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Original Article

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_{18} Sep-Pak cartridge. Isolation and structural elucidation of the pure components was accomplished by high-performance liquid chromatography and spectroscopic methods (UV, ^{1}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, α- and β-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 injectedinto 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_8-C_{20}) 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_{18} 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_{18} 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 λ_{max} (MeOH) 220, 300, 330 nm, ^{1}H- NMR (400MHz, DMSO-d_6): δ 7.48 (d, 1H, *J* = 15.8 Hz, H-β'), 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-α'), 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-α), 2.50 (bs, 2H, H-β), 1.03 (d, 3H, *J* = 6Hz, H-6''), 3.26- 3.45 (remaining sugar protons). Data were in agreement with the published data (26,27).

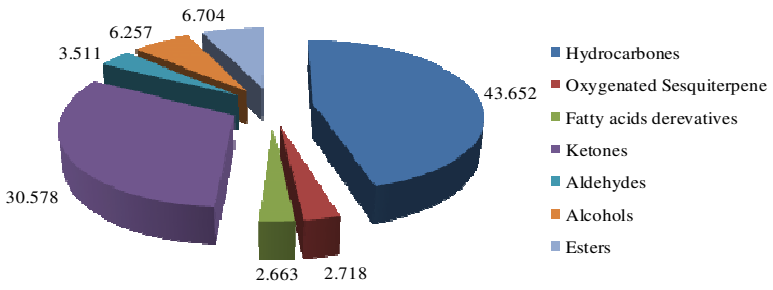

Fig. 1. Identified chemical groups from the essential oil of *E. azerbaijanica*.

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

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

[a]Compounds were reported in order of their elution from a DB-1 column, [b]Identification Method (Is = Kovats retention index based on standard alkanes, Ib = 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_{50} (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 λ_{max} (MeOH) nm: 210, 255, 350 nm (NaOMe) 225, 270, 400 nm ($AlCl_3$) 215, 275, 420 nm, ($AlCl_3$/HCl) 215, 275, 360, 390 nm, (NaOAc) 240, 260, 370 nm, (NaOAc/H_3BO_3) 240, 260, 370 nm, ^{1}H- NMR (400 MHz, DMSO-d_6): δ 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 ^{1}H-NMR and UV spectrum. The ^{1}H-NMR spectrum showed the presence of a tri-substituted phenyl moiety characterized by signals appearing at δ_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 δ_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-α) and 2.50 (bs, 2H, H-β), and olefinic protons (δ_H 7.48, d, 1H, *J* = 15.8 Hz; 6.22, d, 1H, *J* = 15.9 Hz, AX system) ascribable to H-β' and H-α' in the caffeic acid derivative. Anomeric proton signals were observed at δ_H 4.36 (d, 1H, *J* = 7.8 Hz, H-1'') and δ_H 5.02 (bs, 1H, H-1') that were consistent with the β-glucopyranose unit and α-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 ^{1}H-NMR spectrum, which showed characteristic signals at δ_H 6.75 (s, 1H, H-3) and demonstrates that the chromophores are flavones. The anomeric proton signals at δ_H 5.13 (bs, 1H, H-1''') and 5.09 (d, 1H, *J* = 7.2, H-1'') indicated the presence of α-rhamnopyranosyl and β-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 phenylehanoid 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_{50} 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_{50} 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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