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# Isolation and Purification of Two Triterpenoids from the Chinese Medicinal Plant *Fomes officinalis ames*

HUIFENG ZHANG[1,2], BO FENG[1,2], KE LIU[1] and SHUYING GUO[2,*]

[1]College of Life science, Jilin University, Changchun 130013, P.R. China
[2]Pharmaceutical analysis Department, Jilin Medical College, Jilin 132013, P.R. China

*Corresponding author: Fax: +86 432 64560317; Tel: +86 432 64560530; E-mail: shuyingguocn@yeah.net

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A high-speed counter-current chromatography method is developed for the separation and purification of two triterpenoids from the Chinese Uyghur medicinal plant *Fomes officinalis ames*. Crude triterpenoids were obtained from *Fomes officinalis ames* through ethanol extraction. Preparative high-speed counter-current chromatography with two-phase solvent systems made up of *n*-hexane-ethylacetate-methanol-water with a volume ratio of 1:1.5:1.2:1 (v/v) was successfully performed in a stepwise elution. The process yielded two relatively pure triterpenoids in a single run from 500 mg crude extract. The purities of the dehydrosulphurenic acid and 3-keto-dehydrosulphurenic acid were 96.8 % and 92.3 %, respectively.

**Key Words: *Fomes officinalis ames*, Counter-current chromatography, Triterpenoids, Preparative chromatography.**

## INTRODUCTION

*Fomes officinalis ames* is commonly used in traditional Chinese medicine by Uyghur doctors in Xinjiang. This plant, the dry mycelium of Polyporaceae Phellinus (Uigur Pharmacopoeia Fascicule)[1], can be found on the trunks of both living and dead coniferous trees in the northern regions of China, in the Pacific Northwest of the US and Canada and in Europe. *Fomes officinalis ames*, as traditional Chinese medicine, can warm the lungs, reduce phlegm, improve blood circulation and reduce swelling, diuresis and chronic rheumatoid arthritis. Recent studies have shown that this plant enhances immunity[2,3] and stimulates anti-tumor activity[4] as a result of the anti-thrombin role[5] of scavenging oxygen free radicals[6]. The major active constituents of this traditional Chinese medicine are terpinenes, sterols, polysaccharides[7], total triterpenic acids, dehydrosulphurenic acid 8 and flavonoids[9].

In view of these beneficial effects, a large quantity of pure materials should be used for further pharmacological studies, as well as a "marker compound" for the chemical evaluation or standardization of two triterpenoids and their medical products. The preparative separation of two triterpenoids from *Fomes officinalis ames* by classical methods is tedious, time-consuming and requires multiple chromatographic steps with silica gel, polyamide column and others. This method also risks the loss of compounds due to the violent adsorptive effect of the solid matrix[5,10]. Therefore, an efficient method for the preparative separation and isolation of active constituents from natural and cultured *Fomes officinalis ames* is required.

High-speed counter-current chromatography is a unique liquid-liquid partition chromatography that does not require the use of a solid support matrix. The liquid stationary phase of this method is immobilized by centrifugal force, which eliminates the irreversible adsorption of sample into the solid support used in the conventional chromatographic column[11,12]. High-speed counter-current chromatography permits the direct introduction of crude samples into the column without further preparation. Although it has been successfully applied to the analysis and separation of several natural products[13-18], but high-speed counter-current chromatography has not been reported for the separation and purification of terpinenes from *Fomes officinalis ames*. The chemical structures of the two triterpenoids are shown in Fig. 1. The purpose of this study is to develop the efficiency of high-speed counter-current chromatography for the preparative separation and purification of triterpenoids from *Fomes officinalis ames*.

## EXPERIMENTAL

Preparative high-speed counter-current chromatography was conducted using a TBE-300 b high-speed counter-current chromatography system (Tautobiotech, Shanghai, China). The apparatus was equipped with three polytetrafluoroethylene preparative coils connected in a series (tube diameter: 2.6 mm; total volume : 300 mL) and a 20 mL sample

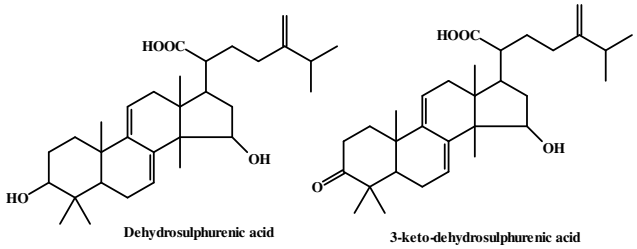


Fig. 1. Chemical of dehydrosulphurenic acid and 3-keto-dehydrosulphurenic acid

loop. The revolution radius or the distance between the holder and the central axis of the centrifuge (R) was 5 cm. The β value varied from 0.5 at the internal terminal to 0.8 at the external terminal (*i.e.*, β = r/R, where r is the distance from the coil to the holder shaft). The high-speed counter-current chromatography system was equipped with a TBP5002 pump, a TBD-2000 UV detector and a Wuhao workstation (Shanghai Wuhao Information Technology Co. Ltd., China). The experimental temperature was adjusted by HX 1050 constant temperature facilitate circulation (Beijing Boyikang Lab Implement, Beijing, China).

The analytical HPLC system used in the study was made up of an LC-20 AT pump and a SPD-20A UV-VIS detector (Shimadzu, Japan) and an LC solution workstation.

**Preparation of crude triterpenoids from *Fomes officinalis ames*:** The dried roots of *Fomes officinalis ames* were ground into powder. Approximately 50 g of powdered *Fomes officinalis ames* was extracted after 2 h of refluxing with 250 mL ethanol twice. After filtering the mixture, 450 mL filtrate was collected. The extract was then concentrated by removing the ethanol content using rotary vapourization at 45 ºC under reduced pressure. The residue was redissolved in water with a total volume of 150 mL diluted with dichloromethane at a 1:2 ratio. The two phases were separated with a separatory funnel. Afterwards, the organic phase was evaporated to dryness by rotary vapourization at 30 ºC. The dry extract of 1.157 g was stored in a refrigerator for the subsequent high-speed counter-current chromatography separation.

**Preparation of two-phase solvent system and sample solution:** Two dual-phase solvent systems were used in the study, an *n*-hexane-ethyl acetate methanol-water (1:1.5:1.2:1, v/v) solution was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated before use. The sample solution was prepared by dissolving the crude sample in the 20 mL lower phase of the solvent system for isolation and purification.

**High-speed counter-current chromatography separation procedure:** First, the coiled column was entirely filled with the upper phase of the solvent system. The apparatus was rotated at 850 rpm, while the lower phase was pumped into the column at a flow rate of 2 mL/min. The mobile phase front then emerged and hydrodynamic equilibrium was established in the column. Afterwards, approximately 20 mL of sample solution containing 500 mg of the crude extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

**HPLC analysis:** The crude sample and the peak fractions obtained by the high-speed counter-current chromatography were analyzed by high-performance liquid chromatography (HPLC). An extend-$C_{18}$ column was used (4.6 mm × 250 mm i.d., 5 μm) (Aglient, USA) with a pre-column equipped with the same stationary phase. The mobile phase was made up of $CH_3CN$-0.4 % phosphoric acid (65:35, v/v)[19]. The flow rate was 0.8 mL/min and the effluent was monitored at 242 nm (Fig. 2).

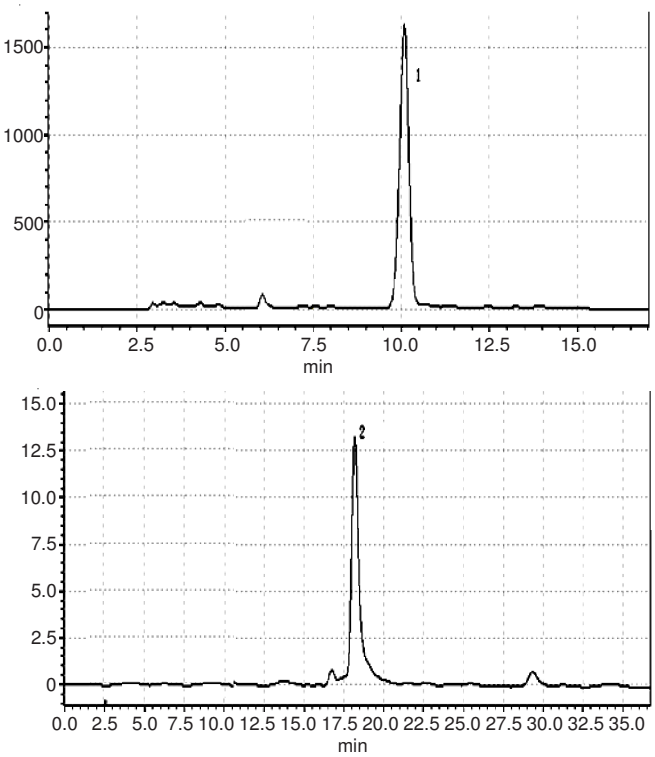


Fig. 2. HPLC chromatogram of triterpinenes purified from *Fomes officinalis ames*. Peaks: 1 = dehydrosulphurenic acid, 2 = 3-keto-dehydrosulphurenic acid. Conditions: column: reversed-phase extend-$C_{18}$ (4.6 mm × 250 mm i.d., 5 μm); mobile phase: $CH_3CN$-0.4 % phosphoric acid (65:35,v/v); flow-rate: 0.8 mL/min; detection at 242 nm

## RESULTS AND DISCUSSION

A suitable two-phase solvent system is critical for the successful isolation and separation in high-speed counter-current chromatography. Several solvent systems, including *n*-hexane-ethyl acetate-methanol-water at different volume ratios (1:1.5:0.9:1, 1:1.5:1:1, 1:1.5:1.1:1, 1:1.5:1.2:1, 1:1.5:1.3:1, v/v) were tested. Testing the five solvent systems revealed that the compounds in solvent system to be made up of *n*-hexane-ethyl acetate-methanol-water at the volume ratios of 1:1.5:0.9:1, 1:1.5:1:1 and 1:1.5:1.1:1 (v/v) with large K values (Table-1). The small K values could be produced in solvent system made up of *n*-hexane-ethyl acetate-methanol-water (1:1.5:1.3:1). The *n*-hexane-ethyl acetate-methanol-water solvent system at the volume ratio of 1:1.5:1.2:1 (v/v) was selected in this present paper. Compared to others, this solvent is most suitable for the isolation and separation process. The influence of mobile phase flow rate, the separation temperature and the revolution speed were also investigated. Results indicate that slow flow speed can produce a good separation, but requires more time, a mobile

phase and an extended chromatogram peak. Based on these aspects, the flow velocity in this study was selected as 2 mL/min. Temperature has a significant effect on the partition coefficient (K) values, stationary phase retention and the mutual solvency of the two phases. Good results were obtained after testing at 15, 20, 25, 30, 35 and 40 ºC, when the separation temperature was controlled at 25 ºC. Revolution speed has a significant influence on stationary phase retention and high rotary speed can increase the retention of the stationary phase. The revolution speed in this study was set at 850 rpm. Two fractions (I-II) were obtained in one-step elution under the optimized conditions; retention of the stationary phase was 53 %. HPLC chromatograms of the fractions obtained by high-speed counter-current chromatography are shown in Fig. 2. The HPLC analysis of each fraction revealed the components eluted in the order of peaks 1 (dehydrosulphurenic acid) and 2 (3-keto-dehydrosulphurenic acid). As shown in Fig. 3, the preparative high-speed counter-current chromatography separation of 500 mg of the crude sample using the *n*-hexane-ethyl acetate-methanol-water solvent system was at the ratio of 1:1.5:1.2:1 in a stepwise elution. The purities of dehydrosulphurenic acid and 3-keto-dehydrosulphurenic acid were 96.8 % and 92.3 %, respectively.

TABLE-1
PARTITION COEFFIIENTS (*K*) OF THE THREE COMPOUNDS IN DIFFERENT SOLVENT SYSTEMS [a]

| *n*-Hexane–ethyl acetate methanol–water | Compound 1 | Compound 2 |
|---|---|---|
| 1:1.5:0.9:1 (v/v) | 3.52 | 4.13 |
| 1:1.5:1:1 (v/v) | 2.87 | 3.16 |
| 1:1.5:1.1:1 (v/v) | 1.95 | 2.36 |
| 1:1.5:1.2:1 (v/v) | 1.42 | 1.69 |
| 1:1.5:1.3:1 (v/v) | 0.31 | 0.54 |

[a]Experimental protocol: 4 mL of each phase of the equilibrated two-phase solvent system was added to approximately 2 mg of crude sample placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase. Compounds 1–2 correspond to dehydrosulphurenic acid and 3-keto-dehydrosulphurenic acid, respectively.

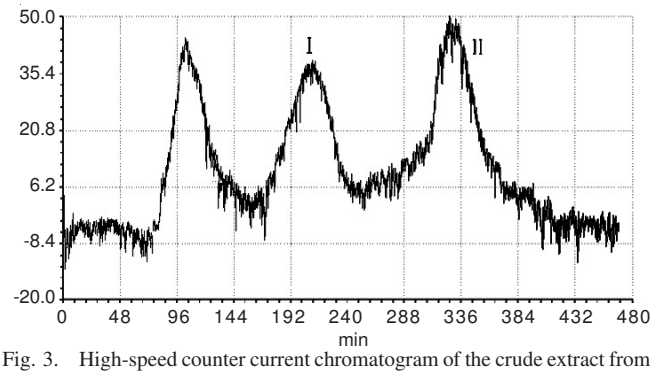


Fig. 3. High-speed counter current chromatogram of the crude extract from *Fomes officinalis ames*. Solvent system: *n*-hexane-ethyl acetate-methanol-water (1:1.5:1.2:1, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2 mL/min; revolution speed: 850 rpm; separation temperature: 25 ºC; sample size: 500 mg; retention of stationary phase: 53 %; sample loop: 20 mL; detection wavelength: 254 nm

Two compounds, dehydrosulphurenic acid and 3-keto-dehydrosulphurenic acid, were successfully isolated and separated from *Fomes officinalis ames* in a one-step high-speed counter-current chromatography process using a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water at the volume ratio of 1:1.5:1.2:1 (v/v).

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