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Original Article

4-O-Caffeoylquinic acid as an antioxidant marker for mulberry leaves rich in phenolic compounds

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ABSTRACT

Mulberry (*Morus alba* L.) leaves are widely used as herbal tea to prevent heat stroke. Potential chemical markers of the antioxidant properties and its correlation with harvesting times and leaf location were explored in this study. A 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay guided isolation of mulberry leaves extract provided five phenolic compounds: 5-O-caffeoylquinic acid (**1**), 4-O-caffeoylquinic acid (**2**), gastrodin (**3**), isoquercetin (**4**) and rutin (**5**). The 50% radical-scavenging concentrations (SC_{50}) of these compounds were 32.76 ± 0.27, 11.41 ± 0.48, 404.30 ± 4.92, 10.63 ± 0.96, and 10.57 ± 0.61 μg/mL, respectively. Chromatographic fingerprinting allowed content analysis of **1**–**5** in samples over a 12-month period. Compounds **1**–**5** were abundance in apical leaves (0–10 cm) in January and February at temperatures < 20 °C. Contents of **2** and **5** were highest in these months and were strongly correlated to the antioxidant property. Therefore, we suggested that the mulberry leaves harvested during January and February have high yield of 4-O-caffeoylquinic acid and this compound can be used as antioxidative marker in mulberry leaves.

1. Introduction

Mulberry (*Morus alba* L.) is globally cultivated due to its economic importance as a food for silkworms (*Bombyx mori* L.) which dates 5000 years ago [1]. Besides of its agronomic value, leaves of mulberry are traditionally used in Asia and Europe either as food in the form of beverage or as decoction remedy for sore throat [2,3]. Several health benefits can be derived using leaves of *M. alba*. Previous studies reported the following biological activities; anti-oxidant, antibacterial, antiviral,

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anticancer, antifatigue, hypolipidemic, hepatoprotective, neuroprotective, inhibitors of α-glucosidase, tyrosinase, hypertension, and arteriosclerosis [4–6]. Phytochemicals in mulberry leaves such as phenolic acids, flavonol glycosides, chalcones, alkaloids, γ-aminobutyric acid, iminosugars, prenylated stilbenes, derivatives of aryl benzofuran and coumarins were responsible for the observed bioactivities [4–6]. The antioxidative property of *M. alba* was due to its ability to prevent lipid peroxidation and adipocytokine dysregulation [7]. Polyphenolic acids and glucosides were characterized from its leaves using HPLC-IT-TOF-MS techniques [8]. Furthermore, variation in flavonol glycosides was observed in different cultivars of mulberry in Japan [9], as much as significant variation in antioxidant potential were determined from three *Morus* species in Pakistan [10].

The surmounting evidence of the ameliorative properties against oxidative stress of the components of mulberry fruits and leaves when consumed as food, leads to increased attention of its application for the treatment of hyperglycemia [11]. Meanwhile as a medicinal material in China, the leaves were collected pass the winter frost [12]. This brings attention on the possible effects of environmental conditions on the content of bioactive substances in mulberry leaves. For an example, increased in applied nitrogen has an inverse effect on the chlorogenic acid but a positive significant effect on 1-deoxynojirimycin content [4]. Growing demand for this raw material as functional food sources due to its high antioxidant capacity would require mulberry farmers to address the good agricultural and collection practice (GACP) requirements to assure quality and effectiveness of harvested products. The aim of the present work was to identify potential chemical markers for the antioxidant properties of *M. alba* leaves using a bioassay guided isolation scheme. This study also determined the best harvest time and leaf location which provide most of these bioactive polyphenols. This method shall help ensure the quality of the material source. To the best of our knowledge, this is the first study conducted to monitor content changes of the antioxidant components in *M. alba* leaves for a period of 1 full year.

2. Materials and methods

2.1. Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu phenol reagent, trifluoroacetic acid (TFA), sulfuric acid, sodium carbonate, vanillin, gallic acid, L-ascorbic acid, (+)-catechin, deuterium oxide (D_2O), and deuterated methanol (CD_3OD) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Purospher STAR RP-18e column (4.0 mm i.d. × 250 mm, 5 μm) was purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile were LiChrosolv and were purchased from Merck. Diaion HP-20 gel (Mitsubishi Chemical Industry, Tokyo, Japan), Sephadex LH-20 gel (GE Healthcare Biosciences, Uppsala, Sweden), LiChroprep RP-18 gel (40–63 μm, Merck), MCI CHP20P gel (Supelco, Bellefonte, PA, USA) and an Oasis HLB cartridge (12 mL, 500 mg, Waters, Milford, MA, USA) were used for the purification and isolation processes. Polyvinylidene difluoride (PVDF) 0.45-μm 13 mm syringe filters was purchased from IT'S Science Corporation (Taipei, Taiwan).

2.2. Plant materials

Commercial dry leaves of *M. alba* L. (locally known as Sang Ye) were purchased from a local traditional Chinese medicine (TCM) store in Taipei, Taiwan. Leaves were pulverized using an electric grinder. Fresh leaves of *M. alba* were collected from the mulberry tree at the botanical garden (N 25°01′30.340, E 121°33′36.287) located in the university campus of Taipei Medical University, from July 2014 to June 2015 (Fig. S1). This tree has a main tree trunk circumference of 57 cm and a crown height of about 450 cm. Leaves were collected from a branch located about 350 cm from the ground. During the 12-month sampling period, changes of the climatic factors over Taipei, Taiwan such as temperature (°C), the amount of precipitation (mm), and sunshine duration (h) were recorded using the Central Weather Bureau's online database [13], Figs. S2–S4. Leaves were classified according to their relative position from the top of a branch. Apical leaves were collected from 0 to 10 cm, followed by middle leaves from 10 to 30 cm and bottom leaves from ≥30 cm. These were rinsed with reversed osmosis (R.O.) purified water and air dried at room temperature for 5–7 days while avoiding direct sunlight exposure. Only air-dried leaves with <15% moisture (AND MX-50, Tokyo, Japan) were pulverized using an electric grinder and kept in vacuum sealed containers avoiding direct sunlight until used for analysis. A voucher specimen was deposited in the School of Pharmacy, College of Pharmacy, Taipei Medical University (Taipei, Taiwan).

Furthermore, another batch of fresh leaves of *M. alba* L. were collected from Yangmingshan National Park (N 25°09′34.230, E 121°32′46.392), a mountainous region at the northern part of Taiwan about 14.95 km away from Taipei on March 2017 (Fig. S5). This tree has a trunk circumference of 58.5 cm and a crown height of 500 cm. Leaves were collected from a branch about 350 cm from the ground. Two groups of leaves were classified as young (budding, translucent light green leaflets) and old (mature, opaque dark green to yellow leaves).

2.3. Extraction and bioassay-guided isolation

Commercial leaf powder (600 g) of *M. alba* was refluxed three times with 6 L of water-methanol (1:1, v/v) at 65 °C for 2 h. After filtration and concentration, the aqueous solution was lyophilized to obtain the 50% methanolic extract (147.03 g). The crude extract was re-suspended in 500 mL deionized water and partitioned with 500 mL *n*-hexane six times to provide an aqueous layer (112.00 g) and an organic layer (35.03 g). Following a DPPH radical scavenging activity-guided isolation, as described in the succeeding sections, the more bioactive aqueous layer (100 g) was chromatographed over a Diaion HP-20 (9.5 cm i.d. × 45 cm) column chromatography (CC), and eluted step-wise with deionized water, followed by methanol/water (2:8, 4:6, 6:4, v/v) and acetone. Resulting fractions were designated as DW (81.38 g), D20M (4.83 g), D40M (3.82 g), D60M (4.84 g), and DA (3.64 g). The most bioactive

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