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Original Research Paper

Synthesis, Ant Proliferative Activity and Docking Study of New Quercetin Derivatives against MDA-MB231 Breast Cancer Cell Lines

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Abstract: MDA-MB231 is a very aggressive and invasive triple negative breast cancer, which present with limited treatment options. Unlike other breast cancer types, it is characterized by absence of hormonal receptors of estrogen, progesterone as well as human epidermal growth factor receptor 2, rendering it unsuitable for hormonal therapy and a perfect candidate for chemotherapy. Quercetin is a common natural flavonoid present in many food items, which have a wide range of biological activities, likes anticancer, antiviral, antibacterial and antioxidant. This study involves the synthesis of new Quercetin derivatives and the investigation their effects against MDA-MB231 cell lines. The structures of the derivatives were established using UV, IR, ¹HNMR, CHNS, EIMS and ESIMS techniques. Their antiproliferative activities were investigated in vitro using Microculture Tetrazolium (MTT) assay. The percentage cell viability following exposure to Quercetin and its derivatives (Compounds 1-5) were measured. Both compounds 1 and 4 show a significant decrease in percentage cell viability from 100% to 43.7% and 38.1% respectively. IC₅₀ value was calculated for compound 1 and 4 and found to be 2.042 and 1.838 μM respectively, indicating that they have a potential anticancer activity against the triple negative breast cancer type. The antiproliferative activity was supported and evidenced by molecular docking study.

Keywords: Quercetin, MDA-MB231, Molecular Docking, MTT Assay

Introduction

Triple Negative Breast Cancer (TNBC) is a cancer type that is devoid of the three typical types of receptors known to stimulate most breast cancer growth; estrogen, progesterone and human Epidermal Growth Factor Receptor 2 (EGFR2). Due to the absence of necessary receptors, common hormonal therapy and drugs that target these receptors are ineffective. However, TNBC is considered perfect candidate for chemotherapy as stated by (Chavez *et al.*, 2010). Although Triple negative breast cancer accounts for only 15% of other breast cancer subtypes, it shows high rate of recurrence and develops resistance to chemotherapy as previously reported by (Rakha *et al.*, 2008). In addition, patients have shown poor prognosis due to the lack of specific targeted therapy (Nguyen *et al.*, 2017). Therefore, it is vital to look for new therapeutic agents that are

effective, less toxic and can prevent relapse emergence. Quercetin is 3, 3', 4', 5, 7-pentahydroxyflavone (Fig. 1) which is a natural poly phenolic compound that belongs to the flavanol class of flavonoids and present within various concentrations in different vegetables and fruits such as onion, broccoli, apple, garlic and tomatoes (Al-Jabban *et al.*, 2015; Hertog *et al.*, 1992). Quercetin characterize by powerful antioxidant activity and wide pharmacological applications including various antiproliferative activities against colon, pancreatic, prostate, brain, bone, gastric and breast cancer (Rauf *et al.*, 2018), in addition to its antimicrobial, anti-inflammatory, antidiabetic, neuroprotective, anti-obesity and the prevention of cardiovascular disease activities (Amic *et al.*, 2007; D'Andrea, 2015; Dajas, 2012; Massi *et al.*, 2017; Moalin *et al.*, 2011; Pietta, 2000; Procházková *et al.*, 2011; Ramos *et al.*, 2006; Wang *et al.*, 2006).

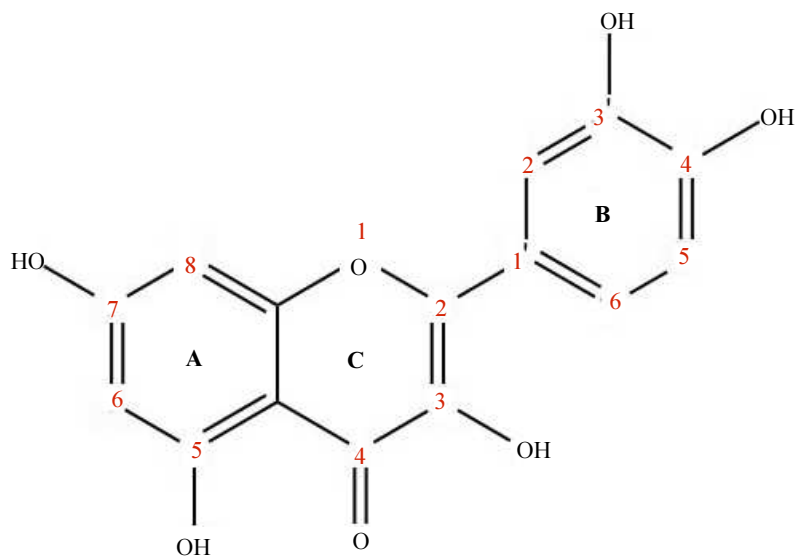


Fig. 1: Quercetin chemical structure

Previous studies have focused on the relationship between anticancer activity of quercetin and the methylation of its five hydroxyl groups and the results have shown the importance of methylation at 4' and 7' position and at 3' and 4' positions on the anticancer activity of Quercetin against SKBR breast cancer cell line (Zhi-Hao *et al.*, 2014) while other studies revealed that 3,3',4',7-tetra-O-methylated Quercetin and 3,3',4',5,7-penta-O-methylated Quercetin have the ability to influence the Breast Cancer Resistance Protein (BCRP) which is considered as potential antimultidrug resistant (MDR) protein (Juvale *et al.*, 2013; Yuan *et al.*, 2012). The insertion of methoxy groups into the Quercetin molecule results in an improvement of its physicochemical properties, such as increasing its metabolic stability, solubility and bioavailability as well as reduction of toxic side effects (Walle, 2009). This study is focused on the methylation of its five hydroxyl groups and the replacement of the carbonyl group by 4-thion, 4-thionmethylidide and 4-C=N-R groups. These modifications in Quercetin structure are likely to induce the most important variations in the general electronic and lipophilic properties of the whole molecule. This study revealed some new potent compounds against MDA-MB231 breast cancer cell line.

The work involves the synthesis of six Quercetin derivatives four of them are novel compounds including three Schiff bases of pentamethoxy-quercetin and a 4-thiomethylidide penta methoxy-quercetin. The synthesized compounds were tested for their anticancer activity against MDA-MB231 breast cancer cell line using MTT assay method. A docking study was conducted to shed more light on their activity.

Materials and Methods

Reagents

Quercetin, dimethyl sulfate, Phosphorous pentasulfide, potassium carbonate, acetone (99%), methanol (99%), toluene (99%), sodium sulfate, anisole, molecular sieve 3Å, ethanol (99%), aniline, methyl iodide and hydrazine hydrate were purchased from Sigma-Aldrich. Ether, chloroform and cyclohexane from BDH, while dichloromethane was obtained from Ajax chemicals Ltd. All chemicals were used as supplied without further purification.

Chemical Synthesis

Synthesis of Compound 1

Compound 1 was synthesized following the procedure described by (Picq *et al.*, 1982) as shown in Fig. 2.

In 1L round bottomed flask connected to a reflux condenser and calcium chloride tube, Quercetin (10 g, 33 mmol), 700 mL acetone (dried on molecular sieves 3Å) and anhydrous potassium carbonate (150 g, 5000 mmol) were placed. Stirring was started and dimethyl sulfate (50 mL, 400 mmol) was added followed by heating the reaction to reflux for 21 h. The reaction mixture was then filtered off and the solid potassium carbonate was washed with hot acetone three times followed by evaporation of the collected filtrate to dryness using rotary evaporator at 50°C. The crude product was then recrystallized from methanol, giving powder of compound 1, 2-(3', 4'-dimethoxyphenyl)-3, 5, 7-trimethoxy - 4H-chromen-4 one.

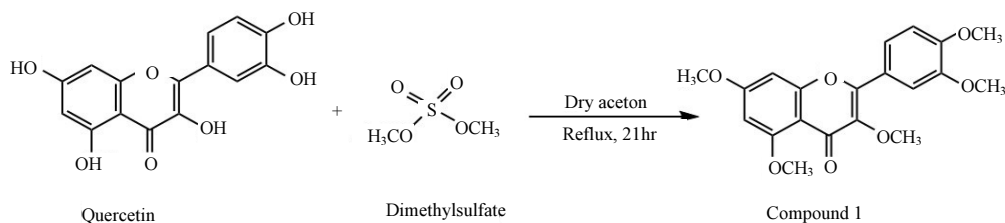


Fig. 2: General Method for the preparation of compound 1

Synthesis of Compound 2

Compound 2 was prepared by the reaction of compound 1 with Lawesson's reagent as described by (Ravishankar *et al.*, 2015) (with modification). Lawesson's reagent has been prepared according to (Thomsen *et al.*, 1984).

Compound 1 (0.6 g, 1.59 mmol) was dissolved in anhydrous toluene (15 mL) was added and the reaction was heated to 110°C followed by Lawesson's reagent (0.63, 1.59 mmol) addition and refluxing for 180 min. After that the reaction mixture was cooled to room temperature, filtered and the precipitate was purified by column chromatography using silica gel and chloroform 100% as eluent to obtain compound 2, 2-(3',4'-Dimethoxyphenyl)-3,5,7-trimethoxy-4H-chromene-4-thione.

Synthesis of Schiff's Bases

Schiff's bases of pentamethoxy quercetin were generally synthesized by two methods: the first involves direct reaction of compound 2 with hydrazine hydrate to obtain compound 3, while the second involves the reaction of appropriate aromatic amine with the methyl iodide derivative of compound 2.

Synthesis of Compound 3

Compound 3 was synthesized by direct addition of hydrazine hydrate to solution of compound 2 in warm ethanol following the procedure described by (Baker *et al.*, 1954; 1952).

In a 100 mL flat bottomed flask, (0.194 g, 0.5 mmol) of compound 2 was dissolved in (10 mL) warm ethanol followed by drop wise addition of 0.5 mL (10 mmol) hydrazine hydrate with stirring till the dark green color of the solution fade giving yellow-orange colored solution. Hydrogen sulfide (H₂S) started to liberate within 5 minutes after the amine addition. Half an hour later, hydrogen sulfide liberation ceased and cold water was added to the reaction mixture and kept in the refrigerator for 24 hrs. The precipitate of compound 3, 2-(3', 4'-dimethoxyphenyl)-3, 5, 7-trimethoxy-4H-chromen-4-ylidene) hydrazine, was then collected.

Synthesis of Compound 4

Compound 4 was synthesized following the procedure described by (Baker *et al.*, 1954; 1952). The reaction of

compound 2 with methyl iodide takes place at room temperature.

In a 100 mL round flask a (0.25 g, 0.64 mmol) of compound 2 was suspended in excess methyl iodide. The reaction mixture color changes from dark green to red. The precipitate was crystallized from the solution, collected after 24 hrs, then washed with ether to give compound 4, 2-(3',4'-dimethoxyphenyl)-3,5,7-trimethoxy-4H-chromen-4-ylidene) methyl sulfonium iodide.

Synthesis of Compound 5 and 6

Compounds 5 and 6 were prepared from compound 4 by mild heating and stirring of the later in methanol with the suitable amine following the procedure described by (Baker *et al.*, 1952).

Synthesis of Compound 5

In a 100 mL round bottomed flask, Aniline (1 mL, 10 mmol) was shaken with a suspension of compound 4 (0.25 g, 0.47 mmol) in 10 mL of warm ethanol. The reaction was completed in within 15 min with the liberation of methanethiol. The product was chromatographed through silica gel and dichloromethane: methanol (9:1) was used as an eluent. The dichloromethane: methanol portion was collected, rotary evaporated to give compound 5, 2-(3', 4'-dimethoxyphenyl)-3, 5, 7-trimethoxy-N-phenyl-4H-chromen-4-imine.

Synthesis of Compound 6

In a 100 mL round flask (0.75 g, 1.4 mmol) of compound 4 was suspended with 5 mL warm methanol and 0.75 g (4.09 mmol) of 3,4,5-trimethoxy aniline dissolved in 5 mL methanol was then added and the red color of the solution suddenly disappeared. The reaction accomplished within 3 hrs to give yellow precipitate. The precipitate was washed with chloroform to give compound 6, 2-(3', 4'-dimethoxyphenyl)-3,5,7-trimethoxy-N-(3", 4", 5"-trimethoxyphenyl)-4H-chromen-4-imine.

Physical Measurements

Melting points were recorded in electrothermal melting point apparatus model Stuart SMP30 (UK) and were uncorrected. Elemental analysis was carried out on elemental Vario Microcube apparatus (Germany). IR spectra were recorded as KBr discs, using Stuart Shimadzu Corporation (Japan) spectrophotometer in the

frequency range 4000-400 cm^{-1} . UV-Visible spectra were recorded at compound concentration of 3×10^{-5} M (in ethanol) using CECIL CE 7500 double beam UV/Visible spectrophotometer (England) 1 cm^3 pathway quartz cells. ^1H NMR spectra were recorded using Bruker 500 MHz-avance III and inova spectrometer at 400 and 500 MHz using Tetramethyl Silane (TMS) as internal standard and, DMSO- d_6 and CDCl_3 as solvents. The chemical shifts being expressed in ppm and coupling constant in Hz. The mass spectra of the studied compounds were obtained on a 5975C VL MSD with Tripe-Axis Detector (Agilent Technologies), using an ionizing potential of 70 eV. The Electrospray Ionization (ESI) mass spectra were obtained with apex-iv detector using methanol and chloroform as solvents.

Preliminary Cytotoxicity Screening

Quercetin and its analogues **1-5** were screened for the preliminary *in vitro* anticancer activity against triple negative breast cancer MDA-MB-231 cell line by MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay reported by (Mosmann, 1983) at department of zoology and cytology, Government college university, Faisalabad 38000, Pakistan. The cultured human breast cancer cells were treated with 0.1 mg/mL of Quercetin and its derivatives **1-5** solutions in DMSO and were tested in triplicate. Cancer cells treated with DMSO only were used as control. After incubation for 48 h at 37°C and 5% CO_2 , the cells were washed with Phosphate Buffer Saline (PBS) to remove dead cells and cell debris and treated with 0.5 mg/mL MTT and further incubated for 4 hrs. at 37°C and 5% CO_2 till formazan crystals were form, these crystals were dissolved in DMSO and absorbance was measured at 570 nm. The percentage of viable cells were then calculated according to Eq. 1:

$$V = ((A_{\text{sample}} - A_{\text{control}}) / A_{\text{control}}) \times 100\% \quad (1)$$

Where:

V = The percentage of cancer cells remaining viable after being treated with anticancer agent (%)

A_{sample} = The absorbance of the tested compound

A_{control} = The absorbance of the positive control (i.e.; untreated cells)

When the sample absorbance values greater than the control this indicate cell proliferation, while lower values suggest cell death.

Calculation of the IC_{50} Value of the Active Compounds

This step involve IC_{50} determination for the compounds that have shown good anticancer activity

against MDA-MB231 cell line from previous step by studying the effect of various concentrations (1, 2, 4, 8, 15.6, 31.25, 62.5, 125, 250, 500 μM) of these compounds on the viability of breast cancer cells and evaluating their effect using MTT assay. The results were analyzed and the IC_{50} values were calculated using Graph Pad Prism 8.1 program and the results were expressed as $\pm\text{SEM}$, with p value <0.05 .

Molecular Docking Studies

In this study Jaunas kinase-2 (JAK2) was used because it is the target to produce the response of many anticancer drugs (Abu Bakar *et al.*, 2018). In the docking study the crystal structure of JAK2 cocrystallized with a potent quinoxaline inhibitor, PDB: 3KRR, was downloaded. In order to investigate the reaction of quercetin and its **1-6** analogues against the targeted proteins a docking study was undertaken between the studied molecules and the JAK2 protein. Docking is a strong computational method for predicting the position of the ligand within the active site of the protein, which reveals the amino acids that interact with binding ligand. This offers valuable information about how the drug positioned within the active site and the coordinates between the different interacted groups.

Molecular Operating System (MOE) docking program was used in this study. As a starting step, the structures of the studied molecules were optimized and prepared for docking using the program SYBYL-X. The structure of the targeted protein is also repaired for the missing parts and for adding missing protons using the same program. The docking calculations will determine the position of the ligand within the active site of the protein as well as calculating the affinity of the ligand to the protein which could be taken as biological activity of the ligand. The binding mode is the top 1 hitting. The MOE program calculates the binding free energy as the affinity between the protein and the ligand. In this program the binding free energy for the ligand is called the score and abbreviated as S and in this case the largest negative value of S means the highest affinity and accordingly the highest biological activity.

Results

The physical properties and UV-visible spectral data of Quercetin and its **1-6** analogues are shown in Table 1, while mass spectra and elemental analysis data are gathered in Table 2 and the IR and the ^1H NMR spectral data are gathered in Tables 3 and 4 respectively.

In vitro antiproliferative activity results of Quercetin and five of its analogues (**1-5**) against breast cancer

MDA-MB-231 cell line showed that Quercetin and compound 5 were inactive, while compound 1-4 shows a good activity and gave a great reduction in the percentage of cell viability, additionally, the IC_{50} value for the most active compound (1 and 4) were calculated and as shown in Table 5.

Theoretically the antiproliferative activities were estimated using Molecular docking assay against Jaunas kinase-2 (JAK2) protein (PDB: 3KRR) for Quercetin and its analogues (1-5) and the results were expressed along with the experimental results in Table 6. Structures of the studied molecules are shown in Fig. 3-9. The correlation between the calculated binding free energies with the experimental biological activities of the studied molecules is shown in Fig. 10. The predicted binding sites of the studied compounds within the target protein are shown in Fig. 11-22 and Table 7.

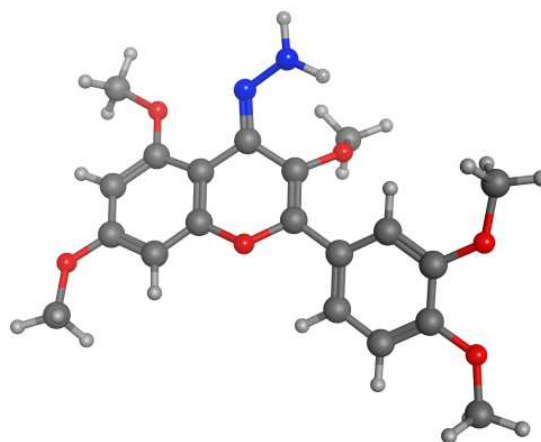


Fig. 5: The molecular structure of compound 3

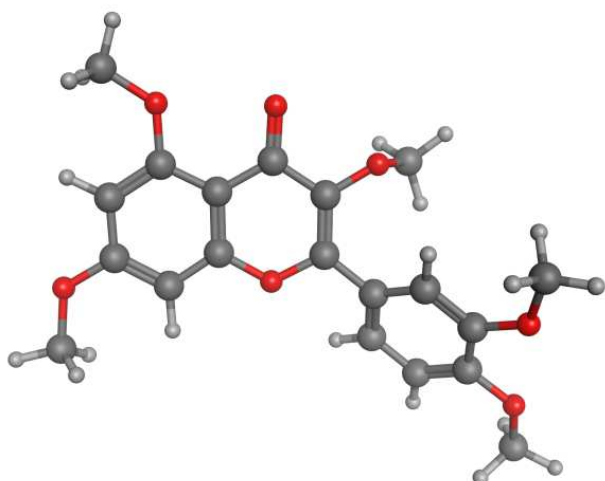


Fig. 3: The molecular structure of compound 1

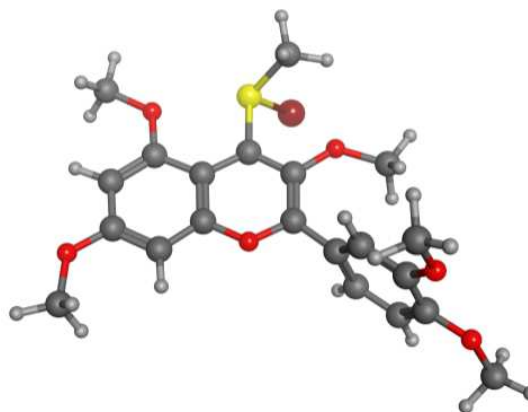


Fig. 6: the molecular structure of compound 4

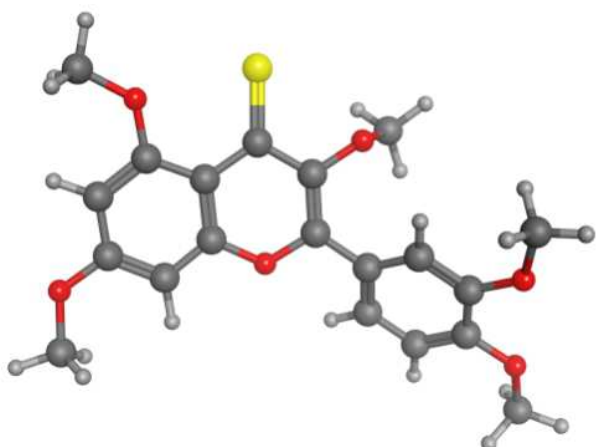


Fig. 4: The molecular structure of compound 2

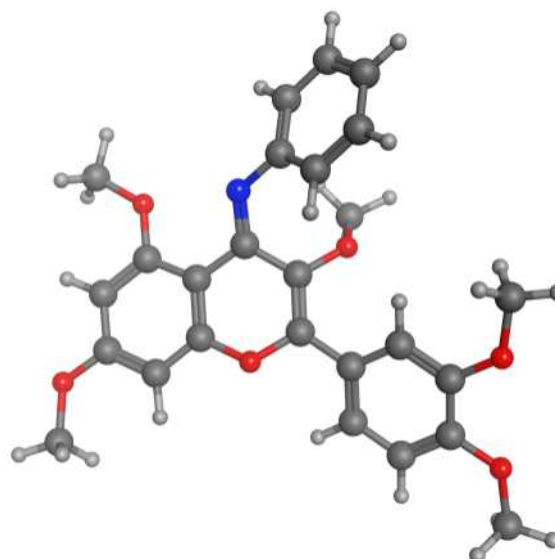


Fig. 7: The molecular structure of compound 5

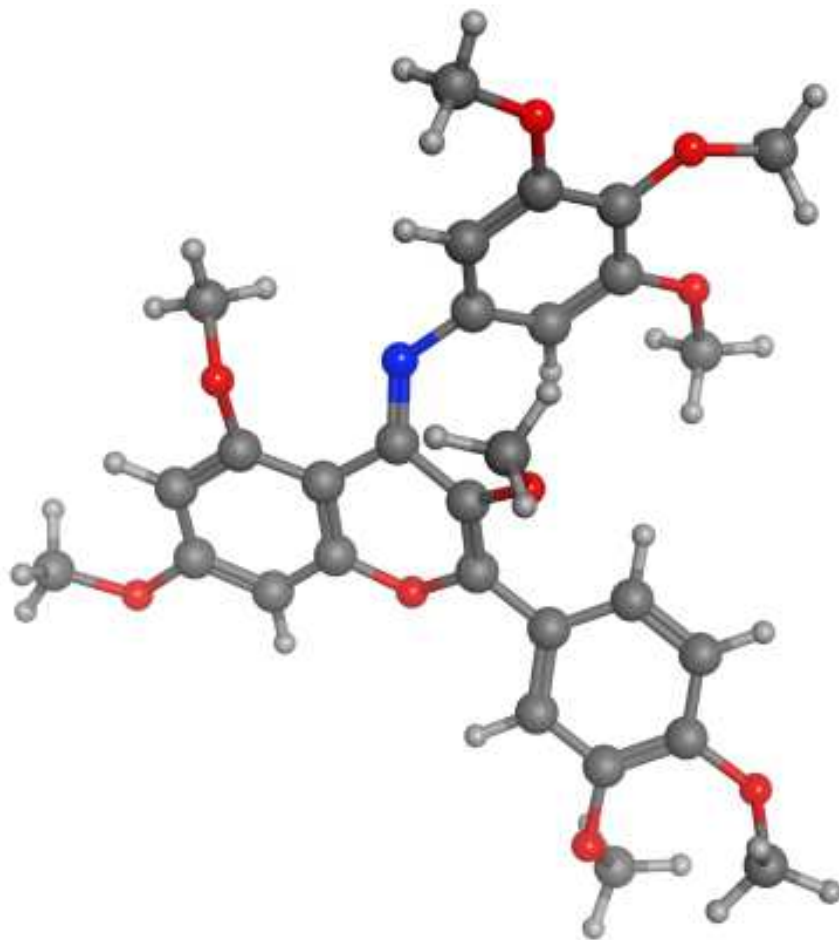


Fig. 8: The molecular structure of compound 6

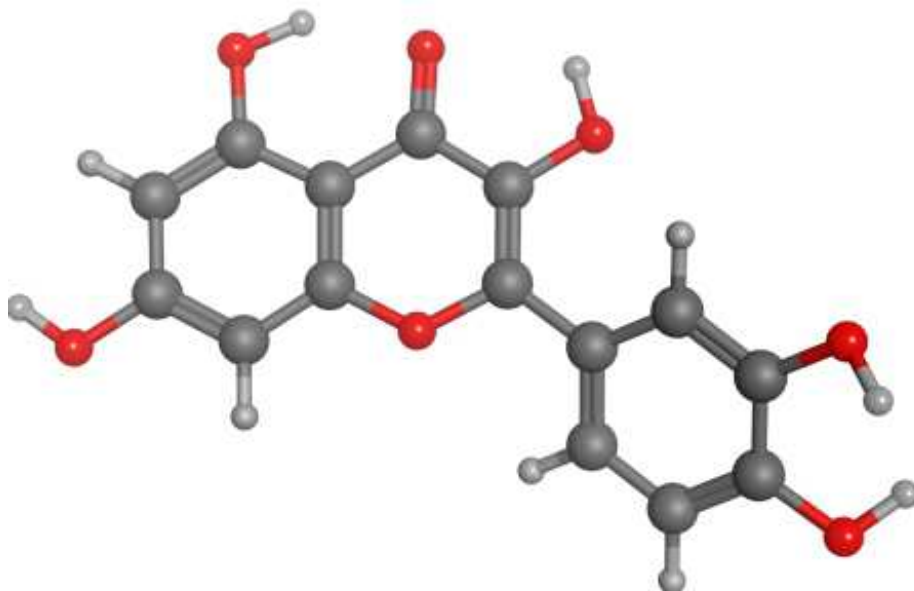


Fig. 9: the molecular structure of Quercetin

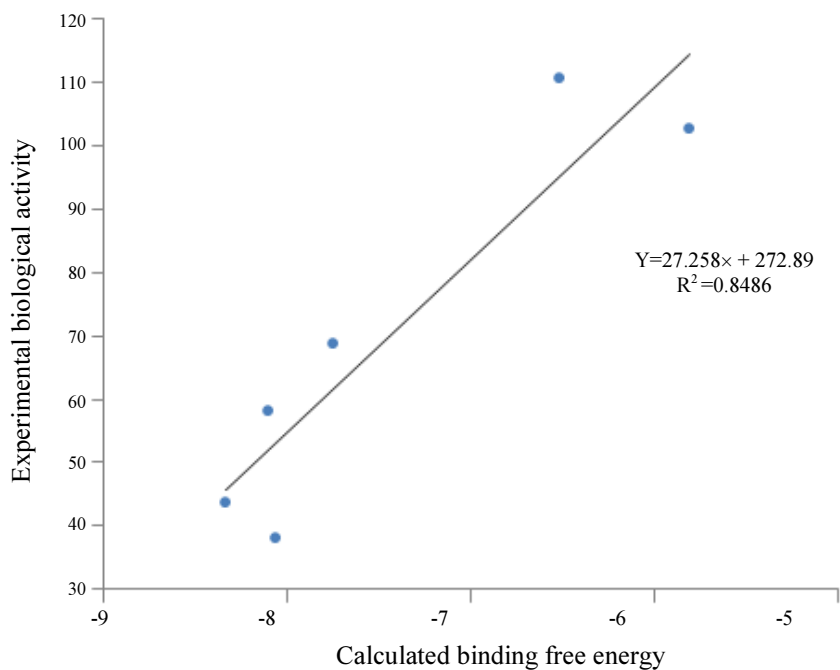


Fig. 10: The calculated binding free energies vs the experimental biological activities of the studied molecules

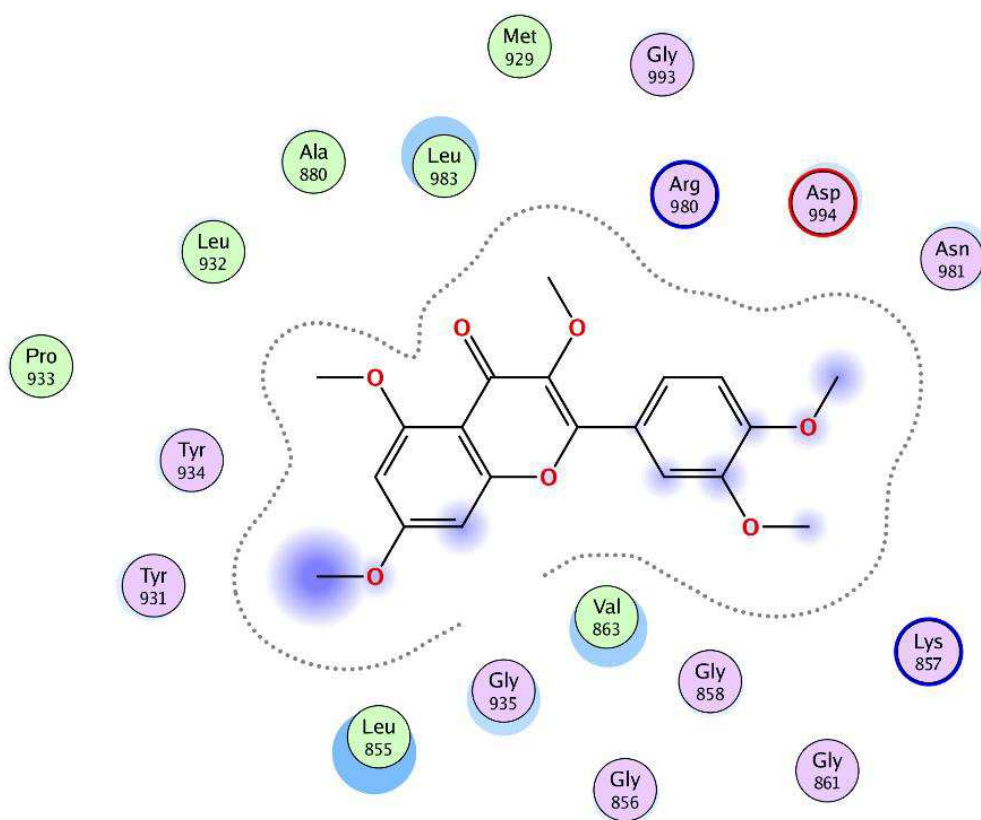


Fig. 11: Ligand interactions of compound 1 with the target protein

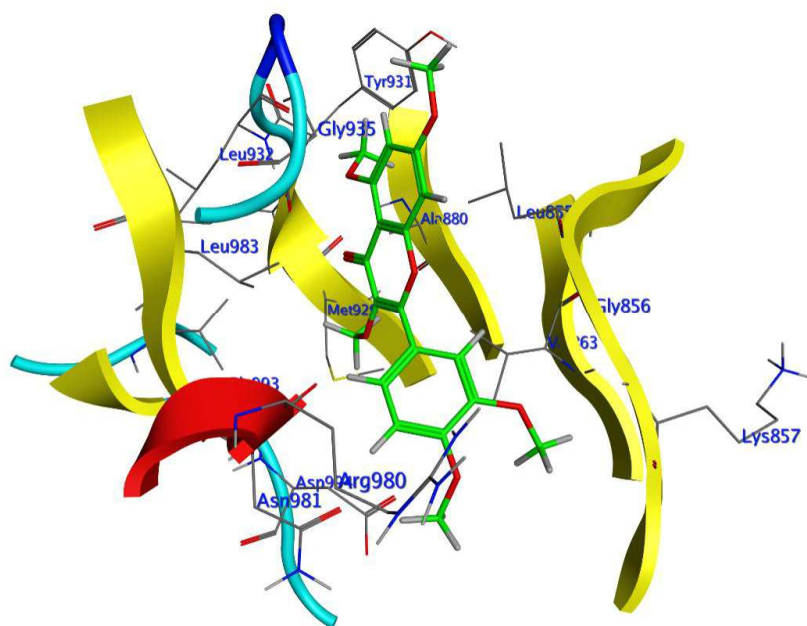


Fig. 12: The active site of compound 1 in the target protein

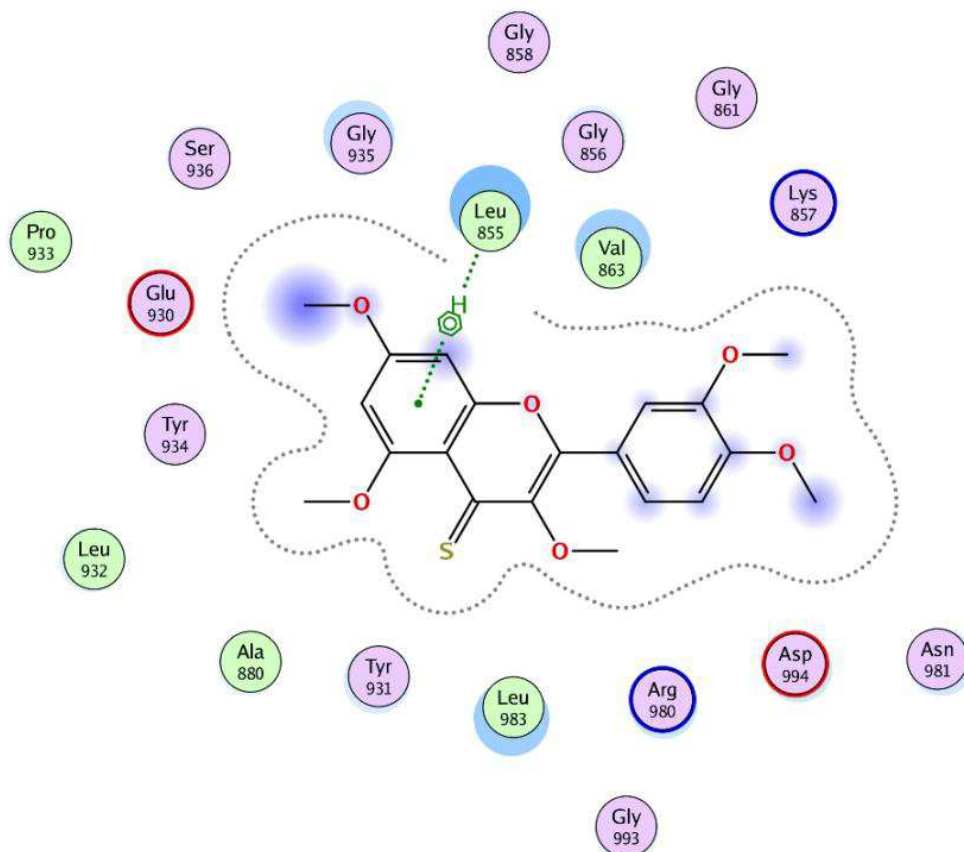


Fig. 13: Ligand interactions of compound 2 with the target protein

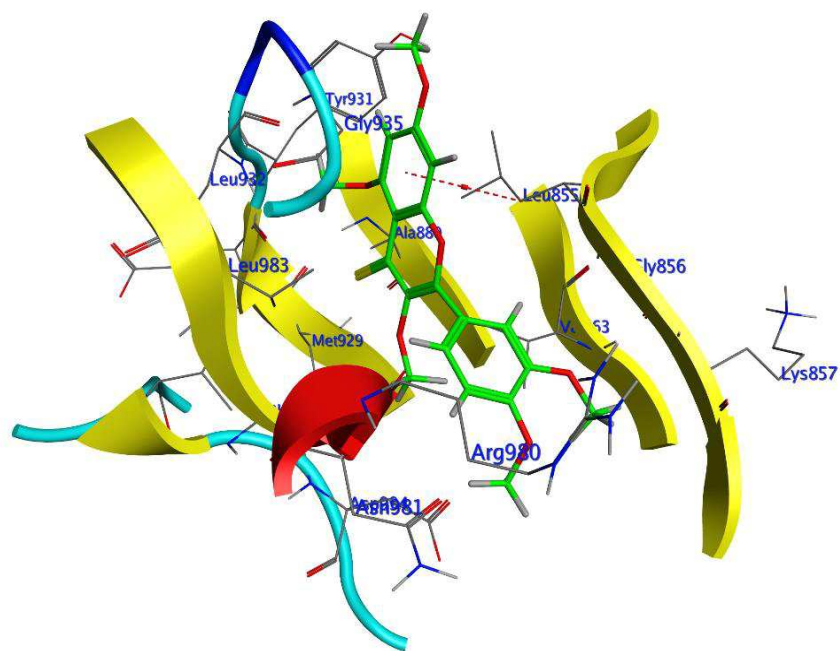


Fig. 14: The active site of compound 2 in the target protein

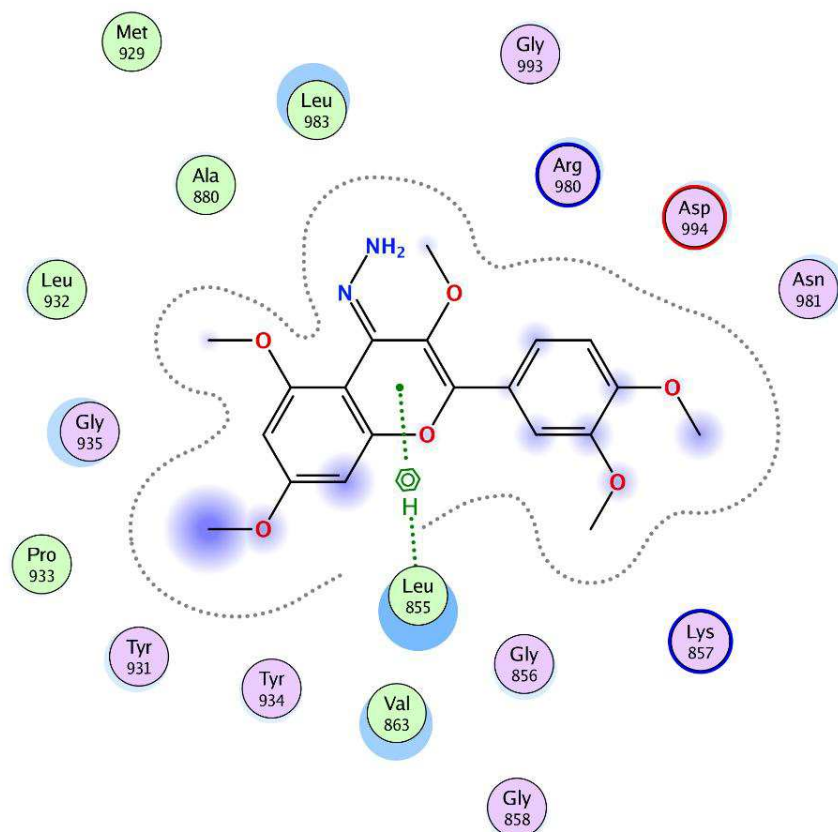


Fig. 15: Ligand interactions of compound 3 with the target protein

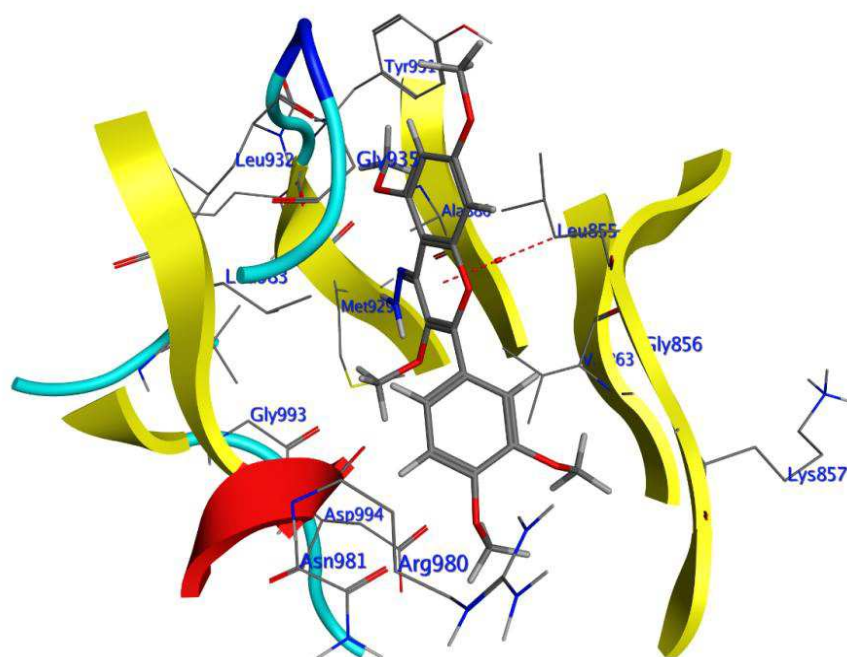


Fig. 16: the active site of compound 3 in the target protein

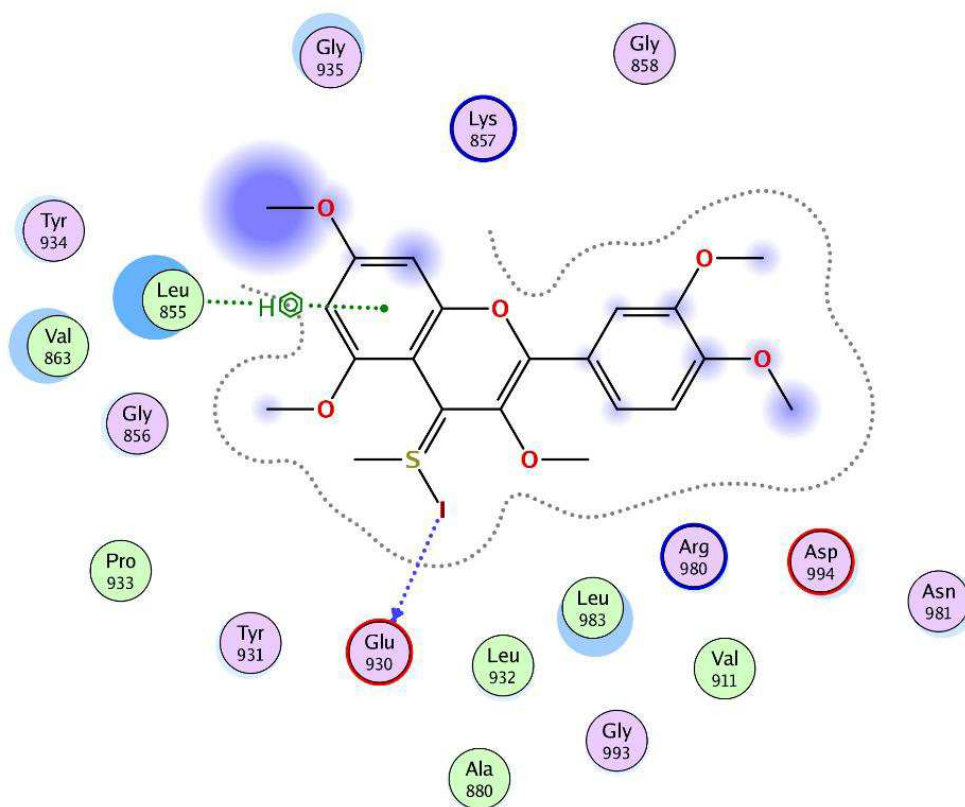


Fig. 17: Ligand interactions of compound 4 with the target

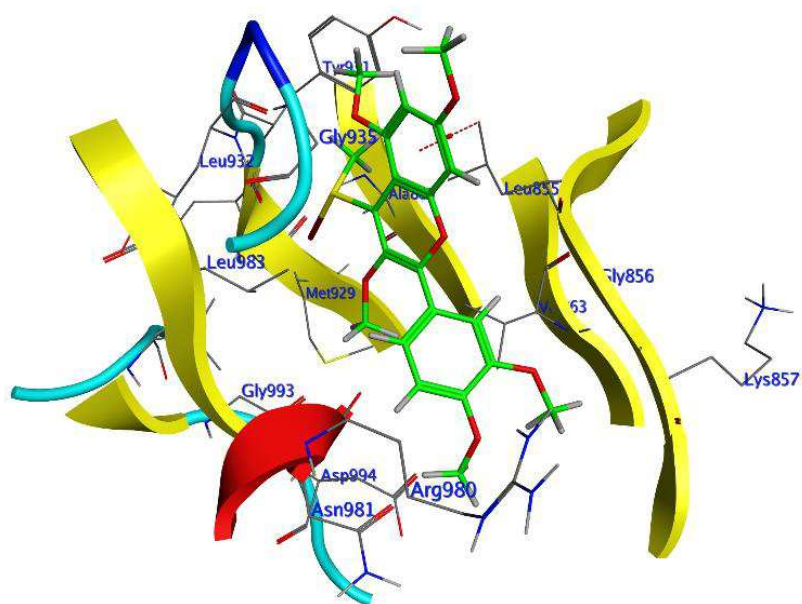


Fig. 18: The active site of compound 4 in the target protein

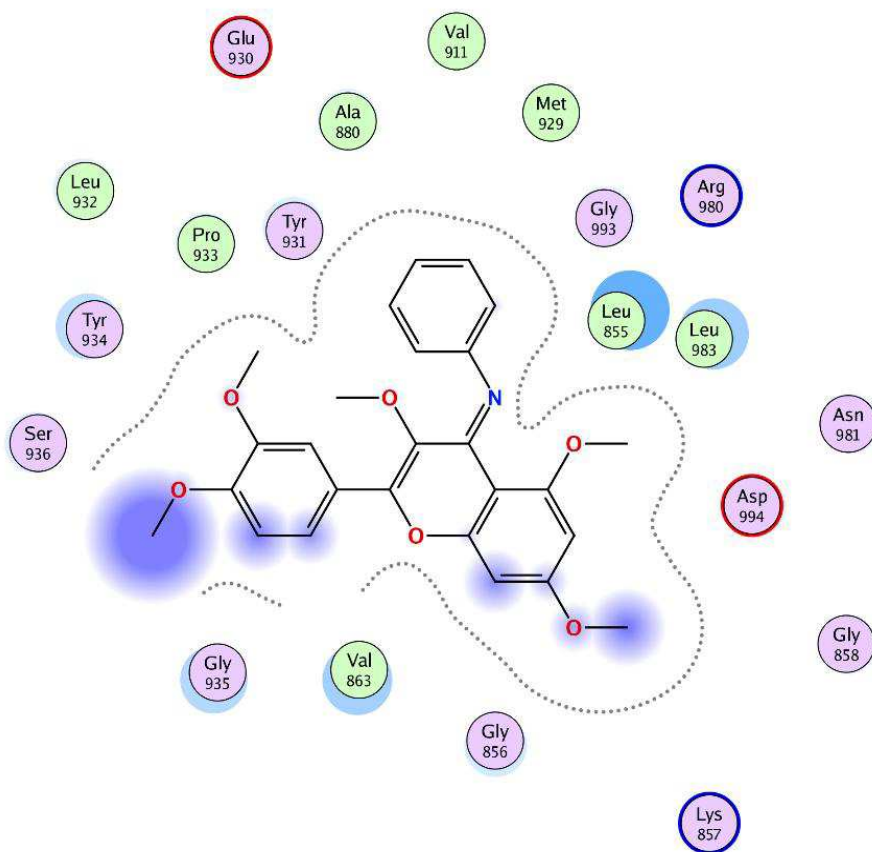


Fig. 19: Ligand interactions of compound 5 with the target protein

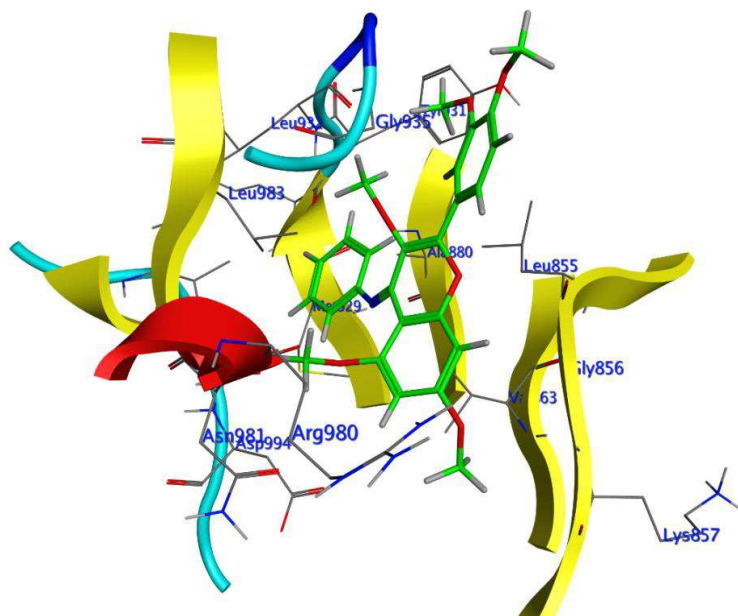


Fig. 20: The active site of compound 5 in the target protein

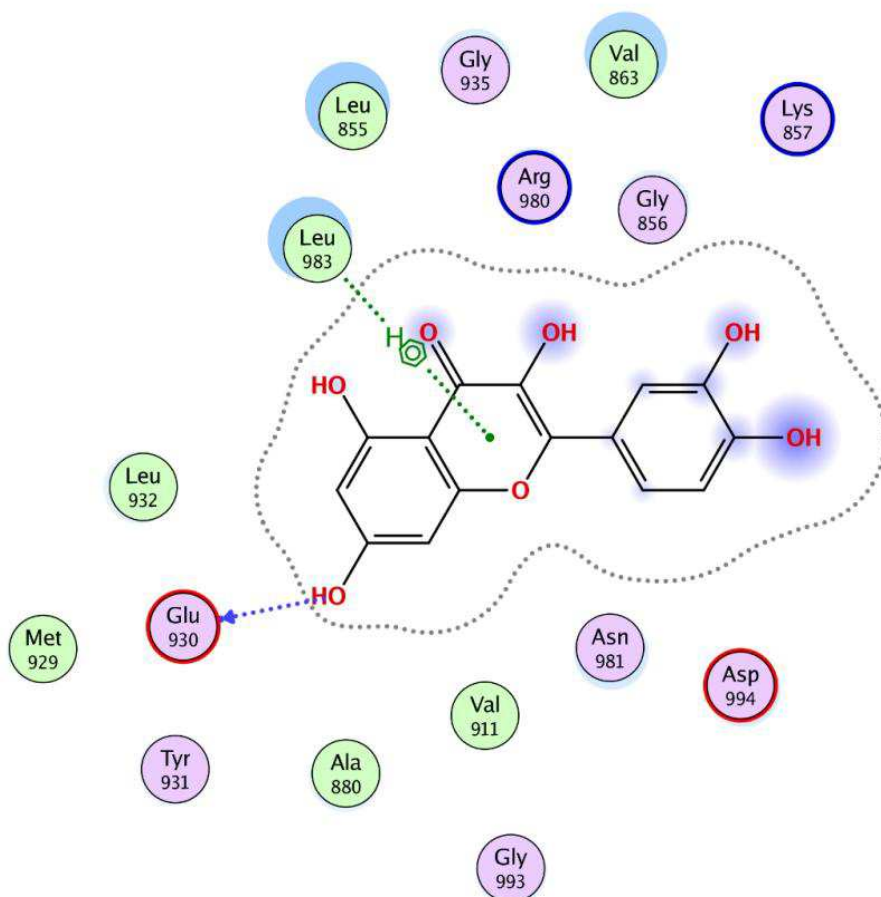


Fig. 21: Ligand interactions of Quercetin with the target protein

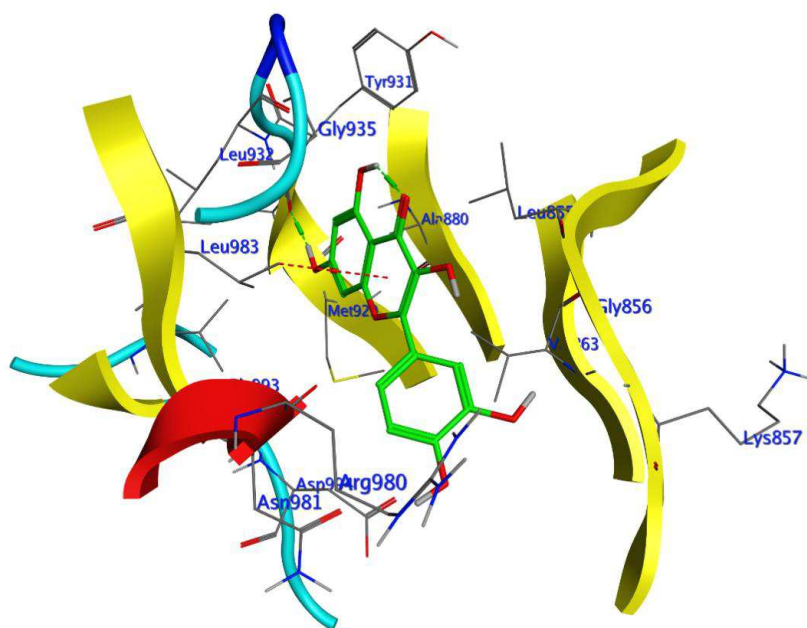


Fig. 22: The active site of Quercetin in the target protein

Table 1: Physical properties and UV-visible spectra of Quercetin and its 1-6 analogues

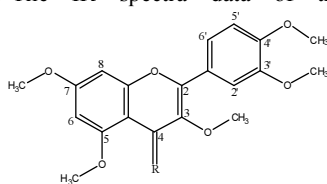
Compounds	Color	Yield %	Melting point °C	λ max nm
Quercetin	yellow	-	314.0-315.0	372.0 257.0
1	white	70	150.0-151.0	339.5 248.0
2	dark green	39	169.2-170.2	397.5 262.5
3	orange	60	163.0-164.0	323.5 289.5
4	dark red	78	156.4-157.4	335.5 265.5
5	yellow	63	126.0-128.0	404.5 265.0
6	yellow	50	149.8-150.1	401.0 265.5

Table 2: Mass spectra and elemental analysis of Quercetin and its 1-6 analogues

Compounds	Molecular weights (g/mole)	Mass (M^+) m/e	Mass ($M+H^+$) m/e	CHNS% Found	CHNS% Calculated
Quercetin	302.40	302 ^a	-	-	-
1	372.00	372.0	-	-	-
2	388.10	388.0	-	C: 60.69 H: 4.958 S: 9.157	C: 61.84 H: 5.19 S: 8.25
3	386.15	386.6	387.15	C: 62.50 H: 5.511 N: 6.68	C: 62.17 H: 5.74 N: 7.25
4	530.00	-	-	C: 46.73 H: 4.38 S: 5.78	C: 47.56 H: 4.37 S: 6.04
5	447.10	447.3	448.17	-	-
6	537.20	537.0	-	-	-

^aas previously reported by (Ice and Wender, 1953)

Table 3: The IR spectra data of the most important absorption bands for Quercetin and its analogues (1-6)



Compounds	OH stretching	NH ₂ stretching	C=O stretching	C=S stretching	C=N stretching	C=C stretching	C-H aliphatic
Quercetin	3437-1800 w, br	-	1666s	-	-	1612vs 1562s 1523vs	-
1	-	-	1627vs	-	-	1604(sh)vs 1516s 1496(sh)vs	2993 2947 2931 2843
2	-	-	-	1253s	-	1620(sh)vs 1589(sh)vs 1519s	2993 2947 2931 2843
3	-	3417m 3387m	-	-	1635s	1600(sh)s 1585(sh)s 1516s	2935 2835
4	-	-	-	1260s	-	1627vs 1573(sh)m 1481s	2981 2935
5	-	-	-	-	1631s	1593s 1554s 1504	2935 2839
6	-	-	-	-	1631s	1593s 1573s 1500s	2839 2939 3001

Vs: very strong, S: strong, m: medium, w: weak, br: broad, sh: shoulder, str: stretching

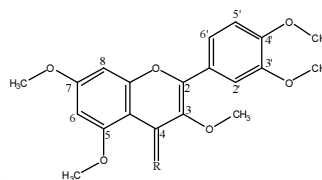


Table 4: ¹HNMR spectra of the Quercetin and its 1-6 analogues

Compounds	Phenolic OH	H6	H8	H2'	H5'	H6'	OCH3	R-substituent
Quercetin	9.25, br s (3H) 10.62, br s (1H) 12.42, s (1H)	6.14s	6.35s	7.63d J _m =1.5	7.87 d J _o =8.4	7.49 dd J _{o,m} =8.4,.5	-	-
1		6.45 d J _m =1.8	6.77 d J _m =1.8	7.62s	7.11d J _o =8.6	7.65 dd J _{o,m} =8.6,1.6	3.70 - 3.87 m	
2		6.42 s	6.53 s	7.86 s	6.99 d J _o =8.5	7.77 d J _o =8.5	3.70 - 3.97 m	
3		6.33 d J _m =1.6	6.46 d J _m =1.8	7.44s	7.0d J _o =9	7.44s	3.51 - 3.83 m	6.9 br,s (NH ₂)
4		5.76s	5.79s	7.08s	6.82d J _o =8.4	7.27d J _o =8.4	3.44 - 3.73 m	1.68s (S-CH ₃)
5		6.37s	6.76s	7.52s	7.09d J _o =8.6	7.6d J _o =8.4	3.7 - 3.83 m	7.14-7.35 m
6		7.07 s	7.46s	7.78s	7.34d J _o =8.8	7.96d J _o =8.4	3.25-4.26 m	7.07s

Table 5: Percentage of breast cancer cell line MDA-MB231 cells that remain viable after being treated with Quercetin and its analogues under appropriate conditions

Compounds	viable cells %		viable cells %		IC ₅₀ value (μM)
	1	2	3	(mean)	
Control	100.00000	100	100	100	-
Quercetin	101.44930	113.42975	117.4668	110.7	-
1	36.56633	42.355372	52.196118	43.7	2.042
2	62.76477	68.698347	74.668029	68.6	-
3	55.74136	57.438017	60.980592	58	-
4	31.21516	38.739669	44.53524	38.1	1.838
5	101.08110	104.34783	102.79627	102.7	-
6	-	-	-	-	-

Table 6: The experimental biological activities, the calculated binding free energies (S) and the predicted biological activities

Compounds	Experimental activity	S (Binding free energy)	Predicted activity	Residue
1	43.7	-8.3382	45.6	-1.9
2	68.6	-7.7526	61.6	7.0
3	58.0	-8.1026	52.0	6.0
4	38.1	-8.0665	53.0	-14.9
5	102.7	-5.811	114.5	-11.8
6	-	-6.5934	-	-
Quercetin	110.7	-6.5222	95.1	15.6

Table 7: The predicted active sites of the studied compounds within the target protein

Compounds	Amino acids that represents the active site
1	Pro933, Leu932, Ala880, Leu983, Met929, Leu855, Val863, Gly993, Arg980, Asp994, Asn981, Tyr934, Tyr931, Gly935, Gly856, Gly858, Gly861, Lys857.
2	Glu930, Pro933, Ser936, Gly935, Leu855, Gly858, Gly856, Val863, Gly861, Lys857, Tyr934, Leu932, Ala880, Tyr931, Leu983, Arg980, Asp994, Asn981, Gly993.
3	Leu932, Ala880, Met929, Leu983, Gly993, Arg980, Asp994, Asn981, Gly935, Pro933, Tyr931, Tyr934, Val863, Leu855, Gly856, Lys857, Gly858.
4	Leu855, Tyr934, Val863, Gly856, Gly935, Lys857, Gly858, Pro933, Tyr931, Glu930, Leu932, Leu983, Arg980, asp994, Ala880, Gly993, Val911.
5	Ser936, Tyr934, Leu932, Pro933, Tyr931, Glu930, Ala880, Val911, Met929, Gly993, Arg980, Leu855, Leu983, Asp994, asn981, Gly858, Gly935, Val863, Gly856, Lys857.
6	Leu983, Leu855, Gly935, Arg980, Val863, Gly856, Lys857, Leu932, Glu930, Met929, Tyr931, Ala880, Val911, asn981, Gly993, Asp994.
Quercetin	Leu983, Leu855, Gly935, Arg980, Val863, Gly856, Lys857, Leu932, Glu930, Met929, Tyr931, Ala880, Val911, asn981, Gly993, Asp994.

Discussion

Synthesis

The unique chemical structure and the characteristic distribution of the hydroxyl groups within the Quercetin molecule made direct conversion of the carbonyl group to its corresponding imine through general Schiff base synthesis method proposed by (Chakraborti *et al.*, 2004) very difficult process, since the efficacy of this method involve the attack of highly electrophilic carbonyl group by highly nucleophilic amine group and thus the carbonyl should be activated first through the addition of Bronsted-Lowery or Lewis acid (LA) that catalyze the amine nucleophilic attack (Chakraborti *et al.*, 2004; da Silva *et al.*, 2011). This activation cannot be obtained within Quercetin molecule, because its carbonyl group form strong hydrogen bonding with C-5 hydroxyl group

along with the presence of the C2=C3, result in strongly polar boundary structure of the Quercetin molecule in which (+) charge on the ring oxygen atom and (-) charge on the carbonyl oxygen atom, thus the carbonyl oxygen atom became easily protonated (Heneczowski *et al.*, 2001). Once the acid catalyst being added, protonation of the carbonyl oxygen would take place and imine formation not possible. Therefore, in order to synthesize Quercetin Schiff base derivatives, Quercetin hydroxyl groups were first methylated, followed by thionation of the carbonyl group using Lawesson's reagent and finally reaction with different primary amines either directly or indirectly by treating the thionated product with methyl iodide in order to get the highly reacted 4-thiomethyl iodide derivative followed by reaction with primary amine as described previously.

Identification

The electronic spectra of quercetin and its 1-6 analogues show similar origin absorption bands of $\pi \rightarrow \pi^*$ transitions source and the differences within the position of these clearly indicate that structural modification within the Quercetin molecule had taken place. The blue shift within the spectra of compound **1** by 32 nm compared to Quercetin is attributed to the replacement of hydroxyl groups by methoxy groups. The hydroxy groups are responsible to the formation of quinone structure that extend the molecule π system and subsequent red shifting of the absorption bands compared to the methoxy groups. In addition, methylation of hydroxyl groups especially that in position C-3 cause a change in the planarity of the B-ring with the rest of Quercetin molecule, which reduce the conjugation between ring B and A with a consequence reduction in the length of the chromophore and λ_{\max} (Heim *et al.*, 2002). On the other hand, a red shift is noticed in the spectrum of compound **2** compared to that of **1** by 58 nm caused by replacing the carbonyl group in compound **1** by thione group in compound **2** (Abboud *et al.*, 1993). A blue shift by 16 nm when going from compound **1** to compound **3** where the position of the long absorption band shift from 339 to 323 nm and this is expected when the carbonyl group is replaced by an imino group, which usually lead to blue shift in the location of band associated with these group (Brodowska *et al.*, 2016), while both compounds **5** and **6** show a red shifting in their long absorption band by about 80 nm compared to compound **3**, that is referred to the aryl group substitution on the imino group nitrogen atom which extend the π system. Compound **4** shows a blue shift within band I by about 4 nm and 62 nm compared to compounds **1** and **2** respectively.

The mass spectra of the compounds **1-3**, **5** and **6** characterized by molecular ion peaks of moderate abundances at m/z 372, 388, 386.6, 447.3 and 537 corresponding to the calculated molecular weights of these compounds which are 372, 388, 386, 447 and 537 g/mole respectively. This confirms the expected molecular structures of studied compounds.

The elemental analysis CHNS results of compounds **2**, **3** and **4** revealed that these compounds were of good purity and in agreement with the expected chemical formula.

The important IR bands of quercetin and compounds **1-6** were shown in Table 3. The absence of the broad wide band of the hydroxyl group in the region 3437-1800 cm^{-1} (Paczowska *et al.*, 2015) of the Quercetin molecule along with the presence of a set of weak bands near 3000 cm^{-1} which attributed to the C-H bond stretching vibration, indicates that all the hydroxyl groups were methylated. Compound **1** is also confirmed by the shift of the carbonyl band at 1666 cm^{-1} of the

Quercetin molecule to 1627 cm^{-1} . On the other hand, the presence of bands at 1253 and 1260 cm^{-1} in compound **2** and **4** respectively confirms the presence of the C=S (Larkin, 2011). Compound **3** shows two medium intensity bands at 3417 and 3387 cm^{-1} that are attributed to the antisymmetric and symmetric N-H stretching vibrations (Karabacak *et al.*, 2009; Larkin, 2011). The disappearance of the C=S band and appearance of the C=N at 1627 cm^{-1} further confirms the formation of compound **3** such finding was also noticed within the IR spectra of compounds **5** and **6** and their C=N bond gave peak at 1631 cm^{-1} (Mughal *et al.*, 2006).

The ^1H NMR spectrum of the quercetin (Table 4) involve the presence of three signals at 9.2, 10.4 and 12.4 ppm that belongs to the phenolic protons at positions (3', 3, 4'), 7 and 5 respectively (Napolitano *et al.*, 2012). The disappearance of the hydroxyl signals in the ^1H NMR spectra of the compounds **1-6** and the existence of new signals at 3.7 ppm which assigned to the methoxy group confirm the methylation of these hydroxyls. Compound **3** shows broad signal at 6.9 ppm that is due to the protons of the amino group, while the spectrum of compound **4** shows a signal at 1.68 ppm due to the protons of the S-CH₃ group (Breinmaier, 2003). The presence of the aromatic groups in the compound **5** and **6** give rise to signals at 7.14-7.35 ppm in compound **5** spectrums and a singlet at 7.07 ppm. Compound **6** also shows additional signals within the aliphatic proton region that belong to the three additional methoxy groups at 3.25-4.26 ppm.

In Vitro Antiproliferative Activity

The MDA-MB231 cell line has been chosen because TNBC represents a real challenge with its limited and non-specific treatment options, aggressiveness, high recurrence and poor survival rate. Patients usually suffer from relapse after 3-5 years of clinical intervention and resistance to chemotherapy can develop, so the need to develop new therapeutic agents to treat this breast cancer type is urgent (Dean and Rhodes, 2014). Since quercetin induces apoptosis and cell cycle arrest in TNBC cells and show advantages including no or less effect on normal cells, effectiveness in killing cancer cells, improvement in cancer relapse as well as enhancement of chemotherapy, it can be considered as a potential therapeutic agent against TNBC cells (Nguyen *et al.*, 2017).

Results of the preliminary antiproliferative activity study of Quercetin and its derivatives (**1-5**) indicates that Quercetin have no antitumor activity against MDA-MB231 cell line of breast cancer cells at tested concentrations, which agrees with previous studies (Ranganathan *et al.*, 2015), however, Quercetin penta methyl ether derivatives (**1-5**) show antitumor activity against MDA-MB231 cell line of breast cancer cells

suggesting a positive correlation between hydroxyl groups protection and antiproliferative activity against MDA-MB231 cell line.

The activity results show the importance of the presence of carbonyl group within the structure of the Quercetin pentamethylated molecule since compound **1** show more potent inhibition (56.3%) than the thio analogue (**2**) and the imino analogues (**3** and **5**) which have potent inhibition values of 31.4%, 42% and 0% respectively. On the other hand, compound **4** gave even better results than compound **1** with 61.9% reduction to the growth of MDA-MB231 cell line, which could be attributed exhibited to the presence of iodine atom within its structure, probably in an antioxidant related mechanism (Rösner *et al.*, 2016). IC₅₀ value results shown in Table 4 clearly indicate that the novel compound **4** is more active than compound **1**, although both are considered moderately active against MDA-MB231 cell line (Prachayasittikul *et al.*, 2015).

Results from Molecular docking assay correlate well with our practical finding and as could be seen from table 6 the program fairly predicted the relative activities of the studied compounds as compared with the experimental activity values. This is also clear in Fig.3 which shows the relationship between the calculated S values and the experimentally measured activities. In this case the R² value of the correlation is 0.849 which is considered very well from the statistical point of view. The correlation equation is:

$$y = 27.258x + 272.89 \quad (2)$$

Where:

y = The biological activity

x = The calculated binding free energy

This equation is used in this study to predict the biological activities of the studied compounds depending on their calculated binding free energies which are the results of the docking calculations. The results are shown in Table 6 under the predicted activity column. The predicted values are fair and can give an approximate idea about the biological activities. The higher activity of compound **1** might be attributed to the presence of bulky groups that are substituted at the carbonyl group in the other compounds which create a steric hindrance. This will lead to a lack of fit in the cavity of the target protein and accordingly show lower interaction with protein.

Conclusion

On the basis of electronic, mass, IR and ¹HNMR spectra and the elemental analysis the compounds **1-6** were characterized. The antiproliferative activity against MDA-MB-231 cell line have shown that the pentamethoxy Quercetin Schiff base and thiomethyl

iodide derivatives have better antiproliferative activity than quercetin itself and that compounds **1** and **4** have potential anticancer therapeutic applications against the triple negative breast cancer type. Moreover, the molecular docking assay correlates well with our experimental findings.

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Author's Contributions

Reham Adnan Taha: Msc student of Prof. Shaker Abdul Salim Neama and Assist prof. Rita S Elias, carried out the experiment and designing the table, figures.

Rita S. Elias: Supervisor of this work/including designing the project of the work, analyzed the data and wrote the paper.

Shaker Abdul Salim Neama: Supervisor of this work/designing the project of the work.

Ethics

This article is original and contains unpublished study. The corresponding author confirms that all the authors have read and approved the manuscript and no ethical issues involved.

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