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Systematic chemical analysis of flavonoids in the Nelumbinis stamen

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ABSTRACT

The stamen of lotus, known as Nelumbinis stamen, has been used as the folk medicine and functional food for a long time, which showed good activities of anti-ulcer, anti-thrombosis, analgesic, anti-diarrhea, strengthen uterine contraction. The bioactivities of Nelumbinis stamen were attributed to the existence of flavonoids, its characteristic chemical constituents. A reliable method for comprehensive chemical analysis of flavonoids in Nelumbinis stamen by HPLC–DAD–MS was developed for the first time. The extraction protocol of flavonoids from Nelumbinis stamen was optimized by an orthogonal design. The chromatographic conditions were optimized, which exhibited similar level than that of the UHPLC platform allowing target compound identification in a shorter time with little solvent consumption. Moreover, similarity analysis, hierarchical clustering analysis and principal components analysis were successfully applied to demonstrate the variability of these Nelumbinis stamen samples.
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Introduction

Traditional Chinese medicine (TCM) has played an important role in prevention and treatment of human diseases for thousands of years, and it was also widely used as functional food with potential health benefits (Chen, 1999). The sophisticated chemical constituents in TCM are the material basic of therapeutic effect and healthy function. Hence, chemical constituent evaluation in TCM is an essential part to the holistic research of TCM complex systems. Nowadays, quantitative analysis of multiple characteristic chemical makers coupled with qualitative analysis of chromatographic fingerprinting provide a promising approach for effective and systematic evaluation of chemical constituents in TCM complex systems (Liang et al., 2004; Fan et al., 2006; Tistaert et al., 2011).

Lotus (*Nelumbo nucifera* Gaerten), is a perennial aquatic herb that has been cultivated for more than 2000 years, which is distributed widely throughout East Asia, Australia and North America (Guo, 2009). It exhibits good traditional efficacy and medicinal application in China with a long history. All of the tissues of *N. nucifera*, including the folium, plumula, semen, receptaculum and rhizomatis nodus, are used as folk medicines with different traditional efficacy and medicinal application, respectively, and all of them are recorded in Chinese Pharmacopoeia (2010 Version). The stamen of *N. nucifera* is usually used for the treatment of seminal emission, spermatorrhea, excessive leucorrhea and frequent urination, and shows the effect of strengthening the kidney and arresting seminal emission. The folium of *N. nucifera* is used for the treatment of dire thirst caused by summer-heat with dire thirst, diarrhea caused by summer-damp or deficiency of the spleen, abnormal uterine bleeding caused by heat in blood and so on. The plumula of *N. nucifera* is usually used for the treatment of impaired consciousness and delirium due to invasion of the pericardium by heat. The semen of *N. nucifera* is usually used for the treatment of protracted diarrhea due to hypofunction of the spleen, leukorrhagia, palpitation and insomnia. The receptaculum of *N. nucifera* is usually used for the treatment of abnormal uterine bleeding and lochiorrhea due to blood stagnation after child birth. The rhizomatis nodus of *N. nucifera* is usually used for the treatment of hematemesis, bleeding from five sense organs or subcutaneous

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tissue and hematuria (Chinese Pharmacopeia Commission, 2010). Owing to its efficacy, Nelumbinis stamen is usually used together with other medicinal materials to compose TCM prescriptions in China. For example, Nelumbinis stamen is the main ingredient of the prescriptions "Jin-suo-gu-jing-wan", "Zhi-zuo-gu-ben-wan" and "Lian-shen-tang", etc. (Deng, 2000; Wang and Wang, 2009). These prescriptions are recorded in the ancient herbal books, which are widely used at TCM hospitals now, and show good clinical efficacy.

Existing results showed flavonoids were the characteristic chemical constituents of Nelumbinis stamen, which exhibited various bioactivities including anti-ulcer, anti-thrombosis, analgesic, anti-diarrhea, strengthen uterine contraction and so on (Zhang et al., 1998; Wu et al., 2003; Zhou et al., 2011). Spectrophotometry, thin layer chromatography, capillary zone electrophoresis with ultraviolet detection and high performance liquid chromatography were the methods used for quality control of Nelumbinis stamen, but the majority of studies were limited to quantitative analysis of little marker compounds in the lotus stamen (Shu et al., 2011; Yang and Zhao, 2011; Jing et al., 2007; Men et al., 2003). Moreover, the chemical quality evaluation of Nelumbinis stamen in Chinese Pharmacopoeia (2010 Version) is still in a blank, so it is necessary to develop an effective method to evaluate its quality accurately and systematically.

An accurate, rapid and systematic high performance liquid chromatography coupled with diode array detection and mass spectrometry (HPLC–DAD–MS) of multiple flavonoids determination in combination with chromatographic fingerprint analysis was developed for chemical quality evaluation of Nelumbinis stamen. The extraction protocol was optimized by an orthogonal experimental design. 11 flavonoids were identified and determined simultaneously. Furthermore, similarity analysis (SA), hierarchical clustering analysis (HCA) and principal components analysis (PCA) were successfully applied to demonstrate the variability of the 11 flavonoids in the 14 batches of Nelumbinis stamen collected from different localities. Multiple kinds of statistical analysis software were successfully applied to data mining to make the results more accurate and reliable.

Materials and methods

Chemical and materials

Eight flavonoid glycosides and three aglycones were obtained from Phytomarker Ltd. (Tianjin, China). Acetonitrile were purchased from Honeywell Burdick & Jackson (Muskegon, USA). Analytical grade of methanol was purchased from Beijing Chemical Works (Beijing, China). Formic acid, acetic acid and phosphoric acid (HPLC grade) were obtained from Tianjin Guang Fu Fine Chemical Research Institute (Tianjin, China). Pure water (18.2 MΩ) for the HPLC analysis was obtained from a Milli-Q System (Millipore, Billerica, MA, USA).

Plant samples

14 batches of Nelumbinis stamen samples were collected from different localities of Jianning County in Fujian province of China. All air-dried samples were ground and sieved (65-mesh), respectively. A sample (1.0000 g) was suspended with 60 ml methanol in a capped conical flask, weighed accurately, and reflux-extracted twice (1 h for each time). The combined extracts were evaporated to 10–15 ml in a rotary evaporator. The residue was transferred to a 25 ml volumetric flask with methanol, and then added methanol to the mark after cooling to room temperature. The sample solution was filtered through a 0.22 μm membrane filter prior to injection into the HPLC system.

HPLC methods

Chromatographic analysis was performed on an Agilent 1260 HPLC system coupled with diode array detector (Agilent Technologies, Palo Alto, CA, USA). Chromatographic data were processed by Agilent Chem Station software. Chromatographic separation was performed on a Poroshell 120 C18-column (100 mm × 4.6 mm, 2.7 μm, Agilent, CA, USA). The mobile phase consisted of 0.7% acetic acid in water (A) and methanol (B), and the flow rate was at 0.6 ml/min. The eluting conditions were optimized as follows: 0–26 min at 15% B; 26–30 min from 15 to 31% B; 30–35 min from 31 to 35% B; 35–42 min at 35% B; 42–45 min from 35 to 90% B and 45–50 min at 90% B with re-equilibration of the column at 50–52 min from 90 to 15% B, and 52–60 min at 15% B. Chromatograms were acquired at 360 nm and diode array spectra were recorded from 210 to 600 nm. The column temperature was maintained at 36 °C and the injection volume was 2 μl.

Identification of flavonoids

HPLC–DAD–MS analysis was carried out with Applied Biosystem 3200 Q-Trap mass spectrometer (Foster City, CA, USA) connected to an Agilent 1200 HPLC system via electrospray ionization interface. The chromatographic conditions were as described above. Electrospray ionization was applied in negative ion mode for MS and MS/MS to give fragmentation information on the molecular weights and aglycone groups. The mass spectrometers were optimized in negative ion mode with an ion spray voltage of 4000 V, curtain gas of 10 psi, nebulizer gas of 60 psi and auxiliary gas 40 psi. The ion source temperature was set at 400 °C. Ultrapure nitrogen was used as nebulizer, heater, curtain and collision-activated dissociation (CAD) gas. Data were processed by the Analyst 1.4 software (Applied Biosystems/MDSSciex). MS data, retention time and UV–Vis spectra were used to identify the flavonoids contained in Nelumbinis stamen. The assignments were validated by co-elution with the corresponding standards and comparison with the published data.

Preparation of standard solutions and method validation

Each standard was accurately weighed, dissolved in methanol, and the standard solutions were then diluted to generate an appropriate concentration range to establish calibration curves. All calibration curves were constructed by using five different concentrations of each standard in triplicate. Analytical method was validated for the calibration curves, limit of detection and quantitation (LOD and LOQ), repeatability, stability, and accuracy of the 11 flavonoids.

Optimization of flavonoids extraction

Optimization of flavonoids extraction conditions from Nelumbinis stamen was studied via an orthogonal (L_9 3^4) experimental design, including three methanol concentrations at 70%, 90% and 100% (v/v), three solvent to sample ratios [20:1, 40:1, 60:1 (v/w)], three different extraction time (0.5 h, 1 h, 1.5 h), and three extraction cycles (1, 2, 3 cycles). Each extract combination was tested in triplicate, and the optimized extraction conditions were as described in 'Plant samples'.

Chemometrics analysis

Similarity analysis was performed by the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese

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