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New Bioflavonoids from *Solanum nigrum* L. by Anticholinesterase and Anti-tyrosinase Activities-guided Fractionation

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Abstract: Two new biflavonoids, (8-hydroxy-3'-*β*-*D*-galactosyl-isoflavone)-2'-8"-(4"'-hydroxy-flavone)-biflavone (**2**); 2',3',5-trihydroxy-5"-methoxy-3"-*O*- *α*-glucosyl-3-4"'-*O*-biflavone (**3**) and along with apigenin (**1**) and quercetin-3-*O*-*β*-glucoside (**4**) were isolated by activity guided fractionation from the whole plant of *Solanum nigrum* L.. The structures were established on the basis of UV, IR, 1D, 2D NMR and HRESI-MS spectroscopic methods. The anticholinesterase activity was performed against acetylcholinesterase and butyrylcholinesterase – the chief enzymes of Alzheimers' disease – using the Ellman method. The tyrosinase inhibitory activity was performed according to L-DOPA method. Since the ethyl acetate (IC_{50}: 90.6±0.3 µg/mL) and *n*-butanol (IC_{50}: 140.6±1.7 µg/mL) extracts exhibited good butyrylcholinesterase inhibitory activity, both were fractionated and the active fractions were used for isolation of the compounds. These both extracts were also exhibited better tyrosinase inhibitory activity (IC_{50}: 76.0±0.6, and 156.8±1.9 µg/mL, respectively). Tested enzyme inhibitory activities of S. *nigrum* were presented in this study for the first time.

Keywords: *Solanum nigrum*; anticholinesterase activity; tyrosinase inhibitory activity; biflavonoid.

1. Introduction

Solanum nigrum L. is a member of the Solanaceae family. S. *nigrum* is commonly called as black nightshade. The plant has been traditionally used in oriental medicines and is believed to have various biological activities [1].

Chemical investigations of the S. *nigrum* afforded steroidal glycosides, steroidal alkaloids, oligoglycosides [2-3], polyphenolic compounds [4], oligosaccharides [5] and flavonoids [6-8]. It also possessed anti-inflammatory [9], antioxidant, antihyperlipidemic [10], antimicrobial [11], cancer chemopreventive [12-13] and hepatoprotective [14]. The leaves are popularly used as a vegetable. The juice of the leaves is mixed with mediums like coconut water, coconut milk, butter milk, cow's milk and fruit juices [15].

The people living in industrialised countries frequently may come across with Alzheimer's disease in older ages. Currently known hypothetical cause of Alzheimer's disease is the acetylcholine deficiency; hence, only the acetylcholinesterase inhibitors (AChE) are used in the treatment of the

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disease. Accordingly, the level of acetylcholine – a neuromediator – remains stable in the biological fluids. Depending on the severity of the disease, different synthetic drugs are given to the patients. These drugs are effective, but most of them have side effects such as toxicity to liver and bradycardia [16]. Therefore, it is even necessary to search novel anticholinesterase drugs.

Tyrosinase inhibitors such as age spots, melisma and sites of actinic damage, arising from accumulation of an excessive level of epidermal pigmentation are important substances to treat abnormal pigmentation disorders. The well-known tyrosinase inhibitors can cause adverse reactions such as skin irritation, dermatitis [17] and skin cancer [18]. Therefore, the development and utilization of the effective tyrosinase inhibitors of natural origin are desired.

The aim of this paper was to isolate and elucidate the secondary metabolites from S. *nigrum* by acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities guided fractionation. In this study, two new biflavonoids (2, 3) (Fig. 1) and two known flavonoids (1, 4) were isolated. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of this plant are presented for the first time in this study.

2. Materials and Methods

2.1. General Experimental Procedures

Melting points were determined on a Yanaco mp apparatus. UV spectra were recorded on a Shimadzu UV-1601 in methanol while IR spectra were on a Shimadzu IR-470 spectrophotometer, and ^{1}H NMR, ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HMBC and HSQC spectra were on a Bruker 600 MHz NMR spectrometer in $CDCl_3$ or CD_3OD. API-ES spectra were measured on LCMS/MS (Agillent 1100 series G1956 BLC/MSD SL). HRESIMS were recorded on Bruker MicrOTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany). Shimadzu LC-8A recycling preparative HPLC coupled with a PDA detector and a reverse phase C_{18} column (Shim-Pack ODS (20x250 mm, 5 μm, Shimadzu Corporation, Japan) was used for the final purification.

Silica gel F_{254} precoated TLC plates and silica gel 60 (0.063-0.200 mm) adsorbent, Ce (IV) sulphate for indication of spots were purchased from Merck Company (Germany) while polyamide-6 DF were from Riedel-De Haen AG (Germany). Sephadex LH-20 (25-100 μM), 3,4-Dihydroxy-D-phenylalanine (L-DOPA), kojic acid, tyrosinase from mushroom (EC 232-653-4, 250 KU, ≥1000 U/mg solid, Sigma), electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U/mg, Sigma), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide (AChI), butyrylthiocholine chloride (BChCl) and galantamine were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). All other chemicals and all solvents were in analytical grade.

2.2. Plant Material

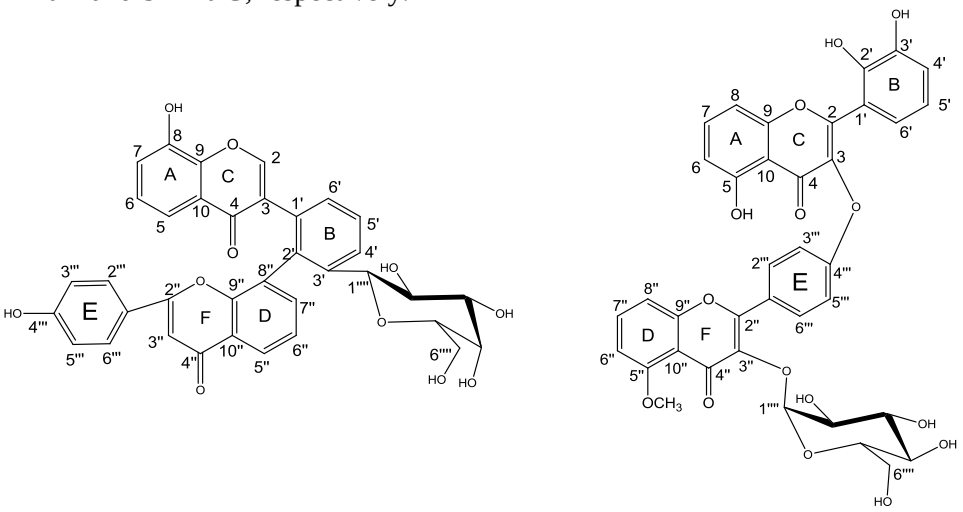
The plant sample was collected from Edirne-Tekirdag, Turkey in August 2013 and provisionally classified by Necmettin Guler, PhD, Trakya University, as *Solanum nigrum* L. (EDTU 13380).

2.3. Extraction and Isolation

The air dried whole plant material (163.31 g) was macerated with *n*-hexane, diethyl ether, ethyl acetate and *n*-butanol at room temperature successively. The extracts were individually concentrated on a rotary evaporator under vacuum. Finally, 5.11 g of *n*-hexane extract, 3.45 g of diethyl ether extract, 9.61 g of ethyl acetate extract and 15.87 g of *n*-butanol extract were obtained.

The four extracts of S. *nigrum* were studied for their anticholinesterase and tyrosinase inhibitory activities [19, 20]. Considering the anticholinesterase activity results, the ethyl acetate extract and *n*-butanol extracts were decided to be used for isolation studies (Table 2). The ethyl acetate extract and *n*-butanol extracts were fractionated using various column chromatographic techniques including reverse phase recycling HPLC.

Firstly, the ethyl acetate extract was loaded onto Silica gel column chromatography (column size: 80 x 10 cm) for fractionation. The column was eluted with gradient mixtures of dicholoromethane to methanol. After the combinations of obtained 201 fractions using TLC procedures, 9 fractions, in total, (SN-Et-A, SN-Et-B, SN-Et-C, SN-Et-D, SN-Et-E, SN-Et-F, SN-Et-G, SN-Et-H, SN-Et-I) were obtained. The anticholinesterase and tyrosinase inhibitory activities of these 9 fractions were determined [19, 20] (Table 3). Among the fractions, SN-Et-E, SN-Et-F and SN-Et-G indicated higher anticholinesterase activity. These fractions were injected to preparative recycling RP-HPLC coupled with a reverse phase C_{18} column (Shim-Pack ODS (20x250 mm, 5 μm) for the purifications. The linear gradient program was 100% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) for 120 min at a flow rate 10 mL/min. The compound **1 (**9 mg) and **2 (**13 mg) were obtained from subfractions SN-Et-E and SN-Et-G, respectively.



(2) **(3)**

Figure 1. Structures of new compounds (**2** and **3**) from *Solanum nigrum* L.

As to *n*-butanol extract, it was loaded onto Silica gel column chromatography (column size: 80 x 10 cm) for fractionation. The column was eluted with gradient mixtures of dicholoromethane to methanol. After the combinations of obtained 201 fractions using TLC procedures, 11 fractions, in total, (SN-A, SN-B, SN-C, SN-D, SN-E, SN-F, SN-G, SN-H, SN-I, SN-J, SN-K) were obtained. The anticholinesterase and tyrosinase inhibitory activities of these 11 fractions were determined [19, 20] (Table 4). Among the fractions, SN-E, SN-H and SN-I indicated higher butyrylcholinesterase inhibitory activity. These fractions were injected to preparative recycling RP-HPLC using the same column and same gradient programme as given above. The compounds **3** (11 mg) and **4** (12 mg) were isolated from SN-I and SN-H, respectively.

2.4. Spectroscopic Data of Compounds

2.4.1. (8-hydroxy-3'-β-D-galactosyl-isoflavone)-2'-8''-(4'''-hydroxy-flavone)-biflavone **(2)**

Yellow amorphous powder, mp: 223-225^{0}C. UV ($CHCl_3$) λ_{max} (log ε): 218 (2.52), 315 (1.82) nm. IR ν_{max} (nujol) cm^{-1}: 3395, 2926, 2857, 1705, 1577, 1451, 1243. ^{1}H and ^{13}C-NMR see Table 1. ^{1}H-^{1}H COSY NMR (600 MHz, CD_3OD): δ_H 8.07/7.45, 7.39 (H-6'/H-4', H-5'); 7.82/7.32 (H-7/H-6); 7.70/7.53 (H-2''', H-6'''/H-3''', H-5'''); 7.32/6.94 (H-6/H-5); 7.11/7.06, 7.57 (H-7''/H-5'', H-6''). API-ES MS m/z (Rel. Int. %): 639 $[M+3]^+$ (20), 437 $[(M-2)-C_6H_{11}O_5$ (sugar unit)$-2xOH]^+$ (100); 472 $[(M-1)-C_6H_{11}O_5$ (sugar unit)$]^+$ (10); 397 $[(M-2)-C_{15}H_9O_3$ (flavonoid unit)$]^+$ (12). HRESIMS *m/z* 636.1705 (calcd. for $C_{36}H_{28}O_{11}$, 636.1630).

2.4.2. 2',3',5-trihydroxy-5''-methoxy-3''-O-α-glucosyl-3-4'''-O-biflavone **(3)**

Yellow powder, mp: 245-248^{0}C. UV (MeOH) λ_{max} (log ε): 228 (2.85), 317 (1.45) nm. IR ν_{max} (nujol) cm^{-1}: 3433, 2975, 1732, 1560, 1456, 1134. ^{1}H and ^{13}C-NMR see Table 1. ^{1}H-^{1}H COSY NMR (600 MHz, CD_3OD): δ_H 7.95/7.42 (H-6/H-7); 7.74/7.65 (H-2''', H-6'''/H-3''', H-5'''); 7.61/7.46 (H-8''/H-6''); 7.58/7.63 (H-6'/H-4'); 7.46/7.17 (H-6''/H-7''). API-ES MS m/z (Rel. Int. %): 702 $[M]^+$ (5), 327 $[(M+2)\text{-}C_{15}H_9O_5$ (flavone unit)-C_6H_5-$OCH_3]^+$ (100), 437 $[(M\text{-}1)\text{-}C_6H_{11}O_6$ (sugar unit)-3xH_2O-$OCH_3]^+$ (8), 689 $[(M+2)\text{-}CH_3]^+$, 653 $[(M+2)\text{-}3xOH]^+$. HRESIMS *m/z* 702.1628 (calcd. for $C_{36}H_{30}O_{15}$, 702.1575).

2.5.Bioassays

2.5.1. Determination of the anticholinesterase activity

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the crude extracts and the subfractions were carried out according to the Ellman's method [19] using a 96-well plate microplate reader [20, 21, 22]. AChE from electric eel and BChE from horse serum were used as enzymes, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB (5,5-0-dithio-bis(2-nitrobenzoic) acid), however, was used to measure the activity. Briefly, 150 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution in ethanol at different concentrations and 20 µL AChE (5.0 mU) or BChE (6.5 mU) solution were mixed. After 15 min of incubation at room temperature, and 10 µL 0.5 µM DTNB were added. The reaction was then initiated by the addition of 10 µL 0.71 mM acetylthiocholine iodide or 0.2 mM butyrylthiocholine chloride.

The results of the reaction were monitored spectrophotometrically at 412 nm. Softmax PRO v5.2 software was used to evaluate measurements. The percentage of inhibitions of AChE or BChE were determined using the formula (E - S)/E x 100, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. Galantamine was used as a reference compound.

2.5.2. Determination of the tyrosinase inhibitory activity

Inhibition of tyrosinase (EC 232-653-4, 250 KU, mushroom tyrosinase, Sigma) was determined using the modified DOPAchrome method with L-DOPA as substrate [21, 23]. Briefly, 150 µL of 50 mM sodium phosphate buffer (pH 6.8), 10 µL of sample in ethanol or control, 20 µL of tyrosinase enzyme solution were added by automatic pipette in a 96-well microplate and incubated for 10 min at 37 ^{0}C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 ^{0}C) due to the formation of the DOPAchrome for 10 min. The percentage of inhibition of tyrosinase was determined by comparison of reaction rates of samples relative to blank sample (ethanol in phosphate buffer pH 6.8) using the formula (E - S)/ E x 100, where E is the activity of enzyme without test sample, and S is the activity of enzyme with test sample. The experiments were carried out in triplicate. Kojic acid was used as a reference compound.

2.5.3. Statistical analysis

All data on enzyme inhibitory activity tests were the averages of triplicate analyses. All antioxidant activity tests were carried out at more than four concentrations, and the results are presented as IC_{50} values. Data were recorded as mean ± S.E.M. (Standard error of the mean). Significant differences between means were determined by student's-*t* test, *p* values <0.05 were regarded as significant.

3. Results and Discussion

3.1. Structure elucidation

The compounds **1** and **2** were isolated from ethyl acetate extract while the compound **3** and **4** were from *n*-butanol extract of S. *nigrum*. The elucidations of the isolated compounds were carried out using spectroscopic techniques; namely, UV, IR, ^{1}H and ^{13}C-NMR, API-ES and HREI-MS, and 2D-NMR techniques.

Among the isolated four compounds, two new biflavonoids (**2** and **3**) and two known flavonoids – apigenin (**1**) and quercetin-3-*O*-*β*-glucoside (**4**) – were obtained.

The elemental composition of compound **2** was concluded as $C_{36}H_{28}O_{11}$ from HRESI-MS 636.1705 (calcd.636.1630). The presence of a fragment peak at 437 $[(M-2)-163$ (sugar unit)-2xOH$)]^{+}$ (%100) in API-ES spectrum of *m/z* 636 showed the cleavage of isoflavone glycoside and flavone. However, a fragment peak at 437 was evidence for a biflavonoid molecule containing two hydroxyl groups and sugar moiety. The ^{1}H NMR spectrum of **2** showed characteristic signals for a biflavonoid, with the signals *δ* 6.94 (1H, *dd*, *J*=1.8, 7.8 Hz, H-5), 7.32 (1H, *dd*, *J*=7.8, 7.8 Hz, H-6), 7.82 (1H, *dd*, *J*=1.8, 7.8 Hz, H-7), 7.06 (1H, *dd*, *J*=3, 7.2 Hz, H-5"), 7.57 (1H, *m*, H-6") and 7.11 (1H, *dd*, *J*=3, 7.2 Hz, H-7") indicating the presence of two monosubstituted A and D rings and two double doublet at *δ* 7.53 (2H, *dd*, *J*=3, 6 Hz, H-3'"/H-5'") and *δ* 7.70 (2H, *dd*, *J*=3, 5.4 Hz, H-2'"/H-6'") showing that the E ring was *para*-substituted. However, the B ring was three substituted at C-1', C-2' and C-3' because the protons were resonated at *δ* 7.45 (1H, *m*, H-4'), 7.39 (1H, *t*, *J*=7.8 Hz, H-5') and 8.07 (1H, *d*, *J*=7.8 Hz, H-6') for B ring (Table1). The one proton singlet at *δ* 8.11 in the C ring was assigned to isoflavone unit. The presence of an anomeric sugar proton at *δ* 5.34 (1H, *d*, *J*=12 Hz) bounded to carbon *δ* 73.4 confirmed that one sugar moiety is C-attached. The glycosyl linkage was determined to be C-C on the basis of the relatively up field anomeric carbon resonances at *δ* 73.4 in contrast with the anomeric carbon of *O*-glycosyl normally resonate at ca. *δ* 100.0 [24]. Flavonoids with C-6 and/or C-8 *C*-glycosides are frequently found in some plant species; however, the *C*-glycosylation in 3' must have occured on the B ring judging from the HMBC correlations between the anomeric proton (δ_H 5.34), and C-4', C-2', C-5' and C-7 and C-2' (δ_c 129.7 and 132.1, respectively) (Fig.2). Thus, the glycoside was determined to be bounded to C-3' of isoflavone.

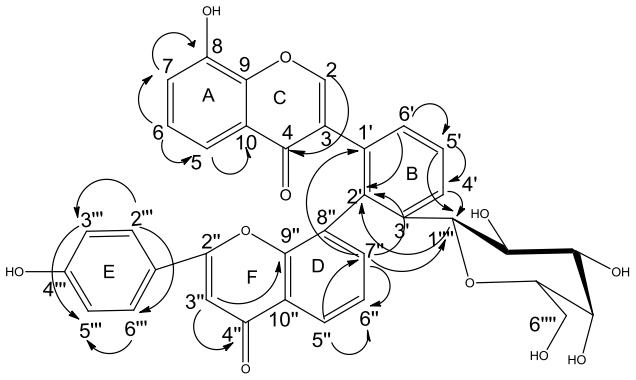


Figure 2. Key HMBC of compound **2**

In addition, the HMBC cross-peak between the H-7" with C-2' (*δ* 132.1) on the B ring indicated that the compound **2** was a biflavonoid consisting of an interflavonoid linkage between C-2 and C-8". The other HMBC correlations observed in Fig. 2 supported the assumed structure. Besides, since the large coupling constant was observed in the ^{1}H NMR spectrum of **2** sugar must take *β*-pyranoside form. After acid hydrolysis [25], of **2** the aqueous layer was separated by HPLC to give only *D*-galactose, optical rotation of the purified galactose was determined. Based on the above data, the structure of compound **2** was determined as a new biflavonoid; (8-hydroxy-3'-*β*-*D*-galactosyl-isoflavone)-2'-8"-(4'"-hydroxy-flavone)-biflavone.

The compound **3** gave a molecular ion peak $[M]^+$ at *m/z* 702 in API-ES and *m/z* 702.1628 in the HRESI-MS (calcd. 702.1575) which was consistent with a molecular formula of $C_{36}H_{30}O_{15}$. The prominent ions at *m/z* 437 $[(M-1)-C_6H_{11}O_6$ (sugar unit)$-3xH_2O-OCH_3]^+$, *m/z* 689 $[(M+2)-CH_3]^+$ and *m/z* 653 $[(M+2)-3xOH]^+$, supported the biflavonoid molecule containing three hydroxyl groups, a methoxy group and sugar moiety. However, the presence of a fragment peak at 327 $[(M+2)-C_{15}H_9O_5$ (flavone unit)$-C_6H_5OCH_3]^+$ (100) in API-ES spectrum of *m/z* 702 showed the cleavage of flavone unit and D ring. The ^{1}H NMR spectrum of compound **3** displayed signals of three H-atoms corresponding to H-6 (δ_H 7.95, 1H, *dd*, *J*=1.2, 8.4 Hz), H-7 (δ_H 7.42, 1H, *t*, *J*=7.2 Hz) and H-8 (δ_H 7.15, 1H, *dd*, *J*=2.4, 8.4 Hz) at the A ring, and methoxy group (δ_H 3.32, 3H, *s*), three H atoms corresponding to H-6" (δ_H 7.46, 1H, *dd*, *J*=1.2, 7.4 Hz), H-7" (δ_H 7.17, 1H, *t*, *J*=6.6 Hz) and H-8" (δ_H 7.61, 1H, *dd*, *J*=1.2, 7.2 Hz) at the D ring of the biflavonoid indicating the presence of the C-5 and C-5" mono substituted A ring moiety and D ring moiety, respectively. The ^{1}H and ^{13}C NMR spectrums have supported with ^{1}H-^{1}H COSY and HSQC experiments (Table 1). The spectra showed its structural fragments to include one set of typical 2',3'-dioxygenated B ring (δ_H 7.63, 1H, *dd*, *J*=1.2, 7.2 Hz, H-4'; δ_H 7.37, 1H, *t*, *J*=7.2 Hz, H-5'; δ_H 7.58, 1H, *dd*, *J*=1.2, 7.8 Hz, H-6') and one set of *para*-oxygenated E ring (δ_H 7.74, 2H, *dd*, *J*=3.6, 6.6 Hz, H-2'''/H-6'''; δ_H 7.65, 2H, *dd*, *J*=3.6, 7.2 Hz, H-3'''/H-5'''). An additional group of signals at δ_H 4.61 (1H, *brd*, *J*=1 Hz, H-1'''') and δ_H 3.4-4.2 (6H, *m*, H-2''''/H-3''''/H-4''''/H-5''''/H-6'''') indicated the presence of an α-glycoside linkage of sugar moiety. The ^{13}C NMR signals corresponding to the sugar moiety (Table 1) were characteristics of a glucopyranoside [26]. The presence of a methoxy group was confirmed by the signal at δ_C 53.7 in the ^{13}C NMR spectra. In the HMBC spectrum of **3** (Fig. 3), the anomeric proton at δ 4.61 (H-1'''') showed cross-peak with the C-3" (δ 134.2) and C-4" (δ 172.1) of ring F that was supporting the placement of one of the sugar moiety as C-3" on the basis of HMBC correlations. The methoxy group (δ_H 3.32) showed a cross-peak with a quarternary carbon signal at δ 101 (C-10") while protons of ring E, H-3''' and H-5''' (δ 7.65) were correlated with a *O*-linked quaternary carbon C-3 (δ 132.5). The above spectral data suggested that **3** could be a biflavonoid consisting of a flavonol unit linked to a flavonol glucoside unit through a C-O-C bond. The HMBC cross-peak between the protons of E ring and C-3 on the C ring indicated that the compound **3** was a biflavonoid consisting of an inter flavonoid ether linkage between C-3 and C-4'''.

Based on the above deductions **3** was elucidated as a new biflavonoid; 2',3',5-trihydroxy-5"-methoxy-3"-*O*- α-glucosyl-3-4'''-*O*-biflavone. On the basis of spectral properties compounds **1** and **4** were identified as apigenin and quercetin-3-*O*-β-glucoside, respectively. However, these **1** and **4** were isolated from S. *nigrum* in previous studies [6-8, 27].

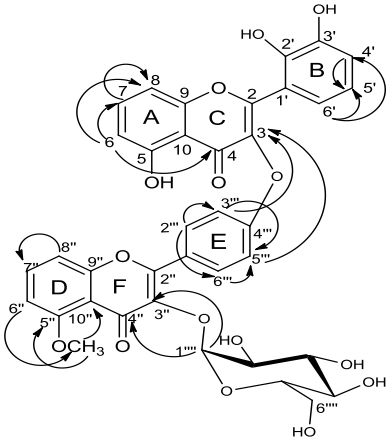


Figure 3. Key HMBC of compound **3**

Table 1. 1D NMR data for compound **2**[a] and compound **3**[b].

	2		**3**	
Position	**δ_H**	**δ_C**	**δ_H**	**δ_C**
2	8.11(s)	153.2		160.0
3		123.5		132.5
4		172.2		172.3
5	6.94 (dd, 1.8, 7.8 Hz)	128.1		157.8
6	7.32 (dd, 7.8, 7.8 Hz)	127.0	7.95 (dd, 1.2, 8.4 Hz)	128.3
7	7.82 (dd, 1.8, 7.8 Hz)	128.8	7.42 (t, 7.2 Hz)	130.1
8		139.8	7.15 (dd, 2.4, 8.4 Hz)	127.2
9		150.7		156.2
10		106.5		110.6
1'		128.4		117.4
2'		132.1		147.6
3'		120.7		143.5
4'	7.45 (m)	129.7	7.63 (dd, 1.2, 7.2 Hz)	132.5
5'	7.39 (t, 7.8 Hz)	130.1	7.37 (t, 7.2 Hz)	127.3
6'	8.07 (d, 7.8 Hz)	130.8	7.58 (dd, 1.2, 7.8 Hz)	128.9
2''		153.4		158.4
3''	5.58 (s)	120.8		134.2
4''		169.8		172.1
5''	7.06 (dd, 3, 7.2 Hz)	128.8		155.8
6''	7.57 (m)	131.2	7.46 (dd, 1.2, 7.4 Hz)	124.3
7''	7.11 (dd, 3, 7.2 Hz)	127.9	7.17 (t, 6.6 Hz)	127.9
8''		104.1	7.61 (dd, 1.2, 7.2 Hz)	131.2
9''		152.8		156.2
10''		104.1		101.0
1'''		128.2		122.5
2'''/6'''	7.70 (dd, 3, 5.4 Hz)	129.7	7.74 (dd, 3.6, 6.6 Hz)	128.4
3'''/5'''	7.53 (dd, 3, 6 Hz)	131.1	7.65 (dd, 3.6, 7.2 Hz)	131.0
4'''		145.6		156.7
1''''	5.34 (d, 12 Hz)	73.4	4.61 (brd, 1 Hz)	102.8
2''''/3''''/4''''/5''''/6''''	3.1-4.2 (m)	71.4/69.1/65.2/63.1/57.3	3.4-4.2 (m)	73.2/61.5/75.6/68.9/76.8
-OCH_3			3.32 (s)	53.7

[a]Recorded in $CDCl_3$; [b]Recorded in CD_3OD.

3.2. Enzymes inhibitory assays

AChE and BChE inhibitory activities of the crude extracts and compounds (**2**, **3**) were performed *in vitro*, and galantamine – used as a drug for the treatment of mild Alzheimer's disease – was used to compare the activity. For tyrosinase inhibitory activity, kojic acid was used as positive standard. The AChE, BChE and tyrosinase inhibitory activities of extracts and compounds (**2**, **3**) were given in Table 2. The hexane and diethyl ether extracts almost demonstrated no activity against AChE. They also exhibited lower activity against BChE. The ethyl acetate and the *n*-butanol extracts moderate activity against BChE (IC_{50}: 90.6 ± 0.3, and 140.6 ± 1.7 µg/mL). At the same conditions, galantamine had 46.0 ± 0.1 µg/mL IC_{50} value. The compound **2** isolated from ethyl acetate extract gave weaker activity (IC_{50}: 195.2 ± 2.2 µg/mL) than that of its crude extract. Likewise, **3** have less activity than that of its extract (IC_{50} > 300 µg/mL). The most active extract was the ethyl acetate extract against tyrosinase enzyme indicating 76.0 ± 0.6 µg/mL IC_{50} value, followed by *n*-butanol extract (IC_{50}: 156.8 ± 1.9 µg/mL). In fact, the ethyl acetate extract exhibited the best inhibitory activity against all enzymes.

Table 2. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of the crude extracts.[a]

Extracts	AChE assay	BChE assay	Tyrosinase Inhibitory assay
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Hexane	>300	217.2±1.9	259.6±2.0
Diethyl ether	>300	175.9±2.1	169.7±1.6
Ethyl acetate	246.8±0.9	90.6±0.3	76.0±0.6
n-butanol	288.9±1.6	140.6±1.7	156.8±1.9
2	>300	195.2±2.2	122.1±2.1
3	>300	299.1±2.5	145.8±2.0
Galantamine[b]	4.5±0.8	46.0±0.1	*NT*
Kojic acid[b]	*NT*	*NT*	67.5±0.1

[a] IC_{50} values represent the means ± S.E.M. of three parallel measurements (p< 0.05).
[b] Reference compounds; *NT*: not tested.

Table 3. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of the fractions of ethyl acetate extract.[a]

Fractions	AChE	BChE	Tyrosinase Inhibitory
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
SN-Et-A	>300	>300	284.3±0.9
SN-Et-B	>300	244.0±1.2	170.9±1.3
SN-Et-C	>300	127.8±0.5	246.5±0.0
SN-Et-D	>300	23.2±1.6	215.8±5.8
SN-Et-E	133.6±3.7	21.3± 0.8	130.8±1.5
SN-Et-F	>300	20.5± 0.7	142.0±1.9
SN-Et-G	>300	26.7± 1.3	120.4±0.7
SN-Et-H	>300	133.2±0.2	*NT*
SN-Et-I	>300	152.2±0.2	*NT*

[a] IC_{50} values represent the means ± S.E.M. of three parallel measurements (p< 0.05).
NT: not tested.

The AChE, BChE and tyrosinase inhibitory activities of fractions of the ethyl acetate were given in Table 3. All fractions exhibited no activity against AChE, except SN-Et-E. The fraction together with SN-Et-F and SN-Et-G also exhibited BChE inhibitory activity. SN-Et-D fraction also had the

same activity; however, SN-Et-G exhibited moderate tyrosinase inhibitory activity. Therefore, SN-Et-E, SN-Et-F and SN-Et-G were selected to study their phytochemistry. The enzymes activities of fractions of *n*-butanol extract were given in Table 4. None of the fractions indicated AChE inhibitory activity (IC_{50} > 300 µg/mL). Conversely, fractions SN-H (IC_{50}: 18.5 ± 1.9 µg/mL) and SN-F (IC_{50}: 22.0 ± 1.3 µg/mL) possessed better activity than that of galantamine (IC_{50}: 46.0 ± 0.1 µg/mL). Fraction SN-E (IC_{50}: 60.6 ± 0.3 µg/mL), however, was comparable with that of galantamine. SN-E also indicated tyrosinase inhibitory activity (IC_{50}: 211.6 ± 3.4 µg/mL).

Table 4. Acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of the fractions of *n*-butanol extract.[a]

Fractions	AChE	BChE	Tyrosinase Inhibitory
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
SN-A	>300	251.8±1.9	>300
SN-B	>300	141.8±3.6	>300
SN-C	>300	80.9±1.5	>300
SN-D	>300	112.3±1.6	>300
SN-E	>300	60.6±0.3	211.6±3.4
SN-F	>300	22.0±1.3	>300
SN-G	>300	170.2±1.6	>300
SN-H	>300	18.5±1.9	>300
SN-I	>300	379.5±6.7	>300
SN-J	>300	167.4±3.3	>300
SN-K	>300	>300	>300

[a] IC_{50} values represent the means ± S.E.M. of three parallel measurements (p< 0.05).

4. Conclusion

Two new biflavonoids, (8-hydroxy-3'-*β*-*D*-galactosyl-isoflavone)-2'-8"-(4"'-hydroxy-flavone)-biflavone (**2**) and 2',3',5-trihydroxy-5"-methoxy-3"-*O*- *α*-glucosyl-3-4"'-*O*-biflavone (**3**) were isolated by activity guided fractionation from ethyl acetate and *n*-BuOH extract of *Solanum nigrum* L., respectively. Their structures were determined by extensive 1D and 2D NMR and MS data analyses. The compound (**2**) and (**3**) have been isolated from this plant species for the first time. There are are two article [28, 29] in connection with isolated biflavonoid from *Solanum* species in the literature. With this publication we hope to contribute additional data to the still puzzling flavonoid chemistry of Solonaceae.

Anticholinesterase activity-guided fractionation was used to isolate all compounds. The tyrosinase inhibitory activity results were also used to select the fractions to be studied. When compared the enzyme inhibitory activities of extracts and compounds **2** and **3**, the extracts exhibited better enzyme inhibitory activities than compounds. This reason may be due to the synergistic effects of the compounds. However, further studies are necessary to reveal the hypothesis.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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