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Two acylated flavonoid glycosides from *Stachys bombycina*, and their free radical scavenging activity

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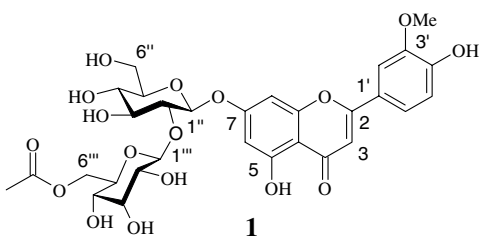
Preparative reversed-phase HPLC analysis of the methanol extract of the aerial parts of *Stachys bombycina* (Lamiaceae) afforded two acylated flavonoids glycosides, chrysoeriol 7-*O*-[6-*O*-acetyl-β-D-allopyranosyl]-(1 → 2)-β-D-glucopyranoside (**1**) and apigenin 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside (**2**), the former being a new natural product. The structures of these compounds were elucidated unambiguously by UV spectroscopic analyses using shift reagents, ESIMS, and 1D and 2D NMR spectroscopic techniques. The free radical scavenging activity of **1** and **2** compounds were assessed by DPPH assay, and the RC_{50} values were 1.25×10^{-2} and 7.69×10^{-4} mg/mL, respectively.

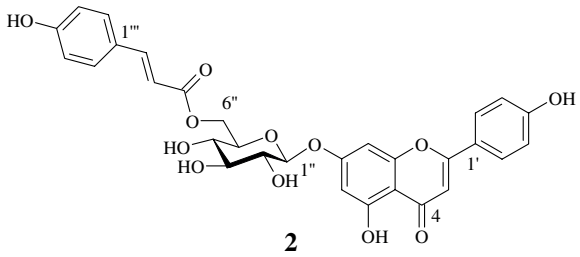
1. Introduction

The subcosmopolitan genus *Stachys* L. comprises more than 270 species and is considered to be one of the largest of the Lamiaceae (Meremeti et al. 2004). *Stachys bombycina* Boiss. is one of the 81 Turkish endemic species of this genus (Bhattacharjee 1982). It grows abundantly only in Antalya, Muğla and Mersin provinces. While there is no report on any previous phytochemical investigation on *S. bombycina* available to date, phytochemical studies on other species of *Stachys* revealed the presence of flavonoid glycosides, phenylethanoid glycosides, iridoid glycosides and terpenoids (Dictionary of Natural Products 2001; ISI Web of Science 2004). We now report on the isolation, structure determination, and free radical scavenging activity of two acylated flavonoid glycosides, chrysoeriol 7-*O*-[6-*O*-acetyl-β-D-allopyranosyl]-(1 → 2)-β-D-glucopyranoside (**1**) and apigenin 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside (**2**), the former being a new natural product, from the aerial parts of *S. bombycina.*



2. Investigations, results and discussion

RP-HPLC analysis of the methanol extract of the aerial parts of *S. bombycina* afforded two acylated flavonoid glycosides which, on the basis of comprehensive spectroscopic analyses (e.g. UV, ESIMS, and 1D and 2D NMR), were characterised as chrysoeriol 7-*O*-[6-*O*-acetyl-β-D-allopyranosyl]-(1 → 2)-β-D-glucopyranoside (**1**) and apigenin 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside (**2**).



Both compounds (**1** and **2**) displayed characteristic UV absorption maxima for a flavone skeleton (Mabry et al. 1970). The ^{1}H NMR and ^{13}C NMR for these compounds (Tables 1 and 2) also confirmed the presence of flavone nucleus in these molecules (Mabry et al. 1970). The ESIMS spectra of **1** revealed $[M + Na]^+$ (positive ion mode) ion peak at m/z 701, and $[M-H]^-$ (negative ion mode) ion peak at m/z 677, suggesting $M_r = 678$ and solving for $C_{31}H_{34}O_{17}$. In the ^{1}H and ^{13}C NMR spectra (Table 1), there were signals for a substituted 5,7,3′,4′-tetrahydroxy flavone nucleus, a glucose moiety, an 6-acetyl allose unit and an aromatic methoxy group. The attachment of the methoxy group at C-3′ was the most feasible option from the biogenetic point of view, and was also supported by the ^{13}C NMR chemical shifts for C-3′ and C-4′ at δ 152.0 and 149.0, respectively. The ^{1}H-^{1}H COSY45 spectrum of **1** (Table 1) revealed ^{1}H-^{1}H couplings and helped to assign all proton resonances. The ^{1}H and ^{13}C NMR data, with the exception of the signal for

Table 1: ^{1}H NMR (coupling constant J = Hz in parentheses) and ^{13}C NMR data, and COSY interaction of compounds 1

Position	Chemical shift δ in ppm		^{1}H-^{1}H interaction obtained from COSY
	δ_H	δ_C	
2	–	165.1	–
3	6.46 s	104.3	–
4	–	182.9	–
5	–	162.0	–
6	6.81 br s	99.3	H-8
7	–	163.7	–
8	6.99 br s	95.9	H-6
9	–	157.7	–
10	–	106.2	–
1′	–	122.1	–
2′	7.59 m	111.1	H-6′
3′	–	152.0	–
4′	–	149.0	–
5′	6.95 m	116.7	H-6′
6′	7.59 m	121.4	H-5′, H-2′
3′-OMe	3.91 s	56.8	–
Glucose moiety			
1″	5.23 d (7.0)	100.4	H-2″
2″	3.20–3.80*	83.6	
3″	3.20–3.80*	78.0	
4″	3.20–3.80*	70.0	
5″	3.20–3.80*	76.7	
6″	3.20–3.80*	61.3	
Allose moiety			
1‴	4.80 d (7.8)	103.4	H-2‴
2‴	3.20–4.10*	72.4	–
3‴	3.20–4.10*	71.7	–
4‴	3.20–4.10*	68.0	–
5‴	3.20–4.10*	72.6	–
6‴	3.20–4.10*	64.9	–
Acyl moiety			
CO	–	171.2	–
CH_3	1.95 s	21.4	–

* Overlapped peaks
Spectra obtained in DMSO-d_6

Table 2: ^{1}H NMR (coupling constant J = Hz in parentheses) and ^{13}C NMR data, and COSY interaction of compounds 2

Position	Chemical shift δ in ppm		^{1}H-^{1}H interaction obtained from COSY
	δ_H	δ_C	
2	–	163.5	–
3	6.49 s	103.7	–
4	–	182.8	–
5	–	157.5	–
6	6.83	100.3	H-8
7	–	165.2	–
8	6.98 s	95.3	H-6
9	–	162.0	–
10	–	106.2	–
1′	–	121.5	–
2′	7.95 d (8.5)	129.4	H-3′, H-6′
3′	6.92 d (8.5)	116.5	H-2′, H-5′
4′	–	162.6	–
5′	6.92 d (8.5)	116.5	H-3′, H-6′
6′	7.95 d (8.5)	129.4	H-2′, H5′
Glucose moiety			
1″	5.18 d (7.5)	100.3	H-2″
2″	3.20–3.90 m	73.8	–
3″	3.20–3.90 m	77.1	–
4″	3.20–3.90 m	70.8	–
5″	3.20–3.90 m	74.7	–
6″	4.47 d (12.15)	64.8	H-5″
Acyl moiety			
1‴	–	125.7	–
2‴	7.38 d (8.3)	131.0	H-3‴, H-6‴
3‴	6.68 d (8.3)	117.0	H-2‴, H-5‴
4‴	–	160.8	–
5‴	6.68 d (8.3)	117.0	H-3‴, H-6‴
6‴	7.38 d (8.3)	131.0	H-2‴, H-5‴
7‴	7.59 d (16.2)	145.8	H-8‴
8‴	6.34 d (16.2)	114.5	H-7‴
9‴	–	167.3	–

* Overlapped peaks
Spectra obtained in DMSO-d_6

the aromatic methoxy (δ_H 3.91 and δ_C 56.8), were comparable with those published for luteolin 7-[6″-acetylallosyl-(1 → 2)glucoside], isolated from *Stachys aegyptiaca* (El-Ansari et al. 1991), and similarly, with the exception of the signals for the acetyl moiety (δ_H 1.95 and δ_C 21.4, 171.2), the NMR data were comparable with published data for chrysoeriol 7-(2″-*O*-β-D-allopyranosyl)β-D-glucopyranoside from *Sideritis grandiflora.* Thus the identity of **1** was confirmed as chrysoeriol 7-*O*-[6-*O*-acetyl-β-D-allopyranosyl]-(1 → 2)-β-D-glucopyranoside, which is a new natural product.

The ESIMS spectra of 2 revealed $[M + Na]^+$ (positive ion mode) ion peak at m/z 601, and $[M-H]^-$ (negative ion mode) ion peak at m/z 577, suggesting $M_r = 578$ and solving for $C_{30}H_{26}O_{12}$. The ^{1}H and ^{13}C NMR spectra (Table 2) of **2**, in addition to the signals associated with the aglycone apigenin, showed signals for a glucose moiety and a *p*-coumaroyl moiety. The deshielded nature of the ^{1}H and ^{13}C NMR signals (δ_H 4.47 and δ_C 64.8) for C-6″ confirmed the attachment of this *p*-coumaroyl moiety at C-6″ of the glucose unit. Apart from the signals associated with the *p*-coumaroyl moiety, all other ^{1}H and ^{13}C NMR signals were comparable to published data for apigenin 7-*O*-β-D-glucopyranoside isolated from *Stachys aegyptiaca* and various other plant sources (El-Ansari et al. 1991; Agarwal and Raghunath 1989). The ^{1}H-^{1}H COSY45 spectrum of **2** (Table 2) displayed ^{1}H-^{1}H couplings and helped to assign key proton resonances. All spectroscopic data including ^{1}H and ^{13}C NMR data of **2** matched perfectly with those published for apigenin 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside (Agarwal and Raghunath 1989; Markham and Geiger 1993; Markham 1982). Thus compound **2** was identified as a known flavone glycoside, 7-*O*-β-D-(6-*p*-coumaroyl)-glucopyranoside.

This is the first report on any phytochemical investigation on *Stachys bombycina*. Previous phytochemical investigations on a number of species of the genus *Stachys* revealed the presence of flavone glucosides, particularly 7-*O*-glucosides, with various kinds of acylation on the sugar moieties (Meremeti et al. 2004). Occurrence of 7-*O*-glucosides of luteolin, apigenin and chrysoeriol are also common in the taxonomically closely related genus *Phlomis* (El-Negoumy et al. 1986; Bucar et al. 1998). Within the genus *Stachys*, the formation of the disaccharyl moiety composed of a glucose and an allose is of common occurrence (Lenherr and Mabry 1987; Lenherr et al. 1984) and has the potential for being used as one of the chemotaxonomic markers for this genus.

Both flavonoids (**1** and **2**) showed prominent free radical scavenging activity (antioxidant activity) in the DPPH assay (Kumarasamy et al. 2001; Takao et al. 1994). The RC_{50} of **1**

and **2** were found to be 1.25×10^{-2} and 7.69×10^{-4} mg/mL, respectively, compared to 2.88×10^{-5} mg/mL for quercetin, a well-known natural antioxidant. The antioxidant activity of these flavonoe glycosides, like other natural phenolic antioxidants is a consequence of the presence of the phenolic moieties in the structures (Kumarasamy et al. 2004). The antioxidant activity of phenolic natural products is predominantly owing to their redox properties, i.e. the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential.

3. Experimental

3.1. General

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV-vis spectrometer. NMR spectra were recorded in CD_3OD on a Bruker 200 MHz NMR Spectrometer (200 MHz for 1 H and 50 MHz for 13 C) using residual solvent peak as internal standard. ESIMS analyses were performed on a Finnigan MAT95 spectrometer. HPLC separation was performed in a Dionex prep-HPLC System coupled with Gynkotek GINA50 autosampler and Dionex UVD340S Photo-Diode-Array detector. A Luna C_{18} preparative HPLC column (10 m, 250 mm × 21.2 mm) was used. Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractionation.

3.2. Plant material

Aerial parts of *S. bombycina* Boiss. were collected in July 2003 from the *Pinus brutia* woods in Antalya, (36 40 910 N, 30 31 730 E), Turkey, about 40 m above sea level. A voucher specimen (Göktürk 5120) representing this collection has been generated in the herbarium of the Department of Biology, Akdeniz University, Turkey.

3.3. Extraction, isolation and structure elucidation

Dried and ground aerial parts (100.0 g) of *S. bombycina* were Soxhlet-extracted successively with *n*-hexane, dichloromethane and MeOH (1.1 L each). All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C. The MeOH extract was fractionated on a Sep-Pak, using a step gradient of 10, 20, 40, 60, 80 and 100% MeOH-water mixture (150 ml each) as eluent. Preparative RP-HPLC (gradient elution, 25–70% MeOH in water in 50 min, 20 ml/min) of the Sep-Pak fraction (60% MeOH in water) yielded compounds **1** and **2** (weight 21.3 and 4.7 mg, and retention time 28.4 and 41.2 min, respectively). Structures of these falvonoids were determined conclusively by UV, ESIMS and 1D and 2D NMR analyses.

3.3.1. Chrysoeriol 7-O-[6-O-acetyl-β-D-allopyranosyl]-(1 → 2)-β-D-glucopyranoside (**1**)

Yellow amorphous solid. UV: λ_{max} (MeOH) nm: 252, 270, 346; +NaOMe: 247 sh, 266, 299 sh, 399; +$AlCl_3$: 264 sh, 270, 298, 356 sh, 390; +$AlCl_3$ + HCl: 274, 292 sh, 358, 380 sh; +NaOAc: 259, 275 sh, 350 sh, 408; +NaOAc + H_3BO_3: 251, 268, 346; ^{1}H- and ^{13}C NMR (Table 1). ESIMS: positive ion mode) m/z 701 $[M + Na]^+$ and negative ion mode) m/z 677 $[M-H]^-$.

3.3.2. Apigenin 7-O-β-D-(6-p-coumaroyl)-glucopyranoside (**2**)

Yellow amorphous solid. UV: λ_{max} (MeOH) nm: 253, 273, 294 sh, 321, 346; +NaOMe: 248 sh, 267, 298 sh, 398; +$AlCl_3$: 264 sh, 271, 298, 356 sh, 390; +$AlCl_3$ + HCl: 274, 292 sh, 358, 381 sh; +NaOAc: 259, 275 sh, 349 sh, 408; +NaOAc + H_3BO_3: 251, 269, 346; ^{1}H- and ^{13}C NMR (Table 2). ESIMS: positive ion mode) m/z 601 $[M + Na]^+$ and negative ion mode) m/z 577 $[M-H]^-$.

3.4. Free radical scavenging activity (DPPH assay)

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Quercetin was obtained from Avocado Research Chemicals Ltd, Shore road, Heysham, Lancs. The method used by Takao et al. (1994) was adopted with suitable modifications (Kumarasamy et al. 2002). DPPH (4 mg) was dissolved in MeOH (50 mL) to obtain a concentration of 80 μg/mL.

Qualitative assay: Test compounds **1** and **2** were applied to a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were noted.

Quantitative assay: Compounds **1** and **2** were dissolved in MeOH to obtain a concentration of 0.5 mg/mL. Dilutions were made to obtain concentrations of 5×10^{-2}, 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6} 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for half an hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the standards (quercetin and trolox).

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