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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF BENZOXAZOLE SUBSTITUTED AZETIDINONE DERIVATIVES AS ANTICANCER AGENTS

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ABSTRACT

Benzoxazole is a heteroaromatic compound, which finds use in research as a starting material for the synthesis of biologically active structures. Azetidinone is a potent, orally active beta lactam which exhibit a number of biological activities like anti-cancer, anti-viral and anti- inflammatory. Among all the heterocycles, benzoxazole and azetidinone are the most important heterocycles exhibiting remarkable anticancer activities. A series of Benzoxazole substituted azetidinone derivatives were synthesized, characterized and evaluated for the anticancer study. The structures were confirmed by ^{1}H NMR, ^{13}C NMR and MASS spectral techniques. The docking study of synthesized compounds were done to find the binding activity of the derivatives to the VEGFR-2receptors.

KEYWORDS: Benzoxazole, Azetidinone, Anticancer activity.

INTRODUCTION

Cancer is a disease in which some of the cell grow uncontrollably and invade to other body parts. It starts almost anywhere in the human body, which is made up of millions of cells. Cancer cells do not have any programming so they are not possessing any physiological function.[1-2] There are 10 basic principles to understand the molecular basis of cancer proposed by Doughs and Hanahan and Robert Weinberg in 2000. The ten hallmarks include activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, degenerating cellular energetics, sustaining proliferative signalling, evading growth suppression, avoiding immune destruction, enabling replicative immunity,

tumor promoting inflammation. The target selected for this study was based on these 10 hallmarks. Sustaining proliferative signalling, genome instability and mutation, inducing angiogenesis, invasion and metastasis were found to be the important hallmarks for different types of solid tumors.[3]

Benzoxazole and its derivatives show different biological activities such as anti-bacterial, anti- fungal, anti-histaminic and anti-cancer properties.[4-5] Azetidinones are the simplest beta lactamexhibiting anti-tubercular, anti-HIV, anti-inflammatory activity etc.[6]

The Vascular Endothelial Growth Factor, a tyrosine kinase receptor is one of the most suitable targets for cancer. Agents which could inhibit VEGFR are directly related to blockade ofregulatory process of cellular proliferation.[7]

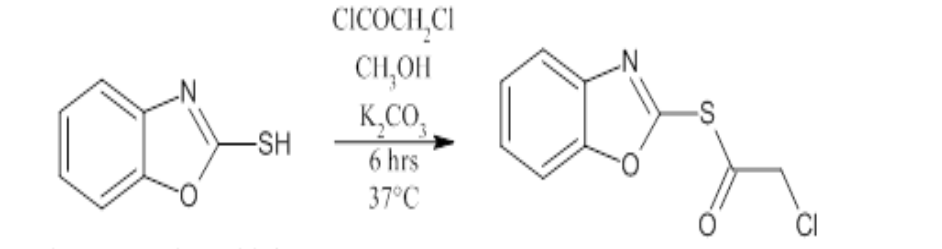
MATERIALS AND METHODS

Melting points of the compounds were recorded using melting point apparatus. IR spectra was determined using JASCO FT-IR-40000cm^{-1} using KBr disc. Analytical grade chemicals and solvents are used in this study. The progress of reaction was checked by TLC plate on suitable solvent system on glass plate coated with silica gel. The solvent system used in this study was petroleum ether: ethyl acetate (4:1).

Procedure for synthesis

Step 1: Synthesis of 2-chloroacetyl Mercapto benzoxazole

An equimolar solution of 2-mercaptobenzoxazole (0.06 mole) and chloroacetyl chloride (0.06 mole) in methanol (30 mL) in the presence of anhydrous potassium carbonate (2 g) was kept at room temperature for about 25 hours. The solvent was removed *in vacuo* and the residue was recrystallized from chloroform to furnish compound.

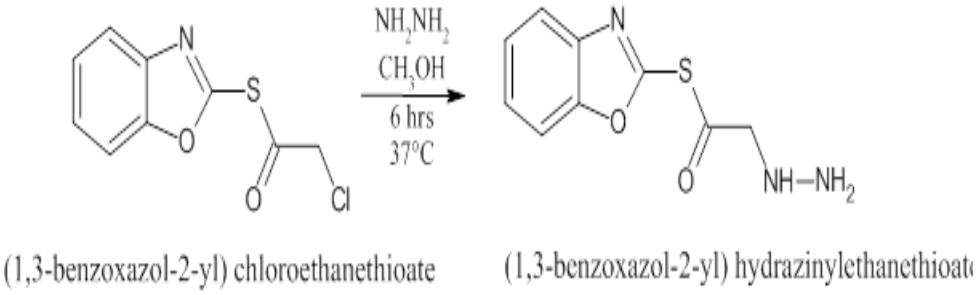


1,3-benzoxazole-2-thiol (1,3-benzoxazol-2-yl) chloroethanethioate

Step 2: Synthesis of (2-Hydrazinoacetyl)-mercaptobenzoxazole.

To a solution of 1 (0.02 mole) and hydrazine hydrate (0.02 mole) in methanol (30 mL) was

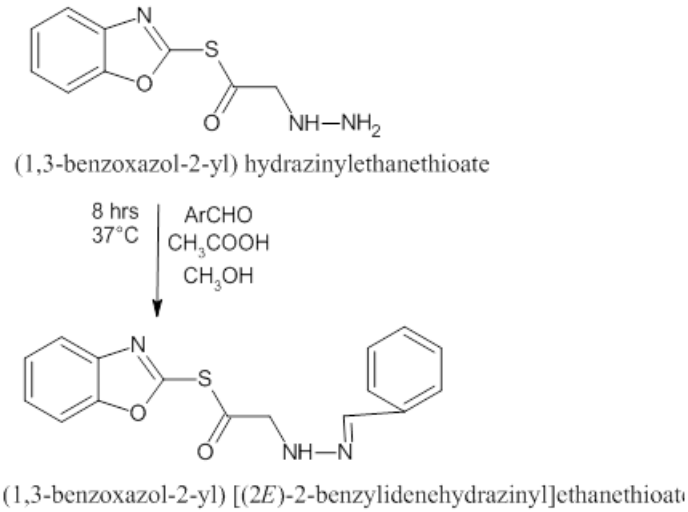
kept at room temperature for about 20 hours. The solvent was removed *in vacuo* and the resulting solidwas dried recrystallized from chloroform to produce analytical pure material.



(1,3-benzoxazol-2-yl) chloroethanethioate (1,3-benzoxazol-2-yl) hydrazinylethanethioat

Step 3: Synthesis of -(Aryliden-hydrazinoacetyl)-mercaptobenzoxazole.

A mixture of compound **2** (0.008 mole) and benzaldehyde (0.008 mole) and 2-3 drops of glacial acetic acid in ethanol (25 mL) was kept at room temperature for about 24 hours. The solvent was removed *in vacuo* and the resulting solid was dried and recrystallized. Likewise, other compoundswere prepared in a similar way using different carbonyl compounds.[8]

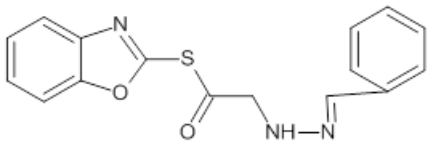


(1,3-benzoxazol-2-yl) hydrazinylethanethioate

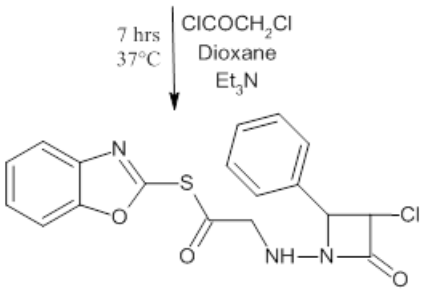
(1,3-benzoxazol-2-yl) [(2*E*)-2-benzylidenehydrazinyl]ethanethioat

Step 4: Synthesis of benzoxazole substituted azetidinone derivatives.

A mixture of Schiffs base (0.01 mole) and triethylamine (3-4 drops) was dissolved in 1,4-Dioxan(50 ml), cooled and stirred. To this well-stirred cooled solution, Chloro acetyl chloride (0.015 mol, ml) was added dropwise. Then stirred for an additional 3 hours at room temperature and refluxit for 7 hrs. The reaction mixture was filtered to remove triethylamine hydrogen chloride and the resultant solution was concentrated, cooled and poured into ice-cold water with stirring. The solid thus obtained was recrystallized from ethanol to yield benzoxazole substituted azetidinone derivatives.

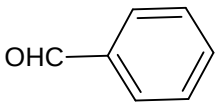
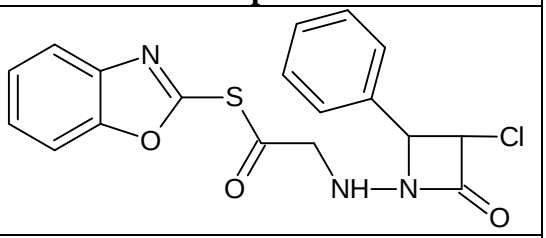
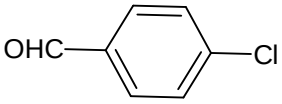
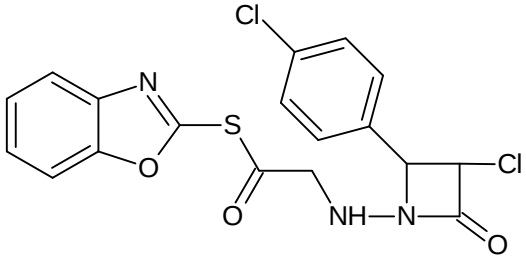
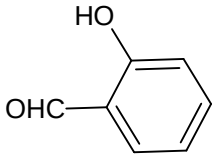
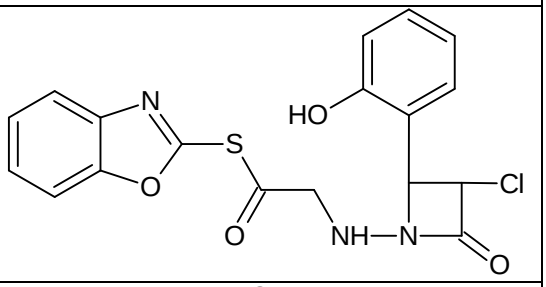
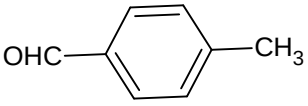
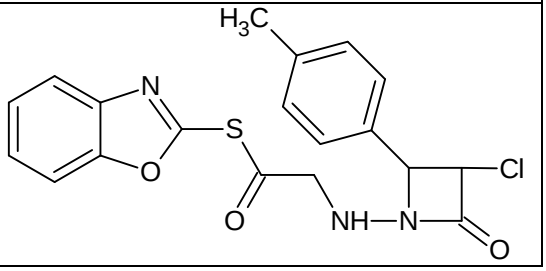


(1,3-benzoxazol-2-yl) [(2*E*)-2-benzylidenehydrazinyl]ethanethioate



(1,3-benzoxazol-2-yl) [(3-chloro-2-oxo-4-phenylazetidin-1-yl)amino]ethanethioat

Table 1: Synthetic compound with various substituents.

Sl. no.	Compound	Ar	Final product
1	BT-1		
2	BT-2		
3	BT-3		
4	BT-4		

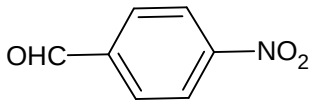
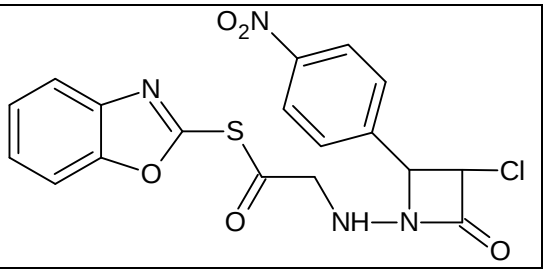
5	BT-5		

Table 2: Physical data of synthesized compounds.

Sl. No.	Compound code	Molecular Formula	Solubility	Melting point (^{0}C)	R*f*	Yield (%)
1.	BT-1	$C_{18}H_{14}ClN_3O_3S$	Methanol	187-189	0.54	60
2.	BT-2	$C_{18}H_{13}Cl_2N_3O_3S$	Methanol, Ethanol	182-185	0.44	64
3.	BT-3	$C_{18}H_{14}ClN_3O_4S$	Methanol	180-184	0.45	60
4.	BT-4	$C_{19}H_{16}ClN_3O_3S$	Methanol	183-187	0.57	62
5.	BT-5	$C_{18}H_{13}ClN_4O_5S$	Methanol, Ethanol	183-187	0.53	68

IR spectral data

Compound BT-1: *S*-1,3-benzoxazol-2-yl [(3-chloro-2-oxo-4-phenylazetidin 1yl) amino] ethane- thioate: IR KBr (cm^{-1}) 3346.53,1550.49, 1594.36, 1619.91, 660 (for benzoxazole nucleus) 2923- 1344.14 (NH stretch), 1727.72(C=O), 750.174 (Cl) 1H NMR: δ6.5-8 (Aromatic proton) δ4.7, 3.4 (aliphatic proton), δ4.4 (CH-Cl). Compound BT-2: *S*-1,3-benzoxazol-2-yl {[3- chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-yl]amino}ethanethioate: IR KBr (cm^{-1}) 3422.53, 1548.56, 1551.45, 1573.63, 1240, 1057.76, 754.995 (benzoxazole nucleus), 3422.06 (NH),1660.41(C=O), 820.563 (Cl) 1H NMR: δ6.9-7.5 (Aromatic proton) δ4.6, 4.2 (aliphatic proton), δ4.5(CH-Cl), 13C NMR: δ117.21-135.73 (Aromatic carbon, aliphatic CH2), 172.34,166.03(C=O), 156.88(C-S), 153.71(C-O), MASS M+2 peak-425.500, Base peak-348.550. Compound BT-3: *S*-1,3-benzoxazol-2-yl {[3-chloro-2-(2-hydroxyphenyl)- 4-oxoazetidin-1-yl] amino} ethanethioate IR KBr (cm^{-1}) 3203.18, 1564.995, 1529.95, 1347.03, 1253.5, 754.995 (benzoxazole), 1700.01(C=O), 3563.81(OH Stretch) 1H NMR: δ6.7-7.5 (Aromatic proton) δ4.8 (aliphatic proton), δ4.2(CH-Cl). Compound BT-4: 3387.35, 1584.24, 1619.91, 1647.88, 1051.01, 668.214(Benzoxazole nucleus), 2956.34-1347.03(NH stretch), 1689.34(C=O), 1H NMR: δ6.8-8.4 (Aromatic proton δ4.7(CH2) δ4.2(CH-Cl) δ3.8(CH2). Compound BT-5:*S*1,3-benzoxazol-2-yl {[3-chloro-2-(4-nitrophenyl)-4- oxoazetidin-1-yl]amino}ethanethioate: IR KBr (cm^{-1}) 3446.6, 1590.66, 1584.68, 1577, 1250.32, 1026.55, 680.5(benzoxazole), 3089-1320.14(NH stretch), 1690.19

(C=O), 1637.75(NO2 stretch) 1H NMR: δ6.5-8 (Aromatic proton) δ4.7, 3.4(aliphatic proton), δ4.4(CH-Cl) δ6.9-8. 7(Aromatic proton), δ4.9 (CH2), δ4.2(CH-Cl),δ3.1(CH2), 13C NMR: δ117.21-135.73 (Aromatic carbon, aliphatic CH2), δ 172.24,156.10(C=O), δ 153.69 (C-S), δ 140.67 (C-O), MASS M+2 peak-435.100, base peak- 282.900.

Anticancer activity

Brine shrimp lethal toxicity assay

Brine shrimp lethality bioassay is a cytotoxicity test of bioactive chemicals based on the killing ability of test compounds on a simple zoological organism-brine shrimp (*Artemia salina*). Dried cysts (1g cyst per litre) were hatched in a hatcher at 28-30°C with strong aeration, under a continuous light regimen. The toxicity was determined by after 24 hours exposure. The number ofsurvivors was counted and percentage death was calculated.

$$Percentage\ Mortality(\%) = Percentage\ of\ control - percentage\ of\ survival\ in\ the\ tretment$$

Various concentrations were prepared by serial dilutions using 2% methanol as solvent. Eachconcentration was tested in triplicate.

Table 3: Acute toxicity of the synthesized Benzoxazole substituted azetidinone derivatives.

Compound Code	Concentration of drug (µg/ml)	Log concentration (ml)	No of initial nauplii	Death after 24 hours	% Mortality
+ve Control	1000	3	10	10	100
	500	2	10	8	80
	250	2.5	10	7	70
BT-1	1000	3	10	10	100
	500	2	10	5	50
	250	2.5	10	5	50
BT-2	1000	3	10	10	100
	500	2	10	6	60
	250	2.5	10	6	60
BT-3	1000	3	10	9	90
	500	2	10	5	50
	250	2.5	10	5	50
BT-4	1000	3	10	9	90
	500	2	10	7	70
	250	2.5	10	6	60
BT-5	1000	3	10	10	100
	500	2	10	7	70
	250	2.5	10	6	60

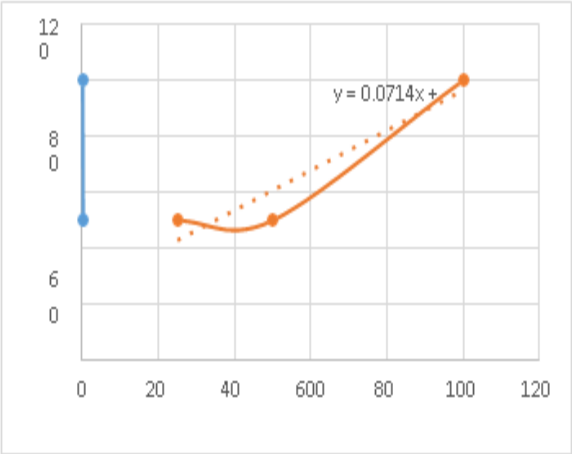


Fig. 1: Determination of LC_{50} of BT-1.

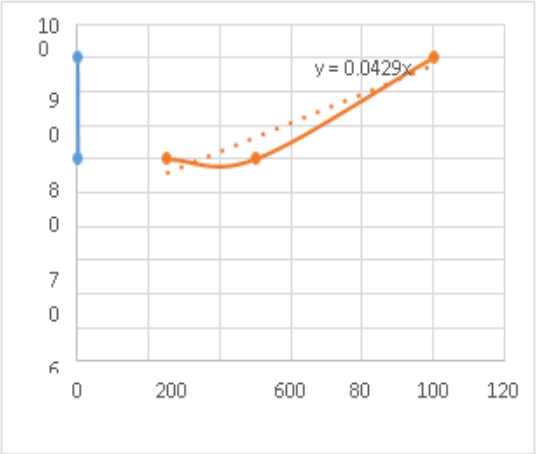


Fig. 2: Determination of LC_{50} of BT-2.

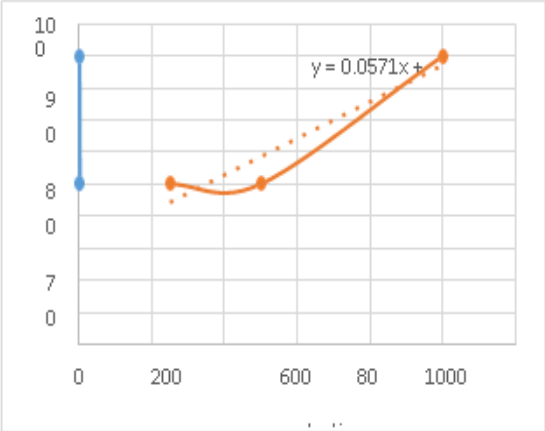


Fig.3: Determination of LC_{50} of BT-3.

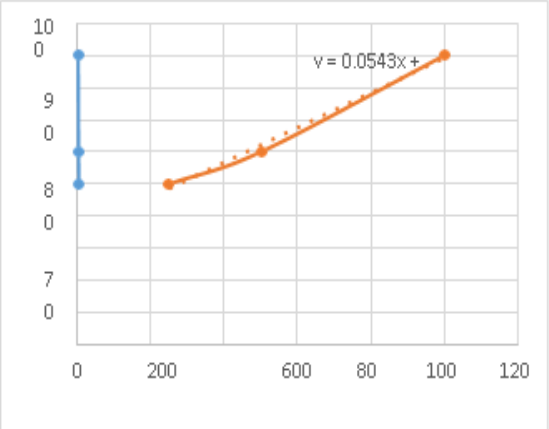


Fig. 4: Determination of LC_{50} of BT-

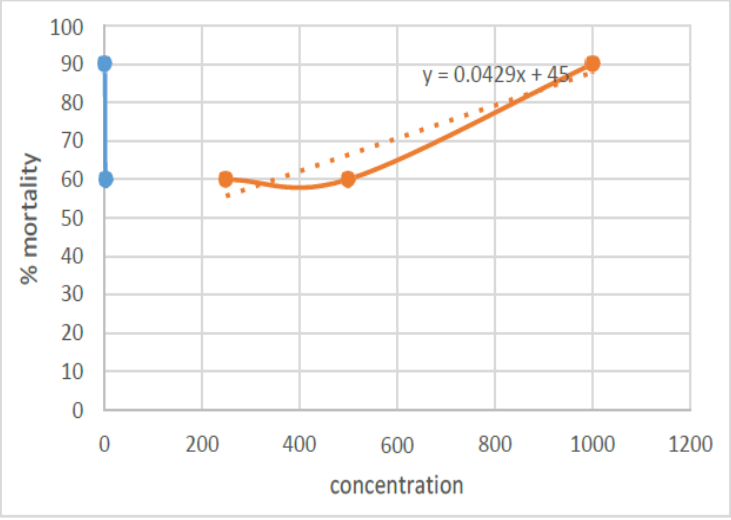


Fig. 5: Determination of LC_{50} of BT-5.

Angiogenesis inhibition assay (Cam assay)

The hallmark of cancer plays a major role in tumor growth and metastasis. Angiogenesis is the formation of new blood vessels from pre-existing blood vessels and from endothelial cell

progenitors. The CAM assay is helpful to determine the anti-angiogenic potential of anticancer drugs.

Neovascularization starts from the 3rd day of incubation and the process were completed by the 10th day day of experiment. On the 10th day, drug is added on it and the response is observed on the 13th day of study. The vessels become less dense if the drug has anti-angiogenic property.

At the 13th day eggs were taken from the incubator and the shells were removed, the contents wereput in to a petridish and photographs were taken.

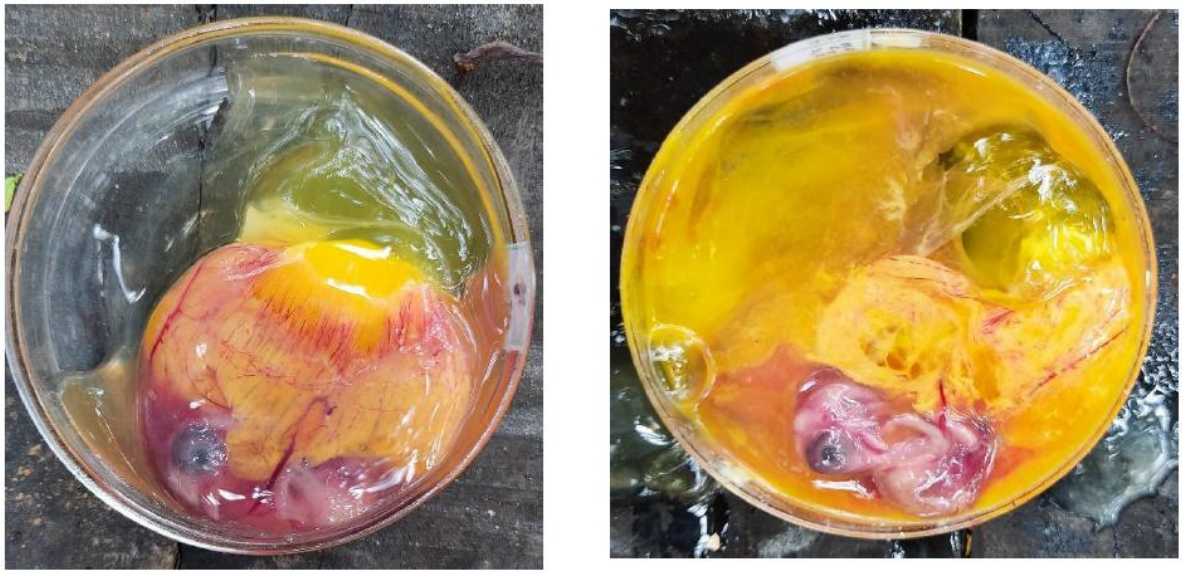
All the synthesized compounds were examined using CAM assay. Methanol is the control used.

Control

BT-1

BT-2

BT-3



BT-4 BT-

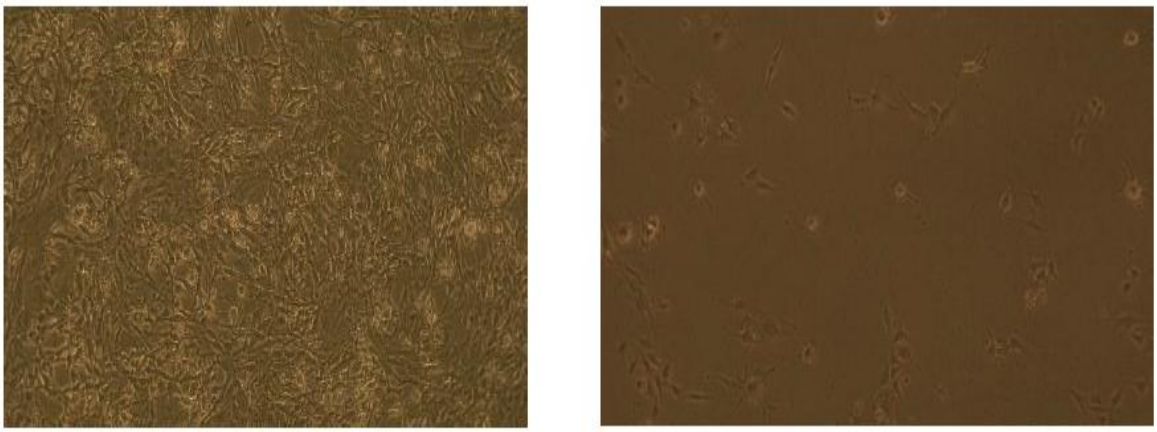
Fig. 6: Representative of various treatments in CAM assay.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals (Insoluble) by living cells, which represents mitochondrial activity. Only the compound BT-5 is opted in MTT assay with various concentrations like 50µg, 100 µg, 200 µg, 400 µg, 800 µg as it reported higher activity in CAM, Brine shrimp assay and good docking score. SKMEL cell is taken for the study.

Percentage viability of each treatment was calculated using the formula:

$$Percentage\ viability = OD\ Of\ \frac{TEST}{CONTROLL} * 100$$



Control 800 µg

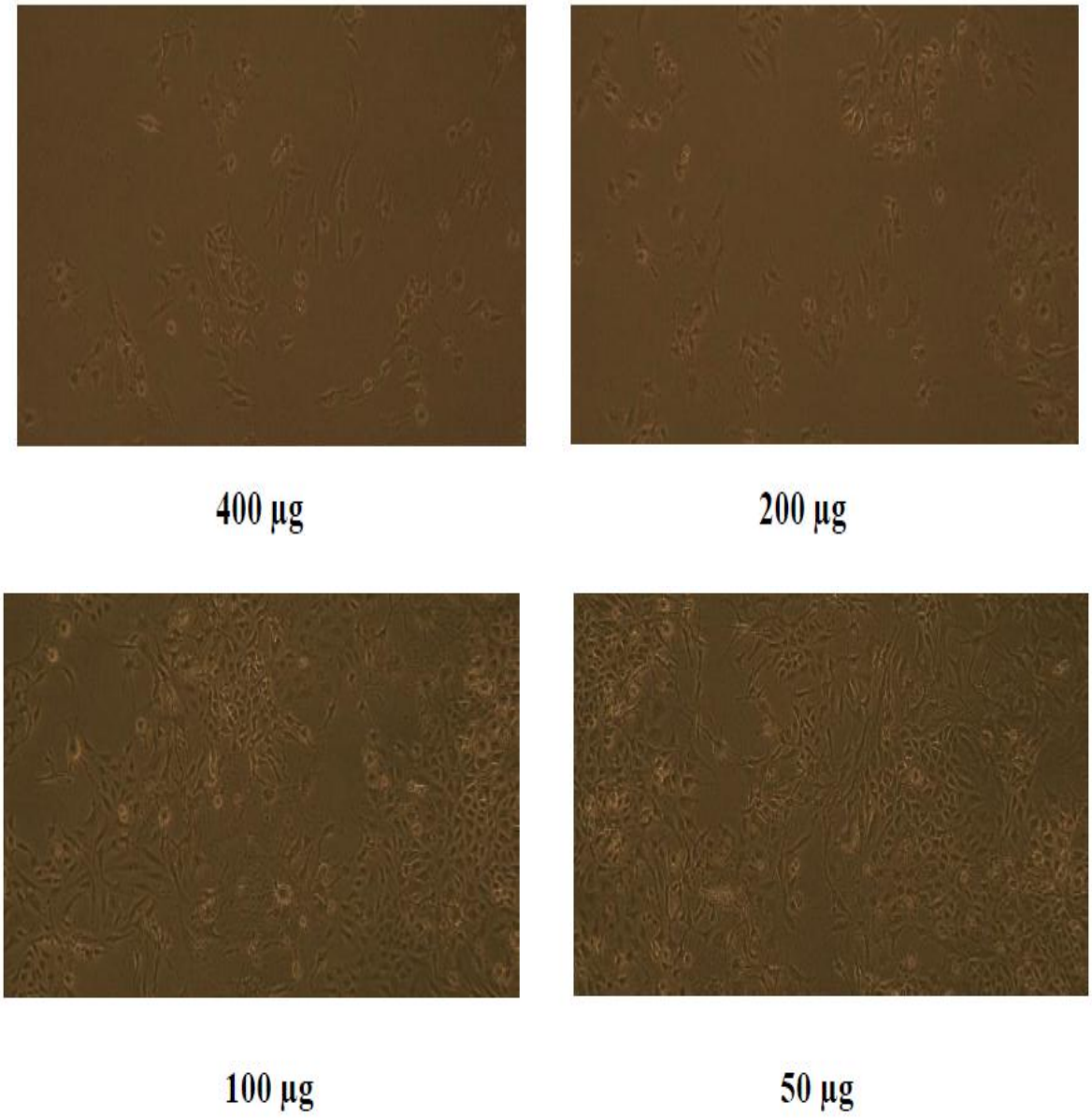


Fig. 7: Images of MTT assay of BT-5 on SKMEL cell line.

Percentage inhibition of each treatment was calculated using the formula

$$\boldsymbol{Percentage\ inhibition = 100 - percentage\ viability}$$

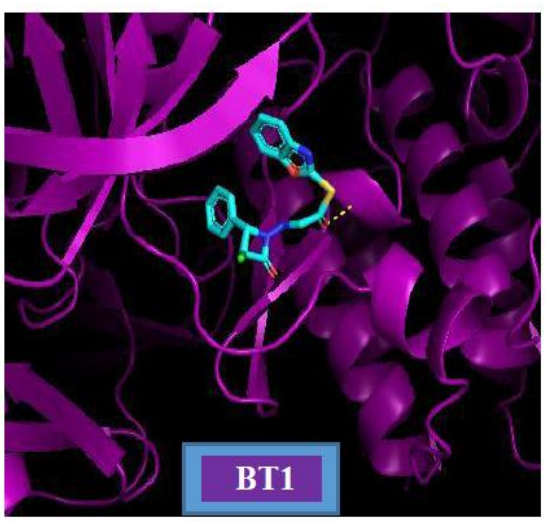
Molecular docking

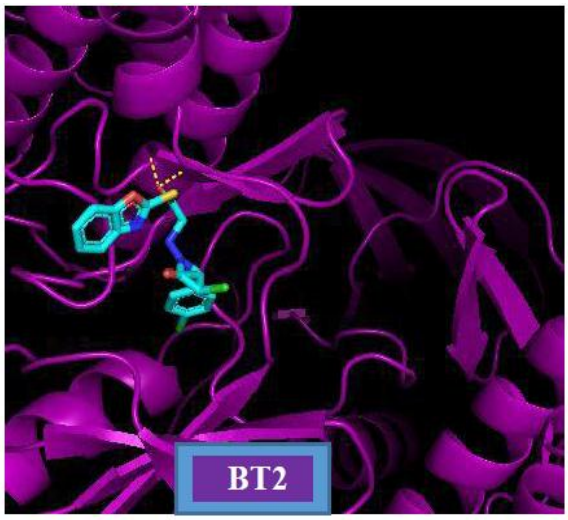
The molecular docking studies of synthesized compounds with VEGFR-2 is done. The schematic 3D representation of compounds with receptor VEGFR-2 (4DBN) was visualized and shown in Figure 6. The docking score of compounds and the standard (sorafenib) with 4DBN is given in table 5. Various hydrogen bond interactions were shown with Ser 535 for derivative BT1, Val 599for derivative BT2, Phe 594 for derivative BT3, Asp 593 for derivative BT4, Phe 582 for derivative BT5. From the docking studies, derivative BT5, BT1 and BT2

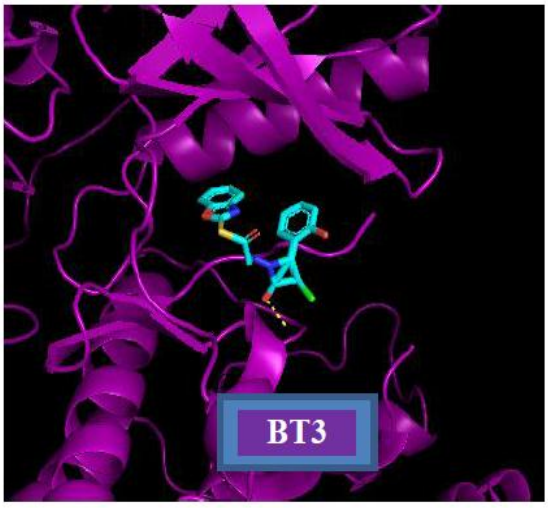
showed high docking score which indicate that these compounds possess high affinity and high polar interaction towards protein4DBN.[9]

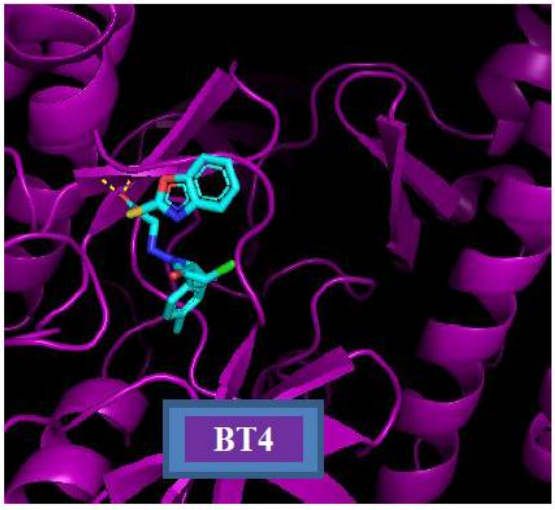
Table 4: Docking score of derivatives and standard (sorafenib) with protein 4DBN (ligandbinding domain of vascular endothelial growth factor.

S. No.	Compound code	Docking score (Kcal/mol)
1.	BT1	-8.6
2.	BT2	-8.4
3.	BT3	-8.1
4.	BT4	-8.0
5.	BT5	-9.3
6.	Standard (Sorafenib)	-10.1









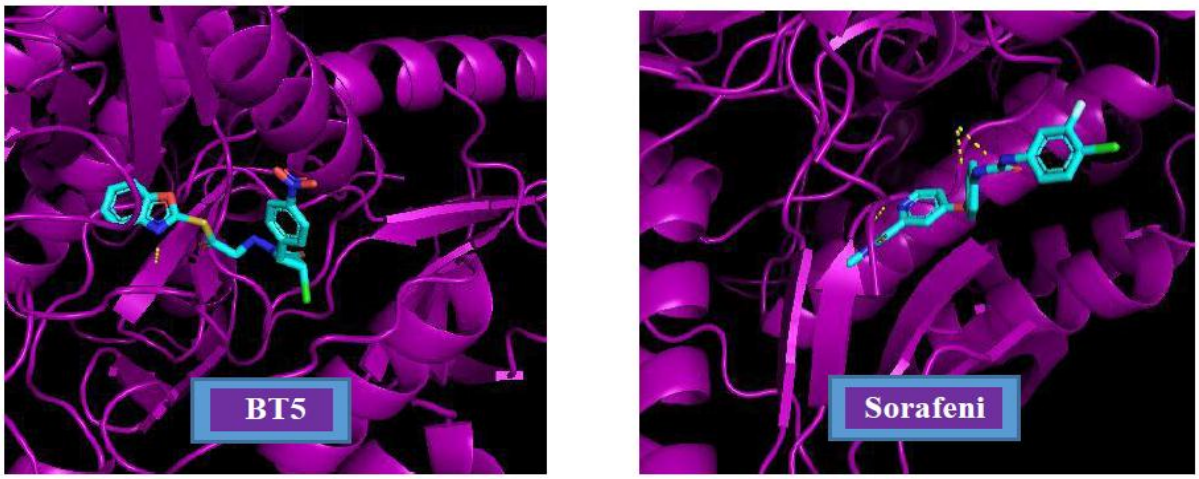


Fig. 8: Docked images of derivatives and standard (sorafenib) with protein 4 DBN (ligand-binding domain of vascular endothelial growth factor 2.

RESULTS AND DISCUSSION

All the compounds were synthesized and purified. The characterization was done by spectral techniques includes IR spectroscopy, proton NMR,[13] C NMR and MASS spectral techniques. The anticancer study of Benzoxazole substituted azetidinone derivatives was carried out by MTT Assay, CAM assay and Brine shrimp lethality assay.

Brine shrimp lethal toxicity assay

Brine shrimp bioassay is considered as a useful tool for preliminary assessment of toxicity. Here all the synthesized derivatives were opted for assay. The results of LC50 values show that the derivatives were safe with low toxicity. The derivatives BT-2, BT-5 has low LC50 value (117.52,92.08) whereas LC50 value of BT-1 is high and is 350.14 g/ml.

Table 5: LC50 value of synthesized Benzoxazole substituted azetidinone derivatives.

SI. No.	Compound Code	LC50
1	BT-1	350.14
2	BT-2	117.52
3	BT-3	350.25
4	BT-4	277.52
5	BT-5	92.08

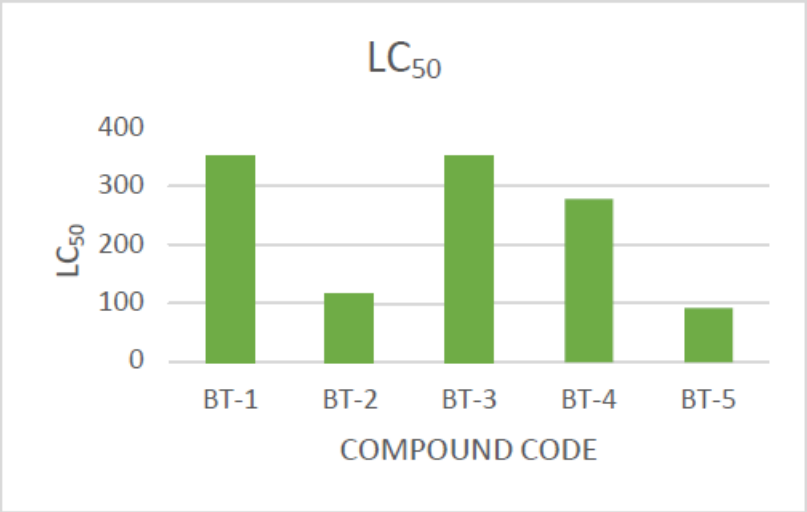


Fig. 9: LC_{50} value of synthesized derivatives.

CAM Assay

Macroscopic observation of control showed normal angiogenesis with dendritic branching pattern of blood vessel formation. They induced abundant blood vessel spouting. However, the treatment of synthesized compounds shows significant visible inhibition in the formation of blood vessels at a concentration of 100 Mcg of 1 Mcg drug. The anti-angiogenetic effect produced is clearly visible in the photographs taken. These results indicate that the BT-5 and BT-2 may have VEGFR inhibiting activity.

MTT Assay

The percentage inhibition of the cell is calculated. It is tabulated in table 6.

Table 6: Anti-neoplastic activity of BT-5 on SKMEL cell lines.

Concentration (µg/ml)	% Viability	% Inhibition
50	80.77	19.33
100	89.72	11.28
200	65.04	34.96
400	37.02	62.98
800	25.81	74.19

DISCUSSION

The benzoxazole substituted azetidinone derivatives (BT1 - BT5) were synthesized and characterized. The purity of the synthesized compounds was checked by melting point using digital melting point apparatus. The chemical structure of the derivatives was confirmed by spectral techniques.

From docking studies, the study concludes that the designed benzoxazole substituted azetidinone derivatives are found to have good interaction in binding pocket of target 4DBN, derivatives possess good anticancer activity with high binding affinity.

The anticancer activity of the synthesized compounds was studied through *invitro* assays like MTT assay, CAM assay and brine shrimp assay. From the biological evaluation the results concluded that the compound BT5 and BT2 exhibit good anticancer activity than others.

Onclusion

The present study is focused on the design, synthesis and biological evaluation of benzoxazole substituted azetidinone derivatives from 2-mercaptobenzoxazole.

The findings are

- ✓ Five derivatives were prepared through conventional method.
- ✓ IR spectra of five compounds predict the expected frequencies.
- ✓ ^{1}H NMR and ^{13}C NMR spectra of five derivatives provide signals.
- ✓ MASS spectra of two derivatives provide peaks.
- ✓ Anticancer study was carried out and the result are given.
- ✓ Molecular docking studies are conducted and the results are shown.

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