Triterpene Saponins from *Calendula arvensis*

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From the aerial parts of *Calendula arvensis* a new triterpene saponin arvensoside C (**1**) was isolated together with four known triterpene saponins. The aglycon moieties had the oleanane skeleton for all of them. Three known flavonol glycosides, isorhamnetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-galactopyranoside were also obtained and characterized. Their structures were elucidated by 1D and 2D NMR experiments including 1D-TOCSY, DQF-COSY, HOHAHA, HSQC and HMBC spectroscopy as well as HRESIMS analysis.

Key words: *Calendula arvensis*, Asteraceae, Triterpene Saponins, Arvensoside C, Flavonol Glycosides

Introduction

Calendula officinalis (Asteraceae), which is commonly called "Marigold", grows as a wild plant and garden plant throughout Europe, North America and Mediterranean region [1, 2]. *Calendula* species especially *C. officinalis* are widely used in European and western Asian traditional medicine for skin complaints, wounds, burn, dysmenorrhoea and duodenal ulcers [3]. Triterpenoid esters, triterpene saponins, flavonol glycosides, ionone and sesquiterpene glycosides have been isolated from the genus *Calendula* as principle secondary metabolites [2, 4 – 10]. The triterpenoids isolated from *Calendula* species have been found to have some pharmacological activities such as hypoglycaemic, gastroprotective, antiviral, antimutagenic and anti-inflammatory [3, 6, 11, 12]. As a part of our studies on the Turkish medicinal plants, we here report the isolation and structure elucidation of five triterpene saponins as well as three flavonol glycosides from the aerial parts of *C. arvensis* which is used as sudorific and for the treatment of menstrual irregularities [13].

Experimental Section

General experimental procedures

Optical rotation was measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm). Bruker AMX 600 instruments (600 MHz for ^{1}H and 150 MHz for ^{13}C) with XWIN NMR software package were used to acquire NMR data. Positive- and negative-mode ESIMS were recorded on a Finnigan TSQ 7000 instrument. Exact masses were measured on a Q-TOF Ultima (Micromass) triple-quadrupole orthogonal time-of-flight (TOF) instrument. Nanospray ionization was used in TOF mode at 8.500 resolving power. Samples were dissolved in pure methanol, mixed with the internal calibrant, and introduced directly into the ion source by direct infusion. Calibration was performed on the peaks of a synthetic peptide (TOF positive ion calibration solution, Bachem), at $m/z = 785.8426$. Sodium-containing molecular ions of analytes were revealed and the elemental composition calculated by the exact mass obtained with four significance numbers. TLC analyses were carried out on silica gel 60 F254 precoated plates (Merck, Darmstadt), detection by 1% vanillin/H2SO4. For medium-pressure liquid chromatographic (MPLC) separations, a Büchi 683 chromatography pump, a Büchi fraction collector C-660, a Rheodyne injector, and Büchi columns (column dimension: 3×25 cm) were used. Silica gel 60 (0.063 – 0.200 mm; Merck, Darmstadt) and was utilized for open column chromatography (CC). LiChroprep C_{18} (Merck) material was used for MPLC and VLC.

Plant material

Calendula arvensis L. (Asteraceae) was collected from Kumluca, Antalya, South Anatolia, Turkey, in January 2005. Voucher specimens (HUEF 05001) have been deposited at

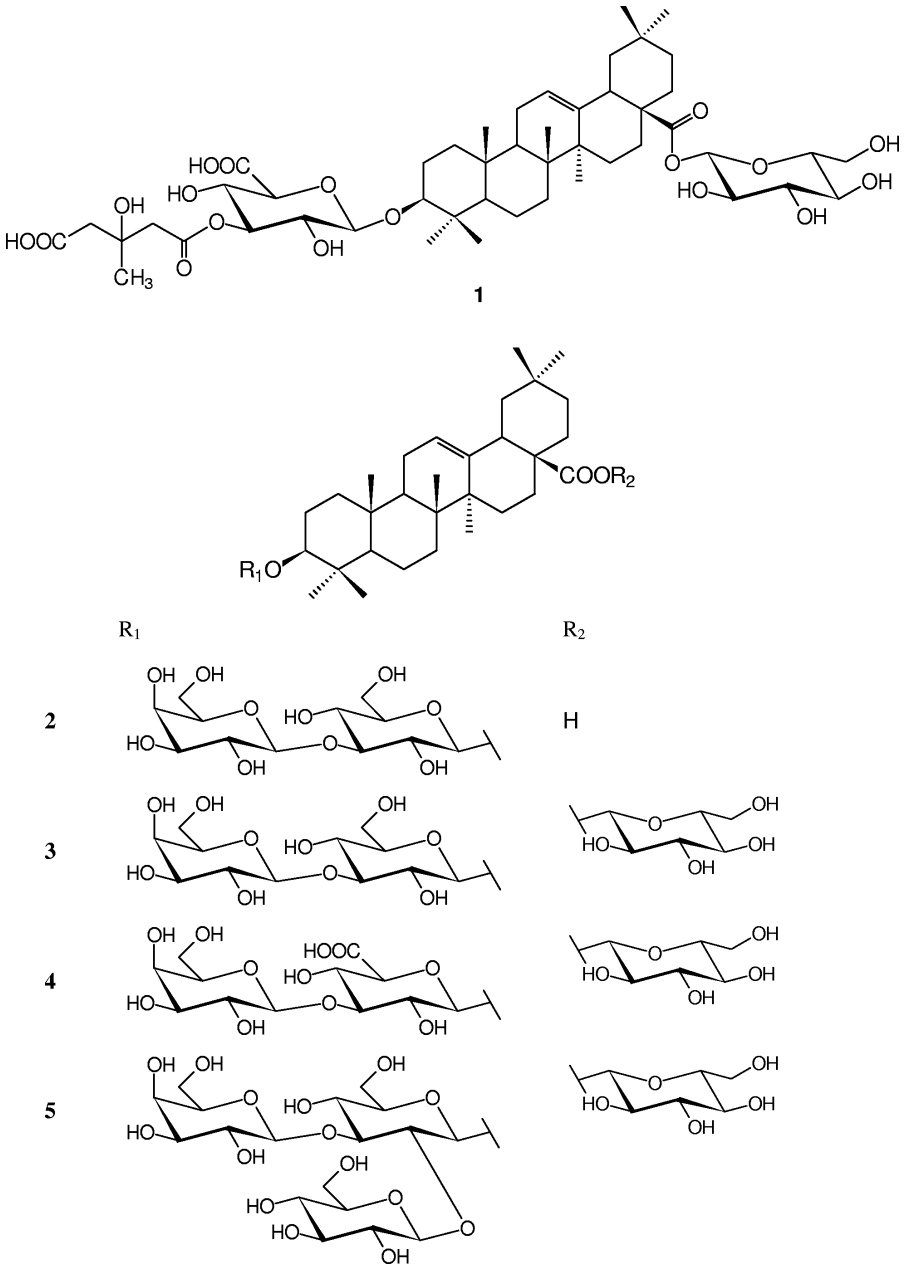

Fig. 1. Triterpene saponins (**1** – **5**) from *C. arvensis*.

the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

Extraction and isolation

The air-dried and powdered aerial parts of *C. arvensis* (400 g) were extracted two times with MeOH (2 × 3.5 l, 4 h) at 45 °C. The combined methanolic extracts were concentrated to a residue (58 g). After diluting with H_2O, the resulting suspension was partitioned with equal volumes of *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH respectively. The *n*-BuOH layer provided 13 g of an extract and was separated by vacuum liquid chromatography (VLC) using reversed-phase material (LiChroprep C_{18}) eluting with MeOH in H_2O to yield eight fractions, A – H. Fraction E (3.5 g) was chromatographed on a SiO_2 column with CH_2Cl_2-MeOH-H_2O (100 : 25 : 2 to 50 : 50 : 10) to give **3** (667 mg) in addition to six subfractions, E_{2-7}. Fraction E_5 (462 mg) was subjected to C_{18}-Medium Pressure Liquid Chromatography

Aglycon			Sugar portion/Dicrotalic acid		
	δ_C ppm	δ_H ppm		δ_C ppm	δ_H ppm, *J* (Hz)
1	39.9	1.63, 1.02	glcA (C-3)		
2	26.9	2.00, 1.70	1	106.2	4.45 d (7.8)
3	90.4	3.23	2	73.4	3.42 dd (7.8, 8.7)
4	39.7	–	3	78.8	4.98 dd (8.7, 8.7)
5	56.8	0.81	4	71.5	3.66†
6	19.7	1.56, 1.43	5	77.4	3.67†
7	34.4	1.42, 1.24	6	173.0	–
8	40.4	–	glu (C-28)		
9	48.8	1.61	1	95.3	5.41 d (8.1)
10	37.4	–	2	73.7	3.35 dd (8.1, 8.8)
11	24.6	1.92	3	78.5	3.38 d (8.8, 9.0)
12	123.7	5.28	4	71.0	3.39 dd (9.0, 8.0)
13	144.7	–	5	78.3	3.43 m
14	42.3	–	6	62.5	3.71 dd (3.5, 12.0)
15	28.8	1.82, 1.11			3.85 dd (1.8, 12.0)
16	23.6	2.08, 1.74	dicrotalic acid (C-3 glcA)		
17	47.9	–	1	172.0	–
18	42.4	2.88	2	47.9	2.66 d (14.1)
19	47.0	1.75, 1.18			2.70 d (14.1)
20	31.2	–	3	71.0	–
21	33.9	1.51, 1.34	4	47.7	2.44 d (14.1)
22	32.6	1.76, 1.65			2.58 d (14.1)
23	28.2	1.07	5	173.4	–
24	16.4	0.86	6	27.9	1.39 s
25	16.0	0.98			
26	17.6	0.83			
27	27.6	1.18			
28	178.0	–			
29	33.7	0.94			
30	24.2	0.96			

Table 1. ^{13}C and ^{1}H NMR spectroscopic data for arvensoside C (**1**), (CD_3OD, ^{13}C: 150 MHz; ^{1}H: 600 MHz)*.

* Assignments based on 1D TOCSY, 2D COSY, HOHAHA, HSQC and HMBC experiments. ^{1}H-^{1}H coupling constants in the sugar spin system were measured from ^{1}H spectrum and are reported in Hz; † overlapped.

(C_{18}-MPLC, column dimension: 3×25 cm) eluting with stepwise H_2O-MeOH gradient (50 to 85% MeOH) to obtain **5** (247 mg). Likewise, fraction E_7 (132 mg) was applied to the same C_{18}-MPLC using 50 – 80% MeOH as solvent mixtures to afford **1** (12 mg) and **4** (70 mg). Compound **2** (15 mg) was purified from fraction G (171 mg) by SiO_2 CC (CH_2Cl_2-MeOH-H_2O, 90 : 10 : 1 to 80 : 20 : 2). In order to isolate the flavonoids the EtOAc extract (1.6 g) was applied to C_{18}-MPLC (column dimensions: 3×25 cm) using a H_2O-MeOH gradient (15 – 100% MeOH) to obtain four fractions, fr. I – IV. Fraction II (93 mg) was rechromatographed on a SiO_2 column ($CHCl_3$-MeOH-H_2O, 85 : 15 : 1 to 80 : 20 : 1) to give the mixture of quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-galactopyranoside (20 mg). Isorhamnetin 3-*O*-β-D-glucopyranoside (6 mg) was purified from fraction III (50 mg) by SiO_2 CC ($CHCl_3$-MeOH-H_2O, 90 : 10 : 1 to 70 : 30 : 3). **2** (4 mg) and **3** (19 mg) were also obtained from fraction IV (80 mg) by SiO_2 CC ($CHCl_3$-MeOH-H_2O, 85 : 15 : 1 to 70 : 30 : 3).

Arvensoside C (**1**): Amorphous powder; $[\alpha]_D^{24} + 71.4$ ($c = 0.07$, MeOH); HRESI-MS: ($C_{48}H_{74}O_{18}$) found $m/z =$ 961.4623 for $[M+Na]^+$, calcd. 961.4652; ESI-MS: $m/z =$ 937 $[M-H]^-$, 793 $[M-H-144]^-$, 631 $[M-H-144-162]^-$, 455 $[M-H-144-162-176]^-$; ^{1}H NMR (600 MHz, CD_3OD): Table 1; ^{13}C NMR (150 MHz, CD_3OD): Table 1.

Results and Discussion

The MeOH extract of *C. arvensis* was partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The *n*-BuOH and EtOAc extracts were subjected to a series of column chromatographic steps, resulting in the isolation of eight compounds one of which is new.

Compound **1** showed a quasi-molecular ion at $m/z = 961.4623$ $[M+Na]^+$ by HRESI-MS, which is consistent with the molecular formula $C_{48}H_{74}O_{18}$. The ^{1}H and ^{13}C NMR spectroscopic data (see Table 1) revealed an ester triterpenoid glycosidic structure. The ^{1}H NMR spectrum of **1** displayed signals arising from seven tertiary methyl groups ($\delta_H = 0.83$, 0.86, 0.94, 0.96, 0.98, 1.07, and 1.18), an oxymethine ($\delta_H = 3.23$) and an olefinic proton ($\delta_H = 5.28$) in the aglycon moiety. These findings along with the 30 resonances observed in the ^{13}C NMR spectrum indicated the aglycon as oleanolic acid [14]. Additionally, the

resonances of anomeric protons observed at $\delta_H = 4.45$ (d, $J = 7.8$ Hz) and 5.41 (d, $J = 8.1$ Hz) in the ^{1}H NMR spectrum were indicative of the presence of two β-linked sugar units. The structures of the sugar units were elucidated as β-D-glucopyranose and β-D-glucuronopyranose by using 1D TOCSY, 2D COSY, HOHAHA and HSQC experiments. The absence of any glycosidation shift for both sugar units suggested that compound **1** was bisdesmosidic. The chemical shifts of anomeric proton ($\delta_H = 5.41$) and carbon ($\delta_C = 95.3$) signals of the β-D-glucopyranose unit indicated that this sugar unit was attached to C-28 carboxyl group *via* an ester linkage which was further confirmed with the long-range correlation between H-1 of the β-D-glucopyranose and C-28 ($\delta_C = 178.0$) in the HMBC experiment. The location of the β-D-glucuronopyranose was found to be at C-3 by the cross-peak between C-3 ($\delta_C = 90.4$) of the oleanolic acid with the anomeric proton. In addition to the proton and carbon resonances assigned to the aglycon and sugar portion, the ^{1}H NMR spectrum of **1** also contained two methylene groups ($\delta_H = 2.66$ and 2.70; $\delta_H = 2.44$ and 2.58) and a tertiary methyl group ($\delta_H = 1.39$) signals. These data along with the corresponding six carbon signals ($\delta_C = 172.0$, 47.9, 71.0, 47.7, 173.4, 27.9) observed in the ^{13}C NMR spectrum led to the identification of dicrotalic acid (3-hydroxy-3-methyl-glutaric acid) [15]. The appearance of downfield signal of H-3 ($\delta_H = 4.98$) and C-3 ($\delta_C = 78.8$) of the glucuronic acid residue and the long-range correlation between the carbonyl carbon ($\delta_C = 172.0$) of the dicrotolic acid and H-3 of the glucuronic acid confirming that the dicrotalic acid was located at C-3 of the glucuronic acid. On the basis of these data, the structure of **1** was identified as 3-*O*-(3-*O*-dicrotaloyl)-β-D-glucuronopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl ester, for which the trivial name arvensoside C is proposed.

In addition to the new compound, four known saponins, arvensoside A (**2**) and B (**3**) [5], glycoside C (**4**) [16], calenduloside D (**5**) [17] and three known flavonol glycosides isorhamnetin 3-*O*-β-D-glucopyranoside [18], quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-galactopyranoside [19] were isolated and identified by comparison of their spectroscopic (NMR and MS) data with those published in the literature.

Arvensoside C (**1**) is also the first ester triterpenoid saponin bearing a dicrotalic acid from the genus *Calendula* while the myristic acid and palmitic acid esters of triterpenoids have previously been isolated from *C. officinalis* [9].

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