Major Constituents and Cytotoxic Effects of
Ajuga chamaecistus ssp. tomentella

Nargess Sadati\(^a\), Kristina Jenett-Siems\(^b\), Karsten Siems\(^c\), Mohammad Reza Shams Ardekan\(^i\), Abbas Hadjiakhoondi\(^d\), Tahmineh Akbarzadeh\(^d\), Seyed Nasser Ostad\(^e\), and Mahnaz Khanavim*\(^f\)

\(^a\) 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

Z. Naturforsch. 67c, 275 – 281 (2012); received May 29, 2011/January 8, 2012

The \(n\)-butanolic fraction of a methanolic extract (80%) from aerial parts of \textit{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 \textit{cis}\-melilotoside (1), \textit{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 \textit{cis}- and \textit{trans}-melilotoside in \textit{Ajuga} species. Cytotoxic evaluation of the \(n\)-butanolic fraction, \textit{cis}- and \textit{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 \(\mu\)g/mL. The results of this study suggest that melilotoside, phenylethyl glycosides, and phytoecdysteroids are the main constituents of the \(n\)-butanolic fraction of \textit{Ajuga chamaecistus} ssp. tomentella.

Key words: \textit{Ajuga chamaecistus} ssp. tomentella, Melilotoside, Cytotoxic Effect

Introduction

More than one hundred species including fifty varieties and subspecies of \textit{Ajuga} (Lamiaceae) are distributed throughout the world. The genus \textit{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 \textit{Ajuga chamaecistus} contains several exclusive subspecies, including \textit{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 \textit{et al}., 2005). A broad range of biological effects has been reported from different species of \textit{Ajuga} such as hypoglycemic (Hilaly and Lyoussi, 2002), treatment of joint disease (Ono \textit{et al}., 2008), anti-inflammatory (Gautam \textit{et al}., 2011), and antimalarial (Kuria \textit{et al}., 2001). Many phytochemical studies on \textit{Ajuga} species were performed which led to the isolation of phytoecdysteroids (Vanyolos \textit{et al}., 2009; Castro \textit{et al}., 2008), diterpenoids (Coll, 2002), iridoids (Manguro \textit{et al}., 2007), and phenylethyl glycosides (Akbay \textit{et al}., 2003). The aim of the present study was a phytochemical investigation of the \(n\)-butanolic fraction obtained from aerial parts of \textit{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.

© 2012 Verlag der Zeitschrift für Naturforschung, Tübingen · http://znaturforsch.com
Material and Methods

General experimental procedures

$^1$H and $^{13}$C NMR spectroscopy of compounds 1 and 2 were performed in CD$_3$OD 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$_3$OD on a Jeol ECX-400 spectrometer (Peabody, MA, USA) (400 MHz, TMS as internal standard). $^1$H- and $^1$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, lipoophilic 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-butanol 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 (B3.1–B3.9). 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$_3$OD): 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$_3$OD): 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$_3$OD): 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
Lavandulifolioside (3): $^1$H NMR (400 MHz, CD$_3$OD): see Table I. – ESI-TOF-MS (positive): $m/z = 779.2384$ [M+Na]$^+$. 

20-Hydroxyecdysone (4): $^1$H NMR (400 MHz, CD$_3$OD): $\delta = 5.79$ (1H, $d$, $J = 2.3$ Hz, H-7), 3.95 (1H, $br$ $s$, H$_{a3}$-3), 3.82 (1H, $m$, H$_{a3}$-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$^+$+Na]$^+$. 

Leonoside B (5): $^1$H NMR (400 MHz, CD$_3$OD): see Table I. – ESI-TOF-MS (positive): $m/z = 807.27$ [M+Na]$^+$, 823.24 [M+K]$^+$. 

Table I. $^1$H NMR spectral data of 3, 5, and 6$^*$. 

<table>
<thead>
<tr>
<th>H</th>
<th>3</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aglycone</td>
<td>H-C(2)</td>
<td>6.68 (d, $J = 2.08$)</td>
<td>6.72 (d, $J = 2.08$)</td>
</tr>
<tr>
<td>H-C(5)</td>
<td>6.66 (d, $J = 7.92$)</td>
<td>6.81 (d, $J = 4.64$)</td>
<td>6.81 (d, $J = 4.4$ )</td>
</tr>
<tr>
<td>H-C(6)</td>
<td>6.56 (dd, $J = 8.12, 2.08$)</td>
<td>6.68 (dd, $J = 8.12, 2.08$)</td>
<td>6.68 (d, $J = 8.12, 2.12$)</td>
</tr>
<tr>
<td>CH$_3$(r)</td>
<td>4.04, 3.77 – 3.23</td>
<td>4.08, 3.73 (m)</td>
<td>4.07, 3.77 – 3.35 (m)</td>
</tr>
<tr>
<td>CH$_3$(β)</td>
<td>2.79 ($br$ $t$, $J = 6.96$)</td>
<td>2.82 ($br$ $t$, $J = 7.44$)</td>
<td>2.82 ($br$ $t$, $J = 7.4$)</td>
</tr>
<tr>
<td>CH$_3$O</td>
<td>-</td>
<td>3.86 (s)</td>
<td>3.86 (s)</td>
</tr>
<tr>
<td>CH$_3$O</td>
<td>-</td>
<td>3.79 (s)</td>
<td>3.79 (s)</td>
</tr>
<tr>
<td>β-Glucose</td>
<td>H-C(1')</td>
<td>4.36 (d, $J = 7.92$)</td>
<td>4.37 (d, $J = 8.12$)</td>
</tr>
<tr>
<td>H-C(2)</td>
<td>3.77 – 3.32</td>
<td>3.70 – 3.47</td>
<td>3.77 – 3.35</td>
</tr>
<tr>
<td>H-C(3)</td>
<td>3.77 – 3.32</td>
<td>3.70 – 3.47</td>
<td>3.77 – 3.35</td>
</tr>
<tr>
<td>H-C(4)</td>
<td>4.92 (t)</td>
<td>4.94 (t)</td>
<td>4.92 (t)</td>
</tr>
<tr>
<td>H-C(5)</td>
<td>3.77 – 3.32</td>
<td>3.70 – 3.47</td>
<td>3.77 – 3.35</td>
</tr>
<tr>
<td>H-C(6_a)</td>
<td>3.77 – 3.32</td>
<td>3.70 – 3.47</td>
<td>3.77 – 3.35</td>
</tr>
<tr>
<td>H-C(6_b)</td>
<td>3.85 (dd, $J = 12.96, 2.4$)</td>
<td>3.85</td>
<td>3.77 – 3.35</td>
</tr>
</tbody>
</table>

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 – 3.32 | 3.70 – 3.47 | 3.77 – 3.35 |
| H-C(4) | 3.77 – 3.32 | 3.70 – 3.47 | 3.77 – 3.35 |
| H-C(5) | 3.77 – 3.32 | 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 – 3.32 | 3.70 – 3.47 | - |
| H-C(3') | 3.77 – 3.32 | 3.70 – 3.47 | - |
| H-C(4') | 3.77 – 3.32 | 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(3') | 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(a') | 6.27 (d, $J = 15.8$) | 6.37 (d, $J = 16$) | 6.37 (d, $J = 16$) |
| H-C(b') | 7.59 (d, $J = 15.88$) | 7.66 (d, $J = 16$) | 7.66 (d, $J = 15.76$) |

* The spectra were measured in CD$_3$OD (400 MHz). Chemical shifts in ppm relative to the internal standard TMS; $J$ in Hz.
Determination of cell viability by the MTT assay
ded IU/mL penicillin and 100
PAA) supplemented with 10% FBS. One hun-
the media. All cell lines were cultured at 37
Cin (Roche, Penzberg, Germany) were added to
Dulbecco’s modifi ed Eagle’s medium (DMEM;
Austria) supplemented with 10% fetal bovine se-
RPMI 1640 cell culture medium (PAA, Pasching,
maintained as exponentially growing cultures in

Cell culture
The colon carcinoma (HT-29), colorectal ad-
enocarcinoma (Caco-2), and breast ductal car-
cinoma (T47D) cell lines, respectively, were
maintained as exponentially growing cultures in
RPMI 1640 cell culture medium (PAA, Pasching,
Austria) supplemented with 10% fetal bovine se-
RPMI 1640 cell culture medium (PAA, Pasching,
maintained as exponentially growing cultures in

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 identifi ed by comparison of their NMR (1H, 13C
NMR, HMBC, HSQC and 1H-1H COSY) and

Compounds 1 and 2 (Fig. 1) were identi-
ced as cis-melilotoside and trans-melilotoside,
respectively. This is the fi rst report on the occur-
cence of cis- and trans-melilotosides in the genus
Ajuga. Melilotoside is a coumaric acid deriva-
tive which was reported for the fi rst time from
Melilotus altissima and M. arvensis (Takaishi,
1968). cis-Melilotoside has been reported from
several plants (Ferreira and Rodriguez-Olivei-
ra, 2010; Yang et al., 2007a, b), also there is an old-
er report on the occurrence of both cis- and trans-
forms of melilotoside in Melilotus altissimus depending
on growing conditions (Kahnt, 1962). Antiproto-
zoal 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 charac-
terized as phenylethanoid glycosides, lavandulifo-
loside (3), leonoside B (5), and martynoside (6),
by comparison of their spectral data with litera-
ture values (Basaran et al., 1988; Calis et al., 1992;
Sasaki et al., 1978). This group of phenolic com-
pounds has interesting biological properties such
as antimicrobial, antibacterial, cytotoxic, antioxi-
dant, enzyme inhibitory, and immunomodulatory.
Lavandulifolioside, a trisaccharide phenylethyl
glycoside, showed inhibition of peroxylipid for-
mation (Jimenez and Riguera, 1994), a negative
chronotropic effect, and decrease of blood pres-
sure (Mlkowska-Leyck et al., 2002). Several phar-
macological 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 identifi ed as ecdysteroids, 20-hydroxy-
edysone (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 polyhydroxy-
steroids 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 iso-
lated from Ajuga species are responsible for their
biological activities. This group of natural pro-
ducts produces a wide range of pharmacological
Fig. 1. Molecular structures of *cis*-melilotoside (1), *trans*-melilotoside (2), lavandulifolioside (3), 20-hydroxyecdysone (4), leoniside B (5), martynoside (6), ajugalactone (7), makisterone A (8), and 24-dehydroprecyasterone (9), isolated from the *n*-butanolic fraction of *Ajuga chamaecictus* ssp. *tomentella*. 

3: R\(^1\) = H, R\(^2\) = arabinose
5: R\(^1\) = CH\(_3\), R\(^2\) = arabinose
6: R\(^1\) = CH\(_3\), R\(^2\) = H
activities in mammals, including adaptogenic and anabolic, and shows increasing protein synthesis in muscles (Gorelick-Feldman et al., 2010), anti-diabetic 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-Hydroxyecdyson 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-hydroxyecdyson, 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.

Acknowledgements

This study was part of a PhD thesis funded and supported by Tehran University of Medical Sciences (TUMS) (grant No. 11302).


