Separation of abietane-type diterpenoids from Clerodendrum kaichianum Hsu by high-speed counter-current chromatography using stepwise elution

Mingfeng Xu, Qin Zhu, Huizhong Wang, Qizhen Du, and Maojun Xu

Key Lab of Medical Plant Genetic Improvement and Quality Control of Zhejiang Province, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China; Institute of Food Chemistry, Zhejiang Gongshang University, Hangzhou, China

ABSTRACT
High-speed counter-current chromatography (HSCCC) was successfully used for the separation of abietane-type diterpenoids from the medicinal plant Clerodendrum kaichianum, which were not separated in our previous study using preparative HPLC. The HSCCC separation employed the lower phases of n-hexane–ethyl acetate–methanol–water (HEMW) 4:5:4:5 and HEMW 4:5:5:4 as the mobile phase for stepwise elution while the upper phase of HEMW 4:5:4:5 was used as the stationary phase. HSCCC separation yielded 90.5 mg of compound 1 (kaichianone A), 137.7 mg of compound 2 (kaichianone B), 125.0 mg of compound 3 (teuvincenone E), and 227.6 mg of compound 4 ( taxusabietane A) with purities of 95.3%, 97.2%, 97.8%, and 98.6%, respectively, as determined by HPLC. Compounds 1–2 are two new abietane-type diterpenoids while Compounds 3–4 are known abietane-type diterpenoids, analyzed by ESIMS and NMR data. The results demonstrated that HSCCC can be an excellent alternative for other separation methods. The two new compounds showed significant cytotoxicity against ileocecal carcinoma HCT-8 and breast adenocarcinoma MCF-7 cells.

GRAPHICAL ABSTRACT
High-speed counter-current chromatography (HSCCC) was successfully used for the separation of abietane-type diterpenoids from the medicinal plant Clerodendrum kaichianum, which were not separated in our previous study using preparative HPLC.

Introduction
The genus Clerodendrum is widely distributed in the tropical and subtropical zones including Africa and southern Asia. A few of species are distributed in eastern Asia, America, and northern Australia. Plants of the genus Clerodendrum are well known for their treatment of different diseases, such as...
as pyretocis, pneumonia, inflammation, and postpartum hemorrhage.\cite{3-5} In China, the leaves and stems of *C. kaichianum* Hsu have long been used as medicinal plants to treat rheumatism, malaria, and other inflammatory diseases. Therefore, the separation and preparation of chemical constituents in *C. kaichianum* Hsu are urgently needed.

Abietane-type diterpenoids are reported as characteristic compounds in the genus *Clerodendrum*. A large amount of abietane-type diterpenoids were obtained by traditional separation methods, such as normal-phase thin-layer chromatography (TLC) or silica gel column chromatography and preparative HPLC.\cite{6-14} Our previous studies also separated more than 40 abietane-type diterpenoids from *C. kaichianum* Hsu using silica gel, Sephadex LH-20, and preparative HPLC on C\textsubscript{18}.\cite{15-18} However, the separation of some fractions from pre-separation on silica gel column chromatography failed to yield pure compounds, even if preparative HPLC was used.

High-speed counter-current chromatography (HSCCC) has been used for separation of natural products with some advantages over other separation methods\cite{19,20} and is considered to be an excellent alternative method. In the present study we applied a stepwise elution HSCCC for the separation of a fraction of abietane diterpenoids obtained from silica gel column chromatography.

**Experimental**

**Apparatus**

Melting points were determined with an X-4 apparatus. UV spectra were obtained with a Shimadzu UV-2550 spectrometer. IR spectra were obtained with a Nicolet 380 FT-IR spectrophotometer using KBr pellets. The nuclear magnetic resonance (NMR) spectroscopic data were recorded on Bruker AVANCE III 500 spectrometers with tetramethylsilane as the internal standard. Chemical shifts are given in ppm; J values are given in Hz. The high-resolution mass analyses were performed with an Agilent 6210 TOF-MS Spectrometer (Agilent, Santa Clara, USA) coupled with an ESI source. Column chromatography was carried out on silica gel (200–300 mesh; Qingdao Marine Chemical Company, Qingdao, China). Analytical thin-layer chromatography (TLC) was performed on Merck RP-C\textsubscript{18} (Merck, Darmstadt, Germany).

The HSCCC separation employed in the present study is a J-type HSCCC designed and constructed in the Institute of Food and Biological Engineering, Zhejiang Gongshang University (Zhejiang China). It holds a separation column at a distance of 10 cm from the central axis of the centrifuge. The column revolves around the central axis of the centrifuge and simultaneously rotates around its own axis at the same angular velocity in the same direction. The column holder hub is 25 cm in length and 6 cm O.D. The multilayer coil column was prepared by winding a 60 m long, 5.0 mm I.D. PTFE (polytetrafluoroethylene) tubing onto the holder hub. The capacity of the column was 1100 mL. The HSCCC system comprises a K-1800 Wellchrom preparative HPLC pump, a 50-mL sample loop made of 5 mm I.D. PTFE tubing, and a B-684 collector with 25-mL tube racks.

**Reagents and pre-separation**

Analytical grade methanol, n-hexane, and ethyl acetate for HSCCC separation were purchased from Hangzhou Chemicals (Hangzhou, China). Methanol used for HPLC was chromatographic grade and purchased from Fisher Chemical (Loughborough, UK). Water was produced by a Milli-Q system (18 MΩ) (Millipore, Bedford, MA, USA). The stems of *C. kaichianum* Hsu were collected on the mountains of Lin'an County, Zhejiang Province, China, in September of 2009. The plant was identified and authenticated by Dr. Chunhui Dai in Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, China. A voucher specimen (No. 20090913) was deposited in the Natural Products Department of Zhejiang Gongshang University, Hangzhou, China.

The stems of *C. kaichianum* (11.6 kg) were air-dried and ground into a uniform powder and then extracted with 75% ethanol (3 × 30 L) three times. The ethanol extract was combined and evaporated to dryness to yield a gummy residue (325 g), which was suspended in H\textsubscript{2}O and extracted with petroleum ether (b.p.: 60–90°C), ethyl acetate, and n-butanol, successively. Part of the ethyl acetate extract (90 g) was subjected to column chromatography through silica gel (2 kg, 100–200 mesh) eluting with petroleum ether–ethyl acetate at various volume ratios (10:1, 4:1, 3:2, 1:1, 1:2, 1:4, and 1:10) to obtain 10 fractions (Fr. 1–Fr. 10, each 10 L) on the basis of TLC analysis. Fr. 5 (2 g), the component containing two new and two known Abietane-type diterpenoids, was subjected to HSCCC separation.

**Preparation of two-phase solvent system and sample preparation**

The two-phase solvent systems were composed of n-hexane, ethyl acetate, methanol, and water. In order to find a suitable volume ratio, the partition coefficient was determined by HPLC analysis.\cite{21} Approximately 1 mg of crude sample was added to a glass tube containing 2 mL of each phase of the two-phase solvent systems. The tubes were shaken for 3 min, and then equal volumes of upper and lower phases were evaporated to dryness, respectively. The residues were dissolved in 1 mL methanol prior to HPLC analysis. The partition coefficient (K) of the target compounds was calculated by the peak areas. The K value was expressed as a peak area of a compound in the stationary (upper) phase divided by the peak area of the compound in the mobile (lower) phase. Four solvent systems composed of n-hexane–ethyl acetate–methanol–water with solvent ratios of 4:5:6:3 (A), 4:5:5:4 (B), 4:5:4:5 (C), and 4:5:3:6 (D) (v/v/v/v) were the candidates for the study of partition coefficient.

**HSCCC separation procedure**

Preparative separation was performed using a stepwise elution with solvent systems B and C in sequence as follows: the coiled column was first entirely filled with the upper organic phase of solvent system B, and then the lower aqueous phase was pumped into the head of the column at a flow-rate of 5.0 mL/min, while the apparatus was rotated at 700 rpm. After the mobile phase...
emerged from the tail outlet and hydrodynamic equilibrium was established in the column. 50 mL of the sample solution containing 1.0 g of Fr. 5 was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 280 nm. Peak fractions were manually collected according to the chromatogram. After 2 hr of elution with solvent system B, the mobile phase was switched to the lower phase of solvent system C to elute the components with large K values in solvent system B.

HPLC analysis

The crude extract of *C. kaichianum* and HSCCC peak fractions were analyzed by HPLC. A Waters HPLC system (Waters, Milford, MA, USA) was composed of an Alliance 2695 LC, a Merck C18 column (5 µm, 250 mm x 4.6 mm), and 2996 photodiode array detector (DAD). A gradient elution was performed for the separations. The gradient was run from 80% water and 20% acetonitrile to 25% water and 75% acetonitrile from 0 to 20 min at the column temperature of 25°C. The flow rate of mobile phase was 1.0 mL/min and the injection volume was 10 µL.

Anti-proliferative assay

The percentage of growth inhibition was determined using an MTT assay to measure viable cells with minor modification. A total of 5000–10,000 exponential phase cells per well was seeded onto a 96-well plate for 24 hr, treated with compounds 1–4 at different concentrations for 72 hr using cisplatin as a positive control. Briefly, 100 µL of an MTT working solution (1 mg/mL) into each well of 96-well plate was added and incubated at 37°C for 4 hr and then the medium was removed. The converted dye formazan was solubilized with 150 µL acidic isopropanol (0.04 M HCl in absolute isopropanol) and each concentration was tested in triplicate. The absorbance was then measured at a wavelength of 570 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). The dose resulting in 50% inhibition of cell growth (IC50) was calculated by NDST software.

Results and discussion

Selection of two-phase solvent system of HSCCC

In HSCCC, the selection of a suitable two-phase solvent system was critical for a successful separation. In our experiment, four kinds of solvent systems including *n*-hexane–ethyl acetate–methanol–water (HEMW) at different volume ratios (4:5:6:3, 4:5:4:5, 4:5:5:4, 4:5:3:6 (v/v/v/v)) were investigated, since these solvent systems have been successfully applied to various samples with a moderate degree of polarity. K values of

| No | HEMW solvent system | Compounds 1+2 | Compound 3 | Compound 4 |
|----|---------------------|---------------|------------|------------|
| A  | 4:5:6:3             | 3.56          | 12.50      | 16.90      |
| B  | 4:5:5:4             | 0.87          | 4.78       | 6.26       |
| C  | 4:5:4:5             | 0.24          | 1.19       | 1.98       |
| D  | 4:5:3:6             | 0.05          | 0.21       | 0.32       |

HEMW means *n*-hexane–ethyl acetate–methanol–water.
The K value was expressed as a peak area of the compounds in the stationary (upper) phase divided by the peak area of the compounds in the mobile (lower) phase.
target compounds are listed in Table 1. A suitable solvent system is one in which the target components have a K value between approximately 0.2 and 5. According to this rule, HEMW 4:5:4:5 was suitable for the separation of compounds 1 and 2, whereas HEMW 4:5:5:4 met the requirement for compounds 3 and 4. Therefore, it would be better using a stepwise elution with the lower phases of HEMW 4:5:4:5 and HEMW 4:5:5:4 as the mobile phase, whereas the upper phase of HEMW 4:5:4:5 was used as stationary phase.

**HSCCC separation**

Figure 1 shows the HSCCC separation of Fr. 5 obtained from silica gel chromatography of the crude extract of C. kaichianum, with a stepwise elution. Peaks I and II were eluted out by the mobile phase of the lower phase of HEMW 4:5:4:5 due to the lower K values of compounds 1 and 2. Peaks III and IV were eluted after the lower phase of HEMW 4:5:4:5 was used mobile phase. The result of this separation demonstrated that the stepwise elution strategy was successful. The fractions corresponding to each peak in HSCCC chromatography of the crude extract of C. kaichianum were successfully separated by partition chromatography of C18 column. However, the two compounds 3 and 4. Therefore, it would be better using a stepwise elution with the lower phases of HEMW 4:5:4:5 and HEMW 4:5:5:4 as the mobile phase, whereas the upper phase of HEMW 4:5:4:5 was used as stationary phase.

Table 2. 

| No. | δC, mult. | δH (in Hz) | HMBC (H-C) | δC, mult. | δH (in Hz) | HMBC (H-C) |
|-----|-----------|------------|------------|-----------|------------|------------|
| 1   | 45.0, CH2 | 2.31, d (16.5) α | 2, 5, 9, 10, 20 | 29.8, CH2 | 0.90, dd (13.4, 6.2) α | 2, 5, 10 |
| 2   | 199.4, qC | –          | –          | 19.6, CH2 | 3.05, dd (13.5, 6.9) β | 1, 3, 4, 10 |
| 3   | 135.9, qC | –          | –          | 18.2, CH  | 0.72, dd (14.8, 6.5) | 1, 19 |
| 4   | 147.5, qC | –          | –          | 15.7, CH  | –          | –          |
| 5   | 160.2, qC | –          | –          | 47.1, qC  | 1.89, dd (13.0, 5.7) | 1, 4, 6, 10, 18 |
| 6   | 124.3, CH | 6.53, s    | 4, 5, 8, 10 | 37.9, CH2 | 2.71, m    | 5, 7, 8    |
| 7   | 188.8, qC | –          | –          | 204.3, qC | –          | –          |
| 8   | 109.0, qC | –          | –          | 110.5, qC | –          | –          |
| 9   | 134.4, qC | –          | –          | 137.5, qC | –          | –          |
| 10  | 42.6, qC  | –          | –          | 37.8, qC  | –          | –          |
| 11  | 131.9, qC | –          | –          | 131.8, qC | –          | –          |
| 12  | 154.3, qC | –          | –          | 155.2, qC | –          | –          |
| 13  | 110.8, qC | –          | –          | 110.8, qC | –          | –          |
| 14  | 154.7, qC | –          | –          | 155.2, qC | –          | –          |
| 15  | 28.6, CH2 | 3.27, dd (15.2, 9.3) α | 13, 14, 16, 17 | 28.6, CH2 | 3.26, dd (15.4, 9.6) α | 12, 13, 14, 16, 17 |
|     |           | 2.93, dd (16.4, 6.1) β |          |           | 2.98, dd (15.4, 7.2) β |          |
| 16  | 86.2, CH  | 5.15, m    | 15, 17     | 86.4, CH  | 5.10, m    | 15, 17     |
| 17  | 64.9, CH2 | 3.86, m    | 15, 16     | 64.8, CH2 | 3.89, m    | 15, 16     |
| 18  | 12.0, CH1 | 2.00, s    | 2, 3, 4, 5 | 22.1, CH2 | 0.53, dd (9.2, 4.2) | 4, 5, 19 |
| 19  | 17.5, CH3 | 2.23, s    | 2, 3, 4, 5 | 22.6, CH3 | 1.05, s    | 3, 4, 5, 18 |
| 20  | 24.8, CH3 | 1.55, s    | 1, 5, 9, 10| 14.4, CH3 | 1.37, s    | 1, 5, 10   |
| 11-OH | 7.45, br s |           |           | 5.18, s   |           | 9, 11, 12, 14 |
| 14-OH | 13.31, s  |           |           | 13.24, s  |           |           |

**Structural identification of the isolated compounds**

Compound 1 (kaichianone A) was obtained as yellowish needles (M.p. 246–248°C) with the molecular formula C20H20O6 by HRESIMS (m/z 355.1180 [M-H]–, calcd. for C20H19O6, 355.1182). The absorption bands in the UV spectrum (214, 296, 377 nm) exhibited the presence of a benzene ring and unsaturated ketone, which was confirmed by the IR spectrum that had absorption bands at 3300, 1680, 1670, and 1597 cm−1 corresponding to phenolic hydroxyl groups, α, β-unsaturated ketone, and benzene moieties, respectively.

The 13C NMR and 13C DEPT NMR spectra indicated that the structure of intermolecular hydrogen between the carbonyl at C-7 and the phenolic hydroxyl group at C-14 was proposed.

This was confirmed by the HMBC spectra. The HMBC spectrum showed the correlations from H-17 to C-15 and the phenolic hydroxyl group at C-14 was proposed.
Compounds 3 and 4 were two known compounds that have been isolated from *Teucrium fruticans* and *Taxus mairei*. Our measured MS and NMR data are in agreement with the reported compounds teuvincenone E and taxusabietane A in the literature.\[^{28,29}\]

**Anti-proliferative activity**

All the diterpenoids from the HSCCC separation were tested for their ability to prevent the cytotoxic effects against HCT-8 and MCF-7 cancer cells using cisplatin as a positive control. Based on the values of IC\(_{50}\) calculated by the cell growth inhibition at concentrations of 1.56 to 50 µM, all of the four compounds showed moderate activity against cancer (IC\(_{50}\) < 20 µM). Among them, compounds 1 and 4 had activity against the two cell lines with IC\(_{50}\) of less than 10 µM (Table 3). In previous studies on the cytotoxicity of abietane diterpenoids, uncinatone showed cytotoxicity against B16, HGC-27, and HEK-293 cell lines, with IC\(_{50}\) values of 6.4, 5.3, and 1.2 µM (positive control: paclitaxel IC\(_{50}\) values: B16, 24.5 µM; HGC-27, 775 nM; HEK-293, 1.4 µM)\[^{11}\], respectively, which suggests that these compounds might have the promising potential to be anticancer agents.

**Conclusions**

High-speed counter-current chromatography was successfully used for the separation of abietane-type diterpenoids from the medicinal plant *C. kaichianum*, which were not separated in our previous study using preparative HPLC. A stepwise separation of the fraction from silica gel chromatography yielded two known and two new abietane-type diterpenoids. The two new compounds showed significant cytotoxicity against ileocecal carcinoma HCT-8 and breast adenocarcinoma MCF-7 cells. Our studies demonstrated that HSCCC can be an excellent alternative for other separation methods.

**Funding**

Financial support from the Project of Zhejiang Provincial Natural Science Foundation (LQ14C010003, LQ13C200007), the Research Foundation of Education Bureau of Zhejiang Province (Y201327898), and the Hangzhou Science and Technology Development Plan (20140432B06) are gratefully acknowledged.

**References**

1. Liu, S.; Zhu, H.; Zhang, S.; Yu, Q.; Xuan, L. Abietane Diterpenoids from *Clerodendrum bungei*. *J. Nat. Prod.* 2008, 71 (5), 755–759.
2. Kumari, G.; Balachandran, J.; Aravind, S.; Ganesh, M. Antifeedant and Growth Inhibitory Effects of Some Neo-Clerodane Diterpenoids Isolated from *Clerodendron bungei*. *J. Nat. Prod.* 2003, 66 (5), 755–759.
3. Thitilertdecha, P.; Rowan, M. Characterisation of Polyphenolic Compounds in *Clerodendrum petasites* S. Moore and Their Potential for Topical Delivery Through the Skin. *J. Ethnopharmacol.* 2014, 154 (2), 400–407.
4. Thitilertdecha, P.; Rowan, M.; Guy, R. Topical Formulation and Dermal Delivery of Active Phenolic Compounds in the Thai Medicinal Plant - *Clerodendrum petasites* S. Moore. *Int. J. Pharmaceut.* 2015, 478 (1), 39–45.
