

## Chemical composition and radical scavenging activity of essential oil and methanolic extract of *Eremostachys azerbaijanica* Rech.f. from Iran

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### Abstract

In the present study, the chemical composition of the essential oil and methanol (MeOH) extract of aerials of *E. azerbaijanica* were identified. Furthermore, the free radical scavenging properties of the volatile oil as well as the MeOH extract of the plant were assessed. The essential oil of the air-dried aerial parts was obtained by hydro-distillation using a Clevenger-type apparatus. The oil was then analyzed by gas chromatography–mass spectrometry and gas chromatography with flame ionization detector. Soxhlet extraction was performed on the aerial parts using n-hexane, dichloromethane and MeOH. The MeOH extract was then subjected to solid-phase extraction using a C<sub>18</sub> Sep-Pak cartridge. Isolation and structural elucidation of the pure components was accomplished by high-performance liquid chromatography and spectroscopic methods (UV, <sup>1</sup>H-NMR). The free radical scavenging properties were determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. A total of 59 components representing 95.9% of the oil constituents were identified which were primarily characterized as terpenoids or aliphatic skeletons. The major components of the oil were hexahydrofarnesyl acetone (27.1%), 2-methyl-6-propyl-dodecane (16.4%) and tricosane (9.3%). One flavonoid (luteolin-7-O-rutinoside) and one phenylethanoid (verbascoside) were also isolated and identified from the MeOH extract. The results of DPPH assays showed that the essential oil of *E. azerbaijanica* possessed weak free radical scavenging activity whereas the MeOH extract and its pure constituents showed significant scavenging activities in comparison with positive controls.

**Keywords:** *Eremostachys azerbaijanica*; DPPH; GC-MS; HPLC; Flavonoid; Phenylethanoid

### INTRODUCTION

The genus *Eremostachys* (Lamiaceae) is represented by about 60 species that occur mainly in central, middle-eastern and western Asian countries and the Caucasus. Of these, 15 species are endemic to Iran (1-3). Several studies have reported local analgesic and anti-inflammatory effects of the plants of this genus (4,5). Biological properties such as antioxidant and antibacterial activities have also been reported (4,6). Moreover, several pharmacological studies have demonstrated the antidepressant and antinociceptive effects of these plants (6-9). Phytochemical studies on the essential oil composition of species of the

genus *Eremostachys*, such as *E. laciniata* (10,11), *E. laevigata* (12,13), *E. macrophylla* (14-16), *E. labiosa* (15) and *E. adenantha* (16) have revealed that terpenoid structures, linear hydrocarbons and their derivatives are major components of these oils at different stages of growth. These reports showed that 1,8-cineol, germacrene-B and -D,  $\alpha$ - and  $\beta$ -phellandrene, spathulenol and caryophyllene derivatives with terpenoid structures, 6,10,14-trimethyl 2 pentadecanone and dodecanal linear hydrocarbons are the most abundant constituents (10-16). Hexadecanoic acid is a common saturated fatty acid found in the volatile oil of some species of this genus (16).

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Other phytochemical studies on the extracts of plants of this genus have demonstrated the presence of different natural compounds. The rhizomes of *E. laciniata* have been identified as a source of iridoid glucosides, flavonoids, and phytosterols (6-8). Ferulic acid derivatives, furanolabdane diterpene glycoside, iridoid glycosides and phenylethanoid glycosides have been identified in the rhizomes of *E. glabra* (4,17-19).

The compounds loasin A and B and loasifolin with flavonoid structure, and eremoside A to C with iridoids skeleton have also been isolated from *E. loasifolia* (20-22). In addition, other iridoid glycosides have been reported in *E. moluccelloides* and an isoflavone compound from *E. vicaryi* (23,24). In the present study, the chemical composition of the essential oil and MeOH extract of the aerial parts of *E. azerbaijanica* were identified. The free radical scavenging properties of the volatile oil and MeOH extract were also assessed.

## MATERIALS AND METHODS

### *Plant material*

Aerial parts of *E. azerbaijanica* Rech.f. were collected during flowering stage from Bostan abad, Eastern Azerbaijan (37° 51' N, 46° 51' E), Iran, in July 2012. A voucher specimen (TBZ-fph-738) of the plant has been deposited in the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences.

### *Isolation of the essential oil*

Essential oil of the powdered air-dried aerial parts of the plant was obtained by hydrodistillation method using a Clevenger-type apparatus for 3 h. The oil was dried over anhydrous sodium sulphate and stored in sealed vials before chemical analyses.

### *Gas Chromatography–Mass Spectrometry*

Gas Chromatography–mass spectrometry (GC–MS) and Gas Chromatography with Flame ionization detector (GC–FID) analysis were performed on a Shimadzu QP-5050A GC–MS system (Japan) and GC-17A equipped with a DB-1 fused silica column (60 m × 0.25 mm i.d., 0.25 µm film thickness); with an oven temperature of 50 °C rising to 270 °C at a rate

of 2 °C/min for a total run time of 114 min. Injector temperature was set at 240 °C and transfer line temperature was 270 °C. Helium was used as the carrier gas at a flow rate of 1.3 ml/min. Sample were diluted 1:10 in n-hexane and 1 µl was injected into the column. Split ratio, ionization energy, scan time, and acquisition mass range were 1:5, 70 eV, 1 s, and 30–600 amu, respectively.

### *Identification of components*

Identification of the constituents was performed based on the direct comparison of the retention times and mass spectral data with standard alkanes (C<sub>8</sub>–C<sub>20</sub>) from Sigma-Aldrich (USA), and computer matching with the NIST 21, NIST 107 and WILEY229 library, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (25).

### *Extraction, separation and identification of non-volatile compounds*

The dried and ground aerial parts of *E. azerbaijanica* (100 g) were Soxhlet-extracted with n-hexane, dichloromethane (DCM) and MeOH (solvents were from Caledon, Canada), successively (1 L each). The MeOH extract (2 g) was subjected to solid-phase extraction (SPE) using a C<sub>18</sub> Sep-Pak cartridge, eluting with a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). All these extracts and fractions were separately concentrated using a rotary evaporator at a maximum temperature of 45 °C.

The Sep-Pak fraction (40% MeOH in water) was further subjected to preparative reversed-phase HPLC (prep-HPLC) conducted on a Knauer HPLC (preparative pump 1800) fitted with a C<sub>18</sub> column (250 mm length, 20 mm i.d, 10 µm particle size, Dr. Maisch, Germany) system. The mobile phase which consisted of 35%-55% MeOH in water for 40% methanol fraction in 70 min ran at flow rate of 8 ml/min and a detector set at 220 nm was used to detect the eluents. The isolated pure compounds were identified by a Bruker Spectrospin 400 MHz NMR-spectrometer. The spectroscopic data of the known compounds were also compared with the respective published data.

### Free radical scavenging activity

Free radical scavenging activity of samples was assessed using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH, obtained from Sigma Aldrich, Germany) free radical. Solutions of DPPH (0.08 mg/ml) were prepared in chloroform for the volatile oil and in MeOH for the extract.

The samples were dissolved in respective solvents to obtain the stock concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.3 and 15.6 µg/ml. Diluted solutions (5 ml each) were mixed with DPPH solution (5 ml) and allowed to stand for 30 min for occurring any reaction. The UV-Visible absorbance was recorded at 517 nm by Spectronic Genesys 5 spectrophotometer. The experiment was performed in triplicate. Trolox and quercetin was used as positive controls (19).

## RESULTS

The air-dried aerial parts of *E. azerbaijanica* were subjected to hydrodistillation for 3 h and exuded colorless oil with the yield of 0.1% (w/w), based on the dry mass. The study on the chemical composition of the essential oil was carried out on a non-polar DB-1 column using GC-MS, GC-FID and the qualitative-quantitative analytical results were compiled in Table 1.

According to Table 1, a total of 59 volatile components were identified in the aerial parts of *E. azerbaijanica* Rech.f. accounting for 95.9% of the total oil. As can be seen in Fig. 1, the essential oil of *E. azerbaijanica* was mostly constituted by linear hydrocarbons (43.6%) with 2-methyl-6-propyl-dodecane (16.4%) and tricosane (9.3%) as the most abundant compounds. The oil also contained

high levels of ketones (30.6%), represented by hexahydrofarnesyl acetone or Phytone (27.1%). Furthermore, the oil presented moderate level of esters (6.7%) and alcohols (6.3%) with 2, 2, 4-trimethyl-1, 3-pentanediol diisobutyrate (6.1%) and 1-dodecanol (1.7%) as the most abundant representative, respectively. Apart from the main components reported above, only hexacosane (4.4%) and heneicosane (2.1%) exceeded a content of 2% of the total oil composition, while the remaining components (52) showed low amount, most of them presenting lower than 1%.

Reversed-phase preparative HPLC analysis of 40% fraction of MeOH extract of *E. azerbaijanica* aerial parts afforded one phenylethanoid glycoside and a flavonoid structure, which were identified unequivocally as verbascoside (11mg,  $t_R = 20.1$  min) and Luteolin-7-O-rutinoside (9 mg,  $t_R = 27.8$  min) on the extensive 1D H-NMR data analyses. The spectroscopic data of the known compounds were also compared with the respective published data.

Verbascoside [1]: brown amorphous powder, UV  $\lambda_{max}$  (MeOH) 220, 300, 330 nm,  $^1H$ - NMR (400MHz, DMSO- $d_6$ ):  $\delta$  7.48 (d, 1H,  $J = 15.8$  Hz, H- $\beta'$ ), 7.02 (bs, 1H, H-2''), 6.99 (d, 1H,  $J = 8.2$  Hz, H-6''), 6.77 (d, 1H,  $J = 8.0$ , H-5''), 6.64 (s, 1H, H-2), 6.62 (bs, 1H, H-5), 6.50 (d, 1H,  $J = 8.0$ , H-6), 6.22 (d, 1H,  $J = 15.9$ , H- $\alpha'$ ), 5.02 (bs, 1H, H-1'), 4.73 (t, 1H,  $J = 9.6$  Hz, H-4'), 4.36 (d, 1H,  $J = 7.8$  Hz, H-1''), 3.73 (m, 1H, H-3'), 3.67 (s, 1H, H-2''), 3.62 (m, 2H), 3.23 (dd, 1H,  $J = 8.4, 8.5$ , H-2'), 3.12 (t, 1H,  $J = 9.6, 9.3$ , H-4''), 2.72 (m, 2H, H- $\alpha$ ), 2.50 (bs, 2H, H- $\beta$ ), 1.03 (d, 3H,  $J = 6$ Hz, H-6''), 3.26- 3.45 ( remaining sugar protons). Data were in agreement with the published data (26,27).

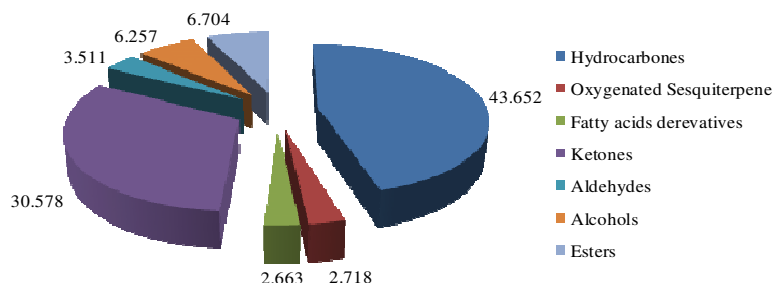


Fig. 1. Identified chemical groups from the essential oil of *E. azerbaijanica*.

**Table 1.** Volatile compounds identified in the aerial part of *E. azerbaijanica*.

NO	<sup>a</sup> Compounds	KI	Percentage	<sup>b</sup> Identification method
1	1-octen-3-ol	962	0.3	GC/MS, I <sub>s</sub>
2	nonanal	1082	0.5	GC/MS, I <sub>s</sub>
3	nonanol	1155	0.3	GC/MS, I <sub>s</sub>
4	N-decanal	1184	0.6	GC/MS, I <sub>s</sub>
5	(Z)-2-decenal	1236	0.2	GC/MS, I <sub>s</sub>
6	1-decanol	1256	0.3	GC/MS, I <sub>s</sub>
7	dihydroedulan I	1282	0.3	GC/MS, I <sub>s</sub>
8	undecanal	1286	0.2	GC/MS, I <sub>s</sub>
9	(E,E)-2,4-decadienal	1288	1.0	GC/MS, I <sub>s</sub>
10	2-undecenal	1339	0.5	GC/MS, I <sub>s</sub>
11	β-damascenone	1360	0.2	GC/MS, I <sub>s</sub>
12	α-copaene	1373	0.5	GC/MS, I <sub>s</sub>
13	β- bourbonene	1381	0.4	GC/MS, I <sub>s</sub>
14	dodecanal	1388	1.5	GC/MS, I <sub>s</sub>
15	tetradecane	1400	0.5	GC/MS, I <sub>s</sub>
16	β-caryophyllene	1414	0.5	GC/MS, I <sub>s</sub>
17	widdrene	1426	0.3	GC/MS, I <sub>s</sub>
18	geranyl acetone	1428	0.6	GC/MS, I <sub>s</sub>
19	2-butyl-1-octanol	1457	0.3	GC/MS, I <sub>s</sub>
20	1-dodecanol	1459	1.7	GC/MS, I <sub>s</sub>
21	β-ionone	1462	0.5	GC/MS, I <sub>s</sub>
22	2-methyl-6-propyl-dodecane	1468	<b>16.4</b>	GC/MS, I <sub>s</sub>
23	2-tridecanone	1476	0.2	GC/MS, I <sub>s</sub>
24	pentadecane	1500	0.4	GC/MS, I <sub>s</sub>
25	nerolidol A (CIS OR TRANS)	1504	0.3	GC/MS, I <sub>s</sub>
26	nerolidol B (CIS OR TRANS)	1547	0.3	GC/MS, I <sub>s</sub>
27	(-)-spathulenol	1561	1.1	GC/MS, I <sub>s</sub>
28	caryophyllene oxide	1567	1.0	GC/MS, I <sub>s</sub>
29	2,2,4-trimethyl-1,3-pentenediol diisobutyrate	1581	6.1	GC/MS, I <sub>s</sub>
30	widdrol	1584	0.4	GC/MS, I <sub>s</sub>
31	octadecanal	1592	0.3	GC/MS, I <sub>s</sub>
32	hexadecane	1600	0.9	GC/MS, I <sub>s</sub>
33	(Z)-linalool Oxide	1616	0.4	GC/MS, I <sub>s</sub>
34	2-hexyl-1-decanol	1658	0.4	GC/MS, I <sub>s</sub>
35	1-tetradecanol	1661	0.6	GC/MS, I <sub>s</sub>
36	2-pentadecanone	1679	0.2	GC/MS, I <sub>s</sub>
37	2-ethylhexyl ester benzoic acid	1685	0.3	GC/MS, I <sub>s</sub>
38	heptadecane	1700	0.6	GC/MS, I <sub>s</sub>
39	3,7,11-trimethyl-1-dodecanol	1723	1.1	GC/MS, I <sub>s</sub>
40	1-hexacosanol	1725	0.5	GC/MS, I <sub>s</sub>
41	octadecane	1800	0.9	GC/MS, I <sub>s</sub>
42	phytane	1814	0.5	GC/MS, I <sub>s</sub>
43	hexahydrofarnesyl acetone (Phytone)	1836	<b>27.1</b>	GC/MS, I <sub>s</sub>
44	4,6,8-trimethyl-1-nonene	1872	0.8	GC/MS, I <sub>s</sub>
45	farnesyl acetone C	1893	0.4	GC/MS, I <sub>s</sub>
46	nonadecane	1900	0.5	GC/MS, I <sub>s</sub>
47	methyl hexadecanoate	1909	0.6	GC/MS, I <sub>s</sub>
48	n-hexadecanoic acid	1949	1.3	GC/MS, I <sub>s</sub>
49	eicosane	2000	0.5	GC/MS, I <sub>s</sub>
50	γ-stearolactone	2061	0.4	GC/MS, I <sub>b</sub>
51	10-octadecynoic acid, methyl ester	2071	0.3	GC/MS, I <sub>b</sub>
52	palmitaldehyde, diallyl acetal	2090	0.3	GC/MS, I <sub>b</sub>
53	heneicosane	2100	2.1	GC/MS, I <sub>b</sub>
54	docosane	2200	0.7	GC/MS, I <sub>b</sub>
55	tricosane	2300	<b>9.3</b>	GC/MS, I <sub>b</sub>
56	tetracosane	2400	1.5	GC/MS, I <sub>b</sub>
57	pentacosane	2500	1.1	GC/MS, I <sub>b</sub>
58	hexacosane	2600	4.4	GC/MS, I <sub>b</sub>
59	heptacosane	2700	0.5	GC/MS, I <sub>b</sub>
	Total compounds		59	
	Total Identified		95.9	

<sup>a</sup>Compounds were reported in order of their elution from a DB-1 column, <sup>b</sup>Identification Method (I<sub>s</sub> = Kovats retention index based on standard alkanes, I<sub>b</sub> = Kovats retention index based on bibliography).

**Table 2.** Free radical scavenging activity of essential oil, MeOH extract and its pure compounds.

Compounds or extract	RC <sub>50</sub> (mg/ml)
Essential oil	15.431
MeOH extract	0.1410
Verbascoside(1)	0.0056
Luteolin-7-O-rutinoside (2)	0.0047
Trolox (positive standard)	0.0030
Quercetin ( positive standard)	0.0039

Luteolin-7-O-rutinoside or scolymoside [2]: brown amorphous powder, UV  $\lambda_{\max}$  (MeOH) nm: 210, 255, 350 nm (NaOMe) 225, 270, 400 nm (AlCl<sub>3</sub>) 215, 275, 420 nm, (AlCl<sub>3</sub>/HCl) 215, 275, 360, 390 nm, (NaOAc) 240, 260, 370 nm, (NaOAc/H<sub>3</sub>BO<sub>3</sub>) 240, 260, 370 nm, <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>):  $\delta$  7.45 (d, 1H,  $J$  = 8.0 Hz, H-6'), 7.42 (bs, 1H, H-2'), 6.91 (d, 1H,  $J$  = 8.1Hz, H-5'), 6.78 (s, 1H, H-8), 6.75 (s, 1H, H-3), 6.44(s, 1H, H-6), 5.13 (bs, 1H, H-1'') 5.09 (d, 1H,  $J$  = 7.2, H-1''), 1.21 (d, 3H,  $J$  = 6.1 Hz, H-6'''), 3.17-3.50 (remaining sugar protons) (28,29).

The free radical scavenging activities of the essential oil, MeOH extract and its pure compounds were evaluated using the DPPH method and the results are displayed in Table 2.

## DISCUSSION

Previous studies on the essential oil of the aerial parts of *E. azerbaijanica* from the Khalkhal mountains in northwestern Iran identified 64.5% carvone as its major component (30). Similar studies on the other Iranian species revealed the presence of dodecanol (72.5%) in the aerial parts of *E. laciniata* (11) and germacrene D (47.1%) in the aerial parts of *E. macrophylla* (14). Esmaeili (12) found 1, 8-cineole, benzaldehyde and piperitenone oxide to be the primary components of the oil of the flowers, stems and roots of *E. laevigata*. The variations in the essential oil components of *E. azerbaijanica* in the present study in comparison with the findings of previous studies could result from various factors such as geographical location, climatic conditions (variations in temperature, humidity, atmospheric pressure), harvesting season and extraction method (31,32).

Solid phase extraction of the polar extract from *E. azerbaijanica* followed by reversed-

phase preparative HPLC analysis of the 40% fraction of MeOH extract led to identification of a phenylethanoid [1] and a flavonoid [2] structure. The compound [1] was identified on the basis of its <sup>1</sup>H-NMR and UV spectrum. The <sup>1</sup>H-NMR spectrum showed the presence of a tri-substituted phenyl moiety characterized by signals appearing at  $\delta_{\text{H}}$  7.02 (bs, 1H, H-2'''), 6.99 (d, 1H,  $J$  = 8.2 Hz, H-6''') and 6.77 (d, 1H,  $J$  = 8.0, H-5'''), a *p*-hydroxyphenethyl alcohol moiety with proton resonances at  $\delta_{\text{H}}$  6.64 (s, 1H, H-2), 6.62 (bs, 1H, H-5), 6.50 (d, 1H,  $J$  = 8 Hz, H6), 2.72 (m, 2H, H- $\alpha$ ) and 2.50 (bs, 2H, H- $\beta$ ), and olefinic protons ( $\delta_{\text{H}}$  7.48, d, 1H,  $J$  = 15.8 Hz; 6.22, d, 1H,  $J$  = 15.9 Hz, AX system) ascribable to H- $\beta'$  and H- $\alpha'$  in the caffeic acid derivative. Anomeric proton signals were observed at  $\delta_{\text{H}}$  4.36 (d, 1H,  $J$  = 7.8 Hz, H-1'') and  $\delta_{\text{H}}$  5.02 (bs, 1H, H-1') that were consistent with the  $\beta$ -glucopyranose unit and  $\alpha$ -rhamnopyranosyl moiety, respectively. The results identified the structure [1] as phenylethanoid, verbascoside or acteoside and are consistent with published spectral data (26,27). Verbascoside has been isolated from other species of the genus *Eremostachys* and the rhizomes of *E. azerbaijanica* (19,33).

UV spectra of the compounds [2] were identical with a flavone moiety (34); this was confirmed by the <sup>1</sup>H-NMR spectrum, which showed characteristic signals at  $\delta_{\text{H}}$  6.75 (s, 1H, H-3) and demonstrates that the chromophores are flavones. The anomeric proton signals at  $\delta_{\text{H}}$  5.13 (bs, 1H, H-1'') and 5.09 (d, 1H,  $J$  = 7.2, H-1'') indicated the presence of  $\alpha$ -rhamnopyranosyl and  $\beta$ -glucopyranose units, respectively. The structure of the compound [2] was identified by comparison of the NMR with published data (28,29) as the diglycosylated flavone luteolin-7-O-rutinoside (scolymoside). To the best of our knowledge, this is the first time that scolymoside has been

isolated from the *Eremostachys* genus. Previous studies on the rhizomes of *E. azerbaijanica* have also demonstrated the presence of iridoid and phenylethanoid glycosides (33,35).

DPPH free radical scavenging assay is based on DPPH as a stable free radical capable of changing color from purple to yellow in the presence of antioxidant agents (36). The free radical scavenging properties of the essential oil, MeOH extract, and isolated compounds are summarized in Table 2. MeOH extract showed a high level of free radical scavenging activity with an RC<sub>50</sub> value of 0.141 mg/ml. It appears that the compounds responsible for this activity are flavonoids and phenylethanoids such as the luteolin-7-O-rutinoside and verbascoside that were isolated from the 40% fraction of the MeOH extract. The results indicate a notable decrease in capacity of these two pure compounds in comparison with the positive control because of the presence of phenolic hydroxyl groups and the number of ortho-dihydroxy positions (37,38). The essential oil of *E. azerbaijanica* showed weak free radical scavenging properties, with an RC<sub>50</sub> value of 15.431mg/ml because of the presence of aliphatic hydrocarbons and the absence of free phenolics and other susceptible scavenging groups in the oil composition.

## CONCLUSION

Phytochemical investigation of the aerial parts of *E. azerbaijanica* demonstrated that this plant is a good source of flavonoids and phenylethanoids that show antioxidant activity.

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