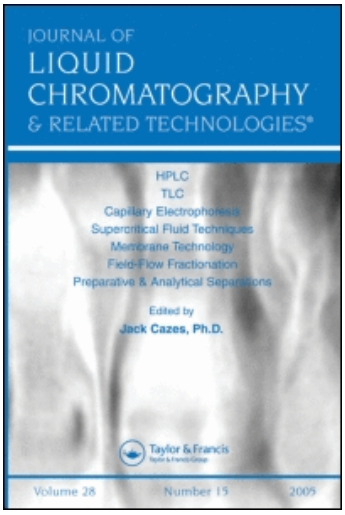

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Preparation of Ursane Triterpenoids from *Centella asiatica* Using High Speed Countercurrent Chromatography with Step-Gradient Elution

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ABSTRACT

Pentacyclic triterpene aglycones and glycosides of the ursane type were successfully separated from 600 mg of a polar extract from *Centella asiatica* (Apiaceae) by high speed countercurrent chromatography (HSCCC), applying a mobile phase gradient with a step-wise increase of elution strength. The lower phase of the biphasic liquid system composed

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of *n*-hexane–*n*-butanol–0.05 M NaOH (5 : 1 : 6, v/v/v) was used as the stationary phase, the upper phase was used as the initial mobile phase. The mobile phase was changed systematically into 1 : 1, 1 : 2 and 1 : 4 consisting of *n*-hexane–*n*-butanol saturated by 0.05 M NaOH. The separation mainly yielded five fractions with asiatic acid (18 mg), madecassic acid (13 mg), asiaticoside (140 mg), and madecassoside (75 mg). The chemical structures of the four compounds were confirmed by means of electrospray ionization ion trap multiple mass spectrometry (ESI–MS–MS) and NMR analysis.

Key Words: *Centella asiatica*; Triterpenoids; Ursane; Step-gradient high speed countercurrent chromatography; Preparative isolation; ESI–MS–MS; NMR.

INTRODUCTION

The prostrate, perennial herb, *Centella asiatica* L., *syn. Hydrocotyle asiatica* L. (Apiaceae) has been used widely for ethnomedicinal purposes in treatment of leprosy, open wounds, and also mental retardance throughout China and India. Several phytochemical studies resulted in the isolation of pentacyclic triterpenoids of the ursane type.[1–12] Asiatic acid, madecassic acid (*syn.* 6β-hydroxy asiatic acid), and their trisaccharides asiaticoside and madecassoside (*syn.* asiaticoside-A) are the principal bioactive compounds, which were inter alia found to accelerate wound healing processes,[5,6] and to improve venous microangiopathy.[7–11] Moreover, positive effects on Alzheimer's disease[12] and radioprotection were reported.[13] Formation of collagen under influence of triterpenoids from *C. asiatica*, and promotion of the metabolic pathway in fibroblasts is still under discussion.[14] Preparation of pure standards of triterpenoids is significant for further scientific studies of these compounds. An important scope of our analysis was to search for an efficient way to isolate pure ursane triterpenoid agylcones and glycosides. Reference material can be used for quantification of active ingredients in fresh and concentrated *C. asiatica* extracts. In recent days, "modern" phytomedicines need to be standardized in order to prevent any risk of overdosing.

The present paper describes the preparative separation of four major ursane triterpenoids from a crude extract of *C. asiatica* by high speed countercurrent chromatography (HSCCC)[15,16] with a novel solvent system applying a three step-gradient with time-depending increase of the eluting strength of the mobile phase. HSCCC is an all-liquid chromatographic system[17] working without solid support, and separation is based on fast partitioning effects of the analytes between two immiscible liquid phases.[17] Superior

separation abilities and excellent recovery rates of this technique are the reasons for the increase in use of HSCCC in natural product isolation.[15,16]

EXPERIMENTAL

Reagents

Organic solvents including *n*-butanol, acetonitrile, methanol, and *n*-hexane used for HSCCC, were of analytical grade, water was nanopure® quality.

Preparation of Sample Solution

Ethanolic extract of *C. asiatica* (50 g) was dissolved in 600 mL water. Then the solution was diluted with 600 mL ethyl acetate, and extracted with 600 mL of *n*-butanol. The *n*-butanol phase was evaporated, in vacuum, at 50°C, and lyophilized to yield 7.8 g of a crude sample for the HSCCC separation.

HSCCC Separation

The HSCCC separation was performed with a multilayer coil countercurrent chromatograph, manufactured by P. C. Inc. (Potomac, MD), equipped with a single 380-mL coil column made of a polytetrafluoroethylene tubing (2.6 mm, I.D.). The mobile phase was delivered by a Biotronik HPLC pump BT 3020 (Jasco, Gross-Umstadt, Germany). In the HSCCC experiment, a biphasic liquid system was applied consisting of *n*-hexane–*n*-butanol–0.05 M NaOH (5 : 1 : 6, v/v/v), where the lower phase was used as the stationary phase. The initially used mobile phase was the upper phase of this solvent system. Changes in mobile phase composition and flow rates throughout the separation are listed in Table 1.

The multilayer coil column was entirely filled with the lower aquatic phase as the stationary phase. Then the apparatus was started to rotate at 800 rpm for equilibration of the system. For a single run, 0.6 g of crude sample was dissolved in 10 mL of mobile phase. Injection of the sample to the HSCCC system was done by a teflon sample loop, followed by immediate pumping of the upper phase, at a flow rate of 4.0 mL/min. After delivering 200 mL of the upper phase, the chromatographic elution was proceeded with three step-gradients composed of 1 : 1, 1 : 2, and 1 : 4 of *n*-hexane–*n*-butanol saturated by 0.05 M NaOH. The effluent stream was collected

Table 1. Mobile phase composition of the step-gradient.

Elution time (min)	Mobile phase composition	Flow rate (mL/min)
0–50	Upper phase of solvent system *n*-hexane–*n*-butanol– 0.05 M NaOH (5 : 1 : 6, v/v/v)	4.0
50–120	*n*-Hexane–*n*-butanol (1 : 1)[a]	3.0
120–220	*n*-Hexane–*n*-butanol (1 : 2)[a]	2.0
220–520	*n*-Hexane–*n*-butanol (1 : 4)[a]	1.5

[a]Saturated with 0.05 M NaOH.

with a Superfrac fraction collector (Pharmacia, Uppsala, Sweden) with 15 mL volume for each tube, and the HSCCC fractions were analyzed by TLC.

TLC Analysis of Fractions

Evaluation of the HSCCC fractions was done by thin-layer chromatography, on normal phase silica gel 60 F_{254} plates Merck (Darmstadt, Germany), developed in ethyl acetate–methanol–water (8 : 2 : 1, v/v/v). Visualization of the triterpenoids was done by spraying with 3% sulfuric acid in ethanol, and subsequent heating to 110°C for 5 min on a hot plate.

HPLC Analysis

The HPLC system for analyzing the triterpenoids was composed of an Agilent 1100 quaternary pump with degasser unit, a thermostated column compartment, a variable wavelength detector, a manual injector, 1100 ChemStation software, and a Zorbax-ODS column (5 mm, 4.6 mm I.D., 325 cm). For the HPLC analysis, initially 20% acetonitrile and 80% water was used, and the gradient increased to 55% acetonitrile in 30 min. The flow rate of the mobile phase was 1.4 mL/min, and the detection wavelength was set to 220 nm.

Electrospray-Ionization–MS–MS (Syringe Pump)

All electrospray ionization (electrospray ionization ion trap multiple mass spectrometry, ESI–MS–MS) experiments were performed on a Bruker Esquire LC–MS ion trap multiple mass spectrometer (Bremen, Germany) in positive and negative ionization mode, analyzing ions up to m/z 2200.

During the ESI–MS and MS–MS fragmentation studies the purified samples were introduced via a syringe pump at a flow rate of 240 μL/min. Drying gas was nitrogen at 7.0 L/min (330°C), and nebulizer pressure was set to 5 psi. The ESI–MS parameters (negative mode): capillary +4500 V, end plate +4000 V, cap exit −90 V, cap exit offset −60 V, skim 1 −30 V, skim 2 −10 V; the ESI–MS parameters (positive mode): capillary −4500 V, end plate −4000 V, cap exit +90 V, cap exit offset +60 V, skim 1 +30 V, skim 2 +10 V. The MS–MS experiments afforded fragmentation amplitude values between 0.8 and 1.2.

NMR Analysis

^{13}C- and DEPT 135-NMR spectra were recorded in MeOH-d_4 on a Bruker AMX 300 (Karlsruhe, Germany) with 300 MHz for ^{1}H- and 75.5 MHz for ^{13}C-measurements, respectively.

RESULTS AND DISCUSSION

Step-Gradient HSCCC Separation

Countercurrent chromatography equipment is frequently operated in isocratic mode with static mobile phase composition. Similar to gradient HPLC, HSCCC can also be employed to a mobile phase gradient, i.e., with linear increase of the elution strength of the mobile phase. Severe loss of stationary phase from the HSCCC coil-system might be a limiting factor of this technique.[16] Certain favorable solvent systems are stable to drastic changes of mobile phase composition during separation. Therefore, a gradient elution mode applied to HSCCC appears to be an elegant way to simplify separation of crude plant extracts, which usually consist of numerous natural products with considerable differences in polarity.[16]

In the present study of an ethanolic extract of *C. asiatica*, a step-gradient instead of a linear gradient was applied (cf. Fig. 1 and Table 1). The lower aqueous phase of the solvent system *n*-hexane–*n*-butanol–0.05 M NaOH (5 : 1 : 6, v/v/v) was delivered as the stationary phase by a single pump, the upper organic phase was used as the initial mobile phase. At this stage, 75% retention of the stationary phase was achieved. During the separation, mobile phase was changed, step-wise, into *n*-hexane–*n*-butanol (1 : 1), (1 : 2), and finally to *n*-hexane–*n*-butanol (1 : 4). The retention of stationary phase during the gradient steps decreased to 67%, to 65%, and to 64% at the end of the separation. All of the three gradient phases applied in the

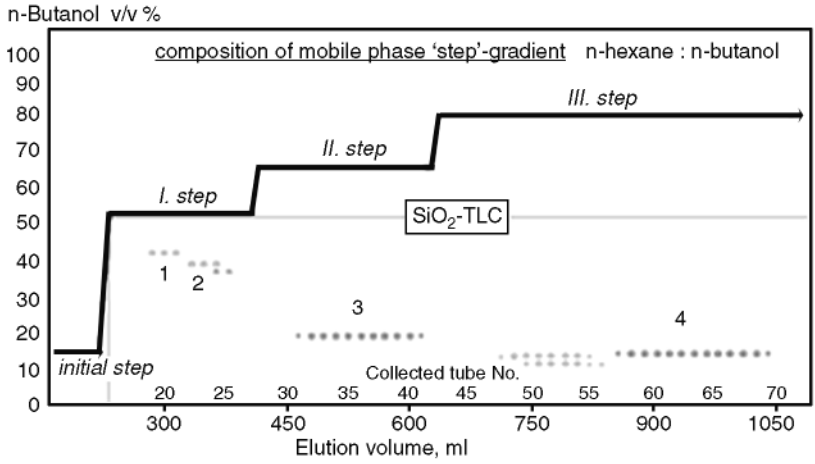


Figure 1. TLC-monitoring of the three step-gradient of the HSCCC separation of the crude extract of *C. asiatica.* The composition of the biphasic liquid system *n*-hexane–*n*-butanol–0.05 M NaOH is graphically correlated to the elution of the four pentacyclic triterpene acids asiatic acid (**1**), madecassic acid (**2**), and triterpene glycosides, asiaticoside (**3**), and madecassoside (**4**). Gradient conditions with 0.05 M NaOH saturation: Initial step: *n*-hexane–*n*-butanol 5 : 1; I step: *n*-hexane–*n*–butanol 1 : 1; II step: *n*-hexane–*n*-butanol 1 : 2; and III step: *n*-hexane–*n*-butanol 1 : 4.

separation were saturated before use by 0.05 M NaOH. To limit the stationary-phase wash-off, the flow rates were significantly reduced, with only 1.5 mL/min for the last gradient step (Table 1).

Figure 1 reflects the TLC analysis of all fractions of *C. asiatica* in relation to the approximate composition of the mobile phase throughout the three step-gradient HSCCC separation. The chromatography mainly yielded five sections, monitored by TLC analysis in which one section (720–855 mL) was a mixture of two spots. Four sections were combined, respectively, to yield asiatic acid (**1**) (elution volume: 270–315 mL), madecassic acid (**2**) (330–360 mL), asiaticoside (**3**) (415–615 mL), and madecassoside (**4**) (870–1065 mL). Substances **1** and **2** were decolorized with charcoal, evaporated under reduced pressure, and lyophilized to yield 18 and 13 mg, respectively. For the glycosides, 140 mg of component **3** and 75 mg of component **4** were recovered. Analytical HPLC of the four substances resulted in single-peaks corresponding to the peaks I, II, III, and IV given in the chromatogram of the crude extract (Fig. 2). The four colorless, powdery substances were directly used for structure elucidation by ^{1}H-, ^{13}C-, DEPT 135-NMR and electrospray–MS–MS experiments.

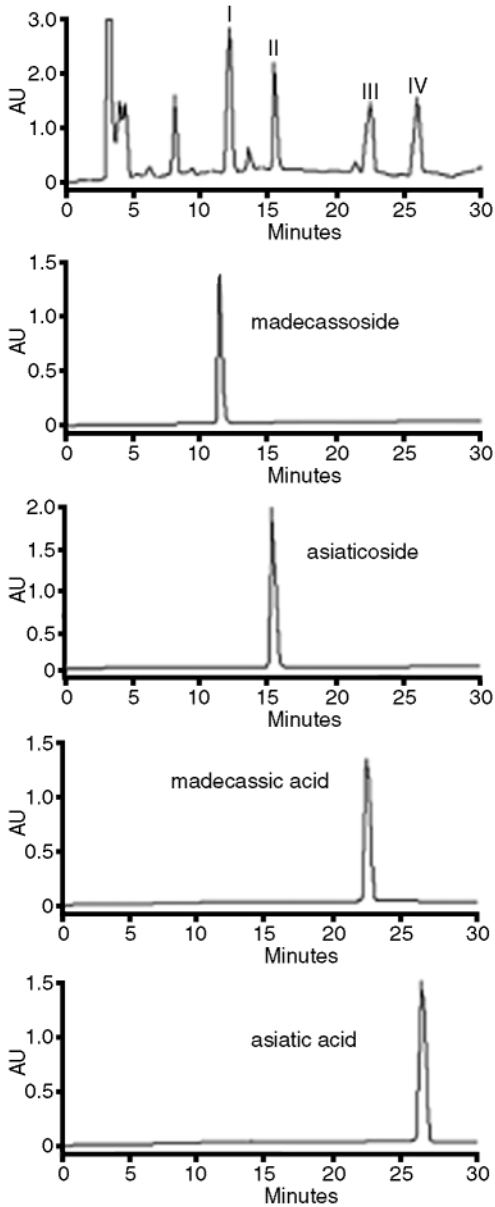


Figure 2. HPLC analysis of the crude sample from *C. asiatica* and the purified components (**1**–**4**) from the step-gradient HSCCC separation: peak I: madecassoside (**4**), peak II: asiaticoside (**3**), peak III: madecassic acid (**2**), and peak IV: asiatic acid (**1**).

At the initial point of the HSCCC separation (Fig. 1), the mobile phase composition is relatively lipohilic, and suitable to focus all components of the extract of *C. asiatica* in the stationary phase of the coil-system. With increasing polarity of gradient step I, effected by higher *n*-butanol content (50% in mobile phase), elution of asiatic acid (**1**) and madecassic acid (**2**) takes place. These pentacyclic triterpene acids only differ slightly in their polarity, due to an additional hydroxylation of substance **2** at position C-6 of the ursane backbone.

With increased eluting strength of gradient step II, with approximately 60% of *n*-butanol in the mobile phase composition, the more polar asiaticoside (**3**), the trisaccharide (glc–glc–rha) of asiatic acid, was eluted as a pure substance.

Madecassoside (**4**) containing the same trisaccharide moiety as **3** was eluted much later from the HSCCC coil-system at gradient step III, employing almost 80% *n*-butanol in the mobile phase.

For performing a step-gradient HSCCC separation, no additional pump is needed, and by cautious control of the given time-constants for change of the gradient steps, reproducibility of the separation is very good.

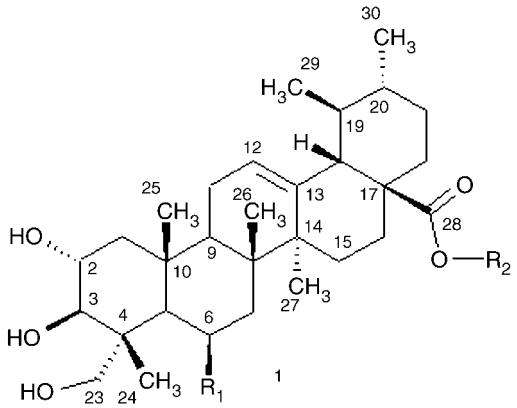
The step-gradient variation of HSCCC is a versatile and economic technique to separate complex crude extracts of a wide polarity range of components. In our investigation, we were able to separate the lipophilic pentacyclic triterpene acids **1** and **2** from their very polar trisaccharide glycosides **3** and **4** in high purity and only one single chromatographic run.

In general, the most valuable attributes of static- or gradient HSCCC separation techniques compared with preparative HPLC are high sample loading capacity, minimum of sample clean-up, no irreversible adsorption effects of analytes to solid phase column material, complete sample recovery, and the much lower mobile phase usage.[17]

Confirmation of Chemical Structures

Several phytochemical investigations already conducted on *C. asiatica* resulted in the isolation of various saponins having urs-12-ene and olean-12-ene type triterpene aglycone moieties.[18] As a slight structural difference, ursane triterpenes have one methyl group attached to C-18 instead of two methyl groups bound to C-20 in oleananes. For the presented HSCCC separation, we only isolated triterpene structures belonging to the urs-12-ene series shown in Fig. 3.

All structures of components **1**–**4** were elucidated by means of modern spectroscopic techniques, including ^{1}H-, ^{13}C-, DEPT 135-NMR and ESI–MS–MS. ^{13}C-NMR data are listed in Tables 2 and 3 and are in excellent accordance to previously published reference data.[3,18–20]



1 : R_1 = H; R_2 = H

2 : R_1 = OH; R_2 = H

3 : R_1 = H; R_2 = trisaccharide unit

4 : R_1 = OH; R_2 = trisaccharide unit

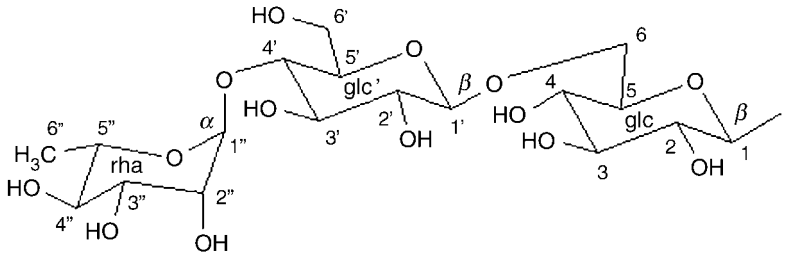


Figure 3. Chemical structures of the triterpenoid components from *C. asiatica* isolated by the step-gradient HSCCC technique: asiatic acid (**1**), madecassic acid (**2**), asiaticoside (**3**), and madecassoside (**4**).

^{13}C-NMR spectral data identified urs-12-ene aglycone moieties for all structures **1**–**4**, and this was also achieved by inspection of δ-values of the olefinic carbons revealing that C-12 is deshielded by 2 ppm, otherwise C-13 is shielded by 5 ppm in comparison with the corresponding carbons of olean-12-enes.[18] The chemical shift differences of the double bond carbons are caused by the spatial proximity of the 19β-(equatorial)-methyl group in the urs-12-ene structure. A further spectroscopical characteristic is position C-18 in the urs-12-enes (**1**–**4**), showing a strong downfield shift of δ

Table 2. ^{13}C- and DEPT 135-NMR spectral data of the four components asiatic acid (**1**), madecassic acid (**2**), asiaticoside (**3**), and madecassoside (**4**) isolated by the gradient HSCCC separation (δ-values in ppm; **1** and **2** in pyridine-d_5; **3** and **4** measured in methanol-d_4).

Carbon	^{13}C (**1**)	^{13}C (**2**)	^{13}C (**3**)	DEPT	^{13}C (**4**)	DEPT
1	47.9	48.2	48.3[a]	CH_2	50.4	CH_2
2	69.1	68.9	69.7	CH_2	69.7	CH
3	78.9	78.7	78.8	CH	78.3	CH
4	42.9	43.1	44.1	C	44.8	C
5	48.8	48.7	48.4[a]	CH	48[a]	CH
6	18.9	67.6	19.1	CH_2	68.5	CH
7	33.6	39.3	33.7	CH_2	41.3	CH_2
8	40.4	39.5	41.0	C	38.5	C
9	48.5	48.9	49.3[a]	CH	48[a]	CH
10	38.6	37.9	39.0	C	40.2	C
11	23.9	25.0	24.5	CH_2	24.6	CH_2
12	125.9	126.0	127.0	CH	127.4	CH
13	138.7	138.6	139.4	C	138.7	C
14	43.8	44.2	43.5	C	43.9	C
15	29.0	28.7	29.3	CH_2	29.3	CH_2
16	25.5	26.0	25.3	CH_2	25.4	CH_2
17	48.1	48.0	49.5[a]	C	48[a]	CH
18	53.3	53.3	54.1	CH	54.2	CH
19	38.7	38.0	40.3	CH	40.2	CH
20	39.6	39.0	40.4	CH	40.5	CH
21	31.3	31.0	31.7	CH_2	31.8	CH_2
22	37.6	37.5	37.6	CH_2	37.6	CH_2
23	67.4	66.5	66.6	CH_2	66.1	CH_2
24	15.5	15.5	13.9	CH_3	15.3	CH_3
25	17.4	17.4	17.9	CH_3	17.6	CH_3
26	19.1	18.7	18.1	CH_3	19.5	CH_3
27	24.3	23.7	24.0	CH_3	24.0	CH_3
28	179.5	179.2	178.0	C	178.1	C
29	17.7	17.7	17.6	CH_3	17.8	CH_3
30	21.4	21.1	21.5	CH_3	21.5	CH_3

Note: The assignments with the same sign may be interchanged in each vertical column.
[a]Signals under methanol-d_4 signal.

11 ppm in comparison with the olean-12-enes due to the missing shielding effect of a 20β-(axial)-methyl-group.[18]

In accordance to reference data, the ^{13}C-NMR resonances for all triterpene moieties in **1**–**4** were almost superimposeable. Madecassic acid (**2**),

Table 3. ^{13}C NMR chemical shifts of sugar moieties of **3** and **4** (δ values in ppm, in methanol-d_4).

3		**4**	
Carbon	^{13}C	Carbon	^{13}C
glc′		glc′	
1	95.9	1	96.0
2	73.8	2	73.8
3	78.0	3	78.1
4	71.1	4	71.3
5	78.4	5	77.9
6	69.7	6	69.8
		(6 → 1)glc	
		(6 → 1)glc	
1	104.5	1	104.5
2	75.3	2	75.3
3	76.8	3	76.7
4	79.7	4	79.7
5	76.9	5	76.9
6	62.0	6	62.0
		(4 → 1)rha	
		(4 → 1)rha	
1	102.9	1	102.9
2	72.5	2	72.5
3	72.3	3	72.3
4	73.9	4	73.9
5	70.7	5	70.7
6	17.8	6	17.8

Note: glc, glucose and rha, rhamnose. The assignments in the vertical column with the same sign might be alternated.

the 6-hydroxyl derivate of asiatic acid (**1**), was elucidated by the strong downfield shift of carbon C-6 with almost δ 50 ppm. As expected, ^{13}C-resonance for the methylene group C-6 observed in substance **1** (δ-value 19 ppm) was not detectable in **2**. C-7, in β-position to the hydroxyl group, was slightly deshielded by δ 6 ppm compared with a chemical shift of δ 33.7 ppm in structure **1**. Confirmation of structure **3** was done by a heteronuclear correlation experiment (HMBC) and corroborated asiatic acid as triterpene moiety, and also esterification of the carboxyl function C-28 by a glucose unit.

Long-range HC-correlation cross peaks from the anomeric protons clearly elucidated the proposed sugar linkages in the trisaccharide of structure **3** (Fig. 3).

The ^{13}C-NMR data for the saccharide units of **3** and **4** were almost identical, and verified the trisaccharide linkages glc(6 → 1) glc (4 → 1)rha for both triterpene glycosides. Indicating the glycosidation at C-4 of the glucose unit, this carbinol resonance is remarkably shifted downfield to δ 79 ppm.

The ESI–MS spectroscopic data of the four components triterpene components are given as below:

- Asiatic acid (**1**): colorless powder, ESI–MS (pos) m/z: 510 $[M + Na]^+$; ESI–MS (neg) m/z: 486 $[M - H]^-$. ^{13}C-NMR data see Table 2.
- Madecassic acid (**2**): colorless powder, ESI (pos) m/z: 526 $[M + Na]^+$; ESI–MS (neg) m/z: 502 $[M - H]^-$. ^{13}C-NMR data see Table 2.
- Asiaticoside (**3**): colorless powder, ESI–MS (pos) m/z: 982 $[M + Na]^+$, 502 $[M + 2Na]^{2+}$, MS^2 fragmentation of m/z 982: 493 [trisaccharide unit + Na]$^+$, MS^3 fragmentation of m/z 493: 475, 405, 349, 331, 289; ESI–MS (neg) m/z: 1004 $[M + 2Na–H]^-$, MS^2 fragmentation of m/z 1004: 958 $[M - H]^-$, MS^3 fragmentation of m/z 958: 469 [trisaccharide unit]$^-$, MS^4 fragmentation of m/z 469: 367, 323 [glucose–glucose]$^-$, 247. ^{13}C-NMR data see Tables 2 and 3.
- Madecassoside (*syn.* asiaticoside-A) (**4**): colorless powder, ESI–MS (pos) m/z: 998 $[M + Na]^+$, 510 $[M + 2Na]^{2+}$, 304, MS^2 fragmentation of m/z 998: 494 [trisaccharide unit + Na]$^+$; ESI–MS (neg) m/z 1020 $[M + 2Na–H]^-$, MS^2 fragmentation of m/z 1020: 974 $[M - H]^-$, MS^3 fragmentation of m/z 974: 469 [trisaccharide unit]$^-$, MS^4 fragmentation of m/z 469: 448, 367, 323 [glucose–glucose]$^-$, 247, 161 [glucose]$^-$. ^{13}C-NMR data see Tables 2 and 3.

The full-scan ESI mass spectrum in positive ionization mode displayed for all triterpenoid components **1**–**4**, prominent sodium cationized quasimolecular ions of the nature $[M + Na]^+$, for glycoside structures **3** and **4**, also addition of two sodium ions, resulting in a double charge $[M + 2Na]^{2+}$. In negative ESI-mode, abundant molecular peaks $[M - H]^-$ corroborated the molecular weights M_r of **1**–**4**. Negative ESI mode also displayed for the glycoside structures **3** and **4** interesting quasimolecular ions of the nature $[M + 2Na–H]^-$, indicating that two neutral sodium molecules had been associated to the structure with abstraction of one proton. Isolation of these ions, and subsequent MS–MS fragmentation, resulted in base peaks for the ion $[M - H]^-$, verifying the previous MS results. We already observed this type of ion formation $[M + 2Na–H]^-$ for polar saponins from *Panax notoginseng*,[21] and we presume an unusual ion-formation for large amphiphilic substances, such as triterpene glycosides.

For the glycosides **3** and **4**, MS–MS fragmentation experiments done in both polarities indicated the cleavage into the trisaccharide and the aglycone unit. Interestingly, for positive and negative mode, the charge remained in the trisaccharide unit, and not as expected in the aglycone moiety.

CONCLUSION

The present study demonstrates that step-gradient elution for HSCCC is a fast and effective methodology suitable for a highly selective preparation of larger amounts of ursane-type pentacyclic triterpenoid acids, and their glycosides, from complex matrices such as a crude extract of *C. asiatica*, yielding asiatic acid (**1**), madecassic acid (**2**), asiaticoside (**3**), and madecassoside (**4**) in a single-step separation.

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