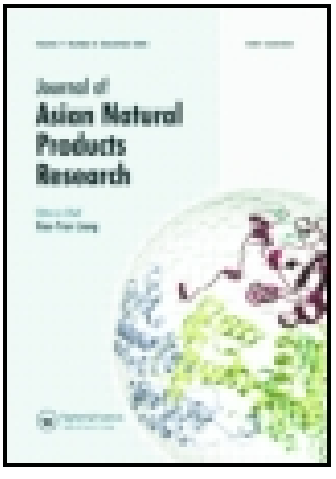

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Two new coumarin glycosides from *Herpetospermum caudigerum*

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Phytochemical investigation on the seeds of *Herpetospermum caudigerum* afforded two new coumarin glycosides A and B (**1** and **2**), together with a known compound herpetolide A (**3**). Their structures were determined by spectroscopic methods, including 2D NMR techniques. Compound **3** exhibited cytotoxicity against HepG2 cells with IC_{50} value of 6.47 μg/ml. Compound **2** showed anti-hepatitis B virus activity with HBsAg secretion by 33.1% at 200 μg/ml.

Keywords: *Herpetospermum caudigerum*; coumarin glycoside; herpetosperin A; herpetosperin B

1. Introduction

Herpetospermum caudigerum WALL (Cucurbitaceae) is distributed in southwest China, Nepal, and northeast India. The dried ripe seeds of *H. caudigerum* have been used for the treatment of liver diseases as a Tibetan folk medicine in China [1]. The ethyl acetate extract from the seeds of *H. caudigerum* showed protective effects on carbon tetrachloride (CCl_4) or thioacetamide-induced acute hepatic injuries in mice [2]. Several phenylpropanoid compounds, including lignans and coumarins, had been previously reported from *H. caudigerum*, and some of them exhibited protective effects on CCl_4-induced hepatocyte injury and anti-Hepatitis B virus effect [2–12]. To find natural bioactive compounds, phytochemical investigation on the seeds of *H. caudigerum* led to the isolation of two new coumarin glycosides, herpetosperins A and B (**1** and **2**, Figure 1), and a known compound, herpetolide A (**3**) [2]. This paper deals with the isolation and structural elucidation of the new compounds as well as the *in vitro* tests on cytotoxicity and anti-hepatitis B virus (HBV) activity of them.

2. Results and discussion

Compound **1** (Figure 1) was obtained as a white powder. The molecular formula was determined as $C_{22}H_{24}O_{11}$ by HR-ESI-MS (*m/z* 487.1202 $[M + Na]^+$), which indicated 11 degrees of unsaturation. The IR spectrum suggested the presence of hydroxyls (3428.6 cm^{-1}) and a carbonyl group (1699.8 cm^{-1}). The presence of a 1,2,4,5-tetrasubstituted benzene ring and a 1,2,3,5-tetrasubstituted benzene ring was indicated from the ^1H NMR spectrum at δ_H 8.12 (1H, s), 7.85 (1H, s), 7.09 (1H, d, $J = 2.6$ Hz), and 6.99 (1H, d, $J = 2.6$ Hz), which was supported by the corresponding 12 downfield signals in the ^{13}C NMR spectrum (Table 1). The ^1H and ^{13}C NMR spectra of **1** also suggested the presence of two methoxyl groups at δ_H 3.86 and 3.99 (each 3H, s), a hydroxymenthyl group [δ_H 4.92 (2H, d, $J = 4.4$ Hz), δ_C 63.8], and a

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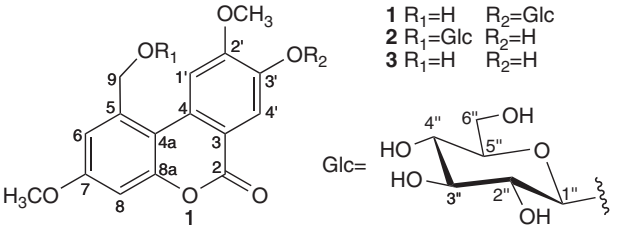

Figure 1. Structures of compounds **1**–**3**.

conjugated carbonyl group at δ_C 160.4. In addition, six proton signals were found in the range of δ_H 3.0–4.0, together with the signal at δ_H 5.12 (1H, d, $J = 6.5$ Hz) assigned as the anomeric proton, suggesting the existence of a sugar moiety. The typical ^{13}C NMR signals at δ_C 100.7 (C-1″), 73.7 (C-2″), 77.7 (C-3″), 69.9 (C-4″), 77.1 (C-5″), and 61.0 (C-6″) confirmed the substructure [13]. The ^{1}H and ^{13}C NMR spectra (Table 1) of the aglycone moiety of **1** were similar to those of **3** which implied that they possessed a similar coumarin skeleton.

The HMBC correlations (Figure 2) of H-6 (δ_H 7.09) with C-7 (δ_C 159.9), C-8 (δ_C 101.5), and C-9 (δ_C 63.8), H-8 (δ_H 6.99) with C-7, C-8a, and C-4a (δ_C 110.7), H_2-9 (δ_H 4.92) with C-6 (δ_C 115.3), C-5 (δ_C 140.5), and C-4a, and the methoxyl at δ_H

Table 1. ^{1}H and ^{13}C NMR spectral data (400 and 100 MHz, respectively, in DMSO-d_6) of compounds **1** and **2**.

	1		**2**	
Position	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C
2		160.4		160.5
3		113.4		113.6
4		131.3		128.7
4a		110.7		111.7
5		140.5		135.5
6	7.09 (1H, d, 2.6)	115.3	7.17 (1H, d, 2.8)	116.7
7		159.9		159.3
8	6.99 (1H, d, 2.6)	101.5	7.01 (1H, d, 2.8)	102.3
8a		153.3		152.8
1′	8.12 (1H, s)	110.0	7.95 (1H, s)	109.5
2′		155.2		154.5
3′		146.5		147.3
4′	7.85 (1H, s)	114.8	7.60 (1H, s)	114.6
9	4.92 (2H, d, 4.4)	63.8	5.34 (1H, d,11.8) 4.91 (1H, d,11.8)	69.8
7-OCH_3	3.86 (1H, s)	56.2	3.86 (1H, s)	56.2
2′-OCH_3	3.99 s	56.4	4.02 (1H, s)	56.7
1″	5.12 (1H, d, 6.5)	100.7	4.43 (1H, d, 7.7)	102.1
2″	3.30–3.32 (1H, m)	73.7	3.07–3.10 (1H, m)	74.1
3″	3.41–3.43 (1H, m)	77.7	3.17–3.19 (1H, m)	77.6
4″	3.23–3.26 (1H, m)	69.9	3.03–3.06 (1H, m)	70.9
5″	3.37–3.39 (1H, m)	77.1	3.14–3.16 (1H, m)	77.3
6″	3.67 (1H, dd, 10.9, 4.7) 3.48–3.54 (1H, m)	61.0	3.74 (1H, dd, 10.3, 7.0) 3.41–3.43 (1H, m)	61.8
9-OH	5.83 (1H, t, 4.7)			
3′-OH			10.53 (1H, br s)	

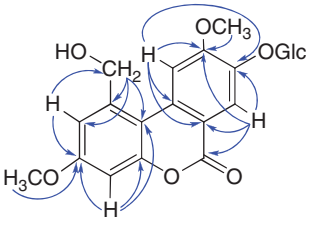

Figure 2. Key HMBC correlations of **1**.

3.86 with C-7 indicated that the hydroxymenthyl group (—CH_2OH) and methoxyl group were substituted at C-5 and C-7, respectively. HMBC cross peaks of H-1′ (δ_H 8.12) with C-3 (δ_C 113.4), C-4 (δ_C 131.3), C-2′ (δ_C 155.2), and C-3′ (δ_C 146.5), H-4′ (δ_H 7.85) with C-2 (δ_C 160.4), C-3, C-2′, and C-3′ deduced that C═O was located at C-2. The methoxyl at δ_H 3.99 with C-2′ in HMBC spectrum confirmed that the methoxyl group was substituted at C-2′. Comparison of the NMR data of **1** with those of **3** showed that the OH group at C-3′ in **3** was replaced by a β-glucopyranosyl in **1**, as confirmed by the HMBC correlations of H-1″ at δ_H 5.12 (1H, d, $J = 6.5$ Hz) with C-3′ at δ_C 146.5. Acid hydrolysis of **1** gave D-glucose, which was identified by comparing with an authentic sample. On the basis of the above evidence, the structure of **1** was elucidated as shown in Figure 1, and it was named as herpetosperin A.

Compound **2** was obtained as a white powder. The molecular formula was determined to be $C_{22}H_{24}O_{11}$ on the basis of HR-ESI-MS (*m/z* 487.1205 $[M + H]^+$), which indicated 11 degrees of unsaturation. The IR spectrum suggested the presence of hydroxyls (3432.4 cm^{-1}) and a carbonyl group (1671.4 cm^{-1}). The ^1H and ^{13}C NMR (Table 1) spectra of **2** showed the proton signals at δ_H 7.95 (1H, s), 7.60 (1H, s), 7.17 (1H, d, $J = 2.8$ Hz), and 7.01 (1H, d, $J = 2.8$ Hz) and 12 downfield carbon signals ranged from δ_C 102.3 to δ_C 159.3, indicating the presence of a 1,2,4,5-tetrasubstituted benzene ring and a 1,2,3,5-tetrasubstituted benzene ring. The ^1H NMR spectrum of **2** also suggested the presence of two methoxyl groups at δ_H 3.86 and 4.02 (each 3H, s), a hydroxylmenthyl group at δ_H 4.91 (1H, d, $J = 11.8$ Hz) and 5.34 (1H, d, $J = 11.8$ Hz), and a conjugated carbonyl group at δ_C 160.5. A sugar group was also observed by typical protons at δ_H 4.43 (1H, d, $J = 7.7$ Hz) and 3.0–4.0 (6H, m), in the ^1H NMR spectrum and six corresponding carbon signals at δ_C 102.1, 74.1, 77.6, 70.9, 77.3, and 61.8 in the ^{13}C NMR spectrum.

The ^1H and ^{13}C NMR spectra of **2** (Table 1) were very similar to those of **1**, except the sugar moiety was substituted at C-9 in **2**, while it was substituted at C-3′ in **1**, which was confirmed by the HMBC correlation of H-1″ (δ_C 4.43, 1H, d, $J = 7.7$ Hz) with C-9 (δ_C 69.8), as well as H_2-9 (δ_H 5.34, 1H, d, $J = 11.8$ Hz and 4.91, 1H, d, $J = 11.8$ Hz) with C-1″ (δ_C 102.1). Thus, the structure of **2** was elucidated as shown in Figure 1, and it was named as herpetosperin B.

Compounds **1**–**3** were evaluated *in vitro* for cytotoxicity against human lung carcinoma A549, human hepatoma HepG2, and human colorectal carcinoma HCT-8 cell lines employing a MTT assay with vincristine sulfate as the positive control. Among them, **3** exhibited cytotoxicity against HepG2 cells with IC_{50} value of 6.47 μg/ml, while the positive control was 1.91 μg/ml. All the compounds were also tested for their antiviral activity against HBV *in vitro*. Among them, **2** showed anti-HBV activity with HBsAg secretion by 33.1% at 200 μg/ml, while the positive control (lamivudine) was 28.6% at the same concentration.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a *P-1020* digital spectropolarimeter (JASCO, Tokyo, Japan) with MeOH as solvent. UV spectra were recorded on a Hitachi U-3010

spectrophotometer (HITACHI, Tokyo, Japan) in MeOH, λ_{max} (log ε) in nm. IR spectra were recorded on a Perkin Elmer Spectrum GX spectrophotometer (Perkin Elmer, Waltham, MA, USA) with a KBr disk. ^{1}H, ^{13}C, and 2D NMR spectra were run on a Bruker DRX400 spectrometer (Bruker, Ettlingen, Germany) in DMSO-d_6, δ in ppm rel. to Me_4Si as internal references, J in Hz. HR-ESI-MS were run on a Bruker Dalonics-BioToF IIIQ mass spectrometer, obtained in positive-ion mode. Semi-prep HPLC was carried out with a Shimadzu LC-20A High Performance Liquid Chromatography (RP-18, 250 × 10 mm, YMC, 10 μm, detector: UV) (Shimadzu, Kyoto, Japan). TLC was performed on silica gel plates GF254 (Yan-Tai Institute of Chemical Technology, Yantai, China). The spots on TLC were visualized by UV light (254 nm) and sprayed with 10% H_2SO_4, followed by heating. Column chromatography (CC) was carried out on a silica gel (200–300 mesh or 300–400 mesh, Qingdao Marine Chemical Factory, Qingdao, China).

3.2. Plant material

Seeds of *H. caudigerum* were collected in Lin-Zhi County, Tibet Autonomous Region, China in July of 2010, and identified by Professor Xiao-Zhong Lan at the Agricultural and Animal Husbandry College, Tibet University. A voucher specimen (No. 2010-CM01) has been deposited in the Herbarium of Medicinal Plants, College of Pharmaceutical Sciences, Southwest University, Chongqing, China.

3.3. Extraction and isolation

The dried and powdered material (22.5 kg) was extracted exhaustively with 95% EtOH for five times, and filtered. The filtrate was evaporated *in vacuo* to give a residue (1860 g), a portion of which (1800 g) was suspended in H_2O (1.5 l) and partitioned with ethyl acetate (3 × 2 l). The combined ethyl acetate solution was concentrated to yield a residue (175 g), which was subjected to CC on silica gel (100–200 mesh, 2 kg, 10 × 120 cm) eluted successively with $CHCl_3$, $CHCl_3$–CH_3OH (30:1, 25:1, 20:1, 15:1, 8:1, 6:1, 3:1, 1:1, v/v) and CH_3OH to yield fractions 1–8. Fr. 2 (4.1 g) was subjected repeated ODS silica gel CC with CH_3OH–H_2O (80:20–100:0, v/v) to yield **3** (8 mg). Fr. 7 (12.0 g) was subjected to ODS silica gel CC with CH_3OH–H_2O (20:80–100:0, v/v) to give four subfractions. Fr. 7-2 (0.8 g) was purified by semi-preparative HPLC with MeOH–H_2O (30:70) to yield **1** (15 mg, t_R 35.7 min) and **2** (13 mg, t_R 31.2 min).

3.3.1. Herpetosperin A (= *5-(hydroxymethyl)-7,2′-dimethoxy-3′-(2″,3″,4″-trihydroxy-5″-(hydroxymethyl)-tetrahydro-2H-pyran-1″-yloxy)-6H-benzo-[c] chromen-2-one;* ***1****)*

White powder; $[\alpha]_D^{25}$ + 84.8 (c = 0.30, CH_3OH); UV (CH_3OH): λ_{max} (log ε) 192 (2.28), 242 (2.38), 261 (2.42), 288 (2.46), 296 (2.47) nm; IR (KBr): ν_{max} 3428.6, 2932.8, 1699.8, 1547.2, 1501.6, 1465.4, 1399.6, 1303.2, 1249.2, 1095.8, 1002.2, 911.4, 846.8, 709.6 cm^{-1}. For ^{1}H and ^{13}C NMR spectral data, see Table 1. HR-ESI-MS: *m/z* 487.1202 $[M + Na]^+$ (calcd for $C_{22}H_{24}NaO_{11}$, 487.1211).

3.3.2. Herpetosperin B (= *3′-hydroxy-7,2′-dimethoxy-9-((2″,3″,4″-trihydroxy-5″-(hydroxymethyl)tetrahydro-2H-pyran-1″-yloxy)methyl)-6H-benzo[c] chromen-2-one;* ***2****)*

White powder; $[\alpha]_D^{25}$ + 98.6 (c = 0.30, CH_3OH); UV (CH_3OH): λ_{max} (log ε) 224 (2.35), 262 (2.42), 276 (2.44), 296 (2.47), 307 (2.49), 337 (2.53) nm; IR (KBr): ν_{max} 3432.4, 2945.6, 1671.4, 1594.6, 1482.2, 1406.8, 1314.6, 1196.2, 1069.2, 1023.2, 998.8, 678.8 cm^{-1}; for ^{1}H and ^{13}C NMR spectral data, see Table 1. HR-ESI-MS:

m/z 487.1205 $[M + Na]^+$ (calcd for $C_{22}H_{24}NaO_{11}$, 487.1211).

3.4. Acid hydrolysis of 1 and 2

The compounds (2 mg each) were hydrolyzed in 2 ml of 2 N HCl for 1 h at 80–90°C. The resulting mixtures were extracted with EtOAc (2 × 2 ml). The aqueous layers were concentrated to dryness. The residue was heated with L-cysteine methyl ester in pyridine at 60°C for 1 h. Sugar standards (D/L) were also derivatized using L-cycteine methyl ester in the same manner. The reaction mixtures were analyzed using C18 HPLC at 35°C with isocratic elution of 25% CH_3CN in 50 mM H_3PO_4 for 40 min and subsequent washing of the column with 90% CH_3CN at a flow rate of 0.8 ml/min with UV detector at 250 nm. By comparing the retention times with the authentic samples with 19.37 min of D-glucose and 18.2 min of L-glucose, the sugar in compounds **1** and **2** was identified as D-glucose.

3.5. Cytotoxicity assay

Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000–10,000 cells per well with compounds added from DMSO-diluted stock. After 3 days culture, attached cells were incubated with cold 10% trichloroacetic acid (MTT, 0.5 mg/ml, 2 h) and subsequently solubilized in DMSO, and the isolates were tested against A549, HepG2, and HCT-8 cancer cell lines using established colorimetric MTT assay protocols [14]. Vincristine sulfate was used as a positive control. All stock cultures were grown in T-25 flasks. The mean IC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant. The absorbance was measured at 550 nm using a microplate reader.

3.6. Anti-HBV test

Drug stock solutions were prepared in DMSO and stored at −70°C. Upon dilution into culture medium, the final DMSO concentration was < 1% (v/v). Cell culture and other procedures were the same as the report [15]. A HepG2-derived human hepatablastoma cell line, 2.2.15, was used in this study, which was transfected with cloned HBV DNA to produce HBV particles. All stock cultures were grown in T-25 flasks containing the Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum, 0.03% (v/v) L-glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 380 μg/ml G418 at 37°C in a humidified atmosphere containing 5% CO_2. After being cultured for 48 h, the HepG2 2.2.15 cells were incubated at 37°C for 9 days in the presence of various concentrations of drugs (200, 100, 50, and 25 μg/ml, respectively) from DMSO-diluted stock, and the medium was refreshed every 3 days. Then the culture supernatants were harvested to detect the HBsAg secretion using diagnostic ELISA kits (Shanghai SIIC KEHUA Biotech Co., Ltd, Shanghai, China) in triplicate, and the standard error of the mean of inhibition values varied no more than 5%. Cell damage was assessed using MTT assay.

Disclosure statement

No potential conflict of interest was reported by the authors.

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