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Isolation and identification of a new saponin from *Cephalaria aytachii*

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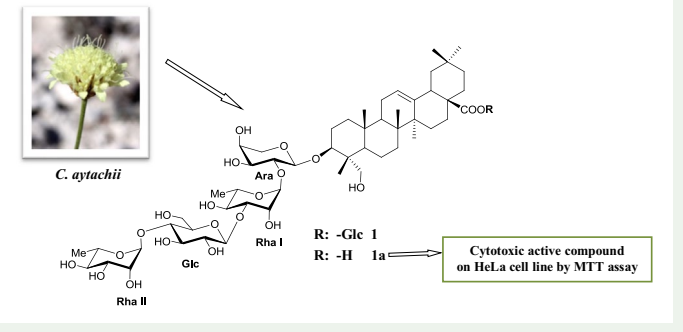
ABSTRACT

A new hederagenin-type triterpenoid glycoside (**1**) named Aytachoside A, along with eight known triterpene glycosides, were isolated from the aerial parts of *Cephalaria aytachii* Gokturk and Sumbul (Dipsacaceae). The structures of compounds **1**–**9** were determined by spectroscopic (1D and 2D NMR, HRESIMS) and chemical examinations. The antimicrobial effect of compound **1** was found considerably active against *Escherichia coli*, *Pseudomonas aeruginosa* and especially *Salmonella typhimurium* microorganisms using the MIC method. Although compound **1** was found not to have a remarkable toxic effect at a concentration lower than 300 µg/mL, cytotoxic activity tests demonstrated that prosapogenin **1a** exhibits a significant cytotoxic activity against HeLa cell lines using the MTT assay for the first time.



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1. Introduction

Cephalaria which is an important genus of the family Dipsacaceae consists of 94 species distributed mainly in the Mediterranean region and the Middle East. Forty *Cephalaria* species are spread out in Turkey and 24 of them are endemic (Davis 1972; Gokturk et al. 2012). As it is clear from the chemical studies of this genus, *Cephalaria* species have lots of natural compounds, including alkaloids (Aliev et al. 1975), iridoids (Gođevac et al. 2006; Mustafaeva et al. 2008), flavonoids (Godjevac et al. 2004; Movsumov et al. 2009), phenolics (Kayce & Kirmizigul 2010) and especially saponins (Kirmizigul et al. 1996; Kirmizigul & Rose 1997;

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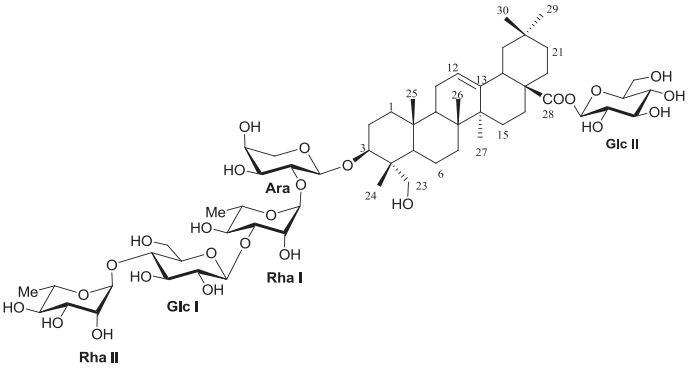

Figure 1. Structure of new compound Aytachoside A (**1**).

Gođevac et al. 2006; Tabatadze et al. 2007; Capanlar & Kirmizigul 2010; Godevac et al. 2010; Sarikahya & Kirmizigul 2010; Sarikahya & Kirmizigul 2012a, 2012b; Kayce et al. 2014; Sarikahya 2014) that have different biological potent, particularly antibacterial (Sarikahya & Kirmizigul 2012a, 2012b), antifungal (Kirmizigul et al. 1996) and cytotoxic activities (Tabatadze et al. 2007; Pasi et al. 2009).

In the course of our ongoing search for cytotoxic active triterpene saponins (Podolak et al. 2010), we have investigated the chemical constituents of the *n*-BuOH extract of the *Cephalaria aytachii*. In this paper, we report the isolation and structural elucidation of a new compound **1**, named Aytachoside A (Figure 1) and eight known glycosides. The chemical structure of the new compound was identified as 3-*O*-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**1**). The structures of eight known triterpenic compounds were determined as; 3-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester (**2**) (Braca et al. 2004), Dipsacoside B (**3**) (Mukhamedziev et al. 1971), Elmalienoside C, Elmalienoside A, Elmalienoside B (**4**–**6**) (Sarikahya & Kirmizigul 2012a), *α*-Hederin (**7**) (Aliev & Movsumov 1976), Macranthoside A (**8**) (Saito et al. 1990) and Davisianoside B (**9**) (Kayce et al. 2014) (Figure S21). Their structures were elucidated using chemical and spectroscopic methods, including 1D, 2D NMR and HRESIMS techniques. Antimicrobial effect of **1** was examined against different bacterial strains and yeast using MIC method. Cytotoxic activity of **1** and prosapogenin **1a** were investigated against the HeLa cell lines by MTT assay for the first time.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. The positive-ion HRESIMS of **1** exhibited an ion peak at *m/z* 1243.6075 $[M + Na]^+$, indicating the molecular formula of $C_{59}H_{96}O_{26}$. The IR spectrum showed absorption bands at 3307, 2948, 1650, 1649, 1449, 1409, 1015 cm^{-1} corresponding hydroxyl, carbonyl, olefin and etheric functions. The ^1H NMR spectrum displayed six tertiary methyl protons at δ_H 0.55 (3H, s, H-24), 0.65 (3H, s, H-26), 0.85 (3H, s, H-27), 0.86 (3H, s, H-29), 0.87 (3H, s, H-30), 0.88 (3H, s, H-25), an olefinic proton signal at δ_H 5.14 (1H, s, H-12), an oxygen-bearing methine proton at δ_H 3.48 (1H, H-3) and one primary alcoholic function at δ_H 3.08 and 3.10 (2H, m, H-23). These signals along with the carbon

resonances in the ^{13}C NMR spectrum for tertiary methyl carbons at δ_C 13.5 (C-24), 17.2 (C-26), 26.0 (C-27), 33.2 (C-29), 23.8 (C-30), 16.1 (C-25), two olefinic carbons at δ_C 122.1, 143.9, the oxygen-bearing methine carbon at δ_C 79.8 (C-3) and the primary alcoholic carbon at δ_C 62.8 suggested that compound **1** possessed hederagenin as an aglycone (Sharma et al. 2013). The downfield shifts of C-3 (79.8) and C-28 (175.7) of the aglycone indicated that compound **1** is a bisdesmosidic glycoside. The ^{13}C NMR spectrum showed 59 carbon signals, of which 30 were assigned to the aglycone moiety and 29 to a sugar portion made up of five sugar units. The ^{1}H NMR spectrum displayed in the sugar region signals corresponding to five anomeric protons at δ_H 4.31 (brs, Ara), 5.01 (brs, Rha II), 5.09 (brs, Rha I), 5.20 (d, J = 7.6 Hz, Glc II) and 4.32 (d, J = 8.0 Hz, Glc I) which were unambiguously correlated by HSQC experiment to the corresponding carbon resonances at δ_C 103.7, 101.6, 100.8, 94.6 and 104.6 indicated the presence of five sugar moieties, respectively. These ^{1}H NMR data were also supported with ^{1}H NMR results in pyridine-d_5 (see supplementary material).

The coupling constants showed two *β*-glycosidic linkages for two glucose units and three *α*-glycosidic linkages for two rhamnose and one arabinose units. This fact is also confirmed by way of acidic hydrolysis and GC-MS analysis. Acid hydrolysis and GC-MS analysis revealed that compound **1** has two D-glucose, one L-arabinose and two L-rhamnose units. The HMBC correlations between H-1 of arabinose at δ_H 4.31 and C-3 of aglycone at δ_C 79.8 and between carbonyl carbon at δ_C 175.7 and H-1 of glucose II at δ_H 5.20, indicated that the glycosidic chains were located at C-3 and C-28 of aglycone, respectively. The other HMBC correlations between H-1 of rhamnose I at δ_H 5.09 and C-2 of arabinose at δ_C 74.5, between H-1 of glucose I at δ_H 4.32 and C-3 of rhamnose I at δ_C 81.9, between H-1 of rhamnose II at δ_H 5.01 and C-4 of glucose I at δ_C 81.2 proved all the linkaging points between sugar to sugar units. After the alkaline hydrolysis of **1**, the ^{1}H NMR data of prosapogenin (**1a**) also verified the structure as 3-*O*-*α*-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl hederagenin 28-*O*-*β*-D-glucopyranosyl ester, namely Aytachoside A (Figure 1).

3. Experimental

3.1. General

Optical rotations of pure compounds were measured at 23 °C using a Rudolph Research Analytical Autopol I automatic polarimeter fitted with a sodium lamp with 1 mL cell. IR spectra were obtained on ATI Mattson 1000 Genesis Series FTIR instrument using KBr discs. NMR experiments were performed on Varian AS 400 MHz and Varian ASP 600 MHz instruments in DMSO-d_6 and in pyridine-d_5, respectively. All chemical shifts (δ) were given in ppm units with reference to tetramethylsilane (TMS) as an internal standard, and the coupling constants (J) were in Hz. HRESIMS analyses were carried out using a Bruker LC micro-Q-TOF mass spectrometer. GC-MS analysis was performed by a Shimadzu GC-MS QP 2010 plus instrument with Rtx-CLP2 cat-42302 apolar column (20 m–0.18 mm ID-0.14 µm df). Medium pressure liquid chromatography (MPLC) was run using a Buchi system (Buchi C-605 pumps, coupled to a UV detector) with Buchi glass column (26/920). Lichroprep RP-18 (25–40 µm; Merck) and silica gel 60 (0.063–0.200 mm; Merck) were used both for column chromatography and MPLC studies. Thin-layer chromatography (TLC) was performed on F254 (Merck) and RP-18 F254s (Merck) precoated aluminium sheets.

3.2. Plant material

The aerial parts of *C. aytachii* Gokturk & Sumbul (Dipsacaceae) were collected from Eskisehir, Sivrihisar-Afyon highway, at 942 m in altitude, in August 2010 and identified by Prof. H. Sumbul and Prof. R. S. Gokturk (Department of Biology, Faculty of Art and Science, Akdeniz University). Voucher specimens (R. S. Gokturk 7483) were kept in the Herbarium Research and Application Centre of Akdeniz University, Antalya in Turkey.

3.3. Extraction and isolation

Air-dried and powdered plant material of *C. aytachii* (whole plant, 1130 g) was extracted with MeOH (4 × 4L) at room temperature overnight. The methanolic solution was then evaporated to dryness under reduced pressure at ~40 °C and gave 135 g of a dark residue. This residue was extracted with (3 × 400 mL) *n*-BuOH:H_2O (1:1) solvent system. After the separation of *n*-BuOH and H_2O phases, the *n*-BuOH fraction was defatted with *n*-hexane (10 × 50 mL) to remove the apolar and oily substances.

The *n*-BuOH extract (60.5 g) was subjected to vacuum liquid chromatography (VLC) using reversed-phase silica gel (Lichroprep RP-18) employing MeOH:H_2O solvent system with a gradient from 0% to 100% MeOH to give 11 main fractions.

The 60% MeOH fraction (20.0 g) of RP-VLC was exposed to MPLC over silica gel using a suitable column (26 mm × 920 mm) and programme (max. pressure: 40 bar, flow rate: 23 mL/min, $CHCl_3$:MeOH:H_2O solvent system, 9:1:0–61:32:7). The 10th fraction of MPLC experiment was loaded into an open silica gel column chromatography with the solvent system $CHCl_3$:MeOH:H_2O (90:10:1–70:30:3) to afford 14 sub-fractions. A new saponin, compound **1** (68.6 mg) was obtained using open column chromatography with RP-18 using MeOH:H_2O (1:4) solvent system from the second sub-fraction. Known compounds **2** (26.6 mg), **3** (37.8 mg), **4** (85.5 mg), **5** (75.4 mg) and **6** (55.4 mg) were purified by RP-18 column chromatography with MeOH:H_2O (1:4) solvent system from the sub-fractions 1, 4, 8, 9 and 11, respectively.

The 70% MeOH fraction (11.5 g) of RP-VLC was exposed to MPLC over silica gel using a suitable column (26 mm × 920 mm) and programme (max. pressure: 40 bar, flow rate: 23 mL/min, $CHCl_3$:MeOH:H_2O solvent system, 9:1:0–61:32:7). Twelve sub-fractions were derived. Compounds **7** (19.5 mg), **8** (136.2 mg) and **9** (333.5 mg) were purified by an open silica gel column chromatography with the solvent system $CHCl_3$:MeOH:H_2O (9:1:0–80:20:2) from the combined third and fourth sub-fractions of MPLC.

3.4. Aytachoside A

Aytachoside A handled as a brown syrup (68.6 mg), $[\alpha]_D^{23}$ − 4.81 (*c* 0.5, MeOH); IR (KBr) ν_{max} 3307, 2948, 1650, 1649, 1449, 1409, 1015 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz): δ 3.48 (1H, m, H-3), 5.14 (1H, s, H-12), 3.08 (1H, m, Ha-23), 3.10 (1Hb, m, H-23), 0.55 (3H, s, H-24), 0.88 (3H, s, H-25), 0.65 (3H, s, H-26), 0.85 (3H, s, H-27), 0.86 (3H, s, H-29), 0.87 (3H, s, H-30), 4.31 (1H, overlapped, H-1Ara), 3.48 (1H, m, H-2Ara), 3.47 (1H, m, H-3Ara), 3.57 (1H, m, H-4Ara), 3.30 (1H, m, Ha-5Ara), 3.65 (1H, m, Hb-5Ara), 5.09 (1H, brs, H-1RhaI), 3.92 (1H, m, H-2RhaI), 3.61 (1H, m, H-3RhaI), 3.65 (1H, m, H-4RhaI), 3.77 (1H, m, H-5RhaI), 1.06 (3H, d, J = 6.6 Hz, H-6RhaI), 4.32 (1H, d, J = 8.0 Hz, H-1GlcI), 3.16 (1H, m, H-2GlcI), 3.17 (1H, m, H-3GlcI), 3.37 (1H, m, H-4GlcI), 3.16 (1H, m, H-5GlcI), 3.46 (1H, m, Ha-6GlcI), 3.62 (1H, m, Hb-6GlcI), 5.01 (1H, brs,

H-1RhaII), 3.47 (1H, m, H-2RhaII), 3.16 (1H, m, H-3RhaII), 3.38 (1H, m, H-4RhaII), 3.86 (1H, m, H-5RhaII), 1.07 (3H, d, $J = 6.6$ Hz, H-6RhaII), 5.20 (1H, d, $J = 7.6$ Hz, H-1GlcII), 3.08 (1H, m, H-2GlcII), 3.16 (1H, m, H-3GlcII), 3.11 (1H, m, H-4GlcII), 3.12 (1H, m, H-5GlcII), 3.42 (1H, m, Ha-6GlcII), 3.56 (1H, m, Hb-6GlcII). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 79.8 (C-3), 122.1 (C-12), 143.9 (C-13), 62.8 (C-23), 13.5 (C-24), 16.1 (C-25), 17.2 (C-26), 26.0 (C-27), 175.7 (C-28), 33.2 (C-29), 23.8 (C-30), 103.7 (C-1Ara), 74.5 (C-2Ara), 73.5 (C-3Ara), 68.5 (C-4Ara), 65.4 (C-5Ara), 100.8 (C-1RhaI), 69.9 (C-2RhaI), 81.9 (C-3RhaI), 71.1 (C-4RhaI), 67.9 (C-5RhaI), 18.3 (C-6RhaI), 104.6 (C-1GlcI), 68.4 (C-2GlcI), 75.1 (C-3GlcI), 81.2 (C-4GlcI), 77.0 (C-5GlcI), 61.1 (C-6GlcI), 101.6 (C-1RhaII), 71.0 (C-2RhaII), 72.6 (C-3RhaII), 71.0 (C-4RhaII), 68.2 (C-5RhaII), 18.2 (C-6RhaII), 94.6 (C-1GlcII), 72.8 (C-2GlcII), 76.9 (C-3GlcII), 69.7 (C-4GlcII), 78.2 (C-5GlcII), 61.1 (C-6GlcII). Positive-ion ESIMS *m/z* 1243.9 $[M + Na]^+$; positive-ion HRESIMS *m/z* 1243.6075, $[M + Na]^+$ (Calcd for $C_{59}H_{96}O_{26}Na$, 1243.6082) (Figure S1–S18).

3.5. Alkaline hydrolysis

Pure compound **1** (10 mg) was refluxed in 5% KOH solution (pH 12–13) at 80 °C for 1 h. The reaction mixture was neutralised with 5% HCl solution and then concentrated to dryness (Calis et al. 1996). The residue was extracted with *n*-BuOH:H_2O (1:1), and the organic layer of pure compound (**1a**) was analysed by ^{1}H NMR and HRESIMS techniques (Figure S19–S20). Alkaline hydrolysis of **1** also afforded a known triterpene glycoside compound **9** (Kayce et al. 2014).

3.6. Acid hydrolysis and sugar analysis

The configurations of the sugar units were determined using GCMS analyses comparing with authentic samples. In this method, firstly compound **1** (5 mg) was hydrolysed with 1N HCl for 6 h at 95 °C. After the extraction with $CHCl_3$, the aqueous layer was evaporated to dryness and kept in drying oven for 48 h at 25 °C. After that the sugars residue and the standard sugar samples were dissolved in anhydrous pyridine (1 mL), and then 1 mL of HMDS-TMCS (hexamethyldisilazane–trimethylchlorosilane, 1:1) was added. Then the mixtures were stirred at 70 °C for 1 h., concentrated under N_2 stream, solved with *n*-hexane (1 µL) and analysed by GCMS (Programme: initial temp. 100 °C rising 10 °C/min. Hold time 2 min. Then rising 30 °C up to 280 °C, hold time 3 min. Total programme time 17.67 min) (Kirmizigul & Anil 1994).While the standard silylated sugar peaks of L-arabinose, L-rhamnose and D-glucose were detected at tR (min) 5.70, 5.76 and 8.40, these peaks were observed at 5.61, 5.71 and 8.30 min for **1**, respectively.

3.7. Antimicrobial activity studies

In vitro antimicrobial activity tests of the new compound **1** was evaluated using Minimum Inhibitory Concentration (MIC) measurements against seven bacterial strains [four Gram-negative; *Escherichia coli* (ATCC 12228), *Klebsiella pneumoniae* (CCM 2318), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (CCM 5445), three Gram-positive; *Staphylococcus aureus* (ATCC6538-P) *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633) and one yeast *Candida albicans* (ATCC 10239)] (see supplementary material). The MIC is defined as the lowest concentration that inhibits microbial cell with TTC addition.

Table 1. Antimicrobial activity results for compound **1**.[a,b]

	Minimum inhibitory concentration							
Compound (mg/mL)	*E. coli*	*S. aureus*	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*	*S. typhimurium*	*B. subtilis*	*C. albicans*
1	1.0	–	1.0	–	–	0.5	–	–
Gentamycin (μg/mL)	1.25	1.25	16.0	2.5	2.5	1.25	1.25	n.t.
Clotrimazole (μg/mL)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	0.75

[a]n.t.: not tested.
[b]'–': ineffective against tested microorganisms.

Table 2. Cytotoxic activity results of compounds **1** and **1a**.[a]

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay		
Sample concentration (mg/mL)	Compound **1**	Compound **1a**
0.001	135.000 ± 14.800	125.000 ± 11.700
0.01	130.000 ± 9.100	45.200 ± 13.500
0.1	118.000 ± 15.500	5.980 ± 0.932
0.25	105.000 ± 7.870	7.330 ± 0.815
0.5	81.000 ± 6.700	8.680 ± 0.950
1.0	57.800 ± 6.730	–

[a]Cell viability (% of control).

Gentamycin (Sigma) and Clotrimazole (Sigma) were used as positive and negative controls, respectively (Atlas et al. 1995).

3.8. Cytotoxic activity studies

Cytotoxic activities of the samples (**1** and **1a**) were tested using the MTT assay with minor modifications (Onay-Ucar et al. 2012), (see supplementary material). The half maximal inhibitory concentration (IC_{50}) of the extracts on HeLa cells were calculated from a graph of cell viability versus the sample concentrations.

Data are given as mean values ± SD with '*n*' denoting the number of experiments. Statistical comparisons were made using one-way analysis of variance (ANOVA) module of GraphPad Prism 5. Difference in mean values were considered significant when $p < 0.05$.

4. Conclusion

One new and eight known glycosides have been isolated from *C. aytachii*. The aglycones of all compounds were identified as hederagenin. Antimicrobial and cytotoxic activity tests of Aytachoside A were examined by MIC and MTT methods, respectively. Comparing the antimicrobial activity results with standards, compound **1** was found considerably active against *E. coli, P. aeruginosa* and especially *S. typhimurium* micro-organisms (Table 1). Prosapogenin **1a** was found as slightly active compound in literature (Kayce et al. 2014). Cytotoxic activity results of compounds **1** and prosapogenin **1a**, demonstrated that prosopagenin **1a** is the most active compound on HeLa cells. Compound **1** did not show a remarkable toxic effect on HeLa cells, at a concentration lower than 300 μg/mL (Table 2, Figure S22).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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