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* Gokturk & 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. aureginosa* 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:

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

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 $\pm$ 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 $P < 0.05$.

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Figure S1. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) spectrum of Aytachoside A (1).

Figure S2. Expanded ^1H NMR (400 MHz, $\text{DMSO}-d_6$) spectrum of Aytachoside A (1).

Figure S3. Expanded ^1H NMR (400 MHz, $\text{DMSO}-d_6$) spectrum of Aytachoside A (**1**).

Figure S4. ^1H NMR (600 MHz, pyridine- d_5) spectrum of Aytachoside A (**1**).

Sample Name:

CAY_6

Data Collected on:

Agilent-NMR-vnmr5600

Archive directory:

Sample directory:

Fidfile: PROTON_01

Pulse Sequence: PROTON (s2pul1)

Solvent: pyridine

Data collected on: Apr 28 2016

Temp. 30.0 C / 303.1 K

Operator: vmmr1

Relax. delay 1.000 sec

Pulse 45.0 degrees

Acq. time 1.704 sec

Width 9615.4 Hz

512 repetitions

OBSERVE H1, 599.7191024 MHz

DATA PROCESSING

PT size 32768

Total time 23 min

Figure S5. Expanded ^1H NMR (600 MHz, pyridine-*d*₅) spectrum of Aytachoside A (**1**).

Figure S6. Expanded ¹H NMR (600 MHz, pyridine-*d*₅) spectrum of Aytachoside A (1).

Figure S7. Expanded ¹H NMR (600 MHz, pyridine-*d*₅) spectrum of Aytachoside A (1).

Sample Name:
CAY_6
Data Collected on:
Agilent-NMR-vnmr600
Archive directory:
Sample directory:
File: PROTON_01
Pulse Sequence: PROTON (s2pul)
Solvent: pyridine
Data collected on: Apr 28 2016
Temp. 30.0 C / 303.1 K
Operator: vnmr1
Relax. delay 1.000 sec
Pulse 45.0 degrees
Acq. time 1.794 sec
Width 9615.4 Hz
512 repetitions
OBSERVE H1, 599.7191024 MHz
DATA PROCESSING
Ft size 32768
Total time 23 min

Figure S8. Expanded ^1H NMR (600 MHz, pyridine- d_5) spectrum of Aytachoside A (1).

CAY6-cl3

exp15 CARBON

```

SAMPLE          PRESATURATION
date Apr 28 2015 satmode n
solvent dms0 wet n
file /home/vmr1/v- SPECIAL
nmrsys/data/CAY6-c- temp 30.0
13_20150428_01/CAR- gain 30
BON_01.fid spin 20
ACQUISITION hst 0.008
sw 25125.6 pw90 10.200
at 1.304 alfa 10.000
np 65536 FLAGS
fb 13800 il n
bs 8 in n
dl 2.000 dp y
nt 25600 hs nm
ct 25072 PROCESSING
TRANSMITTER lb 0.50
tn C13 fn not used
sfrq 100.562 DISPLAY
tof 1519.6 sp -1501.5
tpwr 61 wp 25124.9
pw 5.100 xfl 1502.3
DECOUPLER xfp 0
dn H1 rp -21.1
dof 0 lp -420.1
dm yfy PLOT
decwave w wc 268
dpwr 44 sc 0
dmf 8117 vs 2659
th ai cdc ph 4

```

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

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

Figure S11 COSY spectrum of Aytachoside A (1).

CAY6-2D

exp15 gCOSY

SAMPLE		FLAGS	
date	May 3 2015	hs	nn
solvent	DMSO	sspul	y
sample	CAY6-2D_201- hsglv1		1200
50430_01 SPECIAL			
ACQUISITION		temp	30.0
sw	4310.3	gain	18
at	0.150	spin	0
np	1294	F2 PROCESSING	
fb	not used	sb	-0.075
ss	32	sbs	not used
d1	1.000	fn	4096
nt	16	F1 PROCESSING	
2D ACQUISITION		sb1	-0.080
sw1	4310.3	sbs1	not used
ni	512	proc1	lp
d2	0	fn1	4096
PRESATURATION DISPLAY			
satmode	n	sp	1028.3
wet	n	wp	1178.6
TRANSMITTER		sp1	1024.1
tn	H1	wp1	1222.8
sfrq	399.884	rfl	482.8
tof	-372.6	rfp	0
tpwr	62	rfl1	482.8
pw	15.500	rfd1	0
GRADIENTS PLOT			
gzlvle	1000	wc	241.2
gte	0.001000	sc	6.7
EDratio	1.000	wc2	134.2
gstab	0.000500	sc2	0
DECOUPLER		vs	1103
dn	C13	th	5
dm	nn	ai	cdc
		av

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 (1).

Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name D:\Data\gok\CAY-6-2.d
Method gok_tune_wide.m
Sample Name
Comment

Acquisition Date 10/27/2015 11:08:34 AM

Operator bruker customer
Instrument / Ser# micrOTOF-Q 55

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	5000 V	Set Dry Heater	180 °C
Scan Begin	500 m/z	Set End Plate Offset	-400 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	600.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C40				
Formula, max.	O27-N0-Na-C60				
Measured m/z	1243.61	Tolerance	0.7 ppm	Charge	1
Check Valence	no	Minimum	0	Maximum	0
Nitrogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	no

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

Figure S19 ^1H NMR (400 MHz, $\text{DMSO-}d_6$) spectrum of prosapogenin **1a**

Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name D:\Data\gok\prosopogenin-1.d
 Method gok_tune_wide.m
 Sample Name
 Comment

Acquisition Date 10/27/2015 10:46:59 AM

Operator bruker customer
 Instrument / Ser# microTOF-Q 55

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	5000 V	Set Dry Heater	180 °C
Scan Begin	500 m/z	Set End Plate Offset	-400 V	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste

Generate Molecular Formula Parameter

Formula, min.	C50				
Formula, max.	O22-N0-Na				
Measured m/z	1081.56	Tolerance	0.15 ppm	Charge	1
Check Valence	no	Minimum	0	Maximum	0
Nitrogen Rule	no	Electron Configuration	both		
Filter H/C Ratio	no	Minimum	0	Maximum	3
Estimate Carbon	yes

Figure S20. HRESIMS spectrum of prosapogenin **1a**.

Compounds	R ₁	R ₂
2		
3	-H	
4	-H	
5		
6		
7	-H	-H
8		-H
9		-H

Figure S21. The structures of compounds **2-9**.

a) Compound **1** ($P < 0.0001$, $R^2 = 0.898$),

b) Prosapogenin **1a** ($P < 0.0001$, $R^2 = 0.986$) [Data are mean $\pm$ 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 Microbiology; 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.