



Quick identification of apoptosis inducer from *Isodon eriocalyx* by a drug discovery platform composed of analytical high-speed counter-current chromatography and the fluorescence-based caspase-3 biosensor detection

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ABSTRACT

Analytical high-speed counter-current chromatography (HSCCC), a unique liquid-to-liquid separation technology, has an inherent capability to provide perfect fractionation for tracking active ingredients of medicinal herbs, in a quick, efficient, and high-recovery manner. A high throughput screening (HTS) method which utilizes a novel biosensor that selectively detects apoptosis based on the fluorescence resonance energy transfer (FRET) technique, was newly established and proved to be very sensitive in detecting apoptosis induced by various known anticancer drugs. The first combination of both advanced techniques formed an efficient platform for drug discovery and succeeded in quickly identifying the most potent apoptotic constituent of a Chinese herb namely *Isodon eriocalyx*. The system of *n*-hexane/ethyl acetate/methanol/water was used as the separation solvent. The solvent ratio was first set at 3:5:3:5 to check the water-soluble part of the crude extract, and then 1:1:1:1 was used to isolate the target compounds. The active fraction was tracked and purified continuously using HSCCC which was guided by the apoptosis detection at gradually decreased drug concentrations. As a result, the most potent apoptosis inducer in this herb was discovered by analytical HSCCC equipped with a 16 ml mini-coil column, using less than 50 ml diphase solvent, from about 50 mg active fraction. It was identified as eriocalyxin B, a well-known antitumor natural product, by NMR analysis of the HSCCC purified fraction.

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1. Introduction

Chemotherapy is always one of the most important weapons to fight with cancers. Most chemotherapy drugs, e.g. taxol, exert their anticancer effects by arresting cells and then promoting them into apoptotic cell death [1]. As these drugs cannot discriminate well between cancer cells and noncancer cells, many normal cells

are also killed simultaneously. This nonspecific cytotoxicity damages the patient's immune system and generates many side effects such as vomiting, hair loss, peripheral neurotoxicity [2]. Therefore, developing new anticancer drugs with a higher potency and specificity against cancer cells has become more important. To find more anticancer agents from natural resources is all the time considered as an important strategy. High Throughput Screening (HTS) is the first choice. It requires two things: an efficient cell-based method that can rapidly screen compounds by detecting apoptotic cell death, and a large pool of drug candidates.

We have recently developed a cell-based, high throughput screen method for detecting caspase-3 activation during the process of apoptosis in living cells [3]. The new method, compared to the commonly used *in vitro* caspase activity assay that utilizes cell extracts from a large population of cells, is time-, labor- and cost-consuming. It has shown its power in testing a number of known

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anticancer drugs, including camptothecin, ET, hydroxyurea, paclitaxel and vincristine, and in searching potential anticancer agents from various natural products [4,5].

However, it is not easy to identify the active ingredients from natural materials because each of them has thousands of chemical components. Bioassay-guided fractionation therefore becomes increasingly popular in searching lead compounds. Conventionally, a variety of separation materials such as silica gel and C18 are used to do the fractionation of the crude extract. Unfortunately, all the liquid–solid separation methods often have several disadvantages and limitations: (i) irreversible sample adsorption; (ii) risk of sample denaturation; (iii) peak tailing; and (iv) high consumption of solvents and columns/packing materials [6–10]. Furthermore, the pharmaceutical industries are looking for rapid analytical method, with predictable scaled-up to pilot and process scale with high throughput and high purity [11].

High-speed counter-current chromatography (HSCCC), an advanced separation technique based on solvent partition, is right in such a good position to meet all of these requirements. First of all, HSCCC has satisfactory sample recovery without the use of any solid separation materials. More importantly, the liquid stationary phase, due to the diphasic composition, is tolerant to various crude extracts having different dissolubilities. That makes the sample pretreatment quite easy. Furthermore, the HSCCC separation can be predictable and scaled-up where complicated modeling and algorithms to explain solute behavior are unnecessary [11,12]. It also shows significant advantages in terms of sample stability and consumption of solvents and columns/packing materials.

Since the first paper in Nature by Ito et al. [13], there have been around 1000 scientific papers published regarding this technique [11]. Mostly used in these reports were semipreparative columns which volume was in a range of 200–300 ml. The large scale HSCCC was also quickly developed in recent years which column volume was counted in liters, being able to provide up-to-kilogram sample preparation [11,14]. At the same time, mini CCC at analytical scale, with the column volume less than 20 ml, is newly designed and developed. It has an inherent ability to perform quick and efficient fractionation for high throughput drug screening (HTS). Herein we report its first success in finding the most potent apoptotic component of an anticancer medicinal herb namely *Isodon eriocalyx*, with the aid of the novel caspase-3 biosensor based HTS model [3–5]. *I. eriocalyx* is one of the most representative herbs of the *Isodon* genus that is well known as the natural resource of anticancer *ent*-kauren diterpenoids. It is also argued that its active constituents should be water-soluble since this herb is traditionally used in the form of decoction.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument used in this study was TBE-20A high-speed counter-current chromatograph (Shanghai, Tauto Biotech, China) with three polytetrafluoroethylene preparative coils (diameter of tube, 0.8 mm, total volume, 16 ml). The β value varied from 0.6 at the internal terminal to 0.78 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with an analytical Agilent 1100 HPLC Series.

The preparative HSCCC instrument was TBE-300A high-speed counter-current chromatograph (Shanghai, Tauto Biotech, China) with three polytetrafluoroethylene preparative coils (diameter of tube, 2.6 mm, total volume, 260 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal

to 0.8 at the external terminal. The HSCCC system was equipped with a preparative Agilent 1100 HPLC Series.

HPLC analysis was carried out on an analytical Agilent 1100 Series and Zorbax Eclipse C18 column (4.6 mm \times 250 mm, 5 μ m) at room temperature. The Agilent 1100 HPLC system included a G1311A solvent delivery unit, G1315B UV–vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μ l loop, G1332A degasser and Agilent HPLC workstation.

UPLC–MS analyses were acquired from Waters Acquity UPLC system (Waters Co., Milford, USA) coupled with Waters Q-ToF Premier (Micromass MS Technologies, Manchester, UK) equipped with an electrospray ionization source in positive mode. The system was operated under MassLynx 4.1 software. HPLC/MS analyses were performed using a Waters ACQUITY UPLCTM system (Waters Corp., MA, USA), equipped with a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) operating in positive ion mode and a Waters ACQUITY BEH C₁₈ column (100 mm \times 2.1 mm, 1.7 μ m, Waters Corp., Ireland). MS parameters are as follows: the nebulization gas was set to 600 l/h at temperature of 350 °C, the cone gas set to 50 l/h, and the source temperature set to 100 °C. The capillary voltage and cone voltage were set to 2800 and 35 V, respectively. The molecular masses of the precursor ions and of the product ions were accurately determined with reference compound Leucine-enkephalin ($[M+H]^+$, m/z 556.2615) in the LockSpray mode. The concentration of Leucine-enkephalin was 50 pg/ μ l and the infusion flow rate was 5 μ l/min.

UPLC were performed on a Waters Acquity BEH C18 T3 (2.1 mm \times 100 mm, 1.7 μ m, Wexford, Ireland). The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid with gradient elution: 0–6 min, 5–15.9% B; 6–10 min, 15.9–40% B; 10–15 min, 40–60.5% B; 15–20 min, 60–100% B. The temperature of autosampler was maintained at 10 °C and the injection volume was 2.0 μ l.

¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as internal standard.

2.2. Reagents and sample preparation

Methanol, *n*-hexane, and ethyl acetate of HPLC grade were purchased from TEDIA company, Inc., USA. Water was prepared using MILLI-Q SP reagent water system (Nihon Millipore Kogyo K. K., Japan). The leaves of *I. eriocalyx* (Dunn.) Hara were collected from Weishan Prefecture of Yunnan Province, China, in October 2002, and was authenticated by Prof. H.W. Li (Kunming Institute of Botany). Dried powder of the leaves (20 g) was extracted with 50% aqueous methanol by sonication (3 \times 30 ml \times 30 min). The extracts were combined and concentrated *in vacuo* to give a crude extract (1.1 g). The dried extract was further dissolved in 10 ml lower phase and the sample solution was filtered through a 0.2 μ m syringe membrane filter before HSCCC separation.

2.3. Selection and preparation of two-phase solvent system

Seven major unknown peaks having gradually increasing retention time in the HPLC analysis were firstly selected and treated as targets in order to take all the components into consideration. The commonly used solvent system of *n*-hexane/ethyl acetate/methanol/water was selected in order to suit varied constituents. The solvent system was optimized in solvent ratio by analyzing the K values of these supposed targets. Approximately 2 mg of the crude extract was weighed in a 10 ml test tube to which 4.0 ml of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then, the upper and lower phases were analyzed by HPLC at room temperature. The partition coefficients (K) were expressed as the peak area of target

Table 1The separation coefficients (*K*) of supposed targets 1–7 in varied diphasic solvent systems.

Diphasic solvent system	1	2	3	4	5	6	7
<i>n</i> -Hexane/EtOAc/MeOH/H ₂ O							
1:1:1:1	0.75	0.49	1.03	0.8	0.61	1.13	2.37
4:5:4:5	1.59	1.63	1.67	0.91	1.67	3.14	3.65
3:5:3:5	3.42	3.42	2.94	3.75	3.31	5.81	4.39
2:5:2:5	8.54	9.15	12.9	ND	18.2	ND	ND
1:5:1:5	16.4	14.7	ND	ND	ND	ND	ND
6:4:5:5	0.54	0.38	0.75	0.24	0.35	0.65	1.16
7:3:5:5	0.33	0.21	0.3	0.18	0.17	0.41	0.51
8:2:5:5	0.19	0.12	0.26	0.11	0.1	0.26	0.47
9:1:5:5	0.11	0.07	0.12	0.06	0.05	0.14	0.21
10:0:5:5	0.05	0.04	0.05	0.04	0.04	0.03	0.09

ND means the value is too large to determine.

components in the upper phase divided by that in the lower phase. To obtain more accurate results, every *K* value was measured in triplicate. The results were shown in Table 1.

The selected two-phase solvent system was prepared by adding the solvents to a separation funnel according to the volume ratios and fully equilibrated by shaking repeatedly at room temperature. The upper and lower phases were separated shortly before use and degassed by sonication for 30 min.

2.4. HSCCC separation procedure

The whole procedure of preparative HSCCC separation was carried out as follows: The coil column was entirely filled with the upper phase of the solvent system at 20 ml/min. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at 1.5 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 10 ml of sample solution containing 487.76 mg of crude extract was injected through the injector. The separation temperature was controlled at 25 °C. The effluent from the outlet of the column was continuously monitored at 254 nm by Agilent 1100 HPLC UV–vis detector and was collected by a Spectra/Chrom (USA) CF-1 collector (10 min/tube). According to the chromatogram, the effluent between 70 and 80 min was labeled as the first fraction. When the separation time reached 300 min, the rotation was stopped. All the solvent remained in the coil was pushed out by high pressure gas and labeled as the last fraction, namely fraction No. 24. Each peak fraction was evaporated under reduced pressure.

The subsequent fractionation on the analytical HSCCC had a similar procedure, with only a few modifications. The coil column was entirely filled with the upper phase of the solvent system at 3 ml/min. Then the apparatus was rotated at 1600 rpm, while the lower phase was pumped into the column at 0.3 ml/min. After hydrodynamic equilibrium was established in the column, 1 ml of solution containing 57.11 mg of fraction No. 24 was injected. The effluent from the outlet of the column was collected at 5 min/tube. According to the chromatogram, the effluent between 10 and 15 min was labeled as the first subfraction. When the separation time reached 90 min, the rotation was stopped. All the solvent left in the coil was pushed out by high pressure gas and labeled as the last fraction, namely subfraction 24.12.

2.5. Bioassay of HSCCC fractions for their apoptotic effects

The bioassay method was described in our previous paper with some modifications [3]. All the testing samples were dissolved in DMSO to make stock solutions. The concentration of each stock was at least 1000 times higher than the working concentration. HeLa-C3 cells, which can detect apoptotic cell death involving caspase activation, were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml

streptomycin, in a 5% CO₂ humidity incubator at 37 °C. The sample well for apoptotic activity testing was prepared by seeding a well on a 96-well plate with 7500 HeLa-C3 cells suspended in 100 μl culture medium. The background well was prepared by only adding 100 μl culture medium. After incubation for 12–16 h, the plate was read by a Perkin-Elmer Victor plate reader. The excitation wavelength was set at 440 ± 10 nm. The emission wavelength was at 486 ± 8 nm for CFP (cyan fluorescent protein) and at 535 ± 8 nm for YFP (yellow fluorescent protein). And the data for time point “0 h” was obtained. Then immediately, the old medium was removed and 100 μl freshly prepared culture medium containing the testing sample at a certain working concentration was added to both the sample well and the corresponding background well. Culture medium containing 0.1% DMSO was the negative control while 500 nM taxol was the positive control. After that, the plate was read repeatedly at indicated time points. The data acquisition duration was up to 72 h. The YFP/CFP emission ratio was then calculated. If YFP/CFP emission ratio was reduced below 3, the testing sample was considered as a good apoptotic inducer at that concentration. All samples were

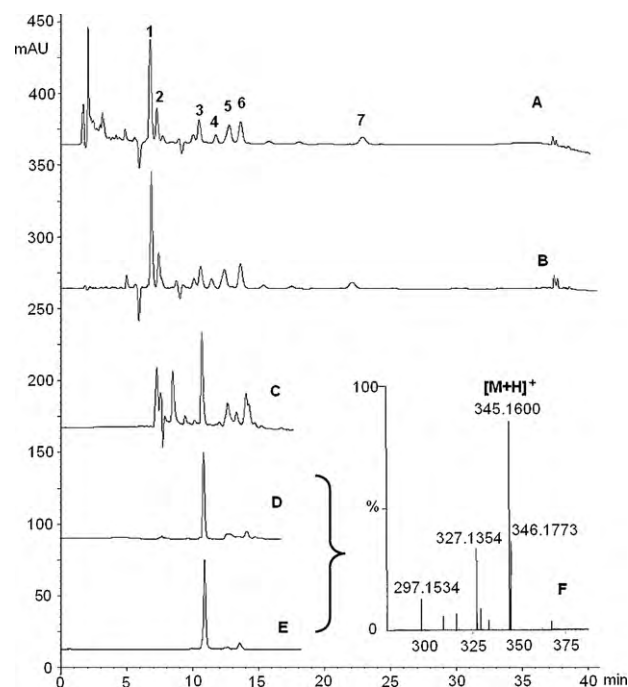


Fig. 1. HPLC chromatograms of the crude extract and active fractions. A, the crude extract; B, fraction 24; C, subfraction 24.3; D, subfraction 24.4; E, subfraction 24.5; F, the positive ESIMS spectrum of subfraction 24.5. HPLC analysis was carried out on an analytical Agilent 1100 series and Zorbax Eclipse C₁₈ column (4.6 mm × 250 mm, 5 μm) at room temperature. Mobile phase: 0–35 min, 0.1% aqueous formic acid/CAN (60:40); 35–40 min, gradually to 100% CAN. UV detection at 254 nm.

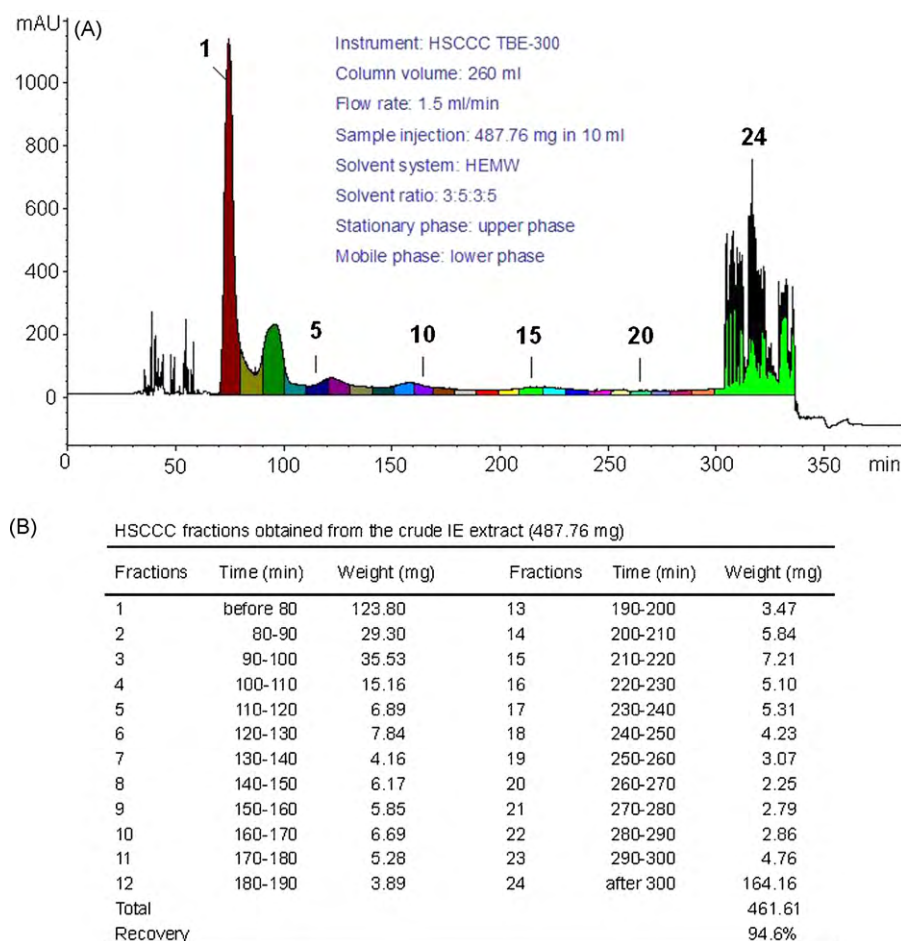


Fig. 2. HSCCC fractionation of the crude extract. A, the HSCCC chromatogram (254 nm) of the crude extract; B, the resulted 24 fractions.

tested in triplicate. The whole experiment was repeated for three times.

2.6. Identification of bioactive CCC peak fractions

The most bioactive HSCCC peak fraction was analyzed by LCMS. Its major constituent was purified using analytical HSCCC and identified by analysis of its ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra.

3. Results and discussion

Bioassay-guided fractionation does not target on any specific compound prior to the blind screening. The herbal extract will be separated into fractions according to their different polarities. There is a debate about the active components of the anticancer herb *I. eriocalyx*. Someone believes that the active components should be water-soluble because this herb is traditionally used in the form of decoction. But modern pharmacological studies indicated that the *ent*-kauren diterpenoids were cytotoxic agents [15]. These diterpenoids are the characteristic constituents of *Isodon* plants and are insoluble in water.

To figure out if any water-soluble chemicals have anticancer activity is one of the tasks of this study. First of all, seven target peaks were supposed (Fig. 1A) in different polarity regions to evaluate the diphasic solvent system. Their individual K values produced by varied solvent systems were determined and shown in Table 1. The ideal K value is always expected to be around 0.5–1.5 [7]. If K is much larger, the separation time will be much pro-

longed. By contrast, small K value will lead to a fast and poor separation. In this study, the solvent system of *n*-hexane/ethyl acetate/methanol/water (1:1:1:1) produced K values in a range of 0.49–2.37, and was selected to separate these seven chromatographic bands. Before that, however, the water-soluble chemicals in the crude extract should be checked, to see if they have anticancer effect. As such, a solvent ratio that can produce larger K values was needed. Finally, the ratio 3:5:3:5 that generated K above 3 was selected to separate the water-soluble fractions first. As a result, 23 fractions in the high polarity range were obtained after the first separation (Fig. 2). It was interesting that none of them showed apoptotic effect at the concentration level of 10 $\mu\text{g}/\text{ml}$, but the last fraction (Fig. 2), the part remained in the coil column when the separation was stopped, was highly active (Fig. 3A). The debate could be ended because the water-soluble part of the crude extract of *I. eriocalyx* did not contain any chemicals showing apoptosis inducing effect. Furthermore, HSCCC proved to be an ideal fractionation method, without missing any active chemicals. As shown in Fig. 2, the sample recovery of the first separation was up to 95%.

Combined with the HSCCC, a HeLa-C3 cell-based apoptotic detection system was used here for a fast screening. This system contained a stable HeLa cell line (HeLa-C3) that expressed a fluorescence resonance energy transfer (FRET)-based biosensor protein CFP-DEVVD-YFP [3]. It consisted of three parts: a donor cyan fluorescent protein (CFP), a peptide linker containing the caspase-3 cleavage site (DEVVD), and an acceptor yellow fluorescent protein (YFP). The detection was based on the FRET effects. Before caspase was activated, energy could be transferred directly from the donor (CFP) to the acceptor (YFP), so that when the donor was excited,

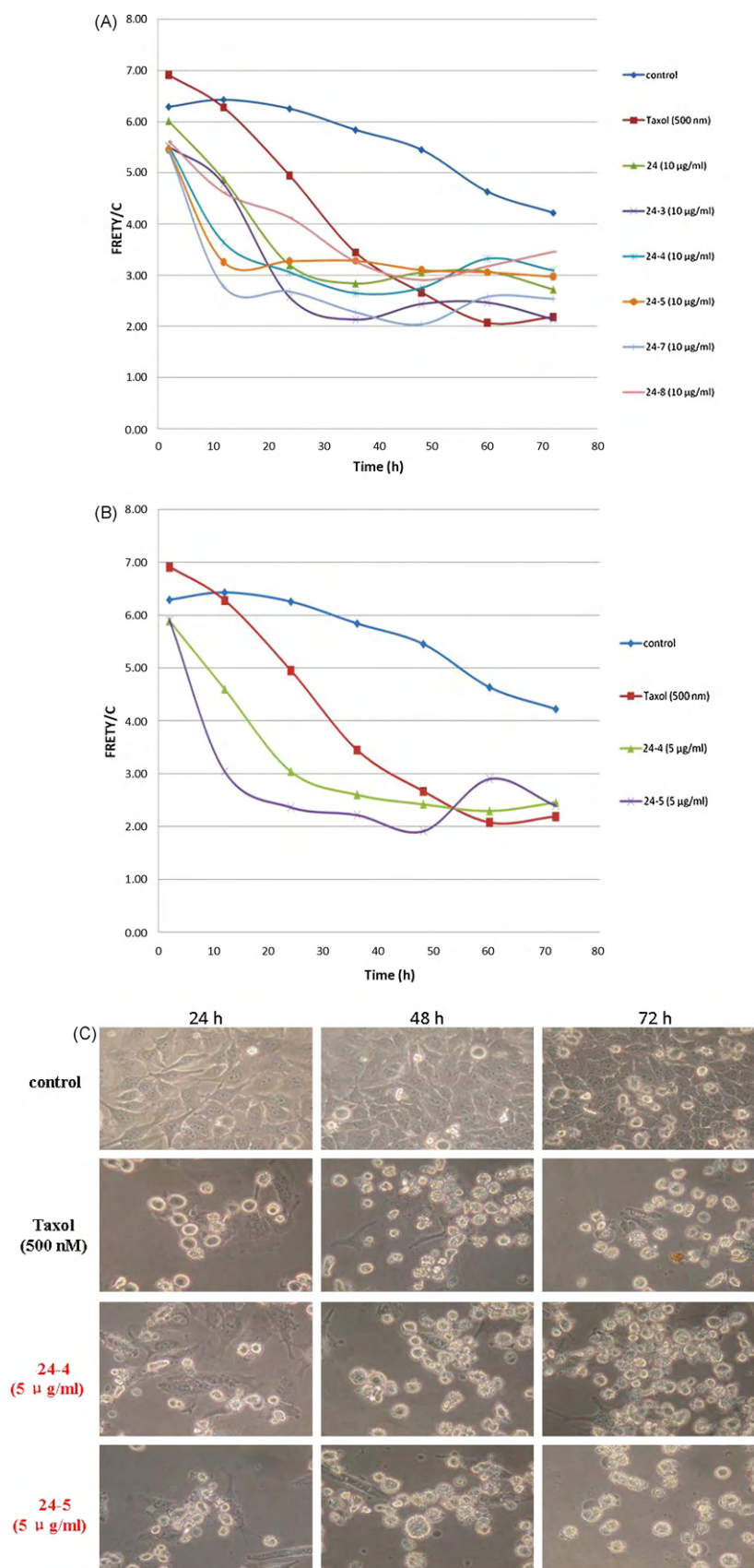


Fig. 3. Apoptosis inducing effects of screened fractions. Those having the YFP/CFP emission ratio below 3 are regarded as active fraction. A, the active fractions at a dose of 10 µg/ml; B, the active fractions at a dose of 5 µg/ml; C, cell morphology of Hela-C3 treated with subfractions 24.4 and 24.5 at 5 µg/ml. Taxol (500 nM) was used here as the positive control.

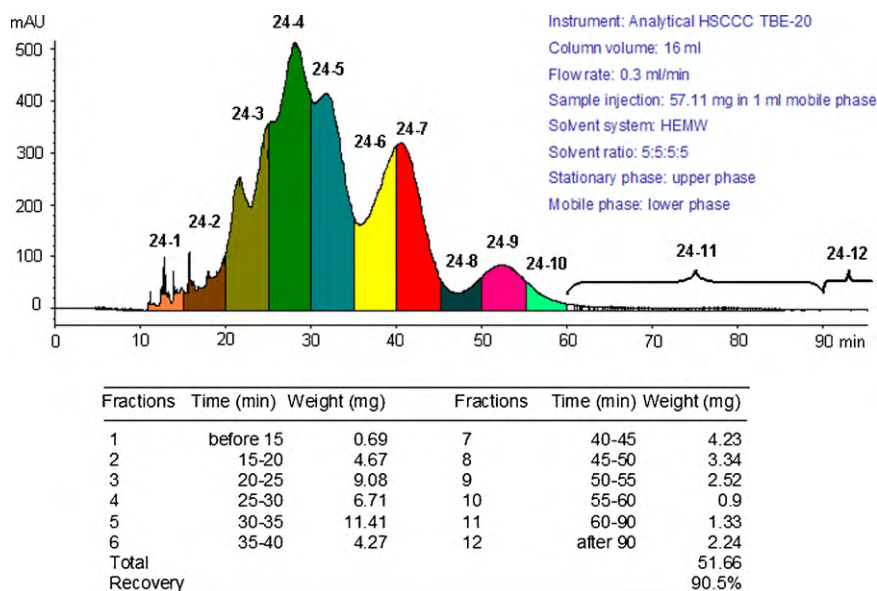


Fig. 4. Further fractionation of the active fraction using analytical HSCCC.

fluorescence emission from the acceptor could be detected. When caspase-3 was activated during apoptosis, it cleaved the biosensor (CFP–DEVD–YFP) and caused the separation of donor and acceptor proteins. Thus, the FRET effect was effectively eliminated, resulting in a reduced emission from YFP and an increased emission from CFP. The fluorescence emission ratio between YFP and CFP reflected the activation of caspase-3 in living cells during apoptosis [3]. Any stimulus that could reduce the fluorescence emission ratio of YFP/CFP to a value lower than 3.0, was considered as a good apoptotic inducer.

Giving the YFP/CFP below 3 at 10 $\mu\text{g}/\text{ml}$ in less than 40 h after drug treatment (Fig. 3A), the 24th fraction was indicated as the active fraction. HPLC examination indicated that this fraction contains all the seven target peaks (Fig. 1B). The HPLC chromatogram is similar to that of the crude extract but does not have any water-soluble component peaks. Therefore the solvent system in a ratio of 1:1:1 was selected to separate the group of seven target peaks. This time the analytical HSCCC was used to make a fast fractionation. Around half of fraction 24 (57.11 mg) was dissolved in 1 ml diphasic solvent and was then injected into the 16 ml coil column. The flow rate was kept at 0.3 ml/min. The total runtime is less than 100 min. After 10 min, the effluents were collected every 5 min and ten subfractions were obtained. The part between 60 and 90 min was subcombined and labeled as subfraction 24.11. All the solvent remained in the coil was pushed out by high pressure gas and labeled as the last subfraction, namely subfraction 24.12. That means the active fraction was quickly separated into 12 subfractions using only a small amount of solvent less than 50 ml (Fig. 4). The sample recovery is still significantly high at 90%.

All these 12 subfractions were screened for their apoptosis inducing effects. At the dose of 10 $\mu\text{g}/\text{ml}$, subfractions 24.3–24.5, 24.7 and 24.8 were active, having a YFP/CFP emission ratio below 3 (Fig. 3A). At a lower dose of 5 $\mu\text{g}/\text{ml}$, however, only subfractions 24.4 and 24.5 were still active (Fig. 3B and C). Both fractions were even stronger than the positive control (taxol, 500 nM). They produced YFP/CFP ratios lower than 3 at less than 30 h after compound treatment, at the same time that of taxol (500 nM) was still around 5.

HPLC analysis showed that subfractions 24.3–24.5 had a common component which possessed the majority of subfractions 24.4 and 24.5 (Fig. 1C–E). Subsequent LCMS analysis presented the exact molecular ion peak $[\text{M}+\text{H}]^+$ at m/z 345.1600, which was consistent

with the molecular formulae $\text{C}_{20}\text{H}_{24}\text{O}_5$ (Fig. 1F). It was proposed to be an *ent*-kauren diterpenoid based on literature research and our previous studies on *Isodon* herbs [15]. Unfortunately, MS/MS analysis on this compound failed to give more structure information due to the instable molecular ion in high collision energy dissociation. Furthermore, isomeric diterpenoids were commonly found in *Isodon* herbs, which were hard to differentiate using MS technology. Thus, NMR analysis is needed to determine the chemical structure. Before that, subfractions 24.4 and 24.5 were combined and purified using analytical HSCCC (Fig. 5). 10 mg white powder was obtained. It showed 20 carbon signals in the ^{13}C and distortionless enhancement by polarization transfer (DEPT) NMR spectra. A skeleton of 7,20-epoxy-*ent*-kaurene was assigned to this compound, based on the characteristic signals of three methines (δ 57.2, 48.3, 34.4 due to C-5, 9, and 13), three quaternary carbons (δ 59.7, 46.5, 35.9 assignable to C-8, 10, and 4), two methyls (δ 30.0 and 24.7 attributable to C-18 and 19), an oxygenated methylene (δ 65.4, assigned as C-20), and a hemiketal quaternary carbon (δ 95.6, C-7). The commonly occurred α,β -unsaturated ketone bearing terminal double bonds was also indicated by the signals at δ 208.4, 152.5, and 118.8 (C-15, 16, and 17). The additional α,β -unsaturated ketone which presented signals at δ 196.9, 127.1, and 161.2

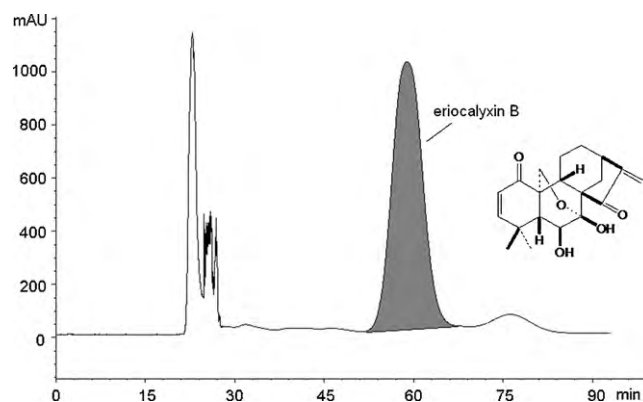


Fig. 5. Purification of the subfractions 24.4 and 24.5 using analytical HSCCC. Instrument: analytical HSCCC TBE-20; coil column volume: 16 ml; flow rate: 0.3 ml/min; sample loaded: 18.12 mg in 200 μl diphasic solvent; diphasic solvent system: HEMW (5.25:5:5.25:5); stationary phase: upper phase; mobile phase: lower phase.

(assignable to C-1, 2, and 3) suggested this compound should be 6,7-dihydroxy-7,20-epoxy-*ent*-kaur-2(3),16(17)-dien-1,15-dione, also called eriocalyxin B. This is confirmed by comparing the ^1H and ^{13}C NMR data with those reported (Supporting information, Fig. 6) [16]. And its antitumor activity has been confirmed by several reports and patent applications that it can induce apoptosis of cancer cells through NF- κ B signaling pathway [17,18].

HSCCC has such a unique separation mode that it is distinguished from all the other separation methods. First of all, theoretically there is no sample loss. Working like a continued solvent partition, it inherits all the strength of this mostly used fractionation method. In this study, the sample recoveries of both preparative and analytical HSCCC reached a high level of up to 95%. The active fraction will not be missed, even if it may not be eluted out but remained in the column. This ensures all the chemicals of the injected sample be screened in fractions as finally obtained, which is the biggest difference between HSCCC and other separation methods. Secondly, the diphasic solvent system is flexible and tolerant by adjusting the solvent ratios. This makes it possible to simultaneously take a check on both water-soluble and alcohol-soluble components of various natural products. And the pretreatment of the crude extract is simplified as much as possible. The crude extract can be directly loaded for separation after dissolved in diphasic solvent and filtrated. In addition, HSCCC also efficiently decreases the risk of sample denature that sometimes happens on solid separation materials. Another advantage is the low consumption of solvent since water is the majority of mobile phase in most cases.

Based on the above mentioned, HSCCC is a preferable tool to provide fractionation for drug discovery in natural resources. With the guidance of exact *K* value measurement, the diphasic solvent system can be designed for specific targets [19]. It can unprecedentedly focus the screening target onto any specific single molecules, if we know the right solvent system. In this study, *n*-hexane/ethyl acetate/methanol/water (3:5:3:5) was used first to separate the water-soluble part into 23 fractions, and then replaced by that of 1:1:1:1 to perform a quick separation of the active fraction and finally to isolate the most active constituent. Furthermore, HSCCC can also make contribution to the identification of active compounds by preparing sufficient samples for NMR analysis. The popularly recommended MS technology always needs some chemical references to summarize the MS fragmentation pattern of the target compound beforehand. And it is helpless in the case of isomers. What MS presents is only a deduction that needs to be

confirmed by NMR data. In this study, the application of analytical HSCCC not only provided fast fractionation, but also helped break the limitation of sample identification using MS technology, which greatly enlarged the advantages of this liquid–liquid separation technique as an important part of HTS. Combined with varied bioassays, it could be used to form a variety of HTS platforms, especially for drug discovery in herbal medicines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.07.036.

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