A Thesis Entitled

SYNTHESIS OF HETEROCYCLIC SCAFFOLDS AS POTENTIAL THERAPEUTIC AGENTS

Submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

CHEMISTRY

By

Ms. SULAKSHA R. DESAI

(MSc.)

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Assagao, Bardez-Goa

SEPTEMBER 2020

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Under the Guidance of

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Goa University

Taleigao Plateau, Goa

SEPTEMBER 2020

DECLARATION

I hereby declare that the work contained in the thesis entitled "**Synthesis of Heterocyclic Scaffolds as Potential Therapeutic agents**" is the result of investigations carried out by me under the guidance of **Prof. Vidya G. Desai** at Department of Chemistry, Dnyanprassarak Mandal's College and Research Centre and that it has not previously formed the basis for the award of any degree or diploma or other similar titles.

In keeping with general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators. I further state that the thesis has been prepared by me and it is my original work and is free of any plagiarism. This document has been duly checked through Plagarism detection tool approved by the Goa University and confirms to the provisions of OA-29.

Mrs. Sulaksha R. Desai,
Ph.D Student
Goa University

Goa University
September 2020

SCHOOL OF CHEMICAL SCIENCES

CERTIFICATE

This is to certify that the thesis entitled **"Synthesis of Heterocyclic Scaffolds as Potential Therapeutic agents"** submitted by **Mrs. Sulaksha R. Desai,** is a record of research work carried out by her during the period of study under my supervision and that it has not previously formed the basis for the award of any degree or diploma or other similar titles. This document has been duly checked through Plagarism detection tool approved by the Goa University and confirms to the provisions of OA-29.

Prof. Vidya G. Desai,
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Goa University
September 2020

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I will be happy to express my special thanks to Department of Science and Technology, Porvorim, Goa, Funds for Improvement of Science and Technology-New Delhi for providing research funding, SAIF, Punjab University, Chandigarh for NMR, HRMS facilities, IIT-Ropar for HRMS Facility, Centaur Pharmaceutical, Goa for IR facility, Karnataka University, Dharwad for NMR facility.

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The acknowledgement would not be complete without thanking my parents and other family members. They have helped me build all good qualities like self-respect, hard work, diligence and to be independent. My sisters Samiksha and Mayuri, my husband Mr. Vaibhav Kadkade, my brother-in-laws Mr. Ankit Bhangui and Mr. Shankar Prabhu who are the reason for the encourangement throughout in all phases.

Lastly, I would thank god for the lovely life, and good health.

.....Sulaksha R. Desai

TABLE OF CONTENTS

Dedicated to My Father

GENERAL REMARKS

- The compound numbers, figure numbers, scheme numbers and reference numbers given in each chapter refer to that particular chapter only.
- Commercial reagents were used.
- Solvents were distilled prior to use and dried if necessary using standard procedures.
- Thin layer chromatography (TLC) were carried out on Silica gel 60 F254 aluminium plates purchased from Merck and were developed in iodine or UV chamber.
- Chromatographic purification were conducted by column chromatography using silica gel (60-120 mesh size) or by flash chromatography using silica gel (230-400 mesh size) on Combiflash Companion instrument.
- All melting points and boiling points were recorded using Thiele's tube and are uncorrected.
- IR spectra were recorded on Shimadzu FT-IR spectrophotometer.
- ^{1}HNMR and ^{13}C NMR spectra were recorded with Bruker Avance DPX500 spectrometer operating at 400MHz, with Me_4Si as an internal standard.
- Mass spectra (HRMS) were recorded on MicroMass ES-QTOF mass spectrometer.

ABBREVIATIONS

GENERAL ABBREVIATIONS			
anhy	Anhydrous	**SAR**	Structure activity relationship
Fig.	Figure	**MIC**	Minimum inhibitory concentration
h	Hours	**PDB**	Protein data bank
dil	Dilute	**OD**	Optical density
aq	Aqueous	**IC_{50}**	Half Maximal Inhibitory Concentration
^{o}C	Degree celcius	**SAR**	Structure activity relationship
r.t.	Room temperature	**MDA-MB-231**	M. D. Anderson for Metastasis Breast Cancer
min	Minutes	**HEK-293**	Human Embryonic Kidney
Sat.	Saturated	**FBS**	Fasting Blood Sugar
Conc.	Concentrated	**et al**	Et alia (and others)
mmol	Millimole	**Cat.**	Catalytic
g	Grams	**%**	Percentage
M. pt	Melting Point	**mL**	Milliliter
MABA	Microplate Alamar Blue Dye Assay	**TNBC**	Triple negative breast cancer
		SOS	Superoxide Scavenging

SPECTROSCOPIC AND CHROMATOGRAPHIC ABBREVIATIONS			
UV	Ultraviolet	**d**	Doublet
NMR	Nuclear Magnetic Resonance	**s**	Singlet
IR	Infra-red	**t**	Triplet
FTIR	Fourier Transform Infrared spectroscopy	**m**	Multiplet
cm^{-1}	Frequency in wavenumber	**dd**	Doublet of doublet
HPLC	High performance liquid chromatography	**dt**	Doublet of triplet
HRMS	High resolution matroscopy	**q**	Quartet
J	Coupling constant	**δ**	Delta(chemical shift)
ppm	Parts per million	**M^{+}**	Molecular ion

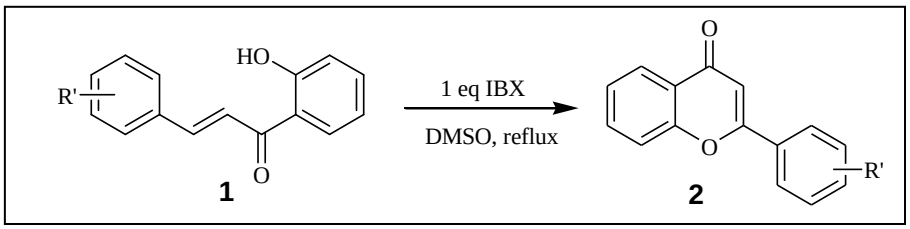
m/z	Mass to charge ratio	**TLC**	Thin layer chromatography

CHEMICAL NAMES AND ABBREVIATIONS			
DMSO	Dimethyl sulfoxide	**H_2SO_4**	Sulphuric acid
EtOH	Ethanol	**$NaIO_4$**	Sodium metaperiodate
IBX	Iodoxybenzoic acid	**Na_2SO_4**	Sodium sulphate
DCM	Dichloromethane	**LISA**	Less Invasive Surgical Approach
NO_2	Nitro	**$ZnCl_2$**	Zinc chloride
COX	Cyclooxegenase	**SO_2Cl_2**	Thionyl chloride
OH	Hydroxyl	**HCl**	Hydrochloric acid
F	Fluoride	**DPPH**	2,2-Diphenyl-1-picrylhydrazyl
Cl	Chloride	**NSAIDs**	Non-steroidal anti-inflammatory drugs
Br	Bromide	**DAPI**	4, 6-Diamino-2-phenylindole
NMe_2	N,N-dimethyl	**NaOH**	Sodium hydroxide
NH_3	Ammonia	**OMe**	Methoxy
K_2CO_3	Potassium carbonate	**NEt_3**	Triethylamine
Glac.	Glacial	**MTT**	3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NADH	Nicotinamide Adenine Dinucleotide Hydrogenase	**$CDCl_3$**	Deuterated chloroform
		Me_4Si	Tetramethyl silane
DMARDS	Disease-modifying antirheumatic drugs		

ABSTRACT OF THESIS

The thesis deals with synthesis of heterocyclic scaffolds which are compounds that have presence of one or more hetero-atom(s) as a part of cyclic system. The biological potential of various heterocyclic scaffolds has drawn special attention of medicinal chemists and hence exhaustive efforts are being carried out in the search of lead molecules pertaining to it. Synthetic heterocycles like flavones, benzoxazoles, quinoxaline derivatives etc. have widespread applications which includes antibacterial, anti-fungal, anti-leishmanial, anti-tubercular, anti-malarial, anti-inflammatory, anti-cancer, anti-convulsant, anti-spasmodic etc.

This thesis is divided into four chapters; the **FIRST** chapter deals with the first ever application of hypervalent iodine reagent towards synthesis of bioactive flavones, hydroxyflavones, haloflavones and aminoflavones. Synthesis of series of differently substituted flavones **2** were prepared from 2-hydroxychalcones **1** via oxidative cyclization, using easily available, non-toxic, hypervalent iodine reagent, IBX. The optimisation studies, proved a convenient IBX-mediated approach towards synthesis of flavones. Synthesizing differently substituted flavones **2**, demonstrates the versatility of the methodology.

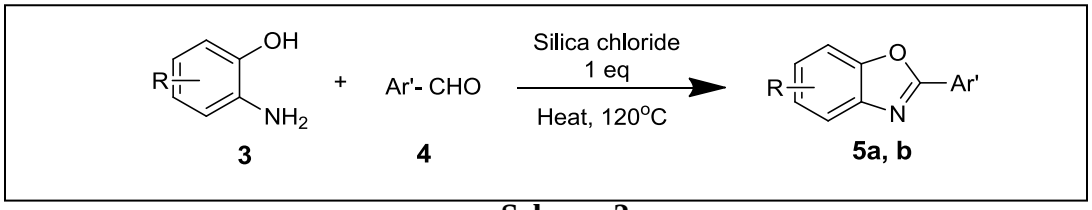


Scheme 1

The selected flavone derivatives were screened against *Mycobacterium tuberculosis* by Alamar Blue dye assay and showed excellent anti-tubercular activity in comparison to the standards Pyrazinamide, Streptomycin and Ciprofloxacin. Thus, have been proved to be potent anti-tubercular agents.

The **SECOND** chapter explains the one-pot solvent free approach towards synthesis of 2-aryl benzoxazole scaffolds. A number of 2-aryl **5a** and heteroaryl benzoxazole scaffolds **5b** were

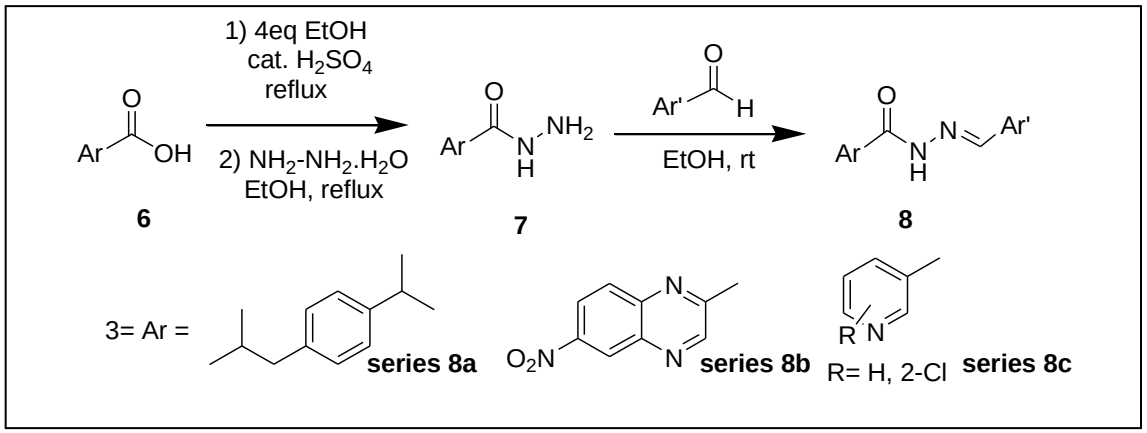
synthesised using silica chloride as a catalyst under neat heat conditions. Silica chloride as highly efficient, recyclable and green catalyst has been well demonstrated here. This chapter also discusses about the molecular docking studies to demonstrate the interaction between the enzyme tyrosine kinase receptor and the synthesized benzoxazole derivatives.



Scheme 2

The enzyme inhibition studies against tyrosine kinase enzyme, along with the apoptosis study by fluorescence microscopy method has been displayed and an *in-vitro* anti-tubercular activity against *Mycobacterium tuberculosis* has also been evaluated. The synthesized benzoxazole scaffolds **5b** has been proved to be better tyrosine kinase inhibitors.

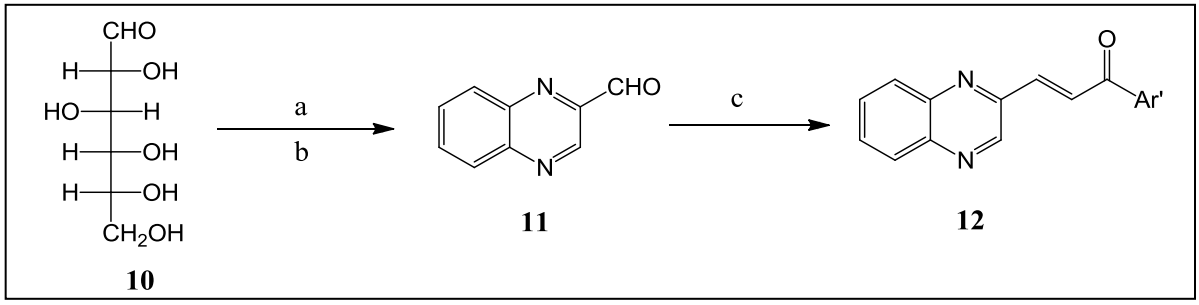
The **THIRD** chapter discusses the synthesis of a series of Ibuprofen based **$8a_1$-a_{11}**, quinoxaline-based **$8b_1$-b_5** and pyridine-based **$8c_1$-c_8**, azomethine derivatives. The synthesized azomethine derivatives series **8a**, **8b**, **8c** having varied pharmacophoric units as well as substitution patterns has been virtually screened for their COX-I and COX-II inhibition, *in-vitro*/*in-vivo* anti-inflammatory activity, COX-I/COX-II enzyme inhibition studies, *in-vitro* anti-tubercular activity, anticancer evaluation against HELA, HEK293 and MCF-7 cell lines, in addition apoptosis studies were also carried out by flow cytometry. These derivatives have been discovered to be better COX-II inhibitors.



Scheme 3

All the synthesized azomethine derivatives have been proved as antioxidant agents, COX inhibitors and as better cancer therapeutics.

The **FOURTH** chapter depicts molecular hybridization of quinoxaline with chalcone moiety representing an interesting class of molecules that can lead to great drug potency. Herein, we report series of novel substituted quinoxalinyl chalcones **12**, its molecular docking studies, and its potential to be good drug targets for the treatment of tuberculosis, cancer and other microbial infections. Such chalcones have also been screened for their antimicrobial activity.



Scheme 4

The key biological activity in this chapter has been antitubercular, targeting an enzyme involved in mycolic acid biosynthesis. The inhibitory action of quinoxalinyl chalcones towards the enzyme enoyl ACP reductase, has been the revealing fact of the above finding.

PUBLICATIONS

S. R. Desai, V. Desai, S. G. Shingade, *In-vitro* anti-cancer assay and apoptotic cell pathway of newly synthesized Benzoxazole-N-heterocyclic hybrids as potent tyrosine kinase inhibitors, Bioorg. Chem., 94(**2020**), 103382**.**

V. Desai, S. R. Desai, IBX-Mediated, Efficient, Metal-Free Approach towards Synthesis of Flavones. Curr. Org Syn., 14(**2017)**, 1-5.

V. Desai, S. Desai, S. NaikGaonkar, U. Palyekar, S. D. Joshi, S. Dixit, Novel Quinoxalinyl Chalcone Hybrid Scaffolds as Enoyl ACP Reductase Inhibitors: Synthesis, Molecular Docking and Biological Evaluation, Bioorg. Med. Chem. Lett., 27(**2017),** 2174-2180.

Conferences and Seminars Attended……

- Participated in International Conference on Green Chemistry: Catalysis, Energy and Environment (ICGC-2015) held at Goa University, from 22-24 January 2015.
- Participated and Presented Poster at17th CRSI National Symposium in Chemistry held at CSIR National Chemistry Pune from 6-8 February, 2015.
- Participated and Presented Poster titled: "Synthesis of novel heterocyclic scaffolds for the treatment of infectious diseases" at National Conference on "New Frontiers in Chemistry-Fundamentals to Application" (NFCFA-2015) organised by Department of Chemistry, BITS-Pilani, K. K. Birla Goa Campus from 18-19 December 2015.
- Participated and presented Poster entitled: "Synthesis of novel Schiff bases and 1, 3, 4-oxadiazole analogues as potential anti-inflammatory and anti-tubercular agents" at 2nd National Conference on "New Frountiers in Chemistry-Fundamentals to Application" (NFCFA-2015) organised by Department of Chemistry, BITS-Pilani, K.K. Birla Goa Campus, from 28-29 January 2017.
- Participated in 21st CRSI National Symposium in Chemistry held at Indian Institute of Chemical Technology, Hyderabad, from 14-16 July 2017.
- Participated in two days National Seminar on "Recent Advances in Applied Chemistry & Electronic Technologies" held at St. Xaviers College, Mapusa from 16-17 January 2018.
- Participated and Presented poster entitled "Metal-free, IBX-mediated Synthesis of Flavones as Potent Anti-tubercular agents" for a one day national symposium on 'Green Chemistry for Better Sustainability' held at Department of Chemistry, Dnyanprassarak Mandal's College and Research Centre, Assagao-Goa on 27th September 2019.
- Participated and Presented poster entitled "In-vitro anticancer assay and apoptotic cell pathway of newly synthesised benzoxazole-N-heterocyclic hybrids as potent Tyrosine Kinase Inhibitors" in Syngenta Agroscience Symposium: Sustainable Chemistry and Technology" organised by Syngenta Biosciences Pvt. Ltd. Goa on 4th November 2019.
- Participated and presented poster entitled "Design, Synthesis and biological evaluation of azomenthine derivatives as enoyl-ACP reductase inhibitors" in the two day national conference organised by Department of chemistry in association with Directorate of Higher Education held at Dnyanprassarak Mandal's College and Research Centre. Assagao-Goa, from 14th-15th February 2020.

CHAPTER 1

An efficient, Metal-free Synthesis of Flavones as Potent Anti-tubercular Agents

1.1. Introduction

Flavones also known as 2-phenyl-4-chromones and represent one of the largest groups of natural products that are highly diverse and important class of compounds belonging to the flavonoid group that occur naturally in fruits, vegetables, nuts, seeds, flowers, and barks. These are widely distributed in plants and are used as synthetic targets due to wide range of activities exhibited by them.

Flavones (2-aryl-4*H*-1-benzopyran-4-ones) are naturally occurring compounds, belonging to the flavonoid family. Such groups of molecules are oxygen containing bicyclic heterocycles having varied biological activities[1] that include anticancer, antioxidant, anti-inflammatory, antiviral, anti-mutagenic, anti-HIV, antibacterial, DNA cleavage etc. Newer 1,2,3-triazole linked chalcone and flavones hybrid compounds have been synthesized and evaluated for their antimicrobial and cytotoxic activities.[2] Flavones also form an important group of naturally occurring alkaloids, Luteolin (**i**) Apigenin (**ii**) and 7-hydroxyflavones (**iii**). All three are known to inhibit AKR1B10, and act as a promising drug target for the treatment of cancer. Similarly, 4'-Bromoflavones (**iv**), 4'-chloroflavones (**v**) and 4'-fluoroflavones (**vi**) exhibit excellent anticancer activity[3] (**Fig.1**).

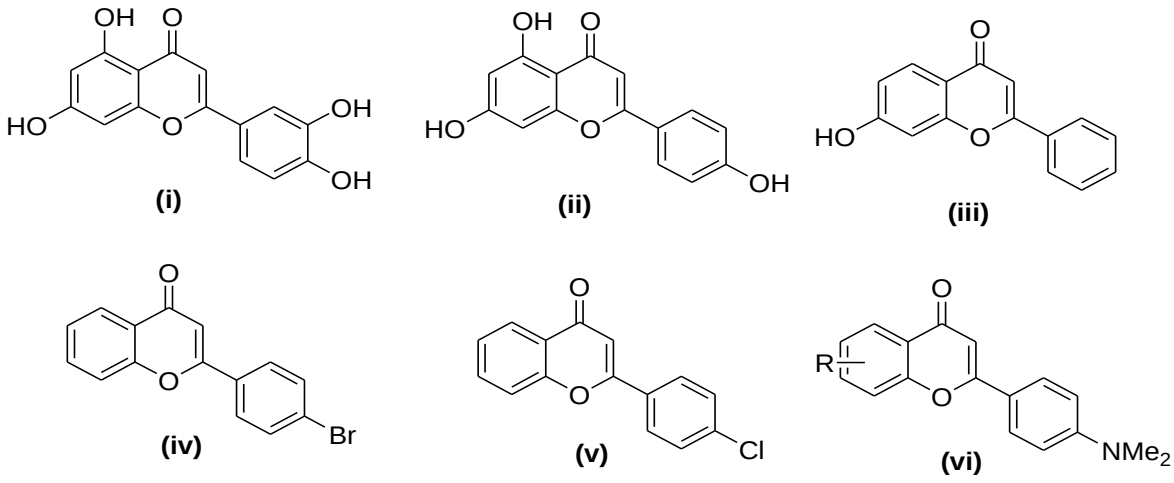


Fig.1. Naturally occurring flavones

Because of their anti-oxidant ability, many of the flavonoids are responsible for health-promoting functions in organism, which are important for the prevention of diseases that are associated with an oxidative damage of membranes, proteins and DNA. This has led to a wide interest among the community of organic chemists to synthesize flavones, both by the development of annulation methodology and by target oriented convergent synthesis. The two

best known methods for flavone synthesis are Baker-Venkatraman rearrangement[4] and oxidative cyclization of 2'-hydroxychalcones.[5]

Flavones being a product of biological importance play a significant role in pharmaceutical industry, so a methodology, which is cost-effective, is of great need. Iodine catalyzed reaction in DMSO is a commonly employed method under refluxing conditions.[6] However, this method is time consuming as it requires removal of iodine by washing with sodium thiosulphate, product purification by column and due to toxicity of direct use of iodine. Recently, ultrasound mediated synthesis of flavones involving use of hazardous iodine monochloride has been reported.[7] Nowadays, there is moreover replacement of iodine by hypervalent iodine reagents in organic synthesis. Reagents like PIDA, PIFA, IBX have made a great impact as powerful and chemoselective oxidants in functional group transformations.

In this regards, IBX (**Fig.2**) as oxidant could be the reagent of choice for better sustainable methodology to synthesize flavones. Thus, we demonstrate herein, how IBX can mimic iodine in having electrophilic character and also being an oxidant to effectively afford differently substituted bioactive flavones.

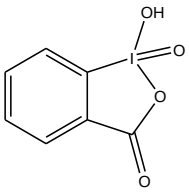


Fig.2. Structure of IBX

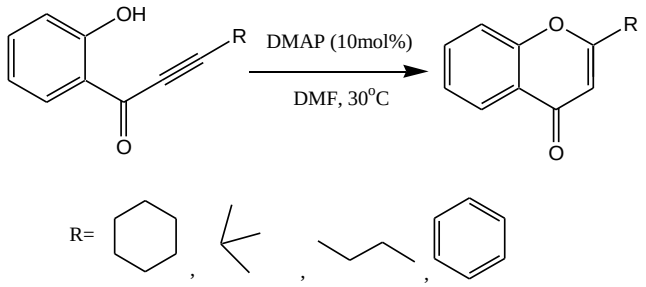
Chemistry of hypervalent iodine has evolved over the years. The precursor of Dess-Martin oxidizing reagent,[8] 1-hydroxy-1λ^5-2-benziodoxol-3(1*H*)-one-1-oxide (IBX), a highly versatile hypervalent iodine(V) reagent has been demonstrated in several diverse applications such as oxidant for various organic functional group transformations, as dehydrogenating agent for aromatization, for oxidative cyclisation of acyclic enone derivatives.[9] This has several advantages such as easy preparation, non-toxic, safe to handle, relatively stable at room temperature for longer period of time, simple reaction conditions and a great degree of chemoselectivity.[10] There has been a successful application of IBX-promoted one pot methodology via oxidative cyclisation leading to benzimidazole.[11] IBX-mediated oxidation of inactivated cyclic amines has been reported.[12] The IBX mediated transformation of amines to aldehydes has also been applied.[13] Due to the insolubility of IBX in most common organic

solvents and also in water, its use has been restricted in organic synthesis. Various methods involving use of water soluble hypervalent iodine(V) reagents has been discussed recently by Zhang *et al.*[14] However, the use of IBX is limited to an oxidant and not as an electrophilic agent. We, in our group developed a one-pot strategy towards the synthesis of trisubstituted isoxazole and isoxazole-3-carboxylic acid using IBX.[15] Thus, further exploring the role of IBX having an electrophilic character towards synthesis of flavones.

1.2. LITERATURE

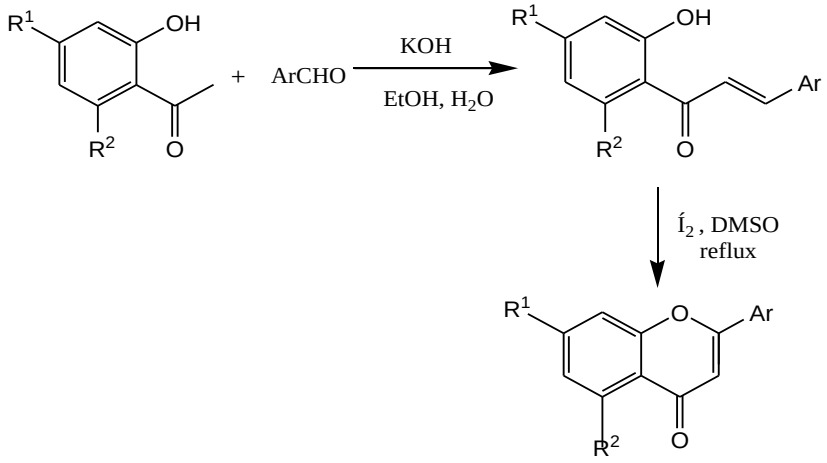
Various methods have been reported for the synthesis of flavones, these methods are associated with limitations like long reaction time, hazardous conditions, use of toxic reagents, long workup procedures, and tedious catalyst preparation. In addition to the above mentioned methods, some of these methods are as follows:

Yoshida M. *et al*[16] have reported a regioselective synthesis of flavone derivatives via DMAP catalysed cyclization of *o*-alkynoylphenol (**Scheme 1**).



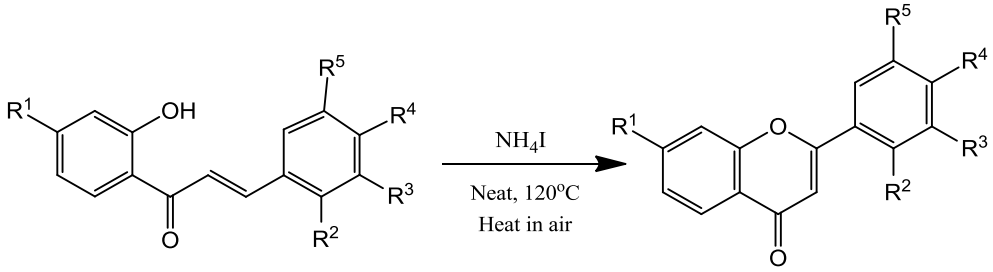
Scheme 1

Dharma Theja N.[17] and his group have established a facile route towards synthesis of flavones derivatives as potent anti-inflammatory agents via iodine catalysed oxidative cyclisation (**Scheme 2)**.



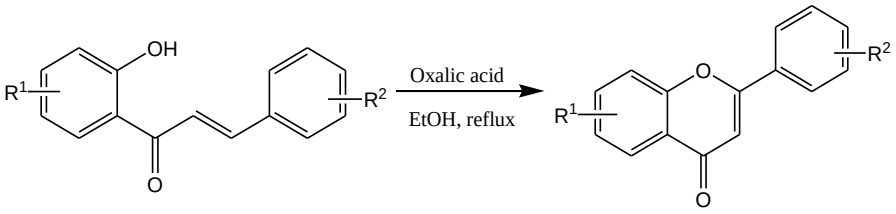
Scheme 2

The oxidative cyclisation towards flavones by *in situ* generated iodine from ammonium iodide in presence of air under solvent free conditions has been reported by Kulkarni *et al*[18] (**Scheme 3).**

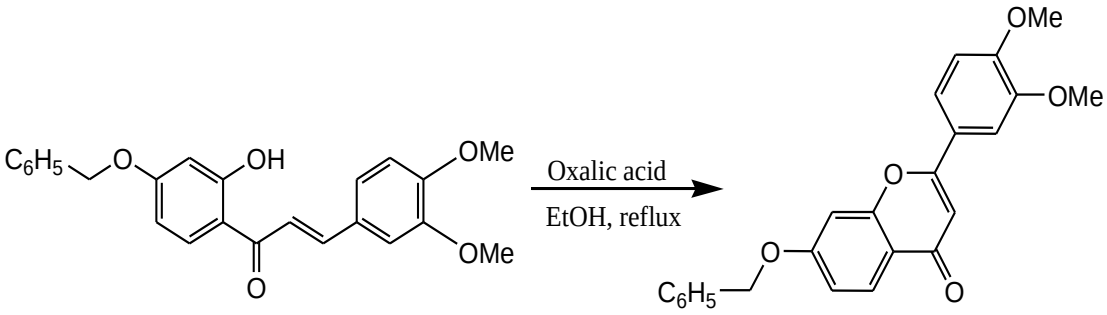


Scheme 3

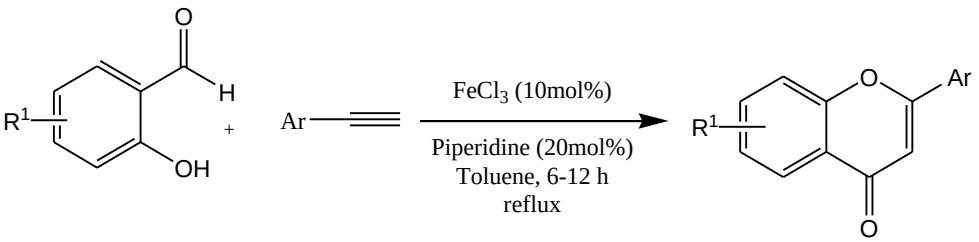
Other oxidants that have been reported in literature include oxalic acid (Shinde *et al*)[19] (**Scheme 4)**, SeO_2/isoamyl alcohol (Alam *et al*)[20] (**Scheme 5)**, $FeCl_3$/ piperidine (Maiti *et al*)[21] (**Scheme 6).**



Scheme 4

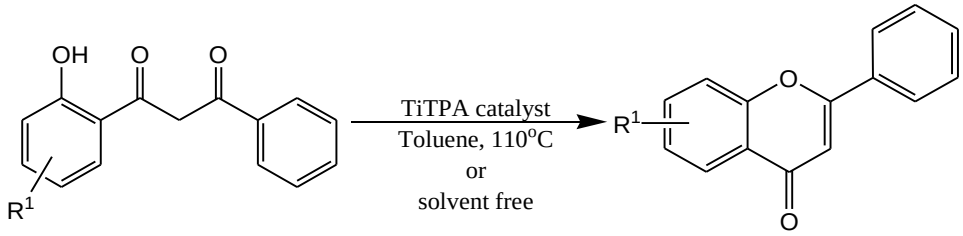


Scheme 5



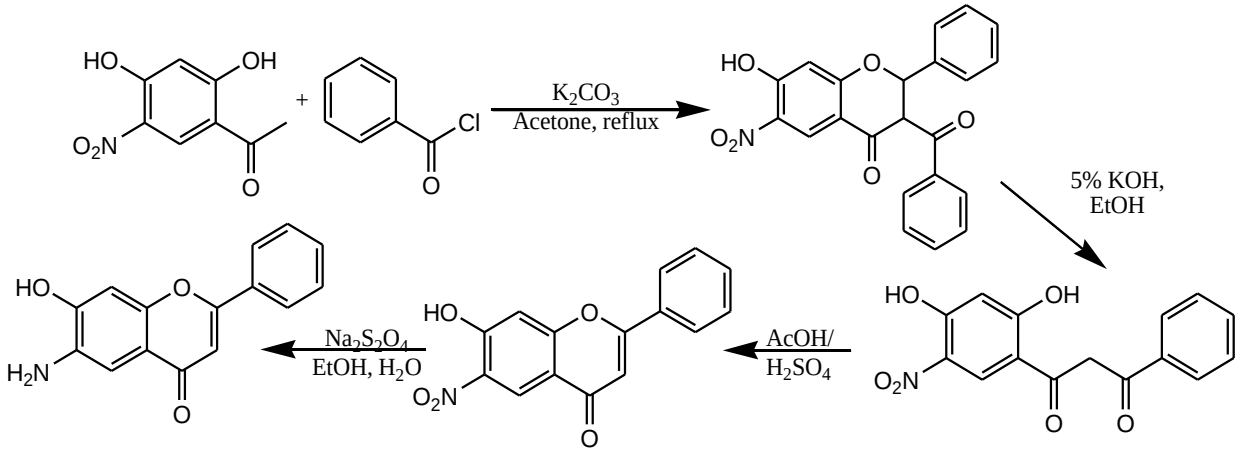
Scheme 6

Perez M. E. *et al*[22] has developed a synthetic route to flavones by using Mesoporous titania/ tungstophosphoric acid composites (**Scheme 7**).



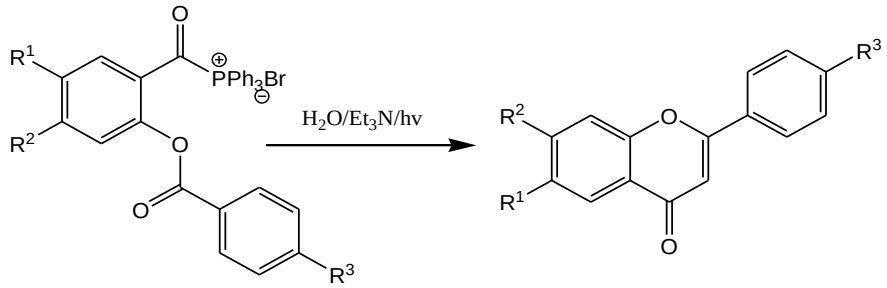
Scheme 7

Zhang S. and his co-workers[23] have reported a novel approach to the synthesis of 6-amino, 7-hydroxy flavones. It has been proved that the hydroxyl group at 5 or 7 positions is required for higher biological activities (**Scheme 8).**



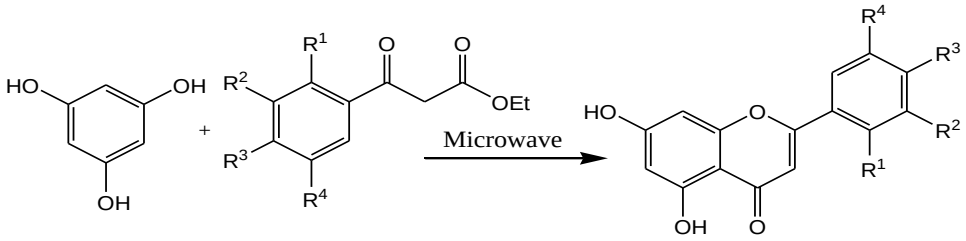
Scheme 8

An intramolecular photochemical Wittig reaction in water towards synthesis of flavones and pyranoflavones has been suggested by Das J. *et al*[24] (**Scheme 9).**



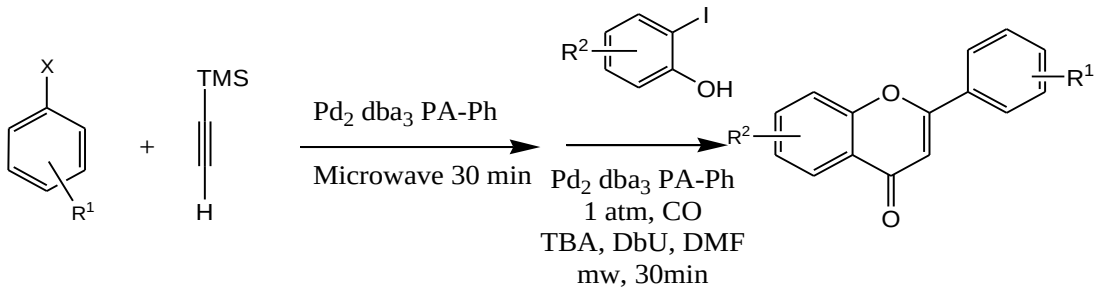
Scheme 9

Similarly, Shaikh R. K. Y. *et al*[25] in his work reports the solvent free synthesis of flavones under microwave conditions by reaction of trihydroxybenzene and 1, 3-dicarbonyl derivative (**Scheme 10).**



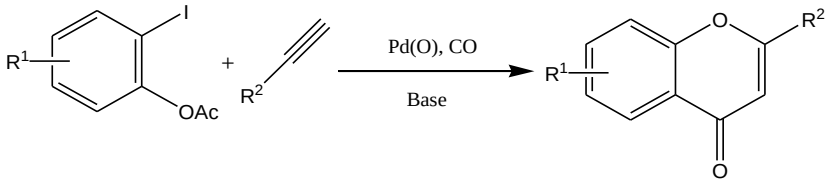
Scheme 10

Another synthesis of flavones has been reported by Awuah *et al*[26] in which microwave assisted one pot Sonogashira carbonylation annulations reactions has been explained towards synthesis of flavones (**Scheme 11).**



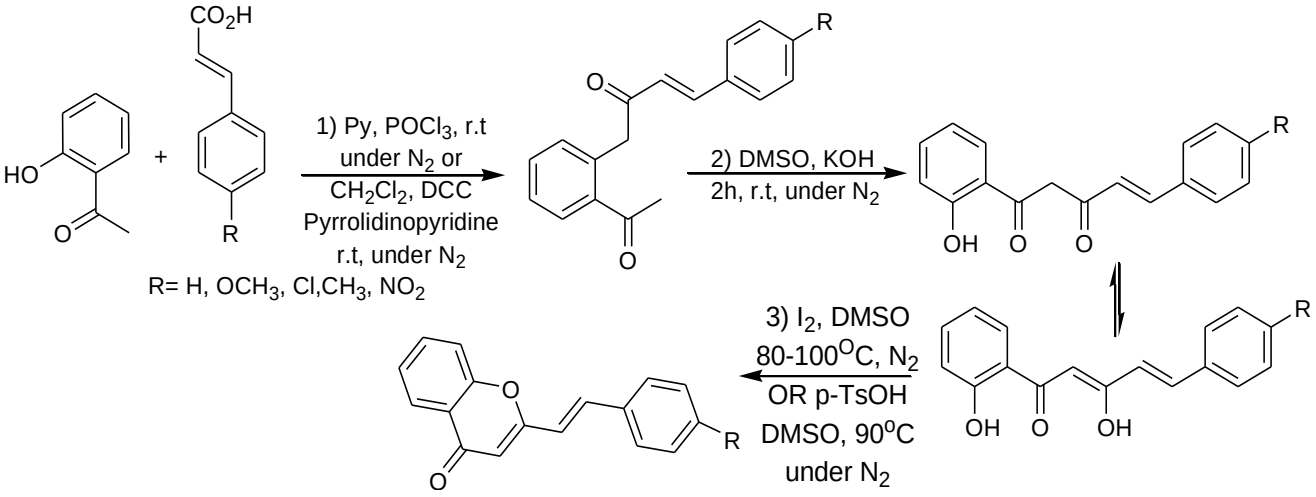
Scheme 11

Palladium catalysed synthesis of flavone in basic environment has been discussed by Miao *et al*[27] by reaction of iodobenzene acetate and acetylene in presence of base (**Scheme 12).**



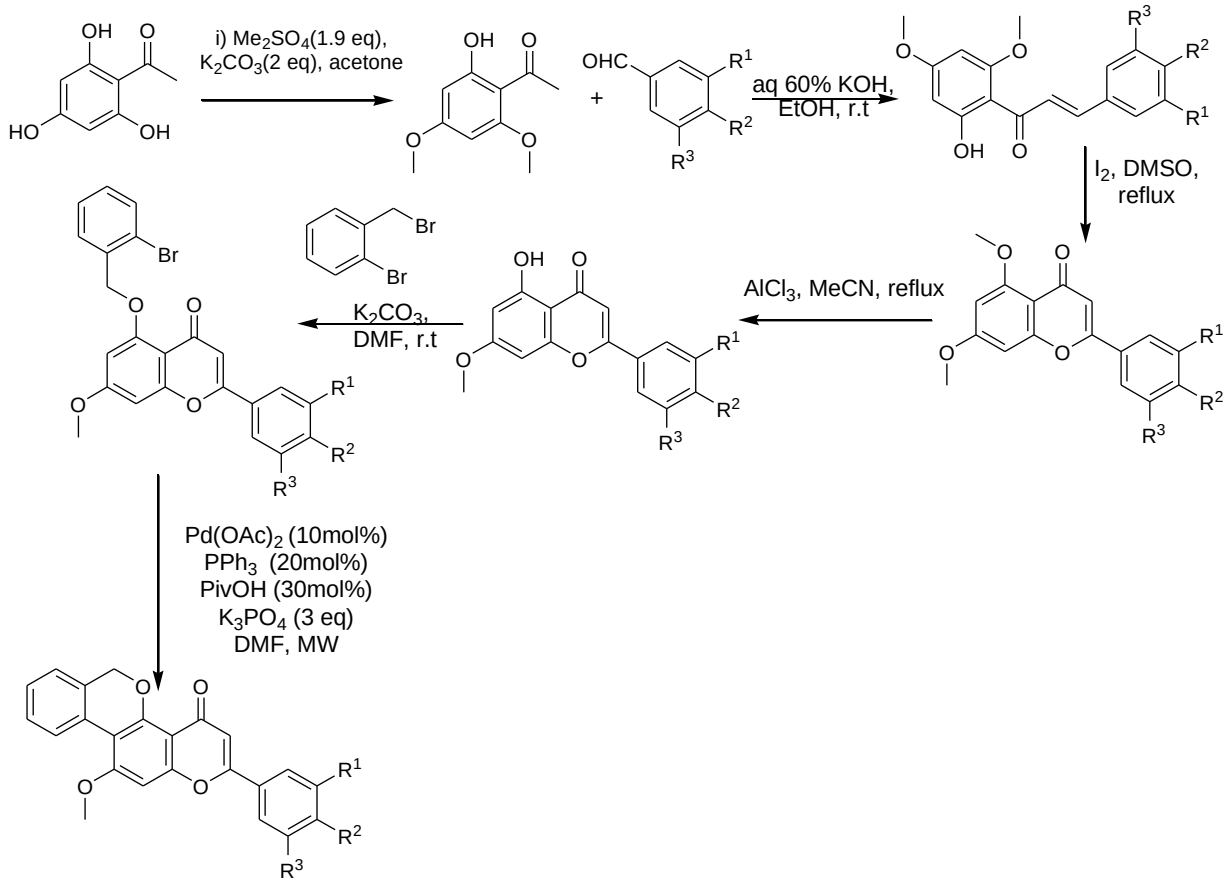
Scheme 12

Silva *et al*[28] has disclosed the low cost, environmentally benign synthesis of styrylchromones and flavones through base catalyzed cyclodehydration route. This synthesis has been carried out in water by both conventional and microwave heating under open and closed vessel conditions (**Scheme 13**).



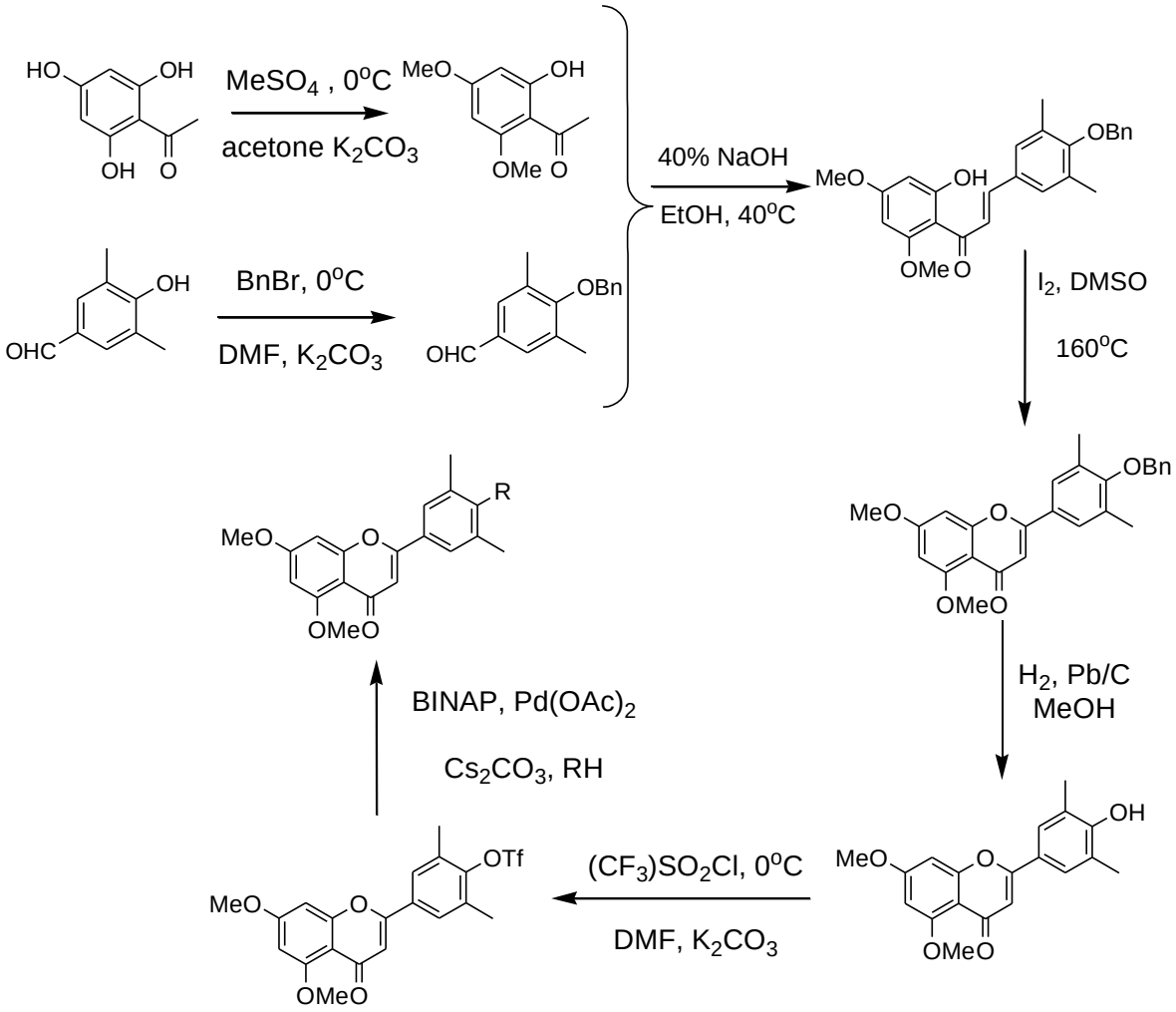
Scheme 13

Sipos *et al*[29] describes the microwave assisted synthesis of benzopyran-fused derivative of flavone, via intramolecular direct arylation method. This flavone derivative has been known to contain natural flavone backbone with different polyalkoxy substitution (**Scheme 14**).



Scheme 14

Design of seventeen flavone derivatives with hydroxyl substitution at 4'-position has been described by Chen *et al*[30]. The synthesis of flavones has been carried out by N-amination. These derivatives have been proved to be anticancer agents (**Scheme 15**).



Scheme 15

All these above reported methods have been associated with several drawbacks such as long reaction time, multiple-steps, high temperature, hazardous conditions, use of toxic reagents, long workup procedures, tedious catalyst preparation etc. So to overcome the limitations associated with the methods, a new strategy has been developed towards synthesis of flavones from 2-hydroxychalcones using hypervalent iodine(V) as an oxidant.

1.3. PRESENT WORK AND DISCUSSION

1.3a. Chemistry

Initially, 1-hydroxy-1λ^5-iodane-2-benziodoxol-3(1*H*)-one-1-oxide (IBX) was prepared from 2-iodobenzoic acid using oxone under aqueous conditions[31] at 70-80^{o}C with mechanical stirring, followed by cooling at 5^{o}C.

A number of substituted flavone derivatives have been synthesized by first ever use of IBX as an oxidant for the oxidative cyclisation of 2'-hydroxy chalcones **1** to their respective flavone derivatives **2**. For the method to be more generalized, we thought of optimizing the reaction conditions based on catalyst amount, temperature and solvents used (**Table 1, entries 1-14).** For optimization, 4-chloro-2'-hydroxychalcone **1b** was selected. Firstly, 1 equivalent of IBX was tried using various solvents. There was no formation of flavone **2b** in acetone, dichloromethane and 1, 4-dioxane due to the solubility problem (Entries 1-3). The conversion to the product **2b** was observed only in DMSO, wherein excellent yield was obtained (Entry 4). Then, the effect of catalyst was studied for the formation of compound **2b**, by varying the amount of catalyst using DMSO (Entries 5-10). No formation of the flavone **2b**, in the presence of 5, 10 and 20 mol% of the catalyst was observed. The conversion of chalcone **1b** into its product **2b** was only observed for 30 mol%, which gave 40% yield (Entry 8). Good yields were obtained using 60 and 80 mol% (Entries 9, 10) whereas, 1 equivalent of the catalyst resulted in 98% yield of the product (Entry 4). Effect of temperature was studied by employing different temperature conditions using 1 equivalent of IBX and DMSO as solvent (Entries 11-14). It was observed that, there was no formation of the product at room temperature even after 48 hours of stirring (Entry 11) and at 80^{o}C and 100^{o}C, after heating for 12 hours (Entries 12, 13). But at 120^{o}C, the product was obtained in 50% yield after 12 hours of heating (Entry14).

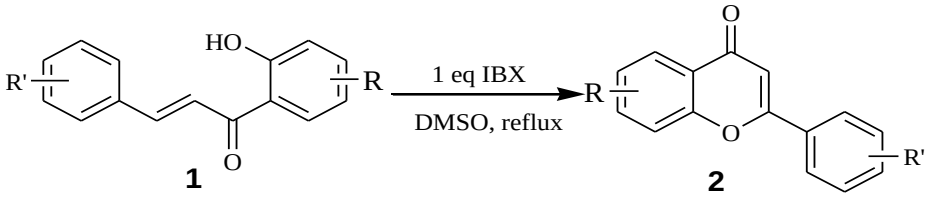
Table 1 Optimisation of reaction conditions for the synthesis of flavone **2b**

Entry	Solvents	Catalyst Mol%	Temp in ^{o}C	Time	% Yield
1	Acetone	1eq	RT	24h	No change
2	DCM	1eq	RT	24h	No change
3	1,4-dioxane	1eq	100^{o}C	8h	No change
4	DMSO	1eq	Reflux	30 min	98%

5	-	5	Reflux	5h	No change
6	-	10	Reflux	4h	No change
7	-	20	Reflux	3h	No change
8	-	30	Reflux	2h	40%
9	-	60	Reflux	1h	78%
10	-	80	Reflux	45min	80%
11	DMSO	1eq	RT	48h	No change
12	-	-	60	12h	No change
13	-	-	100	12h	No change
14	-	-	120	12h	50%

From optimization studies it was clear that, the best efficient conditions for achieving the synthesis of flavones **2b** was refluxing, 4-chloro-2'-hydroxychalcone **1b** using 1 equivalent of oxidant in dimethyl sulphoxide as a solvent.

We thus, extended this method for the synthesis of differently substituted flavones derivatives from 2'-hydroxychalcones **1** which were previously synthesized by Claisen Schmidt condensation[32] of substituted aldehydes with that of 2-hydroxy acetophenone. These chalcones **1** were treated with IBX (1 equivalent), in DMSO under refluxing conditions. Monitoring of the reaction by TLC with the appearance of fluorescent spot in UV light indicated formation of the product **2**. In total, fifteen flavones derivatives were synthesized in very good yields (**Scheme 16A, Table 2**).



Scheme 16A. Synthesis of flavones **2a-o** by oxidative cyclisation

Table 2 Synthesis of flavones **2a-o** from 2'-hydroxychalcones using IBX

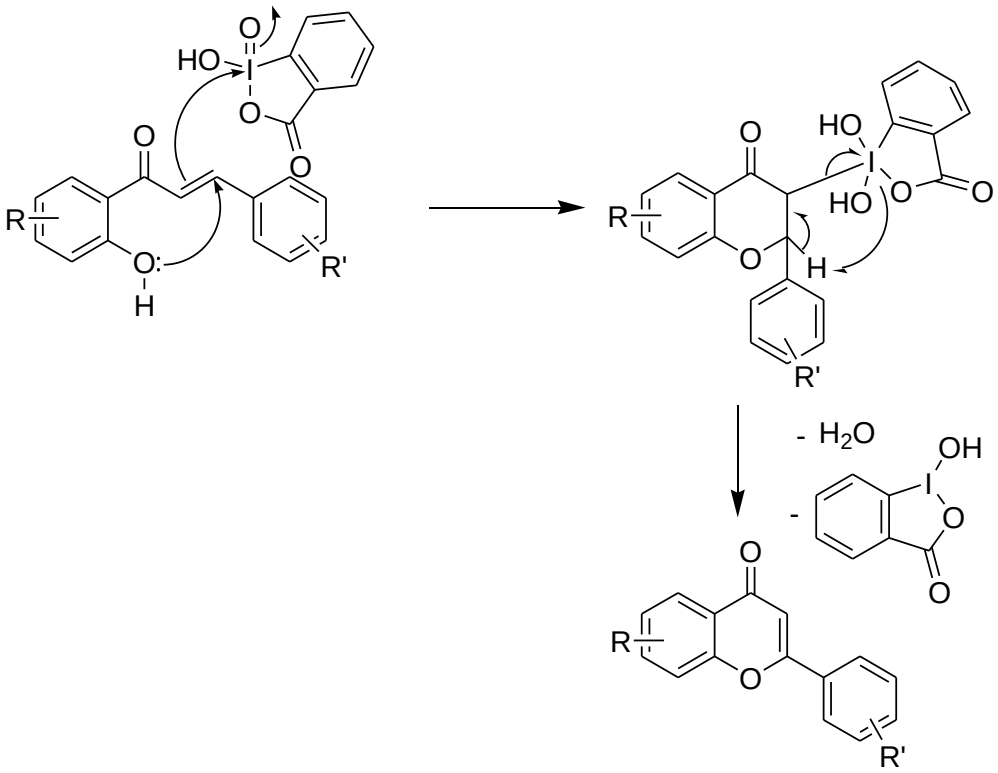
Product[a] **2**	**R'**	**R**	**Time (mins.)**	**Yield**[b] **(%)**	**M. Pt (°C)**	**Lit. M.pt. (°C)** [33]
a	H	H	120	85	90-95	98-100[a]

b	4-Cl	H	30	98	185-187	187-188[b]
c	4-Br	H	30	95	169-170	170-172[c]
d	2,4-Cl	H	30	89	170-171	171-173[d]
e	4-F	H	60	92	132-135	134-135[e]
f	4-OMe	H	30	89	155-158	154-157[a]
g	4-Me	H	45	82	80-82	78-80[b]
h	3-NO_2	H	90	88	260-264	265-267[b]
i	2-Cl	H	120	86	112-115	117-119[a]
j	4-NO_2	H	120	85	271-275	276-278[b]
k	4-NMe_2	H	120	80	159-161	160-163[f]
l	4-Br	7-OH	180	80	278-280	281-283[f]
m	4-Cl	7-OH	180	80	268-270	270-272[g]
n	4-Br	5-Br	120	84	195-198	199-201[h]
o	4-Cl	5-Br	130	82	189-191	190-191[h]

[a]Products were confirmed by ^{1}HNMR and ^{13}CNMR and also by comparing with the literature data. [b]Isolated yields.

The characterization of the synthesized compounds was done by spectroscopic methods such as IR, ^{1}H NMR and ^{13}C NMR spectroscopy. The IR showed disappearance of OH peak, with a shift in the C=O stretching frequency between 1630-1650cm^{-1} thus confirming the formation of product **2.** The ^{1}HNMR showed a singlet peak at δ6.0-6.8ppm which corresponded to 3-H proton of the chromone ring, δ7.2-7.8ppm corresponded to aromatic protons.

In designing a new synthesis of flavone using IBX, the proposed mechanistic pathway[13] could explain the electrophilic character of IBX. It involves attack of the lone pair on the oxygen of the hydroxyl group at the β-carbon of the propen-1-one chain, followed by the attack at the iodine of 1-hydroxy-1λ^5-iodane-2-benziodoxol-3(1*H*)-one-1-oxide (IBX) forming a complex which on hydrolysis could give the 4*H*-Chromen-4-one (**Scheme 16B**).



Scheme 16B. Plausible mechanism of oxidative cyclisation

1.3b. Biological Evaluation:

1.3b.1. Anti-tubercular activity:

Flavones **2** are known to exhibit range of biological activities, more commonly anticancer and antioxidant. However, with limited reports on its anti-tubercular activity, the series of flavone derivatives **2** synthesised during the study could create a platform for its possible action against *Mycobacterium tuberculosis*. Thus, selective flavone derivatives **2** were screened for their *in-vitro* anti-tubercular activity. Anti-tubercular evaluation was done by Microplate Alamar-Blue Dye Assay[34] against the *Mycobacterium tuberculosis* H_{37}Rv strain. The results were compared with the standards used such as Pyrazinamide, Streptomycin and Ciprofloxacin. Inhibition in the activity of the organism was indicated by blue colour, and referred to as sensitive, denoted by letter S, whereas, no inhibition was indicated by pink colour and was referred to as resistance, denoted by letter R (**Table 3, Fig 3**).

Table 3 Anti-tubercular screening[a] of selected flavone derivatives **2b**, **2c**, **2e**, **2j**, **2k**, **2f, 2l, 2m, 2n, 2o**

Compd	Concentration in µg/mL
2b	3.12
2c	3.12
2e	1.6
2j	3.12
2k	1.6
2f	3.12
2l	1.6
2m	1.6
2n	6.25
2o	25

[a]Pyrazinamide and Ciprofloxacin- 3.12 µg/mL(MIC).

The results of the *in-vitro* anti-tubercular activity revealed that all the target flavones derivatives **2** are excellent inhibitors of *Mycobacterium tuberculosis* as that of the standard drugs used with the MIC values in the range of 1.6-25µg/mL.

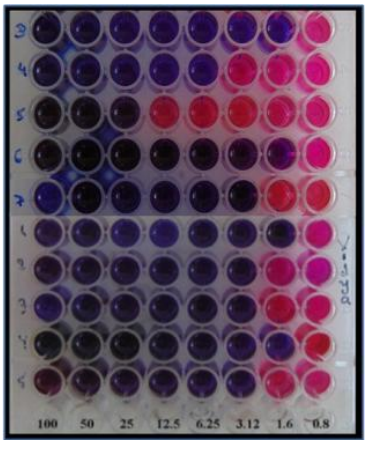
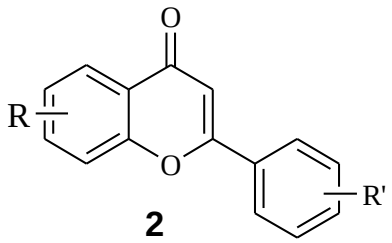


Fig. 3. Alamar Blue Dye Assay: Anti-tubercular screening results of flavones **2b, 2c, 2e, 2k, 2j, 2f, 2l, 2m, 2n, 2o**

1.3b.2. Structure activity relationship studies:

The structure activity relationship studies revealed that presence of electron donating group (NMe_2>OMe) at 4-position enhances the anti-tubercular activity compared to strongly withdrawing NO_2 group.



2

Halogens at 4-position shows significant anti-tubercular activity in the order F>Cl> Br. Replacement of halogen by OH at 7-position increases the potency of anti-tubercular activity.

1.4. CONCLUSION

In conclusion, a new and first approach towards the synthesis of flavones using non-toxic and eco-friendly hypervalent iodine(V) reagent, IBX has been demonstrated. Such a methodology has been well-extended towards the synthesis of hydroxy, and bromo flavones. The work signifies the electrophilic character of IBX, which is an oxidant. The flavone derivatives **2b**, **2c**, **2e**, **2j**, **2k, 2f, 2l, 2m, 2n and 2o** were found to be active against *Mycobacterium tuberculosis*, thus exploring the possibilities of flavones as potent anti-TB drugs. The structure activity relationship has also been predicted.

1.5. EXPERIMENTAL

1.5.1. Chemistry

1.5.1.1. Preparation of Substituted 1, 3-(diphenyl)-prop-2-en-1-one **1**

To a solution of substituted 2-hydroxy acetophenone (1 mmol) in ethanol, aqueous NaOH (2.5 eq, in 10mL H_2O) was added and the reaction mixture was stirred for 15 minutes. To this, substituted benzaldehyde (1mmol) was added and was continued to stir for the required time period. After completion of reaction as monitored by TLC, the reaction mixture was poured in ice cold water and was neutralised by adding concentrated HCl, the solid thus obtained was filtered, dried and recrystallised with ethanol. IR: 3300, 3060, 1690, 1600, 1010, 773, 700, 680cm^{-1}

1.5.1.2. Synthesis of 2-Aryl-4*H*-Chromen-4-one (Flavone) **2a-o**

To a solution of substituted 2'-hydroxychalcone (1mmol) in DMSO (3mL), 1 equivalent IBX was added and the reaction mixture was refluxed for the required period on sand bath. After completion of reaction, ice water was added to it. The solid obtained was filtered, dried and recrystallized from ethanol to give flavones **2**.

1.5.1.2.1. 2-Phenyl-4*H*-chromen-4-one **2a**

IR (KBr): 1660 (C=O), 1465, 1377, 770 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 6.86 (s, 1H, 3-H), 7.57 (m, 3H, 3', 4' & 5'- H), 7.60 (d, 1H, *J*=9.3Hz, 8-H), 7.73 (dt, 1H, *J*=7.4 Hz, 5-H), 7.96 (m, 2H, 2' & 6'- H);^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 162.9, 155.8, 133.4, 131.3, 128.7, 125.9, 125.2, 124.8, 123.6, 117.8, 107.1.

1.5.1.2.2. 2-(4-Chlorophenyl)-4*H*-chromen-4-one **2b**

IR (KBr): 1639 (C=O), 1469, 1409, 1091, 773 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 6.80 (s, 1H, 3-H), 7.46 (dt, 1H, *J*=7.5, 2.3Hz, 6- H), 7.54 (d, 2H, *J*=8.7Hz, 3' & 5'-H), 7.67 (dd, 1H, *J*= 8, 2.1Hz, 8-H), 7.74 (dt, 1H, *J*=7.4, 2.3Hz, 7-H), 7.80 (d, 2H, *J*=8.7Hz, 2' & 6'- H), 8.22 (dd, 1H, *J*= 7.8, 2.1Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 135.7, 131.3, 130.3, 128.9, 127.4, 125.6, 123.8, 123.4, 118.0, 107.3.

1.5.1.2.3. 2-(4-Bromophenyl)-4*H*-chromen-4-one **2c**

IR (KBr): 1640 (C=O), 1478, 1420, 748 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.79 (s, 1H, 3-H), 7.43 (dt, 1H, *J*=8.0, 2.1 Hz, 6- H), 7.50 (d, 2H, *J*=7.6Hz, 3' & 5'-H), 7.54 (dd, 1H, *J*=7.4, 2.1 Hz, 8-H), 7.71 (dt, 1H, *J*=8.4, 2.1 Hz, 7-H), 7.86 (d, 2H, *J*=7.6 Hz, 2' & 6'–H), 8.23 (dd, 1H, *J*=7.8, 2.1 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 132.3, 131.3, 129.6, 126.7, 125.7, 124.0, 123.8, 123.4, 118.0, 107.3.

1.5.1.2.4. 2-(2, 4-Dichlorophenyl)-4*H*-chromen-4-one **2d**

IR (KBr): 1630 (C=O), 1460, 778 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.4 (s, 1H, 3-H), 7.1 (dd, 1H, *J*= 8.4, 2.1 Hz, 8-H), 7.2 (dd, 1H, *J*=7.8, 2.1 Hz, 5'-H), 7.3 (d, 1H, *J*=7.8 Hz, 6'- H), 7.4 (dt, 1H, *J*=7.9, 2.4 Hz, 6- H), 7.7 (dt, 1H, *J*=7.8, 2.1 Hz, 7-H), 7.8 (d, 1H, *J*=2.1 Hz 3'-H), 8.1 (dd, 1H, *J*=8.0, 2.3 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 159.7, 155.7, 135.3, 133.2, 131.4, 129.5, 128.5, 128.0, 127.8, 125.6, 123.8, 123.4, 118.0, 105.6.

1.5.1.2.5. 2-(4-Fluorophenyl)-4*H*-chromen-4-one **2e**

IR (KBr): 1635 (C=O), 1470, 1415, 1078 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.26 (s, 1H, 3-H), 7.24 (dd, 1H, *J*=8.0, 2.2Hz, 8-H), 7.43 (dt, 1H, *J*=7.8, 2.1Hz, 6- H), 7.56 (d, 2H, *J*=7.3Hz, 3' & 5'-H), 7.71 (dt, 1H, *J*=8.2, 2.1Hz, 7-H), 7.94 (dd, 2H, *J*=7.3 Hz, 2' & 6'-H), 8.22 (dd, 1H, *J*=7.9, 2.2Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 163.3, 155.7, 131.3, 129.6, 125.7, 124.9, 123.8, 123.4, 118.1, 115.9, 107.3.

1.5.1.2.6. 2-(4-Methoxyphenyl)-4*H*-chromen-4-one **2f**

IR (KBr): 1625 (C=O), 1460, 1405, 1208 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 3.79 (s, 3H, OCH_3), 6.75 (s, 1H, 3-H), 7.03 (d, 2H, *J*=6.9Hz, 3' & 5'-H), 7.42 (dd, 1H, *J*=8.0, 2.2Hz, 8-H), 7.55 (dt, 1H, *J*=8.0, 2.1Hz, 6- H), 7.68 (dt, 1H, *J*=7.1, 2.1Hz, 7-H), 7.88 (d, 2H, *J*=6.9Hz, 2' & 6' –H), 8.23 (dd, 1H, *J*=8.0, 2.2Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 160.4, 131.4, 128.5, 125.7, 123.8, 123.4, 122.4, 118.0, 114.2, 107.3, 55.6.

1.5.1.2.7. 2-(4-Methylphenyl)-4*H*-chromen-4-one **2g**

IR (KBr): 3080 (Ar-C-H), 1620 (C=O), 1455, 1400 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 2.44 (s, 3H, methyl substituent), 6.79 (s, 1H, 3-H), 7.31 (d, 2H, *J*=7.9 Hz, 3' & 5'-H), 7.41 (dd, 1H, *J*=8.0, 2.1Hz, 8-H), 7.57 (dt, 1H, *J*=8.4, 2.2Hz, 6- H), 7.69 (dt, 1H, *J*=8.2, 2.2Hz, 7-H), 7.83 (d, 2H, *J*=7.9 Hz, 2' & 6' –H), 8.23 (dd, 1H, *J*=8.0, 2.1Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 139.7, 131.3, 129.8, 129.6, 126, 125.6, 123.8, 123.4, 121.3, 118, 107.3.

1.5.1.2.8. 2-(3-Nitrophenyl)-4*H*-chromen-4-one **2h**

IR (KBr): 1646 (C=O), 1530, 1350, 1420 cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 6.56 (s, 1H, 3-H), 7.18 (dd, 1H, *J*= 8.0, 2.2Hz, 8-H), 7.36 (dt, 1H, *J*=8.0 Hz, 6-H), 7.62 (t, 1H, *J*=7.8 & 8.4 Hz, 5'-H), 7.70 (dt, 1H, *J*=7.58 & 8.0 Hz, 7-H), 7.86 (dd, 1H, *J*=8.0, 2.2Hz, 5-H), 8.15 (dd, 1H, *J*=7.8, 2.1Hz, 6'-H), 8.33 (dd, 1H, *J*=8.3, 2.1Hz, 4'-H), 8.67 (s, 1H, 2'-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 140.5, 131.0, 128, 126, 125.6, 124, 123.4, 118, 117.3, 116, 107.

1.5.1.2.9. 2-(2-Chlorophenyl)-4*H*-chromen-4-one **2i**

IR (KBr): 1640 (C=O), 1469, 1409, 773cm^{-1}; ^{1}H NMR(400 MHz, $CDCl_3$): δ 6.52 (s, 1H, 3-H), 7.1 (dd, 1H, *J*=8.1, 2.3Hz, 8-H), 7.35 (dt, 1H, *J*= 7.6, 2.1Hz, 6-H), 7.38 (dt, 1H, *J*=7.4, 2.2Hz, 5'-H), 7.5 (dd, 1H, *J*=8.2, 2.4Hz, 3'-H), 7.64 (dt, 1H, *J*=8.2, 2.3Hz, 4'-H), 7.69 (dt, 1H, *J*=7.8, 2.1Hz, 7-H), 7.76 (dd, 1H, *J*=7.86, 2.3Hz, 6'-H), 7.85 (dd, 1H, *J*=8.2, 2.3Hz, 5–H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 159.7, 155.7, 131.3, 130.7, 130.6, 130.5, 128, 128.3, 127, 125.6, 123.8, 123.4, 118.1, 105.6.

1.5.1.2.10. 2-(4-Nitrophenyl)-4*H*-chromen-4-one **2j**

IR (KBr): 1646 (C=O), 1530, 1350, 1420 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.82 (s, 1H, 3-H), 7.18 (dd, 1H, *J*= 8.1, 2.1Hz, 8-H), 7.36 (dt, 1H, *J*=7.6, 2.3Hz, 6-H), 7.59 (d, 2H, *J*=8.7Hz, 3', 5'- H), 7.6 (dd, 1H, *J*=7.6 & 8.1 Hz, 5-H), 7.70 (dt, 1H, *J*=7.6, 2.1Hz, 7-H), 8.0 (d, 2H, *J*= 8.7Hz, 2', 6'-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 140.5, 131.3, 129.6, 126.4, 125.6, 123.8, 123.4, 118, 117.3, 107.

1.5.1.2.11. 2-(4-Dimethylaminophenyl)-4*H*-chromen-4-one **2k**

IR (KBr): 3200, 3080 (Ar-C-H), 1630 (C=O) cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.86 (s, 6H, methyl substituent), 6.29 (s, 1H, 3-H), 6.63 (d, 2H, *J*=8.8 Hz, 3' & 5'-H), 7.16 (dd, 1H, *J*=8.0, 2.3Hz, 8-H), 7.35 (dt, 1H, *J*=7.6, 2.3Hz, 6-H), 7.57 (d, 2H, *J*=8.8Hz, 2' & 6'-H), 7.68 (dt, 1H, *J*=8.1, 2.6 Hz, 7-H), 7.84 (dd, 1H, *J*=8.1, 2.1Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 163.6, 155.7, 151.4, 135.2, 131.3, 129.6, 126.4, 125.6, 123.8, 123.4, 118, 113.8, 107.3, 40.3.

1.5.1.2.12. 2-(4-Bromophenyl)-7-(hydroxyphenyl)-4*H*-chromen-4-one **2l**

IR (KBr): 3210, 3080 (Ar-C-H), 1650 (C=O), 678 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.44 (s, 1H, 3-H), 6.59 (d, 1H, *J*=1.3Hz, 8-H), 6.84 (dd, 1H, *J*=7.6, 2.1Hz, 6-H), 7.60 (d, 2H, *J*=8.8 Hz, 2' & 6'-H), 7.71 (d, 2H, *J*= 8.8Hz, 3' & 5'–H), 8.1 (d, 1H, *J*=7.8Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.5, 163.6, 155.7, 148.9, 132.3, 129.6, 126.7, 124.02, 119.45, 117.5, 114, 112.32, 107.3.

1.5.1.2.13. 2-(4-Chlorophenyl)-7-(hydroxyphenyl)-4*H*-chromen-4-one **2m**

IR (KBr): 3215, 3085 (Ar-C-H), 1649 (C=O), 773 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ6.5 (s, 1H, 3-H), 6.59 (d, 1H, *J*=1.4Hz, 8-H), 6.84 (dd, 1H, *J*=7.8, 2.3Hz, 6-H), 7.47 (dd, 2H, *J*=8.9 Hz, 3' & 5'-H), 7.72 (dd, 2H, *J*=8.8 Hz, 2' & 6'-H), 8.0 (dd, 1H, J=7.8 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.7, 148.9, 135.7, 130.3, 128.6, 124.2, 119.5, 117.5, 114, 112.2, 107.3.

1.5.1.2.14. 2-(4-Bromophenyl)-5'-(bromophenyl)-4*H*-chromen-4-one **2n**

IR (KBr): 3085 (Ar-C-H), 1590 (C=O), 678 $cm^{-1}$$^{1}H$ NMR (400 MHz, $CDCl_3$): δ6.47 (s, 1H, 3-H), 7.17 (dd, 1H, *J*=8.1Hz, 8-H), 7.46 (t, 1H, *J*=8.1Hz, 7-H), 7.61 (d, 2H, *J*=8.8Hz, 3' & 5'-H), 7.72 (d, 2H, *J*=8.8Hz, 2' & 6'-H), 8.1 (dd, 1H, *J*=7.8, 1.9Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.5, 163.6, 155.7, 148.9, 132.3, 129.6, 126.7, 124.2, 119.5, 117.5, 114.0, 112.2, 107.3.

1.5.1.2.15. 2-(4-Chlorophenyl)-5'-(bromophenyl)-4*H*-chrom-4-one **2o**

IR (KBr): 3090 (Ar-C-H), 1599 (C=O), 773 cm^{-1} ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.52 (s, 1H, 3-H), 7.12 (dd, 1H, *J*=8.1, 2.3Hz, 8-H), 7.47 (t, 1H, *J*=8.1, 2.1Hz, 7-H), 7.48 (dd, 2H, *J*=8.8 Hz, 2' & 6'-H), 7.73 (d, 2H, *J*= 8.8 Hz, 3' & 5'-H), 8.07 (dd, 1H, *J*=7.8, 1.9Hz, 6-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.6, 135.2, 132.3, 129.6, 129.5, 126.7, 124, 117.8, 111.7, 107.5.

1.5.2. Anti-tubercular evaluation:

The anti-mycobacterial activity of synthesized flavone derivatives **2a-o** was assessed against *Mycobacterium tuberculosis* $H_{37}RV$ strain using Microplate Alamar blue dye assay (MABA). Briefly, 200µg/mL of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds was made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µg/mL of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink.

1.6. REFERENCES

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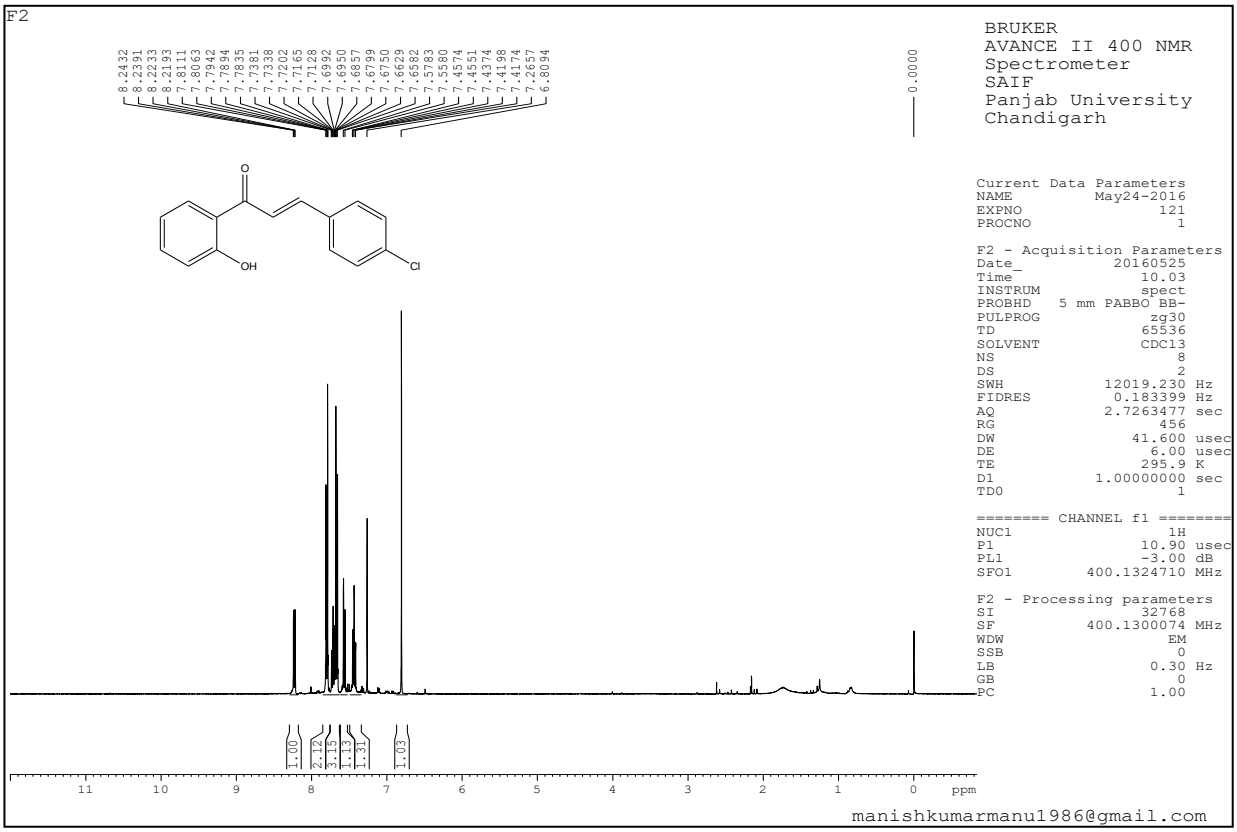
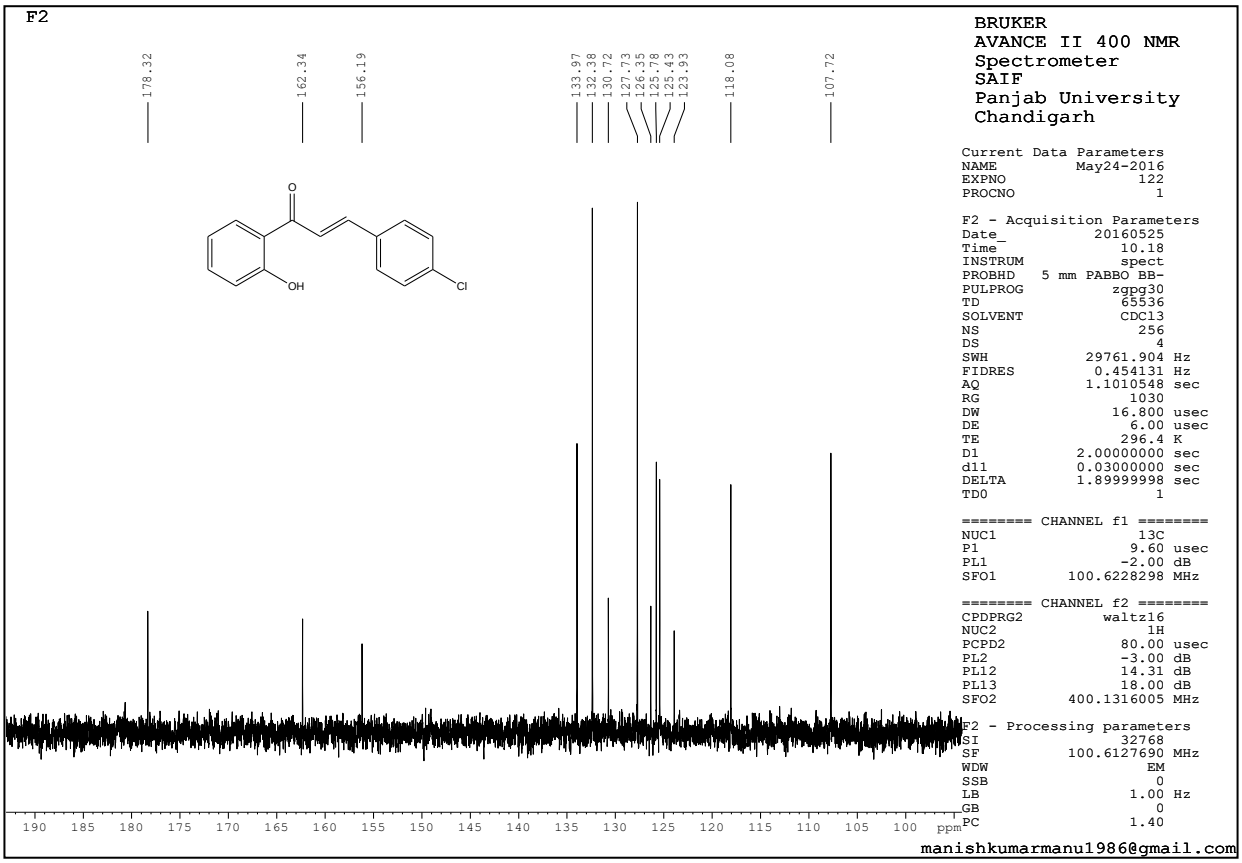
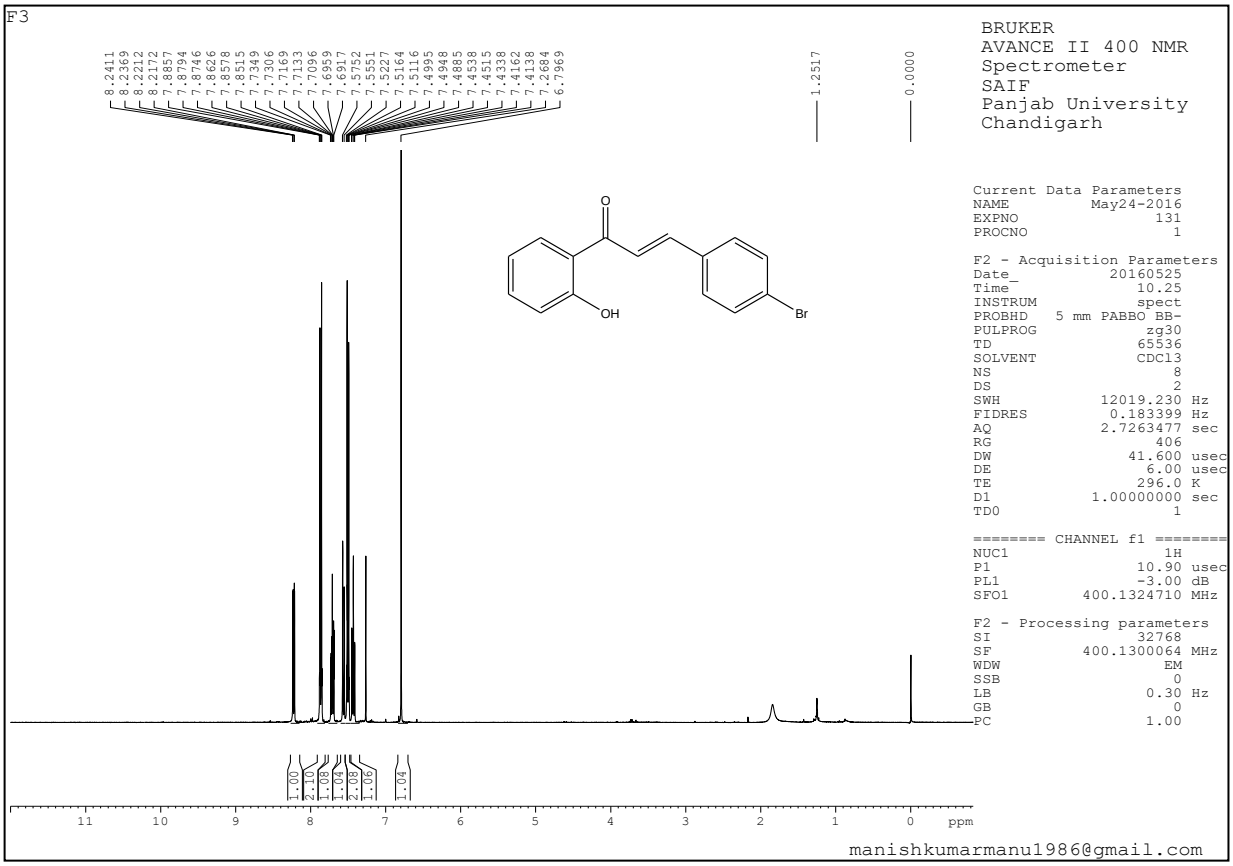
Spectral Data



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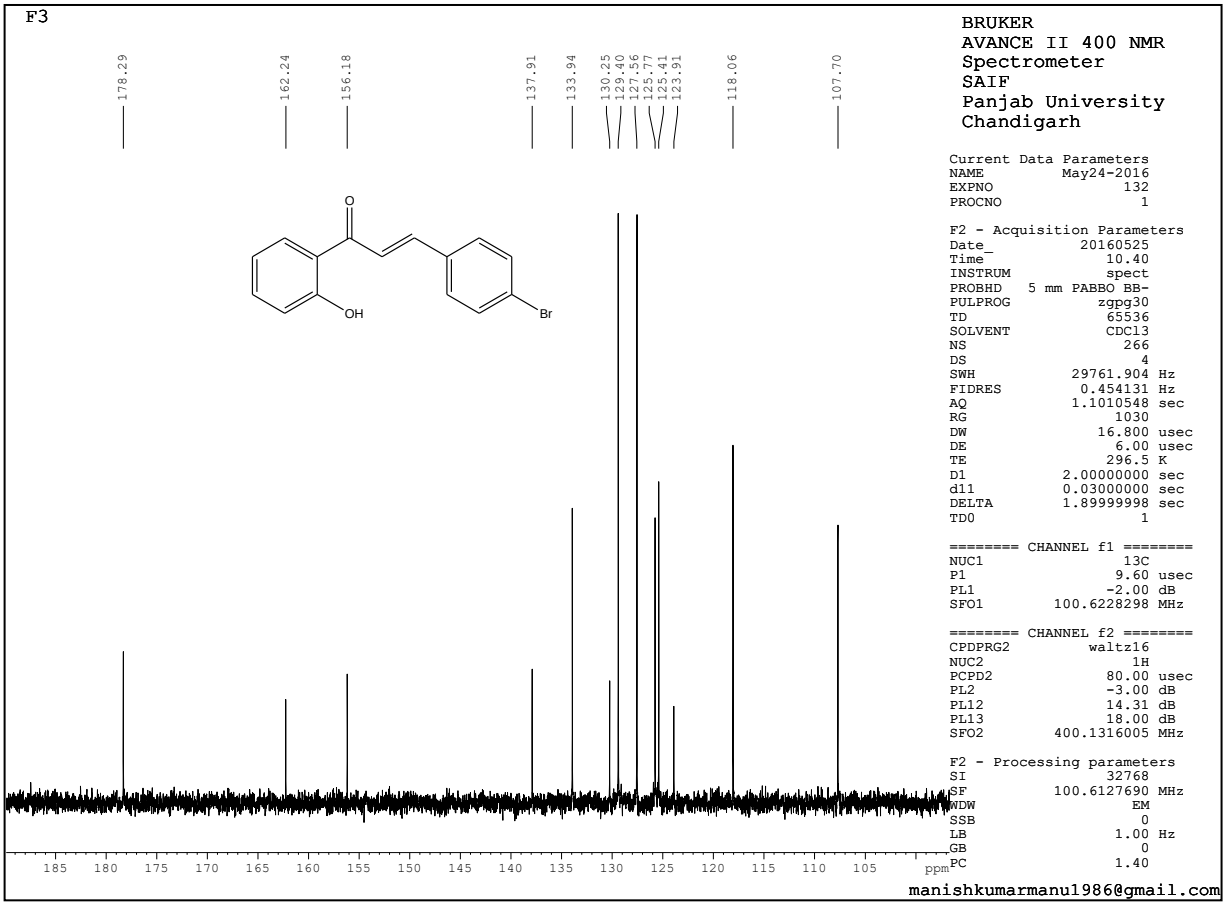
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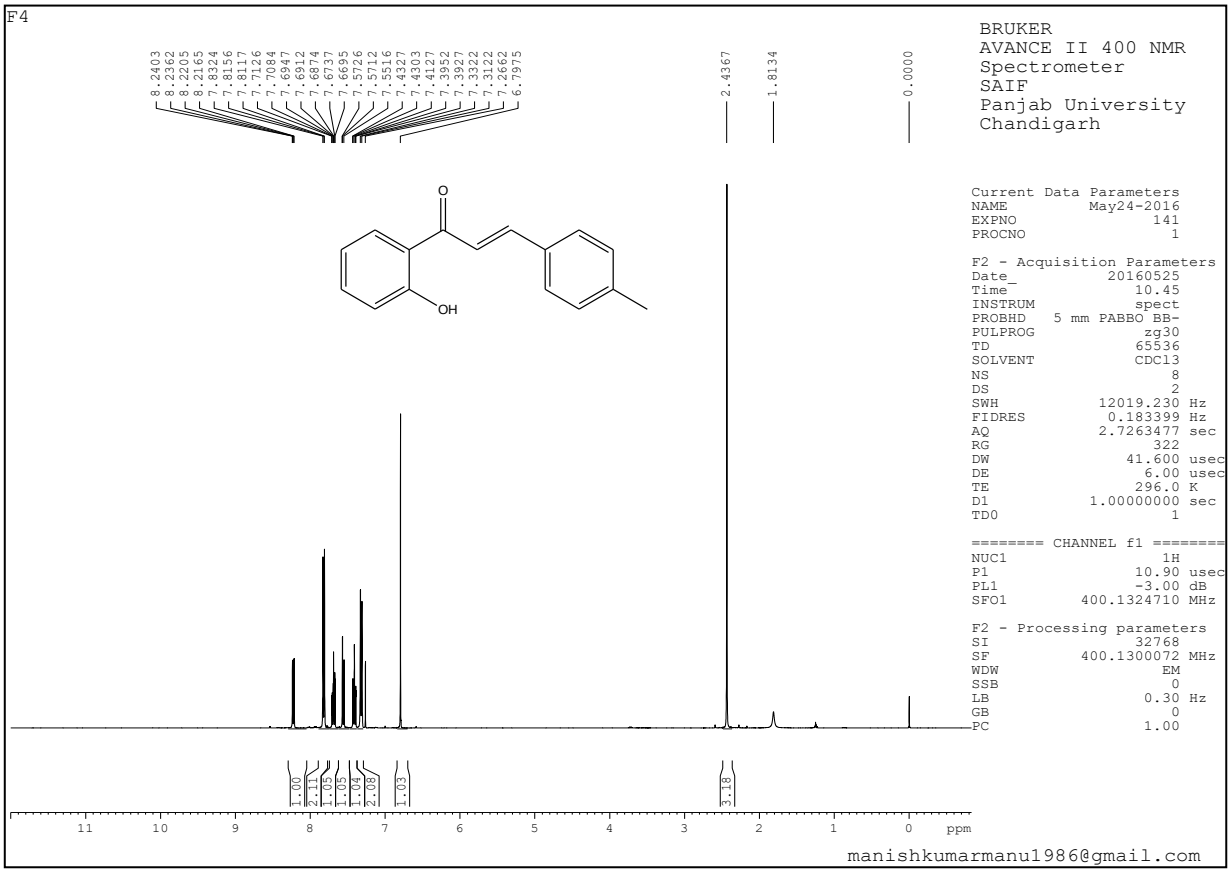
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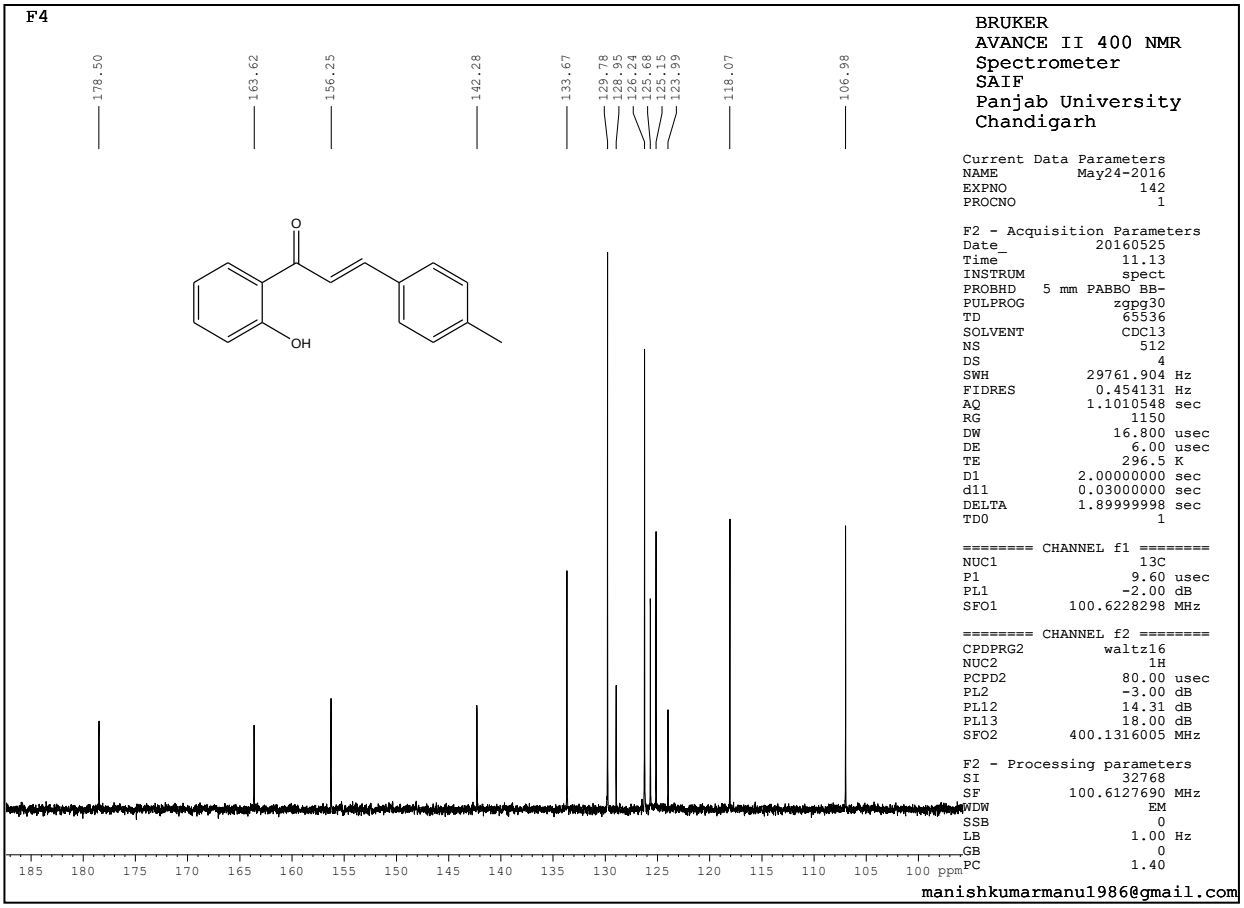
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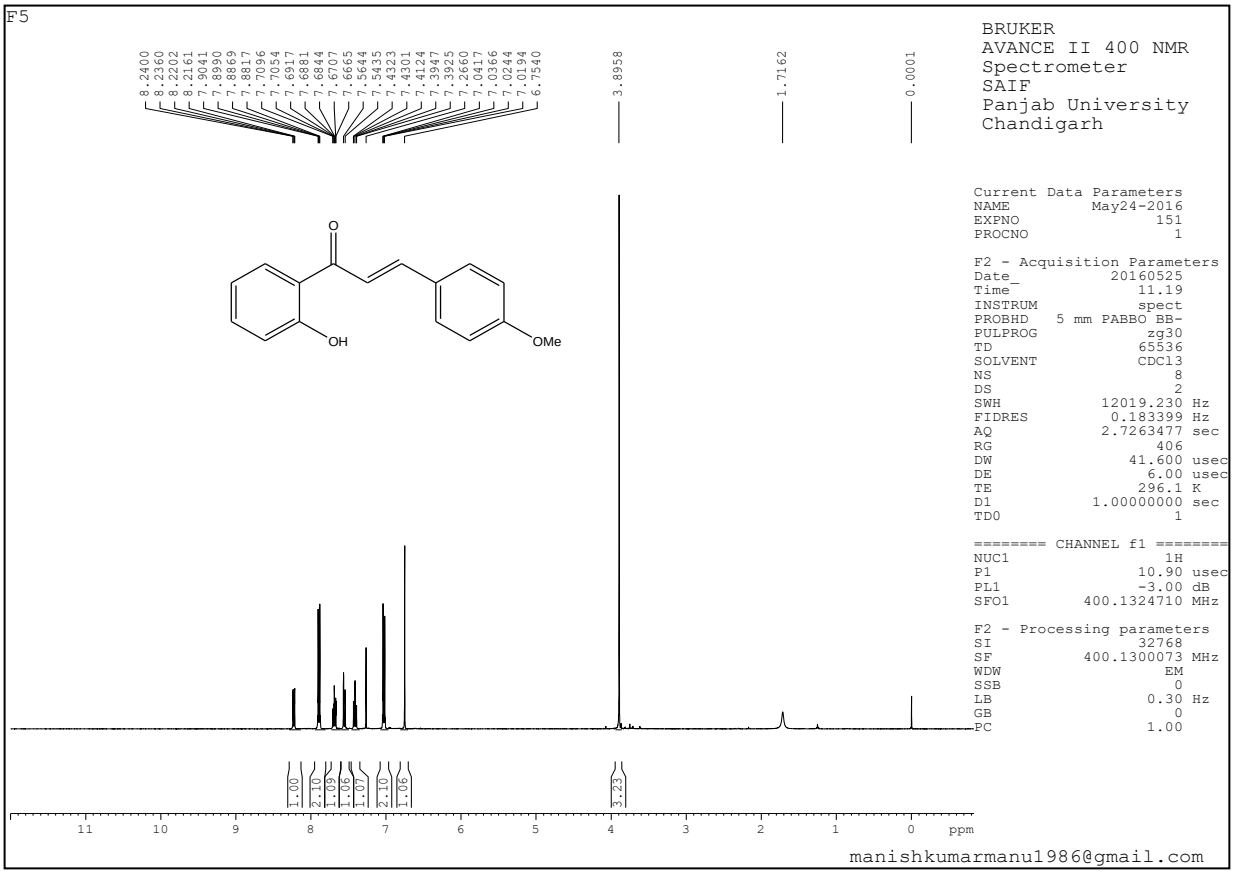
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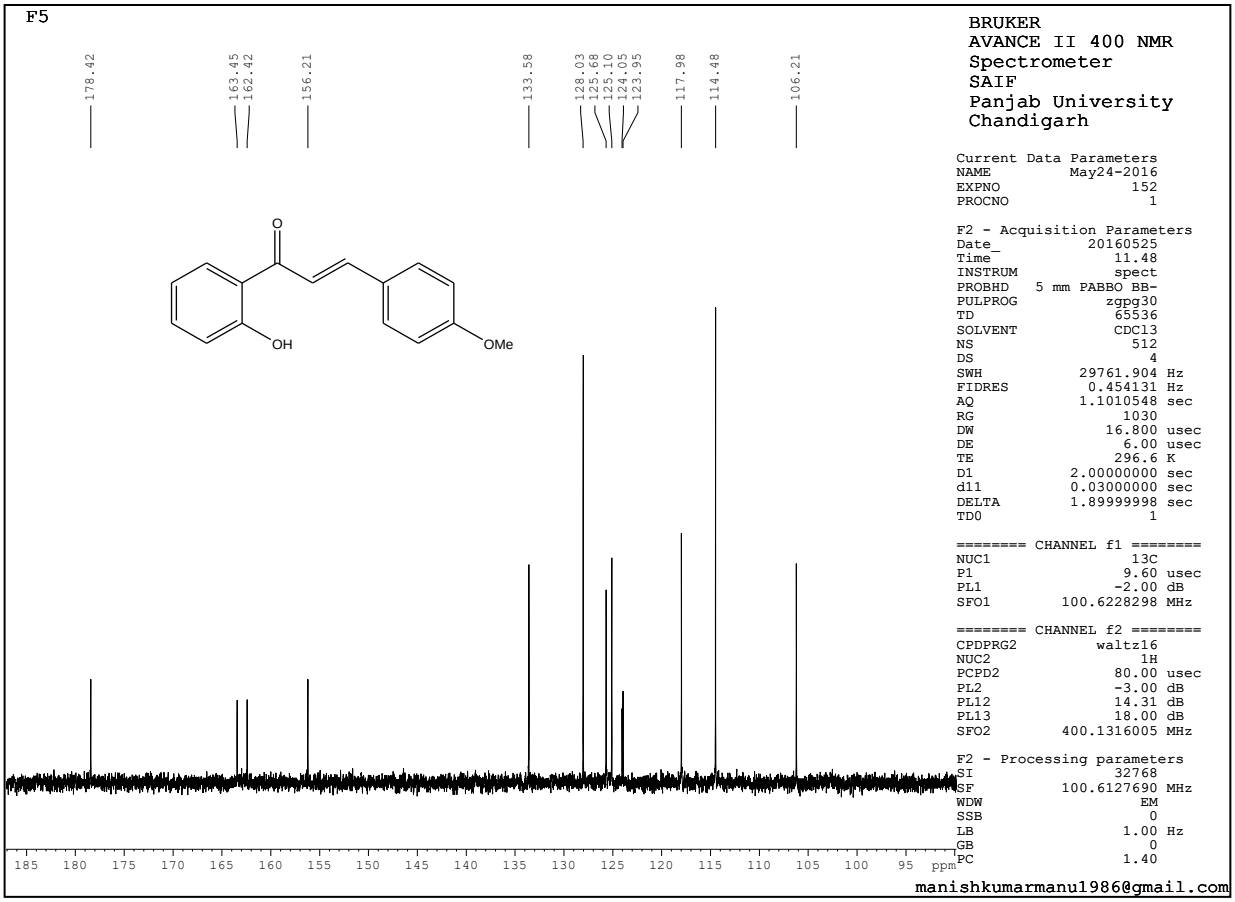
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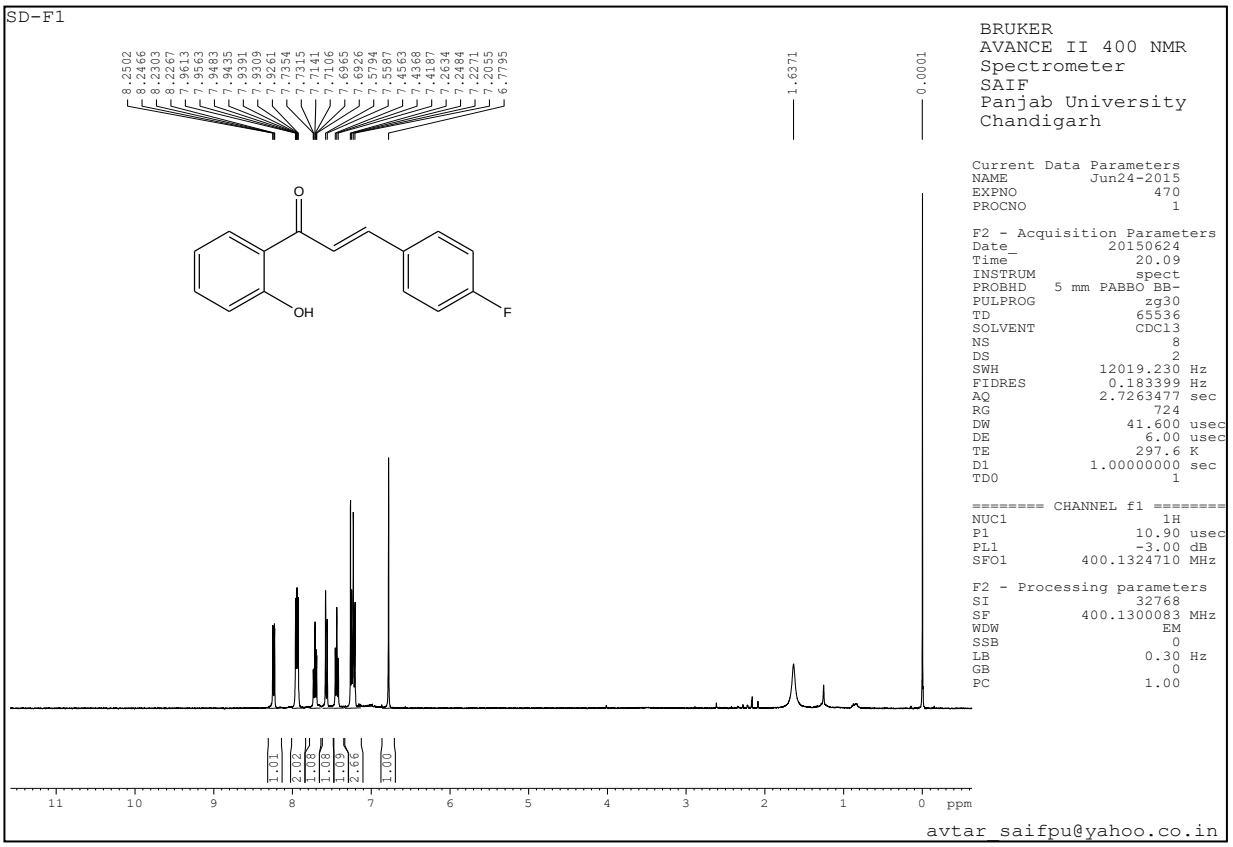
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CHAPTER 2

Synthesis of Benzoxazole-N-Heterocyclic Hybrids as Tyrosine Kinase Inhibitors

2.1. INTRODUCTION

Cancer is affecting millions of lives all around the world[1, 2] with the sophisticated and different changing lifestyle, the growth of cancer disease has erupted rapidly. The most common type of cancers includes lung cancer, oral cancer and breast cancer.[3] Chemotherapy has several limitations and side effects but not exclusively leading to mentioned effects; it has advantageous role for the treatment of cancer particularly in case of late-stage detection where it is the best choice of treatment. Several types of breast cancer carcinoma have been reported that has been responsible for painstaking deaths all around the world. In view of this there is great need to search for newer breast cancer drug targets. Moreover, the scope to develop novel therapy strategies becomes the need and a challenging task. From cancer drug target study, basal like subtypes triple negative breast cancer (TNBC) constitutes to about 80% of basal like tumors and is responsible for 10-15% breast carcinoma and also shows lack of expression of both hormone receptor (Progesterone and Estrogen) and HER2-receptor over expression.[4,5] A drug target for such cancer becomes evident as TNBC and basal like cancers are considered to be in the lead as an aggressive disease with greater chances of metastasis. The therapy for such conditions namely, Trastuzumab (**Fig.4.I**) cuts the risk of recurrence to half-compared to chemotherapy alone. Trastuzumab is given through veins in every 3 weeks for 1 year.[6-9] Scaffold containing heterocyclic conjugates such as 3-Pyrimidinylazaindole analogue (**Fig.4.II),** potent inhibitor of triple negative breast cancer which cuts the risk to upto 90% of tumor growth inhibition as compared to the other leads.[10]

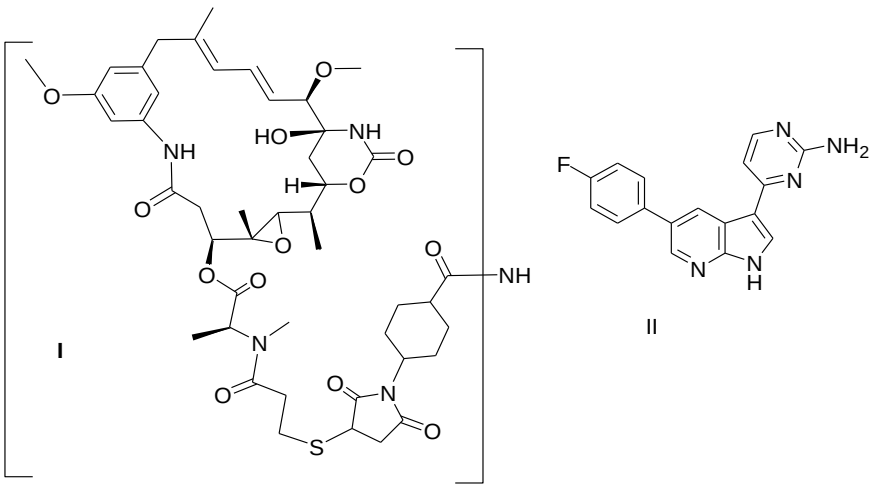


Fig.4. Representative Structures of breast cancer drugs: **I**. Trastuzumab, sold under the brand name Herceptin among others, is a monoclinical antibody, used to treat HER2 positive breast cancer; **II**. 3-Pyrimidinylazaindole, a potent inhibitor of triple negative breast cancer.

Human protein kinase forms a large family of enzymes commonly known as human Kinome encoded by about 1.7% of different types of human genes.[11] In this group of enzymes, tyrosine kinase has been the first protein kinase which has been well identified, described with respect to its location in the cell. Keeping in view the structural features, small molecule kinase inhibitors can be of great help to design and develop specifically targeted therapies against breast cancer. The advantage of such inhibitors is high selectivity, better efficacy and specific action on protein kinase receptors.

In the designing of small drug like molecules, the concept of molecular hybridisation of heterocyclic compounds could be challenging task to gain potency in acting against tyrosine kinase enzymes. In search of new molecular hybrid designs, our focus was on benzoxazole as a core unit as it forms an important class of bicyclic heterocycles having wide spectrum of biological applications.[12] Benzoxazole ring as a core pharmacophoric unit is found in various class of natural and synthetic compounds showing varied biological properties and has been studied for their antibacterial and anti-fungal,[13,14]anticancer[15,16]and HIV-1 reverse transcriptase inhibitors.[17]It constitutes subunits of commercially available drug molecules such as; Flunoxaprofen which is a non-steroidal anti-inflammatory drug,[18] NSC693638- an anticancer agent[19] L-697,661 a reverse transcriptase inhibitor,[20] Nataxazole, an anti-tumor agent[21] related to anti-tumor drugs like UK-1 and AJ19561[22] **Fig.5**. The synthesis of benzoxazolyl-carbohydrazide derivatives and their *in-silico* docking and *in-vitro* anticancer study has been also reported.[23] Combretastin A-4 and benzoxazole analogues have been reported to exhibit more potent anti-cancer activity then the standard drug against A-549 and MCF-7 cell lines.[24]

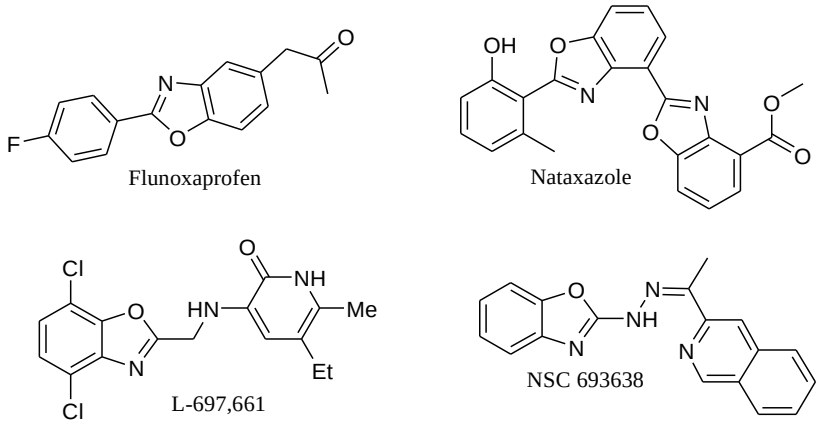
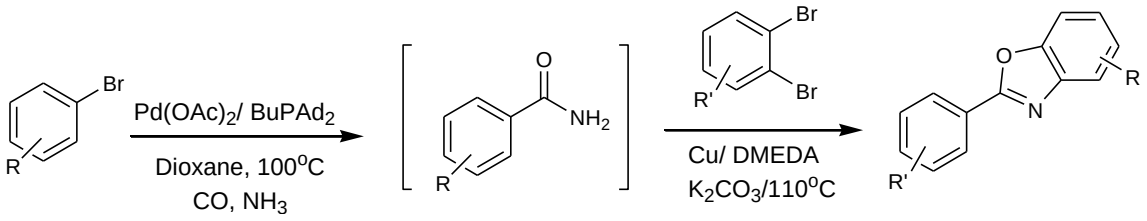


Fig.5. Bioactive Compounds containing Benzoxazole moiety: Flunoxaprofen-NSAIDs, L-697,661 a reverse transcriptase inhibitor, Nataxazole an anti-tumor, NSC693638- an anticancer agent

Nitrogen-based heterocycles are an important class of compounds in pharmaceutical and agrochemical industries. Their structural diversity has made them attractive targets in the synthesis of alkaloids and is important precursors to many biologically important active compounds. They have emerged as integral parts of various drugs known in day to day life. Some of the nitrogen heterocycles of biological importance includes: quinoxaline, indoles, quinolines, pyridines etc. Designing of heterocyclic hybrids[25] has led to newer breakthroughs in the need for treatment of cancer and so it was visualized that heterocyclic hybrids of N-heterocycles with benzoxazole would have a promising effect on the biological activity. Thus, in continuation to our work on designing heterocyclic molecules of biological importance using eco-friendly strategies[26-29] herein, we present the synthesis of benzoxazoles using silica chloride. This methodology was further extended towards synthesis of six novel heterocyclic scaffolds. The molecular docking studies and biological evaluation has also been discussed, revealing our compounds to have the best hits as anti-cancer activity. Development of solid acid catalysed[30-33] synthesis of benzoxazoles and their action as inhibitors of protein kinase enzyme has been the focus of the work.

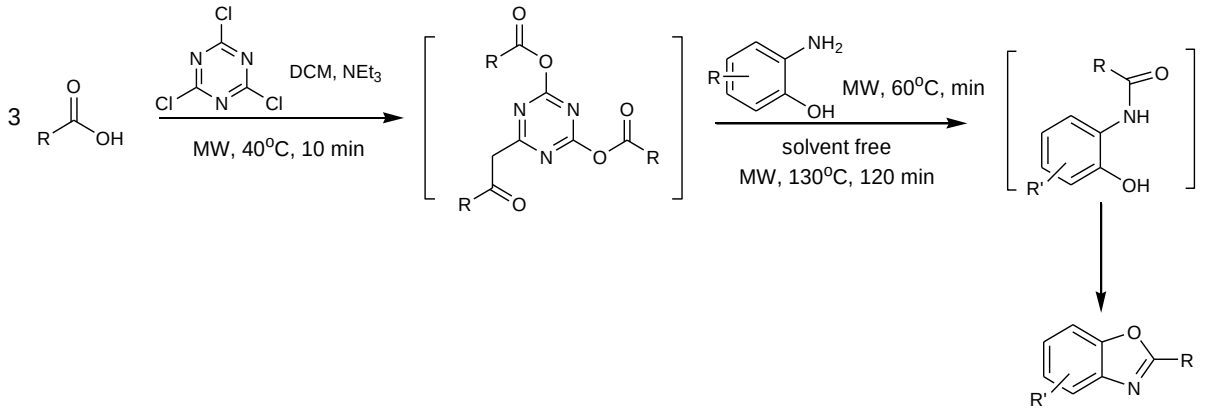
2.2. LITERATURE

Wu X-F. *et al*[34] has reported one pot synthesis of benzoxazoles from arylbromides by successive copper and palladium catalysed reactions involving aminocarbonylation and subsequent copper catalysed coupling (**Scheme 17**).



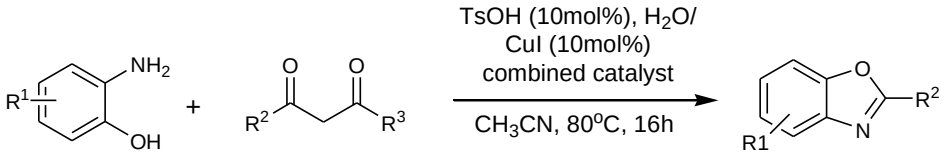
Scheme 17

Microwave assisted synthesis of series of various poly-substituted benzoxazoles were synthesized from carboxylic acids has been demonstrated by Nieddu G. *et al*.[35] It basically involves reaction of three moles of carboxylic acid with triazine under microwave leading to an intermediated which on reacting with *o*-aminophenol under solvent free microwave conditions resulted into polysubstituted benzoxazoles (**Scheme 18**).



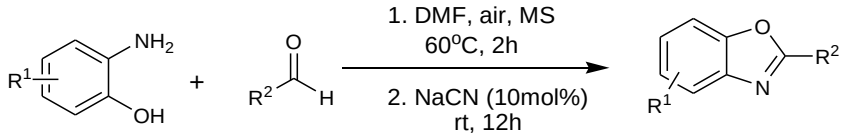
Scheme 18

Yu X. *et al*[36] suggested synthesis of benzoxazoles from *o*-aminophenols and 1,3-diketones catalysed by a combination of bronsted acid and CuI to obtain various substituted benzoxazoles (**Scheme 19**).



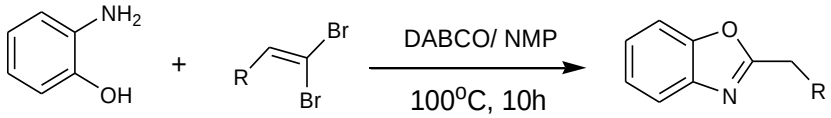
Scheme 19

Facile synthesis of benzofused heteroaromatic compounds via aerobic oxidation in presence of cyanide catalyst is well presented by Cheon C-H *et al*[37] through formation of Schiff bases by a one-pot methodology (**Scheme 20**).



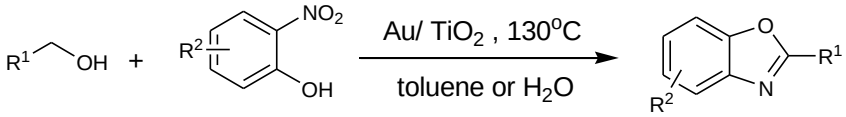
Scheme 20

Direct coupling of 1,1-dibromoethenes with *o*-aminophenols has been achieved by Tao K. *et al*[38] to form benzoxazoles under mildly basic conditions (**Scheme 21).**



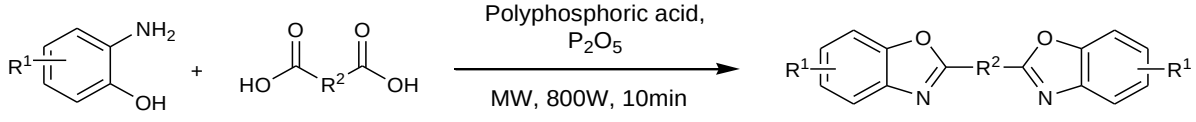
Scheme 21

Tang L. *et al*[39] suggested an efficient synthesis involving *in-situ* reduction of *o*-nitrophenol catalysed by gold nanoparticles supported on TiO_2 followed by condensation with aliphatic alcohol to afford highly selective synthesis of benzoxazoles and benzimidazoles. The reaction has a good tolerance to air and water, a wide substrate scope and represents a new avenue for C-N and C-O bond formation (**Scheme 22)**.



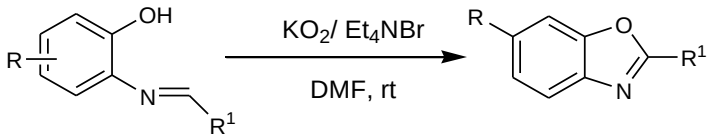
Scheme 22

Lee W-L. *et al*[40] have carried out synthesis of benzoxazoles derivatives faster and effectively under microwave illumination and applied for the phosphors of LEDs. This procedure facilitates the reaction and LEDs with lab-made organic phosphors, which exhibits high thermal stability, low specific gravity and high fluorescent quantum yields (**Scheme 23).**



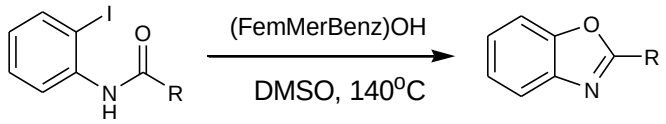
Scheme 23

Singh K. N. *et al*[41] developed an easy and efficient oxidative cyclisation of Schiff bases by condensation of *o*-aminophenol and aromatic aldehydes using *in-situ* generated tetraethylammonium superoxide in aprotic solvent (**Scheme 24).**



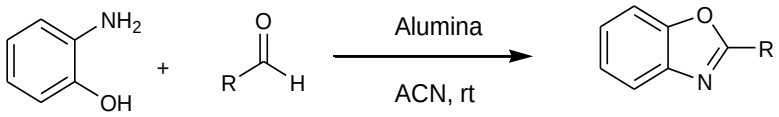
Scheme 24

Jadhav J. *et al*[42] demonstrated herein, the O-arylation of *o*-iodoanilides to corresponding 2-phenylbenzoxazoles using ferrocene tethered polymer supported ionic liquid phase catalyst in DMSO. Their interest in corporating ferrocene is to provide unique geometry to the ligand environment (**Scheme 25**).



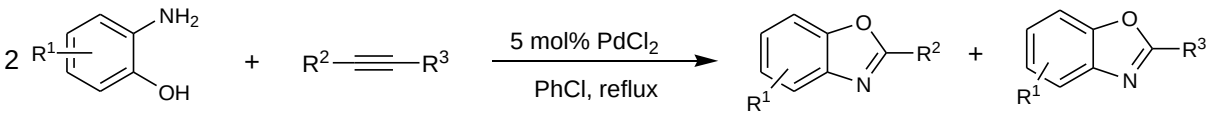
Scheme 25

Suryavanshi A. W. *et al*[43] suggested a novel, highly efficient method for synthesis of benzoxazole derivatives by oxidative cyclisation method. The significant advantage of this method has been greener approach, short reaction time, simple workup, easy preparation and handling of catalyst (**Scheme 26**).



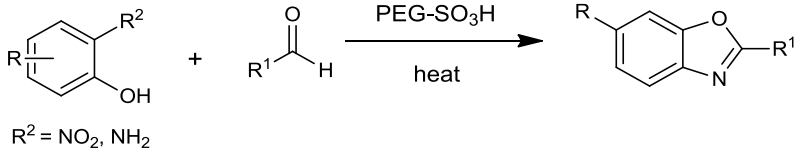
Scheme 26

Xie H-Z. *et al*[44] has developed the palladium catalysed cleavage of C-C triple bonds in the presence of *o*-aminophenol using chlorobenzene as a solvent under the mild reaction conditions, affording benzoxazoles in good to excellent yields (**Scheme 27**).



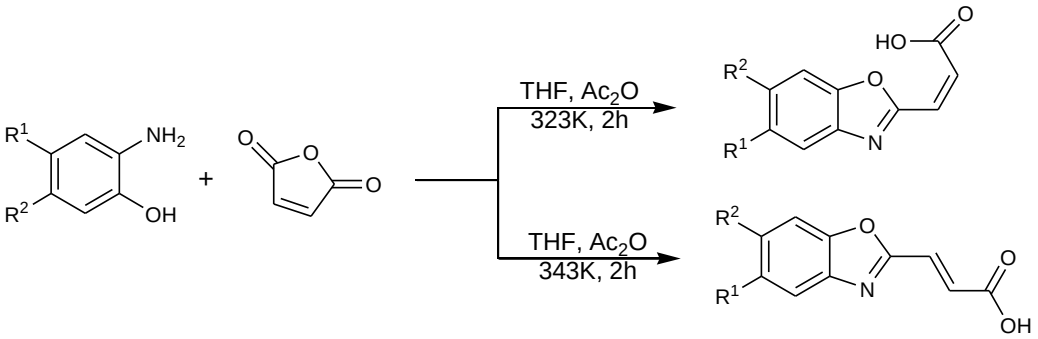
Scheme 27

Chikkhale R. V. *et al*[45] describes a greener route towards benzoxazole synthesis involving the use of highly efficient reusable Poly (ethylene glycol)-bound sulfonic acid catalyst by heating with high purity (**Scheme 28**).



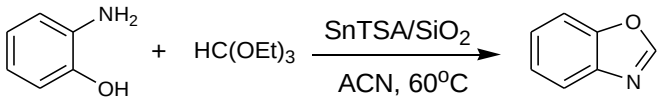
Scheme 28

Flores S. C. A. *et al*[46] established a one pot synthesis for *E* and *Z* stereoisomers derivatives of 3-benzoxazoyl propionic acid by application of thermodynamic and kinetic control (**Scheme 29**).



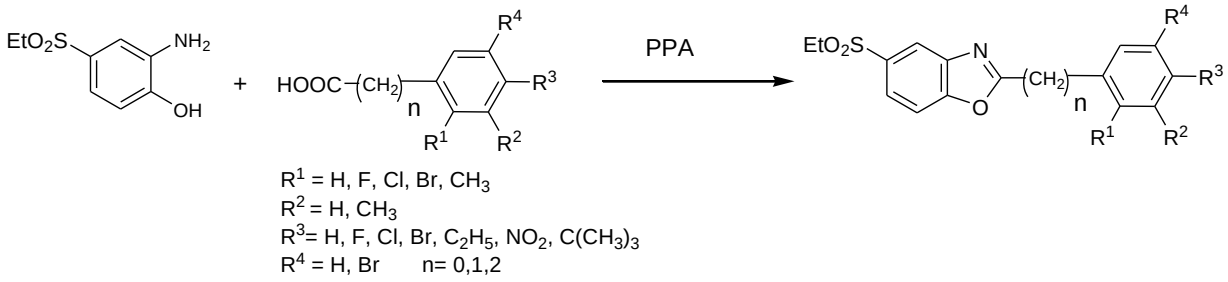
Scheme 29

Srivani A. *et al*[47] demonstrated an efficient solid acid catalyst for the synthesis of benzoxazoles under mild reaction conditions within shorter time. The exchange of tin with the protons of Silico tungstic acid results in generating the lewis acidity and thereby resulting in the increase in catalytic activity for the benzoxazole synthesis (**Scheme 30**).



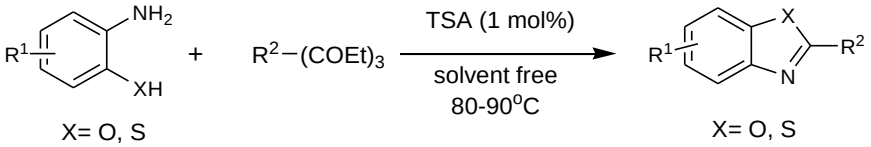
Scheme 30

Temiz-A. *et al*[48] have designed and synthesized the series of various antimicrobial agents in this work, wherein they chose an ethylsulphonyl fragments at the fifth position of benzoxazoles with a substituted phenyl/benzyl/ 2-phenylethyl at the 2nd position of it (**Scheme 31**).



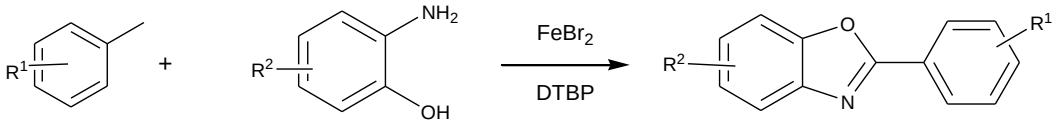
Scheme 31

Use of tungstate sulphuric acid has been employed in the synthesis of benzoxazole derivatives by Farahi M. *et al*[49], via reactions of orthoesters with *o*-aminophenols or *o*-aminothiophenols in high yields (**Scheme 32**).



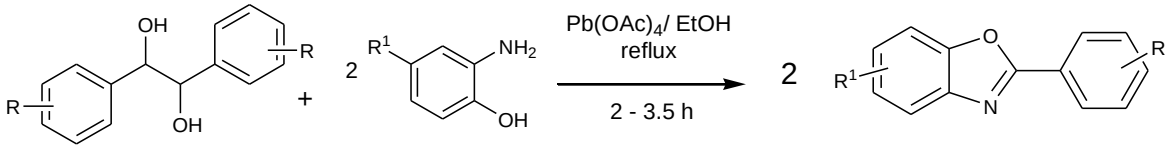
Scheme 32

A practical and simple synthesis of benzoxazoles from easily available substrates was developed by Gu L *et al*[50] in which use of iron as a catalyst to trigger the oxidative process from simple toluene derivatives and aminophenols. A straightforward approach to access substituted benzoxazoles (**Scheme 33**).



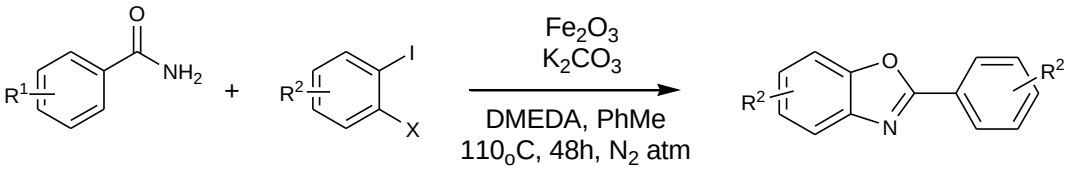
Scheme 33

Kalegowda S. and Narasashetty J.[51] declared a one pot cyclocondensation of 1,2-diols and *o*-aminophenols involving cleavage of C-C bend and formation of C-O and C-N bond in a single synthetic operation in presence of lead tetraacetate under refluxing (**Scheme 34**).



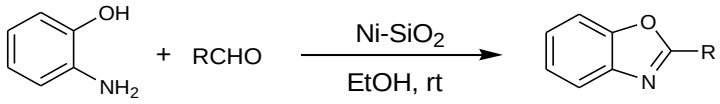
Scheme 34

An eco-friendly and efficient method towards synthesis of *o*-arylbenzoxazoles via a domino iron-catalysed C-N/ C-O cross-coupling reaction has been described by Yang B. *et al*[52] (**Scheme 35**).



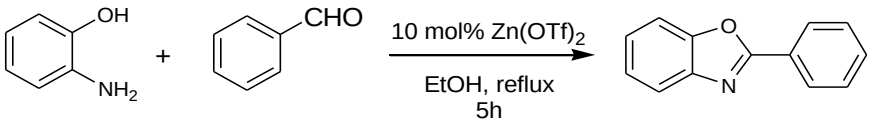
Scheme 35

Madilla S. and Jonnalagadda S.B[53] suggested a simple one-pot synthesis of benzoxazoles derivatives catalysed by nickel supported on silica from *o*-aminophenols and substituted aldehydes (**Scheme 36**).



Scheme 36

Another efficient method has been reported by Ramineni *et al*[54] using zinc triflate under refluxing condition for the series of benzoxazoles (**Scheme 37**).



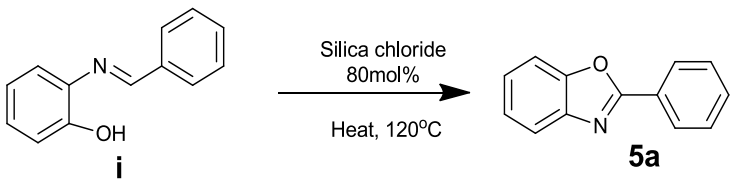
Scheme 37

2.3. PRESENT WORK AND DISCUSSION

2.3a. Chemistry

Our purpose was to design and implement a greener approach for synthesis of *o*-arylbenzoxazoles analogues with the ease of purification, characterisation and biological screening. In our present study, one pot strategy was introduced, to build molecules in short duration of time. The preparation of silica chloride was carried out by literature known procedure.[55] Initially, a two step procedure was adopted wherein, to a previously prepared Schiff base **i** from equimolar ratio of aromatic aldehydes **3** and *o*-aminophenol **4**, was added catalytic amount of silica chloride catalyst and was heated at 120^{o}C for an hour on sand bath under solvent free conditions. The progress of the reaction was monitored by TLC (2:1 hexane/ethylacetate). The fluorescence spot was obtained for the product with no starting reactant. After completion of the reaction, the catalyst was filtered off using ethylacetate and the filtrate was evaporated to dryness by suction. The crude product was further purified using petroleum ether. The structure of the compounds was confirmed by IR, ^{1}H NMR spectroscopy. As we were interested in carrying out one-pot synthesis of benzoxazoles **5**, the optimisation of reaction conditions was performed with different mole ratios of the catalyst and at different temperature on the first step product (Schiff base **i**). The detailed studies on reaction conditions using different mole ratios of catalyst (**Table 4, entries 5-12**) and different temperature condition (**Table 4, entries 1-4**) revealed that, catalyst not in mole ratio but in 1 equivalent and temperature 120^{o}C provided the best yields within the mentioned time. We report here, the usage of silica chloride as a reusable acid catalyst for the synthesis of *o*-arylbenzoxazole analogues. The new protocol doesn't require use of any solvent and can be carried out efficiently. Thus, it can be concluded that silica chloride mediated solvent free approach is the most efficient in terms of ease of isolation and yield of the products.

Table 4 Optimisation of the reaction conditions for the given reaction with variation in temperature and mol ratio of the catalyst.

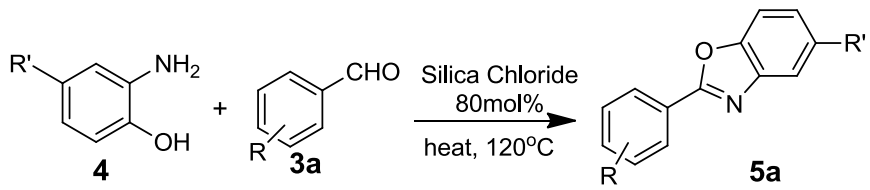


i **5a**

Entry	Catalyst (mol%)	Temp (^{o}C)	Time h	Yield (%)

1	80	Rt	24	-
2	80	80	12	-
3	80	100	12	-
4	80	120	3.5	75
5	5	120	12	-
6	10	120	12	-
7	20	120	12	10
8	30	120	8	40
9	40	120	3.5	70
10	60	120	3	70
11	80	120	3	75
12	1 eq	120	3	82

To establish the scope and generality of this novel approach, reactions of various aldehydes **3a** with *o*-aminophenol **4** was carried out using same reaction conditions (**Scheme 38**). Consequently, a wide variety of aryl benzoxazole derivatives (**5a$_1$-5$_{18}$**) having different functional groups was obtained successfully in yields ranging from 70-80% (**Table 5**). The use of silica chloride serves a crucial role; it activates the aldehyde group followed by imine formation which facilitates the cyclisation to aryl-benzoxazole. To verify the recyclability of silica chloride catalyst, it was filtered after first cycle and washed thoroughly with ethylacetate. Thereafter, the catalyst was reused for the synthesis of compound **5a$_7$** (substrate with electron withdrawing substituent) and **5a$_6$** (substrate with electron donating substituent). However, the catalyst was effective upto four cycles in case of compound **5a$_6$** with no significant decrease in yield of the product and in lesser time whereas, the synthesis of compound **5a$_7$** was successfully carried out upto five cycles (**Fig 6**).



Scheme 38 One-pot synthesis of benzoxazole derivatives (**5a$_1$-a$_{18}$**)

Table 5 Synthesis of benzoxazole derivatives **$5a_1$-a_{18}**

Comp 5a	R	R'	Time h	Yield %	Melting Point ^{o}C
1	2,4- Cl	H	1	75	125-127 (127)
2	4- Cl	H	1.5	75	144-148(148-150)
3	4- Br	H	1.5	71	150-152(157-158)
4	3-NO_2	H	2.5	79	99-102(103-105)
5	2-NO_2	H	2.5	72	97-100(98-102)
6	4-OMe	H	3	77	114-116(113-114)
7	4-NO_2	H	2.5	79	256-260(268)
8	H	H	3.5	78	98-100(101)
9	2-Cl	H	1.5	70	68-72(73)
10	4-Me	H	3	79	104-106(101-103)
11	3-Cl	H	1.5	72	120-122 (122-124)
12	4-OH, 5-NO_2	H	2.5	75	170-174 (178)
13	2-OH, 5-NO_2	H	2.5	71	188-192 (188)
14	4-F	H	1.5	79	90-92 (92-95)
15	3-Br	H	1.5	72	133-137 (136-138)
16	4-NMe_2	H	3	70	179-182(182-183)
17	5-Br, 4-OH, 3-OMe	H	2.5	78	187-190 (190-192)
18	4-OH-3,5- Br	H	2.5	70	166-169 (168-170)

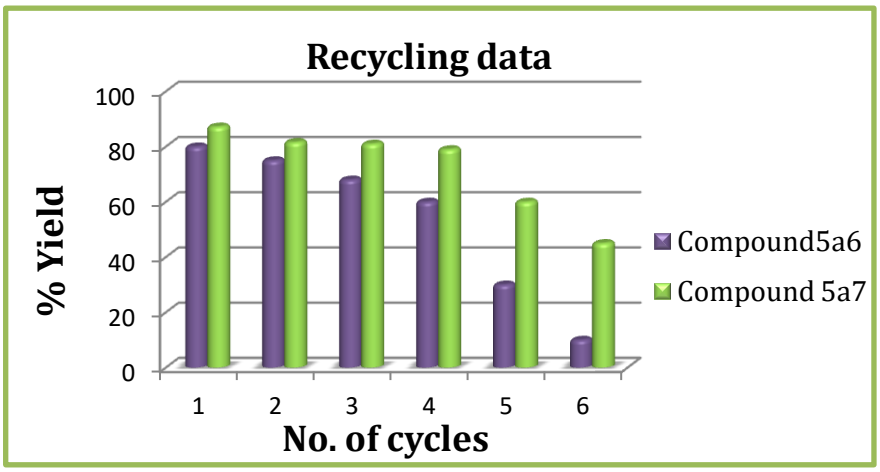
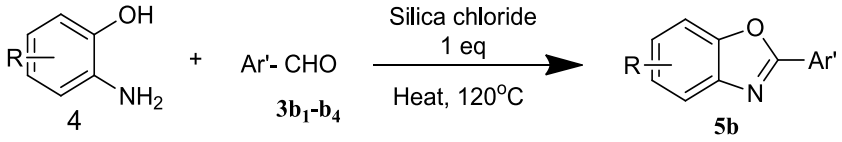


Fig 6. Effect of Recyclability of catalyst on different substrate

However, our focus was on designing of novel benzoxazole-*N*-heterocyclic hybrids **5b** as the core unit as potential kinase inhibitors. For this, we were interested in the molecular hybridization of benzoxazole with an appropriate nitrogen heterocycle. Our previous work on quinoxalinyl chalcones depicted good anti-tubercular and anti-cancer activity.[56]As a continuation to the work on designing of heterocyclic hybrids, an attempt has been made to prepare the new substituted benzoxazole derivatives bearing heterocyclic moiety. Six new benzoxazole-*N*-heterocyclic hybrids **$5b_1$-b_6** has been prepared by the same strategy under solvent free conditions from commercially available aldehydes, previously synthesised quinoxaline-2-carbaldehyde[57]and 2-chloro-3-formyl quinoline[58] (**Scheme 39).** All the six benzoxazole-*N*-heterocyclic hybrids **$5b_1$-b_6** were obtained in good yields (**Table 6).**



R= 4-H,
= 4-Br

$3b_1$, Ar=4-Nitrobenzaldehyde
$3b_2$, Ar=4-Bromobenzaldehyde
$3b_3$, Ar= Quinoxaline-2-carbaldehyde
$3b_4$, Ar=2-Chloro-3-formylquinoline

$5b_1$, R'=5-Br, Ar=4-Nitroophenyl
$5b_2$, R'=5-Br, Ar=4-Bromophenyl
$5b_3$, R'=5-Br, Ar=Quinoxalinyl
$5b_4$, R'=H, Ar=Quinoxalinyl
$5b_5$, R'=H, Ar=2-Chloroquinolinyl
$5b_6$, R'=5-Br, Ar=2-Chloroquinolinyl

Scheme 39 Synthesis of novel *o*-arylbenzoxazole conjugates **$5b_1$-b_6**

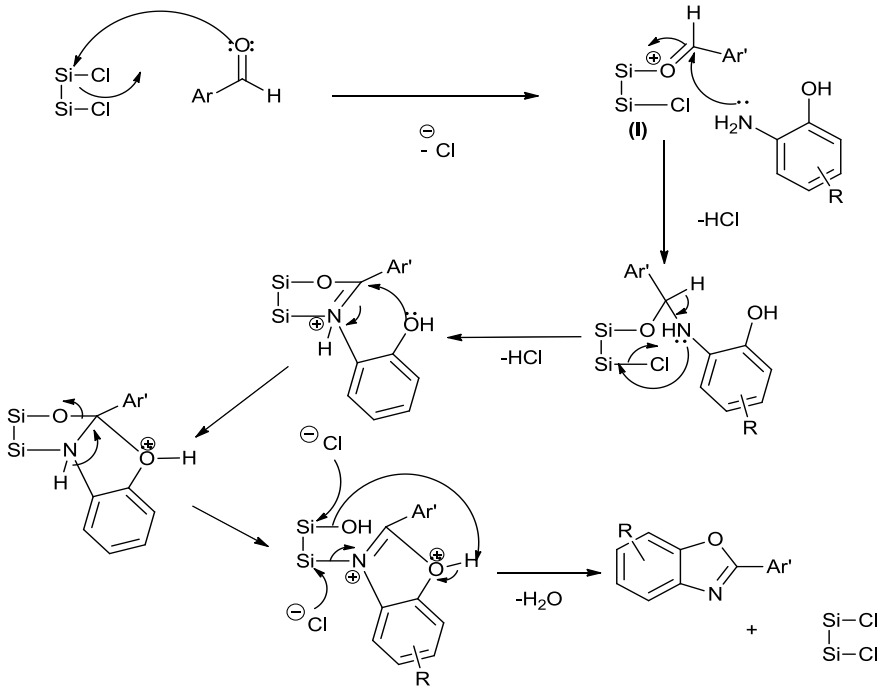
Table 6 One-pot Synthesis of benzoxazolyl conjugates **$5b_1$-b_6.**

Compound 5b	Time h	Yield (%)	Melting Point °C
1	2.5	80	158-162
2	2.5	72	228-232
3	3	74	160-164
4	3	80	189-192
5	3	75	102-108
6	3	76	170-175

Their structures were confirmed by ^{1}HNMR, ^{13}CNMR, and melting points. The IR spectra showed disappearance of OH and C=O peak, C=C peak at 1605cm^{-1}, C=N peak at 1677cm^{-1}

and peak at 1301cm^{-1} attributed to C-O peak. The ^{1}HNMR spectra of the compounds **5a$_1$-a$_{18}$** showed an average spectrum in which appeared two doublets at δ 8.20ppm (Ar-H, 3' & 5'position) and δ 7.51ppm (Ar-H; 2' & 6') . In addition, protons of the fused benzene ring appeared as three multiplets at δ7.79-7.75ppm (–CH=C-N-), δ 7.57-7.61ppm for C5-H, δ7.36-7.39ppm for C7-Hand C6-H; ^{13}C NMR spectra of compounds revealed the presence of C=N at the range of 164.01 C-Cl at the range of 150.3. The structures of N-heterocyclic hybrids **5b$_1$-b$_6$** were confirmed by ^{1}HNMR, ^{13}CNMR, and HRMS data. The calculated M+H ion peak matched with that of the observed M+H ion peak, which confirmed the structure.

In designing the synthesis of benzoxazole derivatives **5a** and **5b**, the mechanistic pathway could be, the attack of lone pair of electrons on oxygen atom of aldehydes on the silica surface resulting in the loss of chlorine atom, leading to formation of intermediate **i** which is then attacked by the amino group of *o*-amino phenol, resulting in a stepwise loss of HCl generating an imine species. The carbon atom of the iminium ion species is then attacked by the hydroxy group (Lone pair on oxygen). The last step could be simultaneous attack of the 2 chloride ions on the silica surface leading to benzoxazole (neutralisation of the positive charge on oxygen and nitrogen is shown by shift of the bonds)**(Scheme 40).**



Scheme 40. Mechanistic pathway showing stepwise formation of benzoxazole

2.3b. Biological Evaluation

2.3b.1. Virtual Screening

The promising approach to cancer therapy has been the targeted therapies which led to beneficial clinical effects. Tyrosine kinase is an important target due to its role in modulation of growth factor signalling, it causes increase in tumor cell proliferation and growth, induces anti-apoptotic effects and promotes angiogenesis and metastasis. Because of all these effects, receptor tyrosine kinase has been a key target for cancer therapy. Tyrosine kinases are enzymes which catalyses the transfer of γ- phosphate group from adenosine triphosphate to target proteins. They play an important role in diverse normal cellular regulatory processes[59], it is characterised by immunoglobulin-like sequences in their amino-terminal extracellular domains, a lipophilic transmembrane segment and an intracellular carboxyl terminal domain which includes its catalytic site. Ligand binding induces dimerisation of these tyrosine kinase receptor and results in autophosphorylation of their cytoplasmic domains and activation in activity of tyrosine kinase. The *o*-aminobenzoxazole and benzimidazole derivatives and pyrazolyl-benzoxazole derivatives have been proved to be active tyrosine kinase inhibitors due to strong binding affinity with enzyme[60, 61]. Hence, we chose enzyme tyrosine kinase as a target for the molecular docking study of the synthesised benzoxazole-*N*-heterocyclic hybrids **5b**.

The crystal structure of the EGFR tyrosine kinase domain (PDB ID: 1m17) complexed with 4-anilinoquinazoline inhibitor was used for the docking studies. The protein file was prepared by the removal of water molecules, addition of polar hydrogens and removal of other bound ligands. The synthesized benzoxazole-hybrids **$5a_1$-a_{18}** and **$5b_1$-b_6** were virtually screened for its anti-cancer activity against tyrosine kinase domain complexed with 4-anilinoquinazoline ligand, the docking score for which has been tabulated in **Table 7**. Docking of the synthesized compounds with EGFR tyrosine kinase domain exhibited well conserved hydrogen bonding with the amino acid residues at the active site.

The molecular docking study revealed that compounds **$5b_1$, $5b_3$ and $5b_4$** act as good inhibitors of tyrosine kinase due to characteristic features **(Fig.7).** However, compounds **$5a_1$-a_{18}** was also found to be active against the target PDB ID: 1M17. The best docked poses of the compounds

are represented in **Fig.8**. Compound **5b$_1$** makes four hydrogen bond interactions at the active site of the enzyme. Three of which were formed with the nitro group present on phenyl ring; one interaction raised from H-bond interaction between nitrogen atom of the NO_2 group with Thr766 (1.55A^o) and two interactions appeared from H-bond interaction between oxygen atom of nitro group and CYS751 (1.52A^o) and Thr830 (1.52A^o). The remaining one has arisen from oxazole ring of benzoxazole moiety. The oxygen atom interacts with Met769 (1.55A^o), as depicted in **Fig.8A**.

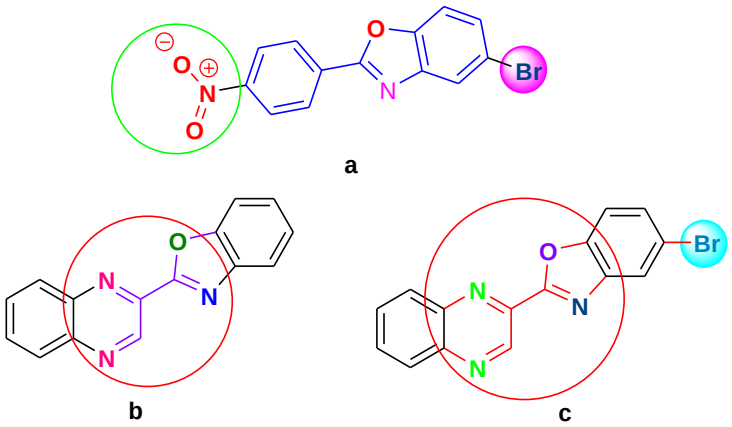


Fig.7. Structural features of compounds responsible for the cytotoxicity: a) Compound **5b$_1$**; b) Compound **5b$_3$**; c) Compound **5b$_4$**

Compound **5b$_3$** makes two hydrogen bond interactions at the active site of the enzyme. One interaction has arisen from nitrogen atom of oxazole ring, where the nitrogen forms H-interaction with Thr766 (1.52A^o). While, the other is raised from H-bond interaction of nitrogen atom of quinoxaline ring with Met769 (1.55A^o) (**Fig.8B)**. Compound **5b$_4$** makes three hydrogen bond interactions at the active site of the enzyme. The interaction arising from nitrogen atom of the quinoxaline, shows H-bonding which interaction with Met769 (1.55A^o). The remaining two interactions are raised from nitrogen and oxygen atom of oxazole ring with Thr766 (1.52A^o) (**Fig.8C).**

The binding interaction of AQ4-1M17 ligand with tyrosine kinase domain active site shows ten hydrogen bonding interaction depicted in **Fig.8D**. It can be seen that the studied compounds showed same type of interactions with amino acid residue Thr766, CYS751, Thr830, Met769 as that of reference AQ4-1M17 ligand. The MolDock score of the screened compounds ranged from -73.0934 to -97.8936. All the compounds showed good docking score, which indicates all

the forces of interaction between ligands and the enzyme in docked compounds. Thus, indicating that molecules preferentially bind to enzyme active sites in comparison to the reference AQ4-1M17 ligand.

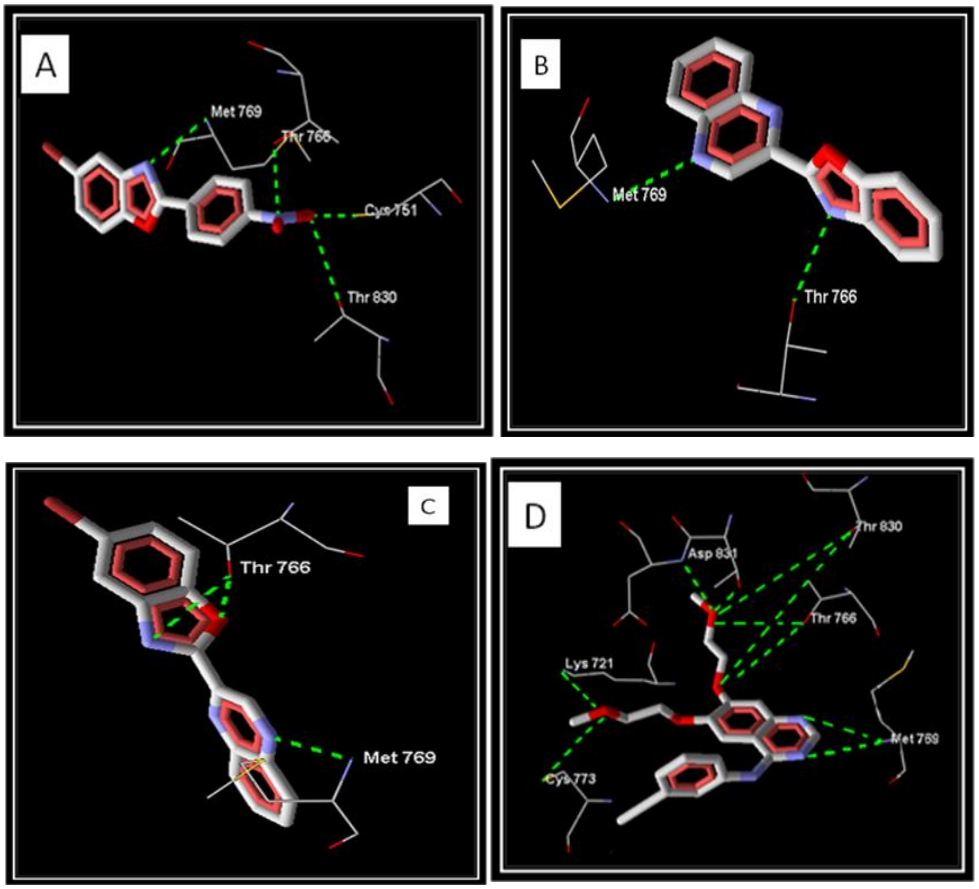


Fig.8. Molecular docking data: the compounds docked in best of its conformation into the binding site of 1M17. (A) Binding mode of compound **$5b_1$** forming four hydrogen bond interactions. (B) Binding mode of compound **$5b_3$** forming two hydrogen bond interactions. (C) Binding mode of compound **$5b_4$** forming three hydrogen bond interactions. (D) Binding interaction of ligand AQ4-1M17 forming 10 hydrogen bond interactions.

Table 7. Molegro docking score for benzoxazole derivatives **$5a_1$- a_{18}** and **$5b_1$- b_6**

Code	MolDock Score	Rerank Score	Protein	H Bond	Heavy atom count	Kcal/mol	Docking Score
$5a_1$	-86.5224	-64.8155	-90.5300	2	19	-5.08955	-86.0269
$5a_2$	-80.5605	-61.4823	-86.9539	1	19	-5.03503	-79.6663
$5a_3$	-80.2853	-62.0941	-86.7351	3	17	-5.01783	-79.4664

$5a_4$	-79.8709	-62.0032	-101.419	3	17	-5.1842	-97.9248
$5a_5$	-96.1274	-66.7314	-99.1471	5	17	-3.7073	-97.9981
$5a_6$	-80.6788	-61.6469	-86.0643	2	17	-4.13724	-83.9878
$5a_7$	-92.6515	-72.1225	-104.053	2	17	-4.8123	-86.6232
$5a_8$	-81.1186	-66.7681	-89.5901	3	16	-4.09693	-76.124
$5a_9$	-80.4031	-54.6825	-89.813	3	16	-3.77365	-71.5362
$5a_{10}$	-73.0934	-56.8369	-79.4259	0	16	-3.5958	-73.2504
$5a_{11}$	-79.7784	-61.9172	-85.919	1	16	-4.01128	-79.2865
$5a_{12}$	-90.1899	-71.7397	-102.058	3	16	-5.0177	-100.482
$5a_{13}$	-97.8936	-71.8246	-101.476	3	19	-5.1523	-96.9324
$5a_{14}$	-75.3016	-58.9796	-81.593	0	19	-3.71931	-80.8615
$5a_{15}$	-78.559	-62.499	-88.8241	0	19	-3.86762	-79.1946
$5a_{16}$	-83.1269	-60.3583	-87.3281	1	17	-3.11882	-87.9088
$5a_{17}$	-81.1726	-45.0394	-100.153	3	19	-4.2722	-78.8718
$5a_{18}$	-79.7915	-58.6621	-88.1242	1	19	-3.59923	-86.9973
$5b_1$	-79.8709	-62.0032	-101.419	4	17	-4.2037	-81.4409
$5b_2$	-80.3296	-63.3705	-87.2282	1	17	-4.9468	-93.4702
$5b_3$	-90.1899	-71.7397	-102.058	2	17	-4.7468	-89.4702
$5b_4$	-92.6515	-72.1225	-104.053	3	17	-4.6325	-91.9065
$5b_5$	-89.4501	-64.1618	-99.8916	1	17	-3.71632	-95.1771
$5b_6$	-89.7198	-70.4287	-97.4172	1	16	-3.24967	-91.2059

2.3b.2. Anticancer Evaluation

On the basis of enzymatic inhibitory potency against PDB ID:1M17, compounds **$5b_1$-b_6** was selectively used as candidates for exploring the mechanisms of anti-cancer. The anti-proliferative activity has been carried out against MDA MB-231 (ER-negative) & MCF-7 (Breast Cancer), A549 (Lung Cancer), KB (Oral Cancer) and HEK293 (Normal Human Kidney Cells) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method.[62] Using graph Pad Prism Version 5.1, the IC_{50} of compounds has been calculated by taking a percentage of cell viability at six different concentrations of treatment (**Fig. 9**).

All the six benzoxazole-*N*-heterocyclic hybrids **5b$_1$-b$_6$** exhibited cell proliferation with IC_{50} values in the range of 0.42-14.39µM. As shown in **Table 8**, the samples showed moderate to good activity comparable to that of standard drugs, especially compounds **5b$_1$, 5b$_3$** and **5b$_4$** showed higher anti-proliferation activity with IC_{50}=0.56±0.07, 0.58±0.05µM, IC_{50}=0.53±0.02, 0.50±0.08µM and IC_{50}=0.73±0.02, 0.60±0.06µM against MDA-MB-231 (TNBC-triple negative breast cancer) and MCF-7 (human breast cancer) cell lines. Compound **5b$_2$**, **5b$_5$** and **5b$_6$** showed moderate activity with IC_{50} values 1.09±0.05, 0.98±0.04 and 0.85±0.01µM against MDA-MB-231 and also against MCF-7 cell lines with the IC_{50} values 1.10±0.03, 0.94±0.04 and 0.91±0.02µM respectively. The higher anti-proliferation against KB (oral cancer) cell line with IC_{50} value of 0.71±0.12 was exhibited by compound **5b$_2$**. Compound **5b$_1$, 5b$_4$** and **5b$_5$** showed comparable anti-proliferation against KB cell line with IC_{50} value 0.82±0.08, 0.86±0.10 and 0.85±0.04µM respectively. Compound **5b$_3$** and **5b$_6$** exhibited moderate anti-proliferation effect. Considering the anti-proliferation effect against A549 (lung cancer), compound **5b$_1$** showed good anti-proliferation with the IC_{50} value 0.70±0.11µM. Compound **5b$_3$**, **5b$_4$, 5b$_5$** and **5b$_6$** showed moderate anti-proliferation against A549 (Lung cancer) cell line with IC_{50} value 0.8µM as compared to the other samples. The results were compatible with the docking studies. The cell viability studies were carried out in dose dependent manner. Surprisingly, the cell viability decreased with the increase in concentration of the compound against five cancer cell lines. The results have been investigated and represented graphically as variation in % cell viability with respect to concentration **Fig 9**.

Table 8 IC_{50} values (µM±S.E.) of compounds **5b$_1$-b$_6$** against four different cell lines and normal human cell lines

Comp 5b	IC_{50} µM				
	MDA MB-231[a]	MCF-7[b]	A549[c]	KB[d]	HEK293[e]
1	0.56±0.07	0.58±0.05	0.70±0.11	0.82±0.08	10.11±0.19
2	1.09±0.05	1.10±0.03	0.97±0.09	0.71±0.12	3.27±0.20
3	0.53±0.02	0.50±0.08	0.82±0.20	0.90±0.05	14.39±0.28
4	0.73±0.02	0.60±0.06	0.87±0.08	0.86±0.10	8.39±0.19
5	0.98±0.04	0.94±0.03	0.89±0.11	0.85±0.04	6.04±0.23
6	0.85±0.01	0.91±0.02	0.83±0.06	0.98±0.03	7.21±0.05

Paclitaxel[f]	0.3±0.02	-	-	-	-

[a]MD A-MB-231 cell line- is an epithelial, human breast cancer cell line established from pleural effusion of a 51-year old Caucasian female with metastatic memmary adenocarcinoma.

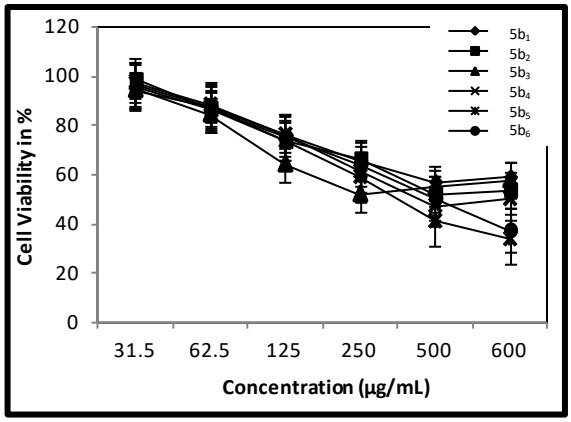
[b]MCF-7 is a human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors. It is derived from pleural effusion of 69-year old Caucasian metastatic breast cancer in 1970

[c]A549 ia a human cancer cell line, derived from the removal and culturing of cancerous lung tissues in the explanted tumor of 58-year old Caucasian metastatic male.

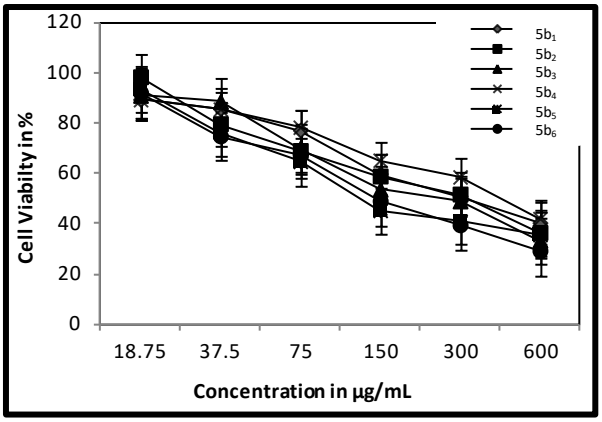
[d]KB is derived from an epidermal carcinoma of the mouth, KB cells contain human papilloma virus18 (HPV-18) sequence;

[e]HEK293 –derived from Human embryonic kidney293 cell lines grown in tissue culture

[f]Paclitaxel- used as standard drug



a



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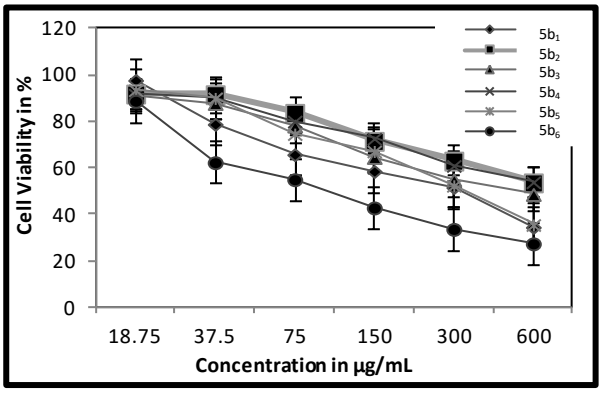

c

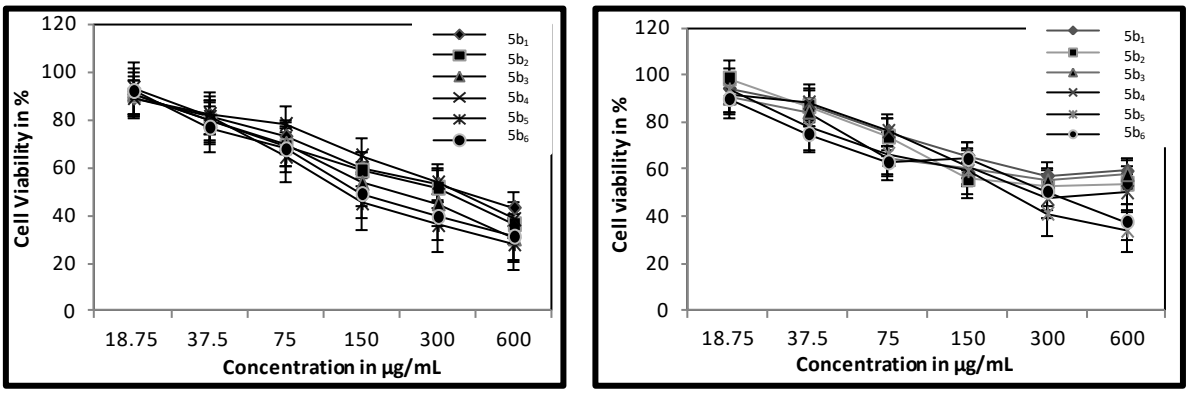

Fig. 9. Graphical representation of cell viability exhibited by **$5b_1$-b_6** for five different cell lines: a)MDA-MB-231, b) MCF-7, c) A549, d)KB, e)HEK293

Following this, the *in vitro* cytotoxicity activity of the tested compounds **$5b_1$-b_6** was analyzed against normal HEK-293 cell lines by MTT colorimetric assay.[62] The colorimetric results revealed that the six evaluated compounds exhibited no significant toxicity effect on normal HEK-293 cells. Selectivity index (SI) reveals the differential activity of a pure compound. Higher SI value is attributed to minimum toxicity and more selectivity. Whereas, a compound with SI value >2 indicates cytotoxicity of the pure compound. As shown in **Table 9** all samples were proved to be cytotoxic to the normal human cells, with maximum SI value exhibited by compound **$5b_3$** followed by compound **$5b_1$**. The effect of benzoxazole-*N*-heterocyclic hybrids **$5b_1$-b_6** on viability of MDA-MB-231 (ER-negative), MCF-7 (Breast Cancer), A549 (Lung Cancer), KB (Oral Cancer) and HEK293 (Normal Human Kidney Cells) was investigated and represented graphically as plot of variation in percentage of viability with respect to concentration (**Fig. 9).**

Table 9. Selective index (SI)^f of compounds **$5b_1$-b_6**

Comp 5b	SI^f-MDA MB-231	SI^f-MCF-7	SI^f-A549	SI^f-KB
1	18.05	17.47	14.44	12.33
2	3.0	2.97	3.37	4.6
3	28.15	28.78	17.55	15.98
4	11.49	13.98	9.64	9.75
5	6.16	6.42	6.78	7.10
6	8.5	7.92	8.68	7.35

[f]**SI**-Selective index, as ratio of IC_{50} value of normal cells to that of IC_{50} value of cancerous cells. IC_{50} values are obtained as the mean±SD(μM) from three different experiments.

2.3b.3. Enzyme Inhibition studies

The effect of benzoxazole-*N*-heterocyclic hybrids **$5b_1$-b_6** on *in vitro* tyrosine kinase activity was carried out using Fetal Bovine Serum (FBS) cell culture treated with indicated amounts of benzoxazole-*N*-heterocyclic hybrids **$5b_1$-b_6** for indicated time. The whole cells were extracted and incubated with tyrosine kinase substrate for the specified time mentioned in the procedure. The plot of relative rate of inhibition in activity of enzyme tyrosine kinase with respect to different concentration of compounds **$5b_1$-b_6** in μg/mL (**Fig. 10)** indicates that compounds **$5b_1$-b_6** induced a dose dependent decrease in the enzyme activity (**Table 10).** It was summarized from the results of the enzyme inhibition studies that, compound **$5b_3$** exhibited higher inhibition in activity of enzyme tyrosine kinase with the IC_{50} value of 0.10±0.16μM, compounds **$5b_1$, $5b_4$** and **$5b_5$** also exhibited good inhibitory action on the activity with IC_{50} value in the range 0.31-0.43μM. Compound **$5b_2$** was found to be least active in inhibiting the growth of enzyme with IC_{50} value 0.86±0.42μM. With decrease in the concentration of compounds **$5b_1$-b_6**, there was decrease in the rate of enzymatic growth. Among the entire compounds studied, compound **$5b_3$** displayed the highest inhibition in the activity of enzyme. All compounds demonstrated good inhibition of tyrosine kinase enzyme activity only at higher concentration; however, effect of inhibition was found to decrease with decrease in concentration.

Table 10. Enzyme inhibition of Tyrosine kinase by compounds **$5b_1$-b_6**

Comp 5b	**IC_{50} value[a]**
1	0.31±0.11
2	0.86±0.42
3	0.10±0.16
4	0.35±0.17
5	0.43±0.21
6	0.56±0.24

[a]IC_{50} values measured in μM

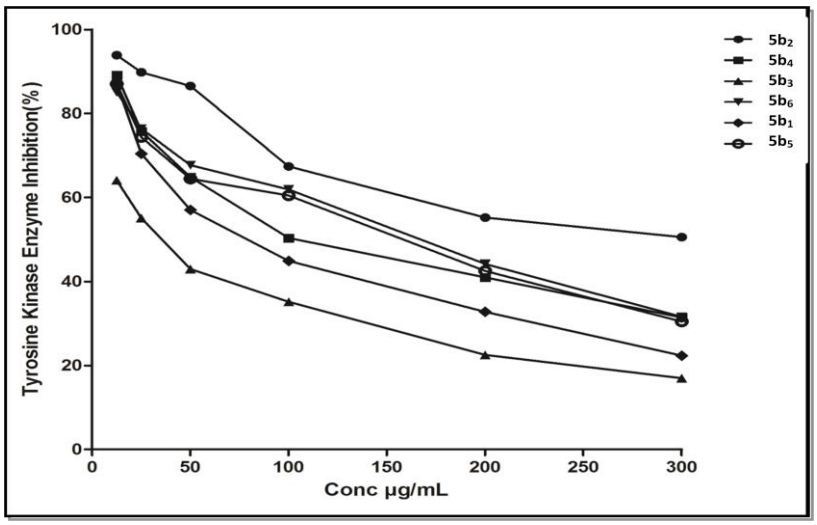

Fig.10. Graphical representation of enzyme Tyrosine kinase inhibition by compounds **$5b_1$-b_6:** Plot of concentration in µg/mL v/s % of inhibition.

2.3b.4. Cell Morphology studies by Fluorescence Microscopy:

2.3b.4.1. Double staining method:

Fluorescence microscopy study was employed to study the mode of cell death induced by synthesized benzoxazole-*N*-heterocyclic hybrids **$5b_1$, $5b_3$** and **$5b_4$** in comparison to the standard Cisplatin by virtue of Acridine orange/ Ethidium bromide staining. The mode of cell death, whether early or late apoptosis can be characterized based on the fluorescence properties. Acridine orange was taken up by both viable and apoptotic (dead) cells by emitting green fluorescence. Cells with disrupted membrane integrity were stained by Ethidium bromide, i.e. late apoptotic cells and necrotic cells. Late apoptotic cells have orange to red nuclei with condensed and fragmented chromatin, and early apoptotic cells show green fluorescence nuclei. Necrotic cells have uniform orange to red nuclei with organised structure. Thus, from the results (**Fig.11)** it was visualised that, viable cells without treating with synthesised compound **$5b_1$, $5b_3$** and **$5b_4$** showed green fluorescent nucleus with acridine orange **Fig.11a**, whereas, late apoptotic cells when treated with compounds **$5b_1$, $5b_3$** and **$5b_4$** exhibited yellow orange fluorescence with nuclear membrane babbling **Fig.11b-d**; thus signifies late apoptotic mode of cell death.

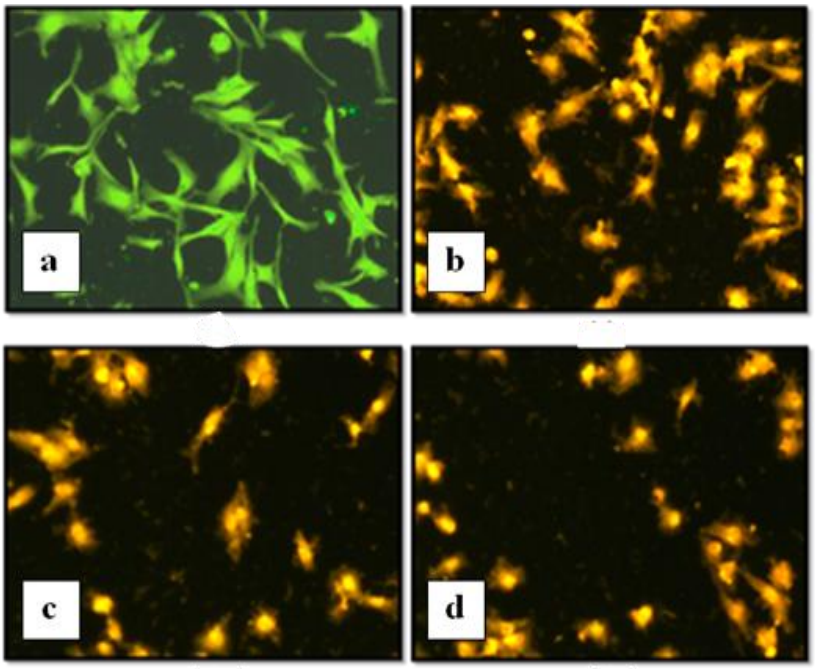

Fig.11. Fluorescence microscopy images of cells stained with Acridine orange/ Ethidium bromide, **a**. viable cells, **b**. Cells treated with compound **$5b_1$**, **c**. cells treated with compound **$5b_3$**, **d**. cells treated with compound **$5b_4$**

2.3b.4.2. DAPI method:

Further confirmation of the apoptotic mode of cell death was done by using DAPI staining method, wherein; cells treated with compounds and the viable cells were stained with 4', 6'-Diamine-2'-phenylindole dihydrochloride (DAPI). Untreated cells showed intact nucleus **Fig.12a** whereas, treated cells showed nuclear condensation and nuclear fragmentation. **Fig.12b-d.**

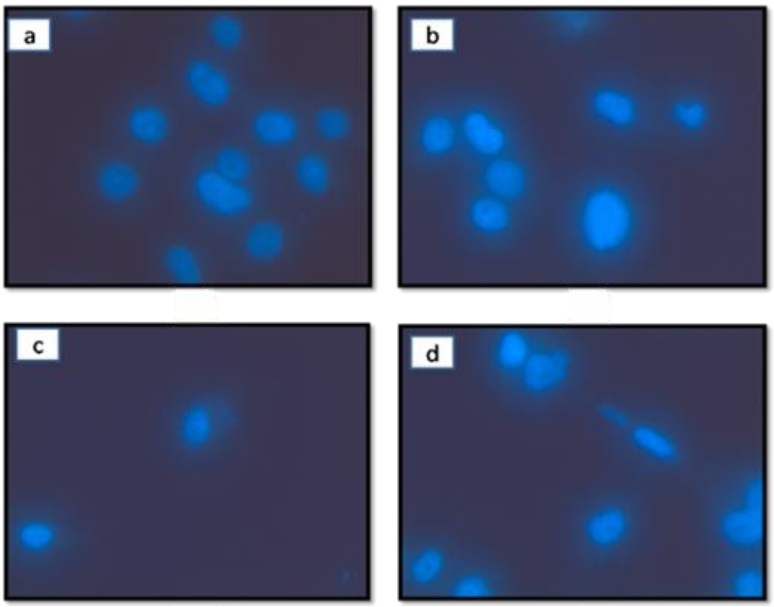

Fig.12. Fluorescence microscopy images of cells stained with DAPI, showing a) viable cells, b) Cells treated with compound **5b$_1$**, c) cells treated with compound **5b$_3$**, d) cells treated with compound **5b$_4$.**

2.3b.5. Anti-tubercular evaluation:

The *in vitro* anti-tubercular screening[63] of the compounds **5a$_1$-a$_{18}$** and **5b$_1$-b$_6$** was carried out against *Mycobacterium tuberculosis* H_{37}Rv strain by Alamar Blue Dye Assay method. This methodology is non-toxic, uses thermally stable reagents and showed good correlation with the proportional and BACTEC radiometric method. All the compounds exhibited better anti-tubercular activity with the MIC value ranging 0.8-50µg/mL compatible with the standard drugs Streptomycin, Pyrazinamide and Ciprofloxacin. Compounds of **5a** series were found to be active at lowest concentration with MIC value 0.8-12.5µg/mL. However, among the **5b** series, only **5b$_1$** and **5b$_2$** were active enough upto MIC value 25 and 12.5µg/mL, whereas, compounds **5b$_3$-b$_6$** were found to be least active with MIC value 50µg/mL (**Fig. 13**).

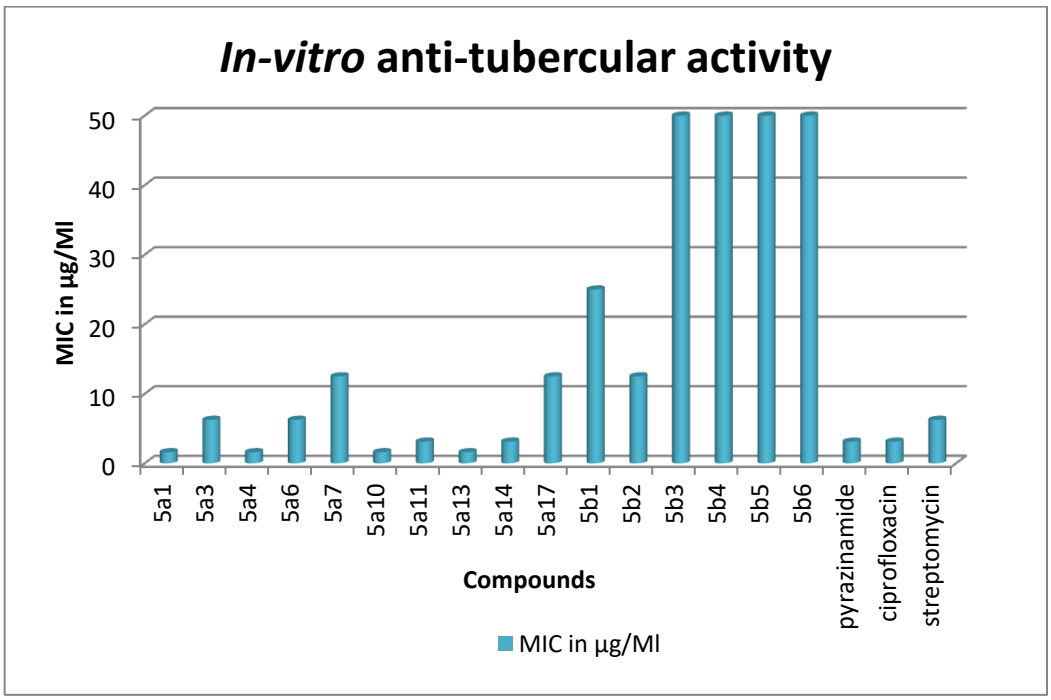

Fig.13. *In-vitro* anti-tubercular evaluation against *Mycobacterium tuberculosis* by Microplate Alamar Blue Dye Assay method

2.3b.6. Structure activity relationship studies

The following Structure activity relationship (SAR) analysis has been executed based on the results of the cytotoxicity studies of benzoxazole-*N*-heterocyclic hybrids **5b$_1$-b$_6$** against five different cancer cell lines (**Fig.14**). All the compounds were found to show good cytotoxicity effect against MDA-MB-231 and MCF-7 breast cancer cell lines as compared to other cell lines. Compound **5b$_1$**, **5b$_3$** and **5b$_4$** exhibited good anti-cancer activity due to strong interaction with the enzyme active site and their characteristic structural features **Fig.7 & 14.** Based on the above observations following structure activity relationship can be demonstrated. As in compound **5b$_1$**, the presence of electron withdrawing group (i.e. Nitro) onto the phenyl ring and also the presence of bromo substituent on the benzoxazole moiety enhance the cytotoxicity effect. Replacement of the nitro group by halogen (i.e.Br) as in compound **5b$_1$,** has moderate effect on cytotoxicity. In case of compound **5b$_3$**, replacing the phenyl ring with quinoxaline ring and also the presence of bromo substituent on the benzoxazole ring exhibits higher effect on cytotoxicity, so also compound **5b$_4$** with quinoxaline moiety exhibited similar effects. Quinoline moiety was found to lower the cytotoxicity effect as in compound **5b$_5$** and **5b$_6$.**

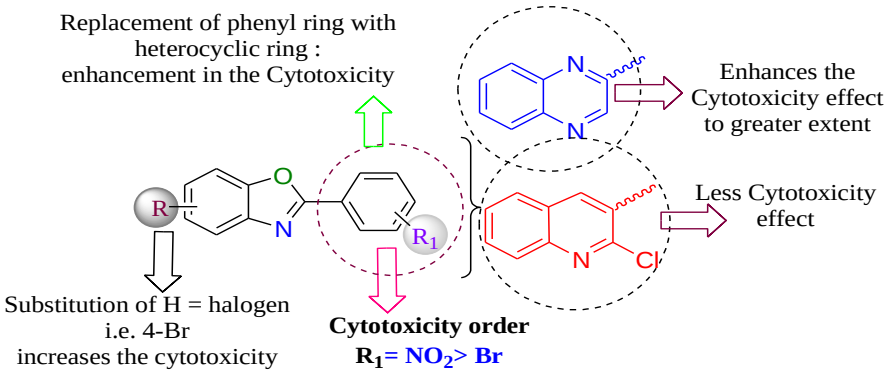

Fig.14. Structural representation of SAR based on cytotoxicity study of benzoxazole hybrids **5b$_1$-b$_6$.**

2.4. CONCLUSION

In conclusion, a newly designed synthesis of benzoxazole has been achieved in an ecofriendly reaction conditions. Such benzoxazole hybrids **5a$_1$-a$_{18}$** and **5b$_1$-b$_6$** have been evaluated as anti-cancer agents, having virtually screened as good inhibitors of enzyme protein tyrosine kinase, the key enzyme that triggers triple negative breast cancer. Comparative cell cytotoxicity studies against five different cancer cell lines MCF-7, MDA-MB-231, KB, A549 and HEK-293 revealed that all six benzoxazole-*N*-heterocyclic hybrids **5b$_1$-b$_6$** showed good inhibitory potency against all cell lines. It was visualized from the anti-cancer activity results that, compounds **5b$_1$**, **5b$_3$** and **5b$_4$** demonstrated excellent inhibitory potency against MDA-MB-231 and MCF-7 breast cancer cell lines with the IC_{50} value in the range of 0.50-0.73µM as compared to standard drug Paclitaxel (IC_{50}: 0.30±0.02µM). Compounds **5b$_1$-b$_6$** were also found to be active against A549 (lung cancer) with IC_{50} values in the range of 0.70-0.97 µM. Comparing the anti-cancer potency against all five cancer cell lines, the target compounds **5b$_1$**, **5b$_3$** and **5b$_4$** exhibited better anti-proliferation effect against MDA-MB-231 and MCF-7 cell lines. Cytotoxicity against HEK-293 (human embryonic kidney) cell line was also evaluated and selectivity was determined. The selective index value in was observed to be between 2.97-28.78 thereby indicating the compounds to be non toxic to human kidney cells. The docking results revealed that compounds **5b$_1$**, **5b$_3$** and **5b$_4$** had good interaction with the active site of enzyme tyrosine kinase. Further, enzyme inhibition study of compounds **5b$_1$-b$_6$** proved that, compound **5b$_3$** exhibits maximum inhibition in activity of enzyme tyrosine kinase with IC_{50} value 0.10±0.16µM. The results were found to be compatible with the docking studies, thus demonstrating that compound **5b$_1$**, **5b$_3$** and **5b$_4$** act as potent inhibitors of tyrosine kinase. Accordingly, a mode of cell death was studied using double staining and DAPI method, which signified the mode of cell death to be late apoptosis. The *in vitro* anti-tubercular activity of the compounds **5a$_1$-a$_{18}$** against *Mycobacterium tuberculosis* H_{37}RV strain exhibited excellent anti-tubercular activity with the MIC value ranging from 1.6-µg/mL compatible with the standard drugs, however, compounds **5b$_3$-b$_6$** were active only upto MIC value of 50µg/mL, thus indicating that these compounds are selective inhibitors of tyrosine kinase enzyme. SAR analysis reveals the influence of benzoxazole-*N*-heterocyclic hybrids in defining its cytotoxicity and anti-cancer activity.

2.5. EXPERIMENTAL

2.5.1. Chemistry:

2.5.1.1.Synthesis of benzoxazole derivatives **$5a_1$-a_{18}** and **$5b_1$-b_6** (Scheme 38 & 39)

To the flask containing a mixture of substituted benzaldehyde (1mmole) **3** and *o*-aminophenol **4** (1mmole) was added silica chloride (1eq) and was heated on a sand bath at 120^{o}C, TLC was taken after every 1 hour. After 4 hours, TLC showed appearance of new spot. The product was isolated by first separating out the catalyst by filtration using organic solvent; the organic layer was dried using anhydrous sodium sulfate and evaporated under vacuum. The solid thus obtained was recrystallized using petroleum ether and its % yield and melting points were determined. The results are tabulated in **Table 5** and **Table 6**.

2.5.1.1.1. 2-(2', 4'-Dichlorophenyl)-1, 3-benzoxazole **$5a_1$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 125-127^{o}C; IR (KBr, cm^{-1}): 3010, 1605 (C=C), 1677 (C=N), 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.0 (s, 1H, *J*=7.6Hz, 3'-H),7.37-8.15(m, 4H, Ar-H), 7.62 (dd, 1H, *J*=8.8, 2.1Hz, 5'-H), 7.24 (d, 1H, *J*= 8.8Hz, 6'-H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.8, 150.1, 141.1, 135.6, 133.9, 129.5, 128.9, 128.1, 127.8, 124.9, 124.1, 119.8, 110.3.

2.5.1.1.2. 2-(4'-Chlorophenyl)-1, 3-benzoxazole **$5a_2$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 144-148^{o}C; IR (KBr, cm^{-1}): 3015, 1657 (C=N), 1607 (C=C), 1035 (C-O), 780; ^{1}HNMR (400MHz, $CDCl_3$): δ7.92 (d, 2H, *J*=8.2, 1.8Hz, 3' & 5'-H), 7.814 (d, 1H, *J*=8.4Hz, 2' & 6'-H), 7.27-8.07(m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 164.8, 151.1, 140.1, 135.7, 129.35, 129.3,128.8, 128.6, 127.5, 124.2, 124.1, 119.5, 110.8.

2.5.1.1.3. 2-(4'-Bromophenyl)-1, 3-benzoxazole **$5a_3$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 71%, m. p.: 150-152^{o}C; IR (KBr, cm^{-1}): 3011, 1640 (C=N), 1600 (C=C), 1550, 1034 (C-O), 730; ^{1}HNMR (400MHz, $CDCl_3$): δ 7.83 (d, 2H, *J*=8.4Hz, 2' & 6'-H); 7.77 (d, 2H, *J*=8.4Hz, 3' & 5'-H); 7.6-7.8(m, 4H, Ar-H), 7.3-7.8 (m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ162.8, 150.8, 142.1, 136.7, 132.5, 132.3, 129.9, 129.8, 127.6, 124.9, 124.2, 119.8, 112.0.

2.5.1.1.4. 2-(3'-Nitrophenyl)-1, 3-benzoxazole **5a$_4$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 99-100^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3010, 1639 (C=N), 1599 (C=C), 1450, 1350, 1036; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.88 (s, 1H, 2'-H), 8.58 (dd, 1H, *J*=7.8, 2.5Hz, 4'-H), 8.18 (dt, 1H, *J*=8.0, 2.1Hz, 6'-H), 7.6-7.8(m, 4H, Ar-H), 7.68 (t, 1H, *J*=7.96, 8.02Hz, 5'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 163.8, 151.3, 142.1, 140.3, 127.4, 127.3, 125.2, 124.9, 124.1, 119.9, 117.3, 116.6, 110.2.

2.5.1.1.5. 2-(2'-Nitrophenyl)-1, 3-benzoxazole **5a$_5$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 98-103^{o}C; IR (KBr, cm^{-1}): 3011, 1632 (C=C), 1677 (C=N), 1550, 1380, 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.78 (dd, 1H, *J*=7.9, 2.3Hz, 3'-H), 8.02 (dd, 1H, *J*=8.6, 7.56 Hz, 4'-H), 7.58-8.2(m, 4H, Ar-H), 8.18 (dt, 1H, *J*=1.6, 8.0Hz, 6'-H), 8.23 (dt, 1H, *J*=7.96, 2.3Hz, 5'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 162.9, 153.3, 142.2, 140.7, 127.2, 127.1, 125.2, 124.9, 124.1, 119.8, 117.3, 116.6, 110.7.

2.5.1.1.6. 2-(2'-Chlorophenyl)-1, 3-benzoxazole **5a$_6$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 68-72^{o}C; IR (KBr, cm^{-1}): 3010, 1657 (C=N), 1607 (C=C), 1035 (C-O), 780; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.17(m, 4H, Ar-H), 7.9 (dd, 1H, *J*=8.2, 1.8Hz, 3'-H), 7.5(dd, 1H, *J*=8.2, 1.8Hz, 6'-H), 7.4(dt, 1H, *J*=8.2, 1.8Hz, 5'-H), 7.3(dt, 1H, *J*=8.2, 1.8Hz, 4'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 163.1, 150.3, 140.5, 135.8, 129.5, 129.1, 128.7, 128.1, 127.7, 125.0, 124.7, 118.8, 113.3.

2.5.1.1.7. 2-(4'-Nitrophenyl)-1, 3-benzoxazole 5**a$_7$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 256-260^{o}C; IR (KBr, cm^{-1}): 3010, 1639 (C=N), 1599 (C=C), 1450, 1350, 1036; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.98 (d, 2H, *J*=8.2, 1.6Hz, 3' & 5'-H), 8.56 (d, 2H, *J*=8.3, 1.7Hz, 2' & 6'-H), 7.58-8.2(m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 164.8, 152.3, 142.5, 140.7, 131.3, 131.2, 125.9, 125.1, 120.9, 118.3, 117.5, 117.5, 113.3.

2.5.1.1.8. 2-Phenyl-1, 3-benzoxazole **5a$_8$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 78%, m. p.: 98-100^{o}C; IR (KBr, cm^{-1}): 3010, 1605 (C=C), 1550 (C=N), ;1030; ^{1}HNMR (400MHz, $CDCl_3$): δ7.37-8.15(m, 4H, Ar-H), 7.17-7.80 (m, 4H, Ar'-H); ^{13}C (100MHz, $CDCl_3$): δ162.8, 150.1, 141.1, 128.9, 128.7, 128.8, 127.9, 127.8, 127.1, 124.9, 124.1, 119.8, 110.3.

2.5.1.1.9. 2-(4'-Methoxyphenyl)-1, 3-benzoxazole **5a$_9$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 77%, m. p.: 114-116^{o}C; IR (KBr, cm^{-1}): 3010, 1620 (C=N), 1590 (C=C), 1210, 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 7.29-7.90 (m, 4H, Ar-H), 7.89 (d, 2H, *J*=8.8 Hz, 2' & 6'- H), 7.34 (d, 2H, *J*=8.8Hz, 3' & 5'-H); 3.84 (s, 3H, OMe); ^{13}C NMR (100MHz, $CDCl_3$): δ 164.4, 160.4, 150.1, 128.9, 128.2, 127.3, 124.9, 124.2, 119.8, 114.7, 114.8, 110.6, 55.8.

2.5.1.1.10. 2-(4'-Methylphenyl)-1, 3-benzoxazole **5a$_{10}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.:104-106^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3030, 1605 (C=N), 1580 (C=C), 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ7.30-8.08(m, 4H, Ar-H), 7.84 (d, 2H, *J*= 2.5, 7.8 Hz, 2' & 6'-H), 7.52 (d, 2H, *J*=7.88, 1.5Hz, 3' & 5'-H); 2.34 (s, 3H, Me); ^{13}C NMR (100 MHz, $CDCl_3$): δ161.1, 150.1, 141.1, 135.6, 133.8, 129.5, 128.9, 128.1, 127.8, 124.9, 124.1, 119.8, 110.3, 22.0.

2.5.1.1.11. 2-(3'-Chlorophenyl)-1, 3-benzoxazole **5a$_{11}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 120-122^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3020, 1635 (C=N), 1600 (C=C), 1030, 760; ^{1}HNMR (400MHz, $CDCl_3$): δ7.88 (s, 1H, 2'-H), 7.82 (dd, 1H, *J*= 8.6, 1.56Hz, 4'-H), 7.78 (dd, 1H, *J*=1.6, 1.8Hz, 6'-H), 7.68 (t, 1H, *J*=7.8Hz, 5'-H) 7.46-8.10 (m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ162.8, 151.1, 140.2, 138.6, 135.8, 127.5, 125.9, 124.8, 124.1, 123.9, 120.1, 119.5, 110.7.

2.5.1.1.12. 2-(4'-Hydroxy, 3'-nitrophenyl)-1, 3-benzoxazole **5a$_{12}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.:170-174^{o}C; IR (KBr, cm^{-1}): 3200, 3010, 1630 (C=N), 1600

(C=C), 1550, 1350, 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.8 (d, 1H, *J*=7.9Hz, 6'-H), 8.25 (s, 1H, 2'-H), 7.52-8.02 (m, 4H, Ar-H),7.35 (d, 1H, *J*=7.9Hz, 5'-H); ^{13}CNMR (100MHz, $CDCl_3$): δ 161.8, 150.1, 141.5, 135.6, 133.8, 129.5, 128.9, 128.2, 127.8, 124.8, 124.1, 119.8, 110.2.

2.5.1.1.13. 2-(2'-Hydroxy, 5'-nitrophenyl)-1, 3-benzoxazole **5a$_{13}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 71%, m. p.: 188-192^{o}C; IR (KBr, cm^{-1}): 3210, 1635 (C=N), 1600 (C=C),1580,1359, 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.79(s, 1H, 6'-H), 8.34(d, 1H, *J*=7.6Hz, 3'-H), 7.52-8.02(m, 4H, Ar-H), 7.38(d, 1H, *J*=7.6Hz, 4'-H);^{13}C NMR (100MHz, $CDCl_3$): δ161.8, 150.1, 141.1, 135.6, 133.9, 129.4, 128.9, 128.2, 127.8, 124.8, 124.1, 119.8, 110.2.

2.5.1.1.14. *2*-(4'-Fluorophenyl)-1, 3-benzoxazole **5a$_{14}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 90-92^{o}C; IR (KBr, cm^{-1}): 3010, 1660 (C=N), 1610 (C=C), 1030 1000; ^{1}HNMR (400MHz, $CDCl_3$): δ7.83(d, 2H, *J*=8.12Hz, 2' & 6'-H), 7.34 (d, 2H, *J*=8.12Hz, 3' & 5'-H) 7.30-8.05(m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 164.4, 163.5, 150.1, 141.5, 133.4, 133.4, 127.5, 124.8, 124.3, 119.5, 117.9, 117.8, 110.6.

2.5.1.1.15. 2-(3'-Bromophenyl)-1, 3-benzoxazole **5a$_{15}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 133-137^{o}C; IR (KBr, cm^{-1}): 3010, 1655 (C=N), 1610 (C=C), 1030, 760; ^{1}HNMR (400MHz, $CDCl_3$): δ7.77 (s, 1H,2'-H), 7.64 (dd, 1H, *J*=8.1Hz, 4'-H), 7.57 (dd, 1H, *J*=7.8, 2.1Hz, 6'-H), 7.51 (t, 1H, *J*=8.0, 7.8 Hz, 5'-H), 7.42-8.1(m, 4H, Ar-H); ^{13}C NMR (100MHz, $CDCl_3$): δ163.6, 150.1, 141.5, 132.5, 130.9, 130.1, 129.9, 127.9, 125.7, 124.8, 124.3, 119.0, 110.9.

2.5.1.1.16. 2-(4'-*N*, *N*-dimethylaminophenyl)-1, 3-benzoxazole **5a$_{16}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 179-182^{o}C; IR (KBr, cm^{-1}): 3100, 3010, 1605 (C=N), 1590 (C=C), 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ7.2-7.8 (m, 4H, Ar-H), 7.8 (d, 2H, *J*=8.8 Hz, 2' &

6'- H), 6.75(dd, 2H, *J*=8.8Hz, 3' & 5'-H), 2.89 (s, 6H, Me); ^{13}C NMR (100MHz, $CDCl_3$): δ164.4, 151.2, 150.1, 141.5, 131.3, 127.7, 124.9, 124.3, 119.5, 111.3, 110.2, 40.5.

2.5.1.1.17. 2-(5'-Bromo-4'-hydroxy-3'-methoxyphenyl)-1,3-benzoxazole **$5a_{17}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 78%, m. p.: 187-192^{o}C; IR (KBr, cm^{-1}): 3250, 3010, 1610 (C=N), 1590 (C=C), 1030, 780; ^{1}HNMR (400MHz, $CDCl_3$): δ 7.16-7.88 (m, 4H, Ar-H), 7.53 (s, 1H, 2'-H), 7.32 (s, 1H, 6'-H), 4.5 (brs, 1H, OH), 3.7(s, 1H, OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.6, 150.1, 149.8, 148.7, 141.2, 130.8, 125.6, 124.3, 124.2, 119.9, 110.9, 110.3, 110.2, 55.2.

2.5.1.1.18. 2-(3',5'-Dibromo-4'-hydroxyphenyl)-1,3-benzoxazole **$5a_{18}$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 166-169^{o}C; IR (KBr, cm^{-1}): 3300, 3010, 1630 (C=N), 1600 (C=C), 1030, 780; ^{1}HNMR (400MHz, $CDCl_3$): δ7.9-8.4 (s, 2H, 2' & 6'-H), 7.16-7.89 (m, 4H, Ar-H), 4.5 (brs, 1H, OH); ^{13}C NMR (100MHz, $CDCl_3$): δ163.6, 152.1, 150.4, 141.3, 130.7, 130.1, 125.4, 124.9, 124.2, 111.8, 110.7.

2.5.1.1.19. 5-Bromo-2-(4'-nitrophenyl)-1, 3-benzoxazole **$5b_1$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 80%, m. p.: 228-232^{o}C; IR (KBr, cm^{-1}): 3010, 1650 (C=N), 1600 (C=C), 1550, 1380, 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.28 (d, 2H, *J*=8.8Hz, 3' & 5'-H), 7.99(d, 1H, *J*= 8.6Hz, 7-H), 7.97 (d, 1H, *J*=8.8Hz, 2' & 6'-H), 7.82 (s, 1H, 4-H), 7.53 (dd, 1H, *J*=8.6, 2.1Hz, 6-H); ^{13}C NMR (100MHz, $CDCl_3$): δ 164.4, 150.3, 142.3, 131.3, 129.7, 128.6, 124.7, 119.6, 118.3, 117.3, 114.7. HRMS: $(M+H)^+$ Obs: 319.3074; Cal: 319.1100.

2.5.1.1.20. 5-Bromo-2-(4'-bromophenyl)-1, 3-benzoxazole **$5b_2$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 158-162^{o}C; IR (KBr, cm^{-1}): 3010, 1655 (C=N), 1620 (C=C), 1030, 780, 760; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.06 (s, 1H, 4-H), 7.87(d, 1H, *J*=8.3Hz, 7-H), 7.83 (d, 1H, *J*=8.6Hz, 2' & 6'-H), 7.70 (d, 2H, *J*=8.6Hz, 3' & 5'-H), 7.58 (d, 1H, *J*=7.9Hz, 6-H); ^{13}C NMR (100MHz, $CDCl_3$): δ164.0, 149.3, 141.3, 132.6, 129.7, 128.62, 119.6, 118.3, 117.8, 117.2, 114.7. HRMS:$(M+H)^+$Obs: 350.0725; Cal: 353.0050.

2.5.1.1.21. 2'-(5-Bromophenyl-1, 3-benzoxazol-2-yl) quinoxaline **$5b_3$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 80%, m. p.: 189-192^{o}C; IR (KBr, cm^{-1}): 3010, 1665 (C=N), 1617 (C=C), 1030, 780; ^{1}HNMR (400MHz, $CDCl_3$): δ 9.3 (s, 1H, 3'-H), 8.47 (s, 1H, 5-H), 8.36 (d, 1H, *J*=8.4Hz, 7-H), 8.06 (dd, 1H, *J*=8.4, 2.3Hz, 8'-H), 7.89 (dd, 1H, *J*=8.5Hz, 5'-H), 7.72 (d, 1H, *J*= 8.4Hz, 6-H), 7.1 (dt, 2H, *J*=7.9, 2.3Hz, 6' & 7'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ161.5, 152.01, 149.7, 145.8, 142.5, 141.9, 141.8, 132.2, 129.8, 128.6, 128.8, 119.4, 117.4. HRMS: $(M+H)^{+}$Obs: 326.2597; Cal: 326.1490.

2.5.1.1.22. 2'-(1, 3-Benzoxazol-2-yl) quinoxaline **$5b_4$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 74%, m. p.: 160-164^{o}C; IR (KBr, cm^{-1}): 3010, 1650 (C=N), 1607 (C=C), 1030; ^{1}HNMR (400MHz, $CDCl_3$): δ 9.4 (s, 1H, 4-H), 8.47 (s, 1H, 3'-H), 8.35 (dd, 1H, *J*=8.4, 2.1Hz, 7-H), 8.1 (dd, 1H, *J*=8.6, 2.3Hz, 8'-H), 8.03 (dd, 1H, *J*=8.6, 2.3Hz, 5'-H), 7.99 (dt, 1H, *J*=8.4, 2.5Hz, 5-H), 7.96 (dt, 1H, *J*=8.4, 2.5Hz, 6-H), 7.89 (dt, 1H, *J*= 8.5, 2.1Hz, 6'-H), 7.72 (dt, 1H, *J*=8.5, 2.1Hz, 5'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ161.4, 152.4, 148.8, 145.4, 142.9, 141.3, 141.1, 131.1, 128.8, 127.7, 125.4, 124.3, 121.4, 115.4, 111.4. HRMS: $(M+H)^{+}$Obs: 247.0866; Cal: 247.2570.

2.5.1.1.23. 3'-(1, 3-Benzoxazol-2-yl)-3'-chloroquinoline **$5b_5$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 102-108^{o}C; IR (KBr, cm^{-1}): 3010, 1665 (C=N), 1637 (C=C), 1030, 830; ^{1}HNMR (400MHz, $CDCl_3$): δ7.37-8.15(m, 4H, Ar-H), 8.2(s, 1H, 4'-H), 8.0(dd, 1H, *J*=7.6, 2.3Hz, 8'-H), 7.64 (dd, 1H, *J*=7.6, 2.3Hz, 5'-H), 7.64-7.62 (dt, 2H, *J*=7.6, 2.3Hz, 6' & 7'-H); ^{13}C NMR(100MHz, $CDCl_3$): δ 161.1, 150.1, 141.5, 135.6, 133.8, 129.4, 128.0, 128.2, 127.8, 124.8, 124.1, 119.9, 110.2. HRMS: $(M+H)^{+}$Obs: 280.1110; Cal: 280.0456.

2.5.1.1.24. 3'-(5-Bromophenyl-1, 3-benzoxazol-2yl)-3'-chloroquinoline **$5b_6$**

This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 76%, m. p.: 170-175^{o}C; IR (KBr, cm^{-1}): 3010, 1660 (C=N), 1620 (C=C), 1030, 830; ^{1}HNMR (400MHz, $CDCl_3$): δ 8.4(s, 1H, 4-H), 8.0(s, 1H, 4'-H), 7.9(d, 1H, *J*=7.8Hz, 7-H), 7.8(dd, 1H, *J*=7.6, 2.1Hz, 8'-H), 7.7(d, 1H, *J*=7.6Hz, 8'-H), 7.62-7.50(m, 3H, *J*=8.2,

2.1Hz, 5',6', 7'-H); ^{13}C NMR (100MHz, $CDCl_3$): δ161.8, 150.1, 141.5, 135.6, 133.9, 129.5, 128.9, 128.2, 127.8, 124.9, 124.1, 119.9, 110.3. HRMS: $(M+H)^+$Obs: 359.0450; Cal: 359.6030.

2.5.2. Biological Evaluation

2.5.2.1. Virtual screening: Protein Structure Preparation

The molecular docking study was performed using Molegro Virtual Docker (MVD-2013, 6.0). The crystal structure of the epidermal growth factor receptor tyrosine kinase domain (PDB ID: 1M17) complexed with 4-anilinoquinazoline inhibitor were downloaded from Protein Data Bank. Molecular docking studies of the synthesized compounds/ligands were performed in order to understand the various interactions between the ligand and enzyme active site in detail. The molecular docking study was performed for the target compounds by using MVD-2013 (Version: 6.0).

2.5.2.2. Molecular Docking Study

The synthesized compounds were built using Chemdraw 11.0. The 2D structures were then converted into energy minimized 3D structures and were saved as MDL Molfile (.mol2). The coordinate files and crystal structures of tyrosine kinase (PDB ID: 1M17) were obtained from the RCSB PDB website. The protein files were prepared by the removal of water molecules, addition of polar hydrogens and removal of other bound ligands. In the present study, the binding sites were selected based on the amino acid residues, which are involved in binding with erlotinib as obtained from protein data bank, which would be considered as the probable best accurate regions as they are solved by experimental crystallographic data. The docking protocol was carried out for the synthesized compounds as listed in **Table 7** using MVD-2013 (6.0) software using the standard operating procedures.

2.5.3. Anti-cancer Evaluation:

2.5.3.1. General procedure for evaluation of anti-cancer inhibitory activity

The anti-cancer activity of the compounds has been accessed by MTT assay. Initially, MTT stock solution has been prepared by taking 5mg in 1 mL of phosphate buffer saline (PBS). The cell viability study was carried out against five different cell lines; MDA-MB-231, MCF-7, A549, KB, HEK-239 cell lines. The cell lines were maintained in 96 wells micro titer plate

containing MEM media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% mixture of Gentamicin (10µg), Penicillin (100units/mL) and Streptomycin (100µg/mL) in presence of 5% CO_2 at 37^oC for 48-72 hours. *In vitro* growth inhibition effect of test compounds was assessed by colorimetric or spectrophotometric determination of conversion of MTT into Formazan blue by living cells. Initially the supernatant from the plate was removed and fresh MEM solution was added and was treated with different concentrations of extract or compound appropriately diluted with DMSO. In our study, 10, 20, 25, 30 and 50µL(10µg/mL prepared in DMSO) of stock solutions were added to respective wells containing 100µL of the medium. Thereby making the final concentrations as 10, 20, 25, 30 and 50µg/mL. After 48hrs of incubation at 37^oC in a humidified atmosphere of 5% CO_2, stock solution of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide was added to each well (20µL, 5mg per mL in sterile phosphate buffered saline) for further 4 hour incubation. The supernatant carefully aspirated, the precipitated crystals of Formazan blue were solubilised by adding DMSO and optical density was measured at wavelength of 570 nm by using LISA plus. The results were represented as mean of five readings upto the concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control. During this assay, reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide was measured by mitochondrial succinate dehydrogenase.

$$\text{Formula: Surviving cells} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \times 100$$

2.5.3.2. Enzyme inhibition studies

Cell Treatment- The cells were seeded at a density of approximately 1×10^5cells/well in a 96-well flat-bottom micro plate and maintained at 37ºC in 95% humidity and 5% CO_2 for overnight. Cells were treated with different concentration of test samples. Then cells were incubated for another 24 hours. The cells in well were washed twice with phosphate buffer solution, and 1 ml of extraction buffer was added. Using cell scraper cells were recovered carefully and centrifuge the cells at 4ºC for 10 min at 10,000 rpm. Collect the supernatant and store as further analysis.

Tyrosine Kinase Assay- Collected supernatant was diluted 25 times with kinase reacting solution provided along with kit. The diluted control was added and treated to sample in each well in duplicate. Then 10 µl of 40 µM ATP-2Na solution was added into each well and mixed

well. Further, incubated for 30 min at 37ºC.Then the samples were removed, the wells were washed 3 times with wash buffer. 100µl of blocking solution was added into each well and incubated for 30 min at 37ºC. The blocking solution was discarded and then 50 µl of Anti-phosphotyrosine - HRP solution was added into each well and incubated for 30 min at 37ºC. Now, the antibody solution was discarded and then each well was washed 4 times with washing buffer. Further, 100 µl of horseradish peroxidase substrate solution was added into each well. Incubated for 30 min at 37ºC. Finally, 100 µl of stop solution was added into each well in the same order as HRP substrate solution. The absorbance was measured at 450 nm with a plate reader.

% Inhibition = 1- (Abs of sample /Abs of control) x 100

2.5.4. Cell morphology studies by Fluorescence microscopy

2.5.4.1.Double staining (Acridine orange-Ethidium bromide):

The cells were seeded at a density of approximately $1x10^4$cells/well in a 24 well flat bottom micro plate containing cover slips and maintained at 37^oC in CO_2 incubator for overnight. More than the IC_{50} of synthesised compounds was treated at 72 hrs. After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30min. 20 µL of dye mixture was incubated for half an hour, examined under fluorescent microscope.

2.5.4.2.DAPI:

The cells were seeded at a density of approximately $1X10^5$cells/well in a 12 well flat bottom micro plate containing cover slips and maintained at 37^oC in CO_2 incubator for overnight. More than the IC_{50} of synthesised compounds was treated at 72 hrs. After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30min. 20 µL of DAPI was incubated for 20min, examined under fluorescent microscopy.

2.5.5. Anti-tubercular activity:

The anti-mycobacterial activity of synthesized benzoxazole derivatives **$5a_1$-a_{18}** and **$5b_1$-b_6** was assessed against *Mycobacterium tuberculosis* H_{37}RV strain using Microplate Alamar Blue assay (MABA). Briefly, 200µg/mL of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds was made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µg/mL of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink.

2.6. REFERENCES

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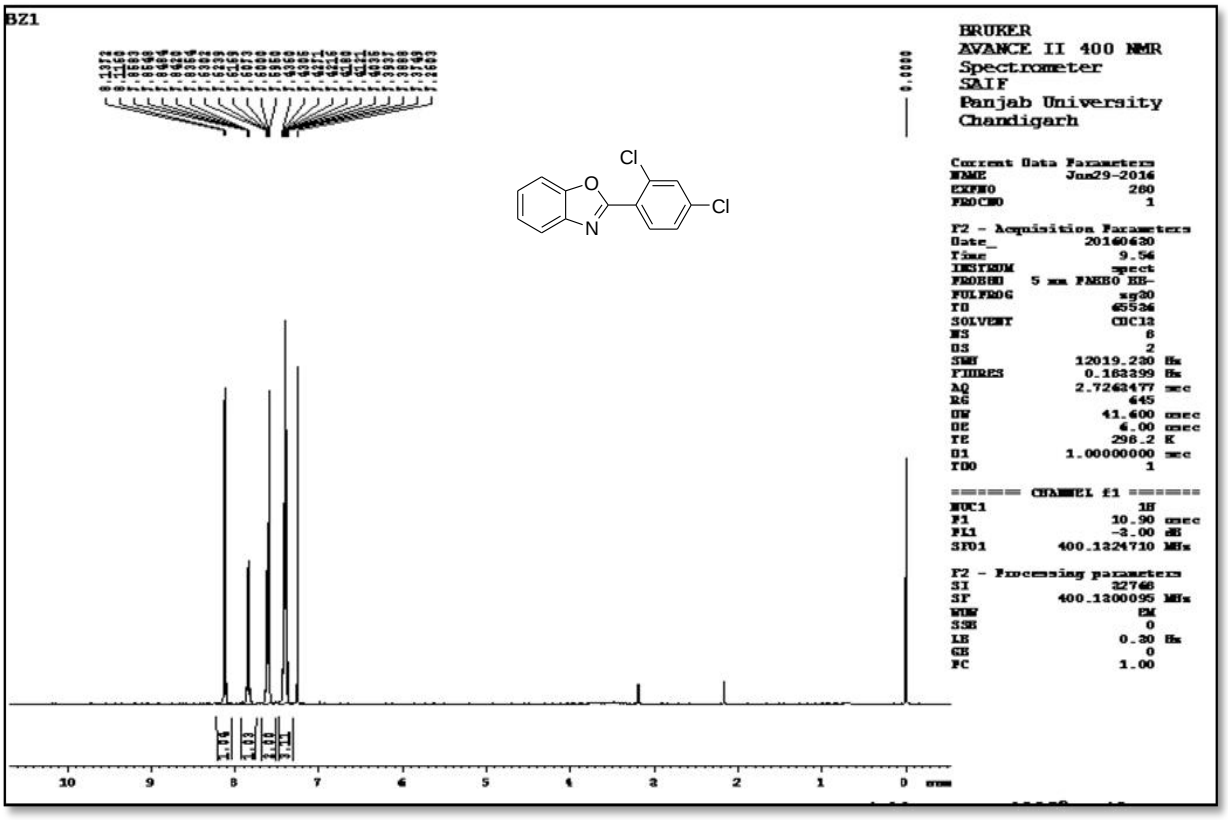

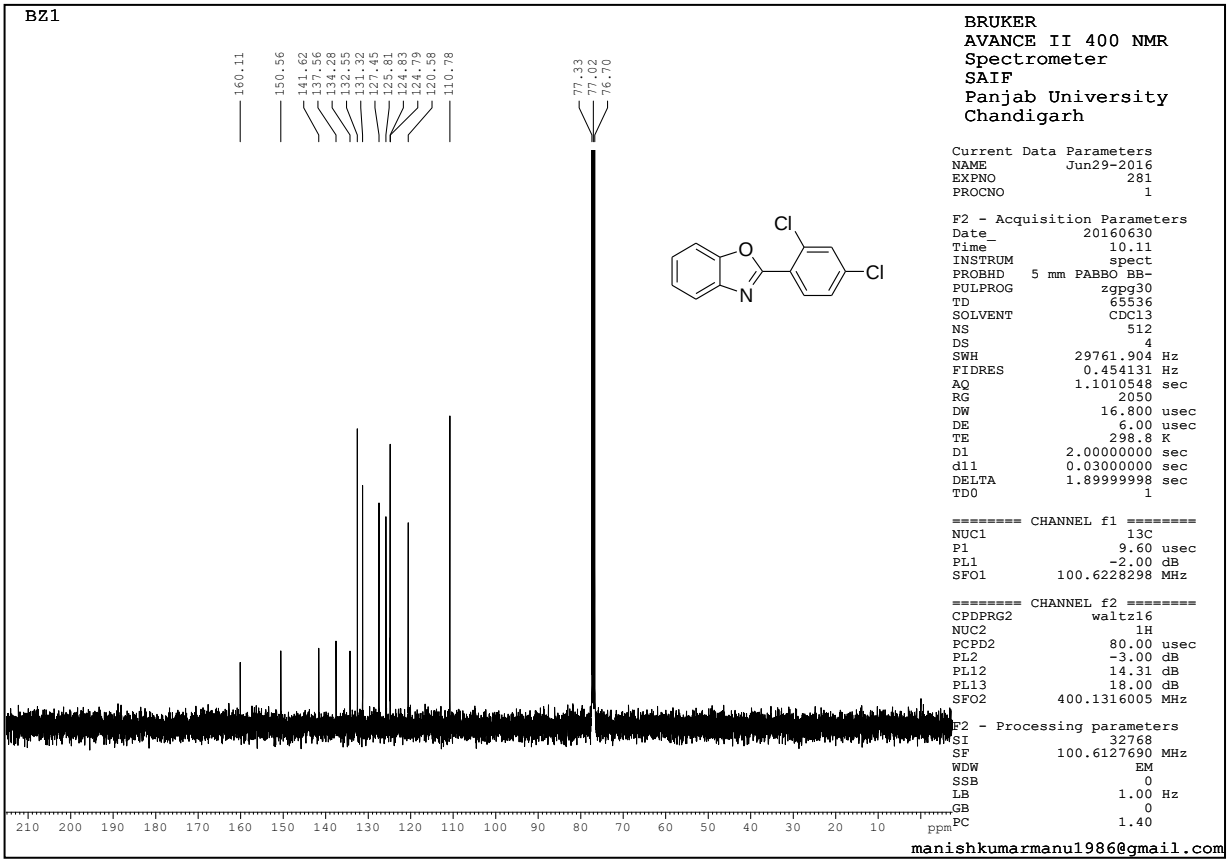

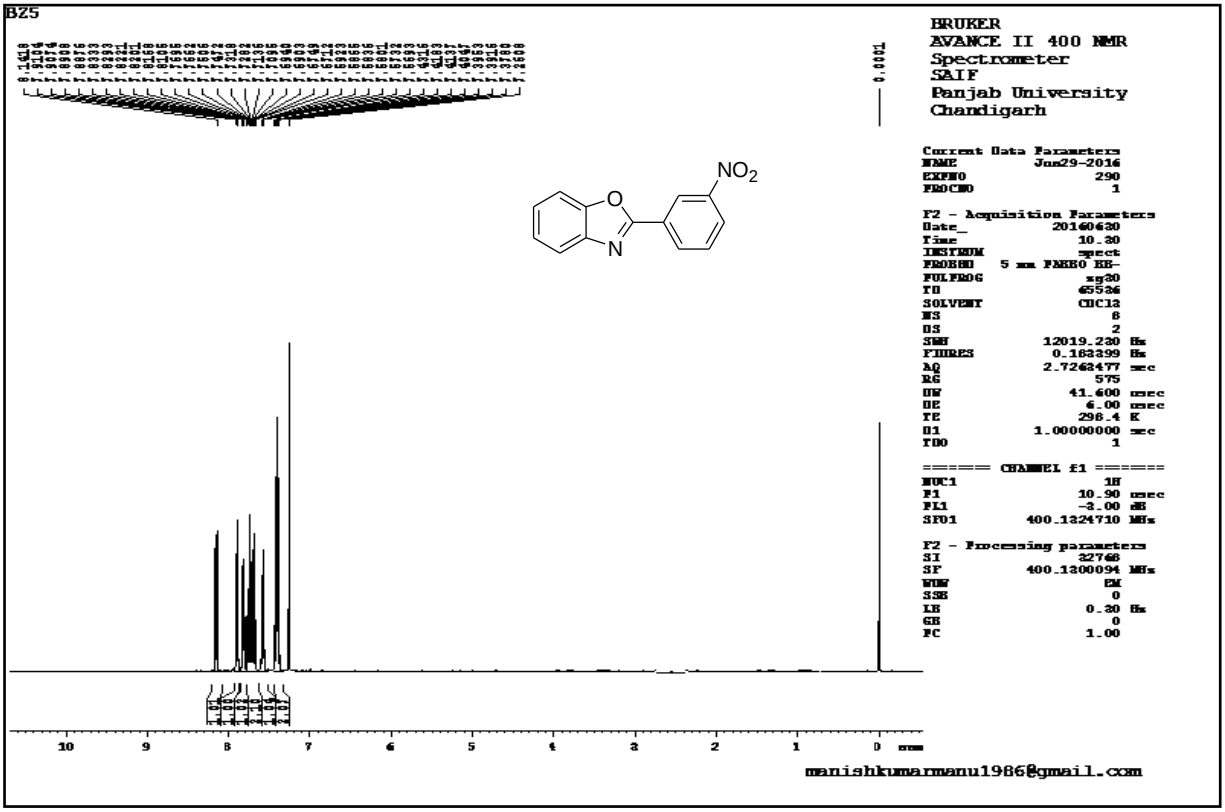
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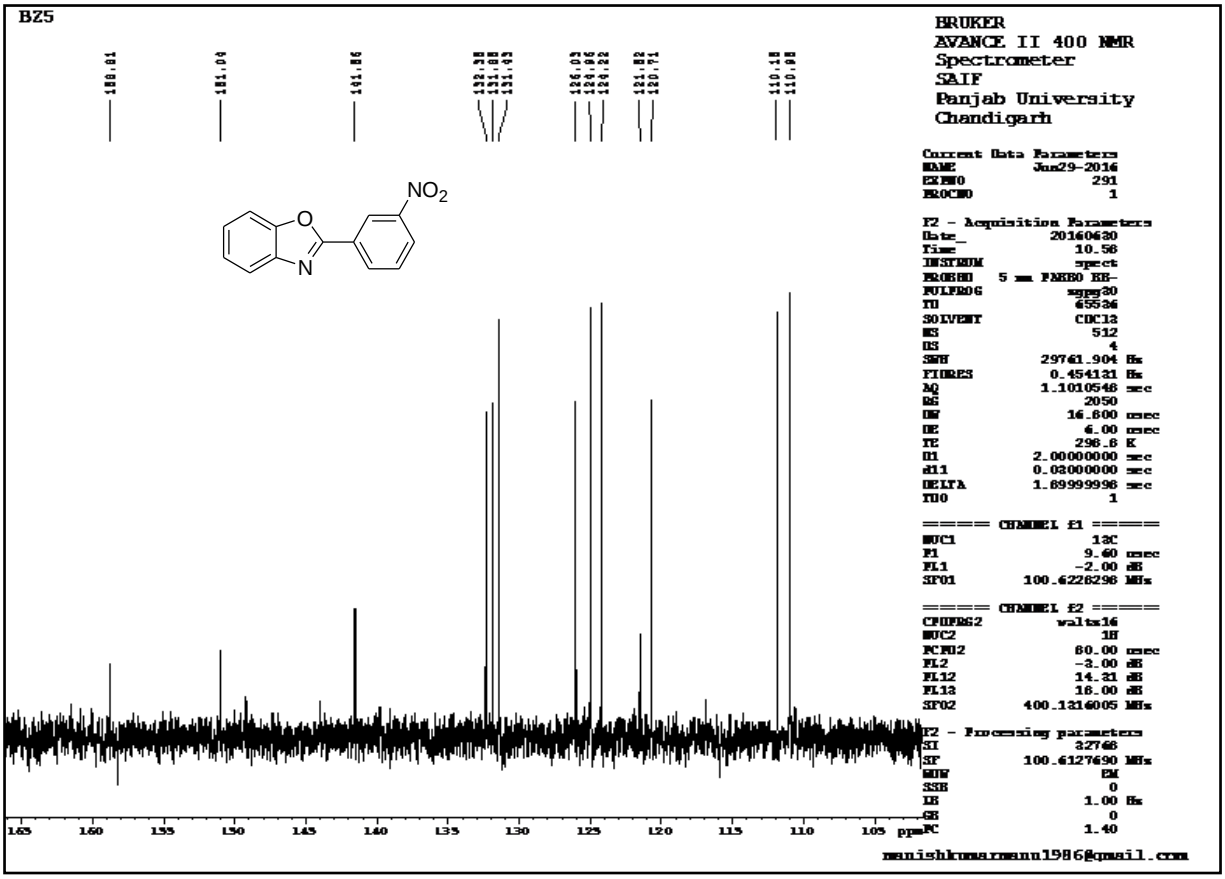
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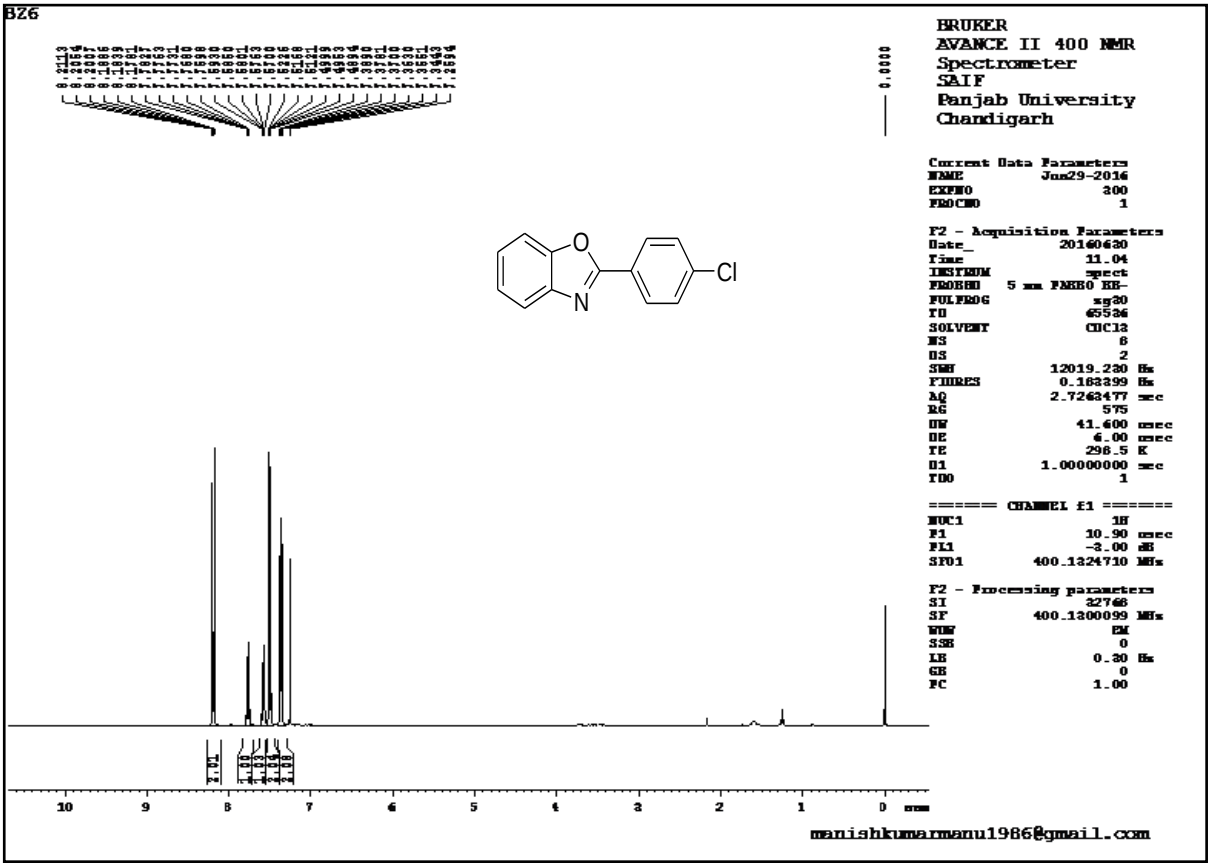
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RG 575
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DE 6.00 usec
TE 298.5 K
D1 1.00000000 sec
TD0 1
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PL1 -3.00 dB
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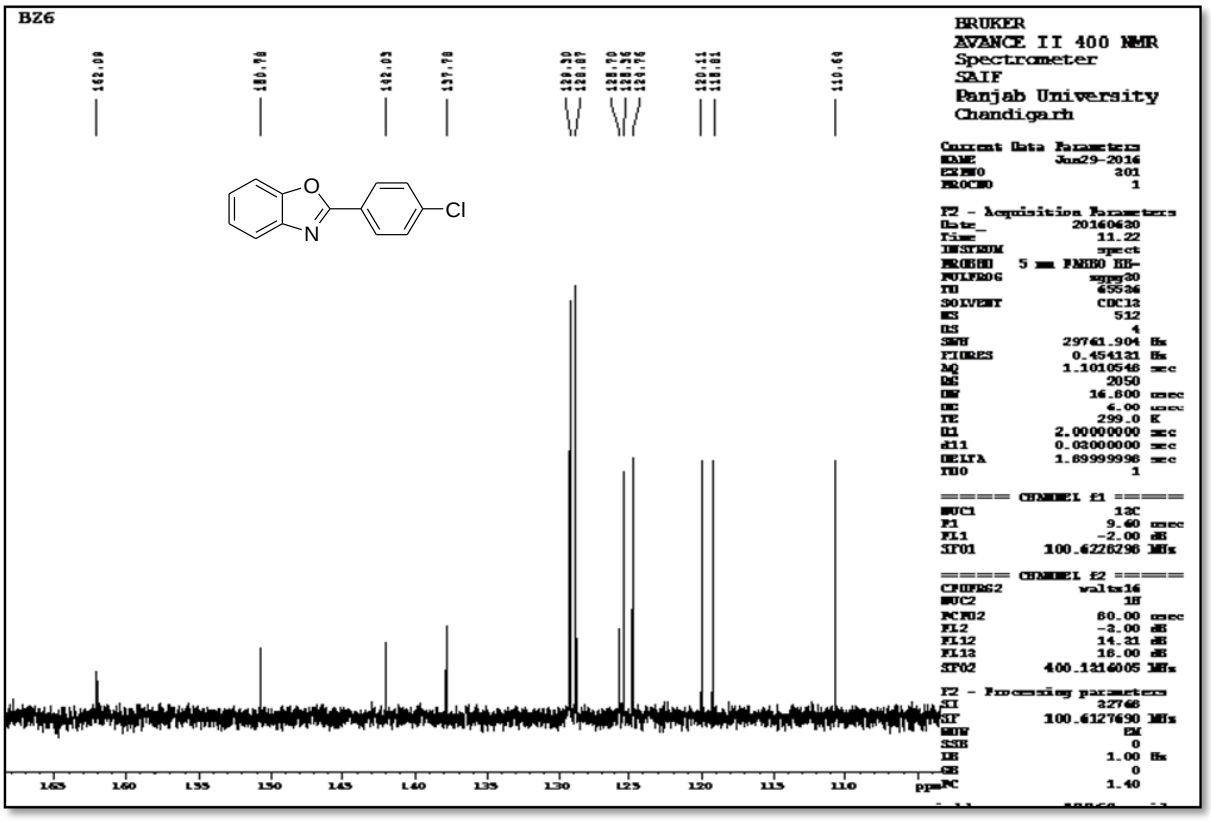
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RG 2050
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DE 6.00 usec
TE 299.0 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999998 sec
TD0 1
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PL1 -2.00 dB
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PL12 14.31 dB
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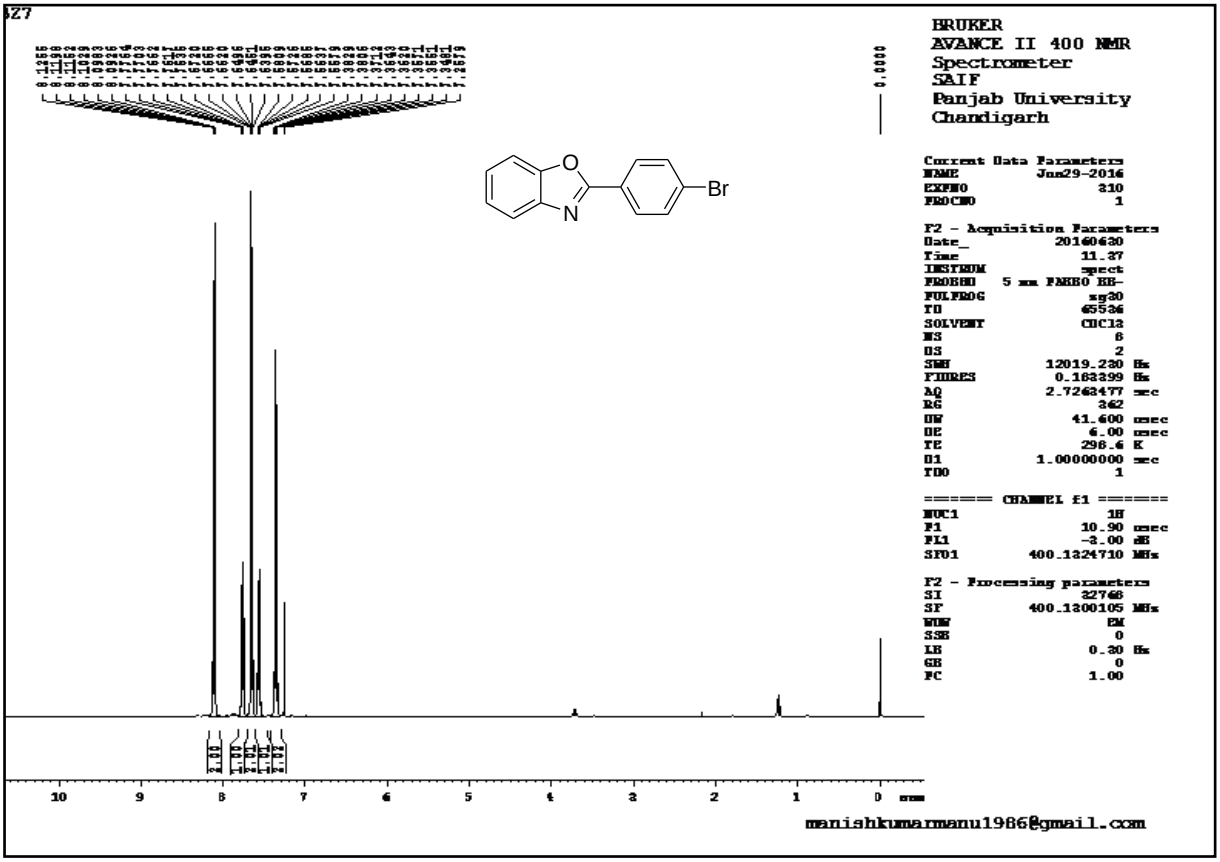
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Panjab University
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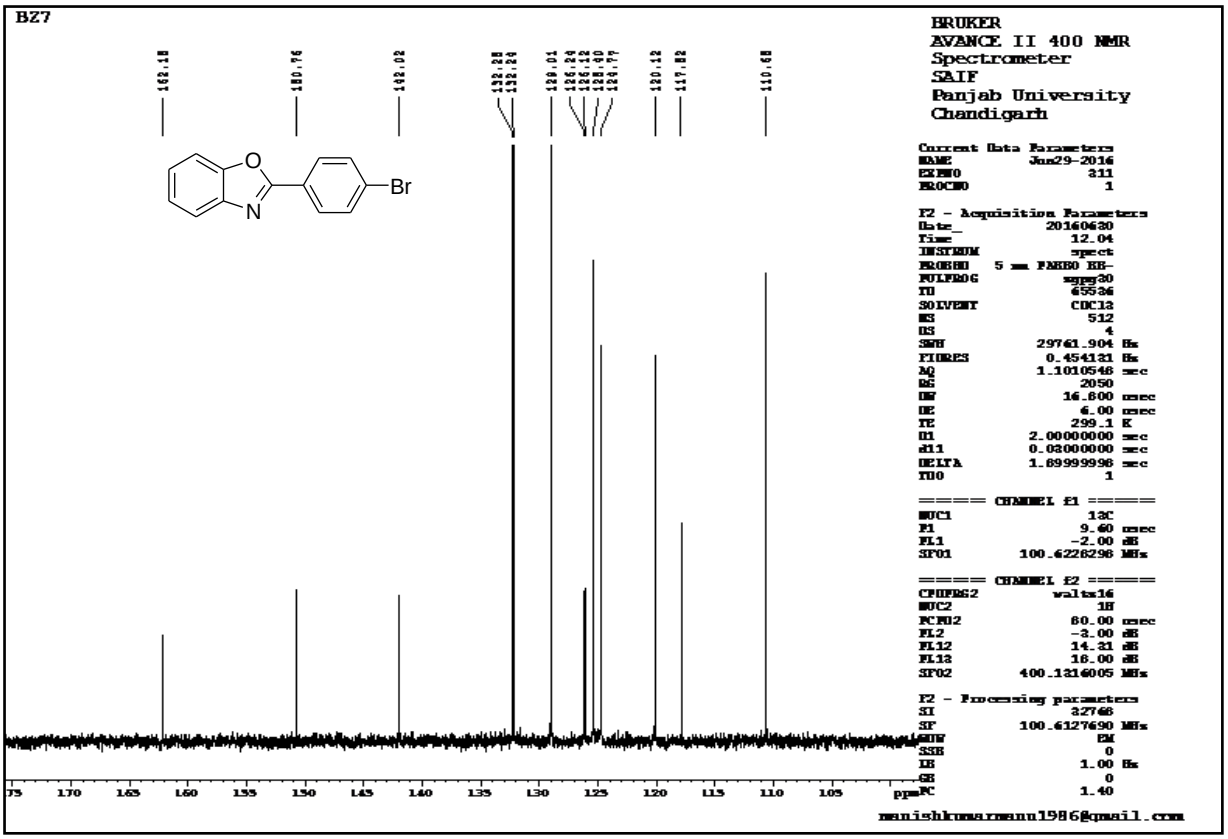
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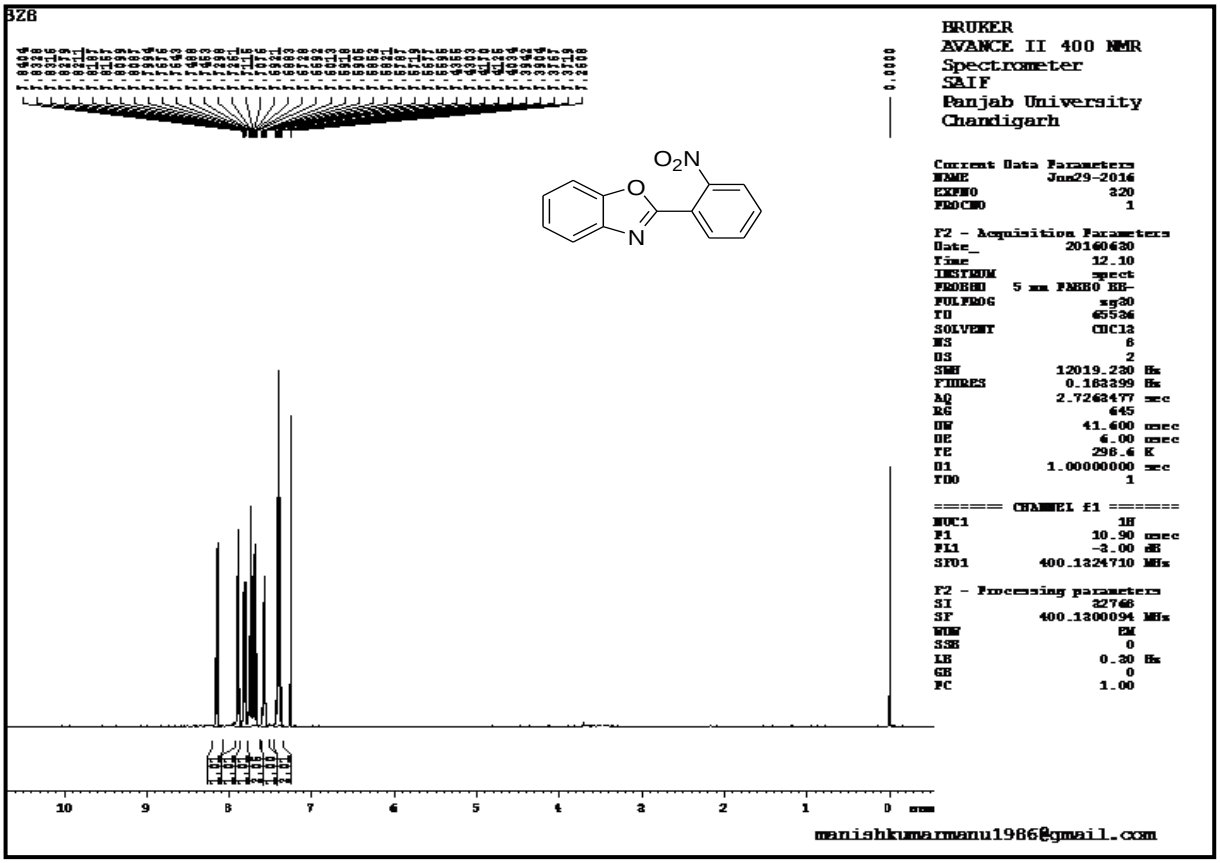
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DE 6.00 usec
TE 298.6 K
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TD0 1
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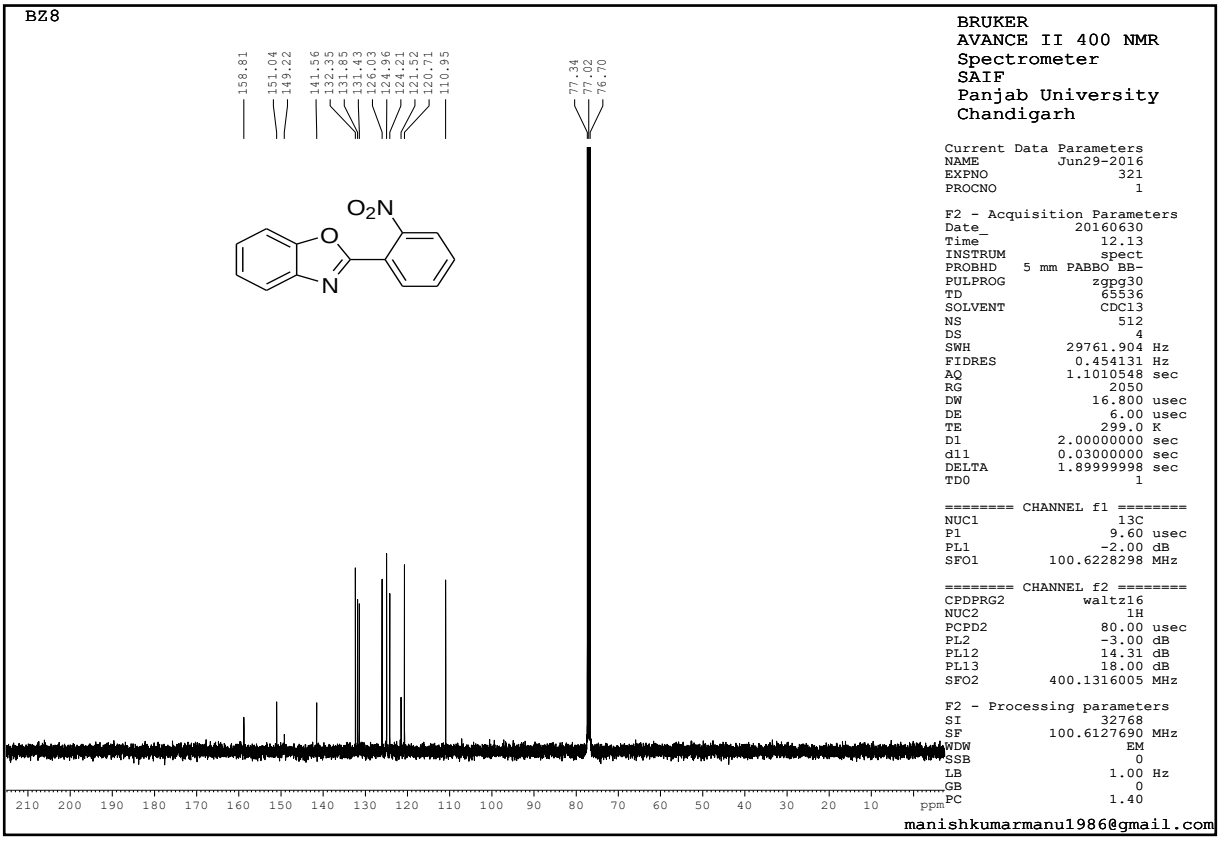
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SAIF
Panjab University
Chandigarh
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DE 6.00 usec
TE 299.0 K
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PL13 18.00 dB
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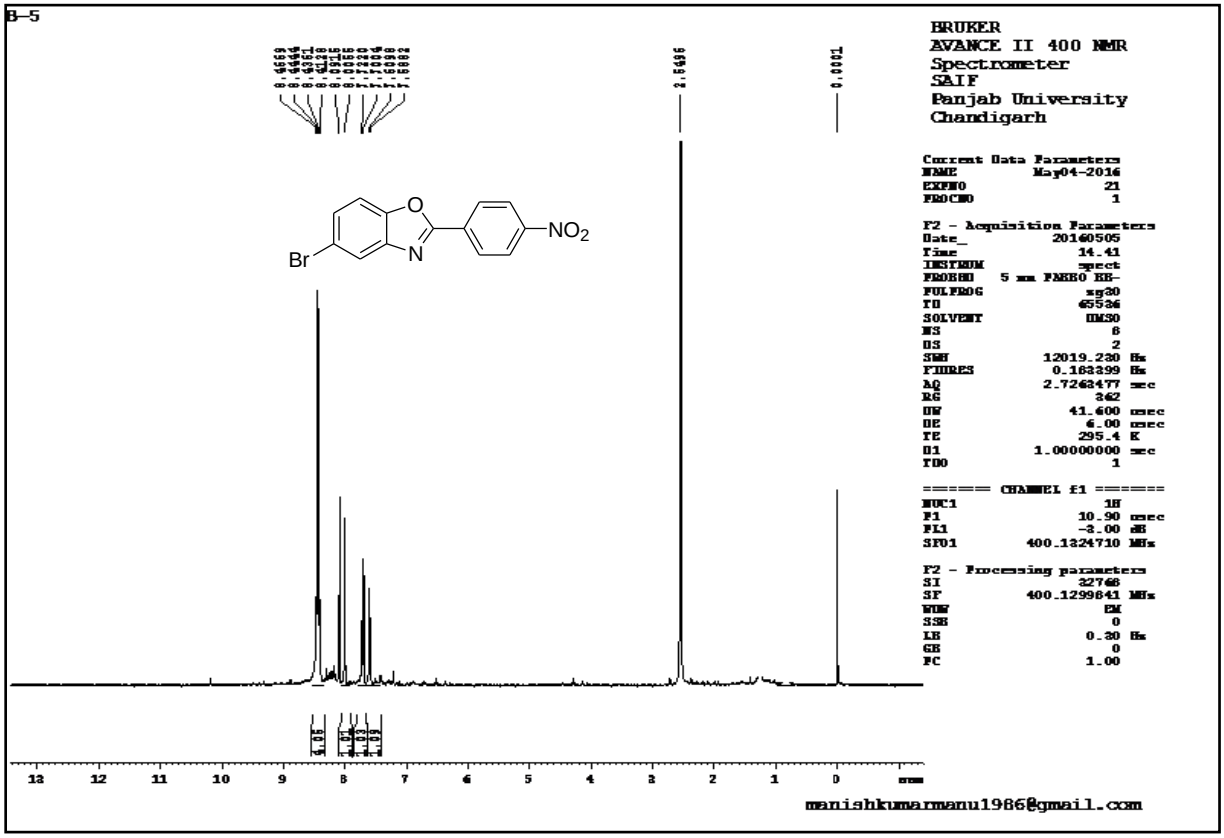
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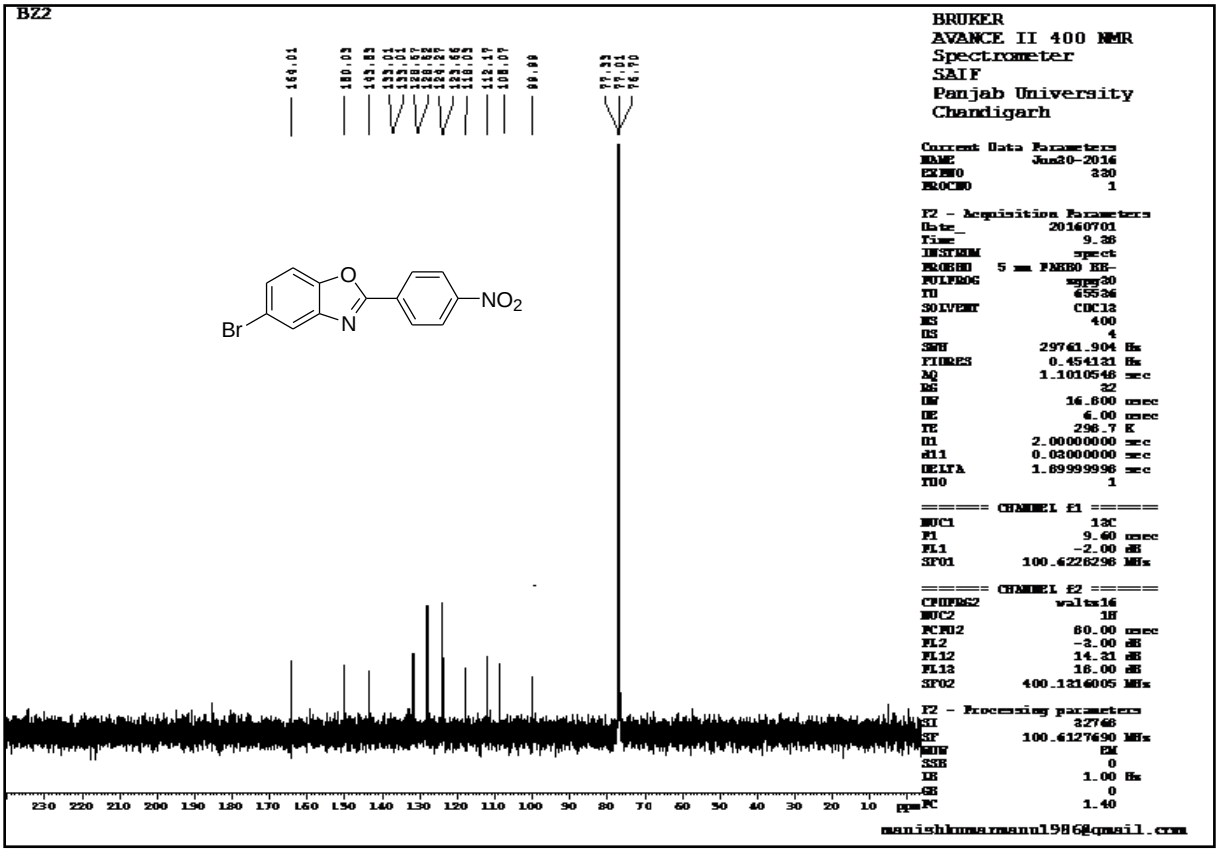
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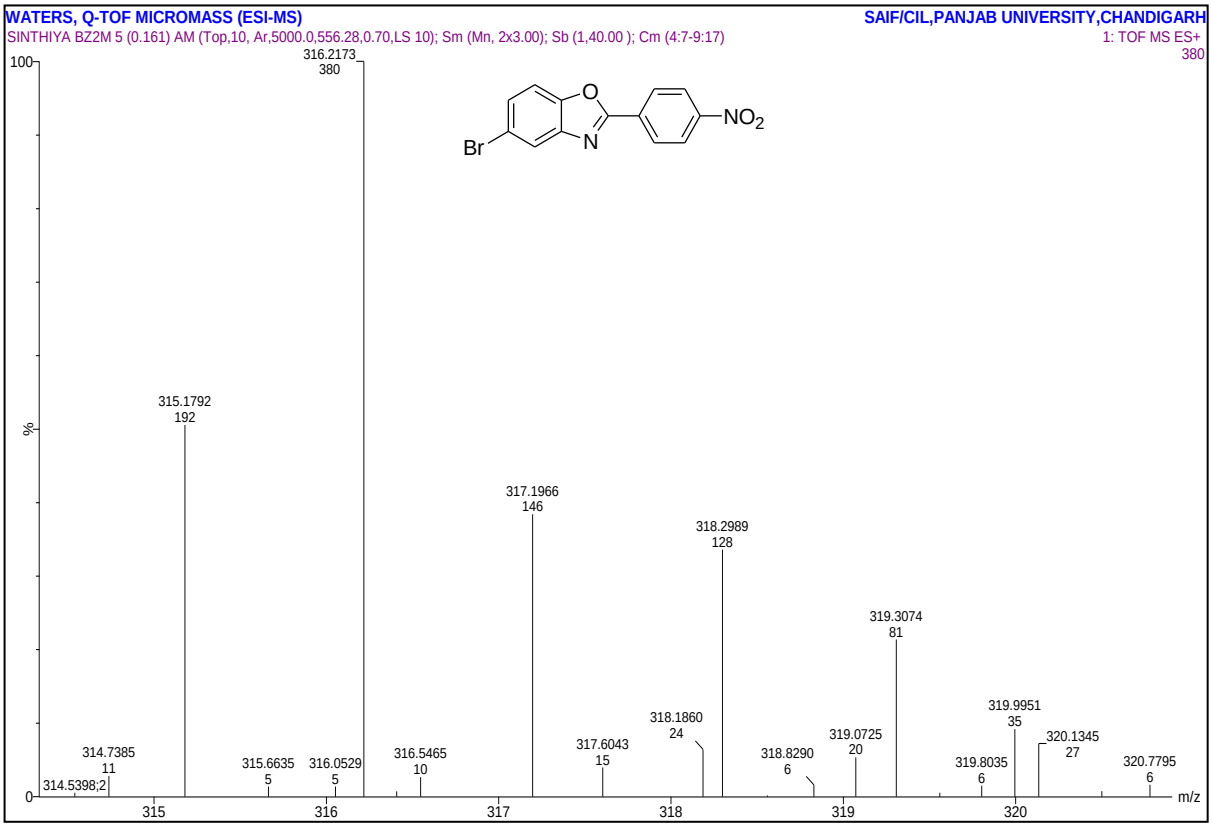
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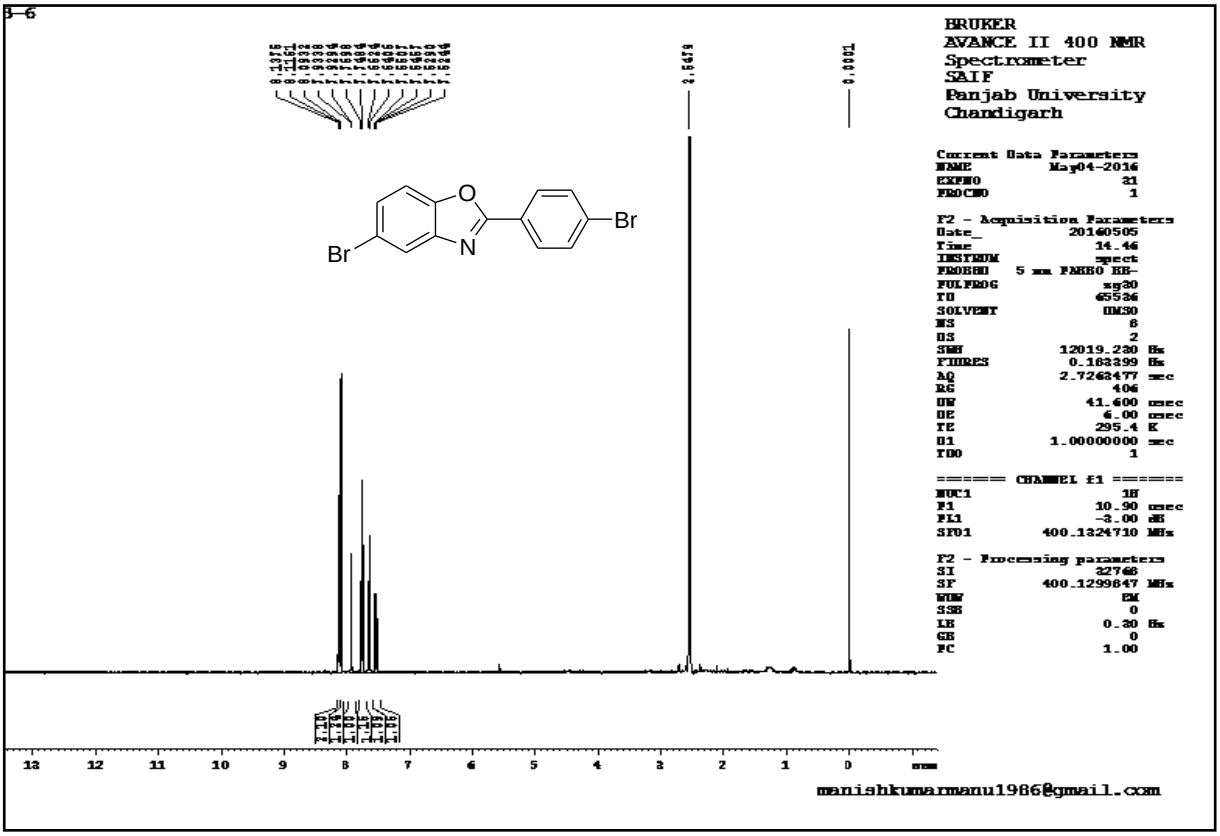
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Panjab University
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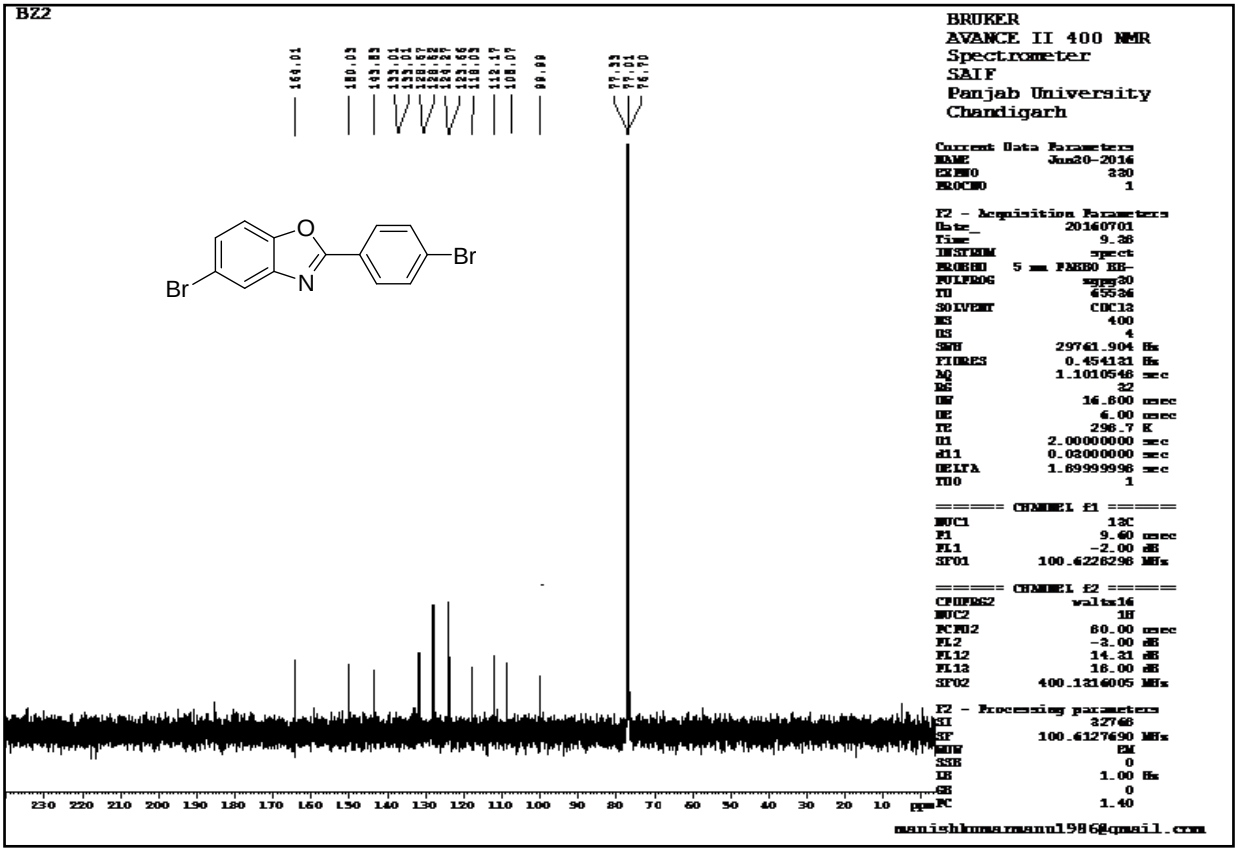
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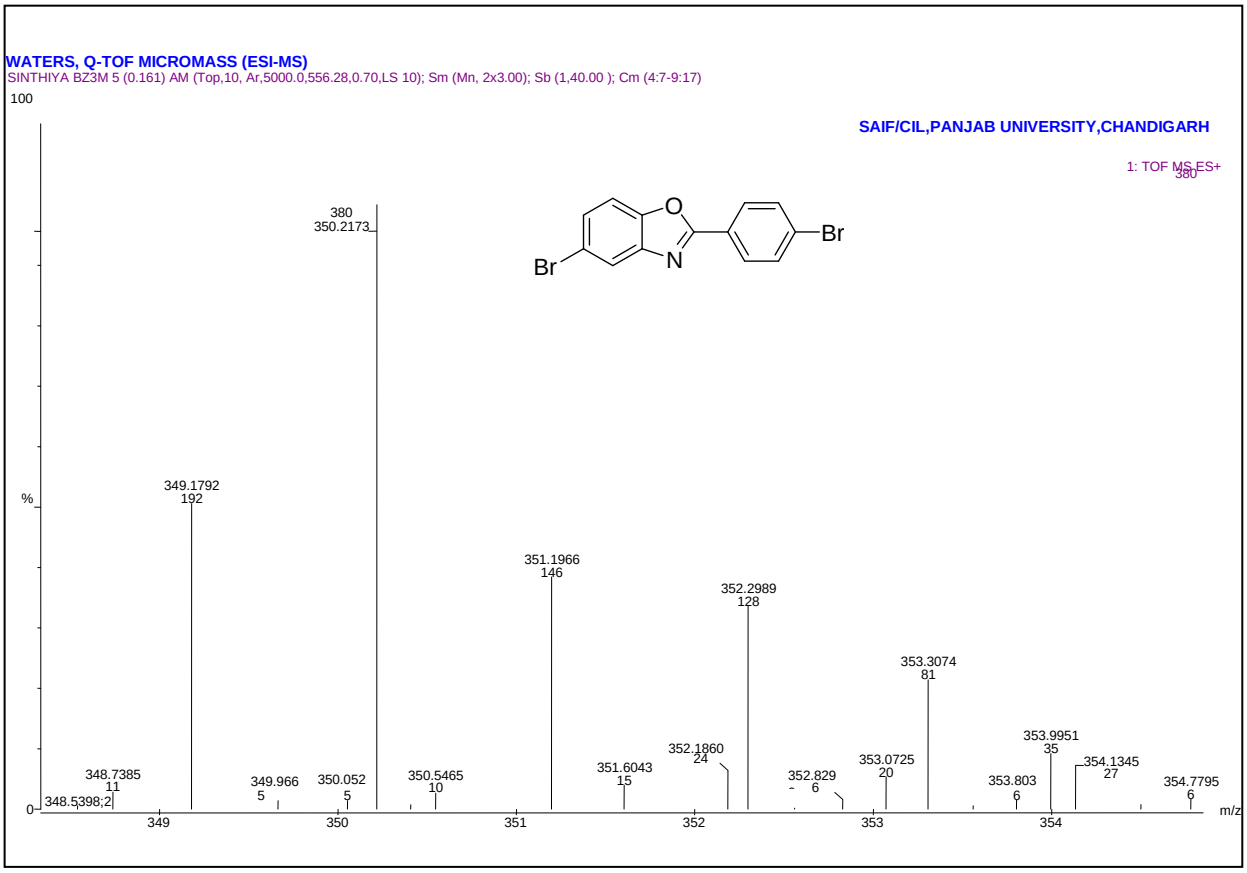
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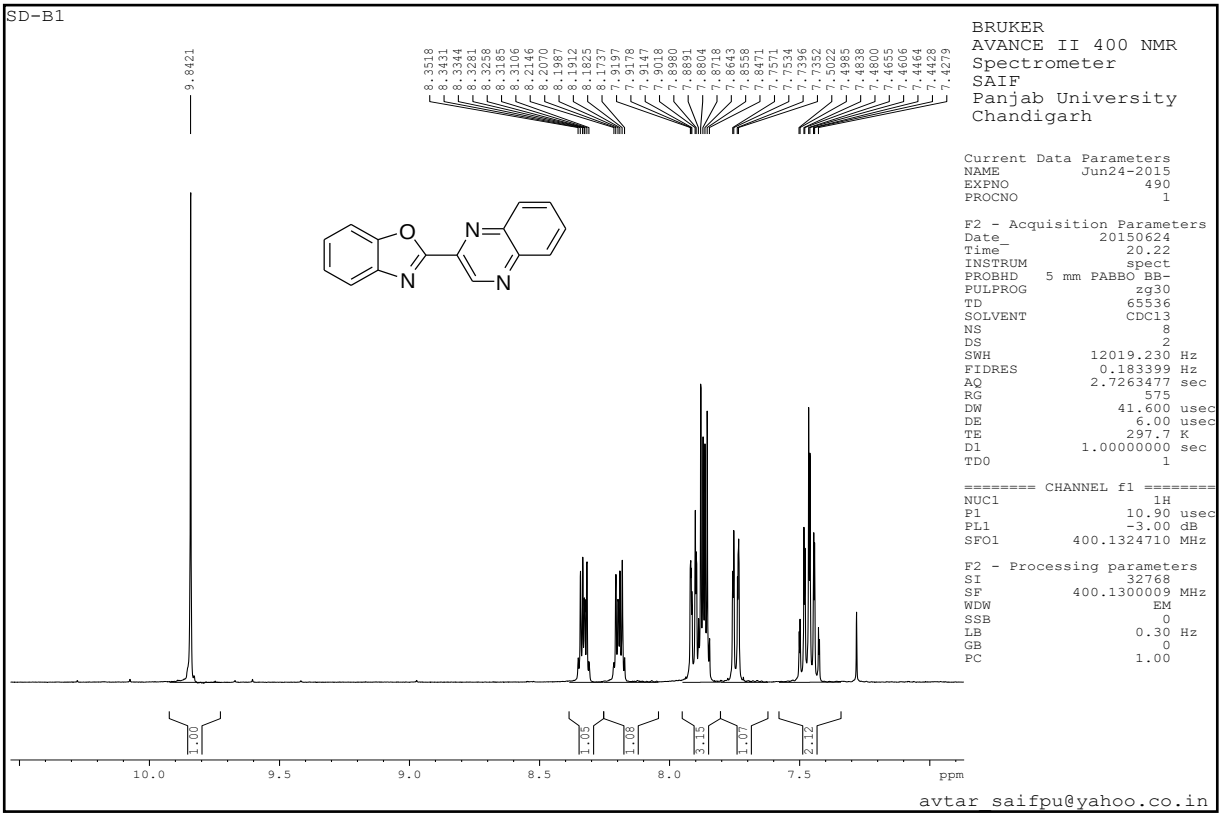
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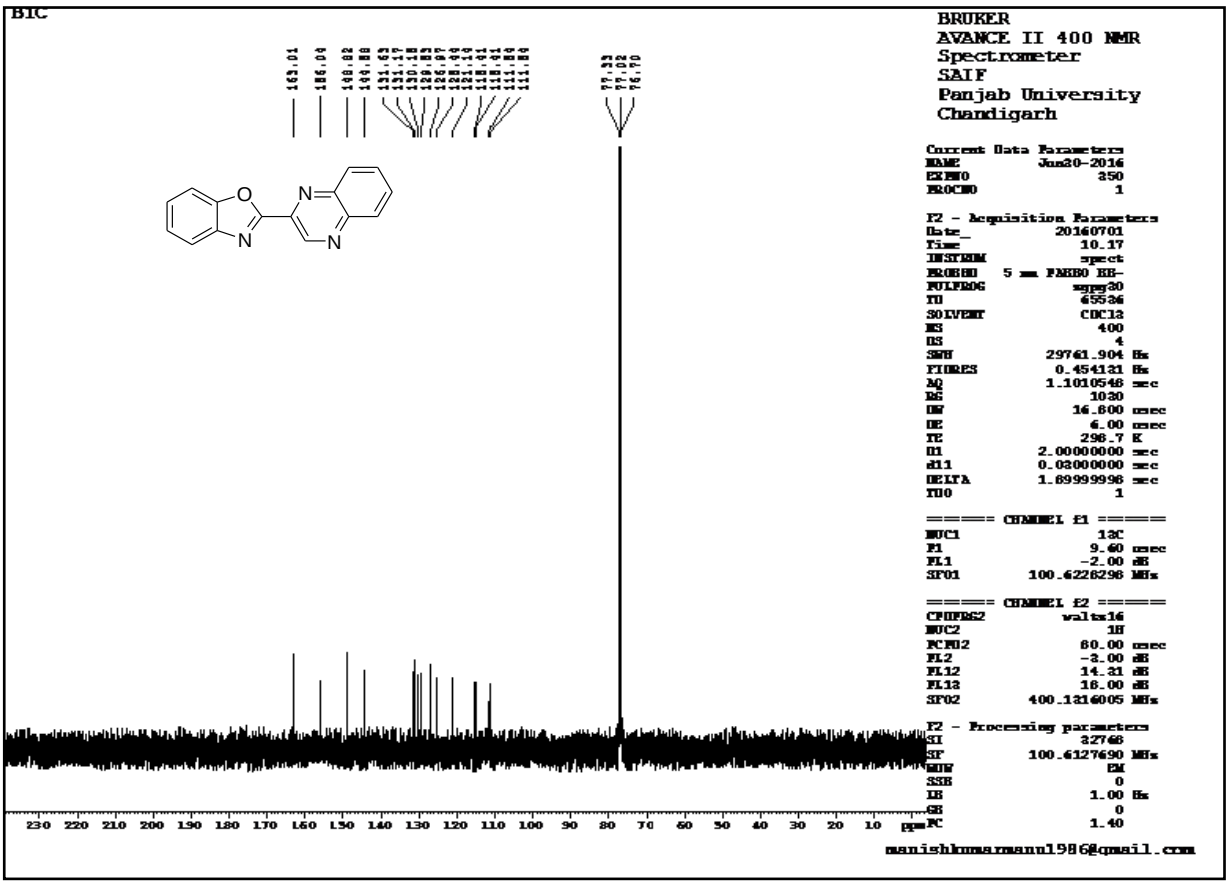
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DELTA 1.89999998 sec
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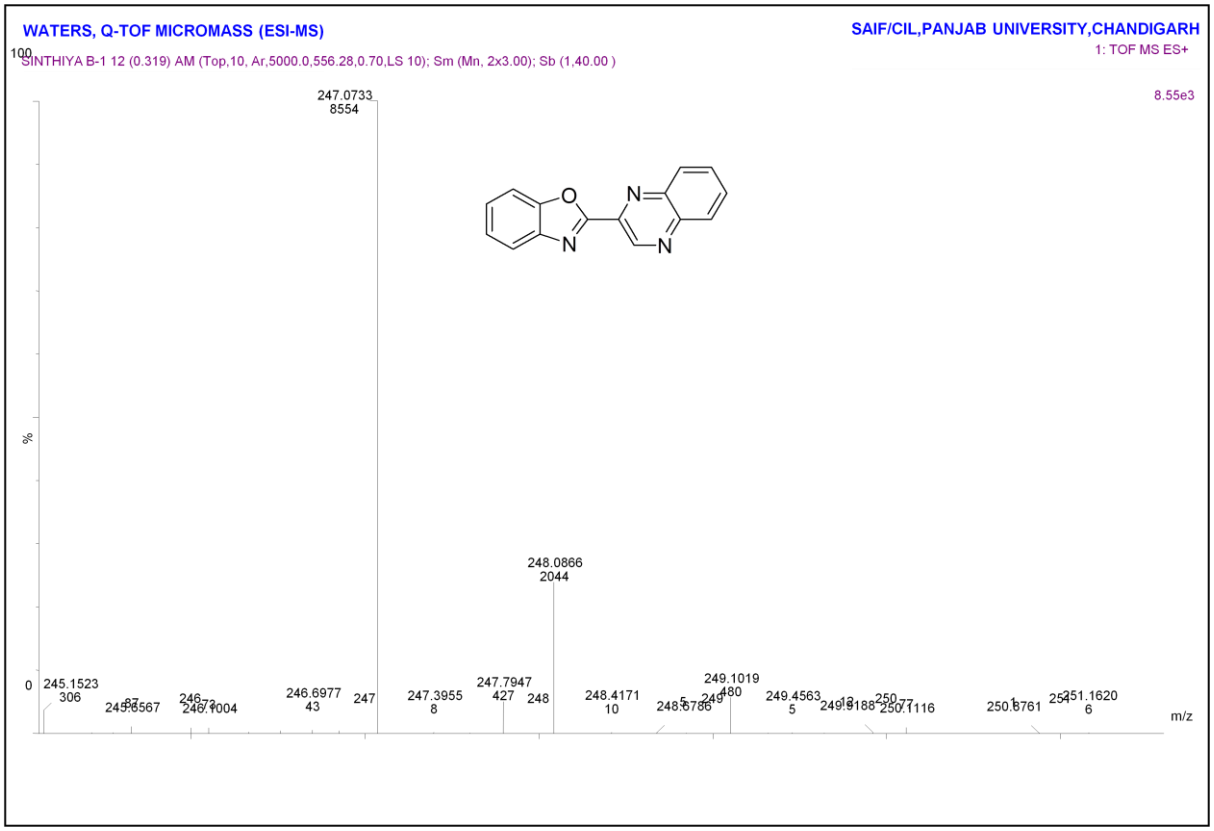
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SAIF/CIL,PANJAB UNIVERSITY,CHANDIGARH
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2044
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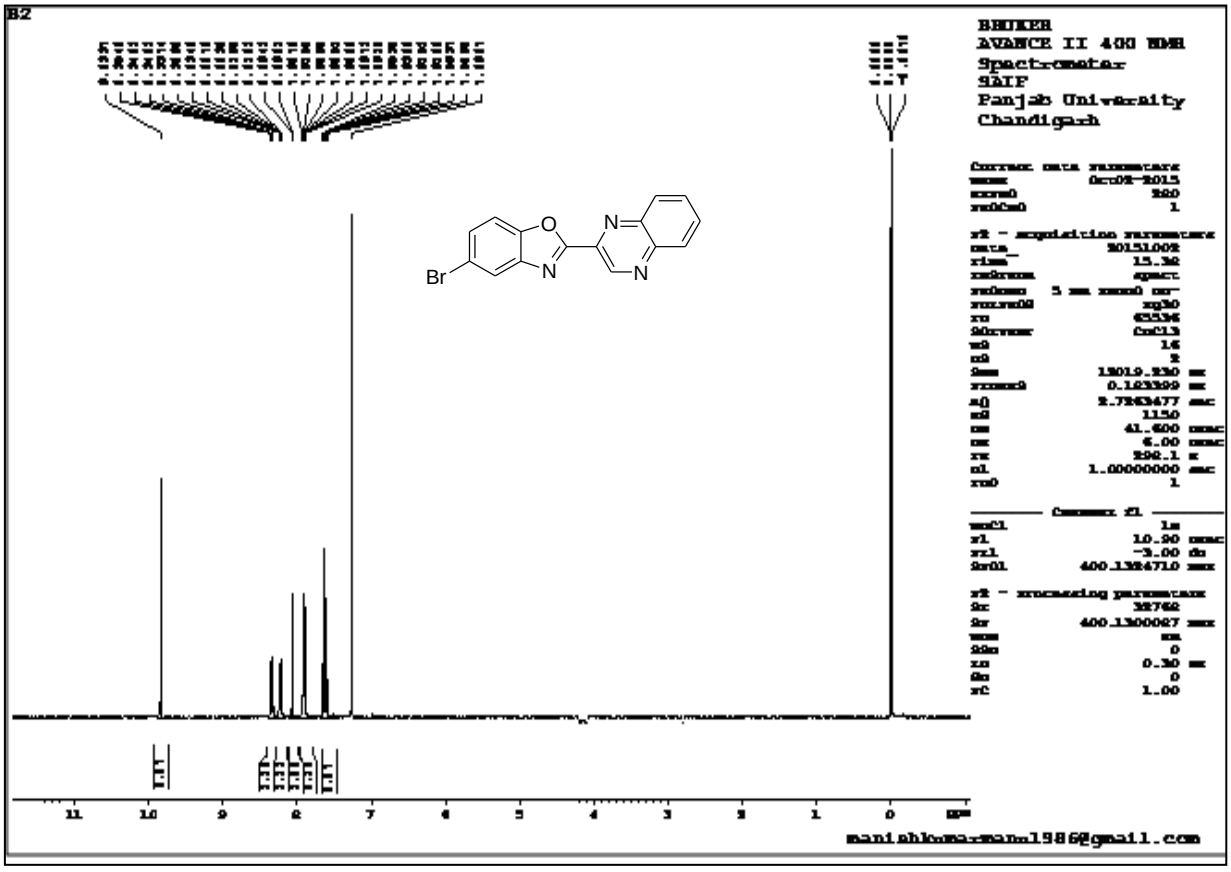
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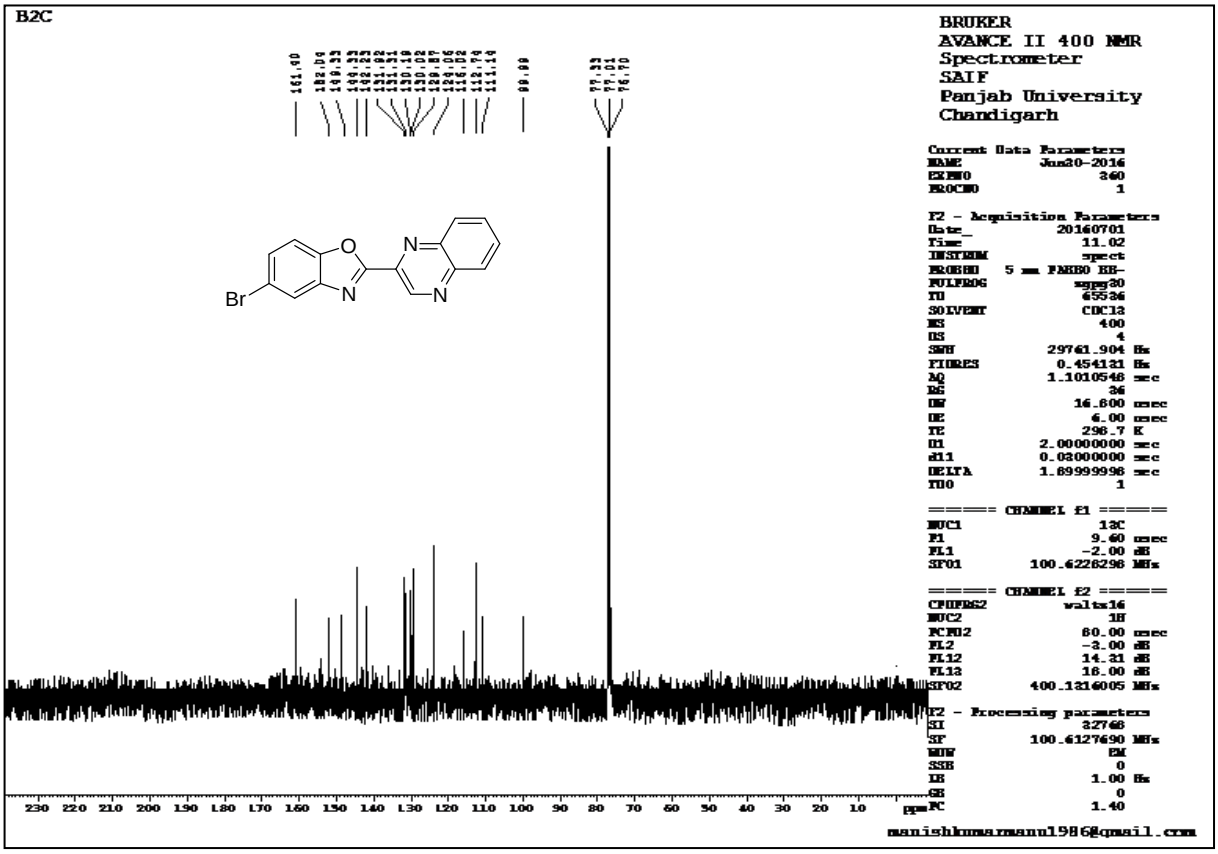
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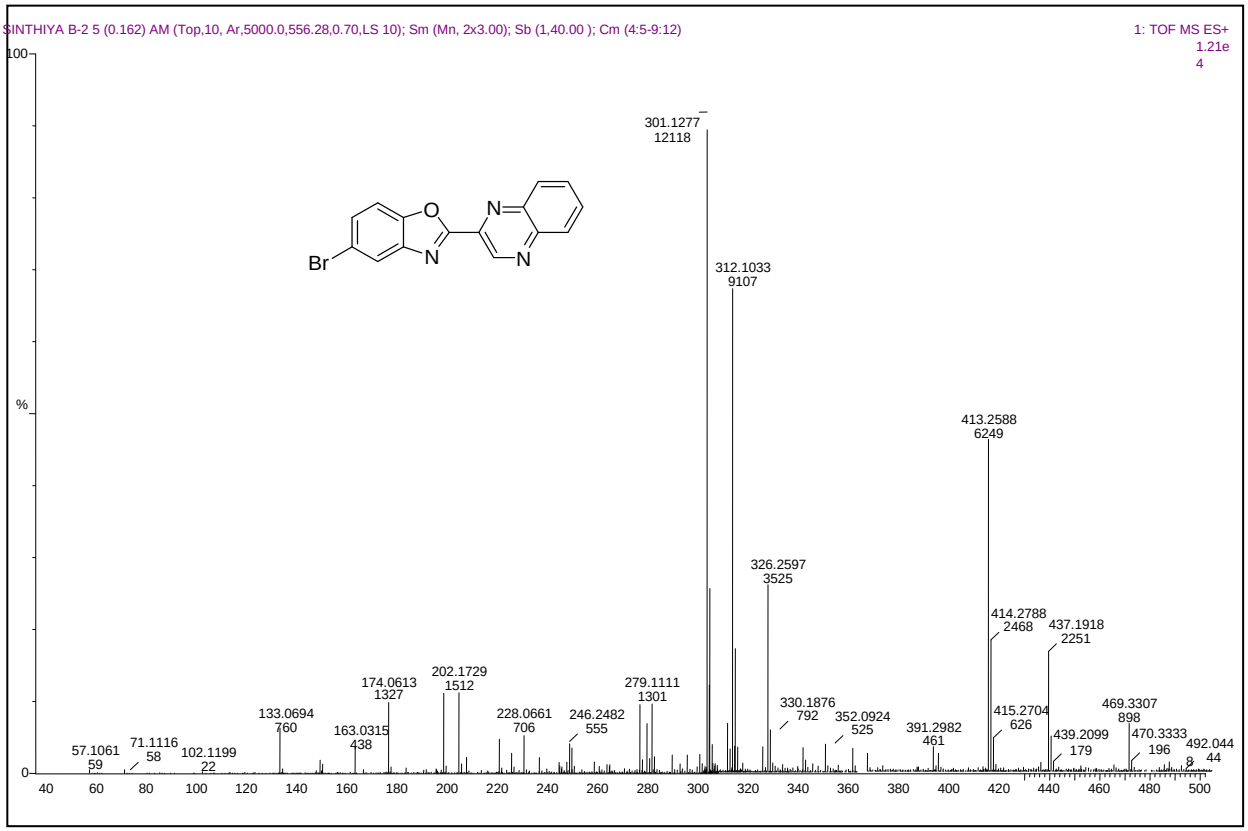
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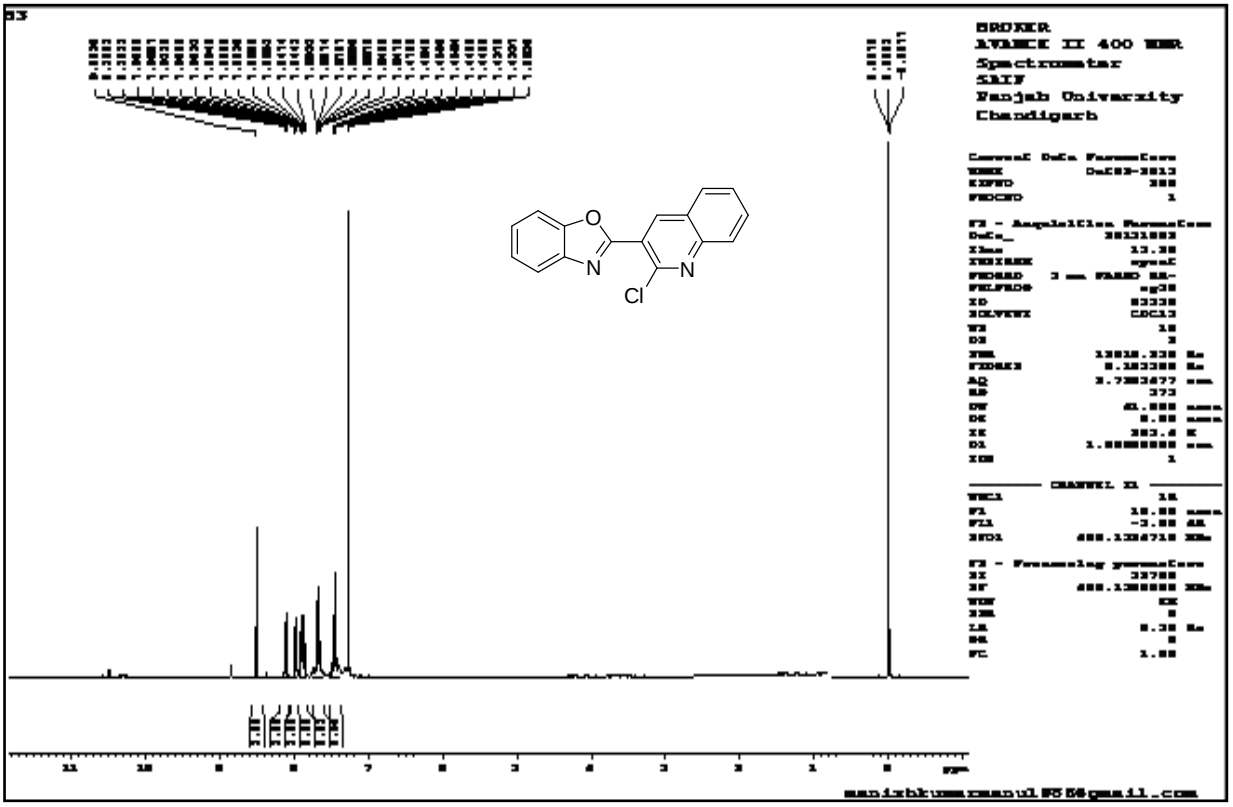
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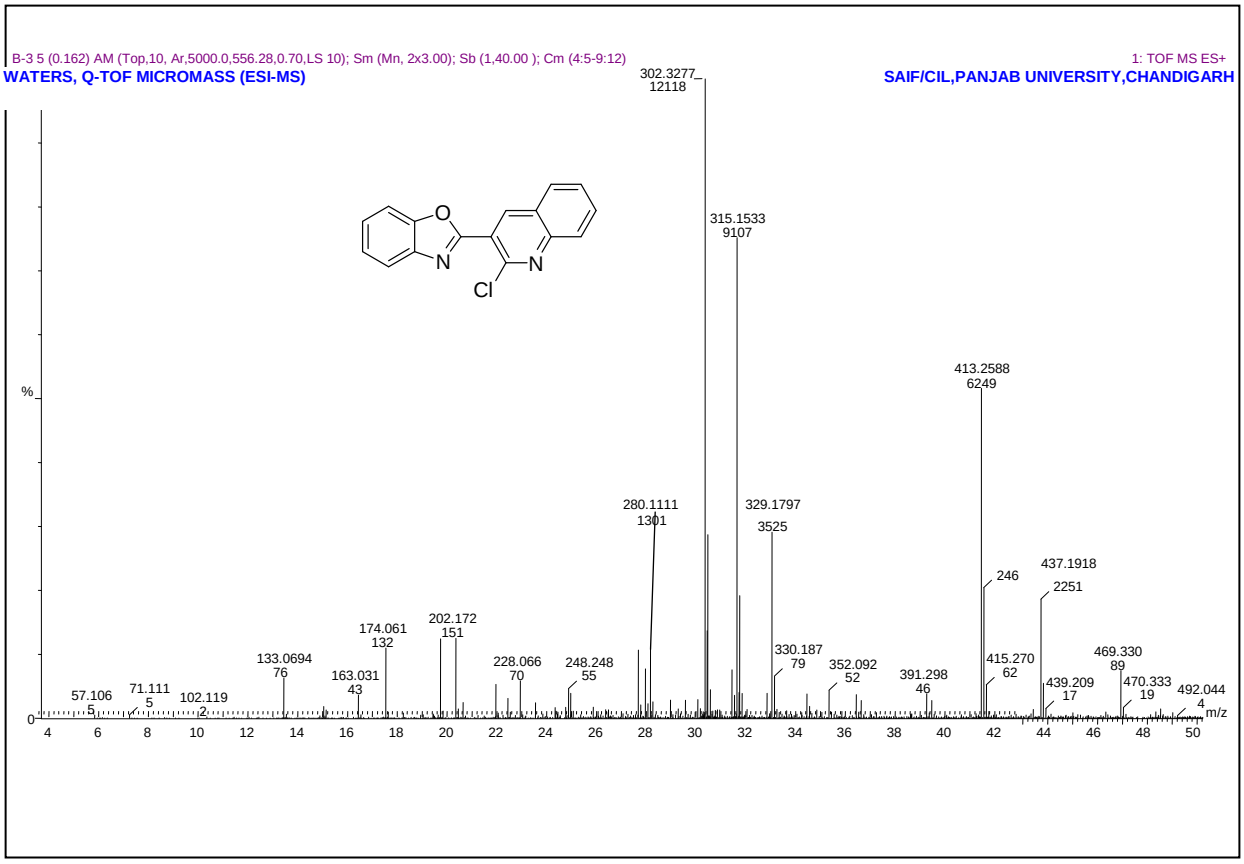
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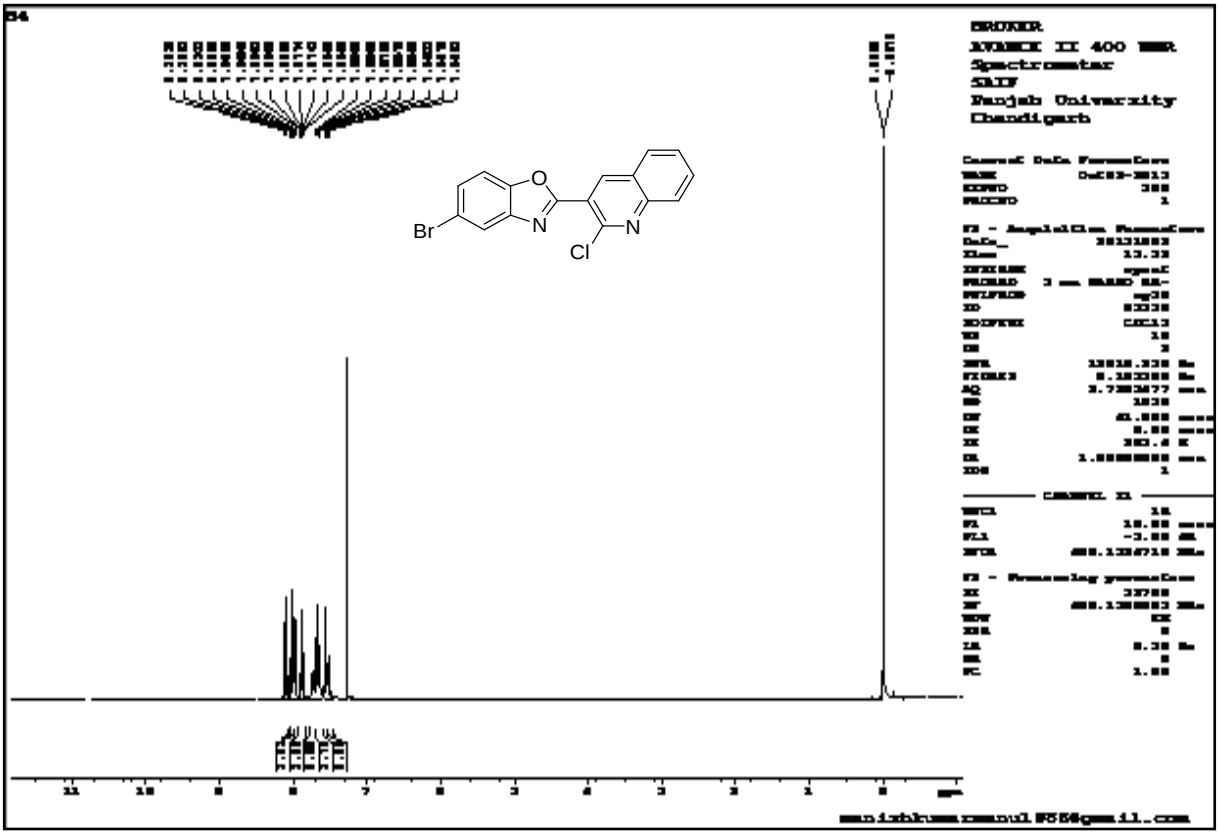
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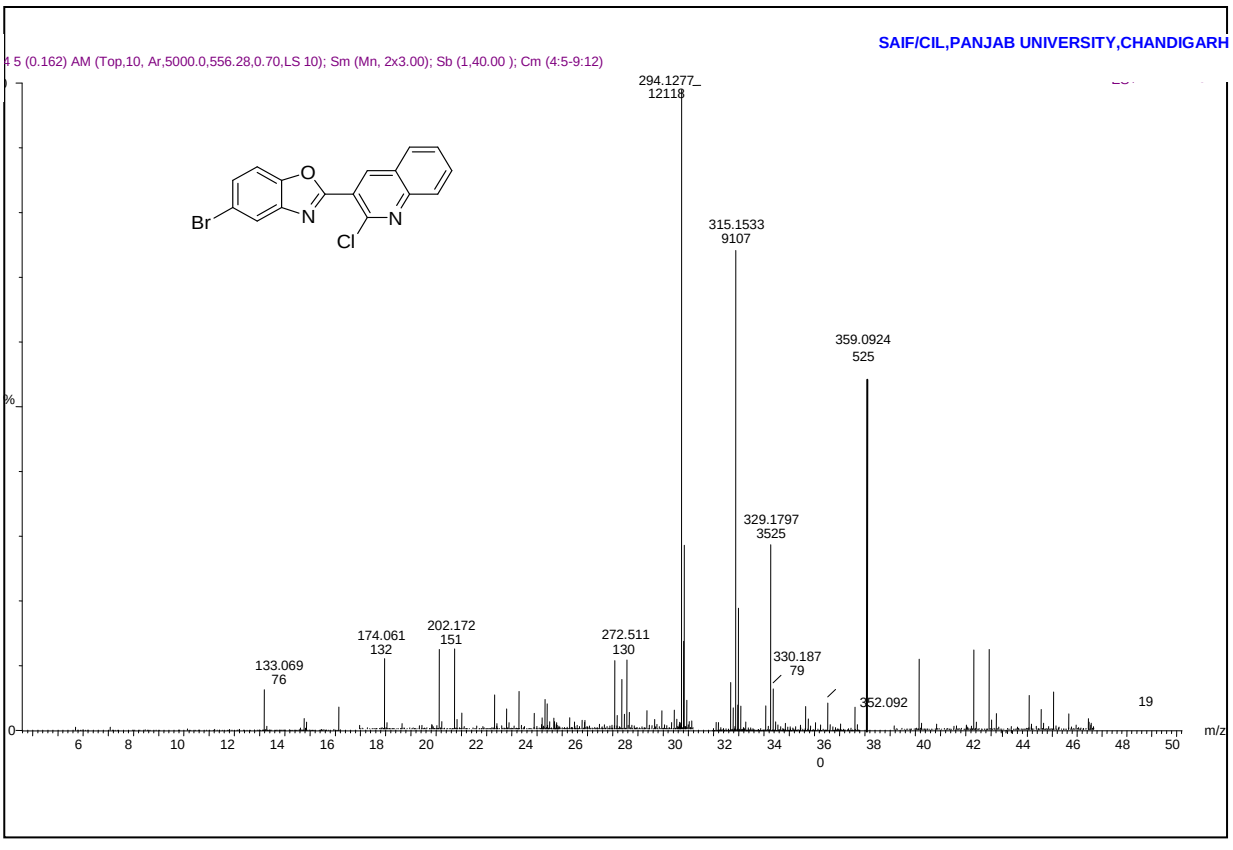
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CHAPTER 3

Synthesis of new Azomethine-based COX Inhibitors as Promising Cancer Therapeutics

CHAPTER 3

3.1. INTRODUCTION

Inflammation is the body's natural response to injury and a part of the body's defense mechanism against foreign invaders such as bacteria, viruses and other infection causing organisms, and also playing an important role in the healing process. It is a sequence of complicated, interrelated events that work to defend the body, ultimately bringing the plasma proteins and phagocytes to the injured area for the purpose of initiating tissue repair.[1-4] Oxidative stress and inflammation are the major cause of chronic diseases including cancers, thereby making it as an essential target for prophylaxis of cancer.

Cancer has been one of the life threatening diseases which have been associated with different molecular changes occurring in the cell.[5-6] It is a genetic disease caused by number of changes in the genes in our body that control cell growth or regulate the detection and repair of DNA damage. The major concern in cancer disease has been the mortality of patients with metastasis. The metastatic process involves primary tumor invading the tumor cell, followed by intravasation, arrest and extravasation of the circulatory system. Finally there is angiogenesis and growth taking place at a distant site. One of the reasons for the growth of tumors gaining metastasis has been cyclooxygenase-2, an inflammation causing protein. The COX-II enzyme is basically responsible for the increased apoptosis resistance as well as growing metastatic potential property of the tumors. Thus, inhibition of enzyme cyclooxygenase-2 can be a promising target for arresting cancer tumor growth and preventing metastasis[7-8] Besides, the development of numerous types of epithelial cell-derived cancers including breast, skin, colon, lung, and prostate is said to be due to the overexpression of COX-II.[9]

Thus, inflammation and cancer are closely related to each other where, inflammation leads to cancer cell development and causes progression. It promotes carcinogenesis by lowering down the response of immune system, causes metastasis, and also reduces response to chemotherapy.[10-11]As also oxidative stress induced by free radicals and its significance in development of inflammation and carcinogenesis has been well established. This close relation of inflammation, oxidative stress and carcinogenesis has been the key in designing anticancer drugs having COX-II inhibitory and antioxidant potential.[12-13] Consequently, inflammation forms the core of human immune response to every infection. By and large the best suitable drugs for autoimmune diseases such rheumatoid arthritis, psoriasis; joint disorders etc. are the non-steroidal anti-inflammatory drugs **(Fig.15A)**, whose use is recommended to relieve pain, reduce swelling and bring down the high body temperature.[14-15]

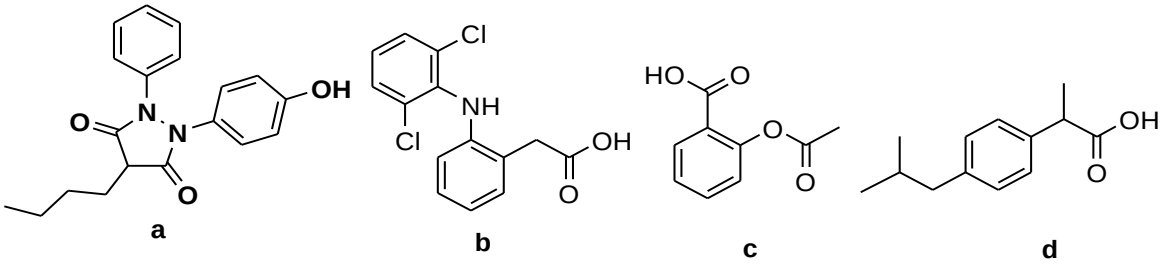


Fig.15.A. Non-steroidal anti-inflammatory drugs: a) Oxaphenbutazone, b) Diclofenac, c) Aspirin, d) Ibuprofen

NSAIDs act by inhibition of cyclooxygenase (COX-I and II) enzymes, which are the key enzymes in the group of cyclooxygenases for the synthesis of biological mediators, including biotransformation of arachidonic acid (AA) to prostaglandins (PGs), prostacyclin and thromboxane A_2 (TxA_2), responsible for various pathophysiological conditions.[16-18] Although both isoforms contribute to the inflammatory process, COX-II is of considerable therapeutic interest as it is induced, resulting in an enhanced formation of prostaglandins, during acute as well as chronic inflammation. Conventionally, NSAIDs are both COX-I and COX-II inhibitors, as a result associated with major side effects of gastric and renal bleeding. To avoid these problems, designing COX-II inhibitors have been of prime importance for more effective and therapeutic anti-inflammatory action as well a possible medicament for metastasis prevention in cancer patients. COX-II inhibitors are class of drugs used for treating autoimmune disorders such as rheumatoid arthritis, acute pain, osteoarthritis, and spondylitis.[19,20] A review on scaffolds derived from various organic molecules as selective COX-II inhibitors has appeared.[21] Coxibs are the NSAIDs, exhibiting selective action against COX-II enzyme **Fig.15B**.

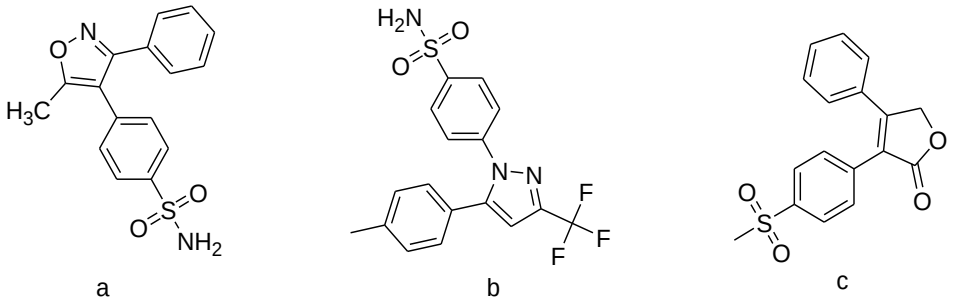
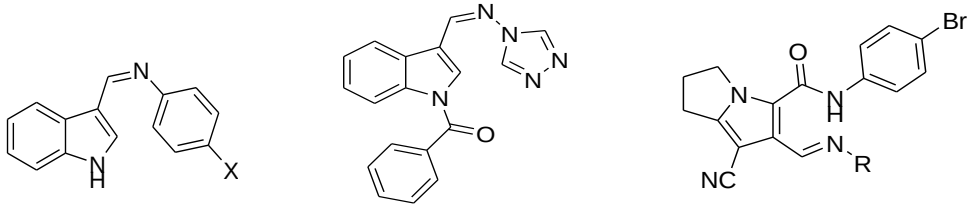


Fig.15B. Selective COX-II inhibitors: a) Valecoxib, b) Celecoxib and c) Rofecoxib

Selective COX-II inhibitors with varied motifs have been designed and screened for their anti-inflammatory action, that include derivatives of 4-thiazolidinone,[22] dihydropyridine,[23] thiadiazole,[24] indole[25] etc. Celecoxib based analogues have been exploited for better COX-II inhibition efficacy.[26-30] Ibuprofen being both COX-I and COX-II inhibitor has been modified to Indoprofen as selective COX-II inhibitor.[31]

NSAID's such as Indomethacin are known to possess anti-cancer activity, which is attributed to reduction of PGE2, generated from bis-oxygenation of arachidonic acid by COX-II.[32] The studies on various cancer cell types including colorectal carcinoma (CRC) proved that Indomethacin inhibits proliferation by induction of apoptosis, enhancing the immune system and inhibits angiogenesis.[33] Use of these drugs causes reduction in risk of cancer.[34-36] In order to primarily design COX-II inhibitors as potential anticancer agents, exploiting the free carboxylic acid group in Ibuprofen was the best target as that group was responsible for less specificity towards COX-II enzyme inhibition. Herein, Ibuprofen could be converted into its hydrazide and further to Schiff base derivatives. Schiff bases (SBs) are important chemical compounds having a significant pharmacological potential with ability to modulate the function of enzymes in various metabolisms.[37-43] The azomethine moiety demonstrates the influence of nitrogen heterocycles such as quinoxaline, quinoline etc as these heterocycles have engaged great attention of the chemists, due to its varied biological activities.[44-47] The search for selective COX-II inhibitors **(Fig.15C)** has also identified azomethine moiety to be one of the scope areas to exploit.[48-52]



a b c

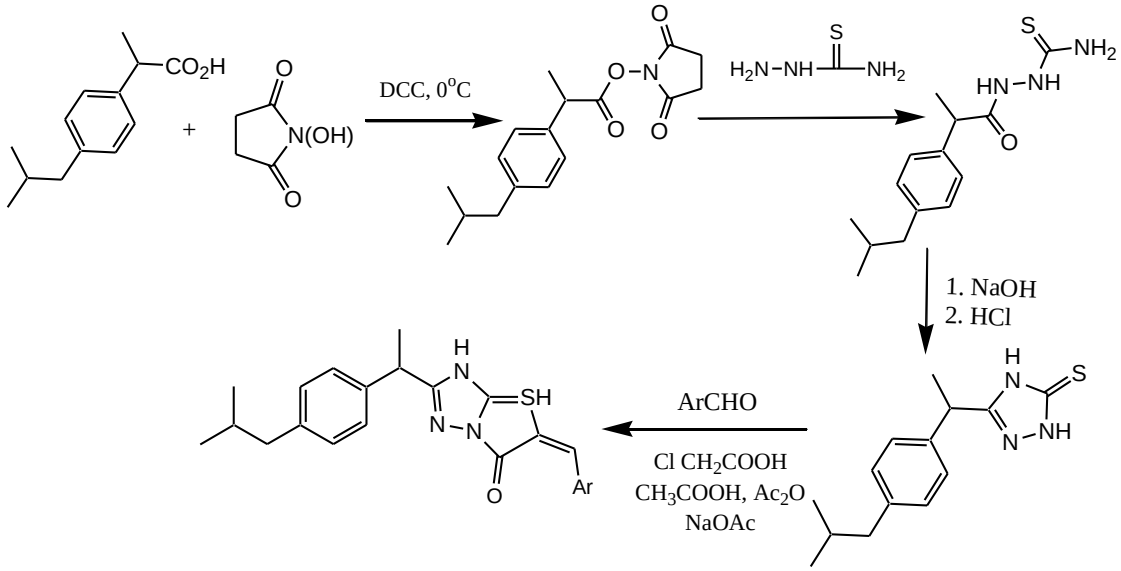
Fig.15C. Schiff bases as COX-II Inhibitors: a. Indole based, b. Indole based with triazole motif, c. Pyrrolizine-based with thiazolidinone motif

Hydrazone compounds are associated with the presence of the active (-CO-NHN=C-) pharmacophore and form a significant category of compounds in medicinal and pharmaceutical chemistry with several biological applications that include analgesics, anticonvulsant, antitubercular, antimicrobial, anti-inflammatory, anticancer, antioxidant and anthelmintics.[53-56] We considered the above literature and planned a strategy to design structural motifs based on

Ibuprofen, quinoxaline and pyridine as template and introducing quinoxaline, quinoline, and indole motifs (**Fig.15C**). We performed preliminary molecular docking studies against enzymes COX-I and COX-II, followed by *in-vitro* COX-I/II enzyme inhibition assay and *in-vivo* anti-inflammatory evaluation on carrageenan paw edema on the selected azomethine derivatives against NSAID standards. For an anti-tubercular activity, molecular docking studies against tuberculosis using enzyme enoyl ACP-reductase were carried out, followed by *in-vitro* anti-tubercular evaluation against *Mycobacterium tuberculosis*. In view of the changes leading to oxidative stress resulting in cancer and inflammation, we screened these azomethine derivatives for *in-vitro* superoxide/DPPH radical scavenging activity and carried out MTT assay against HeLa (human cervical), MCF-7 cell line, along with apoptotic study using flow cytometry method.

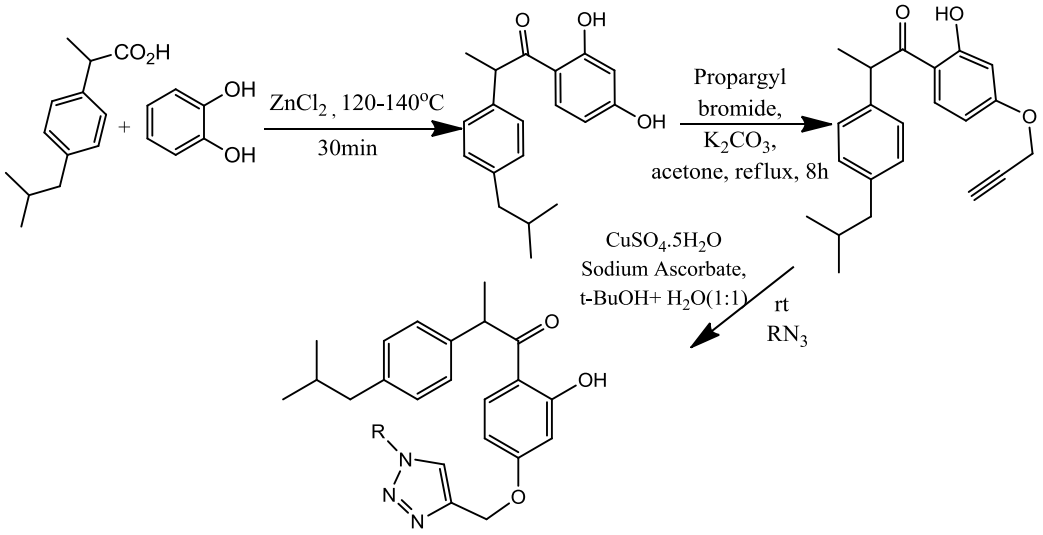
3.2. LITERATURE

An effort towards synthesis of new candidates with analgesics and anti-inflammatory activities and lower ulcerogenic effect has been made by Uzgoren-Baren A. *et al.*[57] A series of thiazolo [3, 2-b]-1,2,4-triazole-5-(6*H*)-one derivatives has been synthesized **(Scheme 41).**



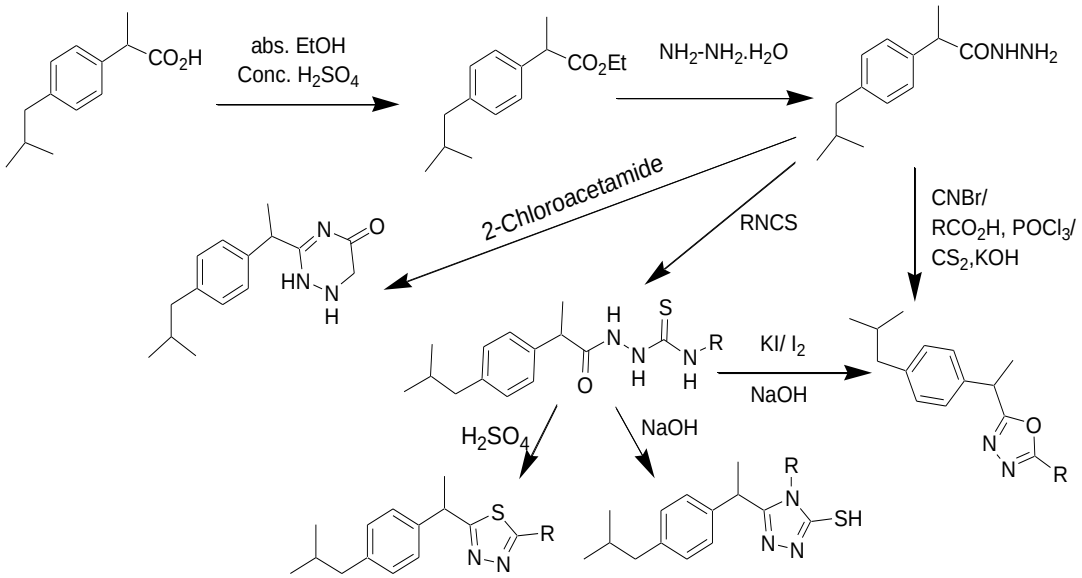
Scheme 41

Angajala K. K. *et al*[58] utilizes an ideal strategy involving the concepts of Click chemistry to synthesise the novel Ibuprofen-based 1,4-disubstituted-1,2,3-triazole containing molecules as an anti-inflammatory and bactericidal agents **(Scheme 42).**



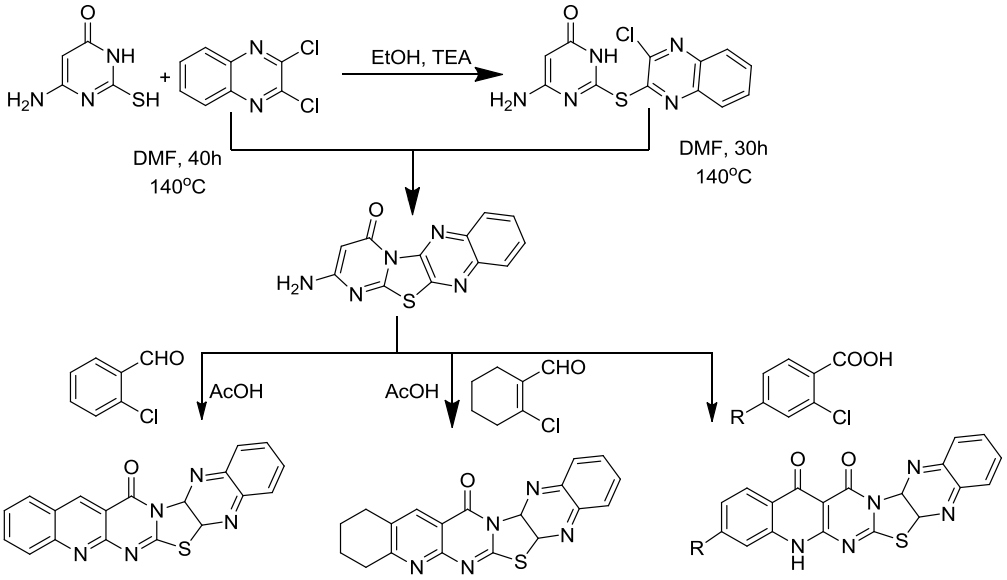
Scheme 42

Amir M., Kumar S.[59] have made an effort to convert the carboxylic acid group of Ibuprofen into 5-membered ring heterocycles thereby reducing ulcerogenic effect of Ibuprofen and these Ibuprofen-based heterocycles were proved to be significant anti-inflammatory agents (**Scheme 43**).



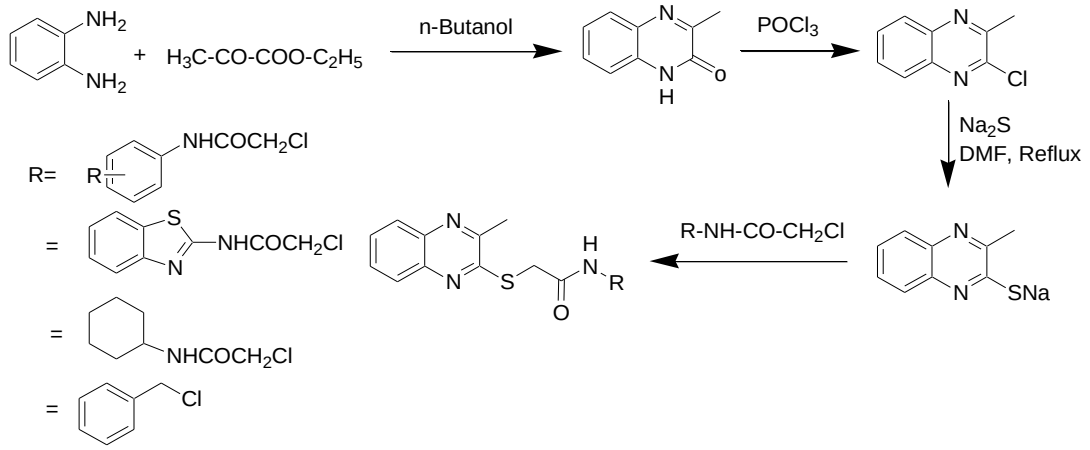
Scheme 43

Gouda M. A. *et al*[60] have established synthesis of poly-condensed new ring systems by effective and rapid new procedures. A simple and convenient route towards new heterocycle-based on quinoxaline for anti-inflammatory and analgesic evaluation (**Scheme 44**).



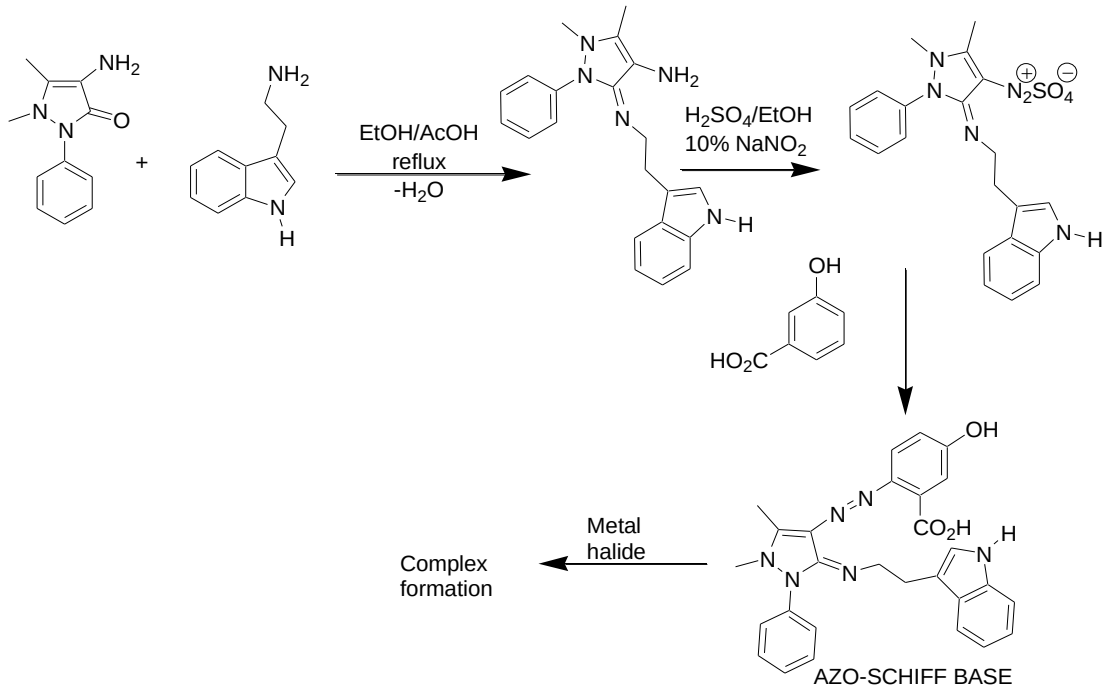
Scheme 44

Singh D. P. *et al*[61] developed synthesis of series of derivatives of 2-Chloro-3-methyl quinoxaline by reacting 3-Methylquinoxalin-2-thiosodium with 2-Chloro-*N*-(substituted aryl/alkyl)-acetamides as an anti-inflammatory agents **(Scheme 45).**



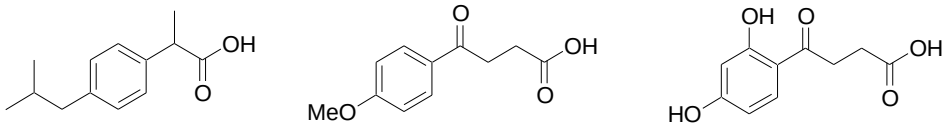
Scheme 45

Zoubi *et al*[62] has reported synthesis, azo-Schiff base complex from *m*-hydroxybenzoic acid with Schiff base of 3-[2-(1*H*-indol-3-yl)-ethylamino]-1,5-dimethyl-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-ylamine and carried out characterization and theoretical studies (**Scheme 46**).



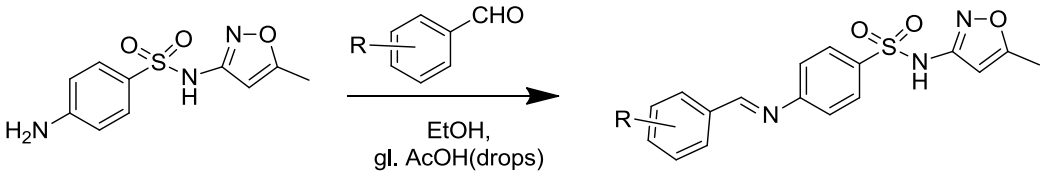
Scheme 46

Bittencourt *et al*[63] integrated the *in silico* studies to evaluate anti-inflammatory potential of 2-benzoylpropionic acid derivatives along with Ibuprofen using molecular docking and their thermodynamic profiles by molecular dynamics, in addition to predicting oral bioavailability, bioactivity and toxicity (**Scheme 47**).



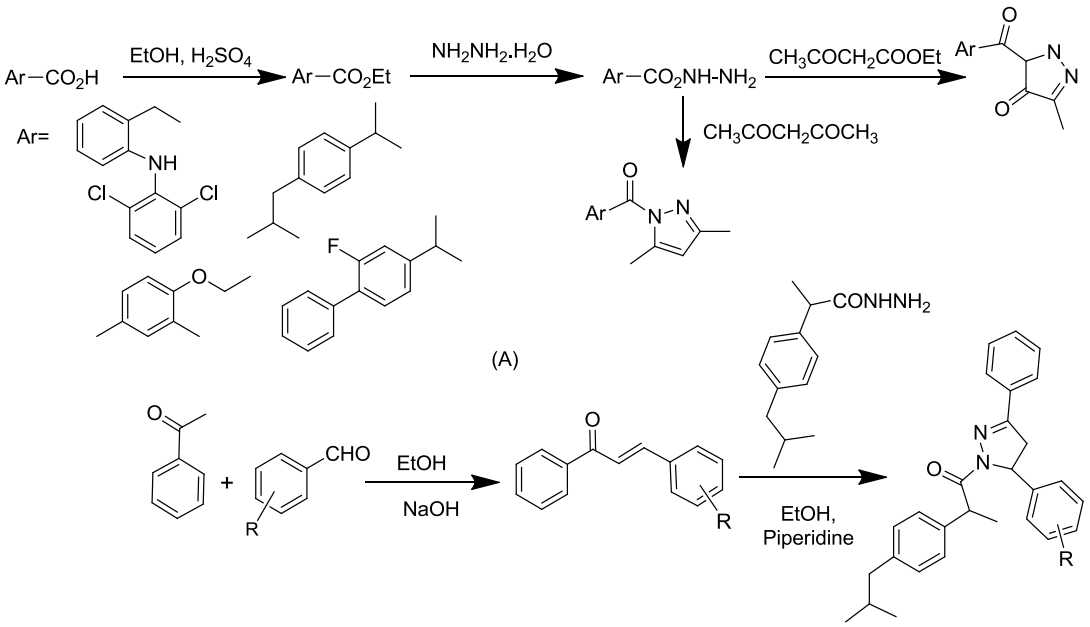
Scheme 47

Hussain *et al*[64] have attempted classical reaction for the synthesis of Schiff bases in ethanolic solution and glacial acetic acid as a catalyst further followed by synthesis of Sulfamethoxazole compounds and characterized them by different physicochemical techniques (**Scheme 48**).



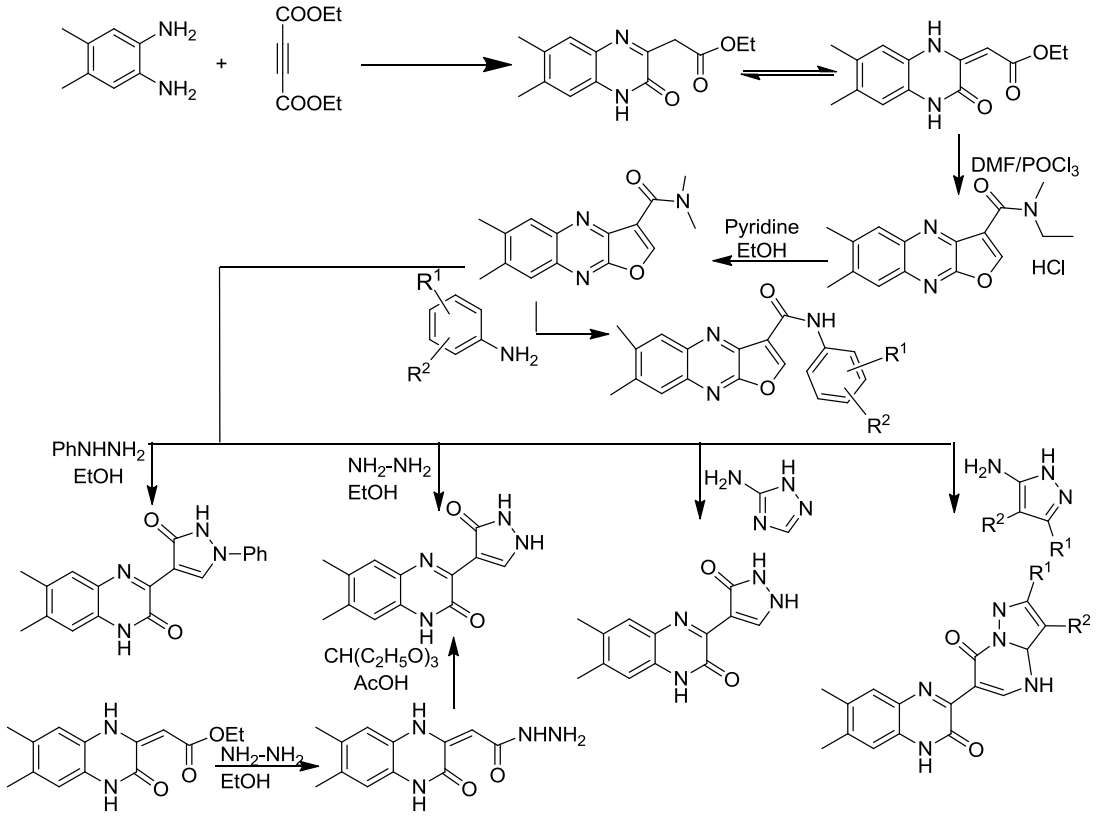
Scheme 48

The synthesis and biological activities of few aryl alkanoic acid derivatives linked with pyrazole, pyrazolone and pyrazoline systems has been developed by Amir, M and Kumar, S.[65] wherein they have mostly employed anti-inflammatory drugs like Diclofenac, Ibuprofen, Flurbiprofen and 2,4-dichlorophenoxyacetic acid (**Scheme 49**).



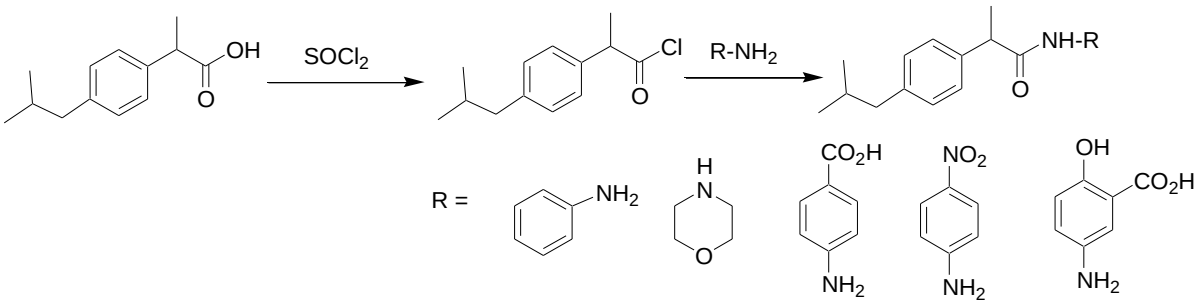
Scheme 49

Ismail *et al*[66] have suggested successful synthesis of some quinoxaline and furo-[2,3-b] quinoxaline derivatives as an anti-inflammatory and analgesic agents known for its ulcerogenic potential involving condensation, Vilsmeir-Hack as one of the steps (**Scheme 50**).



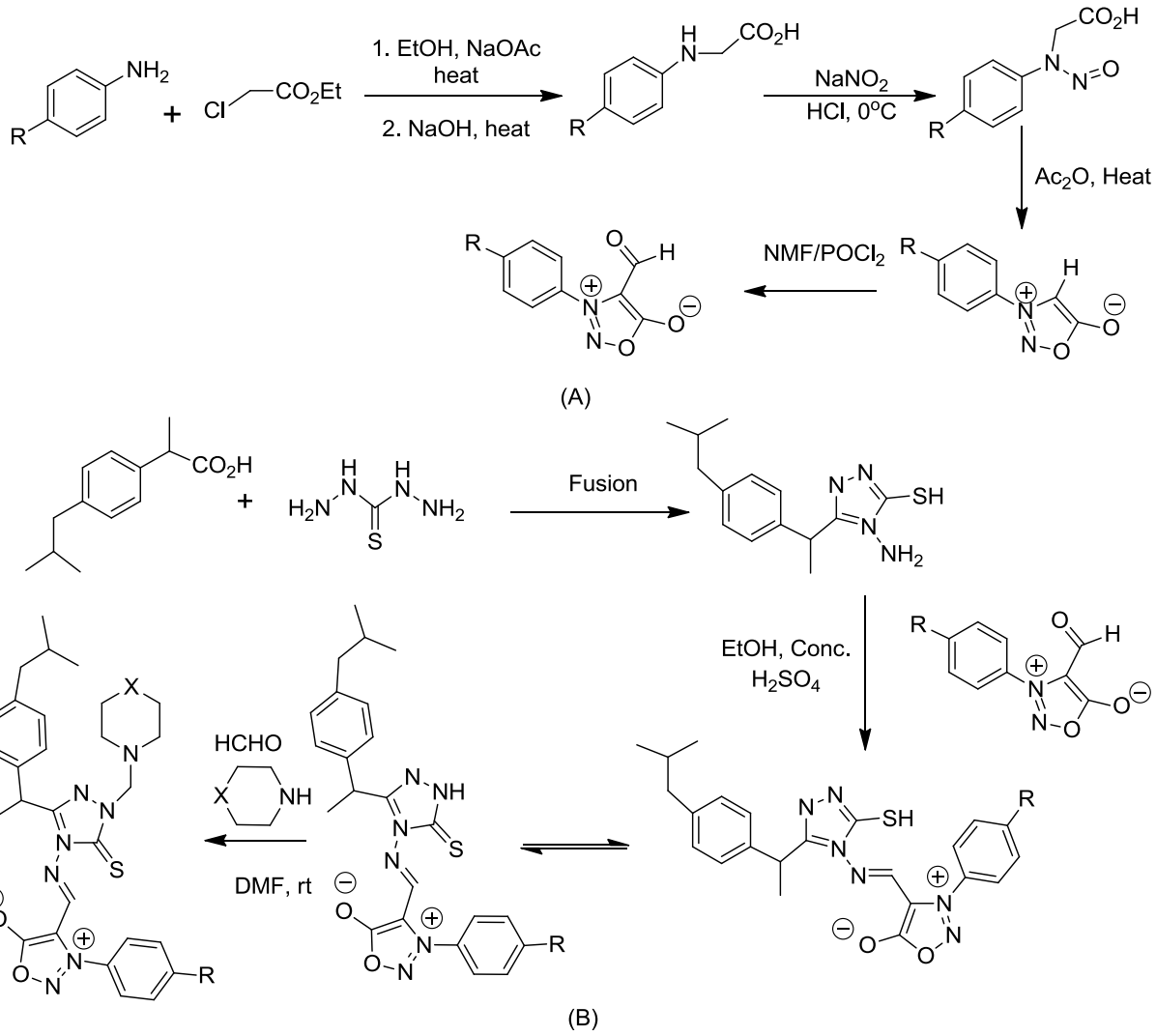
Scheme 50

Kiran *et al*[67] have developed synthesis of (*S*)-Ibuprofen amide derivatives viz. 4-(2-(4-isobutylphenyl)-propanamide benzoic acid, (*S*)-3-(4-Isobutylphenyl)-butan-2-one derivatives with morpholine, (*S)*-2-(4-isobutylphenyl)-*N*-(4-nitrophenyl)-propanamide and (*S*)-3-Hydroxy-5-(2-(4-isobutylphenyl)-propylamino benzoic acid from commercially available Ibuprofen by converting it into its propionylchloride and then reacting with amino substrates. These have been evaluated for their anti-bacterial activities (**Scheme 51**).



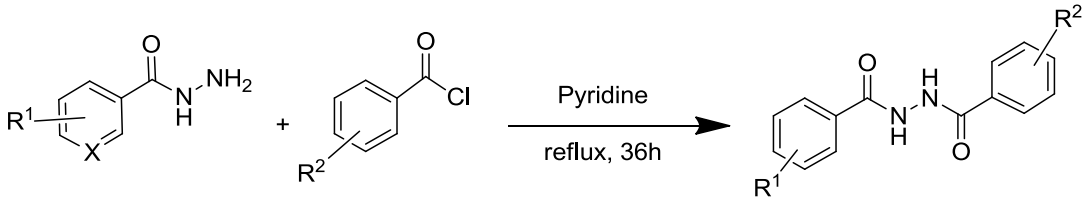
Scheme 51

Nitinchandra *et al*[68] have suggested a regioselective synthesis of novel series of 1-substituted aminomethyl-3-[1-(4-isobutylphenyl)-ethyl]-4-(3-aryl-4-sysnonylidine)-amino-1,2,4-triazol-5-thienes as an anti-inflammatory and analgesic candidates. These compounds have been synthesized by aminomethylation with formaldehyde and secondary amines (**Scheme 52**).



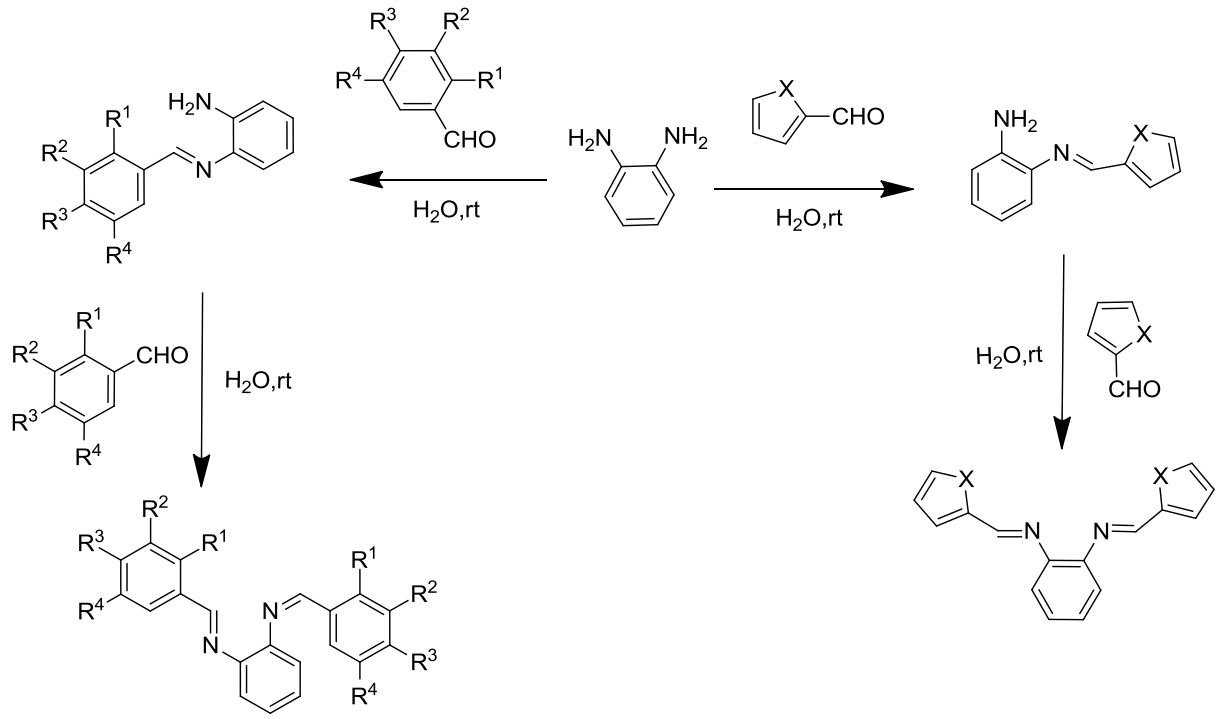
Scheme 52

Abbas *et al*[69] reports the synthesis and *in vitro* urease inhibitory activity of benzohydrazides derivatives, via one-pot reaction of benzohydrazide/Nicotinoyl hydrazide with benzoyl chloride under refluxing using pyridine as a solvent **(Scheme 53).**



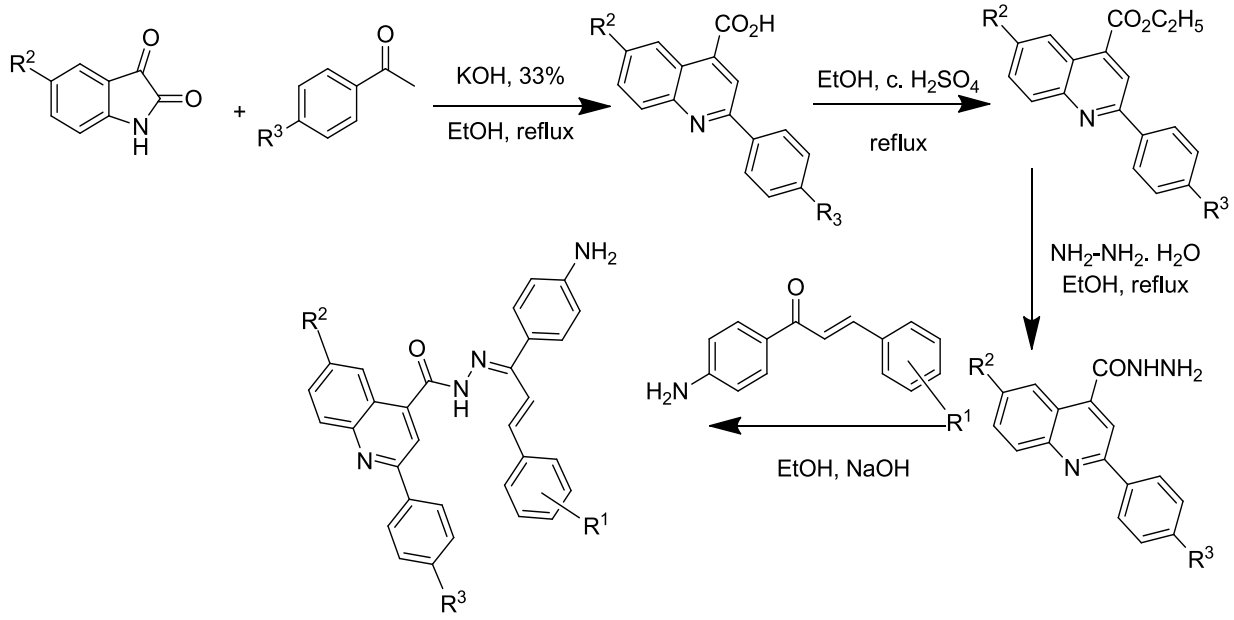
Scheme 53

Rao V.K. *et al*[70] have designed novel route towards synthesis of Schiff bases involving use of highly efficient, eco-friendly solvents by condensation reaction of 1,2-diaminobenzene with various aromatic aldehydes in aqueous medium **(Scheme 54).**



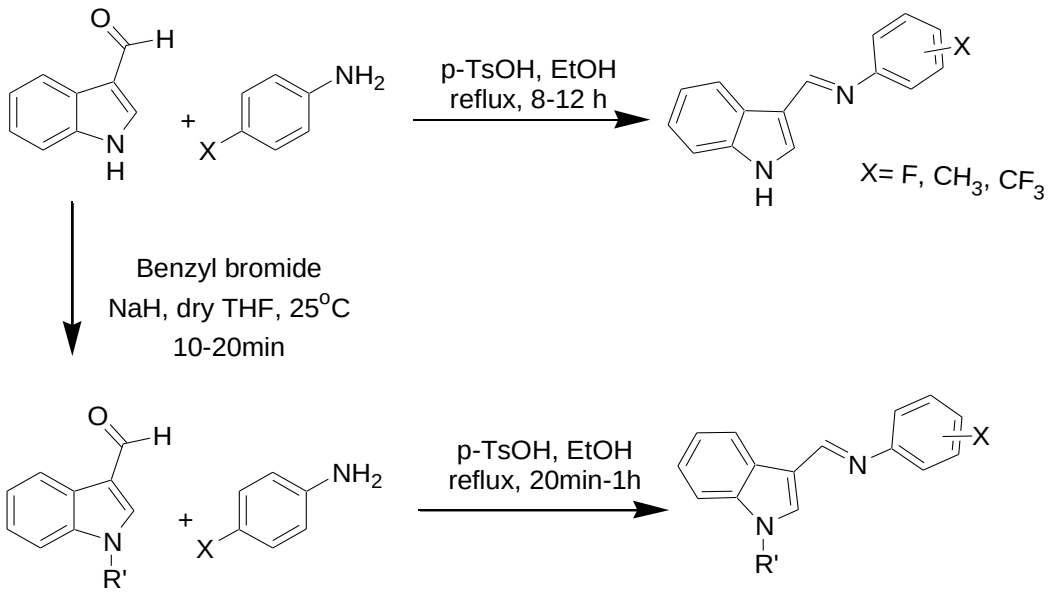
Scheme 54

Kaur J. *et al*[71] have designed selective COX-II inhibitors using indole ring as a template. A series of indole-based Schiff bases focusing on substitution at N-1 and C-3 have been synthesized and their COX-I/COX-II inhibitory activities of compounds were determined (**Scheme 55**).



Scheme 55

Abbas *et al*[72] have directed towards optimizing the use of quinoline and chalcone derivatives and incorporating them into chemotherapeutical agents. A synthesis and evaluation of quinoline-chalcone hybrids was designed as potential anti-cancer candidates (**Scheme 56**).



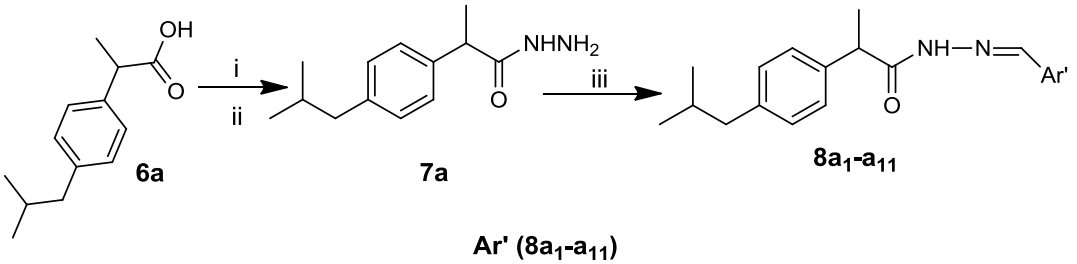
Scheme 56

3.3. PRESENT WORK AND DISCUSSION

3.3a. Chemistry:

As been discussed previously regarding the side effects associated with the carboxylic acid group of Ibuprofen, the exploitation of the acid group as interesting functionality in developing a drug like molecule was visualized**.** The Ibuprofen-based azomethine derivatives **$8a_1$-a_{11}** have been synthesized via a three step synthetic route (**Table 11**), beginning with preparation of ester by carrying out esterification of acid group of Ibuprofen **6a** under acid reflux using ethanol at 100^{o}C for 8 hours.[73] The conversion of an ester to its acid hydrazide **7a** by reaction of ester with hydrazine hydrate under refluxing conditions, and lastly the azomethine derivatives **$8a_1$-a_{11}** were synthesized by a condensation of differently substituted aldehydes with the acid hydrazide **7a** (**Scheme 57**). All the synthesized Ibuprofen-based azomethine derivatives **$8a_1$-a_{11}** have been characterized by NMR (^{1}H and ^{13}C) and HRMS spectroscopic techniques.

The ^{1}H NMR spectrum of Ibuprofen-based azomethine derivatives **$8a_1$-a_{11}** showed following peaks. The two methyl groups $(CH_3)_2$ of isobutyl side chain exhibited one doublet peak at δ 0.8-0.9 ppm due to CH coupling, the methyne –CH appears as multiplet at δ1.4-1.5 ppm due to coupling with $(CH_3)_2$ and CH_2, the CH_2 group shows a doublet peak at δ2.4 ppm, the methyl group of Isopropyl side chain shows a quartet at δ4.12 ppm which is far downfield due to presence of C=O group. The signals corresponding to aromatic protons were observed as doublet, double of doublet, double of triplet due to ortho-, meta- coupling respectively at δ6.0-10.0 ppm along with NH singlet at around δ7.15 ppm and an –N=C-H proton at δ9.0 ppm. ^{13}C NMR spectra of compounds revealed the presence of N=C signal at the range of δ144.0ppm and C=O signal at the range of δ177.3 ppm, in addition, presence of CH_3 peak at δ18.8, 22.1 ppm, along with CH_2 peak at δ30.3 ppm and CH peak at δ41.1, 45.2 ppm. The calculated M+H ion peak matched with that of the observed M+H ion peak, which confirmed the structure.



Ar' ($8a_1$-a_{11})

$8a_1$= 3-(2-chloroquinolinyl)
$8a_2$= 2-quinoxalinyl
$8a_3$= 4-hydroxy-5-nitrophenyl
$8a_4$= 2,4-dichlorophenyl
$8a_5$= 4-N,N-dimethylaminophenyl
$8a_6$=Indol-2-yl
$8a_7$= 4-Fluorophenyl
$8a_8$= 4-bromophenyl
$8a_9$=4-Nitrophenyl
$8a_{10}$= 2-Nitrophenyl
$8a_{11}$=3-Nitrophenyl

Scheme 57. Synthesis of Ibuprofen based azomethine derivatives **$8a_1$-a_{11};** Reagents and conditions: (i) EtOH (4 eq), conc. H_2SO_4 (3-4 drops), 100^{o}C, 8h; (ii) NH_2-NH_2. H_2O (1.5eq), EtOH, 100^{o}C, 10h; (iii) substituted aldehyde derivatives (1 eq), EtOH, gl. AcOH (2 drops), rt, 4-6h.

The synthesis was further extended towards the preparation of more examples of azomethine derivatives involving quinoxaline-series **8b** and pyridine-series **8c** (**Table 12, & 13**). 6-Nitro-quinoxaline-2-carboxylic acid azomethine derivatives **8b** were prepared by first carrying out nitration on previously synthesized quinoxaline-2-carbaldehyde[74] using 5mmol of concentrated nitric acid, warming at 60^{o}C for one hour resulting in desired acid product **6b** in 89% yield after recrystallisation from ethanol. The acid product thus obtained was characterized by IR and melting point. The IR spectrum showed shift in the C=O peak at 1760cm^{-1} which was attributed to COOH group, a broad OH peak and a C-H stretch was observed in the region 3090cm^{-1}.

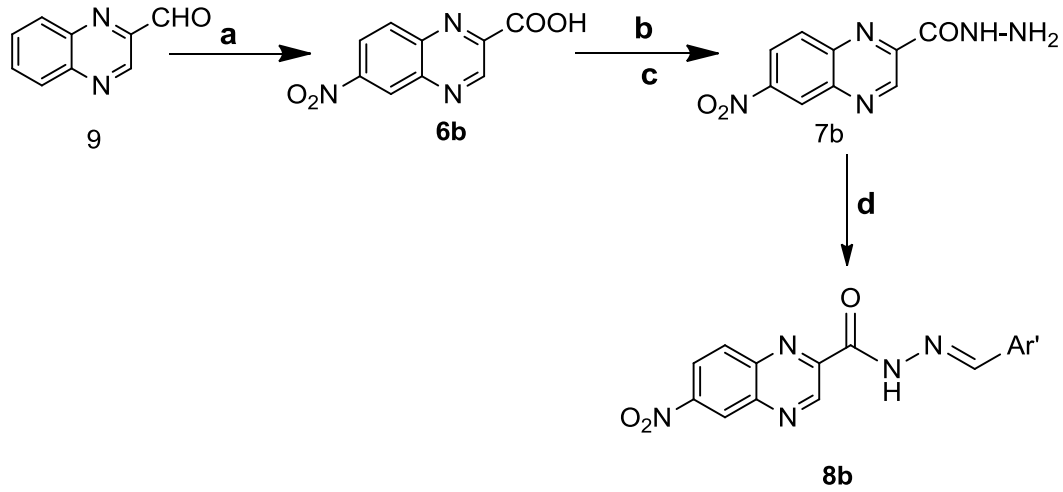
The 6-nitro-quinoxaline-2-carboxylic acid **6b** was then treated with excess ethanol (4 eq) in presence of catalytic amount of concentrated sulphuric acid at 100^{o}C for 4-6h, to give the required ester, which upon subjecting to hydrazine hydrate at 100^{o}C for 10h, was converted to its hydrazide **7b**, whose formation was also confirmed by IR spectroscopy. The IR showed two characteristic at 3200cm^{-1} and 3400cm^{-1} attributed to NH and NH_2(two bends) group, and an amide C=O stretch at 1646cm^{-1}. The final quinoxaline-based azomethine derivatives **8b** were prepared by a condensation reaction of hydrazide **7b** with aryl aldehydes in ethanol (**Scheme 58, Table 12**).

All the synthesized quinoxaline-based azomethine derivatives **$8b_1$-b_6** have been characterized by NMR (^{1}H and ^{13}C) and HRMS spectroscopic techniques. The ^{1}HNMR spectrum of quinoxaline-based azomethine derivatives **$8b_1$-b_6** showed an average spectrum in which appeared one singlet at δ9.0ppm (Quin-H, 3 position), one singlet at δ8.2ppm (azomethine –N=C-H), two doublet at δ8.1ppm (Quin-H, 5 & 8 position) and double of doublet δ7.83-7.86ppm (Quin-H; 7 position), one singlet at δ7.84ppm (NH). In addition, protons of the benzene ring appeared as doublet, double of doublet at δ6.0-7.8ppm (Ar-H); ^{13}C NMR spectra of compounds revealed the presence of N=C signal at the range of δ144.0, 154.2ppm and C=O signal at the range of δ177.3ppm. The calculated M+H ion peak matched with that of the observed M+H ion peak, which confirmed the structure.

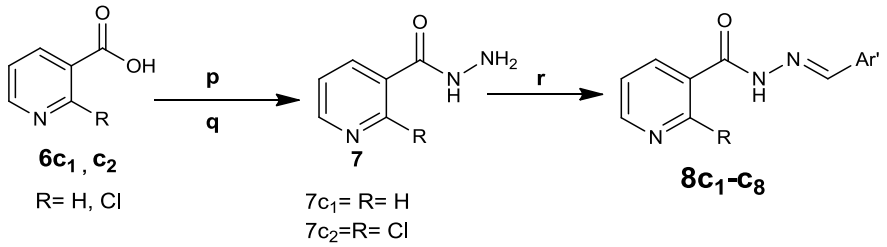
Table 11 List of synthesized Ibuprofen-based azomethine derivatives **$8a_1$-a_{11}**

Code 8a	Ar'	Time[a]	% Yield[b]	Mp. °C[c]
1	2-(3-chloroquinolinyl)	2	80	188-190[d]
2	2-quinoxalinyl	2	78	166-170[d]
3	4-hydroxy-5-nitrophenyl	2	82	148-152[d]
4	2,4-dichlorophenyl	2	77	156-160[d]
5	4-N, N-dimethylaminophenyl	3	67	257-262[d]
6	Indol-2-yl	3	75	170-175
7	4-Fluorophenyl	2	78	134-136
8	4-Bromophenyl	2	82	175-177
9	4-Nitrophenyl	2	80	152-155
10	3-Nitrophenyl	2	78	215-218
11	2-Nitrophenyl	2	76	190-192

[a]Reaction time in hours; [b]Isolated yields;
[c]Determined using Thiels Tube method, [d]Not Reported



Scheme 58. Synthesis of quinoxaline-based azomethine derivatives **$8b_1$-b_6;** Reagents and conditions: (a) conc. HNO_3 (5 mmol), 60°C, 1h; (b) EtOH (4 eq), conc. H_2SO_4 (3-4 drops), 100°C, 8h; (c)NH_2-NH_2. H_2O (1.5eq), EtOH, 100°C, 10 h; (d) substituted aldehyde derivatives (1 eq), EtOH, rt, 2-4h.



Scheme.59. Synthesis of pyridinyl-based azomethine derivatives **$8c_1$-c_8;** Reagents and conditions: (p) EtOH (4 eq), conc. H_2SO_4 (3-4 drops), 100°C, 8 h; (q)NH_2-NH_2. H_2O (1.5eq), EtOH, 100°C, 10 h; (r) substituted aldehyde derivatives (1 eq), EtOH, gl. AcOH (2 drops), rt, 4-6 h.

Similarly, pyridine-based azomethine derivatives **8c** series (**Table 13**) were prepared by same route involving esterification of nicotinic acid derivatives **6c** under acid catalysed reaction in excess ethanol at 100°C for 8 hours to give nicotinyl-ester, followed by reaction with hydrazine hydrate under refluxing condition in ethanol at 100°C for 12 hours which gave nicotinyl hydrazide **7c**, which was characterized by IR spectroscopy. IR spectrum exhibited two characteristic peak at 3200cm^{-1} and 3400cm^{-1} attributed to NH and NH_2 group, and an amide C=O stretch at 1646cm^{-1}. The nicotinyl-hydrazide **7c** was condensed with aryl aldehydes in ethanol at 25°C to give pyridine-based azomethine derivative **8c** (**Scheme 59**).

Table 12. List of synthesized quinoxalinyl-based azomethine derivatives **$8b_1$-b_6**

Code 8b	Ar'	Time[a]	% Yield[b]	Mp. °C[c]
1	4-Bromophenyl	2	70	228-232[d]
2	2-(3-Chloroquinolinyl)	2	80	185-188[d]
3	2, 4-Dichlorophenyl	2	75	240-243[d]
4	4-Hydroxy-5-nitrophenyl	2	80	242-248[d]
5	4-(N,N)-dimethylaminophenyl	2	76	190-194[d]
6	2-Quinoxalinyl	3	78	250-254[d]

[a]Reaction time in hours; [b]Isolated yields; [c]Determined using Thiels Tube method, [d]Not Reported

Table 13. List of synthesized Pyridinyl-based azomethine derivatives **$8c_1$-c_8**

Code 8c	Ar	R	Time[a]	% Yield[b]	Mp. °C[c]
1	4-Nitrophenyl	H	3.5	77	215-217
2	Quinoxalin-2-yl	H	3	80	220-224[d]
3	2,4-dichlorophenyl	H	3	75	120-124
4	3-Nitrophenyl	H	3	78	177-180
5	4-N,N-Dimethylaminophenyl	Cl	4	67	159-162[d]
6	Quinoxalin-2-yl	Cl	3	70	140-144[d]
7	4-Bromophenyl	Cl	2.5	78	156-158[d]
8	4-Nitrophenyl	Cl	4	80	172-174[d]

[a]Reaction time in hours; [b]Isolated yields; [c]Determined using Thiels Tube method, [d]Not Reported

All the synthesized pyridine-based azomethine derivatives **8c$_1$-c$_8$** have been characterized by NMR (^{1}H and ^{13}C) and HRMS spectroscopic techniques. The ^{1}H NMR spectrum of pyridine-based azomethine derivatives **8c$_1$-c$_8$** showed an average spectrum in which appeared a doublet at δ9.62ppm (pyr-H, 6 position), one singlet at δ8.6ppm (Quin-H, 3 position), a double of doublet at δ8.37ppm (pyr-H, 4 position) and double of doublet δ8.29-8.30ppm (Quin-H; 8 position), two double of doublet δ8.12, 8.02ppm (Quin-H; 5 position, pyr-H, 5 position), two double of doublet at δ7.74-7.76ppm (Quin-H; 6 and 7 position), one singlet at δ7.5ppm (azomethine –N=C-H), one singlet at δ7.0ppm (NH). In addition, protons of the benzene ring appeared as doublet, double of doublet at δ6.0-7.8ppm (Ar-H); ^{13}C NMR spectra of compounds revealed the presence of N=C signal at the range of δ150.9, 160.2ppm and C=O signal at the range of δ161.2ppm. The calculated M+H ion peak matched with that of the observed M+H ion peak, which confirmed the structure.

3.3b. Biological Evaluation

3.3b.1. Anti-oxidant assay

3.3b.1.1. DPPH and Superoxide scavenging radical assay:

The anti-oxidant ability of most of the compounds has been determined using DPPH[75] and superoxide scavenging assay.[76] The DPPH radical is a nitrogen centered stable radical, during this process there is change in color from violet to yellow where it gets reduced either by hydrogen or electron donation.[77] Whereas, in superoxide radicals are produced by cellular reactions, using various enzyme system such as lipoxygenase peroxidase, NADPH oxidase and xanthine oxidase.[78] Superoxide has been a weak oxidant and plays role in formation of powerful radicals like hydrogen peroxide, hydroxyl radical and singlet oxygen thereby causing oxidative demage to lipids, proteins and DNA. This method involves generation of superoxide radical through addition of sodium hydroxide to the air saturated dimethyl sulphoxide, which is stable enough to reduce the nitroblue tetrazolium into formazon dye which is further measured at 560nm, against all three series of azomethine derivatives at concentrations of 1000μg/mL. The results of this antioxidant assay[79, 80] revealed that all compounds are better anti-oxidants as compared to the standard Ascorbic acid (%Radical scavenging activity; SOS=75.24%). The azomethine derivatives inhibited the superoxide radical generation and exhibited low to moderate anti-oxidant activity in the range of 48.35-78.34%. On the other hand, when tested against DPPH, these azomethine derivatives displayed lowest-highest anti-oxidant activity

ranging from 43.53-67.01% compared to that of the standard Ascorbic acid (%Radical scavenging activity 85.96±3.00) (**Table 14**). Compounds **$8b_3$**, **$8c_7$** and **$8c_8$** were found to possess mild antioxidant property with % radical scavenging activity between 43.53-58.33% against both superoxide and DPPH radicals. In contrast, compounds **$8a_1$, $8a_2$**, **$8a_4$**, **$8b_1$**, **$8b_2$, $8b_4$**, **$8c_2$** and **$8c_6$** demonstrated highest superoxide scavenging activity with maximum % SOS inhibition shown by compound **$8a_4$** better than standard Ascorbic acid.

% Radical scavenging activity=$\frac{\text{Absorbance of Control- Absorbance of tested compound}}{\text{Absorbance of Control}} X100$

Table14. Anti-oxidant activity of azomethine derivatives against DPPH and Superoxide radical

Compound Code	% Radical scavenging activity	
	Superoxide radical scavenging assay	DPPH scavenging assay
$8a_1$	73.29±1.36	43.93±4.50
$8a_2$	73.03±1.36	46.78±4.20
$8a_3$	66.86±1.16	-
$8a_4$	78.34±2.16	63.28±4.58
$8a_6$	67.25±1.16	-
$8a_{10}$	48.75±3.01	-
$8a_{11}$	63.58±1.06	-
$8b_1$	76.77±1.71	62.56±4.41
$8b_2$	75.91±1.72	-
$8b_3$	53.08±2.17	46.31±4.57
$8b_4$	73.29±1.32	65.02±5.46
$8b_6$	67.65±1.14	60.50±4.80
$8c_2$	75.32±1.72	61.61±4.20
$8c_5$	68.17±1.26	65.97±5.30
$8c_6$	74.08±1.66	67.01±5.62

$8c_7$	48.35±2.43	43.53±4.10
$8c_8$	58.33±2.31	57.81±3.51
Control	100	100
Ascorbic acid	75.24±1.03	85.96±3.00

3.3b.2. Molecular docking studies:

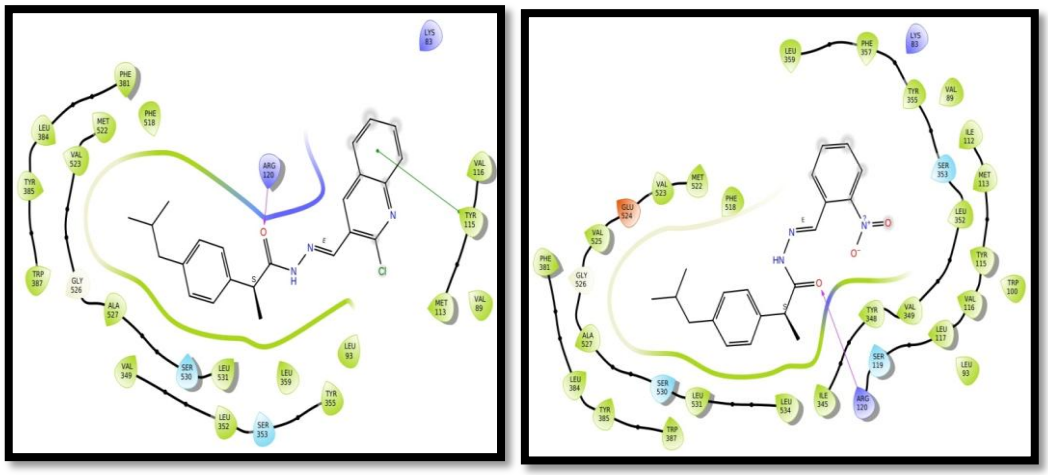
3.3b.2.1.Virtual screening against COX-II enzymes

To demonstrate the mechanism of anti-inflammatory activity and detailed intermolecular interaction between the synthesized compounds and the drug target protein, molecular docking studies were carried out as COX-II inhibitors. The activity and selectivity of the target compounds (azomethines **$8a_1$-a_{11}**, **$8b_1$-b_6 and $8c_1$-c_7**) based on their docking pose and mode with COX-II enzymes was examined by docking studies. For docking against COX-II, a library of the molecules were energy minimized using force field OPLS 2005. The X-ray crystal structure of the enzyme human COX-II (PDB ID), *E.Coli Gyrase B* (PDB 5IKR.) was retrieved from protein data bank with resolution less than 3.0 Å.[81-84]

The docking scores for all the designed compounds rated between -9.00 to -3.00 Kcal/mol. The molecules exhibiting docking scores lower than -8.00 Kcal/mol are depicted in **Table 15**. Molecules **$8a_1$-a_{11}**and **$8c_3$** has maximum affinity for COX-II as compared to the rest of the molecules, with best binding exhibited by **$8a_1$**, **$8a_4$**, and **$8a_{11}$**. The -(C=O)- of the-(C=O)NHNH- functional group is a hydrogen bond acceptor and forms bond with the amino acid Arg120 side chain guanidine group which is a hydrogen bond donor for most of the compounds, while the -NH- of the -(C=O)NHNH- functional group is a hydrogen bond donor and forms bond with the -OH group of the amino acid Tyr355 side chain, which is attributed to the affinity of these molecules. Also in molecule **$8a_1$**, the quinoline ring exhibits π-stacking with phenyl ring of the Tyr115 side chain which can be corroborated with the experimental activity**.** The protein ligand interactions for the molecules **$8a_1$** and **$8a_{11}$** in 2D representation are depicted in **Figure 16,** while the 3D representation for **$8a_1$** is depicted **Figure 17**. Further the docking into the active site of COX-I exhibited binding scores between -6.0 to -7.0 Kcal/mol indicating that the molecules have poor affinity for COX-I thus being selective for COX-II in **Table 16A.**

Table 15. Docking Score (Kcal/mol) for the top ranking compounds binding to the target COX-II.

Ligand Codes	Docking Scores (Kcal/mol)
$8a_{11}$	-8.91
$8a_1$	-8.91
$8a_4$	-8.89
$8a_7$	-8.69
$8a_{10}$	-8.54
$8c_3$	-8.44
$8a_5$	-8.43
$8a_8$	-8.42
$8a_9$	-8.41
$8a_3$	-8.36
$8a_6$	-8.34
$8a_2$	-8.23



$8a_1$ **$8a_{11}$**

Figure 16.The 2D representation of protein ligand interactions for molecules **$8a_1$** and **$8a_{11}$.**

Table 16A. Binding Site interactions for the top ranking ligands with COX-II

Ligands & Interactions	Hydrophobic	Hydrogen Bond Acceptor	Hydrogen Bond Donor	π-Stacking

8a$_{11}$	Val89, tyr115, val116, val349, leu359, ala527, leu531	Tyr355	Arg120	—
8a$_1$	tyr115, val116, val349, leu359, ala527	Tyr355	Arg120	Tyr115
8a$_4$	tyr115, val116, val349, Leu352, Tyr355, leu359, Phe381, Tyr385, ala527, leu531	—-	Arg120	—

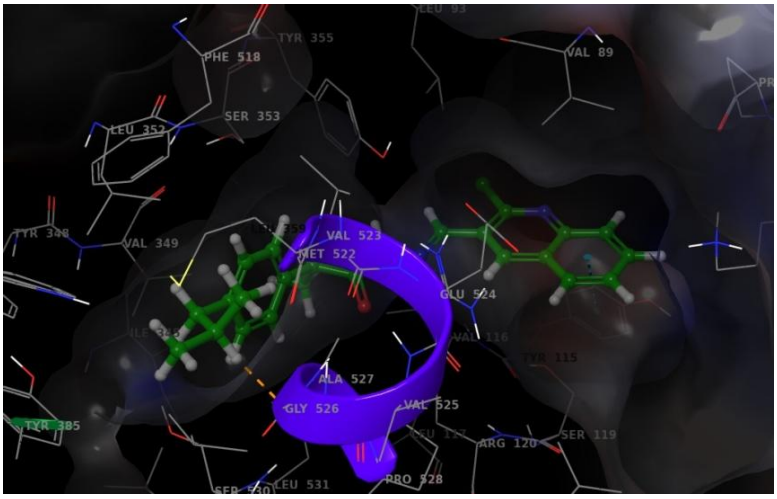


Fig. 17. The 3D representation for protein ligand interactions for the molecule **8a$_1$**.

Also docking of the standard molecules like Celecoxib, Ibuprofen (both isomers) and Indomethacin was performed to compare the results. It was seen that Celecoxib, Ibuprofen (both isomers) and Indomethacin exhibited binding affinity to COX-I while only Celecoxib, Ibuprofen (*R*-isomers) and Indomethacin showed binding to COX-II values for which are tabulated **Table 16B.**

Table 16B. Docking Score (Kcal/mol) for binding to the target Celecoxcib, Ibuprofen (*R*-isomers) and Indomethacin to COX-I and COX-II.

Compound	COX-I	COX-II
Celecoxcib	-9.76	-11.99
Ibuprofen (*R*-isomers)	-9.05	-7.18
Ibuprofen (*S*-isomers)	-8.98	-6.98
Indomethacin	-7.96	-8.81

The designed compounds **8a$_1$-a$_{11}$** along with **8c$_3$** showed docking scores against COX-II as compatible to the NSAID Indomethacin and better than Ibuprofen (*R* and *S* isomers) thus claiming that exploitation of COOH in Ibuprofen should work well to enhance COX-II inhibition.

3.3b.3. In vitro Protein denaturation screening:

3.3b.3.1. Bovine Serum Albumin Protein Denaturation method:

It has been well documented and proved that denaturation of proteins is one of main cause of inflammation. Denaturation of proteins leads to loss of biological functions of proteins. Production of autoantigen in arthritic disease such as rheumatoid arthritis is due to denaturation of protein.[85] NSAIDS corticosteroids and DMARDS are generally used for the treatment of arthritic diseases.[86] Thus, preliminary experiments on protein denaturation can indeed led us to compounds with potent inhibitory action against protein denaturation and further to anti-inflammatory activity. Serum albumin is one of the most extensively known carrier proteins, which plays a very important role in the transportation and disposition of endogenous and exogenous compounds present in blood. It contributes 80% of the colloid atmospheric pressure. It has been understood that the serum albumin is chiefly responsible for maintaining blood pH. Drug-albumin interaction indicates the binding affinities and is very important tool in pharmacodynamics and pharmacology. The study of binding behaviour of proteins with drugs has formed the basis for binding of drug to serum albumin in our *in vitro* protein denaturation studies. When bovine serum albumin is heated, it undergoes denaturation and expresses antigens associated with type II hypertensive reaction associated with diseases like serum sickness, rheumatoid arthritis, and glomerulonephritis. We chose bovine serum albumin (BSA) as our protein model due to its low cost, easy availability, unusual ligand-protein binding properties, behavior analogous to that of human serum albumins. This method provides the basis for new method of assay in the discovery of those drugs which can stabilize the protein from protein by denaturation process. Ibuprofen being an NSAID shows its effect in relieving pain and controls fever but in higher doses exhibits excellent anti-inflammatory properties, thus it was used as standard.

The synthesized compounds of series **8a**, **8b**, and **8c** were screened for protein denaturation using bovine serum albumin method (**Table 17**). The test was performed as described in the literature procedure.[87] The solutions of the target compounds, Ibuprofen as standard drug, and control was prepared with concentration 5µg/mL. The test solution (0.5mL) comprises of

0.45mL of BSA (0.5%W/V aqueous solution) and 0.05mL of test solutions of the compounds. The control solution consists of 0.45mL BSA and 0.05mL of distilled water. The standard solutions consist of 0.45mL BSA and 0.05mL of Ibuprofen solution. The absorbance was measured at 660nm using UV spectrophotometer. The control represents 100% protein denaturation **Fig.18**. The percentage of inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{[\text{Absorbance of Control} - \text{Absorbance of Test solution}]}{\text{Absorbance of control}} \times 100$$

Table 17. *In vitro* Protein denaturation assay by BSA method

Sr. No.	Compound Code	Absorbance	% Inhibition
1	$8a_1$	0.0177	61.85±2.13
2	$8a_2$	0.0182	60.77±3.23
3	$8a_3$	0.0129	72.04±4.98
4	$8a_6$	0.0195	57.95±4.11
5	$8a_7$	0.0242	47.78±3.32
6	$8a_9$	0.0275	40.73±1.09
7	$8a_{10}$	0.0315	32.11±3.02
8	$8a_{11}$	0.0284	38.79±5.32
9	$8b_1$	0.0288	37.9±2.47
10	$8b_2$	0.0251	45.9±1.88
11	$8b_3$	0.0315	12.11±3.56
12	$8b_4$	0.0302	34.9±2.33
13	$8b_5$	0.0355	23.49±4.88
14	$8b_6$	0.0272	41.4±1.32
15	$8c_1$	0.0377	18.7±5.87
16	$8c_2$	0.0345	25.6±3.78

17	$8c_3$	0.0427	7.97±3.99
18	$8c_5$	0.0441	4.9±15.02
19	$8c_6$	0.0382	17.67±2.11
	Control	0.0464	100.0±0.00
Standard	Ibuprofen	0.0232	51.2±1.22

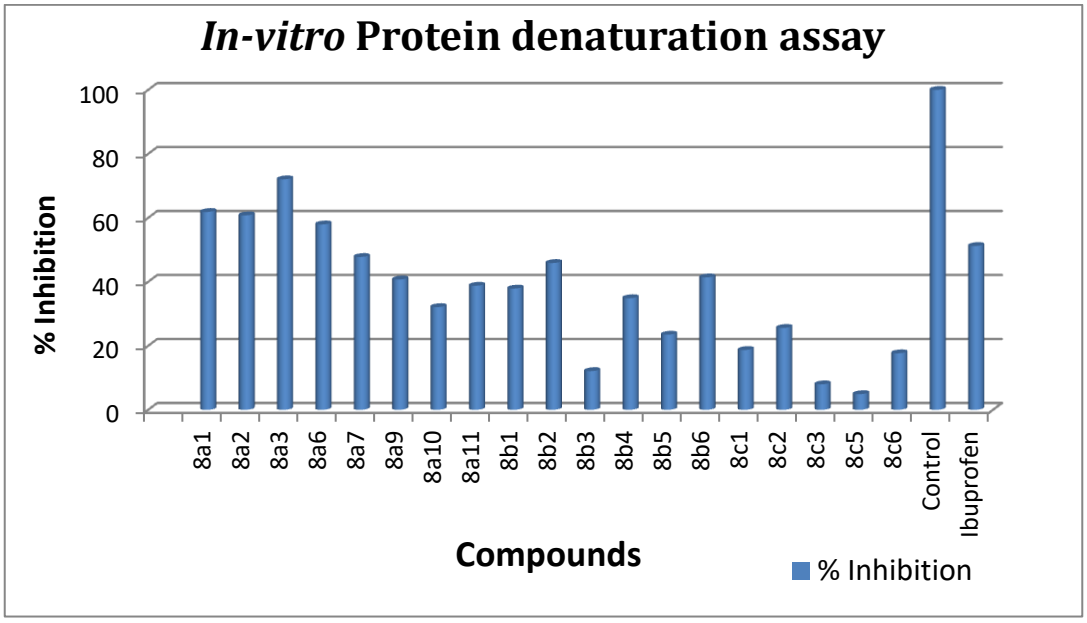


Fig. 18. Plot of % inhibition v/s target compounds along with control and standard drug.

The results were compared with the reference drug. It was summarized from the results that compounds of series **8a** exhibited better % inhibition; with compound **$8a_3$** showing maximum anti-protein denaturation ability as compared to that of standard drug, compounds **$8a_1$**, **$3a_2$** and **$8a_6$** showing good inhibition in the activity than the standard drug. The remaining Ibuprofen-based azomethine derivatives **$8a_5$**, **$8a_7$**, **$8a_9$**, and **$8a_{11}$** along with quinoxalinyl-based compounds **$8b_1$-b_6** exhibited moderate activity with percent inhibition in the range of 22.85-47.78. The reduction in the activity was observed in case of pyridinyl-based azomethine derivatives **$8c_1$-c_6** with inhibition in the activity ranging from 4.9-25.6%.

In view of this, these results of bovine serum albumin method for compounds of **8a** series were validated by performing anti-denaturation assay by both egg albumin protein denaturation and

hemolysis method which would further throw light on possible *in-vitro* anti-inflammatory activity of the compounds.

3.3b.3.2. Egg-Albumin Denaturation and Human RBC membrane stabilization

The selective *in-vitro* screening of the compounds has been carried out in hen's egg albumin protein denaturation method[88] at 100µg/mL concentration using acetyl salicylic acid as standard. The human red blood cell (HRBC) membrane stabilization method has been used as a method to study the *in-vitro* anti-inflammatory activity.[89] In membrane stabilization method, fresh whole blood was collected from healthy male human volunteers by carrying out assay of membrane stabilization erythrocyte suspension and heat-induced hemolysis (**Table 18**). Activity values were expressed in µg/mL and the obtained values were represented graphically as a plot of % inhibition v/s compounds (**Fig.19**).

The results of the *in-vitro* anti-inflammatory studies revealed that of all the compounds which were screened, compounds **8a$_3$**, **8a$_6$**, **8a$_7$**, **8a$_9$** and **8a$_{11}$** showed good inhibition of egg albumin with % inhibition values 79.44±4.23%, 66.42±4.18%, 53.56±5.40, 44.40±1.61, and 37.46±7.06 compatible with standard drug Acetylsalicylic acid (% inhibition 52.15±6.42) and was also compared with Ibuprofen (% inhibition 64.2±1.9). The anti-inflammatory activity was based on substitution on to the acid-hydrazide side chain, and also on the nature of substitution on other phenyl ring. Thus, it was proved that modifying the acid group of Ibuprofen has a remarkable effect on its anti-inflammatory activity.

Table 18. The *in-vitro* Protein denaturation assay by Egg albumin and Human RBCs membrane stabilization method

Sr. No.	Comp Code	R[a]	% Inhibition[b]	
			Egg Albumin Denaturation	Human RBC membrane stabilization
1	8a$_3$	4-OH,5-NO_2	79.44±4.23	77.23±3.15
2	8a$_5$	4-NMe_2	16.43±1.35	14.31±1.51
3	8a$_6$	Ar=Indole ring	66.42±4.18	65.81±1.86

4	$8a_7$	4-F	53.56±5.40	49.84±3.72
5	$8a_8$	4-Br	14.11±6.00	11.86±4.94
6	$8a_9$	4-NO_2	44.40±1.61	37.82±5.03
7	$8a_{10}$	2-NO_2	16.85±2.11	19.35±4.23
8	$8a_{11}$	3-NO_2	37.46±7.06	33.37±5.69
Std[c]	ASA	-	52.15±6.42	59.43 ± 0.87
	Ibuprofen	-	64.2±1.9	-

[a]Order of % inhibition= 4-hydroxy-5-nitrophenyl>2-Indolyl>4-Fluorophenyl>4-Nitrophenyl>3-Nitrophenyl>2-Nitrophenyl>4-N,N-dimethylaminophenyl>4-Bromophenyl

[b]**$8a_3$** and **$8a_6$** exhibited excellent activity by both methods as compared to standard Acetyl salicylic acid

[c] Standard: ASA= acetyl salicylic acid

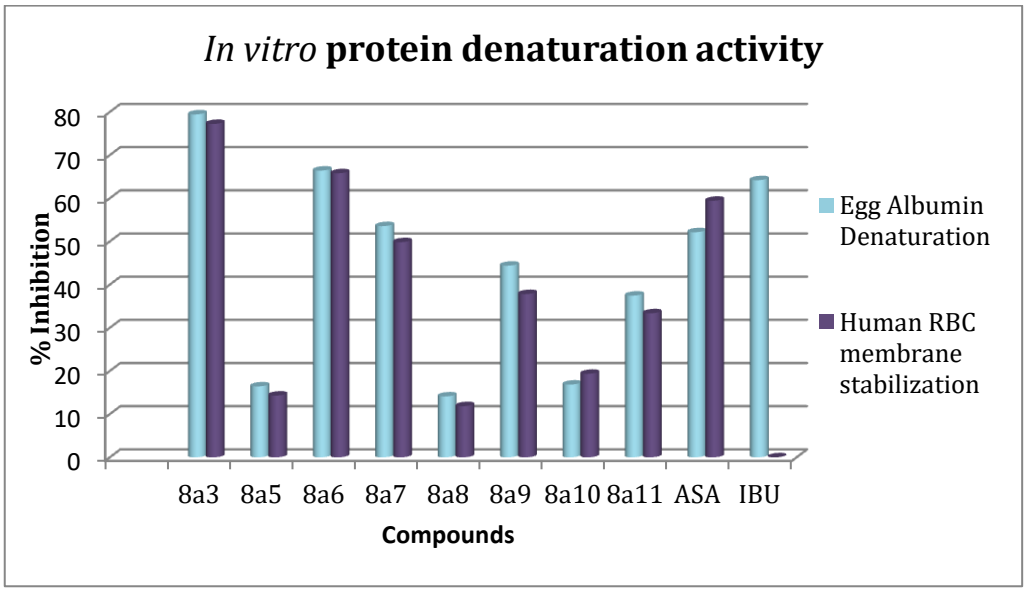


Fig. 19. Graphical representation of anti-denaturation studies: plot of % inhibition against Ibuprofen-based azomethine derivatives

3.3b.3.3. in vitro COX-I and COX-II enzyme inhibition assay:

The *in-vitro* ability of the eighteen test compounds at the concentration of 1000µg/mL to inhibit COX-I and COX-II was determined using a Cayman colorimetric COX inhibitor screening assay kit (catalog number 701080) following the manufacturer's instructions. The COX Inhibitor Screening Assay directly measures PGF2α by stannous chloride reduction of

COX-derived PGH_2 produced in the COX reaction. The inhibition of COX-I & COX-II enzyme by the synthesized compounds was analyzed in the whole blood assay system.[90] The IC_{50} values of the screened compounds and reference drugs, Celecoxib, Ibuprofen and Indomethacin are outlined in **Table 19**. It was revealed from the results that, all compounds showed significant activity against cyclooxygenase enzyme. Compounds **$8a_1$, $8a_2$, $8a_3$, $8a_4$, $8a_6$, $8b_1$, $8b_2$, $8b_4$** and **$8b_6$** showed better activity as compared to one of the reference Celecoxib (IC_{50} =0.151μM) in the range of 0.098-0.142μM. Among these compounds, compound **$8a_1$** bearing 2-(3-chloroquinolinyl) group and compound **$8a_4$** with 2,4-Dichlorophenyl group on Ibuprofen-based azomethine were found to be potent as COX-I inhibitors with IC_{50} of about 0.098 and 0.104μM than other derivatives. This was followed by compounds **$8a_{10}$, $8a_{11}$, $8b_3$, $8b_5$, $8b_6$, $8c_2$, $8c_5$, $8c_7$, $8c_8$** with least COX-I inhibition in the range of 0.166-0.273μM respectively. In contrast, all the synthesized compounds were found to possess significant COX-II inhibition with IC_{50} in the range of 0.058-0.195μM, better than reference drugs Celecoxib (IC_{50} =0.25μM) and Ibuprofen (IC_{50} =0.9μM).

From the observations of COX-I and COX-II enzyme inhibition assay it can be deduced that synthesized compounds of series **8a**, **8b** and **8c** demonstrated better COX-II inhibitory activity than Celecoxib. All compounds exhibited better inhibitory activity against COX-II compared to COX-I, with no selectivity in COX-II inhibition as deduced from lower SI value.

Table 19: *In vitro* Cox-I and Cox-II enzyme inhibition assay of synthesized compounds

Sample Code[a]	Ar'	IC_{50} value of Cox-I Inhibition[b] (μM)	IC_{50} value of Cox-II Inhibition[b] (μM)	SI[c]
8a$_1$	2-(3-Chloroquinolinyl)	0.098±0.007	0.058±0.005	1.69
8a$_2$	2-quinoxalinyl	0.123±0.008	0.063±0.006	1.95
8a$_3$	4-Hydroxy-5-nitrophenyl	0.142±0.007	0.105±0.006	1.35
8a$_4$	2,4-Dichlorophenyl	0.104±0.008	0.098±0.006	1.06
8a$_6$	Indol-3-yl	0.138±0.008	0.064±0.006	2.15
8a$_{10}$	3-Nitrophenyl	0.192±0.007	0.065±0.006	2.95
8a$_{11}$	2-Nitrophenyl	0.175±0.007	0.062±0.006	2.82
8b$_1$	4-Bromophenyl	0.127±0.007	0.115±0.005	1.17
8b$_2$	2-(3-Chloroquinolinyl)	0.139±0.006	0.113±0.005	1.23
8b$_3$	2, 4-Dichlorophenyl	0.166±0.006	0.123±0.005	1.35
8b$_4$	4-Hydroxy-5-nitrophenyl	0.134±0.007	0.144±0.005	0.93
8b$_5$	4-(N, N)-Dimethylaminophenyl	0.188±0.005	0.135±0.006	1.39
8b$_6$	Quinoxalin-2-yl	0.166±0.005	0.088±0.009	1.88
8c$_2$	Quinoxalin-2-yl	0.273±0.006	0.195±0.007	1.40
8c$_5$	4-(N, N)-Dimethylaminophenyl	0.187±0.007	0.116±0.007	1.61
8c$_6$	Quinoxalin-2-yl	0.129±0.010	0.145±0.007	0.88
8c$_7$	4-Bromophenyl	0.188±0.006	0.092±0.006	2.04

$8c_8$	4-Nitrophenyl	0.183±0.007	0.105±0.007	1.74
Celecoxib[d]	-	0.151±0.007	0.25±0.006	0.604
Ibuprofen[d]	-	-	0.9±0.02	-
Indomethacin[d]	-	-	1.56±0.03	

[a]samples of series 4 are Ibuprofen-based, series 9 are quinoxaline-based, series 13 are pyridine-based; [b] IC_{50} in μM was calculated as IC_{50} in μg/ml divided by molecular weight of compounds; [c]SI= selectivity index was calculated as ratio IC_{50} value of COX-I inhibition to that of COX-II; IC_{50} values are obtained as the mean±SD(μM) from three different experiments. [d]Standard NSAID drugs

The *in-vivo* anti-inflammatory screening of compounds on Carrageenan-induced paw edema model have been devised due to its good inhibitory potential. The previous results of molecular docking, anti-denaturation assays, *in-vitro* COX-I/II enzyme inhibition assay proves that the compounds of series **8a** had better inhibitory activity compared to those of **8b** and **8c** series compounds. Based on *in-vitro* COX-II inhibition assay and also due to the presence of high versatile quinoxaline moiety, we selected **$8a_1$, $8a_2$, $8a_3$, $8a_4$, $8a_{10}$, $8a_{11}$, $8b_6$** and **$8c_7$** for its *in-vivo* anti-inflammatory assay.

3.3b.3.4.in-vivo Anti-inflammatory Screening-Carageenan-induced paw edema model

The *in-vivo* anti-inflammatory activity of selected compounds **$8a_1$, $8a_2$, $8a_3$, $8a_4$, $8a_{10}$, $8a_{11}$, $8b_6$** and **$8c_7$** using Diclofenac (negative control) and Ibuprofen (positive control) as standard drug has been carried out on Carrageenan-induced paw edema in male wistar rat according to the reported procedure[91,92] using dose of 100mg/kg body weight. The anti-inflammatory activity was then calculated based on paw volume changes for 1, 2, 3 and 4 hours. The Carrageenan-induced paw edema model is suitable test for evaluating anti-inflammatory drugs which has frequently been used to access the antedematous effect of the drug. Carrageenan is a strong chemical used for the release of inflammatory and pro-inflammatory mediators.

The course of inflammation is biphasic. First phase starts with the release of histamine, serotonin and kinins after injection of phlogistic agent in the first few hours while in the

second phase is related to release of prostaglandins like substance in 2-3 hours. Second phase is sensitive to both the clinically useful steroidal and non-steroidal anti-inflammatory agent. For statistical comparison, the fourth hour reading of percentage in the paw volume inhibition was measured. The statistical analysis was carried out by one way analysis of variance followed by Turkey's multiple comparison test; $p<0.05$ was taken to be statistically significant. The initial paw volumes were measured by using Plethysmometer which served as control. The percent inhibition of paw edema was calculated according to following formula: % inhibition= (a-b)/a x100; where 'a' is mean paw inflammation volume of control and 'b' is the mean paw inflammation volume of test. The results have been summarized in **Table 20**.

The results indicated that all the compounds **$8a_1$, $8a_2$, $8a_3$, $8a_4$, $8a_{10}$, $8a_{11}$, $8b_6$** and **$8c_7$** showed significantly best anti-inflammatory effect lower than the standard Diclofenac sodium[93] after a single dose of 100mg/kg orally for 3 hours in Carrageenan induced paw edema in rats with % inhibition values 49.49-70.70%. The maximum inhibition was displayed by compound **$8a_4$** with % inhibition values 70.70**%** respectively.

Table 20. The *in vivo* anti-inflammatory activity on Carrageenan-induced paw edema model

Comp code	Dose mg/kg	% Inhibition			P values
		1st hour	2nd hour	3rd hour	
$8a_1$	100	50.45±1.71	56.87±1.63	56.56±1.32	<0.05
$8a_2$	100	54.58±1.47	60.66±1.22	65.65±1.24	<0.05
$8a_3$	100	55.04±2.77*	63.03±2.41*	65.65±2.21*	<0.05
$8a_4$	100	62.84±2.43*	71.09±2.13*	70.70±2.41*	<0.05
$8a_{10}$	100	44.49±3.43	47.86±3.13	49.49±3.43	-
$8a_{11}$	100	50.00±2.89*	55.92±2.74*	60.60±2.65*	<0.05
$8b_6$	100	49.54±2.43*	53.55±2.13*	59.09±2.33*	<0.05
$8c_7$	100	46.33±3.22	57.81±2.32	60.60±3.13	<0.05
Ibuprofen	100	66.05±2.12	82.93±2.43	87.87±2.43	-
Diclofenac sodium	100	68.80±2.03	80.56±2.43	83.33±2.13	-

Values represent the mean ± S.E.M of six animals for each group. *p < 0.05: Statistically significant from the control using one-way ANOVA (using Turkey as post hoc test)

On the basis of *in-vitro* COX inhibition assay following Structure Activity relationship can be devised: Presence of Ibuprofen as template is essential for optimal COX-II inhibition, replacement of which by quinoxaline and pyridine reduces the activity. For Ibuprofen-based azomethine derivatives **8a**, presence of nitrogen heterocyclic units, quinoxaline **8a$_2$**, quinoline **8a$_1$**, indole **8a$_6$** as motifs enhances COX inhibition in the order quinoline>quinoxaline≥indole **(Fig.20).** Secondly, disubstitution (OH and NO_2) on phenyl ring is essential for maximum activity as observed in **8a$_3$.** Presence of NO_2 (2-, 3-position) of phenyl ring as in **8a$_{10}$**, **8a$_{11}$** is required for better COX-II inhibition. Presence of Cl group on the aromatic ring as in **8a$_4$** has a marked effect on COX-I/II inhibition. In case of quinoxaline-based azomethine derivatives **8b**, presence of Br substituent at 4-position of phenyl ring as in **8b$_1$** is essential for significant activity, in contrast to Cl substituent as in **8b$_3$** where it lowers activity against COX-1 than COX-II. Presence of electron-donating group (4-NMe_2) on phenyl ring as in **8b$_5$** and **8c$_5$** decreases COX-I but is needed for COX-II inhibition, disubstitution (OH and NO_2) on phenyl ring is essential for maximum activity as observed in **8b$_4$** against COX-II inhibition, replacement of phenyl ring by quinoline and quinoxaline unit as in **8b$_2$**, **8b$_6$** enhances COX-II inhibition in the order quinoline>quinoxaline. Among pyridine-based azomethine derivatives **8c,** replacement of phenyl ring by quinoxaline unit has lowering effect against COX-I as in **8c$_2$.** However, it is required for good COX-I/II inhibition as in **8c$_6$.** Presence of electron withdrawing groups like Br and NO_2 on phenyl ring as seen in **8c$_7$** and **8c$_8$** is proposed enhance COX-II inhibition

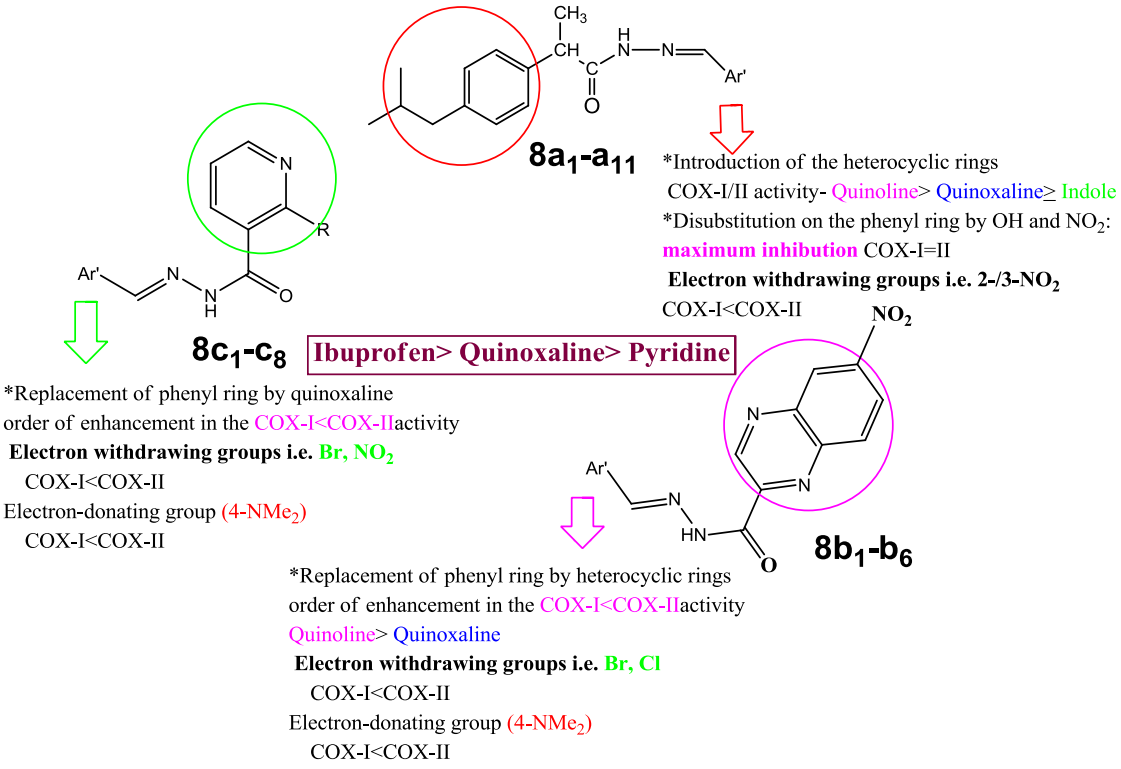


Fig.20. Representation of SAR based on anti-denaturation studies

3.3b.4. Anticancer evaluation*:*

3.3b.4.1. Cytotoxicity assay studies against cancer cell lines

In view of the better anti-inflammatory potency exhibited by compounds **$8a_1$, $8a_2$, $8a_{10}$, $8a_{11}$, $8b_2$, $8b_5$, $8c_5$** and **$8c_6$** we thought of selectively screening these compounds for exploiting their cytotoxicity. The *in-vitro* cytotoxicity of selected compounds has been carried out against MCF-7 (Michigan cancer foundation-7), HeLa (Human cervical) and HEK293 (Human embryonic kidney cells) cancer cell lines by MTT (3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay method.[94-95] This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO, isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reductions of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The IC_{50} values have been calculated using graph pad prism. It has been noticed that, out of eight azomethine derivatives only five compounds **$8a_{10}$, $8a_{11}$, $8b_2$, $8b_5$, $8c_5$** and **$8a_6$** exhibited

better cytotoxicity activity against HeLa cancer cell line with IC_{50} values in the range of 0.09-0.79μM thereby indicating the azomethine group to enhance anti-cancer effects. Compound **8c$_6$** with 2'-quinoxaline ring on 2-chloro pyridine-based azomethine exhibited the more potency among all screened compounds and showed better activity (IC_{50} value 0.09±0.01μM against HeLa cell line and 0.16±0.023μM against MCF-7 cell line) against both cell lines. Excellent anti-cancer effect was been observed for compound **8a$_3$** (IC_{50} values 0.057±0.008μM) against MCF-7 cell line. Compounds **8a$_{11}$** (3-nitrophenyl group) and **8b$_5$** (4-N,N-dimethylaminophenyl group) showed good activity with IC_{50} values 0.11±0.04, 0.14±0.05μM, compounds **8a$_{10}$** (4-nitrophenyl group) and **8b$_2$** (2-quinoxalinyl group) (IC_{50} values 0.25±0.01, 0.20±0.01μM) displayed compatible activity to that of reference drug Paclitaxel against HeLa cancer cell line, but the same compounds **8a$_{10}$, 8a$_{11}$, 8b$_2$** and **8b$_5$** (IC_{50} values 22.25±2.32, 6.40±0.56, 1.23±0.129, 4.05±0.34μM) were found to be least potent against MCF-7 cell line. This was followed by compounds **8a$_1$, 8a$_2$** which were found to be active only against HeLa cancer cell line (IC_{50} values 0.72±0.01 and 0.64±0.02μM) whereas, compound **8c$_5$** exhibited moderate activity against both cell lines (IC_{50} values 0.47±0.07μM against HeLa and 0.58±0.045μM against MCF-7 cancer cell lines). It can be further revealed from the result that compounds exhibited poor to excellent cytotoxicity against HeLa and MCF-7 cancer cell lines although were weakly toxic to human embryonic cells **Table 21**.

Table 21. IC_{50} values (μM±SE) and Selective index (SI)[d] of the azomethine derivatives

Sample code	IC_{50} in μM			SI[d]-	SI[d]-
	HeLa[a]	MCF-7[b]	HEK293[c]	Hela	MCF-7
8a$_1$	0.72±0.01	15.79±1.65	0.87±0.02	1.20	0.05
8a$_2$	0.64±0.02	8.50±1.65	0.44±0.01	0.68	0.05
8a$_3$	NS[e]	0.057±0.008	0.44±0.07	0.68	0.024
8a$_{10}$	0.25±0.01	22.25±2.32	0.99±0.03	3.96	0.04
8a$_{11}$	0.11±0.04	6.40±0.56	0.86±0.03	7.80	0.13
8b$_2$	0.20±0.01	1.23±0.129	0.65±0.04	3.25	0.53
8b$_5$	0.14±0.05	4.05±0.34	0.57±0.05	4.02	0.14
8b$_6$	NS[e]	5.00±0.60	3.56±0.01	0.68	0.71
8c$_5$	0.47±0.07	0.58±0.045	0.92±0.01	1.97	1.43
8c$_6$	0.09±0.01	0.16±0.023	1.05±0.9	11.66	6.56
Paclitaxel	0.23±0.005	0.35±0.005	-	-	-

[a] Human cervical cancer cell line

[b] Michigan cancer foundation-7 cell line

[c] Human embryonic kidney cell line

[d]Selectivity index calculated as ratio of IC_{50} value of normal cells to that of IC_{50} value of cancerous cells

[e]Not significant

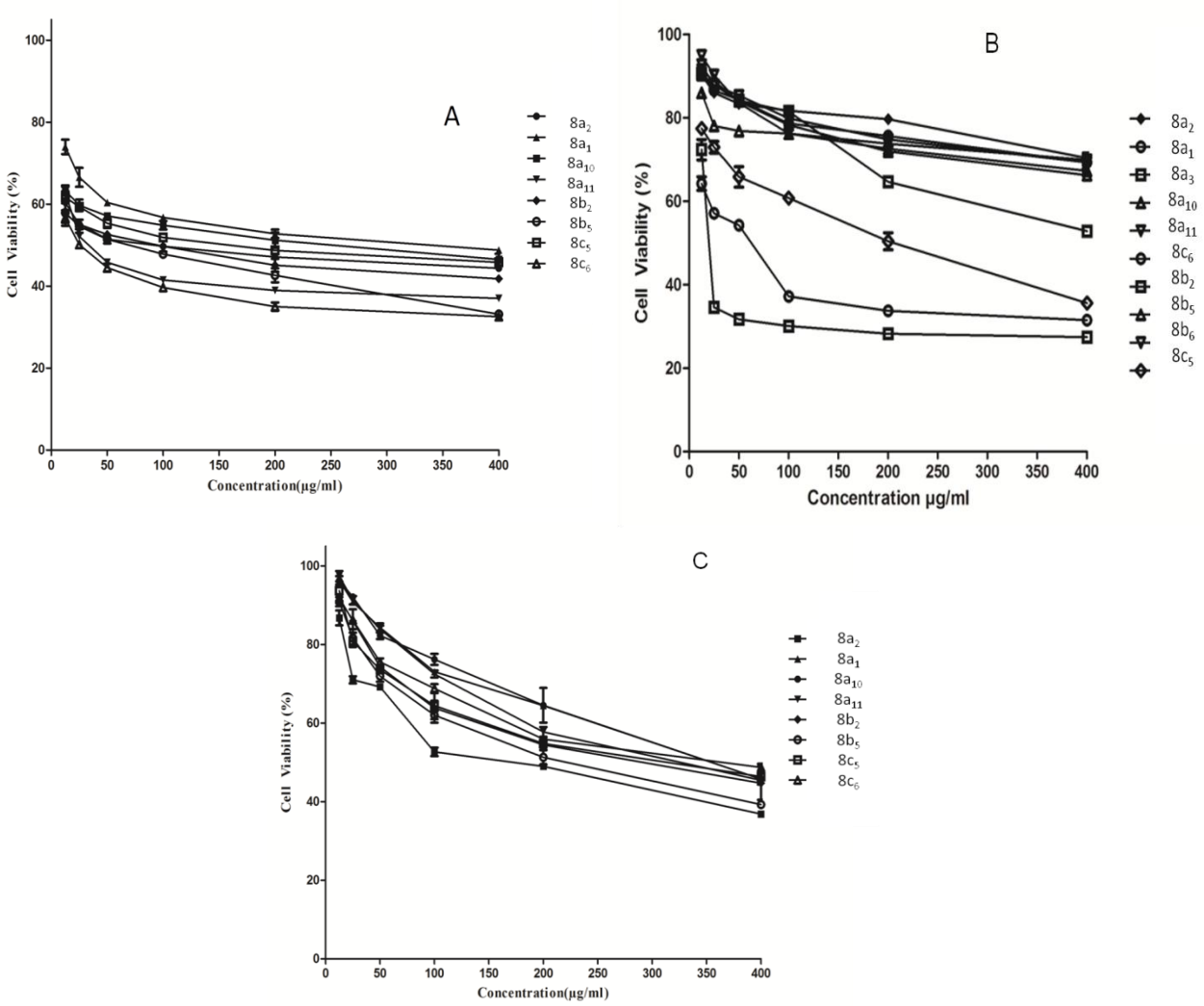


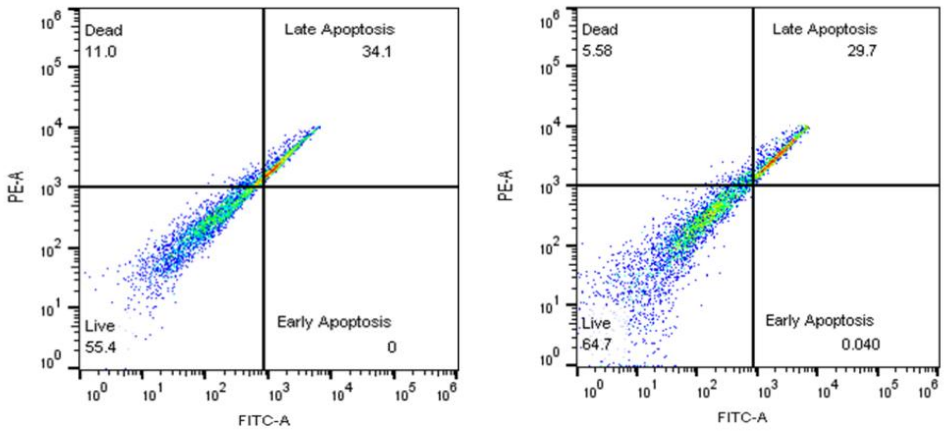
Fig.21. Graphical representation of cell viability of **$8a_1$**, **$8a_2$**, **$8a_{10}$**, **$8a_{11}$**, **$8b_2$**, **$8b_5$**, **$8c_5$** and **$8c_6$** for three different cell lines: **A**. for HeLa cell line, **B.** for MCF-7 cell line and **C.** for HEK-293 cell lines

The cell viability studies were carried out in dose dependent manner. Surprisingly, the cell viability decreased with the increase in concentration of the compound against three cancer cell lines. The results have been investigated and represented graphically as variation in % cell viability with respect to concentration **Fig.21**. ‘

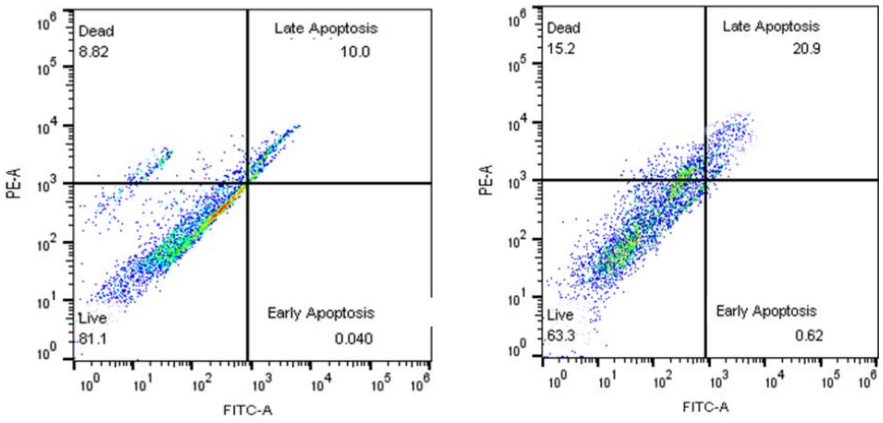
3.3b.4.2.Apoptosis study by Flow cytometry:

Apoptosis is a programmed cell death which occurs in multicellular organisms. In contrast to necrosis, that results from cellular injury, apoptosis is regulated and controlled process and is advantageous during cell cycle. It works on both intrinsic and extrinsic pathway, both of which are catalysed by proteases. In case of intrinsic pathway, cell kills itself as a result of sensing stress whereas, in extrinsic pathway cell kills itself because of other cells. Inspite of its importance in biological phenomenon, it also exhibits defects in variety of diseases. Excessive apoptosis causes atrophy and insufficient apoptosis causes cancer.

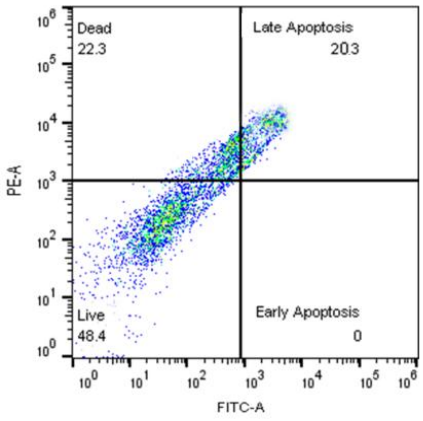
To demonstrate the mechanistic pathway responsible for the anti-proliferative effect of the target compounds **$8a_{10}$, $8a_{11}$, $8b_2$, $8b_5$** and **$8c_6$**, the mode of cell death was discussed by using flow cytometry method[96] on Hela cervical cancer cell lines treated with 100µg/Ml of the above compounds for 24 h. This technique employs use of propidium iodide and annexin V-FITC as a dye to spot viable and dead cells. It detects the live cells (Q4-LL; AV-/PI-), early apoptotic cells (Q3-LR; AV+/PI-), late apoptotic cells (Q2-UR; AV+/PI+) and necrotic cells (Q1-UL; AV-/PI+). The results of flow cytometry (**Fig. 22A**) revealed that, the mode of cell death to be late apoptosis. The percentage of late apoptotic cells exhibited by compounds **$8a_{10}$, $8a_{11}$, $8b_2$, $8b_5$** and **$8c_6$** was 10.0, 34.1, 29.7, 20.9 and 20.3 % respectively.



(a)-$8a_{11}$ (b)-$8b_2$



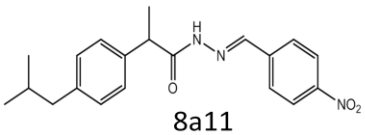
(c) -$8a_{10}$ (d)-$8b_5$



(e)-$8c_6$

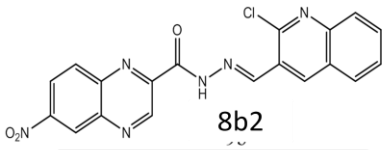
Fig. 22A. Detection of Apoptosis of Hela cells by flow cytometry method

Based on the above biological evaluations, following compounds were considered to have the best antioxidant, anti-inflammatory and anticancer activity (**Fig.22B).**



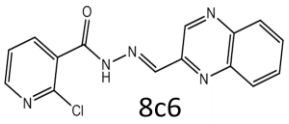
8a11

Cytotoxic against HeLa cell lines
IC_{50} = 0.11±0.04µM; SI= 7.80
Free radical scavenging inhibition
SOS= 63.58%
Anti-inflammatory activity
in-vitro COX-2= 0.062±0.006µM



8b2

Cytotoxic against HeLa cell lines
IC_{50} = 0.20±0.01µM; SI= 3.25
Free radical scavenging inhibition
SOS= 75.91%
Anti-inflammatory activity
in-vitro COX-2= 0.113±0.005µM



8c6

Cytotoxic against HeLa cell lines
IC_{50} = 0.09±0.01µM; SI= 11.66
Free radical scavenging inhibition
SOS= 74.08% DPPH=67.01%
Anti-inflammatory activity
in-vitro COX-2= 0.145±0.007µM

Fig.22B. Compounds with best therapeutic value as anti-inflammatory, anticancer and antioxidant activity.

3.3b.5. Anti-tubercular evaluation:

3.3b.5.1. Virtual Screening of azomethine derivatives as enoyl ACP reductase inhibitors

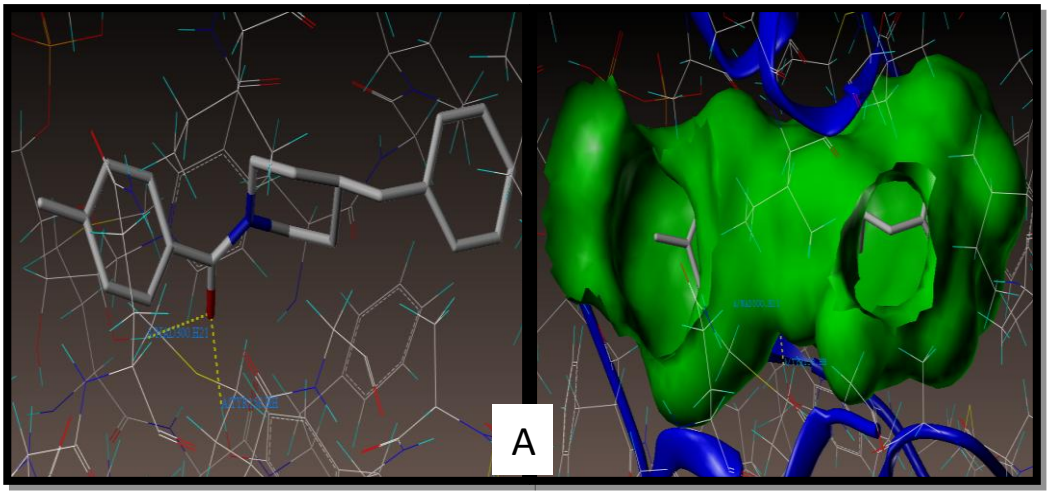
The mechanism of anti-TB activity and detailed intermolecular interactions between the synthesized compounds was investigated using molecular docking studies on the crystal structure of enoyl acyl carrier protein reductase InhA in complex with *N*-(4-methylbenzoyl)-4-benzylpiperidine (PDB ID: 2NSD, 1.9 Å X-ray resolution) using the surflex-dock programmed of sybyl-X 2.0 software.[97-99] All the compounds were docked in the binding site of ENR as shown in **Fig.23B.** The binding energies of the compounds are listed in **Table 22**. The results of docking study revealed that all the compounds display very good docking score against *M. tuberculosis.*

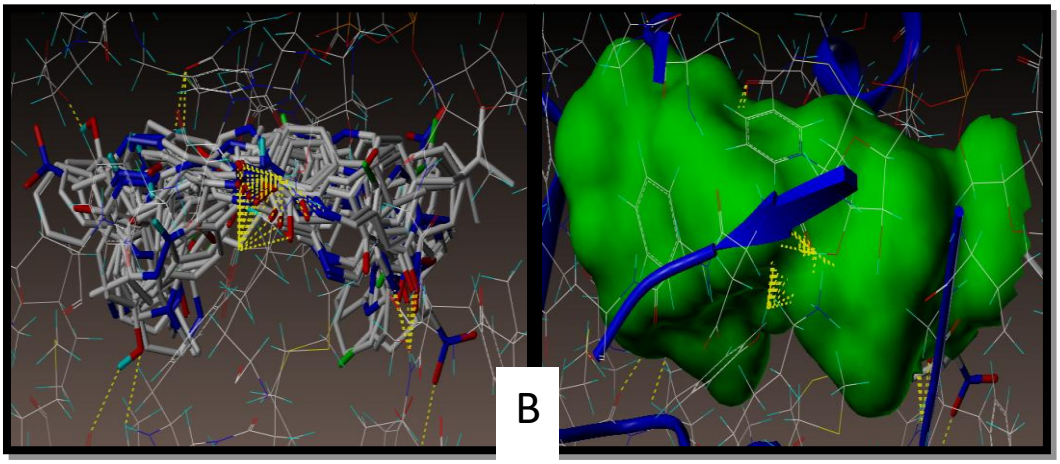
As depicted in the **Fig.23C,** three hydrogen bonding interactions has been observed from compound **$8b_5$** at the active site of the enzyme (PDB ID: 2NSD), of which two interactions were of nitrogen atom present at the 4th position of quinoxaline with hydrogens of TYR158 and NAD300 (N----H-TYR158, 2.06Å; N----H-NAD300, 2.49Å), and one more hydrogen bonding interaction raised from the hydrogen atom of carbohyrazide group with oxygen of NAD300 (CONH----O-NAD300, 2.48Å).

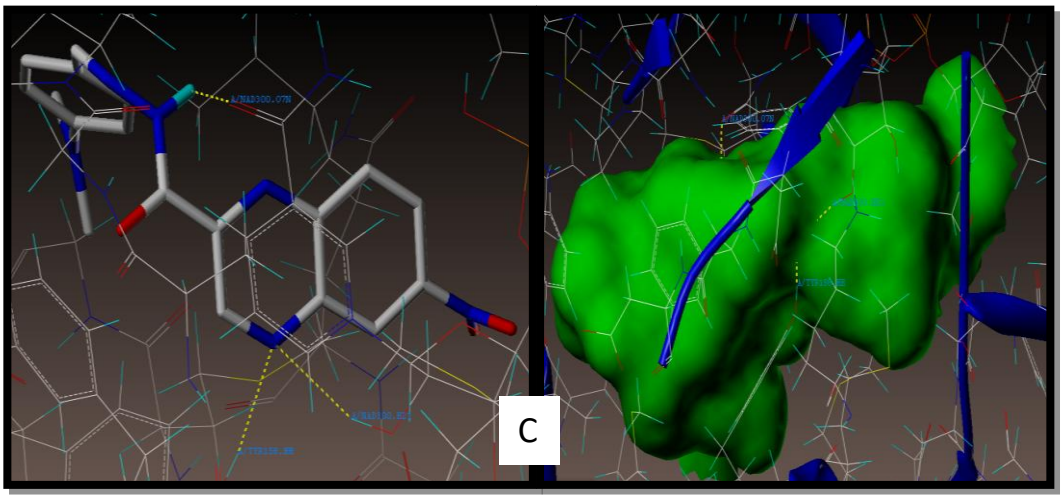
Compound **$8a_{11}$**, exhibits three hydrogen bonding interactions at the active site of the enzyme (PDB ID: 2NSD), among those two interactions were of oxygen atom of carbohydrazide group with hydrogens of TYR158 and NAD300 (C=O----H-TYR158, 2.06Å; C=O----H-NAD300, 1.97Å), and remaining hydrogen bonding interaction is raised from the oxygen atom of nitro group with hydrogen of MET98 (O----N-MET98, 1.92Å) **Fig.23D.**

The binding interaction of 2NSD ligand with enoyl ACP reductase enzyme shows two bonding interaction which has been depicted in **Fig.23A**. It was also seen, that the studied compounds have showed same type of interactions with amino acid residue TYR158, MET98 and co-factor NAD300 as that of reference 2NSD ligand. The hydrophobic and hydrophilic amino acids surrounded to the studied compound **$8b_5$ & $8a_{11}$** has been represented in **Fig.24 (A&B).** All the compounds showed consensus score in the range 9.06-4.40, which indicates all the forces of

interaction between ligands and the enzyme in docked compounds. Thus, indicating that molecules preferentially bind to enzyme in comparison to the reference 2NSD ligand.







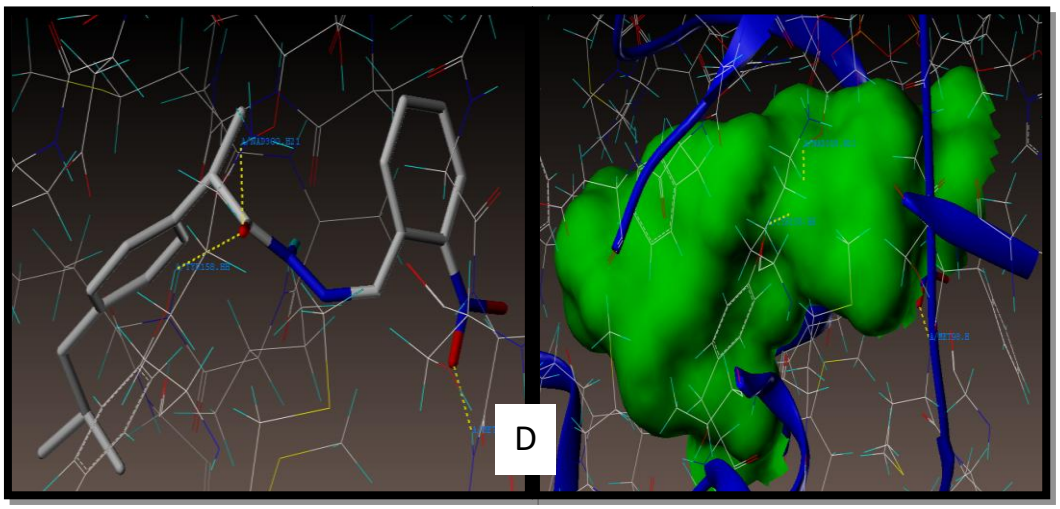


Fig. 23. Docked view at the active site of the enzyme PDB ID: 2NSD with: A) ligand, B) all compounds, C) compound **8b$_5$** and D) compound **8a$_{11}$**

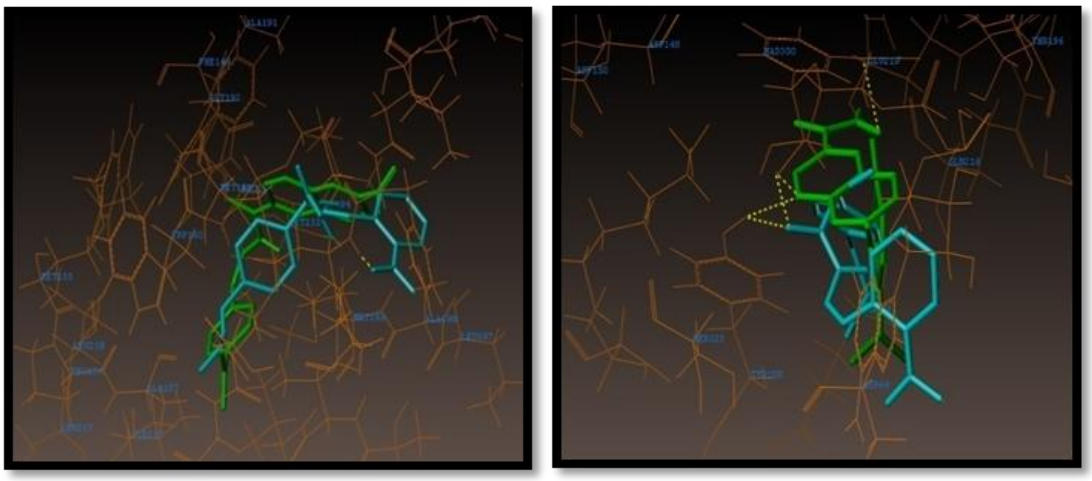


Fig.24. Docked view showing: A) Hydrophobic amino acids surrounded to compounds **8b$_5$** (green colour) and **8a$_{11}$** (cyan colour). B) Hydrophilic amino acids surrounded to compounds **8b$_5$** and **8a$_{11}$**

Table 22. Surflex Docking score (kcal/mol) of the azomethine (Schiff Base) derivatives

Compounds	Total[a]	Crash[b]	Polar[c]	D Score[d]	PMF Score[e]	G Score[f]	Chem Score[g]
2NSD_Ligand	9.25	-0.93	1.54	-150.083	-63.091	-2590.960	-46.922
8b$_5$	9.06	-2.44	1.22	-163.192	-65.611	-297.817	-37.496
8a$_{11}$	8.87	-1.64	1.52	-158.981	-45.854	-294.105	-41.038

8c$_3$	7.99	-2.03	0.00	-164.759	-50.233	-299.542	-39.699
8c$_5$	7.78	-1.09	0.62	-145.416	-27.774	-244.633	-43.425
8c$_6$	7.75	-2.36	2.44	-156.222	-57.675	-266.192	-43.001
8a$_{10}$	7.56	-1.42	0.99	-165.933	-68.485	-280.943	-40.479
8c$_1$	7.44	-1.08	0.65	-145.495	-21.209	-247.009	-41.977
8c$_2$	7.16	-1.70	1.48	-147.392	-49.167	-245.759	-36.079
8b$_6$	7.14	-1.43	2.29	-145.004	-97.809	-221.558	-36.699
8a$_4$	6.95	-2.10	1.18	-154.925	-42.270	-267.174	-32.768
8b$_4$	6.88	-1.66	1.48	-121.404	-53.596	-193.002	-33.480
8a$_1$	6.77	-3.04	0.64	-170.777	-58.299	-272.569	-33.637
8a$_2$	6.62	-1.69	3.03	-137.689	-42.382	-225.524	-32.992
8c$_4$	6.53	-1.28	1.74	-135.417	-54.064	-210.106	-23.614
8c$_7$	6.41	-1.70	1.88	-134.060	-57.128	-211.624	-27.714
8c$_8$	6.22	-2.00	0.00	-153.008	-24.145	-278.038	-25.235
8b$_2$	6.18	-1.55	2.68	-140.408	-49.954	-217.902	-24.942
8b$_1$	6.07	-1.95	2.44	-142.147	-41.334	-220.068	-25.576
8b$_3$	6.01	-0.61	1.59	-117.265	-83.321	-166.226	-24.452
8a$_8$	6.00	-0.69	1.11	-128.322	-74.002	-185.367	-22.437
8a$_7$	5.73	-0.79	2.06	-112.214	-60.078	-149.718	-24.202
8a$_3$	5.08	-1.34	2.34	-126.509	-29.204	-205.042	-30.889
8a$_6$	4.42	-2.47	0.17	-138.965	-38.266	-251.197	-31.674
8a$_9$	4.42	-3.81	2.91	-135.517	-36.105	-186.470	-26.566

8a$_5$	4.40	-0.88	1.18	-97.816	-21.396	-158.299	-23.649

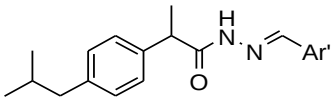
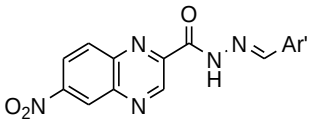
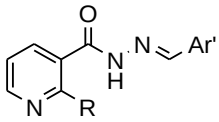
[a]CScore (Consensus Score) integrates total ranking from the affinity of ligands bound to the active site of a receptor and reports the output of total score.
[b]Crash-score reveals the penetration into the binding site. The number close to 0 is favourable. Negative value indicates penetration.
[c]Polar indicating the contribution of the polar interactions to the total score. The polar score may be useful for excluding docking results that make no hydrogen bonds.
[d]D-score for charge and van der Waals interactions between the protein and the ligand.
[e]PMF-score indicating the Helmholtz free energies of interactions for protein-ligand atom pairs (Potential of Mean Force, PMF).
[f]G-score showing hydrogen bonding, complex (ligand-protein), and internal (ligand-ligand) energies.
[g]Chem-score points for H-bonding, lipophilic contact, and rotational entropy, along with an intercept

3.3b.5.2.In-vitro Anti-tubercular Activity:

The results of molecular docking studies demonstrated good binding interaction of all the synthesized compounds with the active site of the enzyme enoyl ACP reductase as shown in **Table 22**. The selective screening of initial thirteen compounds against *Mycobacterium tuberculosis* $H_{37}RV$ strain was carried out by Alamar Blue Dye assay method.[100] This methodology is non-toxic, uses thermally stable reagents and showed good correlation with the proportional and BACTEC radiometric method. All the compounds exhibited better anti-tubercular activity with the MIC value ranging 0.8-12.5µg/mL compatible with the standard drugs Streptomycin, Pyrazinamide and Ciprofloxacin (**Fig.25.B**). The blue colour in the well was interpreted as no bacterial growth whereas pink colour was scored as growth (**Fig.25.A**). The MIC values of the synthesized compounds are represented in **Table 23.**

Table 23. *In vitro* anti-tubercular activity[a] of synthesized Azomethine derivatives:

Sr. No	**Structure**	**Code**	**Ar'**	**MIC[b] in µg/mL**
1		8a$_2$	2-Quinoxalinyl	12.5
2		8a$_3$	4-Hydroxy-5-nitrophenyl	12.5

3		$8a_4$	2,4-Dichorophenyl	1.6
4		$8a_{10}$	2-Nitrophenyl	1.6
5		$8a_{11}$	3-Nitrophenyl	0.8
6		$8b_4$	4-Hydroxy-5-nitrophenyl	1.6
7		$8b_5$	4-*N*,*N*-dimethylaminophenyl	0.8
8		$8b_6$	2-Quinoxalinyl	1.6
9		$8c_1$; R=H	4-*N*,*N*-dimethylaminophenyl	1.6
10		$8c_2$; R=H	2-Quinoxalinyl	1.6
11		$8c_3$; R=H	4-Chlorophenyl	1.6
12		$8c_5$;R=Cl	4-*N*,*N*-dimethylaminophenyl	0.8
13		$8c_6$; R=Cl	2-Quinoxalinyl	0.8
14	Streptomycin[c]			6.25
15	Pyrazinamide[c]		-	3.12
16	Ciprofloxacin[c]			3.12

[a]By Alamar Blue Dye assay method, against H_{37}Rv strain

[b]Minimum inhibitory concentration values

[c]Standard drugs- Streptomycin-belong to aminoglycoside family, Pyrazinamide is First line drug, and Ciprofloxacin-is example of fluroquinolones-Class of Second line drugs

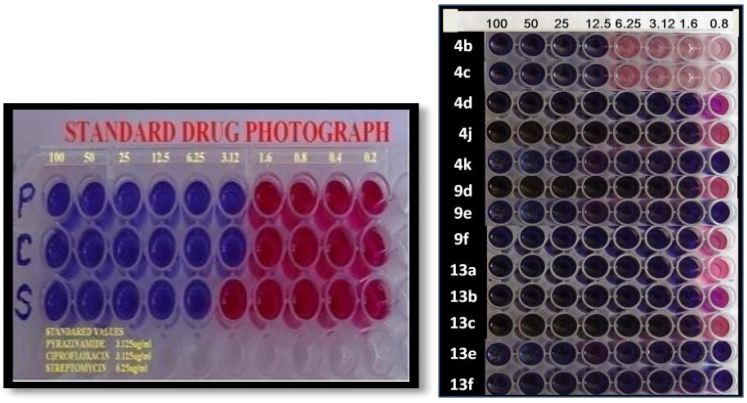


Fig.25A.To the left shows MIC values of standard drugs Streptomycin, Pyrazinamide and Ciprofloxacin while that to the right shows MIC values of synthesized azomethine derivatives.

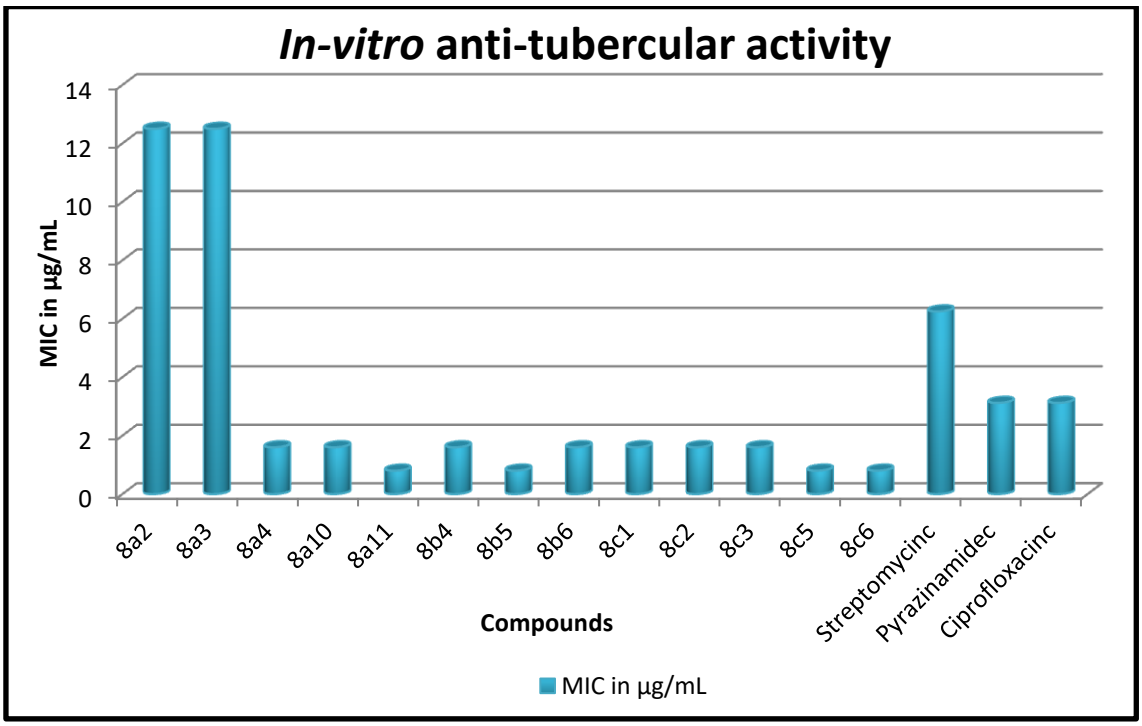


Fig.25B. Graphical representation of anti-tubercular activity: MIC values v/s compounds

Following structure activity relationship can be deduced on the basis of anti-tubercular activity results (**Fig.24**). The acyl hydrazide side chain was the main pharmacophoric unit responsible for the anti-tubercular activity. Substitution on the acyl group by electron deficient substrate and that of the electron rich substrate on the hydrazide NH is essential for better activity, which can be best explained by intact double bond character of C=O group of the -C(O)-NH-NH_2-pharmacophore. All the synthesized derivatives exhibited maximal anti-tubercular activity compatible with the standard drugs except for compound **8a$_2$**, **8a$_3$** and **8b$_4$** which exhibited minimal anti-tubercular activity than the standard drugs. In case of compounds from series **8a**, presence of heterocyclic ring as in **8a$_2$** led to reduction in the activity; whereas, replacement of heterocyclic ring by simple electron deficient ring as in **8a$_3$** and **8a$_{10}$** enhanced the activity; this difference in the activity was attributed to the steric factor. The difference in the activity between **8a$_{10}$** and **8a$_{11}$** was due to –I effect. The nitro group at 3 position exhibits a higher withdrawing effect than at ortho position thus, increasing the activity. Considering compounds of series **8b**, phenyl group substituted on the hydrazide NH with electron donating group as in compound **8b$_3$** exhibited better activity than other compounds **8b$_4$** and **8b$_6$,** which could be predicted to be due to +I (electron donating) effect of the substituents on the phenyl ring. As far as the compounds of series **8c,** were concerned, high activity was shown by compound **8c$_5$** and **8c$_6$** due to the presence of chloro substituent on the pyridine ring which

exhibits high withdrawing effect on the ring thus retaining the double bond character of C=O group of acyl hydrazide side chain. Minimal activity in case of compound **8c$_3$** was attributed to presence of electron deficient ring on the hydrazide (NH) side chain.

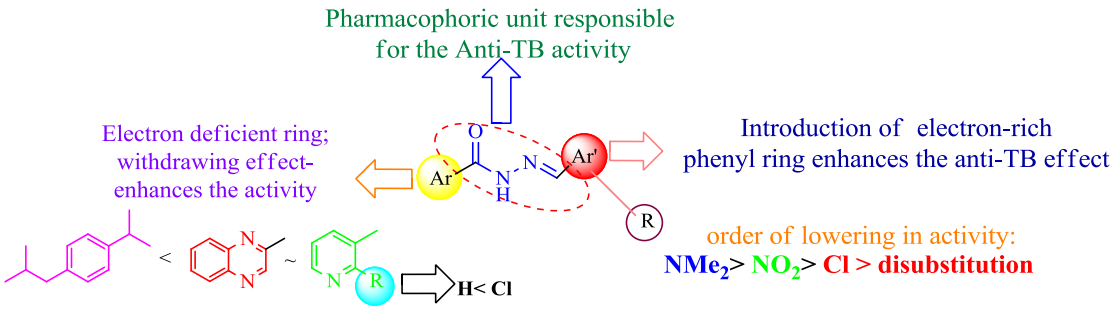


Fig.26. Structural representation of SAR studies of anti-tubercular activity

It can be evident from the above observation that the synthesized azomethine derivatives can be a good lead in development of new drug entities to treat tuberculosis. The dual relationship between inflammation and mycobacterial infection, makes it interesting to study simple drug entities to possess anti-inflammatory properties along with anti-tubercular activity.

3.4. CONCLUSION

A new series of Schiff bases derived from Ibuprofen, quinoxaline and pyridine as template with heterocyclic motifs have been designed and synthesized as compounds **$8a_1$-a_{11}**, **$8b_1$-b_6 and $8c_1$-c_8** respectively. The molecular docking scores of these compounds against COX isoenzymes reveal that, Ibuprofen based Schiff bases are good COX-II inhibitors compared to the parent Ibuprofen, Indomethacin and other Schiff bases. The preliminary *in-vitro* anti-denaturation by bovine serum albumin method, egg-albumin denaturation assay and haemolysis indicates that the **$8a_1$-a_{11}** compounds should exhibit better anti-inflammatory activity. Further *in-vitro* COX-I and COX-II inhibitory assay showed all the compounds to be better COX-II inhibitors with no selectivity in activity as confirmed by lower selectivity index value between 0.88-2.95 as compared to Celecoxib. The *in-vivo* anti-inflammatory activity experiments proved that compounds **$8a_1$, $8a_2$, $8a_3$, $8a_4$, $8a_{10}$, $8a_{11}$, $8b_6$** and **$8c_7$** exhibit values with $p<0.05$ better than both the control. The structure activity relationship studies explain the activity profile of the synthesized compounds against inflammation wherein, the Ibuprofen based Schiff bases have the best COX-II inhibition results and thus optimum anti-inflammatory activity. Ibuprofen based azomethines with the structural motifs, quinoxaline, quinoline and indole favour COX-II inhibition. Compounds **$8b_3$**, **$8c_7$** and **$8c_8$** were found to possess mild antioxidant property with % radical scavenging activity between 43.53-58.33% against both superoxide and DPPH radicals. In contrast, compounds **$8a_3$, $8a_6$, $8a_{10}$, $8a_{11}$, $8b_2$** demonstrated highest antioxidant activity selectively as DPPH scavengers. The anti-cancer evaluation revealed compound **$8c_6$** to possess promising activity against both MCF-7 and HeLa cancer cell lines compared to standard Paclitaxel. Compound **$8a_3$** showed excellent cytotoxicity against MCF-7 cell line with no activity against HeLa (0.057μM). Further the mode of cell death was analysed to be late apoptosis as predicted by flow cytometry. The results of *in vitro* anti-tubercular activity studies demonstrated that all the screened compounds have good anti-tubercular property with the MIC value ranging from 0.8-12.5μg/mL compatible with the standard drugs. Thus, these derivatives could be used as a lead in the development of antitubercular drug. The above findings also proved that presence of heterocyclic moiety such as quinoxaline and quinoline acts as a versatile pharmacophore in the Schiff base designs. With good antioxidant property, COX-I and COX-II inhibitory activity and an excellent anticancer evaluation results, development of potential cancer therapeutics could be achieved.

3.5. EXPERIMENTAL

3.5.1. Synthesis of Ibuprofen-, quinoxalinyl- and pyridinyl-based azomethine derivatives 8a, 8b, 8c

To a solution of respective acids **6a**, **6b** and **6c** (1mmol) in absolute ethanol (15mL) was added catalytic amount of concentrated sulphuric acid (4-6 drops) at 100^{o}C for 6-12 h. After completion, as monitored by TLC (10% Petroleum ether), excess of ethanol was removed by evaporation, the resulting mixture was dissolved in water (10 mL) and it was extracted using diethyl ether (3X 20mL). The ether layer was dried over Na_2SO_4 and evaporated under reduced pressure to give the crude esters **7a, 7b** and **7c**. The ester derivatives were then subjected to reaction with hydrazine-hydrate (99%) in ethanol (10mL) and were refluxed for 3-8 h. The completion of reaction was monitored by TLC (25% Petroleum ether). The excess ethanol was removed by evaporation and to the resulting residue crushed ice was added to precipitate out the crude hydrazide **8a, 8b** and **8c**. The hydrazides were filtered dried over vacuum and were used further for the preparation of hydrazide derivatives (Schiff bases) **8a**, **8b** and **8c**. The preparation of hydrazide derivatives was carried out by reacting 1:1 equivalent of hydrazides (1 mmol) and substituted aromatic aldehydes (1 mmol) in ethanol (10mL) at 25^{o}C for 2-6 h. the product was precipitated out by pouring in ice. The solid obtained was filtered, dried, and recrystallised from ethanol.

3.5.1.1. 2-(4-Isobutylphenyl)-propionic acid-(*o*-chloro-quinolin-3-yl-benzylidene)-hydrazide (**8a$_1$**).

Compound **8a$_1$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 2-chloro-3-formyl quinoline (0.17g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.33g (80%). M.p.188-190^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR ($CDCl_3$): δ 8.37(s, 1H, azomethine), 8.27(s, 1H, pyr), 8.03(dd, *J*=7.7, 2.3Hz, 1H), 7.86(dd, *J*=7.9, 2.1Hz, 1H), 7.70(dt, *J*=7.7, 2.5Hz, 1H), 7.61(dt, *J*=7.9, 2.5Hz, 1H), 7.18(d, *J*=8.2Hz, 2H), 7.05(d, *J*=8.2Hz, 2H), 3.95(q, 1H, isopropyl-CH), 2.44(d, 2H, isobutyl methylene), 1.84 (m, 1H, isobutyl-CH), 1.35(d, 3H, isopropyl-CH_3), 0.86(d, 6H, isobutyl$(CH_3)_2$).^{13}CNMR (400MHz; $CDCl_3$) 175.9, 152.8, 149.9, 143.0, 139.3, 137.8, 133.2, 131.0, 129.1, 129.1, 128.8, 128.8, 128.1, 127.6, 127.2, 126.8, 124.1, 45.8, 42.9, 30.5, 23.2, 23.2, 18.9. HRMS (Electrospray ionization time-of-flight): calc. for $C_{23}H_{24}N_3OCl$, 393.9150; found 393.9300.

3.5.1.2. 2-(4-Isobutylphenyl)-propionic acid-(quinoxalin-2-yl-benzylidene)-hydrazide ***(8a$_2$)***.

Compound **8a$_2$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and quinoxalin-2-carbaldehyde (0.14g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.3g (81%). M.p.166-170^{o}C. TLC(4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz, $CDCl_3$): δ 9.5(s, 1H, pyrazine), 8.7(s, 1H, azomethine), 8.77 (dd, *J*=8.1, 2.9Hz, 1H, quinoxaline), 8.67(dd, *J*=8.0, 2.8Hz, 1H, quinoxaline), 8.28(dt, *J*=8.0, 2.1Hz, 1H, quinoxaline), 8.24(dt, *J*=8.0, 2.1Hz, 1H, qinoxaline), 7.33(d, *J*=8.2Hz, 2H, Ibuprofen), 7.15(d, *J*=8.2Hz, 2H, Ibuprofen), 3.94(q, 1H, isopropyl CH), 2.44(d, 2H, isobutyl methylene), 1.84(m, 1H, isobutyl CH), 1.35(d, 3H, isopropyl-CH_3), 0.91(d, 6H, isobutyl $(CH_3)_2$). ^{13}CNMR (400MHz; $CDCl_3$): 177.0, 148.0, 143.0, 142.8, 142.0, 140.5, 140.0, 138.4, 130.8, 130.8, 129.9, 129.7, 129.4, 127.8, 45.0, 42.0, 30.52, 22.8, 19.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{22}H_{24}N_4O$, 360.4611; found 360.4710.

3.5.1.3. 2-(4-Isobutylphenyl)-propionic acid-(4-hydroxy-3-nitro-benzylidene)-hydrazide (**8a$_3$**).

Compound **8a$_3$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 4-hydroxy-5-nitrobenzaldehyde (0.15g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.28g (85%). M.p.148-152^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz, $CDCl_3$): δ 9.14(s, 1H, azomethine), 8.21(d, *J*=2.4Hz, 1H, Ar), 7.85-7.82(dd, *J*=8.2, 2.1Hz, 1H, Ar), 7.23(d, *J*=8.2Hz, 2H, Ibuprofen), 7.15-7.09(d, *J*=8.2Hz, 1H), 7.08(d, *J*=8.20Hz, 2H, Ibuprofen), 3.67(q, 1H, isopropyl CH), 2.50(d, 2H, isobutyl methylene), 1.86(sept,1H, isobutyl CH), 1.59(d, 3H, isopropyl-CH_3), 0.89(d, 6H, isobutyl $(CH_3)_2$). ^{13}CNMR (400MHz $CDCl_3$) 177.0, 154.7, 143.0, 139.3, 136.7, 135.7, 133.2, 129.1, 128.8, 127.3, 125.5, 116.9, 45.7, 44.9, 29.1, 22.8, 17.4. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{23}N_3O_4$, 369.4211; found 369.4311.

3.5.1.4. 2-(4-Isobutylphenyl)-propionic acid-(2,4-dichloro benzylidene)-hydrazide (**8a$_4$**).

Compound **8a$_4$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 2, 4-dichlorobenzaldehyde (0.16g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.26g (77%). M.p.156-160^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz, $CDCl_3$): δ 8.11(s, 1H, azomethine), 7.43(d, *J*=2.7Hz, 1H, Ar), 7.28(dd, *J*=8.4, 2.5Hz, 1H, Ar), 7.26(d, *J*=8.1Hz, 1H, Ar), 7.18(d, *J*=8.2Hz, 2H,

Ibuprofen), 7.05(d, *J*=8.2Hz, 2H, Ibuprofen), 3.94(q, 1H, isopropyl CH), 2.44(d, 2H, isobutyl methylene), 1.84(sept, 1H, isobutyl CH), 1.35(d, 3H, isopropyl-CH_3), 0.83(d, 6H, isobutyl $(CH_3)_2$). ^{13}C NMR (400MHz; $CDCl_3$) 176.2, 142.0, 139.3, 138.1, 135.4, 134.2, 132.0, 131.5, 130.5, 129.1, 129.1, 128.8, 127.1, 45.8, 40.3, 28.3, 23.1, 17.4. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{22}N_2OCl_2$, 377.3091; found 377.3270.

3.5.1.5. 2-(4-Isobutylphenyl)-propionic acid-(4-(*N*,*N*)-dimethylamino benzylidene)-hydrazide (**8a$_5$**)

Compound **8a$_5$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 4-*N*,*N*-dimethylamino-benzaldehyde (0.135g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.21g (67%). M.p.257-262^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 8.44 (s, 1H, azomethine), 7.58-7.54 (d, *J*=8.8Hz, 2H, Ar), 7.35 (d, *J*=8.04Hz, 2H, Ibuprofen), 7.08 (d, *J*=8.04Hz, 2H, Ibuprofen), 6.74 (d, *J*= 8.8Hz, 2H, Ar), 3.65 (q, 1H, isopropyl CH), 3.03 (s, 6H, -N$(CH_3)_2$), 2.44 (d, 2H, isobutyl methylene), 1.84 (m, 1H, isobutyl CH), 1.59 (d, 3H, isopropyl-CH_3), 0.91 (d, 6H, isobutyl $(CH_3)_2$). ^{13}C NMR (400MHz; $CDCl_3$) 175.9, 151.65, 148.55, 143.61, 140.15, 138.74, 129.91, 128.56, 127.69, 127.57, 111.9, 105.82, 45.15, 40.24, 30.28, 22.51, 18.3. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{22}H_{29}N_3O$, 351.4941; found 351.5120.

3.5.1.6. 2-(4-Isobutylphenyl)-propionic acid (1*H*-indol-3-ylazomethylene)-hydrazide (**8a$_6$**)

Compound **8a$_6$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 3-formyl indole (0.13g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.24g (75%). M.p.257-262^{o}C. TLC (4:1 petroleum ether/EtOAc). ^{1}HNMR(400MHz; $CDCl_3$): δ 8.14 (s, 1H, azomethine), 7.73(s, 1H, pyrrole), 7.55(dd, *J*=8.0, 2.1Hz, 1H, indole), 7.45(dd, *J*=8.0, 2.1Hz, 1H, indole), 7.18(d, *J*=8.2Hz, 2H, Ar), 7.15(dt, *J*=7.9Hz, 2.5, 1H, indole), 7.1(dt, *J*=8.02, 2.3Hz, 1H, indole), 7.05(d, *J*=8.2Hz, 2H, Ar), 3.95(q, 1H, isopropyl CH), 2.44(d, 2H, isobutyl methylene), 1.84(m, 1H, isobutyl CH), 1.35(d, 3H, isopropyl-CH_3), 0.83(d, 6H, isobutyl $(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$): 177.0, 143.0, 139.3, 135.5, 133.2, 130.8, 129.1, 128.8, 128.8, 126.1, 122.2, 120.1, 119.0, 111.2, 46.8, 42.3, 28.9, 22.1, 17.8. HRMS (Electrospray ionization time-of-flight, [M]+): calc.for $C_{22}H_{25}N_3O$, 347.4621; found 347.4722.

3.5.1.7. 2-(4-Isobutyl-phenyl)-propionic acid-(4-fluoro-benzylidene)-hydrazide (**8a$_7$**)

Compound **8a$_7$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 4-Fluoro-benzaldehyde (0.11g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.25g (86%). M.p.134-136^{o}C. TLC(4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz; $CDCl_3$): δ 9.08(s, 1H, azomethine), 7.69-7.62(d, *J*=8.72Hz, 2H, Ar), 7.32-7.26(d, *J*=8.04Hz, 2H, Ar), 7.18-7.10 (d, *J*=8.20Hz, 2H, Ar), 7.09-7.02 (d, *J*= 8.72Hz, 2H), 3.69 (q, 1H, isopropyl CH), 2.47 (d, 2H, isobutyl methylene), 1.86-1.80 (m,1H, isobutyl CH), 1.59 (d, 3H, isopropyl-CH_3), 0.89 (d, 6H, isobutyl $(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$) 176.85, 165.08, 146.61, 142.2, 141.3, 138.5, 130.1, 129.3, 127.6, 116.1, 46.0, 45.1, 30.25, 22.5, 18.6. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{23}N_2OF$, 326.4051; found 326.4153.

3.5.1.8. 2-(4-Isobutyl-phenyl)-propionic acid-(4-bromo-benzylidene)-hydrazide (**8a$_8$**)

Compound **8a$_8$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 4-Bromo-benzaldehyde (0.17g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.30g (85.7%). M.p.125-127^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR ($CDCl_3$): δ 9.09(s, 1H, azomethine), 7.56(dt, *J*=8.57Hz, 2H, Ar), 7.52-7.50(dd, *J*=8.20Hz, 2H, Ar), 7.33-7.31(dt, *J*=8.25Hz, 2H, Ar), 7.18-7.08(dt, *J*= 8.57Hz, 2H), 3.68(q, 1H, isopropyl CH), 2.49-2.42(d, 2H, isobutyl methylene), 1.88-1.79(m, 1H, isobutyl CH), 1.59(d, 3H, isopropyl-CH_3), 0.89(d, 6H, isobutyl $(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$) 176.9, 140.5, 138.4, 135.8, 132.7, 130.1, 129.9, 129.5, 127.5, 124.3, 124.3, 121.7, 121.7, 46.13, 45.0, 41.9, 30.25, 22.4, 22.4, 18.6. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{24}N_2OBr$, 387.4251; found 387.4350.

3.5.1.9. 2-(4-Isobutyl-phenyl)-propionic acid-(4-nitro-benzylidene)-hydrazide (**8a$_9$)**

Compound **8a$_9$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 4-nitro-benzaldehyde (0.14g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.28g (87%). M.p.155-158^{o}C. TLC (4:1 petroleum ether/EtOAc). ^{1}HNMR (400MHz; $CDCl_3$): δ 9.38(s, 1H, azomethine), 8.20-8.13(d, *J*=8.78Hz, 2H, Ar), 7.76-7.69(d, *J*=8.76Hz, 2H,Ar), 7.45-7.23(d, *J*=7.52Hz, 2H, Ibuprofen), 7.10(dt, *J*=7.52Hz, 2H, Ibuprofen), 3.61(q, 1H, isopropyl-CH), 2.42-2.33(d, 2H, isobutyl methylene), 1.77(m,1H, isobutyl CH),

1.51(d, 3H, isopropyl-CH_3), 0.82(d, 6H, isobutyl $(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$) 175.1, 169.9, 143.7, 140.2, 139.8, 138.9, 138.1, 128.6, 126.9, 123.7, 43.8, 39.5, 29.2, 21.8, 18.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{23}N_3O_3$, 353.4221; found 353.4401.

3.5.1.10. 2-(4-Isobutyl-phenyl)-propionic acid-(2-nitro-benzylidene*)*-hydrazide (**8a$_{10}$**)

Compound **8a$_{10}$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 2-nitro-benzaldehyde (0.14g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.26g (86%). M.p.177-180^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR ($CDCl_3$): δ 9.38(s, 1H, azomethine), 8.20-8.13(dd, *J*=8.78, 2.4Hz, 1H, Ar), 7.90-7.96(dd, *J*=8.4, 2.7Hz, 1H, Ar), 7.76-7.69(dt, *J*=8.76, 2.4Hz, 2H, Ar), 7.45-7.23(d, *J*=7.52Hz, 2H, Ar), 7.10-7.00(dt,*J*=7.52Hz, 2H), 3.61(q, 1H, isopropyl CH), 2.42-2.33(d, 2H, isobutyl methylene), 1.77 (m,1H, isobutyl CH), 1.51(d, 3H, isopropyl-CH_3), 0.82(d, 6H, isobutyl $(CH_3)_2$). ^{13}C NMR ($CDCl_3$): 175.1, 169.9, 143.7, 140.2, 139.8, 138.9, 138.1, 128.6, 126.9, 126.6, 123.7, 123.7, 43.8, 39.5, 29.2, 21.8, 18.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{23}N_3O_3$, 353.4221; found 353.4402.

3.5.1.11. 2-(4-Isobutyl-phenyl)-propionic acid-(3-nitro-benzylidene)-hydrazide (**8a$_{11}$**)

Compound **8a$_{11}$** was prepared from Ibuprofen hydrazide **7a** (0.2g, 0.91mmol) and 3-nitro-benzaldehyde (0.14g, 0.91mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.28g (87.5%). M.p.164-168^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR ($CDCl_3$): δ 9.38(s, 1H, azomethine), 8.64(t, *J*=2.6Hz, 1H, Ar), 8.20-8.13(dt, *J*=8.78, 2.3Hz, 1H, Ar), 8.01-8.05(t, *J*=8.0Hz, 1H, Ar) 7.76-7.69(dt, *J*=8.76, 2.3Hz, 1H, Ar), 7.45-7.23(d, *J*=7.52Hz, 2H, Ibuprofen), 7.10-7.00(d, *J*= 7.52Hz, 2H, Ibuprofen), 3.61(q, 1H, isopropyl CH), 2.42-2.33(d, 2H, isobutyl methylene), 1.77(m, 1H, isobutyl CH), 1.51(d, 3H, isopropyl-CH_3), 0.82(d, 6H, isobutyl $(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$): 175.1, 169.9, 143.7, 140.2, 139.8, 138.9, 128.6, 126.9, 126.6, 126.3, 123.9, 123.7, 43.8, 39.5, 29.2, 21.8, 18.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{20}H_{23}N_3O_3$, 353.4220; found 353.4402.

3.5.1.12. 6-Nitro-quinoxaline-2-carboxylic acid-(4-bromo-benzylidene)-hydrazide (**8b$_1$**)

Compound **8b$_1$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and 4-bromo-benzaldehyde (0.18g, 1mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.25g (70%). M.p.228-232^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz; $CDCl_3$): δ 9.53(s, 1H, pyrazinyl H), 8.67(s, 1H, azomethine), 8.24-8.22(d, *J*=8.44Hz, 1H, Ar), 8.18-8.15(d, *J*=2.1Hz, 1H, Ar), 8.08(dd, *J*=8.44, 2.1Hz, 1H), 7.69 (d, *J*=8.44Hz, 2H), 7.59 (d, *J*=8.44Hz, 2H).^{13}C NMR (400MHz; $CDCl_3$): 157.4, 151.7, 147.0, 146.5, 146.0, 143.0, 140.0, 135.0, 135.0, 132.0, 130.5, 126.0, 124.2, 123.2. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{16}H_{10}N_5O_3Br$, 400.1881; found 400.2980.

3.5.1.13. 6-Nitro-quinoxaline-2-carboxylic acid (2-chloro-quinolin-3-yl-benzylidene)-hydrazide (**8b$_2$**)

Compound **8b$_2$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and 2-chloro-3-formyl quinoline (0.157g, 1mmol), and was obtained as pale yellow solid after recrystallisation from EtOH, yield 0.26g (80%). Mp.185-188^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz; $CDCl_3$): δ 9.03(s, 1H, pyrazinyl-H), 8.96(d, *J*=877Hz, 1H, quinoxaline), 8.62(d, *J*=2.1Hz,1H), 8.33(s, 1H, azomethine), 8.18-8.25(s, 1H), 7.85-8.03(dd, *J*=8.77, 2.5Hz, 1H), 7.78-7.82 (dd, *J*=7.9, 2.6Hz, 1H), 7.63-7.53 (m, *J*=7.72, 1.53Hz, 2H), 7.53 (dd, *J*=7.9, 1.89 Hz, 1H). ^{13}C NMR (400MHz; $CDCl_3$):177.9 157.4, 152.8, 151.7, 149.9, 147.0, 146.5, 146.0, 143.0, 139.9, 138.2, 131.5, 130.2, 129.5, 128.3, 127.9, 125.8, 122.3, 120.5. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{19}H_{11}N_6O_3Cl$, 406.7862; found 406.7961.

3.5.1.14. 6-Nitro-quinoxaline-2-carboxylic acid-(2, 4-dichloro-benzylidene)-hydrazide **(8b$_3$**)

Compound **8b$_3$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and 2,4-dichlorobenzaldehyde (0.17g, 1mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.25g (75%). M.p.240-243^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}HNMR (400MHz; $CDCl_3$): δ 9.02(s, 1H, pyrazinyl-H), 8.91(s, 1H, azomethine), 8.57-8.43 (m, *J*=8.8, 2.1Hz, 2H, quinoxaline), 8.08 (d, *J*=8.8Hz, 1H, quinoxaline), 7.43 (t, *J*=2.0Hz, 1H, Ar), 7.31 (dd, J=8.4, 2.5Hz, 1H, Ar), 7.28 (dd, *J*=8.4, 2.5Hz, 1H, Ar).^{13}C NMR

(400MHz; $CDCl_3$) 157.4, 152.0, 148.0, 147.5, 146.9, 143.0, 140.5, 139.0, 137.6, 133.8, 135.0, 132.5, 131.0, 130.5, 127.1, 125.8. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{16}H_9N_5O_3Cl_2$, 390.1801; found 390.1921.

3.5.1.15. 6-Nitro-quinoxaline-2-carboxylic-acid-(4-hydroxy-5-nitrophenyl-benzylidene)-hydrazide (**8b$_4$**)

Compound **8b$_4$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and 4-Hydroxy-5-nitrobenzaldehyde (0.17g, 1mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.25g (80%). M.p.242-246^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 8.6(s, 1H, pyrazinyl H), 8.50(d, *J*=7.5Hz, 1H, quinoxaline), 8.42(d, *J*=8.7Hz, 1H, quinoxaline), 8.31(s, 1H, azomethine), 8.28-8.23(dd, *J*=8.8, 2.3Hz, 1H, quinoxaline), 8.18-8.15(d, *J*=2.5Hz, 1H, Ar), 7.92(d, *J*=8.2Hz, 1H, Ar), 7.88(dd, *J*=7.8, 2.7Hz, 1H, Ar).^{13}CNMR (400MHz; $CDCl_3$): 169.1, 154.7, 152.0, 148.9, 146.5, 143.0, 140.5, 137.6, 133.8, 131.9, 131.4, 130.5, 128.6, 127.1, 125.7, 121.8. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{16}H_{10}N_6O_6$, 382.2920; found 382.0556.

3.5.1.16.6-Nitro-quinoxaline-2-carboxylic-acid-(4-(*N*,*N*)-dimethylamino-benzylidene)-hydrazide (**8b$_5$**)

Compound **8b$_5$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and 4-(*N, N*)-dimethylamino benzaldehyde (0.17g, 1mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.25g (76%). Mp.190-194^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 8.6(s, 1H, pyrazinyl-H), 8.48(d, *J*=2.3Hz, 1H, quinoxaline), 8.4 (d, *J*=2.0Hz, 1H, quinoxaline), 8.28(s, 1H, azomethine), 8.18-8.13(dd, *J*=8.3, 2.1Hz,1H, quinoxaline), 7.82(d, *J*= 8.2Hz, 2H, Ar), 7.58(d, *J*=8.2Hz, 2H, Ar), 3.03(s, 6H, -$N(CH_3)_2$).^{13}C NMR (400MHz; $CDCl_3$) 169.1, 154.7, 152.0, 148.9.0, 146.5, 143.0, 140.5, 137.6, 133.8, 131.9, 131.4, 130.5, 128.6, 127.1, 125.7, 121.8, 15.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{18}H_{16}N_6O_3$, 364.3650; found 364.3750.

3.5.1.17.6-Nitro-quinoxaline-2-carboxylic acid-(quinoxalin-2-yl-methylene)-hydrazide (**8b$_6$**)

Compound **8b$_6$** was prepared from 6-nitro-quinoxaline-2-acid hydrazide **7b** (0.2g, 1mmol) and quinoxalin-2-carbaldehyde (0.17g, 1mmol), and was obtained as white solid after

recrystallisation from EtOH, yield 0.25g (76%). Mp.250-254^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 9.18 (s, 1H, pyrazinyl-H), 9.08(s, 1H, pyrazinyl-H, Ar), 8.8 (d, *J*=1.97Hz, 1H, quinoxaline), 8.4(d, *J*=8.7Hz, 1H, quinoxaline), 8.28(s, 1H, azomethine), 8.18-8.13(dd, *J*=8.8, 2.3Hz,1H, quinoxaline), 8.08-8.05(dd, *J*=7.5, 2.5Hz, 1H, quinoxaline-Ar), 7.82(dd, *J*= 8.2, 2.1Hz, 1H, quinoxaline-Ar), 7.58-7.52(m, *J*=8.7, 1.9Hz, 2H, quinoxaline-Ar). ^{13}C NMR (400MHz; $CDCl_3$) 169.1, 154.7, 152.0, 148.9, 146.5, 143.0, 140.5, 137.6, 133.8, 131.9, 131.4, 130.5, 128.6, 127.1, 125.7, 123.2, 123.6, 121.8. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{18}H_{11}N_7O_3$, 373.3320; found 373.0888.

3.5.1.18. Nicotinic acid-(4-nitro-benzylidene)-hydrazide (**8c$_1$**)

Compound **8c$_1$** was prepared from nicotinic acid hydrazide (**7c**) (0.2g, 1.5mmol) and 4-nitrobenzaldehyde (0.22g, 1.5mmol), and was obtained as pale yellow coloured solid after recrystallisation from EtOH, yield 0.27g (77%). M.p.215-217^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR(400MHz; $CDCl_3$): δ 9.05(s, 1H, pyridine), 8.55(dd, J=8.7, 1.9 Hz, 1H, pyridine), 8.39(s, 1H, azomethine), 8.26(d, *J*=8.7Hz, 2H, Ar), 8.07(dd, *J*= 8.1, 1.9Hz, 1H, pyridine), 7.67(t, *J*=8.1Hz, 1H, pyridine), 7.57(d, *J*=8.7Hz, 2H, Ar).^{13}C NMR(400MHz; $CDCl_3$) 163.0, 153.7, 150.2, 148.3, 144.0, 140.0, 138.5, 130.7, 130.1, 126.7, 121.2. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{13}H_{10}N_4O_3$, 270.2480; found 270.2580.

3.5.1.19. Nicotinic acid-(quinoxalin-2-yl)-methylene-hydrazide (**8c$_2$**)

Compound **8c$_2$** was prepared from nicotinic acid hydrazide **7c** (0.2g, 1.3mmol) and quinoxalin-2-carbaldehyde (0.203g, 1.3mmol), and was obtained as buff coloured solid after recrystallisation from EtOH, yield 0.29g (80%). M.p.220-224^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 9.74(s,1H, pyridine), 8.69(s, 1H, pyrazinyl-H), 8.43(s, *J*=7.6, 1.8Hz, 1H, pyridine), 8.37-8.35(dd, *J*=7.6, 1.8Hz, 1H, pyridine), 8.18-8.15(dd, *J*=7.96, 1.84Hz, 1H, quinoxaline-Ar), 8.10-8.07(dd, *J*=7.86, 1.9Hz, 1H, quinoxaline-Ar), 7.82-7.79 (d, *J*=8.10Hz, 1H, pyridine), 7.50(s, 1H, azomethine), 7.17-7.12(m, *J*=7.86, 4.67, 2H, quinoxaline-Ar).^{13}C NMR (400MHz; $CDCl_3$) 170.0, 154.8, 154.7, 148.9, 145.2, 145.0, 143.8, 143.7, 140.3, 130.3, 130.1, 130.1, 129.1, 129.0, 123.7. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{15}H_{11}N_5O$, 277.2870; found 277.2980.

3.5.1.20. Nicotinic acid-(2, 4-dichloro-benzylidene)-hydrazide (**8c$_3$**)

Compound **8c$_3$** was prepared from nicotinic acid hydrazide **7c** (0.2g, 1.5mmol) and 2, 4-dichlorobenzaldehyde (0.26g, 1.5mmol), and was obtained as pale yellow coloured solid after recrystallisation from EtOH, yield 0.25g (75%). M.p.120-124^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 9.04(s, 1H, pyridine), 8.55(dd, *J*=4.7, 1.9Hz, 1H, pyridine), 8.13(dd, *J*=8.1, 1.9Hz, 1H, pyridine), 8.07(s, 1H, Ar), 7.57(s, 1H, azomethine), 7.43(d, *J*=8.5Hz, 1H, Ar), 7.29(t, *J*=8.5Hz, 1H, Ar) 7.27(d, *J*=1.74, 0.55Hz, 1H, Ar).^{13}C NMR (400MHz; $CDCl_3$) 163.0, 153.7, 148.2, 143.0, 139.0, 138.2, 135.4, 132.0, 131.7, 130.5, 130.0, 127.1, 125.1. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{13}H_9N_3OCl_2$, 294.1350; found 294.1450.

3.5.1.21. Nicotinic acid-(3-nitro-benzylidene)-hydrazide (**8c$_4$**)

Compound **8c$_4$** was prepared from nicotinic acid hydrazide **7c** (0.2g, 1.5mmol) and 3-nitrobenzaldehyde (0.22g, 1.5mmol), and was obtained as pale yellow coloured solid after recrystallisation from EtOH, yield 0.28g (78%). Mp.196-198^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 9.04(s, 1H, pyridine), 8.62(s, 1H, Ar), 8.55(dd, J=7.7, 1.9 Hz, 1H, pyridine), 8.23(dd, J=7.9, 1.9Hz, 1H, pyridine), 8.19(t, J=8.4Hz, 1H, Ar), 8.07(dd, J= 8.1, 2.2Hz, 1H, pyridine), 7.61(dd, J=8.4, 2.1Hz, 1H, Ar), 7.59(s, 1H, azomethine), 7.57(dd, J=8.1, 2.3Hz, 1H, pyridine).^{13}C NMR (400MHz; $CDCl_3$) 163.0, 153.7, 148.5, 147.2, 143.0, 138.2, 135.7, 134.8, 130.7, 129.8, 125.1, 124.1, 123.5. HRMS (Electrospray ionization time-of-flight, [M] +): calc. for $C_{13}H_{10}N_4O_3$, 270.2480; found 270.2580.

3.5.1.22. 2-Chloro-nicotinic acid-(4-*N*,*N*-dimethylamino-benzylidene)-hydrazide (**8c$_5$**)

Compound **8c$_5$** was prepared from 2-chloro nicotinic acid hydrazide **7c** (0.2g, 1.3mmol) and 4-*N*,*N*-dimethylaminobenzaldehyde (0.19g, 1.3mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.30g (67%). M.p.172-174^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz; $CDCl_3$): δ 8.26-8.23(dd, *J*=7.8, 2.0Hz, 1H, pyridine ring), 8.20-8.18(dt, *J*=8.8Hz, 1H, pyridine ring), 8.02(s, 1H, azomethine), 7.60(d, *J*= 8.8Hz, 2H, Ar), 7.45(d, *J*=8.8Hz, 2H, Ar), 7.15(t, *J*=8.8Hz, 1H, pyridine ring), 3.03(s, 6H, -N(CH_3)$_2$).^{13}C NMR (400MHz; $CDCl_3$) 167.0, 152.8, 151.7, 147.7, 144.9, 139.3, 133.3, 129.9, 128.9, 120.7, 15.

HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{15}H_{15}N_3OCl$, 302.7620; found 302.7730.

3.5.1.23.2-Chloro-nicotinic acid-(quinoxalin-2-yl) methylene-hydrazide (**8c$_6$**)

Compound **8c$_6$** was prepared from 2-chloro nicotinic acid hydrazide **7c** (0.2g, 1.3mmol) and quinoxalin-2-carbaldehyde (0.203g, 1.3mmol), and was obtained as buff coloured solid after recrystallisation from EtOH, yield 0.29g (80%). M.p.140-144^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR (400MHz;$CDCl_3$): δ 9.74(s, 1H, pyrazinyl-H), 8.68(dd, *J*=8.3, 2.0Hz, 1H, pyridine), 8.43(dd, *J*=8.3, 2.0Hz, 1H, pyridine), 8.18-8.15(dd, *J*=7.96, 1.84Hz, 1H, Ar), 8.10-8.07(dd, *J*=7.86, 1.9Hz, 1H),7.80(t, *J*=8.10Hz,1H, pyridine), 7.56(s, 1H, azomethine), 7.17-7.12(m, *J*=7.86, 2.67Hz, 2H).^{13}C NMR (400MHz; $CDCl_3$) 170.0, 154.8, 154.7, 148.9, 145.2, 145.0, 143.8, 143.7, 140.3, 130.3, 130.1, 130.1, 129.1, 129.0, 123.7. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{15}H_{10}N_5OCl$, 311.7290; found 311.7390.

3.5.1.24.*2*-Chloro-nicotinicacid-(4-bromo-benzylidene)-hydrazide (**8c$_7$**)

Compound **8c$_7$** was prepared from 2-chloro nicotinic acid hydrazide **7c** (0.2g, 1.3mmol) and 4-bromobenzaldehyde (0.19g, 1.3mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.30g (78%). M.p.156-158^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR(400MHz, $CDCl_3$): δ 8.56-8.53(s,1H, pyridine ring), 8.30(s, 1H, azomethine), 8.26-8.24(dd, *J*=7.8, 2.0Hz, 1H, pyridine ring), 8.20-8.18(d, *J*=8.8Hz, 2H, Ar), 7.60-7.58(d, **J**= 8.8Hz, 2H, Ar), 7.16-7.13(t, J=8.4Hz, 1H, pyridine ring).^{13}C NMR ($CDCl_3$) 167.0, 152.8, 151.7, 147.7, 144.9, 139.3, 133.3, 129.3, 129.9, 128.9, 120.7, 120.7, 117.0. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{13}H_9N_3OClBr$, 338.5850; found 338.6360.

3.5.1.25.2-Chloro-nicotinicacid-(4-nitro-benzylidene)-hydrazide (**8c$_8$**)

Compound **8c$_8$** was prepared from 2-chloro nicotinic acid hydrazide **7c** (0.2g, 1.3mmol) and 4-nitrobenzaldehyde (0.19g, 1.3mmol), and was obtained as white solid after recrystallisation from EtOH, yield 0.30g (80%). M.p.172-174^{o}C. TLC (4:1 petroleum ether/EtOAc).^{1}H NMR ($CDCl_3$): δ 8.66-8.63(s,1H, pyridine ring), 8.40(dd, *J*=7.8, 2.0Hz, 1H, pyridine ring), 8.36-

8.34(d, *J*= 8.8Hz, 2H, Ar), 8.28(d, *J*=8.8Hz, 2H, Ar), 7.98(s, 1H, azomethine), 7.16-7.13(t, *J*=8.8Hz, 1H, pyridine ring).^{13}C NMR ($CDCl_3$) 170.0, 154.8, 154.7, 150.7, 148.9, 140.3, 137.3, 130.3, 129.9, 123.7, 123.0. HRMS (Electrospray ionization time-of-flight, [M]+): calc. for $C_{13}H_9N_4O_3Cl$, 304.6900; found 304.7000.

3.5.2. Biological evaluation

3.5.2.1. Antioxidant activity

Antioxidant activity of synthesized azomethine derivatives was determined using DPPH and superoxide anion radical (SOR) scavenging assays.

3.5.2.1.1. DPPH assay

The free radical scavenging activity of the azomethine derivatives was determined using the DPPH assay as described in literature.[79] azomethine derivatives was assayed at concentrations of 1, 2, 4, 8, 16 and 32 μg/mL. Reaction mixtures containing an ethanol solution of 95 μL DPPH (300 μM) and 5 μL of synthesized compounds in DMSO were placed in a 96-well microplate and incubated at 37°C for 30 min in the dark. The absorbance of the mixture in the 96-well plate was then measured at 517 nm. Ascorbic acid was used as a positive control. Negative control contained DMSO instead of the antioxidant solution while blank contained ethanol instead of DPPH solution. The % inhibition values were calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA) and compared that of the positive control. Experiments were performed in triplicate.

3.5.2.1.2. Superoxide Radical scavenging assay

The SOR scavenging activity of the azomethine derivatives was determined according to a published method with minor modifications[80]. Synthesized azomethine derivatives were assayed at concentrations of 10, 20, 30, 40, 50, 100 μg/mL. The reaction mixture contained 10 mL of azomethine derivatives in DMSO, 90 mL of phosphate buffer (0.1 M, pH 7.4), 40 mL of β-nicotanamide adenine dinucleotide (NADH) (0.2 mM) and 40 mL of nitro blue tetrazolium (NBT) (0.08 mM). The reaction was initiated by the addition of 20 mL of phenazine methosulphate (PMS) (0.008 mM). The solutions of NADH, NBT and PMS were

prepared in phosphate buffer. The rate of NBT reduction was calculated from the differential absorbance at 560 nm (after 5 min of the addition of PMS) with respect to a blank solution in which PMS was replaced by buffer solution. Negative control contained DMSO instead of the antioxidant solution. Ascorbic acid was used as a positive control. The % inhibition values were calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA) and compared that of the positive control. Experiments were carried out in triplicate.

3.5.3. Molecular Docking against Cyclooxygenase 2 (COX-2)

The X-ray crystal structure of the enzyme *human cyclooygenase-2* (PDB ID), *E. Coli Gyrase B* (PDB 5IKR) was retrieved from protein data bank with resolution less than 3.0 Å. The protein as prepared for docking using the Graphical User Interface program AutoDock Tools (ADT). Polar hydrogens, united atom Kollman charges, solvation parameters and fragmental volumes to the proteins were assigned using the ADT GUI. The grid box of dimensions 60 × 60 × 60 in the x, y, z extents was defined around the ligand grid center 39, 2.3, 61.5 with grid spacing of 1.0 Å. The ligands were docked to the active site of the target proteins using the program AutoDock/Vina; the parameter for exhaustive search binding orientations in the grid box set to 8.0.The results less than1.0 Å in positional root-mean-square deviation (RMSD) were clustered together and represented by the result with the most favorable free energy of binding. The ligand docking pose with lowest energy were extracted and aligned with receptor for further analysis of the protein ligand interactions using PLIP.

3.5.4. Molecular Docking against enoyl ACP reductase

Molecular docking was used to clarify the binding mode of the compounds to provide straightforward information for further structural optimization. The crystal structure of enoyl acyl carrier protein reductase InhA in complex with *N*-(4-methylbenzoyl)-4-benzylpiperidine (PDB ID2NSD, 1.9 Å X-ray resolutions) was extracted from the Brookhaven Protein Database (PDB http://www.rcsb.org/pdb). The proteins were prepared for docking by adding polar hydrogen atom with Gasteiger-Huckel charges and water molecules were removed. The 3D structure of the ligands was generated by the SKETCH module implemented in the SYBYL program (Tripos Inc., St. Louis, USA) and its energy-minimized conformation was obtained with the help of the Tripos force field using Gasteiger-Huckel charges and molecular

docking was performed with Surflex-Dock program that is interfaced with Sybyl-X 2.0. and other miscellaneous parameters were assigned with the default values given by the software.

3.5.5. Protein denaturation Activity:

3.5.5.1. *In vitro* Protein denaturation activity of compounds:

3.5.5.1.1. Bovine Serum albumin method:

0.05mL of various tested compounds and standard drug Ibuprofen (100µg/mL) were taken respectively and 0.45mL of bovine serum albumin (0.5%w/V) was mixed. The samples were incubated at 37^{o}C for 20 mins and temperature was increased to keep the sample at 57 ^{o}C for 3 min. After cooling, 2.5mL phosphate buffer was added to above solutions. Absorbance was measured at 255nm. Control represents 100% protein denaturation.

$$\% \text{ inhibition} = \frac{100-[\text{Optical density of test}-\text{Optical density of control}]}{\text{Optical density of control}} X100$$

3.5.5.1.2. Egg albumin denaturation method (Protein denaturation):

The 300µl mixture consisting of 40µl of egg albumin, 260 µl of phosphate buffered saline (PBS, pH 6.4) containing varying concentrations of target compounds so that the final concentrations become 100 µl/mL and double distilled water served as negative control and acetyl salicylic acid was used as positive control. Then the mixtures were incubated at 37±2^{o}C for 15 min and then heated at 70^{o}C for 5 min. After cooling, their absorbance values were measured at 660nm (Shimadzu, UV-1800 spectrophotometer) by using vehicle as blank. The percentage inhibition of protein denaturation was calculated by using following formula:

$$\%\text{Inhibition} = 100x \frac{(\text{Absorbance of control}-\text{Absorbance of sample})}{\text{Absorbance of control}}$$

3.5.5.1.2.1a. Human Red Blood Cell membrane stabilization method:

3.5.5.1.2.1a.1. Collection of blood sample:

Fresh whole blood sample (5mL) was collected from healthy male human volunteers without history of oral contraceptive or anticoagulant therapy or any anti-inflammatory therapy. The collected blood was centrifuged 3000rpm for 10 min and was washed with equal

amount of normal saline and repeated the procedure two times. RBCs were kept in a test tube with an anti-coagulant EDTA under standard conditions of temperature 23±2^{o}C and relative humidity 55±10%

3.5.5.1.2.1a.2. Assay of membrane stabilization Erythrocyte suspension:

The volume of RBC saline solution was reconstituted as a 10% (v/v) suspension with isotonic buffer solution (pH 7.4) which contained in 1L of distilled water: $NaH_{24}.PO_2OH$, 0.26g; $NaHPO_{42,}$ 1.15g; NaCl, 9g (10mM sodium phosphate buffer).

3.5.5.1.2.1a.3. Heat-induced hemolysis:

Aliquots (5ml) of the isotonic buffer, containing 100µg/mL of synthesized compounds were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µl) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54^{o}C for 20 min in water bath. The other pair was maintained at 0-5^{o}C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300rpm and absorbance of the supernatant was measured at 540nm using UV-VIS spectrometer (SHIMADZU-UV-1800). The percentage inhibition or acceleration of hemolysis in tested and was calculated using equation:

% Inhibition of Hemolysis= 100x1-[OD_2-OD_1/ OD_3-OD_1], where, OD_1= test sample unheated, OD_2= test sample heated and OD_3= Control sample heated.

3.5.5.2. Cyclooxygenase (COX)-I/II Inhibition Assay

The *in vitro* ability of the test compounds to inhibit COX-I/II was determined using a Cayman colorimetric COX-inhibitor screening assay kit (catalog number 701080) following the manufacturer's instructions. Briefly, a mixture consisting of 160 µl the assay buffer solutions, 10 µl hemin and, 10 µl diluted COX-I/II enzyme was added to the wells which corresponds to the 100% initial activity wells. A mixture of assay buffer and hemin was taken as background. The different concentration (100, 50, 25, 12.5, 6.25 µg/ml) of the test compounds were made and were added to each wells except the background and the 100% intial activity well. Wells were incubated at 37ºC for 10 min. Quickly, after incubation, 10 µl arachidonic acid was added and mixed well with pipette up and down multiple time and incubated exactly for 2 minutes at

37ºC. The catalysis of the enzyme COX-I/II was stopped by the adding 30 µl of saturated stannous chloride solution to each well. Wells were removed from water bath and incubated at room temperature. Then add 10 µl of prostaglandin (PG) screening AChE tracer was added to each well except 100% initial activity and blank well. After this, 50 µl of PG screening elisa antiserum was added. The plate was kept for overnight incubation. The plate was washed 3 times with buffer to remove unbound reagent and OD was recorded at 405 nm using microplate reader immediately. Then, wells were developed with 200 µL Ellaman's reagent followed by addition of 5µl of PG screening AChE tracer to inhibitor wells. It was incubated for 60 minutes at room temperature in the dark. The OD was measured at 450 nm using microplate reader immediately.

Percent Inhibition (%) = (Activity of Control – Activity of Test) / Activity of Control x 100

The IC_{50} of compounds were calculated using graph Pad Prism Version 5.1, by taking a percentage of inhibition vs COX-II at five different concentrations of treatment.

3.5.5.3. *In-vivo* anti-inflammatory screening

3.5.5.3.1. Experimental animal maintenance

Wistar albino male rats (*Rattus norvegicus*) (180 ± 20 g) were procured from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India) registered animal vendor (M/s National institute of Biosciences, Pune, India) after the approval of the experimental protocols by the Institutional Animal Ethical Committee (IAEC, BiRD) (Registration number 2114/PO/Re/S/20/CPCSEA; IAEC protocol number IAEC/BiRD/2021/03). The animals were housed in the departmental animal house facility at 24 ± 2 °C with a 12 h/12 h day/night cycle and were fed with standard pellet and water was provided ad libitum. All animals were acclimatized for a period of at least 10 days before the initiation of the experiments.

3.5.5.3.2. Measurement of *in-vivo* anti-inflammatory activity

Compounds **$8a_1$**, **$8a_2$, $8a_3$, $8a_4$, $8a_{10}$, $8a_{11}$, $8b_6$,** and **$8b_7$** were screened for their *in vivo* anti-inflammatory activity by the carrageenan-induced paw edema standard method.[93] Mature

wistar male albino rats were obtained from Biocyte institute of Research and Development, Sangli, weighing 150–200 g. Edema was induced in the left hind paw of all rats by subcutaneous injection of 0.1 mL of 1% (w/v) carrageenan in distilled water into their footpads. The carrageenan-induced paw edema model is suitable test for evaluating anti-inflammatory drugs which has frequently been used to access the antedematous effect of the drug. The reduction in the carrageenan induced inflammation was used as a measure of their anti-inflammatory activity. Carrageenan is a strong chemical used for the release of inflammatory and pro-inflammatory mediators. The course of inflammation is biphasic. Rats were divided into six groups of six rats each. The first group was kept as control, and was given the respective volume of the solvent (1% of tween-80 in distilled water). The other groups were orally administered the drugs Diclofenac sodium, ibuprofen at dose of 100mg/kg (reference standards) and the tested compounds suspended in water contain 1% methyl cellulose in dose of 100mg/kg, 1 h before carrageenan injection. The paw volume of each rat was measured using Vernier caliper; before carrageenan injection and then hourly for 4 h post-administration of the drugs. The initial paw volumes were measured by using digital vernier caliper which served as control. The percent inhibition of paw edema was calculated according to following formula: % inhibition= (a-b)/a x100; where 'a' is mean paw inflammation volume of control and 'b' is the mean paw inflammation volume of test. For statistical comparison, the fourth hour reading of percentage in the paw volume inhibition was measured. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Turkey's multiple comparison test; $p<0.05$ was taken to be statistically significant.

3.5.6. *In vitro* anti-tubercular activity:

The anti-mycobacterial activity of compounds were assessed against *M. tuberculosis* using micro plate Alamar Blue assay (MABA). Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds was made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well

was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

3.5.7. Cytotoxicity study

The *in-vitro* cytotoxicity activity of selective compounds **$8a_1$, $8a_2$, $8a_{10}$, $8a_{11}$, $8b_2$, $8b_5$, $8c_5$** and **$8c_6$** was examined by MTT-assay[95] against three different cell lines namely, HeLa, MCF-7, HEK-293 using Paclitaxel as standard. Initially, the cells were seeded in 96 well flat-bottom micro plate comprising Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% heat inactivated fatal bovine serum and 1% antibiotic-antimycotic 100X solution. The cells were then maintained overnight at 37ºC in 95% humidity and 5% CO_2 and were seeded in 96 well. Different concentration (400, 200,100, 50, 25, 12.5, 6.25, µg/mL) of compounds were treated and the cells were incubated for another 48 hours before adding MTT. The wells were washed twice with phosphate buffer saline (PBS) and 20 µL of the MTT staining solution was added to each well and plate was incubated at 37ºC. After 4h, 100 µL of DMSO was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using micro plate reader. Using graph Pad Prism Version5.1, we calculated the IC_{50} of compounds.

Formula:

Surviving cells (%) = Mean OD of test compound /Mean OD of Negative control ×100

3.5.8. Apoptosis studies by flow cytometry

The cells were seeded with 24-well bottom micro plate with coverslips, maintained at 37ºC in CO_2 incubator for overnight. Further, the cells are treated with 20µg/ml of each target compound at 24h. After incubation, cells were washed with phosphate buffer saline and centrifuged for 5 minutes at 500 x g at 4^oC. The supernatant solution was discarded, and cells were resuspended in ice-cold 1X Binding buffer to 1x105 per mL. The tubes were kept on ice and 1µL of annexin V-FITC solution and 5µL PI was added. It was mixed gently and incubated for 15 minutes in the dark. This was followed by addition of 400 µL ice-cold 1X Binding buffer and was mixed gently. The cell preparation was then analysed by flow cytometry within 30 minutes.

3.6. REFERENCES

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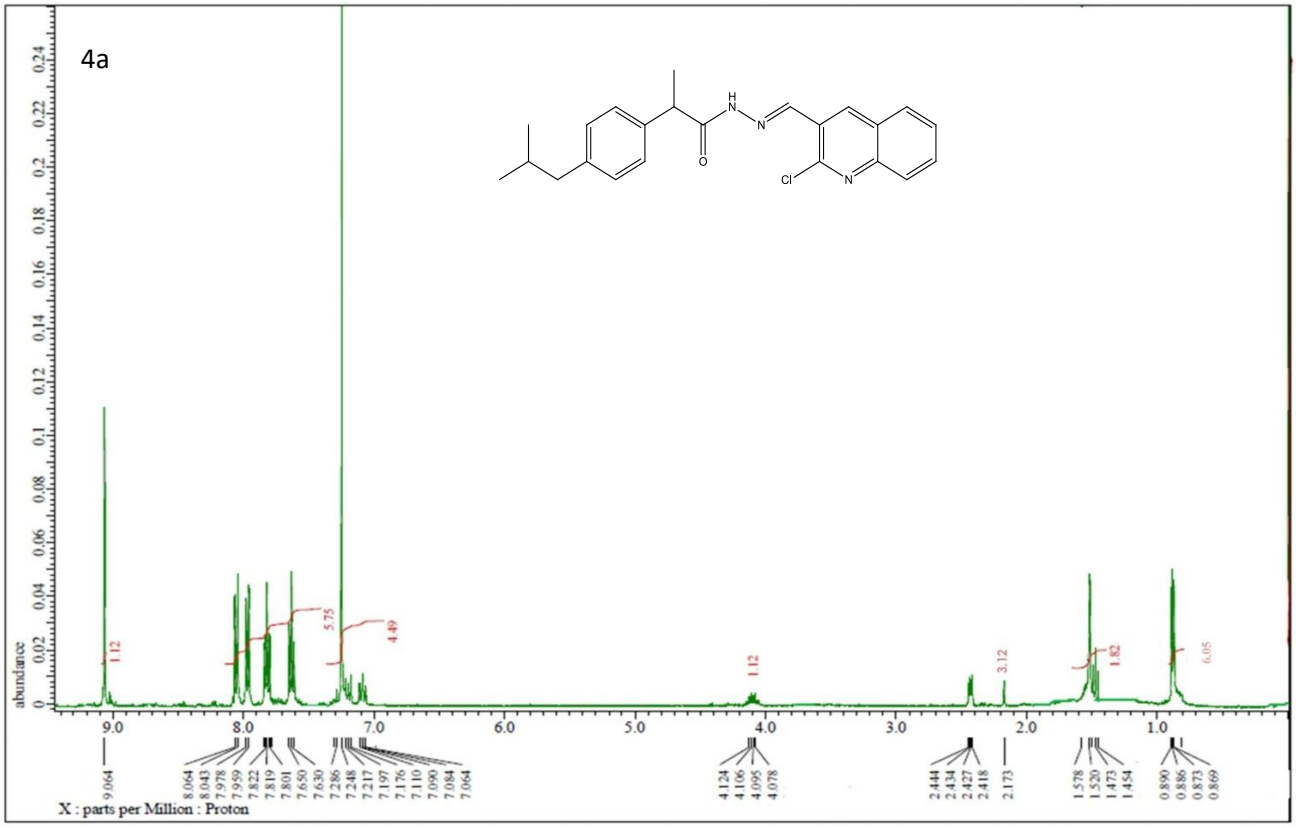

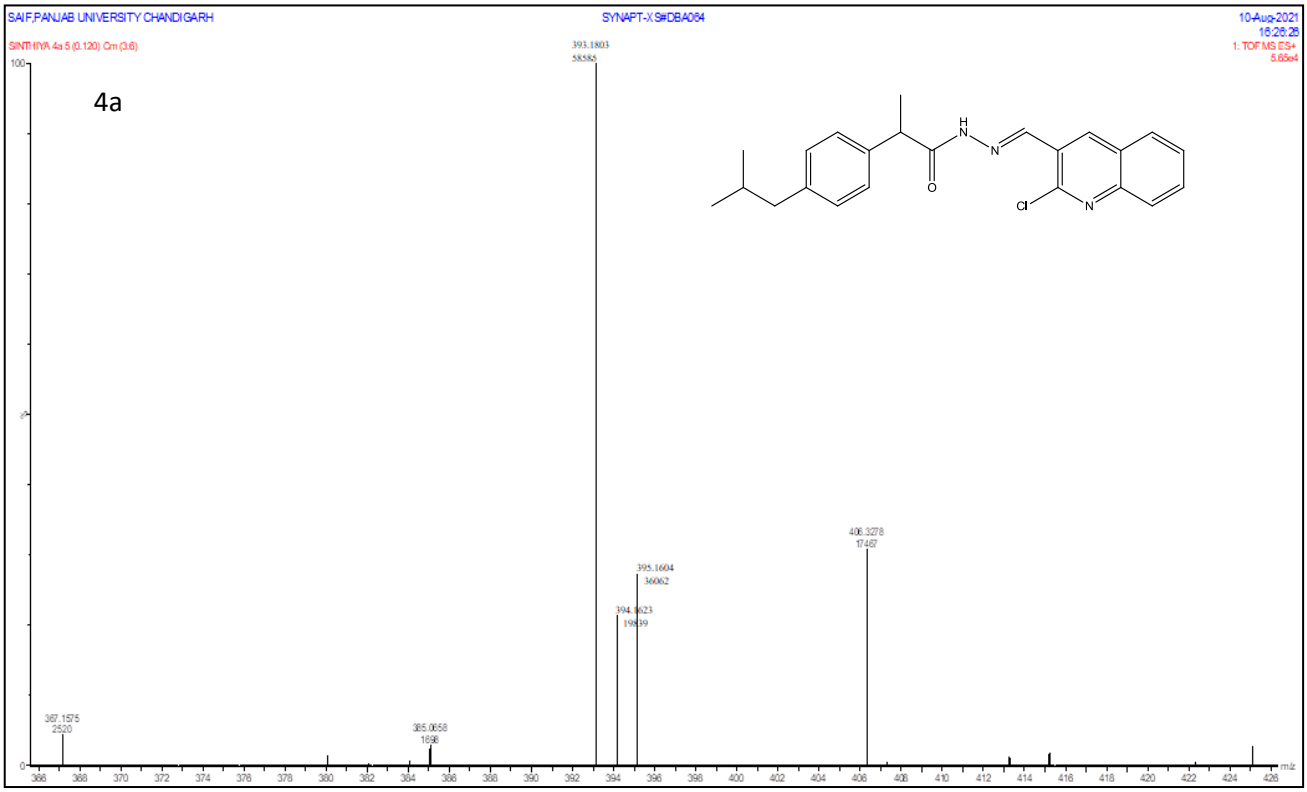

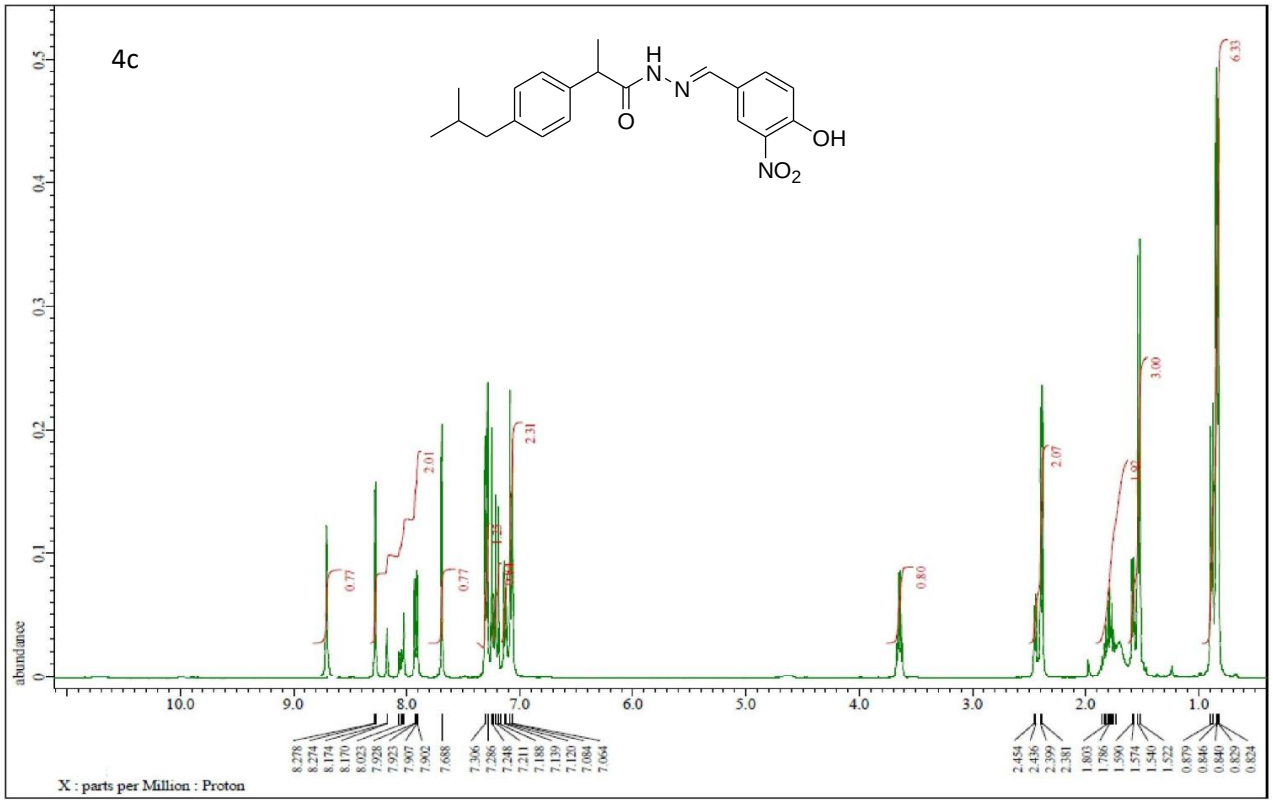
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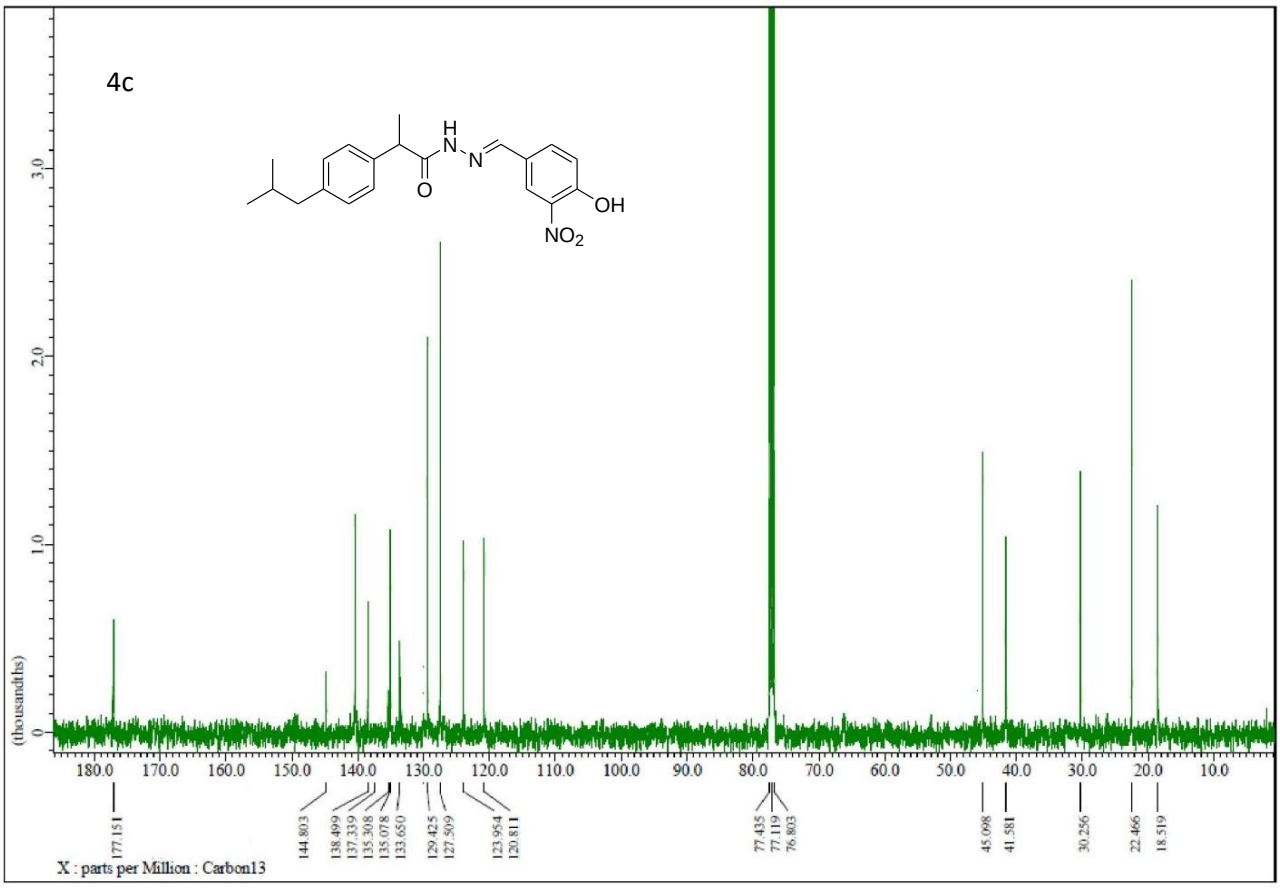
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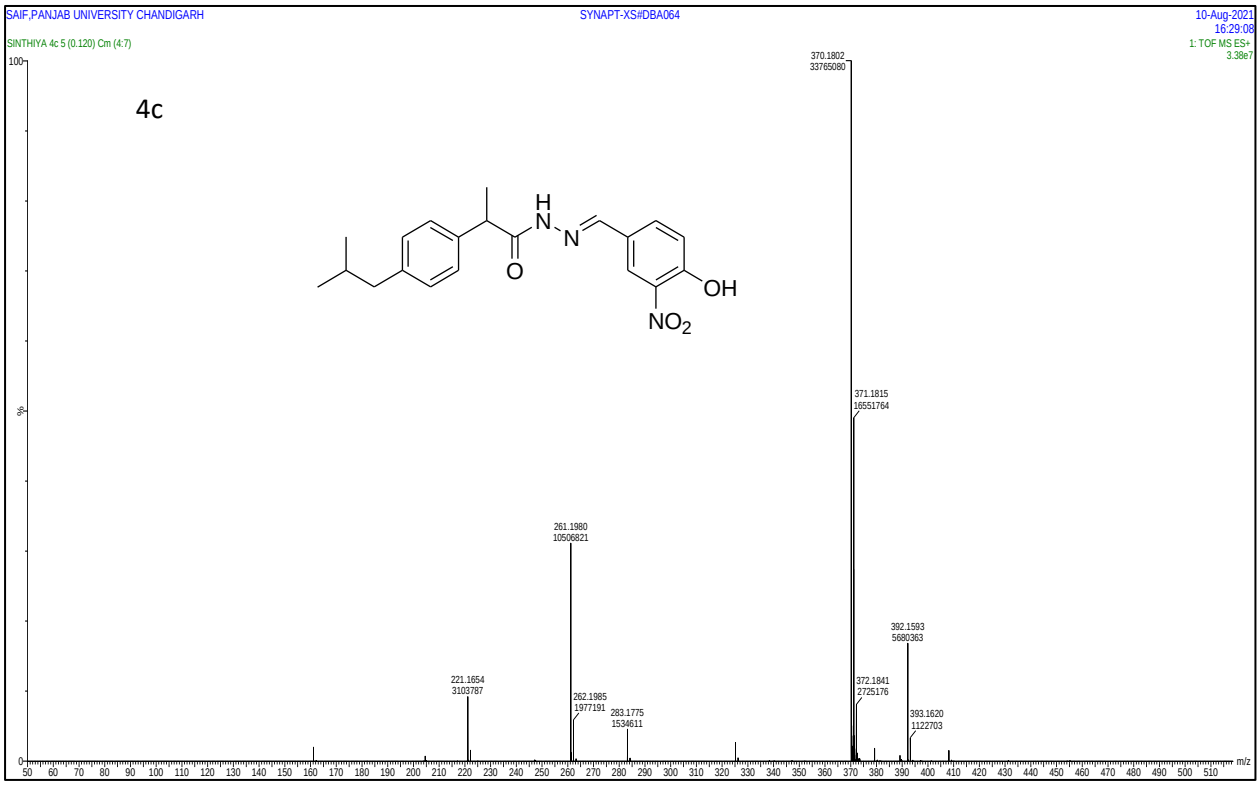
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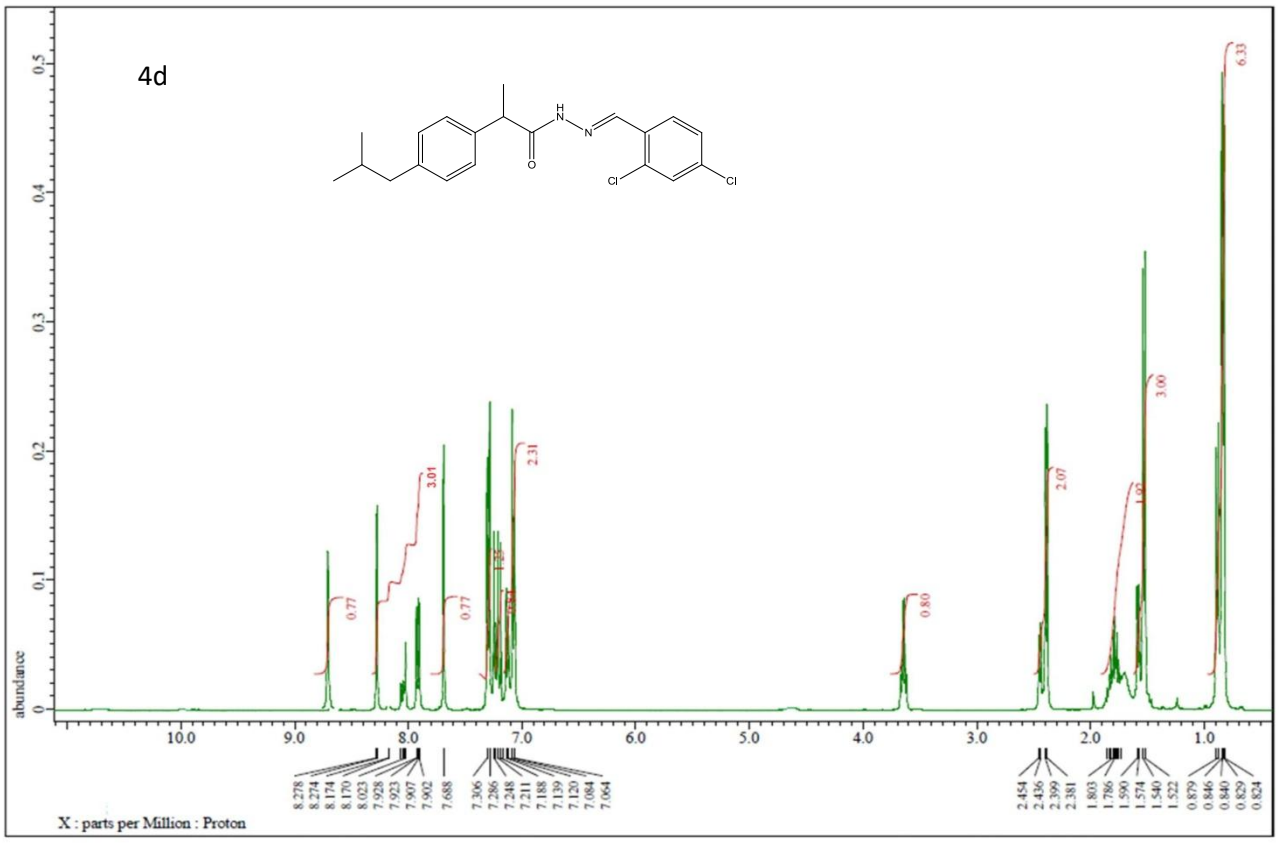
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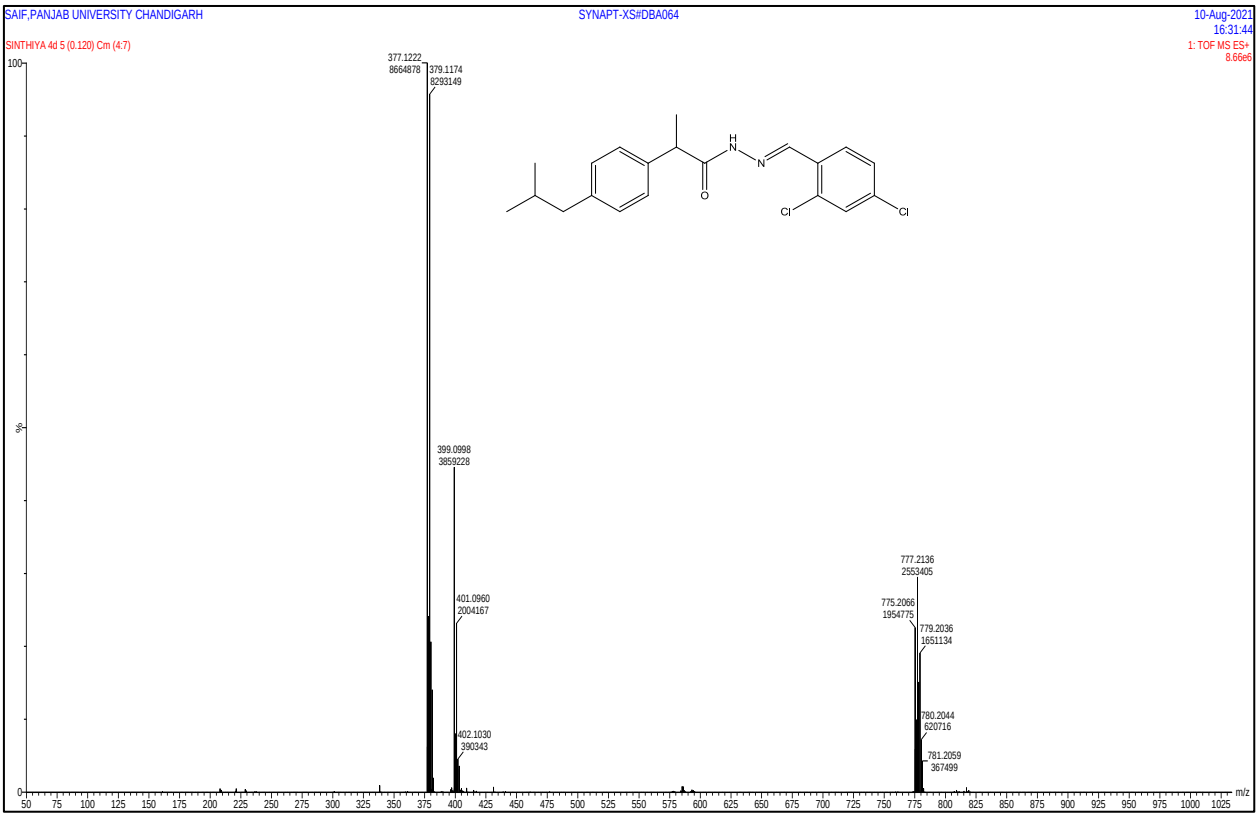
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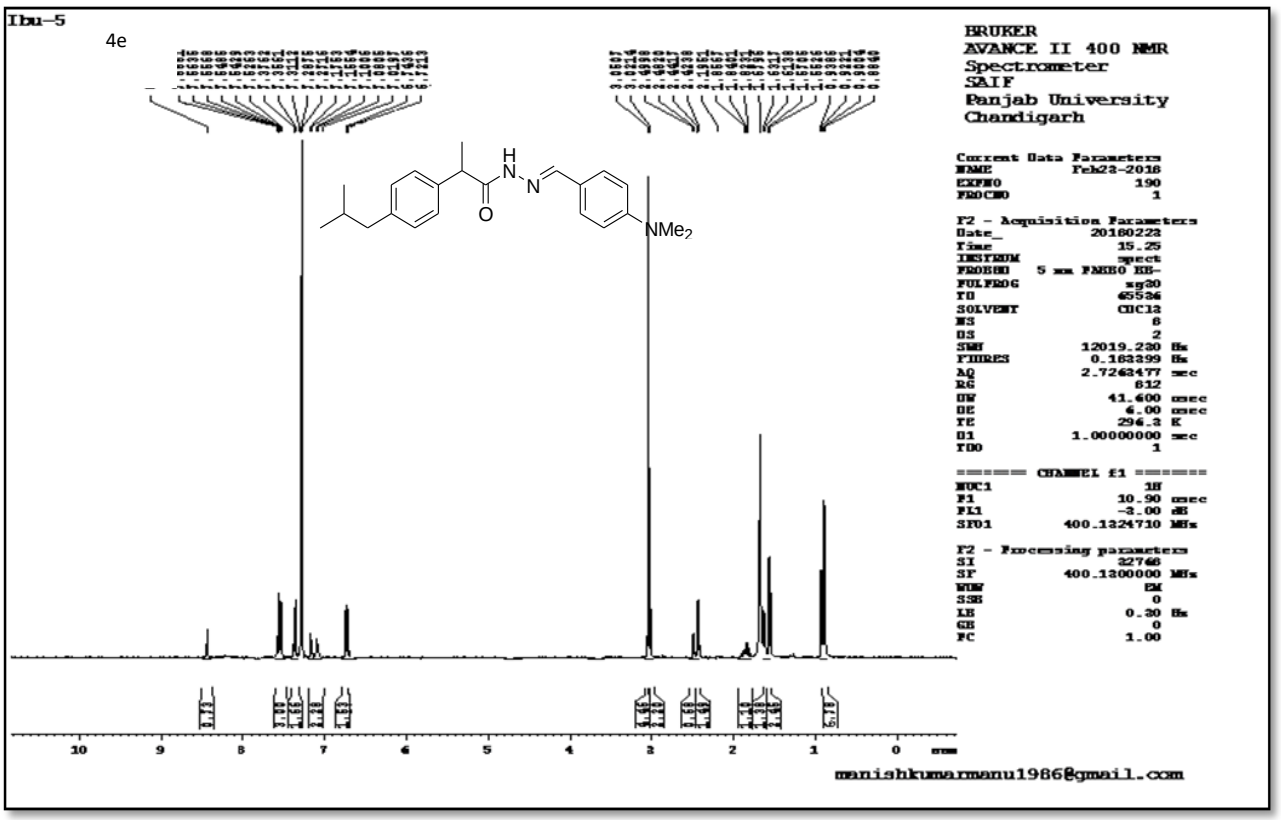
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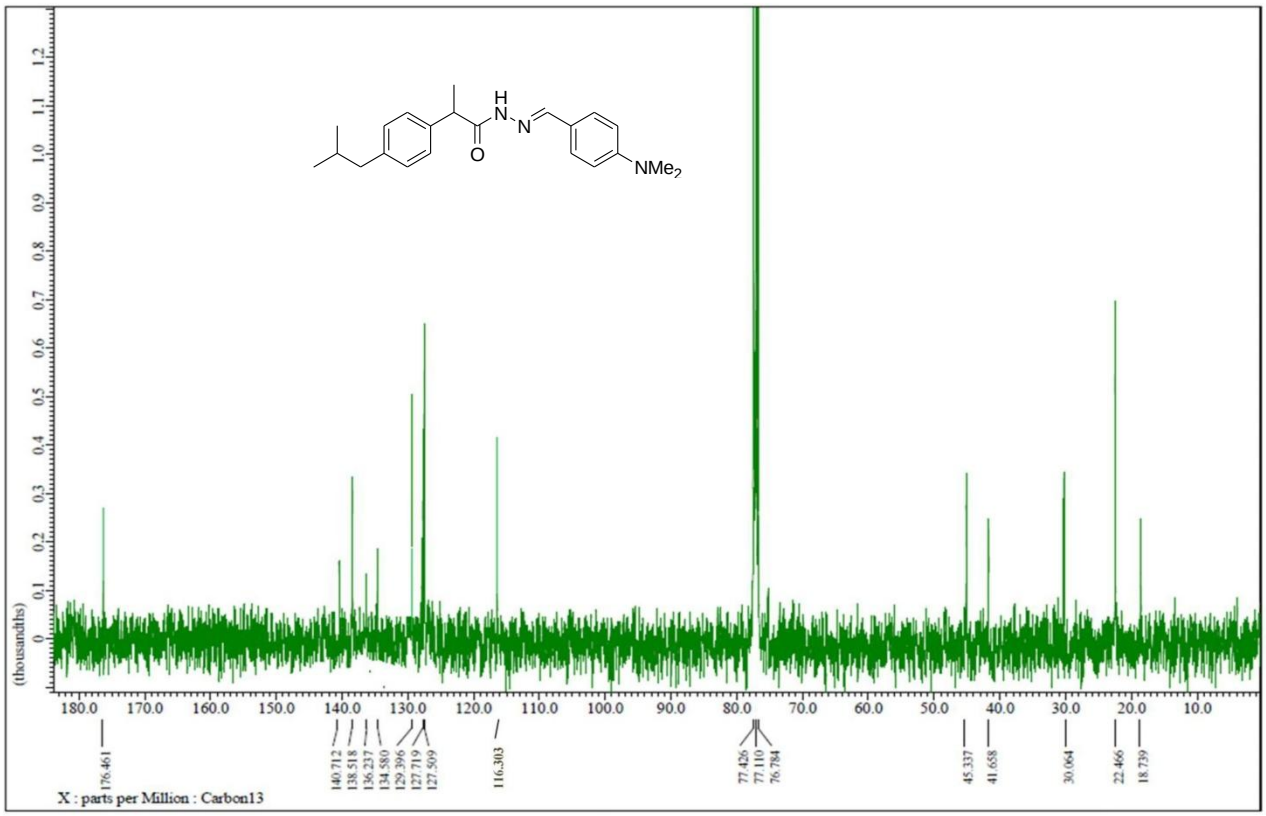
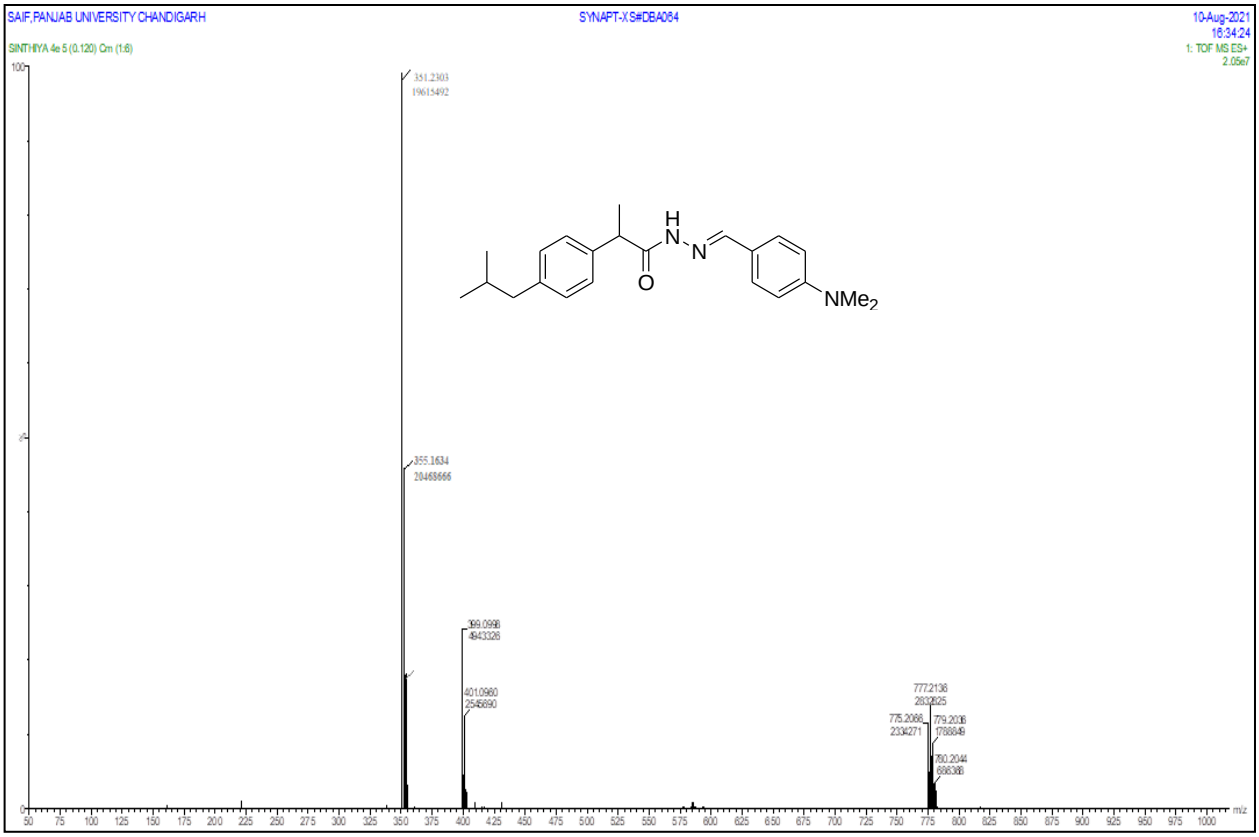
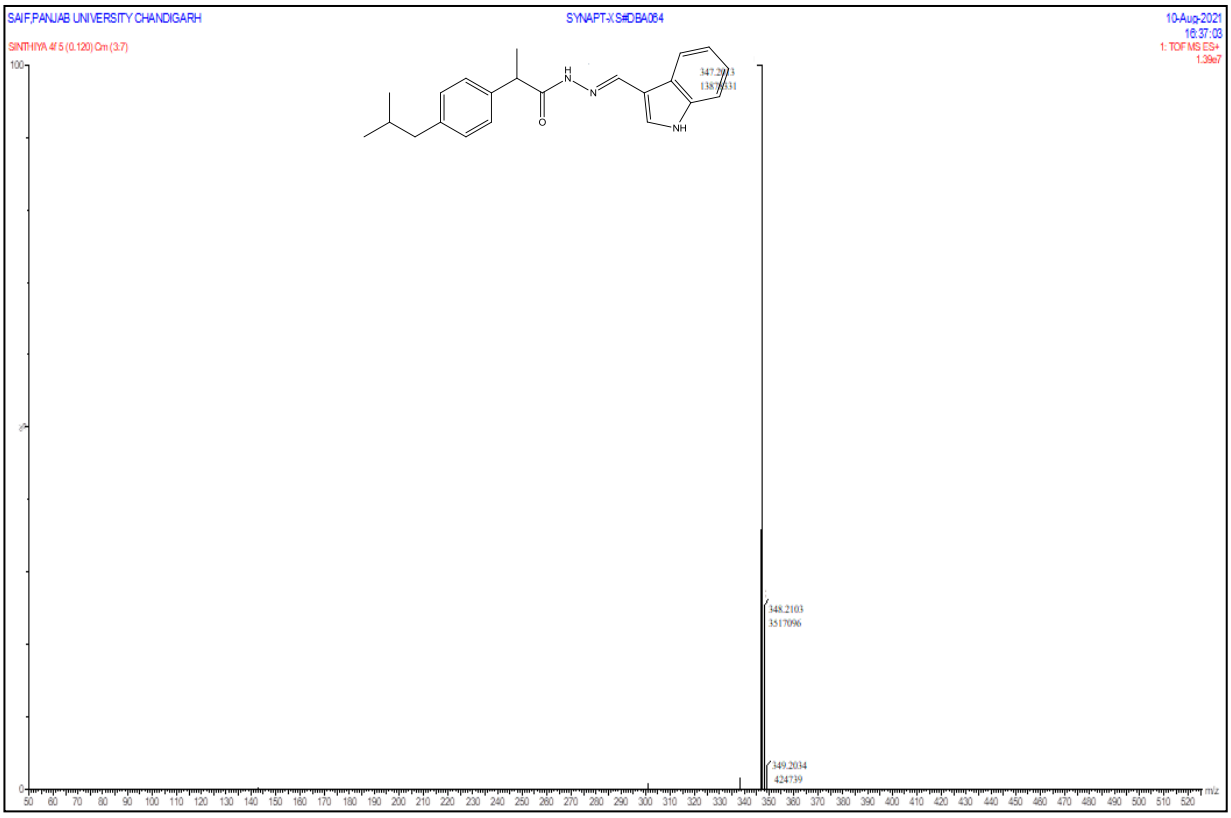
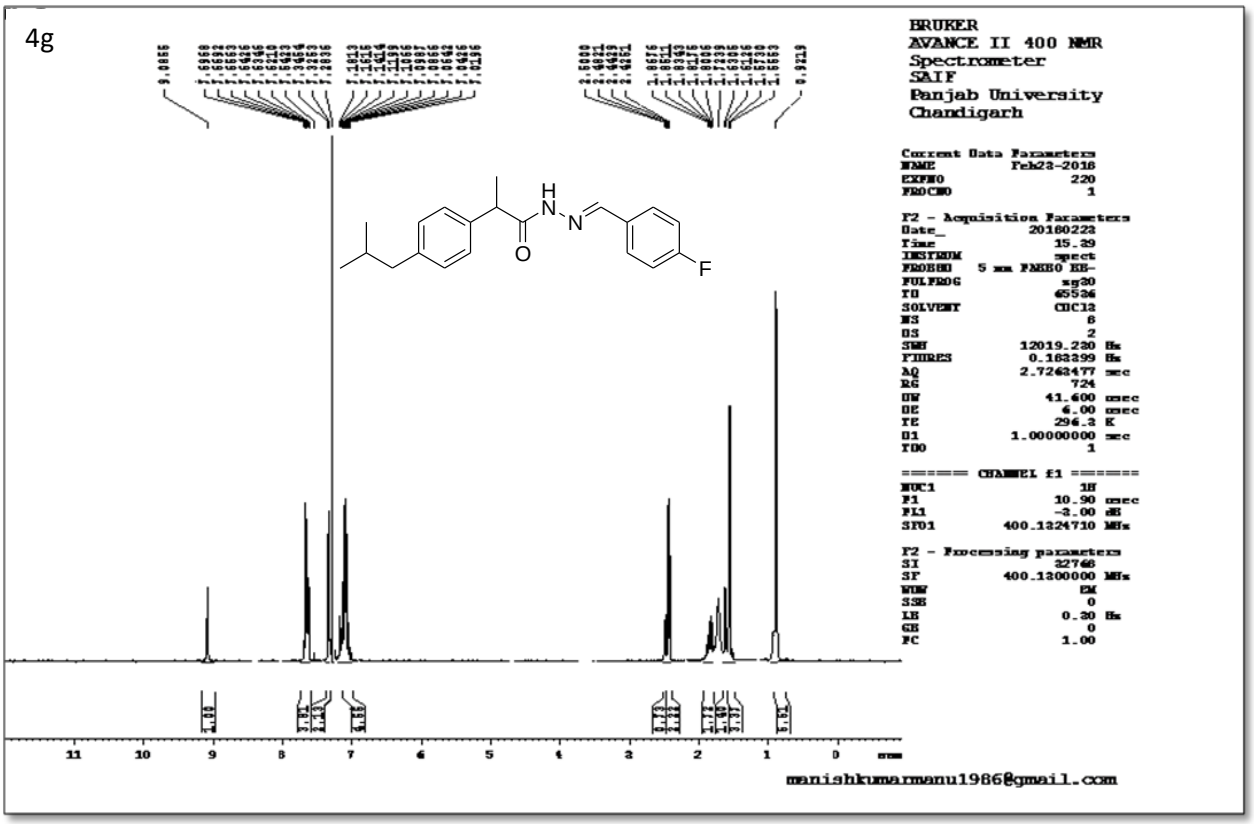
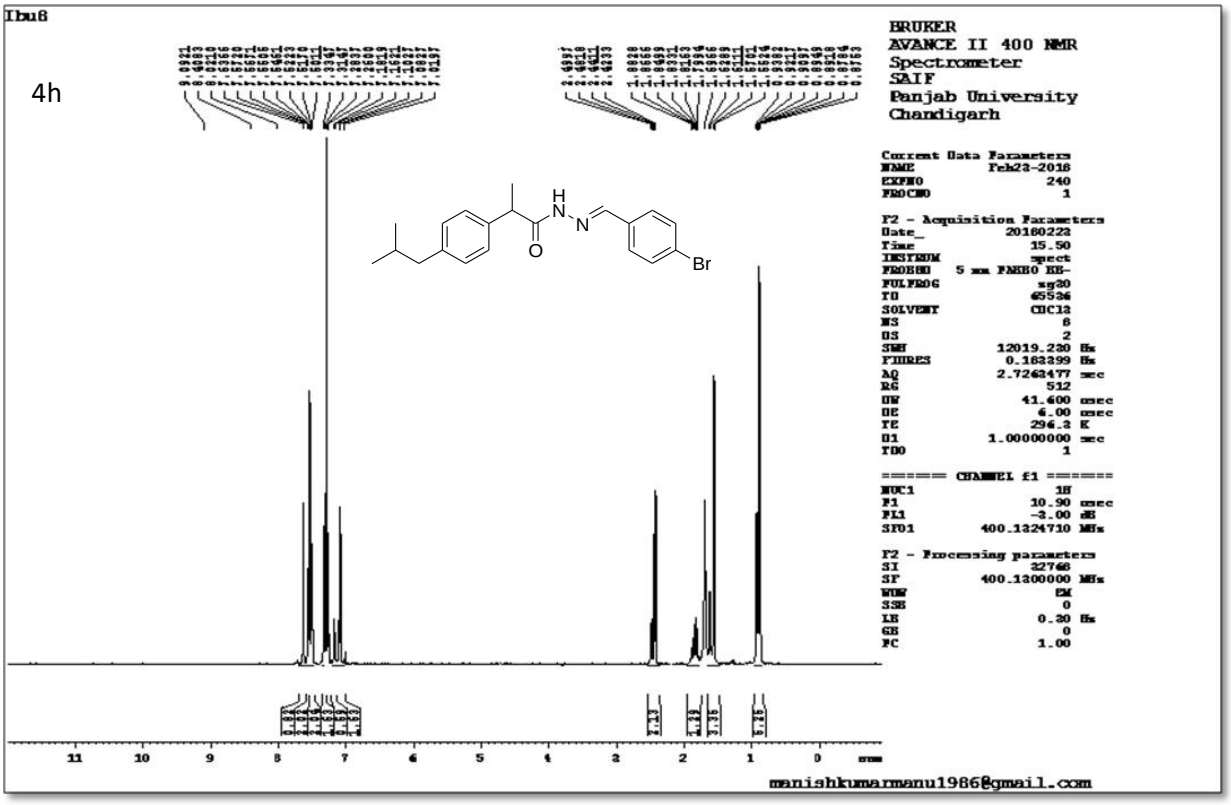
DW 41.600 usec
DE 6.00 usec
TE 294.3 K
D1 1.00000000 sec
TD0 1
====== CHANNEL f1 ======
NUC1 1H
P1 10.90 usec
PL1 -3.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
manishkumarmanu1986@gmail.com

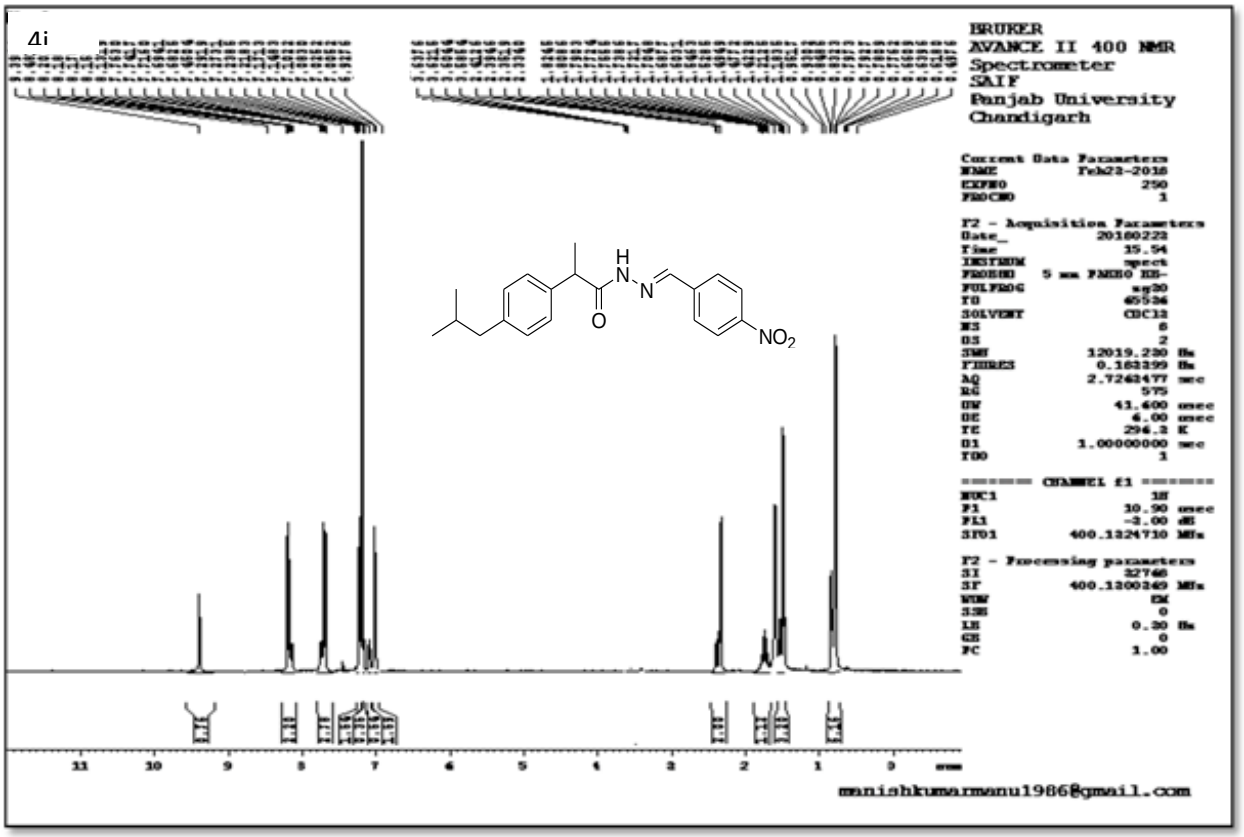

(thousandths)
X : parts per Million : Carbon13
176.461
140.712
138.518
136.237
134.580
129.396
127.719
127.509
116.303
77.426
77.110
76.784
45.337
41.658
30.064
22.466
18.739
NMe2

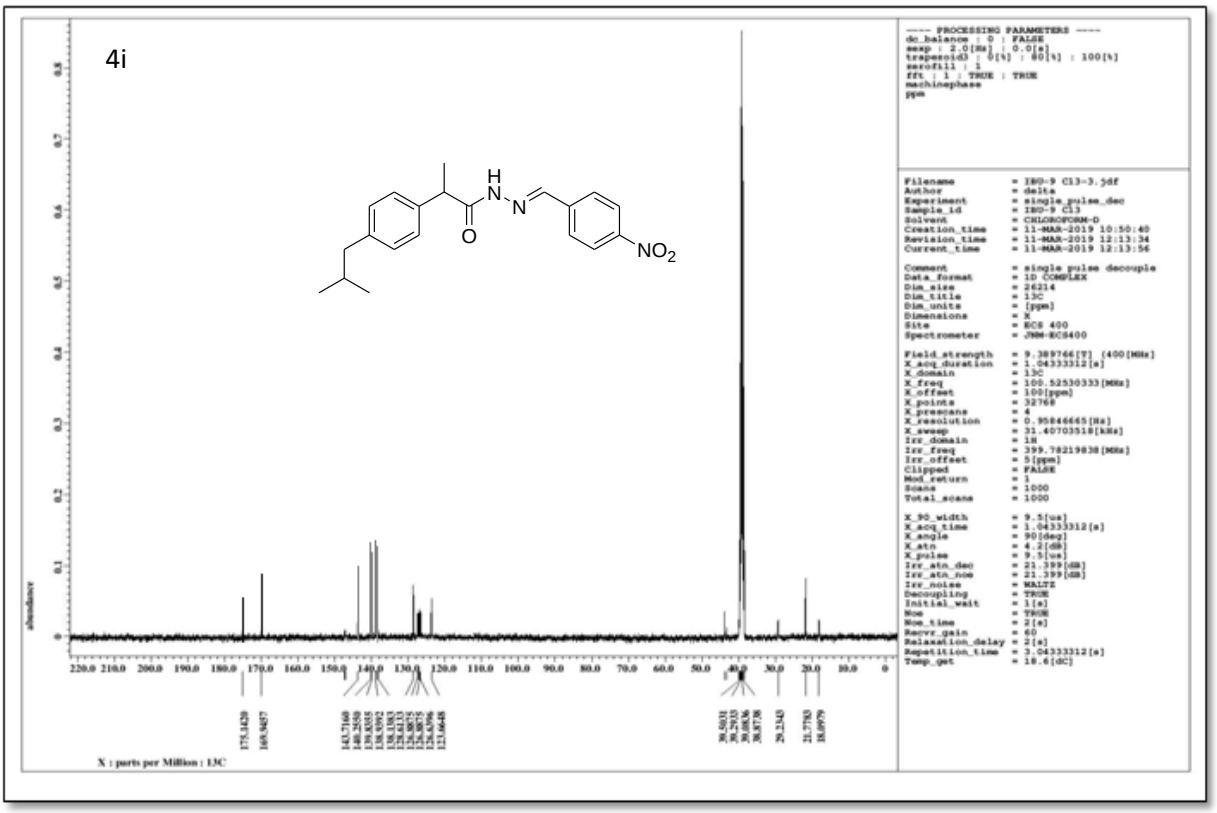

SAIF,PANJAB UNIVERSITY CHANDIGARH
SYNAPT-XS#DBA084
10-Aug-2021
16:34:24
SINTHIYA 4e 5 (0.120) Cm (1:6)
1: TOF MS ES+
2.05e7
351.2303
19615492
355.1634
20468666
399.0998
4943326
401.0960
2545690
777.2136
2602825
775.2088
2334271
779.2036
1788849
780.2044
686368
NMe2

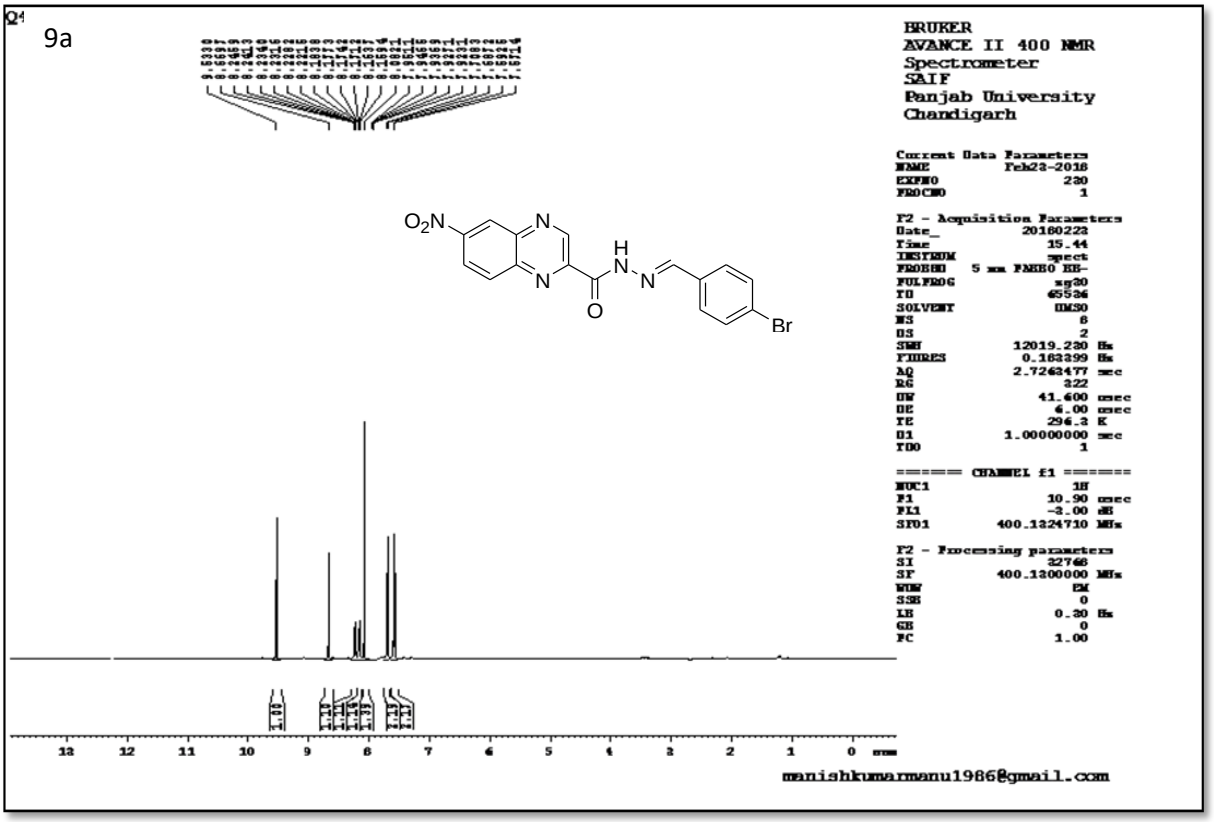

SAIF,PANJAB UNIVERSITY CHANDIGARH
SYNAPT-XS#DBA084
10-Aug-2021
16:37:03
SINTHIYA 4f 5 (0.120) Cm (3:7)
1: TOF MS ES+
1.39e7
348.2103
1517096
349.2034
424739
NH
O
N
H

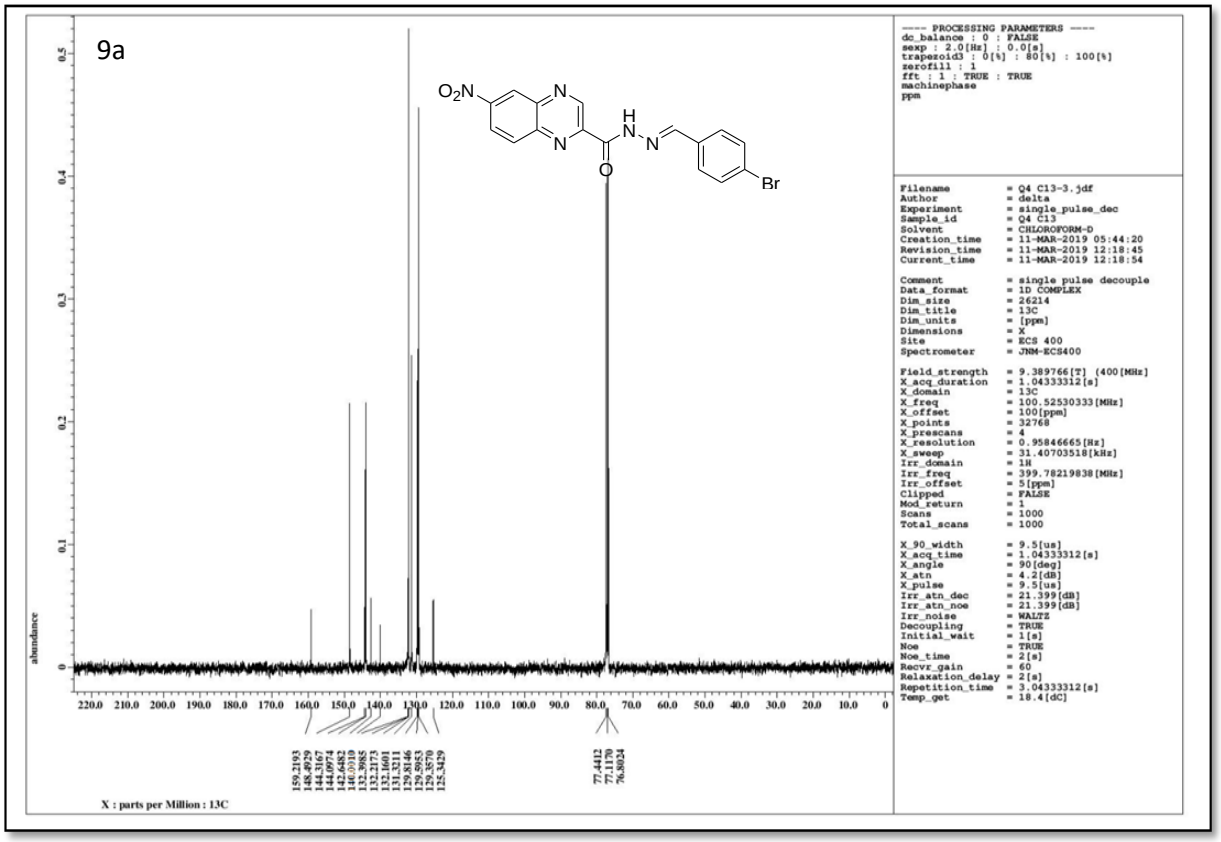

4g
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
Current Data Parameters
NAME Feb23-2016
EXPNO 220
PROCNO 1
F2 - Acquisition Parameters
Date_ 20160223
Time 15.39
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 12019.230 Hz
FIDRES 0.183399 Hz
AQ 2.7263477 sec
RG 724
DW 41.600 usec
DE 6.00 usec
TE 296.3 K
D1 1.00000000 sec
TD0 1
====== CHANNEL f1 ======
NUC1 1H
P1 10.90 usec
PL1 -3.00 dB
SFO1 400.1324710 MHz
F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
F
O
N
H
manishkumarmanu19868@gmail.com

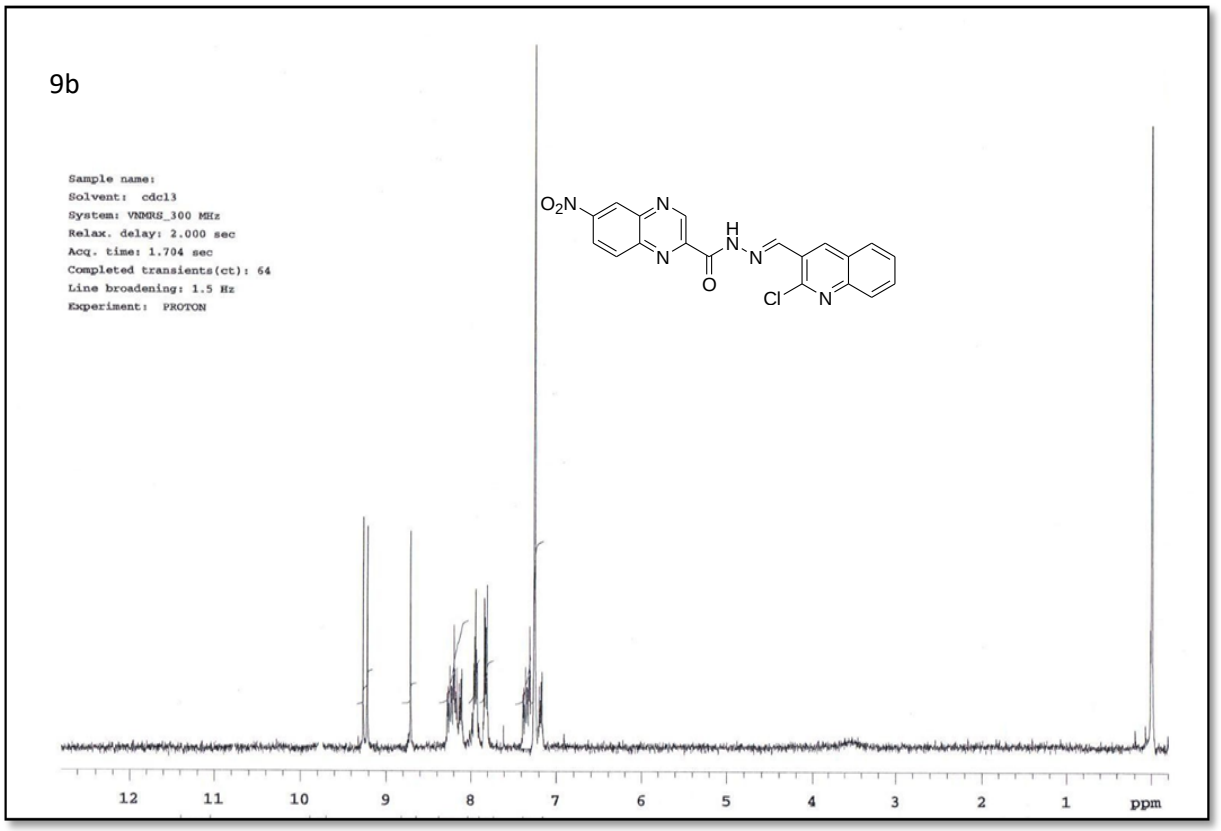

Ibu8
4h
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
Br
manishkumarmanu1986@gmail.com

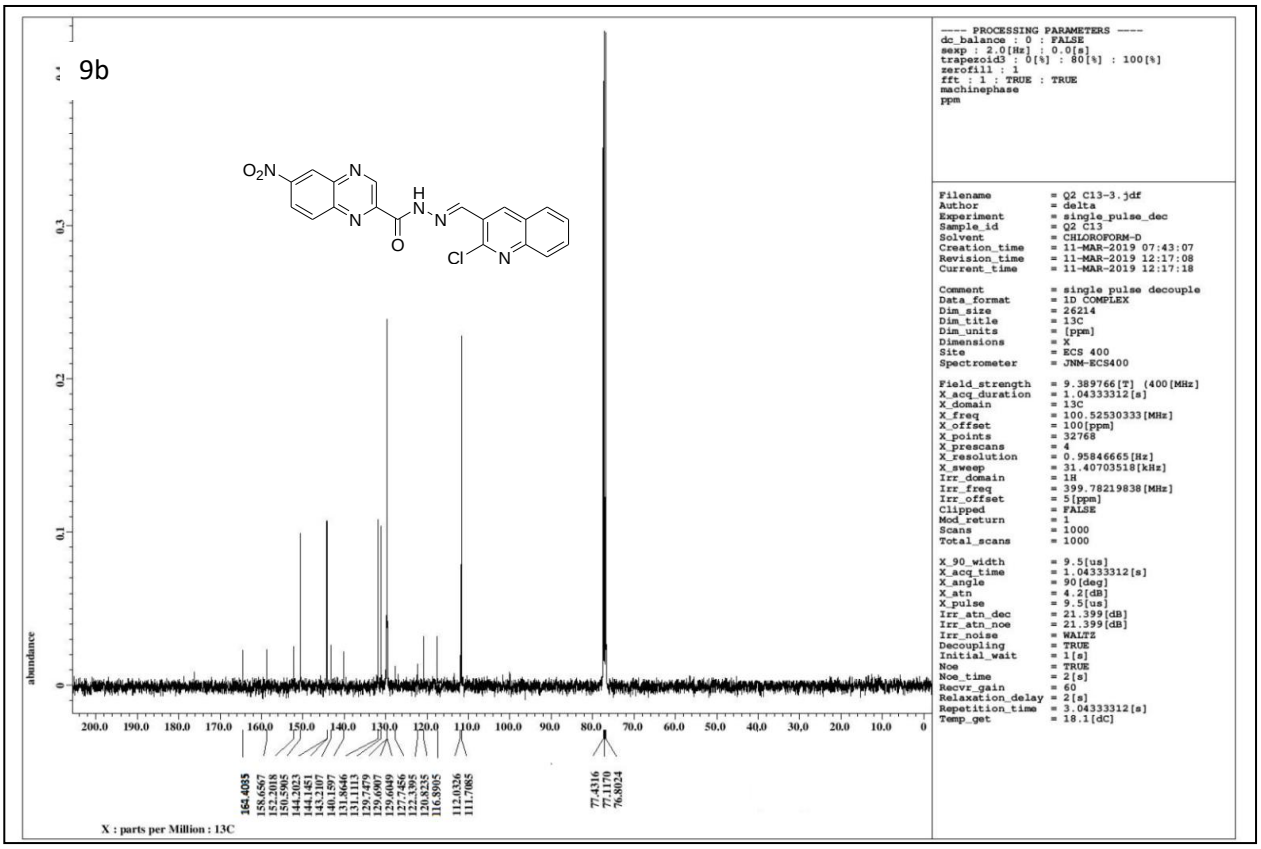

4i
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
NO2
manishkumarmanu1986@gmail.com

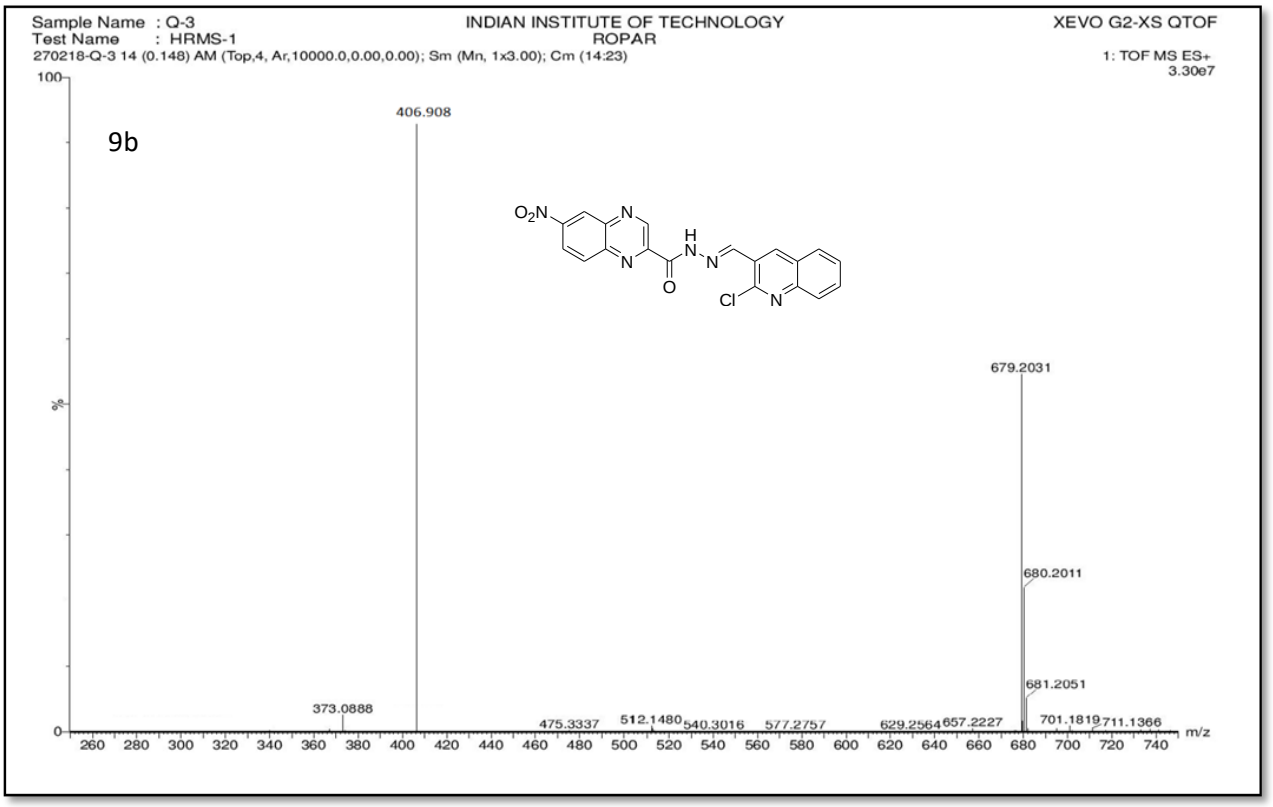

4i
NO2
X : parts per Million : 13C

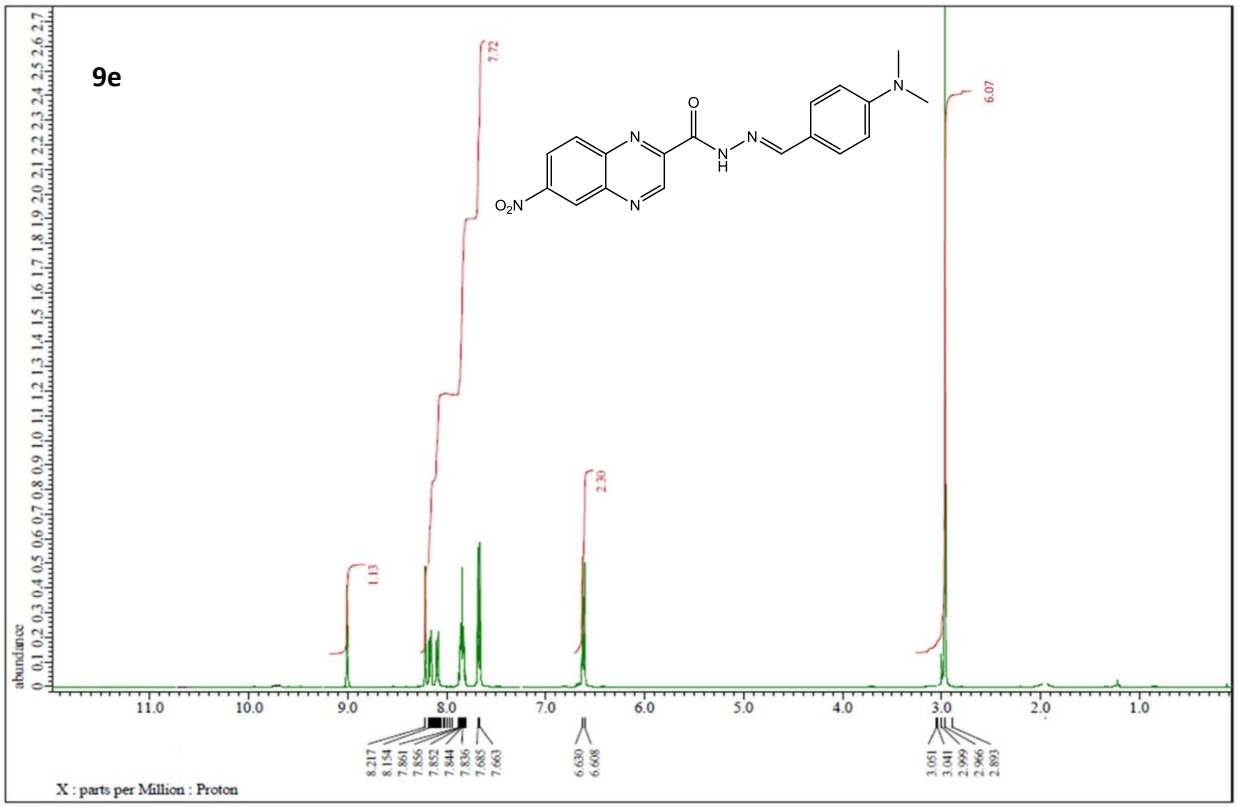

9a
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
O2N
Br
manishkumarmanu1986@gmail.com

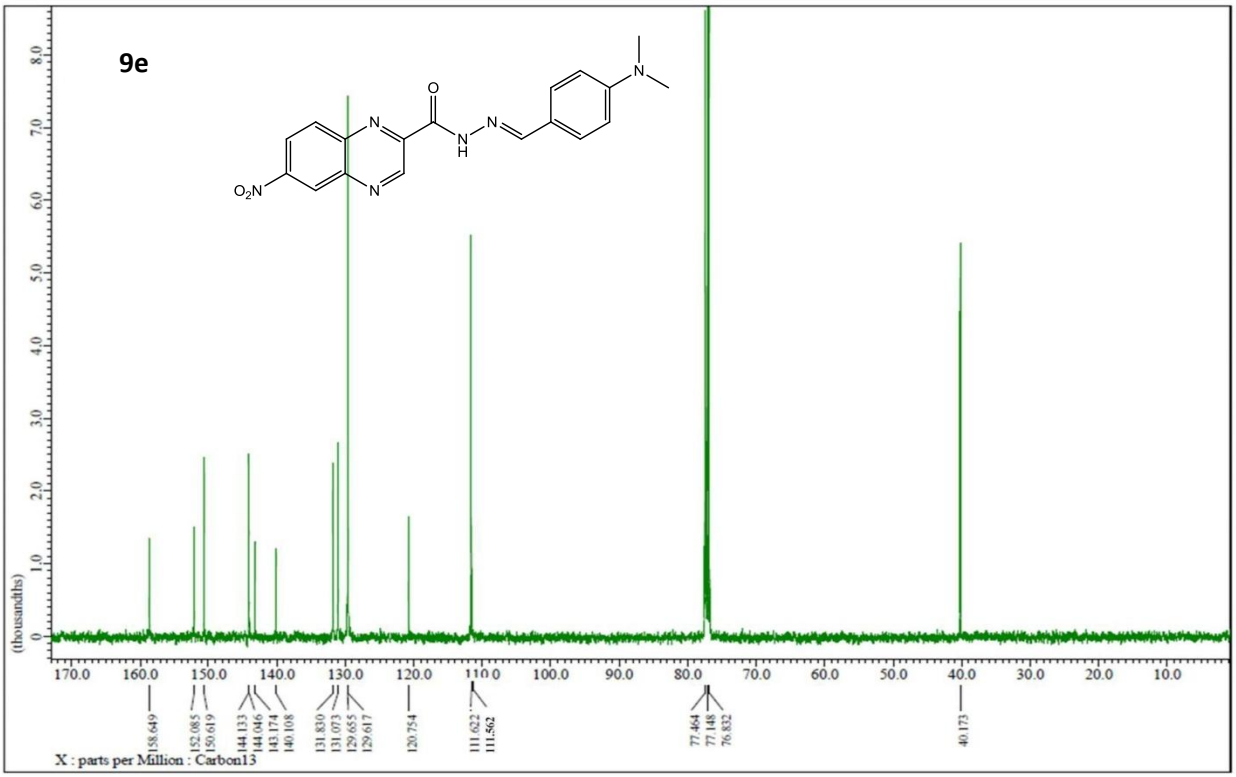

9a
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp : 2.0[Hz] : 0.0[s]
trapezoid3 : 0[%] : 80[%] : 100[%]
zerofill : 1
fft : 1 : TRUE : TRUE
machinephase
ppm
Filename = Q4 C13-3.jdf
Author = delta
Experiment = single_pulse_dec
Sample_id = Q4 C13
Solvent = CHLOROFORM-D
Creation_time = 11-MAR-2019 05:44:20
Revision_time = 11-MAR-2019 12:18:45
Current_time = 11-MAR-2019 12:18:54
Comment = single pulse decouple
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECS 400
Spectrometer = JNM-ECS400
Field_strength = 9.389766[T] (400[MHz]
X_acq_duration = 1.04333312[s]
X_domain = 13C
X_freq = 100.52530333[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.95846665[Hz]
X_sweep = 31.40703518[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 1000
Total_scans = 1000
X_90_width = 9.5[us]
X_acq_time = 1.04333312[s]
X_angle = 90[deg]
X_atn = 4.2[dB]
X_pulse = 9.5[us]
Irr_atn_dec = 21.399[dB]
Irr_atn_noe = 21.399[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 3.04333312[s]
Temp_get = 18.4[dC]
abundance
X : parts per Million : 13C

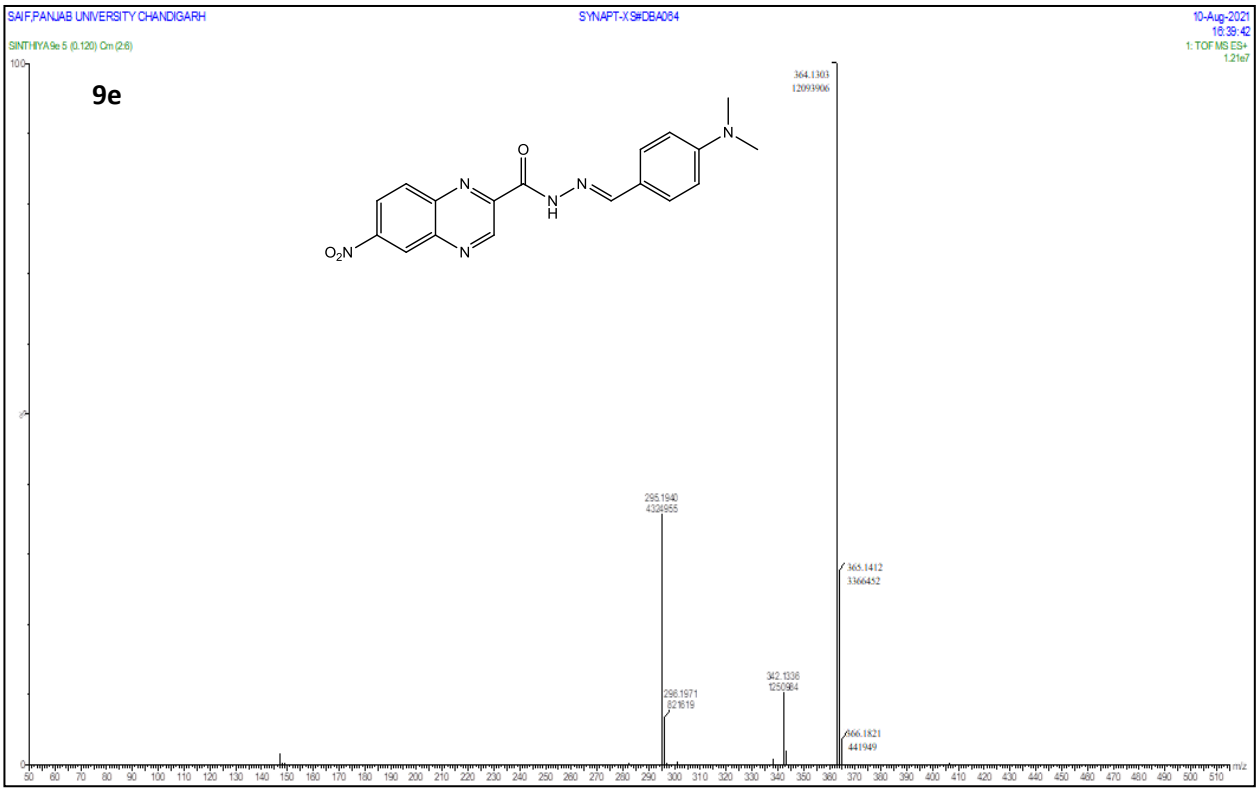

9b
Sample name:
Solvent: cdcl3
System: VNMRS_300 MHz
Relax. delay: 2.000 sec
Acq. time: 1.704 sec
Completed transients(ct): 64
Line broadening: 1.5 Hz
Experiment: PROTON
ppm

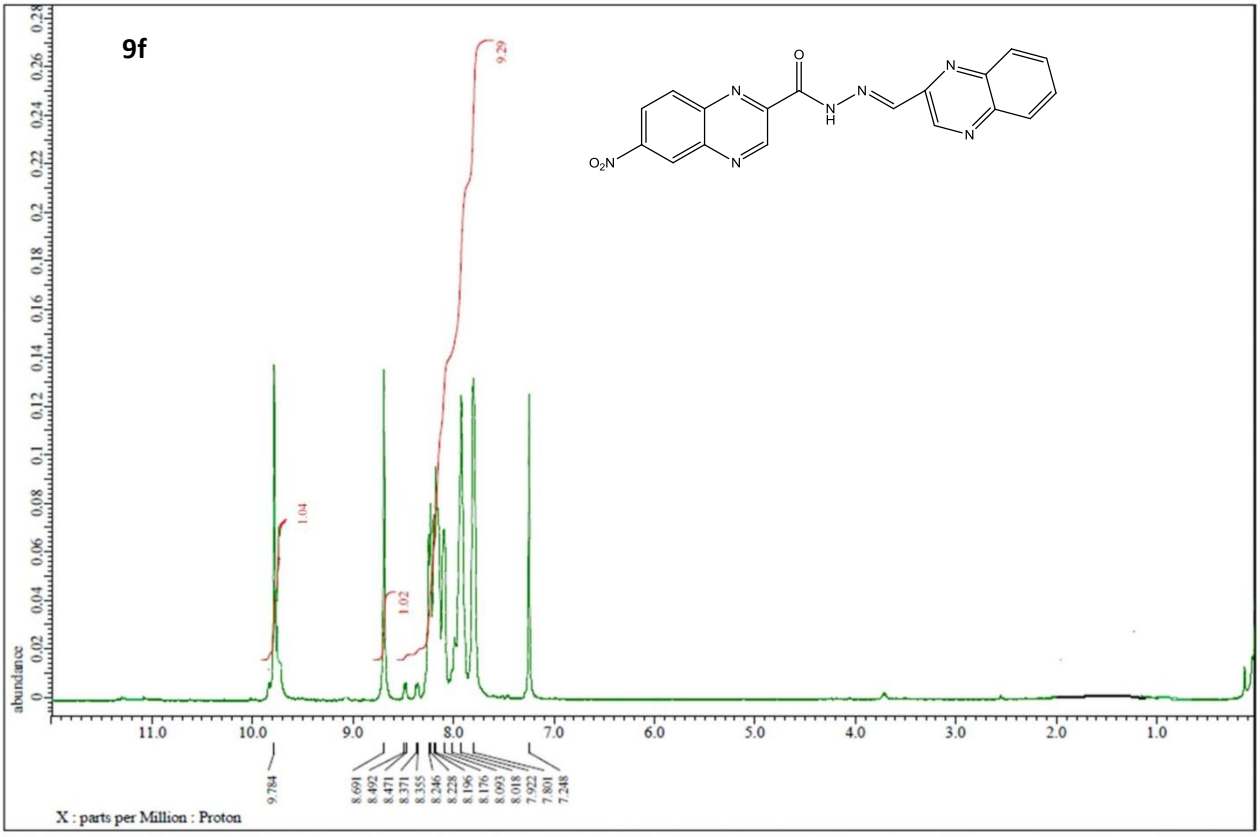

9b
---- PROCESSING PARAMETERS ----
dc_balance : 0 : FALSE
sexp : 2.0[Hz] : 0.0[s]
traperoid3 : 0[%] : 80[%] : 100[%]
zerofill : 1
fft : 1 : TRUE : TRUE
machinephase
ppm
Filename = Q2 C13-3.jdf
Author = delta
Experiment = single_pulse_dec
Sample_id = Q2 C13
Solvent = CHLOROFORM-D
Creation_time = 11-MAR-2019 07:43:07
Revision_time = 11-MAR-2019 12:17:08
Current_time = 11-MAR-2019 12:17:18
Comment = single pulse decouple
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECS 400
Spectrometer = JNM-ECS400
Field_strength = 9.389766[T] (400[MHz]
X_acq_duration = 1.04333312[s]
X_domain = 13C
X_freq = 100.52530333[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.95846665[Hz]
X_sweep = 31.40703518[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 1000
Total_scans = 1000
X_90_width = 9.5[us]
X_acq_time = 1.04333312[s]
X_angle = 90[deg]
X_atn = 4.2[dB]
X_pulse = 9.5[us]
Irr_atn_dec = 21.399[dB]
Irr_atn_noe = 21.399[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 3.04333312[s]
Temp_get = 18.1[dC]
abundance
X : parts per Million : 13C

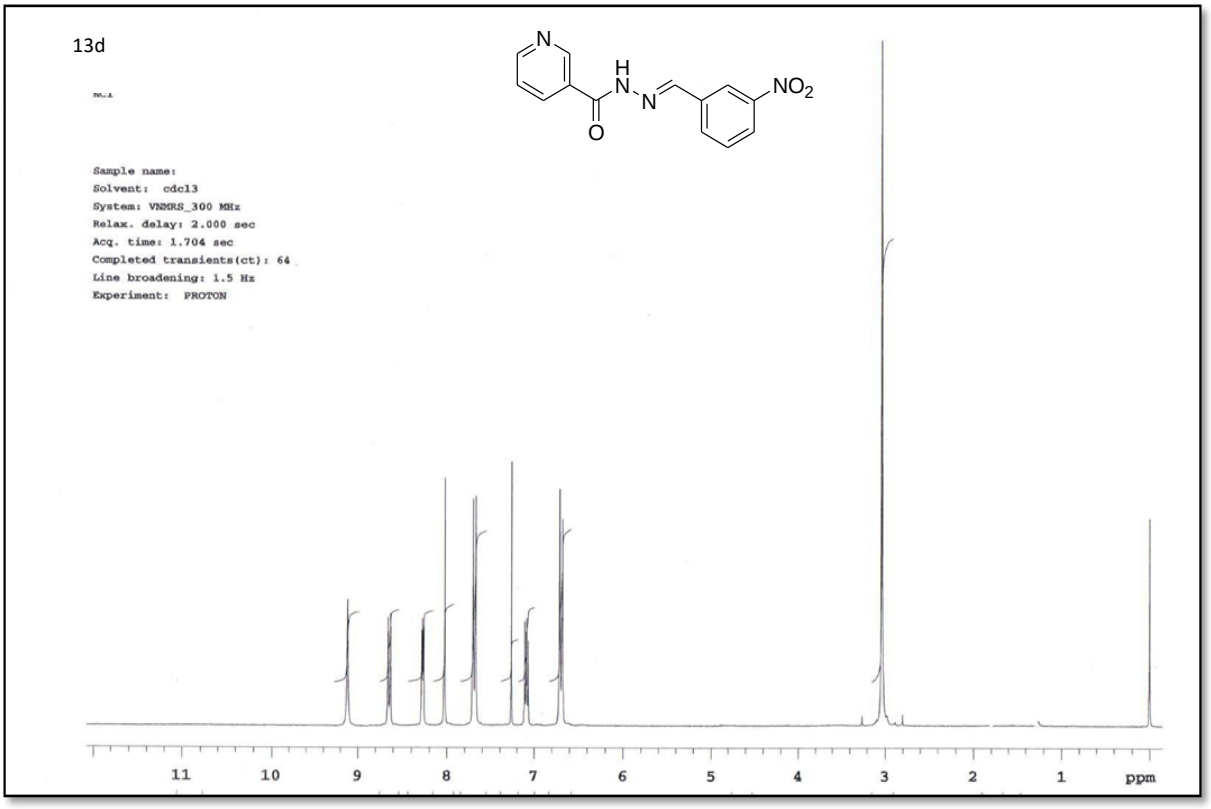

Sample Name : Q-3
Test Name : HRMS-1
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
270218-Q-3 14 (0.148) AM (Top,4, Ar,10000.0,0.00,0.00); Sm (Mn, 1x3.00); Cm (14:23)
1: TOF MS ES+
3.30e7
9b
406.908
679.2031
680.2011
681.2051
373.0888
475.3337
512.1480
540.3016
577.2757
629.2564
657.2227
701.1819
711.1366
%
m/z

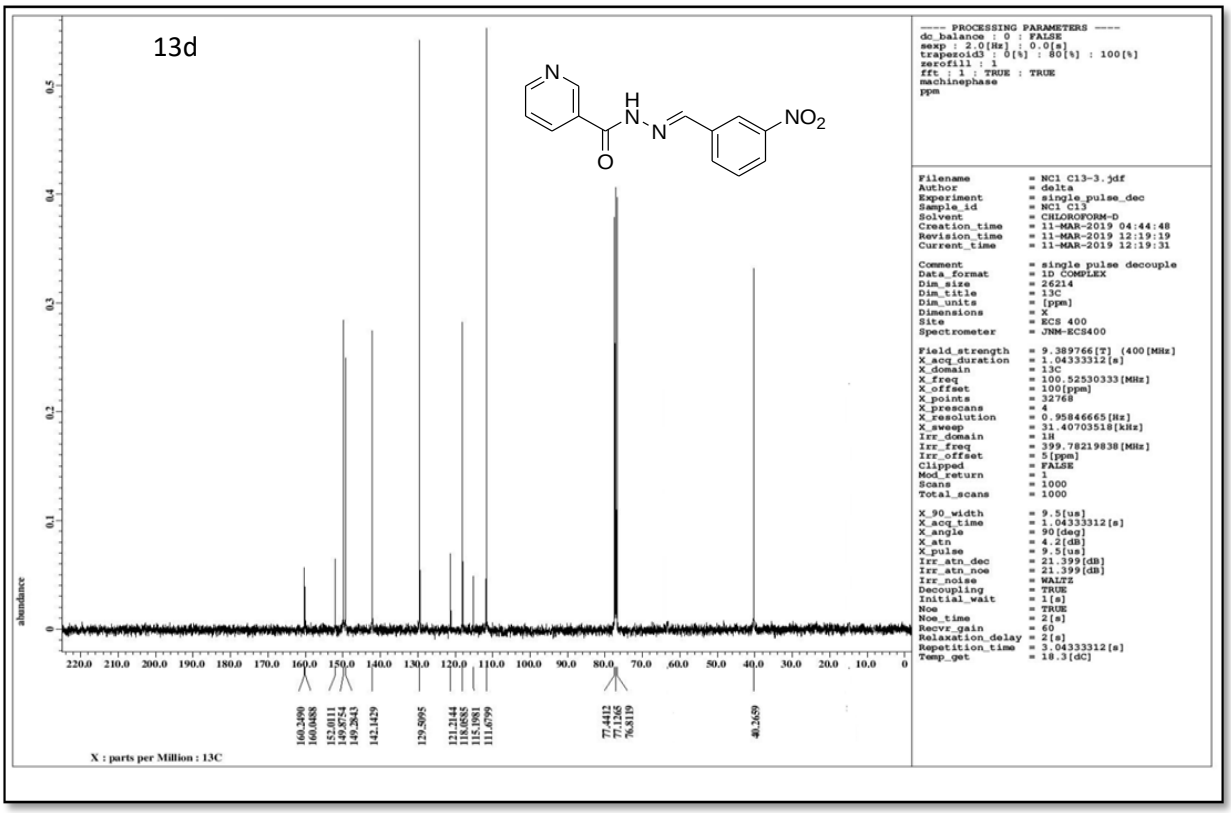

9e
abundance
X : parts per Million : Proton

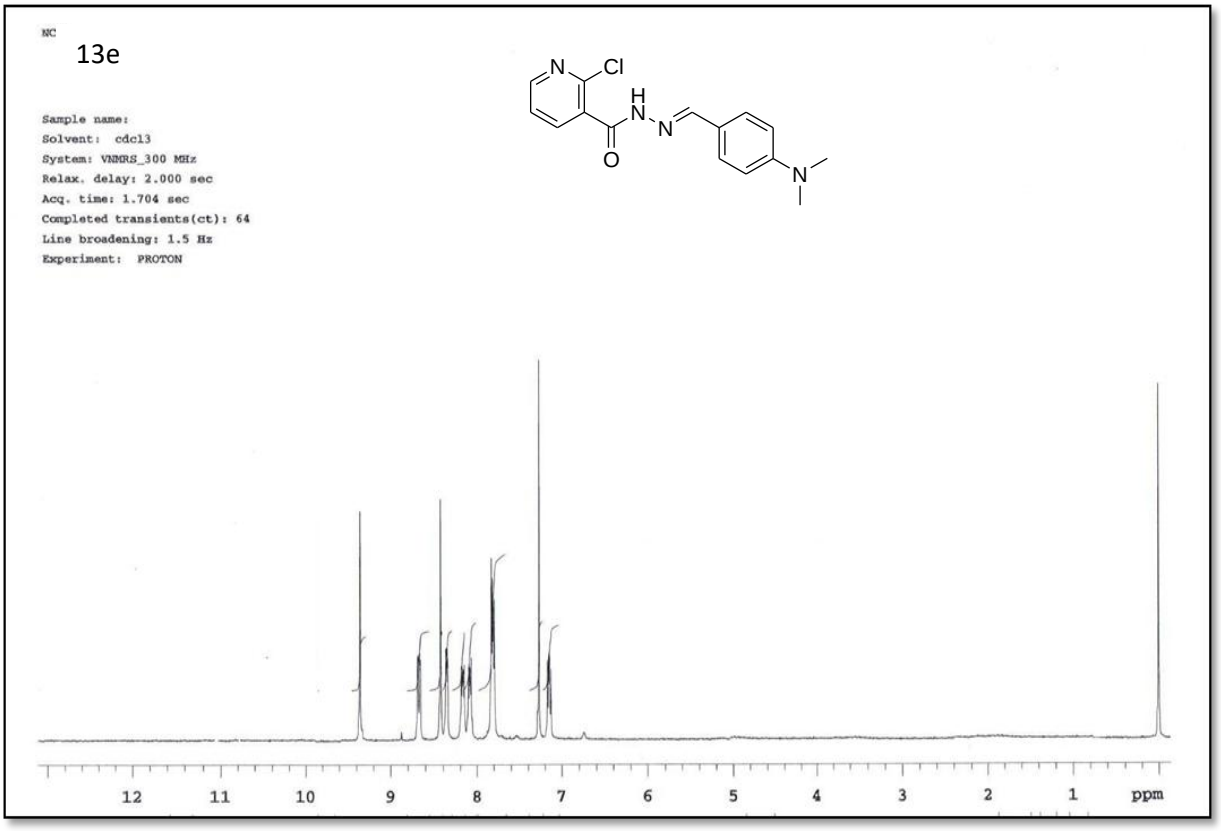

9e
(thousandths)
X : parts per Million : Carbon13

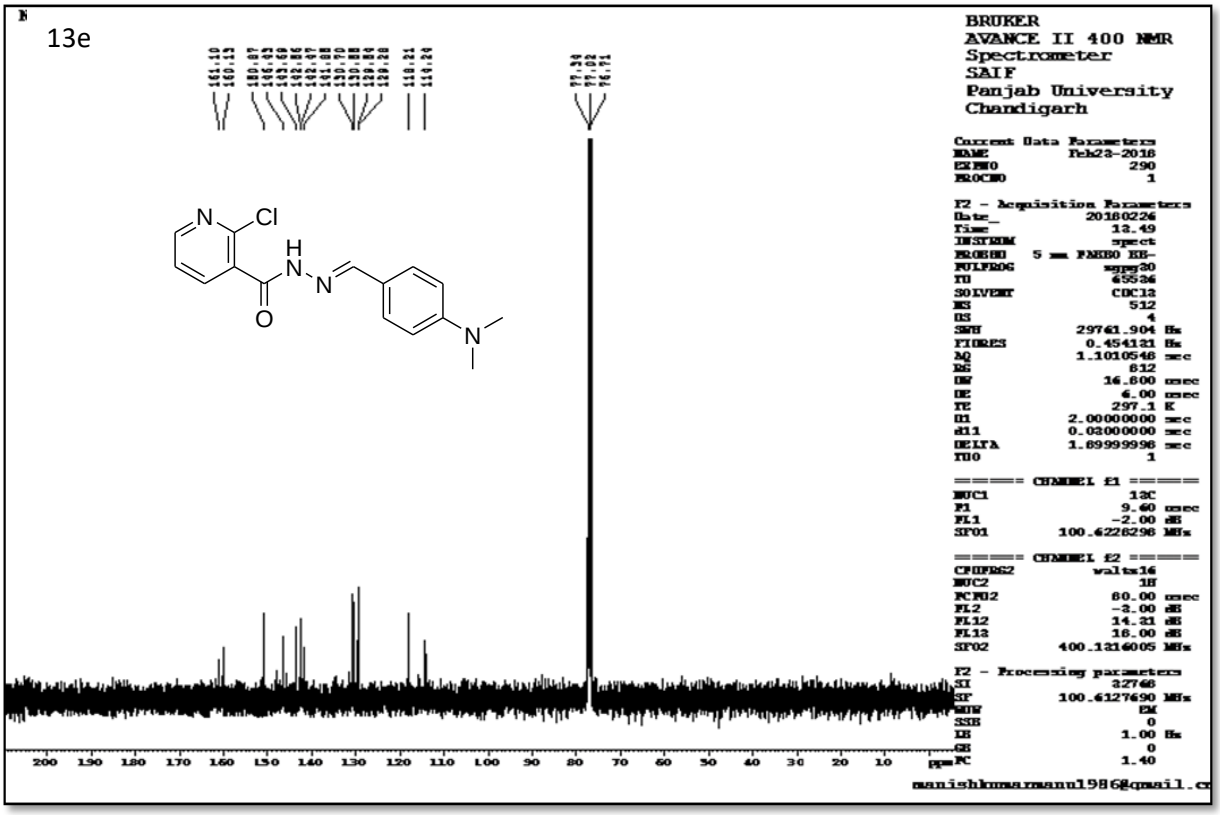

SAIF,PANJAB UNIVERSITY CHANDIGARH
SYNAPT-XS#DBA064
10-Aug-2021
16:39:42
SINTHIYA 9e 5 (0.120) Cm (2:6)
1: TOF MS ES+
1.21e7
9e
364.1303
12093906
295.1940
4324555
365.1412
3366452
342.1336
1250984
296.1971
821619
366.1821
441949
m/z

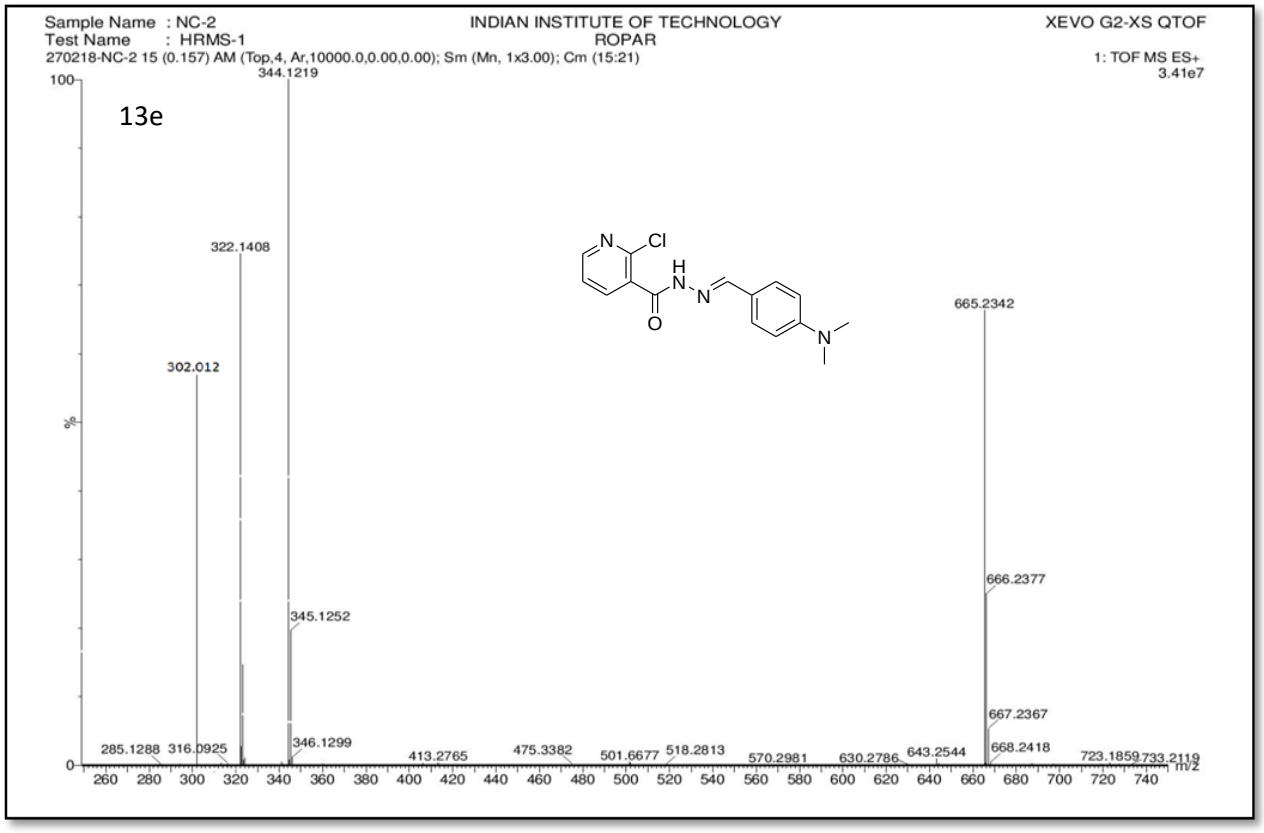

9f
9.29
1.04
1.02
abundance
X : parts per Million : Proton
9.784
8.691
8.492
8.471
8.371
8.355
8.246
8.228
8.196
8.176
8.093
8.018
7.922
7.801
7.248

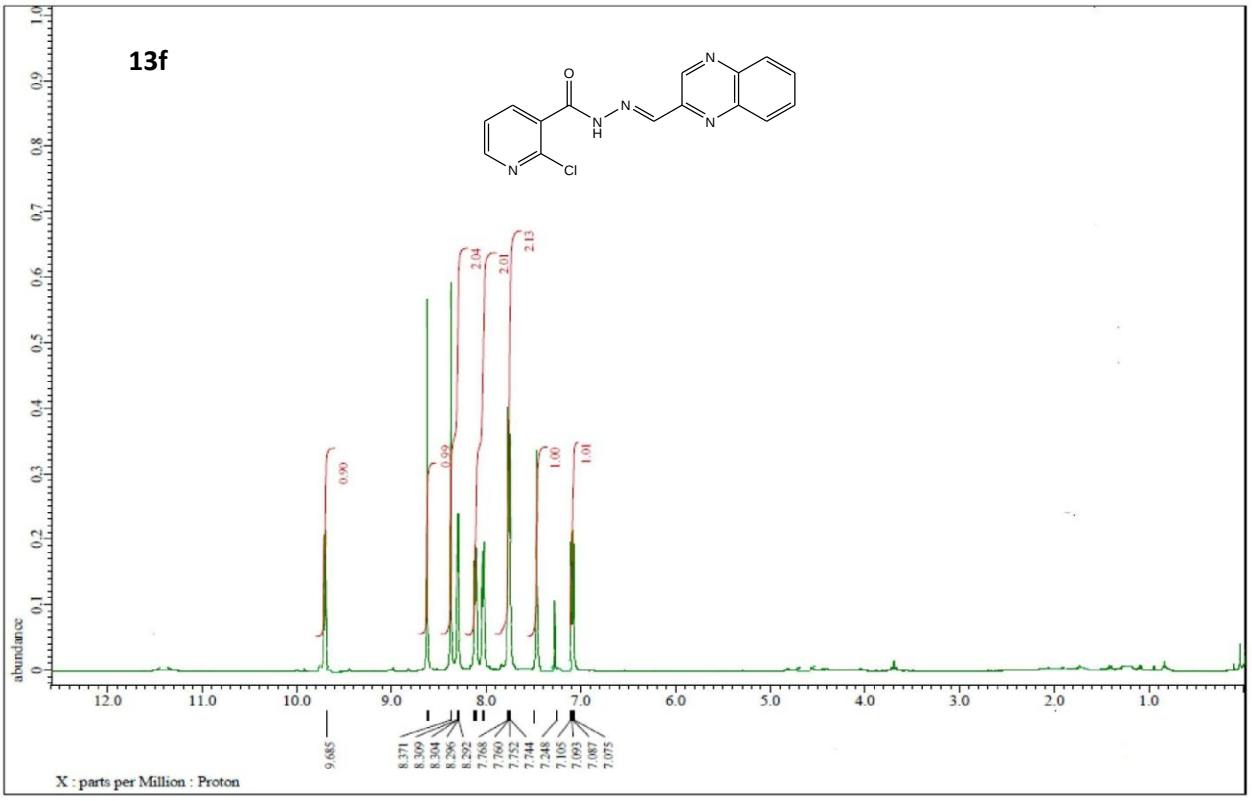

13d
Sample name:
Solvent: cdcl3
System: VNMRS_300 MHz
Relax. delay: 2.000 sec
Acq. time: 1.704 sec
Completed transients(ct): 64
Line broadening: 1.5 Hz
Experiment: PROTON
NO2
11
10
9
8
7
6
5
4
3
2
1
ppm

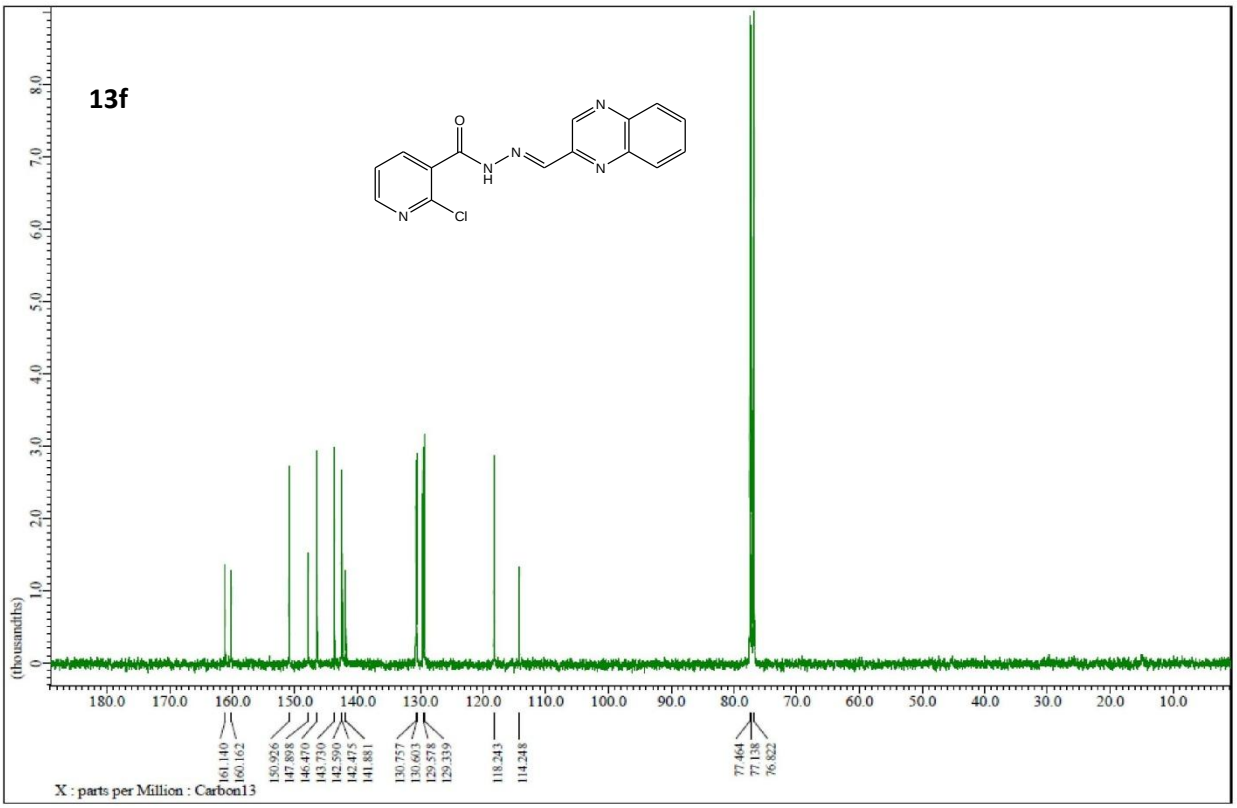

13d
NO2
abundance
X : parts per Million : 13C
Filename = NC1 C13-3.jdf
Author = delta
Experiment = single_pulse_dec
Sample_id = NC1 C13
Solvent = CHLOROFORM-D
Creation_time = 11-MAR-2019 04:44:48
Revision_time = 11-MAR-2019 12:19:19
Current_time = 11-MAR-2019 12:19:31
Comment = single pulse decouple
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECS 400
Spectrometer = JNM-ECS400
Field_strength = 9.389766[T] (400[MHz]
X_acq_duration = 1.04333312[s]
X_domain = 13C
X_freq = 100.52530333[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 0.95846665[Hz]
X_sweep = 31.40703518[kHz]
Irr_domain = 1H
Irr_freq = 399.78219838[MHz]
Irr_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 1000
Total_scans = 1000
X_90_width = 9.5[us]
X_acq_time = 1.04333312[s]
X_angle = 90[deg]
X_atn = 4.2[dB]
X_pulse = 9.5[us]
Irr_atn_dec = 21.399[dB]
Irr_atn_noe = 21.399[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 60
Relaxation_delay = 2[s]
Repetition_time = 3.04333312[s]
Temp_get = 18.3[dC]

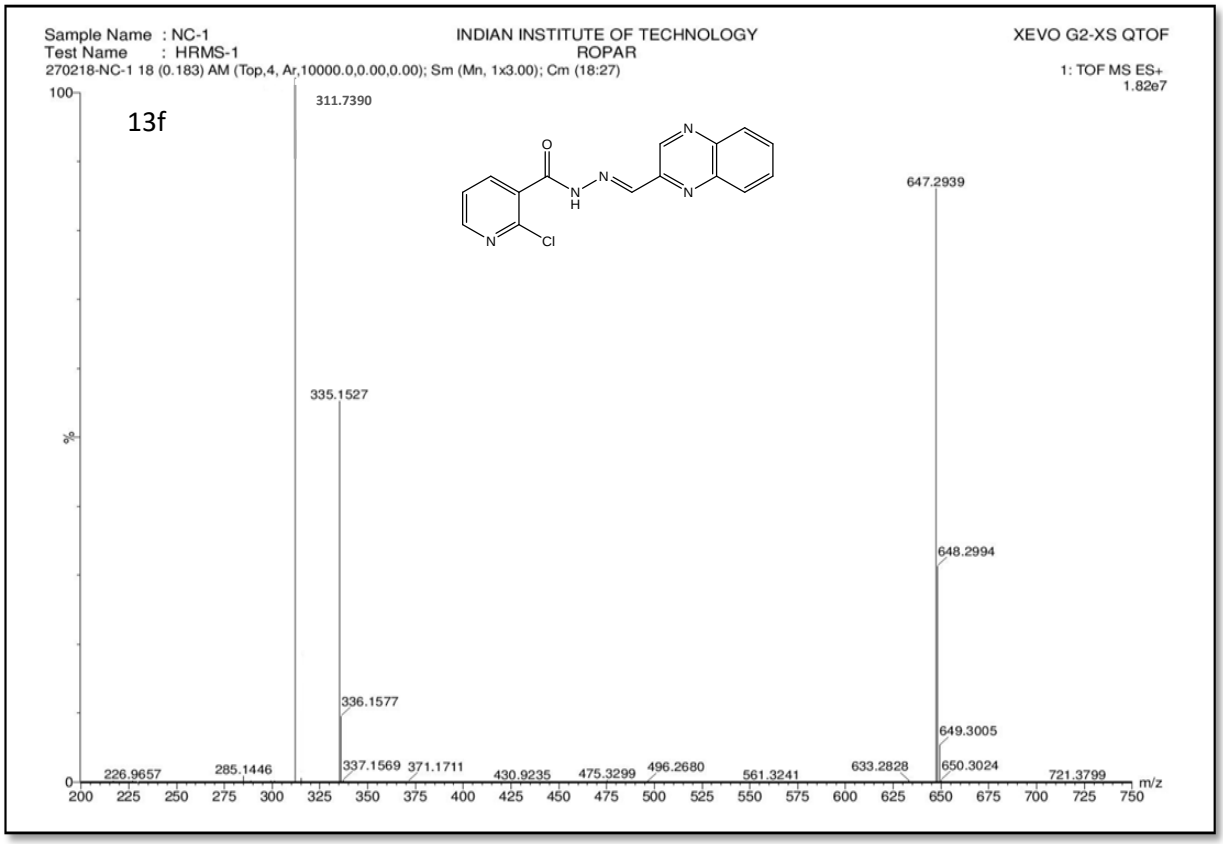

NC
13e
Sample name:
Solvent: cdcl3
System: VNMRS_300 MHz
Relax. delay: 2.000 sec
Acq. time: 1.704 sec
Completed transients(ct): 64
Line broadening: 1.5 Hz
Experiment: PROTON
N
Cl
H
N
N
O
N
12
11
10
9
8
7
6
5
4
3
2
1
ppm


13e
BRUKER
AVANCE II 400 NMR
Spectrometer
SAIF
Panjab University
Chandigarh
Current Data Parameters
NAME Feb23-2018
EXPNO 290
PROCNO 1
F2 - Acquisition Parameters
Date_ 20180226
Time 13.49
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zgpg30
TD 65536
SOLVENT CDCl3
NS 512
DS 4
SWH 29761.904 Hz
FIDRES 0.454131 Hz
AQ 1.1010546 sec
RG 812
DW 16.800 usec
DE 6.00 usec
TE 297.1 K
D1 2.00000000 sec
d11 0.03000000 sec
DELTA 1.89999996 sec
TD0 1
CHANNEL f1
NUC1 13C
P1 9.40 usec
PL1 -2.00 dB
SFO1 100.6228298 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -3.00 dB
PL12 14.31 dB
PL13 16.00 dB
SFO2 400.1316005 MHz
F2 - Processing parameters
SI 32768
SF 100.6127690 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40
manishkumarmanu1986@gmail.c
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm


Sample Name : NC-2
Test Name : HRMS-1
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
270218-NC-2 15 (0.157) AM (Top,4, Ar,10000.0,0.00,0.00); Sm (Mn, 1x3.00); Cm (15:21)
1: TOF MS ES+
3.41e7
13e
344.1219
322.1408
302.012
665.2342
666.2377
345.1252
667.2367
346.1299
668.2418
285.1288
316.0925
413.2765
475.3382
501.6677
518.2813
570.2981
630.2786
643.2544
723.1859
733.2119
m/z


13f
abundance
X : parts per Million : Proton


13f
(thousandths)
X : parts per Million : Carbon13


Sample Name : NC-1
Test Name : HRMS-1
INDIAN INSTITUTE OF TECHNOLOGY
ROPAR
XEVO G2-XS QTOF
1: TOF MS ES+
1.82e7
13f
311.7390
335.1527
336.1577
647.2939
648.2994
649.3005
650.3024
m/z

CHAPTER 4

Novel Quinoxaline-Chalcone Hybrids As Enoyl-ACP Reductase Inhibitors

4.1. INTRODUCTION

One of the major advances in the field of medicinal chemistry and drug discovery has been the molecular hybridization approach. This has risen from "drug evolution", which drug-drug hybridization is leading to drug molecules of potential bioactivity. Konishi *et al* reported hybridization of Benzocaine and Metoclopramide leading to generation of 16 new molecules.[1] The large number of libraries of compounds is an encouragement in the findings for new drug candidates. In the same lines, molecular hybridization is a tool in drug designing, wherein simples molecules can be linked together to construct new hybrid molecules of varied biological interest.[2] This versatile approach of designing new drug entities is the key to achieving large number of hybrid molecules having better affinity and efficacy than the parent molecule from which they are derived. One of the pharmacophoric moieties, which have been more often the target of medicinal chemists, has been naturally occurring as well as synthesized chalcones. Chalcones are biologically important α, β- unsaturated carbonyl compounds, which as excellent building blocks to heterocycles has attracted organic as well as medicinal chemists.[3] Chalcone derivatives have been demonstrated to have wide range of biological activities.[4,5] Chalcone linked hybrids with heterocycles is a step towards achieving new drug targets,[6] for example coumarinyl-chalcone hybrids as a promising bioactive agents showing wide spectrum of biological properties,[7] novel chalcone-thiazole hybrids as having high antibacterial action against *Staphylococcus aureus,* making it a potential candidate to act as antibiotic.[8] Variety of chalcone hybrids such naphthyl, isoxazolyl and indolyl have been known to show potency as anticancer agents.[9-11] *N*-heterocyclic chalcones have been known in literature, and have been biologically evaluated for their anti-microbial and anti-tubercular activity.[12-14]

Tuberculosis (TB) has been affecting more than two million people all around the world and with more than 8 million cases every year.[15] Due to emergence of multi-drug resistant varieties of *Mycobacterium tuberculosis* and aids epidemic, drugs like Isoniazid, Rifampicin, Pyrazinamide, Ethambutol are no more effective.[16] There are several promising clinical trials development programs going on to conduct evaluation of new anti-TB drugs, such as PA-824, which is a Nitroimidazooxazine, an anti-TB drug candidate in the late stage clinical development, showing increased activity against *Mycobacterium tuberculosis*. However, there are few novel compounds known to hit the target. In literature, there are number of chalcones

known to show high inhibitory activity against the growth of *Mycobacterium tuberculosis* strains $H_{37}Rv$, when used in low concentrations.[17] Quinoxaline, a bicyclic nitrogen heterocycle is said to have enough potential to be explored for biological evaluation.[18] Various derivatives of quinoxaline are known to possess wide range of biological activities ranging from anti-microbials, anti-tubercular, kinase inhibitors, anti-viral, anti-inflammatory, analgesics, anticancer, anxiolytics, antihelmintics, anticonvulsants, antioxidants, antidepressant, anti-hypertensives to antiHIVs.[19-22] Thus, being a part of well-known antibiotics, like Echinomycin, Laevomycin and Actinoleutin, the substituted quinoxaline skeleton need to be exploited more in drug discovery.[23]

The combination of these properties of quinoxaline and chalcone in one compound would lead to a drug with potent bioactivity. Molecular hybridization approach from quinoxaline and chalcone include novel 2-acetylquinoxaline derived-chalcones which exhibited i*n vitro* glioma cell proliferation activity.[24] Various other quinoxaline derived derivatives have also been known in literature[25] Quinoxaline derived chalcones has been synthesized and biologically evaluated.[26,27] Chalcone derivatives of quinoxaline-1, 4-dioxides have been screened as anti-TB agents.[28] In his PhD thesis, Mohan *et al* has reported work on synthesis and reactions of quinoxalines involving preparation of quinoxaline-2-carbaldehyde.[29] Interestingly, in literature, quinoxaline-2-carbaldehyde has not being exploited and there are no reports on anti-tubercular activity of quinoxalinylchalcones. In view of this, an approach was designed to get a quinoxalinylchalcone hybrid molecule from acetophenones and quinoxaline-2-carbaldehyde and study its potential as anti-tubercular agents (**Fig. 27**).

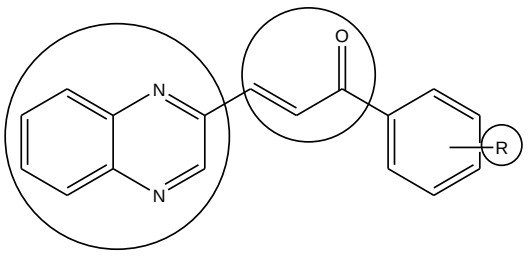
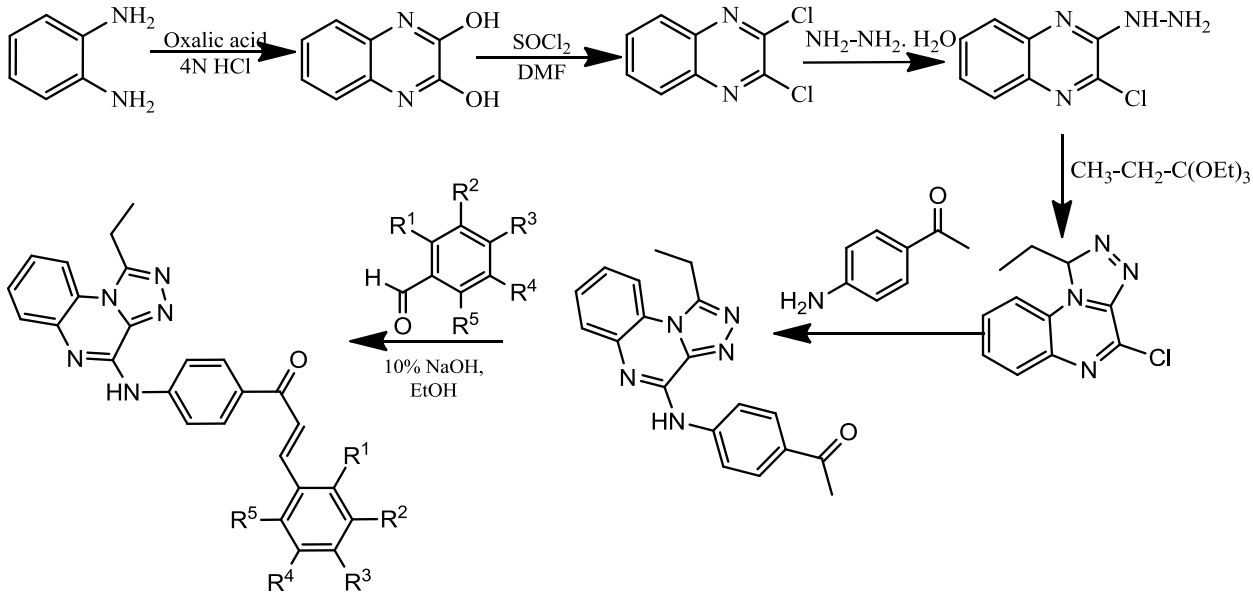


Fig.27. Molecular hybridization of chalcone and quinoxaline having potent activity

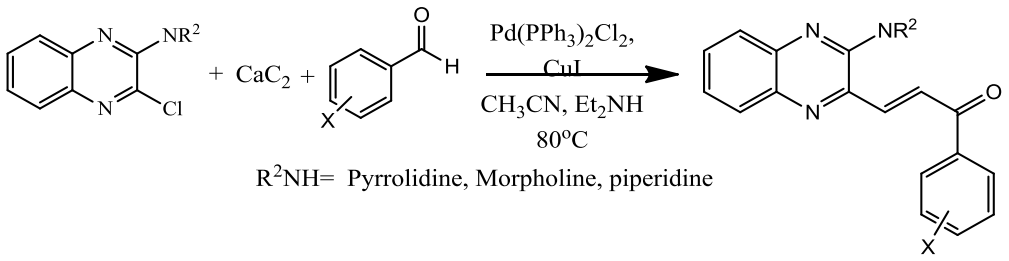
4.2. LITERATURE

Ahmed, *et al*[30] has designed the synthesis and cytotoxic evaluation of novel chalcone derivatives bearing [4, 3-a]-quinoxaline moiety as potent anti-cancer agents with dual EGFR kinase and tubulin polymerization inhibitory effects (**Scheme 60**).



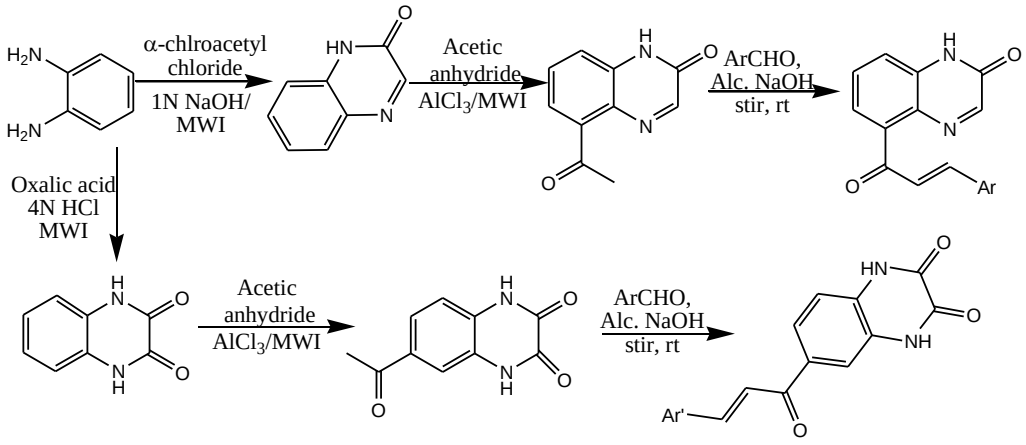
Scheme 60

Keivanloo, *et al*[31] have reported one pot synthesis of quinoxalinyl chalcones from commercially available calcium carbide through Pd-catalysed coupling reaction (**Scheme 61**).



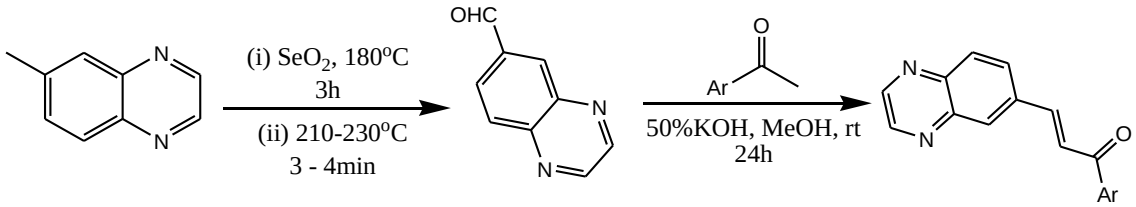
Scheme 61

Geetha, *et al*[32] carried out synthesis of new quinoxalinyl chalcone derivatives and have screened them for its anti-microbial activity (**Scheme 62**).



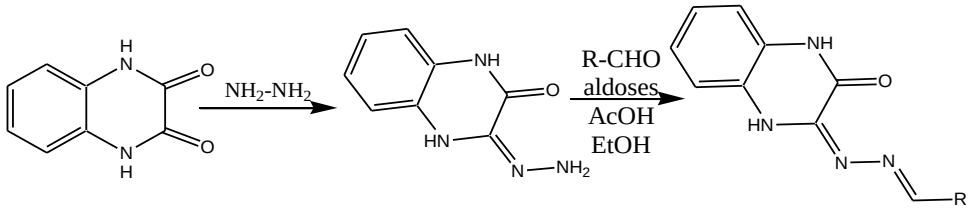
Scheme 62

Gozzi, Winter *et al*[33] describes synthesis of chalcones derivative with quinoxaline moiety as new inhibitors of breast cancer resistance protein (**Scheme 63**).



Scheme 63

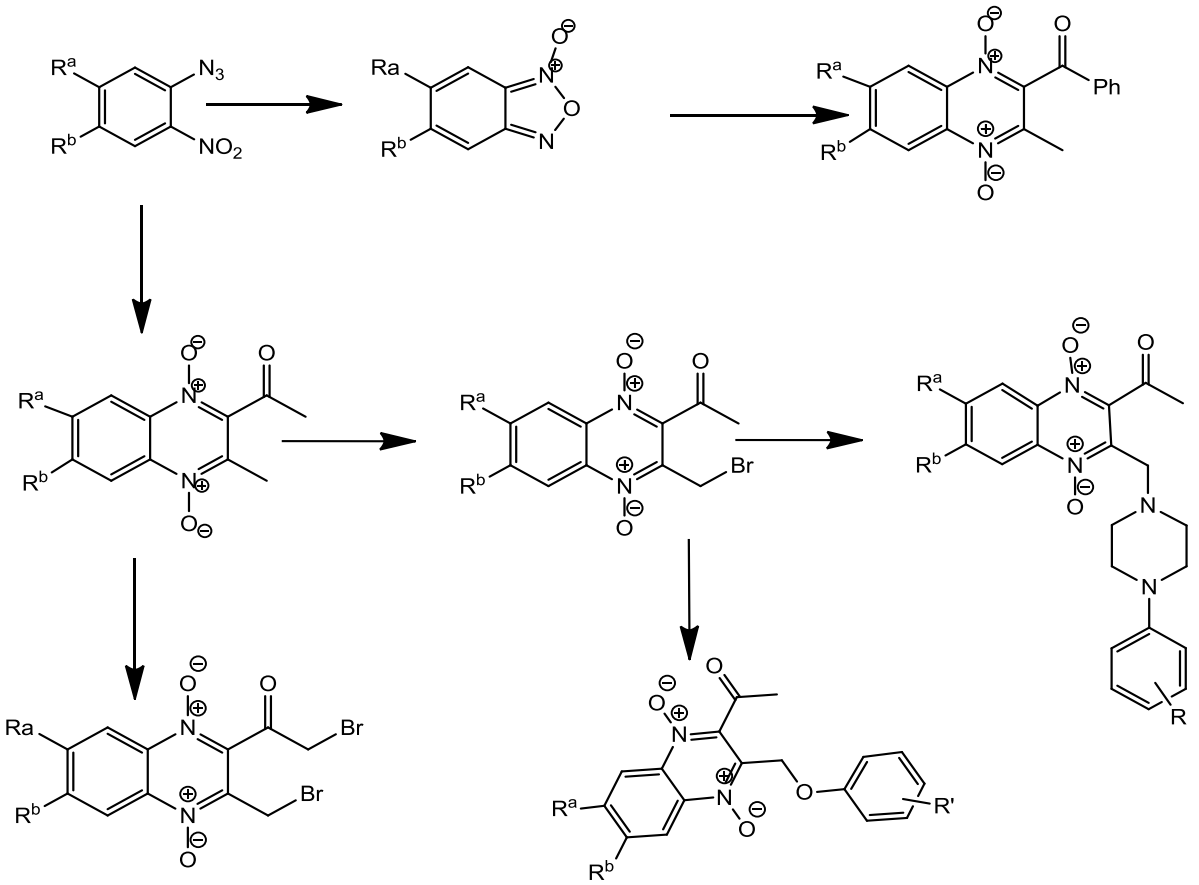
Peraman, *et al* [34] has demonstrated a route towards synthesis of sugar conjugates of quinoxaline as potent anti-tubercular and anti-bacterial agents (**Scheme 64**).



R=aldohexoses: a=Glucose, Mannose, Maltose

Scheme 64

Aldana, *et al*[35] suggested synthesis of quinoxalinyl-1,4-di-*N*-oxide derivatives as anti-mycobacterial tubercular agents (**Scheme 65**)



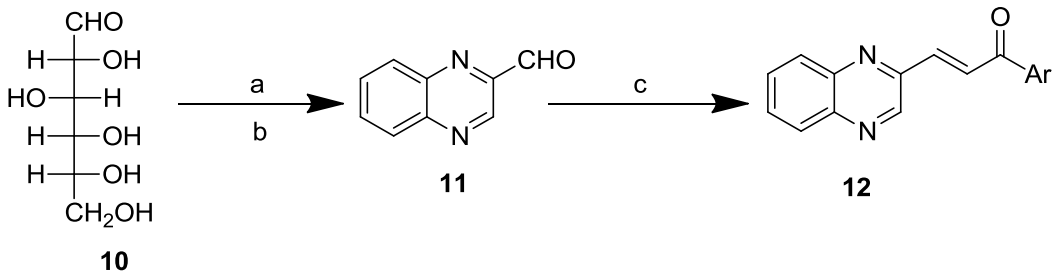
Scheme 65

From the literature it can be seen that quinoxaline-2-carbaldehyde has not been exploited much into any derivative, so we thought of expanding it into an interesting chalcone hybrids.

4.3. PRESENT WORK AND DISCUSSION

4.3a. Chemistry

Quinoxaline-2-carbaldehyde was first synthesized by literature known procedure from glucose, *o*-phenylenediamine, hydrazine hydrate to form an intermediate, followed by oxidation using sodium metaperiodate.[29] This was then reacted with substituted aromatic acetophenones under Claisen Schmidt condensation basic reaction conditions[36, 27] to give corresponding chalcone. derivatives **12a-n** in moderate to good yields (**Scheme 66**, **Table 24**). In all 14 quinoxalinylchalcone derivatives were synthesized.



Scheme 66. Synthetic route towards quinoxalinylchalcones from glucose **(12a-n)** Reagents and conditions: (a) H_2O, *o*-phenylenediamine, glacial CH_3COOH, NH_2-$NH_2.H_2O$, reflux, 5 hours, cool in icebath; (b) H_2O, $NaIO_4$, glacial CH_3COOH, stir, r.t. 16 hours; (c) Aromatic ketones, NaOH, H_2O, EtOH, stir, r.t.; (d) Ice, concentrated HCl

Table 24. Claisen Schmidt condensation of quinoxaline-2-carbaldehyde with various acetophenones to give chalcones **(12a-n)**

Compound 12	Ar	Time[a] h	Yield[b] in %	Melting Point[c] °C
a	4-Hydroxyphenyl	2	78	256-258
b	4-Aminophenyl	2	75	178-180
c	4-Bromophenyl	2	80	124-126
d	4-Methoxyphenyl	4	85	98-100
e	4-Chlorophenyl	3	75	126-128
f	4-Fluorophenyl	2	80	135-138

g	4-Pyridinyl	2	65	150-152
h	3-Hydroxyphenyl	2	80	144-146
i	3-Bromophenyl	2	95	120-122
j	Phenyl	4	70	136-138
k	3-aminophenyl	2	75	156-158
l	4-Nitrophenyl	2	60	164-167
m	3-Nitrophenyl	2	60	205-207
n	Naphthyl	2	90	130-132

[a]Time taken for completion of the reaction monitored by thin layer chromatography.

[b]Calculated from the amount of chalcone obtained after recrystallization.

[c]Determined using thiels tube paraffin method.

The first six compounds 1**2a-f** has been reported by us[27], along with the biological activities. The acetophenones (1 equivalent) was treated with aqueous ethanolic solution of sodium hydroxide, stirred at room temperature for 10 minutes for enolate formation, then to quinoxaline-2-carbaldehyde (1 equivalent) was added and the stirring continued till the completion of the reaction, which was monitored by thin layer chromatography. The reaction was worked up by addition ice and hydrochloric acid. The solid product filtered, was purified by recrystallization using ethanol. All the synthesized compounds have been identified and confirmed by FTIR, ^{1}HNMR and ^{13}CNMR spectroscopy. The molecular mass of the compounds were confirmed by mass spectral analysis.

Further the purification of the chalcones was carried out by HPLC measurements on CXTH-3000 Chromatography Data Handling System (Analytical Technologies Limited). Chromatographic separation was achieved at ambient temperature by using mobile phase consisting of methanol and water in the ratio 90:10 (v/v) by 20 min. The mobile phase was pumped at the rate of 1.0mL/min. The detector wavelength was set at 370nm.The run time was set at 20min and retention time of all chalcones was between 16-22 min. The purity of all the synthesized compounds was found to be 95% and above. The chromatogram of chalcone is shown in **fig 28**.

We were thus successful in achieving synthesis of new quinoxalinyl chalcone hybrids bearing different substituents. This gave us an opportunity to explore the applications of such chalcones for antimicrobial, anticancer and anti-tubercular activity, as well as predict their structure activity relationships (SAR analysis).

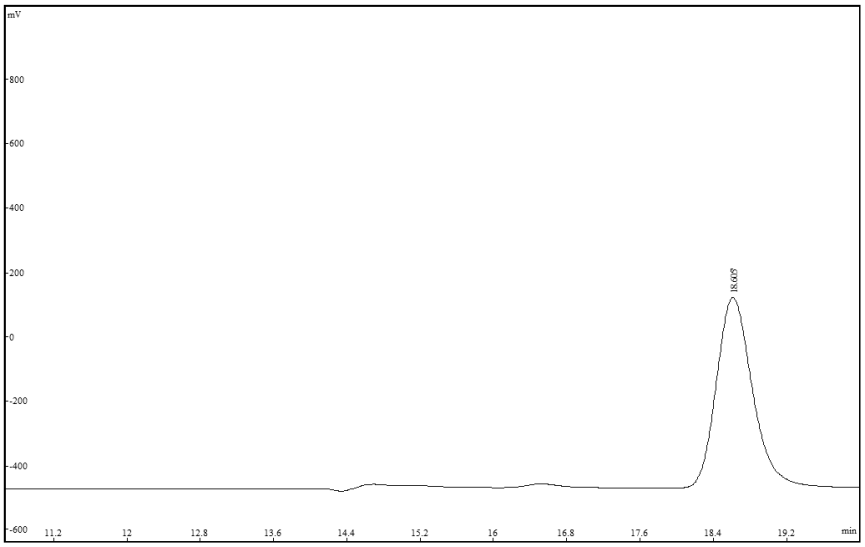


Fig.28. Representative Chromatogram of **Compound 12b**

4.3b. Biological Evaluation:

4.3b.1. Anti-microbial activity studies:

Antibacterial and antifungal activities of the newly synthesized quinoxalinyl chalcone derivatives 1**2a-n** were determined using agar well disc diffusion procedure.[37] In brain heart infusion agar, against gram positive strain *Staphylococcus aureus* and gram negative strains *Escherichia coli*, and in sabouraud agar medium against fungal organism *Candida albicans*. Five wells were made on each plate and 75µL, 50µL, 25µL, 10µL and 5µL of compound were added into the respective wells. Plates were incubated for 18-24 hrs at 37^{o}C in incubator. Diameter of inhibition zone (**Fig.30**) to nearest whole millimeter was measured by holding the measuring device across the zones. Ciprofloxacin and Fluconazole were used as standard drug (**Table 25, Fig.29**).

Table 25. Anti-microbial activity screening[a] of synthesized chalcones **12a-n**

Compound 12	Ar	Minimum inhibitory concentration-MIC (µg/mL)		
		S. aureus	***E. coli***	***C. albicans***
a	4-Hydroxyphenyl	5	5	-
b	4-Aminophenyl	50	25	25
c	4-Bromophenyl	75	25	75
d	4-Methoxyphenyl	50	25	25
e	4-Chlorophenyl	25	10	25
f	4-Fluorophenyl	75	25	25
g	4-Pyridinyl	50	25	50
h	3-Hydroxyphenyl	10	5	10
i	3-Bromophenyl	25	5	25
j	Phenyl	50	50	25
k	3-aminophenyl	50	25	25
l	4-Nitrophenyl	50	25	50
m	3-Nitrophenyl	50	25	25
n	Naphthyl	25	25	25
	Ciprofloxacin[d]	2	2	-
	Fluconazole[d]	-	-	16

[a]Brain heart infusion agar medium is used.

[b]Standard drug used is Ciprofloxacin and Fluconazole.

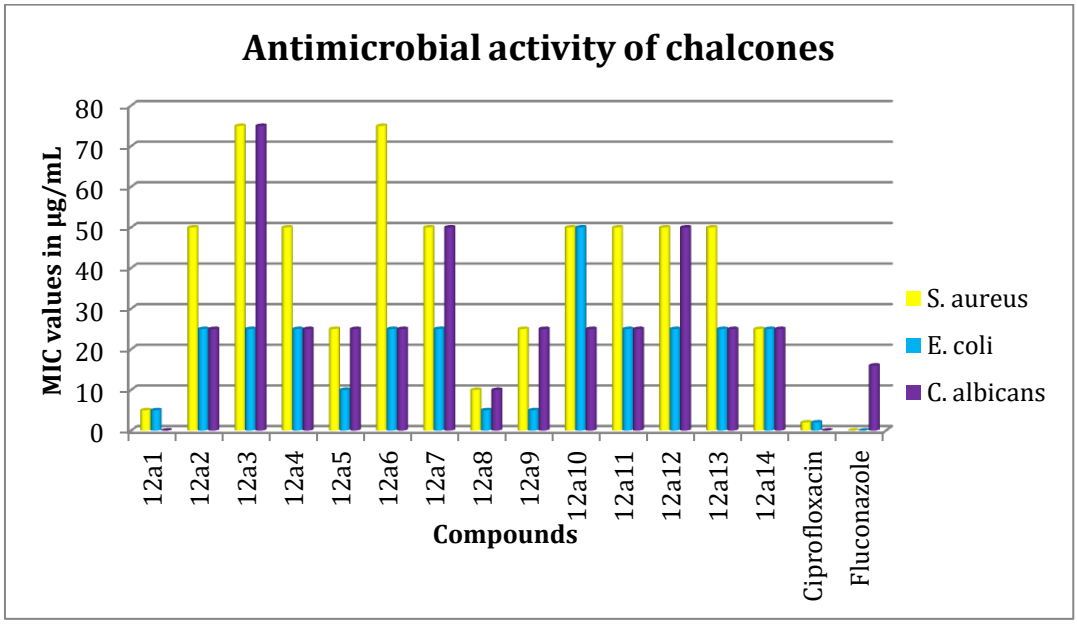


Fig.29. Graphical representation of anti-microbial activity: showing the variation in the minimum inhibitory concentration (µg/mL) of all chalcone derivatives against the standards Ciprofloxacin and Fluconazole.

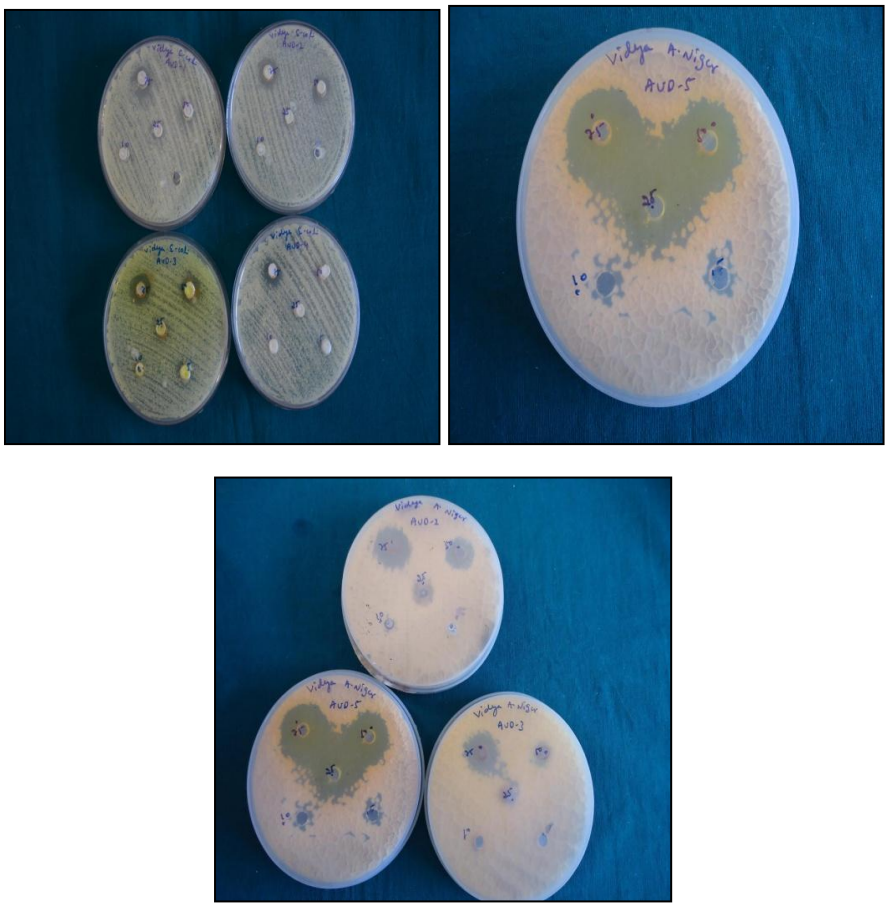


Fig.30. Zone of inhibitions for selected quinoxalinyl chalcones

From the outcomes of the antimicrobial activity of the synthesized quinoxalinyl chalcone derivatives, the following structure activity relationships (SAR) can be established (**Fig.35**). Overall, the quinoxalinyl chalcones (**12a-n**) exhibit better antibacterial activity against gram negative bacterial strains as compared to gram positive bacterial strains. The best activity against gram negative and gram positive bacterial strains for compounds **12a** and **12h** indicates that presence of –OH substituent, is necessary for enhanced antibacterial activity. The position of OH substituent also decides the specificity in (*p*-OH) containing **12a** exhibiting anti-bacterial but no antifungal activity, while (*m*-OH) containing **12h** exhibiting both antibacterial as well as antifungal activity. This was also evident with the comparison of activity of **12c** and **12i** which contains (*p*-Br) and (*m*-Br) substituents respectively. Significant antibacterial and antifungal activity for **12n,** containing naphthyl grouping as compared to **12j** containing phenyl grouping, indicates that increase in hydrophobicity has positive effect on activity. Replacement of phenyl ring **12j** by a heterocyclic pyridinyl ring **12g** does not show any change in activity.

4.3b.2. Cytotoxicity studies:

The cytotoxicity of selected compounds **12a, 12b** and **12f** (**Table 26**) was evaluated using standard MTT assay.[38] In this assay, the reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide was measured by mitochondrial succinate dehydrogenase. The cell line used was MCF-7 cell line, a Michigan cancer foundation-7 cell line, a Human mammary gland adenocarcinoma, which was procured from National Centre for Cell Science (NCCS), Pune, India. IC_{50} is half maximal inhibitory concentration, at which 50% of cells were undergoing cytotoxic cell death due to synthesized compounds treatment. Cisplatin and Doxorubicin was used as standard drugs. Absorbance is measured at wavelength of 570nm by using LISA plus.

Table 26. Preliminary cytotoxicity screening[a] of quinoxalinyl chalcones **12a, 12b** and **12f** by MTT[b] assay

Sample code	IC50[c] µM
12a	**-**

12b	**25**
12f	**50**
Cisplatin[d]	**7.5**
Doxirubicin[d]	**5-7.5**

[a]MCF-7 cell line is Michigan cancer foundation-7 cell line, a Human mammary gland adenocarcinoma, which was procured from National Centre for Cell Science (NCCS), Pune, India

[b]MTT stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

[c]IC_{50} is Half maximal inhibitory concentration.

[d]Standard anticancer drugs used against MCF-7 cell line and their 50% lysis value

The IC_{50} (half maximal inhibitory concentration) value was determined in case of each compound. The least concentration to show 50% inhibition of cell line was found to be 25µg incase of compound **12b** containing $–NH_2$ substituent and 50µg in case of compound **12f** containing halogen substituent whereas, compound 1**2a** containing OH substituent showed no activity against MCF-7 cell lines.

4.3b.3.Anti-tubercular evaluation

4.3b.3.1. Molecular Docking studies:

To generalize the innovative approaches to drug targets, the study related to biology of *Mycobacterium tuberculosis* becomes a key component. Among the several drug targets known for tuberculosis,[39,40] the cell wall biosynthesis related drug target is the most promising one, since their biosynthetic enzymes do not have homologues in the mammalian system. One of the major drug targets for *Mycobacterium tuberculosis* has also been mycolic acid.[41] Mycolic acid, forms an important fatty acid, and a main constituent of the mycobacterial cell wall, which is present in fatty acid synthase system of *Mycobacterium tuberculosis*. The mycolic acid biosynthesis has been carried out by many successsive enzyme catalyzed cycles equivalent to Fatty Acid Synthase (FAS) systems. The enzyme InhA, which is an enoyl acyl carrier protein reductase from *M. tuberculosis* is the key enzyme for the synthesis of type II fatty acid system,

catalyzing the NADH-dependent reduction of 2-trans-enoyl-ACP (acyl carrier protein), which is responsible for the synthesis of mycolic acid. This has been the drug targets for well known anti-tubercular drugs like Isoniazid (INH) and Ethionamide.[42] Chalcones derived enoyl ACP reductase inhibitors have been studied and proved to have high binding affinity to the enzymes wherein the main influencing factors of molecular interaction between ENR and chalcone derivatives determined by this study were H-bond, hydrophobic and electrostatic interaction.[43] We chose enzyme enoyl ACP reductase for our molecular docking studies on quinoxalinylchalcones. The crystal structures used were *Mycobacterium tuberculosis* enoyl reductase (InhA) complexed with 1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) for the docking studies, obtained from the Protein Data Bank. The protein was prepared for docking by adding polar hydrogen atom with Gasteiger-Huckel charges[44] and the molecular docking was performed using Sybyl-X 2.0 software.[45]

The molecular docking study revealed that compound **12a** and **12h** acts as very good inhibitor of enoyl ACP reductase enzyme. As depicted in **Fig. 31**, for compound **12a**, one hydrogen bonding interaction has been observed at the active site of the enzyme (PDB ID: 4TZK). The hydrogen atom of hydroxyl group at 4th position of aromatic ring makes a hydrogen bonding interaction with oxygen of NAD500 (O-H----O-NAD500, 1.86Å). **Table 27** depicts the results of docking studies.

Compound 1**2h,** makes five hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), among them three interactions have arised from the hydrogen of the hydroxyl group present at 3rd position of aromatic ring, with oxygen of NAD500 and THR196 (O-H---- O-NAD500, 1.81 & 2.71 Å; HG-THR196, 2.34 Å) and other two interactions arose from the oxygen of carbonyl group with hydrogen of NAD500 & TYR158 (C=O----H-NAD500, 1.88 Å; H-TYR158, 2.00 Å) **Fig.32.**

The binding interaction of 4TZK ligand with enoyl ACP reductase enzyme shows two bonding interaction which has been depicted in **Fig.33**. It was also seen that the studied compounds have showed same type of interactions with amino acid residue THR196, TYR158 and co-factor NAD500 as that of reference 4TZK ligand. All the compounds showed consensus score in the range 4.10-8.02, which indicates all the forces of interaction between ligands and the enzyme in docked compounds. Thus, indicating that molecules preferentially bind to enzyme in comparison to the reference 4TZK ligand.

Table 27. Surflex Docking score (kcal/mol) of the lists of quinoxalinyl chalcones **(12a-n)**

Code 12	C Score[a]	Crash Score[b]	Polar Score[c]	D Score[d]	PMF Score[e]	G Score[f]	Chem Score[g]
a	6.97	-1.28	3.03	-140.48	-74.20	-222.05	-45.17
b	4.08	-0.62	0.00	-110.13	-53.35	-161.40	-34.34
c	5.56	-0.65	1.91	-117.88	-59.66	-134.90	-39.40
d	6.29	-1.37	2.91	-127.33	-58.88	-205.39	-44.61
e	5.67	-0.51	1.93	-115.39	-57.18	-131.25	-39.22
f	5.65	-0.51	1.82	-109.28	-61.87	-125.36	-36.89
g	5.28	-0.81	0.00	-118.75	-44.64	-184.05	-34.59
h	8.02	-1.71	2.97	-137.23	-57.94	-218.32	-45.35
i	4.97	-0.73	1.81	-117.07	-67.96	-143.90	-40.66
j	5.99	-1.20	1.82	-122.92	-44.00	-168.55	-39.99
k	5.34	-0.88	0.84	-115.44	-60.01	-175.74	-38.99
l	4.10	-0.62	0.00	-118.93	-49.13	-173.90	-33.28
m	4.81	-1.37	0.00	-132.45	-37.36	-209.00	-35.93
n	6.11	-2.11	1.15	-145.14	-64.81	-225.27	-46.90

[a]C Score (Consensus Score) integrates the affinity of ligands bound to the active site of a receptor and reports the output of total score; [b]Crash-score revealing the inappropriate penetration into the binding site. [c]Polar indicating polar interactions contributing to the total score. It excludes docking results that make no hydrogen bonds. [d]D-score indicates for charge and van der Waals interactions. [e]PMF-score indicating the Helmholtz free energies of interactions (Potential of Mean Force, PMF).[f]G-score showing hydrogen bonding, complex (ligand-protein), and internal (ligand-ligand) energies. [g]Chem-score includes H-bonding, lipophilic contact, and rotational entropy, along with an intercept term.

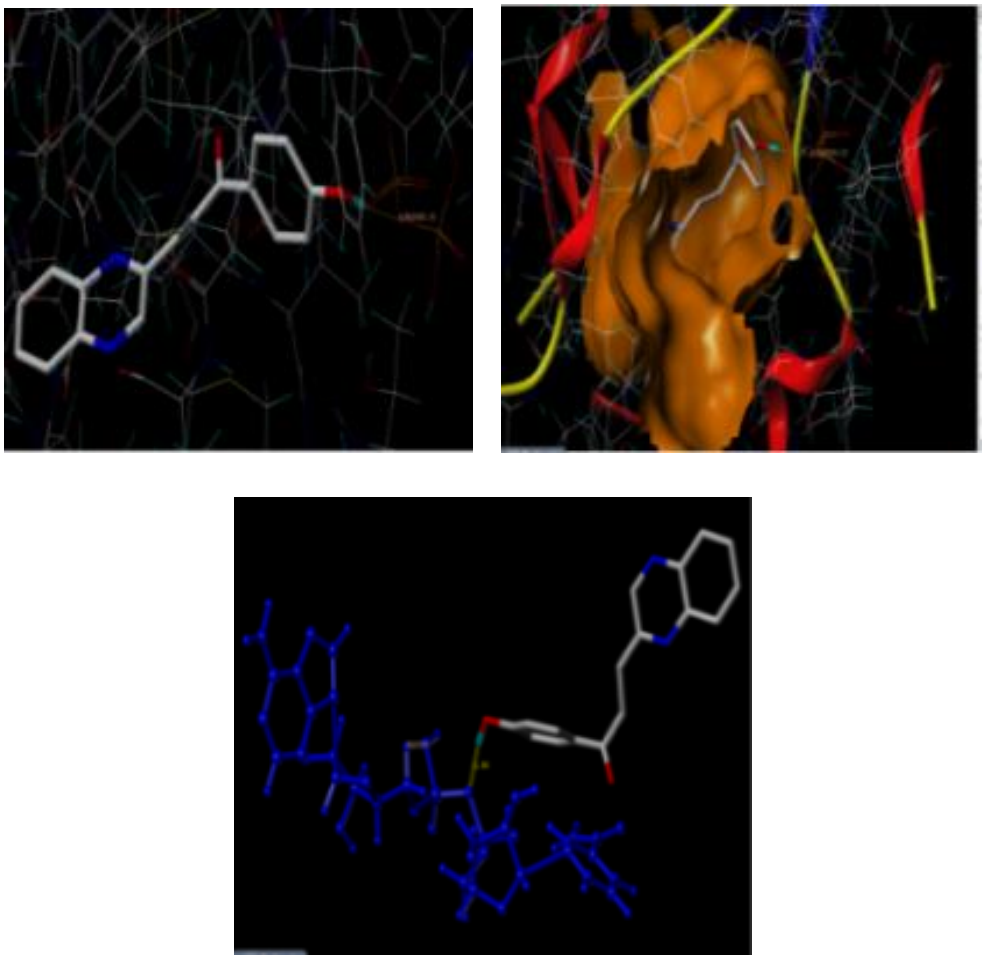


Fig.31. Interaction of **12a** at the binding active site of the enzyme enoylACP reductase (PDB ID: 4TZK). Ligand and key residues are represented as stick models and coloured by atom type, here the proteins are represented by red dotted lines. White: hydrogen atom; red: oxygen atom; dark blue: nitrogen atom; blue: the backbone and carbon atom of compound **12a.** Quinoxalinyl moiety of compound **12a** embedded in the binding pocket of enoylACP reductase. Hydrogen atom of hydroxyl group at 4^{th} position of aromatic ring makes an hydrogen bonding interaction with oxygen of NAD500.

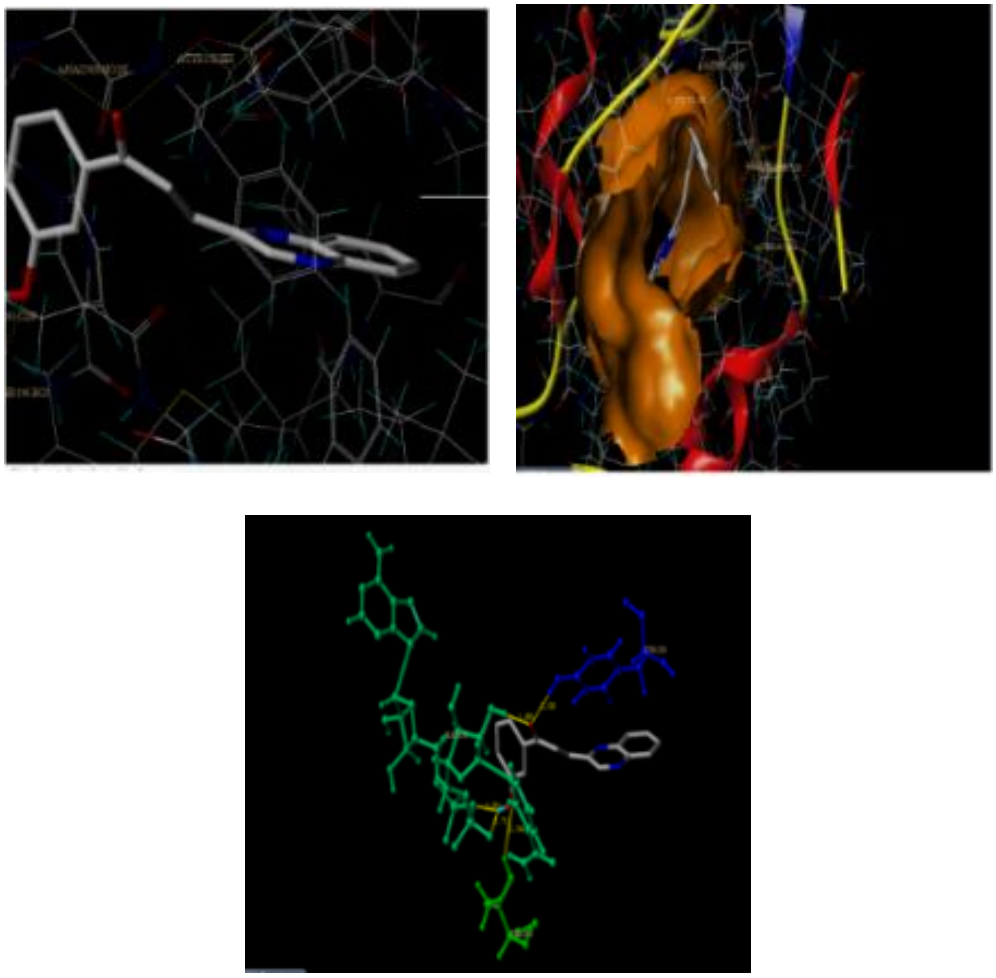


Fig.32. Docked view of compound 1**2h** at the active site of the enzyme PDB ID: 4TZK. Compound 1**2h** makes five hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), among them three interactions are raised from the hydrogen of the hydroxyl group present at 3rd position of aromatic ring.

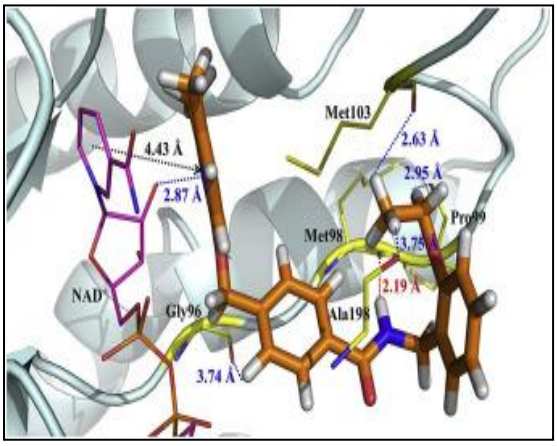


Fig.33.Docked view at active site of enoyl reductase (InhA) complexed with 1-cyclohexyl-*N*-(3, 5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK)

4.3b.3.2. *in-vitro* anti-tubercular screening*:*

The newly synthesized chalcones 1**2a-n** has been screened for their *in vitro* anti-tubercular activity using Alamar Blue Dye method,[46] this methodology is non-toxic, uses thermally stable reagents and showed good correlation with the proportional and BACTEC radiometric method. The *Mycobacterium tuberculosis* strain used was $MtbH_{37}Rv$, while the standard drugs were Streptomycin, Pyrazinamide, and Ciprofloxacin. (**Table 28, Fig 34A**)

Table 28 *in-vitro* anti-tubercular activity[a] of synthesized quinoxalinyl chalcones 1**2a-n**

Sample 12	**Ar**	**Minimum inhibitory concentration-MIC (µg/mL)[b]**
a	4-Hydroxyphenyl	3.12
b	4-Aminophenyl	25
c	4-Bromophenyl	50
d	4-Methoxyphenyl	25
e	4-Chlorophenyl	25
f	4-Fluorophenyl	25
g	4-Pyridinyl	6.25
h	3-Hydroxyphenyl	3.12
i	3-Bromophenyl	25
j	Phenyl	25
k	3-aminophenyl	25
l	4-Nitrophenyl	25
m	3-Nitrophenyl	25
n	Naphthyl	25
Streptomycin[c]		6.25
Ciprofloxacin[c]		3.12

Pyrazinamide[c]	3.12

[a]By Alamar Blue Dye assay method, against H_{37}Rv strain

[b]Minimum inhibitory concentration values

[c]Standard drugs- Streptomycin-belong to aminoglycoside family, Pyrazinamide is First line drug, and Ciprofloxacin-is example of Fluroquinolones- Class of Second line drugs

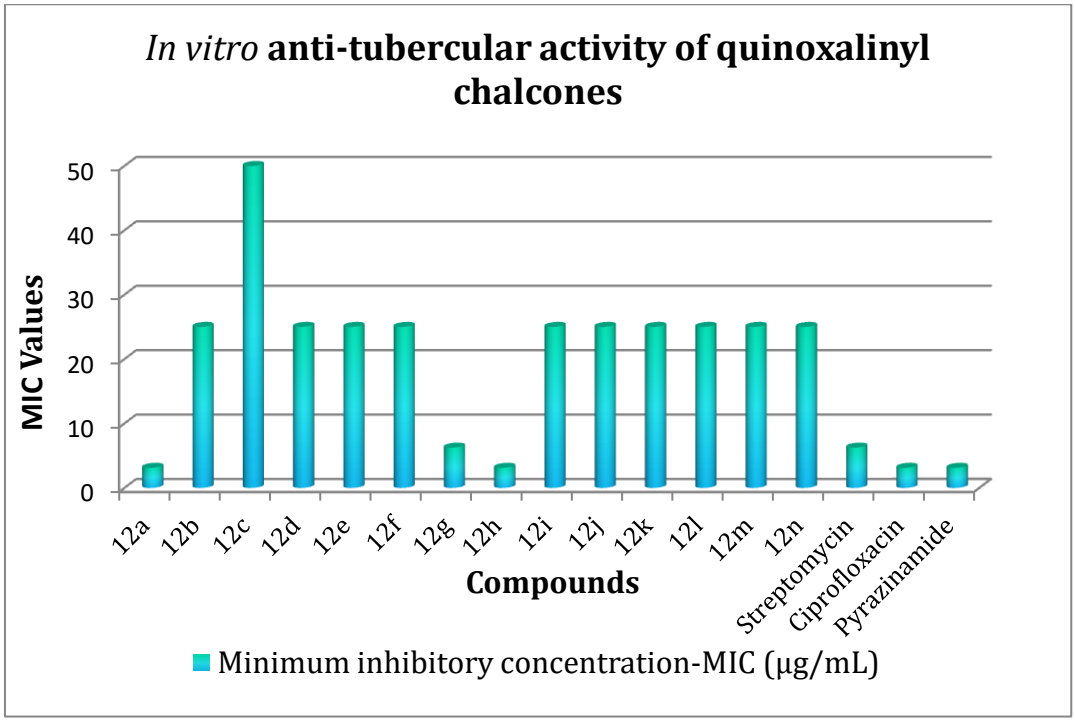


Fig.34A. Graphical representation of anti-tubercular activity: showing the variation in the minimum inhibitory concentration (µg/mL) of all chalcone derivatives **12a-n** against the standards Pyrazinamide, Ciprofloxacin and Streptomycin.

All the synthesized compounds exhibited good to moderate activity, with compounds **12a**, **12g**, **12h**, showing better inhibition in activity compatible with the standard drugs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. (**Fig.34B**)

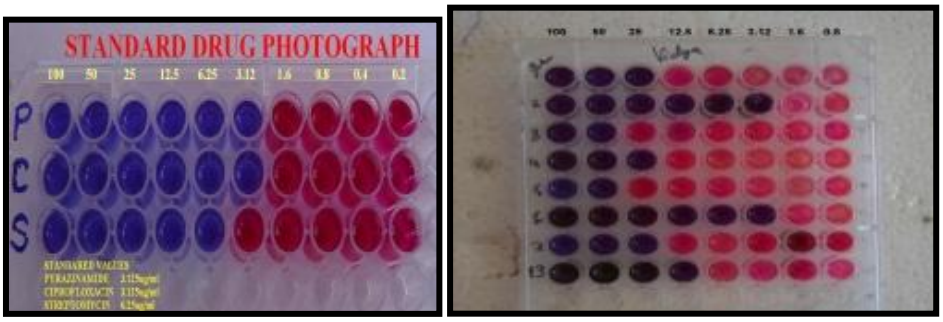


Fig.34B. To the left shows the MIC values for the standard drugs, Streptomycin, Pyrazinamide and Ciprofloxacin, while that to the right shows the MIC values for the synthesized quinoxalinylchalcones; MIC value 3.12µg/mL (2 and 6) is for chalcones **12a** and **12h,** containing the –OH substituent.

From the observations of *in-vitro* anti-tubercular results for the synthesized quinoxalinyl chalcones, following structure-activity relationships (SAR) can be predicted. (**Fig. 35**) The maximal activity exhibited by compounds **12a** and **12h**, is comparable to the standard drugs Pyrazinamide and Ciprofloxacin, indicates that presence of –OH substituent increases the anti-tubercular activity. Replacement of phenyl ring in **12j** by heterocyclic pyridinyl ring in **12g** increases the potency of the compound for anti-tubercular activity. Presence of halogens, methoxy, amino, nitro groups had no significant effect on the anti-tubercular activity. Replacement of phenyl ring in **12j** by naphthyl ring in **12n** decreases the potency of the compound for anti-tubercular activity, indicating that lipophilicity has adverse effect on the activity.

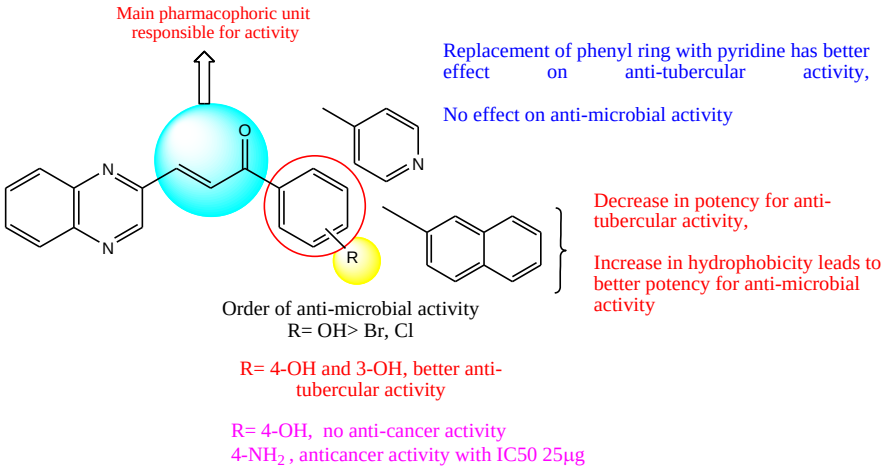


Fig. 35. Structural representation of SAR activity for anti-microbial, anti-tubercular activity.

4.4. CONCLUSION

This work on newly synthesized molecular hybrids, investigates the role of quinoxalinylchalcone moiety as a versatile pharmacophoric unit, having range of biological activities. The study tells us how molecular hybridisation of quinoxaline and chalcone can be exploited in search of novel drug targets to treat highly dreaded diseases like tuberculosis and cancer. The MTT assay studies revealed that chalcone **12b**, containing the $–NH_2$ group, exhibit good anticancer activity. The chalcone **12a,** containing the –OH group showed specifically anti-bacterial and *in vitro* anti-tubercular activity, but were not effective as anticancer agents. *In silico* studies demonstrated that, quinoxalinyl chalcones **12a** and **12h** exhibited good inhibitory action towards the enzyme enoyl ACP reductase, and this has been an additive research on such chalcones. The wide array of bioactivities exhibited by quinoxalinyl chalcones, reflects the importance of such heterocyclic moiety in designing novel drug targets in drug development. These novel findings has thrown light on how a simple quinoxalinyl chalcone hybridisation can lead to a potential drug candidate for treating dreaded diseases like cancer, tuberculosis etc. Further, such chalcones can be excellent building blocks to heterocycles with great diversity, a heterocyclic scaffold, having varied biological applications.

4.5. EXPERIMENTAL

4.5.1. Synthesis of quinoxaline-2-carbaldehyde **11**

Quninoxaline-2-carbaldehyde was synthesized according to the previous report[28] and literature known procedure.[29] At room temperature, glacial acetic acid (1.5 ml), *o*-phenylenediamine (5gm, 46mmol), hydrazine hydrate (1.5ml) and a pinch of Sodium bicarbonate were added to solution of *D*-glucose (8.5gm, 46mmol) in wat.er (12.5ml) and the reaction was heated under reflux for 5hrs on sand bath the product, 2-(*D*-arabinotetrahydroxybutyl)-quinoxaline, which got precipitated on cooling the solution in ice, was filtered and washed with water. It was further purified by recrystallization from hot water. The recrystallized product 2-(*D*-arabinotetrahydroxybutyl)-quinoxaline (0.1gm,0.3mmol) was dissolved in water (6ml) contacting glacial acetic acid (0.2ml) and to it sodium meta periodate (0.87gm,4.6mmole) was added at room temperature. The reaction was stirred for 16 hrs. After 16 hrs, the reaction mixture was filtered and the filtrate was neutralized with sodium bicarbonate. The compound present in neutral solution was extracted with ether. The ether extract after drying with anhydrous sodium sulphate was evaporated to dryness. The resulting residue was recrystallized from pet ether to give pure quinoxaline-2-carbaldehyde. % Yield: 57; Melting Point: 108-110^{o}C

4.5.2. General Procedure for the Preparation of Chalcone Derivatives **(12a-n)**

This procedure is based on our previous report[27] and Vogel's procedure.[30] To a conical flask containing NaOH solution (1.5eq, 10mL H_2O) was added substituted acetophenones (1mmole) in ethanol (10mL), and the reaction mixture was stirred for 10 minutes to allow enolate formation, To this was added quinoxaline-2-carbaldehyde **1** (1mmole) and the reaction mixture was stirred till completion. After completion of the reaction, as monitored by TLC the reaction mixture was poured in an ice bath and was acidified using conc. HCl. The solid obtained was then filtered, dried and recrystallized using Ethanol. The quinoxalinylchalcone 1**2a-n** was then characterized using IR, NMR (^{1}H, ^{13}C) and HR-MS spectroscopy.

4.5.2.1.*(2E)*-1-(4-hydroxyphenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12a).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 4-hydroxyacetophenone (171mg, 1.26mmol) to yield 1**2a**, as pale yellow solid (272 mg, yield 78%) mp 256-260^{o}C; FT-IR (KBr) cm^{-1}: 3250, 3015, 1655, 1606, 1585, 1262; ^{1}HNMR

(400MHz, DMSO) δ 10.48 (brs, 1H, OH), 9.52 (s, 1H, N=C-H), 8.25 (d, 2H, *J*=8.3Hz, C3 & C5 phenyl ring), 8.1-8.14 (m, 4H, C5-C8 quinoxaline ring), 7.89 (dd, 2H, *J*=8.29Hz, C2 & C6 phenyl ring), 7.83 =(d, 1H, *J*=17.7 Hz, H-C=C-C=O), 6.94 (d, 1H, *J*=17.7Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 157.2, 150.3, 145.8, 142.5, 141.8, 132.5, 129.9, 129.8, 129.1, 129.5, 128.3, 128.0, 123.3, 115.2. ESI-HRMS (m/z): calcd. For $C_{17}H_{12}N_2O_2$ $[M+H]^+$: 277.0971; found; 277.0831.

4.5.2.2. *(2E)*-1-(4-aminophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12b).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **1** (200mg, 1.26mmol) and 4-aminoacetophenone (170mg, 1.26mmol) to yield **12b**, as pale yellow solid as orange solid (260 mg, yield 75%)mp 182-185^{o}C; FT-IR (KBr) cm^{-1}: 3354, 3040, 1658, 1609, 1585; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.48 (s, 1H, N=C-H), 7.98-8.30 (m, 4H, C5-8 quinoxaline ring), 7.96-8.01 (dd, 2H, *J*=8.60Hz, C2 & C6 phenyl ring), 7.81-7.86 (dd, 2H, *J*=8.62Hz, C3 & C5 phenyl ring), 7.80 (d, 1H, *J*=17.30Hz, H-C=C-C=O), 6.71(d, 1H, *J*=17.30Hz, C=CH-C=O), 3.5 (brs, 2H, NH_2) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.3, 149.0, 145.8, 142.5, 141.8, 130.8, 129.1, 129.0, 128.3, 128.9, 127.1, 123.3, 113.7. ESI-HRMS (m/z): calcd. For $C_{17}H_{13}N_3O$ $[M+H]^+$: 276.1130; found; 276.1011.

4.5.2.3.*(2E)*-1-(4-bromophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12c)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde 1**1** (200mg, 1.26mmol) and 4-bromoacetophenone (250mg, 1.26mmol) to yield **12c**, as pale yellow solid (362 mg, yield 85%) mp 126-130^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1685, 1580, 1489, 832; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.68 (s, 1H, N=C-H), 7.85-8.35 (m, 4H, C5-8 quinoxaline ring), 7.65 (dd, 2H, *J*=8.65Hz, C2 & C6 phenyl ring), 7.79 (dd, 2H, *J*=8.60Hz, C3 & C5 phenyl ring), 7.23 (d, 1H, *J*=17.30Hz, H-C=C-C=O), 6.85(d, 1H, *J*=17.30Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.3, 145.8, 142.5, 141.8, 136.5, 132.1, 130.9, 129.1, 129.0, 128.3, 128.0, 124.2, 123.3. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_2BrO$ $[M+H]^+$: 339.0120; found; 339.0211.

4.5.2.4. *(2E)*-1-(4-methoxyphenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12d)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 4-methoxyacetophenone (189mg, 1.26mmol) to yield **12d** as pale yellow solid (292mg, yield 80%) mp 94-98^{o}C; FT-IR (KBr) cm^{-1}: 3070, 1671, 1600, 1514, 1259; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.39 (s, 1H, N=C-H), 7.86-8.34 (m, 4H, C5-8 quinoxaline ring), 7.88 (dd, 2H,

J=8.3Hz, C2 & C6 phenyl ring), 7.02 (dd, 2H, *J*=8.3Hz, C3 & C5 phenyl ring), 7.25 (d, 1H, *J*=17.3Hz, H-C=C-C=O), 6.84(d, 1H, *J*=17.3Hz, C=CH-C=O); 3.85 (s, 3H, OCH3) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 160.2, 150.3, 145.8, 142.5, 141.8, 132.5, 130.5, 129.1, 129.0, 128.5, 128.0, 123.3, 114.1, 55.4. ESI-HRMS (m/z): calcd. For $C_{18}H_{14}N_2O_2$ $[M+H]^+$: 291.1120; found; 291.1091.

4.5.2.5.*(2E)*-1-(4-chlorophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12e).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde 1**1** (200mg, 1.26mmol) and4-chloroacetophenone (195mg, 1.26mmol) to yield **12e** as pale yellow solid as pale yellow solid (280mg, yield 75%); mp 124-126^{o}C; FT-IR (KBr) cm^{-1}: 3095, 1683, 1588, 1493, 758; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.30 (s, 1H, N=C-H), 7.86-8.35 (m, 4H, C5-C8 quinoxaline ring), 7.51 (dd, 2H, *J*=8.6Hz, C2 & C6 phenyl ring), 7.7 (dd, 2H, *J*=8.6Hz, C3 & C5 phenyl ring), 7.2 (d, 1H, *J*=17.69Hz, H-C=C-C=O), 6.84 (d, 1H, *J*=17.69Hz, C=CH-C=O).^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.3, 145.8, 142.5, 141.8, 136.6, 132.1, 130.9, 129.8, 129.0, 128.5, 128.0, 124.2, 123.3. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_2ClO$ $[M+H]^+$: 295.0630; found; 295.0560.

4.5.2.6. ***(**2E)*-1-(4-Fluorophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12f).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 4-fluoroacetophenone (174mg, 1.26mmol) to yield **12f** as pale yellow solid (280mg, yield 80%) mp 256-260^{o}C; FT-IR (KBr) cm^{-1}: 3070, 1677, 1597, 1511, 685; ^{1}HNMR (400MHz, $CDCl_3$): δ 9.31(s, 1H, N=C-H), 7.8-8.3(m, 4H, C5-C8 quinoxaline ring),7.4 (dd, 2H, *J*=8.67Hz, C2 & C6 phenyl ring), 7.7 (dd, 2H, *J*=8.67Hz, C3 & C5 phenyl ring), 7.2 (d, 1H, *J*=14Hz, H-C=C-C=O), 6.8 (d, 1H, *J*=14Hz, C=CH-C=O); ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 163.5, 150.3, 145.8, 142.5, 141.8, 134.6, 132.0, 130.5, 129.8, 129.5, 128.3, 128.0, 123.3, 115.55. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_2FO$ $[M+H]^+$: 279.0920; found; 279.0850.

4.5.2.7.***(**2E)*-1-(4-pyridinyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12g).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 4-acetylpyridine (152mg, 1.26mmol) to yield **12g** as light brown solid (215mg, yield 65%) mp 150-152^{o}C; FT-IR (KBr) cm^{-1}: 3080, 1680, 1600, 1560; ^{1}HNMR (400MHz, $CDCl_3$): δ 9.5(s, 1H, N=C-H), 9.02 (dd, 1H, C-5 quinoxaline), 8.64 (d, 2H, C-2 and C-6 phenyl), 8.42 (dd, 1H, *J*=8.67Hz, C-8 quinoxaline), 8.36 (dd, 1H, *J*=7.67Hz, C-6 quinoxaline), 8.22 (dd, 1H, *J*=8.67Hz, C-7 quinoxaline), 7.89 (d, 2H, *J*=8.67Hz, C-3 and C-5 phenyl), 7.52 (d, 1H,

J=15Hz, H-C=C-C=O), 7.4 (d, 1H, *J*=15Hz, C=CH-C=O); ^{13}C NMR (75MHz, $CDCl_3$): δ 196.7, 150.9, 149.6, 143.5, 142.1, 142.2, 141.1, 130.9, 130.6, 129.8, 129.7, 129.4, 128.1, 127.2, 124.3, 121.3. ESI-HRMS (m/z): calcd. For $C_{16}H_{11}N_3O$ $[M+H]^+$: 262.0971; found; 279.0891.

4.5.2.8. *(2E)*-1-(3-hydroxyphenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one (**12h).**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 3-hydroxyacetophenone (171mg, 1.26mmol) to yield **12h**, as brown solid (279 mg, yield 80%) mp 144-146^{o}C; FT-IR (KBr) cm^{-1}: 3300, 3100, 1690, 1600, 1590, 1250; ^{1}HNMR (400MHz, DMSO-d_6): 9.2 (s, 1H, N=C-H), 8.25 (d, 1H, *J*= 8.3Hz, C2 phenyl ring), 7.86 (dd, 1H, *J*=1.88, 8.0Hz, C6 phenyl ring), 7.79 (dd, 1H, *J*=1.88, 1.7Hz, C4 phenyl ring), 7.2-8.2 (m, 4H, C5-C8 quinoxaline ring), 7.89 (dd, 2H, *J*=8.3Hz, 1.88Hz, C5 phenyl ring), 7.9 (d, 1H, *J*=17.7 Hz, H-C=C-C=O), 6.8 (d, 1H, *J*= 17.7Hz, C=CH-C=O), 3.5 (br s, 1H, OH). δ ^{13}C NMR (75MHz, DMSO-d_6): 189.5, 150.3, 147.3, 145.8, 142.5, 142.2, 140.1, 130.8, 129.2, 129.0, 128.5, 128.0, 123.3, 117.0, 115.5. ESI-HRMS (m/z): calcd. For $C_{17}H_{12}N_2O_2$ $[M+H]^+$: 277.0970; found; 277.0800.

4.5.2.9. *(2E)*-1-(3-bromophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12i)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 3-bromoacetophenone (250mg, 1.26mmol) to yield **12i**, as brown solid (400mg, yield: 95%) mp. 120-122^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1690, 1600, 1500, 750; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.37 (s, 1H, N=C-H quinoxaline), 7.86-8.35 (m, 4H, C5-8 quinoxaline ring), 7.86 (dd, 1H, *J*=1.88, 8.0Hz, C6 phenyl ring), 7.79 (dd, 1H, *J*=1.88, 1.7Hz, C2 phenyl ring),7.50 (dd,1H, *J*=7.8, 8.0Hz, C4 phenyl ring), 7.40 (dd, 1H, *J*=7.8, 1.75Hz, C5 phenyl ring), 7.30 (d, 1H, *J*=17.30Hz, H-C=C-C=O), 6.86 (d, 1H, *J*=17.30Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.10, 150.4, 145.8, 142.25, 141.9,140.6, 133.0, 131.1, 130.3, 129.5, 129.0, 129.0, 128.6, 128.1, 127.2, 123.8, 122.8. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_2BrO$ $[M+H]^+$: 339.0121; found; 339.0180.

4.5.2.10. *(2E)*-1-(phenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one**(12j)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (230mg, 1.26mmol) and acetophenone (151mg, 1.26mmol) to yield 1**2j**, as dark brown solid (283mg, yield 70%) mp 136-138^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1680, 1600, 1500; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.0 (s, 1H, N=C-H quinoxaline), 7.9-8.35 (m, 4H, C5-8 quinoxaline ring), 7.76 (d, 1H, *J*=16 Hz, H-

C=C-C=O), 7.35-7.55 (m, 5H, phenyl ring), 6.82 (d, 1H, *J*=16 Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 187.1, 148.4, 145.8, 144.9, 142.0, 141.4, 133.7, 129.9, 128.9, 128.5, 128.5, 128.2, 128.0, 127.3. ESI-HRMS (m/z): calcd. For $C_{17}H_{12}N_2O$ $[M+H]^+$: 261.0940; found; 261.0850.

4.5.2.11. *(2E)*-1-(3-aminophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12k)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 3-aminoacetophenone (170mg, 1.26mmol) to yield 1**2k**, as light red solid (260 mg, yield 75%) mp 156-158^{o}C; FT-IR (KBr) cm^{-1}: 3200, 3300, 3050, 1690, 1600, 1500; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.35 (s, 1H, N=C-H), 7.98-8.40 (m, 4H, C5-8 quinoxaline ring), 8.1 (dd, 1H, *J*=1.8, 1.4Hz, C2 phenyl ring), 7.9 (dd, 1H, *J*=1.8, 8.2Hz, C6 phenyl ring), 7.4 (dd, 1H, *J*=8.3, 8.2Hz, C5 phenyl ring), 7.30 (d, 1H, *J*=17.70Hz, H-C=C-C=O), 7.25 (dd, 1H, *J*=1.4, 8.24Hz, C4 phenyl ring) 6.90 (d, 1H, *J*=17.70Hz, C=CH-C=O), 3.7 (br s, 2H, NH_2) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.3, 147.6, 145.8, 142.5, 141.9, 140.1, 130.8, 129.2, 129.0, 128.3, 128.1, 123.3, 117.0, 115.8, 115.5. ESI-HRMS (m/z): calcd. For $C_{17}H_{13}N_3O$ $[M+H]^+$: 276.1130; found; 276.1010.

4.5.2.12. *(2E)*-1-(4-nitrophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12l)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 4-nitro acetophenone (208mg, 1.26mmol) to yield **12l**, as pale yellow solid (230 mg, yield 60%) mp 164-166^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1700, 1600, 1510, 1400; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.48 (s, 1H, N=C-H), 7.85-8.35(m, 4H, C5-8 quinoxaline ring), 7.75 (dd, 2H, *J*=8.5Hz, C2 & C6 phenyl ring), 7.4 (dd, 2H, *J*=8.60Hz, C3 & C5 phenyl ring), 7.23 (d, 1H, *J*=17.30Hz, H-C=C-C=O), 6.85 (d, 1H, *J*=17.30Hz, C=CH-C=O);^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.2, 145.8, 142.5, 141.8, 139.5, 130.4, 129.1, 129.0, 128.3, 128.0, 128.9, 127.7, 123.3, 117.3. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_3O_3$ $[M+H]^+$: 306.0870; found; 306.0750.

4.5.2.13. *(2E)*-1-(3-nitrophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one **(12m)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 3-nitroacetophenone (208mg, 1.26mmol) to yield 1**2m**, as pale brown solid (230mg, yield 60%) mp 205-207^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1700, 1600, 1510, 1350; ^{1}HNMR (400MHz, $CDCl_3$) δ 9.5 (s, 1H, N=C-H), 8.75 (dd, 1H, *J*=1.82, 1.7 Hz, C2 phenyl ring), 7.85-8.30 (m, 4H, C5-8 quinoxaline ring), 8.34 (dd, 1H, *J*=1.70, 8.2, 1.71 Hz, C4 phenyl ring), 8.1 (dd, 1H, *J*=1.8, 8.0

Hz, C6 phenyl ring), 7.79 (dd, 1H, *J*=1.82, 7.96 Hz, C5 phenyl ring), 7.65 (dd, 1H, *J*=1.82,7.96 Hz, C5 phenyl ring), 7.27 (d, 1H, *J*=17.5Hz, H-C=C-C=O), 6.79 (d, 1H, *J*=17.5Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 187.5, 148.5, 144.0, 145.8, 142.5, 141.9, 140.5, 129.5, 129.2, 128.5, 128.4, 128.0, 127.6, 127.5,117.3,116.5. ESI-HRMS (m/z): calcd. For $C_{17}H_{11}N_3O_3$ $[M+H]^+$: 306.0870; found; 305.0690.

4.5.2.14. *(2E)*-1-(2-naphthyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one**(12n)**

This chalcone was synthesized from quinoxaline-2-carbaldehyde **11** (200mg, 1.26mmol) and 2-acetylnaphthalene (214 mg, 1.26mmol) to yield **12n**, as dark brown solid (350mg, yield 90%) mp 130-132^{o}C; FT-IR (KBr) cm^{-1}: 3100, 1690, 1600, 1500 ^{1}HNMR (400MHz, $CDCl_3$) δ 9.30 (s, 1H, N=C-H), 7.85-8.30 (m, 4H, C5-8 quinoxaline ring), 7.58-8.0 (m, 7H, C1-C7), 7.3 (d, 1H, *J*=17.3Hz, H-C=C-C=O), 6.79 (d, 1H, *J*=15.7Hz, C=CH-C=O) ^{13}C NMR (75MHz, $CDCl_3$) δ 189.5, 150.2, 145.8, 142.25, 141.9, 135.3, 133.7, 132.8, 131.1, 129.7, 129.2, 128.5, 128.3, 128.1, 127.7, 126.5,124.7, 123.3. ESI-HRMS (m/z): calcd. For $C_{21}H_{14}N_2O$ $[M+H]^+$: 311.1181; found; 311.1001.

4.5.3. **Biological evaluation**

4.5.3.1. **Agar Diffusion antibacterial and antifungal activity assay**

Quinoxalinyl chalcones **12a-n** were screened for antimicrobial activity against pathogenic strains Gram positive Staphylococcus aureus and gram negative *Pseudomonas aerugenosa* by agar well diffusion method. Media used for this procedure was brain heart infusion agar. Agar plates were brought to room temperature. For inoculums preparation colonies were transferred to the plates using a loop or swab. Turbidity was then adjusted to equal that of a 0.5 McFarland turbidity standard. Alternatively the suspension was standardized with a photometric device. Within 15 minutes of adjusting the inoculums to McFarland 0.5 turbidity standard a sterile cotton swab was dipped into the inoculums and were rotated against the wall of the tube above the liquid to remove excess inoculum. The entire surface of agar plate was swabbed three times by rotating plates approximately 60^{o}C between streaking. The inoculated plate was allowed to stand for atleast 3 minutes but not longer than 15 min before making wells. A hollow tube of 5mm diameter was heated and pressed above the inoculated agar plate and was removed immediately by making a well in the plate. Five wells were made on each plate and 75µL,

50µL, 25µL, 10µL and 5µL of compound were added into the respective wells. Within 15 min of compound application plates were incubated. Plates were incubated for 18- 24h at 37^{o}C in incubator. Diameter of inhibition zone to nearest whole millimeter was measured by holding the measuring device.

4.5.3.2. Anticancer activity

4.5.3.2a. *In vitro* anticancer activity assay

The MTT assay method[38] was used to evaluate anti-cancer activities of the synthesized chalcones. In this assay, the reduction of yellow 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide was measured by mitochondrial succinate dehydrogenase. This method is based on how the MTT solution enters the cells and passes into mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells were then solubilised with an organic solvent (eg. DMSO, isopropanol) and then released, solubilised formazan reagent was measured spectrophotometrically. The IC_{50} (half maximal inhibitory concentration) value was determined for the selected Chalcones. MTT stock solution was prepared by taking 5mg in 1 mL of PBS. MCF-7 (human procured from NCCS, Pune) cell lines have been used for the study. The cell lines were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum(FCS), containing 5% mixture of Gentamicin (10µg), Penicillin (100units/mL) and streptomycin (100µg/mL) in presence of 5% CO_2 at 37^{o}C for 48-72 hours.

4.5.3.2b. *In-vitro* cytotoxicity assay

In-vitro growth inhibition effect of test compound was assessed by colorimetric or spectrophotometric determination of conversion of MTT into "Formazan blue" by living cells. The supernatant was removed from the plate and fresh MEM solution was added and treated with different concentrations of extract or compound appropriately diluted with DMSO. Control group contains only DMSO. In this study, 10, 20, 25, 30 and 50ul of the stock solution (10mg/ml prepared in DMSO) were added to respective wells containing 100ul of the medium. So, the final concentrations were 10, 20, 25, 30 and 50ug/ml. After 48hrs incubation at 37ºC in a humidified atmosphere of 5% CO_2, stock solution of MTT was added to each well (20µl, 5mg per ml in sterile PBS) for further 4 h incubation. The supernatant was carefully aspirated, the precipitated crystals of "Formazan blue' were solubilised by adding DMSO (100µl) and

optical density was measured at wavelength of 570nm by using LISA plus. The results represent the mean of five readings. The concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control.

$$\text{Surviving cell (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD of control}} \times 100$$

4.5.4. **Anti-tubercular evaluation**

4.5.4.1. Molecular Docking study

The crystal structures used were *Mycobacterium tuberculosis* enoylreductase (InhA) complexed with 1-Cyclohexyl-*N*-(3, 5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) for the docking studies, obtained from the Protein Data Bank. The protein was prepared for docking by adding polar hydrogen atom with Gasteiger-Huckel charges[44] and water molecules were removed. The 3D structure of the ligands was generated by the SKETCH module implemented in the SYBYL program (Tripos Inc., St. Louis, USA) and its energy-minimized conformation was obtained with the help of the Tripos force field using Gasteiger-Huckel charges and molecular docking was performed with Surflex-Dock program that is interfaced with Sybyl-X 2.0.[45] and other miscellaneous parameters were assigned with the default values given by the software.

4.5.4.2. *In vitro* anti-tubercular activity assay

The anti-mycobacterial activity of compounds were assessed against *Mycobacterium tuberculosis* H_{37}RV strain using microplate Alamar Blue assay (MABA).[46] Briefly, 200µl of sterile deionized water was added to all outer perimeter of sterile 96 wells plate to minimized evaporation of medium during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds was made directly on plate.The final drug concentrations tested were 100 to 0.2 µg/ml.Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

4.6. REFERENCES

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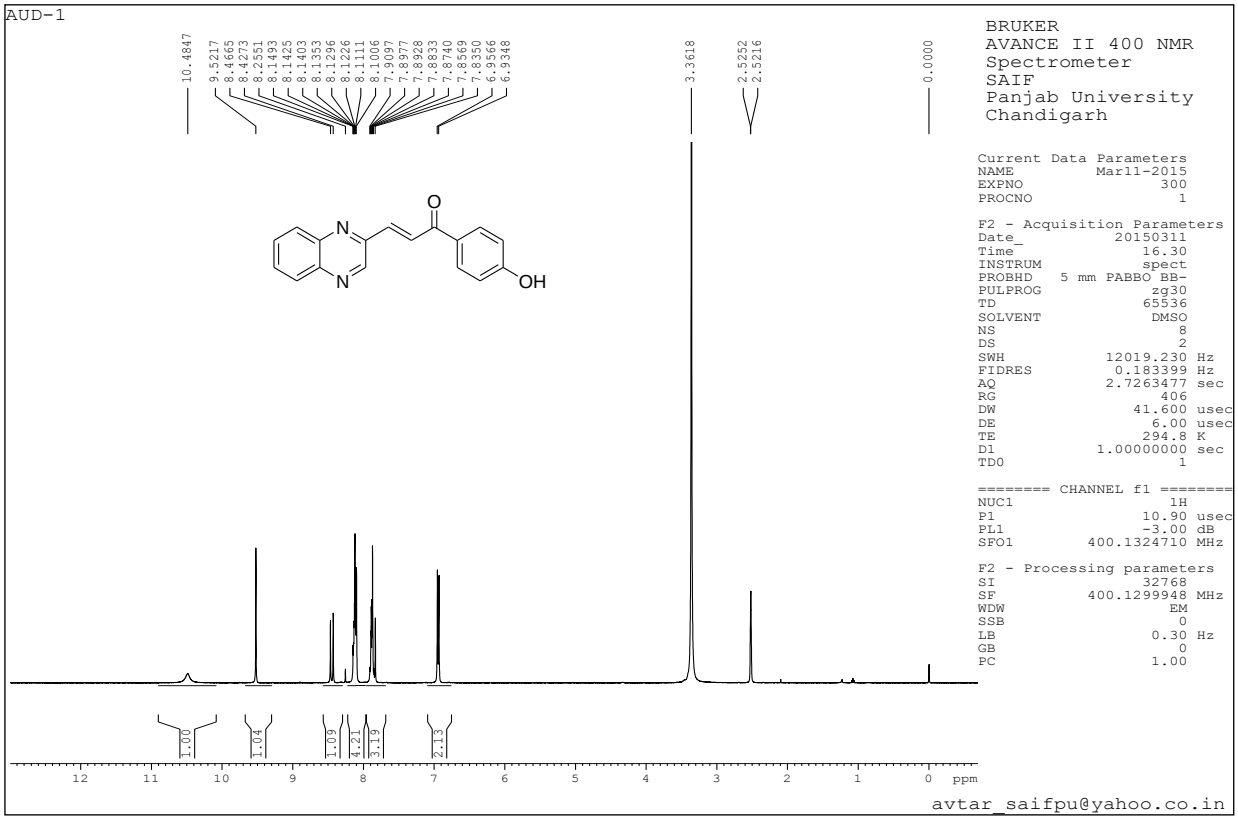

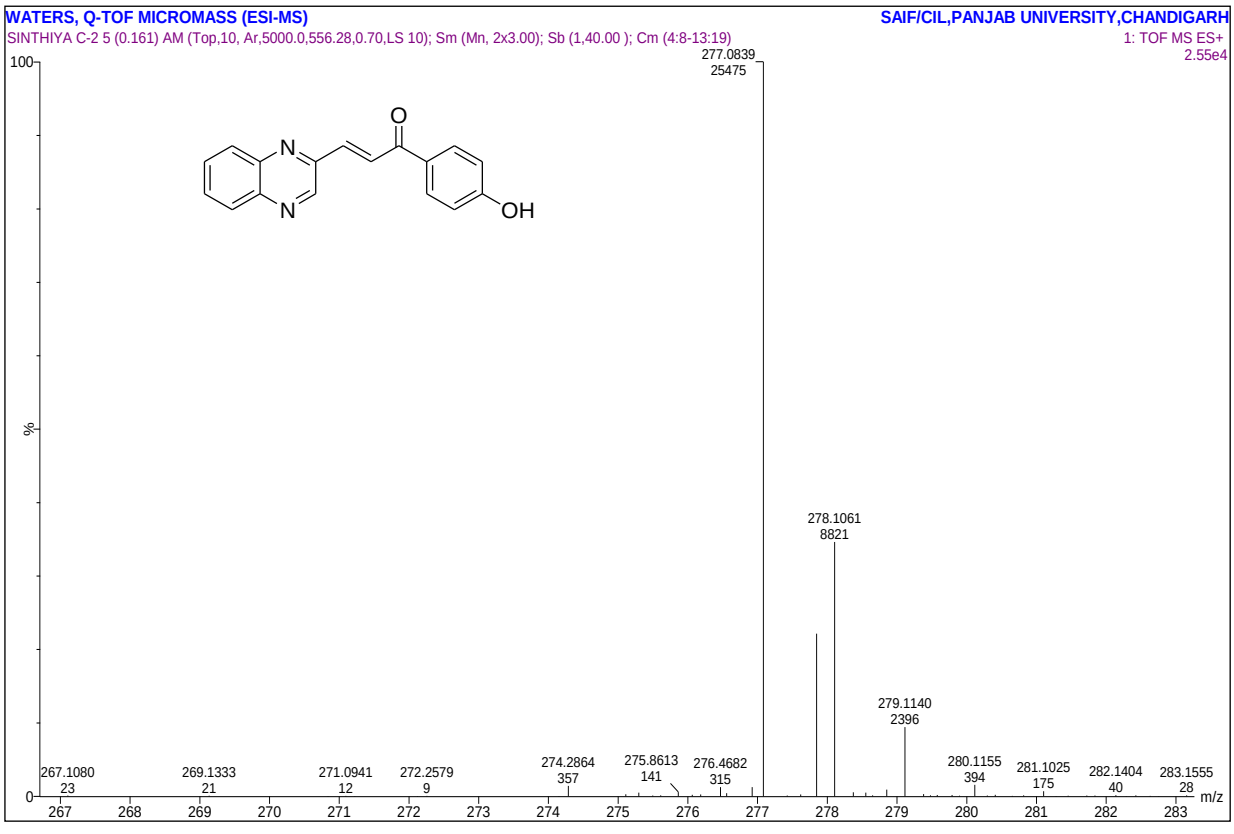

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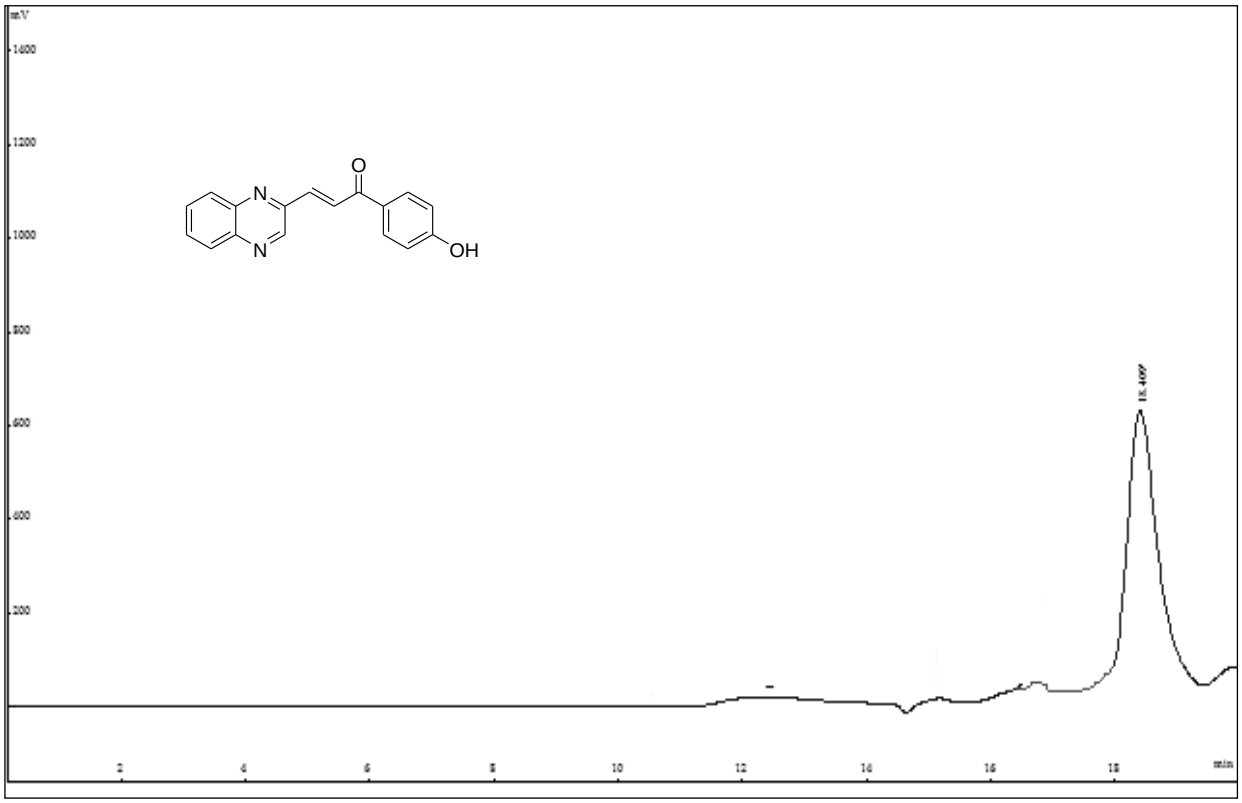

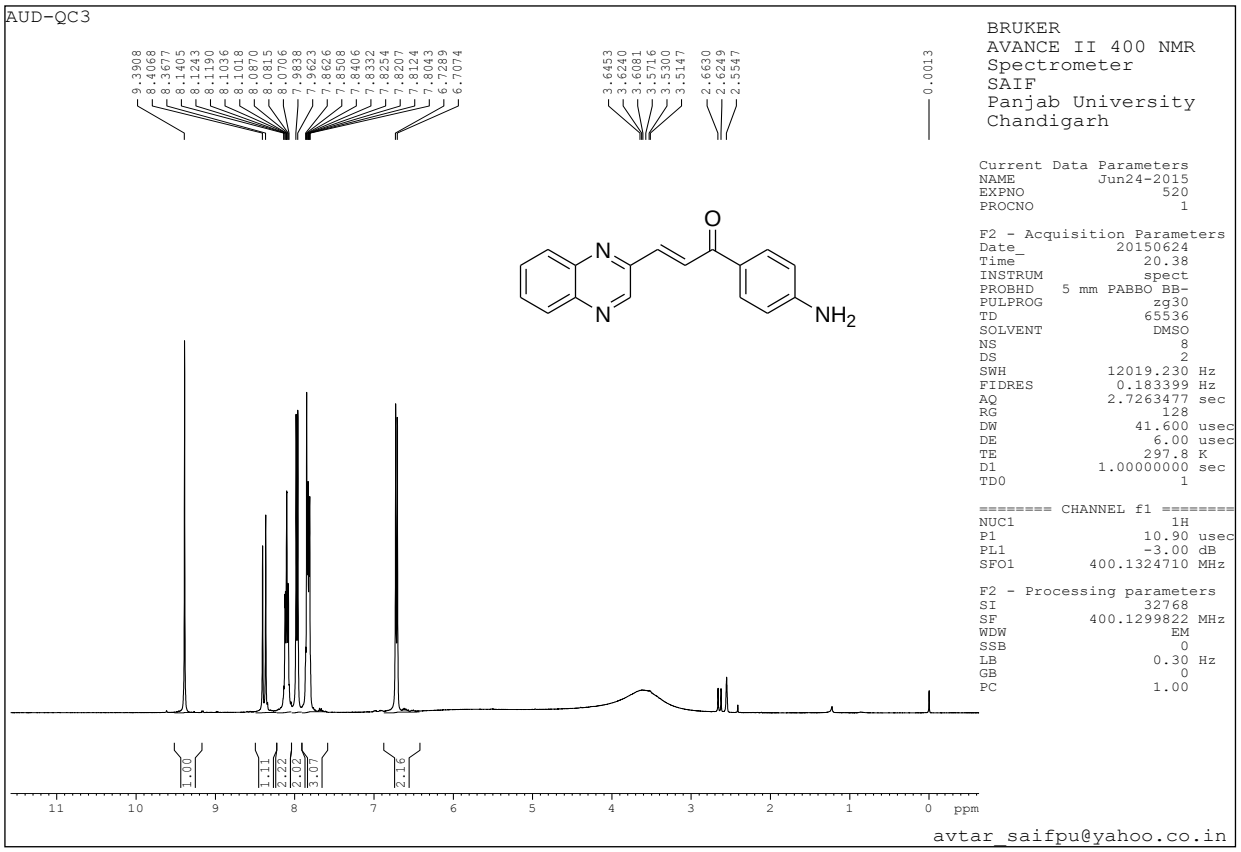
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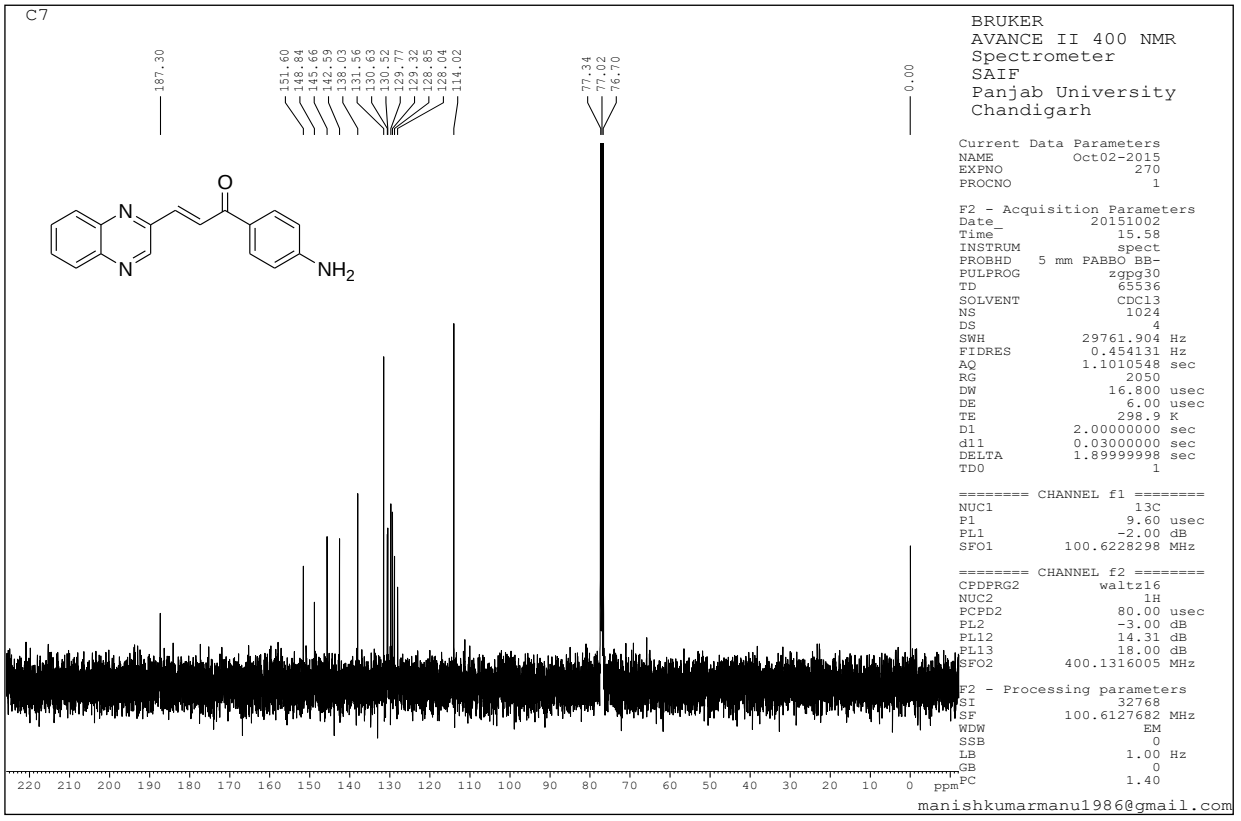
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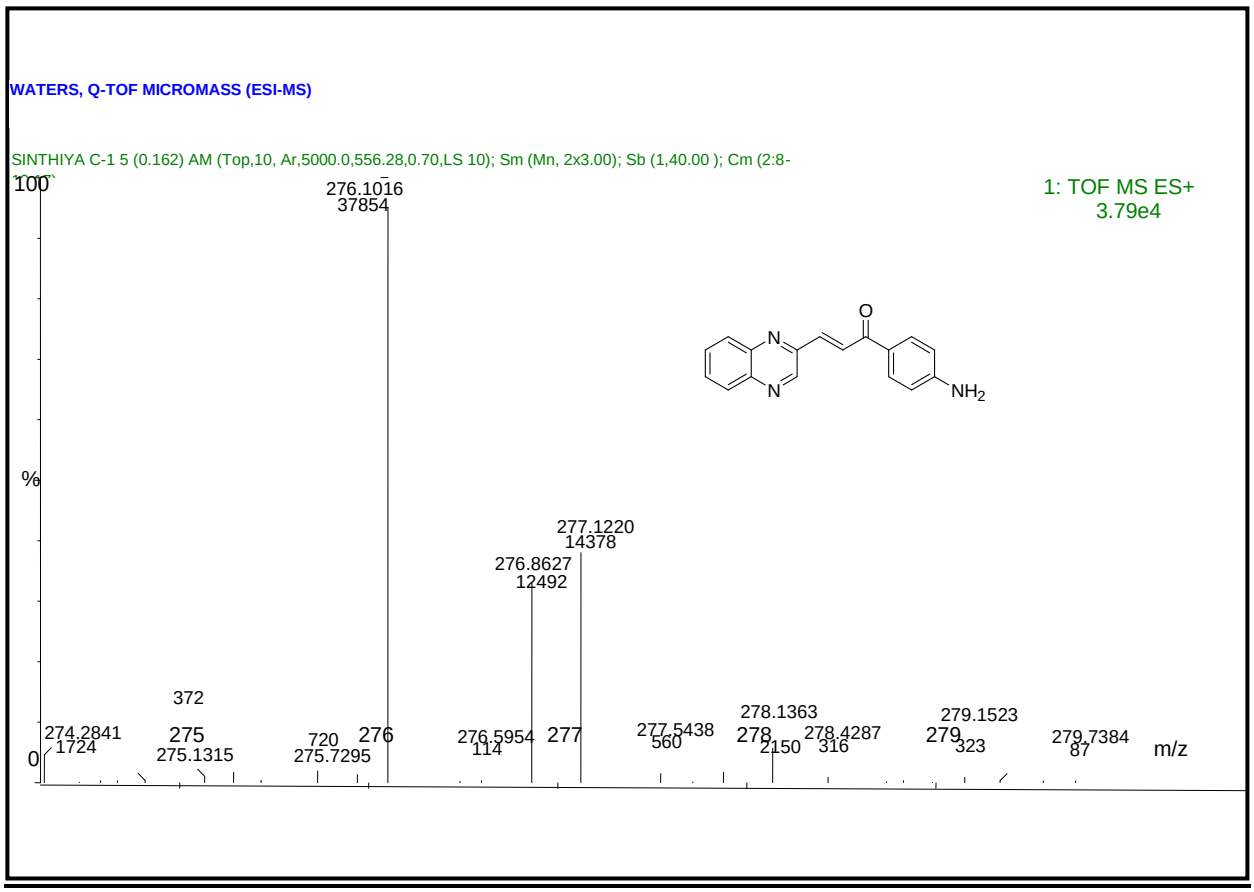
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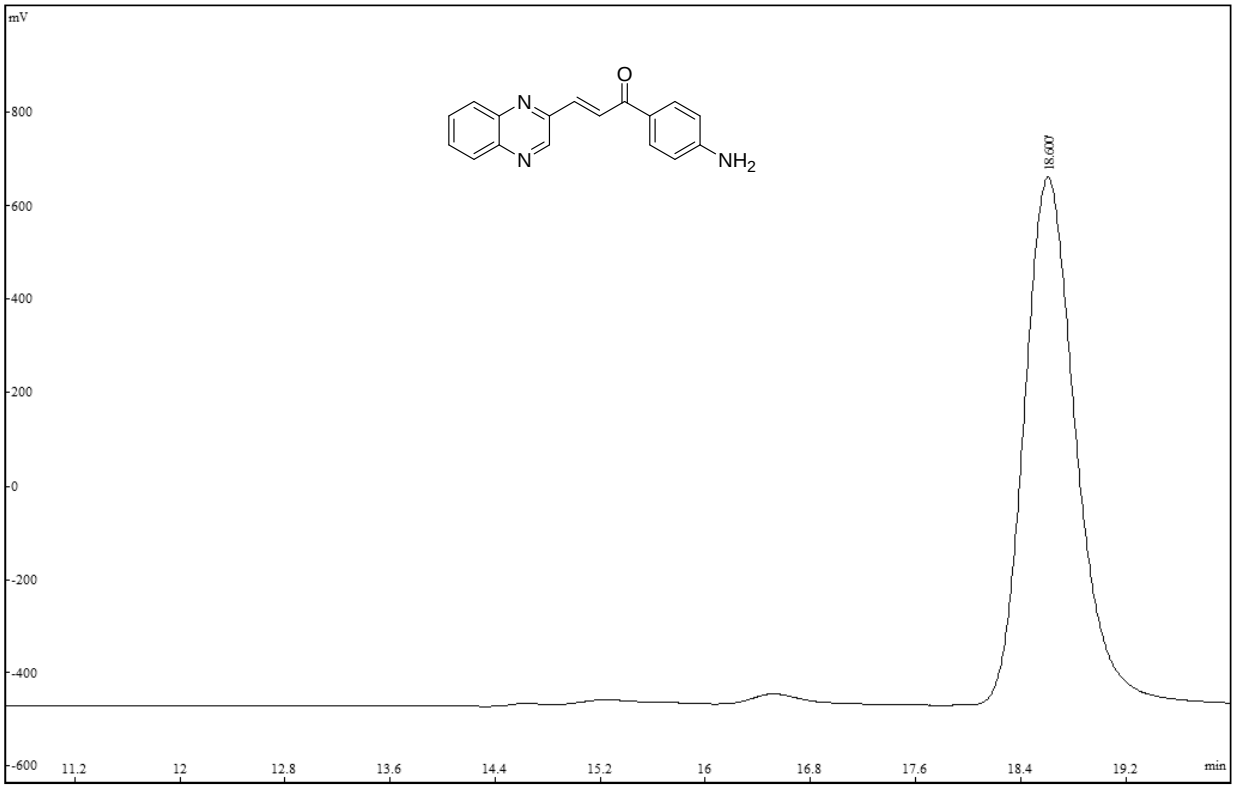
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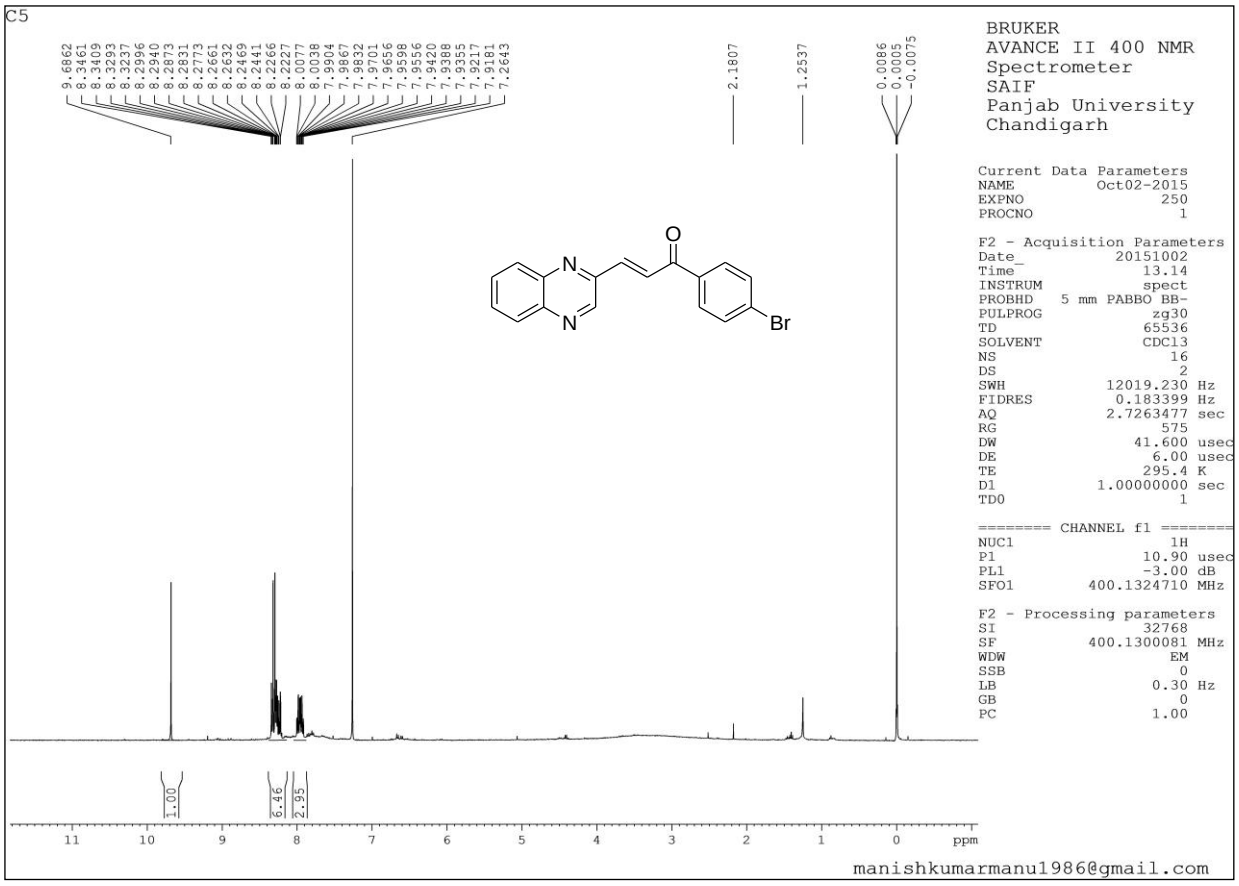
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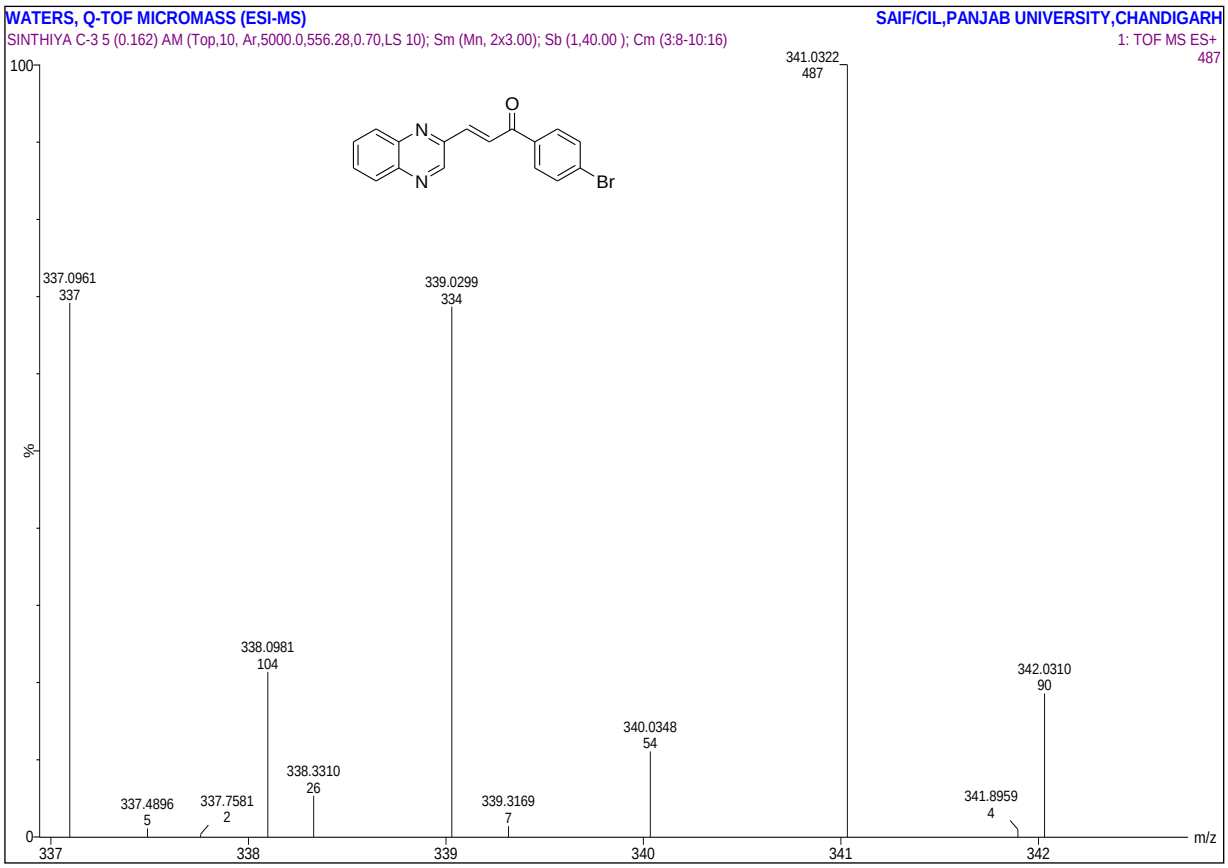
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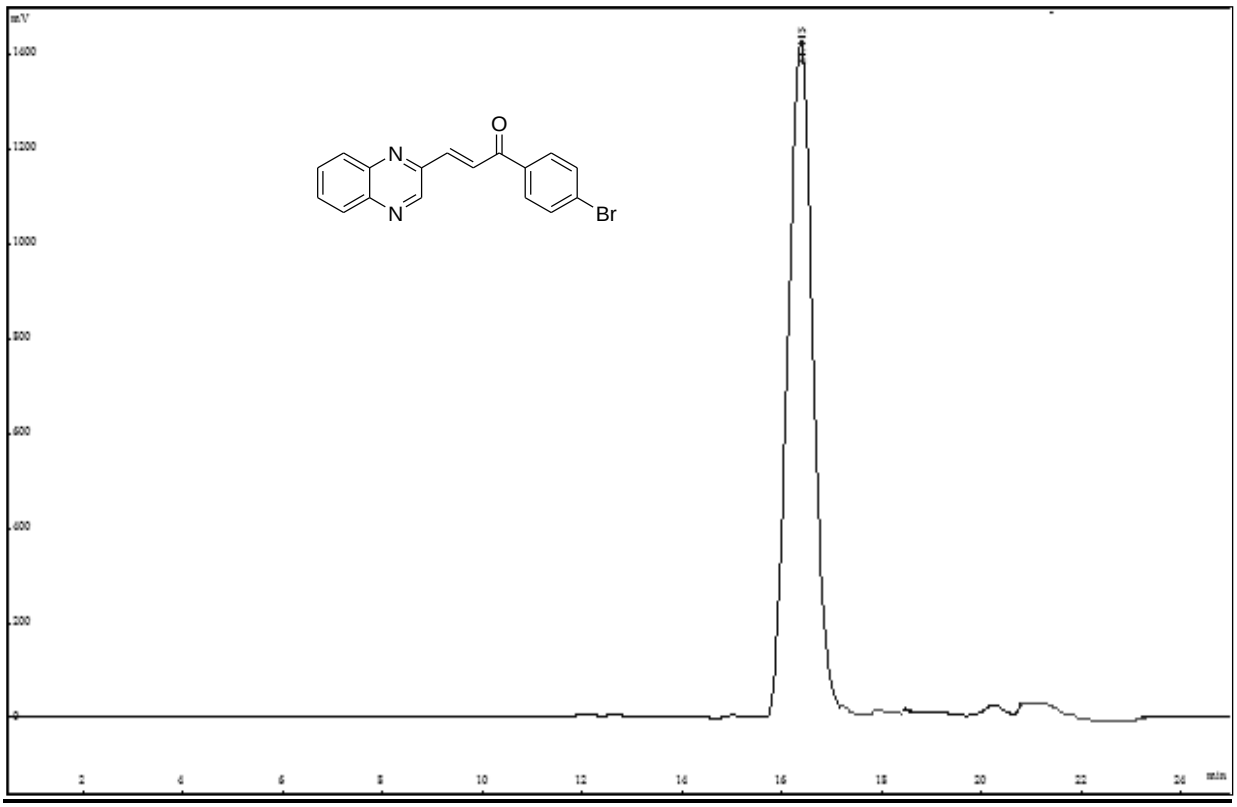
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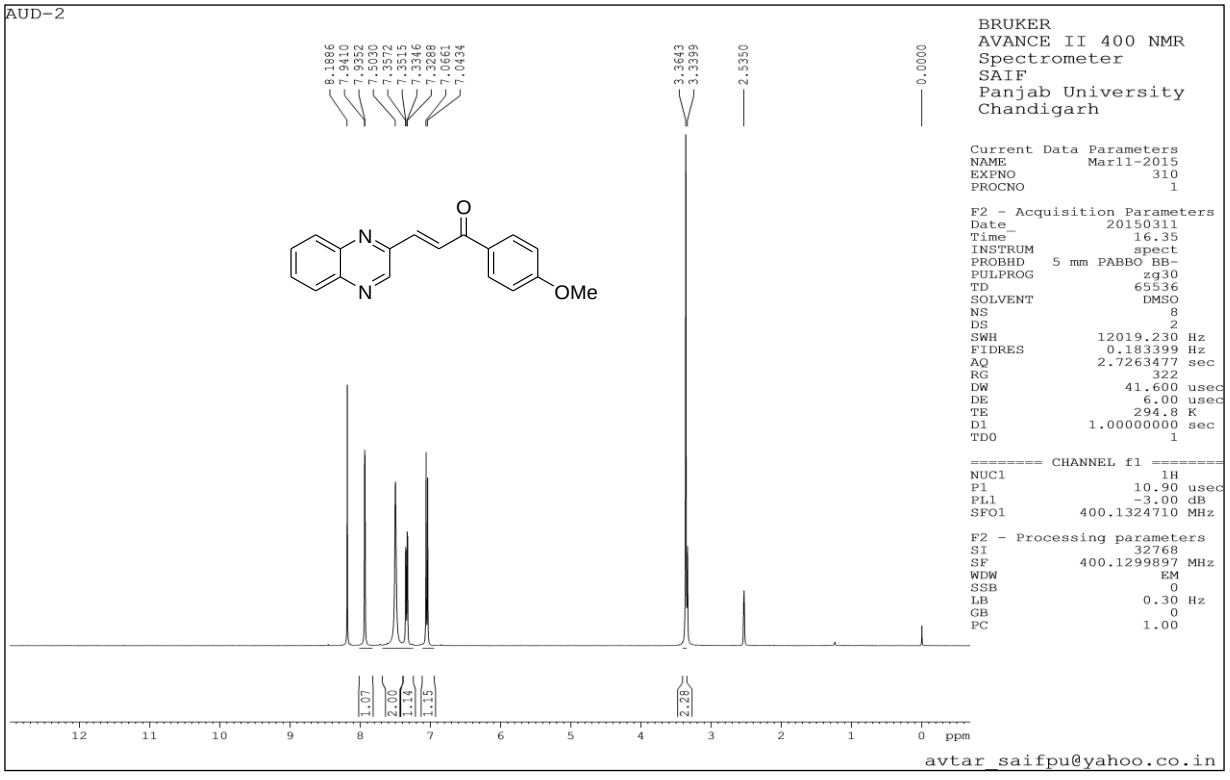
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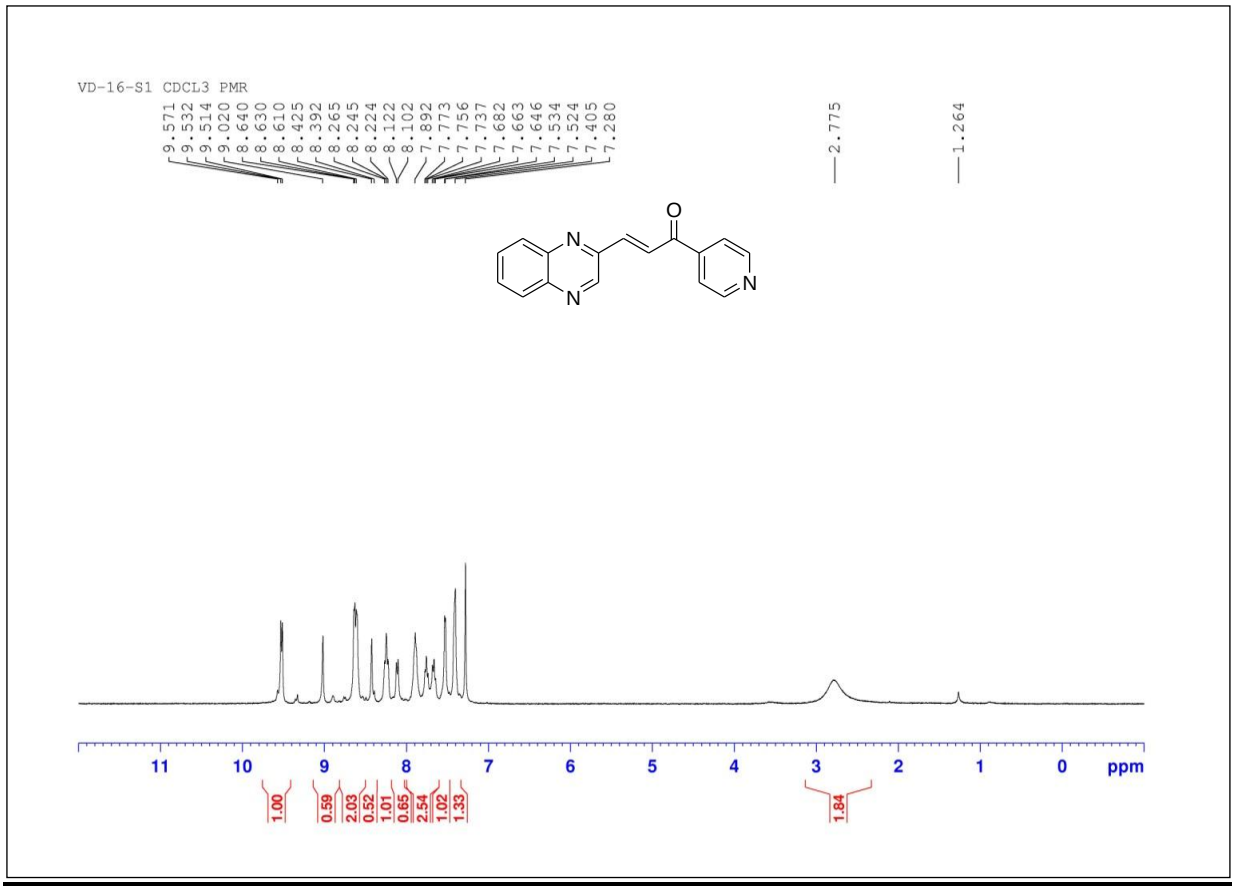
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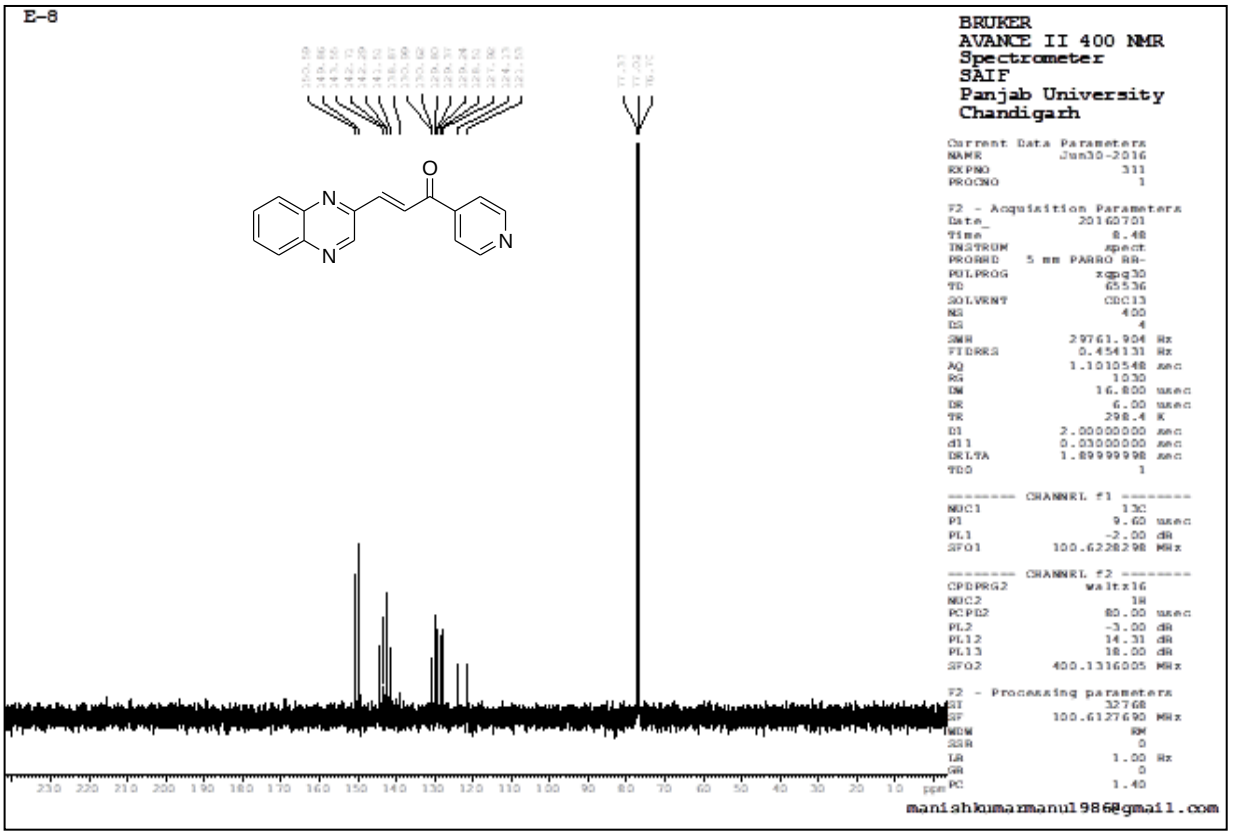
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manishkumarmanul986@gmail.com

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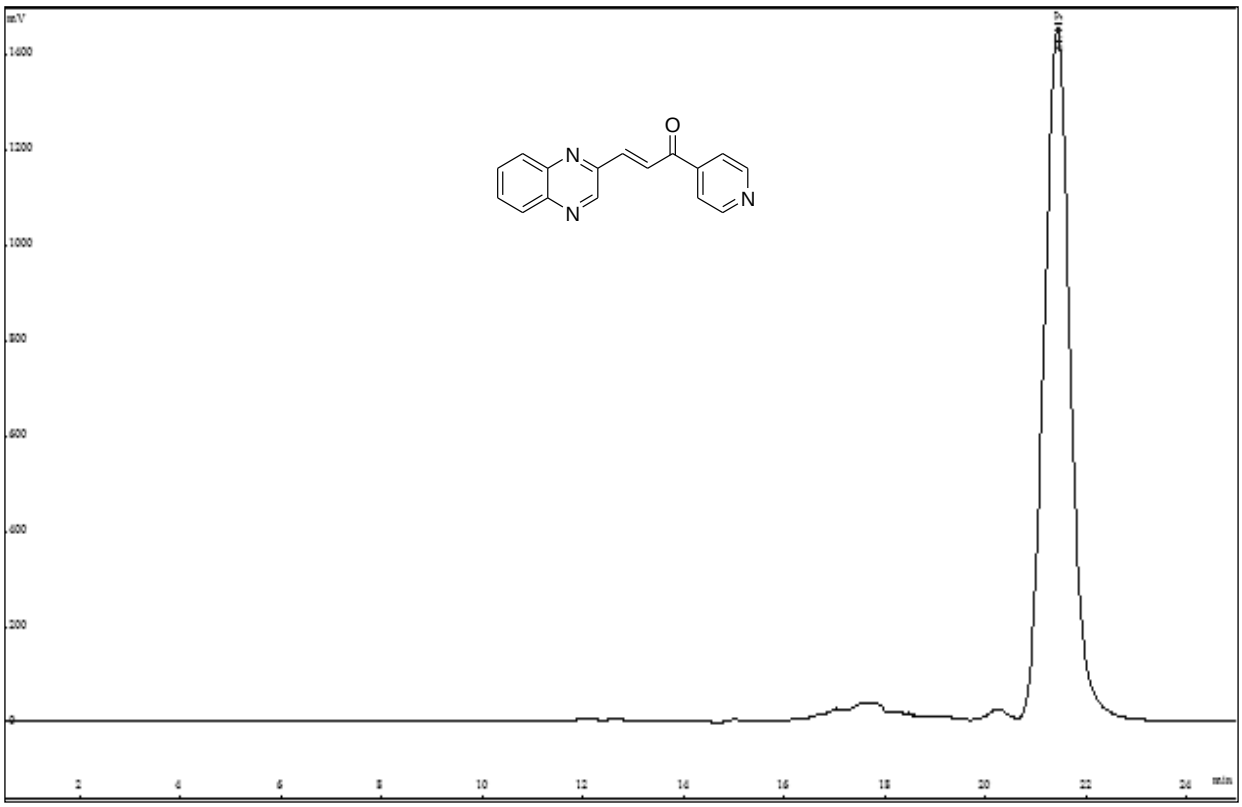
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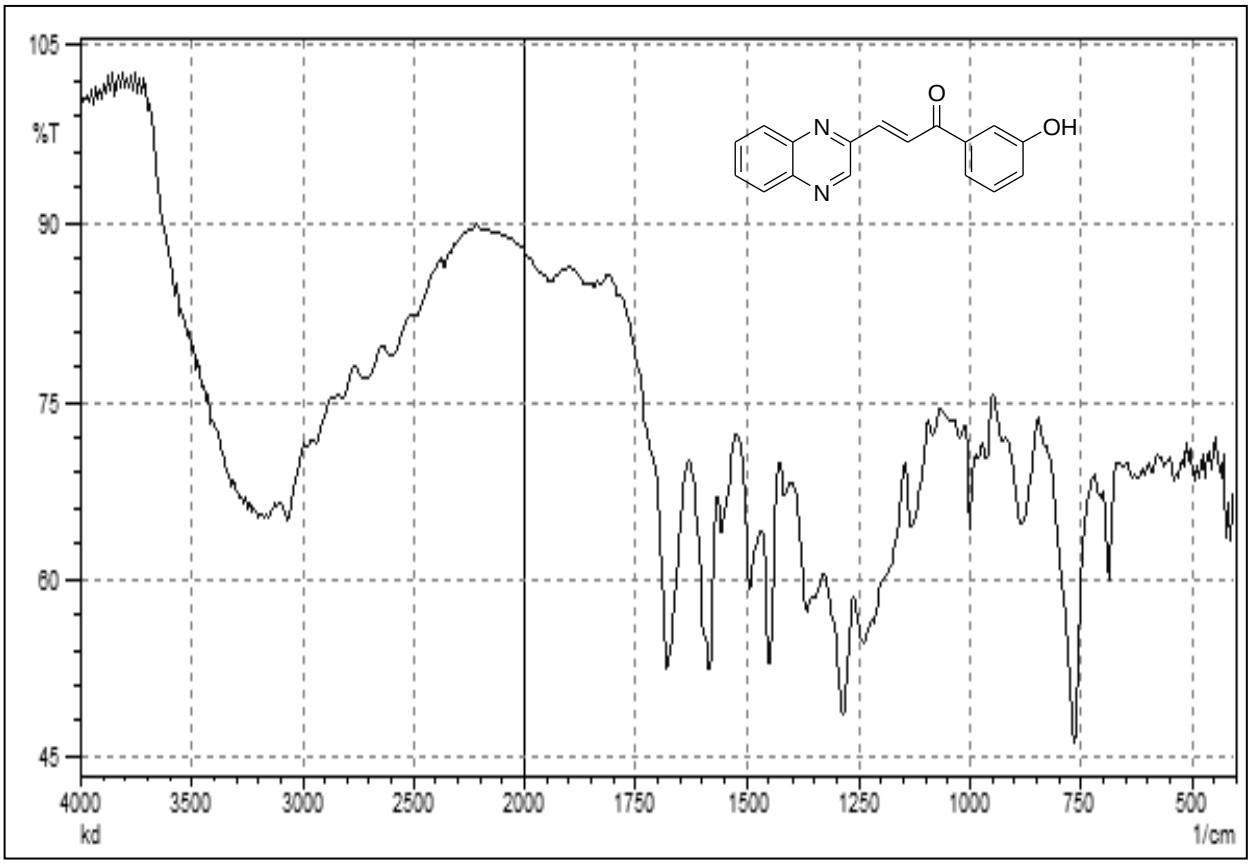
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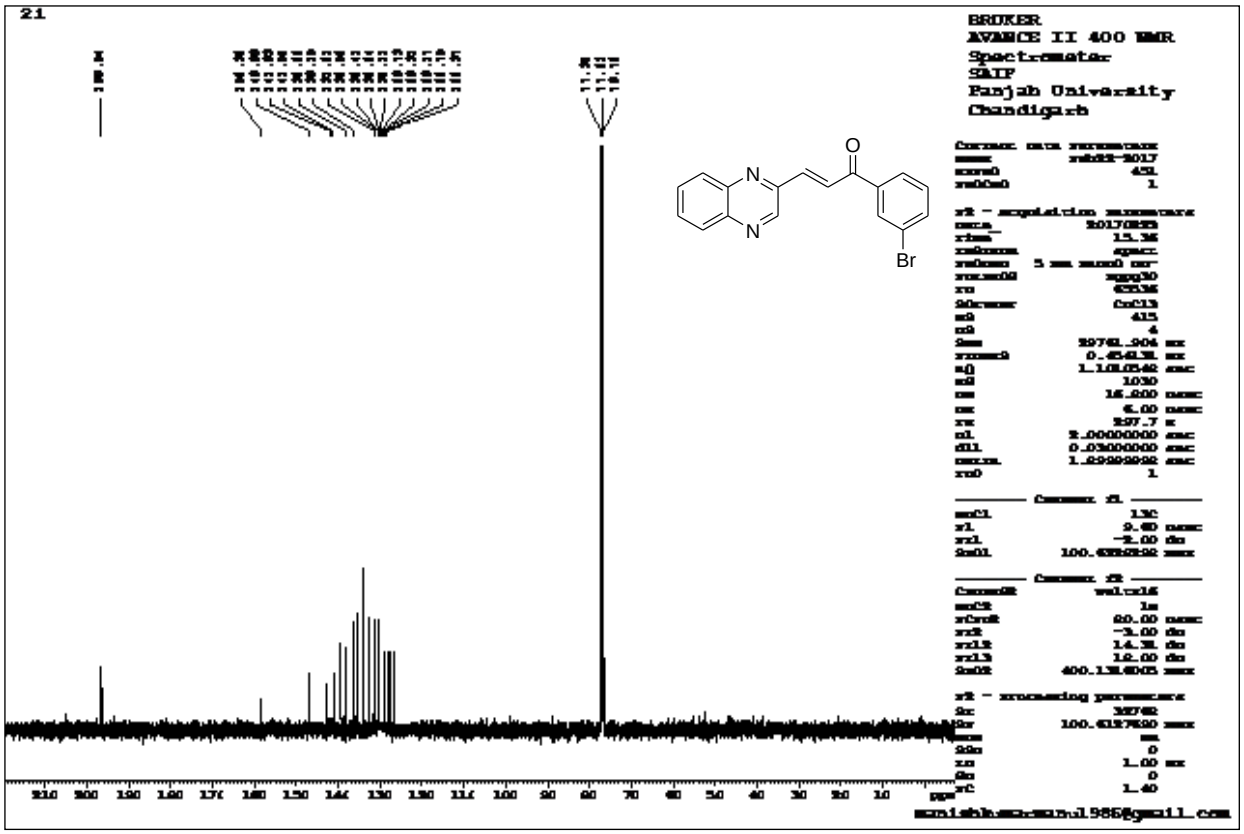
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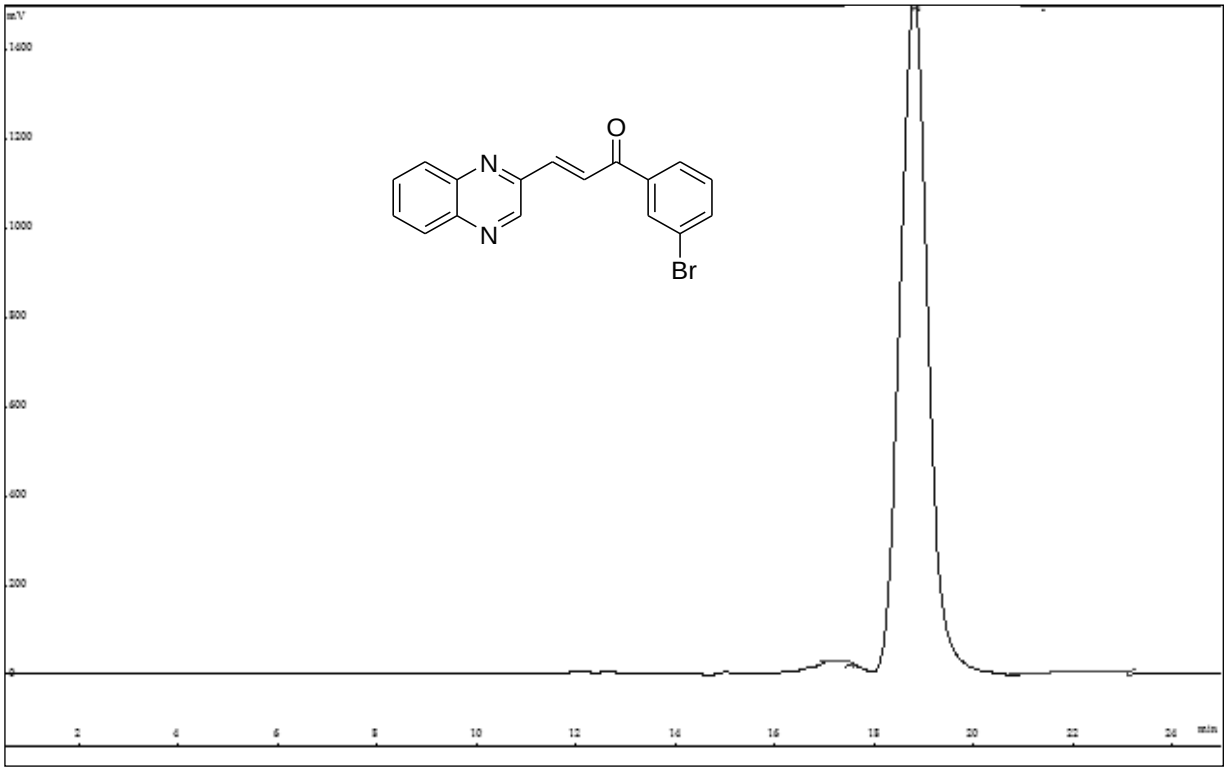
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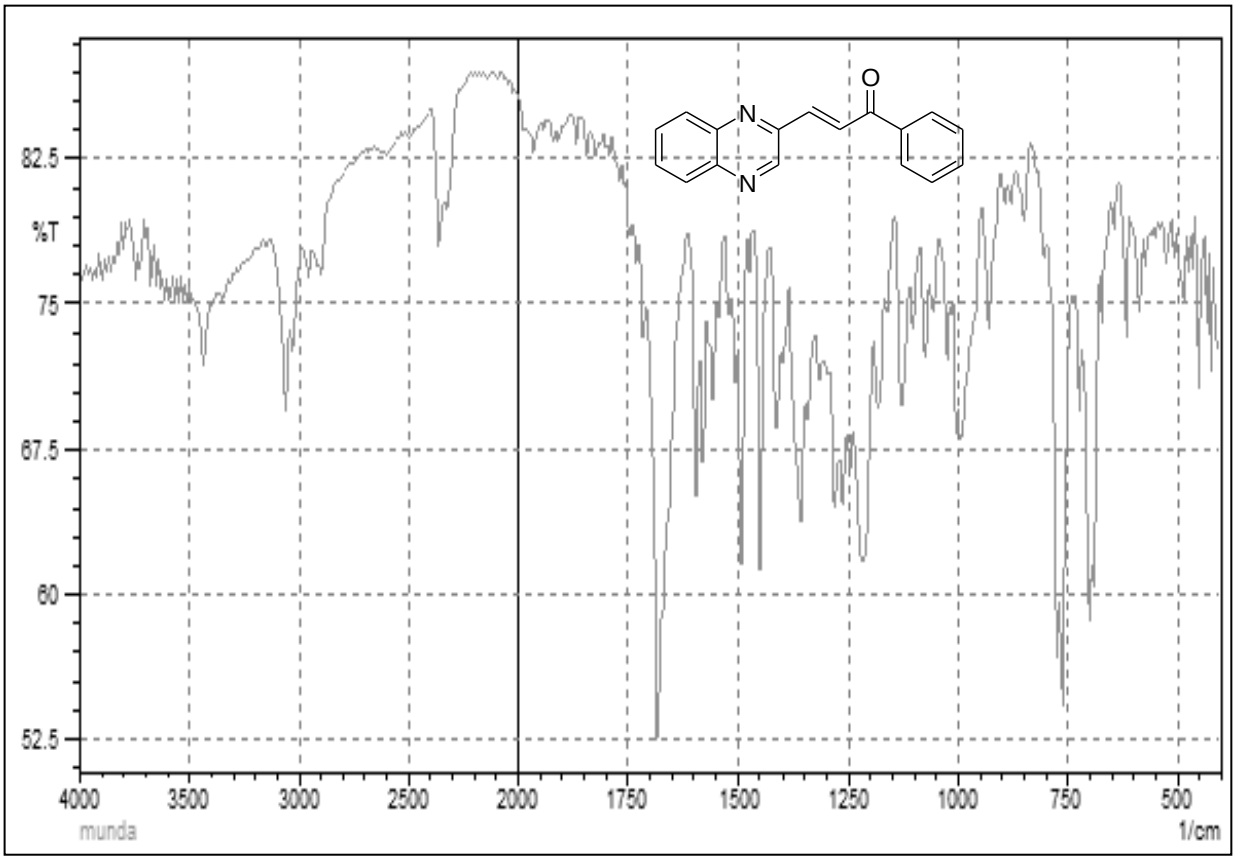
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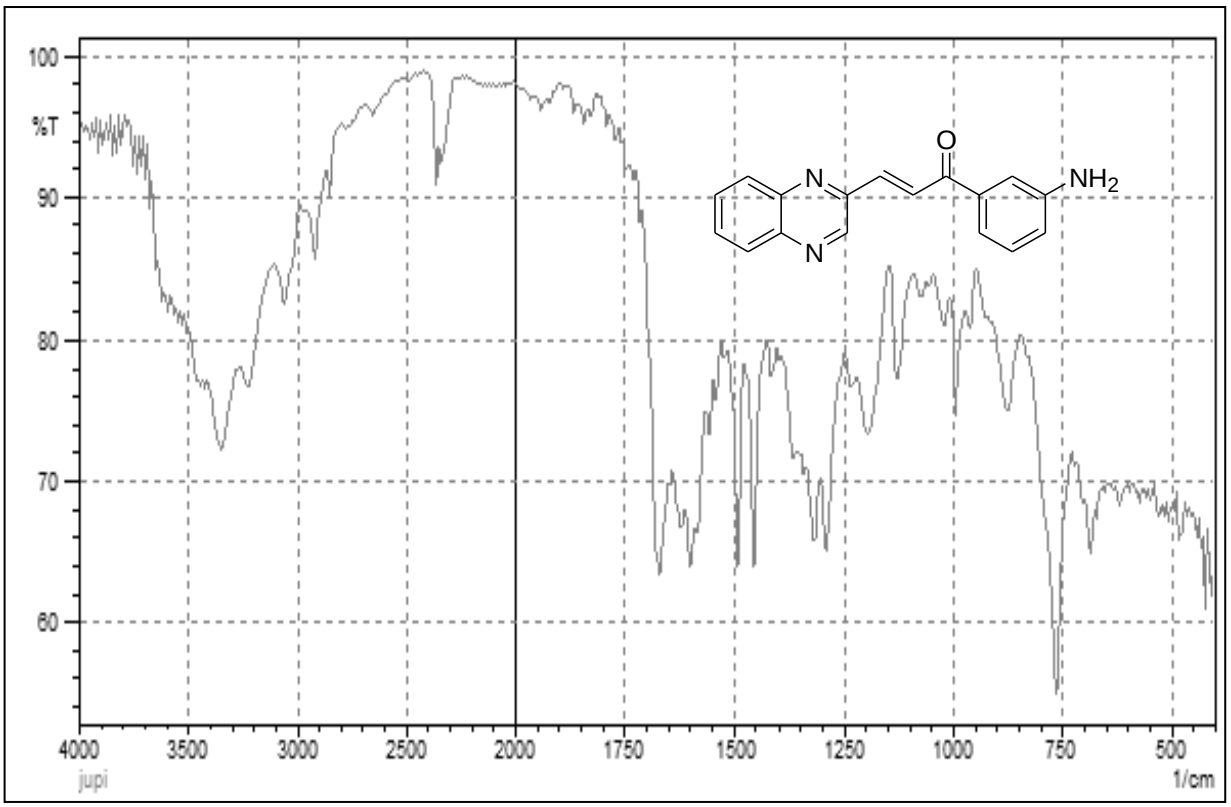
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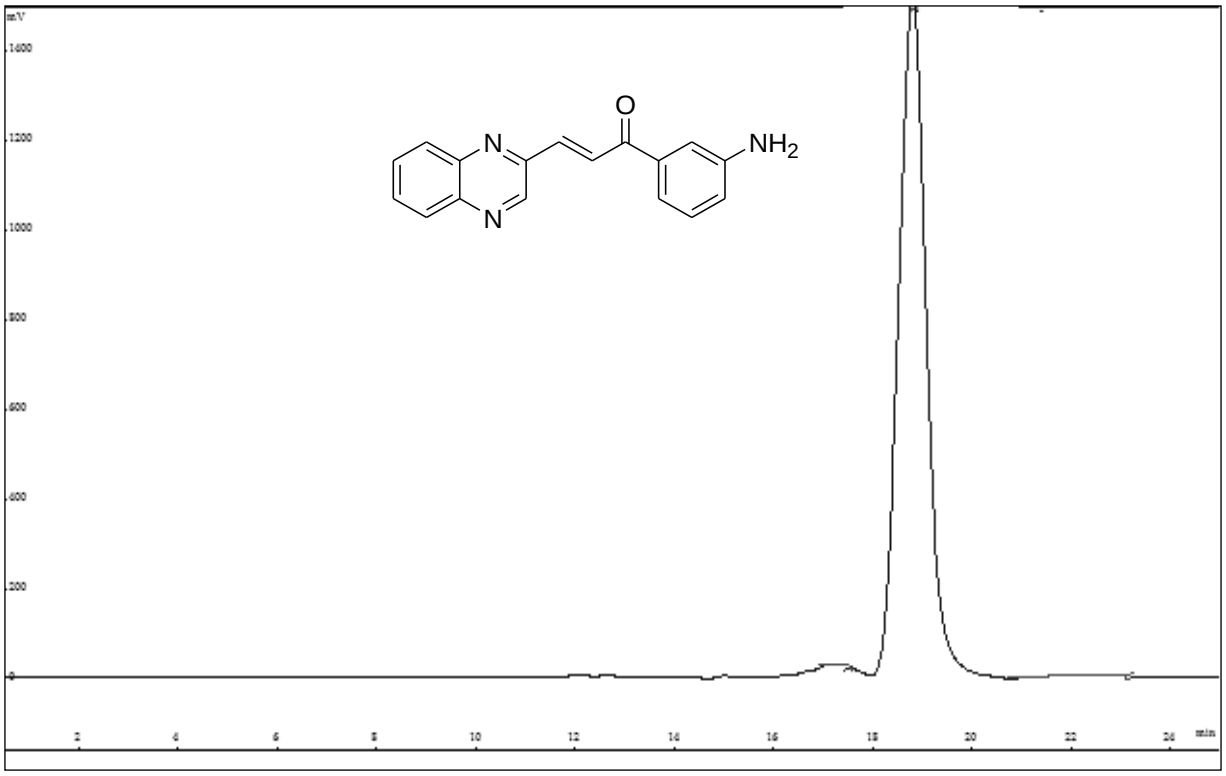
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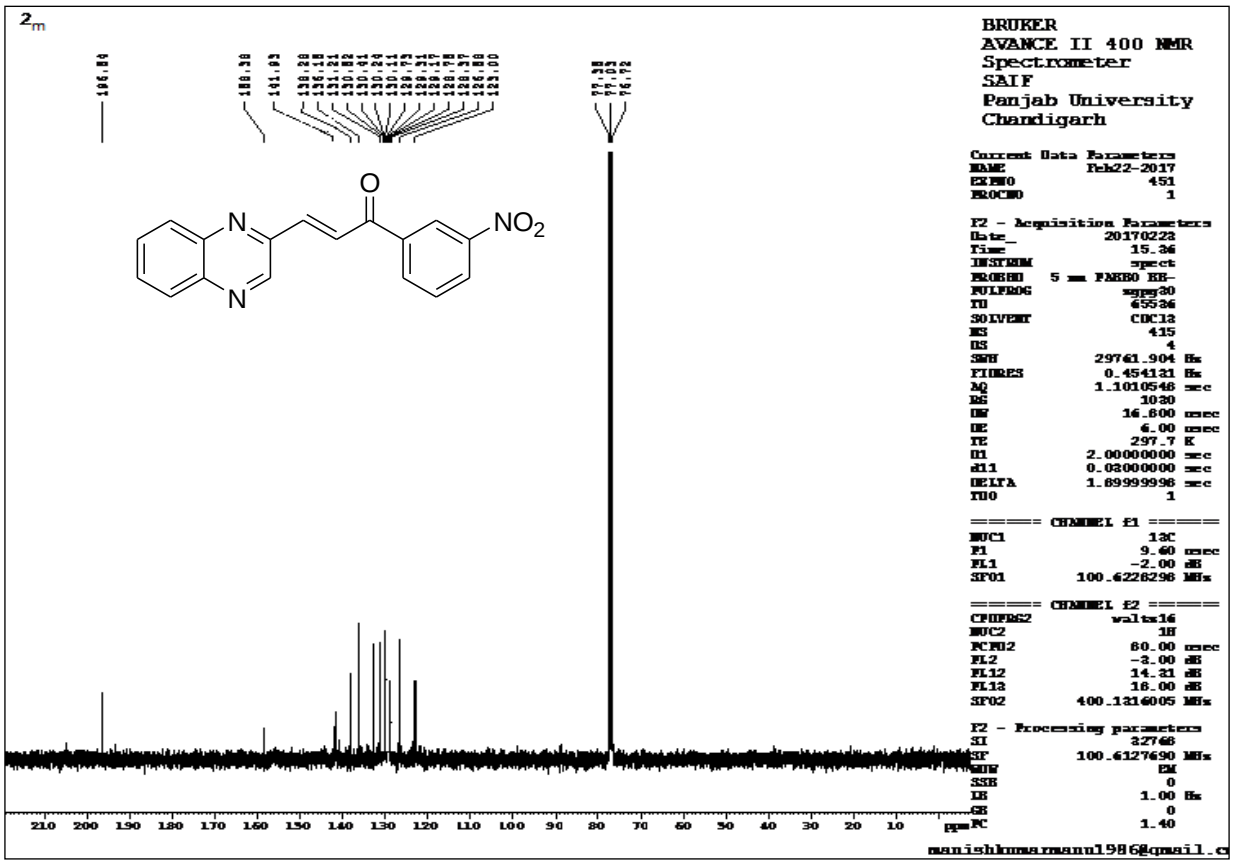
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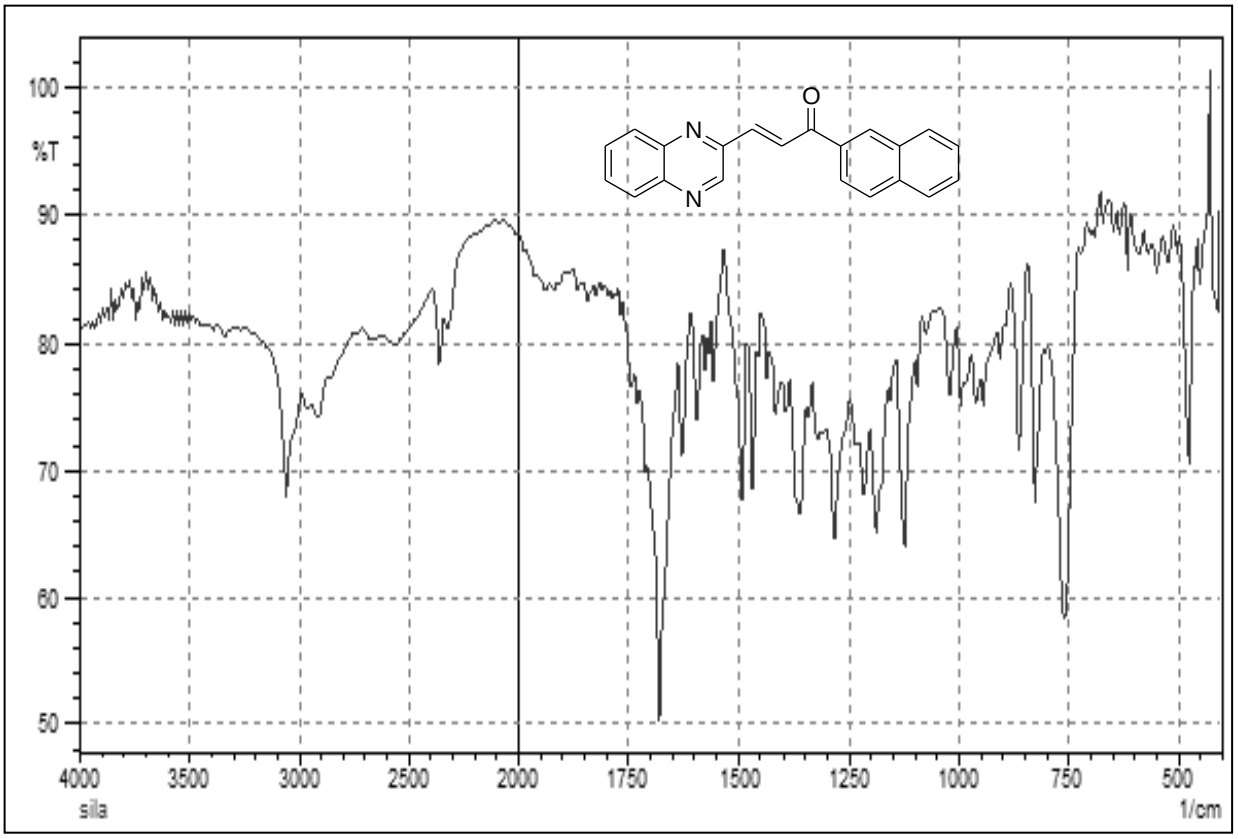
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APPENDIX

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Novel quinoxalinyl chalcone hybrid scaffolds as enoyl ACP reductase inhibitors: Synthesis, molecular docking and biological evaluation

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ABSTRACT

We report herein, first ever synthesis of series of novel differently substituted quinoxalinyl chalcones using Claisen Schmidt condensation, its molecular docking studies, and potential to be good anti-microbial, anti-tubercular and anti-cancer agents. The antimicrobial studies were carried out against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* using disc diffusion procedure. The selected chalcones were tested for anti-cancer and cytotoxicity activity against MCF-7 cancer cell line using MTT assay method. All the synthesized compounds were screened for *in vitro* anti-tubercular screening against MtbH37RV strains by Alamar blue dye method. These results were compared with molecular docking studies carried out on *Mycobacterium tuberculosis* enzyme enoyl ACP reductase using Surflex-Dock program that is interfaced with Sybyl-X 2.0. SAR analysis for antimicrobial and antitubercular activity has also been proposed.

One of the major advances in the field of medicinal chemistry and drug discovery has been the molecular hybridization approach. This has risen from "drug evolution", which drug-drug hybridization is leading to drug molecules of potential bioactivity. Konishi et al. reported hybridization of Benzocaine and Metoclopramide leading to generation of 16 new molecules.[1] The large number of libraries of compounds is an encouragement in the findings for new drug candidates. In the same lines, molecular hybridization is a tool in drug designing, wherein simples molecules can be linked together to construct new hybrid molecules of varied biological interest.[2] This versatile approach of designing new drug entities is the key to achieving large number of hybrid molecules having better affinity and efficacy than the parent molecule from which they are derived. One of the pharmacophoric moieties, which have been more often the target of medicinal chemists has been naturally occurring as well as synthesized. Chalcones are biologically important α,β-unsaturated carbonyl compounds, which as excellent building blocks to heterocycles has attracted organic as well as medicinal chemists.[3] Chalcone derivatives have been demonstrated to have wide range of biological activities.[4,5] Chalcone linked hybrids with heterocycles is a step towards achieving new drug targets,[6] for example coumarinyl-chalcone hybrids as a promising bioactive agents showing wide spectrum of biological properties,[7] novel chalcone-thiazole hybrids as having high antibacterial action against *Staphylococcus aureus,* making it a potential candidate to act as antibiotic.[8] Variety of chalcone hybrids having naphthyl, isoxazolyl and indolyl moieties have been known to show potency as anticancer agents.[9–11] N-heterocyclic chalcones have been known in literature, and have been biologically evaluated for their anti-microbial and anti-tubercular activity.[12–14]

Tuberculosis (TB) is a highly dreaded, chronic, infectious, airborne disease affecting more than two million people all around the world and with more than 8 million cases every year.[15] Due to emergence of multi-drug resistant varieties of *Mycobacterium tuberculosis* and aids epidemic, drugs like isoniazid, rifampicin, pyrazinamide, ethambutol are no more effective.[16] There are several promising clinical trials development programs carried out to conduct evaluation of new anti-TB drugs, such as PA-824, which is a nitroimidazooxazine, an anti-TB drug candidate in the late stage clinical development, showing increased activity against *Mycobacterium tuberculosis*. However, there are few novel compounds known to hit the target. Numbers of chalcones are known to show high inhibitory activity against the growth of *in vitro Mycobacterium tuberculosis* H37Rv strains, when used in low concentrations.[17] Quinoxaline, a bicyclic nitrogen heterocycle is said to have enough potential to be explored for biological evaluation.[18]

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0960-894X/

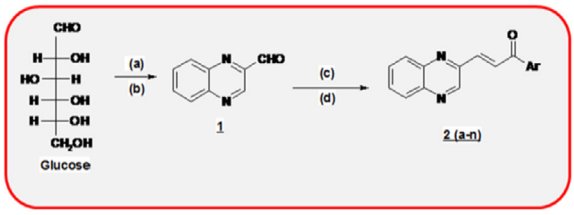
Various derivatives of quinoxaline are known to possess wide range of biological activities ranging from anti-microbials, anti-tubercular, kinase inhibitors, anti-viral, anti-inflammatory, analgesics, anticancer, anxiolytics, antihelmintics, anticonvulsants, antioxidants, antidepressant, anti-hypertensives to antiHIVs.[19–22] Thus, being a part of well-known antibiotics, like echinomycin laevomycin and actinoleutin, the substituted quinoxaline skeleton need to be exploited more in drug discovery.[23]

The combination of these properties of quinoxaline and chalcone in one compound would lead to a drug with potent bioactivity. Molecular hybridization approach from quinoxaline and chalcone include novel 2-acetylquinoxaline derived-chalcones which exhibited *in vitro* glioma cell proliferation activity.[24] Quinoxaline-6-carbaldehyde have also been converted to chalcones using aromatic aldehydes and evaluated for breast cancer.[25] Quinoxaline derived chalcones has been synthesized and biologically evaluated.[26,27] Chalcone derivatives of quinoxaline-1,4-dioxides have been screened as anti-TB agents.[22] In his PhD thesis, Mohan et al. has reported work on synthesis and reactions of quinoxalines involving preparation of quinoxaline-2-carbaldehyde.[28] Interestingly, in literature, quinoxaline-2-carbaldehyde has not been exploited and there are no reports on anti-tubercular activity of quinoxalinyl chalcones. In view of this, an approach was designed to get a quinoxalinyl chalcone hybrid molecule from acetophenones and quinoxaline-2-carbaldehyde and study its potential as antitubercular agents (Fig. 1).

Quinoxaline-2-carbaldehyde was first synthesized by literature known procedure from glucose, phenylenediamine, hydrazine to form an intermediate, followed by oxidation using sodium metaperiodate.[28] This was then reacted with substituted aromatic acetophenones under Claisen Schmidt condensation basic reaction conditions[29,30] to give corresponding chalcone derivatives **2a–n** in moderate to good yields (Scheme 1, Table 1). In all 14 quinoxalinyl chalcone derivatives were synthesized.

The first six compounds **2a–f** have been reported by us,[27] but their molecular docking studies and biological activities have been performed now. The acetophenones (1 equivalent) was treated with aqueous ethanolic solution of sodium hydroxide, stirred at room temperature for 10 min for enolate formation, then to quinoxaline-2-carbaldehyde (1 equivalent) was added and the stirring continued till the completion of the reaction, which was monitored by thin layer chromatography. The reaction was worked up by addition ice and hydrochloric acid. The solid product filtered, was purified by recrystallization using ethanol. All the synthesized compounds have been identified and confirmed by FTIR, ^{1}H NMR and ^{13}C NMR spectroscopy. The purification of the compounds was confirmed by mass spectral analysis and by HPLC measurements on CXTH-3000 Chromatography Data Handling System (Analytical Technologies Limited). Chromatographic separation was achieved at ambient temperature by using mobile phase consisting of methanol and water in the ratio 90:10 (v/v) by 20 min. The mobile phase was pumped at the rate of 1.0 mL/min. The detector wavelength was set at 370 nm. The run time was set at 20 min and retention time of all chalcones was between 16 and 22 min. The purity of all the synthesized compounds was found to be 95% and above. The chromatogram of chalcone is shown in Fig. 2. We were successful in achieving synthesis of new quinoxalinyl chalcone hybrids bearing different substituents. That gave us an opportunity to explore the applications of such chalcones for antimicrobial, anticancer and anti-tubercular activity, as well predict structure activity relationships (SAR analysis).

Antibacterial and antifungal activities of the newly synthesized quinoxalinyl chalcone derivatives **2a–n** were determined using agar well disc diffusion procedure.[31] In brain heart infusion agar, against gram positive strain *Staphylococcus aureus* and gram negative strains *Escherichia coli*, and in sabouraud agar medium against fungal organism *Candida albicans*. Five wells were made on each



Scheme 1. Synthetic route towards quinoxalinyl chalcones from glucose (**2a–n**) Reagents and conditions: (a) H_2O, *o*-phenylene diamine, glacial CH_3COOH, NH_2-NH_2·H_2O, reflux, 5 h, cool in ice bath; (b) H_2O, $NaIO_4$, glacial CH_3COOH, stir, r.t. 16 h; (c) Aromatic ketones, NaOH, H_2O, EtOH, stir, r.t.; (d) Ice, concentrated HCl.

Table 1
Claisen Schmidt condensation of quinoxaline-2-carbaldehyde with various acetophenones to give chalcones **2a–n**.

Compound	Time[a]	Yield[b] in %	Melting Point °C[c]
2a	2	78	256–258
2b	2	75	182–185
2c	2	80	124–126
2d	4	85	98–100
2e	3	75	126–128
2f	2	80	135–138
2g	2	65	150–152
2h	2	80	144–146
2i	2	95	120–122
2j	4	70	136–138
2k	2	75	156–158
2l	2	60	164–167
2m	2	60	205–207
2n	2	90	130–132

[a] Time taken for completion of the reaction monitored by thin layer chromatography.
[b] Calculated from the amount of chalcone obtained after recrystallization.
[c] Determined using thiels tube paraffin method.

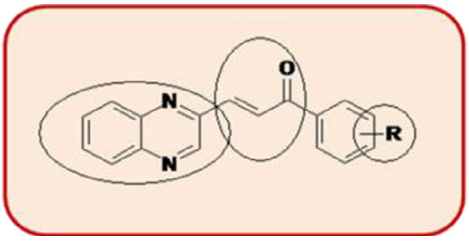


Fig. 1. Molecular hybridisation of quinoxaline and chalcones having drug potency.

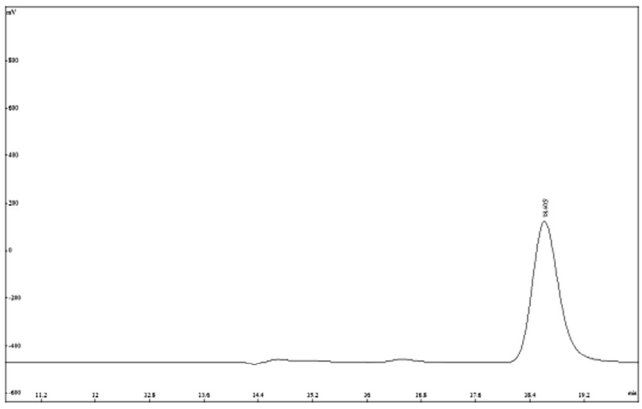


Fig. 2. Representative Chromatogram of chalcone.

plate and 75 μL, 50 μL, 25 μL, 10 μL and 5 μL of compound were added into the respective wells. Plates were incubated for 18–24 h at 37 °C in incubator. Diameter of inhibition zone to nearest whole millimeter was measured by holding the measuring device. Ciprofloxacin and fluconazole was used as standard drug (Table 2).

From the outcomes of the antimicrobial activity of the synthesized quinoxalinyl chalcone derivatives, the following structure activity relationships (SAR) can be established. Overall, the quinoxalinyl chalcones **2a**–**n** exhibit better antibacterial activity against gram-ve bacterial strains as compared to Gram +ve bacterial strains. The best activity against gram −ve and Gram +ve bacterial strains for compounds **2a** and **2h** indicates that presence of —OH substituent, is necessary for enhanced antibacterial activity. The position of OH substituent also decides the specificity. In case of compound **2a** (4-OH) exhibits anti-bacterial but no antifungal activity, while compound **2h** (3-OH) exhibits both antibacterial as well as antifungal activity. This was also evident with the comparison of activity of **2c** and **2i** which contains (*p*-Br) and (*m*-Br) substituents respectively. Significant antibacterial and antifungal activity for **2n**, containing naphthyl group as compared to **2j** containing phenyl group, indicates that increase in hydrophobicity has positive effect on activity. Replacement of phenyl ring **2j** by a heterocyclic pyridinyl ring **2g** does not show change in activity.

The cytotoxicity of selected compounds **2a**, **2b** and **2f** (Table 3) was evaluated using standard MTT assay.[32] In this assay, the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was measured by mitochondrial succinate dehydrogenase. The cell line used was MCF-7 cell line, a Michigan cancer foundation-7 cell line, a Human mammary gland adenocarcinoma, which was procured from National Centre for Cell Science (NCCS), Pune, India. IC50 is Half maximal inhibitory concentration, at which 50% of cells were undergoing cytotoxic cell death due to synthesized compounds treatment. Cis-Platin and Doxorubicin was used as standard drugs. Absorbance is measured at wavelength of 570 nm by using LISA plus.

The **IC50** (half maximal inhibitory concentration) value was determined in case of each compound. The least concentration to show 50% inhibition of cell line was found to be 25 μg in case of compound **2b** containing —NH_2 substituent and 50 μg in case of compound **2f** containing halogen substituent whereas, compound **2a** was found to be inactive against MCF-7 cell lines. Compound **2a** containing OH substituent showed no activity.

The newly synthesized chalcones **2a**–**n** has been screened for their *in vitro* anti-tubercular activity using Alamar Blue Dye method,[33] this methodology is non-toxic, uses thermally stable reagents and showed good correlation with the proportional and BACTEC radiometric method. The *Mycobacterium tuberculosis* strain used was MtbH37Rv, while the standard drugs were Streptomycin, Pyrazinamide, and Ciprofloxacin (Table 4).

A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink (Fig. 3).

From the observations of *in vitro* anti-tubercular results for the synthesized quinoxalinyl chalcones, following structure-activity

Table 2
Anti-microbial activity of synthesized quinoxalinyl chalcones **2a**–**n**.

Compound	Ar	Minimum inhibitory concentration-MIC (μg/mL)		
		S. aureus	*E. coli*	*C. albicans*
2a	4-Hydroxyphenyl	5	5	
2b	4-Aminophenyl	50	25	25
2c	4-Bromophenyl	75	25	75
2d	4-Methoxyphenyl	50	25	25
2e	4-Chlorophenyl	25	10	25
2f	4-Fluorophenyl	75	25	25
2g	4-Pyridinyl	50	25	50
2h	3-Hydroxyphenyl	10	5	10
2i	3-Bromophenyl	25	5	25
2j	Phenyl	50	50	25
2k	3-aminophenyl	50	25	25
2l	4-Nitrophenyl	50	25	50
2m	3-Nitrophenyl	50	25	25
2n	Naphthyl	25	25	25
Ciprofloxacin		2	2	–
Fluconazole		–	–	16

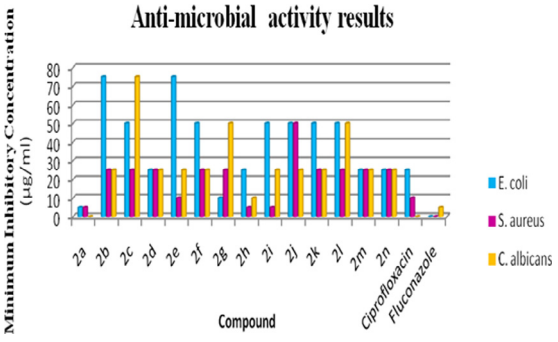
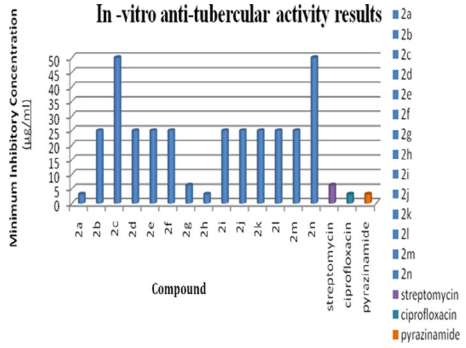


Table 3
Preliminary cytotoxicity screening of quinoxalinyl chalcones **2a**, **2b** and **2f**.

Sample	Conc. μg/mL	Absorbance[d]	Result	IC50 μg
2a	10	0.584	No lysis	
	20	0.581	No lysis	
	30	0.420	No lysis	–
	40	0.399	No lysis	
	50	0.386	No lysis	
2b	10	1.772	Partial lysis	
	20	1.772	Partial lysis	
	30	1.631	50% lysis	25
	40	1.631	>50% lysis	
	50	1.585	>50% lysis	
2f	10	0.836	No lysis	
	20	0.644	No lysis	
	30	0.505	No lysis	50
	40	0.477	No lysis	
	50	0.380	No lysis	
Cis-platin[e]			50% lysis	7.5
Doxorubicin[e]			50% lysis	5–7.5

Table 4
In vitro antitubercular activity of synthesized quinoxalinyl chalcones **2a**–**n**.

Sample	Ar	Minimum inhibitory concentration-MIC (μg/mL) *MtbH37RV*
2a	4-Hydroxyphenyl	3.12
2b	4-Aminophenyl	25
2c	4-Bromophenyl	50
2d	4-Methoxyphenyl	25
2e	4-Chlorophenyl	25
2f	4-Fluorophenyl	25
2g	4-Pyridinyl	6.25
2h	3-Hydroxyphenyl	3.12
2i	3-Bromophenyl	25
2j	Phenyl	25
2k	3-aminophenyl	25
2l	4-Nitrophenyl	25
2m	3-Nitrophenyl	25
2n	Naphthyl	25
Streptomycin[d]		6.25
Ciprofloxacin[d]		3.12
Pyrazinamide[d]		3.12



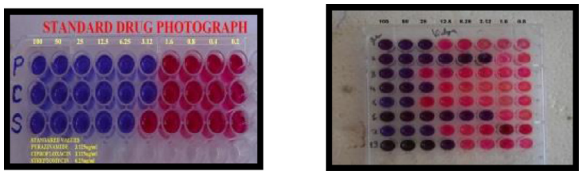


Fig. 3. To the left shows the MIC values for the standard drugs, Streptomycin, Pyrazinamide and Ciprofloxacin, while that to the right shows the MIC values for the synthesized quinoxalinyl chalcones; MIC value 3.12 μg/mL (2 and 6) is for chalcones **2a** and **2h**, containing the —OH substituent.

relationships (SAR) can be predicted. The maximal activity exhibited by compounds **2a** and **2h**, is comparable to the standard drugs Pyrazinamide and Ciprofloxacin, indicates that presence of —OH substituent increases the anti-tubercular activity. Replacement of phenyl ring in **2j** by heterocyclic pyridinyl ring in **2g** increases the potency of the compound for anti-tubercular activity. Presence of halogens, methoxy, amino, nitro groups had no significant effect on the anti-tubercular activity. Replacement of phenyl ring in **2j** by naphthyl ring in **2n** decreases the potency of the compound for anti-tubercular activity, indicating that lipophilicity has adverse effect on the activity.

To generalize the innovative approaches to drug targets, the study related to biology of *Mycobacterium tuberculosis* becomes a key component. Among the several drug targets known for tuberculosis,[34,35] the cell wall biosynthesis related drug target is the most promising one, since their biosynthetic enzymes do not have homologues in the mammalian system. One of the major drug targets for *Mycobacterium tuberculosis* has also been mycolic acid.[36] Mycolic acid, forms an important fatty acid, and a main constituent of the mycobacterial cell wall, which is present in fatty acid synthase system of *Mycobacterium tuberculosis*. The mycolic acid biosynthesis has been carried out by many successive enzyme catalyzed cycles equivalent to Fatty Acid Synthase (FAS) systems. The enzyme InhA, which is an enoyl acyl carrier protein reductase from *Mycobacterium tuberculosis* is the key enzyme for the synthesis of type II fatty acid system, which catalyzes NADH-dependent reduction of 2-*trans*-enoyl-ACP (acyl carrier protein) which is an acyl carrier protein, to yield NAD and reduced enoyl thioester-ACP substrate, which is responsible for the synthesis of mycolic acid. This has been the drug targets for well known anti-Tb drugs like Isoniazid (INH) and Ethionamide.[37] Chalcones derived enoyl ACP reductase inhibitors have been studied and proved to have high binding affinity to the enzymes wherein the main influencing factors of molecular interaction between ENR and chalcone derivatives determined by this study were H-bond, hydrophobic and electro-

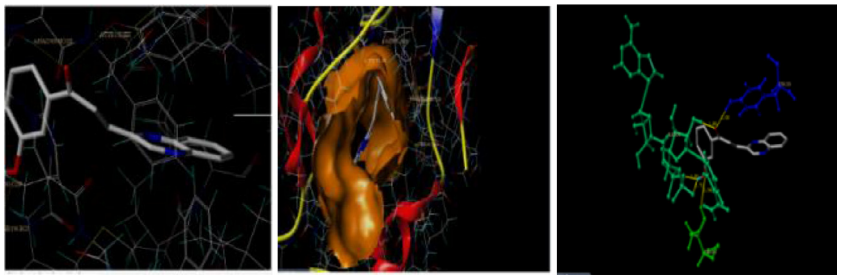


Fig. 4. Interaction of **2a** at the binding active site of the enzyme enoyl acp reductase (PDB ID: 4TZK). Ligand and key residues are represented as stick models and coloured by atom type, here the proteins are represented by red dotted lines. White: hydrogen atom; red: oxygen atom; dark blue: nitrogen atom; blue: the backbone and carbon atom of compound **2a**. Quinoxalinyl moiety of compound **2a** embedded in the binding pocket of enoyl ACP reductase. Hydrogen atom of hydroxyl group at 4th position of aromatic ring makes an hydrogen bonding interaction with oxygen of NAD500.

Table 5
Surflex Docking score (kcal/mol) of the lists of quinoxalinyl chalcones (**2a–n**).

Code	C Score[a]	Crash Score[b]	Polar Score[c]	D Score[d]	PMF Score[e]	G Score[f]	Chem Score[g]
2a	6.97	−1.28	3.03	−140.48	−74.20	−222.05	−45.17
2b	4.08	−0.62	0.00	−110.13	−53.35	−161.40	−34.34
2c	5.56	−0.65	1.91	−117.88	−59.66	−134.90	−39.40
2d	6.29	−1.37	2.91	−127.33	−58.88	−205.39	−44.61
2e	5.67	−0.51	1.93	−115.39	−57.18	−131.25	−39.22
2f	5.65	−0.51	1.82	−109.28	−61.87	−125.36	−36.89
2g	5.28	−0.81	0.00	−118.75	−44.64	−184.05	−34.59
2h	8.02	−1.71	2.97	−137.23	−57.94	−218.32	−45.35
2i	4.97	−0.73	1.81	−117.07	−67.96	−143.90	−40.66
2j	5.99	−1.20	1.82	−122.92	−44.00	−168.55	−39.99
2k	5.34	−0.88	0.84	−115.44	−60.01	−175.74	−38.99
2l	4.10	−0.62	0.00	−118.93	−49.13	−173.90	−33.28
2m	4.81	−1.37	0.00	−132.45	−37.36	−209.00	−35.93
2n	6.11	−2.11	1.15	−145.14	−64.81	−225.27	−46.90

[a] C Score (Consensus Score) integrates a number of popular scoring functions for ranking the affinity of ligands bound to the active site of a receptor and reports the output of total score.
[b] Crash-score revealing the inappropriate penetration into the binding site. Crash scores close to 0 are favorable. Negative numbers indicate penetration.
[c] Polar indicating the contribution of the polar interactions to the total score. The polar score may be useful for excluding docking results that make no hydrogen bonds.
[d] D-score for charge and van der Waals interactions between the protein and the ligand.
[e] PMF-score indicating the Helmholtz free energies of interactions for protein-ligand atom pairs (Potential of Mean Force, PMF).
[f] G-score showing hydrogen bonding, complex (ligand-protein), and internal (ligand-ligand) energies.
[g] Chem-score points for H-bonding, lipophilic contact, and rotational entropy, along with an intercept term.

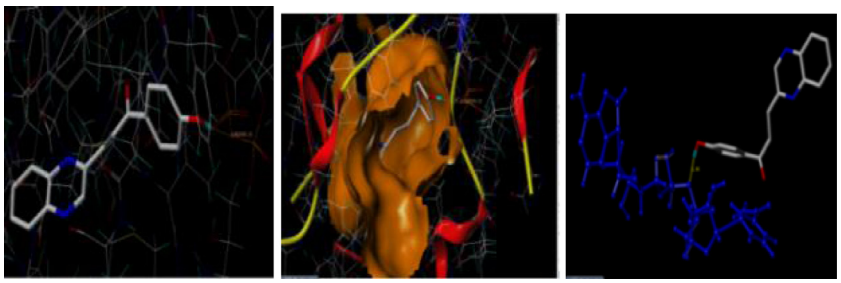


Fig. 5. Docked view of compound **2h** at the active site of the enzyme PDB ID: 4TZK. Compound **2h** makes five hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), among them three interactions are came from the hydrogen of the hydroxyl group present at 3rd position of aromatic ring.

static interaction.[38] So, we chose enzyme enoyl ACP reductase for our molecular docking studies on quinoxalinyl chalcones. The crystal structures used were *Mycobacterium tuberculosis* enoyl reductase (InhA) complexed with 1-cyclohexyl-N-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) for the docking studies, obtained from the Protein Data Bank. The protein was prepared for docking by adding polar hydrogen atom with Gasteiger-Huckel charges[39] and the molecular docking was performed with Surflex-Dock program that is interfaced with Sybyl-X 2.0.[40] The molecular docking study[41] revealed that **2a** and **2h** acts as very good inhibitor of enoyl ACP reductase enzyme. As depicted in Fig. 4, compound **2a**, makes one hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), Hydrogen atom of hydroxyl group at 4th position of aromatic ring makes an hydrogen bonding interaction with oxygen of NAD500 (O—*H*···O-NAD500, 1.86 Å). Table 5 depicts the results of docking studies. As depicted in the Fig. 5, compound **2h** makes five hydrogen bonding interaction at the active site of the enzyme (PDB ID: 4TZK), among them three interactions are came from the hydrogen of the hydroxyl group present at 3rd position of aromatic ring, with oxygen of NAD500 and THR196 (O—*H*···O-NAD500, 1.81 & 2.71 Å; HG-THR196, 2.34 Å) and remaining two interactions raised from

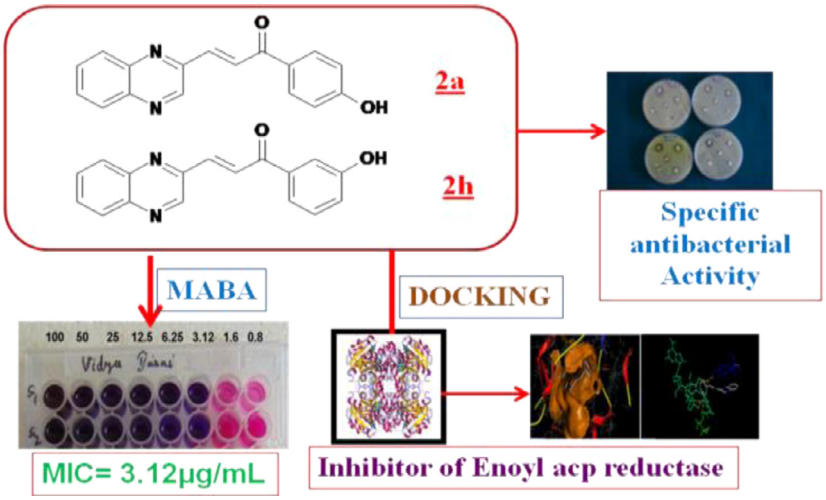


Fig. 6. Quinoxalinyl chalcone **2a** and **2h**, containing —OH substituent shows significant antitubercular activity against MTbH37RV, Chalcone **2a** also exhibits specific antibacterial activity against both Gram +ve as well as Gram −ve bacteria and no activity against antifungal activity, while chalcone **2h** showed both antibacterial as well as antifungal activity.

the oxygen of carbonyl group with hydrogen of NAD500 & TYR158 (C═O···H-NAD500, 1.88 Å; H-TYR158, 2.00 Å). Overall the biological activities of the synthesised quinoxalinyl chalcones is depicted in Fig. 6.

In conclusion, this work on newly synthesized quinoxalinyl chalcones investigates, the role of quinoxalinyl chalcone moiety as a versatile pharmacophoric unit, having range of biological activities. The study tells us about how molecular hybridisation of quinoxaline and chalcone can be exploited to search novel drug targets to treat highly dreaded diseases like tuberculosis and cancer. The MTT assay studies revealed that chalcone **2b**, containing the —NH_2 group, exhibit good anticancer activity. The chalcone **2a**, containing the —OH group showed specifically anti-bacterial and *in vitro* antitubercular activity, but no anticancer activity. The good inhibitory action of quinoxalinyl chalcones **2a** and **2h** towards the enzyme enoyl ACP reductase, has been an additive research on such chalcones. The wide array of bioactivities exhibited by quinoxalinyl chalcones, reflects the importance of such heterocyclic moiety in designing novel drug targets in drug development. This novel findings has thrown light on how a simple quinoxalinyl chalcone hybridisation can lead to a potential moiety as targets to develop new drug entities. Further, such chalcones can be excellent building blocks to heterocycles with great diversity, a heterocyclic scaffold, having varied biological applications.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.03.059.

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30. *General procedures for the preparation of chalcone derivatives* ***(2a–2n):*** To a conical flask containing NaOH solution (1.5 eq, 10 mL H_2O) was added substituted acetophenones (1 mmol) in ethanol (10 mL), and the reaction mixture was stirred for 10 min to allow enolate formation, to this was added quinoxaline-2-carbaldehyde **1** (1 mmol) and the reaction mixture was stirred till completion. After completion of the reaction, as monitored by TLC the reaction mixture was poured in an ice bath and was acidified using conc. HCl. The solid obtained was then filtered, dried and recrystallized using Ethanol. The quinoxalinyl chalcone **2a–n** were obtained in (60–95% yields.*(2E)-1-(4-hydroxyphenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one* (**2a**): This chalcone was

synthesized from quinoxaline-2-carbaldehyde **1** (200 mg, 1.26 mmol) and 4-hydroxyacetophenone (171 mg, 1.26 mmol) to yield **2a**, as pale yellow solid (272 mg, yield 78%) mp 256–260 °C; FT-IR (KBr) cm^{-1}: 3250, 3015, 1655, 1606, 1585, 1262; ^1H NMR (400 MHz, DMSO) δ 10.48 (brs, 1H, OH), 9.52 (s, 1H, N═C—H), 8.25 (d, 2H, *j* = 8.3 Hz, C3 & C5 phenyl ring), 8.1–8.14 (m, 4H, C5-C8 quinoxaline ring), 7.89 (dd, 2H, *j* = 8.29 Hz, C2 & C6 phenyl ring), 7.83 (d, 1H, *j* = 17.7 Hz, H—C═C—C═O), 6.94 (d, 1H, *j* = 17.7 Hz, C═CH—C═O) ^{13}C NMR (75 MHz, $CDCl_3$) δ 189.05, 157.82, 150.23, 145.8, 142.25, 141.8, 132.25, 129.97, 129.97, 129.18, 129.05, 128.53, 128.09, 128.09, 123.3, 115.42, 115.42. ESI-HRMS (*m*/*z*): calcd. For $C_{17}H_{12}N_2O_2$ $[M+H]^+$: 277.097; found; 277.083.*(2E)-1-(4-aminophenyl)-3-(quinoxalin-2-yl)-prop-2-en-1-one* (**2b**): This chalcone was synthesized from quinoxaline-2-carbaldehyde **1** (200 mg, 1.26 mmol) and 4-aminoacetophenone (170 mg, 1.26 mmol) to yield **2b**, as pale yellow solid as orange solid (260 mg, yield 75%) mp 182–185 °C; FT-IR (KBr) cm^{-1}: 3354, 3040, 1658, 1609, 1585; ^1H NMR (400 MHz, $CDCl_3$) δ 9.48 (s, 1H, N═C—H), 7.98–8.30 (m, 4H, C5-8 quinoxaline ring), 7.96–8.01 (dd, 2H, *j* = 8.60 Hz, C2 & C6 phenyl ring), 7.81–7.86 (dd, 2H, *j* = 8.62 Hz, C3 & C5 phenyl ring), 7.80 (d, 1H, *j* = 17.30 Hz, H—C═C—C═O), 6.71 (d, 1H, *j* = 17.30 Hz, C═CH—C═O), 3.5 (brs, 2H, NH_2) ^{13}C NMR (75 MHz, $CDCl_3$) δ 189.05, 150.23, 149.06, 145.8, 142.25, 141.8, 130.08, 130.08, 129.18, 129.05, 128.53, 128.09, 128.09, 127.71, 123.3, 113.17, 113.17. ESI-HRMS (*m*/*z*): calcd. For $C_{17}H_{13}N_3O$ $[M+H]^+$: 276.113; found; 276.101.

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41. *Molecular docking procedure:* The crystal structures used were Mycobacterium tuberculosis enoyl reductase (InhA) complexed with 1-cyclohexyl-N-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB ID: 4TZK) for the docking studies, obtained from the Protein Data Bank. The protein was prepared for docking by adding polar hydrogen atom with Gasteiger-Huckel charges and water molecules were removed. The 3D structure of the ligands was generated by the SKETCH module implemented in the SYBYL program (Tripos Inc., St. Louis, USA) and its energy-minimized conformation was obtained with the help of the Tripos force field using Gasteiger-Huckel charges and molecular docking was performed with Surflex-Dock program that is interfaced with Sybyl-X 2.0. and other miscellaneous parameters were assigned with the default values given by the software.

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RESEARCH ARTICLE

IBX-Mediated, Efficient, Metal-Free Approach Towards Synthesis of Flavones

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Abstract: ***Aim and Objective*:** We explore the role of IBX as an electrophilic agent towards the synthesis of flavones. The main objective was to work within the framework of green chemistry by using eco-friendly, less toxic, iodine-hypervalent reagent to carry out a facile conversion to flavones.

***Material and Methods*:** The use of 1equivalent of IBX under refluxing condition in DMSO was the best employed method for the synthesis of flavones. All the synthesised compounds were characterised by spectroscopic methods such as IR, ^{1}H NMR and ^{13}C NMR spectroscopy.

***Results*:** In total, we successfully synthesized fifteen flavones derivatives in very good yields by using iodine-hypervalent reagent. This method was proved to be the best method as it involved simple workup procedure i.e. by adding water to the reaction mixture followed by filtration of the solid product. The formation of the product was confirmed by IR, NMR and their characteristic melting points.

***Conclusion*:** A new and first approach towards the synthesis of flavones using non-toxic and eco-friendly hypervalent iodine (V) reagent, IBX has been demonstrated. Such a methodology has been well-extended towards the synthesis of hydroxy, and bromo flavones. The work signifies the electrophilic character of IBX, which is an oxidant.

Keywords: Flavones, 2'-hydroxychalcones, hypervalent iodine, o-iodoxybenzoic acid, oxidative cyclization, Claisen-Schmidt condensation.

1. INTRODUCTION

Flavones (2-aryl-4H-1-benzopyran-4-ones) are naturally occurring compounds, belonging to the flavonoid family. Such groups of molecules are oxygen containing bicyclic heterocycles having varied biological activities [1] that include anticancer, antioxidant, anti-inflammatory, antiviral, anti-mutagenic, anti-HIV, antibacterial, DNA cleavage *etc*. Newer 1, 2, 3-triazole linked chalcone and flavone hybrid compounds have been synthesized and evaluated for their antimicrobial and cytotoxic activities [2]. Flavone forms an important group of naturally occurring alkaloids, Luteolin (**i**) Apigenin (**ii**) and 7-hydroxyflavones (**iii**). All three are known to inhibit AKR1B10, and act as a promising drug target for the treatment of cancer.

In view of this, several approaches and reviews on flavones have been reported. The two best known methods for flavone synthesis are Baker Venkatraman rearrangement [3] and oxidative cyclization of 2'-hydroxychalcones [4]. A novel catalytic approach for the synthesis of the flavones under autoclave conditions and its anticancer activity has been studied [5]. The newer methods for the synthesis of flavones in literature, include Pd/C catalyzed cyclocarbonylation of 2-iodophenol with terminal acetylenes [6], of 2-(O-acetyl) derivative of iodobenzene with acetylene [7], besides the iodine catalyzed in DMSO under refluxing conditions [8, 9], oxidative cyclization of 2'-hydroxychalcones has also been carried out using iodine monochloride under ultrasonic irradiation conditions [10], microwave-assisted synthesis, in the presence of dimethyl sulphoxide as solvent [11], solvent free microwave assisted synthesis [12], using phase-transfer catalyst TBATB under basic conditions [13]. Silica-supported $Ce(SO_4)_2.H_2O$ mediated cyclisation of 2'-amino and 2'-hydroxychalcones under solvent free conditions has also been reported. [14] A synthetic route has been reported for prenylated flavones using phenyliododiacetate [15]. Waker oxidation has also been used for the synthesis of flavones [16]. Baker-Venkatraman methodology has been demonstrated using photocyclization [17], microwave irradiations for the synthesis of functionalized flavones has been reported [18]. Recently, K_3PO_4-mediated intra-molecular Rauhut-Currier type reaction for the synthesis of 4H-chromones has been achieved [19]. Review on synthesis and transformation of halochromones has appeared [20]. However, although these known methods are efficient but are associated with drawbacks such as use of expensive and hazardous catalyst, long reaction time and drastic reaction conditions.

Chemistry of hypervalent iodine has evolved over the years. The precursor of Dess-Martin oxidizing reagent [21], 1-hydroxy-1λ^3-2-benziodoxol-3(1H)-one-1-oxide (IBX), a highly versatile hypervalent iodine (V) reagent has been demonstrated in several diverse applications such as oxidant for various organic functional group transformations, as dehydrogenating agent for aromatization, for oxidative cyclisation of acyclic enone derivatives [22]. This has several advantages such as easy preparation, non-toxic, safe to handle, relatively stable at room temperature for longer period of time, simple reaction conditions and a great degree of chemoselectivity [23]. There has been a successful application of IBX-promoted one pot methodology *via* oxidative cyclisation leading to benzimidazole [24]. IBX-mediated oxidation of inactivated cyclic amines has been reported [25]. However, the use of IBX is limited to an oxidant and not as an electrophilic agent. We, in our group developed a one-pot strategy towards the synthesis of trisubstituted isoxazole and isoxazole-3-carboxylic acid using IBX [26]. In continuation to our work on IBX as oxidative cyclising agent we have targeted flavones.

Flavones being a product of biological importance play a significant role in pharmaceutical industry, so a methodology, which is cost-effective, is of great need. Iodine catalyzed reaction in DMSO is a commonly employed method under refluxing conditions [8]. However, this method is time consuming as it requires removal of iodine by washing with sodium dithionate, product purification by column and due to toxicity direct use of iodine has to be avoided. Recently, ultrasound mediated synthesis of flavones involving use of hazardous iodine monochloride has been reported. In such cases,

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1875-6271/17 $58.00+.00

hypervalent iodine reagent like IBX can solve the problem and provide a better eco-friendly method, which can save time with better efficiency. Such reagents have not been used earlier for the synthesis of flavones in any of the literature reports known so far. Thus, we demonstrate herein, how IBX can mimic iodine in having electrophilic character and also being an oxidant to effectively afford differently substituted bioactive flavones.

2. MATERIAL AND METHODS

2.1. Experimental

Melting points were determined by using thiels tube method and have been uncorrected. IR was recorded on SHIMADZU FTIR affinity-1. The NMR spectra were measured in $CDCl_3$ or DMSO at RT on a Brucker AV-II 400 spectrometer. δ is given in ppm relative to tetramethylsilane as an internal reference. Thin layer chromatography was performed using DDC-fertigfolien ALUGRAM^R Xtra SIL G/UV_{254} (Macherey-Nagel GmbH & Co.KG). IBX [27] and 2'-Hydroxychalcones [28] were prepared by literature known procedure.

2.1.1. Preparation of Substituted 1, 3-(diphenyl)-prop-2-en-1-one

To a solution of substituted 2-hydroxyacetophenone (1 mmol) in ethanol, aqueous NaOH (2.5 eq, in 10 mL H_2O) was added, and the reaction mixture was stirred for 15 minutes. To this, substituted benzaldehyde (1 mmol) was added and was continued to stir for the required time period. After completion of reaction as monitored by TLC, the reaction mixture was poured in ice cold water and was neutralised by adding concentrated HCl, the solid thus obtained was filtered, dried, and recrystallised with ethanol. IR: 3300, 3060, 1690, 1600, 1010, 773, 700, 680cm^{-1}

2.1.2. Synthesis of 2-Aryl-4H-Chromen-4-one (Flavone)

To a solution of substituted 2'-hydroxychalcone (1 mmol) in DMSO (3 mL), 1 equivalent IBX was added and the reaction mixture was refluxed for the required period on sand bath. After completion of reaction, ice water was added to it. The solid obtained was filtered, dried and recrystallized from ethanol.

2.1.3. 2-Phenyl-4H-Chromen-4-one (2a)

IR (KBr): 1660 (C=O), 1465, 1377, 770 cm^{-1}; ^{1}HNMR: δ 6.86 (s, 1H, 3-H), 7.57 (m, 3H, 3', 4' & 5'- H), 7.60 (d, 1H, J=9.3Hz, 8-H), 7.73 (dt, 1H, j= 7.4 Hz, 5-H), 7.96 (m, 2H, 2' & 6'- H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 162.9, 155.8, 133.4, 131.3, 128.7, 125.9, 125.2, 124.8, 123.6, 117.8, 107.1.

2.1.4. 2-(4-Chlorophenyl)-4H-Chromen-4-one (2b)

IR (KBr): 1639 (C=O), 1469, 1409, 1091, 773 cm^{-1}; ^{1}HNMR: δ 6.80 (s, 1H, 3-H), 7.46 (t, 1H, J= 7.5 Hz, 6- H), 7.54 (dd, 2H, J=8.7Hz, 3' & 5'-H), 7.67 (d, 1H, J= 8 Hz, 8-H), 7.74 (dt, 1H, J= 7.4 Hz, 7-H), 7.80 (d, 2H, J= 8.7 Hz, 2' & 6'- H), 8.22 (dd, 1H, J= 7.8 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 135.7, 131.3, 130.3, 128.9, 127.4, 125.6, 123.8, 123.4, 118.0, 107.3.

2.1.5. 2-(4-Bromophenyl)-4H-Chromen-4-one (2c)

IR (KBr): 1640 (C=O), 1478, 1420, 748 cm^{-1}; ^{1}HNMR: δ 6.79 (s, 1H, 3-H), 7.43 (t, 1H, J= 8.0 Hz, 6- H), 7.50 (dd, 2H, J=7.6Hz, 3' & 5'-H), 7.54 (dt, 1H, J= 7.4 Hz, 8-H), 7.71 (dt, 1H, J= 8.4 Hz, 7-H), 7.86 (dd, 2H, J=7.6 Hz, 2' & 6' –H), 8.23 (dd, 1H, J= 7.8 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 132.3, 131.3, 129.6, 126.7, 125.7, 124.0, 123.8, 123.4, 118.0, 107.3.

2.1.6. 2-(2, 4-Dichlorophenyl)-4H-Chromen-4-one (2d)

IR (KBr): 1630 (C=O), 1460, 778 cm^{-1}; ^{1}HNMR: δ 6.46 (s, 1H, 3-H), 7.10 (dt, 1H, J= 8.4 Hz, 8-H), 7.158 (d, 1H, 5'-H), 7.208 (d, 1H, 6'- H), 7.35 (dd, 2H, 3'-H), 7.36 (t, 1H, J= 7.9 Hz, 6- H), 7.688 (dt, 1H, J= 7.8 Hz, 7-H), 8.12 (dd, 1H, J= 8.0 Hz, 5-H) ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 159.7, 155.7, 135.3, 133.2, 131.4, 129.15, 128.5, 128.0, 127.8, 125.6, 123.8, 123.4, 118.0, 105.6.

2.1.7. 2-(4-Fluorophenyl)-4H-Chromen-4-one (2e)

IR (KBr): 1635 (C=O), 1470, 1415, 1078 cm^{-1}; ^{1}HNMR: δ 6.26 (s, 1H, 3-H), 7.24 (dt, 1H, J= 8.0 Hz, 8-H), 7.43 (t, 1H, J= 7.8Hz, 6-H), 7.56 (dd, 2H, J=7.3Hz, 3' & 5'-H), 7.71 (dt, 1H, J= 8.2 Hz, 7-H), 7.94 (dd, 2H, J=7.3 Hz, 2' & 6' –H), 8.22 (dd, 1H, J= 7.9 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 163.3, 155.7, 131.3, 129.6, 125.7, 124.9, 123.8, 123.4, 118.1, 115.9, 107.3.

2.1.8. 2-(4-Methoxyphenyl)-4H-Chromen-4-one (2f)

IR (KBr): 1625 (C=O), 1460, 1405, 1208 cm^{-1}; ^{1}HNMR: δ 3.79 (s, 3H, OCH_3), 6.75 (s, 1H, 3-H), 7.03 (dd, 2H, J=6.9 Hz, 3' & 5'-H), 7.42 (dt, 1H, J= 8.0 Hz, 8-H), 7.55 (t, 1H, J= 8.0 Hz, 6- H), 7.68 (dt, 1H, J= 7.1 Hz, 7-H), 7.88 (dd, 2H, J=6.9 Hz, 2' & 6' –H), 8.23 (dd, 1H, J= 8.0 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 160.4, 131.34, 128.5, 125.7, 123.8, 123.4, 122.44, 118.0, 114.42, 107.3, 55.46.

2.1.9. 2-(4-Methylphenyl)-4H-Chromen-4-one (2g)

IR (KBr): 3080 (Ar-C-H), 1620 (C=O), 1455, 1400 cm^{-1}; ^{1}HNMR: δ 2.44 (s, 3H, methyl substituent), 6.79 (s, 1H, 3-H), 7.31 (dd, 2H, J=7.9 Hz, 3' & 5'-H), 7.41 (dt, 1H, J= 8.0 Hz, 8-H), 7.57 (t, 1H, J= 8.4 Hz, 6- H), 7.69 (dt, 1H, J= 8.2 Hz, 7-H), 7.83 (dd, 2H, J=7.9 Hz, 2' & 6' –H), 8.23 (dd, 1H, J= 8.0 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 139.7, 131.3, 129.8, 129.6, 126, 125.6, 123.8, 123.4, 121.3, 118, 107.3.

2.1.10. 2-(3-Nitrophenyl)-4H-Chromen-4-one (2h)

IR (KBr): 1646 (C=O), 1530, 1350, 1420 cm^{-1}; ^{1}HNMR: δ 6.56 (s, 1H, 3-H), 7.18 (dt, 1H, J= 8.0 Hz, 8-H), 7.36 (t, 1H, J= 8.0 Hz, 6- H), 7.62 (dd, 1H, J=7.8 & 8.4 Hz, 5'-H), 7.70 (dt, 1H, J= 7.58 & 8.0 Hz, 7- H), 7.86 (dd, 1H, J=8.0 Hz, 5'- H), 8.15 (dt, 1H, J= 7.8 Hz, 6'-H), 8.33 (dd, 1H, J= 8.3 Hz, 4'-H), 8.67 (s, 1H, 2'–H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 140.5, 131.0, 128, 126, 125.6, 124, 123.4, 118, 117.3, 116, 107.

2.1.11. 2-(2-Chlorophenyl)-4H-Chromen-4-one (2i)

IR (KBr): 1640 (C=O), 1469, 1409, 773cm^{-1}; ^{1}HNMR: δ 6.52 (s, 1H, 3-H), 7.1 (dt, 1H, J= 8.1 Hz, 8-H), 7.35 (t, 1H, J= 7.6 Hz, 6-H), 7.38 (dd, 1H, J=7.4 Hz, 5'-H), 7.5 (dt, 1H, J= 8.2 Hz, 3'- H), 7.64 (dd, 1H, J=8.2 Hz, 4'- H), 7.69 (dt, 1H, J= 7.8 Hz, 7-H), 7.76 (dd, 1H, J= 7.86 Hz, 6'-H), 7.85 (s, 1H, 5–H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 159.7, 155.7, 131.3, 130.7, 130.6, 130.5, 128, 128.3, 127, 125.6, 123.8, 123.4, 118.1, 105.6.

2.1.12. 2-(4-Nitrophenyl)-4H-Chromen-4-one (2j)

IR (KBr): 1646 (C=O), 1530, 1350, 1420 cm^{-1}; ^{1}HNMR: δ 6.82 (s, 1H, 3-H), 7.18 (dt, 1H, J= 8.1 Hz, 8-H), 7.36 (t, 1H, J= 7.6 Hz, 6- H), 7.59 (dd, 1H, J=8.7 Hz, 3', 5'- H), 7.6 (dd, 1H, J=7.6 & 8.1 Hz, 5'-H), 7.70 (dt, 1H, J= 7.6 & 8.0 Hz, 7- H), 8.0 (dt, 1H, J= 8.7 Hz, 2', 6'-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 140.5, 131.3, 129.6, 126.4, 125.6, 123.8, 123.4, 118, 117.3, 107.

2.1.13. 2-(4-Dimethylaminophenyl)-4H-Chromen-4-one (2k)

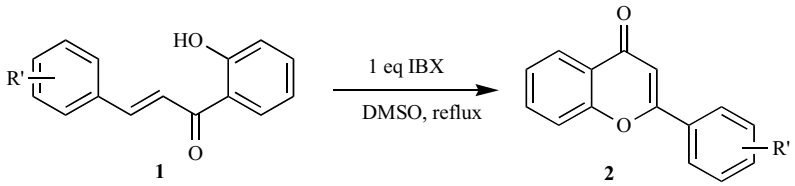
IR (KBr): 3200, 3080 (Ar-C-H), 1630 (C=O) cm^{-1}; ^{1}HNMR: δ 2.86 (s, 6H, methyl substituent), 6.29 (s, 1H, 3-H), 6.63 (dd, 2H, J=8.8 Hz, 3' & 5'-H), 7.16 (dt, 1H, J= 8.0 Hz, 8-H), 7.35 (t, 1H, J= 7.6 Hz, 6- H), 7.57 (dd, 2H, J=8.8 Hz, 2' & 6' –H), 7.68 (dt, 1H, J= 8.1 & 7.6 Hz, 7-H), 7.84 (dd, 1H, J= 8.1 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 163.6, 155.7, 151.4, 135.2, 131.3, 129.6, 126.4, 125.6, 123.8, 123.4, 118, 113.8, 107.3, 40.3.

2.1.14. 2-(4-Bromophenyl)-7-(hydroxyphenyl)-4H-Chromen-4-one (2l)

IR (KBr): 3210, 3080 (Ar-C-H), 1650 (C=O), 678 cm^{-1}; ^{1}HNMR: δ 6.44 (s, 1H, 3-H), 6.59 (d, 1H, J= 1.3 Hz, 8-H), 6.84 (d,

Table 1. Optimisation of reaction conditions for the synthesis of flavone 2b.

Entry	Solvents	Catalyst Mol%	Temp in °C	Time	% Yield
1	Acetone	1eq	RT	24h	No change
2	DCM	1eq	RT	24h	No change
3	1,4-dioxane	1eq	100°C	8h	No change
4	DMSO	1eq	Reflux	30 min	98%
5	-	5	Reflux	5h	No change
6	-	10	Reflux	4h	No change
7	-	20	Reflux	3h	No change
8	-	30	Reflux	2h	40%
9	-	60	Reflux	1h	78%
10	-	80	Reflux	45min	80%
11	DMSO	1eq	RT	48h	No change
12	-	-	60	12h	No change
13	-	-	100	12h	No change
14	-	-	120	12h	50%



Scheme 1. Synthesis of flavones **2a-k** by oxidative cyclisation.

1H, J= 7.6 Hz, 6- H), 7.60 (dd, 2H, J=8.8 Hz, 3' & 5'-H), 7.71 (dd, 2H, J= 8.8 Hz, 2' & 6' –H), 8.1 (dd, 1H, J= 7.8 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.7, 148.9, 132.3, 129.6, 126.7, 124.02, 119.45, 117.5, 114, 112.32, 107.3.

2.1.15. 2-(4-Chlorophenyl)-7-(hydroxyphenyl)-4H-Chromen-4-one (2m)

IR (KBr): 3215, 3085 (Ar-C-H), 1649 (C=O), 773 cm^{-1}; ^{1}HNMR: δ 6.5 (s, 1H, 3-H), 6.59 (d, 1H, J= 1.4 Hz, 8-H), 6.84 (d, 1H, J= 7.8 Hz, 6- H), 7.47 (dd, 2H, J=8.9 Hz, 3' & 5'-H), 7.72 (dd, 2H, J= 8.8 Hz, 2' & 6' –H), 8.0 (dd, 1H, J= 7.8 Hz, 5-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.7, 148.9, 135.7, 130.3, 128.96, 124.02, 119.45, 117.5, 114, 112.32, 107.3.

2.1.16. 2-(4-Bromophenyl)-5'-(Bromophenyl)-4H-Chromen-4-one (2n)

IR (KBr): 3085 (Ar-C-H), 1590 (C=O), 678 cm^{-1} ^{1}HNMR: δ 6.47 (s, 1H, 3-H), 7.17 (d, 1H, J= 8.1 Hz, 8-H), 7.46 (d, 1H, J= 8.1 Hz, 7- H), 7.61 (dd, 2H, J=8.8 Hz, 3' & 5'-H), 7.72 (dd, 2H, J= 8.8 Hz, 2' & 6' –H), 8.1 (dd, 1H, J= 1.9 Hz, 5-H) ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.7, 148.9, 132.3, 129.6, 126.7, 124.02, 119.45, 117.5, 114, 112.32, 107.3.

2.1.17. 2-(4-Chlorophenyl)-5'-(Bromophenyl)-4H-Chrom-4-one (2o)

IR (KBr): 3090 (Ar-C-H), 1599 (C=O), 773 cm^{-11} HNMR: δ 6.52 (s, 1H, 3-H), 7.12 (d, 1H, J= 8.1 Hz, 8-H), 7.47 (d, 1H, J= 8.1 Hz, 7- H), 7.48 (dd, 2H, J=8.8 Hz, 3' & 5'-H), 7.73 (dd, 2H, J= 8.8 Hz, 2' & 6' –H), 8.07 (dd, 1H, J= 1.9 Hz, 5-H) ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.5, 163.6, 155.6, 135.2, 132.3, 129.6, 129.5, 126.7, 124, 117.8, 111.97, 107.35.

3. RESULTS AND DISCUSSIONS

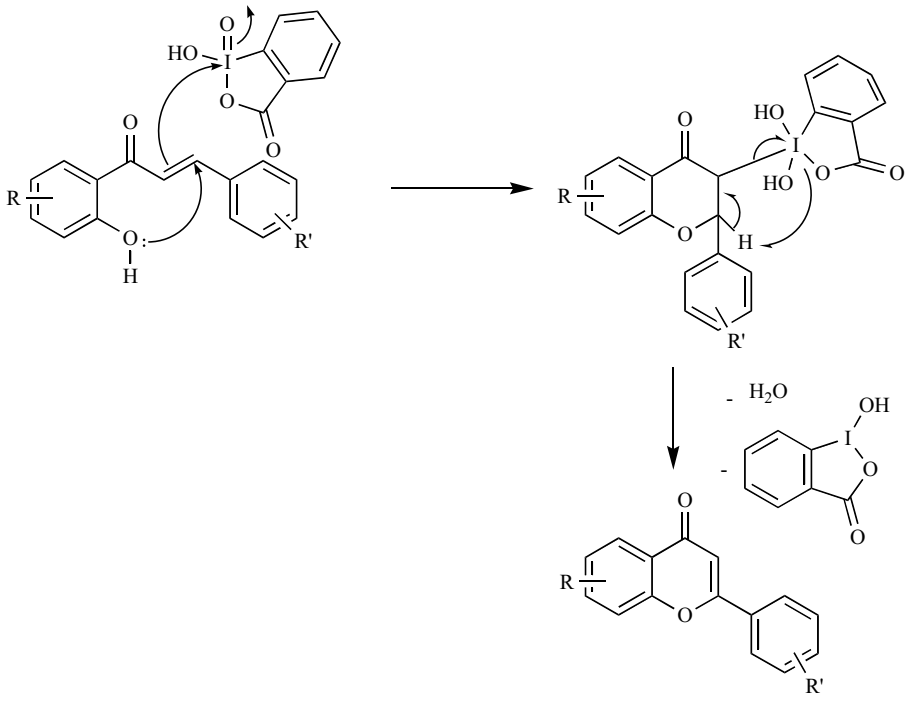
1-hydroxy-1λ^3-iodane-2-benziodoxol-3(1H)-one-1-oxide (IBX) was prepared from o-iodobenzoic acid using oxone under aqueous conditions [27], at 70-80 °C with mechanical stirring, followed by cooling at 5 °C.

A number of substituted flavone derivatives have been synthesised by first ever use of IBX as an oxidant for the oxidative cyclisation of 2'-hydroxy chalcones **1** to their respective flavone derivatives **2**. For the method to be more generalized, we thought of optimizing the reaction conditions based on catalyst amount, temperature and solvents used (Table **1**, entries **1-14**). For optimization, 4'-chloro-2'-hydroxychalcone **1b** was selected. Firstly, one equivalent of IBX was tried using various solvents. There was no formation of flavone **2b** in acetone, dichloromethane and 1, 4-dioxane due to the solubility problem. (Entries 1-3) The conversion to the product **2b** was observed only in DMSO, wherein excellent yield was obtained. (Entry 4) Then, the effect of catalyst was studied for the formation of compound **2b**, by varying the amount of catalyst using DMSO. (Entries 5-10) No formation of the flavone **2b**, in the presence of 5, 10 and 20mol% of the catalyst was observed. The conversion of chalcone into its product **2b** was only observed for 30 mol%, which gave 40% yield. (Entry 8) Good yields were obtained using 60 and 80 mol% (Entries 9, 10) whereas, one equivalent of the catalyst resulted in 98% yield of the product. (Entry 4) Effect of temperature was studied by employing different temperature conditions using one equivalent of IBX and DMSO as solvent. (Entries 11-14) It was observed that, there was no formation of the product at room temperature even after 48 hours of stirring, (Entry 11) and at 80°C and 100°C, after heating for 12 hours. (Entries 12, 13) But at 130°C, the product was obtained in 50% yield after 12 hours of heating (Entry 14).

Table 2. Synthesis of flavones 2a-k from 2'-hydroxychalcones using IBX.

Product[a] 2	R'	R	Time (mins)	Yield[b] (%)	M. Pt (^oC)	Lit. M.pt. (^oC) [29]
a	H	H	120	85	90-95	98-100[a]
b	4-Cl	H	30	98	185-187	187-188[b]
c	4-Br	H	30	95	169-170	170-172[c]
d	2,4-Cl	H	30	89	170-171	171-173[d]
e	4-F	H	60	92	132-135	134-135[e]
f	4-OMe	H	30	89	155-158	154-157[a]
g	4-Me	H	45	82	80-82	78-80[b]
h	3-NO_2	H	90	88	260-264	265-267[b]
i	2-Cl	H	120	86	112-115	117-119[a]
j	4-NO_2	H	120	85	271-275	276-278[b]
k	4-NMe_2	H	120	80	159-161	160-163[f]
l	4-Br	7-OH	180	80	278-280	281-283[f]
m	4-Cl	7-OH	180	80	268-270	270-272[g]
n	4-Br	5-Br	120	84	195-198	199-201[h]
o	4-Cl	5-Br	130	82	189-191	190-191[h]

[a]Products were confirmed by ^{1}HNMR and ^{13}CMR and also by comparing with the literature data
[b]Isolated yields.



Scheme 2. Plausible mechanism of oxidative cyclisation.

Overall, the use of 1equivalent of IBX under refluxing condition in DMSO was the best employed method for the synthesis of flavones. We thus, extended this method for the synthesis of differently substituted 2'-hydroxychalcones **1** which were previously synthesized by Claisen Schmidt condensation [28], of substituted aldehydes with that of 2-hydroxyacetophenone. These chalcones were treated with IBX (1equivalent), in DMSO under refluxing conditions. Monitoring of the reaction by TLC with the appearance of fluorescent spot in UV light indicated formation of the product **2**. In total, fifteen flavones derivatives were synthesized in very good yields (Scheme **1**, Table **2**).

All the synthesised compounds were characterised by spectroscopic methods such as IR, ^{1}H NMR and ^{13}C NMR spectroscopy. The IR showed disappearance of OH peak, with a shift in the C=O stretching frequency between 1630-1650cm^{-1} thus confirming the formation of product **2**. ^{1}HNMR showed a singlet peak at δ 6.0-6.8

ppm which corresponded to the splitting due to 3-*H* proton of the chromone ring, δ 7.2-7.8 ppm corresponded to aromatic protons.

In designing a new synthesis of flavone using IBX, the proposed mechanistic pathway [26] could be, the lone pair on the oxygen of the hydroxyl group attacking the β-carbon of the propen-1-one chain, followed by the attack at the iodine of 1-hydroxy-1λ^3-iodane-2-beniodoxol-3(1*H*)-one-1-oxide (IBX) forming a complex which on hydrolysis could give the 4*H*-Chromen-4-one (Scheme **2**).

CONCLUSION

In conclusion, a new and first approach towards the synthesis of flavones using non-toxic and eco-friendly hypervalent iodine (V) reagent, IBX has been demonstrated. Such a methodology has been well-extended towards the synthesis of hydroxy, and bromo flavones. The work signifies the electrophilic character of IBX, which is an oxidant.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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In-vitro Anti-cancer assay and apoptotic cell pathway of newly synthesized benzoxazole-*N*-heterocyclic hybrids as potent tyrosine kinase inhibitors

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ABSTRACT

A series of benzoxazole-*N*-heterocyclic hybrids have been synthesized by a one-pot strategy. Molecular docking study revealed that such compounds have the ability to inhibit enzyme protein tyrosine kinase. The findings of this work have been the successful synthesis of benzoxazole scaffolds, featuring hybrids of benzoxazole with quinoline and quinoxaline respectively. The molecular docking studies have showed these compounds to be inhibitors of tyrosine kinase enzyme which triggers growth of cancer cells. The cytotoxicity study of compounds **4a-f** showed better potency against breast cancer cell lines MCF-7 and MDA-MB-231 in contrast to oral and lung cancer cell lines KB and A549. The tyrosine kinase activity was measured using Universal Tyrosine Kinase Assay kit using horseradish peroxide (HRP)-conjugated anti-phosphotyrosine kinase solution as a substrate. The compounds **4c** exhibited maximum inhibition in the activity of enzyme tyrosine kinase with IC_{50} value 0.10 ± 0.16 μM, than other compounds which were studied and thus proved to be inhibitors of enzyme tyrosine kinase. The selective index of all four compounds was found out to be greater than two, indicating the non-toxic behaviour, i.e. good anti-cancer activity. Further, fluorescence microscopic study helped to characterize the mode of cell death, which was found to be late apoptosis as indicated by the orange fluorescence. The SAR analysis has also been carried out.

1. Introduction

Cancer is affecting millions of lives all around the world [1,2]. With the sophisticated and different changing lifestyle, the growth of cancer disease has erupted rapidly. The most common type of cancers include lung cancer, oral cancer [3] and breast cancer. Nowadays, due to chemotherapeutic treatment most of them lead to metastasis, disease to liver, bone, lungs and bronchitis. Several types of breast cancer carcinoma have been reported that has been responsible for painstaking deaths all round the world. In view of this there is great need to search for newer breast cancer drug targets. Moreover, the scope to develop novel therapy strategies becomes the need and a challenging task. From cancer drug target study basal like subtypes triple negative breast cancer (TNBC) constitutes to about 80% of basal like tumors and is responsible for 10–15% breast carcinoma and also shows lack of expression of both hormone receptor (Progesterone and Estrogen) and HER2-receptor over expression [4–5]. A drug target for such cancer becomes evident as TNBC and basal like cancers are considered to be in the lead as an aggressive disease with greater chances of metastasis. The therapy for such conditions namely, Trastuzumab (Fig. 1. **I**) cuts the risk of recurrence to half-compared to chemotherapy alone. Trastuzumab is given through veins in every 3 weeks for 1 year [6–9]. Scaffold containing heterocyclic conjugates such as 3-pyrimidinylazaindole analogue, (Fig. 1. **II)** a potent inhibitor of triple negative breast cancer which cuts the risk to upto 90% of tumor growth inhibition as compared to the other leads [10].

Human protein kinase forms a large family of enzymes commonly known as human Kinome encoded by about 1.7% of different types of human genes [11]. In this group of enzymes, tyrosine kinase has been the first protein kinase which has been well identified, described with respect to its location in the cell. Keeping in view the structural features, small molecule kinase inhibitors can be of great help to design and develop targeted therapies against breast cancer. The advantage of such inhibitors is high selectivity, better efficacy and specific action on protein kinase receptors.

In the designing of small drug like molecules, molecular hybridisation of heterocyclic compounds could be challenging task to gain potency in acting against tyrosine kinase enzymes. Thus, our focus was

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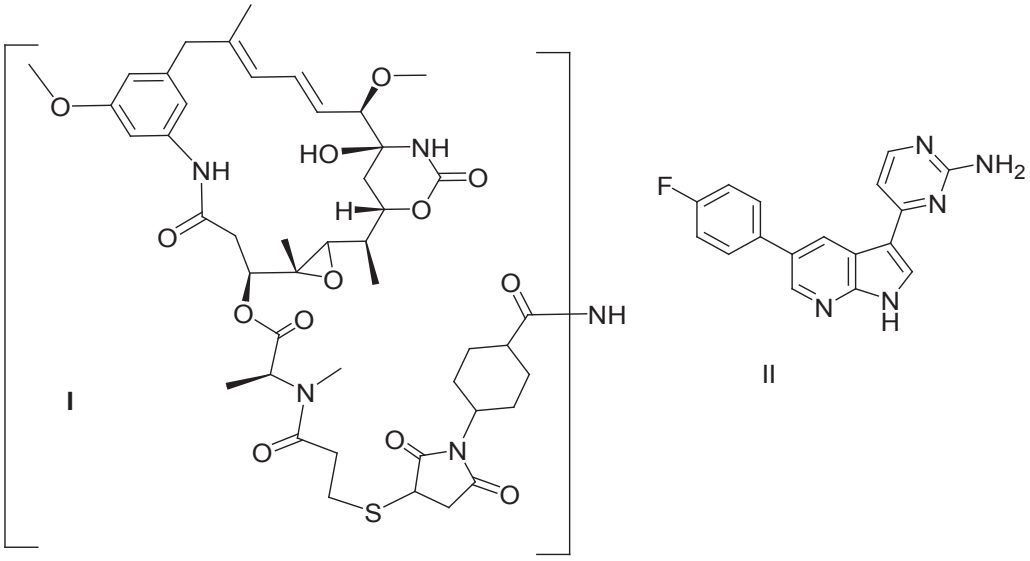


Fig. 1. Representative Structures of breast cancer drugs: I. Trastuzumab, sold under the brand name Herceptin among others, is a monoclinical antibody, used to treat HER2 positive breast cancer; II. 3-Pyrimidinylazaindole, a potent inhibitor of triple negative breast cancer.

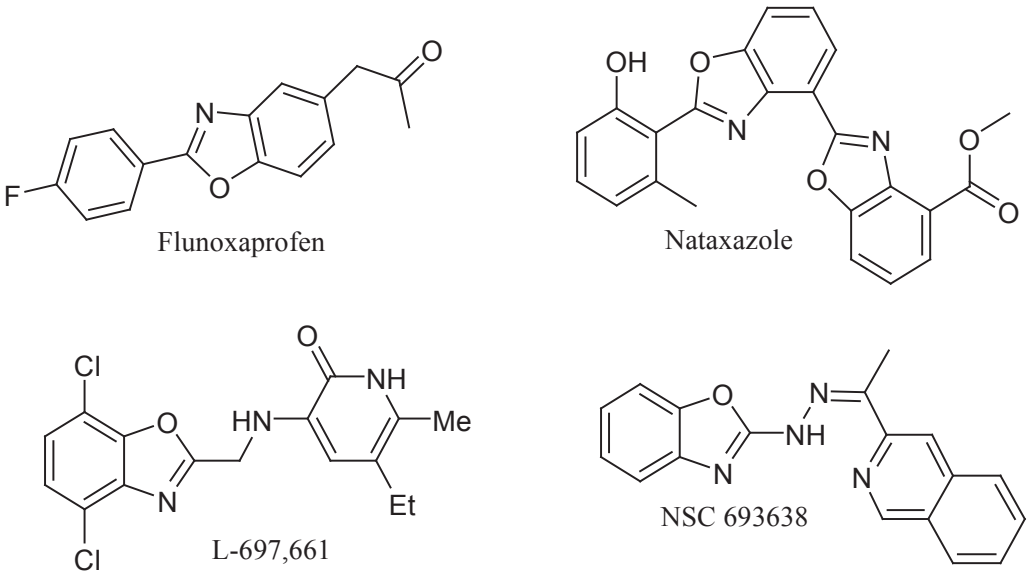
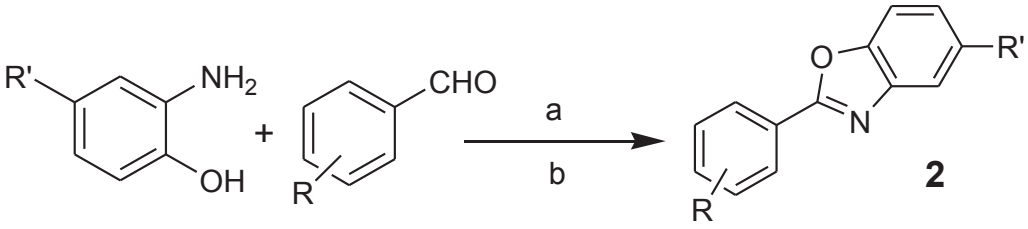


Fig. 2. Bioactive Compounds containing Benzoxazole moiety: flunoxaprofen-NSAIDs, L-697,661 a reverse transcriptase inhibitor, Nataxazole an anti-tumor, NSC693638- an anticancer agent.

on benzoxazole as a core unit as it forms an important class of bicyclic heterocycles having wide spectrum of biological applications [12]. Benzoxazole ring as a core pharmacophoric unit is found in various class of natural and synthetic compounds showing varied biological properties and has been studied for their antibacterial and anti-fungal [13,14], anticancer [15,16] and HIV-1 reverse transcriptase inhibitors [17]. It constitutes subunits of commercially available drug molecules such as; Flunoxaprofen which is a non-steroidal anti-inflammatory drug [18], NSC693638- an anticancer agent [19], L-697,661 a reverse transcriptase inhibitor, [20] Nataxazole, an anti- tumor agent [21] related to anti-tumor drugs like UK-1 and AJ19561 [22]. Fig. 2 The synthesis of benzoxazolyl-carbohydrazide derivatives and their *in-silico* docking and *in-vitro* anti-cancer study has been also reported [23]. Combretastin A-4 and benzoxazole analogues have been reported to exhibit more potent anti-cancer activity then the standard drug against A-549 and MCF-7 cell lines [24].

Nitrogen-based heterocycles are an important class of compounds in pharmaceutical and agrochemical industries. Their structural diversity has made them attractive targets in the synthesis of alkaloids and important precursors to many biologically important active compounds. They have emerged as integral parts of various drugs known in day to day life. Some of the nitrogen heterocycles of biological importance includes: quinoxaline, indoles, quinolines, pyridines etc. Designing of heterocyclic hybrids [25] has led to newer breakthrough in the need for treatment of cancer and so it was visualized that heterocyclic hybrids of *N*-heterocycles with benzoxazole would have a promising effect on the biological activity. Thus, in continuation to our work on designing heterocyclic molecules of biological importance using ecofriendly strategies [26–29] herein, we present the synthesis of benzoxazoles using silica chloride. This methodology was further extended towards synthesis of six novel heterocyclic scaffolds. The molecular docking studies and biological evaluation has also been discussed, revealing our compounds to have the best anti-cancer activity. Development of solid acid catalyzed [30–33] synthesis of benzoxazoles and their action as inhibitors of protein kinase enzyme has been the focus of the work.



Scheme 1. One-pot synthesis of benzoxazole derivatives (2a-r) Reagents and conditions: (a) 1mmole of the 2-aminophenol and substituted benzaldehyde, 1 eq silica chloride, (b) 120 °C, solvent-free.

2. Results and discussion

2.1. Chemistry

The target benzoxazole was synthesized from condensation of 2-aminophenol derivatives with differently substituted aromatic aldehydes via Schiff base intermediate using previously prepared silica chloride [34] under solvent free conditions. The optimization of the reaction conditions revealed that catalyst not in mole ratio but in 1 equivalent and temperature of about 120 °C gave the cyclised product in short time of about 3–4 h. Consequently, a wide variety of 2-aryl benzoxazole derivatives having different functional groups was obtained successfully in good yields in the range of 70–80%. (Scheme 1, Table 1**)**

However, our focus was on designing of novel heterocyclic scaffolds with benzoxazole as the core unit. Our previous work on quinoxalinyl chalcones depicted good anti-tubercular and anti-cancer activity [35]. As a continuation to the work on designing of heterocyclic hybrids, an attempt has been made to prepare the new substituted benzoxazole derivatives bearing heterocyclic moiety. Six new benzoxazole conjugates **4a-f** has been prepared by the same strategy under solvent free conditions from commercially available aldehydes, previously synthesised quinoxaline-2-carbaldehyde [36] and 2-chloro-3-formyl quinolone [37]. (Scheme 2**)** All the six benzoxazole analogues were obtained in good yields. (Table 2**)**

Their structures were confirmed by ^{1}H NMR, ^{13}C NMR, and Melting points. The IR spectra showed disappearance of OH and C=O peak, C=C peak at 1605 cm^{-1}, C=N peak at 1677 cm^{-1} and peak at 1301 cm^{-1} attributed to C–O peak. The ^{1}H NMR spectra of the compounds **2a-r** showed an average spectrum in which appeared two doublets at δ 8.20 ppm (Ar–H, 3′ & 5′position) and δ 7.51 ppm (Ar–H; 2′ & 6′) . In addition, protons of the fused benzene ring appeared as three multiplets at δ7.79–7.75 ppm (–CH=C–N–), δ 7.57–7.61 ppm for C5-H, δ7.36–7.39 ppm for C7-H and C6-H; ^{13}C NMR spectra of compounds revealed the presence of C=N at the range of 164.01C-Cl at the range of 150.3. The structures of *N*-heterocyclic hybrids **4a-f** were confirmed by ^{1}H NMR, ^{13}CNMR, and HRMS data. The calculated M + H ion peak matched with that of the observed M + H ion peak, which confirmed the structure.

Table 1
Synthesis of benzoxazole derivatives **2a-r**[a].

Comp 2	R	R'	Yield[b]	Melting Point[c] °C
a	2,4- Cl	H	75%	125–127 (1 2 7)
b	4- Cl	H	75%	144–148 (148–150)
c	4- Br	H	71%	150–152(157–158)
d	3-NO_2	H	79%	99–102(103–105)
e	2-NO_2	H	72%	97–100(98–102)
f	4-OMe	H	77%	114–116 (113–114)
g	4-NO_2	H	79%	256–260 (2 6 8)
h	H	H	78%	98–100 (1 0 1)
i	2-Cl	H	70%	68–72(73)
j	4-Me	H	79%	104–106 (101–103)
k	3-Cl	H	72%	120–122 (122–124)
l	4-OH, 5-NO_2	H	75%	170–174 (1 7 8)
m	2-OH, 5-NO_2	H	71%	188–192 (1 8 8)
n	4-F	H	79%	90–92 (92–95)
o	3-Br	H	72%	133–137 (136–138)
p	4-NMe_2	H	70%	179–182(182–183)
q	5-Br, 4-OH, 3-OMe	H	78%	187–190 (190–192)
r	4-OH-3,5- Br	H	70%	166–169 (168–170)

[a] Reaction conditions: Schiff base(1mmole), silica chloride 1equivalent.
[b] Isolated yields.
[c] Determined using thiels tube paraffin method.

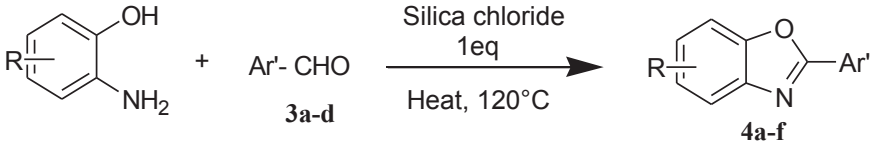
2.2. Biological evaluation

2.2.1. Virtual screening

The promising approach to cancer therapy has been the targeted therapies which lead to beneficial clinical effects. Tyrosine kinase is an important target due to its role in modulation of growth factor signalling, it causes increase in tumor cell proliferation and growth, induces anti-apoptotic effects and promotes angiogenesis and metastasis. Because of all these effects, receptor tyrosine kinase has been a key target for cancer therapy. Tyrosine kinases are enzymes which catalyses the transfer of γ- phosphate group from adenosine triphosphate to target proteins. They play an important role in diverse normal cellular regulatory processes [38]. It is characterised by immunoglobulin-like sequences in their amino-terminal extracellular domains, a lipophilic transmembrane segment and an intracellular carboxyl-terminal domain which includes its catalytic site. Ligand binding induces dimerisation of these tyrosine kinase receptor and results in autophosphorylation of their cytoplasmic domains and activation in activity of tyrosine kinase. The 2-aminobenzoxazole and benzimidazole derivatives and pyrazolyl-benzoxazole derivatives have been proved to be active tyrosine kinase inhibitors due to strong binding affinity with enzyme [39,40]. Hence, we chose enzyme tyrosine kinase as a target for the molecular docking study of the synthesised benzoxazole-*N*-heterocylic hybrids.

The crystal structure of the epidermal growth factor receptor tyrosine kinase domain with 4-anilinoquinazoline inhibitor erlotinib (PDB ID: 1M17) was used for the docking studies, obtained from Protein Data Bank. The protein file was prepared by the removal of water molecules, addition of polar hydrogens and removal of other bound ligands. The synthesized benzoxazole-*N*-heterocyclic conjugates **2a-r** and **4a-f** were virtually screened for its anti-cancer activity against tyrosine kinase domain with 4-anilinoquinazoline inhibitor erlotinib as target enzyme. (Table 3**)** The molecular docking study revealed that compounds **4a**, **4c and 4d** acts as good inhibitors of tyrosine kinase due to characteristic features. (Fig. 3**)** However, compounds **2a-r** were also found to be active with the target PDB ID: 1M17. As depicted in Fig. 4, Compound **4a** makes four hydrogen bond interactions at the active site of the enzyme. Three interaction with the nitro group present on phenyl ring; of which one interactions raised from H-bond interaction between nitrogen atom of the NO_2 group with Thr766 (1.55 A°) and two interaction arised from H-bond interaction between oxygen atom of nitro group and CYS751 (1.52 A°) and Thr830 (1.52 A°). The remaining one arose from oxazole ring of benzoxazole moiety. The oxygen atom interacts with Met769 (1.55 A°).

Compound **4c** makes two hydrogen bond interactions at the active site of the enzyme. One interactions arose from nitrogen atom of oxazole ring, where the nitrogen forms H-interaction with Thr766 (1.52 A°). while the other is raised from H-bond interaction of nitrogen atom of quinoxaline ring with Met769 (1.55 A°). Compound **4d** makes



R= 4-H,
= 4-Br

3a, Ar=4-nitrobenzaldehyde
3b, Ar=4-Bromobenzaldehyde
3c, Ar= quinoxaline-2-carbaldehyde
3d, Ar= 2-chloro-3-formylquinoline

4a, R'=5-Br, Ar=4-Nitrophenyl
4b, R'=5-Br, Ar=4-Bromophenyl
4c, R'=5-Br, Ar= quinoxalinyl
4d, R'=H, Ar=quinoxalinyl
4e, R'=H, Ar=2-chloroquinolinyl
4f, R'=5-Br, Ar=2-chloroquinolinyl

Scheme 2. Synthesis of novel 2-Arylbenzoxazole scaffolds 4a-f.

Table 2
One-pot Synthesis of benzoxazolyl scaffolds **4.**

Entry	Compound 4	Time	Yield[a]	Melting Point[b]°C
1	a	2.5 h	80%	158–162
2	b	2.5 h	72%	228–232
3	c	3 h	74%	160–164
4	d	3 h	80%	189–192
5	e	3 h	75%	102–108
6	f	3 h	76%	170–175

[a] Isolated yields.
[b] Determined using thiels tube paraffin method.

three hydrogen bond interactions at the active site of the enzyme. The interaction arising from nitrogen atom of the quinoxaline, shows H-bonding which interaction with Met769 (1.55 A°). The remaining two interactions are raised from nitrogen and oxygen atom of oxazole ring with Thr766 (1.52 A°). (Fig. 4)

2.2.2. Anticancer evaluation

On the basis of enzymatic inhibitory potency against 1M17, compounds **4a-f** was selectively used as candidates for exploring the mechanisms of anti-cancer. The anti-proliferative activity has been carried out against MDA MB-231 (ER-negative) & MCF-7 (Breast Cancer), A549 (Lung Cancer), KB (Oral Cancer) and HEK293 (Normal Human Kidney Cells) by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method [41]. Using graph Pad Prism Version5.1, the IC_{50} of compounds has been calculated by taking a percentage of Inhibition Tyrosine Kinase Enzyme at six different concentrations of treatment.

All the six benzoxazole-*N*-conjugates **4a-f** exhibited cell proliferation with IC_{50} values in the range of 0.42 to 14.39 μM. As shown in Table 4, the samples showed moderate to good activity comparable to

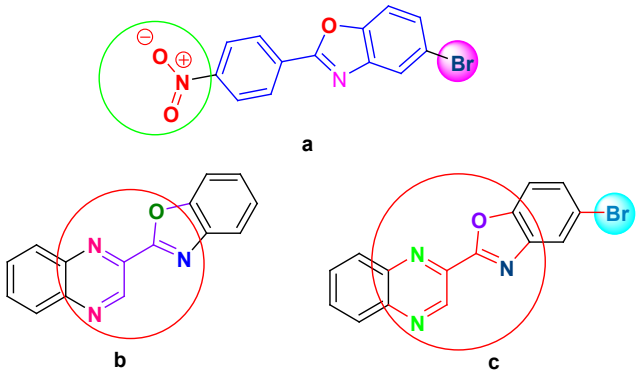


Fig. 3. Structural features of compounds responsible for the cytotoxicity: (a) Compound 4a; (b) Compound 4c; (c) Compound 4d.

Table 3
Molegro docking score for benzoxazole derivatives **2a-r** and **4a-f.**

Code	MolDock Score	Rerank Score	Protein	H bond	Heavy atom count	Kcal/mol	Docking Score
B2a	−86.5224	−64.8155	−90.5300	2	19	−5.08955	−86.0269
B2b	−80.5605	−61.4823	−86.9539	1	19	−5.03503	−79.6663
B2c	−80.2853	−62.0941	−86.7351	3	17	−5.01783	−79.4664
B2d	−79.8709	−62.0032	−101.419	3	17	−5.1842	−97.9248
B2e	−96.1274	−66.7314	−99.1471	5	17	−3.7073	−97.9981
B2f	−80.6788	−61.6469	−86.0643	2	17	−4.13724	−83.9878
B2g	−92.6515	−72.1225	−104.053	2	17	−4.8123	−86.6232
B2h	−81.1186	−66.7681	−89.5901	3	16	−4.09693	−76.124
B2i	−80.4031	−54.6825	−89.813	3	16	−3.77365	−71.5362
B2j	−73.0934	−56.8369	−79.4259	0	16	−3.5958	−73.2504
B2k	−79.7784	−61.9172	−85.919	1	16	−4.01128	−79.2865
B2l	−90.1899	−71.7397	−102.058	3	16	−5.0177	−100.482
B2m	−97.8936	−71.8246	−101.476	3	19	−5.1523	−96.9324
B2n	−75.3016	−58.9796	−81.593	0	19	−3.71931	−80.8615
B2o	−78.559	−62.499	−88.8241	0	19	−3.86762	−79.1946
B2p	−83.1269	−60.3583	−87.3281	1	17	−3.11882	−87.9088
B2q	−81.1726	−45.0394	−100.153	3	19	−4.2722	−78.8718
B2r	−79.7915	−58.6621	−88.1242	1	19	−3.59923	−86.9973
B4a	−79.8709	−62.0032	−101.419	4	17	−4.2037	−81.4409
B4b	−80.3296	−63.3705	−87.2282	1	17	−4.9468	−93.4702
B4c	−90.1899	−71.7397	−102.058	2	17	−4.7468	−89.4702
B4d	−92.6515	−72.1225	−104.053	3	17	−4.6325	−91.9065
B4e	−89.4501	−64.1618	−99.8916	1	17	−3.71632	−95.1771
B4f	−89.7198	−70.4287	−97.4172	1	16	−3.24967	−91.2059

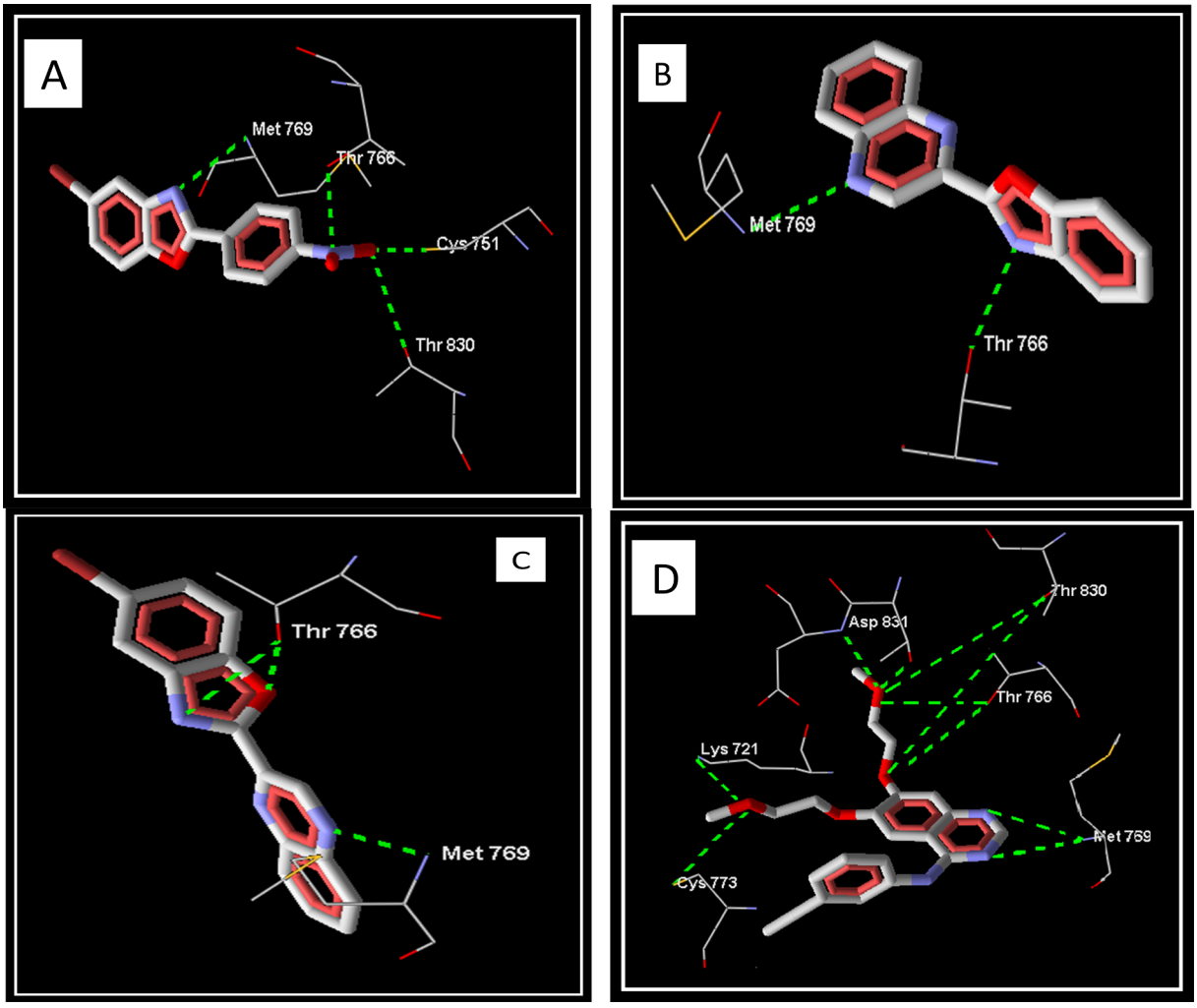


Fig. 4. Molecular docking data: the compounds docked in best of its conformation into the binding site of 1M17. (A) Binding mode of compound 4a forming four hydrogen bond interaction. (B) Binding mode of compound 4c forming two hydrogen bond interaction. (C) Binding mode of compound 4d forming three hydrogen bond interaction. (D) Binding interaction of ligand AQ4-1M17 forming 10 hydrogen bond interaction.

that of standard drugs, especially compound **4a, 4c** and **4d** showed higher anti-proliferation activity with IC_{50} = 0.56 ± 0.07, 0.58 ± 0.05 μM; IC_{50} = 0.53 ± 0.02, 0.50 ± 0.08 μM and IC_{50} = 0.73 ± 0.02, 0.60 ± 0.06 μM against MDA-MB-231(TNBC-triple negative breast cancer) and MCF-7 (human breast cancer) cell lines. Compound **4b**, **4e** and **4f** showed moderate activity with IC_{50}

Table 4
IC_{50} values (μM ± S.E.) of compounds **4a, 4b, 4c, 4d, 4e** and **4f** against four different cell lines and normal human cell lines.

Comp	IC_{50} μM				
	MDA MB-231[a]	MCF-7[b]	A549[c]	KB[d]	HEK293[e]
4a	0.56 ± 0.07	0.58 ± 0.05	0.70 ± 0.11	0.82 ± 0.08	10.11 ± 0.19
4b	1.09 ± 0.05	1.10 ± 0.03	0.97 ± 0.09	0.71 ± 0.12	3.27 ± 0.20
4c	0.53 ± 0.02	0.50 ± 0.08	0.82 ± 0.20	0.90 ± 0.05	14.39 ± 0.28
4d	0.73 ± 0.02	0.60 ± 0.06	0.87 ± 0.08	0.86 ± 0.10	8.39 ± 0.19
4e	0.98 ± 0.04	0.94 ± 0.03	0.89 ± 0.11	0.85 ± 0.04	6.04 ± 0.23
4f	0.85 ± 0.01	0.91 ± 0.02	0.83 ± 0.06	0.98 ± 0.03	7.21 ± 0.05
Paclitaxel[f]	0.3 ± 0.02	–	–	–	–

[a] MDA-MB-231 cell line- is an epithelial, human breast cancer cell line established from pleural effusion of a 51-year old Caucasian female with metastatic memmary adenocarcinoma.

[b] MCF-7 is a human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors. It is derived from pleural effusion of 69-year old Caucasian metastatic breast cancer in 1970.

[c] A549 ia a human cancer cell line, derived from the removal and culturing of cancerous lung tissues in the explanted tumor of 58-year old Caucasian metastatic male.

[d] KB is derived from an epidermal carcinoma of the mouth, KB cells contain human papilloma virus18 (HPV-18) sequence.

[e] HEK293 –derived from Human embryonic kidney293 cell lines grown in tissue culture.

[f] Paclitaxel- used as standard drug.

values 1.09 ± 0.05, 0.98 ± 0.04 and 0.85 ± 0.01 μM against MDA-MB-231 and also against MCF-7 cell lines with the IC_{50} values 1.10 ± 0.03, 0.94 ± 0.04 and 0.91 ± 0.02 μM respectively. The higher anti-proliferation against KB (oral cancer) cell line with IC_{50} value of 0.71 ± 0.12 was exhibited by compound **4b**. Compound **4a, 4d** and **4e** showed comparable anti-proliferation against KB cell line with IC_{50} value 0.82 ± 0.08, 0.86 ± 0.10 and 0.85 ± 0.04 μM respectively. Compound **4c** and **4f** exhibited moderate anti-proliferation effect. Considering the anti-proliferation effect against A549 (lung cancer), compound 4a showed good anti-proliferation with the IC_{50} value 0.70 ± 0.11 μM. Compound **4c**, **4d, 4e** and **4f** showed moderate anti-proliferation against A549 (Lung cancer) cell line with IC_{50} value 0.8 μM as compared to the other samples. The results were compatible with the docking studies.

Following this, the *in-vitro* cytotoxic activity of all the tested compounds was analyzed against normal HEK-293 cell lines by MTT colorimetric assay [41]. The colorimetric results revealed that none of the six evaluated compounds exhibited any significant toxicity effect on normal HEK-293 cells. Selectivity index (SI) reveals the differential activity of a pure compound. Higher SI value is attributed to less toxicity and more selectivity. Whereas, a compound with SI value < 2 indicates cytotoxicity of the pure compound. As shown in Table 5 all samples were proved to be non-toxic on the normal tumor cells. The effect of benzoxazole-*N*-heterocyclic hybrids **4a-f** on viability of MDA MB-231 (ER-negative), MCF-7 (Breast Cancer), A549 (Lung Cancer), KB (Oral Cancer) and HEK293 (Normal Human Kidney Cells) was investigated and represented graphically as variation in percentage of viability with respect to time and concentration. (Fig. 5**)**

2.2.3. Enzyme inhibition studies

The effect of benzoxazole-*N*-heterocyclic hybrids **4a-f** on tyrosine kinase activity *in-vitro* was carried out using Fetal Bovine Serum (FBS) cell culture treated with indicated amounts of benzoxazole-*N*-heterocyclic hybrids for indicated time. The whole cell extracts were extracted and incubated with tyrosine kinase substrate for the specified time mentioned in the procedure. The plot of relative rate of inhibition in activity of enzyme tyrosine kinase with respect to different concentration of compounds **4a-f** (Fig. 6**)** indicates that compounds **4a-f** induced a dose dependent decrease in the enzyme activity. (Table 6**)** It was summarized from the results of the enzyme inhibition studies that, compound **4c** exhibited higher inhibition in activity of enzyme Tyrosine kinase with the IC_{50} value of 0.10 ± 0.16 μM, compounds **4a**, **4d** and **4e** also exhibited good inhibitory action on the activity with IC_{50} value in the range 0.31–0.43 μM. Compound **4b** was found to be least active in inhibiting the growth of enzyme with IC_{50} value 0.86 ± 0.42 μM. With decrease in the concentration of compounds **4a-f,** the rate at which the enzyme grow was decreased, with compound **4c** exhibiting the highest inhibition in the activity of enzyme. All compounds demonstrated good rate of inhibition of tyrosine kinase enzyme at highest concentration but as the concentration decreased the effect of inhibition was found to decrease.

Table 5
Selective index (SI) [f] of compounds.

Comp	SI[f]-MDA MB-231	SI[f]-MCF-7	SI[f]-A549	SI[f]-KB
4a	9.55	9.53	9.41	9.29
4b	2.18	2.17	2.30	2.56
4c	13.86	13.89	13.57	13.49
4d	7.66	7.79	7.52	7.52
4e	5.06	5.10	5.15	5.19
4f	6.36	6.30	6.38	6.23

[f] **SI**-Selective index, calculated as (IC_{50})HEK293 - (IC_{50}) of the respective cell lines.

2.2.4. Cell morphology studies by fluorescence microscopy

Fluorescence microscopy study was employed to study the mode of cell death induced by synthesized benzoxazole-*N*-heterocyclic hybrids **4a**, **4c** and **4d** in comparison to the standard *cis*-platin by virtue of acridine orange/ ethidium bromide staining. The mode of cell death, whether early or late apoptosis can be characterized based on the fluorescence properties. Acridine orange was taken up by both viable and apoptotic (dead) cells by emitting green fluorescence. Cells with disrupted membrane integrity were stained by ethidium bromide, i.e. late apoptotic cells and necrotic cells. Late aoptotic cells have orange to red nuclei with condensed and fragmented chromatin, and early apoptotic cells show green fluorescence nuclei. Necrotic cells have uniform orange to red nuclei with organised structure. Thus, from the results (Fig. 7**)** it was visualised that, viable cells without treating with synthesised compound **4a**, **4c**, **4d** showed green fluorescent nucleus with acridine orange Fig. 7**a**, Whereas, late apoptotic cells when treated with compounds **4a**, **4c** and **4d** exhibited yellow orange fluorescence with nuclear membrane babbling. Fig. 7**b-d**; thus signifies apoptotic mode of cell death.

Further confirmation of the apoptotic mode of cell death was done by using DAPI staining method, wherein; cells treated with compounds and the viable cells were stained with 4′, 6′- diamine-2′-phenylindole dihydrochloride (DAPI). Untreated cells showed intact nucleus Fig. 8**a** whereas, treated cells showed nuclear condensation and nuclear fragmentation. Fig. 8b–d.

2.2.5. SAR studies

The following Structure activity relationship (SAR) analysis has been executed based on the results of the cytotoxicity studies of benzoxazole-*N*-heterocyclic hybrids **4a-f** against five different cancer cell lines. All the compounds were found to show good cytotoxicity effect against MDA-MB- 231 and MCF-7 breast cancer cell lines as compared to other cell lines. Compound **4a**, **4c** and **4d** exhibited good anti-cancer activity due to strong interaction with the enzyme active site and their characteristic structural features. Figs. 3 and 9. As in compound **4a**, the presence of electron withdrawing group (i.e. Nitro) onto the phenyl ring and also the presence of bromo substituent on the benzoxazole moiety enhance the cytotoxicity effect. Replacement of the nitro group by halogen (i.e. Br) as in compound **4b,** has moderate effect on cytotoxicity. In case of compound **4c**, replacing the phenyl ring with quinoxaline ring and also the presence of bromo substituent on the benzoxazole ring exhibits higher effect on cytotoxicity, so also compound **4d** with quinoxaline moiety exhibited similar effects. Quinoline moiety was found to lower the cytotoxicity effect as in compound **4e** and **4f.**

3. Conclusion:

In conclusion, a newly designed synthesis of benzoxazole has been achieved in an ecofriendly reaction conditions. Such benzoxazole hybrids **2a-r** and **4a-f** has been evaluated as anti-proliferative agents, having virtually screened as good inhibitors of enzyme protein tyrosine kinase, the key enzyme that triggers triple negative breast cancer. Comparative cell cytotoxicity studies against five different cancer cell lines MCF-7, MDA-MB-231, KB, A549 and HEK-293 revealed that all six benzoxazole hybrids **4a-f** showed good inhibitory potency against all cell lines. It was visualized from the anti-cancer activity results that, compounds **4a**, **4c** and **4d** demonstrated excellent inhibitory potency against MDA-MB-231 and MCF-7 breast cancer cell lines with the IC_{50} value in the range of 0.50–0.73 μM as compared to standard drug Paclitaxel (IC_{50}; 0.030 ± 0.02 μM). Compounds **4a-f** was also found to be potent against A549 (lung cancer) with IC_{50} values in the range of 0.70 to 0.97 μM.

Comparing the anti-cancer potency against all five cancer cell lines, the target compounds **4a**, **4c** and **4d** exhibited better anti-proliferation effect against MDA-MB-231 and MCF-7 cell lines. Cytotoxicity against HEK-293; human embryonic kidney cell line was also evaluated and

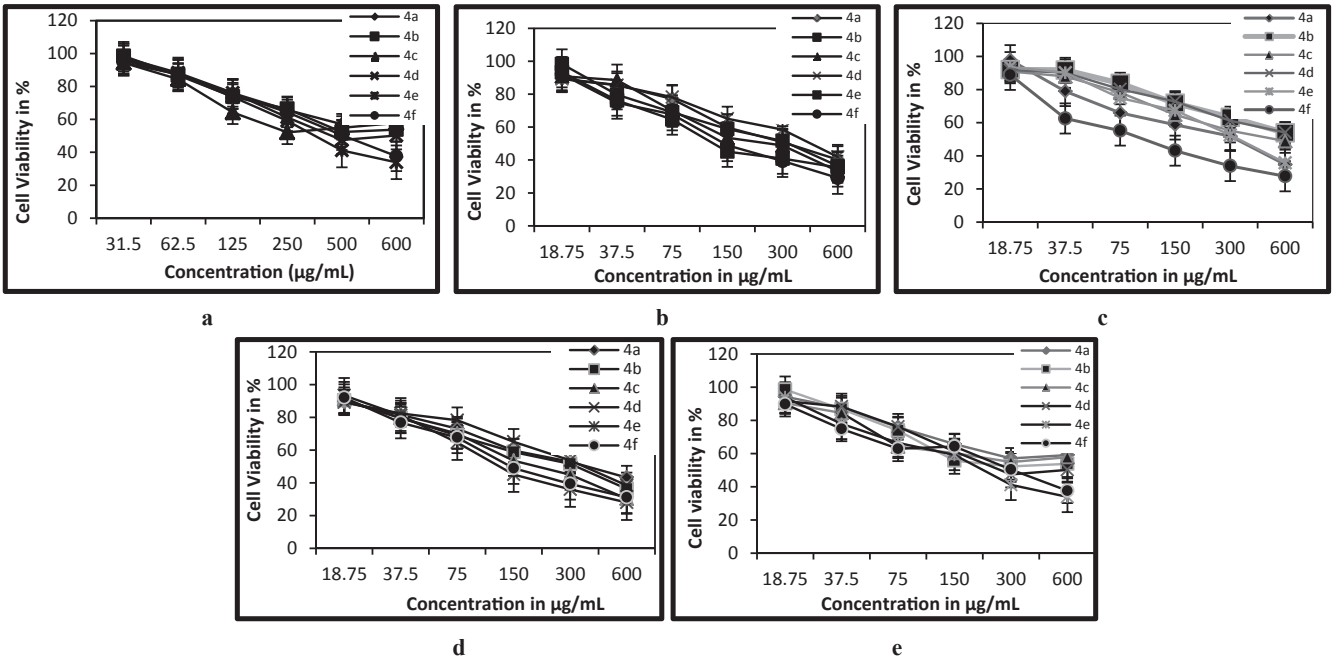


Fig. 5. Graphical representation of cell viability exhibited by 4a, 4b, 4c, 4d, 4e and 4f for five different cell lines: (a)MDA-MB-231, (b) MCF-7, (c) A549, (d)KB, (e) HEK293.

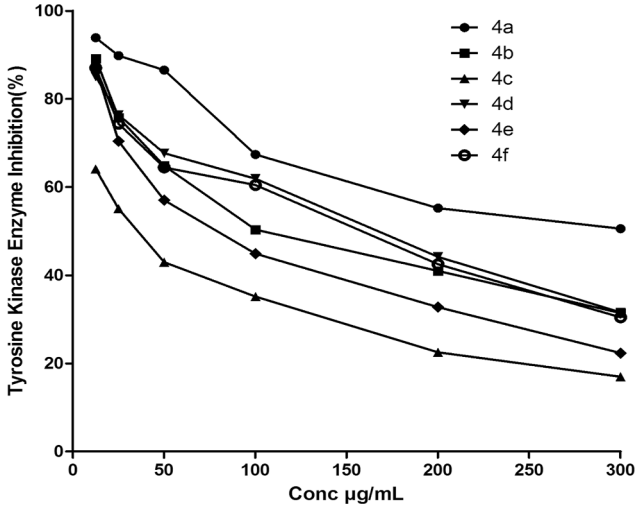


Fig. 6. Graphical representation of enzyme Tyrosine kinase inhibition by compounds 4a-f: Plot of concentration in µg/mL v/s rate of inhibition.

Table 6
Enzyme inhibition of Tyrosine kinase enzyme by compounds **4a-f.**

Compound code	IC_{50} value[a]
4a	0.31 ± 0.11
4b	0.86 ± 0.42
4c	0.10 ± 0.16
4d	0.35 ± 0.17
4e	0.43 ± 0.21
4f	0.56 ± 0.24

[a] IC_{50} values measured in µM.

selectivity was determined. The selective index value in the range of 2.17–13.89 was displayed by the compounds screened. The docking results revealed that compounds **4a**, **4c** and **4d** had good interaction with the active site of enzyme tyrosine kinase. Further, enzyme inhibition study of compounds **4a-f** proved that, all compounds inhibits the activity of enzyme tyrosine kinase with IC_{50} value in the range of 0.10–0.86 µM, with maximum inhibition being exhibited by compound **4c** with IC_{50} value 0.10 ± 0.16 µM. The results were found to be compatible with the docking studies, thus demonstrating that compound **4a**, **4c** and **4d** act as potent inhibitors of tyrosine kinase. Accordingly, a mode of cell death was studies using double staining and DAPI method thus signified the mode of cell death to be late apoptosis. SAR analysis reveals the influence of benzoxazole *N*-heterocyclic hybrids in defining its cytotoxicity and anticancer activity.

On contrary, the *in-vitro* anti-tubercular screening [42] of the compounds **2a-r** against *Mycobacterium tuberculosis* $H_{37}RV$ strain exhibited excellent anti-tubercular activity with the MIC value ranging from 1.6 to 25 µg/mL compatible with the standard drugs Pyrazinamide, Streptomycin and Ciprofloxacin, however, compounds **4a-f** were active only upto MIC value of 50 µg/mL, thus indicating that these compounds are selective inhibitors of tyrosine kinase enzyme. (Fig. 10)

4. Experimental section

4.1. Chemistry

4.1.1. General methods

All the chemicals were purchased from Avra synthesis; Lobachemie, Finar. Apparatus has been purchased from J-Sil and Agarwal. Melting points were determined by using thiels tube method using open capillaries and have been uncorrected. IR was recorded on SHIMADZU FTIR affinity-1. The NMR spectra were measured in $CDCl_3$ or DMSO at RT on a Brucker AV-II 400 spectrometer. δ is given in ppm relative to tetramethylsilane as an internal reference. Thin layer chromatography was performed using DDC-fertigfolien ALUGRAM^{R}Xtra SIL G/UV_{254} (Macherey-Nagel GmbH & Co.KG) Compounds were visualized by illumination under UV light (254 nm) or by use of phosphomolybdic acid stain followed by heating. Melting points were determined using an open capillary tube method and were uncorrected. Chemical shifts are expressed in parts per million relative to tetramethylsilane, which was used as internal standard, coupling constant (J) are in hertz (Hz), and signals are designated as follows: s, singlet; d, doublet; t, triplet; q,

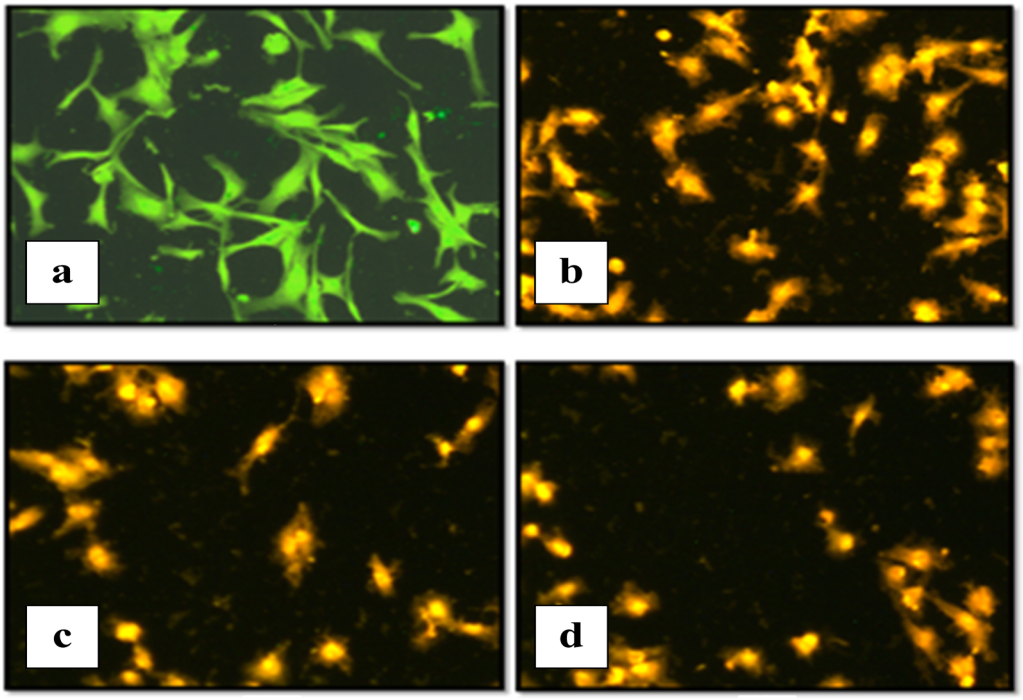


Fig. 7. Fluorescence microscopy images of cells stained with acridine orange/ ethidium bromide, a) viable cells, b) Cells treated with compound 4a, c) cells treated with compound 4c, d) cells treated with compound 4d.

quartet; m, multiplet; dt, doublet of triplet; ddd, double of doublet of doublet, dq, doublet of quartet. All solvents were dried prior to use and stored over 4A^{o} molecular sieves.

4.1.2. Synthesis of benzoxazole derivatives ***2a-r*** *and* ***4a-f*** *(Scheme 1 & 2)*

To the flask containing a mixture of substituted benzaldehyde (1mmole) and 2-aminophenol (1mmole) was added silica chloride (1 eq) and was heated on a sand bath at 120 °C, TLC was taken after every 1 h. After 4 h, TLC showed appearance of new spot. The product was isolated by first separating out the catalyst by filtration using organic solvent; the organic layer was dried using anhydrous sodium sulfate and evaporated under vacuum. The solid thus obtained was

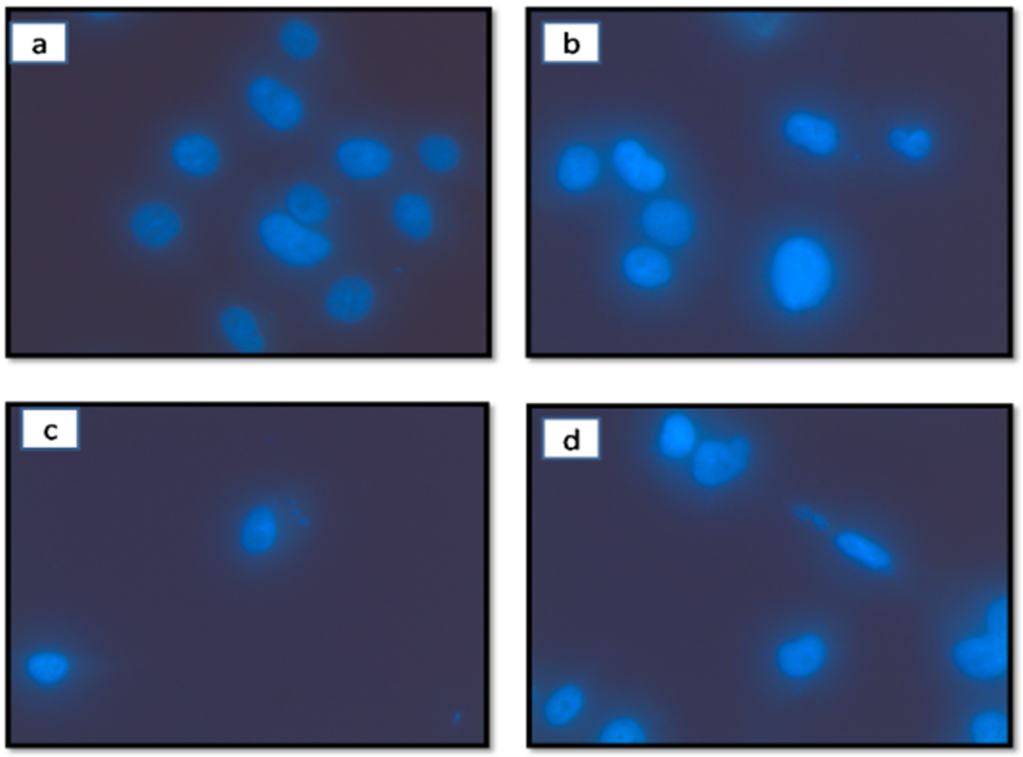


Fig. 8. Fluorescence microscopy images of cells stained with DAPI, showing (a) viable cells, (b) Cells treated with compound 4a, (c) cells treated with compound 4c, (d) cells treated with compound 4d.

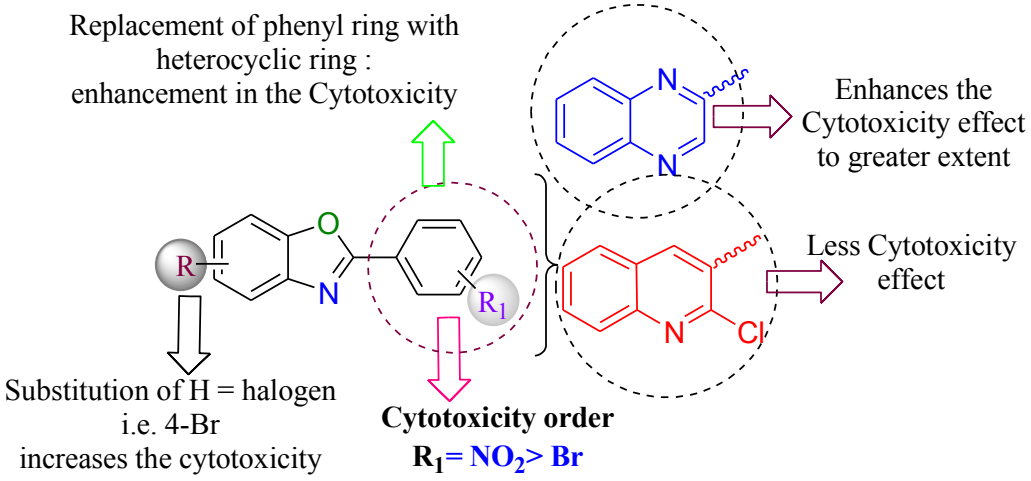


Fig. 9. Structural representation of SAR from cytotoxicity study of benzoxazole hybrids 4a-f.

recrystallized using petroleum ether and its % yield and melting points were determined. The results are tabulated in Table 1 and Table 2.

*4.1.2.1. 2-(2′, 4′-dichlorophenyl)-1, 3-benzoxazole (**2a**).* This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 125–127 °C; IR (KBr, cm^{-1}): 3010, 1605 (C═C), 1677 (C═N), 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.37–8.15(m, 4H, Ar−H), 8.0 (s, 1H, *J* = 6.26 Hz, 3′- H), 7.62 (dd, 1H, *J* = 6.26, 1.8 Hz, 5′-H), 7.24 (dd, 1H, *J* = 1.8 Hz, 6′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372.

*4.1.2.2. 2-(4′-chlorophenyl)-1, 3-benzoxazole (**2b**).* This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 144–148 °C; IR (KBr, cm^{-1}): 3015, 1657 (C═N), 1607 (C═C), 1035 (C−O), 780; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.27–8.07(m, 4H, Ar−H), 7.92 (ddd, 2H, *J* = 8.2, 1.8 Hz, 3′ & 5′-H), 7.814 (ddd, 1H, *J* = 1.6, 8.4 Hz, 2′ & 6′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 164.81, 151.1, 140.125, 135.70, 129.35, 129.35, 128.88, 128.88, 127.57, 124.92, 124.15, 119.859, 110.383.

*4.1.2.3. 2-(4′-bromophenyl)-1, 3-benzoxazole (**2c**).* This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 71%, m. p.: 150–152 °C; IR (KBr, cm^{-1}): 3011, 1640 (C═N), 1600 (C═C), 1550, 1034 (C−O), 730; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.42–8.02(m, 4H, Ar−H), 7.83 (ddd, 2H, *J* = 8.5, 1.76 Hz, 3′ & 5′-H), 7.77 (ddd, 1H, *J* = 1.58, 8.36 Hz, 2′ & 6′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 162.81, 150.8, 142.125, 136.70, 132.35, 132.35, 129.88, 129.88, 127.67, 124.98, 124.20, 119.859, 112.

*4.1.2.4. 2-(3′-Nitrophenyl)-1, 3-benzoxazole (**2d**).* This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 99–100 °C; IR (KBr) (ν_{max}, cm^{-1}): 3010, 1639 (C═N), 1599 (C═C), 1450, 1350, 1036; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 8.88 (ddd, 1H, *J* = 1.6, 1.0, 1.56 Hz, 2′-H), 8.58 (dt, 1H, *J* = 1.8, 8.6, 1.56 Hz, 4′-H), 7.58–8.2(m, 4H, Ar−H), 8.18 (dt, 1H, *J* = 1.6, 1.8, 8.0 Hz, 6′-H), 7.68 (ddd, 1H, *J* = 7.96, 8.02, 1.02 Hz, 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 163.81, 151.3, 142.125, 140.27, 127.29, 127.15, 125.22, 124.99, 124.11, 119.89, 117.30, 116.65, 110.372.

*4.1.2.5. 2-(2′-Nitrophenyl)-1, 3-benzoxazole (**2e**).* This compound was

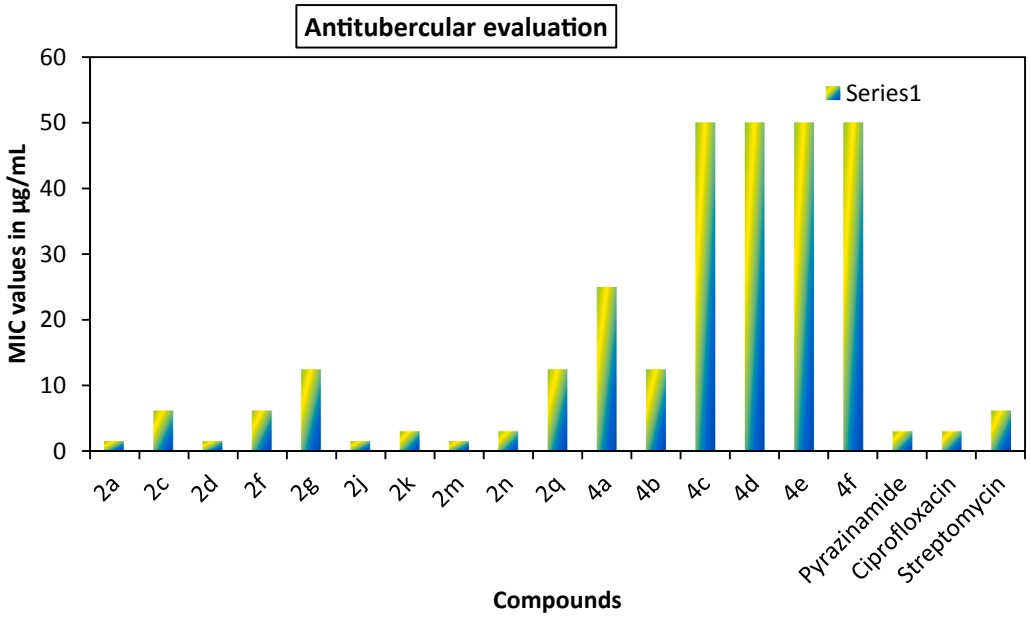


Fig. 10. *In-vitro* anti-tubercular evaluation against *Mycobacterium tuberculosis* by Alamar Blue Dye Assay method.

prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 98–103 °C; IR (KBr, cm^{-1}): 3011, 1632 (C═C), 1677 (C═N), 1550, 1380, 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 8.78 (ddd, 1H, *J* = 1.0, 1.78, 7.9 Hz, 3′- H), 8.02 (dd, 1H, J =, 8.6, 7.56 Hz, 4′-H), 7.58–8.2(m, 4H, Ar−H), 8.18 (dt, 1H, *J* = 1.6, 8.0 Hz, 6′-H), 8.23 (ddd, 1H, *J* = 7.96, 8.02, 1.02 Hz, 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 162.98, 153.3, 142.125, 140.27, 127.29, 127.15, 125.22, 124.99, 124.11, 119.89, 117.30, 116.65, 110.372.

4.1.2.6. 2-(2′-Chlorophenyl)-1, 3-benzoxazole ***(2f)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 68–72 °C; IR (KBr, cm^{-1}): 3010, 1657 (C═N), 1607 (C═C), 1035 (C−O), 780; ^{1}H NMR (400 MHz, δ, ppm $CDCl_3$): 7.32–8.17(m, 4H, Ar−H), 7.9 (dt, 2H, *J* = 8.2, 1.8 Hz, 3′-H), 7.80 (dt, 1H, *J* = 1.6, 8.4 Hz, 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 163.81, 150.3, 140.25, 135.80, 129.85, 129.15, 128.78, 128.18, 127.77, 125.02, 124.75, 118.89, 113.383.

4.1.2.7. 2-(4′-Nitrophenyl)-1, 3-benzoxazole ***(2 g)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 256–260 °C; IR (KBr, cm^{-1}): 3010, 1639 (C═N), 1599 (C═C), 1450, 1350, 1036; ^{1}H NMR (400 MHz, δ, ppm $CDCl_3$): 8.98 (dd, 2H, *J* = 1.6, 8.2 Hz, 3′ & 5′- H), 8.56 (dd, 2H, *J* = 8.3, 1.7 Hz, 2′ & 6′-H), 7.58–8.2(m, 4H, Ar−H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):164.81, 152.3, 142.125, 140.27, 131.21, 131.21, 125.99, 125.11, 120.89, 118.30, 117.65, 117.65, 113.372.

4.1.2.8. 2-Phenyl-1, 3-benzoxazole ***(2 h)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 78%, m. p.: 98–100 °C; IR (KBr, cm^{-1}): 3010, 1605 (C═C), 1550 (C═N), 1030; ^{1}H NMR (400 MHz, δ, ppm $CDCl_3$): 7.37–8.15(m, 4H, Ar-H), 7.17–7.80(m, 4H, Ar'-H); ^{13}C (100 MHz, δ, ppm, $CDCl_3$):162.81, 150.1, 141.125, 128.90, 128.90, 128.88, 127.99, 127.99, 127.1, 124.98, 124.11, 119.859, 110.372.

4.1.2.9. 2-(4′-Methoxyphenyl)-1, 3-benzoxazole ***(2i)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 77%, m. p.: 114–116 °C; IR (KBr, cm^{-1}): 3010, 1620 (C═N), 1590 (C═C), 1210, 1030; ^{1}H NMR (400 MHz, δ, ppm $CDCl_3$): 7.29–7.90 (m, 4H, Ar-H), 7.89 (dd, 2H, *J* = 1.75, 8.8 Hz, 2′ & 6′- H), 7.34 (dd, 2H, *J* = 8.8, 1.75 Hz, 3′ & 5′-H), 3.84 (s, 3H, OMe); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 164.399, 160.42, 150.11, 128.92, 128.92, 127.43, 124.89, 124.211, 119.85, 114.783, 114.783, 110.347, 55.89.

4.1.2.10. 2-(4′-Methylphenyl)-1, 3-benzoxazole ***(2j)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.:104–106 °C; IR (KBr) (ν_{max}, cm^{-1}): 3030, 1605 (C═N), 1580 (C═C), 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.30–8.08(m, 4H, Ar-H), 7.84 (dd, 2H, *J* = 1.5, 7.8 Hz, 2′ & 6′- H), 7.52 (dd, 2H, *J* = 7.88, 1.5 Hz, 3′ & 5′-H), 2.34 (s, 3H, Me); ^{13}C NMR (100 MHz, $CDCl_3$) (*δ*, ppm): 161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372.

4.1.2.11. 2-(3′-Chlorophenyl)-1, 3-benzoxazole ***(2k)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 120–122 °C; IR (KBr) (ν_{max}, cm^{-1}): 3020, 1635 (C═N), 1600 (C═C), 1030, 760; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.88 (dt, 1H, *J* = 1.6, 1.0, 1.56 Hz, 2′- H), 7.82 (dd, 1H, *J* = 8.6, 1.56 Hz, 4′-H), 7.46–8.10 (m, 4H, Ar-H), 7.78 (dt, 1H, *J* = 1.6, 1.8 Hz, 6′-H), 7.68 (dd, 1H, *J* = 7.86, 8.2 Hz, 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):162.81, 151.1, 140.125, 138.66, 135.89, 127.54, 125.90, 124.88, 124.12, 123.98, 120.11, 119.59, 110.72.

4.1.2.12. 2-(4′-Hydroxy, 5′-nitrophenyl)-1, 3-benzoxazole ***(2l)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.:170–174 °C; IR (KBr, cm^{-1}): 3200, 3010, 1630 (C═N), 1600 (C═C), 1550, 1350, 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$):8.8 (dd, 1H, *J* = 1.65, 2.8 Hz, 6′-H), 8.25 (dd, 1H, *J* = 1.6, 7.9 Hz, 2′-H), 7.52–8.02 (m, 4H, Ar-H), 7.35 (dd, 1H, *J* = 7.9,2.6 Hz, 3′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372.

4.1.2.13. 2-(2′-Hydroxy, 5′-nitrophenyl)-1, 3-benzoxazole ***(2m)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 71%, m. p.: 188–192 °C; IR (KBr, cm^{-1}): 3210, 1635 (C═N), 1600 (C═C), 1580,1359, 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 8.79 (dd, 1H, *J* = 3.11, 1.7 Hz, 6′-H), 8.34 (dd, 1H, *J* = 1.7, 7.6 Hz, 3′-H), 7.52–8.02 (m, 4H, Ar-H), 7.38 (dd, 1H, *J* = 8.6,1.4 Hz, 4′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372.

4.1.2.14. 2-(4′-Fluorophenyl)-1, 3-benzoxazole ***(2n)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 79%, m. p.: 90–92 °C; IR (KBr, cm^{-1}): 3010, 1660 (C═N), 1610 (C═C), 1030 1000; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.30–8.05(m, 4H, Ar-H), 7.83 (dd, 2H, *J* = 1.2, 8.12 Hz, 2′ & 6′-H), 7.34 (dd, 2H, *J* = 8.12, 1.02 Hz, 3′ & 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 164.4, 163.35, 150.11, 141.125, 133.4, 133.4, 127.5, 124.98, 124.33, 119.85, 117.8, 117.98, 110.76.

4.1.2.15. 2-(3′-Bromophenyl)-1, 3-benzoxazole ***(2o)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 133–137 °C; IR (KBr, cm^{-1}): 3010, 1655 (C═N), 1610 (C═C), 1030, 760; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$):7.42–8.1 (m, 4H, Ar-H), 7.77 (dt, 1H, *J* = 1.5 Hz, 2′- H), 7.64 (dd, 1H, *J* = 1.5, 8.1 Hz, 4′-H), 7.57 (dt, 1H, *J* = 1.5, 1.4, 7.8 Hz, 6′-H), 7.51 (dd, 1H, *J* = 8.0, 7.8 Hz, 5′-H) ; ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):163.67, 150.11, 141.125, 132.5, 130.89, 130.11, 129.89, 127.99, 125.76, 124.98, 124.311, 119.03, 110.9.

4.1.2.16. 2-(4′-N-dimethylaminophenyl)-1, 3-benzoxazole ***(2p)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 179–182 °C; IR (KBr, cm^{-1}): 3100, 3010, 1605 (C═N), 1590 (C═C), 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$):7.2–7.8 (m, 4H, Ar−H), 7.8 (dd, 2H, *J* = 1.45, 8.8 Hz, 2′ & 6′- H), 6.75 (dd, 2H, *J* = 8.8, 1.5 Hz, 3′ & 5′-H), 2.89 (s, 6H, Me); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):: 164.4, 151.22, 150.11, 141.125, 131.3, 131.3, 127.87, 124.90, 124.311, 119.85, 111.3, 111.3,110.2, 40.5.

4.1.2.17. 2-(5′-Bromo-4′-hydroxy-3′-methoxyphenyl)-1,3-benzoxazole ***(2q)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 78%, m. p.: 187–192 °C; IR (KBr, cm^{-1}): 3250, 3010, 1610 (C═N), 1590 (C═C), 1030, 780; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.16–7.88 (m, 4H, Ar−H), 7.53 (d, 1H, *J* = 2.0 Hz, 2′- H), 7.32 (d, 1H, *J* = 2.0 Hz, 6′-H), 4.5 (brs, 1H, OH), 3.7(s, 1H, OMe); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 163.67, 150.1, 149.8, 148.7, 141.25, 130.87, 125.96, 124.93, 124.112, 119.89, 110.49, 110.3, 110.25, 55.21.

4.1.2.18. 2-(3′,5′-Dibromo-4′-hydroxyphenyl)-1,3-benzoxazole ***(2r)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 70%, m. p.: 166–169 °C; IR (KBr, cm^{-1}): 3300, 3010, 1630 (C═N), 1600 (C═C), 1030, 780; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.16–7.89 (m, 4H, Ar−H), 7.96 (d, 2H, *J* = 2.0 Hz, 2′ & 6′-H), 4.5 (brs, 1H, OH); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):163.67, 152.11, 150.4, 141.3, 130.71, 130.71, 111.08, 111.08,

110.37, 124.97, 124.211, 125.52.

4.1.2.19. 5-Bromo-2-(4′-nitrophenyl)-1, 3-benzoxazole ***(4a)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 80%, m. p.: 228–232 °C; IR (KBr, cm^{-1}): 3010, 1650 (C═N), 1600 (C═C), 1550, 1380, 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 8.28 (dd, 2H, *J* = 8.8 Hz, 3′ & 5′-H), 7.99(d, 1H, *J* = 8.6 Hz, 7- H), 7.97 (dd, 1H, *J* = 8.8 Hz, 2′ & 6′-H), 7.82 (d, 1H, 4-H), 7.53 (dd, 1H, *J* = 8.6 Hz, 6-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):164.04, 150.03, 142.53, 131.3, 131.3, 129.37, 128.62, 124.27, 119.66, 118.03, 117.3, 117.3, 114.7. HRMS: (M + H) Obs: 317.196; Cal: 317.218.

4.1.2.20. 5-Bromo-2-(4′-bromophenyl)-1, 3-benzoxazole ***(4b)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 72%, m. p.: 158–162 °C; IR (KBr, cm^{-1}): 3010, 1655 (C═N), 1620 (C═C), 1030, 780, 760; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$):8.06 (s, 1H, *J* = 2.7 Hz, 4-H), 7.87(dd, 1H, *J* = 2.7, 8.3 Hz, 7- H), 7.83 (dd, 1H, *J* = 8.6 Hz, 2′ & 6′-H), 7.80 (dd, 2H, *J* = 8.6 Hz, 3′ & 5′-H), 7.28 (dd, 1H, *J* = 7.9 Hz, 6-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):164.04, 149.63, 141.23, 132.6, 132.6, 129.37, 128.62, 128.62, 119.66, 118.03, 117.87, 117.2, 114.7. HRMS: (M + H) Obs: 350.90 (100.0%); Cal: 350.89.

4.1.2.21. 2-(5-Bromophenyl-1, 3-benzoxazol-2yl)quinoxaline ***(4c)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 80%, m. p.: 189–192 °C; IR (KBr, cm^{-1}): 3010, 1665 (C═N), 1617 (C═C), 1030, 780; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 9.3 (s, 1H, 3-H), 8.47 (ddt, 1H, *J* = 8.2, 1.9, 3.5 Hz, 5-H), 8.36 (dt, 1H, *J* = 3.5, 1.9, 8.4 Hz, 8- H), 8.06 (dt, 1H, J = 8.4, 7.9, 6-H), 7.87 (dd, 1H, *J* = 8.4, 7.3 Hz, 7′-H), 7.(dd, 1H, *J* = 7.7 Hz, 7-H), 7.89 (dd, 1H, *J* = 8.5 Hz, 6′-H), 7.72 (dt, 1H, *J* = 7.9 Hz, 5′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):161.5, 152.01, 149.7, 145.8, 142.35, 141.9, 141.78, 132.52, 129.28, 128.56, 128.08, 119.43, 117.84. HRMS: $(M + H)^+$ Obs: 326.26; Cal: 326.25.

4.1.2.22. 2- (1, 3-benzoxazol-2-yl) quinoxaline ***(4d)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 74%, m. p.: 160–164 °C; IR (KBr, cm^{-1}): 3010, 1650 (C═N), 1607 (C═C), 1030; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$):9.4 (s, 1H, 3-H), 8.47 (ddt, 1H, *J* = 8.12, 3,48, 1.96 Hz), 8.35 (dd, 1H, *J* = 8.4 Hz, 8- H), 8.1 (dd, 1H, *J* = 8.6 Hz), 8.03 (dd, 1H, J = 8.0, 6-H), 7.99 (dd, 1H, *J* = 8.4, 4.5 Hz, 7′-H), 7.96 (dd, 1H, *J* = 7.7 Hz, 7-H), 7.89 (dd, 1H, *J* = 8.5 Hz, 6′-H), 7.72 (dt, 1H, *J* = 7.9 Hz, 5′-H) ; ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 161.4, 152.04, 148.58, 145.64, 142.29, 141.53, 141.01, 131.10, 128.8, 127.97, 125.44, 124.3, 121.14, 115.41, 111.54. HRMS: $(M + H)^+$ Obs: 247.08; Cal: 247.07.

4.1.2.23. 3-(1, 3-benzoxazol-2yl) – 3-chloroquinoline ***(4e)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 75%, m. p.: 102–108 °C; IR (KBr, cm^{-1}): 3010, 1665 (C═N), 1637 (C═C), 1030, 830; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.37–8.15(m, 4H, Ar—H), 8.0 (s, 1H, *J* = 6.26 Hz, 3′- H), 7.62 (dd, 1H, *J* = 6.26, 1.8 Hz, 5′-H), 7.24 (dd, 1H, *J* = 1.8 Hz, 6′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$): 161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372. HRMS: $(M + H)^+$ Obs: 280.05; Cal: 280.04.

4.1.2.24. 3-(5-Bromophenyl- 1, 3-benzoxazol-2yl) − 3-chloroquinoline ***(4f)***. This compound was prepared according to general procedure and it was obtained as buff coloured solid; yield: 76%, m. p.: 170–175 °C; IR (KBr, cm^{-1}): 3010, 1660 (C═N), 1620 (C═C), 1030, 830; ^{1}H NMR (400 MHz, δ, ppm, $CDCl_3$): 7.37–8.15 (m, 4H, Ar—H), 8.0 (s, 1H, *J* = 6.26 Hz, 3′- H), 7.62 (dd, 1H, *J* = 6.26, 1.8 Hz, 5′-H), 7.24 (dd, 1H, *J* = 1.8 Hz, 6′-H); ^{13}C NMR (100 MHz, δ, ppm, $CDCl_3$):161.81, 150.1, 141.125, 135.66, 133.89, 129.54, 128.90, 128.12, 127.88, 124.98, 124.11, 119.859, 110.372. HRMS: $(M + H)^+$ Obs: 294.07; Cal: 294.06

4.2. Biological evaluation

4.2.1. Virtual screening: Protein structure preparation

The molecular docking study was performed using Molegro Virtual Docker (MVD-2013, 6.0). The crystal structure of the enoyl- ACP reductase (InhA) complexed with an isonicotinic-acyl-NADH inhibitor and epidermal growth factor receptor tyrosine kinase domain with 4-anilinoquinazoline inhibitor erlotinib (PDB ID: 1M17) were downloaded from Protein Data Bank (PDB ID: 1ZID). Molecular docking studies of the synthesized compounds/ligands were performed in order to understand the various interactions between the ligand and enzyme active site in detail. The molecular docking study was performed for the target compounds by using MVD-2013 (Version: 6.0).

4.2.2. Molecular docking study

The synthesized compounds were built using Chemdraw 11.0. The 2D structures were then converted into energy minimized 3D structures and were saved as MDL Molfile (.mol2). The coordinate files and crystal structures of enoyl-acpreductase (InhA, PDB ID: 1ZID) and tyrosine kinase (PDB ID: 1M17) were obtained from the RCSB PDB website. The protein files were prepared by the removal of water molecules, addition of polar hydrogens and removal of other bound ligands. In the present study, the binding sites were selected based on the amino acid residues, which are involved in binding with isonicotinic-acyl-NADH inhibitor of InhA and erlotinib as obtained from protein data bank, which would be considered as the probable best accurate regions as they are solved by experimental crystallographic data. The docking protocol was carried out for the synthesized compounds as listed in Table 3 using MVD-2013 (6.0) software using the standard operating procedures.

4.2.3. Anti-cancer Evaluation:

4.2.3.1. General procedure for evaluation of anti-cancer inhibitory activity. The anti-cancer activity of the compounds has been accessed by MTT assay. Initially, MTT stock solution has been prepared by taking 5 mg in 1 mL of PBS. MCF-7 cell line has been used for the study. The cell line was maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum(FCS), containing 5% mixture of Gentamicin (10 µg), Penicillin (100units/mL) and streptomycin (100 µg/mL) in presence of 5% CO_2 at 37 °C for 48–72 h. In-vitro growth inhibition effect of test compounds was assessed by colorimetric or spectrophotometric determination of conversion of MTT into Formazan blue by living cells. Initially the supernatant from the plate was removed and fresh MEM solution was added and was treated with different concentrations of extract or compound appropriately diluted with DMSO. In our study, 10, 20, 25, 30 and 50 µL (10 mg/mL prepared in DMSO) of stock solutions were added to respective wells containing 100 µL of the medium. Thereby making the final concentrations as 10, 20, 25, 30 and 50 µg/mL. After 48hrs of incubation at 37 °C in a humidified atmosphere of 5% CO_2, stock solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide was added to each well (20 µL, 5 mg per mL in sterile phosphate buffered saline) for further 4 h incubation. The supernatant carefully aspirated, the precipitated crystals of Formazan blue were solubilised by adding DMSO and optical density was measured at wavelength of 570 nm by using LISA plus. The results were represented as mean of five readings upto the concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control. During this assay, reduction of yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide was measured by mitochondrial succinate dehydrogenase.

$$\text{Formula:Surviving cells} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \times 100$$

4.2.3.2. Enzyme inhibition studies. Cell Treatment- The cells were seeded at a density of approximately 1×10^5 cells/well in a 96-well flat-bottom micro plate and maintained at 37 °C in 95% humidity and 5% CO_2 for overnight. Cells were treated with different concentration of test samples. Then cells were incubated for another 24 h. The cells in well were washed twice with phosphate buffer solution, and 1 mL of extraction buffer was added. Using cell scraper cells were recovered carefully and centrifuge the cells at 4 °C for 10 min at 10,000 rpm. Collect the supernatant and store as further analysis.

Tyrosine Kinase Assay- Collected supernatant was diluted 25 times with kinase reacting solution provided along with kit. The diluted control was added and treated to sample in each well in duplicate. Then 10 µL of 40 mM ATP-2Na solution was added into each well and mixed well. Further, incubated for 30 min at 37 °C.Then the samples were removed, the wells were washed 3 times with wash buffer. 100 µL of blocking solution was added into each well and incubated for 30 min at 37 °C. The blocking solution was discarded and then 50 µL of Anti-phosphotyrosine - HRP solution was added into each well and incubatedt for 30 min at 37 °C. Now, the antibody solution was discarded and then each well was washed 4 times with washing buffer. Further, 100 µL of HRP substrate solution (TMBZ) was added into each well. Incubated for 30 min at 37 °C. Finally, 100 µL of stop solution was added into each well in the same order as HRP substrate solution. The absorbance is measured at 450 nm with a plate reader.

$$\%\text{Inhibition} = 1 - (\text{Abs of sample}/\text{Abs of control}) \times 100$$

4.3. Cell morphology studies by fluorescence microscopy

4.3.1. Double staining (Acridine orange-Ethidium bromide)

The cells were seeded at a density of approximately $1x10^4$cells/well in a 24 well flat bottom micro plate containing cover slips and maintained at 37 °C in CO_2 incubator for overnight. More than the IC_{50} of synthesised compounds was treated at 72 hrs. After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. 20 µL of dye mixture was incubated for half an hour, examined under fluorescent microscope.

4.3.2. Dapi

The cells were seeded at a density of approximately $1X10^5$cells/well in a 12 well flat bottom micro plate containing cover slips and maintained at 37 °C in CO_2 incubator for overnight. More than the IC_{50} of synthesised compounds was treated at 72 hrs. After the incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. 20 µL of dye mixture was incubated for 20 min, examined under fluorescent microscope.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary material

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[42] The anti mycobacterial activity of compounds were assessed against M. tuberculosis H37 RV strain using microplate Alamar Blue assay (MABA). Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds were made directly on plate.The final drug concentrations tested were 100 to 0.2 µg/ml.Plates were covered and sealed with parafilm and incubated at 37°C for five days.After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.