Section A-Research paper

ANTICANCER POTENTIAL OF SOME NEWLY SYNTHESIZED TRIPHENYL SUBSTITUTED IMIDAZOLES – DESIGN, SYNTHESIS AND INVITRO CYTOTOXICITY STUDIES

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Article History:

1. Abstract:
In an effort to identify novel anticancer drugs, a series of novel 2,4,5-triaryl imidazole derivatives were designed against the breast cancer targets EGFR and ER. Insilco analysis of these molecules were conducted against the targets EGFR, ER for breast cancer research. The intended compounds were synthesized and characterized using MS, FTIR, and NMR. SRB assay was used to test the synthesized compounds' anticancer activity in vitro against MCF7 cell lines. Out of the 18 compounds synthesized, compounds A9 and D9 demonstrated significant antiproliferative effect with an IC_{50} values of 8.343µg/ml and 11.61µg/ml, respectively.
Keywords: Cancer, Drug design, Imidazole, EGFR, ER

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2. Introduction:

Imidazole is a five-membered heterocyclic ring system containing two nitrogen atoms at position 1 and 3 (Fig. 1). It is an aromatic compound containing a sextet of π electrons. The two nitrogen atoms present make it highly polar and thus soluble in water and many other polar solvents. Imidazole is an amphoteric compound that can act as both an acid and a base [1]. The weak acidic nature is due to the protonated nitrogen at position 1 with a pKa value of 14.9. The strong basic character is due to the nitrogen at position 3 with a pKa value of 7. The basicity is increased because of resonance stabilization of the charge through formation of imidazolium ion [2,3].

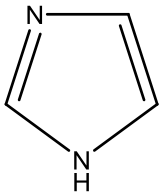


Fig. 1: Structure of imidazole

The structural properties of imidazole are useful for developing interactions such as hydrogen bonds, ionic interactions, Vander Waals interactions, π- π stacking, hydrophobic interactions with receptors and enzymes in the physiological system. These binding interactions are responsible for the diverse biological activities expressed by imidazole derivatives [4,5]. Imidazole scaffold is found to possess anticancer, antimicrobial, antihypertensive, antihistaminic, antipsychotic, neuroleptic, anti-inflammatory, analgesic and antiparasitic activity. Due to the potential of imidazole moiety in medicinal chemistry various imidazole derivatives have been developed as clinically approved drugs over the years.

Anticancer agents such as dacarbazine, azathioprine, nilotinib are some of the potent anticancer agents possessing imidazole scaffold.

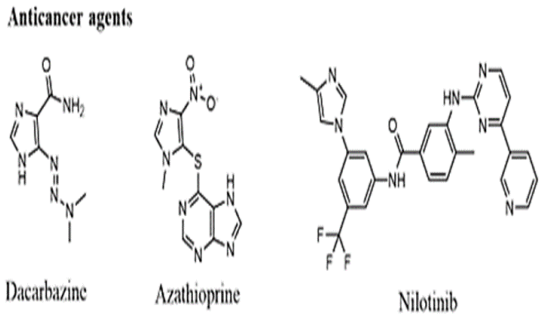


Fig. 2: Marketed anticancer agents with imidazole scaffold.

Cancer is a group of diseases which is characterized by uncontrolled proliferation of abnormal cells [6]. Maintaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion & metastasis constitute the six hallmarks of cancer [7]. Cancer presents as one of the major diseases with a total of 19.3 million cases globally resulting in 10 million deaths in the year 2020. There are various types of cancer, the most common being breast cancer, lung cancer, colon and rectum cancer, prostate cancer, skin cancer and stomach cancer. Out of these, breast cancer was found to be the most prevalent with 2.26 million cases in 2020[8]. The present study focuses on the targeted design of breast cancer. Breast cancer is the most prevalent type of cancer. In the year 2020, 2.26 million new cases of breast cancer were reported globally with 685000 deaths. It is most common in women with only 0.5-1 % of the total cases accounted for in men [9].

With this background it was thought appropriate to design novel anticancer agents and to perform computational studies against two well-known breast cancer targets including EGFR(Endothelial Growth factor receptor) and ER (estrogen receptor). The designed ligands were synthesized and characterized by various physical and spectroscopic methods. Invitro anticancer activity studies were carried out to assess their anticancer potential.

3. MATERIALS AND METHODS:

3.1. Computational studies

All insilico studies, including molecular docking, induced fit docking, RMSD calculation, MMGBSA, and ADMET prediction, were carried out on an HP computer equipped with an Intel

core i3 processor, 4GB RAM, and an Intel Haswell graphics card running Linux Ubuntu 18.04.1 LTS.[10]

3.2 Molecular docking studies

The proposed compounds were subjected to molecular docking investigations at the active sites of the EGFR and estrogenic receptors (PDB ids: 1M17, 3ERT)[11]. The target's crystal structure was retrieved from the protein data library and pre-processed according to normal techniques. The compound structures were drawn, and the energy was reduced. The receptor GRID was generated, and designed ligands were docked at the receptor GRID. RMSD (root mean square deviation) measurements were used to validate the docking5 process. The output findings were examined and recorded, including docking scores, ligand interactions, and 3D interactions [12].

3.2.1 MMGBSA studies

Using prime MM-GBSA, the free binding energy of ligands in protein ligand complexes is predicted. The binding energies of ligands can be calculated using partial charges of the input ligand, an implicit membrane model, or a force field. Protein flexibility can be explained by locating the protein flexibility region. Various sampling procedures, such as minimise, minimise side chains only, and minimise polar hydrogens only, can be used to explain the treatment of the defined flexible region.

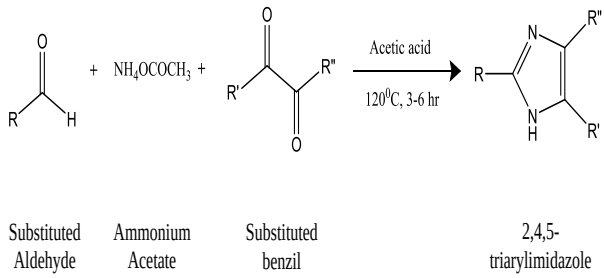
Prime MM-GBSA from the Prime module was used to calculate the ligand binding energies. The Glide XP docking output file, the VSGB solvation model, and the OPLS3e force field were used to calculate binding energy [13].

3.2.2 Induced fit docking

Induced fit docking is a flexible docking method that accounts for protein and ligand flexibility using the Glide and Prime modules. The docking panel Induced fit was used to dock the ligands. The generated ligands were docked onto the protein's binding site using a flexible protein and a flexible ligand. In order to achieve induced fit docking, standard sampling was used. The co-crystallized ligand served as the grid's centroid. The limitations, Glide docking, and prime refinement settings were all left alone. Glide XP docking was completed, as well as XP descriptions[14].

3.3 Synthesis of title compounds

1mmol of aldehyde, 1mmol of substituted benzil, 5mmol of ammonium acetate, and 10ml glacial acetic acid were added to a 50ml round bottom flask. The contents of the RBF were refluxed in an oil bath at 120^{o}C8 for 3-6 hours[15].TLC was used to monitor the completion of the reaction, with Hexane:Ethyl acetate (3:1) as the mobile phase. Following the completion of the reaction, the reaction liquid was allowed to cool to room temperature before being immersed in cold water. The resultant precipitate was vacuum-filtered and dried. With methanol as the solvent, the crude product was recrystallized. The proposed scheme for the synthesis of the title compounds is shown in Scheme 1.



Scheme 1: Reported synthesis of triphenyl imidazole derivatives

3.4 Characterization

The synthesized compounds were characterized by Mass spectrometry, Fourier Transform Infrared spectroscopy and Nuclear Magnetic Resonance spectroscopy.

3.5 Anticancer Assay

3.5.1 SRB assay

The cytotoxic effect of the synthesized compounds on breast cancer cells was determined by SRB assay using MCF7 cell lines obtained from NCCS, Pune.

Preparation of cell culture

DMEM, 10% FBS, 1% of 2 mM l-glutamine, 50 IU/ml penicillin and 50 g/ml streptomycin were used for culturing the cell lines.

3.5.2 Preparation of sample

The solution of the compounds was prepared in DMSO at a concentration of 50mg/ml. Different concentrations of the sample was prepared dilution with the medium. Doxorubicin was used as the standard and their appropriate dilutions were prepared.

3.5.3 Incubation of cells with treatment

MCF7 cells were seeded at a density of 5x103 cells/100µl of medium in each well of a 96-well plate. Compounds of different concentrations were applied in different wells. A well with 200µl of the cells without treatment was used as negative control. The cells were incubated at 37^{o}C in CO2 atmosphere for 48 hours.

3.5.4 Cell fixation and SRB staining

After 48hrs, 100 µl of 40% w/v TCA was added into each well for fixation of the cells and then incubated at 4 °C for 1 hour. Excess TCA was extracted from the wells and the wells were washed with distilled water. 100µl of 0.4% w/v solution of SRB dye in 1% acetic acid was introduced to each well for staining and incubated for 30 minutes at room temperature. SRB dye was removed from the wells and the wells were washed with 1% acetic acid solution. The plates were air dried. 200µl of 10 mM Tris base of pH 10.5 was incorporated into each well after drying. The plates were shaken on a plate shaker for 20 minutes[16].

3.5.5 Final absorbance and cell viability measurement

After proper mixing the absorbance of the cells were measured on ELISA reader at 570nm. The concentration of the compound which showed 50% cell growth inhibition was reported as the IC50 value.

4. RESULTS AND DISCUSSION

4.1 Molecular docking studies and MMGBSA free energy calculation

Molecular docking of 18 designed compounds was performed against two targets EGFR, ER[17].

4.1.1 Molecular docking against EGFR

The ligands were molecular docked against EGFR using PDB Id 1M17. The resolution of the protein was discovered to be 2.6. The docking approach was validated because the RMSD between the co-crystallized ligand and the docked minimized co-crystallized ligand was 1.6654, which should ideally be less than 2.

Table 3 displays the docking results against EGFR, including the docking score and binding energies. When docked against EGFR protein 1M17, ligands D18 and D9 have good docking scores of -5.964 and -5.961, respectively. Erlotinib, the standard medication, has a docking score of -8.813. Because of their minimum binding energies of -50.83, -50.49, and -48.75 Kcal/mol, compounds B9, D8, and D18 bind strongly to EGFR receptors[18].

Ligand	Docking score	MMGBSA binding energy (Kcal/mol)
D18	-5.964	-48.75
D9	-5.961	-44.50
D8	-5.827	-50.49
D11	-5.603	-46.54
C8	-5.569	-45.07
D15	-5.498	-45.38
D19	-5.412	-43.96
B18	-5.362	-42.42
B17	-5.301	-46.97
B11	-5.156	-43.65
B19	-5.130	-42.65
B8	-4.961	-38.56
C19	-4.680	-41.90
B15	-4.622	-34.65
A9	-4.321	-38.95
C9	-4.149	-39.51
C15	-3.995	-45.86
B9	-3.244	-50.83
Erlotinib	-8.813	-64.29

Table 3: Docking and MMGBSA results of designed ligands against EGFR

4.1.2 Molecular docking against ER

Molecular docking of the ligands was performed against ER with PDB Id 3ERT. The protein was found to have a resolution of 1.9 Å. The docking method was validated as the RMSD value between the co-crystallized ligand and the docked minimized co-crystallized ligand was found to be 1.493 Å, which should be ideally less than 2 Å. The molecular docking results against ER are shown in Table 5 where the docking score and binding energies are reported. Ligands A9, D8 and D19 show good docking score of -9.412, -8.556 and -7.028 respectively when docked against ER protein 3ERT. The standard drug tamoxifen showed a docking score of -11.934. Compounds A9, D15 and D18 show strong binding with ER receptors due to their minimum binding energies of -56.86, -56.64 and -50.94 Kcal/mol respectively.

4.1.3 Induced fit docking.

Induced fit docking of all the designed ligands was performed against the three target proteins EGFR, ER[19].

4.1.4 Induced fit docking against EGFR

The induced fit docking results of the ligands against EGFR are shown in Table 9. The IFD scores of the standard drug Erlotinib and designed

ligands were in a close range between -653 to -644. Hydrogen bonding interactions were seen in Erlotinib with MET 769 and CYS 773 amino acid residues. Similar hydrogen bonding interactions were observed in ligands B18, D19, B19, D18, B15 and C15 (Fig. 8). These ligands have the potential to act as EGFR inhibitors.

Ligand	IFD score	Interactions			
		Hydrogen Bond	Halogen bond	Pi-Pi stacking	Pi cation
Erlotinib	-652.48	MET 769, CYS 773			
C19	-649	THR 830, ASP831	LYS 721, LEU 764		
B18	-647.93	MET 769			
C9	-647.69	ASP 831		PHE 699	LYS 721
D19	-647.59	CYS 773, THR 830, ASP831	LYS 721, LEU 764, MET 769		
B9	-647.43	ASP 831		PHE 699	
B19	-647.37	MET 769	THR 830		
D18	-647.3	CYS 773	MET 769		
B11	-646.95	GLU 738	CYS 773		
B15	-646.59	LYS 704, MET 769			
C15	-646.23	LYS 704, MET 769			
D11	-646.19	LYS 704, PRO 770			
D15	-646.03	ASP 831	MET 769		
A9	-645.55	ASP 831			
D9	-645.51	ASP 831		PHE 699	LYS 721
B17	-645.51		MET769		LYS 721
C8	-645.11		ASP 831		
B8	-644.89		LYS 721	PHE 699	
D8	-644.16	TYR 830			

Table 9: Induced fit docking of ligands against EGFR

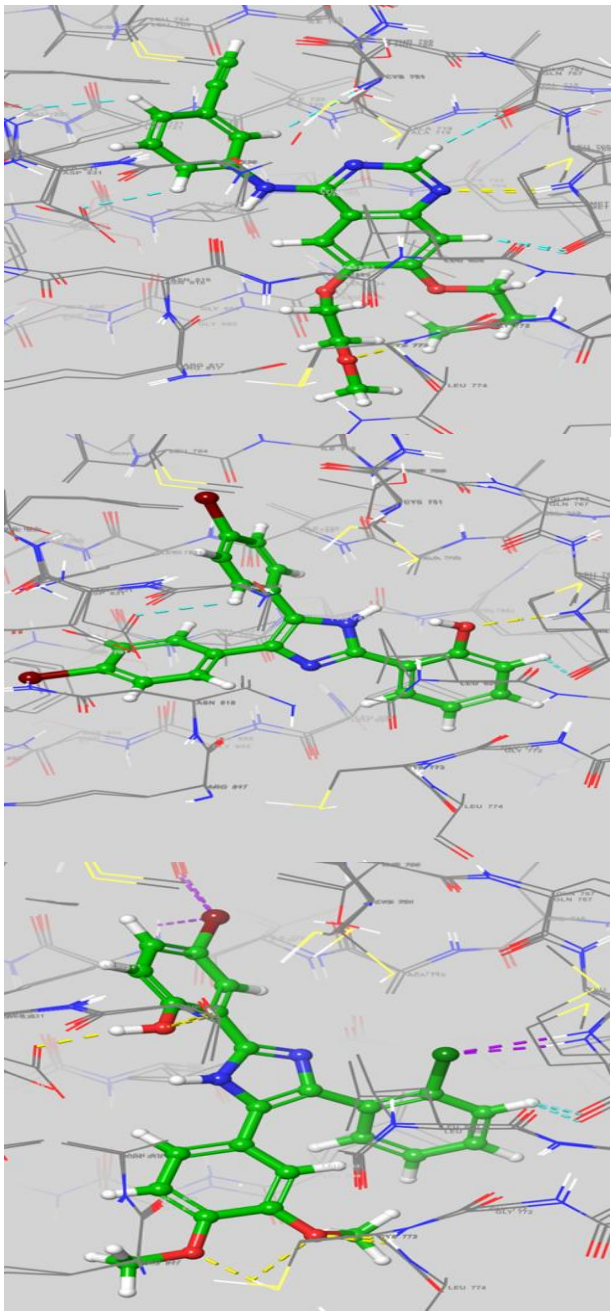


Fig. 8: 3D ligand interaction diagram of (a) Erlotinib (b) B18 (c) D19 with EGFR (PDB Id:1M17)

4.1.5 Induced fit docking against ER

The induced fit docking results of the ligands against ER are shown in Table 10. The IFD scores of the standard drug Tamoxifen and the designed ligands were in a close range between -520 to -515. The standard drug showed hydrogen bonding interaction and salt bridge formation with residue ASP 351 and pi-cation interaction with residue TRP 383. Similar interactions were observed in compounds C9, B19, D11, C19, D9 and D19 (Fig.

9). These compounds can be further developed as Estrogen receptor modulators.

Ligand	IFD score	Interactions		
		Hydrogen Bond	Salt bridge	Pi-Pi stacki
C9	-519.16	LEU 346	ASP 351	
B11	-519.06	GLU 353, ARG 394		
B19	-518.63	ASP 351		PHE 6
B8	-518.56			PHE 4
Tamoxifen	-518.56	ASP 351	ASP 351	
B18	-518.38	LEU 346		PHE 4
C8	-518.37			
B17	-517.87			
D11	-517.62	ASP 351		TRP 3
C19	-517.24	THR 347, ASP 351		TRP 3
B9	-517.24	LEU 346		TRP 3
D8	-516.85	LEU 346		PHE 4
D9	-516.79			TRP 3
D19	-516.6	ASP 351		
B15	-516.54		MET769	TRP 3
D18	-516.22	LEU 346, THR 347		
C15	-516.2			TRP 3
A9	-516.1	LEU 346		
D15	-515.59		GLU 353, ARG 394	

Table 10: Induced fit docking of ligands against ER

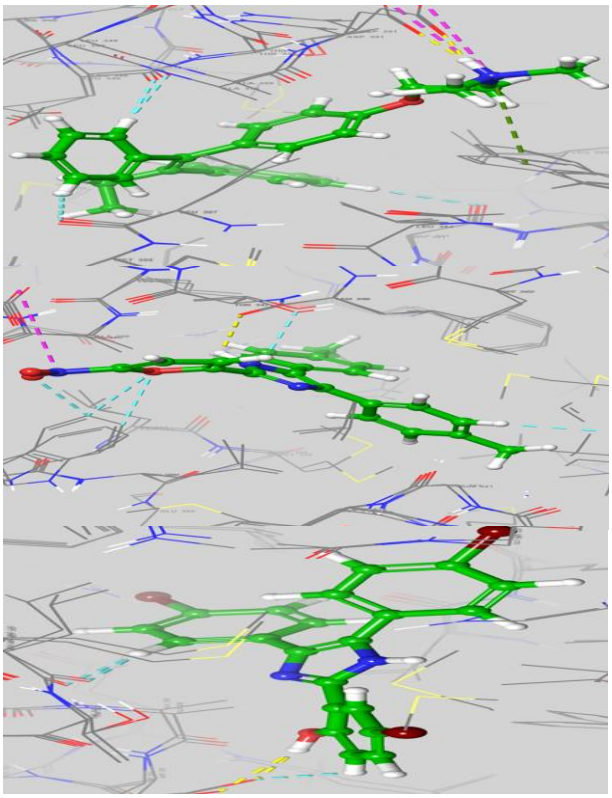


Fig. 9: 3D ligand interaction diagram of (a) Tamoxifen (b) C9 (c) B19 with ER (PDB Id:3ERT)

5 Synthesis

The designed compounds were synthesized, and their physicochemical properties were analyzed. A list of physicochemical properties is given in Table 4

Compound	Molecular formula	Molecular weight (g)	Percentage yield (%)	Rf value	Melting point (°C)
A9	$C_{19}H_{13}N_3O_3$	331.33	98.5	0.53	264-266
B8	$C_{21}H_{12}Br_2ClFN_2$	506.6	74.3	0.68	204-206
B9	$C_{19}H_{11}Br_2N_3O_3$	489.12	95.5	0.15	270-272
B11	$C_{21}H_{14}Br_2N_2O$	470.16	75.1	0.05	222-224
B15	$C_{21}H_{13}Br_2N_3O_2$	499.16	78.9	0.81	278-280
B17	$C_{21}H_{13}Br_2ClN_2$	488.61	82.4	0.68	208-210
B18	$C_{21}H_{14}Br_2N_2O$	470.16	85.5	0.67	238-240
B19	$C_{21}H_{13}Br_3N_2O$	549.06	86.9	0.83	216-218
C8	$C_{23}H_{18}ClFN_2$	376.86	71.3	0.56	118-120
C9	$C_{21}H_{17}N_3O_3$	359.39	94.8	0.11	126-128
C15	$C_{23}H_{19}N_3O_2$	369.42	52.1	0.64	296-298
C19	$C_{23}H_{19}BrN_2O$	419.32	73	0.78	170-172
D8	$C_{23}H_{17}Cl_2FN_2O_2$	443.3	38.8	0.08	88-90
D9	$C_{21}H_{16}ClN_3O_5$	425.83	80.8	0.05	112-114
D11	$C_{23}H_{19}ClN_2O_3$	406.87	60.3	0.07	104-106
D15	$C_{23}H_{18}ClN_3O_4$	435.86	83.9	0.43	238-240
D18	$C_{23}H_{19}ClN_2O_3$	406.87	43.3	0.18	198-200
D19	$C_{23}H_{18}BrClN_2O_3$	485.76	62.7	0.32	84-86

Table 4: Physicochemical properties of the synthesized compounds Characterization

5.1 Characterization

The synthesized test compounds were characterized by IR, MASS and NMR spectroscopic methods. The structure assignment was achieved with the help of both ^{1}H and ^{13}C

NMR techniques. Molecular weight was confirmed by LCMS mass spectroscopy. Important functional groups were assigned using IR spectroscopy. Fig 3 and 4 represent the mass and ^{1}H NMR of the representative compound. The characteristic peaks in ^{1}H NMR spectra were mainly in the aromatic region (7-8 ppm) and corresponding peaks in the aliphatic region from (2-4 ppm).

5.2 Anticancer activity

5.2.1 SRB assay

SRB assay was performed for 10 of the synthesized molecules against MCF7 cell line using Doxorubicin as the standard. The compounds tested were A9, B9, B18, C8, C9, C19, D9, D15, D18 and D19. The percentage inhibition of cells is shown in Table 17. Compounds A9 and D9 showed good activity with IC50 values of 8.343 and 11.61µg/ml respectively. Compounds C9 and B18 showed moderate activity with IC50 values of 16.08 and 16.51µg/ml respectively. The IC50 values of the tested compounds and the standard are shown in Table 18. Three compounds A9, D9 and C9 which showed good activity consist of 2-(5-nitrofuryl) substitution indicating that 5-nitrofuryl substitution improves the activity[20].

By comparing the IC50 values of the compounds in D series consisting of 4-(2-chlorophenyl) and 5-(3,4-dimethoxyphenyl) imidazole substitution, we can infer the effect of various substituents in position 2 of the imidazole ring. The compound with 5-nitrofuryl substitution, D9 showed good activity with IC50 value of 11.61 µg/ml, however, the activity reduced drastically when substituted with 3-nitrophenyl group in compound D15 giving an IC50 value of 129.8 µg/ml . Substitution with 2-hydroxyphenyl group in D18 showed gave an IC50 value of 84.15 µg/ml and it was slightly improved with the substitution of bromine at the 5th position of the phenyl ring reducing the IC50 value to 64.42 µg/ml. The IC50 value of the compounds of C series with 4-methylphenyl substitution at 4th and 5th position of the imidazole ring was comparable to that of the D series compounds. However, the compounds in B series showed drastic variations in the results as compared to compounds in series C and D.

Compound	Concentration in µg/ml						
	125	62.5	31.25	15.625	7.812	3.906	1.953
	Percentage inhibition in %						
A9	74.792	85.00	90.110	89.593	44.414	12.743	-26.877
B9	85.069	53.010	-25.325	-31.66	-73.155	-77.679	-12.786
B18	71.830	86.043	90.332	41.357	30.921	-49.872	-0.512
C8	52.432	53.137	18.822	34.186	9.859	-33.546	-6.466
C9	90.653	92.573	88.092	36.939	-51.216	-53.969	5.121
C19	81.818	53.137	7.5544	-12.42	-16.581	-25.416	-1.664
D9	90.175	90.498	89.206	76.731	21.081	-33.857	5.246
D15	31.099	-15.630	-5.418	2.725	5.763	-13.562	-2.509
D18	67.553	34.913	22.955	-11.752	-73.672	-96.940	-32.694
D19	56.242	45.448	42.604	42.152	25.412	4.858	7.055
Standard	Concentration in µM/ml						
	12.5	6.25	3.125	1.562	0.781	0.39	0.195
	Percentage inhibition in %						
Doxorubicin	89.372	86.875	80.537	62.35595	33.09859	31.37004	21.766

Table 17: Percentage inhibition of MCF7 cells by synthesized compound

6. CONCLUSION

A series of novel 2,4,5- triaryl imidazole derivatives were deigned, synthesized and characterized by various spectroscopic techniques. The designed compounds were further investigated by Insilco studies against the breast cancer targets EGFR and ER. The intended compounds were synthesized and characterized using MS, FTIR, and NMR. SRB assay was used to test the synthesized compounds' anticancer activity in vitro against MCF7 cell lines. Out of the 18 compounds synthesized, compounds A9 and D9 demonstrated significant antiproliferative effect with an IC_{50} values of 8.343µg/ml and 11.61µg/ml, respectively against standard doxorubicin. The derivatives showed preliminary anticancer efficacy, which might be investigated further in animal models in future investigations.

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