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Journal of Chromatography A, 1052 (2004) 217–221
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Preparative isolation and purification of hydroxyanthraquinones and cinnamic acid from the Chinese medicinal herb Rheum officinale Baill. by high-speed counter-current chromatography

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Received 27 May 2004; received in revised form 8 July 2004; accepted 25 August 2004

Abstract

A high-speed counter-current chromatography (HSCCC) method for preparative separation and purification of five hydroxyanthraquinones and cinnamic acid from the Chinese medicinal herb Rheum officinale Baill. was developed by using pH-gradient elution. The purities of rhein, emodin, aloe-emodin, chrysophanol, physcion and cinnamic acid were all over 98%, as determined by high performance liquid chromatography (HPLC). The structures of them were identified by ^{1}H NMR.
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Keywords: Rheum officinale Baill; Hydroxyanthraquinones; Cinnamic acid; High-speed counter-current chromatography; pH-gradient elution

1. Introduction

Da-huang, the duried roots of R. officinale Baill., is one of the most popular traditional medicinal herb and is officially listed in the Chinese Pharmacopoeia [1]. Pharmacological test revealed that R. officinale Baill. extract has strong action on many kinds of bacillis, such as coliform, comma bacillus, staphylococcus aureus and so on, so it has been used for the treatment of dysentery, cholera, uraemia, leukaemia, diabetes and lung cancer [2–4]. The major active components of the herb are hydroxyanthraquinones. They are often used as standards in the quality control of Dahuang products, so high-purity preparation of hydroxyanthraquinones is of great interest. The chemical structures of them and cinnamic acid are shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a form of liquid–liquid partition chromatography which was first invented by Ito [5]. Solute separation is based on partitioning between the two immiscible liquid phases: the mobile phase and the support-free liquid stationary phase. Without any solid matrix, the stationary phase is retained in the column with the aid of a centrifugal force field, so it eliminates irreversible adsorption of samples onto the solid support. Therefore, it is considered as a suitable alternative for separation of phenolic compounds such as flavonoids and hydroxyanthraquinones [6–8]. As an advanced separation technique, it has been widely used for separation of active components from traditional Chinese herbs and other natural products in recent years. Successful application of HSCCC has been reported for the purification of alkaloids [9–11], hydroxyanthraquinones [12,13], flavonoids [14,15], saponins [16], and so on.

The number of carboxylic and phenolic hydroxyl groups and the position of the phenolic hydroxyl groups in the hydroxyanthraquinone molecules determine their acidic–basic characteristics. The characteristic acidity of the hydroxyanthraquinone molecules is different from each other. Thus, they can be separated by using pH-gradient elution method according to their acidity. The purification of hydroxyanthraquinones using pH-modulated stepwise elution by HSCCC has been reported previously [12], only four hydroxy-anthraquinones (emodin, aloe-emodin, chrysophanol and physcion) were purified. The purity of rhein is

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doi:10.1016/j.chroma.2004.08.101

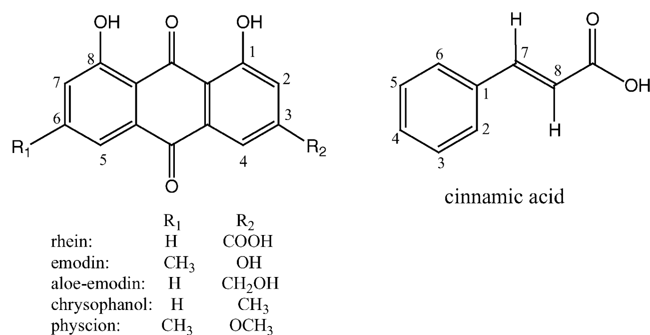

Fig. 1. Chemical structures of hydroxyanthraquinones and cinnamic acid from R. officinale Baill.

very low. In the present study, the continuous pH-gradient elution was employed in HSCCC. The optimum conditions were obtained, which led to successful preparation of five hydroxyanthraquinones and cinnamic acid from crude extract of R. officinale Baill.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β values of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. An ÄKTA prime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd., Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including a G1311A QuatPump, a G1315B DAD, a Rheodyne 7725i injection valve with a 20 μL loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., America).

2.2. Reagents

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water.

The dried roots of R. officinale Baill. were purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of crude sample

Preparation of crude sample was carried out according to the literature [17]. The dried roots of R. officinale Baill. were ground to powder (about 30 mesh). The powder (50 g) was extracted with the mixture of 20% H_2SO_4 and benzene (1:5, v/v) for 1.5 h four times at 81 °C (200 mL each time). The benzene extracts were combined and evaporated under reduced pressure to about 200 mL. Then the extract was extracted with 5% NaOH three times (200 mL each time). The aqueous solution was acidified with 36% HCl to pH 2, and then extracted with aether three times (200 mL each time). The aether extracts were evaporated to dryness. A 1.2 g of crude extract was obtained. It was stored in a refrigerator (4 °C) for further use.

2.4. Preparation of two phase solvent system and sample solutions

One percent NaH_2PO_4 and 1% NaOH solutions were prepared by dissolving suitable amount of NaH_2PO_4 and NaOH in water and then saturated with aether, respectively. Aether saturated with 1% NaH_2PO_4 was used as the stationary phase. The three kinds of solvent were degassed by sonication for 30 min prior to use.

The sample solutions were prepared by dissolving 120 mg of crude extracts in 20 mL of the stationary phase.

2.5. HSCCC separation procedure

HSCCC was performed as follows: aether and 1% NaH_2PO_4 were pumped into the separation column simultaneously by ÄKTA prime with the volume ratio of 40:60, which was controlled by the switch valve. After the column was totally filled with two phases, the HSCCC apparatus was revolved at a speed of 800 rpm while only 1% NaH_2PO_4 was pumped into the column at a flow rate of 2 mL min^{-1}. Half an hour later, hydrodynamic equilibrium was reached. Then the sample solution (120 mg of crude extract dissolved in 20 mL of aether) was injected into the column through the injection valve. At the same time 1% NaH_2PO_4 and 1% NaOH were pumped into the column simultaneously, and the linear gradient elution was started by changing the volume ratio of 1% NaH_2PO_4 and 1% NaOH. The volume ratio of 1% NaH_2PO_4 and 1% NaOH was continuously changed from 100:0–0:100 in 500 min. The separation temperature was controlled at 25 °C. The effluent from the tail end of the column was monitored at 254 nm. The chromatogram was recorded 80 min

after sample injection. Different fractions were collected according to the obtained chromatogram. Each fraction was first acidified with 10% HCl to pH 2, and then extracted with aether.

2.6. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis of R. officinale Baill. crude extract and HSCCC peak fractions was performed with a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. The mobile phase was methanol and 0.1% H_3PO_4 in gradient mode as follows: 0–3 min, 57% methanol; 3–20 min, 57–90% methanol; 20–40 min, 90% methanol. The effluent was monitored at 254 nm and the flow rate was kept at 1.0 ml min^{-1} constantly.

Identification of HSCCC peak fractions was carried out by ^{1}HNMR. ^{1}H NMR spectrum was recorded on a Mercury Plus 400 NMR with TMS as internal standard.

3. Results and discussion

3.1. Optimization of HPLC method

Different kinds of solvent system were used as the mobile phase and different elution modes were employed to analyze crude extracts from R. officinale Baill. by HPLC. The results indicated that when methanol and 0.1% H_3PO_4 were used as the mobile phase in gradient mode (methanol: 0–3 min, 57%; 3–20 min, 57–90%; 20–40 min, 90%), six major peaks can be obtained, and each peak got baseline separation. The peak purity of HPLC was analyzed with Agilent 1100 workstation. The results were satisfactory. HPLC chromatogram of crude extract from R. officinale Baill. was given in Fig. 2(A).

3.2. Optimization of HSCCC conditions

With phenolic hydroxyl groups in molecule, hydroxyanthraquinone is a kind of acid compound. The characteristic acidity of these compounds is determined by the number of carboxylic and phenolic hydroxyl groups as well as the position of the phenolic hydroxyl groups in the molecules. The solubility of them in the solutions with different pH is different from one another. So they can be separated by changing pH value of the mobile phase of HSCCC. A continuous pH-gradient elution mode was employed in this study. A more subtle pH gradient zone and high resolution of HSCCC can be obtained by this method.

The influences of the components of the stationary phase, the concentration of NaH_2PO_4 and NaOH, and the gradient elution time on the separation of hydroxyanthraquinones from R. officinale Baill. by HSCCC were investigated. Good separation results can be obtained when aether was used as the stationary phase, and 1% NaH_2PO_4 and 1% NaOH as the mobile phase in gradient elution mode (1% NaH_2PO_4:1% NaOH = 100:0–0:100 in 500 min). Under the optimum conditions, six major peaks can be obtained and yielded 19 mg of peak I, 19 mg of peak II, 18 mg of peak III, 14 mg of peak IV, 10 mg of peak V, and 6 mg of peak VI from 120 mg of crude extracts. HSCCC chromatogram of crude extracts from R. officinale Baill. was shown in Fig. 3.

3.3. Results of HPLC analysis and identification of each HSCCC fraction

Each fraction of HSCCC was analyzed by HPLC. The purity of each fraction was over 98%. HPLC chromatogram of the purified HSCCC peak fractions was shown in Fig. 2(B–G).

Identification of each HSCCC fraction was carried out by ^{1}H NMR and ^{13}C NMR. each fraction were given as follows:

^{1}H NMR data of HSCCC peak I in Fig. 3: ^{1}H NMR (400 MHz, DMSO): 12.03 (1H, s, C_1–OH), 11.91 (1H, s, C_8–OH), 10.53 (1H, broad, –COOH), 8.08(1H, s, C_2–H), 7.38 (1H, d, J = 8.5 Hz, C_5–H), 7.71 (1H, m, C_6–H), 7.78 (1H, s, C_4–H), 7.81(1H, d, J = 7.5 Hz, C_7–H). Compared with the data given in ref. [18], peak I corresponded to rhein.

^{1}H NMR data of HSCCC peak II in Fig. 3: ^{1}H NMR (400 MHz, C^2HCl_3): 11.01(1H, broad, –COOH), 7.81 (1H, d, J = 16 Hz, C_7–H), 7.57(2H, m, C_2–H, C_6–H), 7.42 (3H, m, C_3–H, C_4–H and C_5–H), 6.47 (1H, d, J = 16 Hz, C_8–H). ^{13}C NMR (400 MHz, C^2HCl_3) data of peak II: 172.06 (–COOH), 147.11 (C_7), 133.99 (C_1), 130.77 (C_4), 128.96 (C_3, C_5), 128.37 (C_2, C_6), 117.17 (C_8). According to ^{1}H NMR and ^{13}C NMR data, peak II was identified as cinnamic acid.

^{1}H NMR data of HSCCC peak III in Fig. 3: ^{1}H NMR (400 MHz, C^2HCl_3): 12.80 (1H, s, C_3–OH), 12.30 (1H, s, C_1–OH), 12.12 (1H, s, C_8–OH), 7.63 (1H, s, C_5–H), 7.29 (1H, d, J = 2.4 Hz, C_4–H), 7.10 (1H, s, C_7–H), 6.68 (1H, d, J = 2.4 Hz, C_2–H), 2.46 (3H, s, CH_3). Compared with the data given in ref. [18], peak III corresponded to emodin.

^{1}H NMR data of HSCCC peak IV in Fig. 3: ^{1}H NMR (400 MHz, C^2HCl_3): 12.12 (1H, s, C_1–OH), 12.11(1H, s, C_8–OH), 7.86 (1H, d, J = 7.2 Hz, C_5–H), 7.81 (1H, s, C_4–H), 7.70 (1H, m, C_6–H), 7.37 (1H, s, C_2–H), 7.32 (1H, d, J = 8.4 Hz, C_7–H), 5.35 (1H, s, –CH_2–OH), 4.85 (2H, s, –CH_2–). Compared with the data given in ref. [18], peak IV corresponded to aloe-emodin.

^{1}H NMR data of HSCCC peak V in Fig. 3: ^{1}H NMR (400 MHz, C^2HCl_3): 12.14 (1H, s, C_1–OH), 12.03 (1H, s, C_8–OH), 7.83 (1H, d, J = 7.2 Hz, C_5–H), 7.68 (1H, m, C_6–H), 7.66 (1H, s, C_4–H), 7.30 (1H, d, J = 8.4 Hz, C_7–H), 7.11(1H, s, C_2–H), 2.47 (3H, s, –CH_3). Compared with the data given in ref. [18], peak V corresponded to chrysophanol.

^{1}H NMR data of HSCCC peak VI in Fig. 3: ^{1}H NMR (400 MHz, C^2HCl_3): 12.34 (1H, s, C_1–OH), 12.14 (1H, s, C_8–OH), 7.64 (1H, s, C_5–H), 7.38 (1H, d, J = 2.4 Hz, C_4–H), 7.10 (1H, s, C_7–H), 6.70 (1H, d, J = 2.4 Hz, C_2–H), 3.95 (3H, s, –OCH_3), 2.46 (3H, s, –CH_3). Compared with the data given in ref. [18], peak VI corresponded to physcion.

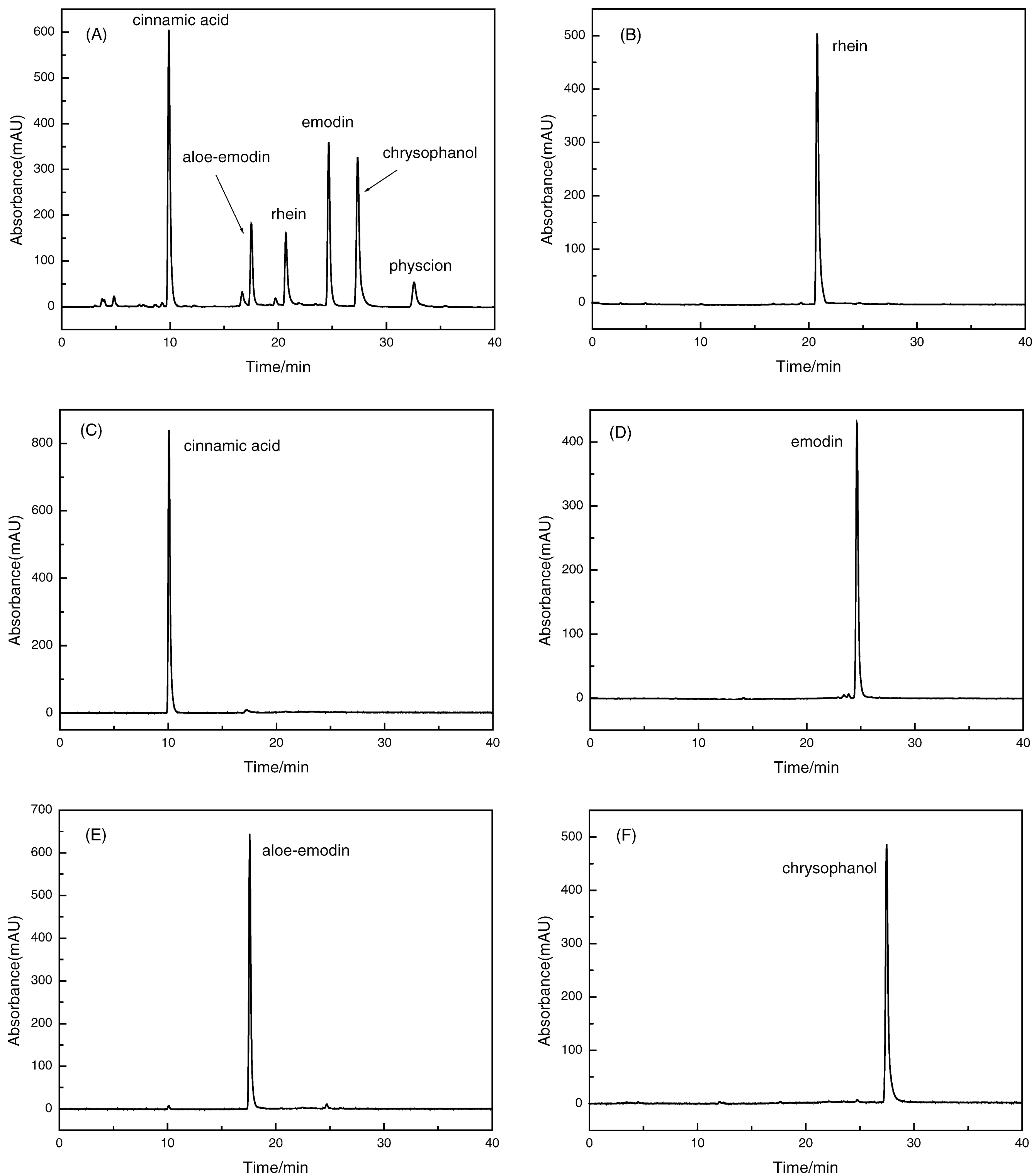

Fig. 2. HPLC chromatogram of crude extract from R. officinale Baill. and HSCCC peak fractions. Column: a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm); mobile phase: methanol and 0.1% H_3PO_4 in gradient mode (methanol: 0–3 min, 57%; 3–20 min, 57–90%; 20–40 min, 90%); flow rate: 1.0 mL min^{-1}; detection wavelength: 254 nm; (A) crude extract from R. officinale Baill; (B) peak I; (C) peak II; (D) peak III; (E) peak IV; (F) peak V; (G) peak VI of Fig. 3.

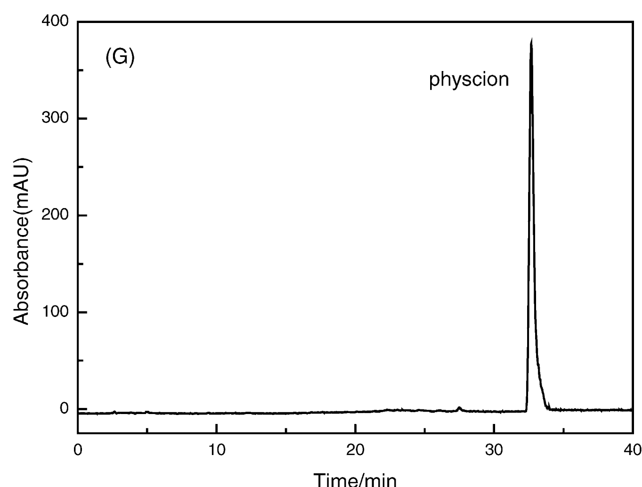

Fig. 2. (*Continued*).

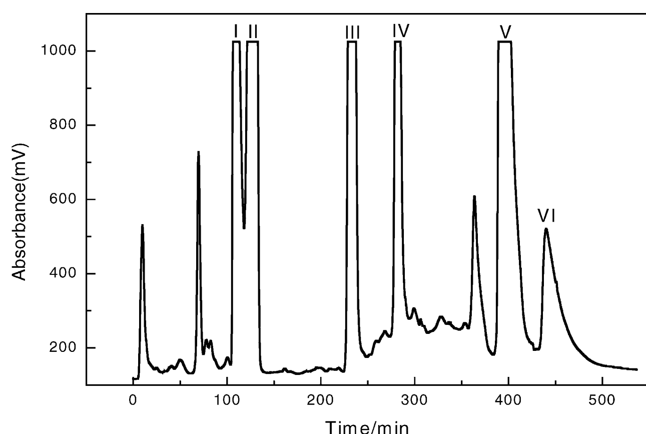

Fig. 3. HSCCC chromatogram of crude extract from R. officinale Baill. Stationary phase: aether; mobile phase: 1% NaH_2PO_4 and 1% NaOH to perform pH-gradient elution (1% NaH_2PO_4:1% NaOH = 100:0–0:100 in 500 min); flow rate: 2.0 mL min^{-1}; revolution speed: 800 rpm; sample size: 120 mg crude extract dissolved in 20 mL of aether; temperature: 25 °C; retention of the stationary phase: 40%. I: Rhein; II: cinnamic acid; III: emodin; IV: aloe-emodin; V: chrysophanol; VI: physcion.

In conclusion, the result of our studies clearly demonstrated that continuous pH-gradient elution method was an efficient way for separation of five hydroxyanthraquinones and cinnamic acid from R. officinale Baill.

Acknowledgement

Jichun Cui was greatly acknowledged for his help in structure identification.

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