International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 4 July-Aug 2025, pp: 1016-1062 www.ijprajournal.com ISSN: 2456-4494

Design, Synthesis, Characterization and Biological Evaluation of Some Novel Heterocyclic Derivatives as Anti Tubercular Agents Targeting Inha (Enoyl Acyl Carrier Protein Reductase) Enzyme

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Date of Submission: 15-08-2025 Date of Acceptance: 25-08-2025

ABSTRACT
Tuberculosis is a transmittable disease which is considered as the major cause of death. Tuberculosis associated deaths, of which181 mostly occurred in developing countries. In addition, recently emerged drug-resistant tuberculosis is a big challenge to control tuberculosis. Therefore, there is an urgent need for the newer molecule development and more efficient evaluation of new TB drugs which may have potential to cure the diseases and shorter treatment regimens. Literature survey evidences that, Imidazole derivatives exhibit various pharmacological activities such as anti-bacterial and Anti-tubercular activity. Thus, a series of imidazole-based molecules were designed and docked against crucial mtb enzyme target Mtfab D (Malonyl COA Acyl Carrier Protein Transacylase). The docked molecules were screened against good docking-score and multiple interactions and opted for synthesis. Synthesized molecules repeatedly recrystallized to meet the purity. All the purified compounds were characterized by various spectral analytical techniques and evaluated for anti-mycobacterial activity against tuberculosis H37RV strain by Microplate Alamar Blue Assay (MABA) method. The experimental results shown that Compound (R2) possesses anti-tubercular activity with an MIC below 3.12 mcg/mL and (R4 and R6) showed moderate anti tubercular activity with an MIC below 12.5mcg/mL. Whereas R7 and RG1 with moderate activity at below 25 µg and 50mcg/mL.

I. INTRODUCTION

Tuberculosis is a chronic infection characterized by caseous necrosis and granuloma formation. It is caused by the bacteria called Mycobacterium tuberculosis. In a majority of the cases, Tuberculosis affects the lungs where it causes the characteristic cough with hemoptysis (bloody sputum)[1]. However, it can also affect other sites of the body known as Extrapulmonary TB. In 1882, Dr. Robert Koch had discovered the organism responsible ie., Bacillus Mycobacterium tuberculosis. This widely known disease is one of the diseases of stigmatization and affects one third of the world population[2]. World Health Organization (WHO) reports that relatively small proportion (5–10%) of the estimated 1.7 billion people infected with M. tuberculosis who will develop TB disease during their lifetime. However, the probability of developing TB disease is much higher among people infected with HIV; it is also higher among people affected by risk factors such as malnutrition, diabetes, smoking and alcohol consumption.

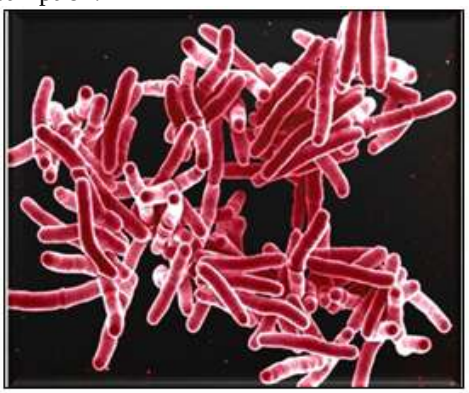

Fig: 1 Mycobacterium tuberculosis[3]

MYCOBACTERIUM GENUS[4]:
Kingdom: Bacteria
Genus: Mycobacterium
Phylum: Actinobacteria Order: Actinomycetales
Suborder: Corynebacterineae
Family: Mycobacteriaceae

Mycobacterium tuberculosis is a small, aerobic, nonmotile, rod-shaped bacillus, 2-4 µm in length and 0.2-0.5µm in width. The human host serves as the only natural reservoir for this bacterium, but it can be cultured in the laboratory. It divides every 16 to 20 hours, which is an extremely slow rate compared with other bacteria[5]. The cell wall of this bacterium is composed of

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complex lipids, such as mycolic acids that make up over half the cell envelope of the mycobacteria. Their hydrophobicity prevents diffusion of many chemicals including drugs into the bacterium. Mycolic acids also play a major role in virulence of M. tuberculosis, by protecting the organism from complement fixation and damage from lysozymes and free radicals in the phagolysosomes of neutrophils. The cell wall also contains acyl glycolipids and other substances such as free lipids and sulfolipids. The high concentration of lipids in the cell wall of this bacterium have been associated with unique clinical characteristics such as impermeability to stains and dyes, resistance to many antibiotics, resistance to killing by acidic and alkaline compounds, resistance to osmotic lysis via complement deposition, resistance to lethal oxidations and survival inside macrophages.

CLINICAL CLASSIFICATION OF MYCOBACTERIA

According to their growth rate, the Mycobacterium genus is usually separated into two major groups:

i) Slow-growing species including M. tuberculosis, M. bovis and M. leprae.
ii) Fast-growing species such as M. smegmatis.[6]

Among the pathogenic species the most relevant for human health are M. tuberculosis and M. leprae, the causative agents of two of the world's oldest diseases, tuberculosis and leprosy, respectively. M. canettii and M. africanum, which also can cause human TB, are most commonly isolated from African patients. M. bovis demonstrates the broadest spectrum of host infection, affecting humans, domestic or wild bovines and goats. M. microti can also cause disease in immuno compromised human patients. M. kansasii, M. malmoense and M. xenopi represent pulmonary opportunists, while M. marinum is the skin pathogen infecting organism by entering through damaged skin.

Sites of TB Disease:

Pulmonary Tuberculosis:

TB disease most commonly affects the lungs; this is referred to as Pulmonary TB. Patients with Pulmonary TB usually have a cough and an abnormal chest radiograph, and may be infectious. Although the majority of TB cases are Pulmonary, TB can occur in almost any anatomical site.

Extrapulmonary Tuberculosis[7]:

Extrapulmonary TB disease occurs in places other than the lungs, including the larynx, the lymph nodes, the pleura, the brain, the kidneys or the bones and joints. In HIV-infected persons, Extrapulmonary TB disease is often accompanied by Pulmonary TB.

Miliary Tuberculosis[8]:

Miliary TB occurs when tubercle bacilli enter the bloodstream and disseminate to all parts of the body, where they grow and cause disease in multiple sites. This condition is rare but serious. "Miliary" refers to the radiograph appearance of millet seeds scattered throughout the lungs. It is most common in infants and children younger than 5 years of age, and in severely immune compromised persons.

PATHOGENISIS[9,10]:

Infection occurs when a person inhales droplet nuclei containing tubercle bacilli that reach terminal alveoli in the lungs. The first step is the recognition of mycobacteria as invading pathogens, followed by activation of innate host defense responses. Cell- mediated immunity is usually developed within approximately two to eight weeks from the initial infection, followed by initiation of adaptive immune responses. The activated T lymphocytes, macrophages, and other immune cells form granulomas that limit further replication and spread of the tubercle bacilli.

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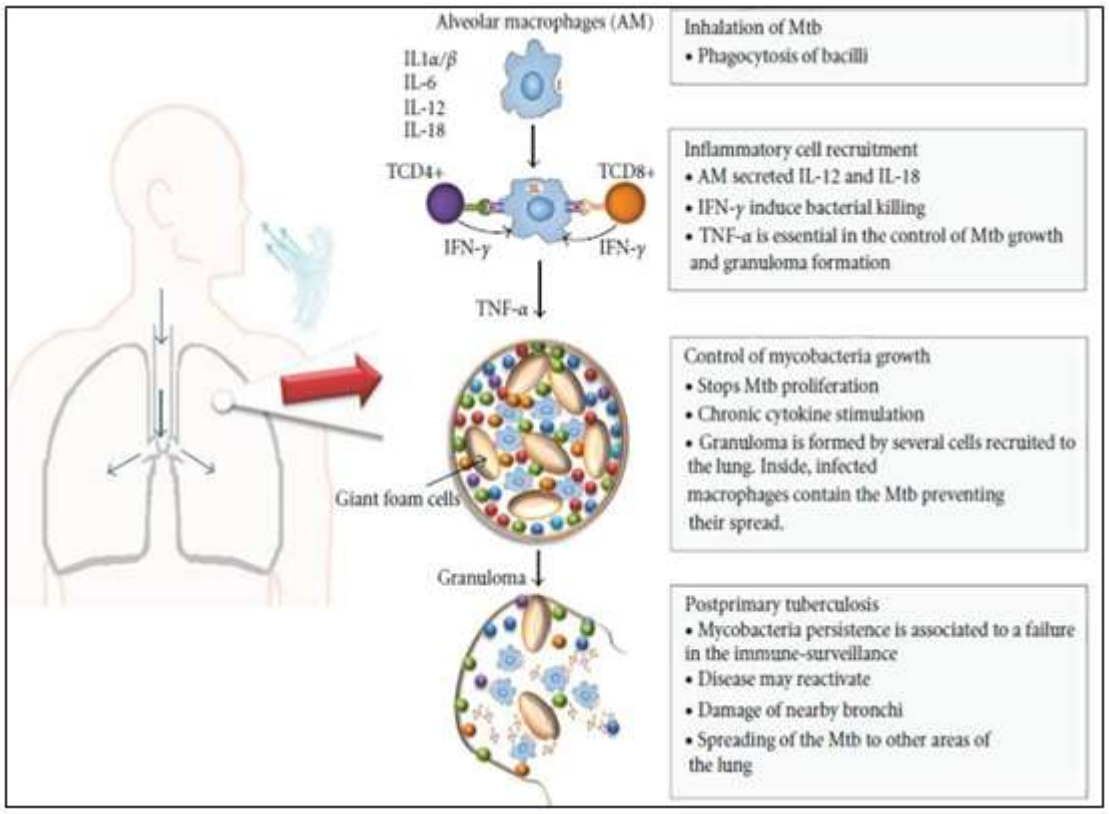

Fig : 2 Pathogenisis of Mycobacterium tuberculosis[11]

LATENT TUBERCULOSIS[12]:

Latent tuberculosis (LTB), also called latent tuberculosis infection (LTBI) Latent TB infection occurs when Mycobacterium tuberculosis run away from the immune system. Latent Tuberculosis infection is generally not contagious and produces no symptoms, but the bacilli may be present in the body. Individuals who have latent infection cannot transmit infection to others. In this infection, most of the individuals do not develop active disease, but the problem occurs when latent infection becomes active. Approximately 10% of the individuals develop active Tuberculosis disease. Some of them develop active disease soon after the infection, whereas, other people develop later, when their immune system becomes weak for one or the other reason. The global prevalence of LTBI broadly reflects the local prevalence of TB. Those with documented contact with a case of transmissible TB are at the highest risk of infection (up to 50% chance). In low-incidence countries, the prevalence of infection in the general population is approximately 1% among young adults, but can increase to 8% in elderly adults, 5and up to 25% in adult migrants from high-incidence countries.

Characteristics of Latent Tuberculosis[13]:

People with latent tuberculosis:

- Have no symptoms of tuberculosis
- Don't feel sick
- Can't spread tuberculosis to others
- Usually have a positive tuberculosis skin test reaction (PPD test)
- In some cases, can develop active tuberculosis if they do not receive treatment for latent tuberculosis.

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PROGRESSION OF TB

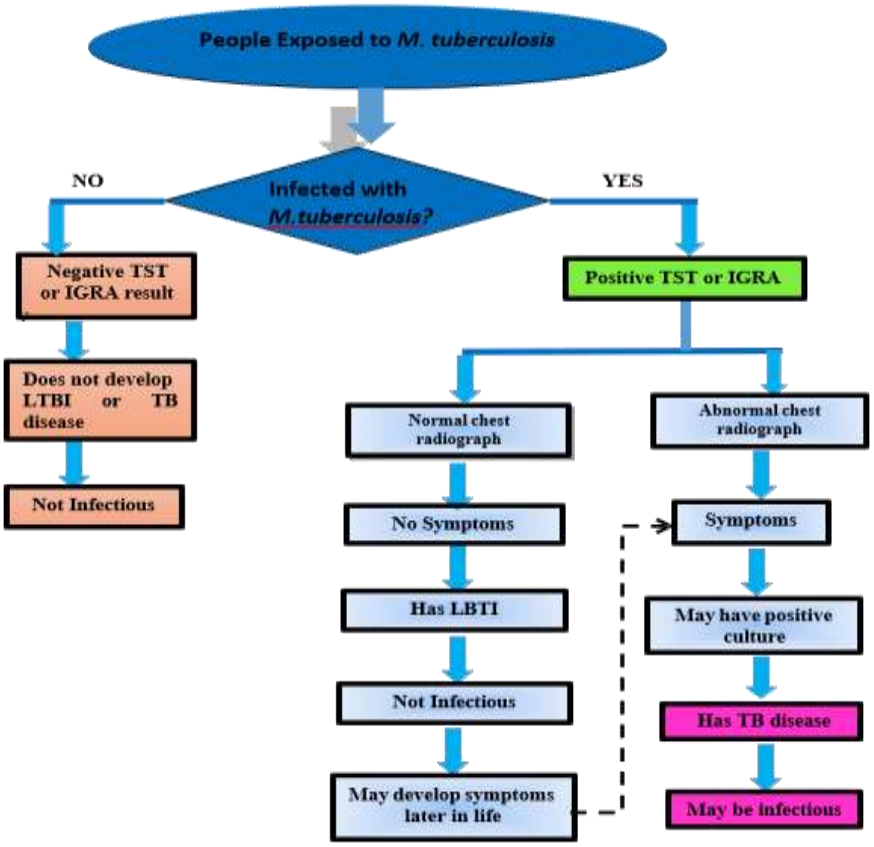

Fig: 3 Progression of TB[14]

SYMPTOMS[15]:

- Coughing with blood sputum
- Loss of appetite
- Weight loss
- Night sweats
- Fever
- Chest pain
- Urine discoloration

DIAGNOSIS[16]:

1. Bacteriological test:

i) **Ziehl-Neelsen stain,** also known as the **Acid-fast stain.** It is the first step in the diagnosis and screening of pulmonary tuberculosis.

ii) **Auramine-rhodamine stain** is a histological technique used to visualize acid fast bacilli using fluorescence microscopy. Acid-fast organisms display a reddish-yellow fluorescence.

2. Sputum culture test:

i) Lowenstein-Jensen solid medium 4 to 18 weeks

ii) Liquid medium 8 to 14 days

iii) Agar medium 7 to 14 days

3. Tuberculin skin test:

The **Mantoux test** is the standard method of determining whether a person is infected with Mycobacterium tuberculosis. The local skin reaction to Tuberculin Purified Protein Derivative (PPD) injected into the skin is used to assess the individual's sensitivity to tuberculin protein.

TB DISEASE BURDEN[17]:

✓ Worldwide, tuberculosis (TB) is one of the top 10 causes of death, and the leading cause from a single infectious agent (above HIV/AIDS); millions of people continue to fall sick with the disease each year.

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- ✓ In 2017, TB caused an estimated 1.3 million deaths among HIV-negative people, and there were an additional 300 000 deaths from TB among HIV-positive people.
- ✓ TB affects all countries and all age groups, but overall the best estimates for 2017 were that 90% of cases were adults (aged ≥15 years), 64% were male, 9% were people living with HIV (72% of them in Africa)
- ✓ And two thirds were in eight countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%).

DRUG-RESISTANT TB (MDR and XDR)[18,19]:

- ✓ Drug-resistant TB is caused by M. tuberculosis organisms that are resistant to the drugs normally used to treat the disease.
- ✓ Drug-resistant TB is transmitted in the same way as drug-susceptible TB, and is no more infectious than drug-susceptible TB. However, delay in the recognition of drug resistance or prolonged periods of infectiousness may facilitate increased transmission and further development of drug resistance.
- ✓ Multidrug-resistant TB (MDR TB) is caused by organisms resistant to the most effective anti-TB drugs, Isoniazid and Rifampin. These drugs are considered first- line drugs and are used to treat most persons with TB disease.
- ✓ Extensively drug-resistant TB (XDR TB) is a relatively rare type of drug-resistant TB. XDR TB is resistant to Isoniazid and Rifampin, plus any Fluoroquinolone and at least one of three injectable second-line drugs (i.e., Amikacin, Kanamycin or Capreomycin). Because XDR TB is resistant to first-line and second-line drugs, patients are left with treatment options that are more toxic, more expensive, and much less effective.

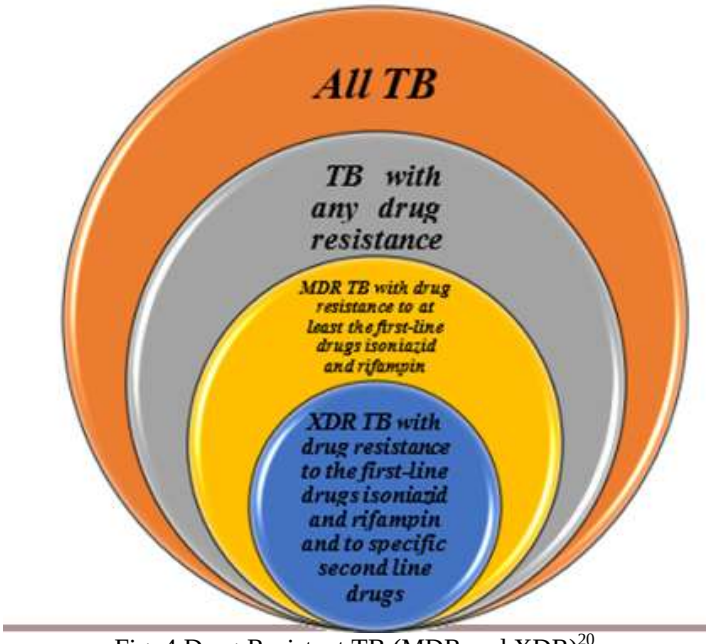

Fig: 4 Drug-Resistant TB (MDR and XDR)[20]

NEED FOR NOVEL ANTI-TUBERCULOSIS AGENTS

- ✓ A number of once active Anti-tuberculosis drugs have now become inactive due to the ever-increasing rise in drug resistant strains of tuberculosis.
- ✓ Drug-resistant TB is a major public health concern.
- ✓ A growing awareness of the increasing drug-resistance and a great need for therapy shortening together with killing also latent forms of M.tb lead to the discovery of more efficient and less toxic treatment regimens.
- ✓ Emergence of Multi-resistant (MDR) strains and high susceptibility of human immunodeficiency virus (HIV) infected persons to the disease forced the scientist to develop novel anti-tuberculosis agents.

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- ✓ It is evident by these facts; there is an ever growing need to develop novel agents for the treatment of tuberculosis. These new agents should be potent, fast acting, have an excellent Pharmacodynamics / Pharmacokinetics profile and have high therapeutic index, and preferably have a novel mechanism of action as to avoid cross-resistant with other agents.

CURRENT TB RESEARCH AND DEVELOPMENT:

- ✓ A small number of technologies emerged in 2017–2018 and several have not demonstrated adequate performance in field evaluation studies.
- ✓ There are 20 drugs in Phase I, II or III trials for the treatment of drug-susceptible TB, multidrug-resistant TB or latent TB infection.
- ✓ Various combination regimens with new or repurposed drugs are in Phase II or Phase III trials[21].
- ✓ Twelve vaccine candidates are in clinical trials: Four in Phase I, Six in Phase II and two in Phase III.
- ✓ They include candidates to prevent the development of TB infection and disease, and candidates to help improve the outcomes of treatment for TB disease.

DRUG DISCOVERY[22]:

Drug discovery is a process, which aims at identifying a compound therapeutically useful in treating and curing a disease. The process of drug discovery involves the identification of ligand, synthesis, characterization, screening, and assays for therapeutic efficacy. The phase between hit identification and lead selections is called the **hit-to-lead phase**. The dominant and the most widely applicable technique for the identification of lead compounds is **HTS** an experimental screening technique based on roboting where large numbers of different compounds are screened in a time as short as possible and at reasonable costs.

Fig: 5 Drug discovery[23]

COMPUTER AIDED DRUG DESIGN IN THE DRUG DISCOVERY PIPELINE[24]:

CADD is capable of increasing the hit rate of novel drug compounds because it uses a much more targeted search than traditional HTS and combinatorial chemistry. It not only aims to explain the molecular basis of therapeutic activity, but also to predict possible derivatives that would improve activity. CADD can be classified into two general categories:

- Structure-based drug design
- Ligand-based drug design

STRUCTURE-BASED DRUG DESIGN[25]:

It relies on the knowledge of the target protein structure to calculate interaction energies for all compounds tested. The core hypothesis of this approach is that a molecule's ability to interact with a specific protein and exert a desired biological effect depends on its ability to favorably interact with a particular binding site on that protein.

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Steps Involved in Structure-based Drug Design

1. Identification of drug target
2. Determination of target structure
3. Identification of binding site
4. Computational drug design methods
5. Evaluation of potential lead candidate

LIGAND-BASED DRUG DESIGN:

It exploits the knowledge of known active and inactive molecules through chemical similarity searches or construction of predictive Quantitative Structure-Activity Relation (QSAR) models. The overall goal is to represent these compounds in such a way that the physicochemical properties most important for their desired interactions are retained, whereas extraneous information not relevant to the interactions is discarded.

MOLECULAR DOCKING[26]:

Molecular Docking is the technique which envisages the "preferred orientation of one molecule to a second when bound to each other to form a stable complex in three dimensional spaces." The success of a docking program depends on two components: the **Search algorithm** and the **Scoring function.**

SEARCH ALGORITHM:

Search algorithm finds different conformations for the ligand. Systemic searches explore all possible binding modes between the ligands and receptor. However, this takes a huge amount of computational time especially for large flexible ligands. The amount of conformational space explored and the computational time required for the search must be balanced.

SCORING FUNCTIONS:

The aim of scoring is to quantify the free energy associated with protein and ligand in the formation of the protein-ligand interactions. They are used to rank the different conformations obtained by the search algorithm. The score in empirical scoring function is derived from individual energy contributions of each component involved in intermolecular interactions.

$$\Delta G_{bind} = \Delta G_0 + \Delta G_{hb} \sum h\text{-bonds} + \Delta G_{ionic} \sum ionic\text{-}int + \Delta G_{lipophilic} \left| A \right| + \Delta G_{rot} NROT$$

Where:

ΔG_0– empirically derived offset that in part corresponds to the overall loss of translational and rotational entropy of the ligand upon binding.

ΔG_{hb}– contribution from hydrogen bonding.

ΔG_{ionic} – contribution from ionic interactions.

ΔG_{lip} – contribution from lipophilic interactions.

$|A_{lipo}|$ is surface area of lipophilic contact between the ligand and receptor.

BIOLOGICAL TARGET[27]

There are various biosynthetic enzymes that are essential for the survival of the Mycobacterium and are considered as potential drug targets. Some of the target enzymes are,

- Mycobacterium tuberculosis InhA, the enoyl acyl carrier protein reductase
- Mycobacterium tuberculosis Glutamine synthetase 1
- Mycobacterium tuberculosis Thymidylate Kinase
- Mycobacterium tuberculosis Protein Kinase G
- Mycobacterium tuberculosis Gyrase Type IIA Topoisomerase
- Mycobacterium tuberculosis L, D Transpeptidase 2.

Enoyl-acyl carrier protein reductase or **InhA** is a key enzyme of the type II fatty acid synthesis (FAS) system, and is the part of a short chain dehydrogenase/reductase family. The direct InhA inhibitors require no mycobacterial enzymatic activation and thus circumvent the resistance mechanism to antitubercular prodrugs such as Isoniazid (INH) and Ethionamide (ETA) that is most commonly observed in drug-resistant clinical isolates.

ENZYME PROFILE:

ENZYME NAME:ENOYL-ACP REDUCTASE
CLASSIFICATION:OXIDO REDUCTASE
POLYMER: 1
TYPE:Protein
CHAINS:A, B
ORGANISM:Mycobacterium tuberculosis
PROTEOME:Chromosome
FUNCTIONAL CATEGORY:Type II fatty acid biosynthesis pathway

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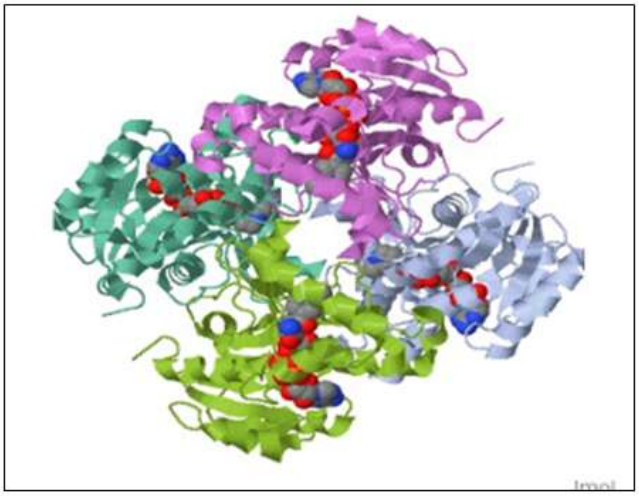

Fig : 6 InhA Enzyme[28]

InhA is an NADH dependent trans enoyl-acyl carrier protein that is part of the fatty acid biosynthesis system and plays a role in the synthesis of Mycolic acid.

Mycolic acid is a long chain fatty acid that is essential in cell wall formation of the human pathogen Mycobacterium tuberculosis.

The InhA enzyme of Mycobacterium tuberculosis is a homotetramer composed of repeating subunit of a single domain with a Rossmann Fold (super-secondary structure that is characterized by an alternating motif of beta-strand-alpha helix-beta strand secondary structure) in the core that provides a NADH binding site.

The single domain can be broken down into two substructures that are connected by short peptide loop. The overall structure exhibits α/β folding with a series of α helices flanking a central β sheet of multiple parallel β strands.

InhA's Function in the Mycolic Acid Pathway:

InhA plays a key role in the synthesis of fatty acids, particularly in M. tuberculosis which, has type one fatty acid synthesis (FASI) and type two fatty acid synthesis (FASII) which together function in the synthesis of mycolic acids. FASI synthesizes C16-18 and C24-26 fatty acids. The fatty acids from FASI are then sent to FASII which promotes chain extension, forming long-chain meromycolic acids that are 56-64 carbons in length. The final step in FAS II is completed by InhA which reduces 2-trans-enoyl-ACPs with chain lengths over twelve carbons in a NADP hydride transfer precedes protonation.

The reaction takes place as follows: initially NADH binds to the active site mediated by van der Waal interactions with the side chains of phenylalanine 41 (F41), leucine 218 and methionine 155 (K218 and M155) to the phosphate group of NADH. There are additional interaction with lysine 165 (K165) that also mediate binding. Binding of NADH causes a conformational change in the Aspartate 42 and Arginine 43 (E42 and R43) side chains and an overall conformational change in InhA. In addition, tyrosine 158 (Y158) plays an important role in aligning the carbonyl substrate, in fact; rotation about its Cα-Cβ bond by 60° brings it into a position where it can hydrogen bond to the carbonyl of the 2-trans enoyl-ACP and provide it with electrophilic stabilization. The substrate binds in a U-shaped conformation with its trans double bond adjacent to the nicotinamide ring of NADH. InhA then reduces the 2-trans double bond of the substrate by forming a enoyl intermediate through the transfer of a hydride ion from NADH to the third carbon of the substrate, followed by protonation of the second carbon. The binding of both the substrate and the cofactor induces another conformational change in InhA that allows for the release of the meromycolic acid product. The meromycolic acids undergo claisen condensation with a C26 fatty acid followed by reduction to a mature mycolic acid.

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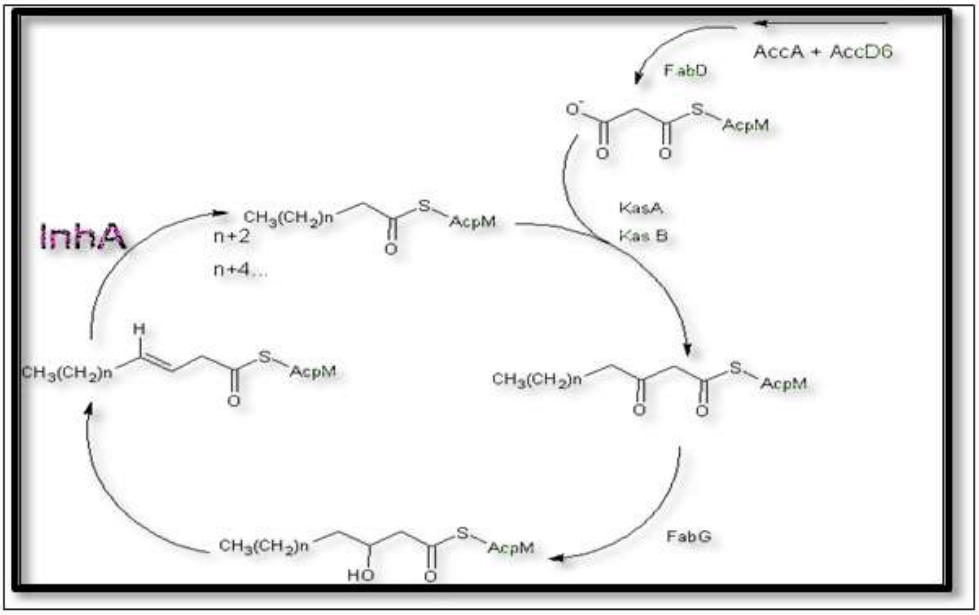

Fig: 7 InhA's Function in the Mycolic Acid Pathway

BASIC NUCLEUS

Heterocyclic structures always are a part in the field of research and development in organic chemistry. Millions of heterocyclic structures are found to exist having special properties and biological importance.

PYRIDINE[29,30,31]

Nucleus are defined by the presence of a six-membered heterocyclic ring consisting of five carbon atoms and one nitrogen atom. The arrangement of atoms is similar to benzene except that one of the carbon−hydrogen ring has been replaced by a nitrogen atom.

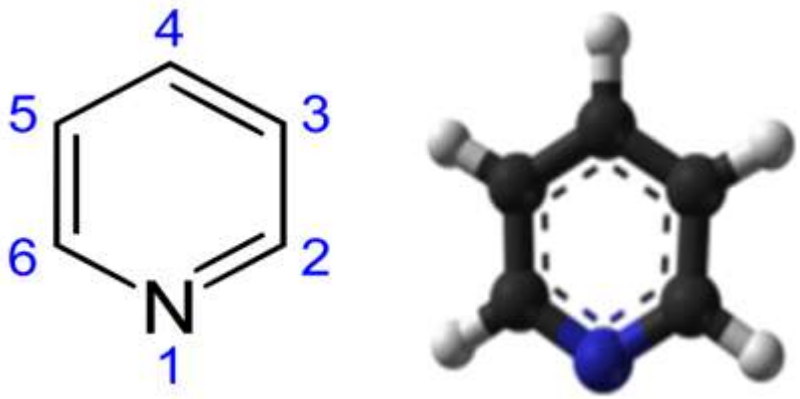

Fig: 8 General structure of Pyridine[32]

Physical properties: C_5H_5N Organic base; flammable, toxic yellowish liquid, with penetrating aroma and burning taste; soluble in water, alcohol, ether, benzene, and fatty oils; boils at 116 °C.

Pyridine derivatives exhibited various types of biological activities viz Antimicrobial, Antibacterial, Antimycobacterial, Analgesic, Antiparkinsonian, Anticonvulsant, Antitumoral, Cytotoxic, Antimalarial, Antidiabetic, Pesticidal, inhibitor and receptor antagonists.

THIADIAZOLE[33,34,35]

Ring system contains five membered heterocyclic compounds containing two nitrogen and a sulfur atom with two double bonds, to give an aromatic ring and the molecular formula is $C_2H_2N_2S$.

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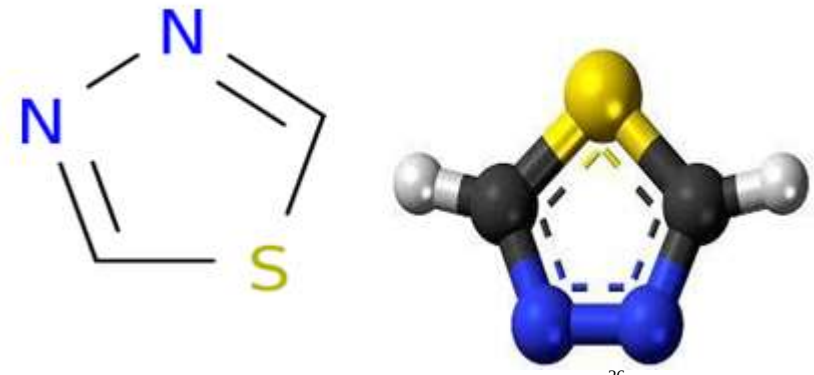

Fig: 9 General structure of Thiadiazole[36]

There are five isomeric forms of thiadiazole which occur in nature as1,3,4-thiadiazoles, 1,2,3-thiadiazole; 1,2,5-thiadiazole; 1,2,4-thiadiazole. 1,3,4-thiadiazoles are important compounds in medicine, agriculture, and in many fields of technology.

It is also known to have unique Antibacterial and Anti-inflammatory activities. Differently substituted thiadiazole moieties have also been found to have other interesting activities such as analgesic, Antimicrobial, Anti-tubercular, Anticonvulsant and Anti- Hepatitis B viral activities. Due to presence of –N=C-S moiety in its structure 1,3,4-thiadiazoles exhibit various biological activities.

II. LITERATURE REVIEW

Review of study about Tuberculosis:

1. **Manishakotadiya et al.,** (2018)[37], reported that the Advances in TB drug development over the past decade are leading to the development of enhanced MDR-TB treatments with simple and short regimens. Additionally, efforts must be made to reduce the development of resistance to these valuable new TB drugs during treatment.
2. **Cheng-Yu Kuo et al.,** (2017)[38], reported that almost no resistance to the tested second- line Antituberculosis drugs among non-MDR-Mtbs. Anti-tuberculosis regimen with Pyrazinamide, Ethambutol, Fluoroquinolone, Kanamycin, Cyclomerizeand p-Aminosalicylic acid can be empirically used for newly diagnosed MDR-TB cases.
3. **Cucunawangsih et al.,** (2015)[39], confirmed that drug resistance, including MDR, observed against all first-line TB drugs was a real threat in the management of TB infection in Indonesia. The resistance pattern identified in this study could assist clinicians in providing appropriate treatment regimen to TB patients and improve their clinical outcome.
4. **Padmanesan Narasimhan et al.,** (2013)[40], summarized that the risk of progression from exposure to the tuberculosis bacilli to the development of active disease. Exogenous factors play a key role in accentuating the progression from exposure to infection among which the bacillary load in the sputum and the proximity of an individual to an infectious TB case are key factors. Similarly endogenous factors lead in progression from infection to active TB disease.
5. **Marcos Abdo Arbexet al.,** (2010)[41], described the mechanisms by which the interactions among the antituberculosis drugs used in the basic regimen can cause drug induced hepatitis, and discussed about the alternatives in this situation.

Review of study about Enoyl acyl carrier protein reductase (InhA) enzyme:

6. **Denise A. Rozwarski et al.,** (1999)[42], reported the Crystal Structure of the Mycobacterium tuberculosis Enoyl-ACP Reductase, InhA, in Complex with NAD^+ and a C16 Fatty Acyl Substrate.**Helmut Berglertet al.,** (1994)[43], reported that the EnvM protein was purified from an overproducing Escherichia coli strain. It showed NADH-dependent enoyl-acyl carrier protein (ACP) reductase activity using both crotonyl-ACP and crotonyl-CoA as substrates. It was concluded that EnvM is the NADH-dependent enoyl-ACP reductase of E. coli and we propose to rename the corresponding gene fabI.

Review of study about 1, 3, 4-Thiadiazole derivatives:

7. **Sevgi et al**., (2010)[44] was synthesized 5-[-4-

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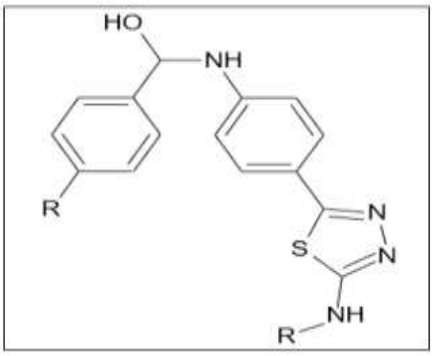
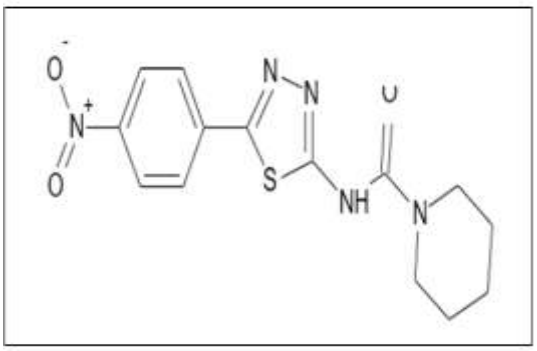
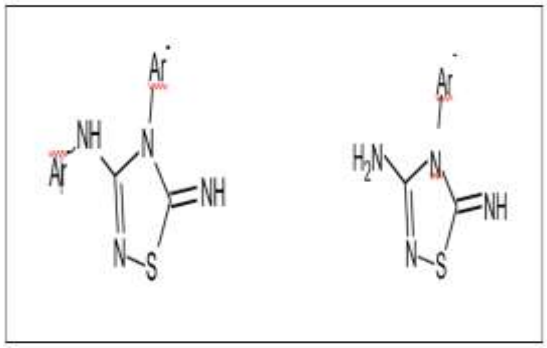
(4fluorobezoylolamino) phenyl]-2-subsitutedamino-1,3,4-thiadiazole 27 and evaluate the cytotoxic activity.



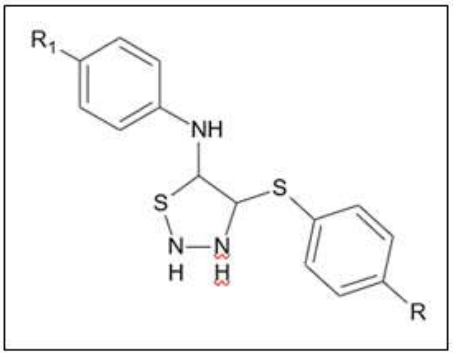
8. **Pattan et al.,** (2009)[45] have been introduced the synthesis of various thiadiazole derivatives compounds and evaluated for anti-diabetic activity.



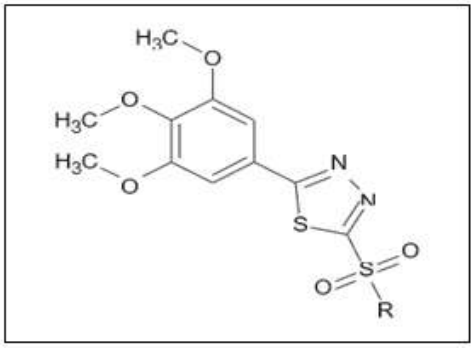
9. **Gupta et al.,** (2008)[46] synthesized a series of 3-aryl amino/amino-4-aryl-5-imino-D2- 1,2,4-thiadiazoline. The Anticonvulsant activity of all the synthesized compounds was evaluated against maximal electroshock induced seizures (MES) and subcutaneous Pentylenetetrazole (ScPTZ) induced seizure models in mice.



10. **Sharma et al.,** (2008)[47] synthesized a new series of selective COX-2 inhibitors with 2-amino-5-sulfanyl-1,3,4-thiadiazole. These compounds were selective inhibitors of COX-2 and potentiated the activity of COX-1 enzyme. The presence of Sulphonamide group is a required pharmacophore for selective inhibition of COX-2 enzyme.



11. **Jun-Chen et al.,** (2007)[48] introduced a series of 5-(3,4,5-trimethoxyphenyl)-2- sulfonyl-1,3,4-thiadiazole derivatives which possess higher Antifungal activities against three kind of fungi Gibberellazeae, Botrytis cinerea, and Sclerotiniasclerotiorum.



12. **Swamy et al.,** (2006)[49] synthesized a series of 4,6-disubstituted 1,2,4-triazolo-1,3,4-thiadiazole derivatives tested for in-vitro antimicrobial activity against Bacillus subtilis, Escherichia coli, Pseudomonas fluorescensetc. and found them to be active with these compounds having maximum activity. The presence of chloro substituent enhances the activity of the compound.

13. **Shafiee et al.,** (2005)[50] A series of 2-(5-nitro-2-furyl) and 2-(5-nitro-2thienyl)-5- substituted-1,3,4-thiadiazole derivatives were synthesized.

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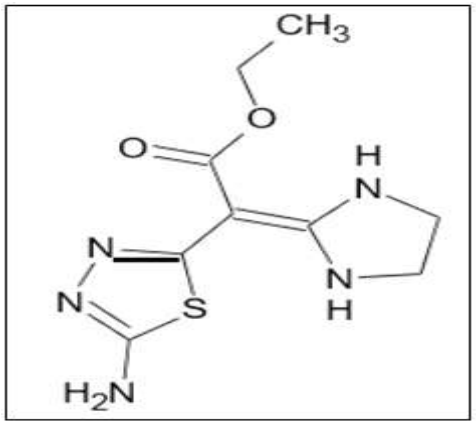
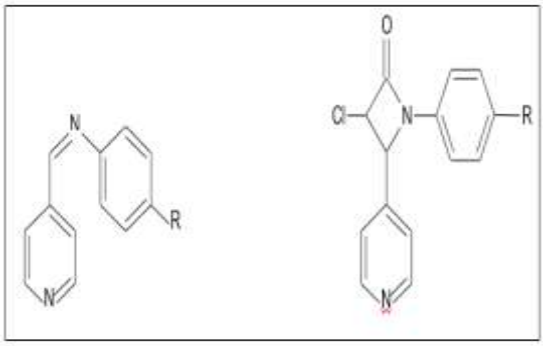
The most active compound was found to be active with an IC50 0.1 μM against Leishmania major promastigotes.

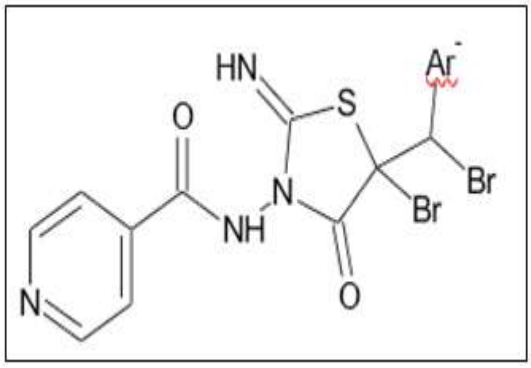


Review of study about Pyridine derivatives:

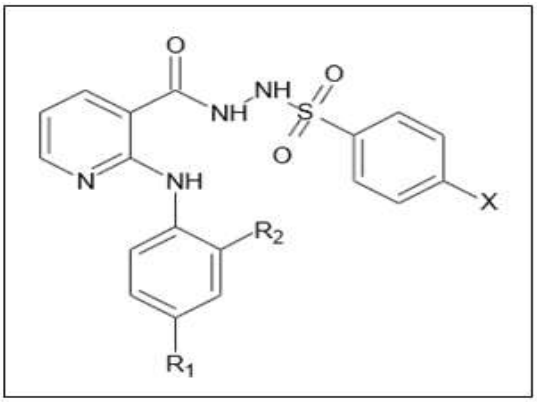
14. **Pramod et al.,** (2018)[51], synthesized a novel derivatives of pyridine containing azetidinone derivatives in simple two-step facile procedure.



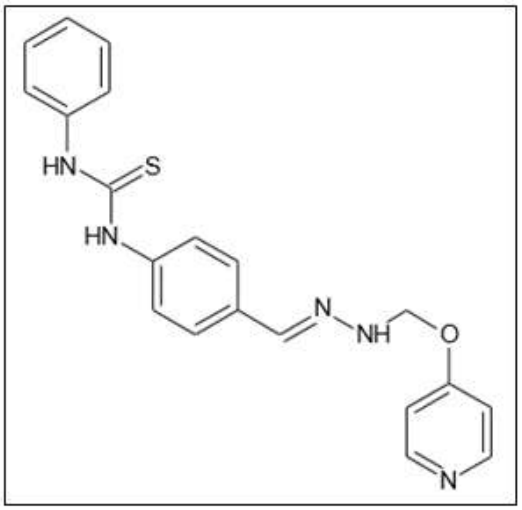
15. **Mishra et al.,** (2007)[52] have synthesized novel 2-Imino-3-(4'-carboxamido pyridyl)- 5-arylidine-4-thiazolidinones as antimicrobial agents.



16. **Kamal et al.,** (2007)[53] synthesized novel 2-Anilino substituted nicotinyl aryl sulfonylhydrazides have synthesized and reported as potential anticancer and antibacterial agents.



17. **Sriram et al.,** (2006)[54] have synthesized some 1-[(4-substituedphenyl]-3-(4-{1- [(pyridine-4-carbonyl) hydrazono] ethyl} phenyl) thiourea and studied their in- vitroantitubercular activity.



18. **Zhuravel et al.,** (2005)[55], synthesized 5-hydroxymethyl-8-methyl-2-(-arylimino)-pyrano[2,3-c]pyridine-3-(-aryl)-carboxamides and studied their antimicrobial activity.

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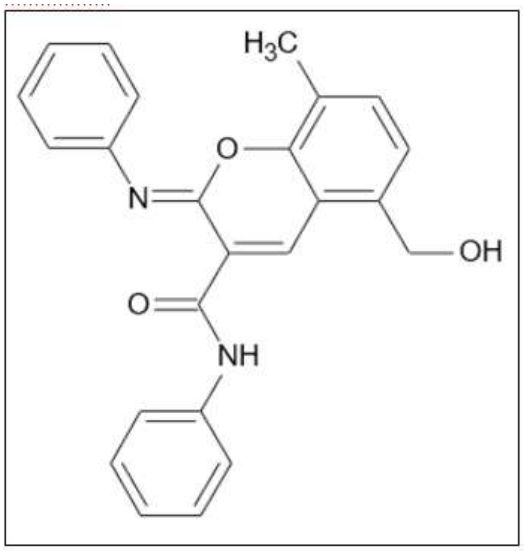
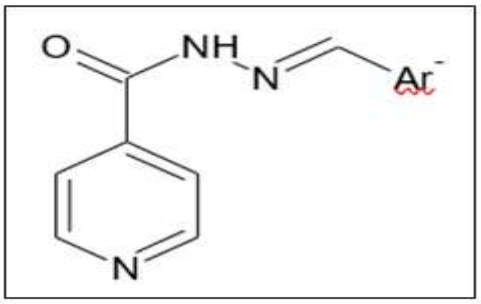
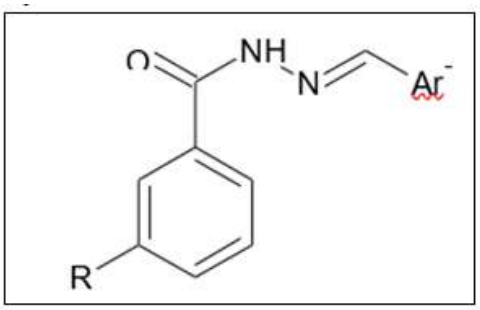


Reviews of study about the Schiff base derivatives:

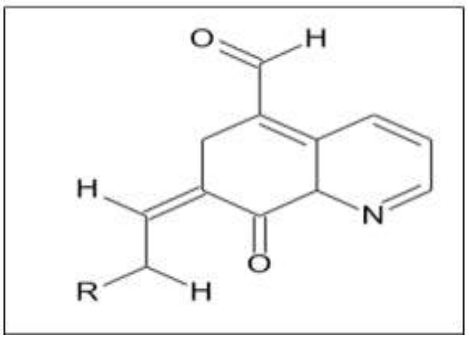
19. **Joginder Kumar et al.,** (2017)[56] summarized that Schiff-base exhibited versatile pharmacological activity, had shown potent antitumor effects against variety of human cell lines and in-vivo animal models. Structure activity relationship of Schiff-base containing derivatives indicated that Schiff-base moiety essential for the pharmacological activity. These results revealed that Schiff-base is an essential pharmacophore for the anticonvulsant activity.
20. **AnuKajal et al.,** (2013)[57], attempt to reviewed all the biological activities reported for Schiff bases in the current literature with an update of recent research findings. This review reflects the contribution of Schiff bases to the design and development of novel lead having potential biological activities with fewer side effects.
21. **A. B. Thomas et al.,** (2011)[58] synthesized Schiff bases of isonicotinoyl hydrazone, N'-[(1Z)-(substituted aromatic) methylidene] pyridine-4-carbohydrazides. Synthesized compounds were evaluated for in vivo antidepressant and nootropic activities. The results revealed that the test compounds substituted with nitro, halogen, and dimethoxy groups exhibited significant antidepressant and nootropic activities. N'-[(1Z)-(2,5dimethoxyphenyl) methylidene]pyridine-4-carbohydrazide was found to exhibit the highest antidepressant activity.



22. **K. S. Kumar et al.,** (2010)[59] synthesized a series of 3-(benzylideneamino)-2 phenyl quinazoline-4(3H)-one, and evaluated for their cytotoxicity and antiviral activity. Compounds having 2-hydroxy substitution showed better antiviral activity.



23. **K. V. Sashidhara et al.,** (2009)[60] synthesized a series of novel keto-enamine Schiff bases, derived from 8-hydroxyquinoline was synthesized and subjected for in-vitro antioxidant, in-vivo antidyslipidemic, and postheparinlipolytic activity.



Reviews of study about the biological evaluation of Anti tubercular activity by MABA:

24. **Sephra N.Ramprasad et al.,** (2012) studied the various applications of Alamar blue as an indicator. The Alamar blue bioassay being utilized to access cell viability and cytotoxicity

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in a biological and environmental system and in a number of cell types including bacteria, yeast, fungi, and protozoa.

25. **Tannaz Birdi et al.,** (2012)[61] Assessed anti-M. tuberculosis activity of five Indian medicinal plants such as Acetone, ethanol and aqueous extracts (prepared sequentially) of Acoruscalamus L. (rhizome), Andrographis paniculate. (leaf), Ocimum sanctum L. (leaf), Piper nigrum L. (seed) and Pueraria tuberosa DC. (tuber) were tested at 1, 10 and 100 µg/ml using the Microplate Alamar Blue Assay. which have been reported in traditional literature for various uses including respiratory ailments.
26. **Melby Mendoza-Aguilar et al.,** (2012)[62] concluded that the MABA appears to be a useful model for the selection of drugs that are effective for the treatment of murine leprosy. To further validate our results, a broader study involving the use of novel anti- mycobacterial agents and other agents, including particular anti-tuberculosis drugs, should be performed.
27. **Jose d Jesus Alba-Romero et al.,** (2011)[63] applied the Alamar blue assay to determine the susceptibility to anti-tuberculosis pharmaceuticals. It is a reliable method to determining the drug susceptibility to pharmaceuticals.
28. **Collins L A et al.,** (1997)[64] reported the high-throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium using Microplate Alamar Blue Assay (MABA) and compared with BACTEC 460 Assay System.

Review of study about Spectroscopy:

29. **Devi Thamizhanban et al.,** (2016)[65] reviewed the highlights of a variety of analytical hyphenated analytical techniques and the role of the instrumentation, analytical methods in assessing the quality of the drugs
30. **Patel Jayvadan et al.,** (2010)[66] reviewed the techniques from the coupling of a separation technique and an on-line spectroscopic detection technology.

Review of study about Drug Design:

31. **Surabhi et al., (**2018)**[67]**overviewed about the computer aided drug design and development of a new drug.
32. **Ram BabuTripathi et al.,** (2016)[68] reviewed about the In-silico expectations of pharmaceutical industry to design of new drug molecules.
33. **Ntie Kang F et al.,** (2015)[69] reported an in-silico evaluation of the ADMET profile of the Streptome DB database. An assessment of the "drug-likeness" and pharmacokinetic profile of >2,400 compounds of natural origin, currently available in the recently published Streptome DB database was also reported.
34. **Salla Virtanen et al.,** (2013)[70] developed a novel method for virtual screening that employs the negative image of the binding site. They were shown that the VS results by this method are often better compared to docking and ligand-based VS.

✓ **Aim and Objectives**

AIM:

To design and synthesize some novel heterocyclic derivatives which will prove to
be effective against Mycobacterium tuberculosis.

OBJECTIVE:

The literature survey reveals the significance of the research in the area of the development of new drugs for tuberculosis, capable of overcoming MDR and XDR-TB. The plan of work includes

- ❖ Design of Enoyl-acyl carrier protein reductase inhibitors by docking studies using Autodock® (1.5.6 version) software
- ❖ In-silico Drug likeness prediction by Molinspiration®
- ❖ In-silico Toxicity Assessment done by OSIRIS®
- ❖ Laboratory synthesis of those compounds with top Docking Scores.
- ❖ Characterization of the synthesized compounds by
  - Thin layer chromatography
  - Melting point.
  - Infrared Spectroscopy
  - ^{1}H Nuclear Magnetic Resonance Spectroscopy.
  - LC-Mass Spectrometry.
- ❖ In-vitro anti-tubercular activity of synthesized compounds (MABA).

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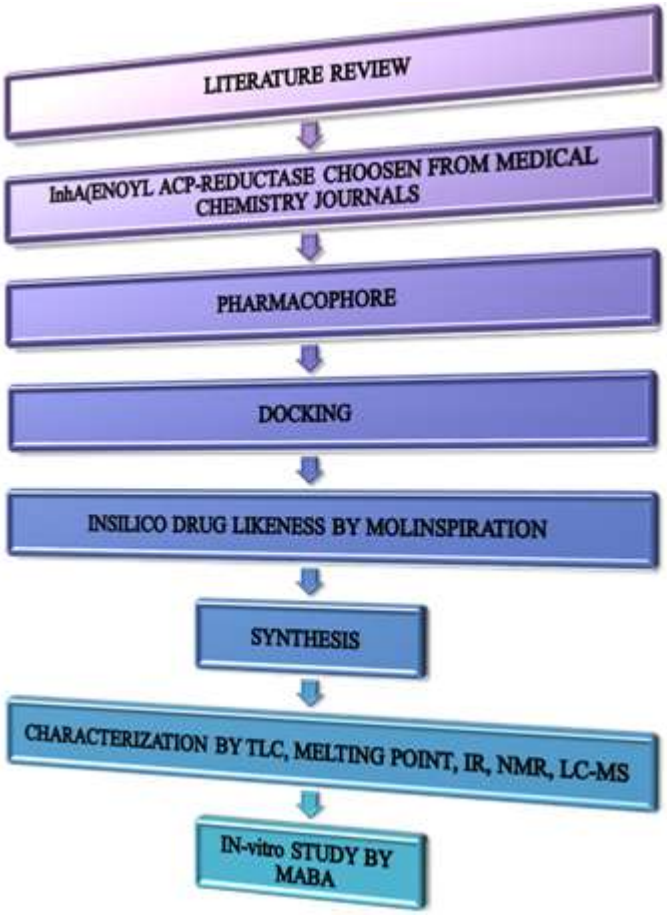
✓ **Plan of Work**



III. MATERIALS AND METHODS

In-Silico Approach

TARGET ENZYME:

The target enzyme InhA, "the enoyl acyl carrier protein reductase" (ENR) from Mycobacterium tuberculosis, was selected as the in-silico target for Mycobacterium tuberculosis. The crystal structure of the enzyme was downloaded from the Protein Data Bank ("An Information Portal to Biological Macromolecular Structures") (PDB ID: 2h9i).

LIGAND:

Molecules based on heterocyclic nucleus and some fused ring systems were created by sketching the molecules using ACD/ChemSketch (freeware)®, Version 12.01 (Build 30815, 10 feb 2009). Then all the ligands were prepared by using "Molegro Molecular viewer-2.5" (Schrodinger®) software to generate the low energy 3D conformers of the ligands.

MOLECULAR DOCKING BY AUTODOCK TOOLS®:

AutoDock tools or ADT is a graphical front end setting up and running Auto Dock and automated docking software designed to predict how small molecules, such as ligands bind to a receptor of known 3D structure[71].

STEPS INVOLVED IN DOCKING PROCEDURE:

- Protein Preparation
- Ligand Preparation
- Receptor Grid Generation
- Ligand Docking (Screening)

Protein preparation:

Open ADT:

- File > Read Molecule> Select Protein File (".pdb" file) Edit > Hydrogen > Add > Polar Only > OK
- Edit > Hydrogen > Merge Non-Polar >

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Continue (in this step if you see any warning please click on "continue")
- Edit > Charges > Compute Gasteiger> Ok
- Edit >Misc> Repair Missing Atoms> Ok/Select all > Dismiss
- File > Save > Write PDB>>Sort Nodes (Check) > OK > (Overwrite) YES

Lig and preparation:
- Ligand > Input > Open> Select Ligand File (".pdb" file) > OK Edit> Charges> Add Kollman charges
- Ligand > Output > Save as PDBQT

Receptor grid preparation:
- Grid > Macromolecule > Choose> Click (Protein) > Select Molecule > OK > Save as PDBQT
- Grid> Set Map types >Choose Ligand> Click (Ligand) > Select Ligand Grid > Grid Box> Set the BOX> File > Close Saving Current
- Grid > Output > Save GPF>grid.gpf> save

Preparation of Docking Parameters:
- Docking > Macromolecule > Set Rigid filename> Select 'Protein.pdbqt' > Open Docking > Ligand > Choose> Click 'Ligand' > Select Ligand> Accept
- Docking > Search Parameters > Genetic Algorithm> Accept
- Docking > Output > Lamarckian GA> Save file as 'dock.dpf' Open cmd (command prompt)
- In command prompt go the destination folder by cd commands and run the following commands
- autogrid4.exe -p grid.gpf -l grid.glg (wait for the response) autodock4.exe -p dock.dpf -l dock.dlg wait for the response)

Visualization / Interpretation of Docking:
- Analyze > Docking > Open > Select 'dock.dlg' > Open >Assign Ligand New Name > OK
- Analyze > Macromolecule > Choose> Click 'Protein' > Select Macromolecule
- Analyze > Conformations> Play, Ranked By Energy or Play > Click on the '&' Button
- Set Play Options >Check 'Build H-Bonds'> View the hydrogen bonds formed > Check 'Show
- Info'> View the Interaction Energy > Build Current Write Complex> Save as 'Result.pdb'.
- Analyze > Load > Information on the predicted interaction energy is shown at the top, and individual conformations
- Analyze > Docking > show interaction: specialized visualization to highlight interactions between the docked conformation of the ligand and the receptor.

In-Silico TOXICITY ASSESSMENT:
- In-silico toxicity Assessment for the molecules were predicted by using **OSIRIS®**, a JAVA based online tool.
- Using this prediction tool, mutagenicity, tumerogenicity, skin irritation and reproductive effects can be calculated.
- The prediction is based on the fragment contribution group present in the structure of the molecule.
- On accessing the OSIRIS®, Property Explorer, which is a JAVA applet, allows us to draw chemical structures.
- On drawing the chemical structures, the toxicity risk parameters are calculating on- the-fly whenever a structure is valid. Prediction results are valued and color coded.
- Properties with high risks of undesired effects are shown in red color. Whereas a green color indicates drug-conform behavior.

In-silico ADME PREDICITIONS:
- The designed and docked molecules are screened in silico using MOLINSPIRATION Cheminformatics® software to evaluate drug likeness.
- This tool is quick and easy to use.
- It is a software available online for calculation of important molecular properties log P, polar surface area, number of hydrogen bond donors and acceptors.

LIPINSKI'S RULE[72]:

Lipinski's rule of five also known as the **Pfizer's rule of five** or **rule of five** is a rule of thumb to evaluate drug likeness. The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including absorption, distribution, metabolism and excretion. The rule states that the active drug has no more than one violation of the following criteria:
- No more than 5 hydrogen bond donors.
- No more than 10 hydrogen bond acceptors
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient log P not

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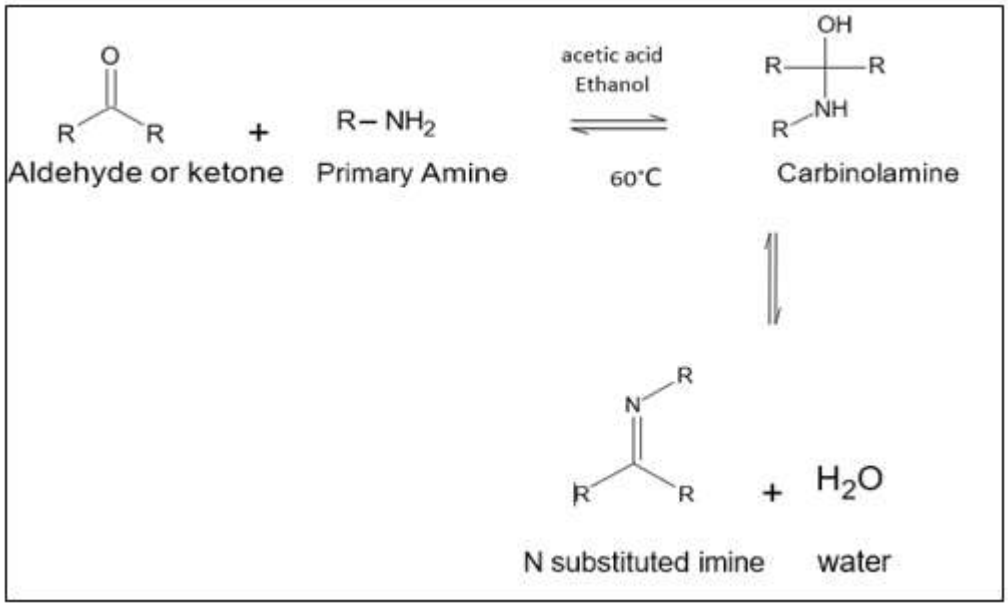
greater than 5

SYNTHETIC METHODOLOGY

Synthesis of Schiff base[73]:



PROCEDURE:

Equimolar quantities of ketone/Aldehyde (0.01mol) and Amine (0.01mol) are added into 20mL of absolute ethanol and 5mL of glacial acetic acid is added to it. Reaction mixture is refluxed for 24hrs at 60ºC. Completion of reaction is confirmed by TLC. The product obtained filtered and dried. Recrystallization is done by using ethanol.

Amines used:

- 3,5-dichloro 4-amino pyridine
- 5 amino 1,3,4 thiadiazole-2 thiol
- Nitro aniline

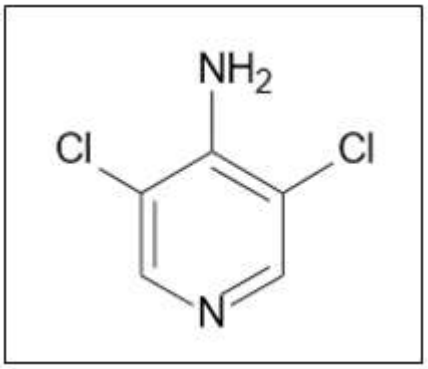
Aldehydes/Ketones used:

- Pyridine 3 carboxaldehyde
- Anisaldehyde
- p-chloroacetophenone
- 3 methyl cyclopentane 1,2 dione
- Isatin

REACTANT PROFILE

1. 3,5-dichloro 4-amino pyridine

1) **Synonym:** 3,5dichloro4pyridinamine
2) **Appearance:** White crystalline powder
3) **Molecular Formula:** $C_5H_4Cl_2N_2$
4) **Molecular weight:** 163 g/mol
5) **Melting point:** 159°C
6) **Solubility:** Chloroform, DMSO, Methanol



2. 5 amino 1,3,4 thiadiazole-2 thiol

1) **Synonym:** 2-amino-5-mercapto-1,3,4-thiadiazole-2-thiol
2) **Appearance:** Pale yellow to cream powder
3) **Molecular Formula:** $C_2H_3N_3S_2$
4) **Molecular weight:** 133.2 g/mol
5) **Melting point:** 235°C
6) **Solubility:** Chloroform, DMSO

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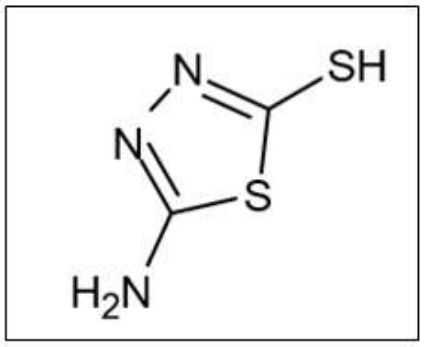
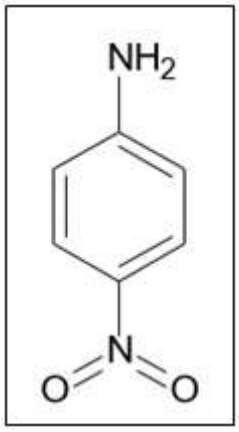
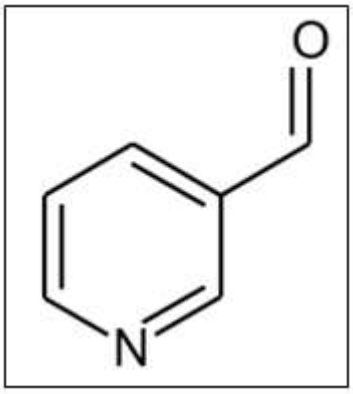


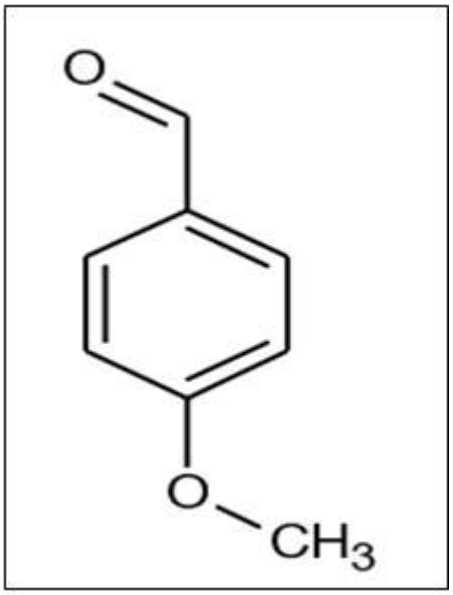
3. Nitro aniline
1) Synonym: 1-amino-4 nitro benzene
2) Appearance : Yellow or brown powder
3) Molecular Formula: $C_6H_6N_2O_2$
4) Molecular weight: 138.12 g/mol
5) Melting point: 145°C
6) Solubility: Chloroform, DMSO, Methanol



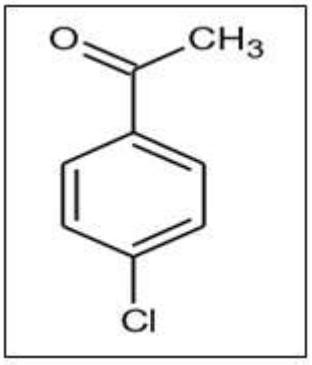
4. Pyridine 3carboxaldehyde:
1. Synonym: Nicotinaldehyde
2. Appearance: Brown oily liquid
3. Molecular Formula: C_6H_5NO
4. Molecular weight: 107.11 g/mol
5. Boiling point: 181°C



5. Anisaldehyde:
1) **Synonym:** 4-Methoxybenzenecarbaldehyde
2) **Appearance:** Clear liquid
3) **Molecular Formula:** $C_8H_8O_2$
4) **Molecular weight:** 136.15 g/mol
5) **Boiling point:** 248°C



6. p-Chloroacetophenone:
1) Synonym: Phenacyl chloride
2) Appearance : Pale yellow liquid
3) Molecular Formula: C_8H_7ClO
4) Molecular weight: 154.59 g/mol
5) Melting point: 145°C
6) Boiling point: 232°C



7. 3 Methyl Cyclopentane 1,2 dione:
1) Synonym: Cyclotene
2) Appearance : White crystalline powder
3) Molecular Formula: $C_6H_8O_2$

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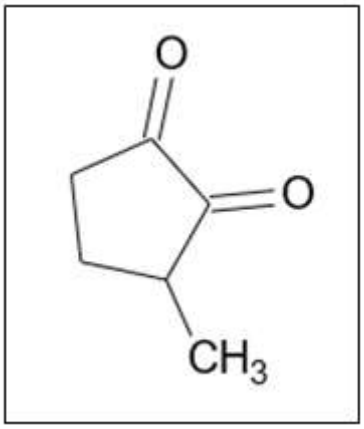
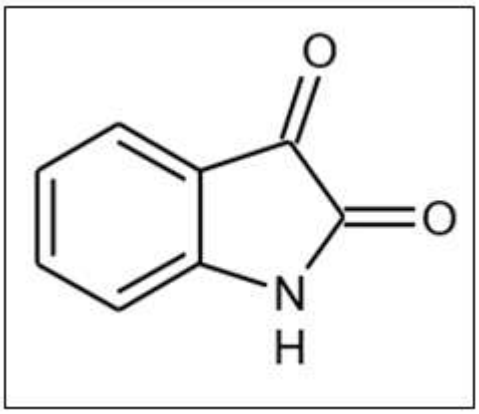
4) **Molecular weight:** 154.59 g/mol
5) **Melting point:** 104-108˚C
6) **Boiling point:** 170˚C
7) **Solubility:** Methanol, Chloroform



8. Isatin:
1) **Synonym:** Tribulin
2) **Appearance:**Orange colour powder
3) **Molecular Formula:** $C_8H_5NO_2$
4) **Molecular weight:** 147.13 g/mol
5) **Melting point:** 200˚C
6) **Boiling point:** 170˚C
7) **Solubility**: Methanol, Chloroform



CHARACTERIZATION PHYSICAL EVALUATION:

1. Physical properties of the synthesized compounds are evaluated, such as
- ✓ Color
- ✓ Nature
- ✓ Solubility
- ✓ Molecular weight
- ✓ Molecular formula
- ✓ Melting point

2. Further the synthesized compounds are characterized by the following Spectroscopic and Spectrometric methods such as
- ✓ IR Spectroscopy by ABB MB 3000-PH FTIR Spectrometer using KBr pellets
- ✓ 1H-NMR Spectroscopy by BRUKER Topspin Advance 500MHz NMR Spectrometer using DMSO d6 and Deuterated chloroform
- ✓ LC MASS Spectrometry by AGILANT Technologies 6230B Time of Flight (TOF).

IR SPECTROSCOPY[74]:

IR spectroscopy is an important and popular tool for structural elucidation and compound identification. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. In IR spectroscopy finger print region is used to compare the two compounds. Infrared spectrum shows percent transmittance versus frequency expressed as wave numbers.

1. 3540-3300 cm^{-1} N-H Stretching Vibration
2. 3670-3230cm^{-1}H Stretching Vibration
3. 1690-1630cm^{-1}C=N Stretching Vibration
4. 2975-2840 cm^{-1}C-H Aliphatic Stretching Vibration

NMR SPECTROSCOPY[75]:

NMR spectroscopy is a powerful analytical technique used to characterize structure of organic molecules by identifying connectivity of elements in molecules. ^1H NMR is used to determine the type and number of H atoms and spatial arrangement of them in a molecule. ^{13}C NMR is used to determine the type of carbon atoms in the molecule and spatial arrangement of them in a molecule. The source of energy in NMR is radio waves which have long wavelengths and thus low energy and frequency. When low-energy radio waves interact with a molecule, they can change the nuclear spins of some elements including ^1H and ^{13}C NMR.

1. Aromatic and hetero aromatic compounds 6-8.5δ
2. Alcoholic hydroxyl protons 1-5.5 δ
3. Aldehyde protons 9-10δ

HYPHENATED TECHNIQUE:

LC-MS:

LC-MS is a hyphenated technique, combining separation power of HPLC with the detection power of Mass Spectrometry. Liquid chromatography separates mixture with multiple components, mass spectrometry provides structural

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identity of the individual components with high molecular specificity and detection sensitivity.

BIOLOGICAL EVALUATION

MICROPLATE ALAMAR BLUE ASSAY (MABA)[76]:

The synthesized compounds were subjected to in-vitro anti-mycobacterial assay in order to find out their in-vitro potency of inhibiting tuberculosis. The desired study was performed by using "Microplate Alamar Blue assay" (MABA) method.

Materials:

The in-vitro antimycobacterial activity of the synthesized compounds was performed using Microplate Alamar Blue assay (MABA) against Mtb (H37RV, ATCC No: 27294). Pyrazinamide, Ciprofloxacin and Streptomycin were used as reference drugs. The results were based upon the minimum inhibitory concentration (MIC)

Principle:

MABA is an indirect colorimetric method for determining the MICs of TB drugs for strains of Mycobacterium tuberculosis. In this assay, the redox indicator Alamar blue monitors the reducing environment of the living cells. It turns from blue to pink in the presence of mycobacterium growth.

Experimental Procedure:

1) The Anti mycobacterial activity of compounds were assessed against M. tuberculosis using "Microplate Alamar Blue assay" (MABA).
2) This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.
3) Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation.
4) The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate.
5) The final drug connotations tested were 100 to 0.2 µg/ml.
6) Plates were covered and sealed with parafilm and incubated at 37ºC for five days.
7) After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs.
8) A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.
9) The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

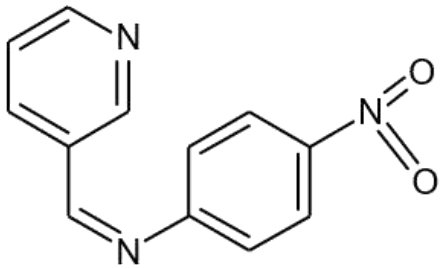
IV. RESULTS AND DISCUSSION

ACTIVITY PREDICTION:

More than 100 compounds sketched using Chemsketch® were docked against the enzyme Enoyl acyl carrier protein reductase using AUTODOCK Tools® 1.5.6 software. The molecules with the good docking scores and favorable interactions were selected, screened and further synthesized.

The molecules with good docking score were mentioned as below:

Table 1: Structure and Docking score

SAMPLE CODE	STRUCTURE	DOCKING SCORE kcal/mol
SN2		-8.54

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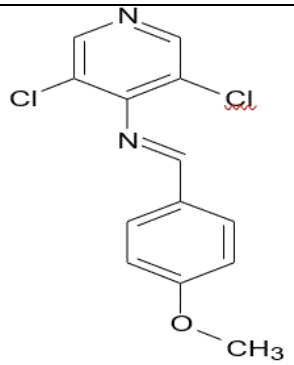
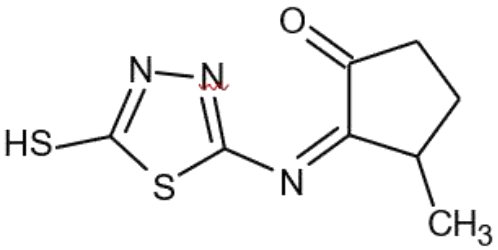
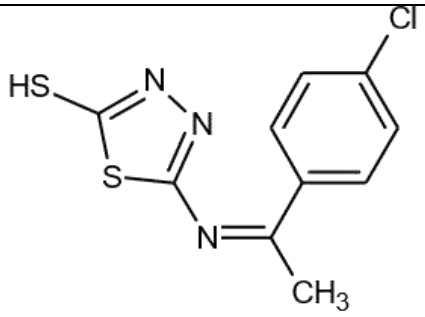
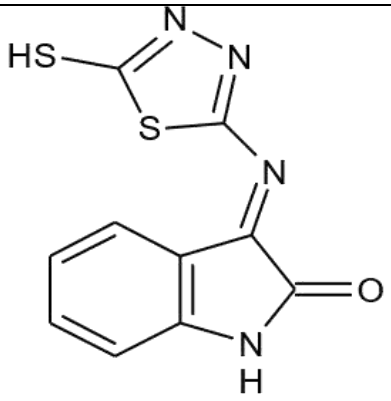
SN5		**-7.22**
MN1		**-7.75**
MN6		**-7.75**
MN7		**-8.96**

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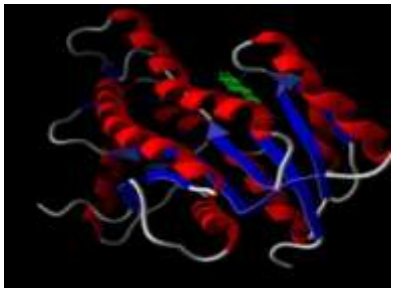
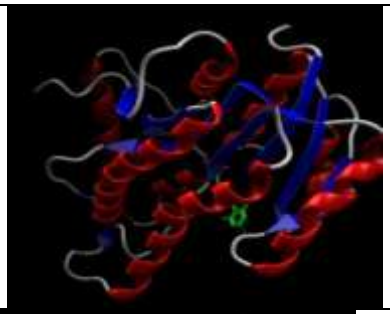
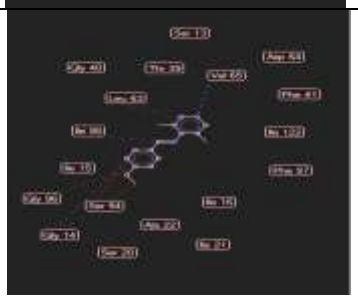
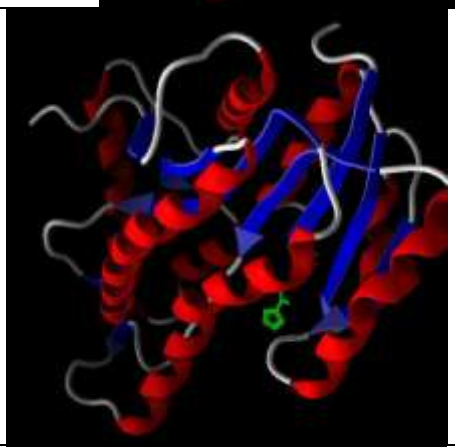
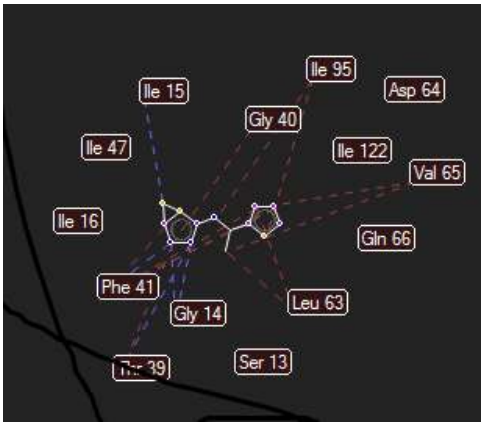
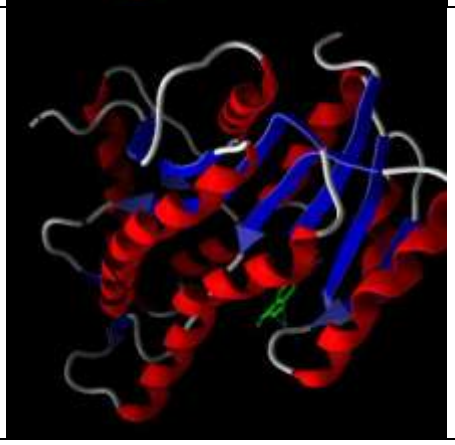
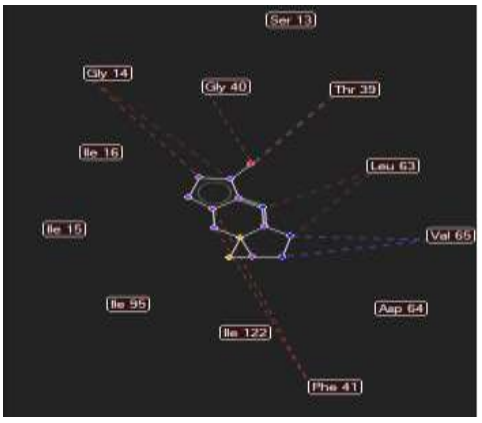
DOCKING VIEW AND INTERACTION OF THE DOCKED MOLECULES WITH THE ENZYME:

Table 2: Docking view and Interaction with Amino acid

SAMPLE CODE	DOCKING VIEW	INTERACTION WITH AMINO ACID
SN2		
SN5		
MN1		
MN6		

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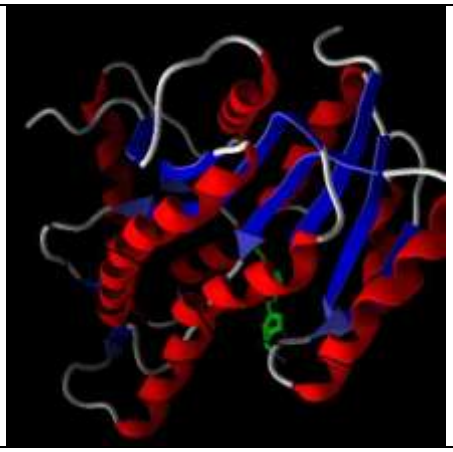
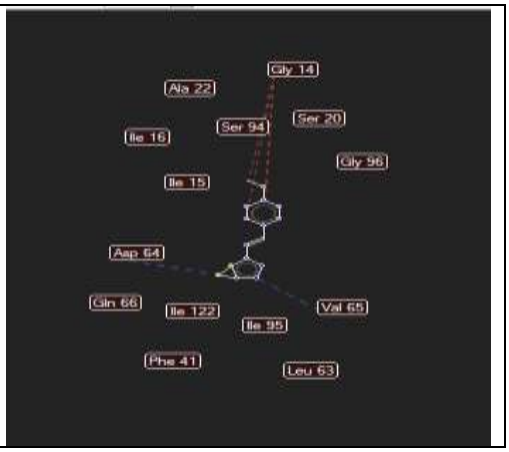
MN7		

In-silico TOXICITY PREDICTION:

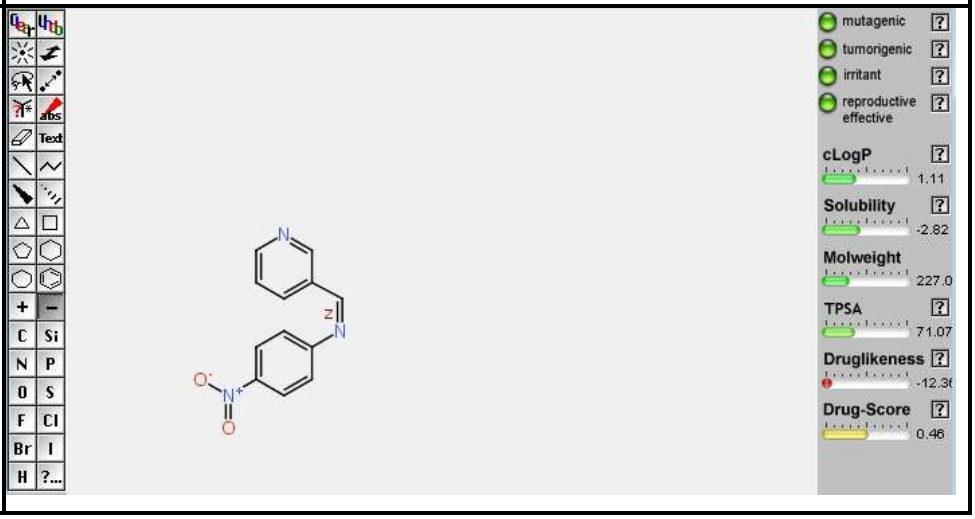
The in-silico toxicity assessment results of the selected 5 ligand molecules were summarized in the **Table 3.** All the molecules were found to be non-toxic in the terms of mutagenicity, tumorigenicity, skin irritancy and reproductive effect based on the in-silico toxicity assessment study.

Prediction of toxicity using OSIRIS® property explorer

Table 3: Prediction of toxicity

TOXICITY RISK	COMPOUND CODE				
	SN2	SN5	MN1	MN6	MN7
MUTAGENIC	---	---	---	---	---
TUMORIGENIC	---	---	---	---	---
IRRITANT	---	---	---	---	---
REPRODUCTIVE EFFECT	---	---	---	---	---

Table 4: In-silico TOXICITY PREDICTION

SAMPLE CODE	In-silico TOXICITY PREDICTION
SN2	

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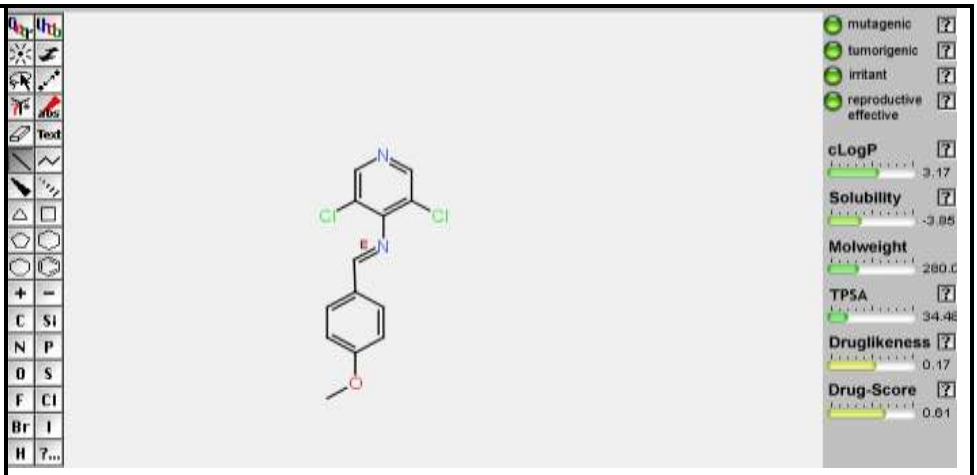
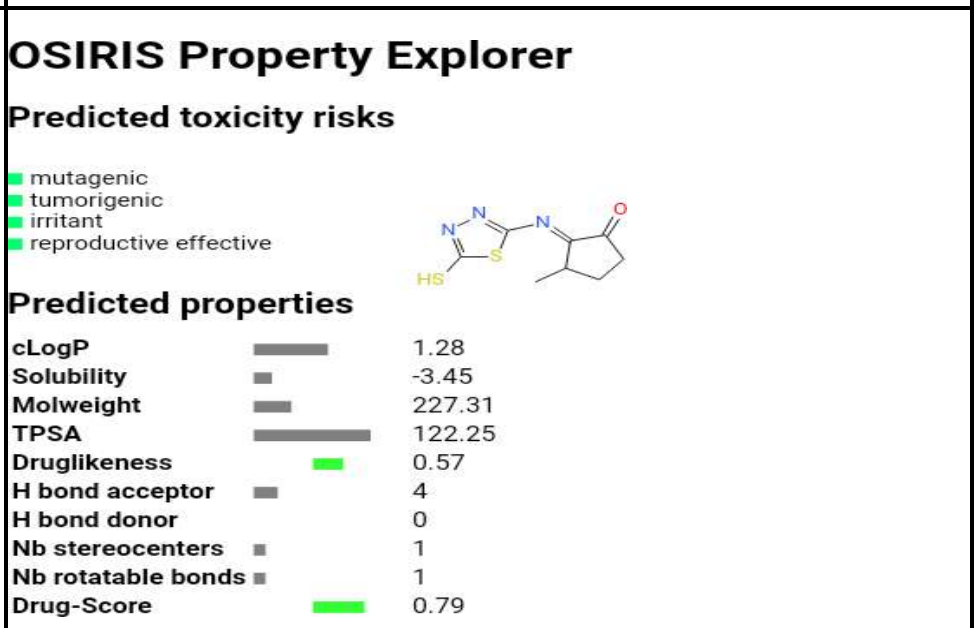
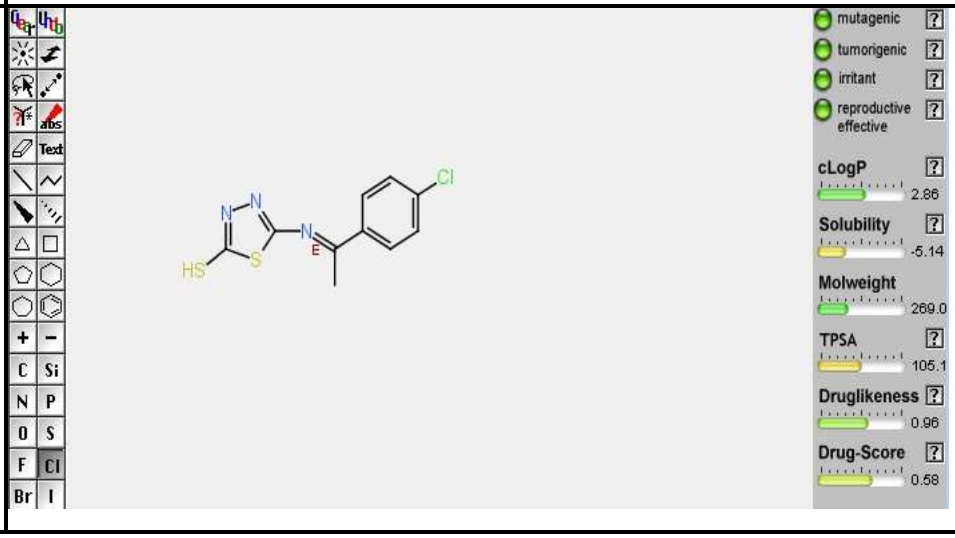
SN5



MN1



MN6



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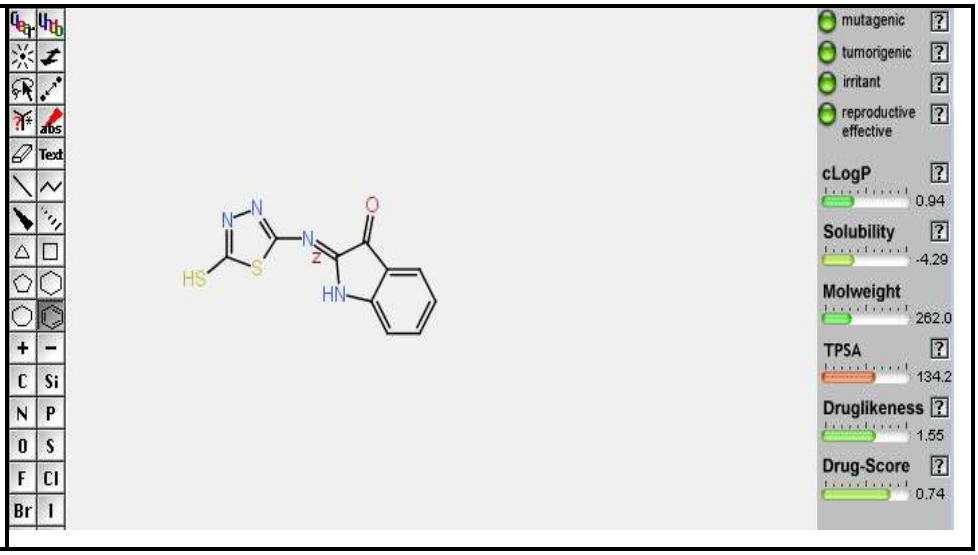
MN7	

PREDICTION OF DRUG LIKENESS:

The designed and docked molecules were screened in-silico using Molinspiration cheminformatics® to evaluate drug likeness. Molinspiration® were used to calculate important physicochemical properties of molecules such as log P, polar surface area, number of hydrogen bond donors and acceptors.

Variants:

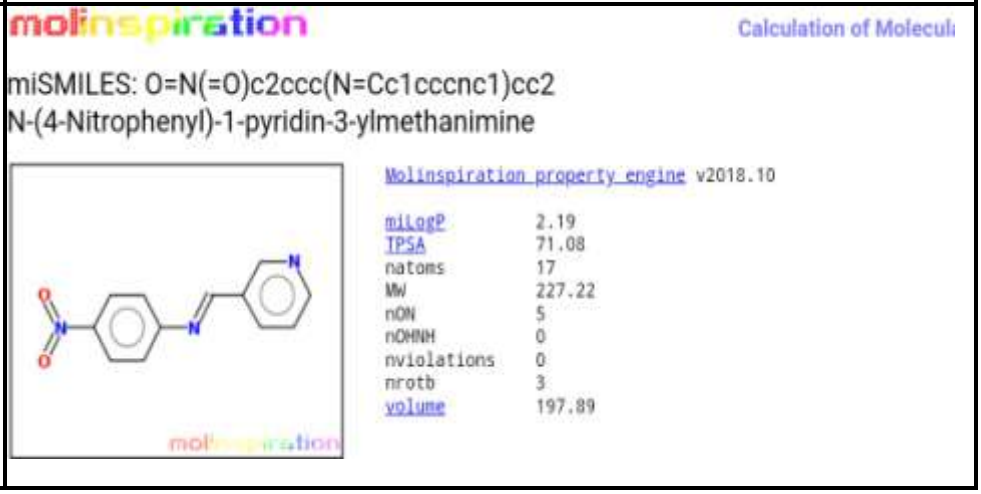
In an attempt to improve the predictions of drug likeness, the rules have spawned many extensions, for example the following:

- ✓ Partition coefficient log P in −0.4 to +5.6 range
- ✓ Molar refractivity from 40 to 130
- ✓ Molecular weight from 180 to 480 Dalton
- ✓ Number of atoms from 20 to 70 (includes H-bond donors [e.g. OHs and NHs] and H-bond acceptors

IN-SILICO SCREENING OF DRUG LIKENESS:

All the novel analogues were checked for conformance to Lipinski's rule using online version of Molinspiration® software & the results are depicted **Table 5.**

Table 5: Prediction of Drug Likeness

SAMPLE CODE	PREDICTION OF DRUG LIKENESS
SN2	

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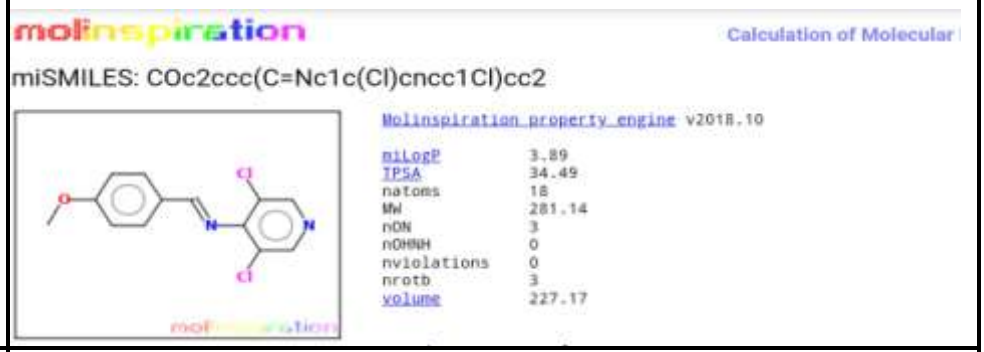
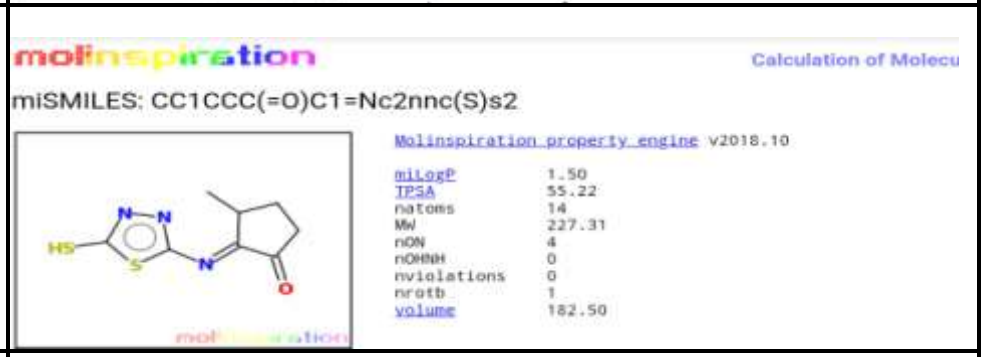
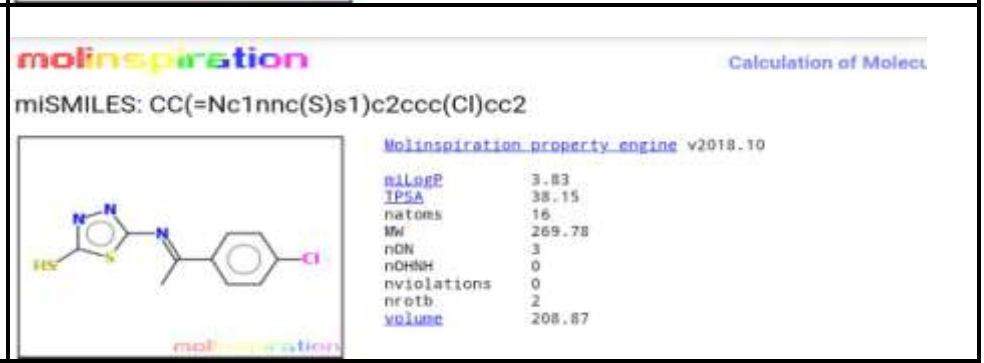
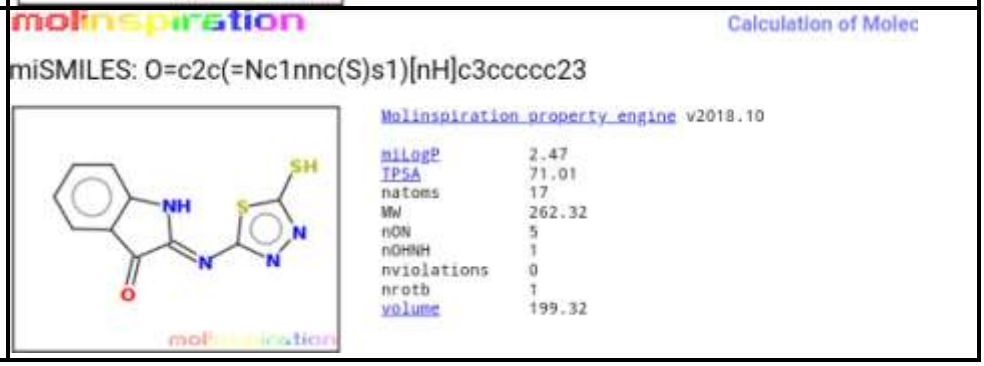
SN5	
MN1	
MN6	
MN7	

RESULTS OF SYNTHESIZED COMPOUNDS:

The selected 5 compounds were synthesized and recrystallised. Then the synthesized compounds were evaluated for their purity through melting point determination and TLC was carried out for the determination of absence of parent compounds and formation of other new compounds.

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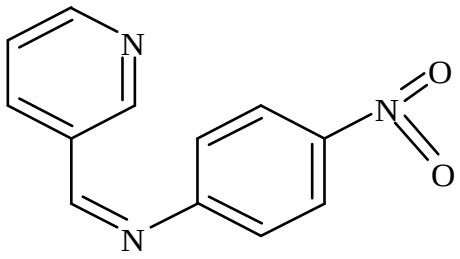
Table No: 6 Results of Synthesized Compounds

Sr. No.	COMPOUND CODE	PERCENTAGE YEILD (%)	MELTING POINT (°C)	Rf VALUE
1	SN2	73%	141-143	0.71
2	SN5	64%	140-142	0.64
3	MN1	78%	196-198	0.66
4	MN6	61%	208-210	0.81
5	MN7	76%	166-167	0.79

The Rf value of the synthesized compounds are varied from the Rf value of the reactants. Hence it is concluded that the reaction was completed. The melting point range confirmed the purity of the product.

The characterization was carried out using sophisticated instruments like IR, NMR, and Mass spectrometry.

PRODUCT PROFILE:
SAMPLE CODE: SN2



4-nitro-N-[(Z)-pyridin-3-ylmethylidene]aniline

Molecular Formula	$C_{12}H_9N_3O_2$ 227.21
Formula Weight	Yellow
Appearance	C(63.43%) H(3.99%) N(18.49%) O(14.08%)
Composition Molar	64.51 ± 0.5 cm^3
Refractivity Molar	183.5 ± 7.0 cm^3
Volume Parachor	493.0 ± 8.0 cm^3
Index of Refraction	1.620 ± 0.05
Surface Tension	52.0 ± 7.0 dyne/cm
Density	1.23 ± 0.1 g/cm^3
Polarizability	25.57 ± 0.5 $10^{-24}cm^3$

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IR SPECTRUM SN2:

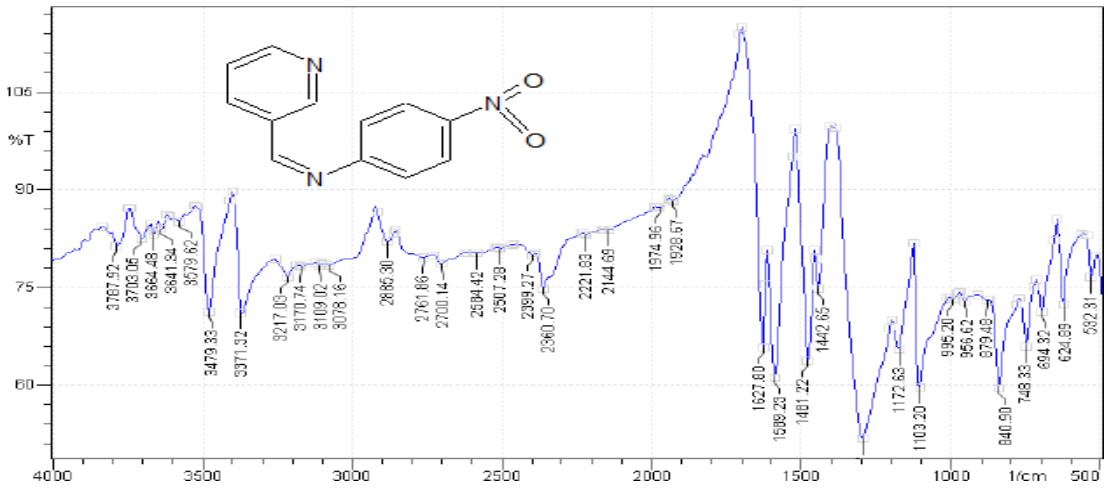
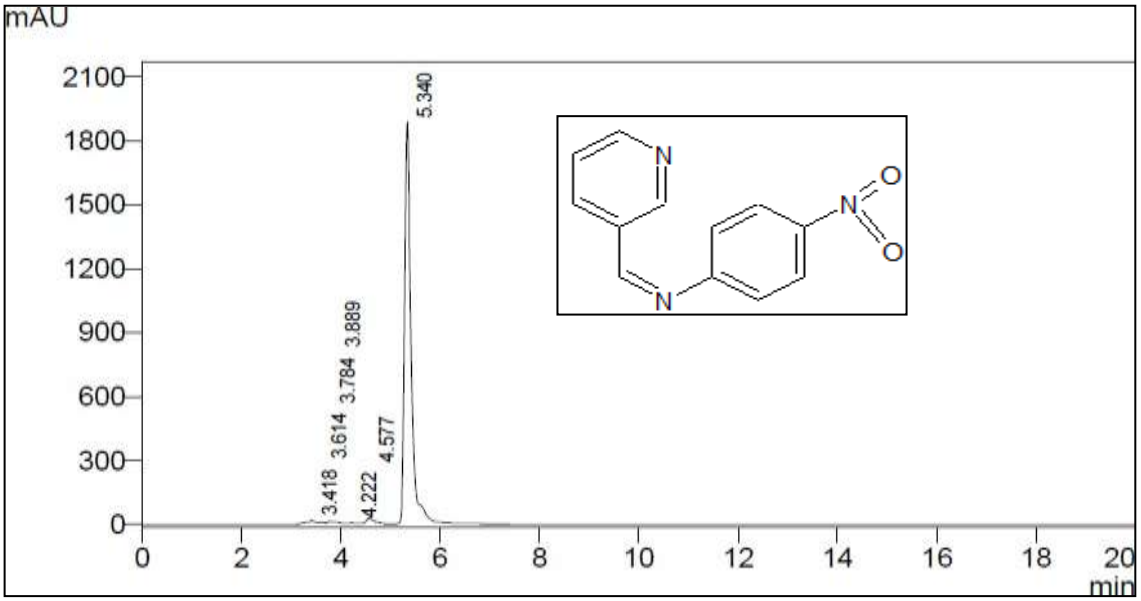


Table No: 7 INTERPRETATION OF IR SPECTRUM SN2:

Sr. No.	Wavenumbers (cm^{-1})	Functional group
1.	3078.16	C-H Stretching (Aromatic)
2.	1589.23	-NO Stretching
3.	1627.80	C=N Stretching

LC-MS CHROMATOGRAM: SN2
Mol.wt-226.20 g/mol



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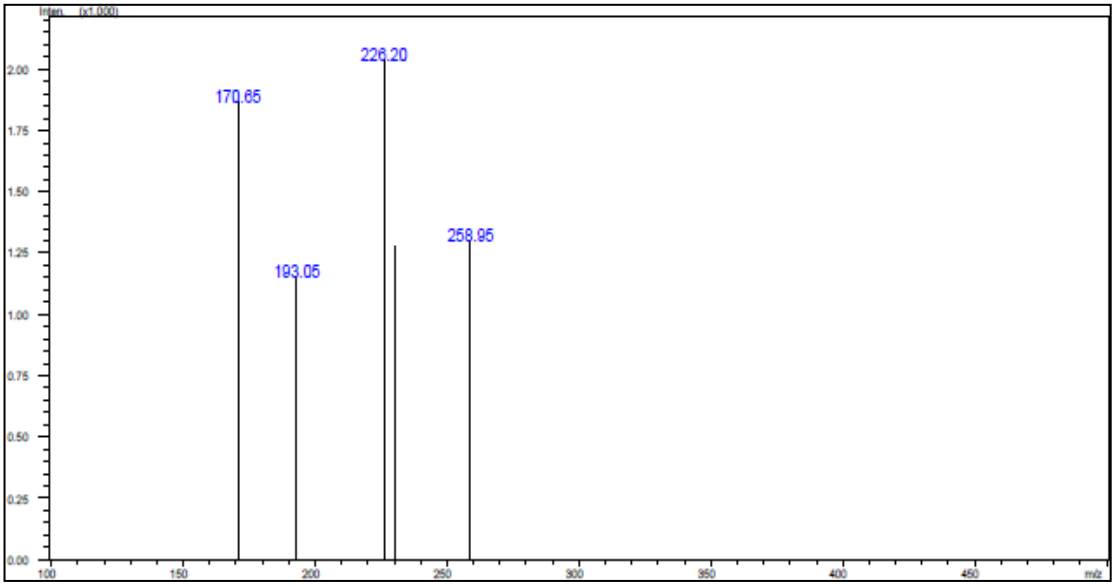

NMR SPECTRUM SN2:

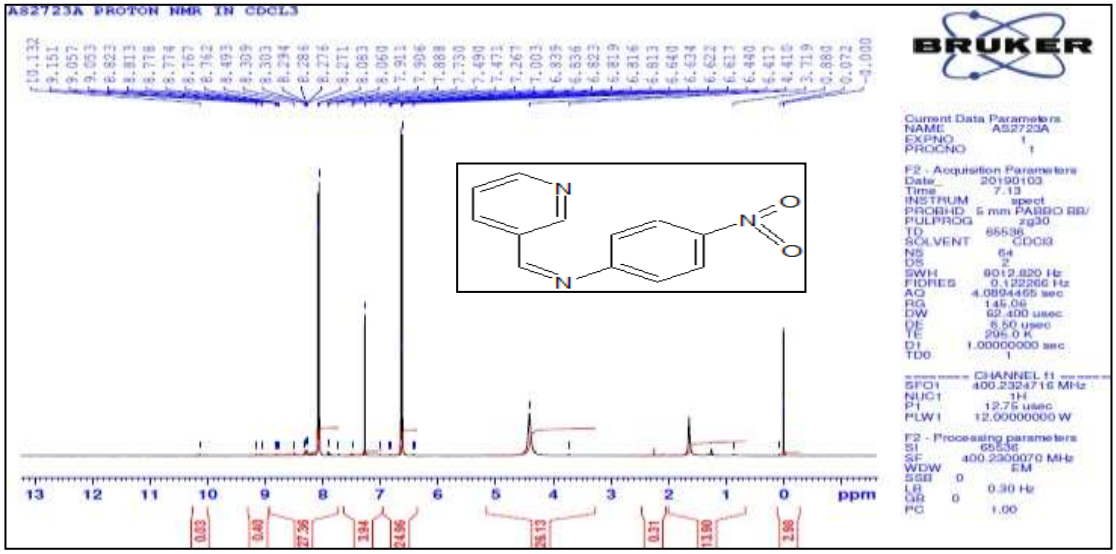

Table No: 8 INTERPRETATION OF NMR SN2

S.NO	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	3.7-6.3	Multiplet	3 Protons
2.	6.9-8	Multiplet	6 Protons

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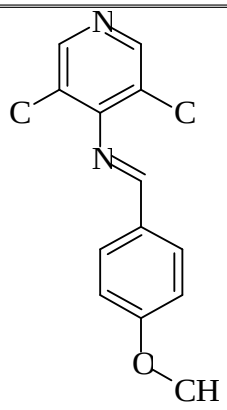
SAMPLE CODE: SN5



3,5-dichloro-*N*-[(*E*)-(4-methoxyphenyl)methylidene]pyridin-4-

Molecular Formula	$C_{13}H_{10}Cl_2N_2$
Formula Weight	O 281.13
Composition	C(55.54%) H(3.59%) Cl(25.22%) N(9.96%) O(5.69%)
Molar Refractivity	73.87 ± 0.5 cm^3
Molar Volume	218.5 ± 7.0 cm^3
Parachor	555.5 ± 8.0 cm^3
Index of	1.591 ± 0.05
	41.7 ± 7.0 dyne/cm

IR SPECTRUM SN5:

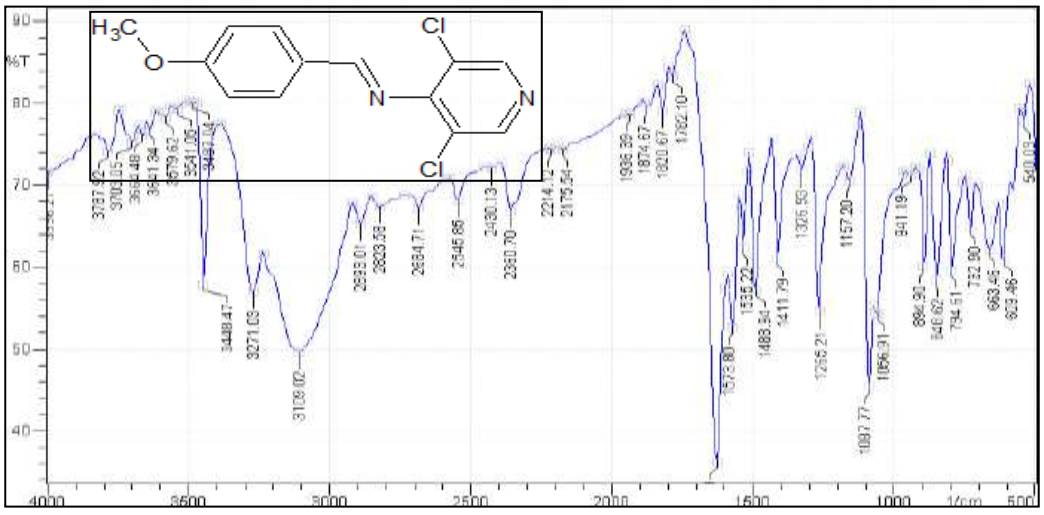


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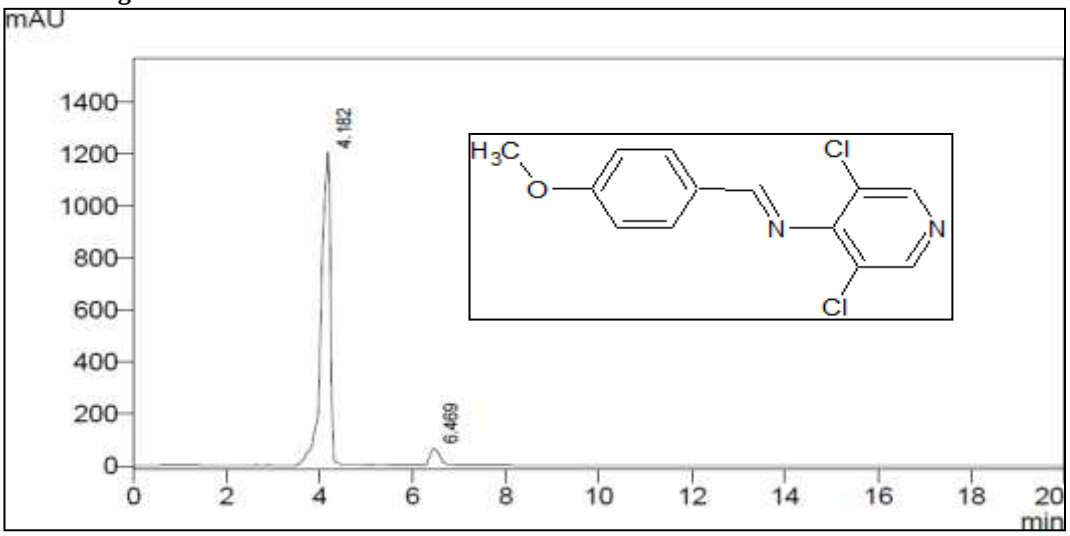
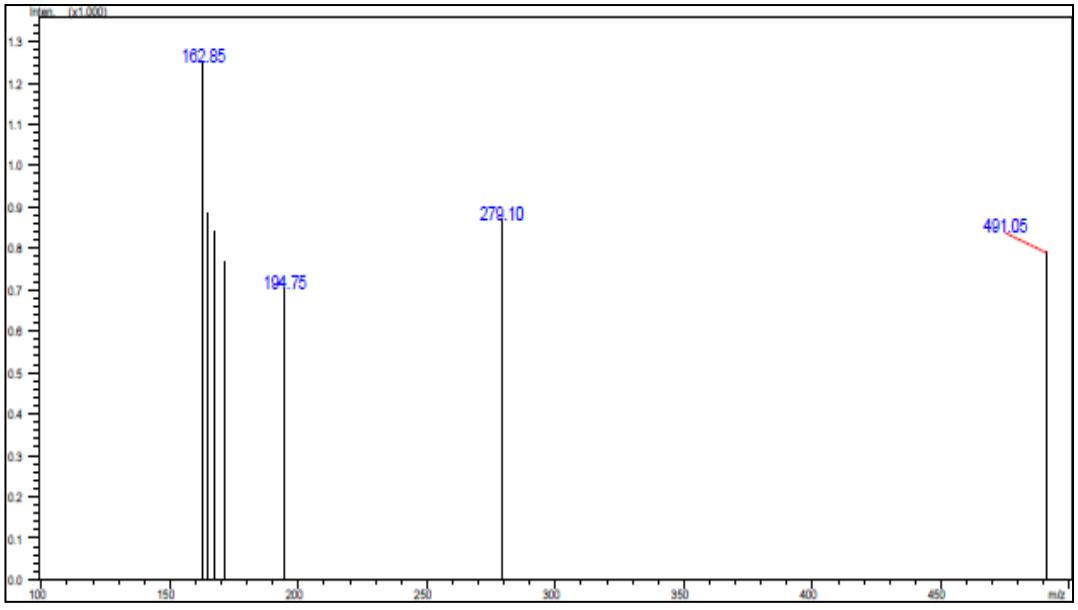
Table No: 9 INTERPRETATION OF IR SPECTRUM SN5

Sr. No.	Wavenumbers (cm^{-1})	Functional group
1.	3109.02	C-H Stretching (Aromatic)
2.	732.90	C-Cl Stretching
3.	1573.80	C=N Stretching

LC-MS CHROMATOGRAM: SN5
Mol.wt-279.10 g/mol





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NMR SPECTRUM SN5:

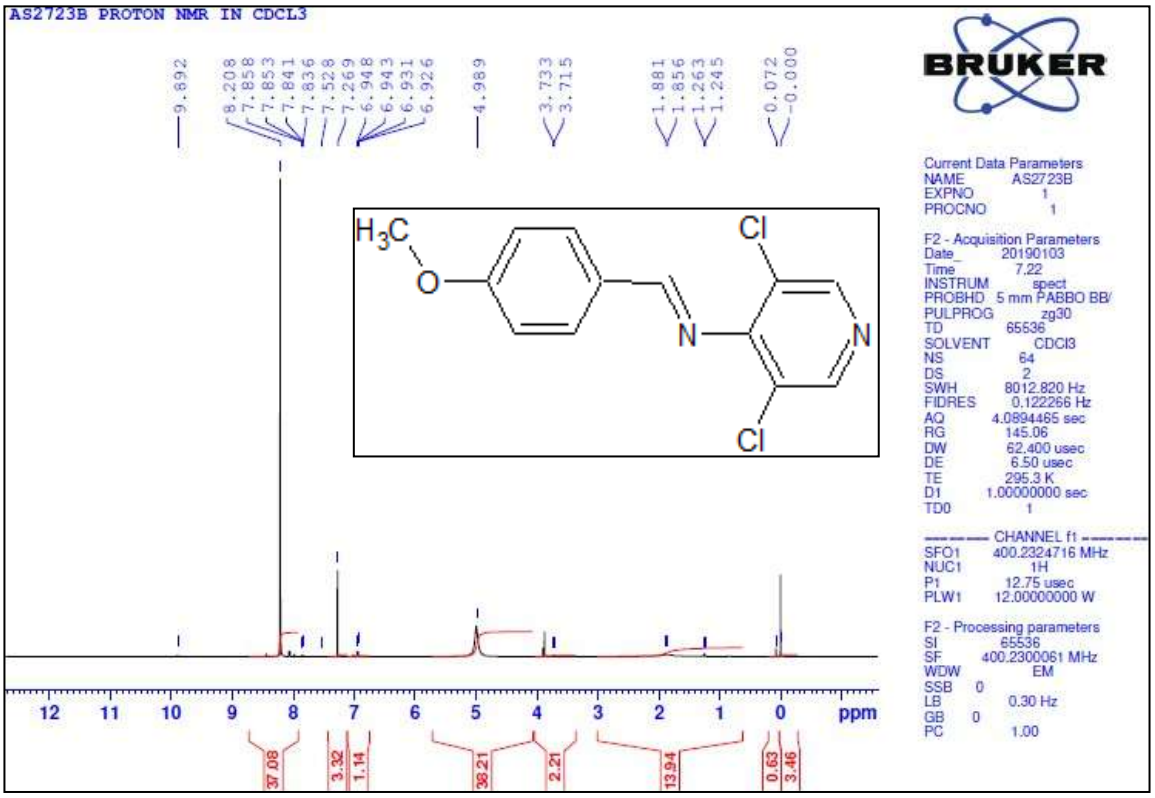

Table No: 10 INTERPRETATION OF NMR SPECTRUM SN5

S.NO	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	3.7-4.9	Multiplet	4 Protons
2	6.9-7.8	Multiplet	6 Protons

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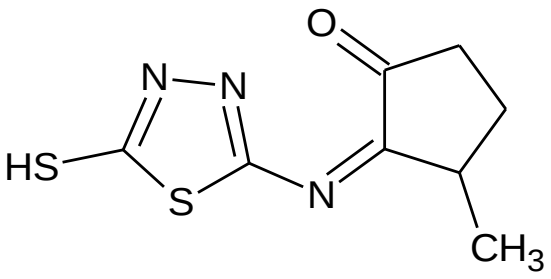
SAMPLE CODE: MN1



(2*Z*)-3-methyl-2-[(5-sulfanyl-1,3,4-thiadiazol-2-yl)imino]cyclopentanone

Molecular Formula	$C_8H_9N_3OS_2$
Formula Weight	227.30
Composition	C(42.27%) H(3.99%) N(18.49%) O(7.04%) S(28.21%)
Molar Refractivity	59.27 ± 0.5 cm^3
Molar Volume	138.0 ± 7.0 cm^3
Parachor	389.3 ± 8.0 cm^3
Index of Refraction	1.804 ± 0.05
Surface Tension	63.2 ± 7.0 dyne/cm
Density	1.64 ± 0.1 g/cm^3
Polarizability	23.49 ± 0.5 $10^{-24}cm^3$

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IR SPECTRUM MN1:

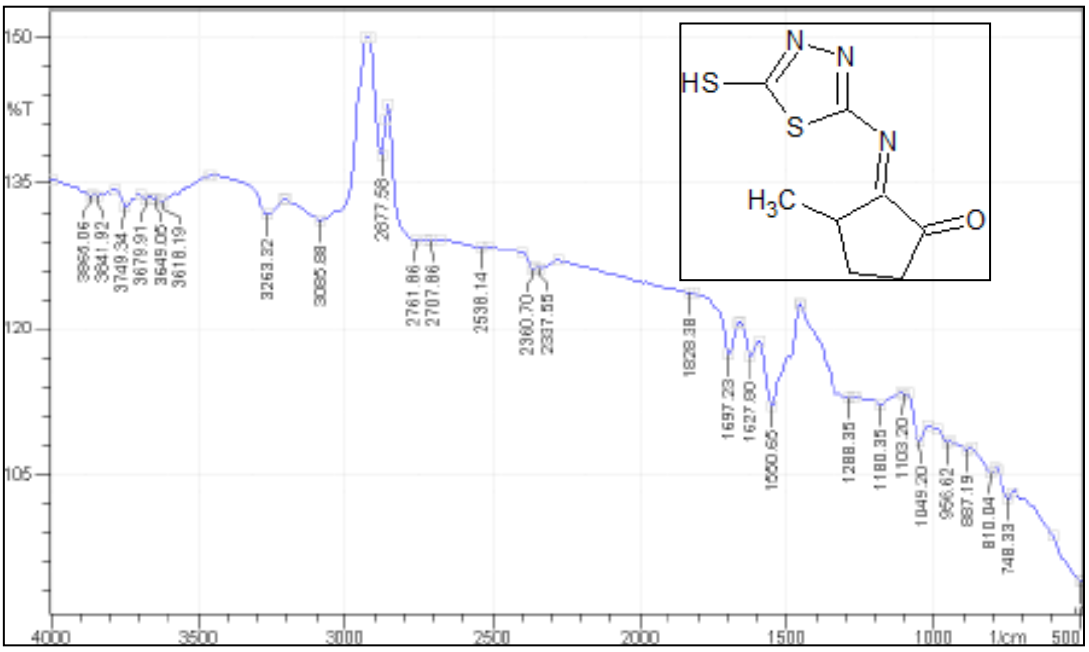
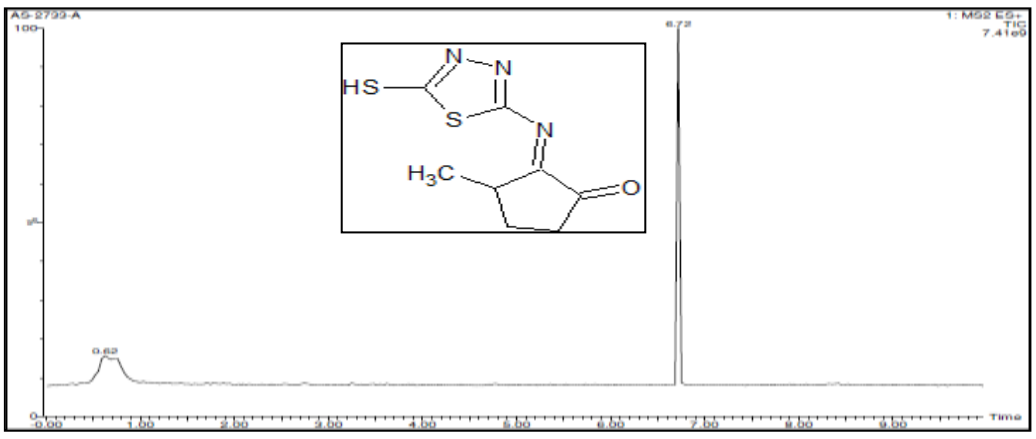


Table No: 11 INTERPRETATION OF IR SPECTRUM MN1

Sr. No.	Wavenumbers (cm^{-1})	Functional group
1.	1627.80	C=O Stretching
2.	2538.14	S-H Stretching
3.	1550.60	C=N Stretching
4.	3085.88	C-H Stretching (Aromatic)

LC MS SPECTRUM MN1:
Mol.wt-227.93 g/mol



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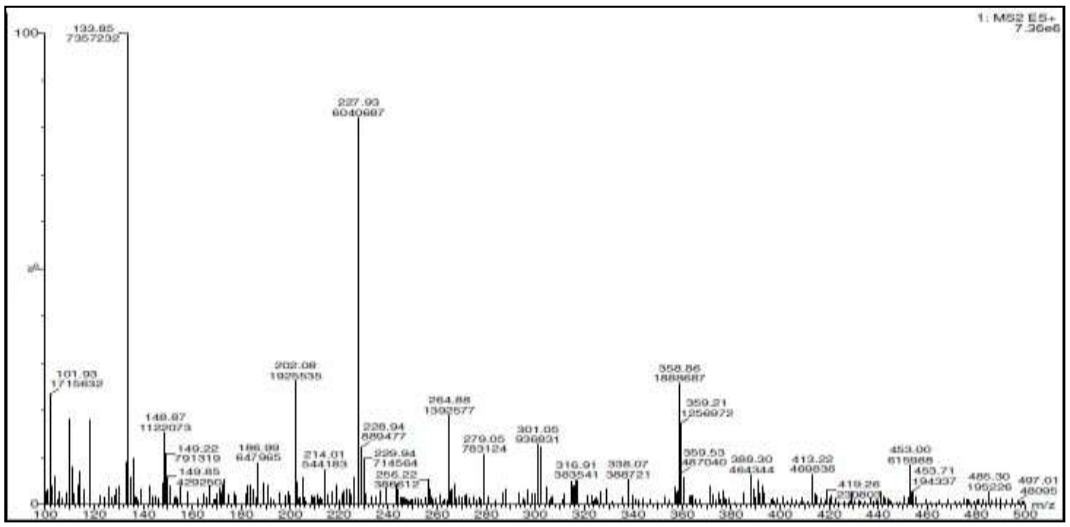

NMR SPECTRUM MN1:

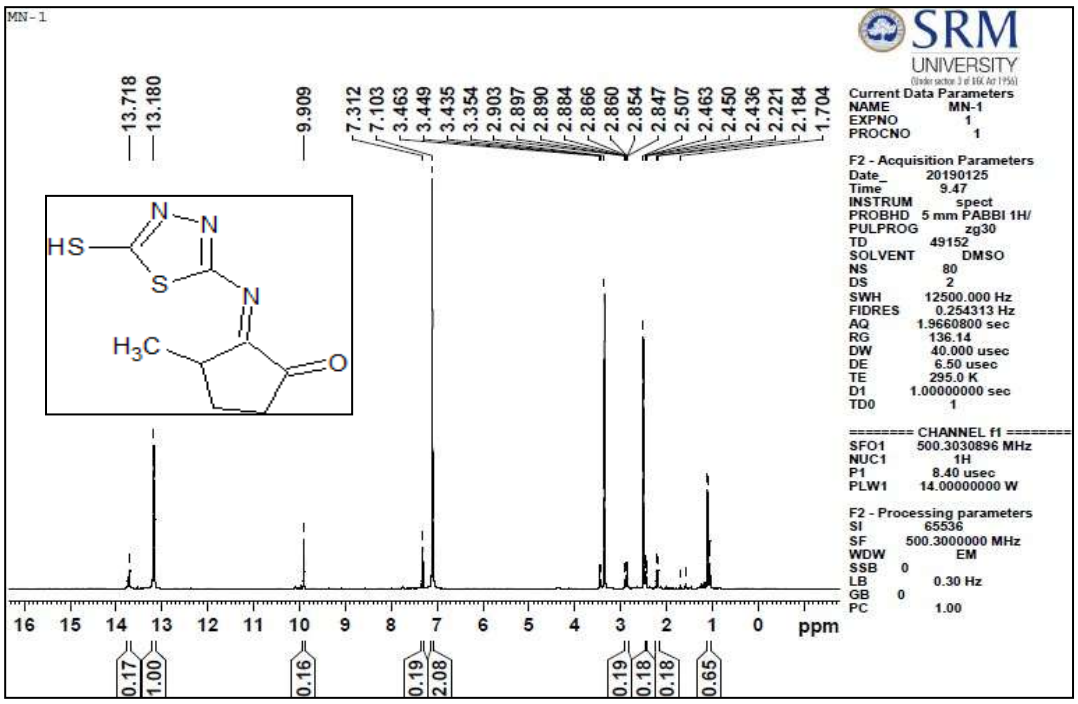

Table No: 12 INTERPRETATION OF NMR SPECTRUM MN1

S.NO	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	1.7-2.4	Multiplet	3 Protons
2.	7.1-7.3	Multiplet	4 Protons
3	9.9	Singlet	2 Protons

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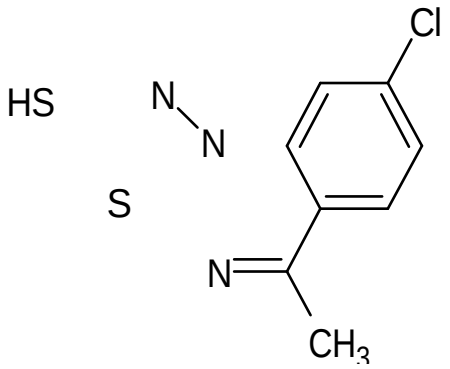
SAMPLE CODE: MN6



5-{[(1*Z*)-1-(4-chlorophenyl)ethylidene]amino}-1,3,4-thiadiazole-2-thiol

Molecular Formula	$C_{10}H_8ClN_3S_2$
Molecular Weight	269.77
Appearance	Light brown colour
Composition	C(44.52%) H(2.99%) Cl(13.14%) N(15.58%) S(23.77%)
Molar Refractivity	72.24 ± 0.5 cm^3
Molar Volume	182.3 ± 7.0 cm^3
Parachor	489.7 ± 8.0 cm^3
Index of Refraction	1.722 ± 0.05
Surface Tension	52.0 ± 7.0 dyne/cm
Density	1.47 ± 0.1 g/cm^3

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IR SPECTRUM MN6:

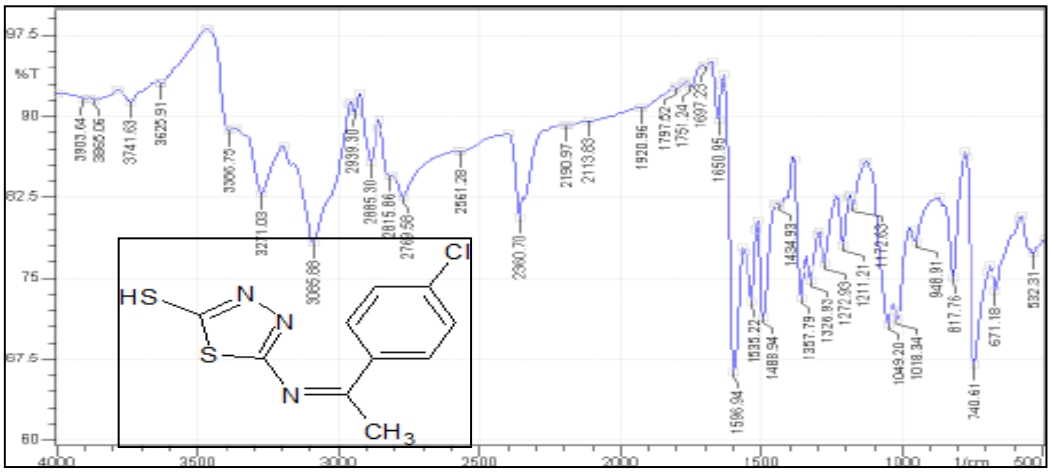
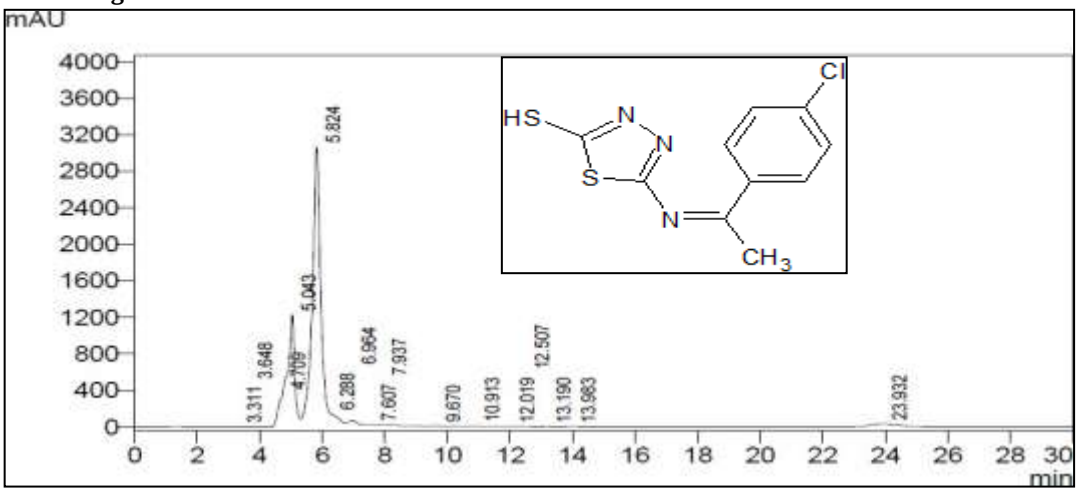


Table No: 13 INTERPRETATION OF IR SPECTRUM MN6

Sr. No.	Wavenumbers (cm^{-1})	Functional group
1.	2561.28	S-H Stretching
2.	740.61	C-Cl Stretching
3.	1535.22	C=N Stretching
4.	3066.88	C-H Stretching (Aromatic)

LCMS SPECTRUM MN6:
Mol.wt-270.70 g/mol



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NMR SPECTRUM MN6:

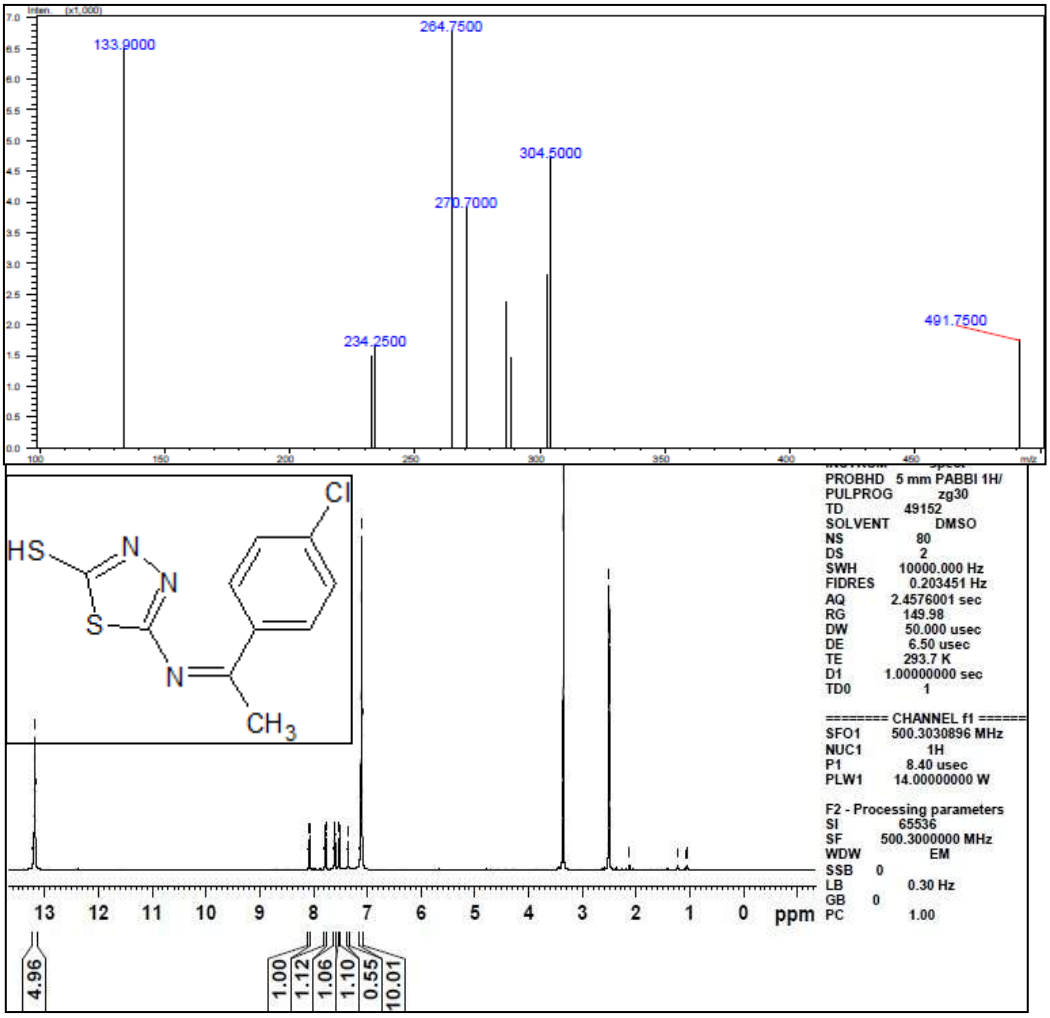

Table No: 14INTERPRETATION OF IR SPECTRUM MN6

Sr. NO.	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	2.5-3.3	Multiplet	3 Protons
2.	7.1-7.7	Multiplet	5 Protons

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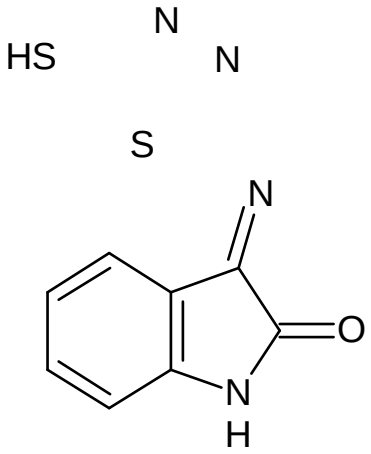
SAMPLE CODE: MN7



(3*E*)-3-[(5-sulfanyl-1,3,4-thiadiazol-2-yl)imino]-1,3-dihydro-2*H*-indol-2-

Molecular Formula	$C_{10}H_6N_4OS_2$
Molecular Weight	262.31
Appearance	Dark red colour
Composition Molar	C(45.79%)H(2.31%)N(21.36%)O(6.10%) S(24.45%)
Refractivity Molar	69.18 ± 0.5 cm^3
Volume Parachor	147.4 ± 7.0 cm^3
Index of Refraction	439.0 ± 8.0 cm^3
Surface Tension	1.911 ± 0.05
Density	78.7 ± 7.0 dyne/cm
Polarizability	1.77 ± 0.1 g/cm^3
	27.42 ± 0.5 $10^{-24}cm^3$

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IR SPECTRUM MN7

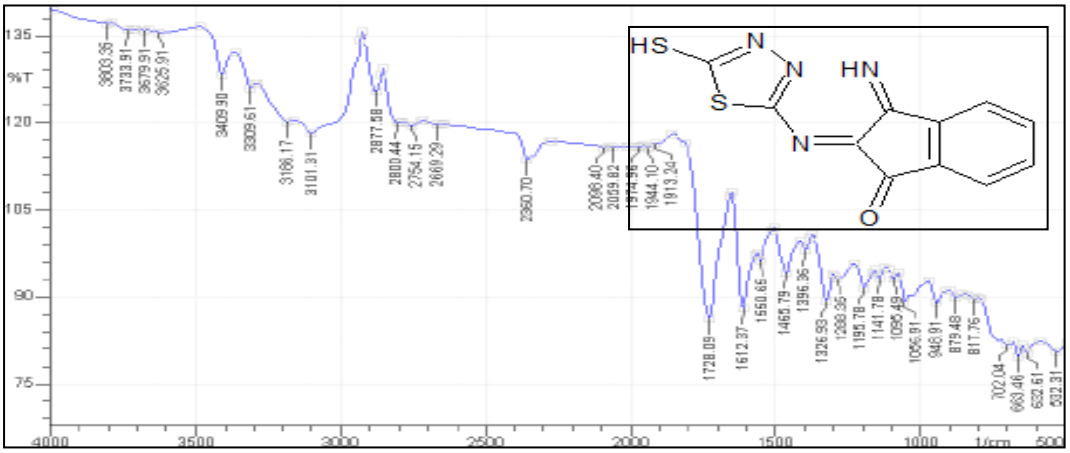
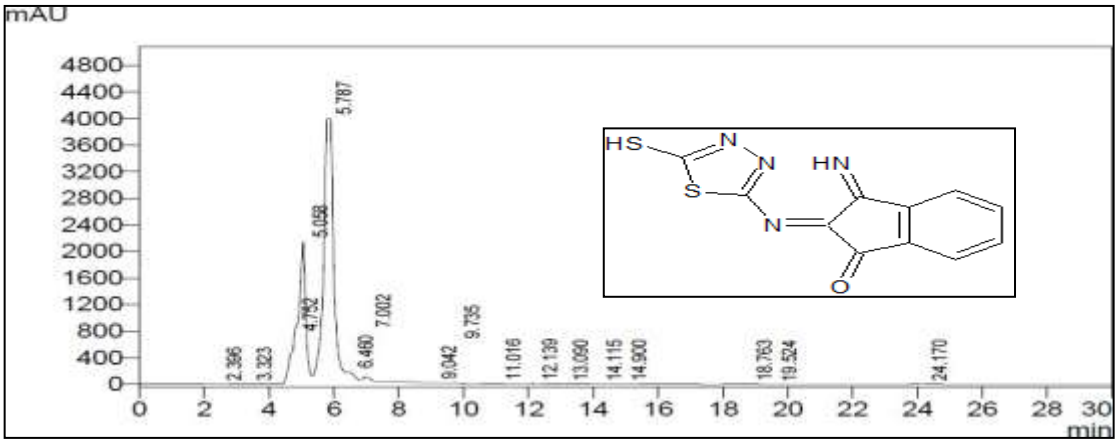


Table No: 15 INTERPRETATION OF IR SPECTRUM MN7

Sr. No.	Wavenumbers (cm^{-1})	Functional group
1.	2669.29	S-H Stretching
2.	1728.09	C-O Stretching
3.	1550.65	C=N Stretching
4.	3101.31	C-H Stretching (Aromatic)

LCMS SPECTRUM MN7
Mol.wt-264.75



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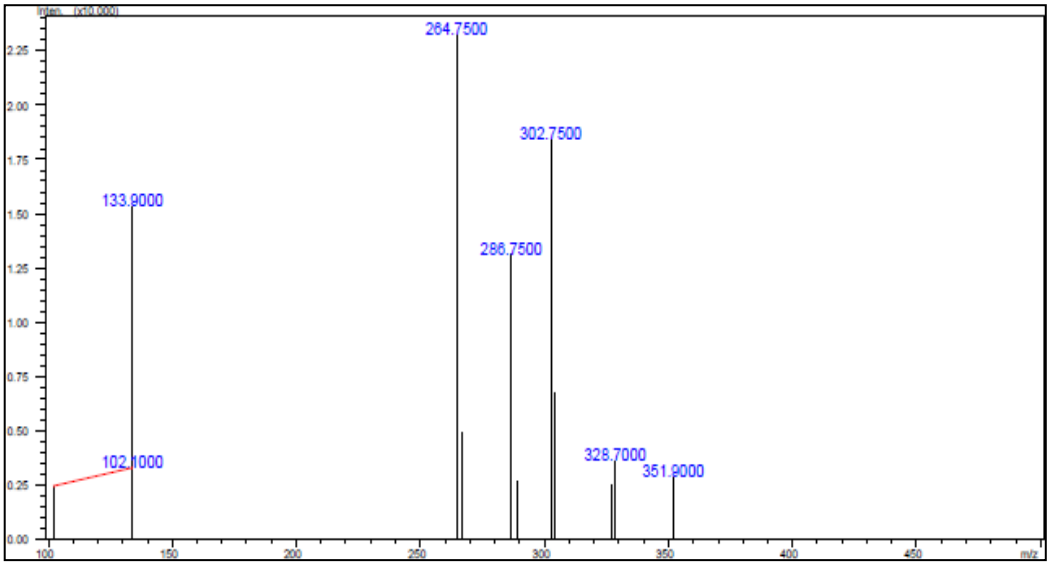

NMR SPECTRUM MN7:

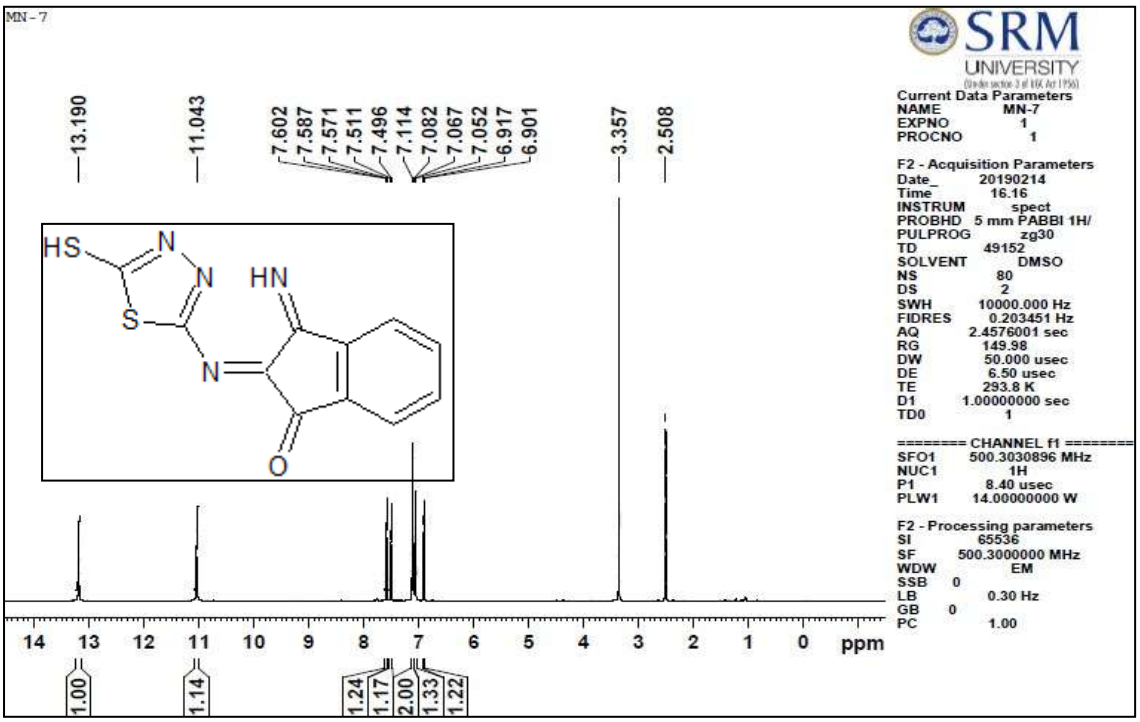

Table No: 16 INTERPRETATION OF NMR MN7

Sr. No.	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	3.3	Singlet	1 Proton
2.	6.9-7.6	Doublet	5 Protons

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INFRARED SPECTROSCOPY:

The IR spectrum of the synthesized compounds were inspected for presence of stretching/bending of the new functional groups and absence of the functional group which induced the changes in the chemical reaction.

All the compounds showed presence of the Imine formation. Also these compounds showed the absence of the stretching/bending for the parent functional groups which underwent the reaction.

The stretching found in the reactant compounds were not found in the IR spectrum of the synthesized compounds.

The reactant compound contains C=O (ketone group), in the resultant product no such stretching is observed instead of that C=N stretching (imine) is observed.

LIQUID CHROMATOGRAPHY:

LC-MS was used to determine the purity of the compounds by looking for additional peaks in a sample that should not be present in the pure compound. Based on the report of LC-MS it was clear that all the compounds were formed as the m+1 peak or m-1 peak and the correct molecular weight was present.

Table No: 17 Molecular Weight Determination by Mass Spectrometry:

Sr. No.	Sample Name	ACTUAL MASS g/mol	CALCULATED MASS g/mol
1.	SN2	226.20	227.22
2.	SN5	279.10	281.14
3.	MN1	227.93	227.31
4.	MN6	270.70	269.78
5.	MN7	264.75	262.32

NMR SPECTROSCOPY:

Proton NMR spectroscopy help us to study the number of equivalent protons and their environment thereby we can ascertain the structure of molecule. The positions of the signals help us to know the nature of protons viz, aromatic, hetero aromatic, aliphatic, vinyl C-H groups. The ^{1}H NMR spectral data of all the synthesized compounds are in conformity with the structure assigned. A singlet at 11.02-11.35 δ was observed for all compounds confirming the presence of N-H proton. All the compounds shows the multiplet and doublet signals for the presence of aromatic protons between (6.2-7.4 δ) hetero aromatic protons between (7.2-7.8δ)**.**

BIOLOGICAL EVALUATION

All the synthesized compounds were screened for their in-vitro antimycobacterial activity against MTB (H37RV, ATCC No: 27294) using Alamar Blue assay (MABA). Pyrazinamide, Ciprofloxacin and Streptomycin were used as reference drugs. The results as Minimum inhibitory concentration (MIC) were presented.

Table No:18 Invitro anti-mycobacterial results

S.No	Sample code	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.	**SN2**	**S**	**S**	**R**	**R**	**R**	**R**	**R**	**R**
2.	**SN5**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
3.	**MN1**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
4.	**MN6**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**R**
5.	**MN7**	**S**	**S**	**S**	**S**	**S**	**R**	**R**	**R**

NOTE: S– Sensitive **R**- Resistant

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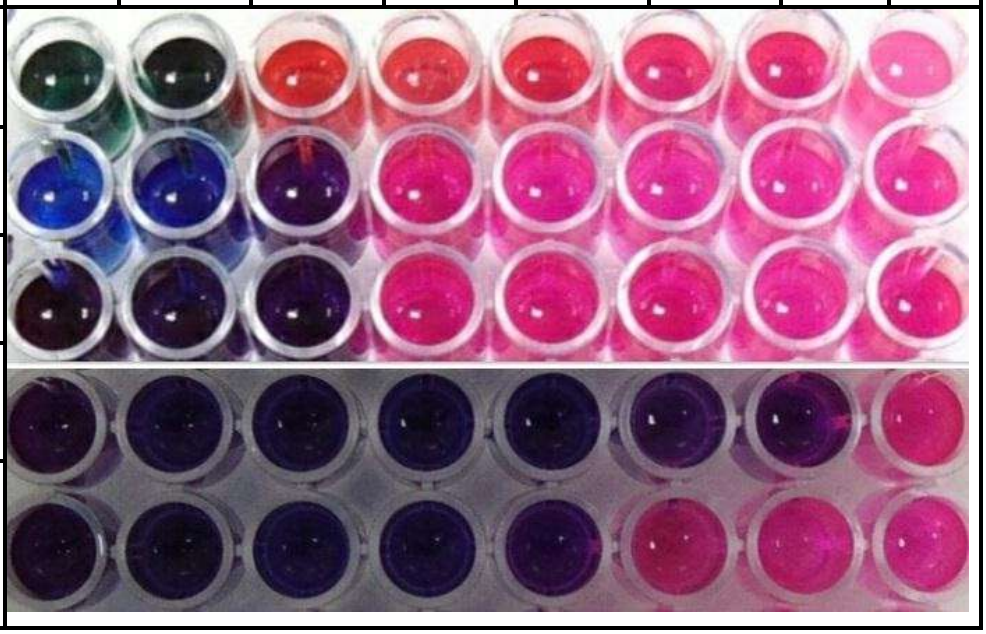
Table No:19 Invitro Anti-mycobacterial report

S. No	Sample Code	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.	SN2								
2.	SN5								
3.	MN1								
4.	MN6								
5.	MN7								

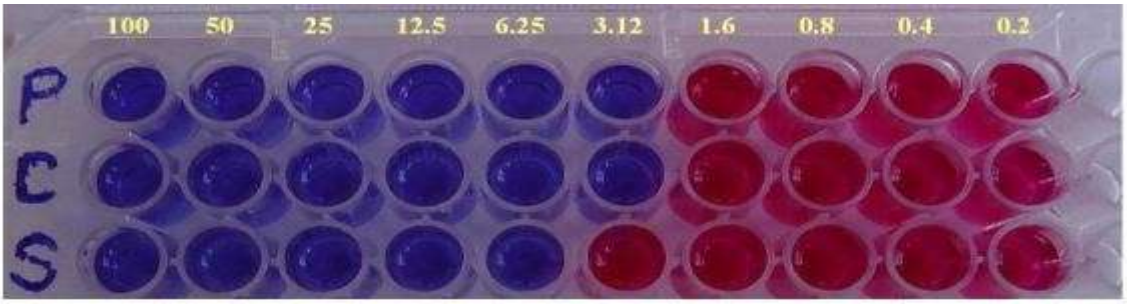


Fig 10: **Standard Drug Photograph**

COMPARATIVE STUDY OF SYNTHESIZIED COMPOUNDS WITHANTI-TB DRUGS

COMPOUND NAME	MOLECULAR WEIGHT g/mol	DOCKING SCORE kcal/mol	MABA ASSAY(MIC) µg/ml
PYRAZINAMIDE	123.11	-5.4	3.125
CIPROFLOXACIN	331.34	-8.13	3.125
SN2	227.21	-8.54	50
SN5	281.13	-7.22	25
MN1	227.30	-7.75	25
MN6	269.77	-7.75	1.6
MN7	262.31	-8.96	6.25

Table 20: Comparative Study with Standard Drugs

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The biological evaluation of the synthesized compounds found that they were sensitive at 50-1.6 µg/mL to the specific organism. Compound MN6 showed better activity when compared to the standard drugs. Other compounds found to have less activity when compared to the standard drugs.

V. SUMMARY

- InhA (enoyl-ACP reductase) (PDB ID – 2h9i) which a critical enzyme for the growth of Mycobacterium tuberculosis was chosen for our study after review of literature.
- Schiff-bases have exhibited versatile pharmacological activity, especially wide range of derivatives had shown potent Anti-tubercular activity.
- Molecules were designed and docked against 2h9i protein using AUTO DOCK® Tools 1.5.6 software.
- Molecules with good Docking score (lower binding energy) and interactions were shortlisted for synthesis. The reaction conditions were optimized.
- Five molecules (SN2, SN5 MN1, MN6, MN7) which were predicted to be effective against Mycobacterium tuberculosis were selected for the synthesis. This was achieved by the molecular docking studies against the target enzyme InhA (PDB ID – 2h9i) of Mycobacterium tuberculosisin-silico ADME assessment and in-silico toxicity predictions.
- All the five molecules were synthesized. The synthesized compounds were purified and characterized.
- The physical characterization and spectral studies like "FT-IR, 1H-NMR, Mass spectrometry confirmed the proposed structure ofthe synthesized compounds.
- All the synthesized compounds were investigated for their in-vitro Anti-tubercular potential using "Microplate Alamar Blue assay" MABA Assay.
- All the compounds showed moderate to potent in vitro activity against MTB with MIC range 0.8-100µg/ml concentrations.
- Compound MN6 shows most potent in-vitro activity with MICs 1.6µg/ml concentration which were comparable into the known anti-TB drugs: Pyrazinamide-3.125µg/ml, Ciprofloxacin - 3.125µg/ml and Streptomycin - 6.25µg/ml.
- The compound MN7 was active at 6.25 µg/ml and the compounds MN1 and SN5 were active at 25 µg/ml. Compound SN2 was active at 50 µg/ml.

VI. CONCLUSION

- ✓ This work concludes that the synthesized molecules are effective in inhibiting Enzyme InhA (enoyl-ACP reductase) (PDB ID – 2h9i) which is important for the growth of Mycobacterium tuberculosis.
- ✓ All the 5 compounds gave Docking score between -7 to -9 kcal/mol. This goes to prove that PDB ID – 2h9i is a critical enzyme for anti-mycobacterial activity.
- ✓ The minimum inhibitory concentration of the 5 synthesized compounds against H37Rv ranged from 1.6 to 50 µg/ml.
- ✓ Further structural refinement to the structure of the synthesized compounds is expected to yield promising molecules against the pathogen Mycobacterium tuberculosis.

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