JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Antioxidant and Tyrosinase Inhibitory Activities of Flavonoids from *Trifolium nigrescens* Subsp. *petrisavi*

Ozlem Demirkiran,*[,†] Temine Sabudak,[‡] Mehmet Ozturk,[§] and Gulacti Topcu[∥]

[†]Department of Pharmacognosy, Faculty of Pharmacy, Trakya University, 22030, Edirne, Turkey

[‡]Department of Chemistry, Faculty of Science and Letters, Namik Kemal University, 59860, Tekirdag, Turkey

[§]Department of Chemistry, Faculty of Science, Mugla Sıtkı Koçman University, 48121, Mugla, Turkey

[∥]Department of Pharmacognosy/Phytochemistry, Faculty of Pharmacy, Bezmialem Vakıf University, 34093, Istanbul, Turkey

Supporting Information

ABSTRACT: *Trifolium nigrescens* was researched for its chemical constituents for the first time. Bioassay-guided isolation of the EtOAc extract from the leaves of *T. nigrescens* resulted in the purification of a new biflavone, 4‴,5,5″,7,7″-pentahydroxy-3′,3‴-dimethoxy-3-*O*-*β*-D-glucosyl-3″,4′-*O*-biflavone (**1**) along with eleven known compounds consisting of three phenolics (**2**−**4**), and eight flavonoid glycosides (**5**−**12**). Their structures were determined by extensive 1D and 2D NMR, and MS data analyses. The isolated compounds were evaluated for their antioxidant activity and inhibitory activity on mushroom tyrosinase. Highly potent inhibitions were found by compounds **7** (IC_{50} = 0.38 mM), **8** (IC_{50} = 0.19 mM), and **12** (IC_{50} = 0.26 mM) when compared with standard tyrosinase inhibitors kojic acid (IC_{50} = 0.67 mM) and L-mimosine (IC_{50} = 0.64 mM). The antioxidative effect of the isolated compounds and the extracts were determined by using *β*-carotene-linoleic acid, DPPH$^{\bullet}$ scavenging, ABTS$^{+\bullet}$ scavenging, superoxide scavenging, and CUPRAC assays. The experimental findings indicated that all the compounds demonstrated activity in all antioxidant activity tests employed except for the compounds **2**−**6**. Compounds **2**−**6** showed moderate activity only in ABTS$^{+\bullet}$ scavenging assay. The new compound **1** exhibited better activity than standard *α*-tocopherol in DPPH$^{\bullet}$ scavenging, and ABTS$^{+\bullet}$ scavenging assays. The results show that *T. nigrescens* can be regarded as a potential source of antioxidant compounds and tyrosinase inhibitors of significance in both the pharmaceutical and food industries.

KEYWORDS: *Fabaceae, T. nigrescens subsp. petrisavi, flavonoids, phenolic compounds, tyrosinase inhibitory activity, antioxidant activity*

INTRODUCTION

Trifolium is a genus of about 300 species of plants in the leguminous pea family Fabaceae. The genus has a cosmopolitan distribution; the highest diversity is found in the temperate Northern Hemisphere. The Mediterranean region is very rich in *Trifolium* species,[1] especially in Turkey, with widespread representation by 103 species.[2] Plants from the genus *Trifolium* have been used in traditional medicine by many cultures. Clovers have been used by Oriental and European cultures for the treatment of eczema and psoriasis.[3] In Turkish folk medicine, some *Trifolium* species such as *T. repens*, *T. arvense*, and *T. pratense* are used as expectorants, analgesics, antiseptics, and against rheumatism aches.[4] Some of the *Trifolium* species are also used as feeding material for sheep and cattle in the Mediterranean region.[5,6] Among them *T. nigrescens* is highly acceptable protein-rich forage for grazing. Its good seed retention allows efficient seed production. There are a number of local ecotypes from which common seed is produced but there are no cultivars. It can be adapted to wide range of soil pH. Very small seeds allow high levels of survival when passing through the gut of grazing animals.[7,8]

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Some chemicals that occur naturally in plants have begun to receive much attention as safe antioxidants since they have been consumed by people and animals for years. Therefore, the development and utilization of more effective antioxidants of natural origin are desired. Compounds possessing different functional groups may display varying polarity, thus potentially leading to scattered antioxidant activity depending on the test employed, so an approach with multiple assays for evaluating the antioxidant potential would be more informative and even necessary. Therefore in this study, antioxidant activities were determined by five complementary tests, namely, the *β*-carotene-linoleic acid assay for lipid peroxidation activity, DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and $O_2^{\bullet-}$ assays for radical-scavenging activity, and CUPRAC assay for reducing antioxidant activity.

Tyrosinase is a copper-containing enzyme widely distributed in nature, and it catalyzes two key reactions in the melanin biosynthesis pathway in melanocytes.[9−11] Melanin production is principally responsible for skin color and plays an important role in prevention of sun-induced skin injury. However, abnormal accumulation of melanin products is responsible for hyperpigmentation. Therefore, tyrosinase inhibitors that suppress melanogenesis have been actively studied for the treatment of hyperpigmentation and for developing whitening

Received: August 19, 2013
Revised: November 21, 2013
Accepted: November 28, 2013
Published: December 16, 2013

Table 1. Antioxidant Activity of Compounds 1−12, the Extracts of *Trifolium nigrescens* and α-Tocopherol, Butylated Hydroxy Toluene (BHT), (+)-Catechin, Quercetin by β-Carotene-Linoleic Acid, DPPH, ABTS, Superoxide, and CUPRAC Assays[a]

	β-carotene-linoleic acid assay	DPPH• assay	ABTS•+ assay	superoxide assay	CUPRAC assay
sample	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	$A_{0.50}$ (μM)[b]
hexane extract	160.29 ± 3.03	398.65 ± 4.01	NT[d]	NT	NT
CH_2Cl_2 extract	99.66 ± 2.56	177.41 ± 3.26	NT	NT	NT
EtOAc extract	31.89 ± 0.86	12.38 ± 0.55	NT	NT	NT
n-BuOH extract	164.25 ± 2.95	92.82 ± 1.20	NT	NT	NT
1	15.66 ± 3.05	12.65 ± 1.59	3.08 ± 0.05	NT	6.89 ± 0.06
2	>200	>200	>200	>100	>200
3	>200	>200	75.95 ± 1.89	>100	>200
4	>200	>200	125.08 ± 3.59	>100	>200
5	>200	>200	128.38 ± 4.59	>100	>200
6	>200	>200	58.70 ± 0.69	>100	173.84 ± 1.86
7	9.30 ± 0.86	4.84 ± 0.19	4.82 ± 0.49	23.29 ± 1.23	6.78 ± 0.13
8	16.26 ± 2.02	185.09 ± 3.45	11.19 ± 0.15	55.00 ± 4.01	28.48 ± 0.26
9	38.42 ± 4.53	4.29 ± 0.86	3.93 ± 0.08	18.37 ± 0.89	6.14 ± 0.11
10	13.30 ± 2.56	4.51 ± 0.69	4.40 ± 0.08	11.95 ± 2.23	6.31 ± 0.12
11	36.59 ± 2.99	67.92 ± 1.25	4.77 ± 0.09	53.11 ± 3.30	12.93 ± 0.06
12	19.99 ± 2.55	6.20 ± 1.09	4.86 ± 0.09	13.49 ± 1.23	8.51 ± 0.11
BHT[c]	1.34 ± 0.09	54.97 ± 0.99	2.91 ± 0.55	19.60 ± 0.00	3.91 ± 0.00
α-tocopherol[c]	2.10 ± 0.09	12.26 ± 0.07	4.87 ± 0.45	11.58 ± 0.19	2.61 ± 0.00
(+)-catechin[c]	8.79 ± 0.89	*NT*	*NT*	1.16 ± 0.02	4.32 ± 0.15
quercetin[c]	1.81 ± 0.11	*NT*	*NT*	1.18 ± 0.03	2.07 ± 0.10

[a]IC_{50} values represent the means ± SEM of three parallel measurements ($p < 0.05$). [b]$A_{0.50}$ values represent the means ± SEM of three parallel measurements ($p < 0.05$). [c]Reference compounds. [d]NT: not tested.

agents.[12−14] Tyrosinase inhibition activity of the isolated compounds was carried out by spectrophotometrically using mushroom tyrosinase according to Hearing Method with slight modification by Khatib et al.[15,16]

T. nigrescens has not been previously investigated for its chemical constituents. Thus, the aim of the present research was to isolate and identify phenolics from the aerial parts of this plant species and to check their possible antioxidant and tyrosinase inhibitory abilities and whether *T. nigrescens* can be considered as a natural and very good commercial source of phenolic-flavonoid compounds.

MATERIALS AND METHODS

General Experimental Procedures. Unless otherwise stated, the following procedures were adopted. Melting points were determined on a Yanaco mp apparatus. UV spectra were measured on a Shimadzu UV-1601 machine in MeOH solutions, and given by λmax nm (log ε). IR spectra were recorded on a Perkin-Elmer One B in nujol and given by cm^{-1}. NMR spectra were done in CD_3OD on a Varian Oxford 300 MHz for ^1H NMR, ^{13}C NMR, DEPT, $^1H-^1H$ COSY, HMBC, and HMQC. ESI-MS was measured on Thermo Finnigan LCQ Advantage Max LC/MS/MS apparatus. HRESIMS spectra were recorded on a Bruker MicrOTOF-Q spectrometer. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (DC-Alugram 60 UV_{254} of E. Merck, Germany), by using ceric sulfate spraying reagent until coloration developed. Purification was carried out on Polyamide-6 DF (Riedel-De Haen AG, Germany), Sephadex LH-20, and silica gel (E. Merck, 230−400 μm mesh, Germany). Recycling preparative HPLC was used for the final purification (Shimadzu LC-8A, Shimadzu Corporation, Japan) with a column Inertsil ODS-4 (20 × 250 mm, 5 μm, GL Sciences Inc., Japan) and a PDA detector (190−800 nm).

Plant Material. *T. nigrescens* was collected from Corlu-Tekirdag, Turkey in June 2009. A voucher specimen is deposited in Biology Department, Trakya University (EDTU 9516).

Extraction and Isolation. The air-dried aerial parts of *T. nigrescens* were macerated with 80% ethanol at room temperature and concentrated in vacuo. The residue (65 g) was diluted with water and then partitioned with hexane, dichloromethane, ethyl acetate, and *n*-butanol, respectively. Considering the lipid peroxidation inhibitory and the free radical scavenging activity results of four extracts, the ethyl acetate extract showed the best activity among the extracts tested (Table 1). For this reason, isolation studies were carried out on ethyl acetate extract. The EtOAc extract of the *T. nigrescens* was purified by different chromatographic methods such as combination of various column chromatographic techniques including reverse phase recycling HPLC.

Ethyl acetate extract (6.1 g) was loaded onto silica gel column chromatography to isolate pure compounds and eluted with gradient mixtures from hexane to EtOAc and from EtOAc to MeOH to obtain 8 fractions (A−H).

Fractions A, B, and C were subjected to silica gel column chromatography using a gradient from 100% hexane to %100 EtOAc. Compounds **2** (18.3 mg), **3** (27.3 mg), and **4** (21.0 mg) were obtained from fractions A, B, and C, respectively. A polyamide column was used for the purification of fraction D. Elution was carried out with 100% $CHCl_3$ to 20% MeOH/$CHCl_3$ to obtain compound **5** (32.1 mg). Latter subfractions of this column were applied to RP-HPLC (ODS-4 column, MeOH:H_2O; 1:1, flow rate 9 mL/min) to obtain compound **6** (23.6 mg). Subfractions of fraction E were applied to RP-HPLC (ODS-4 column, MeOH:H_2O; 1:1, flow rate 9 mL/min) after the polyamide column using a gradient solvent system of MeOH/$CHCl_3$ (0−50%) to obtain compounds **7** (18.6 mg) and **8** (21.5 mg). Fraction F was also loaded to the polyamide column using a gradient solvent from 100% $CHCl_3$ to 50% MeOH/$CHCl_3$. The first subfraction of fraction F was purified by RP HPLC (ODS-4 column, MeOH:H_2O; 1:1, flow rate 9 mL/min) to give compound **9** (24.0 mg). The later subfraction was subjected to sephadex LH-20 column chromatography using H_2O and increasing amounts of MeOH to obtain compound **10** (28.9 mg). Fractions G and H were subjected to sephadex column chromatography using a gradient of H_2O−MeOH. Compounds **12** (13.2 mg) and **1** (25.2 mg) were obtained from fractions G and H, respectively.

Chemical Reagents. Quercetin was obtained from E. Merck (Darmstadt, Germany). For thin-layer chromatography, silica gel F_{254} (Merck 5554) precoated plates were used. Silica gel 60 (0.063−0.200

mm, Merck) and Sephadex LH-20 (25−100 μM, Sigma-Aldrich) were used for column chromatography. TLC plates were developed by spraying with Ce (IV) sulfate in 50% H_2SO_4, followed by heating at 105 °C. Butylatedhydroxytoluene (BHT), α-tocopherol, (+)-catechin were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

Determination of the Antioxidant Activity with the β-Carotene Bleaching Method. The total antioxidant activity of the extracts and the compounds (**1**−**12**) was evaluated using the β-carotene-linoleic acid test system[17] with slight modifications. Ethanol was used as a control. BHT, α-tocopherol, (+)-catechin, and quercetin were used as antioxidant standards for comparison of the activity. The sample concentration providing 50% lipid peroxidation inhibition activity (IC_{50} μM) was calculated from the graph of antioxidant activity percentages (Inhibition %) against sample concentrations (μM).

DPPH Free Radical Scavenging Assay. The free radical scavenging activity of the extracts and the compounds (**1**−**12**) was determined spectrophotometrically by the DPPH assay described by Blois[18] with slight modification. Ethanol was used as a control. BHT and α-tocopherol were used as antioxidant standards for comparison of the activity. The sample concentration providing 50% radical scavenging activity (IC_{50} μM) was calculated from the graph of $DPPH^{\bullet}$ scavenging effect percentages (inhibition %) against sample concentrations (μM).

ABTS Cation Radical Decolorization Assay. The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity of the compounds (**1**−**12**) was determined according to the method of Re et al. with slight modifications.[19] Ethanol was used as a control. BHT and α-tocopherol were used as antioxidant standards for comparison of the activity. The sample concentration providing 50% radical scavenging activity (IC_{50} μM) was calculated from the graph of $ABTS^{\bullet+}$ scavenging effect percentages (inhibition %) against sample concentrations (μM).

Superoxide Anion Radical Scavenging Activity. Measurement of superoxide anion radical scavenging activity of the compounds (**1**−**12**) was based on the method described by Liu et al.[20] with slight modification. Ethanol was used as a control. BHT, α-tocopherol, (+)-catechin, and quercetin were used as antioxidant standards for comparison of the activity. The sample concentration providing 50% radical scavenging activity (IC_{50} μM) was calculated from the graph of $O_2^{\bullet-}$ scavenging effect percentages (inhibition %) against sample concentrations (μM).

Cupric Reducing Antioxidant Capacity (CUPRAC). The cupric reducing antioxidant capacity of the compounds (**1**−**12**) was determined according to the method of Apak et al. with slight modifications.[21] Results were given as $A_{0.50}$ which corresponds to the concentration providing 0.500 absorbance. The sample concentration providing 0.500 absorbance ($A_{0.50}$) was calculated from the graph of the absorbance signal of cupric reducing antioxidant capacity against the sample concentration. BHT, α-tocopherol, (+)-catechin, and quercetin were used as antioxidant standards for comparison of the activity.

Tyrosinase Inhibition Assay. Tyrosinase inhibitory activity of the isolated compounds (**1**−**12**) was determined by spectrophotometrically using mushroom tyrosinase according to Hearing method[15] with slight modification by Khatib et al.[16] L-DOPA was used as substrate, and also kojic acid and L-mimosine were used as standard inhibitors of tyrosinase. The percent inhibition of the enzyme and IC_{50} values of the isolated compounds (**1**−**12**) were calculated using a program developed from the graph of tyrosinase inhibitory activity percentages (inhibition %) against sample concentrations (μM).

Statistical Analysis. All data on antioxidant activity and tyrosinase 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} and $A_{0.50}$ values. Data were recorded as mean ± SEM (standard error of the mean). Significant differences between means were determined by Student's-*t* test and *p* values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Analysis of Phenolics and Flavonoids. The structures of the isolated compounds were elucidated by spectroscopic techniques such as UV, IR, ^1H and ^{13}C NMR, EI and HREI MS, and various 2D NMR techniques. A new compound was identified as an ether linked biflavonoid, 4‴,5,5″7,7″-pentahydroxy-3′,3‴-dimethoxy-3-*O*-β-D-glucosyl-3″,4′-*O*-biflavone (**1**) (Figure 1). The other isolated compounds were

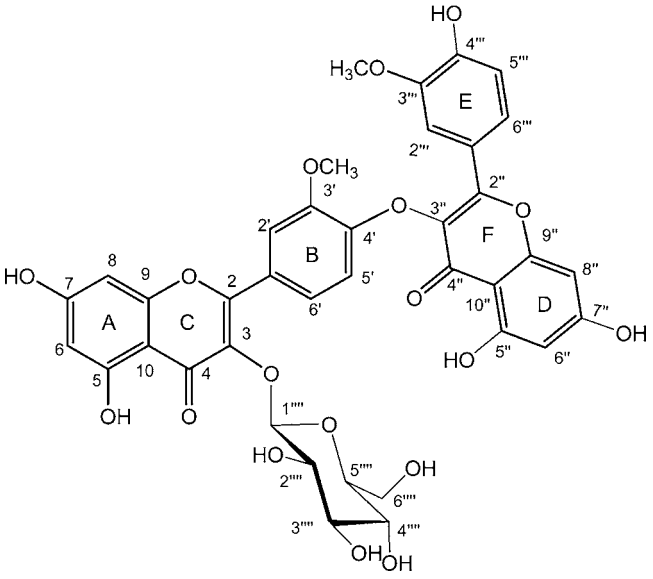
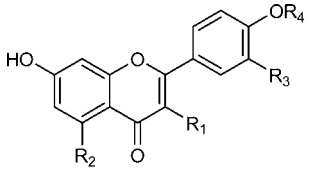


Figure 1. 4‴,5,5″,7,7″-pentahydroxy-3′,3‴-dimethoxy-3-*O*-β-D-glucosyl-3″,4′-*O*-biflavone (**1**).

known phenolic compounds, mainly flavonoid glycosides, namely, 4-hydroxy-3,5-dimethylbenzoic acid (**2**), *p*-hydroxybenzoic acid (**3**), *p*-coumaroic acid (**4**), 7-hydroxy-4′-hydroxyflavon (**5**), 7-hydroxy-4′-methoxyflavanol (**6**) 3′,4′,5,7-tetrahydroxyflavanol-3-*O*-β-glucoside (**7**), 4′,5,7-trihydroxy-3′-methoxyflavanol-3-*O*-β-glucoside (**8**), 3′,4′,5,7-tetrahydroxyflavanol-3-*O*-β-galactoside (**9**), 3′,4′,5,7-tetrahydroxyflavanol-3-*O*-β-glucoside-6″-acetyl (**10**), 4′,5,7-trihydroxy-3′-methoxyflavanol-3-*O*-β-galactoside (**11**), and 3′,4′,7,-trihydroxy-5-methoxyflavanol-3-*O*-β-glucoside (**12**) (Figure 2).

Compound **1** was obtained as yellow powder possessing elemental composition $C_{38}H_{32}O_{18}$ as concluded from HRESIMS 776.15993 (calcd. 776.15886). In the ^1H NMR spectrum, the presence of two sets of *meta* coupled protons as well as two



R_1= H, R_2= H, R_3= H, R_4= H (**5**)
R_1= OH, R_2= H, R_3= H, R_4= OCH_3 (**6**)
R_1= O-β–glucose, R_2= OH, R_3= OH, R_4= H (**7**)
R_1= O-β–glucose, R_2= OH, R_3= OCH_3, R_4= H (**8**)
R_1= O-β–galactose, R_2= OH, R_3= OH, R_4= H (**9**)
R_1= O-β–glucose-6"-acetyl, R_2= OH, R_3= OH, R_4= H (**10**)
R_1= O-β–galactose, R_2= OH, R_3= OCH_3, R_4= H (**11**)
R_1= O-β–glucose, R_2= OCH_3, R_3= OH, R_4= H (**12**)

Figure 2. Isolated flavonoids from *T. nigrescens*.

AMX system protons were indicative for a biflavonoid structure (Table 2). Four protons comprising the overlapped *meta*-coupled dublets appearing at δ 6.16 (d, 2.0 Hz, 2H) and 6.31 (d, 2.0 Hz, 2H) were assigned to H-6/H-6″ and H-8/H-8″ for ring A and ring D of two flavone units, respectively. The two AMX system protons were resonated at δ 8.00 (1H, d, 2.1 Hz, H-2‴), 7.54 (1H, dd, 8.7 and 2.1 Hz, H-6‴), and 6.86 (1H, d, 8.7 Hz, H-5‴) for ring E and at δ 7.53 (1H, s, H-2′), 6.84 (1H, d, 9.0 Hz, H-5′), and 8.06 (1H, d, 9.0 Hz, H-6′) for ring B. The latter splitting pattern was unusual for an AMX system, because a singlet and two doublets with equal coupling constants (9.0 Hz) appeared instead of a doublet with a small coupling constant (ca. 2 Hz), a doublet of doublet (ca. 9 and 2 Hz), and a doublet with a large coupling constant (ca. 9 Hz). The reason for this may be the restricted rotation of ring E due to connection with 4′-flavone unit and 3-*O*-glycoside moiety. The ^{1}H NMR spectrum showed an anomeric proton signal at δ 5.31 (H-1‴) as a doublet of 7.5 Hz indicating a β-glycoside linkage of sugar moiety. Other sugar proton signals were observed at δ 3.44−3.84. The ^{13}C NMR signals corresponding to the sugar moiety (δ, 103.0, 76.1, 73.8, 72.1, 69.2, 61.3) were characteristic of a glucopyranoside.[22] The presence of two methoxy groups was confirmed by the signals at δ_H 3.91 and 3.93 in the ^{1}H NMR, and δ_C 55.9 and 56.0 in the ^{13}C NMR spectra. These two methoxy groups were placed at C-3′ and C-3‴ based on their ROESY correlations with H-2′ (δ_H 7.53, s) and H-2‴ (δ_H 8.00, d, 2.1 Hz), respectively.

Table 2. ^{1}H NMR and ^{13}C NMR Spectral Data of Compound 1

position	δ_H	δ_C
2		157.2
3		134.7
4		178.7
5		162.0
6	6.16 (d, 2.0 Hz)	99.5
7		165.1
8	6.31 (d, 2.0 Hz)	94.4
9		156.2
10		105.0
1′		121.5
2′	7.53 (s)	107.7
3′		147.5
4′		160.1
5′	6.86 (d, 9.0 Hz)	115.2
6′	8.06 (d, 9.0 Hz)	131.2
2″		157.1
3″		138.3
4″		178.2
5″		162.0
6″	6.16 (d, 2.0 Hz)	99.5
7″		165.1
8″	6.31 (d, 2.0 Hz)	94.4
9″		154.3
10″		103.2
1‴		121.8
2‴	8.00 (d, 2.1 Hz)	113.9
3‴		147.0
4‴		149.7
5‴	6.84 (d, 8.7 Hz)	115.2
6‴	7.54 (dd, 8.7, 2.1 Hz)	122.5
1⁗	5.31 (d, 7.5 Hz)	103.0
2⁗	3.28 (m)	72.1
3⁗	3.22 (m)	73.8
4⁗	3.08 (t, 9.0 Hz)	69.2
5⁗	3.51 (m)	76.1
6⁗	3.40 (m)	61.3
OCH_3	3.91 (s)	55.9
OCH_3	3.93 (s)	56.0

In the HMBC spectrum of **1**, the anomeric proton at δ 5.31 (H-1⁗) showed cross-peak with the C-3 (δ 134.7) of ring C while aromatic protons of ring B, H-2′ (δ 7.53) and H-6′ (δ 8.06), were correlated with an *O*-linked quaternary C-2 (δ 157.2) and also another *O*-linked carbon C-4′ (δ 160.1). The H-5′ (δ 6.86) and the methoxy group (δ_H 3.93) showed a cross-peak with *O*-linked carbon signal at δ 147.5 (C-3′) that was supporting the placement of one of the methoxy groups as C-3′ on the basis of HMBC correlations. The protons of ring E, H-2‴ (δ 8.06) and H-6‴ (δ 7.54), were correlated with a *O*-linked quaternary carbon C-2″ (δ 157.1) and also another *O*-linked carbon C-4‴ (δ 149.7). H-6‴ (δ 7.54) also showed a four bond away correlation with an oxygenated carbon at C-3″ (δ 138.3). The H-5‴ (δ 6.86) showed a cross-peak with a quarternary carbon signal at δ 121.8 (C-1‴) as well as an oxygenated carbon signal at δ 147.0 (C-3‴) that was correlated with the methoxy proton at δ 3.91, as well. The overlapped proton signals of H-6 and H-6″ of ring A and ring D, respectively, displayed a crosspeak with a methine signal at δ 93.4 (C-8 and C-8‴) and vice versa H-8 and H-8‴ signals showed a cross peak with the signal at δ 99.3 for C-6 and C-6‴. Comparison of the ^{13}C NMR spectral data of **1** with that of isorhamnetin showed that C-4′ of ring B and C-3″ of ring F in **1** are involved in interflavonoid ether linkage as the resonances of these carbons shifted downfield ca. 11 and 6 ppm, respectively, from the corresponding carbon resonances of isorhamnetin.[23]

Compound **1** (25.2 mg) was hydrolyzed with 2 N HCl (2 mL) at 80 °C for 3 h. The mixture was extracted with $CHCl_3$ (3 × 2 mL). The aqueous layer was neutralized with NaOH (2 N) and evaporated to dryness. The dry powders were dissolved in pyridine (2 mL), and trimethylsilyl imidazole (1.5 mL) was added. The mixture was stirred, and heated at 60 °C for 1 h. An aliquot (4 μL) of the supernatant was removed and directly subjected to GC analysis under the following conditions: A temperature gradient system was used for the oven, starting at 150 °C for 1 min and increasing to 280 °C at rate 3°/min, carrier gas N_2 (1 mL/min), injector and detector temp 250 °C, split ratio 1:50. The configuration of D-glucose was determined by comparison of the retention time of the corresponding derivative with standard D-glucose, giving single peak at 17.20 min. On this basis, the structure of compound **1** was identified as 4‴,5,5″,7,7″-pentahydroxy-3′,3‴-dimethoxy-3-*O*-β-D-glucosyl-3″,4′-*O*-biflavone.

4‴,5,5″,7,7″-Pentahydroxy-3′,3‴-Dimethoxy-3-*O*-β-Glucosyl-3″,4′-*O*-Biflavone (1). $[\alpha]_D^{25}$: −21° (MeOH, *c* 0.5). UV (MeOH) λ_{max} (log ε): 232 (3.436), 315 (1.465) nm. IR (nujol) ν_{max} 3330 (chelated OH), 1620 (>C═O), 1590 (>C═<, aromatic) cm^{-1}. ^{1}H and ^{13}C NMR see Table 2. ESIMS *m*/*z* [M]$^+$ 778.2 (10), [Magl]$^+$ 613.1 (15), [Magl−5xOH]$^+$ 532.7 (80), [Magl−5xOH−OCH_3]$^+$ 502.1 (100). HRESIMS *m*/*z* 776.15993 (calcd for $C_{38}H_{32}O_{18}$, 776.15886).

Antioxidant Activity of Isolated Compounds. The antioxidant activity of individual pure compounds (**1**−**12**) isolated from the ethyl acetate extract was tested by the β-

carotene-linoleic acid assay for lipid peroxidation activity, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and $O_2^{\bullet-}$ assays for radical-scavenging activity, and CUPRAC assay for reducing antioxidant activity. The antioxidant activity test results are given in Table 1.

The phenolic compounds may act as free radical scavengers and chain breaking antioxidants,[24] however, they (**2**−**4**) did not exhibit high antioxidant activity in our assays, except for ABTS cation radical assay. The isolated nine flavonoid compounds (**1**, **5**−**12**) should be most responsible constituents of the ethyl acetate extract, as they were found to be highly active in all complementary five test assays. In the *β*-carotene-linoleic acid assay, compound **7** (IC_{50}: 9.30 ± 0.86 *μ*M) was found to be the most active compound, followed by compounds **10** (IC_{50}: 13.30 ± 2.56 *μ*M) and **1** (IC_{50}: 14.66 ± 3.05 *μ*M). In DPPH assay, compounds **9**, **10**, and **7** exhibited better activity with IC_{50} values of 4.29 ± 0.86, 4.51 ± 0.69, and 4.84 ± 0.19 *μ*M, respectively, than those of BHT (IC_{50}: 54.97 ± 0.99 *μ*M) and *α*-tocopherol (IC_{50}: 12.26 ± 0.07 *μ*M). In the ABTS assay, among the other isolated compounds, the new compound **1** (IC_{50}: 3.08 ± 0.05 *μ*M) showed the best cation radical scavenging activity, and demonstrated higher activity than that of BHT (IC_{50}: 4.87 ± 0.45 *μ*M), and showed activity comparable to that of antioxidant standard *α*-tocopherol (IC_{50}: 2.91 ± 0.55 *μ*M). In superoxide anion radical scavenging activity assay, while compounds **10** (IC_{50}: 11.95 ± 2.23 *μ*M), **12** (IC_{50}: 13.49 ± 1.23 *μ*M), and **9** (IC_{50}: 18.37 ± 0.89 *μ*M) showed activity higher than that of BHT (IC_{50}: 19.60 ± 0.00 *μ*M), compounds **10** and **12** exhibited activities similar to that of *α*-tocopherol (IC_{50}: 11.58 ± 0.19 *μ*M).

These radical scavenging activity results were also supported by the CUPRAC assay. In the CUPRAC assay, compound **9** indicated the best activity ($A_{0.50}$: 6.14 ± 0.11 *μ*M) among the tested compounds, and followed by **10** (IC_{50}: 6.31 ± 0.12 *μ*M), **7** (IC_{50}: 6.78 ± 0.13 *μ*M), and new compound **1** (IC_{50}: 6.89 ± 0.06 *μ*M) (Table 1).

The compounds **5** and **6** have very similar flavonoid structure. The differences between these two compounds are the existence of −OH at C-3 and $-OCH_3$ group at C-4′ in compound **6**, instead of −H and −OH at C-3 and C-4′, respectively, in compound **5**. Compound **6** was found to be more active than the compound **5** which is in accordance with the knowledge that hydroxyl group at C-3 increases the radical scavenging activity.[25]

When the activity of the compounds **7** and **12** were compared, as expected, **7** showed better activity than compound **12** since it has one more hydroxyl group.

Compounds **7** and **9** have also very similar structures. The difference is *O*-*β*-glucoside is bonded at C-3 in compound **7**, instead of *O*-*β*-galactoside in compound **9**. Lipid peroxidation inhibition method revealed that the activity of compound **7** (IC_{50}: 9.30 ± 0.86 *μ*M) is better than that of **9** (IC_{50}: 38.42 ± 4.53 *μ*M). However, compound **9** exhibits better activity than **7** in other assays. The reason for this is the glycosyl moiety may scavenge the singlet oxygen more than galactoside. *β*-carotene lineloic acid assay is a method which reveals the level of inhibition of lipid peroxidation, and it is important to understand the type of antioxidant giving H• radicals to the medium to stop the radical degradation.[26] This method is also important to understand the antioxidants which scavenge singlet oxygen causing radicals in lipids,[27] while in other four methods antioxidant transfers electrons to the media.

Compounds **8** and **11** also have similar structures. The difference is the existence of *O*-*β*-glucoside at C-3 position in compound **8** instead of *O*-*β*-galactoside in compound **11**. The antioxidant activity values of these compounds were correlated with those of compounds **7** and **9**.

In conclusion, the isolated biflavonoid (**1**), phenolic acids (**2**−**4**), flavonoid (**5**), and flavone glycosides (**6**−**12**) were individually found to be more active than their extracts.

Tyrosinase Inhibition Activity of Isolated Compounds. Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skinwhitening and preventive effects. Besides being used in the treatment of some dermatological disorders associated with melanin hyperpigmentation, tyrosinase inhibitors have found an important role in the cosmetic and pharmaceutical industries for their skin-whitening effect and depigmentation after sunburn.[28]

The compounds **7** (IC_{50} = 0.38 mM), **8** (IC_{50} = 0.19 mM), and **12** (IC_{50} = 0.26 mM) exhibited highly potent inhibition against the enzyme tyrosinase, even better than the standard tyrosinase inhibitors kojic acid (KA, IC_{50} = 0.67 mM) and L-mimosine (LM, IC_{50} = 0.64 mM) (Table 2). The compounds **1**, **2**, **3**, **5**, and **6** exhibited good inhibition (range from 0.93 to 1.23 mM) while compounds **4**, **9**, **10**, and **11** exhibited weak tyrosinase inhibition compared to the standard tyrosinase inhibitors (Table 3). In this study, it can be concluded that compounds **7**, **8**, and **12** can be potential candidates for the treatment of melanin biosynthesis related skin diseases, likely hyper-pigmentation of human as well as animals.

Table 3. Tyrosinase Inihbitory Activity of Compounds 1-12

compound	inhibition of tyrosinase (IC_{50}, mM)
1	0.93
2	1.23
3	1.02
4	78.35
5	0.98
6	0.95
7	0.38
8	0.19
9	4.15
10	1.64
11	418.30
12	0.26
kojik acid[a]	0.67
L-mimosine	0.64

[a]Standard tyrosinase inhibitors.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectra of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +90 284 2359592-1232. E-mail: ozdemirkiran@gmail.com, ozlemdemirkiran@trakya.edu.tr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank TUBITAK (The Scientific and Technological Research Council of Turkey) for financial support of the project (TBAG 108T779).

■ REFERENCES

(1) Zohary, M.; Heller, D. *Genus Trifolium*; Ahva Printing Press: Jerusalem, 1984.
(2) Zohary, M. In *Flora of Turkey*, Vol. 3; Davis, P. H., Ed.; University Press: Edinburgh, 1970; pp 384−448.
(3) Kolodziejczyk-Czepas, J. J. Trifolium Species-Derived Substances and Extracts-Biological Activity and Prospects for Medicinal Applications. *J. Ethnopharmacol.* **2012**, *143*, 14−23.
(4) Baytop, T. *Therapy with Medicinal Plants in Turkey*; Istanbul University Press: Istanbul, Turkey, 1984; p 409.
(5) De Rijke, E.; Zafra-Gómez, A.; Ariese, F.; Brinkman, U. A.; Gooije, C. Determination of Isoflavone Glucoside Malonates in *Trifolium pratense* L. (red clover) Extracts: Quantification and Stability Studies. *J. Chromatogr. A* **2001**, *932*, 55−64.
(6) Oleszek, W.; Stochmal, A. Triterpene Saponins and Flavonoids in the Seeds of *Trifolium* species. *Phytochemistry* **2002**, *61*, 165−170.
(7) Knight, W. E. Miscellaneous Annual Clovers. In *Clover Science and Technology*; Taylor, N. L., Ed.; American Society of Agronomy: Madison, WI, 1985; pp 547−562.
(8) Hoveland, C. S.; Evers, G. W. Arrowleaf, Crimson and Other Annual Clovers. In *Forages*, 5th ed., Vol. *1* An Introduction to Grassland Agriculture; Barnes, R. F., Miller, D. A., Nelson, C. J., Eds.; Iowa State University Press: Ames, IA, 1995; pp 249−260.
(9) Hearing, V. J.; Jimenez, M. The Critical Regulatory Control Point in Melanocyte Pigmentation. *Int. J. Biochem.* **1987**, *19*, 1141−1147.
(10) Jimenez, M.; Tsukamoto, K.; Hearing, V. J. Tyrosinase from Two Different Loci Are Expressed by Normal and by Transformed Melanocytes. *J. Biol. Chem.* **1991**, *266*, 1147−1156.
(11) Jimenez-Cervantes, C.; Solano, F.; Kobayashi, T.; Urabe, K.; Hearing, V. J.; Garcia Borron, J. C. A New Enzymatic Function in the Melanogenic Pathway. *J. Biol. Chem.* **1994**, *269*, 17993−18001.
(12) Akiu, S.; Suzuki, Y.; Ashara, T.; Fujinuma, Y.; Fukuda, M. Inhibitory Effect of Arubutin on Melanogenesis: Biochemical Study Using Cultered B16 Melanoma Cells. *Jpn. J. Dermatol.* **1991**, *101*, 609−613.
(13) Hashimoto, A.; Ichihasi, M.; Mishima, Y. The Mechanism of Depigmentation of Hydroquinone; A Study on Supression of Recovery Processes of Tyrosinase Activity in the Pigment Cells *in vivo* and *in vitro*. *Jpn. J. Dermatol.* **1984**, *94*, 794−804.
(14) Khan, V. Effect of Kojic Acid on the Oxidation of DL-DOPA, Norepinephrine, and Dopamine by Mushroom Tyrosinase. *Pigm. Cell Melanoma R.* **1995**, *8*, 234−240.
(15) Hearing, V. J. *Methods in Enzymology*; Academic Press: New York, 1987; Vol. *142*, pp 154−165.
(16) Khatib, S.; Nerya, O.; Musa, R.; Shumel, M.; Tamir, S.; Vaya, J. Chalcones as Potent Tyrosinase Inhibitors: The Importance of 2,4-disubstituted Resorcinol Moiety. *Bioorg. Med. Chem.* **2005**, *13* (13), 433−441.
(17) Marco, G. J. A Rapid Method for Evaluation of Antioxidants. *J. Am. Oil Chem. Soc.* **1968**, *45*, 594−598.
(18) Blois, M. S. Antioxidant Determinations by the Use of a Stable Free Radical. *Nature* **1958**, *181*, 1199−1200.
(19) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.
(20) Liu, F.; Ooi, V. E. C.; Chang, S. T. Free Radical Scavenging Activity of Mushroom Polysaccharide Extracts. *Life Sci.* **1997**, *60*, 763771.
(21) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine: CUPRAC Method. *J. Agric. Food Chem.* **2004**, *52*, 7970−7981.
(22) Agrawal, P. K. NMR Spectroscopy in the Structural Elucidation of Oligosaccharides and Glycosides. *Phytochemistry* **1992**, *31*, 3307−3330.
(23) Fico, G.; Graziella, R.; Flamini, G.; Passarella, D.; Tome, F. Comperative Phytochemical and Morphological Analyses of Three Italian *Primula* species. *Phytochemistry* **2007**, *68*, 1683−1691.
(24) Shahidi, F.; Wanasundara, P. K. J. P. D. Phenolic Antioxidants. *Crit. Rev. Food Sci. Nutr.* **1992**, *32*, 67−103.
(25) Pratt, D. E. Role of Flavones and Related Compounds in Retarding Lipid-oxidative Flavour Changes in Foods. In *Phenolic, Sulfur, and Nitrogen Compounds in Food Flavors*; Vol. *26*, Part 1; Charalambous, G., Katz, I., Eds.; American Chemical Society: Washington, DC, 1976; pp 1−13.
(26) Huang, D.; Ou, B.; Prior, R. L. The Chemistry Behind Antioxidant Capacity Assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.
(27) Öztürk, M.; Kolak, U.; Duru, M. E.; Harmandar, M. GC-MS Analysis of the Antioxidant Active Fractions of *Micromeria juliana* with Anticholinesterase Activity. *Nat. Prod. Comm.* **2009**, *4*, 1271−1276.
(28) Shoukat, P.; Moonkyu, K.; Hwan-Suck, C.; Hyunsu, B. Naturally Occuring Tyrosinase Inhibitors: Mechanism and Applications in Skin Health, Cosmetics and Agriculture Industries. *Phytother. Res.* **2007**, *21*, 805−816.