DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL ISATIN DERIVATIVES AS ANTITUBERCULAR AGENTS

A dissertation submitted to

The Tamilnadu Dr. M.G.R Medical University

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In partial fulfilment of the requirements

for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL CHEMISTRY

Submitted by

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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

COLLEGE OF PHARMACY

MADRAS MEDICAL COLLEGE

CHENNAI-600003

APRIL 2014

CERTIFICATE

This is to certify that the dissertation entitled **"DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL ISATIN DERIVATIVES AS ANTITUBERCULAR AGENTS"** submitted by the candidate bearing the **Reg. No. 261215701** in partial fulfillment of the requirements for the award of the degree of **MASTER OF PHARMACY in PHARMACEUTICAL CHEMISTRY** by **The Tamilnadu Dr. M.G.R Medical University** is a bonafide work done by him during the academic year 2013-2014 at the **Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai-3**.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	EXPANSION
Log P	Partition co-efficient
Log D	Diffusion co-efficient
Da	Daltons
cmaA1	Cyclopropane mycolic acid synthase
TLC	Thin Layer Chromatography
IR	Infrared
NMR	Nuclear magnetic resonance spectroscopy
3D	Three dimensions
MABA	Microplate Alamar Blue Assay
MIC	Minimum inhibitory concentration
GLIDE	Grid Based Ligand Docking Energitics
PSA	Polar Surface Area
TPSA	Total Polar Surface Area
CoMFA	Comparative molecular field analysis
CoMSIA	Comparative molecular similarity index analysis
OSIRIS	Optical spectroscopic and infrared remote imaging system
PDB	Protein Data Bank

CONTENTS

INTRODUCTION

BACTERIOLOGICAL PROFILE OF *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (MTB) is a pathogenic bacterial species in the genus *Mycobacterium* and the causative agent of most cases of tuberculosis (TB) first discovered in 1882 by Robert Koch, *M. tuberculosis* has an unusual, waxy co-antigen, its cell surface (primarily mycolic acid), which makes the cells impervious to Gram staining[1]. Acid-fast detection techniques are used instead. The physiology of *M.tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, MTB infects the lungs *M. tuberculosis* requires oxygen to grow. It does not retain any bacteriological stain due to high lipid content in its wall, and thus is neither Gram-positive nor Gram-negative hence Ziehl-Neelsen staining or acid-fast staining is used [1]. *M. tuberculosis* divides every 15–20 hours, which is extremely slow compared to other bacteria. It is a small bacillus that can withstand weak disinfectants and can survive in a dry state for weeks. Its unusual cell wall, rich in lipids (e.g., mycolic acid), is likely responsible for this resistance and is a key virulence factor.

Tuberculosis, MTB, or TB (short for *tubercle bacillus*) is a common, and in many cases lethal, infectious disease caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* [4]. Tuberculosis typically attacks the lungs, but can also affect other parts of the body. It is spread through the air when people, who haven active TB infection, cough, sneeze, or otherwise transmit their saliva through the air. Most infections are asymptomatic and latent, but about one in ten latent infections eventually progresses to active disease which, if left untreated, kills more than 50% of those so infected. Consumption, phthisis, scrofula, Pott's disease, and the White Plague are all terms used to refer to tuberculosis throughout history. It is generally accepted that the microorganism originated from other, more primitive organisms of the same genus Mycobacterium. Human bones from the Neolithic show a presence of the bacteria, although the exact magnitude (incidence and prevalence) is not known before the 19th century. The first references to tuberculosis in Asian civilization are found in the Vedas [5]. The oldest of them (Rig-Veda, 1500 BC) calls the disease *yaksma*. The Atharvaveda calls it another name: *balasa*. It is in the Atharvaveda that the first description of scrofula

is given. The *Sushruta Samhita*, written around 600 BC, recommends that the disease be treated with breast milk, various meats, alcohol and rest. The Yajurveda advises sufferers to move to higher altitudes. Aretaeus was the first person to rigorously describe the symptoms of the disease in his text *De causis et signis diuturnorum morborum*:"Voice hoarse; neck slightly bent, tender, not flexible, somewhat extended; fingers slender, but joints thick; of the bones alone the figure remains, for the fleshy parts are wasted; the nails of the fingers crooked, their pulps are shrivelled and flat...Nose sharp, slender; cheeks prominent and red; eyes hollow, brilliant and glittering; swollen, pale or livid in countenance; the slender parts of the jaws rest on the teeth as, as if smiling; otherwise of cadaverous aspect..."

PATHOGENESIS OF TUBERCULOSIS

In the lungs, *M. tuberculosis* is taken up by alveolar macrophages, but they are unable to digest the bacterium. Its cell wall prevents the fusion of the phagosome with a lysosome. Specifically, *M. tuberculosis* blocks the bridging molecule, early endosomal auto antigen 1(EEA1); however, this blockade does not prevent fusion of vesicles filled with nutrients. Consequently, the bacteria multiply unchecked within the macrophage [11]. The bacteria also carried the *UreC* gene, which prevents acidification of the phagosome. The bacteria also evade macrophage-killing by neutralizing reactive nitrogen intermediates.

M. tuberculosis usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II Pneumocytes. This cell type is found in greater numbers than macrophages in alveoli, and *M. tuberculosis* can infect and grow in these pneumocytes ex vivo. In addition, dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and presumably play a key role in activating T cells with specific *M. tuberculosis* antigens [7]. Since dendritic cells are migratory, unlike differentiated macrophages, they also may play an important role in dissemination of *M. tuberculosis.* The bacteria are phagocytized in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors. Surfactant protein A, a glycoprotein found on alveolar surfaces, can enhance the binding and uptake of *M tuberculosis* by up regulating mannose receptor activity. On the other hand, surfactant

protein D, similarly located in alveoli, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface, and it is proposed that this prevents *M.tuberculosis* interaction with mannose receptors on the macrophage cell surface. The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake [12]. On entry into a host macrophage, *M. tuberculosis* resides in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle occurs, i.e., phagosome-lysosome fusion, these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides [22].

MICROSCOPY

Owing to their high lipid content in cell walls, *M.tuberculosis* cannot be identified by Gram's staining. Acid-Fast staining or Ziehl-Neelsen staining is employed to identify the organisms. *M. tuberculosis* is characterized by caseating granulomas containing Langhans giant cells, which have a "horseshoe" pattern of nuclei. Organisms are identified by their red colour on acid-fast staining [14].

Fig 01 – *M.tuberculosis*

TYPES OF TUBERCULOSIS

Tuberculosis is a contagious disease that affects almost all the important organs of the body. Clinically, tuberculosis is broadly categorized into three major categories.

Primary Tuberculosis:

When tuberculosis affects a person who had never been exposed to the bacterium earlier, the condition is called primary tuberculosis. In this form of tuberculosis, the source of bacterium is external [17]. In primary tuberculosis the lymph nodes get affected leading to their swelling. Lesions are also formed which are removed during treatment. The removal of the lesion does not indicate bacterial removal as the bacteria may have gone into a dormant phase and if left untreated, it can cause TB when favourable condition comes [19].

Secondary Tuberculosis

It is also known as post-primary tuberculosis. This type of tuberculosis occurs in a person who previously had TB. In primary TB, the bacterium goes into an inactive phase while in secondary tuberculosis; the bacterium regains its active mode and causes the symptoms [17]. Secondary tuberculosis is mostly localized to lungs as oxygen pressure is highest there. Secondary tuberculosis is more infectious than primary tuberculosis. Secondary TB increases the chance of the infection's spread to other organs such as kidneys, heart and brain.

Disseminated Tuberculosis

Disseminated tuberculosis means that the tuberculosis has infected the entire body system. It is a very rare type of disease. Disseminated TB primarily affects the bones of spines, hips, joints and knees, the genital tract of women, the urinary tract and even the central nervous system [23]. It infects the cerebrospinal fluids, the gastro intestinal tract, the adrenal gland, skin of the neck and even the heart.

MEDICINAL CHEMISTRY

Medicinal chemistry is the applied science that is based on the drug design and discovery of new chemical entities and their optimization and development existing as useful drug molecules for the therapeutic purpose [9]. It is also a multi-disciplinary approach drawing on theoretical chemistry, organic chemistry, analytical chemistry, molecular biology, pharmacology and bio-chemistry [9].

DRUG DISCOVERY

The process of drug discovery involves designing, synthesizing, characterization and biological screening of newer chemical entities which would be applicable for therapeutic use(s). This process includes the study of existing drug molecules, reports of biological screening properties and their QSAR. Discovery is the process of identifying the active novel compounds so called *Hits* that are typically found by evaluating more compounds for predicting desired biological properties [24]. Besides a number of approaches in identifying the active *Hits*, the most reliable technique is applicable based on physico-chemical properties and biological screening. The other sources where *Hits* exist namely from natural sources, synthetic chemical libraries from combinatorial chemistry or from collecting historical compounds are evaluated against the biological target [31].

LEAD OPTIMIZATION

Lead optimization is a complex, non-linear process. During this stage of drug discovery, the chemical structure of a confirmed hit is refined to improve its drug characteristics with the goal of producing a preclinical drug candidate. Typically, confirmed hits are evaluated in secondary assays, and a set of related compounds, called analogues, are synthesized and screened. The testing of analogue series results in quantitative information that correlates changes in chemical structure to biological and pharmacological data to establish structure-activity relationships (SARs) [21]. Today, lead optimization often involves a series of standard assays to evaluate toxicity, including P_{450} inhibition and cytotoxicity assays. Toxicity in these relatively simple *in vitro* assays flags hits or leads that could have potential safety concerns. Another characteristic that lead optimization often evaluates is formulation [25]. Formulation and delivery are closely linked. For example, a drug intended to be delivered via intramuscular injection might call for a different formulation than would one intended for oral delivery. Formulation problems and solutions feed back into the iterative lead optimization cycle. Molecular Devices offers a range of products that are particularly well suited for lead optimization studies [32].

DRUG DESIGN

Methodologies used in drug design have changed over time, exploiting and driving new technological advances to solve the varied bottlenecks found along the way. While until the 90s, the major issues were lead discovery and chemical synthesis of drug-like molecules, the emergence of combinatorial chemistry gene technology, and high-throughput tests shifted the focus, with poor absorption, distribution, metabolism, and excretion (ADME) properties of the new drugs capturing more attention today, the field of drug development may seem more fertile than ever before, with vast amounts of information from genomic and proteomic studies facilitating the finding of new targets, the usage of rational combinatorial chemistry for the production of libraries of compounds, the generation of genetically modified animal models for the development and testing of new drugs, and the possibility of using ultra-high-throughput test techniques for the screening of large libraries [3,27]. However, despite all these advances, the revolutionary era of drug design has not arrived yet. A variety of computational approaches can be applied at different stages of the drug-design process: in an early stage, this focus on reducing the number of possible ligands, while at the end, during lead-optimization stages, the emphasis is on decreasing experimental costs and reducing times [26]. Although this is simple to articulate, it has been tried many times with only a few fruitful examples**.**

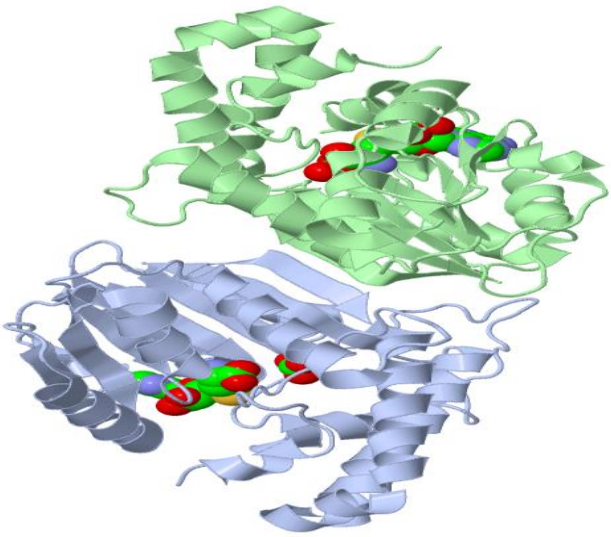
RATIONAL DRUG DESIGN

When the structure of the target protein is known, the drug discovery process usually follows a well-established procedure and it is shown schematically in Figure 2. Virtual screening techniques are applied early during the docking protocol to reduce the size of large compound libraries [25]. Initially, libraries are ‘‘pre-filtered’’ using a series of simple physicochemical descriptors to eliminate compounds not expected to be suitable drugs. Pharmacophore analysis, neural nets, similarity analysis, scaffold analysis, Lipinski’s rule of five and garbage filters are used to sort out molecules according to their ADME properties, among others [34]. This procedure, which reduces the size of the library to a group of molecules more likely to bind the target receptor, is known as enrichment. It is necessary to stress that the selection criteria used during the enrichment steps need to be carefully chosen, as application of too stringent filters may lead to early exclusion of potential leads Similarly, drug-likeness of potential leads may be less important at the early stages than ease of the molecule to experimental validation with in vitro assays and X-ray crystallography [34].

Similar compounds can be further grouped together and arranged in smaller assemblies to assist the screening process. The use of several small libraries is not only a more cost-effective approach, but can usually provide a broader chemical diversity than a single large library [31]. Once an optimum library has been produced, molecules are docked to the target receptor to reduce further the number of candidates. This initial screening makes use of fast, but not very accurate, ranking functions to evaluate the relative stability of the docked complexes. The selected candidates, usually a few hundred, are subject to further docking experiments using more sophisticated scoring functions [32].

Mycolic acids are major components of the cell wall of Mycobacterium tuberculosis. Several studies indicate that functional groups in the acyl chain of mycolic acids are important for pathogenesis and persistence. The mycolic acid cyclopropane synthases (CmaA1) those are responsible for these site-specific modifications of mycolic acids. To derive information on the specificity and enzyme mechanism of the family of proteins, the crystal structures of CmaA1 were solved to 2-A resolution [19]. The enzymes have a seven-stranded alpha/beta fold similar to other methyltransferases with the location and interactions with the cofactor S-adenosyl-l-methionine conserved. The structures of the ternary complexes demonstrate the position of the mycolic acid substrate binding site. Close examination of the active site reveals electron density that we believe represents a bicarbonate ion. The structures support the hypothesis that these enzymes catalyze methyl transfer via a carbocation mechanism in which the bicarbonate ion acts as a general base. In addition, comparison of the enzyme structures reveals a possible mechanism for substrate specificity. These structures provide a foundation for rational-drug design, which may lead to the development of new inhibitors effective against persistent bacteria [19].

Figure-2: 3D view of cmaA1 (1L1E) protein



DOCKING

Docking techniques, designed to find the correct conformation of a ligand and its receptor, have now been used for decades**.** The process of binding a small molecule to its protein target is not simple; several entropic and enthalpic factors influence the interactions between them. The mobility of both ligand and receptor, the effect of the protein environment on the charge distribution over the ligand, and their interactions with the surrounding water molecules, further complicate the quantitative description of the process [8]. The idea behind this technique is to generate a comprehensive set of conformations of the receptor complex, and then to rank them according to their stability. The most popular docking programs include DOCK, Auto Dock, Flex X, GOLD, and GLIDE, among others [4, 13, 20, and 25].

PROTEIN PREPARATION

It is now accepted that the old idea of the ‘‘key and lock’’ interaction of a ligand and its protein Receptor is not an accurate description of most biological complexes. The ligand–protein interactions resemble more a ‘‘hand and glove’’ association, where both parts are flexible and adjust to Complement each other—induced fit [40]. They can modify their shape and mould their complementarity. So as to increase favourable contacts and reduce adverse interactions, maximizing the total binding free Energy. It has been found that active-site regions of enzymes appear to present areas of both low and high conformational stability [9]. Mobile loops that close over the ligand upon binding are included within the flexible parts, while catalytic residues, for example, and are usually structurally stable. This dual character of the active-site environment appears important for optimum binding [9].

RECEPTOR CONFORMATION

The three dimensional (3-D) structures of both ligand and protein are necessary for the application of docking techniques. While the manifold of conformational structures of small molecules may be relatively easy to predict, the lowest energy conformation obtained may not correspond to that of the bound ligand. The structures of proteins present a bigger challenge [37]. Although experimental techniques involving x-ray and NMR Analysis are now in routine, inherent difficulties in the preparation of samples and data collection and interpretation mean we are still far from a complete automated and

High-throughput process. Many proteins targeted for drug design do not have an experimentally determined structure and, therefore, docking studies cannot be performed directly. In some cases, Computational techniques can be used to predict the 3-D structure of a protein provided the structure of a closely related protein homolog is known [41]. Homology modelling or sequence threading Techniques may be used to generate models of protein structure which, although not as good as experimentally determined structures, can be used as docking targets**.**

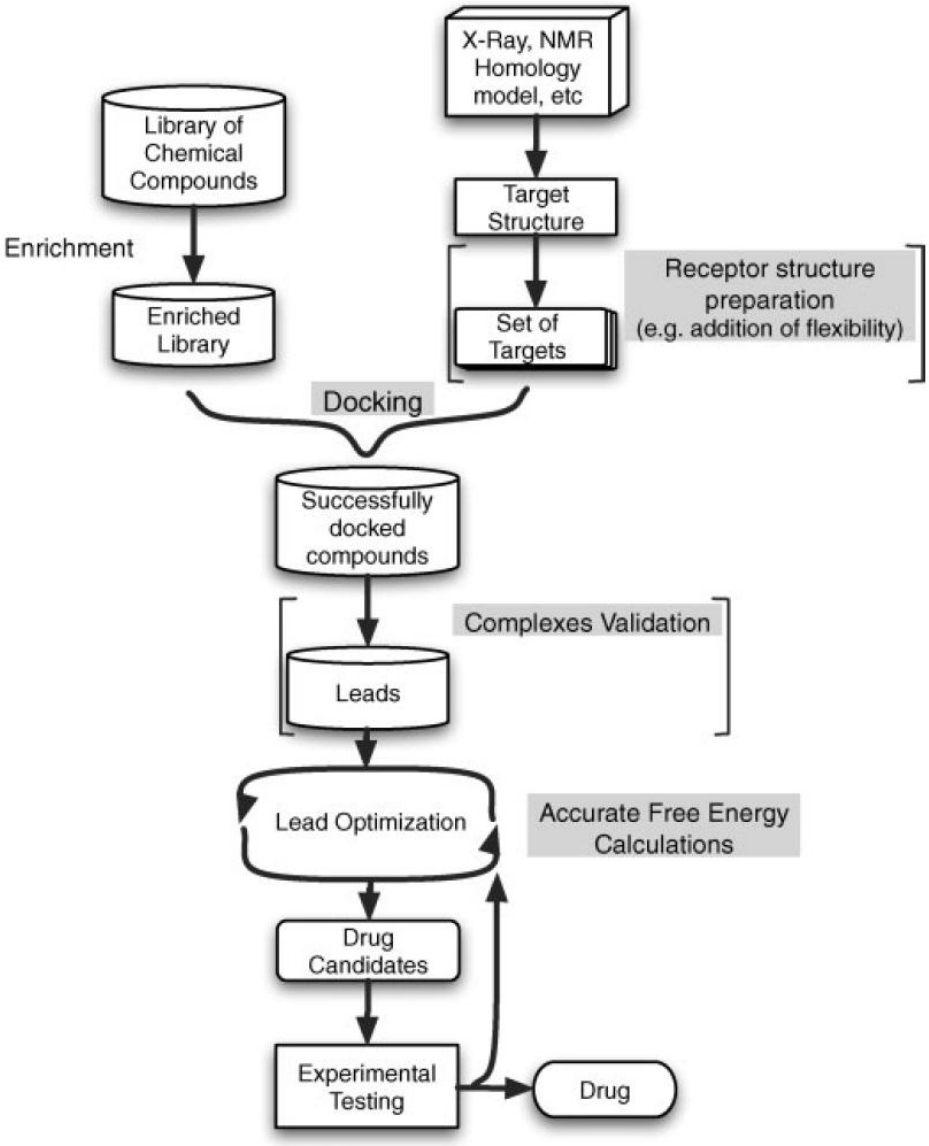


Figure-3: Schematic representation of the protocol commonly followed during a drug-design process, when the structure of the protein target is known [11].

THE FIELD OF HETEROCYCLIC CHEMISTRY

Hetero cycles have been contributed their presence in the field of research and development in organic chemistry. As a result, millions of heterocyclic compounds found to exist in natural source, semi-synthesized and synthesised having special properties with biological importance. Recently, analysis of organic compounds as of June 2007 there were 24,282,284 compounds were registered in chemical abstracts containing cyclic structures with heterocyclic systems making up of many compounds [28]. Among various heterocyclic compounds, we have chosen isatin, it possess an indole nucleus having both the keto and lactam moiety

.

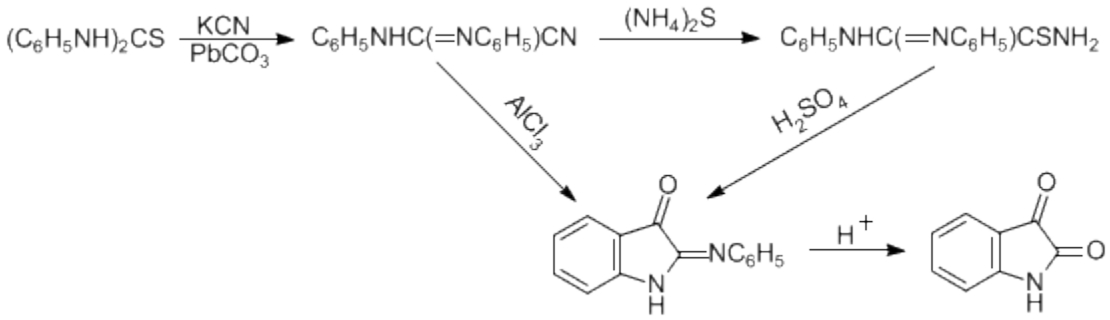
ISATIN

Isatin [1H-indole-2, 3- dione] was first obtained by Erdman and Laurent in 1841 as a product from the oxidation of indigo by nitric and chromic acids. In nature, isatin is found in plants of the genus *isatis* [39]. Substituted isatins are found in plants, for example the melosatin alkaloids (methoxy, phenyl, and pentyl isatins) obtained from the Caribbean tumorigenic plant.

Indole nucleus is found to be very active scaffold in pharmacy field as several natural alkaloids having indole in its structures are found to be therapeutically active agents. Isatin (Indole-2, 3-Dione) is an endogenous compound identified in humans that possesses indole nucleus with wide range of biological activities [40]. Isatin and its synthetic analogues are versatile substrates, which can be used for the synthesis of plentiful heterocyclic derivatives. This review mainly focused on the updated information on the most active isatin derivatives that have been reported to show significant biological actions for instance, Anti-mycobacterial, Anti-HIV, Anticonvulsant, Anticancer, Antiviral, Antimicrobial, Anti-inflammatory and Analgesic. From these outcomes, information for future molecular modifications leading to compounds with significant pharmacological properties may be derived [8, 33]. Indoline-2, 3-dione (isatin) derivatives are reported to show anti-tubercular activities**;** accordingly isatin is a versatile lead molecule for designing of potential anti-tubercular agent [32].

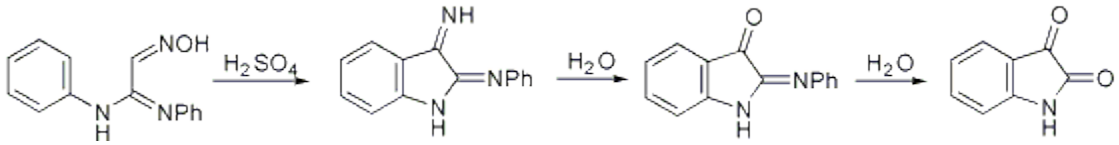
Sandmeyer Diphenylurea Isatin Synthesis [28]

Formation of a cyanoformamidine by treatment of a symmetrical diphenylthiourea with potassium cyanide in alcohol containing lead carbonate, reduction with ammonium sulfide and ring-closure with concentrated sulfuric acid to isatin-2-anil; also formed smoothly by ring closure of the cyanoformamidine with aluminum chloride in benzene or carbon disulfide:



Sandmeyer Isonitrosoacetanilide Isatin Synthesis [30]

Formation of isonitraso aceto diphenyl amidine by condensation of chloral hydrate, hydroxylamine and aniline, cyclization with concentrated sulfuric acid, and quantitative hydrolysis to isatin on dilution:



M-PHENYLENE DIAMINE

***m*-Phenylene diamine**, also called **1, 3-diaminobenzene**, is an organic compound with the formula $C_6H_4(NH_2)_2$. It is an isomer of *o*-phenylene diamine and *p*-phenylene diamine. It is a colourless solid. *m*-Phenylene diamine is produced by hydrogenation of 1, 3-dinitrobenzene [20]. The dinitrobenzene is prepared by di nitration of benzene. *m*-Phenylene diamine is used in the preparation various polymers including aramid fibres, epoxy resins, wire enamel coatings and poly urea elastomers [29]. *m*-phenylene diamine is used as an accelerator for adhesive resins, and as a component of dyes for leather and textiles [35]. Basic Brown 1 (Bismarck Brown), Basic Orange 2, Direct Black 38, and Developed Black BH In hair-dying, *m*-phenylene diamine is a "coupling agent", used to produce blue colours [32].

BIOLOGICAL SCREENING

Microbial assays or microbiological assays is a type of bioassay and are designed to analyse the compounds or substances which have effect on micro-organisms. Microbiological assay is defined as the determination or estimation of concentration or potency of an antibiotic by means of measuring and comparing the area of zone of inhibition or turbidity produced by test substance with that of standard over a suitable microbe under standard conditions [10]. So as definition says the hypothesis is that when an antibiotic is administered, there is inhibition in the growth of microbe as indicated by decrease in area of zone of microbial colony on nutrition media or decrease in turbidity due to decrease in microbial concentration.

Uses of microbial assay:

1. This helps to estimate concentration and potency of antibiotics [9]. This is not always possible by other means of estimations.
2. Help in determination of the best anti-biotic suitable for patient recovery. When microbe in patients phlegm or urine is examined by bioassay the better susceptibility of microbe to the suitable anti-biotic among those available to treat can be decided for proper treatment of infected patient. This determination is possible by immune assays like ELISA test for some diseases [28].

Alamar Blue® ASSAY FOR ESTIMATING THE MICROBIAL VIABILITY

Alamar Blue® is designed to provide a rapid and sensitive measure of cell proliferation and cytotoxicity in various human and animal cell lines, bacteria and fungi. It is simple to use as the indicator dye is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays [27].

The assay incorporates a specially selected oxidation-reduction (REDOX) indicator that both fluoresces and undergoes colorimetric change in response to cellular metabolic reduction. This offers the user a choice of detection method.

Alamar Blue® reagent contains **Resazurin** (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) a blue dye, which itself is non-fluorescent until it is reduced to the pink colour and highly red fluorescent **resorufin**. It is used mainly as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells. Usually it is available commercially as the sodium salt.

PRINCIPLE:

- Growing cells cause a chemical reduction of alamar Blue®.
- Continued growth maintains a reduced environment (fluorescent, red).
- Inhibition of growth maintains an oxidized environment (non-fluorescent, blue).
- Data may be collected using either fluorescence-based or absorbance-based instrumentation.
- Fluorescence is monitored at 530-560 nm excitation wavelength and 590nm emission wavelength.
- Absorbance is monitored at 570 nm and 600 nm.

ASSAY PROTOCOL FOR ESTIMATING ANTI-TB ACTIVITY USING alamarBlue® DYE [27]

1. The anti-mycobacterial activity of compounds were assessed against M. Tuberculosis using alamarblue® micro plate 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 de-ionized 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 Middle brook 7H9 broth and serial dilution of compounds were made directly on plate.

5. The final drug concentrations tested were 100 µg/ml to 0.2 µg/ml.

6. Plates were covered and sealed with paraffin 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 colour change from blue to pink.

SURVEY OF LITERATURE

The purpose of a literature review is to:

- establish a theoretical framework for a topic / subject area
- Define key terms, definitions and terminology
- Identify studies, models, case studies etc. supporting a topic
- Define / establish an area of study.

Literature survey was conducted to have updates about the advances in genomics related to TB, current moieties under research for TB, synthetic pathways, current understanding of the pathophysiology of TB and advances in screening methods.

The following literature was surveyed in depth to provide supporting data for drug design study

- ✓ **Jürgen Bajorath (2001)** did his work on rational drug discovery revisited: Interfacing experimental programs with bio- and chemo-informatics
- ✓ **Maria van Dongen *et al* (2002)** worked on "Structure-based screening and design in drug discovery"
- ✓ **Daniel F. Wyss (2003)** reported his work on "Structure-guided applications in drug discovery"
- ✓ **Alexander Hillisch *et al* (2004)** reported the "Utility of homology models in the drug discovery process"
- ✓ **I.M. Kapetanovic (2008)** published his work on "Computer-aided drug discovery and development (CADDD): In silico-chemico-biological approach".
- ✓ **Sanat K. Mandal *et al* (2009)** published their work on rational drug design.

The following works throws a light upon the various genomic aspects of M.tuberculosis and also various targets intended for drug action

- ✓ **Ashok Rattan *et al* (1998)** published his work on "Multidrug-Resistant *Mycobacterium tuberculosis*: Molecular Perspectives".
- ✓ **J. Brennan (2003)** reported his work on "Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis".
- ✓ **Puneet Chopra *et al* (2003)** reported new drug targets for *Mycobacterium tuberculosis*
- ✓ **James C Sacchettini *et al* (2003)** reported "Mycobacterium tuberculosis: a model system for structural genomics".
- ✓ **C. Terwilliger *et al* (2003)** published his work on "The TB structural genomics consortium: a resource for Mycobacterium tuberculosis biology".
- ✓ **R. Hernandez Pando *et al* (2006)** published their work on the use of mutant mycobacteria as new vaccines to prevent tuberculosis.
- ✓ **Vickery L.Arcus *et al* (2006)** observed the potential impact of structural genomics on tuberculosis drug discovery
- ✓ **Khisimuzi Mdluli and Melvin Spigelman (2006)** reported "Novel targets for tuberculosis drug discovery"
- ✓ **Johan Weigelt *et al* (2008)** published their work correlating "Structural genomics and drug discovery: all in the family".
- ✓ **Nagasuma Chandra *et al* (2008)** reported a target identification pipeline for *Mycobacterium tuberculosis* through an interactome, reactome and genome-scale structural analysis
- ✓ **Yee Siew Choong (2011)** reported the "Effects of Enoyl-Acyl Protein Carrier Reductase Mutations on Physiochemical Interactions with Isoniazid: Molecular Dynamics Simulation".
- ✓ **Anshu Bhardwaj *et al* (2011)** published their work on "Open source drug discovery– A new paradigm of collaborative research in tuberculosis drug development".
- ✓ **T. Cole et al (1992)** worked on "Isolation and characterization of isoniazid resistant mutants of *Mycobacterium smegmatis* and *M. aurum"*

- ✓ **Dorothy Yeboah-Manu *et al* (2012)** conducted a study on "Drug Susceptibility Pattern of Mycobacterium Tuberculosis Isolates from Ghana; Correlation with Clinical Response".

The review on following works provided basic information about the target enzyme, mycolic acid cyclopropane synthase [CmaA1 target (1L1E)] and its function

- ✓ **Chih-chin Huang et al (2002**) conducted a study on "Crystal Structures of Mycolic Acid Cyclopropane Synthases from *Mycobacterium tuberculosis".*
- ✓ **Kuni Takayama et al(2005)** reported a **"**Pathway to Synthesis and Processing of Mycolic Acids in Mycobacterium tuberculosis"

The review on following works provided ideas for synthesis of the desired chemical entities

- ✓ Razieh Sabet, Mehrdad Mohammadpour, Amir Sadeghi, Afshin Fassihi, reported "QSAR study of isatin analogues as in vitro anti-cancer agents", **European Journal of Medicinal Chemistry** 45 (2010) 1113–1118.
- ✓ **Razieh Sabet *et al.,* (2010)** reported a "QSAR study of isatin analogues as in vitro anti-cancer agents".
- ✓ **Isabel Amalia Perillo *et al.,* (2008)** reported a "Simple and Efficient Microwave Assisted *N*-Alkylation of Isatin".
- ✓ **Chaluvaraju KC *et al.,* (2011)** reported a "Synthesis and Biological Evaluation of some Isatin derivatives for Antimicrobial Properties".
- ✓ **Manjusha Verma *et al.,* (2004)** reported a Anticonvulsant activity of Schiff bases of isatin derivatives.
- ✓ **Tarek Aboul-Fadl *et al.,* (2010)** studied about the "Anti-Tubercular Activity of Isatin Derivatives".
- ✓ **Tarek Aboul-Fadl *et al.,* (2010)** studied about the "Schiff bases of indoline-2,3-dione (isatin) derivatives and nalidixic acid carbohydrazide synthesis".
- ✓ **Lin Hou *et al.,* (2008)** reported an "Anti-tumour effect of Isatin on human neuroblastoma cell line (SH-SY5Y)".

- ✓ **E.G. Mesropyan *et al.*, (2005)** reported a "Synthesis of Isatin and 5-Bromoisatin Derivatives".
- ✓ **L.-R. Chen *et al.*, (2010)** studied "Bio-organic synthesis and evaluation of isatin derivatives as effective SARS coronavirus 3CL protease inhibitors".
- ✓ **Ward C. Sumpter (1943)** gave the information about the chemistry of isatin.

The review on following works revealed the basics of Alamar blue assay for evaluating the anti-mycobacterial action

- ✓ **Page *et al* (1993)** conducted "A New Fluorometric Assay for Cytotoxicity Measurements Invitro".
- ✓ **Geier, Steven (1994)** published his work on "Analysis of alamar Blue Overlap: Contribution of Oxidized to Reduced".
- ✓ **Lancaster, M.V. and Fields, R.D. (1996)** carried out "Antibiotic and Cytotoxic Drug Susceptibility Assay's using Resazurin and Poising Agents".
- ✓ **R Hamid *et al* (2004)** carried out "Comparison of alamar blue and MTT assays for high through-put screening"
- ✓ **C. N. Paramasivan *et al* (2004)** carried out "Evaluation of micro plate Alamar blue assay for drug susceptibility testing of *Mycobacterium avium* complex isolates".

AIM AND OBJECTIVES

AIM

To design, synthesis, characterize and evaluate compounds for their potential anti-tubercular activity .The ability of compounds to inhibit cell wall synthesis by inhibiting Mycolic acid cyclopropane synthase [cmaA1 target (1L1E)] is to be evaluated.

OBJECTIVES

DESIGN

In-silico design of Mycolic acid cyclopropane synthase [cmaA1 target (1L1E)] inhibitors.

SYNTHESIS

Based upon the Docking parameters and synthetic feasibility the following compounds will be synthesized.

A. (3*Z*)-3-[(3-{[(*E*)-phenylmethylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one.

B. (3*Z*)-3-[(3-{[(*E*)-(4-methoxyphenyl)methylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one.

C. (3*Z*)-3-[(3-{[(*E*)-(2-hydroxyphenyl)methylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one.

CHARACTERIZATION:

The above synthesised compounds will be identified and characterized by using

- Melting point,
- TLC method,
- Infrared Spectroscopy,
- Nuclear Magnetic Spectroscopy,
- Mass spectroscopy.

BIOLOGICAL EVALUATION

The synthesized compounds will be screened for their anti-tubercular activity by *in-vitro* methods.

TOXICOLOGICAL PREDICTION

Toxicological prediction will be carried out for synthesized compounds by *in-silico* property explorer like OSIRIS.

MATERIALS AND METHODS

DRUG DESIGN

Rational drug design (RDD) is the inventive process of finding new medications based on the knowledge of the biological target. The drug is an organic small molecule which activates or inhibits the function of bio-molecule such as protein, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves design of small molecules that are complementary in shape and charge to bimolecular target to which they interact and therefore will bind to it. Drug design frequently but not necessarily relies in computer modelling techniques.

DOCKING STUDIES (GLIDE)

DOCKING AND SCORING METHODS:

Docking procedure aims to identify the correct binding poses within the binding site of the protein while the scoring function aims to predict binding affinity of ligand for the protein binding region. The scoring function serves three purposes.

1. For ranking the conformations generated by the docking, search for one ligand interacting with a given protein, this aspect is essential to detect the best binding site mode.
2. For ranking different ligands with respect to binding to one protein i.e. prioritizing ligands according to their affinity, this aspect is essential in virtual screening.
3. For ranking one or different ligands with respect to their binding affinity to different proteins their aspect is essential for the consideration of specificity and selectivity.

STEPS IN DOCKING: PROTEIN PREPARATION

A typical PDB file downloaded from protein data bank consists of heavy metals, can contain waters, cofactors, metal ions and can be di-meric or multi-meric. The structure generally has no information on bond orders, topologies, or formal atomic charges. The Terminal groups can also be misaligned because the X-ray structure analysis cannot easily distinguish between O and NH_2 ion. The ionization and tautomeric states are usually unassigned. Generally Glide calculations use an all atom force-field for accurate energy evaluation and thus, Glide requires bond orders and ionization states which to be properly assigned and should performs better when side chains are reoriented, when necessary and steric clashes are relieved.

The steps for the protein preparation carried out are as follows:

1. The ligand/protein co-crystallized structure of **cmaA1** target (1L1E) in the form of PDB was imported into maestro 9.1. The preparation component of a protein preparation facility requires an identified ligand which to be verified.
2. The protein-ligand complex is then identified for its form as dimer or other multimer, containing duplicate binding sites, and other duplicate chains that are redundant. Then remove the redundant binding sites and the associated chains by picking and deleting molecules or chains.
3. The waters to be identified that bridge between the ligand and protein are retained and all the other waters (except those coordinated to metals) are deleted and if waters are added then hydrogen can be automatically added and then the orientations of water molecules are checked once again.
4. The protein, metal ions and cofactors are then adjusted. The structures that are missing residues near the active sites should be repaired. The covalent bonds from the metal ions to the protein should be changed to zero-order bonds. The formal charges on the metal and the ligating groups should be adjusted to their appropriate values.
5. The ligand bond orders and the formal charges are adjusted. In Glide models such interactions are associated with vanderwaals and electrostatic interactions.
6. The restrained minimization of protein structure reorients the side chain containing hydroxyl groups and alleviates the potential steric clashes. The minimization is restrained to the input protein coordinates by a user-selected RMSD tolerance.

LIGAND PREPARATION

The ligand preparation is designed to prepare a high quality 3 D structures for a large numbers of drugs like molecules. The structures which to be docked must have actual ligand structures and should meet the following conditions:

1. The structures must be in three dimensional.
2. The structures must have realistic bond lengths and bond angles.
3. Each structure must consist of a single molecule that has no covalent bonds to the receptor, with not accompanying fragments such as counter ions and solvent molecules.
4. All structures must have their hydrogens.
5. The structures must have an appropriate protonation state for the physiological pH values (around 7).

The Lig-Prep process consists of a series of steps which perform conversions, eliminate unwanted structures, apply corrections to the structures, and optimize the structures. The simple use of Lig-Prep produces a single low-energy 3D structure with correct chiralities for every successfully processed input structure. The Lig-Prep can also produce a large number of structures from each input structure with various ionization states, stereo chemistries, and ring conformations, tautomers, and eliminate molecules using various criteria including molecular weight, specified numbers and types of functional groups which are present.

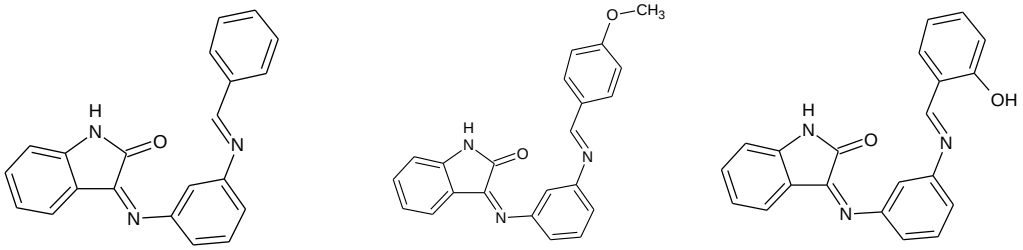
RECEPTOR GRID GENERATION

The grid in which the shape and properties of the receptor are represented by a several different sets of fields which provide progressively more accurate scoring of the ligand poses. Grids must be prepared for each conformation to ensure that possible actives are not missed for receptors that adopt more than one conformation on binding. Receptor grid defines the receptor structure by excluding any co-crystallised ligand which might be present, determine the position and the size of active site as it will be represented by receptor grids, set up the glide constraints, and set up the flexible hydroxyl groups. The receptor grid generation requires a prepared structure where an all-atom structure with appropriate bond orders and formal charges.

LIGAND DOCKING

Glide ligand docking job requires a set of previously calculated receptor grids and one or more ligand structures. The ligand structures must satisfy the criteria listed above in the ligand preparation. The detailed information on setting up grid generation job is given above. The preparation of ligands before docking is strongly recommended. The Lig-Prep or Macro model in Maestro can be used to prepare ligands. If a correct Lewis structure cannot be generated for a ligand, it is skipped by the docking job.

COMPOUND KB01 **COMPOUND KB05** **COMPOUND KB06**



G Score: -7.16 G Score: -6.11 G Score: -8.24

INTERACTIONS

Fig. 04-Interaction between KB01 with 1L1E

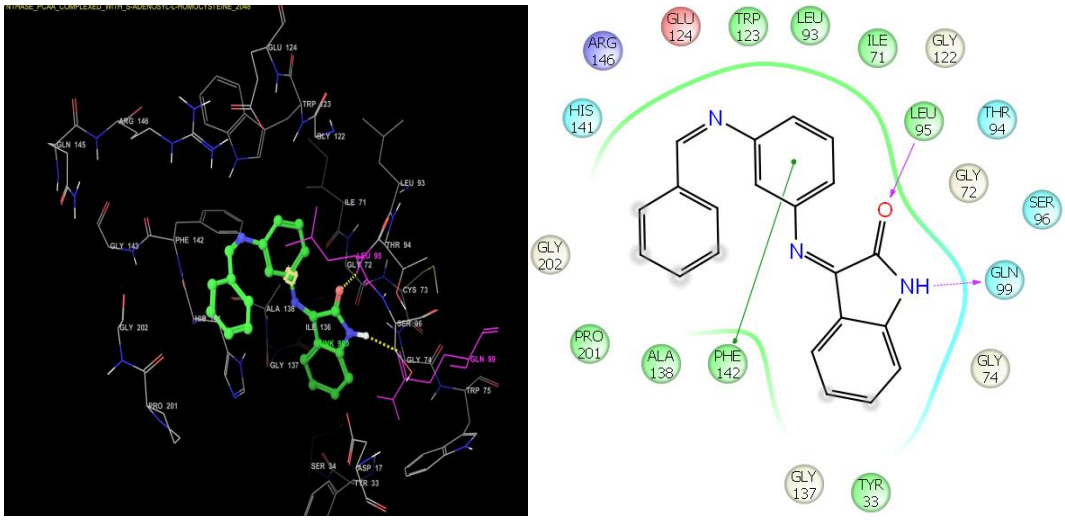


Fig. 05-Interaction between KB05 with 1L1E

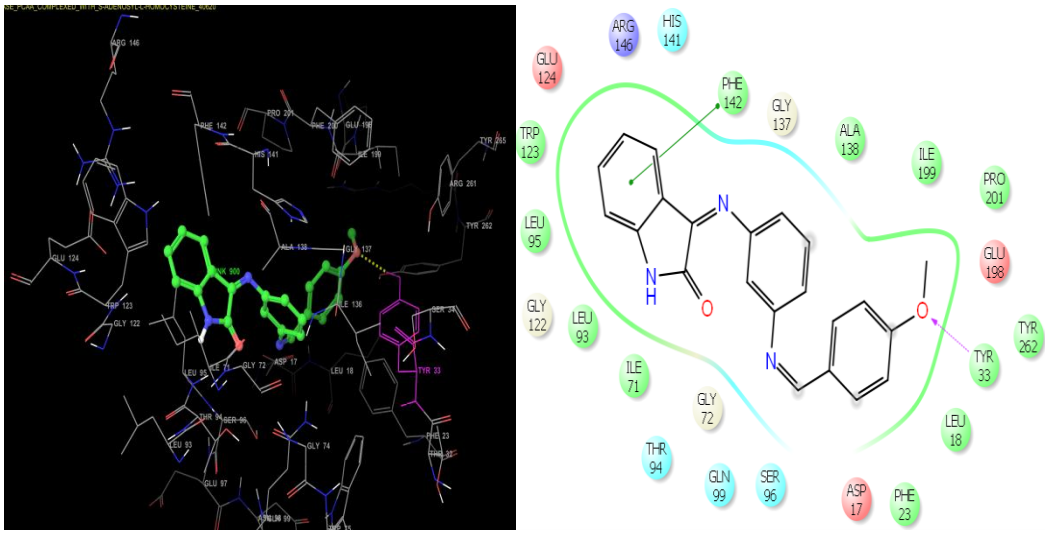


Fig. 06-Interaction between KB06 with 1L1E

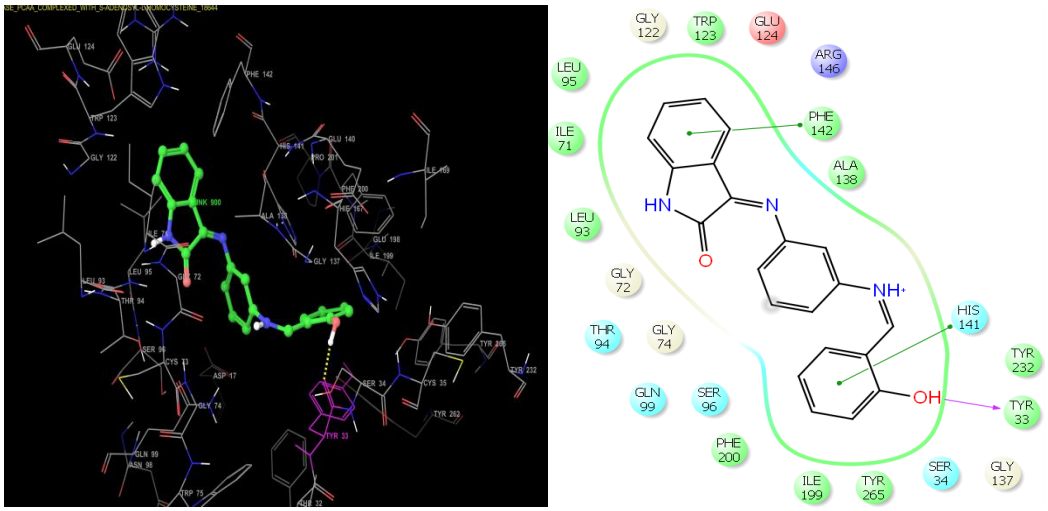


Table -01: Rewards

ligand	G-Score	DockScore	LipophilicEvdW	PhobEn	PhobEnHB	PhobEnPairHB	HBond	Electro	Sitemap	PiCat	ClBr	LowMW
KB01	-7.16	-7.16	-4.61	-0.17	0	0	-1.33	-0.45	-0.41	0	0	-0.42
KB05	-6.11	-6.11	-4.36	-0.3	0	0	-0.42	-0.25	-0.67	0	0	-0.32
KB06	-8.24	-5.52	-5.08	-0.85	0	0	-0.7	-0.58	-0.87	0	0	-0.36

Table-02: Penalties

ligand	Penalties	HBPenal	ExposPenal	RotPenal	EpikStatePenalty	Similarity	Activity
KB01	0	0	0	0.23	0	1	-7.16
KB05	0	0	0	0.19	0	1	-6.11
KB06	0	0	0	0.21	2.72	1	-8.24

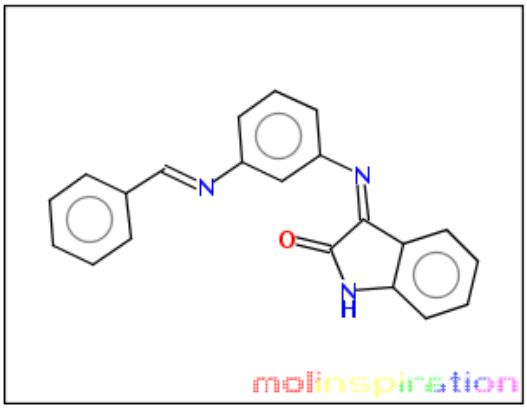
LIPINSKI'S RULE

Lipinski's rule of five is a rule of thumb to evaluate drug likeness, or to determine if a chemical compound with a certain pharmacological or biological activity has the properties that would make it a likely orally active drug in humans. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules. The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion (ADME). However, the rule does not predict if a compound is pharmacologically active. The rule is important for the drug development where a pharmacologically active lead structure is optimized step-wise for increased activity and selectivity, as well as drug-like properties as described by Lipinski's rule. The modification of the molecular structure often leads to drugs with higher molecular weight, more rings, more rotatable bonds, and a higher lipophilicity.

Lipinski's rule says that, an orally active drug has no more than one violation of the following criteria:

- Not more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms).
- Not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms)
- A molecular weight under 500 daltons
- A partition coefficient log *P* less than 5.

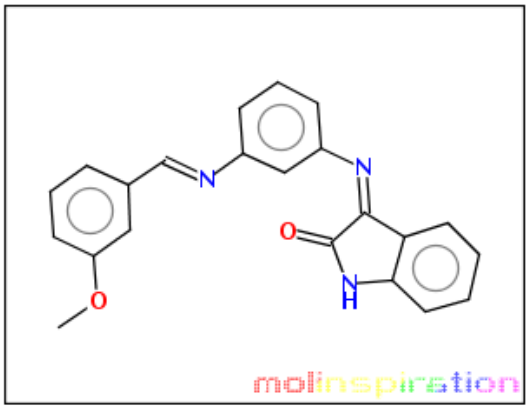
COMPOUND KB01



Molinspiration property engine

miLogP	4.747
TPSA	57.59
natoms	25.0
MW	325.371
nON	4
nOHNH	1
nviolations	0
nrotb	3
volume	293.926

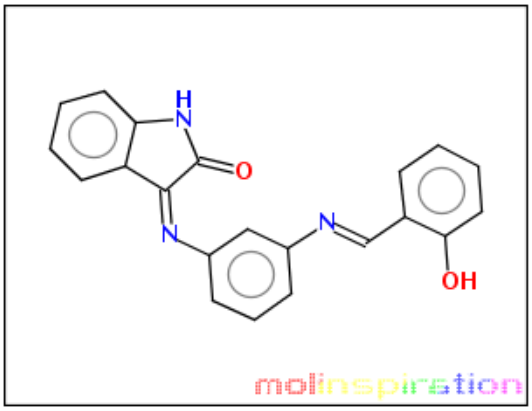
COMPOUND KB05



Molinspiration property engine

miLogP	4.78
TPSA	66.824
natoms	27.0
MW	355.397
nON	5
nOHNH	1
nviolations	0
nrotb	4
volume	319.471

COMPOUND KB06



Molinspiration property engine

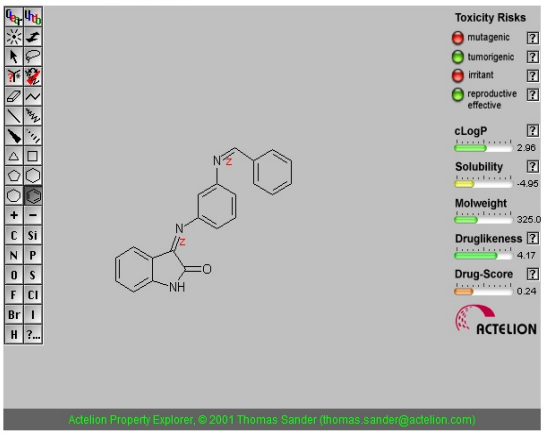
miLogP	4.688
TPSA	77.818
natoms	26.0
MW	341.37
nON	5
nOHNH	2
nviolations	0
nrotb	3
volume	301.943

OSIRIS PROPERTY EXPLORER

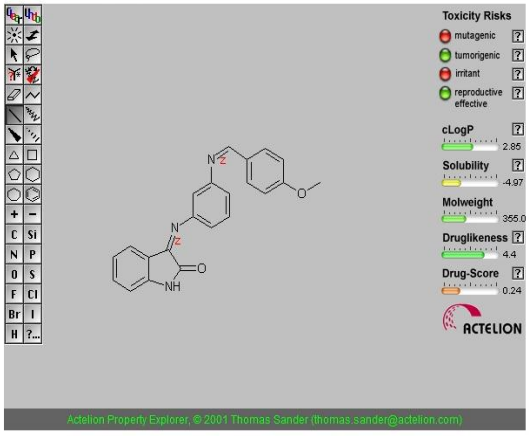
OSIRIS property explorer is used in prediction of toxicity of compounds. Properties with high risks of undesired effects like mutagenicity or a poor intestinal absorption are shown in red. Whereas a green color indicates drug-conform behavior. It provides the information like

- Toxicity risk assessment
- cLogP Prediction
- Solubility Prediction
- Molecular Weights
- Drug-Likeness Prediction
- Overall Drug-Likeness Score

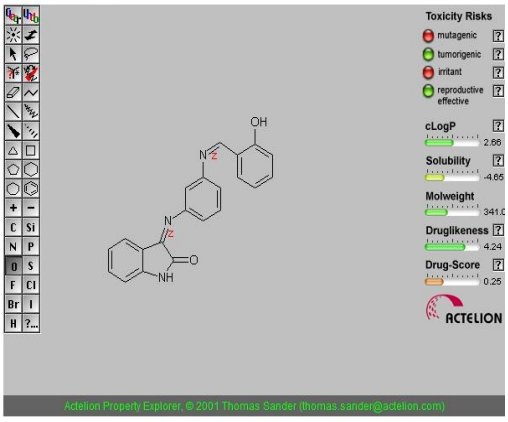
KB01



KB05



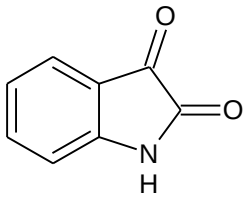
KB06



SYNTHESIS AND CHARACTERISATION

Reactant Profile

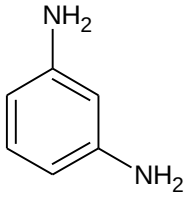
Structure of Isatin



Synonym : 1*H*-indole-2,3-dione
Molecular Formula : $C_8H_5NO_2$
Formula Weight :147.1308
Melting point : 201.5 C
Solubility : Methanol, Ethanol

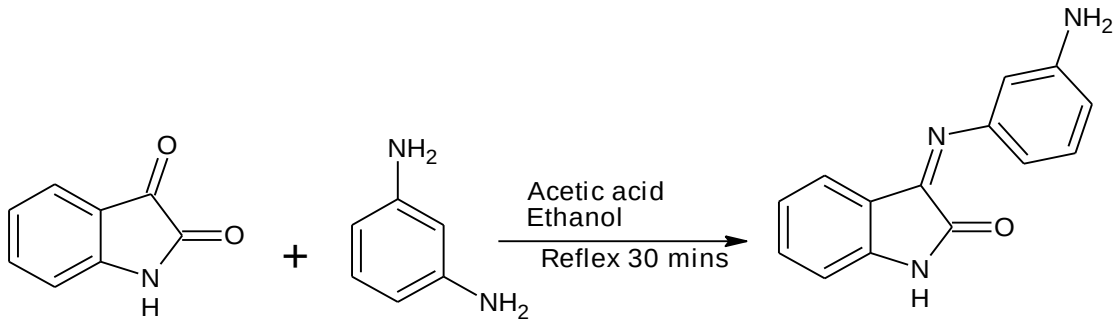
META PHENYLENE DIAMINE

Structure



Synonym : 1H-indole-2, 3-dione
Molecular Formula : $C_8H_5NO_2$
Formula Weight :147.1308
Melting point : 65˚C
Solubility : Methanol, Ethanol

SYNTHETIC SCHEME OF STEP-1

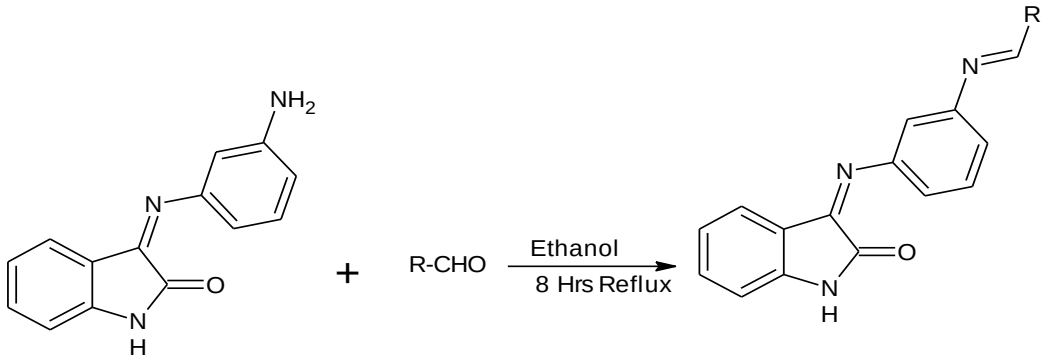


Isatin **m-Phenylene Diamine** **Intermediate**

SYNTHETIC PROCEDURE FOR STEP-1

Equimolar (0.03398 M) quantity of Isatin(5 g) and the aromatic amine i.e. m-phenylene diamine (3.67 g) were taken in a round bottom flask and dissolved in 10-15 ml of warm ethanol and added 3-4 drops of dilute acetic acid. It was heated on a steam bath under reflux for 30-40 mins. Then the setup was kept aside for 24 hrs. at room temperature. The crystalline mass (intermediate) obtained were separated by filtration, vacuum dried and recrystallized from ethanol.

SYNTHETIC SCHEME OF STEP-2

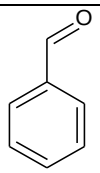
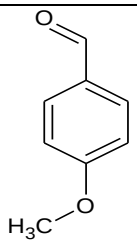
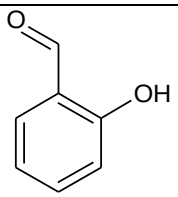


Intermediate from step-1 | **Aromatic Aldehyde (01, 05&06)** | **Product (KB01, 05&06)**

SYNTHETIC PROCEDURE FOR STEP-2

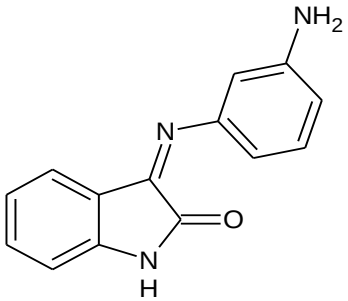
Equimolar (0.002M) quantity of intermediate from step-1 and was treated with various substituted aromatic aldehyde (1-6) in presence of sodium methoxide. Then the reaction mixture was refluxed for 8 hrs. And it was neutralised with dil.Hcl. The resulting precipitates were filtered off and recrystallized from ethanol.

TABLE-03: SHOWING R-SUBSTITUTION

COMPOUND NAME	R - denotes
KB01	 BENZALDEHYDE
KB05	 ANISALDEHYDE
KB06	 SALICYLALDEHYDE

INTERMEDIATE

Structure



Synonym : (3*Z*)-3-[(3-aminophenyl) imino]-1, 3-dihydro-2*H*-indol-2-one

Molecular Formula : $C_{14}H_{11}N_3O$

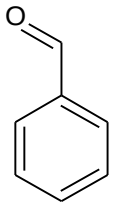
Formula Weight : 237.25664

Melting point : 264˚C

Solubility : Methanol, Ethanol

BENZALDEHYDE

Structure

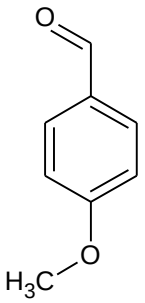


Molecular Formula **:** C_7H_6O

Formula Weight **:** 106.12194

ANISALDEHYDE

Structure



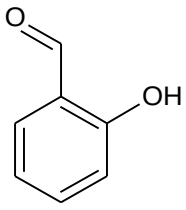
Synonym **:** 4-Methoxybenzaldehyde

Molecular Formula **:** $C_8H_8O_2$

Formula Weight **:** 136.14792

SALICYLALDEHYDE

Structure



Synonym **:** 2-Hydroxybenzaldehyde

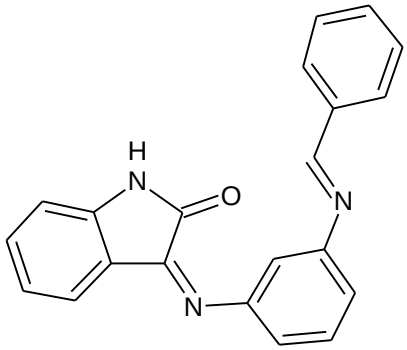
Molecular Formula **:** $C_7H_6O_2$

Formula Weight **:** 122.12134

PRODUCT PROFILE

PHYSICAL PROPERTIES OF COMPOUND KB01

STRUCTURE



IUPAC NAME

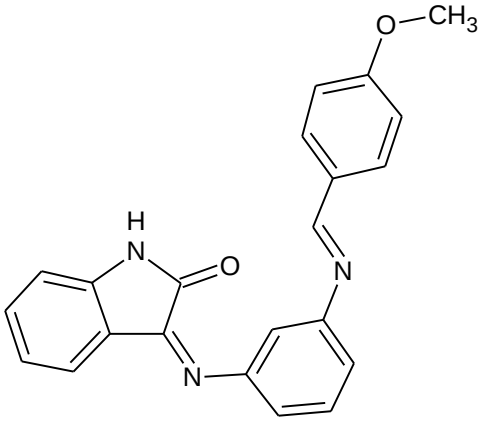
(3*Z*)-3-[(3-{[(*E*)-phenylmethylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one

PROPERTIES

Description	= Brownish red coloured solid mass
Solubility	= Soluble in Methanol, Ethyl acetate, DMSO
Melting point	= 312°C
Molecular Formula	= **$C_{21}H_{15}N_3O$**
Formula Weight	= 325.3633
Composition	= C (77.52%) H (4.65%) N (12.91%) O (4.92%)
Molar Refractivity	= 99.14 ± 0.5 cm^3
Molar Volume	= 267.9 ± 7.0 cm^3
Parachor	= 710.9 ± 8.0 cm^3
Index of Refraction	= 1.661 ± 0.05
Surface Tension	= 49.5 ± 7.0 dyne/cm
Density	= 1.21 ± 0.1 g/cm^3
Polarizability	= 39.30 ± 0.5 $10^{-24}cm^3$

PHYSICAL PROPERTIES OF COMPOUND KB05

STRUCTURE



IUPAC NAME

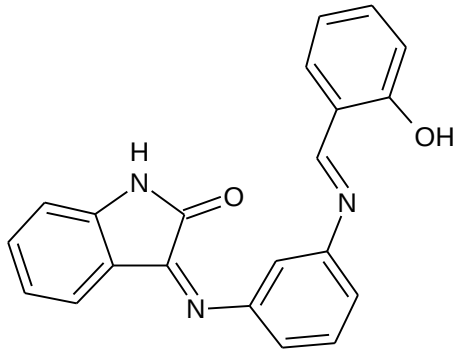
(3*Z*)-3-[(3-{[(*E*)-(4-methoxyphenyl)methylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one

PROPERTIES

Description	= Light brown to black colour solid
Solubility	= Soluble in Methanol, Ethanol, Ethyl acetate, DMSO
Melting point	= 320°C
Molecular Formula	= $\mathbf{C_{22}H_{17}N_3O_2}$
Formula Weight	= 355.38928
Composition	= C (74.35%) H (4.82%) N (11.82%) O (9.00%)
Molar Refractivity	= 104.95 ± 0.5 cm^3
Molar Volume	= 289.5 ± 7.0 cm^3
Parachor	= 761.1 ± 8.0 cm^3
Index of Refraction	= 1.644 ± 0.05
Surface Tension	= 47.7 ± 7.0 dyne/cm
Density	= 1.22 ± 0.1 g/cm^3
Polarizability	= 41.60 ± 0.5 $10^{-24}cm^3$

PHYSICAL PROPERTIES OF KB06

STRUCTURE



IUPAC NAME

(3*Z*)-3-[(3-{[(*E*)-(2-hydroxyphenyl)methylidene]amino}phenyl)imino]-1,3-dihydro-2*H*-indol-2-one

PROPERTIES

Description	= Brownish yellow coloured crystals
Solubility	= Soluble in Methanol, Ethyl acetate, DMSO
Melting point	= 290°C
Molecular Formula	= $\mathbf{C_{21}H_{15}N_3O_2}$
Formula Weight	= 341.3627
Composition	= C (73.89%) H (4.43%) N (12.31%) O (9.37%)
Molar Refractivity	= 99.99 ± 0.5 cm^3
Molar Volume	= 265.1 ± 7.0 cm^3
Parachor	= 716.5 ± 8.0 cm^3
Index of Refraction	= 1.678 ± 0.05
Surface Tension	= 53.3 ± 7.0 dyne/cm
Density	= 1.28 ± 0.1 g/cm^3
Polarizability	= 39.64 ± 0.5 $10^{-24}cm^3$

METHODS OF IDENTIFICATION

The synthesised compounds were identified by using following methods.

Melting point:

The melting points of the compounds were determined by the capillary tube method. The synthesised compounds were started losing its crystallinity at a particular temperature were found and are present without any correction.

Thin layer chromatography:

Pre-coated TLC plates with silicagel GF $_{250}$ were used. Samples of reactants and products were prepared with its suitable solvent. Among the various mobile phases n-Hexane: Ethyl acetate (7:3) were selected based on the trial.

Stationary phase: Pre-coated Silica gel GF_{250}

Mobile phase: n-Hexane: Ethyl acetate (7:3)

Detection: UV Chamber

Location of spots : Spots were visualised by exposing the plates in UV chamber.

CHARACTERISATION

The characterization was carried out using sophisticated methods like Infra-red spectroscopy, Nuclear magnetic resonance spectroscopy and Mass spectroscopy.

INFRA RED ABSORPTION SPECTROSCOPY

Infra-red absorption spectroscopy (region 2.5µ - 15µ) is a powerful tool for identifying pure organic and inorganic compounds because, with the exception of a few homo nuclear molecules such as O_2, N_2, Cl_2 all the molecular species absorb infrared radiation. With the exception of chiral molecules in the crystalline state, each molecular species has a unique infrared absorption spectrum.

NMR SPECTRA

Nuclear magnetic resonance involves the interaction between oscillating magnetic field of electromagnetic radiation and the magnetic energy of the hydrogen nucleus or some other type of nuclei when these are placed in an external static magnetic field.

NMR spectroscopy in which radiofrequency waves induces transitions between magnetic energy levels of nuclei of a molecule. NMR enables us to study the number of equivalent protons and their electronic environment. It reveals the different chemical environment in which the proton is present and helps us to ascertain the structure of molecules.

The number of signals in an NMR spectrum denotes the number of the set of equivalent protons in a molecule. The position of the signals in the spectrum helps us to know the nature of protons such as aromatic, aliphatic, acetylenic, vinyl, adjacent to some electron attracting or electron releasing group etc. The splitting of the signal is due to the different environment of the absorbing proton with respect to the adjacent protons and not with respect to electrons.

MASS SPECTROSCOPY

Mass spectroscopy is an analytical technique used to establish the molecular structure and the molecular weight of the analyte under investigation. In this technique, the compound under investigation is bombarded with a beam of electrons producing ionic fragments of the original species. The relative abundance of the fragment ion formed depends on the stability of the ion and of the lost radical. The resulting charged particles are then separated according to their masses. Mass spectrum is a record of information regarding various masses produced and their relative abundances.

BIOLOGICAL SCREENING FOR ANTI-TB ACTIVITY

This screening is for the compounds that have the potential to be developed in to new drugs against tuberculosis because the compounds inhibit the enzymes required for the formation of cell wall of the tuberculosis bacterium. New drugs are needed in current date, because the rates of cure with present drugs are very slow, and prevalence of mycobacterium tuberculosis resistance to the present drugs is increasing.

ASSAY PROCEDURE FOR ESTIMATING ANTI-TB ACTIVITY USING Alamar Blue® DYE

1. The anti-mycobacterial activity of compounds was assessed against M. *Tuberculosis* using Alamar Blue® micro plate 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 de-ionized 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 Middle brook 7H9 broth and serial dilution of compounds were made directly on plate.

5. The final drug concentrations tested were 100 to 0.2 µg/ml.

6. Plates were covered and sealed with paraffin 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 colour change from blue to pink.

RESULTS AND DISCUSSION

Glide Docking

A promising heterocyclic nucleus isatin (indole2, 3-dione) with proven biological activities was used to prepare a data base of compounds which was then docked against mycolic acid cyclopropane synthase [cmaA1, a target (1L1E)] protein for anti-tubercular activity. Glide software (Maestro 9.1) was used for this purpose

Extra precision (XP) scoring function was utilized to rank the order of compounds. The different derivatives of isatin were docked to the specific target site. The compounds with best G-score were filtered.

Compounds which had the top G-score had the basic nucleus of isatin linked with m-phenylene diamine and they were further attached with different substituted aromatic aldehydes. The top scored compounds were selected and synthesized.

LIPINSKI'S RULE

All the selected compounds of the data base pass the Lipinski's rule and there is no violation in the basic properties. It proves that these molecules have ability to reach the target site for the action. So we concluded that the molecules have positive nature on ADME character.

TOXICITY PREDICTION BY OSIRIS

OSIRIS property explorer was used for prediction of toxicity of compounds. Properties of the synthesized compounds such as high risks of undesired effects like mutagenicity, poor intestinal absorption and drug-like behavior can be predicted by this method. These properties are shown different color indication. It provides information like,

- Toxicity risk assessment
- cLogP Prediction
- Solubility Prediction
- Molecular Weights
- Drug-Likeness Prediction
- Overall Drug-Likeness Score

SYNTHESIS AND CHARACTERIZATION

SYNTHESIS

The compounds KB 01, KB05 & KB 06 were prepared from isatin by 2 steps. In the first step an intermediate compound was synthesized by condensing the isatin and m-phenylene diamine in the presence of few drops of acetic acid. In the 2nd step, the intermediate was further treated with different substituted aromatic aldehydes to give different final (KB 01, KB05&KB 06) products.

CHARACTERIZATION

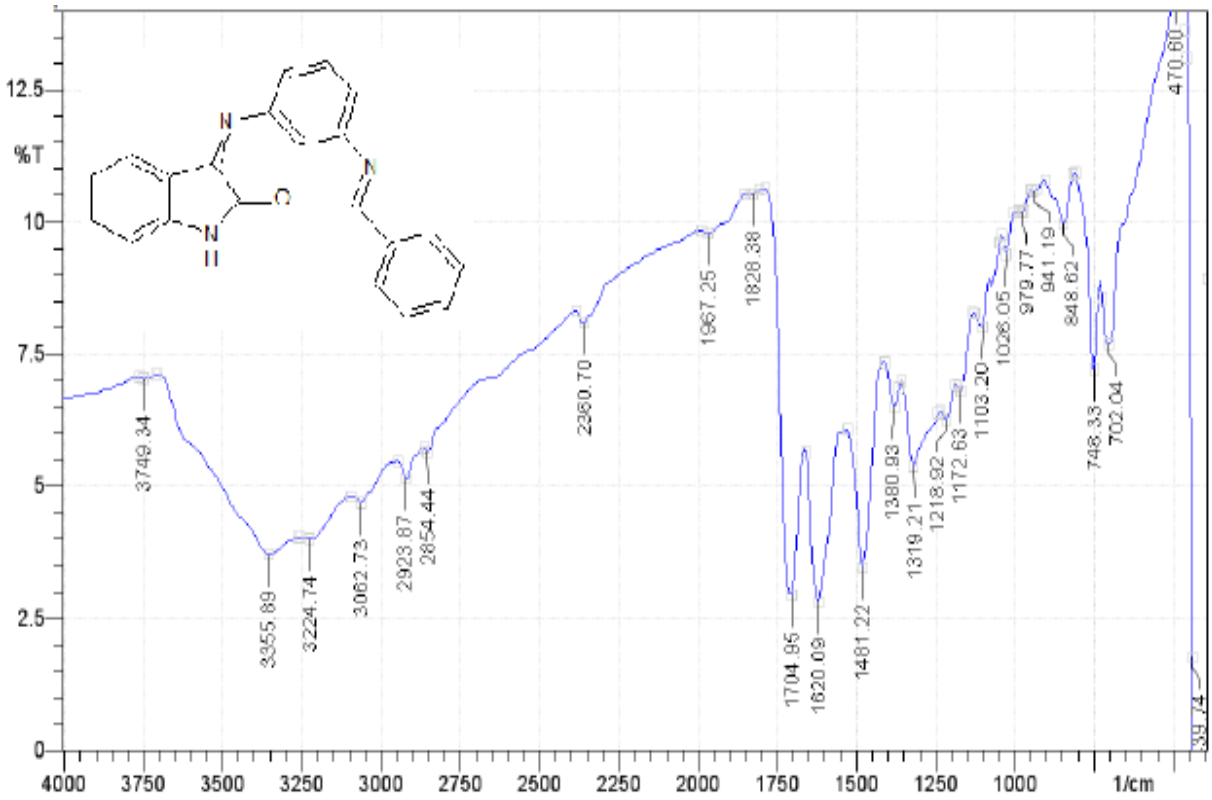
The synthesized compounds were recrystallized and identified as pure by TLC method. The melting points of the compounds were checked and are presented uncorrected. Further characterization was carried out by IR, NMR and Mass Spectroscopy.

IR SPECTROSOPY

An IR spectrum of all the synthesized compounds was taken and was used for identification of the functional groups.

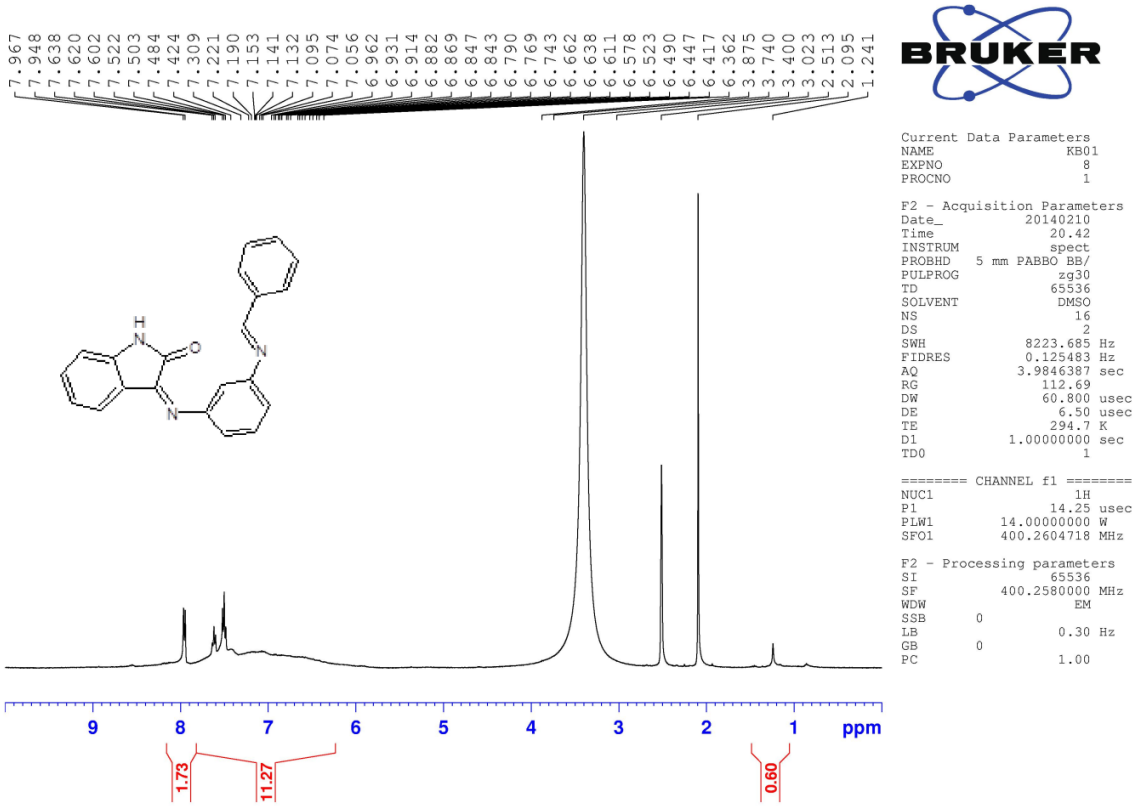
The presence of starting material as impurity was ruled out by examining for the absence of characteristic stretching for the starting materials.

IR SPECTRUM OF KB01



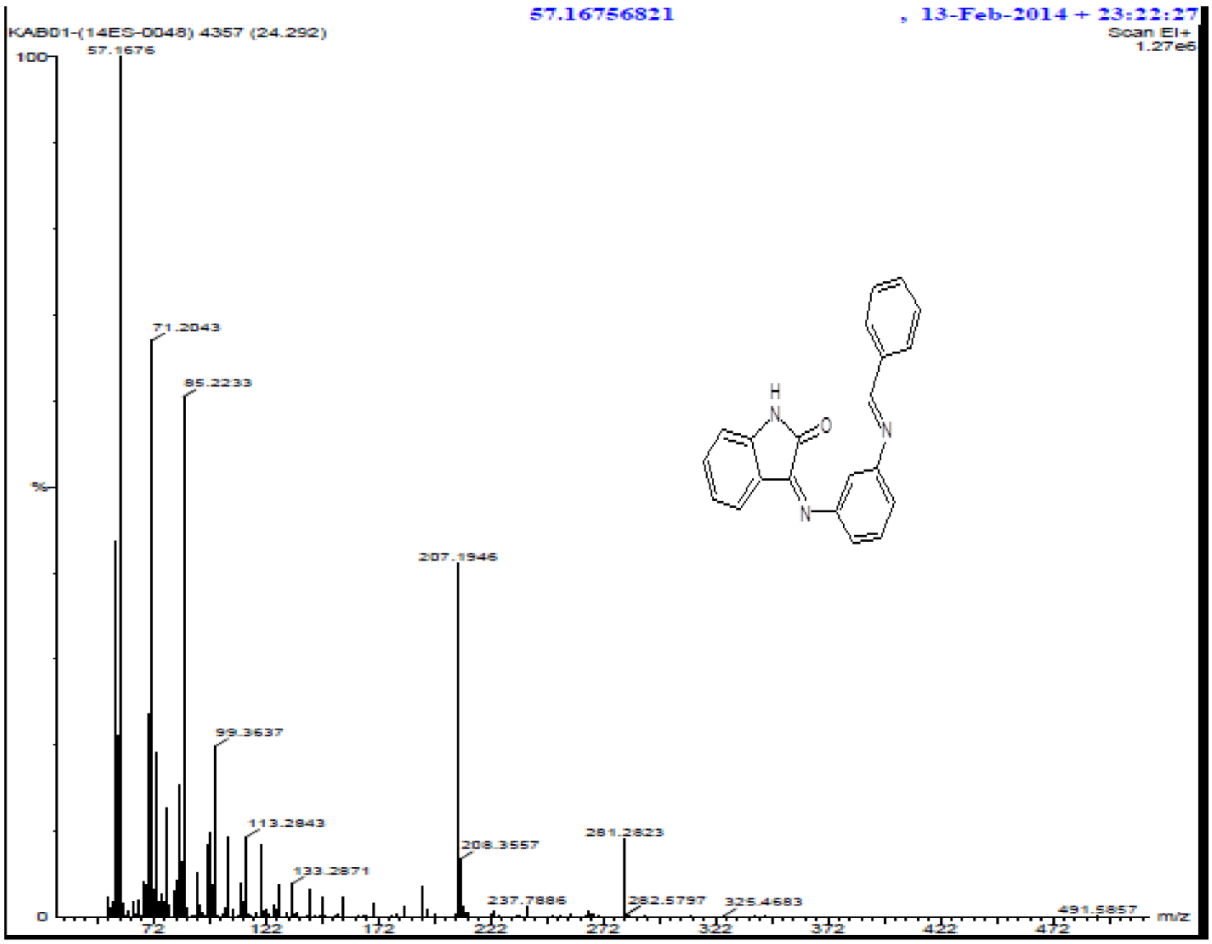
COMPOUND NAME	IR REGION
KB01	3062.73 (Ar-CH), 1481.22 (Ar C=C), 1704.85 (C=O), 3355.89 (N-H), 1103.20 (C-N), 1620.09 (C=N), 2929.87 (Al C-H)

NMR SPECTRUM OF KB01



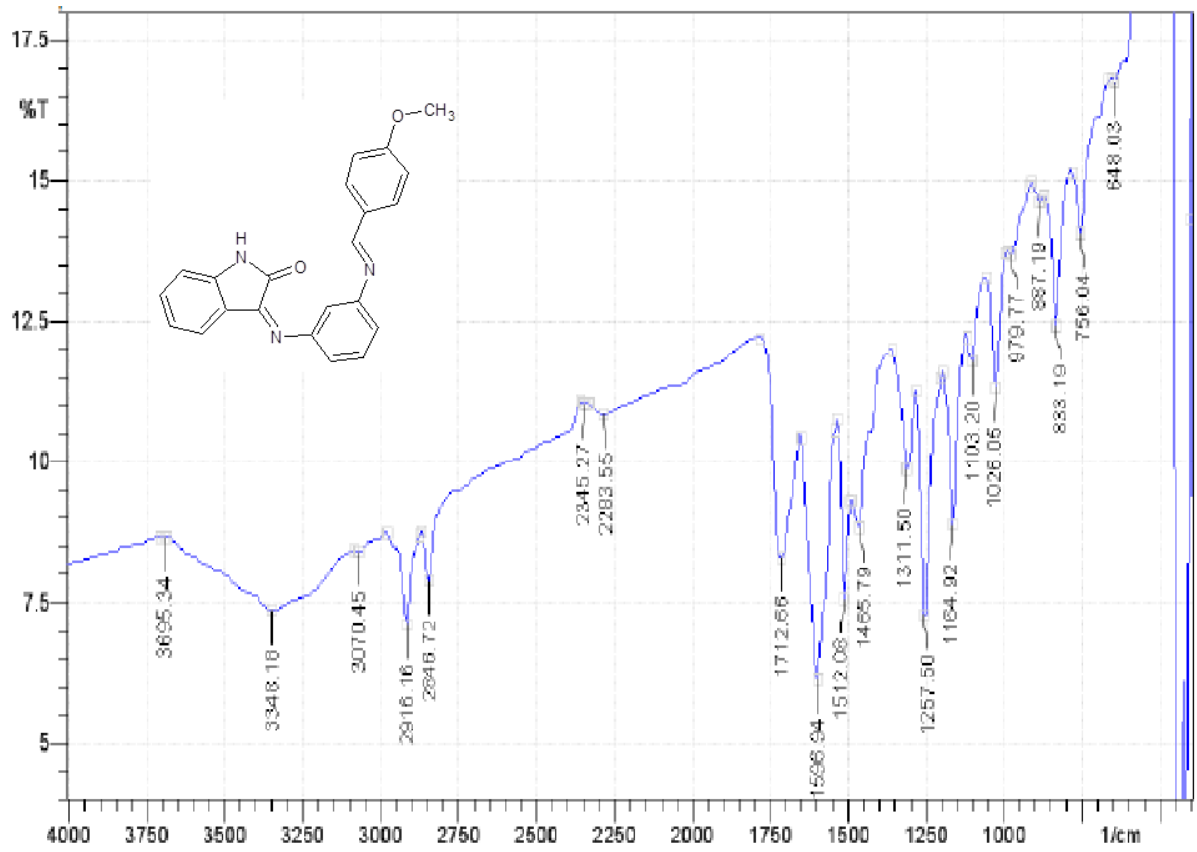
COMPOUND NAME	H^1 NMR
KB01	6.2-7.8δ (11H, m, Ar C-H) 7.8-8.2 δ (2H, s, Ar C-H) 1.4δ (1H, s, Al C-H)

MASS SPECTRUM OF KB01



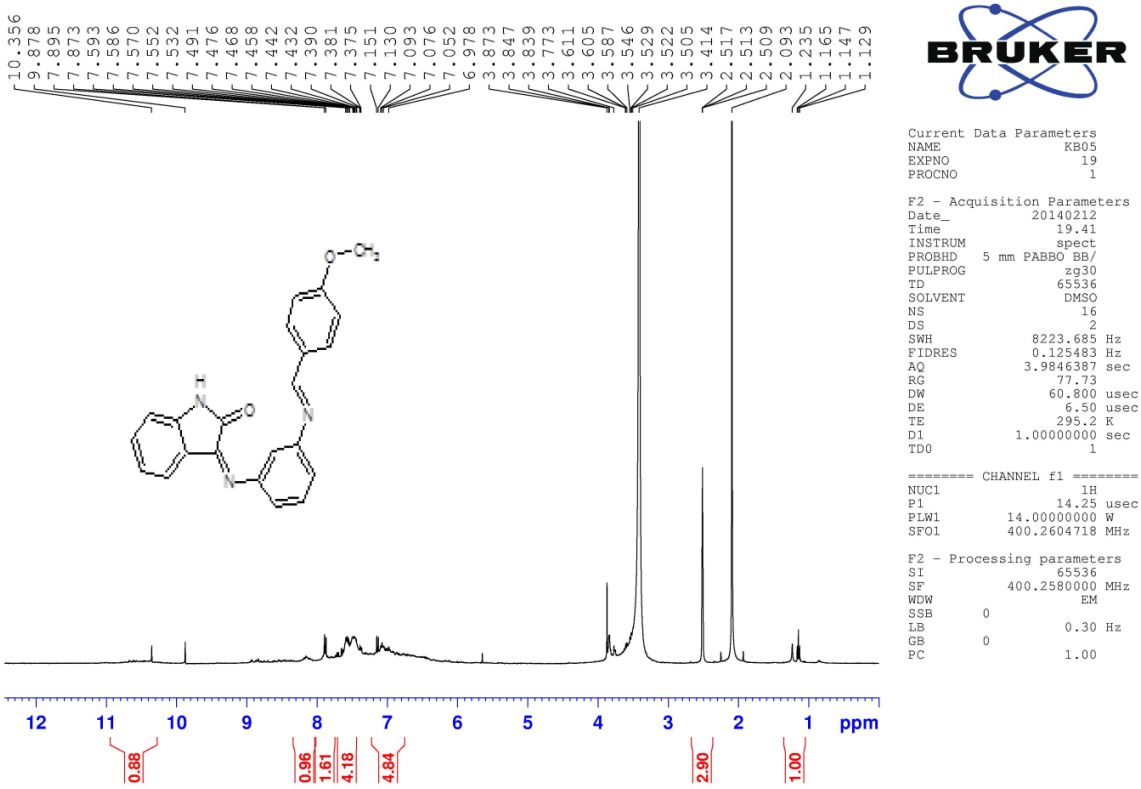
COMPOUND NAME	m/e VALUE
KB01	325.46 (M^+) 57.16 (B)

IR SPECTRUM OF KB05



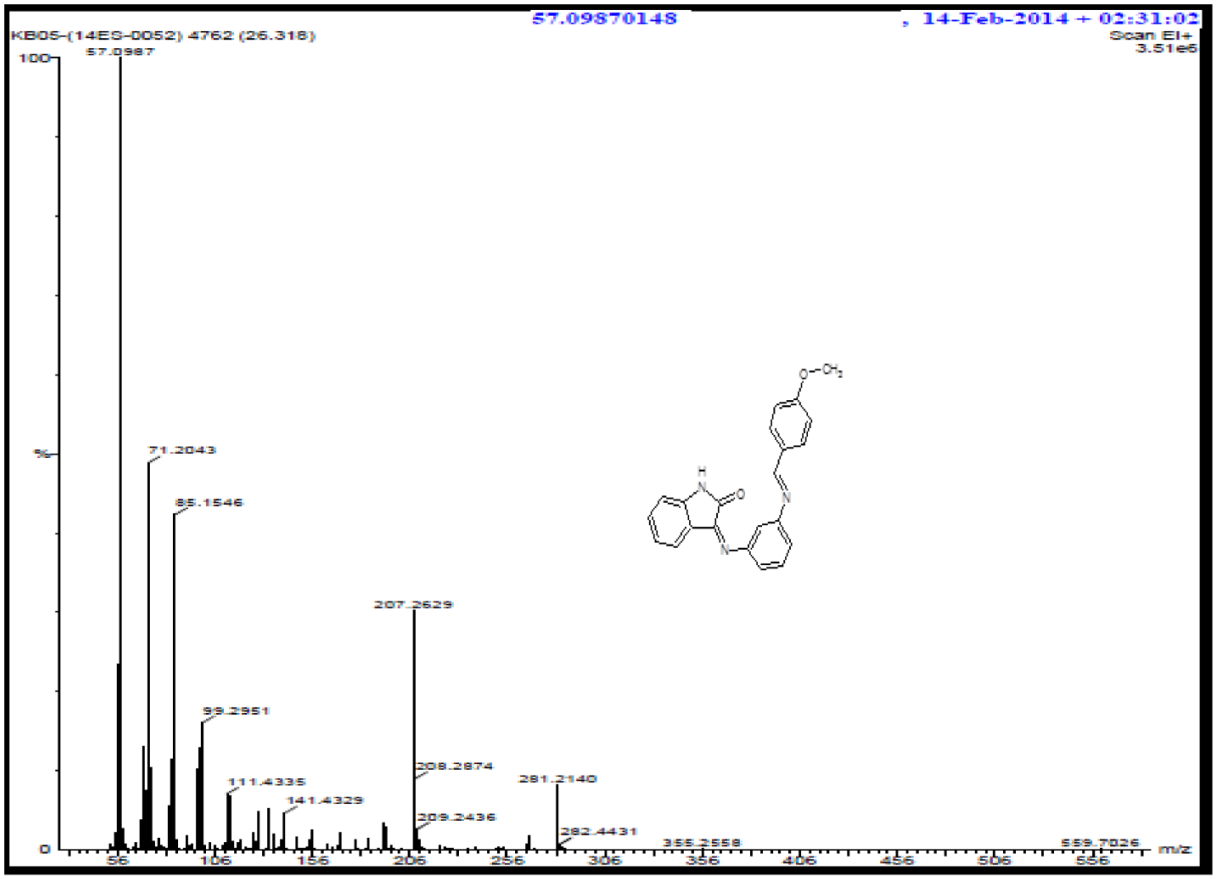
COMPOUND NAME	IR REGION
KB05	3070.45 (Ar-CH), 1465.79 (Ar C=C), 1712.66 (C=O), 1103.20 (C-N), 3348.16 (N-H), 1596.94 (C=N), 2916.16 (Al-CH), 1257.50 (C-O-C).

NMR SPECTRUM OF KB05



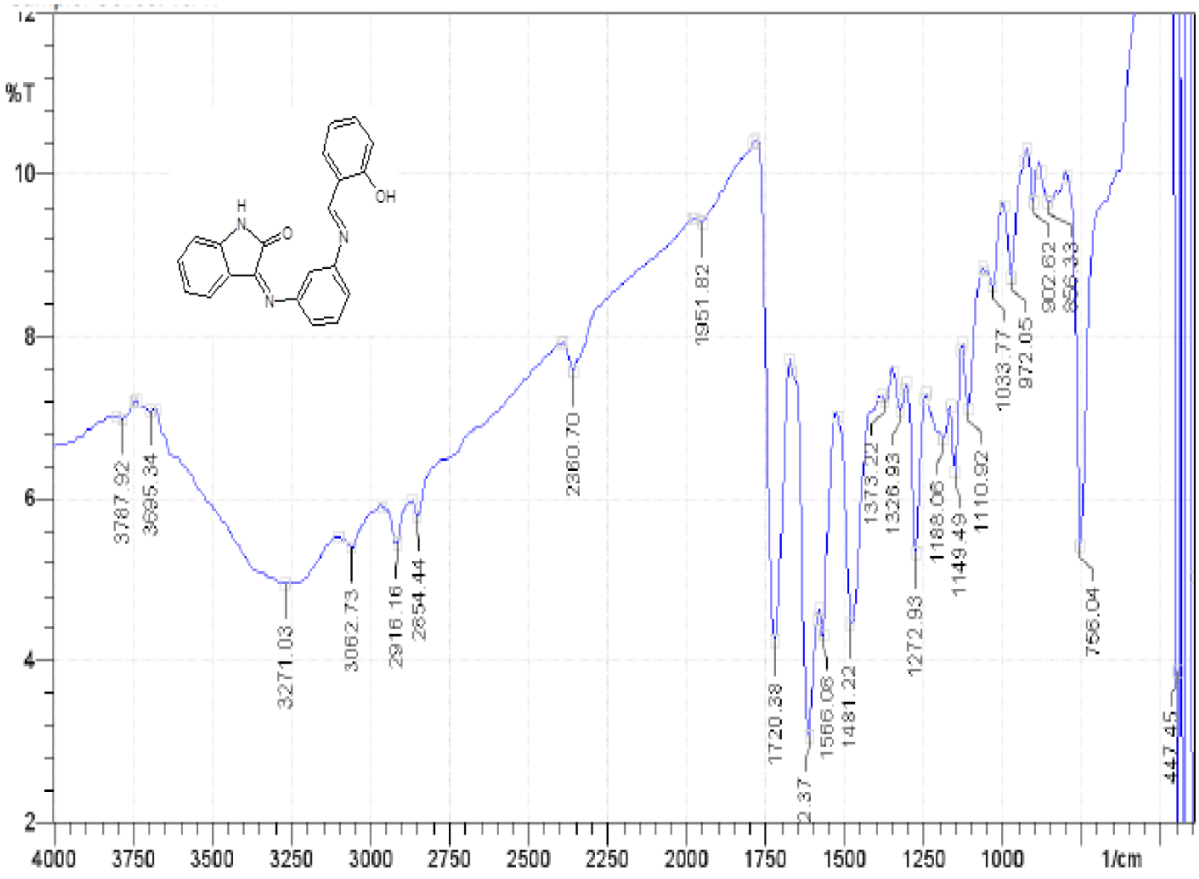
COMPOUND NAME	H^1 NMR
KB05	1.1-1.4δ (1H, s Al C-H) 2.5-2.8δ (3H, s, O-CH_3) 6.8-8.5δ (12H, m, Ar C-H) 10.2-11δ (1H, s, N-H)

MASS SPECTRUM OF KB05



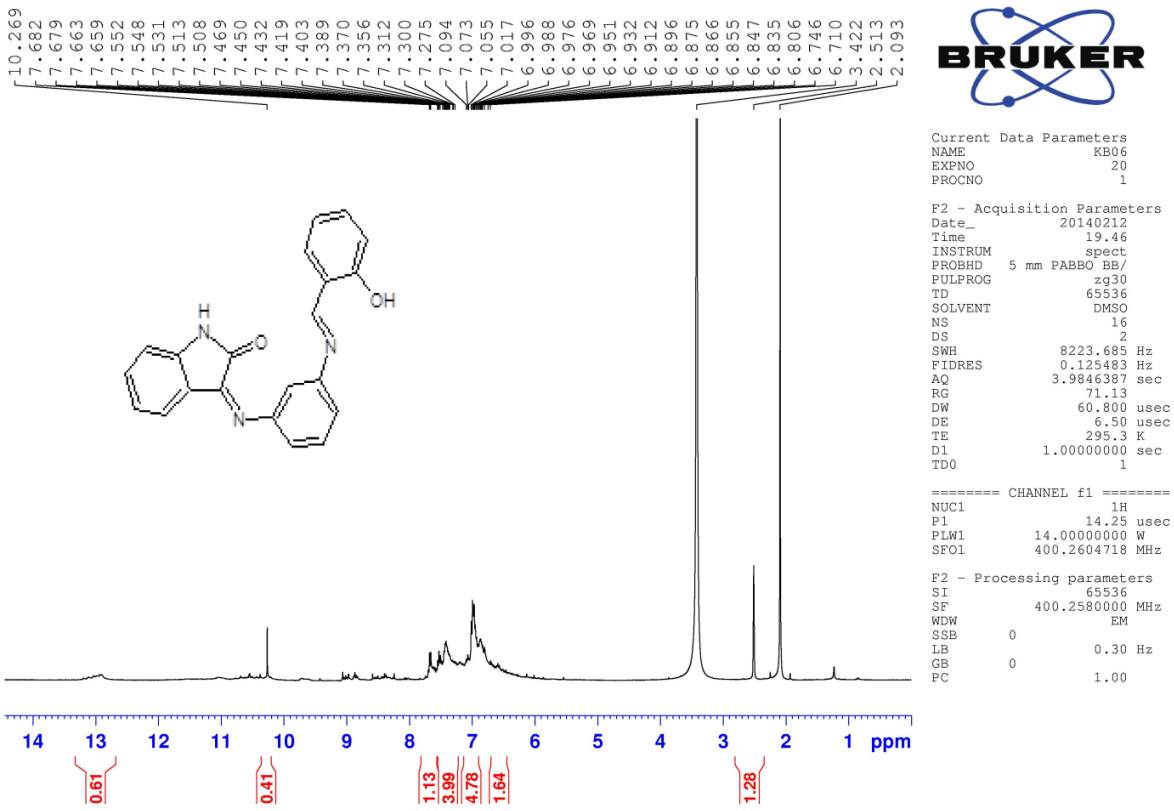
COMPOUND NAME	m/e VALUE
KB05	355.25 (M^+) 57.16 (B)

IR SPECTRUM OF KB06



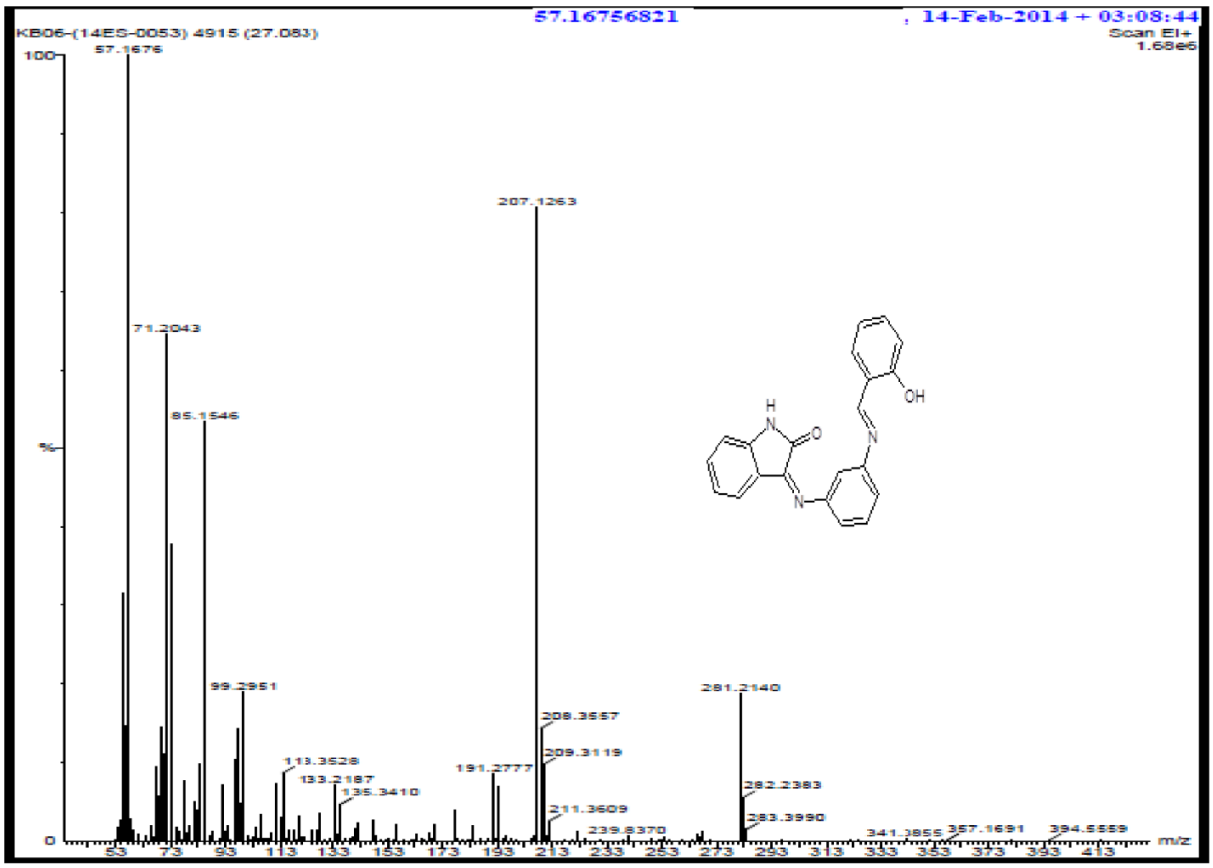
COMPOUND NAME	IR REGION
KB06	3062.73 (Ar-CH), 1481.22 (Ar C=C), 1720.38 (C=O), 1110.92 (C-N), 1612.37 (C=N), 2916.16 (Al-CH), 3695.34 (phenolic OH), 3271.03 (N-H), 1143.49 (C-O).

NMR SPECTRUM OF KB06



COMPOUND NAME	H^1 NMR
KB06	2.2-2.8δ (1H, s, Al C-H) 6.5-7.9 δ (12H, m, Ar C-H) 10.2-10.5 δ (1H, s, Ar O-H) 12.8-13.2 δ (1H, s, N-H)

MASS SPECTRUM OF KB06



COMPOUND NAME	m/e VALUE
KB06	341.38 (M^+) 57.16 (B)

BIOLOGICAL EVALUATION

The anti-tubercular activities of the synthesized compounds were determined by MABA method. Mycobacterium tuberculosis (H37 RV Strain) was used. The pathogen tested was susceptible to all the three compounds at 100µg/ml and 50µg/ml. The data pertaining to those observations are presented in the table and the growth of organism is shown in figure. Inhibition was compared using standard streptomycin [6.25µg/ml] and pyrazinamide [3.12µg/ml] & ciprofloxacin [3.12µg/ml].

TABLE-04: Results for Anti tubercular activity

S.No.	Compound 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	KB01	S	S	R	R	R	R	R	R
2	KB05	S	S	R	R	R	R	R	R
3	KB06	S	S	R	R	R	R	R	R

NOTE:

S - Sensitive

R - Resistant

Strain used: *M.tuberculosis* (H37 RV strain)

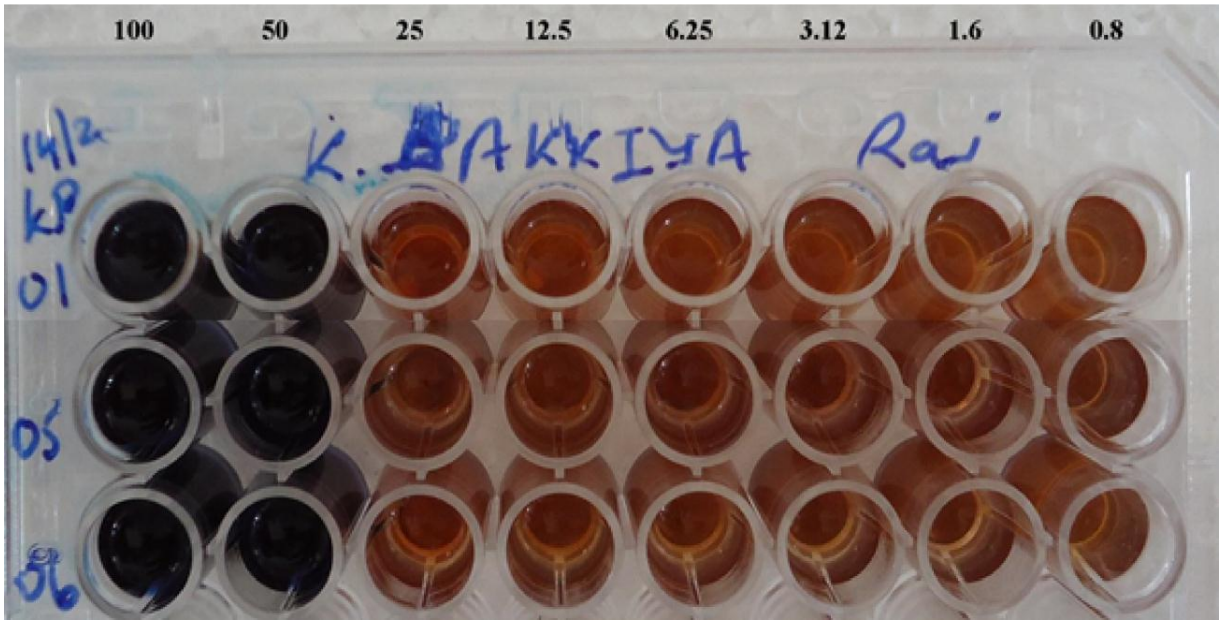
STANDARD VALUES

Pyrazinamide- 3.125µg/ml

Streptomycin- 6.25µg/ml

Ciprofloxacin-3.125µg/ml

Figure-07: Photograph of Alamar Blue Assay



SUMMARY AND CONCLUSION

DRUG DESIGN AND DOCKING

All the designed molecules were docked to the specific target i.e., cmaA1 target (1L1E) mycolic acid cyclopropane synthase using drug design software (GLIDE Maestro 9.1). Those molecules with the top G-Score molecules which possessed synthetic feasibility were selected and others were ruled out.

SYNTHESIS AND CHARACTERIZATION

The chosen compounds were synthesized by the conventional method and checked for purity initially by TLC and melting point methods. The structure of the synthesized compounds was assigned on the basis of spectral studies of IR, H^1NMR and Mass spectroscopy method. All the synthesized compounds comply with the spectral requirements.

BIOLOGICAL SCREENING

Invitro Anti-tubercular activity:

The synthesized compound [KB01, KB05 & KB06] showed the anti-tubercular activity. The pathogen tested was susceptible to all the synthesized compounds at 100µg/ml and 50µg/ml concentration. This proved that the docking method with GLIDE [Maestro 9.1] is ideal and fruitful for predicting biological activity.

Toxicological prediction:

Toxicity prediction was done by the *in silico* approaches using Osiris property explorer software. It shows for all the synthesized compounds are found to be no risk of undesired effects like tumorigenic, reproductive effective. But it shows some extent of undesired effects like mutagenicity and irritant quality.

CONCLUSION

This dissertation concludes that our synthesized compounds are effective in inhibiting the mycolic acid cyclopropane synthase (cmaA1) which is important for the growth of mycobacterium tuberculosis.

The structural improvement in the structure of the synthesized compounds can give new outlook to the development of promising molecules against the pathogen *mycobacterium tuberculosis.*

Acute and chronic toxicity studies can be carried out. These compounds can be evaluated for synergistic properties when combined with other enzyme inhibitors which act along the sequence of all wall synthesis.

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