Bioactive Cycloartane-Type Triterpene Glycosides from *Astragalus elongatus*

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Together with two known cycloartane-type glycosides, askendosides D (3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-6-*O*-β-xylopyranosyl-cycloastragenol, **2**) and G (3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-16-*O*-β-glucopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane, **3**), also a new monodesmosidic cycloartane-type glycoside, elongatoside (**1**), was isolated from the roots of *Astragalus elongatus* and identified as 3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-cycloastragenol. All structures were unambiguously determined by means of spectroscopic and spectrometric methods (1D and 2D NMR, ESI-MS). The isolated compounds were tested for the inhibition of proliferation and ICAM-1 expression *in vitro* using the human microvascular endothelial cell line (HMEC-1). **1** showed weak activity in the ICAM-1 assay.

Key words: Astragalus elongatus, Cycloartane-Type Glycosides, Endothelium

Introduction

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). Cycloartane-type triterpene glycosides are the main secondary metabolites and found to be responsible for diverse biological activities of the roots of *Astragalus* species. Some cycloartane glycosides obtained from *Astragalus* species act as modulators of lymphocyte proliferation (Çalış *et al*., 1997; Verotta *et al*., 2001, 2002). The immunostimulant effects of several cycloartane-type triterpene glycosides on macrophage activation and expression of inflammatory cytokines were investigated (Bedir *et al*., 2000). As diverse effects, the cholagogic (Tursunova *et al*., 2003) and antitrypanosomal activities of cycloartane glycosides have been reported (Özipek *et al.*, 2005; Çalış *et al*., 2006). Further cycloartane-type glycosides isolated from *Astragalus* species were reported to be potential tyrosinase inhibitors (Khan *et al.*, 2006), and cyclosieversigenin (= cycloastragenol) glycosides have been reported to show most promising activity. Therefore, this type of glycosides has been suggested to be lead molecules for the development of new medications of several skin diseases related with the over-expression of the enzyme tyrosinase, like hyperpigmentation. Very recently, cancer chemopreventive effects of cycloartane-type and related triterpenoids have been studied (Kikuchi *et al*., 2007). This promising spectrum of pharmacological effects led us to further search for structurally interesting cycloartane glycosides from the genus *Astragalus*. Under this perspective, we now studied the roots of *A. elongatus*, which is one of the *ca.* 400 species recorded in the flora of Turkey (Chamberlain and Matthews, 1970), reporting the isolation and structure elucidation of three cycloartane glycosides, **1**–**3**, of which compound **1** is new to nature. Due to the use of *Astragalus* species against inflammatory diseases or cancer the potential anti-inflammatory and anti-angiogenic influence of the isolated compounds were investigated on endothelial cells by *in vitro* assays with the human microvascular cell line HMEC-1.

Results and Discussion

The methanolic extract of the roots of *A. elongatus* was separated by a combination of VLC (vacuum

liquid chromatography) and column chromatography (CC) over silica gel using CH_2Cl_2/MeOH/H_2O and EtOAc/MeOH/H_2O mixtures as eluents to yield three cycloartane-type glycosides, **1**–**3**. The NMR data of compounds **1**, **2** and **3** (Tables I and II) revealed characteristic features of cycloartane-type glycosides. Compounds **1** and **2** are cycloastragenol [cyclosieversigenin, (20*R*,24*S*)-3β,6α,16β,25-tetrahydroxy-20,24-epoxy-9,19-cyclolanostane] derivatives (Kitagawa *et al.*, 1983), while compound **3** is a cycloasgenin C [3β,6α,16β,24(*R*),25-pentahydroxycycloartane] derivative.

Compound **1** gave quasimolecular ion peaks in the positive and negative ion ESI-mass spectra at *m/z* 755 $[M+H]^+$, 772 $[M+NH_4]^+$ and 753 $[M-H]^-$, respectively, which are in accordance with the molecular formula $C_{40}H_{66}O_{13}$. The NMR data of **1** were consistent with the presence of a cycloartane-type diglycosidic structure. All NMR assignments (Table I) were based on COSY, HSQC and HMBC experiments. The ^{1}H NMR spectrum of **1** displayed diagnostic signals due to the cyclopropane-ring methylene protons as an AX system (δ 0.54 and 0.37, J_{AX} = 4.2 Hz, H-19a and 19b) and seven tertiary methyl groups (δ 1.30, 1.27, 1.26, 1.21, 1.13, 1.01 and 1.00) for the cycloartane-type aglycone moiety. The ^{13}C NMR spectrum exhibited signals for 40 carbon atoms, 30 of which were assigned to the aglycone moiety, while the remaining were due to two pentapyranose units. The ^{13}C resonance attributed to C-3 (δ 89.6) was only found to be shifted downfield by +8–9 ppm compared to that of cycloastragenol (Kitagawa *et al.*, 1983), indicating the presence of a monodesmosidic structure. The signals for two anomeric protons were observed at δ 4.44 (*d, J* = 7.0 Hz) and 4.46 (*d, J* = 6.9 Hz). The corresponding anomeric carbon resonances were observed at δ 105.8 and 106.5, respectively. The results of the COSY experiment allowed the sequential assignments of all proton resonances within each sugar residue starting from the isolated anomeric proton signals. Thus, on the basis of the chemical shifts, multiplicity of the signals and the coupling constans, the sugar units were identified as an α-arabinopyranosyl and a β-xylopyranosyl unit. The absence of any ^{13}C NMR glycosidation shift for the α-arabinopyranosyl unit suggested this sugar to be terminal. The sites of glycosidations were unambiguously determined by the HMBC experiment which showed long-range correlations between C-3 (δ 89.6) of the aglycone and H-1′ of xylose (δ 4.44), C-2′ of xylose (δ 83.3) and H-1″ of arabinose (δ 4.46). Based on these results, the structure of **1** was established as (20*R*,24*S*)-3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-3β,6α,16β,25-tetrahydroxy-20,24-epoxycycloartane (Fig. 1). This compound has not been reported as a genuine natural compound up to now and was named as elongatoside. This monodesmosidic structure has been obtained as one of the partial hydrolysis products of askendoside D (Isaev *et al.*, 1983). The reported optical rotation value for this compound $\{[\alpha]_D^{21} + (26.4 \pm 2)^\circ$ (*c* 1.06 MeOH)} is almost the same as that of compound **1** $\{[\alpha]_D^{31} + 31.0^\circ$ (*c* 0.1 MeOH)}.

Compound **2** gave quasimolecular ion peaks in the positive and negative ion ESI-mass spectra at *m/z* 887 $[M+H]^+$, 904 $[M+NH_4]^+$ and 885 $[M-H]^-$, respectively, which are in accordance with the molecular formula $C_{45}H_{74}O_{17}$. The signals observed in the ^{1}H and ^{13}C NMR spectra of **2** (Table I) were very similar to those of compound **1** except for the presence of a third sugar unit. The carbon resonances arising from the sugar units clearly indicated the presence of the same biosidic sugar moiety, α-arabinopyranosyl-(1→2)-β-xylopyranosyl, attached to C-3(OH) and an additional xylopyranosyl unit glycosylated at C-6(OH) of the aglycone moiety. Thus, the structure of **2** was a bisdesmosidic glycoside. This was obvious from the ^{1}H NMR spectrum that contained three anomeric protons at δ 4.46 (*d, J* = 7.0 Hz, H-1′), 4.47 (*d, J* = 6.8 Hz, H-1″), and 4.29 (*d, J* = 7.4 Hz, H-1‴). The anomeric proton of the second xylopyranosyl unit (δ 4.29) showed HMBC connectivity to C-6 (δ 79.3). Additional cross-peaks observed in the HMBC experiment between H-1′ and C-3 (δ 89.5) and H-1″ and C-2′ (δ 83.2) revealed that the disaccharide chain at C-3 was the same as in compound **1**. All other structural assignments were further substantiated by DQF-COSY, HSQC and HMBC data. In comparison to NMR data of **1**, the significant differences for **2** were observed for the C-5, C-6, C-7 and C-8 resonances (see Table I) due to the glycosidation at C-6(OH). These results indicated the structure of **2** to be (20*R*,24*S*)-3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-6-*O*-β-xylopyranosyl-3β,6α,16β,25-tetrahydroxy-20,24-epoxycycloartane (Fig. 1), previously reported as askendoside D (Isaev *et al.*, 1983).

The positive and negative ion ESI-mass spectra of compound **3** exhibited a $[M+NH_4]^+$ peak at *m/z* 936 and a $[M-H]^-$ peak at *m/z* 917, respectively, which were compatible with the molecular formula

Table I. ^{13}C and ^{1}H NMR spectroscopic data of **1** and **2** (MeOD; δ_C, 150 MHz; δ_H, 600 MHz).

C/H	DEPT	**1**		**2**	
		δ_C (ppm)	δ_H (ppm), *J* [Hz]	δ_C (ppm)	δ_H (ppm), *J* [Hz]
Agly					
1	CH_2	33.3	1.53 *dt* (13.0, 2.0), 1.24†	32.9	1.55†, 1.28†
2	CH_2	30.6	1.91†, 1.70†	30.3	1.91†, 1.68†
3	CH	89.6	3.23†	89.5	3.20†
4	C	43.2	–	43.2	–
5	CH	54.7	1.38 *d*	53.0	1.62†
6	CH	69.6	3.48†	79.3	3.52†
7	CH_2	38.9	1.46 *dt* (12.2, 4.0), 1.40†	34.4	1.79 *dt* (14.7, 4.5), 1.63†
8	CH	48.7	1.80 *dd* (12.2, 4.2)	45.8	1.90†
9	C	21.9	–	22.1	–
10	C	30.7	–	29.6	–
11	CH_2	26.8		27.0	1.85†, 1.41†
12	CH_2	34.1	1.65†	34.2	1.67†, 1.55†
13	C	46.0	–	46.1	–
14	C	47.0	–	47.1	–
15	CH_2	46.9	1.95 *dd* (12.8, 8.1), 1.40 *dd* (12.8, 6.1)	46.0	2.00 *dd* (12.4, 7.9), 1.38 *dd* (12.4, 6.1)
16	CH	74.6	4.65 *ddd* "*q* like" (8.0, 7.7, 6.0)	74.6	4.65 *ddd* "*q* like" (8.0, 7.7, 6.1)
17	CH	59.1	2.36 *d* (8.0)	58.8	2.36 *d* (7.7)
18	CH_3	22.0	1.27 *s*	21.0	1.24 *s*
19	CH_2	32.2	0.54 *d* (4.2), 0.37 *d* (4.2)	28.4	0.57 *d* (4.4), 0.22 *d* (4.4)
20	C	88.4	–	88.5	–
21	CH_3	28.5	1.21 *s*	28.6	1.21 *s*
22	CH_2	35.5	2.61 *q* like (10.2), 1.65†	35.5	2.61 *q* like (10.4), 1.65†
23	CH_2	27.0	2.06†	26.8	2.02
24	CH	82.7	3.76 *dd* (7.7, 6.4)	82.6	3.76 *dd* (8.4, 5.8)
25	C	72.5	–	72.5	–
26	CH_3	27.6	1.26 *s*	27.6	1.26 *s*
27	CH_3	26.7	1.13 *s*	26.7	1.14 *s*
28	CH_3	28.6	1.30 *s*	28.2	1.24 *s*
29	CH_3	16.3	1.01 *s*	16.4	0.99 *s*
30	CH_3	20.5	1.00 *s*	20.1	1.03 *s*
Xyl					
1′	CH	105.8	4.44 *d* (7.0)	105.6	4.46 *d* (7.0)
2′	CH	83.3	3.42 *dd* (7.0, 9.0)	83.2	3.43 *dd* (7.0, 9.0)
3′	CH	77.0	3.56†	77.0	3.50†
4′	CH	71.1	3.55†	71.0	3.45†
5′	CH_2	66.1	3.85 *dd* (11.3, 4.3), 3.21†	66.1	3.85 *dd* (11.0, 4.6), 3.19†
Ara					
1″	CH	106.5	4.46 *d* (6.9)	106.4	4.47 *d* (6.8)
2″	CH	73.6	3.65 *dd* (6.9, 9.0)	73.5	3.65 *dd* (6.8, 9.0)
3″	C	74.2	3.56 *dd* (9.0, 3.5)	74.2	3.55 *dd* (9.0, 3.5)
4″	CH_2	69.7	3.79 *m*	69.6	3.79 *m*
5″	CH_2	67.4	3.88 *dd* (12.4, 2.8), 3.52†	67.2	3.89 *dd* (12.6, 3.5), 3.51†
Xyl					
1‴	CH			105.5	4.29 *d* (7.4)
2‴	CH			75.4	3.18†
3‴	CH			78.0	3.30†
4‴	CH			71.2	3.46†
5‴	CH_2			66.7	3.82 *dd* (11.0, 4.6), 3.18†

† Signal pattern unclear due to overlapping.

$C_{46}H_{78}O_{18}$. The ^{1}H NMR spectrum of **3** showed signals characteristic of cyclopropane ring methylene protons as an AX system (δ 0.36, 0.51, J_{AX} = 4.2 Hz, H_2-19), six tertiary methyl groups at δ 0.95, 1.01, 1.14, 1.16, 1.16, 1.29, and a secondary methyl group at δ 0.94 (*d*, *J* = 6.6 Hz). Additionally, three anomeric protons at δ 4.27 (*d*, *J* = 7.8 Hz, H-1‴), 4.45 (*d*, *J* = 7.0 Hz, H-1′) and 4.47 (*d*, *J* = 6.8 Hz, H-1″) indicated the presence of two β- and one α-linked sugar units. The ^{13}C NMR spectrum exhibited 46

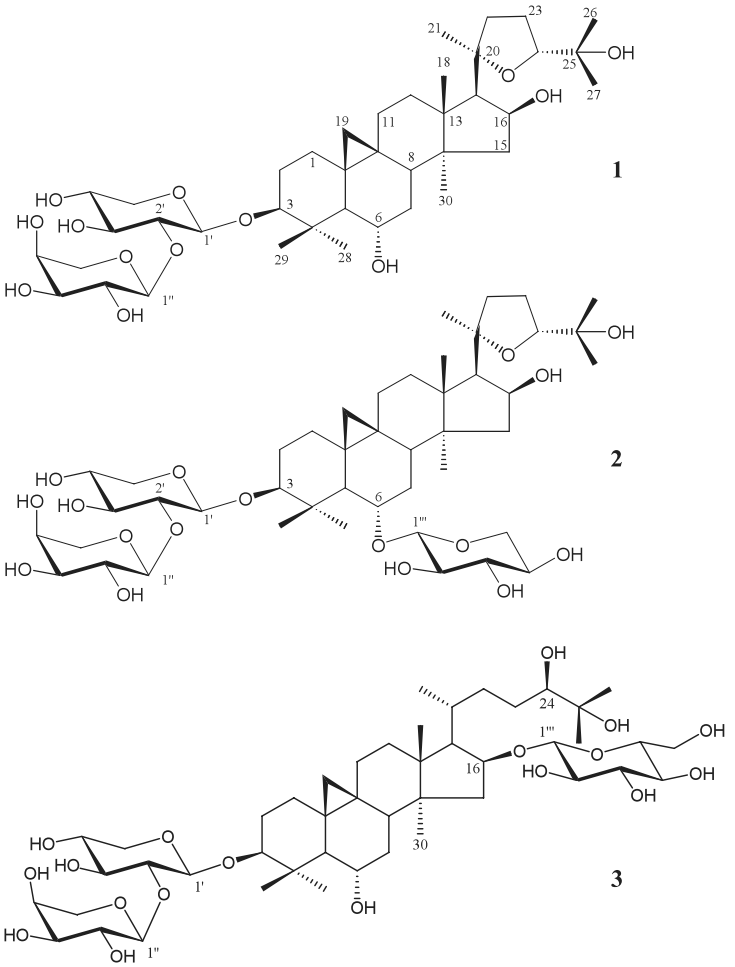

Fig. 1. Chemical structures of elongatoside (**1**), askendoside D (**2**) and askendoside G (**3**).

carbon atoms, 30 of which were assigned to the aglycone moiety while the remaining were due to one hexose and two pentose units. All NMR assignments (Table II) were based on COSY, HSQC, and HMBC experiments. Apart from the anomeric protons, COSY experiments revealed all proton resonances of the sugar units. The chemical shifts and coupling constants of the signals assigned to the sugar moieties indicated the presence of a glucose, a xylose and an arabinose unit. The NMR spectroscopic data (Table II) attributed to the aglycone moiety, especially for the side chain representing a chiral centre at C-24, were in good agreement with those of cycloasgenin C [3β,6α, 16β,24(*R*),25-pentahydroxycycloartane] (Agzamova and Isaev, 2000). Glycosylations at C-3(OH) and C-16(OH) were indicated by the pronounced downfield shifts observed for C-3 (δ 89.7) and C-16 (δ 84.2) relative to the corresponding signals in cycloasgenin C (Agzamova and Isaev, 1999), whereas the C-6 (δ 69.6), C-24 (δ 80.5) and C-25 (δ 73.6) resonances indicated the unglycosylated hydroxy groups at these positions. This suggested that C-3 and C-16 were the sites of glycosidations on the sapogenol moiety, confirming the bisdesmosidic structure of **3**. The HMBC correlations observed from H-1′ (δ 4.45) of xylose and H-1‴ (δ 4.27) of glucose to C-3 (δ 89.7) and C-16 (δ 84.2) of cycloasgenin C, respectively, further proved these assumptions. The third anomeric proton signal at δ 4.47 (H-1″) assigned to the α-arabinopyranosyl unit showed long-range correlation with the C-2′ atom (δ 83.0) of the xylose moiety attached to C-3 of the aglycone. This revealed that C-2′ was the

Table II. ^{13}C and ^{1}H NMR spectroscopic data of **3** (MeOD; δ_C, 150 MHz; δ_H, 600 MHz).

C/H	DEPT	**3**	
		δ_C (ppm)	δ_H (ppm), *J* [Hz]
Agly			
1	CH_2	33.4	1.52 *dt* (13.0, 2.3), 1.20†
2	CH_2	30.6	1.90†, 1.66†
3	CH	89.7	3.20†
4	C	43.2	–
5	CH	54.7	1.36†
6	CH	69.6	3.41†
7	CH_2	38.8	1.48 *dt* (12.2, 3.6), 1.32†
8	CH	49.0	1.78 *dd* (12.2, 4.2)
9	C	22.2	–
10	C	30.4	–
11	CH_2	27.1	1.98†, 1.15†
12	CH_2	33.7	1.65†
13	C	46.4	–
14	C	47.7	–
15	CH_2	49.1	2.08 *dd* (13.8, 8.0), 1.69†
16	CH	84.2	4.21 *q* like (8.0, 7.8, 4.9)
17	CH	58.4	1.80 *dd* (10.6, 8.0)
18	CH_3	19.4	1.16 *s*
19	CH_2	31.8	0.51 *d* (4.2), 0.36 *d* (4.2)
20	CH	32.5	1.92†
21	CH_3	18.4	0.94 *d* (6.6)
22	CH_2	34.7	0.84†, 1.90†
23	CH_2	30.2	1.14, 1.90
24	CH	80.5	3.16†
25	C	73.6	–
26	CH_3	25.1	1.14 *s*
27	CH_3	25.6	1.16 *s*
28	CH_3	16.4	1.01 *s*
29	CH_3	28.6	1.29 *s*
30	CH_3	20.5	0.95 *s*
Xyl			
1′	CH	105.6	4.45 *d* (7.0)
2′	CH	83.0	3.42†
3′	CH	76.5	3.50†
4′	CH	71.0	3.50†
5′	CH_2	65.9	3.86 *dd* (11.9/4.7), 3.18†
Ara			
1″	CH	106.3	4.47 *d* (6.8)
2″	CH	73.6	3.65 *dd* (6.8/9.0)
3″	CH	74.0	3.55 *dd* (9.0/3.5)
4″	CH	69.7	3.79 *m*
5″	CH_2	67.3	3.88 *dd* (12.6/3.0), 3.51†
Glu			
1‴	CH	106.5	4.27 *d* (7.8)
2‴	CH	75.7	3.15†
3‴	CH	78.5	3.28†
4‴	CH	71.8	3.24†
5‴	CH	77.7	3.22†
6‴	CH_2	62.9	3.82 *dd* (11.7/2.2), 3.67 *dd* (11.7/5.2)

† Signal pattern unclear due to overlapping.

site of glycosidation, indicating the presence of the same biosidic sugar moiety at C-3(OH) as in **1** and **2**. Thus, the structure of **3** was identified as 3-*O*-[α-arabinopyranosyl-(1→2)-β-xylopyranosyl]-16-*O*-β-glucopyranosyl-3β,6α,16β,24(*R*),25-pentahydroxycycloartane (askendoside G, Fig. 1), which was first reported from *Astragalus stipulosus* by Agzamova and Isaev (2000) and Karimov *et al.* (2001). Both studies have reported NMR data of **3** in pyridine-d_5. Therefore, the spectroscopic results in CD_3OD obtained in this study are presented together with a brief discussion.

The activity of compounds **1**–**3** against the proliferation of HMEC-1 cells were insignificant in the concentration range 0.05–50 μM (Fig. 2). Inhibition of the ICAM-1 expression was weak, but significant ($p \leq 0.0004$) for compounds **1**–**3** at the concentration of 50 μM (Fig. 3). The observed activity on ICAM-1 is not connected to a cytotoxic effect, due to the fact that MTT and FACS analyses showed no influence on the cell viability for all compounds tested (data not shown). Due to the low observed activity it is questionable whether these compounds can contribute to the anti-inflammatory effects of extracts from *Astragalus* species via inhibition of ICAM-1.

Experimental

General experimental procedures

UV spectra were recorded on a HP Agilent 8453 spectrophotometer. Optical rotations were measured on a Rudolph Autopol IV polarimeter. 1D and 2D NMR spectra were recorded on a Bruker AMX (Avance) 600 MHz spectrometer at 295 K and referenced against the respective residual non-deuterated solvent (see Tables I and II). ESI-mass spectra were measured on a ThermoQuest Finnigan 7000 instrument with a spray voltage of 4 kV. TLC analyses were carried out on silica gel 60 F254 precoated plates (0.2 mm; Merck, Darmstadt, Germany). 1% Vanillin/H_2SO_4 was used as detection reagent.

Plant material

Astragalus elongatus Willd. was collected from Central Anatolia, Ahlatlıbel, Ankara, Turkey in May 2005. A voucher specimen (HUEF 05005) has been deposited at the Herbarium of Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

Extraction and isolation

Air-dried powdered roots of the plant (150 g) were extracted with MeOH (2 × 2 l). The metha-

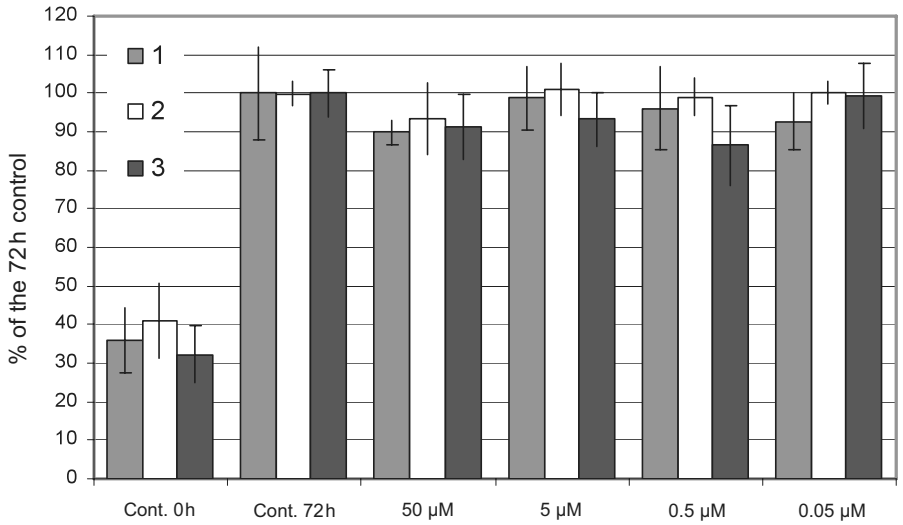

Fig. 2. Antiproliferating activity of compounds **1**–**3** on HMEC-1 cells after 72 h (concentration range: 0.05–50 μM; n = 3; means ± SE).

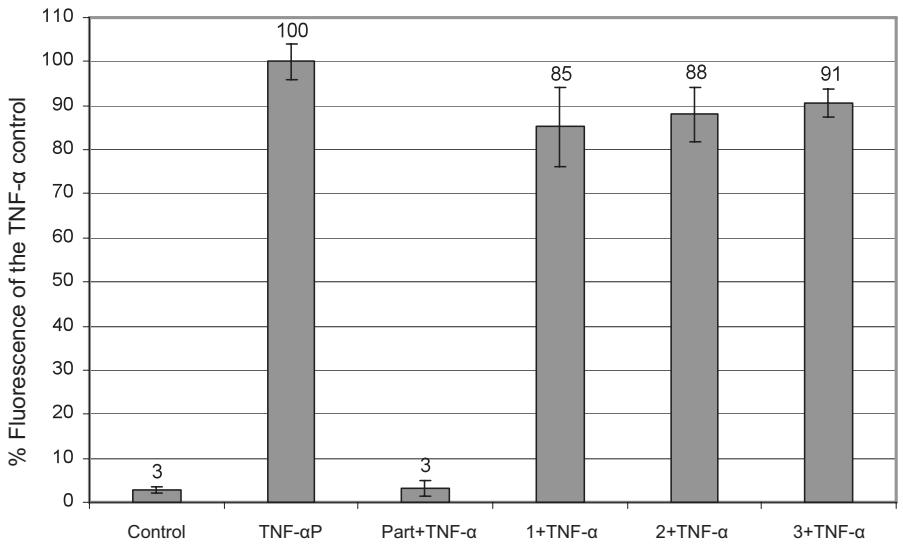

Fig. 3. Inhibition of ICAM-1 expression after 24 h in the presence of compounds **1**–**3** on HMEC-1 cells (50 μM; n = 3; means ± SE); parthenolide (P) was used as positive control.

nolic extract was evaporated under reduced pressure. The water-soluble part of the residue was lyophilized to give 6.09 g of extract which was then fractionated by VLC over silica gel (200 g) using gradient mixtures of CH_2Cl_2/MeOH/H_2O (from 90:10:1 to 50:50:5, 200 ml each) and MeOH (400 ml) to give fifteen fractions. According to their TLC profiles they were combined into six main fractions (A, 241 mg; B, 146 mg; C, 1040 mg; D, 2901 mg; E, 297 mg; and F, 725 mg). Fractions C and D were rich in cyloartane-type compounds. Fraction C (1040 mg) was further subjected to silica gel column chromatography (100 g) using CH_2Cl_2/MeOH/H_2O mixtures (80:20:2, 800 ml; 75:25:2.5, 400 ml; 70:30:3, 500 ml) yielding seventy fractions (20 ml each). Fractions 14–28 were rich in **1**, while fractions 40–51 in **2** and fractions 52–59 in **3**. Fractions 14–28 (226 mg) and 40–51 (190 mg) were

separately rechromatographed over an SiO_2 column (60 g) using EtOAc/MeOH/H_2O mixtures (10:1:0.5, 460 ml; 10:1.5:0.5, 240 ml; 10:2:0.5, 250 ml) as solvent system to give compounds **1** (27 mg) and **2** (60 mg), repectively. Fractions 52–59 (68 mg) were also subjected to an SiO_2 column (35 g) using the same solvent mixtures in different ratio (10:1.5:0.5, 600 ml; 10:2.5:0.75, 400 ml) affording **3** (38 mg).

Elongatoside (**1**): Amorphous white powder; $[\alpha]_D^{31}$ +31.0° (*c* 0.1 MeOH). – ^{1}H NMR (600 MHz, MeOD) and ^{13}C NMR (150 MHz, MeOD): see Table I. – Positive and negative ion ESI-MS: *m/z* = 755 $[M+H]^+$, 772 $[M+NH_4]^+$ and 753 $[M-H]^-$. – HRFABMS: *m/z* = 755.4564 $[M+H]^+$ (calcd. for $C_{40}H_{67}O_{13}$ 755.4582 $[M+H]^+$).

Askendoside D (**2**): Amorphous white powder; $[\alpha]_D^{31}$ +19.0° (*c* 0.1 MeOH). – ^{1}H NMR (600 MHz, MeOD) and ^{13}C NMR (150 MHz, MeOD): see Table I. – Positive and negative ion ESI-MS: *m/z* = 887 $[M+H]^+$, 904 $[M+NH_4]^+$ and 885 $[M-H]^-$. – HRFABMS: *m/z* = 887.4993 $[M+H]^+$ (calcd. for $C_{45}H_{75}O_{17}$ 887.5004 $[M+H]^+$).

Askendoside G (**3**): Amorphous white powder; $[\alpha]_D^{27}$ +30.9° (*c* 0.1 MeOH). – ^{1}H NMR (600 MHz, MeOD) and ^{13}C NMR (150 MHz, MeOD): Table II. – Positive and negative ion ESI-MS: *m/z* = 936 $[M+NH_4]^+$, 917 $[M-H]^-$. – HRFABMS: *m/z* = 919.5263 $[M+H]^+$ (calcd. for $C_{46}H_{79}O_{18}$ 919.5266 $[M+H]^+$).

Cell culture

Human microvascular endothelial cells (HMEC-1) (Ades *et al.*, 1992) were cultivated at 37 °C in 5% CO_2 in ECGM medium (Provitro, Berlin, Germany) containing 10% fetal bovine serum (inactivated), supplements and antibiotics. All experiments were performed three times with three different passages.

Proliferation assay

For the proliferation assay 100 μl of the adherent cells were seeded into collagen-coated 96-well plates ($1.5 \cdot 10^4$ cells/ml, n = 6 for each value). After 24 h, 100 μl of the test compounds dissolved in medium were added to give the final concentrations of 0.05–50 μM. 0 h control cells were stained with an ethanolic solution of crystal violet (CV) for 15 min and washed with water (Kueng *et al.*, 1989). For maximum proliferation control only 100 μl medium were added. The cells were incubated for further 72 h and then stained with CV. For quantification the CV was dissolved with 100 μl of a 0.1 M ethanolic Na citrate solution and the absorbance was measured with a SpectraFluor plus plate reader (Tecan, Crailsheim, Germany) at 560 nm.

ICAM-1 inhibition assay

ICAM-1 expression was measured according to Dirsch *et al.* (2004). Confluent grown HMEC-1 cells were preincubated with test compunds (50 μM in medium), parthenolide (10 μM in medium; positive control) or only with medium (negative control) in 24-well plates (n = 3 for each value). 20 min later TNF-α (10 ng/ml) was added to stimulate ICAM-1 expression. After 24 h cells were removed from the plates, fixed with formalin, washed with PBS, and a FITC-labeled mouse antibody against ICAM-1 (Biozol, Eching, Germany) was added. For quantification the amount of fluorescence was measured by FACS analysis (Becton/Dickinson FACSCalibur, Heidelberg, Germany).

Viability assay

The MTT viability assay was performed according to Mosman (1983) (modified). Confluently grown HMEC-1 cells were incubated with test compounds in 96-well plates (0.05–50 μM in medium; pure medium for negative control; n = 6 for each value). After 24 h the substances and the supernatant were removed and 10 μl MTT (5 mg/ml) in 90 μl medium were added for 3 h. This solution was exchanged for 10% sodium dodecyl sulfate, and 24 h later the absorbance was measured with a SpectraFluor plus plate reader (Tecan, Germany) at 560 nm.

Statistics

All data are expressed as means ± SE. Unpaired t-test was used to determine the significance of the effect in comparison to the untreated controls.

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