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UHPLC-MS^n-assisted characterization of bioactive alkaloids extracted from *Nitraria sibirica* leaves and enriched using response surface method and adsorption on macroporous resin

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ABSTRACT

In Xinjiang, China, *Nitraria sibirica* Pall. is traditionally used to treat hypertension, menstrual disorders, and gastroenteritis. Following process optimization by response surface modeling and Box-Behnken analysis, we extracted 3.83 mg/g of alkaloids from *N. sibirica* leaves in two cycles of extraction for 2.38 h in 89.45% ethanol at liquid-solid ratio 11.381 (mL/g). Subsequent adsorption on HPD-450 resin further enriched the total alkaloid content to 18.08%. Subsequently, 28 compounds, including 15 alkaloids and 13 flavonoids and phenolic compounds, were identified in the extract by UHPLC-Q-Orbitrap-MS. Strikingly, the extracts were strongly antimicrobial against*Candida albicans* and *Staphylococcus aureus*, and significantly antiproliferative against A549, MCF7, and Hela tumor cells, probably due to induction of apoptosis. Finally, the extracts efficiently scavenged radicals with IC_{50} 50.90 ± 6.45 μg/mL.

1. Introduction

Nitraria sibirica Pall. (Nitrariaceae), which is widely distributed in central Asia, promotes ecological health because of superior tolerance to severe drought and high salinity (Zhao et al., 2002). These characteristics are believed to be closely related to active substances the plant produces. As recorded in Chinese Materia Medica, *N. sibirica* fruits and leaves are used to treat hypertension, menstrual disorders, gastroenteritis, and other conditions (Liu and Liu, 1999), including in Uighur, Tibetan, Mongolian, and other traditions (Gu et al., 2017). Of note, *N. sibirica* alkaloids have recently drawn significant research interest, not only because of inhibitory activity against digestive enzymes, but also because of hypoglycemic effects (Kwon et al., 2017). Bakri et al. (2014) also reported that total alkaloids from *N. sibirica* leaves effectively reduce renal inflammation, fibrosis, and hypertension-induced albuminuria in mice treated with angiotensin II and a high-salt diet. However, the pharmacological properties of specific alkaloids, which are the most abundant natural products in *N. sibirica*, are not well-characterized. To the best of our knowledge, alkaloid extraction from *N. sibirica* leaves has also not been optimized through innovative techniques such as response surface methodology (Gopinath et al., 2010), although Box-Behnken design is commonly employed to optimize extraction of alkaloids from plants (Dary et al., 2017). Unlike orthogonal approaches, response surface methodology is mainly used to design experiments, establish models, determine the effects of parameters, and optimize conditions to maximize the desired outcome (Chew et al., 2017). Indeed, systematic extraction, purification, and enrichment of alkaloids, which are found in low levels in plants and other natural products, is essential to improve the efficacy of traditional Chinese medicine.

Abbreviations: DPPH, 2,2-diphenyl-1-picryl-hydrazyl; IC_{50}, 50% inhibitory concentration; UHPLC-Q-Orbitrap-MS, ultra-high-pressure liquid chromatography-quadrupole-orbitrap-mass spectrometry; DMSO, dimethyl sulfoxide; MCF-7, human breast carcinoma cells; Hela, cervical carcinoma cells; A549, lung carcinoma cells; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl; SD, standard deviation

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HPD-450 resin (Zhao et al., 2017a, b) was also used in this study for the first time to enrich *N. sibirica* alkaloids. Because adsorption depends on Van der Waals' forces over large specific surface areas (Ng and Mintova, 2008), organic compounds adsorb to resins to various extents depending on molecular weight and size, and thus can be fractionated and analyzed separately, such as by LC–MS, which is widely used in qualitative and quantitative analysis of secondary metabolites in plants owing to high resolution, sensitivity, and specificity (Tiller et al., 2003). Importantly, LC–MS can be combined with other modalities, such as in UHPLC-Q-Orbitrap-MS, which couples ultra-high performance liquid chromatography to high-resolution tandem mass spectrometry. Accordingly, the combined power provides detailed, high-quality mass spectra, and enables elucidation of the structure of analytes (He et al., 2017). This analysis is also more exhaustive and requires no derivatization, unlike classical chromatographic isolation and purification methods that require tedious and time-consuming column chromatography but nevertheless do not easily separate and identify compounds present at very low concentrations (Erickson, 2006).

In this study, we primarily intended to develop an efficient, green protocol optimized by response surface methodology that could be used as an alternative to conventional methods of extracting plant alkaloids. To this end, ethanol concentration, liquid-solid ratio, extraction time, and number of extraction cycles were optimized to maximize alkaloid yield. UHPLC-Q-Orbitrap-MS was then used to identify alkaloids and other compounds in the extract, following standard chromatographic and MS guidelines. Finally, we investigated the antioxidant, antimicrobial, and antitumor activities of the extract, in search of potent bioactive components for potential use as pharmaceuticals.

2. Experimental

2.1. Plant materials

N. sibirica leaves were collected in July 2017 from Guma, Hetian, Xinjiang Uighur Autonomous Region, China, and identified by Prof. Y. Feng of Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences. A voucher specimen (NS20170915) was deposited at Xinjiang Key Laboratory of Plant Resources and Natural Products Chemistry, Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences. The leaves were dried, powdered, and stored in airtight containers until further use.

2.2. Chemicals and reagents

Atropine sulfate, dimethyl sulfoxide, and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) with purity > 99% were purchased from Sigma (St. Louis, MO, USA), while LC–MS grade methanol and HPLC grade formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and Merck (Darmstadt, Germany), respectively. Deionized water was obtained on a Milli-Q Plus system (Millipore, Bedford, MA, USA). Methanol, *n*-butanol, chloroform, ethyl acetate, and hydrochloric acid were analytical grade. Bromocresol green, ethanol, hydrous sodium carbonate, sodium hydroxide, and other reagents were purchased from Baishi Chemical (Tianjin, China).

2.3. Extraction of N. sibirica alkaloids

Samples (10 g) were extracted under designed extraction conditions covering 100, 80, 60, 40, and 20% ethanol, 8:1, 10:1, 12:1, and 14:1 liquid-solid ratio, 1, 2, and 3 h extraction time, and 1, 2, and 3 cycles of extraction. Extracts from multiple cycles were pooled, condensed, filtered, and diluted to 250 mL for quantification of alkaloids. Extraction runs were conducted in triplicate.

Table 1
Variables and experimental design levels for response surface.

Independent variables	Coded symbols	Levels		
		−1	0	1
Extraction time (h)	X1	1.5	2	3
Ethanol concentration (%)	X2	80	95	100
Ratio of liquid to material (mL/g)	X3	10	12	14

2.4. Analysis of N. Sibirica alkaloids

Alkaloid content was quantified by acid dye colorimetry as previously described (Li et al., 1989), with some modification. Briefly, 1.0 mL samples (40 mg/mL extract or 2–10 μg/mL atropine sulfate) were mixed with 4.0 mL bromothymol blue pH 4.42, 7.0 mL water, and 15.0 mL chloroform, and placed at room temperature with gentle shaking for 3 min, and then without shaking for 60 min. The resulting chloroform layer was recovered, and assayed at 415 nm using a Shimadzu UV-2550 UV–vis spectrophotometer (Japan). The alkaloid concentration in extracts was determined according to a standard curve of atropine sulfate. Each sample was analyzed in triplicate.

2.5. Experimental design

Ethanol concentration, liquid-solid ratio, extraction time, and extraction cycles were initially screened by single-factor experiments as described. Based on preliminary results, a suitable range was determined for X_1 (ethanol concentration), X_2 (liquid-solid ratio), and X_3 (extraction time). The range of independent variables and their levels are listed in (Table 1) as mean ± SD. A second-order polynomial model corresponding to a three-level, three-factor Box-Behnken design (Design Expert Trial Version 8.0, Stat–Ease Inc. Minneapolis, MN, USA) was then fitted to the data to determine the best combination of extraction variables that will maximize alkaloid yields. The model is generally given by

$$(EY) = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum \sum_{i<j=1}^{3} \beta_{ij} x_i x_j + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \varepsilon \quad (1)$$

where EY is the predicted response value, x_i and x_j are values of independent variables, β_0 is a constant term, β_i is a linear coefficient, β_{ij} is an interaction term coefficient, β_{ii} is a quadratic coefficient, and ε is random error. Coefficients were obtained by regression according to the Box-Behnken experimental design.

2.6. Preparation and analysis of alkaloid-rich fractions

Fractions were obtained by reflux extraction in optimal conditions obtained as described, and pooled, concentrated, dissolved in 2% sulfuric acid, and filtered. The filtrate was then alkalinized with Na_2CO_3 to pH 10 and extracted with ethyl acetate (3 times) to remove non-alkaloid impurities, and then further alkalinized with NaOH to pH 14 for extraction with n-butanol (3 times). The first and second extracts were pooled, dried, dissolved in 2% sulfuric acid, adjusted to pH 4, diluted to 0.8 mg/mL alkaloid, adsorbed to a macroporous HPD-450 column (2.5 cm × 45 cm), washed with pure H_2O, and eluted with 1:1 v:v ethanol:H_2O. Eluates were collected, freeze-dried, ground, vacuum-packed, and stored at −20 °C until analysis. This process was repeated twice to get sufficient amounts of alkaloid-rich fractions.

2.7. UHPLC-Q-Orbitrap-MS

Using a Q-Orbitrap-MS system (Thermo Fisher Scientific, Bremen, Germany) coupled to a UHPLC system (Thermo Fisher Scientific, Bremen, Germany) fitted with an Excellect C18 ME column

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