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First synthesis of rugosaflavonoid and its derivatives and their activity against breast cancer†

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Rugosaflavonoid, is a secondary metabolite isolated from the plant *Rosa rugosa* was synthesized in five simple steps from commercially available 3,5-dihydroxy benzoic acid involving domino aldol-Michael-oxidation reaction. This is the first report of the synthesis of rugosaflavonoid (**6a**). A series of its derivatives were also synthesized, characterized and evaluated for the cytotoxicity against the breast cancer MCF-7 and normal NIH3T3 cell lines. The synthetic derivatives of rugosaflavonoid showed comparable activity in both the cell lines and compounds **6d**, **6e** and **6f**, which were found to be cytotoxic towards MCF-7 cell lines but nontoxic to NIH3T3 cell lines at 5 μM concentration. In an attempt to explore the mode of action of the best active compounds, docking on the ATP binding site of EGFR (1M17) was performed considering that EGFR over-expressed in most of the tumors. The docking score (Gscore) of **6f** and standard quercetin was found to be −8.608 and −8.310 respectively.

1. Introduction

Cancer still continues to be one of the major threats to human society because it is widely spreading day by day and there is no complete cure for it. The three most commonly diagnosed types of cancer among women in 2010 were cancers of the breast, lung, and colorectum, accounting for 52% of cancer cases in this group. Breast cancer (BC) represents the most common cancer among women; there were 232 672 estimated new cases and 40 000 estimated deaths in the United States in 2014.[1–3] The toxicity allied with conventional cancer chemotherapy arises primarily from the lack of specificity for tumor cells. It leads to a low therapeutic index, which results in undesirable damage to healthy organs and consequently puts restrictions on the dose of the drug that can be administered. The majority of the currently available anticancer drugs are designed to have specific toxicity toward tumor cells.[4,5] Several trials are being considered to handle this predicament and thus improve the effectiveness and tumor cell specificity of anticancer drugs. Among these approaches, many studies have focused on natural compounds that inhibit precisely the growth of cancer cells more selectively than normal cells. Thus, phytoconstituents have become the dignified category of anticancer drugs. Over 75% of non-biological anticancer drugs approved between 1981 and 2007 were either natural products or were developed based on them.[6] Therefore, the search for new anticancer agents continues to draw attention to the research community. Nature is the biggest lab where millions of chemical reactions are taking place in milliseconds. Medicinal plants are one of the best equipment for the biosynthesis of various drug based molecules. Chromone is a valid scaffold[7] in the field of medicinal chemistry, due to the wide range of its biological activities, and their structure–activity relationships have generated curiosity among medicinal chemists, and this has culminated into the breakthrough of the clinical anticancer agent flavopiridol, as well as several lead molecules in other disease areas.[8] *Rosa rugosa* Thunb. belongs to the family of Rosaceae is a common ornamental flower distributed in the temperate regions of eastern Asia and widely cultivated in Yunnan Province.[9] The petals and buds of *R. rugosa* are frequently used as food, incense, and Chinese medicinal materials for the cure of stomachache, diarrhea, and gynecological ailments.[10] The literature survey has shown the presence of tannins, terpenoids,[11–13] and flavonoids[14,15] in this genus. Anti-inflammatory, cytotoxic and anti-HIV activities have observed with selected chemical ingredients isolated from *R. rugosa*.[16] Hu *et al.*[17] have recently isolated and characterized cytotoxic oxepinochromenone and flavonoids from *R. rugosa*. Rugosaflavonoid is a new flavonoid, isolated from *Rosa rugosa* which showed cytotoxicity against NB4, SHSY5Y, and MCF-7 cells. Till today the synthesis of rugosaflavonoid (Scheme 1) is not reported in the literature. Therefore, we have focused to synthesize recently isolated naturally occurring rugosaflavonoid (**6a**) and its derivatives by simple and convenient method. All the synthesized derivatives were evaluated for their cytotoxicity against the breast cancer cell lines MCF-7 and the normal cell lines NIH3T3.

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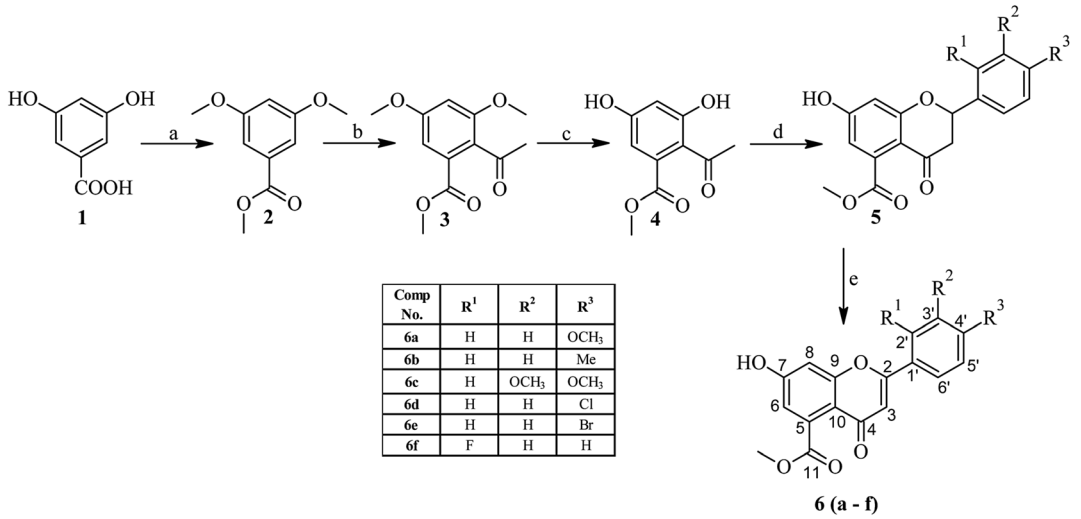


Comp No.	R^1	R^2	R^3
6a	H	H	OCH_3
6b	H	H	Me
6c	H	OCH_3	OCH_3
6d	H	H	Cl
6e	H	H	Br
6f	F	H	H

Scheme 1 Synthesis of rugosaflavonoid and its derivatives using 3,5-dihydroxybenzoic acid as starting material.

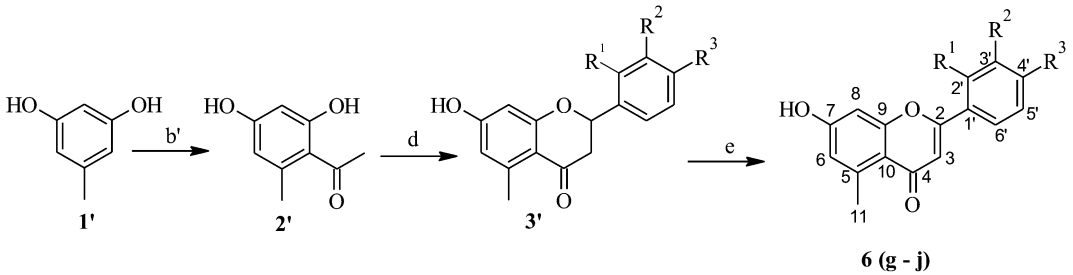
2. Results and discussion

The synthesis of rugosaflavonoid (**6a**) and its derivatives was accomplished in 5 simple steps by selecting 3,5-dihydroxybenzoic acid (**1**) as a building block (Scheme 1). In the first step 3,5-dihydroxybenzoic acid was methylated using dimethyl sulfate in presence of the base,[18] which produced methoxy ester (**2**) with 92% yield. The resultant ester was further acylated using acetyl chloride in carbon disulfide into methyl 2-acetyl-3,5-dimethoxybenzoate (**3**) with 52% yield.[19] Demethylation of methyl 2-acetyl-3,5-dimethoxybenzoate (**3**) using BBr_3 in dichloromethane was attempted as per the procedure reported,[20] however, unfortunately only monomethylated product was obtained. Thus, $AlCl_3$ was used[21] instead of BBr_3 to get the desired demethylated product in good yield. Attempt to cyclize intermediate methyl 2-acetyl-3,5-dihydroxybenzoate (**4**) to rugosaflavonoid (**6a**) by the literature method[22] was unsuccessful, because the reaction resulted into the formation of methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromene-5-carboxylate (**5**) rather than rugosaflavonoid (**6a**), which was accentuated by

Table 1 Docking and cytotoxicity results of compound **6(a–j)** showing cell viability analysis results after MTT assay against MCF-7 and NIH3T3 cell lines. Results are presented in % cell viability. Four different concentrations (5, 10, 15 and 20 μM) of each compound were used for experiments[a]

	Conc. used									
	MCF7 (% cell viability)				NIH3T3 (% cell viability)					
S. no.	5 μM	10 μM	15 μM	20 μM	5 μM	10 μM	15 μM	20 μM	Residue involved in binding with 1M17	Docking score
6a	43	58	60	68	87	74	71	54	Met 769, Leu 768, Asp 831, Gly 772, Leu 694, Glu 738	−5.040
6b	58	47	41	40	90	67	66	62	Met 769, Leu 768, Leu, 694, Gly 772, Asp 831, Thr 830	−6.159
6c	70	52	49	32	73	83	84	82	Met 769, Leu 768, Gln 767, Asp 831, Thr 830, Leu 694	−6.661
6d	64	62	45	36	99	97	93	91	Met 769, Leu 768, Asp 831, Glu 831	−6.549
6e	50	49	41	31	84	79	33	30	Met 769, Leu 768, Asp 831, Glu 738	−6.483
6f	52	45	39	31	96	85	70	62	Met 769, Leu 768, Leu 694, Asp 831, Lys 721, Glu 738	−8.310
6g	56	50	48	44	83	71	64	51	Met 769, Leu 768, Gln 767, Asp 831, Thr 830, Leu 694	−4.557
6h	65	46	43	40	89	74	60	43	Met 769, Leu 768, Leu, 694, Gly 772, Asp 831, Thr 830	−4.743
6i	71	53	48	41	92	88	82	85	Met 769, Leu 768, Asp 831, Glu 831	−4.743
6j	54	49	49	47	55	55	62	65	Met 769, Leu 768, Asp 831, Glu 738	−4.965
Std I quercetin	90	75	67	50	89	76	72	68	Met 769, Lys 721, Glu 738, Asp 831	−8.608

[a] All the samples run in triplicate and average of three results are presented here.



Comp No.	R^1	R^2	R^3
6g	H	H	OCH_3
6h	H	H	CH_3
6i	F	H	H
6j	H	OCH_3	OCH_3

Scheme 2 Synthesis of rugosaflavonoid derivatives using orcinol as starting material.

spectral data. This reaction was attempted 3–4 times to get the required compound (**6a**), but it couldn't be obtained. Eventually, the final step was carried out using the intermediate **5** with I_2 and DMSO (Scheme 1), which provided the desired product (**6a**) during 1 hour only. Rugosaflavonoid was obtained as a yellow solid. A molecular formula of $C_{18}H_{14}O_6$ was confirmed by LC-MS *m/z* 326.30 [M^+] and HRMS *m/z* 327.0853 [M + 1]$^+$. The ^{1}H NMR and ^{13}C NMR data of **6a** were obtained and compared (Table 2) with the reported data[17] and were found in agreement with natural product rugosaflavonoid. It revealed that the compound **6a** has 18 carbon and 14 protons. The ^{1}H NMR of **6a** showed proton signals for methoxycarbonyl group δ (3.81, s, 3H), a methoxy group (3.86, s, 3H), and other proton peaks at δ 6.79 (s, 1H, 3-H), 6.82 (d, 1H, 8-H, J = 1.6 Hz), 7.10 (d, 1H, 6-H, J = 2 Hz), 7.12 (d, 2H, 3′, 5′-H, J = 7.2 Hz), 8.04 (d, 2H, 2′, 6′-H, J = 7.2 Hz), and a phenolic hydroxylic proton at 11.14 (s, 1H, OH). The ^{13}C NMR of compound **6** displayed carbon signals at δ 52.86 (C-11, OCH_3), 55.97 (C-4′, OCH_3), 104.25 (C-8), 105.58 (C-3), 113.55 (C-10), 113.94 (C-6), 114.81 (C-3′), 114.98 (C-5′), 123.49 (C-1′), 127.11 (C-6′), 128.59 (C-2′), 134.55 (C-5), 157.7 (C-9), 158.55 (C-4′) 162.39 (C-2), 162.54 (C-7), 169.19 (C-11), 175.68 (C-4). The IR exhibited the peak at 3446 (OH), and 1735 (C=O), 1624 (C=O). Several derivatives of the rugosaflavonoid using different aromatic aldehydes were also synthesized. Hu *et al.* had reported[17] 13.6 mg of rugosaflavonoid (**6a**) from 8 kg of plant material after several steps of purification. But in the current experiment, 250 mg rugosaflavonoid (**6a**) was obtained from 1 g of methyl 2-acetyl-3,5-dihydroxybenzoate (**4**) *via* the intermediate **5**. The derivatives were also synthesized by replacing the ester group with the methyl group in rugosaflavonoid moiety. These derivatives were synthesized using orcinol (**1′**) as a starting material (Scheme 2), which was acylated followed by the previously stated procedure of cyclization to yield the compounds (**6g–j**). The rugosaflavonoid and its derivatives displayed comparative results in the MTT cytotoxicity assay. The details are presented in the Table 1. The synthetic rugosaflavonoid (**6a**) showed 50% cytotoxicity to MCF-7 cells at 5 μM concentration, but its cytotoxicity reduced after enhancing concentration up to 20 μM with 68% cell viability of

Table 2 Comparative data of synthesized and isolated rugosaflavonoid

Paramater	Rugosaflavonoid synthesized	Rugosaflavonoid isolated
Mp	226–228 °C	Not reported
HRMS	*m/z* 327.0863 [M + 1]$^+$ (calcd for $C_{18}H_{15}O_6$, 327.0863)	HRESIMS *m/z* 349.0682 [M + Na]$^+$ (calcd for $C_{18}H_{14}NaO_6$, 349.0688)
IR (cm^{-1})	3446, 1735, 1624, 1600, 1543, 1436, 1435, 1253, 1180, 1029, 894	3416, 1702, 1657, 1610, 1565, 1456, 1432, 1287, 1182, 1028, 893
^{1}H NMR	(Solvent $DMSd_6$) δ 3.81, (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.79 (s, 1H, 3-H), 6.82 (d, 1H, 8-H, J = 1.6 Hz), 7.10 (d, 1H, 6-H, J = 2 Hz), 7.12 (d, 2H, 3′, 5′-H, J = 7.2 Hz), 8.04 (d, 2H, 2′, 6′-H, J = 7.2 Hz), 11.14 (s, 1H, OH)	(Solvent pyridine-d_5, 500 MHz) δ 3.80 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.68 (s, 1H, 3-H), 6.74 (d, 1H, 8-H, J = 1.8 Hz), 6.89 (d, 1H, 6-H, J = 1.8 Hz), 7.00 (d, 2H, 3′,5′-H, J = 8.8 Hz), 7.76 (d, 2H, 2′,6′-H, J = 8.8 Hz)
^{13}C NMR	52.86 (OCH_3), 55.97 (C-4′, OCH_3) 104.25 (C-8), 105.58 (C-3), 113.55 (C-10), 113.94 (C-6), 114.81 (C-3′), 114.98 (C-5′), 123.49 (C-1′), 127.11 (C-6′), 128.59 (C-2′), 134.55 (C-5), 157.7 (C-9), 158.55 (C-4′), 162.39 (C-2), 162.54 (C-7), 169.19 (C-11), 175.68 (C-4)	52.4 (OCH_3), 55.6 (C-4′, OCH_3), 103.8 (C-8), 105.2(C-3), 113.1 (C-6), 115.6 (C-3′), 115.6 (C-5′), 122.9 (C-1′), 131.0 (C-2′), 131.0 (C-6′), 136.8 (C-5), 158.8 (C-9), 163.2 (C-2), 165.0 (C-7), 168.3 (C-11), 181.5 (C-4)

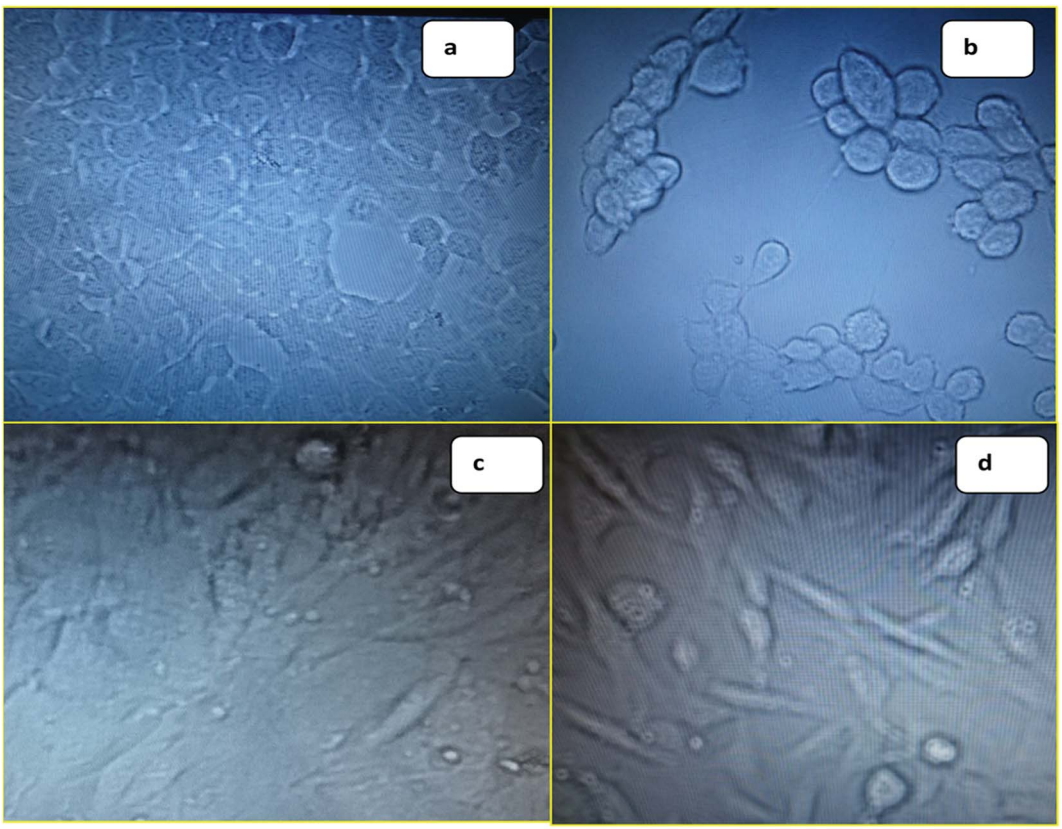


Fig. 1 (a) Image of MCF-7 before treatment with **6f**; (b) image of MCF-7 after treatment with **6f**; (c) image of NIH3T3 before treatment with **6f**; (d) image of NIH3T3 after treatment with **6f**.

MCF-7 cells. It was found to be non-toxic to NIH3T3 normal cell line with 87% cell viability at the lower concentration of 5 μM. However, the toxicity increased with the higher concentration. The derivative **6b** showed dose dependent cytotoxicity towards MCF-7 and NIH3T3 cell lines. Compounds **6c** and **6i** showed marginal cytotoxicity towards MCF-7 and NIH3T3 cell lines at lower concentrations, whereas they displayed high cytotoxicity at 20 μM concentration. When the methoxy substituent at 4′ position of rugosaflavonoid was replaced with halogen, the compounds **6d**, **6e** and **6f** expressed 50% cytotoxicity of MCF-7 cells at the lower concentration. The synthesized derivatives of rugosaflavonoid showed dose-dependent cytotoxicity on MCF-7 cell lines and most of them were non-toxic to NIH3T3 cells. The dimethoxy derivatives **6c** and **6j** showed inhibition of growth of MCF-7, but they were toxic to normal cells. The images of MCF-7 and NIH3T3 cells with **6f** before the treatment and after the treatment are shown in the Fig. 1.

The tyrosine kinase epidermal growth factor receptor (EGFR) is a transmembrane receptor central to numerous cellular process comprising cell migration, adhesion, apoptosis and cell proliferation. The EGFR is over-expressed in almost 90% of tumors.[23,24] Protein–ligand interaction of 1M17 with EGFR-specific inhibitor[25] and anticancer agent, erlotinib, demonstrated computationally that Met 769 formed hydrogen bond with tyrosine kinase inhibitor, whereas Leu 820, Leu 768, Gly 772, Met 769, and Leu 694 indicated hydrophobic interaction with tyrosine kinase inhibitor, erlotinib. Therefore, interaction studies of rugosaflavonoid compounds were carried out with EGFR (1M17) and compared with the molecular docking of quercetin with 1M17. Interestingly, almost all the synthesized compounds showed non bonded interactions (Fig. 2) with the same residues such as Leu 768, Gly 772, Met 769 and Asp 831 as observed in the crystal structure of 1M17 with erlotinib. The protein–ligand interaction profile of **6f** revealed that Lys 721, Glu 738, Met 769 and Asp 831, amino acids involved in the hydrogen bond and π–π interactions in addition to hydrophobic interaction. Molecular docking score of quercetin and **6f** with 1M17 were found to be −8.310 and −8.608 respectively. This result is in agreement with the data published by Singh and Bast.[26] Overall, docking analysis of standard quercetin and rugosaflavonoid derivatives with 1M17 indicated that these derivatives had equal binding affinity which was also well noticed from experimental cytotoxicity results (Table 1).

3. Experimental section

3.1 Materials and method

All the chemicals used during the reactions were procured from Spectrochem, India. ^{1}H NMR and ^{13}C NMR spectra were

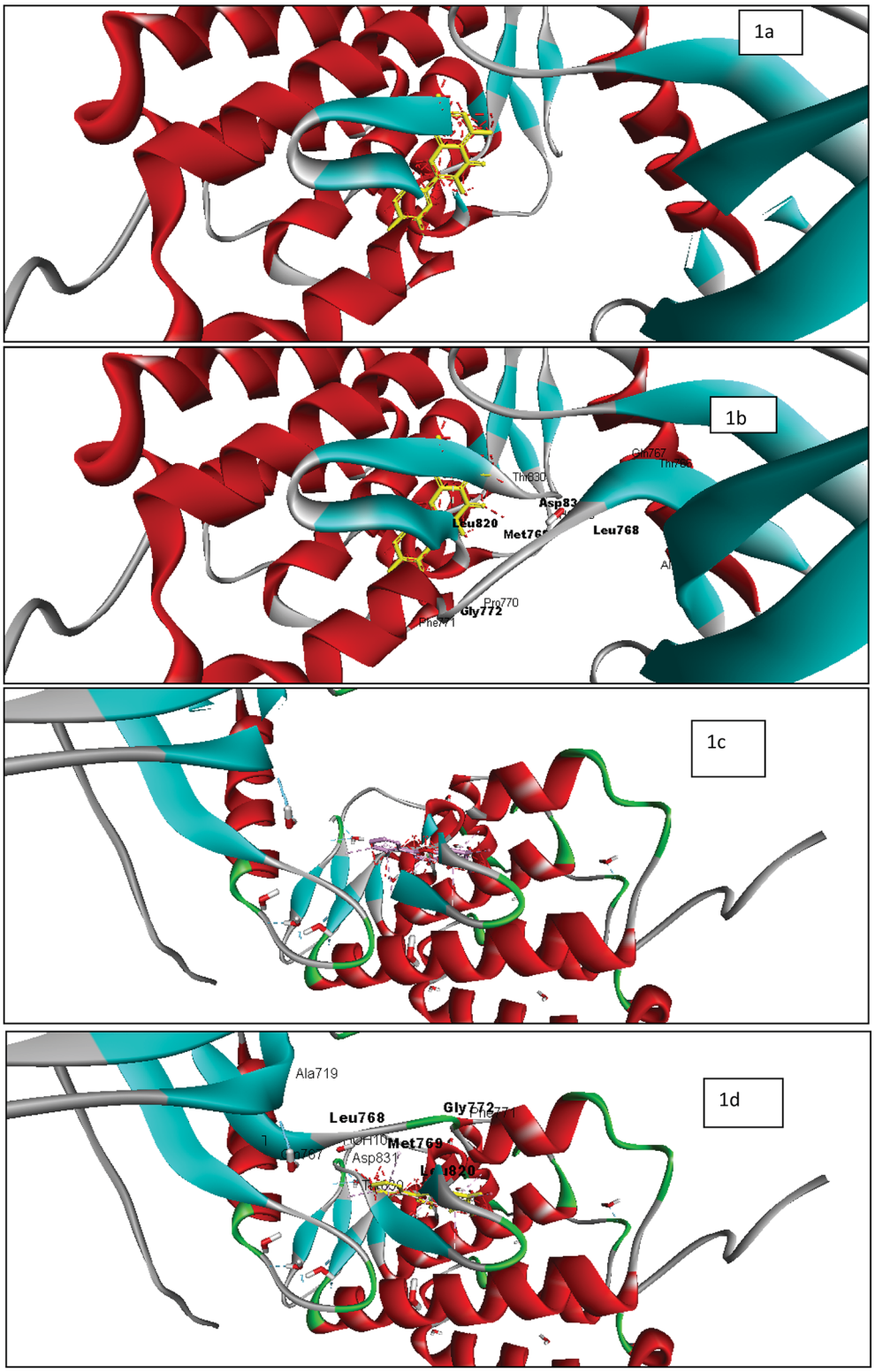


Fig. 2 Docking studies of rugosaflavonoid derivatives with EGFR (1M17) using discovery studio client version 4.0 (a) image of 1M17 with quercetin without active site pocket; (b) image of 1M17 with quercetin with active site pocket (c) image of 1M17 with **6f** without active site pocket; (d) image of 1M17 with **6f** with active site pocket.

recorded at the room temperature on Varian 400 MHz spectrometer and 100 MHz respectively. Chemical shift values were reported with reference to TMS as an internal standard. The samples were prepared by dissolving the synthesized compounds in DMSO-d_6, chemical shifts were expressed in δ (ppm) and coupling constants (J) in hertz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, unresolved multiplet; dd, doublet of doublet. The column chromatography was performed on Merck silica gel 60 (230–400 mesh). Analytical thin layer chromatography was carried out on the precoated Merck silica gel $60F_{254}$ and iodine was used for development of compounds. IR was recorded on FTIR IR Affinity −1 Shimadzu spectrophotometer. CHNS analysis was recorded on Elementar Vario El-III. Dulbecco's Modified Eagle medium (DMEM), Fetal Bovine Serum (FBS) and phosphate buffer saline were procured from Invitrogen. Trypsin and antibiotic solutions were procured from Sigma-Aldrich.

3.2 General procedure

3.2.1 Synthesis of methyl 3,5-dimethoxybenzoate (2). The intermediate **2** was prepared as per reported method by Mc Nulty and Mcleod.[18] In brief, 3,5-dihydroxy benzoic acid (6 mmol) was taken in dry acetone. The mixture was stirred at 40 °C for 15 min, to this K_2CO_3 (2.5 mmol) added and the mixture again stirred at 60 °C for 10 min. Then dimethyl sulfate (2.2 mmol) was added dropwise over a period of 30 min and the temperature was increased slowly to 80 °C. The reaction mixture was allowed to reflux for 6 h. The progress of the reaction was checked by TLC. After completion, reaction was allowed to cool to the room temperature and filtered through the Celite bed. The filtered mixture was concentrated to get crude product. The crude product was slowly poured on crushed ice with constant stirring to obtain solid. The solid obtained was filtered and dried to get methyl 3,5-dimethoxybenzoate in 92% yield.

3.2.2 Synthesis of methyl 2-acetyl-3,5-dimethoxybenzoate (3). The methyl 3,5-dimethoxybenzoate (5 mmol) was mixed with acetyl chloride (25 mmol) and carbon disulfide (2 ml) under dry N_2 in ice bath.[19] To this $AlCl_3$ (15 mmol) was added under vigorous stirring. The reaction was allowed to stir for 15 min. The progress of the reaction was monitored by TLC. After completion, the reaction was quenched with ice and extracted with ethyl acetate. The organic layer was separated, dried over sodium sulfate and concentrated to get a crude product, which was purified by the column chromatography (hexane–ethyl acetate, 70 : 30) to obtain methyl 2-acetyl-3,5-dimethoxybenzoate in 52% yield.

3.2.3 Synthesis of methyl 2-acetyl-3,5-dihydroxybenzoate (4). Methyl 2-acetyl-3,5-dimethoxybenzoate (4 mmol) was appended in chlorobenzene (15 ml), to this $AlCl_3$ (10 mmol) was added slowly at room temperature[20] and the reaction mixture was heated to reflux for 1 h. The progress of the reaction was scrutinized by the TLC. After completion, the reaction was cooled to room temperature and hydrolysed using 1 N HCl. The reaction mixture was extracted with ethyl acetate. The organic layer was separated, dried over sodium sulfate and concentrated to obtain a crude product. The crude product was purified by the column chromatography (hexane–ethyl acetate, 80 : 20) to acquire clean methyl 2-acetyl-3,5-dihydroxybenzoate with 68% yield.

3.2.4 Synthesis of methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-chromene-5-carboxylate (5). Methyl 2-acetyl-3,5-dihydroxybenzoate (4.7 mmol) in DMSO was mixed with anisaldehyde (4.7 mmol), I_2 (0.23 mmol) and pyrrolidine (2.3 mmol) as per the procedure mentioned in the literature[22] and heated at reflux temperature for 8 h. The progress of the reaction was observed by the TLC. After completion, reaction was allowed to cool to the room temperature and quenched with water. The aqueous layer was extracted with ethyl acetate. The organic layer was separated, dried over sodium sulfate and concentrated to get a crude product, which was purified by the column chromatography (hexane–ethyl acetate, 50 : 50) and methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-3,4-dihydro-4*H*-chromene-5-carboxylate was achieved in 45% yield.

3.2.5 Synthesis of methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-5-carboxylate (6a). I_2 (0.15 mmol) was appended to methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-3,4-dihydro-2*H*-1-benzopyran-5-carboxylate (3 mmol) in DMSO (10 ml) and refluxed for 1 h. The progress of the reaction was observed by the TLC. After completion, reaction mixture was cooled to the room temperature and quenched with water. The aqueous layer was extracted with ethyl acetate. The organic layer was separated, dried over sodium sulfate and concentrated and a crude product obtained, which was purified by the column chromatography (hexane–ethyl acetate, 40 : 60) and obtained methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-5-carboxylate in 60% yield.

*Methyl 7-hydroxy-2-(4-methoxyphenyl)-4-oxo-4H-chromene-5-carboxylate (**6a**).* Mp: 226–228 °C; IR (KBr, cm^{-1}), 3446 (OH), 1735 (C=O), 1624 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.81 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.79 (s, 1H, 3-H), 6.82 (d, 1H, 8-H, $J = 1.6$ Hz), 7.10 (d, 1H, 6-H, $J = 2$ Hz), 7.12 (d, 2H, 3′, 5′-H, $J = 7.2$ Hz), 8.04 (d, 2H, 2′, 6′-H, $J = 7.2$ Hz), 11.14 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 52.86 (OCH_3) 55.97 (C-4′, OCH_3), 104.25 (C-8), 105.58 (C-3), 113.55 (C-10), 113.94 (C-6), 114.81 (C-3′), 114.98 (C-5′), 123.49 (C-1′), 127.11 (C-6′), 128.59 (C-2′), 134.55 (C-5), 157.7 (C-9), 158.55 (C-4′), 162.39 (C-2), 162.54 (C-7), 169.19 (C-11), 175.68 (C-4); LCMS (ESI) m/z calculated for $C_{18}H_{14}O_6$: 326.3 and found 327.0; HRMS m/z 327.0863 $[M + 1]^+$ elemental analysis calculated for $C_{18}H_{14}O_6$: C, 66.25, H, 4.32; found: C, 66.31, H, 4.28.

*Methyl 7-hydroxy-2-(4-methylphenyl)-4-oxo-4H-chromene-5-carboxylate (**6b**).* Mp: 240–242 °C; IR (KBr, cm^{-1}), 3516 (OH), 1737 (C=O), 1627 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.39 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.83 (s, 1H, 3-H), 6.84 (s, 1H, 8-H), 7.10 (s, 1H, 6-H), 7.38 (d, 2H, $J = 8.0$ Hz, 3′, 5′-H), 7.95 (d, 2H, $J = 8.0$ Hz, 2′, 6′-H), 11.18 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 21.51 (CH_3), 52.88 (OCH_3), 104.27 (C-8), 106.46 (C-3), 113.43 (C-10), 113.68 (C-6), 126.68 (C-2′, 6′), 128.55 (C-1′), 130.14 (C-3′, 5′), 130.25 (C-4′), 134.58 (C-5), 142.42 (C-9), 157.78 (C-2), 162.49 (C-7) 169.14 (C-11), 175.76 (C-4); LCMS (ESI) m/z calculated for $C_{18}H_{14}O_5$: 310.3 and found 311.0. Elemental

analysis calculated for $C_{18}H_{14}O_5$: C, 69.66, H, 4.54; found: C, 69.61, H, 4.49.

*Methyl 2-(3,4-dimethoxyphenyl)-7-hydroxy-4-oxo-4H-chromene-5-carboxylate (**6c**).* Mp: 233–236 °C; IR (KBr, cm^{-1}), 3444 (OH), 1737 (C=O), 1627 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.79 (s, 1H, 3-H), 6.84 (s, 1H, 8-H), 7.13 (m, 2H, 5′-H, 6-H), 7.52 (s, 1H, 2′-H), 7.63 (d, 1H, 6′-H, $J = 8$ Hz), 11.08 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 52.87 (OCH_3), 56.17 (OCH_3), 56.32 (OCH_3), 104.34 (C-8), 105.89 (C-3), 109.83 (C-6′), 112.14 (C-5′), 113.39 (C-10), 113.53 (C-6), 120.29 (C-1′), 123.59 (C-2′), 134.51 (C-5), 149.47 (C-3′), 152.34 (C-4′), 157.74 (C-9), 162.36 (C-2), 162.48 (C-7), 169.19 (C-11), 175.74 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{19}H_{16}O_7$: 356.32 and found 357.0; elemental analysis calculated for $C_{19}H_{16}O_7$: C, 64.04, H, 4.52; found: C, 64.16, H, 4.59.

*Methyl 2-(4-chlorophenyl)-7-hydroxy-4-oxo-4H-chromene-5-carboxylate (**6d**).* Mp: 262–268 °C; IR (KBr, cm^{-1}), 3645 (OH), 1714 (C=O), 1697 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H, OCH_3), 6.80 (s, 1H, 3-H), 6.89 (s, 1H, 8-H), 7.08 (s, 1H, 6-H), 7.60 (d, 2H, 2′, 6′-H, $J = 8.4$ Hz), 8.07 (d, 2H, 3′, 5′-H, $J = 8.8$ Hz), 11.19 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 52.91 (OCH_3), 104.32 (C-8), 107.48 (C-3), 113.36 (C-10), 113.86 (C-6), 128.60 (C-2′, 6′), 129.62 (C-3′, 5′), 130.28 (C-1′), 134.60 (C-5), 136.99 (C-9), 157.79 (C-4′), 161.24 (C-2), 162.63 (C-7), 169.06 (C-11), 175.75 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{17}H_{11}ClO_5$: 330.71 and found 331.0; elemental analysis calculated for $C_{17}H_{11}ClO_5$: C, 61.73, H, 3.34; found: C, 61.67, H, 3.28.

*Methyl 2-(4-bromophenyl)-7-hydroxy-4-oxo-4H-chromene-5-carboxylate (**6e**).* Mp: 275–277 °C; IR (KBr, cm^{-1}), 3564 (OH), 1737 (C=O), 1627 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H, OCH_3), 6.81 (s, 1H, 8-H), 6.90 (s, 1H, 6-H), 7.07 (s, 1H, 3-H), 7.75 (d, 2H, 2′, 6′-H, $J = 8$ Hz), 8.0 (d, 2H, 3′, 5′-H, $J = 8$ Hz), 11.18 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 53.03 (OCH_3) 104.38 (C-8), 107.41 (C-3), 113.35 (C-10), 113.93 (C-6), 126.01 (C-2′, C-6′), 128.78 (C-3′, 5′), 130.59 (C-1′), 132.62 (C-5), 134.62 (C-9), 157.84 (C-4′), 161.52 (C-2), 162.70 (C-7), 169.19 (C-11), 175.90 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{17}H_{11}BrO_5$: 375.17 and found 376.9, 378.9. Elemental analysis calculated for $C_{17}H_{11}BrO_5$: C, 54.42, H, 2.95; found: C, 54.48, H, 2.95.

*Methyl 2-(2-fluorophenyl)-7-hydroxy-4-oxo-4H-chromene-5-carboxylate (**6f**).* Mp: 222–225 °C; IR (KBr, cm^{-1}), 3645 (OH), 1732 (C=O), 1697 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.81 (s, 3H, OCH_3), 6.65 (s, 1H, 3-H), 6.86 (s, 1H, 8-H), 7.06 (s, 1H, 6-H), 7.41–7.5 (m, 2H, 5′-H, 6′-H), 7.63–7.70 (m, 1H, 4′-H), 8.02–8.06 (m, 1H, 3′-H), 11.22 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 52.81 (OCH_3), 104.15 (C-8), 110.07 (C-6). 110.71 (C-10), 116.12 (C-5′), 116.30 (C-3), 125.21 (C-3′), 128.93 (C-1′), 128.96 (C-6′), 131.36 (C-9), 136.50 (C-5), 159.11 (C-2′), 161.07 (C-4′), 163.43 (C-2), 164.45 (C-7), 169.39 (C-11), 185.83 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{17}H_{11}FO_5$: 314.26 and found 315.0. Elemental analysis calculated for $C_{17}H_{11}FO_5$: C, 64.96, H, 3.52; found: C, 64.91, H, 3.56.

*7-Hydroxy-2-(4-methoxyphenyl)-5-methyl-4-oxo-4H-chromene (**6g**).* Mp: 245–249 °C; IR (KBr, cm^{-1}), 3566 (OH), 1704 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.68 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 6.63 (s, 1H, 3-H), 6.67 (s, 1H, 8-H), 6.81 (s, 1H, 6-H), 7.08 (d, 2H, 2′, 6′-H, $J = 8.4$ Hz), 7.97 (d, 2H, 3′, 5′-H, $J = 8.8$ Hz), 10.59 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 22.92 (CH_3), 55.96 (C-4′, OCH_3), 101.38 (C-8), 106.82 (C-3), 114.95 (C-3′, 5′), 117.19 (C-6), 123.73 (C-1′), 128.27 (C-2′, 6′), 128.56 (C-10), 141.90 (C-5), 159.32 (C-9), 160.71 (C-4′), 161.57 (C-2), 162.26 (C-7) 178.91 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{17}H_{14}O_4$: 282.29 and found 283.0. Elemental analysis calculated for $C_{17}H_{14}O_4$: C, 72.32, H, 4.99; found: C, 72.36, H, 4.93.

*7-Hydroxy-2-(4-methylphenyl)-5-methyl-4-oxo-4H-chromene (**6h**).* Mp: 252–255 °C; IR (KBr, cm^{-1}), 3565 (OH), 1710 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.38 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 6.65 (s, 1H, 3-H), 6.72 (s, 1H, 8-H), 6.82 (s, 1H, 6-H), 7.36 (d, 2H, 3′, 5′-H, $J = 8.4$ Hz), 7.92 (d, 2H, 2′, 6′-H, $J = 7.6$ Hz), 10.62 (s, 1H, OH); ^{13}C NMR, (100 MHz, DMSO-d_6) δ 21.54 (CH_3), 22.92 (CH_3), 101.40 (C-8), 107.65 (C-3), 115.16 (C-1′), 117.27 (C-6), 126.41 (C-3′, C-5′), 128.76 (C-10), 130.12 (C-2′, C-6′), 141.96 (C-4′, C-5), 159.37 (C-9), 160.78 (C-2), 161.67 (C-7), 178.94 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{17}H_{14}O_3$: 266.29 and found 267.0. Elemental analysis calculated for $C_{17}H_{14}O_3$: C, 76.67, H, 5.29; found: C, 76.62, H, 5.26.

*2-(2-Fluorophenyl)-7-hydroxy-5-methyl-4-oxo-4H-chromene (**6i**).* Mp: 252–255 °C; IR (KBr, cm^{-1}), 3564 (OH), 1714 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.69 (s, 3H, CH_3), 6.55 (s, 1H, 3-H), 6.67 (s, 1H, 6-H), 6.78 (s, 1H, 8-H), 7.41–7.48 (m, 2H, 5′, 6′-H), 7.62–7.63 (m, 1H, 4′-H), 7.98–8.0 (m, 1H, 3′-H), 10.71 (s, 1H, OH). ^{13}C NMR, (100 MHz, DMSO-d_6) δ 22.90 (CH_3), 101.34 (C-8), 112.91 (C-6′), 113.01 (C-5′), 114.96 (C-4′), 117.24 (C-6), 117.47 (C-3), 125.71 (C-1′), 129.78 (C-3′), 133.73 (C-2′), 133.82 (C-10), 142.13 (C-5), 156.58 (C-9), 159.54 (C-2), 161.92 (C-7), 178.58 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{16}H_{11}FO_3$: 270.25 and found 271.0. Elemental analysis calculated for $C_{16}H_{11}FO_3$: C, 71.10, H, 4.09; found: C, 71.16, H, 4.17.

*2-(3,4-Dimethoxylphenyl)-7-hydroxy-5-methyl-4-oxo-4H-chromene (**6j**).* Mp: 231–234 °C; IR (KBr, cm^{-1}), 3564 (OH), 1704 (C=O); ^{1}HMR (400 MHz, DMSO-d_6) δ 2.69 (s, 3H, CH_3), 3.86 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.76 (s, 1H, 3-H), 6.79 (s, 1H, 8-H), 6.91 (d, 1H, 6-H), 7.13 (d, 1H, 6′-H, $J = 7.2$ Hz), 7.17 (d, 1H, 2′-H, 7.2 Hz), 7.77 (d, 1H, 5′-H, $J = 7.2$ Hz), 10.60 (s, 1H, OH). ^{13}C NMR, (100 MHz, DMSO-d_6) δ 22.90 (CH_3) 58.90 (OCH_3), 59.30 (OCH_3), 101.33 (C-8), 104.20 (C-3), 110.30 (C-6), 113.35 (C-1′), 114.27 (C-10), 116.57 (C-6′), 120.50 (C-2′), 121.86 (C-5′), 141.86 (C-5), 149.83 (C-3′), 153.23 (C-4′), 159.30 (C-9), 160.80 (C-2), 162.55 (C-7), 178.90 (C-4); LCMS (ESI) *m*/*z* calculated for $C_{18}H_{16}O_5$: 312.31 and found 313.0. Elemental analysis calculated for $C_{18}H_{16}O_5$: C, 69.21, H, 5.15; found: C, 69.17, H, 5.11.

3.3 Biology

3.3.1 Cell lines and culture. Breast cancer MCF-7 and normal NIH3T3 cell lines were obtained from National Center for Cell Science, Pune, India. All cell lines were cultured in a humidified atmosphere of 5% CO_2 in DMEM at 37 °C, supplemented with 10% FBS, penicillin (100 μg ml^{-1}) and streptomycin (100 μg ml^{-1}).

3.3.2 MTT assay for cytotoxicity against MCF-7 and NIH3T3 cells. MCF-7 and NIH3T3 cells were grown in DMEM media containing 10% FBS. The effect of rugosaflavonoid derivatives on the growth of MCF-7 cells (breast cancer cell lines) and

NIH3T3 cells was examined using the MTT assay. Cells were subcultured in 96-well plates at a density of 2.5×10^3 cells per well without test samples for 24 h in a final volume of 150 μl. After 24 h, cells were treated with four different concentrations test specimens (5, 10, 15 and 20 μM in plain DMEM) in triplicates and kept for 24 h in the CO_2 incubator. Next day, treatment media was removed and 20 μl of MTT (1.5 mg ml^{-1} in PBS) was added to the fresh medium. After three hours' of incubation at 37 °C in CO_2 incubator, 180 μl DMSO was added to the each well and plates were agitated for 1 min. Spectrophotometric absorbance at 570 nm was measured. The percentage of viability was calculated as per the following formula: (viable cells)% = (OD of drug-treated sample/OD of untreated sample) × 100. Quercetin was used as the standard.

3.4 Molecular docking studies

The molecular modelling studies were carried out on Windows 7 64-bit operating system using Maestro 11.2 software. The GLIDE docking application of Maestro 11.2 software was used to calculate GScore, which is based on an empirical scoring function and deploy a combination of several parameters. GScore (docking score) was calculated in kcal mol^{-1} and it included ligand–protein interaction energies, hydrophobic interactions, hydrogen bonds, internal energy, π–π stacking interactions, root mean square deviation (RMSD) and desolvation. The X-ray crystallographic structure of erlotinib cocrystallized with EGFR was obtained from the protein data bank (PDB ID: 1M17). The ATP binding site of EGFR was prepared for docking studies in which erlotinib was removed from the active site, hydrogen atoms were added to the structure with their standard geometry. Active sites were observed from the sequence analysis and software run and it was used in predicting interactions at the active site between the selected compounds **6f** and quercetin with EGFR. The 2D structures of the docked compounds were generated, transformed to 3D, protonated, and energy was minimized by using Ligprep application. Grid generation and ligand docking was performed to obtain ligand interaction diagram with 1M17 EGFR and docking score. Receptor–ligand interaction images were obtained from discovery studio client version.

4. Conclusion

In summary, we successfully completed a simple and convenient 5 steps synthesis for a naturally occurring rugosaflavonoid (**6a**) with better yield and evaluated its cytotoxicity against breast cancer cell lines. Molecular docking score of its derivatives with EGFR (1M17) showed that it interacted with active site pocket of 1M17. Some of the synthetic derivatives showed better dose dependent activity against MCF7 than the natural molecule rugosaflavonoid and were found to be non toxic to NIH3T3 cell lines at lower concentration. Though flavonoids are known from the years for their bioactive potential but there is still scope in modifications of natural analogs, which may provide us the lead molecules.

Conflict of interest

There is no conflict of interest among the authors.

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