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IN-SILICO DESIGN, SYNTHESIS AND CHARACTERIZATION OF NOVEL COUMARIN DERIVATIVES AS EGFR INHIBITORS

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Keywords:

Coumarin, EGFR, MTT, docking

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ABSTRACT: In the search for novel anticancer drugs, coumarin derived chalcones were analysed using *in-silico* tools like chemdraw, chemsketch, molinspiration, CASTp, Argus lab etc. Epidermal growth factor receptor protein is involved in cell signaling pathways that control cell division and survival. Molecular docking studies of test compounds with the EGFR tyrosine kinase domain (PDB ID: 6LUD) protein provided the significant docking scores for each test compound (-8.38163 to -11.2691 kcal/mol). In this study, we synthesized and evaluated a novel anticancer compound using MTT assay which measures cell viability by detecting mitochondrial activity. Compound 3M was synthesized through condensation of 6 -acetyl-7-hydroxy-4-methylcoumarin with aromatic aldehyde. The purity was done by recrystallization and structure of compound 3M were confirmed via IR spectroscopy, which provided detailed information on functional groups and structural features. Results demonstrated that 3M exhibited significant cytotoxicity against MCF-7 cell line indicating its potential as a therapeutic agent with a significant inhibition of cell growth with an LC_{50} value of 68.1549µg/mL. The compound's efficacy was assessed using a series of *in-vitro* assays to determine its cytotoxicity and potential mechanism of action. These findings suggest that 3M is a promising candidate for further development in cancer treatment.

INTRODUCTION: Coumarin heterocyclic ring system is widely used in pharmaceutical industry to build various functional groups present in the drug molecules. Significant research has been shown to isolate and purify naturally present biological active coumarins from a range of plants, animals, and microbes and to artificially design and synthesize functionalized coumarin molecules from academic and industry as well with unique heterocyclic structures and characteristics.

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Coumarin is very significant in the treatment of prostate cancer, renal cell carcinoma and leukaemia. Coumarins are oxygenated heterocyclic polyphenolic compounds which can trigger cell cycle arrest, angiogenesis inhibition, kinase inhibition, telomerase inhibition and carbonic anhydrase inhibition on various cancer cells [1-3].

Epidermal growth factor receptor (EGFR) inhibitors are medicines that bind to certain parts of the EGFR and slow down or stop cell growth. EGFR is a protein that is found on the surface of some cells that causes cells to divide when epidermal growth factor binds to it. Physiological function of the epidermal growth factor receptor is to regulate epithelial tissue development and

homeostasis. EGFR is found at abnormally high levels in cancer cells and activation of this protein appears to be important in tumor growth and progression. Non-small-cell lung cancer has the highest prevalence of all types of lung cancer, which is the second most common cancer and the leading cause of cancer-related mortality in many countries. The need for more effective and less toxic treatment options for non-small-cell lung cancer has led to the development of agents targeting the epidermal growth factor receptor–mediated signalling pathway, such as egfr tyrosine kinase inhibitors (egfr-tkis) [4-7].

Although egfr-tkis are less toxic than traditional anti-neoplastic agents, they are commonly associated with acneiform-like rash and diarrhoea. The aim of present study is to develop newer EGFR inhibitors by *in-silico* design using various CADD softwares and further synthesize and characterize the compounds which show least binding energy in drug receptor interaction studies. Primary objective is to design a chemical compound based on the coumarin scaffold that exhibits high affinity and specificity towards the Epidermal Growth Factor Receptor, a crucial protein involved in cancer cell proliferation and survival. Adverse skin reactions occurred in up to 90% of cancer patients treated with EGFR inhibitors, including common skin toxicities such as hair changes and rare fatal skin toxicities (e.g., Stevens–Johnson syndrome).

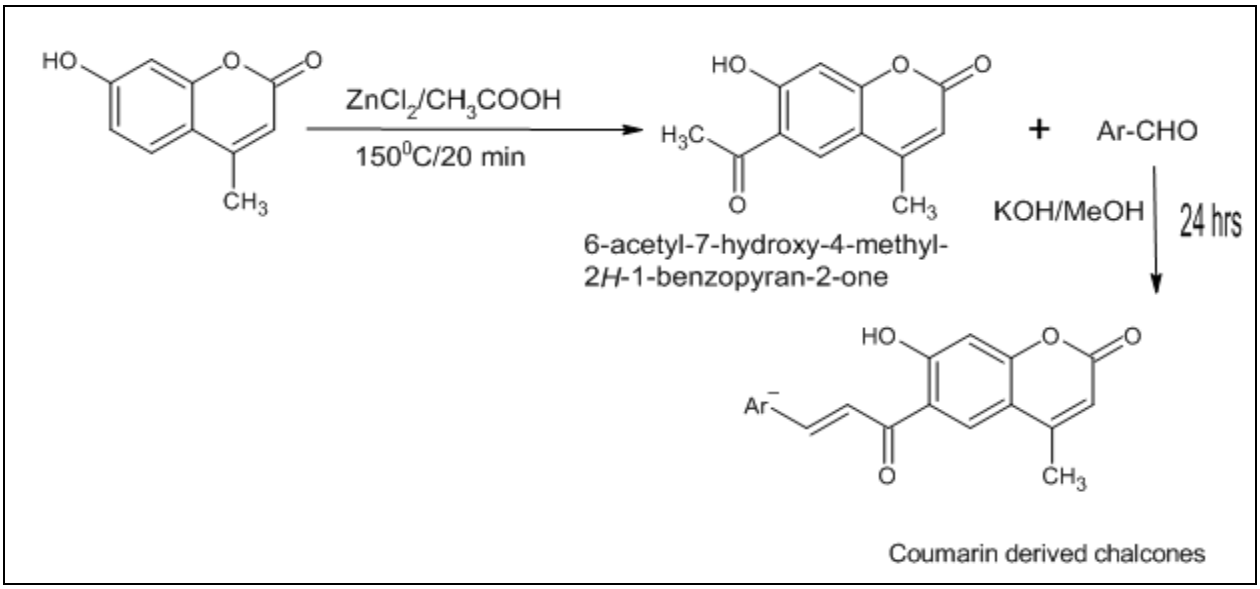
MATERIALS AND METHODS: All the computational procedure has been carried out with the help of windows 10. Software used are Chemsketch, Molinspiration, Chemdraw, PkCSM, CASTp, Argus lab and Molegro Molecular Viewer.

Synthesis:

Step 1: Synthesis of 6 -acetyl-7-hydroxy-4-Methylcoumarin: Freshly fused and powdered zinc chloride (13.629 g, 0.1 mole) is dissolved in glacial acetic acid (12 ml) by heating in beaker on a sand bath. Dry compound 7-hydroxy-4-methyl coumarin is added with stirring to the mixture at 140 °C to obtaincompound 6-acetyl-7-hydroxy-4-methyl coumarin which is then dried and recrystallized by ethanol [8].

Step 2: Synthesis of Novel Substituted Coumarin Derivative: To a mixture of 6-acetyl-7-hydroxy-4-methylcoumarin (1.0 mmol) in ethanol (10 ml) substituted aromatic aldehydes eg. Vanillin (1.0 mmol) and piperidine (1.0 mmol) were added, and then the mixture was refluxed for 24 h. The crude product was obtained by filtration and subsequently by recrystallization by ethanol to give the title compounds.

SCHEME:



Anticancer Assay by MTT Method: Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation

period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours.

After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm [9, 10].

The percentage of growth inhibition was calculated using the formula:

% of viability = Mean OD Samples x 100 / Mean OD of control group

RESULTS AND DISCUSSION: General structure of novel proposed compounds:

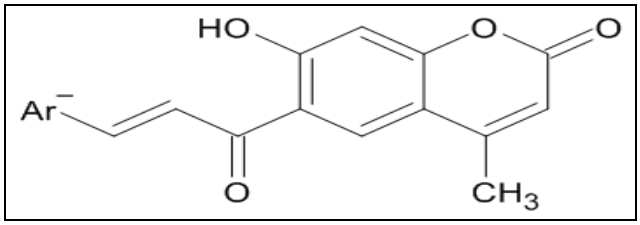


TABLE 1: LIST OF SUBSTITUENTS USE

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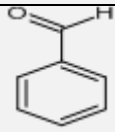
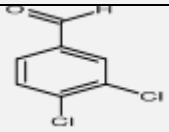
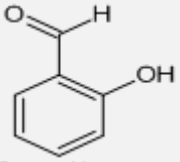
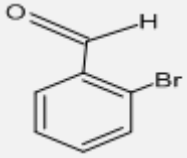
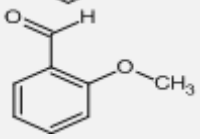
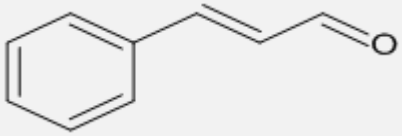
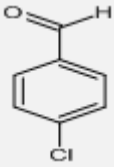
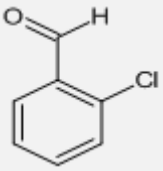
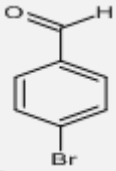
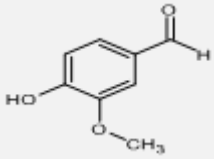
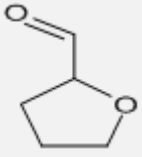
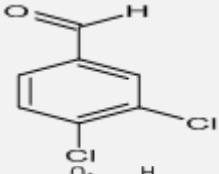
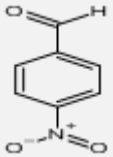
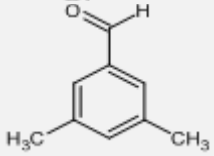
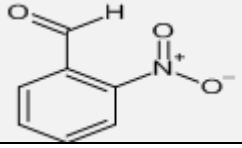
1	A		9	I	
2	B		10	J	
3	C		11	K	
4	D		12	L	
5	E		13	M	
6	F		14	N	
7	G		15	O	
8	H				

TABLE 2: LIPINSKI RULE ANALYSIS OF DESIGNED COMPOUNDS

Compound code	Log P	Molecular weight	Hydrogen Bond Donors	Hydrogen Bond Acceptors	No: of Violations
A	4.16	306.32	1	4	0
B	4.00	350.33	2	4	0
C	4.07	320.34	0	4	0
D	4.37	368.77	1	5	0
E	4.97	385.21	1	4	0
F	2.92	296.28	1	5	0
G	3.28	365.31	1	4	0
H	3.51	289.31	1	3	0
I	5.44	375.21	1	4	1
J	2.81	350.37	2	5	0
K	3.72	334.33	1	5	0
L	4.61	340.76	1	4	0
M	4.74	385.21	1	4	0
N	2.16	304.30	1	4	0
O	4.98	334.37	1	4	0

We have predicted the drug likeliness profile of the compounds through analysis of pharmacokinetics properties of the compound by using mol inspiration online property toolkit. Based on the results obtained from mol inspiration it was observed that all of the proposed compounds except compound I obeyed Lipinski rule of five.

According to Lipinski's rule of five new molecule designed for oral route should have Log P value &<5 molecular weight, <500 dalton < 5 hydrogen bond donor, < 10 hydrogen bond acceptor and should not show any violations [11]. The results are presented in **Table 3.**

TABLE 3: ADME PREDICTIONS BY USING PkCSM SOFTWARE

S. no.	CPD Code	Intestinal Absorption (% Absorbed)	CACO2 Permeability (Log PAPP)	VDss Distribution (Log l/kg)	Fraction Unbound (FU)	Clearance (Log ml/ min/ kg)
1	A	96.129	1.033	-0.237	0.022	0.784
2	B	96.538	1.065	0.018	0.056	0.798
3	C	96.932	1.349	-0.173	0.067	0.944
4	D	95.117	1.092	-0.243	0	-0.051
5	E	95.112	1.042	-0.139	0.027	-0.176
6	F	95.258	1.137	0.1	0.372	0.812
7	G	94.881	0.348	-0.263	0.009	0.754
8	H	94.425	0.264	-0.255	0	0.699
9	I	86.658	0.576	0.011	0.381	-45.457
10	J	91.358	1.004	0.011	0.399	-10.25
11	K	95.976	1.298	0.058	0.219	0.882
12	L	95.771	1.036	-0.068	0.03	-0.146
13	M	95.704	1.032	-0.051	0.028	-0.168
14	N	91.165	-0.113	-0.57	0.	0.717
15	O	95.203	1.104	0.186	0.038	0.801

ADME studies are designed to investigate how a chemical is processed by living organism.

ADME parameter of proposed compounds (A-O) are calculated with the help of pkCSM software. Results shows that most of the derivatives exhibit good ADME properties. **Table 4** presents predicted ADME properties of the compounds. The Caco-2 cell line is composed of human epithelial colorectal adrenocarcinoma cells and is widely used as an *in-vitro* model of the human intestinal mucosa to predict absorption of orally administered drug.

The steady state volume of distribution (VDss) is the theoretical volume that the total dose of a drug would need to be uniformly distributed to give the same concentration as in blood plasma.

The total body clearance and unbound fraction of the drug is also calculated. Predicted value of these parameter for the proposed compound exhibit within the limits.

Thus, it can be suggested that the designed compound may possess a good pharmacokinetics profile, increasing their pharmacological importance.

TABLE 4: TOXICITY PREDICTION OF COMPOUNDS

S. no.	Compound code	Carcinogenicity	Mutagen city
1	A	-VE	-VE
2	B	+VE	-VE
3	C	-VE	-VE
4	D	+VE	+VE
5	E	-VE	-VE
6	F	-VE	-VE
7	G	-VE	-VE
8	H	-VE	-VE
9	I	-VE	-VE
10	J	-VE	-VE
11	K	-VE	-VE
12	L	+VE	+VE
13	M	-VE	-VE
14	N	-VE	-VE
15	O	+VE	+VE

PreADMET is a web based application for predicting toxicity data also and building drug-like library using *in-silico* method. The application of *In-silico* methods increasing with the prediction of toxic risk to human and enivornment.

The mutagenic and carcinogenic effect of the designed compound on human body were predicted by using preADMET software and result showed that all of the compound shows carcinogenicity and mutagenicity.

TABLE 5: BINDING ENERGY OF DESIGNED ANALOGUES

S. no	Compound Code	Binding Energy (K. Cal/Mol)	S. no.	Compound Code	Binding Energy (K. Cal/Mol)
1	A	-10.6895	10	J	-8.93419
2	B	-10.2372	11	K	-10.6702
3	C	-10.3145	12	L	-11.1031
4	D	-10.5049	13	M	-11.2691
5	E	-10.5874	14	N	-9.18639
6	F	-8.38163	15	O	-10.8362
7	G	-9.41893	16	Aesculetin	-6.67487
8	H	-10.9098	17	Gefitinib	-7.63593
9	I	-10.8838			

Binding energy is defined as amount of energy required to separate a particle from a system of particles or to disperse all the particles of the system. Gefitinib is the well known epidermal growth factor receptor (EGFR). EGFR has prove to be an important target of anticancer drugs. In addition to it use for the treatment of other non malignant disease. Based on docking score 3M was found to possess least binding energy and it was synthesized by wet lab method.

DOCKING:

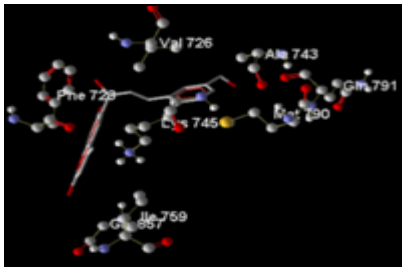


FIG. 1: COMPOUND A BOND LENGTH: 3.1256Å

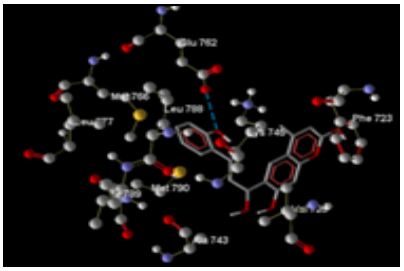


FIG. 2: COMPOUND B BOND LENGTH: 3.6578Å

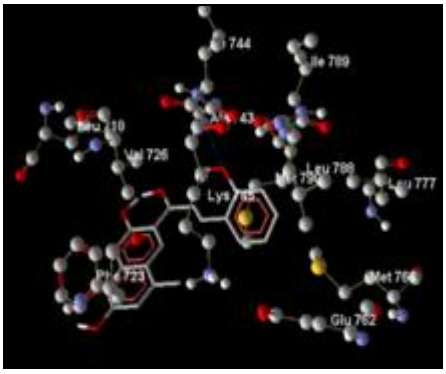


FIG. 3: COMPOUND C
BOND LENGTH: 2.7299Å

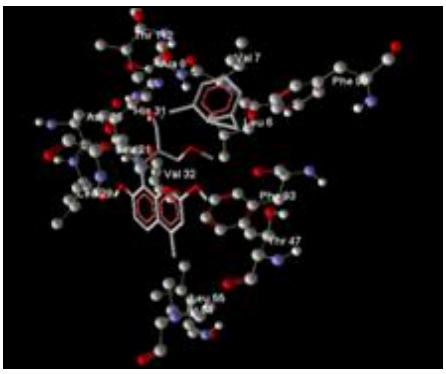


FIG. 4: COMPOUND D
BOND LENGTH: 3.4587Å

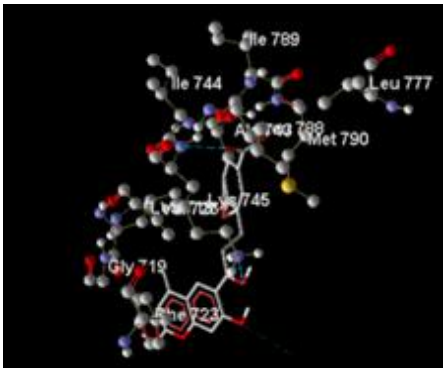


FIG. 5: COMPOUND E
BOND LENGTH: 3.3225 Å

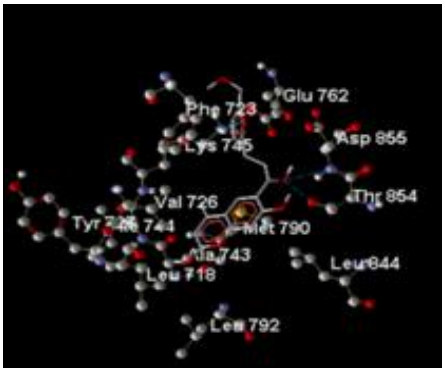


FIG. 6: COMPOUND F
BOND LENGTH: 2.4532 Å

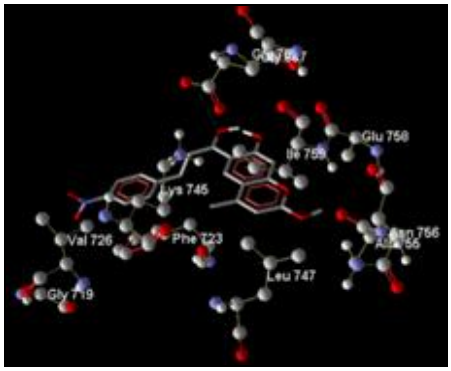


FIG. 7: COMPOUND G
BOND LENGTH: 3.001 Å

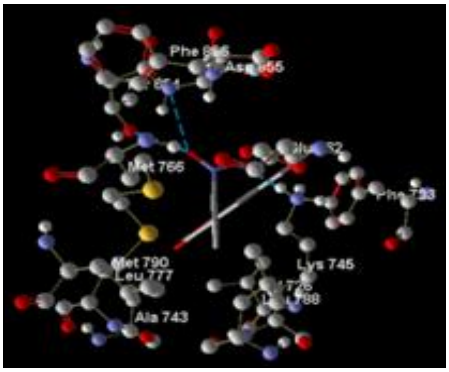


FIG. 8: COMPOUND H
BOND LENGTH: 3.4673 Å

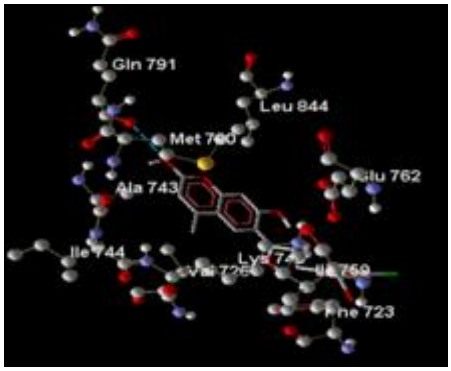


FIG. 9: COMPOUND: I
BOND LENGTH: 3.0563 Å

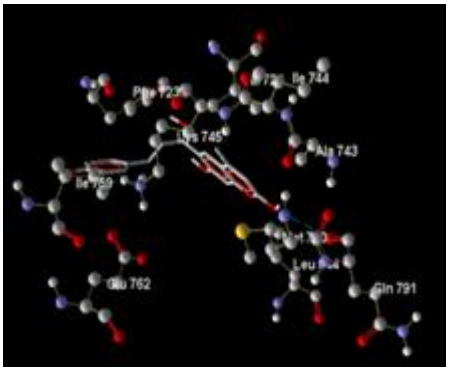


FIG. 10: COMPOUND: J
BOND LENGTH: 2.4590 Å

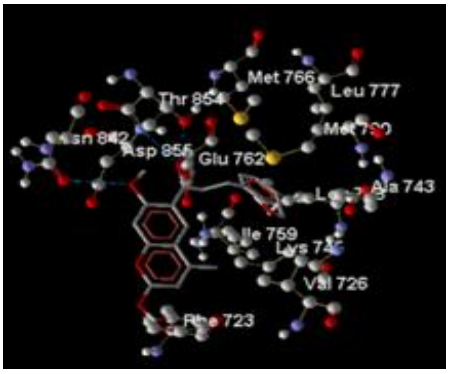


FIG. 11: COMPOUND: K
BOND LENGTH: 3.8521 Å

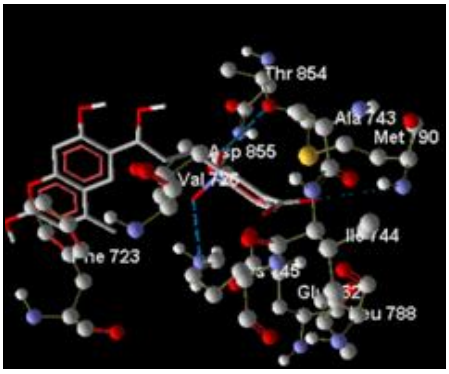


FIG. 12: COMPOUND: L
BOND LENGTH: 3.4132 Å

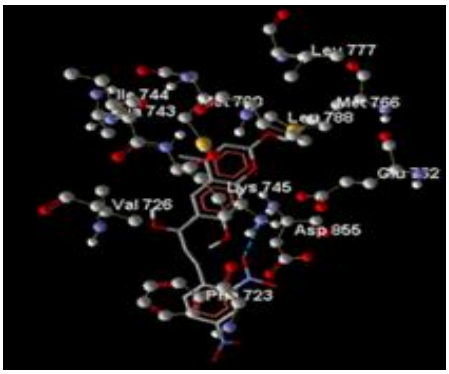


FIG. 13: COMPOUND: M
BOND LENGTH: 2.8711 Å

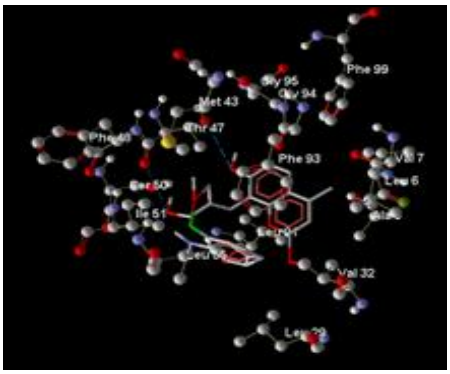


FIG. 14: COMPOUND: N
BOND LENGTH: 3.2465 Å

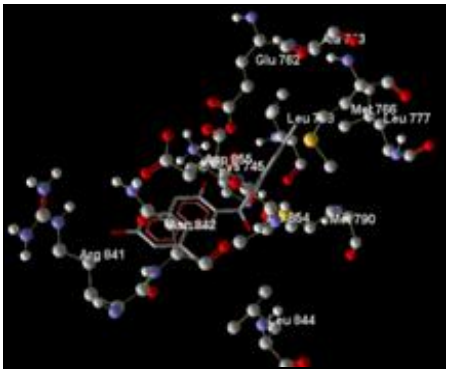


FIG. 15: COMPOUND: O
BOND LENGTH: 3.3410 Å

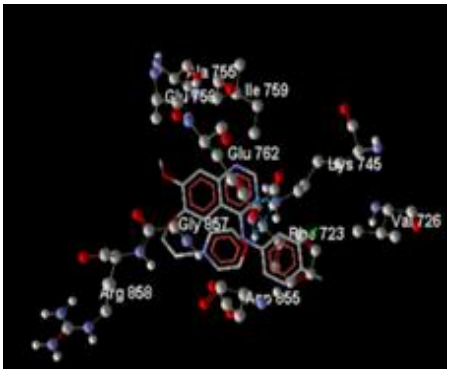


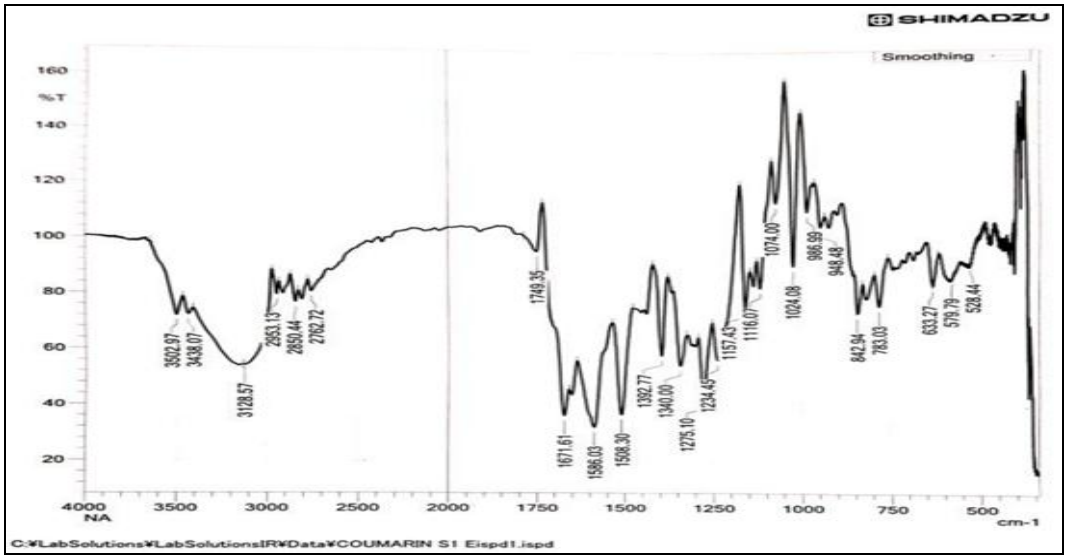
FIG. 16: GEFITINIB
BOND LENGTH: 2.9973 Å

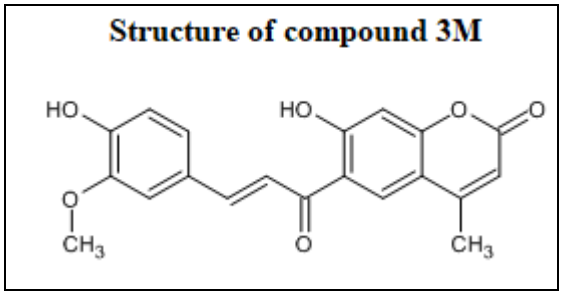
TABLE 6: PHYSICO-CHEMICAL PROPERTIES OF SYNTHESIZED COMPOUND

CPD Code	Colour and apperance	Melting point in ^{o}C
1M	White to Beige	191
2M	Pale brown	69
3M	Crimson red	72

Coumarin chalcone was synthesised by acetylation of 7-hydroxy-4-methyl coumarin and further treatment with aromatic aldehyde in piperidine. Thin layer chromatography was performed using precoated aluminium plates coated with silica gel. Glacial acetic acid: Chloroform: Water in the ratio of 7:6:1 was used as the mobile phase. The spots were visualized in the iodine chamber. Reaction proceeded with good yield.

IR Spectra of Compound 3M: IR spectra was recorded on SHIMADZU FTIR spectrometer using potassium bromide pellets. A Peak at 3502 cm^{-1} indicates (N-H streching), 3128 (C-H streching), 2953 (C-H streching), 2762 (O-H streching), 1749 (C=O streching) and 1508 (N-O streching). The peaks obtained were consistent with the assigned structure.





MTT Assay:

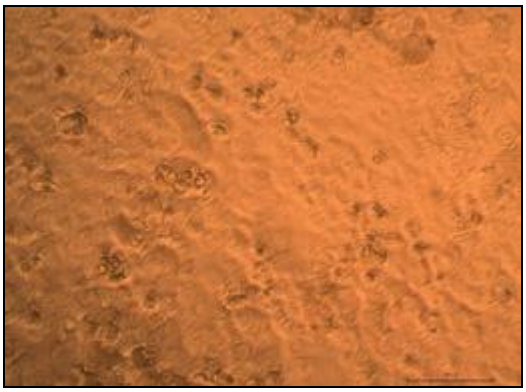


FIG. 17: CONTROL

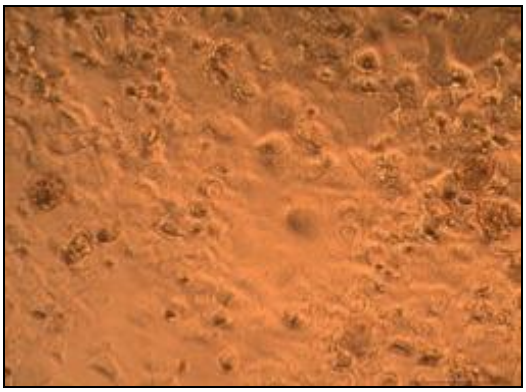


FIG. 18: AT 6.25 µg/ml CONCENTRATION

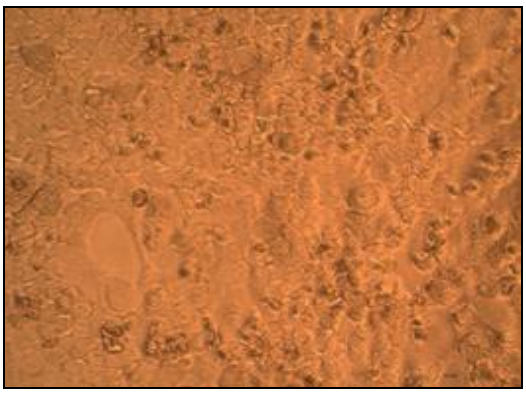


FIG. 19: 12.5 µg/ml

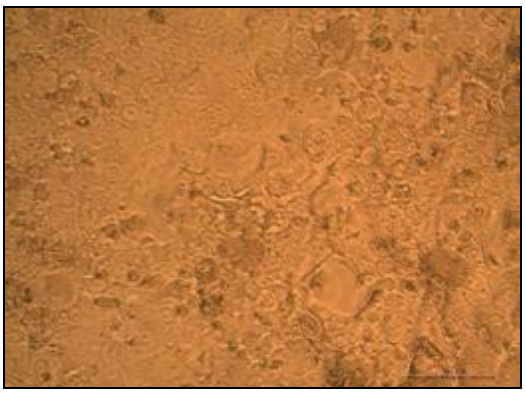


FIG. 20: 25 µg/ml

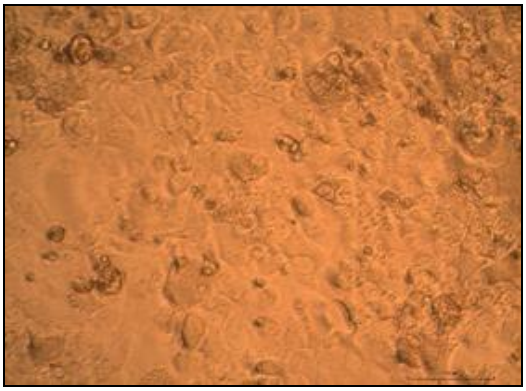


FIG. 21: 50µg/ml

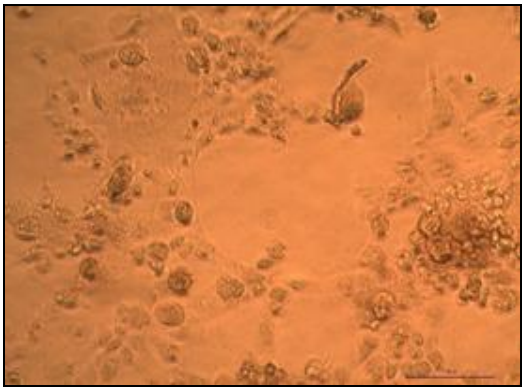


FIG. 22: 100 µg/ml

TABLE 7: PERCENTAGE VIABILITY OF 3M AT VARIOUS CONCENTRACTIONS

Sample Concentration (µg/ml)	OD I	OD II	OD III	Average Absorbance @ 540nm	Percentage Viability
control	0.7458	0.7642	0.7721	0.7607	100.00
6.25	0.7352	0.7215	0.7582	0.7383	97.06
12.5	0.6843	0.6725	0.6652	0.6740	88.60
25	0.6398	0.621	0.6249	0.6286	82.63
50	0.4779	0.4521	0.4432	0.4577	60.17
100	0.2224	0.2058	0.2143	0.2142	28.15

LC_{50} Value of SAMPLE: 68.1549 µg/mL (Calculated using ED50 PLUS V1.0 Software).

TABLE 8: STATISTICAL ANALYSIS

Cell line -MCF-7									
Sample Code: M									
	ODI	ODII	ODIII	% viability 1	% viability 2	% viability 3	Avg	Stdev	Std error
Control	0.7458	0.7642	0.7721	100	100	100	100	0	0
6.25	0.7352	0.7215	0.7582	98.5787	94.4125	98.1997	97.063	2.303	1.330
12.5	0.6843	0.6725	0.6652	91.7538	88.0005	86.1546	88.636	2.853	1.647
25	0.6398	0.621	0.6249	85.7871	81.2614	80.9351	82.661	2.711	1.565
50	0.4779	0.4521	0.4432	64.0788	59.1599	57.4019	60.213	3.460	1.998
100	0.2224	0.2058	0.2143	29.8203	26.9301	27.7555	28.168	1.488	0.859

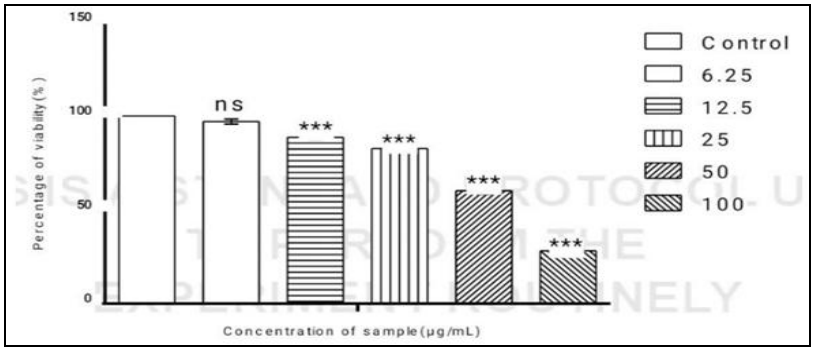


FIG. 23: GRAPHICAL REPRESENTATION OF INHIBITORY ACTIVITY

Graphical representation depicting the anticancer effect of Sample on MCF-7 cell line by MTT assay. Along Y axis Percentage viability and along X axis various concentration of sample were plotted. All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and Dunnets test were performed to analyse data. ***p < 0.001 compared to control groups.

CONCLUSION: Molecular docking was studied to predict the intramolecular interactions of compounds (A-O) with epidermal growth receptor factor (PDB ID:6LUD). The docking stimulation shows that the binding affinity of the coumarin derivative M (-11.2691kcal/mol) was better than that of the std gefitinib (-7.63593kcal/mol). These result are consistent with the *in-vitro* assay

findings. In order to study the drug likeness of the newly designed compounds, *in-silico* Lipinski`s Rule of Five (RO5) and ADME parameters were conducted using molinspiration and pkCSM software. About 90% of orally active compounds satisfy RO5.Among the compound which showed least binding energy M was synthesized by wet lab method as chalcone derivative by condensation of coumarin with aldehyde derivatives. The compound, 3M was synthesized through condensation of 6 – acetyl – 7 – hydroxyl – 4 – methylcoumarin with aromatic aldehyde. The purity was done by recrystallization and structure of 3M were confirmed *via* IR spectroscopy, which provided detailed information on functional groups and structural features. The anticancer potential of the compound was assessed using the MTT assay, which measures cell viability by detecting mitochondrial activity. Results demonstrated that 3M exhibited significant cytotoxicity against MCF-7 cell line indicating its potential as a therapeutic agent. To check the anticancer activity MTT Assay method was adopted using gefitinib as standard drug and LC_{50} Value of sample was found to be 68.1549 µg/mL calculated using ED50 PLUS V1.0 software. The compound's efficacy was assessed using a series of *in-vitro* assays to determine its cytotoxicity and potential mechanism of action. These findings suggest that 3M is a promising candidate for further development in cancer treatment. Using *in-vitro* assays, including MTT we assessed the compound's efficacy against MCF-7 cell line, demonstrating significant cytotoxicity and inhibition of cell proliferation. Infrared (IR) spectroscopy confirmed the structural integrity of 3M, while MTT assays provided insights into its potent anticancer effects.

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CONFLICTS OF INTEREST: Nil

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