Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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To cite this Article Qing, Lv Hui , Rui, Liu , Xing, Wu Tian and Yuan, Lv Gui(2009) 'Isolation and Purification of Two Constitutes from *Dendrobium fimbriatum* Hook by High-Speed Counter-current Chromatography Using Stepwise Elution', Separation Science and Technology, 44: 5, 1218 — 1227

To link to this Article: DOI: 10.1080/01496390902728850

URL: http://dx.doi.org/10.1080/01496390902728850

Separation Science and Technology, 44: 1218–1227, 2009
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ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390902728850

Isolation and Purification of Two Constitutes from *Dendrobium fimbriatum* Hook by High-Speed Counter-current Chromatography Using Stepwise Elution

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Abstract: Preparative high-speed counter-current chromatography (HSCCC) was successfully used for the isolation and purification of 2-hydroxyethyl caffeate and denhydroshizukanolide from *Dendrobium fimbriatum* Hook using stepwise elution with a pair of two-phase solvent systems composed of *n*-hexane-ethyl acetate-methanol-water at (1:1:1:1, v/v) and (3:1:3:1, v/v). Using a preparative unit of the HSCCC centrifuge, about a 100 mg amount of the sample was separated, yielding 13.3 mg of 2-hydroxyethyl caffeate and 18.0 mg of denhydroshizukanolide at a high purity of over 95%. The peak fraction of HSCCC was identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography, *Dendrobium fimbriatum* Hook, plant materials

INTRODUCTION

The traditional Chinese medicine ‘‘Shi Hu,’’ derived from the dried or fresh stems of *Dendrobium fimbriatum* Hook, which belongs to the

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Received 21 July 2008; accepted 27 October 2008.

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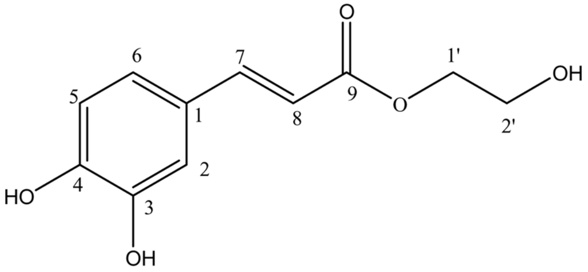
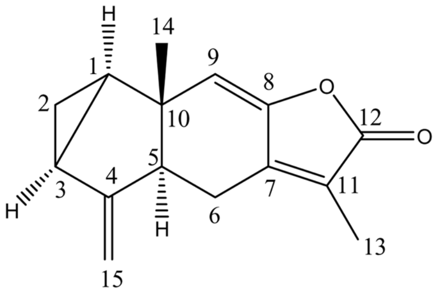
Orchidaceae family, is widely used as both traditional Chinese and folk remedies for treatment of various diseases, such as chronic atrophic gastritis, diabetes, skin aging, and cardiovascular disease, which to a great extent are believed to be closely associated with the metabolic disorders of reactive oxygen species in the human body (1,2). Alkaloids (3,4), bibenzyls (5), fluorenones (5), phenanthrenes (6,7), and sesquiterpenoids (8,9) were reported as the constituents of this plant. Some of these compounds have been reported as having cytotoxic (7), immunoregulatory (8,9), antimutagenic (10) and anti-inflammatory (11) activities.

Counter-current chromatography (CCC) was first introduced by Ito in the late 1960s (12). Since then, it has been widely used in the field of natural product chemistry to effectively separate a large variety of compounds. The technique is an all-liquid method, without solid phases, which relies on the partition of a sample between two immiscible solvents to achieve separation (13). The relative proportions of solute passing into each of the two phases are determined by the respective distribution ratios (*K*). It benefits from great advantages when compared with the traditional liquid-solid separation methods: eliminating the



2-hydroxyethyl caffeate



denhydroshizukanolide

Figure 1. Chemical structures of target compounds.

complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks, and contamination (14). CCC has the unique features of high recovery, high efficiency, and the ease to scale-up, and has been widely used in the separation and purification of various natural and synthetic products (15–19). The purpose of this study, therefore, is to develop a CCC method for preparative isolation and purification of bioactive components (Fig. 1) from *D. fimbriatum* Hook.

EXPERIMENTAL

Apparatus

The isolation and purification of two target compounds from *D. fimbriatum* was performed by an Ito's type-J high-speed counter-current chromatography (HSCCC) instrument constructed in our laboratory. It holds a pair of column holders symmetrically on the rotary frame at a distance of 10 cm from the central axis of the centrifuge. The gear arrangement produces a synchronous planetary motion of each column holder in such a way that it revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. The multiplayer coil separation column was prepared by winding a 26.4 m × 2.6 mm id PTFE tube (six layers) directly onto one of the holders forming multiple coiled layers to give a total capacity of 140 mL, while a counterweight is applied on the other holder to balance the centrifuge. The β-value varied from 0.33 at the internal terminal to 0.60 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft and R the revolution radius or the distance between the holder axis and central axis of the centrifuge).

The revolution speed of the apparatus can be regulated with a speed controller in the range of 0–1000 rpm and up to 60°C with a temperature control unit. In addition, this CCC system is equipped with a Model 2 W-2B constant-flow pump (Beijing Xingda Equipment, Beijing, China), a HD-9704 UV spectrometer operating at 254 and 280 nm, a BSZ-100 fraction collector, a sample injection valve with a 20 mL sample loop, and a N2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

HPLC used was an Agilent 1100 system including a G1311A QuatPump, a G1322 Degasser, a G1314A variable wave detector (VWD), a model 7725i injection valve with a 20 μL loop, a PT100 column oven and Agilent ChemStation for LC.

Reagents

All the organic solvents used for HSCCC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all the solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany. The dried stems of *D. fimbriatum* were purchased from a local drug store.

Preparation of Crude Sample

The stems of *D. fimbriatum* were dried to constant mass at 55°C in a vacuum oven and then pulverized. About 1 kg of pulverized sample was extracted with 4 L of 95% ethanol for 2 h under reflux. The extraction procedure was repeated three times. The extracts were combined and concentrated to dryness, yielding 90 g of the crude ethanol extract. Then the extract was dissolved in 1.5 L water. 1.5 L light petroleum was added and then the mixed liquid was churned up in the rotate speed of 300 rpm for 30 min by churn-dasher. This procedure was repeated four times and the extracts were combined and evaporated by rotary vaporization under reduced pressure, yielding 18 g of the petroleum extract. Then, the petroleum extract was subjected to column chromatography on silica gel (500 g of silica gel H, 100–200 mesh, Qingdao Haiyang Chemica, Qingdao, China) and eluted successively with light petroleum (b.p. 60–90°C)–ethyl acetate solvent mixture of increasingly polarity to obtain six fractions. On concentration, the fraction 5 gave 1.3 g mixture, which was stored in a refrigerator (4°C) for the subsequent CCC separation.

Selection of the Two-Phase Solvent System

The composition of the two-phase solvent system was selected according to the distribution ratio (*K*) and peak resolution of the target compounds. The measurement of the *K* values was performed as follows: a small amount (typically a few milligrams or less depending on its extinction coefficient or absorptivity) of crude sample was added into a test tube to which about 2 ml of each phase of the pre-equilibrated two-phase solvent system were added. After shaken vigorously for 10 min, the mixture was separated by centrifugation for 3 min. Then, an aliquot of each phase (100 μl) was delivered into a test tube separately, each was diluted with an equal volume (1 ml) of methanol and analyzed by HPLC. The *K* value was expressed as the peak area of target compound in the upper phase divided by that in the lower phase (20).

Preparation of the Two-Phase Solvent Systems and Sample Solution

The solvent system utilized in the present study was prepared by mixing *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v) or (3:1:3:1, v/v), and thoroughly equilibrating the mixture in a separatory funnel at room temperature, two phases being separated shortly before use. The sample solutions were prepared by dissolving the crude extract in the equal volumes of upper and lower phase at suitable concentrations according to the preparative purpose.

CCC Separation Procedure

Preparative HSCCC was performed as follows: the multilayer coiled column was first entirely filled with the upper phase as stationary phase. Then the lower phase of system (1:1:1:1, v/v) was pumped into the head end of the inlet column at a flow rate of 2 ml/min, while the apparatus was rotated at 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (100 mg in 2 ml of lower phase and 2 ml of upper phase of solvent system 1) was injected through the injection valve. In the stepwise elution, after certain time of elution using the first solvent system, the lower phase (the mobile phase) was switched to the second solvent. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm. Each peak fraction was collected according to the chromatogram.

HPLC Analysis and Identification of CCC Peak Fractions

HPLC analyses of the crude sample and CCC peak fractions were performed with a Dikma C18 column (250 × 4.6 mm I.D., 5 μm). The mobile phase was methanol (solvent A) and water (solvent B) at the gradient: A from 40% to 60% for 10 min and 60% to 95% for next 35 min. The flow rate was 0.8 ml/min, and the effluent was monitored at 280 nm. Identification of the CCC peak fraction was performed by ^{1}H NMR and ^{13}C NMR. NMR experiments were carried out using a Bruker Advanced DMX 500 NMR spectrometer with pyridine ($CDCl_3$) as solvent and TMS as internal standard.

RESULTS AND DISCUSSIONS

As shown in Fig. 2, the HPLC analysis of the crude extract of *D. fimbriatum* shows several compounds where the purity of 2-hydroxyethyl caffeate and denhydroshizukanolide in crude extract is 18.8% and 23.9%, respectively, based on HPLC peak area percentage.

The main difficulty of the CCC method development arises from the choice of the solvent in an enormous number of possibilities available to the analyst. In order to select a suitable two-phase solvent system for successful CCC separations, the previous articles on the CCC involving separation of similar compounds should be consulted first. Additionally, the two-phase solvent system should satisfy the following requirements:

1. no decomposition or denaturation of the sample;
2. suitable *K* values;
3. good separation factor (>1.5);
4. satisfactory retention of the stationary phase.

In the present work, the preliminary studies were carried out with the two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water at various volume ratios (1:2:1:2, 1:1:1:1, 3:1:3:1, 4:3:4:3), in terms of *K* values and peak resolution (shown in Table 1). The results indicated that when the two-phase solvent system at a volume ratio of 3:1:3:1 was used, denhydroshizukanolide and the impurities nearby could be separated well. However, due to the relatively small *K* values, 2-hydroxyethyl caffeate would be eluted together with the impurities at the very beginning. When the two-phase solvent system at a volume ratio of 1:1:1:1 was employed, 2-hydroxyethyl caffeate could be completely separate with

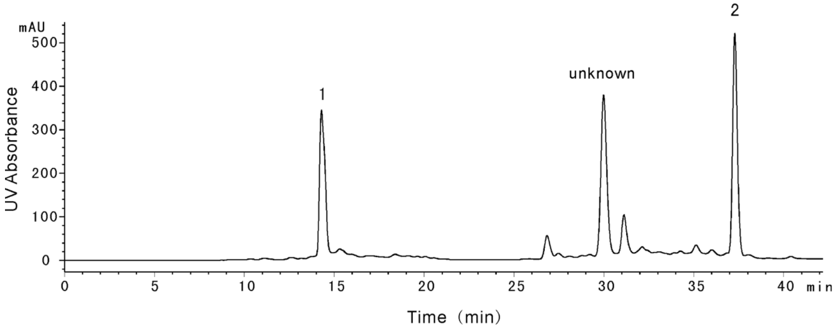


Figure 2. HPLC chromatogram of the crude extract. 1: 2-hydroxyethyl caffeate; 2: denhydroshizukanolide.

Table 1. Distribution ratio of target compounds

	K	
Solvent systems (v/v)	2-Hydroxyethyl caffeate	Denhydroshizukanolide
n-hexane:ethyl acetate:methanol:water (1:2:1:2)	4.24	16.03
n-hexane:ethyl acetate:methanol:water (1:1:1:1)	0.88	7.35
n-hexane:ethyl acetate:methanol:water (3:1:3:1)	0.16	1.82
n-hexane:ethyl acetate:methanol:water (4:3:4:3)	0.53	7.26

the hydrophilic impurities. For saving solvent and time, after 2-hydroxyethyl caffeate was eluted, the mobile phase could be changed to the lower phase of the solvent system at a volume ratio of 3:1:3:1 to separate denhydroshizukanolide and the hydrophobic impurities. Therefore, the present stepwise elution and the combined use of the two solvent systems (1:1:1:1 and 3:1:3:1) were recommended as a practical approach to resolve this problem.

This stepwise elution was applied for the preparative separation of 100 mg of the crude extract of *D. fimbriatum* Hook. As shown in Fig. 3, the separation was started with the solvent system (1:1:1:1) , after most of the polar component were eluted (106 min at the dotted line in Fig. 3), the lower phase (the mobile phase) was switched to the solvent system 3:1:3:1. Then, 2-hydroxyethyl caffeate (peak 1) and denhydroshizukanolide (peak 2) were well resolved and eluted in about 200 min. This separation yielded 13.3 mg of 2-hydroxyethyl caffeate and 18.0 mg of denhydroshizukanolide at a high purity of over 95% based on HPLC analysis.

The structural identification of CCC fraction I in Fig. 3 was carried out by ^{1}H NMR and ^{13}C NMR as follows: ^{1}H NMR(500 MHz, $CDCl_3$): $\delta = 1.71$ (1H, m, H-1), 0.92 (2H, m, H-2), 1.99 (1H, t, H-3), 2.98 (1H, m, H-5), 2.33 (1H, t, H-6), 2.77 (1H, d, H-6), 6.26 (1H, s, H-9), 1.90 (3H, s, H-13), 0.81 (3H, s, H-14), 4.83 (1H, s, H-15), 5.05 (1H, t, H-15). ^{13}C NMR(125 MHz, $CDCl_3$): $\delta = 26.6$ (C-1), 17.2 (C-2), 22.6 (C-3), 150.3 (C-4), 62.2 (C-5), 21.6 (C-6), 148.2 (C-7), 149.8 (C-8), 119.9 (C-9), 40.3 (C-10), 120.1 (C-11), 160.0 (C-12), 8.8 (C-13), 22.3 (C-14), 106.7 (C-15). The structural identification of CCC fraction II in Fig. 3 was carried out by ^{1}H NMR and ^{13}C NMR as follows: ^{1}H NMR (500 MHz, $CDCl_3$): ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.04$ (1H, d, $J = 2.0$ Hz, H-2), 6.77 (1H, d, $J = 7.5$ Hz, H-5),6.95 (1H, dd, $J = 2.0$ & 7.5 Hz, H-6), 7.58 (1H,

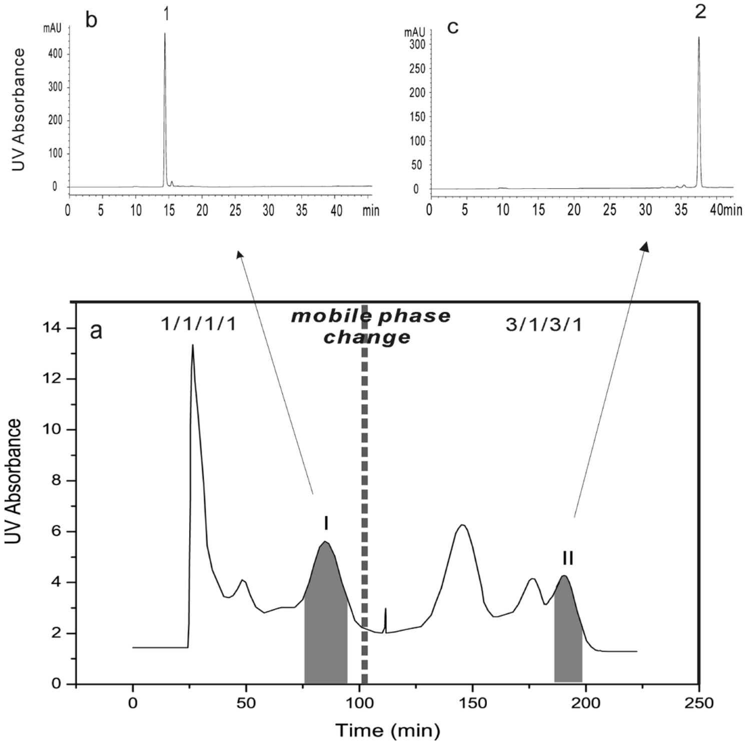


Figure 3. HSCCC chromatogram of the crude extract (a) and HPLC analysis of target analytes (b,c). 1: 2-hydroxyethyl caffeate; 2: denhydroshizukanolide.

d, $J = 16.0$ Hz, H-7), 6.29(1H, d, $J = 16.0$ Hz, H-8), 4.24 (2H, m, H-1′), 3.79 (2H, m, H-2′). ^{13}C NMR (125 MHz, CD_3OD): $\delta = 128.2$ (C-1), 115.5 (C-2), 150.1 (C-3), 147.3 (C-4), 115.6 (C-5), 123.4 (C-6), 147.6 (C-7), 117.0 (C-8), 169.8 (C-9), 67.4 (C-1′), 61.7 (C-2′). According to the literatures,(21,22) fraction I was identified as 2-hydroxyethyl caffeate and fraction II was identified as denhydroshizukanolide.

Here it is worthwhile to discuss the advantage of the above stepwise elution applied in the present studies. As shown in Table 1, one of target peaks with a much greater distribution ratio will be retained in the column for a long period of time. In this case the chromatographic condition should be changed to facilitate elution of the retained peaks without loss of peak resolution. Moreover, due to the use of a biphasic liquid system to separate solutes, gradient elution in CCC is not as straightforward as that in HPLC: any change of the mobile phase composition may change the stationary phase composition, resulting in the possible loss of hydrodynamic equilibrium, as well as loss of the overall separation efficiency. Therefore, the stepwise elution in counter-current chromatography is an efficient way to achieve this goal. While, this method needs a careful choice of the second mobile phase it is always necessary to avoid possible phase disturbance. The results of our studies demonstrated that stepwise counter-current chromatography is a useful method for the preparative separation of 2-hydroxyethyl caffeate and denhydroshizukanolide from a crude extract of *D. fimbriatum* Hook.

CONCLUSIONS

In conclusion, an effective CCC method was developed for the preparative isolation and purification of bioactive components from *D. fimbriatum* Hook. 13.3 mg of 2-hydroxyethyl caffeate (96.0%) and 18.0 mg of denhydroshizukanolide (96.1%) from 100 mg of the crude sample was obtained in a single CCC separation. These results clearly demonstrated that the present CCC method is powerful for the separation of bioactive components from *D. fimbriatum* Hook.

ACKNOWLEDGMENT

Financial support from the Natural Science Foundation of Zhejiang Province (Z206510) is gratefully acknowledged.

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