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Development of a method to screen and isolate potential xanthine oxidase inhibitors from *Panax japlcus var* via ultrafiltration liquid chromatography combined with counter-current chromatography

Sainan Li [a,b], Ying Tang [a], Chunming Liu [a,*], jing Li [c], Liping Guo [b,**], Yuchi Zhang [a]

[a] Central Laboratory, Changchun Normal University, No. 677 North Changji Road, Erdao District, Changchun 130032, China
[b] Faculty of Chemistry, Northeast Normal University, No. 5268 Renmin Street, Nanguan District, Changchun 130024, China
[c] The General Hospital of CNPC, Jilin 132021, China

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ABSTRACT

Panax japlcus var is a typical Chinese herb with a large number of saponins existing in all parts of it. The common methods of screening and isolating saponins are mostly labor-intensive and time-consuming. In this study, a new assay based on ultrafiltration-liquid chromatography–mass spectrometry (UF-LC–MS) was developed for the rapid screening and identifying of the ligands for xanthine oxidase from the extract of *P. japlcus*. Six saponins were identified as xanthine oxidase inhibitors from the extract. Subsequently, the specific binding ligands, namely, 24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd (the purities of them were 95.74%, 96.12%, 93.19%, 94.83%, 95.07% and 94.62%, respectively) were separated by high-speed counter-current chromatography (HSCCC). The component ratio of the solvent system of HSCCC was calculated with the help of a multiexponential function model was optimized. The partition coefficient (*K*) values of the target compounds and resolutions of peaks were employed as the research indicators, and exponential function and binomial formulas were used to optimize the solvent system and flow rate of the mobile phases in a two-stage separation. An optimized two-phase solvent system composed of ethyl acetate, isopropanol, 0.1% aqueous formic acid (1.9:1.0:1.3, v/v/v, for the first-stage) and that composed of methylene chloride, acetonitrile, isopropanol, 0.1% aqueous formic acid (5.6:1.0:2.4:5.2, v/v/v/v, for the second-stage) were used to isolate the six compounds from *P. japlcus*. The targeted compounds isolated, collected and purified by HSCCC were analyzed by high performance liquid chromatography (UPLC), and the chemical structures of all the six compounds were identified by UV, MS and NMR. The results demonstrate that UF-LC–MS combined with HSCCC might provide not only a powerful tool for screening and isolating xanthine oxidase inhibitors in complex samples but also a useful platform for discovering bioactive compounds for the prevention and treatment of gout.

1. Introduction

Gout is a fairly common metabolic disease, which has become a health problem worldwide. The prevalence of gout in the UK and Germany was 1.4% during 2000–2005 [1]. As such, the prevention and treatment of gout have become a global health problem. The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid, which plays a crucial role in gout. During the reoxidation of xanthine oxidase, molecular oxygen acts as electron acceptor, producing superoxide radical and hydrogen peroxide. These reactions can be written as follows: xanthine$+2O_2+H_2O\rightarrow$uric acid$+2O_2\bullet^-+2H^+$; xanthine$+O_2+H_2O\rightarrow$uric acid$+H_2O_2$ [2]. Decreasing the production of uric acid by xanthine oxidase inhibitors has been proven to be one of the most effective treatment strategies for chronic gout. Some synthetic xanthine oxidase inhibitors such as allopurinol [3] and febuxostat [4] have shown remarkable effectiveness against chronic gout. However, they may also cause side effects such as skin rashes, systemic vasculitis, and renal failure [5]. Thus, xanthine oxidase inhibitors from natural products have been explored as viable, harmless, and nontoxic alternatives for the treatment of gout [6]. There is a certain relationship between diabetes and gout since diabetes can cause gout or accelerate the disease. The relationship could therefore provide a guide for the design of anti-diabetic and anti-gout drugs.

The roots of *P. japlcus* (Araliaceae family) are widely used as a Chinese herbal medicine or as a food in Asian countries, for these plants are well-known for their beneficial properties. It is an

* Corresponding author. Fax: +86 431 86168777.
** Corresponding author. Fax: +86 431 85099762.
E-mail addresses: ccsf777@163.com (C. Liu), guolp078@nenu.edu.cn (L. Guo).

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important herb prescribed in traditional Chinese medicine to treat diabetes (wasting-thirst) and exhibits expectorant, antitussive, hemostatic, sedative, and analgesic activities [7]. There are a few studies on the analysis of chemical components of *P. japlcus* and its allied species. Nowadays, saponins obtained in *P. japlcus* have been centrally studied, which have anti-ulcer and anti-obesity effects [8]. Our preliminary in vitro screening study reveals that the extract from *P. japlcus* shows potent xanthine oxidase inhibitory activities. However, it is still unclear that which compounds are the active ingredients in the extract. Furthermore, the degrees of them inhibiting xanthine oxidase activities are still unknown. In the present study we investigated the inhibition of the potent xanthine oxidase activities by the extract and separated the active ingredients according to the initial screening results. The results of this study would explain and support the application of the *n*-butanol extract of *P. japlcus* to the prevention and treatment of gout.

The conventional procedure for screening the compounds with inhibiting xanthine oxidase activities from plants is in vitro screening assay. Unfortunately, the assays based on fractionation require multiple-step isolations of active compounds and conventional analyses for elucidation, which are time-consuming and labor-intensive. To overcome the limitations of in vitro methods and enhance the throughput of the drug discovery, a method based on ultrafiltration liquid chromatography–tandem mass spectrometry (UF-LC–MS) combined with high-speed countercurrent chromatography was proposed. UF-LC–MS has been proven to be a powerful tool for screening and isolating biologically active compounds from botanical extracts because the ultrafiltration step facilitates the separation of ligand–receptor complexes from unbound compounds, and the subsequent LC–MS step could identify the ligands. Low sample consumption, the obviated need for immobilization, and the reuse of enzymes are the most important advantages of UF-LC–MS for the high-throughput screen and identification of active compounds [9–11].

Compared to conventional liquid–solid separation methods, high-speed countercurrent chromatography (HSCCC) is a unique liquid – liquid partition chromatography without solid support in the system. It has been accepted as an efficient preparative technique, and widely used for the separation and purification of various natural and synthetic compounds. HSCCC was employed in this study as the separation unit. However, the current HSCCC technology has two problems that need to be resolved. (i) In the current screening practice of HSCCC solvent systems, a lot of candidate solvent systems are usually prepared, and the optimal solvent systems are selected for the experiment by partition coefficient (K) value examination [12,13]. The screening method above mentioned has a lot of randomness and blindness, and the solvent system so obtained may not be the best solvent system. During the research, it was found that the K values of the target compounds have a nonlinear relationship with the solvent composition ratios. Therefore, it is necessary to use the mathematical method to calculate the relationship between the solvent composition ratio of two-phase solvent system. (ii) Nowadays, researchers in many laboratories have focused their attention on the separation and evaluation of natural products obtained from plants for high-throughput screening [14,15]. However, such compounds have a wide range of polarity. Thus, it becomes imperative that a rapid multistage single-step separation method be developed by which a large number of compounds with varying polarities can be processed. In view of the HSCCC separation principle of the compounds distributed between two immiscible phases [16], the polarity range of the compounds to be separated by HSCCC technique is very narrow. Their treatment needs a novel separation technique with several stages to provide chemical constituents with a wide range of polarities in a relative short time.

In the present work, a simple and rapid method for screening xanthine oxidase inhibitors from the extract of *P. japlcus* was developed by combination of UF-LC–MS with HSCCC. At the same time, six compounds with xanthine oxidase binding activities were purified and separated via HSCCC (Fig. 1), and the optimal HSCCC solvent systems were established by means of a multi-exponential function model. These compounds were identified and characterised by UPLC–MS^n and NMR spectroscopy. The results may explain and support the application of *P. japlcus* extract to the prevention and treatment of gout, and other cardiovascular diseases. UF-LC–HSCCC is a powerful tool for discovering biologically active compounds from natural product extracts.

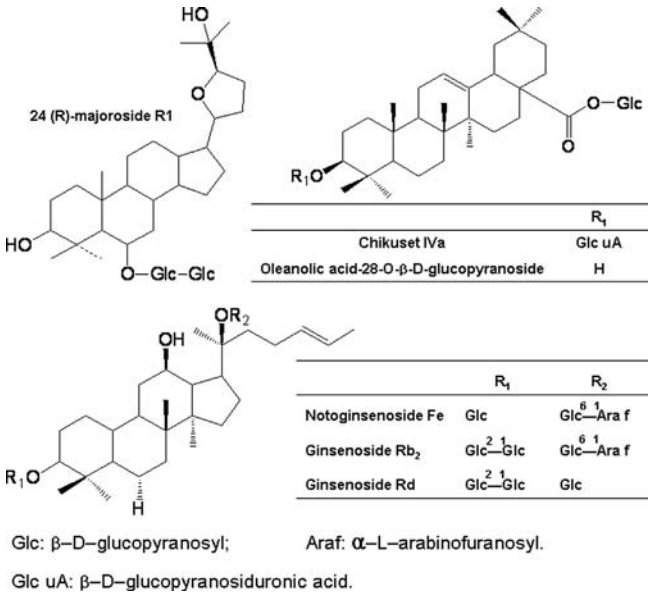

Fig. 1. Chemical structures of target compounds isolated in this study.

2. Material and methods

2.1. Apparatus

High-speed counter-current chromatography was performed on a DE Spectrum HSCCC (Dynamic Extractions, Slough, UK). The multilayer coil separation column was prepared by winding a 28 m × 2.6 mm I.D. PTFE tube directly onto one of the holders forming multiple coiled layers to give a total capacity of 125 mL (for semi-preparative separation column) and 28 mL (for analytical separation column). The β-value varied from 0.33 at the internal terminal to 0.58 at the external terminal (R=8 cm, $\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and the central axis of the centrifuge). The rotation speed was adjusted in a range of 0 to 1600 rpm, and 1400 rpm was used in the present study. The HSCCC system was equipped with a solvent delivery module of BT 8100 (Biotronic, Maintal, Germany), an integrator of D-2500 (Merck Hitachi, Darmstadt, Germany), and an injection valve with a sample loop of 10 mL. Electrospray ionization mass spectrometry was performed on a LCQ FLEET ion-trap mass spectrometer (Thermo Finnigan, San Jose, USA). High-speed liquid chromatography was carried out on a Waters 2695 coupled with a Waters 2998 Diode array detector (Milford, USA). Nuclear magnetic resonance spectra were recorded on a Bruker AV 500 spectrometer operated at 500 MHz for ^{1}H, and at 125 MHz for ^{13}C (Bruker BioSpin, Rheinstetten, Germany).

2.2. Reagents and materials

P. japlcus was purchased from Enshi, Hubei Province, China, and identified by Professor Chunming Liu (Changchun Normal University, Changchun, China). Xanthine oxidase from bovine milk was obtained from Sigma (St. Louis, USA). HPLC-grade acetonitrile and acetic acid were purchased from Fisher Scientific (Loughborough, UK). Solvents and all other chemicals were of analytical grade that were purchased from Beijing Chemical Engineering Company (Beijing, China). Water was purified on a Milli-Q water purification system (Millipore, Boston, USA).

2.3. Preparation of extract of P. japlcus

The dried roots of *P. japlcus* (500 g) were crushed and extracted three times by reflux with 1500 mL of 60% aqueous ethanol for 2.0 h. After filtration, the combined extract was concentrated under reduced pressure and redissolved in water. The crude extract was then eluted on a D101 macroporous resin column with water and *n*-butanol stepwise. Then, the elute with *n*-butanol was concentrated to dryness by rotary vaporisation at 45 °C under reduced pressure, and the residue (18.64 g) was obtained for the analysis.

The powder of *P. japlcus* was accurately weighed and dissolved in methanol (1.0 mg/mL) for analysis and determination by UF-LC–MS combined with HSCCC. It was filtered through a membrane filter (0.45 μm pore size) prior to injection and analyzed in triplicate.

2.4. Determination of xanthine oxidase inhibitory activity

The xanthine oxidase inhibition assay was performed according to a modified method from the literature [17]. Reaction mixture of 100 μL containing 25 μL of 50 mM Tris–HCl buffer (pH 8.7), 25 μL of 0.5 mM EDTA, 25 μL of 0.4 U/mL xanthine oxidase, and 25 μL of 25 μM WST-1 was used. The reaction was initiated by adding xanthine oxidase of an appropriate concentration. The reaction was allowed to proceed at 37 °C for 5 min, and then halted by adding 0.4 mL of methanol to the reaction solution. To the negative control, 25 μL Tris–HCl buffer was added in the reaction mixture as the instead of the xanthine oxidase (without the xanthine oxidase). To the positive control, allopurinol aqueous was added in the reaction mixture as the instead of the tested compound (without the extract of *P. japlcus*). The xanthine oxidase inhibition (%) was calculated via equation $(A_a - A_b)/A_a \times 100\%$, where A_a is the absorbance of the control, and A_b is the absorbance of the sample tested.

2.5. Screening procedure by UF-LC–MS

The principle of UF-LC–MS screening is that the ligands combined with macromolecular receptor could not pass through the ultrafiltration membrane. However, destabilizing conditions, such as addition of an organic solvent, would facilitate the release of bound ligands from the receptor, and the released ligands would pass through the ultrafiltration membrane. Specifically, the components bound with the receptor would be isolated and analyzed via UF-LC–MS. In our experiments, the macromolecular receptors were 24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd. The proposed method was composed of three steps: incubation, ultrafiltration, and characterisation.

A 250 μL of *P. japlcus* sample solution was incubated in 50 μL of 0.4 U/mL xanthine oxidase in 50 mM Tris–HCl buffer (pH 8.7) for 30 min at 37 °C. After incubation, the mixture was filtered through an ultrafiltration membrane (YM-100, the molecular weight cut off of 100,000 Da) according to the method [18], and then centrifuged at 10000 × *g* for 15 min at room temperature. The ultrafiltration membrane was washed three times by centrifugation with 100 μL of Tris–HCl buffer (pH 8.7) to remove the unbound compounds. The bound ligands were released by adding 100 μL of a methanol–water mixture (50:50, v/v, pH 3.30) followed by centrifugation at 10,000 × *g* for 10 min, which was repeated three times. Solvent in the ultrafiltrate was removed under vacuum, and the released ligands were used for further LC–MS analysis. The control experiments were carried out in a similar manner with denatured enzyme. All the binding assays were performed in duplicate and analyzed in triplicate.

2.6. UPLC–DAD–MS^n analysis

Aliquots (5.0 μL each) of each enzymatic reaction solution were analyzed by virtue of UPLC. The UPLC was carried out on an ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm; Waters Corp., MA). The column temperature was controlled at 25 °C. Acetonitrile (A) and 0.05% acetic acid aqueous solution (B) comprised the mobile phase. The flow rate was set to 0.4 mL/min and the eluting gradient was as follows: 95% B at 0–2 min, 95–0% B at 2–17 min, 0% B at 17–20 min. Online UV spectra were obtained over a wavelength range of 203 nm.

A Thermo LCQ-Fleet ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) connected to the UPLC/DAD instrument via an ESI interface was used to carry out MS and MS^n analyses. The mass spectrometer was operated in the negative ion mode. The samples were introduced via a syringe pump at a flow rate of 8.0 μL/min. The capillary voltage was set at −20 V in the negative ion mode. The spray voltage was set at 4.5 kV and the capillary temperature at 250 °C. The scan range was from 100 to 1000 *m*/*z*.

2.7. HSCCC partition coefficient (K) value examination

In this study, saponins of varying polarities from *P. japlcus* were separated by a two-stage HSCCC process. A mathematical model was used to optimize the *K* values of the target compounds. In the first stage, a solvent system composed of ethyl acetate, isopropanol, and 0.1% aqueous formic acid was used to separate the hydrophilic saponins. Approximately 1.0 mL of each phase was delivered into a 10 mL test tube to which about 1.0 mg of the extract of *P. japlcus* was added. The test tube was capped and shaken vigorously for 1.0 min, and allowed to stand until complete separation of the two phases. In the next step, 0.5 mL aliquot of each phase was separately put in a test tube, diluted with 1.0 mL of methanol, and analyzed by UPLC. The *K* value was expressed as the peak area of the target compound in the upper phase divided by that in the lower phase ($K = A_s/A_m$, for polar saponins). A nonlinear correlation was used for analyzing the *K* value of the target compounds and the optimal volume ratio was calculated.

In the second stage, a solvent system composed of methylene chloride, acetonitrile, isopropanol and 0.1% aqueous formic acid was used to separate the moderately hydrophilic saponins. First, 1.0 mL of the upper phase was delivered into a 10 mL test tube, and then the same volume of the corresponding lower phase was added to it. With the same operation, The *K* value was expressed as the UPLC peak area of the target compound in the upper phase (A_1) divided by the peak area in the lower phase (A_2). The diagram of the *K* value tests and the calculations are show in Fig. 2.

2.8. Optimization of the solvent system of the two-stage HSCCC by mathematical modeling

In this study, we used a mathematical model to optimize the solvent system of the two-stage HSCCC. The solvent system of

ethyl acetate, isopropanol, and 0.1% aqueous formic acid was used at the first stage of HSCCC to separate the hydrophilic compounds. Since the target compounds entirely dissolve in the isopropanol, partially dissolve in the ethyl acetate, and dissolve in the 0.1% aqueous formic acid only scarcely, the volume ratios of ethyl acetate/isopropanol and 0.1% aqueous formic acid/isopropanol were considered as independent variables, and the *K* values of the target compounds as dependent variables to calculate the function between independent and dependent variables. At the second stage, the solvent system of methylene chloride, isopropanol, acetonitrile and 0.1% aqueous formic acid was used to separate the moderately hydrophilic compounds. The target compounds can dissolve in acetonitrile, isopropanol easily, in 0.1% aqueous formic acid under certain conditions, and in methylene chloride scarcely. Therefore, the volume ratios of methylene chloride/isopropanol, acetonitrile/isopropanol, and 0.1% aqueous formic acid/isopropanol were considered as independent variables, and the *K* values of the target compounds as dependent variable to calculate the function between independent and dependent variables. A mathematical model was used to define the relationship between the independent and dependent variables in order to optimize the HSCCC solvent system by using the MATLAB software (2007b).

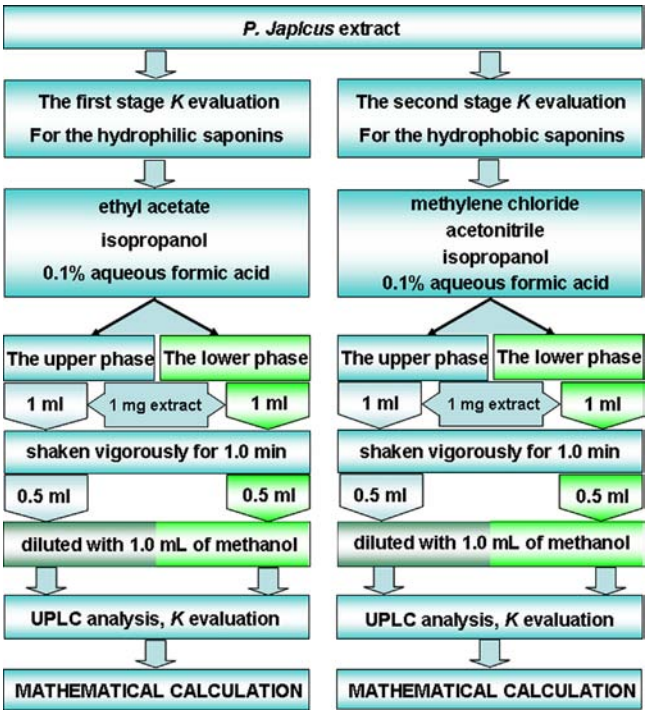

Fig. 2. The diagram of the partition coefficient values test and calculations.

2.9. Separation by HSCCC

To isolate the potential xanthine oxidase inhibitors from *P. japlcus*, separation assays were performed as follows. During the first stage of the HSCCC separation, the multilayer coil was entirely filled with the upper phase of ethyl acetate/isopropanol/0.1% aqueous formic acid (1.9:1.0:1.3, v/v/v) as the stationary phase. The coiled column was rotated at 1200 rpm, and then the lower phase was pumped into the column in the tail to head direction at a flow rate of 2.0 mL/min. After the hydrodynamic equilibrium between the two phases was established in the rotating column, 2.0 mL of a sample solution (a mixture of 1.0 mL of each phase containing 51.07 mg of extract of *P. japlcus* for the semipreparative isolation, and 1.0 mL for the analytical isolation) was injected into the column. At 250 min for the semipreparative isolation, the rotating column was stopped. The eluted compounds were monitored at 203 nm and collected into test tubes with a fraction collector. The fractions were collected according to the elution profile and evaporated with a rotary evaporator. The residues were stored in a refrigerator prior to UPLC and ESI–MS analyses. The retention rate of the stationary phase, relative to the total column capacity, was computed from the volume of the stationary phase collected from the column after the separation was complete.

At the second stage of the HSCCC separation, the solution was injected into the sample loop and pumped into the HSCCC column via the same method as that of the first stage. The fractions were collected according to the elution profile and evaporated via a rotary evaporator. Sample residues were stored in a refrigerator prior to UPLC, MS^n, and NMR analyses. The retention rate of the stationary phase, relative to the total column capacity, was computed from the volume of the stationary phase collected from the column after the separation was complete.

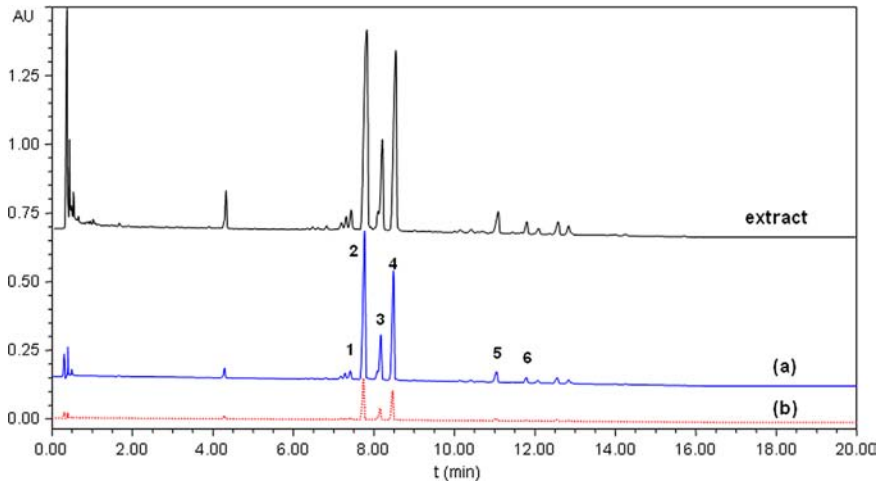

Fig. 3. UPLC chromatograms of the chemical constituents of *P. japlcus* obtained by ultrafiltration (at 203 nm). The UPLC chromatogram obtained by ultrafiltration chromatography of *P. japlcus* extract; analysis was a screening procedure for the ligands binding to xanthine oxidase. The solid line represents the experiment of extract with xanthine oxidase (a), and the dashed line represents the experiment of denatured xanthine oxidase (b). (1) 24 (*R*)-majoroside R_1; (2) chikusetsusaponin IVa; (3) oleanolic acid-28-*O*-β-D-glucopyranoside; (4) notoginsenoside Fe; (5) ginsenoside Rb_2; (6) ginsenoside Rd.

3. Results and discussion

3.1. Evaluation of xanthine oxidase inhibitory activity

The UPLC chromatogram of the extract of *P. japlcus* is shown in Fig. 3. The MS^n data of the chemical compounds from *P. japlcus* were acquired in negative ion mode. In total, 6 peaks appear in the UPLC–MS^n total ion chromatograms. Table 1 lists their retention time (t_R), MS and MS^n fragmentation ions. The result indicates that compounds 1–6 were identified as 24 (*R*)-majoroside R_1, Chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd, respectively. The MS^n data of the compounds mentioned above are consistent with those reported in the literature [19].

The xanthine oxidase inhibitory activity of the extract was 41.81% at 1.0 mg/mL, as evaluated by in vitro assays. It can be seen from Table 2 that the inhibitory activity order of the compounds is: chikusetsusaponin IVa > notoginsenoside Fe > oleanolic acid-28-*O*-β-D-glucopyranoside > ginsenoside Rb_2 > 24 (*R*)-majoroside R_1 > ginsenoside Rd. The obtained data demonstrate that chikusetsusaponin IVa, notoginsenoside Fe, oleanolic acid-28-*O*-β-D-glucopyranoside, ginsenoside Rb_2, 24 (*R*)-majoroside R_1 and ginsenoside Rd had considerable xanthine oxidase inhibitory activity compared with the well-known, synthetic XOD inhibitor, allopurinol (an inhibition rate of 50.90% at 1.0 mg/mL). Therefore, it would be worthwhile to screen and separate the active compounds in the extract of *P. japlcus*.

3.2. Screening of xanthine oxidase ligands in P. japlcus by UF-LC–MS

After the extract was incubated with xanthine oxidase and purified by affinity ultrafiltration, the trapped ligands in the extract from *P. japlcus* were analyzed by UPLC. Compounds specifically bound to xanthine oxidase that were incubated with xanthine oxidase showed higher intensities of the peaks than the control samples incubated with denatured enzyme. As shown in Fig. 3 (a) and (b), larger peaks of the six trapped ligands (chikusetsusaponin IVa, notoginsenoside Fe, oleanolic acid-28-*O*-β-D-glucopyranoside, ginsenoside Rb_2, 24 (*R*)-majoroside R_1 and ginsenoside Rd) were observed in the chromatogram. Other compounds were not considered as xanthine oxidase ligands because they could not be distinguished from the control sample by the ultrafiltration screening assay. Therefore, the six trapped ligands were selected as the target compounds to be isolated in the subsequent experiments.

3.3. Optimization of HSCCC solvent system based on multiexponential function model

A successful HSCCC separation largely depends on a suitable two-phase solvent system, and the solvent system should possess the partition coefficient (K) for target compounds and take a short settling time. The solvent systems of ethyl acetete–isopropanol–0.1% aqueous formic acid at volume ratios of 0.75:1.0:1.5, 1.0:1.0:1.5, 1.25:1.0:1.5, 1.5:1.0:1.5, 1.75:1.0:1.5, 2.0:1.0:1.5, 2.25:1.0:1.5 and 2.50:1.0:1.5, and the solvent systems of methylene chloride–isopropanol–acetonitrile–0.1% aqueous formic acid at volume ratios of 3.5:1.0:0.5:3.0, 4.0:1.0:1.0:3.5, 4.5:1.0:1.5:4.0, 5.0:1.0:2.0:4.5, 5.5:1.0:2.5:5.0, 6.0:1.0:3.0:5.5, 6.5:1.0:3.5:6.0, 7.0:1.0:4.0:6.5, 7.5:1.0:4.5:7.0, and 8.0:1.0:5.0:7.5 were selected for two-stage separation, respectively.

At the first stage, the K values of oleanolic acid-28-*O*-β-D-glucopyranoside, chikusetsusaponin IVa and 24 (*R*)-majoroside R_1, partitioned between the upper and lower phases of the ethyl acetate–isopropanol–0.1% aqueous formic acid mixture at various volume ratios (0.7 5:1.0:1.5, 1.0:1.0:1.5, 1.25:1.0:1.5, 1.5:1.0:1.5, 1.75:1.0:1.5, 2.0:1.0:1.5, 2.25:1.0:1.5, and 2.5:1.0:1.5) were obtained and calculated (Fig. 4). The K values indicate that when the ethyl acetate fraction increased, the K values of the target compounds decreased. The ethyl acetate:isopropanol and 0.1% aqueous formic acid: isopropanol ratios were considered to be the independent variables, and the K values were the dependent variables.

Because there were only two independent variables in the first stage, in the calculation process, we first fixed the volume ratio of 0.1% aqueous formic acid/isopropanol to calculate the function between the K value of the target compound and volume ratio of ethyl acetate/isopropanol, and then to calculate the function between the K value of the target compound and volume ratio of 0.1% aqueous formic acid/isopropanol after determining the optimum volume ratio of ethyl acetate/isopropanol. The results indicate that the K value of the target compound and the ethyl acetate:isopropanol ratio conform to an exponential function. The K values (k) of oleanolic acid-28-*O*-β-D-glucopyranoside ($k_{1,1}$), chikusetsusaponin IVa ($k_{1,2'}$), and 24 (*R*)-majoroside R_1 ($k_{1,3'}$) vary exponentially with the ethyl acetate:isopropanol ratio ($x_{1,1}$) according to Eqs. (1.1–1.3):

$$k_{1,1'} = 8.0998e^{-1.4859x_{1,1}}, \quad R^2 = 0.9969; \tag{1.1}$$

$$k_{1,2'} = 14.404e^{-1.5844x_{1,1}}, \quad R^2 = 0.9925; \tag{1.2}$$

$$k_{1,3'} = 28.527e^{-1.6486x_{1,1}}, \quad R^2 = 0.9972. \tag{1.3}$$

Table 2
Activities of the related compounds to inhibit xanthine oxidase (n = 3).

Compounds	Inhibitory rate[a,b]	IC_{50} (mg/mL)[a]
24 (*R*)-majoroside R_1	23.22% ± 3.21%	2.08 ± 0.18
Chikusetsusaponin IVa	52.87% ± 4.32%	5.03 ± 0.36
Oleanolic acid-28-*O*-β-D-glucopyranoside	36.76% ± 3.55%	3.52 ± 0.21
Notoginsenoside Fe	43.01% ± 3.63%	4.20 ± 0.26
Ginsenoside Rb_2	24.93% ± 2.23%	2.71 ± 0.17
Ginsenoside Rd	22.23% ± 1.86%	1.86 ± 0.15

[a] The result was an average of three determinations.
[b] All of the concentrations were 1.0 mg/mL when the inhibitory rate was determined.

Table 1
Compounds detected in *P. japlcus* by UPLC–MS/MS.

Peak	t_R (min)	MS (m/z)	MS/MS (m/z)	Identification
1	7.41	815.7 $[M–H]^-$	815.7 $[M–H]^-$, 653.2, 491.7	24 (*R*)-majoroside R_1
2	7.76	793.5 $[M–H]^-$	793.5 $[M–H]^-$, 631.3, 455.2	Chikusetsusaponin IVa
3	8.19	617.1 $[M–H]^-$	617.1 $[M–H]^-$, 753.3	Oleanolic acid-28-*O*-β-D-glucopyranoside
4	8.51	915.5 $[M–H]^-$	915.5 $[M–H]^-$, 754.7, 592.4	Notoginsenoside Fe
5	11.09	1077.4 $[M–H]^-$	1077.4 $[M–H]^-$, 945.1, 783.2	Ginsenoside Rb_2
6	11.81	945.6 $[M–H]^-$	945.6 $[M–H]^-$, 784.1, 621.8	Ginsenoside Rd

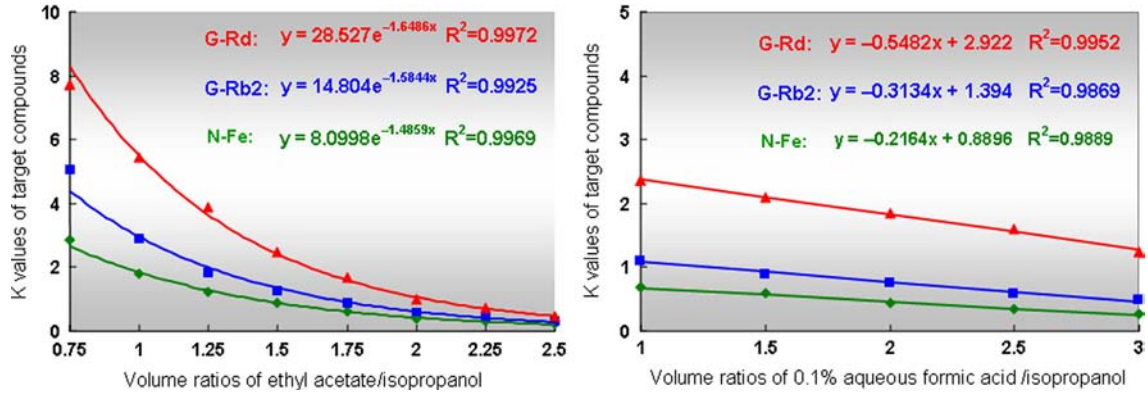

Fig. 4. Correlations between volume ratios of ethyl acetate/isopropanol of the first stage and *K* values of ginsenosides Rd, Rb_2 and notoginsenoside Fe (a); Correlations between volume ratios of 0.1% aqueous formic acid/isopropanol of the first stage and *K* values of ginsenoside Rd, Rb_2 and notoginsenoside Fe (b).

To ensure complete separation and to shorten run time, the qualifications, noted in Eq. (1.4), were set:

$$\begin{cases} 0.5 \le k_{1,1'} \le 2.5; 0.5 \le k_{1,2'} \le 2.5; 0.5 \le k_{1,3'} \le 2.5 \\ k_{1,3'} - 1.5k_{1,2'} \ge 0; k_{1,2'} - 1.5k_{1,1'} \ge 0 \\ Min(k_{1,1'} + k_{1,2'} + k_{1,3'}) \end{cases} \quad (1.4)$$

The $Min(k_{1,1'}+k_{1,2'}+k_{1,3'})$ qualification will ensure that the shortest separation time will be achieved at the minimum *K* value. Based on the MATLAB software, the following results were obtained: $k_{1,1'}=0.5107$, $k_{1,2'}=0.7562$, $k_{1,3'}=1.3074$, $x_{1,1}=1.87$. This result indicates that the optimal ratio of ethyl acetate to isopropanol is 1.87. Using the conditions described above, the theoretical *K* values of oleanolic acid-28-*O*-β-D-glucopyranoside, chikusetsusaponin IVa and 24 (*R*)-majoroside R_1 are 0.5107, 0.7562 and 1.3074, respectively.

After the optimal ratio of ethyl acetate to isopropanol had been calculated, the relationship between *K* of the target compound and the 0.1% aqueous formic acid to isopropanol ratio was calculated. The data indicates that *K* depends linearly on the 0.1% aqueous formic acid to isopropanol ratio. The relationship between each of the *K* values (k) of oleanolic acid-28-*O*-β-D-glucopyranoside, chikusetsusaponin IVa and 24 (*R*)-majoroside R_1 and the 0.1% aqueous formic acid/isopropanol ratio ($x_{1,2}$) is expressed as Eqs. (1.5–1.7):

$$k_{1,1} = -0.2164x_{1,2} + 0.8896, \quad R^2 = 0.9889; \quad (1.5)$$

$$k_{1,2} = -0.3134x_{1,2} + 1.3940, \quad R^2 = 0.9869; \quad (1.6)$$

$$k_{1,3} = -0.5482x_{1,2} + 2.922, \quad R^2 = 0.9952. \quad (1.7)$$

To ensure complete separation and to save time, the *K* values for the analytes, according to the golden rules of HSCCC [20], should fall within the range of $0.5 \le K \le 1.0$. The elution by solvent systems with small *K* values leads to low resolution, while elution by those with large *K* values results in better resolution, but signal broadened. To achieve good separation quickly, y_1 was set to 0.6. According to RULE 5 of the "HSCCC golden rules" [19], the ratio of the two *K* values, or the separation factor ($\alpha = K_1/K_2$, where $K_1 > K_2$), ought to be greater than 1.5 for commercial, semi-preparative, multilayer separation columns. $y_{2,2}-1.5y_{2,1} \ge 0$ and $y_{2,3}-1.5y_{2,2} \ge 0$ needed defining. y_3 was defined as less than 2.5 because more time was needed when this value was greater than 2.5. Therefore, the qualifications, as described in Eq. (1.8), were defined:

$$\begin{cases} 0.6 \le k_{1,1} \le 2.5; 0.6 \le k_{1,2} \le 2.5; 0.6 \le k_{1,3} \le 2.5 \\ k_{1,3} - 1.5k_{1,2} \ge 0; k_{1,2} - 1.5k_{1,1} \ge 0 \\ Max(x_{1,2}) \end{cases} \quad (1.8)$$

The $Max(x_{1,2})$ function will maximize the 0.1% aqueous formic acid:isopropanol ratio, which will reduce the volume of isopropanol required and the cost associated with it. With the aid of the MATLAB software, the following results were obtained: $y_{1,1}=0.6018$, $y_{1,2}=0.9772$, $y_{1,3}=2.1929$, $x_{1,2}=1.33$. The results indicate that the optimal ratio of 0.1% aqueous formic acid to isopropanol was 1.33. Under the conditions described previously, the theoretical *K* values for oleanolic acid-28-*O*-β-D-glucopyranoside, chikusetsusaponin IVa and 24 (*R*)-majoroside R_1 were 0.6018, 0.9772, and 2.1929, respectively. The optimal ethyl acetate:isopropanol:0.1% aqueous formic acid ratio was determined to be 1.87:1.0:1.33.

A solvent system composed of methylene chloride, acetonitrile, isopropanol, and 0.1% aqueous formic acid was used in the second stage. There will be three independent variables at the second stage, and the methods used to determine the optimal solvent system in the first stage cannot be used for these four-component systems. A multivariate exponential model was applied to the system.

The target compounds of the second stage were notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd. The *K* values of the above-mentioned compounds between the upper phase and the lower phase of methylene chloride–isopropanol–acetonitrile–0.1% aqueous formic acid at volume ratios of 3.5:1.0:0.5:3.0, 4.0:1.0:1.0:3.5, 4.5:1.0:1.5:4.0, 5.0:1.0:2.0:4.5, 5.5:1.0:2.5:5.0, 6.0:1.0:3.0:5.5, 6.5:1.0:3.5:6.0, 7.0:1.0:4.0:6.5, 7.5:1.0:4.5:7.0, and 8.0:1.0:5.0:7.5 were obtained which are summarized in Table 3. The *K* values indicate the regularity by increasing or decreasing the portion of methylene chloride, acetonitrile, or isopropanol. The *K* values will be higher by increasing the portion of acetonitrile, and the *K* values will be lower by increasing the portion of methylene chloride or decreasing the portion of isopropanol.

The methylene chloride:isopropanol, acetonitrile:isopropanol, and 0.1% aqueous formic acid:isopropanol ratios were defined as independent variables, and the *K* values of the target compounds were defined as the dependent variables. A mathematical model was used to define the relationship between the independent variable and the dependent variable to optimize the HSCCC solvent system. The ratio of methylene chloride to isopropanol was denoted as $x_{2,1}$, the ratio of acetonitrile to isopropanol as $x_{2,2}$, and the ratio of 0.1% aqueous formic acid to isopropanol as $x_{2,3}$. The *K* value of notoginsenoside Fe distributed in the two-phase

Table 3
Partition coefficient values ($K_{UP/LP}$) of 24 (*R*)-majoroside R_1, chikusetsusaponin IVa and oleanolic acid-28-*O*-β-D-glucopyranoside from *P. japlcus* at different volume ratios of methylene chloride to isopropanol, acetonitrile to isopropanol, and 0.1% aqueous formic acid to isopropanol.

$x_{2,1}$[a]	$x_{2,2}$	$x_{2,3}$	$k_{2,1}$[b]	$k_{2,2}$	$k_{2,3}$
3.5	0.5	3.0	2.36	3.29	5.20
4.0	1.0	3.5	1.86	2.41	4.10
4.5	1.5	4.0	1.23	1.96	3.49
5.0	2.0	4.5	0.85	1.48	2.72
5.5	2,5	5.0	0.62	1.21	2.29
6.0	3.0	5.5	0.52	1.00	1.90
6.5	3.5	6.0	0.36	0.80	1.73
7.0	4.0	6.7	0.22	0.68	1.48
7.5	4.5	7.0	0.21	0.61	1.15
8.0	5.0	7.5	0.12	0.43	1.02
8.5	5.5	8.0	0.10	0.38	0.92

[a] $x_{2,1}$: the ratio of methylene chloride to isopropanol; $x_{2,2}$: the ratio of acetonitrile to isopropanol; $x_{2,3}$: the ratio of 0.1% aqueous formic acid to isopropanol.

[b] $k_{2,1}$: *K* value of 24 (*R*)-majoroside R_1; $k_{2,2}$: *K* value of chikusetsusaponin IVa; $k_{2,3}$: *K* value of oleanolic acid-28-*O*-β-D-glucopyranoside.

solvent system was denoted as $k_{2,1}$, the *K* value of ginsenoside Rb_2 as $k_{2,2}$, and the *K* value of ginsenoside Rd as $k_{2,3}$. With the help of a nonlinear regression function, it was found that functions of $k_{2,1}$, $k_{2,2}$, or $k_{2,3}$ versus $x_{1,1}$, $x_{1,2}$, or $x_{1,3}$ can be expressed as the exponential function $c_1e^{a1\times 1+b1}+c_2e^{a2\times 2+b2}+c_3e^{a3\times 3+b3}$ proved by testing the least square error. Functions concerning $x_{2,1}$, $x_{2,2}$, or $x_{2,3}$ were expressed as Eqs. (2.1–2.3):

$$k_{2,1}=3.4577e^{-0.5967x2,1+1.5815}-3.1265e^{-6.2564x2,2+2.4411}+127.6189e^{-3.1443x2,3+5.1989},\quad R^2=0.9632 \tag{2.1}$$

$$k_{2,2}=3.0731e^{-0.3757x2,1+1.6236}-1.3231e^{-0.376x2,2+0.4263}+6.9316e^{-1.4861x2,3+2.2729},\quad R^2=0.9849 \tag{2.2}$$

$$k_{2,3}=2.9253e^{-0.2935x2,1+2.057}-2.1936e^{-0.2935x2,2+0.8327}+3.5547e^{-0.9833x2,3+1.9897},\quad R^2=0.9795 \tag{2.3}$$

Eqs. (2.1–2.3) were used to optimize the partition coefficients $k_{2,1}$, $k_{2,2}$, and $k_{2,3}$. According to the preliminary results, when $x_{2,1}$ is greater than 7, the partition coefficient of notoginsenoside Fe will be less than 0.25, and that of ginsenoside Rb_2 will be less than 0.60, which means that these two compounds will be eluted easily, but cannot be separated. When $x_{2,1}$ is less than 5.0, the partition coefficient of ginsenoside Rd will be greater than 2.50, which means this compound will be eluted slowly and the separation time will be relatively long. Thus $x_{2,1}$ was set between 5.0 and 7.0. According to the same requirements above mentioned, $x_{2,2}$ was defined between 2.0 and 4.0, and $x_{2,3}$ was defined as greater than 4.5.

The partition coefficients of notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd were denoted as $k_{2,1}$, $k_{2,2}$, and $k_{2,3}$, respectively. The optimization requirements were set as shown in Eq. (2.4):

$$\begin{cases} 5\le x_{2,1}\le 7; 2\le x_{2,2}\le 4; x_{2,3}\ge 4 \\ 0.5\le k_{2,1}\le 0.7; k_{2,2}-1.5k_{2,1}\ge 0; k_{2,3}-1.5k_{2,2}\ge 0; k_{2,3}\le 2.5 \\ Min(k_{2,1}+k_{2,2}+k_{2,3}) \end{cases} \tag{2.4}$$

The $Min(k_{2,1}+k_{2,2}+k_{2,3})$ requirements will ensure the shortest separation time at the minimum *K* value. Based on MATLAB, the results were obtained as Eq. (2.5):

$$\begin{cases} x_{2,1}=5.6; x_{2,2}=2.4; x_{2,3}=5.2 \\ k_{2,1}=0.6123; k_{2,2}=1.1451; k_{2,3}=2.2015 \end{cases} \tag{2.5}$$

The result indicates that the optimal ratio of methylene chloride to isopropanol was 5.6, the optimal ratio of acetonitrile to isopropanol was 2.4, and the optimal ratio of 0.1% aqueous formic acid to isopropanol was 5.2. Under the conditions described above, the theoretical *K* values of notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd were calculated to be 0.6123, 1.1451, and 2.2015, respectively. The optimal ratio of methylene chloride: isopropanol:acetonitrile:0.1% aqueous formic acid for the second stage was determined to be 5.6:1.0:2.4:5.2.

3.4. HSCCC separation of potential xanthine oxidase inhibitors from *P. japlcus*

The separation procedures for HSCCC are described in "Two-stage HSCCC Separation". The relative deviations of the *K* values calculated via the exponential functions from the experimentally determined *K* values did not exceed ±5.0%, indicating that the exponential model can be used reliably to optimize solvent system and predict the *K* values of target compounds. Retention rates of the stationary phases were 57% and 63%, respectively, which are acceptable for HSCCC separations.

During the HSCCC separation with the two-stage, we first used the analytical separation column to test the solvent system. The result indicates that the six target compounds will be separated very well when the solvent system of ethyl acetate/isopropanol/0.1% aqueous formic acid (1.9:1.0:1.3, v/v/v, for the first-stage) and that of methylene chloride/isopropanol/acetonitrile/0.1% aqueous formic acid (5.6:1.0:2.4:5.2, v/v/v/v, for the second-stage) were used for HSCCC separation. Thus, the solvent systems mentioned above were selected as the HSCCC solvent systems. Furthermore, we selected three flow rates to obtain the best semipreparative isolation. When we chose a flow rate of 1.5 mL/min, the complete elution required 350 min. When the flow rate was set at 2.0 mL/min, the elution time was reduced to 250 min. The desired compounds were also entirely eluted, and all the compounds were separated very well. However, when the flow rate was set to 2.5 mL/min, the time was shortened by a lot but some compounds were not separated well. Although this higher flow rate could decrease separation time, many compounds were not separated well and some compounds might be lost. Reducing the flow rate could improve the retention of stationary phase and resolution, but it would extend the total time for the separation. Therefore, we selected 2.0 mL/min as the flow rate for the semipreparative isolation. Finally, a flow rate of 2.0 mL/min, a revolution speed of 1200 rpm, and a temperature of 30 °C were used in the experiment. Fig. 5 shows the semipreparative (containing 51.07 mg of the extract) HSCCC separation of the extract of *P. japlcus* eluted with the two-stage solvent systems. The chromatographic purities of HSCCC fractions 1–6 (24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd) were 95.74%, 96.12%, 93.19%, 94.83%, 95.07% and 94.62%, respectively, determined by UPLC, the isolation rates for compounds 1–6 were 0.24, 1.85, 1.46, 1.73, 0.32 and 0.30 mg/g, respectively, which were directly used for ESI–MS^n analyses. Furthermore, for the comparison, the extract was also separated by conventional column chromatography, the results indicated that only four compounds including chikusetsusaponin IVa, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd were separated, the purities of the four compounds were 90.10%, 87.23%, 90.02% and 84.33%, respectively, and the isolation rates for compounds were1.65, 1.58, 0.21, and 0.12 mg/g, respectively. The result

indicates that the HSCCC have the advantage over conventional preparation method.

3.5. Purity analyses and identification of xanthine oxidase inhibitors by ESI–MS^n

The potential xanthine oxidase inhibitors from the extract of *P. japlcus* by HSCCC were analyzed by UPLC-PDA. The UPLC chromatograms of compounds 1–6 are shown in Fig. 6. With the method developed, the purities of the six target compounds all exceeded 90.0%. Identification of the HSCCC peaks was based on UPLC retention time, MS/MS data, and ^{13}C NMR data. The UPLC/MS and ^{13}C NMR data indicate that target compounds 1–6 are 24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd, respectively.

The ESI–MS and NMR data were provided in the supplementary information. The ^1H and ^{13}C NMR data are in agreement with those of corresponding compounds in the literature [21–26].

4. Conclusions

The present work demonstrates that ultrafiltration liquid chromatography/mass spectrometry coupled with high-speed countercurrent chromatography is a powerful tool for rapid screening, characterizing and isolating xanthine oxidase inhibitors from

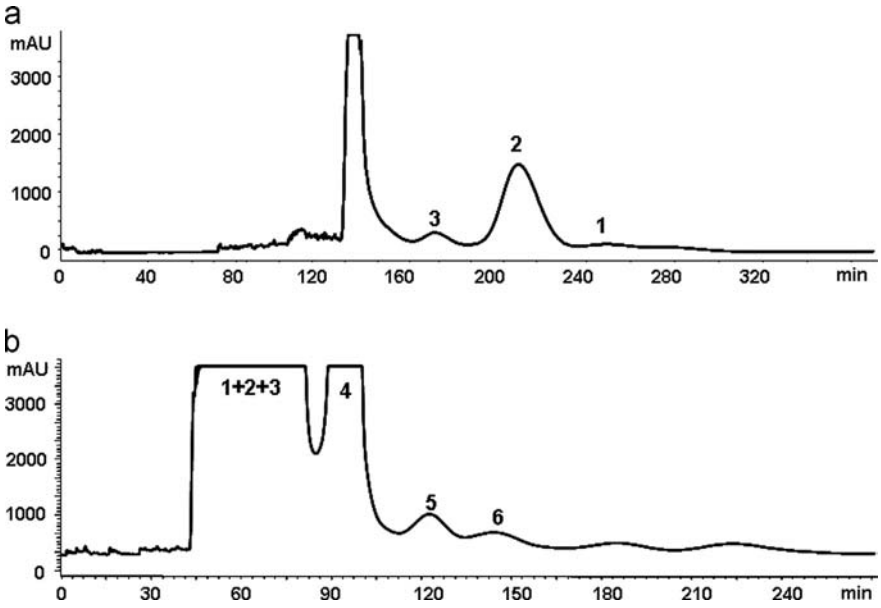

Fig. 5. HSCCC chromatograms of *P. japlcus*. HSCCC chromatogram of *P. japlcus* on a semi-preparative column (125 mL. Mobile phase: the lower phase; flow rate of the mobile phase: 2.0 mL/min; revolution speed: 1200 rpm). The optimized two-phase solvent system at the first stage was composed of ethyl acetate, isopropanol, 0.1% aqueous formic acid (1.9:1.0:1.3, v/v/v), (a); The optimized two-phase solvent system at the second stage was composed of methylene chloride, acetonitrile, isopropanol, 0.1% aqueous formic acid (5.6:1.0:2.4:5.2, v/v/v) (b). (1) 24 (*R*)-majoroside R1; (2) chikusetsusaponin IVa; (3) oleanolic acid-28-*O*-β-D-glucopyranoside; (4) notoginsenoside Fe; (5) ginsenoside Rb2; (6) ginsenoside Rd.

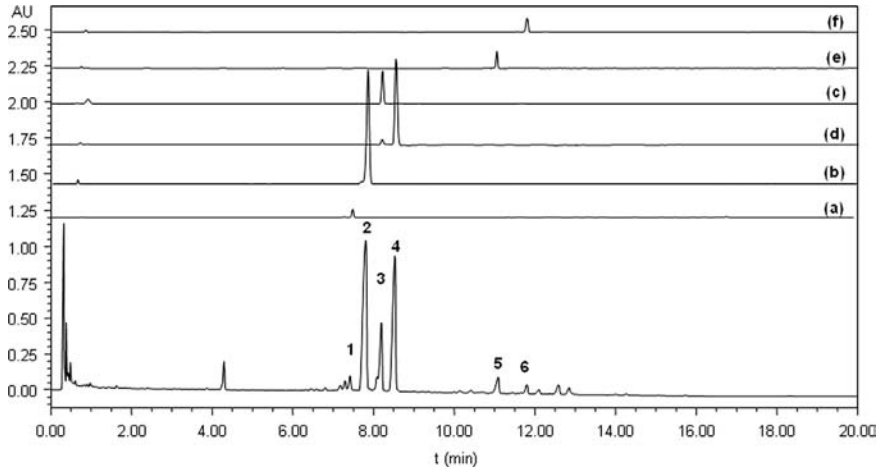

Fig. 6. UPLC chromatograms of xanthine oxidase inhibitors isolated by HSCCC and crude extract of *P. japlcus*. 24 (*R*)-majoroside R_1 (a), chikusetsusaponin IVa (b), oleanolic acid-28-*O*-β-D-glucopyranoside (c) notoginsenoside Fe (d), ginsenoside Rb_2 (e), ginsenoside Rd (f), and crude extract of *P. japlcus*. The UPLC conditions were as follows. A Waters SunFire™ C_{18} column (250 × 4.6 mm id, 5 μm). Column temperature of 25 °C. Acetonitrile (A) and 0.05% phosphoric acid aqueous solution (B) comprised the mobile phase. The flow rate was set to 0.4 mL/min and the solvent gradient adopted was as follows: 95% B at 0–2 min, 95–0% B at 2–17 min, 0% B at 17–20 min. Online UV spectral were obtained in a wavelength range of 203 nm. The chromatographic purities of HSCCC fractions 1, 2, 3, 4, 5 and 6 were 94.83%, 95.07%, 94.62%, 95.74%, 96.12% and 93.19%, respectively. (1) 24 (*R*)-majoroside R_1; (2) chikusetsusaponin IVa; (3) oleanolic acid-28-*O*-β-D-glucopyranoside; (4) notoginsenoside Fe; (5) ginsenoside Rb_2; (6) ginsenoside Rd.

P. japlcus extracts. In our experiment, this method was applied to analyze the extract of *P. japlcus* for the first time, obtaining positive results: six saponins have been screened for studying their activities to inhibit xanthine oxidase, and their structures have been further identified by ESI–MS^n, indicating that the method is effective and applicable in screening xanthine oxidase inhibitors from more complex mixtures. Furthermore, the six ligands 24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside, notoginsenoside Fe, ginsenoside Rb_2 and ginsenoside Rd were isolated via high-speed counter-current chromatography, which respectively accounted for 95.74%, 96.12%, 93.19%, 94.83%, 95.07% and 94.62%, determined by UPLC. To our knowledge, this is the first time that 24 (*R*)-majoroside R_1, chikusetsusaponin IVa, oleanolic acid-28-*O*-β-D-glucopyranoside and notoginsenoside Fe have been separated by HSCCC via a two-stage separation system composed of ethyl acetate–isopropanol–0.1% aqueous formic acid (1.87:1.0:1.33, v/v/v, for the first-stage) and that composed of methylene chloride–isopropanol–acetonitrile–0.1% aqueous formic acid (5.6: 1.0: 2.4: 5.2, v/v/v/v, for the second-stage). The obtained results are expected to be valuable for discovering xanthine oxidase inhibitors from *P. japlcus* and efficiently designing drugs for the prevention and treatment of gout.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.12.005.

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