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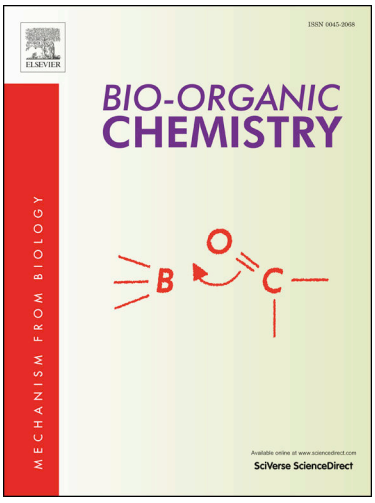

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In-vitro Anti-cancer Assay and Apoptotic Cell Pathway of Newly Synthesized Benzoxazole-N-Heterocyclic Hybrids as Potent Tyrosine Kinase Inhibitors

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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±0.16μM, 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.

Keywords: Benzoxazole, Heterocyclic hybrids, Cytotoxicity, Selective index, Tyrosine Kinase, Apoptosis.

1. Introduction

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lifestyle, the growth of cancer disease has erupted rapidly. The most common type of cancers includes 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 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 synthestic 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]

then the standard drug against A-549 and MCF-7 cell lines.[24]

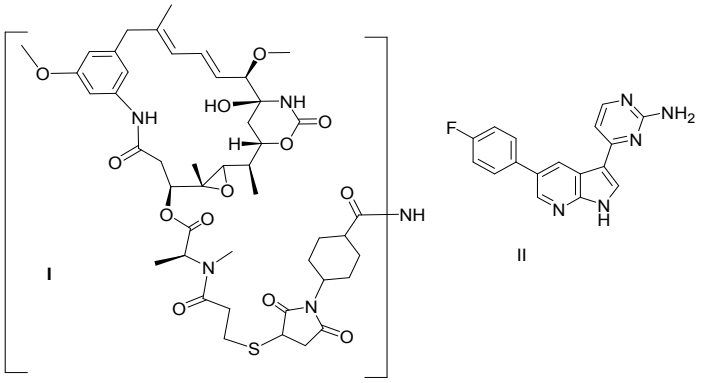


Fig.1. Representative Structures of breast cancer drugs: **I**. Trastuzumab, sold under the brand name Herceptin among others, is a monoclinical antibody, used to treat HER2 positive breast cancer; **II**. 3-Pyrimidinylazaindole, a potent inhibitor of triple negative breast cancer.

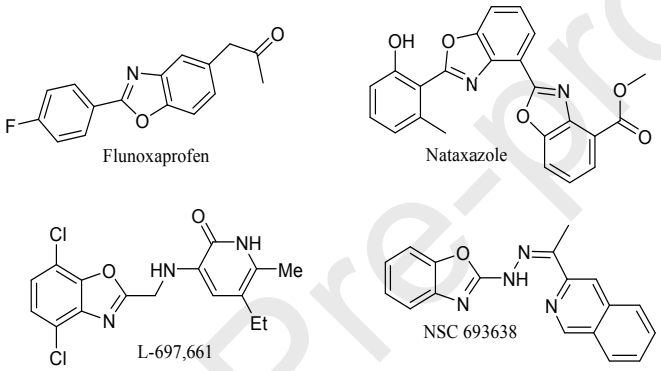
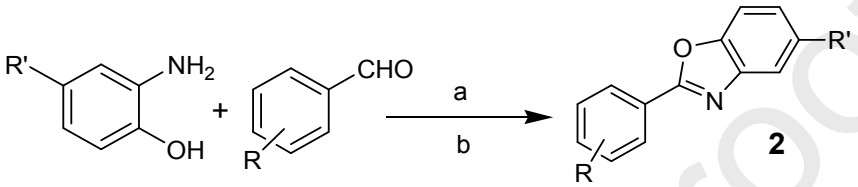


Fig. 2 Bioactive Compounds containing Benzoxazole moiety: flunoxaprofen-NSAIDs, L-697,661 a reverse transcriptase inhibitor, Nataxazole an anti-tumor, NSC693638- an anticancer agent

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 stategies[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 catalysed[30-33] synthesis of benzoxazoles and their action as inhibitors of protein kinase enzyme has been the focus of the work.

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 hours. 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)**



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

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

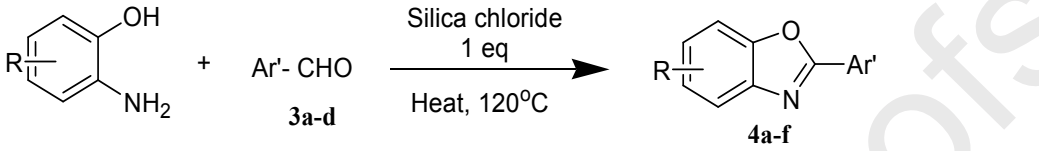
Comp 2	R	R'	Yield[b]	Melting Point[c] °C
a	2,4- Cl	H	75%	125-127 (127)
b	4- Cl	H	75%	144-148 (148-150)
c	4- Br	H	71%	150-152(157-158)
d	3-NO_2	H	79%	99-102(103-105)
e	2-NO_2	H	72%	97-100(98-102)
f	4-OMe	H	77%	114-116 (113-114)
g	4-NO_2	H	79%	256-260 (268)
h	H	H	78%	98-100 (101)
i	2-Cl	H	70%	68-72(73)
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 (178)
m	2-OH, 5-NO_2	H	71%	188-192 (188)
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

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 quinoline[37]. (**Scheme 2)** All the six benzoxazole analogues were obtained in good yields. (**Table 2)**



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-Nitroophenyl
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	3h	74%	160-164
4	d	3h	80%	189-192
5	e	3h	75%	102-108
6	f	3h	76%	170-175

[a]Isolated yields

[b]Determined using thiels tube paraffin method.

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 **2a-r** 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-H and 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 **4a-f** 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.

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^o) and two interaction arised from H-bond interaction between oxygen atom of nitro group and CYS751 (1.52 A^o) and Thr830 (1.52 A^o). The remaining one arose from oxazole ring of benzoxazole moiety. The oxygen atom interacts with Met769 (1.55 A^o).

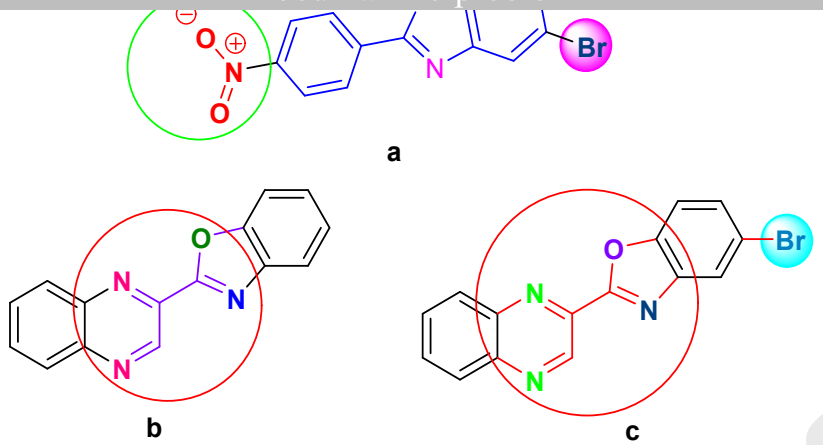


Fig. 3. Structural features of compounds responsible for the cytotoxicity: a) Compound **4a**; b) Compound **4c**; c) Compound **4d**

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^{o}). while the other is raised from H-bond interaction of nitrogen atom of quinoxaline ring with Met769 (1.55 A^{o}). Compound **4d** 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 (1.55 A^{o}). The remaining two interactions are raised from nitrogen and oxygen atom of oxazole ring with Thr766 (1.52 A^{o}). (**Fig. 4)**

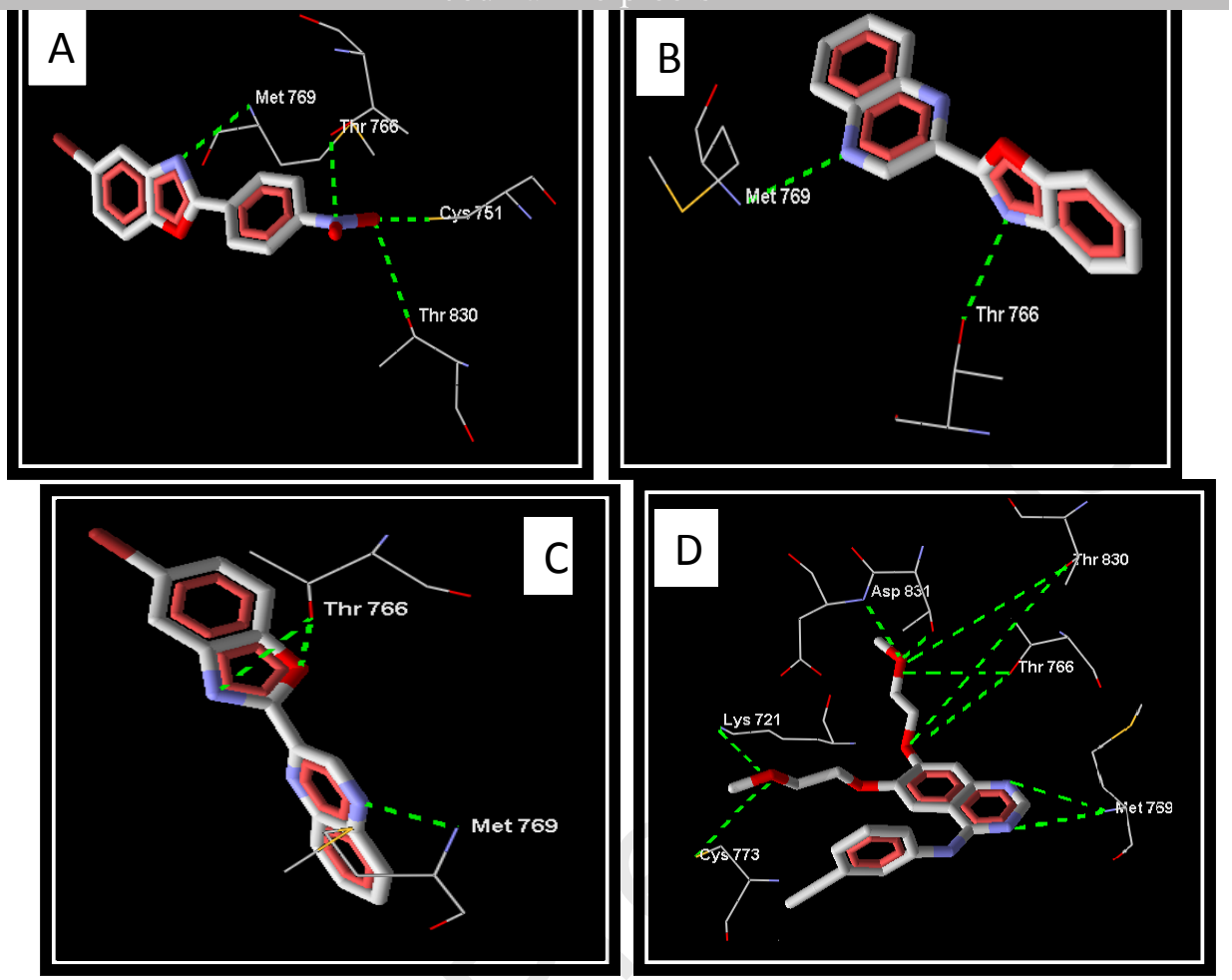


Fig 4. Molecular docking data: the compounds docked in best of its conformation into the binding site of 1M17. (A) Binding mode of compound **4a** forming four hydrogen bond interaction. (B) Binding mode of compound **4c** forming two hydrogen bond interaction. (C) Binding mode of compound **4d** forming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

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

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

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 14.39μM. As shown in **Table 4**, the samples showed moderate to good activity comparable to that of standard drugs, especially compound **4a, 4c** and **4d** showed higher anti-proliferation activity with IC_{50}=0.56±0.07, 0.58±0.05μM; 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 **4b**, **4e** and **4f** 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 **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±0.04μM 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±0.11μM. Compound **4c**, **4d, 4e** and **4f** 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.

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.

	IC_{50} μM

	231*a*				
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

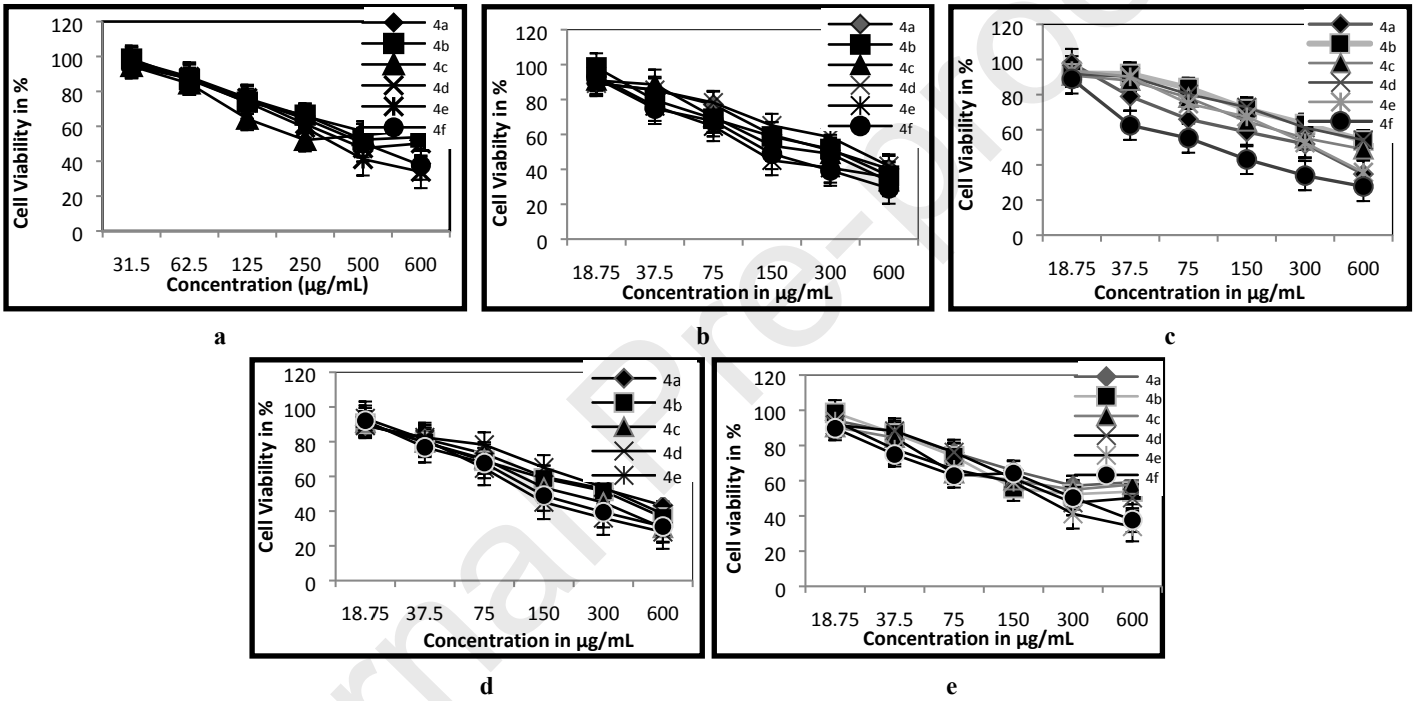


Fig. 5. Graphical representation of cell viability exhibited by **4a**, **4b**, **4c**, **4d**, **4e** and **4f** for five different cell lines: a)MDA-MB-231, b) MCF-7, c) A549, d)KB, e)HEK293

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

(Or as variation in percentage of viability with respect to time and concentration. (**Fig. 5)**

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

2.2.3. 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 0.31-0.43µM. 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.

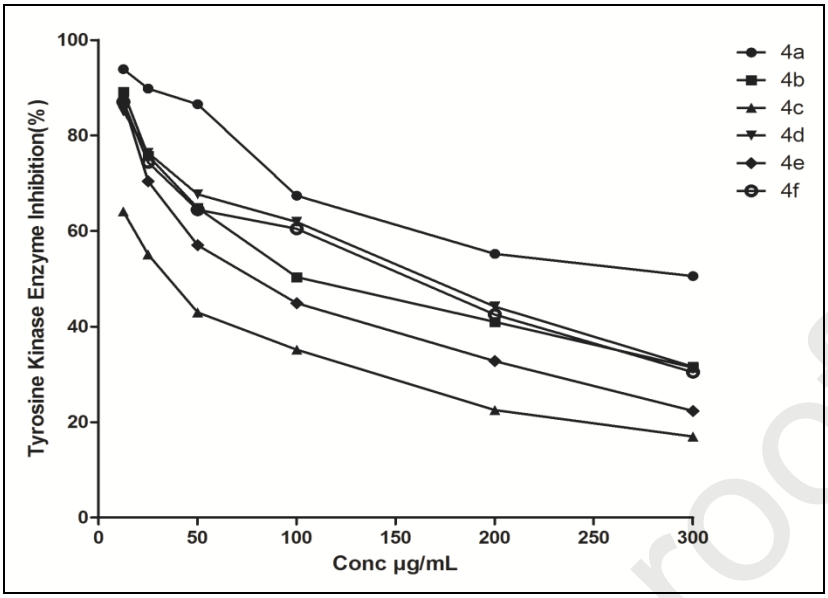


Fig.6. Graphical representation enzyme Tyrosine kinase inhibition by compounds **4a-f:** Plot of concentration in µL/mL v/s rate of inhibition

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

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. 7a**, Whereas, late apoptotic cells when treated with compounds **4a**, **4c** and **4d** exhibited yellow orange fluorescence with nuclear membrane babbling. **Fig. 7b-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. 8a** whereas, treated cells showed nuclear condensation and nuclear fragmentation. **Fig. 8b-d.**

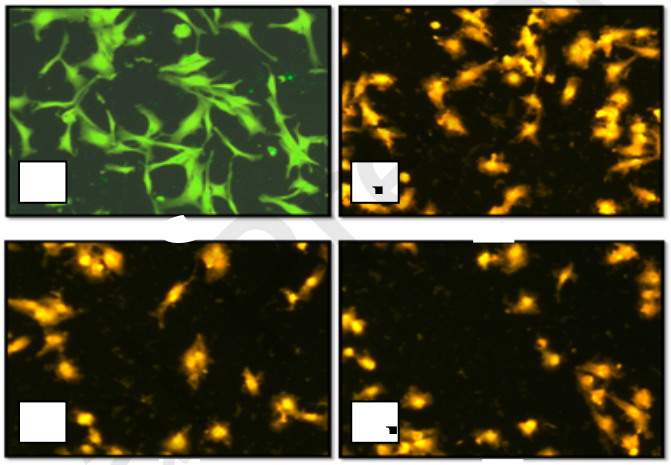


Fig. 7. Fluorescence microscopy images of cells stained with acridine orange/ ethidium bromide, **a**. viable cells, **b**. Cells treated with compound **4a**, **c**. cells treated with compound **4c**, **d**. cells treated with compound **4d**

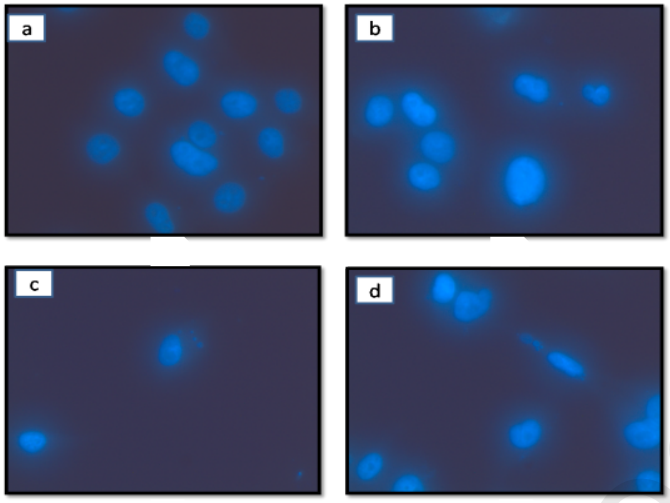


Fig 8. Fluorescence microscopy images of cells stained with DAPI, showing a) viable cells, b) Cells treated with compound **4a**, c) cells treated with compound **4c**, d) cells treated with compound **4d.**

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. **Fig. 3** & **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.**

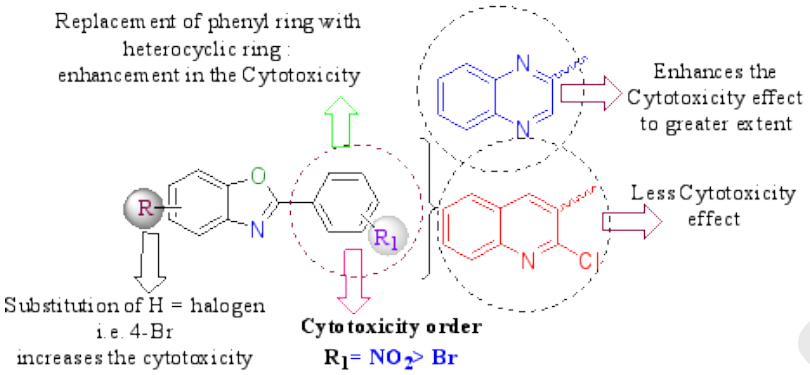


Fig. 9. Structural representation of SAR from cytotoxicity study of benzoxazole hybrids **4a-f**

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 0.50-0.73µM 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 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 0.10-0.86µM, with maximum inhibition being exhibited by compound

4c with IC_{50} value 0.10±0.16µM. 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 1.6-25 µg/mL compatible with the standard drugs Pyrazinamide, Streptomycin and Ciprofloxacin, however, compounds **4a-f** were active only upto MIC value of 50µg/mL, thus indicating that these compounds are selective inhibitors of tyrosine kinase enzyme. (**Fig. 10**)

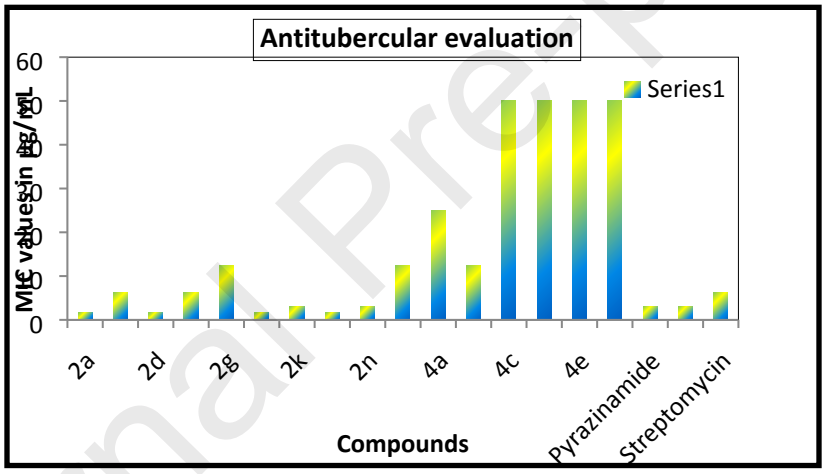


Fig. 10. In-vitro anti-tubercular evaluation against mycobacterium tuberculosis by Alamar Blue Dye Assay method

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 (254nm) 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, 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 (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 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^{o}C; IR (KBr, cm^{-1}): 3010, 1605 (C=C), 1677 (C=N), 1030; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.37-8.15(m, 4H, Ar-H), 8.0 (s, 1H, J= 6.26Hz, 3'-H), 7.62 (dd, 1H, J=6.26, 1.8Hz, 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^{o}C; IR (KBr, cm^{-1}): 3015, 1657 (C=N), 1607 (C=C), 1035 (C-O), 780; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.27-8.07(m, 4H, Ar-H), 7.92 (ddd, 2H, J=8.2, 1.8Hz, 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^{o}C; IR (KBr, cm^{-1}): 3011, 1640 (C=N), 1600 (C=C), 1550, 1034 (C-O), 730; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.42-8.02(m, 4H, Ar-H), 7.83 (ddd, 2H, J=8.5, 1.76Hz, 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^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3010, 1639 (C=N), 1599 (C=C), 1450, 1350, 1036; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 8.88 (ddd, 1H, J= 1.6, 1.0, 1.56Hz, 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,

8.0Hz, 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 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, δ, ppm, $CDCl_3$): 8.78 (ddd, 1H, J= 1.0, 1.78, 7.9Hz, 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 (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^{o}C; IR (KBr, cm^{-1}): 3010, 1657 (C=N), 1607 (C=C), 1035 (C-O), 780; ^{1}HNMR (400MHz, δ, ppm $CDCl_3$): 7.32-8.17(m, 4H, Ar-H), 7.9 (dt, 2H, J=8.2, 1.8Hz, 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 (**2g**)*

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, δ, ppm $CDCl_3$): 8.98 (dd, 2H, J= 1.6, 8.2Hz, 3' & 5'- H), 8.56 (dd, 2H, J=8.3, 1.7Hz, 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 (**2h**)*

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, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1620 (C=N), 1590 (C=C), 1210, 1030; ^{1}HNMR (400MHz, 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, 1.75Hz, 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^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3030, 1605 (C=N), 1580 (C=C), 1030; ^{1}HNMR (400MHz, 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, 1.5Hz, 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^{o}C; IR (KBr) (ν_{max}, cm^{-1}): 3020, 1635 (C=N), 1600 (C=C), 1030, 760; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.88 (dt, 1H, J= 1.6, 1.0, 1.56Hz, 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, 1.8Hz, 6'-H), 7.68 (dd, 1H, J= 7.86, 8.2 Hz, 5'-H); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3200, 3010, 1630 (C=N), 1600 (C=C), 1550, 1350, 1030; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$):8.8 (dd, 1H, J=1.65, 2.8Hz, 6'-H),

8.25 (dd, 1H, J=1.6, 7.9Hz, 2'-H), 7.52-8.02 (m, 4H, Ar-H), 7.35 (dd, 1H, J=7.9,2.6 Hz, 3'-H); ^{13}CNMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3210, 1635 (C=N), 1600 (C=C), 1580,1359, 1030; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 8.79 (dd, 1H, J=3.11, 1.7 Hz, 6'-H), 8.34 (dd, 1H, J=1.7, 7.6Hz, 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 (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1660 (C=N), 1610 (C=C), 1030 1000; ^{1}HNMR (400MHz, 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 (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1655 (C=N), 1610 (C=C), 1030, 760; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$):7.42-8.1 (m, 4H, Ar-H), 7.77 (dt, 1H, J= 1.5Hz, 2'- H), 7.64 (dd, 1H, J=1.5, 8.1Hz, 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 (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3100, 3010, 1605 (C=N), 1590

(C=C), 1030; ^{1}HNMR (400MHz, 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 (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3250, 3010, 1610 (C=N), 1590 (C=C), 1030, 780; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.16-7.88 (m, 4H, Ar-H), 7.53 (d, 1H, J= 2.0Hz, 2'- H), 7.32 (d, 1H, J=2.0Hz, 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^{o}C; IR (KBr, cm^{-1}): 3300, 3010, 1630 (C=N), 1600 (C=C), 1030, 780; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.16-7.89 (m, 4H, Ar-H), 7.96 (d, 2H, J=2.0Hz, 2' & 6'-H), 4.5 (brs, 1H, OH); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1650 (C=N), 1600 (C=C), 1550, 1380, 1030; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 8.28 (dd, 2H, J=8.8Hz, 3' & 5'-H), 7.99(d, 1H, J= 8.6Hz, 7- H), 7.97 (dd, 1H, J=8.8Hz, 2' & 6'-H), 7.82 (d, 1H, 4-H), 7.53 (dd, 1H, J=8.6Hz, 6-H); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1655 (C=N), 1620 (C=C), 1030, 780, 760; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$):8.06 (s, 1H, J=2.7Hz, 4-H), 7.87(dd, 1H, J=

2.7, 8.3Hz, 7- H), 7.83 (dd, 1H, J=8.6Hz, 2' & 6'-H), 7.80 (dd, 2H, J=8.6Hz, 3' & 5'-H), 7.28 (dd, 1H, J=7.9Hz, 6-H); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1665 (C=N), 1617 (C=C), 1030, 780; ^{1}HNMR (400MHz, 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, 8.4Hz, 8- H), 8.06 (dt, 1H, J=8.4, 7.9, 6-H), 7.87 (dd, 1H, J= 8.4, 7.3Hz, 7'-H), 7.(dd, 1H, J= 7.7Hz, 7-H), 7.89 (dd, 1H, J= 8.5Hz, 6'-H), 7.72 (dt, 1H, J=7.9Hz, 5'-H); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1650 (C=N), 1607 (C=C), 1030; ^{1}HNMR (400MHz, 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= 8.4Hz, 8- H), 8.1 (dd, 1H, J= 8.6Hz), 8.03 (dd, 1H, J=8.0, 6-H), 7.99 (dd, 1H, J= 8.4, 4.5Hz, 7'-H), 7.96 (dd, 1H, J= 7.7Hz, 7-H), 7.89 (dd, 1H, J= 8.5Hz, 6'-H), 7.72 (dt, 1H, J=7.9Hz, 5'-H) ; ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1665 (C=N), 1637 (C=C), 1030, 830; ^{1}HNMR (400MHz, δ, ppm, $CDCl_3$): 7.37-8.15(m, 4H, Ar-H), 8.0 (s, 1H, J= 6.26Hz, 3'- H), 7.62 (dd, 1H, J=6.26, 1.8Hz, 5'-H), 7.24 (dd, 1H, J= 1.8 Hz, 6'-H); ^{13}C NMR (100MHz, 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^{o}C; IR (KBr, cm^{-1}): 3010, 1660 (C=N), 1620 (C=C), 1030, 830; ^{1}HNMR (400MHz, 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, 1.8Hz, 5'-H), 7.24 (dd, 1H, J=1.8 Hz, 6'-H); ^{13}C NMR (100MHz, 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 4** 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 5mg 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 (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 (10mg/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.

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

4.2.3.2. 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 mM 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 incubatedt 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 HRP substrate solution (TMBZ) 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 is measured at 450 nm with a plate reader.

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

4.3. Cell morphology studies by Fluorescence microscopy

4.3.1.1. 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^{o}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 30min. 20 µL of dye mixture was incubated for half an hour, examined under fluorescent microscope.

4.3.1.2. 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^{o}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 30min. 20 µL of dye mixture was incubated for 20min, examined under fluorescent microscope

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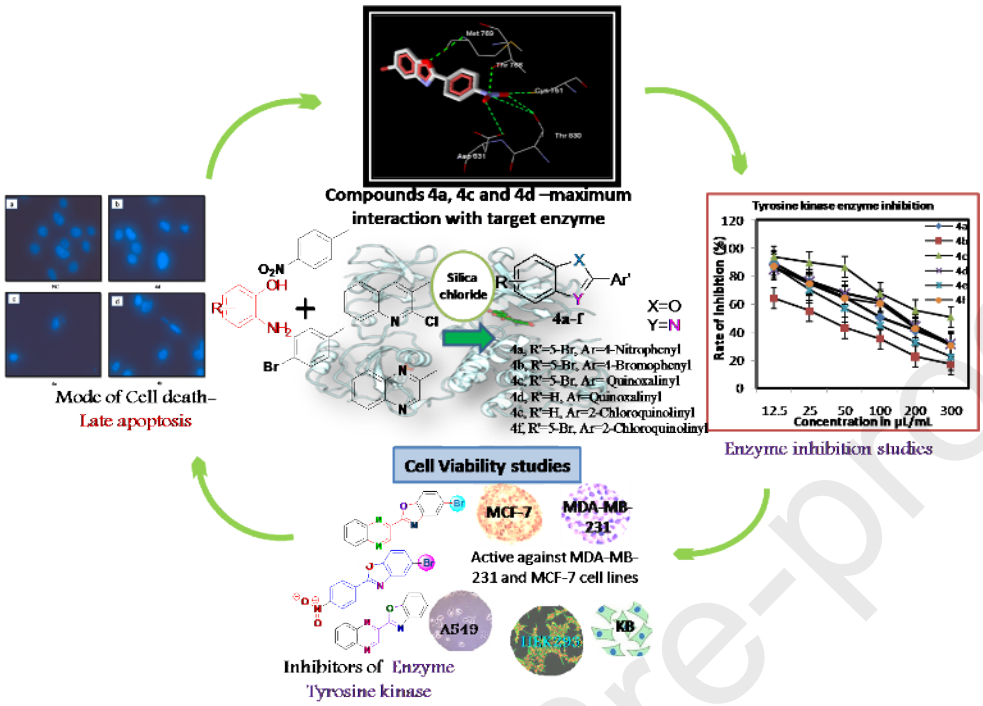
42. The anti mycobacterial activity of compounds were assessed against M. tuberculosis H37 RV strain using microplate Alamar Blue assay (MABA). Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells 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 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.

Highlights of the work

- Synthesis of novel Benzoxazole-N-heterocyclic conjugates as potent tyrosine kinase inhibitors.
- Selective anti-cancer activity of these conjugates to treat breast cancer (MCF-7 and MDA-MB-231 cancer cell lines)
- Molecular docking and structure activity relationship studies of these benzoxazole hybrids.
- One-pot, Solvent-free, silica chloride catalysed synthesis of 2-aryl benzoxazoles.

Graphical abstract



Conflicts of Interest:

- The authors declare no conflicts of interest.