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# Isolation and purification of series bioactive components from *Hypericum perforatum* L. by counter-current chromatography

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ABSTRACT

Counter-current chromatography (CCC) combined with pre-separation by ultrasonic solvent extraction was successively used for the separation of series bioactive compounds from the crude extract of *Hypericum perforatum* L. The petroleum ether extract was separated by the solvent system of n-heptane–methanol–acetonitrile (1.5:0.5:0.5, v/v) and n-heptane–methanol (1.5:1, v/v) in gradient elution, yielding a phloroglucinol compound, hyperforin with HPLC purity over 98%. The ethyl acetate extract was separated by using the solvent system composed of hexane–ethyl acetate–methanol–water (1:1:1:1 and 1:3:1:3, v/v) in gradient through both reverse phase and normal phase elution mode, yielding a naphthodianthrone compound, hypericin with HPLC purity about 95%. The n-butanol extract was separated with the solvent system composed of n-butanol–ethyl acetate–water (1:4:5 and 1.5:3.5:5, v/v) in elution and back-extrusion mode, yielding two of flavones, rutin and hyperoside, with HPLC purity over 95%. HPLC–MS, reference sample and UV spectrum were selectively used in separation to search for target compounds from HPLC-DAD profiles of different sub-extracts. The structures of isolated compounds were further identified by ESI-MS, $^{1}$HNMR and $^{13}$CNMR.



## 1. Introduction

*Hypericum perforatum* L., commonly named St. John's wort in western Europe, is a traditional herbal medicine used in various part of world. The increasing attention on *H. perforatum* L. in recent years relied on its antidepressant activity [1,2]. In addition, studies also have shown that *H. perforatum* L. has antiviral [3], antimicrobial [4], wound healing[5] and anticancer activities [6,7]. The extracts of *H. perforatum* L. contain a variety of pharmacological active constituents including phloroglucinols, naphthodianthrones, and a broad range of flavonoids. The antidepressant activity of *H. perforatum* L. was firstly attributed to the naphthodianthrones hypericin, pseudohypericin, protohypericin and protopseudohypericin [8]. Recent studies revealed that the major phloroglucinol hyperforin and its derivative adhyperforin might be the critical components for its the antidepressant activity [9]. Hypericin, a powerful naturally occurring phytosensizer, has drawn much attention as a potential clinical anticancer agents in recent years. Studies established its powerful in vivo and in vitro antineoplastic activity upon irradiation [10]. Naphthodianthrones were also discovered to act as antiviral agent to inhibit the growth of a variety of neoplasmatic cell types [11]. In addition, flavonoids present in *H. perforatum* L. have also been shown to have antidepressive activities [12] in addition to their antioxidant potential [13].

Numerous papers have been published in qualitative analysis of bioactive constituents in the extract of *H. perforatum* by HPLC [14,15], HPLC–MS [16–19], HPLC–SPE–NMR [20,21], and capillary electrophoresis [22]. Some reports are related to their extraction and isolation including enrichment of hyperforin by supercritical fluid extraction [23], extraction of hyperoside using CTAB reversed micelles [24], purification of hypericin and pseudohypericin using liquid–liquid extraction and SEC [25], purification of total flavonoid glycosides by macroporous adsorption resin [26], separation of naphthodianthrones by preparative high-performance liquid chromatography [27], etc. However, the preparative separation of bioactive components from *H. perforatum* is of great technical challenge due to their low-level amount in the extract and coexistence with their analogues. The commercially acceptable standard for crude extract of *H. perforatum* is that the content of hyperforin and hypericin is no less than 3.0% and 0.3% respectively. Thus better understanding of pharmacological activities of *H. perforatum* and the quality control of related products make it necessary to develop more efficient separation methods for the above mentioned bioactive constitutions.

During recent years, high-speed counter-current chromatography (CCC), a support-free liquid–liquid partition chromatography has been widely used for separation and purification of bioactive components from natural products by eliminating irreversible

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adsorption of sample onto the solid support. The high performance of CCC in the preparative isolation and purification of epigallocatechin and flavonoids from *H. perforatum* L. has been reported [28,29].

In this paper, a systematic separation method was established for four bioactive components from less polar to polar from a crude extract of *H. perforatum* L. by CCC combined with pre-separation by ultrasonic solvent extraction with petroleum ether, ethyl acetate, and n-butanol successively. As a result, one phloroglucinol (hyperforin), one naphthodianthrone (hypericin) and two flavones (rutin and hyperoside) were isolated. HPLC-DAD–MS and NMR were employed for purity analysis and structure elucidation.

## 2. Experimental

### 2.1. Apparatus

The present study employed three CCC units, i.e., a model GS20 analytical (0.8 mm i.d. PTFE tubing, column volume 35 mL, $\beta$ values 0.4–0.72) and a model GS10A3 preparative unit (1.6 mm i.d. PTFE tubing, 220 mL column volume, $\beta$ values 0.5–0.75), both of them were manufactured by Beijing Institute of New Technology Application, Beijing, China. A model TBE-300A preparative unit (1.6 mm i.d. PTFE tubing, 260 mL column volume, $\beta$ values 0.42–0.63, Shanghai Tauto Biotech CO., LTD., Shanghai, China) was also used in some separations.

The HPLC-DAD/MS analyses were performed on an Agilent 1100 HPLC system coupled with diode array detector (DAD) and an Agilent 1100 series MSD trap (SL model) with an electrospray ionization (ESI) interface. The HPLC system is also equipped with a quaternary pump and an autosampler.

NMR analysis was performed on a Bruker AV 600 and ARX 400 NMR instrument in Institute of Chemistry, Chinese Academy of Sciences, Beijing.

### 2.2. Reagents and materials

All organic solvents used for solvent extraction and CCC separation were of analytical grade (Beijing Chemical Reagents Co., Beijing, China). Methanol and tetrahydrofuran used for HPLC analyses is of chromatographic grade (Fisher Scientific, UK), and water for HPLC was prepared with Millipore purifier (Millipore, USA) in our laboratory.

The crude extract powder of *H. perforatum* L. was purchased from Acetar Bio-Tech Inc., Xi-an, China. The content of hypericin is about 0.3%.

**Table 1**
Solvent systems selected for HSCCC separation of different compounds.

| Compounds | Solvent systems |
|---|---|
| Hyperforin (**1**) | n-Heptane–methanol–acetonitrile (1.5:0.5:0.5,v/v)<br>n-Heptane–methanol (1.5:1, v/v) |
| Hypericin (**2**) | Hexane–ethyl acetate–methanol–water (1:3:1:3, v/v)<br>Hexane–ethyl acetate–methanol–water (1:1:1:1, v/v) |
| Rutin (**3**) | n-Butanol–ethyl acetate–water (1:4:5, v/v) |
| Hyperoside (**4**) | n-Butanol–ethyl acetate–water (1.5:3.5:5, v/v) |

### 2.3. Pre-separation of the crude extract by ultrasonic solvent extraction

A 500 g amount of crude extract was extracted with 2.5 L of petroleum ether (b.p. 60–90 °C), ethyl acetate and n-butanol successively under ultrasonication at 30 °C, three times for each solvent, 30 min for each time. After filtration, concentration and drying under vacuum, three parts of sub-extracts with different polarity were achieved as petroleum ether extract (A) 84.5 g, ethyl acetate extract (B) 71.7 g, n-butanol extract (C) 90.8 g. They were subjected to CCC separation for interested components respectively.

### 2.4. Measurement of K values

The selection of solvent systems is usually based on the partition coefficient ($K$), which is the ratio of solute distributed between the mutually equilibrated two solvent phases. It is expressed by the concentration of solute in the stationary phase divided by that in the mobile phase ($K_{U/L} = C_U/C_L$, when upper phase is used as stationary phase). The suitable $K$ values for CCC are $0.5 \leq K \leq 2.0$. The measurement of the $K_{U/L}$ values was performed as follows: an equilibrated solvent system was separated into two phases (2 ml each), a few milligrams of sample was added to the lower phase and thoroughly mixed with a vortex. After settling, pipette 0.5 mL of lower phase and subject it to HPLC analysis for the interested compound by peak height ($H_{L1}$). Then an equal volume of upper phase was added to the residual of lower phase and thoroughly mixed. After settling, pipette another 0.5 mL of lower phase and subject to HPLC analysis for the corresponding peak ($H_{L2}$). $K_{U/L}$ value was calculated as:

$$K_{U/L} = \frac{H_{L1} - H_{L2}}{H_{L2}} \times \frac{V_L}{V_U}, \quad \text{here} \quad V_U = V_L$$

**Table 2**
Mobile phases and detection wavelengths for different compounds in HPLC analyses.

| Compounds | Mobile phases | Detection wavelengths |
|---|---|---|
| Hyperforin (**1**) | A: 2% aqueous acetic acid;<br>B: methanol with 2% acetic acid<br>Elution gradient: 0–5 min, 90–100% B, hold at 100% B to 16 min | 275 nm<br>320 nm |
| Hypericin (**2**) | A: 2% aqueous acetic acid<br>B: tetrahydrofuran with 2% acetic acid<br>Elution gradient: 0–10 min, 15–100% B, hold for 2 min | 590 nm |
| Rutin (**3**) and Hyperoside (**4**) | A: 2% aqueous acetic acid<br>B: methanol with 2% acetic acid<br>Isocratic elution with 40% A and 60% B for 10 min | 254 nm<br>360 nm |

**Table 3**
*K* value of hypericin in different solvent systems.

| Solvent systems (v/v) | $H_{L1}$ | $H_{L2}$ | $K_{U/L}$ |
|---|---|---|---|
| Hexane–ethyl acetate–methanol–water (1:1:1:1) | 68.36548 | 38.25813 | 0.79 |
| Hexane–ethyl acetate–methanol–water (1:1.5:1:1.5) | 69.25871 | 34.12568 | 1.03 |
| Hexane–ethyl acetate–methanol–water (1:1.25:1:1.25) | 58.56981 | 26.00124 | 1.25 |
| Hexane–ethyl acetate–methanol–water (1:3:1:3) | 69.35681 | 20.14568 | 2.44 |