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Determination of caffeoylquinic acid derivatives in *Azolla imbricata* by chitosan-based matrix solid-phase dispersion coupled with HPLC–PDA

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ABSTRACT

Five main components of *Azolla imbricata* were isolated and identified as caffeoylquinic acid derivatives, four of which were isolated from this species for the first time. The five compounds exhibited strong antioxidant activities and were used as chemical markers for quantitative analysis. A chitosan-based matrix solid-phase dispersion (MSPD) extraction coupled with high-performance liquid chromatography (HPLC) was developed for the determination of the five analytes in *A. imbricata*. The optimal conditions for the chitosan-based MSPD extraction were as follows: low viscosity chitosan HL-1 as the dispersant, sample-to-dispersant mass ratio of 1:1, 10 mL of a methanol-sulfuric acid aqueous solution (0.2 M) (7:3, v/v) as the elution solvent, and a grinding time of 1 min. Compared with ultrasonic assisted extraction (UAE), the chitosan-based MSPD extraction exhibited higher extraction efficiency, consumed less solvent and time, and provided a cleaner extract. Compared with C_{18}-based MSPD, the developed method was less expensive and more environmentally-friendly. The validated MSPD–HPLC method was efficient, reliable, and applicable to the quantification of caffeoylquinic acids in *A. imbricata*.

1. Introduction

Azolla imbricata (Roxb.) Nakai, commonly referred to as *Manjianghong* in Chinese, belongs to the Azollaceae family. It is distributed throughout the tropical and warm temperate regions of the world and generally grows on the water surface of ponds, ditches, and rice fields [1,2]. This folk herbal medicine possesses antipyretic, diaphoretic, and diuretic effects [1]. The extract of this herb also possesses antioxidant activity [3]. However, few articles address the bioactive chemical constituents of this plant. Fewer than ten compounds in *A. imbricata* were previously reported. Most of these compounds, including several antioxidants, were only tentatively identified by chromatographic methods, UV and IR spectra [4–6]. Thus, the selection of chemical markers for quantification of *A. imbricata* is difficult, and no papers regarding the quantitative analysis of the caffeoylquinic acid derivatives of this plant have been reported to date.

Sample pretreatment is critical for analysis because of the complex plant matrices. Frequently used extraction methods, such as maceration, Soxhlet extraction, heat reflux extraction, and ultrasonic assisted extraction (UAE), are often followed by a clean-up procedure, such as liquid-liquid or solid-phase extractions. Matrix solid-phase dispersion (MSPD) extraction simultaneously extracts and purifies target analytes from viscous, solid, and semi-solid samples [7,8]. Therefore, this method simplifies the overall process and requires less time. The MSPD technique has been successfully applied to the extraction of various phytochemicals from plants [9–12].

In the MSPD extraction, the dispersant is essential, as it acts as an abrasive and a sorbent. Chitosan, a deacetylated form of a natural polymer chitin [13], is a promising MSPD dispersant. The free hydroxyl and amino groups in chitosan, which can form hydrogen bonds and undergo electrostatic and ion-exchange interactions, allow for the selective adsorption of various compounds [14–16]. In addition, chitosan is readily available, inexpensive, and environmentally-friendly. A chitosan-based MSPD method for

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the extraction of phenols from olive fruits exhibited higher analyte extraction efficiencies than those obtained using MSPD with C_{18} and Florisil as dispersants [17]. This is the only report of the application of chitosan in MSPD, and the results indicate that chitosan-based MSPD is efficient and merits further study.

In this study, the main antioxidant components in *A. imbricata* were isolated and their chemical structures were elucidated as caffeoylquinic acid derivatives (Fig. 1). A chitosan-based MSPD, followed by high-performance liquid chromatography (HPLC), was established for the quantitative analysis of the isolated antioxidants. In addition, the proposed chitosan-based MSPD method was compared with other sample preparation methods, including C_{18}-based MSPD and UAE.

2. Materials and methods

2.1. Materials, chemicals and reagents

Dried herbs of *A. imbricata* (Roxb.) Nakai were collected in 2015 and 2016 from Fujian and Guangdong provinces, China, and were authenticated by Dr. Y. Kang. The plant samples were dried and pulverized into a fine powder (60 mesh).

Chitosan JK-1 (medium viscosity chitosan, viscosity 200–300 mPa.s, deacetylation ≥ 85%), JK-2 (high viscosity chitosan, viscosity 300–800 mPa.s, deacetylation ≥ 85%), and JK-3 (herb extracting chitosan, molecular weight 500,000–1,000,000) were obtained from Golden Shell Pharmaceutical Co, Ltd (Zhejiang, China). ALD-1 (low viscosity chitosan, viscosity < 200 mPa.s, deacetylation ≥ 90%), ALD-2 (medium viscosity chitosan, viscosity 200–400 mPa.s, deacetylation ≥ 80%), and ALD-3 (high viscosity chitosan, viscosity > 400 mPa.s, deacetylation ≥ 80%) were purchased from Aladdin Industrial Co., Ltd. (Shanghai, China). HL-1 (low viscosity chitosan, molecular weight 20,000–22,000, viscosity <10 mPa.s) was provided by Xingchen Biological Product Factory (Nantong, China). C_{18}-bonded silica (40–60 μm) was purchased from Agela Technologies (Tianjin, China).

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was purchased from Aladdin (Shanghai, China). Ascorbic acid and vitamin E were purchased from Adama Reagent Co., Ltd (Shanghai, China). HPLC grade methanol and acetonitrile were obtained from Cinc High Purity Solvents Co., Ltd (Shanghai, China). Other regents were of analytical grade and obtained from Sinopharm chemical reagent Co. Ltd (Shanghai, China).

2.2. Isolation and identification of the main components of A. imbricata

Dried powder of *A. imbricata* (6.9 kg) was extracted with 95% ethanol. The filtrate was evaporated under reduced pressure to afford a crude extract, which was subsequently suspended in water and partitioned successively with petroleum ether and ethyl acetate. The water-soluble fraction was subjected to column chromatography on macroporous resin and MCI gel, and was further purified by semipreparative RP-HPLC to afford compound **1** (67 mg). The ethyl acetate soluble fraction was separated using various column chromatographic methods to yield compounds **2** (20 mg), **3** (195 mg), **4** (30 mg), and **5** (479 mg). The detailed extraction and isolation procedures for compounds **1**–**5** can be found in the Supplementary Information.

The structures of the isolated compounds were identified by their physicochemical properties and various spectroscopic methods. The ^{1}H- and ^{13}C-NMR spectra were recorded with TMS as an internal standard using Bruker Ascend TM 600 MHz and Varian Mercury Plus 400 MHz spectrometers. ESI-MS was performed on an Agilent 1100 LC/MSD. The purities of the compounds were analyzed using the HPLC method described in this study.

2.3. Evaluation of antioxidant activity

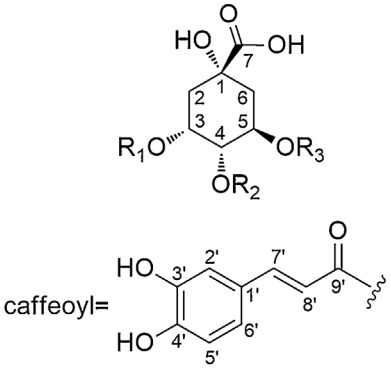
The DPPH radical scavenging assay was performed based on previously reported methods [18,19]. *A. imbricata* extract and five major compounds at different concentrations (5.0–50.0 μg/mL, 1 mL) were separately mixed with DPPH solution (0.8 mg/mL, 1 mL). The mixture was incubated at 37 ° in the dark for 30 min, and its absorbance was subsequently measured at 517 nm. The DPPH solution with the addition of ethanol instead of the sample served as a blank. Ascorbic acid and vitamin E were used as positive controls. Antioxidant activity, which was expressed as the percentage of DPPH radical elimination, was calculated as follows: $[(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$, where A_{blank} and A_{sample} are the absorbances of the blank and sample, respectively. All experiments were performed in triplicate, and the sample concentration providing 50% inhibition (IC_{50}) was calculated using SPSS software.

2.4. Preparation of standard solutions

For method validation, the standard stock solutions of 1 mg/mL for the five caffeoylquinic acids were prepared separately by dissolving the requisite amount of the analytes in methanol. Standard working solutions were prepared by mixing the stock solutions and then diluting with a methanol–0.1% formic acid aqueous solution (50:50, v/v) to obtain different concentrations.

For the DPPH radical scavenging assay, each compound was dissolved in ethanol to prepare the stock solution of 1 mg/mL, and then the stock solution was diluted with ethanol to obtain the working solutions.

All solutions were stored at −20 °C and brought to room temperature (approximately 20 °C) before use.



1	R_1=H	R_2=H	R_3=caffeoyl
2	R_1=caffeoyl	R_2=caffeoyl	R_3=H
3	R_1=caffeoyl	R_2=H	R_3=caffeoyl
4	R_1=H	R_2=caffeoyl	R_3=caffeoyl
5	R_1=caffeoyl	R_2=caffeoyl	R_3=caffeoyl

Fig. 1. Chemical structures of the five compounds studied.