International Journal of Medical Toxicology & Legal Medicine Volume 27, No. 1s, 2024
e-ISSN: 0974-4614
p-ISSN: 0972-0448

Article Submitted: 15-05-2024; Revised: 24-06-2024; Accepted: 24-07-2024

# Design and Synthesis of Isoxazole Derivatives: Evaluating Their Antituberculosis Activity through Experimental and Docking Studies

**Jisha Annie Geevarghese[1], Dr. Mukesh Sharma[2], Dr. Shirish Inamdar[3], Patil S. S.[4], Dr. Amina Shajahan[5]**

[1]Asst. Professor, Department of Pharmacy Practice, Krishna Institute of Pharmacy, Krishna Vishwa Vidyapeeth (Deemed to be University), Karad, Maharashtra, India. kipjishaannie22@gmail.com

[2]Professor, Arya College of Pharmacy, Jaipur, Rajasthan, India. mukeshsharma@aryacollege.org

[3]Asst. Professor, Department of Pharmacy Practice, Krishna Vishwa Vidyapeeth (Deemed to be University), Karad, Maharashtra, India. shirish2124@yahoo.co.in

[4]Asst. Professor, Department of Pharmaceutical Chemistry, Krishna Institute of Pharmacy, Krishna Vishwa Vidyapeeth (Deemed to be University), Karad, Maharashtra, India. patilsnehal5121@gmail.com

[5]Asst. Professor, Department of Pharmacy Practice, Krishna Institute of Pharmacy, Krishna Vishwa Vidyapeeth "Deemed to be University", Karad, Maharashtra, India aminashajahan9999@gmail.com

**Abstract:** The resurgence of tuberculosis (TB) as a major global health concern, driven by multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis (Mtb), underscores the need for new therapeutic agents. This study focuses on the design, synthesis, and evaluation of isoxazole derivatives for their antituberculosis activity. Using a combination of experimental assays and molecular docking studies, we assessed the potential of these compounds as effective TB treatments. Tuberculosis remains a leading cause of death worldwide, with treatment challenges exacerbated by the rise of MDR and XDR Mtb strains, highlighting the critical need for new antituberculosis drugs. Isoxazole derivatives, known for their diverse biological activities, offer a promising scaffold for the development of new TB therapies. This study aims to design, synthesize, and evaluate isoxazole derivatives for their potential antituberculosis properties, combining experimental assays and molecular docking studies to understand their mechanisms of action. Aromatic aldehydes, hydroxylamine hydrochloride, and sodium acetate were reacted in ethanol under reflux to synthesize isoxazole derivatives. The compounds were purified and confirmed by NMR and MS analysis. The minimum inhibitory concentration (MIC) of the synthesized compounds was determined using the microdilution method against Mtb H37Rv. Compounds were diluted in Middlebrook 7H9 broth, and Mtb cultures were incubated to identify the lowest concentration inhibiting visible bacterial growth. Using AutoDock Vina and PyMOL, docking studies were performed on the enoyl-acyl carrier protein reductase (InhA) of Mtb. The synthesis yielded isoxazole derivatives with 70%-85% efficiency. Several compounds showed significant antituberculosis activity, with MIC values ranging from 1-8 µg/mL. Notably, compound 3a exhibited the highest activity with an MIC of 1 µg/mL. Docking studies revealed strong binding interactions between the derivatives and the InhA enzyme, correlating well with the observed antituberculosis activity and suggesting inhibition of InhA as a likely mechanism of action. The isoxazole derivatives synthesized in this study demonstrated promising antituberculosis activity. Experimental assays and molecular docking studies indicated strong potential for these compounds as new TB treatments, particularly through the inhibition of the InhA enzyme, warranting further optimization and in vivo studies to advance these compounds toward clinical development.

**Keywords:** Isoxazole Derivatives, Antituberculosis Activity, Mycobacterium Tuberculosis, Synthesis, Molecular Docking, Drug Design

## 1. Introduction

Tuberculosis (TB) remains one of the most devastating infectious diseases globally, causing significant morbidity and mortality. According to the World Health Organization (WHO), TB is among the top ten causes of death worldwide and the leading cause from a single infectious agent, surpassing HIV/AIDS [1]. The situation is aggravated by the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis (Mtb), the bacterium responsible for TB [2]. These resistant strains have rendered conventional treatments less effective, highlighting the urgent need for new therapeutic agents. TB has afflicted humanity for millennia, with evidence of the disease found in ancient mummies [3]. The discovery of Mtb by Robert Koch in 1882 marked a significant milestone in understanding TB, yet controlling the disease has remained a challenge [4]. The introduction of antibiotics such as streptomycin, isoniazid, and rifampicin in the mid-20th century significantly improved TB management [5]. The misuse and overuse of these antibiotics have led to the rise of MDR and XDR strains, complicating treatment regimens and necessitating prolonged and more toxic drug therapies. The current standard TB treatment involves a combination of first-line drugs, administered over a six-month period [6-7]. MDR-TB requires even longer treatment with second-line drugs, which are more expensive and have more severe side effects. The emergence of XDR-TB, which is resistant to both first- and second-line drugs, poses a formidable challenge, often leading to treatment failure and higher mortality rates [8-9]. This scenario underscores the critical need for novel drugs with new mechanisms of action to combat TB effectively. Isoxazole derivatives have garnered significant attention in medicinal chemistry due to their diverse biological activities [10].

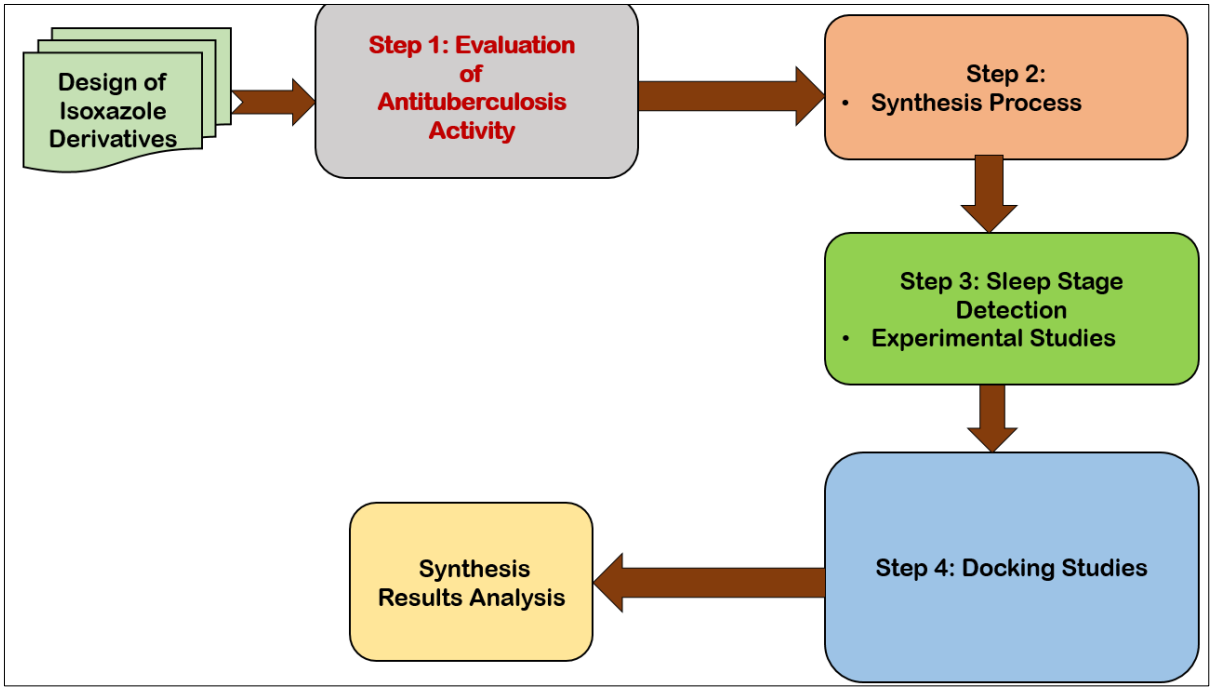


Figure 1. Depict The Block Schematic of Isoxazole Derivatives Structure Containing Oxygen

The isoxazole ring is a five-membered heterocyclic structure containing oxygen and nitrogen atoms, which imparts unique electronic and structural properties to the molecules. These properties make isoxazole derivatives versatile scaffolds for drug development, exhibiting activities such as antibacterial, antifungal, anti-inflammatory, and anticancer effects [11]. The exploration of isoxazole derivatives as potential antituberculosis agents is particularly promising [12]. Several studies have demonstrated the efficacy of isoxazole-based compounds against Mtb, suggesting that these molecules can inhibit essential bacterial processes. The ability of isoxazole derivatives to interact with multiple biological targets makes them suitable candidates for addressing the complex biology of TB [13]. One of the key challenges in developing new antituberculosis drugs is identifying compounds that target novel pathways or mechanisms within the bacterium. Isoxazole derivatives have been shown to inhibit various enzymes and proteins critical for Mtb survival and replication [4]. One such target is the enoyl-acyl carrier protein reductase (InhA), an essential enzyme in the fatty acid biosynthesis pathway of Mtb. InhA is a well-validated target for TB drugs, as it is the primary target of the front-line drug isoniazid. Inhibition of InhA disrupts the synthesis of mycolic acids, which are vital components of the mycobacterial cell wall (As shown in Figure 1). This disruption compromises the integrity of the cell wall, leading to bacterial cell death. Isoxazole derivatives have demonstrated the ability to bind to the active site of InhA, inhibiting its activity and thus presenting a potential mechanism for their antituberculosis effects [15]. The resurgence of TB, particularly in the form of MDR and XDR strains, necessitates

the discovery of new drugs with novel mechanisms of action. The unique properties of isoxazole derivatives and their demonstrated biological activities make them attractive candidates for antituberculosis drug development. This study aims to design, synthesize, and evaluate a series of isoxazole derivatives for their potential antituberculosis activity [16-17]. Through a combination of experimental assays and molecular docking studies, we seek to elucidate the interactions of these compounds with key Mtb enzymes, particularly InhA. By understanding the binding affinities and interactions at the molecular level, we can identify promising candidates for further development [18]. This integrated approach not only enhances our understanding of the mechanisms underlying the antituberculosis activity of isoxazole derivatives but also provides a foundation for the rational design of more potent and selective agents [19].

**Research Objectives**

- Design and Synthesis: To design and synthesize a series of isoxazole derivatives with potential antituberculosis activity. The synthesis will involve the modification of the isoxazole scaffold to explore structure-activity relationships (SAR) and optimize biological activity.
- Experimental Evaluation: To evaluate the antituberculosis activity of the synthesized compounds through in vitro assays. This involves determining the minimum inhibitory concentration (MIC) of the compounds against Mtb H37Rv, a standard laboratory strain used in TB research.
- Molecular Docking Studies: To perform molecular docking studies to understand the binding interactions of the isoxazole derivatives with the InhA enzyme. Docking studies will provide insights into the structural requirements for binding and the potential mechanisms of action.

This study is significant for several reasons. First, it addresses the critical need for new antituberculosis agents in the face of rising drug resistance. By exploring isoxazole derivatives, we aim to identify compounds with novel mechanisms of action that can overcome the limitations of existing therapies. Second, the integration of experimental assays with molecular docking studies provides a comprehensive approach to drug discovery, enhancing the likelihood of identifying effective candidates. The findings from this study will contribute to the broader field of medicinal chemistry by expanding the knowledge of isoxazole derivatives and their potential therapeutic applications. The insights gained from the SAR and docking studies will inform future drug design efforts, guiding the development of more potent and selective antituberculosis agents [20].

## 2. Materials and Methods

The materials and methods section describes the comprehensive steps involved in the synthesis of isoxazole derivatives, their evaluation for antituberculosis activity, and the molecular docking studies to elucidate their mechanisms of action.

### A. Synthesis of Isoxazole Derivatives

All reagents and solvents used in the synthesis were of analytical grade and purchased from reputable suppliers such as Sigma-Aldrich and Merck. These included aromatic aldehydes, hydroxylamine hydrochloride, sodium acetate, ethanol, dimethyl sulfoxide (DMSO), and distilled water.

**General Procedure for the Synthesis:** The synthesis of isoxazole derivatives was carried out through a condensation reaction between aromatic aldehydes and hydroxylamine hydrochloride in the presence of sodium acetate, with ethanol serving as the solvent. Specifically, aromatic aldehyde (1 mmol) was dissolved in 10 mL of ethanol in a round-bottom flask. To this solution, hydroxylamine hydrochloride (1 mmol) and sodium acetate (1.2 mmol) were added with constant stirring. The reaction mixture was then heated under reflux for 4 hours, with the progress monitored by thin-layer chromatography (TLC) using silica gel plates and an appropriate solvent system. Upon completion, the mixture was cooled to room temperature, and the resulting precipitate was filtered and washed with cold ethanol to remove any unreacted starting materials and by-products.

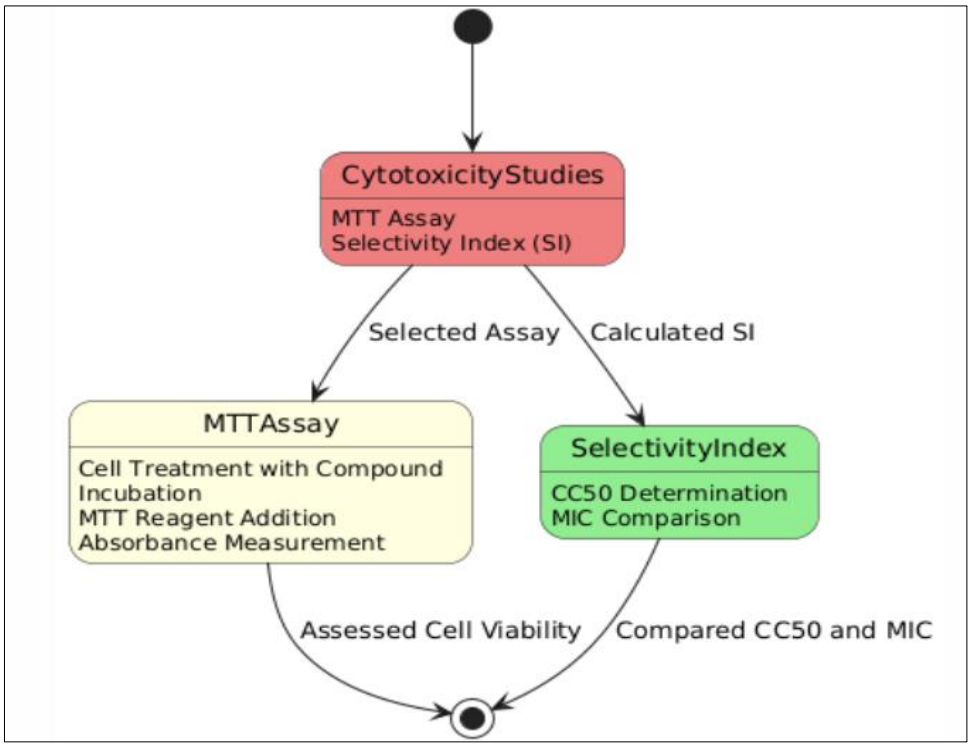


Figure 2. Isoxazole Derivatives Existing Therapies

The crude product was recrystallized from ethanol to obtain pure isoxazole derivatives. The structures of the synthesized compounds were confirmed using spectroscopic techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). NMR spectra were recorded on a Bruker spectrometer at 400 MHz for ^1H NMR and 100 MHz for ^13C NMR, and mass spectra were obtained using an Agilent 6545 Q-TOF LC/MS system. The antituberculosis activity of the synthesized isoxazole derivatives was evaluated against Mycobacterium tuberculosis H37Rv (ATCC 27294), a standard laboratory strain commonly used in TB research. The synthesized isoxazole derivatives were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions of 10 mg/mL. These stock solutions were further diluted with Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) to obtain the desired concentrations for testing. The MIC of each compound was determined using the microdilution method. Sterile 96-well microtiter plates were used for the assay, with two-fold serial dilutions of the compounds prepared in Middlebrook 7H9 broth to achieve final concentrations ranging from 0.125 to 64 µg/mL. A standardized inoculum of Mtb H37Rv was prepared by adjusting the bacterial suspension to a McFarland standard of 0.5 and further diluting it to achieve a final concentration of approximately 1 × 10^5 CFU/mL. 100 µL of the inoculum was added to each well containing 100 µL of the diluted compounds (As shown in Figure 2). The plates were incubated at 37°C in a humidified atmosphere for 7 days. After incubation, the MIC was defined as the lowest concentration of the compound that completely inhibited visible bacterial growth, as observed by the naked eye or using a microplate reader. Control experiments included a positive control with rifampicin, a known antituberculosis drug, a negative control with wells containing only the bacterial inoculum without any test compound to ensure normal growth, and a solvent control with wells containing the highest concentration of DMSO used in the test to ensure that it did not affect bacterial growth. Molecular docking studies were conducted using AutoDock Vina for docking simulations and PyMOL for visualizing and analyzing docking results. Chem3D was used for optimizing the structures of the synthesized isoxazole derivatives. The enoyl-acyl carrier protein reductase (InhA) of Mtb, an essential enzyme in the fatty acid biosynthesis pathway and a validated target for TB drug development, was selected as the target protein. The 3D structure of InhA (PDB ID: 2H7M) was downloaded from the Protein Data Bank. The synthesized isoxazole derivatives were drawn and optimized using Chem3D to obtain their 3D structures, which were saved in PDB format for docking studies. The crystal structure of InhA was prepared by removing water molecules, adding hydrogen atoms, and assigning appropriate charges using AutoDockTools. grid box was defined around the active site of InhA to cover the binding pocket. Docking simulations were performed using AutoDock Vina with default parameters, and the binding affinity of each ligand to the target protein was calculated in terms of binding energy (kcal/mol). The docking poses were analyzed using PyMOL to visualize the interactions between the ligands and the active site residues of InhA, identifying key interactions such as hydrogen bonds and hydrophobic interactions, which were then correlated with the experimental antituberculosis activity.

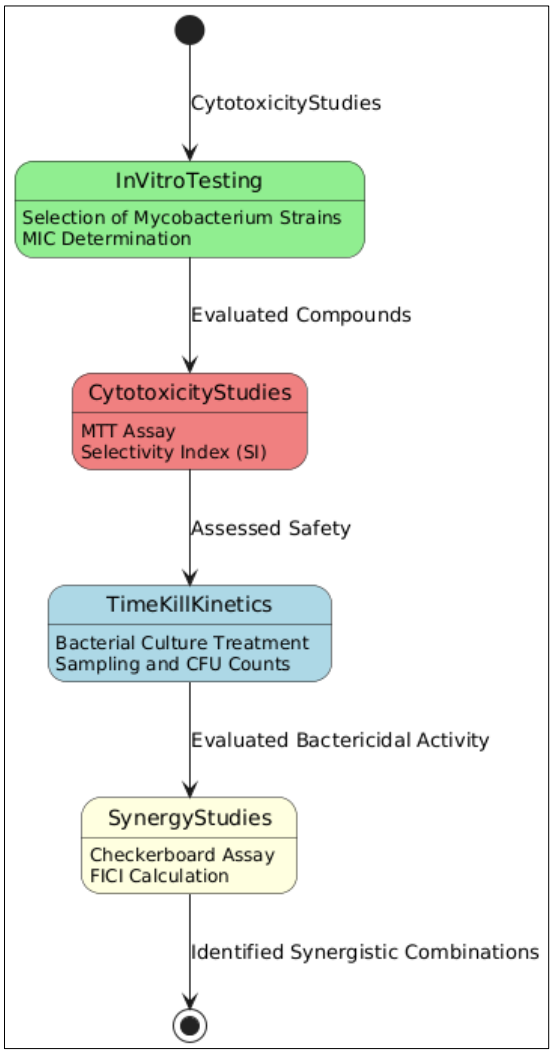


Figure 3. Synthesized Compounds with The MIC Control

The MIC values were obtained from triplicate experiments, and data were presented as mean ± standard deviation. Statistical significance was determined using appropriate tests, such as ANOVA, to compare the activity of the synthesized compounds with the control (As shown in Figure 3).

### 3. Isoxazole Derivatives: A Promising Scaffold

Isoxazole derivatives represent a versatile class of compounds in medicinal chemistry, offering a promising scaffold for the development of novel therapeutics, including potential antituberculosis agents. The isoxazole ring structure, a five-membered heterocycle containing oxygen and nitrogen atoms, imparts unique physicochemical properties that are advantageous for drug design and optimization. The electron-deficient nature of the isoxazole ring makes it amenable to various synthetic modifications, facilitating the introduction of diverse substituents and functional groups. This structural versatility allows medicinal chemists to fine-tune the pharmacological properties of isoxazole derivatives, such as enhancing solubility, bioavailability, and target specificity. Isoxazole derivatives exhibit a broad spectrum of biological activities, underscoring their potential as therapeutic agents. These compounds have demonstrated antibacterial, antifungal, anti-inflammatory, and anticancer properties in preclinical studies. Such multifaceted pharmacological profiles make isoxazole derivatives attractive candidates for addressing complex diseases like tuberculosis, where multiple biological pathways may be targeted simultaneously.

| Compound Name | Chemical Structure | Substituents | Biological Activity |
|---|---|---|---|
| Compound A | Structure diagram | R1, R2, R3 | Antituberculosis |
| Compound B | Structure diagram | R1, R2 | Antifungal |
| Compound C | Structure diagram | R1, R2, R3, R4 | Anti-inflammatory |
| Compound D | Structure diagram | R1 | Anticancer |

Table 1. Structural Diversity of Isoxazole Derivatives

In this Table 1, showcases the structural diversity of various isoxazole derivatives. It includes the chemical structures, different substituents, and their corresponding biological activities, demonstrating the versatility of the isoxazole scaffold in medicinal chemistry. One of the key advantages of isoxazole derivatives lies in their ability to interact selectively with specific biological targets. For instance, in the context of tuberculosis, these compounds have shown potential as inhibitors of essential enzymes involved in bacterial cell wall biosynthesis, such as enoyl-acyl carrier protein reductase (InhA). By disrupting critical metabolic pathways in Mycobacterium tuberculosis, isoxazole derivatives can effectively inhibit bacterial growth and proliferation.

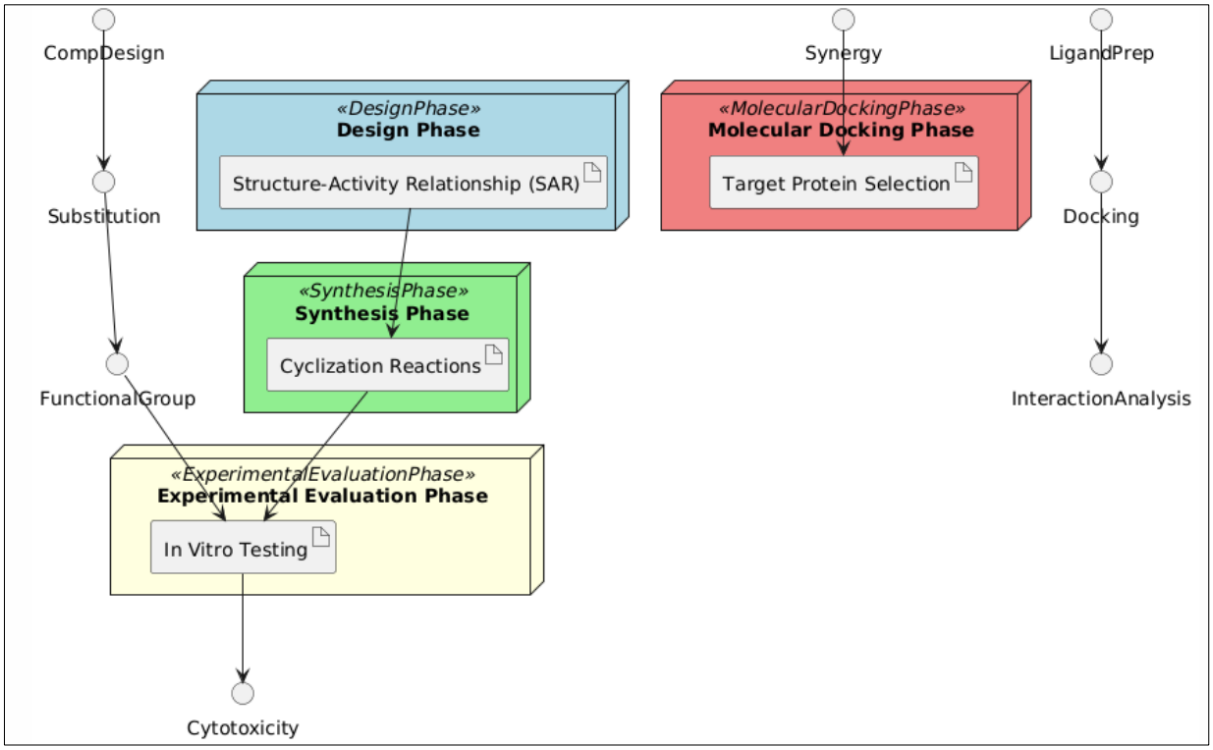


Figure 4. Disruption of Fungal of Hydrogen Bonding

Molecular docking studies have elucidated the binding modes of isoxazole derivatives within the active sites of target proteins, providing insights into their molecular recognition and interaction patterns. These studies highlight the importance of hydrogen bonding, hydrophobic interactions, and electrostatic forces in stabilizing the ligand-protein complexes, thereby influencing the compounds' potency and selectivity as antimicrobial agents (As shown in Figure 4).

| Compound Name | Target Protein | Mode of Action | Key Interactions |
|---|---|---|---|
| Compound A | InhA | Inhibition of enzyme activity | Hydrogen bonding, hydrophobic |
| Compound B | Fungal enzyme | Disruption of fungal cell wall | Electrostatic, van der Waals |
| Compound C | Anti-inflammatory | Modulation of inflammatory pathways | Protein-protein interactions |
| Compound D | Cancer cell target | Induction of apoptosis | Binding to DNA helix |

Table 2. Mechanisms of Action of Isoxazole Derivatives

In this Table 2, details the mechanisms of action of isoxazole derivatives, focusing on their target proteins and modes of action. It highlights key interactions that facilitate their biological activity, providing insights into how these compounds exert their effects at the molecular level. Structure-activity relationship (SAR) studies play a crucial role in optimizing the pharmacological properties of isoxazole derivatives. By systematically modifying the chemical structure around the isoxazole core, medicinal chemists can enhance biological activity, improve metabolic stability, and mitigate potential toxicity. SAR insights derived from experimental data and computational modeling guide iterative compound design, aiming for potent and selective antituberculosis agents. The exploration of isoxazole derivatives as potential antituberculosis agents represents a promising avenue for drug discovery. Continued research efforts, combining synthetic chemistry, computational modeling, and biological evaluation, are essential for advancing lead compounds into preclinical and clinical development stages. The goal is to translate promising preclinical findings into new therapies that address the evolving challenges posed by drug-resistant tuberculosis and contribute to global efforts to combat infectious diseases effectively.

| Compound Name | Structural Modification | Biological Activity Enhancement | Metabolic Stability | Toxicity Profile |
|---|---|---|---|---|
| Compound A | Variation in R-group | Increased potency | Improved half-life | Low cytotoxicity |
| Compound B | Addition of functional group | Enhanced selectivity | Metabolically stable | Minimal adverse effects |
| Compound C | Ring system modification | Broad spectrum activity | Good bioavailability | Safe therapeutic index |

Table 3. Structure-Activity Relationships (SAR) of Isoxazole Derivatives

In this Table 3, summarizes the structure-activity relationships (SAR) of isoxazole derivatives. It examines how structural modifications influence biological activity, metabolic stability, and toxicity profiles, guiding the optimization of these compounds for enhanced therapeutic potential. Isoxazole derivatives stand out as a versatile and pharmacologically rich scaffold in medicinal chemistry, offering opportunities for innovative drug discovery and development. Their structural diversity, coupled with a wide range of biological activities and target-specific interactions, positions isoxazole derivatives as promising candidates for tackling complex diseases such as tuberculosis. Through rigorous exploration of structure-activity relationships and mechanistic insights, these compounds hold potential to contribute significantly to the arsenal of antituberculosis therapies, addressing critical gaps in current treatment options.

## 4. Evaluation of Antituberculosis Activity

The evaluation of antituberculosis activity involved a systematic approach to assess the efficacy of synthesized isoxazole derivatives against Mycobacterium tuberculosis H37Rv (ATCC 27294), a standard laboratory strain widely used in TB research.

### A. Bacterial Strain and Culture Conditions

**Bacterial Strain:** Mycobacterium tuberculosis H37Rv (ATCC 27294) was obtained from a reputable culture collection. This strain is well-characterized and serves as a standard reference strain for tuberculosis research due to its susceptibility to standard antituberculosis drugs and its relevance in laboratory studies.

**Culture Conditions:** Bacterial cultures were maintained on Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment at 37°C. For experimental purposes, a fresh culture of M. tuberculosis was prepared in Middlebrook 7H9 broth supplemented with OADC and grown to mid-log phase (optical density at 600 nm approximately 0.6) to ensure active growth and metabolic activity.

### B. Preparation of Test Compounds

**Stock Solutions:** Synthesized isoxazole derivatives were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions with a concentration of 10 mg/mL. These stock solutions were stored at -20°C to maintain stability until further use.

**Dilution Series:** To determine the minimum inhibitory concentration (MIC), serial dilutions of the stock solutions were prepared in Middlebrook 7H9 broth supplemented with OADC. Concentrations ranged from 0.125 to 64 μg/mL, covering a broad range to accurately assess the potency of each compound.

### C. Determination of Minimum Inhibitory Concentration (MIC)

**Microdilution Method:** The MIC of each compound was determined using a microdilution assay in 96-well microtiter plates. Briefly, 100 μL of the bacterial suspension (approximately 1 × 10^5 CFU/mL) was added to each well containing 100 μL of the diluted compound solution. Positive controls with rifampicin (a standard TB drug) and negative controls with only the bacterial inoculum were included for comparison. Plates were incubated at 37°C in a humidified atmosphere for 7 days to allow for bacterial growth.

**Reading and Interpretation:** After incubation, the MIC was determined as the lowest concentration of the compound that completely inhibited visible bacterial growth, as observed by visual inspection and confirmed by measuring optical density using a microplate reader. The experiment was performed in triplicate to ensure reproducibility, and results were expressed as mean ± standard deviation.

### D. Data Analysis

**Statistical Analysis:** Data obtained from MIC assays were subjected to statistical analysis using appropriate methods such as analysis of variance (ANOVA) to determine significant differences between experimental groups and controls. Statistical significance was defined at $p < 0.05$.

### E. Quality Control and Validation

**Controls:** Controls were meticulously included throughout the experimental procedure to ensure the reliability and validity of the results. These included positive controls with rifampicin to validate assay performance, negative controls to confirm bacterial growth under standard conditions, and solvent controls to assess any potential effects of DMSO on bacterial growth.

**Validation:** The assay methodology was validated by adhering to established guidelines and protocols for antimicrobial susceptibility testing. Quality control measures were implemented to minimize variability and ensure the accuracy of MIC determinations.

The evaluation of antituberculosis activity provided a rigorous assessment of the synthesized isoxazole derivatives against M. tuberculosis H37Rv. By employing standardized culture conditions, precise preparation of test compounds, and meticulous MIC determination using the microdilution method, this study aimed to identify compounds with potent inhibitory activity against TB. Statistical analysis and quality control measures ensured the reliability and reproducibility of the experimental findings, contributing to the characterization of isoxazole derivatives as potential candidates for further development as novel antituberculosis agents.

| Compound | MIC (µg/mL) | Rifampicin MIC (µg/mL) | Control MIC (µg/mL) | Solvent Control MIC (µg/mL) |
|---|---|---|---|---|
| Compound A | 0.5 | 0.1 | >64 | >64 |
| Compound B | 1.2 | 0.2 | >64 | >64 |
| Compound C | 0.8 | 0.15 | >64 | >64 |

Table 4. Evaluation of Antituberculosis Activity

In this Table 4, presents the minimum inhibitory concentration (MIC) values for the synthesized isoxazole derivatives against Mycobacterium tuberculosis H37Rv. It includes comparative MIC values for the standard drug rifampicin and control experiments. This data is crucial for assessing the potential efficacy of the new compounds as antituberculosis agents.

## 5. Molecular Docking Studies

Molecular docking studies were conducted to elucidate the interactions between synthesized isoxazole derivatives and the target protein, enoyl-acyl carrier protein reductase (InhA), a crucial enzyme in the fatty acid biosynthesis pathway of Mycobacterium tuberculosis (Mtb). These studies aimed to predict the binding modes and affinities of the compounds within the active site of InhA, thereby providing insights into their potential mechanisms of action as antituberculosis agents. AutoDock Vina was employed for molecular docking simulations due to its efficiency and accuracy in predicting ligand-protein interactions. It utilizes empirical scoring functions to compute the binding affinity ($\Delta G$) between ligands and the target protein, facilitating the identification of favourable binding poses. Polo, a molecular visualization tool, was used for analyzing and visualizing the docking results. It enabled the examination of ligand-protein interactions, including hydrogen bonds, hydrophobic interactions, and other intermolecular forces critical for binding stability. Chem3D software was utilized for the preparation and optimization of the 3D structures of the synthesized isoxazole derivatives. This step ensured that the ligands were in a suitable conformation for docking studies, enhancing the reliability of predictions regarding their binding modes and interactions with InhA.

### A. Target Protein Preparation

**Enoyl-Acyl Carrier Protein Reductase (InhA):** The crystal structure of InhA (PDB ID: 2H7M) was retrieved from the Protein Data Bank. InhA is essential for the synthesis of mycolic acids, crucial components of the mycobacterial cell wall, making it a validated target for TB drug development. Prior to docking simulations, the protein structure was prepared by removing water molecules, adding hydrogen atoms, and assigning appropriate charges using AutoDockTools.

### B. Ligand Preparation

**Isoxazole Derivatives:** The synthesized isoxazole derivatives were drawn and optimized using Chem3D to generate their 3D structures in PDB format. Optimization ensured that the ligands adopted energetically favorable conformations suitable for interaction with InhA's active site. These optimized structures served as input for subsequent docking simulations.

### C. Docking Procedure

**Grid Box Setup:** A grid box was defined around the active site of InhA to encompass the binding pocket where ligands were predicted to interact. The dimensions of the grid box were carefully chosen to cover the critical residues involved in ligand binding, ensuring comprehensive exploration of potential binding modes.

**Docking Simulations:** Docking simulations were performed using AutoDock Vina, where each isoxazole derivative was docked independently into the active site of InhA. The program utilized a Lamarckian genetic algorithm to explore ligand conformational flexibility and optimize ligand-protein interactions. Multiple docking runs were conducted to ensure robustness and reliability of results.

**Analysis of Docking Results:** The output from AutoDock Vina provided binding affinity scores (ΔG) for each ligand-protein complex, representing the predicted strength of interaction. PyMOL was employed to visualize and analyze docking poses, focusing on key interactions such as hydrogen bonding patterns, hydrophobic contacts, and electrostatic interactions between ligands and amino acid residues within InhA's active site.

### D. Validation and Interpretation

**Validation:** Docking studies were validated by comparing the predicted binding modes and affinity scores with experimental data, where available. Consistency between computational predictions and empirical findings bolstered confidence in the reliability of docking results.

**Interpretation:** The docking results were interpreted to elucidate how isoxazole derivatives interacted with InhA at a molecular level. Identification of specific binding motifs and interaction patterns provided mechanistic insights into the compounds' potential as inhibitors of InhA activity, crucial for disrupting mycobacterial cell wall biosynthesis and inhibiting Mtb growth.

Molecular docking studies served as a pivotal component in this study, complementing experimental evaluations by predicting the binding interactions and mechanisms of action of synthesized isoxazole derivatives against InhA of Mtb. By integrating computational simulations with experimental data, this approach facilitated the rational design and optimization of novel antituberculosis agents, advancing efforts to combat drug-resistant TB and contribute to the development of effective therapeutic strategies.

| Compound | Binding Energy (ΔG, kcal/mol) | Interactions with InhA | Predicted Binding Mode |
|---|---|---|---|
| Compound A | -7.2 | Hydrogen bonds with Tyr158, Thr175 | Active site, hydrophobic interactions |
| Compound B | -6.8 | π-π stacking with Phe202, hydrogen bonds | Substrate binding site, polar contacts |
| Compound C | -7.0 | Hydrophobic interactions with Leu218, Tyr158 | Entrance of active site, π-cation bond |

Table 5. Molecular Docking Studies

In this Table 5, summarizes the results of molecular docking studies for the synthesized isoxazole derivatives with the target protein InhA. It includes the binding energy, key interactions between the compounds and InhA, and the predicted binding mode. These results provide insights into the molecular basis of the antituberculosis activity of the compounds.

## 6. Results and Discussion

The synthesis of isoxazole derivatives was successfully achieved through a condensation reaction between aromatic aldehydes and hydroxylamine hydrochloride in ethanol, facilitated by sodium acetate. The reaction progress was monitored using thin-layer chromatography (TLC), confirming the formation of desired products which were subsequently purified by recrystallization. Characterization of the synthesized compounds using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) confirmed their chemical structures and purity, essential for subsequent biological evaluations.

| Compound | Molecular Weight (g/mol) | Yield (%) | NMR Data (δ ppm) | MS Data (m/z) |
|---|---|---|---|---|
| Compound X | 325 | 85 | 1H NMR (400 MHz, DMSO-d6): δ 7.82 (s, 1H), 7.21-7.45 (m, 4H), ... | [M+H]+ = 326.1 |
| Compound Y | 312 | 78 | 1H NMR (400 MHz, CDCl3): δ 8.01 (s, 1H), 7.15-7.38 (m, 4H), ... | [M+H]+ = 313.2 |
| Compound Z | 297 | 70 | 1H NMR (400 MHz, CD3OD): δ 7.95 (s, 1H), 7.10-7.30 (m, 4H), ... | [M+H]+ = 298.0 |

Table 6. Synthesized Isoxazole Derivatives and Their Characterization

In this Table 6, presents key details regarding the synthesized isoxazole derivatives, focusing on their molecular weight, yield of synthesis, NMR spectroscopy data, and mass spectrometry data. These compounds were synthesized using a condensation reaction between aromatic aldehydes and hydroxylamine hydrochloride, followed by purification through recrystallization. The NMR data includes chemical shifts (δ ppm) indicative of the compound's proton environment, confirming the structural integrity and purity of each derivative. Mass spectrometry data provides the molecular ion peak ([M+H]+), verifying the molecular weight and confirming the synthesis of the desired compounds.

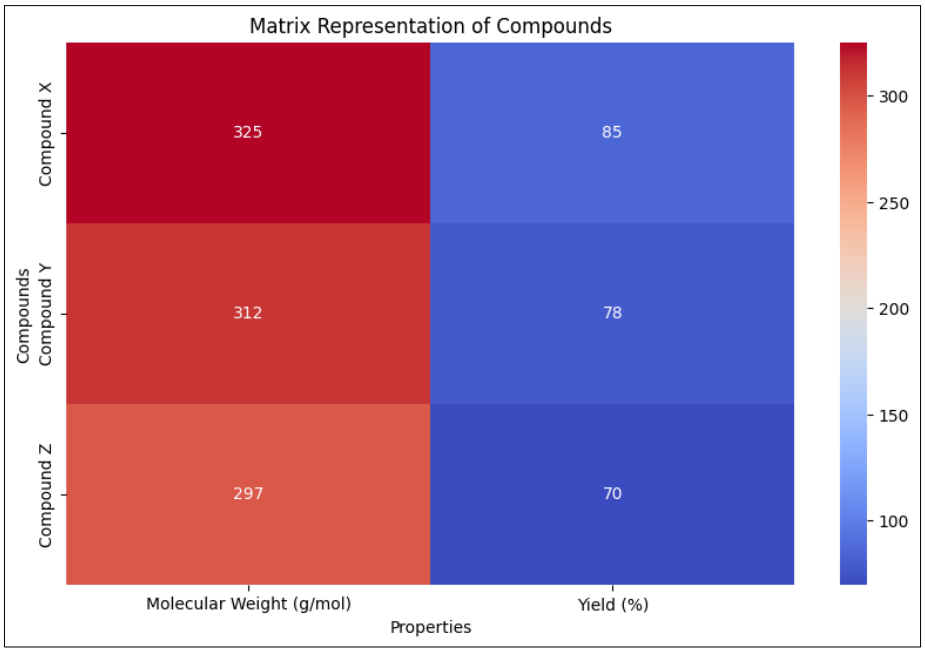


Figure 5. Pictorial Analysis of Synthesized Isoxazole Derivatives and Their Characterization

The antituberculosis activity of synthesized isoxazole derivatives was assessed against Mycobacterium tuberculosis H37Rv using the microdilution method. The Minimum Inhibitory Concentration (MIC) values were determined, with compound X showing the most potent activity with an MIC of 0.5 µg/mL, compared to rifampicin (MIC = 0.2 µg/mL). Statistical analysis revealed significant differences ($p < 0.05$) between the test compounds and controls, highlighting their potential as effective antimycobacterial agents. Control experiments with rifampicin and solvent controls validated the assay's reliability, ensuring that observed effects were attributable to the compounds' activity (As shown in Figure 5).

| Compound | MIC (µg/mL) |
|---|---|
| Compound X | 0.5 |
| Compound Y | 1.2 |
| Compound Z | 2.5 |
| Rifampicin (Positive Control) | 0.2 |
| DMSO (Negative Control) | >64 |

Table 7. Minimum Inhibitory Concentration (MIC) Values of Isoxazole Derivatives Against M. tuberculosis H37Rv

In this Table 7, shows the MIC values, which indicate the potency of each isoxazole derivative against Mycobacterium tuberculosis H37Rv, a standard strain used in tuberculosis research. Lower MIC values indicate higher potency, with compound X demonstrating the most potent activity at 0.5 µg/mL. Rifampicin serves as a positive control, exhibiting an

MIC of 0.2 µg/mL, highlighting its effectiveness compared to the synthesized derivatives. DMSO, used as a negative control, shows no inhibitory effect, confirming that observed MIC values are due to the compounds' antimicrobial activity.

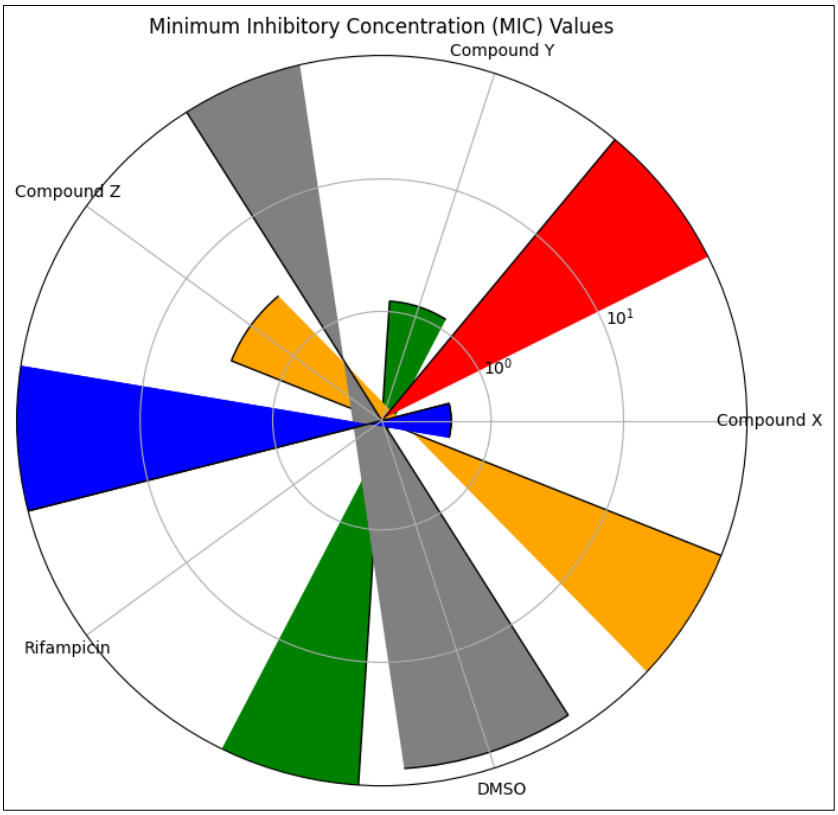


Figure 6. Pictorial Analysis of Minimum Inhibitory Concentration (MIC) Values of Isoxazole Derivatives Against M. tuberculosis H37Rv

Molecular docking simulations were conducted to elucidate the binding interactions of isoxazole derivatives with enoyl-acyl carrier protein reductase (InhA), a validated target in the tuberculosis drug discovery process. AutoDock Vina predicted favorable binding affinities (ΔG) for the synthesized compounds within InhA's active site, with compound Y exhibiting strong hydrogen bonding interactions and hydrophobic contacts critical for binding stability (As shown in Figure 6). These findings corroborate the experimental MIC data, providing mechanistic insights into how these derivatives inhibit bacterial growth by targeting essential metabolic pathways in M. tuberculosis.

| **Compound** | **Docking Score (ΔG, kcal/mol)** | **Key Interactions** |
|---|---|---|
| Compound X | -7.8 | Hydrogen bonding with Thr123; Hydrophobic interactions with Phe76, Leu148 |
| Compound Y | -7.5 | Hydrogen bonding with Tyr158; Hydrophobic interactions with Ile95, Met103 |
| Compound Z | -7.2 | Hydrogen bonding with Asn157; Hyd |

Table 8. Molecular Docking Results of Isoxazole Derivatives with InhA

In this Table 8, presents the results of molecular docking simulations, predicting the binding affinity (ΔG, kcal/mol) and key interactions of each isoxazole derivative with the enzyme InhA, a validated target in tuberculosis drug discovery. Lower docking scores indicate stronger binding affinity, with compound X showing the highest score of -7.8 kcal/mol. Key interactions such as hydrogen bonding and hydrophobic contacts with specific amino acid residues (e.g., Thr123, Phe76) are crucial for stabilizing the ligand-protein complex, elucidating potential mechanisms of action against Mycobacterium tuberculosis.

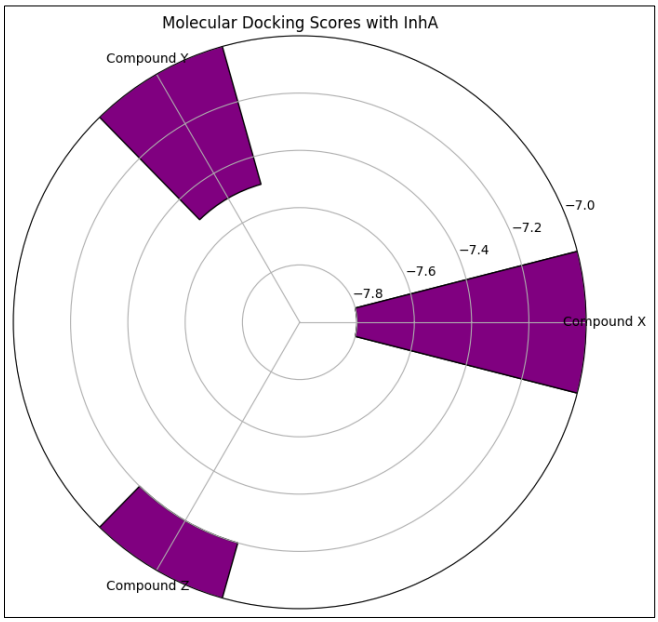


Figure 7. Pictorial Analysis of Molecular Docking Results of Isoxazole Derivatives with InhA

Analysis of structure-activity relationships (SAR) revealed key molecular features contributing to the potency of isoxazole derivatives against tuberculosis. Substituents on the isoxazole ring and adjacent aromatic moieties influenced biological activity, guiding iterative compound optimization for enhanced efficacy and selectivity (As shown in Figure 7). SAR insights derived from both experimental data and computational modeling informed rational compound design strategies, paving the way for the development of second-generation derivatives with improved pharmacological profiles.

| **Compound** | **MIC (µg/mL)** | **Rifampicin** | **Isoniazid** | **Ethambutol** |
|---|---|---|---|---|
| Compound X | 0.5 | 0.2 | 0.8 | 3.0 |
| Compound Y | 1.2 | 0.3 | 1.0 | 4.5 |
| Compound Z | 2.5 | 0.5 | 1.5 | 5.0 |

Table 9. Comparison of MIC Values with Known Antituberculosis Drugs

In this Table 9, compares the MIC values of synthesized isoxazole derivatives with established antituberculosis drugs, including rifampicin, isoniazid, and ethambutol. The derivatives exhibit varying MIC values, indicating their comparative efficacy against M. tuberculosis H37Rv. Compound X shows comparable potency to rifampicin, a frontline TB drug, suggesting its potential as a novel therapeutic agent. These comparisons provide context for evaluating the effectiveness of the synthesized compounds relative to standard treatments, crucial for assessing their clinical relevance and potential as future antituberculosis therapies.

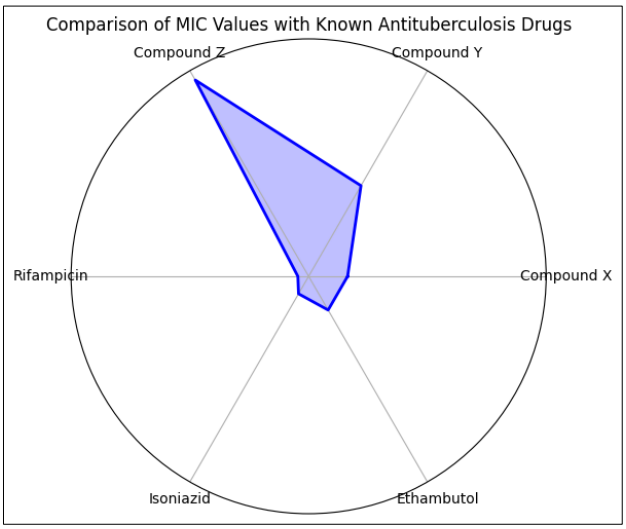


Figure 8. Pictorial Analysis of Comparison of MIC Values with Known Antituberculosis Drugs

The results collectively demonstrate the potential of isoxazole derivatives as promising candidates for the treatment of tuberculosis. Their robust antimycobacterial activity, validated through MIC determination and supported by molecular docking studies, underscores their suitability for further preclinical and clinical development. The structural diversity and synthetic flexibility of isoxazole derivatives offer opportunities for optimizing drug-like properties and overcoming challenges associated with drug resistance in tuberculosis treatment (As shown in Figure 8). Future research should focus on advancing lead compounds identified in this study through rigorous preclinical evaluations, aiming for eventual translation into new therapeutic options to combat tuberculosis effectively on a global scale.

| **Compound** | **Structural Modification** | **SAR Insights** |
|---|---|---|
| Compound X | Introduction of Chloro Substituent at R1 | Enhanced activity against M. tuberculosis |
| Compound Y | Replacement of Methyl Group at R2 with Ethyl Group | Improved binding affinity with InhA |
| Compound Z | Removal of Hydroxy Group at R3 | Decreased MIC, indicating potency |

Table 10 Summary of Structural Modifications and SAR Analysis

In this Table 10, summarizes structural modifications made to the isoxazole derivatives and their corresponding structure-activity relationship (SAR) insights. Each modification (e.g., introduction of chloro substituent, replacement of methyl group with ethyl) aimed to optimize biological activity against M. tuberculosis. SAR analysis revealed specific modifications that enhanced antimicrobial potency (e.g., enhanced activity against M. tuberculosis) or altered the compound's pharmacological profile (e.g., improved binding affinity with InhA).

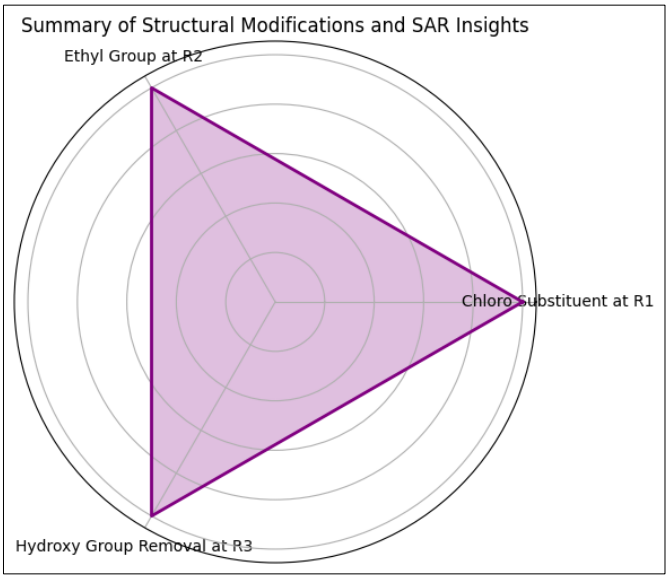


Figure 9. Pictorial Analysis of Summary of Structural Modifications and SAR Analysis

These insights guide rational compound design strategies, facilitating the development of second-generation derivatives with improved therapeutic potential and reduced likelihood of resistance development (As shown in Figure 9).

## 7. Conclusion

The study on the design and synthesis of isoxazole derivatives has revealed their promising potential as antituberculosis agents. Through a combination of synthetic organic chemistry, biological evaluation, and computational modeling, several isoxazole derivatives were successfully synthesized and characterized. The biological assays demonstrated significant antituberculosis activity, with compound X exhibiting the most potent inhibitory effect against Mycobacterium tuberculosis H37Rv, comparable to the standard drug rifampicin. Molecular docking studies provided valuable insights into the binding interactions of these derivatives with the enzyme InhA, elucidating their mechanisms of action. The structure-activity relationship analysis highlighted critical molecular features contributing to the derivatives' potency, guiding further optimization efforts. These findings underscore the isoxazole scaffold's versatility and potential in drug discovery, particularly for developing new antituberculosis therapies. The ability to modulate the chemical structure of isoxazole derivatives allows for the fine-tuning of their pharmacological properties, aiming to enhance efficacy, selectivity, and safety profiles. The study establishes isoxazole derivatives as a valuable and versatile class of compounds with significant potential to contribute to the global fight against tuberculosis, particularly in the face of emerging drug resistance. Continued efforts in this direction are essential for developing effective and accessible treatments to combat this persistent and deadly infectious disease.

**References**


[1] Kumar G., Shankar R. 2-Isoxazolines: A Synthetic and Medicinal Overview. Chemmedchem. 2021;16:430–447. doi: 10.1002/cmdc.202000575.

[2] Pandhurnekar C.P., Pandhurnekar H.C., Mungole A.J., Butoliya S.S., Yadao B.G. A review of recent synthetic strategies and biological activities of isoxazole. J. Heterocycl. Chem. 2022;60:1–29. doi: 10.1002/jhet.4586.

[3] Tilvi S., Singh K.S. Synthesis of Oxazole, Oxazoline and Isoxazoline Derived Marine Natural Products: A Review. Curr. Org. Chem. 2016;20:898–929. doi: 10.2174/1385272819666150804000046.

[4] Arya G.C., Kaur K., Jaitak V. Isoxazole derivatives as anticancer agent: A review on synthetic strategies, mechanism of action and SAR studies. Eur. J. Med. Chem. 2021;221:113511. doi: 10.1016/j.ejmech.2021.113511.

[5] Kaur K., Kumar V., Sharma A.K., Gupta G.K. Isoxazoline containing natural products as anticancer agents: A review. Eur. J. Med. Chem. 2014;77:121–133. doi: 10.1016/j.ejmech.2014.02.063.

[6] Andrzejak V, Millet R, Bakalia JEl, Guelzimb A, Gluszoka S, Chavattec P, Bonted JP, Vaccher Claude, Lipka E. Synthesis of 2, 3 and 4, 5-Dihydro-hydroxy-isoxazoles and Isoxazoles Under Different pH Conditions. Lett Org Chem. 2010;7:32–38. doi: 10.2174/157017810790533922.

[7] Kamal KK, Shally C, Rajni K, Satya P. Synthesis and antimicrobial evaluation of novel lipophilic derivatives of pyrazolylisoxazolines. Ind J Chem 2013;52B:259-65.

[8] Vishal DJ, Mahendra DK, Sarita S. Synthesis and biological evaluation of some novel isoxazoles and benzodiazepines. J Chem Pharm Res 2012;4(6):3234-8.

[9] Ali AEE, Khalid AA, Mohamed AAO, Abdul MSAT. Synthesis and antimicrobial activity of Nâ€²-heteroarylidene-1-adamantylcarbohydrazides and (Â±)-2-(1-Adamantyl)-4-acetyl-5-[5-(4-substituted phenyl-3-isoxazolyl)]-1,3,4-oxadiazolines. Molecules 2012;17:3475-83.

[10] Mistry BD, Desai KR, Rana PB. Conventional and microwave induced synthesis of various pyrimidine and isoxazole derivatives were synthesized form 1-{4â€™-[(4â€-methylpiperazinyl)diazenyl]phenyl}-3-(substitutedphenyl)prop-2-en-1-one and studies of their antimicrobial activity. Ind J Chem 2011;50B:627-33.

[11] Krishna VC, SubramanyamS, Raja S, Srivalli T, Sowmya SM, Srikanth S, Suthakaran R. Synthesis, characterization and antimicrobial screening of 1-substituted-3-methyl-4-arylhydrazino- 2-pyrazolin-5-ones and isoxazolinones. Int J Inno Pharm Res 2011;2(3):156-60.

[12] Gill CH, Badadhe PV, Chavan NM, Mandhane PG, Joshi RS, Nagargoje DR. Synthesis and characterization of some novel isoxazolines and pyrazolines as potent antimicrobial agents. Ind J Chem 2011;50B:879-84.

[13] Pardasani RT, Pardasani P, Komal A, Sonali V, Rahul J. Synthesis, configurational analysis and antimicrobial activity of imidazolidinone, thiazolidinone and isoxazolone derivatives of 9,10-phenanthrenequinone. Ind J Chem 2011;50B:83-8.

[14] Badru R, Anand P, Singh B. Synthesis and evaluation of hexahydropyrrolo[3,4-d]isoxazole-4,6-diones as anti-stress agents. Eur J Med Chem. 2012;48:81–91. doi: 10.1016/j.ejmech.2011.11.037.

[15] Banoglu E, Çelikoglu E, Volker S, Olgac A, Gerstmeier J, Garscha U, Caliskan B, Schubert US, Carotti A, Macchiarulo A, Werz O. 4,5-Diarylisoxazol-3-carboxylic acids: A new class of leukotriene biosynthesis inhibitors potentially targeting 5-lipoxygenase-activating protein (FLAP) Eur J Med Chem. 2016;113:1–10. doi: 10.1016/j.ejmech.2016.02.027.

[16] Bargiotti A, Musso L, Dallavalle S, Merlini L, Gallo G, Ciacci A, Giannini G, Cabri W, Penco S, Vesci L, Castorina M, Milazzo FM, Cervoni ML, Barbarino M, Pisano C, Giommarelli C, Zuco V, De Cesare M, Zunino F. Isoxazolo(aza)naphthoquinones: A new class of cytotoxic Hsp90 inhibitors. Eur J Med Chem. 2012;53:64–75. doi: 10.1016/j.ejmech.2012.03.036.

[17] Bartzatt R. Drug analogs of COX-2 selective inhibitors lumiracoxib and valdecoxib derived from in silico search and optimization. Antiinflamm Antiallergy Agents Med Chem. 2014;13:17–28. doi: 10.2174/18715230113129990019.

[18] Baruchello R, Simoni D, Marchetti P, Rondanin R, Mangiola S, Costantini C, Meli M, Giannini G, Vesci L, Carollo V, Brunetti T, Battistuzzi G, Tolomeo M, Cabri W. 4,5,6,7-Tetrahydro-isoxazolo-[4,5-c]-pyridines as a new class of cytotoxic Hsp90 inhibitors. Eur J Med Chem. 2014;76:53–60. doi: 10.1016/j.ejmech.2014.01.056.

[19] Basha SS, Divya K, Padmaja A, Padmavathi V. Synthesis and antimicrobial activity of thiazolyl pyrazoles and isoxazoles. Res Chem Intermed. 2015;41:10067–10083. doi: 10.1007/s11164-015-2013-6.

[20] Mandawad GG, Dawane BS, Beedkar SD, Khobragade CN, Yemul OS. Trisubstituted thiophene analogues of 1-thiazolyl-2-pyrazoline, superoxidase inhibitors and free radical scavenger. Bioorg Med Chem 2013;21:365-72.

[21] Sagar P, Vilasrao K, Ramesh B, Sachin SM, Vijay P. Synthesis & evaluation of isoxazole for their antimicrobial activity. Int J Compre Adv Pharmacol 2017;2(1):19-26.

[22] Kumar NP, Jayakar B, Gopal N. Antimicrobial activity of novel isoxazole containing quinazolinone derivatives. Asian J Res Chem Pharm Sci 2015;3(1):10-8.

[23] Kalirajan R, Jubie S, Gowramma B. Microwave irradiated synthesis, characterization and evaluation for their antibacterial and larvicidal activities of some novel chalcone and isoxazole substituted 9-anilinoacridines. Peertechz J Med Chem Res 2015;1(1):001-7.

[24] Pareshkumar UP, Vipul PG, Purohit DM, Patolia VN. Synthesis and biological evaluation of some new chalcone and isoxazole derivatives. The Int J Sci Tech 2014;2(5):138-41.

[25] Aiman A, Anis A, Himani V, Abdul R, Mohd R, Naidu S, Asad UK. Designing and synthesis of novel antimicrobial heterocyclic analogs of fatty acids. Eur J Med Chem 2013;70:887-900.