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Journal of Chromatography B, 859 (2007) 119–124
JOURNAL OF CHROMATOGRAPHY B
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Purification and identification of antiviral components from *Laggera pterodonta* by high-speed counter-current chromatography

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Received 6 July 2007; accepted 11 September 2007
Available online 19 September 2007

Abstract

Although *Laggera pterodonta*, a folk medicine has been widely used for several centuries as an antiviral agent, there have been no studies to identify its antiviral components. A bioassay-guided phytochemical examination of *L. pterodonta* disclosed that its aqueous extract, which was made up of three dicaffeoylquinic acids showed significant inhibitory activity of virus replication. Then a simple and efficient preparative high-speed counter-current chromatography (HSCCC) method was successfully established by using ethyl acetate–*n*-butanol–water (3:2:5, v/v) as the two-phase solvent system to isolate and purify three bioactive dicaffeoylquinic acids, 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid. The flow rate was 1.5 ml/min and revolution speed was 800 rpm. The isolation was done in less than 6 h, and 34.6 mg of 3,5-*O*-dicaffeoylquinic acid, 29.4 mg of 3,4-*O*-dicaffeoylquinic acid and 27.1 mg of 4,5-*O*-dicaffeoylquinic acid was yielded from 600 mg of the crude sample in one-step separation with the purity of 98.3%, 96.7% and 96.2%, respectively, as determined by high-performance liquid chromatography (HPLC). The structures of these three bioactive dicaffeoylquinic acids were identified by ultraviolet (UV), electrospray ionization mass spectrometry (ESI-MS), proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR). In the antiviral experiment, three dicaffeoylquinic acids all showed significant inhibitory activity against herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2) and influenza viruses A (IVA) *in vitro* with high selectivity indexes. However, among the three compounds, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid were the more active than 3,4-*O*-dicaffeoylquinic acid against HSV-1, HSV-2 and IVA. This study demonstrated a direct link between the antiviral activity of *L. pterodonta* and the three dicaffeoylquinic acids.

Keywords: *Laggera pterodonta*; High-speed counter-current chromatography; 3,5-*O*-Dicaffeoylquinic acid; 3,4-*O*-Dicaffeoylquinic acid; 4,5-*O*-Dicaffeoylquinic acid; Antiviral activity

1. Introduction

Laggera pterodonta (DC.) Benth is widely distributed in the Southwest of China, especially in Yunnan province. It has been traditionally employed as folk medicine for the treatment of inflammation and bacterial infectious diseases [1]. Numerous of chemical constituents such as eudesmane derivatives, sesquiterpenoid glucosides, flavones, steroids, coumarins, triterpenes as well as phenolic acids have been isolated mainly from the ethanol extract of this plant [2–7]. It has been reported that only the aqueous extract from this plant exhibits antiviral activity. However, the chemical components with the antiviral activity have not been identified yet [8]. So the bioassay-guided purification of active components from *L. pterodonta* was warranted.

Prior to separation, the structures of the antiviral components were poorly known. Based on thin-layer chromatography (TLC), UV and HPLC analysis, it indicated that the aqueous extract of *L. pterodonta* was rich in phenolic compounds, and three dicaffeoylquinic acids were the main compounds [9]. Recent studies have showed that dicaffeoylquinic acids had potent antiviral activity [10–12]. So, further studies on pharmacological and clinical effects necessitate the development of an efficient preparative separation method of these three main compounds. Dicaffeoylquinic acids are naturally occurring phenolic compounds formed by the esterification of quinic acid with two caffeic acids, and the only difference is the position of substituent

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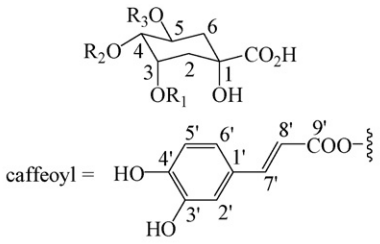
doi:10.1016/j.jchromb.2007.09.016



Compound	R_1	R_2	R_3
3,5-*O*-dicaffeoylquinic acid	caffeoyl	H	caffeoyl
3,4-*O*-dicaffeoylquinic acid	caffeoyl	caffeoyl	H
4,5-*O*-dicaffeoylquinic acid	H	caffeoyl	caffeoyl

Fig. 1. Chemical structures of 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid from *L. pterodonta*.

groups (Fig. 1). Because of similar molecular and instability for these three geometrical isomers, separation and purification of 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid by conventional methods such as column chromatography and HPLC is tedious and usually requires multiple chromatography steps. The overall yields of these methods were poor, because the hydroxyl groups in these compounds make them strongly absorbed onto the solid support during separation. From now, there are not many articles reported their separation at the same time and many samples were the mixture of these three compounds [13–16].

High-speed counter-current chromatography (HSCCC) is a separation method which has been developed to circumvent scaling-up difficulties. This technique is a continuous liquid–liquid partition chromatography based on partitioning of compounds between two immiscible liquid phases with no support matrix. The HSCCC applications were mostly performed using immiscible organic–aqueous two-phase solvent system, and the stationary phase of which is retained in the separation columns by gravity and centrifugal force field, which is continuously eluted by the counter-flowing mobile phase driven by external pumping [17]. Therefore it eliminates irreversible adsorptive loss of samples onto the solid support used in the conventional chromatographic column [18] and it can purify some compounds difficult to separate by other methods. Furthermore, it permits introduction of crude samples directly into the hollow column. Therefore, HSCCC has recently been investigated to effectively separate and purify various natural products [19–24]. HSCCC method is also considered as a suitable alternative for the separation of isomeric compounds [25,26].

The present paper describes an approach using bioassay-guided technique to separate and identify the antiviral components from *L. pterodonta*. An efficient preparative HSCCC was used to isolate and separate 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid with high purity from the enriched dicaffeoylquinic acids fraction from *L. pterodonta* at a single separation. Their structures were elucidated with ESI-MS, ^{1}H NMR and ^{13}C NMR. As far as we know, the three dicaffeoylquinic acids were obtained from *L. pterodonta* for the first time. Their antiviral activity against HSV-1, HSV-2 and IVA were assayed.

2. Experimental

2.1. Chemicals and reagents

D101 macroporous resin was purchased from the Chemical Plant of NanKai University (Tianjin, China), which was cross-linked polystyrene copolymer.

All organic solvents used for preparation of crude extract and HSCCC separation were of analytical grade (The Second Institute of Oceanography, Zhejiang, China). Methanol used for HPLC was of chromatographic grade (Merk, Darmstadt, Germany). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Bedford, MA, USA).

The whole herbs of *L. pterodonta* were collected from Qiubei Country, Yunnan province, China. The plants were identified by Prof. Zhongwen Lin. The voucher specimen (No. 9411122 CLD) was deposited in the State Key Laboratory of Photochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science, Kuming, Yunnan, China.

The 96-well tissue culture plates (Greiner Bio-One GmbH, Essen, Germany), multi-well plate reader (Bio-Tek Instruments, Inc., USA) and inverted microscope (Olympus optical Co., Tokyo, Japan) were used for antiviral analyses.

HSV-1, HSV-2 and IVA virus were all obtained from centers for Disease Control and Prevention, China.

2.2. Bioassay-guided fractionation and isolation of active ingredients

2.2.1. Preparation of H_2O extract

Fresh plants of *L. pterodonta* were dried at 50 °C for 3 days before grinding. About 5.0 kg of the powder was extracted by deionized water (15 l) at 100 °C for three times. All the filtrates were combined and concentrated to dryness under reduced pressure by rotary evaporation at 60 °C. The H_2O extract was then investigated for antiviral activity assessment.

2.2.2. Fractionation of H_2O extract

The H_2O extract was suspended in H_2O (1 l) and then partitioned with *n*-butanol (1 l) three times successively. Then the *n*-butanol solution was evaporated (500 ml, 50 °C) and then subsequently fractionated to isolate the pure compounds responsible for the antiviral activities, which was subjected on column chromatography (20.0 cm × 100 cm, contained 3.0 kg D101 macroporous resin) eluting with H_2O, followed by increasing concentrations of MeOH to give six main fractions: Frs. A–F (Fr. A: 0% MeOH aqueous solution; Fr. B: 20% MeOH aqueous solution; Fr. C: 40% MeOH aqueous solution; Fr. D: 60% MeOH aqueous solution; Fr. E: 80% MeOH aqueous solution; Fr. F: 95% MeOH aqueous solution). The fractionations were

all concentrated to dryness and stored in the refrigerator until required. The recovery of 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid in 40% MeOH aqueous solution were 65.3%, 73.8% and 71.9%, respectively, based on the original concentrations in the crude extracts analyzed by HPLC. The values for the precision and accuracy were better than 6% and 8.5% ($n = 4$).

2.3. HSCCC

2.3.1. Preparation of two-phase solvent system

The following three kinds of solvent system at different volume ratio were tested to select a suitable two-phase solvent system based on the partition coefficient (K) of dicaffeoylquinic acids: (1) chloroform–methanol–water, (2) *n*-butanol–methanol–water, (3) ethyl acetate–*n*-butanol–water. Each solvent mixture was thoroughly equilibrated in separating funnel at room temperature and the two phases separated shortly before use. The K value was expressed as the concentration of dicaffeoylquinic acids in the upper phase divided by that in the lower phase which was determined by HPLC.

2.3.2. HSCCC separation procedures

HSCCC experiments were performed using a coil planet centrifuge equipped with a multiplayer coil column that was designed and fabricated at the Zhejiang Gongshang University, China. The apparatus was equipped with one layer coil column made of convoluted polytetrafluoroethylene (PTFE) tubing with a 1.6 mm average i.d. and a 20 ml sample loop (total volume, 420 ml). The column revolves around its own axis at the angular velocity in the same direction. The revolution radius or the distance between the holder axis and central axis of the centrifuge was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79. $\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The HSCCC system was also equipped with a FMI pump (Zhejiang Instrument Factory, Hangzhou, China) and a variable wavelength PC300 detector operating at 326 nm and a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China).

In each separation, the multiplayer coil column was first entirely filled with the upper stationary phase. Then the lower mobile phase was pumped into the inlet of the column at the flow rate of 1.5 ml/min, while the apparatus was rotated at 800 rpm. After the mobile phase was eluted from the tail outlet and the two phases had established the hydrodynamic equilibrium throughout the column, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 326 nm and the peak fractions were collected. After three peaks were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase.

The sample solution was prepared by dissolving 600 mg of the most active fraction (Fr. C) in 20 ml of the lower phase of the solvent system for isolation and purification.

2.4. HPLC analysis and identification of HSCCC peak fractions

Analytical HPLC was performed on high-performance liquid chromatograph system with Waters Alliance 2695 separations module equipped with 2699 photodiode array detector and *Empower pro* data handling system (Waters Corporation, Milford, MA01757, USA). Samples were conducted on a Symmetry® C_{18} (100 mm × 3.9 mm i.d., 5 μm) column using a isocratic elution of 0.1% acetic acid and methanol (60:40, v/v) as mobile phase at a flow rate of 0.8 ml/min. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks.

MS data were measured on a Bruker Esquire 3000+ instrument (Faellanden, Switzerland). ^{1}H NMR and ^{13}C NMR experiments were performed on a VARIAN INOVA-400 (Varian Corporation, USA) NMR spectrometer using $CDOD_3$ as solvent.

2.5. Antiviral assay

Assay for antiviral activity was based on the inhibition of virus-induced cytopathic effect (CPE) in Vero cells and MDCK cells [27]. After the cells infected by virus for 2 h, media with compounds at different concentrations were added. CPE was observed under the microscope after incubation for 48 h. Acyclovir (ACV) and Ribavrin (RBV) were used as positive reference substance. CPE inhibition data were expressed as the 50% effective (viral CPE-inhibitory) concentration (IC_{50}), 50% cytotoxicity (cell-inhibitory) concentration (CC_{50}) and selectivity index (SI), determined as the CC_{50}/IC_{50}. All values are expressed as means of three independent experiments ± standard errors of mean (SEM).

3. Results and discussion

3.1. Bioassay-guided fractionation of the H_2O extracts

For the scientific evaluation of the claimed effects for *L. pterodonta* in Chinese folk medicine, H_2O extracts were prepared and submitted to biological tests for antiviral activity. As shown in Table 1, H_2O extracts possessed certain antiviral activity against HSV-1, HSV-2 and IVA. The H_2O extracts was partitioned with *n*-butanol to remove impurities such as saccharide,

Table 1
The partition coefficients (K) of the target components in different ratio of volume in ethyl acetate–*n*-butanol–water solvent system (component **1**, 3,5-*O*-dicaffeoylquinic acid; component **2**, 3,4-*O*-dicaffeoylquinic acid; component **3**, 4,5-*O*-dicaffeoylquinic acid)

Ethyl acetate–*n*-butanol–water	Component **1**	Component **2**	Component **3**
1:4:5	2.48	3.93	5.66
2:3:5	1.41	2.29	3.43
3:2:5	0.68	1.21	1.75
4:1:5	0.29	0.47	0.81

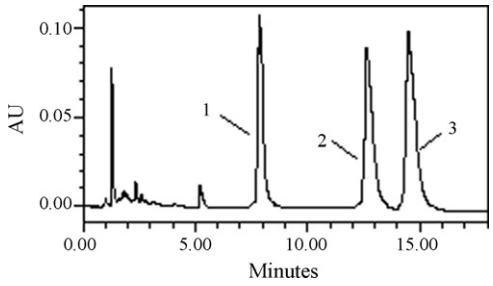


Fig. 2. HPLC chromatogram of enriched extract of *L. pterodonta*. HPLC conditions: column, reversed-phase Symmetry® C_{18} (150 mm × 3.9 mm i.d., 5 μm); mobile phase, 0.1% acetic acid-methanol (60:40, v/v); flow rate, 0.8 ml/min; UV wavelength, 326 nm; column temperature, 30 °C. Peaks 1, 2 and 3 correspond to 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid, respectively.

etc. Then, a bioassay-guided fractionation was performed on the *n*-butanol solution in order to isolate the bioactive compounds contributing to the antiviral activity. The *n*-butanol extract was fractionated over D101 column chromatography to afford six main fractions. Each fraction was then submitted to biological tests for antiviral activity. Since Fr. C (40% MeOH aqueous solution) showed potent activiral activity, further isolation studies were performed using this fraction (Table 2). Successive HSCCC was applicated to isolate the active compounds.

3.2. Screening of HPLC method

HPLC is a popular method to analyze crude extract and HSCCC fractions. The enriched extract after D101 macroporous resin column chromatography contained three main isomeric compounds. Optimization was performed on the enriched extract including composition of mobile phase, flow rate and column temperature. After experiments, 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid could reach baseline separation under the following conditions: the mobile phase composed of 0.1% acetic acid and MeOH (60:40, v/v) was eluted isocratically at a flow rate of 0.8 ml/min, the column temperature and detection wavelength were set at 30 °C and 326 nm, respectively. HPLC chromatogram of the enriched extract is shown in Fig. 2.

3.3. Selection of two-phase solvent system and other conditions of HSCCC

Appropriate solvent system plays an important role in separation by HSCCC. Partition coefficient (*K*) is the most important parameter in solvent system selection, which should be close to 1 to get an efficient separation and a suitable run time. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the *K* value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [28].

In this experiment, three kinds of solvent system at different volume ratios were selected to separate these three similar compounds. Preliminary HSCCC experiments were carried out

Table 2
In vitro cytotoxic and antiviral activities of 40% enriched extract, 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid from *L. pterodonta* against a panel of human pathogenic viruses

Sample	Anti-HSV-1			Anti-HSV-2			Anti-IVA		
	CC_{50}[a]	IC_{50}[b]	SI[c]	CC_{50}[a]	IC_{50}[b]	SI[c]	CC_{50}[a]	IC_{50}[b]	SI[c]
H_2O extract	>2000	204.1 ± 18.6[f]	>9.80	>2000	197.2 ± 20.9	>10.14	>2000	594.5 ± 52.4	>3.36
40% enriched extract	>1000	88.64 ± 9.52	>11.28	>1000	90.05 ± 11.34	>11.11	>1000	258.13 ± 34.51	>3.87
3,5-*O*-Dicaffeoylquinic acid	>2000	79.5 ± 12.6	>25.16	>2000	125.6 ± 11.3	>15.92	>2000	241.4 ± 16.8	>8.29
3,4-*O*-Dicaffeoylquinic acid	>2000	170.4 ± 14.5	>11.74	>2000	229.1 ± 29.6	>8.73	>2000	623.0 ± 54.9	>3.21
4,5-*O*-Dicaffeoylquinic acid	>2000	93.0 ± 7.7	>21.51	>2000	113.6 ± 16.8	>17.61	>2000	202.2 ± 18.3	>9.89
Acyclovir[d]	>1000	0.23 ± 0.05	>4347	>1000	0.17 ± 0.04	>5882	–	–	–
Ribavirin[e]	–	–	–	–	–	–	>400	5.0 ± 0.37	>80.0

[a] CC_{50}: 50% cytotoxic concentration (μmol/ml), except μg/ml for 40% enriched extract.
[b] IC_{50}: concentration required to inhibit viral cytopathic effect by 50% (μmol/ml), except μg/ml for 40% enriched extract.
[c] SI: selectivity index (=CC_{50}/IC_{50}).
[d] Acyclovir which is clinically used as anti-HSV drug was applied as positive control in the antiviral assays.
[e] Ribavirin which is clinically used as anti-IVA drug was applied as positive control in the antiviral assays.
[f] Data are represented as the means ± SEM of three independent experiments.

with the medium polarity, such as chloroform–methanol–water in different volume ratio, however the *K* values were much bigger than 1, and the three target compounds could not be separated. Relatively, *n*-butanol is a good solvent for polar compounds. Thus, various solvent systems based on *n*-butanol and water were conducted partition coefficient tests. Although *K* values of the solvent system composed of *n*-butanol–methanol–water were appropriate, it led to emulsification. In the subsequent studies, this system was replaced with ethyl acetate–*n*-butanol–water. The *K* values of three target compounds are given in Table 1. After comparison of different ratios, ethyl acetate–*n*-butanol–water (3:2:5, v/v) gave the best separation.

Other factors such as the revolution speed of the separation column and the flow rate of the mobile phase were also investigated. Different flow rate (1.0, 1.5, 2.0 and 2.5 ml/min) of the mobile phase and different revolution speed (500, 600, 700 and 800 rpm) of the selected system were examined in the present paper, which can vary the retention of the stationary phase. The retention of the stationary phase is one of the most important parameters in HSCCC. Successful separation in HSCCC largely depends on the amount of stationary phase retained in the column. In general, the higher the retention of the stationary phase, the better the peak resolution. It was clear that high flow rate was unfavorable to the retention of the stationary phase, and the slow flow speed can produce a good separation, but more time and more mobile phase will be needed, and the chromatogram peak

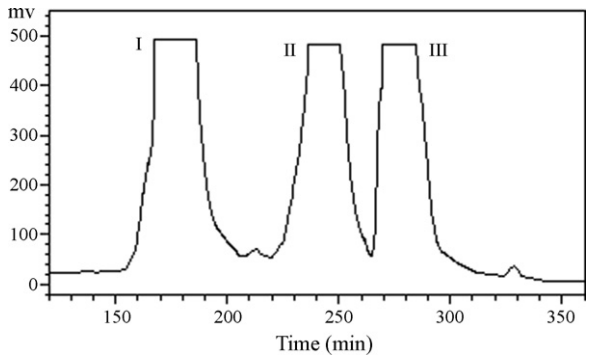


Fig. 3. Preparative HSCCC separation of enriched extract from *L. pterodonta* after cleaning up by D101 macroporus resin. Experimental conditions: revolution speed: 800 rpm; solvent system: ethyl acetate–*n*-butanol–water (3:2:5, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.5 ml/min; retention of the stationary phase: 65%; detection wavelength: 326 nm. Fractions I, II and III correspond to 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid, respectively.

was extended. High rotary speed can increase the retention of the stationary phase. The result showed that when the flow rate was 1.5 ml/min, resolution speed was 800 rpm, retention percentage of the stationary phase could reach 65% and good separation results could be obtained.

Under the optimum conditions, three fractions (I–III) were obtained in one-step elution less than 6 h (HSCCC chro-

Table 3
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of 3,5-*O*-dicaffeoylquinic acid (component **1**), 3,4-*O*-dicaffeoylquinic acid (component **2**) and 4,5-*O*-dicaffeoylquinic acid (component **3**) in CD_3OD (δ in ppm, *J* in Hz)

Position	^{1}H NMR			^{13}C NMR[a]		
	1	**2**	**3**	**1**	**2**	**3**
1				74.9	75.9	76.2
2	2.16 (2H, m)	2.03 (2H, m)	2.26 (2H, m)	37.8	39.6	38.5
3	5.36 (1H, m)	5.68 (1H, br s)	4.35 (1H, br s)	72.1	70.1	69.8
4	3.94 (1H, d, 4.4)	5.16 (1H, br s)	5.09 (1H, d, 7.2)	70.7	75.3	76.1
5	5.39 (1H, m)	4.27 (1H, br s)	5.74 (1H, br s)	72.6	67.0	69.2
6	2.29 (1H, br d, 13.2)	2.16 (2H, m)	2.26 (1H, m)	36.1	37.7	39.8
	2.16 (1H, m)		2.10 (1H, m)			
7				177.8	176.8	176.8
Caffeoyl						
1′				127.9	127.8	127.6
				127.8	127.7	127.5
2′	7.04 (2H, br s)	7.08 (1H, br s)	6.99 (1H, br s)	115.2	115.2	115.2
		7.07 (1H, br s)	6.96 (1H, br s)	115.1	115.2	115.1
3′				146.8	146.8	146.7
				146.8	146.8	146.7
4′				149.6	149.6	149.6
				149.5	149.6	149.6
5′	6.75 (2H, d, 8.4)	6.78 (2H, d, 8.4)	6.71 (2H, m)	116.5	116.5	116.4
				116.5	116.5	116.4
6′	6.94 (2H, br d, 8.4)	6.95 (2H, br d, 8.4)	6.88 (1H, d, 8.0)	123.1	123.2	123.2
			6.85 (1H, d, 8.0)	123.0	123.2	123.2
7′	7.59 (1H, d, 16.0)	7.61 (1H, d, 16.0)	7.56 (1H, d, 5.6)	147.0	147.3	147.7
	7.55 (1H, d, 16.0)	7.57 (1H, d, 16.0)	7.48 (1H, d, 15.6)	146.8	147.3	147.5
8′	6.33 (1H, d, 16.0)	6.32 (1H, d, 16.0)	6.25 (1H, d, 15.6)	115.6	115.2	114.7
	6.24 (1H, d, 16.0)	6.30 (1H, d, 16.0)	6.15 (1H, d, 15.6)	115.1	115.0	114.7
9′				168.9	168.5	168.6
				168.3	168.5	168.3

[a] Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC.

matogram is shown in Fig. 3), which is 34.6 mg of fraction I (collected during 160–210 min), 29.4 mg of fraction II (collected during 225–260 min) and 27.1 mg of fraction III (collected during 270–300 min). The purity of these compounds was 98.3%, 96.7% and 96.2%, respectively, determined by HPLC peak area percentage.

3.4. Antiviral activity

Antiviral activity of the main compounds was again assayed in the same *in vitro* system. As shown in Table 2, the main constituents of the active fraction exhibited antiviral activity at a certain extent. For the three chemical entities, the concentrations of 50% of inhibitory rates against HSV-1 and HSV-2 ranged from 79.5 to 170.4 μmol/ml and 113.6 to 229.1 μmol/ml, respectively. The IC_{50} values on IVA inhibition ranged from 202.2 to 623.0 μmol/ml. Furthermore, the relatively high SI values suggested the safety of medical application. This result supports the claimed effects of *L. pterodonta* being applied as popular herbal medicine by local people in Yunnan province, China. Considered the anti-HIV (human immunodeficiency virus) and anti-RSV (respiratory syncytial virus) activity reported for dicaffeoylquinic acids [11,29], dicaffeoylquinic acids approved the broad-spectrum antiviral activity and *L. pterodonta* is worthy of further study and development as new antiviral medicine.

3.5. Structural elucidation

The structural identification of peak fractions were all performed with UV, ESI-MS, ^{1}H NMR and ^{13}C NMR spectra. Spectroscopic data were determined by photodiode-array detection in the corresponding HPLC solvents.

Peak 1: UV λ_{max} (MeOH) nm: 220, 248, 300 sh, 329; ESI-MS *m/z*: 515 $[M\text{-}H]^-$; ^{1}H NMR and ^{13}C NMR data: Table 3. Comparing the data with the literature [30], the obtained product was identified as 3,5-*O*-dicaffeoylquinic acid.

Peak 2: UV λ_{max} (MeOH) nm: 220, 247, 300 sh, 330; ESI-MS *m/z*: 515 $[M\text{-}H]^-$; ^{1}H NMR and ^{13}C NMR data: Table 3. Their UV, ESI-MS, ^{1}H NMR and ^{13}C NMR data are in agreement with 3,4-*O*-dicaffeoylquinic acid in the literature [29].

Peak 3: UV λ_{max} (MeOH) nm: 218, 247, 300 sh, 326; ESI-MS *m/z*: 515 $[M\text{-}H]^-$; ^{1}H NMR and ^{13}C NMR data: Table 3, which matched with the reported NMR data for 4,5-*O*-dicaffeoylquinic acid [29].

4. Conclusions

Using bioassay-guided fractionation technique, the present study directly linked the antiviral activity of *L. pterodonta* to three dicaffeoylquic acids: 3,5-*O*-dicaffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid. Then, an efficient HSCCC method was developed for the isolation and purification of three dicaffeoylquic acids with a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (3:2:5, v/v) for the first time. The purity of the fractions was over 96% in a one-step separation, which indicated that HSCCC is a powerful technique to isolate and purify isomeric compounds from natural plants. Furthermore, with the method described here, the three dicaffeoylquic acids can be isolated on a large scale which may then be used for further bioactivity studies.

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