DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL ISATIN-UREA SCHIFF BASE DERIVATIVES AS ANTITUBERCULAR AGENTS TARGETING *ATP SYNTHASE*

A Dissertation submitted to
THE TAMIL NADU Dr.M.G.R. MEDICAL UNIVERSITY
CHENNAI - 600 032

In partial fulfillment of the requirements for the award of the Degree of
MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by
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Under the guidance of
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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
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OCTOBER 2021

COLLEGE OF PHARMACY
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CERTIFICATE

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Dr.A.JERAD SURESH, M.Pharm., Ph.D., M.B.A.,
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Dr.A.JERAD SURESH, M.Pharm., Ph.D., M.B.A.,
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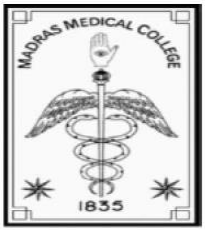

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EXAMINERS

1.

2.

ACKNOWLEDGMENT

First and foremost I thank the Almighty for all the love, grace and blessings he has bestowed upon me, for strengthening me throughout, to successfully complete this dissertation.

I consider this as an opportunity to express my gratitude to all the people, who have been involved directly or indirectly with the successful completion of this dissertation.

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I express my thanks to **Dr.Kishore G Bhat**, Professor and Head, Department of Microbiology,Maratha Mandal's Institute Of Dental Science And Research Institute,Belgaum,Karnataka for his support in carrying out *in vitro* evaluation of anti-tubercular activity.

I would like to express my heartfelt thanks to **Dr.M. Saravana Kumari.,MVsc.,** Special Veterinary officer, MMC and IAEC members for her forceful work for my Acute toxicity study.

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LIST OF ABBREVIATIONS

TB	**Tuberculosis**
WHO	**World Health Organization**
HIV	**Human Immuno Virus**
AIDS	**Acquired Immuno Deficiency Syndrome**
DOTS	**Directly Observed Treatment Short-Course**
MDR-TB	**Multi Drug Resistant- Tuberculosis**
XDR-TB	**Extensively Drug Resistant- Tuberculosis**
ATP	**Adenosine Triphosphate**
IR	**Infrared spectroscopy**
NMR	**Nuclear Magnetic Resonance Imaging**
LC-MS	**Liquid Chromatography-Mass Spectrometry**
MABA	**Micro plate Alamar Blue assay**
DMSO	**Dimethyl Sulfoxide**
CADD	**Computer Aided Drug Designing**
MIC	**Minimum Inhibitory concentration**
LBDD	**Ligand Based Drug Design**

INTRODUCTION [1]

Tuberculosis is an infectious disease considered as a global epidemic [2]. The tuberculosis infection caused by a type of bacterium called Mycobacterium tuberculosis which is an airborne pathogen transmitted among a human which infects macrophages in the lungs. Mycobacterium tuberculosis was first identified by Robert Koch in 1882 and it is curable and preventable. Tuberculosis is the most attractive areas of research because it is the second major cause of death due to an infectious disease in adults worldwide. [2]

LATEST STATUS OF TB EPIDEMIC [4]

TB occurs in every part of the world. Thirty countries of the world have the highest TB burden, among top eight ranking countries are India, china, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh and south Africa. This gravity of situation is understood from the report published in 2020 by WHO, according to this 43% of the largest number of new TB cases occurred in south-east region, followed by 25% of new cases occurred in African region and western pacific with 18% new cases. This alarming situation exposes 0.22 billion people will have acquire TB and 79 million people could die due to TB by the year 2030[3].

MYCOBACTERIUM TUBERCULOSIS [3]

The mycobacterium tuberculosis is under the genus of mycobacterium in which the most important species are mycobacterium tuberculosis (causing TB) and mycobacterium leprae (causing leprae). The mycobacterium tuberculosis complex includes the strains of M.tuberculosis, M.africanum, M.bovis. These species having 99.9% similarity at nucleotide level and differentiate in terms of tropisms, phenotypes and pathogenicity. Mycobacterium bovis is the type of mycobacterium most commonly found in cattle and causes TB infections to humans.

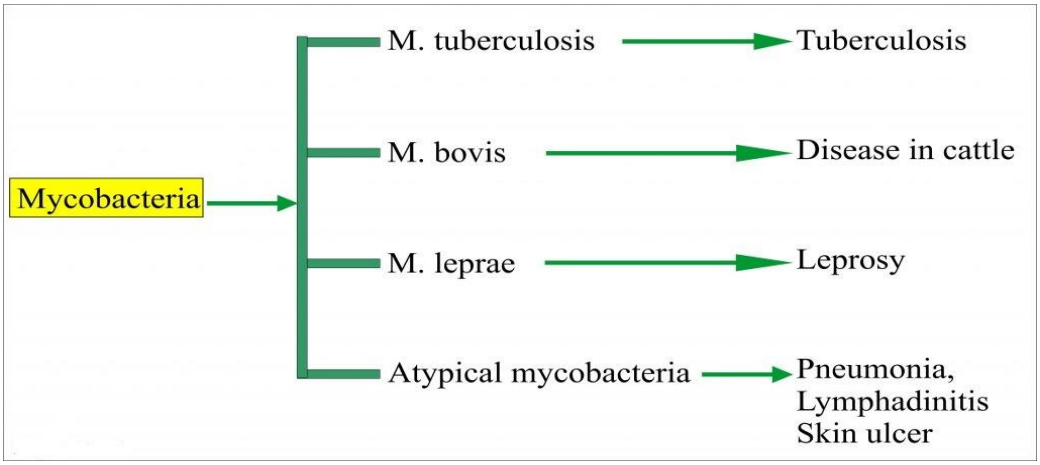


Figure 01: classification of mycobacterium tuberculosis

HISTORY OF TUBERCULOSIS: [3]

- Tuberculosis was known to exist since antiquity. But until 1820, it was not identified as a single disease. The name tuberculosis was coined by 'J.L.Schonlein'.
- In the earlier times, Tuberculosis was commonly known by the terms such as Phthisis, Pulmonalis and White Plague.
- Pulmonary tuberculosis was called as Phthisis which means Consumption in Greek. The common term consumption was used to denote the severe weight loss produced as a result of this deadly disease.
- In 1882, Robert Koch identified the organism causing the disease and described it as ***Mycobacterium tuberculosis***.
- In the 19th and early 20th centuries, TB was more common among the urban poor and the major concern was on the prevention and treatment of the disease.
- Due to this, medical council research initially focused on the tuberculosis research.

CAUSATIVE ORGANISM:

Mycobacterium tuberculosis is gram positive, non-motile, non-spore forming and aerobic bacteria. The curved intracellular rod size 0.2-0.6µm width and 1.0-10µm long. Their cell wall contains mycolic acid rich long chain glycolipids that protect mycobacteria from cell lysosomal attack and also retain red basic fuchsine dye after acid rinsing (acid-fast stain).

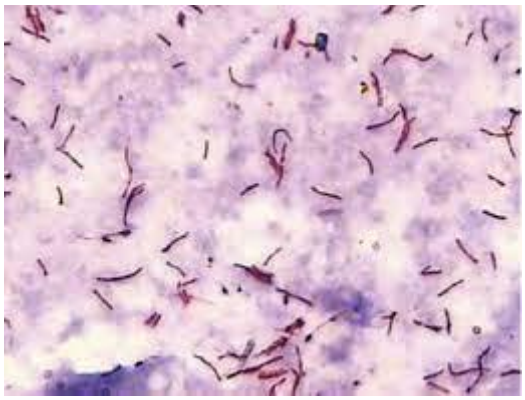


Figure 02: Acid-fast staining of Tb bacteria

HIERARCHICAL CLASSIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

Table 01: Hierarchical classification of *Mycobacterium tuberculosis*

Domain	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Order	Actinomycetales
Family	Mycobacteriaceae
Genus	Mycobacterium
Species	***Mycobacterium tuberculosis***

TRANSMISSION OF TUBERCULOSIS [3]

- Mycobacterium tuberculosis is an airborne pathogen.
- Transmitted among humans which infect macrophages in the lungs.
- Two possible outcomes,
    1. T-cell mediated adaptive immunity enabling the host to eradicate the bacilli at the initial site of infection. The bacilli may further multiply within in the macrophages for 7-21 days.
    2. Alveolar macrophages engulf the bacteria nonspecifically but not activated to kill them.
- Failure of adaptive immunity leads to uncontrolled growth of an organism.
- Subsequent spread through the lymphatic system to secondary sites.
- If not treated it kills more than 50% of the people.

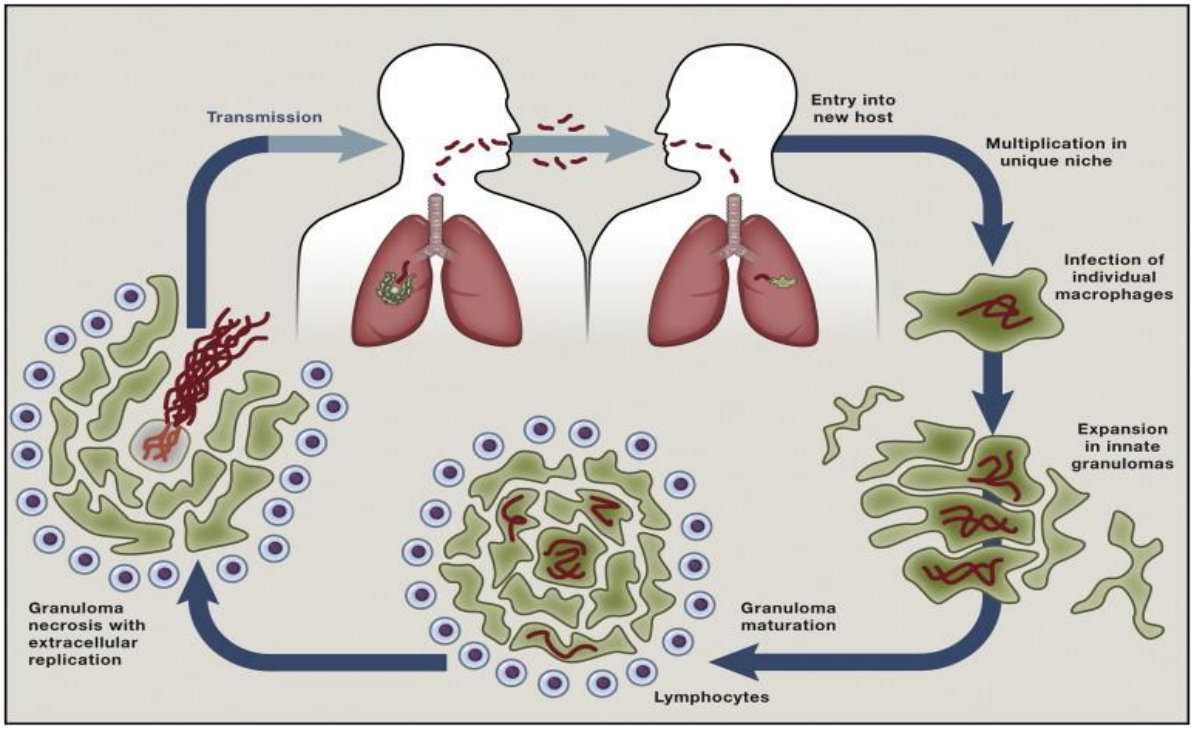


Figure 03[40]: Transmission of Tuberculosis

CLASSIFICATION OF TUBERCULOSIS: [5]

ACTIVE TUBERCULOSIS:

Active tuberculosis is a condition in which Mycobacterium tuberculosis actively causes infection and is contagious. The symptoms of active TB vary depending on whether it is pulmonary tuberculosis or extra pulmonary tuberculosis.

LATENT TUBERCULOSIS:

Latent Tb is a condition in which mycobacterium tuberculosis bacteria are dormant in the body asymptomatically. In this state patient infected with TB but doesn't have active TB condition. 10% people will develop active Tb at a later their life.

MULTIDRUG RESISTANT TUBERCULOSIS:

Resistance to first line anti-tuberculosis drug like isoniazid and rifampicin is known as MDR-TB.

EXTENSIVELY DRUG RESISTANCE TUBERCULOSIS:

Resistance to isoniazid, rifiampicin, fluroquinolones and one of the second line drugs being capable of being injected is known as XDR-TB.

MEDICAL HISTORY OF CURRENT TB CHEMOTHERAPHY [2]

- Treatment of effective chemotherapy for tuberculosis began in 1940s with the discovery and use of streptomycin and para-aminosalicylic acid.
- The first randomized controlled study of STR treatment for TB by the **BMRC (BRITISH MEDICAL RESEARCH CUNCIL)** showed that streptomycin was effective in short-term but develop resistance for the long-term use.
- Later the Isoniazid was discovered and the combined triple drug therapy was introduced.
- In 1993, the WHO declared TB as a global emergency. If only a single drug used for a long period of time resistant mutant will emerge, to avoid this combination of drug therapy used.
- First two months of tuberculosis considered as intensive phase followed by four months considered as continuous phase.
- The hope of eradication of TB arises with the development of an antibiotic Streptomycin in the year 1946. But, the rise of drug resistant strains in 80's has increased the need for newer therapeutic agents.
- Development of resistance to the drugs used in the TB treatment regimen resulted in the emergence of MDR (Multi-Drug Resistant) TB and XDR (Extensively Drug Resistant) TB.
- Co-infections like HIV, COVID-19 and the metabolic disorders like Diabetes worsens the body's natural immunity and renders the management of tuberculosis very challenging.

CELL WALL STRUCTURE: [6]

The structure and composition of the cell wall is unique. It contains a **mycolyl- arabinogalactan-peptidoglycan complex** which maintains the cellular integrity and is responsible for the virulence of the organism. The cell organelles are surrounded by the cytoplasmic membrane and then the peptidoglycan layer. The arabinogalactan layer connects the peptidoglycan layer to the outer mycomembrane composed of the mycolic acids.

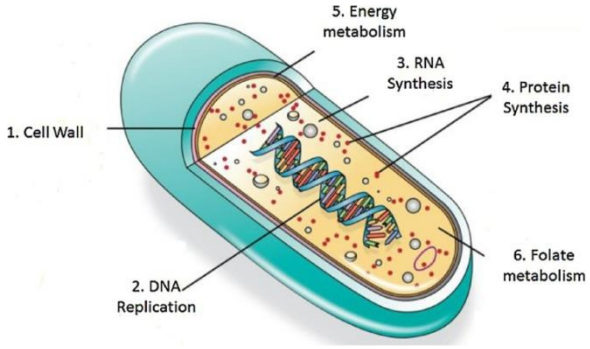


Figure 04[39]: Cell wall structure of Mycobacterium Tuberculosis

A. Peptidoglycan layer:

It comprises of the short peptides and glycan strands that are made up of **N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues** linked by **β-1→4 bonds**. A part of the MurNAc molecules is oxidized to N-glycolyl muramic acid which is also responsible for the cell wall integrity and resistance shown by the bacterium towards the lysozyme and the β-lactam antibiotics. A rigid cell wall is produced by cross linking the peptidoglycan chains.

B. Arabinogalactan layer:

It is a highly branched **heteropolysaccharide** composed of galactose and arabinose in a furanoid form. It is covalently linked with the MurNAc residues of the peptidoglycan layer and connects it with the outer mycolic acid membrane layer.

C. Mycomembrane:

It comprises 50% dry weight of the cell wall. Mycolic acids are the unique **α- branched lipids** which form the homogenous external membrane. They are the highly hydrophobic long chain fatty acids which form a barrier to the hydrophilic drug molecules by forming a lipid shell around the organism and it also acts as a significant determinant of virulence. The external layer maintains the host-symbiont relationship as it contains trehalose monomycolate (TMM) and trehalose dimycolate (TDM/ Cord factor).

Advantages of the high concentration of lipids:

- ✓ The lipid rich outer cell layer makes the organism impermeable to certain stains and dyes.
- ✓ It prevents the antibiotics from entering into the cell.
- ✓ It resists osmotic lysis and killing by acidic and alkaline compounds.
- ✓ It prevents the organism from the lethal oxidation and allows surviving even inside the macrophages.

ANTI-TB TREATMENT:

In India, **Revised National TB Control Program (RNTCP)** was initiated in 1997 to provide proper treatment guidelines across the entire nation. Now, it is updated as RNTCP- II to provide services for TB/HIV & MDR-TB. It utilizes the **Directly Observed Treatment Short course (DOTS)** strategy recommended by the WHO in the mission against Tuberculosis infection. It carries out the five years TB National Strategic Plan (NSP) by the Government of India. The national goal announced in 2017 was '**Elimination of TB in India by 2025'**. In 2020, RNTCP was rechristened as National Tuberculosis Elimination Programme (NTEP).

Table 02: Anti-TB drugs for Drug susceptible Tuberculosis

Drug Susceptible TB	
Intensive phase	**Continuation Phase**
2 months	4 months
Isoniazid, Rifampicin, Ethambutol and Pyrazinamide.	Isoniazid and Rifampicin.

Table 03: Anti-TB drugs for Multi-Drug Resistant Tuberculosis

Drug Resistant TB (MDR TB)	
Intensive Phase	**Continuation Phase**
4 – 6 months	5 months
Ethambutol/Pyrazinamide, Clofazimine/high dose of Isoniazid, Gatifloxacin/Moxifloxacin, Kanamycin/Amikacin	Ethambutol/Pyrazinamide, Clofazimine, Gatifloxacin/Moxifloxacin

CO – INFECTION:

Risk of developing Tuberculosis is more in the Immunocompromised individuals. **HIV/AIDS infection** makes an individual more immunocompromised. A person with HIV Infection is 16.27 times more vulnerable to develop the TB infection than the non-HIV individuals. The risk of death is also twice in these cases. The progress of one disease got speeded by the other.

Providing treatment for both the infection i.e., HIV- Anti RetroViral therapy (ART) & Anti-TB therapy at a time results in several problems such as,

- ✓ Drug-drug interactions
- ✓ Cumulative drug toxicities
- ✓ High pill burden
- ✓ The Immune Reconstitutive Inflammatory Syndrome (IRIS).

At present, the pandemic COVID-19 threatens to reverse the progress made towards the Global TB targets. Due to the national lockdown, there were many restrictions in face to face assessments of patients & infection control strategies which resulted in drop in the new active TB case diagnosis, outpatient settings and monitoring & supply of Anti-TB medications.

UNMET NEEDS OF CURRENT TUBERCULOSIS TREATMENT OF FIRST LINE THERAPY: [8]

The first line TB treatment of standaridized short-course chemotherapy, rifampicin and isoniazid was presecribed for 6 months. Later supplemented with pyrazinamide and ethambutol for following 2 months for drug-susceptible TB.

1. Drug-drug interaction due to high enzyme induction of cytochrome p450.
2. The emergence of drug resistance tuberculosis.
3. Adverse effects from many of the existing drugs.
4. Current TB drugs not active against MDR and XDR tuberculosis.
5. Treatment of latent TB infection.

WHY NEED TB NEW DRUG? [7]

- ✓ Need of novel drug combinations
- ✓ Reducing pill burden
- ✓ Suitable pediatric formulation not available
- ✓ Need for treating HIV-TB co infected patients
- ✓ Available in low price
- ✓ Shorten treatment time
- ✓ To improve the MDR TB treatment

VARIOUS ENZYME TARGETS: [11]

Some of the important enzymes involved in the various cellular processes and remained as the attractive drug targets in the *Mycobacterium tuberculosis* are listed below;

Table 04: Critical Enzymes in *Mycobacterium tuberculosis*

ENZYMES	FUNCTION
Glucosamine-6-phosphate Synthase	Peptidoglycan layer synthesis
Phosphoglucosmaine mutase	Peptidoglycan layer synthesis
Transpeptidases	Cross linking of peptidoglycan chains
Aminoacyl t-RNA synthetases	Menaquinone synthesis
Fatty acyl-AMP ligases	Lipid metabolism
Fatty acyl-coA ligases	Lipid metabolism
Non-ribosomal peptide synthetases	Mycobactin synthesis
Succinate dehydrogenase	Energy production
Glutamine synthetase	Nitrogen metabolism
ATP synthase	ATP production
Rhamnosyl transferase	Arabinogalactan synthesis
α-manno pyranosyl transferase	Phosphatidyl-myo-inositol mannoside synthesis
Fatty acid synthase	Mycolic acid synthesis

For the current research work, **ATP synthase** was chosen as a drug target for ***in-silico*** and ***in-vitro*** studies

BIOLOGICAL TARGET

ENZYME PROFILE- *ATP SYNTHASE* [14] [15][16][17][18]

ATP SYNTHASE is an essential enzyme in the obligate aerobic mycobacterium genus. ATP synthase is membrane-protein complex which is responsible for the production of ATP. It consists of two motor like domains such as F1 motor and F0 motor. ATP driven F1 domain composed of five subunits (α3β3γδε) and it is located in the cytoplasm. Proton-driven F0 domain is embedded in the membrane with three subunits ($_{a1b2c10-15}$). It is an omnipresent enzyme. Mycobacterium can survive by the production of energy molecule ATP by ATP synthase.

Membrane bound ATP synthase converts ADP to ATP by utilizing the transmembrane electrochemical ion (H+ or Na+) gradient. The c subunit of F0 ATP synthase has ion binding sites which transport ions across the membrane and generate the power for ATP synthesis. The catalytic site present in the F1 part of ATP synthase produces ATP by combining ADP and pi.

Since, ATP synthase is essential for the optimal growth of Mycobacterium tuberculosis. It is a key enzyme involved in the energy metabolism. So it is an important drug target for developing novel molecules against the enzyme which can be a promising drug candidate in the anti-tuberculosis therapy. Mycobacterium ATP synthase inhibition of energy metabolism has potential to shorten the treatment time.

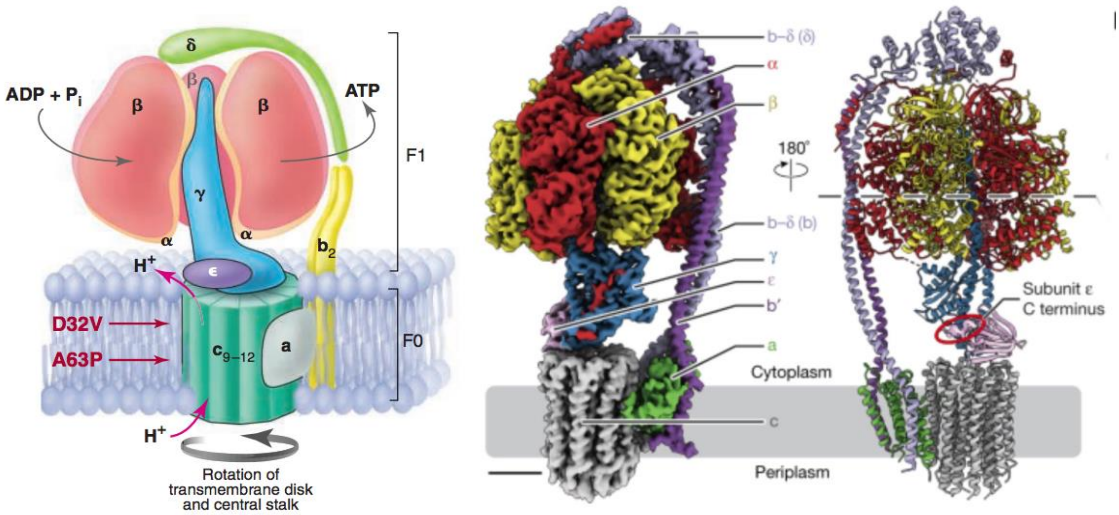


Figure 05: Secondary structure of ATP SYNTHASE (PDB ID– 7JG

BASIC HETERONUCLEAR SCAFFOLD [19][22]

Heterocyclic compounds are those which contain at least one non carbon hetero atom in their ring structure. Many of the drugs contain heterocyclic ring in their structure which have proved to be efficient in their pharmacological activity.

Indole, a bicyclic heteroarene with the Molecular formula C_8H_7N is formed by the fusion of the benzene ring with the pyrole ring. Substituted indoles are the structural elements and synthetic precursors of many compounds. Some of them include tryptophan-derived tryptamine alkaloids, psilocybin (naturally occurring psychedelic drug), Auxin (plant hormone), Indomethacin (Anti-inflammatory drug), Pindolol (Beta-blocker).,etc.,

Isatin (1H-indole-2,3-dione - a diketo derivative of indole) also known as Tribulin, Indenedione or Indolequinone is a substituted indole with two carbonyl groups at 2^{nd} & 3^{rd} positions. In 1840, it was obtained as an oxidation product of Indigo dye by **Otto Linne Erdman & Auguste Laurent**. In humans Isatin is found as a metabolic derivative of the Hormone Adrenaline and Isatin derivatives also occur in plants.

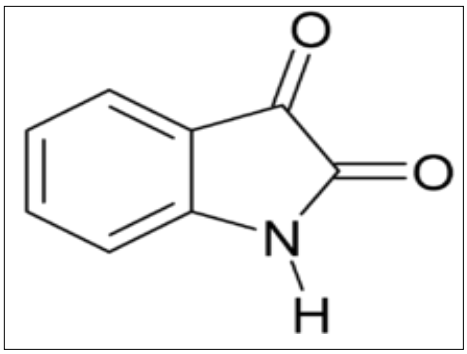
Isatin undergoes numerous substitution (N-substitution, Electrophilic aromatic substitution at Phenyl ring), Addition (Nucleophilic addition at Carbonyl groups), Oxidation, Reduction and Ring expansion reactions. Isatin is one of the valuable building blocks in organic synthesis, due to its unique reactivity. They have a wide range of applications in various fields. Substituted and unsubstituted isatins can be synthesized by **Stolle and Sandmeyer Methodologies.** N-substituted isatins can also be synthesized by simple oxidation procedures using suitable oxidizing agents.

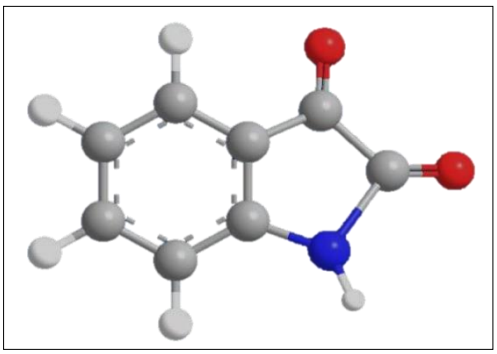
Biological activities of Isatin comprises,

- Anti-cancer
- Anti-bacterial
- Anti-diabetic
- Anti-fungal
- Anti-tubercular
- Anti-malarial

- Anti-inflammatory
- Anti-anxiety
- Anti-oxidant

- Since **Isatin** has wide range of pharmacological activities, it was chosen as a **basic heteronuclear scaffold** in the present study, for designing novel molecules against the enzyme target.
- Structural modifications were performed on the nucleus to get novel isatin derivatives.





REVIEW OF LITERATURE

LITERATURES RELATED TO THE TUBERCULOSIS AND ITS MANAGEMENT:

1. **Chaw, Liling et al., (2020)** [24] assessed the global TB burden and the focus of majority of Countries on the Latent tuberculosis infection by performing a literature survey.
2. **Canetti et al., (2020)** [26] briefly outlined the complication of Anti-TB treatment in the HIV co-infected patients and the new drug regimen for the co-treatment was clearly explained.
3. **Migliori, G. Battista et al., (2020)** [43] evaluated the risks associated with the treatment of Drug-resistant tuberculosis and their clinical management.
4. **Daley, L.Charley (2019**) [47] has outlined the current approaches to control tuberculosis and the several gaps needed to be closed for the eradication of the disease.
5. **Pezella, A.Thomas (2019)** [28] highlighted the epidemiology of TB since ancient times and the evolution of the Tuberculosis treatment.
6. **Adeeb shehzad, Gauhar Rehman et al., (2013)** [7] have explained the challenges in the development of drugs for the treatment of tuberculosis.
7. **Beena and diwan s. rawat (2013)** [3] have reported the overall critical view of Anti-tuberculosis drug research.
8. **Gwendlyn a. mariner et al., (2011)** [2] reported the medicinal chemistry of tuberculosis chemotherapy.
9. **Shruti Rawal, Richa Sood, Nipun Mahajan et al., (2010)** [42] assessed the current status and future prospects for tuberculosis.

LITERATURES RELATED TO THE MYCOBACTERIUM TUBERCULOSIS DRUG TARGETS

10. **Shetye, Gauri et al., (2020)** [25] have elaborated on the novel enzyme targets in the ***Mycobacterium tuberculosis*** that are necessary for the survival of the organism.
11. **Shanib Bhat, Zubair et al., (2018)** [39] have summarized the current TB regimen and the various drug targets exploited in the organism for new drug discovery.
12. **Takushi kaneko et al., (2011)** [43] have reported the novel targets and novel modes of TB drug development.
13. **Zhenkum ma, Christian lienhardt et al., (2010)** [44] assessed the present tuberculosis treatment and their unmed needs.

14. **Sharmila anishetty, mrudula pulimi et al., (2005)** [45] have detailed the potential drug targets in mycobacterium tuberculosis through metabolic pathway analysis.

LITERATURES RELATED TO THE TARGET ENZYME ATP YNTHASE

15. **Martin G. Montgomery, Jessica Petri et.al. (2021)**[15] Described the structure of the ATP synthase from mycobacterium smegmati provides targets for treating tuberculosis.
16. **Hui Guo, Gautier M. Courbon et al., (2021)** [18] briefly outlined the structure of mycobacterium ATP synthase bound to the tuberculosis drug bedaquiline.

17. **McNeil, M. B., Ryburn et al., (2020)** [16] have detailed the transcriptional inhibition of the F1F0-type ATP synthase has bactericidal consequences on the viability of mycobacterium tuberculosis.

18. **Adam Hotra, Priya Ragunathan et al., (2020)** [17] have described the discovery of a novel mycobacterium F-ATP synthase inhibitor and its potency in combination with diaryquinolines.

19. **Sunil kumar, rukmankesh mehra et al., (2017)** [14] highlighted the screening of anti-tubercular compounds library identifies novel ATP synthase inhibitors of mycobacterium tuberculosis.

20. **Aparna bahuguna, Diwan s.rawat (2019)** [11] has assessed the development of new drugs, repurposed drugs and their potential drug targets.

LITERATURES RELATED TO THE BASIC SCAFFOLD

21. **Dogan, D.Sengul(2020)** [29] synthesized various thiourea based derivatives against the **InhA enzyme** and structural characterization were performed. The Minimum Inhibitory Concentration (MIC) and percentage of Enzyme Inhibition was determined. Molecular docking and *in-silico* molecular property calculation for the synthesized molecules were carried out.
22. **Jiang D et al., (2018)** [46] synthesized the various istain derivatives against anti-tubercular activity.

23. **Clay, Charles Michael et al., (2011)** [21] reported the various methods of synthesis of Isatin and their chemical reactivity.
24. **Arockia Rajetal., (2001)** [22] synthesized novel Schiff bases of Isatin by the condensation of isatin and various substituents. The spectral data of the Schiff bases were interpreted.

LITERATURES REGARDING THE COMPUTER AIDED DRUG DESIGN

25. **Fernando, D.Prielo Martinez (2019)** [36] outlined the importance of the Computational drug design methods in the Drug Discovery process.
26. **Hachem, El.Nehme et al., (2017)** [37] have visualized Docking as an essential computation tool for the Structure based drug design.
27. **Tien, Sheng et al., (2015)** [38] established the importance of drug likeness of a molecule and their prediction by various ***in-silico*** filters.

LITERATURES EXPLAINING THE MICROPLATE ALAMAR BLUE ASSAY (MABA) & ACUTE TOXICITY STUDIES

28. **WHO (2018)** [30] published technical manual in which various methods for testing the drug susceptibility in solid and liquid media are clearly explained.
29. **Cho, Sanghyun (2015)** [31] briefly explained the procedure involved in determining the Drug susceptibility by MABA.
30. **Jonsson, martina et al., (2013)** [32] have determined the acute toxicity of the Mycotoxin by following the OECD Guidelines 423 **[Acute Oral Toxic Method]**

AIM & OBJECTIVES

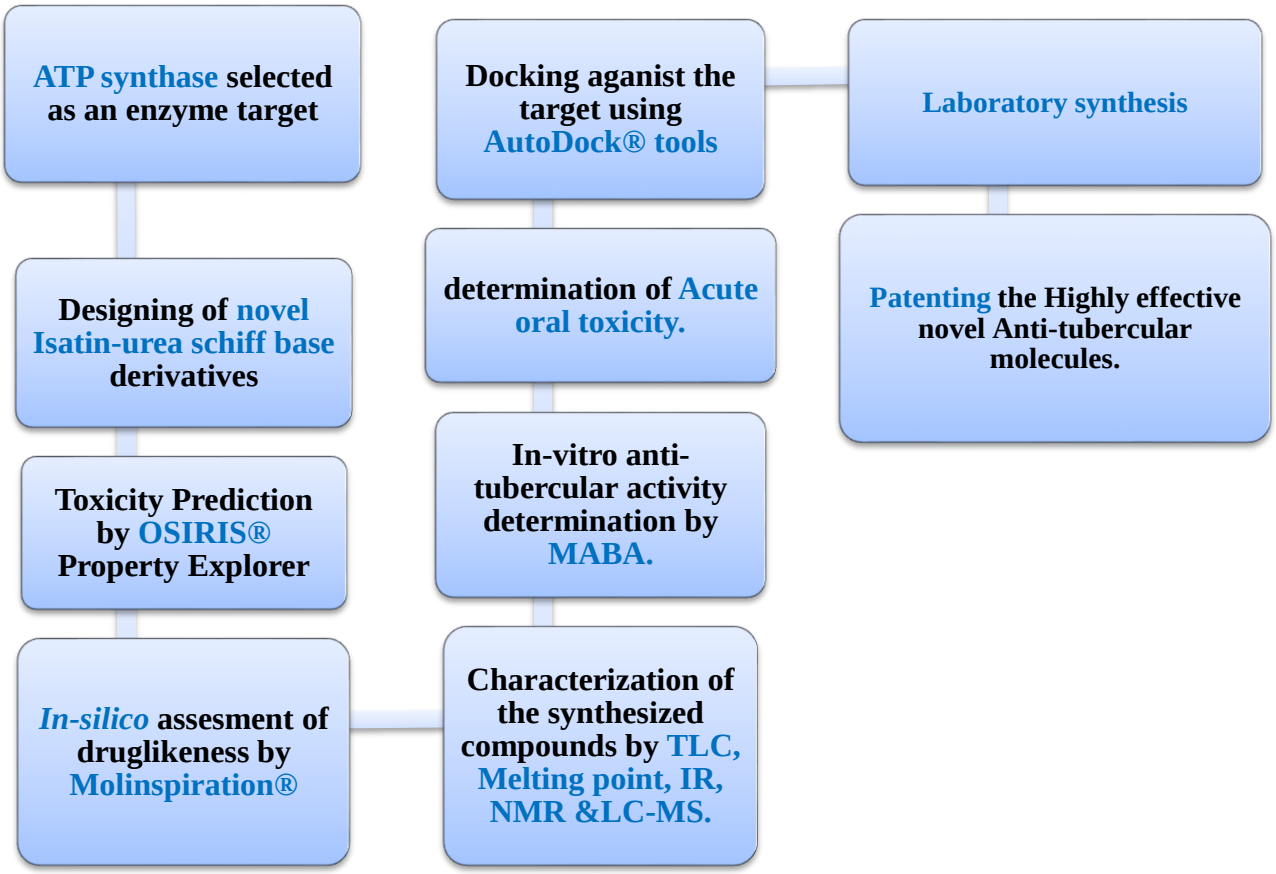
AIM:

The current study aims to develop novel anti-tubercular agents active against the ***Mycobacterium tuberculosis.***

OBJECTIVE:

1. Sketching of isatin novel molecules using chemsketch.
2. Application of,
    - ❖ PASS to determine overall pharmacological activity.
    - ❖ *In-silico* Drug likeness prediction.
    - ❖ *In-silico* Toxicity Assessment.
    - ❖ Design of *ATP synthase* inhibitors by docking studies using Autodock (1.5.6 version) software.
    - ❖ Laboratory synthesis of those compounds with top Docking Scores.
    - ❖ Characterization of the synthesized compounds by
        - ✓ Infrared Spectroscopy.
        - ✓ H1 NMR Spectroscopy.
        - ✓ Melting point.
        - ✓ LC-MS
3. Determination of *In-vitro* anti -tubercular activity of the synthesized compounds by Microplate Alamar Blue Assay (MABA).
4. Acute Toxicity on rats.

PLAN OF THE WORK

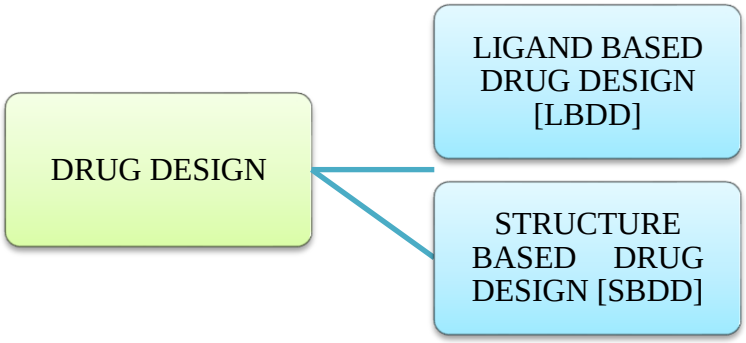


MATERIALS AND METHODS

Drug discovery is a process through which new drug molecules are designed and developed using a combination of Computational, Experimental, Translational and Clinical models. In spite of the advances in Biotechnology and understanding in the biology of systems & diseases, introducing a new drug to the market is still an expensive and time-consuming process due to the proportion of failures in the clinical trials. Computer Aided Drug Discovery (CADD) helps us to save time and money.

COMPUTER AIDED DRUG DESIGN (CADD): [34]

In **Computer Aided Drug Design (CADD)**, many computational approaches are utilized to discover, develop and analyze biologically active molecules. **Virtual screening** is one of the computational techniques used to identify the structures likely to bind the target protein or enzyme by searching libraries of small molecules.



A. LIGAND BASED DRUG DESIGN: [63]

Ligand based drug design is commonly employed when there is no information about the 3D structure of the target receptor or enzyme, is available. It depends on the knowledge of the molecules or ligand that binds to the target protein.

B. STRUCTURE BASED DRUG DESIGN: [63]

When the structural information about the biological drug target is available, this approach is exploited for the development of the molecule inhibiting it. Structure Based Virtual Screening involves the identification of the potential ligand binding site on the drug target. SBDD involves,

- **Docking** (Process of bringing & binding the two molecular structures together) of ligands into the target protein – the conformational space of the ligands which bind to the target molecules is explored.

- **Scoring** i.e., ranking the docked set in accordance to the binding affinity of the ligand to the protein.

AUTODOCK®TOOLS (ADT): [53]

An AUTODOCK tools is used to predict the binding of small molecular ligands to the 3D structure of the known target. **AutoDock® is molecular modeling simulation software developed by Scripps research.** It involves docking the ligand to a set of grids which describes the target protein. Several rotatable bonds in the ligands are altered to get various conformations and the binding affinity of the conformers with the target is predicted. It is an automated random search docking technique which predicts the interaction between the Enzyme-inhibitor complexes, Peptide-antibody complexes and also Protein-protein complexes. It was shown to be useful in Blind Docking where the binding site location is not known.

AutoDock 4.2 version is faster, more accurate, reliable and showed some additional advantages over the older ones.

- **Flexibility** of the side chains in the macromolecule is allowed.
- **New free energy scoring function – AMBER force field** is utilized, which is based on the linear regression analysis.
- Larger set of Protein-ligand complexes with known inhibition constants is available.

The current version of AutoDock® uses the **Lamarckian Genetic Algorithm and empirical free energy scoring function** to provide reproducible docking results for ligands. Docking of ligands with the target protein involves the following steps,

- Preparation of the target protein
- Preparation of the ligands
- Generation of 3D grids around the receptor &ligand
- Docking (screening the binding energy) the ligand

STEPS INVOLVED IN DOCKING:

A. PROTEINPREPARATION:

- ✓ File > Read molecule > Select Protein file in .pdbformat
- ✓ Edit > Charges > Compute Gasteiger (for arbitrary molecules)
- ✓ Edit > Hydrogens > Add > Polaronly

- ✓ Edit > Hydrogens > Merge Non-polar
- ✓ Edit > Misc > Repair Missing Atoms
- ✓ File > Save > Write PDB > Sort Nodes(Check)

B. LIGAND PREPARATION:

- ✓ Ligand > Input > Open > Select Ligand file in pdbformat
- ✓ Edit > Charges > Add Kollman charges
- ✓ Ligand > Torsion > Choose torsion [Rotatable bonds are shown in green, and non-rotatable bonds are shown in red. Bonds that are potentially rotatable but treated as rigid, such as amide bonds and bonds that are made rigid by the user are shown in magenta].
- ✓ Ligand > Torsion > Set number of torsions
- ✓ Ligand > Output > Save as .pdbqt

C. GRID GENERATION:

- ✓ Grid > Macromolecule > choose Protein > Select molecule > Save as.pdbqt
- ✓ Grid > Set map types > choose Ligand > Select ligand
- ✓ Grid > Grid Box > Set the Grid Box dimensions (i.e., XYZ co-ordinates 100 x 100 x 100) > File > Close Saving current
- ✓ Grid > Output > Save as grid.gpf (Grid Parameter file)

D. PREPARATION OF DOCKINGPARAMETERS:

- ✓ Docking > Macromolecule > Set Rigid Filename > Select protein.pdbqt > Open
- ✓ Docking > Ligand > Select Ligand >Accept
- ✓ Docking > Search Parameters > Genetic Algorithm
- ✓ Docking > Output > Lamarckian GA > Save as dock.dpf (Dock Parameter File)

After completing the above steps, cmd (command prompt) was opened and moved to the destination work folder by cd commands. Then the following commands were given,

- ❖ autogrid4.exe –p grid.gpf –l grid.glg (wait for the response)
- ❖ autodock4.exe –p dock.dpf –ldock.dlg.

E. VISUALIZATION OFDOCKING:

- Analyze > Docking > Select dock.dlg
- Analyze > Macromolecule > Select Protein
- Analyze > Conformations > Play ranked by energy
- Set Play options > Build H-Bonds > Show info > Build Current > Write complex > Save asresult.pdb.
- Analysis > Docking > Show interaction (Specialized visualization which highlights the interactions between the docked conformation of the ligand with the receptor).

The Binding energy and the inhibitor constant values were noted from the results. Then the result.pdb file was viewed in the Molegro Molecular viewer. Electrostatic and Steric interactions between the various amino acids in the target protein and ligand were seen.

***IN SILICO* ASSESMENT OF DRUGLIKENESS: [38]**

The undesirable ADMET (Absorption, Distribution, Metabolism, Excretion & Toxicity) profiles are the main cause behind the failure of turning a candidate molecule into a drug. To avoid the risk of attrition in the later stages of drug discovery, the concept of drug likeness was proposed by the Computational medicinal chemists in order to filter out the molecules with undesirable physicochemical properties.

The designed and docked molecules are screened *in silic*o using **MOLINSPIRATION®** Chem informatics software to evaluate drug likeness. This online tool is quick and easy to use. This software used for calculation of important molecular properties log P, polar surface area, number of hydrogen bond donors and acceptors and others, as well as prediction of bioavailability score for the most important drug targets (GPCR ligands, Kinase inhibitors, ion channel modulators, nuclear receptors).

Molecular properties of the designed ligands were predicted by drawing the structures in the online **Molinspiration®tool: https://www.molinspiration.com/cgi-bin/properties**[54]

***IN-SILICO* TOXICITY PREDICTION–OSIRIS® PROPERTY EXPLORER: [55]**

In silico toxicity prediction is done using **OSIRIS®** Property Explorer. It is free software available for access in the Organic Chemistry Portal. Using this online prediction tool, mutagenicity, tumerigenicity, skin irritation and reproductive effects can be determined. The tool detects the toxicity risk alerts and indicated by red colour. The green colour confirms the drug like behavior of the compounds without any toxicity alerts.

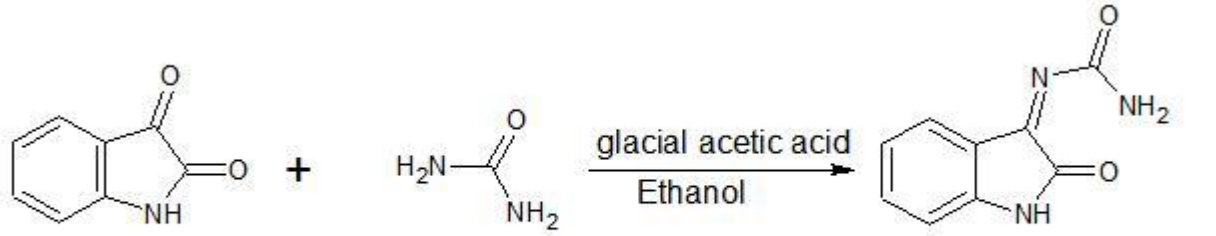
The *in-silico* toxicity of the designed compounds was predicted by drawing the structures in online tool –

http://www.cheminfo.org/Chemistry/Cheminformatics/Property_explorer/index.html#

SYNTHETIC METHODOLOGY:

STEP: 1

The ethanolic solution of (25ml) isatin (0.001M) was mixed with ethanolic solution of (25ml) urea (0.001) and 3-4 drops of glacial acetic acid were added. Then the mixture was stirred for two hours by using magnetic stirrer at 60°C. The solution was filtered and washed with ethanol and recrystallized from ethanol.

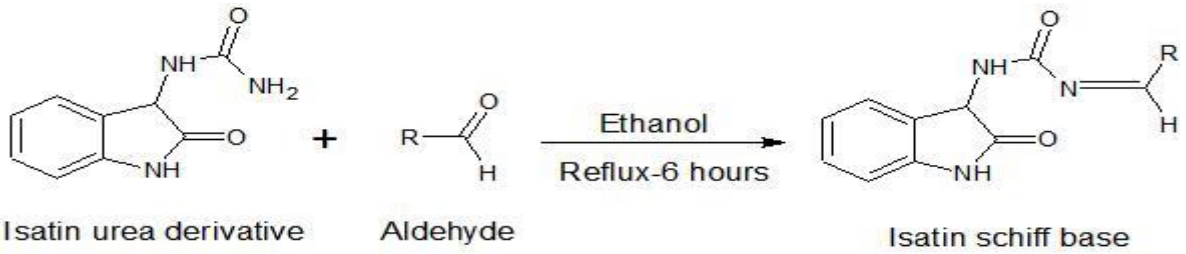


Isatin Urea Isatin urea derivatives

STEP: 2

Equimolar (0.002M) quantity of intermediate step-1 was treated with various substituted aromatic aldehydes in 20ml of ethanol in the presence of glacial acetic acid. The contents were refluxed at 80°C for 6 hours. The progress of reaction was monitored by TLC. After completion of the reaction the contents of the flask were poured over the crushed ice. The solid obtained was filtered, washed with cold water, dried and finally recrystallized from ethanol.

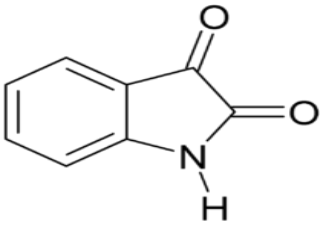
STEP-2 SYNTHETIC SCHEME



Isatin urea derivative Aldehyde Isatin schiff base

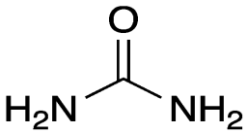
REACTANT PROFILE

- **ISATIN**



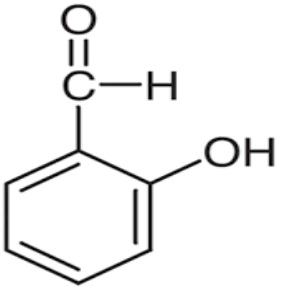
Molecular Formula	:	$C_8H_5NO_2$
Molecular Weight	:	147.13g/mol
Appearance	:	Orange red powder
Melting point	:	200° C
Density	:	1.4 g/cm^3

- **UREA**



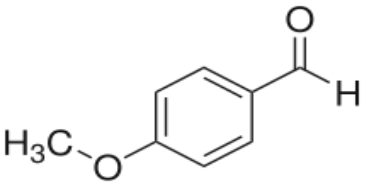
Molecular Formula	:	CH_4N_2O
Molecular Weight	:	60 g/mol
Appearance	:	White crystalline solid
Melting point	:	133° C
Density	:	1.335 g/cm^3

❖ **SALICYALDEHYDE**



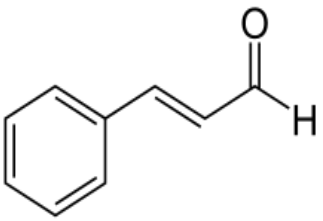
Molecular Formula	:	$C_7H_6O_2$
Molecular Weight	:	122 g/mol
Appearance	:	pale yellow liquid
Boiling point	:	194^o C
Density	:	1.17 g/cm^3

❖ **ANISALDEHYDE**



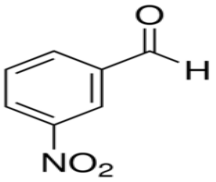
Molecular Formula	:	$C_8H_8O_2$
Molecular Weight	:	136 g/mol
Appearance	:	slightly yellow liquid
Boiling point	:	248^o C
Density	:	1.12 g/cm^3

❖ **CINNAMALDEHYDE**



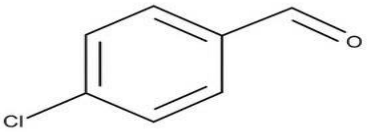
Molecular Formula	:	C_9H_8O
Molecular Weight	:	111.12 g/mol
Appearance	:	yellow oily liquid
Boiling point	:	248^o C
Density	:	1.05 g/cm^3

❖ **3-NITRO BENZALDEHYDE**



Molecular Formula	:	$C_7H_5NO_3$
Molecular Weight	:	151 g/mol
Appearance	:	yellowish to brownish crystalline powder
Melting point	:	58.5^o C
Density	:	1.338 g/cm^3

❖ **4-CHLORO BENZALDEHYDE**



Molecular Formula	:	C_7H_5OCl
Molecular Weight	:	140 g/mol
Appearance	:	white crystalline solid
Melting point	:	48^o C
Density	:	1.196 g/cm^3

CHARACTERIZATION STUDIES:

- **Determination of Melting point:** Purity of the synthesized compounds were evaluated by determining Melting point using the **Digital Melting point apparatus.**
- **Thin Layer Chromatography (TLC):** Pre-coated silica plates were used for TLC. Various proportion of different solvents were tried as Mobile phase and by trial and error method **Methanol:Chloroform in the ratio of 9:1** was chosen as the Mobile phase. TLC was performed to confirm the completion of the synthetic reaction by comparing the R_f value of the reactants and products.

- **Physical Evaluation:** The following physical properties of the synthesized compounds were evaluated,
  - ✓ Color
  - ✓ Nature
  - ✓ Solubility
  - ✓ Melting point
- **Spectroscopic Evaluation:** The synthesized compounds were further characterized by the following Spectroscopic methods,
  - ✓ Infrared Spectroscopy (IR)
  - ✓ Nuclear Magnetic Spectroscopy (^{1}H – NMR)
  - ✓ Liquid Chromatography – Mass Spectrometry(LC-MS)

INFRARED SPECTROSCOPY: [56]

FTIR is a technique which is used to obtain infrared spectrum of absorption, emission and photoconductivity of solid, liquid and gas samples. Fourier transform infrared spectroscopy (FTIR) uses the mathematical process (Fourier transform) to translate the raw data (interferogram) into the actual spectrum. FTIR identifies the presence of organic and inorganic compounds in the sample, depending on the infrared absorption frequency range 600-4000cm$^{-1.}$ It relies on the fact that chemical molecules absorb certain frequencies that are characteristic to their structure. For a vibration to absorb the infrared energy there must be a change in the dipole moment and that influences the intensity of the infrared absorptions. The infrared portion of the electromagnetic spectrum is divided into,

i. Near IR – 14000-4000 cm^{-1} (1.4-0.8μm)
ii. Mid IR -4000-400 cm^{-1} (30-1.4μm)
iii. Far IR – 400-10 cm^{-1} (1000-30μm)

The number of observed absorptions may be increased by combination tones and overtones of the functional vibrations and decreased by molecular symmetry & spectrometer limitations. Unique patterns are found in the region of **1450-600cm^{-1}** and it is referred as **Fingerprint region**. Absorption bands in the **4000-1400cm^{-1}** region are mainly due to the stretching vibrations of the diatomic units and it is referred as **Group frequency or Functional group region.**

Table 05: Group Frequencies in Infrared Region

ABSORPTION (cm^{-1})	APPEARANCE OF PEAK	FUNCTIONAL GROUP	COMPOUND CLASS
3500	Medium	N-H Stretching	Primary amine
3350-3310	Medium	N-H Stretching	Secondary amine
2000-1650	Weak	C-H Stretching	Aromatic compounds
1685-1666	Strong	C=O Stretching	Conjugated ketones
1648-1638	Weak	C=C Stretching	Alkene
1650-1580	Medium	N-H Bending	Amine
1465	Medium	C-H Bending	Alkane
900-700	Strong	C-H Stretching	Benzene derivatives
850-550	Strong	C-Cl Stretching	Chloro compounds

The IR Spectra of the synthesized compounds were obtained from the ABB MB3000 FT-IR Spectrometer.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR):[58]

One of the important techniques used to obtain **the physical, chemical, electronic and structural information about the molecule** is NMR Spectroscopy. This research technique exploits the magnetic properties of certain atomic nuclei including **^{1}H (Proton), ^{13}C (Carbon), ^{15}N (Nitrogen) and ^{19}F (Fluorine)**. The qualitative information about a molecule can be obtained based on the chemical shift produced in the resonance frequencies of nuclear spins in the sample. NMR Spectroscopy is based on the principles such as,

- All atomic nuclei have their own spin and are electrically charged
- In the presence of an external magnetic field, transfer of energy from the base level to the higher energy level occurs at a wavelength that coincides with the radio frequency.
- When the spin returns to its base ground state emission of energy at the same frequency is possible.

- NMR Spectrum for the sample molecule is processed by measuring the signal which matches the transfer frequency.

The amount of energy and the frequency of Electromagnetic radiation required for resonance to occur depends on the strength of the applied external magnetic field and the type of nucleus involved in the study.

CHEMICAL SHIFT:

The NMR Spectrum is displayed as a plot of the applied radio frequency versus the absorption. The position in the plot at which the nuclei absorbs the electromagnetic radiation is called the Chemical shift.

- Left side of the plot – **Downfield or De shielded side**
- Right side of the plot – **Upfield or Shielded side**

TMS (Tetramethylsilane) is commonly used as a standard reference and assigned a chemical shift of zero. The δ-scale is expressed as parts per million (ppm).

In the Proton NMR spectrum, the peaks are splitten into groups due to the coupling between the adjacent protons in the sample molecule. The interaction between the pair of protons is measured by Coupling constant (J).

Thus, in a NMR spectrum

- The location of the peak (chemical shift) provides information about the local chemical environment adjacent to the proton.
- The integration ratio or intensity of the peak depends on the number of equivalent type of protons in the molecule.
- The coupling pattern or multiplicity is based on the number of protons on the adjacent carbon atom.

This information gives us the idea about the basic skeletal information of the molecule.

Table 06: Chemical Shift values in NMR

Types of protons	δ (ppm)
$R\text{-}CH_3$	**0.9**
$R\text{-}CH_2\text{-}R$	**1.3**
$Ar\text{-}CH_3$	**2-3**
$R_2N\text{-}CH_3$	**2.3**
$RO\text{-}CH_3$	**3.8**
$R\text{-}CH_2\text{-}F$	**4.5**
Ar-H	**7.3**

HYPHENATED TECHNIQUES: [33]

Coupling of different analytical techniques i.e., commonly a chromatographic technique is combined with a spectroscopic technique and are known as hyphenated techniques. Components in the sample mixture are separated by the chromatographic technique and then each component enters into the spectroscopic analysis via a suitable inter phase. The term hyphenated technique ranges for the combination of techniques including,

1) Separation – separation
2) Separation – identification
3) Identification – identification.

DOUBLE HYPHENATED TECHNIQUES	TRIPLE HYPHENATED TECHNIQUES
LC-MS	LC-API-MS
LC-NMR	ESI-MS-MS
LC-IR	LC-NMR-MS
GC-MS	APCI-MS-MS
GC-FTIR	LC-PDA-MS

LC-MS [Liquid Chromatography – Mass Spectrometry]

LC-MS is an analytical technique which combines the physical separation of liquid chromatography (HPLC) with the Mass Spectrometry by an interface. To obtain only few fragment ions with the molecular ion, soft ionization techniques are utilized in the LC-MS. For confirming the identity of the compound single LC-MS run is not sufficient. Scanning speed of the MS is influenced by the Chromatographic resolution, therefore to achieve accurate integration, it is suggested to have **10 scans** across the chromatographic peak.

The hyphenated technique LC-MS includes,

- **LC – Liquid chromatography** separates the sample mixture into individual components
- **Interface** – transfers the separated component from LC Column to MS ion source
- **MS – Mass spectrometry** identifies the structure of the individual components

Liquid chromatography involves the separation of components in the liquid mixture by distributing them between the two immiscible phases (Stationary & Mobile phase). Typically in HPLC, 20µl of sample is injected into the mobile phase by a high pressure pump. The sample mixed with the mobile phase permeates through the stationary bed and gets separated based on their differential affinities towards the stationary & mobile phases and gets eluted from the column at different times (Rt**). Reverse Phase Partition**

Chromatography (Non-polar stationary phase and polar mobile phase) is widely used.

The interface is designed to offer adequate nebulization and vaporization of the solvent, ionization of the sample, removal of the excess solvent vapour and the extraction of the ions. The transition of components from the High-pressure environment of HPLC to High vacuum condition in the Mass analyzer is facilitated by the interface. The commonly used interfaces are,

- Electro spray Ionization Interface (ESI),
- Atmospheric Pressure Chemical ionization Interface (APCI).

MS is an analytical technique that measures the Mass to charge ratio (m/z) of the charged ions. The ions produced from the analyte are manipulated by the electric and magnetic fields to determine their m/z ratio. The basic components include,

- **Ion source**: Components in the sample mixture is ionized by electron/photon beams which fragments the sample molecules into gas phase ions and sent to analyzers.
- **Mass analyzer:** Ions are sorted according to their masses.
- **Detector:** Abundance of each ion is measured by the detection through amplification.

The synthesized compounds were characterized by LC-MS instrument – **Agilent technologies 6230B TOF (Time of Flight).**

BIOLOGICAL EVALUATION [60]

The designed and synthesized molecules need to be screened for their activity to inhibit the growth of the ***Mycobacterium tuberculosis***.

MICROPLATE ALAMAR BLUE ASSAY: [35]

The Micro Plate Alamar Blue Assay (MABA) method was used to evaluate anti-tubercular activity of the synthesized compounds ***Mycobacterium tuberculosis* H37Rv** strain. Alamar blue dye is used as an indicator for the determination of viable cells. The oxidized form, Resazurin is non- toxic, non-fluorescent and blue in colour which becomes pink and fluorescent upon reduction of resazurin by viable cells.

PROCEDURE:

1. The anti-mycobacterial activity of compounds were assessed against *M. tuberculosis* using Micro Plate Alamar Blue assay (MABA).
2. This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with BACTEC radiometric method.
3. 200µl of sterile de-ionized water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation.
4. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilutions of compounds were made directly on plate.
5. The final drug concentrations tested were 100 to 0.8µg/ml.
6. Plates were covered and sealed with Para film and incubated at 37ºC for five days.
7. After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs.
8. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth.
9. The MIC was defined as lowest drug concentration, which prevented the color change from blue to pink.

ACUTE ORAL TOXICITY STUDY: [36] [37]

Acute oral toxicity study was designed as per the OECD guidelines (423). **OECD [Organization for Economic Co-operation and Development]** guidelines for the testing of chemicals are an internationally accepted set of specifications for testing the chemicals. It includes five sections,

Table 07: Guidelines under the sections of OECD

S. No	SECTIONS	GUIDELINES
1.	Physical & Chemical Properties	101 – 123
2.	Effects on Biotic Systems	201 – 241
3.	Environmental Fate and Behavior	301 – 317
4.	Health Effects	401 – 493
5.	Other test guidelines	501 – 509

PRINCIPLES AND PURPOSE:

The main purpose of acute toxicity is to evaluate the degree of toxicity in a quantitative and qualitative manner.

SELECTION OF DOSE LEVELS AND ADMINISTRATION OF DOSE:

Being synthetic molecules, the mortality was unlikely at the highest starting dose level (2000mg/kg/b.w). Hence a limit test one dose levels of 2000mg/kg/b.w was conducted in all animals as per the OECD guidelines (423).

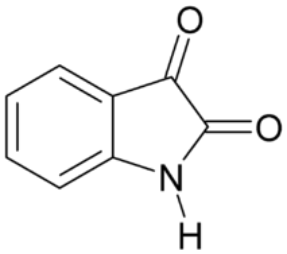
PROCEDURE:

- ✓ The study was performed as per the OECD guidelines 423 (acute toxic class method.
- ✓ Animals were fasted prior to dosing (3-4 hrs.)
- ✓ Animals were weighed and test substance will be administered orally.
- ✓ After dosing animals were observed for 30 mins and special attention was given during first 4 hrs.
- ✓ Finally, animals were obsereved for behavioural signs of toxicity for 14 days.

RESULTS AND DISCUSSION

1. RESULTS OF DRUG DESIGN:

- Five hundred molecules were designed using **ACD/Chem Sketch® (Freeware)** software by performing structural modifications in the basic hetero nuclear scaffold Isatin.
- Energy minimization of the designed molecules was performed in **Chem3D Ultra® software.**
- The designed molecules were coded for identification.

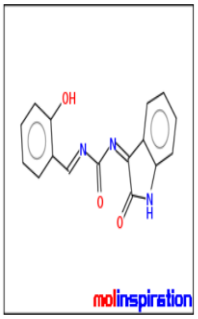
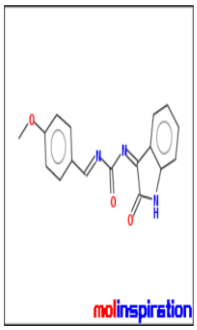
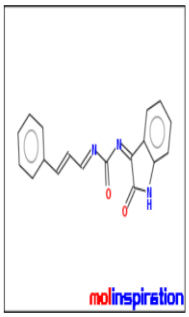


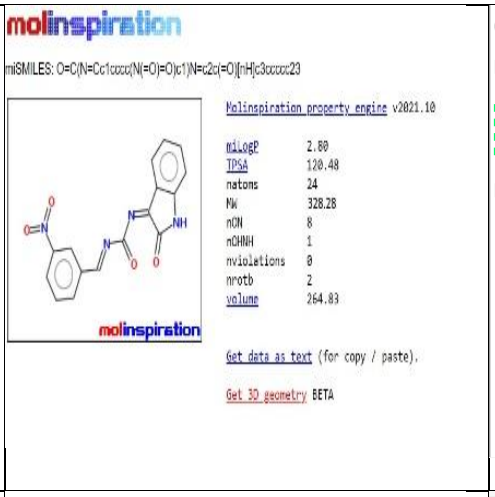
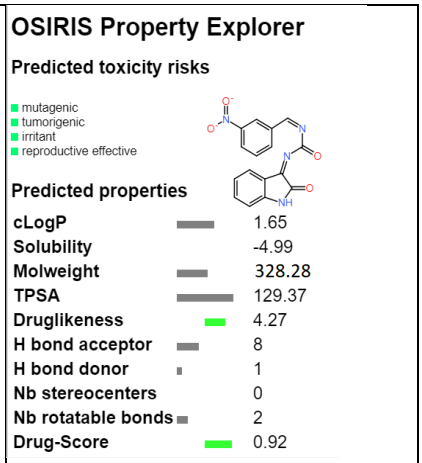
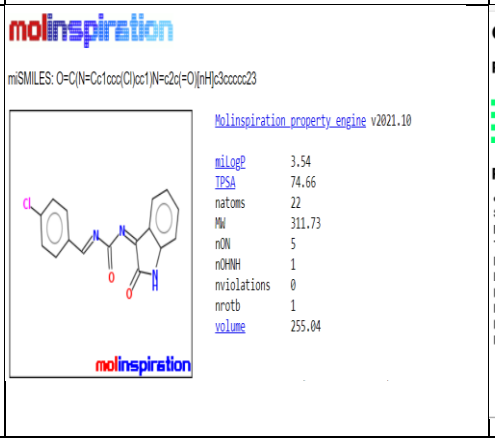
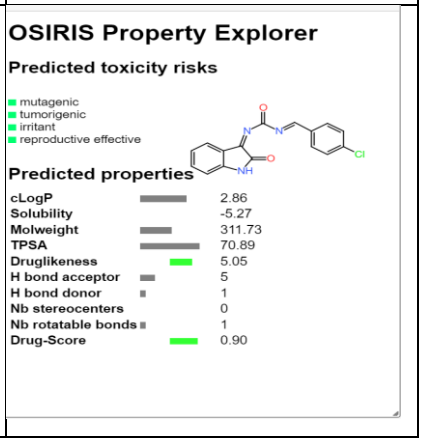
2. RESULTS OF *IN SILICO* DRUG LIKENESS AND TOXICITY PREDICTION:

Lipinski's rule of five explains the ability of the chemical compound with certain physicochemical property to make it an orally active drug. This rule describes the important pharmacokinetics properties like absorption, distribution, metabolism and excretion (ADME).

- For a molecule to be orally active it must have drug likeness properties according to the Lipinski's rule of five and it must be non-toxic.
- The molecules were evaluated for their drug likeness behavior using the online tool – **Molinspiration® Cheminformatics**.
- The toxicity of the molecules was assessed using **OSIRIS® Property Explorer.**

Table 08: *In silico* Drug-likeness and Toxicity prediction

COMPOUND CODE	*IN SILICO* DRUG LIKENESS PREDICTION	*IN SILICO* TOXICITY ASSESSMENT
JP1	molinspiration miSMILES: O=C(N=Cc1ccccc1O)N=c2c(=O)[nH]c3ccccc23  Molinspiration property engine v2021.10 miLogP 2.81 TPSA 94.89 natoms 22 MW 293.28 nON 6 nOHNH 2 nviolations 0 nrotb 1 volume 249.52	OSIRIS Property Explorer Predicted toxicity risks mutagenic tumorigenic irritant reproductive effective Predicted properties cLogP 1.91 Solubility -4.24 Molweight 293.28 TPSA 91.12 Druglikeness 4.24 H bond acceptor 6 H bond donor 2 Nb stereocenters 0 Nb rotatable bonds 1 Drug-Score 0.93
JP2	molinspiration miSMILES: COc3ccc(C=NC(=O)N=c1c(=O)[nH]c2ccccc12)cc3  Molinspiration property engine v2021.10 miLogP 2.92 TPSA 83.89 natoms 23 MW 307.31 nON 6 nOHNH 1 nviolations 0 nrotb 2 volume 267.05	OSIRIS Property Explorer Predicted toxicity risks mutagenic tumorigenic irritant reproductive effective Predicted properties cLogP 2.18 Solubility -4.55 Molweight 307.31 TPSA 80.12 Druglikeness 4.40 H bond acceptor 6 H bond donor 1 Nb stereocenters 0 Nb rotatable bonds 2 Drug-Score 0.92
JP3	molinspiration miSMILES: O=C(N=CC=Cc1ccccc1)N=c2c(=O)[nH]c3ccccc23  Molinspiration property engine v2021.10 miLogP 3.62 TPSA 74.66 natoms 23 MW 303.32 nON 5 nOHNH 1 nviolations 0 nrotb 2 volume 268.92	OSIRIS Property Explorer Predicted toxicity risks mutagenic tumorigenic irritant reproductive effective Predicted properties cLogP 2.27 Solubility -4.82 Molweight 303.32 TPSA 70.89 Druglikeness 3.11 H bond acceptor 5 H bond donor 1 Nb stereocenters 0 Nb rotatable bonds 2 Drug-Score 0.91

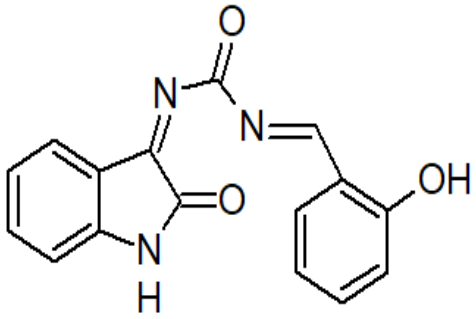
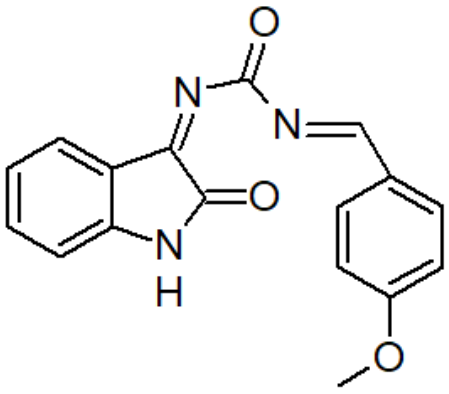
JP4		
JP5		

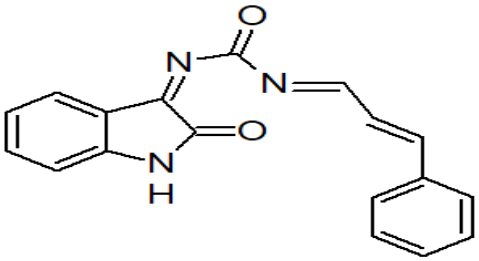
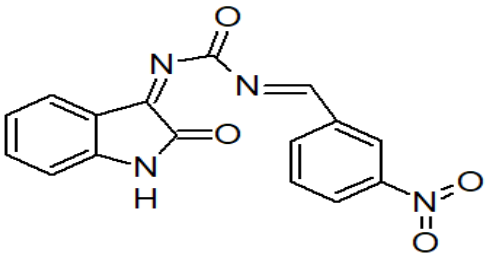
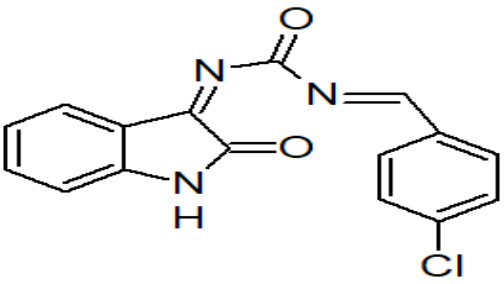
- The above molecules showed **no violations of Lipinski's rule** and **no toxicity risks** (no red colour alert) were shown in OSIRIS® Property Explorer.
- The non-toxic, drug like molecules were subjected to Molecular Docking studies.

3. RESULTS OF MOLECULAR DOCKING:

- The non-toxic molecules were docked against the target enzyme ***ATP SYNTHASE*** **(PDB ID – 7JG5)** of ***Mycobacterium tuberculosis*** to determine their effectiveness as an anti-tubercular agent.
- Five top scored molecules were further synthesized, characterized and evaluated.

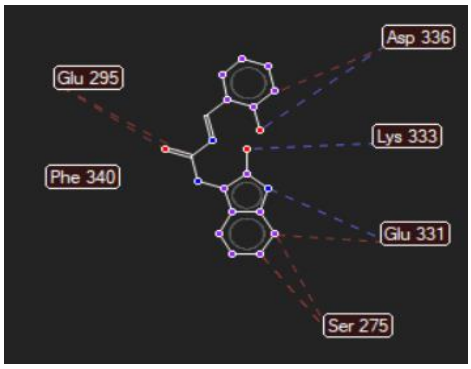
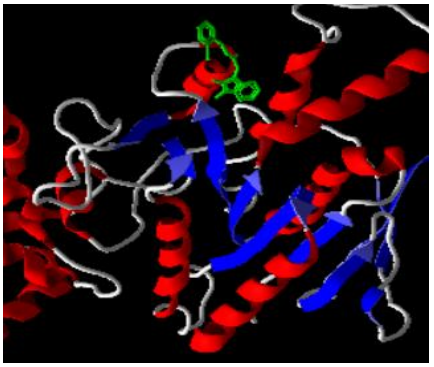
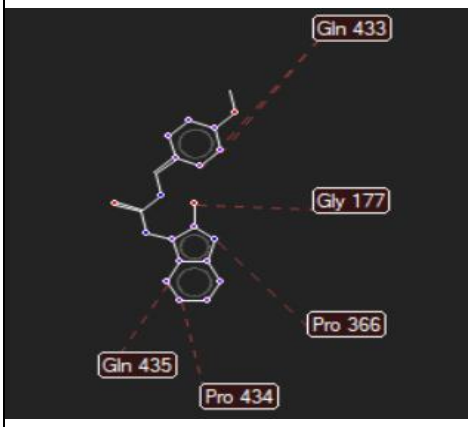
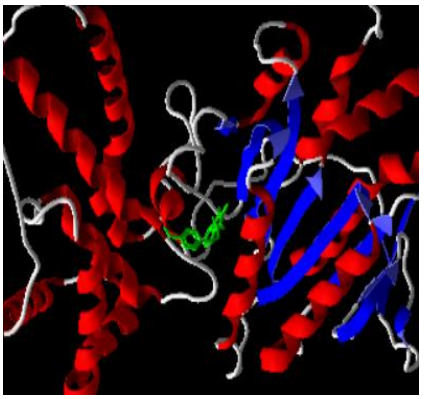
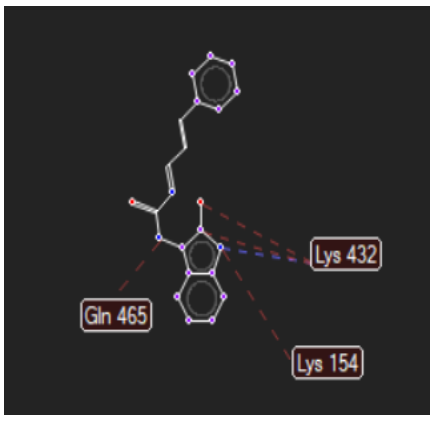
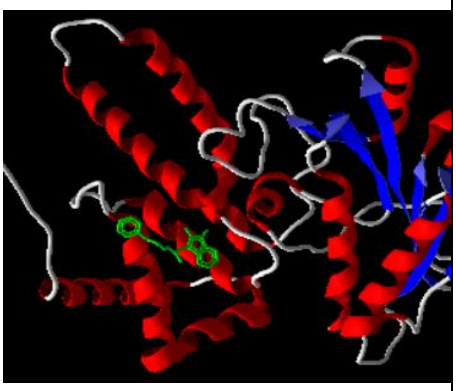
Table 09: Code, Molecular Structure, Binding energy and Inhibition constant value of the top scored compounds.

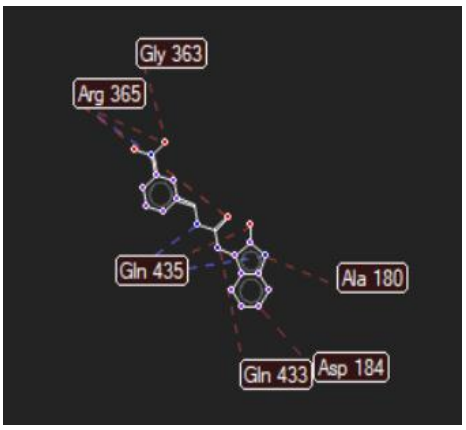
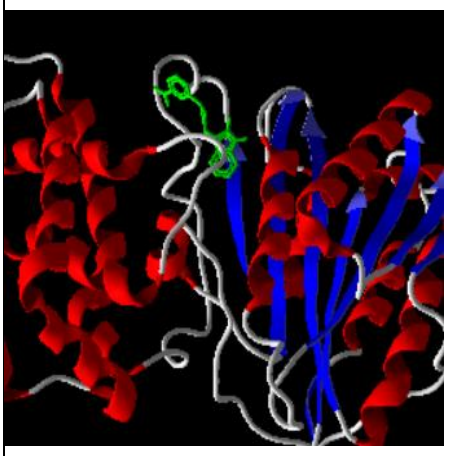
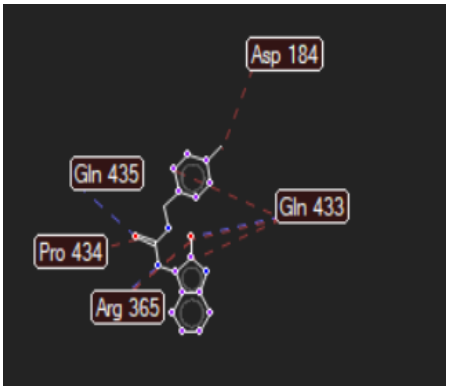
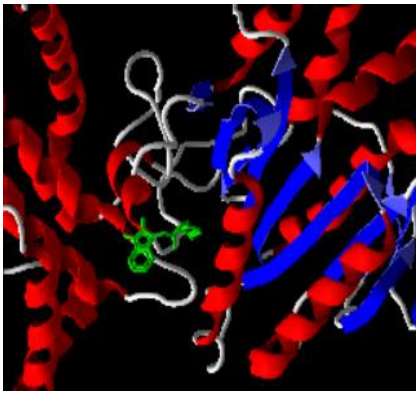
COMPOUND CODE	MOLECULAR STRUCTURE	BINDING ENERGY (kcal/mol)	INHIBITION CONSTANT (nM)
JP1		-7.53	4.39
JP2		-8.86	321.13

JP3		-7.59	2.72
JP4		-9.4	128.18
JP5		-9.29	155.53

- The docking score of the molecules were found to be in the range of **-7 to -9.4 kcal/mol.**
- The inhibition constant value of the molecules was in the range of **2 to 321 nm.**

Table 10: Interactions of molecules with the amino acids and docking view

COMPOUND CODE	INTERACTION WITH AMINOACIDS	DOCKING VIEW
JP1		
JP2		
JP3		

JP4		
JP5		

- The Electrostatic, Hydrogen bond and Steric interactions between the ligands and the amino acids in the target enzyme were observed using the **Molegro Molecular® viewer.**

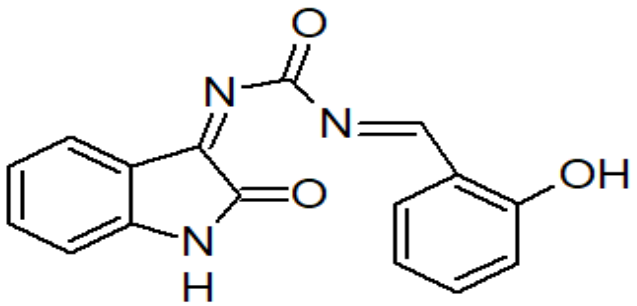
4. PRODUCT PROFILE:

- Molecules which were found to be non-toxic in the *in-silico* toxicity assessment tool were synthesized by suitable laboratory process and recrystallized using ethanol.
- Completion of the synthetic reaction was determined by comparing the R_f values of the reactants and products**. R_f value of the products was found to be different from the R_f value of the reactants.**
- Melting point of the products was determined by Digital Melting Point Apparatus. **Sharp melting point denotes the purity** of the synthesized compound.

Table 11: Data of the synthesized compounds JP1 – JP5

CODE	COLOUR	SOLUBILITY	MELTING POINT	R_f VALUE	PERCENTAGE YIELD
JP1	Reddish brown	Methanol, Ethanol, DMSO	161^o C	0.70	84%
JP2	Reddish brown	Methanol, Ethanol, DMSO	172 ^{o} C	0.64	82%
JP3	Reddish brown	Methanol, Ethanol, DMSO	163 ^{o} C	0.78	78%
JP4	Reddish orange	Methanol, Ethanol, DMSO	114^o C	0.69	88%
JP5	Reddish brown	Methanol, Ethanol, DMSO	170 ^{o} C	0.57	85%

COMPOUND JP1



IUPAC NAME: 1-[(E)-(2-hydroxy phenyl) methylidene]-3-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene] urea

Molecular Formula	:	$C_{16}H_{11}N_3O_3$
Formula Weight	:	293.27684
Composition	:	C (65.53%) H (3.78%) N (14.33%) O (16.37%)
Molar Refractivity	:	80.35 ± 0.5 cm^3
Molar Volume	:	210.6 ± 7.0 cm^3
Parachor	:	584.0 ± 8.0 cm^3
Index of Refraction	:	1.688 ± 0.05
Surface Tension	:	59.1 ± 7.0 dyne/cm
Density	:	1.39 ± 0.1 g/cm^3
Dielectric Constant	:	Not available
Polarizability	:	31.85 ± 0.5 $10^{-24}cm^3$

INFRARED SPECTRUM OF JP1:

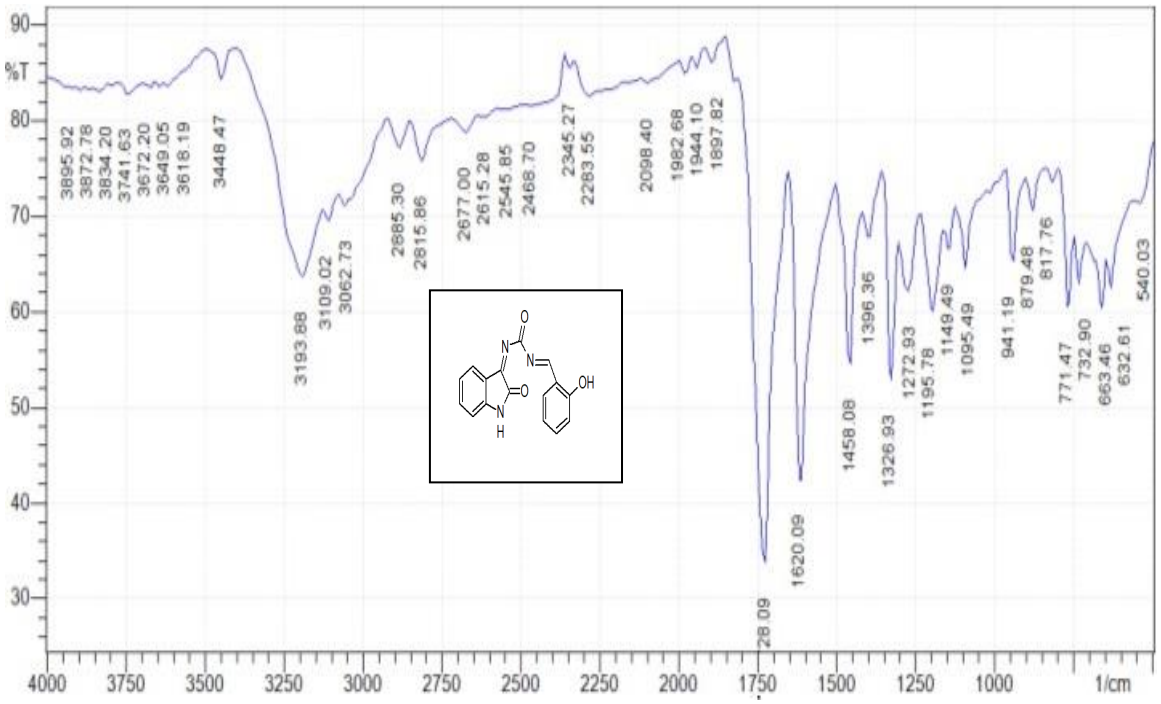


Figure 06: IR Spectrum of Compound JP1

Table 12: IR interpretation of compound JP1

S.NO	WAVE NUMBER (cm^{-1})	FUNCTIONAL GROUP
01.	1620.09	C=N Stretching
02.	3448.47	OH Stretching
03.	1728.09	C=O Stretching
04.	3193.88	N-H Stretching

PROTON NMR SPECTRUM OF JP1:

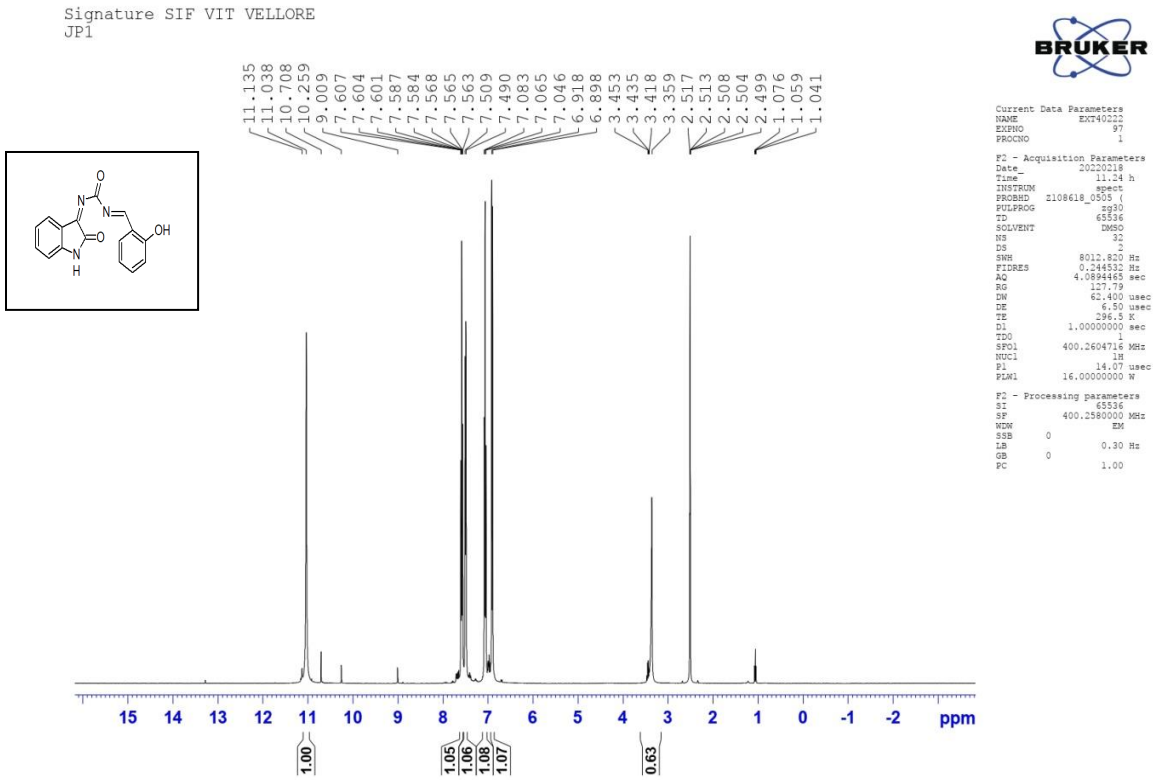


Figure 07: ^{1}H NMR Spectrum of Compound JP1

Table 13: Interpretation of ^{1}H NMR Spectrum of Compound JP1

S.NO	δ VALUE (PPM)	NATURE OF PROTONS	NATURE OF THE PEAK	NUMBER OF PROTONS
01.	11.135	CH=N	Singlet	1
02.	3.45	Ar-OH	Singlet	1
03.	7.2-7.4	Ar-H	Multiplet	4

LC-MS SPECTRUM OF JP1:

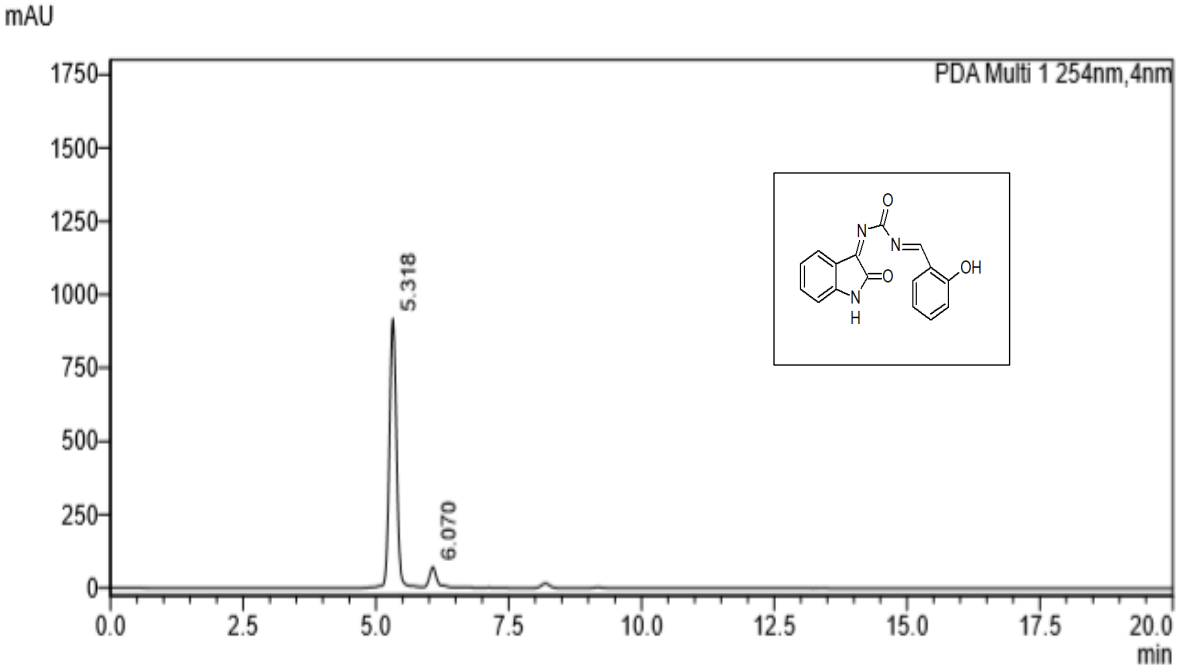


Figure No 08: Chromatogram of compound JP1

Molecular weight of JP1:293.28 g/mol

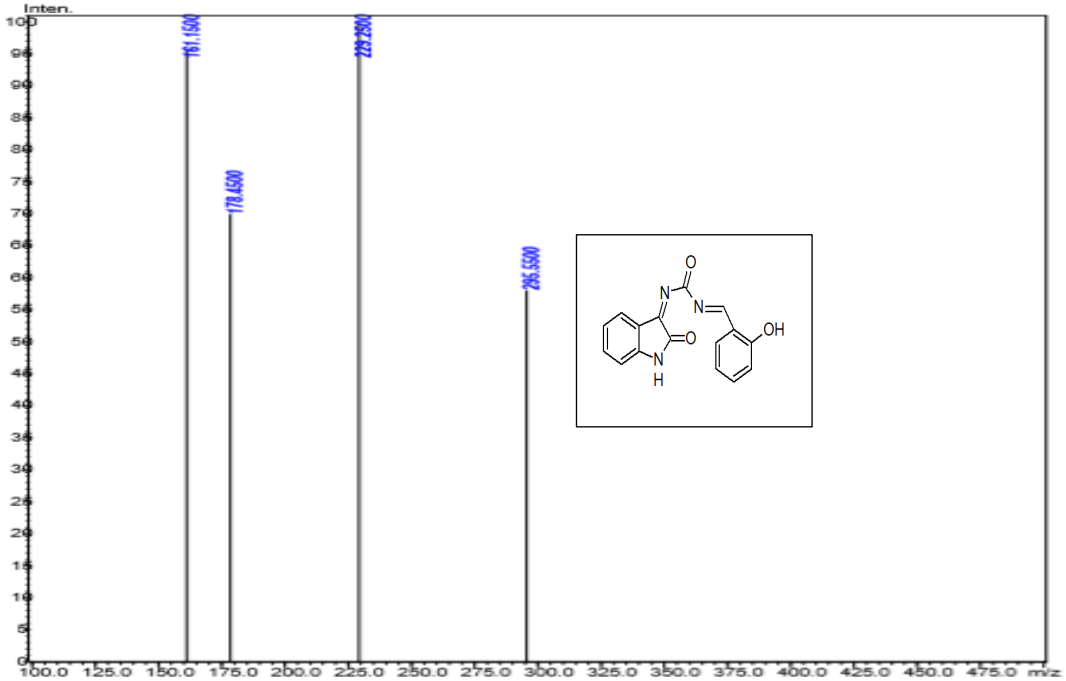
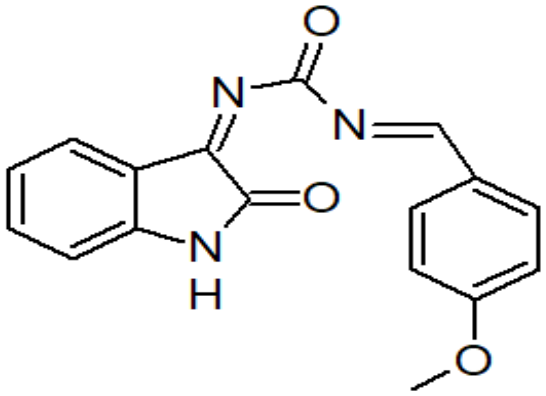


Figure No 09: Mass Spectrum of Compound JP1

COMPOUND JP2



IUPAC NAME: 1-[(E)-(4-methoxy phenyl) methylidene]-3-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene] urea

Molecular Formula	$C_{17}H_{13}N_3O_3$
Formula Weight	307.30342
Composition	C (66.44%) H (4.26%) N (13.67%)
O (15.62%)	
Molar Refractivity	85.31 ± 0.5 cm^3
Molar Volume	235.0 ± 7.0 cm^3
Parachor	628.6 ± 8.0 cm^3
Index of Refraction	1.646 ± 0.05
Surface Tension	51.1 ± 7.0 dyne/cm
Density	1.30 ± 0.1 g/cm^3
Dielectric Constant	Not available
Polarizability	33.82 ± 0.5 $10^{-24}cm^3$

INFRARED SPECTRUM OF JP2:

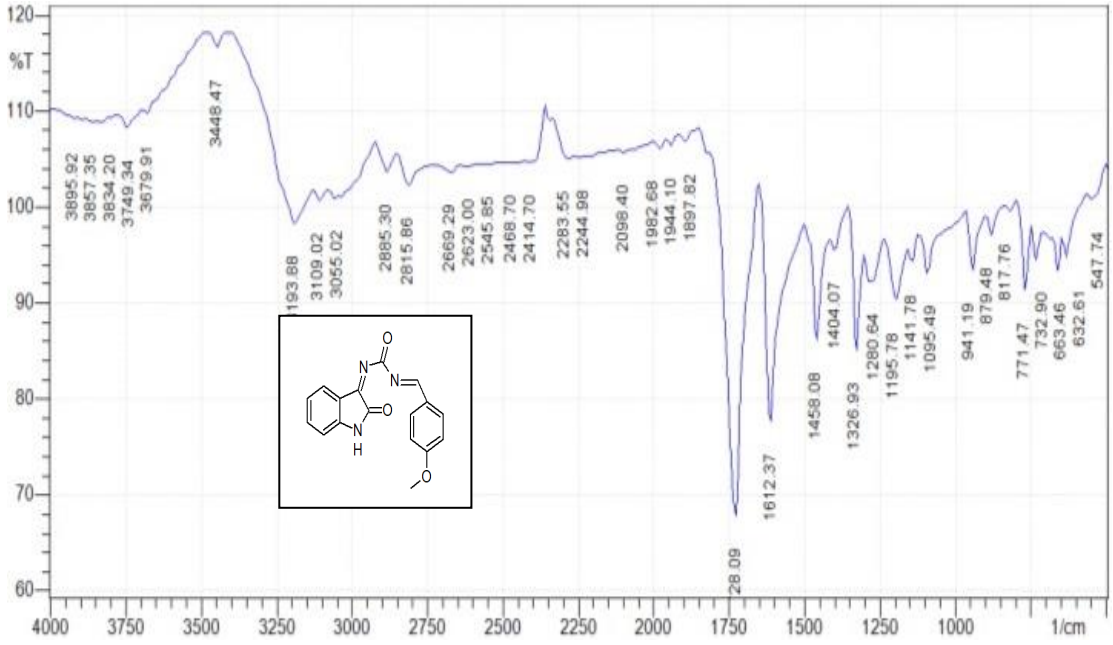


Figure 10: IR spectrum of Compound JP2

Table 14: IR Interpretation of Compound JP2

S.NO	WAVE NUMBER (cm^{-1})	FUNCTIONAL GROUP
01.	1612.37	C=N Stretching
02.	1326.93	C-O-C Stretching
03.	1728.09	C=O Stretching
04.	3193.88	N-H Stretching

PROTON NMR SPECTRUM OF JP2:

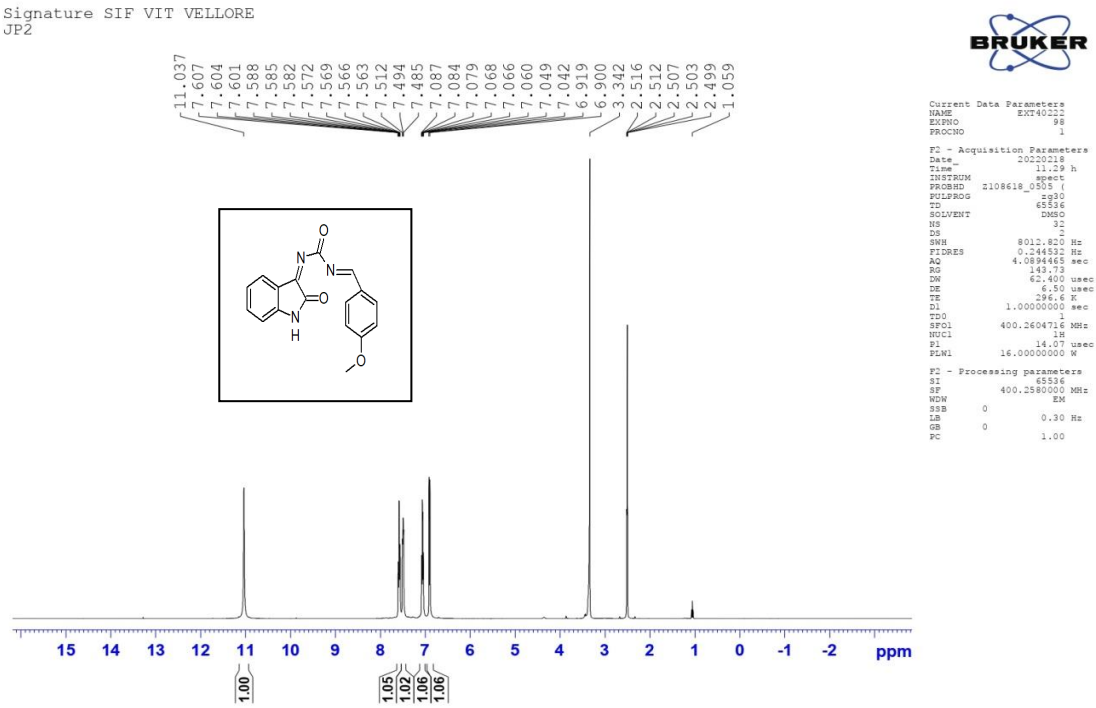


Figure 11: ^{1}H NMR Spectrum of Compound JP2

Table 15: Interpretation of ^{1}H NMR Spectrum of Compound JP2

S.NO	δ VALUE (PPM)	NATURE OF PROTONS	NATURE OF THE PEAK	NUMBER OF PROTONS
01.	11.03	CH=N	Singlet	1
02.	7.2-7.6	Ar-H	Multiplet	4
03.	3.3	-OCH3	Singlet	1

LC-MS SPECTRUM OF JP2:

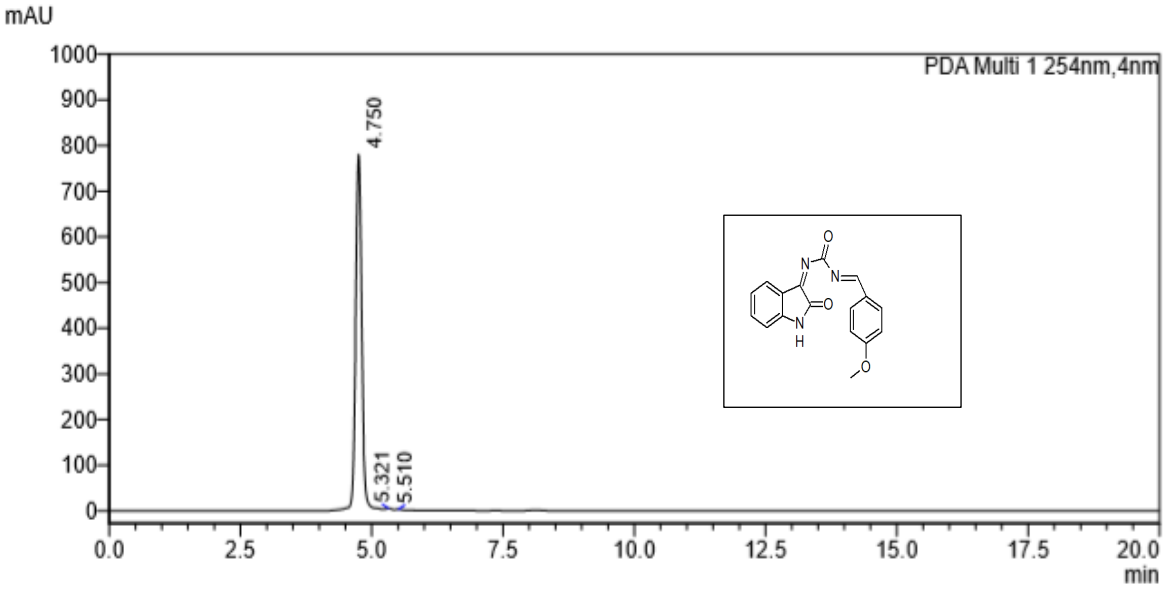


Figure No12: Chromatogram of compound JP2

Molecular Weight of JP2: 307.31g/mol

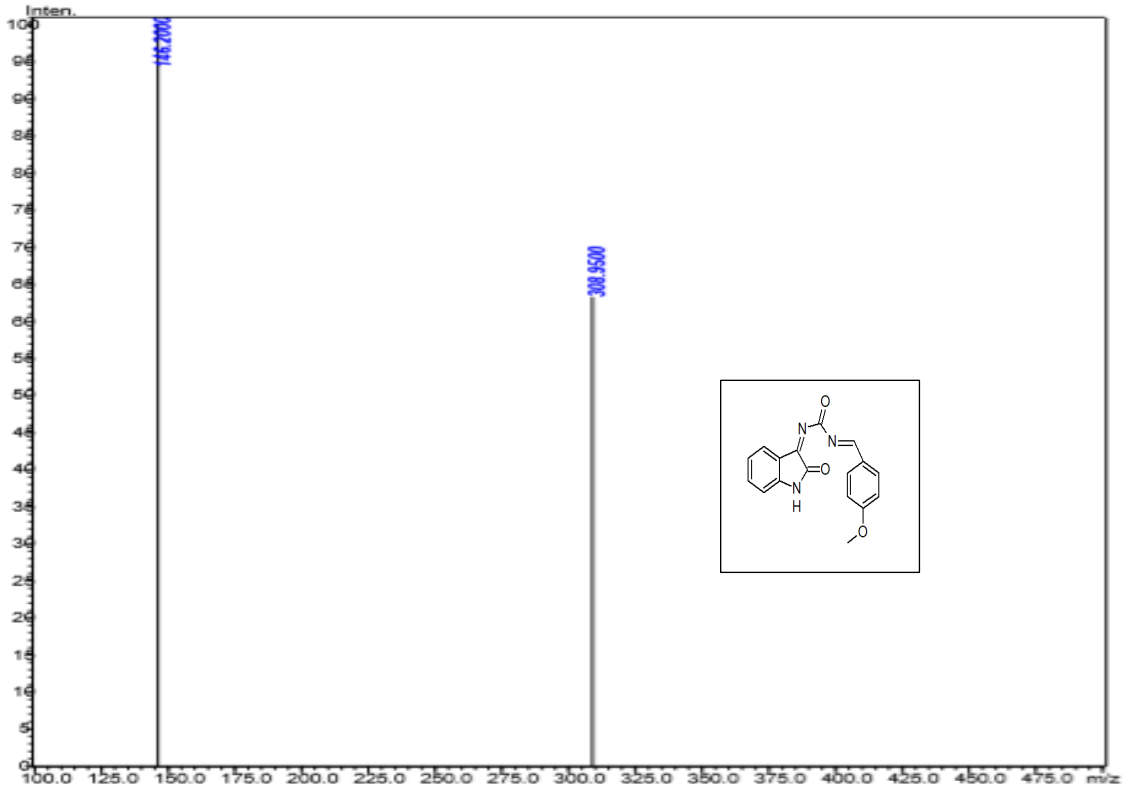
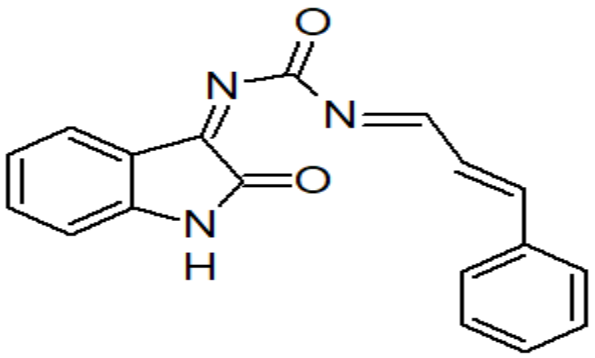


Figure No13: Mass Spectrum of Compound JP2

COMPOUND JP3



IUPAC NAME: 1-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene]-3-[(1E, 2E)-3-phenyl prop-2-en-1-ylidene] urea

Molecular Formula	$C_{18}H_{13}N_3O_2$
Formula Weight	303.31472
Composition	C (71.28%) H (4.32%) N (13.88%) O (10.55%)
Molar Refractivity	88.72 ± 0.5 cm^3
Molar Volume	245.5 ± 7.0 cm^3
Parachor	655.5 ± 8.0 cm^3
Index of Refraction	1.642 ± 0.05
Surface Tension	50.68± 7.0 dyne/cm
Density	1.23 ± 0.1 g/cm^3
Dielectric Constant	Not available
Polarizability	35.17 ± 0.5 $10^{-24}cm^3$

INFRARED SPECTRUM OF JP3:

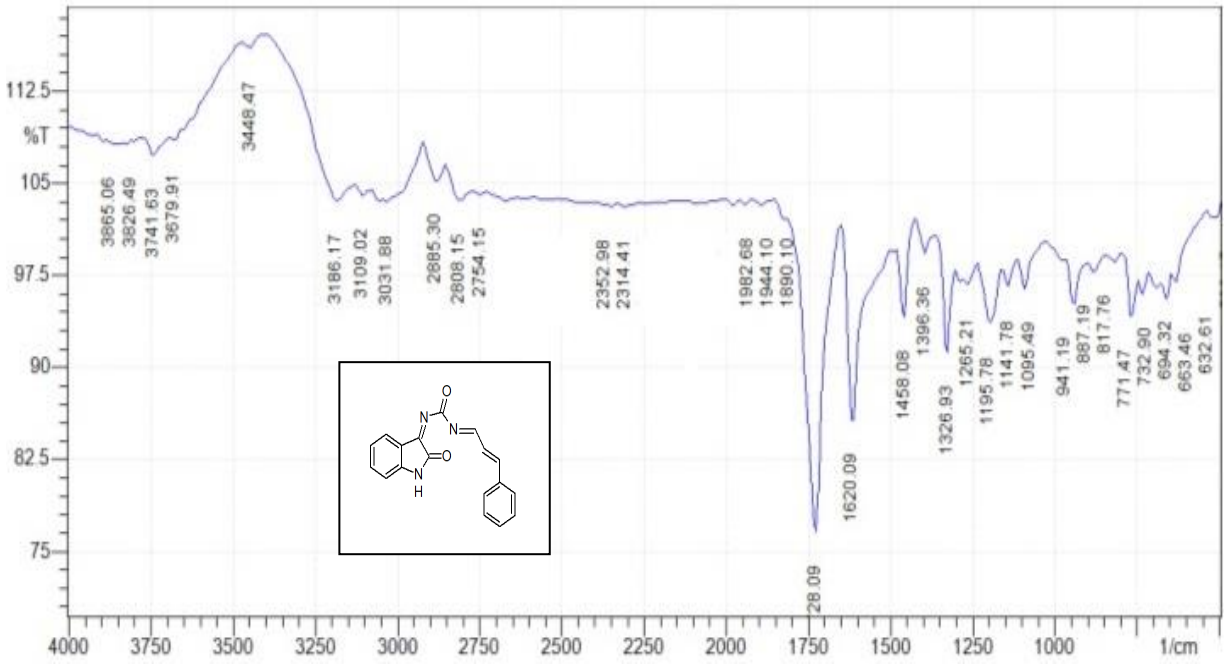


Figure 14: IR Spectrum of Compound JP3

Table 16: IR Interpretation of Compound JP3

S.NO	WAVE NUMBER (cm^{-1})	FUNCTIONAL GROUP
01.	1620.09	C=N Stretching
02.	1458.08	C=C Stretching
03.	1728.09	C=O Stretching
04.	3186.17	N-H Stretching

PROTON NMR SPECTRUM OF JP3:

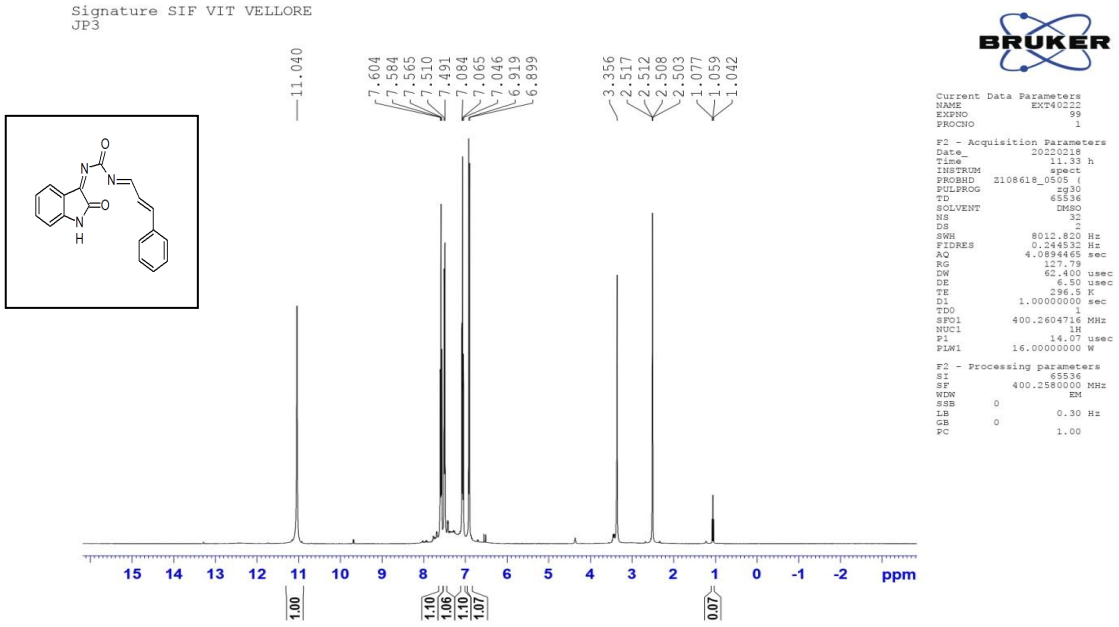


Figure 15: ^{1}H NMR Spectrum of Compound JP3

Table 17: Interpretation of ^{1}H NMR Spectrum of Compound JP3

S.NO	δ VALUE (PPM)	NATURE OF PROTON	NATURE OF THE PEAK	NUMBER OF PROTONS
01.	11.040	N=CH	Singlet	1
02.	7.4-7.6	Ar-H	Multiplet	4
03.	6.8-7.2	CH=CH-CH	Multiplet	3

LC-MS SPECTRUM OF JP3:

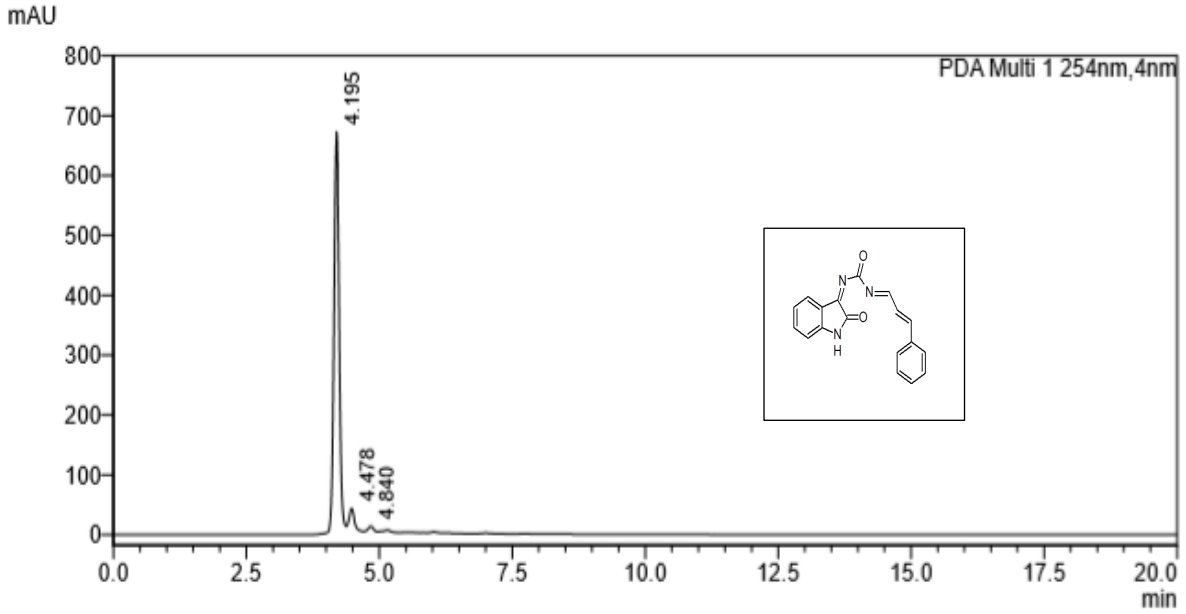


Figure 16: Chromatogram of Compound JP3

Molecular Weight of JP3: 303.32 g/mol

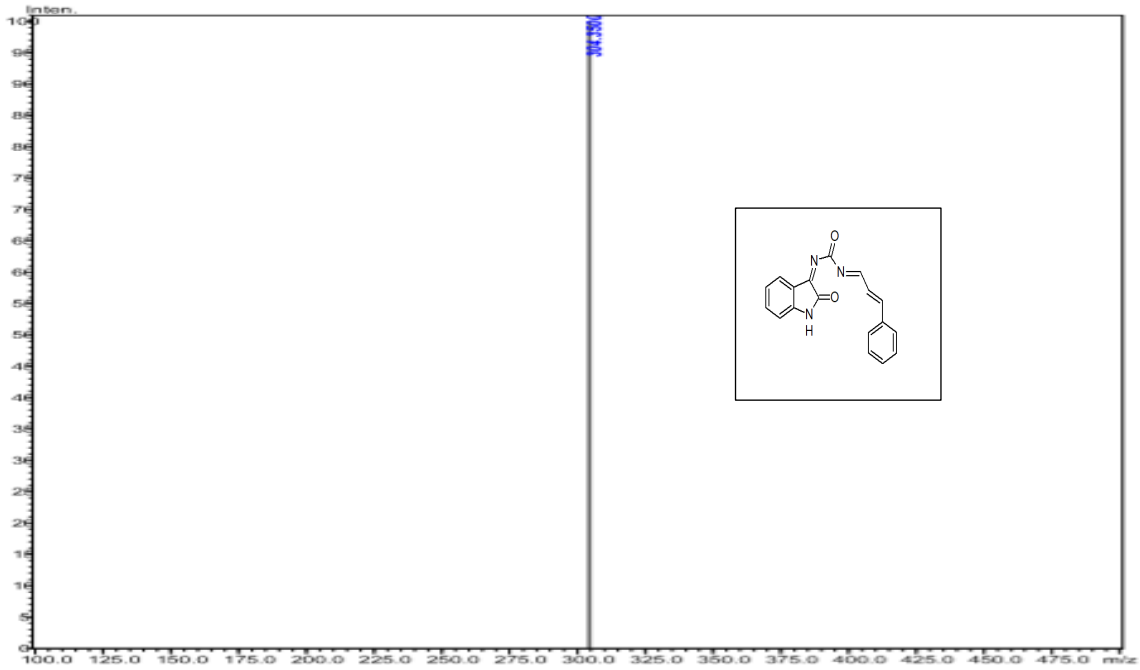
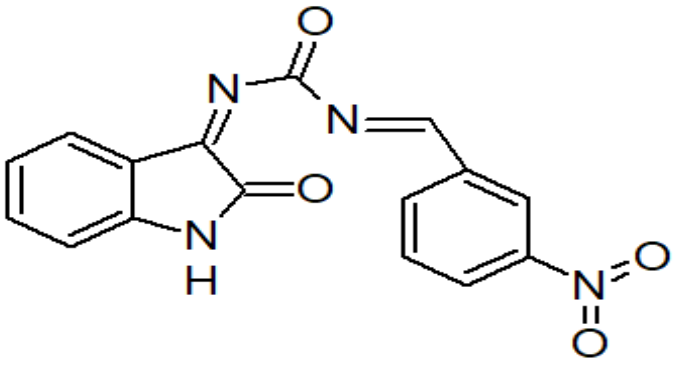


Figure 17: Mass Spectrum of Compound JP3

COMPOUND JP4



IUPAC NAME: 1-[(E)-(3-nitro phenyl) methylidene]-3-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene] urea

Molecular Formula	:	$C_{16}H_{10}N_4O_4$
Formula Weight	:	322.275
Composition	:	C (59.63%) H (3.13%) N (17.38%) O (19.86%)
Molar Refractivity	:	85.16 ± 0.5 cm^3
Molar Volume	:	218.6 ± 7.0 cm^3
Parachor	:	623.8 ± 8.0 cm^3
Index of Refraction	:	1.706 ± 0.05
Surface Tension	:	66.2 ± 7.0 dyne/cm
Density	:	1.47 ± 0.1 g/cm^3
Dielectric Constant	:	Not available
Polarizability	:	33.76 ± 0.5 $10^{-24}cm^3$

INFRARED SPECTRUM OF JP4:

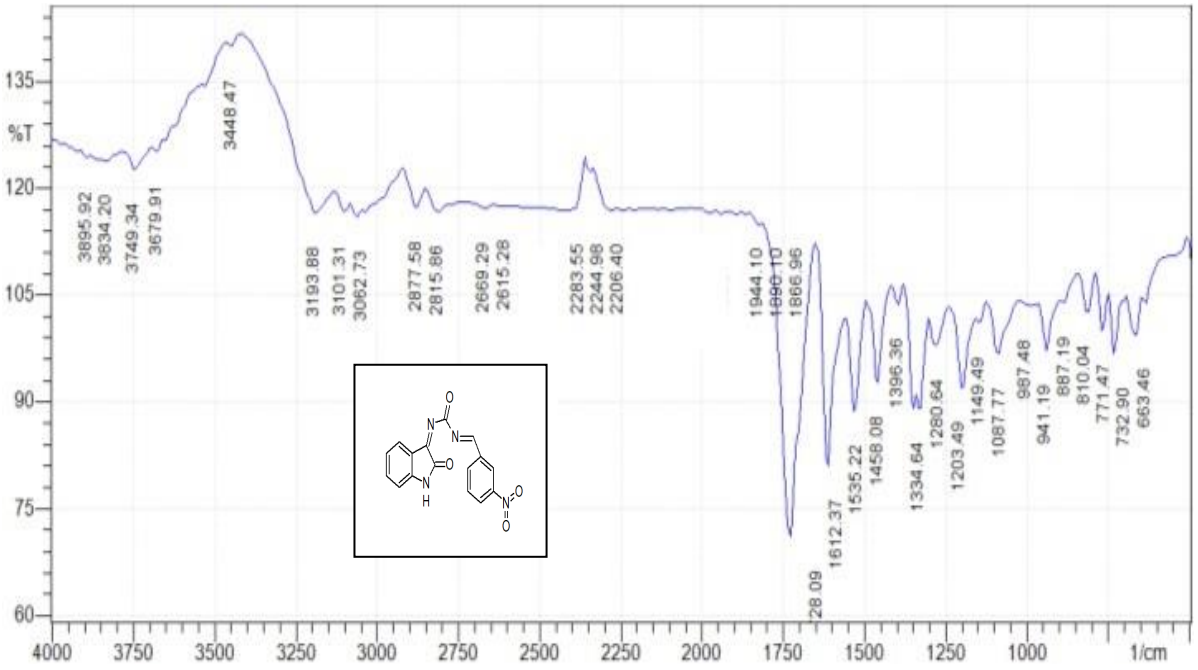


Figure 18: IR Spectrum of Compound JP4

Table 18: IR Interpretation of Compound JP4

S.NO	WAVE NUMBER (cm^{-1})	FUNCTIONAL GROUP
01.	1612	C=N Stretching
02.	3062.73	Ar-CH
03.	1728.09	C=O Stretching
04.	1535	NO2

PROTON NMR SPECTRUM OF JP4:

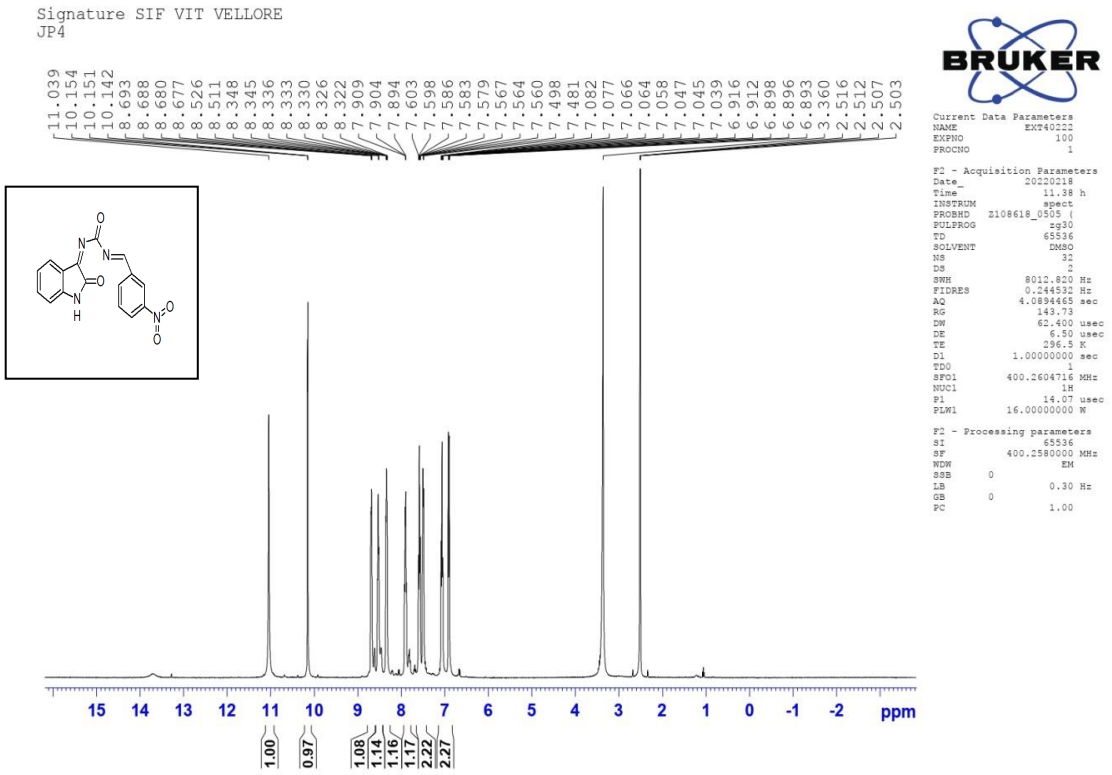


Figure 19: ^{1}H NMR Spectrum of Compound JP4

Table 19: Interpretation of ^{1}H NMR Spectrum of Compound JP4

S.NO	δ VALUE (PPM)	NATURE OF PROTON	NATURE OF THE PEAK	NUMBER OF PROTONS
01.	11	CH=N	Singlet	1
02.	7.2-7.6	Ar-H	Multiplet	4
03.	10.151	-NH	Singlet	1

LC-MS SPECTRUM OF JP4:

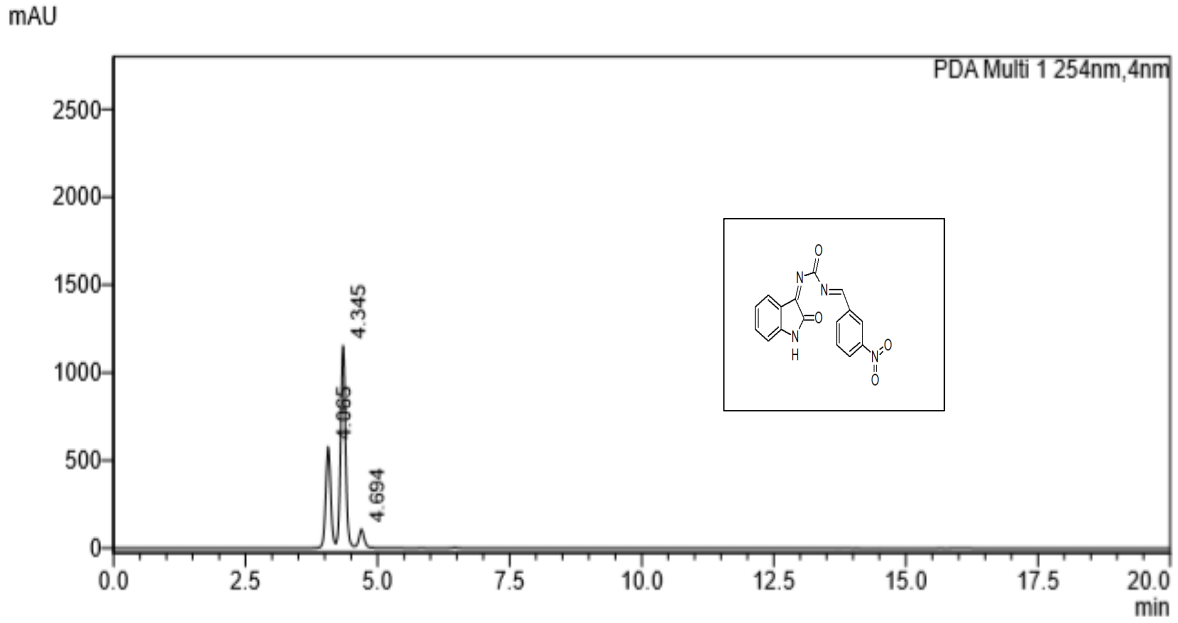


Figure 20: Chromatogram of compound JP4

Molecular Weight of JP4: 328.28 g/mol

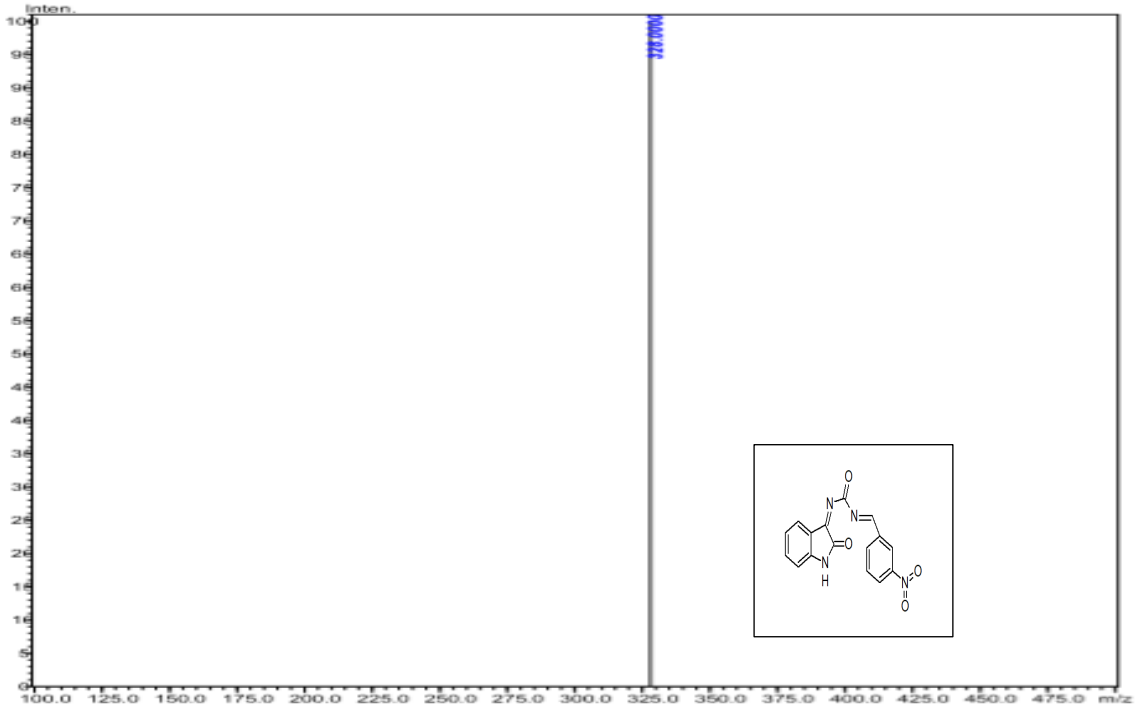
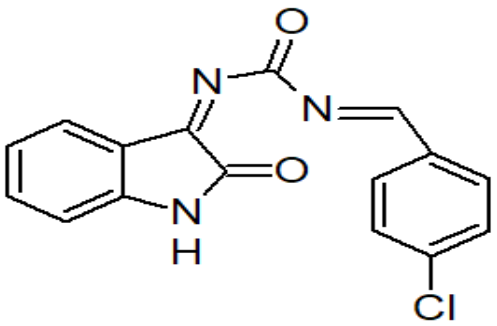


Figure 21: Mass Spectrum of Compound JP4

COMPOUND JP5



IUPAC NAME: 1-[(E)-(4-chloro phenyl) methylidene]-3-[(3Z)-2-oxo-1, 2-dihydro-3H-indol-3-ylidene] urea

Molecular Formula	:	$C_{16}H_{10}ClN_3O_2$
Formula Weight	:	311.7225
Composition	:	C (61.65%) H (3.23%) Cl (11.37%) N (13.48%) O (10.27%)
Molar Refractivity	:	84.10 ± 0.5 cm^3
Molar Volume	:	222.6 ± 7.0 cm^3
Parachor	:	607.1 ± 8.0 cm^3
Index of Refraction	:	1.679 ± 0.05
Surface Tension	:	55.3 ± 7.0 dyne/cm
Density	:	1.40 ± 0.1 g/cm^3
Dielectric Constant	:	Not available
Polarizability	:	33.34 ± 0.5 $10^{-24}cm^3$

INFRARED SPECTRUM OF JP5:

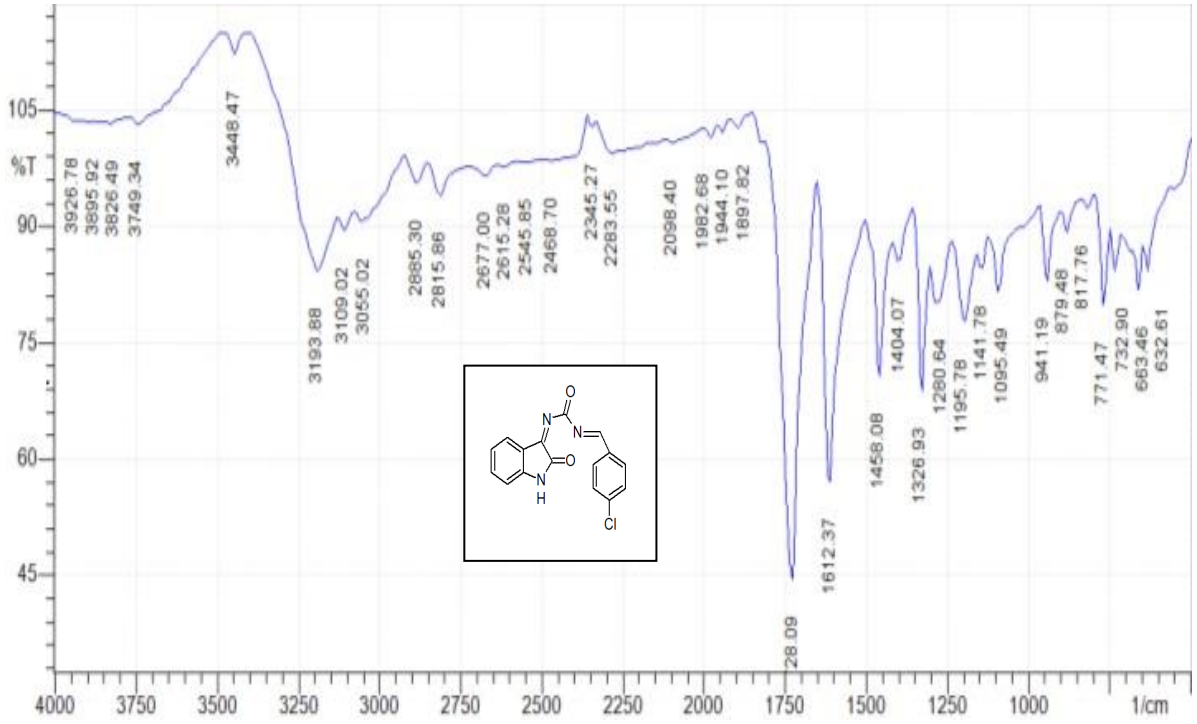


Figure 22: IR Spectrum of Compound JP5

Table 20: IR Interpretation of Compound JP5

S.NO	WAVE NUMBER (cm^{-1})	FUNCTIONAL GROUP
01.	1612.37	C=N Stretching
02.	1728.09	C=O Stretching
03.	3193.88	N-H Stretching
04.	771.47	C-Cl Stretching

PROTON NMR SPECTRUM OF JP5:

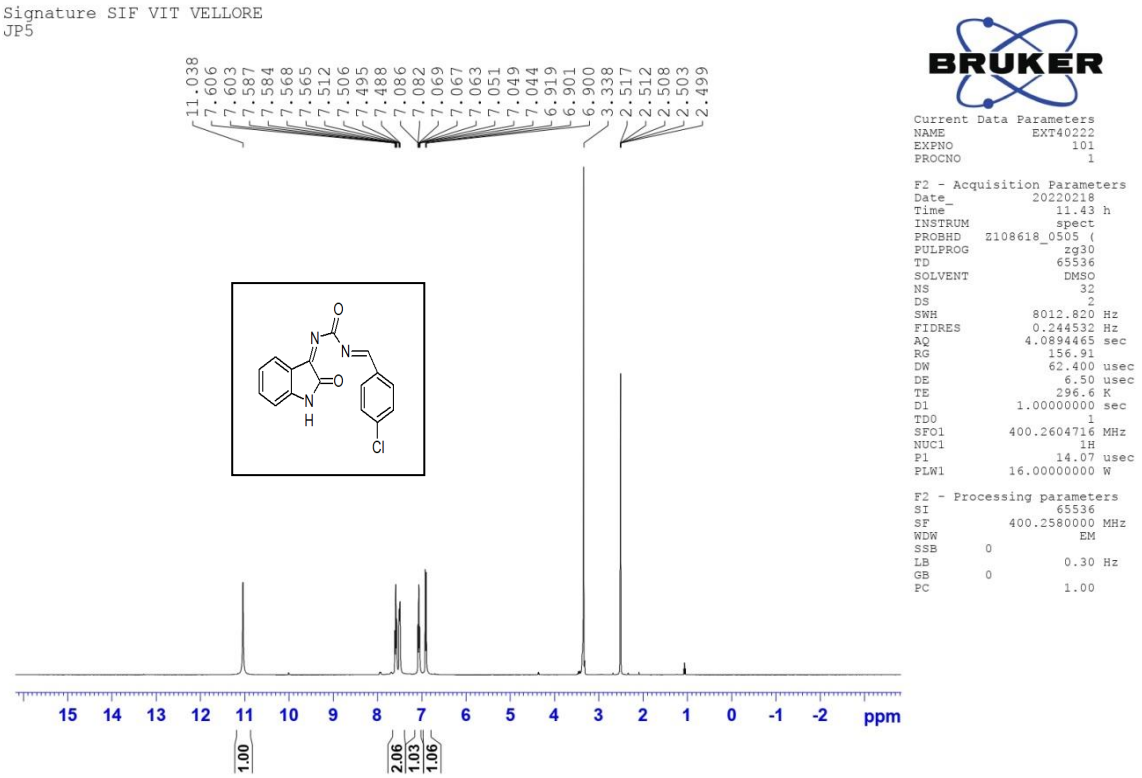


Figure 23: ^{1}H NMR Spectrum of Compound JP5

Table 21: Interpretation of ^{1}H NMR Spectrum of Compound JP5

S.NO	δ VALUE (PPM)	NATURE OF PROTON	NATURE OF THE PEAK	NUMBER OF PROTONS
01.	11.038	CH=N	Singlet	1
02.	6.8-7.2	Ar-H	Multiplet	4
03.	9.009	-NH	Singlet	1

LC-MS SPECTRUM OF JP5:

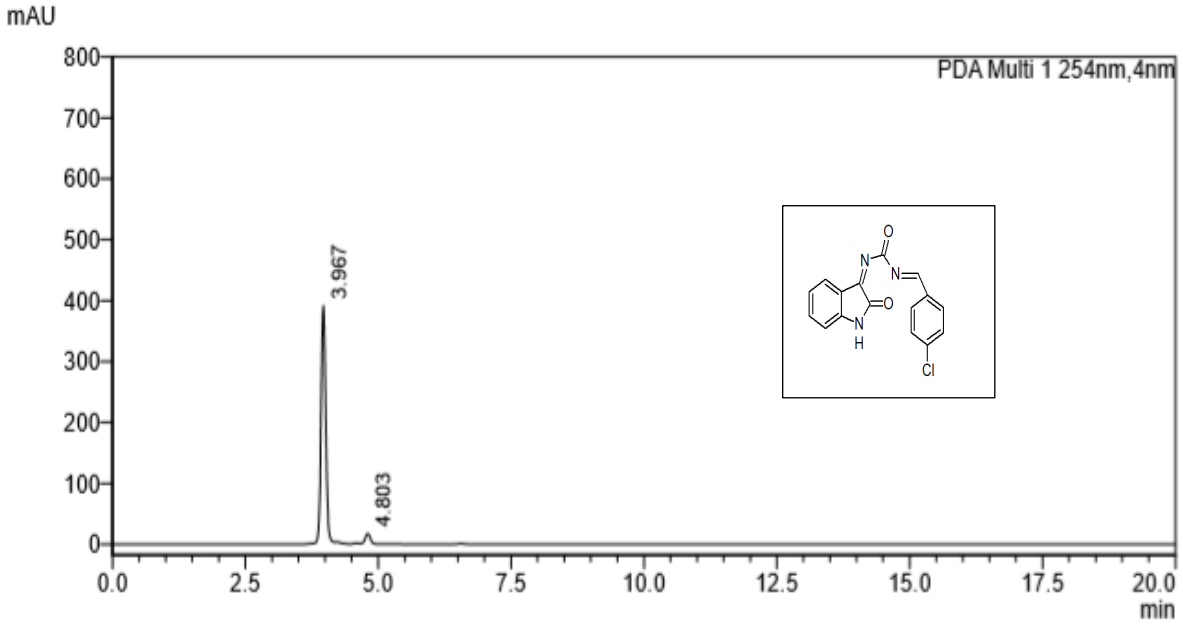


Figure 24: Chromatogram of Compound JP5

Molecular Weight of JP5: 311.73 g/mol

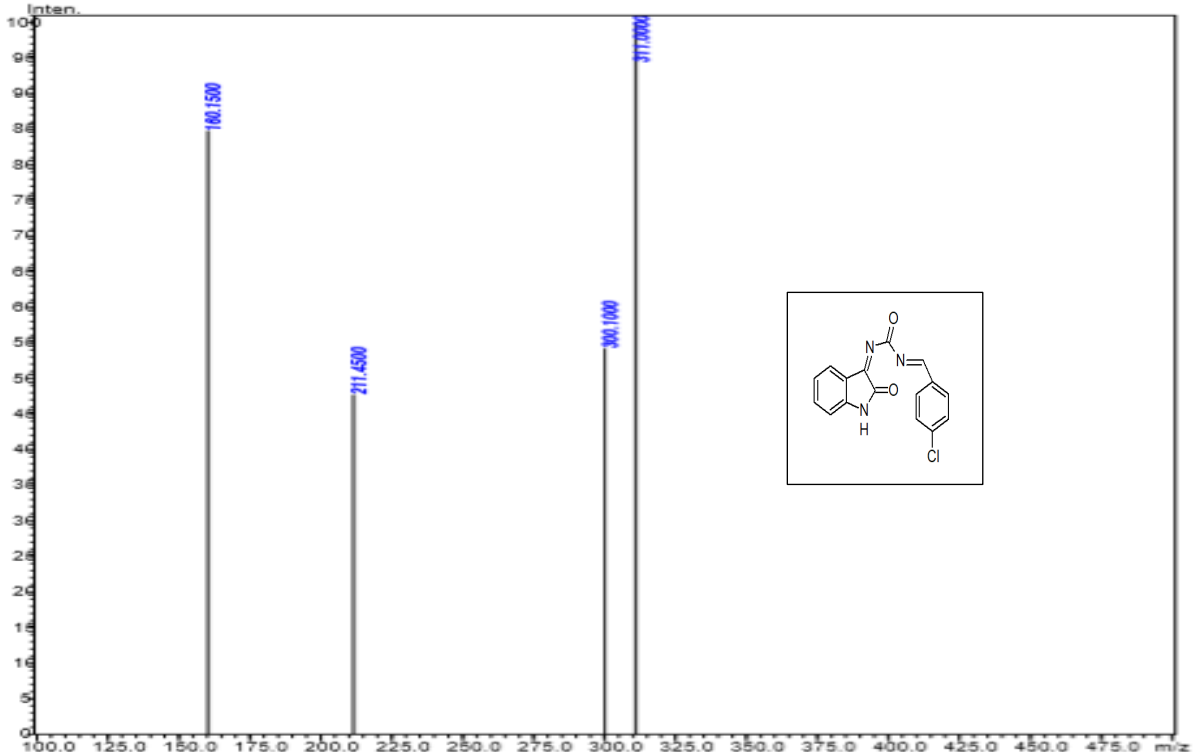


Figure 25: Mass Spectrum of Compound JP5

- All the five synthesized compounds had a different R_f value from the reactant molecules and showed a single spot in the thin layer chromatogram.
- They exhibited a sharp melting point.
- In the IR spectrum, absorptions corresponding to the relevant functional groups were seen.
- From the NMR spectrum, numbers of protons present in the molecular structure were elucidated and the structure of the compound was confirmed.
- In LC-MS analysis, all the compounds were determined as a single peak. The molecular mass of the compounds was obtained from Mass spectrometry.
- The actual mass obtained from the M^{+1} peak of mass spectrum was similarto the calculated mass of the compounds.

Table 22: Comparison of Calculated & Obtained Molecular mass of the Compounds JP1 – JP5.

CODE	CALCULATED MASS (g/mol)	ACTUAL MASS (g/mol)
JP1	293.28	295.55
JP2	307.31	308.95
JP3	303.32	304.35
JP4	328.28	328
JP5	311.73	311

5. RESULTS OF ANTI-TUBERCULAR ACTIVITY:

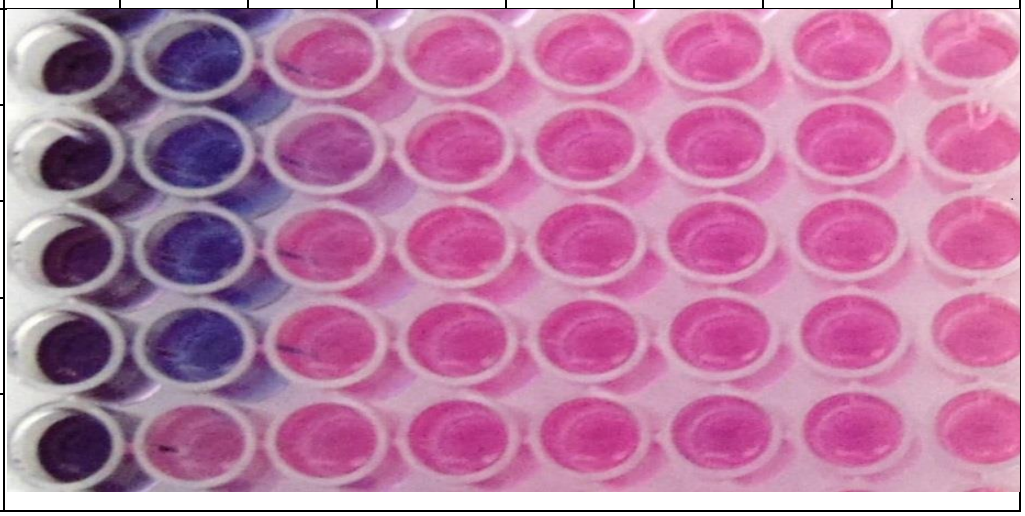
The recrystallized pure compounds were evaluated for their Anti-tubercular activity by *in vitro* Microplate Alamar Blue Assay (MABA). 100 – 0.8 µg/ml dilutions of the compounds were made using Dimethyl sulfoxide (DMSO).

Table 23: Data of MABA results of the compounds JP1 – JP5 (100–0.8 µg/ml)

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
JP1	S	S	R	R	R	R	R	R
JP2	S	S	R	R	R	R	R	R
JP3	S	S	R	R	R	R	R	R
JP4	S	S	R	R	R	R	R	R
JP5	S	R	R	R	R	R	R	R

S – SENSITIVE R – RESISTANT

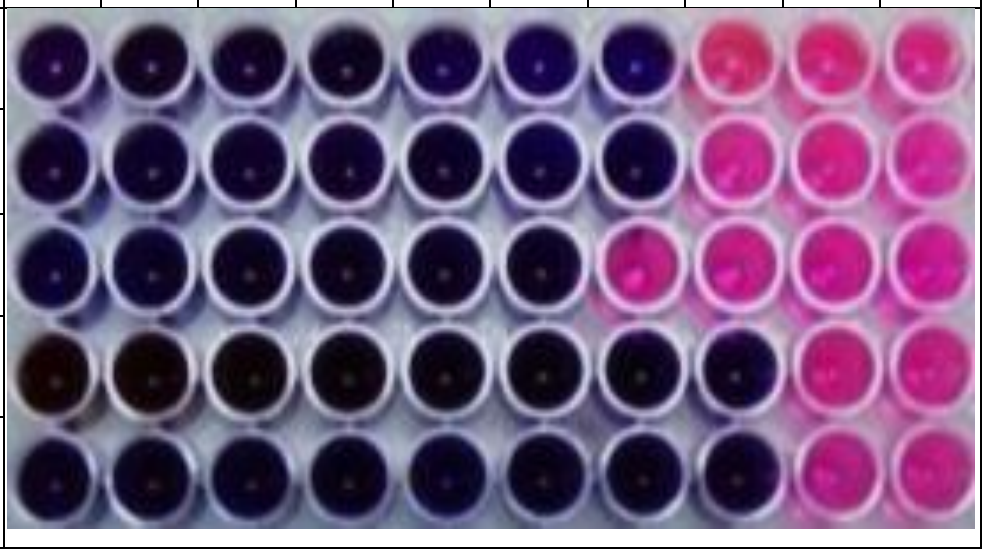
Table 24: Visualization of MABA results of the compounds JP1 – JP5 (100 –0.8µg/ml)

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
JP1								
JP2								
JP3								
JP4								
JP5								

MINIMUM INHIBITORY CONCENTRATION (MIC VALUES) OF STANDARD ANTI-TUBERCULAR DRUGS:

1. Isoniazid – 1.6 µg/ml
2. Ethambutol – 1.6 µg/ml
3. Pyrazinamide- 3.125µg/ml
4. Rifampicin – 0.8 µg/ml
5. Streptomycin- 0.8µg/ml

Table 25: Visualization of MABA results of the Standard Anti-tubercular drugs

STANDARD DRUG	**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**	**0.4 µg/ml**	**0.2 µg/ml**
Isoniazid										
Ethambutol										
Pyrazinamide										
Rifampicin										
Streptomycin										

- All the five synthesized compounds show anti tubercular activity in the MIC values of 50µg/ml and 100 µg/ml.

6. RESULTS OF ACUTE ORAL TOXICITY STUDIES:

The synthesized Compounds JP1-JP5 was selected for acute oral toxicity studies. The study was conducted based on the **OECD Guidelines 423**. A **dose of 2000 mg/kg** was administered to the animals and the behavioral signs of toxicity were observed. Parameters which were evaluated during the study and the observations were tabulated below,

Table 26: Parameters evaluated during the Acute toxicity study

S.NO	PARAMETERS	OBSERVATION
1.	Aggressiveness	Absent
2.	Analgesia	Absent
3.	Body temperature	Normal
4.	Body weight	No significant change
5.	Convulsions	Absent
6.	Diarrhoea	Absent
7.	Lacrimation	Normal
8.	Lighting reflex	Present
9.	Motor activity	Normal
10.	Pre-terminal death	Nil
11.	Respiration	Normal
12.	Restlessness	Absent
13.	Salivation	Normal
14.	Sedation	Absent
15.	Skin colour	Normal
16.	Toxic signs	Absent
17.	Tremors	Absent

- **No significant toxic signs** were observed and **no mortality was reported up to 14 days of study**.
- The five compounds were found to be non-toxic and safe for oral administration up to the dose of 2000mg/kg body weight.

SUMMARY

- **TB** is the most **attractive areas of research,** because it is the Second major cause of death due to an **infectious disease** in adults.
- Twenty-two countries of the world have the highest TB burden, among top five ranking countries are **India, China, Indonesia, South Africa** and **Nigeria.**
- **Drug resistance TB** plays a **major challenge** for the effective control of TB.
- Therefore, the current work aimed to synthesize some novel anti-tubercular compounds.
- Based on the literature review, ***ATP SYNTHASE*** of ***Mycobacterium tuberculosis*** was chosen as the target enzyme against which novel molecules were designed by performing structural modifications in the basic hetero nuclear scaffold Isatin.
- The designed molecules were docked against the target using the **MGL tools**.
- The top scored compounds were assessed for their ***in-silico*** drug likeness property and toxicity profile.
- The non-toxic molecules were synthesized by an appropriate laboratory process and they were coded from JP1 to JP5. Completion of the reaction was determined by TLC.
- The synthesized compounds were subjected to repeated recrystallization using ethanol and Purity of the compounds was ensured by sharp melting point.
- Further the compounds were characterized by spectroscopic analysis such as IR, NMR & LC-MS.
- Anti-tubercular activity of the purified compounds was evaluated **by Microplate Alamar Blue Assay (MABA).** The Compounds showed activity at **50 µg/ml and 100 µg/ml.**
- Acute oral toxicity study was conducted on the Albino rats and the compounds were found to be safe & non-toxic.

CONCLUSION

The current research work concludes that,

1. The synthesized compounds might effectively inhibit the chosen target *ATP synthase* which is involved in the energy metabolism of Mycobacterium Tuberculosis.
2. All the five compounds have the Docking score between **7 to -9.4 kcal/mol.** There is no significant correlation between the score and activity of the compounds.
3. All the synthesized compounds posses anti-tubercular activity at the concentration of **50 µg/ml and 100 µg/ml.**
4. Therefore, further modification of the molecular structure of the compounds is expected to yield promising drug candidates against the deadly disease Mycobacterium Tuberculosis.

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71st Indian Pharmaceutical Congress

Theme: Healthcare System - Role of Regulators

Certificate

This is to certify that Prof./Dr./Mr./Ms.....

of ..... has participated in the 71st Indian Pharmaceutical Congress held at Sri Ramachandra Institute of Higher Education and Research (DU), from 20th to 22nd December 2019, Chennai, Tamil Nadu.

Shri. Ravi Uday Bhaskar	**Dr. P.V.Vijayaraghavan**	**Dr. M. Dhilip Kumar**	**Dr. T.V. Narayana**
President - 71st IPC 2019	Chairman - LOC	Secretary - LOC	General Secretary - IPCA

AIDCOC

Organised by
Indian Pharmaceutical Congress Association

Hosted by
All India Drugs Control Officers' Confederation

One day workshop on Artificial Intelligence in Drug Discovery

Organized by
CSIR-North East Institute of Science & Technology

Sl. No. AIDD1330

Certificate of Participation

This is to certify that

JAYAPRIYA ELANGOVAN

has participated in the one day workshop on "Artificial Intelligence in Drug Discovery" organized by CSIR-North East Institute of Science and Technology, Jorhat on 01-09-2020.

Debabrata Das

Mr. Debabrata Das
Coordinator
CSIR-NEIST, Jorhat

Dr. G. Narahari Sastry
Director, CSIR-NEIST
Jorhat, Assam

Cid: 597.971

Office of the Principal Scientific Adviser to the Government of India

MoE's INNOVATION CELL (GOVERNMENT OF INDIA)

Ministry of Education
Government of India

DRUG DISCOVERY HACKATHON 2020

Certificate

This Certificate is awarded to

JAYAPRIYA ELANGOVAN

for Participating in

Drug Discovery Hackathon 2020

Prof. Vijay Raghavan
Principal Scientific Adviser
Government of India

Prof. Anil Sahasrabudhe
Chairman
AICTE

Prof. B. Suresh
President
Pharmacy Council of India

Dr. Abhay Jere
Chief Innovation Officer
Ministry of Education Innovation Ce

CSIR-SUMMER RESEARCH TRAINING PROGRAM (CSIR-SRTP)2020 ONLINE

CERTIFICATE

Name: JAYAPRIYA ELANGOVAN **Sl. No.:** CSIR/SRTP/2020/NEIST/1583

has completed all the requirements of the CSIR-Summer Research Training Program (CSIR-SRTP) 2020 online during June to August, 2020 coordinated by CSIR-NEIST, Jorhat

DR. G. NARAHARI SASTRY
DIRECTOR
CSIR-NORTH EAST INSTITUTE OF SCIENCE AND TECHNOLOGY

PROF. ALOK DHAWAN
DIRECTOR
CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH

DR. SHEKHAR C. MANDE
DIRECTOR GENERAL, CSIR
SECRETARY, DSIR, GOVT. OF INDIA

MADRAS MEDICAL COLLEGE, CHENNAI – 600003

INSTITUTIONAL ANIMAL ETHICS COMMITTEE

PROCEEDINGS

PRESENT: Dr. A. JERAD SURESH, MPharm., PhD., MBA

Roc. No: 5/AEL/IAEC/MMC/2022 Dated: 01-11-2021

Sub: IAEC, MMC, Ch-3 – Approval of Laboratory Animals – Regarding

Ref: IAEC Meeting held on 21-10-2021

This order is issued based on the approval by the Institutional Animal Ethics Committee Meeting held on 21-10-2021, Thursday.

Project Proposal ID Number	15/2021-2022
CPCSEA Registration Number	1917/GO/ReBi/2016/CPCSEA Valid till 19-9-2026
Name of the Researcher with ID Number	E. JAYAPRIYA 261915705
Name of the Guide	Dr. A. Jerad Suresh, MPharm., PhD
Project Title	Design, Synthesis, Characterization And Biological Evaluation Of Novel Isatin Schiff base derivatives as Anti-tubercular agents targeting ATP Synthase.
Date of submission of proposal to IAEC	07-10-2021
Date of IAEC meeting	21-10-2021
Date of submission of modified proposal to IAEC	22-10-2021
Date of Approval	21-10-2021
Validity of the Approved Proposal	One Year
Number & Species of Laboratory Animals Approved	**30 Wistar Rats Approved**

5/11/21

Chairperson
Institutional Animal Ethics Committee
Madras Medical College
Chennai-600003

PRINCIPAL
COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

To
Dr. A. Jerad Suresh, MPharm., PhD, MBA.
Principal, Prof. & Head, Dept. of Pharmaceutical Chemistry,
College of Pharmacy,
MMC, Ch-3.

Copy to:
Special Veterinary Officer, Animal Experimental Laboratory,
Madras Medical College, Ch-3.