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Ultrasonic Microwave-assisted Extraction Coupled with High-speed Counter-Current Chromatography for the Preparation of Nigakinones from *Picrasma quassioides* (D.Don) Benn

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ABSTRACT:
Introduction – Nigakinones are the main effective compounds of *Picrasma quassioides* (D. Don) Benn and are widely used in traditional Chinese medicine, therefore a rapid and efficient separation and purification method is necessary.
Objective – To develop a new method based on ultrasonic microwave-assisted extraction (UMAE) and high-speed counter-current chromatography (HSCCC) for the rapid separation and purification of nigakinone and methylnigakinone from *P. quassioides* (D.Don) Benn.
Methodology – Response surface methodology (RSM) was used to optimise the extraction conditions of UMAE: 10.0 g of original sample was extracted with 210 mL of 90% (v/v) aqueous methanol at 60°C for 13 min, ultrasonic power was 100 W and microwave power was dynamically adjusted to the given temperature. After extraction, the extract was introduced into the HSCCC and separated with a hexane:ethyl acetate:methanol:2% acetic acid (9:11:9:11, v/v/v/v) solvent system.
Results – About 2.1 mg nigakinone with purity of 96.8% was obtained in one step within 200 min, methylnigakinone was also obtained with a purity of 75.6%. Their chemical structures were identified with ESI-MS and ^{1}H-NMR.
Conclusion – UMAE coupled with HSCCC was found to be a promising and feasible alternative method to separate and purify alkaloids from natural herbs such as *P. quassiodes*.

Supporting information may be found in the online version of this article.

Keywords: High-speed counter-current chromatography; ultrasonic microwave-assisted extraction; nigakinones; *Picrasma quassioides* (D.Don) Benn

Introduction

Picrasma quassioides (D. Don) Benn (Kumu in Chinese), a traditional Chinese medicine widely distributed in China, is a rich source of alkaloids and has long been used to treat chronic dyspepsia, bacillary disease, gastroenteritis, eczema, etc. 4-methoxy-5-hydroxycanthin-6-one (Nigakinone) and 4, 5-dimethoxycanthin-6-one (Methylnigakinone) (Fig. 1) are the main active components in *P. quassioides*, exhibiting important therapeutic activities of significant potency such as promising antifungal, anti-viral, cytotoxic and anti-ulcer activity (Sung *et al*., 1984; Ohmoto *et al*., 1985; Lee *et al*., 2009; Liu *et al*., 2009).

Separation and purification of nigakinone and methylnigakinone are important for both application and further research and development. However, conventional extraction methods such as maceration, percolation and heat reflux along with traditional chromatographic methods are time consuming with low efficiency because of their similar structures (Ohmoto and Koike, 1984; Ohmoto *et al*., 1985). Recently, the use of ultrasonic (Wu *et al*., 2009) and microwave (Halko and Hutta, 2007) energy sources in the sample preparation, which can accelerate the extraction process and reduce the amount of solvent used, has attracted considerable interest. Ultrasonic microwave-assisted extraction (UMAE) is a complementary technology and may significantly accelerate the extraction process, improve selectivity and simplify manipulation (Rostagno *et al*., 2010). Up to now the UMAE method has been used successfully for the extraction of quercetin from *Anoectochilus roxburghii* (Wall.) Lindl. (Huang *et al*., 2008), inulin and phenols-rich dietary fibre powder from burdock root (Lou *et al*., 2009) and phenolic compounds from burdock leaves (Lou *et al*., 2010). Although the method of one-variable-at-a-time was used to discover an optimum condition in UMAE, it does not include the interactive effects among the variables studied, e.g. the interactive effect between ultrasonic power and microwave power, microwave power and extraction time, etc. It also leads to an increase in the number of experiments necessary to conduct the research. Experimental design methodology is an alternative approach allowing the simultaneous variation of all variables studied and accounts for the

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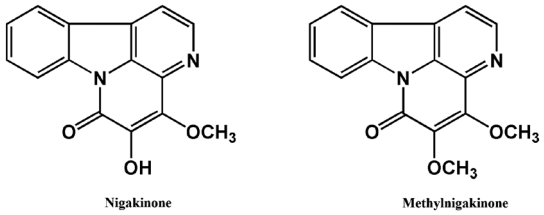

Figure 1. Chemical structures of nigakinone and methylnigakinone.

possible interaction effect between variables (Rodriguez *et al*., 2010). Among these methods, response surface methodology (RSM) is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data (Bezerra *et al*., 2008) that was successfully applied to develop the optimum condition of the UMAE method on the extraction of lycopene from tomatoes (Zhang and Liu, 2008).

On the other hand, thin-layer chromatography and column chromatography are conventional methods for separation and purification of effective compounds from natural products. However, these methods are time consuming and use a lot of solvent. Preparative HPLC (prep-HPLC) is a powerful purification technique in virtue of its excellent efficiency, but plentiful raw samples could overload and pollute the column. Therefore careful sample pretreatment before purification by prep-HPLC is needed (Wang *et al*., 2011). High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography method without any sorbent, which shows high recovery and efficiency as well as elimination of irreversible adsorption of the sample on the solid stationary phase. This method has been applied successfully to the separation and purification of different types of natural product (Xiao *et al*., 2009; He *et al*., 2011).

The aim of this work was to develop a new method based on UMAE and HSCCC for the separation and purification of nigakinones from *Picrasma quassioides* (D.Don) Benn. The effects of extraction temperature, extraction time, liquid/solid ratio and ultrasonic power on the extraction yield of nigakinone were optimised by RSM experiment. The extract obtained from UMAE was then separated and purified by HSCCC under its optimum condition.

Experimental

Reagents and materials

Acetonitrile used in HPLC analysis was of chromatographic grade and purchased from Lab-scan (Dublin, Ireland). All organic solvents used were of analytical grade and obtained from Guangzhou Chemical Factory (Guangdong, China). Deionised water was used throughout the experiments. Dry *Picrasma quassioides* (D.Don) Benn was purchased from the Qingping herbal market in Guangzhou (Guangdong, China).

Apparatus

The UMAE experiments were performed on a UWave-1000 ultrasonic microwave extracting apparatus (Sineo, Shanghai, China). Microwave power, with a maximum of 1000 W at a frequency of 2450 MHz, was dynamically adjusted by temperature and power feedback/control and was able to provide continuous non-pulse microwave heating. Ultrasonic (26–28 KHz) energy was provided by a transducer that could be adjusted between 0 and 800 W. Temperature was monitored by an IR temperature sensor or an advanced Thermowell Pt temperature sensor inside the extraction vessel.

The compounds obtained from the HSCCC were also identified by ^{1}H-NMR (Varian Mercury-plus 300 spectrometer referenced to TMS, USA).

Sample preparation procedures

UMAE. Ten grams of crushed original sample was weighed accurately and then transferred into a flask and 210 mL of 90% (v/v) aqueous methanol was added. The flask was then transferred into the chamber of an apparatus connected with condensing tubes and extraction proceeded at 60°C for 13 min. The speed of magnetic stirrer was set at 1000 rpm, the ultrasonic power was 100 W and microwave power was dynamically adjusted to the given temperature.

Microwave-assistant extraction (MAE). Ten grams of sample were used for extraction with 210 mL of 90% (v/v) aqueous methanol under 60°C for 13 min. Microwave power was maintained at 300 W and the speed of magnetic stirrer was set at 1000 rpm.

Ultrasound-assistant extraction (UAE). Ten grams of sample were used for extraction with 210 mL of 90% (v/v) aqueous methanol under 60°C for 13 min. Ultrasonic power was 100 W and the speed of the magnetic stirrer was set at 1000 rpm. After extraction, the aqueous methanol was evaporated with a rotary evaporator under 50°C, and the extract obtained was then redissolved in the mobile phase of HSCCC and introduced into the HSCCC system for further separation and purification.

Determination of the partition coefficient (*K*)

Hexane:ethyl acetate:methanol:2.0% acetic acid was used as the two-phase solvent system for HSCCC. After the solvent mixtures were thoroughly equilibrated in a separation funnel at room temperature, the two phases were separated and degassed by sonication for 20 min before use. Partition coefficients (*K*) of nigakinone were determined by HPLC as follows: the extract from 0.5 g of the original sample was dissolved in 2 mL of each phase of the pre-equilibrated solvent system. After equilibrium, 1 mL of each phase was concentrated to dryness. The residue was redissolved in 1 mL methanol and analysed by HPLC. The *K* value is defined as the peak area of the target compound in the stationary phase (*AS*) of HSCCC divided by that in the mobile phase (*AM*) at the same retention time in the HPLC chromatogram, that is $K = AS/AM$.

HSCCC separation and purification

The HSCCC instrument was a GS10A HSCCC apparatus (Beijing UE Biotech, Beijing, China). It is fitted with a 110 m × 1.6 mm i.d. PTFE multilayer coil of volume 240 mL with β-values ranging from 0.5 to 0.8. First, the multilayer coil column was filled entirely with the upper organic phase as the stationary phase, using a Model NS-1007 constant-flow pump (Beijing UE Biotech, Beijing, China). Then, the column was rotated at 800 rpm, while the mobile phase, the lower aqueous phase of the solvent system, was pumped into the column at a flow rate of 2.0 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the dried extract dissolved in 5 mL mobile phase was loaded into the injection valve. The effluent from the outlet of the column was continuously monitored at 240 nm with a UV6000 detector (Beijing ChuangXinTongHeng Science & Technology Co., Beijing, China), the peak fractions were collected manually according to the chromatographic profile. After separation, the solvent in the coil was ejected with nitrogen gas to determine the retention of the stationary phase (S_p).

Preparatory HPLC/MS

A Shimadzu LC/MS 2010 system was used for the prep-HPLC separation and MS identification. The prep-HPLC system was equipped with two LC-6AD pumps, a SCL-10Avp system controller, a SIL-10AF auto-injector and a SPD-M10Avp diode array detector (PDA). Samples were separated on a Shim-Pack PREP-ODS (H) KIT column (250 × 20 mm i.d., 5 μm, Shimadazu), the mobile phase was aqueous acetonitrile (42%, v/v) and the flow rate was 5.0 mL/min.

The HPLC system described above was coupled on-line to a single quadruple MS with ESI probe (Q-array-Octapole-Quadruple mass analyser, Shimadzu), a splitting method was used and the split ratio was set at 5:1. Data acquisition and processing were performed with the LC-MS solution Ver 3.0 Workstation software. Negative ion mass spectra of the column eluate were recorded in the range *m/z* 50–1000. High purity nitrogen (N_2) was used both as a drying gas, at a flow rate of 10.0 mL/min, and as a nebulising gas at a pressure of 50 psi. The nebuliser temperature was set at 365°C and a potential of 4500 V was used on the capillary. Ultrahigh-purity helium (He) was used as collision gas and the collision energy was set at 70 V.

HPLC analysis

The extract and fraction of nigakinones from HSCCC were analysed by HPLC. The chromatographic system consisted of a Shimadzu LC-2010 (Shimadzu, Kyoto, Japan), equipped with a SCL system controller, a low-pressure gradient solvent pump, an autosampler equipped with a 10 μL loop, a column oven, a UV–vis detector and CLASS-VP software (Shimadzu, Japan) for registering the detector signal and operating the system. Kromasil C_{18}-column (250 × 4.6 mm, i.d. 5 μm) proceeded by a C_{18} guard column (4.0 × 3.0 mm, i.d. 5 μm; Phenomenex, Torrance, CA, USA) were used. The mobile phase was acetonitrile (A) and 0.3% acetic acid aqueous (B) and it was performed at a flow of 1.0 mL/min. The gradient program was as follows: 18–50% A (0–30 min), 50–50% A (30–35 min). The effluent was monitored at 240 nm.

A series of standard solutions of nigakinone and methylnigakinone within the concentration range of 0.4–31 mg/L and 0.1–37 mg/L, respectively, were prepared to determine the linearity of HPLC analysis. Good linearity was observed with the regression coefficient (*r*) of 0.9999. The limit of detection (LOD) obtained was 0.13 mg/L for nigakinone and 0.03 mg/L for methylnigakinone, respectively, which was evaluated on the basis of a signal-to-noise ratio of 3.0. The limits of quantitation (LOQ) were 0.4 and 0.08 mg/L for nigakinone and methylnigakinone, respectively. The recovery of nigakinone spiked 0.15 mg/g standard in real sample was 96.0% with relative standard deviation (RSD) of 0.8%, while the recovery of methylnigakinone spiked 0.01 mg/g standard was 97.7% with RSD of 3.4% based on the peak area of triplicate analyses. The stability was investigated by determining the varieties of targets on seven separate days. All the RSDs of intraday and interday were less than 2.0% and 3.7%, respectively.

Briefly, the extraction yield was defined as follows:

$$\text{extraction yield } (\mu g/g) = \frac{\text{quantity of target compound in UMAE extract } (\mu g)}{\text{quantity of original sample } (g)}$$

Results and Discussion

Optimisation of UMAE conditions

The correct choice of solvent is of primary significance for obtaining an optimum extraction process. Methanol, ethanol, ethyl acetate, acetone and water were investigated as extraction solvents and the results are shown in Fig. 2. Ninety per cent (v/v) aqueous methanol showed better extraction efficiency for nigakinone and methylnigakinone as well as providing a more convenient sample post-treatment process.

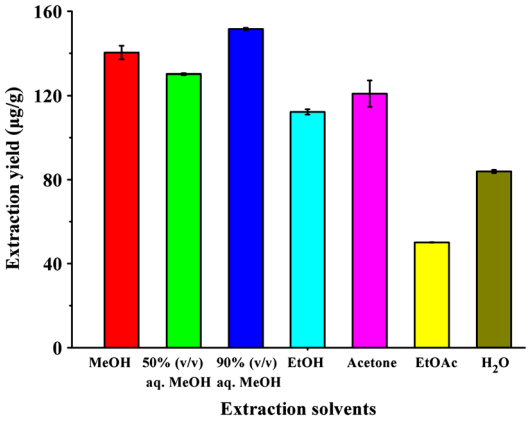

Figure 2. Effect of extraction solvents on the extraction yields of nigakinone.

As well as the extraction solvent, extraction time, extraction temperature and liquid/solid ratio are also significant for the extraction of targets. Among these, some parameters such as the ultrasonic power and extraction time in the extraction process also showed interactions and affected the extraction process. Consequently a two-level fractional factorial design (FFD) experiment was used to select the significant parameters. The FFD method is defined by an experimental domain constituted by a central point and two levels corresponding to the maximum and the minimum values for each factor. The design consisted of eight experiments performed in duplicate and five replicates for the central point in randomised runs. The extraction yield of nigakinone was chosen as the response factor and its RSD in quintuplicate was 3.4%.

According to the preliminary work, four UMAE parameters with potential influence on the extraction process were considered in the FFD design, namely: extraction temperature (Temp, test range 40–60°C), extraction time (Time, test range 2–10 min), ultrasonic power (Power, test range 0–200 W) and liquid/solid ratio (L/S, 8–20 mL/g). The upper and the lower values given to each factor were selected from the available data and the experience gathered in preliminary experiments.

The statistical analysis system (Design-Expert® version 7.0) was used to analyse the experimental data. Analysis of variance (ANOVA) and model residuals were used to check the validity of the mathematical models. Calculations were done at 95% of confidence level. The results of this experimental design are shown in Fig. 3 as standardised Pareto charts displaying the effect (*t* value) of each extraction parameter corresponding to the extraction yield of nigakinone. Those variables showing *t* values higher than the critical *t* value were considered statistically significant. Thus, the liquid/solid ratio was the most significant parameter affecting the extraction yield of nigakinone; therefore increasing the liquid/solid ratio would benefit the extraction of nigakinone. The extraction time was the second most statistically significant parameter. Although both ultrasonic and microwave energy are recognised to enhance chemical reactions such as oxidation as well as to increase the degradation of labile compounds such as fat and oil (Canizares-Macias *et al.*, 2004; Chemat *et al.*, 2004), only ultrasonic power showed a significant positive effect on the extraction of nigakinone. Moreover, although extraction temperature did not show a significant influence in the selected range, it also had positive effect.

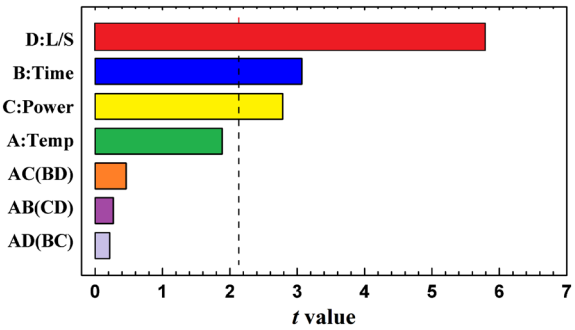

Figure 3. Pareto chart for standardised main effects for the fractional factorial design design of nigakinone. The vertical line indicates the limit at which the influence of the factor is significant at the 95% confidence level. A: Temp, extraction temperature; B: Time, extraction time; C: Power, ultrasonic power; D: L/S, liquid/solid ratio.

The interaction between the extraction temperature with extraction time, power or liquid/solid ratio also did not show significant influence during the extraction process. However, ANOVA results showed that the data of nigakinone did not fit to a linear model, so it was necessary to include some interactions and curvature terms in the model. Consequently, the most significant parameters, including liquid/solid ratio, extraction time and ultrasonic power, were considered for further optimisation using a response surface methodology (RSM) by a central composite design (CCD; Bas and Boyaci, 2007; Bezerra *et al*., 2008).

A central composite design (CCD) with three independent variables (A, extraction time; B, the liquid/solid ratio; C, ultrasonic power) at three levels was performed to optimise the extraction conditions and investigate the effects of the above independent variables on the extraction yield of nigakinone. The experimental domain was defined taking into account the results obtained from the FFD design with some adjustments, that is, the range of liquid/solid ratio was increased to12–24 mL/g, extraction time was increased to 5–15 min and the ultrasonic power test range was increased to 100–300 W, but the extraction temperature was kept constant at 60°C owing to its positive effect on the extraction. The design therefore included 14 experiments in duplicate plus a central point with six replicates in randomised runs. The ANOVA procedure was used to analyse variance and the significances of all terms in the polynomial were considered statistically different when $p < 0.05$. Significance of any differences between groups was evaluated using Duncan's multiple range test.

The process variables and experiment data for extraction yield under different treatment conditions are presented in Table 1. The fitted model for extraction yield (Y) to predict the relationships between the independent variables (A, extraction time; B, the liquid/solid ratio; C, ultrasonic power) and the dependent variables can be expressed by:

$$Y = -21.05781 + 5.65339 \times A + 0.037508 \times \mathrm{B} + 13.95129 \times \mathrm{C} - 2\ .56601 \times 10^{-3} \times \mathrm{A} \times \mathrm{B} - 0.015334 \times \mathrm{A} \times \mathrm{C} - 5.82686 \times 10^{-4} \times \mathrm{B} \times \mathrm{C} - 0.18989 \times \mathrm{A}^2 - 5.19065 \times 10^{-5} \times \mathrm{B}^2 - 0.33431 \times \mathrm{C}^2$$

The data indicated that the proposed regression model for extraction yield of nigakinone was adequate with a satisfactory score of 0.9158, which showed an agreement between the experimental results and the theoretical values predicted by the polynomial model. The full model was made three dimensional and contours were plotted to predict the relationships between the independent variables and the dependent variables. Figure 4 shows the influence of UMAE variables on the extraction yield of nigakinone.

It was found that the liquid/solid ratio and extraction time were the most important factors on the extraction of nigakinone. The extraction yield increased greatly with the increase of extraction time and liquid/solid ratio, but longer extraction time and higher liquid/solid ratio under ultrasonic and microwave irradiation was likely to induce decomposition of nigakinone, resulting in a decrease of its extraction yield (Chen *et al*., 2010). The optimum UMAE conditions were given by RSM as follows: extraction time was 13 min; ultrasonic power was 100 W; liquid/solid ratio was 21 mL/g; extraction temperature was 60°C. Under these conditions, the practical extraction yield of nigakinone was 162.1 μg/g, which was close to the predicted yield (160.2 μg/g) according to the regression model.

Comparison of UMAE with MAE, UAE and HRE

The yields of nigakinone and methylnigakinone obtained by UMAE were compared with microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE) and heat reflux extraction (HRE) methods, and the results are shown in Table 2. ANOVA revealed a significant difference for the extraction yields of nigakinone and methylnigakinone by UMAE compared with other typical methods, which occurred at the 0.05 significance level. With the assistance of the acoustic cavitations and fast heating of microwave irradiation, the extraction in UMAE was obviously improved by shorter extraction time and less volume of solvent needed, with the extraction yields of both nigakinone and methylnigakinone being significantly higher than that in MAE or UAE. These results indicated UMAE was an attractive sample preparation method and had good potential on the extraction of alkaloids from *P. quassioides*.

Optimisation of the HSCCC separation procedure

A suitable solvent system is of primary significance for successful separation and purification in HSCCC. According to the golden rules in selecting optimum conditions introduced by Ito (2005), the solvent system of hexane–ethyl acetate–methanol–water (HEMW), a versatile quaternary solvent system, would be suitable for medium polar compounds such as nigakinone and methylnigakinone. Therefore, several kinds of solvent systems composed of HEMW at different volume ratios were selected and their K values assessed: the results are shown in Table 3.

Although the chemical structures of nigakinone and methylnigakinone are similar, their K values were significantly different in the HEMW solvent systems. In 9:11:9:11 (v/v/v/v) of HEMW, both K values for the selected targets were in the range of 0.5–2.0, indicating an efficient separation and that a suitable run time could be obtained in HSCCC. Moreover, the pH of the solvent system would also affect the separation and K values of alkaloids in HSCCC. When 0.05% $NH_3 \cdot H_2O$ and 2.0% HAc were added into the solvent system of HEMW with a volume ratio of 9:11:9:11, respectively, the K values for both nigakinone and methylnigakinone showed no significant difference.

Table 1. Experiment domain, central composite design matrix and results

Parameter		Code		Level		
				Minimum	Central	Maximum
Extraction time (min)		Time		5	10	15
Ultrasonic power (W)		Power		100	200	300
Liquid/solid ratio (mL/g)		L/S		12	18	24
Experiment	Time (min)	Power (W)	L/S (mL/g)	Extraction yields (μg/g)		
1	5	100	12	118.7		
2	15	100	12	132.4		
3	5	300	12	117.4		
4	15	300	12	128.7		
5	5	100	24	140.7		
6	15	100	24	155.3		
7	5	300	24	140.8		
8	15	300	24	149.6		
9	10	200	18	153.8		
10	5	200	18	148.8		
11	15	200	18	155.9		
12	10	100	18	159.9		
13	10	300	18	153.2		
14	10	200	12	139.1		
15	10	200	24	151.0		

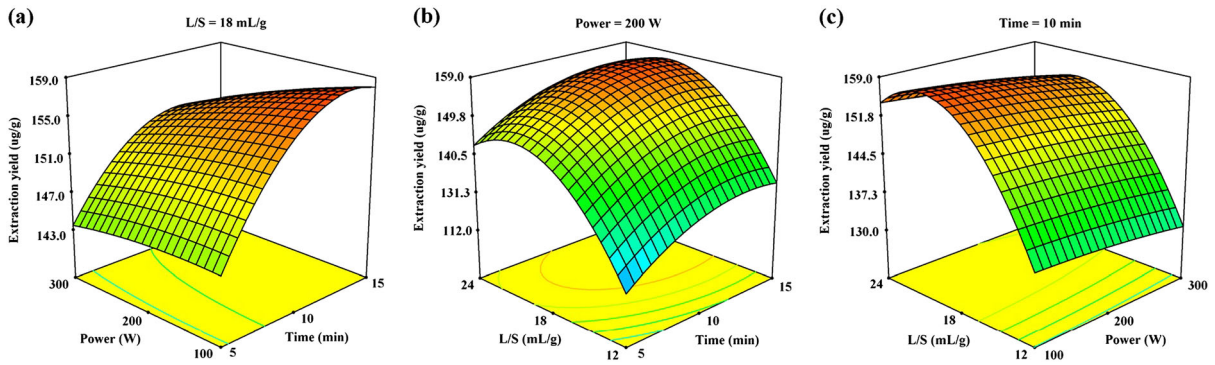

Figure 4. Response surface graph from the central composite design for the extraction of nigakinone from *Picrasma quassioides* samples.

Table 2. Comparison of different methods in extraction of nigakinone and methylnigakinone from *P. quassioides* (n = 5)

Compounds	Ratio of extraction yields (%)			
	UMAE	MAE	UAE	HRE
Nigakinone	100 ± 0.6	95.1 ± 0.8	69.4 ± 4.0	76.0 ± 3.7
Methylnigakinone	100 ± 2.6	92.0 ± 2.9	85.8 ± 3.1	82.3 ± 4.3

Since the composition of alkaloids in *P. quassioides* is rather complex and most of them have similar structures, further experiments were developed with HSCCC in order to select an optimum solvent system. A high purity nigakinone was obtained with the solvent system composed of hexane:ethyl acetate: methanol:2.0% acetic acid at a volume ratio of 9:11:9:11 in an acceptable run time, along with better separation and purification of methylnigakinone. Consequently, hexane:ethyl acetate: methanol:2.0% acetic acid at a volume ratio of 9:11:9:11 was selected as the solvent system for the following experiments.

In addition, other parameters, i.e., revolution speed, flow rate of the mobile phase and stability of the solvent system were also investigated. The results showed that when the flow rate was 2.0 mL/min and resolution speed was 800 rpm, better separation efficiency could be obtained with a satisfactory peak resolution as well as good stability of the solvent system.

Table 3. *K* values and retention of stationary phase (S_p) of nigakinone and methylnigakinone in different hexane-ethyl acetate-methanol–water solvent systems

Volume ratio (v/v/v/v)	S_p (%)	Partition coefficient (*K*)	
		Nigakinone	Methylnigakinone
5:5:5:5	60.5	0.41	0.92
9:11:9:11	61.8	0.95	1.73
4:6:4:6	60.3	1.54	2.98
9:11:9:11 (0.05% $NH_3{\cdot}H_2O$)	62.3	0.92	1.81
9:11:9:11 (2.0% HAc)	58.9	0.95	1.70

On the other hand, the separation efficiency of targets in HSCCC was restricted by the volume of the multilayer coil column, the greater the concentration or volume of the loading sample, the more serious the loss of stationary phase, resulting in poor purity and a low yield of the target compound. The effects of different sample loadings on the purity and yield of nigakinone and methylnigakinone are shown in Table 4. Although the purity of the target fractions obtained was slightly decreased and the yields of nigakinone and methylnigakinone obviously decreased when sample loading increased, 14.0 g of original sample was the acceptable maximum sample loading for separation and purification in HSCCC.

As a result, when 14.0 g of original sample was used for extraction with UMAE and the dried extract was further separated and purified with HSCCC under optimum conditions, the effluents of each fraction from HSCCC (Fig. 5) contained 2.1 mg nigakinone (fraction **I**) and 0.16 mg methylnigakinone (fraction **II**).

The 0.16 mg methylnigakinone (fraction **II**) obtained from HSCCC was dissolved in 0.1 mL methanol and was subjected to further separation by prep-HPLC performed on a Shim-Pack PREP-ODS (H) KIT (250 × 20 mm i.d., 5 μm) column. The optimum chromatographic conditions were that the mobile phase was can: water (42:58, v/v) in isocratic mode at a flow rate of 5.0 mL/min and injection volume was 0.1 mL. The effluent was monitored at 240 nm, peak fractions were collected according to the elution profile and 0.12 mg methylnigakinone fraction was obtained.

HPLC analysis and identification of the separated fractions

HPLC analysis was used for qualitative and quantitative assay of fractions from the HSCCC. The results revealed that fraction **I** corresponds to nigakinone and its purity was 96.8%, and fraction **II** corresponds to methylnigakinone but its purity was 75.6%. With the help of prep-HPLC, fraction **II** from HSCCC was further purified and its purity was increased to 99.0%. The HPLC chromatograms are shown in Fig. 6.

Identification of the nigakinone and methylnigakinone obtained was carried out by ESI-MS and ^{1}H-NMR as follows:

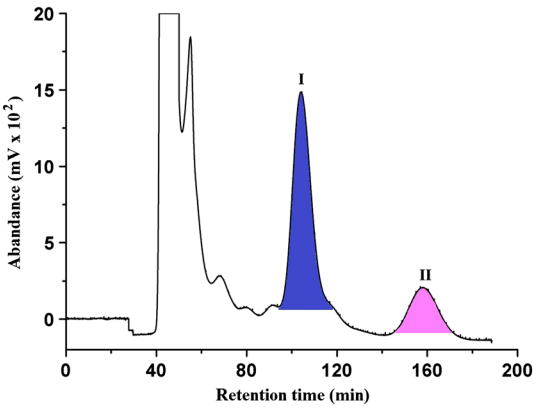

Figure 5. HSCCC chromatogram of extract using the hexane:ethyl acetate:methanol:2.0% acetic acid with volume ratio of 9:11:9:11.

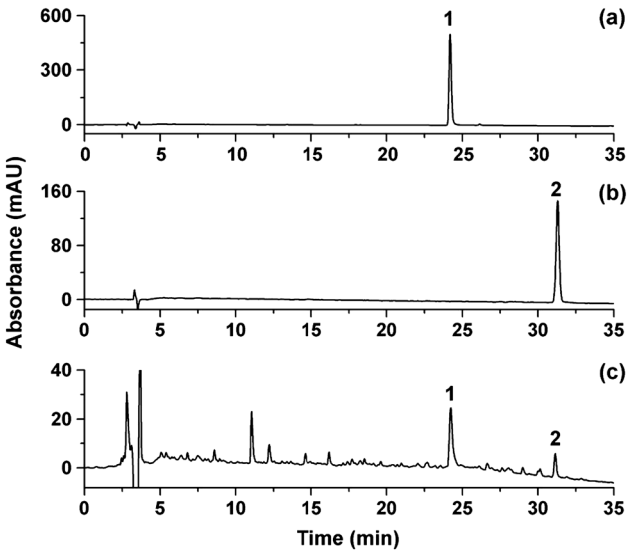

Figure 6. HPLC analysis of nigakinone obtained from (a) HSCCC, (b) methylnigakinone obtained from HSCCC coupled with prep-HPLC and (c) UMAE extract.

Table 4. Effect of sample loading on the purity of obtained nigakinone and methylnigakinone

Sample loading (g)	Nigakinone (NK)				Methylnigakinone (MNK)			
	NK in extract (mg)	Obtained (mg)	Yield (%)	Purity (%)	MNK in extract (mg)	Obtained (mg)	Yield (%)	Purity (%)
8.0	1.5	1.4	90.5	97.5	0.10	0.10	100	79.6
14.0	2.6	2.1	80.8	96.8	0.17	0.16	94.9	75.6
20.0	3.8	2.7	70.9	96.7	0.25	0.23	93.3	75.5

Yield is defined as the amount of target obtained from HSCCC divided by that in the UMAE extract.

(1) Nigakinone: ESI-MS *m/z*: 265 $[M-H]^-$; ^{1}H-NMR (300 Hz, $CDCl_3$) δ: 4.49 (3 H, s, C_4-OCH_3),7.53 (1 H, t, J = 7.7 Hz, 10-H), 7.71 (1 H, t, J = 7.9 Hz, 9-H), 7.91 (1 H, d, J = 5.0 Hz, 1-H), 8.10 (1 H, d, J = 7.9 Hz, 11-H), 8.58 (1 H, d, J = 8.1 Hz, 7-H), 8.86 (1 H, d, J = 5.0 Hz, 2-H). The peak assigned in ^{1}H-NMR corresponded to those reported in previous studies (Sung *et al*., 1984; Lee *et al*., 2009) and it was identified as nigakinone.

(2) Methylnigakinone: ESI-MS *m/z*: 281 $[M+H]^+$; ^{1}H-NMR (300 Hz, $CDCl_3$) δ: 4.09, 4.49 (each 3 H, s, C_4, C_5-OCH_3),7.51 (1 H, t, J = 7.6 Hz, 10-H), 7.68 (1 H, t, J = 7.4 Hz, 9-H), 7.94 (1 H, d, J = 5.0 Hz, 1-H), 8.10 (1 H, d, J = 7.7 Hz, 11-H), 8.66 (1 H, d, J = 8.1 Hz, 7-H), 8.84 (1 H, d, J = 5.0 Hz, 2-H). The peak assigned in ^{1}H-NMR corresponded to those reported in previous studies (Sung *et al*., 1984; Lee *et al*., 2009) and it was identified as methylnigakinone.

Overall, with the present method based on ultrasonic microwave-assisted extraction (UMAE) and high-speed counter-current chromatography (HSCCC), nigakinone and methylnigakinone with high purity can be extracted and separated from *P. quassioides* (D.Don) Benn within 4 h.

Supporting information

Supporting information may be found in the online version of this article.

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