Major Constituents and Cytotoxic Effects of *Ajuga chamaecistus* ssp. *tomentella*

Nargess Sadati[a], Kristina Jenett-Siems[b], Karsten Siems[c], Mohammad Reza Shams Ardekani[a], Abbas Hadjiakhoondi[a], Tahmineh Akbarzadeh[d], Seyed Nasser Ostad[e], and Mahnaz Khanavi[a,*]

[a] Department of Pharmacognosy and Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 14155-6451, Iran. Fax: +98 21 66954706. E-mail: khanavim@sina.tums.ac.ir
[b] Institut für Pharmazie (Pharmazeutische Biologie), Freie Universität Berlin, Berlin, Germany
[c] Analyticon Discovery GmbH, Potsdam, Germany
[d] Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 14155-6451, Iran
[e] Department of Toxicology and Pharmacology and Pharmaceutical Science Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 14155-6451, Iran

* Author for correspondence and reprint requests

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The *n*-butanolic fraction of a methanolic extract (80%) from aerial parts of *Ajuga chamaecistus* ssp. *tomentella* was analysed using different chromatographic methods. Column (CC) and high-performance liquid chromatography (HPLC) were used for isolation and purification. ^{13}C, ^{1}H NMR, H-H COSY, HSQC, HMBC, and ESI-MS were employed for identification of the compounds isolated from this fraction. The structures of the compounds were determined to be *cis*-melilotoside (**1**), *trans*-melilotoside (**2**), lavandulifolioside (**3**), 20-hydroxyecdysone (**4**), leonoside B (**5**), martynoside (**6**), ajugalactone (**7**), makisterone A (**8**), and 24-dehydroprecyasterone (**9**). This is the first report on the presence of *cis*- and *trans*-melilotoside in *Ajuga* species. Cytotoxic evaluation of the *n*-butanolic fraction, *cis*- and *trans*-melilotoside against cancer (T47D, HT-29, and Caco-2) and normal (NIH 3T3) cell lines by the mitochondrial tetrazolium test (MTT) showed no cytotoxic effects up to 400 μg/mL. The results of this study suggest that melilotoside, phenylethyl glycosides, and phytoecdysteroids are the main constituents of the *n*-butanolic fraction of *Ajuga chamaecistus* ssp. *tomentella*.

Key words: Ajuga chamaecistus ssp. *tomentella*, Melilotoside, Cytotoxic Effect

Introduction

More than one hundred species including fifty varieties and subspecies of *Ajuga* (Lamiaceae) are distributed throughout the world. The genus *Ajuga* with the common name bugle is found in China, Korea, Japan, and throughout Europe. Five species of this annual and perennial genus are found in Iran, of which *Ajuga chamaecistus* contains several exclusive subspecies, including *A. chamaecistus* ssp. *tomentella* (Mozaffarian, 2007). Some species belonging to this genus are used in traditional medicine of different countries of the world, including Iran, for treatment of joints pains, gout, and jaundice (Naghibi *et al.*, 2005). A broad range of biological effects has been reported from different species of *Ajuga* such as hypoglycemic (Hilaly and Lyoussi, 2002), treatment of joint disease (Ono *et al.*, 2008), anti-inflammatory (Gautam *et al.*, 2011), and antimalarial (Kuria *et al.*, 2001). Many phytochemical studies on *Ajuga* species were performed which led to the isolation of phytoecdysteroids (Vanyolos *et al.*, 2009; Castro *et al.*, 2008), diterpenoids (Coll, 2002), iridoids (Manguro *et al.*, 2007), and phenylethyl glycosides (Akbay *et al.*, 2003). The aim of the present study was a phytochemical investigation of the *n*-butanolic fraction obtained from aerial parts of *Ajuga chamaecistus* ssp. *tomentella*, collected in Tehran (Iran), which has not been previously reported. Furthermore, we examined the cytotoxicity of the *n*-butanolic fraction and two major constituents, isolated from this fraction, against cancer and normal cell lines (T47D, Caco-2, HT-29, and NIH 3T3) by the MTT assay.

Material and Methods

General experimental procedures

^{1}H and ^{13}C NMR spectroscopy of compounds **1** and **2** were performed in CD_3OD on a Bruker Avance DPX 400 spectrometer (Karlsruhe, Germany) [400 MHz, tetramethylsilane (TMS) as internal standard]. ^{1}H and ^{13}C NMR spectra of compounds **3**–**9** were acquired in CD_3OD on a Jeol ECX-400 spectrometer (Peabody, MA, USA) (400 MHz, TMS as internal standard). ^{1}H-^{1}H COSY, HMBC, and HSQC spectra were obtained on a Bruker DRX 500 MHz spectrometer. ESI-mass spectra were recorded on an Agilent 6210 ESI-TOP spectrometer (Santa Clara, CA, USA). Column chromatography (CC) was performed using Sephadex LH-20 (45 × 4.5 cm, lipophilic Sephadex, 25–100 μm; Sigma, Dorset, UK) and RP-18 (30 × 4.5 cm, Lichroprep RP-18, 40–63 μm; Merck, Darmstadt, Germany) columns. Analytical and preparative high-performance liquid chromatography (HPLC) separations were performed on a Shimadzu LC-10AD pumping system (Kyoto, Japan) with a Shimadzu variable wavelength detector (220 nm) equipped with a Knauer (Berlin, Germany) Eurospher 100 C-18 (7 μm, 250 × 4 mm) and Nucleosil 300-C18 (10 μm, 250 × 16 mm) column, respectively.

Plant material

Aerial parts of *Ajuga chamaecistus* Ging. ssp. *tomentella* (Boiss.) Rech. f. were collected from "Sorkhe Hesar", east of Tehran, Iran, in June 2008 and verified by Prof. G. Amin. A voucher specimen (THE-6697) has been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Extraction and isolation

The air-dried and ground plants of *A. chamaecistus* ssp. *tomentella* (1 kg) were extracted with 80% methanol (7 × 2.5 L) at room temperature and concentrated under reduced pressure to give a dark brown extract (180 g). The extract (150 g) was defatted through repeated extraction with *n*-hexane. The defatted extract was partitioned successively between 80% methanol, diethyl ether, and *n*-butanol. Twenty g of the *n*-butanolic fraction were loaded on a Sephadex LH-20 (150 g) column and eluted with aqueous methanol (60%) to afford 4 fractions. Fraction 2 (10 g) was chromatographed on an RP-18 column, under medium pressure, and eluted with a gradient of water and methanol (80:20–50:50 v/v) to afford 5 fractions (B–F). Fraction B (8 g) was rechromatographed on RP-18 material with a gradient of aqueous methanol (5%, 20%, and 50%) to give 3 fractions (B_1–B_3). B_1 (200 mg) was subjected to semipreparative reversed phase (RP)-HPLC using gradient elution with aqueous methanol (10%–35%; flow-rate, 1 mL/min; time per run, 30 min) to afford compound **1** (14 mg). Compound **2** (20 mg) was obtained by preparative RP-HPLC and a gradient of aqueous methanol (30%–35%) within 40 min from fraction B_2 (190 mg). Fraction B_3 (2.5 g) was chromatographed on an RP-18 column eluted with a gradient of water/methanol (80:20–50:50 v/v) to give 9 fractions ($B_{3.1}$–$B_{3.9}$). Purification of $B_{3.2}$ (200 mg) using preparative RP-HPLC with a gradient of aqueous methanol (30%–60%; flow-rate, 7 mL/min) within 60 min afforded compound **3** (5 mg) and compound **4** (80 mg). Fraction $B_{3.7}$ (144 mg) was subjected to RP-HPLC with aqueous methanol (35%–60%) to give compounds **5** (13.5 mg) and **6** (17.6 mg) within 60 min. Fraction C (200 mg) was purified by RP-HPLC with a gradient of aqueous methanol (30%–60%) within 60 min to obtain compound **7** (8 mg) and compound **6** (11.5 mg). Fraction C (100 mg) was further purified by RP-HPLC using a gradient of water/acetonitrile (80:20–65:35) to afford compound **8** (1 mg) and compound **9** (1 mg). Direct sunlight was excluded during extraction and purification of the compounds.

cis-Melilotoside (**1**): ^{1}H NMR (400 MHz, CD_3OD): δ = 7.52 (1H, *d*, *J* = 7.46 Hz, H-6), 7.31 (1H, *d*, *J* = 12.52 Hz, H-7), 7.28 (1H, *dd*, *J* = 8.56 1.24, Hz, H-4), 7.18 (1H, *d*, *J* = 8.28 Hz, H-3), 6.95 (1H, *t*, *J* = 7.47 Hz, H-5), 5.95 (1H, *d*, *J* = 12.52 Hz, H-8), 4.93 (1H, *d*, *J* = 7.24 Hz, Glu-H-1'), 3.87 (1H, Ha-6'), 3.68 (1H, Hb-6'), 3.47 (1H, H-3'), 3.45 (1H, H-2'), 3.40 (2H, H-4', H-5'). – ^{13}C NMR (CD_3OD): δ = 138.42 (C-7), 130.48 (C-4), 130.27 (C-6), 121.64 (C-5), 120.02 (C-8), 115.27 (C-3); glucose: 101.43 (C-1'), 77.04 (C-5'), 76.95 (C-3'), 73.76 (C-2'), 70.00 (C-4'), 61.32 (C-6'). – ESI-TOF-MS (positive): *m/z* = 349.08 $[M+Na]^+$, 365.05 $[M]^+$.

trans-Melilotoside (**2**): ^{1}H NMR (400 MHz, CD_3OD): δ = 8.14 (1H, *d*, *J* = 16.20 Hz, H-7), 7.64 (1H, *d*, *J* = 7.68 Hz, H-6), 7.42 (1H, *m*, H-4), 7.26

(1H, *m*, H-3), 7.07 (1H, *t*, *J* = 7.48 Hz, H-5), 6.54 (1H, *d*, *J* = 16.2 Hz, H-8), 5.00 (1H, *d*, *J* = 7.68 Hz, Glu-H-1'). – ESI-TOF-MS (positive): *m/z* = 349.08 $[M+Na]^+$, 365.05 $[M]^+$.

Lavandulifolioside (**3**): ^{1}H NMR (400 MHz, CD_3OD): see Table I. – ESI-TOF-MS (positive): *m/z* = 779.2384 $[M+Na]^+$.

20-Hydroxyecdysone (**4**): ^{1}H NMR (400 MHz, CD_3OD): δ = 5.79 (1H, *d*, *J* = 2.3 Hz, H-7), 3.95 (1H, *br s*, H_{eq}-3), 3.82 (1H, *m*, H_{ax}-2), 3.13 (1H, *br t*, H-9), 2.37 (1H, *dd*, H-5), 1.19 (6H, *s*, Me-26,27), 1.18 (3H, *s*, Me-21), 0.94 (3H, *s*, Me-19), 0.86 (3H, *s*, Me-18). – ESI-TOF-MS (positive): *m/z* = 503.298 $[M+Na]^+$, 983.607 $[M_2+Na]^+$.

Leonoside B (**5**): ^{1}H NMR (400 MHz, CD_3OD): see Table I. – ESI-TOF-MS (positive): *m/z* = 807.27 $[M+Na]^+$, 823.24 $[M+K]^+$.

Martynoside (**6**): ^{1}H NMR (400 MHz, CD_3OD): see Table I. – ESI-TOF-MS (positive): *m/z* = 675.23 $[M+Na]^+$, 691.20 $[M+K]^+$.

Ajugalactone (**7**): This is the first report on NMR data of ajugalactone achieved in CD_3OD. ^{1}H NMR (400 MHz, CD_3OD): δ = 5.95 (1H, *J* = 2.56 Hz, H-7), 4.19 (1H, *dd*, *J* = 12.96, 4.16 Hz, H-22), 3.95 (1H, *br s*, H-3), 3.79 (1H, *m*, H-2), 3.55 (1H, *br t*, *J* = 10.9 Hz, H-9), 2.92 (1H, *br t*, *J* = 10.2 Hz, H-17), 2.79 (1H, *q*, H_a-11), 2.38 – 2.32 (2H, *m*, H-23, 28), 2.10 – 2.04 (1H, *m*, H_a-16), 1.76 – 1.72 (1H, *m*, H_b-16), 2.23 – 2.17 (1H, *m*, H_a-15), 1.85 (3H, *br s*, Me-18), 1.76 – 1.72 (1H, *m*, H_b-15), 1.64 (1H, *d*, *J* = 4.4 Hz, H_a-1), 1.47 (1H, *br s*, H_b-1), 1.25 (3H, *s*, Me-26), 1.21 (3H, *s*, Me-18), 1.12 (3H, *t*, *J* = 7.64 Hz, Me-29), 1.07 (3H, *s*, Me-19). – ^{13}C NMR (500 MHz, CD_3OD): δ = 202.1 (C-6), 123.7 (C-7), 162.2 (C-8), 210.2 (C-12), 89.4

Table I. ^{1}H NMR spectral data of **3**, **5**, and **6**[a].

H		**3**	**5**	**6**
Aglycone	H-C(2)	6.68 (*d*, *J* = 2.08)	6.72 (*d*, *J* = 2.08)	6.71 (*d*, *J* = 2.08)
	H-C(5)	6.66 (*d*, *J* = 7.92)	6.81 (*d*, *J* = 4.64)	6.81 (*d*, *J* = 4.4)
	H-C(6)	6.56 (*dd*, *J* = 8.12, 2.08)	6.68 (*dd*, *J* = 8.12, 2.08)	6.68 (*d*, *J* = 8.12, 2.12)
	$CH_2(\alpha)$	4.04, 3.77 – 3.23	4.08, 3.73 (*m*)	4.07, 3.77 – 3.35 (*m*)
	$CH_2(\beta)$	2.79 (*br t*, *J* = 6.96)	2.82 (*br t*, *J* = 7.44)	2.82 (*br t*, *J* = 7.4)
	CH_3 O	–	3.86 (*s*)	3.86 (*s*)
	CH_3 O	–	3.79 (*s*)	3.79 (s)
β-Glucose	H-C(1')	4.36 (*d*, *J* = 7.92)	4.37 (*d*, *J* = 8.12)	4.37 (*d*, *J* = 8.12)
	H-C(2')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C(3')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C(4')	4.92 (*t*)	4.94 (*t*)	4.92 (*t*)
	H-C(5')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C($6'_A$)	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C($6'_B$)	3.85 (*dd*, *J* = 12.96, 2.4)	3.85	3.77 – 3.35
α-Rhamnose	H-C(1'')	5.46 (*br s*)	5.47 (*d*, *J* = 1.4)	5.18 (*d*, *J* = 1.6)
	H-C(2'')	3.92 (*dd*, *J* = 3.24, 1.36)	3.93 (*br s*)	3.92 (*br s*)
	H-C(3'')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C(4'')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	H-C(5'')	3.77 – 3.23	3.70 – 3.47	3.77 – 3.35
	CH_3(6'')	1.05 (*d*, *J* = 6.28)	1.05 (*d*, *J* = 6.28)	1.08 (*d*, *J* = 6.24)
α-Arabinose	H-C(1''')	4.29 (*d*, *J* = 7.2)	4.30 (*d*, *J* = 7.44)	–
	H-C(2''')	3.77 – 3.23	3.70 – 3.47	–
	H-C(3''')	3.77 – 3.23	3.70 – 3.47	–
	H-C(4''')	3.77 – 3.23	3.75 (*br s*)	–
	H-C(5''')	3.77 – 3.23	3.70 – 3.47	–
Caffeic acid	H-C(2'''')	7.03 (*d*, *J* = 1.84)	7.18 (*d*, *J* = 1.8)	7.18 (*d*, *J* = 1.84)
	H-C(5'''')	6.76 (*d*, *J* = 8.12)	6.79 (*d*, *J* = 4.64)	6.79 (*d*, *J* = 4.64)
	H-C(6'''')	6.95 (*dd*, *J* = 8.36, 2.08)	7.07 (*dd*, *J* = 8.36, 1.88)	7.07 (*dd*, *J* = 8.36, 1.88)
	H-C(*α*')	6.27 (*d*, *J* = 15.8)	6.37 (*d*, *J* = 16)	6.37 (*d*, *J* = 16)
	H-C(*β*')	7.59 (*d*, *J* = 15.88)	7.66 (*d*, *J* = 16)	7.66 (*d*, *J* = 15.76)

[a] The spectra were measured in CD_3OD (400 MHz). Chemical shifts in ppm relative to the internal standard TMS; *J* in Hz.

(C-14), 83.3 (C-22), 168.2 (C-27), 154.8 (C-24), 121.5 (C-25). – ESI-TOF-MS (positive): m/z = 539.26 $[M+Na]^+$, 555.23 $[M+K]^+$, 1055.53 $[M_2+Na]^+$.

Makisterone A (**8**): ^{1}H NMR (400 MHz, CD_3OD): δ = 5.79 (1H, *d*, J = 2.5 Hz, H-7), 1.17 (3H, *s*, Me-21), 1.14 (3H, *s*, Me-26), 1.11 (3H, *s*, Me-27), 0.94 (3H, *s*, Me-19), 0.92 (3H, *d*, J = 6.72 Hz, Me-28), 0.87 (3H, *s*, Me-18). – ESI-TOF-MS (positive): m/z = 517.32 $[M+Na]^+$.

24-Dehydroprecyasterone (**9**): ^{1}H NMR (400 MHz, CD_3OD): δ = 5.80 (1H, *d*, J = 2.3 Hz, H-7), 1.83 (3H, *d*, J = 2.0 Hz, Me-26), 1.31 (3H, *s*, Me-21), 1.28 (3H, *d*, J = 6.4 Hz, Me-19), 0.94 (3H, *s*, Me-19), 0.87 (3H, *s*, Me-18). – ESI-TOF-MS (positive): m/z = 541.28 $[M+Na]^+$.

Cell culture

The colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), and breast ductal carcinoma (T47D) cell lines, respectively, were maintained as exponentially growing cultures in RPMI 1640 cell culture medium (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS; PAA) for HT-29 cells and 15% FBS for Caco-2 and T47D cells. The Swiss mouse embryo fibroblast (NIH 3T3) cell line was kept in Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 10% FBS. One hundred IU/mL penicillin and 100 μg/mL streptomycin (Roche, Penzberg, Germany) were added to the media. All cell lines were cultured at 37 °C in air/CO_2 (95:5 v/v) atmosphere.

Determination of cell viability by the MTT assay

Cytotoxic activities of the *n*-butanolic fraction, *cis*- and *trans*-melilotoside from *Ajuga chamaecistus* ssp. *tomentella* against breast ductal carcinoma (T47D), colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), and Swiss mouse embryo fibroblast (NIH 3T3) cell lines were performed according to our previous study (Khanavi *et al*., 2010) by the mitochondrial tetrazolium test (MTT).

Results and Discussion

Isolated compounds **1**–**9** from the *n*-butanolic fraction of the total methanolic extract of aerial parts of *Ajuga chamaecistus* ssp. *tomentella* were identified by comparison of their NMR (^{1}H, ^{13}C NMR, HMBC, HSQC and ^{1}H-^{1}H COSY) and ESI-mass spectral data with those reported in the literature.

Compounds **1** and **2** (Fig. 1) were identified as *cis*-melilotoside and *trans*-melilotoside, respectively. This is the first report on the occurrence of *cis*- and *trans*-melilotosides in the genus *Ajuga*. Melilotoside is a coumaric acid derivative which was reported for the first time from *Melilotus altissima* and *M. arvensis* (Takaishi, 1968). *cis*-Melilotoside has been reported from several plants (Ferreira and Rodriguezde Oliveira, 2010; Yang *et al*., 2007a, b), also there is an older report on the occurrence of both *cis*- and *trans*-forms of melilotoside in *Melilotus albus* depending on growing conditions (Kahnt, 1962). Antiprotozoal activity of melilotoside isolated from *Teloxys graveolens*, a medicinal plant for treatment of dysentery and diarrhea, has been shown (Calzada *et al*., 2003).

Compounds **3**, **5**, and **6** (Fig. 1) were characterized as phenylethanoid glycosides, lavandulifolioside (**3**), leonoside B (**5**), and martynoside (**6**), by comparison of their spectral data with literature values (Basaran *et al*., 1988; Calis *et al*., 1992; Sasaki *et al*., 1978). This group of phenolic compounds has interesting biological properties such as antimicrobial, antibacterial, cytotoxic, antioxidant, enzyme inhibitory, and immunomodulatory. Lavandulifolioside, a trisaccharide phenylethyl glycoside, showed inhibition of peroxylipid formation (Jimenez and Riguera, 1994), a negative chronotropic effect, and decrease of blood pressure (Milkowska-Leyck *et al*., 2002). Several pharmacological activities of martynoside have been reported, including antioxidant (Miao *et al*., 2003) and estrogenic/antiestrogenic properties in breast cancer cells (Papoutsi *et al*., 2006).

The isolated compounds **4** and **7**–**9** (Fig. 1) were identified as ecdysteroids, 20-hydroxyecdysone (**4**), ajugalactone (**7**), makisterone A (**8**), and 24-dehydroprecyasterone (**9**) by comparison of their spectral data with data in the literature (Wessner *et al*., 1992; Imai *et al*., 1968, 1970). The ecdysteroids are a large class of polyhydroxysteroids isolated from both the animal and plant kingdom. Most of the *Ajuga* species have been used in traditional medicine all over the world. Several studies have shown that ecdysteroids isolated from *Ajuga* species are responsible for their biological activities. This group of natural products produces a wide range of pharmacological

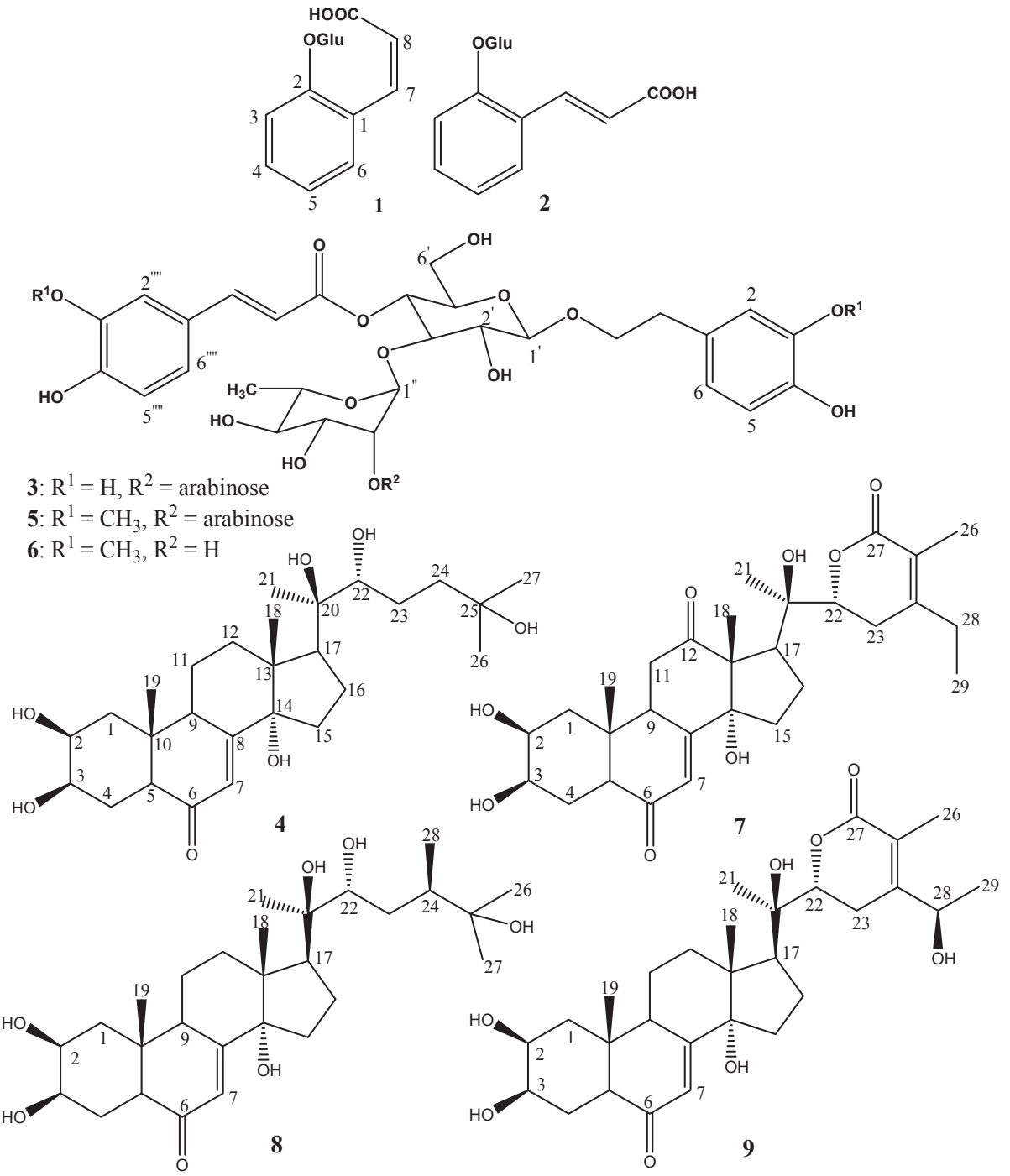

Fig. 1. Molecular structures of *cis*-melilotoside (**1**), *trans*-melilotoside (**2**), lavandulifolioside (**3**), 20-hydroxyecdysone (**4**), leonoside B (**5**), martynoside (**6**), ajugalactone (**7**), makisterone A (**8**), and 24-dehydroprecyasterone (**9**), isolated from the *n*-butanolic fraction of *Ajuga chamaecistus* ssp. *tomentella*.

activities in mammals, including adaptogenic and anabolic, and shows increasing protein synthesis in muscles (Gorelick-Feldman *et al*., 2010), antidiabetic and hypoglycemic (Hamden *et al*., 2008; Kutepova *et al*., 2001), hepatoprotective, immunoprotective, wound-healing (Dinan, 2009), antioxidant, and free radical scavenging effects (Cai *et al*., 2002), and perhaps even antitumour effects (Akbay *et al*., 2002). 20-Hydroxyecdysone and cyasterone, in addition to ajugalactone, seem to be the most common compounds in *Ajuga* species (Ramazanov, 2005).

In the cytotoxic evaluation of the *n*-butanolic fraction, *cis*- (**1**) and *trans*-melilotosides (**2**) did not show cytotoxic effects up to 400 μg/mL against cancer (T47D, HT-29, and Caco-2) and normal (NIH 3T3) cell lines in the MTT assay.

Previous to this study, we isolated three major compounds (20-hydroxyecdysone, cyasterone, and 8-acetylharpagide) from the diethyl ether fraction of *Ajuga chamaecistus* ssp. *tomentella*, which were inactive in the cytotoxicity evaluation (Sadati *et al*., 2012)

In conclusion, our study showed that *cis*- and *trans*-melilotosides, phenylethyl glycosides, and phytoecdysteroids can be considered major constituents of the *n*-butanolic fraction of *Ajuga chamaecistus* ssp. *tomentella*. According to these results, it can be stated that the *n*-butanolic fraction and two major compounds isolated from this fraction are not cytotoxic against cancer and normal cell lines.

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