Efficient Separation of Five Flavonoids from *Oxytropis falcata* Bunge by High-Speed Counter-Current Chromatography and their Anticancer Activity

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Received: 10 June 2019; accepted: 13 Aug 2019

Oxytropis falcata Bunge, known as the “king of herbs” in Tibetan medicine, is used for treatment of hyperpyrexia, pain, wounds, inflammation, and anthrax. However, it is difficult to efficiently isolate compounds with high purity from *O. falcata* because of the complexity of traditional Tibetan medicines. In this study, the 80% ethanol elution fraction from extract by AB-8 macroporous resin column chromatography was demonstrated to have anticancer activity on human hepatoma SMMC-7721 cells in vitro. Then, a high-speed counter-current chromatography (HSCCC) method was successfully established for separation of compounds by using hexane–ethyl acetate–methanol–water (10:4:10:10, v/v/v/v) as the solvent system. Five flavonoids (7-hydroxyflavonone [1], 5,7-dihydroxy-4'-methoxy flavonol [2], 5,7-dihydroxyflavanone [3], 2',4'-dihydroxychalcone [4], and 2',4'-dihydroxydihydrochalcone [5]) were obtained in one-step separation with purities of 97.7%, 98.1%, 98.3%, 99.0%, and 98.3%, respectively. Finally, anticancer activities against the growth of SMMC-7721 cells of 5 flavonoids were confirmed. The IC₅₀ values of the separated compounds were 213.45 µg/mL, 197.74 µg/mL, 375.16 µg/mL, 17.44 µg/mL, and 136.83 µg/mL in 24 h, respectively. The present study provided a basis for further development and utilization of this medicinal herb as a source of a new potential anticancer agent.

Keywords: *Oxytropis falcata* Bunge, high-speed counter-current chromatography, flavonoids

Introduction

Oxytropis falcata Bunge, which belongs to the Leguminosae family, locally known as the “king of herbs” in Tibetan medicine, grows naturally in the northwest part of China at an altitude of 2700–4300 m and has been widely applied in Tibetan and Mongolian medicine for the treatment of ailments such as influenza, hyperpyrexia, pain, wounds, hemorrhage, inflammation, and anthrax [1]. The extracts of *O. falcata* have been reported to exhibit a wide range of biological activities, such as anticancer, antioxidant, and anti-inflammatory, among others [2–5]. In recent years, more and more attention has been drawn to the fact that *O. falcata* has stronger anticancer activity. Yang et al. investigated that essential oil and total flavonoids of *O. falcata* showed antiproliferative activity on SMMC-7721 through down-regulating secretion and expression of MMP-2 in cells [6]. Yang et al. reported that total flavonoid of *O. falcata* is a pro-apoptotic agent for hepatic cancer cells via its modulation of Bcl-2 /Bax balance [7]. Chen et al. investigated that total flavonoids of *O. falcata* influence mitochondrial apoptosis pathway [8]. However, the pharmacological activity measurements were conducted on complex extracts, and what kind of compounds and how each of these compounds contributed to the biological effects are not clearly understood. Therefore, it is very necessary to isolate the active compounds from *O. falcata*.

Previous studies have shown that the major phytochemicals in *O. falcata* are flavonoids, alkaloids, and triterpenoids [9, 10]. With regard to the methods available for the separation of compounds from *O. falcata*, silica and gel chromatography methods are the two classical methods that were applied. However, these two conventional strategies are time-consuming, labor-intensive, and expensive process. Recently, preparative high-performance liquid chromatography (prep-HPLC) has been used for separating compounds from *O. falcata* [11]. However, prep-HPLC has low sample loading capacity and low recovery potential.

High-speed counter-current chromatography (HSCCC) is an optimal choice, which is a continuous liquid–liquid partition chromatography between two immiscible liquid phases without a support matrix. Compared to conventional column chromatography and pre-HPLC, HSCCC has advantages of eliminating irreversible adsorption, lowering the risk of sample denaturation, increasing total sample recovery, raising load capacity, and lowering the cost [12]. HSCCC has been successfully applied to purify many bioactive compounds from natural products [13–15].

To the best of our knowledge, no article has been successfully published about the use of HSCCC for separation and purification of anticancer compounds from *O. falcata*. In this article, 5 flavonoids with high purities were obtained by HSCCC for the first time, and the anticancer activities of these 5 flavonoids were studied. The structures of the 5 flavonoids are shown in Figure 1.

Experimental

Chemicals and Materials. All solvents that were used in HSCCC separation were of analytical grade. 3-(4,5-

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Figure 1. Chemical structures of compounds from *O. falcata* (1: 7-hydroxyflavonone, $C_{15}H_{10}O_4$; 2: 5,7-dihydroxy-4'-methoxy flavonol, $C_{15}H_{10}O_4$; 3: 5,7-dihydroxyflavanone, $C_{15}H_{12}O_4$; 4: 2',4'-dihydroxychalcone, $C_{15}H_{11}O_3$; 5: 2',4'-dihydroxydihydrochalcone, $C_{15}H_{13}O_3$)

Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). AB-8 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjing, China).

The whole plant of *O. falcata* was collected from Qinghai Lake of Qinghai Province, China, in August 2017. The plant was identified by Professor Dejun Zhang (Qinghai University). A voucher herbarium specimen was deposited at Northwest Institute of Plateau Biology (Xining, China) with the voucher number 20180809-O.

Apparatus. The HSCCC system was equipped with 3 polytetrafluoroethylene preparative coils (total volume = 280 mL). The system was equipped with a pump, a UV detector, a constant temperature-circulating implement, a sample loop, and a model N2000 workstation.

HPLC analysis was performed on an Agilent 1200 system (Agilent Technologies Co. Ltd., USA). The Agilent 1200 system was equipped with a pump, a DAD detector, a column thermostat, and an Agilent HPLC workstation.

A Bruker Avance 600 MHz (Bruker, Karlsruhe, Germany) spectrometer was used for the NMR experiments, and tetramethylsilane was used as the internal standard.

Preparation of Crude Sample. The whole fresh plant was air-dried in the shade at room temperature and ground to a fine powder. The dried powder (1 kg) was extracted with 75% ethanol under reflux three times (2 h each time). All extracting solution were combined and concentrated. The process produced 210 g crude extract. The crude extract was then suspended in distilled water and extracted with petroleum ether and ethyl acetate, sequentially, which yielded 48.8 g of the petroleum ether fraction, 102.4 g of the ethyl acetate fraction, and 57.8 g of the water fraction.

The 57.8 g water fraction was separated by AB-8 column chromatography (8 cm $\times$ 120 cm, 4.0 kg AB-8 macroporous resin). Ethanol solutions (20%, 40%, 60%, and 80%) were used consecutively to elute the resin until eluent was nearly colorless. All elution fractions were collected for further research, and 7.2 g 80% ethanol fraction was collected. The 80% fraction was used for HSCCC separation. Figure 2 summarizes the separation process of anticancer compounds from *O. falcata*.

Determination of Anticancer Activity. The *in vitro* anticancer activities of the samples on SMMC-7721 were determined by the MTT-based colorimetric method [16].

Figure 2. The procedure of screening and preparative isolation of compounds from *O. falcata* (7-hydroxyflavonone 1, 5,7-dihydroxy-4'-methoxy flavonol 2, 5,7-dihydroxyflavanone 3, 2',4'-dihydroxychalcone 4, 2',4'-dihydroxydihydrochalcone 5)

Briefly, 2×10^4 cells per well were plated in 200 μ L of medium in 96-well plates and incubated for 24 h. Then, the medium was removed, and fresh growth medium containing different concentrations of the test samples was added to the cells. After 24 h, the growth medium was removed, the MTT reagent (0.2 mg/mL) was added, and the cells were incubated for 4 h. The medium was then discarded, and the intracellular formazan product was dissolved in 150 μ L DMSO. The absorbance of each well was measured at 570 nm using an ELISA reader. Cell viability was expressed as a percentage of the cell viability of control cells.

HSCCC Separation. The selection of the two-phase solvent system was conducted according to the partition coefficient (K) of the target compounds. In the present study, the K value was determined by HPLC as follows. The crude sample powder was dissolved into an appropriate amount of a pre-equilibrated two-phase solvent system and placed into a separation funnel. The funnel was shaken vigorously to equilibrate the sample between the two phases. Then, 2 mL of each phase was evaporated to dryness. The residue was dissolved in 1 mL of methanol and subsequently analyzed by HPLC. The K value was defined as the peak area of component in the upper phase divided by the peak area of component in the lower phase.

The two-phase solvent system used here was composed of hexane–ethyl acetate–methanol–water (10:4:10:10, v/v/v/v). Each solvent was added to a separation funnel and thoroughly equilibrated at room temperature. The upper phase and lower phase were then separated and degassed via ultrasonic bath for 30 min before use. Sample solutions for HSCCC separation were prepared as follows: 200 mg of the sample was dissolved in 10 mL of the lower phase.

For each of the separation processes, the column was firstly filled entirely with the lower phase (stationary phase). Then, the mobile phase was pumped into the column at a flow rate of 1.8 mL/min. This occurred while the apparatus was rotated at 800 rpm.

After the solvent front emerged and the system established a steady-state hydrodynamic equilibrium, the sample solution was injected into the separation column. After the sample injection occurred, data were immediately collected. Fractions were collected according to the chromatogram. They were then evaporated under reduced pressure. Residues were

dissolved in methanol in preparation for purity analysis by HPLC.

HPLC Analysis and Identification of Compounds. An ODS C₁₈ column (4.6 $\times$ 250 mm, 5 μ m) was used to analyze the sample and each fraction of HSCCC. The HPLC mobile phases were H₂O for solvent A and CH₃OH for solvent B. They were eluted from 60% B to 65% B in 5 min, followed by 75% B in 30 min. The flow rate was 1.0 mL/min, and the detection wavelength was 254 nm. Figure 3 shows the HPLC analysis of 80% fraction.

Results and Discussion

Screening of Anticancer Fraction. In order to find anticancer fraction of *O. falcata*, the anticancer activity was estimated with the MTT test. The anticancer activities of petroleum ether, ethyl acetate, water extract, 20% ethanol fraction, 40% ethanol fraction, 60% ethanol fraction, and 80% ethanol fraction were evaluated in vitro. The result showed that the 80% ethanol fraction decreased the viability of SMMC-7721 cells. Based on the anticancer activity, 80% ethanol fraction was treated by HSCCC to obtain active compounds.

Selection of HSCCC Conditions. A successful HSCCC separation depends on the selection of a suitable two-phase solvent system. The suitability of the system depends on the following conditions [17, 18]: (1) the settling time of the solvent system should be less than 20 s; (2) K of the compound should fall within a suitable range ($0.2 < K < 5$); and (3) the separation factor between the 2 compounds should be greater than 1.2.

We optimized the solvent system for HSCCC separation considering the above-stated principles. A series of common solvent systems based on ethyl acetate–methanol–water, *n*-butanol–ethyl acetate–water, and hexane–ethyl acetate–methanol–water were tested by changing the volume ratio of solvents to obtain the optimum conditions that could provide suitable K values. The measured K values are shown in Table 1. The solvent systems with ethyl acetate–methanol–water and *n*-butanol–ethyl acetate–water were tested first; large K values were obtained. The hexane–ethyl acetate–methanol–water (10:10:10:10, 8:10:10:10, and 6:10:10:10, v/v/v/v) were further studied, it was found that the compounds were mainly

Figure 3. HPLC analysis of 80% ethanol elution and the compounds 1, 2, 3, 4, and 5. Conditions: column, ODS C₁₈ column (250 mm $\times$ 4.6 mm i.d., 5 μ m); column temperature, 25 $^{\circ}$ C; mobile phase, H₂O (solvent A) and CH₃OH (solvent B); HPLC analysis, 0–5 min, linear gradient from 60 to 65% B; 5–35 min, linear gradient from 65 to 75% B; flow rate, 1.0 mL/min; detection wavelength, 254 nm

Table 1. The *K* values of the target compounds in different solvent systems

Solvent system	Volume ratio	1	2	3	4	5
ethyl acetate–methanol–water	4:1:5	>5	>5	>5	>5	>5
<i>n</i> -butanol–ethyl acetate–water	4:1:5	>5	>5	>5	>5	>5
<i>n</i> -hexane–ethyl acetate–methanol–water	10:10:10:10	0.06	0.12	0.15	0.23	0.28
<i>n</i> -hexane–ethyl acetate–methanol–water	8:10:10:10	0.11	0.25	0.24	0.37	0.33
<i>n</i> -hexane–ethyl acetate–methanol–water	6:10:10:10	0.18	0.28	0.32	0.41	0.40
<i>n</i> -hexane–ethyl acetate–methanol–water	10:8:10:10	0.22	0.32	0.31	0.43	0.46
<i>n</i> -hexane–ethyl acetate–methanol–water	10:6:10:10	0.34	0.65	0.54	1.42	0.96
<i>n</i> -hexane–ethyl acetate–methanol–water	10:4:10:10	0.41	0.89	0.60	1.79	1.26

distributed in one phase, and good results could not be obtained. When the ratio of hexane–ethyl acetate–methanol–water was fixed at 10:8:10:10, and the *K* of the compound was good, but the separation factor was not good. Therefore, the ratio of ethyl acetate was decreased, and when the ratio of hexane–ethyl acetate–methanol–water was fixed at 10:4:10:10, the *K* of the compounds and the separation factor were good. After consideration, the solvent system of hexane–ethyl acetate–methanol–water (10:4:10:10, *v/v/v/v*) was finally selected for the HSCCC separation.

Under the optimized conditions, good separation results were obtained (Figure 4). In only one round of HSCCC separation, 5 fractions (A–E) were obtained in 260 min: 32.7 mg A, 34.9 mg B, 28.5 mg C, 26.7 mg D, and 22.6 mg E. The HPLC analysis of each fraction revealed that the purities of these 5 compounds (1–5) were 97.7%, 98.1%, 98.3%, 99.0%, and 98.3%, respectively (Figure 3).

Structural Identification. The chemical structures of the separated compounds were identified according to their ^1H and ^{13}C NMR spectra and compared with the literature data. The compounds were identified as follows.

Fraction A: 7-hydroxyflavonone **1**, $\text{C}_{15}\text{H}_{10}\text{O}_4$, ^1H NMR (500 MHz, CD_3OD) δ 7.73 (1H, d, $J = 8.7$ Hz, H-4), 7.49 (2H, d, $J = 7.5$ Hz, H-2',6'), 7.40 (2H, t, $J = 7.5$ Hz, H-3', 5'), 7.35 (1H, d, $J = 7.5$ Hz, H-4'), 6.51 (1H, dd, $J = 8.7, 1.6$ Hz, H-6), 6.39 (1H, d, $J = 1.6$ Hz, H-8), 5.48 (1H, dd, $J = 12.9, 2.3$ Hz, H-2), 3.02 (1H, dd, $J = 16.8, 13.0$ Hz, H-3a), 2.75 (1H, dd, $J = 16.8, 2.7$ Hz, H-3b); ^{13}C NMR (151 MHz, CD_3OD) δ 193.0 (C-4), 166.8 (C-7), 165.4 (C-8a), 140.7 (C-1'), 129.9 (C-3',5'), 129.7 (C-4'), 129.6 (C-5), 127.3 (C-2',6'), 115.0 (C-4a), 111.9 (C-6), 103.8 (C-8), 81.0 (C-2), 45.1 (C-3).

Compared with the reported data, the ^1H NMR and ^{13}C NMR data are in agreement with that of 7-hydroxyflavonone in the literature [19].

Fraction B: 5,7-dihydroxy-4'-methoxy flavonol **2**, $\text{C}_{15}\text{H}_{10}\text{O}_4$, ^1H NMR (500 MHz, CD_3OD) δ 8.02 (2H, d, $J = 8.5$ Hz, H-2',6'), 6.81 (2H, d, $J = 8.5$ Hz, H-3',5'), 6.49 (1H, s, H-6), 6.20 (1H, s, H-8), 3.81 (3H, s, $-\text{OCH}_3$); ^{13}C NMR (151 MHz, CD_3OD) δ 177.5 (C-4), 167.0 (C-7), 162.2 (C-5), 160.7 (C-4'), 158.2 (C-8a), 146.9 (C-2), 137.5 (C-3), 130.8 (C-2',6'), 123.6 (C-1'), 116.3 (C-3',5'), 105.4 (C-4a), 98.6 (C-6), 95.7 (C-8), 92.71, 56.4 ($-\text{OCH}_3$). Compared with the reported data, the ^1H NMR and ^{13}C NMR data are in agreement with that of 5,7-dihydroxy-4'-methoxy flavonol in the literature [20].

Fraction C: 5,7-dihydroxyflavanone **3**, $\text{C}_{15}\text{H}_{12}\text{O}_4$, ^1H NMR (500 MHz, CD_3OD) δ 7.44 (2H, d, $J = 7.4$ Hz, H-2',6'), 7.36 (2H, t, $J = 7.4$ Hz, H-3',5'), 7.32 (2H, t, $J = 7.4$ Hz, H-4'), 5.89 (1H, s, H-6), 5.86 (1H, s, H-8), 5.39 (1H, dd, $J = 12.6, 3.6$ Hz, H-2), 3.03 (1H, dd, $J = 17.0, 12.6$ Hz, H-3a), 2.71 (1H, dd, $J = 17.0, 2.6$ Hz, H-3b); ^{13}C NMR (151 MHz, CD_3OD) δ 197.2 (C-4), 168.8 (C-7), 165.5 (C-5), 164.6 (C-8a), 140.4 (C-1'), 129.7 (C-2',6'), 129.6 (C-4'), 127.3 (C-3',5'), 103.2 (C-4a), 97.2 (C-6), 96.3 (C-8), 80.4 (C-2), 44.1 (C-3). Compared with the reported data, the ^1H NMR and ^{13}C NMR data are in agreement with that of 5,7-dihydroxyflavanone in the literature [21].

Fraction D: 2',4'-dihydroxychalcone **4**, $\text{C}_{15}\text{H}_{11}\text{O}_3$, ^1H NMR (500 MHz, CD_3OD) δ 7.88 (1H, d, $J = 8.9$ Hz, H-9), 7.71 (2H, m, H-1,2), 7.32 (2H, d, $J = 6.7$ Hz, H-2',6'), 7.33 (3H, m, H-3',4',5'), 6.32 (1H, dd, $J = 8.7, 1.5$ Hz, H-8), 6.18 (1H, d, $J = 1.5$ Hz, H-6); ^{13}C NMR (151 MHz, CD_3OD) δ

Figure 4. HSCCC chromatogram of 80% ethanol elution using the *n*-hexane–ethyl acetate–methanol–water solvent system with a volume ratio of 10:4:10:10. Conditions: stationary phase, upper phase; flow rate, 1.8 mL/min; revolution speed, 800 rpm; detection wavelength, 254 nm; retention of the stationary phase, 62%

193.1 (C-3), 167.8 (C-5), 167.7 (C-7), 144.9 (C-1), 136.4 (C-1'), 133.6 (C-9), 131.6 (C-4'), 130.0 (C-(C-2',6')), 129.6 (C-3',5'), 121.9 (C-2), 114.4 (C-4), 109.7 (C-8), 103.9 (C-6). Compared with the reported data, the ^1H NMR and ^{13}C NMR data are in agreement with that of 2',4'-dihydroxychalcone in the literature [10].

Fraction E: 2',4'-dihydroxydihydrochalcone **5**, $\text{C}_{15}\text{H}_{13}\text{O}_3$, ^1H NMR (500 MHz, CD_3OD) δ 7.61 (1H, d, $J = 8.8$ Hz, H-9), 7.16 (4H, m, H-2',3',5',6'), 7.06 (1H, m, H-4'), 6.23 (1H, dd, $J = 8.8, 1.9$ Hz, H-8), 6.14 (1H, d, $J = 1.9$ Hz, H-6), 3.13 (2H, t, $J = 7.6$ Hz, H-1), 2.90 (2H, t, $J = 7.6$ Hz, H-2); ^{13}C NMR (151 MHz, CD_3OD) δ 205.2 (C-3), 166.4 (C-5), 166.4 (C-7), 142.5 (C-1'), 133.6 (C-8), 129.4 (C-3',4',5'), 127.1 (2',6'), 114.0 (C-4), 109.1 (C-7), 103.6 (C-6), 40.5 (C-2), 31.6 (C-1). Compared with the reported data, the ^1H NMR and ^{13}C NMR data are in agreement with that of 2', 4'-dihydroxychalcone in the literature [22].

Anticancer Activities of Compounds. All compounds exhibited dose-dependent activities. The IC_{50} values were 213.45 $\mu\text{g/mL}$, 197.74 $\mu\text{g/mL}$, 375.16 $\mu\text{g/mL}$, 17.44 $\mu\text{g/mL}$, and 136.83 $\mu\text{g/mL}$ for compounds **1–5**, respectively. Compounds **4** and **5** exhibited the higher anticancer activities against the cancer cells compared to the positive control ($p < 0.05$). In this study, 5 compounds isolated from *O. falcata* showed notable anticancer activities, which strongly demonstrated that all these compounds may be partially responsible for *O. falcata*'s anticancer activity [6–8].

Conclusions

In summary, the HSCCC experiment was successfully developed for fast isolation and purification of anticancer compounds from 80% fraction of extract from *O. falcata*. Five flavonoids, namely, 7-hydroxyflavonone **1**, 5,7-dihydroxy-4'-methoxy flavonol **2**, 5,7-dihydroxyflavanone **3**, 2',4'-dihydroxychalcone **4**, and 2',4'-dihydroxydihydrochalcone **5**, were isolated and identified. The isolated flavonoids were evaluated for their anticancer activity, and all compounds showed anticancer activity against the growth of SMMC-7721 cells. The results of the present study indicated that these compounds are worth further investigating.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgements. This work was supported by the Application Basic Research Programs of Qinghai Province (2015-ZI-729) and CAS Key Technology Talent Program.

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