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Essential oil composition and isolation of free-radical-scavenging phenolic glycosides from the aerial parts of *Ajuga chamaepitys* growing in Iran

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Abstract: From the methanolic extract of the aerial parts of *Ajuga chamaepitys* (L.) Schreb., Lamiaceae, one of the Iranian medicinal plants, the phenylethanoid glycoside, acteoside, and two flavone glycosides, chrysoeriol 7-*O*-glucopyranoside (3'-methoxy-luteolin 7-*O*-glucopyranoside) and apigenin 7-*O*-rhamnopyranoside, were isolated by a combination of solid-phase extraction (SPE) and preparative reversed-phase high-performance liquid chromatography (prep-RP-HPLC) methods. Structures of the isolated compounds were elucidated by spectroscopic means. The free-radical-scavenging properties of the extracts, fractions and isolated compounds were determined by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay. While among the extracts, the MeOH extract showed the highest level of free-radical-scavenging activity (RC50 1.15×10^{-1} mg/mL), chrysoeriol 7-*O*-glucopyranoside was the most active (RC50 3.00×10^{-3} mg/mL) among the isolated compounds. The GC-MS and the GC-FID analyses revealed α-pinene (23.66%), β-pinene (9.33%), 1-octen-3-ol (9.72%), β-phellandrene (8.70%) and germacrene-D (7.92%) as the major components of the essential oils derived from the aerial parts of this plant. The presence of phenolic glycosides and the α- and β-pinene-rich essential oils in *A. chamaepitys* may provide some rationale for the traditional medicinal uses of this species in Iran.

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Introduction

The genus *Ajuga* L. of the family Lamiaceae (*alt*. Labiateae) comprises over 300 species with many subspecies and varieties, which are distributed all over the world (Israili & Lyoussi, 2009). *Ajuga chamaepitys* (L.) Schreb. (common name: "yellow bugle") is one of the five *Ajuga* species from the flora of Iran. This species, which has been mentioned as the name of "Kamaphytus" in the old texts of traditional medicine, is one of the species of the Lamiaceae that has been used in the Iranian traditional medicine for centuries (Naghibi et al., 2009). This plant has long been used as a diuretic, tonic, emmenagogue agent and menser remover, and for wound-healing and perspiration (Ulukanli et al., 2005). Applications of this species to treat scorpion and snake bites, hemorrhoids, stomachache, jaundice, inflammatory diseases, such as gout and joint pains, and common colds have also been well documented (Zargari, 1997; Ulukanli et al., 2005; Israili & Lyoussi, 2009; Naghibi et al., 2009). Antimicrobial, antiviral and antifeedant and cytotoxic properties of various extracts of this plant have been reported (Kutas & Nadasy, 2005; Akçin et al., 2006; Orhan et al., 2009; Turkoglu et al., 2010). Previous phytochemical investigations on this plant revealed the presence of cloredan and neocloredan diterpenes in the aerial parts, and β-pinene and germacrene-D as the major components of the essential oils (Hernandez et al., 1980; Camps et al., 1984, 1987; Boneva et al., 1990; Baser et al., 1999; Azizan et al., 2002; Velasco-Negueruela

et al., 2004). In continuation of our phytochemical and pharmacological studies on the medicinal plants of the Iranian flora (Delazar et al., 2004, 2006, 2007, 2009, 2010a,b; 2011a,b; Babaei et al., 2008; Nazemieyh et al., 2008a,b, 2011; Nazifi et al., 2008; Razvi et al., 2008, 2011; Modaressi et al., 2009; Asnaashari et al., 2010), we now report on the isolation, identification and free-radical-scavenging properties of three phenolic glycosides (**1-3**), and the composition of the essential oils of the aerial parts of *Ajuga chamaepitys* (L.) Schreb. growing in Iran.

Material and Methods

General

NMR spectra were obtained using a Bruker Spectrospin 200 and an AMX300 NMR-spectrometers. UV-visible spectra were recorded using a Shimadzu-1600 spectrophotometer. Preparative HPLC was conducted on Shimadzu -10A prep-HPLC coupled with SPDM photo diode array detector (detection at 220 and 280 nm).

Plant material

Aerial parts of *Ajuga chamaepitys* (L.) Schreb., Lamiaceae, were collected during the flowering stage from the "Mishodagh" mountains located in Shabestar (East-Azarbaijan province, Iran) in May 2008. A voucher specimen (TUM-ADE-0318) for this collection has been deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction

Dried and ground aerial parts of *A. chamaepitys* (100 g) were successively Soxhlet-extracted using *n*-hexane, dichloromethane (DCM) and methanol (MeOH) (1.5 L each). All these extracts were separately concentrated under vacuum by rotary evaporator not exceeding the temperature of 50 ºC.

Essential oils extraction

The essential oils of the aerial parts (50 g in 400 mL of H_2O) were obtained by hydro-distillation method using the Clevenger apparatus for about 4 h, dried over anhydrous sodium sulfate and stored at 4 °C until analysis.

Fractionation of the methanolic extract

Dried methanolic extract (2 g × 2) was fractionated by solid-phase-extraction on Sep-Pak (C_{18}, 10 g cartridge) using a step gradient of methanol-water mixture (10:90, 20:80, 40:60, 80:20 and 100:0). All fractions were dried using a rotary evaporator at a temperature not exceeding 50 ºC.

Isolation of phenolic glycosides

The Sep-Pak fraction of 40% MeOH-water (590 mg) was analyzed by prep-RP- HPLC (Dr Maisch ODS preparative column 10 µm, 250 mm x 20 mm, solvent system: linear gradient 0-30 min, 15-25% acetonitrile (ACN) in water; isocratic 25% ACN in water during 30-35 min; linear gradient 35-40 min, 25-5% ACN in water; linear gradient 40-65 min, 5-100% ACN in water; flow rate 8 mL/min; detection at: 220 and 280 nm) to yield the phenylethanoid glycoside, acteoside (1, 30.0 mg, tR = 27.55 min). Similarly, the prep-RP- HPLC analysis of the fraction 60% (70 mg), using the mobile phase: linear gradient 0-57 min, 26-32% ACN in water; isocratic 32% ACN in water during 57-60 min; linear gradient 60-75 min, 32-100% ACN in water; flow rate 8 mL/min; detection at: 220 and 280 nm, resulted in the isolation of chrysoeriol 7-*O*-glucopyranoside (2, 3.5 mg; t_R = 16.4 min) and apigenin 7-*O*-rhamnopyranoside (3, 3 mg; t_R = 29.58 min). The structures of these phenolic glycosides were determined by UV in MeOH and using various shift reagents (Mabry et al., 1970), and NMR (^1H and ^{13}C) spectral analyses as well as by comparison with respective published data.

GC-MS and GC-FID analyses

The essential oils were analyzed using a Shimadzu GCMS-QP5050A gas chromatograph-mass spectrometer (GC-MS) fitted with a fused methyl silicon DB-5 column (60 m x 0.25 mm i.d., 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 0.9 mL/min. The oven temperature was kept at 50 °C for 2 min, and programmed to rise to 230 °C at a rate of 2 °C/min and then kept constant for 8 min. The injector temperature was 250 °C and split ratio was adjusted at 1:51. The mass spectral (MS) data were obtained at the following conditions: ionization potential 70 eV; ion source temperature 200 °C; quadrupole temperature 100 °C; solvent delay 3 min; EM voltage 3000 volts. Identification of compounds was based on direct comparison of the Kovats indices (K. I.) and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library, as well as by comparison (Massada, 1976; Adams, 2004).

For quantitation (area %), the GC analyses were also performed on an Agilent 6890 series apparatus fitted with a FID detector. The FID detector temperature was 300 ºC. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Free-radical-scavenging activity: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free-radical-scavenging effect of the extracts, fractions and isolated compounds was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Kumarasamy et al., 2002, 2007). DPPH was obtained from Fluka Chemie AG, Bucks and a solution of DPPH (0.08 mg/mL) in methanol was used. Dilutions were made to obtain concentrations of 5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.13×10^{-2} and 1.56×10^{-2} mg/mL. Diluted solutions (1 mL each) were mixed with DPPH solution (1 mL) and allowed to stand for 30 min for any reaction to take place. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration.

Results and Discussion

Solid-phase extraction (SPE) of the MeOH extract of the aerial parts of *Ajuga chamaepitys* (L.) Schreb., Lamkiaceae, followed by reversed-phase preparative HPLC analyses of the SPE fractions (40% and 60% aqueous MeOH fractions) resulted in the isolation three free-radical-scavenging phenolic glycosides (**1-3**). Isolated glycosides were identified unequivocally as the phenylethanoid glycoside, acteoside (**1**; Delazar et al., 2005; Nazemiyeh et al., 2008c), and two flavone glycosides, chrysoeriol 7-*O*-glucopyranoside (**2**; Mabry et al., 1970; Wagner et al., 1976; Wenkert & Gottlieb, 1977; Zhang & Li, 2008; Zhao et al., 2009; Zhou et al., 2009) and apigenin 7-*O*-rhamnopyranoside (**3**; Mabry et al., 1970; Wagner et al., 1976; Wenkert & Gottlieb, 1977; Iwashina & Matsumoto, 1994; Chunsriimyatav et al., 2009), by extensive UV spectroscopic analyses using various shift reagents (Mabry et al., 1970) and NMR analyses (Tables 1 and 2). All spectroscopic data were comparable with respective published data. This is the first report on the occurrence of compounds **1-3** in the species of *Ajuga chamaepitys* (L.) Schreb., Lamiaceae, and to the best of our knowledge, none of these phenolic glycosides has ever been reported from the genus Ajuga. However, other flavonoids have previously been reported from only a few other species of the genus *Ajuga*, e.g. *A. reptans* and *A. remota* (Terahara et al., 2001; Manguro et al., 2007).

The free-radical-scavenging activity of the extracts, SPE fractions and isolated glycosides (**1-3**) were determined by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free-radical-scavenging assay (Kumarasamy et al., 2002, 2007) (Table 3). The MeOH extract showed the highest level of activity with a RC50 value of 1.15×10^{-1} mg/mL among all extracts, and most of its activity was contributed by the SPE fractions of 40% and 60% aq. MeOH fractions, and the compounds responsible for this activity were phenolic glycosides (**1-3**), which displayed significant free-radical-scavenging properties with the RC50 values ranging from 8.5×10^{-3} to 3.0×10^{-3} mg/mL (Table 3), quite comparable to that of the positive control quercetin (RC50 2.5×10^{-3} mg/mL). While to the best of our knowledge, this is the first report on the free-radical-scavenging property of *A. chamaepitys* growing in Iran, subspecies *euphratica* from this species that grows in Turkey has recently been shown to possess antioxidant properties (Torkoglu et al., 2010).

Table 1. ^{1}H NMR (200 MHz, CD_3OD, coupling constant J in Hz in parentheses) and ^{13}C NMR (50 MHz, CD_3OD) data of acteoside (1).

Carbon number	Compound **1**	
	δ_H (ppm)	δ_C (ppm)
Aglycone (3-methoxy-4-hydroxyphenylethyl) moiety		
1	-	130.4
2	6.73 d (1.5)	116.1
3	-	145.1
4	-	143.6
5	6.71 d (8.0)	115.3
6	6.60 dd (1.5, 8.0)	120.3
7	2.82 t (5.4)	35.6
8	3.8-4.1*	71.3
Aglycone (trans-3,4-dimethoxycinnamoyl) moiety		
1’	-	126.6
2’	7.29 d (2.0)	113.6
3’	-	145.9
4’	-	148.8
5’	6.81 d (8.0)	115.5
6’	6.99 dd (2.0, 8.0)	122.3
7’	7.63 d (16.0)	147.1
8’	6.30 d (16.0)	115.1
9’	-	167.3
Glucosyl moiety		
1”	4.41 d (7.8)	103.2
2”	3.2-3.8*	75.1
3”	3.2-3.8*	80.7
4”	3.2-3.8*	69.4
5”	3.2-3.8*	75.2
6”	3.2-3.8*	61.3
Rhamnosyl moiety		
1”’	5.2 d (1.5)	102.1
2”’	3.2-3.8*	71.3
3”’	3.2-3.8*	71.1
4”’	3.2-3.8*	72.8
5”’	3.2-3.8*	69.6
6”’	1.12 d (6.1)	17.5

*Overlapped peaks

Table 2. ^{1}H NMR (200 MHz, CD_3OD, coupling constant J in Hz in parentheses) and ^{13}C NMR (50 MHz, CD_3OD) data of chrysoeriol 7-*O*-glucopyranoside (**2**) and apigenin 7-*O*-rhamnopyranoside (**3**).

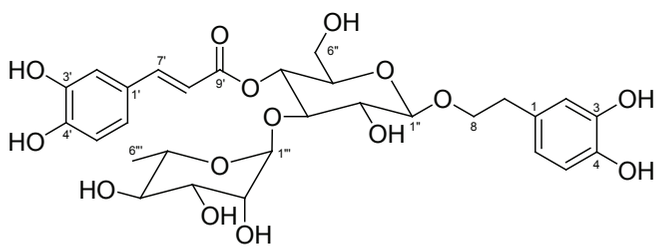
Carbon number	. δ_H (ppm)		. δ_C (ppm)	
	2	**3**	**2**	**3**
Aglycone (flavone) moiety				
2	-	-	162.8	162.2
3	6.75 s	6.61 s	102.6	102.3
4	-	-	182.0	182.9
5	-	-	158.1	158.4
6	6.55 d (2.1)	6.32 br s	99.5	100.1
7	-	-	165.2	165.8
8	6.90 d (2.1)	6.43 br s	95.1	95.0
9			161.3	161.6
10			106.4	106.2
1'	-	-	121.3	121.7
2'	7.56 d (2.0)	7.86 d (7.9)	110.3	128.6
3'	-	6.94 d (7.9)	148.0	116.1
4'	-	-	150.1	161.6
5'	6.98 d (8.4)	6.94 d (7.9)	115.8	115.8
6'	7.60 dd (8.4, 2)	7.86 d (7.9)	120.6	128.3
OMe	4.00 s	-	58.7	-
Glycosyl moiety				
1''	5.52 d (7)	5.41 br s	103.0	103.7
2''	3.30-4.00*	3.30-4.00*	73.7	71.9
3''	3.30-4.00*	3.30-4.00*	77.3	71.3
4''	3.30-4.00*	3.30-4.00*	70.2	73.4
5''	3.30-4.00*	3.30-4.00*	76.8	69.5
6''	3.30-4.00*	1.21 d (7)	61.4	17.4

*Overlapped peaks.

The air-dried aerial parts of *A. chamaepitys* provided 0.1% pale yellow essential oils. The GC-MS and GC-FID analyses of the essential oils led to the separation of 25 peaks accounting for 94% of the total oils. Out of 25 separated peaks, 22 components (~88%) could be identified (Table 4). The majority of components present in the oils were hydrocarbon monoterpenes constituting about 49% of the total oils. There were also significant amounts (~19%) of hydrocarbon sesquiterpenes present in the oils. The absence of any oxygenated mono- or sesquiterpenes in the oils was particularly noticeable. Among the identified components, α-pinene was the major component representing about 24% of the total oils. β-Pinene (9.33%), 1-octen-3-ol (9.72%), β-phellandrene (9.70%) and germacrene D (7.92%) were four other noteworthy components which were present in significant amounts. The composition of the essential oils of the aerial parts of the subspecies and varieties of *A. chamaepitys* varies quite significantly in different regions. For example, in *A. chamaepitys* ssp. chamaepitys growing in Spain, γ-muurolene (40.3%), limonene (20.5%) and germacrene B (7.8%) were identified as the major components (Velasco-Negueruela et al., 2004), which were significantly different from the composition of *A. chamaepitys* as stated above. However, the presence of α- and β-pinene in significant amounts appears to be common in the subspecies and varieties of this species that grow in north of Iran (Gilan province) and Turkey (Baser et al., 1999; Azizan et al., 2002). The predominance of α- and β-pinene in the essential oils of other *Ajuga* species has also been noted (Javidnia et al., 2010; Mohammadhosseini et al., 2011).

Table 3. Free-radical-scavenging activities of the extracts, fractions and isolated compounds (**1-3**) from the aerial parts of *Ajuga chamaepitys* (L.) Schreb.in the DPPH assay.

Extracts/fractions/compounds	RC50 value (mg/mL)
n-Hexane extract	14.5×10^{-1}
DCM extract	6.34×10^{-1}
MeOH extract	1.15×10^{-1}
SPE fraction 10% MeOH-H_2O	4.33×10^{-1}
SPE fraction 20% MeOH-H_2O	1.45×10^{-1}
SPE fraction 40% MeOH-H_2O	1.50×10^{-2}
SPE fraction 60% MeOH-H_2O	6.80×10^{-2}
SPE fraction 80% MeOH-H_2O	1.42×10^{-1}
SPE fraction 100% MeOH	2.36×10^{-1}
Acteoside (**1**)	8.50×10^{-3}
Chrysoeriol 7-*O*-glucopyranoside (**2**)	3.00×10^{-3}
Apigenin 7-*O*-rhamnopyranoside (**3**)	4.80×10^{-3}
Quercetin (positive control)	2.50×10^{-3}



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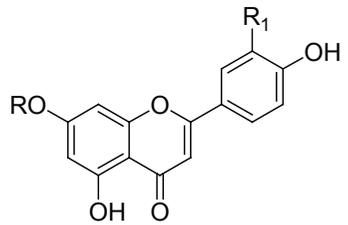

2 R=glucosyl; R_1=OMe
3 R=rhamnosyl; R_1=H

Table 4. GC-MS and GC-FID data of the essential oils of the aerial parts of *Ajuga chamaepitys* (L.) Schreb.

No.	Compounds	Real % area	K. I.	Molecular mass	Molecular formula
1	α-Pinene	23.66	931	136	$C_{10}H_{16}$
2	1-Hepten-3-one	3.04	948	126	$C_8H_{14}O$
3	β-Pinene	9.33	971	136	$C_{10}H_{16}$
4	1-Octen-3-ol	9.72	971	128	$C_8H_{16}O$
5	β-Myrcene	1.83	987	136	$C_{10}H_{16}$
6	β-Phellandrene	8.70	1022	136	$C_{10}H_{16}$
7	δ-Carene	2.56	1023	136	$C_{10}H_{16}$
8	β-Ocimene	1.20	1042	136	$C_{10}H_{16}$
9	α-Terpinolene	1.29	1081	136	$C_{10}H_{16}$
10	β-Damascenone	3.35	1377	190	$C_{10}H_{18}O$
11	Unidentified	1.15	1379	136	-
12	α-Bourbonene	1.10	1381	204	$C_{15}H_{24}$
13	*trans*-α-Bergamoptene	1.82	1328	204	$C_{15}H_{24}$
14	Aromadendrene	3.91	1433	204	$C_{15}H_{24}$
15	α-Humulene	2.81	1447	204	$C_{15}H_{24}$
16	Unidentified	1.92	1461	136	-
17	Germacrene D	7.92	1477	204	$C_{15}H_{24}$
18	β-Curcumene	0.60	1508	204	$C_{15}H_{24}$
19	Junipene	1.28	1652	204	$C_{15}H_{24}$
20	Unidentified	2.95	1716	220	-
21	Hexadecanoic acid	3.21	1951	256	$C_{16}H_{32}O_2$
22	Eicosane	1.05	2000	282	$C_{20}H_{42}$
23	Hexacosane	1.78	2600	366	$C_{26}H_{54}$
24	Octacosane	2.55	2800	394	$C_{28}H_{58}$
25	Nonacosane	1.27	2900	408	$C_{29}H_{60}$
	Hydrocarbon monoterpenes	48.57			
	Hydrocarbon sesquiterpenes	19.44			
	Hydrocarbon nonterpenes	6.65			
	Oxygenated nonterpenes	19.32			
	Unidentified	6.02			
	Total identified	87.96			

The phytochemical investigation of the aerial parts of *A. chamaepitys* has demonstrated that this plant is a good source of phenolic glycosides with significant free-radical-scavenging property, and the essential oils of the aerial parts predominantly contain hydrocarbon monoterpenes. While flavonoids and phenyl propanoid glycosides (*e.g.* acteoside, **1**) are well known biologically active natural products, plant essential oils possess, among others, notable antimicrobial properties. Thus, it is reasonable to assume, yet not being too much speculative, that the presence of phenolic glycosides **1-3**, and the α- and β-pinene-rich essential oils in *A. chamaepitys* may provide some rationale for the traditional medicinal uses of this species in Iran.

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