SUPPLEMENTARY MATERIAL

Chemical Profiling of Anti-Hepatocellular Carcinoma Constituents from *Caragana tangutica* Maxim. by Off-line Semi-preparative HPLC-NMR

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ABSTRACT

An EtOAc fraction from the roots of *Caragana tangutica* Maxim. (CTEA) displayed promising anti-hepatocellular carcinoma (HCC) activity during screening of a traditional Chinese ethnic herb library against HepG2 and Hep3B cell lines. HPLC-based activity profiling of CTEA by combination of MS-guided large scale semi-preparative HPLC and NMR methods led to the identification of a new pterocarpan glycoside, (-)-maackiain 3-O-6'-O-methyl malonyl- β -D-glucopyranoside (**1**), together with three known pterocarpan glycosides, (-)-maackiain 3-O- β -D-glucopyranoside (**2**), 3-O-6'-O-acrylyl- β -D-galactopyranoside (**3**), and (-)-maackiain 3-O-6'-O-acetyl- β -D-glucopyranoside (**4**). Compound **1** was isolated during a drug discovery program aimed at identifying new anti-HCC leads from a natural product library. Anti-HCC study showed that all four compounds exhibited cytotoxic activity with IC₅₀ values range of 29.1–53.5 μ g/mL against HepG2 and Hep3B cell lines.

Key words: anti-hepatocellular carcinoma; tibetan medicines; *Caragana tangutica*; pterocarpan glycosides

1. *In vitro* anti-HCC assay of compounds 1-4

Compounds **1–4** isolated from this Tibetan herb were evaluated against HepG2 and Hep3B HCC cell lines. A human embryonic kidney cell line HEK293 was used to investigate the preliminary toxicity toward human cells. Four isolated compounds **1–4** displayed cytotoxicity against HepG2 and Hep3B with IC₅₀ value range between 29.1 and 53.5 µg/mL (Table S1), and showed no cytotoxicity against HEK293 up to 200 µg/mL. Our results showed that all four compounds exhibited cytotoxic activity against HepG2 and Hep3B cell lines.

2. Experimental

2.1. General experimental procedures

Semi-preparative HPLC was carried out on a Waters 2535 HPLC fitted with a 2998 Photodiode Array Detector and a 2707 Autosampler (Waters). Separations were performed on two Waters Sunfire™ C18 columns (5 µm, 10 × 150 mm; 5 µm, 20 × 250 mm) (Waters, Ireland). Direct injection ESIMS and LC-PDA-ESIMS analyses were recorded on a Waters ACQUITY SQD MS system (Waters, USA) connected to a Waters 1525 HPLC with a 2998 Photodiode Array Detector (Waters, USA). The NMR spectra were recorded on an AVANCE III 600 MHz spectrometer (Bruker BioSpin, Germany). Optical rotations were recorded on a Jasco P-1020 polarimeter. CD spectra were recorded on a JASCO J-720W spectrophotometer (JASCO, Japan). UV and IR spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer (Agilent, USA) and a Nicolet Magna FT-IR 750 spectrometer (Nicolet, USA), respectively. All the solvents used for chromatography were of high performance liquid chromatography (HPLC) grade and all the other chemicals were of analytical-reagent grade. HPLC-grade acetonitrile (MeCN) was purchased from Merck Chemical Company (Darmstadt, Germany). Sephadex LH-20 gel was obtained from GE Health Care (Uppsala, Sweden).

2.2. Plant Material

The roots of *Caragana tangutica* Maxim. were collected in July, 2013 in Qilian County, Qinghai province, China, and identified by Prof. Jingyuan Yuan, Guangxi

Botanical Garden of Medicinal Plants. A voucher specimen (No. CT20130711) is deposited in School of Pharmaceutical Sciences, South-Central University for Nationalities, Wuhan, China.

2.3. Sample Preparation

Air-dried roots of *C. tangutica* (30 g) were ground and then extracted sequentially by maceration at room temperature with *n*-hexane (3 × 500 mL, 3 h each), followed by ethyl acetate (3 × 500 mL, 3 h each) and methanol (3 × 500 mL, 3 h each). The solvents were evaporated at reduced pressure to yield 0.58 g, 1.37 g, and 2.35 g of *n*-hexane, ethyl acetate and methanol fractions, respectively.

2.4. Microfractionation for Activity Profiling

The semi-preparative HPLC was performed with a linear gradient of acetonitrile in water from 10% to 100% in 20 min, to 100% in 25 min delivered at 5.0 mL/min (two mobile phases contained 0.1% formic acid). 20 µL of extract (100 mg/mL in DMSO) were injected. A total of 11 time-based microfractions of 120 s each were collected. Solvent removal of microfractions was carried out with a centrifugal evaporator (Virtis Vac-Freeze EL).

2.5. ESIMS Method

ESIMS analyses by direct injection were recorded on a Waters ACQUITY SQD MS system (Waters, USA). ESIMS were recorded in both positive and negative ion modes. The capillary voltage was 4000 V, the capillary exit voltage was 140.0 V, and the skimmer voltage was 40 V. The nebulizer gas pressure was set to 40 psi, the dry gas flow to 10.0 L/min and the dry temperature to 320 °C. Mass range was set from *m/z* 120–1500. Data acquisition and processing were achieved with MassLynx™ 4.0 software (Waters, USA).

2.6. NMR Experiments

The NMR spectra were recorded in DMSO-*d*₆ on an AVANCE III 600 MHz spectrometer equipped with 1.4 mm heavy wall Micro NMR tubes (NORELL, USA). The active volume of the 1.4 mm heavy wall Micro NMR tube is about 150 µL. Typically, ¹H spectra were obtained at 128 scans. ROESY spectra were obtained at the scan range of 4–32. The HSQC and HMBC spectra were obtained at the scan range of

16–96.

2.7. Large Scale Semi-preparative HPLC and Sephadex Micro-column Method

1.10 g of the ethyl acetate fraction of *C. tangutica* was preadsorbed to C₁₈-bonded silica (1.0 g) and then packed into a stainless steel cartridge (20 mm × 30 mm) and attached to the Waters Sunfire C₁₈ semipreparative HPLC column (250 mm × 20 mm, 5 μm). Isocratic HPLC conditions of 90% H₂O (0.1% formic acid)/10% MeCN (0.1% formic acid) were initially employed for the first 5 min, and then a linear gradient to 60% MeCN (0.1% formic acid) was run over 45 min, followed by a linear gradient to 100% MeCN (0.1% formic acid) for a further 10 min, all at a flow rate of 10 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min, then analyzed by ESIMS with direct injection. Fractions 21–25 contained the target ion (*m/z* 447/469/893), fractions 26–30 contained the target ion (*m/z* 501/523/1023), and fractions 31–35 contained two other target ion (*m/z* 547/1093/1115 and 489/511/977). Fractions containing target ions were combined and evaporated and to dry, respectively. The residue combined by fractions 31–35 was subjected to semi-preparative HPLC (CH₃CN in H₂O from 20% to 70%, 50 min) with the Waters Sunfire C₁₈ semi-preparative HPLC column (150 mm × 10 mm, 5 μm) to yield **1** (1.8 mg, *t_R* 24.3 min) and **4** (4.7 mg, *t_R* 26.7 min), respectively. Two other residues combined by fractions 21–25 and 26–30 respectively were purified through a Sephadex LH-20 micro-column (300 mm × 10 mm) eluted by methanol (containing 0.1% formic acid) with the flow rate at 0.5 ml/min. The eluents were analyzed by the analytical HPLC and combined to yield pure compounds, **2** (2.6 mg) and **3** (3.9 mg). Compounds **1–4** were dissolved in DMSO-*d*₆ for further NMR tests.

2.8. (-)-*maackiain* 3-*O*-6'-*O*-methyl malonyl-β-*D*-glucopyranoside

Light yellow powder, C₂₆H₂₆O₁₃; [α]_D²⁰ - 105.7 (*c* = 0.1, MeOH); CD (*c* = 0.1, MeOH) Δε: - 8.9 (231 nm), - 2.2 (279 nm), + 3.9 (311 nm); UV (MeOH) λ_{max} (log ε) (nm): 280 (3.21), 285 (3.28), 311 (3.66); IR (KBr) ν_{max} (cm⁻¹): 3402, 1725, 1621, 1599, 1498, 1251, 1162, 1058; ESIMS *m/z*: 569 [M + Na]⁺, 547 [M + H]⁺; HRESIMS *m/z*: 547.1448 (calcd for C₂₆H₂₇O₁₃, 547.1446); ¹H-NMR (DMSO-*d*₆, 600 MHz): δ_H 7.37

(1H, d, $J = 8.5$ Hz, H-1), 6.98 (1H, s, H-7), 6.67 (1H, dd, $J = 8.5, 2.1$ Hz, H-2), 6.53 (1H, d, $J = 2.1$ Hz, H-4), 6.52 (1H, s, H-10), 5.94 (1H, s, OCH₂O), 5.90 (1H, s, OCH₂O), 5.55 (1H, d, $J = 6.8$ Hz, 11a), 4.26 (1H, dd, $J = 10.2, 3.7$ Hz, 6 α), 3.61 (1H, t, $J = 10.2$ Hz, 6 β), 3.60 (1H, m, 6a), 3.46 (1H, d, $J = 16.1$ Hz, H-2''), 3.51 (1H, d, $J = 16.1$ Hz, H-2''), 3.59 (3H, s, OMe), 4.87 (d, $J = 7.6$ Hz, Glu H-1'), 3.23 (1H, m, H-2'), 3.28 (1H, m, H-3'), 3.16 (1H, m, H-4'), 3.61 (1H, m, H-5'), 4.36 (1H, d, $J = 11.7$ Hz, H-6' β), 4.10 (1H, d, $J = 11.7, 7.0$ Hz, H-6' α); ¹³C-NMR (DMSO-*d*₆, 150 MHz): 131.9 (C-1), 110.3 (C-2), 158.1 (C-3), 104.0 (C-4), 156.1 (C-5), 65.9 (C-6), 40.1 (C-6a), 118.2 (C-6b), 105.3 (C-7), 141.1 (C-8), 147.5 (C-9), 93.3 (C-10), 153.6 (C-10a), 77.6 (11a), 114.2 (11b), 101.0 (OCH₂O), 99.9 (Glu C-1'), 73.0 (C-2'), 73.5 (C-3'), 69.7 (C-4'), 76.2 (C-5'), 64.3 (C-6'), 166.4 (C-1''), 40.7 (C-2''), 166.8 (C-3''), 52.1 (OMe).

2.9. Cytotoxicity Assay

Cancer cell lines HepG2 (human hepatocellular carcinoma), Hep3B (human hepatocellular carcinoma), and HEK293 (human embryonic kidney) were purchased from the American Type Culture Collection (ATCC, USA). The cytotoxic assay was performed as previously described (Song et al., 2010, 2015). Briefly, three cell lines suspended in RPMI 1640 containing 10% Fetal Bovine Serum (FBS) were seeded at 1×10^4 cells (200 μ L) per well in a flat 96-well plate, and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. After 24 h, the medium containing different concentrations of microfractions or pure compounds was added, and 1% DMSO was used as solvent control. After that, the cells were fixed with EtOH-H₂O (95:5, v/v), stained with crystal violet solution, and lysed with a solution of 0.1 N HCl in MeOH. The absorbance in control and drug treated wells was measured in an automated microplate reader (Bio-Rad 550) at 550 nm.

3. References

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HPLC-based Activity Profiling of Anti-Hepatocellular Carcinoma Constituents

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Table S1 Cytotoxic activity of compounds 1-4 from *C. tangutica*

Compounds	Cells		
	HepG2	Hep3B	HEK293
1	40.3 ± 3.6	36.4 ± 2.8	>200
2	39.8 ± 3.4	53.5 ± 4.1	>200
3	29.1 ± 2.2	34.5 ± 2.8	>200
4	36.5 ± 3.1	38.4 ± 3.6	>200
5-FU^a	7.5 ± 1.8	9.3 ± 1.5	14.7 ± 0.4

^a 5-FU was used as positive control.

Figure S1 The chromatogram of the semi-preparative HPLC separation of CTEA is shown at 254, 280 and 320 nm

Figure S2 The off-line ¹H NMR spectra of compounds 1-4 in DMSO-d₆ by 1.4 mm heavy wall Micro NMR tubes with 128 scans

Figure S3 The key long range correlations from The HMBC spectrum of **1** (1.5 mg) in DMSO-d₆ by 1.4 mm heavy wall microprobe NMR tube with 128 scans

Figure S4 ^1H NMR spectrum of compound 1

Figure S5 ¹³C NMR spectrum of compound 1

Figure S6 ^1H - ^1H COSY spectrum of compound **1**

Figure S7 HSQC spectrum of compound **1**

Figure S8 HMBC spectrum of compound **1**

Figure S9 ROESY spectrum of compound **1**