Physocalycoside, a New Phenylethanoid Glycoside from *Phlomis physocalyx* Hub.-Mor.

Tayfun Ersöz[a,*], Kalina Iv. Alipieva[b], Funda Nuray Yalçın[a], Pınar Akbay[a], Nedjalka Handjieva[b], Ali A. Dönmez[c], Simeon Popov[b], and İhsan Çalış[a]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University TR 06100, Ankara, Turkey. Fax: +90-312-3114777; E-mail: tersoz@hacettepe.edu.tr
[b] Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] Department of Biology, Faculty of Science, Hacettepe University TR 06532, Ankara, Turkey

* Author for correspondence and reprint requests

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A new phenylethanoid tetraglycoside, physocalycoside (**2**), was isolated from the aerial parts of *Phlomis physocalyx*. Its structure was identified as 3-hydroxy-4-methoxy-β-phenylethoxy-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)]-4-*O*-feruloyl-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside, on the basis of spectroscopic evidence. In addition, one known iridoid glucoside, lamiide (**1**) and five known phenylethanoid glycosides, wiedemannioside C (**3**), verbascoside (= acteoside) (**4**), leucosceptoside A (**5**), martynoside (**6**), and forsythoside B (**7**) were also characterized. Compounds **2–7** demonstrated radical scavenging properties towards the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

Key words: Phlomis physocalyx, Iridoid and Phenylethanoid Glycosides, Radical Scavenging Activity

Introduction

The genus *Phlomis* (Lamiaceae) is represented by 34 species in the Flora of Turkey (Huber-Morath, 1982). Some members of the genus possess medicinal properties (Saracoğlu *et al*., 1995) and are used as tonics and stimulants in the Anatolian folk medicine (Baytop, 1999). As a part of our ongoing search on secondary metabolites of Turkish *Phlomis* species (Başaran *et al*., 1991; Çalış *et al*., 1990a,b, 1991; Ersöz *et al*., 2001a–c, 2002a–c; Harput *et al*., 1998, 1999; Saracoğlu *et al*., 1995, 1997, 1998, 2002), we studied the Turkish endemic, *Phlomis physocalyx* Hub.-Mor. This plant is an eglandular herb to 30 cm, growing on steppes and calcareous hills at elevations of 950–1730 m in Inner Anatolia (Huber-Morath, 1982). It was found that the methanolic extract of aerial parts of the title plant exhibits antioxidant effects, based on the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. The present paper deals with the isolation and structure elucidation of the new phenylethanoid glycoside, physocalycoside (**2**), as well as of six known compounds, the iridoid glucoside, lamiide (**1**) and the phenylethanoid glycosides, wiedemannioside C (**3**), verbascoside (= acteoside) (**4**), leucosceptoside A (**5**), martynoside (**6**), and forsythoside B (**7**). Antioxidant activity of the phenylethanoid glycosides (**2–7**) is also presented.

Material and Methods

General experimental procedures

Optical rotations were measured on a Rudolph autopol IV Polarimeter using a sodium lamp operating at 589 nm. UV (MeOH) spectra were recorded on a Shimadzu UV-160A spectrophotometer. IR spectra (KBr) were determined on a Perkin-Elmer 2000 FTIR spectrometer. NMR measurements in CD_3OD were performed on a Bruker AMX 300 (^{1}H: 300.13 and ^{13}C: 75.5 MHz) and Varian Unity 500 (^{1}H: 500 and ^{13}C: 125 MHz) spectrometers. Positive mode HR-MALDI MS data were taken on a Ionspec.-Ultima-FTMS instrument with DBH as matrix substance. ESIMS were recorded in the positive ion modes on a Finnigan TSQ 7000 spectrometer. For open-column chromatography (CC), Polyamide (Polyamid-MN-Polyamid SC-6, Machery-Nagel, Düren), Kiesel gel 60 (0.063–0.200 mm, Merck) and Sephadex LH-20 were used. Low-pressure liquid chromatography (RP-8 LPLC) was performed on Lobar pre-

packed columns (310–25 and 240–10), LiChroprep RP-8 (40–63 mμ, Merck). Analytical and preparative-TLC were carried on pre-coated Kieselgel 60 F_{254} aluminum sheets (Merck). Compounds were detected by UV and 1% vanillin/H_2SO_4 followed by heating at 105 °C for 1–2 min. For radical scavenging assay, DPPH (= 2,2-diphenyl-1-picrylhydrazyl, Fluka) was used. Absorbance at 517 nm was measured with an automated microplate reader (Bio-Tek Instruments Inc.) spectrophotometer.

Plant material

Phlomis physocalyx Hub.-Mor. (Lamiaceae) was collected in July 2001 at Sivas (1550 m) near Gürün-Kangal-Kocakurt crossing, Inner Anatolia, Turkey. Voucher specimens have been deposited in the Herbarium of the Biology Department, Faculty of Science, Hacettepe University, Ankara, Turkey (AAD 9555).

Extraction and isolation

The air-dried and powdered aerial parts of *P. physocalyx* (600 g) were extracted with MeOH (3×2500 ml) at 40 °C. After evaporation of the combined extracts *in vacuo* 51.6 g of crude MeOH extract was obtained. The crude extract was dissolved in water (250 ml) and the water-insoluble material was removed by filtration. The filtrate was then extracted succesively with *n*-BuOH (3×150 ml) to obtain the *n*-BuOH fraction (29.4 g). An aliquot of the *n*-BuOH fraction (10 g) was separated on a polyamide column (100 g). Elution with H_2O (500 ml) and gradient MeOH–H_2O (25 to 100%, each 500 ml) mixtures afforded 8 main fractions (A–H). Fraction A (930 mg) was purified on a Lobar RP-8 column (310–25) using increasing amounts of MeOH in H_2O (0 to 65%) to afford fractions A_1 and A_2. Fraction A_2 (99 mg) was separated on a Lobar RP-8 column (240–10) using 5 to 25% MeOH in H_2O as eluent to give lamiide (**1**, 75 mg). Fr. B (420 mg) was fractionated over a Si gel (30 g) column eluting with $CHCl_3$–MeOH–H_2O (80:20:0 to 80:20:3 v/v/v) mixture to yield six fractions (frs. B_1–B_6). Fraction B_5 (74.4 mg) was purified on a Lobar column (240–10) employing a 5% stepwise gradient elution of MeOH in H_2O (0 to 60%) to get a mixture of compounds **2** and **3** (25 mg). Furter fractionation of this mixture by LPLC with a 5% stepwise gradient mixture of MeOH in H_2O (5 to 45%) afforded physoalycoside (**2**, 10 mg) and wiedemannioside C (**3**, 5 mg). Fr. D (117 mg) yielded fractions D_1–D_3 after separation on a Si gel (20 g) column using $CHCl_3$–MeOH–H_2O (80:20:1 to 70:30:3 v/v/v) mixture. Fr. D_2 (33.6 mg) was rechromatographed on Si gel (10 g) with $CHCl_3$–MeOH (98:2 to 90:10 v/v) mixture to yield martynoside (**6**, 3 mg). An aliquot (340 mg) of fraction E (1.52 g) was subjected to Si gel CC (30 g) with $CHCl_3$–MeOH–H_2O (80:20:1 to 60:40:4 v/v/v) mixture to obtain nine fractions E_1–E_9. Fraction E_7 (64.4 mg) was purified by Sephadex CC (MeOH) to give leucosceptoside A (**5**, 10 mg). 540 mg of fraction F (1.52 g) was subjected to Si gel CC (30 g) employing $CHCl_3$–MeOH–H_2O (80:20:1 to 80:20:3 v/v/v) mixtures to give six fractions (frs. F_1–F_6). Fr. F_4 yielded verbascoside (**4**, 28 mg) after purification on a Lobar column (240–10) employing 5% stepwise gradient elution of MeOH in H_2O (25 to 60%). Fraction F_5 was likely separated on a Lobar column (240–10) with a 5% stepwise gradient elution of MeOH in H_2O (15 to 60%) to give a mixture (40 mg) of verbascoside (**4**) and forsythoside B (**7**). This mixture was then separated by PTLC with a $CHCl_3$–MeOH–H_2O (61:32:7 v/v/v) solvent system to yield verbascoside (**4**, 13.2 mg) and forsythoside B (**7**, 23.9 mg).

Physocalycoside (**2**): Amorphous yellowish powder; $[\alpha]_D^{20}$ −43.9° (*c* = 0.1, MeOH); positive-ion HR-MALDI-MS *m/z*: calcd. for $C_{43}H_{60}O_{24}Na$: 983.3473. Found: 983.3358; UV $\lambda_{max.}$ (MeOH, nm) 219, 237, 287 (sh), 328; υ_{max} (KBr, cm^{-1}) 3400 (OH), 1699 (C=O), 1630 (olefinic C=C), 1605 and 1508 (arom. ring); ^1H NMR (CD_3OD, 500 MHz): Table I; ^{13}C NMR (CD_3OD, 125 MHz): Table I.

Reduction of DPPH radical: Methanolic solutions (0.1%) of the phenylethanoid glycosides (**2–7**) were chromatographed on a Si gel TLC plate using $CHCl_3$–MeOH–H_2O (61:32:7) mixture as solvent system. After drying, TLC plates were sprayed with a 0.2% DPPH (Fluka) solution in MeOH. Compounds showing yellow-on-purple spot were regarded as antioxidant (Cuendet *et al*., 1997).

DPPH assay in vitro: The radical scavenging activity of the phenylethanoid glycosides was examined with the DPPH radical, as described by Cuendet *et al*. (2001). Ascorbic acid was used as control. 50 μl 0.02% DPPH in MeOH, 200 μl MeOH and

30 µl of sample solution in MeOH were mixed in a 96-well plate. After incubation of the plate for 30 min at room temperature, optical density was measured at 517 nm and the inhibition percentage (%) of the radical scavenging activity was calculated using the following equation:

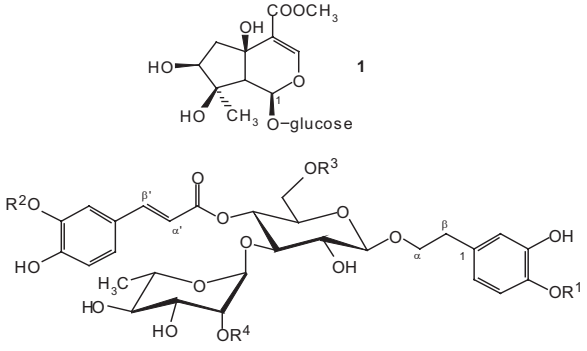
$$\text{Inhibition (\%)} = \frac{A_o - A_s}{A_o} \times 100$$

where A_o is the absorbance of the control and A_s is absorbance of the sample at 517 nm. IC_{50} was determined as the amount of the sample (µM) reducing the absorbance by 50%.

Results and Discussion

The methanolic extract of aeriale parts of *P. physocalyx* was suspended in water and partitioned with *n*-BuOH. Chromatographic separations of the *n*-BuOH extract by polyamide column chromatography followed by low-pressure liquid chromatography and column chromatography on Si gel and Sephadex LH-20 resulted in the isolation of compounds **1–7** (Fig. 1).

The structures of compounds **1** and **3–7** were established as the known lamiide (Bianco *et al.*, 1977; Assaad *et al.*, 1992), wiedemannioside C (Abou Gazar *et al.*, 2003), verbascoside (= acteoside; Sticher and Lahloub, 1982), leucosceptoside A (Miyase *et al.*, 1982), martynoside (Çalış *et al.*, 1984), and forsythoside B (Endo *et al.*, 1982), respectively, by comparison of their ^1H and ^{13}C NMR spectroscopic properties and ESI-MS data with those reported in the literature.



	R^1	R^2	R^3	R^4
2	CH_3	CH_3	glucose	rhamnose
3	H	CH_3	glucose	H
4	H	H	H	H
5	H	CH_3	H	H
6	CH_3	CH_3	H	H
7	H	H	apiose	H

Fig. 1. Iridoid (**1**) and phenylethanoid (**2–7**) glycosides from *P. physocalyx*.

Physocalycoside (**2**) was obtained as a yellowish amorphous powder, $[\alpha]_D^{20}$ −43.9° (*c* = 0.1, MeOH). The positive-ion HR-MALDI mass spectrum exhibited molecular ion peaks $[M+Na]^+$ and $[M+K]^+$ at *m/z* 983 and 999, respectively, consistent with the molecular formula $C_{43}H_{60}O_{24}$. UV absorption bands at λ_{max} 219(sh), 237, 287(sh), and 237 indicated the polyphenolic nature of **2**. The IR spectrum showed absorption bands due to hydroxyl (3400 cm^{-1}), α,β-unsaturated ester carbonyl (1699 cm^{-1}), olefinic double bond (1630 cm^{-1}), and aromatic rings (1605, 1508 cm^{-1}). The ^{13}C NMR and DEPT data (*see* Table I) exhibited 43 carbon resonances, in which 4 methyl, 4 methylene, 28 methine, and 7 quaternary carbon resonances could be assigned for **2**. The ^1H NMR spectrum of compound **2** (see Table I) exhibited characteristic signals arising from (*E*)-ferulic acid and 3-hydroxy-4-methoxyphenylethanol moieties: six aromatic proton signals (2 × ABX systems, in the region of δ_H 7.20–6.76), two *trans*-olefinic proton signals (AB system, δ_H 7.67, *d*, J_{AB} = 15.8 Hz and 6.38, *d*, J_{AB} = 15.8 Hz), and β-methylene (δ_H 2.88, 2H, *t*, *J* = 7.6 Hz) proton signals together with two non-equivalent proton signals (δ_H 4.08, *m* and 3.77, overlapped) attributed to the side-chain of the phenethyl alcohol moiety. Additionally, four anomeric proton resonances at δ_H 5.36 (*d*, *J* = 1.7 Hz, H-1″ of an α-rhamnose), 4.90 (overlapped, H-1‴ of an α-rhamnose), 4.41 (*d*, *J* = 7.8 Hz, H-1′ of a β-glucose), and 4.28 (*d*, *J* = 7.8 Hz, H-1‴ of a β-glucose) indicated the tetraglycosidic nature of **2**. The secondary methyl signals appeared at δ_H 1.07 (*d*, *J* = 6.3 Hz, H-6″) and 1.28 (*d*, *J* = 6.3 Hz, H-6‴) supported the presence of two rhamnose moieties in **2**. Moreover, the ^{13}C NMR data confirmed the tetraglycosidic sugar chain, exhibiting four anomeric carbon resonances at δ_C 104.7 (C-1‴ of a β-glucose), 104.2 (C-1′ of a β-glucose), 103.8 (C-1‴ of an α-rhamnose) and 101.7 (C-1″ of an α-rhamnose), which showed correlations with the anomeric protons of the related sugar units. The complete assignments of all proton and carbon resonances were based on the results of ^1H-^1H COSY, ^1H-^{13}C HSQC and HMBC experiments. The feru-

Table I. ^{13}C and ^{1}H NMR (CD_3OD, ^{13}C: 125 MHz and ^{1}H: 500 MHz) data and HMBC correlations for **2***.

C/H Atom	d_C ppm**	DEPT	d_H ppm, *J* [Hz]	HMBC (C→H)
Aglycone				
1	131.6 s	C	–	H-2, H-6
2	117.1 d	CH	6.76 d (2.2)	H-6
3	147.6 s	C	–	H-5
4	147.8 s	C	–	H-2, H-6,4-OMe
5	113.7 d	CH	6.81 d (8.2)	
6	121.2 d	CH	6.69 dd (8.2/2.2)	H-2, H_2-β
α	36.7 t	CH_2	4.08 m/3.77†	H-1′, H_2-β
β	72.3 t	CH_2	2.88 t (7.6)	
4-OMe	56.4 q	CH_3	3.85 s	
Glucose				
1′	104.2 d	CH	4.41 d (7.8)	H-2′
2′	76.1 d	CH	3.39 dd (7.8/9.0)	
3′	81.6 d	CH	3.78†	H-1″, H-2′, H-4′
4′	70.6 d	CH	5.01 t (9.0)	
5′	77.9 d	CH	3.30†	
6′	69.4 t	CH_2	3.95†/3.64†	H-1‴″
Rhamnose				
1″	101.7 d	CH	5.36 d (1.7)	
2″	80.2 d	CH	3.94 dd (1.7/3.4)	H-1‴″
3″	71.7 d	CH	3.63†	
4″	74.0 d	CH	3.30†	H_3-6″, H-1″
5″	70.5 d	CH	3.57 dq (6.3/10.0)	H_3-6″
6″	17.9 q	CH_3	1.07 d (6.3)	
Glucose (→C-6′)				
1‴	104.7 d	CH	4.28 d (7.8)	H-2″
2‴	75.1 d	CH	3.20 dd (7.8/9.0)	
3‴	77.9 d	CH	3.32†	
4‴	71.4 d	CH	3.27† (9.5)	
5‴	78.1 d	CH	3.24 m	
6‴	62.6 t	CH_2	3.84†/3.63†	
Rhamnose (→C-2″)				
1‴″	103.8 d	CH	4.90†	
2‴″	72.0 d	CH	3.92 dd (1.7/3.4)	
3‴″	72.2 d	CH	3.62†	
4‴″	74.0 d	CH	3.30†	H-1‴″, H_3-6‴″
5‴″	70.2 d	CH	3.72†	H_3-6″
6‴″	18.6 q	CH_3	1.28 d (6.3)	
Feruloyl				
1‴″	127.6 s	C	–	H-β′, H-5‴″
2‴″	112.8 d	CH	7.20 d (1.8)	H-β′, H-6‴″
3‴″	149.4 s	C	–	H-2‴″, H-5‴″, 3‴″-OMe
4‴″	150.9 s	C	–	H-2‴″, H-5‴″, H-6‴″
5‴″	116.5 d	CH	6.81 d (8.2)	
6‴″	124.4 d	CH	7.09 dd (8.2/1.8)	H-β′, H-2‴″
α′	115.1 d	CH	6.38 d (15.8)	H-β′
β′	148.1 d	CH	7.67 d (15.8)	H-2‴″, H-6‴″
C=O	168.4 s	C	–	H-α′, H-β′, H-4′
3‴″-OMe	56.4 q	CH_3	3.89 s	

* All ^{1}H and ^{13}C assignments are based on 2D NMR (COSY, HSQC and HMBC) experiments.
** Multiplicites are based on DEPT-135 experiment.
† Signal patterns are unclear due to overlapping.

loyl group was supposed to be positioned at C-4′ of the core glucose due to the strong deshielding of the H-4′ resonance of the glucose unit (δ_H 5.01, *t*, *J* = 9.0 Hz). This assumption was supported by the heteronuclear long-range copuling observed between the carbonyl carbon resonance (δ_C 168.4) of the acyl moiety and H-4′ (δ_H 5.01). On the other hand, a HMBC cross-peak observed from C-α carbon reso-

nance (δ_C 72.3) of the phenylethyl alcohol unit to the anomeric proton of the core glucose (δ_H 4.41, H-1′) indicated the attachment of the core glucose to be the C-α carbon atom of the aglycone. The structure of the tetrasaccharide unit was elucidated by means of DQF-COSY and HSQC experiments. Although, the highly deshielded carbon signals arising from the core glucose and the first rhamnose units suggested that the glucose unit to be glycosylated at C-3′ (δ_C 81.6) and C-6′ (δ_C 69.4), whereas the rhamnose unit at C-2″ (δ_C 80.2), however, a prominent HMBC experiment allowed us to assign unambiguously all the interglycosidic connectivities of the sugar sequence. Thus, the correlations were observed between C-3′ (δ_C 81.6) of the core glucose and H-1″ (δ_H 5.36) of the first rhamnose, C-6′ (δ_C 69.4) of the core glucose and H-1‴ (δ_H 4.28) of the second glucose, as well as C-2″ (δ_C 80.2) of the first rhamnose and H-1⁗ (δ_H 4.90) of the second rhamnose moiety. Some significant long-range correlations confirming the proposed structure were given in Fig. 2. Thus, on the basis of its NMR data, the structure of compound **2** was established as 3-hydroxy-4-methoxy-β-phenylethoxy-*O*-[α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)]-4-*O*-feruloyl-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside for which the trivial name physocalycoside is proposed.

In recent years papers on the isolation and characterization of numerous mono-, di-, and trisaccharide phenylethanoids have been published. As far as we know, till now only four tetraglycosidic phenylethanoids, magnolioside C (Hasegawa *et al.*, 1988), ballotetroside (Seidel *et al.*, 1997), trichosanthoside B (Çalış *et al.*, 1999), and marrubioside (Sahpaz *et al.*, 2002) have been described. Therefore, physocalycoside (**2**) appears as the fifth representative of this class. On the other hand, all previously isolated tetrasaccharidic glycosides are caffeic acid esters and contain a 3,4-dihydroxyphenethyl alcohol moiety, while, physocalycoside (**2**) is a ferulic acid ester and contains a 3-hydroxy-4-methoxyphenethyl alcohol unit as the aglycone.

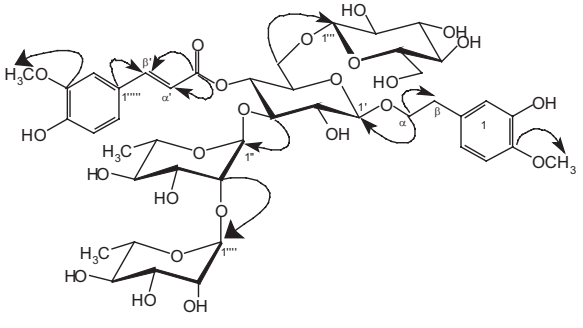


Fig. 2. Selected heteronuclear multiple bond correlations (HMBC) for physocalycoside (**2**). Arrows point from carbon to proton.

Phenylethanoid glycosides (**2–7**) were screened for antioxidant activities by a TLC autographic assay with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Cuendet *et al.*, 1997; Takao *et al.*, 1994) and showed antioxidant properties based on their ability to scavenge free radicals. Then the free-radical scavenging effects of the phenylethanoid glycosides (**2–7**), corresponding to the intensity of quenching the DPPH radical were evaluated (Cuendet *et* al., 2001). Compounds **2–7** exhibited a dose-dependent reduction on DPPH. Results are given in Table II.

Table II. Free radical scavenging effects of the phenylethanoid glycosides **2–7** on DPPH.

Compounds	Concentration [μM]					IC_{50}
	10	25	50	100	200	[μM]
Ascorbic acid[a]	0.86[b]	2.30	9.77	35.63	79.02	112
2	2.30	16.09	30.75	42.82	59.77	50
3	14.37	18.68	30.46	57.76	78.16	79
4	28.45	33.62	54.31	79.00	79.31	49
5	18.39	22.99	28.16	65.50	79.02	76
6	13.51	17.53	25.57	33.91	55.40	101
7	17.53	28.16	55.75	75.86	78.74	43

[a] Reference compound.
[b] % inhibition.

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