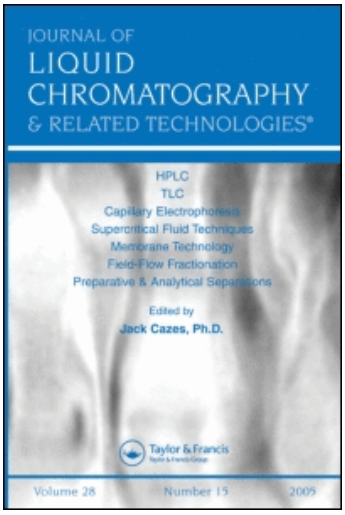

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Single-Step Preparative Separation of Barbinervic Acid and its Epimer (Rotungenic Acid), Along with Two Other Pentacyclic Triterpene Acids from the Leaves of *Diospyros kaki* Using HSCCC

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Abstract: The leaf of *Diospyros kaki* is a traditional Chinese medicine, which has been used for treatment of stroke or syndrome and as a hypotensive drug. The pentacyclic triterpene acids, isolated from the leaves of *Diospyros kaki*, are their main bioactive compounds, which are very difficult and tedious to be obtained by conventional chromatography methods, especially for the separation of barbinervic acid and its epimer (rotungenic acid). In the present report, for the first time a one step preparative high speed countercurrent chromatography (HSCCC, with a 1000 mL coil column) method was developed for the separation and purification of four bioactive pentacyclic triterpene acids from this plant with a solvent system composed of n-hexane–ethyl acetate–methanol–water (3:6:4:2, v/v/v/v). In a single operation, 49.6 mg of barbinervic acid (3α,19α,24-trihydroxy-urs-12-en-28-oic acid) and its epimer, 32.2 mg of rotungenic acid (3β,19α,24-trihydroxy-urs-12-en-28-oic acid) along with 11.0 mg of 24-hydroxy ursolic acid (3β,24-dihydroxy-urs-12-en-28-oic acid), which was reported for the first time from the leaves of *Diospyros kaki*, and 18.0 mg of ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) were obtained from 750.0 mg of the extract of the leaves of *Diospyros kaki*. The chemical structure of the four compounds was elucidated by EI-MS, ESI-MS, ^{1}H NMR, and ^{13}C NMR.

Keywords: Preparative chromatography, High speed countercurrent chromatography, *Diospyros kaki*, Pentacyclic triterpene acid, Barbinervic acid, Ursolic acid

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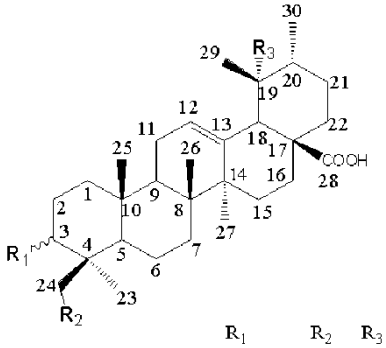
INTRODUCTION

Traditional Chinese medicines (TCM) have been used to treat human diseases in China for centuries. People are becoming increasingly interested in TCM's because of their low toxicity and good therapeutic performance. The leaf of *Diospyros kaki* is one of the widely used traditional Chinese medicines, which has been used for years in the treatment of stroke or syndrome of apoplexy in clinics in China to improve the outcome of ischemia stroke,[1–3] and is utilized as a hypotensive drug in Japanese traditional medicine. The isolation and identification of various compounds from the *Diospyros kaki* leaves have been reported; the pentacyclic triterpene acids isolated from the leaves of *Diospyros kaki* exhibit suppressing stimulus induced superoxide generation and tyrosyl phosphorylation,[4] and are used as preventives and remedies for type IV allergy induced inflammation.[5] In order to further study the biological activities of these pentacyclic triterpene acids and to control the quality of this traditional Chinese medicine and derived extracts, a large quantity of pure materials are urgently needed. However, the preparative separation and purification of these bioactive components from the leaves of *Diospyros kaki* by classical methods are very difficult, and tedious, and usually requires multiple chromatography steps,[6,7] especially for the separation of barbinervic acid and its epimer (rotungenic acid). High speed countercurrent chromatography (HSCCC) is a unique form of liquid–liquid partition chromatography with a liquid stationary phase. The method provides an advantage over the conventional column chromatography by eliminating the use of a solid support where an amount of stationary phase is limited and dangers of irreversible adsorption from the support are inevitably present.[8]

HSCCC has recently been investigated to separate and purify, effectively, a number of natural products.[9–11] However, no report has been published on the use of HSCCC for the separation and purification of pentacyclic triterpene acids from the leaves of *Diospyros kaki*. The aim of this study, therefore, was to develop an efficient HSCCC method for the preparative separation and purification of the four pentacyclic triterpene acids simultaneously in one step from the leaves of *Diospyros kaki* in larger amounts.

The present report deals with the one step isolation and purification of barbinervic acid (3α,19α,24-trihydroxy-urs-12-en-28-oic acid) (component **1**), rotungenic acid (3β,19α,24-trihydroxy-urs-12-en-28-oic acid) (component **2**), 24-hydroxy ursolic acid (3β,24-dihydroxy-urs-12-en-28-oic acid) (component **3**) and ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) (component **4**) using high speed countercurrent chromatography from the leaves of *Diospyros kaki* for the first time (see Figure 1). Component **1** is an epimer of component **2**, the difference between them is only the configuration of a hydroxyl group at position C3: one contains an axial hydroxyl group and the other an equatorial hydroxyl group. The content of component **3** is very small in the leaves of *Diospyros kaki*, which is so easy to be irreversibly absorbed on the support that it is very difficult to be obtained by conventional



	R_1	R_2	R_3
1 barbinervic acid	3a-OH	OH	OH
2 rotungenic acid	3ß-OH	OH	OH
3 24-hydroxy ursolic acid	3ß-OH	OH	H
4 ursolic acid	3ß-OH	H	H

Figure 1. Structure of pentacyclic triterpene acids 1–4 isolated from the leaves extract of *Diospyros kaki.*

methods. This is the first report about 3β,24-dihydroxy-urs-12-en-28-oic acid (component **3**) in the leaves of *Diospyros kaki*. The structures of all the compounds are determined by spectral methods of MS and NMR.

EXPERIMENTAL

Reagents and Materials

HPLC grade methanol (E. Merck, Darmstadt, Germany) was used for the HPLC analysis. Deionized water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). HSCCC and extraction reagents were of analytical reagent grade. The leaves of *Diospyros kaki* were collected in autumn 2004 from Hangzhou (Zhejiang, China). Dried leaves were crushed into pieces prior to extraction.

Apparatus

HSCCC

Preparative HSCCC was performed using a Model TBE-1000A HSCCC system manufactured by Tauto Biotech Co., Ltd., Shanghai, China, equipped with a 1000 mL coil column made of polytetrafluoroethylene tubing (i.d. 1.6 mm). The β value of the preparative column varied from 0.51 at the internal layer to 0.71 at the external layer ($\beta = r/R$, where r is the distance from the coil to

the holder shaft, and R is the revolution radius or the distance between the holder axis and the central axis of centrifuge.). The revolution speed of the apparatus could be adjusted in a range between 0 and 600 rpm. The solvent was pumped into the column by a Model SD−9002 constant-flow pump (Beijing Shengyitong Technology Development Co., Ltd.), and continuously delivered by 213.9 nm absorption with a Model 8823B UV detector (Beijing Institute of New Technology Application). The data were displayed and analyzed simultaneously on a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by an HX−2050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). A manual injection valve with an 80 mL loop was used to introduce the sample solution into the column.

HPLC

HPLC was performed on an Agilent 1100 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler, and UV detector. The column configuration consisted of Agilent Zorbax SB-C_{18} reserved-phase column (5 μm, 250 mm × 4.6 mm, i.d.).

Preparation of Sample

The powder of the leaves of *Diospyros kaki* (100 g) was degreased by hot petroleum ether (bp 60−90°C), followed by extraction twice each with 400 mL ethyl acetate for 2 hours under constant stirring at 70°C. The ethyl acetate extracts were combined and evaporated to dry under reduced pressure to yield 6.7 g ethyl acetate soluble residue. The residue was degreased by hot petroleum ether (bp 60−90°C), and then was dissolved in ethanol and decolorized by active carbon thrice each for 2 hours. The ethanol solution was evaporated to dryness under reduced pressure. The residue was recrystallized from ethanol and filtrated to remove most of the ursolic acid,[12] and the filtrate was evaporated to dryness under reduced pressure to yield 3.3 g of a light yellow powder, which was directly used for HSCCC separation.

Selection of Two-Phase Solvent System

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. The K was defined as the peak area of component in the upper phase divided by the peak area of component in the lower phase. The K values were determined by HPLC.[13] In brief, a suitable amount of crude extract was dissolved in a 20 mL test tube to which 5 mL each of pre-equilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for several minutes to

thoroughly equilibrate the sample with two phases. Then, equal volumes (1 mL) of the upper and lower phases were evaporated to dryness, separately, under vacuum at $<40^{\circ}$C. The residues were diluted with methanol to 1 mL and analyzed by HPLC to determine the K of each component.

HSCCC Separation

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water. After thoroughly equilibrating the mixtures in a separatory funnel at room temperature, two phases were separated shortly before use.

The multilayer coil column was first entirely filled with the upper phase (stationary phase). The lower phase (mobile phase) was then pumped into the head end of the inlet column at a flow rate of 5.0 mL min^{-1}, while the apparatus was rotated at 450 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (750.0 mg of the crude extract in 40 mL of the upper phase of the solvent system) was injected into the column through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 213.9 nm and the chromatogram was recorded. The reasons for selecting 213.9 nm as the detection wavelength are as follows: (1) this could avoid the interferences of absorbance of the mobile phase to some extent; (2) the maximum absorbance of these triterpene acids is close at 210 nm.

Eluates were collected into test tubes at 2 min intervals per tube. After the separation, the solvents in the column were pushed out and the retention of stationary phase was measured. Analysis of HSCCC fractions was done by TLC. TLC monitoring of fractions was necessary because the absorbance of the pentacyclic triterpene acids was very weak. Final purity of pentacyclic triterpene acids **1**–**4** was verified by HPLC.

Thin-Layer Chromatography (TLC) Analysis

Evaluation of the HSCCC fractions was done by TLC on normal-phase silica gel plates (Qingdao Haiyang Chemical plant, Qingdao, China), and *n*-hexane–acetone–ethyl acetate (4:2:1, v/v/v) as the developing system.

HPLC Analysis and Identification of Fractionated Compounds

After optimizing some separation parameters, the ratio 88:12 (v/v) of the solvent system of A-B (A, methanol; B, 0.1% aqueous H_3PO_4) at a flow rate of 1.0 mL/min was performed so as to ensure that each run of analysis

was completed within 20 min with good resolution of adjacent peaks and low solvent consumption. As the maximum absorbance of these triterpene acids is close at 210 nm, it was chosen as the detection wavelength.

^{1}H and ^{13}C NMR spectra were recorded in [$^{2}H_6$] dimethyl sulfoxide (DMSO-d_6) on a Bruker AMX 500 spectrometer (Karlsruhe, Germany) using TMS as internal standard.

Electron impact mass spectrometry (EI-MS) experiments were performed on a Finnigan Trace DSQ Single Quadrupole GC/MS system (Thermo Electron Corporation, USA) by direct inlet probe at 75ev. Electrospray ionization mass spectrometry (ESI-MS) experiments were performed on a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Electron Corporation, USA) equipped with a Finnigan electrospray, in negative mode.

RESULTS AND DISCUSSION

HSCCC Solvent System

HSCCC is very useful for the separation and purification of natural products. The selection of the two-phase solvent system is the most important, and is also the most difficult step because any change of the mobile phase composition is likely to change the stationary phase composition or volume; it is estimated that about 90% of the entire work in HSCCC is invested in solvent system selection.[9]

In order to select a suitable system, previous articles on HSCCC should be carefully consulted, and some rules need to be considered.[14,15] For example, the target compound should be soluble and stable in the solvent system; the settling time of the solvent system should be short (<30 s); the partition of the target compounds between two phases should be appropriate; and the retention of the stationary phase should be satisfactory.[16]

Du et al.[17] used a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (3:6:2:1, v/v/v/v) to separate two pentacyclic triterpene acids (ursolic acid and oleanolic acid) from the fruit of *L. lucidum Ait* by HSCCC. However, we tried it and found that this system was not suitable for the separation of the four pentacyclic triterpene acids in the leaves of *Diospyros kaki*, the retention of the stationary phase relative to the total column volume was much lower than 50% and loss of stationary phase from the HSCCC coil system was very severe due to the rather long settling time of the two-phase solvent system.

The two-phase solvent system containing *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v) was a versatile system, which was used to separate diterpenoids,[18] coumarins,[19] isoflavones,[20] alkaloids,[21] and mevinolinic acid.[22] However, this system was not suitable for separation of components **3** and **4** in the leaves of *Diospyros kaki*, because the two components were mainly partitioned in the upper phase and the

separation time would be too long, and the HSCCC peak will be extended seriously.

Therefore, other three two-phase solvent systems (*n*-hexane-ethyl acetate-methanol-water) were tested by changing the volume ratio of the solvent to obtain the optimum composition that gave an ideal range of partition coefficient (K) values and an appropriate settling time of the two-phase solvent system. Their K values were measured and summarized in Table 1. When *n*-hexane–ethyl acetate–methanol–water (3:7:4:2, v/v/v/v) was used as the two-phase solvent system, the K values were suitable, but the settling time of the solvent system was considerably longer than 30 s. A system containing *n*-hexane–ethyl acetate–methanol–water (3:6:5:2, v/v/v/v) was found to have a short settling time, however, it was not suitable for separation of components **2** and **3** in the leaves of *Diospyros kaki*, because the K values of the system are very close between components **2** and **3**. Finally, when *n*-hexane–ethyl acetate–methanol–water (3:6:4:2, v/v/v/v) was used, good separation results could be obtained and the separation time was acceptable.

In conclusion, the ratio 3:6:4:2 (v/v/v/v) of the two-phase solvent system containing *n*-hexane–ethyl acetate–methanol–water was the best to separate and purify the extract of the leaves of *Diospyros kaki*, after trying all the above solvent systems.

HSCCC Separation

The ethyl acetate extract of the leaves of *Diospyros kaki* was separated by HSCCC using a single solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:6:4:2, v/v/v/v). (The HSCCC chromatogram is shown in Figure 2). The solvent system possessed good retention character at the operation condition, i.e., 53.5% of retention rate of the stationary phase after the separation was finished. The analytical results were monitored by TLC, all fractions with the same value of R_f were combined

Table 1. The partition coefficients (K) of the target components in different ratio of volume in *n*-hexane–ethyl acetate–methanol–water solvent system

n-hexane–ethyl acetate–methanol–water ratio of volume	Component 1	Component 2	Component 3	Component 4
1:1:1:1	0.84	1.96	8.26	37.04
3:7:4:2	0.81	1.20	1.91	3.06
3:6:5:2	0.19	0.63	0.76	1.99
3:6:4:2	0.42	0.76	1.39	2.90

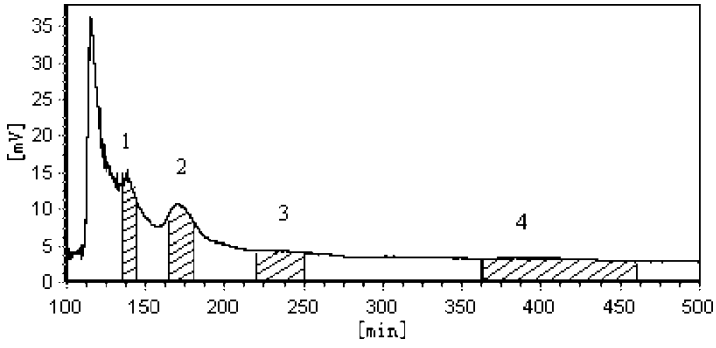


Figure 2. HSCCC separation chromatogram of 750.0 mg crude sample. Solvent system: *n*-hexane–ethyl acetate–methanol–water (3:6:4:2, v/v/v/v); stationary phase: upper phase; flow-rate of the mobile phase: 5.0 mL min^{-1}; revolution: 450 rpm; retention of stationary phase: 53.5%; UV wavelength: 213.9 nm; column temperature: 20°C.

to evaporated to dryness under reduced pressure, and then yielded component **1** (49.6 mg) upon recrystallization from MeOH and component **2** (32.2 mg), 3 (11 mg), 4 (18.0 mg) upon recrystallization from EtOH. Component **1** with R_f 0.18, elution time 134–144 min. Component **2** with R_f 0.31, elution time 164–180 min. Component **3** with R_f 0.47, elution time 220–250 min. Component **4** with R_f 0.76, elution time 375–460 min. Each component was determined by HPLC (see Figure 3), and their purities were above 98%.

Elucidation of Chemical Structure

Structures of component **1**, **2**, **3**, and **4** were confirmed by means of modern spectroscopic techniques, including ^1H, ^{13}C NMR, EI-MS and ESI-MS. All ^{13}C-NMR data are in very good agreement with previously published reference data.

Component **1**: barbinervic acid, colorless needles (MeOH), EI-MS m/z: 488 $[M]^+$, 470, 452, 442, 426, 264, 246, 231, 219, 206, 187, 175, 151, 146, 119. Negative ESI-MS *m/z*: 487 $[M\text{-}H]^-$. ^1H NMR (500 MHz, DMSO-d_6), δ: 5.16267 (1H, *m*, C_{12}-H), 3.43642, 3.21610 (2H, AB-*q*, $J = 10.8$ Hz each *d*, *br*, C_{24}-H), 3.16879 (H, *s*, *br*, C_3-βH), 2.36219 (1H, *s*, C_{18}-H), 1.29988 (3H, *s*, $-CH_3$), 1.27818 (3H, *s*, $-CH_3$), 1.07841 (3H, *s*, $-CH_3$), 0.88874 (3H, *s*, $-CH_3$), 0.83309 (3H, *d*, $J = 6.7$ Hz, C_{30}-H), 0.67139 (3H, *s*, $-CH_3$). ^{13}C NMR (500 MHz, DMSO-d_6), δ: 179.4300 (C-28), 139.0300 (C-13), 127.3950 (C-12), 72.1376 (C-19), 68.8881 (C-3), 64.6500 (C-24), 53.6860 (C-18), 49.3936 (C-5), 47.3988 (C-17), 47.1160 (C-19), 42.9481 (C-4), 41.8962 (C-20), 41.5887 (C-14), 39.9755 (C-8), 37.757 (C-22), 36.9541 (C-10), 33.5868 (C-7), 33.3641 (C-1), 28.4987 (C-15), 26.9080

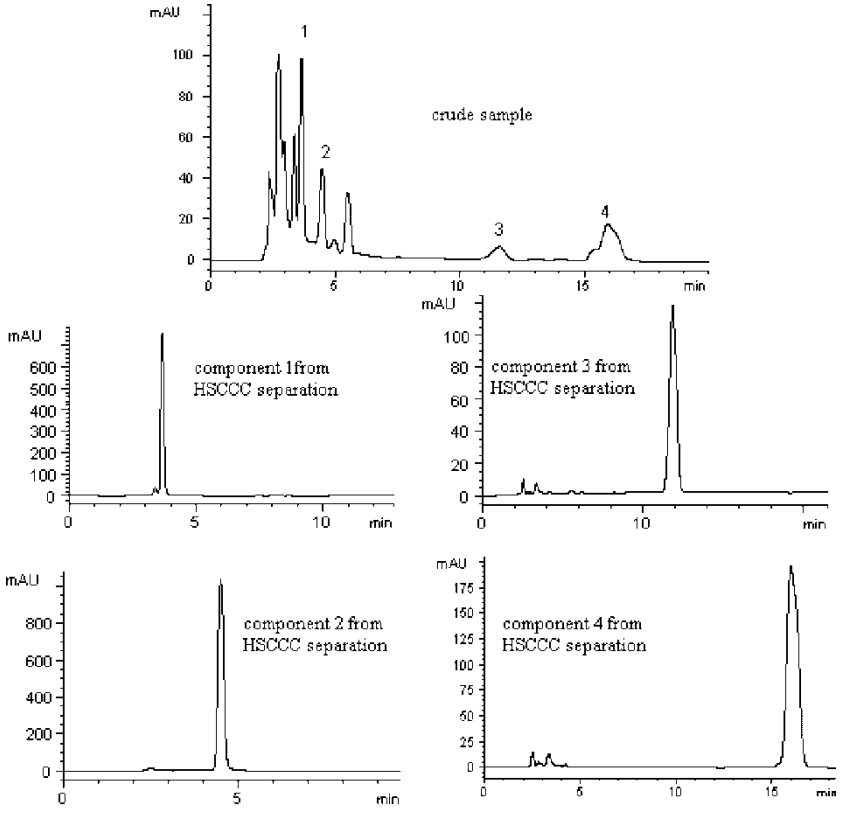


Figure 3. HPLC analysis of crude sample and the components obtained from HSCCC separation. Experimental conditions: Agilent Zorbax SB-C_{18} reserved-phase column (5 μm, 250 mm × 4.6 mm, i.d.), mobile phase: MeOH: 0.1%H_3PO_4 aqueous solution (88: 12, v/v); Flow rate: 1.0 mL min^{-1}; UV wavelength: 210 nm; column temperature: 30°C.

(C-29), 26.4321 (C-21), 25.6692 (C-2), 25.6692 (C-16), 24.4913 (C-27), 23.7504 (C-11), 23.1500 (C-23), 18.6751 (C-6), 16.9557 (C-26), 16.7702 (C-30), 15.8101 (C-25). These data were consistent with the literature[23–25] about 3α, 19α, 24-trihydroxy-urs-12-en-28-oic acid.

Component **2**: rotungenic acid, colorless needles (EtOH), EI-MS m/z: 488 $[M]^+$, 470, 452, 442, 426, 264, 246, 202, 189, 175, 146, 119. Negative ESI-MS *m/z*: 487 $[M\text{-}H]^-$. ^1H NMR (500 MHz, DMSO-d_6), δ: 5.15640 (1H, *m*, C_{12}-H), 3.84781 (1H, d, $J = 10.83$ Hz, C_{24}-H), 3.18728 (1H, *d*, *br*, $J = 10.40$ Hz, C_{24}-H), 3.16879 (H, *s*, *br*, C_3-αH), 2.36334 (1H, *s*, C_{18}-H), 1.27801 (3H, *s*, $-CH_3$), 1.07396 (6H, *s*, 2 × $-CH_3$), 0.84877 (3H, *s*, $-CH_3$), 0.83659 (3H, *d*, $J = 6.1$ Hz, C_{30}-H), 0.67661 (3H, *s*, $-CH_3$). ^{13}C NMR

(500 MHz, DMSO-d_6), δ: 179.3840 (C-28), 139.0370 (C-13), 127.2160 (C-12), 79.2140 (C-3), 72.1120 (C-19), 63.5020 (C-24), 55.9490 (C-5), 53.6470 (C-18), 47.3646 (C-17), 47.2507 (C-19), 42.5528 (C-4), 41.8691 (C-20), 41.4804 (C-14), 39.8225 (C-8), 38.5454 (C-1), 37.7127 (C-22), 36.8395 (C-10), 33.5322 (C-7), 28.5548 (C-15), 27.6879 (C-2), 26.8707 (C-29), 26.3996 (C-21), 25.6601 (C-16), 24.3339 (C-27), 23.8169 (C-11), 23.3681 (C-23), 19.1733 (C-6), 16.8779 (C-25), 16.7536 (C-26), 15.8885 (C-30). These data were consistent with the literature[26–29] about 3β, 19α, 24-trihydroxy-urs-12-en-28-oic acid.

Component **3**: 24-hydroxy ursolic acid, colorless cuboids (EtOH), EI-MS m/z: 472 $[M]^+$, 454, 436, 426, 408, 393, 300, 248, 223, 203, 189, 175, 133. Negative ESI-MS m/z: 471 $[M\text{-}H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6), δ: 5.12211 (1H, *m*, C_{12}-H), 3.83622 (1H, d, $J = 10.91$ Hz, C_{24}-H), 3.17680 (1H, *d*, *br*, $J = 9.76$ Hz, C_{24}-H), 3.26561 (H, *s*, *br*, C_3-αH), 2.11331 (1H, *d*, $J = 11.20$ Hz, C_{18}-H), 1.06904 (3H, *s*, $-CH_3$), 1.03150 (3H, *s*, $-CH_3$), 0.91082 (3H, *s*, $-CH_3$), 0.86984 (3H, *d*, $J = 5.375$ Hz, C_{30}-H), 0.81331 (3H, *d*, $J = 6.30$ Hz, C_{29}-H), 0.72141 (3H, *s*, $-CH_3$). ^{13}C NMR (500 MHz, DMSO-d_6), δ: 178.7420 (C-28), 138.645 (C-13), 124.9990 (C-12), 79.1430 (C-3), 63.5330 (C-24), 55.8835 (C-5), 52.8729 (C-18), 47.5699 (C-9), 47.3241 (C-17), 42.58668 (C-14), 42.0630 (C-1), 39.5987 (C-8), 38.9958 (C-4), 38.9529 (C-20), 38.6567 (C-19), 36.8278 (C-10), 36.8048 (C-22), 33.5890 (C-7), 30.6865 (C-21), 28.0365 (C-2), 27.7033 (C-15), 24.3056 (C-16), 23.5437 (C-11), 23.5439 (C-27), 23.4022 (C-23), 21.5593 (C-30), 19.0861 (C-6), 17.4908 (C-26), 17.2280 (C-29), 16.0231 (C-25). These data were consistent with literatures[30,31] about 3β, 24-dihydroxy-urs-12-en-28-oic acid, an un-reported compound in the chemical studies of the leaves of *Diospyros kaki.*

Component **4**: ursolic acid, colorless needles (EtOH), EI-MS m/z: 456 $[M]^+$, 439, 423, 411, 248, 203 189, 191, 133. Negative ESI-MS m/z: 455 $[M\text{-}H]^-$, ^{1}H NMR (500 MHz, DMSO-d_6), δ: 5.12364 (1H, *m*, C_{12}-H), 2.99997 (H, *s*, *br*, C_3-αH), 2.11539 (1H, *d*, $J = 11.28$ Hz C_{18}-H), 1.03608 (3H, *s*, $-CH_3$), 0.91040 (3H, *d*, $J = 8.965$ Hz, C_{29}-H), 0.89247 (3H, *s*, $-CH_3$), 0.86369 (3H, *s*, $-CH_3$), 0.81502 (3H, *d*, $J = 6.30$ Hz, C_{30}-H), 0.74567 (3H, *s*, $-CH_3$), 0.67412 (3H, *s*, $-CH_3$). ^{13}C NMR (500 MHz, DMSO-d_6), δ: 178.7420 (C-28), 138.6720 (C-13), 125.0810 (C-12), 77.3490 (C-3), 55.3094 (C-5), 52.8783 (C-18), 47.5353 (C-9), 47.3175 (C-17), 42.1301 (C-14),, 39.6058 (C-8), 38.9996 (C-4), 38.9630 (C-19), 38.8656 (C-20), 38.7595 (C-1), 37.0232 (C-10), 36.8140 (C-22), 33.2199 (C-7), 30.7019 (C-21), 28.7486 (C-23), 28.0419 (C-15), 27.4748 (C-2), 24.3140 (C-16), 23.7626 (C-27), 23.3483 (C-11), 21.5627 (C-30), 18.5063 (C-6), 17.4944 (C-29), 17.3916 (C-26), 16.5485 (C-24), 15.7124 (C-25). These data were consistent with literature[32–34] about 3β-hydroxy-urs-12-en-28-oic acid.

CONCLUSION

The present study demonstrates that preparative HSCCC (with a 1000 mL coil column) is a fast and effective methodology, suitable for highly selective preparation of larger amounts of pentacyclic triterpene acids in the leaves of *Diospyros kaki* in a complex matrix. Barbinervic acid, 49.6 mg, (3α,19α,24-trihydroxy-urs-12-en-28-oic acid) (**1**), 32.2 mg of rotungenic acid (3β,19α,24-trihydroxy-urs-12-en-28-oic acid) (**2**), 11.0 mg of 24-hydroxy ursolic acid (3β,24-dihydroxy-urs-12-en-28-oic acid) (**3**), which was reported for the first time from the leaves of *Diospyros kaki*, and 18.0 mg of ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) (**4**) were obtained from 750.0 mg of crude extract from the leaves of *Diospyros kaki* after only a one step separation, with *n*-hexane–ethyl acetate–methanol–water (3:6:4:2, v/v/v/v) as solvent system for the first time. The purities of these triterpene acids were above 98%. The overall results of the present study indicate that HSCCC is a powerful technique in separating and purifying bioactive compounds from natural sources.

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