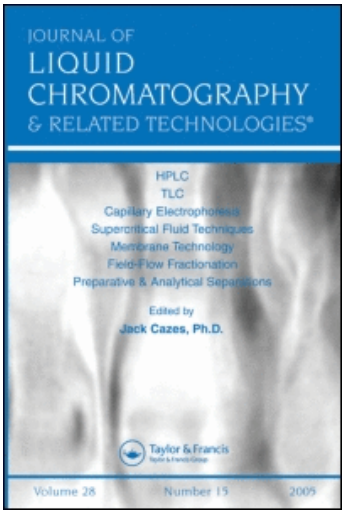

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Separation and Identification of Flavonoids in an Extract from the Seeds of *Oroxylum indicum* by CCC

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ABSTRACT

Oroxylum indicum (*O. indicum*) is widely used as a medicinal plant in China and Japan. The seeds are used as the crude drug "Mu Hu DIE" in China. Flavonoids are present in the seeds of *O. indicum*. The composition of an ethyl acetate extract of the seeds of *O. indicum* is completed using high speed analytical counter current chromatography and preparative high-speed countercurrent chromatography (HSCCC). The HSCCC separation of the extract used a solvent system of hexane/ethyl acetate/methanol/water. Optimal distribution coefficients (K_D) were determined by use of high performance liquid chromatography (HPLC). An HPLC method was developed to analyse the extract. Using

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an isocratic elution, effective CCC separation of three components was achieved. A step-gradient elution was then developed that produced a faster separation with better resolution. Separation of the components was achieved using 20 mg of extract, and the peaks were examined by LC–MS and NMR. The flavonoids, chrysin, and baicalein, were identified. Another flavonoid, possibly baicalein-7-o-glucoside was suspected.

Key Words: Flavonoids; *Oroxylum indicum*; HSCC; Analysis; Isocratic and gradient elution.

INTRODUCTION

Oroxylum indicum is a widely used medicinal plant in China and Japan. Its seeds are used as a folk remedy for hysteria and broncho-pneumonis. It has also been found to be a useful purgative. The seeds have been used as an analgesic, antitussive, and anti-inflammatory agent for the treatment of cough, bronchitis, and other diseases. In the seeds of *O. indicum*, there are a large amount of bioactive flavonoids such as baicalein, baicalin, chrysin, apigenin.[1] Recent studies have reported baicalein and chrysin to show anti-inflammatory, anti-allergic,[2] antioxidant, and anticancer activities.[3–5]

High-speed countercurrent chromatography (HSCCC), being a support-free liquid chromatography, eliminates complications such as irreversible adsorption onto the solid support, tailing of the solute peaks, etc. It has been extensively used for the separation and purification of natural products. Its basic principles have already been described in the Conway's book.[6] A large number of polar natural products have been separated by CCC; many of them are polyphenolics. Flavonoids are well suited to be separated and purified by this method.[7–10]

In this paper, high-speed analytical countercurrent chromatography was used to select a suitable solvent system and HSCCC was applied to the purification of the three flavonoids, followed by the identification of these using LC–MS electrospray ionisation and NMR.

EXPERIMENTAL

Solvents and Reagents

All organic solvents used were of analytical grade and purchased from Fisher scientific UK, Loughborough, Leics, UK. The plant *O. indicum* was purchased from Chengdu, the crude drug market of Sichuan Province in China. Baicalein and chrysin standards were purchased from the Sigma Company.

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High-Speed Countercurrent Chromatography Separation

An experimental prototype J-type coil planet centrifuge HSCCC preparative instrument was supplied by Brunel Institute for Bioengineering, Brunel University, UK. This machine was equipped with an HP1100 pump, a UV spectrophotometer detector, and a sample collector. A manual sample injection valve was used to introduce the samples into the column. The HSCCC has four coils on two separate bobbins; each bobbin containing two concentrically wound coils of PTFE tubing with a total volume of 495 mL. In this study, the column (49.9 mL volume, β range at 0.7–0.83) was used. The other three coils were filled with methanol/water = 50 : 50 to maintain balance. Biphasic mixtures of hexane/ethyl acetate/methanol/water were prepared in the ratio (v/v) of 1/1.2/1/1, 1/1.2/3/1, and 0.1/1.6/0.2/1.6, respectively. Each solvent mixture was thoroughly equilibrated in a separator funnel by repeated vigorous shaking and degassed at room temperature by helium purge for 30 min.

The column was first filled with the upper organic (hexane/ethyl acetate) phase of the biphasic mixture; then 20 mg of the extract dissolved in 1 mL mobile phase was loaded. The coils were rotated in a reverse direction at a constant speed of 800 rpm, and the system purged with lower aqueous (methanol/water) mobile phase up to the head of the coil. The mobile phase was then pumped into the coil from head to tail at a flow rate of 1.5 mL/min by a HP1100 pump (Hewlett Packard), and the resulting effluent was collected at the tail in a graduated burette. The equilibration point of the system was determined when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be calculated by subtracting the volume of stationary phase eluted at the end of the equilibration process from the total volume of the system. The effluent from the tail end of the column was continuously monitored with a UV detector at 275 nm and the retention of the stationary phase, relative to the total column capacity, was computed from the volume of the stationary phase collected from the column after the separation was completed. Peak fractions were collected into test tubes with a sampler controller (Gilson, France) for analysis by high performance liquid chromatography (HPLC). Both gradient and isocratic reverse phase liquid chromatography were used to analyze the fractions.

Extraction of Plant Sample

Fifty grams of seeds of *O. indicum* were refluxed for five hours in 90% methanol, the extract was then filtered and evaporated. The residue was redissolved in 200 mL H_2O and extracted three times with ethyl acetate. Final evaporation yielded 6.5 g of a yellow powder (13%).

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HPLC-ESI-MS/MS Analysis and Identification of Unknown Components

The peak fraction analyses and identification were carried out using an Agilent 1100 HPLC apparatus, which was interfaced with a LCQ Ion trap mass spectrometer (Finnigan MAT, San Jose, and CA). A cosmosil C_{18} RP column (150 × 4.6 mm i.d. Phenomenex) was used at a temperature of 30°C, flow rate of 1.0 mL/min, and wavelength of 275.5 nm. A gradient elution of formic acid 0.2% (A) and 100% CH_3CN (B) was used: 0–10 min 80% A and 20% B; 10–12 min 55% A, 45% B; 12–20 min 20% A, 80% B. The +ESI conditions: sheath gas flow rate: 60 arbitrary; aux gas 10 arbitrary; flow rate 1.0 mL/min; capillary temperature 220°C; capillary voltage 10.0 V, and tube lens offset 5.0 V. A split of 1/10 was used.

A Thermoquest Finnigan AQA mass spectrometer interfaced with an analytical HSCCC of coil volume 25.0 mL was used to rapidly develop a suitable solvent system and to identify the molecular weight of unknown components. A flow rate of 1.0 mL/min was used with a split 1/10 to the AQA mass spectrometer.

RESULTS AND DISCUSSION

Selection of Two-Phase Solvent System

Successful separation requires the careful search for a suitable two-phase solvent system to provide an ideal range of distribution coefficients (K_D) ($0.1 < K_D < 3$) for the sample. Generally speaking, a two phase solvent system should satisfy the following requirements: (I) for ensuring satisfactory retention of the stationary phase, the settling time of the solvent system should be considerably shorter than 30 sec; (II) for efficient separation, the K_D of target compounds should be close to 1, and the separation factor (α, the ratio of K_D between two components) should be greater than 1.5. If the K_D value is much smaller than 1, the solutes are eluted close together near the solvent front, which will result in a loss of peak resolution; furthermore, if K_D is larger than 1, the solutes are highly retained and eluted in broad peaks. The K_D value for a pure compound may be determined simply by measuring the UV absorbance of each phase after distribution between the two phases. When the compounds to be separated are not available in a pure form, as in the present case, their K_D values obviously cannot be determined by this method. In this case, one can use the HPLC method described below.

As shown in Fig. 1, HPLC can separate the ethyl acetate extract into four peaks. So, HPLC can be employed to determine the distribution coefficients. Approximately 1 mg of the test sample was weighted in a

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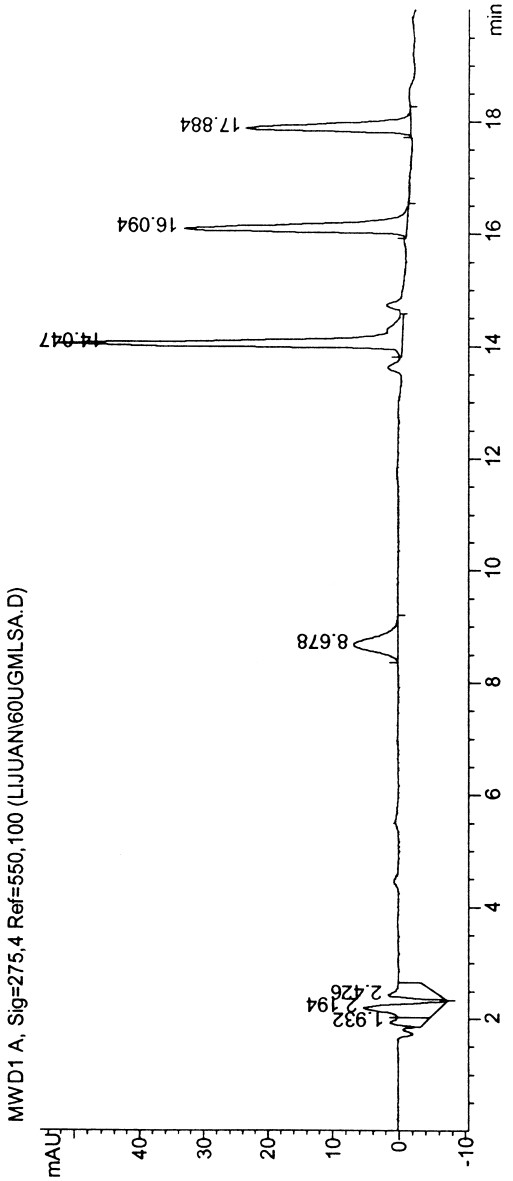

Figure 1. High performance liquid chromatography chromatograms of the initial ethyl acetate extract from *O. indicum*. Cosmosil RP18, 15 cm column, mobile phase water–acetonitrile 0.2% formic acid. Gradient elution from 20% acetonitrile to 80% acetonitrile in 20 min. ESI-MS detection (total current).

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10 mL test tube to which 2 mL, each, pre-equilibrated two-phase solvent system was added. The test tube was closed and shaken vigorously for 1 min to thoroughly equilibrate the sample between the two phases. Then, equal volumes (about 100 μL) of the upper and lower phases were diluted with methanol to 1 mL, and analyzed by HPLC to determine the distribution coefficient of each component.

Figure 1 shows that three major components, one minor component, and two very small components appeared in the ethyl acetate extract. High-speed countercurrent chromatography interfaced mass spectrometry was employed to rapidly select a solvent system. Analytical CCC could finish the elution procedure in 30 min and avoid the waste of solvent in having to select a solvent system. A selection of solvent systems was tried by this method to separate the sample such as butanol/water, butyronitrile/methanol/water, hexane/ethyl acetate/methanol/water (H/E/M/W) and hexane/butanol/methanol/water at various ratios. A composition of the H/E/M/W solvent system was found to be the most suitable for separation of the extract. Figure 2 shows the results of separation by HSCCC–mass spectrometry.

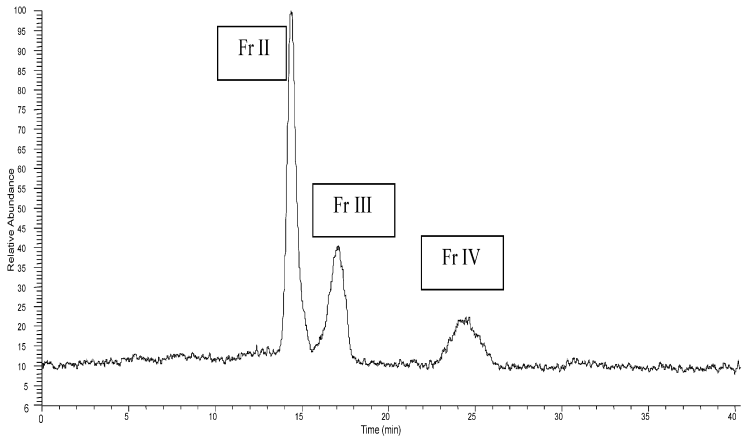

Figure 2. High-speed analytical countercurrent chromatography/Mass spectrometry for the separation of ethyl acetate extract. Separation conditions: speed 1200 rpm; head to tail; flow rate 1.0 mL/min; hexane/ethyl acetate/methanol/water = 1/1.2/2.5/1; coil volume 27.1 mL; Stationary phase: upper phase; mobile phase: lower phase; retention of stationary phase = 78.5%; sample concentration 40 μg/mL; isocratic elution. Mass spectrometry: +ESI full scan with probe temperature 250°C, AQA 40 V, probe voltage 4.5 kV.

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Preparative High-Speed Countercurrent Chromatography Separation

High-speed analytical countercurrent chromatography–mass spectrometry indicates that the solvent system of hexane/ethyl acetate/methanol/water was the best solvent system, and three components were well resolved. Their molecular weights were 432, 270, and 254, respectively. This result could be applied to preparative HSCCC. Figures 3 and 4 show the separation of ethyl acetate extract by HSCCC with different elution modes.

Figures 3 and 4 show that three compounds are well resolved both in isocratic and gradient elusions and shortened elution time could be obtained using gradient CCC than that with isocratic elution. No stationary phase wash off was observed in the solvent system since the aqueous phase of system B was richer in methanol and actually dissolved part of the organic phase (stationary phase) of system A. Fractions III and IV yielded 95% and 98% pure components. Fraction II only yielded an 85% pure component II, 10% compound I, and other impurities. In order to separate the two components, a modified solvent system (H/E/M/W) 0.2/1.6/0.2/1.6 was employed. After fraction II was further separated by HSCCC, the purity of component II was increased to

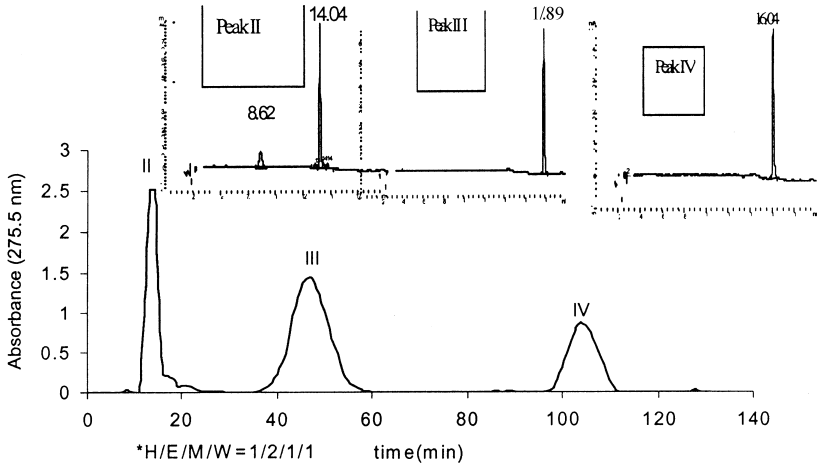

Figure 3. Separation of the ethyl acetate extract by HSCCC and HPLC analyses of the fractions. Brunel HSCCC machine, coil volume: 49.9 mL; injection volume 1.0 mL (10 mg/mL sample); liquid system: H/E/M/W 1/1.2/1/1 (v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase in head-to-tail direction; retention of stationary phase, $S_f = 72\%$; flow rate: 1.0 mL/min; rotation speed: 800 rpm; rotation stopped at 120 min.

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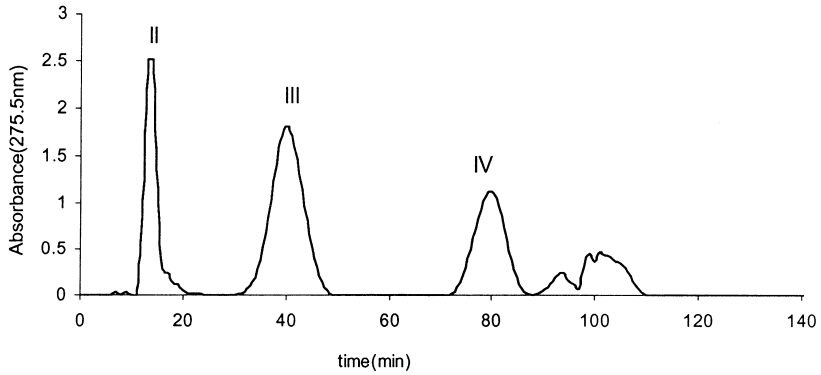

Figure 4. Separation of the ethyl acetate extract by CCC in gradient elution. Machine and sample, see Fig. 3. Two biphasic liquid systems were used: A: H/E/M/W = 1/1.2/1/1 (v/v/v/v) and B: H/E/M/W = 1/1.2/3/1 (v/v/v/v). Step gradient program: step 1: 0–20 min, 100% A; step 2: 20–60 min, from 0% lower phase of B to 60% lower phase of B; step 3: 60–130 min, 100% lower phase of B.

92% and 75% pure minor component I was also obtained. Figure 5 shows the second separation of fraction II by CCC and HPLC analysis result.

Identification of Fractions II, III, and IV by HPLC/ESI/MS and HPLC/ESI MS/MS

In order to identify the isolated components of component II, fractions III, and IV, an on line LC/MS system was used to obtain their ESI mass spectra. For component II, the $[M+H]^+$ molecule ion at $m/z = 433$ can be clearly observed, and the molecular weight is 432. The ESI–MS spectrum of component II showed an intense aglycone ion $[A+H]^+$ at $m/z = 271$ by loss of a neutral fragment of mass 162 mu [Fig. 6(A)]. The loss of 162 mu between these peaks confirmed the presence of a hexose residue. The daughter MS/MS spectra of the ion at $m/z = 271$ exhibits five main diagnostic fragment ions at $m/z = 253$, 225, 169, 140.7, and 103 [Fig. 6(B)]. Intensive fragment at $m/z = 103$ proved that B ring (Sch. 1) has no OH group, and fragment at $m/z = 169$ shows the substitution of the A ring by 3 OH groups. These fragment ions are very important to identify the structure of component II. According to a previous report,[1] component II was possibly baicalein-7-o-glucoside. In order to confirm this conclusion, component II was refluxed for 2 h at 90°C in 1.2 mol/mL HCl (methanol) solution and hydrolyzed in order to give the corresponding aglycones. After evaporation

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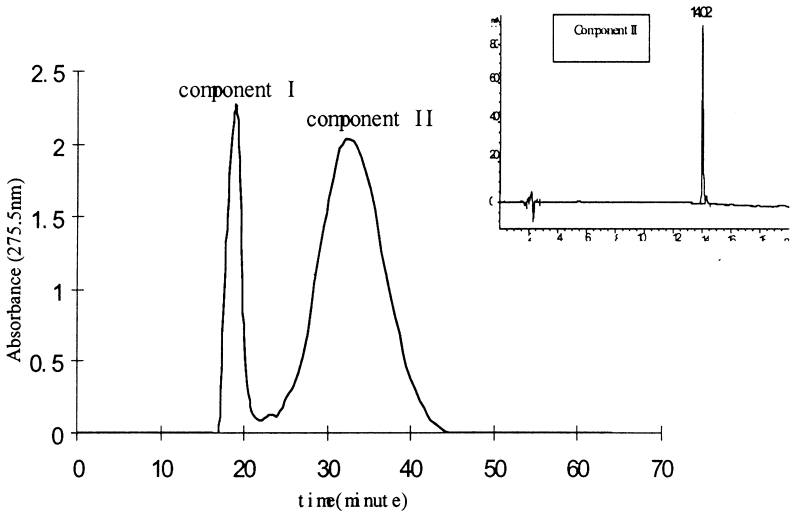

Figure 5. Second step separation of peak II by HSCCC and its HPLC analysis. Machine volume 49.9 mL, solvent system: H/E/M/W 0.2/1.6/0.2/1.6 (v/v/v/v); stationary phase: organic upper phase; mobile phase: aqueous lower phase in the head-to-tail direction, 1 mL/min. $S_f = 71\%$. Inset: HPLC chromatogram of peak II.

to dryness using a rotary evaporator at a 35°C water bath, the residue was dissolved in methanol and then subjected to LC/MS/MS analysis. The hydrolyzed component II gave the same retention time and fragmentations as that of the baicalein at MS^2 $m/z = 271$. In view of the common occurrence of particular sugar moieties in flavonoids,[10] component II is probably baicalein-7-o-glucoside. Further work on component II would be required to fully characterize it. Figure 6 shows the spectra of component II and Sch. 1 shows the proposed mechanism of its main fragmentation routes. For fraction III, the proposed fragmentation mechanism at $m/z = 271$ is similar to Sch. 1. The daughter MS/MS spectra at $m/z = 271$, displayed exactly the same fragmentation patterns as standard baicalein sample. The LC retention time of fraction III also confirmed its identity as baicalein.

From the MS/MS data and retention time analysis, fraction IV was identified as chrysin. Scheme 2 showed its proposed mechanism.

^{1}H NMR and ^{13}C NMR Confirmed Fraction II, III, and IV

In the ^{13}C NMR spectrum of fraction III, the signals appeared at almost the same positions as those of a pure standard of baicalein, indicating that

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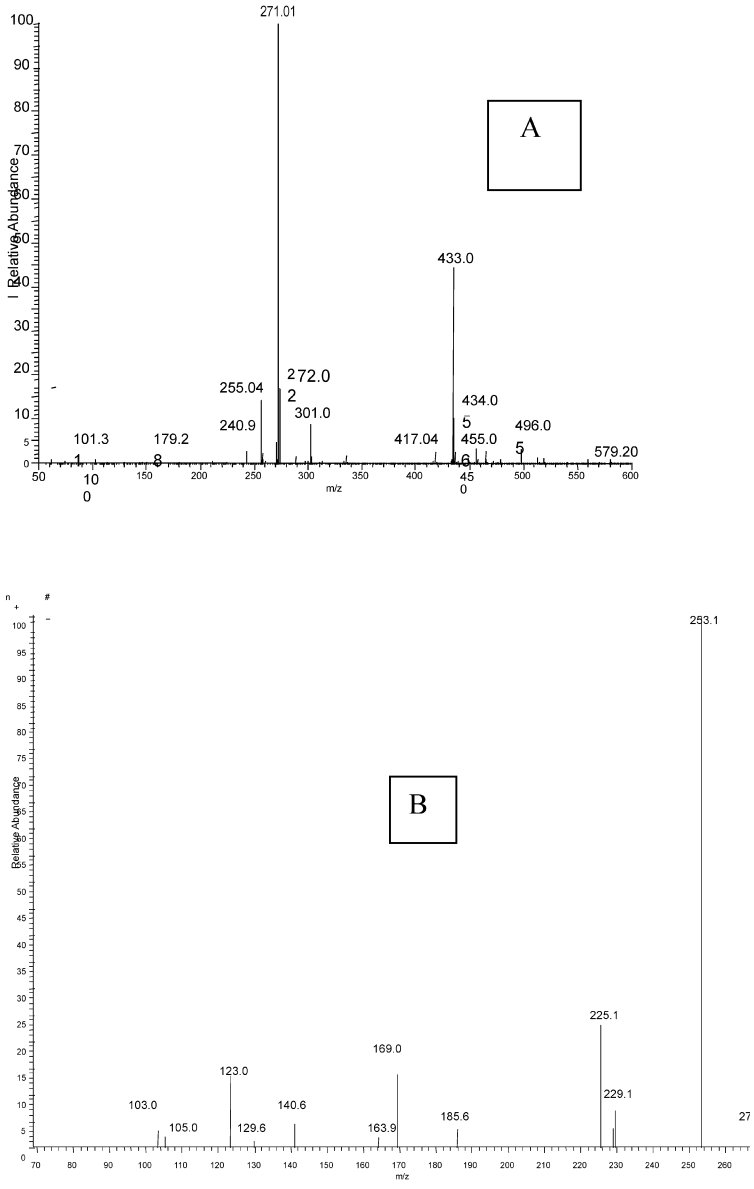

Figure 6. (A) LC/MS +ESI full scan spectra of component II (see Fig. 5) and (B) MS/MS scan of fragment 271 of component II on LCQ with 40% CID (collision induced dissociation).

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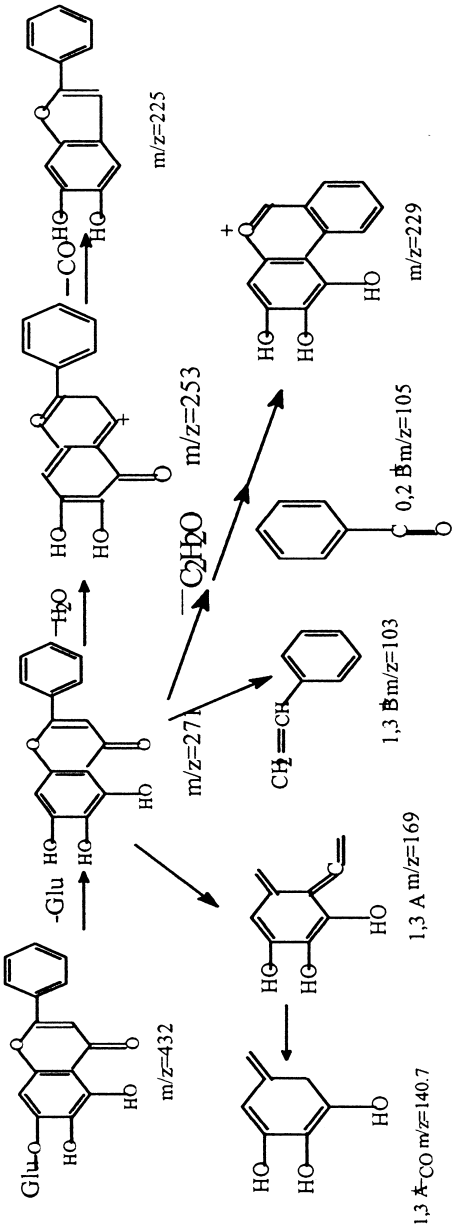

Scheme 1. Main fragmentations of protonated fragment at $m/z = 271$ by ESI–MS^2.

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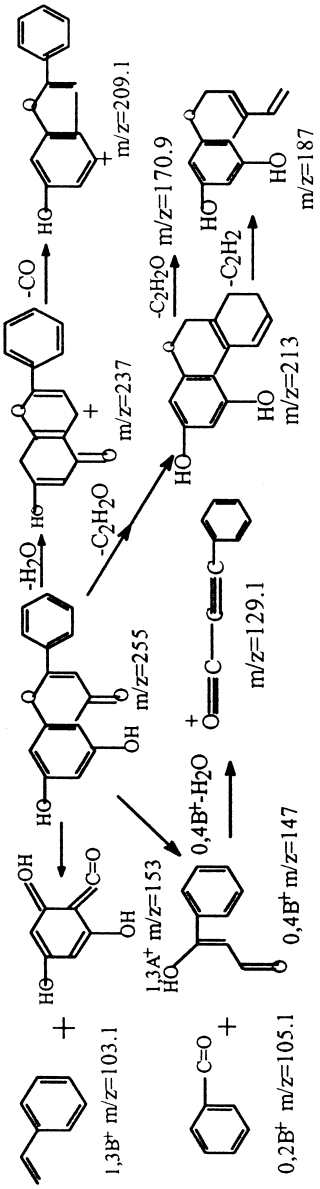

Scheme 2. Main fragmentations of protonated fragment at $m/z = 255$ by CID 40%.

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fraction III is baicalein. [^{1}H NMR (CD_3CD, TMS) δ ppm: 7.8 (2H, H-2′, H-6′); 7.3 (3H, H-3′, H-4′, H-5′); 6.5 (1H, H-3) 6.4 (1H, H-8); ^{13}C NMR 0] δ ppm: 95.45 (C-8), 105.75 (C-10), 106.24 (C-3), 127.77 (C-2′, C-6′), 130.61 (C-3′, C-5′), 131.15 (C-6), 133.32 (C-4′), 133.13 (C-1′), 148.30 (C-9), 152.56 (C-5), 155.55 (C-2), 165.98 (C-7), 184.64 (C-4). Fraction IV [^{1}H NMR (CD_3CD, TMS) δ ppm: 7.9 (2H, H-2′, H-6′); 7.5 (3H, H-3′, H-4′, H-5′); 6.4 (1H, H-3); 6.5 (1H, H-8); 6.1 (1H, H-6) ^{13}C NMR 0] δ ppm: 95.45 (C-8), 99.27 (C-6), 104.31 (C-10), 104.90 (C-3), 126.30 (C-2′, C-6′), 129.10 (C-3′, C-5′), 131.43 (C-1′), 131.93 (C-4′), 158.44 (C-9), 162.16 (C-5), 164.50 (C-2), 165.57 (C-7), 182.74 (C-4). The ^{13}C NMR and ^{1}HNMR signals obtained for fraction IV were identical to those of standard chrysin. Therefore, fraction IV was confirmed as chrysin. For fraction II, this compound has yet to be positively confirmed, but is tentatively assigned as baicalein-7-o-glucoside.

CONCLUSION

Our study demonstrates that HSCCC is a valuable method in separating, purifying, and identifying bioactive components from natural products. Using HSCCC, three flavonoids, baicalein-7-o-glucoside, baicalein, and chrysin were separated and purified from the *O. indicum* seeds with a two-phase solvent system comprised of hexane/ethyl acetate/methanol/water in less than 2 hours. From 20 mg crude ethyl acetate extract, 3.2 mg of 92.0% pure baicalein-7-o-glucoside, 6.8 mg of 95% pure baicalein, and 4.2 mg 98% pure chrysin were obtained in a single run.

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