# Chemical Constituents of *Saniculiphyllum guangxiense*

by **Chang-An Geng**[a]), **Xiao-Yan Huang**[a]), **Li-Gong Lei**[b]), **Xue-Mei Zhang**[a]), and **Ji-Jun Chen***[a])

[a]) State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, 132#, Lanhei Road, Heilongtan, Kunming 650201, P. R. China (phone: +86-871-5223265; fax: +86-871-5227197; e-mail: chenjj@mail.kib.ac.cn)

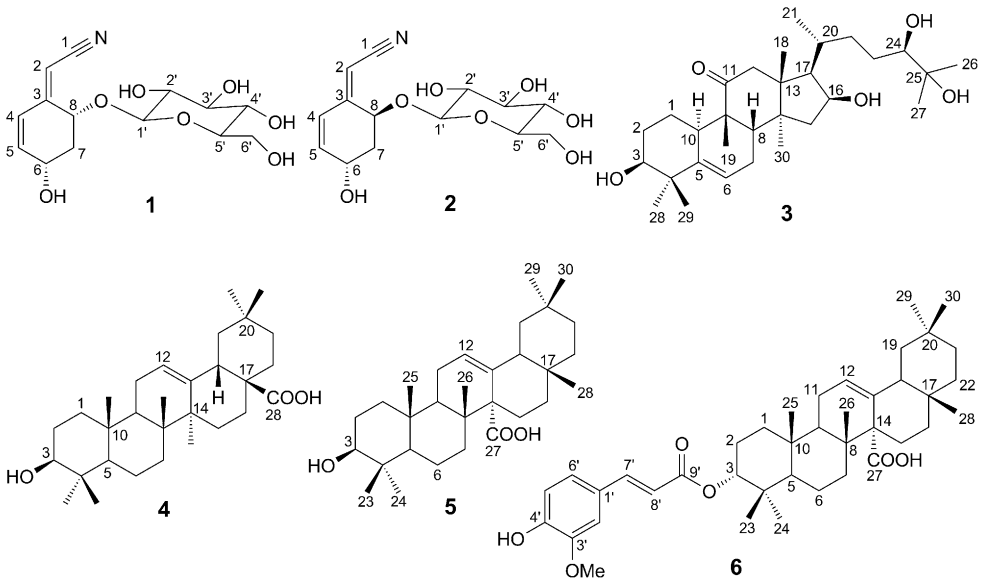
[b]) Key Laboratory of Biodiversity and Biogeography of Chinese Academy of Sciences, Kunming Institute of Botany, Chinese Academy of Sciences, 132#, Lanhei Road, Heilongtan, Kunming 650201, P. R. China

The first phytochemical investigation on *Saniculiphyllum guangxiense* resulted in the isolation of two new triterpenoids, 16$\beta$-hydroxybryodulcosigenin (**3**) and 3$\alpha$-*O*-feruloylolean-12-en-27-oic acid (**6**), together with six known compounds, menisdaurin (**1**), purshianin (**2**), oleanolic acid (**4**), 3$\beta$-hydroxyolean-12-en-27-oic acid (**5**), $\beta$-sitosterol (**7**), and daucosterol (**8**), which were characterized by extensive spectroscopic analyses and in one case by X-ray diffraction. According to this primary investigation, *S. guangxiense* is rich in nitrile glucosides and triterpenoids, of which menisdaurin (**1**; 0.06%) and purshianin (**2**; 0.015%) are the main constituents. Compounds **1**–**6** were assayed for their anti-hepatitis B virus (HBV) activities against the secretion of HBsAg and HBeAg, as well as HBV DNA replication on Hep G 2.2.15 cell line *in vitro*. The most active compound, menisdaurin (**1**), inhibits HBV DNA replication with an $IC_{50}$ value of 0.32 mM ($SI > 11.97$).

**Introduction.** – *Saniculiphyllum guangxiense* (Chinese name: *Bian-Dou-Ye-Cao*), the only species of the genus *Saniculiphyllum* of the single-genus tribe Saniculiphylleae (Saxifragaceae), is a perennial herb mainly distributed in Yunnan and Guangxi provinces of China [1]. Most of the taxonomic characteristics of *S. guangxiense* is similar to those of tribe Saxifrageae, except for the long and creeping rhizomes and palmatiparted basal leaves [2]. *S. guangxiense* is little-known due to being rarely collected or observed, and its taxonomy is still disputed. To date, no chemical and biological study on the title plant has been reported. Therefore, it will be worthy to clarify its chemical constituents from the phytochemical and chemotaxonomic perspectives.

Our phytochemical investigation of this plant led to the isolation of two new triterpenoids, 16$\beta$-hydroxybryodulcosigenin (**3**) and 3$\alpha$-*O*-feruloylolean-12-en-27-oic acid (**6**), together with six known compounds, menisdaurin (**1**), purshianin (**2**), oleanolic acid (**4**), 3$\beta$-hydroxyolean-12-en-27-oic acid (**5**), $\beta$-sitosterol (**7**), and daucosterol (**8**). Their structures were characterized by extensive spectroscopic analyses and, in one case, by X-ray crystallographic diffraction. This article mainly deals with the isolation and structure elucidation of compounds **1**–**8**, as well as their anti-HBV properties on the Hep G 2.2.15 cell line *in vitro*.

**Results and Discussion.** – *Structure Elucidation*. Compound **3** was obtained as a white powder, and its molecular formula $C_{30}H_{50}O_5$ was determined by HR-ESI-MS




analysis (positive-ion mode; calc. for $C_{30}H_{50}O_5^+$, 490.3658; found *m/z* 490.3661). The presence of OH (3427 $cm^{-1}$), C=O (1691 $cm^{-1}$), and C=C (1640 $cm^{-1}$) groups was deduced from the IR spectrum. The $^{13}$C-NMR (DEPT) spectrum (*Table 1*) displayed 30 C-atom signals assigned to seven quaternary C-atoms, and eight CH, seven $CH_2$, and eight Me groups. All H-atoms were readily assigned to their bonding C-atoms based on a HSQC experiment. Four OH signals were observed at $\delta$(H) 3.99 (*d*, $J=3.4$, HO–C(16)), 3.77 (*d*, $J=4.1$, HO–C(24)), 3.63 (*d*, $J=5.0$, HO–C(3)), 3.35 (*s*, HO–C(25)), respectively, which were exchangeable with $D_2O$. The locations of all the OH groups were unambiguously determined based on the $^1$H,$^1$H-COSY and HMBC analyses. In the $^1$H- and $^{13}$C-NMR spectra, signals of one CO group ($\delta$(C) 213.4 (*s*, C(11))), one trisubstituted C=C bond ($\delta$(C) 143.5 (*s*, C(5)), 118.7 (*d*, C(6)); $\delta$(H) 5.68 (br. *d*, $J=5.8$, H–C(6))), three O-bearing CH groups ($\delta$(C) 76.4 (*d*, C(3)), 72.1 (*d*, C(16)), 76.8 (*d*, C(24)); $\delta$(H) 3.08–3.14 (*m*, H–C(3)), 4.50–4.56 (*m*, H–C(16)), 3.41 (br. *d*, $J=11.3$, H–C(24))), and one O-bearing quaternary C-atom ($\delta$(C) 72.6 (*s*, C(25))) were detected. Eight Me signals were observed in the upfield part of the $^1$H-NMR spectrum ($\delta$(H) 0.92–1.14), of which one *doublet* at $\delta$(H) 0.93 (*d*, $J=6.5$, Me(21)) was assigned to a tertiary Me group. Two adjacent Me groups ($\delta$(H) 1.12 (*s*, Me(26)), 1.10 (*s*, Me(27)); $\delta$(C) 25.0 (*q*, C(26)), 24.8 (*q*, C(27))) and one quaternary C-atom ($\delta$(C) 72.6 (*s*, C(25))) indicated a $Me_2C$–OH group. With the structural characteristics discussed above, compound **3** was proposed to possess a cucurbitane-triterpenoid skeleton [3]. The $^{13}$C-NMR data of compound **3** were similar to those of bryodulcosigenin and its glycosides [4][5], except for the obvious downfield shift of C(16) (from 28.1 (*t*) in bryodulcosigenin to 72.1 (*d*) in **3**). This suggested that compound **3** is the 16-OH substituted derivative of bryodulcosigenin, which was also consistent with the molecular weight of compound **3**, which was 16 amu higher than that of bryodulcosigenin.

Table 1. *$^1$H- and $^{13}$C-NMR Data* ($CDCl_3$) *of Compounds* ***3*** *and* ***6*** *(δ* in ppm, *J* in Hz*)*

| Position | **3** | | Position | **6** | |
|---|---|---|---|---|---|
| | δ(H) | δ(C) | | δ(H) | δ(C) |
| $CH_2$(1) | 1.50–1.55 (*m*), 0.94–1.02 (*m*) | 25.8 (*t*) | $CH_2$(1) | 1.25–1.31 (*m*), 1.40–1.48 (*m*) | 36.4 (*t*) |
| $CH_2$(2) | 1.63–1.69 (*m*), 1.45–1.49 (*m*) | 31.3 (*t*) | $CH_2$(2) | 1.16–1.20 (*m*), 1.60–1.64 (*m*) | 22.9 (*t*) |
| H–C(3) | 3.08–3.14 (*m*) | 76.4 (*d*) | H–C(3) | 4.77 (br. *s*) | 77.8 (*d*) |
| C(4) | | 43.0 (*s*) | C(4) | | 40.2 (*s*) |
| C(5) | | 143.5 (*s*) | H–C(5) | 1.31–1.37 (*m*) | 50.0 (*d*) |
| H–C(6) | 5.68 (br. *d*, *J*=5.8) | 118.7 (*d*) | $CH_2$(6) | 1.35–1.39 (*m*), 1.45–1.49 (*m*) | 18.1 (*t*) |
| $CH_2$(7) | 2.33–2.39 (*m*), 1.96–2.02 (*m*) | 24.5 (*t*) | $CH_2$(7) | 1.40–1.46 (*m*), 1.45–1.49 (*m*) | 33.8 (*t*) |
| H–C(8) | 2.00–2.06 (*m*) | 44.3 (*d*) | C(8) | | 37.1 (*s*) |
| C(9) | | 47.7 (*s*) | H–C(9) | 2.06–2.10 (*m*) | 48.8 (*d*) |
| H–C(10) | 2.25–2.35 (*m*) | 36.0 (*d*) | C(10) | | 36.7 (*s*) |
| C(11) | | 213.4 (*s*) | $CH_2$(11) | 1.70–1.74 (*m*), 2.00–2.06 (*m*) | 22.7 (*t*) |
| $CH_2$(12) | 3.03 (*d*, *J*=14.5, $H_\beta$), 2.34 (*d*, *J*=14.5, $H_\alpha$) | 49.2 (*t*) | H–C(12) | 5.76 (br. *s*) | 127.1 (*d*) |
| C(13) | | 49.9 (*s*) | C(13) | | 138.8 (*s*) |
| C(14) | | 49.2 (*s*) | C(14) | | 56.4 (*s*) |
| $CH_2$(15) | 2.04–2.08 (*m*), 1.30–1.36 (*m*) | 47.0 (*t*) | $CH_2$(15) | 1.68–1.72 (*m*), 1.87–1.93 (*m*) | 22.2 (*t*) |
| H–C(16) | 4.50–4.56 (*m*) | 72.1 (*d*) | $CH_2$(16) | 0.75–0.81 (*m*), 1.91–1.99 (*m*) | 27.4 (*t*) |
| H–C(17) | 1.80–1.84 (*m*) | 54.8 (*d*) | C(17) | | 33.2 (*s*) |
| Me(18) | 0.93 (*s*) | 18.3 (*q*) | H–C(18) | 2.04–2.08 (*m*) | 47.7 (*d*) |
| Me(19) | 1.01 (*s*) | 20.4 (*q*) | $CH_2$(19) | 0.95–1.03 (*m*), 1.45–1.49 (*m*) | 44.7 (*t*) |
| H–C(20) | 1.97–2.01 (*m*) | 28.2 (*d*) | C(20) | | 30.9 (*s*) |
| Me(21) | 0.93 (*d*, *J*=6.5) | 17.9 (*q*) | $CH_2$(21) | 1.08–1.12 (*m*), 1.35–1.39 (*m*) | 34.2 (*t*) |
| $CH_2$(22) | 1.77–1.83 (*m*), 1.10–1.14 (*m*) | 32.7 (*t*) | $CH_2$(22) | 1.40–1.46 (*m*), 1.70–1.78 (*m*) | 35.9 (*t*) |
| $CH_2$(23) | 1.57–1.63 (*m*), 1.36–1.40 (*m*) | 27.4 (*t*) | Me(23) | 0.87 (*s*) | 27.8 (*q*) |
| H–C(24) | 3.41 (br. *d*, *J*=11.3) | 76.8 (*d*) | Me(24) | 0.91 (*s*) | 21.8 (*q*) |
| C(25) | | 72.6 (*s*) | Me(25) | 1.00 (*s*) | 16.2 (*q*) |
| Me(26) | 1.12 (*s*) | 25.0 (*q*) | Me(26) | 1.04 (*s*) | 17.9 (*q*) |
| Me(27) | 1.10 (*s*) | 24.8 (*q*) | C(27) | | 177.4 (*s*) |
| Me(28) | 1.14 (*s*) | 26.2 (*q*) | Me(28) | 0.84 (*s*) | 28.2 (*q*) |
| Me(29) | 0.92 (*s*) | 20.9 (*q*) | Me(29) | 0.84 (*s*) | 32.7 (*q*) |
| Me(30) | 1.06 (*s*) | 19.8 (*q*) | Me(30) | 0.85 (*s*) | 23.6 (*q*) |
| HO–C(3) | 3.63 (*d*, *J*=5.0) | | C(1′) | | 127.1 (*s*) |
| HO–C(16) | 3.99 (*d*, *J*=3.4) | | H–C(2′) | 7.08 (br. *s*) | 110.0 (*d*) |
| HO–C(24) | 3.77 (*d*, *J*=4.1) | | C(3′) | | 146.7 (*s*) |
| HO–C(25) | 3.35 (*s*) | | C(4′) | | 147.7 (*s*) |
| | | | H–C(5′) | 6.91 (*d*, *J*=8.2) | 114.6 (*d*) |
| | | | H–C(6′) | 7.12 (*d*, *J*=8.2) | 122.6 (*d*) |
| | | | H–C(7′) | 7.60 (*d*, *J*=15.9) | 144.7 (*d*) |
| | | | H–C(8′) | 6.28 (*d*, *J*=15.9) | 116.1 (*d*) |
| | | | C(9′) | | 166.9 (*s*) |
| | | | MeO | 3.96 (*s*) | 56.1 (*q*) |

2D-NMR Analyses supported the above deduction. The ketone was proposed to be positioned at C(11) based on the HMBC from the signals of H–C(12) and Me(19) to that of C(11) (*Fig. 1*). Similarly, the C=C bond was deduced to be located between C(5) and C(6) according to the HMBC experiment (H–C(6)/C(4) and C(10), and H–C(1)/C(5)). Two OH groups were deduced to be located at C(16) and C(24) by means of

$^1$H,$^1$H-COSY correlations (HO–C(24)/H–C(24)/$CH_2$(23)/$CH_2$(22)/H–C(20)/H–C(17)/H–C(16)/HO–C(16); *Fig. 1*). The OH group at C(3) was confirmed by cross-peaks between the signals of HO–C(3)/H–C(3)/$CH_2$(2)/$CH_2$(1) in the $^1$H,$^1$H-COSY spectrum; HO–C(25) was determined based on the HMBC cross-peaks from the signal of HO–C(25) to those of C(25), C(26), and C(27).

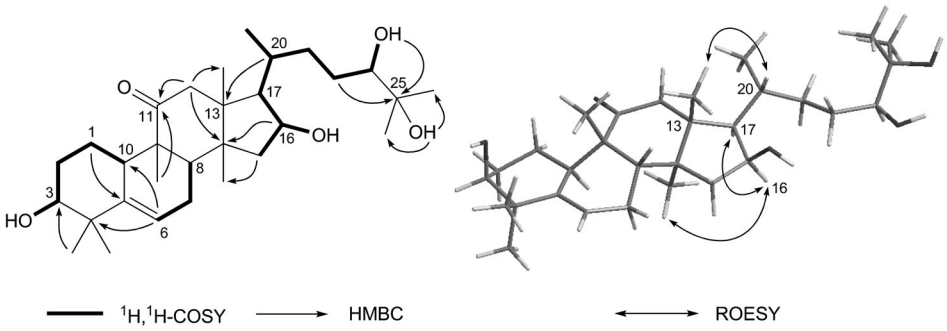



Fig. 1. *Key 2D-correlations of compound* **3**

In the ROESY spectrum, the observed correlations between the signals of H–C(16) and H–C(17), and those of Me(18) and H–C(20) indicated the $\alpha$-orientation of H–C(16) and H–C(17). Therefore, compound **3** was determined as 16$\beta$-hydroxybryodulcosigenin as shown in *Fig. 1*.

Compound **6**, a white powder, had a molecular formula $C_{40}H_{56}O_6$, determined by a HR-ESI-MS spectrum (negative-ion mode), which showed the *quasi*-molecular-ion peak $[M-H]^-$ at *m/z* 631.3982 (calc. for 631.3998), with 13 degrees of unsaturation. The IR spectrum displayed characteristic bands of OH (3415 $cm^{-1}$), C=O (1708, 1686 $cm^{-1}$), C=C (1631 $cm^{-1}$) groups, and of a phenyl moiety (1594, 1516, and 1465 $cm^{-1}$). The $^{13}$C-NMR (DEPT) spectrum (*Table 1*) displayed 40 C-atom resonances due to twelve quaternary C-atoms, and ten $CH_2$, ten CH, and eight Me groups. The presence of a feruloyl group was readily deduced from the $^1$H-NMR ($\delta$(H) 7.60 (*d*, $J=15.9$, H–C(7′)), 7.12 (*d*, $J=8.2$, H–C(6′)), 7.08 (br. *s*, H–C(2′)), 6.91 (*d*, $J=8.2$, H–C(5′)), 6.28 (*d*, $J=15.9$, H–C(8′)), and 3.96 (*s*, MeO)) and $^{13}$C-NMR data ($\delta$(C) 127.1 (*s*, C(1′)), 110.0 (*d*, C(2′)), 146.7 (*s*, C(3′)), 147.7 (*s*, C(4′)), 114.6 (*d*, C(5′)), 122.6 (*d*, C(6′)), 144.7 (*d*, C(7′)), 116.1 (*d*, C(8′)), 166.9 (*s*, C(9′)), 56.1 (*q*, MeO)). Besides the feruloyl group, the residual 30 C-atoms including a trisubstituted C=C bond ($\delta$(C) 127.1 (*d*, C(12)), 138.8 (*s*, C(13))), one C=O group ($\delta$(C) 177.4 (*s*, C(27))) and seven Me groups ($\delta$(C) 27.8 (*q*, Me(23)), 21.8 (*q*, Me(24)), 16.2 (*q*, Me(25)), 17.9 (*q*, Me(26)), 28.2 (*q*, Me(28)), 32.7 (*q*, Me(29)), 23.6 (*q*, Me(30))) indicated an oleanane-type triterpenoid. The $^1$H- and $^{13}$C-NMR data of compound **6** were similar to those of 3$\alpha$-*O*-caffeoylolean-12-en-27-oic acid, except for an additional MeO group [6–8]. Therefore, compound **6** was determined as 3$\alpha$-*O*-feruloylolean-12-en-27-oic acid, which was supported by 2D-NMR ($^1$H,$^1$H-COSY and HMBC) analyses (*Fig. 2*).

Compounds **1**, **2**, **4**, and **5** were identified as menisdaurin (**1**) [9], purshianin (**2**) [10], oleanolic acid (**4**) [11], and 3$\beta$-hydroxyolean-12-en-27-oic acid (**5**) [6], by comparison of their spectroscopic data with those reported; menisdaurin (**1**) was additionally confirmed by X-ray single crystallographic analysis (*Fig. 3*). $\beta$-Sitosterol (**7**) and

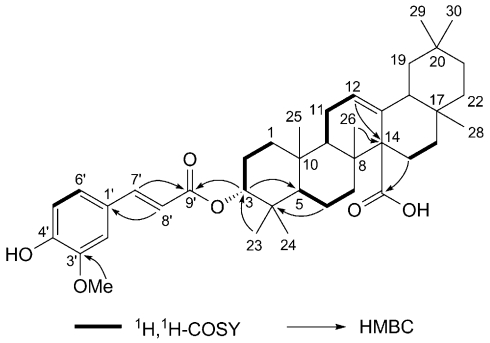



Fig. 2. *Key 2D correlations of compound* **6**

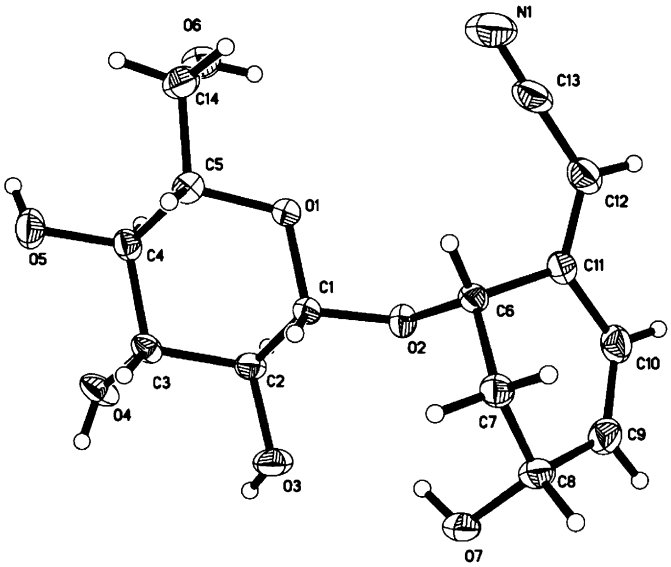



Fig. 3. *X-Ray crystal structure of compound* **1**

daucosterol (**8**) were determined by comparing with authentic samples on TLC (silica gel).

*Anti-HBV Activity.* Compounds **1**–**6** were assayed on Hep G 2.2.15 cell line *in vitro* for their inhibition on the secretion of HBsAg and HBeAg, as well as HBV DNA replication (tenofovir was used as positive control). Menisdaurin (**1**) showed activity inhibiting HBV DNA replication with an $IC_{50}$ value of 0.32 mM ($SI > 11.97$), however, its diastereoisomer, purshianin (**2**), exhibited no anti-HBV effect. Both menisdaurin (**1**) and purshianin (**2**) displayed no activity against the secretion of HBsAg and HBeAg, as well as cytotoxicity at the highest tested concentration of 3.83 and 2.20 mM, respectively. In contrast to the nitrile glucosides, the triterpenoids **3**–**6** possessed obvious cytotoxicities leading to low selectivity indices. Of these triterpenoids,

Table 2. *Anti-HBV Activities of Compounds* **1**–**6**[a])

| Compounds | $CC_{50}$ [mM] | HBsAg | | HBeAg | | HBV DNA | |
|---|---|---|---|---|---|---|---|
| | | $IC_{50}$ [mM] | *SI* | $IC_{50}$ [mM] | *SI* | $IC_{50}$ [mM] | *SI* |
| **1** | >3.83[b]) | >3.83[c]) | – | >3.83 | – | 0.32 | >11.97 |
| **2** | >2.20 | >2.20 | – | >2.20 | – | >2.20 | – |
| **3** | 0.12 | 0.16 | <1 | >1.59 | – | >0.40 | – |
| **4** | 0.034 | 0.038 | <1 | >1.97 | – | 0.069 | <1 |
| **5** | 0.016 | 0.010 | 1.60 | 0.032 | <1 | 0.017 | <1 |
| **6** | 0.033 | 0.048 | <1 | >1.99 | – | 0.16 | – |
| Tenofovir | >1.56 | 1.35 | >1.16 | 1.13 | >1.38 | 0.00044 | >3545.45 |

[a]) All values are the mean of two independent experiments. $CC_{50}$: 50 % cytotoxic concentration; $IC_{50}$: 50 % effective concentration; *SI* (selective index) = $CC_{50}/IC_{50}$; HBsAg: HBV surface antigen; HBeAg: HBV e antigen. [b]) The $CC_{50}$ values were not reached at the highest tested concentration. [c]) The $IC_{50}$ values were not reached at the highest tested concentration.

compound **5** displayed a moderate effect against the secretion of HBsAg ($IC_{50}$ = 0.010 mM, $SI$ = 1.60).

**Conclusions.** – We found in this primary phytochemical investigation, that *S. guangxiense* is rich in nitrile glucosides and triterpenoids, of which menisdaurin (**1**) and purshianin (**2**) are the main constituents whose content is up to 0.06 and 0.015%, respectively. Generally, flavonoids are widely present in Saxifragaceae plants [12–15], however, no flavonoid has been isolated from *S. guangxiense* in the present study. In addition, bergenin as a characteristic component in Saxifragaceae was also not detected in the title plant [16]. Therefore, from a chemotaxonomic point of view, this primary investigation provides valuable clues for the taxonomy of *S. guangxiense* as a new tribe.

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## Experimental Part

*General Experimental Procedures.* Column chromatography (CC): silica gel ($SiO_2$, 200–300 mesh; *Qingdao Makall Chemical Company*, Qingdao, P. R. China) and *Sephadex LH-20* (20–150 μm; *Pharmacia Fine Chemical Co. Ltd.*, Uppsala, Sweden). Optical rotations: *Jasco model 1020* polarimeter (*Horiba*, Tokyo, Japan). UV Spectra: *Shimadzu UV-2401A* spectrophotometer (*Shimadzu*, Kyoto, Japan). IR Spectra: *Bio-Rad FTS-135* spectrometer (*Bio-Rad*, California, USA), measured in KBr. 1D- and 2D-NMR Spectra: *Bruker AM-400* NMR or *DRX-500* spectrometers with TMS as an internal standard (*Bruker*, Bremerhaven, Germany). MS Spectra: *VG Auto Spec-3000* spectrometer (*VG*, Manchester, England). X-Ray diffraction data: *D/max-3B* X-ray diffractometer (*Rigaku*, Tokyo, Japan) with a graphite monochromator, Mo$K_\alpha$ radiation.

*Plant Material.* The whole plants of *Saniculiphyllum guangxiense* were collected in Funing county, Wenshan prefecture, Yunnan Province, in March 2010. The sample was identified as *Saniculiphyllum*

*guangxiense* by *L.-G. L.* A voucher specimen (No. L.-G. Lei 20090403 (KUN)) was deposited with the Laboratory of Antivirus and Natural Medicinal Chemistry, Kunming Institute of Botany.

*Extraction and Isolation.* Air-dried and powdered whole plants of *S. guangxiense* (200.0 g) were extracted with MeOH (1.0 l) under reflux for three times, each time for 2 h. After removing the solvent *in vacuo*, the crude extract (6.0 g) was dissolved in 90% MeOH/$H_2O$ (150 ml), and defatted with petroleum ether (PE; 200 ml × 3), to give a residue (5.0 g). The residue was fractionated over a $SiO_2$ column (100 g; 3.0 × 20.0 cm) with $CHCl_3$/MeOH (from 95 : 5 to 80 : 20 (*v*/*v*)), to afford fractions *A*–*D*. *Fr. A* (70 mg) was chromatographed over a $SiO_2$ column (20 g; 1.4 × 20 cm), eluted with PE/acetone (from 90 : 10 to 70 : 30 (*v*/*v*)), and further purified by recrystallization (PE/acetone 3 : 1 (*v*/*v*)) to give compounds **7** (20 mg) and **5** (15 mg). *Fr. B* (1.2 g) was subjected to a $SiO_2$ column (30 g; 1.7 × 21 cm) and eluted with $CHCl_3$/acetone (from 90 : 10 to 75 : 25, *v*/*v*), and then purified by repeated CC involving *Sephadex LH-20* (50 g; 1.4 × 145 cm, MeOH), $SiO_2$ (20 g; 1.4 × 20 cm, $CHCl_3$/MeOH 95 : 5 (*v*/*v*)). After recrystallization (MeOH), compounds **6** (13 mg), **4** (20 mg), and **3** (23 mg) were obtained. *Fr. C* (2.0 g) was loaded on a $SiO_2$ column (30 g; 1.7 × 21 cm, MeOH/$H_2O$ 90 : 10, *v*/*v*), and further chromatographed on *RP-18* gel (40 g; 2 × 21 cm, MeOH/$H_2O$ 5 : 95 → 25 : 75 (*v*/*v*)) and a *Sephadex LH-20* column (70 g; 2.5 × 40 cm, MeOH) to afford compounds **8** (20 mg), **1** (120 mg), and **2** (30 mg).

In vitro *Anti-HBV Assay.* The anti-HBV assay was carried out according to our previous reports [17–21]. Compounds **1**–**6** were evaluated for their anti-HBV activities and cytotoxicity on the Hep G 2.2.15 cell line, which was stably transfected with HBV genome using *Lipofectamine 2000* reagent (*Invitrogen*, Carlsbad, CA, USA). All the tested samples were dissolved in DMSO (*Gibco*, solvent control) for the anti-HBV activity and cytotoxicity assays. The concentration of DMSO in the culture was kept below 2.5 µl/ml to ensure that the growth of the cells was not affected.

*Assay for HBV Antigen Secretion.* The anti-HBV antigen secretion activities were assayed by enzyme-linked immunosorbent assay (ELISA; *Autobio Diagnostics Co., Ltd.*, P.R. China). Hep G 2.2.15 Cells were seeded in a 48-well microplate at a density of $3 \times 10^4$ cells/well and cultured for 72 h at 37° under 5% $CO_2$. The culture medium was replaced with fresh medium (with or without the tested compounds, or with the positive control) and the cells were cultured for an additional 72 h. The culture media were collected and tested for HBsAg and HBeAg levels using ELISAs (*Autobio Diagnostics Co., Ltd.*). The absorbance (*A*) of each well was measured at 490 nm with a microplate reader (*Model 680*, *Bio-Rad, Inc.*, USA).

*Assay for HBV DNA Replication.* Hep G 2.2.15 Cells were seeded in a 24-well microplate at a density of $5 \times 10^5$ cells/well and cultured for 72 h at 37° under 5% $CO_2$. After refreshing the culture medium, the cells were cultured for additional 48 h, and this procedure was repeated once again. Cells were collected and total DNA was isolated by using a *TIANamp Gemomic DNA Kit* (*TIANGEN*, *Biotech Co., Ltd.*, P. R. China) according to the manufacturer's instructions. The real-time PCR assay was used to detect the HBV DNA. Briefly, 10 µl of DNA sample was amplified in a mixture (25 µl) containing *2 × SYBR Green PCR Master Mix* (*Applied Biosystems*, USA) and two primers specific for HBV: a forward primer (HBV-t1: 5′-CAA GGA ACC TCT ATG TAT CCC TCC-3′) and reverse primer (HBV-t2 : 5′-TCC GTC CGA AGG TTT GGT AC-3′) covering the 50-base pair insertion from 541 bp to 591 bp. Amplification and detection were performed in the *Mastercycler Ep Realplex System* (*Eppendorf*, *Masteraycler Eprealplex*, Germany) with incubation at 95° for 2 min and, subsequently, 40 three-step cycles (20 s at 95°; 15 s at 58°; 20 min at 72°) were performed. The standard was prepared by serial dilutions of a known amount of the cloned HBV plasmid pCP10, carrying two head-to-tail copies of the HBV genome as positive control. The specificity of two primers (HBV-t1 and HBVt2) was confirmed in every PCR run by dissociation curve analysis.

*Cytotoxicity Assay.* Cytotoxicity was assayed with a modified MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (*Gibco Invitrogen*, Carlsbad, CA, USA). After the culture medium used for the HBV antigen secretion assay was removed, the plates were air dried and MTT (400 µg/ml, 200 µl/well) was added. 4 h later, the MTT was removed and replaced with DMSO (300 µl/well), and the samples were incubated for 10 min. The supernatants (100 µl) were then transferred to 96-well plates, and the absorbance (*A*) was measured at 490 nm with an automatic plate reader (*Model 680*, *Bio-Rad Inc.*).

*Cell Line and Cell Culture.* Hep G 2.2.15 Cells used widely to assay anti-HBV activities are derived from the Hep G 2 hepatoma cell line (American Type Culture Collection, Manassas, VA, USA). In this study, Hep G 2.2.15 cells were cultured in *RPMI-1640* medium (*Gibco*) supplemented with 10% fetal calf serum (*Gibco*), 100 µg/ml G148 (*Gibco*), 100 IU/ml penicillin (*Gibco*), and 100 IU/ml streptomycin (*Gibco*), and maintained at 37° in a moist atmosphere containing 5% $CO_2$.

*16β-Hydroxybryodulcosigenin* (*=(1*S*,4*R*,9β,16β,24*R*)-1,16,24,25-Tetrahydroxy-9,10,14-trimethyl-4,9-cyclo-9,10-secocholest-5-en-11-one*; **3**). White powder. $C_{30}H_{50}O_5$, $M_w$ 490. $[\alpha]_D^{24.9} = +112.0$ ($c = 0.16$, $CHCl_3$). IR (KBr): 3427, 2964, 2928, 1691, 1640, 1466, 1376, 1023, 961. $^1$H- and $^{13}$C-NMR: see *Table 1*. EI-MS: 491 (1, $[M+H]^+$), 472 (1, $[M-H_2O]^+$), 457 (14), 436 (7), 421 (8), 395 (8), 309 (22), 270 (15), 243 (22), 175 (28), 119 (24), 105 (29), 59 (100). HR-ESI-MS (pos.): 490.3661 ($M^+$, $C_{30}H_{50}O_5^+$; calc. 490.3658).

*3α-*O*-Feruloylolean-12-en-27-oic Acid* (*=(3α)-3-{[(2*E*)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-enoyl]oxy}olean-12-en-27-oic Acid*; **6**). White powder. $C_{40}H_{56}O_6$. $M_w$ 632. $[\alpha]_D^{25.2} = -3.8$ ($c = 0.24$, $CHCl_3$). UV ($CHCl_3$): 321 (4.08), 297 (3.97). IR (KBr): 3415, 2950, 2861, 1708, 1686, 1631, 1594, 1516, 1465, 1386, 1267, 1169, 1159, 1035, 979. $^1$H- and $^{13}$C-NMR: see *Table 1*. EI-MS: 632 (3, $M^+$), 588 (10), 573 (7), 379 (22), 241 (28), 190 (46), 177 (100), 145 (22). ESI-MS (neg.): 631 ($[M-H]^-$). HR-ESI-MS (neg.): 631.3982 ($M^-$, $C_{40}H_{55}O_6^-$; calc. 631.3998).

*Crystallographic Data of Compound* **1**[1]). $C_{14}H_{23}NO_9$ ($C_{14}H_{19}NO_7 + 2\ H_2O$), $M_w$ 349.33; monoclinic, space group $P2_1$; $a = 8.9160(13)$, $b = 6.6620(10)$, $c = 13.908(2)$ Å, $\alpha = 90$, $\beta = 93.642(2)$, $\gamma = 90°$, $V = 824.4(2)$ Å$^3$, $Z = 2$, $d = 1.407$ g/cm$^3$, crystal dimensions $0.32 \times 0.20 \times 0.12$ nm$^3$ were used for measurement on a SHELXL-97 with a graphite monochromator, Mo$K_\alpha$ radiation. The total number of reflections measured was 3631, of which 2811 were observed, $I > 2\sigma(I)$. Final indices: $R_1 = 0.0253$, w$R_2 = 0.0509$.

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[1]) The crystal structure of compound **1** was solved by direct method of SHLXS-97 [22] and expanded using difference *Fourier* technique, refined by the program SHLXL-97 [23] and the full-matrix least-squares calculations. Crystallographic data for compound **1** have been deposited with the *Cambridge Crystallographic Data Centre* (deposition number: CCDC-816432). Copies of these data can be obtained free of charge *via* www.ccdc.cam.ac.uk/conts/retrieving.htm (or from the *Cambridge Crystallographic Data Centre*, 12, Union Road, Cambridge CB21EZ, UK; fax: +44 1223-336-033; or desposit@ccdc.cam.ac.uk).

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