Med Chem Res
DOI 10.1007/s00044-015-1345-5

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

New antimicrobial and cytotoxic acylated triterpenoidal saponins from *Gleditsia aquatica*

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Received: 18 September 2014 / Accepted: 19 February 2015

Abstract Phytochemical investigation on the fruits of *Gleditsia aquatica* resulted in the isolation and identification of two new bisdesmosidic triterpenoidal saponins, aquaticasaponin A (**1**) and aquaticasaponin B (**2**) acylated with two and one monoterpenic acids, respectively, and one known cytokinin, aquaticine C (**3**). The structural elucidation of isolated metabolites was established on the basis of 1D, 2D NMR, and MS spectral analyses. The antimicrobial activity of the isolated compounds [**1**–**3**] was evaluated. Compound **1** exhibited the highest degree of activity against *Syncephalastrum racemosum* with an MIC value of 9.2 μM, whereas compound **2** exhibited the highest degree of activity against *Escherichia coli* with an MIC value of 67.3 μM. The isolated compounds also exhibited good cytotoxic activity against human breast cancer (MCF-7) and human colon cancer (HCT-116) cell lines with values of IC_{50} from 0.5 to 1.0 μM. Compound **1** was found to be the most active against colon cancer HCT-116 cell line with IC_{50} value of 0.5 μM.

Keywords *Gleditsia aquatica* · Fabaceae · Acylated oleanane-type triterpenoidal saponins · Antimicrobial activity · Cytotoxic activity

Introduction

Genus *Gleditsia* L. (Fabaceae) has long been known in traditional Chinese folk medicine and used for the treatment of apoplexy, as an expectorant and as a pesticide (Zhong and Dian, 1977). Saponins (Zang *et al.*, 1999a, b, c, d) and cytokinins (Hosny *et al.*, 2009) have been reported from some species of this genus. In the flora of Egypt, the genus *Gleditsia* is represented by three species: *G. aquatica* March., *G. caspia* Desf, and *G. triacanthos*. L. *Gleditsia aquatica* March. is a perennial shrub distributed throughout Egypt. Two new acylated triterpenoidal saponins, aquaticosides A and B, and two new cytokinins, aquaticine A and B were isolated from the fruits of *G. aquatic*a (Ragab *et al.*, 2010). Further, phytochemical study of the fruits of this plant has led to the isolation of another two new saponins, termed aquaticasaponin A (**1**) and aquaticasaponin B (**2**) and one known cytokinin, named aquaticine C (**3**) (Fig. 1). This paper deals with the elucidation of their structures by extensive NMR studies, including DEPT, DQF-COSY, HMQC, and HMBC experiments and the results of hydrolytic cleavage. The two saponins consisting of the same aglycon and the same sugar sequence were acylated with one or two monoterpenic acid units at C-2 position or at C-2 and C-3 positions of the rhamnose moiety, which is attached to C-6 position of the glucose moiety, which is directly connected to the C-28 carbonyl group of the aglycon. Antimicrobial and cytotoxic activities of the isolated compounds (**1**–**3**) have been studied and significant results were obtained.

Electronic supplementary material The online version of this article (doi:10.1007/s00044-015-1345-5) contains supplementary material, which is available to authorized users.

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Published online: 28 February 2015

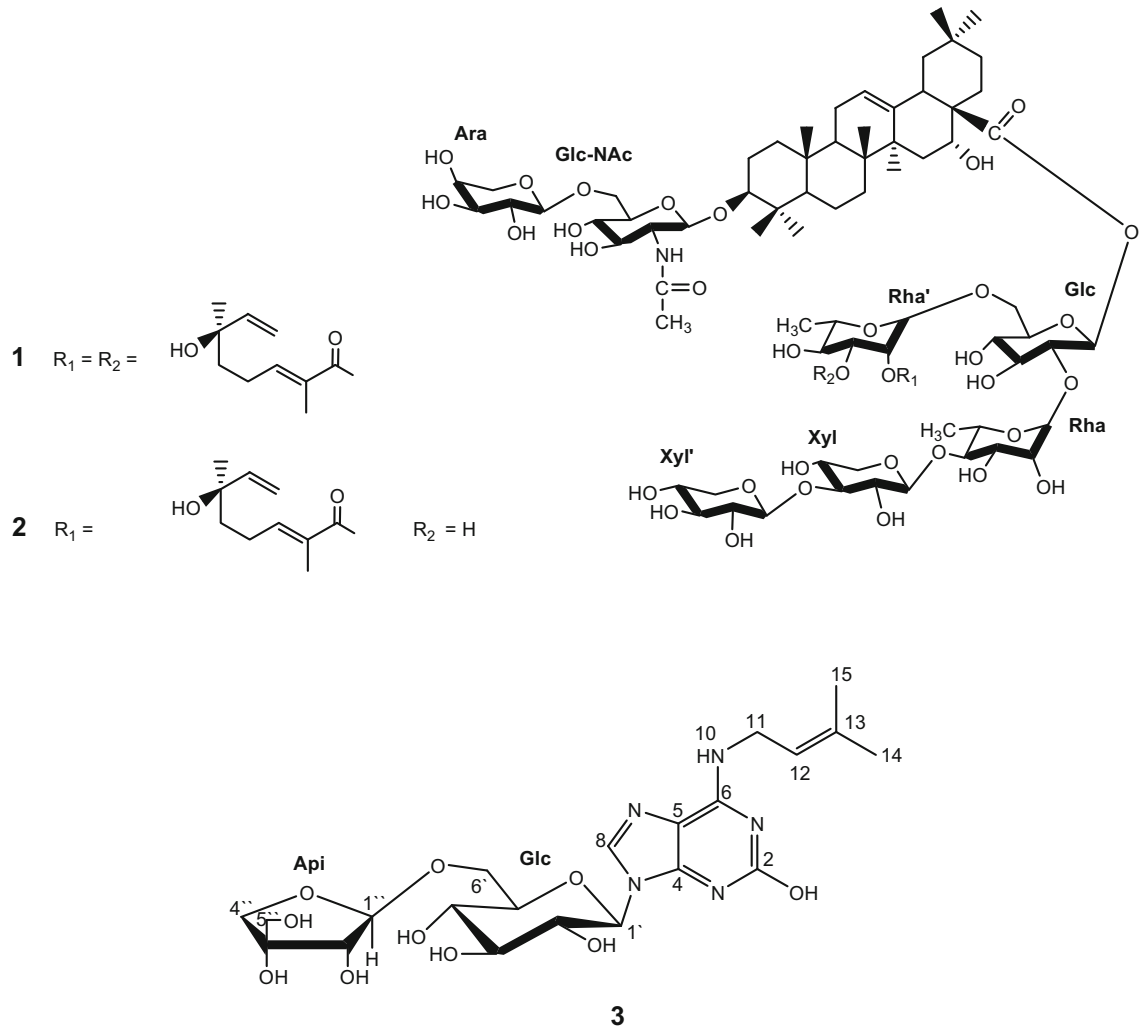

Fig. 1 Compounds **1–3**

Materials and methods

General experimental procedures

UV spectra were determined with a Hitachi 340 spectrophotometer; IR spectra were carried out on a Nicolet 205 FT-IR spectrometer connected to a Hewlett–Packard Color Pro. Plotter. The ^{1}H- and ^{13}C-NMR measurements were obtained with a Bruker NM spectrometer operating at 600 and 400 MHz (for ^{1}H) and 100 MHz (for ^{13}C), a Bruker ARX-500, and a Jeol JNM ECA 500 NMR spectrometer operating at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C) in DMSO-d_6 or CD_3OD solutions, and chemical shifts were expressed in δ (ppm) with reference to TMS, and coupling constant (J) in Hertz. ^{13}C multiplicities were determined by the DEPT pulse sequence (135°). DQF-COSY, HMBC, and HMQC NMR experiments were carried out using a Bruker AMX-600 and a Bruker ARX-500 high field spectrometer equipped with an IBM Aspect-2000 processor and with software VNMR version 4.1 or NUTS program for NMR. HRESIMS spectra were measured using a Bruker Bioapex-FTMS with electrospray ionization (ESI). ESIMS was carried out on a TSQ700 triple quadruple instrument (Finnegan, Santos, CA, USA) mass spectrometer. EIMS was carried out on Scan EIMS-TIC, VG-ZAB-HF, X-mass (158.64, 800.00) mass spectrometer (VG Analytical, Inc.). Polyamide (ICN Biomedicals) and Si gel (Si gel 60, Merck) were used for open column chromatography. Flash column liquid chromatography was performed using J.T. Baker glassware with 40 μm Si gel (Baker) and Sepralyte C_{18} (40 μm) as the stationary phase. TLC was carried out on precoated silica gel 60 F254 (Merck) plates. Developed chromatograms were visualized by spraying with 1 % vanillin-H_2SO_4, followed by heating at 100 °C for 5 min or spraying the developed plates with 2 % ninhydrin in acetone.

Plant material

The fruits of *G. aquatica* March. were collected from agricultural Museum-Dokki, Giza, Egypt in March 2001 and were kindly identified by Engineer Badeia Hassan Aly Dewan, Consultant of Egyptian Flora, Agricultural Museum, Dokki, Giza, Egypt, and by Mrs. Terase Labib, Taxonomist of Orman Garden, Giza, Egypt. A voucher specimen {G-01} has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Antimicrobial assays

Antimicrobial activities of compounds **1–3** were investigated in vitro against different bacteria and fungi. Two standard strains of Gram-positive bacteria [*Streptococcus pneumonia* (RCMB 010010) and *Bacillus subtilis* (RCMB 010067)] and two standard strains of Gram-negative bacteria [*Pseudomonas aeruginosa* (RCMB 010043) and *Escherichia coli* (RCMB 010052)] were used for antibacterial assay. Four clinical pathogenic fungi [*Aspergillus fumigates* (RCMB 02568), *Syncephalastrum racemosum* (RCMB 05922), *Geotricum candidum* (RCMB 05097), and *Candida albicans* (RCMB 05036)] were used for antifungal assay. The microbial species are environmental and clinically pathogenic microorganisms obtained from Regional Center for Mycology and Biotechnology antimicrobial unit (RCMB), Al-Azhar University. Antimicrobial tests were carried out by the agar well diffusion method (Scott, 1989), using 100 µL of suspension containing 1×10^8 colony forming units (CFU)/mL for tested bacteria and 1×10^4 spore/mL fungi spread on nutrient agar and malt extract agar, respectively. After the media had cooled and solidified, wells (6 mm in diameter) were made in the solidified agar and loaded with 100 µL of tested compound solution in 1 mL dimethyl sulfoxide (DMSO) with concentrations of 13.5, 14.7, 48.7 µM for compounds **1–3**, respectively. Negative controls were prepared using DMSO employed for dissolving the tested compound, while ampicillin, gentamycin, and amphotericin B were used as positive controls for Gram-positive bacteria, Gram-negative bacteria, and fungi, respectively. The inoculated plates were then incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi, and the diameter of any resulting zones of inhibition of growth was measured in millimeter (mm). Each experiment was performed in triplicates, and the data were expressed as mean ± SD. The minimal inhibitory concentrations (MICs) were determined by using the twofold serial dilution technique (Rajbhandari and Schopke, 1999). The twofold serial dilutions of the tested compound solutions were prepared. The final concentrations of the solutions were 269.2–0.003, 295.6–0.004, and 974.6–0.013 µM for compounds **1–3**, respectively. The tubes were then inoculated with the test organisms, grown in their suitable broth for tested pathogenic bacteria (1×10^8 CFU/mL for bacteria and 1×10^4 spore/mL for fungi); each 0.5 mL received 100 µL of the above inoculum and was incubated at 37 °C for 24 h for bacteria and after 48 h of incubation at 28 °C for fungi. MIC values were taken as the lowest compound concentration that prevents visible bacterial growth. Each experiment was made three times. The results are presented in (Table 4).

Cytotoxicity assays

The compounds (**1–3**) were tested for cytotoxicity against two human tumor cell lines: breast cancer (MCF-7) and colon cancer (HCT-116) cell lines. The cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown on Roswell Park Memorial Institute (RPMI) 1640 medium (Nissui Pharm. Co., Ltd., Tokyo, Japan) supplemented with 10 % inactivated fetal calf serum and 50 µg/mL gentamycin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and were sub-cultured 2–3 times a week. The cytotoxic activity was determined by using cell viability assay method as described previously (Mosmann, 1983). The experiments were performed in triplicates, and the percentage of cell viability was calculated as the mean absorbance of control cells/mean absorbance of treated cells. Dose–response curves were prepared, and the IC_{50} value was determined. The results are presented in (Table 5).

Statistical analysis (Woodson, 1987)

All data were expressed as mean ± SE. Student's *t* test was applied for detecting the significance of difference between each sample; $P < 0.05$ was taken as the level of significance.

Chemistry

Extraction and isolation

The dried powdered fruits (3.0 kg) of *G. aquatica* were subjected to exhaustive extraction with 95 % EtOH (8 L ×4) to yield 230 g of a solid extract, which was then suspended in water and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH to obtain petroleum ether (16.5 g), EtOAc (7 g), and *n*-BuOH (47 g) fractions after removing solvent under reduced pressure. The *n*-BuOH-soluble fraction was applied to a column of polyamide and eluted with H_2O and 25, 50, 75, and 100 %

MeOH. The H_2O fraction (28 g) was chromatographed over Si gel and Si gel flash CC eluted with $CHCl_3$–MeOH (90:10–70:30) to give four fractions of A (950 mg), B (2.6 g), C (3.3 g), and D (4.3 g). Fraction D (4.3 g) was chromatographed over a reversed-phase Sepralyte RP-18 CC using a gradient of MeOH–H_2O (50:50–60:40) to yield D1 (1.9 g), and D2 (2 g). Fraction D1 was rechromatographed over Si gel and Si gel flash CC eluted with $CHCl_3$–MeOH (80:20–75:25) to give four fractions of D1a (360 mg), D1b (350 mg), D1c (210 mg), and D1d (90 mg). Fraction D1c was purified on a reversed-phase Sepralyte RP-18 CC using MeOH–H_2O (57:43) and then finally purified by Sephadex LH-20 CC eluted with MeOH to afford **1** (50 mg). By the same method, fraction D2 was rechromatographed over Si gel and Si gel flash CC eluted with $CHCl_3$–MeOH (80:20–75:25) to give three fractions of D2a (200 mg), D2b (425 mg), and D2c (390 mg). Fraction D2b was purified on a reversed-phase Sepralyte RP-18 CC using MeOH-H_2O (59:41) and then finally purified by Sephadex LH-20 CC eluted with MeOH to afford **2** (40 mg). Fraction C was rechromatographed over Si gel CC eluted with $CHCl_3$–MeOH (85:15–80:20) to yield C1 (800 mg), C2 (750 mg), C3 (530 mg), and C4 (420 mg). Fraction C2 was repeatedly subjected to Si gel CC eluted with $CHCl_3$–MeOH (85:15) and a reversed-phase Sepralyte RP-18 CC eluted with MeOH–H_2O (80:20) and then finally purified by Sephadex LH-20 CC eluted with MeOH to afford **3** (45 mg).

Aquaticasaponin A (**1**): An amorphous solid from MeOH; IR (KBr) υ_{max} 3445, 1740, 1690 cm^{-1}; HRESIMS *m/z* 1856.8787 $[M–H]^-$ (calcd. for $C_{91}H_{142}NO_{38}$, 1856.9210); ^1H NMR and ^{13}C NMR data see Tables 1, 2, and 3.

Aquaticasaponin B (**2**): An amorphous solid from MeOH; IR (KBr) υ_{max} 3450, 1735, 1690 cm^{-1}; HRESIMS *m/z* 1690.7631 $[M-H]^-$ (calcd. for $C_{81}H_{128}NO_{36}$, 1690.8216); ^1H NMR and ^{13}C NMR data see Tables 1, 2, and 3.

Aquaticine C (**3**): White amorphous powder from MeOH; UV (MeOH) λ_{max}: 220, 270 nm; IR (KBr) υ_{max} 3440, 1635 cm^{-1}; ESIMS *m/z* 514 $[M + H]^+$, 382 $[M + H\text{-apiose}]^+$, and 763 $[2M + H\text{-2 apiose}]^+$; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.06 (1H, s, H-8), 5.40 (1H, d, $J = 8.5$ Hz, H-1′), 5.26 (1H, t, $J = 6.8$ Hz, H-12), 4.86 (1H, d, $J = 3.1$ Hz, H-1″), 4.53 (2H, d, $J = 6.8$ Hz, H-11), 3.87 (1H, d, $J = 9.3$ Hz, H-4″a), 3.85 (1H, d, $J = 3.1$ Hz, H-2″), 3.84 (1H, dd, $J = 11.5$, 5.5 Hz, H-6′a), 3.68 (1H, m, H-5′), 3.64 (1H, dd, $J = 11.5$, 2.8 Hz, H-6′b), 3.59 (1H, d, $J = 9.3$ Hz, H-4″b), 3.49 (1H, t, $J = 9.3$ Hz, H-4′), 3.40 (1H, d, $J = 11.2$ Hz, H-5″a), 3.34–3.32 (3H, m, H-2′, H-3′, H-5″b), 1.77 (3H, s, H-14), 1.64 (3H, s, H-15), 7.02, 5.55, 5.38, 5.32, 5.14, 5.11 and 4.50 (brs, NH and OHs); ^{13}C MNR (DMSO-d_6, 100 MHz) δ 154.67 (C, C-2), 153.30 (C, C-4), 153.16 (C, C-6), 142.91 (CH, C-8), 134.59 (C, C-13), 119.99 (CH, C-12), 109.14 (CH, C-1″), 102.25 (C, C-5), 86.25 (CH, C-1′), 78.58 (C, C-3″), 77.65 (CH, C-5′), 76.25 (CH, C-3′), 75.90 (CH, C-2″), 73.23 (CH_2, C-4″), 72.25 (CH, C-2′), 68.01 (CH, C-4′), 65.36 (CH_2, C-6′), 62.40 (CH_2, C-5″), 39.89 (CH_2, C-11), 25.29 (CH_3, C-15), 17.89 (CH_3, C-14).

Acid hydrolysis of compounds **1** and **2**

A 5 mg of each compound was refluxed separately with 2 M HCl in MeOH (5 mL) at 80 °C for 6 h in a water bath. The reaction mixture was evaporated, and the hydrolysate after dilution with H_2O (10 mL) was extracted with $CHCl_3$ (3 × 10 mL). The $CHCl_3$ extracts were evaporated to afford the aglycons, which were identified as echinocystic acid (*m/z* 472 by EIMS and its NMR data) from **1** and **2**. The aqueous layer was neutralized with 2 N KOH solution and concentrated to 1 mL under reduced pressure. The concentrated aqueous layer showed spots at the same Rf as glucose, xylose, arabinose and rhamnose for 1 and 2 on TLC silica gel (30:12:4 $CHCl_3$:MeOH:H_2O), 9 mL of lower layer and 1 mL of HOAc, and on PC (iso-PrOH:n-BuOH:H_2O 7:1:2), using aniline hydrogen phthalate as a detecting reagent.

Echinocystic acid: An amorphous solid from MeOH; EIMS *m/z* 472 $[M]^+$; ^1H NMR (DMSO-d_6, 500 MHz) δ 5.20 (1H, brs, H-12), 4.30 (1H, brt, H-16), 3.00 (1H, dd, $J = 11.0$, 5.0 Hz, H-3), 1.30 (s, H_3-27), 0.90 (s, H_3-30), 0.89 (s, H_3-23), 0.85 (s, H_3-25), 0.83 (s, H_3-29), 0.68 (s, H_3-26), 0.67 (s, H_3-24); ^{13}C NMR data (DMSO-d_6, 125 MHz) δ 177.0 (C-28), 144.1 (C-13), 121.2 (C-12), 76.8 (C-3), 72.9 (C-16), 54.8 (C-5), 47.3 (C-17), 46.3 (C-19), 46.2 (C-9), 41.0 (C-14), 40.0 (C-18), 38.3 (C-4), 39.1 (C-8), 38.1 (C-1), 36.5 (C-10), 34.7 (C-15), 35.1 (C-21), 32.8 (C-29), 32.6 (C-7), 31.3 (C-22), 30.2 (C-20), 28.2 (C-23), 26.9 (C-2), 26.4 (C-27), 24.1 (C-30), 22.7 (C-11), 18.0 (C-6), 16.8 (C-26), 16.0 (C-24), 15.1 (C-25).

Acid hydrolysis of compound **3**

5 milligram of **3** was hydrolyzed with 0.1 N H_2SO_4 (1.0 mL) at 100 °C for 2 h. After neutralization with $NaHCO_3$, solvent was evaporated. The water-soluble part of the residue showed a spot at the same *Rf* as glucose and apiose for **3** on TLC (silica gel 14:6:1 $CHCl_3$–MeOH–H_2O).

Results and discussion

Dried fruits of *G. aquatica* were extracted with ethanol, and then the dried ethanolic extract was suspended in water and fractionated with petroleum ether, ethyl acetate, and

Table 1 ^{1}H and ^{13}C NMR data (δ in ppm) for the aglycon moieties of compounds **1** and **2** (500 MHz for ^{1}H and 125.0 MHz for ^{13}C)

Position	1			2		
	δ_C^a	δ_H^a	δ_H^b	δ_C^a	δ_H^a	δ_H^b
1	38.14	1.46, 0.74	1.63, 0.95	38.15	1.48, 0.76	1.64, 0.98
2	25.54	1.79, 1.55	1.90, 1.65	25.50	1.80, 1.55	1.90, 1.65
3	88.15	2.99	3.10	88.14	2.99	3.10
4	38.18	–	–	38.22	–	–
5	55.12	0.64	0.75	55.08	0.62	0.76
6	18.07	1.39, 1.21	1.55, 1.30	17.96	1.41, 1.23	1.55, 1.31
7	32.41	1.33, 1.10	1.45, 1.20	32.43	1.34, 1.12	1.46, 1.20
8	38.45	–	–	38.45	–	–
9	46.09	1.45	1.60	46.09	1.46	1.61
10	36.28	–	–	36.28	–	–
11	22.95	1.76	1.88	22.95	1.76	1.89
12	121.46	5.16, brs	5.28, brs	121.44	5.14, brs	5.29, brs
13	143.41	–	–	143.38	–	–
14	41.91	–	–	41.91	–	–
15	34.82	1.56, 1.20	1.61, 1.45	34.85	1.56, 1.21	1.62, 1.45
16	72.51	4.32	4.47	72.51	4.30	4.47
17	48.19	–	–	48.17	–	
18	39.72	2.80	2.90	39.74	2.81	2.92
19	46.65	2.20, 0.94	2.30, 1.05	46.64	2.21, 0.95	2.30, 1.05
20	30.15	–	–	30.16	–	–
21	34.95	1.82, 1.05	1.90, 1.17	34.96	1.83, 1.07	1.91, 1.19
22	30.73	1.84, 1.55	1.95, 1.72	30.68	1.86, 1.56	1.93, 1.71
23	27.55	0.85, s	0.96, s	27.55	0.85, s	0.96, s
24	16.31	0.61, s	0.75, s	16.32	0.60, s	0.76, s
25	15.36	0.83, s	0.92, s	15.35	0.83, s	0.93, s
26	16.60	0.61, s	0.74, s	16.60	0.62, s	0.75, s
27	26.18	1.27, s	1.34, s	26.19	1.28, s	1.35, s
28	174.85	–	–	174.85	–	–
29	32.91	0.80, s	0.85, s	32.87	0.81, s	0.86, s
30	24.21	0.83, s	0.93, s	24.21	0.84, s	0.94, s

The assignment was based upon DEPT, DQF-COSY, HMQC, and HMBC experiments

[a] DMSO-d_6

[b] CD_3OD

n-butanol. The *n*-butanol fraction was subjected to subsequent purification using several chromatographic techniques (polyamide, repeated silica gel, RP-18, and Sephadex LH-20 columns); two new and one known compounds were isolated (Fig. 1).

Aquaticasaponin A (**1**) was obtained as a white amorphous solid. The molecular formula was deduced as $C_{91}H_{143}NO_{38}$ from an $[M-H]^-$ ion at *m/z* 1856.8787 in the HRESIMS and from the ^{13}C-NMR spectrum. The ^{1}H and ^{13}C-NMR spectra displayed resonances due to the seven tertiary methyl groups, and the two olefinic carbons indicated the aglycon of **1** possessed an olean-12-ene skeleton. After an extensive 2D NMR study, the aglycon was identified as echinocystic acid. The ^{13}C-NMR spectrum of **1** showed 91 carbon signals (Tables 1, 2, 3), from which 30 signals were attributed to the aglycon and 41 signals were attributed to the sugar moieties. The remaining 20 signals were consistent with the presence of two monoterpene carboxylic acids. It was apparent from the chemical shifts of the C-3 (δ 88.15) and C-28 (δ 174.85) that **1** was a bisdesmosidic glycoside. In the ^{1}H-NMR spectrum of **1**, seven sugar anomeric proton signals appeared at δ 4.21 (1H, d, J = 5.7 Hz, ara), 4.24 (1H, d, J = 8.0 Hz, glc-NAc), 4.34 (1H, d, J = 7.4 Hz, xyl′), 4.43 (1H, d, J = 7.4 Hz, xyl), 4.69 (1H, br s, rha′), 5.11 (1H, br s, rha), and 5.29 (1H, d, J = 7.2 Hz, glc). The corresponding seven anomeric carbons were observed at δ 103.23,

Table 2 ^{1}H and ^{13}C-NMR data (δ in ppm) for the sugar moieties of compounds **1** and **2** (500 MHz for ^{1}H and 125.0 MHz for ^{13}C)

Position	1			2		
	δ_C^a	δ_H^a	δ_H^b	δ_C^a	δ_H^a	δ_H^b
C_3-Glc-Nac						
1	103.54	4.24, d, 8.0	4.44, d, 8.5	103.53	4.25, d, 8.0	4.43, d, 8.2
2	55.70	3.41	3.66, m	55.69	3.40	3.65
3	73.71	3.26	3.44	73.72	3.28	3.44
4	70.74	3.03	3.32	70.74	3.05	3.32
5	73.71	3.22	3.42	73.72	3.23	3.42
6	68.10	3.48, 3.88	3.71, 4.07	68.09	3.49, 3.91	3.71, 4.07
NH	–	7.72, d, 9.2	–	–	7.72, d, 9.2	–
CO	168.92	–	–	168.84	–	–
CH_3	23.18	1.76, s	1.96, s	23.19	1.77, s	1.96, s
Ara						
1	103.23	4.21, d, 5.7	4.33, d, 6.6	103.23	4.22, d, 5.7	4.33, d, 6.3
2	70.55	3.31	3.58	70.55	3.32	3.58
3	72.28	3.32	3.51	72.25	3.34	3.51
4	68.02	3.58	3.78	68.01	3.60	3.78
5	64.63	3.30, 3.67	3.52, 3.84	64.62	3.31, 3.66	3.52, 3.84
C_{28}-Glc						
1	92.78	5.29, d, 7.2	5.35, d, 7.2	92.82	5.30, d, 7.2	5.35, d, 7.0
2	75.46	3.34	3.58	75.47	3.34	3.58
3	77.18	3.22	3.46	77.14	3.24	3.46
4	69.52	3.40	3.61	69.52	3.41	3.61
5	76.01	3.24	3.51	76.03	3.25	3.51
6	65.79	3.71, 3.14	3.92, 3.26	65.79	3.71, 3.16	3.92, 3.26
Rha						
1	99.99	5.11, brs	5.39, brs	100.01	5.12, brs	5.39, brs
2	69.64	3.74	3.95	69.68	3.76	3.95
3	70.55	3.64	3.85	70.55	3.62	3.85
4	83.01	3.38	3.53	82.98	3.37	3.52
5	67.18	3.52	3.80	67.17	3.53	3.80
6	17.95	1.17, d, 6.3	1.32, d, 6.3	17.95	1.17, d, 6.0	1.32, d, 5.8
Xyl						
1	104.92	4.43, d, 7.4	4.53, d, 7.5	104.96	4.44, d, 7.2	4.53, d, 7.5
2	73.49	3.22	3.48	73.49	3.23	3.48
3	86.33	3.36	3.47	86.24	3.38	3.47
4	69.30	3.26	3.50	69.38	3.27	3.50
5	65.43	3.70, 3.02	3.22, 3.89	65.46	3.72, 3.03	3.22, 3.89
Xyl′						
1	104.60	4.34, d, 7.4	4.46, d, 7.2	104.59	4.34, d, 7.4	4.46, d, 7.2
2	73.83	3.07	3.35	73.83	3.07	3.34
3	76.01	3.12	3.52	76.10	3.13	3.52
4	69.30	3.25	3.48	69.30	3.27	3.48
5	65.79	3.71, 3.10	3.90, 3.33	65.79	3.73, 3.10	3.90, 3.33
Rha′						
1	96.57	4.69, brs	4.85, d, 1.5	96.50	4.52, brs	4.71, brs
2	69.73	5.05	5.27, dd, 3.5, 1.5	72.10	4.93	5.06
3	71.50	4.94	5.13, dd, 9.8, 3.5	69.21	3.62	3.87
4	69.42	3.36	3.54	72.28	3.18	3.40

Table 2 continued

Position	1			2		
	δ_C^a	δ_H^a	δ_H^b	δ_C^a	δ_H^a	δ_H^b
5	68.25	3.61	3.78	68.22	3.46	3.63
6	17.95	1.18, d, 6.0	1.29, d, 6.3	17.91	1.16, d, 6.0	1.25, d, 6.0

The assignment was based upon DEPT, DQF-COSY, HMQC, and HMBC experiments

[a] DMSO-d_6

[b] CD_3OD

Table 3 ^{1}H and ^{13}C NMR data (δ in ppm) for the monoterpenic acid moieties of compounds **1** and **2** (500 MHz for ^{1}H and 125 MHz for ^{13}C)

Position	**1**			**2**		
	δ_C^a	δ_H^a	δ_H^b	δ_C^a	δ_H^a	δ_H^b
MT_1						
1	166.31	–	–	166.06	–	–
2	126.61	–	–	126.56	–	–
3	142.95	6.52, t, 6.9	6.83, t, 7.6	142.77	6.53, t, 7.6	6.80, t, 7.6
4	23.11	2.17–2.05, m	2.32–2.20, m	23.09	2.18–2.09, m	2.30–2.25, m
5	40.59	1.47–1.35, m	1.62–1.52, m	40.58	1.48–1.42, m	1.60–1.55, m
6	71.33	–	–	71.32	–	–
7	145.66	5.88, dd, 17.2, 10.5	5.89, dd, 17.3, 10.7	145.68	5.85, dd, 17.2, 10.6	5.90, dd, 17.3, 10.7
8	111.39	4.98, dd, 10.6, 1.9	5.07, dd, 11.0, 1.2	111.38	4.98, dd, 10.6, 1.9	5.05, dd, 10.7, 2.2
		5.16, dd, 17.2, 1.9	5.23, dd, 17.3, 1.6		5.17, dd, 17.2, 1.9	5.22, dd, 17.3, 2.2
9	12.18	1.65, s	1.75, s	12.18	1.74, s	1.85, s
10	27.80	1.14, s	1.28, s	27.81	1.14, s	1.27, s
MT_2						
1	166.99	–	–			
2	126.44	–	–			
3	143.61	6.69, t, 7.9	6.67, t, 7.6			
4	23.26	2.17–2.05, m	2.32–2.20, m			
5	40.59	1.47–1.35, m	1.62–1.52, m			
6	71.33	–	–			
7	145.66	5.82, dd, 17.2, 10.5	5.87, dd, 17.3, 10.7			
8	111.39	4.96, dd, 10.6, 1.9	5.04, dd, 11.0, 1.2			
		5.14, dd, 17.2, 1.9	5.20, dd, 17.3, 1.6			
9	12.00	1.73, s	1.83, s			
10	27.80	1.15, s	1.25, s			

The assignment was based upon DEPT, DQF-COSY, HMQC, and HMBC experiments

[a] DMSO-d_6

[b] CD_3OD

103.54, 104.60, 104.92, 96.57, 99.99, and 92.78, respectively (Table 2). The presence of two methyl signals at δ_C 17.95, δ_H 1.17 (d, $J = 6.3$ Hz) and δ_C 17.95, δ_H 1.18 (d, $J = 6.0$ Hz) in the ^{1}H and ^{13}C-NMR spectra suggested that two of the seven monosaccharides are being deoxy sugar. Acid hydrolysis of **1** afforded echinocystic acid, which was identified by comparison with the reference data (Akai *et al.*, 1985, Maillard *et al.*, 1992) and with the D-glucose, D-xylose, L-arabinose, and L-rhamnose which were identified by co-TLC and PC analysis with authentic sugars. The structures of the oligosaccharide moieties were determined through DQF-COSY, HMQC, and HMBC experiments. Starting from the well-resolved anomeric proton signals or the methyl group proton signals for the deoxy sugars or the acylated proton signals of the acylated sugar, the sequential assignments of all the proton resonances to individual monosaccharide were

Table 4 Antimicrobial activity as MICs (μM) of compounds **1–3**

Tested microorganisms	MIC (μM)			
	1	**2**	**3**	Standard
G +ve bacteria				Ampicillin
S. pneumonia	36.9	67.3	30.5	1.4
B. subtilis	4.6	8.4	15.2	0.17
G −ve bacteria				Gentamycin
P. aeruginosa	NA	NA	NA	13.5
E. coli	73.9	67.3	121.8	27.1
Fungi				Amphotericin B
A. fumigates	73.9	67.3	60.9	1.1
S. racemosum	9.2	67.3	60.9	16.9
G. candidum	18.5	33.7	30.5	0.12
C. albicans	NA	NA	NA	0.25

NA no activity

achieved using DQF-COSY spectrum. On the basis of the assigned proton signals, a HMQC experiment then gave the corresponding carbon assignments, and these were further clarified by HMBC experiment. Accordingly, the assignments of the protons and protonated carbons were established (Table 2), and the seven sugar units were identified as one glucose, two xyloses, two rhamnoses, one arabinose, and one *N*-acetylglucosamine. The ^{13}C-NMR data for the sugar moieties indicated that all the monosaccharides were in the pyranose forms. The anomeric configurations for the sugar moieties were fully defined from their chemical shifts and $^{3}J_{H1,H2}$ coupling constants (Table 2). Accordingly, the glucopyranosyl unit, the two xylopyranosyl units, and the *N*-acetylglucosamine unit were established to be in the *β*-configuration, while the arabinopyranosyl unit and the two rhamnopyranosyl units were in the α-configuration (Gorin and Mazurek, 1975; Overend, 1972). The linkage of the sugar units at C-3 was established from the following HMBC correlation: H-1 (δ 4.21) of Ara with C-6 (δ 68.10) of Glc-NAc. The attachment of the disaccharide moiety to C-3 of the aglycon was confirmed by the long-range correlation between H-1 (δ 4.24) of Glc-NAc and C-3 (δ 88.15) of the aglycon. The sequence of the sugar chin at C-28 was deduced from the following HMBC correlations: H-1(δ 5.11) of Rha with C-2 (δ 75.46) of Glc; H-1 (δ 4.43) of xyl with C-4 (δ 83.01) of Rha; H-1 (δ 4.34) of xyl′ with C-3 (δ 86.33) of xyl; and H-1 (δ 4.69) of Rha′ with C-6 (δ 65.79) of Glc, while the attachment of the pentasaccharide chain to C-28 of the aglycon was based on a correlation of H-1 (δ 5.29) of Glc with the C-28 (δ 174.85) of the aglycon. The presence of two monoterpenic units in **1** was indicated by various NMR data and by comparison of these data with those reported in the literature (Zang *et al.*, 1999a, b, c, Okada *et al.*, 1980), which confirmed that both monoterpenoid moieties in **1** were the same and were characterized as (2*E*)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid. The stereochemistry of the $\Delta^{2,3}$ double bond was determined as *E* from the chemical shifts of H-3, since the olefinic proton of the *Z*-isomer appeared at a higher field (Okada *et al.*, 1980). The absolute configuration of C-6 position was not established. The binding sites of the two monoterpenic acids MT_1 and MT_2 were revealed by two acylation shifts observed at δ 5.05 and 4.92. Using both COSY and HMBC experiments, these signals were assigned to H-2 and H-3 of Rha′, respectively. Further, the HMBC spectrum exhibited significant cross-correlations between H-2 of Rha′ and the carbonyl carbon (δ 166.31) of the monoterpenic acid MT_1 and between H-3 of Rha′ and the carbonyl carbon (δ 166.99) of the monoterpenic acid MT_2. Therefore, the two monoterpenic acids MT_1 and MT_2 were located at C-2 and C-3 of Rha′, respectively. The foregoing evidence led to the elucidation of the structure of compound **1** as 3-*O*-α-L-arabinopyranosyl-(1 → 6)-2-acetamido-2-deoxy-*β*-D-glucopyranosyl echinocystic acid 28-*O*-*β*-D-xylopyranosyl-(1 → 3)-*β*-D-xylopyranosyl-

Table 5 Cytotoxicity of compounds **1–3** against cultured MCF-7 and HCT-116 cancer cell lines

Compounds	Growth inhibition constant (IC_{50})[a] (μM)	
	MCF-7	HCT-116
1	0.80 ± 0.3	0.5 ± 0.21
2	1.0 ± 0.3	0.76 ± 0.4
3	>10	>10
Doxorubicin[b]	0.81 ± 0.06	0.86 ± 0.1

[a] IC_{50} is defined as the concentration that resulted in a 50 % decrease in cell number, and the results are mean ± standard deviation of three independent replicates. The $IC_{50} > 10$ μM was considered to be no cytotoxicity

[b] Positive control substance

(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[(2*E*)-6-hydroxy-2,6-dimethyl-2,7-octadienoyl-(1 → 2) and (2′*E*)-6′-hydroxy-2′,6′-dimethyl-2′,7′-octadienoyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyra-nosyl ester.

Aquaticasaponin B (**2**), a white amorphous solid, gave an $[M-H]^-$ ion at 1690.7631 in the HRESIMS, consistent with a molecular formula of $C_{81}H_{129}NO_{36}$, 166 mass units lower than that of **1**, implying **2** was a derivative of **1** with one less monoterpenic acid. Acid hydrolysis of **2** allowed the identification of the same aglycon (echinocystic acid) and the same sugar components as **1**, that is, D-glucose, D-xylose, L-arabinose, and L-rhamnose based on the TLC and PC analysis. Detailed analysis of the ^{13}C-NMR data obtained for **2** indicated the chemical shifts for the aglycon part and sugar moieties of **2** bore a close resemblance to those of **1**, suggesting that both compounds had the same aglycon and the same sugar structures and sequence at both the C-3 and C-28 positions (Tables 1, 2). A comparison of the NMR spectroscopic data for the acylated oligosaccharide chain at C-28 of **1** and **2** showed that **2** lacked the monoterpenic acid MT_2 connected to C-3 of Rha′ as found in **1** and the remaining monoterpenic acid MT_1 connected to C-2 of Rha′ was identified as (2*E*)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid. As observed in the HMBC spectrum, the long-range correlation of H-2 (δ 4.93) of Rha′ with C-1 (δ 166.06) of the monoterpenic acid MT_1 established that the monoterpenic acid MT_1 was attached to C-2 of Rha′. The downfield shift of H-2 of Rha′ also indicated it was the position of acylation (Table 3). On the basis of above evidence, the structure of compound **2** was concluded to be 3-*O*-α-L-arabinopyranosyl-(1 → 6)-2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid 28-*O*-β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[(2*E*)-6-hydroxy-2,6-dimethyl-2,7-octadienoyl-(1 → 2)-α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranosyl ester.

The known compound (**3**) was identified by comparison of its spectroscopic data with reported data in the literature (Hosny *et al.*, 2009) as 6-*N*-(3-methylbut-2-enylamino)-2-hydroxy-9-[β-D-apiofuranosyl-(1″ → 6′)-β-D-glucopyra-nosyl] purine and was named aquaticine C.

Compounds **1–3** were evaluated for their antimicrobial and cytotoxic activities. The results of the MIC determinations (Table 4) showed noticeable MIC values for the tested compounds (**1–3**) against the entire set of the tested organisms except for *P. aeruginosa* and *C. albicans*. All the tested compounds exhibited both antibacterial and antifungal activities. The obtained MICs varied from 4.6 to 121 μM for the tested compounds. The lowest MIC values (4.6, 8.4, 15.2 μM) were observed with the compounds **1–3** against *B. subtilis*, respectively. When regarding the activity of the positive standard against the tested microbial species, compound **1** showed the highest level of activity against *S. racemosum* with an MIC 9.2 μM (nearly twofold lower than that of amphotericin B), followed by compound **2** against the G −ve bacteria *E. coli* with an MIC 67.3 μM (nearly only twofold greater than that of gentamycin). Compound **3** showed the lowest level of activity against all strains. Compounds **1** and **2** exhibited good cytotoxicity against breast cancer (MCF-7) and colon cancer (HCT-116) cell lines and gave IC_{50} in the range 0.5–1.0 μM. On the other hand, compound **3** showed no cytotoxicity against these cancer cell lines (>10 μM) (Table 5).

Acknowledgments Many thanks are due to Dr. Mohammed Hosny, Professor of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt and Dr. Ehab M. Mostafa, Lecturer of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, for 2D NMR and Ms Facilities.

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