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Letter to the Editor

Characteristics of fruit growth, component analysis and antioxidant activity of mulberry (*Morus* spp.)

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ABSTRACT

The chemical constituents including β-sitosterol, scopoletin, rutin, quercetin, and kaempferol in *Morus atropurpurea* fruits were purified using column chromatography in this study. Moreover, in which, scopoletin, rutin, quercetin, and kaempferol were evaluated as indicator components to analyze multiple components simultaneously by HPLC. The HPLC method ensured the accuracy and efficiency, and could be used for analyzing the quantity of the constituents of the leaves, branches, and fruits which were in colors of green, red, and black of three *Morus* species, *M. atropurpurea*, *Morus laevigata*, and *Morus alba*. The results indicated that the fruits had highest content of scopoletin, leaves had highest content of rutin, black fruit had highest content of quercetin, and the branches had highest content of kaempferol. Analysis of various species revealed that the most content of selected compounds, total phenolic and flavonoids were found in the branches and fruit of *M. atropurpurea*; meanwhile, it also had the highest DPPH scavenging ability. However, the leaves of *M. laevigata* had the highest antioxidant activity.

1. Introduction

Mulberry (Moraceae, *Morus*) is a perennial deciduous fruit, and is one of abundant fruits during spring in Taiwan. Mulberry flowers in January, fruits in January to February, and can be harvested from March to April. The growing locations of mulberry are in eastern and mid-northern lower sea level mountainsides of Taiwan. Among those places, the biggest planting areas are at Rei Suei, Hwalian County, followed by Miaoli County (Chang et al., 2005).

Mulberry not only produces many fruits, but also its leaves, branches, and root skin are famously used as traditional Chinese medicinal and as crude drugs with names such as Mori Folium, Mori Ramulus, and Mori Cortex. Many reports had studied the protein, carbohydrate, amino acid, organic acid, carotene, dry matter and anthocyanin content of some mulberry species, as well as their flavor, total acidity, ascorbic acid and pH (Elmaci and Altug, 2002; Gerasopoulos and Stavroulakis, 1997; Lale and Ozcagiran, 1996; Ozdemir and Topuz, 1998; Jia et al., 1999). The mulberry fruits contain numerous chemical constituents, including polyphenolics, phytosterols, triterpenes, cardiac glycosides, saponins, alkaloids, anthocyanins, volatile oils, amino acids, fatty acids, and the minerals in the fruits including N, P, K, Ca, Mg, Na, Fe, Cu, Mn, and Zn were found (Ercisli and Orhan, 2007; Du et al., 2008; Aramwit et al., 2010; Wu et al., 2011). However, no simultaneous analytical method for scopoletin, rutin, quercetin, kaempferol in mulberry has been reported. These nutritional and chemical compositions have some pharmacological activities such as reducing cholesterol levels, coronary sclerosis and high blood sugar levels, and relieving coughing and sputum. They also have other pharmaceutical applications (Bondada et al., 2001). A number of studies have conducted antioxidant activity of leaves, twigs, root bark, different cultivars, and alcoholic fermentation process in mulberry (Andallu and Varadacharyulu, 2003; Katsube et al., 2006; Chang et al., 2011; Bae and Suh, 2007; Pérez-Gregorio et al., 2011); however, no report has been made to discern the effects of various ripeness fruits on the antioxidant activity of mulberry.

This study used three species, *Morus alba* L., *Morus laevigat*, and *Morus atropurpurea* to investigate the physiological and chemical nature during their fruits growing process. This investigation examines the chemical constituents of the fruits of mulberry using column chromatography. Index standards including scopoletin, rutin, quercetin, kaempferol were also established by the simultaneous analytical HPLC method to ensure accuracy and efficiency. Meanwhile, the antioxidant activity was also evaluated. The same approach was used to analyze compositional variations among mulberry tree species (*M. atropurpurea*, *M. laevigata* and *M. alba*) and with fruits of various ripeness.

2. Materials and methods

2.1. Materials

Three perennial *Morus* species (*M. atropurpurea*, *M. laevigata* and *M. alba*) were randomly collected from 60 plants in each block of the three blocks of plants (90 plants/block of each variety, separately) in the Fruit Garden of the Department of Plant Industry, National Ping-tung University of Science & Technology (NPUST). The identities of the materials were confirmed by Professor Chung-Ruey Yen. Voucher specimens were maintained in the laboratory of the corresponding author at the Department of Plant Industry, NPUST. They were then transplanted in the farm of NPUST when they were one year old. The plants were grown for two years in the

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field before harvesting. The tested parts of mulberry plant were the leaves, branches, and fruits which were in color of green, red, and black.

2.2. Chemicals and reagents

Baicalein, used for internal standard, was purchased from Fluka (Buchs, Switzerland). The 95% ethanol was purchased from Taiwan Tabacco and Wine Board (R.O.C.). 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, and Folin–Ciocalteu were purchased from Sigma Chem. Co., (USA). Acetonitrile and methanol (HPLC grade) were purchased from Mallinckrodt, Inc. (USA). Phosphoric acid (analytical-reagent grade) was purchased from Kanto Chemical (Japan). Other reagents that used in this study were all of analytical grade.

2.3. Measurement of physical and chemical properties of mulberry fruits at different maturing stages

2.3.1. Dry weight and hydrous content

Ten grams each of the three species fresh mulberry fruits in different stages were frozen dried to a constant weight separately. The dry weight and hydrous content were calculated using the equations listed below.

Dry weight (%) = (Dry weight of fruit meats/fruit meats before drying) × 100%.

Hydrous content = ((fruit flesh weight before drying − fruit flesh weight after drying)/fruit flesh weight before drying) × 100%.

2.3.2. pH value

Fifty grams each of fresh mulberry fruits in different stages of three species were made into juice using blender and then were centrifuged. The pH of suspension was tested at room temperature using a pH meter.

2.3.3. Sugar content

Different stages of three species fresh mulberry fruits were made into juice by using a blender and were centrifuged at 4 °C, 13,000 *g* for 20 min. Then, sugar content of suspension was calibrated by refractometer, and the data were shown in °Brix (Mitchell et al., 1991; Young et al., 1993).

2.3.4. Hue of fruits

Fresh mulberry fruits of three species, at different making stages, were made into juice of which the *L* value (brightness), α value, β value, θ value (hue angle), and *C* value (chroma) were measured. *L* value ranges between 100 and 0. The higher is the *L* value, the brighter is the appearance. If α value is positive, the hue is toward red. If α value is negative the hue is toward green. If α value is zero the hue is toward gray. Meanwhile, if β value is positive, the hue is toward yellow. If β value is negative, the hue is toward blue. If β value is zero, the hue is toward gray. The θ value was calculated in $\tan^{-1}(\beta/\alpha)$ to present the changes in hue of fruits' color. The value of θ represents that 0 means red, 90 means yellow, 180 means green, and 270 means blue. Chroma was calculated in $(\alpha^2 + \beta^2)^{1/2}$. The higher is the *C* value, the higher is the depth of color (Pelembe et al., 2002).

2.4. Isolation of components

Dried mulberry fruit was pulverized and each of 3.3 kg of the powder thus obtained was extracted using 30 L of methanol in three containers separately at room temperature for two days before being filtered. The methanol extracts were pooled and mixed and then was vacuum evaporated to obtain a dry residue. The residue was partitioned with water/ethyl acetate to obtain ethyl acetate layer and a water layer. The ethyl acetate layer was charged into a silica gel (70–230 mesh) open column (10 cm × 60 cm). Development was carried out using an *n*-hexane–ethyl acetate mixture as the eluting solvent to obtain eight fractions. Fractions 3, 5, 6, 7 were chromatographed on silica gel (230–400 mesh) and a sephadex LH-20 column using MeOH to yield β-sitosterol (white needless from MeOH; mp 138 °C), scopoletin (colorless needles from MeOH; mp 209 °C), rutin (yellow needless from MeOH; mp 16 °C), quercetin (yellow needless from MeOH; mp 280 °C), and kaempferol (yellow needless from chloroform; mp 277 °C). NMR spectrometry, mass spectrometry, and melting point determinations were used to characterize the compounds. All analytical values were completely consistent with those in the literature (Yu and Chen, 2000; Lin et al., 2002; Ardhaoui et al., 2004; Jiang et al., 2000; Chen et al., 2004).

2.5. HPLC instruments and conditions

HPLC analysis was carried out on a Hitachi system that was equipped with a DG-2410 degasser, an L-7100 pump, an L-7420 UV/vis detector, and an L-7200 auto-sampler. Peak areas were calculated with D-7000 HSM software. A Waters ODS-2 column (4.6 mm I.D. × 250 mm) was used. The mobile phase was a mixture of 10% acetonitrile (solvent *A*), 50% acetonitrile (solvent *B*) and 90% acetonitrile (solvent *C*) and a linear gradient elution was performed. The conditions were 0–5 min, 6% *B*; 5–10 min, 6–7% *B*; 10–35 min, 7% *B*; 35–45 min, 7–30% *B*; 45–55 min, 30% *B*; 55–60 min, 30–35% *B*; 60–85 min, 35–40% *B*; 85–90 min, 40–50% *B*; 90–100 min, 50–55% *B*; 100–110 min, 90% *C*; 110–120 min, 90–100% *C*. The flow rate was 1.0 mL/min. The detection wavelength was UV 360 nm. The column temperature was 30 °C. A 20 μL of each sample solution, prepared as described above, was injected into the HPLC column for analysis. The results were obtained by interpolation using the linear regression plot from the standard component solution.

2.6. Preparation of standard and internal standard component solutions

All of the standards were weighed and dissolved in 70% methanol to give concentrations of scopoletin 200.0 μg/mL, rutin 1000.0 μg/mL, quercetin 200.0 μg/mL, and kaempferol 100.0 μg/mL. The baicalein (720.0 μg/mL) was prepared as the standard and internal standard stock solution.

2.7. Preparation of sample solutions

All samples were dried at 60 °C for 24 h. Each sample (1 g) was extracted using 100 mL methanol at 80 °C for 2 h. The solution was filtered, evaporated, and dissolved to 5 mL by adding 70% methanol, while internal standard, baicalein, was simultaneously added to each solution to a concentration of 360 μg/mL. These test solutions were used for subsequent HPLC analysis after being filtered through 0.45 μm membrane filters.

2.8. Calibration method

Each standard component stock solution was diluted using 70% methanol to give sequential concentrations of scopoletin: 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, 50.0, 100.00 μg/mL; rutin: 3.9, 7.8, 15.6, 31.3, 62.5, 125.0, 250.0, 500.0 μg/mL; quercetin: 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, 50.0, 100.0 μg/mL; kaempferol: 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25.0, 50.0 μg/mL.

Each dilution contained the internal standard solution, baicalein, at 360 μg/mL. After filtering through a 0.45 μm membrane filter, 20 μL of each concentration solution was injected into the HPLC column for analysis. The calibration curve was plotted by using the ratio of the peak areas that corresponded to each