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Isolation and purification of flavonoid glycosides from *Trollius ledebouri* using high-speed counter-current chromatography by stepwise increasing the flow-rate of the mobile phase

Xin Zhou, Jinyong Peng, Guorong Fan *, Yutian Wu

Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, No. 325 Guohe Road, Shanghai 200433, China

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Abstract

Three flavonoid glycosides including orientin, vitexin, quercetin-3-*O*-neohesperidoside and one unknown compound were isolated and purified by high-speed counter-current chromatography (HSCCC) and semi-preparative HPLC from *Trollius ledebouri Reichb*., a traditional Chinese medicine. Preparative HSCCC with a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) was successfully performed by increasing the flow-rate of the mobile phase from 1.5 to 2.5 ml/min after 190 min. Consequently, 95.8 mg orientin, 11.6 mg vitexin, 9.3 mg unknown compound with purities of over 97% and one partially purified peak fraction (contained quercetin-3-*O*-neohesperidoside at 85.1% purity) were obtained from 500 mg of the crude extract. Then the partially purified fraction was further purified by reversed-phase semi-preparative high-performance liquid chromatography. The structure identification of all pure fractions was carried out by UV, MS, ^{1}H NMR and ^{13}C NMR.

Keywords: *Trollius ledebouri Reichb*.; Flavonoid glycoside; Preparative chromatography; Counter-current chromatography; Macroporous resin

1. Introduction

Trollius ledebouri Reichb. (Jin Lianhua in Chinese), a member of Ranunculaceae's family, is mainly distributed in the northern regions of China. It is one of well-known herbs that possess antimicrobial and antiviral actions and have been used to treat colds, high fevers, chronic tonsillitis, and acute tympanitis [1]. The major active compounds are considered to be flavonoids including orientin, vitexin and quercetin-3-*O*-neohesperidoside [2–4]. Now pharmacological tests revealed that quercetin-3-*O*-neohesperidoside exhibited anti-inflammatory properties [5], while orientin and vitexin demonstrated antiviral, antimicrobial, antioxidant and radioprotection activities [6,7]. Their structures are shown in Fig. 1.

In view of their pharmacological activities and suitable chromophores for UV detection, a large quantity of pure materials is urgently needed for further pharmacological studies and as "marker compounds" for the chemical evaluation or standardization of *T. ledebouri Reichb*. and its medical products [8–10]. So, an effective method for the isolation and purification of orientin, vitexin and quercetin-3-*O*-neohesperidoside from natural sources is warranted.

The separation and purification of flavonoids using conventional methods such as column chromatography requires several steps resulting in low recoveries of the products. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, has an excellent sample recovery compared to some conventional methods, and widely used for separation and purification of various natural and synthetic products [11–15]. Although vitexin has been purified from *Trollius Chinen-*

* Corresponding author. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: guorfan@yahoo.com.cn (G. Fan).

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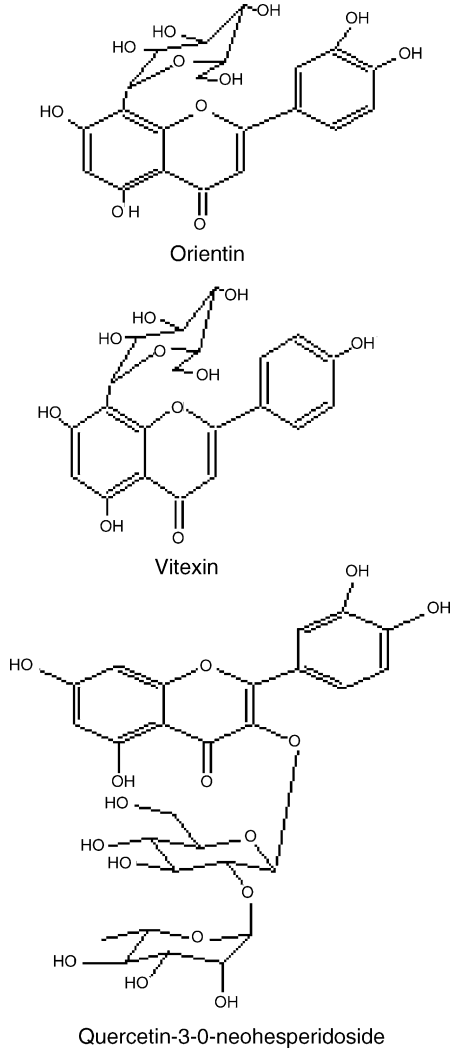

Fig. 1. The chemical structures of vitexin, orientin and quercetin-3-*O*-neohesperidoside.

sis Bunge by HSCCC [16], no paper has been reported on the use of HSCCC for the isolation and purification orientin, vitexin, and quercetin-3-*O*-neohesperidoside from *T. ledebouri Reichb*.

Therefore, the aim of this study was to develop an efficient method for the isolation and purification of orientin, vitexin and quercetin-3-*O*-neohesperidoside with high purities from *T. ledebouri Reichb*. by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a model TBE–300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 254 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). The analytical HPLC system used throughout this study consisted of LC–10AT pump (Shimadzu, Japan), a SPD–10A UV–vis detector (Shimadzu, Japan), and a model N2000 workstation (Zhejiang University, Hangzhou, China). The semi-preparative HPLC system consisted of Waters 515 pump, 2487 detector (Waters, USA), a 1000 μl sample loop and the model N2000 workstation. D101 macroporous resin (outer appearance: cream white opaque ball pellet resin; grain length: (0.3–1.25 mm) ≥90%; moisture: 65–75%; moisture looking density: 0.65–0.75 g/ml; comparing specific surface area: 500–550 m^2/g; even hole diameter: 90–100 Å; polar nature: non-polarity) was purchased from Shanghai Institute of Pharmaceutical Industry (Shanghai, China).

2.2. Reagents

Ethyl acetate, aluminium trichloride, *n*-butanol, methanol, ethanol, acetic acid, chloroform were analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Aceonitrile, isopropanol and tetrahydrofuran were HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18.2 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The *T. ledebouri Reichb.* was purchased from a local drug store.

2.3. Preparation of the crude extract

The dried flowers (300 g) were extracted three times (2.0 h for each time) with 3000 ml 60% ethanol. Then the extract was filtered and evaporated to dryness by rotary evaporation at 60 °C under reduced pressure. The residue was dissolved in water and the water solution was chromatographed on D101 macroporous resin. In the macroporous resin column chromatography, water and different concentration of aqueous ethanol were tested by a gradient elution program and each collection elution was analyzed by TLC, which performed with chloroform–acetone–methanol–acetic acid (2:1:2:0.05, v/v) on polyamide plates (WuLian Chemical Factory, Shanghai, China) in saturated chambers. The detection was achieved by spray 1% aluminium trichloride ethanol solution.

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

The two-phase solvent system used in the present study was prepared by mixing ethyl acetate–*n*-butanol–water (2:1:3, v/v/v). After thoroughly equilibrating the mixtures in a separated funnel at room temperature, the two phases were separated shortly before use. The sample solution was prepared by dissolving the crude extract in the lower phase of solvent system for isolation and purification.

2.5. HSCCC separation procedure

In the crude sample isolation and separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.5 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 15 ml sample solution containing 500 mg of the crude extract was injected through the injection value. After 190 min, the flow-rate of the mobile phase was increased to 2.5 ml/min. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 25 °C.

2.6. Preparative HPLC purification procedure

The partially purified fraction was evaporated to dryness under reduced pressure at 60 °C and dissolved with mobile phase (about 30 mg/ml for fraction "I"). The separation was performed with a YWG C_{18} (200 mm × 10.0 mm, i.d., 10 μm) (Dalian Elite Scientific Instruments Dalian, China) column with a pre-column equipped with the same stationary phase, at a column temperature of 25 °C. The mobile phase composed of acetonitrile–0.1% aqueous acetic acid (15:85, v/v) was isocratically eluted at a flow-rate of 1.2 ml/min and injection volume was 750 μl. The effluent was monitored at 254 nm and peak fraction was collected according to the elution profile.

2.7. HPLC analysis and identification of HSCCC peak fractions and preparative HPLC fraction

The crude sample and each peak fraction obtained by HSCCC and preparative HPLC were analyzed by HPLC. The column used was a Hypersil C_{18} (200 mm × 4.0 mm i.d. 5 μm) (Dalian Elite Scientific instruments, Liaoning province, China) with a pre-column equipped with the same stationary phase, the mobile phase was 0.1% acetic acid solution–tetrahydrofuran–isopropanol–aceonitrile (420:30:20:30, v/v). The flow rate was 0.5 ml/min, and the effluent was monitored at 254 nm. Identification of the

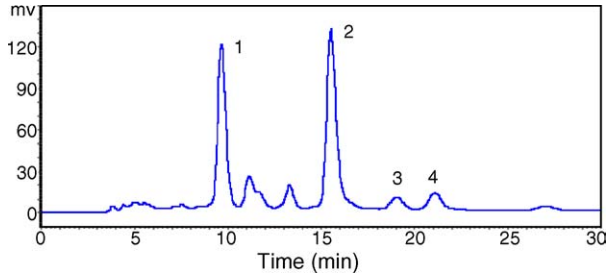

Fig. 2. HPLC chromatogram of the crude extract from *Trollius ledebouri Reichb.* Conditions: column, reversed-phase Hypersil C_{18} (200 mm × 4.0 mm i.d., 5 μm); mobile phase, 0.1% acetic acid solution–tetrahydrofuran–isopropanol–aceonitrile (420:30:20:30, v/v); flow rate, 0.5 ml/min; UV wavelength, 254 nm; peaks 1, 2, and 4 correspond to quercetin-3-*O*-neohesperidoside, orientin, and vitexin; peak 3: unknown component.

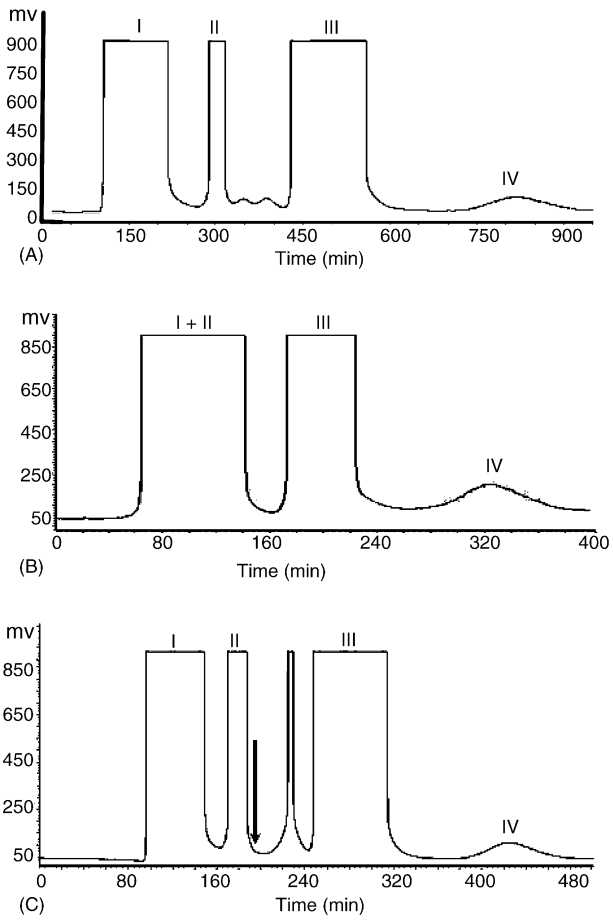

Fig. 3. Chromatogram of the crude extract by preparative HSCCC. Conditions: solvent system–ethyl acetate–*n*-butanol–water (2:1:3, v/v/v); stationary phase: upper phase; mobile phase: lower phase; revolution speed: 800 rpm; sample size: 500 mg; sample loop: 20 ml; detection wavelength: 254 nm. (A) Flow rate: 1.5 ml/min, retention of stationary phase: 49%; (B) flow rate: 2.5 ml/min, retention of stationary phase: 42%; (C) flow rate: 0–190 min, 1.5 ml/min and 190–480min, 2.5 ml/min; retention of stationary phase: 49%. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.5 to 2.5 ml/min after 190 min. (I) Collected during 98–148 min; (II) collected during 170–188 min; (III) collected during 248–315 min; and (IV) collected during 395–465 min.

HSCCC peak fractions and preparative HPLC fraction was carried out by UV, MS and NMR.

3. Results and discussion

In our present study, D101 macroporous resin was used to obtain crude extract in place of organic solvent extraction, which are unfriendly to our environment. The result indicted that target compounds mainly existed in 10%, 20% and 30% ethanol solution. So, water was first used to remove some hydrosoluble chemicals, which had no or little retention on D101 macroporous resin. Second, 30% ethanol was used to elute target compounds and the crude extract was obtained for further isolation and purification. At last, 95% ethanol was used to activate the resin for another use. Consequently, 17.6 g crude extract was obtained from 300 g *T. ledebouri Reichb.* using this extraction protocol.

Subsequently, the crude extract was analyzed by HPLC. When 0.1% acetic acid solution–tetrahydrofuran–isopropanol–aceonitrile (420:30:20:30, v/v) was used as the mobile phase, the temperature of the column was set at 30 °C and isocratically eluted at a flow rate of 0.5 ml/min, good result could be obtained. HPLC chromatogram was shown in Fig. 2. Peaks 1–4 are corresponding to quercetin-3-*O*-neohesperidoside, orientin, unknown component and vitexin.

Successful separation by HSCCC largely depends upon the selection of a suitable two-phase solvent system, which requires the following considerations [17–19]: (1) for satisfactory retention of the stationary phase; (2) the settling time of the solvent system should be shorter than 30 s; (3) the partition coefficient (K) should be close to 1 and the separation factor (α, $\alpha = K_2/K_1$, $K_2 > K_1$) between two compounds should be greater than 1.5. In general, small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening.

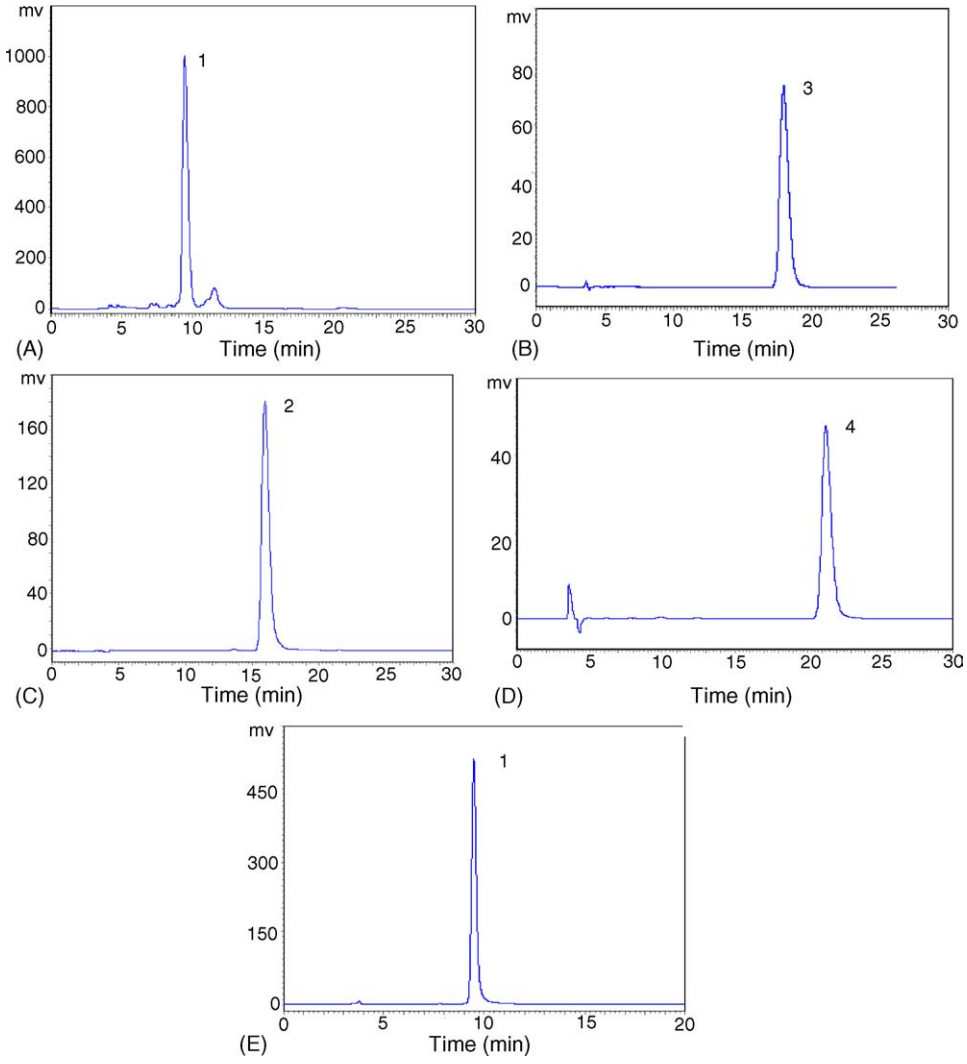

Fig. 4. HPLC chromatogram of HSCCC and preparative HPLC fractions. Conditions, column, reversed-phase Hypersil C_{18} (200 mm × 4.0 mm i.d., 5 μm); mobile phase, 0.1% acetic acid solution–tetrahydrofuran–isopropanol–aceonitrile (420:30:20:30, v/v); flow rate, 0.5 ml/min; UV wavelength, 254 nm. (A): HSCCC fraction "I", (B): HSCCC fraction "II", (C): HSCCC fraction "III", (D): HSCCC fraction "IV", (E): preparative HPLC fraction "V"; peaks 1, 2, and 4 correspond to quercetin-3-*O*-neohesperidoside, orientin, and vitexin; peak 3: unknown component.

So, some two-phase solvent systems were tested including ethyl acetate–*n*-butanol–water at various volume ratios (2:1:3, 2:3:5, v/v/v), *n*-butanol–ethanol–water (4:1:4, v/v/v), ethyl acetate–methanol–water (4:1:5, v/v/v) and *n*-butanol–water (1:1, v/v). When *n*-butanol–ethanol–water (4:1:4, v/v/v) was used, the settling time of the solvent system was long (>30 s), and if ethyl acetate–methanol–water (4:1:5, v/v/v) or *n*-butanol–water (1:1, v/v) was utilized as the two-phase solvent system, orientin would be eluted together with other compounds with similar properties near the solvent front and resulting in a poor resolution. When acetate–*n*-butanol–water (2:3:5, v/v/v) was tested, the retention of the stationary phase was poor. Finally, the two-phase solvent system of ethyl acetate–*n*-butanol–water at a ratio of (2:1:3, v/v/v) was found to be suitable for the separation of flavonoid glycosides from the crude extract. Preliminary HSCCC experiment was carried out at a constant flow-rate of 1.5 ml/min. As a result, although all target compounds could be completely separated, vitexin was retained in the column over 15 h (Fig. 3A). In the subsequent studies, different constant flow-rates (2.0 and 2.5 ml/min) were tested. It was found that separation time could be reduced to less than 6 h, but it was difficult to separate peaks 1 and 2 (Fig. 3B). So, the elution protocol with stepwise increasing the flow-rate of the mobile phase was used: 0–190 min, the flow-rate was set at 1.5 ml/min and 190–480 min, it was increased to 2.5 ml/min. Consequently, four fractions were obtained from 500 mg crude extract in less than 8 h and the retention of stationary phase was 49% (Fig. 3C).

As shown in Fig. 4, the HPLC analysis of each HSCCC fraction revealed that three pure flavonoid glycosides could be obtained from the crude extract. The purities of fraction "II" (unknown component, 9.3 mg), fraction "I" (orientin, 95.8 mg), fraction"IV" (vitexin, 11.6 mg) were 97.6%, 99.6% and 98.1%, respectively. Fraction "I" (80.6 mg) was partially purified peak fraction contained quercetin-3-*O*-neohesperidoside with purity at 85.1% (Fig. 5).

In order to obtain quercetin-3-*O*-neohesperidoside with high purity, preparative HPLC was used to further purify fraction "I". The effluent of target compound (fraction "V") was collected and analyzed by HPLC (Fig. 4F). The results indicated that quercetin-3-*O*-neohesperidoside with purity at 99.8% could be obtained successfully by preparative HPLC separation under the above optimized conditions.

Identification of HSCCC pure fractions and preparative HPLC pure fraction were carried out by UV, MS, NMR analysis as follows.

Orientin, yellow powder, UV λ_{nm}^{MeOH}: 345, 270, 255. ESI-MS: 448 (M^+). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 3.22–3.88 (6H, m, glucosyl-H), 4.72 (1H, d, J = 9.0 Hz, H-1″), 6.25 (1H, s, H-6), 6.65 (1H, s, H-3), 6.90 (1H, d, J = 8.2 Hz, H-5′), 7.44 (1H, d, J = 2.1 Hz, H-2′), 7.50 (1H, dd, J = 2.1, 8.0 Hz, H-6′), 13.15 (1H, s, 5-OH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 164.16 (C-2), 102.41 (C-3), 182.03 (C-4), 160.47 (C-5), 98.31 (C-6), 162.80 (C-7), 104.65 (C-8), 156.01 (C-9), 103.99 (C-10), 121.97 (C-1′), 114.07 (C-2′), 145.95 (C-3′), 149.90 (C-4′), 115.78 (C-5′), 119.45 (C-6′), 73.50 (C-1″), 70.90 (C-2″), 78.88 (C-3″), 70.83 (C-4″), 82.04 (C-5″), 61.76 (C-6″).

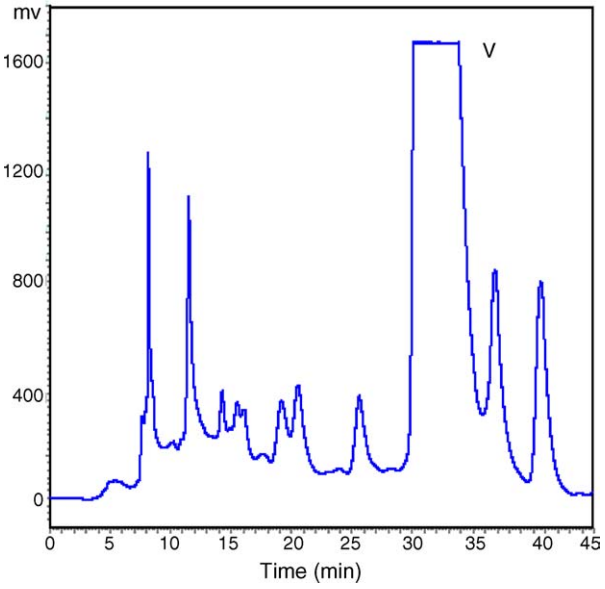

Fig. 5. Preparative HPLC chromatogram of HSCCC fraction "I". Conditions, column, YWG C_{18} (200 mm × 10.0 mm, i.d. 10 μm); mobile phase, acetonitrile–0.1% aqueous acetic acid (15:85, v/v); flow rate, 1.2 ml/min; UV wavelength, 254 nm; inject volume, 750 μl.

Vitexin, yellow powder, UV λ_{nm}^{MeOH}: 330, 301, 269. ESI-MS: 432 (M^+). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 4.94 (1H, d, J = 9.8 Hz, H-1″), 6.44 (1H, s, H-6), 6.94 (1H, s, H-3), 7.05 (2H, d, J= 8.7 Hz, H-3′,5′), 8.26 (2H, d, J = 8.7 Hz, H-2′, 6′), 10.35 (1H, s, 4′-OH), 10.83(1H, s, 7-OH), 13.17 (1H, s, 5-OH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 164.98 (C-2), 102.51 (C-3), 182.73 (C-4), 155.64 (C-5), 98.45 (C-6), 162.31 (C-7), 104.56 (C-8), 160.28 (C-9), 104.07 (C-10), 122.07 (C-1′), 128.99 (C-2′, 6′), 115.01 (C-3′, 5′), 161.32 (C-4′), 73.93 (C-1″), 71.03 (C-2″), 79.01 (C-3″), 70.20 (C-4″), 81.29 (C-5″), 61.36 (C-6″).

Quercetin-3-*O*-neohesperidoside, yellow powder, UV λ_{nm}^{MeOH}: 354, 296, 266, 255. ESI-MS: 610 (M^+). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 0.72 (3H, d, J = 6.2 Hz, rha-CH_3), 3.90–4.47 (10H, m, glucosyl-H), 5.17 (1H, s, rha-C_1-H), 5.73 (1H, d, J = 7.2 Hz, GluC_1-H), 6.25 (1H, d, J = 1.8 Hz, H-6), 6.59 (1H, d, J = 1.8 Hz, H-8), 6.85 (1H, d, J = 8.5 Hz, H-5′), 7.82 (1H, dd, J = 1.8, 8.5 Hz, H-6′), 7.74 (1H, d, J = 1.8, H-2′), 12.67 (1H, s, 5-OH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 156.32 (C-2), 133.50 (C-3), 177.58 (C-4), 161.42(C-5), 98.78 (C-6), 164.02 (C-7), 93.44 (C-8), 156.74 (C-9), 103.87 (C-10), 121.96 (C-1′), 115.84 (C-2′), 144.95 (C-3′), 148.18 (C-4′), 116.16 (C-5′), 121.15 (C-6′), 98.48 (C-1″), 76.76 (C-2″), 77.34 (C-3″), 69.86 (C-4″), 77.15 (C-5″), 60.52 (C-6″), 100.76 (C-1‴), 70.36 (C-2‴), 70.83 (C-3‴), 71.90 (C-4‴), 68.57 (C-5‴), 17.41 (C-6‴).

Comparing with the reported data, the MS, ^{1}H NMR and ^{13}C NMR data were in agreement with those reported in literatures [4,5,20–25].

In conclusion, an HSCCC method for the preparative separation and purification of orientin, vitexin, quercetin-3-*O*-neohesperidoside and one unknown compound from the medicinal plant *T. ledebouri Reichb*. was developed by stepwise increasing the flow-rate of the mobile phase. The present study indicates that HSCCC is a very powerful technique for the preparative separation and purification of bioactive components from plant materials.

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