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Synthesis, characterization, and anticancer activity of benzoxazole derivatives

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ABSTRACT

A series of benzoxazole and derivatives of heterocyclic bearing nitrogen, oxygen and oxazole moieties constitutes the core structure of a several biological active compounds. Based on these findings, a series of benzoic acid substituted benzoxazole were synthesized by refluxing CS_2 and o-aminophenol. Compounds were characterized by IR, ^1H NMR, and ^{13}C NMR. All the compounds were tested for their anticancer activity against MCF-7 breast cancer cell line with MTT assay. Most of the compounds showed moderate to good anti-breast cancer activity. Docking studies of the synthesized compounds was done with the help of iGEMDOCKv2.1 software using GRIP batch docking method to study their observed activity.

Keywords: Anticancer activity, Synthesis, Docking, MCF-7 cell line.

INTRODUCTION

Synthetic organic chemistry has always been a vital part of the highly integrated and multidisciplinary process of anticancer drug development. However, the nature of its major contribution has varied over time. In recent years, efforts have been made to synthesize potential anticancer drugs. Consequently, hundreds of chemical variants of known classes of cancer therapeutic agents have been synthesized. Recent advances in biomedical sciences and combinatorial chemistry have resulted in the design and synthesis of hundreds of new antineoplastic agents with potential activity against wide range of therapeutic targets. If our understanding of the drug action and pathogenesis of different types of neoplasm becomes clearer, more rational approaches to the design of newer drugs which selectively target the tumor with no or reduced side effects may emerge. However, the exact biology of cancer still remain senigmatous at large offering a lot of scope for the research to develop newer compounds to targetthe malignant cells. The small and simple benzoxazole nucleus is present in compounds involved in research possess interesting biological activities like antifungal[1], antihistaminic[2], antitumor[3], antinuclear[4], anti-inflammatory[5] anti-HIV[6] and anti-cancer[7]. Benzoxazole is used primarily in industry and research, and has no household use. Being a heterocyclic compound, benzoxazole finds use in research as a starting material for the synthesis of larger, usually bioactive structures [8-9]. Benzoxazole can be considered as structural isosteres1 of the naturally occurring nucleic bases adenine and guanine, which allow them to interact easily with polymers of living systems[10].In the present study is it planned to synthesize benzoxazole compounds and characterize these compounds by IR, ^1H NMR and ^{13}C NMR spectral analysis. Since these compounds contain highly biological active benzoxazole nucleus, it is also aimed at carrying out anti-cancer activity [11].

EXPERIMENTAL SECTION

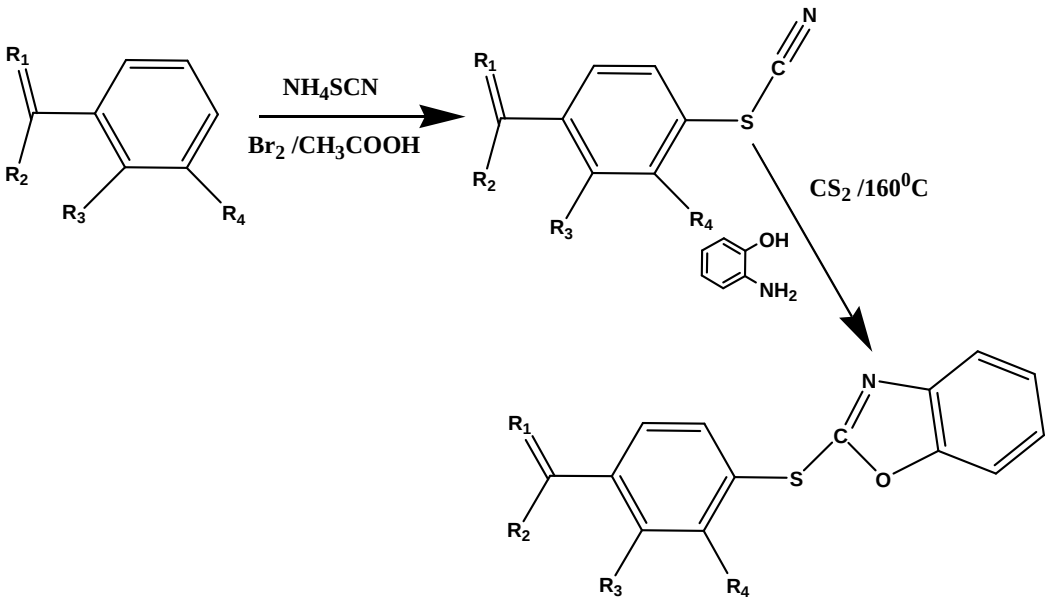
All the chemicals and solvents used were of AR-grade obtained from Sigma- Aldrich, Sisco Research Laboratories, Qualingens, Hi-media, nice chemicals, Spectrochem and were used without further purification. All melting points were taken in open capillaries and are uncorrected. Elemental analysis was performed on a Perkin-Elmer analyzer. IR spectral[12] were recorded in KBr on Shimadzu spectrometer, ^{1}H-NMR and ^{13}C-NMRin DMSO-d6 on a Bruker AC-400 spectrometer using TMS as an internal standard. The microorganisms were obtained from National Chemical Laboratory, Pune. Thin-layer chromatography (TLC) was performed on pre-coated aluminium plates (silica gel 60F254, Merck). Plates were visualized by UV light and iodine vapor.

Synthesis of thiocyanate (TC1-TC5)

The substituted/unsubstituted benzoic acid (0.5 mol) was dissolved in acetic acid (125 ml) and the solution was added to the solution of ammonium thiocyanate (1.05mol, 80 g) in glacial acetic acid (250 ml). This solution was cooled to 10-20º C. To this well stirred solution, a solution of bromine (0.5 mol, 25.7 ml) in acetic acid (250 ml) was added dropwise for thirty minutes and the temperature was maintained below 20ºC. After the addition of bromine, it was kept at room temperature for ten minutes and then it was diluted with an equal amount of water. The solid material was filtered, washed, dried and recrystallized from ethanol.

Synthesis of benzoxazole (Compound BOX 1-BOX 5)

A mixture of thiocyanate TC1-TC 5 (0.01 mol), o-aminophenol (0.01mol, 1.08g) and carbon disulphide (0.1 mol, 8 ml) was heated in an oil bath at 160^{o}C for 6 hours. The resultant benzoxazole was cooled and recrystallized from ethanol.



	R1	R2	R3	R4
TC1, BOX1,	O	OH	H	H
TC2, BOX2,	O	OH	Cl_2	H
TC3, BOX3,	O	OH	Br	H
TC4, BOX4,	O	OH	H	NO_2
TC5, BOX5,	O	OH	OCH_3	H

RESULTS AND DISCUSSION

CompoundTC-1 4-thiocyanatobenzoicacid
Anal. Calcd. For $C_8H_5SN_3O_2$: C, 53.62; H, 2.81; N, 7.82; O 17.86; S 17.82 Found: C, 53.60; H, 2.88; N, 7.88; O, 17.87; S, 17.86; Yield %(76), ES (+) 179 (M+H); IR KBr (cm ¯1): - ^{v}C≡N: 2220cm-1.

CompoundTC-2 2-chloro-4-thiocyanatobenzoicacid
Anal. Calcd. For $C_8H_4ClSNO_2$: C, 44.98; H, 1.89; N, 6.56; O 14.98; Cl, 16.59; S 15.01 Found: C, 44.07; H, 1.94; N, 6.60; O, 15.05; Cl, 16.60; S, 15.04; Yield %(62), ES (+) 213 (M+H); IR KBr (cm ¯1): - ^{v}C≡N: 2231cm-1.

CompoundTC-3 2-bromo-4-thiocyanatobenzoicacid
Anal. Calcd. For $C_8H_4BrSNO_2$: C, 37.23; H, 1.53; N, 5.43; O 12.40; Br, 30.96; S 12.42 Found: C, 37.27; H, 1.63; N, 5.48; O, 12.45; Br, 30.87; S, 12.49; Yield %(87), ES (+) 258 (M+H); IR KBr (cm ¯1): - ^{v}C≡N: 2221cm-1.

CompoundTC-4 3-nitro-4-thiocyanatobenzoicacid
Anal. Calcd. For $C_8H_4SN_2O_4$: C, 42.86; H, 1.80; N, 12.50; O 28.55; S 14.30 Found: C, 42.90; H, 1.84; N, 12.56; O, 28.59; S, 14.34; Yield %(75), ES (+) 224 (M+H); IR KBr (cm ¯1): - ^{v}C≡N: 2210cm-1.

CompoundTC-5 2-methoxy-4-thiocyanatobenzoicacid
Anal. Calcd. For $C_9H_7SNO_3$: C, 51.67; H, 3.37; N, 06.69; O 22.94; S 15.33 Found: C, 51.70; H, 3.41; N, 06.73; O, 22.99; S, 15.38; Yield %(80), ES (+) 209 (M+H); IR KBr (cm ¯1): - ^{v}C≡N: 2192cm-1.

Compound BOX 1:4-(benzo[d]oxazol-2-ylthio) benzoic acid
Anal. Calcd. For $C_{14}H_9SNO_3$: C, 61.98; H, 3.34; N, 05.16; O 17.69; S 11.82. Found: C, 62.12; H, 3.39; N, 05.21; O, 17.74; S, 11.88; Yield %(89), ES (+) 271(M+H); 1H NMR (DMSO-d6) δ 7.85 (Ar-H, multiplet), δ 11.2 (Ar-OH, singlet), ^{13}C-NMR: δ 178 (OH), δ 149(C=N), IR KBr (cm ¯1): 1658(C=Nstr), 1294 (C-Ostr), 3007 (OH str).

Compound BOX 2:**4-(benzo[d]oxazol-2-ylthio)-2-chlorobenzoic acid**
Anal. Calcd. For $C_{14}H_8SNClO_3$: C, 55.00; H, 2.64; N, 04.58; O 15.70; Cl, 11.60; S 10.49. Found: C, 55.05; H, 2.64; N, 04.61; O, 15.74; Cl, 11.66 S, 11.66; Yield %(76), ES (+) 305 (M+H); 1H NMR (DMSO-d6)δ 7.69 (Ar-H, multiplet), δ 10.40 (Ar-OH, singlet). ^{13}C-NMR: δ 160 (OH), δ 152(C=N). IR KBr (cm ¯1): 1657(C=Nstr), 1294 (C-Ostr), 3009 (OH str).

Compound BOX 3:4-(benzo[d]oxazol-2-ylthio)-2-bromobenzoic acid):
Anal. Calcd. For $C_{14}H_8SNBrO_3$: C, 48.02; H, 2.30; N, 04.00; O 13.71; Br, 22.82; S 09.16. Found: C, 48.08; H, 2.34; N, 04.06; O, 13.76; Br, 22.89; S, 09.23; Yield %(67), ES (+) 350 (M+H); ^{1}H-NMR (DMSO-d6)δ 8.01 (Ar-H, multiplet), δ 11.05 (Ar-OH, singlet).δ 10.40 (Ar-OH, singlet). ^{13}C-NMR: δ 174 (OH), δ 149(C=N). IR KBr (cm ¯1): 1632(C=Nstr), 1249 (C-Ostr), 2862 (OH str).

Compound BOX 4:**4-(benzo[d]oxazol-2-ylthio)-3-nitrobenzoic acid):**
Anal. Calcd. For $C_{14}H_8SN_2O_5$: C, 53.16; H, 2.55; N, 08.86; O 25.89; S 10.14. Found: C, 53.20; H, 2.63; N, 08.93; O, 25.95; S, 10.22; Yield %(75), ES (+) 316 (M+H); ^{1}H-NMR (DMSO-d6)δ 7.48 (Ar-H, multiplet), δ 11.3 (Ar-OH, singlet).^{13}C-NMR: δ 181 (OH), δ 153(C=N), IR KBr (cm ¯1): 1687(C=Nstr), 1292 (C-Ostr), 3008 (OH str).

Compound BOX 5:**4-(benzo[d]oxazol-2-ylthio)-2-methoxybenzoic acid):**
Anal. Calcd. For $C_{15}H_{11}SNO_4$: C, 59.79; H, 3.68; N, 04.65; O 21.24; S 10.64. Found: C, 60.05; H, 3.73; N, 04.72; O, 21.31; S, 10.71; Yield %(72), ES (+) 301 (M+H); ^{1}H-NMR (DMSO-d6)δ 7.97 (Ar-H, multiplet), δ 10.8 (Ar-OH, singlet).^{13}C-NMR: δ 180 (OH), δ 151(C=N), IR KBr (cm ¯1): 1633(C=Nstr), 1274 (C-Ostr), 3026 (OH str).

Biological evaluation
Anticancer activity
Growth of breast cancer cells was quantitated by the ability of living cells to reduce the yellow MTT to purple formazan products [13]. The amount of formazan product formed is directly proportional to the number of living cells. Synthesized compounds were prepared as 4.0 mM top stock solutions, dissolved in DMSO. MCF-7 human breast cancer cells (human breast adenocarcinoma cell line originally obtained in 1973 from Michigan Cancer Foundation) were cultivated at 37 °C in an atmosphere of 5% CO2 in Dubecco's modified Eagle's minimal medium

(DMEM) supplemented with 3.0 mM L glutamine with 10% fetal bovine serum were routinely sub cultured twice weekly to maintain in continuous logarithmic growth. Cells were trypsinized for the passage into the well plate and plated at 10,000 cells/well in 100 µL of medium in 96-well plates. Cells were allowed to adhere to the surface of well plates. After 24 h, medium was removed and 100 µL of drug solutions (prepared at 10, 50, 100 and 250 µM concentrations) were added into the wells. 100 µL of fresh medium without cells was added as control. 4 wells were used for each concentration of drug solution, while 4 wells were reserved for cell culture control, which contained the corresponding amount of DMSO. The total drug exposure was 48 h. After 48 h, contents of the well were removed and 20 µL of MTT solution (5 mg in 1 mL of phosphate buffer saline) was added to each well. Incubation at 37 °C for 4 h allowed reduction of MTT by mitochondrial dehydrogenase to an insoluble formazan product. Well contents were removed and the formazan product was solubilized by addition of 100 µL DMSO. The purple colour was produced. Absorbance of each well was read on Tenac 200 plate reader at 570 nm. From the absorbance, the % inhibition was calculated as % growth inhibition = Ac – At / Ac X 100.Where *A*c is the mean absorbance of control and *A*t is the mean absorbance of test (Table 1).

Table 1% Growth inhibition of MCF-7 cells

Compound	% Growth inhibition				
	0.25uM	2.5uM	25uM	50uM	100uM
BOX 1	0.39874	1.89651	17.59423	26.23456	30.23651
BOX 2	0.56234	2.2368	16.56823	29.85641	32.56871
BOX 3	0.47869	3.1456	25.65847	32.46689	45.56891
BOX 4	0.29541	1.9964	21.5242	27.7445	42.23651
BOX 5	0.59874	2.89652	18.5742	29.13557	31.44675

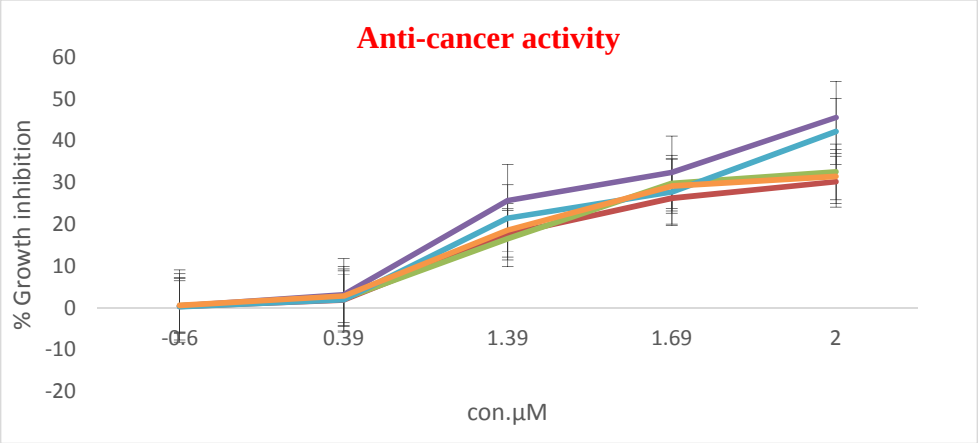


Fig.1% Growth of inhibition of synthesized benzoxazole

Anti-microbial Activity

The anti-microbial activity for the sample was carried out by Disc Diffusion Technique [14]. The test microorganisms (Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus Niger) maintained by periodical subculturing on nutrient agar and sabouraud dextrose agar medium for bacteria and fungi respectively. The test microorganisms were obtained from National Chemical Laboratory NCL), Pune and maintained by periodical sub culturing on nutrient agar and sabouraud dextrose agar medium for bacteria and fungi respectively. The effects produced by the sample were compared with the effect produced by the positive control (Reference standard ciprofloxacin 5 µg/disc for bacteria; Nystatin 100 units/disc for fungi).

Table 2 Anti-microbial activity of the synthesized compounds

S.No	Name of the microorganisms	Zone of Inhibition in mm					
		BOX 1	BOX 2	BOX 3	BOX 4	BOX 5	Std
1	*Staphylococcus aureus*	22	16	23	30	18	35
2	*Bacillus Subtilis*	19	24	19	24	26	40
3	*Escherichia Coli*	18	23	20	21	19	38
4	*Pseudomonas aeruginosa*	26	22	21	27	31	40
5	*Candida Albicans*	18	16	17	20	22	25
6	*Aspergillus Niger*	20	18	24	19	21	30

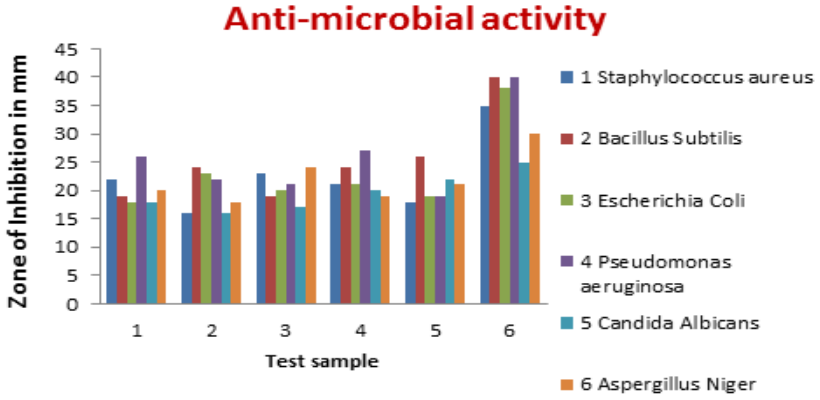


Fig.2 Zone of inhibition of synthesized benzoxazole against anti-microbial activity

Anti inflammtory activity

Carrageenan induced hind paw edema:

Albino rats of either sex weighing 150-200gms were divided into six groups of six animals each. The dosage of the drugs administrated to the different groups were as follows: Group 1 – Control received normal saline, Group 2 to 16 received test in a dose of 50 mg/kg and Group 17-Indomethacin(10mg/Kg).All the drugs were administrated orally.

After one hour of the administration of the drugs, dose 0.1 ml of 1% w/v carrageenan solution in normal saline was injected into the subplantar tissue of the left hind paw of the rat and the right hind paw served as the control. The paw volume of the rats were measured in the digital plethysmograph(Ugo basile, Italy) at the end of 0, 60, 120 and 180 min.The increase in paw edema of the treated group was compared with that of the control and the inhibitory effect of the drugs were studied. The relative potency of the drungs under investigations were calculated based upon the percentage inhibition of the inflammation [22].

$$\text{Percentage Inhibition} = \frac{\text{Control (increase in paw volume in 3}^{rd}\text{ hour)} - \text{Test(increase in paw volume in 3}^{rd}\text{ hour)}}{\text{Control (increase in pawvolume in 3}^{rd}\text{ hour).}} \times 100$$

Table 3 Anti-inflammatory activity of the synthesized compounds

Treatment	Dose mg/kg p.o.	Paw volume Increase after 3 hrs (ml)	Percentage Inhibition
Control	5 ml/kg	111.61±10.56	-
BOX 1	50mg/kg	67.52±6.42	38.09
BOX 2	50mg/kg	68.24±4.81	37.97
BOX 3	50mg/kg	72.75±6.52	32.09
BOX 4	50mg/kg	61.46±5.55	44.05
BOX 5	50mg/kg	78.16±6.17	27.41
Indomethacin	10mg/kg	40.4±3.62	63.80

P< 0.001 values are expressed as ±SEM. Number of an imals using are 6 in each group

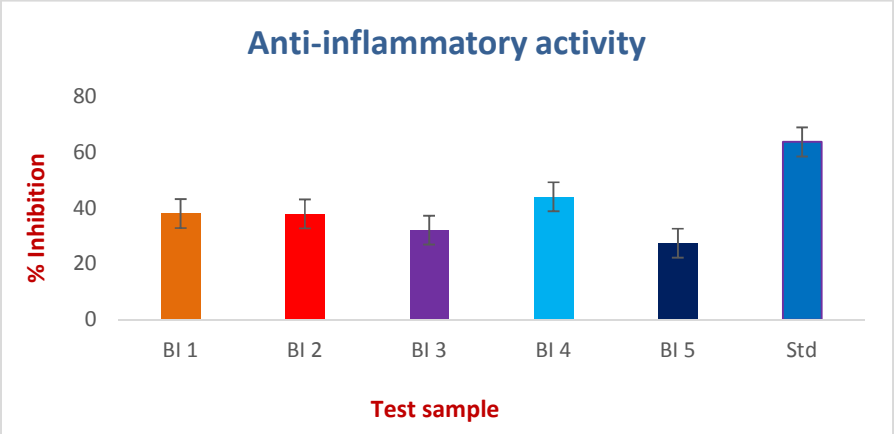


Fig.3%of inhibition of synthesized benzoxazole

Docking studies

Over activation of receptor tyrosine kinase (RTK) signaling pathways is strongly associated with carcinogenesis. So it is becoming increasingly clear that impaired deactivation of RTKs may be a mechanism in cancer. On this basis, we selected RTK as a biological target for docking study of synthesized compounds. The crystal structure of EGFR kinase domain (PDB ID: 2a91) in complex with an irreversible inhibitors was obtained from the protein data bank [15-16]. The crude PDB structure of receptor was then refined by completing the incomplete residues. The crystallized ligand lying within the receptor was modified by assigning missing bond order and hybridization states. The side chain hydrogen was then added to the crystal structure and their positions were optimized up to the rms gradient 1 by aggregating the other part of the receptor.

Target Protein Structure

The structure of the target protein was downloaded from PDB

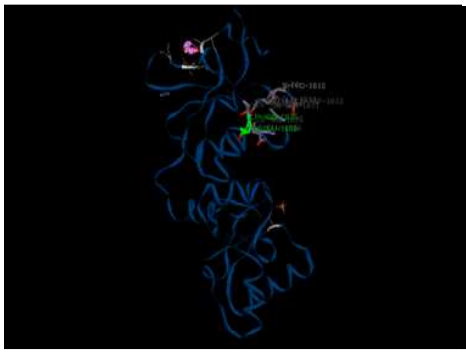
PDB ID: 3PXB. Structures of the compounds. The structures of the different compounds were drawn using ChemSketch software and the files were processed and saved as MOL files.

The PDB structure with the ID 1FOL was loaded in to the iGEMDOCK software. The binding site for the target was prepared with the radius of 4 A^0. The different ligands were drawn, prepared and uploaded into the software. The following parameters were set. Population size: 100,Generations: 50,Number of solutions: 2. the output path was set. 'Start docking' option was clicked and when docking was complete post analysis of the docked ligands was done. The predicted poses and the energy list of these poses will be outputted into the "best Pose" and "fitness.txt" of the output location, respectively. The predicted poses and scores of ligands are saved in the user defined output path. Fitness is the total energy of a predicted pose in the binding site. The empirical scoring function of iGEMDOCK is estimated as: Fitness = vdW + Hbond + Elec.Here, the vdW term is van der Waal energy. Hbond and Elect terms are hydrogen bonding energy and electro statistic energy, respectively.

The interaction residues and energy values of the synthesis compounds with the target.

Table 4 Energy values of the synthesized compounds

Compound	Total Energy	VDW	H Bond	Elec
BOX 1	-95.974	-86.349	-9.630	0
BOX 2	-91.415	-89.643	-1.770	0
BOX 3	-92.456	-89.168	-3.236	0
BOX 4	-100.38	-100.38	0	0
BOX 5	-109.36	-97.670	-11.69	0



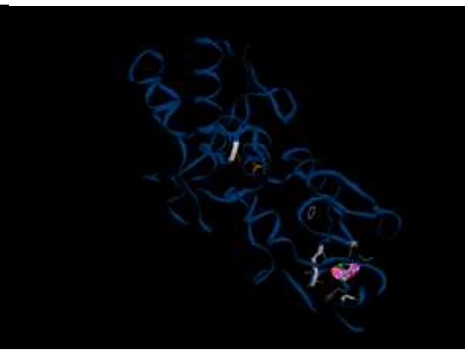
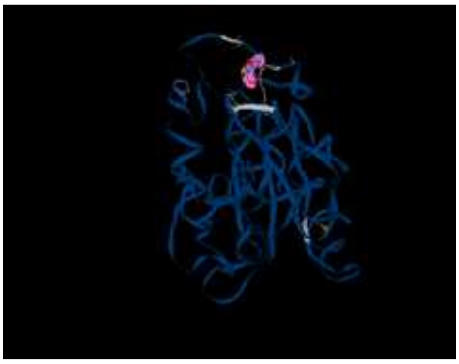


Fig.4 Interaction of BOX-1 with *BRCA1*Fig.5 Interaction of BOX-2 with *BRCA1*



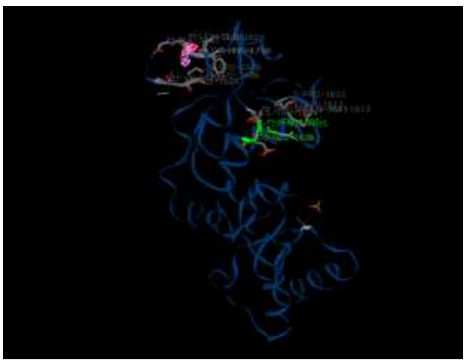


Fig.6 Interaction of BOX-3 with *BRCA1* **Fig.7 Interaction of BOX-4 with *BRCA1***

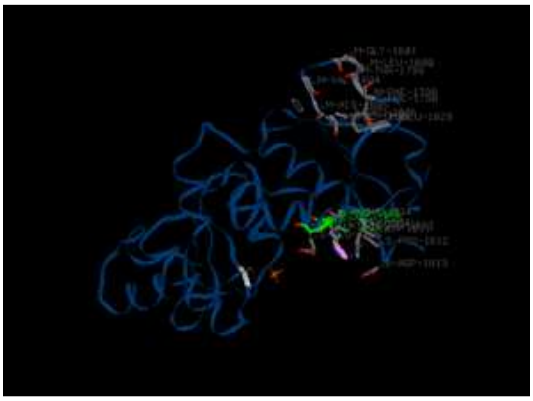


Fig.3 8Interaction of QZ-5 with *BRCA1*

DISCUSSION

All the synthesized compounds were screened for cytotoxicity on MCF-7 cell lines by MTT method. Cytotoxicity was checked at 24 hours and 48 hours duration. It was found that the activity of the compounds was increased after 48 hours as compared to 24 hours. Among the tested compounds BOX 3 and BOX 4 showed potent activity and their % growth inhibition was 45.568 and 42.236 at 100 μM/ml. Compounds BOX 3 and BOX 4 were showed IC50 50 μM/ml and 32.466 μM/ml. Docking studies was carried out by taking tyrosine kinase domain as a target for anticancer activity, the compound BOX 5 was found to have highest negative dock score (109.36). It means that it can fit well in the receptor cavity forming energetically most stable drug receptor complex. From the microbiological data, it was observed that compounds BOX 1 and BOX 3 showed marginal activity, while compound BOX 5 proved to be the most active among the tested compounds. The anti-inflammatory activity study showed that compound BOX 4 has significant effect over carrageenan induced hind paw edema.

CONCLUSION

As the concentration of compound being tested increased, the *in- vitro* anticancer activity also increased. The docking score of the synthesized compounds could not be correlated with the *invitro* anticancer activity and conclusion could not be drawn on their exact mechanism of action. So further molecular modification is required in order to arrive at more accurate structure activity relationship with their anticancer activity on breast cancer cell lines or different crystal structure of tyrosine kinase domain could be selected from PDB to study their mechanism of action.A study of the anti-microbial activity was carried out and the results are given. A study of the anti-inflammatory activity was also made and the results are tabulated.

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