SUPPLEMENTARY MATERIAL

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

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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* Goktuk & 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 *E. coli*, *P. aeruginosa* and especially *S. thyphimirium* 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.

**Keywords:** Aytachoside A; Dipsacaceae; *Cephalaria aytachii*; triterpene saponin; antimicrobial activity; cytotoxic activity
Experimental 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)]. The bacterial strains were inoculated on Mueller–Hinton broth (MHB, Difco) and incubated for 24 h at 37 ± 0.1 °C. The inocula from 24 h broth cultures were adjusted to 0.5 McFarland standards. The dilution series of the compound was prepared in test tube then transferred to the broth in 96-well microtiter plate. Final concentration was ranged from 5 to 0.3 mg/mL in the medium. The last well containing 100 μL of MHB without compound and 10 μL of the inocula on each strip was used as a negative control. All plates were covered with a sterile plate sealer and incubated at 37 °C for 24 h. The MIC is defined as the lowest concentration that inhibits microbial cell with TTC addition. Gentamycin (Sigma) and Clotrimazole (Sigma) were used as positive and negative controls, respectively (Atlas et al. 1995).

Cytotoxic activity studies
Cytotoxic activities of the samples were tested by using the MTT assay with minor modifications (Onay-Ucar et al. 2012). The assay based on the reduction of MTT (the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a colored formazan product by mitochondrial dehydrogenase, which is active only in living cells. The stock solutions of the samples were diluted with EMEM. The cells were maintained in 96 well-plates (each well contained 200 μl cell suspension at a density of 1×10^5 cells/ml). After reaching confluence (1 day later), the cells were treated with increasing concentrations (1 μg/mL-1000 μg/mL) of the samples diluted with EMEM. After growth of the cells for 48h at 37°C in a humidified 5% CO₂ atmosphere, the adherent cells were washed with phosphate buffered saline (PBS), then 10 μL of MTT stock solution (5 mg/mL) and 90 μl PBS buffer was added to each well and the plates were further incubated at 37°C for 4h. At the end of this period, supernatants were discarded, DMSO (200 μ) was added to each well to solubilize the water-insoluble purple formazan crystals. The absorbance was measured at 570 and 690 nm in a microplate reader.
(μQuant, Bio-Tek Instruments, Inc. Highland Park, USA). The cell viability was calculated using the following equation:

Cell viability (%) = \( \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \)

The half maximal inhibitory concentration (IC₅₀) 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.
List of Supplemental Material

Figure S1. $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1) (page 5).

Figure S2. Expanded $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1) (page 6).

Figure S3. Expanded $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1) (page 7).

Figure S4. $^1$H NMR (600 MHz, Pyridine-$d_5$) spectrum of Aytachoside A (1) (page 8).

Figure S5. Expanded $^1$H NMR (600 MHz, Pyridine-$d_5$) spectrum of Aytachoside A (1) (page 9).

Figure S6. Expanded $^1$H NMR (600 MHz, Pyridine-$d_5$) spectrum of Aytachoside A (1) (page 10).

Figure S7. Expanded $^1$H NMR (600 MHz, Pyridine-$d_5$) spectrum of Aytachoside A (1) (page 11).

Figure S8. Expanded $^1$H NMR (600 MHz, Pyridine-$d_5$) spectrum of Aytachoside A (1) (page 12).

Figure S9. $^{13}$C NMR (100 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1) (page 13).

Figure S10. Expanded $^{13}$C NMR (100 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1) (page 14).

Figure S11. COSY spectrum of Aytachoside A (1) (page 15).

Figure S12. Expanded COSY spectrum of Aytachoside A (1) (page 16).

Figure S13. Expanded COSY spectrum of Aytachoside A (1) (page 17).

Figure S14. HSQC spectrum of Aytachoside A (1) (page 18).

Figure S15. Expanded HSQC spectrum of Aytachoside A (1) (page 19).

Figure S16. HMBC spectrum of Aytachoside A (1) (page 20).

Figure S17. Expanded HMBC spectrum of Aytachoside A (1) (page 21).

Figure S18. HRESIMS spectrum of Aytachoside A (1) (page 22).

Figure S19. $^{13}$H NMR (400 MHz, DMSO-$d_6$) spectrum of prosapogenin 1a (page 23).

Figure S20. HRESIMS spectrum of prosapogenin 1a (page 24).

Figure S21. The structures of compounds 2–9 (page 25).

Figure S22. Cytotoxic effects of the compound 1 on HeLa cells (page 26).
Figure S1. $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1).
Figure S2. Expanded $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1).
Figure S3. Expanded $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1).
Figure S4. $^1$H NMR (600 MHz, pyridine-$d_5$) spectrum of Aytachoside A (1).
Figure S5. Expanded $^1$H NMR (600 MHz, pyridine-$d_5$) spectrum of Aytachoside A (I).

Sample Name: CAY_6
Data Collected on: Agilent-NMR-vnhr600
Archive directory:
Sample directory:
File: PROTON_61
Pulse Sequence: PROTON (s2pul)
Solvent: pyridine
Data collected on: Apr 28 2016

Temp. 30.0 C / 303.1 K
Operator: vinri

Relax. delay 1.000 sec
Pulse 45.0 degrees
Avg. time 1.764 sec
Width 6615.6 Hz
512 repetitions
OBSERVE XL, 599.7191024 MHz
DATA PROCESSING:
PT size 32768
Total time 23 min
Figure S6. Expanded $^1$H NMR (600 MHz, pyridine-$d_5$) spectrum of Aytachoside A (1).
Figure S7. Expanded $^1$H NMR (600 MHz, pyridine-$d_5$) spectrum of Aytachoside A (1).
Figure S8. Expanded $^1$H NMR (600 MHz, pyridine-$d_5$) spectrum of Aytachoside A (1).
Figure S9. $^1^C$ NMR (100 MHz, DMSO-$d_6$) spectrum of Atachoside A (1).
Figure S10 Expanded $^{13}$C NMR (100 MHz, DMSO-$d_6$) spectrum of Aytachoside A (1).
Figure S11 COSY spectrum of Aytachoside A (I).
Figure S12 Expanded COSY spectrum of Aytachoside A (1).
Figure S13 Expanded COSY spectrum of Aytachoside A (1).
Figure S14. HSQC spectrum of Aytachoside A (1).
Figure S15. Expanded HSQC spectrum of Aytachoside A (1).
Figure S16. HMBC spectrum of Aytachoside A (1).
Figure S17. Expanded HMBC spectrum of Aytachoside A (I).
Figure S18. HRESIMS spectrum of Aytachoside A (1).
Figure S19 $^1$H NMR (400 MHz, DMSO-$d_6$) spectrum of prosapogenin 1a
Figure S20. HRESIMS spectrum of prosapogenin 1a.
| Compounds | $R_1$ | $R_2$ |
|-----------|-------|-------|
| 2         | ![Structure](image1) | ![Structure](image2) |
| 3         | -H    | ![Structure](image3) |
| 4         | -H    | ![Structure](image4) |
| 5         | ![Structure](image5) | ![Structure](image6) |
| 6         | ![Structure](image7) | ![Structure](image8) |
| 7         | -H    | -H    |
| 8         | ![Structure](image9) | -H    |
| 9         | ![Structure](image10) | -H    |

Figure S21. The structures of compounds 2-9.
a) Compound 1 (P<0.0001, R²=0.898),

b) Prosapogenin 1a (P<0.0001, R²=0.986) [Data are mean ±SD of percent changes compared with untreated controls (n=6)]

Figure S22. Cytotoxic effects of the compound 1 and 1a on HeLa cells.

References
Atlas RM, Parks LC, Brown AE. 1995. Laboratory Manual of Experimental Microbiolog; Mosby-Year Books: St. Louis, p. 341.
Onay-Ucar E, Erol O, Kandemir B, Mertoglu E, Karagoz A, Arda N. 2012. *Viscum album* L. extracts protects HeLa cells against nuclear and mitochondrial DNA damage. eCAM. 2012:1-7.