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Analytical Methods

Isolation and purification of phenylethanoid glycosides from *Cistanche deserticola* by high-speed counter-current chromatography

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Abstract

Five phenylethanoid glycosides (PhGs), echinacoside, cistanoside A, acteoside, isoacteoside and 2′-acetylacteoside, were isolated and purified from *Cistanche deserticola* for the first time by high-speed counter-current chromatography (HSCCC) using two biphasic systems, one consisting of ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v) and another of ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1, v/v/v/v). A total of 28.5 mg of echinacoside, 18.4 mg of cistanoside A, 14.6 mg of acteoside, 30.1 mg of isoacteoside and 25.2 mg of 2′-acetylacteoside were purified from 1412 mg of the *n*-butanol extract of *C. deserticola*, each at over 92.5% purity as determined by HPLC. The structures were identified by their retention time, UV, LC–ESI-MS in the negative ion mode, and confirmed by NMR experiments. The characteristic LC–ESI-MS″ fragmentation pattern of the five compounds is discussed, and found to be a very specific and useful tool for the structural identification of PhGs from this important medicinal plant.

Keywords: *Cistanche deserticola*; Phenylethanoid; Echinacoside; Cistanoside A; Acteoside; Isoacteoside; 2′-Acetylacteoside; HSCCC; LC–ESI-MS; NMR

1. Introduction

Cistanche deserticola Y.C. Ma is a well known Chinese herbal medicine and has been widely used in traditional preparations similar to today's functional food ingredients or food supplements (Wong, Li, Cheng, & Chen, 2006). The major bioactive constituents in *C. deserticola* are phenylethanoid glycosides (PhGs), including echinacoside, acteoside, isoacteoside, 2′-acetylacteoside, and cistanoside A (Kobayashi, Karasawa, & Miyase, 1984; Kobayashi et al., 1987). PhGs are widely distributed in the plant kingdom, and have been extensively studied for their various biological functions such as hepatoprotective (Xiong et al., 1998), anti-inflammatory, antinociceptive activity (Schapoval et al., 1998) and antioxidant activities (Cheng, Wei, Guo, Ni, & Liu, 2005; He, Lau, Xu, Li, & But, 2000; Li, Wang, & Wang, 1997; Li, Wang, Zheng, Liu, & Jia, 1993; Wang, Jiang, Wu, & Wang, 2001; Xiong, Kadota, Tani, & Namba, 1996), improving sexual function (Xie & Wu, 1993; Zong, He, Wu, & Chen, 1996), and sedative effect (Lu, 1998).

Owing to the above significant bioactivities, large quantities of pure compounds are urgently needed as reference standards and for various *in vitro* and *in vivo* studies related to the use of traditional Chinese medicines. Effective methods for the isolation, purification and structural characterization of PhGs, therefore become necessary. However, such work usually requires the use of multiple chromatographic steps for sample clean up and isolation (Gross, Lahloub, Anklin, Schulten, & Sticher, 1988; Nishimura, Sasaki, Inagaki, Chin, & Mitsuhashi, 1991; Ravn, Nishibe, Sasahara, & Li, 1990; Shoyama, Matsumoto, & Nishioka, 1987), which usually results in low recovery rates due to

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irreversible adsorptions of PhGs onto the solid support during separation (Lei et al., 2001). In contrast, high-speed counter-current chromatography (HSCCC) has become an effective alternative to the conventional chromatographic techniques for the separation of some PhGs from plant extracts (Lei et al., 2001; Li et al., 2005). Lei et al. successfully separated acteoside and 2′-acetylacteoside from *Cistanches salsa* (C.A. Mey.) G. Beck by using HSCCC (Lei et al., 2001). The authors of this paper have previously reported the separation of acteoside and isoacteoside from *Plantago psyllium* L. by HSCCC (Li et al., 2005). However, no report has been published on the separation and purification of multiple PhGs from *C. deserticola* using HSCCC. Due to the lack of standards, LC–MS methods have been developed and used as a powerful analytical tool for rapid characterization and identification of some PhGs in plant extract (Li et al., 2005; Wang et al., 2000).

In this paper, we report a HSCCC method developed for the preparation of echinacoside, cistanoside A, acteoside, isoacteoside and 2′-acetylacteoside from *C. deserticola*. Characterization and analysis of the five PhGs separated were accomplished by use of LC coupled with in-line ESI mass spectrometry and NMR experiments. The retention time, molecular weight and the characteristic fragment ions of the five PhGs are presented and discussed in this paper. The structures of the five PhGs identified in this investigation are shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

Acteoside was purchased from Sigma–Aldrich (Oakville, ON), echinacoside was purchased from ChromaDex (Santa Ana, CA). Isoacteoside was isolated from *P. psyllium* L. (Li et al., 2005). *C. deserticola* was purchased from Beijing TongRenTang Medicinal Store (China). All solvents were of HPLC grade and purchased from Caledon Laboratories Ltd. (Georgetown, ON).

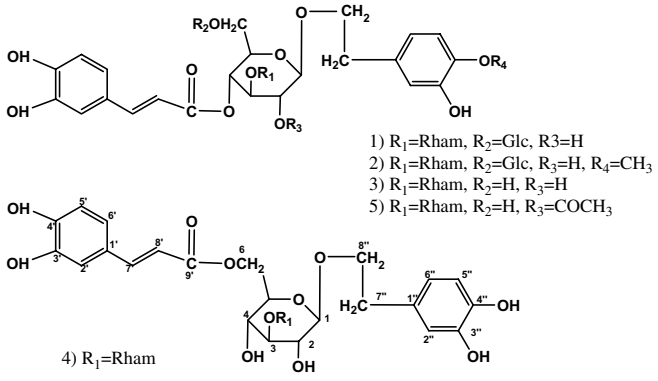


Fig. 1. Structures of phenylethanoid glycosides in plant extract of *C. deserticola*. (1) Echinacoside; (2) cistanoside A; (3) acteoside; (4) isoacteoside; (5) 2′-acetylacteoside.

2.2. Sample preparation

C. deserticola (20 g) was extracted five times at room temperature for 12 h each with 100 mL of 80% aqueous ethanol. Each time the extraction mixture was filtered through a Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England). Combined filtrate was concentrated to 100 mL *in vacuo* at < 40 °C. The resulting aqueous solution was defatted twice, each with 100 mL of hexane and then extracted successively for five times, each with 100 mL *n*-butanol. The *n*-butanol layers were combined and concentrated to dryness *in vacuo* at < 40 °C, which yielded 2.2 g of *n*-butanol extract. The extract was stored at -20 °C before HSCCC separation.

2.3. HSCCC separation procedure

The preparative HSCCC was carried out in a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, Maryland USA). This apparatus had three preparative coils, connected in series (total volume, 325 mL). The revolution speed of the apparatus could be regulated between 0 and 2000 rpm. The HSCCC system was equipped with an HPLC pump (Pharma-Tech Research, Baltimore, Maryland, USA), a Model 450 UV detector (Alltech, USA), a Model L 120 E flat-bed recorder (Linseis Inc., Princeton Jct, USA), a fraction collector (Advantec MFS Inc., USA) and a sample injection valve with a 10-mL sample loop.

A mixture of ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v) was shaken vigorously in a separatory funnel and let stand and separate at room temperature. The two phases were used in the HSCCC after they reached equilibrium. The entire coiled column was first filled with the upper layer, which serves as the stationary phase. The lower layer (mobile phase) was then pumped into the head-end of the column at a flow-rate of 1.5 mL/min. The rotation speed was set at 1050 rpm. A sample (ca. 230 mg each time) dissolved in 8 mL of the mixture of ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v) was loaded into the injection valve after the system reached hydrodynamic equilibrium. This biphasic solvent system was selected based on the partition coefficient (K), which was 0.87, 1.11 and 1.32 for acteoside, isoacteoside and 2′-acetylacteoside, respectively. The K value was the ratio of the concentrations in the top and bottom layers of the same compound as determined by HPLC (Fig. 2). The effluent from the outlet of the column was continuously monitored by a UV detector at 254 nm and collected into test tubes with a fraction collector set at 4 min for each tube. Fractions from the HSCCC that had the same single peak as determined by HPLC were combined, and freeze-dried (Fig. 3). Those containing a mixture of compounds 3 and 4 were pooled, concentrated and re-injected to the same system for further purification. Tubes of fraction 1 (Fig. 3A) were pooled, concentrated and re-injected to a new system containing ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1, v/v/v/v) for further

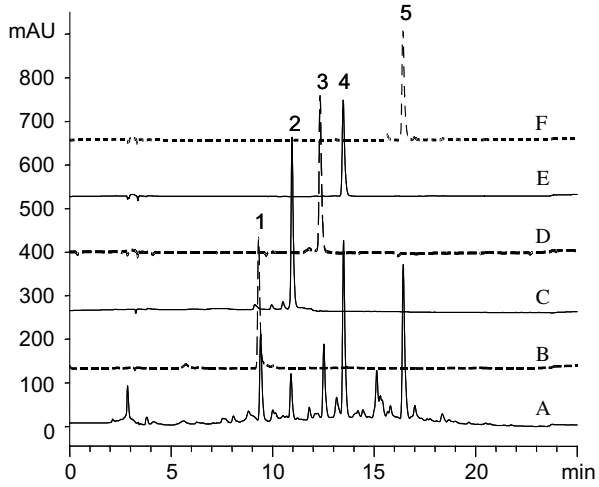


Fig. 2. HPLC profiles of the *n*-butanol extract of *C. deserticola* (A) and purified compounds 1–5 (B–F). Experimental conditions: column, Phenomenex C_{18}-ODS column (250 × 4.6 mm, 5 μm) and a C_{18} guard column; mobile phase, acetonitrile (A) and 2% acetic acid (B); gradient program, 90% B to 60% B 0–20 min, 60% B to 0% B 20–22 min, and 0% B to 90% B 22–25 min; sample injection volume, 10 μL; flow-rate, 1.0 mL/min; detection, 320 nm.

separation. This system provided a *K* value of 0.52 and 0.92 for compounds 1 and 2 (Fig. 3C), respectively. All fractions of the same pure compound were combined, and freeze-dried. The purified compounds were stored at −20 °C before HPLC–ESI-MS and NMR analyses.

2.4. LC conditions

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD) was used for the analysis of PhGs in the *n*-butanol extract of *C. deserticola* and fractions collected from the HSCCC separation. The separation was carried out in a Phenomenex ODS-C_{18} column (250 × 4.6 mm, 5 μm) with a C_{18} guard column. The binary mobile phase consisted of acetonitrile (solvent A) and water containing 2% acetic acid (solvent B). All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 1.0 mL/min for a total run time of 25 min. The system was run with a gradient program: 0–20 min: 90% B to 60% B; 20–22 min: 60% B to 0% B; and 22–25 min, 0% B to 90% B. The sample injection volume was 10 μL. Peaks of interest were monitored at 320 nm by a DAD detector.

2.5. LC–ESI-MS experiments

LC–MS experiments were carried out using a Finnigan LCQ DECA ion trap mass spectrometer (ThermoFnnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The samples were analyzed in the same chromatographic condition. A negative mode was used for data collection. The sheath gas and auxiliary flow-rates were set at 96 and 7 (arbitrary unit), respectively. The capillary voltage was set at −29 V and its temperature was controlled at 350 °C. The entrance lens voltage was fixed at 40 V and the multipole RF amplitude was set at 540 V. The ESI needle voltage was controlled at 4.5 kV. The tube lens offset was 16 V, the multipole lens 1 offset was 8.20 V and the multipole lens 2 offset was 10.5 V. The electron multiplier voltage was set at −980 V for ion detection.

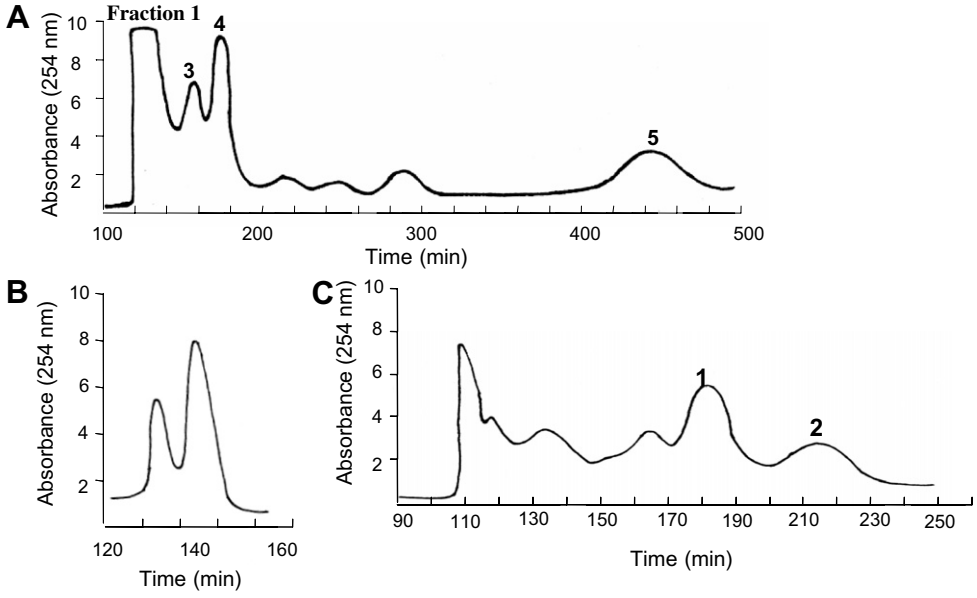


Fig. 3. HSCCC chromatograms of the *n*-butanol extract of *C. deserticola*. (A) *n*-butanol extract; Experimental conditions: coil volume: 325 mL; rotation speed: 1050pm; flow-rate: 1.5 mL/min; sample injection volume: 10 mL; sample injected: 250 mg extract dissolved in 8 mL of mixture of ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v). (B) Combined fractions containing both compounds 3 and 4 using the same solvent system as A. (C) Fraction 1 containing both compounds 1 and 2 using a biphasic solvent system of ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1, v/v/v/v).

2.6. NMR for Identification

NMR spectra were recorded on a Bruker Avance-600 spectrometer (Bruker BioSpin Ltd., Milton, Canada). Only compounds 2 and 5 (no standards available) were subjected to NMR experiments. Samples were dissolved in CD_3OD.

3. Results and discussion

3.1. HSCCC separation

The *n*-butanol extract of *C. deserticola* and the fractions corresponding to each peak isolated by HSCCC were analyzed by HPLC, and the results are given in Fig. 2. Five major compounds (peaks 1–5) were separated and detected with retention times at 9.2 min, 10.7 min, 12.3 min, 13.3 min and 16.2 min, respectively.

A successful HSCCC separation depends largely on a suitable two-phase solvent system that provides an ideal partition coefficient (*K*) around 1 for the desired compound. Such a biphasic system should also yield a reasonably short settling time (Chen, Games, & Jones, 2003; Foucault & Chevolot, 1998; Oka, Oka, & Ito, 1991). In our experiment, we selected four series of solvent systems according to the solubility of the target compounds in *C. deserticola*. HPLC was used to measure the concentration in each phase, from which the *K* values of the target compounds were calculated. Two systems, ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5, v/v/v/v) and ethyl acetate–water (1:1, v/v), have been previously used in HSCCC to separate acteoside and 2′-acetylacteoside from *C. salsa*, and acteoside and isoacteoside from *P. Psyllium*, respectively (Lei et al., 2001; Li et al., 2005). Although the first system had a relatively short settling time, it had poor performance in separating the PhGs of *C. deserticola*, due to the low *K* values for compounds 1 and 2, and high *K* values for compounds 3–5. The *K* values were very low for compounds 1–4 in the second system, but very high for compound 5 (Table 1). A modified system containing ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v), gave an ideal *K* value for compounds 3–5 at 0.87, 1.11 and 1.32, respectively, and resulted in a good separation of these three compounds (Fig. 3A and B). This system, however, produced too small a *K* value for compounds 1 and 2, causing the two compounds to co-elute near the solvent front (fraction 1 in Fig. 3A). A further modification to the system (ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1, v/v/v/v) elevated the *K* values for compound 1 and 2 to 0.52 and 0.92, respectively, and led to complete separation (Fig. 3C).

Fig. 3A shows the HSCCC separation of a sample containing 230 mg of the *n*-butanol extract of *C. deserticola* using ethyl acetate–ethanol–water (5:0.5:4.5, v/v/v). Fractions that were confirmed by HPLC to contain only compounds 3, 4 or 5 were combined separately, and those containing compounds 3 and 4 were pooled, freeze-dried and re-subjected to the HSCCC for further separation (Fig. 3B). The two-step HSCCC separation described above yielded a total of 14.6 mg, 30.1 mg and 25.2 mg of compounds 3–5 from 1412 mg *n*-butanol extract. Fig. 3C shows the HSCCC separation of a sample containing compounds 1 and 2 (fraction 1 in the first separation) using ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1, v/v/v/v). A total of 28.5 and 18.4 mg of compounds 1 and 2 were obtained. The chromatographic purities of the freeze-dried compounds 1–5 were over 92.5%, which were directly used for LC–ESI-MS and NMR analyses.

3.2. Structural identification by LC–ESI-MS and NMR

Tentative identification of compounds 1, 3 and 4 were achieved by congruent retention times and UV spectral data with those of the authentic echinacoside, acteoside and isoacteoside (Fig. 2). Compounds 2 and 5 were unknown, however, the UV spectra of all five compounds were highly similar, indicating similar structural features.

To further investigate the structures of these five compounds, LC–ESI-MS″ experiments were attempted and the results are shown in Fig. 4 and Table 2. Compounds related to the peaks (1–5) in Fig. 2 exhibited intense deprotonated molecular ions $[M-H]^-$ at *m*/*z* 785, 799, 623, 623 and 665, respectively, in the negative mode. Dimeric $[2M-H]^-$ ions were also observed for peaks 1–4 in Fig. 2. These confirmed the molecular weights of peaks 1–5 to be 786, 800, 624, 624, and 666, respectively. The LC–MS″ data (Table 2) provided highly useful structural information for the five PhGs, such as the neutral loss of

Table 1
The *K* (partition coefficient) values of compounds 1–5 in different solvent systems[a]

Solvent system	1	2	3	4	5
Ethyl acetate–*n*-butanol–ethanol–water (4:0.6:0.6:5)	0.17	0.20	1.98	2.44	7.57
Ethyl acetate–water (1:1)	0.22	0.21	0.26	0.25	1.35
Ethyl acetate–ethanol–water (5:0.5:4.5)	0.04	0.06	0.87	1.11	1.32
Ethyl acetate–*n*-butanol–ethanol–water (0.5:0.5:0.1:1)	0.52	0.92			

[a] Experimental procedure: approximately 1 mg of each sample was weighed in a 10 mL test tube into which 1 mL of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. An aliquot of 100 μL of each layer was taken out and evaporated separately to dryness *in vacuo* at <40 °C. The residue was dissolved in 10 μL methanol and analyzed by HPLC for determining the partition coefficient (*K*) of compounds 1–5. The *K* value was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase.

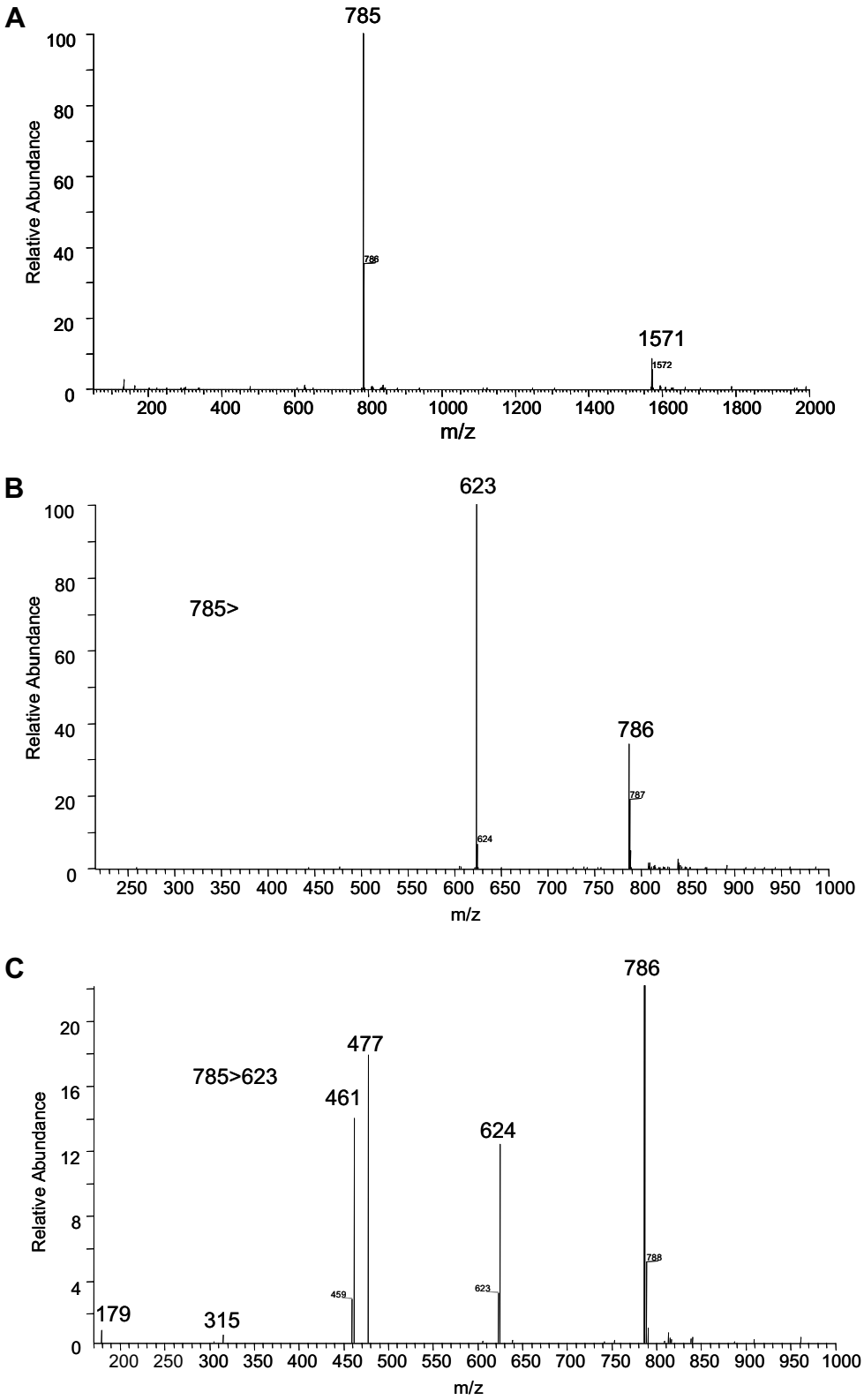
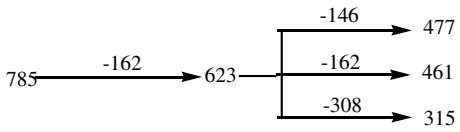
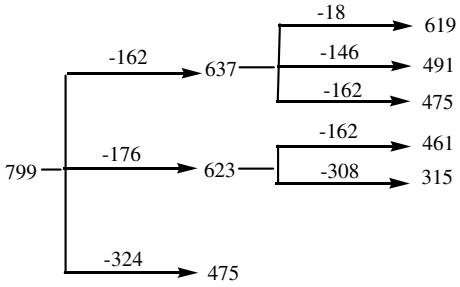
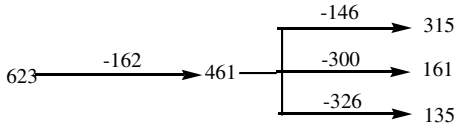
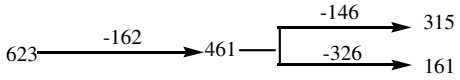
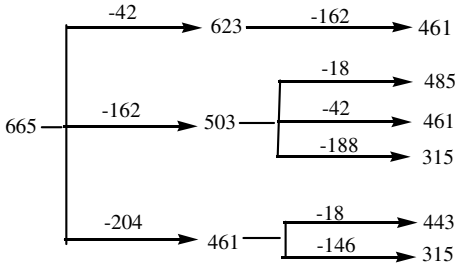


Fig. 4. (A) LC–ESI-MS spectrum of *m*/*z* 785 of compound 1; (B) LC–ESI-MS^2 spectrum of the parent ion *m*/*z* 785 of compound 1; (C) LC–ESI-MS^3 spectrum of the parent ion *m*/*z* 623 of compound 1.

the caffeoyl moiety (162), the glucose moiety (162), the rhamnose moiety (146), a CH_2 radical (14) and the $COCH_2$ group (42).

The LC–ESI-MS of peak 1 is shown in Fig. 4A. The deprotonated molecular ion $[M-H]^-$ at *m*/*z* 785 with a high abundance and a dimeric deprotonated molecular

Table 2
LC–ESI-MS^n data of peaks 1–5 shown in Fig. 2

Peak #, identification	t_R (min)[a]	$[M-H]^-$ (m/z)[b]	$[2M-H]^-$ (m/z)[c]	MS^2 (m/z)[d]	MS^3 (m/z)[d]
1, Echinacoside	9.37	785	1571		
2, Cistanoside A	10.87	799	1599		
3, Acteoside	12.49	623	1247		
4, Isoacteoside	13.46	623	1247		
5, 2′-acetyacteoside	16.39	665			

[a] Retention time.
[b] Deprotonated molecular ion.
[c] Dimeric deprotonated molecular ion.
[d] MS″ fragment ions.

ion $[2M-H]^-$ at m/z 1571 were observed in the negative mode, suggestive of a molecular weight of 786, which was the same as that of echinacoside. Further investigation in the LC–MS^2 experiment of the m/z 785 ion yielded one main daughter ion at m/z 623 (Fig. 4B) produced directly from m/z 785 by loss of a caffeoyl moiety or a hexose moiety as $[M-162-H]^-$. The LC–MS^3 spectrum of m/z 623 exhibited two major ions at m/z 477 and 461, and two minor ions at m/z 315 and 179 (Fig. 3C, Table 2). The mass differences between m/z 623 and the fragment ions m/z 477 and 461 were 146 and 162, respectively, corresponding to the loss of a rhamnose unit and a glucose moiety or a caffeoyl moiety $[M-162-H]^-$. The m/z 623 ion also lost a caffeoyl moiety and a rhamnose moiety to produce the m/z 315 ion. The ion at m/z 179 was produced from the cleavage of the caffeoyl moiety, with the negative charge remaining on the part of the caffeoyl moiety. LC–ESI-MS″ experiment on the authentic echinacoside showed the same fragmentation pattern. Peak 1 was therefore confirmed to be echinacoside.

For peak 2, the LC–ESI-MS showed m/z 799 as the deprotonated molecular ion $[M-H]^-$ and m/z 1599 as its dimeric ion, suggestive of a molecular mass of 800. During MS^2 experiments, the m/z 799 ion formed three daughter

ions at *m/z* 637, 623 and 475 (Table 1). The ion at *m/z* 637 was produced directly from the parent ion of *m/z* 799 again due to the neutral loss of the caffeoyl $[M-162-H]^-$ or a glucose moiety $[M-162-H]^-$. The ion at *m/z* 623 resulted from loss of a CH_2 radical. The ion at *m/z* 475 was formed from the neutral loss of both the caffeoyl moiety $[M-162-H]^-$ and the glucose moiety from the parent ion. The MS^3 experiment on *m/z* 637 produced three ions at *m/z* 619, 491 and 475, corresponding to the losses of one water, a rhamnose unit and a glucose moiety, respectively. The *m/z* 623 daughter ion produced *m/z* 461 and 315 in the MS^3 study, which followed the same fragmentation pathways as echinacoside as discussed above. The LC–ESI-MS^n data supported a tentative identification for peak 2 as cistanoside A.

LC–ESI-MS experiments were also conducted for peaks 3 and 4 (t_R 12.49 and 13.46 min in Fig. 2). Both peaks showed the same $[M-H]^-$ ion at *m/z* 623 and dimer at *m/z* 1247 in the negative mode (Table 1), indicating they are possibly isomers with the same molecular weight of 624, the same as acteoside and isoacteoside. The MS^2 spectra of the $[M-H]^-$ ions also showed one same daughter ion of *m/z* 461, which indicated the loss of the caffeoyl moiety from the parent ion *m/z* 623 (Table 1). Similar MS^3 spectra were obtained for the two compounds. For peak 3, the MS^3 spectrum of the ion at *m/z* 461 formed three ions at *m/z* 315, 161 and 135. The *m/z* 315 is formed after losing a rhamnose as discussed earlier. The ion *m/z* 161 was produced from the cleavage of the caffeoyl moiety, followed by a further loss of one water; the charge remained on the part of the caffeoyl moiety. The ion at *m/z* 135 $[aglycone-18-H]^-$ arose from the cleavage of the glycosidic bond at C1 position with an additional loss of one water, leaving the charge to be on the part of the aglycone moiety. The MS^3 spectrum of the peak 4 followed the same fragmentation pathway as peak 3 except for the missing ion at *m/z* 135. The molecular ion and the fragmentation patterns of these two compounds are consistent with the literature data on acteoside and isoacteoside (Wang et al., 2000), although an additional ion *m/z* 153 was found and

Table 3
Proton NMR data of cistanoside A and 2′-acetylacteoside

Moiety	Carbon number	Cistanoside A	2′-Acetylacteoside
		δ_H (*J*)	δ_H (*J*)
Hydroxytyrosol	1		
	2	6.865 (d, 1.8)	6.613 (d, 1.8)
	3		
	4		
	5	6.695 (d, 8.4)	6.629 (d, 7.8)
	6	6.687 (dd, 8.4, 1.8)	6.490 (dd, 7.8, 1.8)
	7	3.689 (dd, 9.6, 7.2) 4.015 (dd, 9.6, 7.2)	3.605 (dd, 9.6, 6.6) 3.983 (dd, 9.6, 6.6)
	8	2.834 (m)	2.680 (m)
Caffeic acid	1		
	2	7.060 (d, 1.8)	7.042 (d, 1.8)
	3		
	4		
	5	6.770 (d, 7.8)	6.769 (d, 8.4)
	6	6.592 (dd, 7.8, 1.8)	6.916 (dd, 8.4, 1.8)
	7	7.592 (d, 15.6)	7.566 (d, 15.6)
	8	6.273 (d, 15.6)	6.298 (d, 15.6)
Glucose (inner)	1	4.394 (d, 7.8)	4.467 (d, 7.8)
	2	3.387 (dd, 9.0, 7.8)	4.788 (dd, 9.6)
	3	3.806 (t, 9.0)	3.664 (t, 9.6)
	4	4.061 (dd, 9.6, 2.4)	3.485 (dd 9.6)
	5	3.753 (m)	3.571 (m)
	6	3.941 (dd, 11.4, 2.4), 3.634 (dd, 11.4, 2.4),	4.493 (dd, 12.0, 1.8), 4.354 (dd, 12.0)
Rhamnose	1	5.176 (d, 1.8)	4.790 (d, 1.8)
	2	3.618 (m)	3.664 (dd, 3.0, 1.8)
	3	3.580 (dd, 7.8, 3.0)	3.613 (dd, 9.6, 3.0)
	4	3.280 (dd, 7.8)	3.366 (dd, 9.6)
	5	3.534 (q, 6.6)	3.942 (q, 6.0)
	6	1.074 (d, 6.0)	1.224 (d, 6.0)
Glucose (outer)	1	4.282 (d, 7.8, 2.4)	
	2	3.181 (dd, 9.0, 7.8)	
	3	3.321 (t, 9.0)	
	4	3.532 (dd, 9.0)	
	5	3.205 (m)	
	6	3.899 (dd, 12.0), 3.662 (dd, 12.0)	
$-CH_3$			1.962 (s)
$-OCH_3$		3.840 (s)	

δ_H – chemical shift in ppm; splitting patterns and *J* values (Hz) are in parentheses. See Fig. 1 for carbon numbers of each moiety.

assigned as [aglycone−H]$^-$ by Wang et al. (Wang et al., 2000). This ion may have been unstable and lost a water to give m/z 135 in our experiment. Based on the MS data and the congruent retention times of peaks 3 and 4 with the standards, they are therefore identified as acteoside and isoacteoside, respectively.

The LC–ESI-MS data of peak 5 are shown in Table 2. A deprotonated molecular ion [M−H]$^-$ (m/z 665) was the only ion found in the negative mode, implying a molecular mass of 666. Three daughter ions were observed at m/z 623, 503 and 461 in the MS^2 experiment (Table 2). The daughter ions at m/z 623 and 503 were formed directly from the parent ion by loss of a $COCH_2$ group and a caffeoyl moiety, respectively. The ion at m/z 461 came from loss of both the caffeoyl and the $COCH_2$ moiety [M−162−42−H]$^-$ from the parent ion. In the MS^3 experiment, m/z 623 produced m/z 461, and m/z 503 yielded three ions at m/z 485, 461 and 315. The MS^3 spectrum of the daughter ion m/z 461 gave two ions at 443 and 315. By comparing the LC–MS″ fragmentation pattern of peak 5 with other compounds reported in this study and with other reported (Li et al., 2005; Wang et al., 2000), we concluded that peak 5 was structurally highly related to acteoside with the only difference being the $COCH_3$ unit on the R_3 position. A tentative identity was therefore given to peak 5 as 2′-acetylacteoside (Fig. 1).

The structures of the two tentatively identified compounds, peak 2 (as cistanoside A) and peak 5 (as 2′-acetylacteoside) were confirmed of their structures by ^{1}H NMR. The chemical shifts and coupling constants of all protons in compounds 2 and 5, as shown in Table 3., matched with the reported NMR data for cistanoside A and 2′-acetylacteoside, respectively (Kobayashi et al., 1984, 1987). 2D NMR experiments (long-range COSY, ROESY, and CH correlation) were also conducted in the present study and they further confirmed the identification (data not shown).

4. Conclusions

In the present paper, HSCCC was successfully used for the isolation and purification of echinacoside, cistanoside A, acteoside, isoacteoside and 2′-acetylacteoside from the *n*-butanol extract of *C. deserticola*. It is therefore a proven means for semi-preparative separation of bioactives. Meanwhile, the structures of the five PhGs in *C. deserticola* have been investigated by means of LC–ESI-MS″; some characteristic features of PhGs were found, which allowed us to determine the functional groups in the structures. The LC–ESI-MS″ method is therefore a powerful tool for rapid identification of phenylethanoids and their glycosides in *C. deserticola* extracts, especially when substantiated by NMR data.

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