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Flavonoids analysis about mulberry fruit of Korean mulberry cultivar, 'Daeshim'

Wan-Taek Ju*, O-Chul Kwon, Yong-Soon Kim, Hyun-Bok Kim, Gyoo-Byung Sung, and Jong-gil Kim

National Institute of Agricultural Science, RDA, Wanju-gun, 55365, Republic of Korea

Abstract

Mulberry fruit is a new income product in Korea sericulture due to the increase of fruit consumption. However, flavonoids of Korean mulberry cultivar for fruit production did not reported yet. In this study, the typical mulberry cultivar, 'Daeshim' was analyzed using ultrahigh performance liquid chromatography coupled with diode array detection and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF/MS) technique for flavonoids analysis. Nine flavonoids were isolated and analyzed from Daeshim using UPLC-DAD-QTOF/MS chromatogram. According to quantitative analysis, rutin (66.1 mg/100g DW) and quercetin 3-O-(6"-O-malonyl) glucoside (26.7 mg/100g DW) were abundant in mulberry fruit. Our results might be used as basic information for mulberry consumption.

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Introduction

Plants have received a lot of attention as a source of biologically active substances, including antioxidants, antimutagens and anticarcinogens (Dillard and German, 2000). Many herbs, cereals, fruits, vegetables and other plant materials possess antioxidant properties, mainly because of the content of phenolic compounds. Phenolic compounds: flavonoids, phenolic acids, anthocyanins, tannins, lignans, and lignins, are important for normal plant growth and development as well as a defense against infection and injury. Especially, flavonoids are a large group of polyphenolic compounds (Enkhmaa *et al*., 2005), and it has distributed into six subclasses including flavonols, flavanones, isoflavones, flavan-3-ols, flavones, and anthocyanins (Haminiuk *et al*., 2012). Flavonoids have found applications in food and pharmaceutical industries for their valuable properties, and adsorbent resins have been utilized to separate and concentrate these products from the natural matrixes (Fu *et al*., 2005; Qi *et al*., 2007).

Mulberry (*Morus* sp.) an deciduous tree belonging to the family of Moraceae, has been cultivated in many asian countries such as Korea, China, India, Japan and where the leaves were used as food for silkworms (Agarwal and Kanwar, 2007; Nuengchamnong *et al*., 2007). It has historically been used for leaf yield in sericulture, and its fruit, leaves, branches and roots with good sources of bioactive compounds have been used in traditional medicine to treat diabetes, hypotention, anemia and arthritis (Ozgen *et al*., 2009). Some researchers have found that mulberry leaves are rich in phenylpropanoids, flavonoids, alkaloids and many other bioactive compounds (Wanyo *et al*., 2011, Sugiyama *et al*., 2016). Few species of mulberry were evaluated for their

***Corresponding author.**
Wan-Taek Ju
Department of Agriculfural Biology, National Institute of Agricultural Science, RDA, Wanju-gun 55365, Republic of Korea
Tel: +82632382855 / FAX: +82632383832
E-mail: wantaek@korea.kr

edible fruits. Flavonoids and flavonoids glycosides are the most important components in mulberry fruits. Flavonoids, as compounds originating from plants, are part of the human diet and have many positive impacts on the human organism. They act as natural antioxidants and have an effect on many diseases. Rutin, quercetin, kaempferol have also been isolated from mulberry fruits (Yang *et al*., 2012). Pawlowska *et al*. (2008) have analysis for HPLC profile of flavonoid for *M. nigra* and *M. alba* fruits, and this compounds have identified by NMR and ESI-MS. They also studied that *M. nigra* fruits contains cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and pelargonidin 3-*O*-rutinoside by HPLC/PDA/ESI-MS analysis. Also, Naderi *et al*. (2004) found that extract of *M. nigra* fruits have protective action against peroxidative damage to biomembranes and biomolecules.

With the rapid increase of mulberry fruit production in Korea, the demand for mulberry cultivars, techniques, and pest control methods for fruit production have been stronger. Korean mulberry cultivar, 'Daeshim' is a variety for high-yield mulberry fruit, and it was developed in 2014 through local adaptability test. It has over the 70% productivity when we were comparing with popular Korean mulberry cultivar, 'Chungil' (Kim and Ryu, 2000), and its fruit size is bigger than the other varieties. However, flavonoids about this mulberry fruit have not been previously studied and its composition is unknown. Also, the utilization of different *Morus* species was attempted and inter-specific hybridization was conducted to incorporate the desirable characters for crop improvement. In this study, the flavonoids about mulberry fruit of Daeshim cultivated in Korea are a quality characteristics study in order to draw attention to the nutrient profiles of Daeshim fruits and promoting the further development of the Korean mulberry resources.

Materials and Methods

Plant material and reagents

Mulberry fruits were collected from Korean mulberry cultivar, Daeshim in Sericultural and Apicultural Materials Division, RDA, Jeon-Ju, Republic of Korea. The samples of mulberry fruits were dried in lyophilizer. All dried samples were crushed and stored below -18°C prior to analysis. HPLC-grade solvents (acetonitrile, methanol, and water) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All reagents and standards were prepared using Milli-Q deionized water (Millipore, Bedford, USA). All other chemicals and reagents were of analytical reagent grade.

Sample preparation

Daeshim fruit (100 g) was prepared for this experiment, and extraction conducted according to the method described by Kim *et al*. (2012) with minimum modifications. The powdered fruits was mixed with 10 mL of acidified hydro alcoholic solvent (methanol: water: formic acid (50:45:5,v/v/v) containing 100 ppm of galangin as internal standard.). The mixture was first vortex, stirred with shaker for 5 min at 200 rpm and then centrifuged for 15 min at 3,000 rpm and 10°C. The supernatant was filtered using syringe filter (0.45 μm, PTFE, Whatman, Kent, England). 0.5 mL of filtrate was diluted with water to 5 mL of final volume. The flavonoid extract then purified and isolated by solid phase extraction method using sep-pak C-18 (Waters Co., Milford, MA, USA). Sep-pak activation was done by washing the cartridge with 2 mL of methanol, followed by 2 mL of water for conditioning. Then the diluted extract was loaded on the sep-pak and impurities were removed by washing with 2 mL of water. Finally total flavonoids mixture was eluted from sep-pak by using 3 mL of methanol. The purified extract was concentrated using N_2 gas, and then re-dissolved with 0.5 mL of the extract solvents without internal standard prior to instrument analysis.

UPLC conditions

An ultra performance liquid chromatography (UPLC) equipped with photo diode array detector set at 280 and 320 nm was coupled with quadrupole time-of-flight mass spectroscopy (Waters Co., Milford, MA, USA) used for analysis. UV spectra were taken in the region of 210-600 nm. Chromatographic condition was conducted: column, Luna Omega 1.6 μm C18, 150 × 2.1 mm, Phenomenex; Pre-column: SecurityGuard ULTRA Cartridges, UHPLC C18 for 2.1 ID column, Phenomenex, column temperature 30°C; mobile phase was used 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B); flow rate 0.3 ml/min; injection volume 5 μL; total running time 60

min; the gradient elution had the following profile: 0 - 2 min 7% B, 24 min 15% B, 40 min 30% B, 48 - 50 min 60% B, 53 - 54 min 90% B, 55 - 60min 7% B. Mass analysis condition: ion source temperature 120°C, desolvation temperature 400°C, desolvation gas 1000 L/h, cone gas 30 L/h, capillary voltage 3500 V, sampling cone voltage 40 V, ion mode positive ion mode and mass range *m/z* 50-800.

Results and Discussion

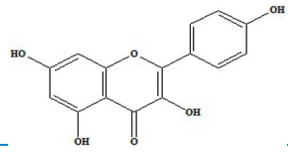
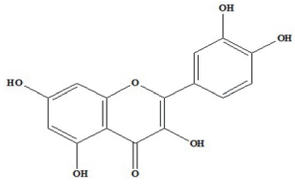
Isolation and identification of flavonoids from mulberry fruit

The major type of flavonoid in mulberry fruits is glycoside, with mainly quercetin and kampferol aglycones (Table 1). We identified nine flavonoids from mulberry fruit, Peak 1, quercetin 3-*O*-rutinoside-7-*O*-glucoside (morkotin A); Peak 2, quercetin 3,7-di-*O*-glucoside; Peak 3, quercetin 3-*O*-rutinoside (rutin); Peak 4, quercetin 3-*O*-glucoside (isoquercitrin); Peak 5, quercetin 3-*O*-(6"-*O*-malonyl)glucoside; Peak 6, kaempferol 3-*O*-rutinoside; Peak 7, kaempferol 3-*O*-glucoside; Peak 8, Kaempferol 3-*O*-(6″-*O*-malonyl)glucoside; Peak 9, Quercetin 3-*O*-(2″-*O*-malonyl) glucoside(morkotin C). Especially, Quercetin 3-*O*-rutinoside-7-*O*-glucoside (morkotin A) and quercetin 3-*O*-(2"-*O*-malonyl) glucoside (morkotin C) were identified as new compounds and further research will be need to purify a these compounds and evaluate their biological activity. In case of the other report, Qin *et al*. (2010) indicated that the molecular structure of anthocyanins from mulberry fruit pigment matched with cyanidin 3-O-rutinoside, cyanidin 3-O-glucoside, pelargonidin 3-O-glucoside, and pelargonidin 3-O-rutinoside from mulberry fruits using mass spectra from HPLC-PAD-MS. This study has shown that mulberry fruit constituents have high antioxidant capacity and that their ability to reduce the risk of or reverse oxidative stress associated diseases is largely due to the presence of polyphenols. On the other hands, the nonanthocyanin phenolics were identified from two mulberry by HPLC-DAD-ESI-MS/MS method (Katsube *et al*., 2006). Also, six nonanthocyanin phenolics were searched for procatechuic acid, chlorogenic acid, 4-caffeoylquinic acid, taxifolin, rutin, quercetin and 3,5-diCQA, taxifolin-hexoside, kaempferol-hexoside (Zhang *et al*., 2008). Thus, no information on the isolation and identification of the nine flavonoids from this fruit produced in Korea is available.

The content of total flavonoids in mulberry fruit

To determine the contents of flavonoids in mulberry fruit, it was measured nine flavonoids' contents. As shown in Table 2, total flavonoids contents from Dae-shim fruits was determined on 119.9 mg. Quercetin 3-*O*-rutinoside (Peak 3) and quercetin 3-*O*-(6"-*O*-malonyl) glucoside (Peak 5) was detected on 66.1 mg and 26.7 mg, respectively. Ju *et al.* (2017) reported that total

Table 1. Flavonoids isolated from mulberry fruits of Korean mulberry cultivar, 'Daeshim'(***Morus alba*** L.) and their mass spectrometric data.

Aglycones		Peak no.	Individual flavonols	MW	Fragment ions (m/z)
Kaempferol (m/z 287)		7	Kaempferol 3-*O*-glucoside (astragalin)	448	471, 449, 287
		8	Kaempferol 3-*O*-(6″-*O*-malonyl)glucoside	534	557, 535, 287
		6	Kaempferol 3-*O*-rutinoside (nicotiflorin)	594	617, 595, 449, 287
Quercetin (m/z 303)		4	Quercetin 3-*O*-glucoside (isoquercitrin)	464	487, 465, 303
		5	Quercetin 3-*O*-(6″-*O*-malonyl)glucoside	464	573, 551, 465, 303
		9	Quercetin 3-*O*-(2″-*O*-malonyl)glucoside (morkotin C)*	550	573, 551, 303
		3	Quercetin 3-*O*-rutinoside (rutin)	610	633, 611, 465, 449, 303
		2	Quercetin 3,7-di-*O*-glucoside	626	649, 627, 465, 303
		1	Quercetin 3-*O*-rutinoside-7-*O*-glucoside (morkotin A)*	772	795, 773, 627, 611, 465, 303

All samples analyzed in positive ion mode ($[M+H]^+$) using UPLC-DAD-QTOF/MS. * New flavonoid identified in mulberry fruit.

Table 2. Contents (mg/100g DW) of fruit flavonoids in Korean mulberry cultivar, 'Daeshim'

Peak No.	Individual flavonols	Content (mg/100g)
1	Quercetin 3-O-rutinoside-7-O-glucoside (morkotin A)a	1.6 ± 0.2
2	Quercetin 3,7-di-O-glucoside	0.6 ± 0.1
3	Quercetin 3-O-rutinoside (rutin)	66.1 ± 4.1
4	Quercetin 3-O-glucoside (isoquercitrin)	11.0 ± 0.5
5	Quercetin 3-O-(6″-O-malonyl)glucoside	26.7 ± 1.4
6	Kaempferol 3-O-rutinoside (nicotiflorin)	5.3 ± 0.3
7	Kaempferol 3-O-glucoside (astragalin)	3.1 ± 0.4
8	Kaempferol 3-O-(6″-O-malonyl)glucoside	4.1 ± 0.5
9	Quercetin 3-O-(2″-O-malonyl)glucoside (morkotin C)a	1.4 ± 0.1
Total		119.9 ± 7.0

* Each value calculated as means ± SD of three replicates using internal standard (galangin)

flavonoids amounts from Korean mulberry cultivar, 'Suhyang' were determined on 79.6 mg. Generally, it was known that rutin and isoquercitirin was contains and main flavonoid in mulberry. Isoquercitrin content in the results of the present study was lower than that in a study by Pawlowska *et al*. (2008). Isoquercitrin is a natural flavonoid glucoside that is distributed in medicinal and dietary plants, such as vegetables, herbs, and flowers and, together with rutin, is one of the major glycosidic forms of the natural flavonol quercetin. It was reported that the major flavonoid compounds in the 8 cultivars of mulberry fruits were quercetin (5.36–58.42 mg/100 g DW) and rutin (18.73–26.90 mg/100 g DW) (Butkhup *et al*., 2013). In the results of our study confirmed that major flavonoids in mulberry fruits were quercetin 3-*O*-rutinoside (rutin) (peak 8), and the content of these compounds were higher.

HPLC–DAD–ESI/MS analysis

The molecular masses of flavonoids isolated from mulberry fruit was analyzed by HPLC–DAD–ESI/MS system. It was analyzed in positive ion mode (m/z, $[M+H]^+$) using UPLC-DAD-QTOF/MS and display chromatograms of major flavonoids isolated from mulberry fruit (Fig. 1.). Nine mulberry flavonoids were identified on the chromatogram between 5 and 40 min. High performance liquid chromatography coupled with a photodiode-array detector and mass spectrometry (HPLC-DAD-MS) provides a powerful and economical tool for polyphenol analysis in crude plant extracts, while HPLC-MS provides information about the polyphenol molecular weight and the molecular structure from its fragmentation data (Price *et al.*, 1999; Alonso-Salces *et al.*, 2001).

In Fig.2, the chemical structures of the individual flavonoids were determined by analysis of fragment ion patterns. All the flavonoids also gave information about $[M+Na]^+$ or $[M+H]^+$ ions depending on the mass of the compound. Total compounds of mulberry fruit were identified as flavonoid compounds in the analysis range time 5–40 min, and they were all kaempferol and quercetin glycosides. Peaks 1, 2, 3, 4, 5, 9 corresponded to quercetin derivatives confirmed with MS via the ion m/z $[quercetin+H]^+$, while the four others were kaempferol derivatives defined with MS ion at m/z $[kaempferol+H]^+$. The major peak 3 with high flavonoid content, generating MS fragments m/z of 633, 611, 465, 449, and 303 were assigned (Fig. 2A). This compound was already identified and described in *M. alba* dried leaves using NMR and MS techniques (Katsube *et al*., 2006). These compounds are found in various fruits and vegetables and other anatomical parts of plants (Wiczkowski and Piskuła, 2004). Quercetin is distributed in distributed in different parts of plants of the plant not only as aglycones but also as glycosides and is known to impart luxuriant color to the fruits,

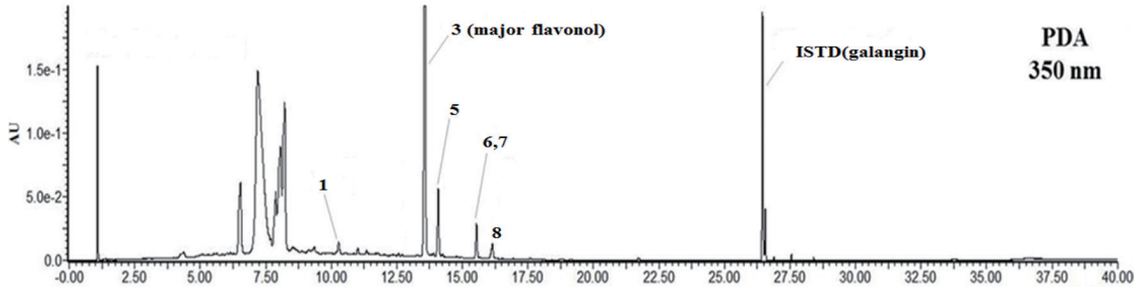


Fig. 1. LC chromatograms of mulberry fruit flavonoids isolated from samples of Korean mulberry cultivar, 'Daeshim' (*Morus alba* L.)

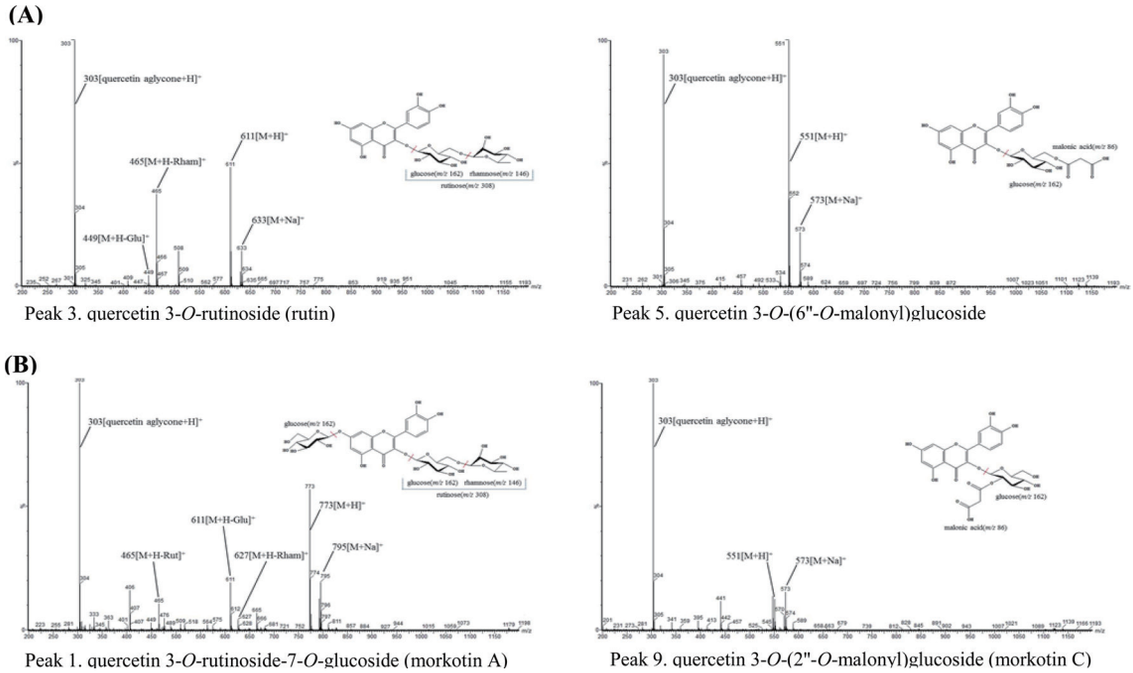


Fig. 2. Mass spectra of flavonoids detected in extracts of Korean mulberry fruits. (A) Higher flavonoids detected from Daeshim; (B) Two new flavonoids detected from Daeshim

flowers, leafy parts etc.

In this study, we achieved quantification of flavonoids components extracted with mulberry fruit from Korea cultivar. An UPLC-DAD-QTOF/MS system was used, and identification of mulberry fruit constituents was carried out on the basis of the complementary information obtained from LC spectra, MS ions, and MS/MS fragments. Rutin is a phenolic compound with glycosidic linkage and occur as monosaccharides with glucose, galactose, rhamnose or xylose. It is reported to exhibit significant pharmacological activities, including anti-oxidation, anti-inflammation, anti-diabetic etc. To the best of our knowledge, rutin was detected on the highest content in fruit and further research will be devoted to evaluate their biological activity. In conclusion, obtaining information about the concentration of functional materials in mulberry fruit could contribute to the development and promotion of processed, functional products and offer possible industrial use of Daeshim, holding promises to enhance the overall profitability of sericulture.

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