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Isolation and purification of series bioactive components from *Hypericum Perforatum* L. by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) 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, ¹HNMR and ¹³CNMR.

Keywords

High-speed counter-current chromatography; *Hypericum perforatum* L.; hyperforin; hypericin; rutin; hyperoside

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 *Hypericum perforatum* L. in recent years relied on its antidepressant activity[1–2]. In addition, studies also have shown that *Hypericum perforatum* L. has antiviral[3], antimicrobial[4], wound healing[5] and anticancer activities[6–7]. The extracts of *Hypericum perforatum* L. contain a variety of pharmacological active constituents including phloroglucinols, naphthodianthrone, and a broad range of flavonoids. The antidepressant activity of *Hypericum perforatum* L. was firstly attributed to the naphthodianthrone 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 phytosensitizer, 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]. Naphthodianthrone were also discovered to act as antiviral agent to inhibit the growth of a variety of neoplastic cell types[11]. In addition, flavonoids present in *Hypericum 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 *Hypericum 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 naphthodianthrone by preparative high-performance liquid chromatography[27] etc.. However, the preparative separation of bioactive components from *Hypericum 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 *Hypericum 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 *Hypericum perforatum* and the quality control of related products make it necessary to develop more efficient separation methods for the above mentioned bioactive constituents.

During recent years, high-speed counter-current chromatography (HSCCC), a support-free liquid-liquid partition chromatography has been widely used for separation and purification of bioactive components from natural products by eliminating irreversible adsorption of sample onto the solid support. The high performance of HSCCC in the preparative isolation and purification of epigallocatechin and flavonoids from *Hypericum 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 *Hypericum perforatum* L by HSCCC 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 HSCCC units, i.e., a model GS20 analytical (0.8 mm i.d. PTFE tubing, column volume 35 mL, β values 0.4 to 0.72) and a model GS10A3 preparative unit (1.6 mm i.d. PTFE tubing, 220 mL column volume, β 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, β 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 HSCCC 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 *Hypericum perforatum* L. was purchased from Acetar Bio-Tech Inc., Xi-an, China. The content of hypericin is about 0.3%.

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, 30min 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 HSCCC 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 HSCCC 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 $K_{U/L} = [(H_{L1} - H_{L2})/H_{L2}] (V_L/V_U)$, where $V_U=V_L$.

2.5. HSCCC separation

2.5.1 Preparation of two-phase solvent and sample solution—The solvent system utilized in the HSCCC separation was prepared by mixing the selected solvent system (Table 1), and thoroughly equilibrating in a separator funnel at room temperature. The two phases were separated shortly before use.

The sample solutions for HSCCC were prepared by dissolving the samples intending to be separated in mobile phase at suitable concentration according to the separation purpose.

2.5.2 Separation procedure—In each separation, the coiled column was first entirely filled with the stationary phase (usually upper phase unless specified), and then the mobile phase was pumped into the column at a flow-rate of 1.0 mL/min and 2.0 mL/min with column rotation of 1600 rpm and 800 rpm for analytical and preparative HSCCC respectively. After the mobile phase front emerged and hydrodynamic equilibrium was established, the sample solution was injected through the sample loop (2 mL for analytical and 20 mL for preparative unit). The effluent from the coil column was continuously monitored with a UV detector at 280 nm. Each peak fraction was manually collected according to the chromatographic profile displayed on the recorder or the workstation. The retention of the stationary phase was calculated according to the volume of the stationary phase collected before sample injection.

2.6. HPLC-DAD/MS analyses

Each sub-extracts and fractions collected from HSCCC were analyzed by HPLC using Agilent Zorbax SB-C18 column (250×4.6 mm i.d., 5 μm). The different mobile phases were employed for different target compounds as listed in Table 2 at a flow-rate of 1.0 ml/min. A split valve was used to connect the outlet of the HPLC/DAD and the inlet of ESI and the mobile phase flow-rate was reduced to 0.5 mL/min for HPLC-MS analysis. The MS system was operated in the following conditions: nebulizer pressure 50 psi, drying gas flow 10 L/min, drying gas temperature 350°C, scan range from m/z 100 to 2200.

3. Results and discussion

3.1. Separation of hyperforin (1) from the petroleum ether extract

Due to the low content of hyperforin and no reference samples available, HPLC-DAD-MS was employed to search for the possible target compound in petroleum ether extract (A). As seen in Fig. 1, HPLC-MS BPC chromatogram displayed a remarkable peak at 12.3 min (Fig. 1A), the MS spectrum in both positive and negative mode indicate it has the same molecular weight with hyperforin, m/z 537. While at the same retention time in HPLC-DAD chromatogram, only a small peak can be observed (Fig. 1B). Thus the peak at 12.3min was locked as target hyperforin tentatively in the following separation.

As hyperforin is a less polar compound, two solvent systems composed of n-heptane-methanol (1.5:1, v/v) and n-heptane-acetonitrile (1:1, v/v) were firstly selected for the test of partition coefficient (K) of hyperforin. As a result, two K values, 1.28 and 2.07, were obtained corresponding to the two solvent systems. Based on fast test on the analytical HSCCC and taken the resolution and separation time into account, a mediate solvent system composed of n-heptane-methanol-acetonitrile (1.5:0.5:0.5, v/v) was chosen as the start separation solvent system, lower phase as mobile phase, and change to the lower phase of n-heptane-methanol (1.5:1, v/v) at point M, as illustrated in Fig. 2A. Through this step of separation, the concentration of hyperforin can be increased from 14% in petroleum ether extract to 85% (HPLC 320 nm) (Fig. 1C). Then a slightly different solvent system composed of n-heptane-methanol-acetonitrile (1.5:0.5:0.1, v/v/v) was employed to further purify hyperforin together with n-heptane-methanol (1.5:1, v/v) in gradient, as seen in Fig. 2B. Finally, high pure of hyperforin (>98%) was obtained as shown in Fig. 1D, which was confirmed by HPLC-MS again. Its chemical structure was confirmed with ¹HNMR and ¹³CNMR later.

3.2. Separation of hypericin (2) from the ethyl acetate extract

The content of hypericin is even lower than hyperforin in *Hypericum perforatum* L. extract. The screening of hypericin in ethyl acetate extract (B) was conducted by comparing the retention time of each peak in the HPLC chromatograms of the ethyl acetate extract (Fig. 3A) with that of hypericin reference sample (Fig. 3 B). The characteristic absorption wavelength of hypericin 359nm was used for detection.

In order to isolate peak 2 from ethyl acetate extract by HSCCC, a medium polar solvent system composed of hexane-ethyl acetate-methanol-water in series volume ratio were examined for K value of hypericin, the results were showed in Table 3. To meet the requirement of high resolution and short separation time, a gradient elution mode with solvent system of hexane-ethyl acetate-methanol-water (1:3:1:3, v/v) (I) and (1:1:1:1, v/v) (II) was selected for the HSCCC separation of the hypericin.

Firstly, a reverse phase elution mode was used, starting with solvent system (I), lower phase as mobile phase, to achieve a good resolution between hypericin and other components, and

then change to the lower phase of solvent system (II) at M point to shorten the elution time of hypericin, as shown in Fig. 4A. Through this separation, most of the interferences have been separated from hypericin, only a peak co-existed with it as seen in Fig. 3C. The concentration of hypericin can be increased from 5% in ethyl acetate extract to 75%. Thus secondly, a normal phase elution mode was employed, starting with solvent system (II), upper phase as mobile phase, then change to the upper phase of solvent system (I) as shown in Fig. 4B. Through this purification, high pure hypericin (about 95% at 590nm) was produced, see in Fig. 3D. Its UV spectrum is the same as that of reference sample, while its purity is higher. As the limit amount of pure hypericin, its chemical structure was not verified by NMR.

3.3. Separation of rutin (3) and hyperoside (4) from the n-butanol extract

Our aim for this polar sub-extract is to find some active flavones especially hyperoside. HPLC-MS BPC chromatogram of the n-butanol extract displayed a peak at 3.1min (Fig. 5A), its MS spectrum in negative mode presented two main molecular fragments m/z 463.1 and m/z 609.2. The former is consistent to the molecular ion of hyperoside $[M-H]^-$, while the latter was proved to be that of lutein $[M-H]^-$ later. However, they were co-eluted in HPLC, as seen in both HPLC-MS and HPLC-DAD chromatogram (Fig. 5B). The UV spectrum of the peak at 3.1min also illustrated the characteristic of flavones. Hence this peak was our target peak to be separated. Because of the polarity of the n-butanol extract, a polar solvent system composed of n-butanol-ethyl acetate-water (1:4:5, v/v) was chosen. As shown in Fig. 6A, from starting point, lower phase was served as mobile phase eluting from head to tail, then change to back-extrusion mode at M point by eluting upper mobile phase from tail to head. Through this separation, two completely separated flavone peak fractions 3 and 4 were obtained, although they were eluted almost in the same retention time in HPLC. After further purification, seen in Fig. 6B and 6C, high pure of two flavones were prepared (>95%) according to HPLC analysis, seen in Fig. 5C and 5D. They were confirmed as lutein (3) and hyperoside (4) respectively by ESI-MS, 1H NMR and ^{13}C NMR later.

3.4. Identification of separated compounds

Compound **1** (hyperforin): ESI-MS: m/z 537.5 $[M+H]^+$ and m/z 535.4 $[M-H]^-$ give the molecular weight of 536, which is in agreement with the molecular formula of hyperforin ($C_{35}H_{52}O_4$).

1H NMR (600MHz, CD_3OD), δ_H : 1.75(1H, m, H-4), 1.92(1H, m, 1H-5), 1.27(1H, m, 1H-5), 2.11(1H, m, H-11), 1.15(6H, dd, 3H-12, 3H-13), 0.98(3H, s, 3H-14), 1.80(2H, m, 2H-15), 2.02(1H, m, 1H-16), 1.80(1H, m, 1H-16), 4.76(H, qt, 7.2, 1.2, H-17), 1.64(3H, s, 3H-19), 1.47(3H, s, H-20), 2.02(2H, m, 2H-21), 5.11(1H, tt, 7.2, 1.2, H-22), 1.50(3H, s, 3H-24), 1.50(3H, s, 3H-25), 3.20(2H, sep, 7.2, 2H-26), 5.18(1H, tt, 7.2, 1.2, H-27), 1.50(3H, s, 3H-29), 1.70(3H, s, 3H-30), 2.49(2H, dd, 7.2, 4.8, 2H-31), 4.76(2H, qt, 7.2, 1.2, H-17, H-32), 1.60(6H, s, 3H-34, 3H-35).

^{13}C NMR (150MHz, CD_3OD), δ_C : 208.35 (C-1), 82.42 (C-2), 47.81 (C-3), 42.57 (C-4), 40.27 (C-5), 58.27 (C-6), 181.59 (C-7), 120.25 (C-8), 183.18 (C-9), 209.64 (C-10), 41.57 (C-11), 20.44 (C-12), 19.36 (C-13), 15.8 (C-14), 37.68 (C-15), 27.66 (C-16), 122.31 (C-17), 133.36 (C-18), 24.88 (C-19), 17.92 (C-20), 24.0 (C-21), 126.61 (C-22), 131.61 (C-23), 24.90 (C-24), 18.01 (C-25), 22.40 (C-26), 122.71 (C-27), 133.19 (C-28), 24.99 (C-29), 18.10 (C-30), 30.03 (C-31), 121.21 (C-32), 132.93 (C-33), 25.01 (C-34), 18.14 (C-35).

Based on our analysis and comparison with the NMR data reported in literature [30], compound **1** was confirmed as hyperforin, its structure is shown in Fig. 7.

Compound **2** (hypericin): As the limit amount of pure product, chemical structure was not verified by NMR for Compound **2**. It was identified as hypericin through the comparison of retention time and UV spectrum in HPLC with hypericin reference sample. Its structure is also shown in Fig. 7.

Compound **3** (rutin): ESI-MS: m/z 633.1 $[M+Na]^+$ in positive and m/z 609.1 $[M-H]^-$ in negative mode provide the molecular weight of 610, which is in agreement with the molecular formula of rutin ($C_{27}H_{30}O_{16}$).

1H NMR (400MHz, DMSO- d_6): 12.62 (1H, s, OH-5), 10.83(1H, s, OH-7), 9.60(1H, s, OH-4'), 9.15(1H, s, OH-3'), 7.57(1H, d, H-6'), 7.54(1H, s, H-2'), 6.85(1H, d, H-5'), 6.40(1H, s, H-8), 6.20(1H, s, H-6), 5.46(1H, d, H-1''), 5.37(1H, s, OH-2''), 5.26(1H, s, OH-3''), 5.10(1H, s, OH-4''), 5.04(1H, s, OH-2'''), 4.93(1H, s, OH-3'''), 4.85 (1H, s, OH-4'''), 4.34(1H, brs, H-1'''), 1.05(3H, d, H-6'''). The overlapping multiple peaks in the range of 3~3.5 ppm are attributed to the other ten hydrogen atoms of glucose and rhamnose pyran ring.

^{13}C NMR (DMSO- d_6): 177.9 (C-4), 164.5 (C-7), 161.7 (C-5), 156.8 (C-9), 156.6 (C-2), 148.9 (C-4'), 145.2 (C-3'), 133.8 (C-3), 122.0 (C-6'), 121.6 (C-1'), 116.7 (C-2'), 115.7 (C-5'), 104.4 (C-10), 102.3 (C-1''), 101.4(C-1'''), 99.1 (C-6), 93.9 (C-8), 78.0 (C-3''), 77.0 (C-5''), 76.3 (C-2''), 74.5 (C-4'''), 73.6 (C-4''), 71.7 (C-2'''), 70.4 (C-3'''), 68.3 (C-5'''), 65.0 (C-6''), 18.9 (C-6''').

The above data of compound **3** are in agreement with that of rutin [31]. Its structure was also shown in Fig. 7.

Compound **4** (hyperoside): ESI-MS: m/z 487.0 $[M+23]^+$ in positive and m/z 463.1 $[M-H]^-$ in negative mode provide the molecular weight of 464, which is in agreement with the molecular formula of hyperoside ($C_{21}H_{20}O_{12}$).

1H NMR (400MHz, DMSO- d_6), δH : 12.62 (1H, s, OH-5), 10.83(1H, s, OH-7), 6.20(1H, brs, H-6), 6.40(1H, brs, H-8), 9.69(1H, s, OH-4'), 9.18(1H, s, OH-3'), 7.54(1H, s, H-2'), 6.84(1H, d, H-5'), 7.65(1H, d, H-6'), 5.34(1H, d, H-1''), 5.27(1H, brs, OH-2''), 5.09(1H, s, OH-3''), 5.11(1H, s, OH-4''). The overlapping multiple peaks in about 3.5ppm are attributed to the six hydrogen atoms of glucose ring.

^{13}C NMR (DMSO- d_6), δC : 156.6 (C-2), 133.8 (C-3), 177.9 (C-4), 161.7 (C-5), 99.1 (C-6), 164.5 (C-7), 93.9 (C-8), 156.7 (C-9), 104.4 (C-10), 121.6 (C-1'), 115.6 (C-2'), 145.2 (C-3'), 148.9 (C-4'), 116.5 (C-5'), 122.4 (C-6'), 101.4 (C-1''), 71.7 (C-2''), 73.7 (C-3''), 68.4 (C-4''), 76.3 (C-5''), 60.5 (C-6'').

Compared with the reported data, the MS, 1H NMR and ^{13}C NMR data of compound **4** are in agreement with that of hyperoside in the literatures [29,32]. Its structure is shown in Fig. 7.

4. Conclusions

A systematic method has been established for the separation of series of bioactive components from a crude extract of *Hypericum perforatum* L by HSCCC combined with pre-separation by ultrasonic solvent extraction with petroleum ether, ethyl acetate, and n-butanol successively. Four high pure (over 95%) of bioactive compounds including hyperforin, hypericin, rutin and hyperosid were separated successfully from three sub-extracts with different polarity. In the study, HPLC-MS, reference sample and UV spectrum were selectively used to search for target compounds from HPLC-DAD profiles of different

sub-extracts. The structure of isolated compounds were identified by ESI-MS, ¹HNMR and ¹³CNMR except for hypericin, it was confirmed tentatively by comparing HPLC-DAD retention time and UV spectrum with that of hypericin reference sample. The above results illustrated the effectiveness of HSCCC as a semi-preparative separation technique for the isolation and purification of bioactive components from *Hypericum perforatum* L.

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Fig. 1.

HPLC analysis of petroleum ether extract and target hyperforin (**1**) fractions from HSCCC. A and B. HPLC-MS BPC and HPLC-DAD chromatogram of petroleum ether extract; C and D. HPLC-DAD chromatograms of target fractions from first and second HSCCC separation (Fig. 2A and 2B).

Conditions: Column: Zorbax SB-C18 (250×4.6mm i.d., 5μm); Mobile phase: 2% aqueous acetic acid (A), methanol with 2% acetic acid (B), elution with the linear gradient: 0–5min, 90%–100% B, hold at 100% B to 16min; Detection: 275, 320 nm; Flow-rate: 1mL/min.

Fig. 2.

HSCCC separation of hyperforin (**1**) from the petroleum ether extract.

Conditions: A. GS10A3 preparative HSCCC; Solvent systems: n-heptane-methanolacetonitrile (1.5:0.5:0.5, v/v) change to n-heptane-methanol (1.5:1, v/v) at point M, lower phase as mobile phase; Flow-rate: 2.0 mL/min; Rotation speed: 800 rpm; Sample: 1.0 g of the petroleum ether extract. B. GS20 analytical HSCCC; Solvent systems: n-heptane-methanol-acetonitrile (1.5:0.5:0.1, v/v) change to n-heptane- methanol (1.5:1, v/v) at point M, lower phase as mobile phase; Flow-rate: 1.0 mL/min; Rotation speed: 1800 rpm; Sample: 40 mg of peak **1** fraction from A.

Fig. 3.

HPLC analysis of the ethyl acetate extract (A), reference sample of hypericin (B), target peak fraction from first HSCCC (C) and second HSCCC (D).

Conditions: Column: Zorbax SB-C18 (250×4.6mm i.d., 5μm); Mobile phase: 2% aqueous acetic acid (A), tetrahydrofuran with 2% acetic acid (B), elution gradient: 0–10 min, 15% – 100% B, hold for 2min; Detection: 590 nm; Flow-rate: 1mL/min.

Fig. 4.

HSCCC separation of hypericin (**2**) from the ethyl acetate extract.

Conditions: Tauto TBE-300A HSCCC; solvent systems: hexane-ethyl acetate-methanol-water (1:3:1:3, v/v) (I) and (1:1:1:1, v/v) (II); Flow-rate: 2.0 mL/min;

Rotation speed: 850 rpm; A. Reverse phase mode, starting with solvent system (I), lower phase as mobile phase, then change to the lower phase of solvent system (II) at M point;

Sample: 104mg of the ethyl acetate extract; B. Normal phase mode, starting with solvent system (II), upper phase as mobile phase, then change to the upper phase of solvent system (I) at M point; Sample: 20mg of peak **2** fraction from A.

Fig. 5.

HPLC analysis of *n*-butanol extract and target lutein (**3**) and hyperoside (**4**) fractions from HSCCC. A and B. HPLC-MS BPC and HPLC-DAD chromatogram of petroleum ether extract; C and D. HPLC-DAD chromatograms of purified lutein (**3**) and hyperoside (**4**) after HSCCC separation (Fig. 6B and 6C).

Conditions: Column: Zorbax SB-C18 (250×4.6mm I.D., 5μm); Mobile phase: 2% aqueous acetic acid (A), methanol with 2% acetic acid (B), isocratic elution with 40% A and 60% B for 10min; Detection: 254, 360 nm; Flow-rate: 1mL/min.

Fig. 6.

HSCCC separations of lutein (**3**) and hyperoside (**4**) from n-butanol extract.

Conditions: Tauto TBE-300A HSCCC; Flow-rate: 2.0 mL/min; Rotation speed: 850 rpm; A. Solvent system: n-butanol-ethyl acetate-water (1:4:5, v/v), lower mobile phase eluted from head to tail and change to upper mobile phase back-extrusion from tail to head at M point; Sample: 110mg of the n-butanol extract. B. Solvent system: n-butanol-ethyl acetate-water (1.5:3.5:5, v/v), lower mobile phase eluted from head to tail; Sample: 206mg of accumulated peak 3 fraction from HSCCC separation (A). C. the same condition as A; Sample: 121mg of accumulated peak 4 fraction from HSCCC separation (A).

Fig. 7.
Chemical structures of hyperforin (1), hypericin (2), rutin (3) and hyperoside (4)

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)
	n-heptane-methanol (1.5:1, v/v)
Hypericin (2)	hexane-ethyl acetate-methanol-water (1:3:1:3, v/v)
	hexane-ethyl acetate-methanol-water [1:1:1:1, v/v)
Rutin (3) and Hyperoside (4)	n-butanol-ethyl acetate-water (1.4:5, v/v)
	n-butanol-ethyl acetate-water (1.5:5.5:5, v/v)

Table 2

Mobile phases and detection wavelengths for different compounds in HPLC

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

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)	63.36548	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)	53.56981	26.00124	1.25
hexane-ethyl acetate-methanol-water (1:3:1:3)	69.35681	20.14568	2.44