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Rapid determination of flavonoids in plumules of sacred lotus cultivars and assessment of their antioxidant activities

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ABSTRACT

As recorded in "Chinese Pharmacopoeia", lotus plumule can be used as medicine. In present study, flavonoids in plumules of 38 lotus (*Nelumbo nucifera* Gaertner) cultivars were evaluated and quantified by ultra-performance liquid chromatography with photodiode array (UPLC-PDA) and ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS/MS) for the first time. In total, twenty flavonoids were detected and identified, among which fourteen were found to be flavonoid *C*-glycosides. These cultivars were clustered into three clusters characterized by sum of detected flavonoids. Furthermore, our study showed that the correlation between total polyphenol content and antioxidant activity which was measured by DPPH and FRAP assays was significant positive. Among them, 'Taikonglian', 'Jinqi', 'Yinqiu', and 'Hongtailian' exhibited the greater potential of antioxidant activity and could be utilized in the production of healthcare products. This research is of great value to more comprehensive understanding of medicinal property of lotus plumule. Also, it will be helpful in the development of commercial exploitation of lotus plumule.

1. Introduction

Flavonoids are important polyphenolic compounds occurring in plant kingdom and have received much attention for their potential role in human health in recent years. Usually, there are two ways that sugars are linked to flavonoid skeletons resulting in *C*- or *O*-glycosides (Abad-García et al., 2008). In *C*-glycosides, sugars are attached directly to the nucleuses through a C—C bond that is stable towards acid hydrolysis, while the sugars of *O*-glycosides are connected to aglycones by an acid hydrolyzable O—C bond (Du et al., 2010). Generally, *C*-glycosides are less common than *O*-glycosides (Brazier-Hicks et al., 2009). They are only present in some specific plant groups with substitution occurring singly or doubly at the *C*-8 and/or *C*-6 position (Brazier-Hicks et al., 2009 Harborne, 1993). These flavonoid *C*-glycosides have various biological properties, such as antimicrobial (Dinda et al., 2006), antifungal (McNally et al., 2003), antioxidant (Kitta et al., 1992; Ramarathnam et al., 1989), and UV-protective (Les and Sheridan, 1990). In medicinal perspective, some activities also have been confirmed, such as counteracting inflammation and cancer development (Manthey et al., 2001), acting as antihypertensive (Prabhakar et al., 1981) and anti-obesity agent (Kim et al., 2010).

Sacred lotus, an ancient dicotyledonous plant, belonging to *Nelumbo* genus, consists of only two species all over the world, namely *Nelumbo nucifera* Gaertn. and *Nelumbo lutea* Pers. In China, it has a history of cultivation over 2000 years and has been widely cultivated in the majority of provinces (Guo, 2008). As one of the top ten famous Chinese traditional flowers, lotus is highly appreciated for its ornamental value and unique cultural and religious significance (Li et al., 2010). As recorded in "Chinese Pharmacopoeia", plumule, stamen, leaf and other parts of lotus can be used as medicines. Virtually, it is employed as not only foodstuff but also herbal medicine (Shen-Miller et al., 2002).

Lotus plumule is the green germ of a mature lotus seed with bitter taste, located between the two cotyledons (Kato et al., 2015). With so many kinds of bioactive compounds including alkaloids, flavonoids, polysaccharides, sitosterols, volatile oils and microelements in it, lotus plumule can be used in the treatment

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of hypertension, arrhythmia, platelet aggregation, lipid peroxidation, cancer and radicals scavenging (Zeng et al., 2005). Liensinine, isoliensinine and neferine are thought to be the three major biologically active compounds in lotus plumule (Yu et al., 2013). And the pharmacological properties of these bisbenzylisoquinoline alkaloids including antioxidant, antidepressant, antiarrhythmic, and anti-HIV have been demonstrated (Dong et al., 2012; Itoh et al., 2011; Kashiwada et al., 2005; Zhou et al., 2007). In previous studies, more attention has been given to the investigation of alkaloids and little is known about the flavonoids. Recently, accumulated *C*-glycosyl flavonoids have been found in lotus plumule (Li et al., 2014). To comprehend the influence of the newly discovered compounds on medicinal value of lotus plumule, *C*-glycosyl flavonoids in lotus cultivars were assessed in this study. A fast and efficient method was established to evaluate the flavonoid profiles in lotus plumules by UPLC and two complementary methods were used to estimate the antioxidant activity. This work will also be helpful in clarifying flavonoids metabolic pathways in lotus plumule.

2. Materials and methods

2.1. Plant materials

Seeds at their full maturity of 38 lotus cultivars were collected at the lotus germplasm resources garden (latitude 39°48′N, longitude 116°28′E, and altitude 76 m) in Beijing Botanical Garden, Institute of Botany, the Chinese Academy of Sciences (IBCAS) in mid-August, 2014. These cultivars were all planted in the same size cylinders (diameter 40 cm, height 30 cm) and had been cultivated under the same conditions for more than 3 years. Plumules were peeled from seeds and dried at room temperature, then ground into fine powder in liquid nitrogen using mortars and pestles, and stored in desiccator for later use. All concentrations used in this study were calculated from dry weight (DW).

2.2. Chemicals and materials

Flavone *C*-glycosides standards of luteolin 8-*C*-β-D-glucopyranoside (orientin), luteolin 6-*C*-β-D-glucopyranoside (isoorientin), apigenin 8-*C*-β-D-glucopyranoside (vitexin), and apigenin 6-*C*-β-D-glucopyranoside (isovitexin) were purchased from Shanghai Tauto Biotech (Shanghai, China). Apigenin 6-*C*-glucoside-8-*C*-arabinoside (schaftoside) and apigenin 6-*C*-arabinoside-8-*C*-glucoside (isoschaftoside) were purchased from Beijing Bio-function Biotech (Beijing, China). Flavonol standard of quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (rutin) and gallic acid (GA) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Folin-Ciocalteu phenol reagent and 2,4,6-tripyridyl-*S*-triazine (TPTZ) were purchased from Sigma–Aldrich (St. Louis, USA). 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH·) was obtained from Tokyo Chemical Industry (Shanghai) Development Company, Limited. Formic acid and acetonitrile used for chromatographic analysis were of chromatographic grade and purchased from Alltech Scientific (Beijing, China). The rest of chemicals, including sodium carbonate, sodium acetate, ferric chloride, methanol, hydrochloric acid, acetic acid were of analytical grade and obtained from Beijing Chemical Works (Beijing, China). Double distilled water was produced by a Milli-Q System (Millipore, Billerica, MA, USA) and 0.22 μm millipore membrane filters were purchased from ANPEL Scientific Instrument Corporation (Shanghai, China).

2.3. Flavonoids extraction

The method of flavonoids extraction referred to that of Chen et al. (2012) with some modifications. Approximate 50 mg of plumule powder was mixed with 1 mL methanol-water (70:30, v/v), shaken in a QL-861 vortex (Kylinbell Lab Instruments, Jiangsu, China) for 30 s, sonicated in KQ-500DE ultrasonic cleaner (Ultrasonic instruments, Jiangsu, China) at room temperature for 20 min, centrifuged in SIGMA 3K30 (Sigma Centrifuges, Germany) (12,000 rpm, 10 min), and the supernatant was collected. The above steps were repeated for 3 more times. The collected supernatant was pulled together and supplemented up to 4 mL with methanol-water (70:30, v/v), and then placed at −20 °C for 24 h to precipitate chlorophyll. The extract was filtered through 0.22 μm millipore membrane after centrifugation. Then the clear supernatant was analyzed on UPLC-PDA and UPLC-MS/MS. Three replicates were performed for each sample.

2.4. UPLC-PDA system and conditions

Chromatographic separations were performed on a 10 cm × 2.1 mm Waters® ACQUITY™ 1.7 μm BEH C_{18} column (Waters, Milford, MA, USA) using a Waters ACQUITY Ultra Performance Liquid Chromatograph (UPLC I-CLASS, Waters) system. Chromatograms were acquired at 350 nm and photodiode array spectra were recorded from 200 to 800 nm. Eluent A was 0.1% formic acid aqueous solution, eluent B was absolute acetonitrile. A gradient elution protocol as follows was used: 5% B at 0 min, 13% B at 1 min, 24% B at 4 min, and 33% B at 7 min. The flow rate was 0.2 mL min^{-1} and the column temperature was maintained at 25 °C. The injection volume of each sample was 1 μL.

2.5. UPLC–MS/MS system and conditions

UPLC–MS/MS analysis for flavonoids were performed using a Xevo™ TQ-MS triple quadrupole mass spectrometer (Waters, Milford, MA, USA) connected to an ACQUITY Ultra Performance Liquid Chromatograph (UPLC I-CLASS, Waters). The UPLC separation conditions were the same as mentioned above. The flavonoids were employed both in positive ion (PI) and in negative ion (NI) mode and MS detection conditions were as follows: capillary voltage, 3.00 kV for PI mode and 2.50 kV for NI mode; cone voltage, 10 V for PI mode and 70 V for NI mode; desolvation gas flow, 650 L/h; cone gas flow, 50 L/h; collision gas flow, 0.12 mL/min; collision energy, 15 eV for PI mode and 30 eV for NI mode; desolvation temperature, 350 °C; source temperature, 150 °C; scan range, 100–1000 (*m*/*z*). Analytical software (MassLynx, version 4.1) was used for the system control and data processing.

2.6. Quantitative and qualitative analysis of flavonoids

Quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (rutin) was used as standard for the semi-quantitative analysis of the flavonoids. Six concentrations of rutin were prepared with methanol-water (70:30, v/v) and detected with UPLC-PDA at the wavelength of 350 nm. The regression equation was Y = 132,759 X (r^2 = 0.9981), showing good linearity between concentrations and peak areas. The results were presented in the form of milligrams of standard per 100 g DW. The other standards were dissolved in methanol-water (70:30, v/v) for qualitative analysis of samples by co-elution.

2.7. Total polyphenol content

The total polyphenol content (TPC) of extract was measured by means of Folin-Ciocalteu method (Wang et al., 2014). GA was