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Optimizing the extraction of anti-tumor alkaloids from the stem of *Berberis amurensis* by response surface methodology

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ABSTRACT

Response surface methodology (RSM) using a Box–Behnken design (BBD) was employed to optimize the conditions for extraction of anti-tumor alkaloids from the stem of *Berberis amurensis* Rupr. (BAAs). Four independent variables (ethanol concentration, pH value, ratio of liquid to material and extraction time) were investigated and the optimal conditions for BAAs were evaluated by means of response surface methodology (RSM). Moreover, the *in vitro* anti-tumor activity of BAAs was investigated. The results showed that the experimental data could be fitted to a quadratic polynomial model using correlation analysis of the mathematical regression model. Response surface plots showed that all independent variables significantly influenced the extraction yield of BAAs. The optimum extraction conditions were as follows: ethanol concentration of 67.28%, pH value of 1.81, the ratio of liquid to material of 11.24:1 (mL/g), and extraction time of 1.58 h. The average experimental BAAs yield under the optimum conditions was found to be 24.63 ± 0.28 mg/g, which agreed with the predicted value of 24.33 mg/g. UPLC-PDA analysis showed that berberine was the principal alkaloid compound in *B. amurensis* Rupr stem. Additionally, BAAs could inhibit MCF-7 and HEPG2 cell proliferation *in vitro*, and the 50% inhibitory concentration (IC50) at 48 h was around 402.25 and 477.17 μg/mL, respectively. The anti-tumor activity of BAAs were dose-dependent.

1. Introduction

Protoberberine alkaloids belong to benzyltetrahydroisoquinolines alkaloids, which widely distribute in the vegetable kingdom, *e.g.*, in Ranunculaceae plant *Coptis chinensis* Franch., Berberidaceae plant *Berberis amurensis* Rupr., Rutaceae plant *Phellodendron amurense* Rupr., *etc.* (Chen et al., 2008; Li et al., 2006; Xu et al., 2006). In recent years, an increasing number of protoberberine alkaloids have been used in medicine and health-care food due to their various biological activities, such as antidiabetic (Hsu et al., 2013), antidepressive (Sun et al., 2013a), memory-enhancing (Dhingra and Kumar, 2012), and neuroprotective effects (Luo et al., 2012). At the same time, a growing amount of research has shown that protoberberine alkaloids possess anti-tumor effect by inducing cell cycle arrest, p53-dependent apoptosis, inhibiting AP-1 activity or other means (Cao et al., 2013).

B. amurensis Rupr. is an important medicinal plant that grows mainly in Northeast and North China, the middle and northern part of Korean peninsula and the far East of Russia. The roots, stems, fruits and leaves of *B. amurensis* have long been used as traditional medicinal herb for the treatment of hypertonia, dysentery, eczema and diseases of liver, intestine and also as a hemostatic agent (Lee and Kim, 1997; Yusupov et al., 1993). To date, more than ten protoberberine alkaloids, including berberine, jatrorrhizine, palmatine, have been isolated from *B. amurensis* (Lee and Kim, 1997; Karimov, 1993; Yusupov et al., 1993). In addition, eight phenolic constituents including six lignan derivatives and two phenylpropanoids have been isolated from this plant (Park et al., 2009).

Generally, the prepared sample of crude drug could be affected by some parameters although the quality of raw material is constant. For example, pH value is a critical factor for extraction of total alkaloids owing to the special structure of the compounds. Moreover, a higher content could be obtained by increasing time,

Abbreviations: BBD, Box–Behnken design; RSM, response surface methodology; BBAs, alkaloids of *Bereris amurensis* Rupr.

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but the value may be invariant or decreased when the time reaches to a stable level. In the course of extraction, the content of active constituents would be decreased and the total volume of prepared samples would be increased along with the ratio of liquid to raw material and extraction number increasing. Finally, ethanol concentration is also a significant factor for extraction of alkaloids and appropriate concentration of ethanol should be chosen. Therefore, the five parameters exhibit important effects on the extraction of alkaloids.

In order to confirm the optimal operating conditions while the others are kept at a constant level. One-factor-at-a-time technique has been widely used to achieve higher extraction yields. The major disadvantage of this method is that it could not shows interactive effects among the variables and depict the optimal parameters on the process (Baş and Boyacı, 2007). Compared with a one-factor-at-a-time design, mathematical and statistical techniques are mainly adopted for designing experiments, building models, evaluating the effects of parameters and confirming optimum condition of factors for desirable responses in response surface methodology. The most common design, Box–Behnken design (BBD), has been widely used to optimize technical parameters. In terms of these advantages, BBD is commonly employed in optimizing the extraction of bioactive constituents including alkaloids (Teng and Choi, 2014), flavonoids (Sheng et al., 2013), ginsenosides (Sun et al., 2013b), and polysaccharides (Zhang et al., 2014).

Up to now, there is no information on optimization of protoberberine alkaloids extraction from the stem of *B. amurensis*. In addition, there are no detailed investigations to explore the antitumor activity of alkaloids in the stem of *B. amurensis* (BAAs). In the present study, the alkaloids content was considered as response value while pH value, ethanol concentration, the ratio of liquid to material, extraction time and number were selected as optimization parameters. BBD, followed by canonical and ridge analysis, was employed to optimize the process parameters of BAAs extraction. Furthermore, the anti-tumor effect of BAAs on MCF-7 and HEPG2 cell was evaluated in the search for high quality bioactive constituents for use in the pharmaceutical industry.

2. Materials and methods

2.1. Plant materials

The *B. amurensis* Rupr. was collected from the Shangzhi city (N45°14′33.62″, E127°33′54.23″) of Heilongjiang Province, China in October, 2013 and authenticated by professor Chen Wang, biological department, Harbin Normal University, People's Republic of China. The material was dried in air and cut into slices, then ground to powder with an approximate size of 0.2–0.5 mm. The powder was kept in sealed polyethylene bags at 4 °C until required.

2.2. Chemicals and reagents

Acetonitrile of chromatographic grade was purchased from Merck Serono Pharmaceutical R&D Co., Ltd. (Beijing, China). Ethanol and ammonium acetate were of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China). Reverse osmosis Milli-Q water (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Standards of berberine hydrochloride, jatrorrhizine hydrochloride and palmatine hydrochloride, all in 98% purity, were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.3. Extraction of BAAs

Samples of three grams were extracted by ethanol solvent in a designed extraction concentration, pH value, the ratio of liquid to material, extraction time and number. The extraction solution was centrifuged at 4000 rpm for 10 min to collect the supernatant. After repeated extraction, all the supernatant was filtrated and diluted to 100 mL for determination of BAAs.

2.4. Determination of BAAs

Content of three protoberberine alkaloids was measured applying a previously described method (Kong et al., 2009) with some modification. Briefly diluted sample (1 mL) was evaporated to dryness in an evaporating dish in water bath (30 °C). Then the residue was dissolved with MeOH–HCl (100:1, v/v) in a 100 mL volumetric flask and filtered through a 0.22 μm nylon filter membrane prior to injection into the UPLC system.

2.5. UPLC analysis of BAAs

In the present work, BAAs were quantified simultaneously by the Waters ACQUITY UPLC® system (Waters Corporation, Milford, USA). Chromatographic separation was performed on an ACQUITY UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm, Waters Corporation, Milford, USA) with the oven temperature of 35 °C. The chromatographic peaks were identified with the Waters PDA eλ Acquity UPLC Detector. The detection wavelength was set at 345 nm. All chromatographic experiments were performed in an isocratic mode. The mobile phase consisted of a mixture of solvents of chromatographic purity, acetonitrile/water (2 mM ammonium acetate and 0.05% formic acid, 25:75, v/v, pH 3.20) (Chen et al., 2013; Qiu et al., 2012). The flow rate was set to 0.4 mL/min. Compound samples were weighed on analytical scales with an accuracy of 0.01 mg. All the samples were prepared in 5, 10, 25 and 100 mL volumetric flasks. Solution volume injections of 2.0 μL were performed with the use of an autosampler. Each sample was analyzed three times and the run time was 5 min. All solutions were filtered through a 0.22 μm membrane filter. The final result was presented as an arithmetical mean. The control of the UPLC system and detection was achieved by the Empower II Waters® software. The method was validated in terms of precision, accuracy, specificity and stability for BAAs.

2.6. Experimental design and statistical analyzes

The main factors affecting extraction efficiency, including pH value, ethanol concentration, extraction time, the ratio of liquid to material and extraction number, had significant effects on BAAs production, so the five parameters were screened by single-factor experiment.

Based on the preliminary results, the proper range of the extraction variables including X_1 (ethanol concentration), X_2 (pH value), X_3 (ratio of liquid to material), and X_4 (extraction time) was determined. Then, a three-level-four-factor BBD (Design Expert software, Trial Version 7.0.0, Stat–Ease Inc., Minneapolis, MN, USA) was applied to determine the best combination of extraction variables for the yields of BAAs. The range of independent variables and

Table 1
Variables and experimental design levels for response surface.

Independent variables	Coded symbols	Levels		
		−1	0	1
Ethanol concentration (%)	X_1	60	65	70
pH value	X_2	1	2	3
Ratio of liquid to material (mL/g)	X_3	8	10	12
Extraction time (h)	X_4	1	1.5	2