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Synthesis, docking study and anticancer activity of coumarin substituted derivatives of benzothiazole

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ABSTRACT

During the last 2 decades, the study of the biological activities of Coumarin derivatives has been the aim of many researchers. Based on these findings, a series of Coumarin substituted benzothiazoles were synthesized by refluxing Coumarin-4-carboxaldehyde and o-aminothiophenol in acetic acid. Compounds were characterized by IR, 1H NMR and mass spectroscopy. 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 VLife MDS 3.0 software using GRIP batch docking method to study their observed activity.

Keywords: Anticancer, Synthesis, Docking, Tyrosine kinase, 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 [1].

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 remains enigmatous at large offering a lot of scope for the research to develop newer compounds to target the malignant cells.

Coumarins (known as 1, 2-benzopyrones or *o*-hydroxycinnamic acid-8-lactones) comprise a very large class of phenolic derivatives found in plants and consist of fused benzene and α-pyrone rings [2,3]. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. Owing to their importance in many fields of everyday life, such as pharmaceutical, cosmetic, perfume, and nutrition, their chemistry has been widely investigated and many natural and nonnatural coumarins have been synthesized.

Coumarin is a natural substance that has shown anti-tumor activity *in- vivo*, with the effect believed to be due to its metabolites (e.g. 7-hydroxycoumarin). Therefore, the focus will be on these relevant compounds and their therapeutic importance. A recent study has shown that coumarin substituted benzothiazole inhibits the protein tyrosine kinase. This knowledge may lead to its use in cancer therapy [4].The most recent work involves 2 cell-lines, MCF-7 a breast carcinoma and A549 lung carcinoma. Unsubstituted coumarins appear to be toxic because of their oxidative decarboxylation, resulting in the formation of *o*-hydroxyphenylacetaldehyde or *o*-hydroxyphenyl acetic acid derivatives which form very stable complexes with heavy metals inside the body.

Benzothiazoles comprise a novel class of therapeutic compounds shown to exert a wide range of biological activities. Although they have been known from long ago to be biologically active [5, 6], their varied biological features are still of great scientific interest. Benzothiazoles show antitumor activity, especially the phenyl-substituted benzothiazole [7, 8] while condensed pyrimido benzothiazoles and benzothiazolo quinazolines exert antiviral activity [9]. Recently, Racane *et al.* [10] have described the synthesis of bis-substituted amidino benzothiazoles as potential anti HIV agents. Substituted 6-nitro-and 6-aminobenzothiazoles [11] show antimicrobial activity. Benzothiazoles have been reported to be the molecules of interest, with potent anticancer activity and they act by binding to ATP binding site of protein kinase. ATP binding site of protein kinase provided an extensive opportunity to design newer analogs. With this background, we report an attempt to discern the structural and physicochemical requirements for inhibition of p56lck target.

Based on these findings, we describe the synthesis of some compounds featuring heterocyclic ring (benzothiazole) fused onto the coumarin moiety with the aim of obtaining more potent pharmacologically active compounds.

MATERIALS AND METHODS

Reagents

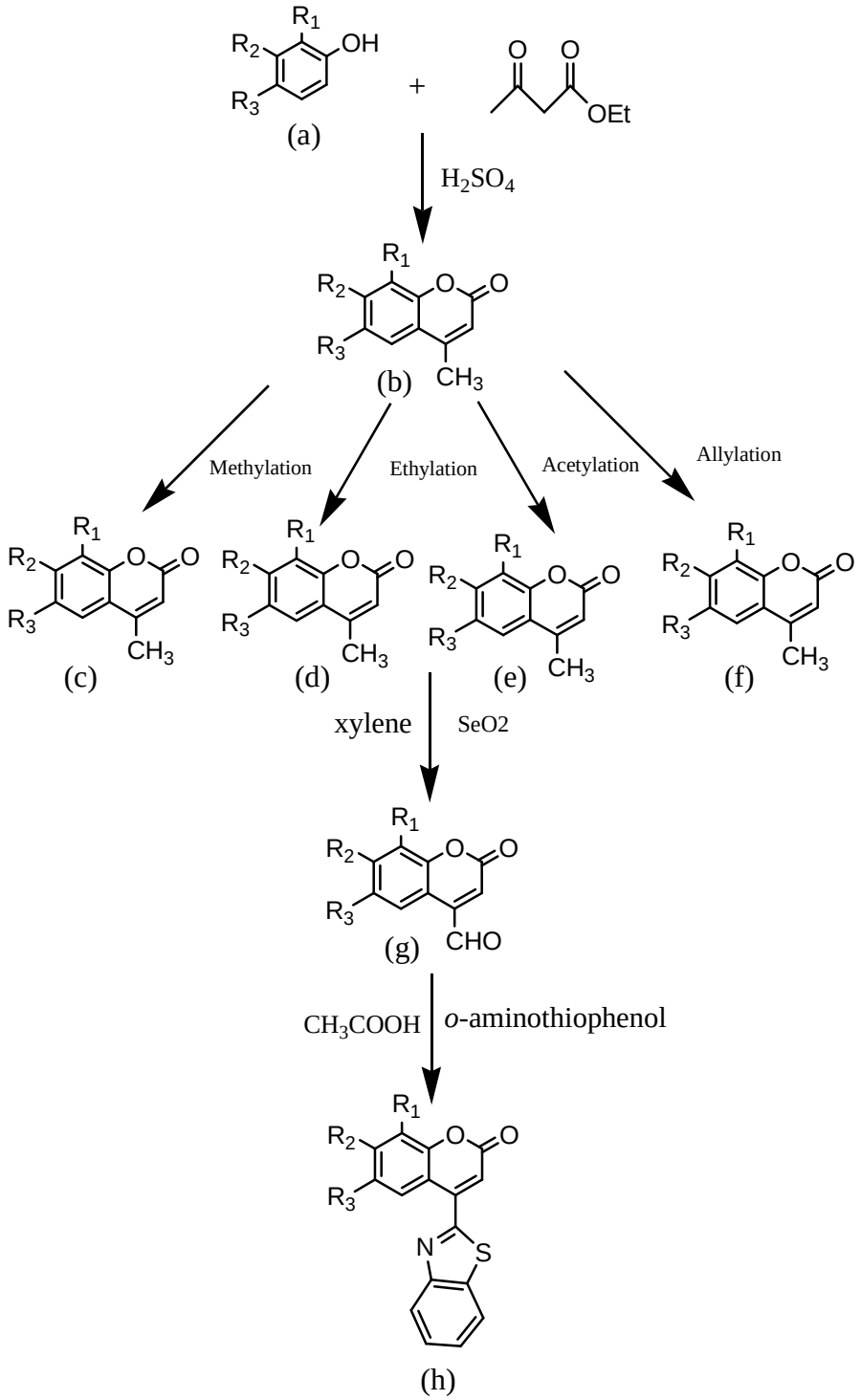
All the chemicals and solvents used were of AR-grade and LR-grade and obtained from Sigma-Aldrich, Sisco Research Laboratories, Qualingens, Hi-media, Nice chemicals, Spectrochem and were used without further purification.

Equipment

Melting points were measured on a electrothermal melting point apparatus (Shital scientific industries, Mangalore, India). Infrared (IR) spectra were recorded as KBr pellets with an FTIR-8310 spectrophotometer (Shimadzu, Japan). Proton magnetic resonance (1H NMR) spectra were recorded in DMSO-d6 (Merck) on a AMX-400 NMR Spectrometer (IISc, Bangalore, India). Mass spectra were recorded on a GCMS (QP 5050A, Shimadzu Corporation, Japan). Absorption maxima were taken on a UV-Visible spectrophotometer-1650 (Shimadzu, Japan).

TLC analysis

Thin-layer chromatography (TLC) was performed on pre-coated aluminium plates (silica gel 60 F254, Merck). Plates were visualized by UV light and iodine vapor.



Synthesis of substituted coumarin [12]: A mixture of the substituted resorcinol (0.01mol) and concentrated sulphuric acid (10ml) was taken in a flask. To this ethylacetoacetate (0.01mol) was added dropwise by maintained the temperature below 10^0C. Kept the reaction mixture at room temperature for 18h and then poured into a mixture of crushed ice (20g) and water (30ml). The precipitate was collected, washed with water. This solid was dissolved in 5% sodium hydroxide

solution (15ml), filtered and 2M sulphuric acid was added with stirring until the solution was acid to litmus. Substituted coumarin was collected, washed with water and dried.

Methylation [13]: A mixture of the coumarin (0.01mol), dimethyl sulphate (0.15g), anhydrous potassium carbonate (0.4g) and acetone (0.7ml) was taken in a microwave flask. The flask was placed in microwave oven for 10 min. The contents of the flask were poured into ice water and neutralized using 10% hydrochloric acid and then extracted with ether. The organic layer was washed with aqueous bicarbonate solution and brine respectively and dried over anhydrous sodium sulphate. Ether was removed to get o-methylated product.

Ethylation [14]: A mixture of the substituted coumarin (0.002mol), ethyl iodide (0.022mol), anhydrous potassium carbonate (0.005mol) and acetone (50ml) was refluxed for 24h. The reaction mixture was poured into water. The precipitate was collected, washed with water and dried.

Acetylation [15]: A mixture of substituted coumarin (0.01mol), anhydrous sodium acetate (0.049mol) and acetic anhydride (0.06mol) was heated on a water bath for 8h and poured into water. The precipitate was collected, washed with 1% aqueous sodium hydroxide solution and dried.

Allylation [16]: A mixture of substituted coumarin (40g), allyl bromide (2.1ml) and anhydrous potassium carbonate (8g) in acetone (100ml) was refluxed on a water bath for 10h and poured into water. The precipitate was collected, washed with aqueous sodium hydroxide.

Formylation [17]: Substituted coumarin (1g) was dissolved in hot xylene (50ml). The solution was cooled and selenium dioxide (1g) was added. The mixture was refluxed for 12h and filtered in hot condition. Solvent was removed to get desired product.

Synthesis of coumarin substituted benzothiazole [18]: The coumarin-4-carboxaldehyde (0.055mol) and o-aminothiophenol (0.05mol) was refluxed in acetic acid (7ml) for 5h. The solution was cooled and the product precipitated. This solid was collected, washed with water and recrystallized from methanol. Yields and physical characteristics are listed in Table 1.

4-(benzo[d]thiazol-2-yl)-7-methoxy-2H-chromen-2-one (SC1) IR (KBr): 2922.25(C-H), 1616.40(C=C), 1734.06(C=O), 1546.96(C=N), 1383.01(C-N), 756.12(C-S) cm^{-1}. 1H NMR (DMSO): δ = 6.75(s, 1H, H-3), 8.20(d, 1H, J = 8.22Hz, H-5), 7.07(d, 1H, J = 7.07 Hz, H-6), 7.59(s, 1H, J = 7.62, H-8), 7.04(d, 1H, J = 7.01Hz, H-4'), 7.64(t, 1H, J = 7.64Hz, H-5'), 8.22(t, 1H, J = 8.22 Hz, H-6'), 8.53(d, 1H, J = 8.50Hz, H-7'), 3.89(s, 3H, CH_3). GC-MS: 309(M^+).

4-(benzo[d]thiazol-2-yl)-7-ethoxy-2H-chromen-2-one (SC2) IR (KBr): 2976.26(C-H), 1606.76(C=C), 1714.77(C=O), 1546.96(C=N), 1383.01(C-N), 765.77(C-S) cm^{-1}. 1H NMR(DMSO): δ = 6.74(s, 1H, H-3), 8.23(d, 1H, J = 8.22 Hz, H-5), 7.03(d, 1H, J = 7.07 Hz, H-6), 7.65(s, 1H, J = 7.62 Hz, H-8), 7.0(d, 1H, J = 7.01 Hz, H-4'), 7.62(t, 1H, J = 7.64 Hz, H-5'), 8.24(t, 1H, J = 8.22 Hz, H-6'), 8.51(d, 1H, J = 8.50 Hz, H-7'), 4.18(s, 2H, CH_2), 1.38(s,3H, CH_3). GC-MS: 323(M^+).

4-(benzo[d]thiazol-2-yl)-6-methoxy-2H-chromen-2-one (SC5) IR (KBr): 2945.40(C-H), 1604.83(C=C), 1716.70(C=O), 1558.45(C=N), 1479.45(C-N), 756.12(C-S) cm^{-1}.

4-(benzo[d]thiazol-2-yl)-6-ethoxy-2H-chromen-2-one (SC6)IR (KBr): 2972.40(C-H), 1604.83(C=C), 1716.70(C=O), 1558.54(C=N), 1483.31(C-N), 769.62(C-S) cm^{-1}. 1H NMR (DMSO): δ = 6.95(s, 1H, H-3), 8.17(s, 1H, H-5), 7.57(d, 1H, H-7), 7.41(d, 1H, H-8), 7.30(d, 1H, H-4'), 7.42(t, 1H, H-5'), 8.17(t, 1H, H-6'), 8.24(d, 1H, H-7'),), 4.09(s, 2H, CH_2), 1.36(s,3H, CH_3).
4-(benzo[d]thiazol-2-yl)-7, 8-dimethoxy-2H-chromen-2-one (SC8) IR (KBr): 2931.90(C-H), 1600.93(C=C), 1726.35(C=O), 1552.75(C=N), 1475.59(C-N), 750.33(C-S) cm^{-1}.

4-(benzo[d]thiazol-2-yl)-7-methoxy-8-methyl-2H-chromen-2-one (SC11) IR (KBr): 2968.55(C-H), 1600.93(C=C), 1726.35(C=O), 1554.68(C=N), 1475.59(C-N), 752.26(C-S) cm^{-1}.

4-(benzo[d]thiazol-2-yl)-6-chloro-7-methoxy-2H-chromen-2-one (SC13)IR (KBr): 2985.91(C-H), 1614.69(C=C), 1735.99(C=O), 1546.96(C=N), 1487.17(C-N), 752.26(C-S) cm^{-1}.

4-(benzo[d]thiazol-2-yl)-6, 7-dimethoxy-2H-chromen-2-one (SC14)IR (KBr): 2982.91(C-H), 1614.47(C=C), 1728.28(C=O), 1546.96(C=N), 1456.30(C-N), 758.05(C-S) cm^{-1}.

Table 1 Physical data of the Coumarin substituted benzothiazole derivatives

Compound	R1	R2	R3	%Yield	Mt.pt (^oC)	Rf*
SC1	H	OCH_3	H	74	152-154	0.70
SC2	H	OC_2H_5	H	74	150-155	0.71
SC3	H	OAc	H	68	177-180	0.68
SC4	H	$OCH_2CH=CH_2$	H	70	180-183	0.72
SC5	H	H	OCH_3	72	172-174	0.62
SC6	H	H	OC_2H_5	70	170-173	0.66
SC7	H	H	OAc	60	170-172	0.79
SC8	OCH_3	OCH_3	H	66	210-212	0.68
SC9	OAc	OAc	H	62	190-193	0.64
SC10	H	N $(C_2H_5)_2$	H	56	300-303	0.62
SC11	CH_3	OCH_3	H	72	212-215	0.74
SC12	CH_3	OAc	H	63	220-222	0.72
SC13	H	OCH_3	Cl	67	200-203	0.62
SC14	H	OCH_3	OCH_3	75	200-202	0.76
SC15	H	OAc	OAc	65	176-180	0.72
SC16	H	OC_2H_5	OC_2H_5	73	178-181	0.70
SC17	H	$OCH_2CH=CH_2$	$OCH_2CH=CH_2$	63	212-216	0.67
SC18	NO_2	OCH_3	H	68	176-180	0.64
SC19	H	$SCOCH_3$	H	65	202-206	0.68
SC20	H	SCH_3	H	72	189-192	0.72

** n-Hexane: ethyl acetate, 7:3*

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 [19]. 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% CO_2 in Dubecco's modified Eagle's minimal medium (DMEM) supplemented with 3.0 mM L-

glutamine with 10% fetal bovine serum were routinely subcultured 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 solubilised 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 = $A_c - A_t / A_c$ X 100. Where A_c is the mean absorbance of control and A_t is the mean absorbance of test (Table 2).

Table 2 % Growth inhibition of MCF-7 cells

Compound	% Growth inhibition			
	10µM	50 µM	100 µM	200 µM
SC1	1.32	2.56	6.32	19.53
SC2	2.21	5.64	7.89	19.53
SC3	-	-	2.58	7.64
SC4	1.68	4.56	10.65	17.55
SC5	2.65	3.89	12.65	19.32
SC6	5.20	8.26	12.58	17.83
SC7	46.89	66.47	73.64	78.68
SC8	3.01	4.61	6.82	19.07
SC9	6.54	13.58	16.06	34.53
SC10	-	1.96	5.65	15.66
SC11	-	-	4.16	11.60
SC12	16.78	23.80	45.23	78.71
SC13	1.48	1.64	11.08	26.50
SC14	11.98	17.32	22.98	58.03
SC15	3.54	9.24	12.65	36.24
SC16	7.65	16.93	36.45	64.92
SC17	18.54	25.34	43.78	70.34
SC18	1.25	5.64	7.89	14.84
SC19	6.54	15.34	37.56	60.85
SC20	11.67	69.00	75.15	79.40

Docking studies

Over activation of receptor tyrosine kinase (RTK) signalling 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 [20]. The crude PDB structure of receptor was then refined by completing the incomplete residues. The co-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.

The optimized receptor was then saved as mol file and used for docking simulation. The 2D structure of the compounds were built and then converted into the 3D with the help of vLife MDS 3.0 software. The 3D structures were then energetically minimized upto the rms gradient of 0.01 using Merck Molecular Force Field (MMFF). Conformers of all the synthesized ligands were selected and number of seeds used for searching the conformational space was set as 5. All conformers were then energetically minimized upto the rms gradient of 0.01 and then saved in seperate folder. The active site selection was done by choosing the cavity having maximum hydrophobic surface area.

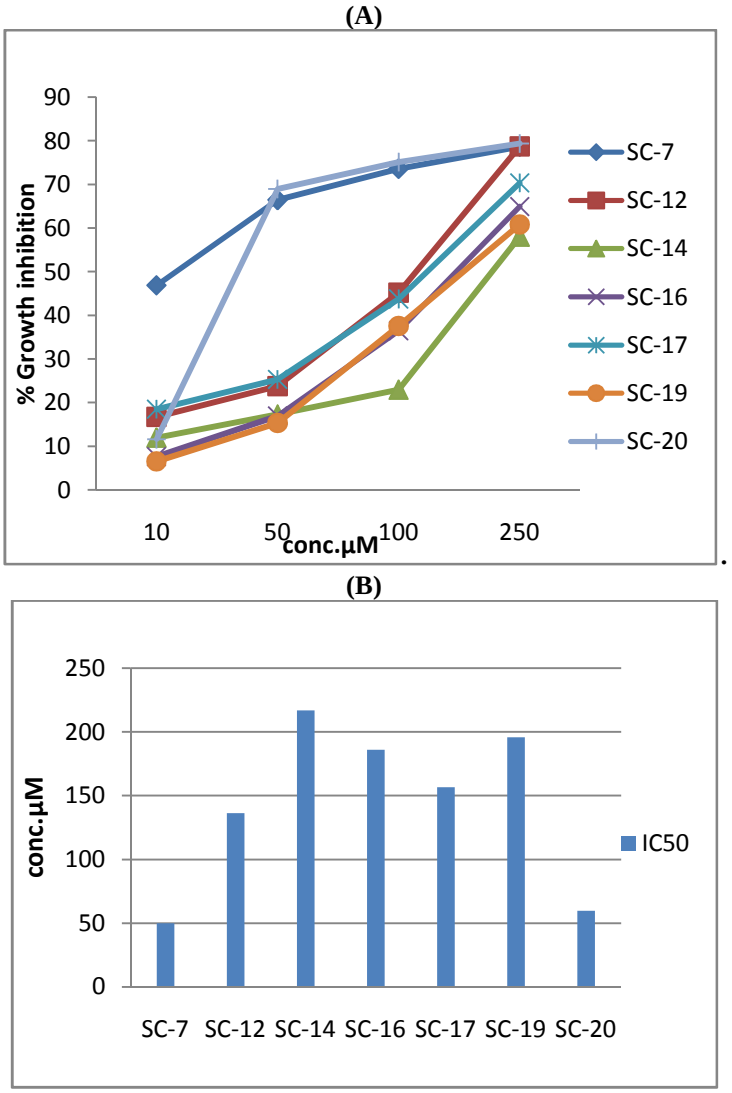


Fig.1. Graph of % growth inhibition (A) and IC50 (B) of MCF-7 cells

Docking simulation was done by GRIP batch docking method. In this, all generated conformers of one ligand were put as one batch in GRIP docking wizard. Likewise, the batches for all other ligands were put. All the conformers were virtually docked at the defined cavity of the receptor. The parameters fixed for docking simulation was like this number of placements: 30, rotation angle: 30°, exhaustive method, scoring function: dock score. By rotation angle, the ligand gets rotated for different poses. By placements, the method will check all the 30 possible placements into the active site pocket and results out few best placements out of 30. For each ligand, all the conformers with their best placements and their dock score will be saved in output folder. The method also highlights the best placement of best conformer of one particular ligand which is

having best (minimum) dock score. In the results of docking, we have listed only best conformers and its dock score for each ligand in table 3. The ligand forming most stable drug-receptor complex is the one which is having minimum dock score.
After docking simulation, the best docked conformer of each ligand and receptor were merged and their complexes were then energetically optimized by defining the radius of 10Å measured from the docked ligand. Stepwise energy optimization was done by first hydrogen, second side chains and finally the backbone of receptor [21]. The optimized complexes were then checked for various interaction of ligand with receptor like hydrogen bonding, hydrophobic bonding and van der Waal's interaction.

Table 3 Docking scores of the synthesized compounds

Compound	Dock score
SC1	-34.6749
SC2	-42.4280
SC3	-37.4230
SC4	-39.2946
SC5	-54.7300
SC6	-61.0300
SC7	-56.9200
SC8	-36.4051
SC9	-38.9500
SC10	-36.4400
SC11	-26.4984
SC12	-34.1472
SC13	-45.4894
SC14	-45.4547
SC15	-63.2771
SC16	-54.3518
SC17	-64.6781
SC18	-31.9916
SC19	-58.0969
SC20	-32.7429

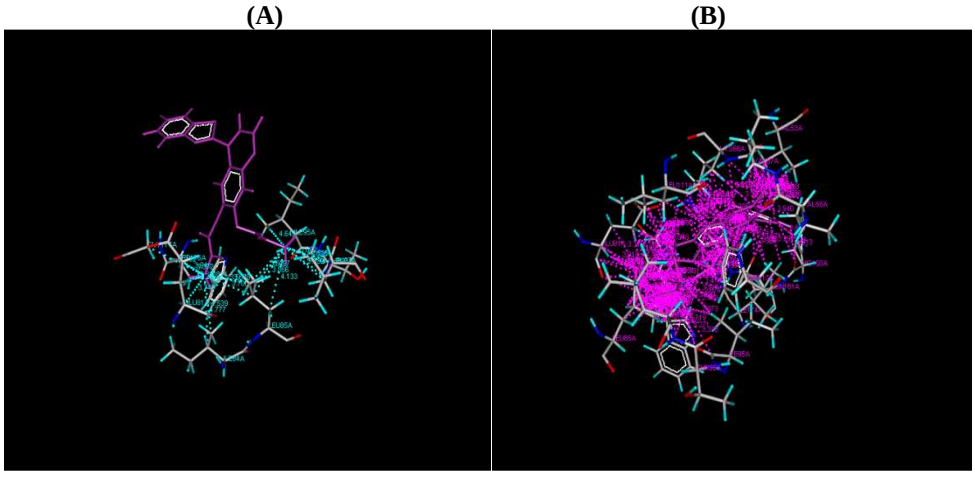


Fig.2. (A) Hydrophobic interaction of SC17 with the receptor residues by 'C' atom of allyloxy methylene group shown by light green dotted lines(B) Van der Waal's interaction of SC17 with the receptor residues shown by pink colour dotted lines.

The compound SC-17 was not showing hydrogen bonding with the receptor. It was found to form hydrophobic bonding by C- atom of allyloxy methylene group. Some of the residues involved in this type of interaction are Glu81, Leu85, Thr96, and Gly177 (Fig. 2A). It was exhibited large number of van der Waal's bonding with wide range of residues. Some of the residues involved in this type of interaction are Val53, Lys68, Glu81, Leu85, Thr96, and Gly177 etc. (Fig. 2B).

RESULTS AND 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 SC7 and SC20 showed potent activity and their % growth inhibition was 78.68 and 79.40 at 250 µM/ml. Compounds SC7 and SC20 were showed IC_{50} 50 µM/ml and 59.80µM/ml. Docking studies was carried out by taking tyrosine kinase domain as a target for anticancer activity, the compound SC17 was found to have highest negative dock score (-64.67). It means that it can fit well in the receptor cavity forming energetically most stable drug receptor complex.

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 *in-vitro* 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.

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