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IN-SILICO DESIGN AND DIHYDROFOLATE REDUCTASE INHIBITORY ACTIVITY OF NOVEL COUMARIN BUTANAMIDE DERIVATIVES

Shiny George*, Pooja S. Panicker, Jini Anna Thomas, Anit Tomy, Shilpa Shivan, Sruthy O. D., Prasanth A. R., A. J. Chacko

Department of Pharmaceutical Chemistry, Hindustan College of Pharmacy, Kanjirapally, Kerala, India.

***Corresponding Author: Dr. Shiny George**
Department of Pharmaceutical Chemistry, Hindustan College of Pharmacy, Kanjirapally, Kerala, India.

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ABSTRACT

Molecular docking is one of the best data-based screening methodology of virtual screening for ligand which minimized the work cost by filtering and helps to predict the toxicity study for designing the formulation or synthesis of New Chemical Entity in now a day of pharmaceutical research developments. Heterocyclic compounds are widely distributed in nature and they were found to possess various physiological activities. Coumarin and related fused heterocycles are of interest as potential bioactive molecules. The present work has focused on incorporation of coumarin and its related derivatives and evaluates dihydrofolate reductase inhibitory activity. A new series of coumarin derivatives were designed as DHFR inhibitors based on Lipinski rule evaluation and docking. *Insilico* molecular docking was carried out using Argus Lab. To identify the potential anti-bacterial agent lead compounds among compounds 3A-3Y docking calculations were performed in to the 3D structure of the catalytic site of DHFR enzyme (PDB CODE:3SRQ). Docking score of the novel compound showed good fit against DHFR when compared with standard inhibitor Trimethoprim. *Invitro* antibacterial activity of synthesized compound against *S. Aureus* by agar well diffusion method shows good activity when compared to standard drug.

KEYWORDS: Coumarin, DHFR, Antibacterial, Docking.

INTRODUCTION

Increasing incidences of microbial infection by the development of microbial resistance of most antibiotics through either genome of microbial mutations or an evolved mechanism of resistance of action is a major health problem. In order to combat medication resistance on clinically important infections, it is necessary to find novel compounds with antibacterial activity that may function through mechanisms of action that are different from those of well-known classes of antimicrobial drugs. The demands of the current health scenario witnessing morbidity and mortality due to microbial infections and multidrug-resistant bacterial strains. Coumarin (2*H*-1-benzopyran-2-one) derivatives are a large class of highly important lactones containing a fused structure of benzene ring and α-pyrone. In pursuit of searching new antimicrobial agents, coumarin linked to butanamide derivatives have been developed and evaluated for their antimicrobial properties against Gram positive bacteria. Natural and synthetic coumarins demonstrated a broad range of therapeutic applications including antimicrobial,[1] anti-HIV,[2] antioxidant,[3] anticoagulant,[4] anti-infammatory[5] anticonvulsant,[6] anticancer[7] and antiviral[8-10] activity. In the past few years, attempts have been reported towards the optimization, synthesis, and evaluation of novel coumarin analogues as antimicrobial agents.

Dihydrofolate reductase has attracted a lot of attention as a molecular target for bacterial resistance over several decades resulting in a number of useful agents. Dihydrofolate reductase, is an enzyme that catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH) - dependant reduction of dihydrofolate to tetrahydrofolate in microbial and eukaryotes cells. The crucial role of DHFR is related to biosynthesis pathways of the thymidylate and purines, as well as other amino acids.[11] Trimethoprim is the well- known dihydrofolate reductase inhibitor and one of the standard antibiotics used in urinary tract infections. The pharmacokinetic profile of a compound defines its absorption, distribution, metabolism, and excretion (ADME) properties. While optimal binding properties of a new drug to the therapeutic target are crucial, ensuring that it can reach the target site in sufficient concentrations to produce the physiological effect safely is essential for the introduction into the clinic. pkCSM provides a platform for the analysis and optimization of pharmacokinetic and toxicity properties implemented in a user-friendly, freely available web interface (http://structure.bioc.cam.ac.uk/pkcsm), a valuable tool to help medicinal chemists to find the balance between potency, safety, and pharmacokinetic properties. In present study a series of coumarin butanamide derivatives were developed as DHFR inhibitors based on various *insilico* tools for docking and ADMET. Further synthesized compound was confirmed by IR spectra and enzyme inhibitory activity was checked by *invitro* method.

MATERIALS AND METHODS

Preparation of ligand files

Ligand files for the molecular docking studies were prepared in Chem Draw Ultra software, Cambridge Soft Corporation, USA. Version-12.0, 1997-2010. It is a Chem Tech tool used for the drawing of ligand molecules. Compound structures were drawn in ChemDraw software and converted to mol format (.mol file) with the standard settings and further used for docking studies.

Chemical structures and SMILES notations of the compounds were also obtained by using ACD labs Chemsketch version 12.0 (www.acdlabs.com/resources/freeware/chemsketch/).

Drug likeness assessment

In silico Lipinski Rule of Five (RO5)) analysis was conducted using Molinspiration (accessed 24 February 2018), an online cheminformatics software that provides web-based interactive calculation of molecular properties, including molecular weight, hydrogen bond donors and acceptors, and calculated partition coefficients of the molecules.

Selection of target protein

The 3D crystal structure of DHFR receptor[11] (entry code: 3SRQ) used for docking was recovered from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/home). Reference compound such as trimethoprim was directly downloaded from DrugBank 2.5 database and it is potential competitive inhibitor against target protein.

Active site prediction

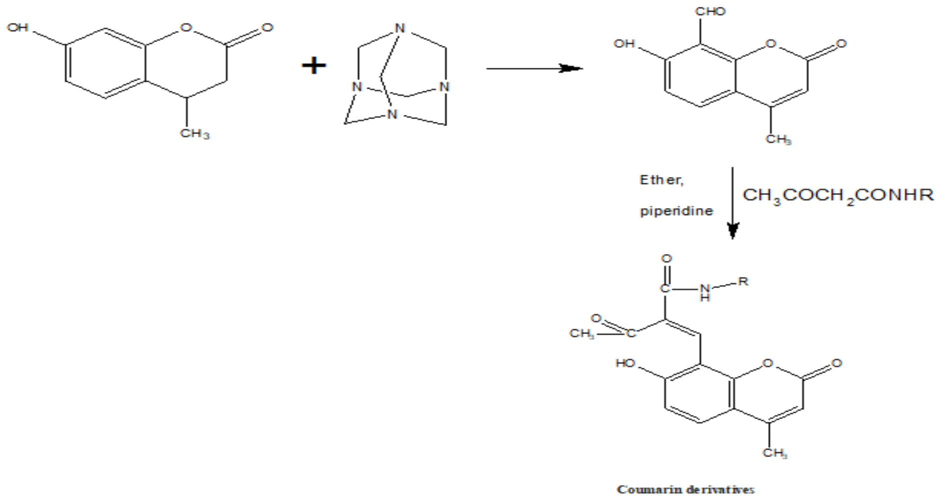
A prediction of active site and ligand binding sites of 3D protein structure was done by using Computed Atlas of Surface Topography of proteins (CASTp) which is a web server that provides online services for locating, delineating and measuring geometric and topological properties of protein structures.

Docking study

Molecular docking studies performed with target DHFR receptor and novel compounds were carried out by using Arguslab and viewed in Molegro Molecular Viewer asper previous literature.

Chemistry

Chemicals used were purchased Isochem Laboratories. All the chemicals were used without further purification. Melting points were determined in open capillary tube and were uncorrected. Purity of the synthesized compounds was routinely checked by TLC on silica gel G with solvent system – chloroform and ethanol (8:2) using iodine vapour for detection. The infrared (IR) spectra (expressed in wave number υ [cm^{-1}]) were recorded by the SHIMADZU FT/IR Spectrometer (Shimadzu Europa GmbH, Duisburg, Germany) using potassium bromide (KBr) discs at Ezhuthachan College of Pharmacy, Trivandrum.



Scheme for synthesis of coumarin derivative

a) Synthesis of 4-methyl-7-hyroxy-8-formyl coumarin (2)

7-hydroxy-4-methyl coumarin (0.001 mol) was dissolved in glacial acetic acid (20 ml) and hexamine (0.003 mol) was added to the reaction mixture. Heated to 80-85 ° C in a water bath for 6 hr. A hot mixture of 5ml water and 30ml dil. Hydrochloric acid was added, kept for 30 min, and cooled to room temperature. It was extracted with 20ml of diethyl ether and on evaporation of ether, a pale-yellow solid was obtained.

b) Synthesis of N, N-di substituted acetamide derivatives (2E)

Diphenylamine (0.001 mol) was dissolved in 10 ml ethanol, 0.0012 moles of ethyl acetoacetate was added and stirred for 5 hr at reflux. Cooled the mass to 0-5 ° C and filtered, which were used directly in the next step. Yield was varied from 60-70%.

c) Synthesis of novel coumarin derivatives by condensation of formyl Derivatives and Substituted acetamide (3E)

8-formyl-4-methyl-7- hydroxy coumarin (0.001 mol) was dissolved in 10ml ethanol containing N, N di substituted acetamide derivatives (0.001 mol) and catalytic amount of piperidine. Refluxed for 2 hr, cooled to 0-5 ° C and filtered the solid. Pale yellow to orange red color solid were obtained. Yields varied from 50-60%.

Agar well diffusion method

Antimicrobial tests were conducted via the agar well diffusion method[3] using 100 µL of suspension containing 1 × 10^8 CFU/mL pathological tested *S. Aureus*. After the media cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 µL of the tested compound solution prepared by dissolving 10 mg of the chemical compound in 1 mL dimethyl sulfoxide (DMSO). The inoculated plates were then incubated for 24 h at 37 °C for bacteria. DMSO served as negative control. Trimethoprim (200 mg/mL)) was used as standards for antibacterial activity. After incubation, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms and compared with that of the standard. Antimicrobial activities were expressed as the diameter of the inhibition zone (IZ) in mm. The experiment was carried out in triplicate and the average zone of inhibition was calculated.

RESULTS AND DISCUSSION

General structure of novel proposed compound

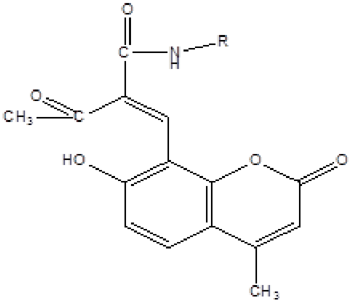


Table 1: List of substituents

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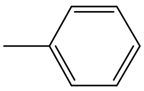
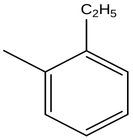
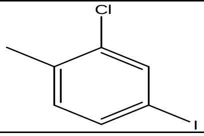
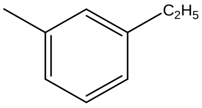
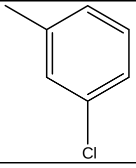
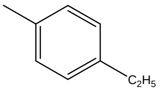
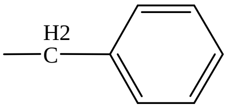
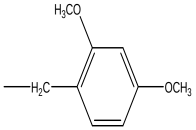
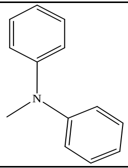
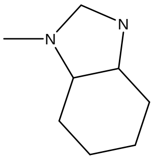
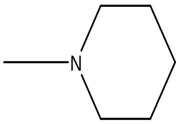
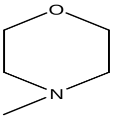
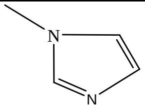
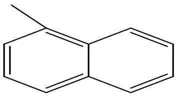
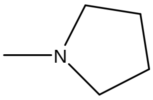
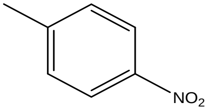
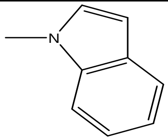
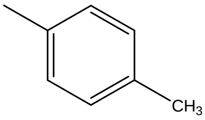
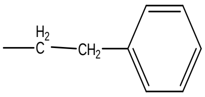
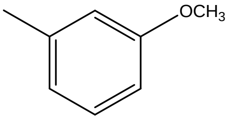
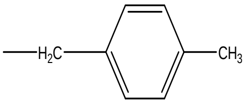
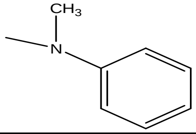
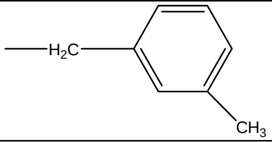
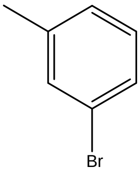
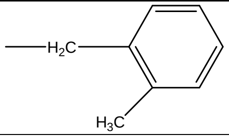
1	3A		14	3N	
2	3B		15	3O	
3	3C		16	3P	
4	3D		17	3Q	
5	3E		18	3R	

6	3F		19	3S	
7	3G		20	3T	
8	3H		21	3U	
9	3I		22	3V	
10	3J		23	3W	
11	3K		24	3X	
12	3L		25	3Y	
13	3M				

We have predicted the drug likeliness profile of the compounds through analysis of pharmacokinetic properties of the compounds by using molinspiration online property toolkit. Based on the results obtained from molinspiration it was observed that all of the proposed compounds obeyed Lipinski rule of five. According to the Lipinski's rule of five new molecule designed for oral route should have Log P value <5, Molecular weight <500 dalton, hydrogen bond donor <5, hydrogen bond acceptor <10. None of the proposed derivatives show violation of rule. The results are presented in Table 2.

Table 2: Lipinski rule analysis

Compound code	Log P	Molecular weight	Hydrogen Bond Donors	Hydrogen Bond Acceptor	No. of violations
3A	3.12	363.37	2	6	0
3B	5.06	439.47	1	6	1
3C	3.77	397.81	2	6	0
3D	2.82	377.40	2	6	0
3E	4.80	523.71	2	6	1
3F	2.57	355.39	1	6	0
3G	1.34	339.31	1	8	0
3H	2.07	341.36	1	6	0
3I	4.11	387.39	1	6	0
3J	3.22	391.42	2	6	0
3K	3.27	391.42	2	6	0
3L	3.24	391.42	2	6	0
3M	3.22	391.42	2	6	0
3N	3.98	391.42	2	6	0
3O	4.01	391.42	2	6	0

3P	4.03	391.42	2	6	0
3Q	2.86	437.45	2	8	0
3R	2.69	396.44	2	7	0
3S	1.51	357.36	1	7	0
3T	4.30	413.43	2	6	0
3U	3.08	408.37	2	9	0
3V	3.56	377.40	2	6	0
3W	3.15	393.39	2	7	0
3X	3.36	377.40	1	6	0
3Y	3.90	442.26	2	6	0

Table 3: ADME predictions by 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	3A	85.549	1.004	0.011	0.381	-48.069
2	3B	98.287	0.934	-0.892	0.229	0.401
3	3C	90.729	0.944	0.206	0.02	-0.287
4	3D	86.078	0.93	-0.036	0.028	0.657
5	3E	90.13	1.208	0.226	0.003	-0.47
6	3F	96.404	0.951	0.359	0.199	0.759
7	3G	83.155	0.935	-0.195	0.168	0.846
8	3H	96.793	0.942	0.318	0.214	0.762
9	3I	96.921	1.046	0.003	0.123	0.785
10	3J	85.236	0.913	-0.027	0.021	0.677
11	3K	87.752	1.237	0.255	0.024	0.644
12	3L	87.845	1.253	0.21	0.023	0.651
13	3M	87.943	1.324	0.185	0.016	0.655
14	3N	90.099	1.127	0.265	0.008	0.512
15	3O	83.407	0.581	0.011	0.381	-49.697
16	3P	90.009	1.0	0.308	0.017	0.498
17	3Q	80.378	1.467	0.141	0.056	1.019
18	3R	82.964	1.038	1.309	0.336	0.841
19	3S	86.643	0.262	0.032	0.225	0.776
20	3T	95.802	0.877	-0.479	0.115	0.456
21	3U	84.722	1.031	0.011	0.383	-31.94
22	3V	89.922	1	0.265	0.03	0.466
23	3W	91.293	0.954	0.146	0.03	0.511
24	3X	97.916	1.054	-0.13	0.056	0.662
25	3Y	84.28	0.585	0.011	0.381	-43.659

ADME studies are designed to investigate how a chemical is processed by living organism. ADME parameters of proposed compounds (3A-3Y) are calculated with the help of pkCSM software. Results shows that most of the derivatives exhibit good ADME properties. Table 3 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. Total body clearance and unbound fraction of the drug is also calculated. Predicted value of these parameters for the proposed compounds exhibit within the limits. Thus, it can be suggested that the designed compounds may possess a good pharmacokinetic profile, increasing their pharmacological importance. PreADMET is a web based application for predicting ADME data and building drug-like library using *in-silico* method.

Table 4: Toxicity prediction of designed derivatives

S. NO.	CPD Code	Carcinogenicity	Mutagenicity
1	3A	- ve	+ve
2	3B	-ve	-ve
3	3C	-ve	+ve

4	3D	-ve	+ve
5	3E	-ve	-ve
6	3F	-ve	+ve
7	3G	-ve	+ve
8	3H	-ve	+ve
9	3I	-ve	+ve
10	3J	-ve	+ve
11	3K	-ve	+ve
12	3L	-ve	+ve
13	3M	-ve	+ve
14	3N	-ve	+ve
15	3O	-ve	+ve
16	3P	-ve	-ve
17	3Q	-ve	+ve
18	3R	-ve	-ve
19	3S	-ve	+ve
20	3T	-ve	+ve
21	3U	+ve	+ve
22	3V	-ve	+ve
23	3W	-ve	+ve
24	3X	-ve	+ve
25	3Y	-ve	+ve

The application of *in-silico* methods is increasing with the prediction of toxic risks to human and the environment. The mutagenic and carcinogenic effects of designed compounds on human body were predicted using pkCSM software and results showed (Table 4) that all of the compounds are non-carcinogenic but shows mutagenicity.

Table 5: Binding energy of designed analogues

S. No	Compound code	Binding energy (kcal/mol)	S. No	Compound code	Binding energy (kcal/mol)
1	3A	-11.7879	14	3N	-12.6982
2	3B	-11.2411	15	3O	-12.2428
3	3C	-11.6422	16	3P	-12.4741
4	3D	-11.9422	17	3Q	-10.8798
5	3E	-12.8894	18	3R	-9.82705
6	3F	-10.0174	19	3S	-9.17285
7	3G	-9.92754	20	3T	-12.2125
8	3H	-10.3108	21	3U	-11.908
9	3I	-10.5013	22	3V	-12.0781
10	3J	-12.3676	23	3W	-10.9214
11	3K	-12.2478	24	3X	-11.0773
12	3L	-12.9006	25	3Y	-11.8725
13	3M	-12.2758	26	Trimethoprim	-5.4636

Trimethoprim is the well- known *dihydrofolate reductase* inhibitor. Inhibition of *Dihydrofolate reductase* (DHFR) depletes the available tetrahydrofolate and blocks the formation of thymidylate, purines, amino acids- methionine and glycine and several other cell constituents. DHFR has proven to be an important target of antineoplastic, antiprotozoal, antifungal, and antimicrobial drugs in addition to its use for the treatment of other non-malignant diseases. All the compound show good inhibition against 3SRQ (Table 5). Based on docking score 3E was found to possess least binding energy and it was synthesized by wet lab method.

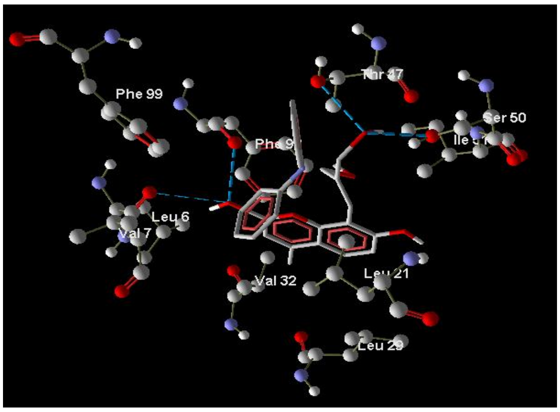


Fig. 1: Interaction of 3B with 3SRQ.
Best ligand pose energy: -11.2411Kcal/mol
Bond Length : 2.77711 A^0

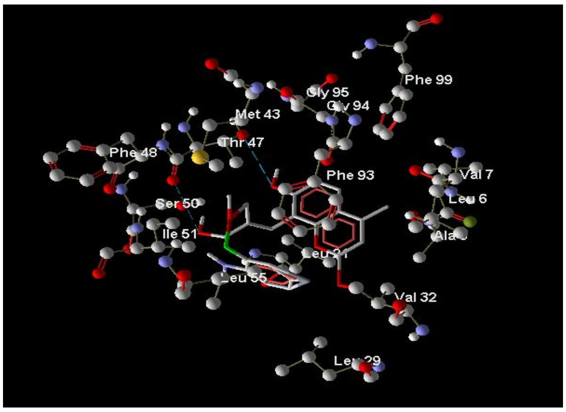


Fig. 2: Interaction of 3E with 3SRQ.
Best ligand pose energy: -12.8894 Kcal/mol
Bond length : 3.35482 A^o

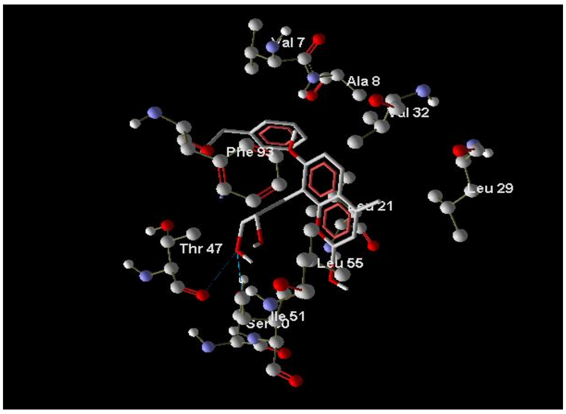


Fig. 3: Interaction of 3J with 3SRQ.
Best ligand pose energy : -12.3676 kcal/mol
Bond length:2.44866A°

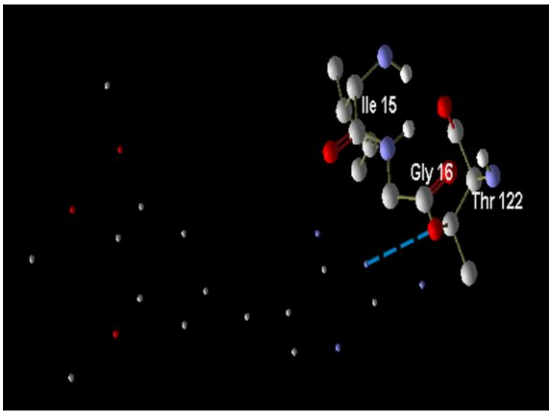


Fig. 4: Interaction of std with 3SRQ.
Best ligand pose energy : -5.46366 kcal/mol
Bond length:2.93001A°

Table 6: Physico- chemical Properties of synthesized compound.

CPD code	Colour and apperance	Melting point in °C	Percentage yield
2	Brownish yellow	65	76
2E	Greenish white	63	74
3E	Pale yellow	60	69

IR spectra shows a Peak at 3405cm^{-1} indicates N-H str, 1666cm^{-1}indicates C=O str, 1546cm^{-1} indicates C-H str, 1386cm^{-1} indicates C-OH str, 1116cm^{-1} indicates C-O str, 773cm^{-1} indicates m–disubstituted compounds recognized by two medium bands.

Anti–bacterial activity was determined by well diffusion method using standard Drug Trimethoprim (200mg/ml) and test was 100µg/ml. When compared to std zone of inhibition of 3E was found to be 14 mm and standard 10mm which indicate good antibacterial activity of 3E against *S. aureus*.

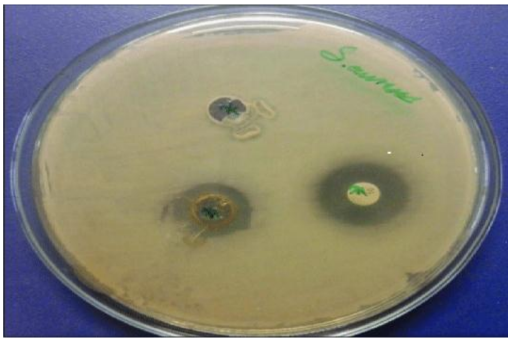


Fig. 5: Antibacterial activity of 3E against *S. Aureus* by Well diffusion method.

CONCLUSION

Molecular docking was studied to predict the intermolecular interactions of compounds (3A-3Y) with dihydrofolate reductase enzyme (PDB ID: 3SRQ). The docking simulation shows that the binding affinity of the coumarin derivative 3E (−12.8894 kcal/mol) was better than that of the standard trimethoprim (−-5.46366 kcal/mol). These results 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. This rule states that a molecule likely to be developed as an orally active drug candidate should not show more than one violation of the following four criteria: Molecular weight ≤ 500, octanol-water coefficient (LogP) ≤ 5, H-bond donors (n-OHNH) ≤ 5, and H-bond acceptors (n-ON) ≤ 10. Despite the importance of RO5 for labelling a molecule as "drug-like", these criteria are restricted to the topic of oral bioavailability via passive transport alone. Among the compounds which showed least binding energy 3E was synthesized by wet lab method by condensation of formyl derivative of coumarin with di substituted acetamide derivative. Synthesized compound was identified by melting point and IR spectroscopy. To check the antibacterial activity agar well diffusion method was adopted using trimethoprim as standard drug. 3E ((e)-2-((7-hydroxy-4-methyl-2-oxo-2h-chromen-8-yl)methylene)-3-oxo-N,N-diphenylbutanamide) showed good activity against *S. Aureus* when compared to standard drug. From the present study it can be concluded that the coumarin derivatives were found to possess good DHFR inhibition in bacterial infections.

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