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Preparative isolation and purification of alkaloids from *Picrasma quassiodes* (D. Don) Benn. by high-speed countercurrent chromatography

Wenna Zhao¹, Jiao He¹, Yongmin Zhang², Yoichiro Ito³, Qi Su¹, and Wenji Sun^{1,*}

¹Biomedicine Key Laboratory of Shaanxi Province, Northwest University, Xi'an 710069, P.R. China

²Institut Parisien de Chimie Moléculaire, Université Pierre et Marie Curie-Paris 6 (UMR CNRS 7201), 4 place Jussieu, 75005 Paris, France

³Bioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Abstract

By using a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (2:2:2:2, v/v/v/v), a high-speed counter-current chromatography technique was successfully used for isolation and purification of three alkaloids from *Picrasma quassiodes* (D. Don) Benn. for the first time. A total of 22.1 mg of 3-methylcanthin-2,6-dione, 4.9 mg of 4-methoxy-5-hydroxycanthin-6-one and 1.2 mg of 1-methoxycarbonyl- β -carboline were obtained from 100 mg of crude extract of *Picrasma quassiodes* (D. Don) Benn. in less than 5 h, with purities of 89.30%, 98.32% and 98.19%, respectively. The target compounds were identified by ESI-MS, ¹H NMR and ¹³C NMR.

Keywords

high-speed countercurrent chromatography; *Picrasma quassiodes* (D. Don) Benn.; alkaloids

INTRODUCTION

Picrasma quassiodes (D. Don) Benn. called Kumu in Chinese is widely distributed in most areas of mainland China. The branches and leaves of the plants are used as traditional folk medicine for treatment of gastroenteritis, eczema and snakebite, and other diseases^[1,2]. The total alkaloids including β -carbolines and canthin-6-ones alkaloids are considered as the major biologically active components which were reported to inhibit cAMP phosphodiesterase^[3], and have anti-inflammatory and antiviral activities^[4]. At present, the conventional separation method of alkaloids from *Picrasma quassiodes* (D. Don) Benn. consisted of silica gel column chromatography and other column chromatography which require several steps and consume large amounts of solvent. Therefore, it's highly desirable to find a green and preparative separation and purification method.

High-speed counter-current chromatography (HSCCC) is a support-free liquid-liquid partition chromatography, which has a variety of advantages over conventional column

*Corresponding author: Wenji Sun. Biomedicine Key Laboratory of Shaanxi Province, Northwest University, No. 229 Taibai North Road, Xi'an 710069, People's Republic of China. cxbml@nwu.edu.cn.

chromatography such as an excellent sample recovery, shorter isolation time, and wider range of selection of two-phase solvent systems [5,6]. HSCCC has been widely used for separation and purification of alkaloids from Chinese herbal medicine for years [6–8]. However, to our knowledge, no report was focused on the isolation and purification of alkaloids from *Picrasma quassiodes* (D. Don) Benn. by HSCCC.

In this paper, we report an efficient new method for separation and purification of three alkaloids including 3-methylcanthin-2,6-dione, 4-methoxy-5-hydroxycanthin-6-one, and 1-methoxycarbonyl- β -carboline (Figure 1) from the Chinese medicinal plant *Picrasma quassiodes* (D. Don) Benn.

EXPERIMENTAL

Reagents and Plant Materials

Organic solvents such as n-hexane, ethyl acetate and methanol used for preparation of crude samples and HSCCC separation were of analytical grades and purchased from Tianjin Chemical Factory (Tianjin, China). The water used for the experiment was treated with a Milli-Q plus water purification system (Millipore, Madrid, Spain). Acetonitrile used for HPLC analyses was of chromatographic grade and also purchased from Tianjin Chemical Factory.

Dried branches of *Picrasma quassiodes* (D. Don) Benn. were purchased from Xi'an Wanshou Road Chinese crude drug market (Shaanxi, China) and the identification was made by Professor Yazhou Wang, College of Life Science, Northwest University, China.

Apparatus

The HSCCC instrument used in the present study was TBE-300A high-speed countercurrent chromatograph (Tauto Biotech Co. Ltd, Shanghai, China), equipped with a 260 mL coil column made of polytetrafluoroethylene (PTFE) tubing of 1.5 mm I.D. The β -value of this preparative column ranged from 0.5 at the internal to 0.8 at the external layer ($\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed of the apparatus could be ranged from 0 to 1000 rpm, while 800 rpm was used in the present study. The solvent was pumped into the column with a Model TBE5002 constant flow pump (Tauto Biotech Co. Ltd, Shanghai, China). Continuous monitoring of the effluent was achieved with a Model 500A-UV Monitor (Tauto Biotech Co. Ltd, Shanghai, China) at 254 nm. A manual sample injection valve with a 20 mL loop was used to introduce the sample into the column. The data were collected and analyzed simultaneously on a Model N2000 chromatography workstation (Zhejiang University, Hangzhou, China).

The analytical HPLC equipment analysis used throughout this study was a Waters Alliance 2695 system (Waters, Milford, MA, USA), which consisted of a vacuum degasser, a low pressure quaternary pump, an auto sampler and a dual- λ absorbance detector, controlled by "Empower" software and a Waters 2487 UV dual λ absorbance detector (Waters, USA). A Welchrom C18 column (250 $\times$ 4.6 mm, 5 μ m) was used for analysis of alkaloids.

Preparation of the Crude Extract

Powdered branches of *Picrasma quassiodes* (D. Don) Benn. (2.0 kg) were first extracted by refluxing in 16 L of 80% ethanol for three times. Then the ethanol extracts were pooled and concentrated at 70°C under reduced pressure and the residues (97.5 g) were redissolved in water (500 mL, pH = 2), and then extracted with 1200 mL of ethyl acetate (repeated eight times). The lower acidic phase was separated and adjusted pH at 10 with sodium hydroxide.

This alkaline aqueous solution was extracted with dichloromethane (ten times) and the organic phases were combined and evaporated under reduced pressure. This yielded 30.1 g of crude extract which was submitted to HSCCC separation.

Selection of Two-Phase Solvent System

The two-phase solvent system was selected according to the partition coefficient (K) of the target components. The K values were defined by the peak area of components in the upper phase divided by the that in the lower phase, which was determined by HPLC analysis. Firstly, different volume ratios of chloroform-methanol-water, petroleum ether-ethyl acetate-methanol-water and *n*-hexane-ethyl acetate-methanol-water were shaken and equilibrated in a separation funnel at room temperature. Then, 2.0 mg of crude sample was weighed in a 10 mL test tube to which 2 mL of each phase of the equilibrated two-phase solvent system was added. The capped tube was shaken vigorously for several minutes to thoroughly equilibrate the sample between two phases. And 0.5 mL of the upper and lower phases were each evaporated to dryness and the residues were redissolved in 2 mL of methanol, and analyzed by HPLC to determine K value of each components. The peak area of the upper phase was recorded as A_U and that of the lower phase was recorded as A_L . The K value was calculated according to the following equation: $K = A_U/A_L$.

Preparation of Two-Phase Solvent System and Sample Solution

The two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (2:2:2:2, $v/v/v/v$) was mixed and equilibrated thoroughly in a separatory funnel at room temperature overnight. Then the two phases were separated and each degassed by sonication for 30 min prior to use. The sample solution for HSCCC separation was prepared by dissolving 100 mg of the crude extract in a mixture of 5 mL of each phase of the solvent system used for separation.

HSCCC Separation Procedure

The preparative separation was performed on the Model TBE300A HSCCC with the selected solvent system composed of *n*-hexane-ethyl acetate-methanol-water (2:2:2:2, $v/v/v/v$). The multilayer coil column was first entirely filled with the upper phase (stationary phase). The lower phase (mobile phase) was then pumped into the head end of the column at a suitable flow rate of 2.0 mL/min while revolution speed was set at 800 rpm. After a clear mobile phase eluting at the tail outlet indicating hydrodynamic equilibrium was reached, the sample solution (100 mg of the crude extract in 5 mL of each phase) was injected through the injection valve. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and the chromatogram was recorded. Each peak fraction was collected into the test tubes at 5 min/tube. Peak fractions were analyzed by HPLC, ESI-MS and NMR.

HPLC Analysis and Identification of HSCCC Peak Fraction

Each purified fraction from the HSCCC separation was analyzed by HPLC on a Welch Materials C18 column (250 mm×4.6 mm, i.d., 5 μ m) with the column temperature at 30°C. The mobile phase was acetonitrile-water (0.1% HCOOH) in a gradient mode as follows: acetonitrile: 0–7 min, 25%; 7–8 min, 25% to 30%; 9–40 min, 30%, the flow rate was 1.0 mL/min. All solvents were filtered through a 0.45 μ m filter before use. The effluent was monitored at 254 nm by a UV detector. Each peak fraction was collected according to the obtained chromatogram, evaporated under reduced pressure, and then dissolved in methanol for HPLC analysis. The area normalization method was used to determine the purity of each ingredient on HPLC.

To identify each peak of the HSCCC fractions, ESI-MS experiment was carried out using a Thermo Scientific LTQ XL ion trap mass spectrometer (Thermo Fnnigan, San Jose, CA, USA) equipped with an electrospray ionization source, and NMR spectra were recorded with a INOVA spectrometer (Varian Co. Ltd, America)

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

Several elution systems were tested to separate the crude extracts on HPLC, such as methanol-water, methanol-water (0.1% HCOOH), acetonitrile-water (0.1% phosphoric acid) and acetonitrile-water (0.1% HCOOH) etc. The optimum HPLC mobile phase was found to be acetonitrile-water (0.1% HCOOH) in a gradient mode as follows: acetonitrile: 0–7 min, 25%; 7–8 min, 25% to 30%; 9–40 min, 30%, each peak achieved baseline separation. The crude extracts and peak fractions separated by HSCCC were analyzed by HPLC under these optimum conditions. The HPLC chromatograms of the crude extract and HSCCC peak fractopms 1 – 3, are shown in Fig. 2 (a–d).

Selection of Two-Phase Solvent System and Other Conditions of HSCCC

The selection of two-phase solvent system is the critical step in HSCCC separation. The partition coefficient (K) is the ratio of solute distributed between the mutually equilibrated two solvent phases, the suitable K values for HSCCC are between 0.5 and 1.0. Solutes with smaller K values elute near the solvent front with lower peak resolution while solutes with larger K values tend to give better resolution but broader, more dilute peaks due to a longer elution time^[5]. In order to achieve an ideal separation of target compounds, a series of experiments were performed to optimize the two-phase solvent system for HSCCC. Several two-phase solvent systems such as: petroleum ether-ethyl acetate-methanol-water; chloroform-methanol-water and n-hexane-ethyl acetate-methanol-water were tested, and their K values were listed in Table 1.

A number of alkaloids had been separated by HSCCC using chloroform-methanol-water (containing different proportions of hydrochloric acid) ^[9,10]. In order to avoid a risk of damage of our HSCCC system which contains 316L stainless steel We tested a similar but neutral solvent systems such chloroform-methanol-water (4:2:2, v/v/v) and chloroform-methanol-water (4:4.5:2, v/v/v), but they gave unsatisfactory K values. Further studies showed that petroleum ether-ethyl acetate-methanol-water system (20:27:23:17, v/v/v/v), (22:25:23:17, v/v/v/v) and (18:29:21:19, v/v/v/v) together with n-hexane-ethyl acetate-methanol-water (2:2:2:2, v/v/v/v) were all suitable for the separation (Table 1). But, among those n-hexane-ethyl acetate-methanol-water (2:2:2:2, v/v/v/v) seem to be the best with an acceptable separation time.

We also tested the effect of flow rate of the mobile phase and the revolution speed on the separation. When the flow rate was 1.5 mL/min, the separation results were almost the same as 2.0 mL/min, but the time delayed about 100 min and the chromatography peak became broader. When the revolution speed was increased from 800 to 900 rpm the separation efficiency was decreased. The optimum separation conditions were determined as follows: the revolution speed at 800 r/min and the flow rate at 2.0 mL/min which gave satisfactory stationary phase retention at 61% (Figure 3). Three kinds of alkaloids were successfully separated in one-step operation yielding 22.1 mg of 3-methylcanthin-2,6-dione (compound 1), 4.9 mg of 4-methoxy-5-hydroxycanthin-6-one (compound 2), and 1.2 mg of 1-mthoxycarbonyl- β -carboline (compound 3) from 100 mg of the crude sample.

Structural Identification

The chemical structure of each peak of HSCCC was identified by IR, ESI-MS, ^1H NMR, and ^{13}C NMR.

Compound **1**: orange-red needle crystal, ESI-MS (m/z): 273 $[\text{M}+\text{Na}]^+$. ^1H NMR (400 MHz, CDCl_3): δ 7.90 (1H, d, $J = 8.0$ Hz, H-8), 8.67 (1H, d, $J = 8.4$ Hz, H-9), 7.49 (1H, t, $J = 8.0$ Hz, H-10), 7.67 (1H, t, $J = 8.0$ Hz, H-11), 7.13 (1H, d, $J = 7.2$ Hz, H-5), 7.26 (1H, s, H-1), 6.10 (1H, s, H-4), 3.88 (3H, s, 3-N- CH_3); ^{13}C NMR (100 MHz, CDCl_3): δ 140.6, 140.4, 133.2, 131.3, 129.8, 128.8, 127.4, 127.1, 126.9, 126.7. The data were compared with those reported in the literature (Taichi, O., 1982), and compound **1** was identified as 3-methylcanthin-2,6-dione.

Compound **2**: yellow needle crystal, ESI-MS (m/z): 266 $[\text{M}]^+$. ^1H NMR (400 MHz, CDCl_3): δ 8.86 (1H, d, $J = 4.8$ Hz, H-1), 7.91 (1H, d, $J = 5.6$ Hz, H-2), 8.57 (1H, d, $J = 8.0$ Hz, H-8), 7.72 (1H, t, $J = 8.0$ Hz, H-9), 7.55 (1H, t, $J = 7.6$ Hz, H-10), 8.10 (1H, d, $J = 8.0$ Hz, H-11), 4.48 (3H, s, 4- CH_3); ^{13}C NMR (100 MHz, CDCl_3): δ 145.9, 138.7, 136.3, 130.7, 130.3, 125.8, 125.5, 122.8, 116.7, 114.7, 61.0. The data were compared with those reported in the literature^[11], and compound **2** was identified to be 4-methoxy-5-hydroxycanthin-6-one.

Compound **3**: light yellow needle crystal, ESI-MS (m/z): 226 $[\text{M}]^+$. ^1H NMR (400 MHz, CDCl_3): δ 8.59 (1H, d, $J = 4.8$ Hz, H-3), 8.15 (1H, d, $J = 4.4$ Hz, H-4), 8.17 (1H, d, $J = 5.2$ Hz, H-5), 7.59 (1H, t, $J = 7.6$ Hz, H-6), 7.34 (1H, t, $J = 6.4$ Hz, H-7), 7.64 (1H, d, $J = 8.0$ Hz, H-8), 9.91 (1H, s, 9-NH), 4.13 (1H, s, 12- OCH_3); ^{13}C NMR (100 MHz, CDCl_3): δ 167.2, 140.7, 138.9, 137.1, 131.5, 129.5, 121.9, 120.8, 120.7, 118.7, 111.8, 52.8. The data were compared with those reported in the literature^[12], and compound **3** was identified as 1-methoxycarbonyl- β -carboline.

CONCLUSION

Three alkaloids, 3-methylcanthin-2,6-dione, 4-methoxy-5-hydroxycanthin-6-one and 1-methoxycarbonyl- β -carboline were separated from crude extracts of *Picrasma quassioides* (D. Don) Benn. by HSCCC using n-hexane-ethyl acetate-methanol-water (2:2:2:2, v/v/v/v) as the two-phase solvent system. This is the first example to use HSCCC for the separation and purification of the alkaloids from *Picrasma quassioides* (D. Don) Benn. The method was proved to be efficient, simple and fast.

Acknowledgments

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(1) 3-methylcanthin-2,6-dione

(2) 4-methoxy-5-hydroxycanthin-6-one

(3) 1-methoxycarbonyl- β -carboline**Fig.1.**

Chemical structures of three alkaloids: (1) 3-methylcanthin-2,6-dione; (2) 4-methoxy-5-hydroxycanthin-6-one; (3) 1-methoxycarbonyl- β -carboline.

Fig.2.

HPLC chromatograms of crude extracts and HSCCC peak fractions. Conditions: column, Welch C18 column (250×4.6 mm, 5 μ m); mobile phase: acetonitrile-water (0.1% HCOOH) in a gradient mode as follows: acetonitrile: 0–7 min, 25%; 7–8 min, 25% to 30%; 9–4 min, 30%; flow rate: 1.0 mL/min; detection wavelength: 254 nm; column temperature: 30 °C; injection volume: 20 μ L.

(a) Crude sample; (b) combined fractions, peak 1; (c) combined fractions, peak 2; (d) combined fractions, peak 3

Fig. 3. HSCCC separation chromatogram of the crude extract from *Picrasma quassiodes* (D. Don) Benn. Solvent system: n-hexane-ethyl acetate-methanol-water (2:2:2:2, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase. flow rate of the mobile phase: 2.0 mL/min; revolution speed: 800 rpm; column temperature: 25 °C; sample: 100 mg of crude extract dissolved in 10 mL of two-phase solvent system; detection wavelength: 254 nm. (peak 1: 3-methylcanthin-2,6-dione); (peak 2: 4-methoxy-5-hydroxycanthin-6-one); (peak 3: 1-methoxycarbonyl- β -carboline)

Table 1

The partition coefficient (K) of the target compounds in different systems

solvent system	ratio	K	K	K
		(compound 1)	(compound 2)	(compound 3)
petroleumether-ethyl	20:27:23:17	0.69	0.62	0.42
acetate-methanol	22:25:23:17	0.84	0.58	0.65
-water	18:29:21:19	1.50	0.88	0.62
chloroform-methanol-	4:2:2	11.8	-----	-----
water	4:4.5:2	0.1	0.03	-----
	2:2:2:2	1.24	0.71	0.53
n-hexane-ethyl	2:2.5:2:2	0.19	0.70	0.97
acetate-methanol-wate	2:2:1.5:2	-----	0.58	1.20
r	2:1.5:1.5:2	-----	0.58	0.83