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Comprehensive separation of major compositions from *Sophora japonica var. violacea* by counter-current chromatography using a liquid-liquid extraction strategy

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ABSTRACT

In the present study, a liquid-liquid extraction strategy for sample pretreatment and solvent system selection was established to assist comprehensive separation of major active compositions from natural product by counter-current chromatography. The two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water at the ratio of 10:0:8:2, 5:5:5:5 and 3:5:3:5 (v/v) were used for sample pretreatment, in which the major compositions were almost entirely distributed in the upper phase of two-phase solvent systems. After extraction, the enriched components FI, FV and FVI were obtained. According to this result, the two-phase solvent systems were slightly adjusted to lower polarities and used for HSCCC separation. As a result, 62 mg of compound 6 was isolated from 200 mg FI. 15 mg of compound 4 and 13 mg of compound 5 were isolated from 70 mg FV. 15 mg of compound 3, 51 mg of compound 1 and 16 mg of compound 2 were isolated from 200 mg FVI. The purities of all isolated compounds were over 93%. Their structures were identified as *p*-hydroxybenzoic acid, kaempferol-3-O-β-D-glucopyranoside, kaempferol 7-O-α-L-rhamnopyranoside, formononetin, 4′-O-methyl kaempferol, and di-butyl phthalate by ^{1}H NMR and ^{13}C NMR.

1. Introduction

High–speed counter–current chromatography (HSCCC) has been used for the separation of the active ingredients from natural products for many years. It is a support-free liquid–liquid partitioning chromatographic technique and has advantages in avoiding low yield, low recovery and time-consuming operation (Friesen et al., 2015; Sutherland and Fisher, 2009; Sun et al., 2016).

Because HSCCC employs two immiscible liquid phases without a solid support for separation, the samples will not be lost on the stationary phase due to irreversible adsorption. So, it is beneficial to photochemical studies using HSCCC to separate as many components as possible. However, not all the compounds in the complex mixture can be as the “suitable compounds” separated by HSCCC due to the interruption by their properties and surrounding environment. It is a challenge to enrich the “suitable compounds” one by one from the complex extraction for HSCCC separation.

In HSCCC, the most important step was the selection of two-phase solvent system for the target compound (Ito, 2005). In the previous studies, several statistic or mathematic methods were developed to assist rapid prediction of solvent systems (Berthod et al., 2005; Lu et al., 2009; Liang et al., 2015). Besides, TLC method was also developed to predict HSCCC solvent system (Friesen and Pauli, 2005; Yang et al., 2012). The generally useful estimate of solvent systems (GUESS) method was a practical approach for the prediction of CCC distribution constants, K values, by standard TLC (Friesen and Pauli, 2005; Liu et al., 2015). The solvent system, function of mobile phase, the choice of normal or reverse phase can be predicted and required according to TLC Rf values, equivalent solvent systems and calibration with the GUESS standard compounds (Friesen and Pauli, 2005). There is no doubt that these methods are very effective in the HSCCC separation of limited target compounds, because they select the solvent systems based on partition coefficient (K) which is the ratio of the solute distributed between the mutually equilibrated two solvent phases (Ito, 2005; Wang et al., 2016). However, if we are going to separate all “suitable compounds” with the very different polarities in the complex extraction, it's very difficult and complicated to evaluate all K values with these methods.

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In addition, the impurities in the mixture also need to be paid attention to in the comprehensive separation of "suitable compounds". They will interrupt the HSCCC separation, the settling time of two-phase solvent system, reducing the retention of stationary phase, and reducing the yield and purity of the product, even causing separation failure. As we have known, before HSCCC separation, the efficient sample pretreatment methods, such as macroporous resin chromatography (Liang et al., 2011), Sephadex LH–20 column(Yang et al., 2009), silica gel column (Yang et al., 2012), and liquid–liquid extraction (Liu et al., 2014), were usually applied to increase the content of target compound in the complex natural extracts. These methods can not only increase the yield and purity of HSCCC product efficiently, but also improve the retention of stationary phase of HSCCC. Among these methods, liquid–liquid extraction without the adsorptive loss of samples is the simplest method (Wang et al., 2005; Du et al., 2009). It will be helpful to establish a method which can select the suitable solvent systems for sample pretreatment and HSCCC separation simultaneously. Recently, a rational liquid–liquid extraction–assisted sample pretreatment method was developed to enrich the target compounds and remove the impurities (Wang et al., 2017). In this method, a quaternary system composed of *n*-hexane–ethyl acetate–methanol–water (HEMW) was selected for the sample pretreatment. Most of the impurities can be removed by this method. As a result, the HSCCC separation efficiency, the yields and purities of products were all improved. The quaternary system was chosen according to the reported practical table for HSCCC separation, and the polarities of solvent systems in the table increased gradually from the top to the bottom (Ito, 2005). It reminds us that if the solvent systems which were list in the reported practical table according to the polarity in order are used for the crude sample extraction one by one, the chemical compositions in the crude sample will be fractioned according to their polarities in order. Besides, the corresponding solvent systems can be as the two-phase solvent system for their HSCCC separation directly or with a slightly modification of the polarity.

Sophora japonica var. *violacea* (SJV), a medical plant, is a variant of *Sophora japonica* L. The biological activities of its flowers and extracts include bleeding hemorrhoids, hematuria, hematemesis, hemorrhinia, uterine or intestinal hemorrhage, metrorrhagia, leukorrhea, conjunctivitis, pyoderma, arteriosclerosis, hypertension, and dizziness (Han et al., 1996; Chinese medicine company, 1994; Ishida et al., 1989; Frédérich et al., 2009; Pharmacopoeia Commission, 2010; Ji et al., 2012; Liang et al., 2013). The major chemical compositions are flavonoids, triterpene glycosides, phospholipids, alkaloids, amino acids, polysaccharides, and fatty acids (Lo et al., 2009).

In order to explore the active secondary metabolites, a combination method of liquid-liquid extraction and HSCCC was established in the present study. Consequently, six active compositions, including *p*-hydroxybenzoic acid, kaempferol-3-O-β-D-glucopyranoside, kaempferol 7-O-α-L-rhamnopyranoside, formononetin, 4′-O-methyl kaempferol, and dibutyl phthalate, were isolated from SJV by HSCCC using a liquid-liquid extraction strategy for the sample pretreatment and solvent system selection. p-hydroxybenzoic acid and dibutyl phthalate possess antibacterial activity and protein tyrosine phosphatase 1B inhibitory activity, respectively (Chen, 2008; Wang et al., 2016). The flavonoids and isoflavone have widely bioactivities, including antioxidant, antibacterial, anticancer, and so on. These compounds probably play an important role in the biological activity of SJV. The separation of these active compounds has laid the foundation for further pharmacological and application research of this plant.

2. Materials and methods

2.1. Apparatus

The HSCCC separation system includes a model TBE-300C high–speed countercurrent chromatograph (Tauto Biotech, Shanghai, China) with three polytetrafluoroethylene (PTFE) coils (internal tube diameter: 1.9 mm), a model TBP5002 constant–flow pump (Tauto Biotech, Shanghai, China), a model UV2000D monitor at 254 nm (Sanotac Scientific Instruments CO., LTD, Shanghai, China) and a model V2.0.2B workstation (Tauto Biotech, Shanghai, China). The total volume is 300 mL.

HPLC equipment (Yilite company, Dalian, China) includes a UV230II detector, two 230 P pumps and a Rehodyne model 3725i-038 sample injector. The analysis was carried out with a SinoChrom ODS-BP C18 column (5 μm, 4.6 mm × 200 mm). The data were processed on EC2006 workstation (Yilite company, Dalian, China).

2.2. Chemicals and plant material

HPLC–grade methanol was purchased from Young Metal Co. Ltd (Seoul, South Korea). All analytical grade solvents were purchased from Beijing Chemical Works (Beijing, China).

The flowers of *Sophora japonica* var. *violacea* (SJV) were collected from Dalian, China, which was authenticated by Guanmian Shen, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences (Editorial Committee of Flora Reipublicae Popularis Sinicae, 1994).

2.3. Preparation of crude sample

The dried flowers of SJV (106 g) were refluxed with light petroleum (b.p. 30–60°C) (2.5 L, 3 times) and ethyl acetate (2.5 L, 3 times) for 3 h. All ethyl acetate extracts were combined and evaporated under reduced pressure. 1.50 g of ethyl acetate extracts were obtained as the crude sample.

2.4. Selection of extraction solvent systems by liquid-liquid microextraction

3 mg ethyl acetate extracts were dissolved in the lower phase (0.5 mL) of *n*–hexane–ethyl acetate–methanol–water (10:0:8:2, v/v), and then the upper phase (0.5 mL) was added and the mixture was vortexed. After equilibration, the upper phase was taken out by syringe (Fig. 1). The process was repeated twice. The upper phase was combined and evaporated to dryness. The solid was dissolved in methanol as the FI, which was analyzed to find the major composition by HPLC.

The fractions FII–FVII were obtained with the same operation, just the two-phase solvent systems composed of *n*–hexane–ethyl acetate–methanol–water at the ratios of 10:0:6:4, 9:1:5:5, 7:3:5:5, 5:5:5:5,

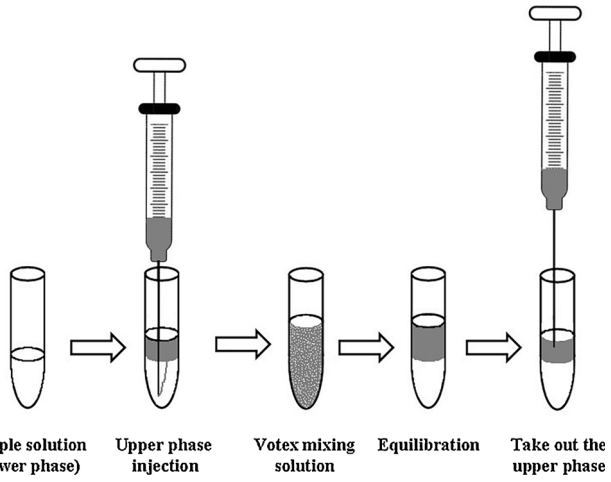

Fig. 1. Schematic diagram for liquid-liquid microextraction procedure of ethyl acetate extracts. Ethyl acetate extracts: 3 mg; Composition of solvent system: *n*–hexane–ethyl acetate–methanol–water; Upper phase: 0.5 mL; Lower phase: 0.5 mL.