DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL HETEROCYCLIC DERIVATIVES AS ANTI TUBERCULAR AGENTS TARGETING L, D TRANSPEPTIDASE 2

A Dissertation submitted to
THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY
CHENNAI-600032

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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CERTIFICATE

This is to certify that the dissertation entitled **"DESIGN, SYNTHESIS CHARACTERIZATION AND BIOLOGICAL EVALUATION OF SOME NOVEL HETEROCYCLIC DERIVATIVES AS ANTI TUBERCULAR AGENTS"** submitted by the candidate bearing the Register No:**261515710** in partial fulfillment of the requirements for the award of degree of **MASTER OF PHARMACY in PHARMACEUTICAL CHEMISTRY** by the TamilNadu **Dr. M.G.R Medical University** is a bonafide work done by her during the academic year 2016-2017 in the **Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai- 600 003**.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
DOTS	Directly Observed Treatment Schedule
LC-MS	Liquid Chromatography and Mass Spectroscopy
GC-MS	Gas Chromatography and Mass Spectroscopy
TLC	Thin Layer Chromatography
IR	Infra-Red
pdb	Protein Data Bank
Rf	Retention Factor
m.p	Melting Point
Mol.For	Molecular Formula
Mol.Wt	Molecular Weight
CADD	Computer Aided Drug Design
DMSO	Dimethyl Sulphoxide
◦C	Degree Celsius
m\e	Mass per charge Ratio
STD	Standard
CFU ML-1	Colony Forming Unit per Milliliter
UV	Ultra Violet
MIC	Minimum Inhibitory Concentration
mg\kg	Milligram per kilogram
Mcg	Microgram
Min	Minutes
Mtb	MycobacteriumTuberculosis
MDR-TB	Multi Drug Resistance TuBerculosis
MABA	Microplate Alamar Blue Assay

INTRODUCTION

TUBERCULOSIS

Tuberculosis is a contagious disease caused by bacteria called Mycobacterium tuberculosis and it is an air borne disease. Tuberculosis generally affects the lungs, but can also affect the other parts of the body. Tuberculosis is spread through the air when people who have active TB in their lungs cough, spit, speak, or sneeze. People with latent TB do not spread the disease. Active infection occurs more often in people with HIV/AIDS and in those who smoke. Tuberculosis is curable and preventable disease. [1]

BACKGROUND

Tuberculosis is the second most deadly disease (first HIV/AIDS) and continues to be a major worldwide problem. According to the World health Organization (WHO) about one-third of the World's population has latent TB. The WHO also declared that TB is only second to HIV/AIDS as the most dreaded disease. In 2015, 10.4 million people fell ill with TB disease and 1.8 million people died from TB (including 0.4 million among people with HIV). Over 95% of TB deaths occur in low and middle income countries.

In 2015, an estimated 1 million children became ill with TB and 1, 70,000 children died of TB (excluding children with HIV). Globally in 2015, an estimated 4,80,000 people developed multidrug- resistant TB (MDR-TB).TB incidence has fallen by an average of 1.5% per year since 2000.This needs to accelerate to a 4-5% annual decline to reach the 2020 milestone of the "END TB STRATEGY". Ending

the TB epidemic by 2030 is among the health targets of the newly adopted Sustainable Development Goals. [2]

HISTORY [3]

M. Tuberculosis, then known as the "Tubercle Bacillus", was first described on 24 march1882 by Robert Koch, who subsequently received the Nobel Prize in physiology or medicine for this discovery in 1905. Tuberculosis has existed throughout history, but the name has changed frequently over time. As the Physician Benjamin Marten described in his "A Theory of Consumption", Tuberculosis may be caused by small living creatures transmitted through the air to other persons. The historical term "**consumption**" came about due to the weight loss.

M. tuberculosis is carried in airborne particles, called droplet nuclei, of 1– 5 microns in diameter. Infectious droplet nuclei are generated when persons who have pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. Depending on the environment, these tiny particles can remain suspended in the air for several hours. *M. tuberculosis* is transmitted through the air, not by surface contact. Transmission occurs when a person inhales droplet nuclei containing *M. tuberculosis*, and the droplet nuclei traverses the mouth or nasal passages, upper respiratory tract, bronchi to reach the alveoli of the lungs. [4]

MYCOBACTERIA

Mycobacterium Tuberculosis is a pathogenic bacterial species of the family mycobacteriaceae and the causative agent of most cases of TB. M. Tuberculosis is the rod- shaped, spore forming aerobic bacterium. Mycobacterium typically measure 0.5µm to 3µm, are classified as acid fast bacilli and have an unusual waxy coating on their cell

surface (Primarily due to the presence of mycolic acid). The **Ziehl- neelsen or acid fast stain** is used.

TYPES OF TUBERCULOSIS

- Active tuberculosis
- Latent tuberculosis

ACTIVE TUBERCULOSIS

Active tuberculosis means the bacteria are active in the body i.e., the immune system is unable to stop these bacteria from causing illness.

LATENT TUBERCULOSIS

It is also known as inactive TB. If a person has a latent TB, their body has been able to successfully fight against the bacteria and stop them from causing illness. People who have latent TB do not feel sick, do not have symptoms and cannot spread tuberculosis. In the people who have HIV, the inactive TB may become active TB if their immune system becomes weakened. [5]

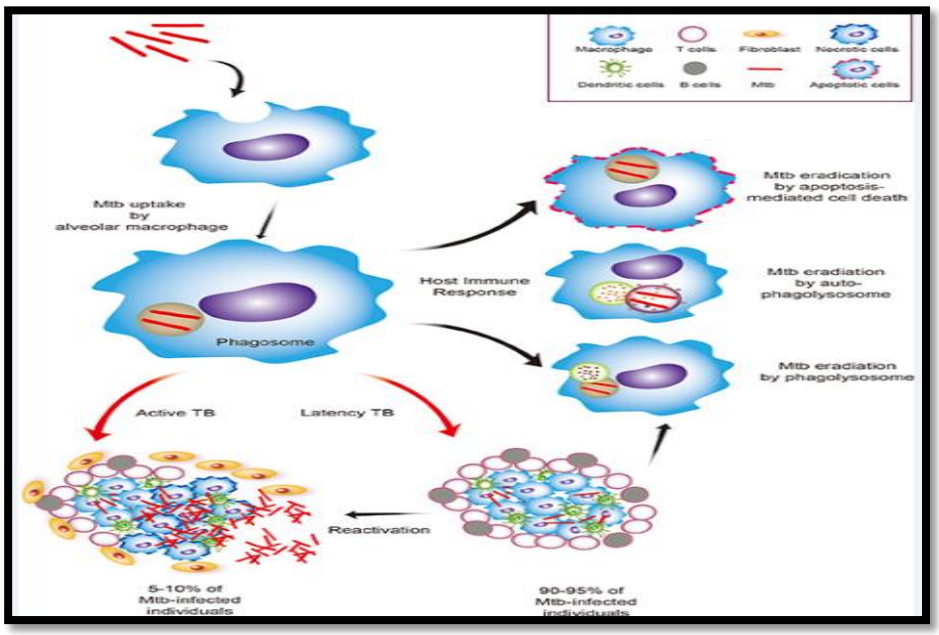


Figure 1[6]

MYCOBACTERIAL CELL WALL

Mycobacterium Tuberculosis has a tough cell wall that prevents passage of nutrients and therefore giving it the characteristic of slow growth rate. The cell wall of the pathogen looks like a gram- positive cell wall. The cell envelop contains a polypeptide layer, a peptidoglycan layer, and free lipids. It also contains complex structure of fatty acids such as mycolic acid that appear glossy. The Mycobacterium tuberculosis cell wall contains three classes of mycolic acids: Alpha, keto, and methoxy mycolates. The cell wall contains lipid complexes including acyl glycolipids and sulfolipids. These are porins in the membranes to facilitate transport. Beneath the cell wall there are layer of arabinogalactone and peptidoglycan that lie just above the plasma membrane. .[7]

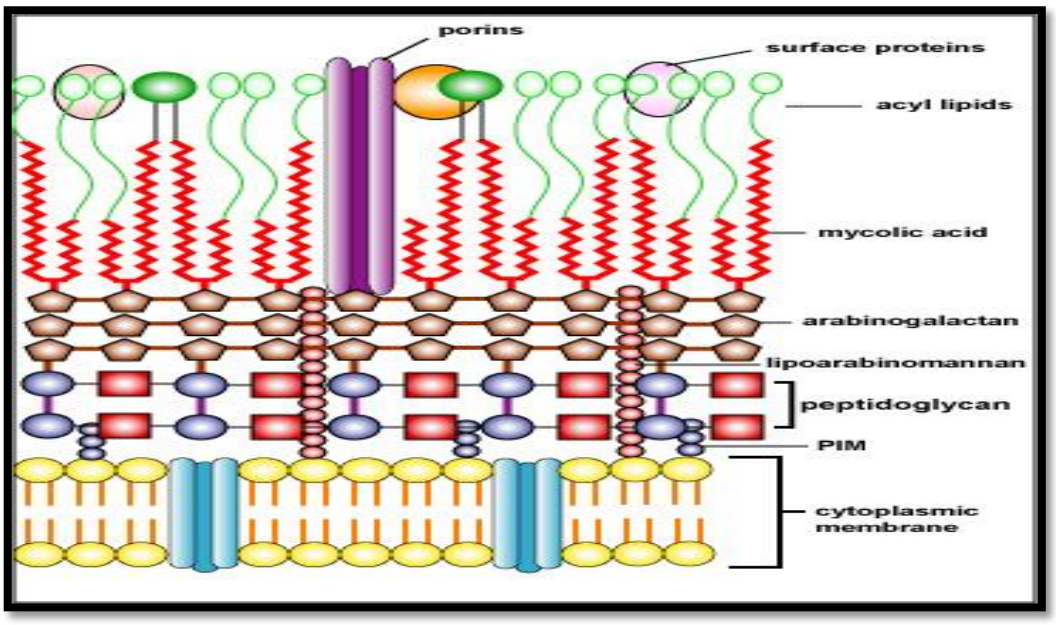


Figure 2[8] MYCOBACTERIAL CELL WALL

GENOME

The mycobacterium tuberculosis genome encodes about 190 transcriptional regulators, including sigma factors, two- components system and more than 140 transcription regulators. Several regulators have been found to environmental distress, such as extreme cold or heat, iron starvation and oxidative stress. To survive in these harsh conditions for a long period in the host, Mycobactrerium Tuberculosis has learnt to adapt to the environment by allowing an inhibiting transcription according to its surrounding. [9]

GENOME STRUCTURE

Mycobacterium Tuberculosis has circular chromosomes of about 4,200,000 long nucleotide. [10] The genome was studied generally using the strain Mycobacterium tuberculosis H37RV. The genome comprises 4,411,529 base pairs, contains around 4,000 genes, and has a very high guanine + cytosine content that is reflected in the biased amino-acid content of the proteins. Genes that code for lipid metabolism are very important part of the bacterial genome and 8% of the genome is involved in its activity.[11]

Plasmids are also important in transferring virulence because genes on the plasmids are more easily transferred than genes located on the chromosome. One such 18kb plasmid in the mycobaterium tuberculosis H37RV strain was proven to conduct gene transfer. [12]

CHOLESTEROL METABOLISM

Cholesterol metabolism has been studied exclusively because of its possible therapeutic applications in TB infections. It has been shown numerous times that TB

requires cholesterol for virulence in vivo, because mycobacterium tuberculosis the causative agent utilizes cholesterol as a source of carbon, energy, and steroid-derived metabolites throughout the course of infection. [13]

ROLE OF CHOLESTEROL IN TB INFECTION

Tuberculosis infection has unique virulence factors compared to most pathogens. They infect host cell and persist inside phagosome, where there are limited nutrients. Tuberculosis has ability to utilize cholesterol, which is a common component of human cell membrane, plays a role in its persistence, [7] because the cholesterol catabolism pathway requires oxygen that TB infect the lungs where oxygen concentrations are high.[14]

PATHOPHYSIOLOGY

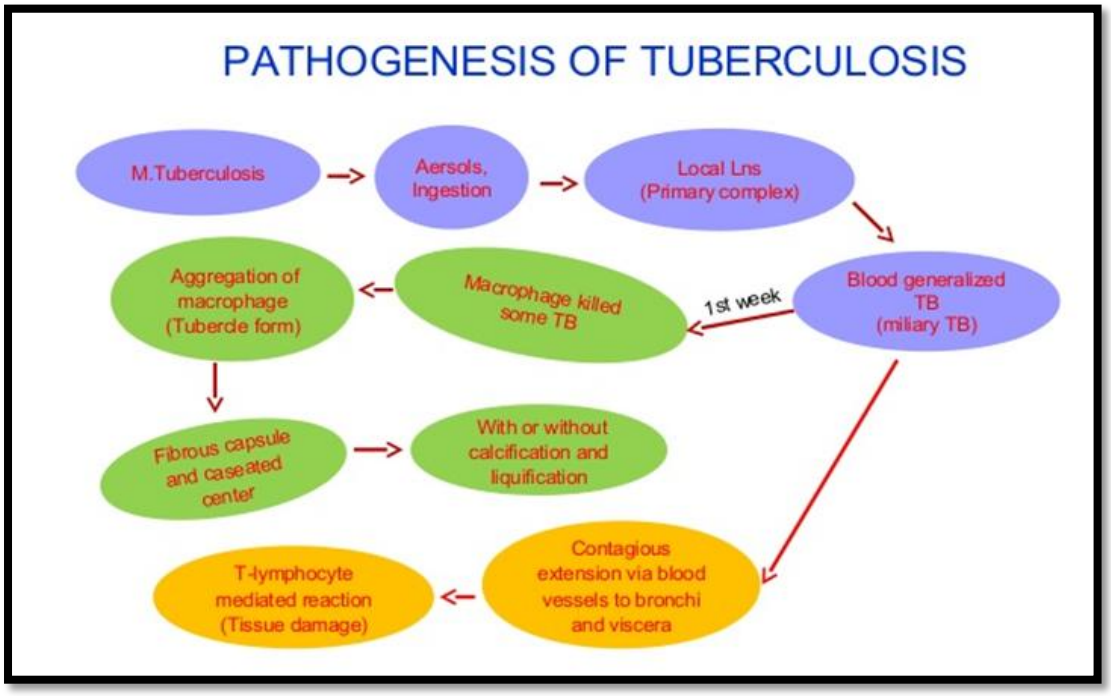


FIGURE 3[15]

LIFECYCLE OF MYCOBACTERIUM TUBERCULOSIS [16]

The five Stages of Tuberculosis

1) Onset (1-7 Days): The Bacteria is inhaled.

2) Symbiosis (7-21 Days): If the Bacteria does not get killed then it reproduces.

3) Initial Caseous Necrosis (14-21 Days): Tuberculosis starts to develop when the Bacteria slows down at reproducing; they kill the surrounding non activated Macrophages and run out of cells to divide in. The Bacteria then produces anoxic conditions and reduces the P^H. The Bacteria can't reproduce anymore but can live for a long time.

4) Interplay of Tissue Damaging and Macrophage Activating Immune Response (After 21 days): Macrophages surround the tubercle but some may be inactive. Tuberculosis then uses it to reproduce which causes it to grow. The tubercle can break off and spread around. If it spreads in the blood one can develop tuberculosis outside the lungs, this is called Miliary Tuberculosis.

5) Liquification and Cavity Formation: The tubercles at one point will liquefy, which will make the disease spread faster, not everyone will get to this stage. Only a small percent of people will get to this stage.

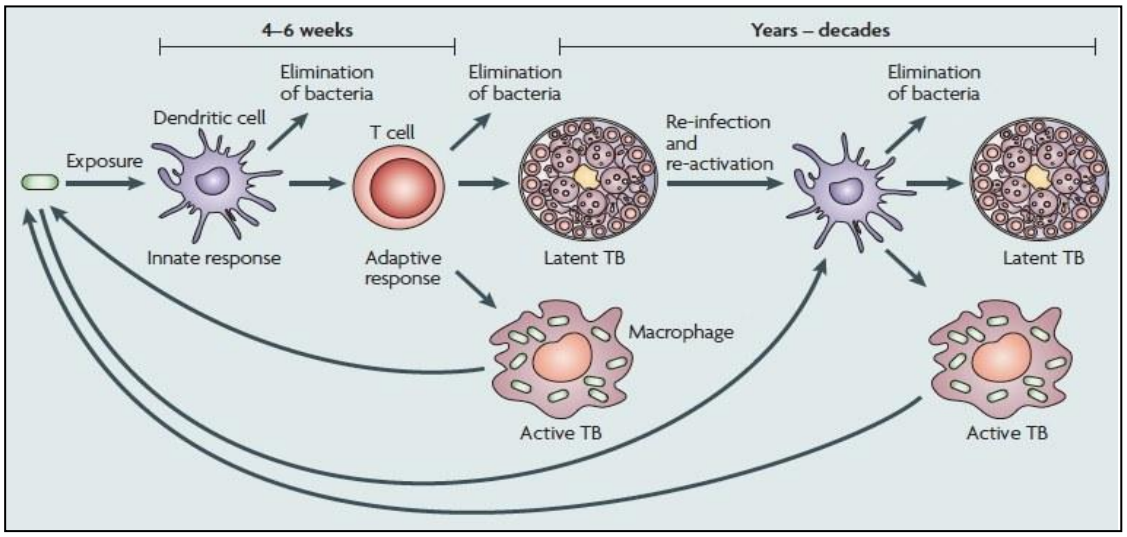


FIGURE 4[17]

TB treatment [18]

The aims of TB treatment are:

- To cure the patient of TB and restore their quality of life and productivity;
- To prevent relapse of TB;
- To reduce the transmission of TB to others;
- To prevent the development and transmission of drug resistant TB.

There are more than twenty drugs available for TB treatment. They are used in differing combinations in different circumstances. So for example, some TB drugs are only used for the treatment of new patients when there is no suggestion of any drug resistance. Others are only used for the treatment of drug resistant TB. The new TB drugs bedaquiline and delamanid are also now available to be used for the treatment of MDR-TB when there aren't any other drugs available. More than 90% of people with drug susceptible TB (that is TB which is not drug resistant) can be cured in six months using a combination of "first line" TB drugs.

NEED FOR THE NEW ANTI-TB DRUGS

- The new rise in TB cases and especially the increase of drug resistant mycobacteria indicate an urgent need to develop new anti-TB drugs.
- The long duration of TB therapy is a consequence of persistent mycobacterium Tuberculosis, not effectively killed by current anti-TB agents.[19]
- Recent advances in the knowledge of the biology of organism and the availability of the genome sequence give an opportunity to explore a wide range of novel targets for drug design.
- There is a need to design new drugs that are more active against slowly growing and non-growing persistent bacilli to meet the population at risk of developing active disease through reactivation.
- It is important to achieve a shortened therapy schedule to encourage patient compliance and to slow down the development of drug resistance in mycobacteria.[20]
- To improve the treatment of MDR-TB.
- To provide more effective treatment of latent tuberculosis infection.
- XDR TB disease is resistant to first-line and second-line drugs, patients are left with treatment options that are more toxic, more expensive, and much less effective.[21]
- Discovery of a compound that would reduce both the total duration of treatment and the frequency of drug administration.
- With multidrug resistant cases of tuberculosis increasing globally, better antibiotic drugs and novel drug targets are becoming an urgent need.[22]

ENZYME PROFILE

TARGET: L, D-TRANSPEPTIDASE-2 [23]

Transpeptidase may refer to: DD-transpeptidase, a bacterial enzyme that cross-links the peptidoglycan chains to form rigid cell walls. Peptidyl transferase, which acts as an enzyme in ribosomes. Gamma-glutamyl transpeptidase, a liver enzyme .D-glutamyl transpeptidase .Sortase, and possibly archaeosortase and exosortase, enzymes involved in protein sorting in prokaryotes.

- Traditional β-lactam antibiotics that inhibit D, D-transpeptidases are not effective against mycobacteria, because mycobacteria rely mostly on L, D-trans peptidases for biosynthesis and maintenance of their peptidoglycan layer.
- This reliance plays a major role in drug resistance and persistence of Mycobacterium tuberculosis (Mtb) infections.
- The crystal structure at **1.7 Å** resolution of the M -tb L, D-transpeptidase containing a bound peptidoglycan fragment, provides information about catalytic site organization as well as substrate recognition by the enzyme.
- Based on structural, kinetics, and calorimetric data, a catalytic mechanism for LdtMt2 in which both acyl- acceptor and acyl-donor substrates reach the catalytic site from the same, rather than different, entrance has been proposed by Sabri B. Erdemli et al.[44]
- This information provides vital insights that facilitate development of drugs targeting this validated yet unexploited enzyme.

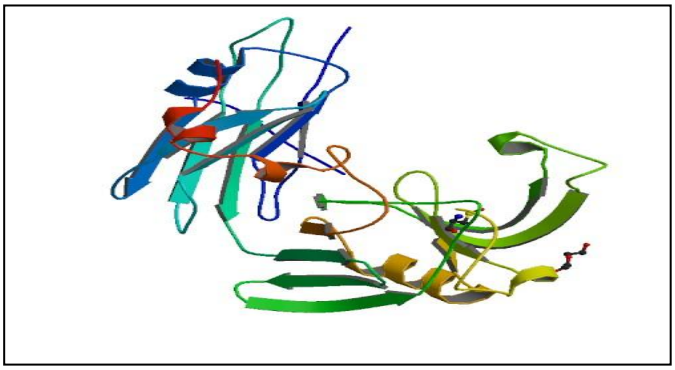


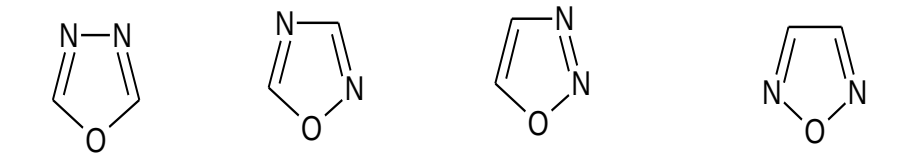
FIGURE 5[25]

HETEROCYCLIC CHEMISTRY

Millions of heterocyclic structures are found to exist having special properties and biological importance. Among various compounds, we have chosen 1, 3, 4-oxadiazole. This ring system possesses important biological activity and many drugs contain an oxadiazole ring.

1, 3, 4- OXADIAZOLE- NUCLEUS [26]

1, 3, 4-Oxadiazole is a heterocyclic compound containing an oxygen atom and two nitrogen atoms in a five- membered ring. It is derived from furan by substitution of two methylene groups (=CH) with two pyridine type nitrogens (-N=).



Isomers of oxadiazole

Of these 1, 3, 4-oxadiazole and 1, 2, 4-oxadiazole are better known, and more widely studied by researcher because of their many important chemical and biological properties.

Among heterocyclic compounds, 1, 3, 4-oxadiazole has become an important construction motif for the development of new drugs. Compounds containing 1, 3, 4-oxadiazole cores have a broad biological activity spectrum including antibacterial, antifungal, analgesic, anti-inflammatory, antiviral, anticancer, antihypertensive, anticonvulsant, and anti-diabetic properties. They have also attracted interest in medicinal chemistry as surrogates (bioisosteres) for carboxylic acids, esters and carboxamides . The ability of 1, 3, 4-oxadiazole heterocyclic compounds to undergo various chemical reactions has made them important for molecule planning because of their privileged structure, which has enormous biological potential. Two examples of compounds containing the 1, 3, 4-oxadiazole unit currently used in clinical medicine are: Raltegravir, an antiretroviral drug and Zibotentan an anticancer agent.

The synthesis of novel 1, 3, 4-oxadiazole derivatives, and investigation of their chemical properties and biological behavior has accelerated in the last two decades. In recent years the number of scientific studies with these compounds has increased considerably. Considering the period from 2002 to 2012, the Scifinder Scholar database records 2,577 references to 1, 3, 4-oxadiazole, demonstrating its relevance for heterocyclic chemistry. Figure 5 shows the number of publications over the past twelve years involving 1, 3, 4-oxadiazole. The graph is of course not linear, there is a decrease from 2002 (169 articles) to 2003 (146 articles), and then a gradual increase from 2003 to 2006 (219 articles), again a small decline from 2006 to 2007 (214 articles), and an

increase from 2007 to 2011 (319 articles).Taking into account the importance of these compounds to both heterocyclic and medicinal chemistry, we have decided to present the main synthetic approaches used for obtaining the heterocyclic nucleus, as well as the broad spectrum of pharmacological activities reported in the literature over the past twelve years.

Number of publications in the last twelve years involving 1,3,4-oxadiazole.

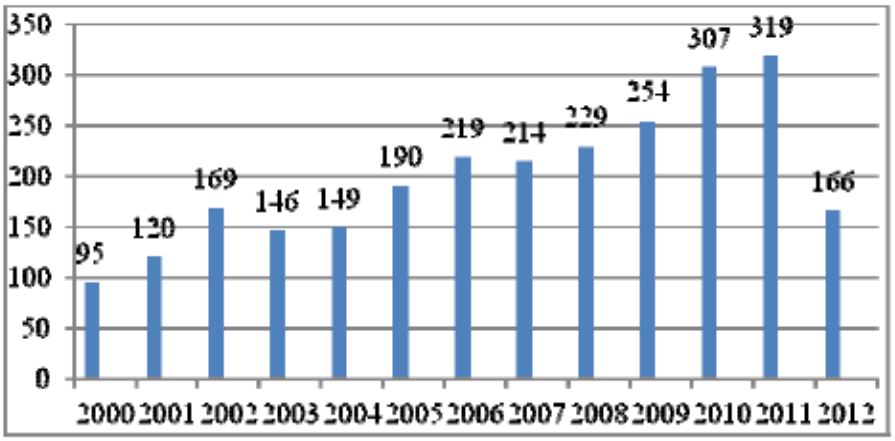


Figure 6[26]

Literature survey reveals that among heterocyclic compounds, 1, 3, 4-oxadiazole is an important moiety for development of new drugs.

Compounds containing 1, 3, 4-oxadiazole moiety has been found to exhibit wide range of biological activities including

- Anticancer
- Antibacterial
- Antifungal
- Analgesic
- Anti-inflammatory
- Anticonvulsant

1, 3, 4-Oxadiazoles have also gained interest in medicinal chemistry as surrogates for carboxylic acids, esters and carboxamides. Because of their privileged structure, oxadiazoles possess great biological potential and thus has made them important for molecule planning. Variety of therapeutically active compounds currently being used in clinical medicine are: HIV – integrase inhibitor Raltegraviran antiretroviral drug .Zibotentan an anticancer agent , a nitrofuran antibacterial furamizole , antihypertensive agents tiodazosin and nesapidil are based on 1,3,4-Oxadiazole moiety. Since many of 1, 3, 4-oxadiazoles display a remarkable biological activity, their synthesis and transformations have been receiving particular interest for a long time.

In recent years the number of scientific studies with these compounds has increased Considerably. Considering the importance of these compounds to both heterocyclic and medicinal chemistry, we have decided to discuss the main synthetic approaches used for obtaining the oxadiazole moiety, as well as the broad spectrum of pharmacological activities reported in the literature.

It was observed from the literature that certain five membered heterocyclic compounds possess interesting biological activity. Among them the compounds bearing 1, 3, 4-oxadiazole and pyrazole nucleus have wide applications in medicinal chemistry. These compounds also have been reported to have significant antitubercular activity.

REVIEW OF LITERATURE

Literature related to synthetic methodologies of scaffolds of interest, Drug Design and QSAR methods, target and screening methods for antitubercular activity was reviwed.

1. **Robert Koch.** (2008) [3] He was the first microbiologist to establish that mycobacterium is the cause of tuberculosis.

2. **Rahul Jain et al.** (2005) [27] Established that tuberculosis is the second leading infectious causes of mortality today behind only HIV/AIDS.

3. **Van der Geize R et** a**l.** (2007) [15] Worked on the Gene cluster encoding cholesterol catabolism a Soil Actinomycetes provides insight into mycobacterium tuberculosis survival in Macrophages.

4. **Thomas ST et al.** (2011) [7] Carried out pathway profiling in mycobacterium tuberculosis and elucidation of cholesterol derived catabolite and enzymes that catalyze its metabolism.

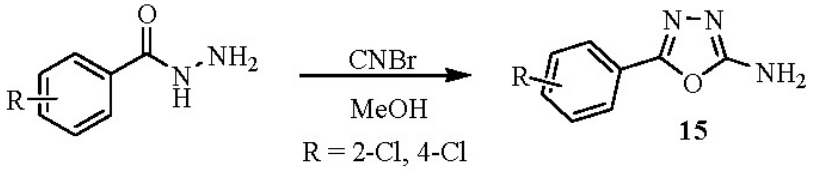
THE REVIEW OF LITERATURE RELATED TO THE TARGET ENZYME, 3VYP AND ITS FUNCTION:

5. **Kim et al. (2013)** [24] worked on the Structural basis for the inhibition of Mycobacterium tuberculosis.

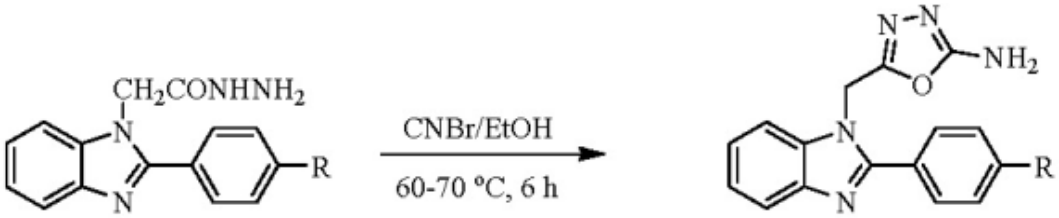
6. **Hyoun Sook Kim et al. (2012**) [25] reported the structural basis for the inhibition of Mycobacterium Tuberculosis.

A LITERATURE REVIEW OF THE AVAILABLE 1, 3, 4 OXADIAZOLE WAS CONDUCTED

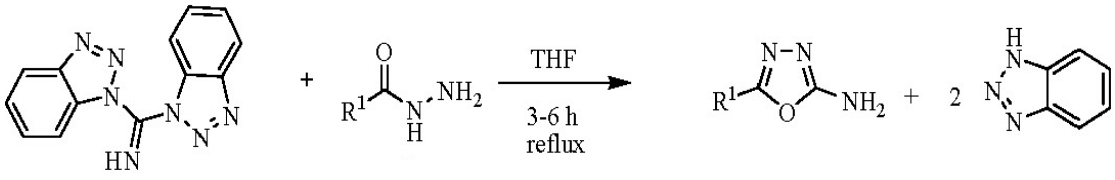
7. **Patel and Patel et al.** (2014) [28] synthesized 5-aryl-2-amino-1, 3, 4-oxadiazole compounds in good yield.



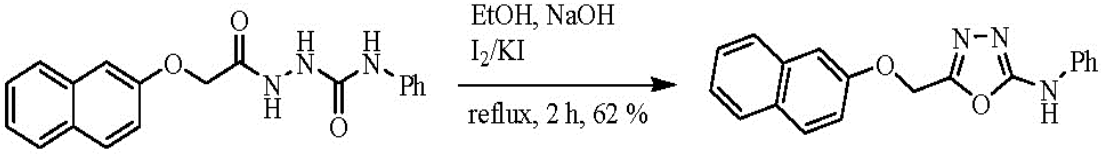
8. **Kerimov et al. (**2014) [29] synthesized a new series of 2-amino-1, 3, 4-oxadiazoles 17 carrying a benzimidazole moiety in 33%–60% yield from the reaction between 2-(2-(4-substituted-phenyl)-1H-benzo[d]imidazol-1-yl) acetohydrazide and cyanogen bromide.



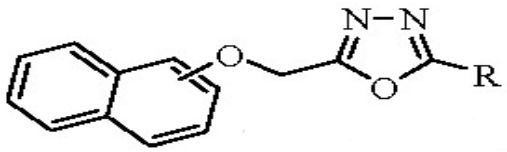
9. **Katritzky et al.** (2014) [30] prepared 5-aryl-2-amino-1, 3, 4-oxadiazole compounds in excellent yields from the reaction between di(benzotriazol-1-yl)methanimine and arylhydrazides.



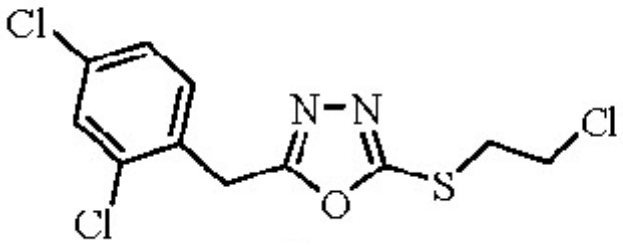
10. **El-Sayed et al.** [31] synthesized 5-((naphthalen-2-yloxy)methyl)-Nphenyl-1, 3, 4-oxadiazol-2-amine 24 in 62% yield, by heating compound 23 in ethanol in the presence of sodium hydroxide and iodine in potassium iodide.



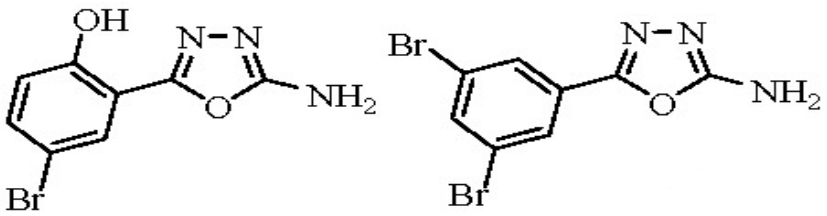
11. **Sahinet al. (2014)**[32] synthesized a series of new derivatives of 5-(1-/2-naphthyloxymethyl)-1,3,4-oxadiazol-2(3H)-thione (R=SH), 5-(1-/2-naphthyloxymethyl)-1,3,4-oxadiazole-2-amino (R=NH2), and 5-(1-/2-naphthyloxymethyl)-1,3,4-oxadiazol-2(3H)- ones (R=OH) and evaluated their antimicrobial activity. All were active against *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans* and *C. parapsilosis* at a minimum concentration of 64–256 mg/mL.



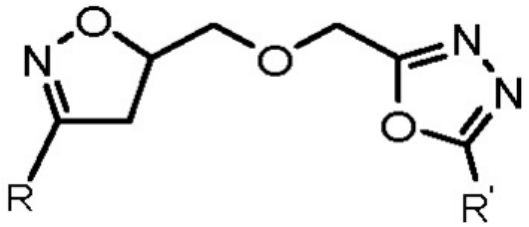
12. **Bakal et al. (2014)** [33] investigated anti-tubercular activity for a series of 2, 5-disubstituted oxadiazoles against *M. tuberculosis* H337Rv. The compound with a MIC50 = 0.04 ± 0.01 μM was comparable with Isoniazid.



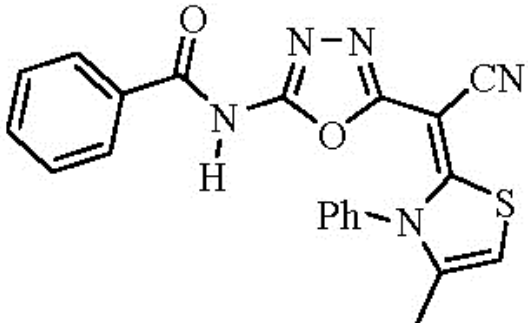
13. **Kumar et al. (2014)**[34] investigated antibacterial and antifungal activity of 2-(5-amino-1,3,4-oxadiazol-2-yl)-4-bromophenol , and 5-(3,5-dibromophenyl)-1,3,4-oxadiazol-2-amine against two strains of Gram-positive bacteria; Streptococcus aureus, Bacillus subtilis, two strains of Gram-negative bacteria; Klebsiella pneumoniae and Escherichia coli, and two fungal species; Aspergillus Niger and C. Pannical. The tests showed activities which were approximately equal to the standard drugs of treatment streptomycin and griseofulvin, respectively.



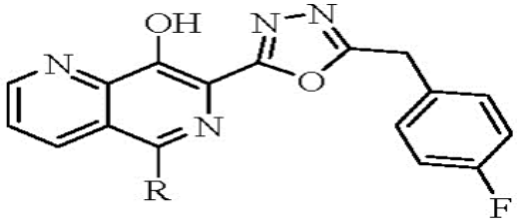
14. **Jayashankar B et al. (2014)** [35] synthesized a series of novel ether-linked bis(heterocycle)s. All the synthesized compounds were screened for anti-inflammatory and analgesic activities. Some of them showed excellent anti-inflammatory and analgesic activities.



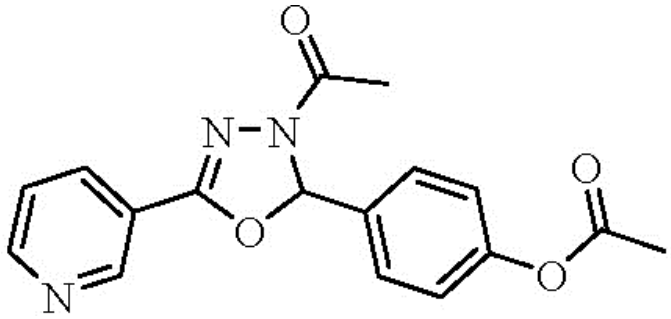
15. **Samir Bondock et al. (2014)** [36] synthesized a new series of 1, 3, 4-oxadiazole-based heterocycles.The compounds were screened for their antitumor activity. Some synthesized compounds exhibited good anti-tumor activity.



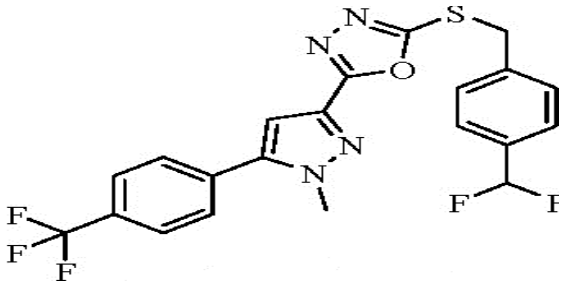
16. **Johns et al. (2014)** [37] reported antiviral activity (through inhibition of viral DNA integration) for new derivatives containing the 1, 3, 4-oxadiazole unit in combination with a ring system of 8-hydroxy-1,6-naphthyridine.



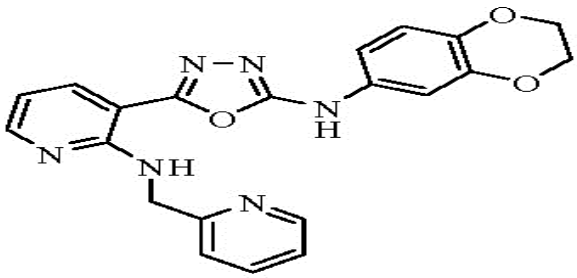
17. **Bankar et al. (2014)** [38] reported Vasorelaxant effect of compound, 4-(3-acetyl-5-(pyridin-3-yl) - 2, 3-dihydro-1, 3, 4-oxadiazol-2-yl) phenyl acetate in rat aortic rings through calcium channel blockage.



18. **Puthiyapurayil et al. (2014)** [39] synthesized a novel series of 1, 3, 4-oxadiazoles bearing N- methylpyrazoles. Compounds were tested for their cytotoxic activity by MTT assay.



19. **Tuma et al. (2014)** [40] synthesized and evaluated various 1, 3, 4-oxadiazole derivatives as to their ability to inhibit tubulin polymerization and block the mitotic division of tumor cells and exhibited potent activity. In vitro studies of this compound indicated that at nano-concentrations it interrupted mitotic division in breast carcinoma and squamous cell tumors, which included multi-drug resistant cells.



LITERATURE REVIEW RELATED TO THE ENZYME L, D-TRANSPEPTIDASE

20. **Wen-Juan Li et al.** [41] worked on the crystal structure of L, D-transpeptidase LdtMt2 in complex with meropenem reveals the mechanism of carbapenem against Mycobacterium tuberculosis.

21. Sabari b erdemli et al. [42] studied that the traditional β-lactam antibiotics that disrupt the D, D-transpeptidases are not effective against mycobacteria. As mycobacteria rely mostly on β -lactam insensitive L, D-transpeptidases for biosynthesis and maintenance of their peptidoglycan layer. Based on the structural, kinetic and calorimetric data, catalytic mechanism for LdtMtb2 has been proposed.

22. Lauriane Lecoq et al. [43] worked on the dynamics Induced by β-Lactam Antibiotics in the Active Site of Bacillus subtilis L, D-Transpeptidase.

23. **Soumya De et al.** [44] investigated the structural and dynamic basis for the unexpected inhibition of peptidoglycan crosslinking L, D-transpeptidases by carbapenam antibiotics.

THE REVIEW RELATED TO THE DRUG DESIGN STUDY:

24. **Deepak D Borkar, et al. (2012)** [45] performed on the Design and Synthesis of *p*-hydroxy benzohydrazide Derivatives for their Antimycobacterial Activity.

25. **Andrew Worth et al. (1998)** [46] reported on Distribution, Metabolism and Excretion (ADME) properties, which are often important in discriminating between the toxicological profiles of parent compounds and their metabolites/degradation products.

26. **Lipinski CA et al. (2001)** [47] investigated on the experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings.

27. Lipinski CA. (2004) [48] escribed a Lead and drug-like compounds and the role of fine resolution.

LITERATURE RELATED TO THE EVALUATION OF ANTI TUBERCULAR ACTIVITY BY MABA WAS REVIWED

28. **Jose de Jesus Alba-Romero et al.** [49] applied the Alamar Blue Assay to determine the susceptibility to anti-tuberculosis pharmaceuticals. The result showed that the MABA test is fast and easy to apply. It is a very reliable method of determining the drug susceptibility to pharmaceuticals.

29. **Sephra N Ramprasad et al.** [50] studied the multiple application of Alamar Blue as an Indicator of metabolic function and cellular health in cell viability Bioassays.

30. **Scott G Franzblau et al**. [51] studied MIC determination by MABA. A colorimetric, Microplate Based Alamar Blue Assay (MABA) method was used to determine the MICs of Isoniazid, Rifampin, Streptomycin and Ethambutol for Mycobacterium tuberculosis and the H37Rv strain by using bacterial suspensions prepared directly from media. The MABA is a simpe, rapid, low cost, appropriate technology which does not require extensive instrumentation and which makes use of a nontoxic, temperature stable reagent.

AIM AND PLAN OF WORK

AIM

The aim of this project is to develop potential antimycobacterial agents.

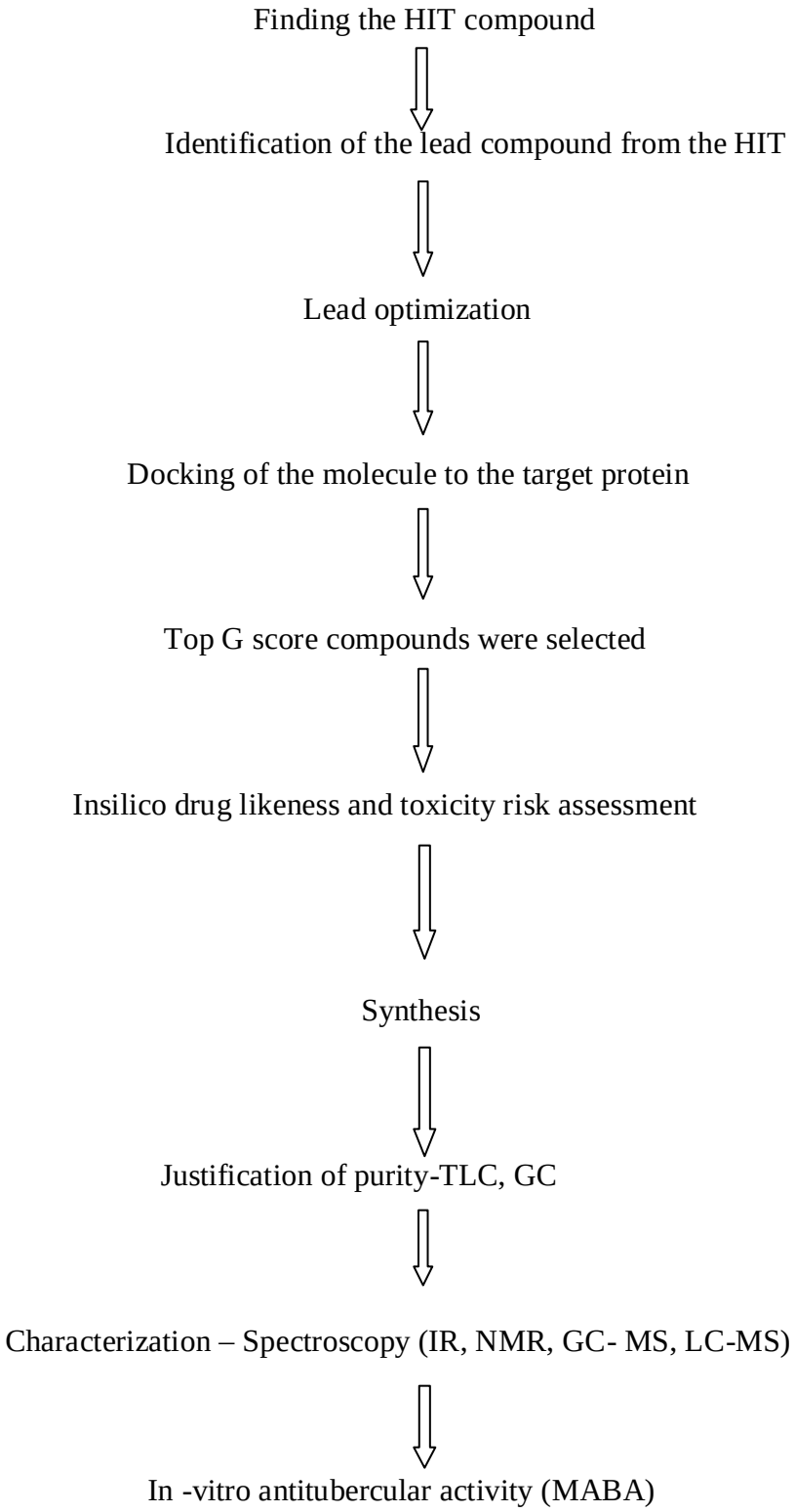
OBJECTIVES

The objective of the project is to design and synthesize some compounds which will act on L, D Transpeptidase 2 and inhibit the cell wall synthesis of *M*. tuberculosis.

THE PLAN OF WORK

- Design of L,D Transpeptidase 2 inhibitors by docking studies
- Insilico Prediction of Drug Likeness and Toxicity
- Laboratory synthesis of the top G score compounds
- Characterization of the synthesized compounds by
  - ✓ Infrared Spectrometry
  - ✓ H^1Nuclear Magnetic Resonance Spectroscopy
  - ✓ C^{13}Nuclear Magnetic Resonance Spectroscopy
  - ✓ Mass Spectrometry
- Determination of In-vitro anti tubercular activity of synthesized compounds(MABA)

The present study was carried out based on the below flow chart



MATERIALS AND METHODS

DOCKING STUDIES

DRUG DESIGN

Molecules for a particular target can now be designed using a number of commercial softwares. The software includes GLIDE® of schrodinger, Autodock® tools and Argus lab®. Molecules from data base can be docked on to relevant targets by using these softwares and determine the energetically more reliable conformations. The molecules are refined as ligands and receptor protein as target.

TYPES

There are two major types of drug design.

- Ligand based drug design
- Structure based drug design

1. LIGAND BASED DRUG DESIGN

Ligand based drug design relies on knowledge of other molecules that bind to the biological target of interest. These molecules may be used to derive a pharmacophore model that defines the minimum necessary structural characteristics, a molecule must possess in order to bind to the target. [53] In other words, a model of the biological target may be built based on the knowledge of what binds to it, and this model in turn may be used to design new molecular entities that interact with the target. Alternatively, a quantitative structural activity relationship (QSAR), in which correlation between calculated properties of molecules and their experimentally determined biological activity, may be derived. These QSAR relationships in turn may be used to predict the activity of new analogues.

1. STUCTURE BASED DRUG DESIGN

Structure based drug design is a direct approach which relies on knowledge of the three dimensional structure of the biological target obtained through methods such as x-ray crystallography and NMR spectroscopy [54]. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive and selectivity to the target using interactive graphics and the intuition of a medicinal chemist or various automated computational procedures to suggest new drug candidates. [55]

COMPUTER-AIDED DRUG DESIGN [56]

Computer-aided drug design uses computational chemistry to discover, enhance or study drugs and related biologically active molecules. Molecular mechanics or molecular dynamics are most often used to predict the conformation of the small molecule and to model conformational changes in the biological target that may occur when the small molecule binds to it.

Molecular mechanics methods may also be used to provide semi-quantitative prediction of the binding affinity. Also, knowledge- based scoring function may be used to provide binding affinity estimates.

Drug design with the help of computers may be used at any of the following stages of drug discovery:

1. Hit identification using virtual screening(structure or ligand- based design)
2. Hit-to-lead optimization of affinity and selectivity (structure- based design, QSAR etc.)

3. Lead optimization: Optimization of other pharmacokinetic properties while maintaining affinity.

In order to overcome the insufficient prediction of binding affinity calculated by recent scoring functions, the protein-ligand interaction and a compound's 3D structure information are used for analysis. [57]

ACTIVE SITE IDENTIFICATION

Active site identification is the first step in this program. It analyzes the protein to find the binding pocket, derives key interaction sites within the binding pocket, and then prepares the necessary data for ligand fragment link. The basic input for this step are the 3D structure of the protein and a pre docked ligand in PDB format, as well as their atomic properties. Both ligand and protein atoms need to be classified and their atomic properties should be defined, basically into four types:

1. Hydrophobic atom: All carbons in hydrocarbon chains are in aromatic groups.
2. H bond donor: Oxygen and Nitrogen atoms bonded to hydrogen atoms.
3. H bond acceptor: oxygen sp2 and sp hybridized nitrogen atoms with lone pair of electrons.
4. Polar atom: Oxygen and nitrogen atoms that are neither H bond donor nor H bond acceptor, sulfur, phosphorous, halogen, metal, and carbon atoms bonded to heteroatoms.[58]

DOCKING

Docking involves the fitting of a molecule into the target structure in a variety of positions, conformations and orientations. Molecular docking is used to predict the structure of the intermolecular complex formed between two molecules. The small molecule called ligand usually interacts with the protein binding site. There are several possible mutual conformations in which binding may occur. These are commonly called

binding modes. It also predicts the strength of the binding, the energy of the complex; the types of the complex; the types of the signal produced and calculates the binding affinity between two molecules using scoring functions. The most interesting case is the type of protein-ligand interaction, which has its applications in medicine.[59]

TYPES OF DOCKING

- **Lock And Key or Rigid Docking -** In rigid docking, both the internal geometry of the receptor and ligand is kept fixed and docking is performed.
- **Induced Fit or Flexible Docking -** An enumeration on the rotations of one of the molecules (usually smaller one) is performed. Every rotation the surface cell occupancy and energy is calculated; later the most optimum pose is selected.

ARGUS LAB®

Argus lab® 4.0 distributed freely us made available for windows platforms by Planaria Software.It is an introductory molecular modeling package for students. The Argus docking engine implementry in A.L 4.0 approximates an exhaustive search method with similarities to AUTODOCK® and GLIDE®.

Flexible ligand docking is possible with Argus lab® where the ligand is described as torsion tree and grids are constructed that overlay the binding site. The accuracy of the Argus lab® docking algorithm takes into account, the key features such as "the nature of binding site and the number of rotatable bonds in the ligand." [58]

STEPS INVOLVED IN DOCKING

Docking is done by using ARGUS LAB® Software.

1. Protein preparation.
2. Selection of active site (Q-Site finder).
3. Ligand Preparation.
4. Docking Procedure.
5. Visualization / Interpretation of Docking.

1. PROTEIN PREPARATION:

Step: 1

- Enter protein (pdb) ID in the protein data bank. (1TPY)
- Go to download files and select pdb as text file.
- Save the download pdb (text file) to the desktop.

Step: 2

- Open Argus lab file Open Import pdb file from the desktop.
- 3D Structure of the protein will appear in the workspace of Argus lab.
- Left side of the screen shows molecular tree view.
- Open pdb Open 'residues' 'Open misc'
- From 'Misc' delete the inhibitor and hetero residues [Note: Do not delete Co-factor]
- Open water press shift, select all water molecules and delete.
- Add hydrogen atoms.
- Go to Calculation on the toolbar energy by UFF method start.
- Save the prepared protein as *.agi file format in the desktop.

2. Q-SITE FINDER [59]

The following set of operation was carried for the Q-site finder

Step: 1

- Open Q-Site finder through online.
- Upload / Import the PDB format of the Protein.
- Find all the active site and make a list out of the common amino acid residues.

Step: 2

- Open residues open Amino acids.
- Press control and select the amino acids which were listed from the Q-Site finder.
- Make sure that all amino acid residues listed are selected.
- Right click on the mouse make a group from the selected residues give name
- Binding site Ok.

3. LIGAND PREPARATION:

The operation for the ligand preparation that was followed is given below.

- Draw the structure from Chem sketch and save as MDL Mol format.
- Import the ligand into workspace of Argus lab.
- Clean Geometry Clean Hybridization.
- Select the ligand, Right click on the mouse Make a group from the residues give
- name ligand Ok.

4. DOCKING PROCEDURE

The operation for the ligand preparation that was followed is given below.

- Select the set up a Dock Ligand calculation from the toolbar.
- Argus Dock as the Docking Engine.
- Dock was selected as calculation type.
- Flexible for the scoring function.
- Calculation size.
- Start docking.
- Save the Docked protein Ligand complex as Brookhaven pdb files (*.pdb).

5. VISUALIZATION / INTERPRETATION OF DOCKING

Molegro Molecular viewer will help in analyzing the energies and interaction of the Binding.

- View Docking view & Secondary Structure view.
- View Hydrogen bond interaction.
- Ligand map Interaction overlay.

INSILICO SCREENING OF DRUG LIKENESS

A drug to be pharmacologically active and exert the action it should possess pharmacokinetic properties like absorption, distribution, metabolism and excretion. In the field of drug research and development many drug failures occurs due to unfavorable ADME properties. This has to be ruled out early in the process of drug discovery. Some computational methods have been evolved to investigate the most suitable drug molecules before synthesis. Lipinski rule of five it is also known as Pfizer's rule of five is a rule used to evaluate drug likeness. It is used to predict whether a molecule is likely to be orally bio-available or not.

LIPINSKI'S RULE [60]

Lipinski's rule is used to predict if a molecule is likely to be orally bioavailable or to evaluate drug likeness. The rule was formulated by Christopher A. Lipinski in1997. The rule states that for drug likeness the molecule should have the following properties.

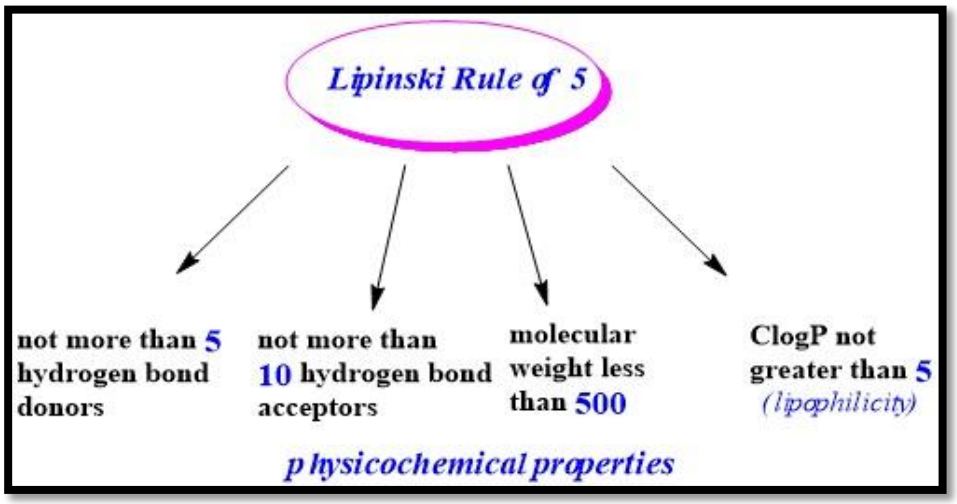


FIGURE 7 [62]

The designed and docked molecules are screened insilico using Molinspiration®, a cheminformatics software to evaluate drug likeness. This tool is quick and easy to use. It is software available in online for calculation of important molecular properties such as

- Log P
- Polar surface area
- Number of hydrogen bond donors
- Number of hydrogen bond acceptors

As well as prediction of bioactivity score for the most important drug targets

- GPCR ligands
- Kinase inhibitors
- Ion channel inhibitors
- Nuclear acceptors[62]

ADME ANALYSIS [63]

A deeper understanding of the relationships between important ADME parameters and molecular structure and properties has been used to develop in silico models that allow the early estimation of several ADME properties. Among other important issues, prediction of properties that provide information about dose size and dose frequency such as oral absorption, bioavailability, brain penetration clearance and volume of distribution also needed.

ABSORPTION

A Compound crossing a membrane by purely passive diffusion, a reasonable permeability estimate can be made using single molecular properties, such as log D

(diffusion co-efficient) or hydrogen-bonding capacity. The simple insilico models for estimating absorption are based on a single descriptor, such as log P (partition coefficient), log D, polar surface area which is a descriptor of hydrogen- bonding potential. Different multivariate approaches such as multiple linear regressions, partial least squares and artificial neural networks have been used to develop quantitative structure-human-intestinal-absorption relationships.

BIOAVAILABILITY

Important properties for determining permeability seem to be the size of the molecule, as well as its capacity to make hydrogen bonds, its overall liphophilicity and possible its shape and flexibility.

BLOOD-BRAIN BARRIER

Drugs that act in the CNS need to cross the blood brain barrier (BBB) to reach their molecular target, by contrast for drugs with a peripheral target, little or no BBB penetration might be required in order to avoid CNS side effects. In order for a drug to cross the blood brain barrier (molecule targeted to brain). Rule of thumb says log P values should be closer to 2 with a molecular mass of <450 Da and or with a polar surface area (PSA) <100 A are likely to possess.

Dermal and Ocular Penetration

For dermal and ocular route it should satisfy the existing parameters like log P (partition coefficient) for aqueous solubility, molecular weight and molecular flexibility.

Metabolism

Various *in silico* approaches exist in evaluating the metabolism namely QSAR and 3D QSAR etc. apart from those computational chemists have updated the structural details in the data bases and tools for predicting metabolism. Simultaneously it reveals

the metabolic information as well as the toxicity related to the molecular fragments by which the drug molecule undergoes the metabolism.

TOXICITY PREDICTION [64]

Toxicity is one of the major criteria to be considered for a molecule to shine as a successful clinical candidate in the pharmaceutical research. The Osiris® property explorer is an integral part of Actelion's in house substance registration system. Commercial *in silico* tools estimates toxicity and provides information by the use of QSAR (parameters and descriptors), scientific literatures and to some extent in abstracting issues from humans.

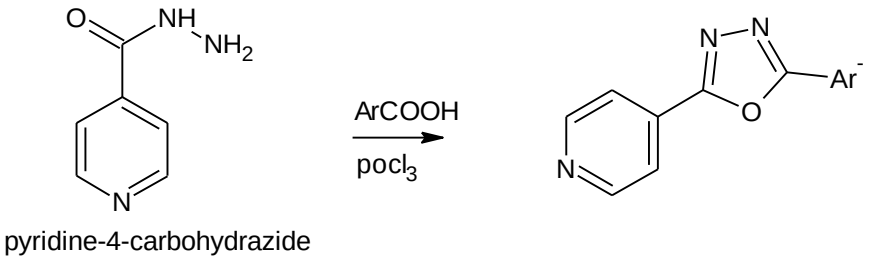
In silico approaches like OSIRIS® property explorer predicts carcinogenicity, mutagenicity, teratogenicity, immune toxicology, irritation, sensitization etc. In addition, hepato, neuro and cardio toxicity are evaluated in newly updated *in silico* tools. Prediction results indicates color coded in which the red color shows high risks with undesired effects like mutagenicity or a poor intestinal absorption and green color indicates drug-conform behavior.

SYNTHETIC METHODOLOGY

SYNTHESIS

The compounds with top docking score were selected for synthesis as per the scheme below. The necessary chemicals of laboratory grade for the synthesis were procured from Sigma Aldrich and synthesis was carried out.

SCHEME [65]



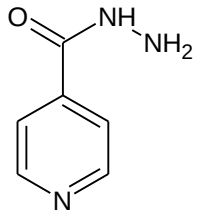
pyridine-4-carbohydrazide

PROCEDURE

A mixture of 0.01 mole pyridine-4-carbohydrazide and 0.01 mole of aromatic acid is dissolved in phosphorus oxychloride and refluxed for 18-22 hr. The reaction mixture is slowly poured over crushed ice and kept overnight. The solid mass thus separated is filtered, dried, and purified by recrystallization from ethanol.

REACTANT PROFILE

ISONICOTINIC ACID HYDRAZIDE



pyridine-4-carbohydrazide

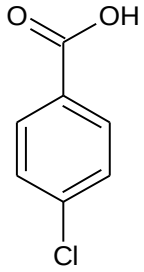
Molecular formula- $C_6H_7N_3O$

Molecular weight -137.13

Melting point -171.4ºC

Appearance - Colorless or white crystalline powder

4-CHLORO BENZOIC ACID



4-chlorobenzoic acid

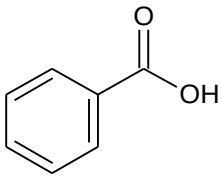
Molecular formula- $C_7H_5ClO_2$

Molecular weight -156.56

Melting point -243ºC

Appearance - White powder or coarse crystal

BENZOIC ACID



benzoic acid

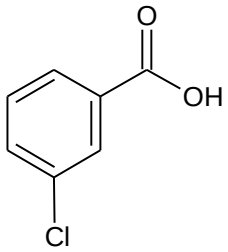
Molecular formula- $C_7H_5ClO_2$

Molecular weight -156.56

Melting point -122.41ºC

Appearance -White crystalline needles.

3-CHLOROBENZOIC ACID



3-chlorobenzoic acid

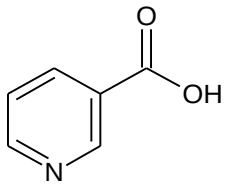
Molecular formula- $C_7H_5ClO_2$

Molecular weight -156.56

Melting point - 243ºC

Appearance - White powder or coarse crystal

NICOTINIC ACID



pyridine-3-carboxylic acid

Molecular formula- $C_6H_5NO_2$

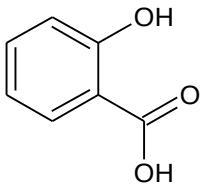
Molecular weight -123.11

Melting point - 237ºC

Boiling point - 238ºC

Appearance -white translucent crystals

SALICYLIC ACID



2-hydroxybenzoic acid

Molecular formula- $C_7H_6O_3$

Molecular weight -138.12

Melting point -158.6ºC

Boiling point -200ºC

Appearance -colorless to white crystals

CHARACTERIZATION STUDIES

THIN LAYER CHROMATOGRAPHY [66]

Pre-coated silica plates are used for the TLC .Various mobile phase are used , after the trial and error method, Benzene: Acetone in the ratio 3:2 is used to determine the Rf value of the synthesized compounds.

IR SPECTROSCOPY [67]

The IR spectrum of the synthesized compound will be verified for the presence of the newly created functional groups and absence of the functional group of the parent compounds, involved in the reaction.

Presence of new functional groups

- C=N stretching in the region of 1730-1650 cm^{-1}
- C-O-C stretching in the region of 1200-1300cm^{-1}

H^1 NMR SPECTROSCOPY

The proton NMR spectrum will be obtained using BRUKER ADVANCE III 500Hz NMR spectrometry using the appropriate solvent i.e. Deuterated Dimethyl Sulphoxide.

BIOLOGICAL EVALUATION OF ANTI-TUBERCULAR ACTIVITY

The designed and synthesized molecules need to be screened for their activity to inhibit the growth of the Mycobacterium tuberculosis. There are various high throughput assays available for screening of new chemical entities against tuberculosis. They are:

- Resazurin Micro plate Assay (REMA)
- Nitrate Reductase Assay (NRA)
- Micro plate Alamar Blue Assay (MABA)

- 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)
- Middle Brook 7H11 Agar dilution Assay
- Broth Micro dilution Method
- BACTEC system
- Luciferase Reporter Phage Assay

THE ALAMAR BLUE ASSAY [50]

Alamar Blue monitors the reducing environment of the living cell. The active ingredient is resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one), also known as diazoresorcinol, azoresorcin, resazoin, resazurine, which is water-soluble, stable in culture medium, is non-toxic and permeable through cell membranes. Continuous monitoring of cells in culture is therefore permitted. Growth is measured quantitatively by a visual color change and the amount of fluorescence produced is proportional to the number of the living cells which is determined by colorimetric and fluorimetric methods.

ADVANTAGES

- It has accurate time course measurement.
- It has high sensitivity and linearity.
- It involves no cell lysis.

IN VITRO ANTITUBERCULAR ACTIVITY

MICROPLATE ALAMAR BLUE ASSAY (MABA) [51]

- The anti-mycobacterial activities of the synthesized compounds are determined by MABA method. The organism used is M.tuberculosis H37Rv
- Alamar blue dye is used as an indicator for the determination of viable cells.

PRINCIPLE

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

PROCEDURE

1) The anti-mycobacterium activity of compounds was assessed against M. tuberculosis using Micro Plate Alamar Blue assay (MABA).

2) This methodology is non-toxic, and uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.

3) 200µl of sterile deionized water is 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 receives 100 µl of the Middle brook 7H9 broth and serial dilution of compounds is placed directly on plate.

5) The final drug concentrations tested is 100 to 0.8 µg/ml.

6) Plates are covered and sealed with 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 is added to the plate and incubated for 24 hrs.

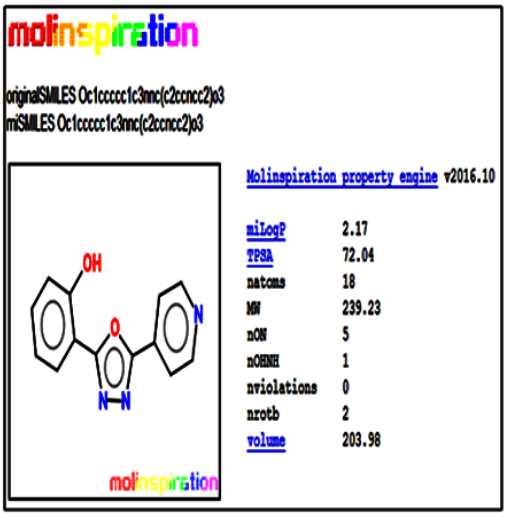
8) A blue color in the well is interpreted as no bacterial growth, and pink color was scored as growth.

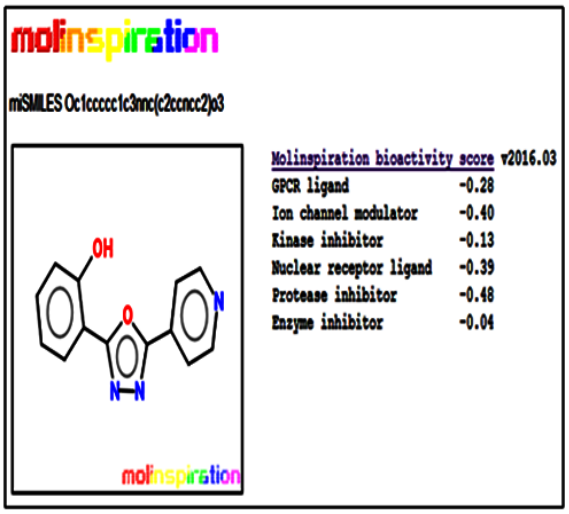
The MIC is defined as lowest drug concentration which prevents the color change from blue to pink.

INSILICO DRUG LIKENESS PROPERTY

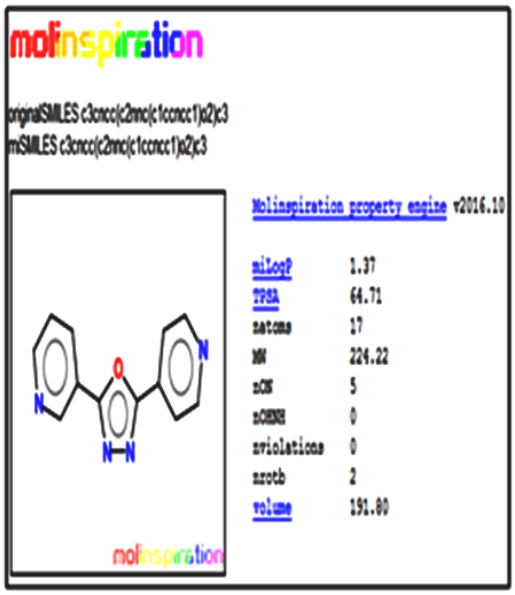
Insilico drug likeness property is used to predict whether a molecule is likely to be orally bio-available or to evaluate drug likeness.This property prediction was done using the software Molinspiration®.The output is presented here with SA, NA, VS1, VS2, VS4 and VS5.

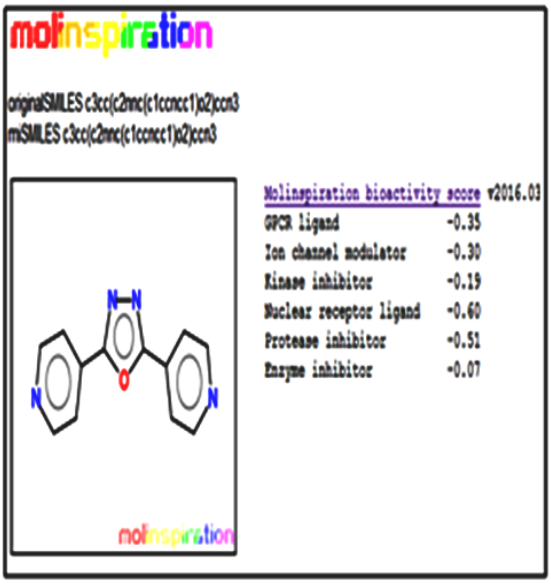
CODE: SA



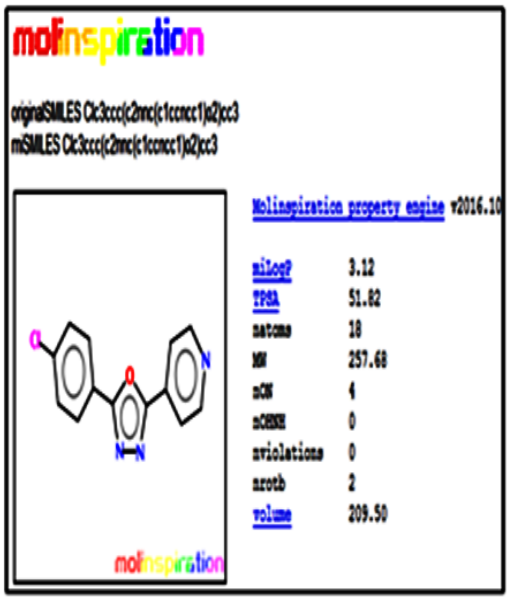


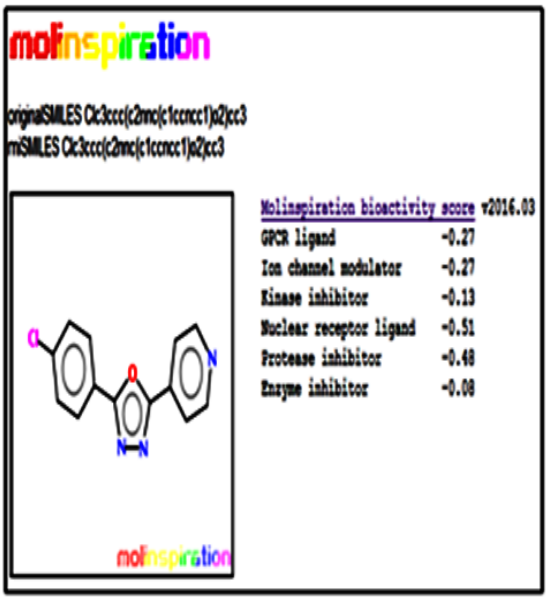
CODE: NA



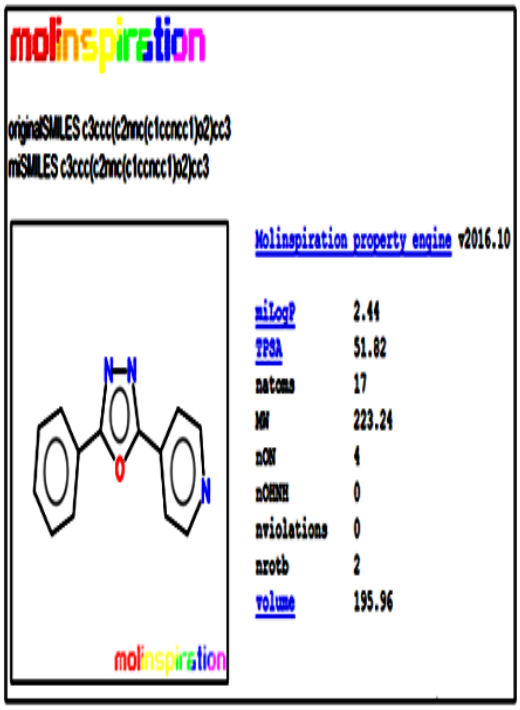


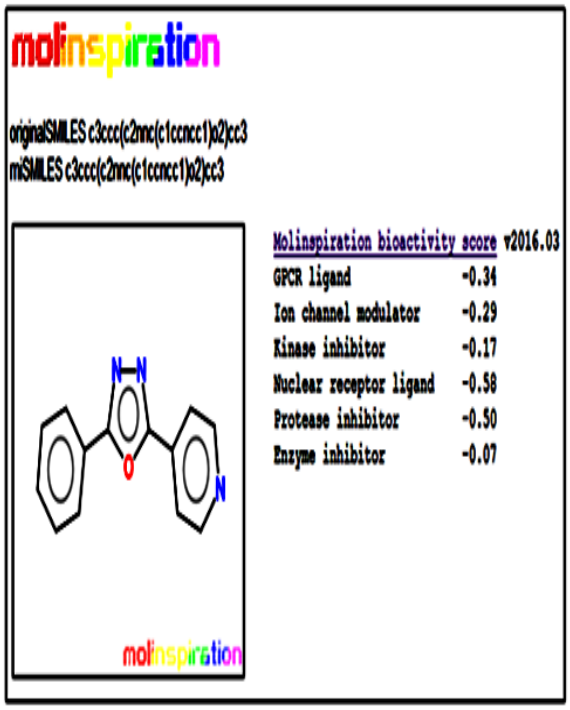
CODE: VS1



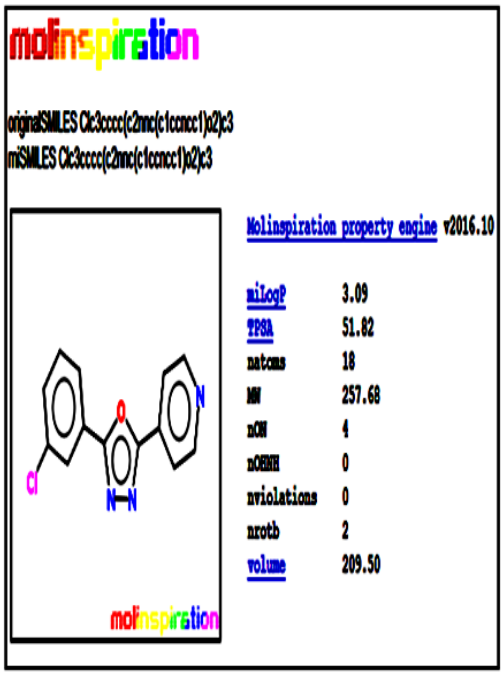


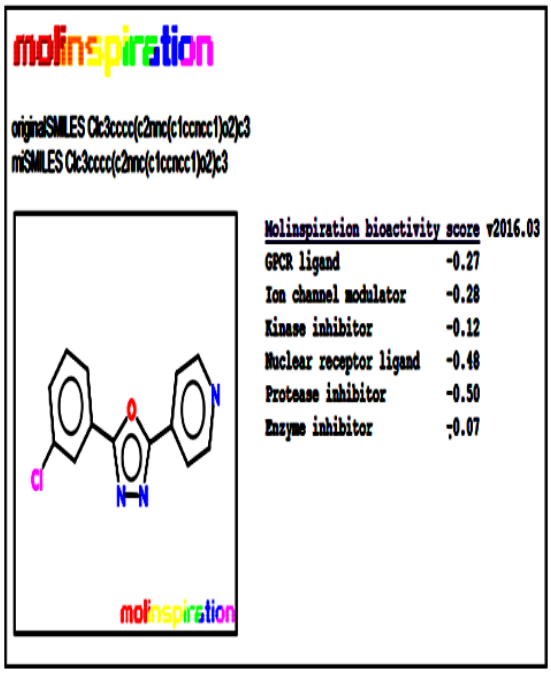
CODE: VS2



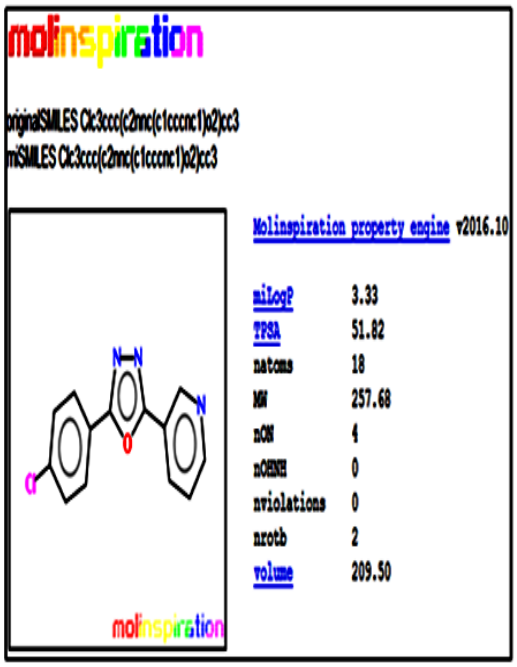


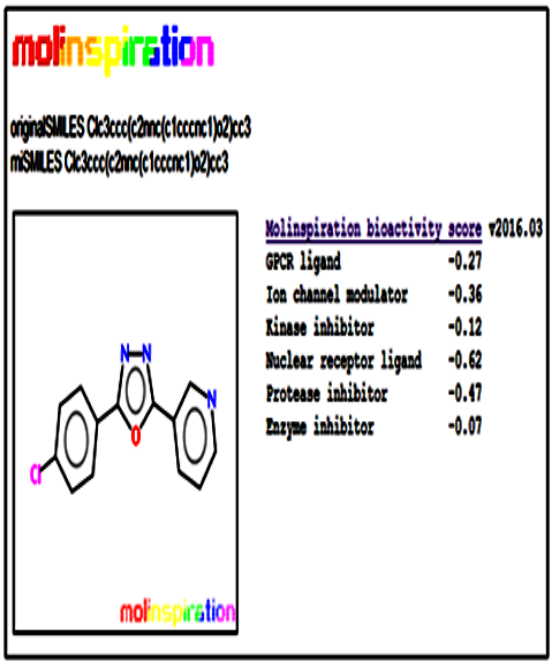
CODE: VS4





CODE:VS5

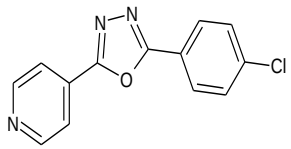
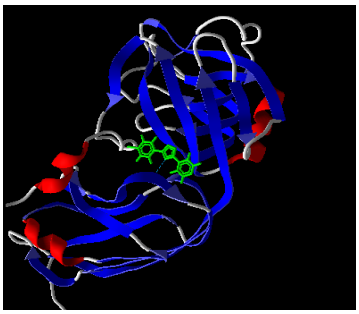
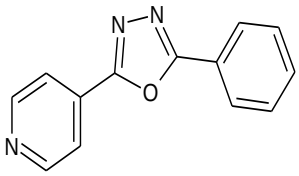
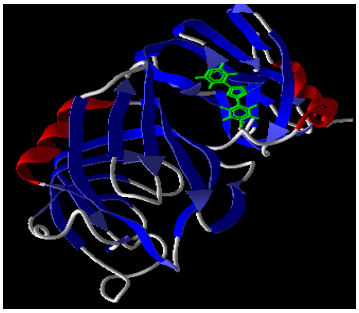
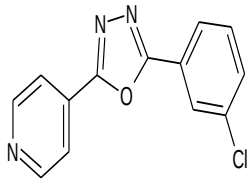
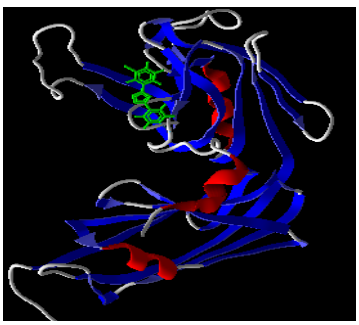




DOCKING

The derivatives of the selected 100 compounds were docked against 3VYP (Mtb enzyme).The molecules were screened for good docking score and interactions. The results of the docking for the different compounds are presented below:

TABLE 1- DOCKING SCORES AND VIEW

CODE	CHEMICAL STRUCTURE	DOCKING SCORE	DOCKING VIEW
VS1	 4-[5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl]pyridine	-9.796 Kcal/mol	
VS2	 4-(5-phenyl-1,3,4-oxadiazol-2-yl)pyridine	-9.672 Kcal/mol	
VS4	 4-[5-(3-chlorophenyl)-1,3,4-oxadiazol-2-yl]pyridine	-9.541 Kcal/mol	

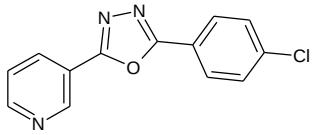
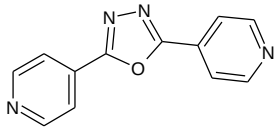
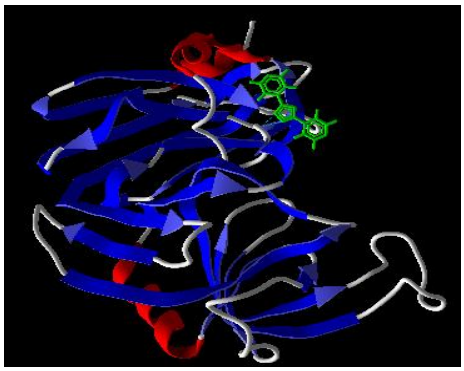
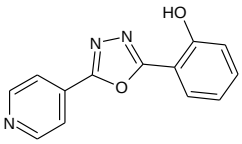
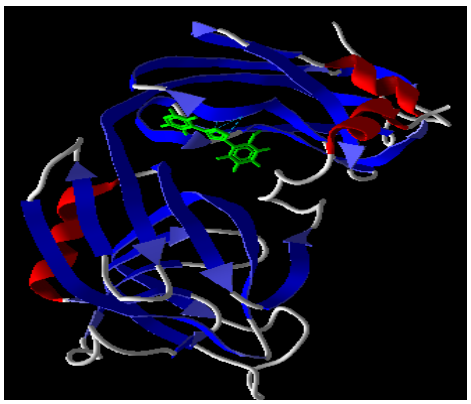
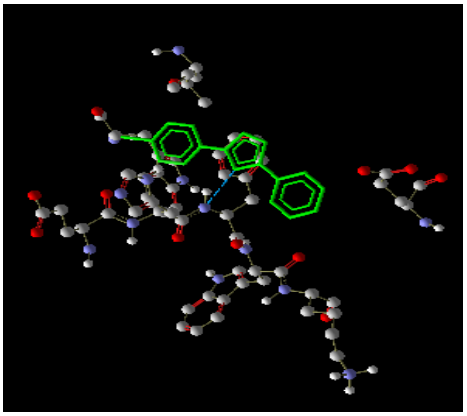
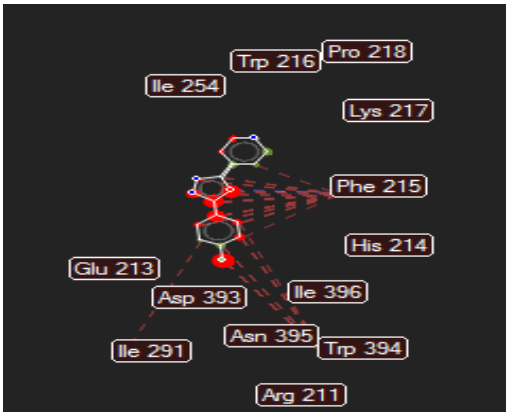
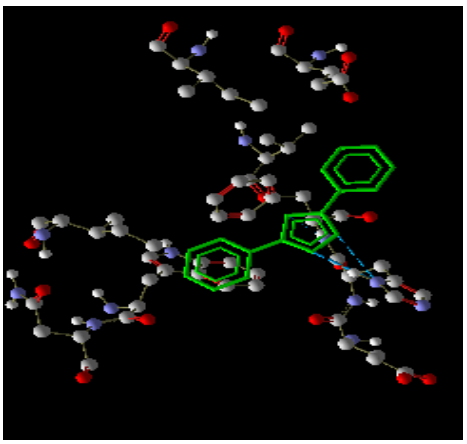
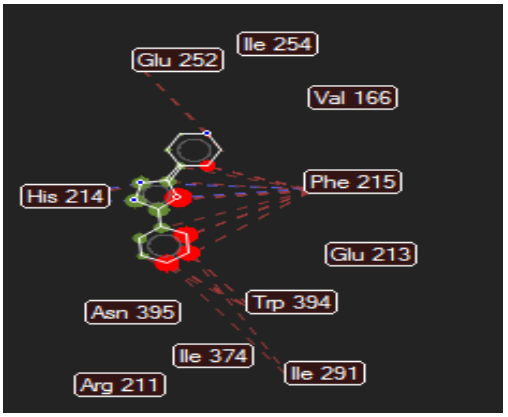
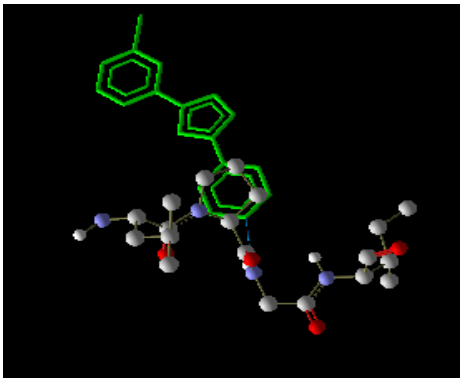
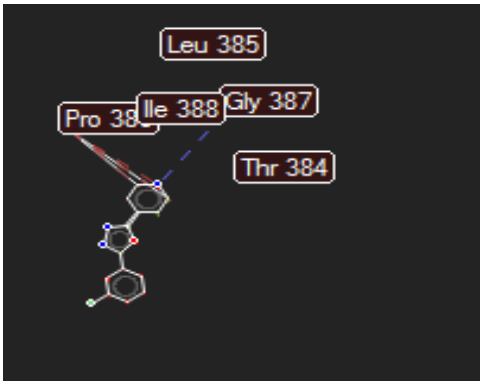
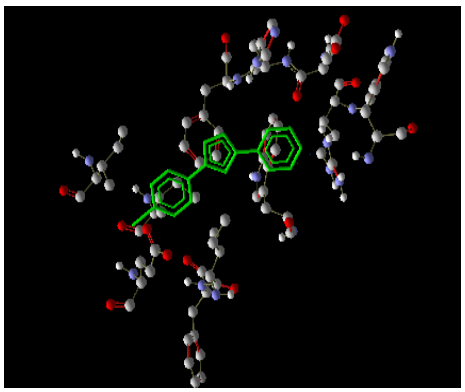
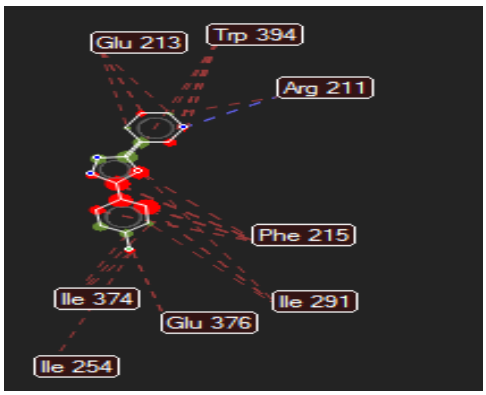
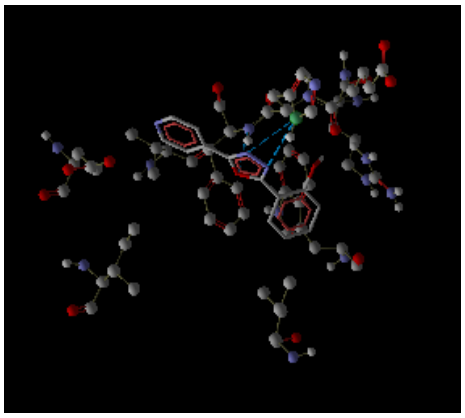
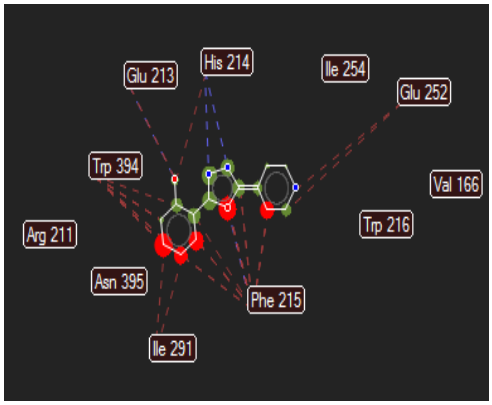
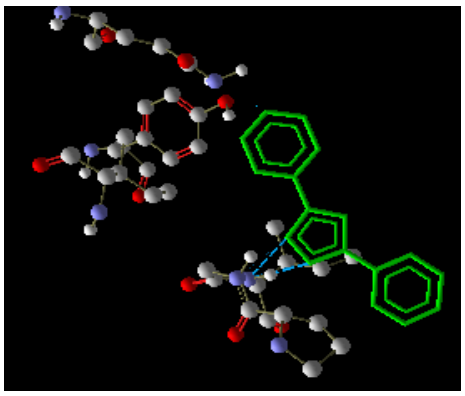
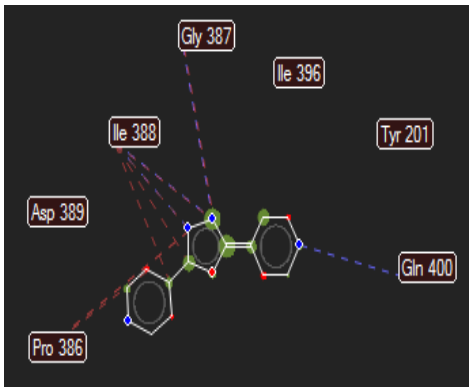
CODE	CHEMICAL STRUCTURE	DOCKING SCORE	DOCKING VIEW
VS5	 3-[5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl]pyridine	-9.452 Kcal/mol	
NA	 4,4'-(1,3,4-oxadiazole-2,5-diyl)dipyridine	-8.351 Kcal/mol	
SA	 2-[5-(pyridin-4-yl)-1,3,4-oxadiazol-2-yl]phenol	-9.310 Kcal/mol	

TABLE 2-INTERACTIONS WITH HYDROGEN BOND AND AMINO ACIDS

CODE	HYDROGEN BOND INTERACTIONS	INTERACTION WITH AMINO ACIDS
VS1		
VS2		
VS4		

CODE	HYDROGEN BOND INTERACTIONS	INTERACTION WITH AMINO ACIDS
VS5		
SA		
NA		

TOXICITY PREDICTION (OSIRIS PROPERTY EXPLORER)

All the data set molecules were subjected to the toxicity risk assessment by using Osiris program.Prediction results are color coded in which the red color shows high risks with undesired effects like mutagenicity or a poor intestinal absorption and green color indicates drug-conform behavior.

SAMPLE CODE: SA

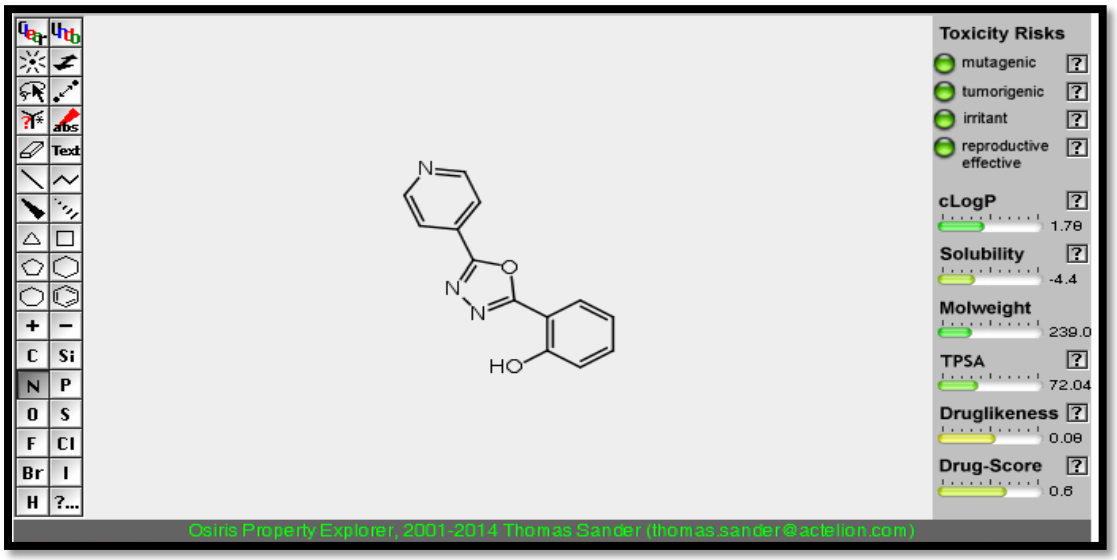


FIGURE 8- TOXICITY PREDICTION OF SAMPLE SA

SAMPLE CODE: NA

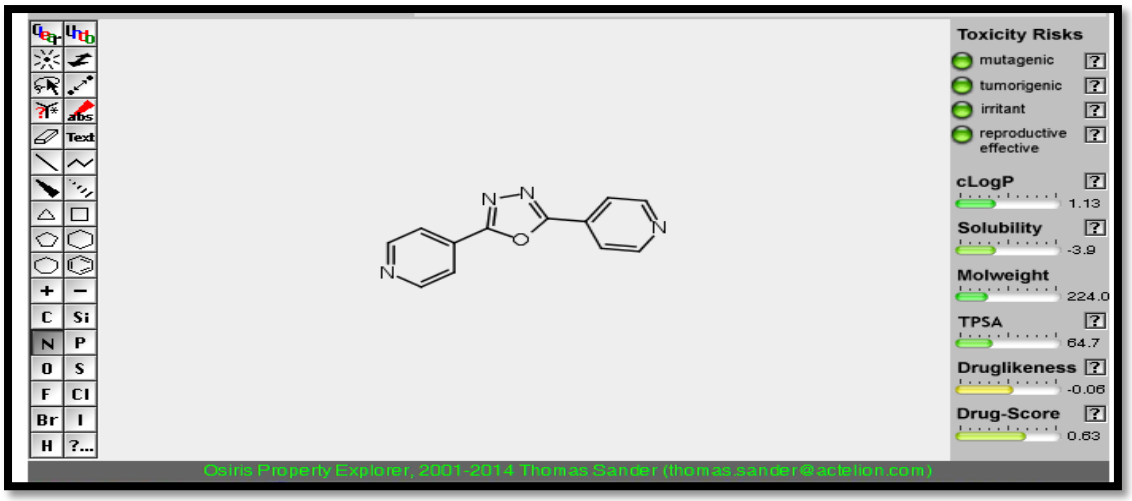


FIGURE 9- TOXICITY PREDICTION OF SAMPLE NA

SAMPLE CODE: VS1

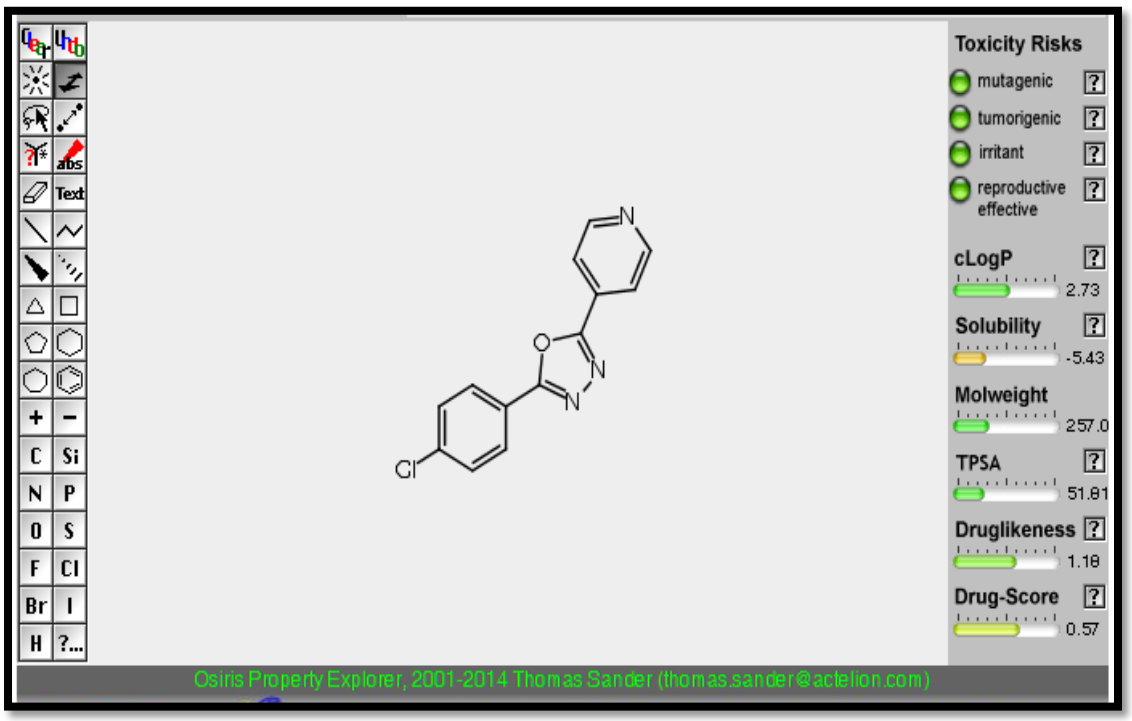


FIGURE 10 - TOXICITY PREDICTION OF SAMPLE VS1

SAMPLE CODE: VS2

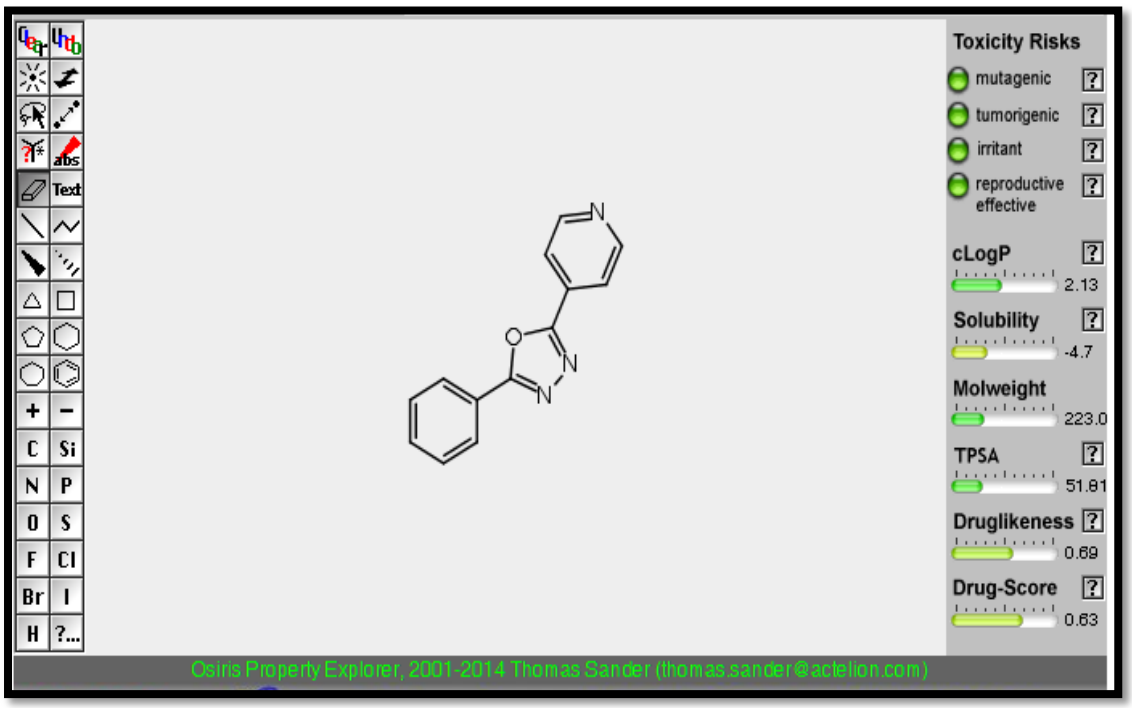


FIGURE 11- TOXICITY PREDICTION OF SAMPLE VS2

SAMPLE CODE: VS4

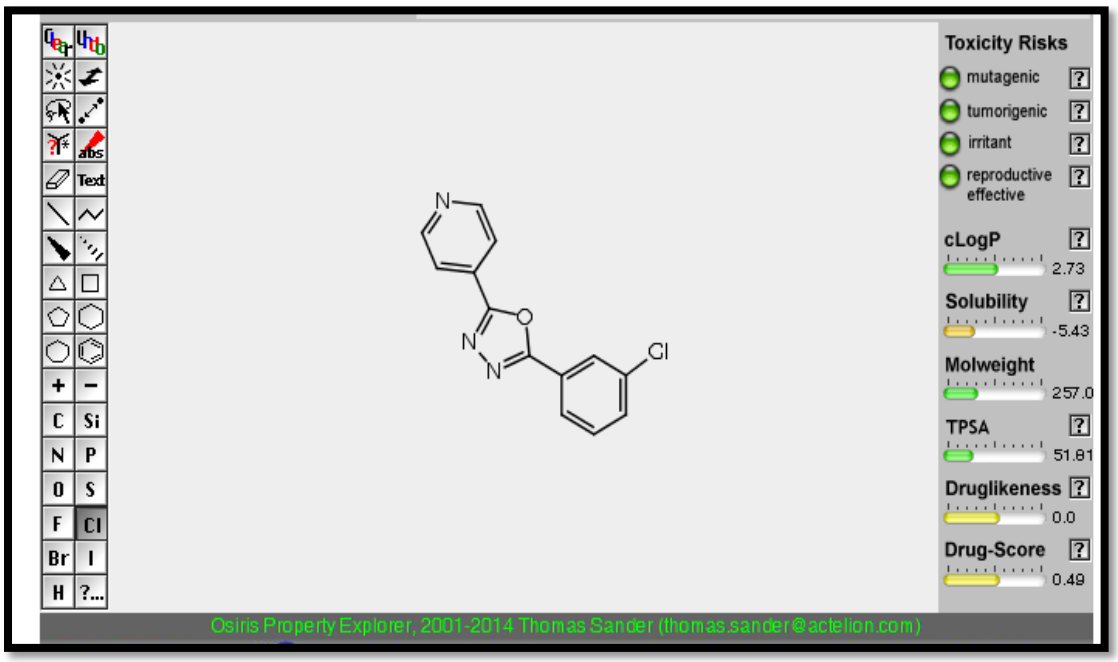


FIGURE 12- TOXICITY PREDICTION OF SAMPLE VS4

SAMPLE CODE: VS5

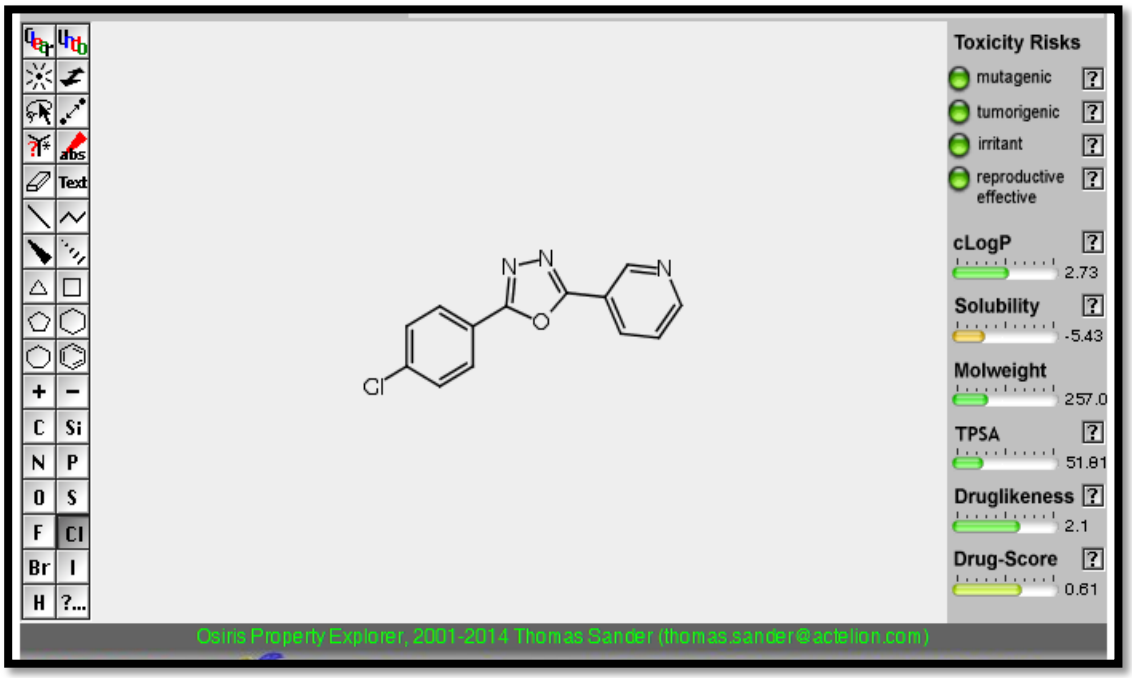


FIGURE 13- TOXICITY PREDICTION OF SAMPLE VS5

PRODUCT PROFILE

The synthesized compounds were evaluated for their purity through melting point determination and checking absence of parent compounds or other new compounds by TLC. The mobile phase used for all the synthesized compounds was benzene: Acetone in the ratio of 3:2. The selected 6 scaffolds were synthesized in an appropriate manner and recystallized. Then the purity of the compounds was determined by sharp melting point and single spot obtained in the TLC.

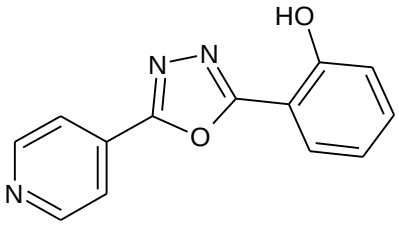
TABLE 3

SAMPLE CODE	MOLECULAR WEIGHT	MELTING POINT	Rf VALUE	YIELD (%)
SA	239	196^{o}C	0.71	80%
NA	224	157 ^{o}C	0.51	72%
VS1	257	166 ^{o}C	0.61	70%
VS2	257	92 ^{o}C	0.55	75%
VS4	257	108 ^{o}C	0.44	82%
VS5	257	165 ^{o}C	0.53	74%

The Rf value of the synthesized compounds was varied from the Rf value of the reactants.It is concluded that the reaction was completed.The compounds were obtained in acceptable yield.

CODE SA

IUPAC NAME: 2-[5-(pyridin-4-yl)-1, 3, 4-oxadiazol-2-yl] phenol



Molecular Formula	-$C_{13}H_9N_3O_2$
Molecular Weight	-239.22
Appearance	-Yellow color
Melting Point	-196^{0}C
Yield	-80%
Composition	- C (65.27%) H (3.79%) N (17.56%) O (13.38%)
Molar Refractivity	- 63.89 ± 0.3 cm3
Molar Volume	- 180.8 ± 3.0 cm3
Parachor	-505.4 ± 4.0 cm3
Index of Refraction	-1.624 ± 0.02
Surface Tension	-57.8 ± 3.0 dyne/cm
Density	- 1.322 ± 0.06 g/cm3
Polarizability	-25.32 ± 0.5 10- 24 cm3

IR SPECTRUM: SA

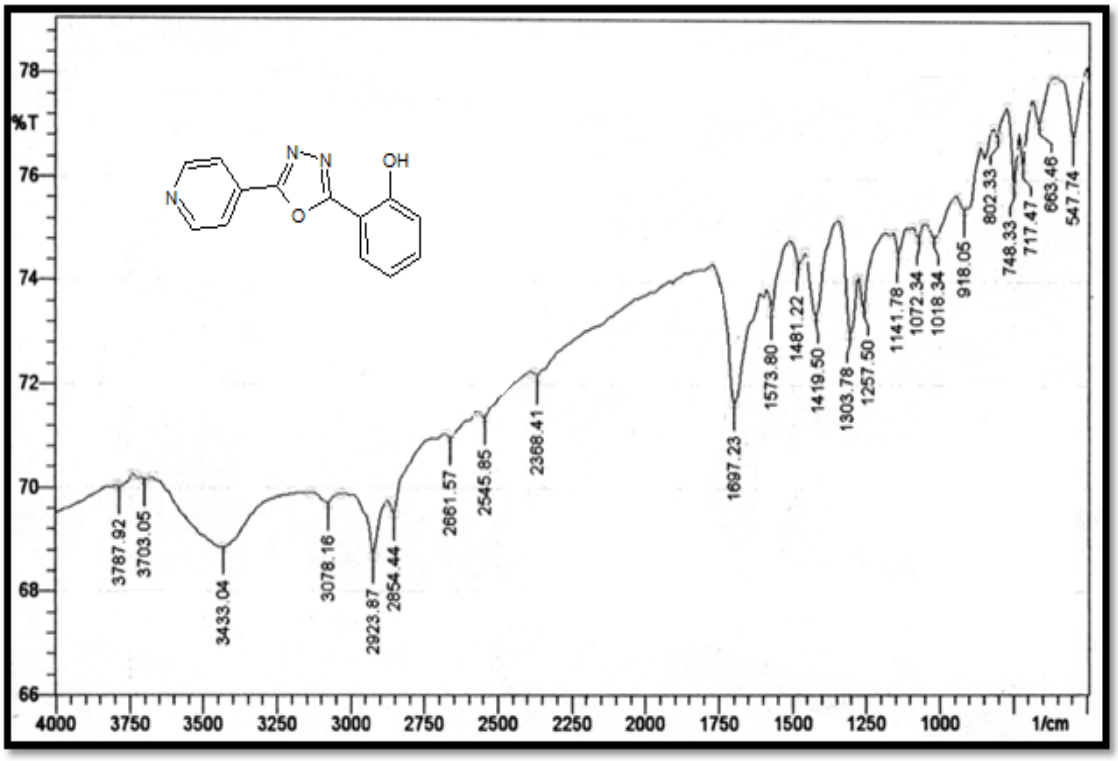
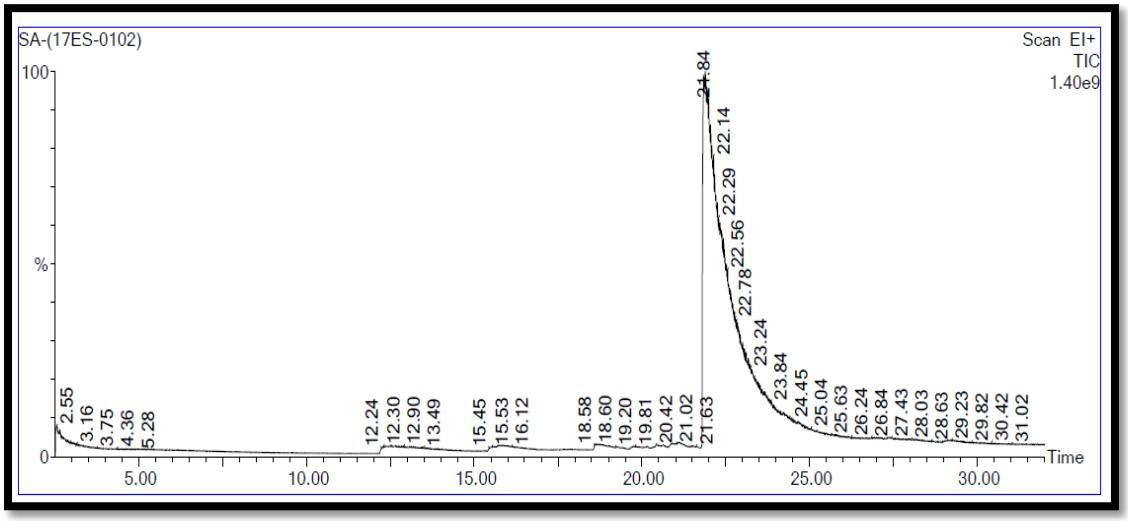


FIGURE 14- IR SPECTRUM OF SAMPLE SA

TABLE 4- IR INTERPRETATION OF SAMPLE SA

S.No	Wavenumber (cm^{-1})	Functional group
1.	3078 cm^{-1}	Ar C-H stretching
2.	1257 cm^{-1}	C-O-C Stretching
3.	1573 cm^{-1}	Ar C=C Stretching
4	1697 cm-1	C=N Stretching
5.	3433 cm^{-1}	O-H Stretching

GC-MS : SA



#	RT	Scan	Height	Area	Area %	Norm %
1	21.886	3876	1,365,966,592	1,159,584,640.0	100.000	100.00

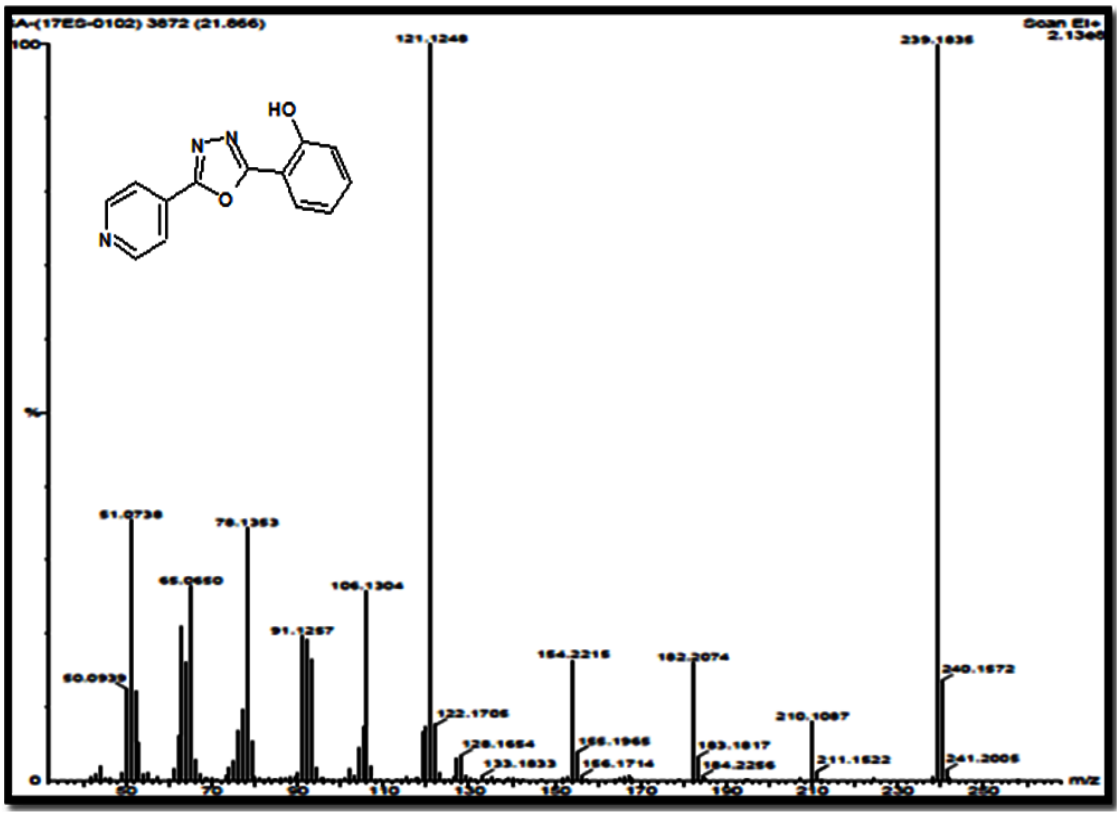


FIGURE 15- GC-MS SPECTRUM OF SAMPLE CODE SA

H1NMR SPECTRUM: SA

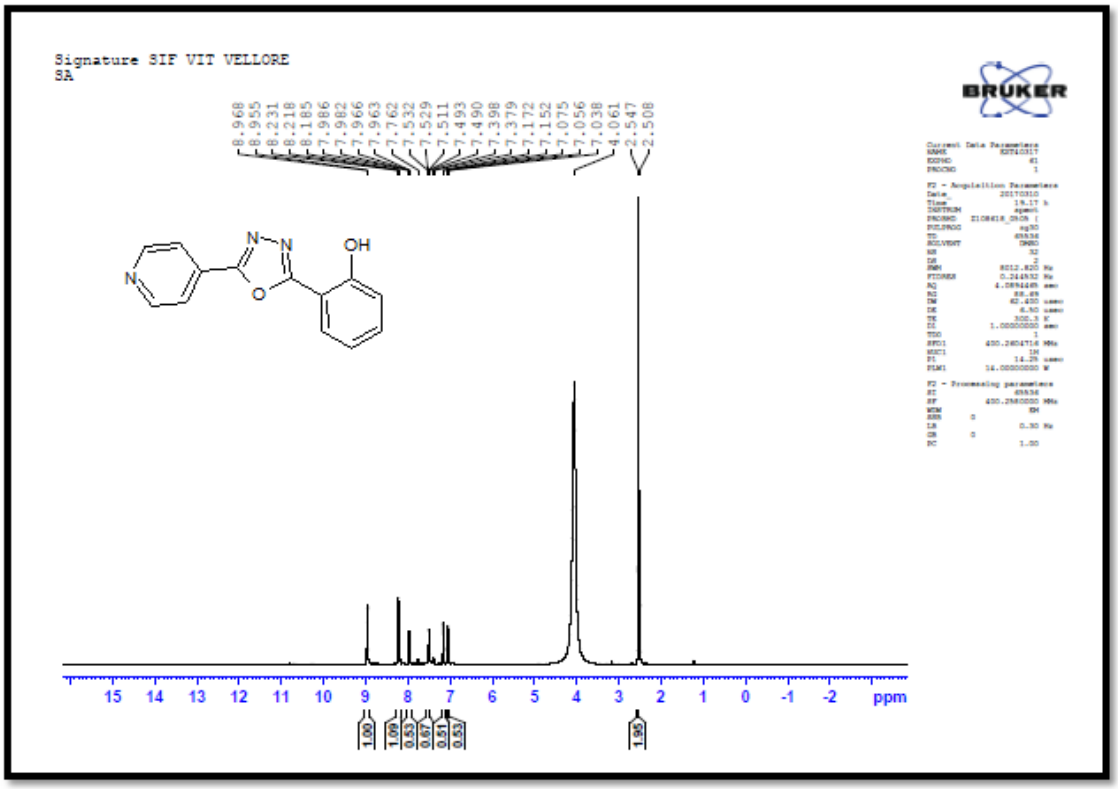


FIGURE 16-H1 NMR SPECTRUM OF SAMPLE SA

TABLE 5- H1 NMR INTERPRETATION OF SAMPLE SA

S.NO	δ VALUE(PPM)	NATURE OF PEAK	NUMBER OF PROTONS
1.	2.54-2.55ppm	Singlet	2
2.	7.03-7.17ppm	Triplet	2
3.	7.37-7.53 ppm	Triplet	1
4.	7.96-7.98 ppm	Doublet	1
5.	8.18-8.23 ppm	Doublet	2
6.	8.95-8.96 ppm	Doublet	1

C13 SPECTRUM OF SA

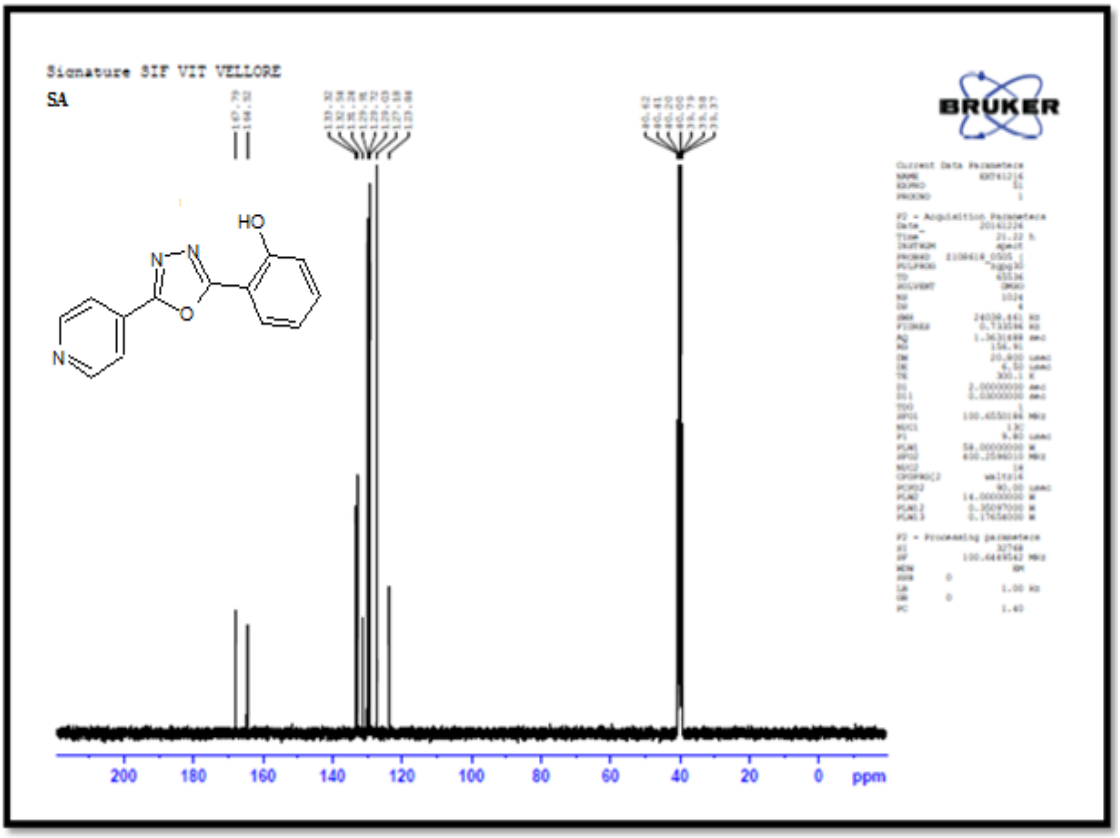
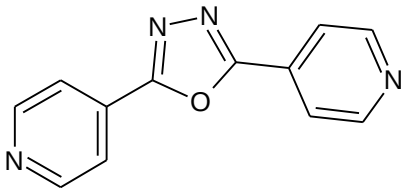


FIGURE 17- C13 SPECTRUM OF SA

CODE: NA

IUPAC NAME: 4, 4'-(1, 3, 4-oxadiazole-2, 5-diyl) dipyridine



Molecular Formula	-$C_{13}H_8N_4O$
Molecular Weight	-224.21
Appearance	-Brown color
Melting Point	-157^{0}C
Yield	-72%
Composition	- C (64.10%) H (3.60%) N (24.99%) O (7.14%)
Molar Refractivity	- 60.10 ± 0.3 cm3
Molar Volume	- 194.4 ± 3.0 cm3
Parachor	-484.5 ± 4.0 cm3
Index of Refraction	-1.599 ± 0.02
Surface Tension	-57.8 ± 3.0 dyne/cm
Density	- 1.276 ± 0.06 g/cm3
Polarizability	-23.82 ± 0.5 10- 24 cm3
Nominal mass	-224Da
Monoisotopic mass	-224.04Da

IR SPECTRUM: NA

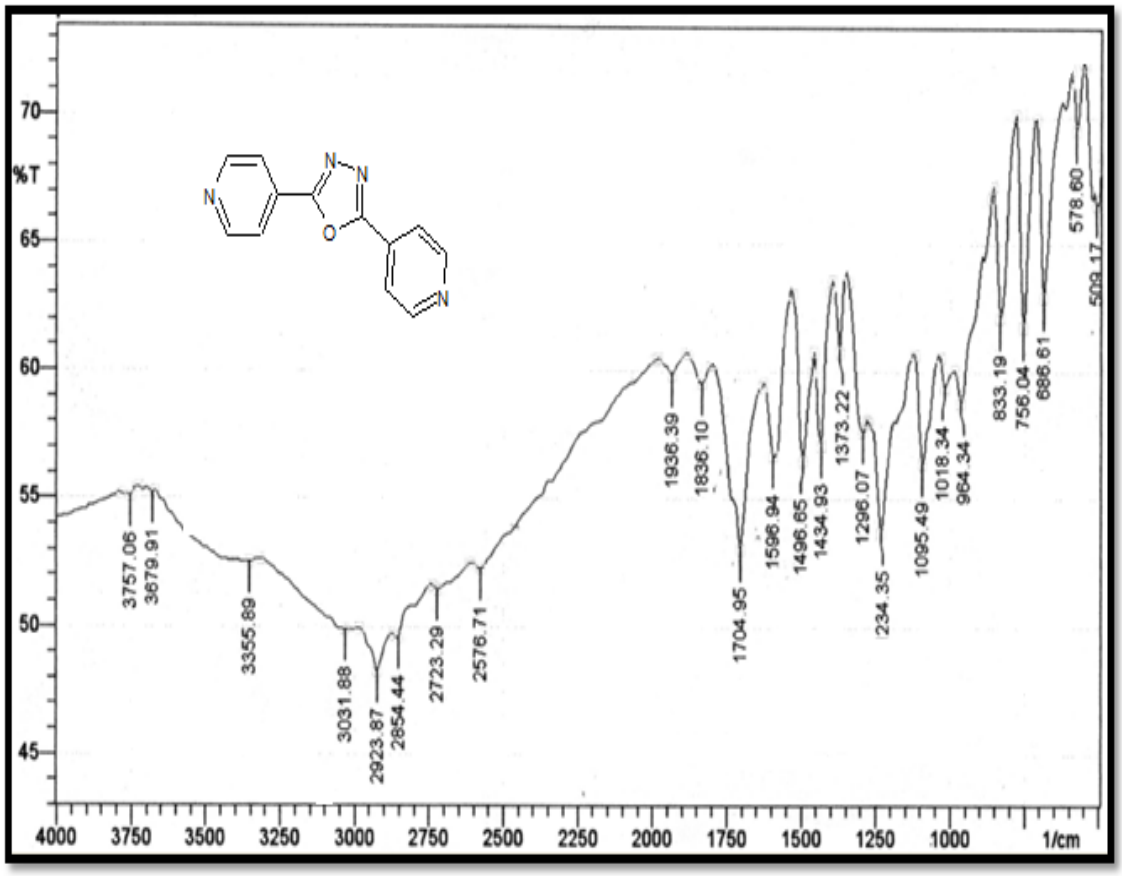
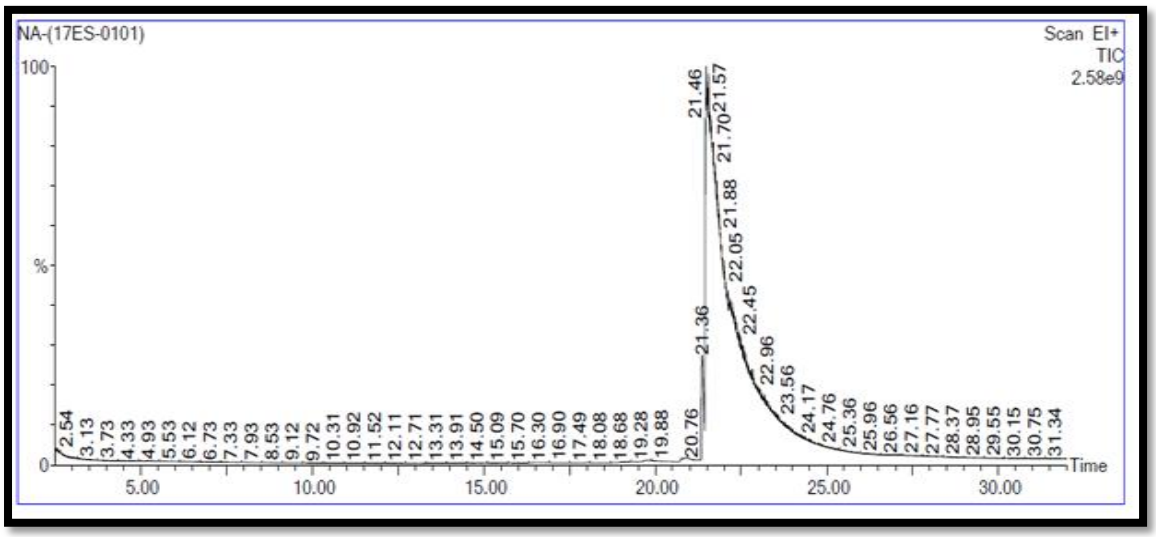


FIGURE 18- IR SPECTRUM OF SAMPLE NA

TABLE 6- IR INTERPRETATION OF SAMPLE NA

S.no	Wavenumber (cm^{-1})	Functional group
1.	3031 cm^{-1}	Ar C-H stretching
2.	1296 cm^{-1}	C-O-C Stretching
3.	1596 cm^{-1}	Ar C=C Stretching
4.	1704 cm-1	C=N Stretching

GC-MS SPECTRUM: NA



#	RT	Scan	Height	Area	Area %	Norm %
1	21.486	3796	2,358,435,328	1,460,902,784.0	100.000	100.00

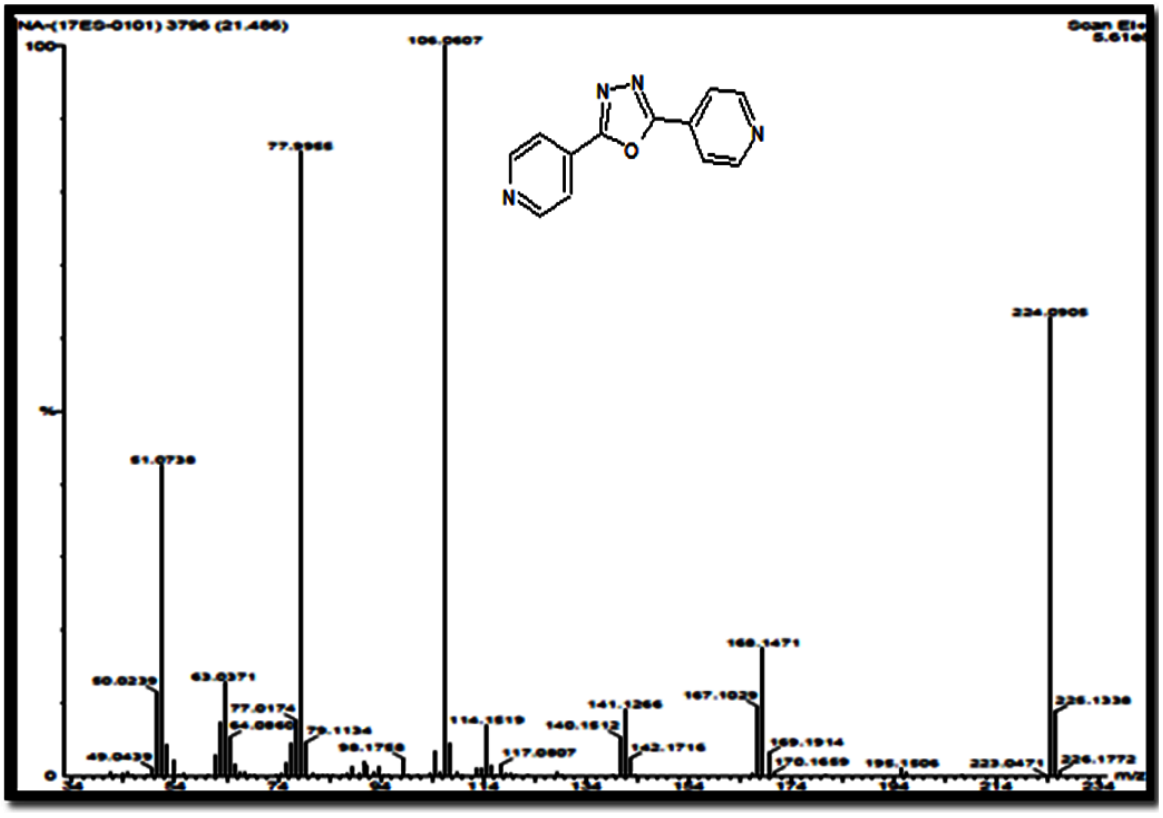


FIGURE 19- GC-MS SPECTRUM OF SAMPLE NA

H1NMR SPECTRUM: NA

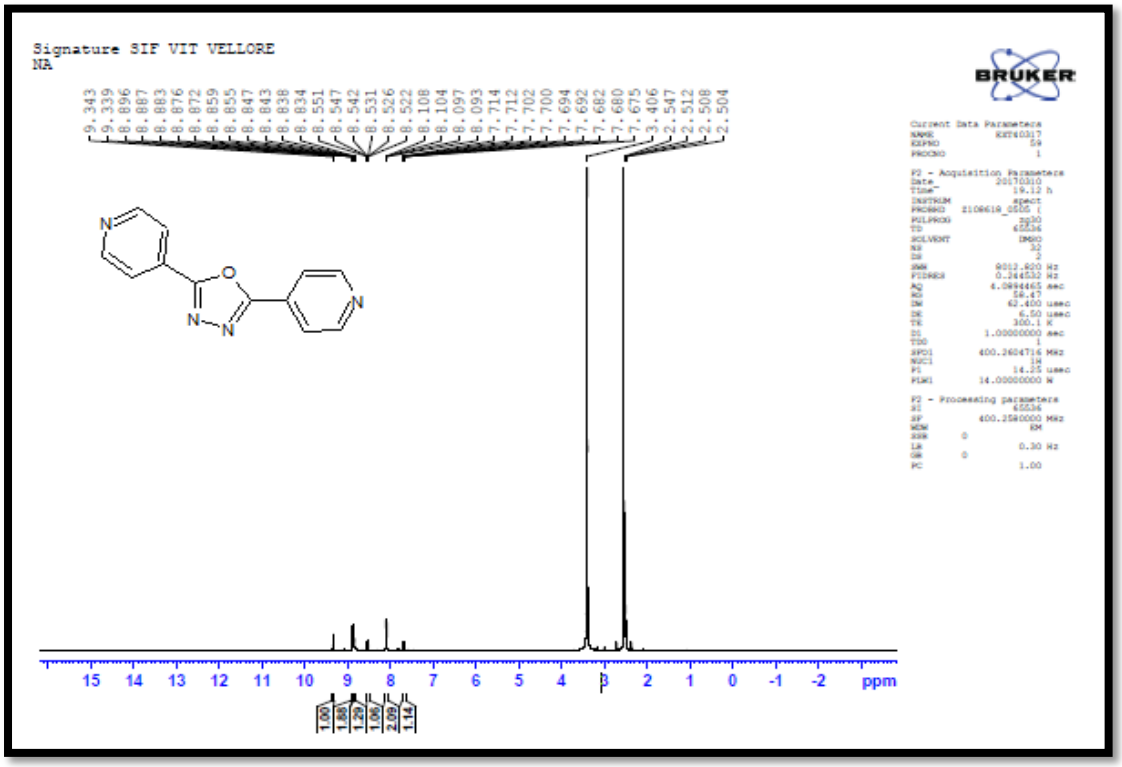


FIGURE 20-H1 NMR SPECTRUM OF SAMPLE NA

TABLE 7- H1NMR INTERPRETATION OF SAMPLE NA

S.NO	δ VALUE(PPM)	NATURE OF PEAK	NUMBER OF PROTONS
1.	7.68-7.71ppm	Quartet	1
2.	8.09-8.10ppm	Triplet	2
3.	8.52-8.55ppm	Multiplet	1
4.	8.83-8.89ppm	Multiplet	3
5.	9.33-9.34ppm	Doublet	1

C13 SPECTRUM OF NA

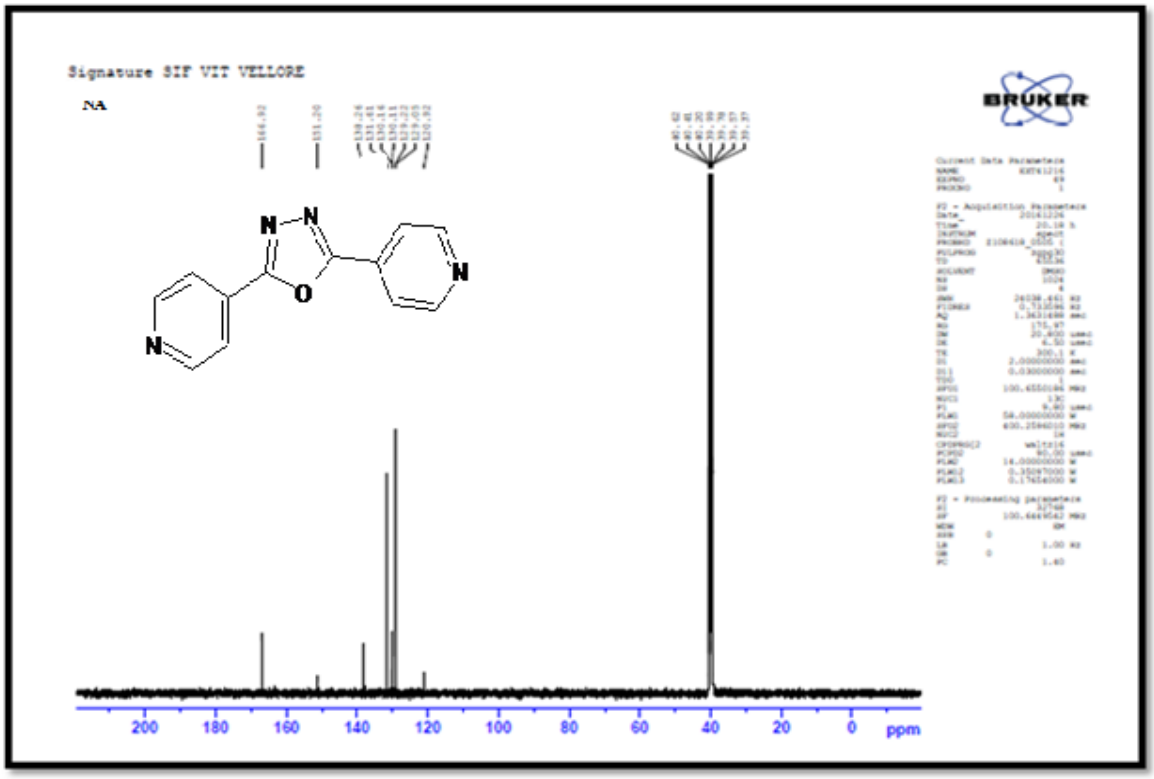
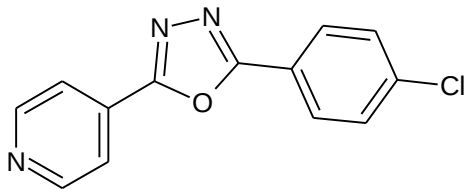


FIGURE 21- C13 SPECTRUM OF NA

CODE: VS1

IUPAC NAME: 4-[5-(4-chlorophenyl)-1, 3, 4-oxadiazol-2-yl] pyridine



Molecular Formula	-$C_{13}H_8ClN_3O$
Molecular Weight	-257.67
Appearance	-White color
Melting Point	-166^{0}C
Yield	-70%
Composition	- C (60.60%) H (3.13%) Cl (13.76) N (16.31%) O (6.21%)
Molar Refractivity	- 66.90 ± 0.3 cm^3
Molar Volume	-194.4 ± 3.0 cm^3
Parachor	-526.2 ± 4.0 cm^3
Index of Refraction	-1.604 ± 0.02
Surface Tension	-53.6 ± 3.0 dyne/cm
Density	- 1.325 ± 0.06 g/cm^3
Polarizability	-26.52 ±0.5 10- 24 cm^3
Nominal mass	-257Da
Monoisotopic mass	-257.033Da

IR SPECTRUM: VS1

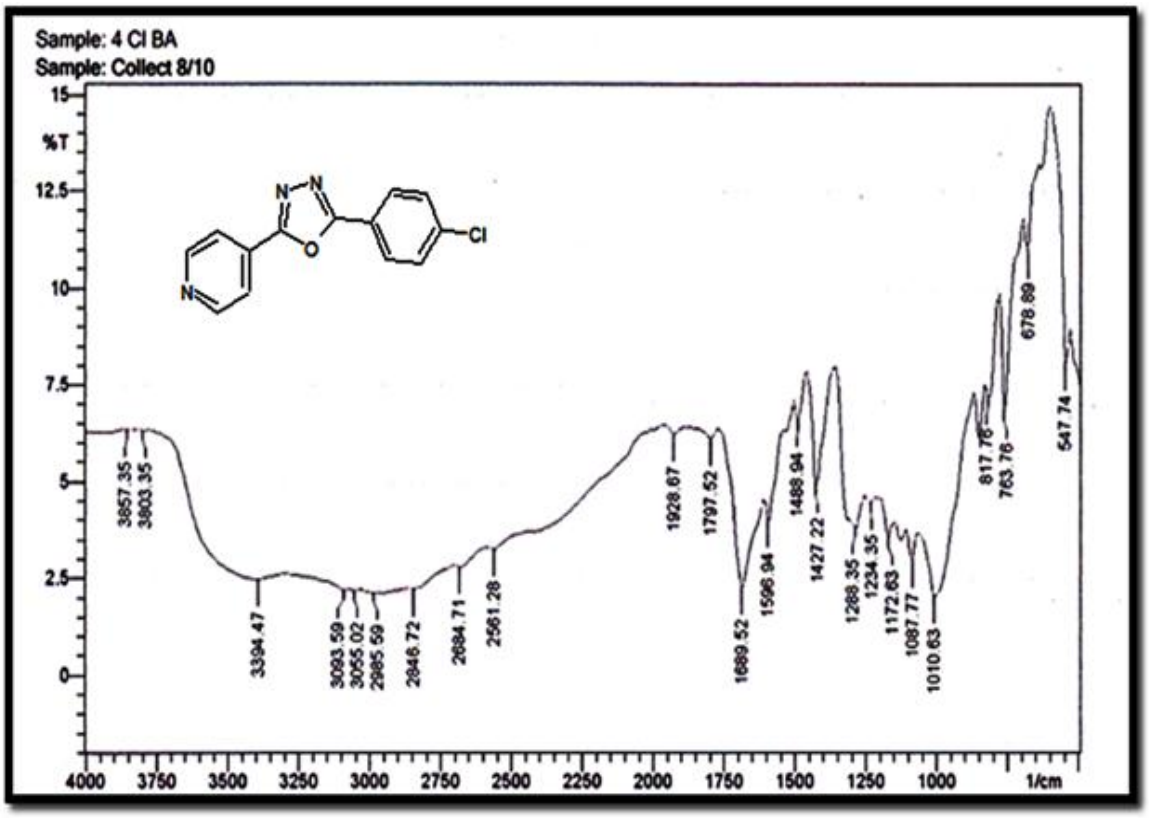


FIGURE 22- IR SPECTRUM OF SAMPLE VS1

TABLE 8- IR INTERPRETATION OF SAMPLE VS1

S.no	Wavenumber (cm^{-1})	Functional group
1.	3055 cm^{-1}	Ar C-H stretching
2.	1288 cm^{-1}	C-O-C Stretching
3.	1596 cm^{-1}	Ar C=C Stretching
4.	1689 cm-1	C=N Stretching
5.	753cm^{-1}	C-Cl Stretching

LC-MS SPECTRUM: VS1

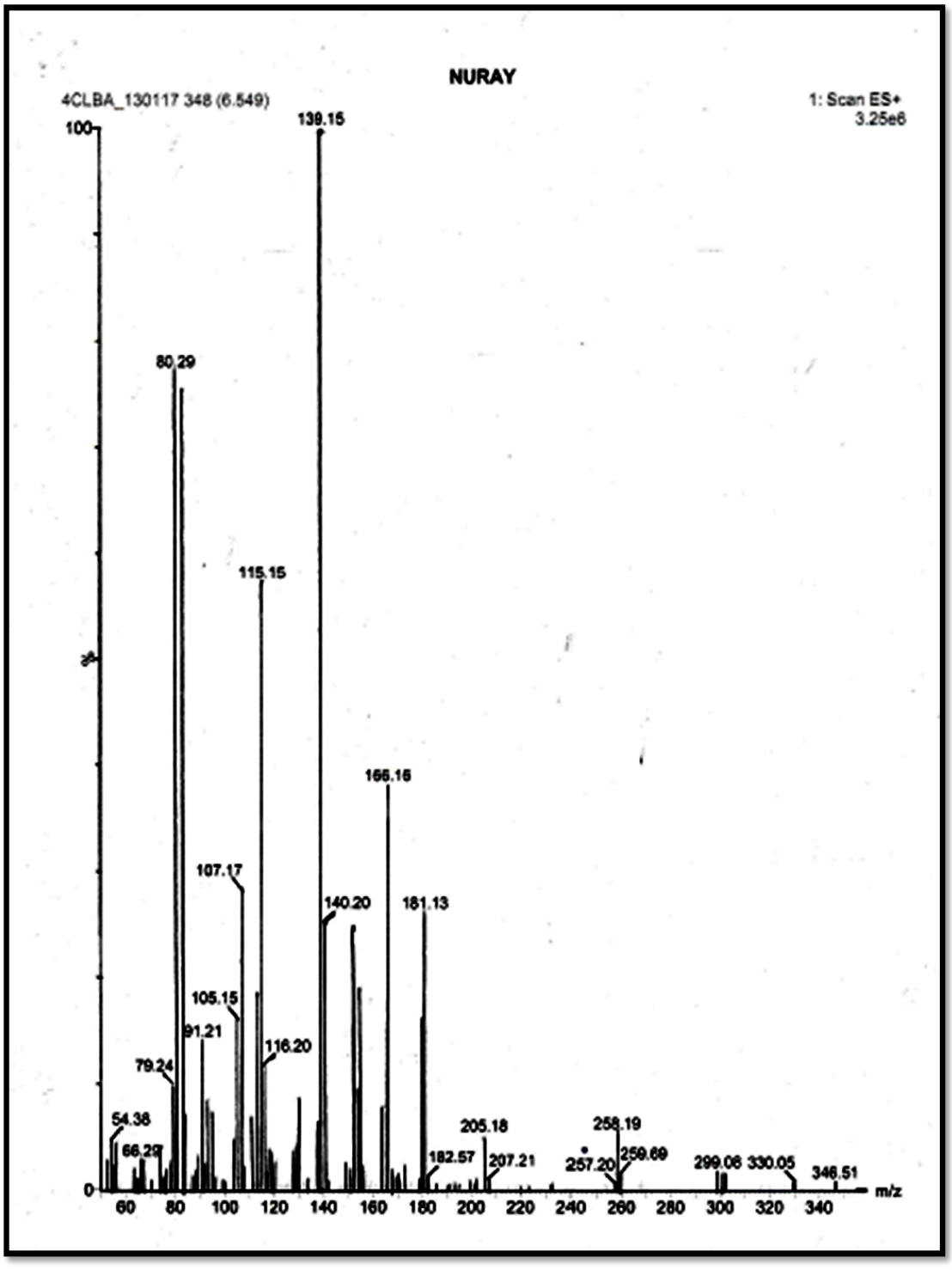


FIGURE 23- LC-MS SPECTRUM OF SAMPLE VS1

H^1NMR SPECTRUM: VS1

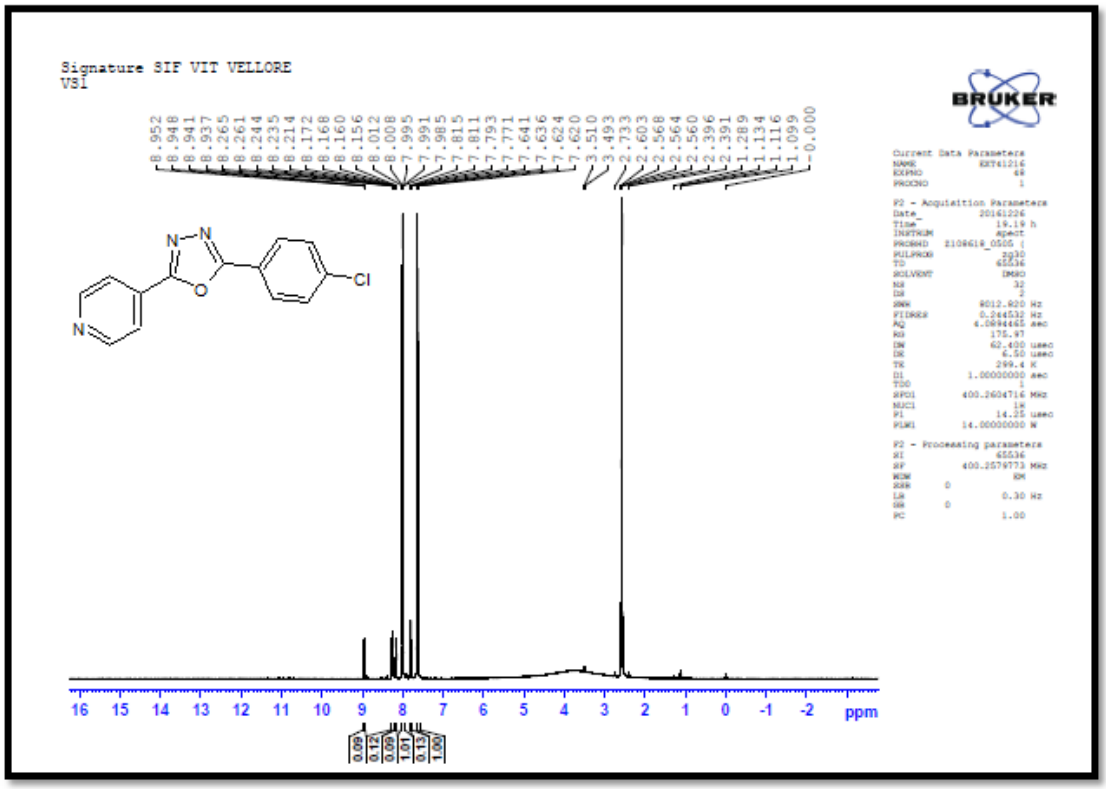


FIGURE 24- H1 NMR SPECTRUM OF SAMPLE VS1

TABLE 9- H1NMR INTERPRETATION OF SAMPLE VS1

S.NO	δ VALUE(PPM)	NATURE OF PEAK	NUMBER OF PROTONS
1.	7.62-7.64ppm	Doublet	2
2.	7.77-7.81ppm	Doublet	1
3.	7.98-7.8.01ppm	Doublet	2
4.	8.15-8.26ppm	Doublet	2
5.	8.93-8.95ppm	Doublet	1

C13NMR SPECTUM: VS1

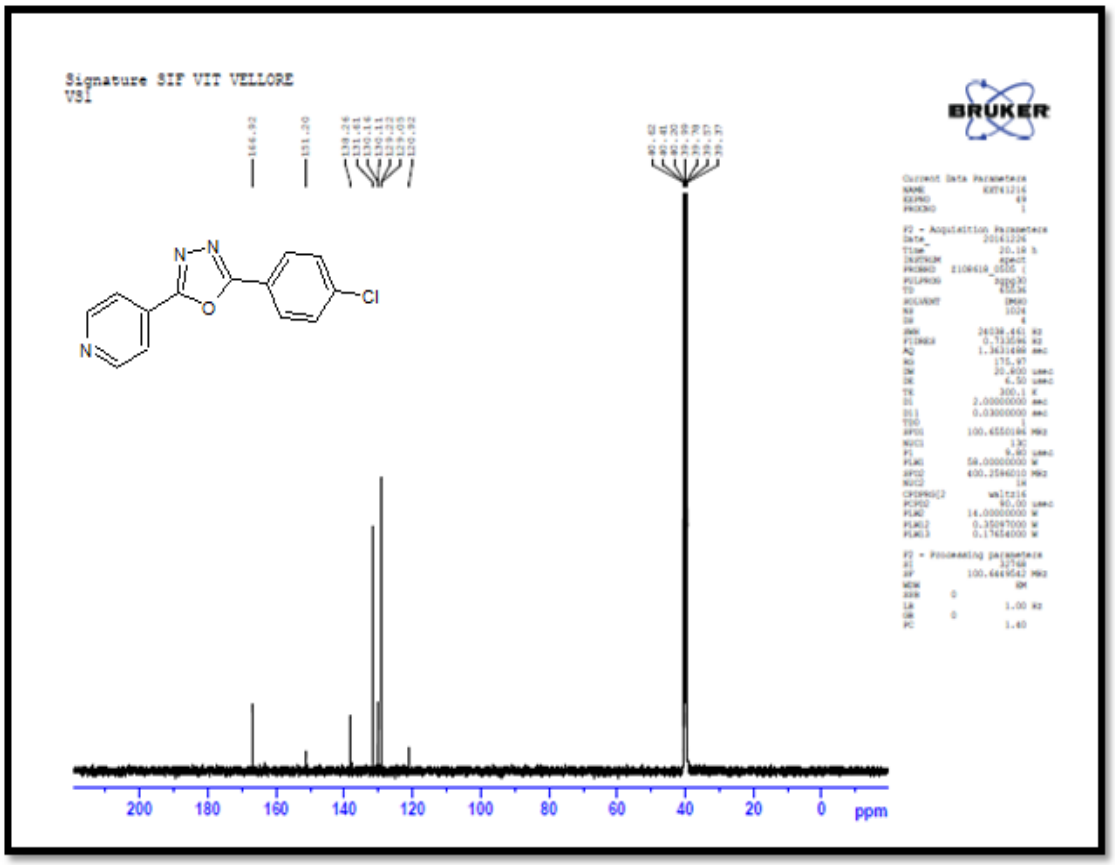
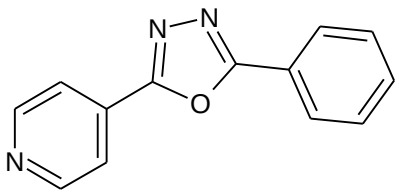


FIGURE 25-C13 NMR SPECTRUM OF SAMPLE VS1

CODE: VS2

IUPAC NAME: 4-(5-phenyl-1, 3, 4-oxadiazol-2-yl) pyridine



Molecular Formula	-$C_{13}H_9N_3O$
Molecular Weight	-223.23
Appearance	- White color
Melting Point	-92^{0}C
Yield	-75%
Composition	- C (69.95%) H (4.06%) N (18.82%) O (7.17%)
Molar Refractivity	- 62.01 ± 0.3 cm3
Molar Volume	- 182.4 ± 3.0 cm3
Parachor	-490.4 ± 4.0 cm3
Index of Refraction	-1.595 ± 0.02
Surface Tension	-52.1 ± 3.0 dyne/cm
Density	- 1.223 ± 0.06 g/cm3
Polarizability	-26.52 ±0.5 10- 24 cm3
Nominal mass	-223Da
Monoisotopic mass	- 223.03Da

IR SPECTRUM: VS2

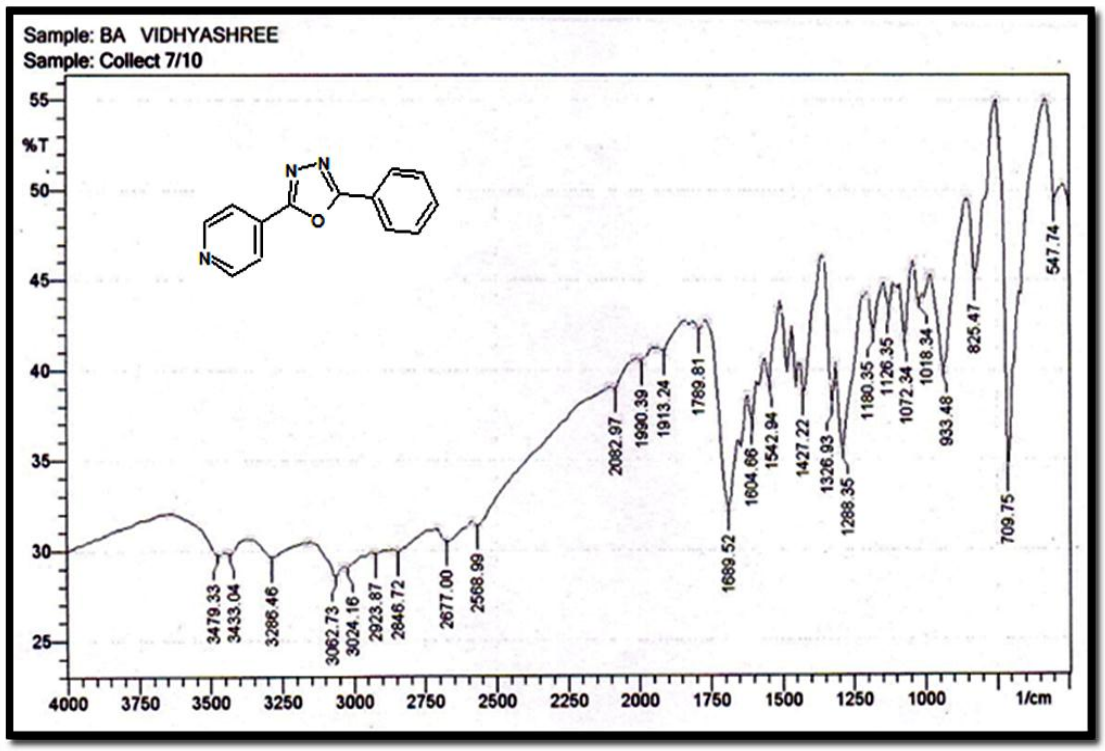


FIGURE 26- IR SPECTRUM OF SAMPLE VS2

TABLE 10- IR INTERPRETATION OF SAMPLE VS2

S.no	Wave number (cm^{-1})	Functional group
1.	3062 cm^{-1}	Ar C-H stretching
2.	1288 cm^{-1}	C-O-C Stretching
3.	1542 cm^{-1}	Ar C=C Stretching
4.	1606 cm-1	C=N Stretching

LC-MS SPECTRUM: VS2

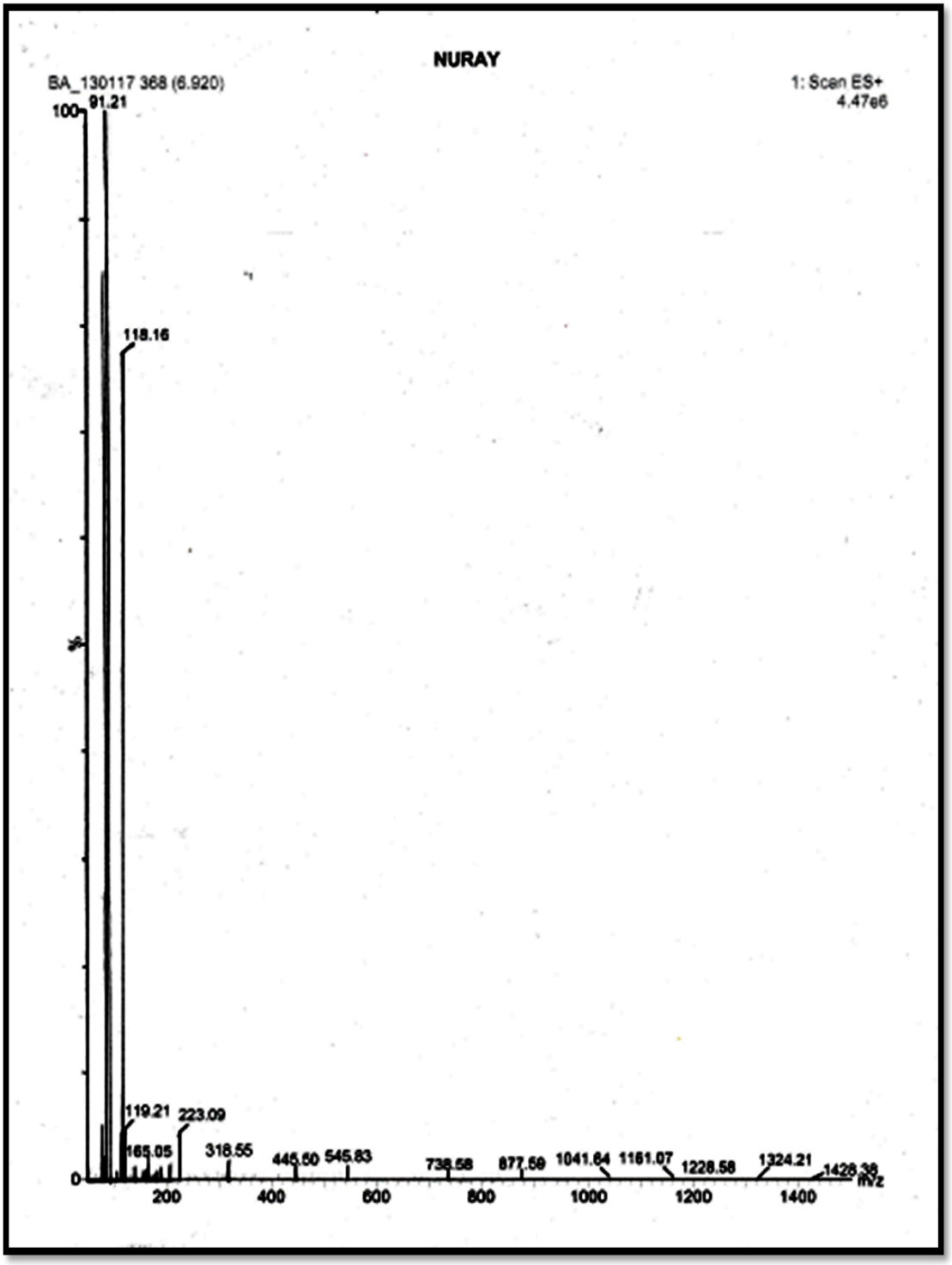


FIGURE 27- IR SPECTRUM OF SAMPLE VS2

H^1NMRSPECTRUM: VS2

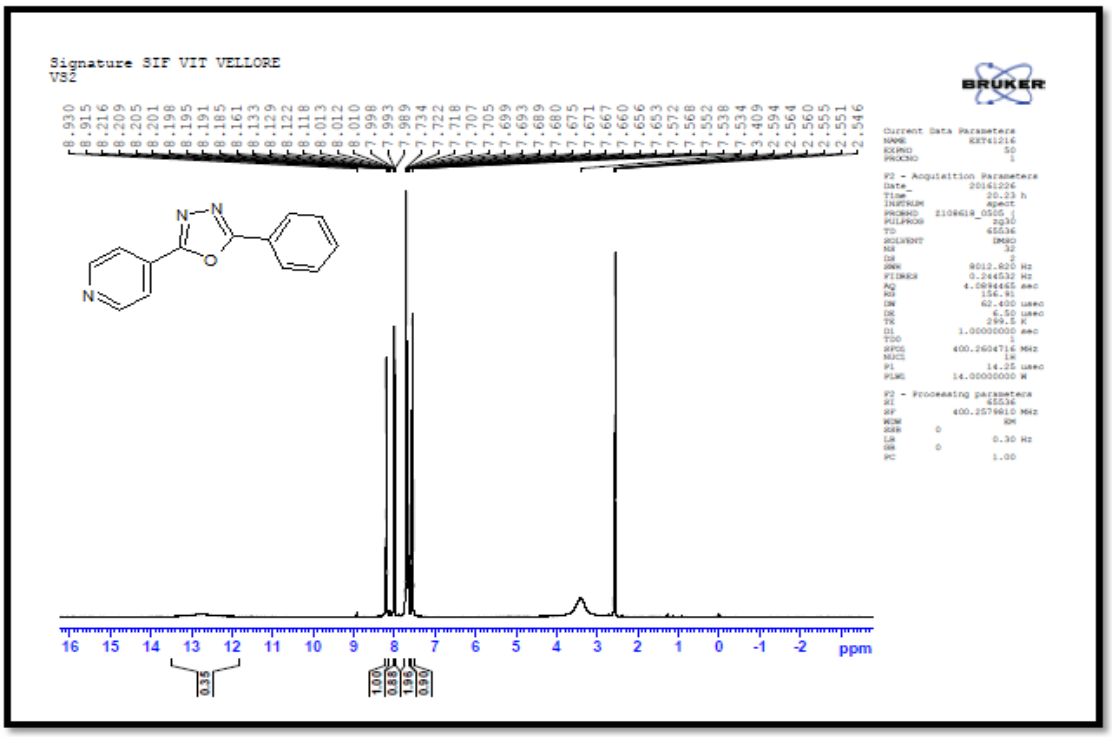


FIGURE 28- H1 NMR SPECTRUM OF SAMPLE VS4

TABLE 11-H1 NMR INTERPRETATION OF SAMPLE VS4

S.NO	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	7.53-7.57ppm	Triplet	1
2.	7.65-7.73ppm	Multiplet	4
3.	7.98-7.8.01ppm	Triplet	2
4.	8.18-8.2ppm	Triplet	2

C13 NMR SPECTRUM: VS2

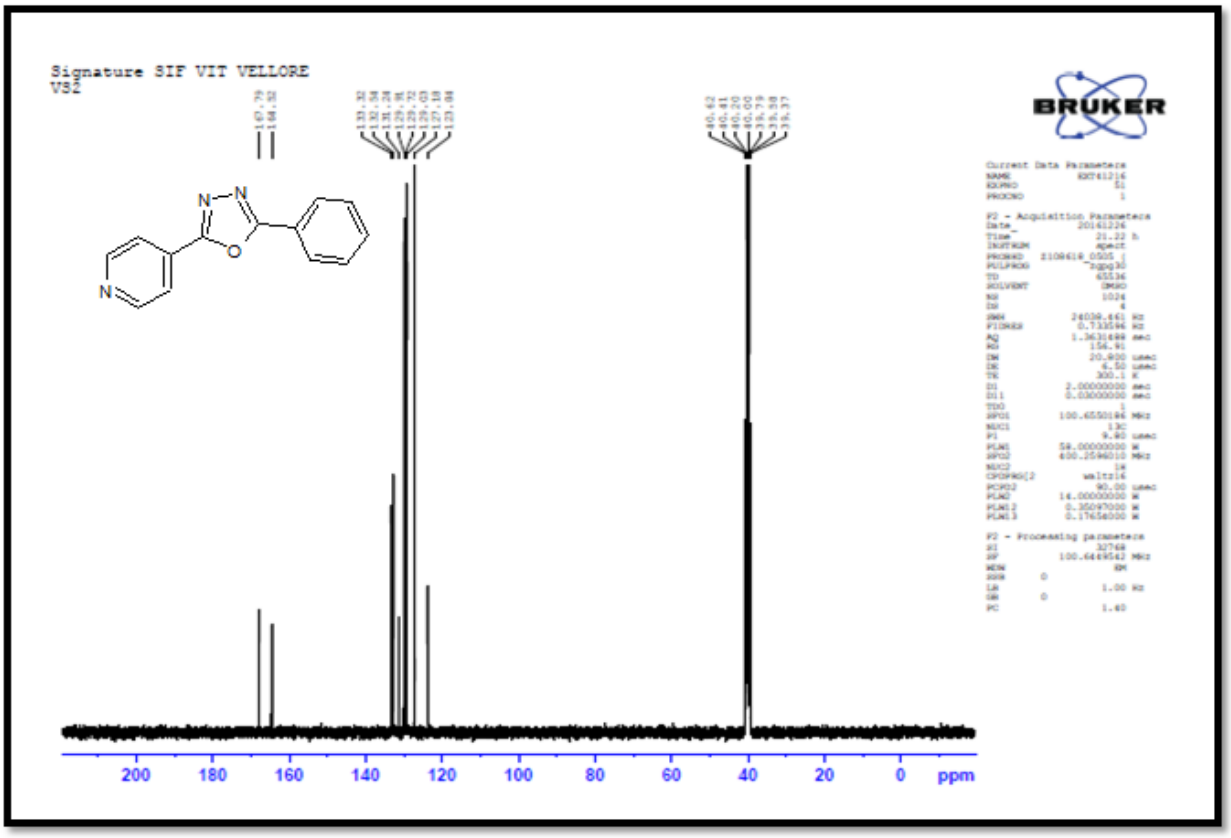
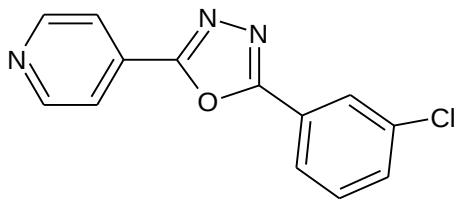


FIGURE 29- C13 NMR SPECTRUM OF SAMPLE VS2

CODE: VS4

IUPAC NAME: 4-[5-(3-chlorophenyl)-1, 3, 4-oxadiazol-2-yl] pyridine



Molecular Formula	-$C_{13}H_8ClN_3O$
Molecular Weight	-257.67
Appearance	- White color
Melting Point	-108^0C
Yield	-82%
Composition	- C (60.60%) H (3.13%) Cl (13.76) N (16.31%) O (6.21%)
Molar Refractivity	- 66.90 ± 0.3 cm3
Molar Volume	- 194.4 ± 3.0 cm3
Parachor	-526.2 ± 4.0 cm3
Index of Refraction	-1.604 ± 0.02
Surface Tension	-53.6 ± 3.0 dyne/cm
Density	- 1.325 ± 0.06 g/cm3
Polarizability	-26.52 ±0.5 10- 24 cm3
Nominal mass	-257Da
Monoisotopic mass	-257.033Da

IR SPECTRUM: VS4

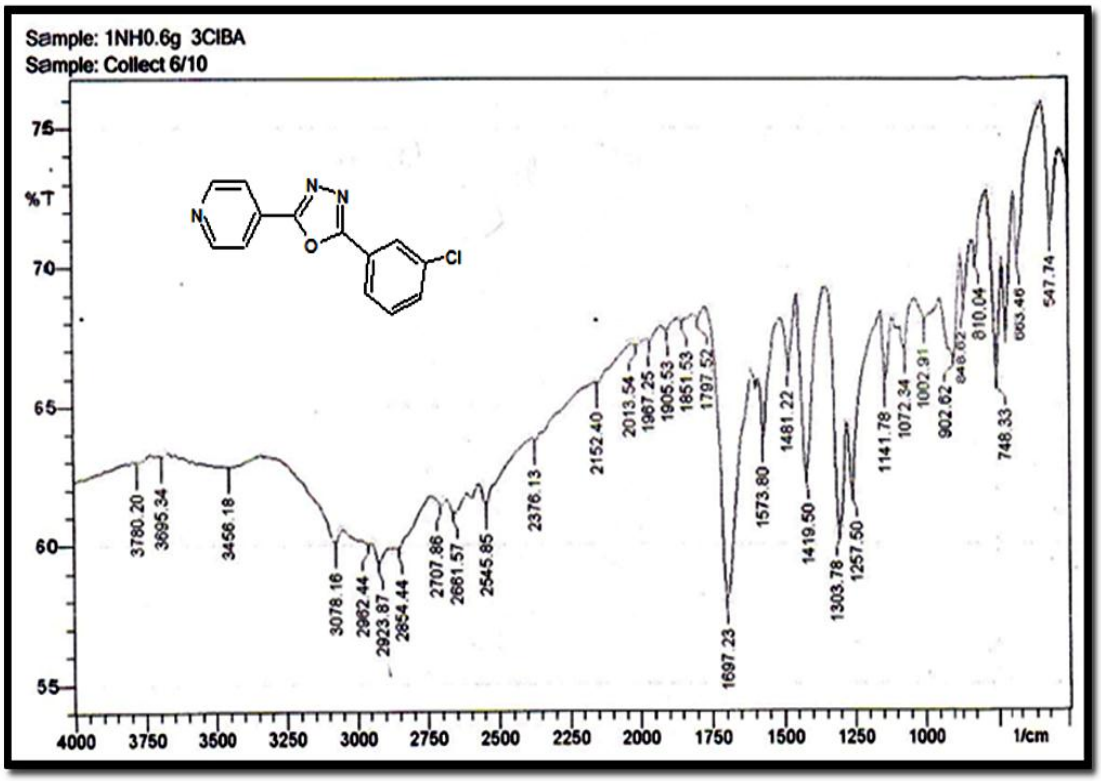
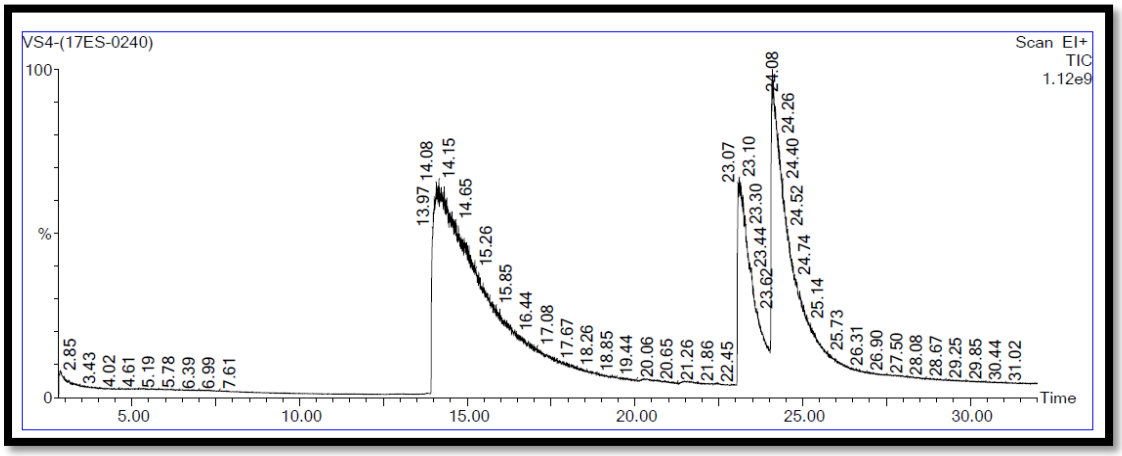


FIGURE 30- IR SPECTRUM OF SAMPLE VS4

TABLE 12- IR INTERPRETATION OF SAMPLE VS4

S.no	Wavenumber (cm^{-1})	Functional group
1.		
2.	3078 cm^{-1}	Ar C-H stretching
3.	1257 cm^{-1}	C-O-C Stretching
4.	1573 cm^{-1}	Ar C=C Stretching
5.	1697 cm-1	C=N Stretching
	748cm^{-1}	C-Cl Stretching

GC-MS SPECTRUM: VS4



#	RT	Scan	Height	Area	Area %	Norm %
1	14.153	2270	728,964,096	1,266,300,032.0	63.168	100.00
2	23.101	4059	699,447,744	292,924,992.0	14.612	23.13
3	24.097	4258	966,183,360	445,439,552.0	22.220	35.18

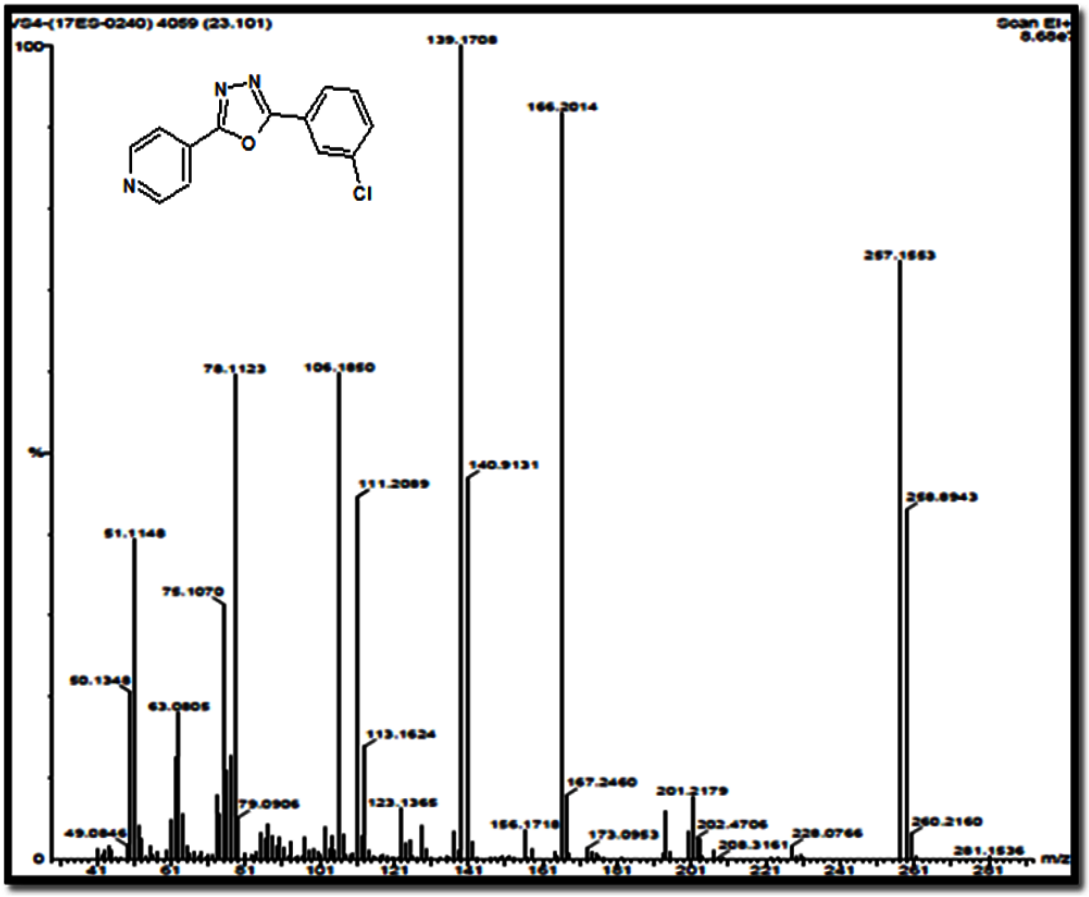
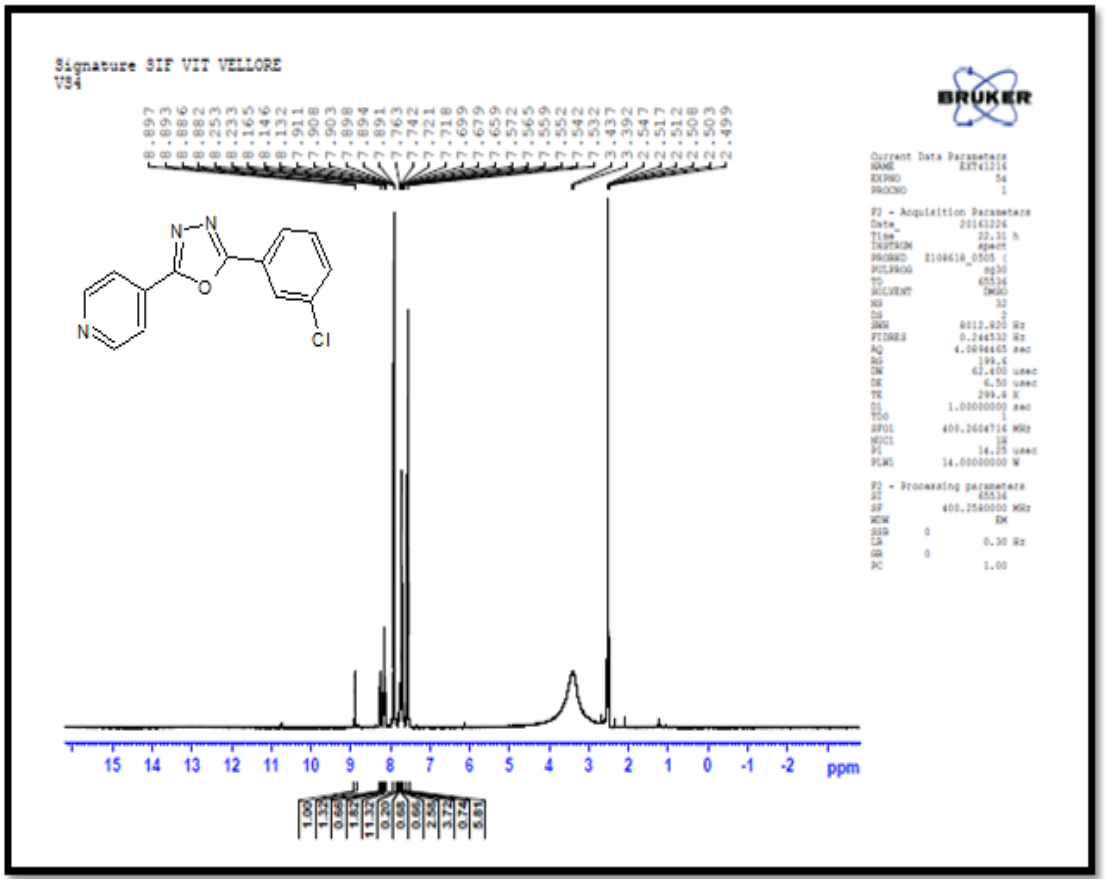


FIGURE 31- GC-MS SPECTRUM OF SAMPLE VS4

H^1NMR SPECTRUM: VS4



FIFURE 32- H1 NMR SPECTRUM OF SAMPLE VS4

TABLE 13- H1 INTERPRETATION OF SAMPLE VS4

S.NO	δ VALUE	NATURE OF PEAK	NUMBER OF PROTONS
1.	7.53-7.57	Triplet	3
2.	7.89-7.91	Multiplet	4
3.	8.88-8.89	Doublet	2

C^{13}NMR SPECTRUM: VS4

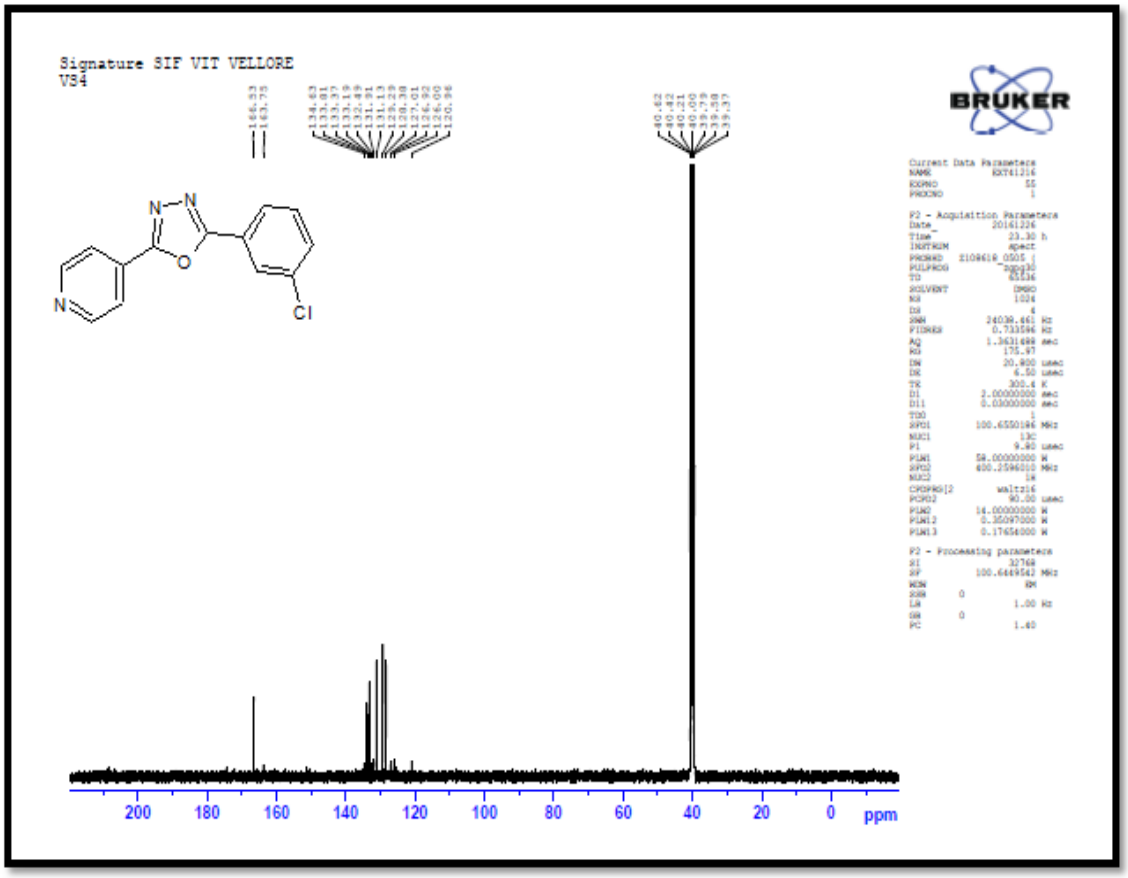
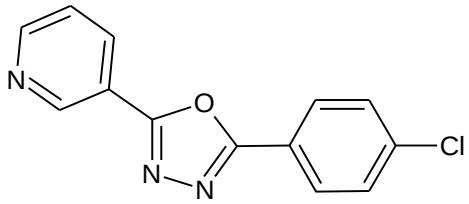


FIGURE 33- C13 NMR SPECTRUM OF SAMPLE VS4

CODE: VS5

IUPAC NAME: 3-[5-(4-chlorophenyl)-1, 3, 4-oxadiazol-2-yl] pyridine



Molecular Formula	-$C_{13}H_8ClN_3O$
Molecular Weight	-257.67
Appearance	-White color
Melting Point	-165^{0}C
Yield	-74%
Composition	- C (60.60%) H (3.13%) Cl (13.76) N (16.31%) O (6.21%)
Molar Refractivity	- 66.90 ± 0.3 cm3
Molar Volume	- 194.4 ± 3.0 cm3
Parachor	-526.2 ± 4.0 cm3
Index of Refraction	- 1.604 ± 0.02
Surface Tension	-53.6 ± 3.0 dyne/cm
Density	- 1.325 ± 0.06 g/cm3
Polarizability	-26.52 ±0.5 10- 24 cm3
Nominal mass	-257Da
Monoisotopic mass	-257.033Da

IR SPECTRUM: VS5

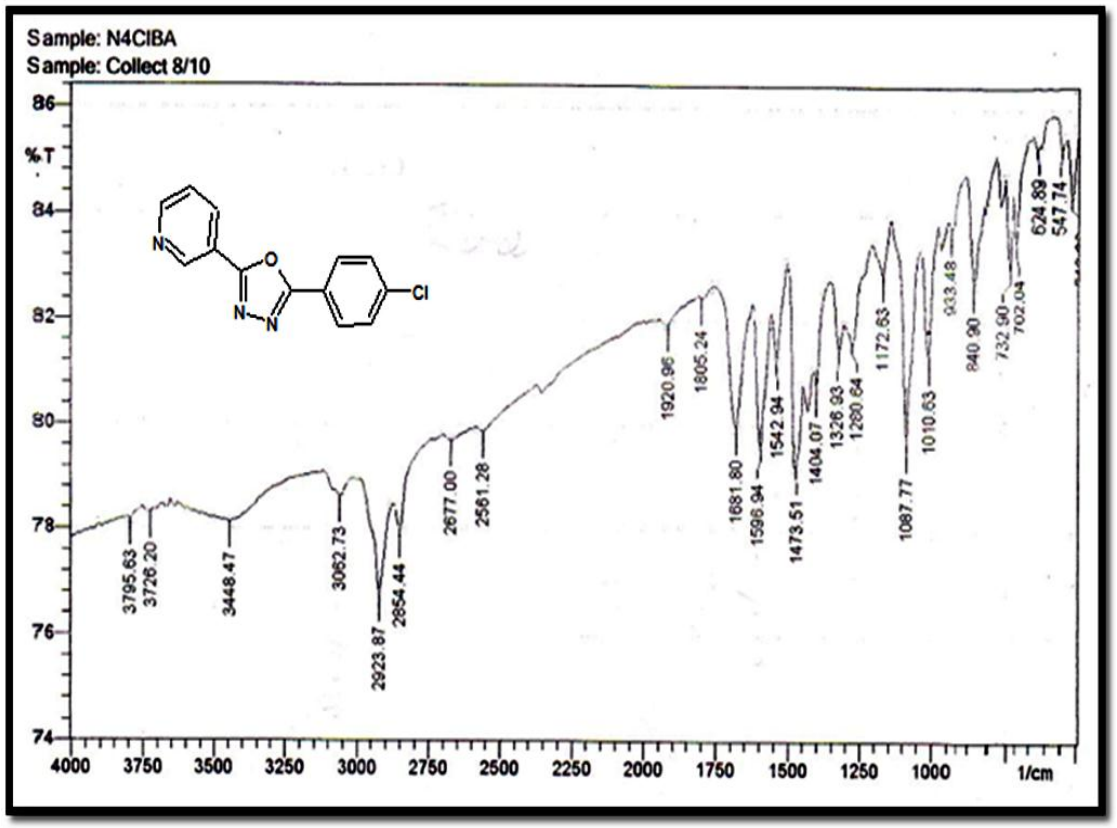
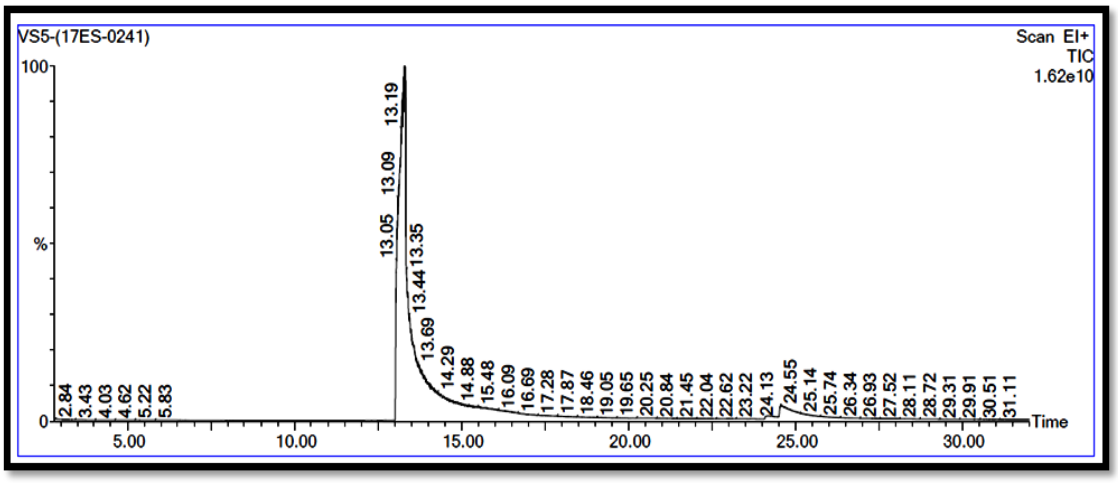


FIGURE 34- IR SPECTRUM OF SAMPLE VS5

TABLE 14- IR INTERPRETATION OF SAMPLE VS5

S.No	Wavenumber (cm^{-1})	Functional group
1.	3055 cm^{-1}	Ar C-H stretching
2.	1288 cm^{-1}	C-O-C Stretching
3.	1596 cm^{-1}	Ar C=C Stretching
4.	1689 cm-1	C=N Stretching
5.	753cm^{-1}	C-Cl Stretching

GC-MS SPECTRUM: VS5



#	RT	Scan	Height	Area	Area %	Norm %
1	13.303	2100	16,009,319,424	5,924,636,672.0	95.865	100.00
2	24.562	4351	545,990,208	255,539,376.0	4.135	4.31

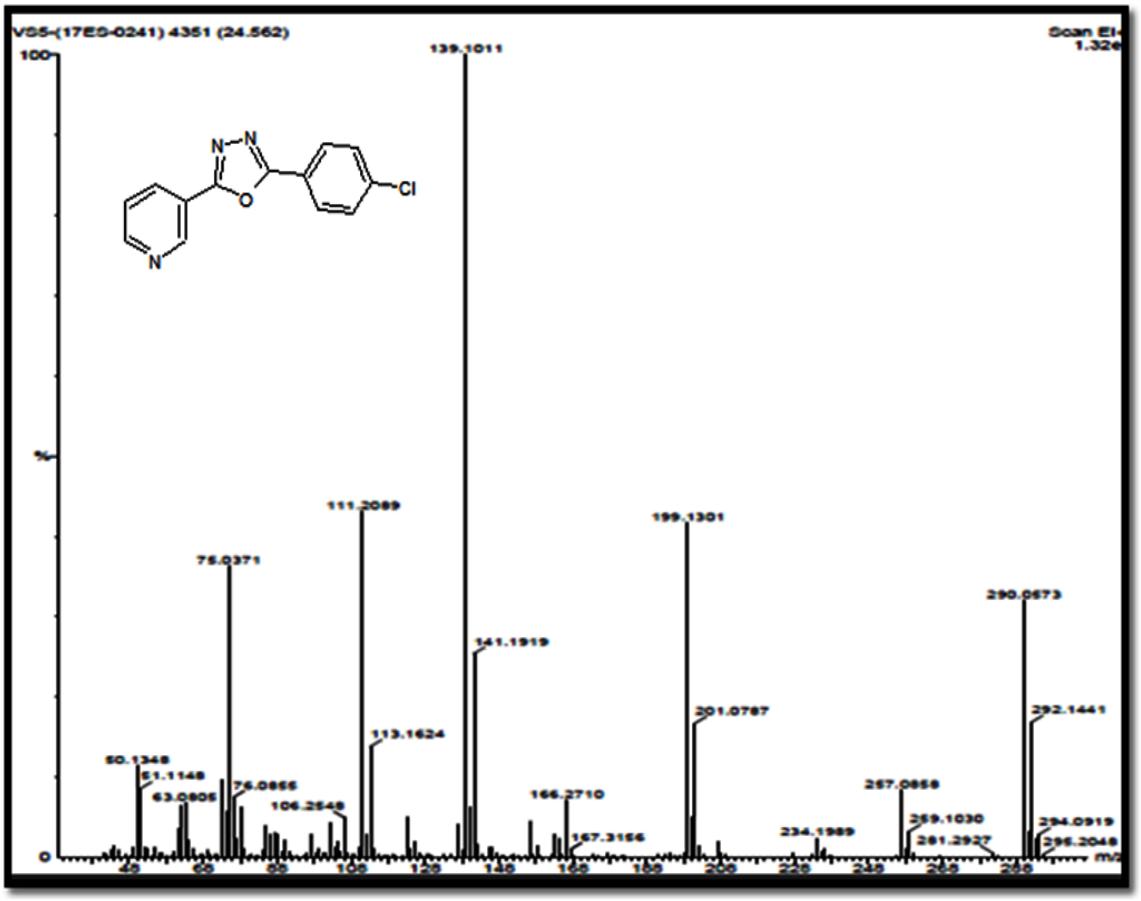


FIGURE 35- GC-MS SPECTRUM OF SAMPLE VS5

H^1NMR SPECTRUM: VS5

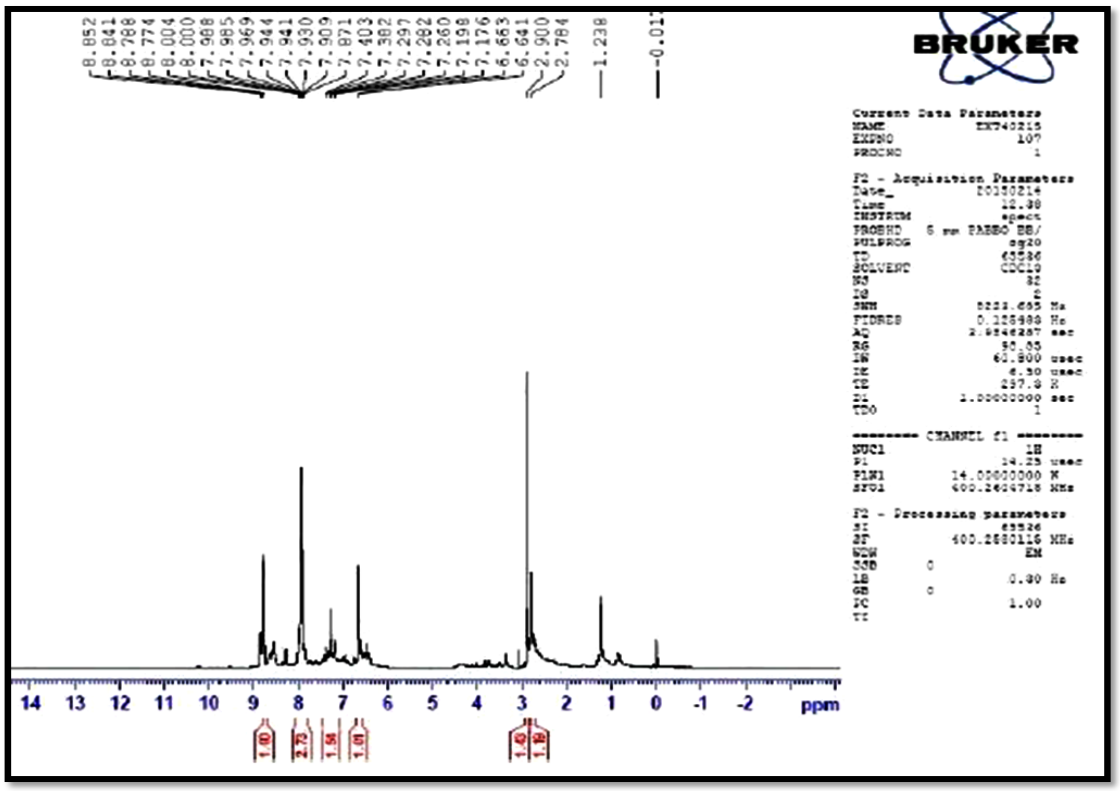


FIGURE 36- H1 NMR SPECTRUM OF SAMPLE VS5

TABLE 15- HI INTERPRETATION OF SAMPLE VS5

S.NO	δ VALUE(PPM)	NATURE OF PEAK	NUMBER OF PROTONS
1.	6.64-6.66ppm	Doublet	1
2.	7.17-7.40ppm	Multiplet	2
3.	7.87-8.00ppm	Multiplet	3
4.	8.77-8.85ppm	Multiplet	2

C^{13}NMR SPECTRUM: VS5

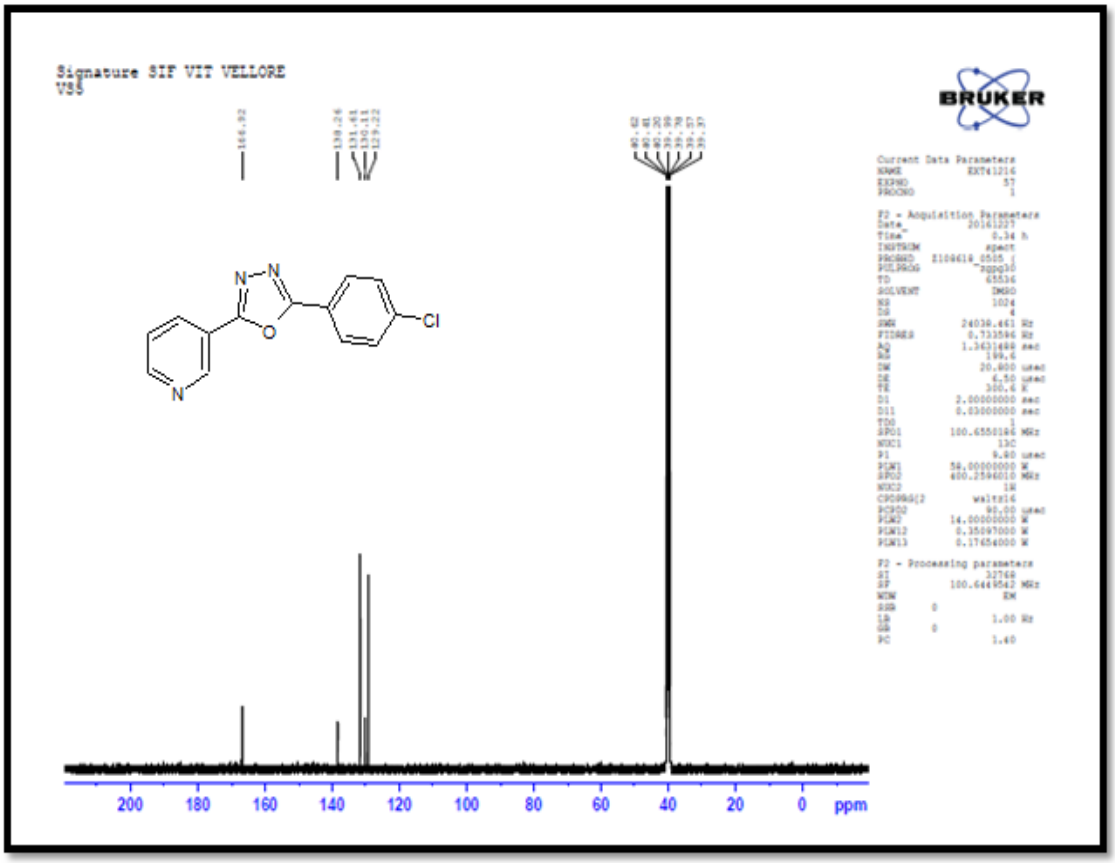


FIGURE 37-C13 NMR SPECTRUM OF SAMPLE VS5

MOLECULAR MASS OF THE SYNTHESIZED COMPOUNDS

TABLE 16- MOLECULAR MASS OF THE SYNTHESIZED COMPOUNDS

SAMPLE CODE	CALCULATED MASS	ACTUAL MASS
SA	239.18 g/mole	239.22 g/mole
NA	224.09g/mole	224.21 g/mole
VS1	257.20 g/mole	257.67 g/mole
VS2	223.09 g/mole	223.23 g/mole
VS4	257.15 g/mole	257.67 g/mole
VS5	257.08 g/mole	257.67 g/mole

ANTI-TB RESULTS

The recrystallized compounds were screened for Anti-mycobacterial activity in vitro method called Microplate Alamar Blue Assay (MABA). The results are tabulated below:

TABLE 17-ANTI-TB RESULTS

S.NO	SAMPLE CODE	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
1	**VS1**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
2	**VS2**	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
3	**VS4**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**
4	**VS5**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**
5	**SA**	**S**	**S**	**S**	**S**	**R**	**R**	**R**	**R**
6	**NA**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**

None of the compounds was found to be as sensitive as the standard compounds. However the docking score for Pyrazinamide is -6.6Kcal/mol which is higher than that all the synthesized compounds. This is a way indicates the limitation of the software. It must also be mentioned that compounds with the lowest energetic i.e. VS4, VS5 and SA have shown to be more active than the compounds which are energetically less stable.

NOTE:

S-Sensitive

R-Resistant

Stain used: M. tuberculosis (H37 RV Strain): ATTCC No- 27294

Here are the standard values for the Anti-Tb test which was performed.

Pyrazinamide- 3.125 µg/ml

Streptomycin- 6.25 µg/ml

Ciprofloxacin- 3.125 µg/ml

SAMPLE DRUG PHOTOGRAPH:

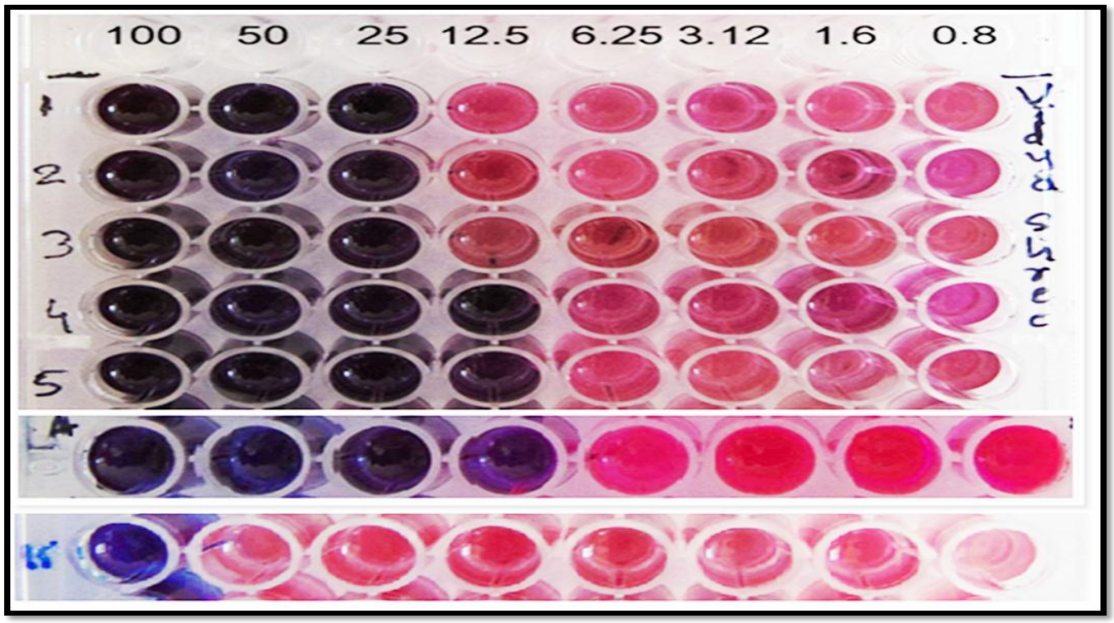


FIGURE 38- SAMPLE DRUG PHOTOGRAPH

STANDARD DRUG PHOTOGRAPH:

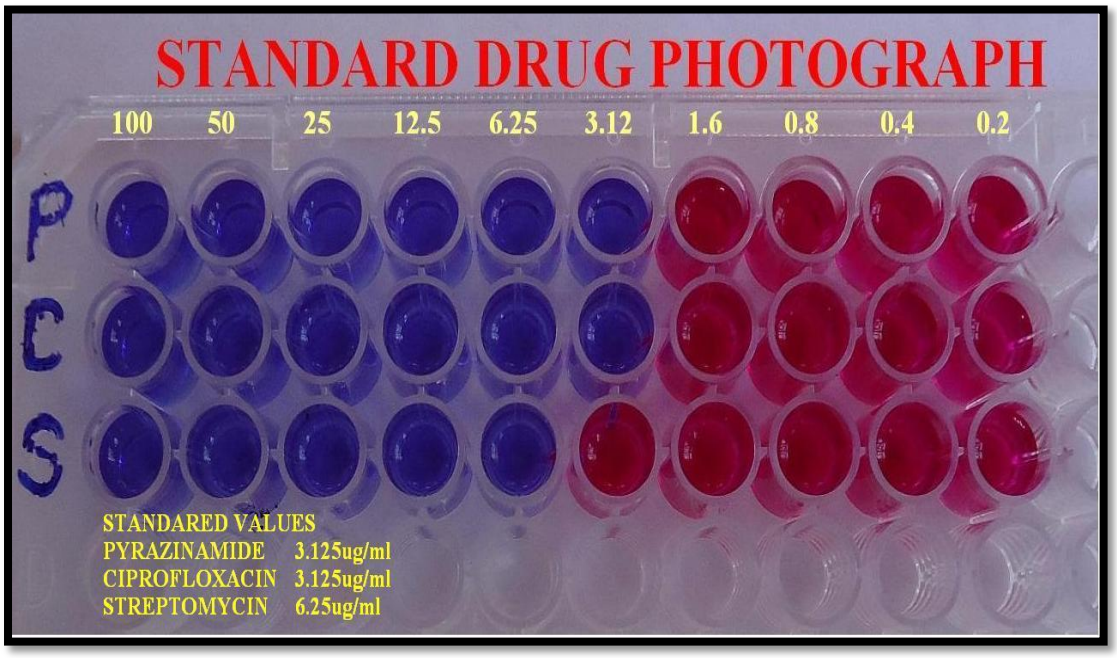


FIGURE 39- STANDARD DRUG PHOTOGRAPH

MOLECULES DOCKED AGAINST DIFFERENT TARGETS

The compounds were also docked against other critical enzyme of the mycobacterium tuberculosis Diaminopimelate epimarase (dapF) and Glutamine synthetase. The docking scores are listed below.

TABLE 18

SAMPLE CODE	L,D TRANSPEPTIDASE 2	DIAMINOPIMELATE EPIMERASE	GLUTAMINE SYNTHETASE
SA	**-9.310Kcal/mol**	**-8.262 Kcal/mol**	**-8.926 Kcal/mol**
NA	**-8.351Kcal/mol**	**-8.212 Kcal/mol**	**-9.121 Kcal/mol**
VS1	**-9.796Kcal/mol**	**-9.525 Kcal/mol**	**-9.421 Kcal/mol**
VS2	**-9.672Kcal/mol**	**-9.321 Kcal/mol**	**-8.924 Kcal/mol**
VS4	**-9.541Kcal/mol**	**-9.528 Kcal/mol**	**-9.121 Kcal/mol**
VS5	**-9.452Kcal/mol**	**-9.312 Kcal/mol**	**-8.731 Kcal/mol**

SUMMARY AND CONCLUSION

- L, D-Transpeptidase-2 (3VYP) is a critical enzyme for the cell wall synthesis of Mycobacterium tuberculosis was chosen for study after review of literature.
- Selected molecules were designed and docked against L, D-Transpeptidase-2 (3VYP) using Argus lab® 4.0software.
- Molecules with good Docking score (lower binding energy) and interactions were shortlisted for synthesis. The reaction conditions were optimized.
- The selected molecules were subjected to Toxicity prediction assessments by OSIRIS® software. The results are color coded as green color which predicts the drug likeness.
- Compounds were synthesized by conventional method and labeled as SA, NA, VS1, VS2, VS4 and VS5.
- The purity of the synthesized compounds was ensured by repeated recrystallization. Further the compounds were evaluated by TLC and Melting point determination.
- The characterization of the synthesized compounds was done using Infra-red, Nuclear Magnetic Resonance (H^1 NMR &C^{13}NMR) and Mass spectroscopic methods (LC-MS, GC-MS)
- The pure compounds were screened for In-vitro Anti- tubercular activity by Micro plate Alamar Blue Assay (MABA). All compounds showed a significant anti-mycobacterium activity.
- Docking with other critical enzyme was carried out.

CONCLUSION

- Our work concludes that our synthesized molecules are effective in inhibiting the target enzyme L, D-Transpeptidase 2, which is important for the growth of Mycobacterium tuberculosis Cell wall.
- All the 6 compounds gave Docking score between -8.30 to -9.74kcal/mol. There is correlation between the score and activities of all the 6 compounds which were tested and compared with the standard drugs.
- Multi target docking was done for different critical enzymes of mycobacterium tuberculosis using Argus lab®. From the results, the targeted enzyme l,d-transpeptidase 2 score was closer to the other two target ie., diaminopimelate epimerase and glutamine synthase. There was not much significant difference between the other two targeted compounds.
- The minimum inhibitory concentration of the 6 synthesized compounds against H37RV ranged from 12.5 µg/ml which is better compared to that of the certain known Anti-TB agents. Pyrazinamide- 3.125µg/ml, Ciprofloxacin- 3.125µg/ml and Streptomycin- 6.25µg/ml. Further structural refinement to the structure of the synthesized compounds is expected to yield promising molecules against the pathogen Mycobacterium tuberculosis.

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