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Research Article

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Quali-Quantitative Analysis of Flavonoids for Mulberry Leaf and Fruit of 'Suhyang'

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Abstract

BACKGROUND: Globally, mulberry (*Morus* sp.) is exploited for feeding leaf to silkworms in order to obtain silk fiber or for animal feedstock production. Also, mulberry fruit is known to a by-product that was produced from mulberry tree after harvesting leaves for silkworm rearing, as a yield and consumption of mulberry fruit was increased, it has been fixing to a new income crop. Mulberry leaves and fruits are used for the health benefits of human beings. Mulberry contains various bioactive components, such as alkaloids and flavonoids. Mulberry flavonoids are an important part of the diet because of their effects on human nutrition. The flavonoids in mulberry leaf and fruit of 'Suhyang'(*Morus alba* L.) were determined.

METHODS AND RESULTS: Flavonoids for mulberry leaf and fruit of 'Suhyang' were analysed using ultrahigh performance liquid chromatography coupled with diode array detection and quadrupole time-of-flight mass spectrometry (UPLC-DAD-QTOF/MS) technique. An UPLC-DAD-QTOF/MS system was used, and identification of mulberry leaves constituents was carried out on the basis of the complementary information obtained from LC spectra, MS ions, and MS/MS fragments. The mulberry leaf (16 flavonoids) and fruit (9 flavonoids) were isolated and analyzed from Suhyang using UPLC-DAD-QTOF/MS chromatogram. To the best of our knowledge, Quercetin 3-*O*-(6"-*O*-malonyl) glucoside and quercetin 3-*O*-rutinoside (rutin) was detected on the highest content in leaf and fruit, respectively and further research will be devoted to evaluate their biological activity.

CONCLUSION: Obtaining information about the concentration of functional materials in mulberry leaves could contribute to the development and promotion of processed, functional products and offer possible industrial use of 'Suhyang', holding promises to enhance the overall profitability of sericulture.

Key words: Flavonoids, *Morus alba* L., Mulberry leaves, UPLC-DAD-QTOF/MS

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Introduction

Mulberry (*Morus* sp.) an deciduous tree belonging to the family of Moraceae, is a genus of 10–16 species of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, North America, and southern Europe (Agarwal & Kanwar, 2007). Mulberry leaves contain many nutritional components that are the best feed for silkworms. It is exploited for feeding leaf to silkworms in order to obtain silk fiber or for animal feedstock production. Especially, mulberry fruit is known to a by-product that was produced from mulberry tree after harvesting leaves

for silkworm rearing, as a yield and consumption of mulberry fruit was increased, it has been fixing to a new income crop.

Mulberry has been used in East Asia (Korea, China, and Japan) as an herbal medicine due to their various pharmacological effects, such as anti-hyperglycemic (Singab *et al.*, 2005), anti-allergic (Chai *et al.*, 2005), and immuno-modulatory (Bharani *et al.*, 2010). Mulberry leaves and fruits possess hypoglycemic, hypotensive, diuretic, bacteriostatic and antivirotic properties. It has been demonstrated that mulberry leaves exert an antihyperglycemic effect by reducing blood glucose in streptozotocin-induced diabetic mice (Kim *et al.*, 1999), and mulberry fruit contains not only high amounts of anthocyanins, but non-anthocyanin phenolics including rutin and quercetin known to have multi-bioactive functions including neroprotective effects (Kim *et al.*, 1996). Also, mulberry have recognized as a source due to their biologically active compounds, such as flavonoids (anthocyanin, rutin, quercetin, and isoquercitrin), steroids, amino acids, polysaccharides, γ-aminobutyric acid (GABA), vitamins, and 1-deoxynojirimycin (DNJ) (Kim *et al.*, 2003; Choi and Hwang, 2005; Wang *et al.*, 2008; Zhang *et al.*, 2016).

Flavonoids are a large group of polyphenolic compounds found in fruits, vegetables, and herbs (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). They are important for humans not only because they contribute to plant color but also because they show many biological and pharmacological activities, including antioxidative, antiinflammatory, and antiviral effects (Ross and Kasum, 2002). Plants of the genus *Morus* are known to be rich in flavonoids, including quercetin 3-(O-malonylglucoside), rutin, isoquercitin (Katsube *et al.*, 2006), cyanidin 3 rutinoside, and cyanidin 3-glucoside (Chen *et al.*, 2006). Zhishen *et al.*, (1999) reported that the flavonoid contents for mulberry leaves of 19 varieties of species in two different seasons (spring and autumn) varied from 9.84 to 26.6 mg/g (dry weight). They also found that two of least four flavonoids are rutin and quercetin by high performance liquid chromatography (HPLC) analysis of mulberry leaves. 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 founded that in the red pigment of *M. nigra* fruits revealed the presence of cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, pelargonidin 3-*O*-glucoside, and pelargonidin 3-*O*-rutinoside by HPLC/PDA/ESI-MS analysis. More recently, Thabti *et al.*, (2014) reported that the highest total flavonoid contents among leaves of three mulberry species were detected in *M. rubra*.

In Korea, flavonoids about a various of mulberry leaves and fruits has 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. Sung *et al.*, (2014) reported that 'Suhyang' (*Morus alba* L.) was high yielding cultivar by 12% and higher in sugar content of mulberry fruit compared to control cultivar 'Chungil'. In this study, it has evaluated the HPLC flavonoid profiles of mulberry leaf and fruit of 'Suhyang'(*Morus alba* L) using a quali-quantitative comparative study in order to draw attention to the nutrient profiles of Suhyang mulberry leaf and fruit and promoting the further development of the Korean mulberry resources.

Materials and Methods

Plant material and reagents

Mulberry leaf and fruit of 'Suhyang'(*Morus alba* L) were collected from the Sericulture and Apiculture Division for Department of Agricultural Biology, RDA, Jeon-Ju, Republic of Korea. All mulberry leaf samples were cleaned, dried in lyophilizer. All dried samples were pulverized and stored below -18℃ prior to analysis. HPLC-grade solvents (acetonitrile, methanol, and water) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Junsei Chemical (Tokyo, Japan). Galangin (Sigma Aldrich Co., St. Louis, MO, USA) was used as internal standard solution (ISTD).

Preparation of samples for instrument analysis

Sample extraction conducted according to the method described by Kim *et al.*, (2012) with minimum modifications. The powdered fruits (1gm) was mixed with 10 ml of acidified hydro alcoholic solvent (methanol: water: formic acid (50:45:5,v/v/v) containing 100ppm of galangin as internal standard.). The mixture was first vortex, stirred with shaker for 5 min at 200rpm and then centrifuged for 15 min at 3,000 rpm and 10℃. 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.

Instrumentation

Ultra performance liquid chromatography (UPLC) 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: Security Guard ULTRA Cartridges, UHPLC C18 for 2.1 ID column, Phenomenex, column temperature 30℃; moblie 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℃, desolvation temperature 400℃, desolvation gas 1000 l/hrs, cone gas 30 l/hrs, capillary voltage 3500 V, sampling cone voltage 40 V, ion mode positive ion mode and mass range *m/z* 50-800. All data are expressed as mean±standard deviation (SD) of at three independent experiments.

Results and Discussion

Isolation and identification of flavonoids from mulberry leaves

The mulberry leaf (16 flavonoids) and fruit (9 flavonoids) were isolated and analyzed from Suhyang (Table 1). The following flavonoid compounds from leaf and fruit have been isolated and identified in common: quercetin 3-*O*-rutinoside-7-*O*-glucoside (morkotin A) [peak 1], quercetin 3,7-di-*O*-glucoside [peak 2], quercetin 3-*O*-rutinoside (rutin) [peak 8], quercetin 3-*O*-glucoside (isoquercitrin) [peak 10], quercetin 3-*O*-(6''-*O*-malonyl)glucoside [peak 11], kaempferol 3-*O*-rutinoside (nicotiflorin) [peak 12], kaempferol 3-*O*-glucoside (astragalin) [peak 14], quercetin 3-*O*-(2''-*O*-malonyl)glucoside (morkotin C) [peak 15], kaempferol 3-*O*-(6''-*O*-malonyl)glucoside [peak 16] (Table 1). 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 devoted to evaluate their biological activity. The majority of kaempferol and quercetin in mulberry leaves naturally exist as glycosides (Thabti *et al.*, 2012). The other study was reported that Katsube *et al.*, (2006) identified quercetin-3-*O*-(6-*O*-malonyl)-β-D-glucoside (QMG) and kaempferol-3-*O*-(6-*O*-malonyl)-β-D-glucoside (KMG), from mulberry leaves, of which QMG was found more abundantly. Also, Qualitative analysis of the nonanthocyanin phenolics from 2 mulberry cultivars was performed using HPLC-DAD-ESI-MS/MS method. As a result of the analysis, six nonanthocyanin phenolics were identified (procatechuic acid, chlorogenic acid, 4-caffeoylquinic acid, taxifolin, rutin, quercetin) and three others (3,5-diCQA, taxifolin-hexoside, kaempferol-hexoside) were tentatively identified (Zhang *et al.*, 2008). Thus, no information on the isolation and identification of the 16 flavonoids and 9 flavonoids from Suhyang leaf and fruit produced in Korea is available.

Quantification of flavonoids in mulberry leaves

To determine the contents of flavonoids in Suhyang leaf and fruit, HPLC was carried out using 16 flavonoids and 9 flavonoids isolated from mulberry leaf and fruit. As shown in Table 2, total flavonoids contents was determined on 1014.4 (leaf) and 79.6 (fruit) mg, respectively. Specially, in leaf and fruit, Quercetin 3-*O*-(6''-*O*-malonyl) glucoside (Peak 11) and quercetin 3-*O*-rutinoside (Peak 8) was detected on the highest content, respectively. Thabti *et al.*, (2012) reported that total flavonoids amounts ranged from 193.87 to 398.33 mg RE/100 g DW on *Morus* rubra and were quantified at 450mg (Aqueous extrats) in the stem bark of *Morus alba* var. alba. When ethanol extraction was use on Cheong Il, the quercetin 3-*O*-(6''-*O*-malonyl) glucoside what was known main bioactive substance of antidiabetic and antiarteriosclerosis was 143.25 mg/100 g and quantitative changes of the six polyphenols in Cheongil, a mulberry cultivar widely

Table 1. Isolated 17 flavonoids and their mass spectrometric data in the leaves and fruits of Suhyang

Aglycones	Glycosides	Acylations	Peak No.	Individual flavonols [Retention Time, min]	MW	Fragment ions (*m/z*)	Type	Reference
kaempferol (m/z287)	Mono	Mal	14	kaempferol 3-*O*-glucoside(astragalin)[16.07]	448	471, 449, 287	Leaf, Fruit	Dugo *et al.*, 2009 Katsube *et al.*, 2006
			16	kaempferol 3-*O*-(6''-*O*-malonyl)glucoside[17.88]	534	557, 535, 287	Leaf, Fruit	Thabti *et al.*, 2012
		Mal	17	kaempferol 3-*O*-(2''-*O*-malonyl)glucoside(moragrolD) [18.32]	534	557, 535, 287	Leaf	NF
	Di		12	kaempferol 3-*O*-rutinoside(nicotiflorin)[15.50]	594	617, 595, 449, 287	Leaf, Fruit	Dugo *et al.*, 2009 Pawlowska *et al.*, 2008
			6	kaempferol 3,7-di-*O*-glucoside[12.55]	610	633, 611, 449, 287	Leaf	Dugo *et al.*, 2009
		Mal	13	kaempferol 3-*O*-(6''-*O*-malonyl)glucoside-7-*O*-rhamnoside(moragrolC)NF[15.89]	680	703, 681, 535, 433, 287	Leaf	NF
			7	kaempferol 3-*O*-rutinoside-7-*O*-rhamnoside (moragrolB)NF[12.68]	740	763, 741, 595, 449, 433, 287	Leaf	NF
	Tri		4	kaempferol 3-*O*-rutinoside-7-*O*-glucoside (moragrolA)[11.74]	756	779, 757, 611, 595, 449, 287	Leaf	NF
			10	quercetin 3-*O*-glucoside(isoquercitrin)[14.01]	464	487, 465, 303	Leaf, Fruit	Dugo *et al.*, 2009 Katsube *et al.*, 2006 Pawlowska *et al.*, 2008 Thabti *et al.*, 2012
quercetin (m/z303)	Mono	Mal	11	quercetin 3-*O*-(6''-*O*-malonyl)glucoside[15.42]	550	573, 551, 303	Leaf, Fruit	Dugo *et al.*, 2009 Katsube *et al.*, 2006 Thabti *et al.*, 2012
			15	quercetin 3-*O*-(2''-*O*-malonyl)glucoside (morkotinC)[16.21]	550	573, 551, 303	Leaf, Fruit	NF
	Di		5	quercetin 3-*O*-rhamnoside-7-*O*-glucoside[12.28]	610	633, 611, 465, 303	Leaf	Thabti *et al.*, 2012
			8	quercetin 3-*O*-rutinoside(rutin)[13.50]	610	633, 611, 465, 449, 303	Leaf, Fruit	Dugo *et al.*, 2009 Katsube *et al.*, 2006 Pawlowska *et al.*, 2008 Thabti *et al.*, 2012
			2	quercetin 3,7-di-*O*-glucoside[10.99]	626	649, 627, 465, 303	Leaf, Fruit	Dugo *et al.*, 2009 Thabti *et al.*, 2012
			3	quercetin 3-*O*-rutinoside-7-*O*-rhamnoside (morkotinB)[11.31]	756	779, 757, 611, 465, 449, 303	Leaf	NF
	Tri		1	quercetin 3-*O*-rutinoside-7-*O*-glucoside (morkotinA)[10.25]	772	795, 773, 627, 611, 465, 303	Leaf, Fruit	NF

*All samples analyzed in positive ion mode (*m/z*, $[M+H]^+$) using UPLC-DAD-QTOF/MS
*NF, new flavonoid in mulberry

used as a material in mulberry leaf tea, were investigated according to three different heat pre-treatments: steaming, roasting, and microwaving (Choi *et al.*, 2015). The each other reason was due to other cultivation region and climate even though same variety phenol compound. Generally, it was known that rutin and isoquercitirin was contain and main flavonoid in mulberry. But, in case of Suhyang, its contents was 139.5 (peak8; rutin) and 92.9 (peak10: isoquercitrin) in leaf, respectively (Fig. 1A.). It was found that in the case of dry fruits and vegetables, the rutin content show little variation in range (from 0.15% to 0.18%) (Kalinova *et al.*, 2006). In case of Suhyang fruit, it was contained a highest quercetin 3-*O*rutinoside (Peak 8) content and also detected Quercetin 3-*O*(6''-*O*malonyl) glucoside (Peak 11) (Fig. 1B.). Isoquercitrin content in the results of the present study was lower than that in a study by

Table 2. Contents (mg/100g DW) of leaf and fruit flavonoids in Suhyang

Peak No.	Leaf	Fruit
1	10.6±0.9	1.6±0.1
2	17.3±1.7	1.0±0.1
3	6.8±0.8	ND
4	6.6±1.9	ND
5	3.6±0.6	ND
6	4.5±0.3	ND
7	5.4±0.4	ND
8	139.5±12.7	38.1±1.7
10	92.9±14.9	7.5±0.2
11	312.5±24.9	20.8±0.7
12	103.2±8.2	2.4±0.1
13	4.2±0.6	ND
14	75.1±10.4	4.1±0.1
15	20.8±0.8	1.0±0.1
16	202.7±15.5	3.1±0.1
17	8.7±0.2	ND
Total	1014.4±93.1	79.6±2.9

* Each value calculated as means ± SD of three replicates using internal standard (galangin), * ND : Non-deteted

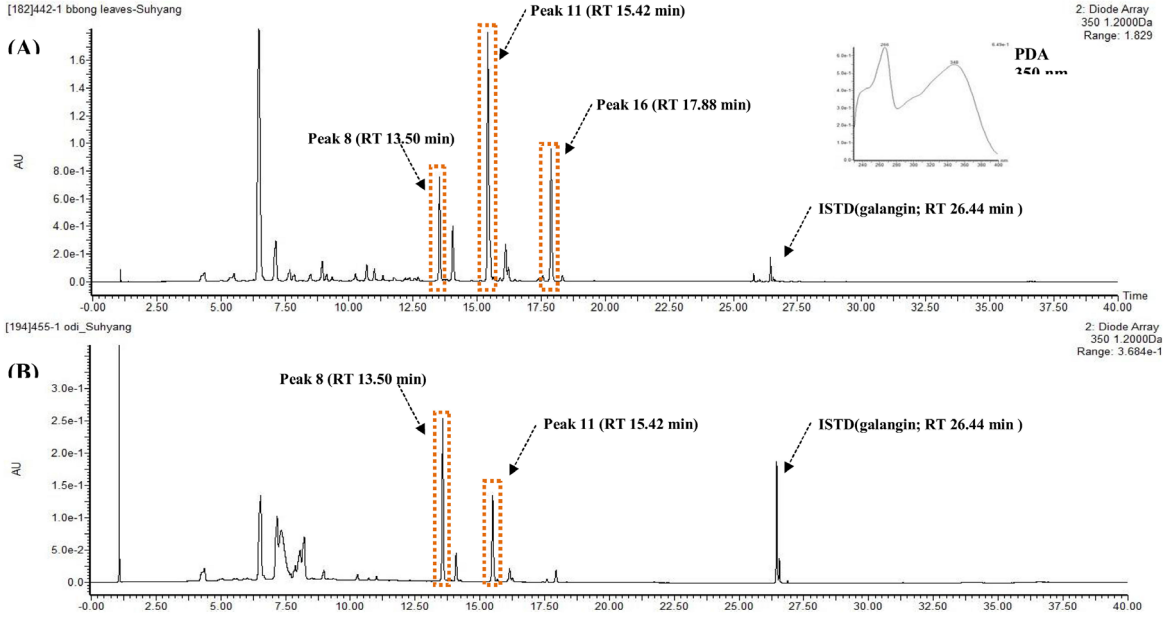

Fig. 1. LC chromatograms at 350 nm of flavonoids in the leaf of Suhyang leaf (A) and fruit (B). The flavonoid names are listed in Table 1.

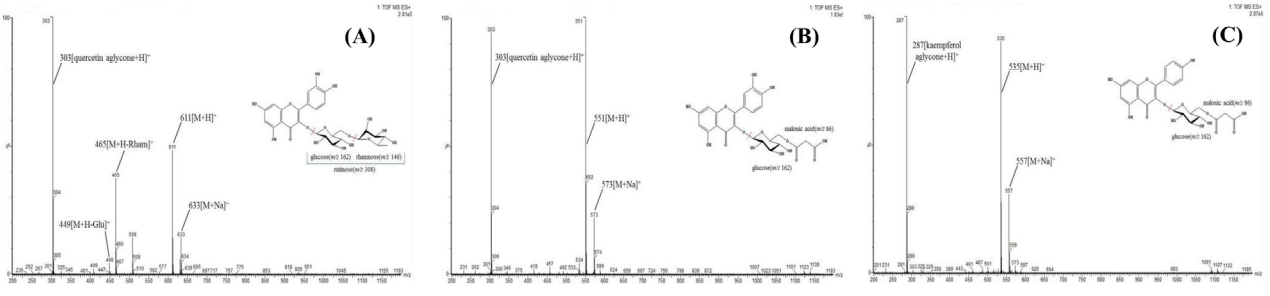

Fig. 2. LC-MS spectra of 3 major flavonoids from Suhyang leaf. (A) quercetin 3-*O*-rutinoside; (B) quercetin 3-*O*-(6"-*O*-malonyl)glucoside; (C) kaempferol 3-*O*-(6"-*O*-malonyl)glucoside.

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. Zhishen *et al.*, (1999) reported that mulberry leaves of all species of mulberry contain flavonoids and was worth pointing out that autumn mulberry leaves, even thoug possessing less flavonoids, provide useful material for extraction, as such leaves are generally not used to feed silkworms. Butkhup *et al.*, (2013) reported that the major flavonoid compounds in the 8 cultivars of mulberry fruits were quercetin (5.36–58.42 mg/100g DW) and rutin (18.73–26.90 mg/100g DW). 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 this compound was higher than that in a study by Butkhup *et al.*, (2013).

In order to get information about the molecular masses of flavonoids detected by HPLC–DAD, HPLC–ESI-MS analysis of the fractionated extract was carried out. All samples analyzed in positive ion mode (m/z, $[M+H]^+$) using UPLC-DAD-QTOF/MS. It was shown about UPLC-DAD-QTOF/MS chromatogrms of major flavonols detected from Suhyang leaf (Fig. 1.). In case of fruit, flavonol contents was much less compare with leaf. So, Fruit flavonol's chromatogram was not data shown. As shown on Fig. 2., almost all the flavonoids also gave information about [M+Na] + or $[M+H]^+$ions depending on the mass of the compound. Total compounds from leaf and 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, 5, 8, 10, 11, and 15 corresponded to quercetin derivatives confirmed with MS via the ion m/z [quercetin+H]$^+$ while the nine others (peaks 4, 6, 7, 12, 13, 14, 16, and 17) were kaempferol derivatives defined with MS ion at m/z [kaempferol+H]$^+$. The major peak 11 with high flavonol content, 16 of Suhyang leaf, generating MS fragments m/z of 573, 551, and 303 were assigned (Fig. 2B.). Generally, most common compound in mulberry leaf, the MS fragments m/z of peak 8 (rutin) was assigned at 633, 611, 465, 449, 303. This compound was already identified and described in *M. alba* dried leaves using NMR and MS techniques (Katsube *et al.*, 2006). Quercetin O-glycosides are quercetin derivatives with at least one O-glycosidic bond which are widely distributed in the plant. Quercetin 3-*O*-glycosides occur as monosaccharides with glucose, galactose, rhamnose or xylose. These compounds are found in various fruits and vegetables and other anatomical parts of plants (Wiczkowski and Piskuła, 2004). In *Triumfetta argentea*, vincetoxicoside, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside, kaempferitrin, isoquercitrin, quercitrin, kaempferol 3-*O*-glucoside (astragalin), kaempferol 3-*O*-rhamnoside (afzelin) and quercetin rhamnosylxyloside were isolated together with tiliroside (Hegnauer, 1973).

In this study, we achieved quantification of flavonoids components extracted with Suhyang leaf and fruit from Korea cultivar. An UPLC-DAD-QTOF/MS system was used, and identification of mulberry leaves constituents was carried out on the basis of the complementary information obtained from LC spectra, MS ions, and MS/MS fragments. To the best of our knowledge, Quercetin 3-*O*-(6''-*O*-malonyl) glucoside and quercetin 3-*O*-rutinoside (rutin) was detected on the highest content in leaf and fruit, respectively and further research will be devoted to evaluate their biological activity. In conclusion, obtaining information about the concentration of functional materials in mulberry leaves could contribute to the development and promotion of processed, functional products and offer possible industrial use of Suhyang, holding promises to enhance the overall profitability of sericulture.

Notes

The author declare no conflict of interest.

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