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Antiproliferative, antimicrobial and antioxidant activities of the chemical constituents of *Ajuga turkestanica*

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Abstract

Ajuga turkestanica Rgl. Brig (Lamiaceae) is a medicinal plant from Uzbekistan. Methanol, chloroform, butanol, and water extracts as well as isolated phytoecdysteroids and iridoids were evaluated for their antioxidant, cytotoxic and antibacterial activities. Water and butanol extracts exhibited good antioxidant activity with IC_{50} values of 7.24 ± 0.82 and 14.57 ± 1.64 µg/mL. The chloroform extract showed potent cytotoxic effects against the cancer cell lines HeLa, HepG-2, and MCF-7 with IC_{50} values of 7.13 ± 0.85, 9.03 ± 0.92, and 10.77 ± 1.44 µg/mL, respectively. Compared to the extracts, isolated phytoecdysteroids and iridoids showed weak cytotoxic activity. The chloroform extract has antimicrobial properties even against multiresistant strains like *Staphylococcus aureus* MRSA 1000/93 and *Streptococcus pyogenes* ATCC 12344. The methanol and chloroform extracts of *A. turkestanica* were further investigated for their GLC-volatile components using GLC/FID and GLC/MS. Pregna-4,9 (11)-dien-20-ol-3-on-19-oic acid lactone (19.58%), 20-methyl-pregna-5,17-dien-3β-ol (12.93%), 3,7-dioxocholan-24-oic acid (10.53%) and betulin (10.18%) were detected as the major compounds.

Keywords: *Ajuga turkestanica;* phytoecdysteroids; iridoids; HPLC; GLC; activity

Introduction

More than *45* species of the genus *Ajuga* L. (Lamiaceae) are found in temperate regions of the Old World and have been used in folk medicine because of their anthelmintic, antifungal, hypoglycemic, antitumor, and antimicrobial properties (Mabberley, 2008; Israili et al., 2009). Plants of the genus *Ajuga* produce a variety of biological active secondary meta-

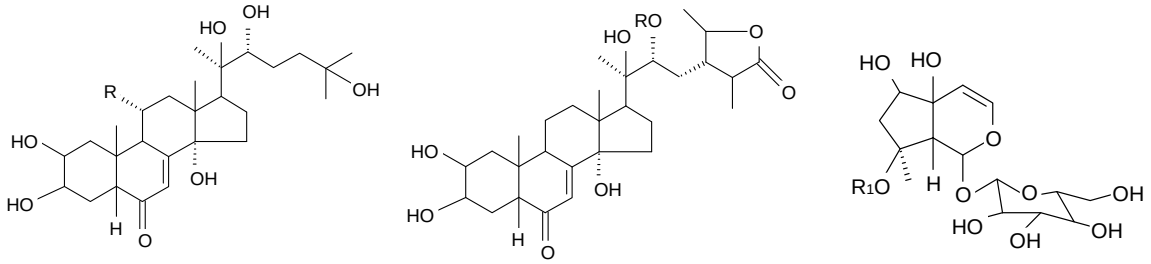
bolites including phytoecdysteroids, iridoids, neoclerodane diterpenoids, sterols, withanolides, anthocyanins, flavonoids, ionones, and quinones (Turkoglu et al., 2010).

In the flora of Uzbekistan, *Ajuga* is represented by two species: *Ajuga genevensis* L., and *Ajuga turkestanica* Rgl. Brig. (Sokolov et al., 1991). *A. turkestanica* is an endemic perennial plant and grows in areas at 600-1000 m above sea level in Southern Pamir-Alay mountains on Southwest slobes of the Hissar Mountain (Ganiev et al., 1990; Sokolov et al., 1991). *A. turkestanica* has *been* widely used *in* folk medicine for enhancement of muscular strength, against heart disease, muscle and stomach aches (Grace et al., 2008).

A. turkestanica produces a rich amount of bioactive phytoecdysteroids: 20-hydroxyecdysone (0.25% of dry weight), turkesterone (0.22%) (Usmanov et al., 1973, 1975; Abdukadyrov et al., 2005), cyasterone (Usmanov et al., 1971), 22-acetylcyasterone (Usmanov et al., 1978), ajugalactone (Saatov et al., 1977), ajugasterone B (Usmanov et al., 1977), α-ecdysone, ecdysone 2,3-monoacetonide (Saatov et al., 1999). Further secondary metabolites are iridoid glucosides, such as harpagide, 8-*O*-acetylharpagide (Kotenko et al., 1994) and carbohydrates (Abdukadyrov et al., 2004). From aerial parts six neo-clerodane diterpenoids were isolated: 14,15-dihydroajugachin B, 14-hydro-15-methoxyajugachin B, chamaepitin, ajugachin B, ajugapitin, and lupulin A (Grace et al., 2008).

The phytoecdysteroids show low *in vivo* toxicity to vertebrates (LD_{50} values of 20-hydroxyecdysone is 6.4 g/kg and >9 g/kg, i.p. and p.o. to mice). Since ecdysteroids function as moulting hormones in insects, they can be considered as natural insecticides. Some phytoecdysteroids strengthened lactation especially in conditions of hypolactation (Khalitova et al., 1998), and possess the hypoglycemic activity (Kutepova et al., 2001). The iridoids harpagide (5) and 8-*O*-acetylharpagide (6) promote bile secretion (Syrov et al., 1986). These iridoids can be found in many other plants and have been used in phytomedicine against inflammation, pain, and microbial infections (Van Wyk and Wink, 2004).

In the present study, we report on the chemical composition of polar and non-polar extracts and the antimicrobial, antioxidant and cytotoxic activities of root extracts (methanol, chloroform, butanol and water) from *A. turkestanica* in comparison to four isolated phytoecdysteroids 20-hydroxyecdysone (syn. ecdysterone) (**1**), turkesterone (**2**), cyasterone (**3**), 22-acetylcyasterone (**4**), and two iridoid glucosides harpagide (**5**), 8-*O*-acetylharpagide (**6**) (Fig. 1).



1 R=H
2 R=OH

3 R=H
4 R=Ac

5 R=H
6 R=Ac

Figure 1. Chemical structures of the phytoecdysteroids and iridoids from *A. turkestanica.*

Materials and methods

Plant material

Roots of *A. turkestanica* were collected in the Surkhan-Darya region of Uzbekistan in the summer of 2009 and identified at the Department of Herbal Plants (Institute of the Chemistry of Plant Substances, Uzbekistan) by Dr. O.A. Nigmatullaev (voucher specimen number 20077092).

Preparation of A. turkestanica extracts

Roots were air-dried at room temperature before grinding them to a powder with a Warring blender. After grinding, 100 g of plant material was extracted with solvents of methanol, chloroform, butanol and water; yields of these extracts were 5.1, 1.6, 4.2 and 8.3%, respectively (from the weight of the air dried roots). Extraction with each solvent was carried out for one day at room temperature. The solvent was evaporated in a rotary vacuum evaporator at 40 °C. The extracts were then kept under refrigerated conditions until further use.

Chemicals and reagents

Cell culture media, supplements, and dimethylsulfoxide (DMSO) were purchased from Roth (Karlsruhe, Germany) and Greiner Labortechnik (Frickenhausen, Germany). Doxorubicin (≥ 98 %) and quercetin (≥ 98 %) were obtained from Gibco (Invitrogen, Karlsruhe, Germany). Authentical phytoecdysteroids and iridoids were obtained from the Institute of the Chemistry of Plant Substance, Tashkent, Uzbekistan. The purity of the tested compounds were > 95 %, as determined by HPLC.

HPLC analysis

The contents and quantity of the phytoecdysteroids and iridoids from the roots of *A. turkestanica* were investigated by HPLC. Chromatographic profiles of *A. turkestanica* extracts were generated using a high performance liquid chromatograph LC-10ATvp connected to a UV-VIS detector SPD-10Avp (Shimadzu Co, Kyoto, Japan). *A. turkestanica* extracts were diluted to 1 mg/ml, filtered through 0.22 µm and 20 µl were injected. For separation of these extracts, a Nucleosil 100-5 C18 column with a size 250 mm × 4 mm (Macherey-Nagel GmbH & Co, KG) was used. Elution was carried out by a mobile phase consisted of A (water) and solvent B (acetonitrile) and the gradient profile was as follows: from 0% B to 5% B in 8 min, from 5% B to 85% B at 8-30 min, from 95% B to 100% B% at 30-35 min and at 100% B% until 40 min. Flow rate was 1 ml/min and detection was at 247 nm and 200 nm (Abdukadyrov et al., 2005). The quantifications of 20-hydroxyecdysone (**1**), turkesterone (**2**), cyasterone (**3**), harpagide (**5**) and 8-*O*-acetylharpagide (**6**) in the extracts of *A. turkestanica* were carried out using a calibration curve of corresponding standards at different concentrations.

GLC/FID analysis

High-resolution GLC analyses were carried out on a Focus GC (Thermo Fisher Scientific, Milan, Italy) equipped with TR1-MS fused bonded column (30 m × 0.25 mm × 0.25 µm) (Thermo Fisher Scientific®, Florida, USA) and FID detector; carrier gas was nitro-

gen (1.5 ml/ min). The operating conditions were: initial temperature 40 ºC, for 1 min isothermal followed by linear temperature increase till 230 ºC at a rate of 4 ºC/min 230 ºC, then 5 min isothermal. Detector and injector temperatures were 300 ºC and 220 ºC, respectively. The split ratio was 1:20. Chrom-card® chromatography data system ver. 2.3.3 (Thermo Electron Corp®, Florida, USA) was used for recording and integrating of the chromatograms.

GLC/MS analysis

The analyses were carried out on Focus GC (Thermo Fisher Scientific, Milan, Italy) equipped with the same column and conditions mentioned for GLC/FID. The capillary column was directly coupled to a quadrupole mass spectrometer Polaris Q (Thermo Electron Corp®, Milan, Italy).The injector temperature was 220 ºC. Helium carrier gas flow rate was 1.5 ml/min. All the mass spectra were recorded under the following conditions: filament emission current, 100 mA; electron energy, 70 eV; ion source, 250 ºC; diluted samples were injected with split mode (split ratio, 1:15). Compounds were identified by comparison of their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), our own laboratory database and the literature (Adams, 2004; Budzikiewicz et al., 1964; Nibret and Wink, 2010).

Antioxidant activity

DPPH radical-scavenging activity*

The antioxidant and radical scavenging activities of the isolated compounds and extracts were evaluated according to Brandwilliams et al. (1995) using diphenyl picryl hydrazyl (DPPH*). Equal volumes of sample solutions containing 0.02–10 mg/mL of the samples and 0.2 mM methanolic solution of DPPH* were pipetted into 96-well plates. The absorbance was measured against a blank at 517 nm using a Tecan Safire II Reader after incubation in the dark for 30 min at room temperature compared with DPPH* control after background subtraction. Quercetin was used as a positive control. The percent inhibition was calculated from three different experiments using the following equation:

$$\text{RSA (\%)} = [(\text{Abs}_{517\text{control}} - \text{Abs}_{517\text{sample}})/ \text{Abs}_{517\text{control}}] \times 100$$

where RSA = radical scavenging activity; Abs_{517} = absorption at 517 nm; control = non-reduced DPPH*.

Cytotoxicity studies

Cell cultures

HeLa (cervical cancer), HepG-2 (hepatic cancer), and MCF-7 (breast cancer) human cell lines were maintained in DMEM complete media (L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin) in addition to 10 mM non-essential amino acids. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Cytotoxicity assay

Sensitivity of the cancer cells to drugs was determined in triplicate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Mosmann, 1983). The extracts and individual substances were dissolved in dimethylsulfoxide (DMSO) and further serially diluted with the medium in two-fold fashion into ten different concentrations so as to attain final concentrations ranging from 0.977 to 500 µg/mL for extracts and from 0.977 to 500 µM for isolated substances, in 96-well plates; each well contained 100 µL medium. The concentration of the solvent, DMSO, did not exceed 0.05% in the medium that contained the highest concentration of extract or compound tested. Wells containing the solvent and wells without the solvent were included in the experiment. Exponentially growing cells were seeded in a 96-well plate (2×10^4 cells/well), the cells were cultivated for 24 h and then incubated with various concentrations of the serially diluted tested samples at 37 °C for 24 h and then with 0.5 mg/mL MTT for 4 h. The formed formazan crystals were dissolved in 100 µL DMSO. The absorbance was detected at 570 nm with a Tecan Safire II Reader. The cell viability rate (%) of three independent experiments was calculated by the following formula:

Cell viability rate (%) = ((OD of treated cells − OD of media (blank) / (OD of control cells − OD of media (blank)) × 100 %

where OD = optical density

Antimicrobial activity

Test microorganisms

The antimicrobial activity was evaluated against standard strains which included Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* MRSA ATCC 10442, vancomycin- resistant *Enterococcus VanB* VRE ATCC 31299 and *Streptococcus pyogenes* ATCC 12344, two clinical isolates *Staphylococcus aureus* MRSA 1000/93 and *Enterococcus VanB* VRE 902291, Gram-negative bacteria such as *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853, and yeasts such as *Candida albicans* ATCC 90028, and *Candida glabrata* ATCC MYA 2950. All microorganism cultures were supplied by Medical Microbiology Laboratory, Hygiene Institute, Heidelberg University, Germany.

Culture media

Columbia with 5% sheep blood (BD) and Mueller-Hinton Broth (MHB) (Fluka) were used in bacterial tests. All bacterial cultures were incubated at 37 °C for 24 h. CHROM agar Candida (BD) and Sabouraud Dextrose broth (SDB) (Oxid) were used in fungal tests. All fungal cultures were incubated at 25 °C for 48 h.

Inoculum preparation

One or two bacterial or fungal colonies from an 18-24 h agar plate were suspended in saline to a turbidity matching 0.5 McFarland $\approx1\times10^8$ CFU/mL; 1:100 dilution was performed

from this suspension using 900 µL broth to get 1×10^6 CFU/mL.

Diffusion method

1×10^6 CFU/mL of bacterial and fungal suspensions was spread on Colombia with 5% sheep blood and CHROM agar Candida, respectively. Wells with diameter of 6 mm were cut off and delivered with 40 µL of each extract (40 mg/ml) and of each pure substance (0.5 mM). DMSO, ampicillin (1 mg/mL), vancomycin (1 mg/mL), and nystatin (1 mg/mL) were used as controls. All the plates were observed for zone of inhibition at 37 °C for 24 h (bacteria) and at 25 °C for 48 h (yeasts).

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)

Microdilution method was used to determine MIC as described by NCCLS (2006). Plant extracts were first of all dissolved in DMSO 5% to concentration of 8 mg/8 mg/ml and the pure substances to the concentration of 1 mM and then were diluted two-fold with MHB (bacteria) and SDB (fungi) in 96-well plates to obtain a range of concentrations between (8-0.015 mg/mL) for plant extracts, and between (1000 and 1.5 µM) for pure substances. The bacterial and fungal suspensions of 1×10^6 CFU/mL were subsequenly added and the plates were incubated at 37 °C for 24 h (bacteria) and at 25 °C for 48 h (yeasts). MIC was defined as the first concentration did not give visible turbidity comparing to a negative control. Each test was performed in duplicate for each extract and substance. 3 µL of each clear well was inoculated in appropriate agar media and incubated in the appropriate conditions. MMC was determined as the concentration that did not yield growth on agar after incubation.

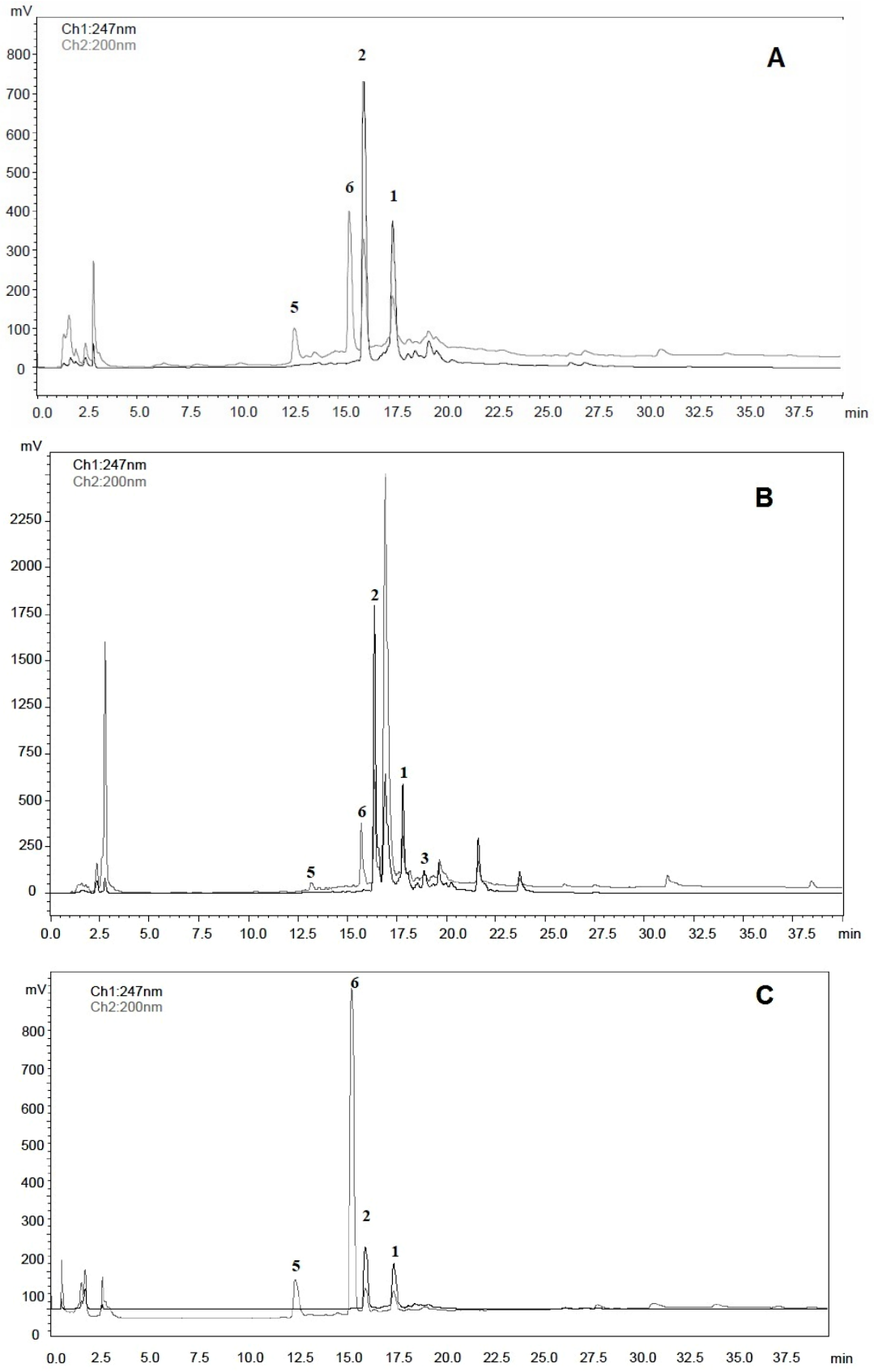
Statistical analysis

All experiments were carried out three times unless mentioned in the text. Continuous variables were presented as mean ± SD. IC_{50} values were calculated using a four parameter logistic curve (SigmaPlot 11.0) and all the data were statistically evaluated using Student's t-test or the Kruskal–Wallis test (GraphPad Prism 5.01; GraphPad Software, Inc., San Diego, USA) followed by Dunn's post-hoc multiple comparison test when the significance value is <0.05 using the same significance level.

Results

HPLC analysis of the extracts of A. turkestanica

Chromatographic profiles for butanol, methanol, chloroform and water extracts were generated by HPLC (Fig. 2). HPLC analysis revealed the presence of phytoecdysteroids and iridoids as the most abundant metabolites. The following phytoecdysteroids and iridoids could be identified unequivocally: 20-hydroxyecdysone (**1**) (t_R=17.7 min), turkesterone (**2**) (t_R=16.7 min), cyasterone (**3**) (t_R=19.0 min), harpagide (**5**) (t_R=12.7 min), and 8-*O*-acetylharpagide (**6**) (t_R=15.6 min) (Fig. 2). The composition of the extracts is reported in Table 1.



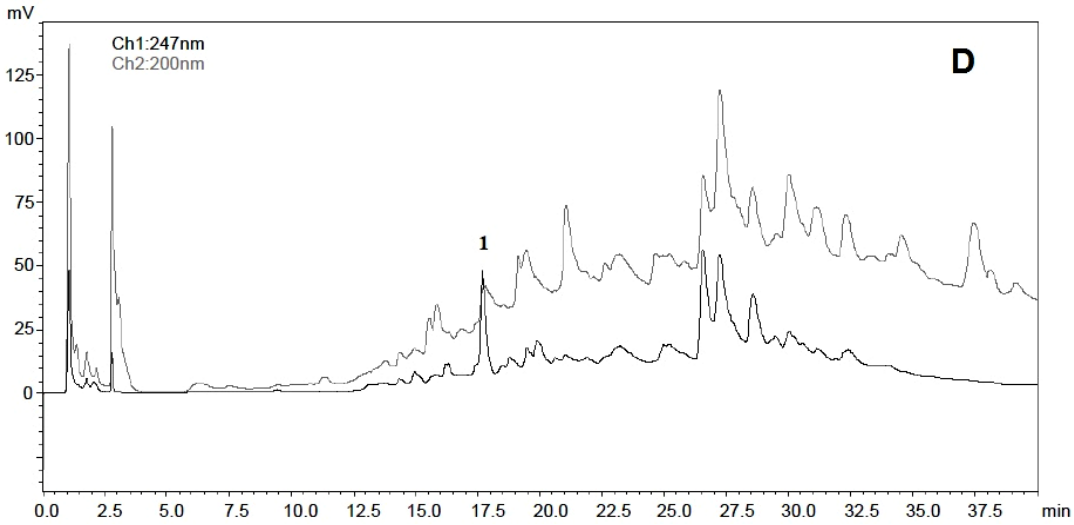


Figure 2 Representative HPLC chromatogram of *A. turkestanica* (**A**) methanol, (**B**) butanol, (**C**) water and (**D**) chloroform extracts. Peaks correspond to: **1:** 20-hydroxyecdysone, **2:** turkesterone, **3:** cyasterone, **5:** harpagide, **6**: 8-*O*-acetylharpagide.

Table 1**.** Composition of water, methanol, butanol and chloroform extracts from *A. turkestanica*

Compound	Retention time (t_R, min)	Content of extracts (mg/ml)			
		Water	Butanol	Methanol	Chloroform
20-Hydroxyecdysone (1)	17.7	0.01	0.065	0.046	0.034
Turkesterone (2)	16.7	0.012	0.193	0.114	n/d
Cyasterone (3)	19.0	n/d	0.027	n/d	n/d
Harpagide (5)	12.7	0.061	0.016	0.049	n/d
8-*O*-Acetylharpagide (6)	15.6	0.148	0.098	0.125	0.002

n/d – not determined

Table 2. Identified compounds in chloroform extracted volatiles of the roots of *A. turkestanica.*

N	Retention time (min)	Compound name	Retention index[a] (RI)	Abundance [b] %
1	36.36	1,2,7,8,8a,9,10,10a-Octahydro-2,2,7,7-tetramethylphenanthrene	2045	0.58
2	38.06	4β-18-Norkaur-16-ene	2144	0.66
3	39.11	Abieta-9(11),8(14),12-trien-12-ol (Ferruginol)	2205	0.24
4	40.43	Unknown	2280	0.47
5	40.51	Abieta-6,8,11,13-tetraen-12-yl acetate	2286	0.41
6	40.63	Totarol	2293	0.36
7	40.89	Stigmast-5-en-3-ol (β-Sitosterol)	2308	0.33
8	42.32	16α,17-Epoxypregn-4-ene-3,20-dione*	2391	0.57
9	42.49	3,17-Dihydroxypregn-5-en-20-one	2401	0.76
10	43.42	Unknown	2455	0.23
11	44.64	20-Methyl-pregna-5,17-dien-3β-ol	2526	12.93
12	44.68	Unknown	2537	0.78
13	46.81	16-Dehydropregnenolone	2542	2.19
14	45.00	Pregnane-3,11,20-trione*	2550	0.98
15	48.89	5-Pregnen-3β-ol-7,20-dione*	2582	0.19
16	49.83	3,7-Dioxocholan-24-oic acid*	2587	10.53
17	50.91	Pregna-4,9 (11)-dien-20-ol-3-on-19-oic acid	2673	19.58
18	51.55	Ajuforrestine A*	2731	3.33
19	52.19	11b-Hydroxy-3,11a-dimethyl-1,9-dioxo-3a,4,5,5a,5b,9,10,11,11a,11b,12,13-dodecahydro-3H-naphtho[2',1':4,5]indeno[1,7a-c]furan-12-yl acetate*	2933	1.55
20	52.39	4,18-Epoxy-6,19-dihydroxy-13-cleroden-15,16-olide-19-	3103	0.98

		acetate*		
21	53.68	Stigmast-4-en-3-one	3206	3.34
22	53.74	Unknown	3225	0.83
23	53.97	4,4-Dimethylcholesta-7,9(11)-dien-3-ol	3283	8.43
24	54.18	Olean-12-en-3-one	3370	1.76
25	54.65	Betulin	>3400	10.18
26	55.42	Barrigenol	>3400	4.37

[a] - the Kovats index was calculated on TR1-MS column; [b] - average of three analyses; * - tentatively identified

GLC analysis

The chloroform extract was analysed in more detail because of its pronounced biological activities. Results of the GLC analysis of the chloroform extract are presented in Table 2 and Fig. 3. A total of 22 components were identified in this extract. The most abundant components were mainly sterols and oxo steroids, and triterpenes. Furthermore, abieta-, nor- and cleroden diterpenes; meroterpene and polycyclic aromatic hydrocarbons: pregna-4,9 (11)-dien-20-ol-3-on-19-oic acid (19.58%), 20-methyl-pregna-5,17-dien-3β-ol (12.93%), 3,7-dioxocholan-24-oic acid (10.53%), betulin (10.18%), 4,4-dimethylcholesta-7,9(11)-dien-3-ol

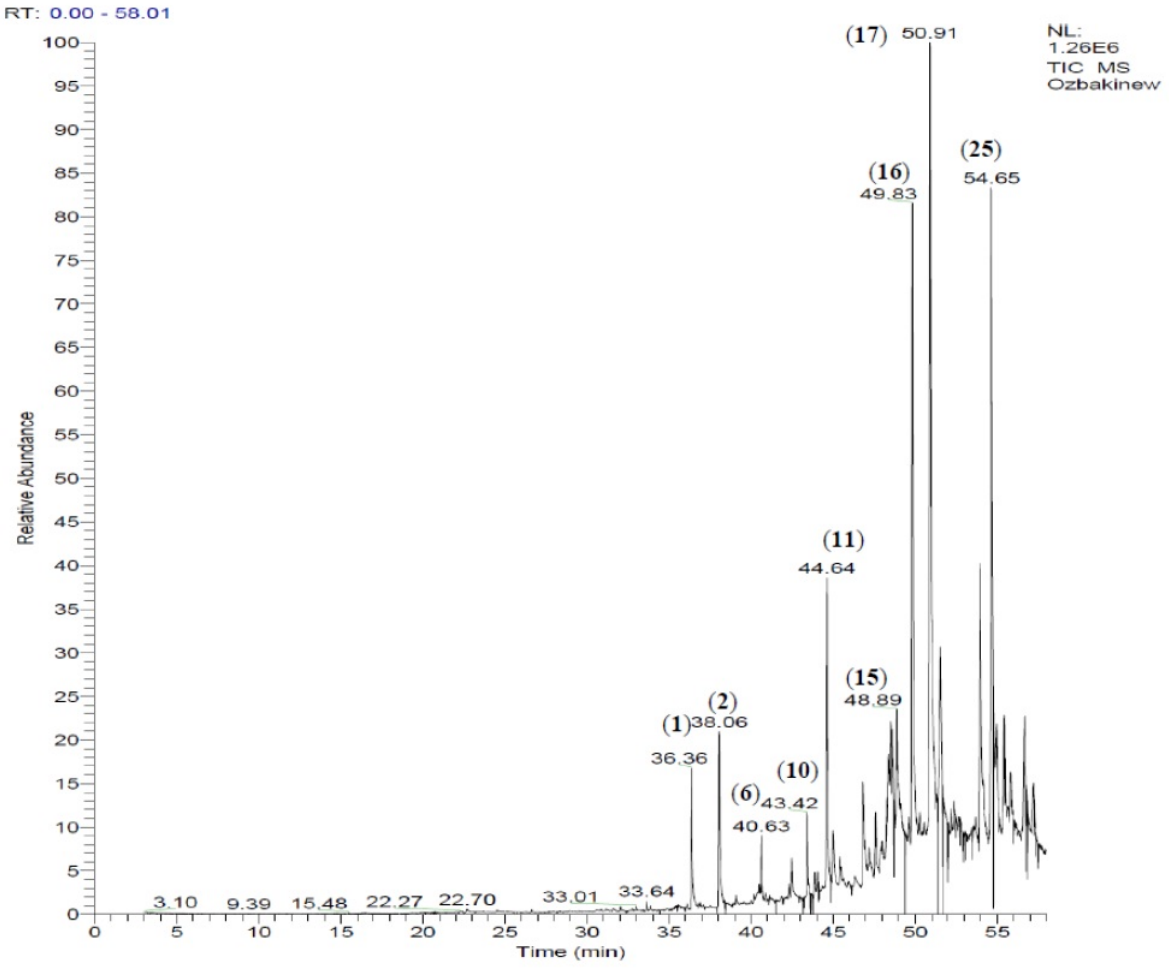


Figure 3. Chromatogram of a chloroform extract of *A. turkestanica* by GLC.

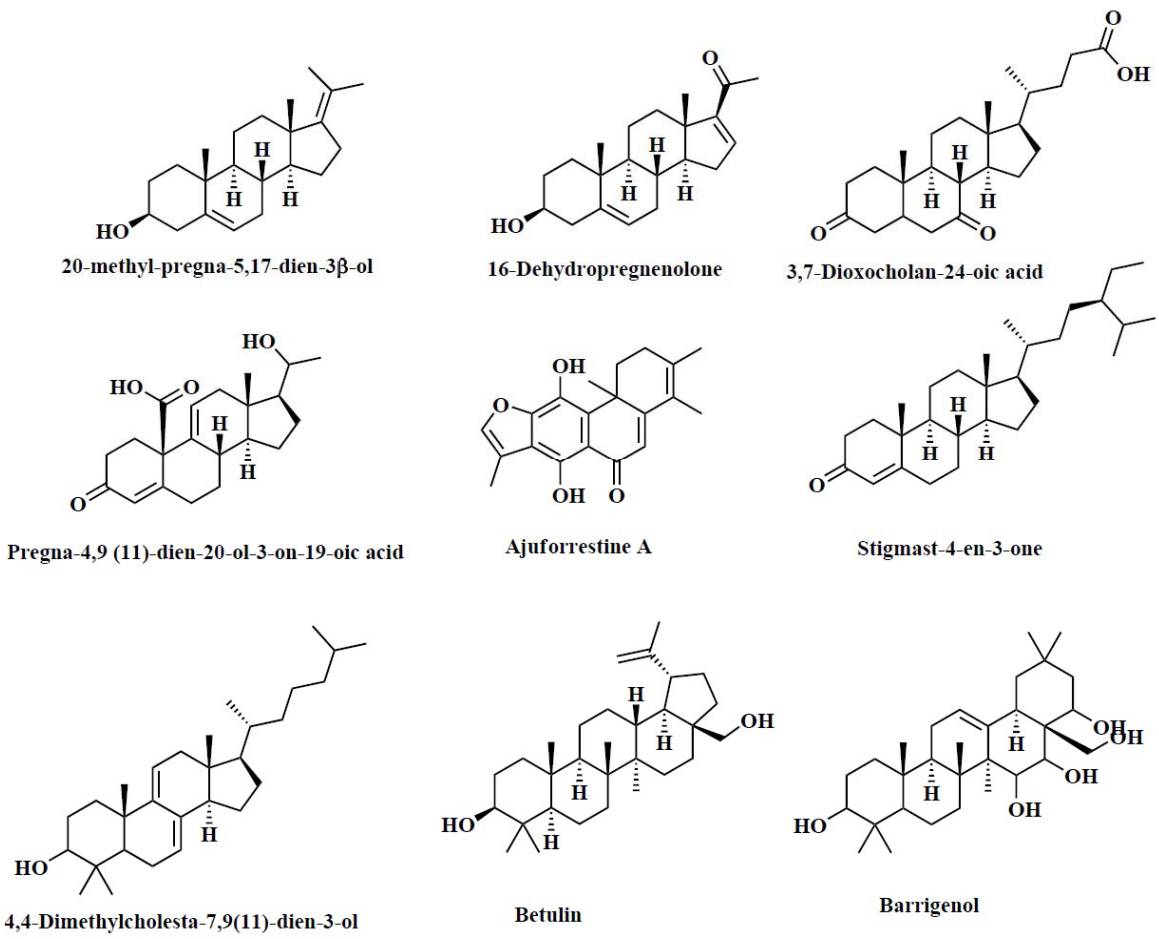


Figure 4. Structures of selected identified compounds from the root extract of *A. turkestanica*

(8.43%), barrigenol (4.37%), stigmast-4-en-3-one (3.34%), ajuforrestine A (3.33%) and 16-dehydropregnenolone (2.19%) were identified unambiguously as major compounds (Fig. 4).

Antioxidant test

The DPPH* radical-scavenging activities of the reference substance (quercetin), extracts and isolated secondary metabolites are documented in Table 3 and Fig. 5. All ecdysteroids and iridoids exhibited weak DPPH* radical scavenging activity with IC_{50} values above 100 μM. Among the tested extracts, water and butanol extracts had a higher antiradical capacity with a IC_{50} value 6.13 ± 0.71 and 12.23 ± 1.42 μg/mL, respectively.

Cytotoxicity analysis

A cytotoxicity screening of the methanol, chloroform, butanol extracts and isolated phytoecdysteroids **1-4**, and iridoid glucosides **5**, **6** was carried out in HeLa, HepG-2, and MCF-7 cells. The IC_{50} values of corresponding extracts and isolated secondary metabolites are reported in Table 4 and Fig. 6. The phytoecdysteroids and iridoids showed a moderate in-

Table 3. Antioxidant activity of phytoecdysteroids, iridoids, and extracts isolated from *A. turkestanica* using the DPPH* radical scavenging assay. The data are represented as IC_{50} values (mean ± SD).

Compounds and extracts	IC_{50} (μg/mL)
Extracts	
Water	6.13 ± 0.71
Butanol	12.23 ± 1.42
Methanol	57.84 ± 4.19
Chloroform	100.5 ± 8.42
Phytoecdysteroids	
20-Hydroxyecdysone (**1**)	142.90 ± 10.43
Turkesterone (**2**)	140.92 ± 12.01
Cyasterone (**3**)	106.31 ± 9.12
22-Acetylcyasterone (**4**)	114.23 ± 9.84
Iridoids	
Harpagide (**5**)	173.48 ± 15.72
8-O-Acetylharpagide (**6**)	294.94 ± 30.25
Control	
Quercetin	3.37 ± 0.77

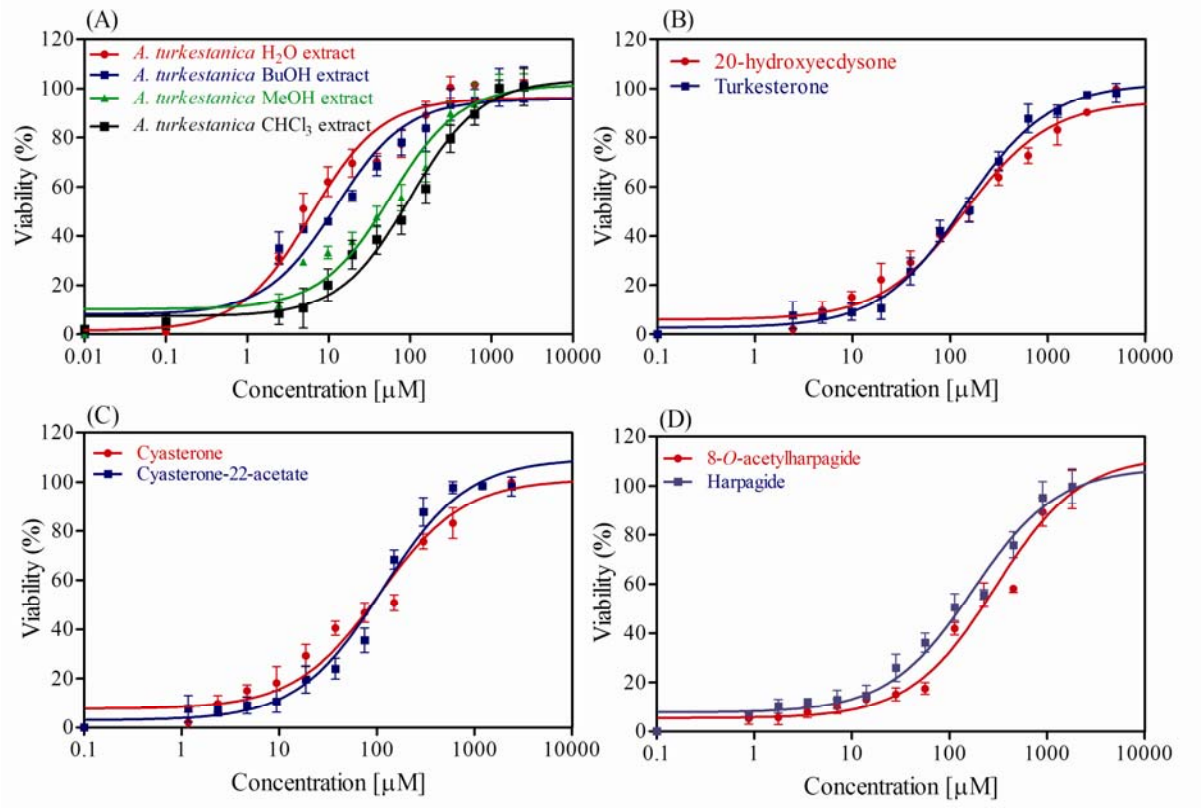
Table 4. Antiproliferative activities of phytoecdysteroids, iridoids and extracts isolated from *A. turkestanica* on HeLa, HepG-2 and MCF-7 cell lines. The data are represented as IC_{50} values (mean ± SD).

Sample	IC_{50} of compounds and extracts (μg/mL)		
	HeLa	HepG-2	MCF-7
Extracts			
Water	234.25 ± 18.34	144.42 ± 10.07	193.04 ± 10.59
Butanol	133.11 ± 10.24	119.96 ± 8.57	130.56 ± 10.53
Methanol	72.34 ± 2.78	75.04 ± 5.80	81.94 ± 2.59
Chloroform	7.13 ± 0.85	9.03 ± 0.92	10.77 ± 1.44
Phytoecdysteroids			
20-Hydroxyecdysone (**1**)	85.57 ± 3.25	57.10 ± 10.77	73.81 ± 10.71
Turkesterone (**2**)	75.17 ± 4.14	63.01 ± 7.53	105.21 ± 10.96
Cyasterone (**3**)	77.24 ± 10.15	52.03 ± 7.85	82.07 ± 11.69
22-Acetylcyasterone (**4**)	67.49 ± 8.47	71.38 ± 2.74	115.45 ± 0.38
Iridoids			
Harpagide (**5**)	58.31 ± 10.58	51.79 ± 12.85	94.96 ± 19.07
8-O-Acetylharpagide (**6**)	61.59 ± 8.17	68.14 ± 11.35	86.09 ± 12.04
Control			
Doxorubicin (μg/ml)	1.07 ± 0.11	0.39 ± 0.04	0.28 ± 0.02

hibition of cell proliferation with IC_{50} values above 50 μg/mL. Methanol and chloroform extracts exhibited the highest level of cytotoxicity. Especially the chloroform extract strongly inhibited cell growth in all tested cell lines (IC_{50} = 7.13 ± 0.85 μg/mL in HeLa, 9.03 ± 0.92 μg/mL in HepG-2, and 10.77 ± 1.44 μg/mL in MCF-7 cells).

Antimicrobial test

The extracts were tested for antimicrobial activity against several human pathogenic bacteria and yeasts at various concentrations, ranging from 8 to 0.015 mg/mL. The correspo-



Fig**ure**. 5 Dose-response curve for DPPH* scavenging activity of **A** H_2O, BuOH, MeOH, and $CHCl_3$ extracts of *A. turkestanica*, **B** 20-hydroxyecdysone, turkesterone, **C** cyasterone, 22-acetyl-cyasterone, and **D** harpagide, 8-*O*-acetylharpagide. The data shown are means ± SD obtained from three independent experiments.

nding MIC and MMC values are reported in Table 5. Pure phytoecdysteroids and iridoids were tested at concentrations from 1.5 to 1000 µM. Growth of *Enterococcus VanB* VRE ATCC 902291, *E. VanB* VRE ATCC 31299, *Staphylococcus aureus* MRSA ATCC 1000/93, and *S. aureus* MRSA ATCC 10442 were not inhibited by any of the isolated secondary metabolites. Only compounds **1**, and **4-6** showed a MIC of 0.5 mM against *E. VanB* VRE ATCC 31299, and compound **1** inhibited *S. aureus* MRSA ATCC 10442 (MIC 0.5 mM). Compounds **1-6** have MIC values corresponding to 0.5 mM in both *S. pyogenes* ATCC 12344 and *C. albicans* ATCC 90028. Other phytoecdysteroids and iridoids were inactive against *Candida glabrata* ATCC MYA 2950 except cyasterone (**3**) (MIC > 0.5 mM and MMC > 0.5 mM). *Klebsiella pneumonia* ATCC 700603 was inhibited by all the compounds (MIC = 0.25 mM), except compound **1** (MIC = 0.5 mM). *P. aeruginosa* ATCC 27853 was inhibited by the phytoecdysteroid **4**, which showed the strongest activity (MIC = 0.125 mM), whereas other compounds showed MIC values from 0.25 to 0.5 mM (Table 5).

The chloroform extract showed strong antimicrobial activity against *S. aureus* MRSA ATCC 1000/93 and *S. pyogenes* (MIC = 0.06 mg/mL and MMC = 0.03 mg/mL), respectively. *C. glabrata* was not inhibited by any of the tested plant extracts, whereas only chloroform extract showed a weak inhibition (MIC>4 mg/mL) against this yeast. Butanol and methanol extracts (MIC = 4 - 8 mg/mL) were less active.

Table 5a. Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) of the extracts and compounds from the plant *A. turkestanica* against different pathogens

Sample		*Staphylococcus aureus* MRSA ATCC 10442	*Enterococcus Vanb* VRE ATCC 31299	*Staphylococcus aureus* MRSA ATCC 1000/93	*Enterococcus Vanb* VRE ATCC 902291	*Streptococcus pyogenes* ATCC 12344
A. t. H_2O extract	I.z.	0	NA	6.05±0.05	5.1±0.1	8.8±0.1
	MIC	8	NA	8	>8	4
	MMC	>8	NA	8	>8	>8
A. t. BuOH extract	I.z.	0	NA	4.05±0.05	3.1±0.1	4.8±0.1
	MIC	4	NA	4	>4	2
	MMC	>4	NA	8	>4	4
A. t. MeOH extract	I.z.	3.15±1.15	3.8±0.2	3.9±0.1	3.8±0.1	5.9±0.1
	MIC	>4	2	4	>4	2
	MMC	>4	8	>4	>4	4
A. t. $CHCl_3$ extract	I.z.	7.1±0.1	3.4±0.2	6.85±0.15	4.8±0.2	8.2±0.2
	MIC	>4	>4	0.06	4	0.03
	MMC	>4	>4	0.5	>4	0.25
20-Hydroxy-ecdysone (**1**)	I.z.	0	0	NA	NA	4.9±0.1
	MIC*	0.5	0.5	NA	NA	0.5
	MMC*	>0.5	>0.5	NA	NA	>0.5
Turkesterone (**2**)	I.z.	NA	NA	NA	NA	3.05±0.05
	MIC*	NA	NA	NA	NA	0.5
	MMC*	NA	NA	NA	NA	>0.5
Cyasterone (**3**)	I.z.	NA	NA	NA	NA	4.1±0.1
	MIC*	NA	NA	NA	NA	0.5
	MMC*	NA	NA	NA	NA	>0.5
22-*O*-Acetyl-cyasterone (**4**)	I.z.	NA	3	NA	NA	3.9±0.1
	MIC*	NA	0.5	NA	NA	0.5
	MMC*	NA	>0.5	NA	NA	>0.5
8-*O*-Acetyl-harpagide (**5**)	I.z.	NA	0	NA	NA	3.95±0.05
	MIC*	NA	0.5	NA	NA	0.5
	MMC*	NA	>0.5	NA	NA	>0.5
Harpagide (**6**)	I.z.	NA	0	NA	NA	3.9±0.1
	MIC*	NA	0.25	NA	NA	0.5
	MMC*	NA	0.5	NA	NA	>0.5
Ampicillin [a]	I.z.	14.5±0.5	15	13.5±0.5	NA	25±1
	MIC	25	1	50	NA	0.05
	MMC	>25	7	>50	NA	0.1
Vancomycin [a]	I.z.	10±0.2	NT	NT	NA	15±1
	MIC	0.8	25	7	NA	0.1
	MMC	12.5	>50	12.5	NA	0.4
Nystatine [a]	I.z.	NT	NT	NT	NT	NT
	MIC	NT	NT	NT	NT	NT
	MMC	NT	NT	NT	NT	NT

A.t. – *A. turkestanica*; I.z. – inhibition zone, mm; MIC - mg/mL; MIC* - mM; MMC - mg/mL; MMC* - mM; NT – not tested; NA – not active; [a] = MIC and MMC values in µg/ml

Discussion

A. turkestanica accumulates high levels of phytoecdysteroids (Abdukadyrov et al., 2005; Usmanov et al., 1975; Saatov et al., 1977) and has therefore been exploited as an indu strial source for the production of phytoecdysteroids. The main major phytoecdysteroids are 20-hydroxyecdysone (**1**) and turkesterone (**2**). The chemical analysis of the present study confirms already reported (Abdukadyrov et al., 2005; Usmanov et al., 1971, 1975, 1978; Saatov et al., 1977). Due to the substantial bioactivity of the $CHCl_3$ extract (Table 3-5), such extract is characterized by both HPLC and GLC. In addition to compounds **1** and **6**, chloroform extract contains a number of oxo sterols (pregna-4,9 (11)-dien-20-ol-3-on-19-oic acid lactone, 3,7-dioxocholan-24-oic acid and 16δ-pregnenolone), sterols (20-methyl-pregna-5,17-dien-3β-ol and 4,4-dimethylcholesta-7,9(11)-dien-3-ol) and triterpenes (betulin and barrigenol) (Table 2).

Table 5b. Minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) of the extracts and compounds from the plant *A. turkestanica* against different pathogens.

Sample		*Escherichia coli* ATCC 25922	*Klebsiella pneumonia* ATCC 700603	*Pseudomonas aeruginosa* ATCC 27853	*Candida albicans* ATCC 90028	*Candida glabrata* ATCC MYA 2950
A. t. H_2O extract	I.z.	0	0	4.5±0.1	0	NA
	MIC	8	8	8	8	NA
	MMC	>8	>8	>8	>8	NA
A. t. BuOH extract	I.z.	0	0	3	0	NA
	MIC	8	8	4	4	NA
	MMC	>8	>8	8	>4	NA
A. t. MeOH extract	I.z.	3.15±0.15	4.85±0.15	4.4±0.2	3.8±0.2	NA
	MIC	8	4	4	>4	NA
	MMC	>8	>4	8	>4	NA
A. t. $CHCl_3$ extract	I.z.	3.3±0.1	4.8±0.2	4.9±0.1	5.75±0.25	3.9±0.1
	MIC	8	8	4	2	>4
	MMC	>8	>8	>4	>4	>4
20-Hydroxy-ecdysone (**1**)	I.z.	3.1±0.1	3	3.4	3.9±0.1	NA
	MIC*	1	0.5	0.25	>0.5	NA
	MMC*	>1	>0.5	0.5	>0.5	NA
Turkesterone (**2**)	I.z.	3	3.8±0.2	3	4.8±0.2	NA
	MIC*	1	0.25	0.5	0.5	NA
	MMC*	>1	0.5	1	>0.5	NA
Cyasterone (**3**)	I.z.	3±0.1	3.9±0.1	3.05±0.15	3	3.9±0.1
	MIC*	1	0.25	0.5	0.5	>0.5
	MMC*	>1	0.5	1	>0.5	>0.5
22-*O*-Acetyl-cyasterone (**4**)	I.z.	3.05±0.15	3.85±0.15	3.4±0.4	3	NA
	MIC*	1	0.25	0.125	>0.5	NA
	MMC*	>1	0.5	0.25	>0.5	NA
8-*O*-Acetyl-harpagide (**5**)	I.z.	3.05±0.05	3	3.1±0.1	3.85±0.15	NA
	MIC*	0.5	0.25	0.25	0	NA
	MMC*	>0.5	0.5	0.5	0	NA
Harpagide (**6**)	I.z.	3	3.2±0.1	3.1±0.1	NA	NA
	MIC*	1	0.25	0.25	NA	NA
	MMC*	>1	0.5	0.5	NA	NA
Ampicillin [a]	I.z.	14	-	NA	NT	NT
	MIC	12.5	25	NA	NT	NT
	MMC	25	25	NA	NT	NT
Vancomycin [a]	I.z.	NA	NT	NA	NT	NT
	MIC	NA	25	NA	NT	NT
	MMC	NA	50	NA	NT	NT
Nystatine [a]	I.z.	NT	NT	NT	10±1.2	12±1
	MIC	NT	NT	NT	0.2	0.2
	MMC	NT	NT	NT	0.4	0.2

A.t. – *A. turkestanica*; I.z. – inhibition zone, mm; MIC - mg/mL; MIC* - mM; MMC - mg/mL; MMC* - mM; NT – not tested; NA – not active; . [a] = MIC and MMC in µg/ml

These phytochemicals were completely different from those of previously published studies performed on lipophilic extractions of other *Ajuga* species (Azizan et al., 2002; Baser et al., 1999; 2001; Javidnia et al., 2010; Velasco-Negueruela et al., 2004; Sa-jjadi et al., 2004).

Isolated phytoecdysteroids and iridoids of *A. turkestanica* were ineffective for DPPH radical scavenging activity (Table 3). This is not surprising because the structure of ecdysteroid molecules is unlikely to exert an antioxidant effect, as compared to the common antioxidative flavonoids (Harborne and Williams, 2000). In our experiments polar extracts such as butanol and methanol extracts were more active; this activity could be due to phenolics or other antioxidants that were not identified in our analysis. This finding is in agreement with other studies (Miliauskas et al., 2005; Turkoglu et al., 2010).

As shown in Table 4 none of the isolated secondary metabolites exhibited a high cytotoxicity (IC_{50} above 100 µM or 50 µg/ml). The higher activity of the chloroform extract is probably due to additional compounds that were identified by GLC and GLC-MS (Fig. 3). Since individual substances of the $CHCl_3$ extract were not available, we could not identify the underlying cytotoxic principle. Lagova and Valueva (1981) found that 20-hydroxyecdysone was ineffective to inhibit the growth of several tumour types, whereas it stimulated that of mammary gland carcinomas. In this specific case, because ecdysteroids structurally resemble sex hormones, they may bind to steroid hormone receptors in mammals and stimulate hormone-dependent tumors. Takasaki et al. (1999) reported that phytoecdysteroids and iridoids from *Ajuga decumbens* have anticancer properties. In their study cyasterone, polypodine B, decumbesterone A, especially 8-*O*-acetylharpagide (**6**) showed strong tumour preventive activities *in vivo* in a mouse-skin model, using 7,12-dimethylbenz[a]anthracene as tumour initiator and TPA as promoter.

As shown in Table 5, phytoecdysteroids and iridoids of *A. turkestanica* had weak antimicrobial activity against Gram-positive bacteria, *C. glabrata* ATCC MYA 2950, except *S. pyogenes* ATCC 12344. Only cyasterone (**3**) showed activity (MIC > 0.5 mM and MMC > 0.5 mM) against *C. glabrata* ATCC MYA 2950. Compounds **1**-**6** showed stronger activity against all Gram-negative bacteria. Acetyl group containing phytoecdysteroids such as **4** and **5** inhibited the growth of bacteria used. The maximum inhibition was observed against *K. pneumonia* ATCC 700603 and *P. aeruginosa* ATCC 27853 having of MIC values of 0.125-0.25 mM. According to Shirshova et al. (2006) and Volodin et al. (1999) true phytoecdysteroids such as ecdysone, inokosterone and 20-hydroxyecdysone (**1**) have weak antimicrobial activity. But the introduction of acetyl groups into the molecule **1** can increase antimicrobial activity against *Bacillus cereus*, *Proteus rettgeri* and *Saccharomyces cerevisiae* in the sequence from 2-acetate-20-hydroxyecdysone<2,3,22-tri-acetate-20-hydroxyecdysone < 2,3,22,25-tetraacetate-20-hydroxyecdysone (Shirshova et al., 2006; Politova et al., 2001). Whereas iridoid glucosides are rather inactive, some iridoid aglycones from the *Cymbaria mongolica* showed antibacterial activity against *Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus*. Among them, 1β-methoxylmussaenin A possessed significant activity similar to that of chloramphenicol (Dai et al., 2002). Iridoid glucosides are stored inactive prodrugs in plant vacuoles; only after treatment with beta-glucosidase an aglycone is formed in which the lactol rings opens. Then two reactive aldehyde groups are generated; these can interfere with amino groups of proteins and nucleic acids (Wink, 2008; Wink and van Wyk, 2008).

Our antimicrobial tests revealed that the isolated ecdysteroids are hardly antimicrobial. However, the chloroform extract has antimicrobial activity even against multiresistant strains with known resistance against antibiotics, like *Staphylococcus aureus* MRSA ATCC 1000/93 and *Streptococcus pyogenes* ATCC 12344 (Table 5).

We assume that cytotoxic and antibacterial activities of the chloroform extract of *A. turkestanica* may be due to the presence of nonpolar compounds such as oxo sterols, sterols, diterpenes, and triterpenes. According to the literature, some of pregnene and pregnadiene derivatives were potential inhibitors of 5α-reductase type II, inhibited cell proliferation of LNCap and PC-3 prostate cancer cells and were the most active in the 5AR2 inhibitory test (Kim and Ma, 2009). Also some triterpenes such as betulin could act as potent antitumour

promoters, being active against colorectal (DLD-1), breast (MCF-7), prostate (PC-3) and lung (A549) cancer cell lines (Gauthier et al., 2009).

In conclusion, whereas isolated ecdysteroids and iridoid glucosides of *A. turkestanica* do not function as antioxidants or substantial cytotoxic or antimicrobial agents, the chloroform extract with more lipophilic compound was more active. In a next step further studies should be performed on the isolation and identification of the active compounds of the chloroform extract of *A. turkestanica*.

Conflict of interest

The authors declare no conflict of interest.

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