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Antioxidant capacity of extract from edible flowers of *Prunus mume* in China and its active components

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ABSTRACT

Prunus mume flowers are used as traditional edible and medicinal materials in China. In this study, phenolic compounds and antioxidant activity of ethanolic extract from flowers of *P. mume* in China were investigated for the first time. The total phenol content was estimated as gallic acid equivalents by the Folin–Ciocalteu reagent method. The antioxidant activity was measured by DPPH$^{\bullet}$, ABTS$^{\bullet+}$, and OH$^{\bullet}$ free radicals scavenging and ferric-reducing antioxidant power (FRAP). Three chlorogenic acid isomers, namely, 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids, were isolated and purified by preparative HPLC from the ethanolic extract and identified by UV, MS and NMR. The contents of these isolated compounds were quantified by HPLC. Results showed that 5-*O*-caffeoylquinic acid was of the highest level in these three isomers. The ethanolic extract demonstrated activity to some degree in all the antioxidant assays. In all tested assays, all of the isolated chlorogenic acid isomers exhibited strong antioxidant activities, which were almost the same. The results showed that chlorogenic acid isomers are the key phenolic compounds which are responsible for antioxidant activity of the ethanolic extract from Chinese *P. mume* flowers.

1. Introduction

Prunus mume Sieb. et Zucc, originated in China, has been widely cultivated in Asia for about 3000 years. *P. mume* fruits are often consumed as preserved fruit and wine. In particular, *P. mume* flowers are used to make various delicious foods since ancient times, such as porridge, soup and drink. In Chinese traditional medicine, various parts of *P. mume* (*i.e.*, immature fruit, flowers, leaves, branches, seeds and roots) have been used as herbal medicine materials. For example, the fruit has been used as the medicine to cure fever, cough and intestinal disorder. Recently, the concentrated fruit juice has been found to markedly improve human blood fluidity *in vitro* and also to relieve tension in experimental menopausal rats (Ina, Yamada, & Matsumoto, 2004; Utsunomiya et al., 2002). Particularly, flowers of *P. mume* have been prescribed for stomachic, alexipharmic, expectorant, and sedative purposes and for the treatment of eye pain and skin disorders. Recently, Yoshikawa et al. (2002) reported that the methanol extract from flowers of *P. mume* in Japan exhibited inhibitory activity on aldose reductase and platelet aggregation *in vitro*.

It is well known that oxidative damage of biological molecules in human body is involved in degenerative or pathological process such as aging, stroke, diabetes mellitus, coronary heart disease and cancer. Consequently, the traditional function of *P. mume* flowers may result from its antioxidant activity. Recently, phenolic compounds have received growing attention, because they have been reported to have anti-mutagenic, anti-carcinogenic and antioxidant activities. Concerning the phenolic compounds, 2″-*O*-acetylrutin, 2″-*O*-acetyl-3′-*O*-methylrutin, quercetin 3-*O*-(2″,6″-α-L-dirhamnopyranosyl)-β-D-galactopyranoside, quercetin 3-*O*-rhamnosyl (1 → 6) galactoside, quercetin 3-*O*-neohesperidoside, isorhamnetin 3-*O*-rhamnoside, chlorogenic acid and rutin were detected from the flowers of *P. mume* in Japan (Matsuda et al., 2003; Yoshikawa et al., 2002), but there have been no studies on flowers of *P. mume* cultivated in China. The profile of phenolic compounds may vary with country of origin (Zheng & Clifford, 2008).

In order to supply more scientific evidences for the research and development of the edible flowers of *P. mume* in China in the field of pharmaceutical and functional foods, this study mainly investigated the antioxidant activity of the ethanolic extract from flowers of *P. mume* in China and identified their phenolic compounds for the first time. Furthermore, the contents and the antioxidant activities of the identified phenolic compounds were also compared and evaluated.

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2. Materials and methods

2.1. General

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich (Steinheim, Germany). 5-*O*-Caffeoylquinic acid, Folin–Ciocalteu reagent, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,4,6-Tri(2′-pyridyl)-1,3,5-triazine (TPTZ) and 3-aminophthalhydrazide were purchased from Sigma chemical Co. (St. Louis, MO, USA). 3-*O*-Caffeoylquinic acid and 4-*O*-caffeoylquinic acid were purchased from Chengdu Purification Technology Development Co. Ltd (Chengdu, China). ^{1}H NMR and ^{13}C NMR spectrometry was recorded on an Avance DMX-500 (500 MHz) NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). UV data were obtained from HPLC with DAD analysis (Waters, Milford, MA, USA). Mass spectral data were obtained with an APEX III Fourier-transform ion cyclotron resonance mass spectrometry (Bruker Daltonics Inc., Billerica, MA, USA). Ultrapure water made by the Milli-Q system (Millipore, Bedford, USA) was used during both preparative and analytical HPLC analysis. All solvents used for chromatography were of HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Plant material

Fresh flowers of *P. mume* were collected from Xiaoshan, Zhejiang, China, during February of 2006. Botanical identification was performed by Xiaoshan Research Institute of *P. mume* (Xiaoshan, Zhejiang, China).

2.3. Extraction

Heat reflux method was employed in the process of extraction. The air-dried and ground flowers of Chinese *P. mume* were extracted with ethanol/water (30:70, v/v) solution (1:10, w/v) for 1 h at 60 ± 1 °C. The sample was extracted twice under same condition. Then the filtered extracts were mixed and concentrated at 40 ± 1 °C in a rotary evaporator under reduced pressure. At last, the concentrated extract was lyophilized to yield a dark-brown powder. It was denoted as the ethanolic extract.

2.4. Determination of total phenol content

Total phenol content of the ethanolic extract was determined using the Folin–Ciocalteu reagent method with a little modification (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, the ethanolic extract was dissolved in ethanol/water (30:70, v/v) solution, 100 μL aliquot of the sample solution was transferred to a 10 mL volumetric flask, containing 6.0 mL distilled water, to which was subsequently added 500 μL undiluted Folin–Ciocalteu reagent. After 3 min, 2 mL of Na_2CO_3 (15 g/100 mL) was added and the volume was made up to 10 mL with distilled water. After a 2 h incubation period at room temperature, the absorbance was measured at 760 nm. Gallic acid was used as an analytical standard for total phenol quantification. The total phenol content was determined as mg of gallic acid equivalents (GAE)/g dry weight of the ethanolic extract.

2.5. Isolation and purification of phenolic compounds

The ethanolic extract (3.00 g) was dissolved in methanol and filtered through a 0.45 μm membrane filter. This solution was fractionated by preparative reverse-phase HPLC. The chromatographic separation was performed on a Waters DeltaPrep 600 Preparative Chromatography system equipped with Waters Prep LC Controller and Waters 2996 Photodiode Array Detector (Waters, Milford, MA, USA). The preparative reverse-phase HPLC was performed on a Hedera® ODS-2 preparative column (10 μm, 300 mm × 30 mm I.D., Hanbon Sci. & Tech, Jiangsu, China). The mobile phases included glacial acetic acid/water (2:98, v/v) (solvent A) and methanol (solvent B). A gradient elution program was used for preparative separation as follows: 88:12(A/B) to 55:45(A/B) in 50 min, 0:100 (A/B) in a further 40 min. The flow rate was 10 mL/min while the monitored wavelength was 328 nm. The eluate was collected in three fractions. These fractions were further isolated separately by preparative reverse-phase HPLC [Luna C_{18} column (10 μm, 250 mm × 10 mm I.D., Torrance, CA, USA), acetonitrile: glacial acetic acid/water (2:98, v/v) = 15:85 (v/v), flow rate: 3 mL/min, the monitored wavelength: 328 nm] to yield three pure compounds from the 3 g ethanolic extract as follows: compound **1** (10.6 mg), compound **2** (33.8 mg) and compound **3** (14.8 mg).

2.6. Analytical HPLC detection

The ethanolic extract and each purified compound from the preparative HPLC separation were analyzed by analytical HPLC, which was performed on a Waters 2695 HPLC chromatograph (Waters, Milford, MA, USA) with a Luna C_{18} column (5 μm, 250 mm × 4.6 mm I.D.) purchased from Phenomenex (Torrance, CA, USA). A gradient elution programme was used with the mobile phase, combining solvent A [glacial acetic acid/water (2:98, v/v)] and solvent B (acetonitrile) as follows: 100:0 to 90:10(A/B) in 10 min, 90:10 to 75:25 (A/B) in 30 min, 75:25 to 35:65 (A/B) in1 min, held for 10 min, 35:65 to 100:0 (A/B) in 1 min, held for 10 min. The flow rate was 0.7 mL/min. The column temperature was maintained at 40 °C. Signal was monitored at 328 nm with the diode array detector (DAD).

Standard solutions of compounds **1**–**3** were prepared at 1 mg/mL in methanol/water (50:50, v/v). They were diluted to make seven concentrations (5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100 μg/mL) calibration curves. The ethanolic extract was dissolved in methanol/water (50:50, v/v) (1 mg/mL). The solutions were filtered through a 0.45 μm membrane filter, and aliquots of the filtrate (15 μL) were injected. All of the above experiments were replicated three times. These tested compounds in the ethanolic extract were quantified from the calibration curve (Table 1).

Table 1
Calibration curves[a] and LOD[b] for 3 standard compounds under the proposed HPLC method

Name of the compounds	Slope, *a*	Intercept, *b*	R^2	LOD (μg/mL)
3-*O*-Caffeoylquinic acid	66,701 (±288)	−138,015 (±26,248)	0.9994	0.21
5-*O*-Caffeoylquinic acid	60,900 (±221)	37,052 (±4350)	0.9996	0.16
4-*O*-Caffeoylquinic acid	51,372 (±123)	−27,128 (±3176)	0.9999	0.29

[a] For each calibration curve, the equation is $y = ax + b$, where y is the peak area, x is the concentration of the analyte (μg/mL), a is the slope, b is the intercept and R^2 is the correlation coefficient. SD values are given in parenthesis.
[b] LOD: the limits of detection correspond to concentrations giving a signal-to-noise ratio of 3.