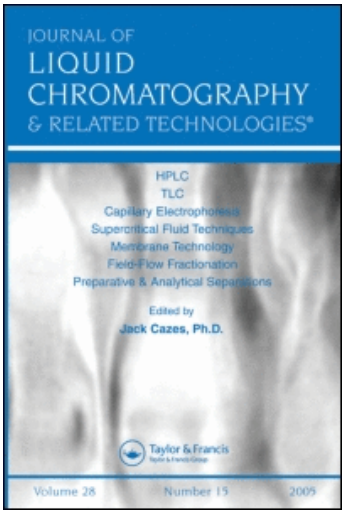

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PREPARATIVE ISOLATION AND PURIFICATION OF FLAVONE C-GLYCOSIDES FROM THE LEAVES OF *FICUS MICROCARPA* L.f BY MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY, HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY, AND PREPARATIVE LIQUID CHROMATOGRAPHY

Xiaohong Wang[a]; Yong Liang[a]; Licai Zhu[a]; Huichun Xie[a]; Hang Li[a]; Junting He[a]; Man Pan[a]; Tianyou Zhang[b]; Yoichiro Ito[c]
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PREPARATIVE ISOLATION AND PURIFICATION OF FLAVONE C-GLYCOSIDES FROM THE LEAVES OF *FICUS MICROCARPA* L.f BY MEDIUM-PRESSURE LIQUID CHROMATOGRAPHY, HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY, AND PREPARATIVE LIQUID CHROMATOGRAPHY

Xiaohong Wang,[1] Yong Liang,[1] Licai Zhu,[1] Huichun Xie,[1] Hang Li,[1] Junting He,[1] Man Pan,[1] Tianyou Zhang,[2] and Yoichiro Ito[3]

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□ *Combined with medium pressure liquid chromatography (MPLC) and preparative high performance liquid chromatography (perp-HPLC), high speed countercurrent chromatography (HSCCC) was applied for the separation and purification of flavone C-glycosides from the crude extract of leaves of Ficus microcarpa L.f. HSCCC separation was performed on a two-phase solvent system composed of methyl tert- butyl ether–ethyl acetate –1-butanol–acetonitrile–0.1% aqueous trifluoroacetic acid at a volume ratio of 1:3:1:1:5. Partially resolved peak fractions from HSCCC separation were further purified by preparative HPLC. Four well separated compounds were obtained and their purities were determined by HPLC. The purities of these peaks were 97.28%, 97.20%, 92.23%, and 98.40%. These peaks were characterized by ESI-MS^n. According to the reference, they were identified as orientin (peak I), isovitexin-3″-O-glucopyranoside (peak II), isovitexin (peak III), and vitexin (peak IV), and yielded 1.2 mg, 4.5 mg, 3.3 mg, and 1.8 mg, respectively.*

Keywords *Ficus microcarpa* L.f, flavone C-glycosides, high speed countercurrent chromatography, medium pressure liquid chromatography, preparative high pressure liquid chromatography

INTRODUCTION

Ficus microcarpa L.f belongs to the genus Ficus of the Moraceae family. It is widely distributed in the tropical and subtropical regions. In China, it

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mainly distributes in Guangdong and Guangxi. Its aerial root and leaves can be used as medicine. Due to its various pharmaceutical effects such as clearing heat, detoxifying, diminishing swelling, removing dampness, and relieving pain, *Ficus microcarpa* L.f has been used widely for the treatment of chronic bronchitis, influenza, whooping cough, tonsillitis, toothache, bacillary dysentery, enteritis, and bruises.[1] Many triterpenoids,[2–11] phenolic compounds,[12] isoflavones,[13–15] and flavones[17,19] have been isolated from aerial roots, bark, leaves, and fruits of *Ficus microcarpa* L.f.

Separation and purification of chemical constituents from *Ficus microcarpa* L.f by the classical methods requires multiple chromatographic steps using a silica gel, polyamide column,[3–15,17–19] in which a large amount of using organic solvents are unfriendly to our environment and these conventional separation methods are tedious, time consuming, and have the peril of loss of sample due to the highly adsorptive effect onto the solid matrix. Therefore, more efficient separation methods need to be explored further.

High speed countercurrent chromatography (HSCCC) is a form of liquid–liquid partition chromatography with no support solid matrix[16] Solute separation is based on partitioning between the two immiscible liquid phases: the mobile phase and the liquid stationary phase, the latter of which is retained in the column by the aid of a centrifugal force provided by the planetary motion of the column. Since the system eliminates irreversible adsorption of samples onto the solid support, it yields excellent sample recovery and is very suitable for separation of active components from traditional Chinese medicinal herbs and other natural products.[20–22] Although vitexin and orientin have been purified from *Trollius ledebouri* and isovitexin from *Patrinia villosa* Juss by HSCCC,[23,24] no paper has been reported on the use of HSCCC for the isolation and purification of vitexin, orientin, isovitexin, and isovitexin-3″-O-glucopyranoside from *Ficus microcarpa* L.f.

Preparative high performance liquid chromatography (prep-HPLC) is a powerful tool in virtue of its excellent efficiency and high recovery. In the present study, HSCCC was combined with MPLC and prep-HPLC to achieve rapid isolation and purification of orientin (1.2 mg), isovitexin-3″-O-glucopyranoside (4.5 mg), isovitexin (3.3 mg), Vitexin (1.8 mg). The structures of these compounds (Fig. 9) were identified by ESI-MS^n.

EXPERIMENTAL

Apparatus

The HSCCC instrument employed in the present study was a GS10A high speed countercurrent chromatograph (Beijing Institute of Technology Application, Beijing, China), with a multilayer coil separation column

(i.d. of the tubing = 1.6 mm, total volume = 230 mL) and a 20 mL sample loop. The β value of the preparative column varied from 0.5 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The system was also equipped with an S-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm or 280 nm, and a Yokogawa 3057 recorder.

The MPLC equipment was an EZ purifier with a dual λ UV absorbance detector (Shanghai LiSui E-Tech Co. Ltd., Shanghai). AB-8 macroporous resin was purchased from Tianjin University. The analytical HPLC equipment was a Shimadzu LC-20AT consisting of a Shimadzu SPD-M20A UV detector, a sample injection valve (Model 7725i) with a 20 mL loop, and a Shimadzu LC Solution Workstation (Shimadzu, Kyoto, Japan). The column applied in this work was a Diamonsil C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Dikma Technologies China). The preparative HPLC equipment was a Waters 600 model with a Waters 2487 dual λ absorbance detector (Waters, USA). The column applied in this work was a YMC-Pack ODS-A column (250 mm × 10 mm, i.d., 5 μm, YMC Co. Ltd., Japan). ESI-MS^n analysis was carried out with an LCQ Advantage (Thermo Finnigan, USA), and the NMR with a Varian NMR 400 System (Varian, USA).

Reagents

All organic solvents used for HSCCC were of analytical grade (Tianjing Damao Reagent Factory, Tianjing, China). Methanol used for HPLC analysis was of chromatographic grade (DIKMA filiale in Guangzhou, China). The dried leaves of *Ficus microcarpa* L.f were offered by Department of Pharmaceutical Analysis of Guangdong Pharmaceutical University, Guangzhou, China.

Preparation of Crude Sample

The dried leaves of *Ficus microcarpa* L.f were crushed with a disintegrator (60°C, 6 h) to give about 300 g of powder, which was extracted three times each for 5 hours with 20 L of 75% ethanol at 80°C. The pooled extract was dried under a reduced pressure and suspended in H_2O (1.5 L), followed by extraction with petroleum ether (boiling point: 60°C–90°C), ethyl acetate, and 1-butanol in this sequence. The 1-butanol extract was evaporated to dryness and stored in a refrigerator at 4°C for further analysis.

MPLC Separation

AB-8 macroporous resin was soaked by 95% ethanol for 24 h and subsequently rinsed by pure water thoroughly before use, and then loaded into the chromatographic column (three plastic columns of 180 mm × 30 mm connected by the head-tail way) with wet packed method, the connected column volume (CV) is 380 mL.

The 1-butanol extract was subjected to MPLC, the column filled with AB-8 macroporous resin was first washed by distilled water with four times CV, and then eluted by ethanol-water (10/90, 70/30, v/v) solutions. Each part of desorption solutions was analyzed by HPLC and then concentrated to dryness under vacuum. Each fraction was evaporated to dryness and stored in a refrigerator at 4°C for further purification.

HSCCC Separation

In this work, we mainly studied the concentrated 70% ethanol-water eluate. The concentrated 70% sample was separated by HSCCC with a two-phase solvent system composed of methyl *tert*-butyl ether–ethyl acetate–1-butanol–acetonitrile–0.1% aqueous trifluoroacetic acid at a volume ratio of 1:3:1:1:5. After thoroughly equilibrating the mixture in a separatory funnel at room temperature, two-phases were separated shortly before use. The upper organic phase was used as stationary phase, and the lower aqueous phase as mobile phase.

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow-rate of 1.5 mL/min, while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (50 mg dissolved in 2 mL mixture solution of lower phase and upper phase (1:1, v/v) of the solvent system) was injected through the sample port, after that 15 mL of the stationary phase was injected in the same way. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm. The flow rate of the lower phase (mobile phase) was regulated at 2.0 mL/min after the fraction 1 was eluted (about 2.5 hour). Each peak fraction was manually collected according to the elution profile and subsequently analyzed by HPLC.

Preparative HPLC Separation

When the HSCCC separation yielded only partially resolved peaks as fractions 1, 2 and 3 from the 70% ethanol-water eluate, these peak fractions

2 and 3 were further purified by prep-HPLC. The purification and separation were performed with a YMC-Pack ODS-A column as follows: methanol −0.05% phosphoric acid was used as the mobile phase in an isocratic mode at a flow-rate of 3.0 mL/min.

HPLC, ESI-MS Analysis and Identification of Target Compounds

Crude samples and all chromatographic fractions were analyzed by HPLC. A Diamonsil C_{18} column was employed. Methanol and 0.05% aqueous phosphoric acid were used to constitute the mobile phase in gradient mode. The flow rate of the mobile phase was 1.0 mL/min and the analytes were monitored by a diode-array detection (DAD).

Identification of target compounds was carried out by ESI-MS using a Finnigan LCQ Deca ion trap mass XP MAX spectrometer equipped with an electrospray ionization source (Thermo Finnigan, San Jose, CA, USA).

RESULTS AND DISCUSSION

HPLC Chromatogram of 1-Butanol Extract

The crude 1-butanol extract was analyzed by HPLC with a mobile phase composed of methanol-water (gradient elution, methanol: 25%, 2 min; 60%, 42 min; 85%, 50 min). As shown in Fig. 1, there are many peaks eluted from 5 min to 30 min of the retention times (RT), indicating that the extract with 1-butanol was highly complex and it may be necessary to further purify by MPLC before being subjected to HSCCC separation.

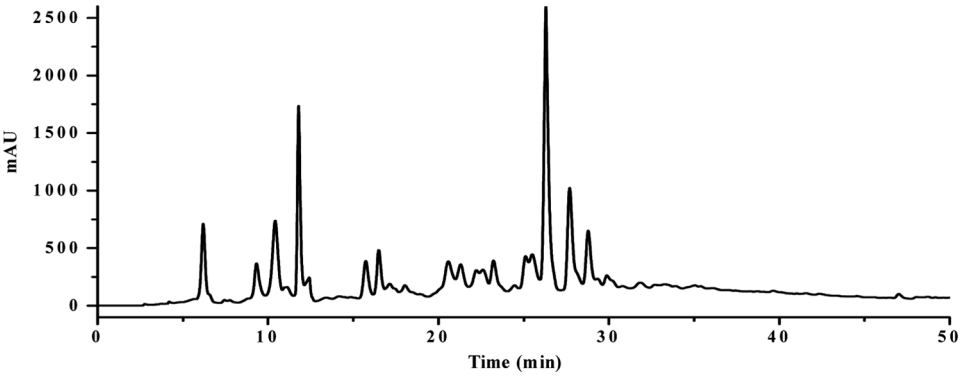


FIGURE 1 HPLC chromatogram of the 1-butanol extract. column: Diamonsil C18 (25 mm × 4.6 mm) at room temperature; mobile phase: methanol-water (gradient elution, methanol, 25%, 2 min; 25–60%, 42 min; 60–85%, 50 min); flow rate: 1.0 mL/min; detection wavelength: 330 nm.

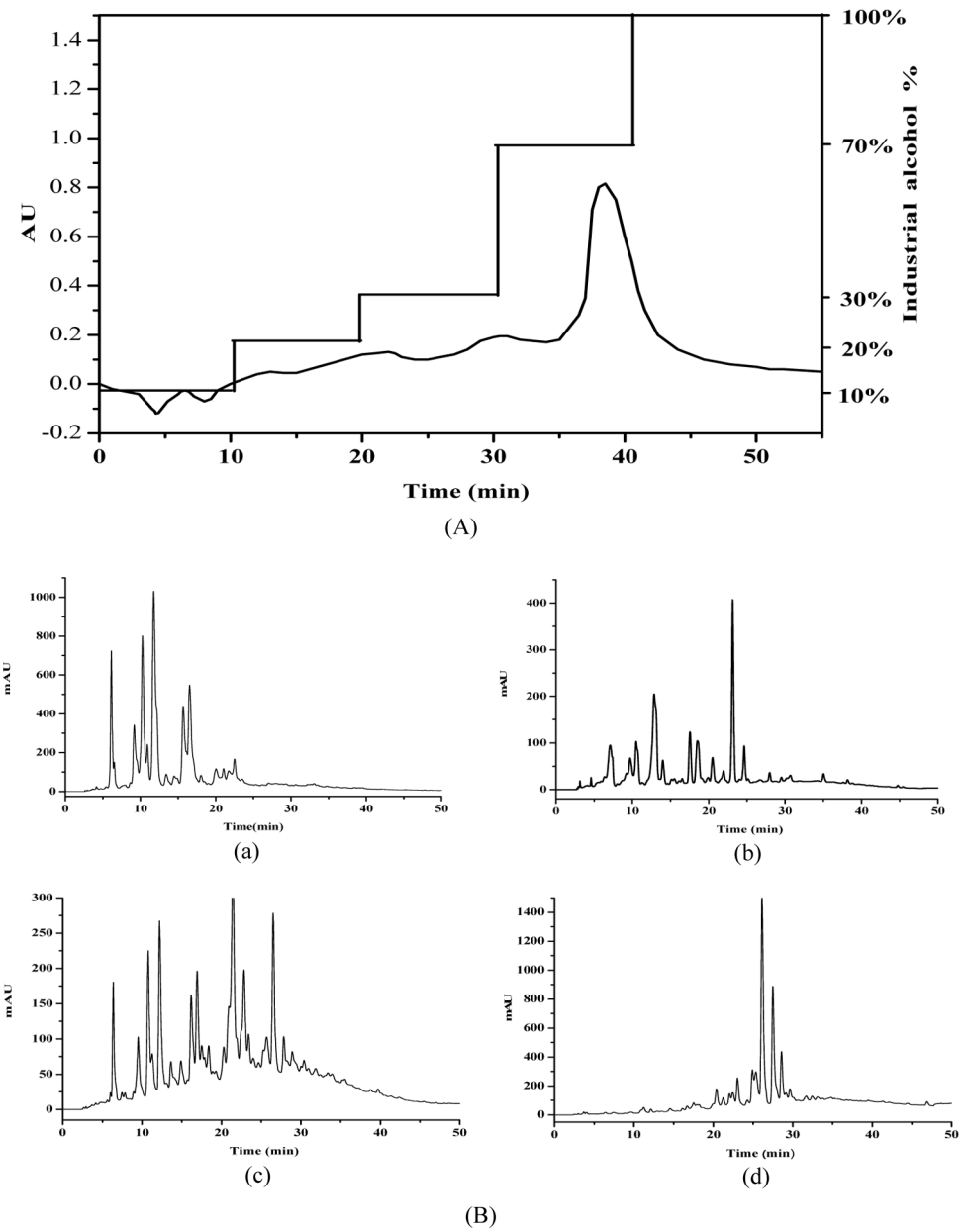


FIGURE 2 (A):MPLC chromatograms of the eluate column: three plastic connected column filled with AB-8 macroporous resin; mobile phase: industrial alcohol-water (gradient elution, methanol, 10%, 10 min; 20%, 10 min; 30%, 10 min; 70%, 10 min; and 100%, 15 min); flow rate: 18 mL · min^{-1}; detection wavelength: 330 nm. (B): HPLC chromatograms of the eluate. (a), (b), (c) and (d) were 10%, 20%, 30%, 70% industrial alcohol aqueous solution elutes, respectively; column: Diamonsil C18 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol-water (gradient elution, methanol, 25%, 2 min; 25–60%, 42 min; 60–85%, 50 min); flow rate: 1.0 mL · min^{-1}; detection wavelength: 330 nm.

Optimization of MPLC Conditions

Ethanol-water solution was used as eluent for AB-8 macroporous resin. Initially, the condition of MPLC was set as industrial alcohol-water (industrial alcohol, 10%, 20%, 30%, 70%, each for 10 min, and 100% for 15 min). The results of MPLC elution and HPLC analysis were shown in the Fig. 2.

From Fig. 2A, we can find that the 1-butanol extract was mostly eluted by 70% ethanol-water, and the results of HPLC analysis revealed that the peaks of 10%, 20%, and 30% at the retention time between 0 and 20 min were similar, but along with the increase of the % concentration of industrial alcohol, the peaks between 20 and 30 min of 20% and 30% ethanol fractions started to overlap with those of the 70% eluate, leading to unsuccessful prefractionation, and, therefore, we chose the 10% and 70% to achieve our aim. The MPLC and HPLC chromatograms were shown in Fig. 3.

It can be seen from Fig. 3a that the substances were mostly distributed in 10% and 70% eluate by extending the elution time from 10 to 30 min. Their retention time in Fig. 3b were 7 min to 17 min for the 10% industrial alcohol eluent; 20 to 30 min for the 70% industrial alcohol fractions indicating that the prefractionation was achieved successfully.

Optimization of HPLC Conditions

Several elution systems were tested in HPLC separation of the concentrated 70% ethanol-water eluate such as methanol-water, methanol-0.1%

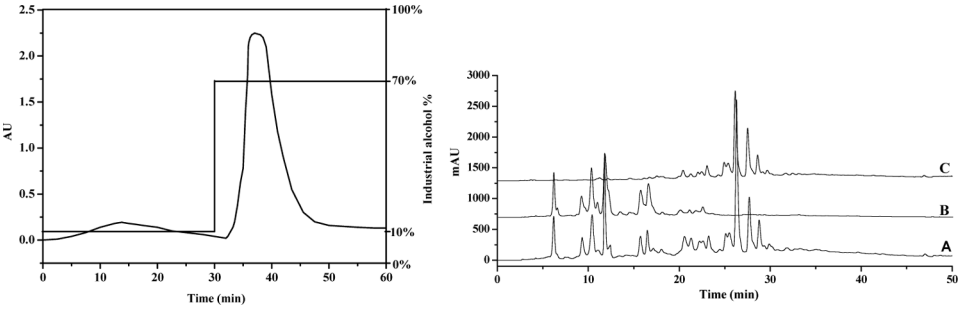


FIGURE 3 (a): MPLC chromatograms of the eluate. column: three plasti connected-column filled with AB-8 macroporous resin; mobile phase: industrial alcohol-water (gradient elution, methanol, 10%, 30 min; 70%, 30 min); flow rate: 18 mL/min; detection wavelength: 330 nm. (b): HPLC chromatograms A, B and C were obtained from the crude 1-butanol extracts, 10% and 70% industrial alcohol fractions, respectively; column: Diamonsil C18 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol-water (gradient elution, methanol, 25%, 2 min; 25–60%, 42 min; 60–85%, 50 min); flow rate: 1.0 mL/min; detection wavelength: 330 nm.

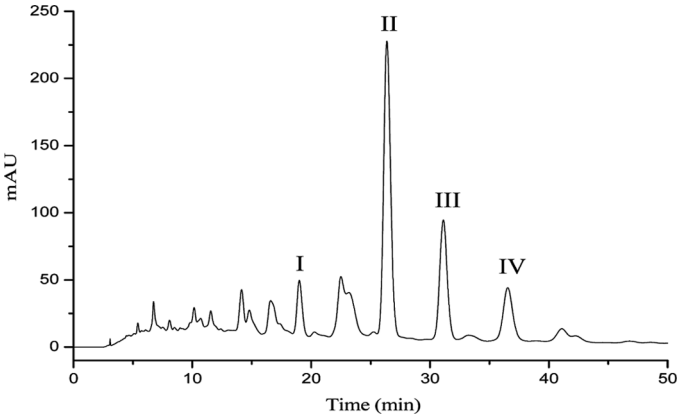


FIGURE 4 HPLC chromatogram of the concentrated 70% industrial alcohol aqueous solution eluate. column: Diamonsil C18 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol-0.05% aqueous phosphoric acid (isocratic elution, methanol, 34%, 50 min); flow rate: 1.0 mL/min; detection wavelength: 330 nm.

aqueous acetic acid, gradient elution of method-0.1% aqueous acetic acid, etc. When methanol-0.05% aqueous phosphoric acid was used as the mobile phase in isocratic mode (methanol: 34%, 50 min), a good result could be obtained. The optimized chromatogram was shown in Fig. 4.

Selection of Two-Phase Solvent System for HSCCC

Successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K) for the targeted compounds. The ideal K value of the compound separated by HSCCC is between 0.5 and 2.0. If the K values are bigger than 2.0, the separation time will be too long and the HSCCC peak will be extended seriously.

TABLE 1 *K*-Values of the Compounds in Different Two-Phase Solvent Systems

	K-value			
Solvent System (v/v)	I	II	III	IV
A: TFA-B-MBE-ACN(5:2:2:1, v/v/v/v)	3.91	0.79	6.48	3.71
B: TFA-B-MBE-ACN-EA(5:1.5:1:1:2, v/v/v/v/v)	4.15	0.66	5.36	3.31
C: TFA-B-MBE-ACN-EA(5:1:1:1:2, v/v/v/v/v)	3.21	0.42	2.18	2.69
D: TFA-B-MBE-ACN-EA(5:1:1:1:3, v/v/v/v/v)	1.52	0.32	2.76	1.63
E: TFA-B-MBE-ACN-EA(5:0.5:1:1:3.5, v/v/v/v/v)	0.32	0.01	0.44	0.15

TFA: 0.1% aqueous trifluoroacetic acid, B: 1-butanol, MBE: methyl *tert*-butyl ether.
ACN: acetonitrile, EA: ethyl acetate.

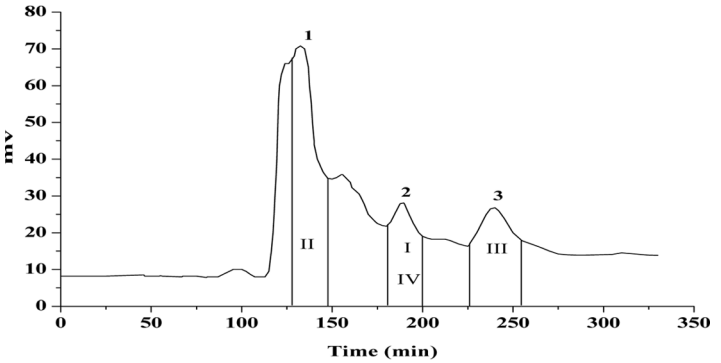


FIGURE 5 HSCCC chromatogram of the 70% industrial alcohol aqueous solution eluate. Solvent system: methyl *tert*-butyl ether-ethyl acetate-1-butanol-acetonitrile-water (0.1% trifluoroacetic acid aqueous solution) at a ratio of 1:3:1:1:5 (D in Table 1); stationary phase: the upper organic phase; mobile phase: the lower aqueous phase; flow rate: 1.8 mL/min; revolution speed: 800 rpm; detection wavelength: 280 nm; sample size: 50 mg crude sample dissolved in 2 mL of lower phase and upper phase mixture (1:1, v/v) of the solvent system.

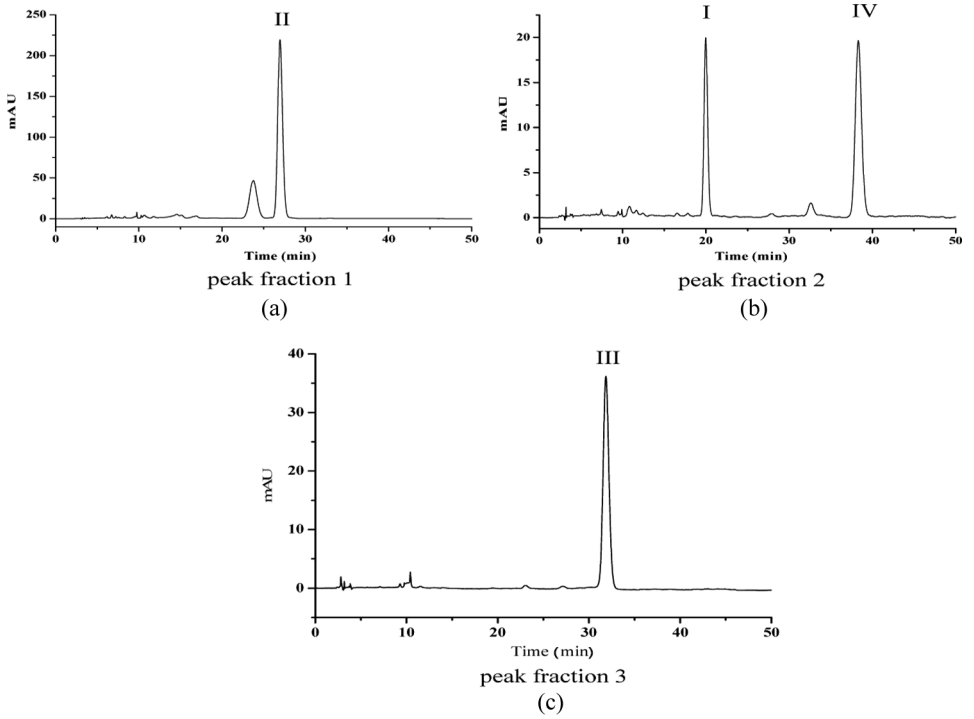


FIGURE 6 HPLC chromatograms of peak fractions from HSCCC. Peak fraction 3 was the target compound, which purity was 92.23%. column: Diamonsil C18 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol-water (isocratic elution, methanol, 34%, 50 min); flow rate: 1.0 mL/min; detection wavelength: 330 nm.

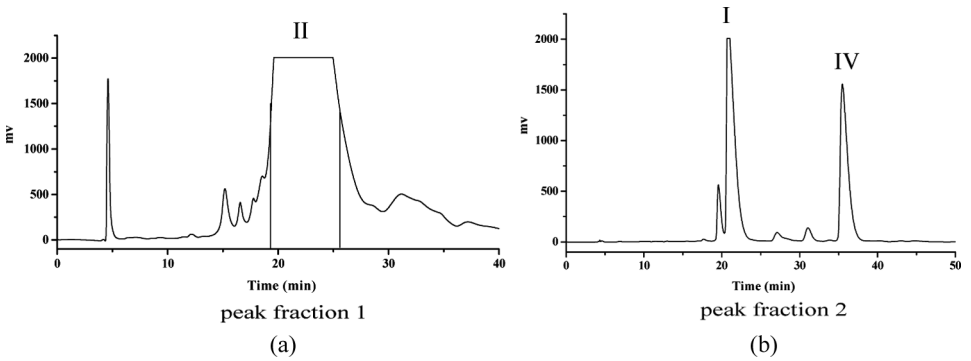


FIGURE 7 Preparative HPLC chromatograms of peak fractions 1 and 2 from HSCCC separation. column: YMC-Pack ODS-A column (250 mm × 10 mm); mobile phase: methanol-0.05% aqueous phosphoric acid, flow rate: 3.0 mL/min; detection wavelength: 330 nm.

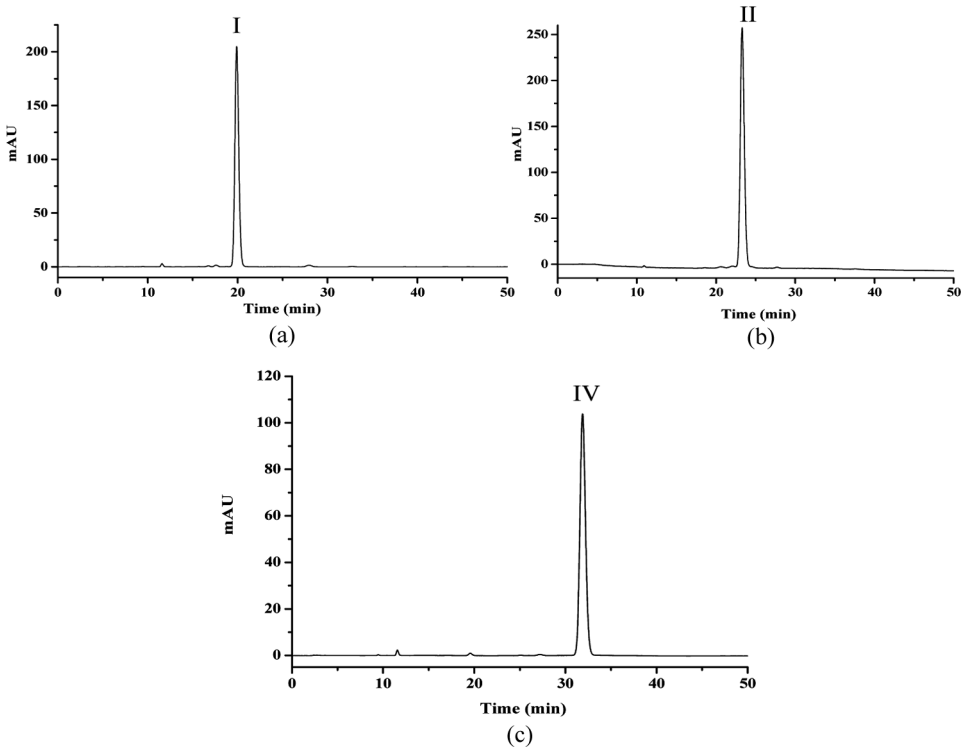


FIGURE 8 HPLC chromatograms of the further purified target compounds from prep-HPLC. Column: Diamonsil C18 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol-0.05% aqueous phosphoric acid(isocratic elution, methanol, 34%, 50 min); flow rate: 1.0 mL/min; detection wavelength: 330 nm.

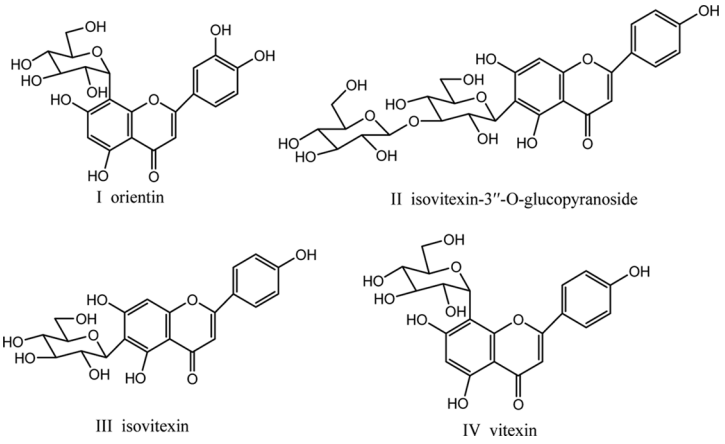


FIGURE 9 Chemical structures of target compounds from *Ficus microcarpa*.

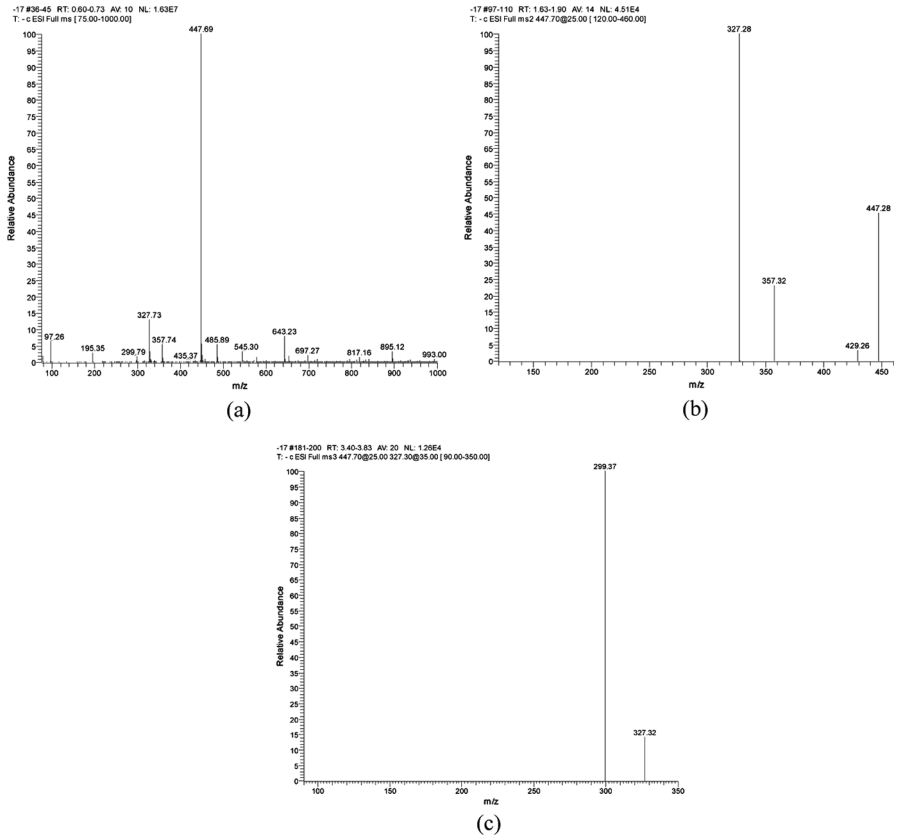
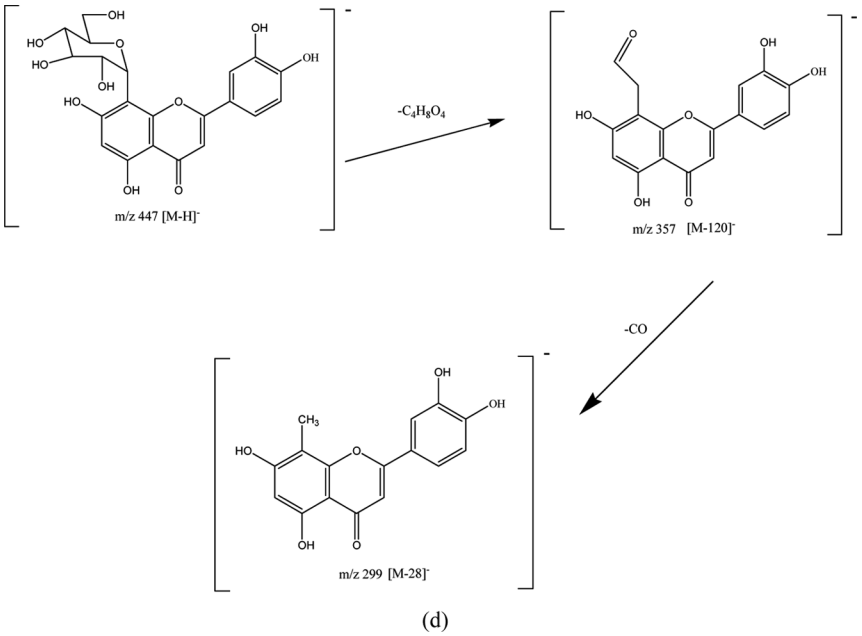


FIGURE 10 ESI-MS^n mass spectra of compound I (a) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound I; (b) MS^2 on product ion m/z 429, 357, 327; (c) MS^3 on product ion m/z 299; (d) Proposed fragmentation mechanism of the $[M\text{-}H]^-$ ion of compound.



(d)

FIGURE 10 Continued.

In this study, several two-phase solvent systems were tested to optimize the K values of four target compounds for HSCCC separation based on HPLC chromatograms. The K values were determined by HPLC as follows: a suitable amount of crude sample was dissolved in 5 mL of aqueous phase of the pre-equilibrated two-phase solvent system and the solution was analyzed by HPLC, the peak area being recorded as A_1. Then, the equal volume of the organic phase was added to the solution and mixed thoroughly. After the equilibration was established, the aqueous phase was analyzed by HPLC again, the peak area being recorded as A_2.

The partition coefficient (K) was obtained from the pair of corresponding peaks by the following equation:

$$K = (A_1 - A_2)/A_2.$$

Since the target compounds were in the 1-butanol extract (polar solvent), several relatively polar solvent systems were examined as listed in Table 1.

The k-values of the target compounds in the 0.1% aqueous trifluoroacetic acid-1 -butanol-methyl *tert*-butyl ether-acetonitrile solvent system (A) was slightly too large, which indicates that the target compounds would be partitioned more in the upper phase requiring long separation times by HSCCC. When the solvent system was modified with adding ethyl

acetate and reducing the 1-butanol (B), we found that the K values became smaller. The solvent volume ratio at 5:0.5:1:1:3.5 (E) investigated next showed too small k-values. Finally, 0.1% aqueous trifluoroacetic acid-1-butanol-methyl *tert*-butyl ether-acetonitrile-ethyl acetate (5:1:1:1:3, v/v/v/v/v) (D) gave suitable K values and was selected for HSCCC separation. Using this two-phase solvent system, the effect of the flow rate of the mobile phase was examined. When the flow rate was increased from 1.5 mL/min to 2 mL/min, the separation time became acceptable, but the retention of the stationary phase was only 45%. To solve this problem, 15 mL of the stationary phase was injected into the sample port after the crude sample to maintain an acceptable amount of the stationary phase in the column.

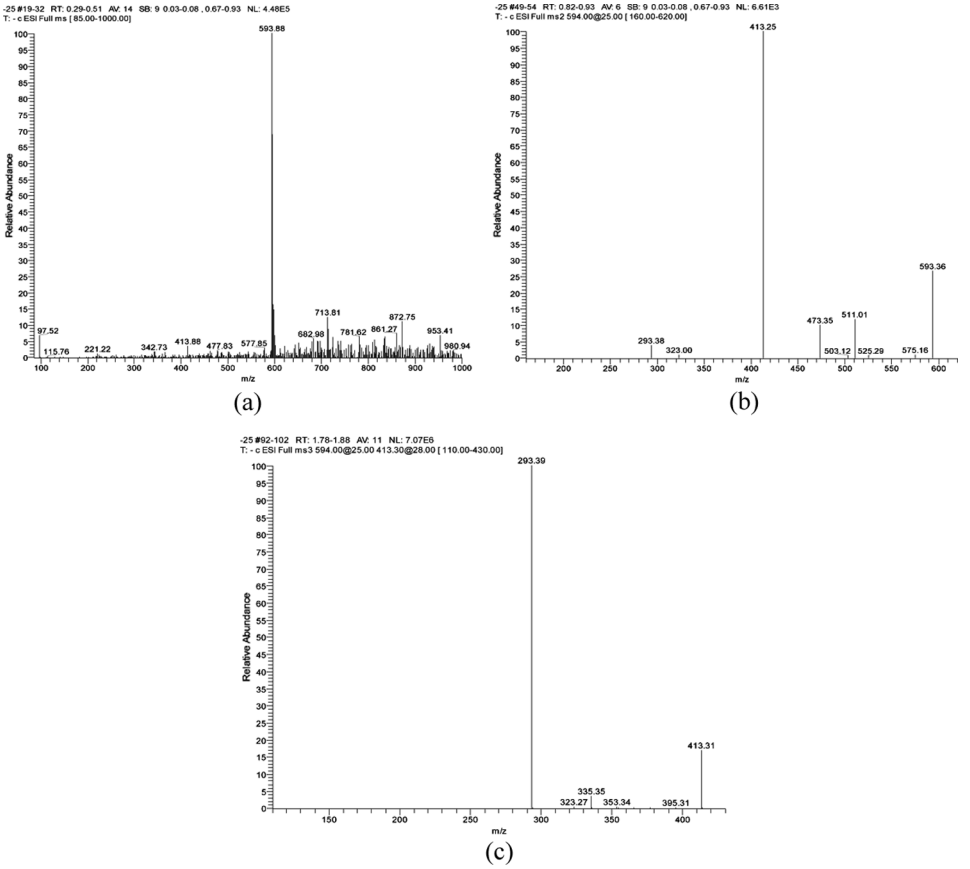


FIGURE 11 ESI-MS^n mass spectra of compound II (a) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound II; (b) MS^2 on product ion m/z 511, 473, 413; (c) MS^3 on product ion m/z 293; (d) Proposed fragmentation mechanism of the $[M\text{-}H]^-$ ion of compound.

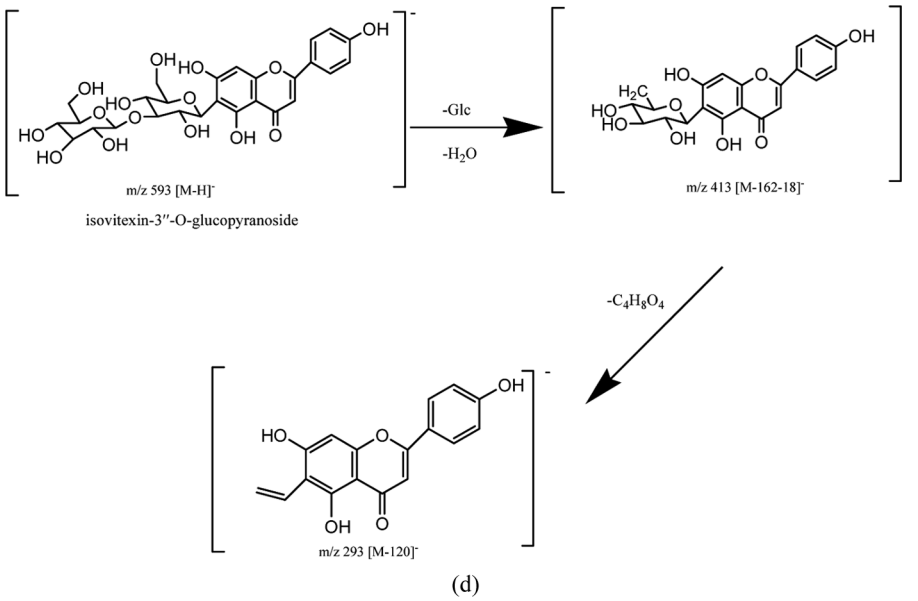


FIGURE 11 Continued.

The HSCCC separation was carried out as follows. The revolution speed was selected at 800 rpm, the flow rate of the mobile phase was set at 1.5 mL/min at the beginning. When the fraction 1 was eluted (in about 2.5 h) the flow rate of the mobile phase was increased to 2.0 mL/min to shorten the separation time. The concentrated 70% ethanol fraction from MPLC was separated under this optimized HSCCC condition. The typical HSCCC chromatogram and HPLC chromatogram were shown in Figs. 5 and 6, respectively. Only peak fraction 3 was a pure peak by this one step HSCCC separation, while peak fractions 1 and 2 required further purification by prep-HPLC.

Optimization of Prep-HPLC Conditions

In order to obtain pure materials, the peak fraction 1 and 2 from HSCCC separation were subjected to prep-HPLC to be purified further (Fig. 7). The prep-HPLC mobile phase conditions of the peak fractions 1 and 2 were methanol-0.05% aqueous phosphoric acid (isocratic elution, methanol, 32%, 40 min); methanol-0.05% aqueous phosphoric acid (isocratic elution, methanol, 30%, 50 min), respectively.

Analysis the Result of Preparative HPLC Separation

Three compounds purified by the combined use of HSCCC and P-HPLC were orientin (compound I), isovitexin-3″-O-glucopyranoside

(compound II), and vitexin (compound IV). The purities of these compounds were 97.28%, 97.20%, 98.40%, respectively, by HPLC analysis (Fig. 8).

Identification of the Target Compounds

The chemical structure of each peak from P-HPLC was identified according to EIS-MS^n data.

Peak I: negative ESI-MS, m/z 447.69 $[M\text{-}H]^-$, EIS-MS^2: m/z 429.26, 357.32, 327.28, EIS-MS^3: m/z 299.37 (Fig. 10).

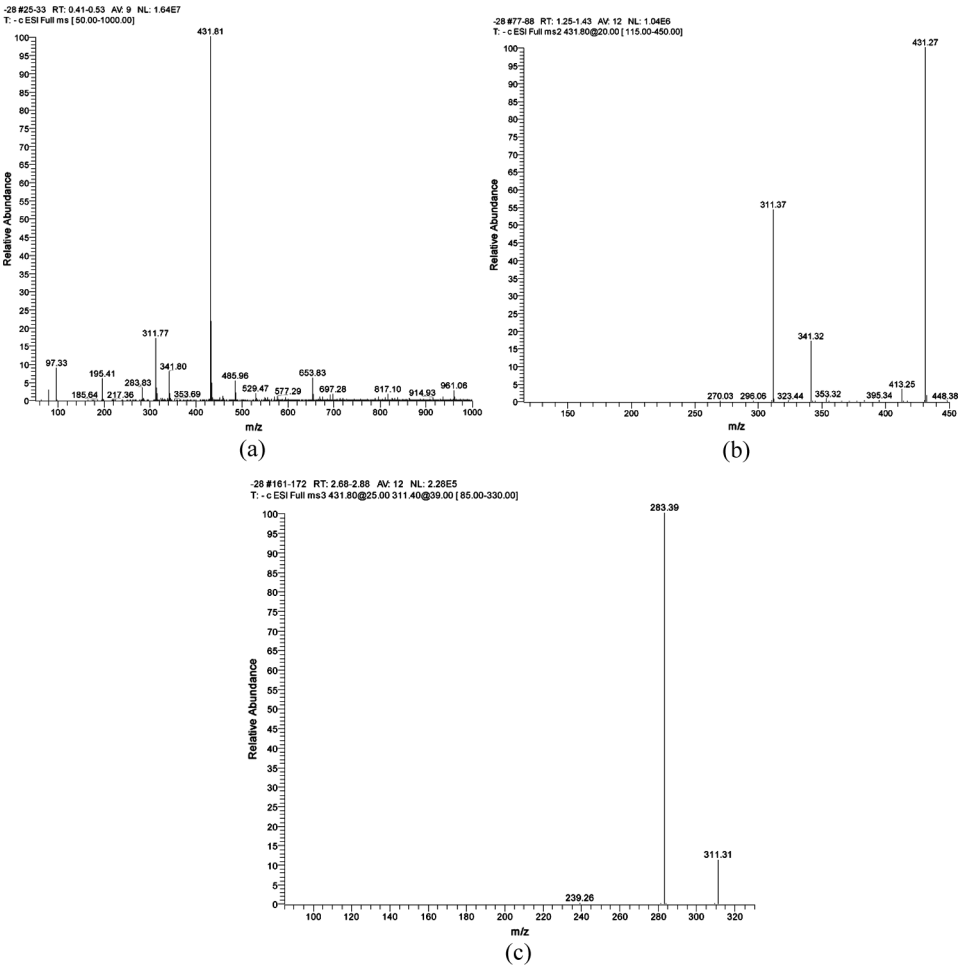
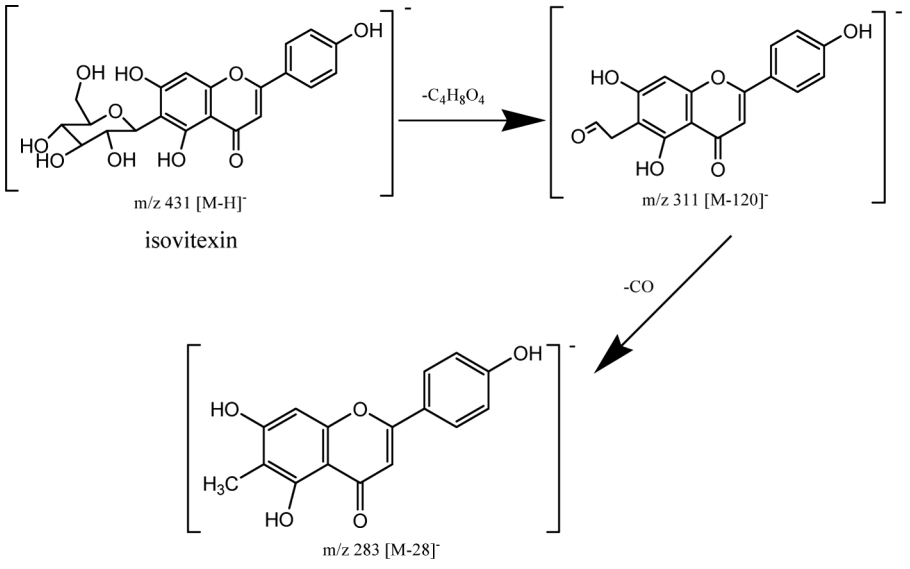


FIGURE 12 ESI-MS^n mass spectra of compound III (a) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound III, m/z 431; (b) MS^2 on product ion m/z 341, 311; (c) MS^3 on product ion m/z 283. (d) Proposed fragmentation mechanism of the $[M\text{-}H]^-$ ion of compound III.



(d)

FIGURE 12 Continued.

Peak II: negative ESI-MS, m/z 593.88 $[M\text{-}H]^-$, EIS-MS^2: m/z 511.01, 473.35, 413.25. EIS-MS^3: m/z 413.31, 293.39 (Fig. 11).

Peak III: negative ESI-MS, m/z 431.81 $[M\text{-}H]^-$, EIS-MS^2: m/z 341.32, 311.37. EIS-MS^3: m/z 283.39 (Fig. 12).

Peak IV: negative ESI-MS, m/z 431.94 $[M\text{-}H]^-$, EIS-MS^2: m/z 383.39, 341.35, 311.29. EIS-MS^3: m/z 283.42 (Fig. 13).

The ESI-MS mass spectra of compound I (peak I) in the negative mode gave m/z 447 as the deprotonated molecular ion [*M*-H], which confirmed the molecular mass as 448, the same as that for orientin.[16] Further experiments in MS^2 of the m/z 447 ion ([*M*-H]) produced a main fragment at m/z 327. The ion at m/z 327 is considered to be the loss of the $C_4H_8O_4$ [*M*-H-120] from the parent ion m/z 447. The MS^3 spectrum of the ion at m/z 327 yielded one ion at m/z 299 by losing a CO unit. Possible fragmentation pathways of orientin are illustrated in Fig. 10d.

The ESI-MS mass spectra of compound II (peak II) in the negative mode gave m/z 593 as the deprotonated molecular ion [*M*-H], which confirmed the molecular mass as 594, the same as that for isovitexin-3″-O-glucopyranoside.[17] Further experiments in MS^2 of the m/z 593 ion ([*M*-H]) produced a main fragment at m/z 413. The ion at m/z 413 is considered to be the loss of Glc and H_2O [*M*-H-162-18] from the parent ion m/z 593. To our knowledge, the MS^3 spectrum of the ion at m/z 413 yielded

one ion at m/z 293 by losing a $C_4H_8O_4$ unit [*M*-H-120] from the parent ion m/z 413. Possible fragmentation pathways of anthraquinone are illustrated in Fig. 11d.

For compound III (peak III) and IV (peak IV), the ESI-MS data provided m/z 431 as the deprotonated molecular ion [*M*-H], which indicate that they have the same molecular mass of 432, The MS^2 spectrum of the mainly ion at m/z 341 and MS^3 spectra of the ion at m/z 283 were all the same, suggesting that these two compounds are isomers with similar structures. They were confirmed as isovitexin and vitexin.[18]

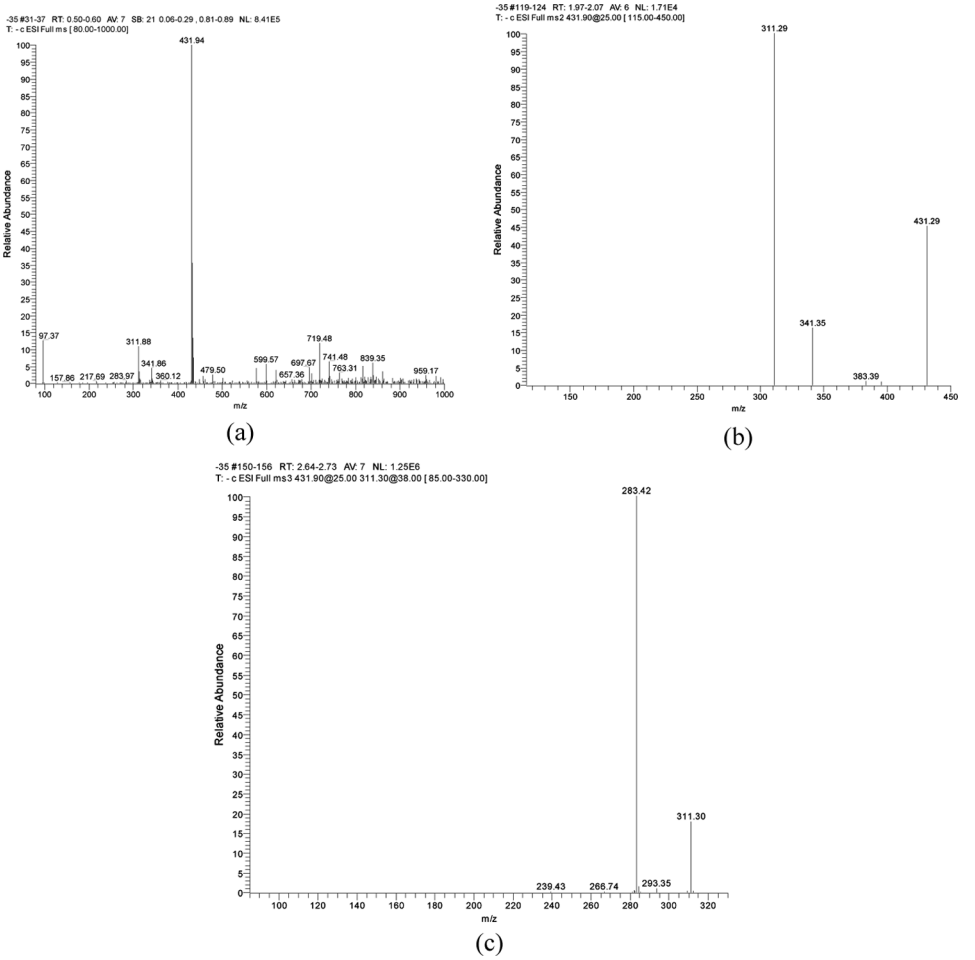


FIGURE 13 ESI-MS^n mass spectra of compound IV (a) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound IV, m/z 431; (b) MS^2 on product ion m/z 341, 311; (c) MS^3 on product ion m/z 283; (d) Proposed fragmentation mechanism of the $[M\text{-}H]^-$ ion of compound IV.

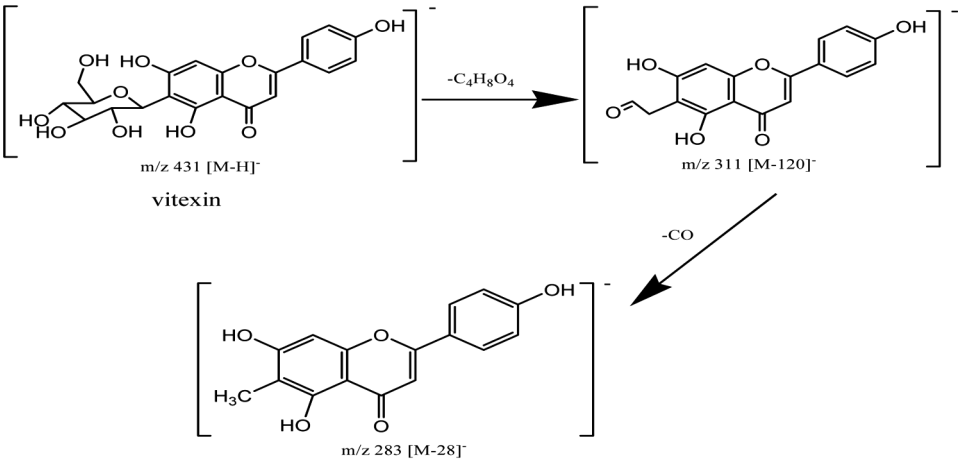


FIGURE 13 Continued.

CONCLUSIONS

In this paper, a rapid method using MPLC followed by HSCCC and prep-HPLC was established for separation and purification of chemical constituents in the *Ficus microcarpa* L.f. First, prefractionation of the 1-butanol extract was achieved by MPLC; and then the concentrated fractions were undertaken by HSCCC. Some pure substances were obtained in this step. Nevertheless, most peak fractions contained impurity, which were needed to be further purified using prep-HPLC. The peaks were individually collected and their identities were studied by EIS-MS^n. In this method, it is not necessary to carefully optimize the HSCCC conditions that would consume a considerable length of time. Therefore, this method will be useful for separation of target compounds from a complex mixture of natural products.

REFERENCES

1. Fan, M.S.; Ye, G.; Huang, C.G. The advances of chemistry and pharmacological study of Ficus genus. Nat. Prod. Res. Devel. **2005**, *17* (4), 497–504.
2. Higa, M.; Yogi, S.; Hakama, K. Studies on the constituents of *Ficus micracarpa* L.f. I. Triterpenoids from the leaves. Bull. Coll. Sci. Univ. Ryukyrus **1987**, *13*, 75–86.
3. Kuo, Y.H.; Chiang, Y.M. Five new taraxastane-type triterpenes from the aerial roots of *Ficus micracarpa*. Chem. Pharm. Bull. **1999**, *47*, 498–500.
4. Kuo, Y.H.; Chiang, Y.M. Six new ursane- and oleanane-type triterpenes from the aerial roots of *Ficus micracarpa*. Chem. Pharm. Bull. **2000**, *48*, 593–596.
5. Chiang, Y.M.; Su, J.K.; Liu, Y.H.; Kuo, Y.H. New cyclopropyl-triterpenoids from the aerial roots of *Ficus micracarpa*. Chem. Pharm. Bull. **2001**, *49*, 581–583.

6. Chiang, Y.M.; Kuo, Y.H. Taraxastane-triterpenes from the aerial roots of *Ficus micracarpa*. J. Nat. Prod. **2000**, *48*, 898–901.
7. Chiang, Y.M.; Kuo, Y.H. New peroxy triterpenes from the aerial roots of *Ficus micracarpa*. J. Nat. Prod. **2001**, *49*, 436–439.
8. Kuo, Y.H.; Li, H.Y. Two Novel Triterpenes from the leaves of *Ficus micracarpa*. Helvetica Chimica Acta **2004**, *87*, 1071–1076.
9. Chiang, Y.M.; Kuo, Y.H. Two novel alpha-tocopheroids from the aerial roots of *Ficus micracarpa*. Tetrahedron Lett. **2003**, *44*, 5125–5128.
10. Chiang, Y.M.; Kuo, Y.H. Novel Triterpenoids from the aerial roots of *Ficus micracarpa*. J. Org. Chem. **2002**, *67*, 7656–7661.
11. Chiang, Y.M.; Chang, J.Y.; Kuo, C.C.; Chang, C.Y.; Kuo, Y.H. Cytotoxic triterpenes from the aerial roots of *Ficus micracarpa*. Phytochemistry **2005**, *66*, 495–501.
12. Li, Y.C.; Kuo, Y.H. A monoterpenoid and two simple phenols from heartwood of *Ficus micracarpa*. Phytochemistry **1998**, *49*, 2417–2419.
13. Li, Y.C.; Kuo, Y.H. Two new isoflavones from the bark of *Ficus micracarpa*. J. Nat. Prod. **1997**, *60*, 292–293.
14. Kuo, Y.H.; Li, Y.C. Constituents of the bark of *Ficus micracarpa* L. f. J. Chin. Chem. Soc. **1997**, *44*, 321–325.
15. Xu, H.; Wang, X.M.; Wei, X.; Li, J.Y.; Liu, K. A new chalcone from the aerial roots of *Ficus micracarpa*. Chin. Chem. Lett. **2009**, *20*, 576–578.
16. Li, Y.W. Studies on the Chemical Components and Quality Standard of *Ficus microcarpa*. Linn. f. Doctoral Dissertation of Beijing University of Chinese Medicine. 2008.
17. Gao, G.H.; Deng, X.Y.; Liu, J.; Li, F.M. Flavone C-glycosides from *Folium isatidis*. J. of Shenyang Pharmaceut. Univ. **2007**, *24*, 748–750.
18. Dai, Z. Studies on the Chemical Constituents and Quality Control of *Folium fici microcarpae*. Master's Degree Dissertation of Guangdong Pharmaceutical University 2008.
19. Ito, Y. Efficient preparative countercurrent chromatography with a coil plant centrifuge. J. Chromatogr. A **1981**, *214*, 122–125.
20. Liu, R.M.; Sun, Q.H.; Sun, A.L.; Cui, J.C. Isolation and purification of coumarin compounds from *Cortex fraxinus* by high speed countercurrent chromatography. J. Chromatogr. A **2005**, *1072*, 195–199.
21. Sun, Q.H.; Sun, A.L.; Liu, R.M.P. Preparative isolation and purification of four compounds from the Chinese medicine herb *Rhizoma anemarrhenae* by high-speed counter-current chromatography. J. Chromatogr. A **2006**, *1104*, 69–74.
22. Han, X.; Ma, X.F.; Zhang, T.Y.; Zhang, Y.B.; Liu, Q.H.; Ito, Y. Isolation of high purity casticin from *Artemisia annua* L. by high speed countercurrent chromatography. J. Chromatogr. A **2007**, *1151*, 180–182.
23. Zhou, X.; Peng, J.Y.; Fan, G.R.; Wu, Y.T. Isolation and purification of flavonoid glycosides from *Torllius ledebouri* using high speed countercurrent chromatography by stepwise increasing the flow rate of the mobile phase. J. Chromatogr. A **2005**, *1092*, 216–221.
24. Peng, J.Y.; Fan, G.R.; Hong, Z.Y.; Chai, Y.F.; Wu, Y.T. Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high speed countercurrent chromatography. J. Chromatogr. A **2005**, *1074*, 111–115.