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Leishmanicidal cycloartane-type triterpene glycosides from *Astragalus oleifolius*

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Abstract

Two new cycloartane-type glycosides oleifoliosides A (**1**) and B (**2**) were isolated from the lower stem parts of *Astragalus oleifolius*. Their structures were identified as 3-*O*-[β-xylopyranosyl-(1 → 2)-α-arabinopyranosyl]-6-*O*–β-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane and 3-*O*-[β-xylopyranosyl-(1 → 2)-α-arabinopyranosyl]-6-*O*–β-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane, respectively, by means of spectroscopic methods (IR, 1D and 2D NMR, ESI-MS). Three known cycloartane glycosides cyclocanthoside E (**3**), astragaloside II (**4**) and astragaloside IV (**5**) were also isolated and characterized. All five compounds were evaluated for in vitro trypanocidal, leishmanicidal and antiplasmodial activities as well as their cytotoxic potential on primary mammalian (L6) cells. Except for the compound **5**, all compounds showed notable growth inhibitory activity against *Leishmania donovani* with IC_{50} values ranging from 13.2 to 21.3 μg/ml. Only weak activity against *Trypanosoma brucei rhodesiense* was observed with the known compounds astragaloside II (**4**, IC_{50} 66.6 μg/ml) and cyclocanthoside E (**3**, IC_{50} 85.2 μg/ml), while all compounds were inactive against *Trypanosoma cruzi* and *Plasmodium falciparum*. None of the compounds were toxic to mammalian cells (IC_{50}'s > 90 μg/ml). This is the first report of leishmanicidal and trypanocidal activity of cycloartane-type triterpene glycosides.

Keywords: *Astragalus oleifolius*; Leguminosae; Cycloartane-type glycoside; Oleifoliosides A and B; *Trypanosoma*; *Leishmania*; *Plasmodium*

1. Introduction

In the flora of Turkey the genus *Astragalus* (Leguminosae) is represented by 380 species (Davis, 1970). The roots of several *Astragalus* species are well-known in traditional medicine for the treatment of nephritis, diabetes, leukemia, uterine cancer and as an antiperspirant, diuretic and tonic (Tang and Eisenbrand, 1992). *Astragalus microcephalus* is used primarily in Turkey for the production of the economically important gum, tragacanth (Dogan et al., 1985). *Astragalus* species are rich in cycloartane-type triterpene glycosides that possess diverse biological activities. Some cycloartane glycosides have been shown to have antitumor and AIDS antiviral activity (Gariboldi et al., 1995). The immunostimulant effects of several cycloartane-type triterpene glycosides on macrophage activation and expression of inflammatory cytokines were investigated (Bedir et al., 2000a). Some other cycloartane glycosides obtained from *Astragalus* species act as modulators of lymphocyte proliferation (Çalış et al., 1997; Verotta et al., 2001, 2002). However, the anti-protozoal activity of cycloartane

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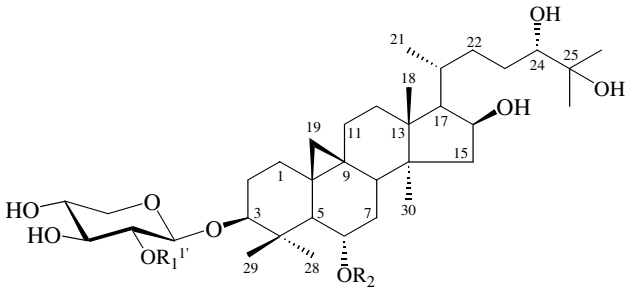
glycosides has remained uninvestigated. Previously we reported five cycloartane-type triterpenic glycosides, macrophyllosaponins A–E from the roots of *Astragalus oleifolius* DC. (Çalış et al., 1996; Bedir et al., 2000b). In the continuation of our investigations on this genus, we now studied the lower stem parts of the same plant collected from a different region that yielded two new cycloartane-type glycosides oleifoliosides A (**1**) and B (**2**), as well as three known glycosides cyclocanthoside E (**3**) astragaloside II (**4**) and astragaloside IV (**5**). This report describes the isolation, structure elucidation, broad spectrum anti-protozoal activity and cytotoxic potential of the compounds **1**–**5** (see Fig. 1).

2. Results and discussion

The methanolic extract of the lower stem parts of *A. oleifolius* was separated by a combination of RP-VLC (reversed-phase vacuum liquid chromatography) and silica gel column chromatography (CC) to yield two new (**1** and **2**) and three known cycloartane-type glycosides, **3**–**5**.

The ESI-MS spectrum of **1** exhibited a $[M + Na]^+$ peak at *m/z* 911 which was compatible with the molecular formula $C_{45}H_{76}O_{17}$. The ^1H NMR spectrum of **1** showed signals characteristic of cyclopropane-ring methylene protons as an AX system (δ 0.12, 0.63, J_{AX} = 4.4 Hz, H_2-19), six tertiary methyl groups at δ 1.18, 1.35, 1.37, 1.46, 1.49, 1.80 and a secondary methyl group at δ 1.09 (*d*, *J* = 6.5 Hz). Additionally, three anomeric protons at δ 4.79 (*d*, *J* = 7.1 Hz, H-1′), 4.85 (*d*, *J* = 7.2 Hz, H-1‴) and 5.20 (*d*, *J* = 6.6 Hz, H-1″) indicated the presence of two β- and one α-linked sugar units. The ^{13}C NMR spectrum exhibited 45 carbon atoms, 30 of which were assigned to the aglycone moiety while the remaining were due to three pentose units. All NMR assigments (Tables 1, 2) were based on DQF-COSY, HSQC, HMBC and ROESY experiments. The chemical shifts and coupling constants of the signals assigned to the sugar moieties revealed the presence of two



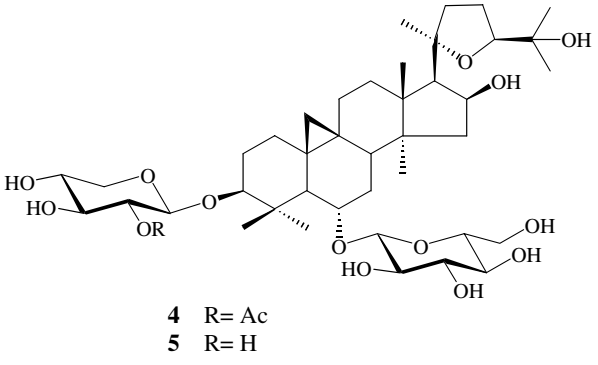


Fig. 1. Structures of the compounds **1**–**5**.

Table 1
^1H and ^{13}C NMR spectroscopic data for aglycones of oleifoliosides A (**1**) and B (**2**) (pyridine-d_5; δ: ppm; *J*: Hz)

Atom	DEPT	**1**		**2**	
		δ_H	δ_C	δ_H	δ_C
1	CH_2	1.60 *m*	31.8	1.60 *m*	32.1
		1.34*m*		1.33*m*	
2	CH_2	2.00 *m*	30.1	2.02 *m*	30.2
		2.30 *m*		2.33 *m*	
3	CH	3.39 *dd* (11.6/4.4)	87.7	3.46 *dd* (11.3/4.1)	87.9
4	C		42.7		42.7
5	CH	1.87 *d*(7.2)	51.9	1.88 *m*	52.3
6	CH	3.80 *m*	77.2	3.80 *m*	78.4
7	CH_2	2.09 *m*	33.0	2.04 *m*	33.8
		2.13 *m*		2.22 *m*	
8	CH	2.13 *m*	43.2	2.02 *m*	44.5
9	C		21.5		21.5
10	C		27.9		28.3
11	CH_2	1.69 *m*	26.4	1.76 *m*	26.3
		1.54 *m*		1.35 *m*	
12	CH_2	1.66 *m*	33.2	1.64 *m*	33.2
		2.31 *m*			
13	C		45.9		45.8
14	C		46.8		46.9
15	CH_2	2.41 *dd* (12.8/8.0)	47.2	2.43 *m*	47.6
		1.84 *m*		1.85 *m*	
16	CH	4.77 *m*	72.0	4.74 *m*	72.0
17	CH	1.88 *m*	56.9	1.85 *m*	57.1
18	CH_3	1.37 *s*	17.5	1.40 *s*	18.1
19	CH_2	0.12 *d* (4.4)	25.0	0.18 *d* (4.2)	26.9
		0.63 *d* (4.4)		0.61 *d* (4.2)	
20	CH	2.41[a]	28.7	2.43 *m*	28.6
21	CH_3	1.09 *d* (6.5)	18.4	1.09 *d* (6.4)	18.4
22	CH_2	1.51 *m*	32.8	1.47[a]	32.9
				2.33 *m*	
23	CH_2	1.85 *m*	27.8	1.85 *m*	27.8
		1.99 *m*		2.02 *m*	
24	CH	3.94 br *d* (10.6)	77.1	3.95 *dd* (11.1/2.2)	77.1
25	C		72.5		72.5
26	CH_3	1.46 *s*	26.5	1.47 *s*	26.5
27	CH_3	1.49 *s*	25.8	1.49 *s*	25.8
28	CH_3	1.80 *s*	27.5	1.91 *s*	27.9
29	CH_3	1.35 *s*	16.4	1.40 *s*	16.4
30	CH_3	1.18 *s*	19.5	1.06 *s*	19.7

[a] Signal pattern unclear due to the overlapping.

xylose and an arabinose unit. The NMR spectroscopic data (Table 1) attributed to the sapogenol moiety were characteristic for a cycloartane-type triterpenoid skeleton and in good agreement with cyclocanthogenin (Isaev et al., 1992). However, the carbon resonances attributed to C-3 (δ 87.7) and C-6 (δ 77.2) were found to be shifted downfield by +8–9 ppm, in comparison with those of cyclocanthogenin. This suggested that C-3 and C-6 were the sites of glycosidations on the sapogenol moiety, thus confirming the bisdesmosidic structure of **1**. The HMBC correlations observed between H-1′ (δ_H 4.79) of xylose and C-3 (δ_C 87.7), and H-1‴(δ_H 4.85) of arabinose and C-6 (δ_C 77.2) further proved these assumptions. The third anomeric proton signal at δ 5.20 (H-1″) assigned to the α-arabinopyranosyl unit showed long-range correlation with the C-2′ atom (δ 83.6) of the first xylose moiety attached to C-3 of the sapogenol. This revealead that C-2′ was the site of the glycosidation. All these observations were supported by a ROESY experiment which showed correlations between the protons of H-1′ of xylose (δ_H 4.79) and H-3 of aglycone (δ_H 3.39); H-1‴ of the second xylose (δ_H 4.85) and H-6 of aglycone (δ_H 3.80) and finally, H-1″ of arabinose (δ_H 5.20) and H-2′ of the first xylose linked to the aglycone at C-3 (δ_H 4.10). Thus, **1** was identified as 3-*O*-[β-xylopyranosyl-(1 → 2)-α-arabinopyranosyl]-6-*O*-β-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane, and named oleifolioside A.

The ESI-MS spectrum of compound **2** showed an ion peak at *m/z* 941 $[M + Na]^+$ in agreement with the molecular formula $C_{46}H_{78}O_{18}$. The signals observed in the ^1H- and ^{13}C NMR spectra of **2** were very similar to those of compound **1**. Based on chemical shift and coupling constant analyses, compound **2** was found to contain the same aglycone, cyclocanthogenin, as compound **1** (Table 1). The only difference between **1** and **2** originated from the sugar moiety attached at the C-6 of the sapogenol. Compound **1** contained a β-xylose group at this position, whereas **2** had a β-glucose unit attached at C-6. This was obvious from the ^1H NMR spectrum that contained three anomeric protons at δ 4.84 (*d*, *J* = 7.0 Hz, H-1′), 4.93 (*d*, *J* = 7.6 Hz, H-1‴) and 5.21 (*d*, *J* = 6.6 Hz, H-1″), which were readily assigned to a β-xylopyranosyl, an α-arabinopyranosyl and a β-glucopyranosyl unit, respectively. The anomeric proton of the latter β-glucopyranosyl moiety (δ_H 4.93, H-1‴) showed HMBC connectivity to C-6 (δ_C 78.4) confirming the ^1H NMR data. Additional cross peaks observed in the HMBC spectrum between the anomeric proton of α-arabinopyranosyl unit (δ_H 5.21, H-1″) and C-2′ (δ_C 83.6) of the β-xylopyranosyl unit revealed that the disaccharide chain at C-3 was the same as in compound **1**, i.e., (α-arabinopyranosyl-(1 → 2)-β-xylopyranoside). All other structural assignments (Tables 1, 2) were further substantiated by DQF-COSY, HSQC, HMBC and ROESY data. Consequently, the structure of **2** was determined as 3-*O*-[β-xylopyranosyl-(1 → 2)-α-arabinopyranosyl]-6-*O*-β-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxy-cycloartane with the trival name oleifolioside B.

Additionally, three known compounds cyclocanthoside E (**3**), astragaloside II (**4**) and astragaloside IV (**5**) were also isolated and identified on the basis of their ESI-MS, IR,1D- and 2D NMR (DQF-COSY, HSQC,

Table 2
^1H and ^{13}C NMR spectroscopic data for sugar moieties of oleifoliosides A (**1**) and B (**2**) (pyridine-d_5; δ: ppm; *J*: Hz; Xyl: Xylose; Ara: Arabinose; Glc: Glucose)

Atom	**1**		Atom	**2**	
	δ_H	δ_C		δ_H	δ_C
Xyl-1′	4.79 *d* (7.1)	105.3	Xyl-1′	4.84 *d* (7.0)	105.4
2′	4.10 *dd* (8.6/7.1)	83.6	2′	4.13 *dd* (8.8/7.0)	83.6
3′	4.16[a]	77.5	3′	4.17[a]	77.5
4′	4.17[a]	71.0	4′	4.17[a]	71.0
5′	3.59 *dd* (11.3/9.5)	66.0	5′	3.58 *dd* (11.0/9.5)	66.6
	4.27 *dd* (11.3/5.3)			4.27 *dd* (11.0/5.2)	
Ara-1″	5.20 *d* (6.6)	106.8	Ara-1″	5.21 *d* (6.6)	106.7
2″	4.61 *dd* (8.5/6.6)	73.7	2″	4.61 *dd* (8.3/6.6)	73.7
3″	4.23 *dd* (8.5/3.2)	74.3	3″	4.22 *dd* (8.3/3.1)	74.3
4″	4.30 *m*	69.1	4″	4.30 *m*	69.2
5″	3.81 *dd* (12.0/1.8)	67.1	5″	3.82 *dd* (12.1/1.7)	67.1
	4.43 *dd* (12.0/3.2)			4.44 *dd* (12.1/3.1)	
Xyl-1‴	4.85 *d* (7.2)	105.8	Glc-1‴	4.93 *d* (7.6)	105.2
2‴	3.99 *t* (7.9)	75.5	2‴	4.03 *t* (8.1)	75.7
3‴	4.14 *t* (8.4)	78.4	3‴	4.20[a]	79.0
4‴	4.17[a]	71.1	4‴	4.17[a]	72.0
5‴	3.69 *dd* (11.2/9.6)	66.9	5‴	3.92 *ddd* (9.0/5.4/2.5)	78.1
	4.31 *dd* (11.2/5.0)		6‴	4.31 *dd* (11.4/5.4)	63.3
				4.49 *dd* (11.4/2.5)	

[a] Signal pattern unclear due to the overlapping.

HMBC and ROESY) data (Isaev et al., 1992; Kitagawa et al., 1983).

Compounds **1**–**5** were evaluated for their in vitro anti-protozoal activity against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. In order to determine their selective toxicity, the pure compounds were also tested on rat skeletal myoblasts (L6 cells). As shown in Table 3, all pure isolates were inactive against *T. cruzi* and *P. falciparum* at tested concentrations. Only the known compounds astragaloside II (**4**) and cyclocanthoside E (**3**) exhibited weak inhibitory activity against *T. b. rhodesiense* with the IC_{50} values of 66.6 μg/ml and 85.2 μg/ml, respectively. All compounds, except for **5**, revealed leishmanicidal activity against *L. donovani* with IC_{50} values ranging from 13.2 to 21.3 μg/ml (Table 3). None of the five compounds possessed cytotoxicity on mammalian L6 cells, indicating their leishmanicidal effect to be selective.

Astragalus membranaceus has been recently shown to have growth inhibitory activity against *T. cruzi* (Schinella et al., 2002), but its active principles have not been identified. Thus, it appears that the current study is the first report dealing with the anti-protozoal activity of the constituents of the genus *Astragalus*. To the best of our knowledge, this is also the first report of antileishmanial and antitrypanosomal activity for cycloartane-type triterpene glycosides.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on an Autopol IV Rudolph Research Analytical polarimeter using a sodium lamp operating at 589 nm. IR spectra were measured on a Jasco FT/IR-420 spectrometer on KBr pellets. The 1D- and 2D NMR spectra were obtained in pyridine-d_5 on a Bruker DRX 600 MHz spectrometer operating at 600 (^{1}H NMR) and 150 (^{13}C NMR) MHz. The chemical shift values are reported as parts per million (ppm) units relative to tetramethylsilane (TMS). ESI-mass spectra were taken on a Bruker Esquire-LC–MS (ESI mode) spectrometer. For vacuum liquid chromatography (VLC), reversed phase material LiChroprep C_{18} was used. Column chromatography was carried out on silica gel (Kieselgel 60, 60–230 mesh).

3.2. Plant material

A. oleifolius was collected from Şırnak: Uludere–Habur junction towards Hakkari in June 2003. The voucher specimen (AAD 11299) has been deposited at the Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey.

3.3. Extracton and isolation

Air dried powdered lower stem parts of the plant (290 g) were extracted with MeOH (2 × 1.5 l). The methanolic extract was evaporated under vacuum. The residue (35 g) was dissolved in water and then subjected to RP-VLC using step-gradient H_2O–MeOH mixtures (8:2, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, each 200 ml) and MeOH (400 ml) to give twelve fractions (A–L). Fraction G (4.5 g) eluted with H_2O–MeOH (2:8) was further subjected to silica gel column chromatography (170 g) using CH_2Cl_2:MeOH:H_2O (90:10:1, 80:20:2, 70:30:3, 65:35:3.5) solvent systems yielding 22 fractions (G-1 → G-22). Fr. G-13 (270 mg) was applied to a silica gel column (25 g) using CH_2Cl_2:MeOH (9:1, 8:2) and CH_2Cl_2:MeOH:H_2O (8:2:0.1) mixtures to give five fractions (G-13a → 13e). Fr.G-13d (152 mg) was rechromatographed over a silica gel column (15 g) employing CH_2Cl_2:MeOH (8:2) solvent system. The fractions (130 mg) eluted from this column were combined and reapplied to a silica gel column (14 g) using EtOAc:MeOH:H_2O mixtures (10:1:0.1, 10:1:0.2). Astragaloside IV (**5**) was obtained as cyristals from the fractions which were

Table 3
Anti-protozoal and cytotoxic activity of the cycloartane glycosides **1**–**5** from *Astragalus oleifolius* (IC_{50} in μg/ml)

Compound	*Trypanosoma b. rhodesiense*	*Trypanosoma cruzi*	*Leishmania donovani*	*Plasmodium falciparum*	L-6 cells
Standard	0.0032[a]	0.50[b]	0.087[c]	0.086[d]	0.008[e]
1	>90	>30	13.2	>5	>90
2	>90	>30	13.7	>5	>90
3	85.2	>30	14.1	>5	>90
4	66.6	>30	21.3	>5	>90
5	>90	>30	>30	>5	>90

[a] Melarsoprol.
[b] Benznidazole.
[c] Miltefosine.
[d] Chloroquine.
[e] Phodophyllotoxin.

left overnight (9.3 mg). Fr. G-16 (750 mg) was subjected to silica gel CC (70 g) with CH_2Cl_2:MeOH:H_2O (80:20:2, 75:25:2.5) mixtures to give six fractions (G-16a → G-16f). Fr. G-16f (332 mg) was rechromatographed over a SiO_2 column (40 g) with EtOAc:MeOH:H_2O (10:1:0.5) solvent system to give cyclocanthoside E (**3**, 13.5 mg) and oleifolioside A (**1**) (15 mg). Fr. H (1.8 g) eluted from initial RP-VLC was subjected to SiO_2 CC (100 g) with CH_2Cl_2:MeOH:H_2O (9:1:0.2, 8:2:0.2) solvent systems to give 19 fractions (H-1 → H-19). Fr. H-7 (65 mg) was purified by SiO_2CC (10 g, CH_2Cl_2:MeOH:H_2O 9:1:0.1) to give astragaloside II (**4**, 13 mg). Fr. H-19 (100 mg) was subjected to a column packed with SiO_2 (11 g) eluting with CH_2Cl_2:MeOH:H_2O (90:10:1, 85:15:1) solvent systems to give oleifolioside B (**2**) (14 mg).

3.4. Oleifolioside A (*1*)

Amorphous white powder. $[\alpha]_D^{27}$ +18.9 (*c* 0.1 MeOH); IR ν_{max}(KBr) cm^{-1}: 3427 (OH), 2922 (CH), 1048; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5), see Table 1 (aglycone moiety) and Table 2 (sugar moieties); ESI-MS: (*m*/*z*) 911 $[M + Na]^+$.

3.5. Oleifolioside B (*2*)

Amorphous white powder. $[\alpha]_D^{27}$ +21.9 (*c* 0.1 MeOH); IR ν_{max}(KBr) cm^{-1}: 3423 (OH), 2923 (CH), 1167, 1078; ^1H NMR (500 MHz, pyridine-d_5) and ^{13}C NMR (125 MHz, pyridine-d_5), see Table 1 (aglycone moiety) and Table 2 (sugar moieties); ESI-MS: (*m*/*z*) 941 $[M + Na]^+$.

3.6. Biological assays

3.6.1. Trypanosoma b. rhodesiense and cytotoxicity assays

Minimum Essential Medium (50 μl) supplemented according to (Baltz et al., 1985) with 2-mercaptoethanol and 15% heat-inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 μg/ml. Then 10^4 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 μl were added to each well and the plate incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. 10 μl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) were then added to each well and incubation continued for a further 2–4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Räz et al., 1997). Fluorescence development was expressed as percentage of the control, and IC_{50} values determined. Cytotoxicity was assessed using the same assay and rat skeletal myoblasts (L6 cells).

3.6.2. Trypanosoma cruzi

Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtiter plates at 2000 cells/well in 100 μl RPMI 1640 medium with 10% FBS and 2 mM L-glutamine. After 24 h the medium was removed and replaced by 100 μl per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the ß-galactosidase (Lac Z) gene. Forty-eight h later the medium was removed from the wells and replaced by 100 μl fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 90 to 0.123 μg/ml. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 μl) was added to all wells. A colour reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were transferred into a graphic programme (e.g. EXCEL), sigmoidal inhibition curves were determined and IC_{50} values calculated.

3.6.3. Leishmania donovani

Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium (Cunningham, 1977) at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. One hundred μl of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtiter plates. Seven 3-fold dilutions were used covering a range from 30 to 0.041 μg/ml. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Ten μl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) were then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (=inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated.

3.6.4. Plasmodium falciparum

Antiplasmodial activity was determined using the K1 strain of *P. falciparum* (resistant to chloroquine and pyrimethamine). A modification of the [^3H]-hypoxanthine incorporation assay was used (Matile and Pink, 1990). Briefly, infected human red blood cells in RPMI 1640 medium with 5% Albumax were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37 °C in a reduced oxygen atmosphere, 0.5 μCi ^3H-hypoxanthine was added to each well. Cultures were

incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a BetaplateTM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. From the sigmoidal inhibition curves IC_{50} values were calculated.

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References

Baltz, T., Baltz, D., Giroud, C., Crockett, J., 1985. Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. Rhodesiense* and *T. gambiense*. EMBO J. 4, 1273–1277.

Bedir, E., Pugh, N., Çalış, İ., Pasco, D.S., Khan, I.A., 2000a. Immunostimulatory effects of cycloartane-type triterpene glycosides from *Astragalus* species. Biol. Pharm. Bull. 23, 834–837.

Bedir, E., Çalış, İ., Khan, I.A., 2000b. A novel compound from the roots of *Astragalus oleifolius*. Chem. Pharm. Bull. 48, 1081–1083.

Cunningham, I., 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. 24, 325–329.

Çalış, İ., Zor, M., Saracoglu, I., Isimer, A., Ruegger, H., 1996. Four novel cycloartane glycosides from *Astragalus oleifolius*. J. Nat. Prod. 59, 1019–1023.

Çalış, İ., Yuruker, A., Tasdemir, D., Wright, A.D., Sticher, O., Luo, Y.-D., Pezzuto, J.M., 1997. Cycloartane-type triterpene glycosides from the roots of *Astragalus melanophrurius*. Planta Med. 63, 183–186.

Davis, P.H., 1970Flora of Turkey and East Aegean Islands, vol. 3. University Press, Edinburg, pp. 49–254.

Dogan, M., Ekim, T., Anderson, D.M.W., 1985. The production of gum tragacanth from *Astragalus microcephalus* in Turkey. A contributions towards a balanced environments. Biol. Agric. Hortic. 2, 329–334.

Gariboldi, P., Pelizzoni, F., Tato, M., Verotta, L., El-Sebakhy, N.A., Asaad, A.M., Abdallah, R.M., Toaima, S.M., 1995. Cycloartane triterpene gylcosides from *Astragalus trigonus*. Phytochemisry 40, 1755–1760.

Isaev, M.I., Imomnazarov, B.A., Fadeev, Y.u.M., Kintya, P.A., 1992. Triterpene glycosides of *Astragalus* and their genins XLII. Cycloartanes of *Astragalus tragacantha*. Chem. Nat. Comp., 315–320.

Kitagawa, I., Wang, H.K., Saito, M., Takagaki, A., Yoshikawa, M., 1983. Saponin and sapogenol. XXXV. Chemical constituents of *Astragalus* radix, the root of *Astragalus membranaceus* Bunge (2). Astragalosides I, II and IV, acetylastragaloside I and isoastragalosides I and II. Chem. Pharm. Bull. 31, 698–708.

Matile, H., Pink, J.R.L., 1990. *Plasmodium falciparum* malaria parasite cultures and their use in immunology. In: Lefkovits, I., Pernis, B. (Eds.), Immunological Methods. Academic Press, San Diego, pp. 221–234.

Räz, B., Iten, M., Grether-Bühler, Y., Kaminsky, R., Brun, R., 1997. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. Acta Trop. 68, 139–147.

Schinella, G.R., Tournier, H.A., Prieto, J.M., Rios, J.L., Buschiazzo, H., Zaidenberg, A., 2002. Inhibition of *Trypanosoma cruzi* growth by medical plant extracts. Fitoterapia 73, 569–575.

Tang, W., Eisenbrand, G., 1992. Chinese Drugs of Plant Origin. Springer-Verlag, Berlin, pp. 191–197.

Verotta, L., Guerrini, M., El-Sebakhy, N.A., Asaad, A.M., Toaima, S.M., Abou-Sheer, M.E., Luo, Y.-D., Pezzuto, J.M., 2001. Cycloartane saponins from *Astragalus peregrinus* as modulators of lymphocyte proliferation. Fitoterapia 72, 894–905.

Verotta, L., Guerrini, M., El-Sebakhy, N.A., Asaad, A.M., Toaima, S.M., Radwan, M.M., Luo, Y.-D., Pezzuto, J.M., 2002. Cycloartane and oleanane saponins from Egyptian *Astragalus* spp. as modulators of lymphocyte proliferation. Planta Med. 68, 986–994.