"Design, synthesis, characterization and biological evaluation of Schiff bases of 5-amino -4-[2-(4-nitro-1, 3-benzothiazol-2 yl) hydrazinylidene]-2, 4-dihydro-3*H* -pyrazol -3-one derivatives"

D to

The Tamil Nadu Dr. M.G.R Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Chemistry)

Submitted by

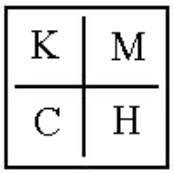
RAJALAKSHMI.G

Under the guidance of

Mr.K.K.Sivakumar, M. Pharm., (Ph.D).,

Asst.professor, Department of Pharmaceutical Chemistry.

April-2012



DEPARTMENT OF PHARMACEUTICAL CHEMISTRY,

KMCH COLLEGE OF PHARMACY,

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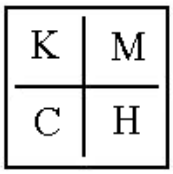

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY,

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COIMBATORE 641-048.

Certificates

Dr. A Rajasekaran, M. Pharm., Ph.D.,
Principal,
KMCH College of Pharmacy,
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Coimbatore - 641 048. (T.N).

CERTIFICATE

This is to certify that the dissertation work entitled **"Design, synthesis, characterization and biological evaluation of Schiff bases of 5-amino -4-[2-(4-nitro-1,3-benzothiazol-2yl) hydrazinylidene]-2,4-dihydro-3*H* -pyrazol -3-one derivatives"** submitted by **Ms.Rajalakshmi.G (Reg.No.26107136)** is a bonafide work carried out by the candidate under the guidance of **K.K.Sivakumar, M.Pharm., (Ph.D).,** Asst. professor, to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Chemistry** at the Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Coimbatore, during the academic year 2011-2012.

Date: **Dr. A. Rajasekaran, M.Pharm., Ph.D.**
Place: **Principal**

K.K. Sivakumar, M.Pharm,(Ph.D),
Assistant Professor,
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Kovai Estate, Kalapatti Road,
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Mr.K.K.Sivakumar, M.Pharm., (Ph.D).,
Asst.prof.Dept of Pharmaceutical Chemistry.

DECLARATION

I do hereby declare that the dissertation work entitled **"Design, synthesis, characterization and biological evaluation of Schiff bases of 5-amino -4-[2-(4-nitro-1,3-benzothiazol-2 yl)hydrazinylidene]-2,4-dihydro-3*H* -pyrazol -3-one derivatives"** submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Chemistry** at the Department of Pharmaceutical Chemistry was done by me under the guidance of **Mr.K.K.Sivakumar, M.Pharm., (Ph.D).,** Asst. professor, at the Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Coimbatore, during the academic year 2011-2012.

RAJALAKSHMI.G

(26107136)

EVALUATION CERTIFICATE

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Internal Examiner **External Examiner**

Convener of Examinations

Examination Center : KMCH College of Pharmacy,
Coimbatore.

Date :

Acknowledgement

ACKNOWLEDGEMENT

This dissertation entitled **"Design, synthesis, characterization and biological evaluation of Schiff bases of 5-amino-4-[2-(4-nitro-1,3-benzothiazol-2 yl)hydrazinylidene]-2,4-dihydro-3*H* -pyrazol -3-one derivatives"** would not have been feasible one would not have been a feasible one without the grace of god almighty who gave me moral till the completion of my project.

First and foremost I am extremely beholden to my esteemed guide, **Mr. K.K. Sivakumar, M.Pharm., (Ph.D)., Asst. Professor,** Dept. of Pharmaceutical Chemistry, for his constant insight, personal advice, countless serenity and pain taking effort in all stages of study.

With great pleasure I wish to place my indebtedness to **Dr. A. Rajasekaran, M. Pharm., Ph.D.**, **Principal** for his support and for giving me an opportunity to do my project work.

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My respectful regards to our beloved Managing trustee **Dr.Thavamani.D.Palanisamy** and our respected Chairman **Dr.Nalla.G.Palanisamy,** KMCH College of Pharmacy, Coimbatore.

My special thanks to the library staff for providing library facilities. My sincere thanks to all other teaching and nonteaching staff of KMCH College of Pharmacy, especially
Mrs. Banu, lab assistant, Dept. of pharmaceutical pharmacology and **Mrs. Ananthi, Mrs. Lavanya.,** lab assistant, Department of Pharmaceutical Chemistry and others who directly or indirectly gave a helping hand to me while carrying out this study.

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I express my heartful thanks to my friends **G.Indiragandhi, M.Govindaraj, G.Ashokkumar, Sabbashini Bugga Reddy, K.Sheejadevi, S.M.J.Guptha, R.S.Shanmugarajan, P.Parasuraman, T.Aravazhi, T.Nilofernisha, S.Saranya, Smylin Ajitha Rani** for their support and encouragement during the course of my works. Their encouragement was highly inspirational throughout the course of this work.

Above all I dedicate myself before the unfailing presence of ***GOD*** and constant love and encouragement given to me by my beloved **Parents, Guide, Brother and Friends** who deserves the credit of success in whatever work I did.

RAJALAKSHMI .G
(Reg.No.26107136)

ABBREVIATIONS

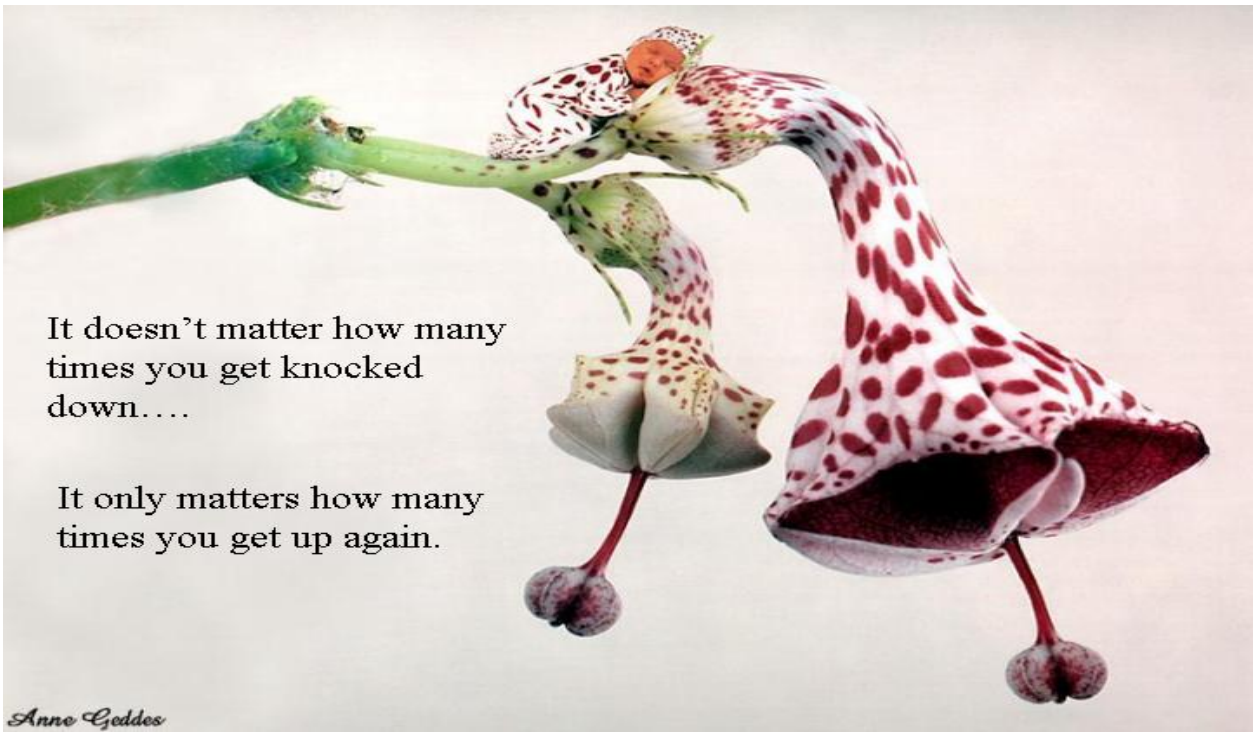
ROS	Reactive Oxidative Stress
%	Percentage
^{1}HNMR	Nuclear Magnetic Resonance
Mg	Milligram
Ml	Milliliter
Mm	Millimole
µg	Microgram
mm	Millimeter
µg/ml	Microgram per liter
Hrs	Hours
^{0}C	Degree centigrade
Fig.	Figure
Tab.	Table
UV-VIS	Ultraviolet and visible spectroscopy
Min.	Minutes
IR	Infrared spectroscopy
H_2O_2	Hydrogen peroxide
STD	Standard
TLC	Thin layer chromatography
KBr	Potassium bromide
FTIR	Fourier transforms infrared spectrometer
IC	Inhibitory concentration

EC	Effective concentration
Cont	Control
DMSO	Dimethyl sulfoxide
DPPH	Diphenyl picryl hydrazyl
FRAP	Ferric reducing antioxidant power
ABTS	Azinodiethyl benzthiazoline sulphonic acid
MTT	Dimethyl thiazolyl diphenyl tetrazolium bromide
REMA	Resazurin microplate assay

CONTENTS

Dedicate all your actions to God. You have control only on your actions and not on the result- Five factors govern the outcome all actions (good or bad): Context, individual, tools at one's disposal, how the tools are used, and providence (luck/fate/chance).

"The greatest mantra is Wisdom + Action = Success"



DEDICATED TO MY BELOVED ALMIGHTY, PARENTS, BROTHER, GUIDE & FRIENDS

Chapter 1

Introduction

1. INTRODUCTION

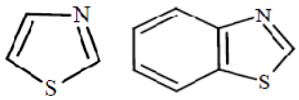
1.1. BENZOTHIAZOLE NUCLEUS

A heterocyclic compound is one which possesses a cyclic structure with at least two different kinds of hetero atoms in the ring. Nitrogen, oxygen and sulphur are the most common heteroatoms. Heterocyclic compounds are very widely distributed in nature and are essential to life in various ways. Most of the sugars and their derivatives, including vitamin C, for instance, exist in the form of five-membered (furan) or six-membered (pyran) rings containing one oxygen atom. Most member of vitamin B group possess heterocyclic ring containing nitrogen. One example is vitamin B6 (pyridoxine), which is a derivative of pyridine, essential in amino acid metabolism. [1]

Benzothiazole moieties are part of compounds showing numerous biological activities such as antimicrobial, [2-6] anticancer, [7-9, 10] anthelmintic, [11] anti-diabetic [12] activities. They have also found application in industry as anti-oxidants, vulkanisation accelerators. Various benzothiazoles such as 2-aryl benzothiazole received much attention due to unique structure and its uses as radioactive amyloid imagining agents,[13] and anticancer[14] agents. Benzothiazoles are bicyclic ring system with multiple applications.

In the 1950s, a number of 2-nitrobenzothiazoles were intensively studied, as the 2- nitro benzothiazole scaffold is one of privileged structure in medicinal chemistry and reported cytotoxic on cancer cells [15]. It must be emphasized that combination of 2- nitrobenzothiazoles with other heterocyclic is a well known approach to design new drug like molecules, which allows achieving new pharmacological profile, action, toxicity lowering. The 2-nitrobenzothiazoles are novel class of potent and selective antitumor agents and display characteristic profile of cytotoxic response across the cell lines. In addition, benzothiazole ring is present in various marine or terrestrial natural compounds, which have useful biological properties. In last few years it was reported that benzothiazole, its bioisosters and derivatives had antimicrobial activities against gram-negative, gram-positive bacteria and fungi.

Benzothiazoles (1-thia-3-azaindene) are fused membered rings, which contain the heterocycles bearing thiazole. Sulphur and nitrogen atoms constitute the core structure of thiazole and many pharmacologically and biologically active compounds.



I-1 I-2

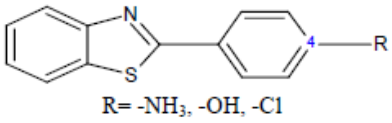
Thiazole is structurally related to thiophene and pyridine, but in most of its properties it resembles to the latter. Thiazole was first described by Hantzsch and Waber in 1887. Popp confirmed its structure in 1889. The basic structure of benzothiazole consist of benzene ring fused with 4, 5 position of thiazole. The two rings together constitute the basic nucleus 1, 3-benzothiazole.

1.1.1. MEDICINAL IMPORTANCE OF BENZOTHIAZOLE NUCLEUS

A large number of therapeutic agents are synthesized with the help of Benzothiazole nucleus. During recent years there have been some interesting developments in the biological activities of benzothiazole derivatives. These compounds have special significance in the field of medicinal chemistry due to their remarkable pharmacological potentialities.

1.1.2. STRUCTURAL ACTIVITY RELATIONSHIP STUDY

Presence of hydrophobic moieties in molecule is conductive for cytotoxic activity of benzothiazole derivatives against cancer cell lines. The amino, hydroxyl, and chloro group containing benzothiazole shows better anticancer activity. [16]



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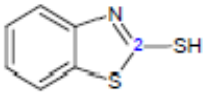
The substituent's at second position of benzothiazole ring like mercapto group and hydrazine group are responsible for marked bactericidal activity and anti-inflammatory activity. [17]



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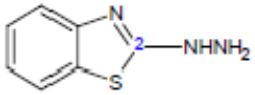
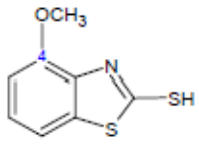


I-5

Introduction of methoxy group (-OCH3) at position 4 of 2-mercaptobenzothiazole increase antibacterial activity and introduction of chloro group (-Cl) at same position increase antifungal activity. [18]



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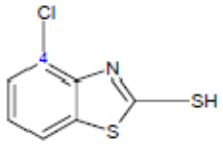
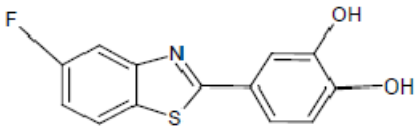


I-7

Minor modification of dihydroxyphenyl group, removal of fluro group or its replacement with other halogens had a profoundly dyschemotherapeuitic effect with respect to *in vitro* cancer cell growth inhibitory activity. [19, 20]



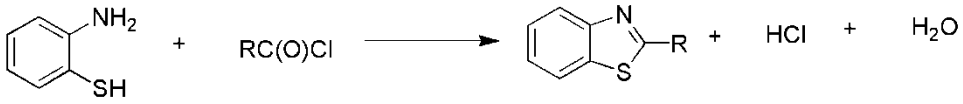
I-8

The thiazole ring has been extensively studied and it forms a part of vitamin B, penicillins and the antibacterial thiazoles. Given below is a brief account of various structural modifications done on benzothiazole ring and their associated biological activities.

1.1.3. SYNTHESIS OF BENZOTHIAZOLE

Benzothiazoles consist of a 5-membered 1, 3-thiazole ring fused to a benzene ring. The eight atoms of the bicycle and the attached substituent's are coplanar.

1. Benzothiazole are prepared by treatment of 2-aminobenzenethiol with acid chlorides.[21]



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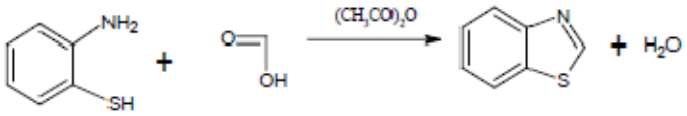
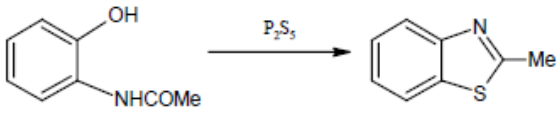
2. Benzothiazole may be prepared by action of acid anhydrides (or) chlorides on o-amino phenols and formic acid in presence of acetic anhydride.[22]



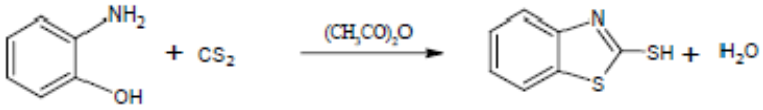
I-10

3. Benzothiazoles are also formed by action of phosphorus pentasulfide on o-acylaminophenoles.[23]



I-11

4. 2-Mercaptobenzothiazole is vulkanisation accelerator it may be prepared as follows.[24]

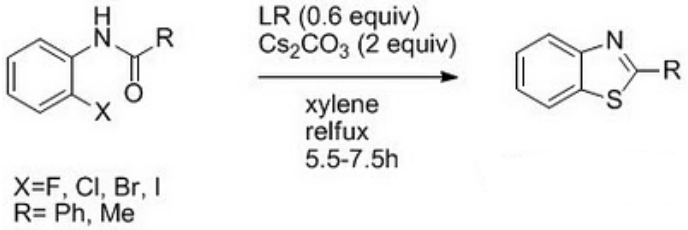


I-12

5. Benzothiazoles prepared from simple thioanilides and using Dess-Martin periodinane to promote the cyclization in 15 minutes.[25]

I-13

6. Preparation of 2-(Alkyl)arylbenzothiazoles from the Corresponding o-Halobenzanilides.[26]



I-14

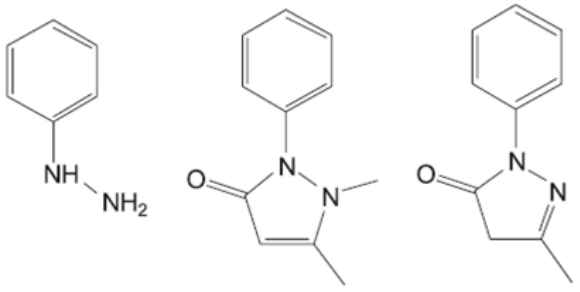
1.2. PYRAZOLONE NUCLEUS

Pyrazolones are well known and important nitrogen containing five-membered heterocyclic compounds. Various methods have been worked out for their syntheses. Synthesis and characterization of pyrazolone derivatives has been a developing field within the realm of heterocyclic chemistry for the past several decades because of their ready accessibility through synthesis, wide range of chemical reactivity and broad spectrum of biological activity and variety of industrial applications. [27]

The pyrazolone function is quite stable, and has inspired chemists to utilize this stable fragment in bioactive moieties to synthesize new compounds possessing biological activities. Pyrazolones have been reported to show a broad spectrum of biological activities including antibacterial [28], antioxidant [29], and anti-inflammatory [30] activities. Its derivatives possess a wide range of biological and physiological activities such as antitumor [31], analgesic [32] activities and industrial applications. Survey of literature in the recent past reveals that some pyrazolone derivatives also possess effect.

After the work of Fischer and Knovenagel [33] in the late 19th century, the reaction of α, β-unsaturated aldehydes and ketones with hydrazines became one of the most popular methods for the preparation of 2-pyrazolones. As a result, numerous substituted 2-pyrazolones have been synthesized, which has made possible structural activity relationship investigations of these substances.

In view of the ongoing interest in the synthesis of nitrogen containing heterocyclic, the synthesis of biologically active new pyrazolone derivatives have been undertaken.



I-15 I-16 I-17

The above three structures represents the tautomeric forms of pyrazolone nucleus. The application of present heterocyclic nomenclature to pyrazolone requires that nitrogen atoms be numbered one and two in each structure.

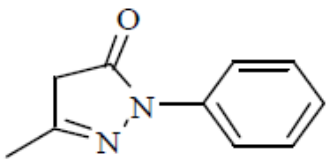
The chemistry of pyrazolone began in 1883 when Knorrreported the first pyrazolone derivative. The reaction of phenyl hydrazine and ethylacetoacetate resulted in novel structure identified in 1887 as 1-phenyl-3-methyl-5-pyrazolone. The Knorr pyrazole synthesis is the reaction of hydrazines with 1, 3 dicarbonyl compounds to provide the pyrazole or pyrazolone ring system. Pyrazolone is a five membered lactum ring containing two nitrogen and a ketone group in its ring. The prototype molecule, antipyrine was synthesized for clinical use in 1883. The methylated nitrogen derivative aminopyrine was introduced in 1897 and taken off from the market in the 1970s because of its property to form nitrosamines. Pyrazolone is an important pharmacophore which exhibits wide spread pharmacological properties. [34]

1.2.1. MEDICINAL IMPORTANCE OF PYRAZOLONE NUCLEUS

A large number of therapeutic agents are synthesized with the help of pyrazolone nucleus. During recent years there have been some interesting developments in the biological activities of pyrazolone derivatives. These compounds have special significance in the field of medicinal chemistry due to their remarkable pharmacological potentialities. [35]

1.2.2. STRUCTURAL ACTIVITY RELATIONSHIP STUDY

- Antioxidant studies shows that among pyrazolones having ring deactivating substituents, the highest activity was obtained with substituent having highest lipophilicity, lowest electron withdrawing power and highest polarisability (i.e., bromo compound).[36]
- Among pyrazolone, substituents exerting activity in descending order of potency were found to be: m-NO_2 >o, p-(CH3)2 > p-Cl >p-NO2 > o-COOH.

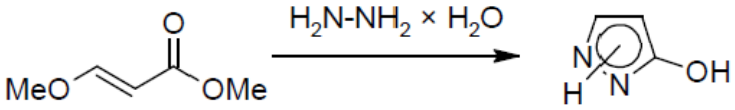


- Among o-substituted phenyl derivatives and o,p-disubstituted phenyl derivatives of pyrazolones, highest activity was observed with substituent possessing highest lipophilicity (i.e., dimethyl compound).[37]
- Considering the effect of orientation of nitro group in the phenyl ring of pyrazoles and pyrazolones on better activity, it was observed that compounds having nitro group in meta orientation gave better activity than o-isomer.
- Among carboxyl derivatives of pyrazolones, p-isomer gave better activity.[38]

1.2.3. SYNTHESIS OF PYRAZOLONE

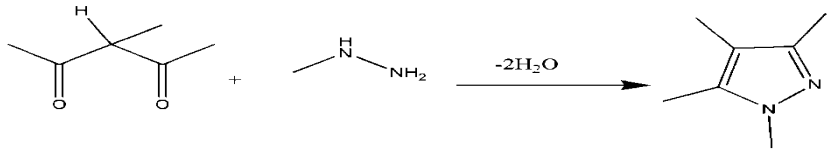
Pyrazolone consist of 5-membered containing two nitrogen, one ketone as hetero atoms.

1. Preparation of the fully unsubstituted pyrazolone system from hydrazine hydrate and methyl (2*E*)-3-methoxyacrylate.[39]



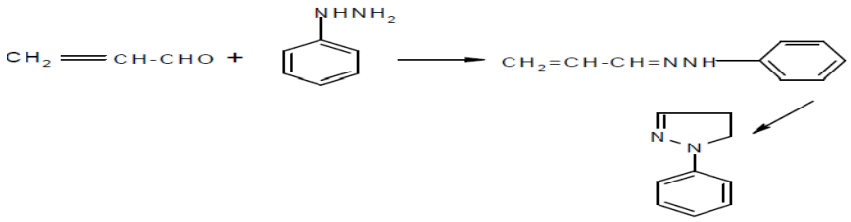
I-19

2. Pyrazoles can be made from a 1,3-dicarbonyl component and a hydrazine or hydroxylamine respectively.[40]



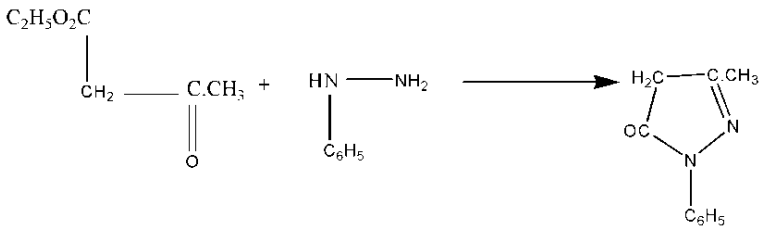
I-20

3. 1-Phenyl, 2-pyrazolines was synthesized from the reaction between acrolein and phenyl hydrazine.[40]



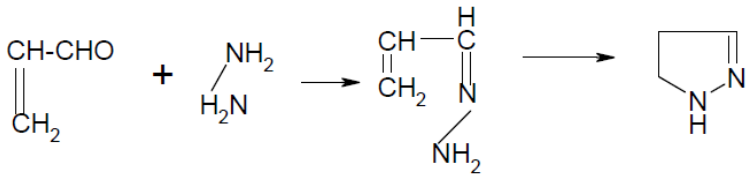
I-21

4. Synthesis of 1-phenyl-3-methyl-5-pyrazolone by esters of acetic acid and phenyl hydrazine.[40]



I-22

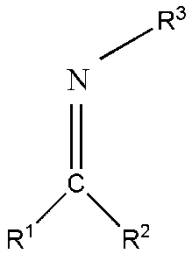
5. 1,2- pyrazolines was synthesized from the reaction between acrylaldehyde and hydrazine.[40]



I-23

1.3. SCHIFF BASE

A Schiff base, named after Hugo Schiff is a compound with a functional group that contains a carbon-nitrogen double bond with the nitrogen atom connected to an aryl or alkyl group, not hydrogen. Schiff bases in a broad sense have the general formula $R^1R^2C=NR^3$, where R is an organic side chain. In this definition, Schiff base is synonymous with azomethine. Some restrict the term to the secondary aldimines (azomethines where the carbon is connected to a hydrogen atom), thus with the general formula RCH=NR'.

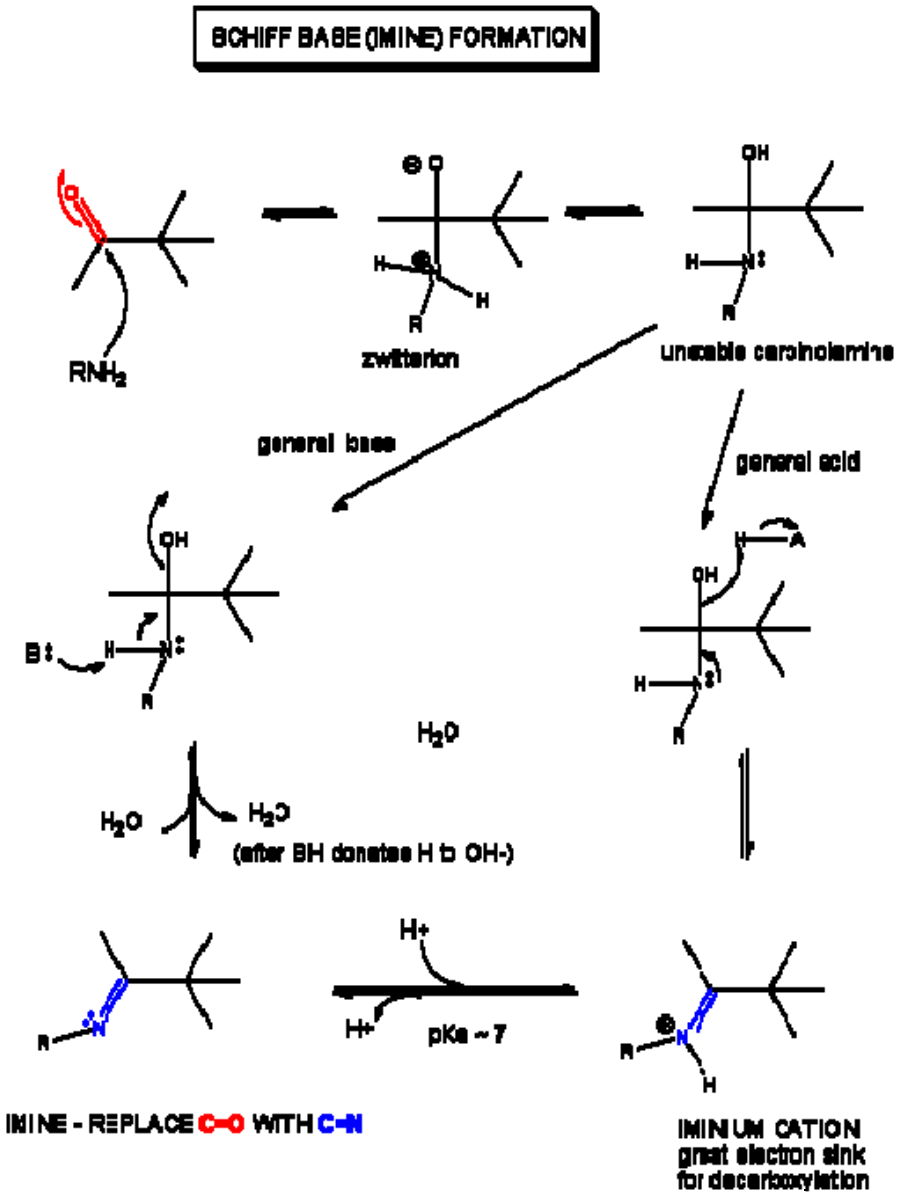


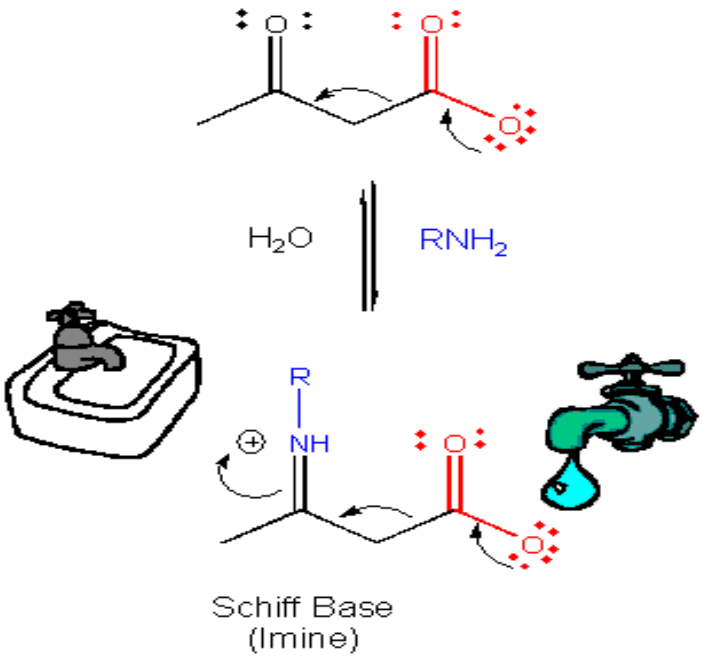
I-24

The chain on the nitrogen makes the Schiff base a stable imine. A schiff base derived from an aniline, where R^3 is a phenyl or a substituted phenyl, can be called an anil.[41]

1.3.1. MECHANISM OF SCHIFF BASE

An imine or schiff base forms, This is easily protonated to form a positively charged N at the former carbonyl O center. This serves as an excellent electron sink for decarboxylation reactions of beta-keto acids and illustrates an important point. Electrons in chemical reactions can be viewed as flowing from a source (such as a carboxyl group) to a sink (such as an nucleophilic carbonyl O or a positively charged N in a Schiff base). [42]

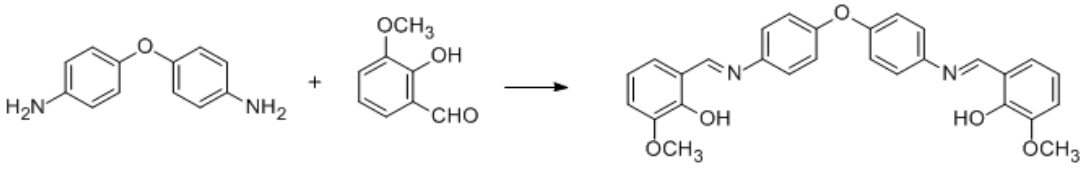




I-26

1.3.2. SYNTHESIS

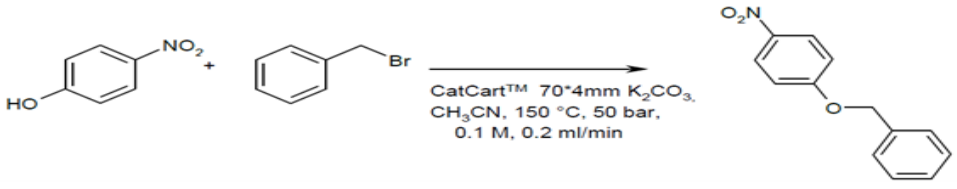
1. Schiff bases can be synthesized from an aromatic amine and a carbonyl compound by nucleophilic addition forming a hemiaminal, followed by a dehydration to generate an imine. In a typical reaction, 4,4'-diaminodiphenyl ether reacts with o-vanillin.[43]



I-27

2. Alkylation:

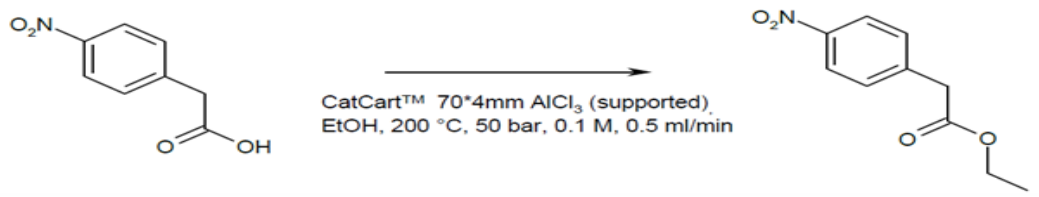
   1-Benzyloxy-4-nitro-benzene [44]



I-28

3. Esterification:

   (4-Nitro-phenyl)-acetic acid ethyl ester [45]



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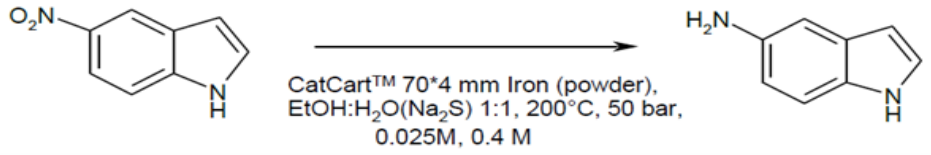
4. Béchamp-reduction:[46]

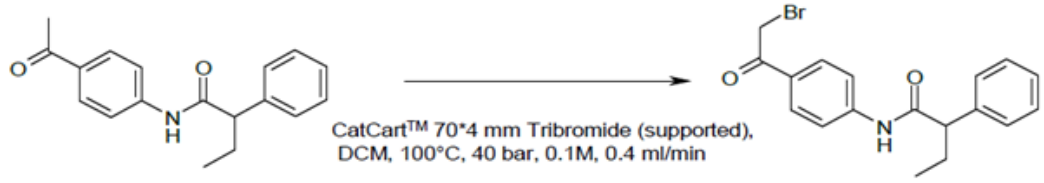
   5-Aminoindole



I-30

5. Bromination:[47]

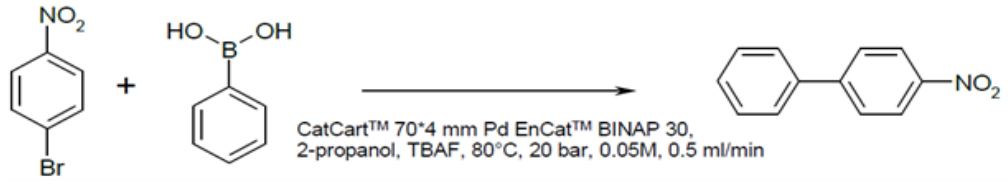
   N-[4-(2-Bromo-acetyl)-phenyl]-2-phenyl-butyramide



I-31

6. C-C Coupling:[48]

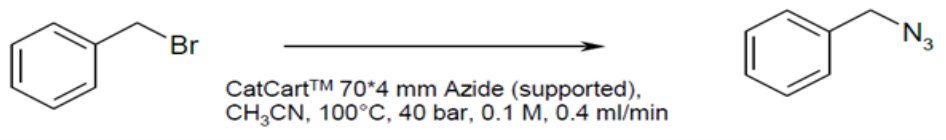
   4-Nitro-biphenyl



I-32

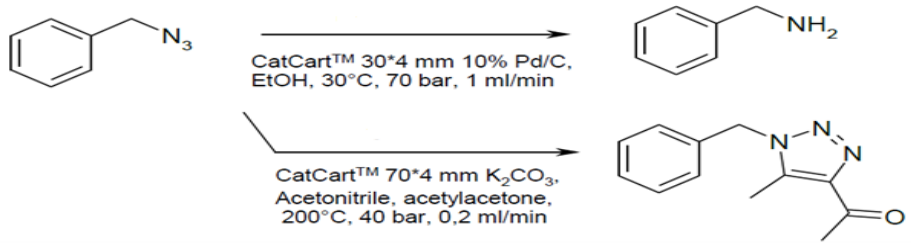
7. In situ organic azide reagent synthesis:[49]

   Benzyl azide



I-33

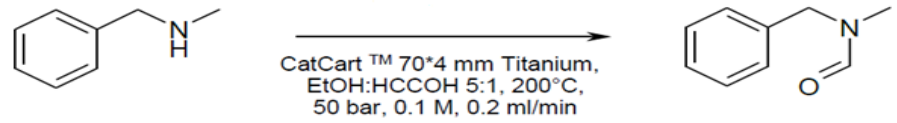
8. Benzyl amine and triazole synthesis from crude azide product[50]



I-34

9. N-formylation:[51]

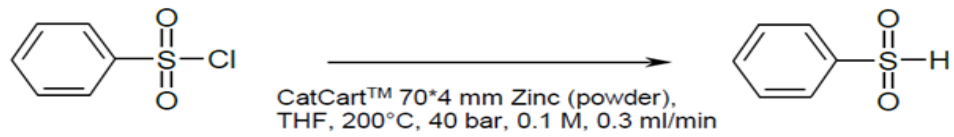
   N-Benzyl-N-methyl-formamide



I-35

10. Dehalogenation:[52]

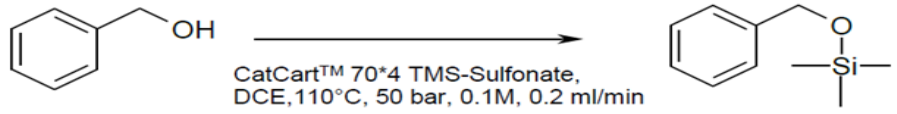
    Benzenesulfinic acid



I-36

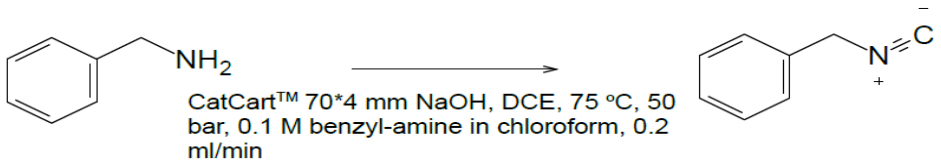
11. Trimethylsilylation:[53]

    Benzyloxy-trimethyl-silane



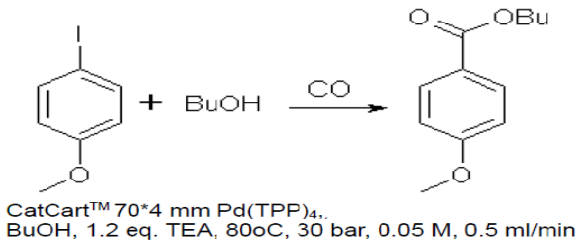
I-37

12. Isonitrile formation:[54]



I-38

13. Reactions with CO:[55]



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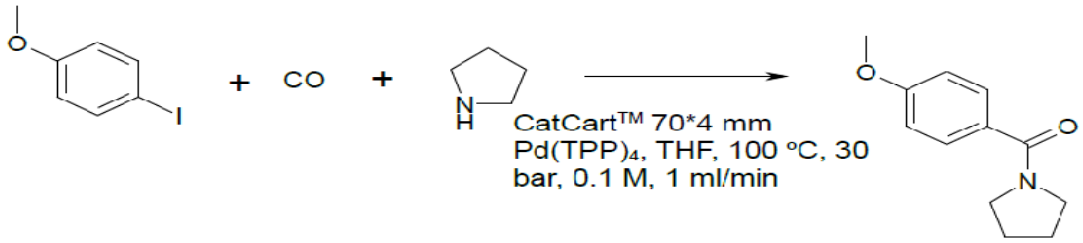


I-40

1.4. DOCKING

Molecular modelling encompasses all theoretical methods and computational techniques used to model or mimic the behaviour of molecules. The techniques are used in the fields of computational chemistry, computational biology and materials science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies. The simplest calculations can be performed by hand, but inevitably computers are required to perform molecular modelling of any reasonably sized system. The common feature of molecular modelling techniques is the atomistic level description of the molecular systems; the lowest level of information is individual atoms or a small group of atoms. This is in contrast to quantum chemistry (also known as electronic structure calculations) where electrons are

considered explicitly. The benefit of molecular modelling is that it reduces the complexity of the system, allowing many more particles (atoms) to be considered during simulations. [56, 57]

In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using for example scoring functions. [58]

The associations between biologically relevant molecules such as proteins, nucleic acids, carbohydrates, and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs antagonism). Therefore docking is useful for predicting both the strength and type of signal produced. [59]

Docking is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs. Given the biological and pharmaceutical significance of molecular docking, considerable efforts have been directed towards improving the methods used to predict docking. [60]

Some glossary for docking,

• **Receptor** or **host** or **lock** – The "receiving" molecule, most commonly a protein or other biopolymer.

• **Ligand** or **guest** or **key** – The complementary partner molecule which binds to the receptor. Ligands are most often small molecules but could also be another biopolymer.

• **Docking** – Computational simulation of a candidate ligand binding to a receptor.

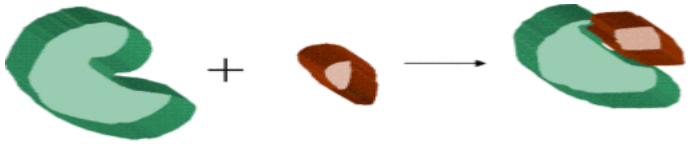
• **Binding mode** – The orientation of the ligand relative to the receptor as well as the conformation of the ligand and receptor when bound to each other.

• **Pose** – A candidate binding mode.

• **Scoring** – The process of evaluating a particular pose by counting the number of favorable intermolecular interactions such as hydrogen bonds and hydrophobic contacts.

• **Ranking** – The process of classifying which ligands are most likely to interact favorably to a particular receptor based on the predicted free-energy of binding. [61]

Schematic diagram illustrating the docking of a small molecule ligand (brown) to a protein receptor (green) to produce a complex.



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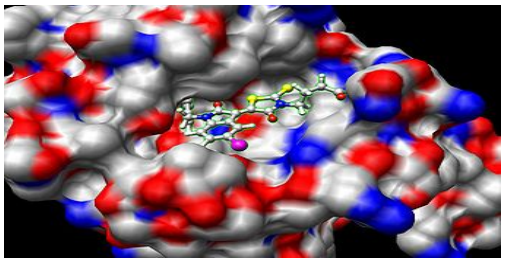
Small molecule docked to a protein



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1.4.1. DOCKING APPROACHES [62]

Two approaches are particularly popular within the molecular docking community. One approach uses a matching technique that describes the protein and the ligand as complementary surfaces. The second approach simulates the actual docking process in which the ligand-protein pairwise interaction energies are calculated. Both approaches have significant advantages as well as some limitations. These are outlined below.

- ❖ **Shape complementarity**

Geometric matching, shape complementarity methods describe the protein and ligand as a set of features that make them dockable. These features may include molecular surface,

complementary surface descriptors. In this case, the receptor's molecular surface is described in terms of its solvent-accessible surface area and the ligand's molecular surface is described in terms of its matching surface description. The complementarity between the two surfaces amounts to the shape matching description that may help finding the complementary pose of docking the target and the ligand molecules. Another approach is to describe the hydrophobic features of the protein using turns in the main chain atoms. Yet another approach is to use a fourier shape descriptor technique, where as the shape complementarity based approaches are typically fast and robust, they cannot usually model the movements or dynamic changes in the ligand, protein conformations accurately, although recent developments allow these methods to investigate ligand flexibility. Shape complementarity methods can quickly scan through several thousand ligands in a matter of seconds and actually figure out whether they can bind at the protein's active site, and are usually scalable to even protein-protein interactions. They are also much more amenable to pharmacophore based approaches, since they use geometric descriptions of the ligands to find optimal binding.

- **Simulation**

The simulation of the docking process as such is a much more complicated process. In this approach, the protein and the ligand are separated by some physical distance, and the ligand finds its position into the protein's active site after a certain number of "moves" in its conformational space. The moves incorporate rigid body transformations such as translations and rotations, as well as internal changes to the ligand's structure including torsion angle rotations. Each of these moves in the conformation space of the ligand induces a total energetic cost of the system, and hence after every move the total energy of the system is calculated. The obvious advantage of the method is that it is more amenable to incorporate ligand flexibility into its modeling whereas shape complementarity techniques have to use some ingenious methods to incorporate flexibility in ligands. Another advantage is that the process is physically closer to what happens in reality, when the protein and ligand approach each other after molecular recognition. A clear disadvantage of this technique is that it takes longer time to evaluate the optimal pose of binding since they have to explore a rather large energy landscape. However grid-based techniques as well as fast optimization methods have significantly ameliorated these problems.

1.4.2. MECHANICS OF DOCKING [63]

To perform a docking screen, the first requirement is a structure of the protein of interest. Usually the structure has been determined using a biophysical technique such as x-ray crystallography or less often, NMR spectroscopy. This protein structure and a database of potential ligands serve as inputs to a docking program. The success of a docking program depends on two components: the search algorithm and the scoring function.

- **Search algorithm**

The search space in theory consists of all possible orientations and conformations of the protein paired with the ligand. However in practice with current computational resources, it is impossible to exhaustively explore the search space, this would involve enumerating all possible distortions of each molecule (molecules are dynamic and exist in an ensemble of conformational states) and all possible rotational and translational orientations of the ligand relative to the protein at a given level of granularity. Most docking programs in use account for a flexible ligand, and several attempt to model a flexible protein receptor. Each "snapshot" of the pair is referred to as a pose.

A variety of conformational search strategies have been applied to the ligand and to the receptor. These include:

- Systematic or stochastic torsional searches about rotatable bonds.
- Molecular dynamics simulations.
- Genetic algorithms to "evolve" new low energy conformations.

- **Ligand flexibility**

Conformations of the ligand may be generated in the absence of the receptor and subsequently docked or conformations may be generated on-the-fly in the presence of the receptor binding cavity or with full rotational flexibility of every dihedral angle using fragment based docking. Force field energy evaluation are most often used to select energetically reasonable conformations, but knowledge-based methods have also been used.

- **Receptor flexibility**

Computational capacity has increased dramatically over the last decade making possible the use of more sophisticated and computationally intensive methods in computer-assisted drug design. However, dealing with receptor flexibility in docking methodologies is still a thorny issue. The main reason behind this difficulty is the large number of degrees of freedom that have to be considered in this kind of calculations. Neglecting it, however, leads to poor docking results in terms of binding pose prediction.

Multiple static structures experimentally determined for the same protein in different conformations are often used to emulate receptor flexibility. Alternatively rotamer libraries of amino acid side chains that surround the binding cavity may be searched to generate alternate but energetically reasonable protein conformations.

1.4.3. SCORING FUNCTION [64]

The scoring function takes a pose as input and returns a number indicating that, the pose represents a favorable binding interaction.

Most scoring functions are physics-based molecular mechanics force fields that estimate the energy of the pose; a low (negative) energy indicates a stable system and thus a likely binding interaction. An alternative approach is to derive a statistical potential for interactions from a large database of protein-ligand complexes, such as the Protein Data Bank, and evaluate the fit of the pose according to this inferred potential.

There are a large number of structures from X-ray crystallography for complexes between proteins and high affinity ligands, but comparatively fewer for low affinity ligands as the later complexes tend to be less stable and therefore more difficult to crystallize. Scoring functions trained with this data can dock high affinity ligands correctly, but they will also give plausible docked conformations for ligands that do not bind. This gives a large number of false positive hits, i.e., ligands predicted to bind to the proteins that actually don't when placed together in a test tube.

One way to reduce the number of false positives is to recalculate the energy of the top scoring poses using (potentially) more accurate but computationally more intensive techniques such as generalized born or poisson-boltzmann methods.

1.4.4. APPLICATIONS [65]

A binding interaction between a small molecule ligand and an enzyme protein may result in activation or inhibition of the enzyme. If the protein is a receptor, ligand binding may result in agonism or antagonism. Docking is most commonly used in the field of drug design, most drugs are small organic molecules, and docking may be applied to:

- Hit identification – docking combined with a scoring function can be used to quickly screen large databases of potential drugs insilico to identify molecules that are likely to bind to protein target of interest (virtual screening).
- Lead optimization – docking can be used to predict in where and in which relative orientation a ligand binds to a protein (also referred to as the binding mode or pose). This information may in turn be used to design more potent and selective analogs.
- Bioremediation – Protein ligand docking can also be used to predict pollutants that can be degraded by enzymes.

1.5. ANTIMICROBIAL

1.5.1. ANTIBACTERIAL

Synthetic antibacterial compounds are divided into two major classes: typical agents and systemic agents. The typical agents are termed disinfectants, antiseptics, and preservatives, depending on how they are used. Antiseptics and disinfectants differ from systemic agents in that they show little selective toxicity between the microbes and the host. Furthermore, most of them do not aid wound healing and may even impair it. Nevertheless, there are indispensable uses for disinfectants in hospital sanitation, including sterilization of surgical instruments, public health methods, and in the home. Antiseptics have important applications in the preoperative preparation of the both surgeons and patients. They also are used in treating local infections caused by microorganisms refractive to systemic antimicrobial agents. [66]

- **Mechanisms of bacterial resistance**

The outer memberane of gram-negative bacteria is composed of lipopolysaccharide, proteins, and lipids. It presents a barrier to many chemical agents, including quaternary ammonium compounds and triphenylmethane dyes. In contrast, the cytoplasmic membrane of gram-positive bacteria, excepting spores and mycobacteria, has greater permeability to most agents. Spores have a special coat and/or cortex that may not be permeable to hydrogen peroxide and chlorine disinfectants. Bacterial cells may also have efflux mechanisms that extrude agents such as quaternary ammonium compounds, dyes, and mercury compounds from their interiors. [67]

- **Systemic synthetic antibacterial**

Prontosil rubrum, sulfonamido-azo dye, was the first clinically useful systemic antibacterial agent to be discovered. This discovery was done in the early 1930s and the development of sulfonamides and sulfones as a class of antibacterial agents. Their broad antimicrobial spectrum provided, for the first time, drugs for the cure and prevention of a variety of bacterial infections; their widespread clinical use brought about a sharp decline in morbidity and mortality of treatable infectious diseases, and thus proved of great medical and public health importance. Recognition of the inhibition of the action of sulphonamides by extracts which was down to be attributed to the presence of p-aminobenzoic acid (PABA), required in folate biosynthesis, was the first clear demonstration of metabolites antagonism as a mechanism of drug action; this provided the long sought after mechanistic basis for drug action.[68]

1.5.2. ANTIFUNGAL

The first antifungal discovered in 1939 was griseofulvin. The introduction of griseofulvin was followed in 1960 by that of amphotericin B, which is still the "gold standard "for the treatment of severe systemic mycoses.

Fungal infection are caused by eukaryotic organisms and for that reason they generally present more difficult therapeutic problems than do bacterial infections. The fungal cell wall may be considered to be a prime target for selectively toxic antifungal agent because of its chitin structure, absent from human cells. Day by day number of antifungal agent are preparing and against fungi.

Of the five fundamental kingdoms of Life, the kingdom fungi are arguably the most diverse and prevalent. Unlike the kingdom monera (containing bacteria), fungi are eukaryotic organisms.

Thus the issue of selectivity predominates in the guest for safe and effective chemotherapeutic remedies for diseases caused by fungi. Chemotherapy, there is a risk-reward ratio to be taken into account; in the context of fungal infections, this ratio may vary greatly, from minor irritations such as athlete's foot to life-threatening systemic infections such as those caused by *Aspergillus fumigatus*. This addresses medicinal aspects of the treatment of fungal diseases of all types, but because most recent research has been directed toward the treatment of systemic infections, emphasis is placed on this aspect. [69]

1.6. ANTIOXIDANT

The World Health Organization (WHO) has estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts and their active components. Under stress, our bodies produce more reactive oxygen species (ROS) than enzymatic antioxidants and non-enzymatic antioxidants. This imbalance leads to cell damage and health problems. A lack of antioxidants, which can quench the reactive free radicals, facilitates the development of degenerative diseases, including cancers and inflammatory diseases. One solution to this problem is to supplement the diet with antioxidant compounds. The antioxidants can therefore serve as a type of preventive medicine. Recent reports indicate that there is an inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases. However, synthetic antioxidants have been widely used as antioxidants in the food industry. [70]

Antioxidants, including pyrazolone compounds have diverse biological effects, such as anti-inflammatory, anti-cancer effects, as a result of their antioxidant activiy.

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents. [71]

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and damage or kill cells.

As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stoke and inflammatory diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease.

Reactive oxygen species (ROS), capable of causing damage to DNA, has been associated with carcinogenesis, coronary heart disease, inflammatory disease and many other health problems related to advancing age. In low concentrations, synthetic antioxidants are also in use for many industrial processes e.g. inhibition of radical formation for preventing premature polymerization during processing, storage and transportation of unsaturated monomers. They exert their effects by scavenging or preventing he generation of ROS which can protect the formation of free radicals and retard the progress of many chronic diseases including cancer and inflammation diseases. [72]

Some of the methods available for knowing antioxidant activity are,

- **Determination of DPPH (1-1-diphenyl 2-picryl hydrazyl) radical-scavenging activity**
- **Determination of hydroxyl radical-scavenging activity**
- **Determination of nitric oxide radical-scavenging activity**
- **ABTS radical scavenging assay**
- **FRAP radical scavenging assay**

1.7. CANCER

Normal cells in our body follow an orderly path of growth, division and death. Cancer is a class of diseases characterized by out-of-control cell growth which harms the body by forming lumps or masses of tissue called tumors. Tumors are invasive, aggressive and mostly metastatic. Tumors that stay in one spot and show limited growth are called benign which can often be removed, and, in most cases, they do not come back. Also, cells in benign tumors do not spread to other parts of the body. Cancer is, thus, the result of cells that uncontrollably grow and do not die. [73]

1.7.1. CAUSES OF CANCER

Cancer is a diverse class of diseases which differ widely in their causes and biology. The common thread in all known cancers is the acquisition of abnormalities in the genetic material of the cancer cell and its progency. Research into the pathogenesis of cancer can be divided into

three broad areas of focus. The first area of research focuses on the agents and events which cause or facilitate genetic changes in cells destined to become cancer. Second, it is important to uncover the precise nature of the genetic damage, and the genes which are affected by it. The third focus is on the consequences of those genetic changes on the biology of the cell, both in generating the defining properties of a cancer cell, and in facilitating additional genetic events, leading to further progression of the cancer.

- **Chemical carcinogens:** Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis.Substances that cause DNA mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Particular substances have been linked to specific types of cancer. Tobacco smoking is associated with lung cancer and bladder cancer. Prolonged exposure to asbestos fibers is associated with mesothelioma. Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen. Such chemicals are thought to promote cancers through their stimulating effect on the rate of cell mitosis.
- **Ionizing radiation:** Sources of ionizing radiation, such as radon gas, can cause cancer. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies.
- **Hormonal imbalance:** Some hormones can act in a similar manner to non mutagenic carcinogens in that they may stimulate excessive cell growth. A well established example is the role of hyper estrogenic states in promoting endometrial cancer.
- **Immune system dysfunction:** HIV is associated with a number of malignancies, including kaposi's sarcoma, non-hodgkin's lymphoma, and HPV associated malignancies such as anal cancer and cervical cancer. AIDS defining illnesses have long included these diagnoses. The increased incidence of malignancies in HIV patients points to the breakdown of immune surveillance as a possible etiology of cancer. Certain other immune deficiency states (e.g. common variable immunodeficiency and IgA deficiency) are also associated with increased risk of malignancy.
- **Heredity:** Most forms of cancer are "sporadic", and have no basis in heredity. There are, however, a number of recognised syndromes of cancer with a hereditary component, often a defective tumor suppressor allele.

- **Other causes:** A few types of cancer in non-humans have been found to be caused by the tumor cells themselves. This phenomenon is seen in sticker's sarcoma, also known as canine transmissible venereal tumor.[74]

1.7.2. TYPES OF CANCER

- **Carcinoma** - cancer of the skin or tissues that line or cover internal organs.
- **Sarcoma** - cancer that starts in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow.
- **Lymphoma and myeloma** - cancers that begin in the cells of the immune system.
- **Central nervous system cancers** - cancers that begin in the tissues of the brain and spinal cord.
- **Adenoma** - cancers that arise in the thyroid, the pituitary gland, the adrenal gland, and other glandular tissues.

1.7.3. TREATMENT

- Surgery
- Radiation therapy
- Immunotherapy
- Hormone therapy
- Gene therapy
- Chemotherapy

1.7.4. CANCER CHEMOTHERAPY

Chemotherapy refers to the treatment of an ailment by chemicals especially by killing micro-organisms or cancerous cells. Antineoplastic drugs are used in cancer chemotherapy. A single "cure" for cancer has proved elusive since there is not a single type of cancer but more than hundred different types of cancer. Though the available anticancer drugs have distinct mechanisms of action which may vary in their effects on different types of normal and cancer cells, their toxicity to normal rapidly growing cells in the intestinal and bone marrow areas and

the problem of resistance pose a great problem. For this reason cancer chemotherapy may consist of using a combination of several drugs for varying lengths of time. [75]

Based on their mechanism of action, chemotherapy agents can be divided into three main categories as follows:

- **Drugs that stop the synthesis of DNA building blocks**
  e.g. methotrexate, fluorouracil, hydroxyurea, mercaptopurine etc.
- **Drugs that directly damage the DNA**
  e.g. cisplatin, antibiotics – daunorubicin, doxorubicin, etoposide etc.

- **Drugs that affect the synthesis or breakdown of the mitotic spindles**
  e.g. vinblastine, vincristine, paclitaxel etc.

1.8. TUBERCULOSIS

Tuberculosis (TB), which was referred to as "consumption" in the early days due to the wasting nature of this disease , is an infectious disease caused by bacteria whose scientific name is *Mycobacterium tuberculosis*. It was first isolated by a german physician named robert koch in 1882, who received the nobel prize for this discovery. Tuberculosis is transmitted primarily from person to person by breathing infected air during close contact and it mainly affects the lungs but also can involve almost any organ of the body. Another form of tuberculosis called atypical tuberculosis is transmitted by drinking unpasteurized milk contaminated with *Mycobacterium bovis*. [76]

1.8.1. TREATMENT OF TB

Standard "short" course treatment for TB consists of a six-month regimen (two months treatment with isoniazid, rifampin and pyrazinamide followed by four months of isoniazid and rifampin). Ethambutol or streptomycin may be added based on the drug sensitivity of the patient. Multiple drug therapy is adopted due to the rapid development of resistance to single drug regimen. For latent tuberculosis, the standard treatment of six to nine months of isoniazid alone is preferred. [77]

Chapter 2

Literature Review

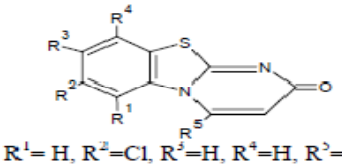
2. LITERATURE REVIEW

Literature survey suggest that extensive synthetic work on benzothiazole containing pyrazolone derivatives is going on in recent years because of their pronounced biological activities such as anti-microbial, antitubercular, antioxidant and cytotoxic activities.

2.1. BENZOTHIAZOLE NUCLEUS

2.1.1. Antimicrobial activity

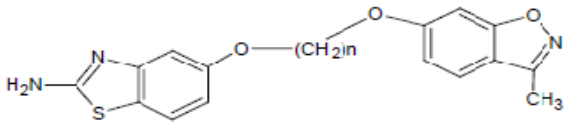
1. Gupta *et al.,* 2009[78] has reported synthesis of series of pyrimido [2,1-b] benzothiazoles exhibiting as potent antimicrobial activity against *E.coli* and *Enterobacter* as test organisms at conc 100µg per disc using vancomycine and meropenam as standard drug.



R^1= H, R^2=Cl, R^3=H, R^4=H, R^5=Ph.

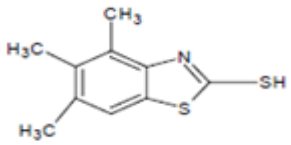
II-1

2. Kumbhare *et al.,* 2009[79] has synthesized new benzothiozole and benzisoxazole from 2-amino 5, 6-hydroxybenzothiazole, 6-hydroxy-3-methyl-1, 2-benzisoxal and different dihaloalkanes showed as antimicrobial activity against *Staphylococcus aureus,* and *E. coli* by disc diffusion method. Ciprofloxacin (10µg/ml) were used as standard drug.



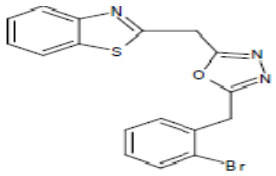
II-2

3. Murthi *et al.,* 2008[80] has synthesized some new 2-mercaptobenzothiazoles.This benzothiazole ring exhibit as antimicrobial activity against *Aspergillus flavus,*and *Candida albicans.* Fluconazole (10µg/ml) were used as standard drug.



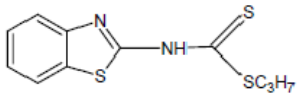
II-3

4. Rajeeva *et al.*, 2009[81] has synthesized some new 2-substituted benzothiazole derivatives exhibit as antimicrobial activity against *E. coli*, *S. aureus*. Ofloxacine (100µg/ml) were used as standard drug.



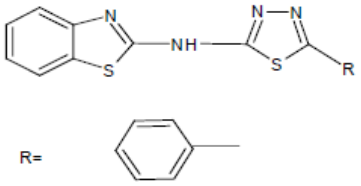
II-4

5. Maharan *et al.*, 2007[82] has synthesized series of benzothiazole-2-yl-dithiocarbamates showed as potent antimicrobial activity against *Aspergillus flavus* and *Candida albicans*. griciofulvin (100µg/ml) were used as standard drug.



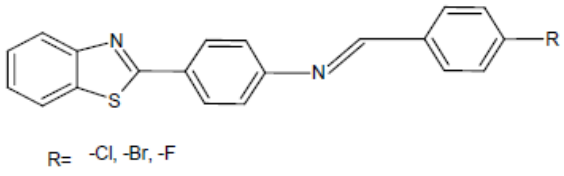
II-5

6. Amir *et al.*, 2009[83] has synthesized 1, 3, 4-thiadiazole and imidazolline derivatives exhibit as antimicrobial activity against *B. subtilis, E. coli and P.aeruginosa* by disc diffusion method at conc. 100µg/ml.



II-6

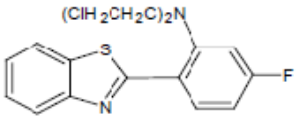
7. Nagarjuna *et al.*, 2009[84] has synthesized benzothiazole substituted thiazolidinone. Compounds showed as antimicrobial activity against *E. coli*, *S. aureus* and *C. albicans* *Aspergillus flavus.* Ofloxacine (50µg/ml) and ketokonazole (50µg/ml) were used as standard.



II-7

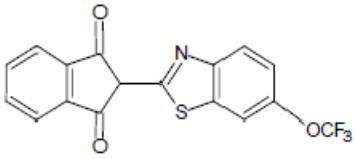
2.1.2. Anti-cancer activity

8. Kini *et al.*, 2007[85] has reported refluxed o-aminophenols with substituted benzoic acid in presence of polyphosphoric acid at higher temperature to get aryl substituted benzothiazoles which showed as anticancer activity on HL-60 cell lines.



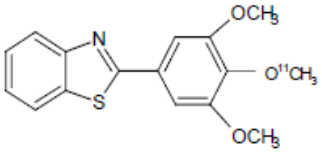
II-8

9. Stanton *et al.*, 2008[86] has synthesized benzothiazole containing phthalimide and studied their anti-cancer activity on human carcinoma cell lines.



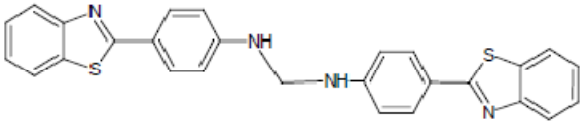
II-9

10. Wang *et al.*, 2006[87] has synthesized carbon 11 labeled fluorinated 2-aryl benzothiazoles used for protein emission tomography (PET) to image tyrocinekinese in cancer and studied their anti-cancer activity on human carcinoma cell lines.



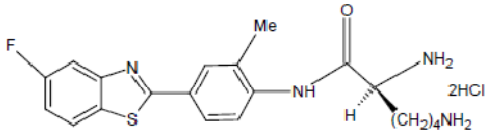
II-10

11. Gupta *et al.*, 2010[88] has synthesized benzothiazole derivatives and evaluated for *in vitro* cytotoxic activity against HL-60 and U- 937 cell lines using 5-flurouracil, and cisplatin as std. The QSAR study showed that increase in hydrogen donor count is conductive for cytotoxic activity of benzothiazole derivatives against HL-60 cell lines.



II-11

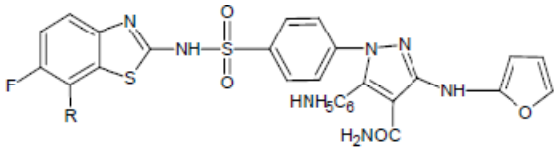
12. Hutchinson *et al.,* 2001[89] have been synthesized Fluorinated analogues of 2-(4-aminophenyl) benzothiazoles, 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole exhibit selective and potent anticancer activity on U- 937 cell lines.



II-12

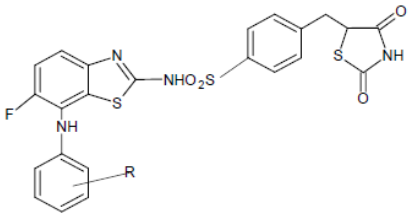
2.1.3. Anti-oxidant activity

13. Sreenivasa *et al.,* 2009[90] has synthesized fllurobenzothiazole comprising sulfonamide pyrazole derivitives. They screened synthesized for antioxidant activity by using DPPH method.



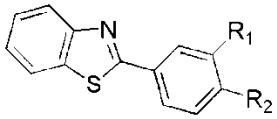
II-13

14. Pattan *et al.,* 2005[91] has synthesized 2-amino [5` (4-sulphonylbenzylidine)-2, 4-thiazolidnedione]-7-chloro-6-flurobenzothiazole series and screened for their antioxidant activity by FRAP method.



II-14

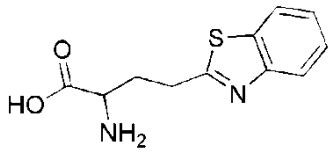
15. Radchenka *et al*., 2009[92] was reported benzthiazole containing compounds as potent anti-oxidant agents by using DPPH method.



R_1=OCH_3, R_2=H

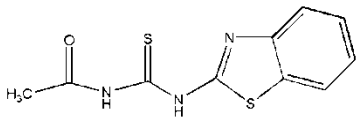
II-15

16. Smith *et al.,* 2003[93] was reported benzthiazole containing peptides as potent anti-oxidant agents by using DPPH method.



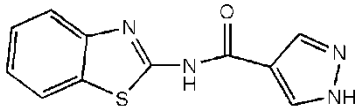
II-16

17. Sohail Saeed *et al.,* 2010[94] has reported a series of thiourea derivatives bearing benzthiazole moiety were efficiently synthesized and evaluated for antioxidant agents by using ABTS method.



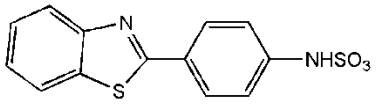
II-17

18. Samir Bondock *et al.,* 2001[95] has reported synthesis of substituted heterocyclic incorporating benzthiazole moiety as potent antioxidant agents by using DPPH method.



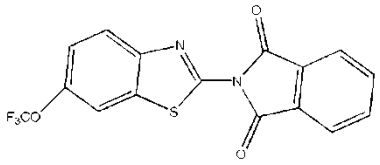
II-18

19. Stevens *et al.,* 2007[96] has reported a series of sulfamate salt derivatives of the potent and selective 2-(4-aminophenyl) benzothiazole as antioxidant agents by using ABTS method.



II-19

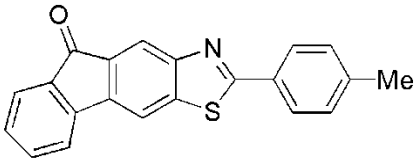
20. Albert Sun *et al.,* 2008[97] has reported synthesis of benzothiazole containing phthalic imide derivative exhibiting in-vitro antioxidant agents by using DPPH method.



II-20

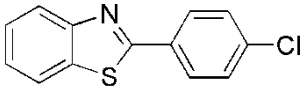
2.1.4. Antitubercular activity

21. Evindar *et al.,* 2006[98] has reported synthesis of benzothiazole moiety showed as antitubercular activity by using amplified mycobacterium tuberculosis direct assay method.



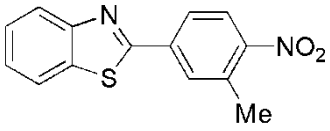
II-21

22. Joyce *et al.,* 2004[99] has reported synthesis of benzothiazole derivatives exhibit as antitubercular activity by using agar dilution assay method.



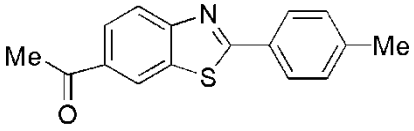
II-22

23. Batey *et al.,* 2003[100] has reported synthesis of benzothiazole showed as antitubercular activity by using microplate alamar blue assay method.



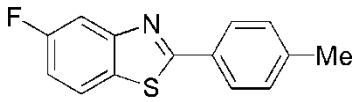
II-23

24. Rudawar *et al.,* 2005[101] has reported synthesis of benzothiazole compounds exhibit as antitubercular activity by using resazurin microplate assay method.



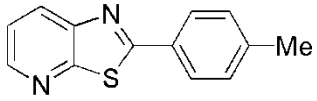
II-24

25. Lawesson *et al.,* 1977[102] has reported synthesis of benzothiazole derivatives exhibit as antitubercular activity by using amplified mycobacterium tuberculosis direct assay method.



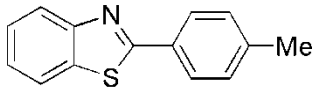
II-25

26. Cava *et al*., 1985[103] has reported synthesis of benzothiazole derivatives exhibit as antitubercular activity by using agar dilution assay method.



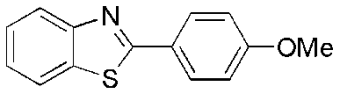
II-26

27. Foreman *et al.,* 2000[104] has reported synthesis of benzothiazole derivatives exhibit as antitubercular activity by using microplate alamar blue assay method.



II-27

28. Willis *et al.,* 2007[105] has reported synthesis of benzothiazole derivatives exhibit as antitubercular activity by using resazurin microplate assay method.

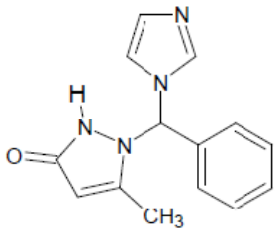


II-28

2.2. PYRAZOLONE NUCLEUS

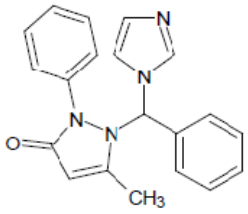
2.2.1. Antimicrobial activity

29. Furniss *et al.,* 1998[106] has reported synthesis of 1-[1*H*-imidazol-1-yl(phenyl)methyl]-5-methyl-1,2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *E.coli* and *Enterobacter* as test organisms at conc 100µg per disc using vancomycine and meropenam as standard drug by disc diffusion method.



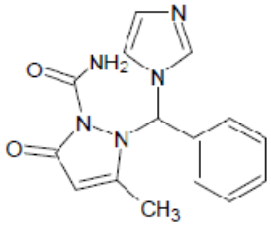
II-29

30. Harish *et al.,* 2007[107] has reported synthesis of 1-[1*H*-imidazol-1-yl(phenyl)methyl]-5-methyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *Staphylococcus aureus* and *E. coli* by disc diffusion method. Ciprofloxacin (10µg/ml) were used as standard drug.



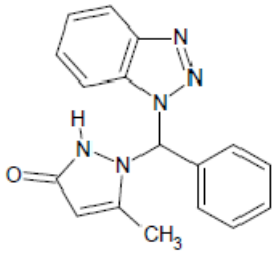
II-30

31. Dubois *et al.,* 2004[108] has reported synthesis of 2-[1*H*-imidazol-1-yl(phenyl)methyl]-3 methyl-5-oxo-2,5-dihydro-1*H*-pyrazole-1-carboxamide showed as antimicrobial activity against *Aspergillus flavus*,and *Candida albicans* by disc diffusion method. Fluconazole (10µg/ml) were used as standard drug.



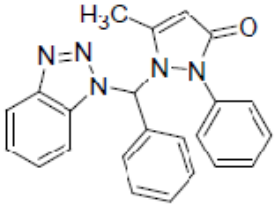
II-31

32. Sushma *et al.,* 2007[109] has reported synthesis of 1-[1*H*-benzotriazol-1-yl(phenyl)methyl]-5-methyl-1,2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *E. coli, S. aureus*. Ofloxacine (100µg/ml) were used as standard drug.



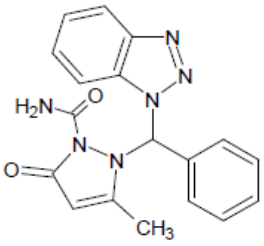
II-32

33. Winter *et al.,* 1962[110] has reported synthesis of 1-[1*H*-benzotriazol-1-yl (phenyl) methyl]-4-methyl-2-phenyl-1, 2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *Aspergillus flavus* and *Candida albicans*. griciofulvin (50µg/ml) were used as standard drug.



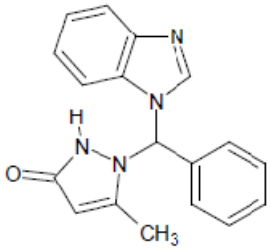
II-33

34. Arpana *et al*., 2007[111] has reported synthesis of 2-[1*H*-benzotriazol-1-yl(phenyl)methyl]-4-methyl-5-oxo-2,5-dihydro-1*H*-pyrazole-1-carboxamide showed as antimicrobial activity against *B. subtilis, E. coli and P.aeruginosa* by disc diffusion method at conc. 100µg/ml..



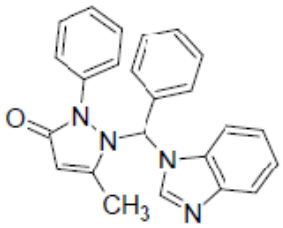
II-34

35. Pramila *et al.,* 2007[112] has reported synthesis of 1-[1*H*-benzimidazol-1-yl(phenyl)methyl]-5-methyl-1,2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *E. coli, S. aureus* and *C. albicans Aspergillus flavus.* Ofloxacine (10µg/ml) and ketokonazole (10µg/ml) were used as std.



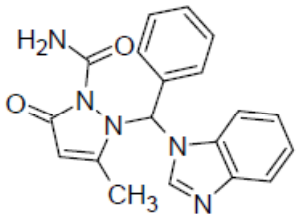
II-35

36. Melmed *et al.,* 2004[113] has reported synthesis of 1-[1*H*-benzimidazol-1-yl(phenyl)methyl]-5-methyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one showed as antimicrobial activity against *Aspergillus flavus* and *Candida albicans*. griciofulvin (10µg/ml) were used as standard drug..



II-36

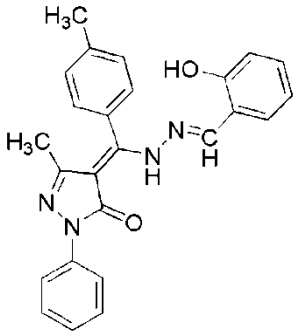
37. Rainsford *et al.,* 2001[114] has reported synthesis of 2-[1*H*-benzimidazol-1-yl(phenyl)methyl]-3-methyl-5-oxo-2,5-dihydro-1*H*-pyrazole-1-carboxamide showed as antimicrobial activity against *E. coli.* Ofloxacine (100µg/ml) were used as standard drug.



II-37

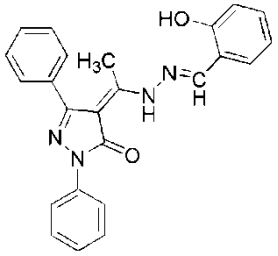
2.2.2. Anti-cancer activity

38. Pan *et al.*, 2001[115] has reported synthesis of 1-phenyl-3-methyl-4- (2 -hydroxylbenzylidenehydrazine)-p-methylbenzylidene-5- pyrazolone exhibit selective and potent anticancer activity by using MTT assay method.



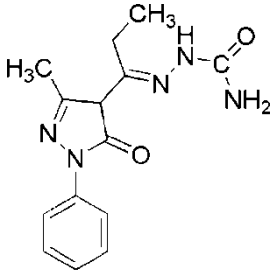
II-38

39. Bertenshaw *et al.,* 1997[116] has reported synthesis of 1, 3-di phenyl-4- (2 - hydroxylbenzylidenehydrazine)-ethidine-5- pyrazolone exhibit selective and potent anticancer activity by using flow cytometric assay for cell apoptosis.



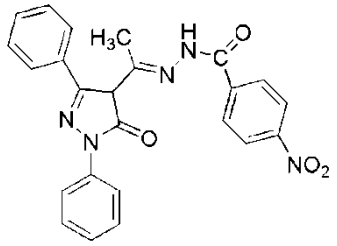
II-39

40. Cirillo *et al.,* 2002[117] has reported synthesis of 1-Phenyl-3- methyl-4-propionyl-5- pryazolone semicarbazone exhibit selective and potent anticancer activity by using lactate dehydrogenase release assay method.



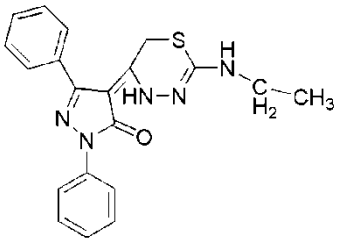
II-40

41. Amici *et al*., 2004[118] has reported synthesis of 1, 3-di phenyl-4- (p - nitrobenzoylhydrazide)-ethidine-5- pyrazolone exhibit selective and potent anticancer activity by using p-glycoprotein pump in MCF-7R cells assay method.



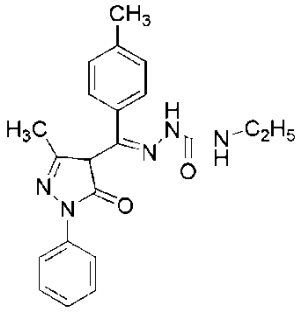
II-41

42. Sasaki *et al.,* 2004[119] has reported synthesis of 1,3-di phenyl-4- (2 –ethylamino-6H -1, 3,4-th iadiazine-5- yl ene)-5- pyrazolone exhibit selective and potent anticancer activity by using insulin-like growth factor-I induced kinase receptor activation assay .



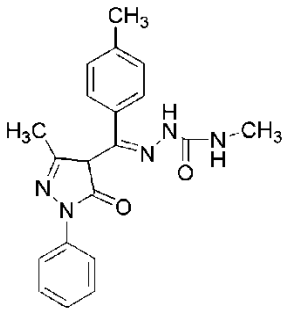
II-42

43. Ashour *et al*., 2005[120] has reported synthesis of 1-Phenyl-3- methyl-4-(ethylaminoformylhydrazide)-p- methylbenzylidene-5- pyrazolone exhibit selective and potent anticancer activity by using insulin-like gD trkA induced kinase receptor activation assay method.



II-43

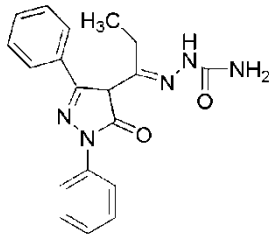
44. Zhang *et al.,* 2006[121] has reported synthesis of 1-phenyl-3- methyl-4-(methylaminoformylhydrazide)-p- methylbenzylidene-5- pyrazolone exhibit selective and potent anticancer activity by using tumor endothelial cell tube formation assay.



II-44

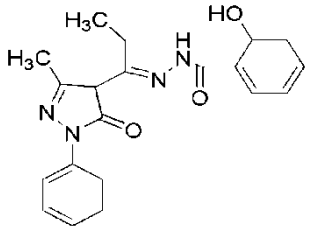
2.2.3. Anti-oxidant activity

45. Ovalles *et al.,* 2006[122] has reported synthesis of 1,3-di phenyl-4- propionyl-5-pyrazolone semicarbazone as antioxidant agents by using ABTS method.



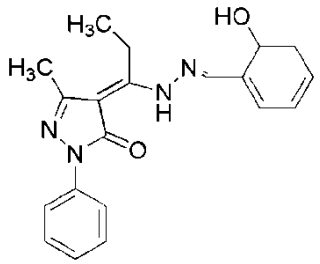
II-45

46. Naito *et al*., 2002[123] has reported synthesis of [CuL(EtOH)]1-Phenyl-3- methyl-4-(salicyclidenehydrazide)-propylidene-5- pyrazolone as antioxidant agents by using FRAP method.



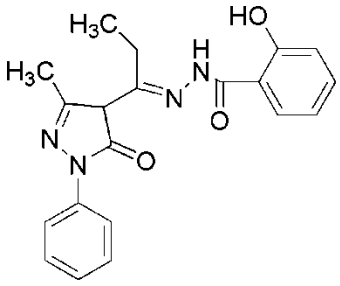
II-46

47. Deng *et al*., 2004[124] has reported synthesis of [CuL(EtOH)]1-Phenyl-3- methyl-4- (2 -hydroxylbenzylidenehydrazine)-propylidene-5- pyrazolone as antioxidant agents by using ABTS method.



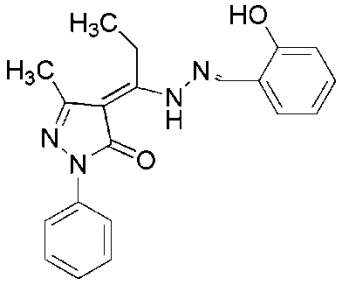
II-47

48. Liang *et al.*, 2002[125] has reported synthesis of 1-Phenyl-3- methyl-4- (2 -hydroxylbenzoylhydrazide)-propylidene-5- pyrazolone as antioxidant agents by using ABTS method.



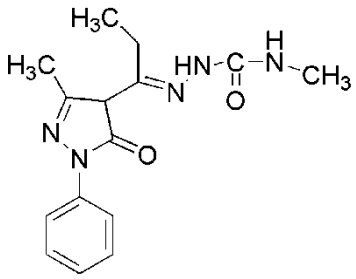
II-48

49. Jia *et al.*, 2007[126] has reported synthesis of 1-Phenyl-3- methyl-4- (2 -hy droxylbenzylidenehydrazine)-propylidene-5- pyrazolone as antioxidant agents.



II-49

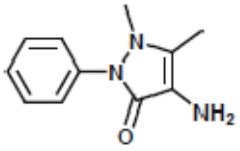
50. Tu *et al.*, 2003[127] has reported synthesis of 1-Phenyl-3- methyl-4- (methylaminoformylhydrazide)-propylidene-5- pyrazolone as antioxidant agents by using DPPH method. .



II-50

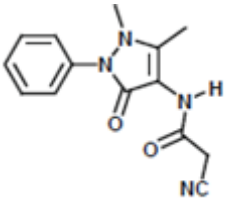
2.2.4. Antitubercular activity

51. Sandrini *et al.,* 2002[128] has reported synthesis of 4-amino-2,3-dimethyl-1-phenyl-1,2-dihydropyrazol-3-one showed as antitubercular activity by using resazurin microplate assay method.



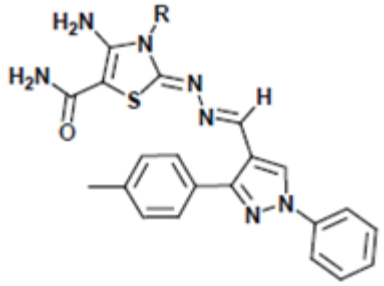
II-51

52. Erol *et al.,* 2001[129] has reported synthesis of 2-chloro-N-(2, 5-dihydro-2, 3-dimethyl-5-oxo-1-phenyl-1H-pyrazol-4-yl)-acetamide showed as antitubercular activity by using amplified mycobacterium tuberculosis direct assay method.



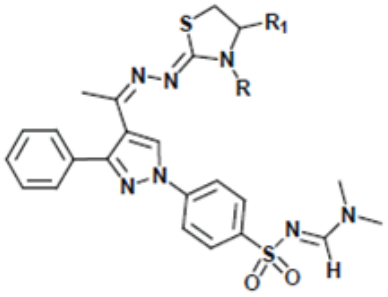
II-52

53. Fahmy *et al.,* 2003[130] has reported synthesis of pyrazolone compounds showed as antitubercular activity by using resazurin microplate assay method.



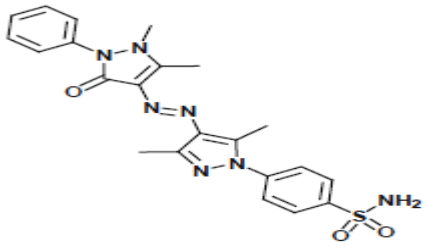
II-53

54. Bekhit *et al.*, 2004[131] has reported synthesis of pyrazolone moiety showed as antitubercular activity by using microplate alamar blue assay method.



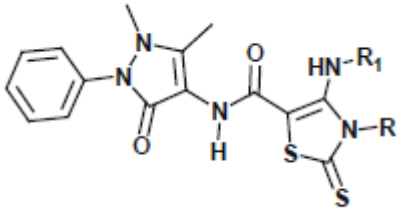
II-54

55. Sahin *et al.*, 2004[132] has reported synthesis of pyrazolone moiety exhibit as antitubercular activity by using microplate alamar blue assay method.



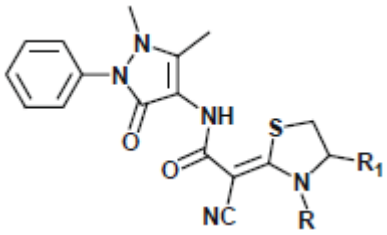
II-55

56. Hawash *et al.*, 2006[133] has reported synthesis of pyrazolone moiety exhibit as antitubercular activity by using amplified mycobacterium tuberculosis direct assay method.



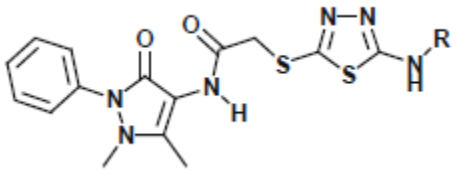
II-56

57. Rismondo *et al.,* 2007[134] has reported synthesis of pyrazolone moiety showed as antitubercular activity by using resazurin microplate assay method.



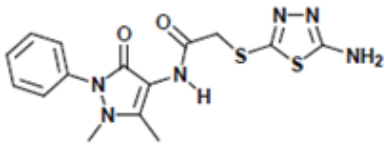
II-57

58. Varga *et al.,* 2008[135] has reported synthesis of pyrazolone moiety exhibit as antitubercular activity by using agar dilution assay method.



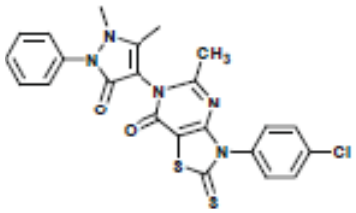
II-58

59. Dannhardt *et al.,* 2001[136] has reported synthesis of 2-(5-amino-1, 3, 4-thiadiazol-2-ylthio)-N-(2,5-dihydro-2,3-dimethyl-5-oxo-1-phenyl-1H-pyrazol-4-yl) acetamide showed as antitubercular activity by using microplate alamar blue assay method.



II-59

60. Lown *et al.,* 2002[137] has reported synthesis of 2-cyano-N-(2, 5-dihydro-2, 3-dimethyl-5-oxo-1-phenyl-1H-pyrazol-4-yl) acetamide showed as antitubercular activity by using amplified mycobacterium tuberculosis direct assay method.



II-60

Chapter 3

Aim and Objectives

3. AIM AND OBJECTIVES

Tuberculosis is a major health problem worldwide, with approximately 1.7 million people dying annually from the disease. The long current drug regimen, the emergence of drug resistant strains and HIV co-infection have resulted in a resurgence in research efforts to address the urgent need for new antituberculosis drugs. The success of chemotherapy is strictly related to the use of drug combinations. In the initial treatment, it is a fundamental rule to use atleast three drugs, to avoid the emergence of bacterial resistance, which is the principle cause of therapeutic failure. The development of resistance to antibiotics and the changing clinical pattern of bacterial infections are constant challenge to drug research.

From the chemotherapeutic point of view, there are two sources of new chemical entities. The first is the extraordinary diversity provided by natural products. The second results from the design of new or the modification of synthetic transformations.

Benzothiazole, Pyrazolone have attracted continuing interest because of their varied biological activities.

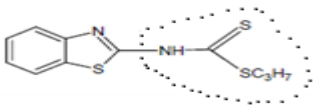
Benzothiazoles are bicyclic ring system with multiple applications. Various benzothiazoles such as 2-aryl benzothiazole received much attention due to unique structure and its exhibit as cytotoxic on cancer cells, antimicrobial, antioxidant, antitubercular [14] agents.

Pyrazolone derivatives are an important class of heterocyclic compounds. In recent years, the pyrazolone nucleus containing compounds involved in research aimed at evaluating new products that possess biological activities, such as antimicrobial, *in-vitro* antioxidant, antitubercular and anticancer agents.

Based on the literatures we are planning to link both Benzthiazole and Pyrazolone nucleus for better biological activities like anti-microbial, *in-vitro* anticancer, antitubercular and antioxidant agents.

Maharan *et al.,* 2007[82] has synthesized series of benzothiazole-2-yl-dithiocarbamates showed as potent antimicrobial activity. Particularly, NH group present at second position which exhibit as anti-microbial, antitubercular, antioxidant and cytotoxic agents.

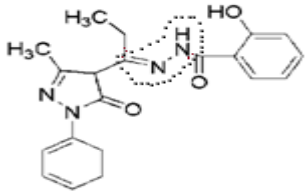
Previous reported compound



III-1

Naito *et al*., 2002[123] has reported synthesis of [CuL(EtOH)]1-Phenyl-3- methyl-4-(salicyclidenehydrazide)-propylidene-5- pyrazolone as antioxidant agents. Especially, the third and fourth position groups may show anti-microbial, antitubercular, antioxidant and cytotoxic agents.

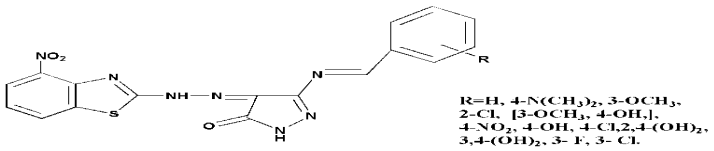
Previous reported compound



III-2

Consequently, the combination of benzothiazole containing pyrazolone as promising approach in drug-like molecules design. Hence it is expected that these compounds may show antimicrobial, anticancer, antitubercular and antioxidant activities.

Newly synthesized compounds



III-3

The aim of our work is, in connection with the above diverse biological activities we turned our attention to the synthesize, some newer benzothiazole containing pyrazolone bearing various aromatic aldehydes and related compounds, with the carefully selected auxophores like bromo, chloro, nitro and methoxy aldehydes groups and to facilitate biological activities of lead molecule by the appropriate synthetic route for anti-microbial, antitubercular, antioxidant and cytotoxic agents.

The purpose of research was to synthesize the better activity compounds using different substituted aromatic aldehydes are chosen as the starting material for the synthesis of schiff's bases with pyrazolone. [138]

3.1. MOLECULAR DOCKING STUDIES

The objective of the project is to implement the grid generation and rigid docking steps of dock as described. Implementation of a carefully tuned form of grid calculation should be fairly easy to accomplish; an implementation of rigid docking will be somewhat challenging, but should also be possible to finish in the time allowed for the project. The grid generation implementation will be benchmarked using a set of target proteins ranging in size from 100 to 2000 atoms, and rigid docking of ligands will be benchmarked on a set of ligands ranging from 5 to 20 atoms, docked into a pregenerated set of 76 target points. Analysis of the limitations of the hardware for this problem is required, including a comparison of the different bottlenecks encountered for the grid generation and conformational sampling steps. Molecular docking study was on one tubercular protein Thymidylate Kinase of benzothiazole containing pyrazolone derivatives. [139]

Chapter 4

Plan of work

4. PLAN OF WORK

The plan of the present work,

- The review of literature and scientific survey of the title compounds was done from 1950's.From that developed new methodology.
- Design and docking study of designed compounds by autodock software module version 4.0.
- To synthesis the title Schiff base compounds, based on the reported literature procedure by using various aldehydes.
- The purity of the recrystallized compounds check by melting point, TLC and solubility.
- Characterization of synthesized compounds by various analytical techniques like UV-Visible, FT-IR, ^{1}H NMR and Mass spectral studies.
- Screening of synthesized compounds for their preliminary antimicrobial activity of zone of inhibition by disc diffusion method, minimum inhibitory concentration by serial dilution method and bactericidal, fungicidal by minimum bactericidal, fungicidal concentration method.
- Screening of synthesized compounds for *in vitro* anti-tubercular activity against *Mycobacterium tuberculosis* H_{37}Rv by Resazurin Microplate Assay method.
- The *in vitro* anti-oxidant activity of synthesized compounds to be determined by using DPPH, FRAP and ABTS method.
- Screening of synthesized compounds for *in vitro* cytotoxic activity by MTT assay method.

Chapter 5

Experimental Work

5. EXPERIMENTAL WORK

EXPERIMENTAL PROTOCOL

The experimental work conducted under the following sub headings.

EXPERIMENTAL PART I (Chapter V)

5.1. Docking studies.

5.2. Synthesis.

5.2.1. Step I Preparation of 4-nitro-1, 3-benzothiazol-2-amine.

5.2.2. Step II Preparation of ethyl cyano[2-(4-nitro-1,3-benzothiazol-2-yl) hyrazinylidene] acetate.

5.2.3. Step III Preparation of 5-amino-4-[2-(4-nitro-1, 3-benzothiazol-2-yl) hydrazinylidene]-2, 4-dihydro-3H-pyrazol-3-one.

5.2.4. Step IV Preparation of schiff base.

5.3. Physicochemical studies and Characterization of synthesized compounds.

EXPERIMENTAL PART II (Chapter VI)

6. Biological screening

6.1. Evaluation *In-vitro* antimicrobial study of zone of inhibition by disc diffusion method, minimum inhibitory concentration by serial dilution method and bactericidal, fungicidal by minimum bactericidal concentration and minimum fungicidal concentration method.

6.2. Evaluation *In-vitro* antitubercular activity by REMA method.

6.3. Evaluation *In-vitro* antioxidant study by DPPH, FRAP, ABTS assay method.

6.4. Evaluation *In-vitro* cytotoxic activity by MTTS method.

5.1. DOCKING STUDIES

5.1.1. AUTODOCK

Autodock is a suite of C programs used to simulate interactions between small flexible ligands and macromolecules of known structure. Docking is achieved through a search of conformational space using a Lamarckian genetic algorithm coupled with energy assessments using autodock method. The combination of these two functions produces a family of molecular coordinates detailing possible docked ligand conformations which can then be used as a starting

point for theoretical ligand design and study. Confidence in the docked conformation is represented by an energy value based on both quantum and molecular mechanical modelling of atomic forces. The success of a docking program depends on the Lamarckian genetic algorithm. [140]

- **Lamarckian genetic algorithm**

The vast majority of genetic algorithms mimics the major characteristics of darwinian evolution and apply mendelian genetics. This is illustrated on the one way transfer of information from the genotype to the phenotype. However, in those cases where an inverse mapping function exists, one which yields a genotype from a given phenotype, it is possible to finish a local search by replacing the individual with the result of the local search. This is called the Lamarckian genetic algorithm (LGA). The most important issues arising in hybrids of local search (LS) techniques with the GA revolve around the developmental mapping, which transforms genotypic representations into phenotypic ones. The genotypic space is defined in terms of the genetic operator's mutation and cross over in our experiments by which parents of one generation are perturbed. The phenotypic space is defined directly by the problem, namely, the energy function being optimized. [141]

In our case, the fitness or energy is calculated from the ligand's coordinates, which together form its phenotype. The genotypic representations of the ligand, and its mutation and cross over operators, have already been described. The developmental mapping simply transforms a molecule's genotypic state variables into the corresponding set of atomic coordinates. This means that the developmental mapping does not need to be inverted. None the less, this molecular variation of the genetic algorithm still qualifies as Lamarckian, because any ''environmental adaptations'' of the ligand acquired during the local search will be inherited by its offspring. At each generation, it is possible to let a user defined fraction of the population undergo such a local search. We have found improved efficiency of docking with local search frequencies. [142]

5.1.2. AUTODOCK PROCEDURE[143]

Docking studies of compounds BTZP3a-BTZP3l were performed using one antimycobacterial protein Thymidylate Kinase (PDB ID: 1MRS) obtained from the RCSB protein data

bank,http://www.rcsb.org/pdb.Experiments were performed using the program Autodock module version 4.0.

AutoDock includes 3 steps:

1. Preparation of receptor & ligand files.

2. Calculation of affinity maps by using a 3D grid around the receptor & ligand.

3. Defining the docking parameters and running the docking simulation.

The preparation step starts with pdb files of receptor (R.pdb) and ligand (L.pdb), which are added hydrogens and then saved as RH.pdb & LH.pdb. The calculation of affinity maps in the "Grid" section requires the above pdb files to be assigned charges & atom types, and also that the nonpolar hydrogens are merged. This is done automatically by ADT, and the resulting files need to be saved as RH.pdbqt & LH.pdbqt, which is the only format AutoGrid & AutoDock can work with. Calculation of affinity maps is done by AutoGrid, and then docking can be done by AutoDock. The newest docking algorithm is LGA (Lamarckian Genetic Algorithm).

Preparing and Running a Docking

1. Preparing the protein

- Opening file: [Right-click "PMV molecules"] → [choose file].
- Color by atom: [Click under "Atom"].
- Eliminate water: Select → Select from string → [write HOH in "Residue" line and in the "Atom" line] → Add → Dismiss → Edit → Delete → Delete Atom Set.
- Find missing atom and repairing them: File → Load module → [Pmv; repair Commands] → Edit → Misc. → Check for missing atoms → Edit → Misc. → Repair missing atoms.
- Add hydrogen's: Edit → Hydrogen's → Add → [choose "All hydrogen's", "no bond order", and "Yes" to renumbering].

- Hide protein: [Click on the gray under "show Molecules"]. (Note: if you are planning rigid docking (i.e. no flexible parts in the protein), save the protein as RH.pdb for now)

2. Preparing the ligand

- Make sure the ligand has all hydrogens added before working with ADT.
- Toggle the "AutoDock Tools" button.
- Opening file: Ligand → Input → Open → All Files → [choose file] → Open. (ADT now automatically computes Gasteiger charges, merges nonpolar hydrogens, and assigns Autodock Type to each atom.)
- Define torsions:

  * Ligand → Torsion Tree → Detect Root (this is the rigid part of the ligand)

  * Ligand → Torsion Tree → Choose Torsions → [either choose from the viewer specific bonds, or use the widget to make certain bond types active (rotatable) or inactive (non-rotatable). Amide bonds should NOT be active (colored pink)] → done.

  * Ligand → Torsion Tree → Set Number of Torsions → [choose the number of rotatable bonds that move the 'fewest' or 'most' atoms].

  Save ligand file:

  * Ligand → Output → Save as PDBQT → [save with L.pdbqt].

  Hide the ligand, as explained in (A5) for the protein.

3. Preparing the flexible residue file (Note: if you are planning rigid docking, ignore this section and do the following: Grid → Macromolecule → Open → [choose RH.pdb]. AutoDock will automatically add charges and merge hydrogens. Save the object as RH.pdbqt and move to next section.)

- Flexible residues → Input → Choose molecule → [choose the original protein R.pdb] → Yes to merge nonpolar hydrogens (AutoDock assigns charges + atom types to R.pdb, and merges nonpolar hydrogens).

- Select the residues to be flexible: Select → Select from string → ARG8 → Add → Dismiss.
- Define the rotatable bonds: Flexible residues → Choose torsions in currently selected residues → [click on rotatable bonds to inactivate them, or vice versa].
- Save the flexible residues: Flexible residues → Output → Save flexible PDBQT → [save as R_flex.pdbqt].
- Save the rigid residues: Flexible residues → Output → Save rigid PDBQT → [save as R_rigid.pdbqt].
- Delete this version of protein: Edit → Delete → Delete Molecule → [choose protein (R)] → Delete → Dismiss.

4. Running AutoGrid calculation the purpose of this section is to define the search grid and produce grid maps used later by Autodock.

- Open the rigid protein: Grid → Macromolecule → Open → [choose the rigid protein] → Yes to preserving the existing charges. (Note: if you are doing rigid docking, choose RH.pdbqt)
- Prepare grid parameter file: Grid → Set Map Types → Choose Ligand → [choose the ligand already opened] → Accept.
- Set grid properties: Grid → Grid Box → [Set the grid dimensions, spacing, and center] → File → Close Saving Current.
- Save the grid settings as GPf file: Grid → Output → Save GPF → [save as R.gpf].
- Running: Run → Run AutoGrid → [make sure the program name has the right path, and that it is where the input files are] → Launch → [in the command prompt prompt, type "tail –f hsg1.glg" to follow the process] (Note: the AutoGrid calculation can be started directly from the command prompt by typing "autogrid4 –p hsg1.gpf –l hsg1.glg ")

5. Preparing the docking parameter file (.dpf)

- Specifying the rigid molecule: Docking → Macromolecule → Set Rigid Filename → [choose R_rigid.pdbqt]. (or RH.pdbqt for rigid docking)
- Specifying the ligand: Docking → Ligand → Choose → [choose L.pdbqt] → [here you can set the initial location of the ligand] → Accept.
- Specifying the flexible residues: Docking → Macromolecule → Set flexible Residues Filename → [choose R_flex.pdbqt].
- Setting the parameters for the chosen docking method: Docking → Search Parameters → Genetic Algorithm → [for 1st time, use the short number of evaluations (50,000), and for other runs choose the medium or long] → Accept. 5. Setting docking parameters: Docking → Docking Parameters → [choose the defaults].
- Specifying the name of the ligand dpf file to be formed, containing the docking instructions: Docking → Output → Lamarckian GA → [type L.dpf].
- Confirming the details of docking: Docking → Edit DPF → [make sure the right ligand pdbqt file name appears after the word "move", and that the right number of active torsions is specified].

6. Running AutoDock

- Make sure the AutoDock executable is in the same directory as the macromolecule, ligand, GPF, DPF and flex files (in case of flexible docking).
- Running: Run → Run AutoDock... → Launch.

7. When RH and LH already exist

- Protein: Grid → Macromolecule → choose RH.pdb → (charges & atom types assigned, nonpolar hydrogen merged) → File → save → write PDBQT → save as RH.pdbqt
- Ligand: Ligand → Input → Open → All Files → choose LH.pdb → (charges & atom types assigned, nonpolar hydrogen merged) → save as LH.pdbqt
- Set the rest of the grid parameters & calculate map

- Setting Docking parameters: Docking → Macromolecule → Set Rigid Filename → choose either RH.pdbqt or RH_rigid.pdbqt → Docking → Ligand → Choose → choose LH.pdbqt → set the rest of the docking parameters.
- Running docking simulation.

8. Viewing Docking Results

A. Reading the docking log file (.dlg)

- Analyze → Dockings → Open → [choose L.dlg].
- Analyze → Conformations → Load → [double-click on each conformation to view it on screen].

B.Visualizing docked conformations

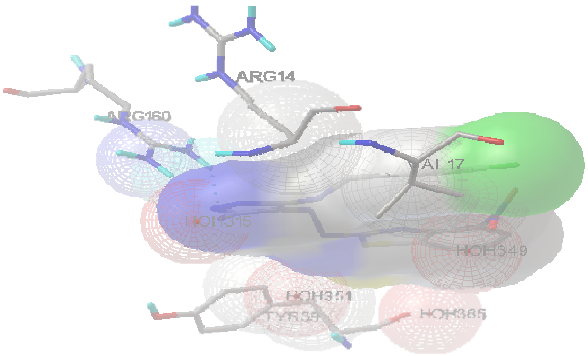
- Analyze → Conformations → Play... (Note: & allows changing the ligand's color) .The docking score and graph was given in the **table 1 and Figure 1.**

Table 1. Docking score of synthesized compounds

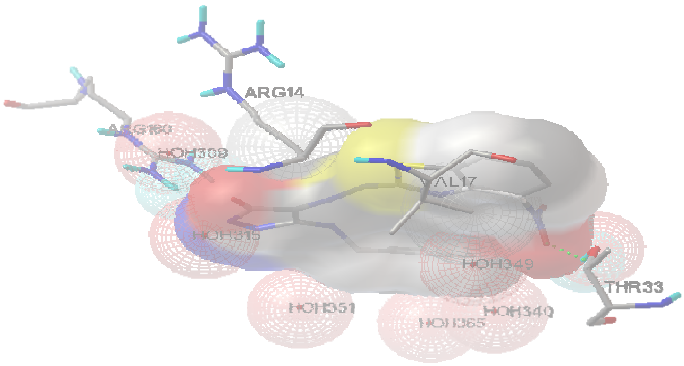
Compound code	Autodock (pose) score Kcal/mol
	1MRS
BTZP3a	-6730000.0
BTZP3b	-6980000.00
BTZP3c	-5.22
BTZP3d	-5.68
BTZP3e	-6710000.0
BTZP3f	-7010000.0
BTZP3g	-5.65
BTZP3h	-5.82
BTZP3i	-6910000.0
BTZP3j	-6450000.0
BTZP3k	-5.46
BTZP3l	-5.59

Figure 1. Docked ligand of synthesized compounds

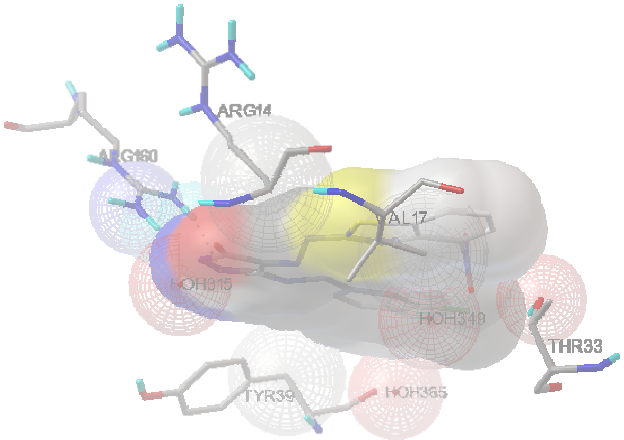
BTZP3h



BTZP3k

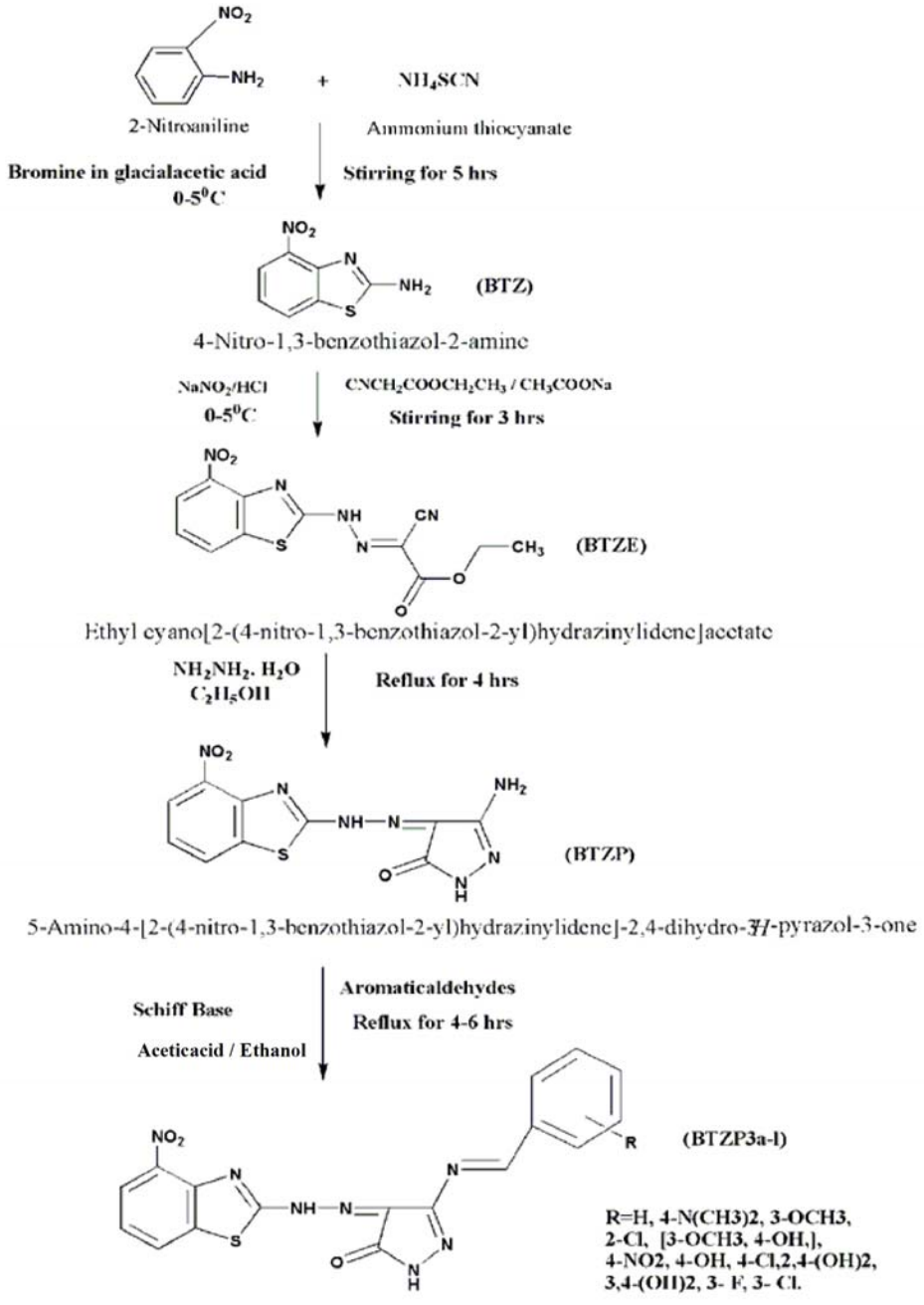


BTZP3l

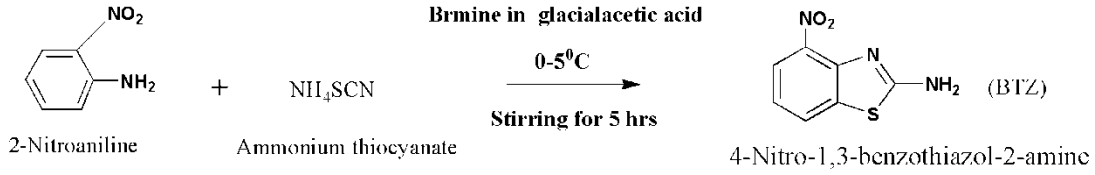


5.2. SYNTHESIS

SCHEME



5.2.1. STEP 1: SYNTHESIS OF 4-NITRO-1, 3-BENZOTHIAZOL-2-AMINE FROM 2-NITRO ANILINE:

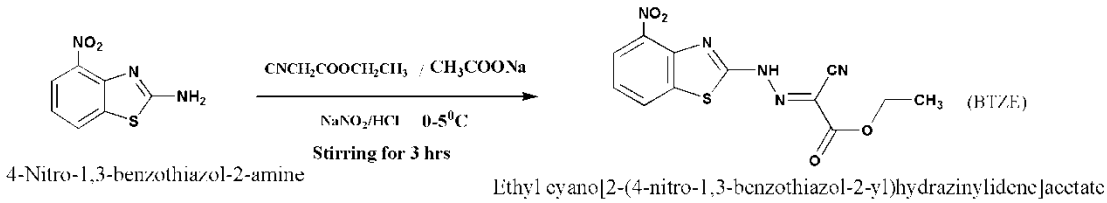


V-2

PROCEDURE [144]

To glacial acetic acid [150ml], precooled to 5^0c, ammonium thiocyanate [0.06mol, 4.56gm] and 2-nitroaniline [0.06mol, 5.58gm] were added. The mixture was placed in freezing with addition of bromine [0.02mol, 1.01mol in 10ml glacial acetic acid] from a dropping funnel at such rate that temperature does not rise above 5^0c, stirring was continued for 5 hrs at 0-10^0c and neutralized with aqueous ammonia solution, kept it in refrigerator for overnight, filtered, washed with water and dried, recrystallised from ethanol to obtained 4-nitro-1, 3-benzothiazol-2-amine (BTZ). The solvent system used for TLC was Benzene: Methanol (9:1) and Yield: 90%w/w, m.p: 148˚C, R_f = 0.63. The physicochemical properties of synthesized compounds were given in the **table 2.**

5.2.2. STEP 2: SYNTHESIS OF ETHYL CYANO [2-(4-NITRO-1, 3-BENZOTHIAZOL-2-YL) HYRAZINYLIDENE] ACETATE FROM 4-NITRO-1, 3-BENZOTHIAZOL-2-AMINE:



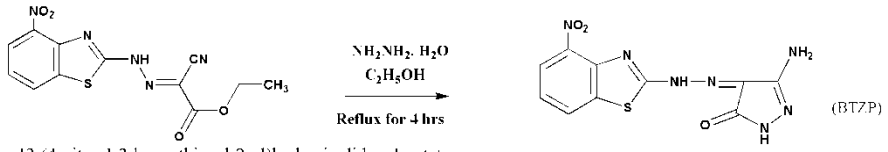
V-3

PROCEDURE [145]

A mixture of 4-nitro-1,3-benzothiazol-2-amine [0.02 mol, 5.8gm] in conc.HCl [10ml in 10ml of water] was cooled to 0-5^0c under dry ice and cooled sodium nitrite solution [1.5gm in 10ml of water] added to it drop wise during 10mts. The reaction mixture was then stirred for 30 mts.

To an ice-cold mixture of ethylcyanoacetate [0.01mol, 1.13ml] and saturated solution of sodium acetate [0.05mol,10ml] in ethanol [50ml], was added dropwise with stirring a solution of diazonium salt compound over 15 mts. The stirring was continued for 30 mts at 0-5^{0}c and the reaction mixture then left for 1.45 hrs at room temperature. The solid product was collected and recrystallised from ethanol to give ethyl cyano[2-(4-Nitro-1,3-benzothiazol-2-yl)hyrazinylidene]acetate (BTZE). The solvent system used for TLC was Benzene: Methanol (9:1) and Yield: 70%w/w, m.p: 122˚C, R_f = 0.74. The physicochemical properties of synthesized compounds were given in the **table 2.**

5.2.3. STEP 3: SYNTHESIS OF 5-AMINO-4-[2-(4- NITRO-1,3-BENZOTHIAZOL-2-YL)HYDRAZINYLIDENE]-2,4-DIHYDRO-3H-PYRAZOL-3-ONE FROM ETHYL CYANO[2-(4-NITRO-1,3-BENZOTHIAZOL-2 YL)HYRAZINYLIDENE]ACETATE :



Ethyl cyano[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]acetate

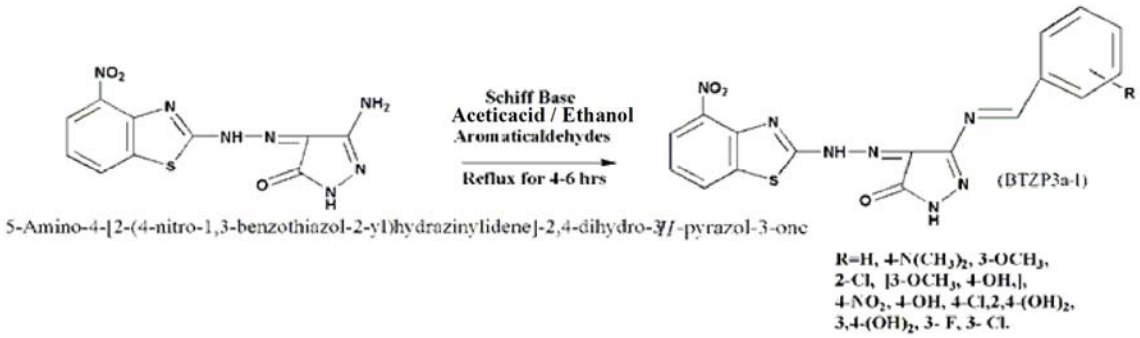
5-Amino-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3*H*-pyrazol-3-one

V-4

PROCEDURE [146]

A mixture of ethyl cyano[2-(4-Nitro-1,3-benzothiazol-2-yl)hyrazinylidene]acetate [0.005mol,1.64gm], hydrazines hydrate [0.01mol,0.32ml] in ethanol [30ml] was heated under refluxed for 4 hrs. The solvent was concentrated and the reaction product was allowed to cool. The separated product was filtered off, washed with water, dried and recrystallised from methanol to obtained 5-amino-4-[2-(4-Nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H-pyrazol-3-one (BTZP) . The solvent system used for TLC was Benzene: Methanol (9:1) and Yield: 95%w/w, m.p: 171˚C, R_f = 0.79. The physicochemical properties of synthesized compounds were given in the **table 2.**

5.2.4. STEP 4: SYNTHESIS OF SCHIFF BASE

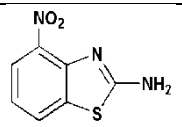
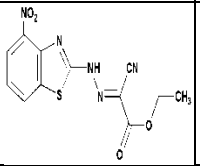
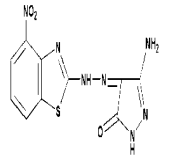


V-5

PROCEDURE [147]

Mixture of 5-amino-4-[2-(4-Nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H-pyrazol-3-one (0.01 mol, 1.1gm) and substituted aromatic aldehydes (0.01 mol.1.1gm) in ethanol containing aceticacid (0.1mol,0.5 ml) was refluxed for 4-6 hrs. Excess of solvent was distilled off, concentrated and cooled. The solid thus separated was filtered, washed and recrystallised from ethanol. The physicochemical properties of synthesized compounds were given in the **table 3.**

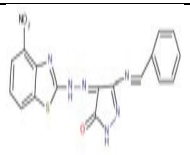
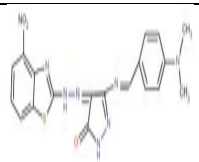
Table 2. Physico chemical data of intermediate compounds

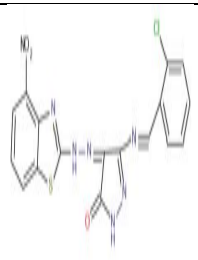
CODE NO	STRUCTURE	IUPAC NAME	MOLECULAR FORMULA	MOL. WT	MP (^{O}C)	Rf value	Log P	POLARIZABILITY	COLOUR	% YIELD	SOLUBILITY
BTZ		4-nitro-1,3-benzothiazol-2-amine	$C_7H_5N_3O_2S$	195.20	148	0.63	1.91	18.67	Brownish orange	90	CHLOROFORM
BTZE		ethyl cyano[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]acetate	$C_{12}H_9N_5O_4S$	319.30	122	0.74	3.87	29.34	Dark red	70	BENZENE
BTZP		5-amino-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{10}H_7N_7O_3S$	305.27	171	0.79	1.80	27.42	Muddy brown	95	CHLOROFORM

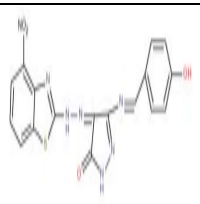
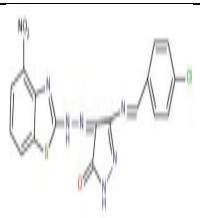
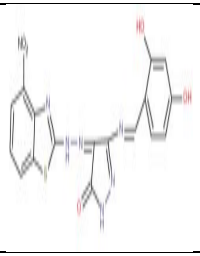
Solvent system: Benzene: Methanol (9:1)

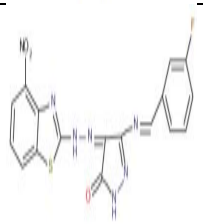
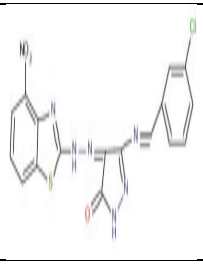
Detecting agent: Iodine vapours.

Table 3. Physico chemical data of synthesized compounds

CODE NO	STRUCTURE	IUPAC NAME	MOLECULAR FORMULA	MOL. WT	MP (^{O}C)	Rf value	Log P	POLARIZABILITY	COLOUR	% YIELD	SOLUBILITY
BTZP 3a		4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-5-{[(E)-phenylmethylidene] amino}-2,4-dihydro-3H -pyrazol-3-one	$C_{17}H_{11}N_7O_3S$	393.38	107	0.77	4.08	38.31	Brown	90	BENZENE, CHLOROFORM
BTZP 3b		5-({(E)-[4-(dimethylamino)phenyl]methylidene}amino)-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H-pyrazol-3-one	$C_{19}H_{16}N_8O_3S$	436.45	120	0.64	4.18	43.13	Brownish red	94	CHLOROFORM, BENZENE
BTZP 3c		5-{[(E)-(3-methoxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one	$C_{18}H_{13}N_7O_4S$	423.41	113	0.69	3.92	40.86	Dark brown	91	BENZENE, CHLOROFORM

BTZP 3d		5-{[(E)-(2-chlorophenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one	$C_{17}H_{10}ClN_7O_3S$	427.82	112	0.40	4.68	40.25	Dark red	93	CHLOROFORM, BENZENE
BTZP 3e		5-{[(E)-(4-hydroxy-3-methoxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one	$C_{18}H_{13}N_7O_5S$	439.40	115	0.55	3.61	41.52	Light brown	85	BENZENE, CHLOROFORM
BTZP 3f		4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-5-{[(E)-(4-nitrophenyl)methylidene]amino}-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{10}N_8O_5S$	438.38	114	0.50	4.02	40.30	Brown	88	CHLOROFORM, BENZENE

BTZP 3g		5-{[(E)-(4-hydroxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{11}N_7O_4S$	409.38	109	0.81	3.77	38.96	Dark red	86	BENZENE, CHLOROFORM
BTZP 3h		5-{[(E)-(4-chlorophenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{10}ClN_7O_3S$	427.82	111	0.52	4.68	40.24	Brownish orange	87	CHLOROFORM, BENZENE
BTZP 3i		5-{[(E)-(2,4-dihydroxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{11}N_7O_5S$	425.38	110	0.63	3.47	39.62	Dark brown	80	CHLOROFORM, BENZENE

BTZP 3j		5-{[(E)-(3,4-dihydroxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{11}N_7O_5S$	425.38	116	0.70	3.47	39.62	Dark red	82	BENZENE, CHLOROFORM
BTZP 3k		5-{[(E)-(3-fluorophenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{10}FN_7O_3S$	411.37	106	0.56	4.22	38.06	Brownish yellow	95	CHLOROFORM, BENZENE
BTZP 3l		5-{[(E)-(3-chlorophenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one	$C_{17}H_{10}ClN_7O_3S$	427.82	117	0.74	4.68	40.24	Brown	92	BENZENE, CHLOROFORM

Solvent system: Methanol: Chloroform (1:1)

Detecting agent: Iodine vapours.

5.3. PHYSICOCHEMICAL STUDIES AND CHARACTERIZATION OF SYNTHESIZED COMPOUNDS

Determination of R_f values, chromatography is an important technique to identify the formation of synthesized compound and to determine the purity of compound. R_f value is the characteristic for each of the compound.

R_f = Distance travelled by solute / Distance travelled by solvent

For the determination R_f value the precoated silica plate of 5cm long and 2cm width were used. Chromatogram were developed by ascending technique when solvent front travelled appropriate distance, plates were taken out and dried. The location of spot was detected by using iodine vapours.

The melting points of the compounds were determined in one end fused capillary tubes on a THERMONIC MODEL-C-LMP-1, campbell melting point apparatus and are uncorrected. IR spectra were recorded in KBr pellets on JASCO FT-IR-1700 spectrometer infrared spectrophotometer. ^{1} H-NMR spetra were determined at 400 MHZ on DSX-300/AV-III 400/DRX-500/AV-III 500(S)/ (L) AV-700 NMR spectrometer with tetramethyl silane as internal standard. Mass spectra were recorded on JEOL GCmate at sophisticated instrumental analytical laboratory (SAIL) IIT MADRAS, Chennai. UV/Visible spectra were taken in the region of 200-800nm, on Shimadzu UV-1700 Spectrometer. DMSO was used as a solvent. Thin layer Chromatography on a silicagel-G coated glass plates visualized by iodine vapours. The accurate weights of the chemicals were taken on the electronic balance and in analytical balance [78, 89]. The spectral data was given in the **table 4, 5 and Figure 2, 3, 4, 5, 6, 7& 8.**

Table 4. Spectral data of intermediate compounds

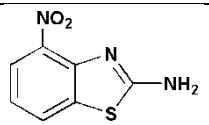
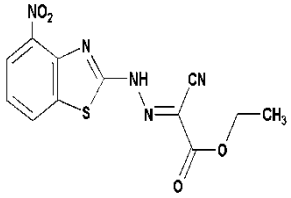
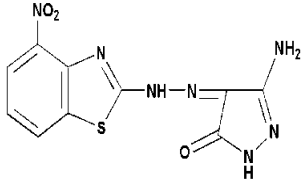
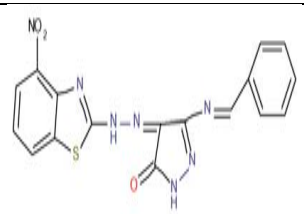
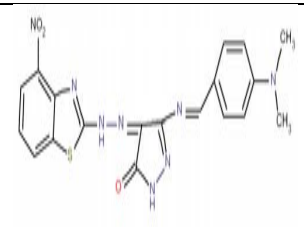
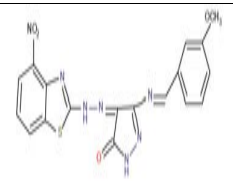
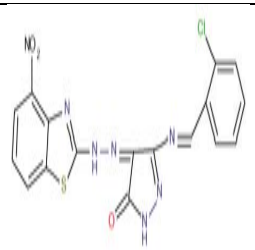
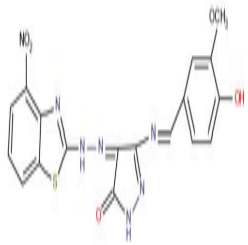
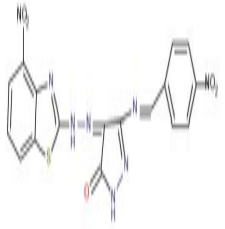
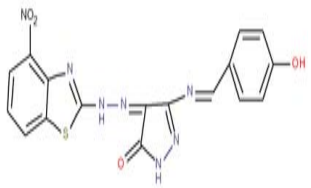
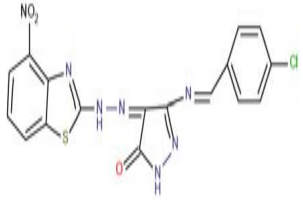
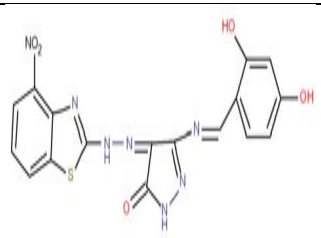
CODE NO	STRUCTURE	λ_{max} (nm)	(IR) υ_{max} (KBr/cm^{-1})	^{1}H NMR (DMSO, δ, ppm)	Molecular ion (M+) peak
BTZ		514	3132.79(NH_2 stretching) 2936,1502.28,745.35(aromatic stretching) 1348.32(NO_2) 1629.55(C=Nstretching) 700(C-S-Cstretching)	-	-
BTZE		475.5	3132.79(NH stretching) 2926,1592,749.20,(aromatic stretching) 2856(CH stretching) 1346.63(NO_2) 1718.26(C=Ostretching) 1401.03(C-Nstretching) 1624.73(C=Nstretching) 2360.44(−C≡Nstretching) 700(C-S-Cstretching) 1786.44(C=O of ester stretching)	-	-
BTZP		484	3132.79(NH stretching) 3343.05(NH_2 stretching) 2920,1597,747,(aromatic stretching) 1340.43(NO_2) 1715.62(C=Ostretching) 1401.03(C-N stretching) 1618.95(C=N stretching) 671.10(C-S-C stretching)	6.60-8.30(m,3H,Ar proton) 6.15 (s,1H,NH –N proton) 3.87(s,1H,NH proton) 8.32(s,2H,NH_2 proton)	-

Table 5. Spectral data of synthesized compounds

CODE NO	STRUCTURE	λ_{max} (nm)	(IR) υ_{max} (KBr/cm^{-1})	^{1}H NMR (DMSO, δ, ppm)	Molecular ion (M+)peak
BTZP 3a		513	3127.97(NH stretching) 2922.59,1572,745,(aromatic stretching) 1344.14(NO_2) 1700.91(C=Ostretching) 1401(C-N stretching) 1601.59(C=N stretching) 699.06(C-S-Cstretching)	6.60-8.30(m,8H,Ar proton) 6.15 (s,1H,NH-N proton) 3.86(s,1H,NH proton) 8.32(s,1H,N=CH proton)	-
BTZP 3b		699	3133.76(NH stretching) 2926,1522.52,773.31,(aromatic stretching) 1331.61(NO_2) 1592.91(C=Ostretching) 1401.03(C-Nstretching) 1698.02(C=Nstretching) 699.06(C-S-Cstretching) 1321($N(CH_3)_2$)	6.62-7.69(m,7H,Ar) 6.59(s,1H,NH-Nproton) 3.57(s,1H,NH proton) 8.60(s,1H,N=CH proton) 3.04(s,6H,$N(CH_3)_2$)	-

BTZP 3c		512.5	3135.69(NH stretching) 2921.63,1524.45,756.92,(aromatic stretching) 1331.61(NO_2) 1599.66(C=Ostretching) 1401.03(C-N stretching) 1695.12(C=Nstretching) 697.14(C-S-C stretching) 1070.3(C-O-C stretching)	6.70-8.59 (m,7H,Ar) 6.68 (s,1H,NH-N proton) 3.88(s,1H,NH proton) 8.64 (s,1H,N=CH proton) 3.35(s,3H,(OCH_3)	-
BTZP 3d		455	3128.94(NH stretching) 2962,1509.99,772.36,(aromatic stretching) 1311.36(NO_2) 1596.77(C=O stretching) 1400.07(C-N stretching) 1679.69(C=Nstretching) 699.06(C-S-C stretching) 830.20(C-Cl)	6.32-8.30(m,7H,Ar) 6.15(s,1H,NH-N proton) 3.89(s,1H,NH proton) 8.32 (s,1H,N=CH proton)	-
BTZP 3e		479	3127.01(NH stretching) 2960,1512.88,733.78,(aromatic stretching) 1348.18(NO_2) 1623.77(C=O stretching) 1401.03(C-N stretching) 1686.44(C=Nstretching) 696(C-S-C stretching) 1081.62(C-O-C stretching) 3373(OH stretching)	7.39-8.33(m,6H,Ar) 7.36 (s,1H,NH-N proton) 3.89(s,1H,NH proton) 8.85 (s,1H,N=CH proton) 3.34(s,3H,(OCH_3) 9.87(s,1H,OH)	-
BTZP 3f		512.5	3127.01(NH stretching) 2960,733.78,(aromatic stretching) 1512.88,1348.18(NO_2) 1623.77(C=O stretching) 1401.03(C-N stretching) 1686.44(C=Nstretching) 696(C-S-C stretching)	6.60- 8.21(m,7H,Ar) 6.15 (s,1H,NH-N proton) 3.89 (s,1H,NH proton) 8.23 (s,1H,N=CH proton)	-

BTZP 3g		514	3133.79(NH stretching) 2932,1502.28,745.35,(aromatic stretching) 1348.72(NO_2) 1602.28(C=O stretching) 1401.03(C-N stretching) 1629.55(C=Nstretching) 686(C-S-C stretching) 3378.67(OH stretching)	6.94-7.76(m,7H,Ar) 6.92(s,1H,NH-N proton) 3.86(s,1H,NH proton) 7.77(s,1H,N=CH proton) 9.79(s,1H,OH)	409.38
BTZP 3h		401	3141.47(NH stretching) 2920,1492.63,740.53(aromatic stretching) 1339.36(NO_2) 1573.63(C=Ostretching) 1401.03(C-N stretching) 1681.62(C=N stretching) 686(C-S-C stretching) 821(C-Cl)	6.61-8.25(m,7H,Ar) 6.60(s,1H,NH-N proton) 3.86(s,1H,NH proton) 8.31(s,1H,N=CH proton)	-
BTZP 3i		511	3152.08(NH stretching) 2921.63,1501.34,740.53(aromatic stretching) 1337.5(NO_2) 1699(C=O stretching) 1400.03(C-N stretching) 1622.84(C=N stretching) 780(C-S-C stretching) 3433.62,3364.21(OH stretching)	6.60-8.30(m,6H,Ar) 6.15(s,1H,NH-N proton) 3.86(s,1H,NH proton) 8.32(s,1H,N=CH proton) 9.87(s,2H,OH)	-

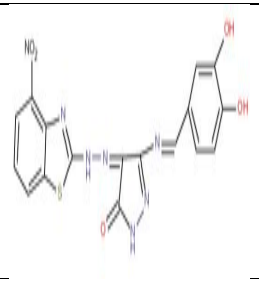
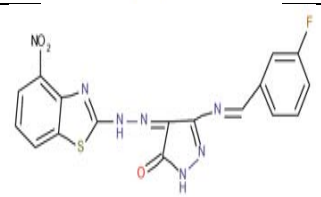
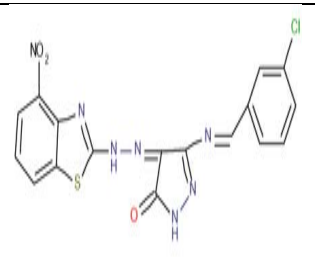
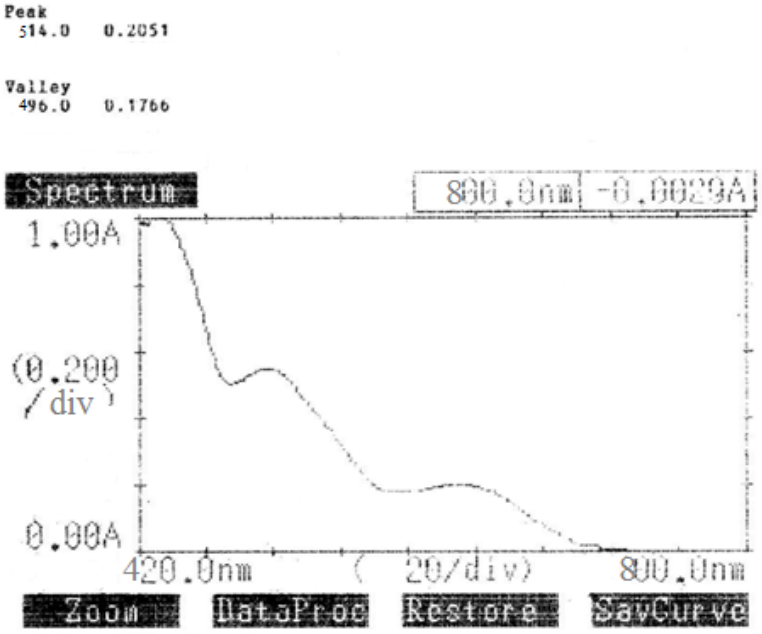
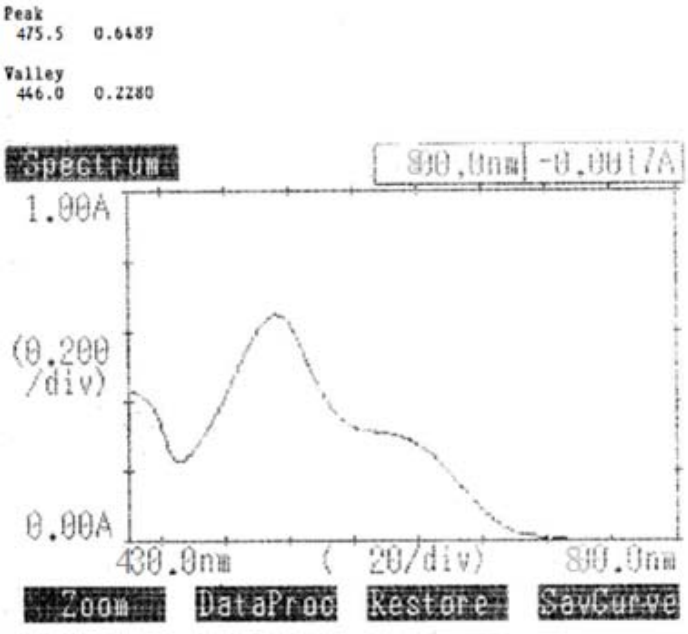
BTZP 3j		514	3151.11(NH stretching) 2920,1502,742.46,(aromatic stretching) 1333.57(NO_2) 1623.77(C=O stretching) 1401.03(C-N stretching) 1677.77(C=N stretching) 686(C-S-C stretching) 3463.2,3378.67(OH stretching)	6.60-7.39(m,6H,Ar) 6.15(s,1H,NH-N proton) 3.86(s,1H,NH proton) 7.40(s,1H,N=CH proton) 9.87(s,2H,OH)	-
BTZP 3k		513	3160.75(NH stretching) 2920,1588,733.17,(aromatic stretching) 1336.32(NO_2) 1598.7(C=O stretching) 1400.07(C-N stretching) 1676.8(C=N stretching) 603.6(C-S-C stretching) 1416(C-F strecting)	6.68-8.59(m,7H,Ar) 6.15(s,1H,NH-N proton) 3.87(s,1H,NH proton) 8.64(s,1H,N=CH proton)	411.37
BTZP 3l		477.5	3149.19(NH stretching) 2920,1494.91,737,(aromatic stretching) 1343.03(NO_2) 1590.02(C=O stretching) 1401.03(C-N stretching) 1675.84(C=N stretching) 686(C-S-C stretching) 837.19(C-Cl)	7.02-7.95(m,7H,Ar) 7.00(s,1H,NH-N proton) 3.86(s,1H,NH proton) 7.96(s,1H,N=CH proton)	-

Figure 2. UV-Visible spectra of intermediate compounds

BTZ



BTZE



BTZP

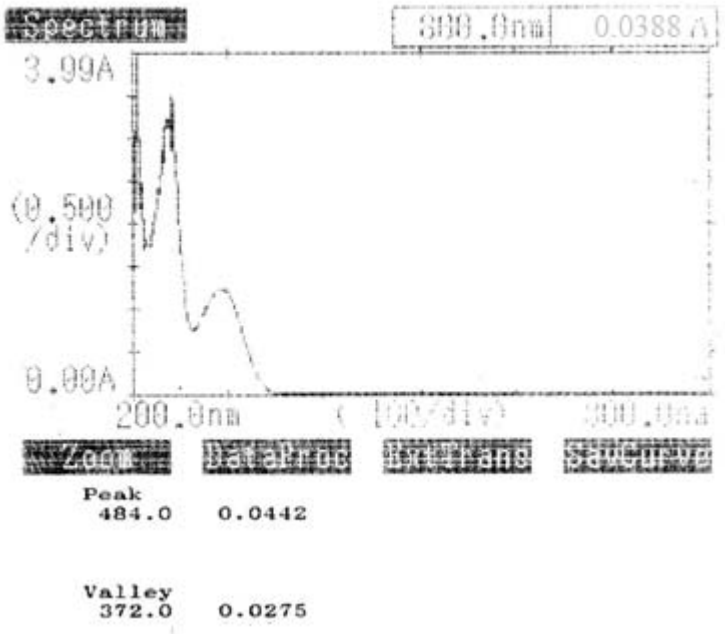
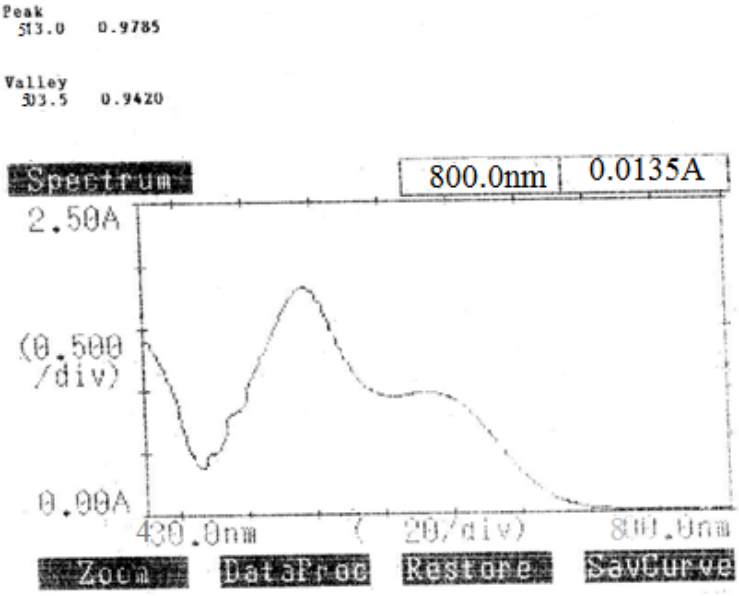
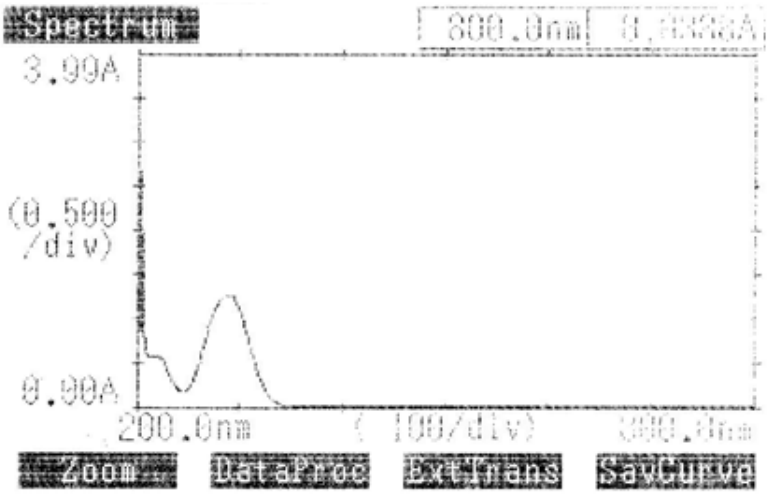


Figure 3. UV-Visible spectra of synthesized compounds

BTZP3a



BTZP3b



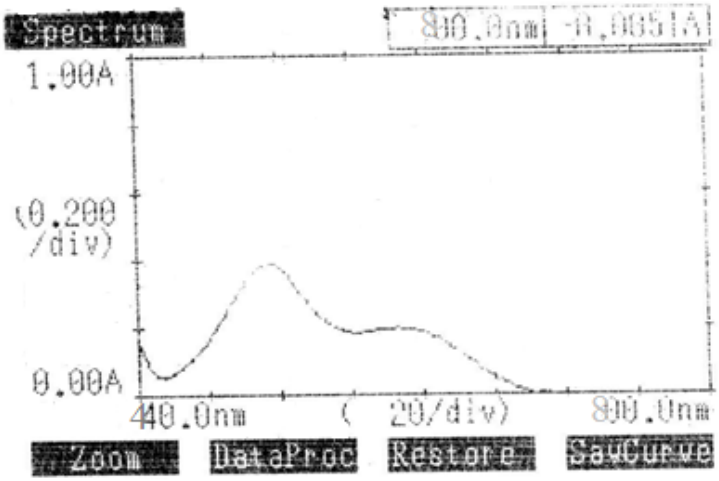
Peak
699.0 0.0397

Valley
371.0 0.0220

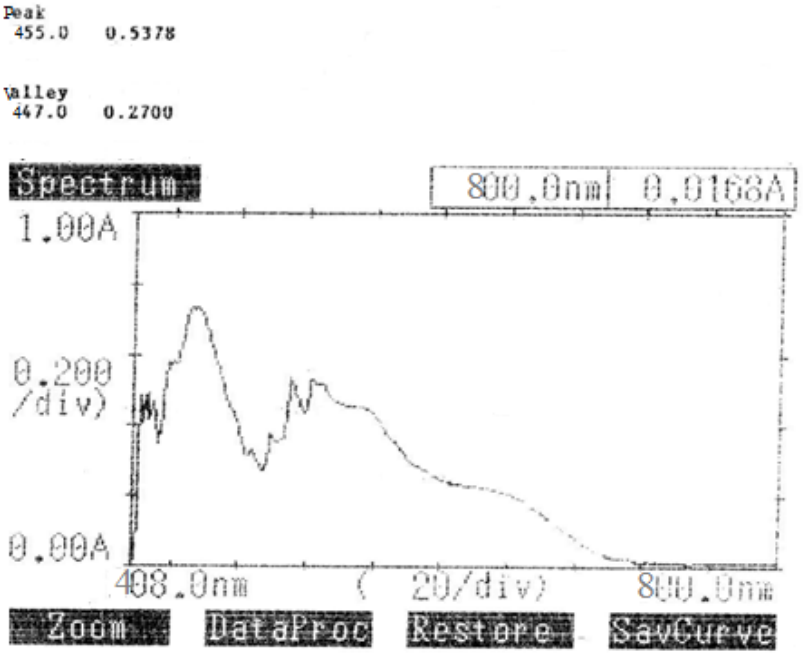
BTZP3c

Peak
512.5 0.4997

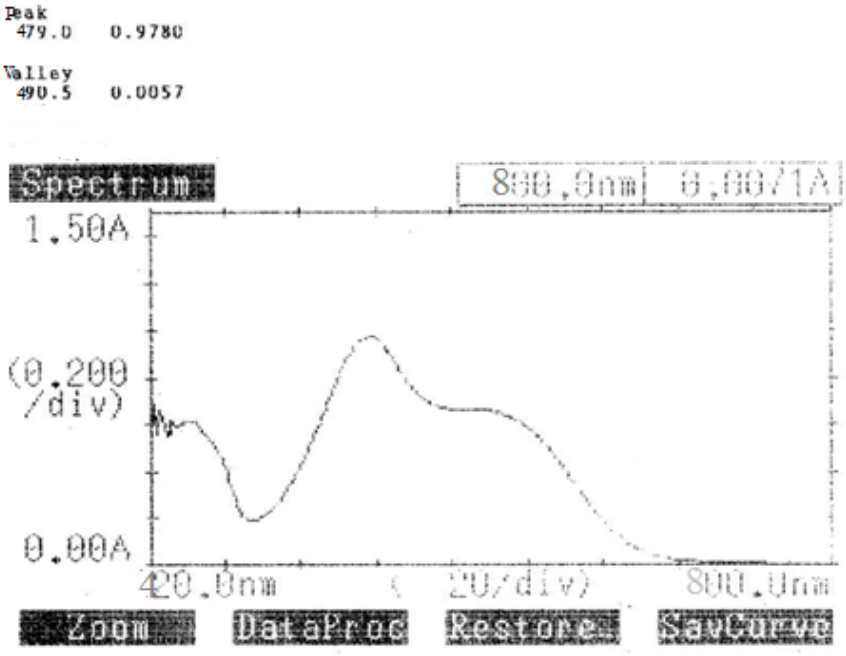
Valley
574.0 0.0077



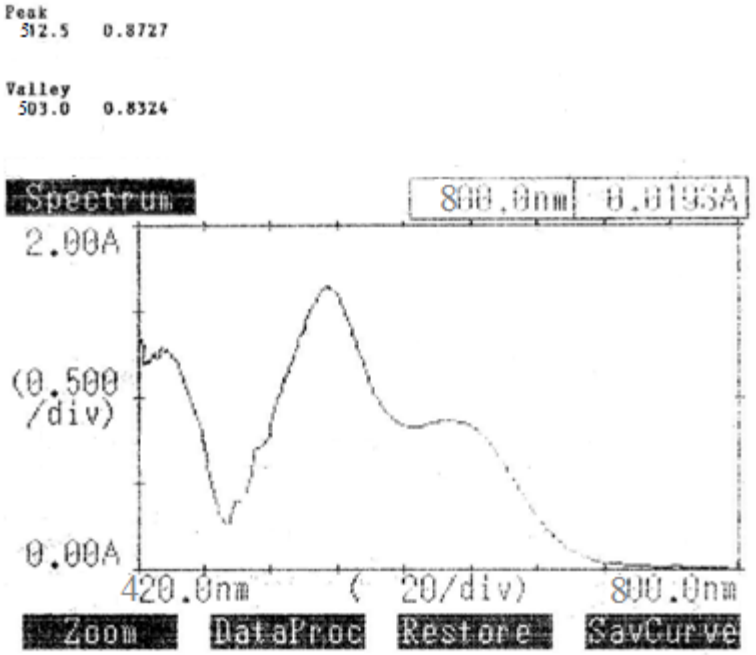
BTZP3d



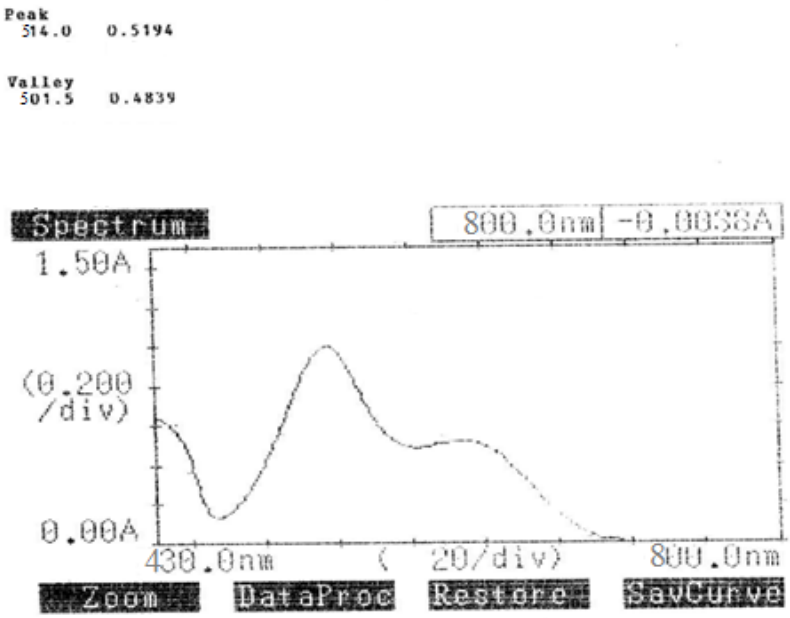
BTZP3e



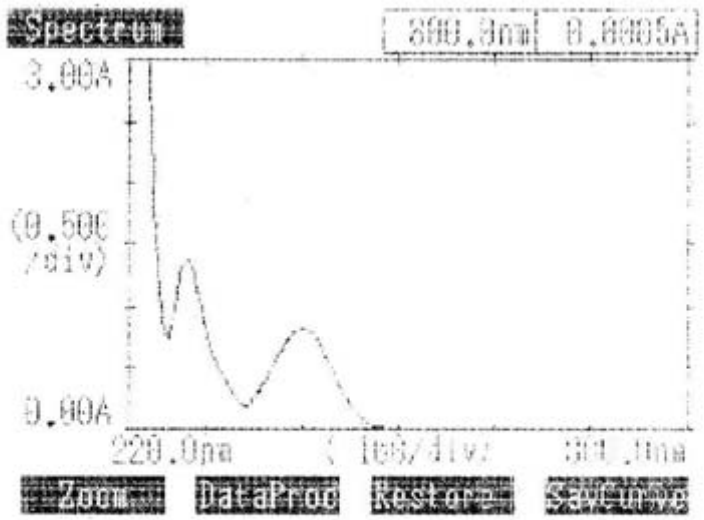
BTZP3f



BTZP3g



BTZP3h



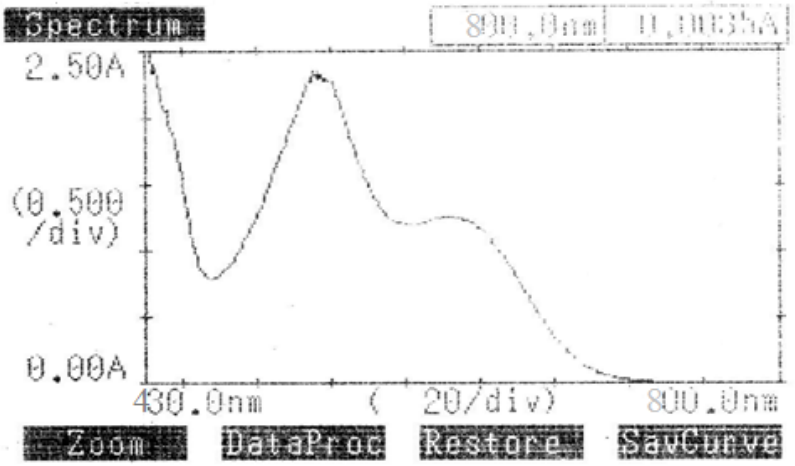
Peak
401.0 0.8218

Valley
342.0 0.1901

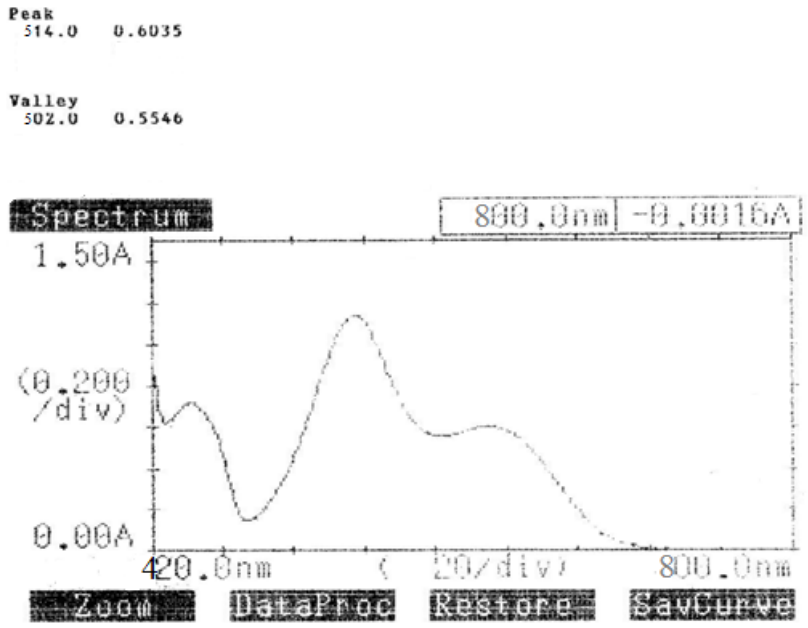
BTZP3i

Peak
511.0 1.2535

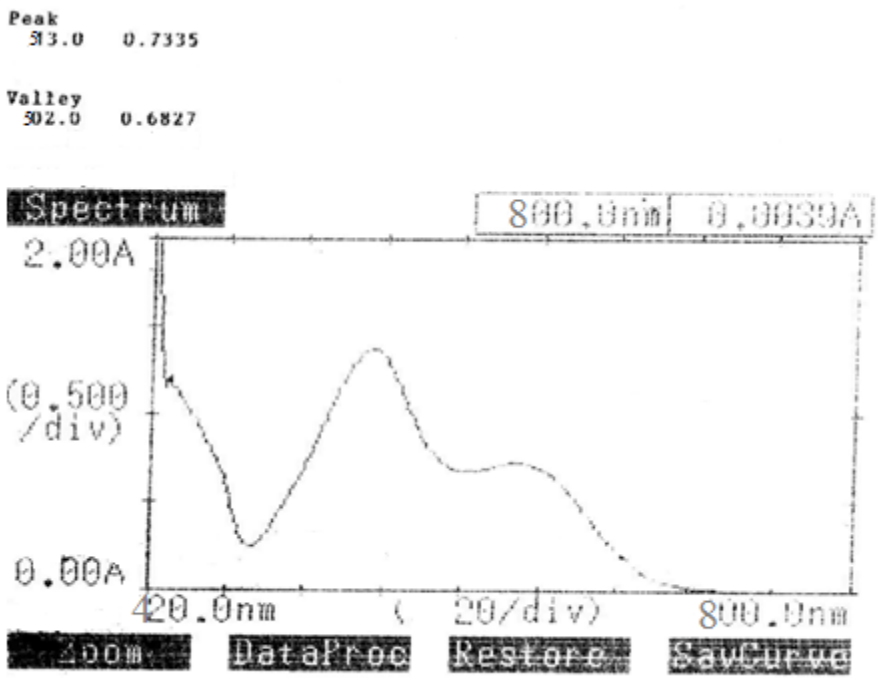
Valley
502.0 1.1914



BTZP3j



BTZP3k



BTZP3l

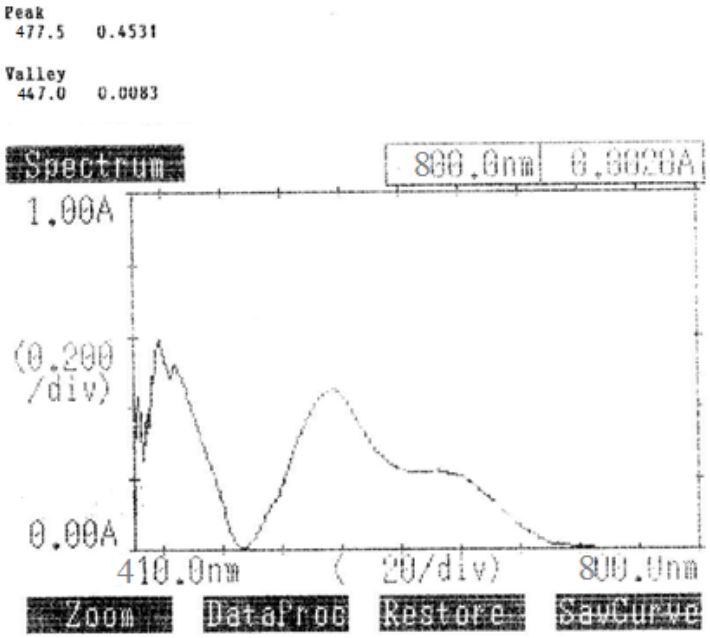
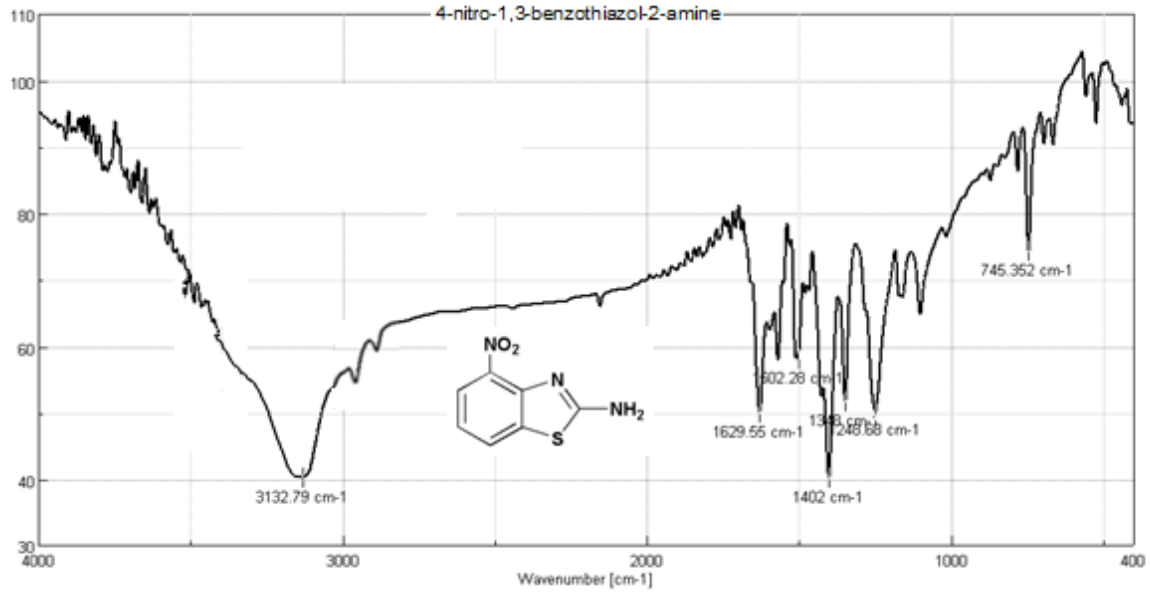
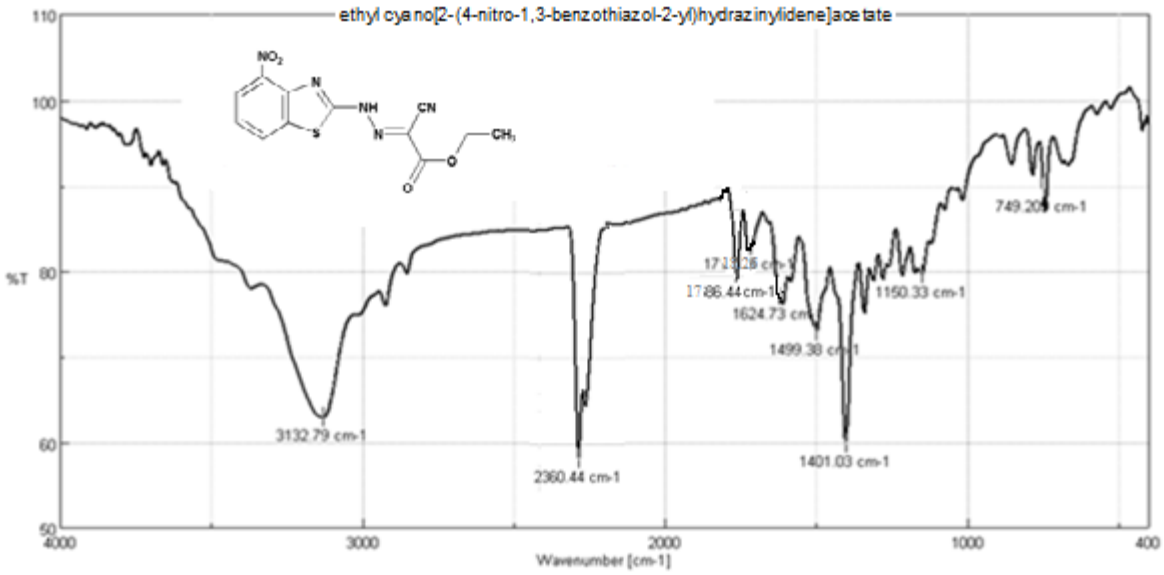


Figure 4. IR Spectra of intermediate compounds

BTZ



BTZE


ethyl cyano[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]acetate
%T
Wavenumber [cm-1]
3132.79 cm-1
2360.44 cm-1
1401.03 cm-1
1150.33 cm-1
1624.73 cm-1
1499.38 cm-1

BTZP

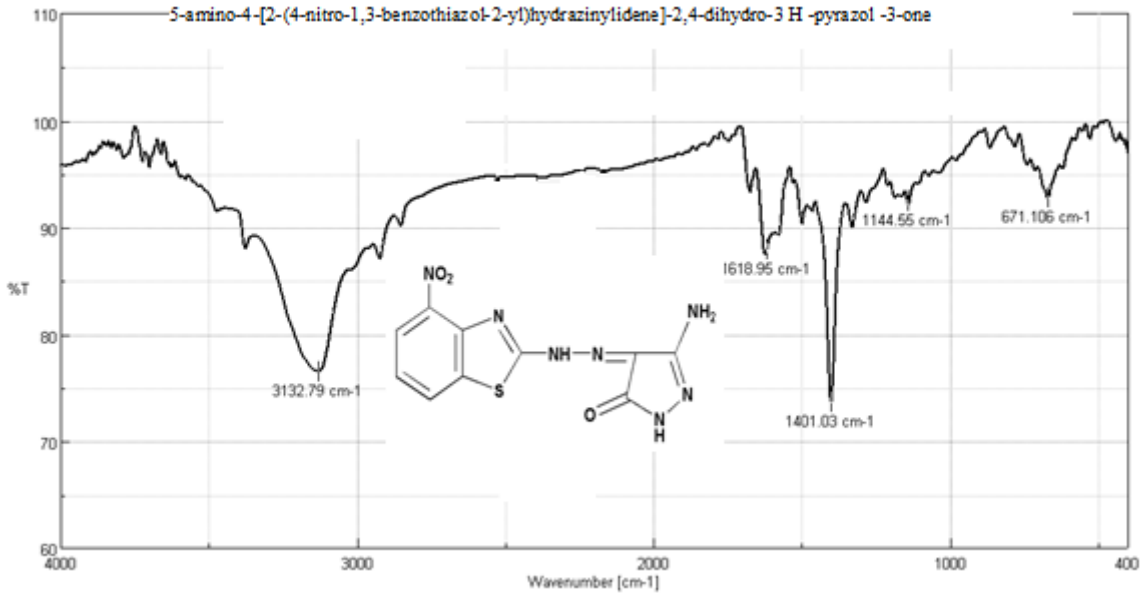
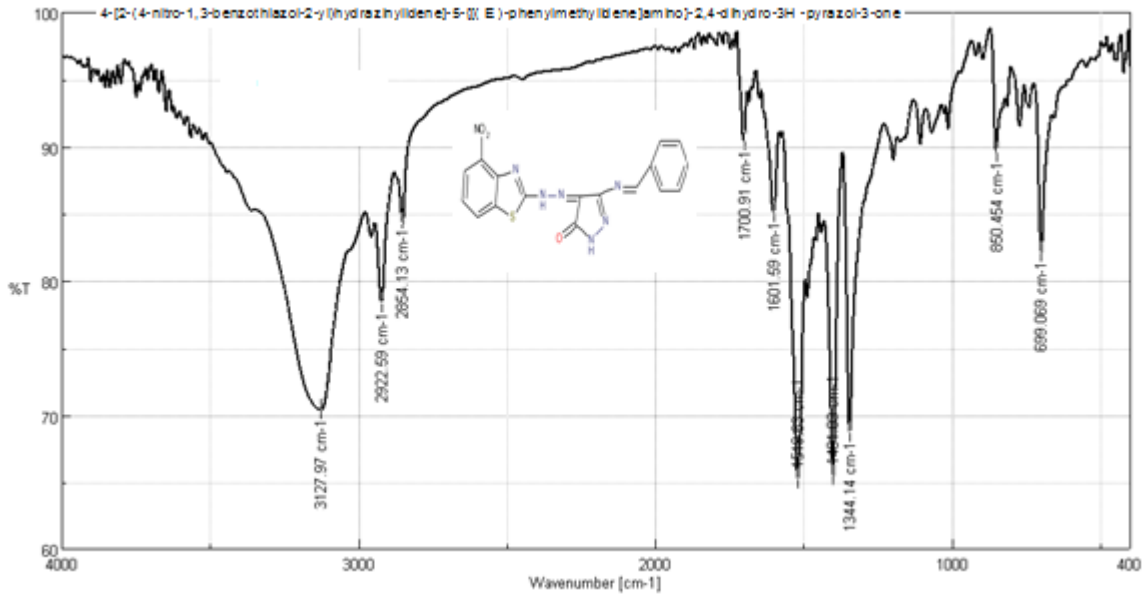
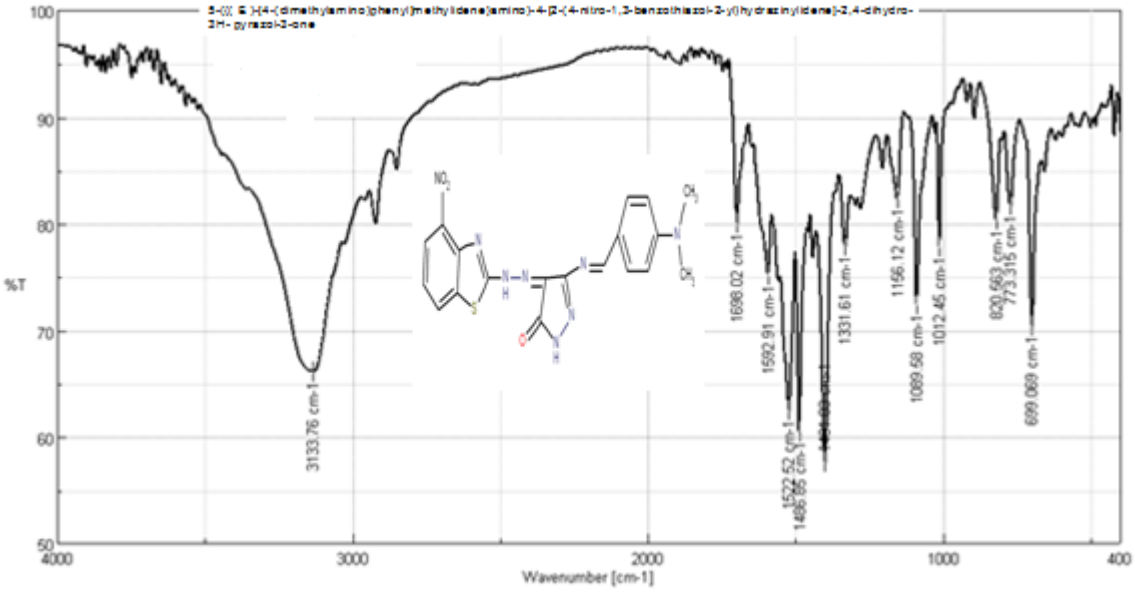

5-amino-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one
%T
Wavenumber [cm-1]
3132.79 cm-1
1618.95 cm-1
1401.03 cm-1
1144.55 cm-1
671.106 cm-1

Figure 5. IR Spectra of synthesized compounds

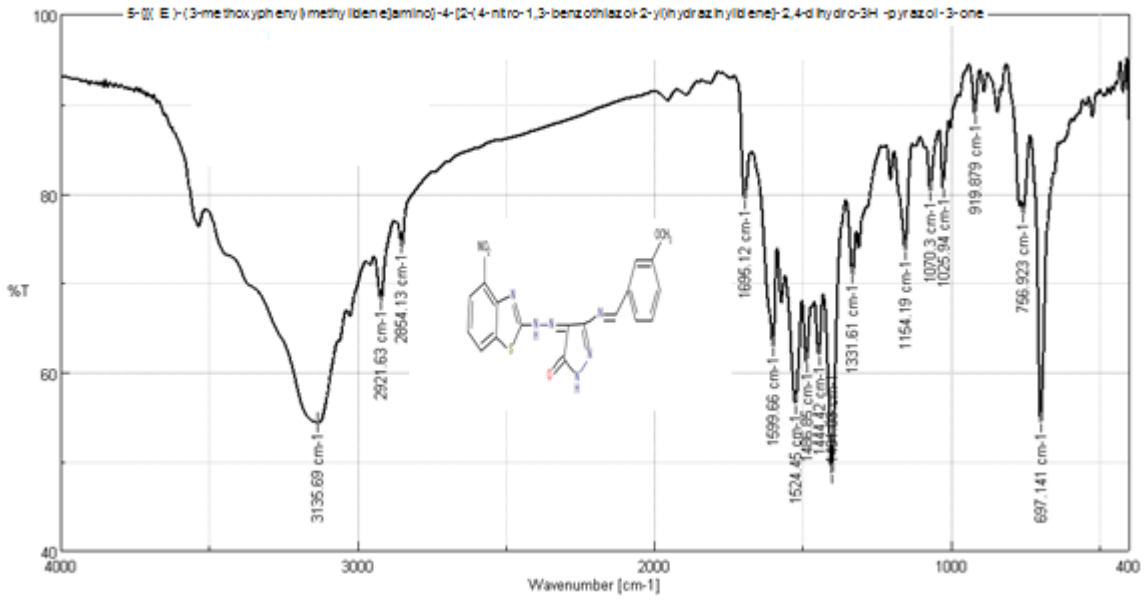
BTZP3a



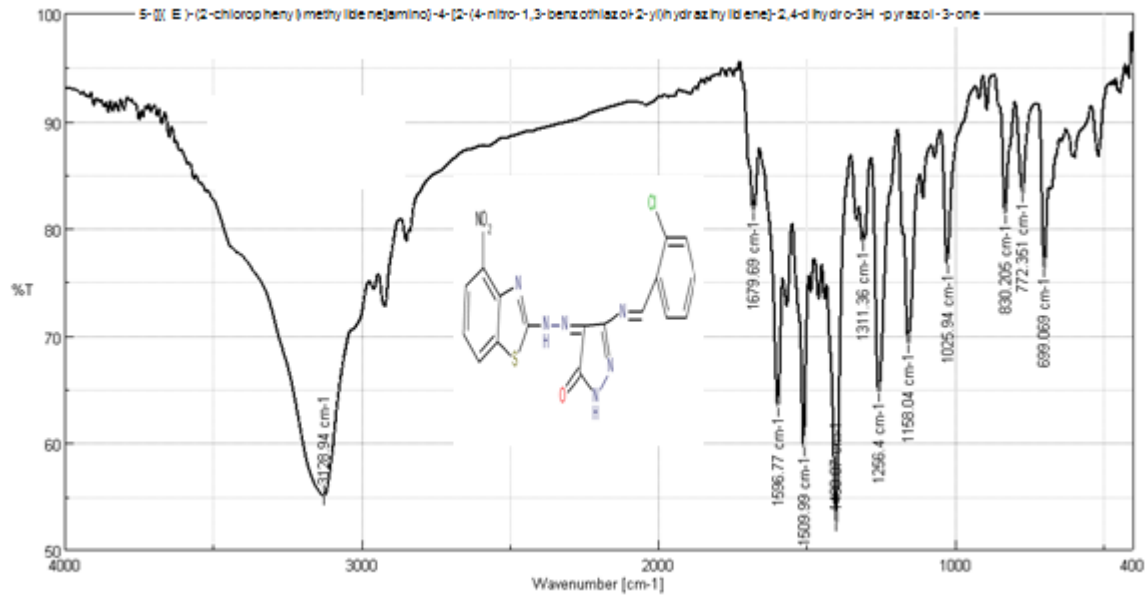
BTZP3b



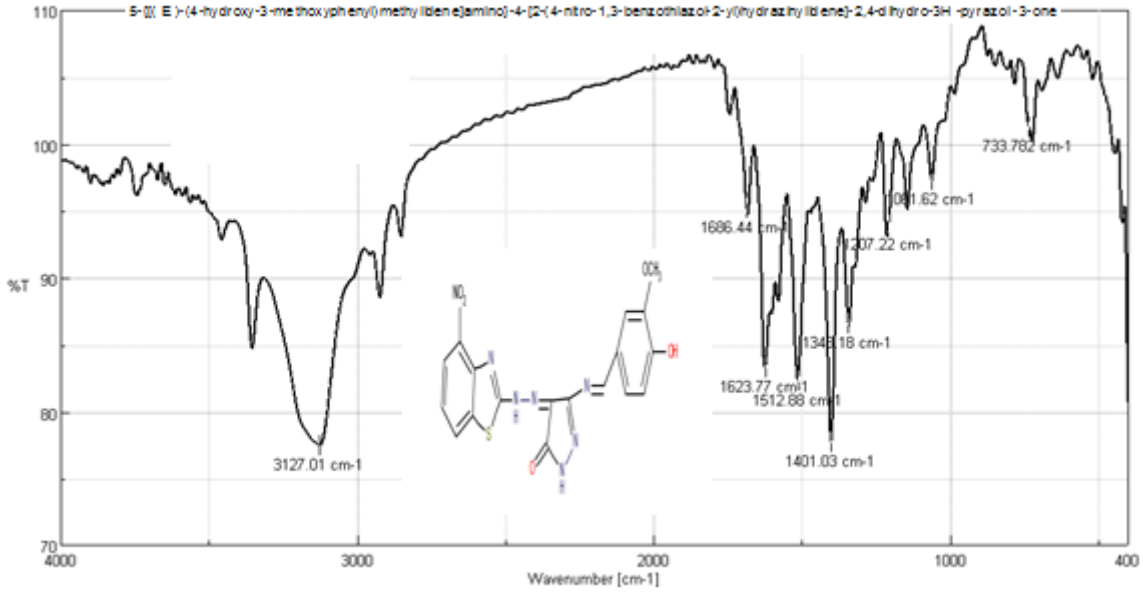
BTZP3c



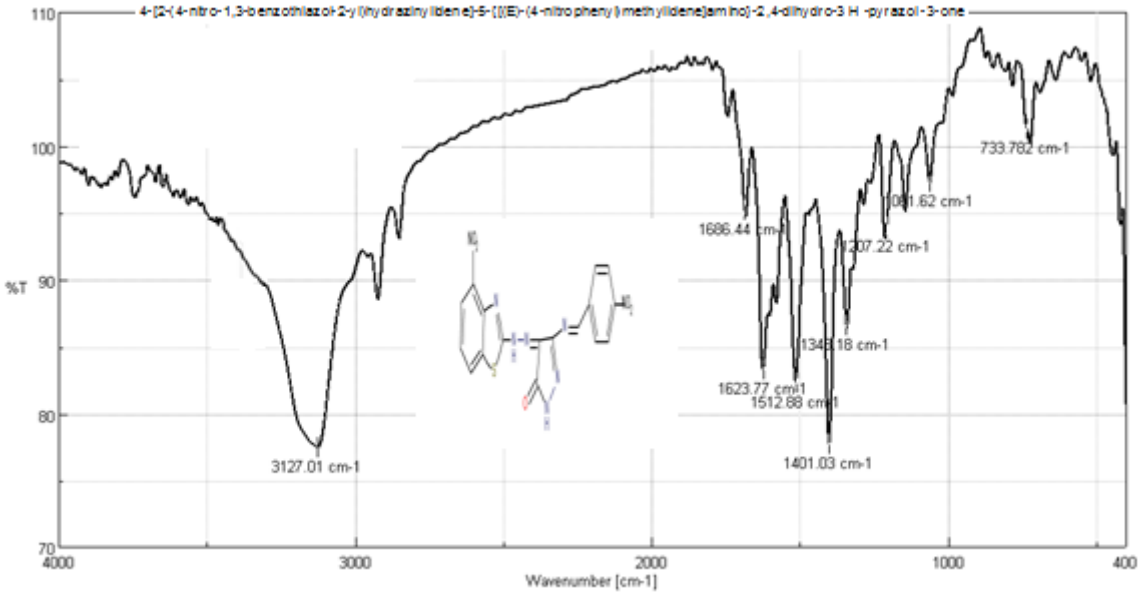
BTZP3d



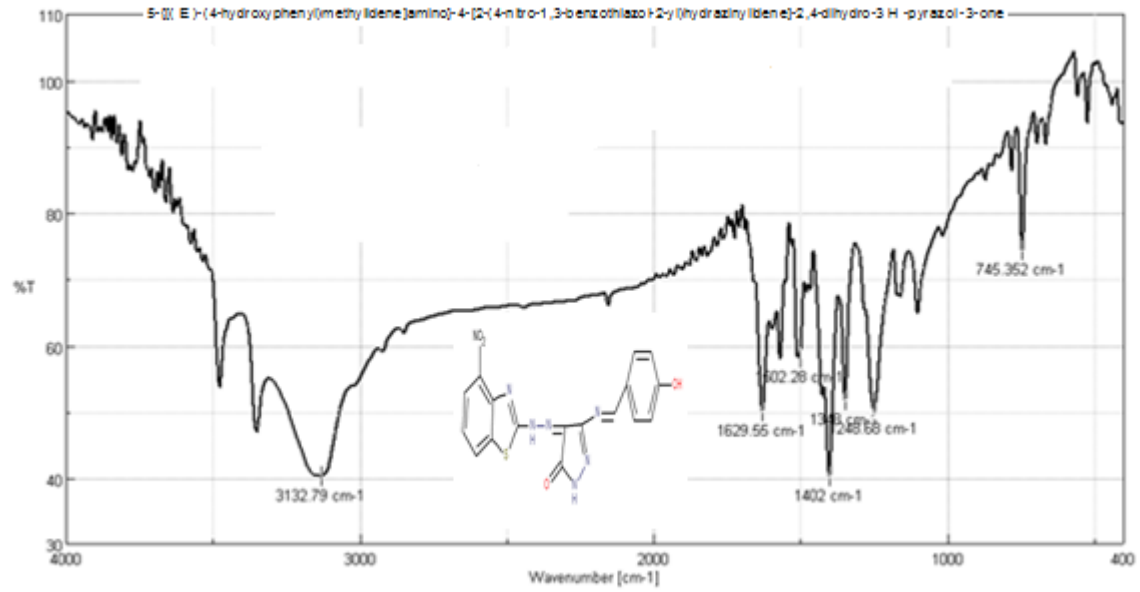
BTZP3e



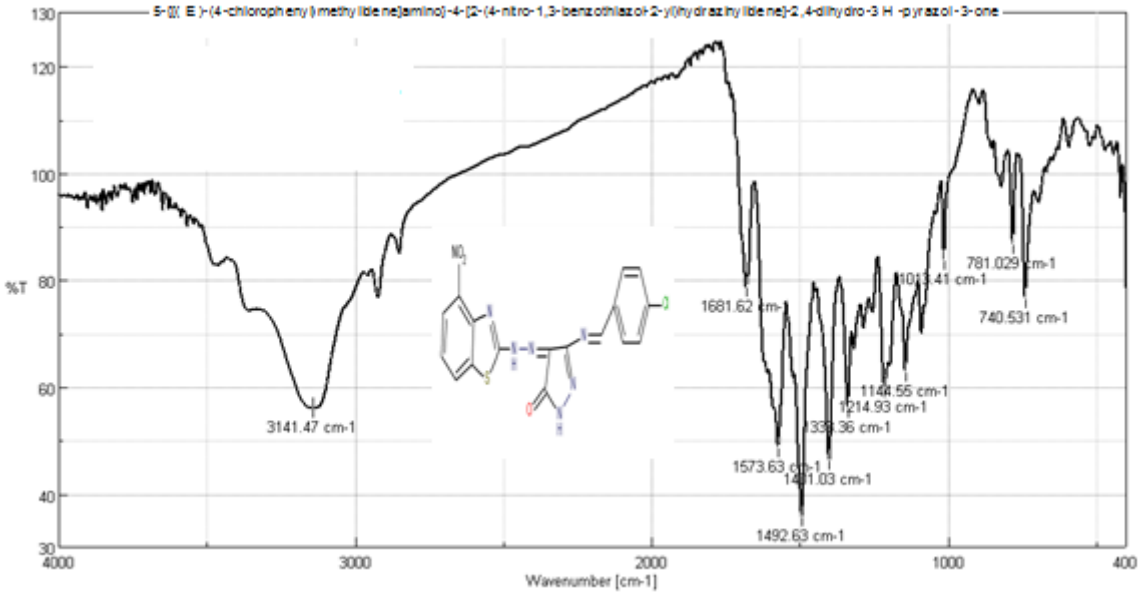
BTZP3f



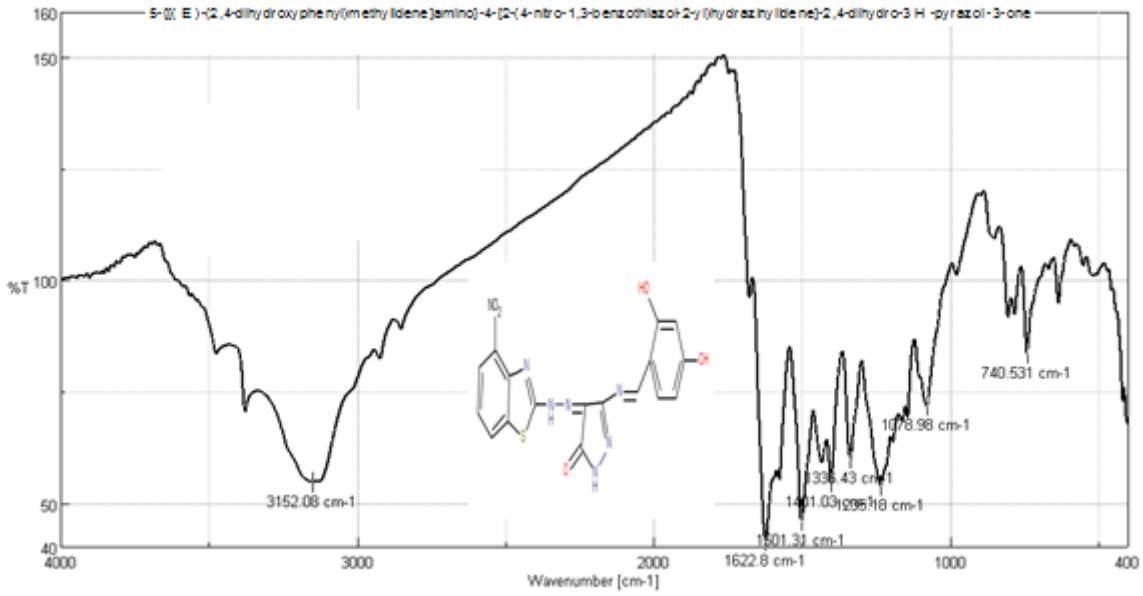
BTZP3g



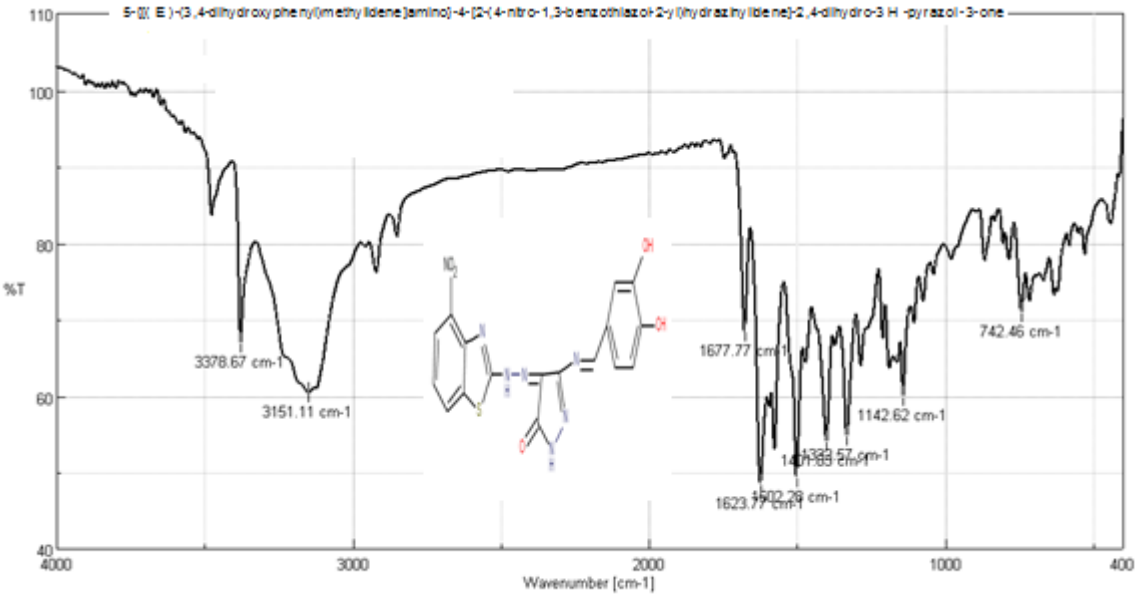
BTZP3h



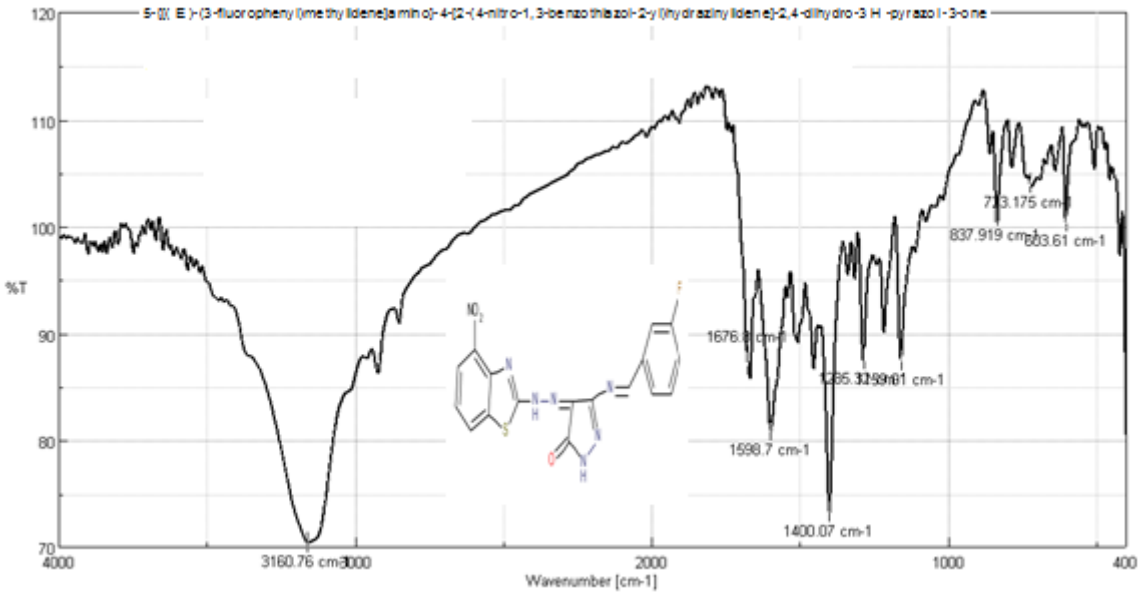
BTZP3i



BTZP3j



BTZP3k



BTZP3l

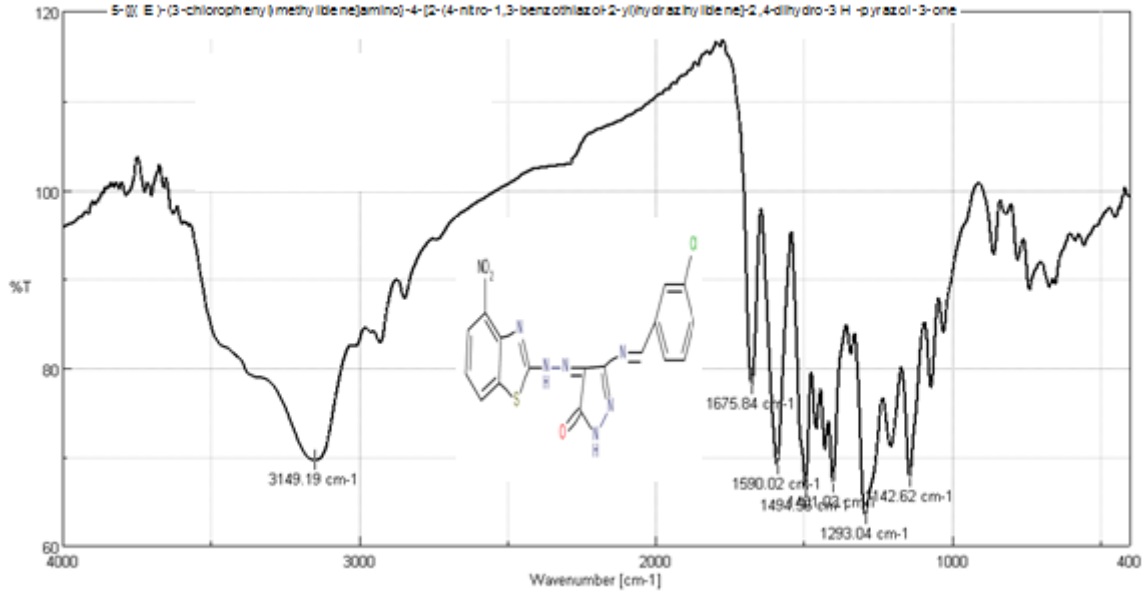
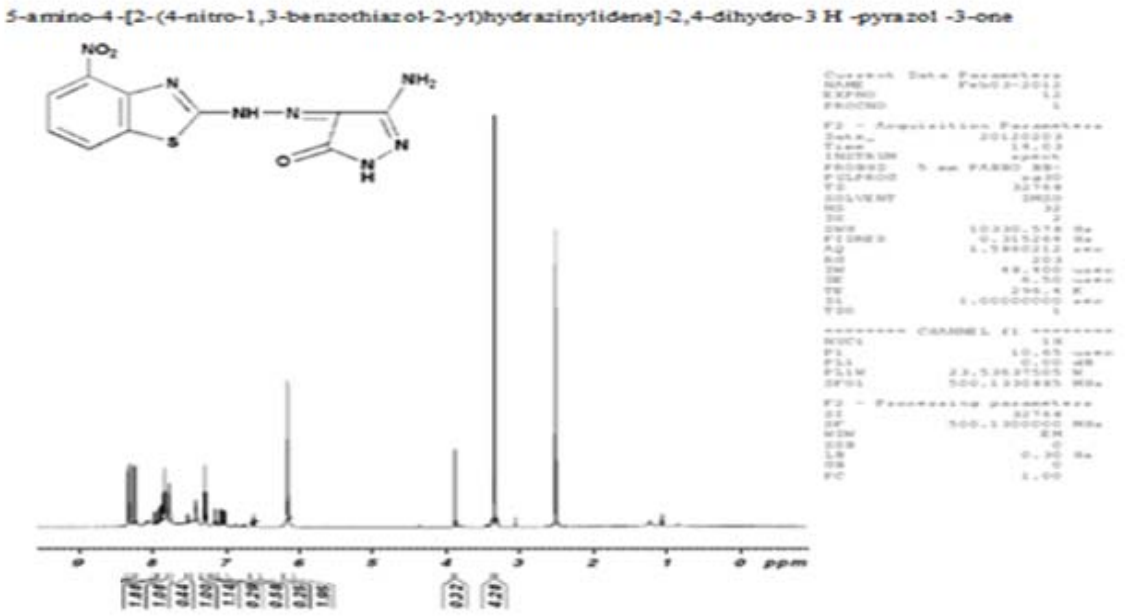


Figure 6. NMR spectra of intermediate compounds

BTZP



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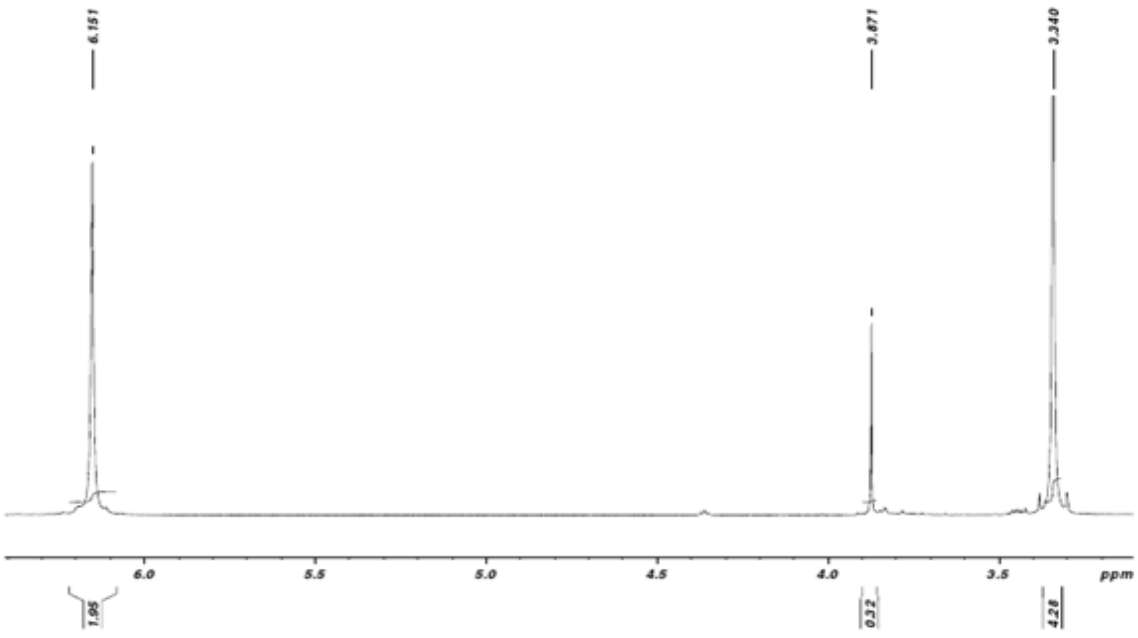

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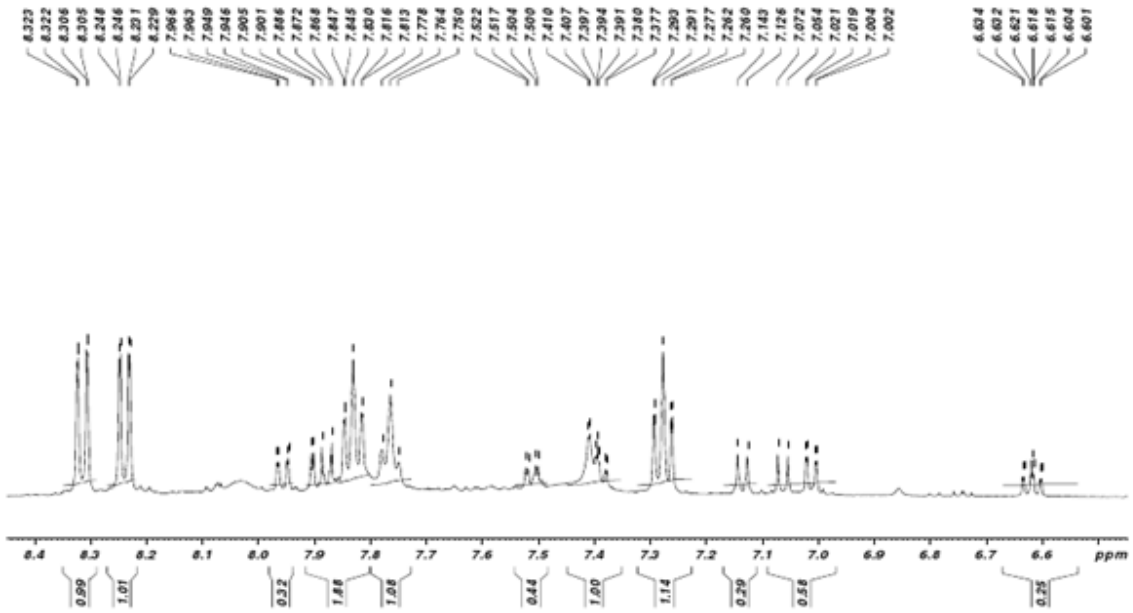

Figure 7. NMR spectra of synthesized compounds

BTZP3a

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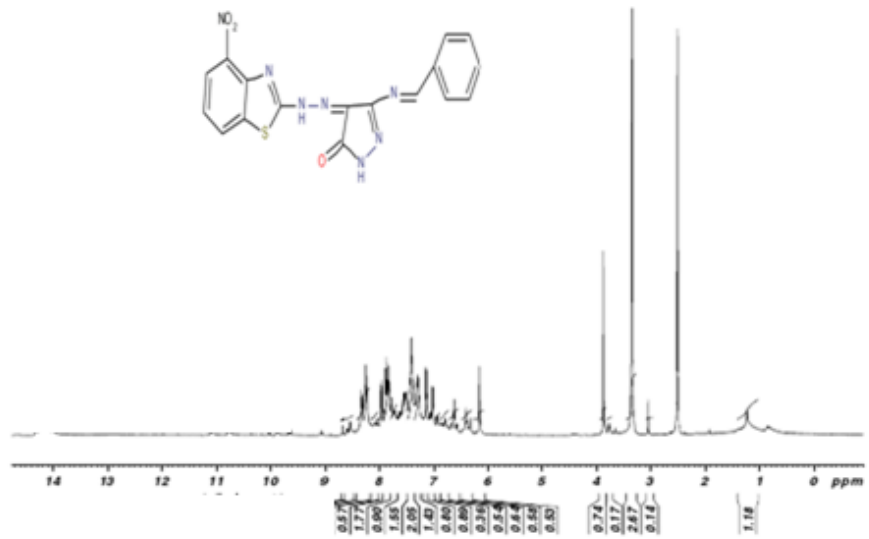

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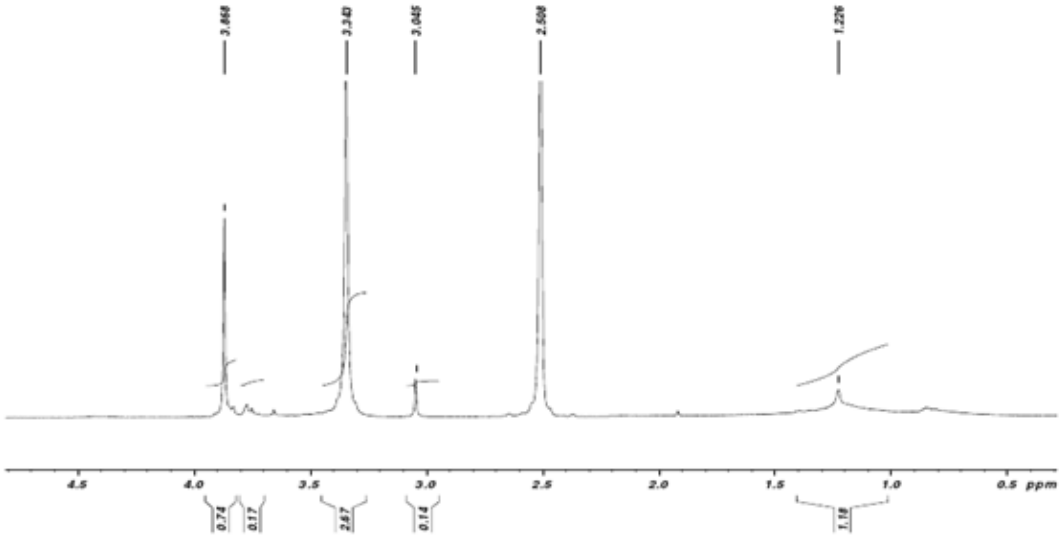

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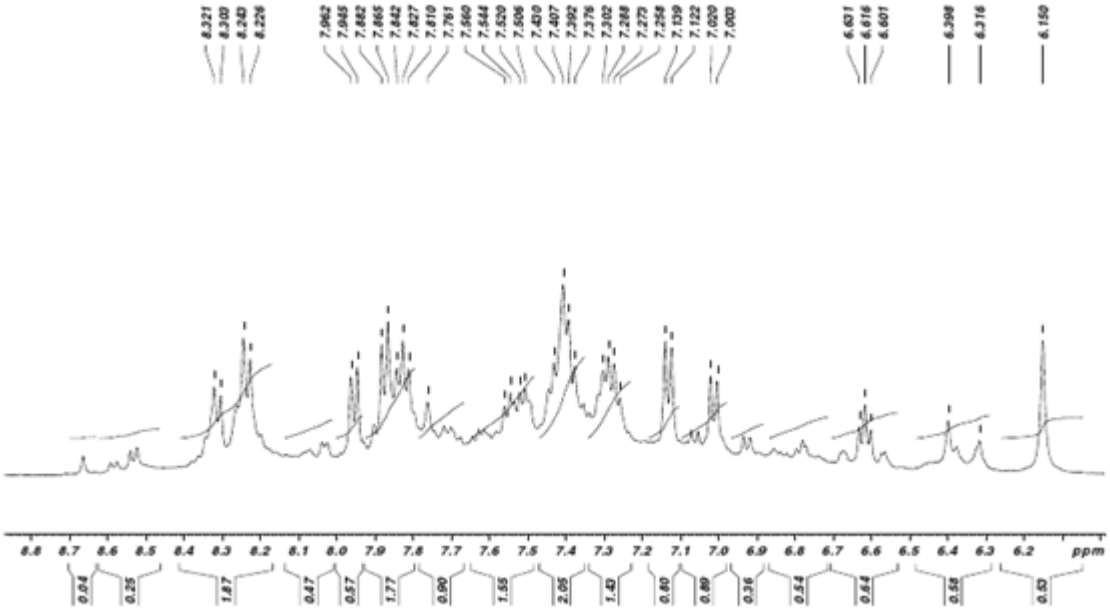

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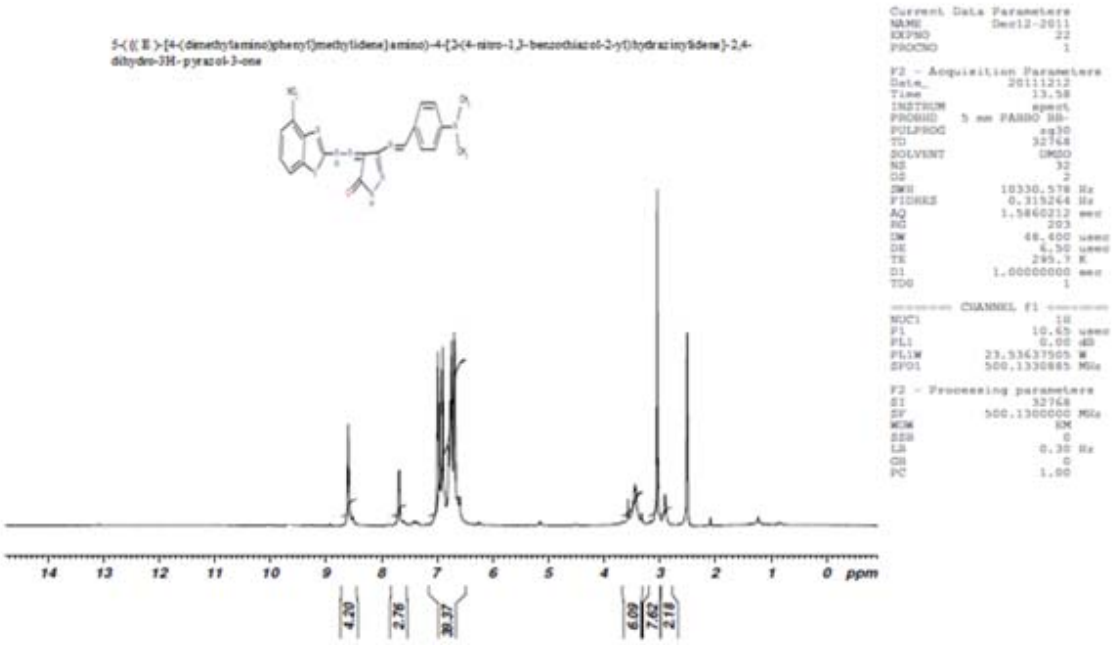

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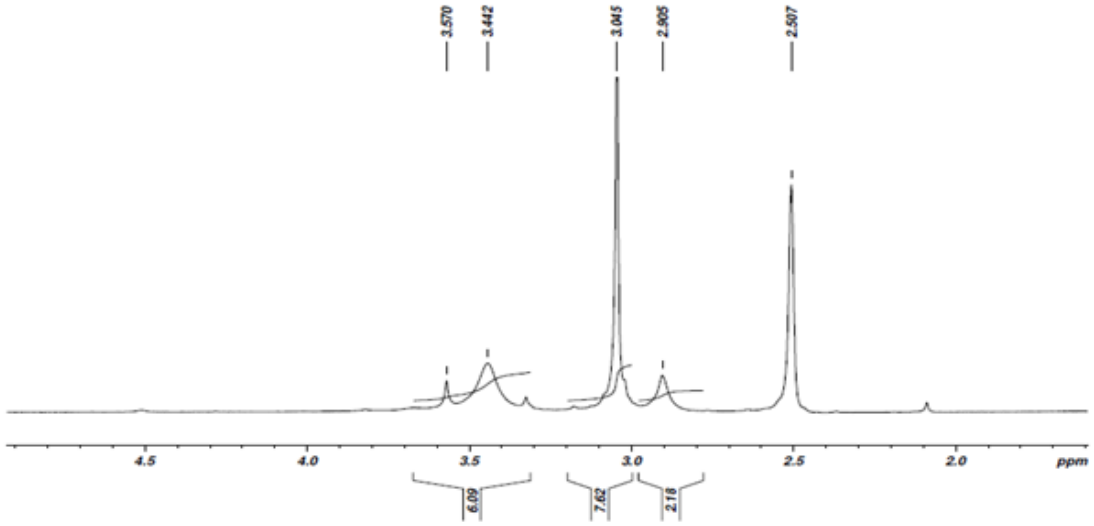

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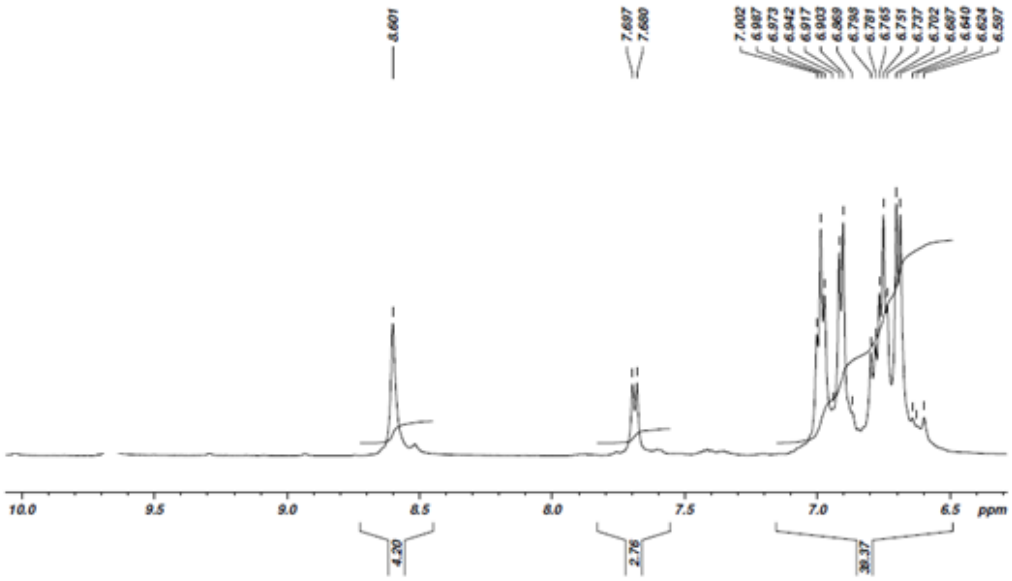

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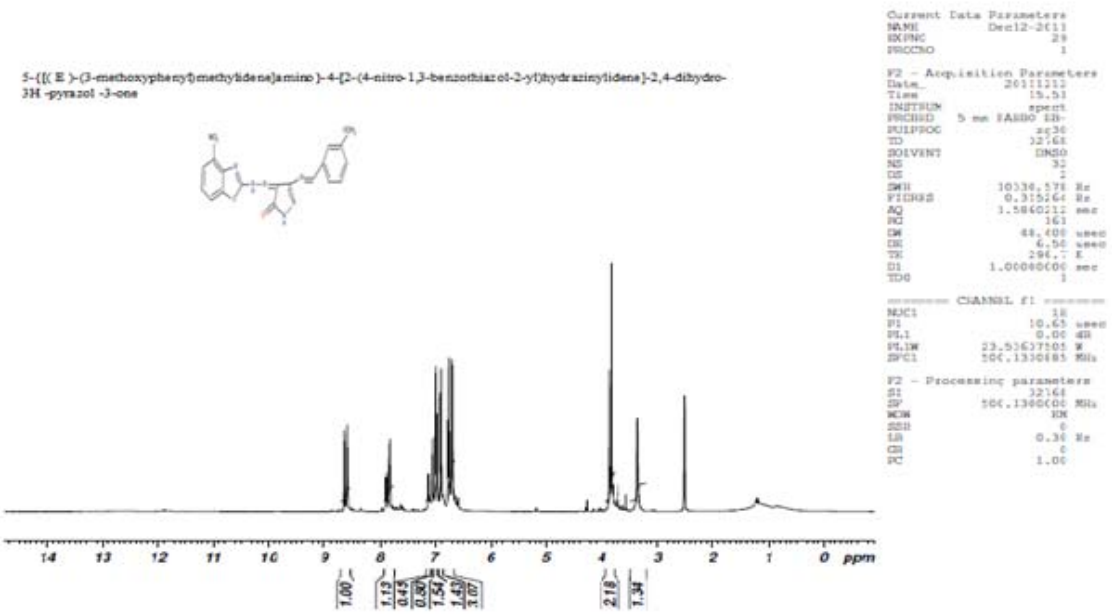

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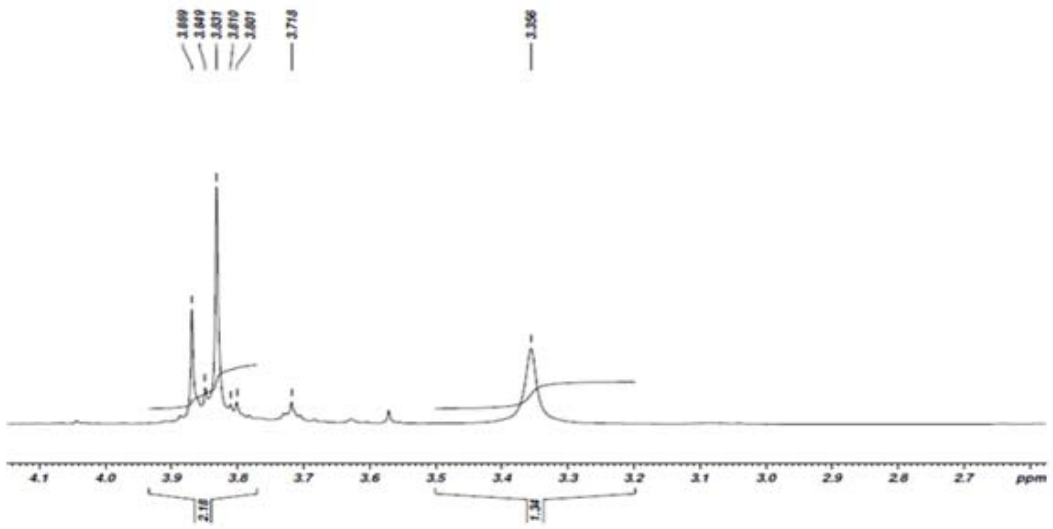

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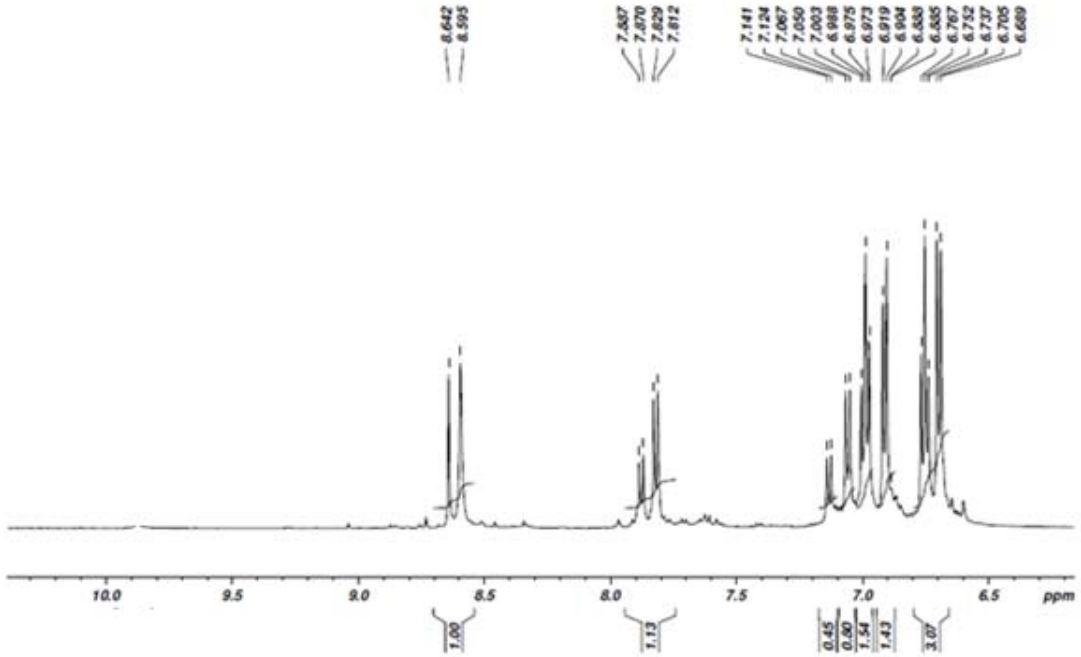

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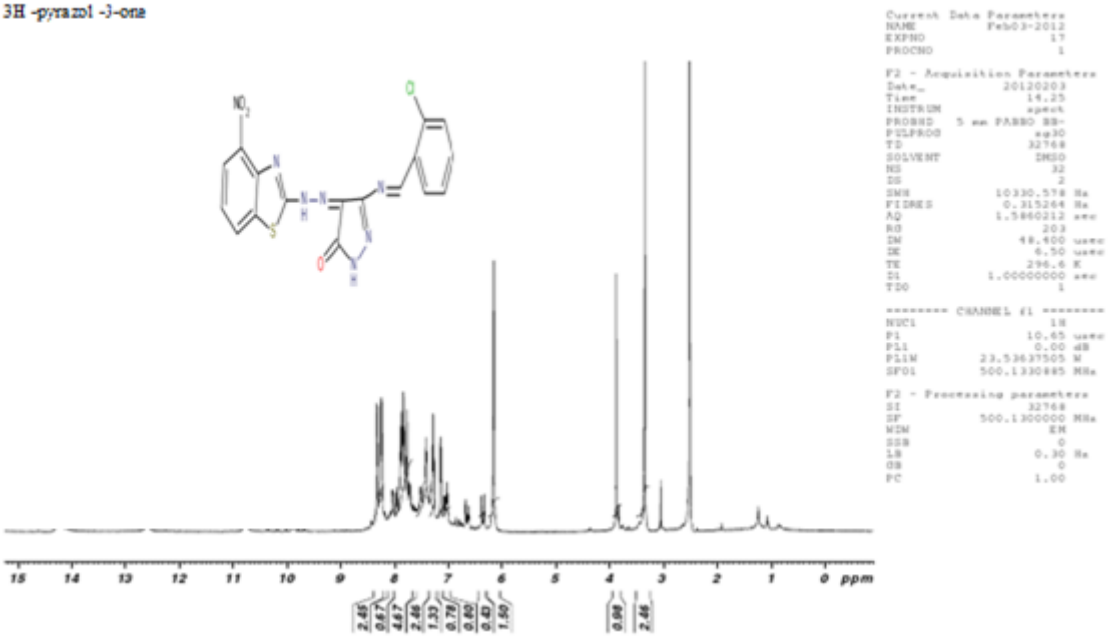

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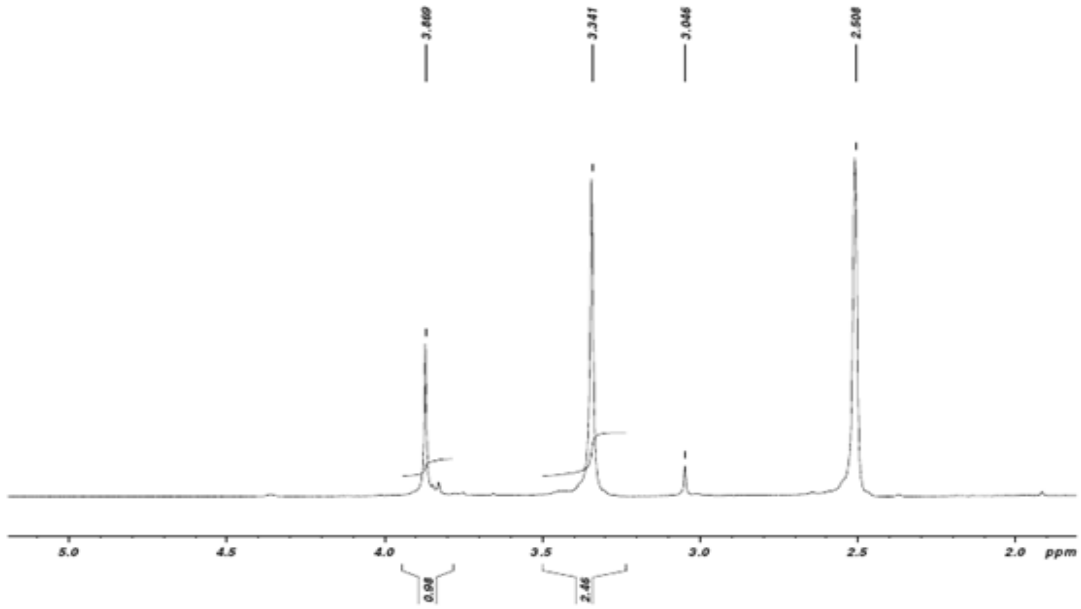
BTZP3d



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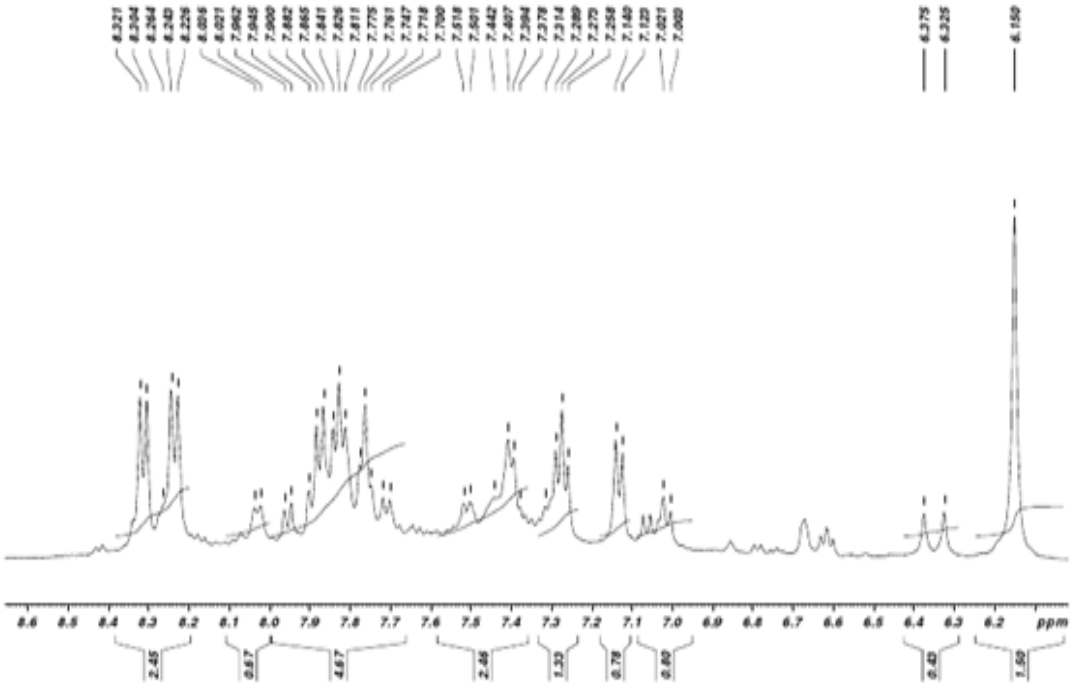

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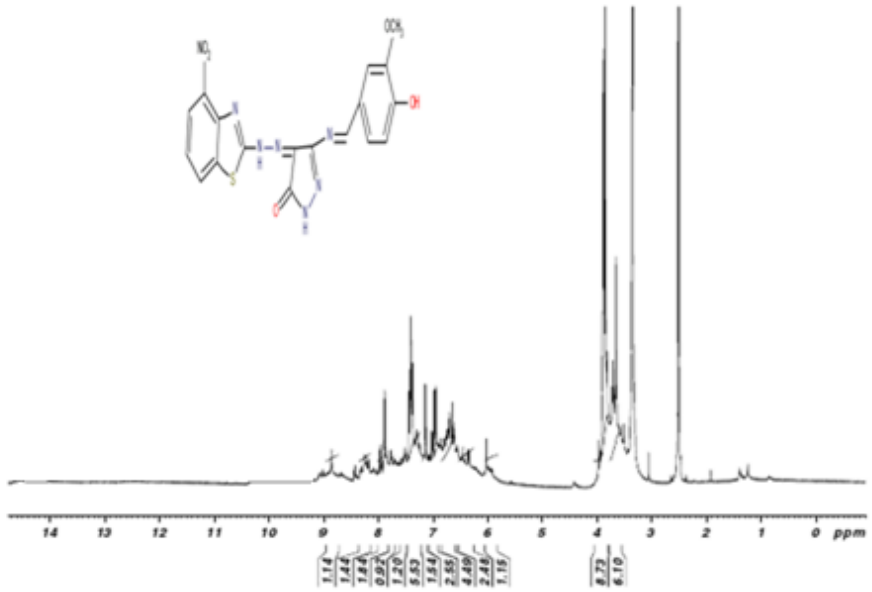
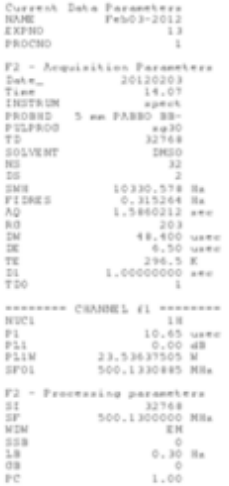
5-{[(E)-(4-hydroxy-3-methoxyphenyl)methylidene]amino }-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one





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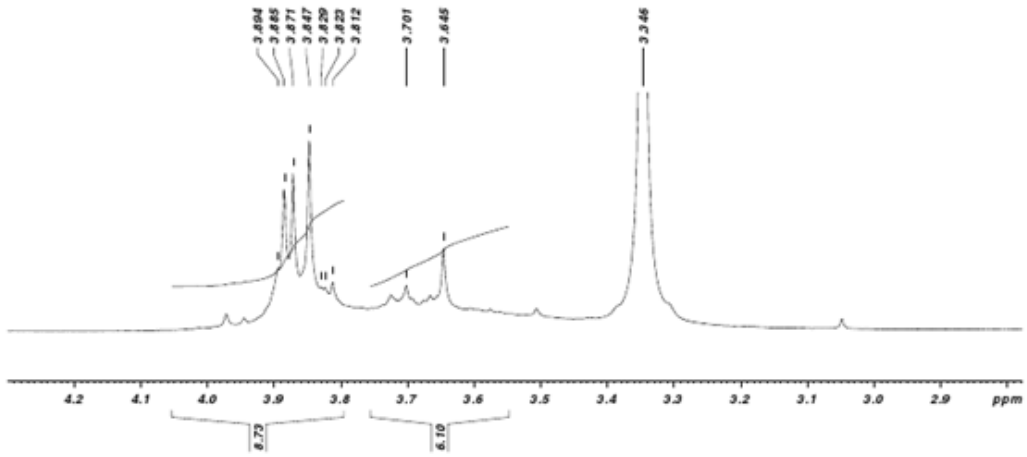

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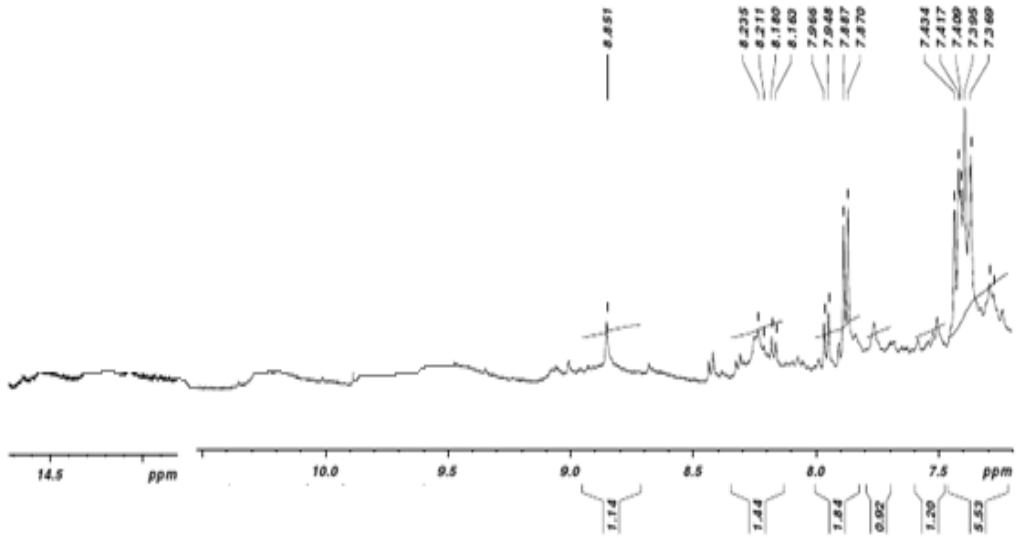

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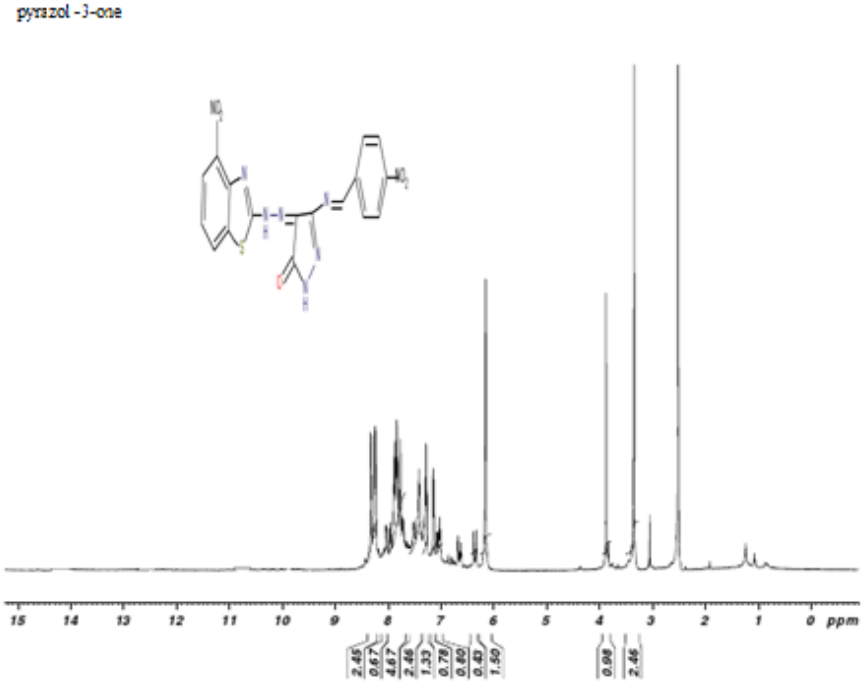
4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-5-{[(E)-(4-nitrophenyl)methylidene]amino}-2,4-dihydro-3 H-pyrazol-3-one

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FIDRES              0.315264 Hz
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RG                        203
DW                   48.400 usec
DE                    6.50 usec
TE                     296.6 K
D1                  1.00000000 sec
TD0                        1

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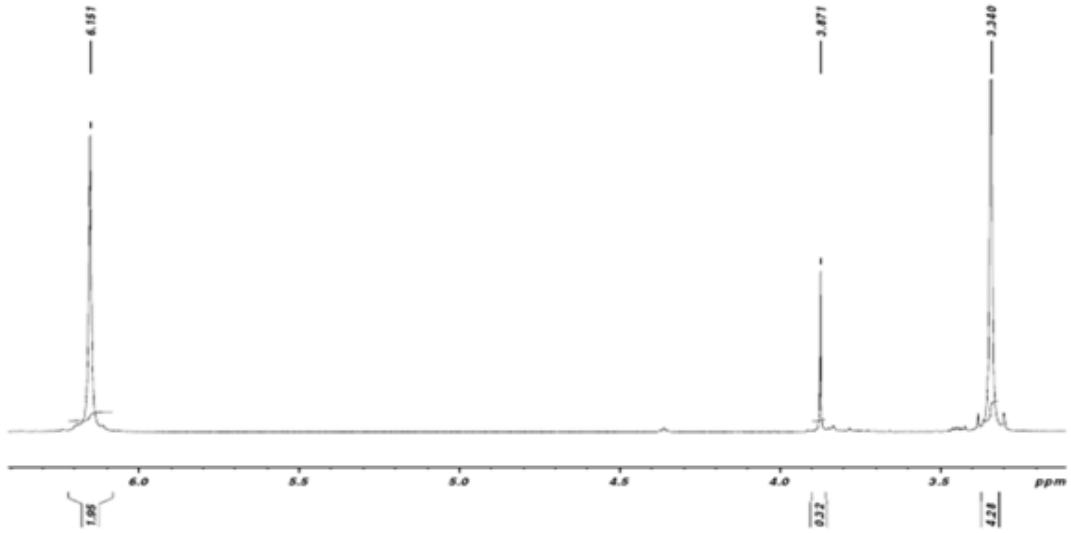

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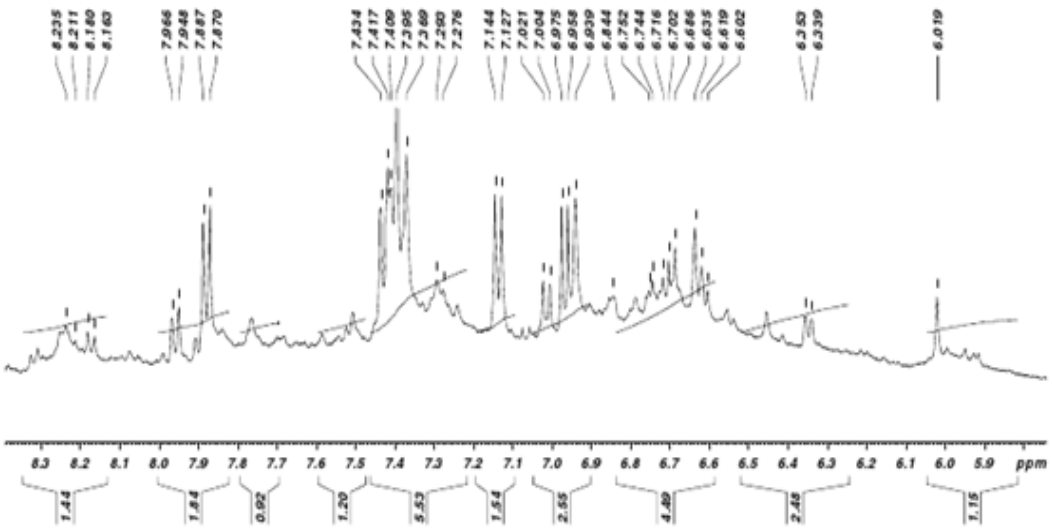

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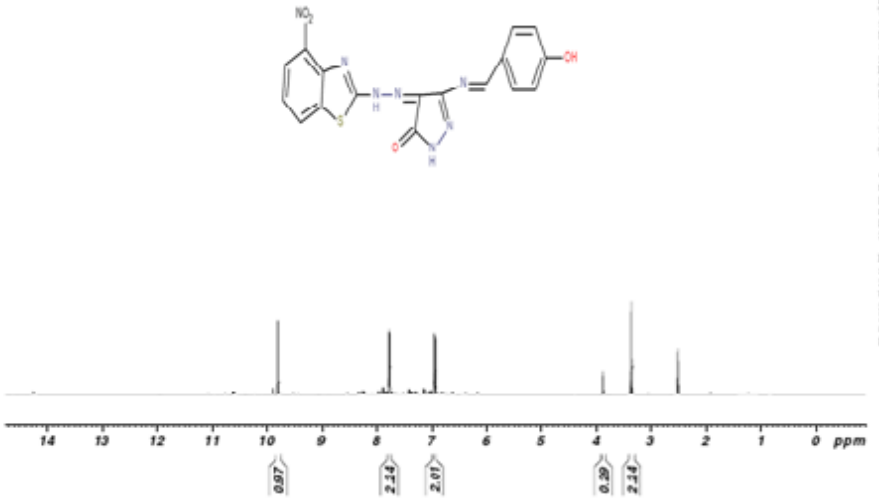


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DE                      6.50 usec
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D1                  1.00000000 sec
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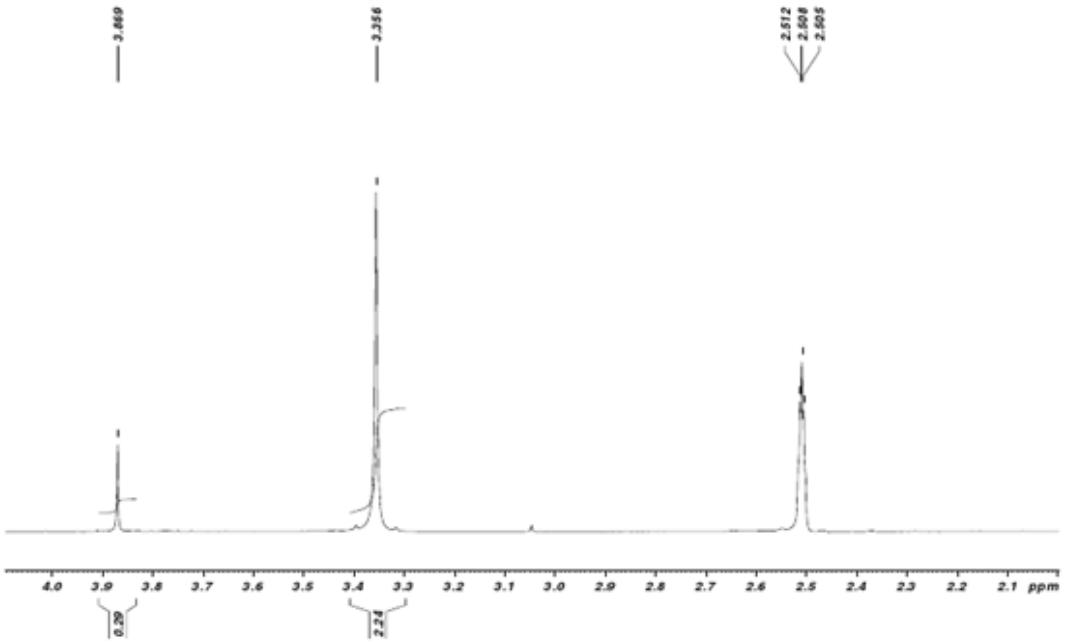

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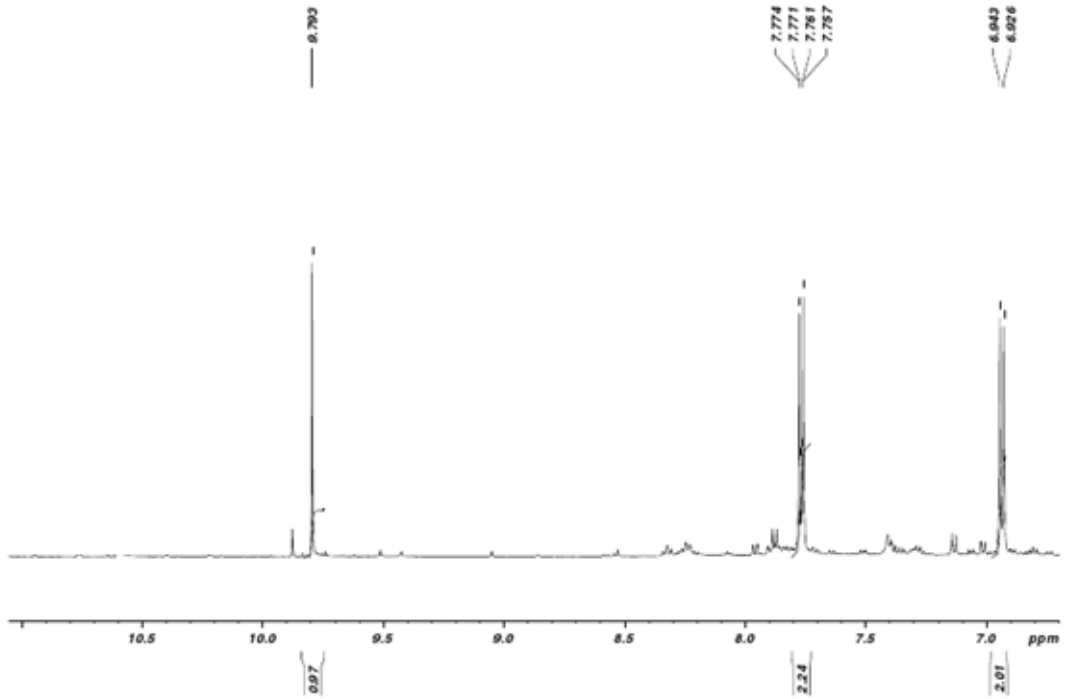

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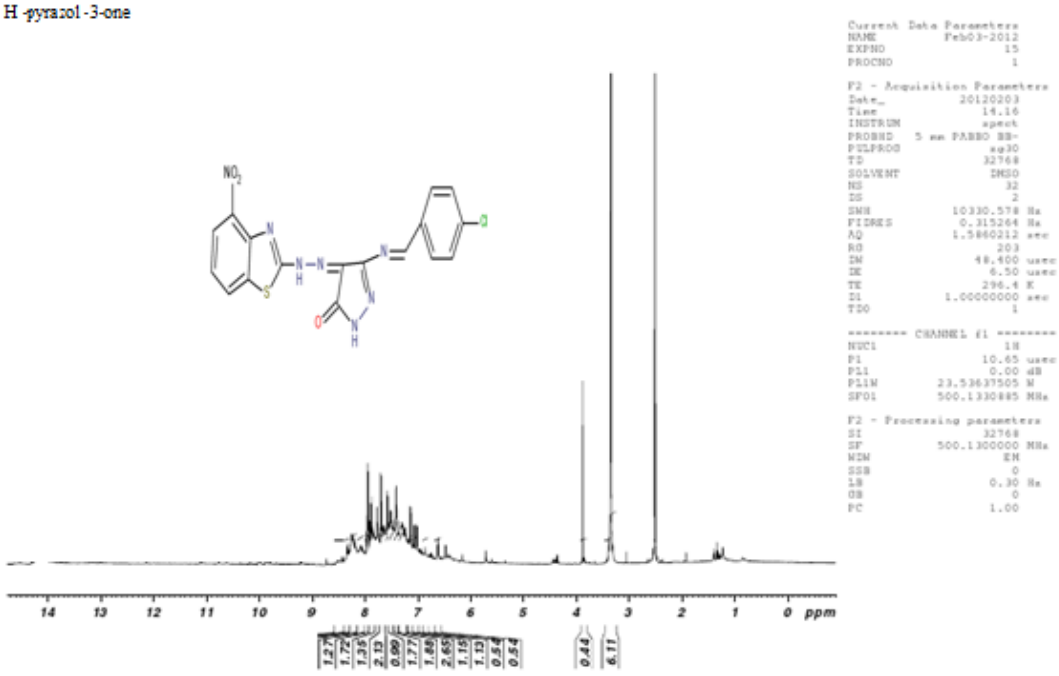

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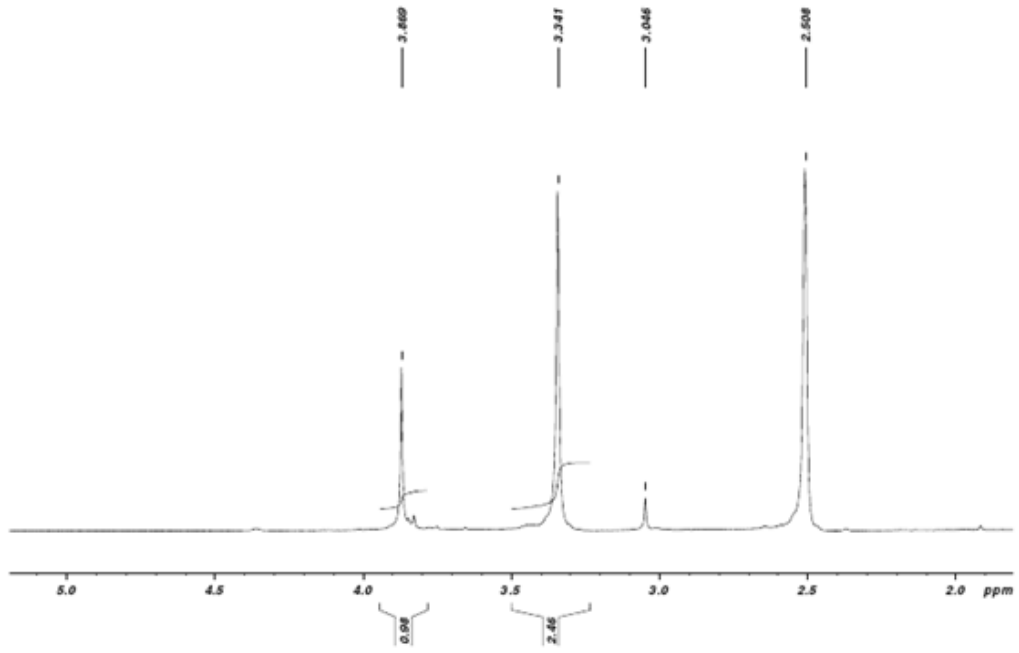

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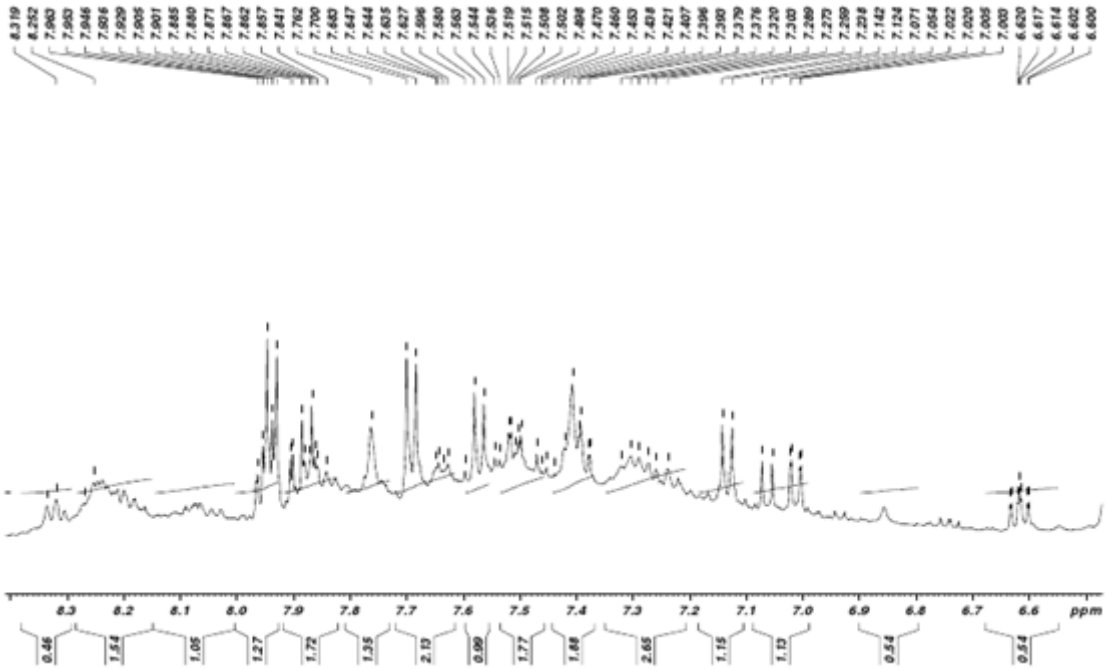

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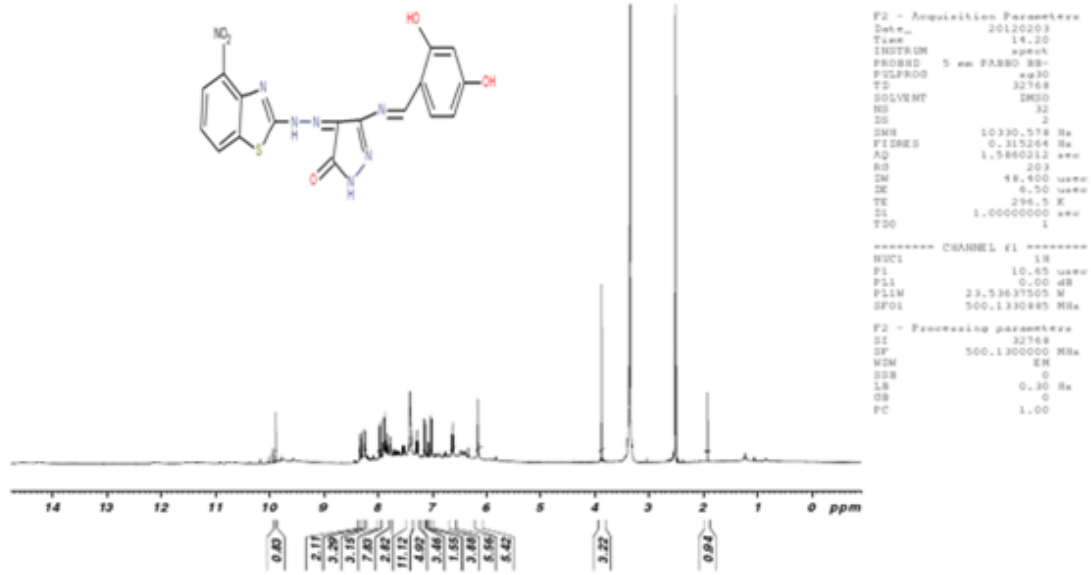

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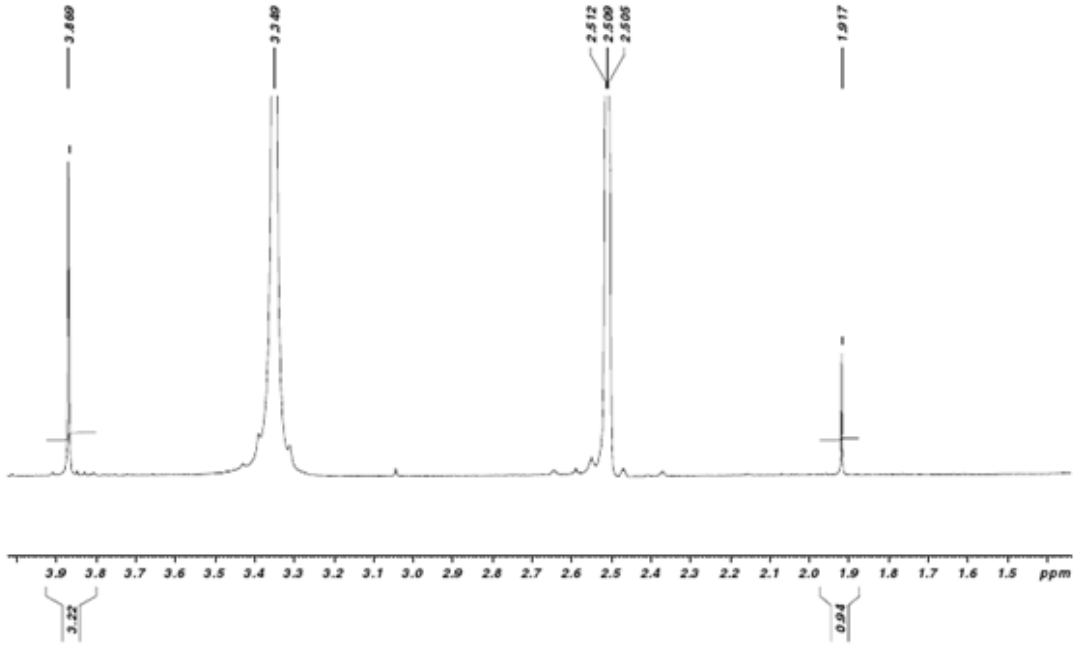

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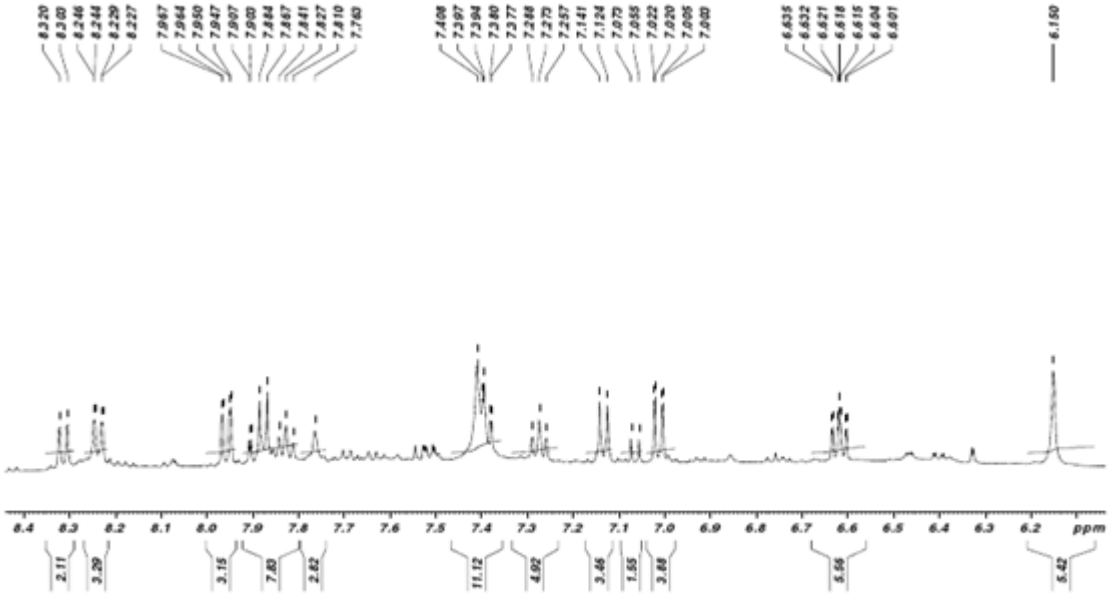

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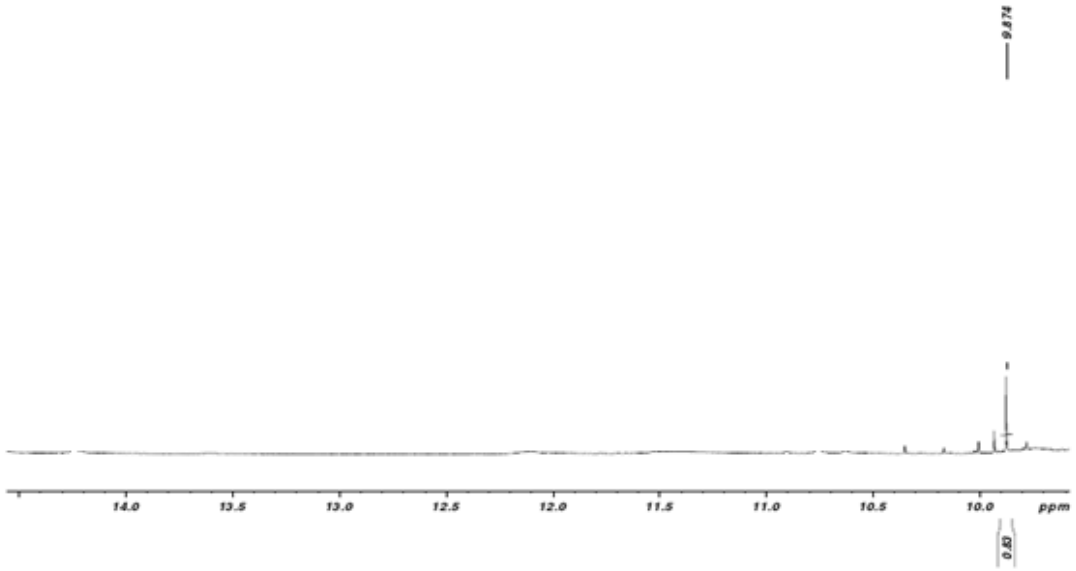

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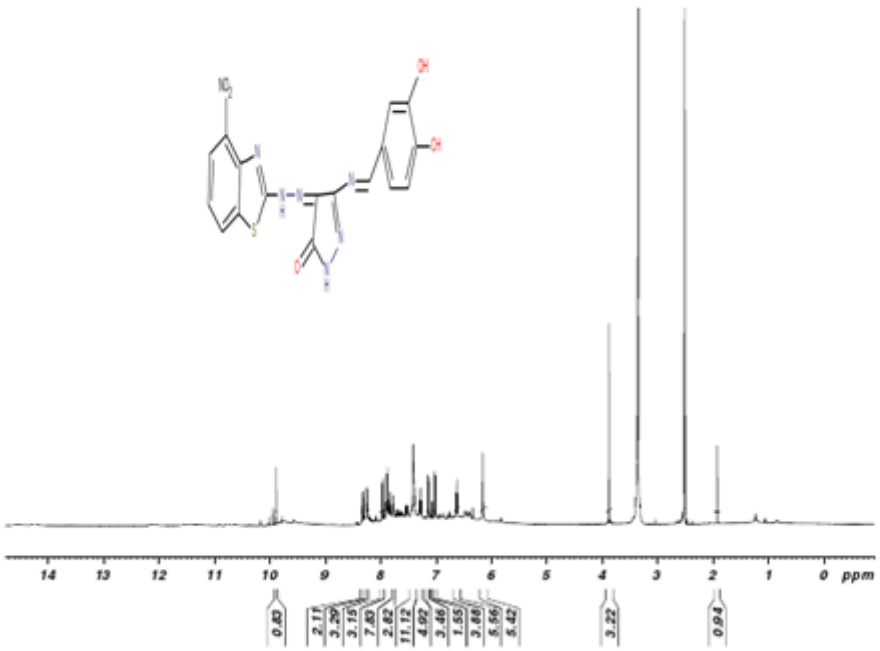
5-{[(E)-(3,4-dihydroxyphenyl)methylidene]amino}-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol -3-one

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RG                   203
DW              48.400 usec
DE               6.50 usec
TE              296.5 K
D1          1.00000000 sec
TD0                    1

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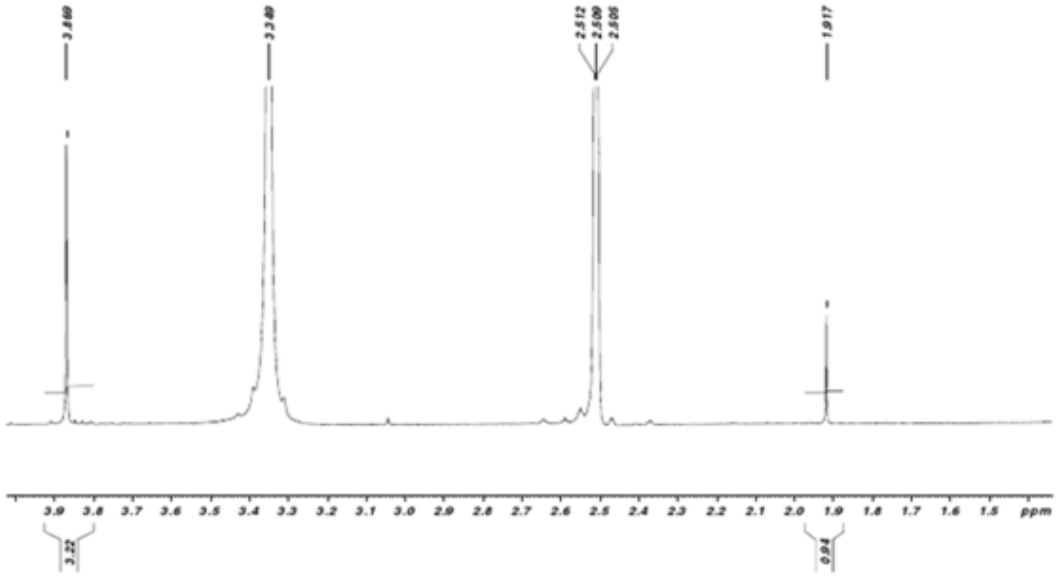

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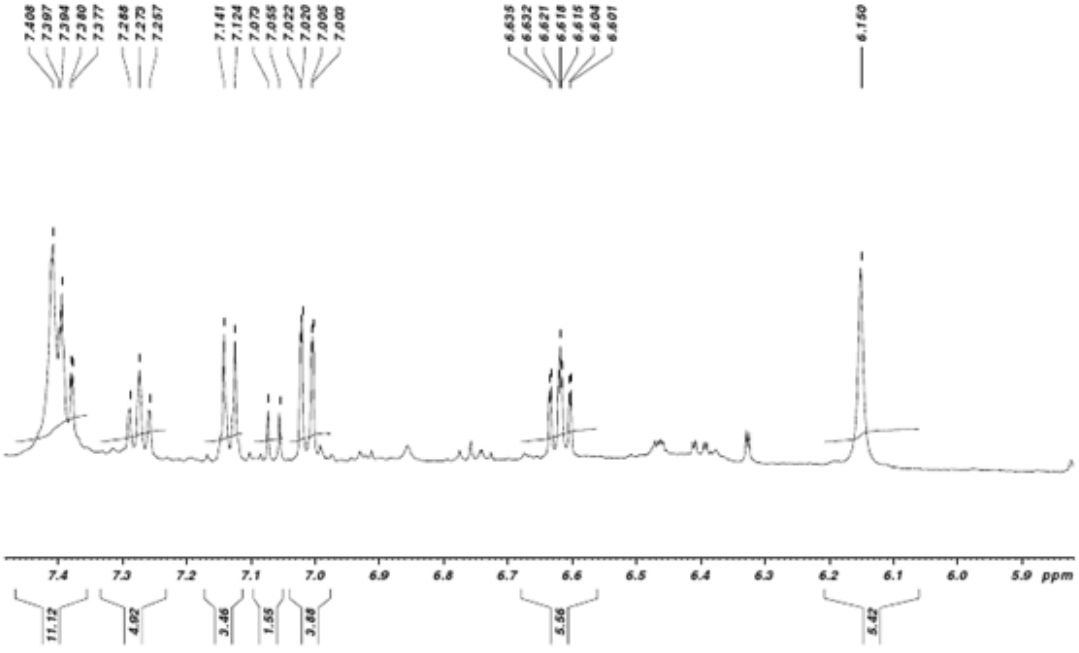

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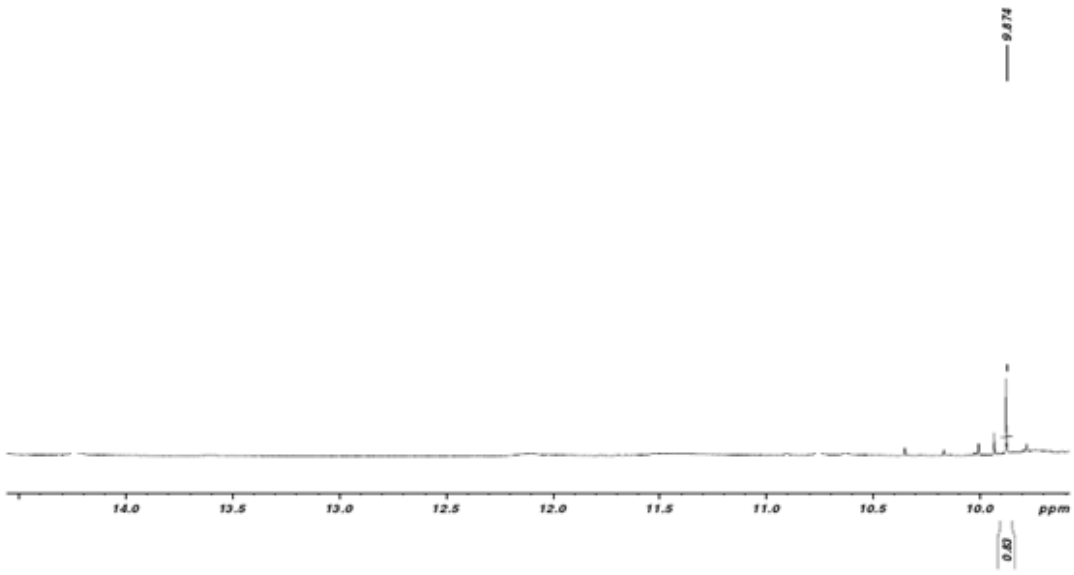

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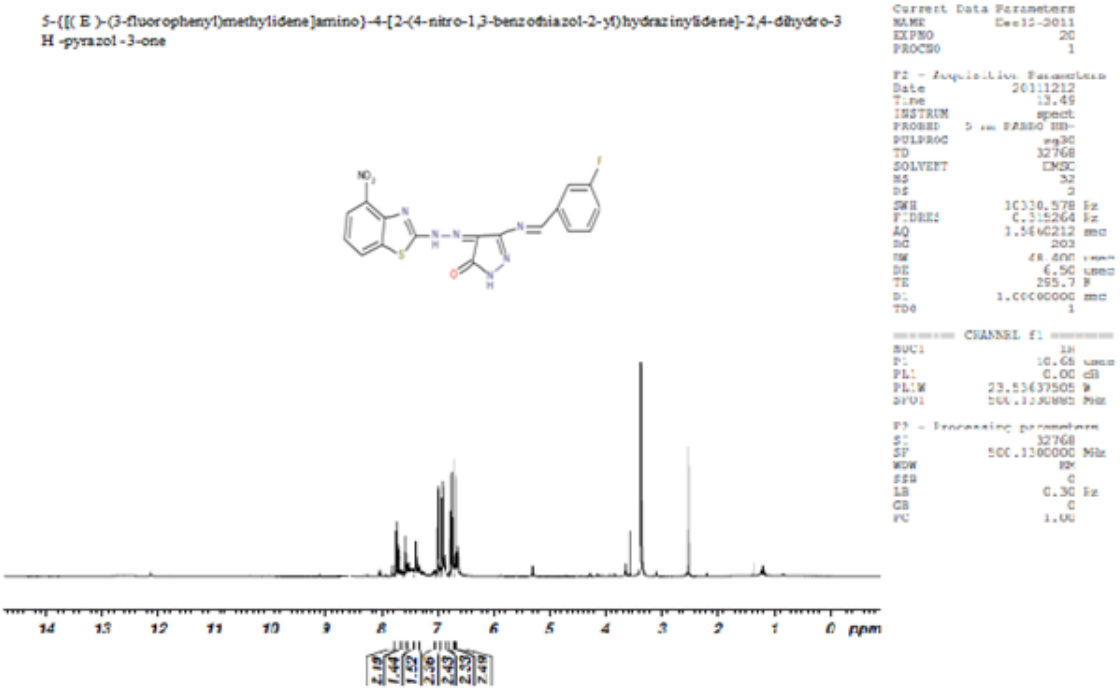

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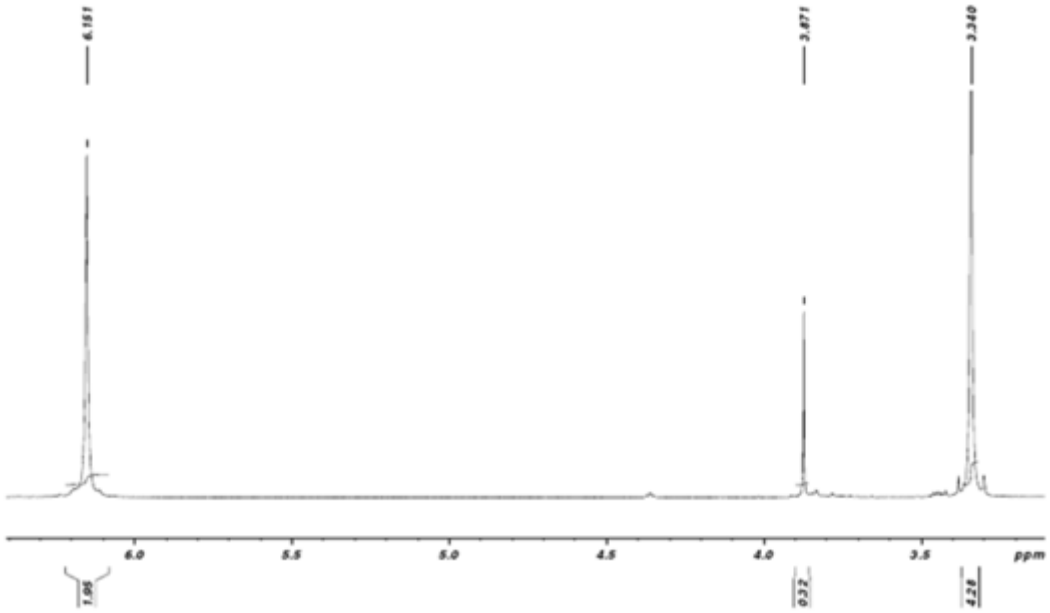

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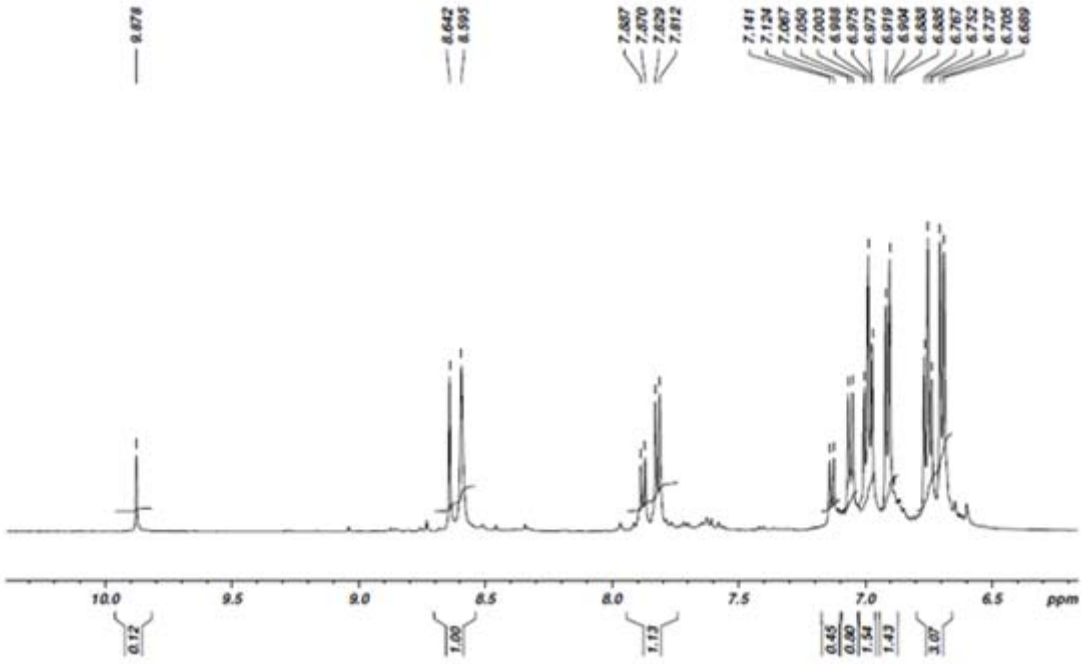

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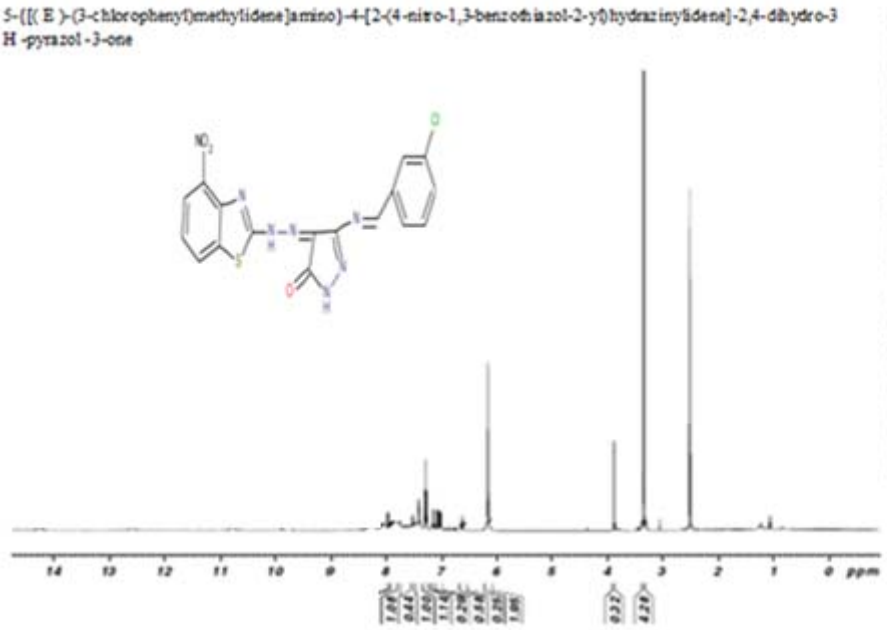
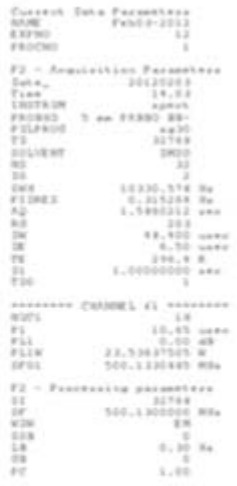
5-([(E)-(3-chlorophenyl)methylidene]amino)-4-[2-(4-nitro-1,3-benzothiazol-2-yl)hydrazinylidene]-2,4-dihydro-3 H -pyrazol-3-one





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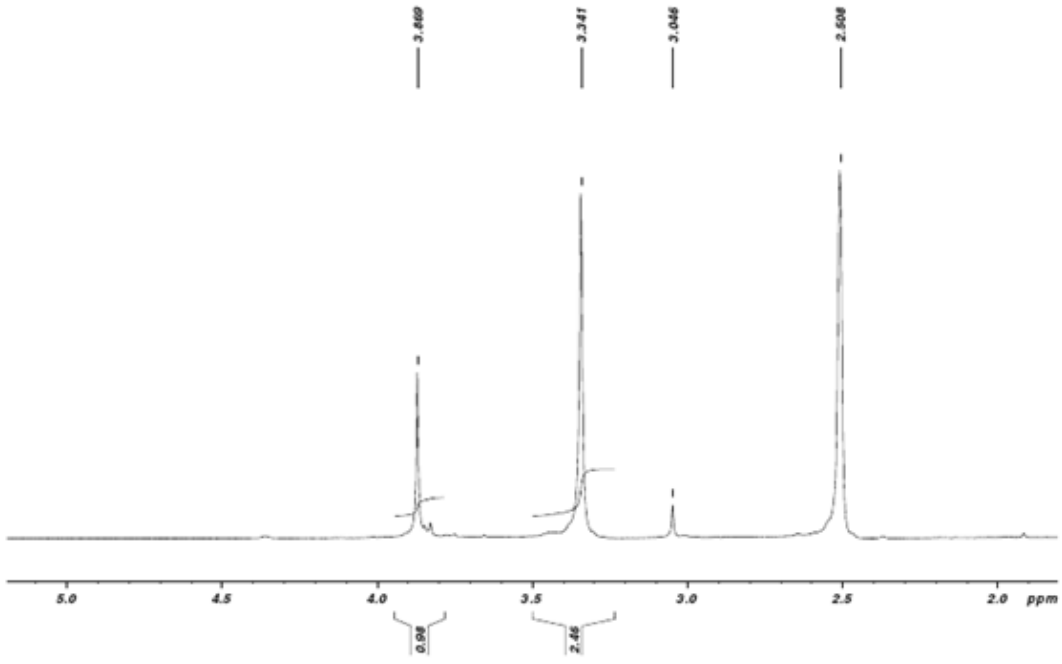

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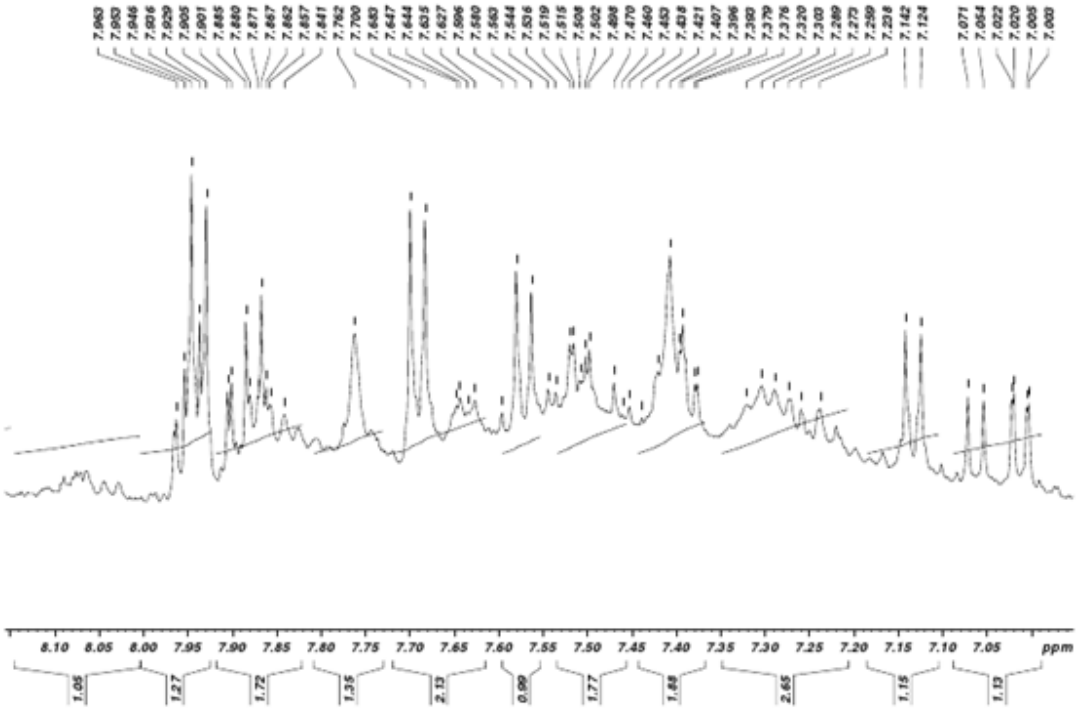
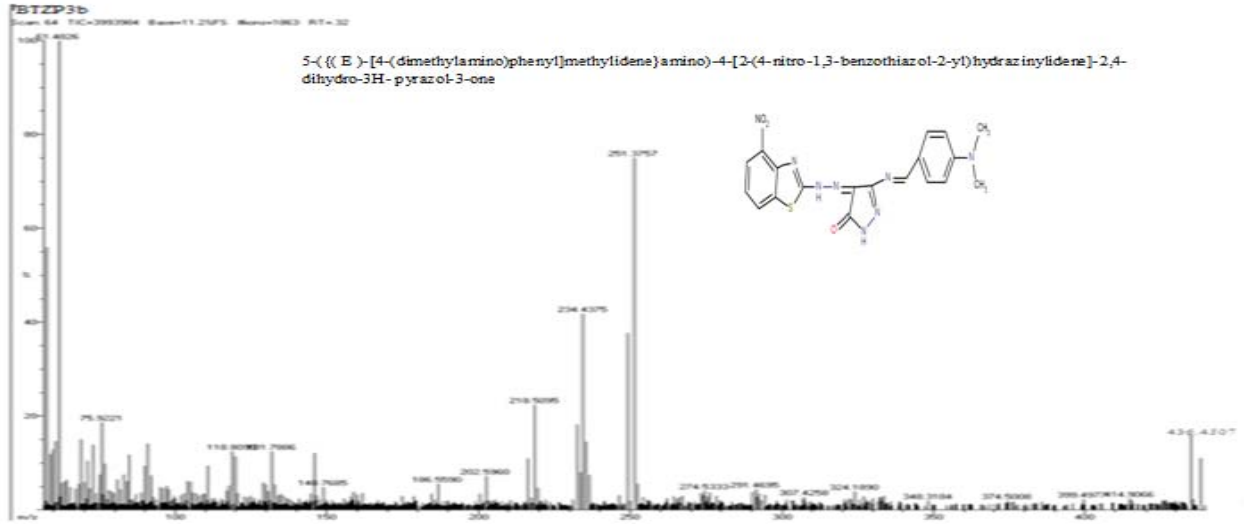


Figure 8. Mass spectra of synthesized compounds

BTZP3b



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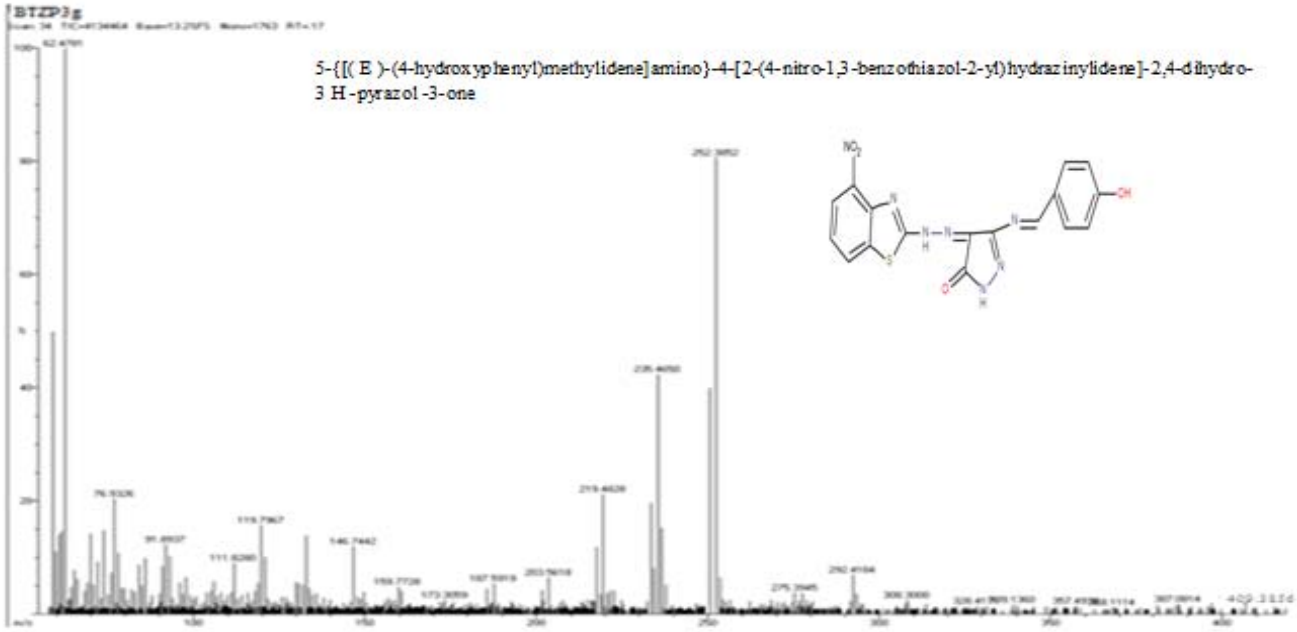

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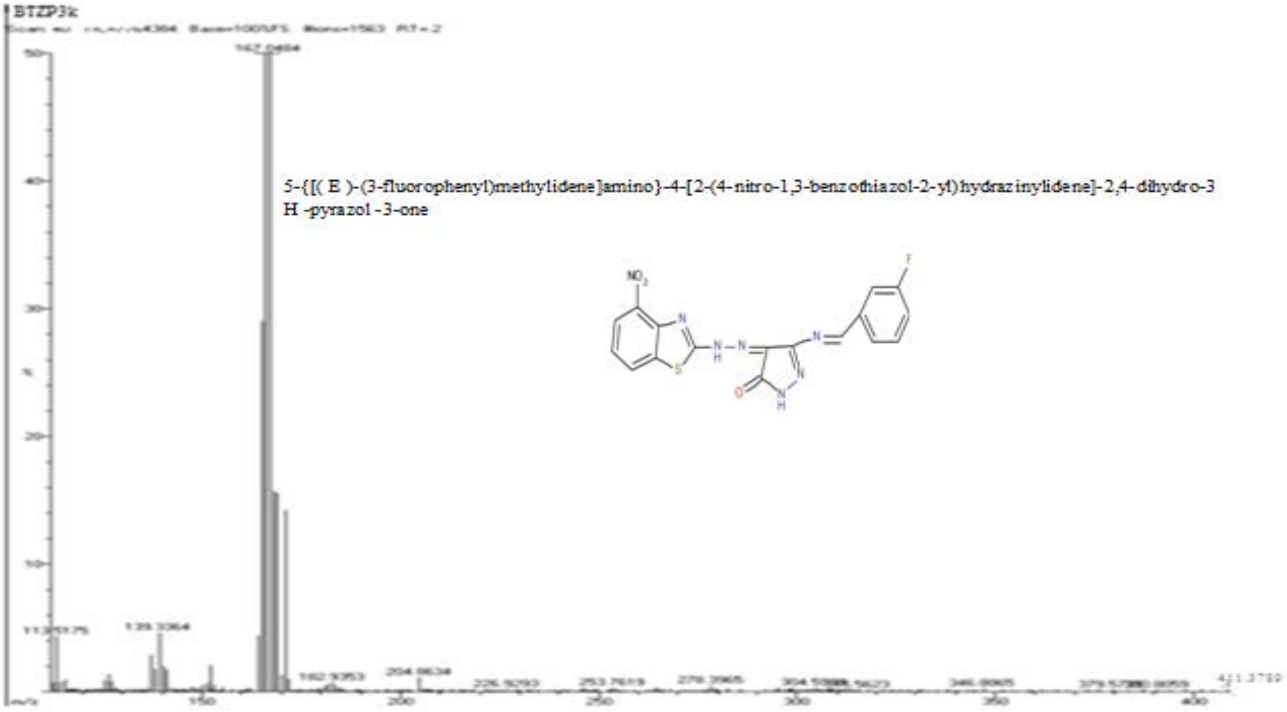


Chapter 6

Biological Screening

6. BIOLOGICAL SCREENING

6.1. Evaluation *In vitro* antimicrobial study of zone of inhibition by disc diffusion method, minimum inhibitory concentration by serial dilution method and bactericidal, fungicidal by minimum bactericidal concentration and minimum fungicidal concentration method.

6.2. Evaluation *In vitro* antitubercular activity by REMA method.

6.3. Evaluation *In vitro* antioxidant study by DPPH, FRAP, ABTS assay method.

6.4. Evaluation *In vitro* cytotoxic activity by MTTS method.

6.1. ANTIMICROBIAL STUDIES

Antimicrobial testing is indicated for any organism that contributes to an infectious disease warranting antimicrobial chemotherapy, if its susceptibility cannot be reliably predicted from pre-existing antibiograms. The introduction of a variety of antimicrobials today makes it a necessity to perform the antimicrobial susceptibility test, the results of which can guide us to the selection of appropriate antimicrobial drug with unquestionable benefit and least problem of antimicrobial resistance. Two methods were used for the antimicrobial studies.

- **Zone of inhibition (Disc diffusion method)**
- **Minimum inhibitory concentration (Serial dilution method)**

6.1.1. DISC DIFFUSION METHOD

Principle

When a filter paper disc impregnated with a chemical is placed medium containing agar, the chemical will diffuse from the disc into the agar. This diffusion places the chemical in the agar around the disc. The area of chemical infiltration around the disc is determined by the solubility of the chemical and its molecular size. An organism placed on the agar fails to grow in the area around the disc if it is susceptible to the chemical. This area of around the disc where no growth is observed is known as a “zone of inhibition”.

The drug potency is established by comparing the inhibition produced by the test compound with that produced by known concentration of reference standard. When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions

that must be constant from test to test include the agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature, and atmosphere).

6.1.2. ANTIBACTERIAL SCREENING BY DISC DIFFUSION METHOD

The antibacterial activities of synthesized compounds were screened in the concentration of 1 µg/µl in dimethyl sulphoxide (DMSO) against the listed microorganisms, obtained from National Collection of Industrial Microorganisms (NCIM), Pune and Microbial Type Culture Collection (MTCC), Chandigarh, in the Muller Hinton agar medium by disc diffusion method using ciprofloxacin (0.5 µg/µl) as standard. The antibacterial activity was evaluated by measuring zone of inhibition in mm.

Table 6. List of bacterial strains used in the study

GRAM POSITIVE	GRAM NEGATIVE
Micrococcus luteus MTCC 2169	*Escherichia coli* MTCC 2065
Staphylococcus aureus MTCC 2079	*Pseudomonas aruginosa* MTCC 2200
Bacillus subtills MTCC 2063	*Rhodosporum ruberum* MTCC 5128
Corney bacterium MTCC 2640	*Viberio cholera* MTCC 1738
Bacillus lintus NCIM 2018	*Salmonella Paratypic* NCIM 2501
Staphylococcus albus MTCC 2178	*Klebsiella pneumoniae* NCIM 2707

Preparation of inoculums

Preparation of inoculums of bacteria was carried out by Muller Hinton Broth and transferred to test tube and kept it for sterilization in autoclave at 120^0 C for 15 min. Then added culture of each bacterium to each tube (this step was carried out in aseptic room near laminar air flow) then kept it for incubation in incubator for 18-24 h at 37^0 C.

Procedure

Muller Hinton agar medium was prepared by dissolving 21 gm of Muller Hinton agar in 1000 ml of distilled water and agar-agar 1-2 gm for solublization. Then kept it for sterilization in autoclave for 121^0 C for 15 min. The petri plates were cleaned, sterilized and marked. These medium (Muller Hinton agar) were poured into petri-plates under aseptic conditions and allowed to solidify. Standardized bacterial inoculum was spread uniformly over the surface of medium by using a sterile non-absorbent cotton swab and finally the swab was passed around the edge of the

medium. The inoculated petri plates were closed with the lid and allowed to dry at room temperature. The sample impregnated discs and standard discs were placed on the inoculated agar medium. All petriplates were incubated at 37^0C for 24 h. After the incubation, diameter of zone of inhibition produced by the sample and standard was measured. The details are tabulated in **Table 8, 9 and Figure 9, 10, 11 and 12.**

6.1.3. ANTIFUNGAL SCREENING BY DISC DIFFUSION METHOD

The antifungal activities of synthesized compounds were screened in the concentration of 1µg/µl in dimethyl sulphoxide (DMSO) against the listed fungal strains, obtained from National Collection of Industrial Microorganisms (NCIM), Pune and Microbial Type Culture Collection (MTCC), Chandigarh, in the Sabourand's Dextrose Broth by disc diffusion method using Clotrimazole (10 µg disc) as standard. The antifungal activity was evaluated by measuring zone of inhibition in mm.

Table 7. List of fungal strains used in the study
Candida albicans MTCC 3100
Monococcus purpureus MTCC 1090
Aspergillus niger MTCC 1344
Trichophyton rubrum MTCC 3272
Aspergillus fumigates MTCC 1811
Aspergillus parasites MTCC 2796

Preparation of inoculums

Preparation of inoculums of fungus was carried out by Sabourand's Dextrose Broth and transferred to test tube and kept it for sterilization in autoclave at 120^0 C for 15 min. Then added culture of each fungal to each tube (this step was carried out in aseptic room near laminar air flow) then kept it for incubation in incubator for 24-48 h at 29^0 C.

Procedure

Sabourand's Dextrose agar medium was prepared by dissolving 1 gm of peptone and 4 gm of dextrose in 100 ml of distilled water and agar-agar 1-2 gm for solublization. Then kept it for

sterilization in autoclave for 121^0 C for 15 min. The petri plates were cleaned, sterilized and marked. These medium (Sabourand's Dextrose agar) were poured into petri-plates under aseptic conditions and allowed to solidify. Standardized fungal inoculum was spread uniformly over the surface of medium by using a sterile non-absorbent cotton swab and finally the swab was passed around the edge of the medium. The inoculated petri plates were closed with the lid and allowed to dry at room temperature. The sample impregnated discs and standard discs were placed on the inoculated agar medium. All petriplates were incubated at 29^0C for 24 - 48 h. After the incubation, diameter of zone of inhibition produced by the sample and standard was measured.[148] The details are tabulated in **Table 10 and Figure 13, 14.**

6.1.4. SERIAL DILUTION METHOD

Principle

Minimum inhibitory concentration (MIC), in microbiology, is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Dilution tests are used to determine the MIC of an agent required to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial agent in either agar or broth media. The potency of drug is based on inhibition of microbial growth as indicated by measurement of turbidity of a suspension of a suitable microorganism in a fluid medium to which graded amount of test compound have been added. The changes in turbidity produced by known concentration of reference materials are compared with the results.

6.1.5. MIC STUDIES

Procedure for determination of minimum inhibitory concentration for synthesized compounds against bacteria by serial dilution method

The serial dilution of compound solutions were made from the stock (1000 µg/ml) by using Muller Hinton broth using the method described below.

- The tubes were labeled 1 to 8 and 1 ml of Muller Hinton broth was added to the first 5 tubes and 8th tube, and then added 0.5 ml Muller Hinton broth to 6th and 7th tubes.
- One ml of different synthesized compounds was added to the 1st tube, mixed and transferred 1 ml serially up to tube 6. Mixed and transferred 0.5 ml to the 7th tube so that each tube, 1 to 7 contained 1 ml diluted extracts. The 8th tube served as the control.

- With a standardized micro pipette, added a drop of the diluted broth culture approximately 0.01 ml of the test organism to all tubes, including the control, gently mixed and incubated at 37 ^{0} C for 18 h.
- After incubation the turbidity was observed. The highest dilution of particular compounds showing no turbidity and recorded. This was taken as the end point, and this dilution was considered to contain the concentration of drug equivalent to MIC [149]. The details are tabulated in **Table11, 12 and Figure 15, 16**.

Procedure for determination of minimum inhibitory concentration for synthesized compounds against fungi by serial dilution method

The serial dilution of compound solutions were made from the stock (1000 µg/ml) by using Sabourand's dextrose broth using the method described below.

- The tubes were labeled 1 to 8 and 1 ml of Sabourand's dextrose broth was added to the first 5 tubes and 8th tube, and then added 0.5 ml Muller Hinton broth to 6th and 7th tubes.
- One ml of different synthesized compounds was added to the 1st tube, mixed and transferred 1 ml serially up to tube 6. Mixed and transferred 0.5 ml to the 7th tube so that each tube, 1 to 7 contained 1 ml diluted extracts. The 8th tube served as the control.
- With a standardized micro pipette, added a drop of the diluted broth culture approximately 0.01ml of the test organism to all tubes, including the control, gently mixed and incubated at 27^{0} C for 24 - 48 h.
- The highest dilution of particular compounds showing no turbidity was observed and recorded. This was taken as the end point, and this dilution was considered to contain the concentration of drug equivalent to MIC. [150] The details are tabulated in **Table 13 and Figure 17**.

6.1.6. BACTERICIDAL/FUNGICIDAL

Principle

Minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC), in microbiology, is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Dilution tests are used to determine the MBC,

MFC of an agent required to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial agent in either agar or broth media.

- ⇒ Bactericidal and fungicidal agents are those which are able to kill bacteria and fungi.
- ⇒ Bacteriostatic and fungiostatic agents are those which are able to prevent the multiplication of bacteria and fungi.
- ⇒ A chemical agents which is bactericidal and fungicidal at a particular concentration, may only the bacteriostatic and fungiostatic agents at a higher dilution.

Procedure for determination of minimum bactericidal concentration (MBC)
Procedure

Muller Hinton agar medium was prepared by dissolving 21 gm of Muller Hinton agar in 1000 ml of distilled water and agar-agar 1-2 gm for solublization. Then kept it for sterilization in autoclave for 121^0 C for 15 min. The petri plates were cleaned, sterilized and marked. These medium (Muller Hinton agar) were poured in the sterile petri plates to this add 100µl of muller hinton broth from the MIC tube (after determining Minimum inhibitory concentration (MIC) value) and mixed well, allowed to solidify. Three more petri plates also prepared from the MIC tube with high concentration. The petri plates were closed with the lid and allowed to dry at room temperature. All petri plates were allowed to incubation at37^0C for 24 hrs. The minimum bactericidal concentration (MBC) were determined by sub culturing 100 µL of culture from each tube that remained clear in the MIC determination into fresh medium. MBC values represent the lowest concentration of compound that produces a 99.9% end point reduction. [151]

Procedure for determination of minimum fungicidal concentration (MFC)
Procedure

Sabourand's Dextrose agar medium was prepared by dissolving 1 gm of peptone and 4 gm of dextrose in 100 ml of distilled water and agar-agar 1-2 gm for solublization. Then kept it for sterilization in autoclave for 121^0 C for 15 min. The petri plates were cleaned, sterilized and marked. These medium (Sabourand's Dextrose agar) were poured in the sterile petri plates to this add 100µl of sabourand's dextrose broth from the MIC tube (after determining Minimum inhibitory concentration (MIC) value) and mixed well, allowed to solidify. Four more petri plates also prepared from the MIC tube with high concentration. The petri plates were closed with the lid and allowed to dry at room temperature. All petri plates were allowed to incubation at29^0C for 48 hrs.The minimum fungicidal concentration (MFC) were determined by sub

culturing 100 µL of culture from each tube that remained clear in the MIC determination into fresh medium.MFC values represent the lowest concentration of compound that produces a 99.9% end point reduction. [151]

ANTIMICROBIAL STUDIES

Table 8. Antibacterial screening of synthesized compounds (gram positive strains) by disc diffusion method

Compound code	Mean zone of inhibition (in mm) and percentage inhibition					
	Micrococcus Luteus	***Staphylococcus Aureus***	***Bacillus Substils***	***Corney Bacterium***	***Bacillus Lintus***	***Staphylococcus Albus***
BTZP3a	10 (83%)	11 (73%)	9 (60%)	9 (60%)	12 (80%)	9 (60%)
BTZP3b	9 (75%)	9 (60%)	9 (60%)	11 (73%)	12 (80%)	9 (60%)
BTZP3c	9 (75%)	11 (73%)	11 (73%)	10 (66%)	12 (80%)	10 (66%)
BTZP3d	10 (83%)	10 (66%)	11 (73%)	10 (66%)	12 (80%)	10 (66%)
BTZP3e	9 (75%)	9 (60%)	9 (60%)	9 (60%)	11 (73%)	9 (60%)
BTZP3f	9 (75%)	9 (60%)	9 (60%)	11 (73%)	11 (73%)	9 (60%)
BTZP3g	10 (83%)	9 (60%)	10 (66%)	11 (73%)	13 (86%)	11 (73%)
BTZP3h	10 (83%)	9 (60%)	10 (66%)	9 (60%)	13 (86%)	11 (73%)
BTZP3i	9 (75%)	10 (66%)	10 (66%)	10 (66%)	11 (73%)	11 (73%)
BTZP3j	9 (75%)	9 (60%)	11 (73%)	9 (60%)	11 (73%)	10 (66%)
BTZP3k	9 (75%)	11 (73%)	9 (60%)	10 (66%)	11 (73%)	9 (60%)
BTZP3l	9 (75%)	11 (73%)	9 (60%)	10 (66%)	10 (66%)	9 (60%)
Ciprofloxacin	12	15	15	15	15	15

BTZP3a-l = Synthesized compounds in the concentration of 100 µg/disc.
Standard = Ciprofloxacin in the concentration of 5 µg/disc.
Solvent = DMSO.
Media = **Bacteria: Muller Hinton Agar.**

Figure 9. Antibacterial screening of synthesized compounds (gram positive strains) by disc diffusion method

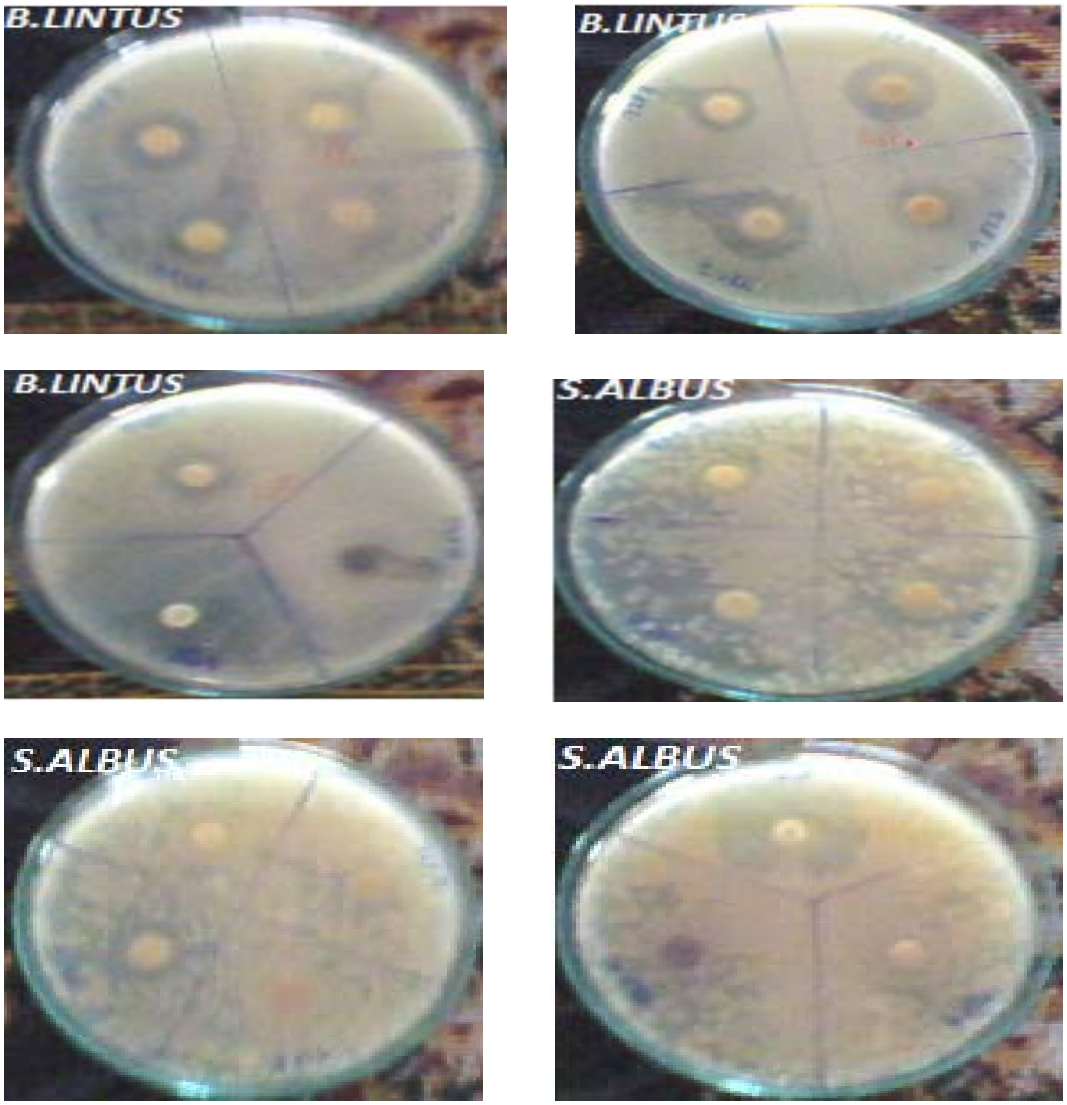


Figure 10. Antibacterial screening of synthesized compounds (gram positive strains) by percentage inhibition.

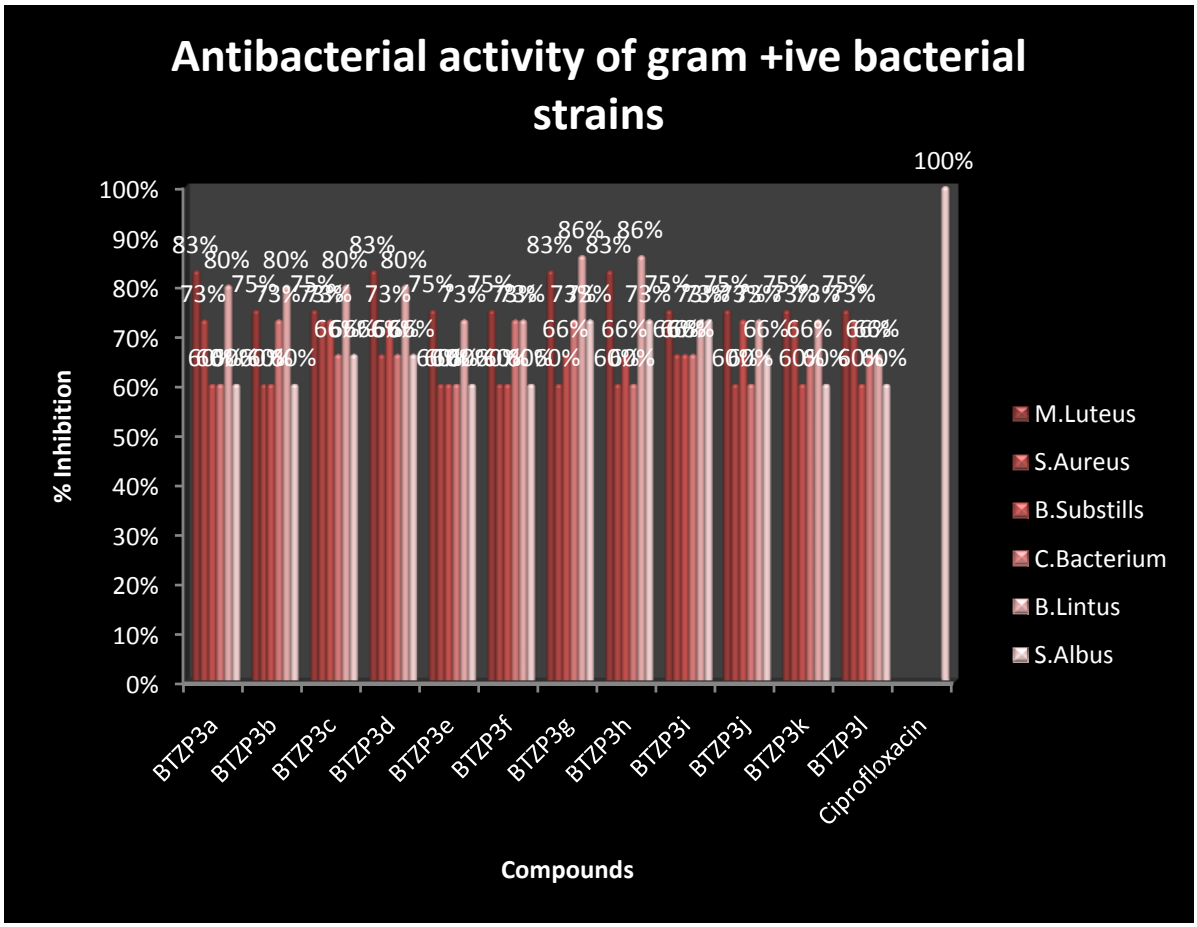


Table 9. Antibacterial screening of synthesized compounds (gram negative bacteria) By disc diffusion method

Compound code	Mean zone of inhibition (in mm) and percentage inhibition					
	Escherichia Coli	***Pseudomonas Aruginosa***	***Rhodosporum Ruberum***	***Viberio Cholera***	***Salmonella Paratyphii***	***Klebsiella Pneumoniae***
BTZP3a	9 (56%)	8 (62%)	8 (80%)	10 (45%)	10 (125%)	10 (111%)
BTZP3b	10 (62%)	8 (62%)	11 (110%)	10 (45%)	13 (162%)	10 (111%)
BTZP3c	11 (68%)	8 (62%)	8 (80%)	10 (45%)	13 (162%)	10 (111%)
BTZP3d	10 (62%)	8 (62%)	10 (100%)	13 (59%)	14 (175%)	12 (133%)
BTZP3e	9 (56%)	8 (62%)	9 (90%)	13 (59%)	12 (150%)	11 (122%)
BTZP3f	10 (62%)	8 (62%)	10 (100%)	13 (59%)	12 (150%)	11 (122%)
BTZP3g	10 (62%)	8 (62%)	11 (110%)	13 (59%)	10 (125%)	11 (122%)
BTZP3h	9 (56%)	11 (85%)	10 (100%)	13 (59%)	13 (162%)	14(155%)
BTZP3i	10 (62%)	8 (62%)	9 (90%)	13 (59%)	13 (162%)	13(144%)
BTZP3j	8 (50%)	11 (85%)	8 (80%)	13 (59%)	13 (162%)	11 (122%)
BTZP3k	8 (50%)	9 (69%)	8 (80%)	13 (59%)	10 (125%)	10 (111%)
BTZP3l	8 (50%)	9 (69%)	8 (80%)	13 (59%)	10 (125%)	10 (111%)
Ciprofloxacin	16	13	10	22	8	9

BTZP3a-l = Synthesized compounds in the concentration of 100 µg/disc.
Standard = Ciprofloxacin in the concentration of 5 µg/disc.
Solvent = DMSO.
Media = **Bacteria : Muller Hinton Agar.**

igure 11. Antibacterial screening of synthesized compounds (gram negative strains) by disc diffusion method

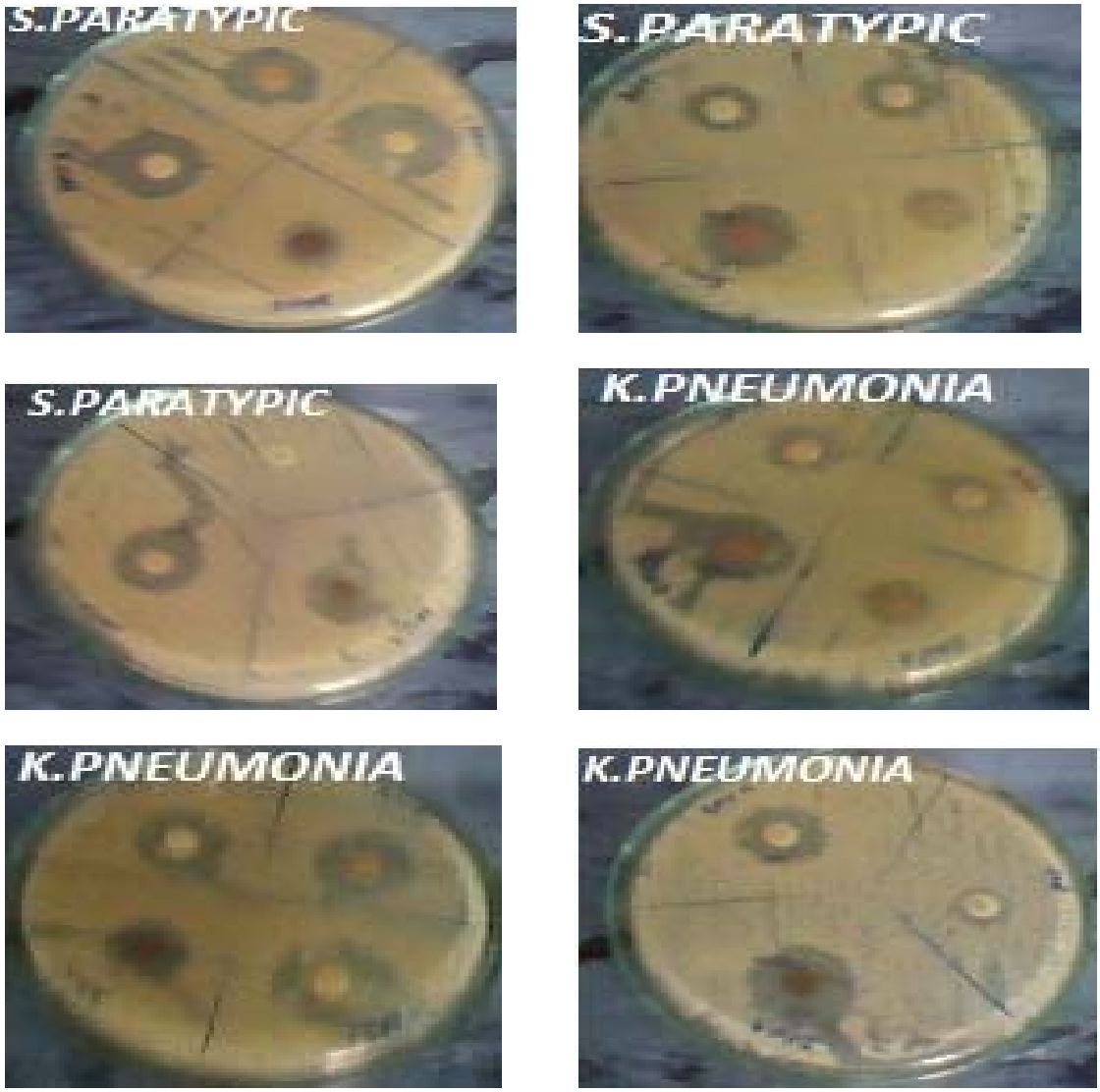


Figure 12. Antibacterial screening of synthesized compounds (gram negative strains) by percentage inhibition.

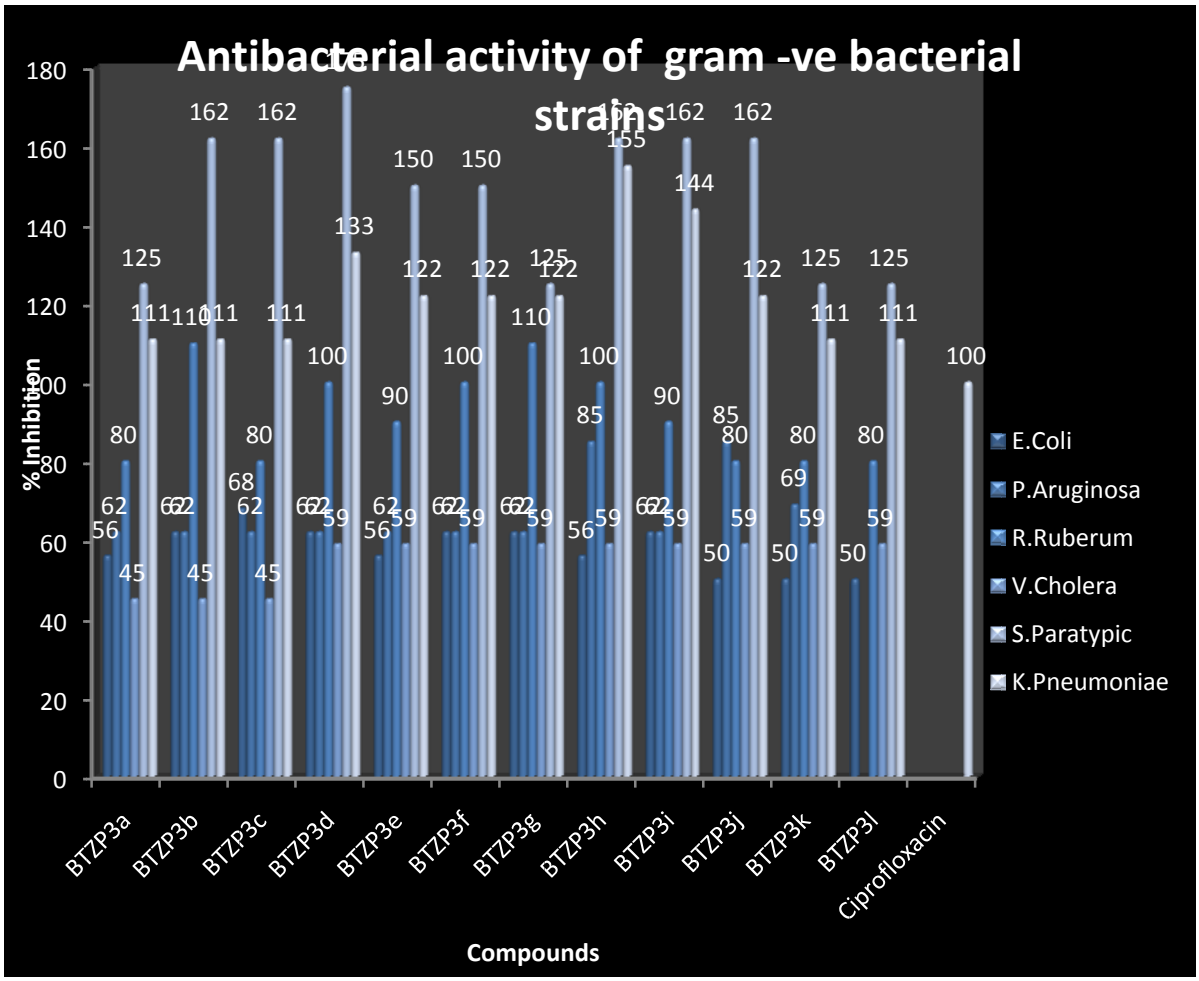


Table 10. Antifungal screening of synthesized compounds (fungi strains) by disc diffusion method

Compound code	Mean zone of inhibition (in mm) and percentage inhibition					
	Candida Albicans	***Monococcus Purpureus***	***Aspergillus Niger***	***Trichophyton rubrum***	***Aspergillus Fumigates***	***Aspergillus Parasites***
BTZP3a	9 (75%)	9 (82%)	10 (77%)	11 (85%)	8 (80%)	13 (130%)
BTZP3b	9 (75%)	9 (82%)	10 (77%)	9 (69%)	8 (80%)	9 (90%)
BTZP3c	11 (91%)	9 (82%)	10 (77%)	9 (69%)	8 (80%)	9 (90%)
BTZP3d	9 (75%)	9 (82%)	11 (85%)	9 (69%)	13 (130%)	8 (80%)
BTZP3e	11 (91%)	9 (82%)	10 (77%)	9 (69%)	11 (110%)	8 (80%)
BTZP3f	12 (100%)	9 (82%)	13 (100%)	9 (69%)	8 (80%)	8 (80%)
BTZP3g	13 (108%)	14 (127%)	13 (100%)	13 (100%)	10 (100%)	9 (90%)
BTZP3h	11 (91%)	14 (127%)	13 (100%)	13 (100%)	10 (100%)	11 (110%)
BTZP3i	9 (75%)	14 (127%)	10 (77%)	11 (85%)	8 (80%)	9 (90%)
BTZP3j	11(91%)	9 (82%)	13 (100%)	13 (100%)	8 (80%)	9 (90%)
BTZP3k	13 (108%)	14 (127%)	11 (85%)	9 (69%)	8 (80%)	8 (80%)
BTZP3l	11 (91%)	14 (127%)	10 (77%)	9 (69%)	8 (80%)	8 (80%)
Clo-trimazole	12	11	13	13	10	10

BTZP3a-l = Synthesized compounds in the concentration of 100 µg/disc.
Standard = Clotrimazole in the concentration of 5 µg/disc
Solvent = DMSO.
Media = **Fungi : Sabourand's Dextrose Agar.**

Figure 13. Antifungal screening of synthesized compounds at 100µg/discs by disc diffusion method

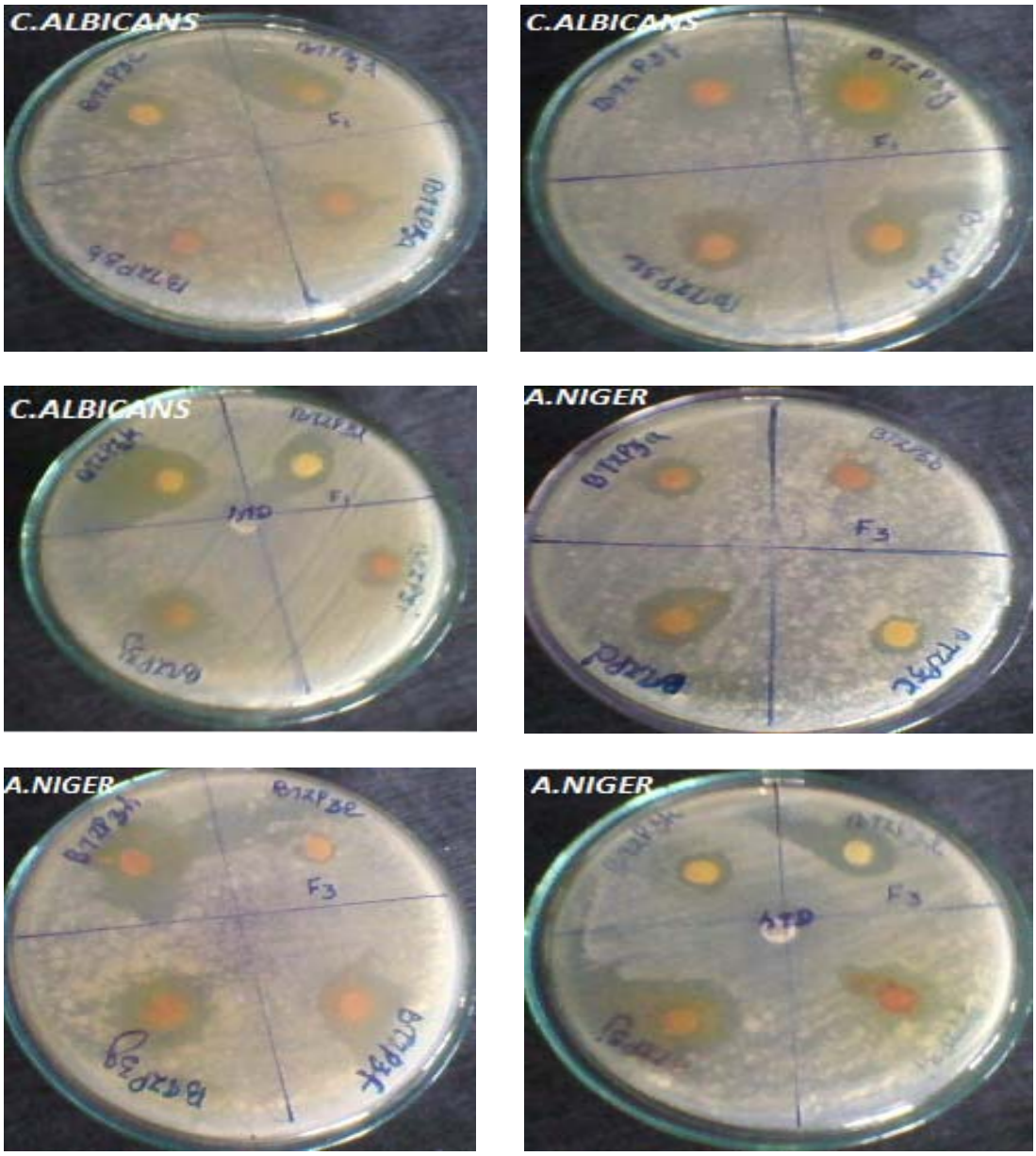


Figure 14. Antifungal screening of synthesized compounds at 100µg/discs by percentage inhibition.

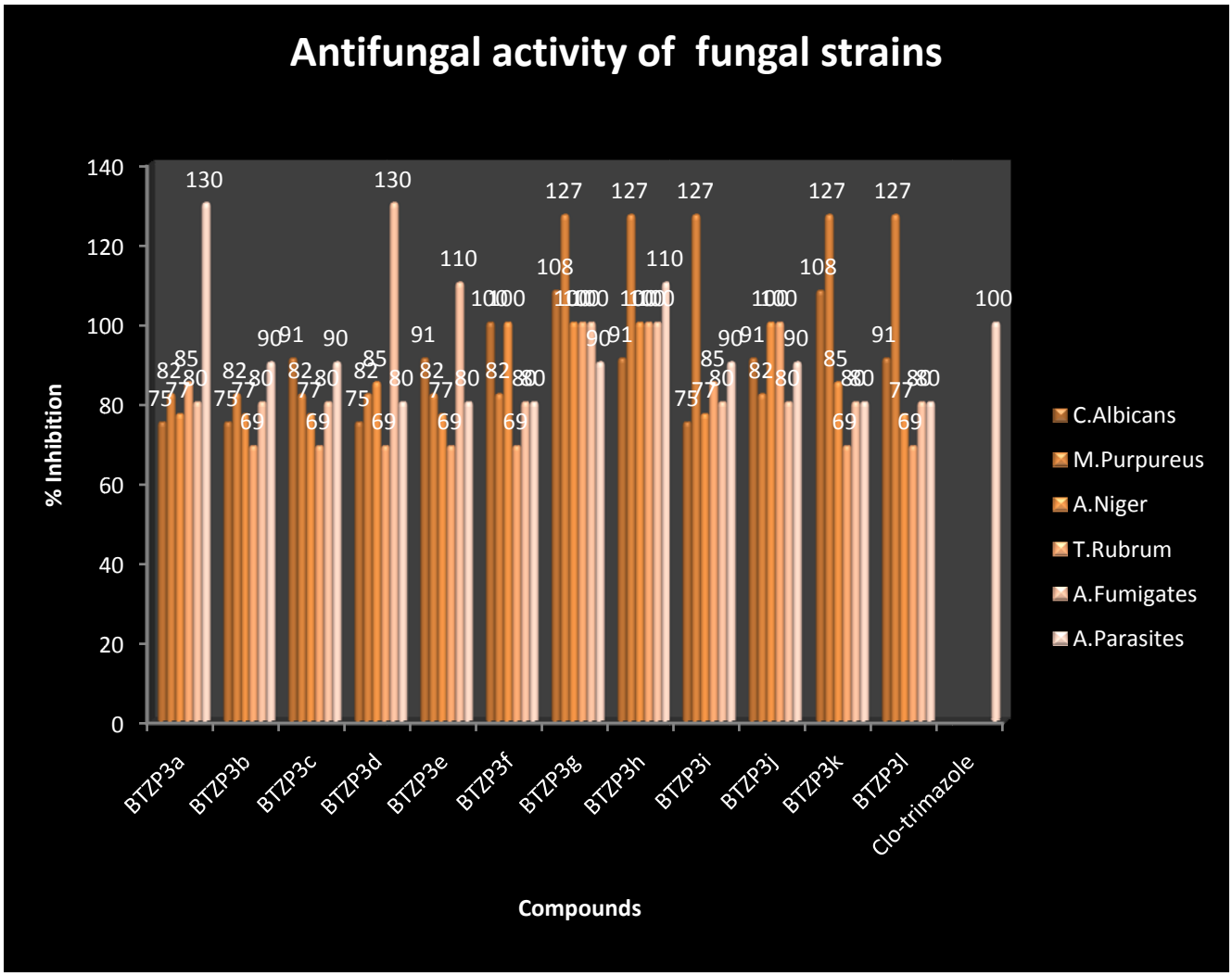


Table 11. Minimum inhibitory concentration of synthesized compounds (gram positive strains) by serial dilution method

Compound code	Minimum inhibitory concentration (in microgram/ml)					
	Micrococcus Luteus	***Staphylococcus Aureus***	***Bacillus Substils***	***Corney Bacterium***	***Bacillus Lintus***	***Staphylococcus Albus***
BTZP3a	12.5	12.5	25	25	6.25	25
BTZP3b	25	25	25	12.5	12.5	25
BTZP3c	25	12.5	12.5	25	12.5	25
BTZP3d	12.5	25	12.5	25	6.25	25
BTZP3e	25	25	25	25	25	25
BTZP3f	25	25	25	12.5	25	25
BTZP3g	12.5	25	25	12.5	6.25	12.5
BTZP3h	12.5	25	25	25	6.25	12.5
BTZP3i	25	25	25	25	25	12.5
BTZP3j	25	25	12.5	25	25	25
BTZP3k	25	12.5	25	25	25	25
BTZP3l	25	12.5	25	25	25	25
Ciprofloxacin	1.56	1.56	1.56	1.56	1.56	1.56

BTZP3a-l = Synthesized compounds in the concentration of 100-1.56 µg/ml.
Solvent = DMSO.
Media = **Bacteria : Muller Hinton Broth.**

Table 12. Minimum inhibitory concentration of synthesized compounds (gram negative strains) by serial dilution method

Compound code	Minimum inhibitory concentration(in microgram/ml)					
	Escherichia Coli	***Pseudomonas Aruginosa***	***Rhodosporum Ruberum***	***Viberio Cholera***	***Salmonella Paratyphii***	***Klebsiella Pneumoniae***
BTZP3a	25	25	12.5	25	25	25
BTZP3b	25	25	1.56	25	6.25	25
BTZP3c	25	25	12.5	25	6.25	25
BTZP3d	25	25	3.12	25	3.12	6.25
BTZP3e	25	25	6.25	25	12.5	12.5
BTZP3f	25	25	3.12	25	12.5	12.5
BTZP3g	25	25	3.12	25	25	12.5
BTZP3h	25	12.5	3.12	25	6.25	3.12
BTZP3i	25	25	6.25	25	6.25	3.12
BTZP3j	25	12.5	12.5	25	6.25	12.5
BTZP3k	25	25	12.5	25	25	25
BTZP3l	25	25	12.5	25	25	25
Ciprofloxacin	1.56	1.56	1.56	1.56	1.56	1.56

BTZP3a-l = Synthesized compounds in the concentration of 100-1.56 µg/ ml.
Solvent = DMSO.
Media = **Bacteria : Muller Hinton Broth.**

Table 13. Minimum inhibitory concentration of synthesized compounds (fungi strains) by serial dilution method

Compound code	Minimum inhibitory concentration(in microgram/ml)					
	Candida Albicans	***Monococcus Purpureus***	***Aspergillus Niger***	***T.Rubrum***	***Aspergillus Fumigates***	***Aspergillus Parasites***
BTZP3a	25	6.25	25	12.5	12.5	3.12
BTZP3b	25	6.25	25	25	12.5	6.25
BTZP3c	12.5	6.25	25	25	12.5	6.25
BTZP3d	25	6.25	12.5	25	3.12	12.5
BTZP3e	12.5	6.25	12.5	25	3.12	12.5
BTZP3f	6.25	6.25	6.25	25	12.5	12.5
BTZP3g	3.12	3.12	6.25	3.12	6.25	6.25
BTZP3h	12.5	3.12	6.25	3.12	6.25	3.12
BTZP3i	25	3.12	12.5	12.5	12.5	6.25
BTZP3j	12.5	6.25	6.25	3.12	12.5	6.25
BTZP3k	3.12	3.12	12.5	25	12.5	12.5
BTZP3l	12.5	3.12	12.5	25	12.5	12.5
Clo-trimazole	1.56	1.56	1.56	1.56	1.56	1.56

BTZP3a-l = Synthesized compounds in the concentration of 100-1.56 µg/ ml.
Solvent = DMSO.
Media = **Fungi : Sabourand's Dextrose Broth.**

Figure 15. Minimum inhibitory concentration of synthesized compounds (gram positive strains) by serial dilution method

Figure 16. Minimum inhibitory concentration of synthesized compounds (gram negative strains) by serial dilution method

Figure 17. Minimum inhibitory concentration of synthesized compounds (fungal strains) by serial dilution method

6.2. ANTITUBERCULAR SCREENING

The slow growth of *Mycobacterium tuberculosis*, *M. avium*, and related species and longer incubation times limits the use of drug susceptibility assays which rely on the development of colonies or turbidity for antitubercular screening. Susceptibility can be determined more rapidly with the radiometric BACTEC 460 system, the clinical drug susceptibility system of choice for most of the last decade, but it has the disadvantages of high cost, high volume, lack of high-throughput format, and requirement for radioisotope disposal.

6.2.1. RESAZURIN MICROPLATE ASSAY

Principle

Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue dye, itself nonfluorescent until it is reduced to the pink colored and highly red fluorescent resorufin by oxidoreductases within viable cells. It is used mainly as an oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells, the principle being that resazurin does not fluoresce when exposed to green light while resorufin fluoresces.

Procedure

The anti-TB activity of the compounds was tested by resazurin microplate assay. *M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Difco BBL, Sparks, MD, USA) supplemented with 10 % OADC (Becton Dickinson, Sparks, MD, USA) and 0.5% glycerol. The optical density of the bacterial culture was adjusted to McFarland 1.0 unit and 50 µl from this suspension was used as the inoculums. Stock solutions of the test compounds were prepared in dimethyl formamide (DMF) and were added to fresh medium in the wells of a 96-well microplate to which 50 µl inoculum was added making the total assay volume 200 µl. The final concentrations of the test molecules were 1, 10 and 100 µg/ml. Growth control wells contained medium and *M. tuberculosis* H37Rv alone. Rifampicin (1.0 µg/ml) served as positive control for inhibition of growth. Negative control wells contained the highest volume of DMF used in test wells without any compound. After incubation at 37ºC for 7 days, 15 µl of 0.01% resazurin (Sigma, St. Louis. MO, USA) solution in sterile water was added to the first growth control wells and incubated for 24 h. Once the first set of growth controls turned pink, the dye solution was added to the second set of growth controls and the test wells, and incubated for 24 h at 37ºC. Blue color in the wells

containing the test compounds indicated inhibition of growth and pink indicated lack of inhibition of growth of *M. tuberculosis*. [152] The details are tabulated in **Table14** and **Figure 18**.

IN VITRO ANTITUBERCULAR STUDIES

Table 14. *Invitro* antitubercular screening by REMA method

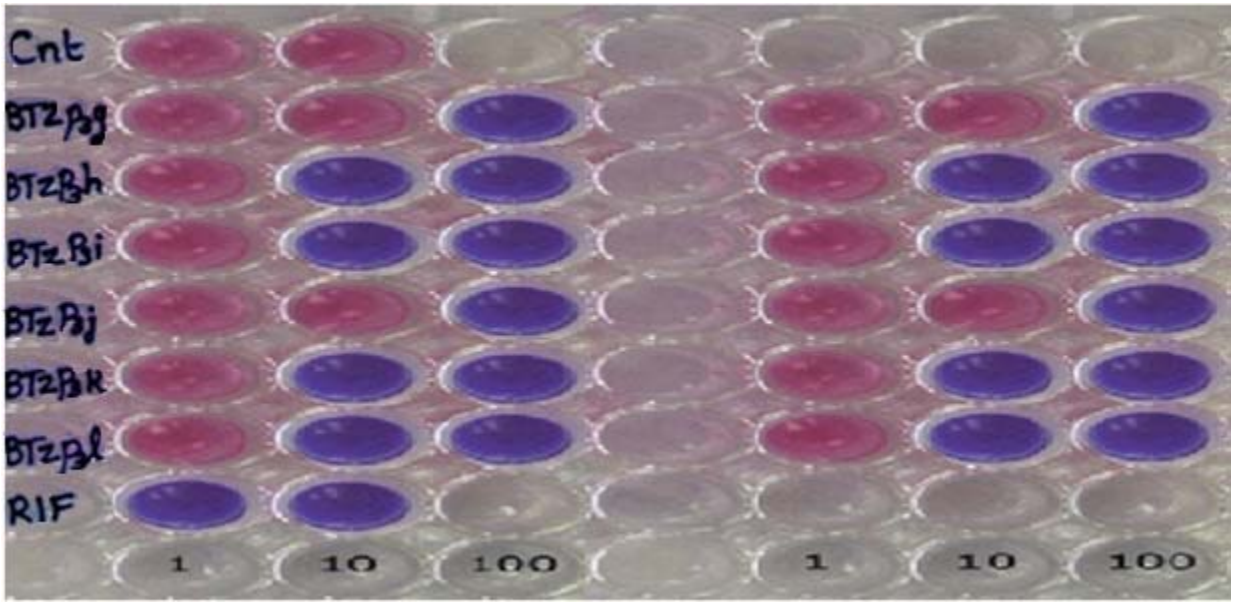
Compound code	Concentration (µg /ml)		
	1	**10**	**100**
BTZP3g	N	N	P
BTZP3h	N	P	P
BTZP3i	N	P	P
BTZP3j	N	N	P
BTZP3k	N	P	P
BTZP3l	N	P	P
Control	N	N	
Rifampicin	P	P	

N = No inhibition
P = Inhibition

Reagents used

Reagents : Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide.
Concentration : 1, 10 and 100 µg/ml.
Standard : Rifampicin
Media : Middlebrook 7H9 broth.
Solvent : DMF.

Figure 18. *Invitro* antitubercular screening by REMA



6.3. *IN VITRO* ANTIOXIDANT STUDIES

An antioxidant is the one capable of slowing or preventing the oxidation where it protects the cell damage by reactive oxygen species that are produced during redox reactions in the cell. Antioxidants found in the body can be small molecules such as glutathione, vitamins, or macromolecules such as catalase, glutathione peroxidase. As oxidative stress contributes to the development of many diseases including Alzheimer's disease, Parkinson's disease, diabetes, rheumatoid arthritis and neurodegeneration, the use of antioxidants in pharmacology is intensively studied. The antioxidant assays can roughly be classified into two types:

- **Assays based on hydrogen atom transfer (HAT) reactions**

The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. Some examples of such assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays.

- **Assays based on electron transfer (ET)**

These assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The sample's antioxidant concentration is correlated with the degree of its color change. Examples of such assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), "total antioxidant potential" assay using a Cu(II) complex as an oxidant and DPPH radical scavenging assay.In addition, there are also other assays intended to measure a sample's scavenging capacity of biologically relevant oxidants such as singlet oxygen, superoxide anion, peroxynitrite etc.[153] In-vitro Anti-oxidant screening of synthesized compounds were done by using three methods

- ❖ DPPH method
- ❖ FRAP method and
- ❖ ABTS method

6.3.1. DPPH RADICAL SCAVENGING METHOD

The free radical scavenging activity of a compound determined by this method is based on a reaction between a chomogen compound and antioxidant, and the concentration of the chomogen which is unreacted determined spectrophotometrically or colorimetrically. Most often used chomogen compound is DPPH (2, 2-Diphenyl-1-Picryl-hydrazyl).

Principle

DPPH molecule is characterized as a stable free radical by virtue of delocalization of the unpaired electron which gives rise to the deep violet colour, characterized by an absorption at about 518 nm.When solution of DPPH mixed with that of a substance that can donate a hydrogen atom (ie an antioxidant) it is converted to its reduced form with the loss of this violet color to pale yellow color. The primary reaction involved in this assay is given below

$$\mathbf{Z^{\cdot} + AH = ZH + A^{\cdot}}$$

Where,

$Z^{\cdot}$ = DPPH radical

AH = Donor molecule

ZH = Reduced form

$A^{\cdot}$ = Free radical

The result of the antioxidant efficiency is expressed as EC_{50}, determined as the concentration of substrate that causes 50% loss in absorbance.

Reagents used

Radical : DDPH

Solvent : Ethanol

Standard : Ascorbic acid

Preparation of 0.3 mM DPPH solution:

It was prepared by dissolving DPPH (5.91 mg) in 50 ml of ethanol. This stock solution was prepared freshly and kept in the dark at ambient temperature when not used.

Preparation of sample stock solution:

The sample stock solution was prepared by dissolving the compound in suitable solvent (ethanol) with a final concentration of 1 mg/ml.

Preparation of standard stock solution:

The standard stock solution was prepared by dissolving the Ascorbic acid in suitable solvent (ethanol) with a final concentration of 1 mg/ml.

Procedure:

The effect of compound on DPPH radical was assayed using this method. Sample stock solutions (1.0 mg/ml) were diluted to appropriate final concentrations in ethanol. An ethanolic solution of 1 ml of DPPH (0.3 mM) was added to 0.5 ml of the compound and allowed to react at room temperature in a dark place for 30 minutes. After 30 minutes the absorbance values were measured at 518 nm. All the measurements were taken as a triplicate values. From the average of the absorbance values, lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH radical scavenging capability was calculated using the following equation:

$$\textbf{\% inhibition} = (ABS_{control} - ABS_{test}) / ABS_{control} \times 100$$

Where,

$ABS_{Control}$ =Absorbance of ethanol+DPPH

ABS_{Test} =Absorbance of DPPH + Compound /Standard

The percentage antioxidant activity (% inhibition) was extrapolated against concentration of the compound and EC_{50} was determined graphically [154]. The results are tabulated in **Table15 and Figure 19.**

6.3.2. FRAP RADICAL SCAVENGING METHOD

FRAP (Ferric Reducing Ability of Plasma) is one of the most rapid test and very useful for routine analysis. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2, 4, 6 – tri (2 – pyridyl) – s – triazine) and $FeCl_3 6H_2O$.

Principle

The Ferric Reducing/Antioxidant Power assay measures the ability of antioxidants to reduce the complex of Fe(III)- 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta- 1,4-diene chloride (TPTZ) to intense blue Fe(II) TPTZ complex at optimum pH (3.6). The reduction is quantitated by change in absorption at 593 nm.

Reagents used

Radical : FRAP

Solvent : Ethanol

Standard: Ferrous sulphate

Preparation of FRAP reagent

FRAP reagent was prepared by mixing10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM $FeCl_3$. $6H_2O$ solution.

Preparation of test solution

10 mg of each of the drug samples were accurately weighed separately and dissolved in 1ml of ethanol. These solutions were serially diluted with ethanol to obtain the lower dilutions**.**

Procedure

FRAP assay was carried out 0.2 ml of the compound is added to 3.8 ml of FRAP reagent and the reaction mixture was incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. $FeSO_4$ is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as Mmol $FeSO_4$ equivalents per gram of sample [155]. The results are tabulated in **Table16 and Figure 20.**

Percentage inhibition= [1− (absorbance of test/absorbance of control)] × 100

6.3.3. ABTS RADICAL SCAVENGING METHOD

The ABTS assay is based on the inhibition of the absorbance of radical cation, $ABTS^+$, which has a characteristic wavelength at 745 nm, by antioxidants. In this assay, the ABTS radical which is a blue green chromogen, is generated in a stable form using potassium persulphate. The formed colored radical is then mixed with antioxidant in the reaction medium and the colored radical is converted back to colorless ABTS. Most often used chomogen compound is ABTS [2, 2'azino-bis (3-ethyl benzthiazoline-6-sulphonic acid)].

Principle

A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, $ABTS^{-+}$, that is green in color. Antioxidants supress this reaction by

electron donation radical scavenging and inhibit the formation of the colored ABTS radical. Antioxidants inhibit the oxidation of ABTS by electron transfer radical scavenging.

$$\text{metmyoglobin} + H_2O_2 \longrightarrow {}^{\cdot}\text{ferryl myoglobin} + H_2O$$

$$\text{ABTS} + {}^{\cdot}\text{ferryl myoglobin} \longrightarrow ABTS^{-+} + \text{metmyoglobin}$$

Reagents used

Radical : ABTS

Solvent : Ethanol

Standard : Ascorbic acid

Preparation of ABTS solution

ABTS was dissolved in de-ionized water to 7 mM concentration, and potassium persulphate added to a concentration of 2.45 Mm**.** The reaction mixture was left to stand at room temperature overnight in dark before use.

Preparation of test solution

10 mg of each of the drug samples were accurately weighed separately and dissolved in 1 ml of ethanol. These solutions were serially diluted with ethanol to obtain the lower dilutions**.**

Preparation of standard solution

The standard stock solution was prepared by dissolving the Ascorbic acid in suitable solvent (1 ml of ethanol) and serially diluted with ethanol to obtain the lower dilutions.

Procedure

The ABTS + assay was carried out using the improved assay of 1 ml of distilled ethanol was added to 0.2 ml of various concentrations of the drug samples or standard, and 0.16 ml of ABTS solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 745 nm. Blank was maintained without ABTS. The assay was performed at least in triplicates.IC_{50} value obtained is the concentration of the sample required to inhibit 50 % ABTS radical mono cation. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition} = (ABS_{control} - ABS_{test}) / ABS_{control} \times 100$$

Where,

$ABS_{Control}$ =Absorbance of ethanol+ABTS

ABS_{Test} =Absorbance of ABTS + Compound /Standard

The percentage antioxidant activity (% inhibition) was extrapolated against concentration of the compound and EC_{50} was determined graphically [156]. The results are tabulated in **Table 17 and Figure 21.**

ANTIOXIDANT STUDIES

Table 15. DPPH radical scavenging activity of synthesized compounds

Compound code	Concentration (µg /ml)	% Inhibition	EC_{50} Value
Standard (Ascorbic acid)	25	48.41	28
	50	71.82	
	75	81.97	
	100	91.07	
BTZP3a	25	11.39	87
	50	24.21	
	75	39.56	
	100	70.67	
BTZP3b	25	23.75	66
	50	37.76	
	75	59.92	
	100	74.76	
BTZP3c	25	14.16	75
	50	32.51	
	75	50.18	
	100	90.25	
BTZP3d	25	11.93	96
	50	28.42	
	75	38.72	
	100	55.07	
BTZP3e	25	36.50	47
	50	51.44	
	75	63.07	
	100	85.99	
BTZP3f	25	31.97	53
	50	48.42	
	75	65.08	
	100	91.09	

BTZP3g	25	41.72	32
	50	69.37	
	75	87.75	
	100	91.19	
BTZP3h	25	35.04	44
	50	56.74	
	75	76.13	
	100	95.03	
BTZP3i	25	29.75	56
	50	46.19	
	75	61.97	
	100	90.83	
BTZP3j	25	15.71	66
	50	37.46	
	75	56.98	
	100	83.30	
BTZP3k	25	13.71	73
	50	24.91	
	75	46.36	
	100	89.93	
BTZP3l	25	12.55	82
	50	25.27	
	75	43.63	
	100	64.84	

Reagents used

Reagents : DPPH
Solvent : DMSO
Standard : Ascorbic acid
Absorbance : 518nm

Table 16. FRAP radical scavenging activity of synthesized compounds

Compound code	Concentration (µg /ml)	Absorption	R^2
Standard ($FeSO_4.7H_2O$)	25	1.0020	0.972
	50	1.1843	
	75	1.2743	
	100	1.3784	
BTZP3a	25	0.5506	0.961
	50	0.6780	
	75	0.7896	
	100	0.8342	
BTZP3b	25	0.5519	0.980
	50	0.5612	
	75	0.5789	
	100	0.5876	
BTZP3c	25	0.9612	0.989
	50	0.9636	
	75	0.9658	
	100	0.9673	
BTZP3d	25	0.1123	0.994
	50	0.1387	
	75	0.1576	
	100	0.1783	
BTZP3e	25	0.0023	0.998
	50	0.0046	
	75	0.0065	
	100	0.0087	
BTZP3f	25	0.6011	0.659
	50	0.6056	
	75	0.6089	
	100	0.7089	
BTZP3g	25	0.2022	0.992
	50	0.2045	
	75	0.2061	
	100	0.2087	
BTZP3h	25	0.0012	0.999
	50	0.0035	
	75	0.0056	
	100	0.0078	
BTZP3i	25	0.0020	0.980
	50	0.0037	
	75	0.0049	
	100	0.0058	

BTZP3j	25	0.3313	0.983
	50	0.3338	
	75	0.3353	
	100	0.3384	
BTZP3k	25	0.7432	0.978
	50	0.8990	
	75	0.9848	
	100	1.0775	
BTZP3l	25	0.0198	0.967
	50	0.0347	
	75	0.0518	
	100	0.0827	

Reagents used

Reagents : FRAP
Solvent : DMSO
Standard : $FeSO_4.7H_2O$
Absorbance : 593nm

Table 17. ABTS radical scavenging activity of synthesized compounds

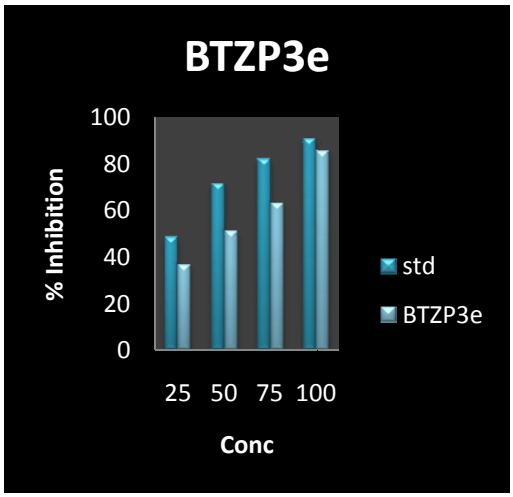
Compound code	Concentration (µg /ml)	% Inhibition	EC_{50} Value
Standard (Ascorbic acid)	25	47.72	26
	50	59.43	
	75	74.08	
	100	82.31	
BTZP3a	25	38.73	70
	50	38.86	
	75	46.35	
	100	51.21	
BTZP3b	25	48.05	32
	50	59.71	
	75	72.49	
	100	81.65	
BTZP3c	25	39.35	54
	50	49.38	
	75	56.99	
	100	58.41	

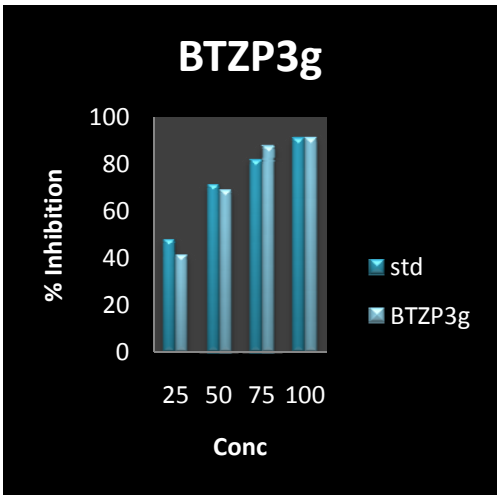
BTZP3d	25	42.33	38
	50	54.55	
	75	61.27	
	100	71.30	
BTZP3e	25	14.45	81
	50	38.79	
	75	49.70	
	100	56.55	
BTZP3f	25	49.39	30
	50	62.67	
	75	74.31	
	100	87.98	
BTZP3g	25	26.09	59
	50	45.35	
	75	62.79	
	100	70.37	
BTZP3h	25	43.16	37
	50	55.67	
	75	63.76	
	100	76.26	
BTZP3i	25	12.96	71
	50	38.87	
	75	51.70	
	100	57.23	
BTZP3j	25	14.43	85
	50	32.68	
	75	46.15	
	100	59.08	
BTZP3k	25	37.02	43
	50	56.60	
	75	74.43	
	100	87.77	
BTZP3l	25	49.19	34
	50	51.11	
	75	58.13	
	100	64.51	

Reagents used

Reagents : ABTS
Solvent : DMSO
Standard : Ascorbic acid
Absorbance : 745nm

Figure 19. DPPH radical scavenging activity of synthesized compounds





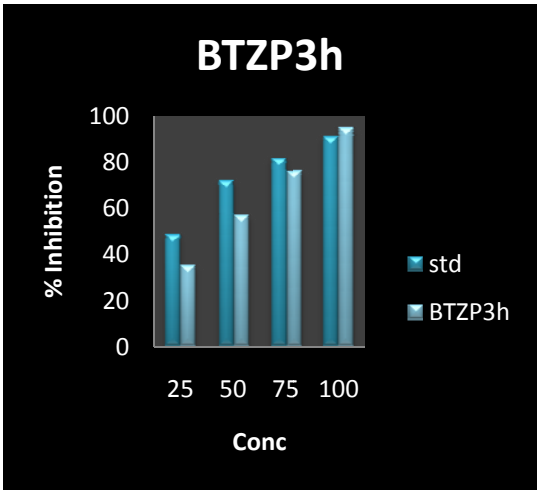
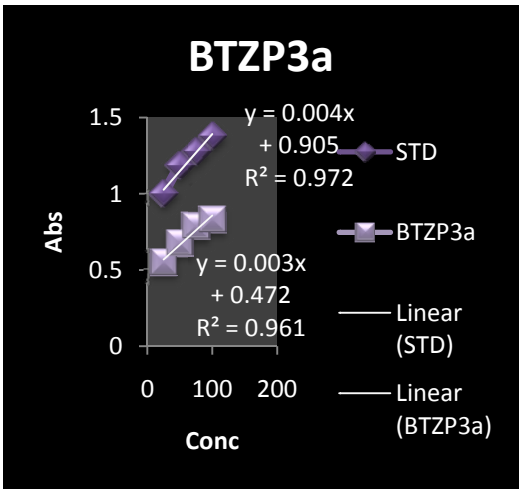
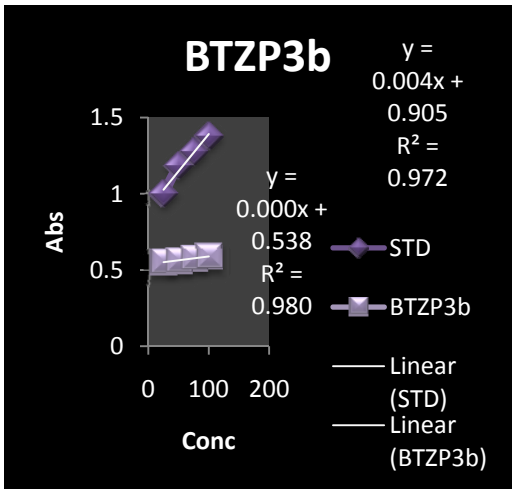
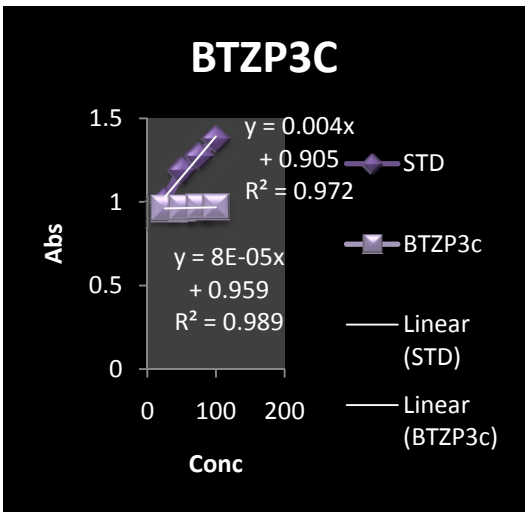
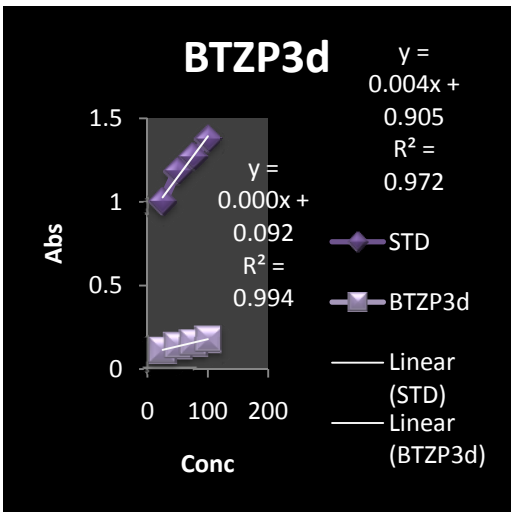


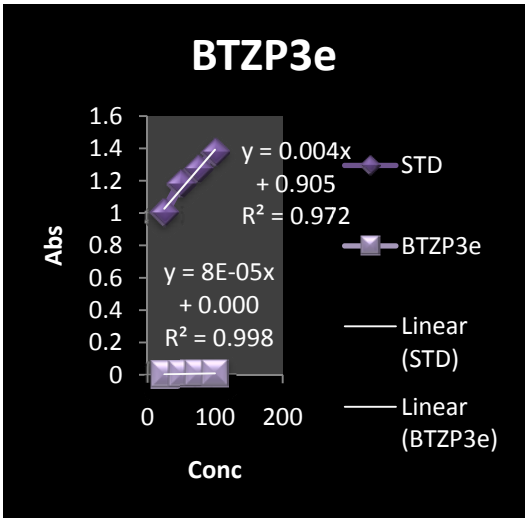
Figure 20. FRAB radical scavenging activity of synthesized compounds

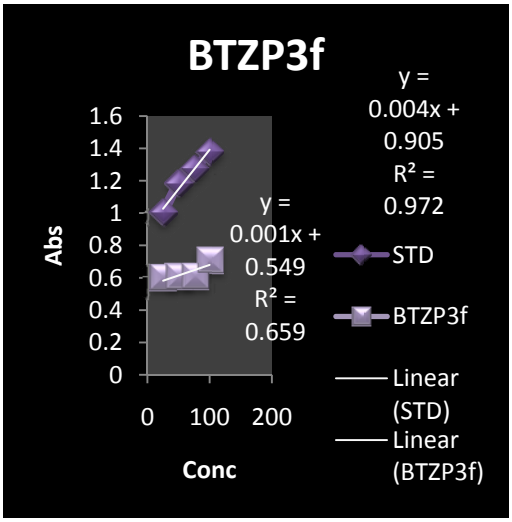


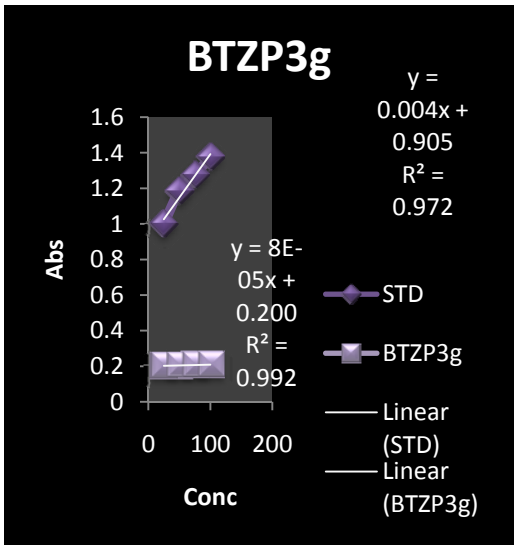








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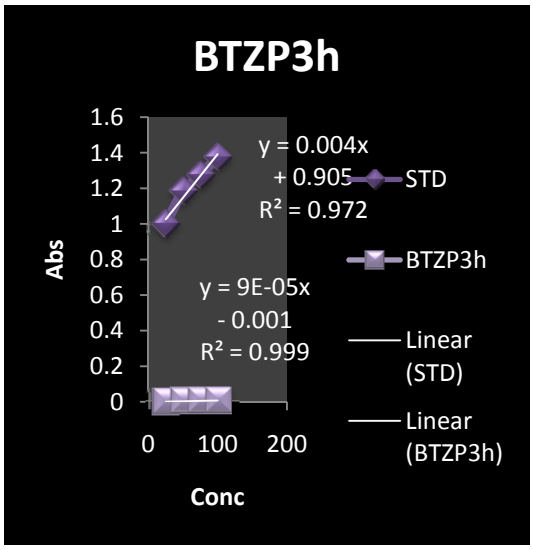
R² = 0.972
y = 8E-05x + 0.200
R² = 0.992
STD
BTZP3g
Linear (STD)
Linear (BTZP3g)
Abs
Conc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200


BTZP3h
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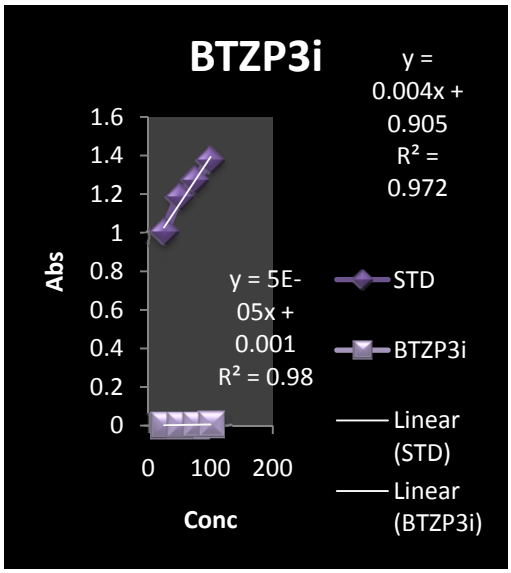
R² = 0.972
y = 9E-05x - 0.001
R² = 0.999
STD
BTZP3h
Linear (STD)
Linear (BTZP3h)
Abs
Conc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200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R² = 0.972
y = 5E-05x + 0.001
R² = 0.98
STD
BTZP3i
Linear (STD)
Linear (BTZP3i)
Abs
Conc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
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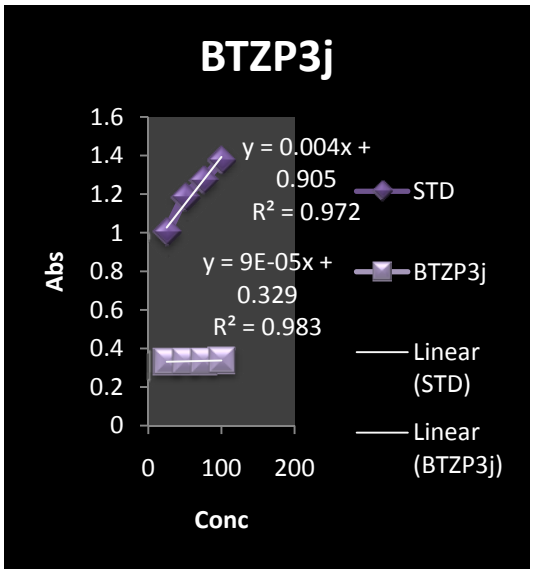
BTZP3j
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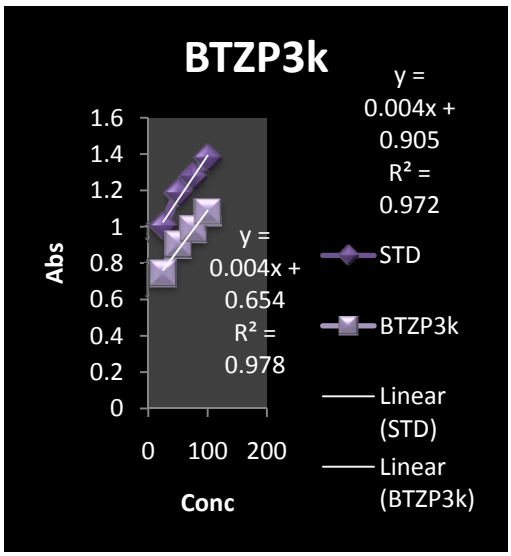
R² = 0.972
y = 9E-05x + 0.329
R² = 0.983
STD
BTZP3j
Linear (STD)
Linear (BTZP3j)
Abs
Conc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200


BTZP3k
y = 0.004x + 0.905
R² = 0.972
y = 0.004x + 0.654
R² = 0.978
STD
BTZP3k
Linear (STD)
Linear (BTZP3k)
Abs
Conc
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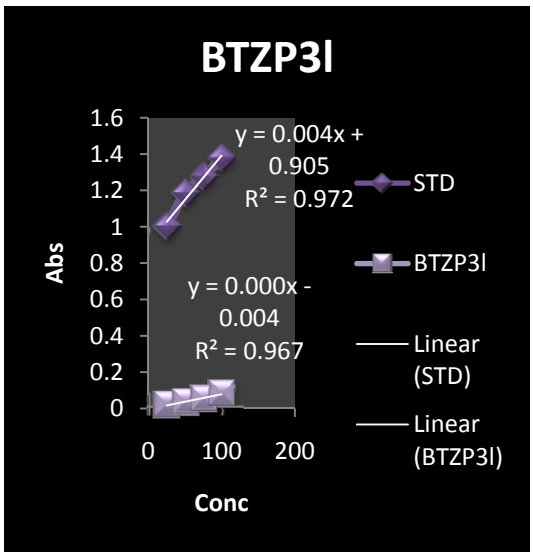
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200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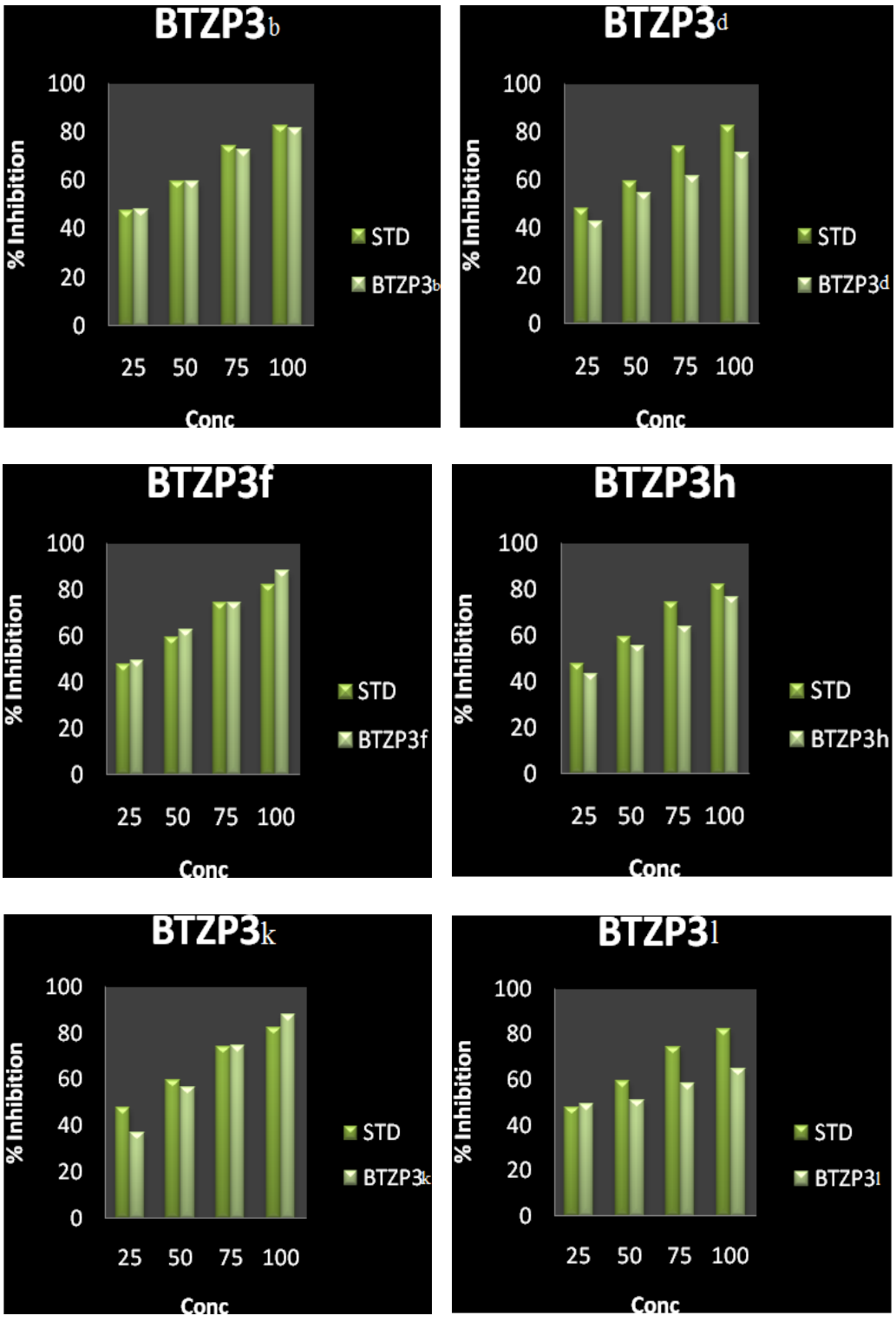
R² = 0.972
y = 0.000x - 0.004
R² = 0.967
STD
BTZP3l
Linear (STD)
Linear (BTZP3l)
Abs
Conc
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200

Figure 21. ABTS radical scavenging activity of synthesized compounds



6.4. *IN VITRO* CYTOTOXIC SCREENING

Several approaches have been used in the past for the measurement of cell viability and growth. Though trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death), the method is not sensitive and cannot be adapted for high-throughput screening. The uptake of radioactive substances, usually tritium-labeled thymidine, can be measured. This method is accurate but is also time-consuming and involves handling of radioactive substances. [157]

6.4.1. MTT ASSAY

Principle

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan by mitochondrial succinate dehydrogenase in living cells. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The insoluble purple formazan product is converted to a colored solution using a solubilization agent (usually either DMSO, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid). The absorbance of this colored solution is then quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. Since reduction of MTT can only occur in metabolically active cells, this assay gives a direct measure of the viability of cells.

Procedure

The mouse embryonic fibroblasts cell line (NIH 3T3) was obtained from National Centre for Cell Science (NCCS), Pune. NIH 3T3 cell line was grown in Dulbeccos modified Eagles medium containing 10% fetal bovine serum (FBS).

For screening experiment, the cells were seeded into 96-well plates in 100 µl of medium containing 5% FBS, at plating density of 10,000 cells/well and incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of samples. The samples were solubilized in Dimethylsulfoxide and diluted in serum free medium. After 24 h, 100 µl of the medium containing the samples at various concentration (eg: 0.01, 0.1, 1, 10, and 100 µM)

was added and incubated at 37^0C, 5% CO2, 95% air and 100% relative humidity for 48 h. Triplicate was maintained and the medium containing without extracts were served as control. After 48h, 15 µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100

Non linear regression graph was plotted between percentage cell inhibition and Log_{10} concentration and IC_{50} was determined using graph pad prism software [158] .The results are tabulated in **Table18, 19 and Figure 22**.

IN VITRO CYTOTOXIC STUDIES

Table 18. Absorbance of various concentrations of samples

Compound code	Conc(uM)	0.01	0.1	1	10	100	Cont
BTZP3b	ABS	0.141	0.142	0.133	0.114	0.002	0.145
		0.15	0.141	0.135	0.118	0.008	0.143
		0.144	0.146	0.139	0.113	0.005	0.147
	Avg	0.145	0.143	0.135	0.115	0.005	0.146
BTZP3e	ABS	0.148	0.14	0.136	0.12	0	0.145
		0.142	0.139	0.141	0.126	0.002	0.148
		0.144	0.133	0.138	0.127	0.004	0.147
	Avg	0.144	0.137	0.138	0.124	0.002	0.146
BTZP3f	ABS	0.145	0.144	0.138	0.135	0.04	0.145
		0.142	0.145	0.141	0.136	0.038	0.148
		0.148	0.143	0.14	0.131	0.033	0.147
	AVS	0.145	0.144	0.139	0.134	0.037	0.146
BTZP3g	ABS	0.64	0.618	0.238	0.036	0	0.661
		0.649	0.616	0.245	0.031	0	0.635
		0.622	0.6	0.231	0.036	0	0.594
	AVS	0.637	0.611	0.238	0.034	0	0.63
BTZP3i	ABS	0.145	0.142	0.141	0.133	0.102	0.145
		0.148	0.146	0.144	0.136	0.11	0.148
		0.146	0.146	0.14	0.138	0.09	0.147
	Avg	0.146	0.144	0.141	0.135	0.103	0.146
BTZP3l	ABS	0.31	0.22	0.045	0.035	0.003	0.285
		0.308	0.225	0.057	0.034	0.004	0.286
		0.319	0.215	0.059	0.028	0.004	0.273
	Avg	0.312	0.22	0.053	0.032	0.003	0.281

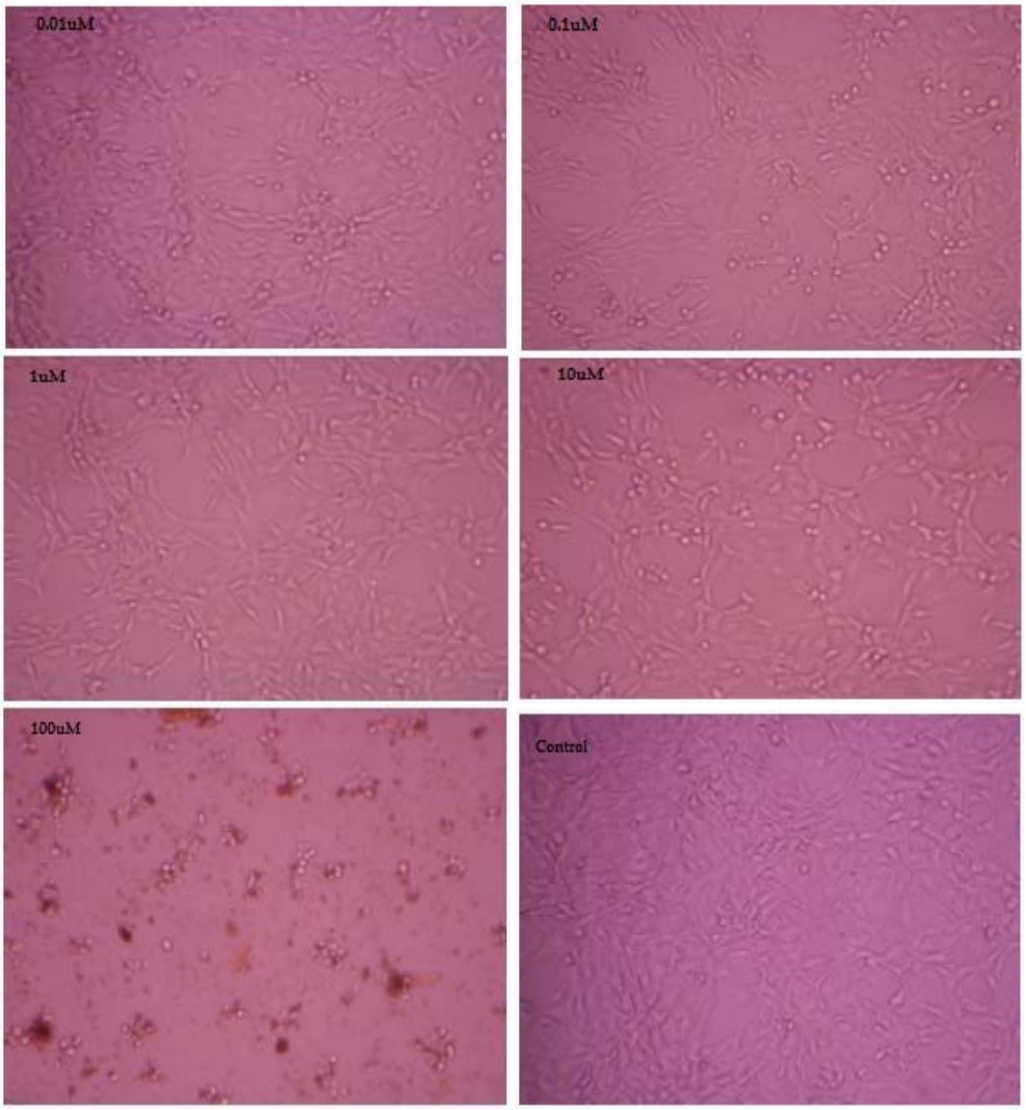
Table 19. Percentage cell inhibition produced by synthesized compounds at varying concentrations

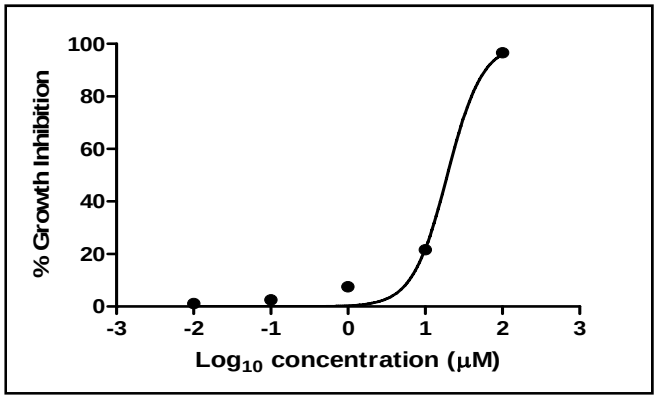
Compound code	Conc (µM)	% Cell Inhibition	IC_{50} (µM)
BTZP3b	0.01	1.13	19.34
	0.1	2.5	
	1	7.5	
	10	21.59	
	100	96.59	
BTZP3e	0.01	1.36	19.6
	0.1	6.36	
	1	5.68	
	10	15.22	
	100	98.68	
BTZP3f	0.01	1.13	48
	0.1	1.81	
	1	4.77	
	10	8.63	
	100	74.77	

BTZP3g	0.01	-1.11	22.78
	0.1	2.96	
	1	62.22	
	10	94.55	
	100	100	
BTZP3i	0.01	0.22	>100
	0.1	1.36	
	1	3.40	
	10	7.5	
	100	29.77	
BTZP3l	0.01	-11.01	17.6
	0.1	21.80	
	1	80.92	
	10	88.50	
	100	96.69	

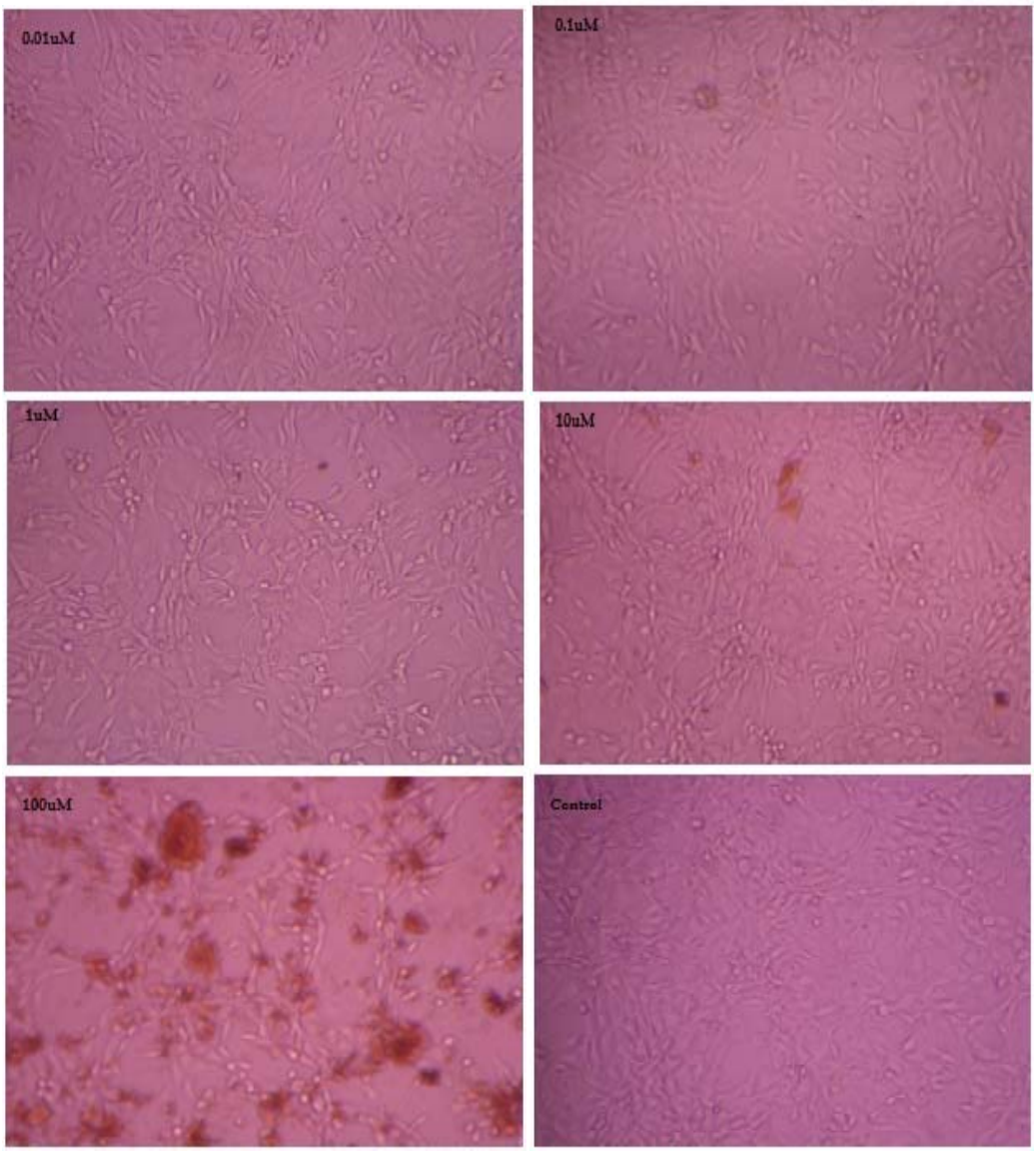
Figure 22. ***Invitro*** **cytotoxic screening by MTT assay**

BTZP3b





BTZP3f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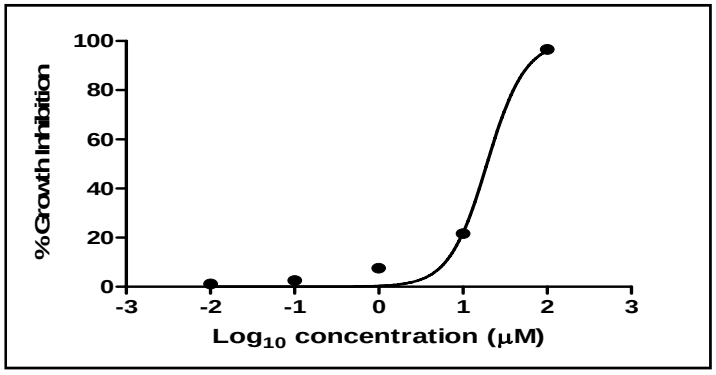
0.1uM
1uM
10uM
100uM
Control


100
80
60
40
20
0
-3
-2
-1
0
1
2
3
Log10 concentration (μM)

Chapter 7

Results and Discussion

7. RESULTS AND DISCUSSION

7.1. CHEMISTRY

In the present work 12 different schiff bases were synthesised in 4 steps.

Step: 1

4-Nitro-1, 3-benzothiazol-2-amine (BTZ) has been synthesized by precooled mixture of 2-nitroaniline, ammonium thiocyanate and glacial acetic acid with bromine were stirred. The resulting mixture was neutralized with aqueous ammonia solution to form BTZ.

Step: 2

Ethyl cyano[2-(4-Nitro-1,3-benzothiazol-2-yl)hyrazinylidene]acetate (BTZE) was synthesized by diazotization of 4-Nitro-1,3-benzothiazol-2-amine (BTZ) with sodium nitrite in concentrated hydrochloric acid and the resulting diazonium salt was coupling with ethylcyanoacetate in presence of sodium acetate using ethanol as solvent.

Step: 3

The 5-amino-4-[2-(4-nitro-1, 3-benzothiazol-2 yl) hydrazinylidene]-2, 4-dihydro-3H -pyrazol -3-one (BTZP) was synthesized by cyclising ethyl cyano [2-(4-Nitro-1, 3-benzothiazol-2-yl) hyrazinylidene] acetate (BTZE) with hydrazine hydrate using ethanol as solvent.

Step: 4

The title compounds (Schiff base of 5-amino-4-[2-(4-nitro-1,3-benzothiazol-2 yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one) were synthesized by refluxing 5-amino-4-[2-(4-nitro-1,3-benzothiazol-2 yl)hydrazinylidene]-2,4-dihydro-3H -pyrazol -3-one (BTZP) with 12 different aromatic aldehydes in presence of acetic acid using ethanol as solvent. The percentage yield of the synthesized were found to be in the range of 70- 95%. Purity of all the newly compounds were checked by melting point and TLC Analysis and the structures were confirmed by UV, IR, ^{1}HNMR and MASS spectral data were given in Table : 2,3,4&5.

MOLECULAR DOCKING STUDY

Docking study was performed by using ligand structure preparation by using build module then preparation of ligand in 3D structure form and the preparation of protein by removing unwanted molecule for using molecular modelling calculation than generated a receptor grid, further legend was docked using Autodock.

Docking studies of designed and synthesized compounds (BTZP3a-l) were carried out using Autodock module version 4.0.The pose score of synthesized compounds benzothiazole

containing pyrazolone derivatives were calculated on one tubercular protein, Thymidylate Kinase (PBD ID is 1MRS).

Docking calculation revealed that among all the investigated compounds a similar trend in binding mode was observed.Synthesized compounds BTZP3h, BTZP3k, BTZP3l was shown H-bond interaction with Arg160:HH111,Arg160:HH211.Tyr34:HN-1,Arg160:HH11-1,gln41:HE221, Val8:HN-1, LYS13:H32-1 of active site of thymidylate kinase (1MRS) inhibitor. The molecular docking study was tabulated in Table: 1and Fig: 1.

7.2. DETERMINATION OF PHYSICOCHEMICAL PROPERTIES OF SYNTHESIZED COMPOUNDS

Melting point analysis

Melting points of all the newly synthesised compounds were checked and uncorrected and the values were given in Table: 2&3.

Thin layer chromatography analysis

The reaction time for all the compounds were determined by running TLC and a single spot was obtained. R_f Values of all the newly synthesised compounds were checked and were tabulated in Table: 2&3.

Solubility

Solubility was checked for all the newly synthesised compounds and found that all the compounds were soluble in non polar solvents (Chloroform, Benzene) and were tabulated in Table: 2&3.

Polarizability

Polarizability of all the newly synthesised compounds was calculated by using marvin sketch software and the values were tabulated in Table: 2&3.

Log P

LogP Values of all the newly synthesised compounds were calculated by using marvin sketch software and were tabulated in Table: 2&3.

7.3. CHARACTERIZATION

Ultraviolet and visible spectroscopy

The structures of intermediates and all the newly synthesised compounds were determined and showed absorbance between 400-700nm and were tabulated in Table: 4&5 and Fig: 2&3.

Infrared spectral analysis

The structures of intermediates, compound BTZ confirmed by the presence of characteristic peaks in the region 700 cm^{-1},1629.55 cm^{-1}, 3132.79 cm^{-1}, 1348.32 cm^{-1} and 2936 cm^{-1}, 1502.28 cm^{-1} and 745.35 cm^{-1} associated for –C-S-C-, -C=N, -NH_2, N=O and Ar stretching respectively. The compound BTZE showed the disappearance of the characteristic bands of the -NH_2 stretching and also shows the stretching for cyanide group, ester group in the region 2360.44 cm^{-1}, 1786.44 cm^{-1} an respectively. The compound BTZP confirmed the presence of -C-N, -C=O, -C=N, -NH stretching, by the peaks at 1401.03 cm^{-1}, 1715.62 cm^{-1}, 1618.95 cm^{-1}, 3132.79 cm^{-1}, reappearance of the characteristic bands of the -NH_2 stretching in the region 3343.05 cm^{-1} respectively and also showed the disappearance of the characteristic bands of the cyanide group and ester group.

All the Schiff base derivative compounds showed the characteristic peaks in the region 3400-3100 cm^{-1} for associated NH stretching, 3100-2850,1600-1500,840- 790 cm^{-1} for Ar stretching,700-600 cm^{-1} for C-S-C stretching, 1690-1640 cm^{-1} for C=N stretching,<1500 cm^{-1} for C-N stretching, 1550 cm^{-1} - 1500 cm^{-1} for NO_2 group and 1700-1590 $cm^{-1}$for C=O stretching. Compound BTZP3b containing – N $(CH_3)_2$ group showed absorption bands at 1321 cm^{-1}. Compound BTZP3c, BTZP3e containing C-O-C group showed absorption bands at 1070.3 cm^{-1}, 1081.62 cm^{-1}. Compound BTZP3f containing NO_2 group showed absorption bands at 1512.88 cm^{-1}, for the N=O stretching. The peak at 830.20 cm^{-1}, 821 cm^{-1}, 837.19 cm^{-1} could be assigned to C-Cl stretching in the compound BTZP3d, BTZP3h, BTZP3l. Presence of hydroxyl group was confirmed by the appearance of broad peak at 3430-3161 cm^{-1} in the compound BTZP3e, BTZP3g, BTZP3i, BTZP3j. Compound BTZP3k containing C-F group showed absorption bands at 1416 cm^{-1} and were tabulated in Table: 4&5 and Fig: 4&5.

Nuclear magnetic resonance spectral analysis

The structures of the newly synthesized compounds (BTZP, BTZP3a-l) were confirmed by ^{1}H-NMR spectra. The ^{1}H-NMR spectra of all the synthesized compounds showed the absence of the

peaks for the $-NH_2$ proton signal at δ 8.32 ppm. All the synthesized compounds showed multiplets in the range δ 6.32-8.59 for the protons of aromatic ring and a singlet at δ 6.15, 3.57-3.89, 7.4-8.64 which may be assigned to -NH-N proton,-NH proton of pyrazolone,-N=CH proton of Schiff base. The spectrum of BTZP3b, BTZP3c, revealed a singlet at δ 3.04, 3.35 which may be assigned to -N $(CH_3)_2$, OCH_3 proton. The spectra of compound BTZP3g showed singlet at δ 9.87 corresponding to OH group and were tabulated in Table: 4&5 and Fig: 6&7.

Mass spectral analysis

Electron impact mass spectral analysis was carried out on randomly selected compounds BTZP3b, BTZP3g and BTZP3k. Mass spectrums of the compounds were in full agreement with their molecular weights and were tabulated in Table: 4&5 and Fig:8.

7.4. BIOLOGICAL EVALUATION

***In vitro* antibacterial screening and bactericidal**

All the newly synthesised compounds (BTZP3a-l), at 100µg/disc concentration level were investigated for preliminary antibacterial activity against twelve bacterial strains (six gram positive and six gram negative) by the disc diffusion method, to determine the zone of inhibition. The results were compared with the standard drug (Ciprofloxacin) at 5µg/disc concentration level. All the newly synthesized compounds (BTZP3a-l) were screened minimum inhibitory concentration at 100-1.56µg/ml against all twelve bacterial strains (six gram positive and six gram negative) by serial dilution method followed by to determine the synthesized compounds are bactericidal / bacteriostatic agent. All the synthesized compounds are bactericidal in nature.

All the synthesised compounds (BTZP3a-l) were shown mild to moderate activity against all the screened microbacterial (gram positive) strains. Among the screened gram positive bacterial strains, all the synthesised compounds (BTZP3a-l) shown moderate activity against (gram positive bacteria) with the percentage of inhibition range from 73-86 % and the MIC range at 6.25 µg/ml concentration level. On the other hand, all the synthesized compounds (BTZP3a-l) showed mild activity against all the tested gram positive bacterial strains with the percentage of inhibition range 60-66% and the MIC range at 25 µg/ml concentration level.

All the synthesized compounds (BTZP3a-l) shown more potent or equal activity aganist *Rhodosporum ruberum, Salmonella paratypic and Klebsellia pneumonie* with the zone of inhibition range 80-175% and the MIC value at 3.12µg/ml concentration level. Among the synthesised compounds (BTZP3h & BTZP3j) shown moderate activity against *Pseudomonas aruginosa,* (gram negative bacteria) with the percentage of inhibition range at 85% and the MIC range at 12.5 µg/ml concentration level. On the other hand, all the synthesized compounds shown mild activity against *Escherichia coli, Viberio cholera* (gram negative strains) with the percentage of inhibition range 45- 62% and the MIC range at 25 µg/ml concentration level.

The preliminary antibacterial activity of synthesized compounds result shows better activity against gram negative bacteria than gram positive bacteria.

***In vitro* antifungal screening and fungicidal**

All the newly synthesised compounds (BTZP3a-l), at 100µg/disc concentration level were investigated for preliminary antifungal activity against six fungal strains by the disc diffusion method, to determine the zone of inhibition. The results were compared with the standard drug (Clotrimazole) at 5µg/disc concentration level. All the newly synthesised compounds (BTZP3a-l) showed MIC at 25-3.12 µg/ml concentration against all the screened fungal strains by serial dilution method and followed by determine whether the synthesized compounds are fungicidal / fungiostatic agents. Almost all newly synthesized compounds are fungicidal in nature.

All the synthesised compounds were shown moderate to significant activity against all the screened fungal strains. The compounds BTZP3g and BTZP3h showed more potent or equal activity against all the tested fungal strains at 100µg/disc concentration level compared with standard clotrimazole at 5µg/disc concentration level with the zone of inhibition range 90-127% percentage and the MIC value at 3.12 µg/ml concentration level.

***In vitro* antimycobacterial screening**

All the newly synthesised compounds were investigated for *In-vitro* anti mycobacterial activity by performing the resazurin microplate assay method**.** *In-vitro* antimycobacterial screening of six randomly selected samples using REMA revealed that, the compounds BTZP3g and BTZP3j possess antimycobacterial activity at a concentration of 100µg/ml. The compounds BTZP3h, BTZP3i, BTZP3k, and BTZP3l possess antimycobacterial activity at a concentration of 10 µg/ml respectively.

***In vitro* antioxidant screening**

All the newly synthesised compounds were investigated for *in-vitro* antioxidant activity by DPPH, FRAP and ABTS assay methods**.** The ascorbic acid was used as standard reference for DPPH and ABTS assay methods. The ferrous sulphate was used as standard reference for FRAP assay methods. In vitro antioxidant DPPH assay method result showed that the compounds BTZP3g, BTZP3h and BTZP3e, were appreciable DPPH radical scavenging activity with EC_{50} values of 47, 32 and 44 µg/ml respectively. In vitro antioxidant ABTS assay method result shown that the compounds BTZP3b, BTZP3d, BTZP3f, BTZP3h, BTZP3k, BTZP3l were appreciable ABTS radical scavenging activity with EC_{50} values of 32,38,30,37,43,34 µg/ml respectively. In FRAP assay method all the synthesized compounds showed appreciable FRAP radical scavenging activity compared with standard except the compound (BTZP3f).

***In vitro* cytotoxic screening**

The *in-vitro* cytotoxic studies were performed on randomly selected compounds (BTZP3b, BTZP3e, BTZP3f, BTZP3g, BTZP3i and BTZP3l) using MTT assay against normal cell line. The percentage cell Inhibition were calculated by using formula, 100- Abs (sample)/Abs (control) x100. Non linear regression graph was plotted between percentage cell inhibition and Log_{10} concentration and IC_{50} was determined using graph pad prism software. *In-vitro* cytotoxic MTT assay method results indicated that, the compounds BTZP3f and BTZP3i show less cytotoxic activity against normal cell line with IC_{50} value of 48µM and >100 µM respectively. Other compounds (BTZP3b, BTZP3e, BTZP3g, and BTZP3l) show IC_{50} value of 19.34, 19.6, 22.78, and 17.6 µM respectively.

Chapter 8

Summary & Conclusion

8. SUMMARY AND CONCLUSION

8.1. CHEMISTRY

In the present work totally twelve 5-amino substituted -4-[2-(4-nitro-1, 3-benzothiazol-2 yl) hydrazinylidene]-2, 4-dihydro-3H -pyrazol -3-one (BTZP3a-l) compounds were synthesised. The compound 4-nitro-1, 3-benzothiazol-2-amine (BTZ) was synthesized from 2-nitroaniline, ammonium thiocyanate and glacial acetic acid with bromine. Ethyl cyano [2-(4-Nitro-1, 3-benzothiazol-2-yl)hyrazinylidene]acetate (BTZE) was prepared by coupling through diazonium salts of 4-nitro-1,3-benzothiazol-2-amine (BTZ) with ethylcyanoacetate. BTZE which was cyclised with hydrazine hydrate and the resulting product (BTZP) was subjected to schiff base reaction with 12 different aldehydes. The yield was found to be 70-95% in all the stage.

The final compounds were found to be soluble in non polar solvents. Thin layer chromatography was used to find out the reaction time to completion the reaction and purity of the synthesized compounds. Melting points were taken in open glass capillary tubes and were uncorrected. The UV and IR spectra of synthesized compounds appeared in the exhibited regions. The structures of intermediates, the compound BTZ confirmed by the presence of characteristic peaks in the region 3132.79 cm^{-1}associated for $-NH_2$ stretching respectively. BTZE showed the disappearance of the characteristic bands of the $-NH_2$ stretching and also shows the stretching for cyanide group, ester group in the region 2360.44 cm^{-1}, 1786.44 cm^{-1} an respectively. The compound BTZP confirmed, the disappearance of the characteristic bands of the cyanide group and ester group and also showed the reappearance of the characteristic bands of the $-NH_2$ stretching in the region 3343.05 cm^{-1} respectively.

Compound BTZP3b containing – N $(CH_3)_2$ group showed absorption bands at 1321 cm^{-1}. Compound BTZP3c, BTZP3e containing C-O-C group showed absorption bands at 1070.3 cm^{-1}, 1081.62 cm^{-1}. Compound BTZP3f containing NO_2 group showed absorption bands at 1512.88 cm^{-1}, for the N=O stretching. The peak at 830.20 cm^{-1}, 821 cm^{-1}, 837.19 cm^{-1} could be assigned to C-Cl stretching in the compound BTZP3d, BTZP3h, BTZP3l. Presence of hydroxyl group was confirmed by the appearance of broad peak at 3430-3161 cm^{-1} in the compound BTZP3e, BTZP3g, BTZP3i, BTZP3j. Compound BTZP3k containing C-F group showed absorption bands at 1416 cm^{-1}.

The structures of the newly synthesized compounds (BTZP, BTZP3a-l) were confirmed by ^{1}H-NMR spectra. The ^{1}H-NMR spectra of all the synthesized compounds showed the absence of the peaks for the $–NH_2$ proton signal at δ 8.32 ppm. All the synthesized compounds showed multiplets in the range δ 6.32-8.59 for the protons of aromatic ring and a singlet at δ 6.15, 3.57-3.89,7.4-8.64ppm which may be assigned to –NH-N proton,-NH proton of pyrazolone,-N=CH proton of Schiff base. The spectrum of BTZP3b, BTZP3c, revealed a singlet at δ 3.04, 3.35 ppm which may be assigned to -N $(CH_3)_2$, OCH_3 proton. The spectra of compound BTZP3g showed singlet at δ 9.87 ppm corresponding to OH group.

Mass spectrums of the synthesized compounds were in full agreement with their molecular weights and studies showed satisfactory results.

MOLECULAR DOCKING STUDY

Molecular docking study was performed on one tubercular protein Thymidylate Kinase (1MRS).Synthesized compounds (BTZP3h, BTZP3k, BTZP3l) were shown pose score -5.82,-5.68,-5.65 on thymidylate kinase inhibitor.

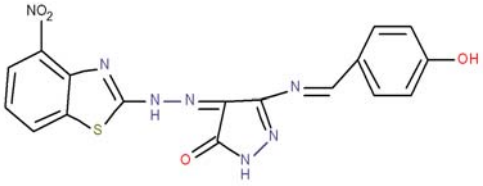
8.2. BIOLOGICAL EVALUATION

***In vitro* antibacterial screening**

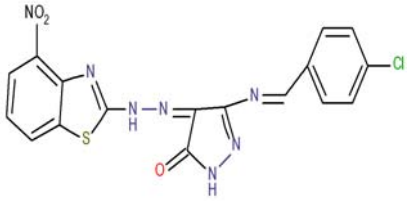
All the newly synthesized compounds were screened for their preliminary antibacterial activity against *Micrococcus luteus, Staphylococcus aureus, Bacillus substils, Corney bacterium, Bacillus lintus, Staphylococcus albus* (gram positive bacterial strains) *Escherichia Coli, Pseudomonas aruginosa, Rhodosporum ruberum, Vibrio cholera, Salmonella paratyphii and Klbsellia pneumonia* (gram negative bacterial strains) at a concentration of 100µg/disc and the result compared with standard ciprofloxacin 5 µg/discs concentration level.

All the newly synthesized compounds (BTZP3g and BTZP3h) showed moderate activity with the percentage of inhibition range at 86% against *Bacillus Lintus* (gram positive bacterial strains) when compared with standard drug ciprofloxacin (5µg/disc) with MIC range at 6.25µg/ml concentration level.

BTZP3g

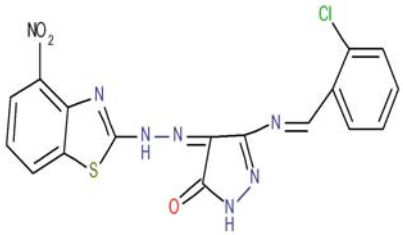


BTZP3h



All the newly synthesized compounds (BTZP3d) demonstrated more zone of inhibition with the percentage of inhibition range at 175% against *Salmonella paratyphii* (gram negative bacterial strains) when compared with standard drug ciprofloxacin (5µg/disc) with MIC range at 3.12µg/ml concentration level.

BTZP3d

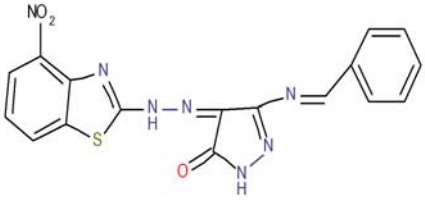


***In vitro* antifungal screening**

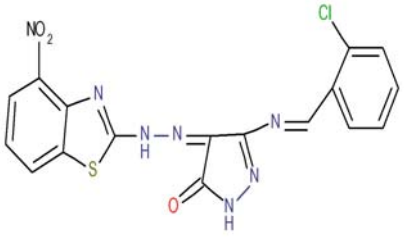
All the newly synthesized compounds were screened for their preliminary antifungal activity against *Candida albicans, Monococcus purpureus, Aspergillus niger, Trichophyton rubrum, Aspergillus fumigates, Aspergillus parasites at* a concentration of 100µg/disc.

All the newly synthesised compounds (BTZP3a and BTZP3d), demonstrated more zone of inhibition with the percentage of inhibition range at130% against *Aspergillus fumigates, Aspergillus parasites* (fungal strains) when compared with standard drug clotrimazole (5µg/disc) and the MIC range at 3.12 µg/ml concentration level.

BTZP3a



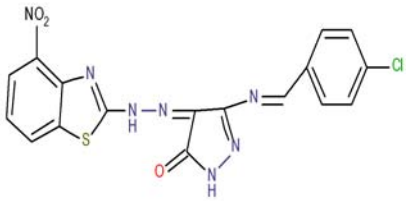
BTZP3d



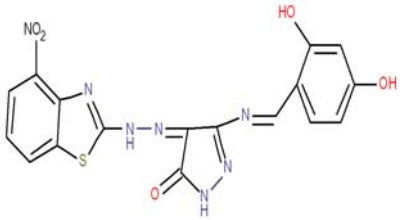
***In vitro* antitubercular screening**

Synthesized compounds (six) screened, four compounds BTZP3h, BTZP3i, BTZP3k, and BTZP3l were found to possess potent *invitro* anti -mycobacterial activity at 10 µg/ml concentration level.

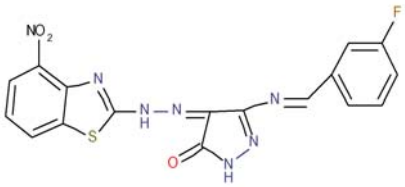
BTZP3h



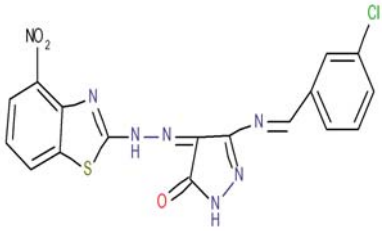
BTZP3i



BTZP3k



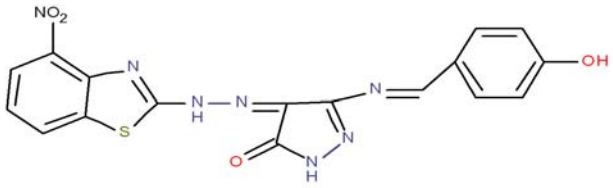
BTZP3l



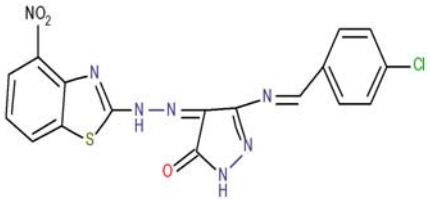
***In vitro* antioxidant screening**

All the newly synthesised compounds were investigated for *in-vitro* antioxidant activity by DPPH, FRAP and ABTS assay methods**.** The ascorbic acid was used as standard reference for DPPH and ABTS assay methods. The ferrous sulphate was used as standard reference for FRAP assay methods. The compounds BTZP3g was found to possess DPPH radical scavenging antioxidant activity with an EC_{50} of 32µg/ml, BTZP3h was found to possess FRAP radical scavenging antioxidant activity with an R^2 of 0.999 and BTZP3f was found to possess ABTS radical scavenging antioxidant activity with an EC_{50} of 30µg/ml.

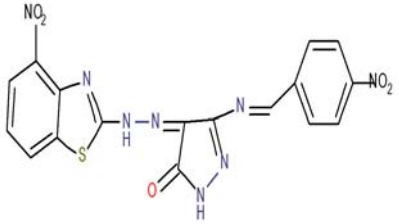
BTZP3g



BTZP3h



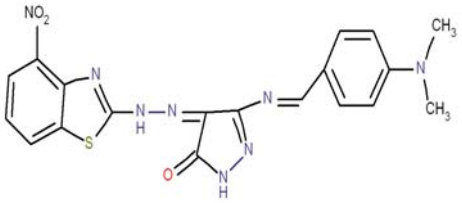
BTZP3f



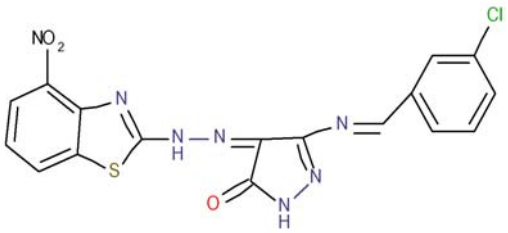
***In vitro* cytotoxic screening**

Six synthesized compounds were screened for *in vitro* cytotoxic study by MTT Assay method, the compounds BTZP3b, BTZP3l, were found to possess potent *invitro* cytotoxic activity.

BTZP3b



BTZP3l



8.3. CONCLUSION

The 2-aryl benzothiazole moieties are already known for different biological activities. In the present investigation an attempt has been made for the synthesis of some novel benzothiazole containing pyrazolone analogues combining with different substituted aldehydes by schiff base reaction to get a good antimicrobial, bactericidal, fungicidal, *in vitro* anti-tubercular, antioxidant and cytotoxic activity with high toxic effects.

Biological screening results clearly indicated that the compounds of the scheme have shown good *in vitro* antimicrobial, anti-tubercular, antioxidant activity compared with the standard drugs and almost all the newly synthesized compounds are bactericidal and fungicidal in nature. Unfortunately, the overall results indicate that they were weekly active with a low selectivity index as indicate by the cytotoxic effect. Even though the synthesized compounds showed good antimicrobial activity with less safety index, due to compounds have high toxicity towards normal cell lines.

The result obtained, taking into account the significant activities of the examined compounds, it is believed that further optimization of these identified chemical leads can probably lead to the development of more active molecules. Future studies are proposed reduced toxicity and to establish their *in vivo* efficacy and receptor interaction, after making suitable structural modifications.

Chapter 9

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ABSTRACT

This project was aimed for the synthesis of Schiff Bases of 5-amino-4-[2-(4-nitro-1, 3-benzothiazol-2 yl) hydrazinylidene]-2, 4-dihydro-3*H* -pyrazol -3-one. All the synthesized compounds were purified and characterized on the basis of spectral data (IR, ^{1}HNMR & Mass spectra). Molecular docking study was performed for synthesized compounds (BTZP3a-l) on one tubercular protein, thymidylate kinase (PDB ID: 1MRS) by using Autodock module version 4.0.Among the synthesized compounds docked, the compounds BTZP3h, BTZP3k, BTZP3l on 1MRS shown -5.82, -5.68, -5.65 pose score. The title compounds were screened for their preliminary anti-microbial activity against gram positive, gram negative strains and fungal strains by disc diffusion method for determining zone of inhibition and serial dilution method was followed to determine minimum inhibitory concentration (MIC), followed by to determine minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC). The preliminary antibacterial activity of synthesized compounds result shows better activity against gram negative bacteria than gram positive bacteria. Almost all the newly synthesized compounds (BTZP3a-l) are bactericidal and fungicidal in nature. Anti-tubercular activity by Resazurin microplate assay method against mycobacterium tuberculosis H_{37}Rv using rifampicin as standard at 1 µg/ml concentration level. The synthesized compounds BTZP3h, BTZP3i, BTZP3k, BTZP3l were shown significant activity against *mycobacterium tuberculosis* $H_{37}Rv$ at 10µg/ml. Anti-oxidant activity by DPPH, FRAP, ABTS assay method. The synthesized compounds BTZP3g, BTZP3h, BTZP3f were shown significant antioxidant activity. Cytotoxic activities by MTT assay method, the synthesized compounds BTZP3b, BTZP3l were found to possess potent *invitro* cytotoxic activity.