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Preparative isolation and purification of phillyrin from the medicinal plant *Forsythia suspensa* by high-speed counter-current chromatography

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Abstract

Forsythia suspensa (Thunb.) Vahl. has been used widely in traditional medicines to treat gonorrhea, erysipelas, inflammation, pyrexia and ulcer. It has also shown antioxidant activity, as well as antibacterial, antiviral, choleretic and antiemetic effects. A high-speed counter-current chromatography (HSCCC) method was developed for the preparative separation and purification of the bioactive molecule phillyrin from *F. suspensa* (Thunb.) Vahl. The crude phillyrin was obtained by extraction with 50% ethanol from the dried fruits of *F. suspensa* (Thunb.) Vahl. under sonication. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (1:9:1:9, v/v/v/v) was successfully performed, and the components purified and collected were analyzed by high-performance liquid chromatography. The method yielded 5.6 mg phillyrin at 98.6% purity from 500 mg of the crude extract (1.2% phillyrin) with the recovery of 92% in a one-step separation.

Keywords: *Forsythia suspensa*; Counter-current chromatography; Preparative chromatography; Plant materials; Phillyrin

1. Introduction

Forsythia suspensa (*F. suspensa*) (Thunb.) Vahl. is widely distributed in China, Korea and Japan. Its fruits have long been used in Chinese and Japanese folk medicines to treat gonorrhea, erysipelas, inflammation, pyrexia and ulcer [1–3]. Moreover, the extract of the fruits showed antioxidant activity, as well as antibacterial, antiviral, choleretic and antiemetic effects [4–7]. Phillyrin is the main bioactive component of *F. suspensa* (Thunb.) Vahl., and its chemical structure is shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid-liquid partition chromatography that uses a liquid stationary phase, which has been applied to the separation of a number of natural products [8–19]. However, no report has been published on the use of HSCCC for the isolation and purification of phillyrin from plants. The aim of this study, therefore, was to develop an efficient method for the isolation and purification of phillyrin from the medicinal plant *F. suspensa* (Thunb.) Vahl. by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (inner diameter of tube, 2.6 mm; total volume, 325 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β-value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$ where r is the distance from the coil

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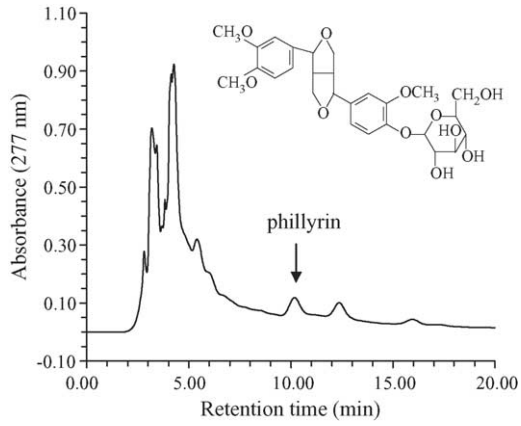

Fig. 1. Chromatogram of the crude extract from *Forsythia suspensa* (Thunb.) Vahl. by HPLC analysis as well as the chemical structure of phillyrin. Conditions: column, reversed-phase μBondapak C_{18} column (300 mm × 3.9 mm i.d., 10 μm); mobile phase, acetonitrile–water (25:75, v/v); flow-rate, 1.0 ml min^{-1}; detection at 277 nm.

to the holder shaft). The HSCCC system was equipped with a Model Series II HPLC pump (Pharma-Tech Research), a Model SPD-10Avp UV–vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10 ml sample loop.

2.2. Reagents

All solutions were prepared with analytical grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethyl acetate, *n*-hexane, methanol, absolute ethanol and acetonitrile were obtained from BDH (Poole, UK). The standard phillyrin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The phillyrin stock solution was 1.0 mg ml^{-1} which was prepared by dissolving 5.0 mg of phillyrin in 5.00 ml methanol and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with methanol.

The dried fruits of *F. suspensa* (Thunb.) Vahl. was obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.3. Preparation of crude extracts from F. suspensa (Thunb.) Vahl.

Preparation of crude extracts was carried out according to the literature [20,21]. In brief, the dried fruits of *F. suspensa* (Thunb.) Vahl. were ground to powder. The powder (100 g) was extracted with 500 ml of 60% ethanol under sonication for 1 h. The mixture was filtered, and then the residue was repeatedly extracted twice (500 ml each time). The filtrate was combined, and the extract was evaporated to dryness by rotary vaporization. The residue (14.51 g) was stored in a refrigerator for the subsequent HSCCC separation.

2.4. Preparation of two-phase solvent system and sample solution

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios. Each 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 extract in the lower phase of the solvent system used for HSCCC separation.

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) was pumped into the column in the head-to-tail elution mode at a flow-rate of 1.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, approximately 10 ml of the sample solution containing 500 mg of the crude extract was injected into the head of the column through the injection valve. The effluent of the column was continuously monitored with a UV–vis detector at 277 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis

The crude extract and each peak fraction obtained by HSCCC were analyzed by HPLC according to Xu and Su [22]. The HPLC system used throughout this study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium chromatography data system (Waters). The column used was a reversed-phase μBondapak C_{18} column (300 mm × 3.9 mm i.d., 10 μm, Waters). The mobile phase was acetonitrile–water (25:75, v/v). The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 277 nm. The peak identification was based on the retention time and the UV spectrum against the standard. Routine sample calculation was made by comparison of the peak area with that of the standard.

3. Results and discussion

The crude extract obtained from *F. suspensa* (Thunb.) Vahl. was analyzed by HPLC, and the chromatogram is shown in Fig. 1. The extract contained a number of different compounds, and the content of phillyrin was low (1.2%).

In order to select a suitable two-phase solvent system, the previous articles on the HSCCC should be consulted, and some rules need be considered [8]. Preliminary HSCCC

Table 1
The *K* (partition coefficient) values of phillyrin in several solvent systems

No.	Solvent system	*K* value
1	*n*-Butanol–ethyl acetate–water (2:3:5)	6.25
2	*n*-Butanol–ethyl acetate–water (1:4:5)	3.87
3	*n*-Hexane–ethanol–water (5:1:4)	0.011
4	*n*-Hexane–ethyl acetate–ethanol–water (5:5:3:7)	0.099
5	*n*-Hexane–ethyl acetate–ethanol–water (3:7:3:7)	0.246
6	*n*-Hexane–ethyl acetate–ethanol–water (3:7:1:9)	0.312
7	*n*-Hexane–ethyl acetate–ethanol–water (1:9:1:9)	0.799

Notes: the solvent system 7 was selected. The peak resolution was poor with the solvent systems 3–6, and the elution time was too long with the two solvent systems 1 and 2.

experiments were carried out with the two-phase solvent system composed of *n*-butanol–ethyl acetate–water, which has been successfully used for the separation and purification of salidroside from the herbal medicine *Rhodiola sachalinensis* A. Bor. [23]. Firstly, *n*-butanol–ethyl acetate–water at a volume ratio of 2:3:5 was tested. It was, however, very difficult to elute phillyrin from the stationary phase. As shown in Table 1, the *K* value of phillyrin in the two-phase solvent system was too big (6.25). In order to decrease the polarity of the mobile phase, *n*-butanol–ethyl acetate–water at a volume ratio of 1:4:5 was attempted. Although the *K* value was decreased from 6.25 to 3.87, it was still difficult to elute phillyrin from the stationary phase, and the time it was retained in the column was more than 7 h. In the subsequent studies, another two-phase solvent system was investigated.

A two-phase solvent system composed of *n*-hexane–ethanol–water at a volume ratio of 5:1:4 was evaluated. It was very difficult to purify phillyrin from the crude extract, because the retention time of phillyrin was short. As shown in Table 1, the *K* value of phillyrin in the two-phase solvent system was too small (0.011). Subsequently, a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 5:5:3:7 was tested. The time phillyrin was retained in the column was still too short, and the *K* value remained small (0.099). With the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 3:7:3:7, the overlap of chromatographic peaks of phillyrin with other compounds was still a problem, although the *K* value was increased from 0.099 to 0.246. Then, the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 3:7:1:9 was attempted because the mobile phase had stronger polarity. The peak resolution was improved, and the *K* value was increased from 0.246 to 0.312. Finally, the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 1:9:1:9 was tested. The separation of phillyrin with other components was achieved with good peak resolution, and the retention of the stationary phase was satisfactory (approximately 56%). After phillyrin was eluted out, in order to save solvents and time, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with

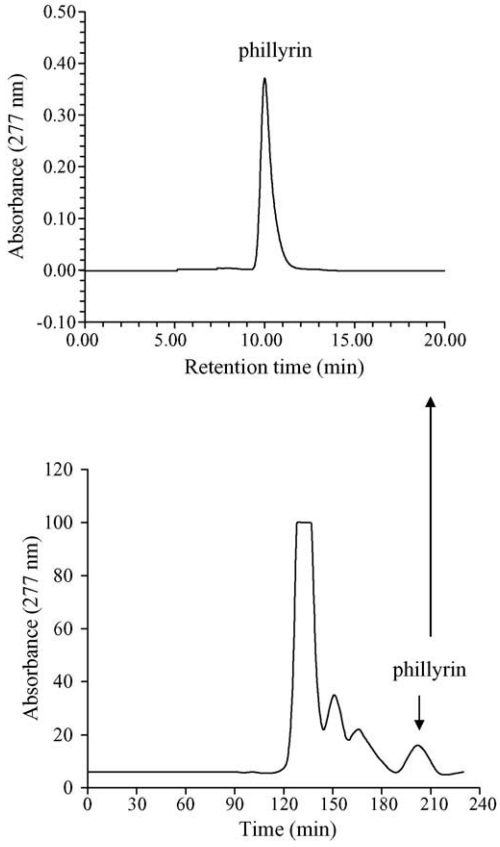

Fig. 2. Chromatogram of the crude extract from *Forsythia suspensa* (Thunb.) Vahl. by HSCCC separation, along with the HPLC chromatogram of the purified phillyrin from HSCCC. HSCCC conditions: column, multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–ethanol–water (1:9:1:9, v/v/v/v); mobile phase, lower phase (water phase); flow-rate, 1.0 ml min^{-1}; detection at 277 nm; sample size, 500 mg; retention of the stationary phase, 56%. HPLC conditions: column, reversed-phase μBondapak C_{18} column (300 mm × 3.9 mm i.d., 10 μm); mobile phase, acetonitrile–water (25:75, v/v); flow-rate, 1.0 ml min^{-1}; detection at 277 nm.

the mobile phase because the stationary phase was not to be reused. Fig. 2 shows the preparative HSCCC separation of 500 mg of crude extract using the solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 1:9:1:9. The separation yielded 5.6 mg of phillyrin at 98.6% purity according to HPLC analysis, and the recovery of phillyrin was 92%. The HPLC chromatogram of phillyrin as purified from the preparative HSCCC is shown in Fig. 2. Phillyrin was identified based on the retention time and the UV spectrum against the standard because it is a known structure compound typically present in *F. suspensa* (Thunb.) Vahl., and is also the main bioactive component of the plant.

In conclusion, an HSCCC method was developed for the preparative isolation and purification of bioactive molecule phillyrin from the medicinal plant *F. suspensa* (Thunb.) Vahl. Phillyrin with high purity could be obtained from the crude extract in a one-step separation, and its recovery was also high. The present study indicates that HSCCC is

a very powerful technique for the preparative isolation and purification of natural products.

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