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Secondary Metabolites from *Phlomis syriaca* and Their Antioxidant Activities

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An iridoid glucoside, lamiide (**1**); 4 phenylethanoid glycosides, acteoside (**2**), β-OH acteoside (**3**), leucosceptoside A (**4**) and samioside (**5**); a caffeic acid ester, chlorogenic acid (**6**); 2 flavone glucosides, luteolin-7-*O*-glucopyranoside (**7**) and chrysoeriol-7-*O*-glucopyranoside (**8**); and a flavanone aglycone, naringenin (**9**), were isolated from the aerial parts of *Phlomis syriaca.* The structures of the isolated compounds were elucidated by means of spectroscopic (UV, IR, 1D- and 2D-NMR, and FAB-MS) methods. Free radical scavenging activity of the isolated compounds was determined using the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), spectroscopically.

Key Words: *Phlomis syriaca,* Lamiaceae, Iridoid glucosides, Phenylethanoid glycosides, Flavonoids, Free radical scavenging activity, DPPH.

Introduction

The genus *Phlomis* L. (Lamiaceae) is a well-known genus in Turkey and is represented by 34 species in Turkish flora, of which 21 are endemic[1]. Some of the *Phlomis* species are used as tonics and stimulants in Anatolia and are locally known as "çalba" and "şalba"[2]. In Turkey and several other countries, it is also used as an antipyretic, antidiabetic, and diuretic, and for treatment of allergy[2,3]. To date, we have studied almost all the members of the genus growing in Turkey and described the isolation and characterization of their iridoids, phenylethanoid glycosides, lignans, neolignans, monomeric phenylpropanoids, monoterpene glucosides, diterpenoids, triterpenes, and nor-triterpene glycosides[4−8]. In a continuation of the research of the secondary metabolites of *Phlomis* species, we report here the isolation and structure of 9 compounds from the aerial parts of *Phlomis syriaca.* The free radical scavenging effect of the isolated compounds was determined using the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging system. In this assay, antioxidants react with the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which gives a strong absorption at

517 nm, resulting in the production of colorless 2,2-diphenyl-1-picrylhydrazine; the degree of decoloration in the DPPH solution indicates the free radical scavenging activity of the substances[9].

Experimental

General Experimental Procedures: The UV (MeOH, λ_{max}, nm) and IR (KBr, υ_{max}, cm^{-1}) spectra were recorded with Shimadzu UV-240 and Perkin Elmer 2000 FTIR spectrophotometers, respectively. NMR measurements were performed on a JEOL JNM-A 500 spectrometer in methanol-d_4 and DMSO (^{1}H: 500 MHz; ^{13}C: 125 MHz). Chemical shifts were given in ppm with tetramethylsilane (TMS) as an internal standard. FAB-MS were recorded in NBA matrix in the positive ion mode on a JEOL JMS-DX300 spectrometer. Column chromatography was carried out on silica gel (Merck, Kieselgel 60, 60-230 mesh), polyamide (Fluka, 50-160 μm), and Sephadex LH-20 (Pharmacia). Medium pressure liquid chromatography (MPLC) was realized on Labomatic (18.5 × 352 mm) and Büchi (25 × 460 mm) glass columns filled with Li Chroprep RP-18 (Merck), using Lewa M5 peristaltic and Büchi B-684 pumps. Thin layer chromatography (TLC) was conducted on pre-coated, commercial silica gel (Merck, 60 F_{254}) plates with $CHCl_3$-MeOH-H_2O (61:32:7, 70:30:3, 80:20:2) as a developing solvent system. Compounds **1-9** were detected by UV fluorescence and/or spraying with 1% vanillin/H_2SO_4, followed by heating at 100 °C for 5 min. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) was obtained from Aldrich Co. (Milwaukee, WI, USA) and 3-*tert*-butyl-4-hydroxyanisole (BHA) was purchased from Nacalai Tesuque Co. (Kyoto, Japan).

Plant Material: *Phlomis syriaca* Boiss. (Lamiaceae) was collected 12 km from Gaziantep traveling toward Nizip in South Anatolia. A voucher specimen has been deposited in the Herbarium of the Biology Department, Hacettepe University, Ankara, Turkey (AAD 10941).

Extraction and Isolation: The air-dried aerial parts of *Phlomis syriaca* (450 g) were extracted with MeOH at 40 °C for 12 h (×3, 2 L). The combined extracts were evaporated under vacuum to give 42 g of crude extract. The MeOH extract was dissolved in H_2O (0.2 L). H_2O-insoluble material was completely removed by filtration. The filtrate (31 g) was subjected to polyamide (164 g) column chromatography and eluted with H_2O, followed by increasing concentrations of MeOH to give 5 main fractions (Frs.) [Fr. A: (H_2O), 15.3 g; Fr. B: (25% MeOH), 4.5 g; Fr. C: (50% MeOH), 0.97 g; Fr. D: (75% MeOH), 1.5 g; Fr. E: (MeOH), 0.4 g].

The fraction eluted with H_2O from the polyamide column (Fr. A) was fractionated between water and n-BuOH to remove sugars. After evaporation, n-BuOH fraction (2 g) was subjected to a silica gel column eluting with $CHCl_3$-MeOH (95:5 → 80:20) and purified by Sephadex LH 20 using MeOH to yield compound **1** (14.7 mg). Fr. C was fractionated over MPLC eluting with increasing concentrations of MeOH (25% → 60%) to give compounds **2** (44.3 mg), **3** (7 mg), **4** (19 mg)**, 5** (22.4 mg), and **6** (17 mg), in pure form. Fr. E, rich in flavonoid glycosides, was also applied to MPLC using increasing concentrations of MeOH (10% → 70%) to yield compounds **7** and **8,** in a mixed form (20.5 mg), and a fraction rich in compound **9**, the latter of which was purified passing through a silica gel column (7 mg).

Lamiide $C_{17}H_{26}O_{12}$ (1): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR data were identical to those reported in the literature[10].

Acteoside $C_{29}H_{36}O_{15}$ (2): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR

data were identical to those reported in the literature[11].

β-Hydroxyacteoside $C_{29}H_{36}O_{16}$ (3): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR data were identical to those reported in the literature[12].

Leucosceptoside A $C_{30}H_{38}O_{15}$ (4): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR data were identical to those reported in the literature[13].

Samioside $C_{33}H_{42}O_{16}$ (5): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR data were identical to those reported in the literature[14].

Chlorogenic acid $C_{16}H_{18}O_9$(6): UV, IR, ^{1}H (500 MHz, CD_3OD), and ^{13}C (125 MHz, CD_3OD) NMR data were identical to those reported in the literature[7].

Luteolin-7-*O*-glucopyranoside $C_{21}H_{20}O_{10}$ (7): UV, IR, ^{1}H (500 MHz, DMSO-d_6), and ^{13}C (125 MHz, DMSO-d_6) NMR data (Table 1) were identical to those reported in the literature[15,16].

Table 1. ^{13}C and ^{1}H NMR spectral data for compounds **7-9** (in DMSO-d_6; ^{13}C: 125 MHz; ^{1}H: 500 MHz).

		7			8			9		
C/H	DEPT	δ_C	δ_H	(J)	δ_C	δ_H	(J)	δ_C	δ_H	(J)
2	C†	164.45			164.10			78.52	5.43 dd	(3.1/12.8)
3	CH††	102.89	6.73 s		103.28	6.94 s		42.09	3.25 dd	(12.8/17.1)
									2.67 dd	(3.1/17.1)
4	C	181.74			181.93			196.41		
5	C	161.02			160.99			163.59		
6	CH	99.43	6.44 br.s		99.40	6.45 br. s		95.90	5.90 br.s	
7	C	162.83			162.87			166.82		
8	CH	94.60	6.78 br.s		94.92	6.87 br.s		95.52	5.90 br.s	
9	C	156.85			156.82			163.03		
10	C	105.24			105.23			101.84		
1′	C	120.88			121.07			128.94		
2′	CH	113.25	7.41 d	(2.1)	110.18	7.58 br.s		128.40	7.31 d	(8.9)
3′	C†	145.83			148.00			115.25	6.81 d	(8.9)
4′	C	150.37			151.00			157.82		
5′	CH	115.87	6.89 d	(8.3)	115.73	6.93 d	(8.2)	115.25	6.81 d	(8.9)
6′	CH	119.13	7.44 dd	(8.3/2.1)	120.45	7.59 d	(8.5)	128.40	7.31 d	(8.9)
1″	CH	99.80	5.07 d	(7.3)	99.91	5.05 d	(7.3)			
2″	CH	73.03	3.26 d	(8.9)	73.02	3.25 d	(9.2)			
3″	CH	76.30	3.30 dd	(8.5/7.0)	76.30	3.29 dd	(8.8/5.2)			
4″	CH	69.47	3.18 t	(9.8)	69.51	3.17 t	(8.7)			
5″	CH	77.06	3.46 d	(9.1)	77.10	3.48*				
6″	CH_2	60.53	3.44 dd	(10.3/5.8)	60.52	3.48 dd	(10.4/5.1)			
			3.71 d	(10.4)		3.71 d	(10.1)			
OCH_3	CH_3	-			55.87	3.88 s				

* Signal patterns are unclear due to overlapping.

†CH and ††CH_2 for compound **9**

Chrysoeriol-7-*O*-glucopyranoside $C_{22}H_{22}O_{11}$ (8): UV, IR, ^{1}H (500 MHz, DMSO-d_6), and ^{13}C (125 MHz, DMSO-d_6) NMR data (Table 1) were identical to those reported in the literature[15,16].

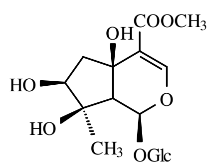
Naringenin $C_{15}H_{11}O_5$ (9): UV, IR, ^{1}H (500 MHz, DMSO-d_6), and ^{13}C (125 MHz, DMSO-d_6) NMR data (Table 1) were identical to those reported in the literature[15,16].

DPPH free radical scavenging activity: MeOH solution (100 μL) of the sample at various concentrations was added to 100 μL of 120 μM DPPH in MeOH. The reaction mixture was shaken vigorously

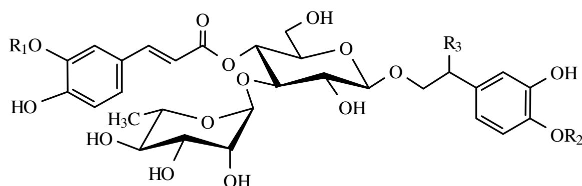
and the absorbance of remaining DPPH was measured at 517 nm after 30 min using a microplate reader (Biotek Instruments Inc. Elx800, Vermont, USA). The radical scavenging activity was determined by subtracting the absorbance with that of blank (100%) containing only DPPH and solvent. BHA and ascorbic acid were used as standards and samples were prepared using the same dilution procedures[9,17].

Results and Discussion

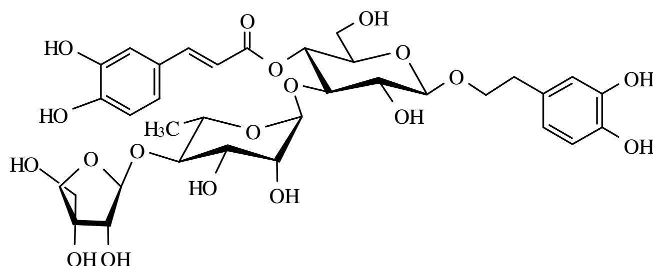
The water soluble part of the methanolic extract prepared from the aerial parts of *P. syriaca* was subjected to successive column chromatography (polyamide, normal/reverse phase silica gel, and Sephadex LH 20) to give compounds **1-9** in pure form (Figure). Compound **1** was isolated as a colorless, amorphous powder. Its UV spectrum showed an absorption peak (λ_{max} 232 nm) characteristic of a 4-substituted iridoid enol ether system. The IR absorption bands were indicative of the presence of hydroxyl groups (3400 cm^{-1}), a carbonyl (1700 cm^{-1}), and an enolic double bond (1640 cm^{-1}). Its ^{1}H-NMR (CD_3OD, 400.133 MHz) spectral data were identical to those reported for iridoid glucoside lamiide in the literature[10]. Compounds **2-5** were obtained as colorless, amorphous powders. Their UV spectra confirmed polyphenolic natures. In the IR spectrum, IR bands for hydroxyl groups, α,β-unsaturated ester, and aromatic rings were observed. The structures of compounds **2-5** were identical to the ^{1}H and ^{13}C-NMR data, which were superimposeable to those of the data published for acteoside (**2**), β-OH-acteoside (**3**), leucosceptoside A (**4**), and samioside (**5**)[11−14]. Compound **6** was identified as chlorogenic acid by direct comparison with the authentic samples on TLC and by comparing its spectral data with those reported in the literature[7]. The structure elucidation of compounds **7-9** was realized based on the evidence that follows. Compounds **7** and **8** were each isolated as an amorphous yellow powder. ^{1}H and ^{13}C NMR spectra of compound **7** (Table 1) showed signals belonging to the aromatic system and sugar moieties. The IR absorption bands for hydroxyl (3385 cm^{-1}), γ-pyrone carbonyl (1661 cm^{-1}), and aromatic rings (1608, 1508 cm^{-1}), and UV spectrum (λ_{max} 269, 345) suggested that compound **7** was a flavone glycoside. In the ^{1}H-NMR spectrum, 3 aromatic protons at δ_H 7.41, δ_H 6.89, and δ_H 7.44, which were observed as an ABX system, suggested the presence of an *o*-disubstituted B ring. Moreover, 2 meta-coupled signals in the aromatic region at δ_H 6.44 (s, H-6) and 6.78 (s, H-8) were consistent with a 5,7-dihydroxy-substituted A ring of flavonoid. A singlet signal at δ_H 6.73 (1H) was attributed to the H-3 of the aglycone. The anomeric proton signal at δ_H 5.07 (d, J=7.3 Hz) and long-range correlation between the anomeric proton and C-7 (δ_C 162.83) assigned to the location of the sugar unit, which attached to C-7 of the aglycone. After the complete interpretation of the NMR data based on the ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, and HMBC experiments, and comparison of these data with those reported in the literature, compound **7** was determined to be luteolin-7-*O*-glucopyranoside[15,16]. ^{1}H- and ^{13}C-NMR spectra of **8** were similar to those of **7**, except for the presence of a methoxyl group at δ 3.89 (3H, s). It is easy to presume this methoxyl group attached to the 3' position of the B ring from the HMBC spectrum. From these results, the structure of compound **8** was identified as chrysoeriol-7-*O*-glucopyranoside, which was confirmed by the comparison of its spectral data with those reported in literature[15,16]. Compound **9** was also isolated as an amorphous powder. Its UV absorption, together with ^{1}H- and ^{13}C-NMR spectra, indicated that compound **9** has a non-glycosidic flavanone structure (Table 1). Its molecular formula $C_{15}H_{11}O_5$was established by FAB-MS, and this was in good agreement with the observation of the 1 methylene, 7 methine, and 7 quaternary carbon resonances in the ^{13}C-NMR and DEPT spectra. In the ^{1}H-NMR spectrum, 3 signals at δ_H 6.81 and



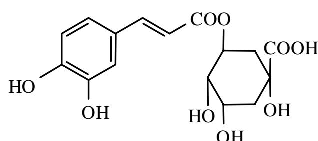
Lamiide (**1**)



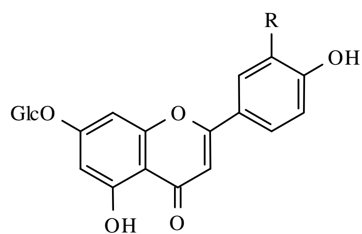
	R_1	R_2	R_3
Acteoside (**2**)	H	H	H
β-OH acteoside (**3**)	H	H	OH
Leucosceptoside A (**5**)	CH_3	H	H

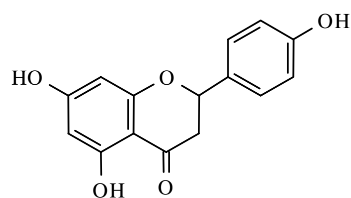


Samioside (**4**)



Chlorogenic acid (**6**)





Naringenin (**9**)

	R
Luteolin-7-*O*-glucopyranoside (**7**)	OH
Chrysoeriol-7-*O*-glucopyranoside (**8**)	OCH_3

Figure. Isolated compounds (**1-9**) from *P. syriaca*.

δ_H 7.31 (each 2H, d), as an AA′BB′ system, and δ_H 5.90 (2H, s, H-6 and H-8) were observed for 6 aromatic protons. However, the absence of the aromatic singlet for C-3 of the aglycone suggested the saturated C-3 position for compound **9**. Instead, the signals at δ_H 3.25 (dd, J= 12.9/17.1) and 2.67 (dd, J= 3.05/17.1) for geminally coupled protons were ascribed to H-3α and H-3β of the aglycone. These data suggested that the structure of compound **9** was the same as the naringenin, and this was confirmed by the comparison of its spectral data with those reported in the literature[15,16,]. Isolation of the flavanone aglycone, naringenin, and its spectral data for *Phlomis* species were demonstrated for the first time in this study.

Monoglycosidic iridoid glucosides are widely observed in *Phlomis* species. Lamiide is the most common iridoid compound, which has been previously isolated from 20 different *Phlomis* species. The second most important group of *Phlomis* species is the phenylethanoid glycosides. Phenylethanoid di- and tri-glycosides, and rarely tetra-glycosides were found in our previous studies. In addition, flavonoid glycosides are also isolated frequently from the many *Phlomis* species (Table 2)[5,18,19].

Table 2. Distribution of the isolated compounds in the genus *Phlomis*.

	Phlomis species*										
Isolated compounds	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Lamiide		+	+	+	+	+			+		+
Acteoside	+	+	+	+	+	+	+	+	+	+	+
β-OH acteoside	+	+									
Leucosceptoside A	+		+	+							
Samioside				+	+						
Chlorogenic acid						+	+	+	+		
Luteolin-7-*O*-glucopyranoside								+		+	
Chrysoeriol-7-*O*-glucopyranoside			+					+	+	+	+

***1.** *P. sieheana*, **2.** *P. viscosa*, **3.** *P. integrifolia*, **4.** *P. nissoli*, **5.** *P. samia*, **6.** *P. lycia*, **7.** *P. longifolia* var. *longifolia*, **8.** *P. brunneogaleata*, **9.** *P. capitata*, **10.** *P. lunariifolia*, **11.** *P. oppositiflora*

Table 3. Free radical scavenging activity of compounds **1-6**, BHA, and AA, on the DPPH radical (120 μM)*

Compounds	Scavenge %				
	200 μM	100 μM	50 μ M	25 μM	10 μM
Lamiide (**1**)	8.8	8.4	8.1	8.1	7.2
Acteoside (**2**)	38.1	34.4	33.6	34.5	30.1
$\beta-$ OH acteoside (**3**)	47.2	40.3	37.2	34.2	32.5
Leucosceptoside A (**4**)	41.8	33.1	32.6	31.2	17.9
Samioside (**5**)	41.5	38.1	37.7	34.3	31.4
Chlorogenic acid (**6**)	68.6	62.7	48.7	37.3	33.7
BHA	35.6	34.7	33.8	31.3	25.0
AA	39.9	37.7	36.2	34.6	20.3

*Each value is the average of duplicate determinations. The inhibitory ratio of each compound is expressed as follows: scavenge % = 100 $\times$ [(Abs_{blank}-Abs_{sample})/Abs_{blank}].

Blank: in the absence of sample

BHA: 3-*tert*-butyl-4-hydroxy-anisole

AA: Ascorbic acid

Several biological activities of *Phlomis* extracts and the isolated compounds, such as antioxidant, cytotoxic, and antimicrobial, have been reported previously[20,21]. On the basis of this information, free radical scavenging activities of compounds **1-6** were determined using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging system, by comparing 3-*tert*-butyl-4-hydroxy-anisole (BHA), a widely used synthetic antioxidant, and ascorbic acid, a commonly used natural antioxidant. Although antioxidant activities of flavonoids are well known[22], we could not test the radical scavenging effects of compounds **7-9** because of their small quantities. Compound **1** did not show any scavenging activity against the DPPH radical because of its non-phenolic structure. As shown in Table 3, compounds **2-5** have potent radical scavenging activities, more than that of BHA and ascorbic acid. The scavenging activity of β-OH acteoside (**3**) was higher than that of acteoside (**2**), leucosceptoside A (**4**), and samioside (**5**), at the concentration of 200 μM. This activity may be due to the hydroxylation of phenylethanol moiety. Compared to the phenylethanoid glycosides, chlorogenic acid (**6**) showed the strongest activity in each tested concentration. The radical scavenging effects of antioxidants on the DPPH radical are thought to be due to their hydrogen donating ability. Phenolic hydroxyls and carboxylic acid moieties have been recognized to function as electron or hydrogen donors[9]. Thus, the DPPH radical scavenging activity of chlorogenic acid may be mostly related to the phenolic hydroxyls and carboxylic acid group in its structure. Our results supported the previously reported antioxidant activity of phenylethanoid glycosides[21]. Further research to identify the different biological activities of *Phlomis* species and related compounds are in progress.

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