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ISOLATION OF GLYCOSIDES FROM THE BARKS OF *ILEX ROTUNDA* BY HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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Abstract

Semi-preparative and preparative high-speed counter-current chromatography (HSCCC) were successfully used for isolation of glycosides from 50% ethanol extract of the dried barks of *Ilex rotunda* Thunb. (Aquifoliaceae) by using a two-phase solvent system composed of ethyl acetate-*n*-butanol-water (1:6:7, v/v/v). From 1.0 g of the extract, syringaresinol 4',4''-di-*o*- β -D-glucopyranoside (**I**, 20.2 mg), syringin (**II**, 56.8 mg), sinapaldehyde glucoside (**III**, 26.2 mg), syringaresinol 4'-*o*- β -D-glucopyranoside (**IV**, 20.4 mg), and pedunculoside (**V**, 45.1 mg) were obtained by one run of TBE-1000A HSCCC instrument with 1000 mL of column volume. Their structures were identified by IR, MS, and ¹H and ¹³C NMR studies. Glycoside **I** was isolated from this plant for the first time.

Keywords

Ilex rotunda; High-speed counter-current chromatography (HSCCC); Glycoside; Syringaresinol 4', 4''-di-*o*- β -D-glucopyranoside

INTRODUCTION

Jiubiyang, the dried barks of *Ilex rotunda* Thunb. (Aquifoliaceae), has been used as herbal tea and traditional Chinese medicine for the treatment of fever, throat-swelling, eczema, diarrhea, and furuncle^[1]. The *n*-butanol extract of Jiubiyang can depress both normal blood pressure and arterial hypertension in rats^[2], has protective effect on arrhythmia, and prevent the damage of myocardial ischemia in rats and mice^[3]. The ethanol extract of Jiubiyang can reduce coronary blood flow, weaken myocardial contractility, slow down heart rate, prolong survival time, and prevent arrhythmias in mice^[4]. A number of chemical components have been isolated from the barks of *I. rotunda*, including pedunculoside (**V**), syringin (**II**)^[5,6,7], 3 β -hydroxy-oleanane, rotundic acid, rotundic acid isopropylidene, sinapaldehyde,

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sinapaldehyde glucoside (**III**), stearic acid, syringaldehyde^[8], ilexrotunin, rotundanonic acid, rotundioic acid, rotungenic acid^[9], 3 β -[(α -L-arabinopyranosyl)oxy]-19 α -hydroxyolean-12-en-28-oic acid 28-*o*- β -D-glucopyranosyl ester, caffeic acid 4-*o*- β -D-glucopyranoside, β -daucosterol, kudinoside H, pomolic acid, β -sitosterol, syringaresinol 4'-*o*- β -D-glucopyranoside (**IV**), vanillic acid 4-*o*- β -D-glucopyranoside^[10], abbeokutone, 3 β ,19 α -dihydroxyurs-12-en-24,28-dioic acid, 19 α ,24-dihydroxyurs-12-en-3-one-28 oic acid, disyringin ether, friedelin, glucose, 28-hydroxy-friedelin, 3 β -hydroxy-oleanane, inositol, nonadecylic acid, oleanolic acid, rotundaol, and sugereoside^[11,12].

High-speed counter-current chromatography (HSCCC), a support-free liquid-liquid partition chromatographic technique, has been widely used for isolation and purification of active components from traditional Chinese medicines and natural products^[13–15]. Most of the studies focus on several main compounds in some parts, such as flavones^[16,17], phenols^[18–21], saponins^[22–24], and alkaloids^[25,26]. However, only few studies have focused on the direct separation and purification of chemical constituents from crude extracts of natural products^[27–29].

In this study, five glycosides were directly obtained by HSCCC from 50% ethanol extract of Jiubiyang, using two separation columns with total capacities of 260 mL of semi-preparation and 1000 mL of preparation.

EXPERIMENTAL

Apparatus

TBE-300A model HSCCC (Tauto Biotechnology Company, Shanghai, China) for semi-preparative separation has three PTFE (polytetrafluoroethylene) coils (*I.D.* of the tubing = 1.8 mm, column volume = 260 mL) and a 20 mL manual injection sample loop. The β value ($\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the distance between the holder axis and central axis of the centrifuge) of the multilayer coil varies from 0.60 (internal terminal) to 0.80 (external terminal). The revolution speed of the apparatus was regulated at 0–1000 rpm with an electronic speed controller. The solvent was pumped into the column with a Tauto TBP50A pump (Tauto Biotechnology Company, Shanghai, China) and the eluent was continuously monitored by a TBD-2000 UV detector (Tauto Biotechnology Company, Shanghai, China). The separation temperature was controlled by a DTY-20A water-circulating constant temperature implement (Tauto Biotechnology Company, Shanghai, China). The chromatogram was recorded by a Jinda biochemical chromatography workstation V4.0 (Tauto Biotechnology Company, Shanghai, China).

TBE-1000A model HSCCC for preparative separation has three PTFE coils (*I.D.* of the tubing = 3.0 mm, column volume = 1000 mL) and an 80 mL manual injection sample loop. The β value of the multilayer coil varies from 0.60 (internal terminal) to 0.78 (external terminal). The revolution speed of the apparatus was regulated at 0–600 rpm with an electronic speed controller. The solvent was pumped into the column with a Tauto TBP50A pump (Tauto Biotechnology Company, Shanghai, China) and the eluent was continuously monitored by a TBD-2000 UV detector (Tauto Biotechnology Company, Shanghai, China). The separation temperature was controlled by a TC-1050 water-circulating constant

temperature implement (Beijing Detianyou Science and Technology Development Company, Beijing, China). The chromatogram was recorded by a Jinda biochemical chromatography workstation V4.0 (Tauto Biotechnology Company, Shanghai, China).

Samples were analyzed by a Shimadzu LC-20AT high performance liquid chromatography (HPLC) instrument (Shimadzu, Japan) equipped with an SPD-M20A diode array detector (DAD), a SIL-20A auto sampler, a DGU-20As degasser, a CTO-10ASvp column oven, and a Shimadzu LC-solution workstation.

The ^1H and ^{13}C NMR spectra were measured by a Bruker AV400 spectrometer. The chemical shift values are reported as δ in ppm relative to tetramethylsilane (TMS) or sodium trimethylsilylpropionate (TSP) and the coupling constants (J) are in hertz (Hz). High resolution electron spray ionization mass spectra (HR-ESI-MS) were recorded on a Waters Xevo G2 Qtof mass spectrometer with positive ion mode. IR spectra were recorded on a Shimadzu FTIR-8400s spectrometer with KBr pellets.

Reagents and Materials

All organic solvents used for HSCCC separation, K -value determination, and extraction were of analytical grades (Sinopharm Chemical Reagent Beijing Co., Ltd, Beijing, China). Acetonitrile for HPLC was of chromatographic grade (Merck, Germany). Dried barks of *I. rotunda* were purchased from Guangzhou Caizhitang Pharmaceutical Co., Ltd (Guangdong Province, China) and identified by Professor Shilin Hu, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. A voucher specimen was deposited in Department of Chemistry, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences with the specimen number of 20111016.

Preparation of Jiubiyang Extract

The dried barks (1 kg) of *I. rotunda* were extracted 3 times with 10 L of 50% ethanol-water solution at 80°C. The extract was concentrated to a volume of 5 L in a rotary evaporator device (RE-201D, Henan Yuhua Instrument Co., Ltd, China) and centrifuged at 6000 rpm for 10 min using an LD5-10 centrifuge (Beijing Jinli Centrifuge Co., Ltd, China). The supernatant fluid was dried with a rotary evaporator to yield 175 g of Jiubiyang extract.

Measurement of Partition Coefficients (K)

The solvent mixtures were thoroughly equilibrated in a test tube and the upper phase and the lower phase were separated. The lower phase (2.0 mL) and 10 mg of Jiubiyang extract were delivered into a 10 mL test tube, mixed thoroughly, and stood for several minutes. The solution (5 μL) was taken directly for HPLC determination and the peak area was recorded as A_{initial} . Then, the upper phase (2.0 mL) was added to the solution, mixed thoroughly, and stood until two clear layers were formed. The lower phase solution (5 μL) was taken directly for HPLC determination and the peak area was recorded as A_{lower} . The partition coefficients (K) of glycosides were obtained by the following equation: $K = (A_{\text{initial}} - A_{\text{lower}}) / A_{\text{lower}}$.

Preparation of Two-Phase Solvent System and Sample Solution

The two-phase solvent system composed of ethyl acetate-*n*-butanol-water (1:6:7, v/v/v) were thoroughly mixed in a separatory funnel and allowed to stand until two clear layers were formed at 25°C. Then, the upper phase and lower phase were separated. The sample solution (25 mg/mL) for HSCCC separation was made by dissolving 1.0 g of Jiubiyang extract in the mixture of 20 mL lower phase and 20 mL upper phase of the two-phase solvent systems.

Separation Procedure

TBE-300A model HSCCC semi-preparation: A head-tail elution mode was selected for the initial separation. The upper phase was filled into the coil column as a stationary phase at a flow rate of 40 mL/min for 7 min. Then the apparatus was rotated at 850 rpm, meanwhile the lower mobile phase was pumped into the coil column at a flow rate of 1.0 mL/min. After hydrodynamic equilibrium was established, 12 mL of sample solution containing 300 mg of Jiubiyang extract was injected into the column. After several peaks were eluted at 312 min, the elution mode was turned to the tail-head mode and the upper phase was pumped into the coil column at a flow rate of 1.0 mL/min. During the separation process, the column temperature was controlled at 25°C. The effluent from the column was monitored at 254 nm. The fractions were collected by time-controlled collection method.

TBE 1000A model HSCCC preparation: A head tail elution mode was applied for the initial separation. The upper phase was filled into the coil column as a stationary phase at a flow rate of 50 mL/min for 20 min. Then the apparatus was rotated at 450 rpm, meanwhile the lower mobile phase was pumped into the coil column at a flow rate of 3.0 mL/min. After the hydrodynamic equilibrium was established, 40 mL of sample solution containing 1.0 g of Jiubiyang extract was injected into the column. After several peaks were eluted at 436 min, the elution mode was turned to the tail-head mode and the upper phase was pumped into the coil column at a flow rate of 3.0 mL/min. During the separation process, the column temperature was controlled at 25°C. The effluent from the column was monitored at 254 nm. The fractions were collected by time-controlled collection method.

HPLC Analysis and Purification of HSCCC Fractions

Jiubiyang extract and HSCCC fractions were analyzed by HPLC method. The analysis was performed using a Shimadzu LC-20AT chromatographic system with a Shiseido UG120-C18 reversed phase column (4.6 mm×150 mm, 5 µm, Shiseido Co. Ltd., Japan). The mobile phase was acetonitrile (A)-water (B) for gradient elution: 0–10 min, 10% of A; 10–20 min, 10%–40% of A; 20–30 min, 40% of A; with a flow rate of 1.0 mL/min. The column temperature was controlled at 30°C. The detection wavelength was 210 nm.

The fractions **I** and **III** of preparative HSCCC were purified by preparative HPLC with a Varian prepstar preparative HPLC apparatus and a Mightysil RP-18 GP column (20 mm×250 mm, 5 µm, Kanto Chemical Co, Inc., Japan). The mobile phase was acetonitrile-water (15:85, v/v) with a flow rate of 15 mL/min and the detection wavelength was 210 nm.

Structural Identification of Glycosides

The infrared radiation (IR) spectra, positive ion model HR-ESI-MS, and ^1H and ^{13}C NMR spectra of glycosides were measured. The chemical structures of glycosides were identified on the base of these spectra.

RESULTS AND DISCUSSION

Selection of Two-Phase Solvent System

The most important factor for a successful HSCCC separation is the selection of a suitable two-phase solvent system [30], which provides an ideal range of the partition coefficient (K) for the target compounds. Because pedunculoside (**V**) and syringin (**II**) were two main chemical constituents in Jiubiyang [7] and the peaks of other chemical constituents were situated between syringin (**II**) and pedunculoside (**V**) in the HPLC chromatogram of Jiubiyang extract as shown in Figure 4, it was possible to select the suitable two phase solvent system according the properties of pedunculoside of the lowest polarity and syringin of the highest polarity. On the basis of HBAW method [31], medium polar or polar solvent systems were tested. A series of experiments were performed to determine the optimal solvent two-phase system for the HSCCC separation and the results were summarized in Table 1.

As shown in Table 1, the K -values of glycosides were different. The K -value of glycoside **I** was very small and that of glycoside **V** was very large. Therefore, the head-tail elution mode was chosen to separate the polar compounds firstly and then the tail-head elution mode was chosen to obtain the lower polar compounds.

As shown in Table 1, the very small K -values of glycosides **I**, **II**, **III**, and **IV** meant that they would be maintained in the upper phase of *n*-heptane-*n*-butanol-acetonitrile-water (2:3.8:1.2:4, v/v/v/v) used as the stationary phase of HSCCC. The K -values of these glycosides increased in two-phase systems of *n*-heptane-*n*-butanol-acetonitrile-water (1:4.4:0.6:4, v/v/v/v) and *n*-butanol-water (1:1, v/v). The solvent system of *n*-butanol-water (1:1, v/v) system seemed to be suitable but the retention of the upper stationary phase would be unsatisfactory. Therefore, ethyl acetate was added into *n*-butanol-water system according to the proportion of Oka [32] and Ito [33] methods. As the decrease of the ethyl acetate proportion, the K -values were gradually become to more ideal. Finally, ethyl acetate-*n*-butanol-water (1:6:7, v/v/v) was chosen for HSCCC to separate the glycosides in Jiubiyang extract.

The chemical structures of glycosides were shown in Figure 1.

Optimization of Operational Parameters on Semi-Preparative HSCCC

Besides the two-phase solvent systems and their compositions, the flow rate of mobile phase, the revolution speed of separation column, and the separation temperature were also investigated. The retention of stationary phase with 35% was obtained with revolution speed of 850 rpm, flow rate of 1.0 mL/min, and column temperature at 25°C for TBE-300A model HSCCC separation with 260 mL of column volume. As shown in Figure 2, five glycosides were separated and obtained from 300 mg of Jiubiyang extract using the two-phase solvent

system composed of ethyl acetate-*n*-butanol-water (1:6:7, v/v/v). However, the amount of these glycosides was too small to be identified.

Optimization of Operational Parameters on Preparative HSCCC

After being further investigated, a retention of stationary phase with 25% was obtained using a solvent system composed of ethyl acetate-*n*-butanol-water (1:6:7, v/v/v) with revolution speed of 450 rpm, flow rate of 3.0 mL/min, and column temperature at 25°C for TBE-1000A model HSCCC instrument with 1000 mL of column volume. As shown in Figure 3, the HSCCC separation of Jiubiyang extract yielded the fractions contained syringaresinol 4',4''-*di-o-β-D*-glucopyranoside (**I**, 20.2 mg), syringin (**II**, 56.8 mg), sinapaldehyde glucoside (**III**, 26.2 mg), syringaresinol 4'-*o-β-D*-glucopyranoside (**IV**, 20.4 mg), and pedunculoside (**V**, 45.1 mg) from 1.0 g of Jiubiyang extract.

HPLC Analysis and Purification of HSCCC Fractions

Jiubiyang extract and the fractions of preparative HSCCC were analyzed by the HPLC method. As shown in Figure 4, the purities of glycosides in their fractions by an area normalization method at 210 nm detection were 55.4%, 98.1%, 68.6%, 95.3%, and 97.3%, respectively. The fractions contained glycosides **I** and **III** were further purified by preparative HPLC.

Structural Identification of Glycosides

The chemical structures of each glycosides were identified on bases of IR, HR-ESI-MS, and ¹H and ¹³C NMR studies.

Syringaresinol 4',4''-*di-o-β-D*-glucopyranoside (**I**) IR (KBr). $\nu_{\max}$: 3420, 2966, 2941, 1635, 1458, 1419, 1384, 1126, 1076 cm⁻¹. HR-ESI-MS [M+NH₄]⁺ *m/z*: 760.3037 (C₃₄H₄₆O₁₈NH₄, Calcd. for 760.3028). ¹H NMR (400 MHz, CD₃OD) δ ppm: 6.71 (4H, s, 2', 2'', 6', 6''), 4.94 (2H, d, *J*=6.4 Hz, Glc-i-1, Glc-ii-1), 4.80 (2H, d, *J*=3.2 Hz, 2, 6), 4.23 (2H, dd, *J*=9.2, 6.0 Hz, 4 α , 8 α), 3.90 (2H, dd, *J*=9.2, 3.2 Hz, 4 β , 8 β), 3.80 (12H, s, OCH₃×4), 3.73 (2H, dd, *J*=12.4, 2.4 Hz, Glc-i-6, Glc-ii-6), 3.64 (2H, dd, *J*=12.4, 5.2 Hz, Glc-i-6, Glc-ii-6), 3.49 (2H, m, Glc-i-2, Glc-ii-2), 3.48 (2H, m, Glc-i-3, Glc-ii-3), 3.42 (2H, m, Glc-i-4, Glc-ii-4), 3.26 (2H, m, Glc-i-5, Glc-ii-5), 3.18 (2H, m, 1, 5). ¹³C NMR (100 MHz, CD₃OD) δ ppm: 153.0 (3', 3'', 5', 5''), 137.3 (1', 1''), 133.9 (4', 4''), 104.0 (2', 2'', 6', 6''), 102.9 (Glc-i-1, Glc-ii-1), 85.2 (2, 6), 77.4 (Glc-i-5, Glc-ii-5), 76.4 (Glc-i-3, Glc-ii-3), 73.8 (Glc-i-2, Glc-ii-2), 71.5 (4, 8), 70.1 (Glc-i-4, Glc-ii-4), 60.6 (Glc-i-6, Glc-ii-6), 56.5 (3'-, 3''-, 5'-, 5''-, OCH₃×4), 53.5 (1, 5). The chemical structure was identified on the base of these spectra and references [34,35].

Syringin (**II**) IR (KBr) $\nu_{\max}$: 3564, 3390, 1589, 1508, 1419, 1132, 1093, 1028, 966, 617 cm⁻¹. HR-ESI-MS [M+NH₄]⁺ *m/z*: 390.1774 (C₁₇H₂₄O₉NH₄, Calcd. for 390.1764). ¹H NMR (400 MHz, CD₃OD) δ ppm: 6.76 (2H, s, 3, 5), 6.56 (1H, d, *J*=16.0 Hz, 7), 6.34 (1H, dt, *J*=16.0, 5.6 Hz, 8), 4.88 (1H, d, *J*=7.2 Hz, Glc-1'), 4.24 (2H, d, *J*=5.6 Hz, 9), 3.87 (6H, s, 2-, 6-, OCH₃×2), 3.80 (1H, dd, *J*=12.0, 2.0 Hz, Glc-6'), 3.69 (1H, dd, *J*=12.0, 5.2 Hz, Glc-6'), 3.51 (1H, m, Glc-2'), 3.45 (1H, m, Glc-3'), 3.43 (1H, m, Glc-4'), 3.22 (1H, m, Glc-5'). ¹³C NMR (100 MHz, CD₃OD) δ ppm: 152.9 (2, 6), 134.5 (1), 133.9 (4), 129.9 (8),

128.7 (7), 104.1 (3, 5), 103.9 (Glc-1'), 76.9 (Glc-3'), 76.4 (Glc-5'), 74.3 (Glc-2'), 69.9 (Glc-4'), 62.2 (9), 61.1 (Glc-6'), 55.7 (2-, 6-, OCH₃×2). The chemical structure was identified on the base of these spectra and references [8,12].

Sinapaldehyde glucoside (**III**) IR (KBr) $\nu_{\max}$: 3420, 1683, 1506, 1456, 1419, 1338, 1245, 1132, 1070, 669 cm⁻¹. HR-ESI-MS [M+H]⁺ m/z : 371.1282 (C₁₇H₂₂O₉H, Calcd. for 371.1298). ¹H NMR (400 MHz, D₂O) δ ppm: 9.49 (1H, d, $J=7.6$ Hz, 9), 7.61 (1H, d, $J=15.6$ Hz, 7), 7.01 (2H, s, 3, 5), 6.72 (1H, dd, $J=16.0, 8.0$ Hz, 8), 3.84 (6H, s, 2-, 6-, OCH₃×2), 5.06 (1H, d, $J=7.2$ Hz, Glc-1'), 3.76 (1H, dd, $J=12.4, 2.0$ Hz, Glc-6'), 3.65 (1H, dd, $J=12.4, 5.2$ Hz, Glc-6'), 3.52 (1H, m, Glc-2'), 3.48 (1H, m, Glc-3'), 3.42 (1H, m, Glc-4'), 3.31 (1H, m, Glc-5'). ¹³C NMR (100 MHz, D₂O) δ ppm: 194.5 (9), 153.4 (7), 152.9 (3, 5), 136.5 (4), 129.5 (1), 127.9 (8), 106.9 (2, 6), 103.1 (Glc-1'), 76.5 (Glc-3'), 75.8 (Glc-5'), 73.8 (Glc-2'), 69.4 (Glc-4'), 60.2 (Glc-6'). The chemical structure was identified on the base of these spectra and reference [12].

Syringaresinol 4'-*o*- β -D-glucopyranoside (**IV**) IR (KBr) $\nu_{\max}$: 3390, 1635, 1456, 1418, 1385, 1120, 1078 cm⁻¹. HR-ESI-MS [M+NH₄]⁺ m/z : 598.2493 (C₂₈H₃₆O₁₃NH₄, Calcd. for 598.2500). ¹H NMR (400 MHz, CD₃OD) δ ppm: 6.74 (2H, s, 2'', 6''), 6.67 (2H, s, 2', 6'), 4.86 (1H, m, Glc-1'), 4.78 (1H, d, $J=4.0$ Hz, 2), 4.74 (1H, d, $J=4.0$ Hz, 6), 4.30 (1H, m, 4 β), 4.27 (1H, m, 8 β), 3.93 (2H, dd, $J=2.8, 9.2$ Hz, 4 $\alpha, 8\alpha$), 3.88 (6H, s, 3'', 5'', OCH₃×2), 3.86 (6H, s, 3', 5', OCH₃×2), 3.79 (1H, dd, $J=11.6, 2.0$ Hz, Glc-6'), 3.68 (1H, dd, $J=11.6, 4.4$ Hz, Glc-6'), 3.49 (1H, m, Glc-2'), 3.44 (1H, m, Glc-3'), 3.42 (1H, m, Glc-4'), 3.23 (1H, m, Glc-5'), 3.15 (2H, m, 1, 5). ¹³C NMR (100 MHz, CD₃OD) δ ppm: 152.6 (3'', 5''), 147.6 (3', 5'), 137.3 (1''), 134.3 (4'), 133.8 (4''), 128.9 (1'), 104.2 (2'', 6''), 103.1 (2', 6'), 102.8 (Glc-1'), 86.8 (6), 81.5 (2), 77.0 (Glc-5'), 76.4 (Glc-3'), 74.2 (Glc-2'), 70.4 (Glc-4'), 70.0 (4), 69.1 (8), 61.0 (Glc-6'), 55.7 (3'', 5'', OCH₃×2), 55.4 (3', 5', OCH₃×2), 53.9 (5), 49.4 (1). The chemical structure was identified on the base of these spectra and references [34,36].

Pedunculoside (**V**) IR (KBr) $\nu_{\max}$: 3420, 2932, 1734, 1652, 1471, 1386, 1074, 1047, 650 cm⁻¹. HR ESI MS [M+Na]⁺ m/z : 673.3923 (C₃₆H₅₈O₁₀Na, Calcd. for 673.3928). ¹H NMR (400 MHz, C₅D₅N) δ ppm: 6.34 (1H, d, $J=7.6$ Hz, Glc-1'), 5.59 (1H, s, 12), 4.50 (1H, m, Glc-6'), 4.45 (1H, m, Glc-6'), 4.40 (1H, t, $J=8.6$ Hz, Glc-4'), 4.34 (1H, t, $J=8.8$ Hz, Glc-3'), 4.26 (1H, t, $J=8.4$ Hz, Glc-2'), 4.22 (1H, d, $J=6$ Hz, 3), 4.19 (1H, d, $J=10.0$ Hz, 23), 4.08 (1H, m, Glc-5'), 3.73 (1H, d, $J=10.0$ Hz, 23), 3.10 (1H, td, $J=13.2, 3.2$ Hz, 16), 2.96 (1H, s, 18), 2.50 (1H, td, $J=13.2, 3.2$ Hz, 15), 2.09 (2H, m, 11), 2.07 (1H, m, 1), 2.02 (3H, m, 16, 21 α , 21 β), 1.94 (1H, m, 9), 1.92 (2H, m, 2), 1.87 (1H, m, 1), 1.72 (1H, td, $J=11.2, 2.8$ Hz, 7), 1.67 (3H, s, 27), 1.65 (1H, m, 6), 1.59 (1H, m, 22), 1.55 (1H, m, 5), 1.46 (1H, m, 7), 1.42 (3H, s, 29; 1H, m, 6), 1.38 (1H, m, 20), 1.26 (3H, s, 26), 1.24 (1H, m, 15), 1.09 (3H, s, 24; 1H, m, 22), 1.08 (3H, d, $J=5.6$ Hz, 30), 1.06 (3H, s, 25). ¹³C NMR (100 MHz, C₅D₅N) δ ppm: 177.9 (28), 140.2 (13), 129.4 (12), 96.8 (Glc-1'), 80.2 (Glc-5'), 79.9 (Glc-3'), 75.0 (Glc-2'), 74.5 (3), 73.6 (19), 72.2 (Glc-4'), 69.0 (23), 63.3 (Glc-6'), 55.4 (18), 49.6 (5), 49.6 (17), 48.8 (9), 43.8 (4), 43.0 (14), 43.0 (20), 41.5 (8), 39.8 (22), 38.6 (1), 38.1 (10), 34.2 (7), 30.2 (15), 28.6 (2), 27.9 (29), 27.6 (21), 27.0 (16), 25.5 (27), 25.0 (11), 19.7 (6), 18.4 (26), 17.6 (29), 17.0 (25), 14.0 (24). The chemical structure was identified on the base of these spectra and references [8,37].

CONCLUSIONS

Five glycosides were successfully separated by semi-preparative (260 mL column volume) and preparative (1000 mL column volume) HSCCC with a two-phase solvent system composed of ethyl acetate-*n*-butanol-water (1:6:7, v/v/v) from 50% ethanol extract of the dried barks of *I. rotunda*. From 1.0 g of Jiubiyang extract, syringaresinol 4',4''-*di-o*- β -D-glucopyranoside (**I**, 20.2 mg), syringin (**II**, 56.8 mg), sinapaldehyde glucoside (**III**, 26.2 mg), syringaresinol 4'-*o*- β -D-glucopyranoside (**IV**, 20.4 mg), and pedunculoside (**V**, 45.1 mg) were obtained in 550 min by a single separation procedure of preparative HSCCC. Their chemical structures were identified by means of IR, MS, and ^1H and ^{13}C NMR studies.

The peak order of glycoside **I** was located before those of glycoside **II** and **III** in Figures 2 and 3, because glycoside **I** had the biggest polarity for its smallest partition coefficient (*K*) in Table 1. However, it was interesting that the peak order of glycoside **I** was located after those of glycosides **II** and **III** in Figure 4. The irreversible adsorption was taken place between glycoside **I** and the solid support in HPLC column.

This was the first report that glycosides were isolated from the plant of *I. rotunda* by HSCCC method and syringaresinol 4',4''-*di-o*- β -D-glucopyranoside (**I**) was found from this plant for the first time.

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FIGURE 1.

Chemical structures of syringaresinol 4',4''-di- β -D-glucopyranoside (**I**), syringin (**II**), sinapaldehyde glucoside (**III**), syringaresinol 4'- β -D-glucopyranoside (**IV**), and pedunculoside (**V**).

FIGURE 2.
Semi-preparative HSCCC chromatogram of Jiubiyang extract.

FIGURE 3.
Preparative HSCCC chromatogram of Jiubiyang extract.

FIGURE 4.
HPLC chromatogram of Jiubiyi extract and HSCCC fractions.

TABLE 1The *K*-values of glycosides in different two-phase solvent systems

Solvent system (v/v/v)	<i>K</i> -value				
	I	II	III	IV	V
<i>n</i> -Heptane- <i>n</i> -butanol-acetonitrile-water (2:3.8:1.2:4)	0.01	0.15	0.24	0.75	15.32
<i>n</i> -Heptane- <i>n</i> -butanol-acetonitrile-water (1:4.4:0.6:4)	0.01	0.33	0.53	2.71	71.53
<i>n</i> -Butanol-water (1:1)	0.13	0.76	1.03	10.24	165.0
Ethyl acetate- <i>n</i> -butanol-water (4:1:5)	0.06	0.12	0.25	0.63	196.3
Ethyl acetate- <i>n</i> -butanol-water (3:2:5)	0.01	0.33	3.72	7.88	220.2
Ethyl acetate- <i>n</i> -butanol-water (1:4:5)	0.10	0.54	1.43	6.69	66.50
Ethyl acetate- <i>n</i> -butanol-water (1:6:7)	0.09	0.66	1.63	10.35	96.48