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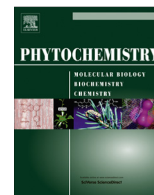

Phenolic compounds from *Trifolium echinatum* Bieb. and investigation of their tyrosinase inhibitory and antioxidant activities

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ABSTRACT

Two bischromones, 3,3′-dimethoxy-2′-oxychromone (**1**), 3,3′-dihydroxy-2,2′-oxychromone (**2**) and a biflavone, 5,7,4′,5″,3‴,4‴′-hexahydroxy-3″-*O*-*β*-glucosyl-3′,7″-*O*-biflavone (**3**) have been isolated from whole plant of *Trifolium echinatum* Bieb. together with five known flavonoids. The structures of the compounds were elucidated by 1D and 2D NMR analysis as well as HRESIMS. The isolated compounds were investigated for their antioxidant activity and tyrosinase inhibitory activity. Highly potent inhibition was found for compounds **1** (IC_{50} = 0.41 mM), **5** (IC_{50} = 0.47 mM) and **8** (IC_{50} = 0.45 mM) compared to those of standard tyrosinase inhibitors kojic acid (IC_{50} = 0.67 mM) and L-mimosine (IC_{50} = 0.64 mM). The antioxidative effect of the extracts was determined by using *β*-carotene–linoleic acid, $DPPH^{\cdot}$ scavenging, $ABTS^{+\cdot}$ scavenging, and CUPRAC assays. The experimental findings indicated that the compounds **2** and **8** were found to be active in radical scavenging and CUPRAC assays.

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1. Introduction

The Mediterranean region is very rich in *Trifolium* (Fabaceae) species, especially in Turkey where they are spread out as distinct 103 species (Zohary and Heller, 1984; Zohary, 1970). Plants from the genus *Trifolium* have been used in traditional medicine by many cultures (Sabudak and Guler, 2009). In Turkish folk medicine, some *Trifolium* species such as *Trifolium repens*, *Trifolium arvense*, *Trifolium pratense* are used as expectorant, analgesic, antiseptic and against rheumatism aches (Baytop, 1984).

Tyrosinase is a copper-containing enzyme, which is widely distributed in microorganisms, animals and plants and is a key enzyme in melanin biosynthesis, involved in determining the color of mammalian skin and hair. Melanin plays a crucial protective role against skin photo carcinogenesis; however, the production of abnormal melanin pigmentation is a serious esthetic problem in human beings (Priestley, 1993). Furthermore, tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects (Maeda and Fukuda, 1991; Seiberg et al., 2000). Recently, tyrosinase inhibitors have attracted concern due to hyperpigmentation (Friedman, 1996) resulting from the increased use of tyrosinase enzyme in medicinal (Mosher et al., 1983) and cosmetic products (Maeda and Fukuda, 1991), and also their identification and isolation from natural sources are increased (Son et al., 2000). Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low costs, especially when rich sources are identified.

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.

The aim of the present study focuses on the isolation and structure elucidation of phenolics from *Trifolium echinatum* Bieb. (Fabaceae) and investigation of their tyrosinase inhibitory and antioxidant activities. The antioxidant properties of the both crude extracts and isolated compounds were investigated by five complimentary tests; namely, lipid peroxidation inhibition activity, DPPH radical scavenging activity, ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) assay. As a chemical investigation of T. *echinatum*, two new bischromones (**1** and **2**), a new biflavonoid (**3**) (Fig. 1) and five known flavonoids (**4**, **5**, **6**, **7** and **8**) were isolated (Fig. 1). The structures of isolated compounds were elucidated by high-resolution mass spectrometry and multidimensional NMR spectroscopy. There is only one article

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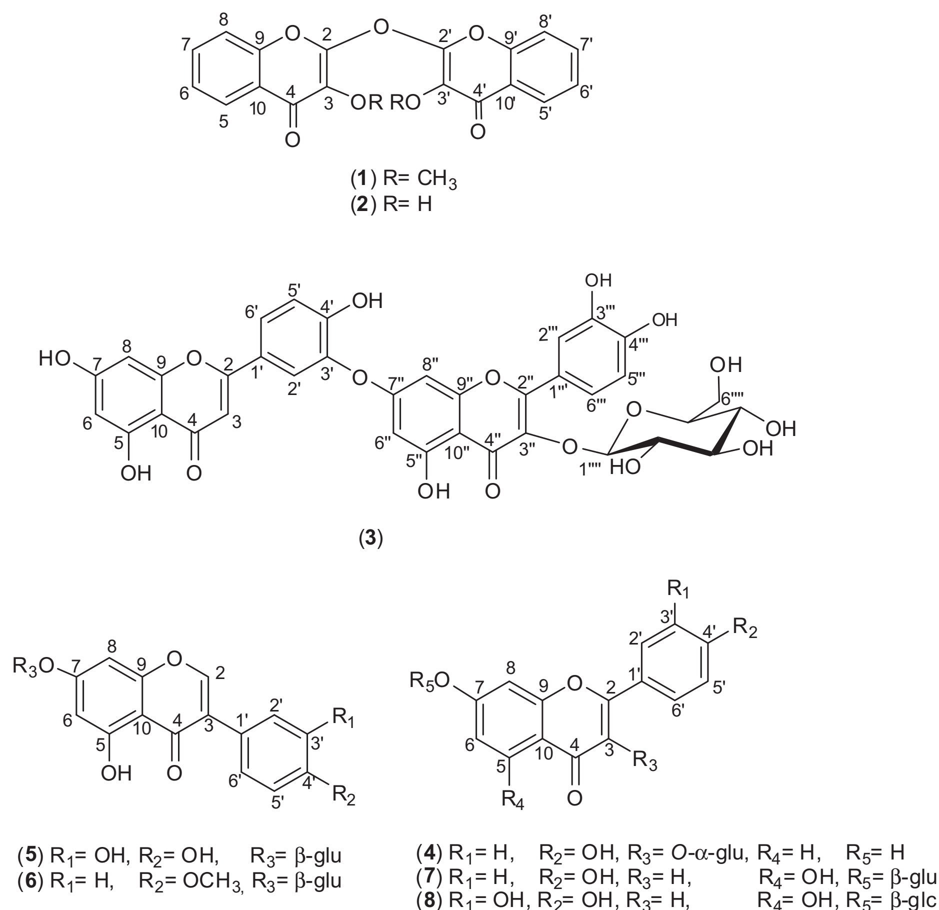

Fig. 1. Structures of isolated compounds (**1**–**8**) from *Trifolium echinatum*.

in connection with chemistry of *T. echinatum* in the literature (Shalashvili, 1993). Accordingly, this is the first report about bioactivity of the chemical constituents of *T. echinatum* Bieb. Furthermore, the known compounds (**4**–**8**) have been isolated from this plant species for the first time.

2. Results and discussion

2.1. Isolation and structure elucidation

The air dried aerial parts of *T. echinatum* were extracted with 80% ethanol in room temperature. The EtOH extract were concentrated *in vacuo*, and the residue was diluted with water and then partitioned with hexane, dichloromethane, ethyl acetate, and *n*-butanol, respectively. These extracts of *T. echinatum* were evaluated for their *in vitro* antioxidant activity by four complimentary tests. Considering the antioxidant activity results, ethyl acetate and *n*-butanol extracts of *T. echinatum* were showed better activity than the other extracts (Table 1). For this reason, ethyl acetate and *n*-butanol extracts of *T. echinatum* were studied. The ethyl acetate extract was purified by open column chromatography combined with semi preparative HPLC–UV affording compounds **1**–**6** and **8**, while the purification of the *n*-BuOH extract using the same technique afforded compound **7**.

The compound **1** gave a molecular ion peak $[M]^+$ at *m*/*z* 366 in positive ESI-MS and at *m*/*z* 366.0823 in the HRESIMS, consistent with a molecular formula of $C_{20}H_{14}O_7$ (Calcd. 366.0739). The ^{1}H NMR spectrum (**1**) displayed two signals corresponding to *meta* and *orto*-coupled protons at δ_H 6.67 (*dd*, *J* = 8.0 and 1.0 Hz, H-8/H-8′) and δ_H 7.72 (*dd*, *J* = 7.3, 1.4 Hz, H-5/H-5′). The ^{1}H NMR spectrum demonstrated two protons as *ddd* at δ_H 7.16 (*ddd*, *J* = 7.3, 7.3, 1.9 Hz) and δ_H 6.65 (*ddd*, *J* = 8.0, 8.0, 1.0 Hz), assignable to H-7/H-7′ and H-6/H-6′ protons, respectively. The presence of a methoxy group was observed at δ_H 3.5 with a singlet signal corresponding to three protons. The HMBC spectrum of compound **1** suggested that the methoxy group was attached to the C-3′ position. The ^{13}C NMR, DEPT and HMQC spectra revealed 10 carbon signals including four sp^2 quaternary carbons, four sp^2 methines, one carbonyl and one methoxy carbon. Among them, two quaternary carbons (δ_C 158.0, and 160.2) and the carbonyl carbon (δ_C 175.6) were ascribed to those bearing an oxygen atom. The molecular ion peak $[M]^+$ at *m*/*z* 366 in positive ESI-MS were suggested that the compound **1** has a symmetrical bischromone structure. Based on these results, **1** was identified as 3,3′-dimethoxy-2,2′-oxychromone which is a new natural product.

Spectral data of **2** showed that it possessed a similar structure to that of **1**, but it differentiated at C-3′ position. The difference between these two compounds is that absence of methoxy signal in ^{1}H NMR spectrum of compound **2**. In its ^{1}H NMR spectrum, aromatic proton signals at δ_H 6.77 (1H, *dd*, *J* = 8.0, 1.0 Hz), 7.25 (1H, *ddd*, *J* = 7.3, 7.3, 1.9 Hz), 6.76 (1H, *ddd*, *J* = 8.0, 8.0, 1.0 Hz), and 7.83 (1H, *dd*, *J* = 7.8, 1.4 Hz) were observed, suggesting that **2** has a bischromone skeleton with disubstituted hydroxyl groups. The full assignment of ^{1}H NMR of compound **2** was achieved with the aid of ^{13}C NMR, DEPT, HMQC and HMBC spectra. A detailed study on the ^{13}C NMR, DEPT and HMQC spectra revealed 9 carbon signals due to four sp^2 quaternary carbons, four sp^2 methines and one carbonyl carbon.

Table 1
Antioxidant activity of the compounds (**1–8**), all extracts of *Trifolium echinatum* and α-tocopherol, butylatedhydroxy toluene (BHT) by the β-carotene–linoleic acid, DPPH, ABTS, and CUPRAC assays.[a]

Extracts and samples	β-Carotene–linoleic acid assay IC_{50} (μM)	DPPH· assay IC_{50} (μM)	$ABTS^{\cdot+}$ assay IC_{50} (μM)	CUPRAC $A_{0.50}$ (μM)[b]
Hexane extract	129.1 ± 1.76	211.9 ± 2.00	NT	NT
Dichloromethane extract	52.62 ± 1.22	126.9 ± 1.60	NT	NT
Ethyl acetate extract	15.00 ± 0.76	10.32 ± 0.67	NT	NT
Butanol extract	18.35 ± 0.84	16.44 ± 0.96	NT	NT
1	>200	>200	360.77 ± 6.59	296.93 ± 0.01
2	44.46 ± 1.67	12.79 ± 1.01	4.90 ± 0.09	6.36 ± 0.01
3	36.24±0.99	304.89 ± 3.09	82.93 ± 0.95	111.73 ± 0.01
4	>200	>200	100.47 ± 2.09	401.82 ± 0.02
5	56.24 ± 1.66	495.81 ± 5.29	148.38 ± 3.89	101.07 ± 0.02
6	>200	>200	100.90 ± 2.69	397.64 ± 0.00
7	106.38 ± 0.95	195.07 ± 2.19	45.67 ± 0.89	105.63 ± 0.02
8	84.41±1.51	101.42 ± 1.59	19.28 ± 0.07	59.71 ± 0.01
BHT[c]	1.34 ± 0.09	54.97 ± 0.99	4.10 ± 0.06	3.80 ± 0.00
α-Tocoferol[c]	2.10 ± 0.09	12.26 ± 0.07	4.87 ± 0.45	10.20 ± 0.01
(+)-Catechin[c]	8.79 ± 0.89	4.32 ± 0.15	1.16 ± 0.02	NT
Quercetin[c]	1.81 ± 0.11	2.07 ± 0.10	1.18 ± 0.03	NT

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

The signal at δ_C 177.6 for the carbonyl carbon (C-4/C-4′), four other signals of sp^2 aromatic carbons at δ_C 130.4 (C-5/C-5′), 117.7 (C-6/C-6′), 132 (C-7/C-7′), 115.8 (C-8/C-8′) and four querternary carbons at 160.2 (C-2/C-2′), 132.4 (C-3/C-3′), 161.5 (C-9/C-9′), 112.8 (C-10/C-10′) were characteristic of a chromone structure. The compound **2**, exhibited a molecular ion peak at *m/z* 338 $[M]^+$ which was indicative for the symmetrical chromone structure similar to compound **1**. The HRESIMS of compound **2** showed the molecular ion peak at *m/z* 338.0452 in agreement with molecular formula $C_{18}H_{10}O_7$ (Calcd. 338.0426). Thus, the structure of the new compound **2** was elucidated as 3,3′-dihydroxy-2,2′-oxychromone.

The ^{1}H NMR spectrum of **3** showed a two ABX coupling systems, one set of signals at δ_H 7.41 (1H, *d*, *J* = 1.9 Hz), 6.91 (1H, *d*, *J* = 8.2 Hz) and 7.42 (1H, *dd*, *J* = 8.2, 1.9 Hz) for H-2′, H-5′ and H-6′ of B-ring, respectively, the other set of signals at δ_H 7.84 (1H, *d*, *J* = 2.4 Hz), 6.87 (1H, *d*, *J* = 8.7 Hz) and 7.58 (1H, *dd*, *J* = 8.7, 2.4 Hz) for, H-2‴, H-5‴ and H-6‴ of E-ring respectively, indicating the presence of the C-3′, C-4′ disubstituted B-ring moiety and C-3‴ and C-4‴ disubstituted E-ring moiety. Two pair of *meta*-protons at δ_H 6.80, 6.51, 6.19 and 6.39 were located at the H-6, H-8, H-6″ and H-8″, respectively. The sharp signal at δ_H 6.61 (1H, *s*) was assigned to be H-3 of C-ring, an additional group of signals at δ_H 5.1 (H-1⁗, *d*, *J* = 7 Hz) and δ_H 3.4–4.8 (H-2⁗, H-3⁗, H-4⁗, H-5⁗, H-6⁗) indicated the presence of a sugar unit at C-3″ in ring F (Agrawal, 1992; Kajjout and Rolando, 2011; Wang et al., 2011). The identity of the sugar was determined by the analysis of a combination of its HMQC and HMBC spectra (Fig. 2). The β-anomeric configuration for the glucose unit was determined from its large $^3J_{H1,H2}$ coupling constant (*ca.* 7 Hz). The HMBC spectrum showed cross peak between C-3″ (δc 134.4) and H-1⁗ (δ_H 5.1) (Fig. 2).

The above spectral data suggested that **3** could be a biflavonoid consisting of a flavone unit linked to a flavonol glucoside unit through a C–*O*–C bond. The supporting evidence came from the ^{13}C NMR spectral data of **3** which showed a close resemblance to those of apigenin (Markham, 1978) and quercetin-3-*O*-glucoside (Isoquercitrin) (Tatsis et al., 2007; Wang et al., 2012). Further comparison of ^{13}C NMR spectral data of **3** with those of apigenin and quercetin-3-*O*-glucoside showed that C-3′ of ring B should be involved in the interflavonoid ether linkage with C-7″ of ring D as the resonance of C-3′ which was shifted downfield by 28.7 ppm from the corresponding carbon resonance of apigenin. The HMBC spectrum of **3** further confirmed the formation of an interflavonoid ether linkage between C-3′ and C-7″ based on three-bond away correlations from H-6′ to C-3′ and from H-8′ to C-3′, as well as from H-5′ to C-3′.

The compound **3** gave a molecular ion peak $[M]^+$ at *m/z* 732 in negative ESI-MS and at *m/z* 732.1345 in the HRESIMS which was consistent with a molecular formula of $C_{36}H_{28}O_{17}$ (Calcd. 732.1326). However, negative ESI-MS spectrum of **3** exhibited some characteristic flavonoid fragments at *m/z* 447 (100) and *m/z* 464 (40). The $[M-C_{15}H_9O_6]^+$ ion at *m/z* 447 and $[(M+1)-C_{15}H_{19}O_5]^+$ ion at *m/z* 464 were evidence for a biflavonoid containing a interflavonyl linkage at C-3′–O–C-7″. Based on the above deductions **3** was elucidated as a novel biflavonoid 5,7,4′,5″,3‴,4⁗-hexahydroxy-3″-*O*-β-glucosyl-3′,7″-*O*-biflavone.

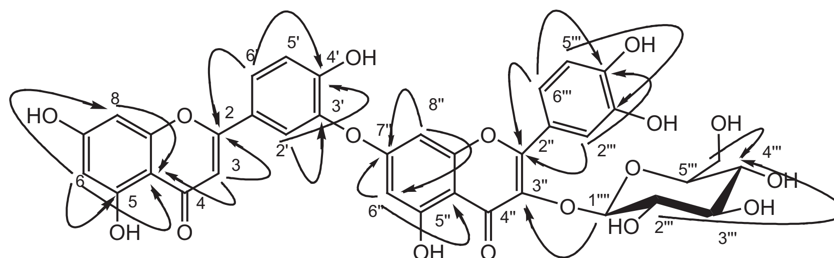

Fig. 2. Key HMBC of compound **3**.

Table 2
Tyrosinase inhibitory activity of compounds (**1–8**)[a].

Compounds	IC_{50}(mM)
1	0.41
2	1.00
3	0.95
4	0.70
5	0.47
6	1.25
7	1.12
8	0.45
Kojik asit[b]	0.67
L-Mimosine[b]	0.64

[a] Values are means ± S.E.M. of three separate experiments.
[b] Standard tyrosinase inhibitor.

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

Position	Compound (**1**)		Compound (**2**)	
	gCOSY	gHMBC	gCOSY	gHMBC
5	H-6, H-7	C-4, C-7, C-9	H-6, H-7	C-4, C-7, C-9
6	H-5, H-7, H-8	C-8, C-10	H-5, H-7, H-8	C-5, C-7, C-8
7	H-5, H-6, H-8	C-5, C-9	H-5, H-6, H-8	C-5, C-9
8	H-6, H-7	C-6, C-10	H-6, H-7	C-6, C-7
5′	H-6′, H-7′	C-4′, C-7′, C-9′	H-6′, H-7′	C-4′, C-7′, C-9′
6′	H-5′, H-7′, H-8′	C-8′, C-10′	H-5′, H-7′, H-8′	C-5′, C-7′, C-8′
7′	H-5′, H-6′, H-8′	C-5′, C-9′	H-5′, H-6′, H-8′	C-5′, C-9′
8′	H-6′, H-7′	C-6′, C-10′	H-6′, H-7′	C-6′, C-7′
$-OC\underline{H}_3$	–	C-3, C-3′	–	–

[a] Recorded in CD_3OD.

On the basis of spectral properties compounds **4–8** were identified as 7,4′-dihydroxy-flavonol-3-*O*-α-glucoside; orobol-7-*O*-*β*-glucoside; biochanin A-7-*O*-*β*-glucoside; apigenin-7-*O*-*β*-glucoside and luteolin-7-*O*-*β*-galactoside, respectively. The 1D and 2D NMR and MS data of **4** (Markham, 1978), **5** (Polasek et al., 2007), **6** (Polasek et al., 2007), **7** (Geissberger and Sequin, 1991; Markham, 1978) and **8** (Li et al., 2008; Markham, 1978) were in good agreement with those of data given in the literature. These known flavonoids were isolated from this plant species for the first time.

2.2. Antioxidant and tyrosinase inhibitory activities of the isolated compounds

There are several methods for determining the antioxidant activity. In this study, antioxidant activities were determined by four complimentary tests, namely, the *β*-carotene–linoleic acid assay for lipid peroxidation activity, and DPPH· and $ABTS^{\cdot+}$ assays for radical-scavenging activity, and CUPRAC assay for reducing antioxidant activity. Considering the lipid peroxidation inhibitory and the free radical scavenging activity results of four extracts, the ethyl acetate and the *n*-butanol extracts showed better activity than the others extracts (Table 1). For this reason, further antioxidant activity tests were carried out on all the individual pure compounds **1**–**8** isolated from the ethyl acetate and *n*-butanol extracts. The antioxidant activity test results are given in Table 1.

Phenolic compounds are known to be powerful chain breaking antioxidants (Shahidi and Wanasundara, 1992). Therefore, the compounds (**1**–**8**), especially the aglycons of the compounds must be responsible for the antioxidant activity of the extract. The most active compounds of the ethyl acetate extract concerning the lipid peroxidation inhibition were the flavonoids. Compound **2** was found to be the most active compound, followed by compound **8**, except for the *β*-carotene–linoleic acid assay. In the ABTS assays, compound **2** (IC_{50}: 4.90 ± 0.09 μM) showed close activity to those of antioxidant standards α-tocopherol and BHT. In DPPH assay this compound exhibited better activity (IC_{50}: 12.79 ± 1.01 μM) than that of BHT (IC_{50}: 54.97 ± 0.99 μM) and showed close activity to that of α-tocopherol (IC_{50}: 12.26 ± 0.07 μM). These radical scavenging activities are supported by the CUPRAC assay. In the CUPRAC assay, however, compound **2** indicated better activity ($A_{0.50}$: 6.36 ± 0.01 μM) than that of α-tocopherol ($A_{0.50}$: 10.20 ± 0.01 μM) (Table 1).

The compounds **1** and **2** have the chromone structure, but **2** is a phenolic compound due to OH groups at C-3 and C-3′. Compound **2** is able to give electrons or H· to the media via these groups. Therefore, it is not surprising for **2** that it can act as antioxidant. Compound **1**, however, has no hydroxyl groups and it was unable to scavenge the radicals (Table 1).

Compound **3** is a biflavonoid which is a bulky molecule. It has inhibited the lipid peroxidation similar to those of compound **2** due to its hydroxyl groups. Another advantage of **3** is that it has better solubility in water than **2**. As the reaction medium of *β*-carotene–linoleic acid assay is oxygenated water compound **3** indicated better lipid peroxidation inhibition than **2**. However, because of steric hindrance **3** was unable to approach DPPH free radical. It also demonstrated that $ABTS^{\cdot+}$ scavenging activity of **3** was more than compounds **1**, **4**–**6**, but less than **2**, **7**–**8** (Table 1).

Compounds **4** and **7** are flavonoids. The difference between two compounds is the position of the glucose moiety. While in **4** the glucose is attached to C-3, in compound **7** the glucose is attached to C-7. Compound **7** was found to be more active than the compound **4** in accordance with the knowledge of hydroxyl group at C-3 increases the radical scavenging activity, in contrast the sugar at C-3 decreases the antioxidant activity (Pratt, 1976). When the compounds **5** and **8** were compared, the differences between these two compounds are that **5** is a isoflavanoid, and *O*-*β*-glucoside is bonded instead of *O*-*β*-galactoside at C-**7** position. The antioxidant data of **5** and **8** revealed that compound **5** indicated better lipid peroxidation inhibitory activity than the compound **8**, while compound **8** demonstrated better radical scavenging and reducing power activities than the compound **5** Compound **8**, however, as expected it showed better activity than compound **7** since it has one more hydroxyl group at C-4′ in the structure (Table 1).

In conclusion, the isolated bischromones, biflavonoid and flavone glycosides were not found to be more active alone than their extracts as expected. These results indicate that the antioxidant activity of the extracts is probably explained by a synergy of the phenolic compounds.

The isolated compounds were also screened for their tyrosinase inhibitory activity. The compounds **1**, **5** and **8** exhibited highyl potent inhibition against tyrosinase, even better than the standard tyrosinase inhibitors kojic acid and L-mimosine (Table 2). The compound **4** (IC_{50} = 0.70 mM) exhibited almost equal potent inhibition while compounds **2**, **3**, **6**, **7** exhibited lower tyrosinase inhibition compared to the standard tyrosinase inhibitors (Table 2).

3. Conclusions

From the EtOAc and *n*-BuOH extracts of whole plant of *Trifolium echinatum* eight compounds were isolated: two new bischromones (**1** and **2**), a new biflavonoid (**3**) and five known flavonoids (**4**, **5**, **6**, **7** and **8**). All of the compounds have been isolated from this plant species for the first time.

The antioxidant activity and tyrosinase inhibitory activity of *T. echinatum* for the first time in this study, either. The compounds **1**, **5** and **8** 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 antioxidant activity, however, were determined by four complimentary methods, and compound **2** was found to be the most active compound, followed by compound **8**. Compound **3** showed the best lipid peroxidation inhibitory activity on the β-carotene–linoleic acid assay (Table 1).

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic industry due to their skin whitening 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 (Shoukat et al., 2007). In this study, it can be concluded that compounds **1**, **5** and **8** can be potential candidate for the treatment of melanin biosynthesis related skin diseases, likely hyper- pigmentation of human as well as animals.

4. Experimental

4.1. General

NMR spectra were done in CD_3OD on a Varian Unity Inova (Varian, Palo Alto, CA, USA) 500 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 (Basel, Switzerland). Recycling preparative HPLC was used for the final purification (Shimadzu LC-8A, Shimadzu Corporation, Japan) with a PDA detector and a column Shim-Pack ODS (C-18) (20 × 250 mm, 5 μm, Shimadzu Corporation, Japan) using flow rate as 10 mL min^{-1}. HRESIMS were recorded on Bruker MicrOTOF-Q spectrometer (Bruker Daltonics, Bremen, Germany). The tyrosinase inhibitory activity was monitored using a microplate reader (Molecular Devices, SpectraMax 190, USA). Thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (DC-Alugram 60 UV_{254} of E. Merck, Germany), by using ceric sulphate spraying reagent until coloration developed. Sephadex LH-20, and silica gel (E. Merck, 230–400 μm mesh, Germany). Methanol, chloroform, quercetin, copper (II) chloride, ammonium acetate and potassium persulfate were obtained from E. Merck (Darmstadt, Germany). For thin layer chromatography, silica gel F_{254} (Merck 5554) precoated plates were used. Polyamide-6 DF (Riedel-De Haen AG, Germany), silica gel 60 (0.063–0.200 mm, Merck), Sephadex LH-20 (25–100 μM, Sigma–Aldrich) were used for column chromatography. TLC plates were developed by spraying with Ce(IV) sulphate in 50% H_2SO_4, followed by heating at 105 °C. β-carotene, linoleic acid, polyoxyethylenesorbitan monopalmitate (Tween-40), butylated hydroxytoluene (BHT), α-tocopherol, neocuproin, (+)-catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (Sigma–Aldrich GmbH, Sternheim, Germany). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from FlukaChemie (FlukaChemie GmbH, Sternheim, Germany). All other chemicals and solvents were in analytical grade.

4.2. Plant material

The plant sample was collected from Corlu-Tekirdag, Turkey in May–June 2009 and provisionally classified by Necmettin Guler, PhD, Trakya University, as *Trifolium echinatum* Bieb. (EDTU 9516).

4.3. Extraction

The air dried whole plant material (950 g) was macerated with 80% ethanol in room temperature. The ethanol extract were concentrated on a rotary evaporator under vacuum. The residual extract (137.4 g) was diluted with water and then extracted by *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), successively. After the solvents evaporated, 15.87 g of *n*-hexane extract, 3.4 g of dichloromethane extract, 7.2 g of ethyl acetate extract and 19.2 g of *n*-butanol extract were obtained.

4.4. Isolation

As the ethyl acetate and the *n*-butanol extracts showed better antioxidant activity results, these extracts were chosen to study for the isolation and structure elucidation of pure compounds.

The ethyl acetate extract (7.2 g) was subjected to CC (Silica gel-Merck 1.77734; glass column size: 80 × 5 cm) and eluted with 100% CH_2Cl_2 to 100% EtOAc and then 100% EtOAc to 100% MeOH by 10% increase in the polarity to give 170 fractions. The 15 fractions (F1–F15) were obtained according to TLC results of 170 fractions. Fraction 8 (F8) was purified by semi-preparative RP-HPLC using a Shim-Pack ODS C-18 column (20 × 250 mm, 5 μm) using a linear gradient from 10% MeOH (0.1% TFA): 90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) for 45 min at a flow rate 10 mL min^{-1} and yielded **6** (21.8 mg). Fraction F11 was purified by semi-preparative RP-HPLC (Shim-Pack ODS C-18 column, 20 × 250 mm, 5 μm) using a linear gradient from 10% MeOH: 90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) for 45 min at a flow rate 10 mL min^{-1} and yielded compound **2** (7.7 mg). A linear gradient from 10% MeOH: 90% H_2O (0.1% TFA) to 65% MeOH: 35% H_2O (0.1% TFA) at a flow rate of 9 mL/min over 60 min was run and totally 60 fractions were collected for each min. from F12. Subfractions 24, 35, and 43 contained pure compounds **1** (15.0 mg), **4** (12.1 mg), and **5** (17.8 mg), respectively. Fraction F13 applied to RP-HPLC (Shim-Pack ODS (C-18) column, flow rate 10 mL min^{-1}) using a linear gradient from 10% MeOH: 90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) for 45 min to obtain compound **8** (19.4 mg). F14 was purified using a linear gradient from 10% MeOH (0.1% TFA): 90% H_2O (0.1% TFA) to 100% MeOH (0.1% TFA) for 45 min at a flow rate of 9 mL/min and yielded compound **3** (17.1 mg).

In this study, *n*-butanol extract (19.2 g) was subjected to CC (Poliamid-Fluka-02395; glass column size: 60 × 2 cm). Elution with the mixtures of 100% H_2O to 100% MeOH gave 32 fractions. The four fractions (A–D) were obtained according to TLC results of 32 fractions. Frac. B was applied to the RP-HPLC using a linear gradient from 10% MeOH: 90% H_2O to 100% MeOH for 45 min to obtain compound **7** (17.6 mg).

4.5. Compound characterisation

4.5.1. 3,3′-Dimethoxy-2,2′-oxychromone (*1*)

Amorphous white powder, mp 135–138 °C. UV (MeOH) λ_{max} (log ε) nm: 202 (4.1), 227 (0.9), 297 (2.8). ^1H NMR (500 MHz, CD_3OD): δ_H 6.67 (2H, *dd*, *J* = 8.0, 1.0 Hz, H-8/H-8′), 7.16 (2H, *ddd*, *J* = 7.3, 7.3, 1.9 Hz, H-7/H-7′), 6.65 (2H, *ddd*, *J* = 8.0, 8.0, 1.0 Hz, H-6/H-6′), 7.72 (2H, *dd*, *J* = 7.3, 1.4 Hz, H-5/H-5′), 3.5 (6H, *s*, OMe). ^{13}C NMR (125 MHz, CD_3OD): δc 158.0 (C-2/C-2′), 138.6 (C-3/C-3′), 175.6 (C-4/C-4′), 131.0 (C-5/C-5′), 118.0 (C-6/C-6′), 132.4 (C-7/C-7′), 115.8 (C-8/C-8′), 160.2 (C-9/C-9′), 119.1 (C-10/C-10′), 63.1(–OCH_3). Positive ESI-MS *m/z* (Rel. Int. %): 366 $[M]^+$ (8), 300 $[(M-2)-(2\times OCH_3)]^+$ (50); HRESIMS *m/z* 366.0823 (Calcd. for $C_{20}H_{14}O_7$ 366.0739).

4.5.2. 3,3′-Dihydroxy-2,2′-oxychromone **(2)**

Amorphous white powder, mp 158–161 °C. UV (MeOH) λ_{max} (log ε) nm: 203 (3.8), 238 (1.2), 307 (2.5). ^1H NMR (500 MHz, CD_3OD): δ_H 6.77 (1H, *dd*, *J* = 8.0, 1.0 Hz, H-8/H-8′), 7.25 (1H, *ddd*, *J* = 7.3,

7.3, 1.9 Hz, H-7/H-7′), 6.76 (1H, *ddd*, *J* = 8.0, 8.0, 1.0 Hz, H-6/H-6′), 7.83 (1H, *dd*, *J* = 7.8, 1.4 Hz, H-5/H-5′). ^{13}C NMR (125 MHz, CD_3OD): δ_C 160.2 (C-2/C-2′), 132.4 (C-3/C-3′), 177.6 (C-4/C-4′), 130.4 (C-5/C-5′), 117.7 (C-6/C-6′), 132.0 (C-7/C-7′), 115.8 (C-8/C-8′), 161.0 (C-9/C-9′), 112.0 (C-10/C-10′. Positive ESI-MS *m/z* (Rel. Int. %): 338 $[M]^+$ (8), 300 $[(M-2)-(2\times H_2O)]^+$ (100); HRESIMS *m/z* 338.0452 (Calcd. for $C_{18}H_{10}O_7$ 338.0426).

4.5.3. 5,7,4′,5″,3‴,4⁗-Hexahydroxy-3″-O-β-glucosyl-3′,7″-O-biflavone (3)

Amorphous yellow powder, mp 153 °C. UV (MeOH) λ_{max} (log ε) nm: 254 (2.8), 368 (3.2). ^{1}H NMR (500 MHz, CD_3OD): δ_H 6.51 (1H, *d*, *J* = 1.9 Hz, H-8), 6.80 (1H, *d*, *J* = 1.9 Hz, H-6), 6.61 (1H, *s*, H-3), 7.41 (1H, *d*, *J* = 1.9 Hz, H-2′), 6.91 (1H, *d*, *J* = 8.2 Hz, H-5′), 7.42 (1H, *dd*, *J* = 8.2, 1.9 Hz, H-6′), 6.39 (1H, *d*, *J* = 1.9 Hz, H-8″), 6.19 (1H, *d*, *J* = 1.9 Hz, H-6″), 7.84 (1H, *d*, *J* = 2.4 Hz, H-2‴), 6.87 (1H, *d*, *J* = 8.7 Hz, H-5‴), 7.58 (1H, *dd*, *J* = 8.7, 2.4 Hz, H-6‴), 5.1 (1H, *d*, *J* = 7 Hz, H-1⁗), 3.4–4-8 (6H, *m*, H-2⁗/H-3⁗/H-4⁗/H-5⁗/H-6⁗). ^{13}C NMR (125 MHz, CD_3OD): δ_C 166.1 (C-2), 103.7 (C-3), 178.7 (C-4), 158.0 (C-5), 100.2 (C-6), 164.1 (C-7), 95 (C-8), 155.8 (C-9), 106.5 (C-10), 122.0 (C-1′), 112.7 (C-2′), 146.0 (C-3′), 150.4 (C-4′), 116.2 (C-5′), 119.2 (C-6′), 155.1 (C-2″), 134.3 (C-3″), 178.2 (C-4″), 159.5 (C-5″), 104.0 (C-6″), 163.2 (C-7″), 94.5 (C-8″), 154.3 (C-9″), 103.2 (C-10″), 122.8 (C-1‴), 116.9 (C-2‴), 144.0 (C-3‴), 148.0 (C-4‴), 115.6 (C-5‴), 121.0 (C-6‴), 101.5 (C-1⁗), 72.4 (C-2⁗), 74.7 (C-3⁗), 71.0 (C-4⁗), 76.3 (C-5⁗), 65.1 (C-6⁗). ^{1}H–^{1}H COSY (CD_3-OD): δ_H 6.39/6.19 (H-8″/H-6″); 7.42/7.41, 6.91 (H-6′/H-2′, H-5′); 6.51/6.80 (H-8/H-6); 7.58/6.87, 7.84 (H-6‴/H-5‴, H-2‴); 5.1/3.4–4.48 (H-1⁗/H-2⁗). Negative ESI-MS *m/z* (Rel. Int. %): 732 $[M]^+$ (5), 447 $[M-C_{15}H_9O_6]^+$ (100), 464 $[(M+1)-C_{15}H_{19}O_5]^+$ (40); HRESIMS *m/z* 732. 1345 (Calcd. for $C_{36}H_{28}O_{17}$, 732.1326).

4.6. Bioassays

4.6.1. Determination of the antioxidant activity with the -carotene bleaching method

The antioxidant activity was evaluated using -carotene–linoleic acid test system (Miller, 1971) with slight modifications. -Carotene (0.5 mg) in 1 mL of chloroform was added to 25 L of linoleic acid, and 200 mg of Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL of distilled water saturated with oxygen, were added by vigorous shaking. Four thousand microliter of this mixture was transferred into different test tubes containing different concentrations of the sample in ethanol. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of -carotene, was prepared for background subtraction. (+)-catechin, quercetin, BHT and -tocopherol were used as standards.

The bleaching rate (R) of -carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

where: ln = natural log, *a* = absorbance at time zero, *b* = absorbance at time *t* (120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$AA = \frac{R_{\text{control}} - R_{\text{sample}}}{R_{\text{control}}} \times 100$$

4.6.2. DPPH free radical scavenging activity

The free radical scavenging activity of the extracts of *T. echinatum* and the isolated compounds was determined by the DPPH assay described by Blois (1958) with slight modification. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solutions in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH}^{\cdot}\ \text{Scavenging Effect}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

The sample concentration providing 50% free radical scavenging activity (IC_{50}) was calculated from the graph of DPPH· scavenging effect percentage against sample concentration. BHT, α-tocopherol, (+)-catechin and quercetin were used as antioxidant standards for comparison of the activity (Table 3).

4.6.3. ABTS cation radical decolorization assay

The spectrophotometric analysis of $ABTS^{\cdot+}$ scavenging activity was determined according to the method of Re et al. (1999) with slight modifications. The $ABTS^{\cdot+}$ was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with ethanol. Then, 2 mL of $ABTS^{\cdot+}$ solution was added to 1 mL of sample solution in ethanol at different concentrations (5–50 μg/mL). After 30 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). The scavenging capability of $ABTS^{\cdot+}$ was calculated using the following equation:

$$\text{ABTS}^{\cdot+}\text{scavenging effect}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

The sample concentration providing 50% cation radical scavenging activity (IC_{50}) was calculated from the graph of $ABTS^{\cdot+}$scavenging effect percentage against sample concentration. BHT, α-tocopherol, (+)-catechin and quercetin were used as antioxidant standards for comparison of the activity.

4.6.4. Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity was determined according to the method of (Apak et al. 2004), with slight modifications. To each well, in a 96 well plate, 50 μL 10 mM Cu (II), 50 μL 7.5 mM neocuproine, and 60 μL NH_4Ac buffer (1 M, pH 7.0) solutions were added. Forty microliter extract at different concentrations were added to the initial mixture so as to make the final volume 200 μL. After 1 h, the absorbance at 450 nm was recorded against a reagent blank by using a 96-well microplate reader. Results were given as $A_{0.50}$ which corresponds the concentration providing 0.500 absorbance. The sample concentration providing 0.500 absorbance ($A_{0.50}$) was calculated from the graph of the absorbance of cupric reducing antioxidant capacity against the sample concentration. BHT and α-tocopherol were used as antioxidant standards for comparison of the activity.

4.6.5. Tyrosinase inhibition activity of the purified compounds

Tyrosinase inhibitory activity of the isolated compounds were determined by spectrophotometrically using mushroom tyrosinase according to Hearing method (Hearing, 1987) with slight modification by Khatib (Khatib et al., 2005). L-DOPA was used as substrate

and also kojic acid and L-mimosine were used as standard inhibitors of tyrosinase.

Potassium phosphate buffer (0.07 mL, 50 mM) at pH 6.5, 0.3 mL tyrosinase (333 units/mL) and 2 μL of the tested compounds (0.5–500 μM), dissolved in absolute ethanol were inserted into 96-well plates. After 5 min incubation at room temperature, 12 mM L-DOPA were added and incubated for additional 20 min. The optical densities of the samples were measured at 492 nm, and relative to control containing ethanol (2 μL) and without inhibitor, demonstrating a linear colour change with time during the 20 min of the experiment.

The percent inhibition of the enzyme and IC_{50} values of the isolated compounds were calculated using a program developed with Microsoft Excel. The following equation has been followed:

$$\%\text{Inhibition} = [A - B/A] \times 100$$

Here A and B are the absorbances of the control and the samples, respectively.

4.6.6. 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 ± 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.

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