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Chemical constituents of *Swertia yunnanensis* and their anti-hepatitis B virus activity

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ABSTRACT

Four new triterpenoids, sweriyunnangenin A (**1**), sweriyunnanosides A (**2**), B (**3**) and C (**4**), along with nineteen known compounds (**5**–**23**) were isolated from *Swertia yunnanensis*. Based on extensive spectroscopic analyses (1D- and 2D-NMR, HRESIMS, UV, IR, $[\alpha]_D$), the structures of sweriyunnangenin A (**1**), sweriyunnanosides A (**2**), B (**3**) and C (**4**) were elucidated as taraxer-14-ene-3α,6β-diol, oleanolic acid 28-O-β-D-glucopyranosyl-(1 → 2)-O-β-D-glucopyranoside, 2α,3β-di-hydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl (1 → 6)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside and hederagenin 28-O-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside, respectively. Twenty-two compounds were evaluated for their anti-HBV activities on the HepG 2.2.15 cell line *in vitro*, of which nine compounds showed potent anti-HBV activities. Compounds **1**, **5**–**6**, **14**–**16** and **19** showed activities against the secretion of HBsAg (IC_{50} values from 0.10 to 1.76 mM) and HBeAg (IC_{50} values from 0.04 to 1.41 mM), and compounds **11** and **13**–**16** exhibited significant inhibition on HBV DNA replication (IC_{50} values from 0.01 to 0.09 mM).

1. Introduction

The genus *Swertia* (Gentianaceae) contains about 170 species widely distributed in the world, 79 of which are found in China, and more than 30 species have been used as medicinal herbs for treating hepatitis, cholecystitis, pneumonia, osteomyelitis, dysentery and scabies in China [1–4]. The main constituents of this genus are iridoids, secoiridoids, xanthones, flavonoids, and triterpenoids, which possess hepatoprotective, antihepatotoxic, antioxidant, mutagenic, antidiabetic, anti-ulcer and antigastritic activities [4–7]. *Swertia mileensis* (Qing-ye-dan) is a famous traditional Chinese medicine (TCM) documented in Chinese Pharmacopeia for the treatment of hepatitis [8]. Our previous investigation on *S. mileensis* resulted in a series of novel skeletons with anti-hepatitis B virus (HBV) activity [9]. *Swertia yunnanensis*, the congener plant of *S. mileensis*, is also used for treating jaundice, hepatitis, and cholecystitis in Yunnan, Sichuan, and Guizhou provinces of China [2–4]. Previous phytochemical studies on *S. yunnanensis* resulted in eleven compounds, including xanthones, triterpenoids, secoiridoids, and steroids [10,11]. Our *in vitro* anti-HBV bioassay manifested that the ethanol extracts of *S. yunnanensis* showed significant inhibition on the secretion of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) with the IC_{50} values of 0.79 mg/mL (SI 2.41) and 0.34 mg/mL (SI 5.36), respectively, and on HBV DNA replication with the IC_{50} value of 0.08 mg/mL (SI 18.9). However, the active substances responsible for the anti-HBV property were unclear. In order to clarify its active constituents, extensive investigation on the ethanol extract of *S. yunnanensis* yielded four new triterpenoids (**1**–**4**) (Fig. 1),

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along with nineteen known compounds, including triterpenoids, xanthones, flavonoids, secoiridoids, steroids, nucleotide, and phenol. Herein, we described the isolation and structure elucidation of the isolates, as well as their anti-HBV activity.

2. Experimental procedure

2.1. General

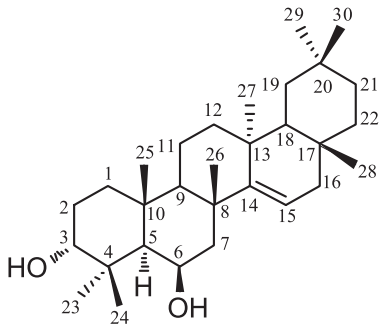
Optical rotations were obtained on a JASCO Model 1020 Digital Polarimeter (HORIBA, Tokyo, Japan) at room temperature. UV spectra were recorded using a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets (Bio-Rad, Hercules, California, USA). 1D (^{1}H, ^{13}C) and 2D NMR experiments (^{1}H–^{1}H COSY, HSQC, HMBC and ROESY) were recorded on Bruker AM-400, DRX-500 or AVANCE III-600 spectrometers (Bruker, Bremerhaven, Germany). The chemical shifts were given in δ (ppm) scale and referenced to the solvent signal. Mass spectra were acquired on a LCMS-IT-TOF spectrometer (Shimadzu, Kyoto, Japan). Column chromatography was performed on silica gel (200–300 mesh; Qingdao Makall Chemical Company, Qingdao, PR China). Semi-preparative HPLC was carried out by Waters Alliance 2695 liquid chromatography with a ZORBAX SB-C_{18} (5 μm, 9.4 × 250 mm) column (Agilent, USA) at a flow rate of 3.0 mL/min. Sephadex LH-20 (20–150 μm) for chromatography was purchased from Pharmacia Fine Chemical Co. Ltd. (Pharmacia, Uppsala, Sweden), and Rp-18 (40–63 μm) from Fuji Silysia Chemical Ltd. (Fuji, Japan). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

2.2. Plant material

The whole plants of *S. yunnanensis* were collected in Lijiang, Yunnan province, PR China, in September 2008 and identified by Prof. Dr. Li-Gong Lei, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 20081118) was deposited at the Laboratory of Antivirus and Natural Medicinal Chemistry, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried whole plants of *S. yunnanensis* (5.2 kg) were powdered and extracted with 90% EtOH (50 L) at room temperature for 3 times. The combined EtOH extract was concentrated under reduced pressure to give a dark-brown residue, which was suspended in H_2O (5 L) and partitioned with petroleum ether (PE, 5 L × 3), EtOAc (5 L × 4) and *n*-butanol (4 L × 3), successively. The EtOAc part (217 g) was subjected to silica gel column chromatography (CC, 18 × 28 cm, 2500 g), eluting with a gradient of increasing MeOH in $CHCl_3$, to give 5 fractions (Frs. A1–A5). Fr. A1 was further divided into 3 subfractions (Frs. A1-1–A1-3), by silica gel CC (9 × 30 cm, 750 g) using PE–EtOAc (90:10, 80:20, 70:30, *v*/*v*) as the eluent. Fr. A1-2 was chromatographed on silica gel CC (2 × 36 cm, 36 g, PE–Me_2CO, 92:8) to yield compounds **1** (64 mg), **5** (156 mg) and **20** (342 mg). Fr. A1-3 was subjected to silica gel CC (2 × 30 cm, 30 g) eluted with PE–EtOAc (75:25) and further purified over a Sephadex LH-20 CC (1.3 × 135 cm, 53 g) with an isocratic solvent system of $CHCl_3$–MeOH (50:50) to yield compounds **10** (17 mg), **11** (32 mg) and **18** (7 mg). Compounds **6** (31 g) and **19** (587 mg) were obtained from Fr. A2 by silica gel CC (9 × 30 cm, 750 g) using $CHCl_3$–Me_2CO (95:5, 90:10) as the eluent and recrystallized from $CHCl_3$–MeOH (50:50). Fr. A3 and Fr. A4 were further separated by CC over Rp-18 (2.54 × 50 cm, 125 g), eluting with a gradient of increasing MeOH in H_2O (25:75–100:0) and further purified using preparative HPLC (SB-C_{18}, 5 μm, 9.4 × 250 mm, Agilent) to afford compounds **12** (27 mg), **13** (9 mg), **14** (16 mg), **15** (14 mg) and **16** (23 mg) eluted with MeOH–H_2O. Fr. A5 was subjected to silica gel CC (4 × 135 cm, 150 g) eluted with



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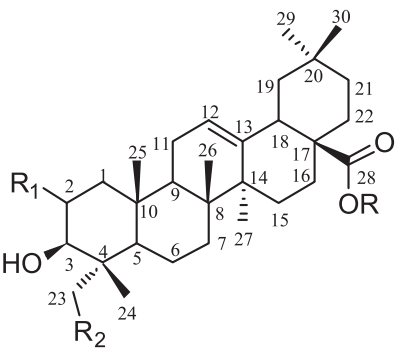

	R_1	R_2	R_3
2	—H	H	glc$\overset{2}{-}$glc
3	····OH	H	glc$\overset{2}{-}$glc$\overset{6}{-}$glc$\overset{6}{-}$glc
4	—H	OH	glc$\overset{2}{-}$glc$\overset{6}{-}$glc$\overset{6}{-}$glc

Fig. 1. Chemical structure of compounds **1**–**4**.

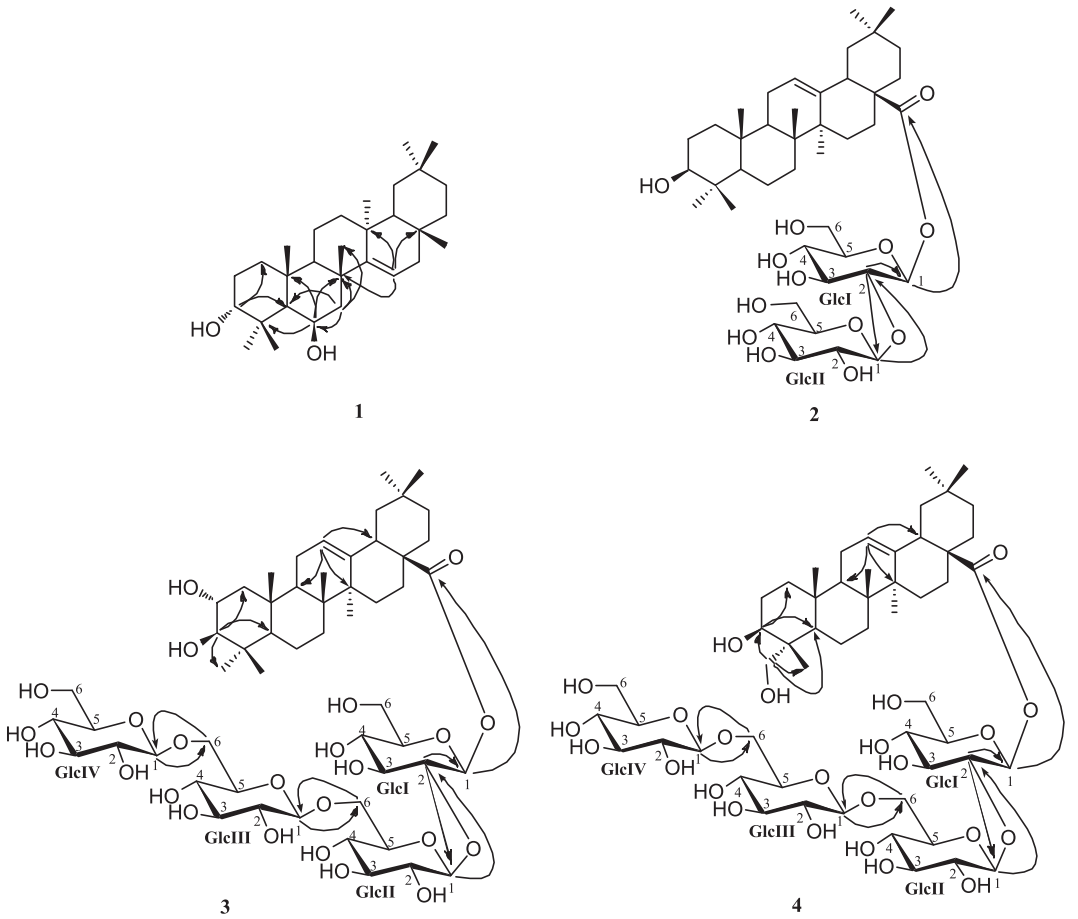

Fig. 2. Key HMBC (H → C) correlations of compound **1**–**4**.

$CHCl_3$–MeOH–H_2O (87:13:1), followed by Sephadex LH-20 column (1.3 × 135 cm, 53 g) eluted with $CHCl_3$–MeOH (30:70) to give compounds **2** (38 mg) and **7** (78 mg). The *n*-butanol part (107 g) of residue was chromatographed on a silica gel column (9 × 40 cm, 1000 g), successively eluted with $CHCl_3$–MeOH–H_2O (95:5:0, 90:10:1, 80:20:2, 60:40:4), to yield four sub-fractions (Frs. B1–B4). Fr. B1 was subjected to silica gel CC (9 × 40 cm, 1000 g) eluting with PE–Me_2CO (93:7) and recrystallized from MeOH (70:30) to give compound **17** (73 g). Fr. B2 was chromatographed on silica gel CC (2 × 35 cm, 36 g) using $CHCl_3$–MeOH–H_2O (85:15:1.5) as the eluent and further purified by a Sephadex LH-20 column (1.3 × 135 cm, 53 g) eluted with MeOH, to afford compounds **8** (183 mg) and **23** (247 mg). Fr. B3 was repeatedly subjected to silica gel CC (6 × 40 cm, 400 g) eluting with $CHCl_3$–MeOH–H_2O (80:20:2) to give compounds **9** (10.3 g) and **21** (786 mg). Fr. B4 was chromatographed on silica gel CC (2.5 × 36 cm, 50 g) eluted with $CHCl_3$–MeOH–H_2O (70:30:3) and further purified using preparative HPLC (MeOH–H_2O, 70:30) to afford compounds **3** (18 mg) and **4** (14 mg).

Sweriyunnangenin A (**1**): colorless needle crystals; mp 314–315 °C; [α]24 D: −51.6 (*c* 0.18, C_5H_5N); UV (MeOH): λ_{max} (log ε) = 205 (3.7) nm; IR (KBr): ν_{max} = 3441, 2950, 2920, 1638, 1462, 1378, 1220, 1043 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; positive HREIMS: *m/z* =442.3802 $[M]^+$ (calcd for $C_{30}H_{50}O_2$, 442.3811).

Sweriyunnanoside A (**2**): white amorphous power; [α]24 D: +4.0 (*c* 0.17, DMSO); UV (MeOH): λ_{max} (log ε) = 205 (3.7) nm; IR (KBr): ν_{max} = 3396, 2928, 1728, 1632, 1462, 1367, 1261, 1085, 1046 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; negative EIMS: *m/z* = 779 $[M-H]^-$, 455 $[M-H-162-162]^-$, HRESIMS 779.4471 $[M-H]^-$ (calcd for $C_{42}H_{68}O_{13}$, 779.4587).

Sweriyunnanoside B (**3**): white amorphous power; [α] 17 D: −15.1 (*c* 0.10, MeOH); UV (MeOH): λ_{max} (log ε) = 202 (3.8) nm; IR (KBr): ν_{max} = 3426, 2926, 1731, 1629, 1460, 1385, 1366, 1261, 1163, 1077, 1033, 587 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; negative HRESIMS 1119.5465 $[M-H]^-$ (calcd for $C_{42}H_{68}O_{13}$, 1119.5593).

Sweriyunnanoside C (**4**),: white amorphous power; [α] 17 D: −9.8 (*c* 0.10, MeOH); UV (MeOH): λ_{max} (log ε) = 202 (3.9) nm; IR (KBr): ν_{max} = 3419, 2929, 1739, 1633, 1462, 1388, 1365, 1262, 1162, 1077, 1034, 616 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR data, see Tables 1 and 2; negative

Table 1
^{1}H-NMR data of compounds **1**–**4** (δ_H values, **1** in C_5D_5N and **2**–**4** in CD_3OD) [a,b].

Position	1	2	3	4
1	1.82[c], 1.42[c]	1.62[c]; 0.97[c]	1.71[c]; 1.14[c]	1.62[c]; 0.98[c]
2	2.24 (t, J = 14.3), 1.81[c]	1.58[c]	3.62[c]	1.65[c]; 1.59[c]
3	3.67 (br s)	3.14 (t, J = 9.4)	2.90 (d, J = 9.5)	3.60 (dd, J = 11.7, 4.5)
5	1.84[c]	0.74[c]	0.84[c]	1.63[c]
6	4.77 (br s)	1.54[c]; 1.44[c]	1.59[c]	1.47 (t, J = 11.2); 1.42[c]
7	2.58 (d, J = 13.9)	1.66[c]	1.68[c]	1.67[c]
9	1.91[c]	1.57[c]	1.63[c]	1.63[c]
11	1.82[c], 1.84[c]	1.89[c]	2.00[c]; 1.90[c]	2.00 (td, J = 13.5, 3.6)
12	1.64[c]	5.24 (br s)	5.25 (t, J = 3.4)	5.23 (t, J = 3.5)
15	5.61 (br s)	1.76[c]; 1.02[c]	1.73[c]; 1.09 (br d, J = 14.2)	1.73[c]; 1.09 (d, J = 14.2)
16	1.98 (d, J = 13.9), 1.63[c]	1.88[c]	1.94[c]	1.90[c]
18	1.02[c]	2.83 (br d, J = 10.7)	2.83 (dd, J = 13.8, 4.0)	2.82 (dd, J = 13.8, 4.0)
19	1.32[c]	1.69[c]; 1.13[c]	1.93[c]; 0.88[c]	1.71[c]; 1.72 (d, J = 10.7)
21	1.66[c]	1.40[c]; 1.20[c]	1.42[c]; 1.23[c]	1.42[c]; 1.23 (t, J = 11.7)
22	1.82[c], 1.43[c]	1.45[c]; 1.35[c]	1.49[c]; 1.39[c]	1.57[c]; 1.35 (t, J = 6.0)
23	1.40 (s)	0.96 (s)	1.01 (s)	3.51[c]; 3.29[c]
24	1.62 (s)	0.77 (s)	0.81 (s)	0.69 (s)
25	1.69 (s)	0.94 (s)	1.01 (s)	0.98 (s)
26	1.73 (s)	0.79 (s)	0.79 (s)	0.79 (s)
27	0.93 (s)	1.17 (s)	1.16 (s)	1.16 (s)
28	0.88 (s)	0.91 (s)	0.92 (s)	0.92 (s)
29	0.99 (s)	0.93 (s)	0.94 (s)	0.93 (s)
30	0.97 (s)			
GlcI-1		5.45 (d, J = 8.0)	5.42 (d, J = 8.2)	5.43 (d, J = 8.2)
GlcI-2		3.87[c]	3.89[c]	3.88[c]
GlcI-3		3.36[c]	3.66[c]	3.65[c]
GlcI-4		3.38[c]	3.51[c]	3.50[c]
GlcI-5		3.65[c]	3.51[c]	3.50[c]
GlcI-6		3.80 (dd, J = 14.9, 6.0) 3.67[c]	3.87 (m); 3.68 (m)	3.87[c]; 3.67[c]
GlcII-1		4.80 (d, J = 7.7)	4.81 (d, J = 7.8)	4.80 (d, J = 7.8)
GlcII-2		3.21 (t, J = 8.4)	3.24[c]	3.24[c]
GlcII-3		3.29[c]	3.51[c]	3.45[c]
GlcII-4		3.13[c]	3.29[c]	3.27[c]
GlcII-5		3.36[c]	3.36[c]	3.35[c]
GlcII-6		3.90 (d, J = 11.4); 3.62[c]	4.12 (d, J = 11.3); 3.79, m	4.11 (d, J = 11.2); 3.78, m
GlcIII-1			4.35 (d, J = 7.8)	4.35 (d, J = 7.8)
GlcIII-2			3.24[c]	3.24[c]
GlcIII-3			3.30[c]	3.29[c]
GlcIII-4			3.25[c]	3.27[c]
GlcIII-5			3.24[c]	3.24[c]
GlcIII-6			4.20 (d, J = 10.1) 3.79 (m)	4.19 (dd, J = 11.5, 1.5) 3.72 (t, J = 6.6)
GlcIV-1			4.44 (d, J = 7.8)	4.43 (d, J = 7.8)
GlcIV-2			3.24[c]	3.24[c]
GlcIV-3			3.30[c]	3.30[c]
GlcIV-4			3.25[c]	3.27[c]
GlcIV-5			3.36[c]	3.35[c]
GlcIV-6			3.87, m; 3.68, m	3.87 (m); 3.67 (m)

[a] Based on ^{1}H-, ^{13}C-NMR (DEPT), COSY, HSQC, HMBC, NOESY experiments.
[b] Coupling constants (J) in Hz were given in parentheses.
[c] Signal patterns are unclear due to overlapping.

EIMS: m/z =1120 $[M]^-$, HRESIMS 1119.5457 $[M-H]^-$ (calcd for $C_{42}H_{68}O_{13}$, 1119.5593).

2.4. Acid hydrolysis of **2**–**4**

Compounds **2**–**4** (5 mg each) were refluxed with 2 N HCl (5 mL) at 80 °C for 5 h. After neutralization with $NaHCO_3$ and extraction with EtOAc, the aqueous layer was concentrated and detected by TLC over silica gel ($CHCl_3$–MeOH–H_2O, 65:35:4). The presence of glucose was confirmed by comparison with authentic samples of glucose (R_f 0.33). The aqueous part was purified by column chromatography over silica gel (EtOAc–MeOH–H_2O, 75:25:3) and identified to be D-glucose based on its $[a]_D$ value.

The anti-HBV assay was performed according to our previous report [9]. Tenofovir purchased from Jiangxi Chenyang Pharmaceutical Co. Ltd., China (purity > 97.6%) was used as the positive control.

3. Results and discussion

Sweriyunnangenin A (**1**) was obtained as colorless needles (mp 314–315 °C). The molecular formula was deduced as $C_{30}H_{50}O_2$ by the HREIMS (m/z: 442.3802 $[M]^+$, calcd for

Table 2
^{13}C-NMR data of compounds **1**–**4** (δ_C values, **1** in C_5D_5N and **2**–**4** in CD_3OD).

Position	1	2	3	4
1	35.3 (t)	39.9 (d)	47.4 (t)	39.7 (t)
2	26.4 (t)	27.9 (d)	69.6 (d)	27.6 (t)
3	76.9 (d)	79.7 (d)	84.6 (d)	74.0 (d)
4	39.1 (s)	38.2 (s)	40.7 (s)	43.1 (s)
5	49.9 (d)	56.8 (d)	56.9 (d)	48.9 (d)
6	68.3 (d)	19.5 (t)	19.7 (t)	19.2 (t)
7	49.8 (t)	33.0 (t)	33.2 (t)	33.7 (t)
8	39.4 (s)	40.8 (s)	40.9 (s)	40.9 (s)
9	50.1 (d)	49.2 (d)	49.3 (d)	49.2 (d)
10	38.3 (s)	39.9 (s)	39.4 (s)	38.1 (s)
11	17.9 (t)	23.5 (d)	23.9 (t)	23.9 (t)
12	34.3 (t)	123.6 (d)	123.5 (d)	123.6 (d)
13	37.7 (s)	145.0 (s)	145.4 (s)	145.4 (s)
14	158.8 (s)	42.8 (d)	43.1 (s)	43.4 (s)
15	117.0 (d)	29.8 (t)	29.2 (t)	29.7 (t)
16	37.9 (t)	24.6 (t)	24.8 (t)	24.7 (t)
17	36.0 (s)	48.0 (d)	48.2 (s)	48.2 (s)
18	49.2 (d)	42.5 (d)	42.6 (d)	42.7 (d)
19	36.9 (t)	47.2 (t)	48.3 (t)	47.4 (t)
20	29.0 (s)	31.6 (s)	31.7 (s)	31.7 (s)
21	33.4 (t)	34.9 (t)	35.1 (t)	35.1 (t)
22	35.3 (t)	34.1 (t)	34.1 (t)	33.2 (t)
23	29.2 (q)	28.8 (q)	29.5 (q)	67.3 (t)
24	24.8 (q)	16.4 (q)	17.7 (q)	12.9 (q)
25	16.9 (q)	16.0 (q)	17.4 (q)	16.4 (q)
26	27.6 (q)	17.8 (q)	17.9 (q)	18.0 (q)
27	21.7 (q)	26.5 (q)	26.6 (q)	26.7 (q)
28	30.0 (q)	178.2 (s)	178.4 (s)	178.4 (s)
29	33.3 (q)	33.6 (q)	33.7 (q)	33.7 (q)
30	29.9 (q)	24.1 (q)	24.3 (q)	24.3 (q)
GlcI-1		93.9 (d)	93.8 (d)	94.0 (d)
GlcI-2		78.2 (d)	77.6 (d)	77.8 (d)
GlcI-3		78.6 (d)	78.9 (d)	78.9 (d)
GlcI-4		70.8 (d)	70.7 (d)	70.7 (d)
GlcI-5		78.8 (d)	77.9 (d)	77.9 (d)
GlcI-6		62.3 (t)	62.8 (t)	62.8 (t)
GlcII-1		103.7 (d)	103.7 (d)	103.8 (d)
GlcII-2		75.8 (d)	75.3 (d)	75.2 (d)
GlcII-3		78.2 (d)	77.6 (d)	77.3 (d)
GlcII-4		72.5 (d)	71.6 (d)	71.6 (d)
GlcII-5		78.0 (d)	78.2 (d)	78.2 (d)
GlcII-6		63.6 (t)	69.4 (t)	69.4 (t)
GlcIII-1			104.8 (d)	104.8 (d)
GlcIII-2			75.2 (d)	75.3 (d)
GlcIII-3			78.1 (d)	78.1 (d)
GlcIII-4			72.0 (d)	71.9 (d)
GlcIII-5			78.2 (d)	78.2 (d)
GlcIII-6			70.8 (t)	70.7 (t)
GlcIV-1			105.2 (d)	105.2 (d)
GlcIV-2			75.9 (d)	75.9 (d)
GlcIV-3			78.2 (d)	78.1 (d)
GlcIV-4			71.9 (d)	72.0 (d)
GlcIV-5			78.2 (d)	78.2 (d)
GlcIV-6			63.0 (t)	63.1 (t)

Assignments were established by interpretation of ^{1}H-, ^{13}C-NMR (DEPT), HSQC, HMBC and COSY.

442.3811) requiring six degrees of unsaturation. Its IR spectrum indicated the presence of hydroxyl (3441 cm^{-1}) and olefinic (1638 cm^{-1}) groups. The ^{1}H NMR spectrum showed eight tertiary methyl signals at δ 0.88 (3H, s, H-28), 0.93 (3H, s, H-27), 0.97 (3H, s, H-30), 0.99 (3H, s, H-29), 1.40 (3H, s, H-23), 1.62 (3H, s, H-24), 1.69 (3H, s, H-25), and 1.73 (3H, s, H-26). In the ^{13}C NMR (DEPT) spectrum, thirty carbons including eight methyls, six methylenes, nine methines, seven quaternary carbons including one double bond (δ 158.8, and δ 117.0) groups were indicative of a taraxerane skeleton [12,13]. Detailed comparison of the spectroscopic data of **1** with those of methyl-3-episumaresinolate revealed that they were similar in rings A and B [12]. Furthermore, two hydroxyl groups at C-3 and C-6 were corroborated by the HMBC of a long-range correlation of δ 3.67 (br s, H-3) with carbon signals at δ 24.8 (C-24), 35.3 (C-1) and 49.9 (C-5), as well as δ 4.77 (br s, H-6) with two quaternary carbons δ 39.1 (C-4) and 39.4 (C-8) (Fig. 2). The α orientation of the hydroxyl group at C-3 and the β orientation of the hydroxyl group at C-6 were supported by ROESY correlation of H-3 with H-24, and H-6 with H-5 and H-23 (Fig. 3). The presence of a double bond was proposed between C-14 and C-15, by HMBC correlation of H-15 with C-8, C-13 and C-17. Therefore, the structure of sweriyunnangenin A (**1**) was established as taraxer-14-ene-3α,6β-diol.

Sweriyunnanoside A (**2**) was isolated as white amorphous power. A molecular formula of $C_{42}H_{68}O_{13}$ was assigned from the negative HRESIMS (*m/z*: 779.4471 $[M-H]^-$, calcd for 779.4587). Its ESI-MS showed a $[M-H]^-$ at *m/z* 779 and a prominent fragment ion $[M-H-162-162]^-$ at *m/z* 455 attributed to the sequential loss of two hexoses. The D-glucose was confirmed by comparison with authentic samples on silica TLC ($CHCl_3$–MeOH–H_2O, 65:35:4, R_f 0.33), in combination with $[\alpha]_D$ experiment ($[\alpha]25$ D: +46.7, *c* 0.14, H_2O) [14]. The β configurations for the glucose were determined from the coupling constants (δ 5.45, J = 8.0 Hz and 4.80, J = 7.7 Hz) [15]. The ^{1}H NMR spectrum exhibited the signals of seven singlet methyl groups (δ 0.77, 0.79, 0.91, 0.93, 0.94, 0.96 and 1.17) and trisubstituted olefinic proton (δ 5.24, br s). The ^{13}C NMR spectrum also revealed the presence of seven quaternary carbons (δ 16.0, 16.4, 17.8, 24.1, 26.5, 28.8 and 33.6), a pair of olefinic carbons (δ 123.6 and 145.0), one ester carbonyl carbon (δ 178.2), and two anomeric carbons (δ 103.7 and 93.9). The NMR data of **2** were consistent with the triterpene of olean-12-ene type. A detailed inspection of 1D and 2D NMR spectra led to the identification of aglycone part as oleanolic acid [12]. Comparing the ^{13}C NMR spectrum of **2** with that of oleanolic acid, C-28 was obviously up-field shifted 3.0 ppm, indicating the glycosylation at C-28. The sugar sequence and the linkage sites were determined by the HMBC correlations between δ 5.45 (H-1$_{GlcI}$) and δ 178.2 (C-28), δ 4.80 (H-1$_{GlcII}$)

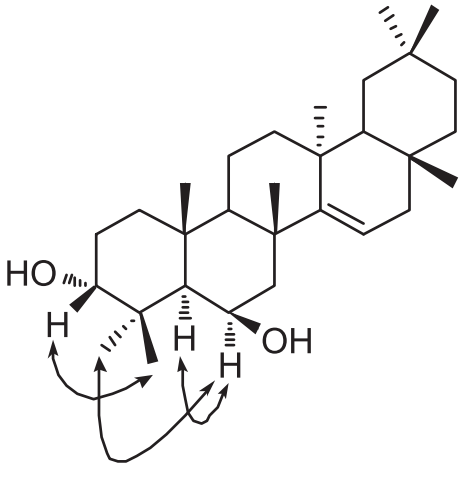

Fig. 3. Key ROESY (H ↔ H) correlations of compound **1**.

and 78.2 (C-2_{GlcI}), δ 3.82 (H-2_{GlcI}) and δ 93.9 (C-1_{GlcI}) and 103.7 (C-1_{GlcII}). Thus, sweriyunnanoside A (**2**) was identified as oleanolic acid 28-O-β-D-glucopyranosyl-(1 → 2)-O-β-D-glucopyranoside (Fig. 4).

Sweriyunnanoside B (**3**) was obtained as white amorphous powders. Its molecular formula was determined as $C_{54}H_{88}O_{24}$ from negative-ion HRESIMS (*m/z* 1119.5465 $[M-H]^-$, calcd. 1119.5593) corresponding to eleven degrees of unsaturation. The ^{13}C NMR spectrum of compound **3** displayed 54 carbon signals, 30 of which were assigned to a triterpenoid skeleton. The ^1H NMR spectrum showed the signals of seven methyl groups (δ 0.79, 0.81, 0.92, 0.94, 1.01 × 2 and 1.16), one trisubstituted olefinic proton δ 5.25 (t, $J = 3.4$ Hz). The ^{13}C NMR spectrum revealed the presence of seven quaternary carbons (δ 17.4, 17.7, 17.9, 24.3, 26.6, 29.5 and 33.7), two oxygenated methane carbons (δ 69.6 and 84.6), a pair of olefinic carbons (δ 123.5 and 145.4), and one ester carbonyl carbon (δ 178.4). Detailed inspection of its 1D and 2D NMR spectrum led to the identification of aglycone part as 2α,3β-dihydroxyolean-12-en-28-oic acid (maslinic acid) [16]. In the ^1H and ^{13}C NMR spectrum, signals ascribable to four anomeric protons at δ 5.42 (1H, d, $J = 8.2$ Hz), 4.81 (1H, d, $J = 7.8$ Hz), 4.44 (1H, d, $J = 7.8$ Hz) and 4.35 (1H, d, $J = 7.8$ Hz), and four anomeric carbons at δ_C 93.8, 103.7, 104.8 and 105.2 were noted. The chemical shifts of the rest of the individual protons and their attached carbons of the four sugar units were ascertained from the COSY, HSQC and HMBC experiments. Acid hydrolysis of **3** showed only glucose, which was confirmed as D-glucose by comparison with an authentic sample on TLC, and $[\alpha]_D$ experiment ($[\alpha]25$ D: +49.8, *c* 0.16, H_2O) [14]. The β configurations for the glucose were determined from their $^3J_{H1,\,H2}$ coupling constants (7.8–8.2 Hz) [15]. In the HMBC spectrum, correlative peak from δ 5.42 (H-1_{GlcI}) to δ 178.4 (C-28) indicated that the sugar chain was located at C-28. The linkage sequence was established by the HMBC correlations from δ 3.89 (H-2_{GlcI}) to 103.7 (C-1_{GlcII}), δ 4.81 (H-1_{GlcII}) to 77.6 (C-2_{GlcI}), δ 4.35 (H-1_{GlcIII}) to 69.4 (C-6_{GlcII}), and δ 4.44 (H-1_{GlcIV}) to 70.8 (C-6_{GlcIII}). From all the above given data, the structure of sweriyunnanoside B (**3**) was established as 2α,3β-dihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside.

Sweriyunnanoside C (**4**) was isolated as a white amorphous powder with the molecular formula $C_{54}H_{88}O_{24}$ from negative HRESIMS (*m/z* 1119.5457 $[M-H]^-$, calcd. 1119.5593). The ^{13}C NMR spectrum of **4** displayed 54 carbon signals, indicative of a triperpenoid glycoside. The assignment of ^1H and ^{13}C NMR spectroscopic data of **4** was based on HSQC, HMBC and COSY. The aglycone of **4** was identified as hederagenin by comparison of ^1H and ^{13}C data (Tables 1 and 2) with literature [17]. The ^1H and ^{13}CNMR spectrum of **4** exhibited four anomeric proton resonances at δ 5.43 (1H, d, $J = 8.2$ Hz), 4.80 (1H, d, $J = 7.8$ Hz), 4.43 (1H, d, $J = 7.8$ Hz) and 4.35 (1H, d, $J = 7.8$ Hz), and four anomeric carbons at δ 94.0, 103.8, 104.8 and 105.2. Acid hydrolysis of **4** yielded only D-glucose which was identified by comparison with an authentic sample on TLC,

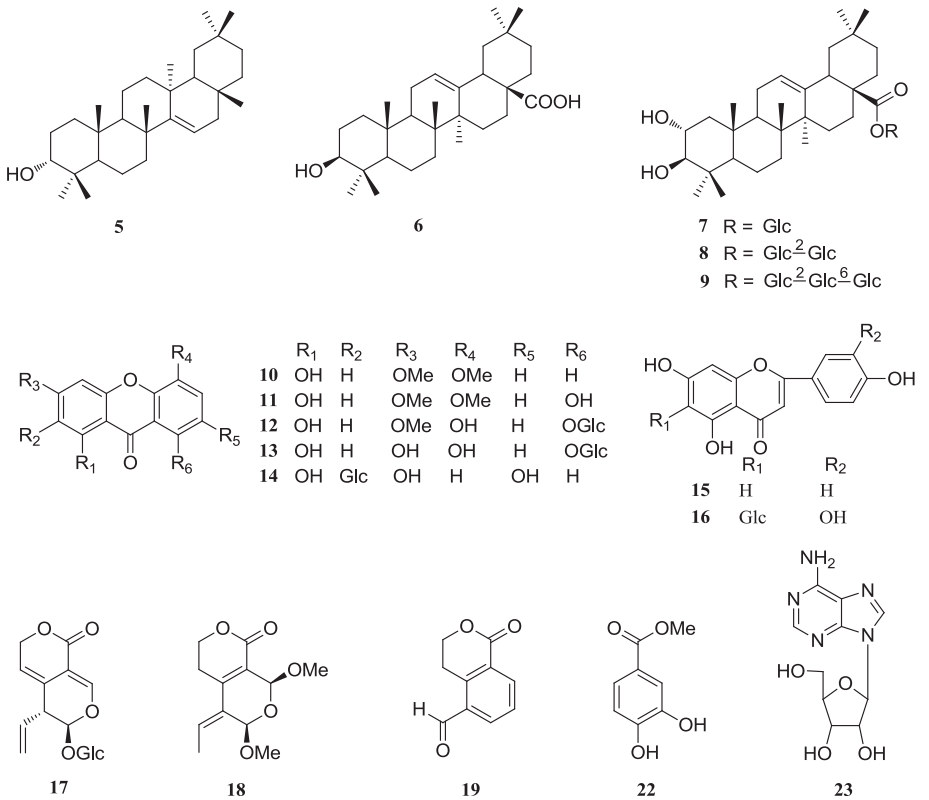

Fig. 4. hemical structures of compounds 5–23.

Table 3
Anti-HBV activities of **1**, **5**, **6**, **11**, **13–16**, **19** and **22**[a].

Compounds	CC_{50} [mM]	HBsAg[b]		HBeAg[c]		HBV DNA[d]	
		IC_{50} [mM]	SI[e]	IC_{50} [mM]	SI	IC_{50} [mM]	SI
1	>1.93	0.28	>6.89	0.29	>6.66	–	–
5	>2.16	0.70	>3.08	1.41	>1.53	–	–
6	>2.22	1.26	>1.76	0.94	>2.36	–	–
11	>5.00	–	–	–	–	0.07	>71.4
13	>2.69	–	–	–	–	0.01	>269.0
14	2.04	0.21	9.71	0.04	51.0	0.09	22.7
15	0.10	0.10	1.0	<0.03	>3.33	<0.01	>10.0
16	0.99	1.51	<1.0	0.23	4.30	0.05	19.8
19	1.76	1.30	1.35	1.14	1.54	0.76	2.32
22	>6.47	–	–	4.03	>1.60	1.55	>4.17
Tenofovir[f]	>1.39	1.25	>1.11	1.21	>1.15	0.00046	>3021.7

[a] All values are the mean of two independent experiments.
[b] HBsAg: HBV surface antigen.
[c] HBeAg: HBV e antigen.
[d] DNA: HBV DNA replication.
[e] CC_{50} = 50% cytotoxic concentration, IC_{50} = 50% inhibition concentration, SI (selectivity index) = CC_{50}/IC_{50}.
[f] Tenofovir, an antiviral agent used as a positive control. The other compounds exhibited no anti-HBV activity at the maximal testing concentration.

and $[\alpha]_D$ experiment ([α]25 D: +58.6, *c* 0.14, H_2O) [14]. The β configurations of the four glucosyl groups were determined from their $^3J_{H1,\ H2}$ coupling constants (7.8–8.2 Hz) [15]. The unambiguous determination of the glucose sequence and linkage sites was obtained from the HMBC spectrum, which showed key correlative peaks between δ 5.42 ($H\text{-}1_{Glcl}$) and δ 178.4 (C-28), δ 3.88 ($H\text{-}2_{Glcl}$) and 103.7 ($C\text{-}1_{GlclI}$), δ 4.80 ($H\text{-}1_{GlclII}$) and 77.8 ($C\text{-}2_{Glcl}$), δ 4.35 ($H\text{-}1_{GlclIII}$) and δ 69.4 ($C\text{-}6_{Glcl}$), δ 4.43 ($H\text{-}1_{GlclV}$) and δ_C 70.7 ($C\text{-}6_{GlclII}$). On the basis of these evidence, the structure of sweriyunnanoside C (**4**) was established as hederagenin 28-O-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl (1 → 2)-β-D-glucopyranoside.

By comparing the spectral data with those in the literatures, compounds **5–23** were identified as 3-epitaraxerol (**5**) [13], oleanolic acid (**6**) [12], 2α,3β-dihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranoside (**7**) [18], 2α,3β-dihydroxyolean-12-en-28-oic acid 28-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (**8**) [16], swerticinctonide (**9**) [19], 1-hydroxy-3,5-di-methoxyxanthone (**10**) [20], 1,8-dihydroxy-3,5-dimethoxyxanthone (**11**) [20], swertianolin (**12**) [21], norswertianolin (**13**) [21], neolancerin (**14**) [22], luteolin (**15**) [23], isovitexin (**16**) [24], gentiopicroside (**17**) [25], (*Z*)-5-Ethylidene-3,4,5,6-tetrahydro-*cis*-6,8-dimethoxy-1*H*,8*H*-pyrano-[3,4-*c*]pyran-1-one (**18**) [26], erythrocentaurin (**19**) [25], β-sitosterol (**20**) [27], daucosterol (**21**) [27], 3,4-dihydroxybenzoic acid methyl ester (**22**) [28] and adenosine (**23**) [29].

Compounds **1–22** were evaluated for their anti-HBV activity on HepG 2.2.15 cells *in vitro*, namely inhibiting the secretion of HBsAg, HBeAg and HBV DNA replication using tenofovir as positive control. As shown in Table 3, the triterpenoids **1**, **5** and **6** exhibited activities against the secretion of HBsAg (IC_{50} 0.28, 0.70 and 1.26 mM) and HBeAg (IC_{50} 0.29, 1.41 and 0.94 mM), respectively. The other six triterpenoids showed no anti-HBV activities at the maximal testing concentration, which might be due to the presence of the glycosyls in their structure. The xanthones **11** and **13–16** possessed potent anti-HBV activity. Compounds **14–16** showed activities inhibiting the secretion of HBsAg (IC_{50} 0.21, 0.10 and 1.51 mM) and HBeAg (IC_{50} 0.04, <0.03 and 0.23 mM), and remarkable inhibition on HBV DNA replication (IC_{50} 0.09, <0.01 and 0.05 mM). However compounds **11** and **13** exhibited only inhibitory activity against HBV DNA replication with IC_{50} values of 0.07 mM and 0.01 mM, which might be due to methylation or glycosidation of hydroxy group in their structure. In the case of secoiridoids, only compound **19** with a formyl group displayed activities against the secretion of HBsAg (IC_{50} 1.30 mM) and HBeAg (IC_{50} 1.14 mM), and against HBV DNA replication (IC_{50} 0.76 mM).

Acknowledgments

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Appendix A. Supplementary data

1D- and 2D-NMR, MS, IR, UV and $[a]_D$ spectra of compounds **1–4**, as well as the procedure for anti-HBV assay and the anti-HBV data of compounds from two independent experiments are available as Supporting information. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fitote.2013.05.023.

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