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Short communication

Preparative isolation and purification of two phenylbutenoids from the rhizomes of *Zingiber Cassumunar* by upright counter-current chromatography

Yanbin Lu, Cuirong Sun, Yu Wang, Yuanjiang Pan *

Department of Chemistry, Zhejiang University, Hangzhou, Zhejiang Province 310027, China

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Abstract

Two phenylbutenoids, (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene, were separated from the rhizomes of *Zingiber Cassumunar* using a preparative upright counter-current chromatography (CCC). With a two-phase solvent system composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:2:1, v/v), 150 mg of (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and 175 mg of (*E*)-4-(3′,′-dimethoxyphenyl)but-1,3-diene with the purity of 98.7 and 95.1%, respectively, were obtained from 600 mg of the crude sample of *Z. Cassumunar* in a single-step separation. Structures of these two compounds were identified by ESI-MS, ^{1}H NMR and ^{13}C NMR.

Keywords: *Zingiber Cassumunar*; Counter-current chromatography; Phenylbutenoids; Plant materials

1. Introduction

Zingiber Cassumunar is a traditional medicinal plant in southeast Asia, especially in Thailand and Indonesia. It has been used as an embrocation for long times [1]. The pharmacological studies have demonstrated that the rhizomes of Z. Cassumunar have antioxidant [2] and antifungal activities [3]. Phenylbutenoids are typical non-polar substances in the rhizomes of *Z. Cassumunar* and recent studies have reported that some phenylbutenoids also have anti-inflammatory activities [4] and can be used as insecticidal constituents [5]. The preparative separation and purification of phenylbutenoids from the rhizomes of *Z. Cassumunar* by conventional methods is tedious and usually requires multiple chromatography steps [6–9]. Because of the irreversible adsorption, it is always difficult to obtain pure compounds.

Counter-current chromatography (CCC) is a unique liquid–liquid partition chromatography without use of solid support matrix [10]. Therefore, it eliminates the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks and contamination. The method has been successfully applied to the analysis and separation of various natural and synthetic products [11–13]. So far, no report has been published on the use of CCC for the isolation and purification of phenylbutenoids. The purpose of this study, therefore, is to develop a CCC method for preparative isolation and purification of phenylbutenoids from the rhizomes of *Z. Cassumunar*.

2. Experimental

2.1. Apparatus

The CCC isolation and purification of two phenylbutenoids from the rhizomes of *Z. Cassumunar* was performed by upright coil planet centrifuge with four multilayer coils connected in series. Its design principle and dimensions

* Corresponding author. Tel.: +86 571 87951264; fax: +86 571 87951264.
E-mail address: panyuanjiang@zju.edu.cn (Y. Pan).

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were described in the literature [14]. The upright CCC apparatus holds four identical multilayer coil columns in the symmetrical positions around the rotary frame at distance of 9 cm from the central axis of the centrifuge to maintain perfect balance of centrifuge system without the use of a counterweight. Each separation column was made by winding a single piece of 4 mm I.D. and 1 mm wall thickness polytetrafluroethylene (PTFE) tubing directly onto the holder hub of 5 cm diameter forming three layers of right-handed and left-handed coils alternating in each layer between a pair of flanges spaced 35 cm apart. The β-value (ratio of helical radius of the coil and revolution radius) of the multilayer coil varies from 0.28 at the internal terminal to 0.48 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). These multilayer coils are connected in series on the rotary frame using a flow tube (PTFE, 1.6 mm I.D. and 0.7 mm wall thickness) to give a total capacity of 1600 ml while the unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal.

The apparatus can be operated up to maximum speed of 800 rpm with a speed Sunwind control unit (Shenduo Electric Corp., Shanghai, China) and up to 60 °C with a temperature control unit. In addition, this CCC system is equipped with a Type-J-W metering pump (Zhijiang Petroleum Equipment, Hangzhou, China), a HD-9704 UV spectrometer operating at 254 and 280 nm, Shimadzu C-R1B Chomatopac recorder, BSZ-100 fraction collector, a sample injection valve with a 30 ml sample loop and NT2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

The high-performance liquid chromatography (HPLC) used was Agilent 1100 system including G1311A QuatPump, G1322 Degasser, G1314A variable wave detector (VWD), a model 7725 injection valve with 20 μl loop, a PT100 column oven and Agilent ShemStation for LC.

2.2. Reagents

All organic solvents used for CCC 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 solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany.

The ethanol extracts of rhizomes of *Z. Cassumunar* were kindly provided by Dr. L. Broto and S. Kardono from Research Center for Chemistry, Indonesian Institute of Sciences.

2.3. Preparation of crude sample

The ethanol extracts of the rhizomes of *Z. Cassumunar* (800 g) were dissolved in 1.5 l water. The aqueous solution was again extracted with 4.5 l light petroleum (b.p. 60–90 °C) for five times. The extracts were combined and evaporated under reduced pressure and 40 °C, yielding 189 g of the crude sample. It was stored in a refrigerator (4 °C) for the subsequent CCC separation.

2.4. Preparation of the two-phase solvent systems and sample solution

The two-phase solvent system used was composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water at various volume ratios. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in a solvent mixture consisting of equal volumes of both upper and lower phases at suitable concentration according to the preparative scale of CCC separation.

2.5. CCC separation procedure

Preparative CCC was performed as follows: the four upright multilayer coil columns connected in series were first entirely filled with upper phase as stationary phase, and then the sample solution was injected through the sample port and the lower phase as a mobile phase was pumped in head-to-tail elution mode at flow rate of 5.0 ml min^{-1} while the column was rotated at 500 rpm. The effluent was monitored on-line at 254 nm and automatically collected in 20 ml test tube per 3 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile and HPLC detection.

2.6. HPLC analysis and identification of CCC peak fractions

HPLC analyses of the crude sample and CCC peak fractions were performed with a Zorbax Extend-C18 column (150 mm × 4.6 mm I.D., 5 μm, Agilent). The mobile phase was methanol (solvent A) and water (solvent B) at the gradient: A from 50 to 90% and B from 50 to 10% for 40 min. The flow rate was 1.0 ml min^{-1}, and the effluent was monitored at 254 nm.

Identification of the CCC peak fraction was performed by ESI-MS, ^{1}H NMR and ^{13}C NMR. Positive ESI-MS analyses were performed using Bruker Esquire 3000 plus spectrometer with an electrospray ionization (ESI) interface. NMR experiments were carried out using a Bruker Advanced DMX 500 NMR spectrometer with chloroform ($CDCl_3$) as solvent and TMS as internal standard.

3. Results and discussions

The crude sample obtained from rhizomes of *Z. Cassumunar* was first analyzed by HPLC, and the chromatogram

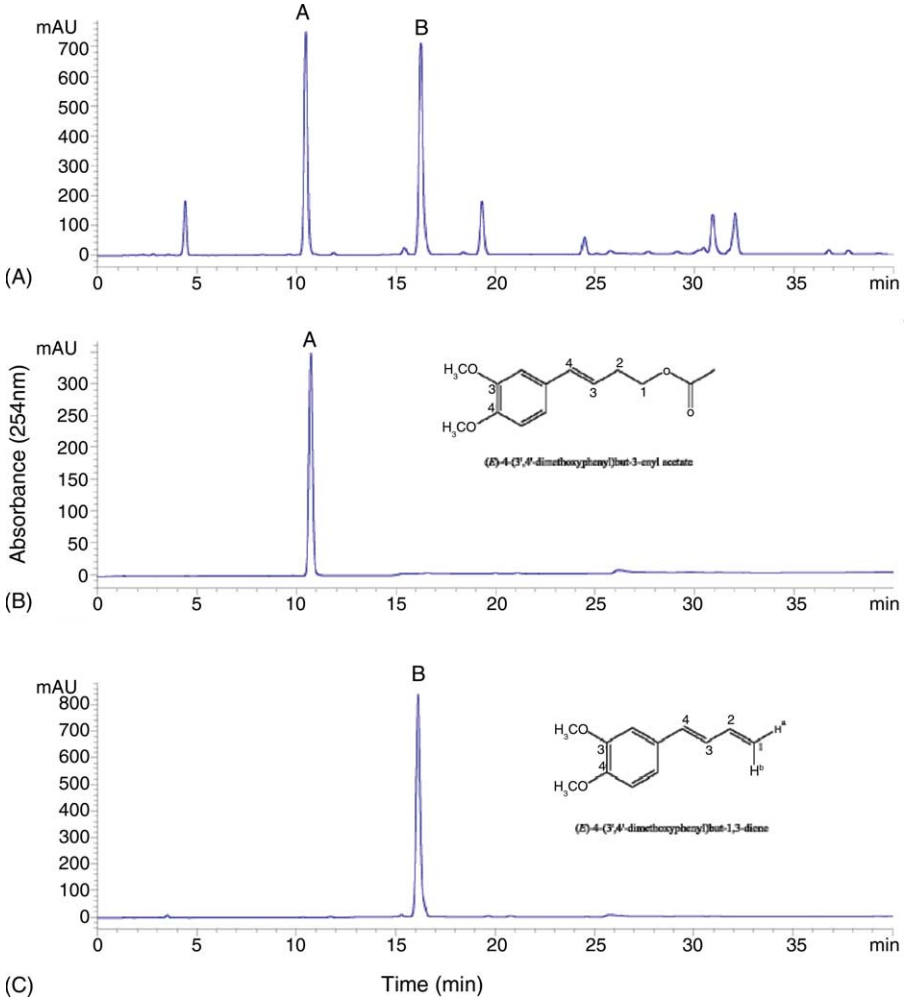

Fig. 1. HPLC chromatograms and chemical structures of target compounds. (A) Crude sample from the rhizomes of *Z. Cassumunar*; (B) CCC fraction of peak A; (C) CCC fraction of peak B. Conditions: column, Zorbax Extend-C18 column (150 mm × 4.6 mm I.D., 5 μm, Agilent); column temperature, 25 °C; mobile phase, A (methanol) and B (water) at the gradient: A from 50% to 90% and B from 50% to 10% for 40 min; flow rate, 1.0 ml min^{-1}; detection, 254 nm.

is shown in Fig. 1A. Peak A corresponds to (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and peak B corresponds to (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene. The contents of A and B were 29.3 and 32.2%, respectively.

The successful separation by CCC largely relies on the selection of a suitable two-phase solvent system. In order to achieve an efficient separation of target compounds, the previous articles on the CCC involving separation of similar compounds should be consulted. In addition, the two-phase solvent system should satisfy the following requirements: (1) short settling time (<30 s); (2) no decomposition or denaturation of the sample; (3) sufficient sample solubility; (4) suitable partition coefficient (*K*) values (usually between 0.5 and 2); (5) satisfactory retention of the stationary phase [12]. Generally speaking, the higher the retention of the stationary phase, the better the peak resolution. And a smaller *K* value elutes the solute close to the solvent front with lower resolution while a larger *K* value tends to give better resolution but broader, more dilute peaks due to a longer elution time [15].

Table 1
The *K* (partition coefficient) values of the target compounds in several solvent systems

Solvent systems (v/v)	K_A	K_B
Light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:0.5:1)	0.223	0.769
Light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:1:1)	0.237	0.663
Light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (6:4:1:1)	0.241	0.819
Light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:2:1)	0.356	0.754
Light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (6:4:2:1)	0.416	0.987

A: (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate; B: (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene.

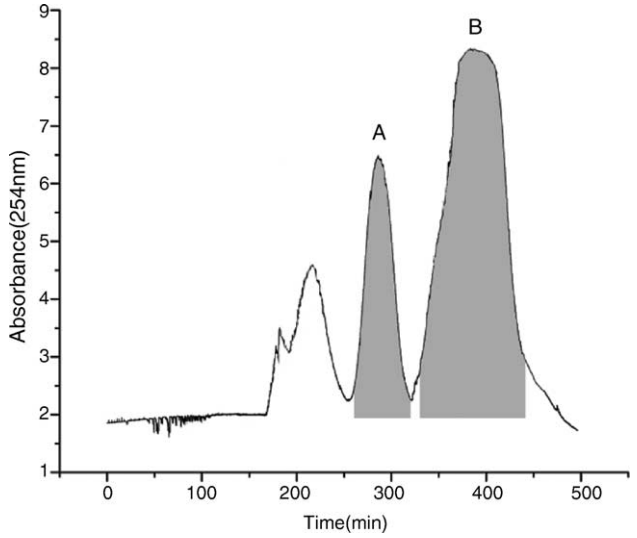

Fig. 2. Preparative CCC separation of the crude sample from the rhizomes of *Z. Cassumunar*; A: (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate; B: (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene. CCC separation conditions: column, multilayer coil of 4.0 mm I.D. PTFE tube with a total capacity of 1600 ml; rotary speed, 500 rpm; column temperature, 35 °C; solvent system, light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:2:1, v/v); mobile phase, lower phase; flow rate, 5.0 ml min^{-1}; detection, 254 nm; sample size, 600 mg of crude sample dissolved in 10 ml upper phase and 10 ml lower phase; retention of the stationary phase, 50.1%.

In the present study, the crude extracts have lipophilic physical properties and are soluble in non-polar solvent. According to the literature [16], the two-phase solvent system composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:0.5:1, v/v) was very efficient for CCC separation of non-polar substances from the essential oil of the rhizomes of *Curcuma wenyujin*. Thus, the two-phase solvent systems composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water at various volume ratios (5:4:0.5:1, 5:4:1:1, 6:4:1:1, 5:4:2:1, 6:4:2:1, v/v) were evaluated in terms of *K* values and peak resolution. The results indicated that when the two-phase solvent system at a volume ratio of 5:4:2:1 was used, the separation of the target compounds was achieved with satisfactory peak resolution, and the retention of the stationary phase was good (50.1%). Therefore, the two-phase solvent system composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:2:1, v/v) was selected for separation of the two phenylbutenoids from the crude sample of *Z. Cassumunar* in the present study.

Fig. 2 shows the preparative CCC separation of 600 mg of the crude sample using the solvent system composed of light petroleum (b.p. 60–90 °C)–ethanol–diethyl ether–water (5:4:2:1, v/v). In order to save solvents and time, other eluting substances after the target compounds were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was used only once. Each CCC peak fraction was analyzed by HPLC and the chromatograms are shown in Fig. 1B and C. As a result, 150 mg of (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and 175 mg of (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene with the purity of 98.7 and 95.1%, respectively, were yielded in a single-step separation, which clearly indicated the two-phase system was very efficient for CCC separation of the phenylbutenoids.

The *K* values of target compounds in several solvent systems were measured according to the literature [13], and are given in Table 1.

Identification of each CCC fraction was carried out by positive ESI-MS, ^{1}H NMR and ^{13}C NMR. Compared with the data given in literatures [6,8,9], peak A was identified as (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and peak B was identified as (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene.

4. Conclusion

In conclusion, upright CCC was successfully used for preparative isolation of two phenylbutenoids from the rhizomes of *Z. Cassumunar*, and 150 mg of (*E*)-4-(3′,4′-dimethoxyphenyl)but-3-enyl acetate and 175 mg of (*E*)-4-(3′,4′-dimethoxyphenyl)but-1,3-diene with over 95% purity were yielded from 600 mg of the crude extract in a one-step separation. These results clearly demonstrated that the present CCC method is powerful for the separation of phenylbutenoids from the rhizomes of *Z. Cassumunar*.

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