Two New Cytotoxic Triterpene Glycosides from the Sea Cucumber *Holothuria scabra*

Authors **Hua Han**[1,2], **Yanghua Yi**[1], **Qiangzhi Xu**[3], **Mingping La**[1], **Hongwei Zhang**[1]

Affiliations
[1] Research Center for Marine Drugs, School of Pharmacy, Second Military Medical University, Shanghai, P. R. China
[2] College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang, P. R. China
[3] Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai, P. R. China

Key words
- sea cucumber
- *Holothuria scabra*
- triterpene glycoside
- scabraside A
- scabraside B
- cytotoxity
- Holothuriidae

Abstract

Two new triterpene glycosides, scabraside A (**1**) and B (**2**), and a structurally known compound (**3**), were isolated from the sea cucumber *Holothuria scabra* (Holothuriidae) collected from the South China Sea. Structure of these compounds was elucidated by spectroscopic and chemical methods. The glycosides **1** and **2** exhibit the same common structural features, i. e., the presence of 12- and 17-hydroxy groups in the holostane-type triterpene aglycone with a 9(11)-ene bond, but are different in the side chains of the triterpene aglycone. The glycosides **1** and **2** had significant *in vitro* cytotoxicity against four human tumor cell lines in comparison to 10-hydroxycamptothecin.

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Bibliography
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Correspondence
Prof. Yanghua Yi
Research Center for Marine Drugs
School of Pharmacy
Second Military Medical University
325 Guo-He Road
Shanghai 200433
People's Republic of China
Phone: + 86 21 65 38 49 88
Fax: + 86 21 65 48 36 62
hhan05@126.com

Introduction

Triterpene glycosides are the typical predominant secondary metabolites in sea cucumbers. More than 100 of these glycosides have been reported. The majority of these saponins have a sugar chain of up to six monosaccharide units linked to the C-3 of the aglycone, which is usually a triterpene of the lanosterol type with an 18(20)-lactone [1,2]. The saponins have a wide spectrum of biological functions, including antifungal, cytotoxic, hemolytic, cytostatic and immunomodulatory activities [3]. Sea cucumber, *Holothuria scabra* (Holothuriidae), is distributed abundantly in the South China Sea, and is used as a tonic in China [4]. As part of our research on biological secondary metabolites from echinoderms [5,6], we have focused our attention on the polar extracts of *H. scabra*. In this paper, we report the isolation and structure elucidation of two new sulfated triterpene glycosides, scabraside A (**1**) and B (**2**) (**Fig. 1**), as well as their cytotoxicity against four human tumor cell lines.

Materials and Methods

General experimental procedures

Melting points were determined on an XT5-XMT apparatus. Optical rotations were measured on a Perkin-Elmer-341 polarimeter. IR spectra were recorded on a Bruker Vector-22 apparatus. NMR spectra were recorded in C_5D_5N on a Bruker Avance-II-600 spectrometer with TMS as internal standard. ESI- and HR-ESI-MS were acquired using a Q-TOF Micro LC-MS-MS mass spectrometer. GC-MS were acquired using a Finnigan Voyager GC/MS apparatus with an ULTRA-2 column (50 m × 0.2 mm). HPLC was carried out on an Agilent 1100 liquid chromatograph equipped with a refractive index detector using a Zorbax 300 SB-C_{18} column (250 × 9.4 mm). Column chromatographic separations were performed on silica gel H (200–300 mesh, 10–40 µm; Qingdao Marine Chemical Inc.) and Lobar Lichroprep RP-C_{18} (40–63 µm; Merck). Fractions were monitored by TLC on precoated silica gel $HSGF_{254}$ plates ($CHCl_3$-EtOAc-MeOH-H_2O, 4 : 4 : 2.5 : 0.5) or RP-C18 (MeOH-H_2O, 1 : 1), (Qingdao Marine Chemical, Inc.) and spots were visualized by spraying with 15% H_2SO_4-EtOH solution, followed by heating.

Animal material

Specimens of *H. scabra* were collected from offshore waters in the South China Sea (Hainan, P. R. China) in May, 2006, and its taxonomic classfication was identified by Prof. Yu-Lin Liao (Institute of Oceanology, Chinese Academy of Science, P. R. China). A voucher specimen (No. HYSC-2006-05) was deposited at the Research Center for Marine Drugs, School of Pharmacy, Second Military Medical University (Shanghai, P. R. China).

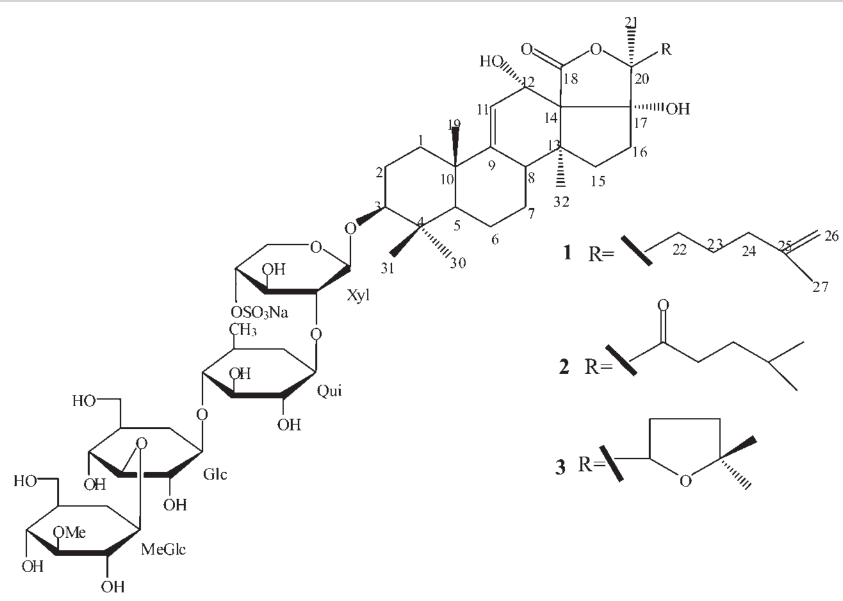

Fig. 1 Structures of compounds **1–3**.

Extraction and isolation

The sea cucumbers (3 kg, dry weight) were powdered and refluxed four times each for 1 h with 60% ethanol (6 L × 4). The extract was concentrated, and the residue (420 g) was suspended in H_2O, passed through a DA101 resin column (2 kg, 105 × 15 cm, i. d., Nankai University) and then eluted with H_2O (5 L), 70% EtOH (10 L), 95% EtOH (5 L), respectively. The glycoside fraction was eluted with 70% ethanol. The combined extracts were concentrated. The glycoside fraction (crude glycoside-containing mixture, 70 g) was chromatographed over silica gel CC (200–300 mesh, 2100 g, 85 × 15 cm, i. d.), stepwise eluted with a $CHCl_3$-MeOH-H_2O (8 : 2 : 1 to 6.5 : 3.5 : 1, lower phase) gradient to give fraction A (2.43 g), B (3 g), C (1.13 g), D (3.8 g) and E (2.23 g).

Fractions D and E were further purified by MPLC with a column of reversed-phase silica (Lichroprep RP-C_{18}, 40–63 µm, 70 × 3 cm, i. d.). Final purification of the glycosides in fractions D and E was achieved by HPLC (Zorbax 300 SB-C_{18}, 5 µm; 250 × 9.4 mm i. d.). Fraction D afforded 10.7 mg of pure glycoside **1** (t_R = 21 min) using MeOH-H_2O (62 : 38) as the mobile phase and a flow rate of 1.5 mL/min. Fraction E gave 19 mg of pure glycoside **2** (t_R = 28.7 min) using MeOH-H_2O (57 : 43) as the mobile phase and a flow rate of 1.5 mL/min.

Scabraside A (**1**): colorless amorphous powder, m. p. 234–236 °C, $[\alpha]_D^{20}$: − 11.3 (*c* 0.4, MeOH); IR (KBr): ν_{max} = 3418, 1760, 1652, 1228, 1069 cm^{-1}; ESI-MS (+) mode: *m/z* = 1227 $[M + Na]^+$, (−) mode: *m/z* = 1181 $[M - Na]^-$; HR-ESI-MS (+) mode: *m/z* = 1227.4849 $[M + Na]^+$ (calcd. for $C_{54}H_{85}Na_2O_{26}S^+$: 1227.4845). For ^{1}H- and ^{13}C-NMR data, see **Tables 1** and **2**.

Scabraside B (**2**): colorless amorphous powder, m. p. 236–238 °C, $[\alpha]_D^{20}$: − 14.9 (*c* 0.35, MeOH); IR (KBr): ν_{max} = 3407, 1775, 1778, 1631, 1266, 1074 cm^{-1}; ESI-MS (+) mode: *m/z* = 1243 $[M + Na]^+$, (−) mode: *m/z* = 1197 $[M - Na]^-$; HR-ESI-MS (+) mode: *m/z* = 1243.4792 $[M + Na]^+$ (calcd. for $C_{54}H_{85}Na_2O_{27}S^+$: 1243.4794). For ^{1}H- and ^{13}C-NMR data, see **Tables 1** and **2**.

Acid hydrolysis of the compounds 1–3

Each of the glycosides (1 mg) was heated with 2 mol/L trifluoroacetic acid (1 mL) at 120 °C for 2 h. The reaction mixture was evaporated to dryness, and the residue was partitioned between CH_2Cl_2 and H_2O. The aqueous phase was concentrated under reduced pressure. Then pyridine (1 mL) and $NH_2OH{\cdot}HCl$ (2 mg) were added to the dried residue, and the mixture was heated at 90 °C for 30 min. Then, Ac_2O (0.8 mL) was added, and heating was continued at 90 °C for 1 h. The solution was concentrated, and the resulting aldononitrile peracetates were analyzed by GC-MS using standard aldononitrile peracetates (Sigma) as reference samples. D-xylose, D-quinovose, D-glucose and D-3-*O*-methylglucose were identified in a 1 : 1 : 1 : 1 ratio for all three glycosides.

Bioassay

The cytotoxicities of **1** (95% purity) and **2** (95% purity) against human leukemia (HL-60, MOLT-4), human lung cancer (A-549), and human hepatoma (BEL-7402) cells (Shanghai Institute of Materia Medica Chinese Academy of Sciences) were evaluated by the sulforhodamine-B (SRB) assay [7], with the anticancer agent 10-hydroxycamptothecin (98% purity; Knowshine Pharmachemicals, Inc.) as a positive control. The target tumor cells at log phase were cultured with RPMI-1640 medium containing 10% fetal bovine serum (FBS). After dilution to 4×10^4 cells/mL with complete medium, 90 µL of the cell suspension were added to each well of a 96-well culture plate. Cultures were pre-incubated for 24 h in a humidified 5% CO_2 atmosphere at 37 °C. Control or test solution (10 µL) was pipetted into each well and incubated for additional 72 h. At the end of the exposure time, 50 µL of 50% CCl_3COOH (cold) was added to each well to fix the cells (at 48 °C for 1 h), and the plates were washed with tap water (5×), and air-dried. Then, 0.4% SRB solution (50 µL) in 1% AcOH was added, and the staining was allowed to proceed for 30 min. The residual dye was washed out with 1% AcOH (4×), and the plates were air-dried. To each well, 10 mM non-buffered Tris solution (150 µL) were added, and the optical density of each well was measured with a microplate reader at 520 nm. The activities of **1, 2** and the positive control at 100, 10, 1, 0.1, and 0.01 µM were determined (three replicates for each concentration). The percent inhibition (I) was calculated according to the formula: $I = [100 - (OD_t/OD_s) \times 100]$, where OD_t is the mean value of the optical density of the test compounds and OD_s that of the solvent control. The concentration inducing 50% inhibition of cell growth (IC_{50}) was

Table 1 ^{1}H- (600 MHz) and ^{13}C-NMR(150 MHz) data (δ value, *J* in Hz) for the aglyconemoiety of glycosides **1** and **2** in pyridine-d_5.[a]

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
1	36.3	1.39 m, 1.81 m	36.7	1.37 m, 1.79 m
2	27.0	1.86 m, 2.05 m	27.1	1.91 m, 2.01 m
3	88.7	3.13 dd (4.8, 12.0)	88.6	3.14 dd (4.8, 12.0)
4	40.0		40.0	
5	52.7	0.97 d (10.2)	52.6	0.82 d (10.2)
6	28.3	1.69 m, 1.52 m	28.1	1.68 m, 1.50 m
7	21.2	1.45 m, 1.74 m	21.1	1.48 m, 1.72 m
8	40.9	3.33 brd (10.4)	40.9	3.29 brd (10.2)
9	154.0		154.2	
10	39.7		39.7	
11	115.6	5.57 d (4.2)	115.3	5.59 d (4.2)
12	71.3	4.95 dd (5.6,12.0)	70.9	4.92 dd (5.6, 11.4)
13	58.6		58.5	
14	46.4		46.2	
15	36.7	1.79 m, 1.36 m	37.0	1.89 m, 1.40 m
16	36.1	2.28 m, 2.61 m	36.3	2.75 m, 2.87 m
17	89.3		92.1	
18	174.7		173.6	
19	22.6	1.36 s	22.5	1.34 s
20	87.1		87.4	
21	23.0	1.66 s	21.4	1.89 s
22	38.2	1.79 m, 1.85 m	208.5	
23	22.4	0.83 m, 0.89 m	39.0	2.10 m, 2.39 m
24	38.4	1.92 m, 1.99 m	32.4	1.78 m, 1.82 m
25	145.5		27.7	1.50 s
26	110.8	4.66 s	22.4	0.83 s
27	22.2	1.67 s	22.5	0.84 s
30	16.7	1.03 s	16.7	1.01 s
31	28.1	1.23 s	28.1	1.22 s
32	20.1	1.65 s	19.8	1.61 s

[a] Assignments aided by DQFCOSY, TOCSY, HMQC, HMBC and NOESY experiments

determined graphically for each experiment by curve fitting using Prism 4.0 software (GraphPad Software, Inc.) and the equation derived by DeLean et al. [8]. The IC_{50} values for each treatment (**1, 2** and the control) were expressed as mean ± S.D. (n = 3), and the difference in the IC_{50} values between the tested compound (**1** or **2**) and control was examined using Student's t-test. $P < 0.05$ was accepted as a significant difference.

Results and Discussion

The 60% EtOH extract of *H. scabra* was successively chromatographed on DA-101 resin, silica gel, and reversed-phase silica (Lichroprep RP-18, 40–63 µm, Merck). Finally, reversed-phase HPLC on Zobax SB C-18 afforded scabraside A (**1**), scabraside B (**2**) and a structurally known compound **3**.

Scabraside A (**1**) was positive in the Liebermann-Burchard and Molish tests. Its molecular formula was determined as $C_{54}H_{85}O_{26}SNa$ from the pseudomolecular ion peak at *m/z* = 1227.4849 $[M + Na]^+$ in the positive-ion mode HR-ESI-MS and at *m/z* = 1181 $[M - Na]^-$ in the negative-ion mode ESI-MS. A fragment ion peak at *m/z* = 1107 $[M - OSO_3Na + Na - H]^+$ indicated the presence of a sulfate group in **1**, which was confirmed by the IR spectrum with absorption bands at 1255 and 1071 cm^{-1}. An examination of the ^{1}H- and ^{13}C-NMR spectra of **1** indicated the presence of a triterpene aglycone with six methyls, two olefinic bonds and one lactone carbonyl group, which had a close similarity to the aglycone of hemoiedemoside A [3], but **1** differed from hemoiedemoside A at C-17 and C-16. The hydroxy group was located at C-17 on the basis of a cross-peak [$\delta_H = 1.66/\delta_C = 89.3$ (H-21/C-17), $\delta_H = 2.28/\delta_C = 89.3$ (H-16/C-17)] in the HMBC spectrum (**Fig. 2**). The location of the methylene at C-16 was deduced from the chemical shift of the H-16 signal (δ = 2.28), which showed coupling to signals at δ = 1.79 (H-15α), 1.36 (H-15β) in the TOCSY spectrum, and correlation with the methylene signal at δ = 36.1 in the HMBC spectrum. The positions of a double bond at $\Delta^{9(11)}$ and a disubstituted terminal double bond at $\Delta^{25(26)}$ were deduced from the NMR signals at $\delta_C = 154.0$ (C-9), 115.6 (C-11); $\delta_H = 5.57$ (*d, J* = 4.2, H-11) and $\delta_C = 145.5$ (C-25), 110.8 (C-26); $\delta_H = 4.66$ (*s*, H-26) together with the analysis of the TOCSY and HMBC experiments (**Fig. 2**). The HMBC spectrum showed the cross-peaks H-7/C-9, H-19/C-9, H-8/C-11, H-12/C-11, H-23/C-25, H-24/C-25, H-27/C-25 and H-27/C-26, and in the TOCSY spectrum, two protons [δ = 5.57 (H-11) and 4.95 (H-12)] and four protons [δ = 1.36 (H-15), 1.79 (H-15), 2.28 (H-16) and 2.61 (H-16)] comprising a two-spin and a four-spin system, respectively. The ^{1}H-NMR spectrum also showed an olefinic methyl signal [δ = 1.67 (*s*, H-27)] and five methyl groups [δ = 1.23 (*s*, H-31), 1.03 (*s*, H-30), 1.65 (*s*, H-32), 1.36 (*s*, H-19) and 1.66 (*s*, H-21)]. A signal characteristic for an oxygenated methine [$\delta_C = 71.3$ (C-12); $\delta_H = 4.95$ (br. s, H-12)] in the holostane nucleus indicated α-configuration of the allylic OH group at C-12 [9]. Therefore, a 12-hydroxylated $\Delta^{9(11)}$ terpenoid aglycone was identified.

The presence of four β-sugar units in **1** was deduced from the ^{13}C- and ^{1}H-NMR spectra, which showed four anomeric carbon and four anomeric protons (doublets) resonances with *J* values of

Position	1		2	
	δ_C	δ_H	δ_C	δ_H
Xyl				
1	105.2	4.65 d (7.2)	105.1	4.63 d (7.2)
2	83.4	4.05 m	83.2	4.02 m
3	75.3	4.26 m	75.0	4.24 m
4	75.5	5.03 m	75.4	5.01 m
5	64.3	3.69 m	64.2	3.67 m
		4.72 m		4.68 m
Qui				
1	105.4	5.01 brs	105.3	4.48 d (7.8)[b]
2	76.3	3.95 m	76.3	3.94 m
3	75.8	4.03 m	75.8	4.05 m
4	86.8	3.59 m	87.0	3.58 m
5	71.9	3.67 m	71.9	3.66 m
6	18.1	1.69 d (5.4)	18.0	1.69 d (5.4)
Glc				
1	104.9	4.94 (overlapped)	104.8	4.34 d (7.8)[b]
2	73.9	4.01 m	74.0	4.06 m
3	87.9	4.21 m	87.8	4.18 m
4	69.5	3.99 m	69.5	4.06 m
5	77.8	3.91 m	77.5	3.92 m
6	61.8	4.46 m	62.0	4.43 m
		4.27 m		4.22 m
MeGlc				
1	105.8	5.30 d (7.8)	105.6	5.30 d (7.8)
2	75.0	4.02 m	75.0	3.97 m
3	88.0	3.68 m	88.0	3.69 m
4	70.6	4.02 m	70.5	4.00 m
5	78.3	3.94 m	78.2	3.96 m
6	62.1	4.19 m	61.7	4.19 m
		4.26 m		4.40 m
OMe	60.8	3.84 s	60.8	3.83 s

Table 2 ^{1}H- (600 MHz) and ^{13}C-NMR(150 MHz) data (δ value, *J* in Hz) for the sugar moiety of glycosides **1** and **2** in pyridine-d_5.[a]

[a] Assignments aided by DQFCOSY, TOCSY, HMQC, HMBC and NOESY experiments; [b] Measured at 600 MHz in DMSO-d_6

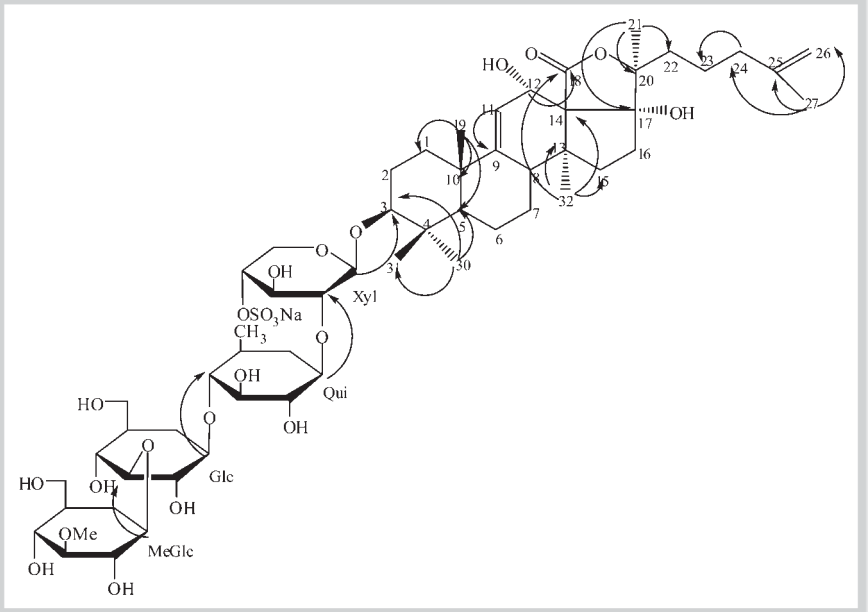

Fig. 2 Key HMBC correlations of compound **1**.

7.2–7.8 Hz. The sugar moiety was confirmed to be D-xylose (Xyl), D-quinovose (Qui), D-glucose (Glc) and 3-*O*-methylglucose (MeGlc) in a ratio of 1 : 1 : 1 : 1 by acidic hydrolysis (aqueous 2 M CF_3COOH) followed by GC/MS analysis of the corresponding aldononitrile peracetates and by comparing the GC retention time of the corresponding aldononitrile peracetates with those of the authentic samples prepared in the same manner [9]. The common D-configurations for the four carbohydrate units were assumed according to those most often encountered among triterpene glycosides from sea cucumbers [1].

Table 3 *In vitro* cytotoxicity (IC_{50}: μM) of glycosides **1** and **2** against four tumor cell lines (mean ± S. D., n = 3).

Cell line	1	2	HCP[a]
A-549	5.62 ± 1.08*	3.40 ± 0.73*	0.84 ± 0.05
HL-60	0.05 ± 0.02*	0.25 ± 0.04*	0.41 ± 0.13
MOLT-4	0.09 ± 0.04	0.08 ± 0.02	0.12 ± 0.01
BEL-7402	2.07 ± 0.44*	2.26 ± 0.56*	0.72 ± 0.20

* $P < 0.05$ vs. Control; [a] HCP, 10-hydroxycamptothecine, a positive control

The ^{1}H- and ^{13}C-NMR signals attributable to the sugar unit were assigned by the 2D NMR experiments and the data indicated that sugar residues were all in pyranose form. The sequence of the sugar residues in **1** was determined by analysis of HMBC correlations: Xyl H-1/C-3 of the aglycone, Qui H-1/Xyl C-2, Glc H-1/Qui C-4 and MeGlc H-1/Glc C-3. The position of the sulfate group was determined by comparing ^{13}C-NMR data of **1** with those of known glycosides [10]. A downfield esterification shift was observed for the signal of Xyl C-4 (from δ 68.2 to 75.5). On the basis of the above data, the structure of **1** was deduced as 3-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-quinovopyranosyl-(1 → 2)-4-*O*-sulfate-*β*-D-xylopyransyl]-holosta-9(11),25-diene-3*β*,12*α*,17*α*-triol sodium salt and named scabraside A.

The molecular formula of **2** was determined as $C_{54}H_{85}O_{27}SNa$ from the pseudomolecular ion peaks at $m/z = 1243.4792$ $[M + Na]^+$ in the positive-ion mode HR-ESI-MS and at $m/z = 1197$ $[M - Na]^-$ in the negative-ion mode ESI-MS. The fragment ion peak at $m/z = 1123$ ($[M - OSO_3Na + Na - H]^+$) indicated the presence of a sulfate group in **2** which was confirmed by the IR (KBr) spectrum with absorption bands at 1266 and 1074 cm^{-1}. The ^{13}C-NMR spectral data of the aglycone moiety were closely similar to those of leucospilotaside A [11], from which **2** differed only by the replacement of a hydroxy group (δ = 27.7, C-25) with the signal of a methine (δ = 69.0) and downfield or upfield shifts of the neighboring carbon signals. The absence of a hydroxy group linked to C-25 in **2** was confirmed on the basis of analysis of 2D NMR spectra, especially the cross-peaks H-26/C-25, H-23/C-25 and H-24/C-25 in the HMBC spectrum. Thus, the aglycone of **2** was determined to be a 22-oxo-9(11)-ene-3*β*,12*α*,17*α*-triol. Comparing the NMR data of **2** and **1** suggested that these two compounds bore the same sugar moiety, same sequence of the sugar residues and same β-D-xylose residue linked to C-3 of aglycone. This conclusion was further confirmed by chemical evidence. That is the sugar moieties of **2** were determined as D-xylose (Xyl), D-quinovose (Qui), D-glucose (Glc) and 3-*O*-methylglucose (MeGlc) in a ratio of 1 : 1 : 1 : 1, as supported by acidic hydrolysis (aquous 2 M CF_3COOH) and chemical derivatization to the corresponding aldononitrile peracetates, which were analyzed using GC/MS. Hence, the structure of **2** was determined as 3-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1 → 3)-*β*-D-glucopyranosyl-(1 → 4)-*β*-D-quinovopyranosyl-(1 → 2)-4-*O*-sulfate-*β*-D-xylopyransyl]-holosta-22-ketone-9(11)-ene-3*β*,12*α*,17*α*-triol sodium salt and named scabraside B.

Cytotoxicities of compounds **1** and **2** against four tumor cell lines (A-549, HL-60, MOLT-4 and BEL-7402) were evaluated using the sulforhodamine B (SRB) protein assay with 10-hydroxycamptothecine (HCP) as reference compound [7]. Compounds **1** and **2** were cytotoxic, with low IC_{50} value of cell viability after drug exposure of 72 h, against all four cell lines tested.

Compounds **1** and **2** had higher activities towards HL-60 (IC_{50} = 0.05 μM for **1** and 0.25 μM for **2**) and MOLT-4 (IC_{50} = 0.09 μM for **1** and 0.08 μM for **2**), but lower activities towards A-549 and BEL-7402, than that of the positive control 10-hydroxycamptothecin (**Table 3**). These results suggest that scabraside A (**1**) and scabraside B (**2**) are potential antitumor agents that deserve further investigation, and evaluation in other cancer models.

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