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New steroidal saponins with cytotoxic activities from *Smilax trinervula*

Fang Liang, Jun-Wei He, Gen-Hua Zhu, Rong-Hua Liu, Ji-Cheng Shu, Feng Shao, Hui-Lian Huang*

Key Laboratory of Modern Preparation of TCM, Jiangxi University of Traditional Chinese Medicine, Ministry of Education, Nanchang 330004, China

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ABSTRACT

Three new steroidal saponins, namely, trinervulosides A–C (**1–3**), were isolated from the rhizomes and roots of *Smilax trinervula*, together with four known compounds, **4–7**. Their structures were determined through chemical evidence, NMR spectroscopy, mass spectrometry and comparison with the literature. Compounds **1–7** were assayed for cytotoxic activities, and only trinervuloside B (**2**) showed activity against SGC-7901 and HCT-116 cell lines.

1. Introduction

Smilax (Smilaceae) comprises approximately 370 species of shrubs which grow in tropical and temperate areas around the world. The rhizomes of *S. china* ("Ba Qia" in Chinese) and *S. glabra* ("Tu Fu Ling" in Chinese) are commonly used as herbal materials in traditional Chinese medicine (TCM). Based on previous phytochemical investigation on *S. riparia*, *S. menispermoidea* and *S. china*, it can be concluded that the genus *Smilax* is rich in steroidal saponins (Sashida et al., 1992; Nikaidoa and Ohmotoa, 1992; Jia and Ju, 1992; Belhouchet et al., 2008; Zhang et al., 2012; Shao et al., 2007). Steroidal saponins from Smilaceae exhibit a range of bioactivities, such as anti-inflammatory, cytotoxicity and anti-tumor effects (Ivanova et al., 2011; Wu et al., 2010).

As part of our continued interest in steroidal saponins in *Smilax* (Smilaceae) plants (Huang et al., 2009; Lin et al., 2012), we have undertaken the chemical investigation of *Smilax trinervula*, an evergreen climbing shrub mainly distributed in the south of China (i.e., Zhejiang, Jiangxi, Hunan and Guizhou provinces). Following our previous work on this plant, which led to the isolation and characterization of phenylpropanoids and neolignans (Shu et al., 2015), the current article reports three new steroidal saponins and four known relatives from the rhizomes of *S. trinervula.*

2. Results and discussion

Seven compounds including three new steroidal saponins were identified from the extracts of the air-dried rhizomes of *S. trinervula*. Compound **1** (Fig. 1) was obtained as a white amorphous powder with a molecular formula of $C_{51}H_{84}O_{23}$, determined from the positive ion at *m/z* 1087.5265 $[M+Na]^+$ in the HR-ESI–MS. The structure of **1** was identified by comparison of its ^{1}H- and ^{13}C NMR (Tables 1 and 2), HSQC and HMBC data with those of congeners from *Solanum* genera plants (Zhu et al., 2001), *Dioscorea collettii* (Hu et al., 1999a, 1999b) and *Dioscorea anthaica* (Dong et al., 2001).

The ^{1}H NMR spectrum of **1** showed signals belonging to six methyl proton groups at δ_H 1.09(s, H-19), 1.22 (s, H-18), 1.45(d, J = 5.8, H-21), 0.95 (d, J = 6.6, H-27), 1.65(d, J = 6.2, H-6‴), 1.77 (d, J = 6.1, H-6″) and a olefinic proton at δ_H 5.29 (*br s*, H-6), as well as protons attributable to an oxymethylene H-26 at δ_H 3.56 (dd, J = 9.5, 6.0) and 3.95 (dd, J = 9.4, 6.5), observed in the ^{1}H NMR spectrum (Table 1). The ^{13}C NMR spectra of **1** showed 51 signals, 24 of which were assigned to the saccharide protons and 27 to the aglycone moiety. Among them, the aglycone moiety had three angular methyl groups at δ_C 13.4 (C-18), 19.5 (C-19), 24.2 (C-21), one secondary methyl group at d_C 17 (C-27), two olefinic carbons at d_C 141 (C-5), 121.8 (C-6), two hydroxyl carbon signals at d_C 75.2 (C-16), 62.3 (C-20) and a carbonyl carbon signal at d_C 173.2 (C-22). We determined that the aglycone possessed a pregnane-5-en skeleton through analysis of the ^{1}H- and ^{13}C NMR spectra. In addition, HMBC experiments revealed correlations between H-21/C-17, H-21/C-20; H-27/C-24, H-27/C-25, H-27/C-26, H-23/C-22, H-24/C-22, H-25/C-24, H-25/C-26 (Fig. 2). By comparison of the ^{13}C NMR spectroscopic signals of the aglycone moiety of **1** with values reported in the literature (Zhu et al., 2001; Hu et al., 1999a, 1999b; Dong et al.,

* Corresponding author.
E-mail address: huilianh@163.com (H.-L. Huang).

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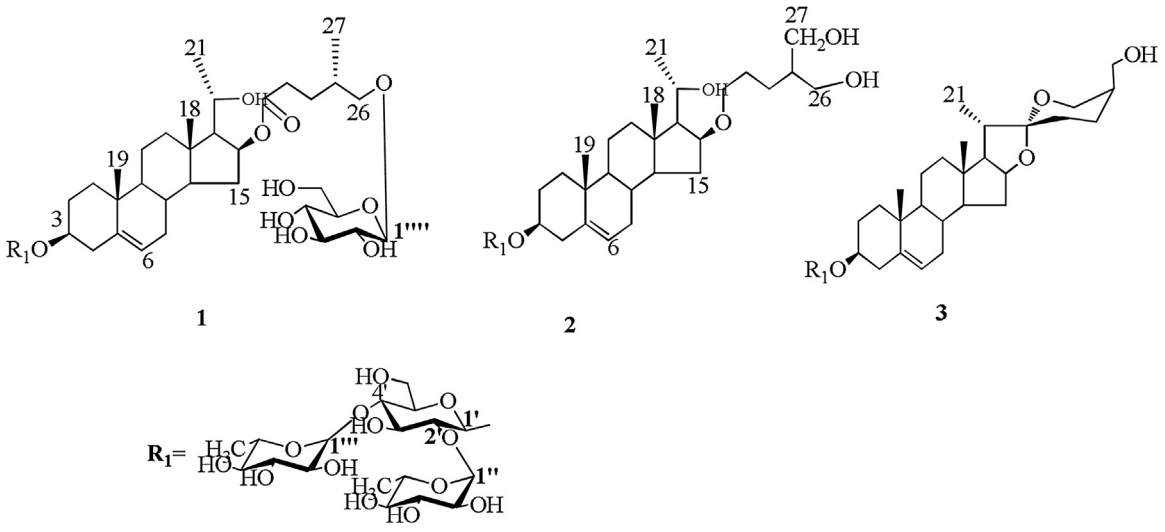

Fig. 1. Structures of compounds **1–3**.

2001), and extensive HMQC and HMBC data analyses, the aglycone of **1** was shown to be 3β, 20, 26-trihydroxy-20,22-seco-25(R)-furosta-5-en-22-one.

The assignments of the sugar moieties were similarly interpreted. The ^{1}H- and ^{13}C NMR spectra of **1** exhibited four sugar anomeric protons at d_H 4.97 (d, J = 7.5, H-1′), 6.44 (s, H-1″), 5.89 (s, H-1‴), 4.86 (d, J = 7.8, H-1⁗) (Table 2), and anomeric carbon atoms at d_C 100.3 (C-1′), 102.1 (C-1″), 102.9 (C-1‴) and 105 (C-1⁗), respectively. Acid hydrolysis of **1** yielded D-glucose and L-rhamnose, as revealed by HPLC analysis and comparison with authentic standards. HSQC spectrum analysis showed signals for four sugar anomeric protons, which were correlated with four anomeric carbon signals, respectively. The connectivity of each sugar unit from C-1 to C-6 was determined using HSQC and HMBC. HMBC experiments revealed the following correlations: H-1′/C-3; H-1″/C-2′; H-1‴/C-4′, and H-1⁗/C-26 (Fig. 2). These analyses revealed the presence of 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D- glucopyranosyl and 26-*O*-β-D-glucopyranosyl groups in compound **1** (Fig. 2). Based on these data, it was established that the structure of compound **1** was 26-*O*-β-D-glucopyranosyl-3β,20,26-trihydroxy-20,22-seco-25(*R*)-furosta-5-en-22-one 3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]- β-D-glucopyranoside, and given the rivial name trinervuloside A.

Compound **2** was isolated as a white amorphous powder and had the molecular formula of $C_{45}H_{76}O_{18}$ as determined by ^{13}C NMR and HR-ESI–MS. The HR-ESI–MS showed an $[M+Na]^+$ peak at m/z 927.6610, corresponding to $C_{45}H_{76}O_{18}Na$. The ^{1}H NMR spectrum of **2** showed the presence of five methyl groups at d_H 1.08 (s, H-18), 1.08 (s, H-19), 1.52 (d, J = 6.2, H-21), 1.79 (d, J = 6.1, H-6″) and 1.65 (d,

Table 1
^{1}H and ^{13}C NMR data of the aglycone moieties of **1–3** in pyridine.

Aglycone moiety position	**1**		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	37.1	1.00 m,1.71 m	37.5	0.98 m,1.75 m	37.5	0.99 m, 1.72 m
2	30.2	1.84 m,2.08 m	30.2	1.87 m,2.07 m	30.2	1.89 m, 2.08 m
3	78.1	3.89 m	78.1	3.89 m	78.1	3.90 m
4	39	2.8 m,2.77 m	39	2.76 m,2.8 m	39.0	2.74 (t, 11.3), 2.82 (dd, 13.2, 5.2)
5	141	–	140.8	–	140.8	–
6	121.8	5.29 br.s	121.9	5.33 br.s	121.8	5.32 (br.d 4.9)
7	32.1	1.90 m,2.02 m	32.3	1.90 m,2.03 m	32.3	1.86 m, 2.03 m
8	31.6	1.55 m	31.4	1.54 m	31.7	1.56 m
9	50.6	0.98 m	50.5	0.90 m	50.3	0.89 m
10	37.5	–	37.1	–	37.1	–
11	21	1.53 m	20.9	1.45 m	21.1	1.41 m
12	40.2	1.38 m,2.70(m	39.5	1.05 m,1.84 m	39.9	1.09 m, 1.67 m
13	43	–	41.3	–	40.5	–
14	54.3	0.91 m	54.5	0.89 m	56.6	1.03 m
15	36	1.26 m,2.46 m	36.5	1.57 m,2.29 m	32.2	1.39 m, 1.46 m
16	75.2	4.07 m	75.3	4.04 m	81.2	4.52 m
17	62.3	1.63 m	63.8	1.46 m	62.8	1.79 (br.d, 6.2)
18	13.4	1.22 s	14.2	1.08 s	16.4	0.83 s
19	19.5	1.09 s	19.4	1.08 s	19.4	1.06 s
20	65.2	4.47 m	66	4.5 m	42.5	1.90 m
21	24.2	1.45(d,5.8)	24.1	1.52(d, J = 6.2)	15.0	1.13 (d, 6.9)
22	173.2	–	71.7	4.27 m	109.9	–
23	32.5	2.48 m	31.1		27.3	1.51 m, 1.91 m
24	29.3	1.60 m,2.01 m	29.9	1.27 m,2.1 m	21.7	1.90m
25	33.5	1.93 m	33.4		36.3	1.90 m
26	74.8	3.56(dd, 9.5,6.0), 3.95(dd, 9.4,6.5)	64.9	4.25 m	60.7	4.02 (br.d,11.3), 4.15 (dd, 11.3, 2.6)
27	17	0.95(d, J = 6.6)	62.9	4.42 m,4.58 m	61.3	4.24 m

Table 2
^{1}H and ^{13}C NMR data of the sugar portion of 1–3 in pyridine.

Sugar moiety	**1**		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1′	100.3	4.97(d, 7.5)	100.3	4.98(d, *J* = 7.5)	100.3	4.99 (overlapped)
2′	78.6	4.43 m	78	4.23 m	78.0	4.25 m
3′	77	3.64 m	77	3.65 m	77.0	3.65 m
4′	78.6	3.97 m	78.6	3.97 m	78.5	4.41 m
5′	78	4.22 m	77.8	4.23 m	77.8	4.25 m
6′	61.3	4.12 m,4.24 m	61.3	4.10 m,4.22 m	61.5	3.97 m, 4.19 m
1″	102.1	6.44 s	102.1	6.45 s	102.1	6.49 br.s
2″	72.6	4.71 m	72.6	4.71 m	72.6	4.66 m
3″	72.8	4.56 m	72.9	4.57 m	72.8	4.56 m
4″	74.1	4.36 m	74	4.37 m	74.2	4.37 m
5″	69.6	4.99 m	69.6	4.99 m	69.6	4.95 m
6″	18.7	1.77(d, 6.1)	18.7	1.79(d,6.1)	18.7	1.78 (d, 6.2)
1‴	102.9	5.89 s	102.9	5.9 s	102.9	5.89 br.s
2‴	72.6	4.86 m	72.8	4.87 m	72.6	4.86 m
3‴	72.9	4.67 m	73	4.65 m	72.9	4.71 m
4‴	74	4.39 m	74.2	4.39 m	74.0	4.42 m
5‴	70.4	4.95 m	70.5	4.97 m	70.4	4.95 m
6‴	18.5	1.65(d, 6.2)	18.5	1.65(d,6.2)	18.5	1.65 (d, 6.2)
1⁗	105	4.86(d, 7.8)				
2⁗	75.3	5.37 m				
3⁗	78.6	4.27 m				
4⁗	71.7	4.25 m				
5⁗	77.8	4.22 m				
6⁗	62.9	4.41 m,4.58 m				

J = 6.2, H-6‴), and an olefinic proton at 5.33 (*br s*, H-6). The DEPT and ^{13}C NMR spectra showed 45 carbon signals, of which 18 were assigned to the saccharide protons and 27 to the aglycone moiety. ^{1}H- and ^{13}C NMR data indicated that compound **2** had a similar aglycone component as was found in compound **1**, but differed in the d_C 71.7 (C-22), 64.9 (C-26) and 62.9(C-27). With the help of HSQC and HMBC spectra and the comparison of congeners from *Solanum* genus plants (Zhu et al., 2001), the skeleton of **2** was determined to be 26,27-ol-pentanosyl-pregn-5-en-3β,16β,20-trihydroxy, supported by the following HMBC correlations: H-16/C-22, H-21/C-17, H-21/C-20, H-22/C-16, H-22/C-24, H-27/C-24, H-27/C-26 (Fig. 3).

Acid hydrolysis experiments and HPLC analysis revealed that compound **2** has L-rhamnose and D-glucose sugars, which were confirmed by the chemical shifts of two methyl groups at d_C 18.7 (C-6″) and 18.5 (C-6‴). Moreover, the ^{1}H NMR spectrum showed three sugar anomeric protons at d_H 4.98 (d, H-1′), 6.45 (s, H-1″), and 5.9 (s, H-1‴) and two methyl groups at 1.79 (d, H-6″), and 1.65 (d, H-6‴) (Table 2). A combination of COSY and HMBC spectra confirmed connectivity between C-1 through C-6 sugar units. HMBC revealed correlations between H-1′/C-3; H-1″/C-2′; H-1‴/C-4′ (Fig. 3). Therefore, connectivity of the saccharide moieties were determined to be 3-*O*-α-L-rhamnopyranosyl-(1 → 2)- [α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranosyl. Combined, these data determined that the structure of **2** is 26,27-ol-pentanosyl-pregn-5-en-3β,16β,20-trihydroxy 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, and was named trinervuloside B.

Compound **3** was obtained as a white amorphous powder. The molecular formula established by HR-ESI–MS was identified as $C_{45}H_{72}O_{17}$. The ^{1}H NMR spectrum of **3** showed two tertiary methyl groups observed at d_H 0.83 (s, H-18) and 1.06 (s, H-19), while three secondary methyl groups resonated as a doublet at d_H 1.13 (d, *J* = 6.9, H-21), 1.65 (d, *J* = 6.2, H-6‴), and 1.78 (d, *J* = 6.2, H-6″). It also displayed the signals of one trisubstituted olefinic proton at d_H 5.32 (*br.d*, *J* = 4.9, H-6)), two methylene protons at d_H 2.74 (t, *J* = 11.3, H-4) and 2.82 (dd, *J* = 13.2, 5.2, H-4), as well as two oxymethylene proton at d_H 4.02 (*br.d*, *J* = 11.3, H-26) and 4.15 (dd, *J* = 11.3, 2.6, H-26). The ^{13}C NMR and DEPT^{135} spectra revealed 45 carbon signals, attributable to five methyls, 12 methylenes, 24 methines, and four quaternary carbons. Three signals at d_C 15.0 (C-21), 16.4 (C-18), and 19.4 (C-19) were assigned to angular methyl groups. Two signals at d_C 121.8 (C-6) and 140.8 (C-5) were assigned to olefinic carbons. With the aid of HSQC and HMBC correlations the planar structure was established and all of the ^{1}H and ^{13}C NMR signals of **3** were assigned (Table 1). The ^{1}H and ^{13}C NMR signals were very

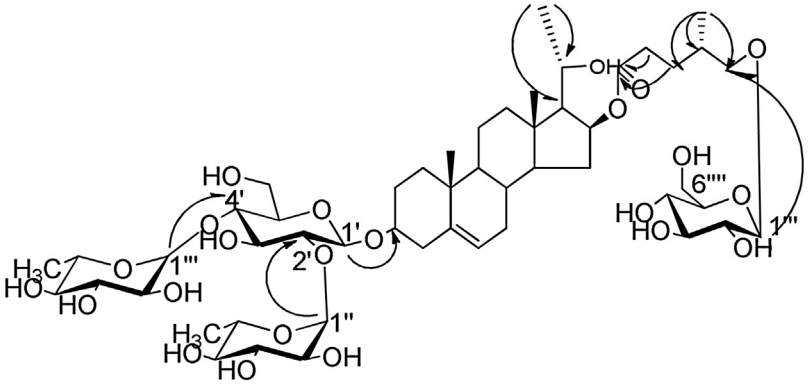

Fig. 2. Key HMBC correlations (H → C) of compound **1**.

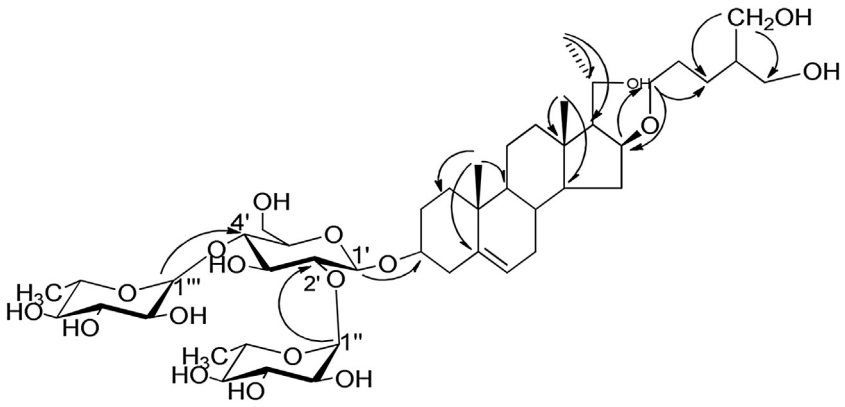

Fig. 3. Key HMBC correlations (H → C) of compound **2**.

similar to those of the known compound isonarthogenin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside (Sashida et al., 1992) except for the signals from the F ring [C-23 (ΔC −4.3), C-24 (ΔC −2.4), C-25 (ΔC −3.0), C-26 (ΔC −3.4), and C-27 (ΔC −3.2)]. The downfield chemical shifts of C-23 to C-27 were ascribed to the γ-gauche effect, suggesting that the C-27 substituent was β-orientated in **3**. Thus, the structure of **3** was established as narthogenin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, and named trinervuloside C.

Additionally, four known compounds were isolated. Based on the NMR spectroscopic data and comparison with the literature or authentic samples, their structures were determined to be 3-*O*-[bis-α-L-rhamnopyranosyl-(1 → 2 and 1 → 4)-β-D-glucopyranosyl]-26-*O*-β-D-glucopyranosyl-20(*R*)–methoxy-25R- furosta-5,22 (23)-diene-3β,26-dio, dioscoreside E (**4**) (Dong et al., 2004), (25S) 26-*O*-β-D-glucopyranosyl-3β,20α,26-trihydroxyfurostan-5,22-diene 3-*O*-α-L- rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-*O*-β-D-glucopyranoside, smilaxchinoside A (**5**) (Shao et al., 2007), 26-*O*-β-D-glucopyranosyl-25*R*- furost-5,20 (22)-dien-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, pseudoprotodioscin (**6**) (Ju and Jia, 1992), 3-O-β-chacotriosyl kryptogenin 26-*O*-β-D-glucopyranoside, Anguiviosides XV (**7**) (Honbu et al., 2002).

The cytotoxicities of compounds **1–7** were tested against SH-SY5Y, SGC-7901, HCT-116 and Lovo cell lines. The results showed that only trinervuloside B (**2**) had activity against SGC-7901 and HCT-116 (IC_{50} value of 8.1 μM and 5.5 μM, respectively). The other compounds were inactive (IC_{50} > 100 μM, respectively).

3. Experimental

3.1. General

NMR Spectra: a Bruker AM-400 spectrometer (400-MHz) in Pyridine-d_5 at room temperature (25 °C); Electrospray ionization (ESI) mass spectra were acquired in the positive ion mode on a LCQ DECAXP instrument (Thermo Finnigan, San Jose, CA, USA) equipped with an ion trap mass analyzer. HR-ESI–MS were obtained in the positive ion mode using a Waters UPLC Premier Q-TOF system. For further purification of samples, preparative HPLC was run with a pump and a detector using a Prevail C_{18} column (5 μm, 10.0 mm I.D × 250 mm) at rate of 3.0 mL/min and a detection wavelength of 210 nm. HPLC analysis was carried out on an Agilent 1200 system equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. The column was a Thermo C_{18} (5 μm, 4.6 mm I.D × 250 mm). Column chromatography: MCI GEL (Mitsubishi chemical corporation, Japan). Sephadex LH-20 (Amersham Bio-sciences AB, Uppsala, Sweden); Silica gel (200–300 mesh); $HSGF_{254}$ for TLC were produced by Qingdao Ocean Chemical Group Co. of China and C_{18} SPE by Bulk Sorbent (Grace Davison Discovery Sciences).

3.2. Plant material

The rhizomes of *S. trinervula* (Smilacaceae) were collected from Yichun city, Jiangxi province, China, in May 2011. The plant was identified by Chengxin Fu, a professor at the Laboratory of Systematic and Evolutionary Botany and Biodiversity, College of Life Sciences, Zhejiang University. A voucher specimen (No.20110502) was deposited at the Key Laboratory of Modern Preparation of TCM, Jiangxi University of TCM, China.

3.3. Extraction and isolation

Air-dried and powdered rhizomes and roots (30 kg) were extracted three times with 70% EtOH and concentrated in vacuo to give crude extract, which was then suspended in H_2O and partitioned successively with EtOAc and *n*-BuOH. The *n*-BuOH soluble portion (470.3 g) was first chromatographed on a HP-20 macroporous adsorption resin eluted with 30%, 50%, 70% and 95% EtOH, successively, to obtain four fractions (Fr. A, B, C and D). Fr. A (64.6 g) was subjected to a MCI column afterwards using a stepwise gradient of MeOH:H_2O (followed by 20%, 40%, 60%, 80%, 100% elution) to obtain five subfractions. Fr.A.3 (6 g) was subjected to silica gel column using a stepwise gradient of CHCl3: MeOH (from 5:1 to 0:1), purified by Sephadex LH-20 column, and eluted with MeOH to give two parts Fr.A.3-1 and Fr.A.3-2. Fr.A.3-1 (127.8 mg) was further purified by preparative HPLC with CH_3CN: H2O (22%) to get compound **1** (18.8 mg), **2** (25.4 mg), **5** (4 mg), and **7** (9.5 mg). Likewise, Fr.A.3-2 (69.2 mg) was further purified by preparative HPLC with CH_3CN:H2O (30%) to obtain **3** (6.4 mg), **4** (12.7 mg), **6** (21.8 mg).

3.3.1. Trinervuloside A (*1*)

White amorphous powder, $[\alpha]_D^{25}$-15.3 (*c* 0.105, MeOH), IR (KBr) V_{max} cm^{-1}: 3400, 1710, 1045; UV(MeOH) λ_{max} (logε): 245(2.1), 203 (3.08); for NMR data see Tables 1 and 2; HR-ESI–MS: 1087.5265 $[M+Na]^+$(calcd. For $C_{51}H_{84}O_{23}$, 1087.5301)

3.3.2. Trinervuloside B (*2*)

White amorphous powder, $[\alpha]_D^{25}$-9.8 (0.1, MeOH), IR (KBr) V_{max} cm^{-1}: 3405, 1720, 1045; UV(MeOH) λ_{max} (logε): 265(0.12), 203

(1.8); for NMR data see Tables 1 and 2; HR-ESI–MS: 927.6610 $[M+Na]^+$(calcd. For $C_{45}H_{76}O_{18}$, 927.4929).

3.3.3. Trinervuloside C (**3**)

White amorphous powder, $[\alpha]_D^{25}$-23.6 (0.1, MeOH), IR (KBr) V_{max} cm^{-1}: 3402, 1710, 1040; UV(MeOH) λ_{max} (logε): 203(4.09); NMR data see Tables 1 and 2; HR-ESI–MS: 885.4808 $[M+H]^+$(calcd. For $C_{45}H_{72}O_{17}$, 885.4803).

3.4. Acid hydrolysis of **1-3**

Compounds **1–3** (1.5 mg, each) were hydrolyzed with 2 M CF_3COOH (5 mL), and the hydrolyzed products were treated and detected through HPLC following the methods of Lin et al. (2012) and Guo et al. (2004).

3.5. Cytotoxic activities

The cytotoxicity of the compounds against four human tumor cell lines (SH-SY5Y, SGC-7901, HCT-116, Lovo) with Vero as a positive control was evaluated using the MTT method. Cells were seeded in 96-well microplates at a density of 150 per well and were cultured in cell culture medium (RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 g/ml streptomycin) for 12 h, then treated with the test compounds added in DMSO dissolved stock solution. The final DMSO concentration never exceeded 0.2% (v/v). After 48 h of cultivation, cells were incubated with MTT (0.5 mg/mL, 4 h) and subsequently resolved in DMSO. The absorbance in the control and drug-treated wells was measured by an automated microplate reader at 570/630 nm. All experiments were carried out in triplicate and repeated twice. The cytotoxicity was expressed as IC_{50} values (50% inhibitory concentration).

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