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Rutin and isoflavones with cytotoxic activity from soybean, and it's *in silico* docking study against ERK2 kinase.

Ahmed Sami, Ali M. S. Hebishy, and Mohamed S. Abdelfattah*.

Natural Products Research Unit (NPRU), Chemistry Department, Faculty of Science, Helwan University, Egypt.

ABSTRACT

Rutin (3,3',4',5,7 pentahydroxy flavone-3-rhamnoglucoside) and isoflavones (daidzein and genistein) has been isolated from the ethanolic extract of soybean. The anticancer activities of the compounds **1**-**3** were evaluated against human breast adenocarcinoma cell line (MCF-7), liver cancer cell line (HepG-2) and epithelial colorectal adenocarcinoma cells (CaCO-2). The results of the cytotoxic activity revealed that the genistein (**3**) with was active against CaCO-2 cancer cells (IC_{50} = 13.8 µg/ml). Rutin was also active against HepG-2 cell line (IC_{50} = 23.3 µg/ml). A computational docking study for compounds **1**-**3** against ERK2 kinase was performed to formulate a hypothetical mechanism by which the tested compound inhibits the growth of cancer cells.

Keyword: Soybean, isoflavone, chemical analysis, anticancer activities.

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Corresponding author

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INTRODUCTION

Flavonoids are a large family of over 5,000 hydroxylated polyphenolic compounds that carry out important functions in plants, including attracting pollinating insects; combating environmental stresses, such as microbial infection; and regulating cell growth [1]. In higher plants, they are involved in symbiotic nitrogen fixation and floral pigmentation. Several interest in flavonoid derivatives has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [2,3]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both in vivo and in vitro systems [4]. Flavonoids have ability to induce human protective enzyme systems. The number of studies has suggested protective effects of flavonoids against many such as cancers [5]. As per literature, flavonoids are classified into 12 major subclasses based on chemical structures, six of which, namely anthocyanidins, flavan-3-ols, flavonols, flavones, flavanones, and isoflavones are of dietary significance [6]. Glycosylated flavonols (bound to at least one sugar molecule) are the most widely distributed flavonoids in the diet [7,8].

Soybean (Glycine max) is a rich source of multiple classes of bioactive components with isoflavones (primarily genistein and daidzein) receiving considerable attention regarding the inhibition of inflammation and cancer prevention [9]. The soy components that have stimulated the most research interest are flavones and isoflavones, which are polyphenols with estrogenic properties highly contained in soybeans. In our study, we isolate rutin and isoflavones from soybean crude extract and evaluate of their anticancer activities against different cancer cells as well as docking their structures inside the active site of human ERK2 kinase to detect their binding mode and docking score.

MATERIALS AND METHODS

Sample collection

Soybean seeds was purchased from the Egyptian local markets during May 2018. Seeds were collected, air dried and grounded into powder by using grinder.

Preparation of the crude extract

Fifty grams of soybean flour was mixed with 250 ml of 70% ethanol (v/v) under reflux at 55 ºC with continuous stirring for 24 hours. The extract was then centrifuged at 3000 rpm for 10 minutes to remove the residues. The ethanol was evaporated under vacuum.

Isolation of rutin and isoflavones from the aqueous layer of soybean extract

After evaporation the ethanol under reduced pressure, the aqueous layer was collected and leave to stand for three days. A yellow precipitate separated out of the solution was observed. The precipitate was filtrated and washed three times with dichloromethane: ethyl acetate (2:1) to give 500 mg of a yellow solid. The residue of the aqueous layer was adsorbed on silica gel and subjected to column chromatography (30 × 500 mm) on silica gel. The elution was performed using DCM only, then DCM-MeOH gradients (1%, 2%, 4%, 6%, 8%, 10%, 15%, 20%, 30%, 50%) and finally washed with MeOH. After controlling by TLC (DCM/ 5% MeOH), four fractions were obtained. Compound **2** was purified by PTLC (3 plates, 20 × 20 cm, DCM/5%MeOH). Fraction **III** showed on TLC a one strong UV absorbing zone at 254 nm. The fraction was dissolved in a minimum volume of MeOH and applied to PTLC (5 plates, 20 × 20 cm, DCM/12%MeOH) to give compound **3**.

Compound 1: Yellow solid, ^{1}H NMR (DMSO-d_6, 100 MHz) δ 12.60 (s, 1H), 7.55 (dd, *J* = 2.1 Hz, 7.5 Hz, 2H), 6.83 (d, *J* = 8.7 Hz, 1H), 6.38 (d, *J* = 2.1 Hz, 1H), 6.19 (d, *J* = 2.1 Hz, 1H), 5.30 (t, *J* = 7.2 Hz, 9.9 Hz, 2H), 5.09 (t, *J* = 7.8 Hz, 5.4 Hz, 2H), 4.38 (brs, 4H), 3.70 (d, *J* = 9.9 Hz, 1H), 3.29-3.06 (m, 6H), 0.99 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 157.1 (C-2),133.8 (C-3),177.8 (C-4), 157.1 (C-5), 99.1 (C-6), 164.6 (C-7), 94.1 (C-8), 161.7 (C-9), 104.4 (C-10), 122.1 (C-1'), 115.7(C-2'),145.2 (C-3'), 148.9 (C-4'), 116.7 (C-5'),121.6 (C-6'),101.2 (C-1''),74.5 (C-2''), 76.9 (C-3''), 72.3 (C-4''), 76.4 (C-5''), 67.5 (C-6''), 101.6 (C-1'''), 70.5 (C-2'''), 71.0 (C-3'''), 71.8 (C-4'''), 68.7 (C-5'''), 18.2 (C-6''').

Compound 2: White solid, (+)-ESI-MS **:***m/z* = 255.05 ([M+H]$^+$) and 531.05 ([2M+Na]$^+$); **^{1}H-NMR ([D$_6$] DMSO, 400 MHz**): δ_H 10.89 (1H, 7-OH), 9.54 (1H, 4'-OH), 8.27 (1H, s, H-2), 7.96 (1H, d, *J* = 6.6 Hz, H-5), 7.37 (2H, dd, *J* = 6.8 and 1.2 Hz, H-2', H-6'), 6.93 (1H, dd, *J* = 6.6 and 1.3 Hz, H-6), 6.84 (1H, d, *J* = 1.3 Hz, H-8), 6.75 (1H , dd , *J* = 6.8 and 1.2 Hz, H-3', H-5').

Compound 3: White solid, EI-MS (70 eV) **:***m/z* = 270 (M$^+$), **^{1}H-NMR (acetone-d$_6$):** δ_H 13.02 (1H, s, 5-OH), 8.22 (1H, s, H-2), 7.49 (2H, d, *J* = 8.2 Hz, H-2', H-6'), 6.69 (2H, d, *J* = 8.2 Hz, H-3', H-5'), 6.45 (1H, d, *J* = 1.15 Hz, H-8), 6.29 (1H, d, *J* =1.15 Hz, H-6).

Cytotoxicity study

Human breast adenocarcinoma cell line (MCF-7), liver cancer cell line (HepG-2) and epithelial colorectal adenocarcinoma cells (CaCO-2) were obtained from the American Type Culture Collection. The cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L of glucose, 4 mmol/L of l-glutamine, and 10% heat-inactivated fatal calf serum.

Cell viability assay

The cytotoxic activity of compounds (**1**-**3**) was evaluated against the human breast adenocarcinoma cell line (MCF-7), liver cancer cell line (HepG-2) and epithelial colorectal adenocarcinoma cells (CaCO-2) using MTT method [10]. The cells were seeded in 96-well plates (4×10^4 cells per well) with 100 µl of Dulbecco's modified Eagle's medium. Cells were incubated at 37°C in a 5% CO_2 incubator for 24 h. Compounds at different doses were added to each well. After 24 h incubation, the culture media was removed and the cells were washed with 100 µl phosphate-buffered saline. Cells were fixed by adding 100 µl of 70% ethanol and incubated at room temperature for 15 min. After removal of ethanol, 100 µl of methylene blue dye was added. The plates were incubated at room temperature for 15 min. To remove the excess of dye, the plate washed three times with tap water and then incubated for 2 h at 37 °C. Dye was eluted from the attached cells by adding 100 µl of 0.1 mol/L HCl in each well and then incubated for 5 min at room temperature. The developed blue color was measured using a microplate reader at 630 nm. Controls referred to wells containing only cells and medium with and without 10% dimethyl sulfoxide. Doxorubicin was used as a positive control.

Docking experiment

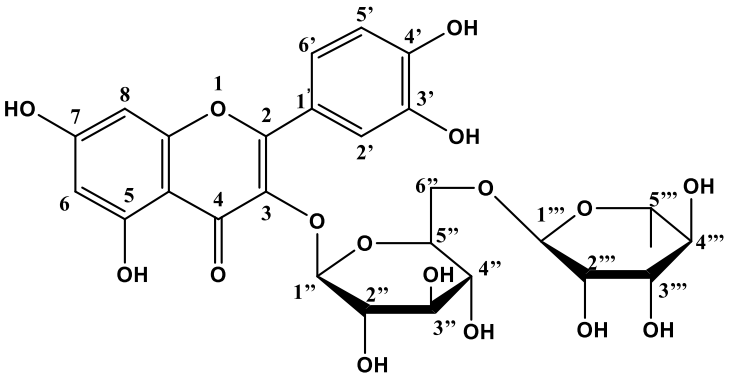
Crystal structure of the human extracellular signal-regulated protein kinase (ERK2) was obtained from the Protein Data Bank with PDB code of 5BUJ. Docking of compounds 1-3 was carried out using Schrodinger 16.4 software Glide's Extra Precision (XP) [11]. Compounds were assembled by using Maestro 9.2 and LigPrep 2.4 software. Parameters of the molecular docking were set to the default hard potential function. The active positions were set within 10 Å radius around the ligand found in ERK2 structures. All docking calculations were carried out using the grid-based MolDock score (GRID) function with 0.30 Å grid resolution. The binding sites of the protein were determined using MVD cavities prediction. The binding site was set inside a restriction sphere of 20 Å radius.

RESULTS AND DISCUSSION

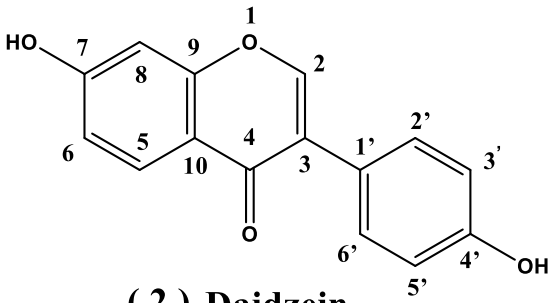
Isolation and structure elucidation of compounds 1-3

Compound **1** was isolated from soybean flour as a yellow precipitate. The UV spectrum of **1** (Figure S1) exhibited absorption bands at 210, 255 and 362 nm. The ^{1}H NMR spectrum (Figure S2) of **1** showed the presence of one chelated hydroxyl group at δ 12.60 (s, 1H) and five aromatic protons at δ 7.55 (dd, *J* = 2.1 Hz, 7.5 Hz, 2H), 6.83 (d, *J* = 8.7 Hz, 1H), 6.38 (d, *J* = 2.1 Hz, 1H), 6.19 (d, *J* = 2.1 Hz, 1H). Additionally, several signals for two sugar units at δ 3.70-3.21 (m, 6H of sugar moieties), 3.70 (d, 1H-Rham), 0.99 (3H, d, CH_3 -Rham), 5.09-4.38 (4H, H-1 Glu) and 5.30 (1H, d, H-6) were observed. The ^{13}C NMR spectrum (Figure S3) of **1** depicted twenty-seven carbon signals, fifteen of them may be due to the flavonol moiety. The spectrum also showed nine oxygenated carbon atoms (δ_c 76.9-67.5), two anomeric carbon signals (δ_c 101.6 and 101.2) and one methyl group (δ_C 18.2) of rhamnose. Based on the above data, compound **1** was identified as rutin (Figure **1**) and confirmed by comparing its spectroscopic data with the literature [12]. Compound **2** was isolated from fraction **II** as a white solid. The molecular weight of **2** was established to be 254 Daltons by ESI-MS (Figure S4). It gave a *quasi*-molecular ion at

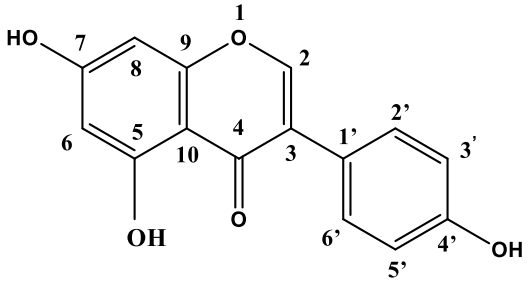
m/z = 255 $[M+H]^{+1}$ and 531 $[2M+Na]^{+1}$, respectively. The ^{1}H NMR spectra of compound **2** (Figure S5) showed the presence of two OH groups at δ 9.54 (s, 4'-OH), 10.89 (s, 7-OH), one proton at δ 8.27 (s, H-2), two sets of aromatic protons at δ 7.37 (2H, dd, *J* = 6.8 and 1.2 Hz, H-2', H-6') and 6.75 (1H , dd , *J* = 6.8 and 1.2 Hz, H-3', H-5'). Additionally, the spectra revealed three aromatic protons at δ 7.96 (d, *J* = 6.6 Hz, H-5), 6.93 (dd, *J* = 6.6 and 1.3 Hz, H-6) and 6.84 (d, *J* = 1.3 Hz, H-8) of a 1,2,4-trisubstituted benzene moiety. Based on the molecular weight and ^{1}H NMR data, the isolated compound could be identified as daidzein (**2**) (Figure **1**). The structure of **2** was confirmed by comparison with the literature value [13]. The isolated isoflavone had antifungal activity [14], and induced DNA breaks in the human tumor cell line HT29 effects [15]. Some studies showed that, daidzein stimulate the growth of breast cancer cells [16-17]. Fraction **III** was purified by PTLC (DCM/12% MeOH) to give a colorless solid with a strong absorbance under UV light at 254 nm. The molecular weight of **3** was found to be 270 Daltons based on EI-mass data. The ^{1}H NMR spectrum of **3** (Figure S6 and S7) revealed three OH groups [□□12.96 (s, 5-OH), 10.88 (brs, 7-OH) and 9.63 (brs, 4'-OH)], one singlet [□□8.30 (s, H-2)], two doublets each with the relative intensity two [□□7.38 (d, *J* = 8.2 Hz, H-2', H-6') and □□6.83 (d, *J* = 8.2 Hz, H-3', H-5')] as well as two *meta* coupled aromatic protons [□□6.39 (d, *J* = 1.15 Hz, H-8) and □□6.23 (d, *J* =1.15 Hz, H-6)]. Searching in Dictionary of Natural Products using molecular weight and ^{1}H NMR data led to genistein (**3**) (Figure **1**). Genistein is an isoflavone present in soy and is known to have multiple molecular effects, such as the inhibition of inflammation, promotion of apoptosis, and modulation of steroidal hormone receptors and metabolic pathways [18].



(1) Rutin



(2) Daidzein



(3) Genistein

Figure 1: Structure of compounds 1-3.

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Anticancer activity of compounds 1-3

The *in vitro* cytotoxic activity of compounds (**1**-**3**) were evaluated against liver cancer cell line (HepG-2), human breast adenocarcinoma cell line (MCF-7) and epithelial colorectal adenocarcinoma cells (CaCO-2) (Figure **2** and Table **1**). Different concentrations of the tested compounds were used to calculate the values of IC_{50} (the half-maximal inhibitory concentration). Doxorubicin (DOX) has been used as a positive control.

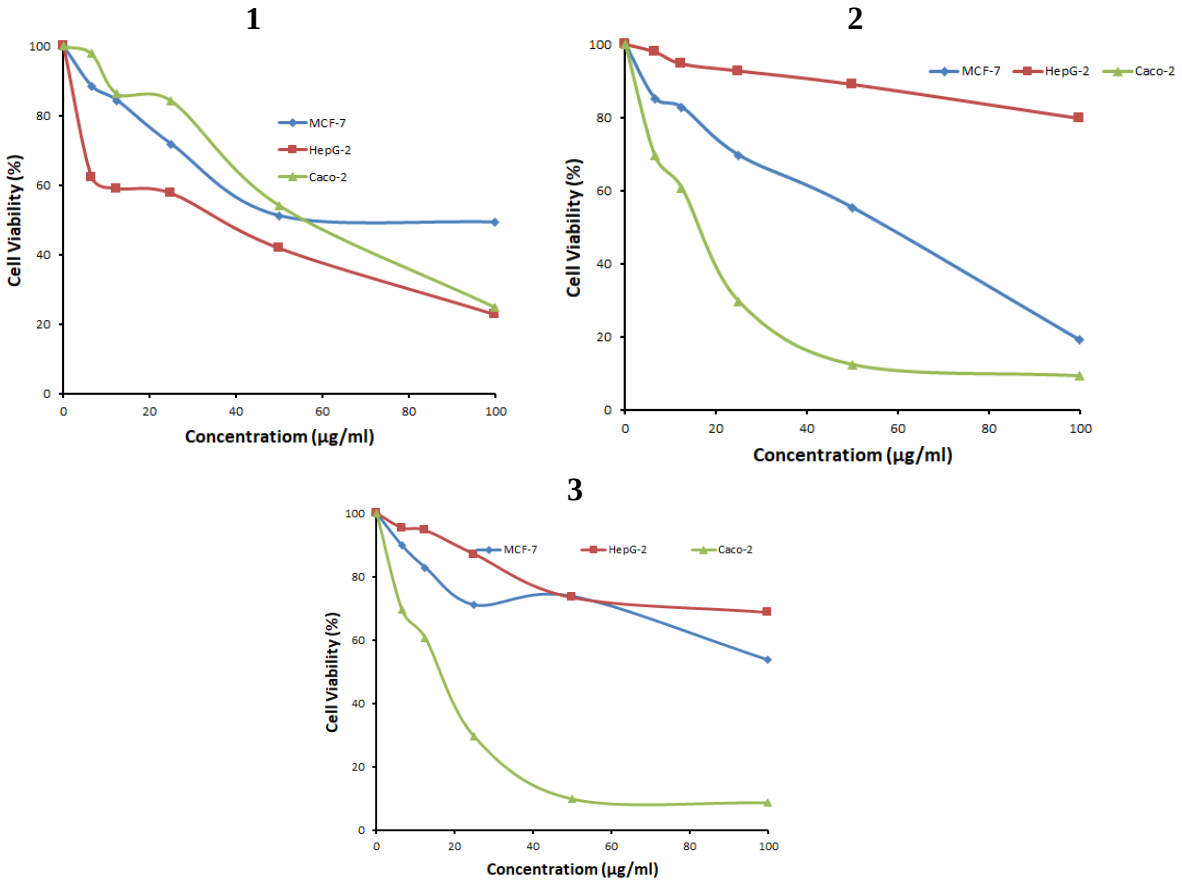

Figure 2: Cytotoxic activity of compounds 1-3 against HepG-2, MCF-7 and CaCo-2 cell lines.

Table 1. Cytotoxic activity (IC_{50}, µg/ml) of compounds 1-3 against HepG-2, MCF-7 and CaCo-2 cells

Compound No.	MTT assay IC_{50} (µM)		
	HepG-2	MCF-7	CaCo-2
1	23.3	76.9	55.2
2	522.7	45.9	30.1
3	235.0	149.0	13.8
Doxorubicin	1.28	0.318	0.332

Data were reported as mean ± S.D. (n = 5).

The MTT assay results showed that compound **1** had the strongest activity on HepG-2 cell lines with IC_{50} values of 23.3 µg/ml. Concerning MCF-7 cell line, the most active compound as daidzein **2** (IC_{50} value of 45.9 µg/ml) followed rutin **1** (IC_{50} value of 76.9 µg/ml). On the other hand, evaluation for compounds **1-3** against epithelial colorectal adenocarcinoma cells (CaCO-2) showed higher activity for compound **3** with IC_{50} = 13.8 µg/ml.

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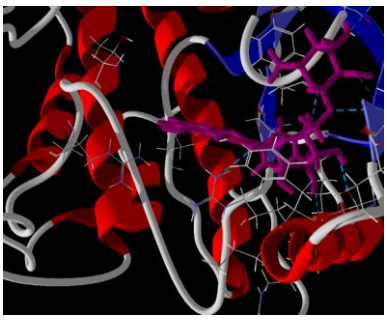
Molecular Docking Studies

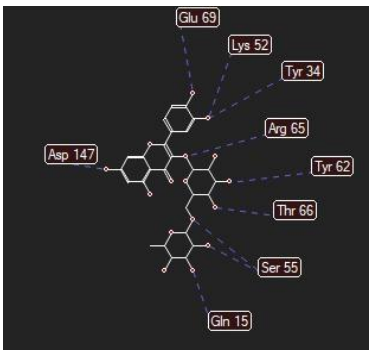
To expect the binding modes of compounds **1**-**3** in the active sites of the ERK2 kinase (PDB ID: 5buj), molecular docking was performed based on MolDock score and Hydrogen bond interaction (Table 2). The ERK2 can be considered one of the primary effectors kinases of ERK cancer signaling pathway and reported in different types of cancers [19]. The ERK pathway is the most commonly considered mammalian MAPK cascade and is identified to be the main driver of cellular proliferation [20]. In our study, we found that the ERK2 protein can bind with compounds **1**-**3** with docking score in the range of -88.60 to -128.48 kcal mol^{-1}. Docking studies showed that Glu69, Arg65, Asp147, Lys52, Tyr34, Tyr62, Thr66, Ser55 and Gln15 in the ERK2 kinase likely play a major role in substrate binding. The docking model revealed that the hydroxy groups of rutin aglycon interact with Asp147, Lys52, and Tyr34. The oxygen atom of the glyosidic linkage binds with Arg65. The sugars moieties interact through hydrogen bonds with Tyr62, Tyr66, Ser55 and Gln15 (Figure 3). Based on the *in silico* and in vitro data, compound **1** seem to show pronounced inhibitory activity against the tested cancer cells and soybean is a good source of bioactive compounds.

Table 2: In silico docking study of compounds 8-29 with ERK2 protein (PDB: 5BUJ)

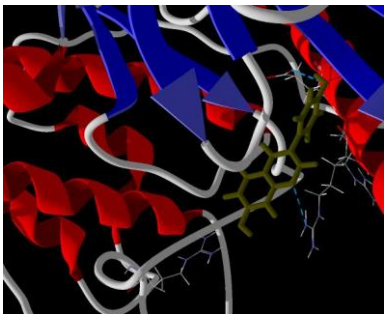
Compounds No.	Docking Score (Kcal/mol)	No. of Hydrogen Bonds	Hydrogen Bonding
1	-128.48	10	Asp147, Glu69, Lys52, Tyr34, Arg65, Tyr62, Thr66, Ser55, Gln15
2	-87.24	2	Glu69, Arg65
3	-88.60	3	Glu69, Arg65, Asp165

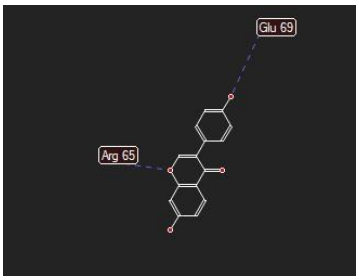
Compound 1





Compound 2





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Compound 3

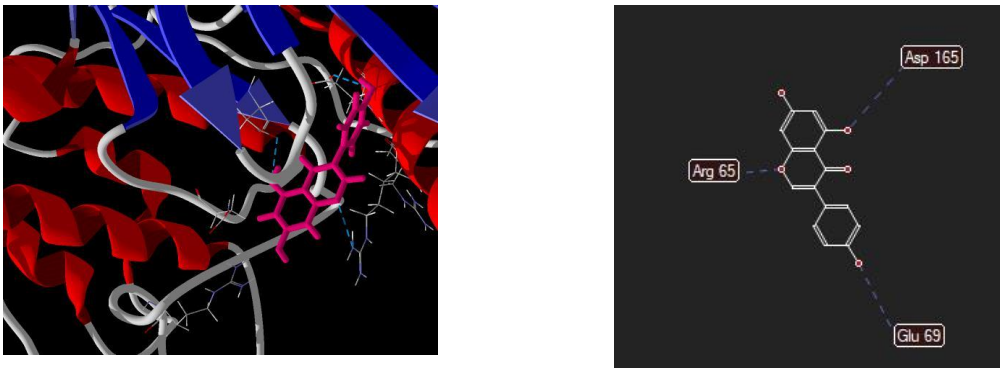

Figure 3. Docking interaction of some active compounds 1-3 with human extracellular signal-regulated protein kinase (ERK2) (PDB: 5BUJ)

Contents

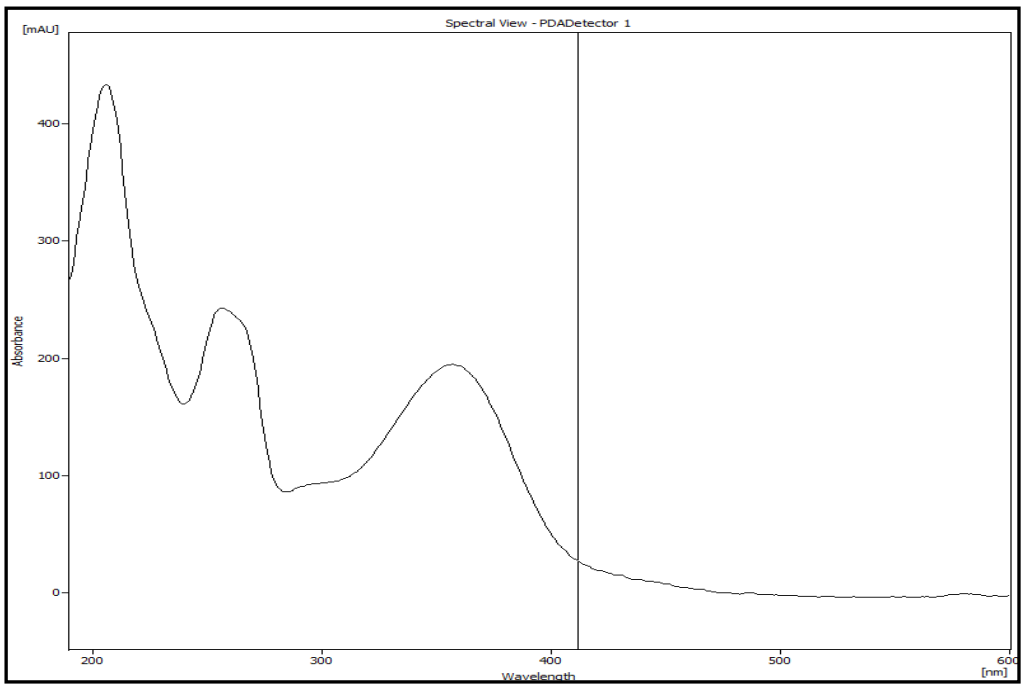

Figure S1: UV-spectra of rutin from the HPLC-PDA.

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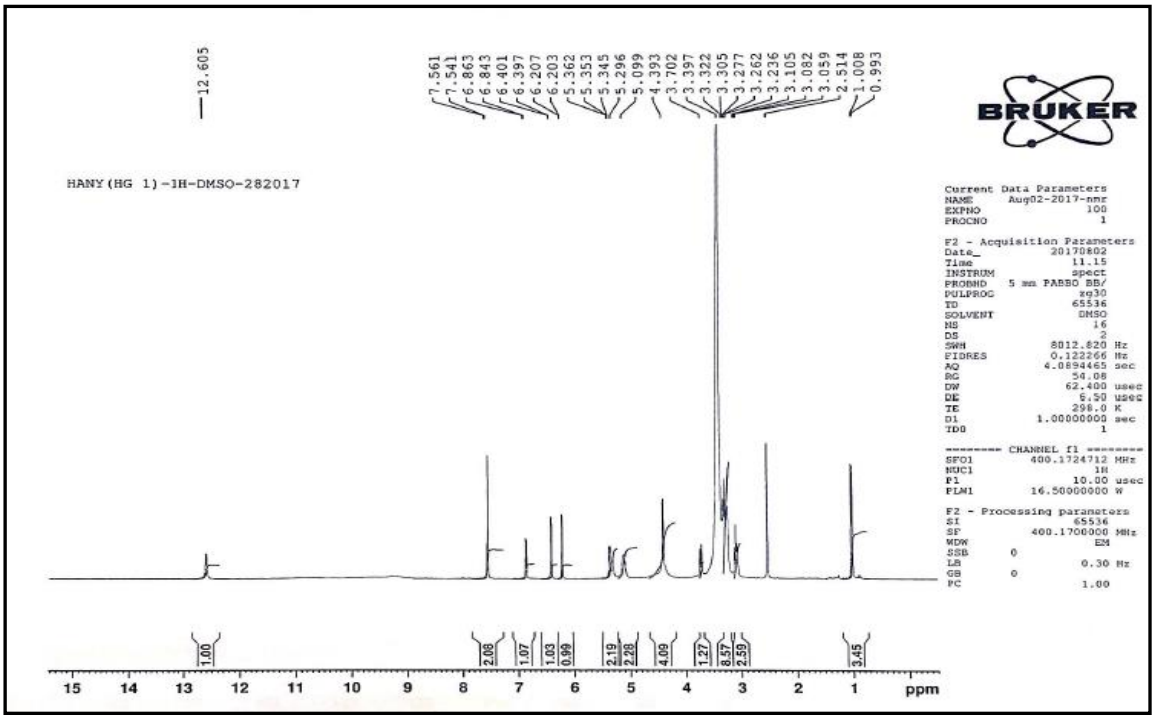

Figure S2:[1]H NMR spectrum of rutin (DMSO-d_6, 400 MHz).

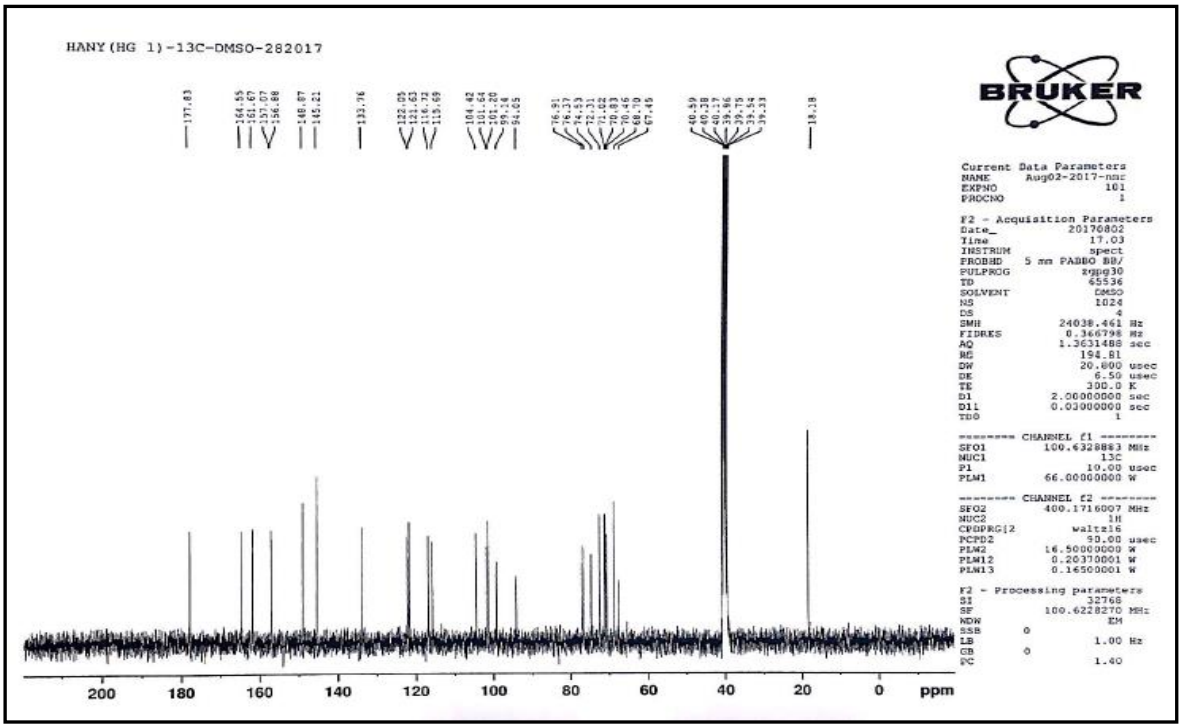

Figure S3:[13]C NMR spectrum of rutin (DMSO-d_6, 100 MHz).

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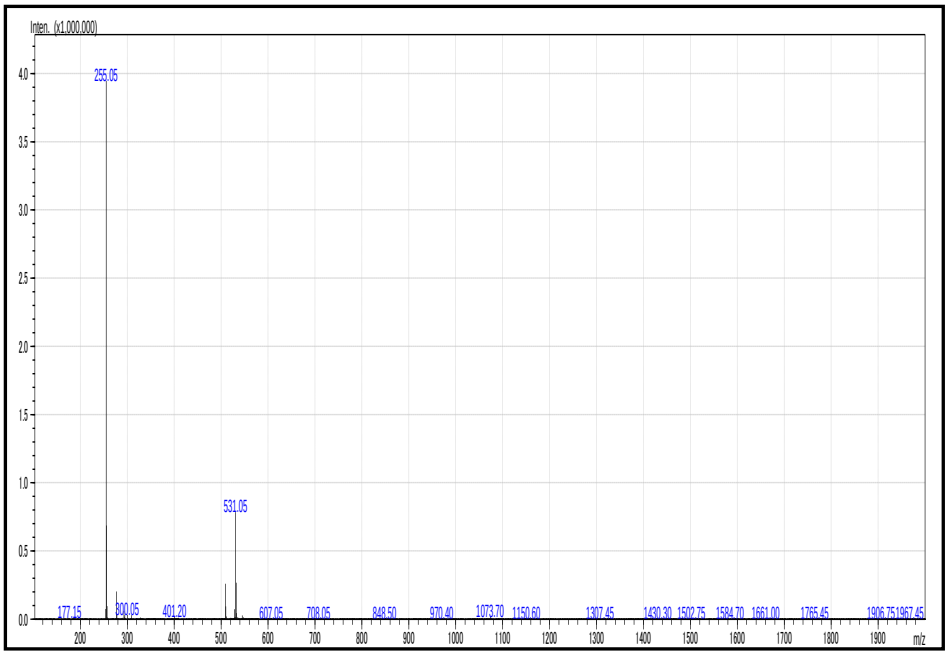

Figure S4: (+)-ESI-MS of daidzein (2)

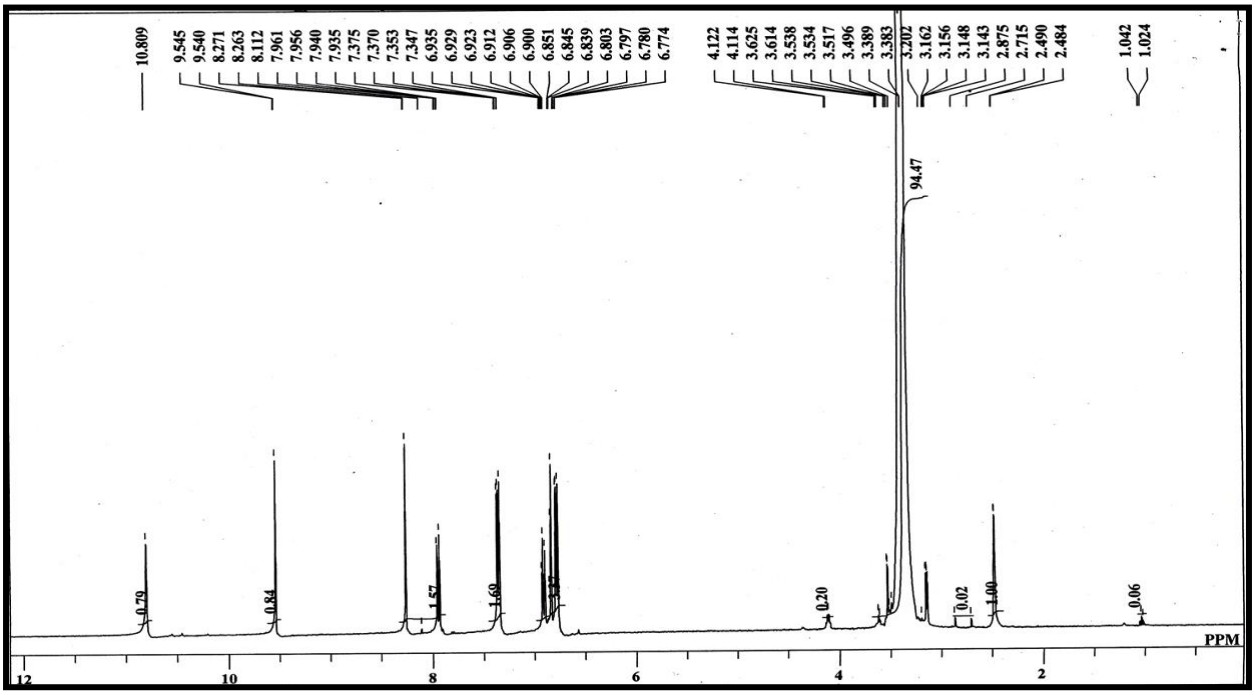

Figure S5: ^{1}H NMR Spectrum (DMSO-d_6,400 MHz) of daidzein (2)

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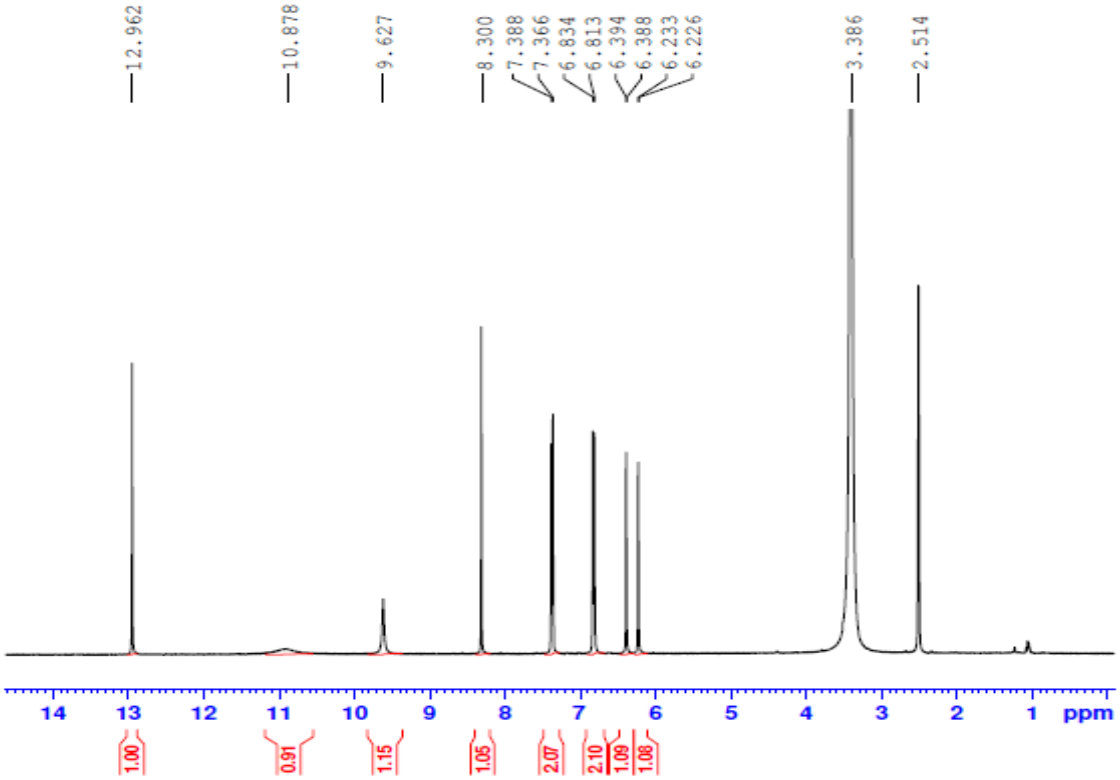

Figure S6: ^{1}H NMR Spectrum (DMSO-d_6,400 MHz) of genistein (3).

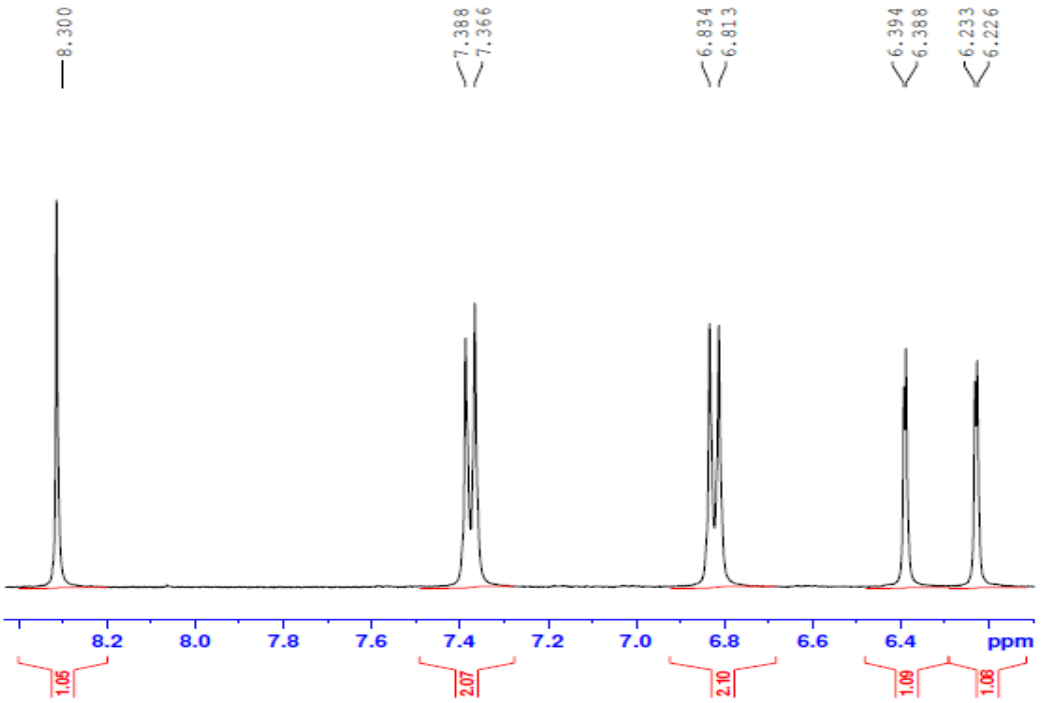

Figure S7: Expanded ^{1}H NMR Spectrum (DMSO-d_6,400 MHz) of genistein (3).

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