Triterpene Saponins from *Knautia integrifolia* var. *bidens*

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Two new triterpene saponins (**1** and **2**), named bidenosides A and B, were isolated from the roots of *Knautia integrifolia* var. *bidens*. The structures were identified as 3-*O*-α-L-rhamnopyranosyl-28-*O*-[β-D-allopyranosyl-(1 → 6)-β-D-glucopyranosyl]-pomolic acid, and 3-*O*-α-L-rhamnopyranosyl-28-*O*-[β-D-allopyranosyl-(1 → 6)-β-D-glucopyranosyl]-oleanolic acid on the basis of spectroscopic evidence.

Key words: Knautia integrifolia var. *bidens*, Dipsacaceae, Saponin, Oleanolic Acid, Pomolic Acid

Introduction

Knautia integrifolia var. *bidens* (Dipsacaceae) is an annual herbaceous plant with lavender-blue flowers. It mainly grows in W. Turkey, S. Anatolia, Aegean Islands, W. Syria, and E. Mediterranean countries [1]. No previous phytochemical or biological study has been reported on *K. integrifolia* var. *bidens*. On the other hand, earlier studies performed on *K. arvensis* resulted in the isolation of two new saponin glycosides, named knautiosides A and B [2]. Additionally, caffeic acid derivatives were determined in complex plant extracts before and after acid hydrolysis in some Dipsacaceae family plants including *K. arvensis*, *K. drymeia* and *K. tatarica* by using thin-layer chromatography and densitometry [3]. The present paper deals with the isolation and structure elucidation of two new triterpene-type saponins.

Material and Methods

General experimental procedures

Optical rotations were measured using JASCO DIP-370 digital polarimeter in MeOH at ambient temperature. IR spectra were measured on a Perkin Elmer 1600 series FT-IR spectrometer in KBr pellets. The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C), using TMS as internal standard. Multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were run using a standard Bruker pulse programs. The delay for long-range coupling in the HMBC ($1/2J$) was set to 63 msec. The HRMS were obtained by direct injection using Bruker Bioapex-FTMS with electrospray ionization (ESI). LC-MS data were obtained using a Finnigan AQA ThermoQuest instrument in the ESI mode. Column chromatography was carried out on Kieselgel 60 (Merck 7734), Sephadex LH-20 (Pharmacia 17-0090-02) and Li Chroprep RP (C-18) (Merck 9303). Analytical TLC was conducted on pre-coated Kieselgel 60 F_{254} aluminium sheets (Merck 5554 and Merck 5560). Compounds were detected by UV and 1% vanillin/H_2SO_4 followed by heating at 105 °C for 1 – 2 min. Preparative TLC was performed on Kieselgel 60 HF_{254} (Merck 7747) and pre-coated RP (C-18) glass-plates (Merck 5434).

Plant material

Knautia integrifolia var. *bidens* was collected from Bornova, Izmir (Turkey) in April, 2001. A voucher specimen (EGE 37213) is deposited in the Herbarium Center of Faculty of Science, Ege University, Izmir, Turkey.

Extraction and isolation

The air-dried and powdered roots of the plant material (1.1 kg) were extracted with MeOH (2 × 5 l) at room temperature and filtered. The filtrate was concentrated to dryness *in vacuo* and defatted with cyclohexane. The resulting residue (56.61 g) was suspended in H_2O and partitioned between CH_2Cl_2 and *n*-BuOH sequentially. An aliquot of the *n*-BuOH

Table 1. ^{1}H and ^{13}C Assignments of **1** (in DMSO-*d6* + C_5D_5N + CD_3OD) and **2** (in C_5D_5N + CD_3OD).

C/H	**1** (δ_C)	**2** (δ_C)	**1** (δ_H, *J* Hz)	**2** (δ_H, *J* Hz)
1	38.9 t	39.1 t	1.42 m, 0.95 m	1.51 m, 0.96 m
2	26.6 t	26.9 t	1.65 m, 1.86 m	2.13 m, 1.86 m
3	88.9 d	89.0 d	3.10 dd (4.5, 11.0)	3.33 dd (4.0, 11.5)
4	39.7 s	39.8 s		
5	55.9 d	56.2 d	0.68 d (11.0)	0.79 brd (11.5)
6	18.7 t	18.8 t	1.22 m, 1.40 m	1.26 m, 1.43 m
7	33.4 t	33.3 t	1.34 m, 1.46 m	1.33 m, 1.46 m
8	40.4 s	40.2 s		
9	47.7 d	48.4 d	1.59 m	1.63 dd (7.0, 10.0)
10	37.0 s	37.3 s		
11	24.0 t	23.7 t	1.39 m, 1.87 m	1.20 m, 2.06 m
12	129.4 d	123.1 d	5.32 brs	5.41 brs
13	139.1 s	144.5 s		
14	42.1 s	42.4 s		
15	29.1 t	28.6 t	1.02 m, 2.08 m	1.17 m, 2.31 m
16	26.0 t	24.1 t	1.73 m, 2.79 m	1.88 m, 2.22 m
17	48.6 s	47.3 s		
18	54.1 d	42.0 d	2.63 s	3.19 dd (4.5, 14.0)
19	72.4 s	46.5 t		1.25 m, 1.75 m
20	42.0 d	31.0 s	1.25 m	
21	26.6 t	34.3 t	1.73 m	1.11 m, 1.33 m
22	37.6 t	32.7 t	1.73 m, 1.82 m	1.76 m, 1.89 m
23	28.2 q	28.5 q	1.09 s	1.26 s
24	16.7 q	17.2 q	0.80 s	0.96 s
25	15.7 q	15.9 q	0.80 s	0.88 s
26	17.3 q	17.8 q	0.88 s	1.09 s
27	24.5 q	26.4 q	1.44 s	1.24 s
28	177.3 q	176.9 q		
29	27.0 q	33.4 q	1.20 s	0.87 s
30	16.9 q	24.0 q	0.89 d (6.7)	0.88 s
Ara-1	106.9 d	107.8 d	4.42 d (6.8)	4.76 d (7.0)
2	72.4 d	73.2 d	3.92 †	4.42 dd (7.0, 8.5)
3	75.4 d	74.9 d	3.95 †	4.17 †
4	68.5 d	69.7 d	3.74 †	4.28 †
5	66.2 t	67.0 t	3.60 brd (10.5), 4.02 †	3.83 brd (11.0), 4.31 †
Glu-1	95.5 d	96.0 d	5.79 d (8.0)	6.23 d (8.0)
2	74.1 d	74.2 d	3.74 †	4.13 †
3	78.0 d	78.9 d	3.79 †	4.21 t (8.5)
4	70.5 d	71.2 d	3.80 †	4.30 †
5	77.6 d	78.2 d	3.78 †	4.10 †
6	69.2 t	69.8 t	3.95 †, 4.35 brd (10.5)	4.30 †, 4.70 brd (10.0)
All-1	102.4 d	103.1 d	4.45 d (7.5)	5.44 d (8.0)
	71.9 d	72.5 d	3.55 dd (2.5, 7.5)	3.92 m
	73.1 d	73.2 d	4.27 t (2.7)	4.65 t (2.5)
	69.0 d	69.1 d	3.76 †	4.13 †
	75.4 d	76.2 d	3.95 †	4.35 †
	62.5 t	63.0 t	3.92 †, 4.01 †	4.28 †, 4.38 †

Assignments confirmed by COSY, HMQC and HMBC experiments. Multiplicites are based on DEPT-135 experiment. †: Signal pattern was unclear due to overlapping.

fraction (6.13 g) was dissolved in MeOH and filtrated over Al_2O_3 in order to remove phenolics. After evaporation, the resultant crude extract (4.23 g) was subjected to vacuum liquid chromatography (VLC) over reversed phase material [Li

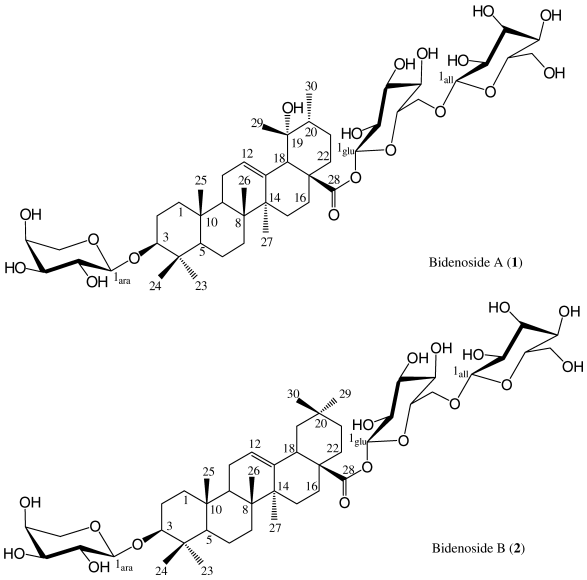

Fig. 1. Saponin glycosides (**1** and **2**) from *Knautia integrifolia* var *bidens.*

Chroprep, C-18, 40 μm] using H_2O (300 ml), H_2O-MeOH (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 50:50, 40:60, 35:65, 30:70, 20:80, 10:90; the volume of each solvent mixture 100 ml) and MeOH (300 ml) as eluents to yield 40 fractions (fraction volume: 50 ml). Fractions 13 – 34 were combined (2.54 g) and resubjected to VLC using reversed-phase material (LiChroprep, C-18, 40 μm, 150 g, 7×17 cm) using H_2O (200 ml), gradient mixtures of MeOH in H_2O (%10-90, 1800 ml) and MeOH (200 ml). A total of 22 fractions was collected. The fractions rich in saponins (1.50 g) were pooled and chromatographed on a normal phase Si gel column (480 g, 4×75 cm) using mixtures of CH_2Cl_2-MeOH-H_2O (80:20:1, 80:20:2, 70:30:3, 61:32:7, 64:50:10) to give 1434 fractions. The fractions 950 – 1434 were combined (633 mg) and applied to normal phase PTLC ($CHCl_3$-MeOH-H_2O; 61:32:7 solvent system) to give 6 bands (A-F). The bands C and D (182 mg) were separated on RP-PTLC with MeOH-H_2O; 8:2 to afford 2 major bands (Band 1, 2). Band 1 (23 mg) was subjected to a sephadex column, (80 g, 4×15 cm, 80% MeOH) to give a pure saponin (**2**; 11.3 mg). Band 2 (62 mg) was further subjected to RP-PTLC (MeOH-H_2O; 6.5:3.5) to give **1** (18.6 mg).

Bidenoside A (**1**): Amorphous white powder; $[\alpha]_D^{26}$ -2.52° ($c = 0.1$, MeOH); positive-ion HR-ESIMS *m/z*: calcd. for $C_{47}H_{76}O_{18}$: 928.5032; found: 929.5020 $[M+H]^+$; IR: ν_{max} (KBr, cm^{-1}) 3387 (OH), 1738 (C=O, ester group), 1250 (C-O); ^{1}H NMR (DMSO-*d6* + C_5D_5N + CD_3OD, 500 MHz): Table 1; ^{13}C NMR (DMSO-*d6* + C_5D_5N + CD_3OD, 125 MHz): Table 1.

Bidenoside B (**2**): Amorphous white powder; $[\alpha]_D^{26}$ -2.84° ($c = 0.1$, MeOH); positive-ion HR-ESIMS *m/z*:

calcd. for $C_{46}H_{72}O_{18}$: 912.5083; found: 913.5160 $[M+H]^+$; IR: ν_{max} (KBr, cm^{-1}) 3400 (OH), 1727 (C=O, ester group), 1210 (C-O); ^1H NMR (C_5D_5N + CD_3OD, 500 MHz): Table 1; ^{13}C NMR (C_5D_5N + CD_3OD, 125 MHz): Table 1.

Results and Discussion

Two triterpene type glycosides were isolated and purified by a combination of chromatographic methods from the roots of *K. integrifolia* var *bidens*.

Compound **1** was obtained as an amorphous powder. The HR-ESI mass spectrum of **1** showed a quasimolecular $[M+H]^+$ peak at *m/z* 929.5020 corresponding to a molecular formula of $C_{47}H_{76}O_{18}$.

The ^1H NMR spectrum of **1** exhibited the presence of six tertiary methyl signals at $\delta = 0.80\,(\times 2)$, 0.88, 1.09, 1.20 and 1.44, a secondary methyl signal at $\delta = 0.89$ (d, $J = 6.7$ Hz), and an olefinic proton signal at $\delta = 5.32$ (brs). Additionally, three anomeric proton signals at $\delta = 4.42$ (d, $J = 6.8$ Hz), 4.45 (d, $J = 6.7$ Hz), and 5.79 (d, $J = 8.0$ Hz) were observed in the low-field region implying trisaccharide nature of the molecule.

Inspection of ^{13}C NMR spectrum of **1** revealed 47 carbon signals seventeen of which were assigned to a pentose and two hexose units, and the left over 30 signals to a triterpenoid skeleton. Furthermore, the ^{13}C NMR spectrum of **1** had three anomeric carbon resonances at $\delta = 106.9$, 102.4 and 95.5 suggesting that **1** possessed two ether-type and one ester-type glycosidic linkages.

A detailed analysis of the NMR spectral data of **1** displayed the features of a 19-oxygenated urs-12-ene type triterpene saponin, pomolic acid [4, 5]. Full assignment of the ^1H and ^{13}C signals of **1**, which were secured by G-DQF-COSY and G-HMQC spectra, showed marked glycosylation shifts for C-3 ($\delta = 88.9$; $\sim 9-10$ ppm) and C-28 ($\delta = 177.8$; ~ -2.7 ppm). All connectivities within **1** were also confirmed by a G-HMBC experiment (Fig. 2). These results suggested that **1** was a bisdesmosidic saponin in which the sugar residues were linked to C-3 and C-28 of urs-12-ene skeleton.

A G-DQF-COSY spectrum of **1** allowed unambiguous assignment of all proton sugar signals and G-HMQC spectrum correlated each proton signal to the corresponding carbon resonances. Based on 2D data the sugar residues were assigned to be α-L-arabinose, β-D-glucose and β-D-allose which were in good accordance to those reported [4–6]. The absence of any

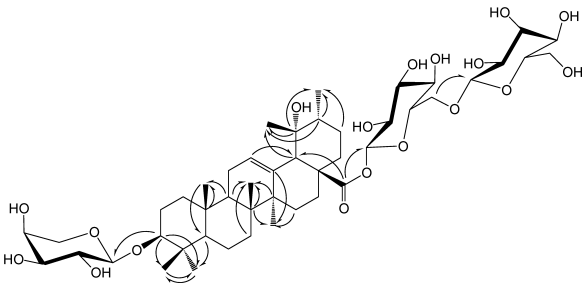

Fig. 2. Selected heteronuclear multiple bond correlations (HMBC) for bidenoside A (**1**). Arrows point from carbon to proton.

glycosylation shifts for the carbon resonances of α-L-arabinose and β-D-allose suggested that these sugar moieties must be terminal.

All connectivities, including the sites of attachment of sugar moieties on the aglycone as well as the interglycosidic linkage, were determined by a G-HMBC experiment. In the HMBC spectrum, the anomeric proton signal at $\delta = 4.42$ assigned to the $H\text{-}1_{ara}$ showed long-range correlation with the carbon at $\delta = 88.9$ (C-3). The anomeric proton of β-D-glucose unit displayed long-range correlation with the carbon resonance at $\delta = 177.3$ (C-28), while H-1 of β-D-allose moiety ($\delta = 4.45$) exhibited correlation with the C-6 of glucose moiety. Thus, α-L-arabinose unit attached to C-3, while disaccharide residue linked to C-28.

From the above evidence, the structure of **1** was concluded to be 3-*O*-α-L-arabinopyranosyl-28-*O*-[β-D-allopyranosyl($1 \rightarrow 6$)-β-D-glucopyranosyl]-pomolic acid, for which the trivial name bidenoside A is proposed.

The HR-ESI mass spectrum of **2** displayed a quasimolecular ion peak, corresponding to a molecular formula of $C_{47}H_{76}O_{17}$, at *m/z* 913.5060 $[M+H]^+$ indicating a molecular weight 16 mass unit less than **1**.

The ^1H NMR spectrum of **2** showed signals due to seven tertiary methyl signals [$\delta = 0.87$, 0.88 ($\times 2$), 0.96, 1.09, 1.24, 1.26], and an olefinic proton ($\delta = 5.41$, brs). A detailed inspection of the ^1H and ^{13}C NMR spectra of **2** indicated that saponin **2** is the 3,28-bidesmoside of oleanolic acid possessing three monosaccharide units [7, 8].

In the ^{13}C NMR spectrum the signals arising from the sugar moieties were in good agreement with those of **1**. Moreover, 2D-NMR data of **2** (G-DQF-COSY and G-HMQC) supported that compound **2** had the same sugar residues as in saponin **1** (terminal α-

L-arabinopyranose and β-D-allopyranose, and β-D-glucopyranose). The C-H long-range couplings were observed in the G-HMBC spectrum to locate the sugar sequences on the molecule. The correlation peaks between H-1_{ara} ($\delta = 4.76$, d, $J = 7.0$ Hz) and C-3 of the aglycone ($\delta = 89.0$); H-1_{glu} ($\delta = 6.23$, d, $J = 8.0$ Hz) and C-28 ($\delta = 176.9$); H-1_{all} ($\delta = 5.44$, d, $J = 8.0$ Hz) and C-6_{glu} ($\delta = 69.8$) confirmed the sites of glycosidation [α-L-arabinopyranosyl moiety at C-3 and β-D-allopyranosyl($1 \rightarrow 6$)-β-D-glucopyranosyl unit at C-28].

Consequently, the structure of **2** was established as 3-*O*-α-L-arabinopyranosyl-28-*O*-[β-D-allopyranosyl($1 \rightarrow 6$)-β-D-glucopyranosyl]-oleanolic acid, for which the trivial name bidenoside B is proposed.

In recent years papers on the isolation and characterization of numerous triterpene glycosides have been published in Dipsacaceae family. As far as we know, up to now only two saponin glycosides, knautosides A and B [2] have been described from *Knautia* genus. Therefore, bidenosides A and B appear to be third and fourth representatives of saponin class. On the other hand, previously isolated saponin glycosides were ursane-type, while bidenosides A and B were oleanolic and pomolic acid derivatives, respectively, first entries of their class in the genus *Knautia*.

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