H37 RV strain): ATCC No-27294.

Standard values for the Anti-Tb test are as follows:
Isoniazid – 1.6 µg/ml, Ethambutol – 1.6 µg/ml, Pyrazinamide- 3.125µg/ml, Rifampicin – 0.8 µg/ml, Streptomycin- 0.8µg/ml
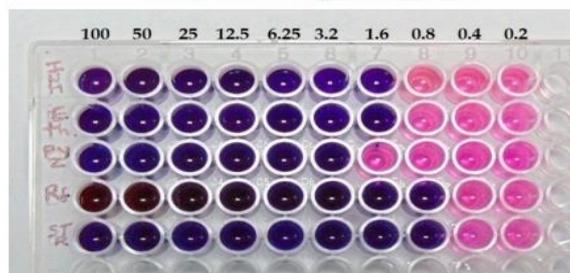
Anti-mycobacterial activity of Standard drugs

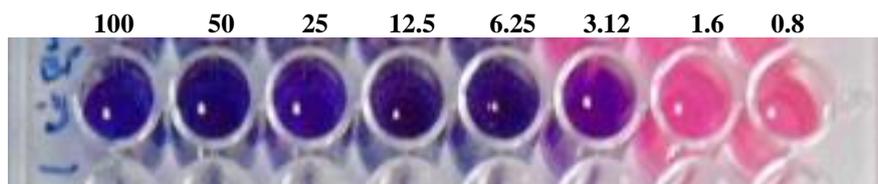Among the synthesized compounds, IBF1 was selected for evaluation of biological activity.

Results

Sl.No.	Sample	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	IBF1	S	S	S	S	S	S	R	R

Results of biological evaluation of compound IBF3

Note:
S- Sensitive
R- Resistant

Anti-mycobacterial activity of IBF1

4. CONCLUTION
From this study we concluded that, during the docking studies, all the developed compounds were able to successfully interact with the target enzyme within a favourable range. The results of molecular docking indicated that IBF1, IBF2 and IBF3 displayed efficient interactions with the active site, surpassing the interactions observed with the co-crystallized ligand. Notably, (E)-N'-(4-chlorobenzylidene) isonicotinohydrazide, which possesses an electronegative chlorine atom and two aromatic rings, exhibited specific interactions with Asp A 148, Ala A 191, Phe A 149, Met A 155.Trp A 222, Leu A 218 and Pro A 193. Furthermore, IBF 3 demonstrated the highest activity among the developed compounds against *Mycobacterium tuberculosis*. The presence of heterocyclic ring on both the end of designed compound IBF3 may shows the least binding affinity towards 2NSD protein and the presence of electronegative halogen atoms on the compound IBF3 may be responsible for the highest binding affinity of that compound towards 2NSD.

A simple and efficient method for synthesizing the designed compounds, identified through molecular modelling studies, has been successfully developed. The synthesis of all the compounds involved magnetic stirring at room temperature for a duration of 1 to 2 hours. The progress of the reactions was monitored using TLC (thin-layer chromatography) and the determination of melting points. The yields of the compounds ranged from 60% to 72%, and they exhibited reasonable purity. To ensure higher purity required for spectral studies, all the synthesized compounds underwent two rounds of recrystallization in ethanol.

The formation of isoniazid derivatives was confirmed by conducting spectral studies, including IR (infrared spectroscopy), ^{1}H NMR (proton nuclear magnetic resonance), and ^{13}C NMR (carbon-13 nuclear magnetic resonance). These spectroscopic analyses provided evidence for the structural characteristics of the synthesized compounds. Additionally, mass spectrometry was employed to determine the molecular mass of the compounds, further confirming the identification and structure of the isoniazid derivatives.

The compound IBF1, which exhibited the highest docking score among the synthesized compounds, was selected for in-vitro analysis. Through Microplate Alamar Blue Assay(MABA), it was discovered that IBF1 demonstrated significant activity against the *Mycobacterium tuberculosis*. The compound IBF1 exhibited an MIC value of 3.12µg/ml.

To determine the efficacy of the synthesized molecules, it is necessary to conduct in vivo evaluations using small animals. This step is crucial for establishing the effectiveness of the compounds. Our study aims to offer valuable insights to researchers involved in the development of anti-tubercular drugs.

5. ACKNOWLEDGEMENT

REFERANCE
1. World Health Organization Global Tuberculosis Report, 2022. https://www.who.int/news-room/fact-sheets/detail/tuberculosis
2. van Soolingen D, Hoogenboezem T, de Haas PE, et al. A novel pathogenic taxon of the Mycobacterium tuberculosis complex, Canetti: characterization of an exceptional isolate from Africa. Int J Syst Bacteriol, 1997; 47: 1236.
3. Jordao L and Vieira OV. Tuberculosis: New Aspects of an Old Disease. Int J Cell Biol, 2011; 1-13.
4. Currently, there are 10 drugs that are approved by the FDA for the treatment of active TB of these approved drugs, the first-line pharmacological

intervention that forms the core treatment regimen includes isoniazid, rifampin, ethambutol, and pyrazinamide.

5. Bloom, B. R., & Murray, C. J. L. Tuberculosis: Commentary on a Reemergent Killer. Science, 1992; 257(5073): 1055–1064.

6. INH is a pro-drug, which is oxidatively activated in vivo by the katG-encoded mycobacterial catalase peroxidase to generate an isonicotinoyl radical Zhao, X., Yu, H., Yu, S., Wang, F., Sacchettini, J. C., & Magliozzo, R. S. Hydrogen Peroxide-Mediated Isoniazid Activation Catalyzed by Mycobacterium tuberculosis Catalase−Peroxidase (KatG) and Its S315T Mutant†,‡. Biochemistry, 2006; 45(13): 4131–4140.

7. Argyrou A, Vetting MW, Aladegbami B, Blanchard JS. Mycobacterium tuberculosis dihydrofolate reductase is a target for isoniazid. Nat Struct Mol Biol, 2006; 13: 408-413.

8. Talavera, W., R. Miranda, K. Lessnau, and L. Klapholz. Extrapulmonary tuberculosis, In L. N. Friedman (ed.), Tuberculosis: current concepts and treatment, CRC Press, Inc., Boca Raton, Fla, 2001; 2: 139-190.

9. Wallgren, A. The time-table of tuberculosis. Tubercle, 1948; 29(11): 245–251.

10. Kamholz, S. L. Pleural tuberculosis, In W. N. Rom and S. Garay (ed.), Tuberculosis. Little, Brown and Co., Boston, Mass, 1996; 483-491.

11. Garay. S.Pulmonary tuberculosis. In W. N. Rom and S. Garay (ed.), Tuberculosis. Little, Brown and Co., Boston, Mass, 1996; 373-412.

12. Jagirdar, J., and D. ZagZag. Pathology and insights into pathogenesis of tuberculosis, p. In W. N. Rom and S. Garay (ed.), Tuberculosis. Little, Brown and Co., Boston, Mass, 1996; 467-497.

13. Center for Disease Control and Prevention https://www.cdc.gov/tb/education/corecurr/pdf/chapter2.pdf.

14. Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., & Behar, S. M. In search of a new paradigm for protective immunity to TB. Nature Reviews Microbiology, 2014; 12(4): 289–299.

15. Bandera A, Gori A, Catozzi L, Degli Esposti A, Marchetti G, Molteni C, et al. Molecular epidemiology study of exogenous reinfection in an area with a low incidence of tuberculosis. J Clin Microbiol, 2001; 39: 2213–8.

16. Hunter, R. L., Jagannath, C., & Actor, J. K. Pathology of postprimary tuberculosis in humans and mice: Contradiction of long-held beliefs. Tuberculosis, 2007; 87(4): 267–278.

17. Hunter RL, Olsen MR, Jagannath C, Actor JK. Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavitary tuberculosis, including a revised description of the pathology of secondary disease. Ann Clin Lab Sci, 2006; 36: 371–86.

18. Hunter RL, Actor JK, Hwang S, Karev V, Jagannath C. Pathogenesis of post primary tuberculosis: immunity and hypersensitivity in the development of cavities. Ann Clin Lab Sci, 2014; 44: 365–87.

19. Yoder M, Lamichhane G, Bishai W. Cavitary pulmonary tuberculosis: The Holy Grail of disease transmission. Current Sci, 2004; 86: 74–81.