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Full Length Research Paper

Acylated triterpenoidal saponins and cytokinins from *Gleditsia aquatica*

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Two new bisdesmosidic triterpenoidal saponins acylated with one and two monoterpenoid units; aquaticoside A and B (1 and 2), two new cytokinins, aquaticine A and B (3 and 4), and one known cytokinin; adenosine (5), were isolated from the fruits of *Gleditsia aquatica*. Their structures were established by means of extensive 1D and 2D NMR studies and chemical degradation. Cytotoxicities (ED_{50}) of various extracts and isolated pure saponins (1 and 2), were measured against various tumor cell lines. The isolated pure saponins (1 and 2), showed the most potent cytotoxicities with ovary adenocarcinoma (MDAH 2774, NIH: OVCAR-3), uterus mesodermal tumor (SK-UT-1), skin malignant melanoma (G-361) and colon colorectal adenocarcinoma (LS 174 T) cell lines.

Key words: NMR, acylated triterpenoidal saponins, cytokinins, *Gleditsia aquatica*.

INTRODUCTION

Genus *Gleditsia* L. (Leguminosae) has long been known in traditional Chinese folk medicine and used for the treatment of apoplexy, as an expectorant and as a pesti-cide (Zhong and Dian, 1977). Several oleanane - type triterpenoidal saponins acylated with monoterpenic acids were isolated from the fruits of *Gleditsia sinensis* Lam. and *Gleditsia japonica* Miq. (Zang et al., 1999a - d). 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 shrup distributed throughout Egypt. Surprisingly no inten-sive research work has been reported on this species. In this paper, we describe the isolation and elucidation of two new triterpenoidal saponins, named aquaticosides A and B (1 and 2) Figure 1, two new cytokinins, named aquaticine A and B (3 and 4) Figure 2, and one known cytokinin, adenosine (5), by using various NMR tech-niques, including, DEPT, DQF-COSY, HMQC and HMBC experiments and MS analysis as will as some chemical degradation. The two saponins were acylated with one or two monoterpenic units at C-6 position or at C-3 and C-6 positions of the glucose moiety, which are directly connected to the C-28 carbonyl group of the aglycon. Various extracts and the isolated saponins (1 and 2) were evaluated for their cytotoxicities.

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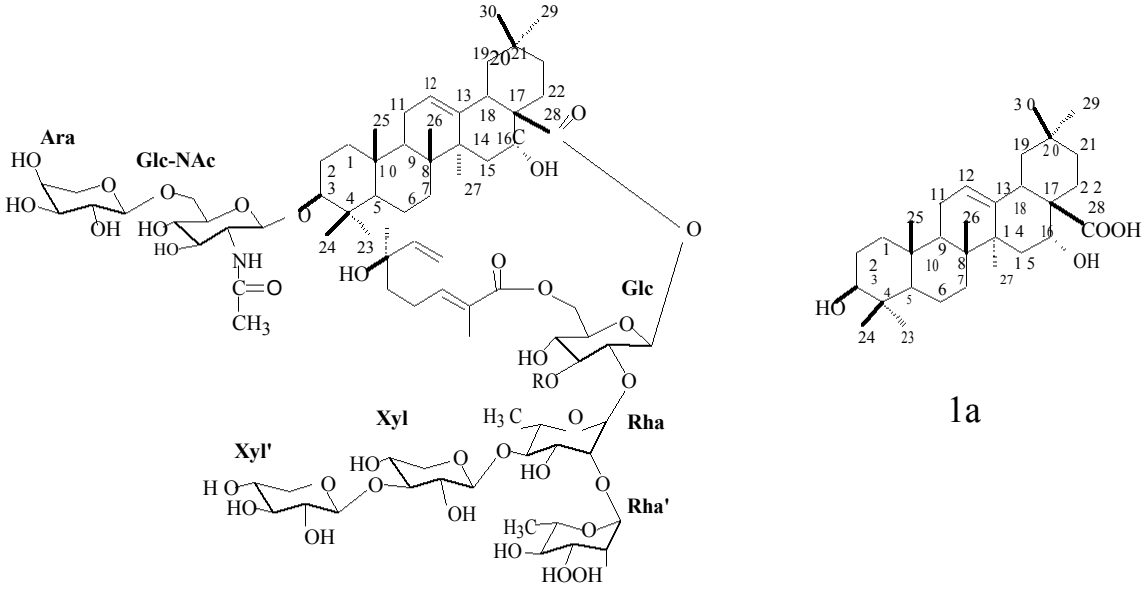
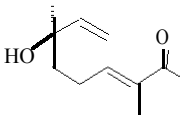
MATERIALS AND METHODS

General experimental procedures

Optical rotations were performed with an Autopol III automatic polarimeter (Rudolph Research Co., Flanders, NJ, USA). 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) in DMSO-d_6 solution, 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 high field spectrometer equipped with an IBM Aspect-2000 processor and with software VNMR version 4.1 or NUTS program for NMR. HRFAB mass spectra were performed on a VGZAB-HF reversed geometry (BE configuration, where B is a magnetic sector and E is an electrostatic analyzer) mass spectrometer (MS) (VG Analytical, Inc.). ESIMS (positive and negative ion acquisition mode) was carried out on a TSQ700 triple quadruple instrument (Finnegan, Santos, CA, USA) mass spectrometer. MALDI -TOFMS was conducted using perceptive Biosystems, Voyager DE-STR mass spectrometer. EIMS was carried 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 chrom-



Compound 1 R =



Compound 2 R =H

Figure 1. Structure of compounds 1and 2.

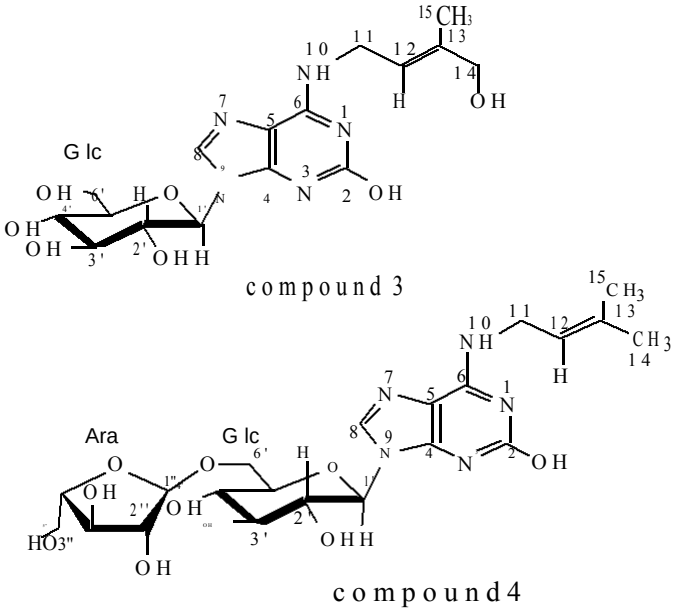


Figure 2. Structure of compounds 3 and 4.

atography 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 F_{254} (Merck) plates. Developed chromatgrams were visualized by spraying with 1%

vanillin-H_2SO_4, followed by heating at 100 for 5 min or spraying the developed plates with 2% ninhydrin in acetone.

The following material and reagents were used for cell culture and cytotoxic assays. Human liver adenocarcinoma (NCI-H1755, SK-HEP-1), kidney carcinoma (A-498), kidney hypernephroma (SW 156), ovary adenocarcinoma (MDAH 2774, NIH:OVCAR-3, uterus mesodermal tumor (SK-UT-1), skin malignant melanoma (G-361, RPMI-7951, Hs 908.Sk), prostate carcinoma (DU 145) and colon colorectal adenocarcinoma (HCT-8 [HRT-18], LS 174T, COLO 320DM) cell lines were purchased from the American Type Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island NY, USA). Eagle Minimum Essential Medium (EMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium were from Nissui Pharm. Co., Ltd., Tokyo, Japan. Flat-bottom plates, 96 well were from Iwaki Glass Co., Ltd (Funabashi-Chiba-Ken, Japan). The (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT), for colorimetric assay was from Sigma (St. Louis, Mo., USA).10 % Fetal Bovine serum (FBS) was from Gibco BrL (Rockville, MD, USA). All other chemicals used were of analytical reagent grade.

Plant materials

The fruits of *G. aquatica* March. were collected from agricultural Museum - Dokky, Giza, Egypt in March, 2001, and were identified by late professor Nabil El-Hadidy (Faculty of Science, Cairo University, Egypt). A voucher specimen has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

Extraction and Isolation

The powdered fruits (3.0 Kg) of *G. aquatica* were subjected to exhaustive extraction with 95% EtOH (4 x 10 L). The alcoholic extract was concentrated (230 g), suspended in H_2O, and then partitioned successively with pet. Ether (16.5 g), EtOAc (7 g) and *n*-BuOH (47 g).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 column chromatography 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 reversed-phase Sepralyte C_{18} flash column chromatography to yield D_1 (1.9 g), and D_2 (2 g). Fraction D_2 was repeatedly subjected to Sepralyte C_{18} flash CC and Sephadex LH 20 CC to afford 1 (26 mg) and 2 (34 mg). Fraction C (3.3 g) was rechromatographed over Si gel column chromatography to yield C_1 (800 mg), C_2 (750 mg), C_3 (530 mg) and C_4 (420 mg). Fraction C_1 (800 mg) was repeatedly subjected to reversed - phase Sepralyte C_{18} flash CC and Sephadex LH 20 CC to afford 3 (22 mg) and 4 (43 mg). By the same method, fraction B furnished 5 (38 mg).

Aquaticoside A (1):

An amorphous solid from MeOH; $[\alpha]^{25}D$ -13.8º ($_c$ 1.0, MeOH); IR (KBr) υ_{max} 3450, 1740, 1690 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) aglycon δ 5.18 (1H, brs, H-12), 4.35 (1H, brt, H-16), 2.98 (1H, dd, *J* = 11.9, 5.1 Hz, H-3), 1.28 (3H, s, H_3-27), 0.87 (3H, s, H_3-23),0.84 (6H, s, H_3-25 and H_3-30), 0.80 (3H, s, H_3-29), 0.63 (6H, s, H_3-24 and H_3-26); monoterpenic acid Mt_1 δ 6.54 (1H, t, *J* = 7.5 Hz, H-3), 5.89 (1H, dd, *J* = 17.2, 10.6 Hz, H-7), 5.16 (1H, dd, *J* = 17.2, 1.9 Hz, H-8b), 4.97 (1H, dd, *J* = 10.6, 1.9 Hz, H-8a), 2.23-2.14 (2H, m, H_2-4), 1.75 (3H, s, H_3-9), 1.51-1.42 (2H, m, H_2-5), 1.15 (6H, s, H_3-10); monoterpenic acid Mt_2 δ 6.72 (1H, t, *J* = 7.5 Hz, H-3), 5.86 (1H, dd, *J* = 17.2, 10.6 Hz, H-7), 5.18 (1H, dd, *J* = 17.2, 1.9 Hz, H-8b), 4.98 (1H, dd, *J* = 10.6, 1.9 Hz, H-8a), 2.23-2.14 (2H, m, H_2-4), 1.66 (3H, s, H_3-9), 1.51-1.42 (2H, m, H_2-5), 1.15 (6H, s, H_3-10); ^{13}C NMR (DMSO-d_6, 100.0 MHz) monoterpenic acid Mt_1 δ 166.14 (C, C-1), 145.55 (CH, C-7), 142.79 (CH, C-3), 126.47 (C, C-2), 111.55 (CH_2, C-8), 71.16 (C, C-6), 40.48 (CH2, C-5), 27.66 (CH3, C-10), 23.10 (CH2, C-4), 12.01 (CH3, C-9); monoterpenic acid Mt_2 δ 165.92 (C, C-1), 145.55 (CH, C-7), 143.46 (CH, C-3), 126.29 (C, C-2), 111.19 (CH2, C-8), 71.11 (C, C-6), 40.45 (CH2, C-5), 27.65 (CH3, C-10), 23.10 (CH_2, C-4), 11.84 (CH3, C-9); other NMR data are listed in Tables 1 and 2; ESIMS m\z 1880 $[M+Na]^+$; MALDI-TOFMS (positive ion mode) m\z 1880 $[M+Na]^+$, 1748 $[M+Na\text{-}xyl]^+$; HRFABMS *m/z* 1880.9193 $[M+Na]^+$ (calcd for 1880.9186).

Aquaticoside B (2)

An amorphous solid from MeOH; $[\alpha]^{25}D$ -21.2º ($_c$ 1.0, MeOH); IR (KBr) υ_{max} 3500, 1728, 1695 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) aglycon δ 5.17 (1H, brs, H-12), 4.36 (1H, brt, H-16), 2.98 (1H, dd, *J* = 11.5, 5.1 Hz, H-3), 1.28, 0.87, 0.86, 0.85, 0.81, 0.64, 0.63 (each 3H, s, H_3-27, 23, 30, 25, 29, 26 and 24, respectively); monoterpenic acid Mt δ 6.70 (1H, t, *J* = 7.6 Hz, H-3), 5.83 (1H, dd, *J* = 17.2, 10.6 Hz, H-7), 5.14 (1H, dd, *J* = 17.2, 1.9 Hz, H-8b), 4.96 (1H, dd, *J* = 10.6, 1.9 Hz, H-8a), 2.20-2.12 (2H, m, H_2-4), 1.75 (3H, s, H_3-9), 1.60-1.47 (2H, m, H_2-5), 1.17 (3H, s, H_3-10); ^{13}C NMR (DMSO-d_6, 100.0 MHz) monoterpenic acid Mt δ 166.31 (C, C-1), 145.59 (CH, C-7), 142.62 (CH, C-3), 126.67 (C, C-2), 111.20 (CH_2, C-8), 71.17 (C, C-6), 40.46 (CH2, C-5), 27.66 (CH3, C-10), 22.78 (CH2, C-4), 12.09 (CH3, C-9); other NMR data are listed in Tables 1 and 2; ESIMS m\z 1714 $[M+Na]^+$,1568 $[(M+Na)\text{-}rha`]^+$, 1436 $[(M+Na)\text{-}rha`\text{-}xyl`]^+$, 1270 $[(M+Na)\text{-}rha`\text{-}xyl`\text{-}Mt]^+$ and 1138 $[(M+Na)\text{-}rha`\text{-}2\ xyl\text{-}Mt]^+$; MALDI-TOFMS (positive ion mode) m\z 1715 $[M+Na+H]^+$, 1568 $[(M+Na)\text{-}rha`]^+$, 1437 $[(M+Na+H)\text{-}rha`\text{-}xyl`]^+$, and 1270 $[(M+Na)\text{-}rha`\text{-}xyl`\text{-}Mt]^+$; HRFABMS *m/z* 1714.8198 $[M+Na]^+$ (calcd for 1714.8192).

Aquaticine A (3)

colorless needles from MeOH; $[\alpha]^{25}D$-6.78 ($_c$ 1.0, MeOH); UV (MeOH) λ_{max}: 212, 268 nm; IR (KBr) υ_{max} 3450, 1640 cm^{-1}; ^1H (DMSO-d_6, 600 MHz) and ^{13}C (DMSO-d_6, 100 MHz) NMR data are given in Table 3; ESIMS m\z 398 $[M+H]^+$, 420 $[M+Na]^+$, 795 $[2M+H]^+$ and 817 $[2M+Na]^+$; HRFABMS *m/z* 420.1499 $[M+Na]^+$ (calcd for 420.1495).

Aquaticine B (4)

colorless needles from MeOH; $[\alpha]^{25}D$-16.5 ($_c$ 1.0, MeOH); UV (MeOH) λ_{max}: 215, 270 nm; IR (KBr) υ_{max} 3400, 1632 cm^{-1}; ^1H (DMSO-d_6, 600 MHz) and ^{13}C (DMSO-d_6, 100 MHz) NMR data are shown in Table 4; ESIMS m\z 514 $[M+H]^+$, 536 $[M+Na]^+$, 1049 $[2M+Na]^+$, 382 $[M+H\text{-}ara]^+$, 404 $[M+Na\text{-}ara]^+$ and 785 $[2M+Na\text{-}2\ ara]^+$; HRFABMS *m/z* 536.1975 $[M+Na]^+$ (calcd for 536.1969).

Adenosine (5)

colorless needles from MeOH; UV (MeOH) λ_{max}: 210, 265 nm; IR (KBr) υ_{max} 3400, 1680, 1615 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 8.34 (1H, s, H-8), 8.13 (1H, s, H-2), 7.33 (2H, s, NH_2), 5.88 (1H, d, *J* = 6.2 Hz, H-1`), 3.97 (1H, dd, *J* = 6.2, 5.4 Hz, H-2`), 3.68 (1H, dd, *J* = 12.0, 2.9 Hz, H-5`a), 3.56 (1H, dd, *J* = 11.6, 3.6 Hz, H-5`b), 3.52 (1H, ddd, *J* = 5.4, 2.9, 3.6 Hz, H-4`), 3.43 (1H, t, *J* = 5.4 Hz, H-3`);^{13}C NMR (100.0 MHz, DMSO-d_6) δ 156.06 (C, C-6), 152.26 (CH, C-2), 148.95 (C, C-4), 139.79 (CH, C-8), 119.25 (C, C-5), 87.79 (CH, C-1`), 85.77 (CH, C-4`), 73.32 (CH, C-2`), 70.54 (CH, C-3`), 62.97 (CH_2, C-5`); ESIMS m\z 268 $[M+H]^+$, 557 $[2M+Na]^+$.

Table 1. ^{13}C NMR Spectroscopic Data (100.0 MHz in DMSO-d_6) for the aglycon moieties of Aquaticoside A (1) and B (2) and Echinocystic acid (1a)

1a	2	1	Position
δ_C, mult.	δ_C, mult.	δ_C, mult.	
37.80	38.00	38.05	1
26.60	25.27	25.27	2
77.19	87.98	87.97	3
38.27	38.89	38.88	4
54.24	54.97	54.97	5
18.10	17.90	17.91	6
32.70	32.69	32.70	7
38.19	38.30	38.29	8
46.05	45.94	45.95	9
36.49	36.14	36.13	10
22.90	22.91	22.95	11
121.07	121.30	121.25	12
144.80	143.22	143.25	13
40.89	41.00	40.99	14
34.80	34.79	34.82	15
73.06	72.35	72.35	16
47.66	48.02	48.04	17
39.89	40.01	39.99	18
46.41	46.52	46.50	19
30.28	30.00	30.00	20
34.23	34.15	34.13	21
31.40	30.51	30.50	22
27.89	27.41	27.40	23
15.55	16.16	16.13	24
15.12	15.20	15.19	25
16.46	16.45	16.45	26
26.24	26.04	26.03	27
178.60	174.69	174.67	28
32.99	32.71	32.75	29
24.26	24.06	24.08	30

Acid hydrolysis of aquaticosides A and B (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 x 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 2N KOH solution and concentrated to 1 ml under reduced pressure. The residue was compared with standard sugars by Si gel TLC [($CHCl_3$-MeOH-H_2O: 30:12:4), 9 ml of lower layer and 1 ml of HOAc] and by PC (iso-PrOH-*n*-BuOH-H_2O: 7:1:2); detection with aniline hydrogen phthalate, which indicated the sugars of (1 and 2) to be glucose, xylose, arabinose and rhamnose.

Echinocystic acid (1a)

An amorphous solid from MeOH; $[\alpha]^{25}D$ +39.5º ($_c$ 1.0, MeOH); IR (KBr) υ_{max} 3450, 2940, 1685 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) δ 5.24 (1H, brs, H-12), 4.55 (1H, brt, H-16), 2.88 (1H, dd, *J* = 11.5, 5.0 Hz, H-3), 1.32, 0.91,0.90, 0.85, 0.82, 0.69, 0.66 (each 3H, s, H_3-27, 30, 25, 29, 23, 26 and 24, respectively); ^{13}C NMR data are given in Table 1; EIMS m\z 472 $[M]^+$.

Acid hydrolysis of aquaticines A and B (3 and 4)

Samples (5 mg) of [3] and [4] were separately 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 R_f as glucose for [3] and glucose and arabinose for [4] on TLC (silica gel 14:6:1 $CHCl_3$-MeOH-H_2O).

Cytotoxicity assays

Cytotoxicity assays were carried out by the same method of Goren et al. (1996) and Hosny and Rosazza (2002). The 50% effective dose (ED_{50}) obtained by measuring growth inhibition with MTT are shown in (Table 5).

Statistical analysis (Woodson, 1987)

All cytotoxic 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.

RESULTS AND DISCUSSION

Aquaticoside A (1), an amorphous solid, had a molecular formula of $C_{91}H_{143}NO_{38}$ deduced from the $[M+Na]^+$ ion at m\z 1880.9193 in the positive ion HRFABMS and $[M+Na]^+$ ions at m\z 1880 in the positive ion ESIMS and MALDI-TOFMS (positive ion mode) as well as from its ^{13}C, DEPT, NMR data. The IR spectrum showed a carbonyl group (1740 cm^{-1}), *α, β*-unsaturated carbonyl group (1690 cm^{-1}) and hydroxyl group (3450 cm^{-1}) absorptions. The seven tertiary methyl carbon signals at δ 15.19, 16.13, 16.45, 24.08, 26.03, 27.40, 32.75 and the two olefinic carbon signals at δ 121.25, 143.25, coupled with the ^1H information (seven methyl proton singlets at δ 0.63 [6H], 0.80, 0.84 [6H], 0.87, 1.28 and a broad singlet vinyl proton at δ 5.18) indicated the aglycon possessed an olean-12-ene skeleton. After an extensive 2D NMR study, the aglycon was identified as echinocystic acid (Table 1). The chemical shifts of C-3 (δ 87.97) and C-28(δ 174.67) indicated that 1 was a bisdesmosidic glycoside. The ^1H and ^{13}C NMR of 1 displayed seven sugar anomeric protons at δ 4.23 (d, *J* = 5.7 Hz), 4.26 (d, *J* = 8.0 Hz), 4.36 (d, *J* = 7.4 Hz), 4.45 (d, *J* = 7.4 Hz), 4.69 (brs), 5.12 (brs), and 5.32 (d, *J* = 7.2 Hz) and carbons at δ 92.66, 96.46, 99.85, 103.06, 103.40, 104.44, and 104.82 (Table 2). Among the 91 carbon signals in the ^{13}C NMR spectrum, 30 signals were assigned to the aglycon, 41 to the oligosaccharide moiety, and the remaining 20 to

Table 2. NMR Spectroscopic data (600 and 400.0 MHz in DMSO-d_6) for the sugar moieties of aquaticoside A (1) and B (2).

Position	1		2	
	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
		C_3-Glc-NAc		
1	4.26 (8.0)	103.40	4.26 (8.5)	103.39
2	3.39	55.57	3.39	55.57
3	3.14	76.97	3.18	76.96
4	3.37	70.40	3.40	70.40
5	3.26	75.87	3.27	75.88
6	5.01, 5.05	67.97	5.03, 5.08	67.98
NH	7.72 (9.2)	-	7.71 (9.0)	-
CO	1.77	168.65	1.76	168.65
CH_3		23.04		23.03
		Ara		
1	4.23 (5.7)	103.06	4.23 (5.4)	103.06
2	3.44	72.35	3.41	72.34
3	3.24	73.55	3.23	73.55
4	3.75	68.09	3.76	68.12
5	3.32, 3.67	65.29	3.33, 3.66	65.30
		C_{28}-Glc		
1	5.32 (7.2)	92.66	5.31 (7.2)	92.82
2	4.59	76.19	4.39	76.50
3	4.99	71.36	3.30	77.14
4	3.30	73.69	3.24	71.00
5	3.43	75.72	3.62	75.33
6	5.04, 5.07	64.42	5.38, 5.86	65.33
		Rha		
1	5.12 (brs)	99.85	5.11 (brs)	99.83
2	4.89	73.34	4.93	73.34
3	4.03	70.39	4.06	70.41
4	3.40	82.59	3.41	82.60
5	3.55	67.87	3.55	67.88
6	1.18 (6.0)	17.75	1.18 (6.2)	17.80
		Xyl		
1	4.45 (7.4)	104.82	4.45 (7.2)	104.84
2	3.35	74.03	3.36	74.04
3	3.38	86.13	3.38	86.11
4	3.70	69.09	3.70	69.09
5	3.23, 3.64	65.64	3.24, 3.65	65.64
		Xyl'		
1	4.36 (7.4)	104.44	4.36 (7.3)	104.43
2	3.39	74.20	3.40	74.21
3	3.44	75.33	3.44	75.34
4	3.84	69.36	3.84	69.37
5	3.27, 3.76	67.77	3.28, 3.74	67.74

Table 2. Cont'd.

		Rha'		
1	4.69 (brs)	96.46	4.63 (brs)	96.49
2	3.91	70.65	3.89	70.64
3	3.50	70.87	3.50	70.87
4	3.68	73.69	3.67	73.68
5	3.59	69.15	3.60	69.16
6	1.17 (6.0)	17.80	1.17 (6.0)	17.81

two monoterpenic units. Acidic hydrolysis of 1 furnished echinocystic acid identified from the $[M]^+$ ion at m/z 472 in the EI-MS and its NMR data, and the sugar components were identified as glucose, xylose, arabinose and rhamnose by co-TLC and PC analysis with an authentic sample. The identity of the monosaccharides and the sequence of the oligosaccharide chain were determined by a combination of DQF-COSY, DEPT, HMQC, and HMBC experiments. Starting from the anomeric proton of each sugar unit, all the hydrogen's within each spin system were assigned 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 confirmed by an HMBC experiment. After the assignments of the protons and protonated carbons were established (Table 2). The seven sugar units were identified as one glucose, two xyloses, two rhamnoses, one arabinose and one N-acetylglucosamine. All the monosaccharides were determined to be in the pyranose form from their ^{13}C NMR data. The anomeric configurations for the sugar moieties were fully defined from their chemical shifts and $^{3}J_{H1,\ H2}$ coupling constants. Accordingly the glucose, xylose and N-acetylglucosamine were established to be in the *β*-configuration, while the arabinose and the rhamnose were in the *α*-configuration (Altona and Haasnoot, 1980). The linkage of the sugar units at C-3 was established from the following HMBC correlation: H-1(δ 4.23) of Ara with C-6 (δ 69.35) of Glc-NAc. The attachment of the disaccharide moiety to C-3 of the aglycon was confirmed by the long-rang correlation between H-1 (δ 4.26) of Glc-NAc and C-3 (δ 87.97) of the aglycon. The sequence of the sugar chin at C-28 was deduced from the following HMBC correlations: H-1(δ 5.12) of Rha with C-2 (δ 76.97) of Glc; H-1 (δ4.69) of Rha` with C-2 (δ 73.34) of Rha; H-1 (δ 4.45) of xyl with C-4 (δ 82.59) of Rha; H-1 (δ 4.36) of xyl` with C-3 (δ 86.13) of xyl, while the attachment of the pentasaccharide chain to C-28 of the aglycon was based on a correlation of H-1(δ 5.32) of Glc with the C-28 (δ 174.67) of the aglycon. As observed in the HMBC spectrum the long-rang correlation of H-3 (δ 4.99) of Glc with C-1 (δ 166.14) of the monoterpenoid unit MT_1 and H_2-6 (δ5.07, 5.04) of Glc with C-1 (δ 165.92) of the monoterpenoid unit MT_2, established that two monoterpenoid units, MT_1 and MT_2, were attached to C-3 and C-6 of Glc, respectively. The downfield shifts of H-3 and H_2-6 of Glc, also indicated they were positions of acylation. 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, d; 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 absolute configuration of C-6 position was not established. On the basis of the above evidence, the structure of aquaticoside A (1) was elucidated as 3-*O*-*α*- L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyrano-syl echinocystic acid 28-*O*-*β*-D-xylopyranosyl(1→3)-*β*-D-xylopyranosyl-(1→4)[*α*-L-rhamnopyranosyl-(1→2)]-*α*-L-rhamnopyranosyl-(1→2)-[(2*E*)-6-hydroxy-2,6-dimethyl 2,7- octadienoyl -(1→ 3) and (2`*E*)-6`-hydroxy-2`,6`-dimethyl-2`,7`-octadienoyl-(1→6)]-*β*-D-glucopyranosyl ester.

Aquaticoside B (2) had the molecular formula $C_{81}H_{129}NO_{36}$ as deduced from the $[M+Na]^+$ ion at m\z 1714.8198 in the positive ion HRFABMS and the $[M+Na]^+$ ion at m\z 1714 in the positive ion ESIMS and the $[M+H+Na]^+$ ion at m\z 1715 in the MALDI-TOFMS (positive mode), and from its DEPT, ^{13}C NMR data. The IR spectrum of 2 exhibited carbonyl group (1728 cm^{-1}) and *α,β*-unsaturated carbonyl group (1695 cm^{-1}) absorptions. It was apparent from the chemical shifts of C-3 (δ 87.77) and C-28 (δ 174.69) of the aglycon in the ^{13}C NMR spectrum that 2 was also a bisdesmosidic triterpenoid glycoside. The ^{1}H and ^{13}C NMR spectra displayed seven anomeric proton and carbon signals (Table 2) and a monoterpenoid unit. Acid hydrolysis of 2 gave the aglycon echinocystic acid, identified by its $[M]^+$ ion at m\z 472 in the EIMS and its NMR data, and the sugar units were determined by co-TLC and PC analysis with an authentic sample to be D-glucose, D-xylose, L-arabinose, and L-rhamnose. Extensive NMR data obtained for 2 showed that this compound possessed the same sugar structures at both C-3 and C-28 positions as did 1.

The monoterpenoid unit was identified as (2*E*)-6-hydroxy-2,6-dimethyl-2,7-octadienoic acid by its NMR data and was determined to be linked to C-6 of the Glc unit, from the downfield shifts of H_2-6 (δ 5.38, 5.86) and C-6 (δ 64.43) due to an acetylation effect (Zang et al., 1999a). The major fragmentation peaks at m\z 1568 $[(M+Na)\text{-rha`}]^+$, 1436 $[(M+Na)\text{-rha`-xyl`}]^+$, 1270 $[(M+Na)\text{-rha`-xyl`-Mt}]^+$, 1138 $[(M+Na\text{-rha`-xyl`-Mt-xyl}]^+$ in the positive ion

Table 3. 1D and 2D NMR Spectroscopic data (600 and 400.0 MHz in DMSO-d_6) for Aquaticine A (3).

Position	Aquaticine A (1)			
	δ_C, mult.	δ_H (*J* in Hz)	DQF-COSY	HMBC
2	154.74, $_qC$	-	-	-
4	102.44, $_qC$	-	-	-
5	153.40, $_qC$	-	-	-
6	152.86, $_qC$	-	-	-
8	142.79, CH	8.09, s	H-1`	C-1`, 4, 5
11	39.30, CH_2	4.59, d (7.1)	H-12	C-6, 12, 13, 15
12	120.85, CH	5.26, t (7.1)	H-11, 14, 15	C-11, 13, 14, 15
13	139.27, $_qC$	-	-	-
14	59.83, CH_2	4.11, d (5.6)	H-12, 14-OH	C-12, 13, 15
15	21.24, CH_3	1.68, s	H-12, 14	C-12, 13, 14
1`	86.26, CH	5.42, d (8.5)	H-8, 2`	C-4, 8, 2`, 3`, 5`
2`	72.36, CH	3.32*	H-1`, 3`	C-1`,3`, 4`
3`	76.20, CH	3.34*	H-2`, 4`	C-1`, 2`, 4`, 5`
4`	67.90, CH	3.47, t (9.4)	H-3`, 5`	C-2`, 3`, 5`, 6`
5`	79.22, CH	3.51, m	H-4`, 6`a, 6`b	C-1`, 3`, 4`, 6`a,b
6`a.	58.78, CH_2	3.71, dd (11.2, 2.8)	H-5,6`b	C-4`,5`
6`b.	-	3.66, dd (11.2, 5.0)	H-5, 6`a	C-4`,5`
2-OH	-	7.20, brs	-	-
NH	-	7.20, brs	-	-
14-OH	-	4.79, t (5.6)	H-14	C-13, 14
2`-OH	-	5.54, brs	-	-
3`-OH	-	5.36, brs	-	-
4`-OH	-	5.29, brs	-	-
6`-OH	-	5.01, brs	-	-

* The splitting pattern is masked by the signal of water.

ESIMS and 1568 $[M+Na\text{-}rha`]^+$ ,1437 $[(M+H+Na)\text{-}rha`\text{-}xyl`]^+$and 1270 $[(M+Na)\text{-}rha`\text{-}xyl`\text{-}Mt]^+$ in the MALDI-TOFMS of 2 were also in agreement with the structure deduced above. From the above evidence the structure of 2 was elucidated as 3-*O*-*α*-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D gluco-pyranosyl echinocystic acid 28-*O*-*β*-D-xylopyranosyl (1→3)- *β*-D-xylopyranosyl-(1→4)[*α*-L-rhamnopyranosyl (1→2)]-*α*-L-rhamnopyranosyl-(1→2)-[(2*E*)-6-hydroxy-2,6-dimethyl 2,7-2,7- octadienoyl -(1→ 6)]-*β*-D-glucopyranosyl ester.

Aquaticine A (3) was analyzed by positive ion HRFABM to give an $[M+Na]^+$ ion peak at m\z 420.1499 and by positive ion ESIMS to give an $[M+H]^+$ and an $[M+Na]^+$, ion peaks at m\z 398 and 420, respectively, suggesting the molecular formula of 3 to be $C_{16}H_{23}N_5O_7$. Its UV spectrum showed absorption bands at 210 and 270 nm which are indicative of N6-substituted adenine derivatives (Leonard and Deyrup, 1962). The IR spectrum showed absorptions at 3450 cm^{-1} (hydroxyl group) and 1640 cm^{-1} (conjugated C = N group). As aromatic proton, one singlet at δ8.09 was found in the ^1H NMR spectrum. This one charac-teristic singlet, as well as the ^{13}C NMR spectrum, which indicates the presence of five sp^2 carbons at [δ 102.44 (C-4), 142.79 (C-8), 152.86 (C-6), 153.40 (C-5), 154.74 (C-2)], and the presence of five nitrogen atoms from ESIMS, suggested the presence of hydroxyl adenine skeleton. An olefinic proton at δ 5.26 (1H, t, *J* = 7.1 Hz, H-12), geminal methylene protons at δ 4.11 (2H,d, *J* = 5.6 Hz, H_2-14), and a sharp singlet due to methyl protons at δ 1.68 (CH_3-15), and two sp^2 carbons at [δ 120.85 (C-12), 139.27 (C-13)], one methyl carbon at δ 21.24 (C-15) and two sp^3 carbons at [δ 39.30 (C-11), 59.83 (C-14)] suggested the presence of hydroxyl isopentenyl side chain. Combined 2D NMR (COSY, HMQC and HMBC) experiments determine the structure of the aglycon moiety of 3 as 2-hydroxyzeatin (Table 3). The ^{13}C NMR spectrum of 3 indicated the presence of one anomeric carbon at δ 86.26 (C-1`). The $^1H$ NMR spectrum correspondingly showed the signal of one anomeric proton at δ 5.42 (1H, d, *J* = 8.5 Hz, H-1`), suggesting the presence of one

Table 4. 1D and 2D NMR Spectroscopic data (600 and 400.0 MHz in DMSO-d_6) for Aquaticine B (4).

	Aquaticine B (2)			
Position	**δ_C, mult.**	**δ_H (*J* in Hz)**	**DQF-COSY**	**HMBC**
2	154.72, $_q$C	-	-	-
4	102.34, $_q$C	-	-	-
5	153.36, $_q$C	-	-	-
6	153.01, $_q$C	-	-	-
8	142.73, CH	8.07, s	H-1`	C-1`, 4, 5
11	39.89, CH_2	4.52, d (6.8)	H-12	C-6, 12, 13
12	120.07, CH	5.25, t (6.8)	H-11, 14, 15	C-11, 13, 14, 15
13	134.69, $_q$C	-	-	-
14	17.89, CH_3	1.78, s	H-12, 15	C-12, 13, 15
15	25.29, CH_3	1.65, s	H-12, 14	C-12, 13, 14
1`	86.23, CH	5.42, d (8.6)	H-8, 2`	C-4, 8, 2`, 3`, 5`
2`	72.35, CH	3.34*	H-1`, 3`	C-1`,3`, 4`
3`	76.26, CH	3.33*	H-2`, 4`	C-1`, 2`, 4`, 5`
4`	67.94, CH	3.47, t (9.3)	H-3`, 5`	C-2`, 3`, 5`, 6`
5`	77.66, CH	3.68, m	H-4`, 6`a, 6`b	C-1`, 3`, 4`, 6`a,b
6`a.	65.33, CH_2	3.86, dd (11.5, 5.3)	H-5,6`b	C-4`,5`
6`b.		3.64, dd (11.5, 4.0)	H-5, 6`a	C-4`,5`
1``	109.14, CH	4.87, d (3.1)	H-2``	C-6`, 2``, 3``, 5``
2``	79.22, CH	3.86, dd (3.7, 3.1)	H-1``, 3``	C-1``,3``, 4``
3``	76.05, CH	3.60, t (3.7)	H-2``, 4``	C-1``, 2``, 4``, 5``
4``	86.26, CH	3.94, m	H-3``, 5``	C-2``, 3``, 5``
5``a.	62.41, CH_2	3.84, dd (11.2, 2.8)	H-4``, 5``b	C-1``, 3``, 4``
5``b.	-	3.38, dd (11.2, 5.0)	H-4``, 5``a	C-1``, 3``, 4``
2-OH	-	7.02, brs	-	-
NH	-	7.02, brs	-	-
2`-OH	-	5.55, brs	-	-
3`-OH	-	5.24, brs	-	-
4`-OH	-	5.32, brs	-	-
2``-OH	-	5.52, brs	-	-
3``-OH	-	5.44, brs	-	-
5``-OH	-	4.98, brs	-	-

* The splitting pattern is masked by the signal of water.

sugar moiety. The identity of the sugar moiety was determined by a combination of DQF - COSY, DEPT, HMQC, and HMBC experiments. The coupling constants of ^{1}H NMR and the chemical shifts of ^{13}C NMR suggested the presence of one *β*-D-glucopyranosyl moiety (Altona and Haasnoot, 1980). Acid hydrolysis of 3 confirmed the sugar component as glucose by TLC analysis. The attachment of the glucose moiety to N6- position of the hydroxyl zeatin moiety was deduced from the three-bond long-range correlation observed between the glucosyl anomeric proton (δ 5.42) and methin carbon at δ 142.69 (C-8), and the quaternary carbon at δ 102.44 (C-4) in the HMBC spectrum. Additionally by HMQC experiment, the singlet aromatic proton at δ 8.09 (s, H-8) of the hydroxy zeatin was identified by its connectivity with C-8 methin carbon at δ 142.69, which in turn show the cross-peak with anomeric C-1` (δ 86.26) by HMBC, clearly indicated the attachment of the sugar moiety to the hydroxyzeatin at N6-position. The configuration of the hydroxyl-isopentenyl group of the hydroxyzeatin moiety was determined to be trans by comparison of the chemical shifts of the ^{1}H and ^{13}C NMR signals with those of trans-zeatin riboside (Kobayashi et al., 1997). Accordingly, the structure of aquaticine A was characterized as 6-N-(trans-4-hydroxy-3-methylbut-2-enylamino)-2-hydroxy-9-*β*-D-glucopyranosyl purine.

Aquaticine B (4) had a pseudo molecular ion peak at m\z 536.1975 $[M+Na]^+$, in the positive ion HRFABMS and pseudo molecular ion peaks at m\z 514 $[M+H]^+$ and 536 $[M+Na]^+$, as indicated from the positive-ion mode ESIMS, which compatible with a molecular formula $C_{21}H_{31}N_5O_{10}$. Its UV and IR spectra showed absorption bands similar to 2

Table 5. Cytotoxicity of *Gleditsia aquatica* extracts and isolated saponins (1and 2) against selected liver, kidney, ovary, uterus, skin, prostate and colon tumor cell lines.

Tumor cell lines	*Gleditsia aquatica* extracts and pure compounds (1 and 2) ED_{50} (µg/ ml)				
	EtOH	ETOAc	n-BuOH	1	2
Liver adenocarcinoma NCI-H1755	77.39(± 0.64)	> 100	80.65 (± 0.76)	70.87 (± 0.58)	83.66 (± 0.71)
SK-HEP- Tumor Cell Lines 1	> 100	65.93 (± 0.53)	> 100	54.93 (± 0.29)	73.18 (± 0.53)
Kidney carcinoma A-498	> 100	> 100	> 100	> 100	> 100
Kidney hypernephroma SW 156	92.45 (± 0.82)	65.91 (± 0.60)	> 100	48.94 (± 0.22)	64.23 (± 0.45)
Ovary adenocarcinoma MDAH 2774	17.47 (± 0.09)	13.03 (± 0.08)	30.75 (± 0.16)	4.86 (± 0.05)	5.45 (± 0.06)
NIH:OVCAR	23.81 (± 0.14)	26.19 (± 0.15)	39.15 (± 0.19)	3.11 (± 0.05)	14.66 (± 0.11)
Uterus mesodermal tumor SK-UT-1	8.93 (± 0.04)	12.17 (± 0.08)	27.68 (± 0.14)	6.23 (± 0.06)	3.68 (± 0.05)
Skin malignant melanoma G-361	13.34 (± 0.10)	16.43 (± 0.12)	25.72 (± 0.16)	10.15 (± 0.10)	9.95 (± 0.08)
RPMI-7951	35.65 (± 0.19)	12.76 (± 0.10)	28.49 (± 0.18)	11.40 (± 0.10)	12.15 (± 0.08)
Hs 908. SK	48.14 (± 0.23)	55.15 (± 0.26)	45.85 (± 0.22)	58.82 (± 0.26)	65.25 (± 0.42)
Prostate carcinoma DU 145	42.12 (± 0.20)	28.14 (± 0.16)	25.00 (± 0.13)	36.81 (± 0.23)	32.03 (± 0.18)
Colon colorectal adenocarcinoma HCT8(HRT-8)	67.74 (± 0.60)	59.26 (± 0.25)	45.75 (± 0.22)	42.70 (± 0.20)	40.38 (± 0.20)
LS 174T	40.78 (± 0.20)	32.18 (± 0.15)	29.80 (± 0.17)	4.18 (± 0.05)	3.66 (± 0.05)
COLO 320 DM	82.05 (± 0.69)	75.15 (± 0.63)	90.35 (± 0.77)	48.60 (± 0.25)	52.85 (± 0.25)

The gross features of its NMR spectra (Table 4), indicated a close structural relationship between 3 and 4, the difference in the ^{1}H NMR spectrum between 4 and 3, were the presence of signals for geminal methylene protons at δ 4.52 (2H, d, J = 6.8 Hz, H_2-11), a methine proton at δ 5.25 (1H, t, J = 6.8 Hz, H-12) and two methyl singlet protons at δ 1.65 and 1.78 (H_3-15, H_3-14, respec-tively), indicated the presence of isopentenyl group in 4 versus hydroxyl-isopentenyl group in 3. This functionality was further confirmed by ^{13}C NMR resonances at δ 17.89 (C-14), 25.29 (C-15), 39.89 (C-11), 120.07 (C-12) and 134.69 (C-13). The sugars of 4 were evidenced by two anomeric proton and carbon signals in the ^{1}H and ^{13}C NMR spectra at [δ_H 5.42 (H-1`), $\delta_C$ 86.23 (C-1`), δ_H 4.87 (H-1``), $\delta_C$ 109.14 (C-1``)]. The *β*-anomeric configuration for the glucose residue was evident from the J_{H1-H2}value of (8.6 Hz) (Kobayashi et al., 1997). The presence of *α* – L-arabinofuranoside was shown by a J_{H1-H2} coupling con-stant of (3.1 Hz). A comparison of the anomeric chemical shifts and coupling constants for the arabinose residue in 4 and methyl *α* and *β* – arabinofuranoside (Colquhoun et al., 1989), indicated that the ring is in the furanose form and that it is *α* –linked to glucose, which was further confirmed by the resonance of arabinofuranosyl C-1 at δ 109.14 in 4. Acid hydrolysis of 4 confirmed the sugar components as glucose and arabinose by TLC analysis. The downfield shift of CH_2-6` of the glucopyranoside in the $^{1}H$ NMR ($\delta_H$ 3.64 and 3.86) and in the $^{13}C$ NMR ($\delta_C$ 65.33) indicated the arabino-furanosyl unit is located at C-6` of the glucose moiety, which was supported by an HMBC correlations observed between H-1`` (δ 4.87) of Ara and C-6` (δ 65.33) of Glc, $H_2$-6` (δ 3.64, 3.86) of Glc and C-1`` (109.14) of Ara. The assignment of arabinose as a terminal sugar was indicated from the ESIMS frag-ment ions at m\z 382 $[M+H\text{-arabinose}]^+$, 404 $[M+Na\text{-arabonose}]^+$ and 785 $[2M+Na+2\ \text{arabinose}\ ]^+$. Charac-teristic correlations observed in the HMBC spec-trum between the glucosyl anomeric proton (δ 5.42) and methine carbon at δ 142.73 (C-8), and quaternary carbon at δ 102.34 (C-4), confirmed the glucose was linked to the 9-N position of the aglycon. The foregoing evidence led to the elucidation of the structure of 4 6-N-(3-methylbut-2-enylamino)-2-hydroxy-9- [*α*-L-arabinofuranosyl- (1``→ 6`)-*β*-D-glucopyranosyl] purine.

Adenosine (5) has the molecular formula $C_{10}H_{13}N_5O_4$ as deduced from the positive-ion mode ESIMS. Its ^{1}H NMR showed two one-proton singlets at δ 8.13 and 8.34, and one two-proton singlet at δ 7.33 in the aroma-tic region, and one-proton doublet at δ 5.88 (J=6.2 Hz).

These signals are characteristic for H-2, H-8, NH2 an-danomeric proton, respectively, of adenosine (Son et al., 1991). Other physical data (UV, IR and 13C NMR) (Son et al., 1991) being in agreement with those reported for adenosine, confirmed the structure for 5. Some *in vivo* studies (Zhong et al., 2003; Zhong et al., 2004) reported that pure saponins or extracts of *Gleditsia sinensis* showed significant cytotoxicity against human leukemia (HL-60) cells, breast cancer (MCF-7) cells and other tumor cell lines and caused induction of apoptosis, and inhibition of DNA topoisomerase. The 50% effective dose (ED_{50}) obtained by measuring growth inhibition with MTT (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide) (Goren et al., 1996; Woodson, 1987) are shown in Table 5. The activities observed with crude extracts

were surprising. Saponins (1 and 2) were more cytotoxic versus ovary adenocarcinoma, the uterus mesodermal tumor, the G-361 and RPMI-7951 skin malignant melanoma, and the LS 174T colon colorectal adenocacinoma lines. While compound 1 was more potent than 2 with NIH: OVCAR ovary adenocarcinoma and 2 was more potent than 1 with SK-UT-1 uterus mesodermal tumor lines. Relatively limited information exists on the structure - activity relationships for the cytotoxicities of these compounds.

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