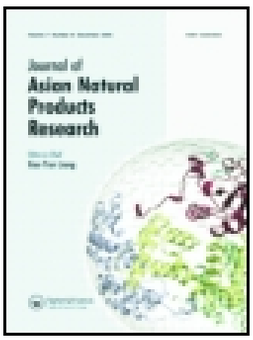

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Two new steroidal saponins from the roots of *Cynanchum limprichtii*

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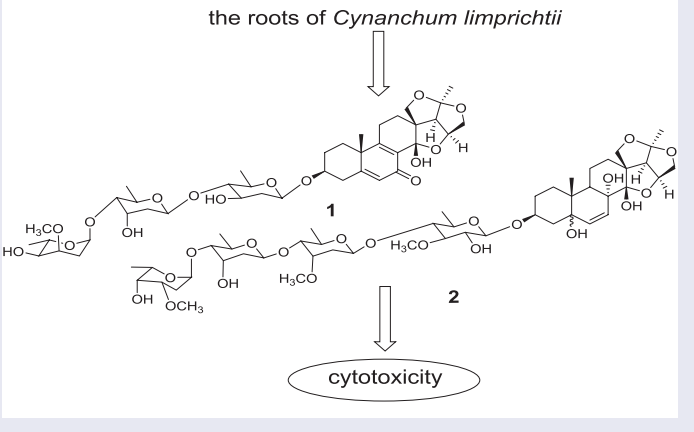
ABSTRACT

As a part of our continuing research for bioactive constituents from *Cynanchum limprichtii* Schltr., two new C_{21} steroidal glycosides limproside A (**1**) and limproside B (**2**) were isolated from the roots of *Cynanchum limprichtii*. Their structures were elucidated on the basis of 1D- and 2D-NMR spectroscopic data as well as HR-ESI-MS analysis. The cytotoxicity of two compounds against two selected human cancer cell lines was assayed.



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Asclepiadaceae; *Cynanchum limprichtii*; steroidal glycoside; limproside; cytotoxicity

1. Introduction

Plants of the genus *Cynanchum* belong to the family Asclepiadaceae, and their roots contain steroidal glycosides with various biological activities such as anti-tumor, immunosuppressive, antidepressant, neuroprotective, antivirus, and appetite- suppressing effects [1].

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In previous studies, essential oils [2], flavonoids, alkaloids, terpenes, and steroidals were isolated from this genus [3]. Chemical investigation revealed that they contain characteristic C_{21} steroidal glycosides, especially with the normal four-ring C_{21} steroid skeleton or the aberrant 13, 14:14, 15-disecopregnane-type skeleton or 14, 15-secopregnane-type skeleton [4]. As a kind of characteristic and bioactive constituents, more than 200 C_{21} steroidal glycosides have been isolated from *Cynanchum* species [5]. As part of our continuing chemical investigation of the steroidal constituents of *Cynanchum* plants, a phytochemical screening of the roots of *Cynanchum limprichtii* was performed. We report the discovery of two steroidal glycosides limproside A **(1)** and limproside B **(2)** (Figure 1). Extensive spectroscopic studies were conducted to determine the structures of the new compounds, including two-dimensional NMR, HR-ESI-MS, and hydrolytic cleavage followed by chromatographic and spectroscopic analysis. Additionally, the cytotoxicity of two compounds was evaluated against selected human cancer cell lines, including HL-60 (human leukemic promyelocytic cell) and Caco-2 (human Caco-2 colon cancer cell).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined as $C_{40}H_{58}O_{15}$ by its pseudo molecular ion peak at *m/z* 801.3618 $[M + Na]^+$ in the positive HR-ESI-MS experiment, indicating 12 degrees of unsaturation. The IR spectrum showed the absorption bands for hydroxyl (3439 cm^{-1}), carbonyl (1655 cm^{-1}) and olefinic (1632 cm^{-1}) groups. The ^1H NMR spectrum of aglycone portion exhibited two tertiary methyl protons at δ_H 1.18 (3H, s, H-19) and 1.61 (3H, s, H-21), one olefinic proton signal at δ_H 6.31 (1H, brs, H-6), two oxygen-substituted methine protons at δ_H 3.80–3.84 (1H, m, H-3) and 4.24 (1H, dd, J = 7.0, 3.4 Hz, H-16), and two methylene groups at δ_H 3.73 (1H, dd, J = 10.4, 3.4 Hz, H_α-15) and 4.31 (1H, d, J = 10.4 Hz, H_β-15), and at δ_H 3.89 (1H, d, J = 9.3 Hz, H_α-18) and 5.22 (1H, d, J = 9.3 Hz, H_β-18). The ^{13}C NMR spectrum showed two oxygenated tertiary carbon signals at δ_C 105.5 (C-14) and 118.3 (C-20), four olefinic carbon signals at δ_C 164.7 (C-5), 126.3(C-6), 130.8 (C-8), and 162.1 (C-9), one conjugated ketonic carbonyl carbon signal at δ_C 186.8 (C-7). Comparison of ^1H and ^{13}C NMR data (Table 1) with those of stauntoside G [6], a known steroidal glycoside isolated from the roots of *C. stauntonii*, revealed that compound **1** contained a same aglycone. Three anomeric protons and carbon signals at δ_H 4.86 (1H, d, J = 9.6 Hz, H-1′)/98.1 (C-1′), 5.23 (1H, d, J = 9.3 Hz, H-1″)/99.6

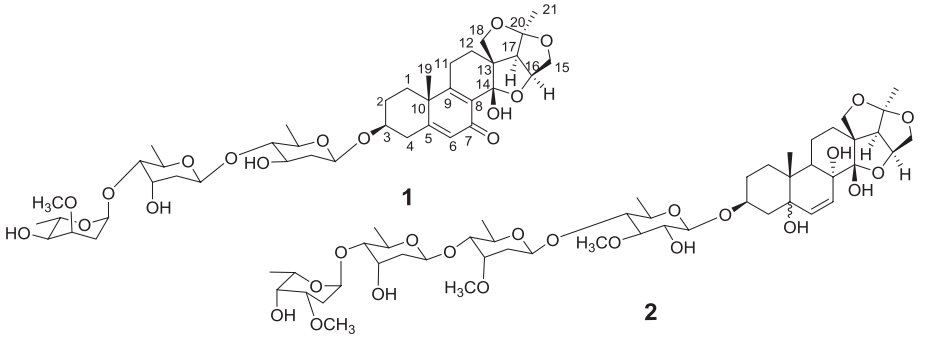

Figure 1. The structures of compounds **1** and **2.**

Table 1. ^{1}H and ^{13}C NMR spectral data of compounds **1** and **2** in C_5D_5N (at 400 and 100 MHz, resp.).

	1		**2**	
Position	δ_C	δ_H(J Hz)	δ_C	δ_H(J Hz)
1	33.5	1.92–1.94 (m), 1.21–1.25 (m)	32.7	2.08–2.12 (m),1.60–1.63 (m)
2	28.8	2.15–2.18 (m), 1.81–1.85 (m)	29.9	2.21–2.25 (m),1.87–1.91 (m)
3	76.7	3.80–3.84 (m)	75.4	4.71–4.74 (m)
4	38.7	2.80 (brdd, 14.1, 2.2), 1.94–1.96 (m)	39.5	2.41–2.45 (m), 2.00–2.03 (m)
5	164.7		73.1	
6	126.3	6.31 (brs)	137.7	6.01 (d, 9.8)
7	186.8		133.4	6.64 (d, 9.8)
8	130.8		76.2	
9	162.1		52.3	2.89 (dd, 12.4, 6.0)
10	42.3		42.2	
11	22.2	2.40–2.45 (m), 1.54–1.56 (m)	20.0	1.82–1.84 (m), 1.68–1.71 (m)
12	27.1	1.88–1.91 (m), 2.05–2.08 (m)	35.6	2.23–2.28 (m)
13	58.0		58.6	
14	105.5		111.5	
15	72.0	4.31(d,10.4), 3.73(dd, 10.4, 3.4)	73.8	4.22 (o), 3.75 (dd, 9.8, 4.2)
16	79.2	4.24 (dd, 7.0, 3.4)	83.6	5.14 (o)
17	59.0	3.00 (d, 7.0)	69.5	3.12 (d, 7.8)
18	74.7	5.22 (d, 9.3), 3.89 (d, 9.3)	77.0	5.11 (o), 4.07 (o)
19	22.8	1.18 (s)	19.0	1.31 (s)
20	118.3		118.7	
21	22.9	1.61 (s)	23.2	1.60 (s)
14-OH				8.01 (s)
	β-Dcan	*β*-D-can	*β*-D-the	*β*-D-the
1′	98.1	4.86 (d, 9.6)	102.8	4.77 (d, 7.7)
2′	39.7	2.51–2.55 (m), 1.97–2.02 (m)	75.0	3.93–3.95 (m)
3′	69.1	4.17–4.21 (m)	86.2	3.63–3.67 (m)
4′	88.2	3.29–3.32 (m)	83.6	3.48–3.50 (m)
5′	70.8	3.54–3.56 (m)	72.0	3.50–3.53 (m)
6′	18.3	1.32 (d, 6.2)	18.9	1.41 (d, 6.3)
3-OCH_3			60.6	3.93 (s)
	β-D-digt	*β*-D-digt	*β*-D-cym	*β*-D-cym
1″	99.6	5.23 (d, 9.3)	99.9	5.14 (d, 9.5)
2″	38.0	2.40–2.44 (m), 1.97–2.00 (m)	35.3	2.39–2.42 (m), 1.68–1.71 (m)
3″	67.3	4.47–4.51 (m)	77.8	3.92–3.96 (m)
4″	80.5	3.42–3.46 (m)	83.2	3.69–3.73 (m)
5″	69.8	3.95–3.99 (m)	69.7	4.19–4.25 (m)
6″	18.1	1.35 (d, 6.2)	19.0	1.31 (d, 6.2)
3-OCH_3			57.7	3.55 (s)
	α-L-cym	*α*-L-cym	*β*-D-digt	*β*-D-digt
1‴	98.3	5.03 (d, 3.8)	99.2	5.51 (d, 9.7)
2‴	32.0	2.32–2.35 (m), 1.82–1.86 (m)	40.1	2.38–2.40 (m), 1.97–2.00 (m)
3‴	76.2	3.67–3.70 (m)	69.2	4.28–4.33 (m)
4‴	72.3	3.61–3.63 (m)	82.6	3.40 (dd, 9.6, 2.6)
5‴	67.2	4.44–4.48 (m)	68.1	4.30–4.34 (m)
6‴	18.1	1.42 (d, 6.3)	19.0	1.40 (d, 6.2)
3-OCH_3	56.5	3.36 (s)		
			α-L-dign	*α*-L-dign
1⁗			101.5	5.21 (d, 3.2)
2⁗			31.3	2.40–2.43 (m), 2.07–2.10 (m)
3⁗			76.2	3.84–3.87 (m)
4⁗			68.1	4.62–4.66 (m)
5⁗			68.0	4.06–4.09 (m)
6⁗			18.1	1.57 (d, 6.2)
3-OCH_3			55.4	3.32 (s)

(C-1″), 5.03 (1H, d, J = 3.8 Hz, H-1‴)/98.3(C-1‴) in the ^{1}H and ^{13}C NMR spectra, along with the presence of three secondary methyl proton signals at δ_H 1.32 (3H, d, J = 6.2 Hz, H-6′), 1.35 (3H, d, J = 6.2 Hz, H-6″) and 1.42 (3H, d, J = 6.3 Hz, H-6‴), suggested that **1**

contained three 6-deoxysugar. The sugar moieties were defined as digitoxose, cymarose, and canaropyranose by their ^{13}C NMR resonance data, which matched well with those of stauntoside P [7]. The splitting patterns of anomeric proton signals indicated that **1** had two sugar units with β-linkages and one with α-linkage. The linkage positions and sequence of these sugars were ascertained by HMBC correlations (Figure 2) from δ_H 5.03 (H-1‴ of α-cymaropyranose) to δ_C 80.5 (C-4″), from δ_H 5.23 (H-1″ of β-digitoxopyranose) to δ_C 88.2 (C-4′), from δ_H 4.86 (H-1′ of β-canaropyranose) to δ_C 76.7 (C-3). Compound **1** was subjected to acid hydrolysis and HPLC analysis, which gave one D-canarose, one L-diginose, and one D-cymarose. Therefore, **1** was characterized to be stauntogenin A 3-*O*-α-L- cymaropyranosyl-(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-canaropyranoside and was given the trivial name limproside A.

Compound **2** was obtained as white amorphous powder. The positive mode HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 995.4910 $[M + Na]^+$, revealing its molecular formula to be $C_{48}H_{76}O_{20}$. The IR spectrum showed the absorption bands for hydroxy (3425 cm^{-1}) and olefinic (1632 cm^{-1}) groups. The ^1H NMR spectrum of **2** revealed the diagnostic signals of steroidal glycoside, with a 14, 15-secopregnane-type skeleton aglycone being exhibited typically by two tertiary methylic groups at δ_H 1.31 (3H, s, H-19) and 1.60 (3H, s, H-21) , two oxygen-substituted methine groups at δ_H 4.71–4.74 (1H, m, H-3) and 5.14 (1H, overlap, H-16), and two methylene groups at δ_H 3.75 (1H, dd, J = 9.8, 4.2 Hz, H_α -15) and 4.22 (1H, overlap, H_β-15), and at δ_H 4.07 (1H, overlap, H_α-18) and 5.11 (1H, overlap, H_β-18), and with four 6-deoxypyranoses being shown by four anometic proton signals at δ_H 4.77 (1H, d, J = 7.7 Hz, H-1′), 5.14 (1H, d, J = 9.5 Hz, H-1″), 5.51 (1H, d, J = 9.7 Hz, H-1‴) and 5.21 (1H, d, J = 3.2 Hz, H-1‴) , which correlated to the corresponding anomeric carbon signals at δ_C 102.8 (C-1′), 99.9 (C-1″), 99.2 (C-1‴) and 101.5 (C-1‴) respectively, in the HSQC spectrum, and four methyls at δ_H 1.41 (3H, d, J = 6.3 Hz, H-6′), 1.31 (3H, d, J = 6.2 Hz, H-6″), 1.40 (3H, d, J = 6.2 Hz, H-6‴) and 1.57 (3H, d, J = 6.2 Hz, H-6‴). In addition, three methoxyl signals at δ_H 3.93 (3H, s), 3.55(3H, s), 3.32 (3H, s) were also observed in the ^1H NMR spectrum, which were compatible with three methylated 6-deoxypyranoses. These proton signals together with distinctive olefinic ptotons at δ_H 6.01(1H, d, J = 9.8 Hz, H-6) correlating to the carbon at δ_C 137.7 (C-6), showing HMBC

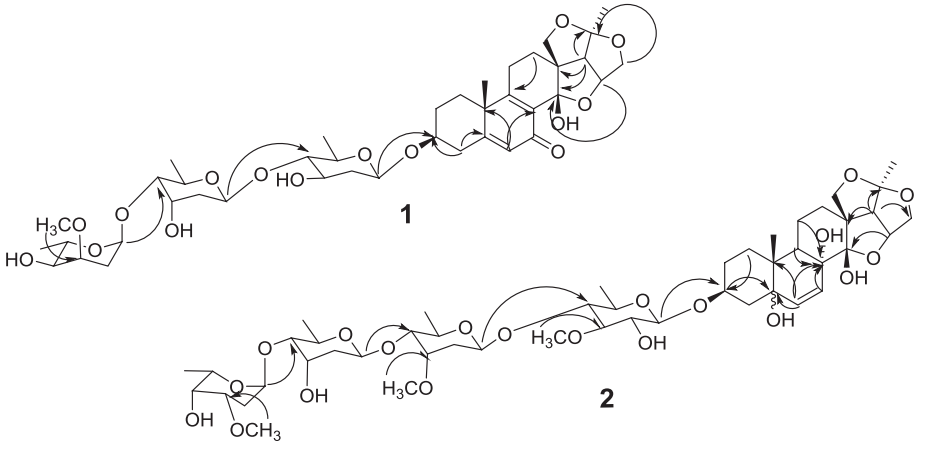

Figure 2. Key HMBC correlations of compounds **1** and **2.**

correlations (Figure 2) with the carbons at δ_C 42.2 (C-10), 73.1 (C-5), 76.2 (C-8) and 39.5 (C-4), the other olefinic proton at δ_H 6.64 (1H, d, J = 9.8 Hz, H-7) correlating to the carbon at δ_C 133.4 (C-7), showing HMBC correlations with the carbons at δ_C 52.3 (C-9), 111.5 (C-14) and 76.2 (C-8), indicated the presence of a 5, 8, 14-trihydroxyl-14, 15-secopregn-6-ene type skeleton aglycone moiety. Further, by consideration of the molecular formula and ^{13}C NMR spectroscopic data (Table 1), three hydroxyls were proposed to be linked at C-5, C-8, and C-14, respectively. C14-OH was confirmed by the HMBC correlations from 14-OH at δ_H 8.01 to the carbons at δ_C 58.6 (C-13), 76.2 (C-8), and 111.5 (C-14), and based on the generally accepted β-orientation of Me-19 in 14, 15-secopregnane-type skeleton, the β-orientation of 14-OH could be confirmed by the NOE correlation between 14-OH and H-19 (Figure 3). Because of the failure of detecting the labile protons from hydroxyls, 8-OH was confirmed as α-orientation by comparing with stauntoside H [6]. But the configuration of 5-OH was not able to be confirmed using this method. The linkage positions and sequence of the four sugars were ascertained by HMBC correlations from δ_H 5.21 (H-1‴ of α-diginopyranose) to δ_C 82.6 (C-4‴ of β-digitoxopyranose) , from δ_H 5.51 (H-1‴ of β-digitoxopyranose) to δ_C 83.2 (C-4″ of β-cymaropyranose), from δ_H 5.14 (H-1″ of β-cymaropyranose) to δ_C 83.6 (C-4′ of β-thevetopyranose) and from δ_H 4.77 (H-1′ of β-thevetopyranose) to δ_C 75.4 (C-3). The splitting patterns of anomeric proton signals indicated that **2** had three sugar units with β-linkages and one with α-linkage. The β-D-digitoxopyranose, β-D-thevetopyranose, β-D-cymaropyranose and α-L-diginopyranose were speculated by comparing the ^{1}H and ^{13}C NMR spectroscopic data of **2** with those in the literature [6]. Acid hydrolysis of **2** afforded four sugars (digitoxose, cymarose, diginose and thevetose). Identification of D-digitoxose, D-thevetose, D-cymarose and L-diginose were performed by HPLC analysis with an optical rotation detector. Thus, the structure of **2** was determined to be 5, 8, 14-trihydroxyl-3-*O*-α-L- diginopyranosyl -(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-thevetopyranosyl-14, 16:15, 20: 18, 20-triepoxy-14, 15-secopregn-6-ene, and was given the trivial name limproside B.

Limprosides A and B were tested for their cytotoxicity against HL-60 and Caco-2 cell lines using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) colorimetric assay. Unfortunately, the two compounds exhibited no cytotoxicity.

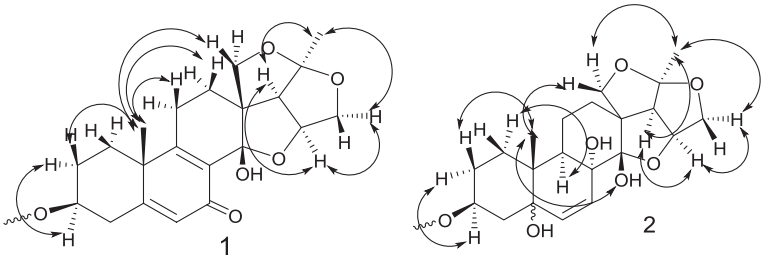

Figure 3. Key NOESY correlations of compounds **1** and **2.**

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter (JASCO international Co., Ltd., Tokyo, Japan). IR spectra were obtained from a Bruker IFS-55 spectrometer (Bruker Co., Karlsruhe, Germany). HR-ESI-MS were acquired using a Micro-mass Autospec-Untima TOF mass spectrophotometer (Waters, Milford, U.S.A.). One- and two-dimensional NMR spectra were recorded on a Bruker Avance-400/-600 spectrometer (Bruker Co., Karlsruhe, Germany) with tetramethylsilane as an internal standard. CD spectra were recorded with a Biologic MOS-450 spectrometer (Bio-Logic Co., Claix, France). Sugars analytical HPLC was carried out on a Jasco PU-4180 pump and an OR-4090 detector (JASCO international Co., Ltd., Tokyo, Japan). HPLC was performed with an Asahipak NH2P-50 4E column (4.6 mm × 250 mm, 5 μm). Silica gel GF254 (10–40 μm) prepared for TLC and silica gel (200–300 mesh) for column chromatography (CC) were obtained from Qingdao Marine Chemical Factory (Qingdao, China). Sephadex LH-20 was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, U.K.). C_{18} reversed-phase (RP-18) silica gel (50 μm, YMC Co. Ltd., Kyoto, Japan) was used for CC. RP-18 F_{254S} (Merck, Darmstadt, Germany) was used for reversed phase plates.

3.2. Plant materials

The dried roots (20 kg) of *Cynanchum limprichtii* Schltr. were bought from Anhui Economy People Pharmaceutical Co., Ltd. A voucher specimen was identified by Prof. Jincai Lu of Shenyang Pharmaceutical University and has been deposited in the School of Traditional Chinese Materia Medica of Shenyang Pharmaceutical University (No. 2013052).

3.3. Extraction and isolation

The dried roots of *C. limprichtii* (20 kg) were extracted with 95% EtOH for three times (each 2 h) and the combined solution evaporated to dryness by a vacuum rotary evaporator to afford a crude extract (3500 g). The crude extract was successively partitioned with petroleum ether, ethyl acetate, and *n*-butanol to yield three extracts. The ethyl acetate extract (300 g) was subjected to silica gel CC using a gradient of CH_2Cl_2–MeOH (100:0–0:100, v/v) to obtain five fractions (Fr.A-E) based on TLC analyses.

Fr.B (92 g) was separated by silica gel CC eluted with petroleum ether-CH_3COCH_3 (100:10–100:100, v/v) to afford nine fractions (Fr.B1-B9). Fr.B9 (38 g) was further separated by silica gel CC using a gradient of petroleum ether-CH_3COCH_3 (100:15–100:100, v/v) to obtain ten fractions (Fr.B9A-B9 J). Fr.B9F (10 g) yielded four fractions (Fr.B9F1-B9F4) by silica gel CC eluted with CH_2Cl_2–MeOH (100:2–100:10, v/v). Fr.B9F1(5 g) further produced nine fractions (Fr.B9F1A-B9F1I) by silica gel CC eluted with petroleum ether-CH_3COCH_3 (100:10–100:50, v/v). Fr.B9F1E (1 g) was subjected to Sephadex LH-20 gel CC eluted with MeOH to afford five fractions (Fr.B9F1E1-B9F1E5). Fr.B9F1E2 (350 mg) was subjected to RP-C_{18} CC by using MeOH-H_2O (50:50–80:20, v/v) as the eluting solvent, and followed by purification on the silica gel CC using CH_2Cl_2–CH_3COCH_3–MeOH (30:2:1, v/v) to provide compound **1** (16 mg). Fr.B9H (12 g) was subjected to silica gel CC eluted with petroleum ether-CH_3COCH_3 (100:20–100:40, v/v) to give nine fractions (Fr.B9H1-B9H9).

Fr.B9H7 (4 g) further produced six fractions (Fr.B9H7A-B9H7F) by silica gel CC eluted with petroleum ether-EtOAc (1:3, 1:5, 1:7, v/v). Fr.B9H7F (1.8 g) was separated by silica gel CC eluted with petroleum ether-CH_3COCH_3 (100:50–100:100, v/v) to afford eight fractions (Fr. B9H7F1-B9H7F8). Fr.B9H7F7 (330 mg) was subjected to RP-C_{18} CC eluted with MeOH-H_2O (60:40–80:20, v/v) to give compound **2** (10 mg).

3.3.1. *Limproside A* (1)

White amorphous powder. $[\alpha]_D^{20}$ −84.5 (c 0.2, MeOH). CD (MeOH)$\Delta\varepsilon_{249\,nm}$ + 2.85. IR (KBr) ν_{max}: 3439, 2933, 2659, 1696, 1655, 1632, 1403, 1384, 1271, 1062, 833 cm^{-1}. ^1H (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) spectral data see Table 1. HR-ESI-MS (positive-ion mode) *m/z*: 801.3618 $[M + Na]^+$ (calcd for $C_{40}H_{58}O_{15}Na$, 801.3668).

3.3.2. *Limproside B* (2)

White amorphous powder. $[\alpha]_D^{20}$ −43.3 (c 0.05, CH_3COCH_3). CD ($CHCl_3$) $\Delta\varepsilon_{205\,nm}$ + 8.83, $\Delta\varepsilon_{215\,nm}$ −3.00. IR (KBr) ν_{max}: 3425, 2932, 2659, 1649, 1632, 1403, 1384, 1271, 1069, 832 cm^{-1}. ^1H NMR (pyridine-d_5, 400 MHz) and ^{13}C NMR (pyridine-d_5, 100 MHz) spectral data see Table 1. HR-ESI-MS (positive-ion mode) *m/z*: 995.4910 $[M + Na]^+$ (calcd for $C_{48}H_{76}O_{20}Na$, 995.4828).

3.4. Acid hydrolysis of compounds 1 and 2

Each solution of **1** and **2** (each 6 mg) in MeOH (5 ml) was added 0.1 N H_2SO_4 (5 ml) and kept at 50 °C for 30 min. The solution was diluted with 10 ml of water and then concentrated to 10 ml. The solution was kept at 60 °C for another 30 min, and then neutralized with aqueous saturated $Ba(OH)_2$ and the precipitates were filtered off. The filtrate was concentrated and analyzed by TLC with three solvent systems: solvent A, $CHCl_3$–CH_3OH (9:1); solvent B, CH_2Cl_2–C_2H_5OH (9:1); and solvent C, PE-acetone (3:2). The R_f values of cymarose, diginose, digitoxose, thevetose, and canaropyranose, in the order of 0.56, 0.40, 0.21, 0.59, and 0.31 with solvent A; 0.47, 0.28, 0.15, 0.53, and 0.27 with solvent B; and 0.28, 0.18, 0.10, 0.37, and 0.17 with solvent C, respectively [8–10].

3.5. Determination of absolute configurations of sugar moieties in 1 and 2

The determination of absolute configurations of the deoxysugars obtained from water layer of the hydrolysis solution of the compounds by HPLC under the following conditions: column, Asahipak NH2P-50 4E column; flow rate, 1.0 ml/min; solvent, 75% MeCN-H_2O; detection, OR-4090 detector, identification of D-cymarose, L-cymarose, D-digitoxose, L-diginose, D-canaropyranose, and D-thevetose in each sugar fraction was carried out by comparison of the retention times and polarities with those of authentic samples (the standards of sugars were obtained by acid hydrolysis of known compounds with the same method of those new compounds). D-cymarose (t_R 8.76 min, positive polarity), L-cymarose (t_R 9.15 min, negative polarity), D-digitoxose (t_R 11.5 min, positive polarity), L-diginose (t_R 7.21 min, negative polarity), D-canaropyranose (t_R 14.9 min, positive polarity), and D-thevetose (t_R 6.02 min, positive polarity).

3.6. Cytotoxic assay

The MTT assay [11,12] was used to determine the cytotoxicity of each compound against two cultured human cancer cell lines HL-60 and Caco-2. 5-Fluorouracil was used as a positive control. The cytotoxicity of 5-fluorouracil against the HL-60 and Caco-2 cell lines was estimated by their IC_{50} values of 6.38 and 17.01 μM, respectively, for these two cell lines.

Disclosure statement

No potential conflict of interest was reported by the authors.

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