# Isolation and Purification of Isoaloeresin D and Aloin from *Aloe vera* by High-speed Counter-current Chromatography

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**Abstract**: **Objective** To develop an efficient method to isolate and purify the main components isoaloeresin D and aloin from *Aloe vera* for its industrial production. **Methods** High-speed counter-current chromatography was used to isolate isoaloeresin D and aloin in a one-step separation from dried crude extract of *A. vera*. The biphasic solvent system composed of hexane-ethyl acetate-acetone-water (0.2∶5∶1.5∶5) was used at a flow rate of 1.0 mL/min, while the lipophilic phase was selected as the mobile phase and the apparatus was rotated at 840 r/min. The effluent was detected at 254 nm. **Results** Isoaloeresin D (53.1 mg) and aloin (106.9 mg) were separated from the crude extract (384.7 mg) with the purities of 98.6% and 99.5%, respectively. **Conclusion** HSCCC is a powerful technique for isolation and separation of chemical composition from aloe.

**Key words**: *Aloe vera*; aloin; high-speed counter-current chromatography; isoaloeresin D; extraction and purification
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## Introduction

The species in *Aloe* L. have been used as ethnic medicines in many different countries for centuries, possessing functions, such as anti-cancer, anti-inflammatory, anti-virus, evacuating, protecting liver, and increasing immunity (Lu *et al*, 2008). *Aloe vera* L. has been most commonly used as medicine, healthy foods, and cosmetics nowadays. The active components of aloe include anthraquinones, chromones, polysaccharides, enzymes and, so on (Chen *et al*, 2002). It is reported that anthraquinones and chromones are responsible for the anti-cancer activity, anti-inflammatory, and evacuating (Cao *et al*, 2004). However, most aloe preparations are mixtures of multiple components, usually lead to composition confusion, functional ambiguous, and uncontrollable quality. The conventional methods including solvent extraction, column chromatographic separation with silica gel and/or polyamide are often used to isolate pure anthraquinones and chromones from aloe (Yuan *et al*, 1994; Saeda *et al*, 2007), but they are tedious, time consuming, and easily contaminated. And it is very difficult to separate these compounds respectively from aloe in industrial scale, so the separation and purification of active components from aloe has become a pressing issue. High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatography without solid support matrix. It eliminates the risk of irreversible adsorption, deactivation and absorbance contamination, resulting in higher recovery and larger scale of products than conventional techniques (He *et al*, 2007; Zhao and He, 2007; Zhang and Yang, 2008). Aloin (Fig. 1) was isolated and purified from aloe by HSCCC (Huang *et al*, 2006), while isoaloeresin D has not been isolated by HSCCC yet. Yet yielding these two components by HSCCC in one-step separation has not been reported. In this study, a simple and efficient method was developed to isolate and purify the major anthraquinones and chromones in aloe by HSCCC in one-step

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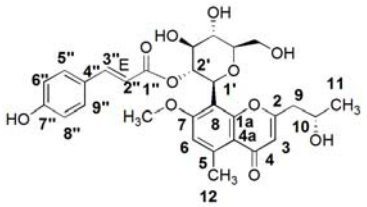
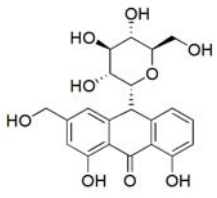
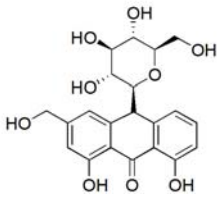


isoaloeresin D



aloin A



aloin B

**Fig. 1 The chemical structures of isoaloeresin D, aloin B, and aloin A**

separation. HPLC-DAD was used to select the two-phase solvent system and determined the purities of the compounds. The structures of the compounds were characterized by UV, IR, LC-MS, TOF-MS, $^{1}$H-NMR and $^{13}$C-NMR. The results can be employed as a more efficient method to separate these compounds from aloe and can be transferred to use in relevant medicinal industrial production.

## Materials and methods

### Reagents and materials

All organic solvents used for HSCCC were of analytical grade and purchased from Baishi Chemical Industrial Co. Ltd. (Tianjin, China). Ultrapure water was used for all solutions, dilutions, and HPLC analysis. Methanol used for HPLC analysis was of chromatographic grade and purchased from Tedia (Bangkok, Thailand).

The standard aloin A (Batch no: 110787-200504) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The aloe powder (Batch no: 20061001) was purchased from Yunnan Yuanjiang Evergreen Biological Co., Ltd. (Yuxi, China).

### Apparatus

HSCCC experiments were performed using a model Mk5 QuilkPrep500 HSCCC (AECS-Quik. Prep, S. Wales, Britain). The separating device consisted of three preparative coils connected in series. The polytetrafluoroethene tubes had an inner diameter of 2.16 mm and a total volume of 120 mL. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge ($R$), was 10 cm, and the $\beta$ values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where $r$ is the distance from the coil to the holder shaft). The rotation speed was adjustable and in the range of 0–860 r/min. The HSCCC system was equipped with a Series Ⅱ constant pump (Scientific System Co., MA, USA), a SPD-10Avp UV detector (Shimadzu, Hangzhou, China) and an injection valve with a 5 mL sample loop. The data were collected with a model N2000 chromatography workstation (Zhejiang University, Hangzhou, China).

The HPLC system used was a model Waters 1525 binary gradient pump, a model 717 automatic sampler, a model 2996 diode array detector and the Empower workstation (Wacters, MA, USA). The analyses were performed using a Nucleodur 100-5 $C_{18}$ column (250 mm × 4.6 mm, 5 μm), which was purchased from Düren (Germany), at ambient temperature. The mobile phase consisted of methanol (A) and 0.34% acetic acid (B) using a gradient program as follow: 0–30 min, 40%A–80%A,60%B–20%B; 30–40 min, 80%A–95%A, 20%B–5%B. The flow rate was 1.0 mL/min. The diode array detector was set to the range of 190–370 nm, and the elution was monitored at 254 nm and 356 nm.

The LC-MS system was performed using a TQS system with a Finnigan Surveyor MS pump, a Finnigan Surveyor Autosampler (Thermo Fisher Scientific, MA, USA), which was controlled by Xcalibur1.3 workstation. The data were recorded by LC Quan software. The MS analyses of aloin were performed in the negative ion mode and in a full scan mode. The spectra were recorded in the range of 50–1000 $m/z$. Nitrogen was used as the drying gas at a flow rate of 11 mL/min and the nebulizing gas at a pressure of 241.325 Pa. The drying temperature was set at 350 ℃. The MS analyses of isoaloeresin D were performed on LC-MS-IT-TOF high resolution mass spectrometry (Shimadzu, Kyoto, Japan) in the negative ion mode.

IR spectra of the compounds were recorded by a TENSOR37 IR spectrometer (Bruker Co., Bremen, Germany). $^{1}$H-NMR and $^{13}$C-NMR experiments were performed on a Bruker AvanceIII 400 MHz spectrometer at 400 and 100 MHz, respectively, with $CD_3OD$ as solvent.

**Preparation of samples**

The standard substance was dissolved in methanol as standard solution, of which concentration was 80.0 μg/mL.

Aloe powder (25.0 g) was extracted with 500 mL of acetone by sonication for 30 min, and then centrifuged. The supernatant was concentrated under the reduced pressure at 60 ℃ in a rotary evaporator. The slurry was dried in vacuum at 60 ℃. Extract (16.3 g) was obtained and stored under below 10 ℃ for the HSCCC separation and isolation.

Acetone extract (513.0 mg) was dissolved by methanol and transferred to a 10 mL volumetric flask. The solution was diluted with four times of methanol and the concentration was 12.8 mg/mL. The solutions were filtered through a 0.45 μm membrane filter before injected into HPLC.

**Selection of the biphasic solvent system**

The selection of the biphasic solvent system is the most important step in HSCCC. It is evaluated by the partition coefficient [$K$ = $C$ (stationary phase) / $C$ (mobile phase)] of the target compounds and the stationary phase retention. Meanwhile, the settling time of the two phases should not exceed 20 s after inverting for several seconds. And the sample should have a sufficient solubility in the solvent, which would not lead to a decomposition or denaturation of the sample. According to the golden rules in selecting optimum solvent system (Yoichiro, 2005), the suitable $K$ values of the target compounds should be in the range of 0.5–1.0, which means the $K_{u/l}$ [$K_{u/l}$= $C$ (upper phase) / $C$ (lower phase)] should be in the range of 0.5–2.0; and the ratio of the two $K$ values ($\alpha = K_1 / K_2$, where $K_1 > K_2$) should be greater than 1.5; The stationary phase retention should be greater than 50%. After testing chloroform-methanol-water, hexane-ethyl acetate-methanol-water, and hexane-ethyl acetate-acetone-water systems, the hexane-ethyl acetate-methanol-water and hexane-ethyl acetate-acetone-water systems were selected. Testing different volume ratios of the solvent, hexane-ethyl acetate-acetone-water (0.2∶5∶1.5∶5) had a better $K$ value and stationary phase retention. The results of the tests were shown in Table1.

**Preparation of the biphasic solvent system and HSCCC separation procedure**

The biphasic solvent system consisting of hexane-ethyl acetate-acetone-water (0.2∶5∶1.5∶5) was applied for the HSCCC separation and isolation. The solvent mixture was thoroughly equilibrated in a separatory funnel at ambient temperature. The two phases were separated and degassed by sonication. The preparative column of HSCCC was entirely filled with the lower aqueous phase as stationary phase and the elute was conducted in tail-to-head mode. Then the upper organic phase was pumped at a flow rate of 1.0 mL/min while the apparatus was rotated at 840 r/min. The aloe extract was dissolved with lower aqueous phase, of which the concentration was 85.5 mg/mL. After the mobile phase front emerged and the system had reached hydrodynamic equilibrium, sample solution (4.5 mL) was injected into the column. The stationary phase retention was 79.1%.

**Table 1 The $K_{u/l}$ of the three compounds in different solvent systems**

| hexane-ethyl acetate-acetone-water | $K_{u/l}$ | | |
|---|---|---|---|
| | isoaloeresin D | aloin A | aloin B |
| 0.2∶5∶1∶5 | 1.00 | 0.58 | 0.66 |
| 0.2∶5∶1.5∶5 | 1.03 | 0.62 | 0.64 |
| 0.5∶5∶0.5∶5 | 0.97 | 0.40 | 0.44 |

The effluent was monitored at 254 nm and the fractions were collected at the intervals of 5 min. The total time of HSCCC separation was 320 min. The fractions were combined according to the chromatograms of the HSCCC (Fig. 2) and HPLC. As the result, the fraction 1 was collected from 99 to 144 min, while the fraction 2 was collected from 152 to 250 min.

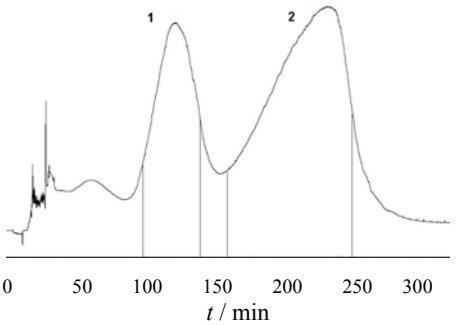



**Fig. 2 HSCCC chromatogram of acetone extract**

## Results

The fractions 1 and 2 were evaporated to yield 53.1 mg compound Ⅰ and 106.9 mg compounds Ⅱ and Ⅲ, of which the recovery rates (the ratio of the weight of compounds and crude extract) were 13.8% and 27.8%,

respectively. The purities of HSCCC peak fractions were determined by HPLC. It was shown that the purity of fraction 1 was 98.6%, and the fraction 2 was a mixture of compounds Ⅱ and Ⅲ, in which the total purity was 99.5%. The HPLC chromatograms of each HSCCC peak fraction are shown in Fig. 3 (c–d). And the structures were identified according to UV, IR, LC-MS, TOF-MS, $^{1}$H-NMR, and $^{13}$C-NMR spectra.

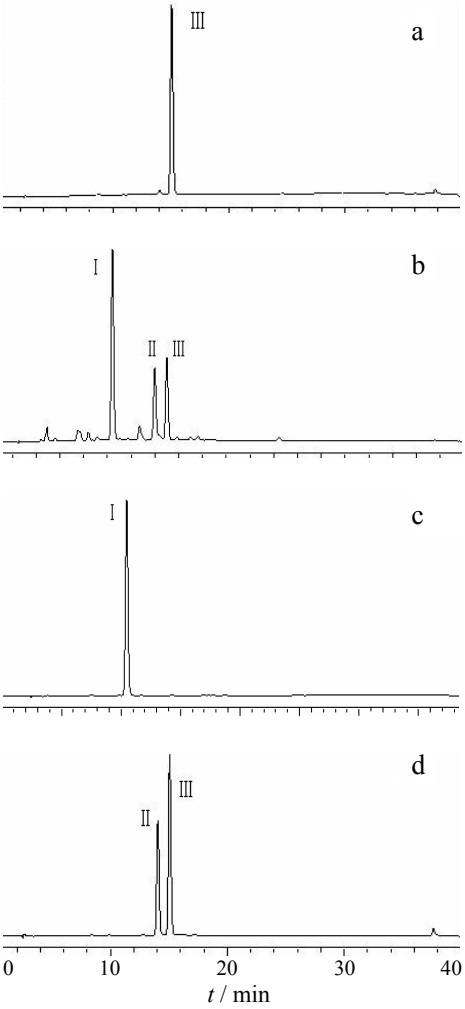



**Fig.3 HPLC chromatograms of standard aloin A (a), the crude extract (b), fraction 1 (c), and fraction 2 (d)**

Ⅰ: isoaloeresin D Ⅱ: aloin A Ⅲ: aloin B

Compound Ⅰ: UV $\lambda_{max}^{MeOH}$ nm: 229.1, 299.9; IR$\nu_{max}^{KBr}$ ($cm^{-1}$): 3446, 1649, 1602, 1514, 1383; HR-MS *m/z*: 555.1891[M－H]$^{-}$.$^{1}$H-NMR (400 MHz, $CD_3OD$) $\delta$: 6.11 (1H, s, H-3), 6.79 (1H, s, H-6), 3.88 (3H, s, 7-$OCH_3$), 2.76 (2H, d, $J$ = 8.3 Hz, H-9), 4.50 (1H, m, H-10), 1.35 (3H, d, $J$ = 6.2 Hz, H-11), 2.72 (3H, s, H-12), 5.19 (1H, d, $J$ = 10.1 Hz, H-1′), 5.73(1H, t, $J$ = 9.6 Hz, H-2′), 6.04(1H, d, $J$ = 15.9 Hz, H-2″), 7.36(1H, d, $J$ = 16.8 Hz, H-3″), 7.33(2H, d, $J$ = 8.9 Hz, H-5″ and H-9″), 6.75(2H, $J$ = 8.6 Hz, H-6″ and H-8″). $^{13}$C-NMR (100 MHz, $CD_3OD$) $\delta$: 167.4 (C-2), 112.5 (C-3), 182.3 (C-4), 116.8 (C-4a), 144.6 (C-5), 112.6 (C-6), 162.0 (C-7), 111.8 (C-8), 159.6 (C-1a), 44.6 (C-9), 66.0 (C-10), 23.7 (C-11), 23.6 (C-12), 57.1 (7-OCH3), 72.3 (C-1′), 73.8 (C-2′), 77.9 (C-3′), 72.8 (C-4′), 83.0 (C-5′), 63.1 (C-6′), 168.0 (C-1″), 114.7 (C-2″), 146.6 (C-3″), 127.0 (C-4″), 131.1 (C-5″ and C-9″), 117.2 (C-6″ and C-8″), 161.3 (C-7″). Compared with the data given in references (Nobuyuki *et al*, 1996; Xiao *et al*, 2000), it was identified as isoaloeresin D.

Compounds Ⅱ and Ⅲ: UV $\lambda_{max}^{MeOH}$ nm: 223.3, 270.3, 297.5, 357.0, IR$\nu_{max}^{KBr}$ ($cm^{-1}$): 3442, 1619, 1458, 1386, 1261. EI-MS *m/z*: 417.14[M－H]$^{-}$, 296.99. Besides the above data, compound Ⅲ had the same retention time in HPLC with the reference substance of aloin A, so compound Ⅲ was identified as aloin A. According to the similar data with aloin A except the retention time, compound Ⅱ was identified as the isomer of aloin A, named aloin B.

## Discussion

According to the literatures, the solvent systems used for separating the chemical composition in HSCCC mostly contained chloroform (Saeda *et al*, 2007; He *et al*, 2007), which has not only low separation proficiency but also a deleterious solvent. For these reasons, the hexane-ethyl acetate-acetone-water system was selected in this study as the solvent system, which had better separation efficiency and less pollution. Testing different volume ratios of the solvent, hexane-ethyl acetate-acetone-water (0.2∶5∶1.5∶5) was chosen as the optimal solvent system for separation and isolation. The lipophilic phase (upper phase) was selected as the mobile phase, because it had better separation efficiency and was easier for drying of the fractions.

Isoaloeresin D is one kind of cinnamoyl-chromones, which showed topical anti-inflammatory activity (John *et al*, 1996). However, the conventional methods including column chromatographic separation with silica gel and/or polyamide used to isolate isoaloeresin D were tedious, time consuming, and inefficient. XIAO Zhi-yan only obtained 56 mg isoaloeresin D from 80 g ethyl acetate extracts by repeated gel column chromatography and Sephadex LH-20 column (Xiao *et al*, 2000). Also, it was KAMURA Nobuycjkoi who yielded the compound

from ethanol extracts and chromatographed over Sephadex LH-20 and MCI-gel CHP 20P, repeatedly. With the HSCCC method we developed, isoaloeresin D (53.1 mg) was yielded from acetone extracts (384.7 mg). The method was more efficient and productive, and could be used in industry.

Our research demonstrates that HSCCC is a powerful technique for isolation and separation of chemical composition from aloe. Isoaloeresin D and aloin were successfully obtained from aloe powder by HSCCC with high purities and good recovery ratios. The compounds were yielded on a larger scale, with higher separation efficiency and better reproducibility, which could be used as reference substances and for bioavailability and bioactivity studies. The method we developed with stable conditions and standardizing materials could even be used in industry. The results of the research have laid a firm foundation for our further investigation on the pharmacological studies of aloe and exploiting high quality aloe products.

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