CHEMICAL COMPOSITION, FREE-RADICAL-SCAVENGING AND INSECTICIDAL ACTIVITIES OF THE AERIAL PARTS OF *STACHYS BYZANTINA*

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Abstract – Stachys byzantina K. Koch. is an Iranian endemic species of the genus Stachys L., which comprises about 300 species, and is one of the largest genera of the family Lamiaceae. A combination of solid phase extraction (SPE) and high pressure liquid chromatography (HPLC) of the methanolic extract of the aerial parts of *S. byzantina* afforded three phenylethanoids, 2'-O-arabinosyl verbascoside (1), verbascoside (2), aeschynanthoside C (3) and three flavones apigenin 7-O-glucoside (4), apigenin 7-O-(6-*p*-coumaroyl)-glucoside (5) and apigenin (6). The structures of these compounds were determined by spectroscopic methods. Free-radical-scavenging and insecticidal properties of the crude extracts, the fractions and the isolated compounds were assessed.

Keywords: Stachys byzantina, Lamiaceae, phenylethanoid, flavone, insecticidal, free-radical-scavenger

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INTRODUCTION

Stachys byzantina K. Koch. (Lamiaceae alt. Labiatae), commonly known as 'lamb's ear' or 'lamb's tongue' or 'sonbolehe noghrehi or zabanehe bare' in Farsi, is one of the Iranian species of the genus Stachys L., and is found in the north and north-west of Iran, and is also distributed in Turkey, Caucasia and Afghanistan (Ghahreman et al., 1970; GRIN Database, 2010). Species of the genus Stachys including S. byzantina have been used as an anti-inflammatory, antitumor, anticancer, antispasmodic, sedative and diuretic agent, and in the treatment of digestive disorders, wounds, infections, asthma, rheumatic and inflammatory disorders, dysentery, epilepsy, common cold and neuropathy in traditional medicine (Maleki et al., 2001; Naghibi et al., 2005; Morteza-Semnani et al., 2006; Dulger and Aki, 2009; Güven et al., 2009). While previous bioactivity studies revealed anti-inflammatory, antioxidant and antimicrobial activities of the essential oils (Takeda et al., 1997; Skaltsa et al., 1999), phytochemical analysis established the presence of megastigmane glycosides and phenylethanoids in the aerial parts of this plant (Takeda et al. 1997). As part of our continuing phytochemical and pharmacological studies on the species of the genus *Stachys* (Nazemieyh et al., 2006; 2007; Delazar et al., 2005a; 2010), we now report the isolation and characterization of three phenylethanoids (1-3) and three flavonoids (4-6) from the aerial parts of *S. byzantina*, as well as the free-radicalscavenging and the insecticidal properties of the crude extracts, different fractions and isolated compounds.

MATERIALS AND METHODS

General

UV spectra were obtained in MeOH using Shimadzu UV160-1600. NMR spectra were recorded in CD₃OD on a Bruker 200 MHz NMR spectrometer (200 MHz for ¹H and 50MHz for ¹³C) using residual solvent peak as the internal standard. Methanolic fractions were

purified by a Kanuwer preparative HPLC coupled with a UV-Visible- PDA detector (190-400 nm).

Plant Materials

The aerial parts of *Stachys byzantina* K. Koch. were collected from the Shabestar region located in the west Azerbaijan province during August 2008. A voucher specimen (no. TUM-ADE-695) representing this collection has been retained in the herbarium of the School of Pharmacy, Tabriz University of Medical Sciences, Iran.

Extraction of plant materials and isolation of compounds

The dried and ground aerial parts of S. byzantina (100 g) were Soxhlet-extracted, successively, with *n*-hexane, dichloromethane (DCM) and methanol (MeOH) (1.2 L each). All these extracts were separately concentrated using a rotary evaporator at a maximum temperature of 45° C. A portion of the MeOH extract (2 g) was subjected to solid-phase extraction (SPE) on a Sep-Pak 10g C₁₈ cartridge using a step gradient of MeOH-water mixture (20:80, 40:60, 60:40, 80:20 and 100:0). The preparative HPLC (Dr. Mainsch GmbH ODS column 20 μ m, 250 mm \times 20 mm; mobile phase: 0-20 min, MeOH from 35% to 65% in water; 20-25 min, 65% MeOH in water, 25-27 min MeOH from 65% to 35% in water, 27-30 min 35% MeOH, flow rate = 8 mL/min) purification of the 40% MeOH SPE fraction (598.2 mg) yielded three phenylethanoid glycosides: 1 (22.7 mg, $t_{\rm R}$ = 10.00 min), 2 (46.6 mg, $t_{\rm R}$ = 10.57 min), **3** (3.1 mg, $t_{\rm R}$ = 16.25 min), as well as one flavonoid 4 (4.1 mg, $t_{\rm R}$ = 17.72 min). Again, the preparative HPLC (mobile phase: 0-20 min, MeOH from 60% to 70% in water; 20-25 min, 70% MeOH in water, 25-27 min MeOH from 70% to 60% in water, 27-30 min 60% MeOH, flow rate = 8 mL/min) purification of the 60% MeOH SPE fraction (205.1 mg) yielded the two flavonoids: 5 (3.7 mg, $t_{\rm R}$ = 14.12 min) and 6 (2.5 mg, $t_{\rm R} = 16.85$ min).

Free-radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) (molecular formula $C_{18}H_{12}N_5O_6$), was obtained from Fluka

Chemie AG, Bucks. Quercetin and Trolox[®] were obtained from Avocado Research Chemicals Ltd, Shore Road, Heysham, Lancs, UK. 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 sample solutions were applied on a TLC plate and sprayed with DPPH solution using an atomizer. Color was allowed to develop for 30 min. The color changes (purple on white) were noted.

Quantitative assay: The extracts/fractions were dissolved in MeOH to obtain the test concentration 10 mg/mL as stock. The test compounds as well as the positive control were used as 1 mg/mL stock. Serial dilutions were carried out with the stock solutions (10 mg/mL) of the plant extracts, fractions and pure compounds to obtain concentrations of 5x10⁻¹, 5x10⁻², 5x10⁻³, 5x10⁻⁴, 5x10⁻⁵, 5x10⁻⁶ mg/mL. Diluted solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur. UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The RC₅₀ value, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as mg/mL. The same procedure was followed for the positive controls, quercetin and Trolox*.

Insecticidal assay

Adults of *Oryzaephilus mercator* were collected from a laboratory culture. *Oryzaephilus mercator* was reared on a mixture of whole wheat flour and maize flour at the ratio of 1:1 in glass containers containing 0.5 kg of the mixture. All insect species were reared at 27±2°C, 12% moisture content in continuous darkness for about 3 weeks without exposing to insecticides. Adults used in the experiments were 1–3 weeks old and of mixed sex.

Different fractions of methanolic extract were dissolved in MeOH to obtain concentrations of

Position		¹ H ch	emical shifts (^{13}C chemical shifts ($\delta_{\!H})$ in ppm								
	1	2	3	4	5	6	1	2	3	4	5	6
Aglycone												
1	-	-	-	-	-	-	130.5	130.5	131.1	-	-	-
2	6.74 d (1.8)	6.73 d (1.7)	6.77 d (1.9)	-	-	-	113.7	113.7	113.2	162.2	163.3	162.
3	-	-	-	6.70 s	6.59 s	6.68 s	145.8	145.8	148.5	102.3	101.1	102.
4	-	-	-	-	-	-	145.1	145.1	145.6	182.9	182.9	182.
5	6.72 d (7.8)	6.72 d (8.0)	6.85 d (8.0)	-	-	-	115.5	115.5	115.7	158.4	159.0	158.
6	6.60 dd (1.8, 7.8)	6.60 dd (1.7, 8.0)	6.52 dd (1.9, 8.0)	6.53 d (2.1)	6.46 d (2.1)	6.25 d (2.1)	120.2	120.2	121.8	100.1	100.1	100.
7	2.85 m	2.83 m	2.72 m	-	-	-	35.5	35.5	36.4	165.8	167.0	165.
8	3.90 m	3.90 m	3.96 m	6.86 d (2.1)	6.73 d (2.1)	6.50 d (2.1)	71.8	71.8	72.4	95.0	94.2	95.0
9	-	-	-	-	-	-	-	-	-	161.6	161.6	161
10	-	-	-	-	-	-	-	-	-	106.2	106.0	106
3-OMe	-	-	3.86 s	-	-	-	-	-	56.8	-	-	-
1'	-	-	-	-	-	-	126.7	126.7	127.2	121.7	122.0	121
2'	7.10 d (1.7)	7.10 d (2.0)	7.12 d (2.0)	7.93 d (8.8)	7.89 d (8.8)	7.93 d (8.8)	114.2	114.2	111.3	128.6	128.7	128.
3'	-	-	-	6.96 d (8.8)	6.97 d (8.8)	6.96 d (8.8)	147.0	147.0	149.8	116.1	116.1	116
4'	-	-	-	-	-	-	148.7	148.7	150.3	161.6	161.6	161
5'	6.82 d (8.1)	6.81 d (8.0)	6.77 d (8.0)	6.96 d (8.8)	6.97 d (8.8)	6.96 d (8.8)	116.1	116.1	116.5	116.1	116.1	115
6'	7.00 dd (1.7, 8.1)	7.01 dd (2.0, 8.0)	7.01 dd (2.0, 8.0)	7.93 d (8.8)	7.89 d (8.8)	7.93 d (8.8)	122.2	122.2	123.8	128.6	128.7	128
7'	7.63 d (15.9)	7.63 d (16.0)	7.48 d (16.0)	-			147.3	147.3	147.6	-	-	-
8'	6.30 d (15.9)	6.30 d (16.0)	6.21 d (16.0)	-			115.3	115.3	116.6	-	-	-
9'	-	-	-	-			167.3	167.3	168.9	-	-	-
3"-OMe	-	-	3.91 s	-			-	-	56.1	-	-	-
Glucosyl unit												
G-1	4.42 d (7.9)	4.42 d (7.9)	4.43 d (7.9)	4.80 d (7.8)			101.3	102.0	102.9	103.0	103.10	-
G-2	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*			73.3	75.2	74.3	75.2	73.6	-

Table 1. ¹H (coupling constant J in Hz in parentheses) and ${}^{13}C$ NMR data for compounds 1-6.

Table	1.	Ctd.
I ubic		olu.

Position		¹ H c	hemical sh	ifts (δ_{H}) in	ppm	^{13}C chemical shifts ($\delta_{\text{H}})$ in ppm						
	1	2	3	4	5	6	1	2	3	4	5	6
G-3	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*			81.7	80.7	85.8	79.9	76.8	
G-4	4.98 t (9.0)	4.98 t (9.0)	4.98 t (9.0)	4.98 t (9.0)			70.9	69.6	71.3	70.1	70.81	
G-5	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*			75.0	75.0	75.5	75.2	76.0	
G-6	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*	3.20 - 3.80*			61.3	61.4	62.8	62.1	60.6	
Rhamnosyl unit			-	-								
R-1	5.51 d (1.5)	5.51 d (1.5)	-	-			103.2	103.2	-	-	-	
R-2	3.20 - 3.80*	3.20 - 3.80*	-	-			71.2	71.3	-	-	-	-
R-3	3.20 - 3.80*	3.20 - 3.80*	-	-			70.9	71.1	-	-	-	
R-4	3.20 - 3.80*	3.20 - 3.80*	-	-			73.2	72.8	-	-	-	
R-5	3.20 - 3.80*	3.20 - 3.80*	-	-			69.3	69.4	-	-	-	
R-6	1.12 d (6.1)	1.13 d (6.0)	-	-			17.4	17.4	-	-	-	
Arabinosyl unit										-		
A-1	4.34 d (6.7)	-	-	-	-	-	106.4	-	-	-	-	
A-2	3.40 - 3.90*	-	-	-	-	-	71.8	-	-	-	-	
A-3	3.40 - 3.90*	-	-	-	-	-	81.3	-	-	-	-	-
A-4	3.40 - 3.90*	-	-	-	-	-	69.5	-	-	-	-	
A-5	3.40 - 3.90*	-	-	-	-	-	66.3	-	-	-	-	
Xylosyl unit												
X-1	-	-	4.40 d (7.8)	-	-	-	-	-	105.9	-	-	-
X-2	-	-	3.10 – 3.90*	-	-	-	-	-	75.4	-	-	-
X-3	-	-	3.10 - 3.90*	-	-	-	-	-	77.8	-	-	
X-4	-	-	3.10 – 3.90*	-	-	-	-	-	70.9	-	-	-

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Position		$^{1}\mathrm{H}$	chemical shi	fts (δ _H) iı	n ppm			¹³ C o	chemical shi	fts (δ_{H}) ir	n ppm	
	1	2		1	2		1	2		1	2	
X-5	-	-	3.10 - 3.90*	-	-	-	-	-	67.8	-	-	-
<i>p-</i> Coumaroyl unit												
C-1	-	-	-	-	-	-	-	-	-	-	126.0	-
C-2 & C-6	-	-	-	-	6.93 d (8.7)	-	-	-	-	-	131.5	-
C-3 & C-5	-	-	-	-	6.59 d (8.6)	-	-	-	-	-	122.0	-
C-4	-	-	-	-	-	-	-	-	-	-	167.0	-
C-7	-	-	-	-	6.52d (12.8)	-	-	-	-	-	144.1	-
C-8	-	-	-	-	5.80d (12.8)	-	-	-	-	-	114.6	-
C-9	-	-	-	-	-	-	-	-	-	-	167.0	-

* Overlapped peaks.

20, 10 and 5 mg/mL. The filter paper (9 cm of diameter) received 1 mL of these extracts, and was placed on a Petri dish (9 cm of diameter). The control was treated with pure solvents. After the solvent evaporation, 10 adults of Oryzaephilus surinamensis L. (Silvanidae) were placed in each Petri dish, maintained at 27±0.5°C, 12% moisture content and a 12 h photo phase. The experimental design was completely randomized, with three replicates. Insect mortality was evaluated after 4, 8, 24 and 48 h of exposure to impregnated filter paper. The procedure used was that described by Loschiavo et al. (1963) and modified by Freedman et al. (1982). Beetle responses to treated discs versus control discs were converted to "percentage of mortality" (Figure 2 and Table 3).

RESULTS

The dried and ground aerial parts of *S. byzantina* (100 g) were Soxhlet-extracted, successively, with *n*-

hexane, dichloromethane (DCM) and methanol (MeOH) (1.2 L each) to yield 2.6, 3.3 and 7.4 g of dried extracts, respectively. The solid-phase extraction (SPE) of a portion of the MeOH extract (2 g) on a Sep-Pak 10g C₁₈ cartridge using a step gradient of MeOH-water mixture, e.g. 20:80, 40:60, 60:40, 80:20 and 100:0, produced 1.0, 0.6, 0.2, 0.057 and 0.044 g dried fractions, respectively. A combination of solid phase extraction (SPE) and high pressure liquid chromatography (HPLC) of the methanolic extract of the aerial parts of S. byzantina resulted in the isolation of three phenylethanoids: 2'-O-arabinosyl verbascoside (1), verbascoside (2), aeschynanthoside C (3), and three flavones: apigenin 7-O-glucoside (4), apigenin 7-O-(6-*p*-coumaroyl)-glucoside (5) and apigenin (6) (Figure 1). The structures of these compounds were determined by spectroscopic methods (Table 1) and by comparison of the spectroscopic data with previously published data (Mabry et al., 1970; Markham et al., 1978; Agrawal, 1989; Delazar et al., 2005b; Li et al., 2008).

Extracts/Fractions/Compounds	Antioxidant activity ^a					
	Qualitative	Quantita	tive (RC50)			
		mg/mL	Mol/L			
<i>n</i> -Hexane	+	1.66	NA			
DCM	+	2.35	NA			
MeOH	+	1.5 x 10 ⁻²	NA			
Solid-phase extraction fraction (20% MeOH in water)	+	9.6 x 10 ⁻²	NA			
Solid-phase extraction fraction (40% MeOH in water)	+	1.4 x 10 ⁻²	NA			
Solid-phase extraction fraction (60% MeOH in water)	+	2.1 x 10 ⁻²	NA			
Solid-phase extraction fraction (80% MeOH in water)	+	1.2 x 10 ⁻¹	NA			
Solid-phase extraction fraction (100% MeOH in water)	+	5.2 x 10 ⁻¹	NA			
1	+	9.6 x 10 ⁻³	1.28 x 10 ⁻⁵			
2	+	8.8 x 10 ⁻³	1.41 x 10 ⁻⁵			
3	+	1.2 x 10 ⁻¹	2.0 x 10 ⁻⁴			
4	+	3.1 x 10 ⁻¹	7.22 x 10 ⁻⁴			
5	+	4.5 x 10 ⁻¹	7.89 x 10 ⁻⁴			
6	+	2.1 x 10 ⁻¹	7.90 x 10 ⁻⁴			
Quercetin	+	2.78 x 10 ⁻⁵	9.20 x 10 ⁻⁸			
Trolox	+	2.6 x 10 ⁻³	1.04 x 10 ⁻⁵			

Table 2. Free-radical-scavenging activity of different extracts, fractions and isolated compounds (1-6) from Stachys byzantina

^aDetermined by the DPPH assay. + = Activity; NA =Not applicable

In the TLC-based qualitative antioxidant assay using the DPPH spray all extracts, fractions and isolated compounds (1-6) showed antioxidant properties, indicated by the presence of a yellow/white spot on a purple background on the TLC plates (Table 2). The RC_{50} values of all extracts, fractions and isolated compounds determined by the quantitative DPPH assay are presented in Table 2.

The insecticidal property of the solid-phase extraction fractions of the MeOH extract of S. byzantine has been evaluated by the assay described by Loschiavo et al. (1963) and modified by Freedman et al. (1982). The results are shown in Table 3.

DISCUSSION AND CONCLUSION

Compounds (1-6) isolated from the MeOH extract of *S. byzantina* are quite typical chemical classes for

the genus Stachys. In fact, phenylethanoids and flavonoids are of common occurrence in this genus as well as in the family Lamiaceae (Delazar et al., 2005b). Within the family Lamiaceae, in addition to the Stachys, verbascoside (also known as acteoside, 2) and related phenylethanoids were reported previously from a number of other genera, e.g. Eremostachvs. Faradaya, Lamium, Leonurus, Marrubium. Phlomis. Prostanthera. Oxera. Scutellaria and Sideritis (Delazar et al., 2005b; ISI Web of Knowledge, 2010). However, to the best of our knowledge, phenylethanoids 1 and 3 have never reported from been the genus Stachys. Aeschynanthoside C (3) and related compounds have recently been isolated from the family Gesneriaceae (Li et al., 2008). Apigenin (6) and its glycoside (4) occur, not only in the Lamiaceae, but also widely in other plant families. However, the distribution of apigenin 7-O-(6-p-coumaroyl)-

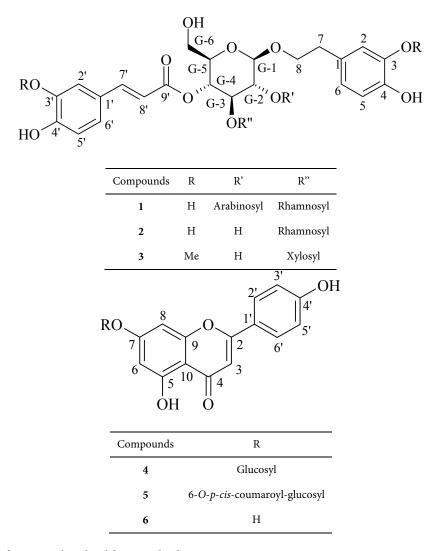


Figure 1. Structures of compounds isolated from Stachys byzantina

glucoside (5) is not that widespread. It is interesting to note that compounds 1-3 and 5 have a caffeic acid (or its methyl ether or *cis* isomer) moiety. Caffeic acid conjugates are considered to be chemotaxonomically important characters within the family Lamiaceae (Delazar et al., 2005b). Verbascoside (2) and related compounds which contain a caffeic acid moiety, sugars and a phenylethyl group occur characteristically in the ajugoid Lamiaceae. Therefore, isolation and identification of caffeic acid conjugates (1-3 and 5) from the aerial parts of *S. byzantina* of the family Lamiaceae might be chemotaxonomically significant. As these compounds showed high freeradical-scavenging activity it can be assumed that they might provide plants with some protection against any oxidative stress.

The DPPH antioxidant assay is based on the principle that 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, is decolorized in the presence of free radical scavengers (antioxidants). The odd electron in the DPPH radical is responsible for the absorbance at 517 nm, and also for the visible deep purple color (Kumarasamy et al., 2002; 2007). When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively mea-

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Hours .	Solid-phase extraction fractions of the MeOH extract of S. byzantina												
	20% MeOH in water		40% MeOH in water		60% MeOH in water		80% MeOH in water		100% MeOH in water				
	mg/mL	% Mortality	mg/mL	% Mortality	mg/mL	% Mortality	mg/mL	% Mortality	mg/mL	% Mortality			
4	5	0.0	5	0.0	5	0.0	5	0.0	5	0.0			
	10	13.3	10	6.6	10	0.0	10	6.6	10	6.6			
	20	26.6	20	16.0	20	0.0	20	16.0	20	23.3			
8	5	0.0	5	3.3	5	0.0	5	0.0	5	3.3			
	10	16.6	10	10.0	10	0.0	10	6.6	10	10.0			
	20	30.0	20	23.3	20	3.3	20	16.0	20	23.3			
24	5	10.0	5	3.3	5	0.0	5	3.3	5	10.0			
	10	16.6	10	10.0	10	0.0	10	10.0	10	16.6			
	20	30.0	20	26.6	20	6.6	20	20.0	20	33.3			
48	5	13.3	5	3.3	5	0.0	5	3.3	5	10.0			
	10 20	26.6	10 20	13.3	10 20	0.0	10 20	13.3	10 20	16.6			
	20	33.3	20	26.6	20	6.6	20	20.0	20	33.3			

Table 3. Percent mortality of *O. surinamesis* exposed to 1 mL of solid-phase extraction fractions at concentrations of 5, 10 and 20 mg/ mL after 1, 4, 8 and 24 hours

sured from the changes in absorbance. Among the extracts, the MeOH extract was found to be the most active, with a RC₅₀ value of $1.5 \times 10^{-2} \text{ mg/mL}$. This extract was, therefore, subjected to solidphase extraction resulting in five fractions which were also tested in the DPPH assay. The fractions eluted with 40 and 60% MeOH in water were the most potent fractions (RC₅₀ = 1.4×10^{-2} and 2.1×10^{-2} 10⁻² mg/mL, respectively), but the fraction eluted with 20% of MeOH in water was also significantly active (Table 2). The fractions eluted with 80% and 100% MeOH in water were the least active. The DPPH-scavenging capacity of the extracts was compared with known antioxidants, quercetin and Trolox[®]. The activity profile of these fractions indicated that compounds responsible for the freeradical-scavenging activity were mainly polar compounds. As the fractions eluted with 40 and 60% MeOH in water were the most active, they were subjected to preparative HPLC analyses leading to the isolation of compounds 1-6. The DPPH assay was also carried out with these compounds (Table 2). It was established that compounds 1 and 2 were the most active among all the compounds (RC₅₀ = 1.4×10^{-2} and 2.1×10^{-2} mg/mL, respectively). Compounds 3-6 were active at higher concentrations. The free-radical scavenging (antioxidant) activity and total phenolics content, based on, respectively, the ferric reducing ability of plasma (FRAP) and the Folin-Ciocalteu assays, of the MeOH extracts of a number of other Stachys species, including S. byzantina, have been reported recently (Khanavi et al., 2009). However, in that study no attempt was made to find the compounds responsible for the antioxidant activity. This is the first report on the isolation and identification of compounds responsible for freeradical-scavenging activity.

This is also the first report on the insecticidal property of the fractions of the MeOH extract of *S. byzantina* (Table 3). The highest percent mortality was observed with the solid-phase extraction fraction eluted with 20% MeOH in water, and it was concentration dependent. At the concentration of 20 mg/mL, the percent mortality rates due to this fraction were 26.6, 30.0, 30.0 and 33.3 % after 4, 8, 24 and 48 h. A similar profile was also observed with the fraction eluted with 100% MeOH.

The presented findings provide a possible scientific basis for the ethnobotanical uses of this plant, particularly its use as an anti-inflammatory, antitumor and anticancer agent, and as well as its insecticidal properties.

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