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Saponins from *Astragalus hareftae* (NAB.) SIRJ.

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ABSTRACT

Four cycloartane- (hareftosides A–D) and oleanane-type triterpenoids (hareftoside E) were isolated from *Astragalus hareftae* along with fifteen known compounds. Structures of the compounds were established as 3,6-di-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**1**), 3,6,24-tri-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**2**), 3-*O*-β-D-xylopyranosyl-3β,6α,16β,25-tetrahydroxy-20(*R*),25(*S*)-epoxycycloartane (**3**), 16-*O*-β-D-glucopyranosyl-3β,6α,16β,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane (**4**), 3-*O*-[β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucuronopyranosyl]-soyasapogenol B (**5**) by the extensive use of 1D- and 2D-NMR experiments along with ESI-MS and HR-MS analyses.

1. Introduction

The Fabaceae (Leguminosae) is a family of flowering plants comprising 269 genera and 5100 species (Davis, 1970). *Astragalus* L., the largest genus in the family Leguminosae, is represented by 445 species in the flora of Turkey and 224 of these species are endemic (Davis, 1970; Aytaç, 2000).

Gum tragacanth, a very well known foodstuff and pharmaceutical emulsifier, is an economically important natural product obtained from *Astragalus* species growing wild in Turkey (Tang and Eisenbrand, 1992). In Turkish folk medicine, the aqueous extracts of some *Astragalus* species (Sevimli-Gür et al., 2011) are used to treat leukemia as well as healing wounds (Calis et al., 1997; Bedir et al., 2000). Roots of these plants are also used as antiperspirant, diuretic, tonic and for the treatment of nephritis, diabetes, leukemia and uterine cancer (Tang and Eisenbrand, 1992).

Polysaccharides and saponins are the major classes of chemical compounds isolated from *Astragalus* species; however, the most investigated constituents are saponins. Cycloartane- and oleanane-type glycosides from *Astragalus* species show interesting biological properties, including immune-stimulating (Bedir et al., 2000; Yesilada et al., 2005; Nalbantsoy et al., 2011, 2012), antiprotozoal (Özipek et al., 2005), wound healing (Sevimli-Gür et al., 2011) antiviral and cytotoxic activities (Tian et al., 2005).

Several cycloartane and oleanane-type triterpene glycosides were isolated from Turkish *Astragalus* species (Ríos and Waterman, 1997; Verotta and El-Sebakhy, 2001; Bedir et al., 1998a,b, 1999; Polat et al., 2009, 2010; Horo et al., 2010; Gülcemal et al., 2011).

As part of our studies on the new bioactive compounds from Turkish *Astragalus* species, we carried out a study on *Astragalus hareftae* (NAB.) SIRJ. (Leguminosae). This paper reports the isolation of four new cycloartane-type (**1**–**4**) and a new oleanane-type triterpene glycosides from the methanolic extract of the whole plant of *A. hareftae* along with 11 known cycloartane-type glycosides (**6**–**16**) and four known oleanane-type triterpene glycosides (**17**–**20**). Their structures were elucidated by extensive spectroscopic methods including 1D- (^{1}H, ^{13}C and TOCSY) and 2D-NMR (DQF-COSY, HSQC, HMBC, and ROESY) experiments as well as ESI-MS and HR-MS analyses.

2. Results and discussion

The HR-MALDI-TOF mass spectrum of **1** (*m/z* 779.4564 $[M+Na]^+$, calcd. for $C_{40}H_{68}O_{13}Na$, 779.4558) supported a molecular formula of $C_{40}H_{68}O_{13}$. The ESI-MS showed the major ion peak at *m/z* 779 which was assigned to $[M+Na]^+$. The MS/MS of this ion showed a peak at *m/z* 647 $[M+Na-132]^+$, corresponding to the loss of a pentose unit. In the MS^3 spectrum, a peak at *m/z* 515 $[M+Na-132-132]^+$, corresponding to the loss of an additional pentose unit, was observed.

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In particular, the ^{1}H NMR spectrum of **1** showed characteristic signals due to a cyclopropane methylene as an AX system at δ_H 0.58 and 0.24 (each 1H, d, J = 4.2 Hz), six tertiary methyl groups at δ_H 1.16, 1.17, 1.19, 1.26, 1.02 and 1.00, which were correlated in HSQC with carbon signals at δ_C 18.1, 25.2 (x2), 28.0, 16.5 and 19.7, respectively. A secondary methyl group at δ_H 1.06 (3H, d, J = 6.5 Hz), and four oxygen bearing methine proton signals at δ_H 4.48 (ddd, J = 8.0, 8.0, 5.2 Hz), 3.53 (ddd, J = 9.5, 9.5, 4.5 Hz), 3.41 (dd, J = 10.5, 2.2 Hz) and 3.23 (dd, J = 11.3, 4.0 Hz), which were indicative of secondary alcoholic functions (Table 1), were readily noticed in the ^{1}H NMR spectrum.

The NMR data of the aglycon moiety of **1** was in good agreement with those reported for cyclocanthogenin (Bedir et al., 1998a), with glycosidation shifts for C-3 (δ 89.8) and C-6 (δ 79.3) (Table 1), thus, confirming the bisdesmosidic structure of **1** (Fig. 1). Additionally, signals of two anomeric protons at δ_H 4.310 (d, J = 7.5 Hz) and 4.312 (d, J = 7.5 Hz) were observed with ^{13}C chemical shifts of their relative attached carbons (δ 107.5, 105.6, respectively). The chemical shifts of the rest of the individual protons of the two sugar units were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analyses, and ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (Table 2), substantiating the presence of two β-xylopyranoside units. Unambiguous determination of the sequence and linkage sites was obtained from the HMBC spectrum, which showed key correlation peaks between the proton signal at δ_H 4.310 (H-1′$_{xyl}$) and the carbon resonance at δ_C 89.8 (C-3), and the proton signal at δ_H 4.312 (H-1″$_{xyl}$) and the carbon resonance at δ_C 79.3 (C-6). The D configuration of the xylose units was established after the hydrolysis of **1** followed by GC analysis (De Marino et al., 2003).

On the basis of all the evidence, the structure of compound **1** was established as 3,6-di-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*), 25-pentahydroxycycloartane, named hareftoside A.

The HR-MALDI-TOF mass spectrum of **2** (*m/z* 911.4984 [M+Na]$^+$, calcd. for $C_{45}H_{76}O_{17}Na$, 911.4980) supported a molecular formula of $C_{45}H_{76}O_{17}$. The ESI-MS spectrum showed the major ion peak at *m/z* 911 which was assigned to [M+Na]$^+$. The MS/MS of this ion showed peaks at *m/z* 779 [M+Na−132]$^+$, 647 [M+Na−132−132]$^+$ and 515 [M+Na−132−132−132]$^+$, corresponding to the loss of three pentose units, respectively.

The HSQC, HMBC and COSY spectra of **2** in comparison with those of **1** showed that **2** differed from **1** by the presence of an additional xylose unit. The anomeric protons were observed at δ_H 4.32 (d, J = 7.5 Hz), 4.33 (d, J = 7.5 Hz) and 4.40 (d, J = 7.5 Hz) with ^{13}C chemical shifts of their relative attached carbons at δ 107.8, 105.9 and 105.7, respectively. Moreover signal C-24 was found about +10 ppm downfield shifted, in comparison to that of compound **1**. The HMBC correlation between the proton signal at δ_H 4.33 (H-1′$_{xyl}$) and the carbon resonance at δ 89.9 (C-24) allowed us to determine the linkage site of the additional sugar unit. The configuration of the three xylose units was established as D after the hydrolysis of **2** followed by GC analysis (De Marino et al., 2003).

Thus, the structure of **2** was established as 3,6,24-tri-*O*-β-D-xylopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane, for which the trivial name hareftoside B is proposed.

The molecular formula of **3** was established as $C_{35}H_{58}O_9$ by the HR-MALDI-TOF mass spectrum (*m/z* 645.3982 [M+Na]$^+$, calcd. for

Table 1
^{13}C and ^{1}H NMR data of the aglycon moieties of compounds **1–5** (600 MHz, CD_3OD).

Position	**1**		**2**		**3**		**4**		**5**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	32.7	1.56, 1.30, m	32.7	1.63, 1.30, m	33.2	1.56, 1.31, m	33.1	1.59, 1.29, m	39.6	1.66, 1.07, m
2	30.2	1.95, 1.69, m	30.2	1.97, 1.64, m	30.3	1.95, 1.69, m	30.8	1.74, 1.65, m	26.9	2.15, 1.80, m
3	89.8	3.23, dd (11.3, 4.0)	90.7	3.22, dd (11.3, 4.0)	89.5	3.23, dd (11.3, 4.0)	79.3	3.24, dd (11.3, 4.0)	91.4	3.47, dd (11.5, 4.5)
4	42.8	–	42.8	–	43.0	–	42.5	–	44.3	–
5	52.9	1.65, d (9.5)	53.1	1.65, d (9.5)	54.6	1.38, d (9.5)	54.3	1.37, d (9.5)	57.3	0.98, m
6	79.3	3.53, ddd (9.5, 9.5, 4.5)	79.2	3.53, ddd (9.5, 9.5, 4.5)	69.4	3.47, ddd (9.5, 9.5, 4.5)	69.6	3.48, ddd (9.5, 9.5, 4.5)	19.5	1.67, 1.40, m
7	39.9	1.72, 1.68, m	39.9	1.71 (2H), m	38.7	1.46, 1.37, m	38.7	1.51, 1.37, m	34.4	1.58,1.43, m
8	45.8	1.91, dd (11.9, 4.2)	45.7	1.92, m	48.6	1.83, dd (11.9, 4.2)	48.4	1.88, dd (11.9, 4.2)	40.6	–
9	22.1	–	22.0	–	21.7	–	21.8	–	48.9	1.61, m
10	29.3	–	29.3	–	30.4	–	30.3	–	37.4	–
11	26.8	1.84, 1.44, m	27.5	1.84, 1.42, m	26.9	2.03, 1.21, m	26.9	2.01, 1.25, m	24.8	1.91, (2H) m
12	33.7	1.79 (2H), m	33.8	1.81 (2H), m	34.5	1.92, 1.73, m	33.6	1.79 (2H), m	123.2	5.28, t (3.5)
13	46.7	–	46.6	–	47.3	–	47.3		145.4	–
14	46.8	–	46.8	–	47.3	–	47.5	–	43.3	–
15	47.6	2.08, dd (12.7, 8.0) 1.39, dd (12.7, 5.2)	48.2	2.08, dd (12.7, 8.0) 1.41, dd (12.7, 5.2)	48.3	1.99, dd (12.7, 8.0) 1.49, dd (12.7, 5.2)	48.1	2.10, dd (12.7, 8.0) 1.81, dd (12.7, 5.2)	26.9	1.84, 1.05, m
16	73.2	4.48, ddd (8.0, 8.0, 5.2)	72.8	4.44, ddd (8.0, 8.0, 5.2)	74.8	4.64, ddd (8.0, 8.0, 5.2)	84.4	4.36, ddd (8.0, 8.0, 5.2)	29.8	1.78 (2H), m
17	57.7	1.73, dd (9.9, 8.0)	58.1	1.74, dd (9.9, 8.0)	61.2	2.01, dd (9.9, 8.0)	60.2	2.35, dd (9.9, 8.0)	38.6	–
18	18.1	1.16, s	18.0	1.17, s	21.2	1.45, s	21.6	1.45, s	46.7	2.07, m
19	27.7	0.58, d (4.2) 0.24, d (4.2)	27.7	0.60, d (4.2) 0.25, d (4.2)	32.1	0.57, d (4.2) 0.43, d (4.2)	32.1	0.56, d (4.2) 0.39, d (4.2)	47.4	1.77, 0.99, m
20	29.6	1.91, m	31.2	1.87, m	80.6	–	88.1	–	37.0	–
21	18.0	1.06, d (6.5)	18.0	0.97, d (6.5)	28.3	1.55, s	26.0	1.44, s	42.1	1.47, 1.36, m
22	33.8	1.81, 1.65, m	33.8	1.81, 1.65, m	26.9	2.64, 1.21, m	38.5	2.03, 1.93, m	76.8	3.42, m
23	28.2	1.64, 1.43, m	29.2	1.65 (2H), m	23.8	2.23, 1.72, m	25.8	1.96, 1.88, m	23.1	1.27, s
24	78.6	3.41, d (10.3, 2.2)	89.9	3.48, dd (10.3, 2.2)	69.7	3.50, brs	84.6	3.80, t (6.0)	64.2	4.17, d (11.5) 3.25, d (11.5)
25	73.6	–	73.4	–	76.5	–	72.6	–	16.5	0.94, s
26	25.2	1.17, s	24.7	1.18, s	28.6	1.32, s	25.8	1.21, s	17.6	1.01, s
27	25.2	1.19, s	26.4	1.18, s	28.2	1.22, s	26.7	1.19, s	25.4	1.15, s
28	28.0	1.26, s	28.1	1.28, s	28.6	1.33, s	28.6	1.33, s	20.5	0.87, s
29	16.5	1.02, s	16.5	1.02, s	16.4	1.05, s	15.6	0.98, s	32.6	0.95, s
30	19.7	1.00, s	19.7	1.00, s	20.5	0.97, s	20.6	1.00, s	29.2	1.04, s

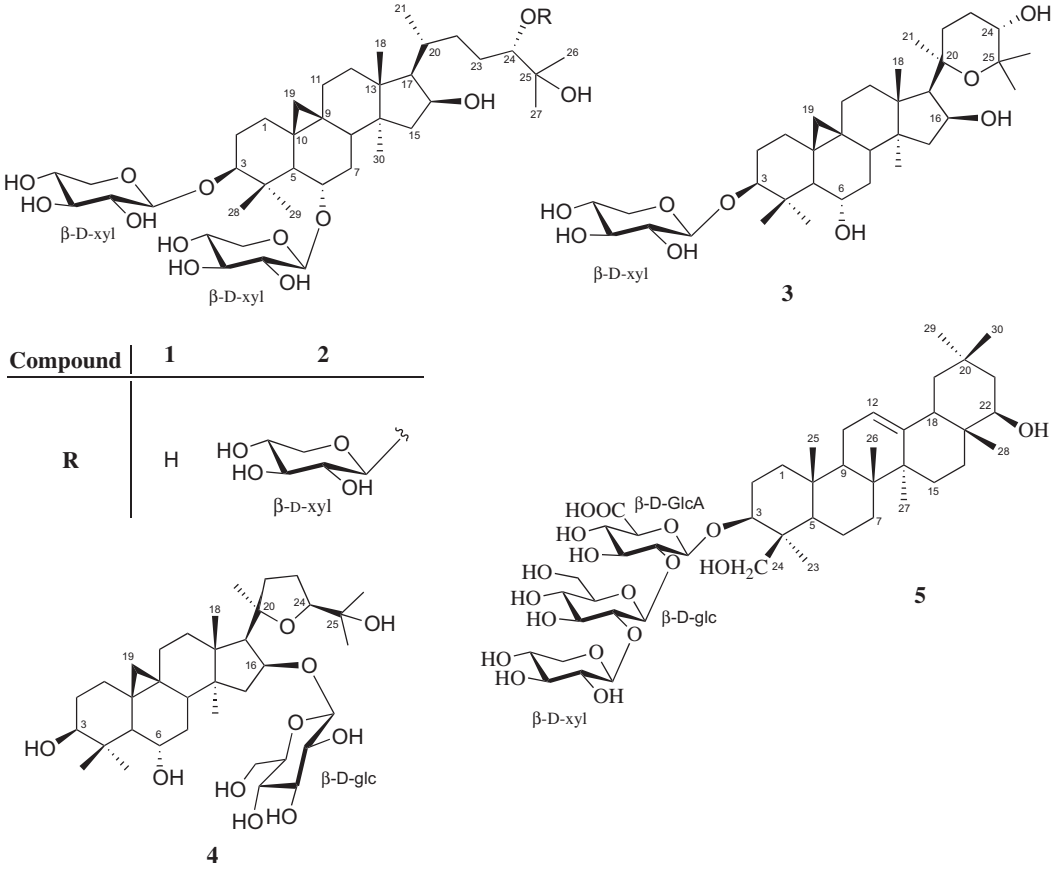

Fig. 1. Structures of compounds **1–5**.

Table 2
^{13}C and ^{1}H NMR data of the sugar moieties of compounds **1–5** (600 MHz, CD_3OD).

	1		**2**		**3**		**4**		**5**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
	β-D-Xyl at C-3		β-D-Xyl at C-3		β-D-Xyl at C-3		β-D-Glc (at C-16)		β-D-GlcA (at C-3)	
1′	107.5	4.31 d (7.5)	107.8	4.32 d (7.5)	106.7	4.30 d (7.5)	106.2	4.28, d (7.5)	104.4	4.51, d (7.5)
2′	75.3	3.21 dd (9.2, 7.5)	75.3	3.21 dd (9.2, 7.5)	75.1	3.21 dd (9.2, 7.5)	75.2	3.20 dd (7.5, 9.0)	81.1	3.59, dd (7.5, 9.0)
3′	77.8	3.32 t (9.2)	77.9	3.33 t (9.2)	77.6	3.31 t (9.2)	78.0	3.36, t (9.0)	76.8	3.64, dd (9.0, 9.0)
4′	71.0	3.51 m	71.4	3.51 m	71.0	3.49 m	71.5	3.32, t (9.0)	72.7	3.51, dd (9.0, 9.0)
5′	66.5	3.85 dd (11.7, 5.2) 3.20 t (11.7)	66.8	3.85 dd (11.7, 5.2) 3.20 t (11.7)	66.4	3.84 dd (11.7, 5.2) 3.20 t (11.7)	77.5	3.26, m	76.6	3.61, d (9.0)
6′							62.1	3.86, dd (3.5, 12) 3.71, dd (4.5, 12)	176.7	–
	β-D-Xyl at C-6		β-D-Xyl at C-6						β-D-Glc (at C-2_{GlcA})	
1″	105.6	4.312 d (7.5)	105.7	4.40 d (7.5)					103.0	4.84, d (7.5)
2″	75.3	3.21 dd (9.2, 7.5)	75.3	3.21 dd (9.2, 7.5)					83.0	3.75, dd (7.5, 9.0)
3″	77.8	3.32 t (9.2)	77.9	3.33 t (9.2)					74.5	3.68, t (9.0)
4″	71.0	3.51 m	71.4	3.51 m					70.6	3.86, t (9.0)
5″	66.5	3.85 dd (11.7, 5.2) 3.20 t (11.7)	66.8	3.85 dd (11.7, 5.2) 3.20 t (11.7)					76.2	3.52, m
6″									62.4	3.82, dd (3.5, 12) 3.75, dd (4.5, 12)
			β-D-Xyl at C-24						β-D-Xyl at (at C-2_{Glc})	
1‴			105.9	4.33 d (7.5)					106.6	4.58 d (7.5)
2‴			75.3	3.21 dd (9.2, 7.5)					75.4	3.31 dd (9.2, 7.5)
3‴			77.9	3.33 t (9.2)					76.8	3.41 t (9.2)
4‴			71.4	3.51 m					70.4	3.55 m
5‴			66.8	3.85 dd (11.7, 5.2) 3.20 t (11.7)					67.0	4.04 dd (11.7, 5.2) 3.29 t (11.7)

$C_{35}H_{58}O_9Na$, 645.3979). The positive ESI-MS spectrum showed the sodiated ion peak at *m/z* 645 $[M+Na]^+$. Its MS/MS fragmentation showed a peak at *m/z* 513 $[M+Na-132]^+$ due to the loss of a pentose unit.

The ^{1}H NMR spectrum of **3** showed signals due to a cyclopropane methylene at δ_H 0.57 and 0.43 (each 1H, d, J = 4.2 Hz), seven tertiary methyl groups at δ_H 1.45 (3H, s), 1.55 (3H, s), 1.32 (6H, s), 1.22 (3H, s), 1.33 (3H, s), 1.05 (3H, s) and 0.97 (3H, s), and four oxymethine proton signals at δ_H 4.64 (ddd, J = 8.0, 8.0, 5.2 Hz), 3.47 (ddd, J = 9.5, 9.5, 4.5 Hz), 3.50 (br s) and 3.23 (dd, J = 11.3, 4.0 Hz). In the ^{1}H NMR spectrum, an anomeric proton at δ_H 4.30 (d, J = 7.5 Hz) was observed, implying a monosaccharidic cycloartane-type glycoside. Complete assignments of the ^{1}H and ^{13}C NMR signals of the sugar portion were accomplished by 1D-TOCSY, HSQC, HMBC and DQF-COSY experiments, which led to the identification of a β-xylopyranosyl unit. To clarify the intermolecular connectivities of the partial structures in **3,** HMBC was used. Two oxygenated quaternary carbons with their characteristic chemical shifts at δ 78.9 and 75.2 (C-20 and C-25, respectively), implying a monohydroxypyran derivative, were in agreement with those reported for 3β,6α,16β,24α-tetrahydroxy-20(*R*),25-epoxycycloartane named cyclocephalogenin, previously reported from *Astragalus* spp. (Bedir et al., 1998a; Horo et al., 2010; Calis et al., 2001; Alaniya et al., 2008). The linkage site was deduced based on the key HMBC correlation between the proton signals at δ_H 4.30 (H-1'$_{xyl}$) and the carbon resonance at δ 89.5 (C-3). The hydrolysis of **3** followed by GC analysis allowed establishing the D configuration of the xylose unit (De Marino et al., 2003).

Thus, **3** was elucidated as 3-*O*-β-D-xylopyranosyl-3β,6α,16β,25-tetrahydroxy-20(*R*),25(*S*)-epoxycycloartane, and named as hareftoside C.

The HR-MALDI-TOF mass spectrum of **4** showed a major ion peak at *m/z* 675.4086 [M+Na]$^+$ ascribable to the molecular formula $C_{36}H_{60}O_{10}$ (calcd. for $C_{36}H_{60}O_{10}Na$, 675.4084). The positive ESI-MS spectrum showed the sodiated ion peak at *m/z* 675 [M+Na]$^+$. Its MS/MS fragmentation showed a peak at *m/z* 513 [M+Na−162]$^+$ due to the loss of a hexose unit.

The ^{1}H NMR spectrum of **4** also showed characteristic signals due to a cycloartane-type triterpenoid framework: cyclopropane methylene protons at δ_H 0.56 and 0.39 (each 1H, *d*, J = 4.2 Hz), seven tertiary methyl groups at δ_H 1.45 (3H, s), 1.44 (3H, s), 1.33 (3H, s), 1.21 (3H, s), 1.19 (6H, s), 1.00 (3H, s) and 0.98 (3H, s), and four oxygen bearing methine proton signals at δ_H 4.36 (ddd, J = 8.0, 8.0, 5.2 Hz), 3.80 (t, J = 6.0 Hz), 3.48 (ddd, J = 9.5, 9.0, 4.5 Hz) and 3.24 (dd, J = 11.3, 4.0 Hz) (Table 1). The NMR data of the aglycon of **4** were superimposable with those reported for cycloastragenol (Kitagawa et al., 1983a), with a glycosidation shift observed for C-16 (δ 84.4) (Table 1), confirming the monodesmosidic nature of **4**. Additionally, in the ^{1}H NMR spectrum, a signal ascribable to an anomeric proton at δ_H 4.28 (d, J = 7.5 Hz) was observed. Chemical shifts of the sugar unit, ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analyses, were consistent with the presence of a β-glucopyranosyl unit. Determination of the linkage site was obtained from the HMBC spectrum, which showed key correlation peak between the proton signal at δ_H 4.28 (H-1'$_{Glc}$) and the carbon resonance at δ 84.4 (C-16). The D configuration of the glucose unit was established after the hydrolysis of **4** followed by GC analysis (De Marino et al., 2003).

On the basis of these evidence, the structure of compound **4**, which was named as hareftoside D, was established as 16-*O*-β-D-glucopyranosyl-3β,6α,16β,25-tetrahydroxy-20(*R*),24(*S*)-epoxycycloartane.

The HR-MALDI-TOF mass spectrum of **5** showed a major ion peak at *m/z* 951.4932 [M+Na]$^+$ ascribable to the molecular formula $C_{47}H_{76}O_{18}$ (calcd. for $C_{47}H_{76}O_{18}Na$, 951.4929). The ESI-MS spectrum showed the major ion peak at *m/z* 951, which was assigned to [M+Na]$^+$. The MS/MS of this ion showed peaks at *m/z* 819 [M+Na−132]$^+$, corresponding to the loss of a pentose unit, at *m/z* 657 [M+Na−132−162]$^+$ due to the loss of a hexose unit and at *m/z* 481 [M+Na−132−162−176]$^+$, ascribable to the loss of a glucuronopyranosyl unit.

In the ^{1}H and ^{13}C NMR spectra of **5**, signals derived from seven methyl groups as singlet at δ_H 1.27, 1.15, 1.04, 1.01, 0.95, 0.94 and 0.87, an olefinic proton at δ_H 5.28 (t, J = 3.5 Hz), two oxygen bearing methine protons at δ_H 3.47 (dd, J = 11.5, 4.5 Hz), 3.42 (m), and a primary alcoholic function at δ_H 3.25 and 4.17 (d, J = 11.5 Hz) were observed. The NMR spectral data of **5** were consistent with a triterpene of the oleanane-type (Kapusta et al., 2005). A detailed inspection of the 1D and 2D NMR spectra led to the identification of the aglycon part as soyasapogenol B possessing three sugar residues (Woldemichael and Winka, 2002). Only the glycosidation shift was noted for C-3 (δ 91.4) (Table 1), confirming the monodesmosidic nature of **5**. Additionally, in the ^{1}H NMR spectrum, signals ascribable to three anomeric protons at δ_H 4.51 (d, J = 7.5 Hz), 4.84 (d, J = 7.5 Hz) and 4.58 (d, J = 7.5 Hz) were noted. The chemical shifts of the rest of the individual protons of the three sugar units were ascertained from a combination of 1D-TOCSY and DQF-COSY spectral analyses, and ^{13}C chemical shifts of their relative attached carbons were assigned unambiguously from the HSQC spectrum (Table 2). The data showed the presence of β-glucuronopyranosyl, β-glucopyranosyl and β-xylopyranosyl units. Determination of the sugar residue sequence and linkage site was obtained from the HMBC spectrum, which showed key correlation between the proton signal at δ_H 4.51 (H-1'$_{GlcA}$) and the carbon resonance at δ 91.4 (C-3), helping to locate β-glucuronopyranosyl unit at the C-3 position of the soyasapogenol B. The C-2′ signal of β-glucuronopyranosyl (81.1) was found to be shifted downfield about +5 ppm in comparison with a terminal β-glucuronopyranosyl moiety (Horo et al., 2010). In the HMBC spectrum, a cross peak between this carbon and H-1″$_{Glc}$ (4.84, d, J = 7.5 Hz) confirmed the inner sugar as glucopyranosyl. Also, the C-2″$_{glc}$ signal at δ 83.0, shifted downfield about +8 ppm, displayed a cross-peak with the terminal sugar's anomeric signal at δ_H 4.58 (d, J = 7.5 Hz), facilitating β-xylopyranosyl unit at C-2″. The D configuration of glucose, glucuronic acid and xylose units was established after the hydrolysis of **5** followed by GC analysis (De Marino et al., 2003). On the basis of the evidence, the structure of **5** was established as 3-*O*-[β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucuronopyranosyl]-soyasapogenol B, for which the trivial name hareftoside E is proposed.

Additionally, eleven known cycloartane-type glycosides (**6**–**16**) cyclocanthoside E (**6**) (Bedir et al., 1998b), macrophyllosaponin B (**7**) (Calis et al., 1996), 3-*O*-β-D-xylopyranosyl-6,25-di-*O*-β-D-glucopyranosyl-3β,6α,16β,24(*S*),25-pentahydroxycycloartane (**8**) (Gülcemal et al., 2011), oleifoloside B (**9**) (Ríos and Waterman, 1997), cyclocephaloside I (**10**) (Bedir et al., 1998b), astrasieversianin X (**11**) (Gan et al., 1986), trojanoside B (**12**) (Bedir et al., 1999), cycloastragenol (**13**) (Kitagawa et al., 1983a), astragaloside IV (**14**) (Kitagawa et al., 1983a), brachyoside B (**15**) (Bedir et al., 1998a), cyclodissectoside (**16**) (Sukhina et al., 2007), and four known oleanane-type triterpene glycosides, azukisaponin V (**17**) (Kitagawa et al., 1983a), dehydroazukisaponin V (**18**) (Mohamed et al., 1995), wistariasaponin D (**19**) (Konoshima et al., 1991), astragaloside VIII (**20**) (Kitagawa et al., 1983b), were isolated.

Consequently, as part of our ongoing project on the *Astragalus* genus, we investigated the glycosidic constituents of *A. hareftae*. We have shown that *A. hareftae*, which belongs to Acanthophace section, contains cycloartane and oleanane type triterpenoids. Until now, 25 out of 445 Turkish *Astragalus* species, from 13 different sections, have been investigated for their secondary metabolite contents. Cycloastragenol, possessing 20(*R*),24(*S*)-epoxy side chain, is the chief aglycone in the *Astragalus* genera. However, cyclocephalogenol is more unusual in the genus so far reported only from 3 sections, viz., Rhacophorus, Adiaspastus and Acanthophace. From

the Rhacoporus section, which is the largest section of *Astragalus* with about 100 members, three out of eight studied Turkish species gave cyclocephalogenol (*A. microcephalus*, Bedir et al., 1998b; *A. zahlbruckneri*, Calis et al., 2001; *A. schottianus*, Karabey et al., 2012), whereas *A. aureus*, single examined species of Adiaspastus section, provided the same aglycone. Only two members of Acanthophace section, viz., *A. hareftae* and *A. icmadophilus,* have been studied so far, and it is noteworthy that cyclocephalogenol is encountered in both members. This suggests that the presence of cyclocephalogenol in Acanthophace section could be of taxonomic importance. Further studies are required to confirm the above assumption, and our continuing studies will be of use in clarifying chemotaxonomical classification of the genus *Astragalus*.

3. Experimental

3.1. General

Optical rotations were measured on a JASCO DIP 1000 polarimeter. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpinGmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbeat 300K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma–Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, and HMBC spectra. The NMR data were processed using UXNMR software. Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. A mixture of analyte solution and a-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard. ESI-MS analyses were performed using a ThermoFinnigan LCQ Deca XP Max iontrap mass spectrometer equipped with Xcalibur software. GC analysis was performed on a Termo Finnigan Trace GC apparatus using a l-Chirasil-Val column (0.32 mm × 25 m).

3.2. Plant material

A. hareftae (NAB.) SIRJ. was collected from the altitude of 3200 m in Başet Mountain in Gürpınar Village, Van, Eastern Anatolia, Turkey on July 25, 2008. The plant material was identified by F. Özgökçe (Department of Biology, Faculty of Science & Art, Yüzüncü Yıl University, Van, Turkey). Voucher specimen has been deposited in the Herbarium of Yüzüncü Yıl University, Van, Turkey (VANF 13189).

3.3. Extraction and isolation

Part of the air-dried and powdered plant material of *A. hareftae* (whole plant; 1.6 kg out of 2.8 kg) was first extracted with *n*-hexane (1 × 4 L) under reflux at 68 °C, and then with MeOH (3 × 4 L) under reflux at 64 °C for 6 h (each). After filtration and evaporation procedures, *n*-hexane (3.85 g) and MeOH (50 g) extracts were obtained respectively. The MeOH extract (50 g) was dissolved in water (300 ml) and partitioned with CH_2Cl_2 (3 × 200 ml) and *n*-BuOH (3 × 200 ml), respectively. The *n*-BuOH phase (5.45 g), which was rich in saponins, was subjected to open column chromatography by using Sephadex LH-20 (150 g), eluted with MeOH to give seven main fractions (Fr.1–Fr.7). Fr.1 (4.05 g) was subjected to vacuum liquid chromatography (VLC), using reverse-phase material (Lichroprep RP-18, 25–40 μm, 130 g). Elution was performed with water (600 ml), H_2O–MeOH (8:2, 900 ml; 6:4, 1300 ml; 4:6, 1000 ml; 2:8, 800 ml) and MeOH (1000 ml) to afford six main fractions (Fr.1A–Fr.1F).

Fr.1E (30 mg) was applied to si gel (Merck, 7734, 20 g) column chromatography. Elution was carried out under isocratic condition with $CHCl_3$–MeOH–H_2O (90:10:1) to give **11** (4.6 mg) and **14** (15 mg). Fr.1D (1.695 g) was subjected to si gel (65 g) open column chromatography, employed with $CHCl_3$–MeOH (95:5 and 90:10) and $CHCl_3$–MeOH–H_2O mixtures (90:10:1 and 80:20:2) to afford six sub-fractions (Fr.1Da–Fr.1Df).

Fr.1Dc (49 mg) was chromatographed on RP (C-18) (22 g) by using MeOH–H_2O (6:4) to yield **2** (7.4 mg) and **12** (6 mg). Fr.1De (28 mg) was subjected to open column chromatography using RP (C-18) (28 g), eluted with MeOH–H_2O (4:6) to give **5** (4 mg), **18** (5 mg) and **19** (5 mg). Fr.1Df (150 mg) was applied to reverse-phase material (C-18, 28 g), eluted with H_2O–MeOH (5:5) to afford **17** (6.5 mg) and **20** (6.5 mg).

The other fractions to be studied were lost due to a laboratory accident. Therefore, the remaining part of the plant material (1.2 kg) was studied by using the same methodology (*n*-BuOH extract: 25 g) to obtain the other saponins. The BuOH extract was subjected to VLC using reverse-phase material (300 g) eluted with water (1200 ml), H_2O–MeOH (7:3, 1800 ml; 5:5, 4000 ml; 3:7, 3000 ml and MeOH 1000 ml) to give six main fractions (Fr.G–Fr.L).

Fr.L (1.5 g) was chromatographed over si gel (240 g), eluted with CH_2Cl_2–MeOH–H_2O (90:10:1) yielding six sub-fractions. Fr.L1 (48 mg) was subjected to si gel (17 g) column chromatography. Elution was carried out with CH_2Cl_2–MeOH (95:5) to obtain **13** (12 mg). Fr.L3 (103 mg) was chromatographed on the medium pressure liquid chromatography (MPLC) by using RP-18 with the solvent system H_2O–MeOH (4:6) to afford **15** (14.3 mg). Fr.L4 (48 mg) was subjected to si gel (32 g) column chromatography, eluted with CH_2Cl_2–MeOH–H_2O (90:10:1) to give **6** (10.4 mg).

Fr.J (4.8 g) was subjected to MPLC. Elution was started with the solvent system H_2O–MeOH (5:5), increased step wise to H_2O–MeOH (2:8) to yield six subfractions (Fr.J1–Fr.J6). Fr.J4 (170 mg) was subjected to si gel (45 g) column chromatography, employed with CH_2Cl_2–MeOH–H_2O (90:10:1) to give **1** (16 mg) and a subfraction Fr.J4.1 (30 mg). Fr.J4.1 was applied to si gel (17 g) once more with solvent system CH_2Cl_2–MeOH–H_2O (80:20:2) to afford **9** (21 mg). Fr.J3 (850 mg) was subjected to si gel (50 g) column chromatography with the solvent system CH_2Cl_2–MeOH–H_2O (90:10:1) to give **3** (3 mg), **4** (3.1 mg), **16** (4.4 mg), **8** (20.5 mg), and a sub-fraction Fr.J3.1 (190 mg). Fr.J3.1 was subjected to MPLC (RP, C-18), employed with solvent system H_2O–MeOH (5:5) to afford **10** (94 mg) and **7** (21.4 mg).

3.4. 3,6-Di-O-β-D-xylopyranosyl-3β,6α,16β,24(S),25-pentahydroxycycloartane (*1*)

Amorphous white solid; $[\alpha]_D^{25}$ +27.3 (*c* 0.1 MeOH); IR ν_{max}^{KBr} cm^{-1}: 3460 (>OH), 3035 (cyclopropane ring), 2945 (>CH), 1260 and 1058 (C–O–C); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of aglycone moiety, see Table 1; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of the sugar portion, see Table 2; ESI-MS *m/z* 779 $[M+Na]^+$; MS/MS *m/z* 647 $[M+Na-132]^+$, *m/z* 515 $[M+Na-132-132]^+$; HR-MALDI-TOF-MS *m/z* $[M+Na]^+$ calcd. for $C_{40}H_{68}O_{13}Na$, 779.4558, found 779.4564.

3.5. 3,6,24-Tri-O-β-D-xylopyranosyl-3β,6α,16β,24(S),25-pentahydroxycycloartane (*2*)

Amorphous white solid; $[\alpha]_D^{25}$ +24.3 (*c* 0.1 MeOH); IR ν_{max}^{KBr} cm^{-1}: 3470 (>OH), 3030 (cyclopropane ring), 2950 (>CH), 1264 and 1060 (C–O–C); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of aglycone moiety, see Table 1; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of the sugar

portion, see Table 2; ESI-MS *m/z* 911 $[M+Na]^+$; MS/MS *m/z* 779 $[M+Na-132]^+$, *m/z* 647 $[M+Na-132-132]^+$, *m/z* 515 $[M+Na-132-132-132]^+$; HR-MALDI-TOF-MS *m/z* $[M+Na]^+$ calcd. for $C_{45}H_{76}O_{17}Na$, 911.4980, found 911.4984.

3.6. 3-O-β-D-xylopyranosyl-3,6,16,25-tetrahydroxy-20(R),25(S)-epoxycycloartane (**3**)

Amorphous white solid; $[\alpha]_D^{25}$ +19.8 (*c* 0.1 MeOH); IR ν_{max}^{KBr} cm^{-1}: 3480 (>OH), 3030 (cyclopropane ring), 2945 (>CH), 1260 and 1055 (C–O–C); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of aglycone moiety, see Table 1; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of the sugar portion, see Table 2; ESI-MS *m/z* 645 $[M+Na]^+$; MS/MS *m/z* 513 $[M+Na-132]^+$; HR-MALDI-TOF-MS *m/z* $[M+Na]^+$ calcd. for $C_{35}H_{58}O_9Na$, 645.3979, found 645.3982.

3.7. 16-O-β-D-glucopyranosyl-3,6,16,25-tetrahydroxy-20(R),24(S)-epoxycycloartane (**4**)

Amorphous white solid; $[\alpha]_D^{25}$ +20.8 (*c* 0.1 MeOH); 3460 (>OH), 3040 (cyclopropane ring), 2935 (>CH), 1250 and 1050 (C–O–C); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of aglycone moiety, see Table 1; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of the sugar portion, see Table 2; ESI-MS *m/z* 675 $[M+Na]^+$; MS/MS *m/z* 513 $[M+Na-162]^+$; HR-MALDI-TOF-MS *m/z* $[M+Na]^+$ calcd. for $C_{36}H_{60}O_{10}Na$, 675.4084, found 675.4086.

3.8. 3-O-[β-D-xylopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→2)-O-β-D-glucuronopyranosyl] soyasapogenol B (**5**)

Amorphous white solid; $C_{53}H_{84}O_{24}$; $[\alpha]_D^{25}$ +12.1 (*c* 0.1 MeOH); IR ν_{max}^{KBr} cm^{-1}: 3448 (>OH), 2934 (>CH), 1658 (C=C); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of aglycone moiety, see Table 1; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data of the sugar portion, see Table 2; ESI-MS *m/z* 951 $[M+Na]^+$; MS/MS *m/z* 819 $[M+Na-132]^+$, *m/z* 657 $[M+Na-132-162]^+$, 481 $[M+Na-132-162-176]^+$; HR-MALDI-TOF-MS *m/z* $[M+Na]^+$ calcd. for $C_{47}H_{76}O_{18}Na$, 951.4929, found 951.4932.

3.9. Acid hydrolysis

The configurations of sugar units were established after the hydrolysis of **1**–**5** with 1 N HCl, trimethylsilation and determination of the retention times by GC operating in the experimental conditions previously reported by De Marino et al. (2003).

The peaks of the hydrolysate of **1–3** were detected at 10.99 and 12.02 (D-xylose). For the hydrolysate of **4** a peak at 14.73 min (D-glucose) was detected. The peaks of the hydrolysate of **5** were detected at 15.82 min (D-glucuronic acid), 14.73 min (D-glucose), and 10.98 and 12.02 (D-xylose). Retention times for authentic samples after being treated in the same manner with 1-(trimethylsilyl)-imidazole in pyridine were detected at 10.98 and 12.00 min (D-xylose), at 14.71 min (D-glucose), and at 15.81 min (D-glucuronic acid).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.07.015.

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