Quantitative Changes of Polyphenolic Compounds in Mulberry (*Morus alba* L.) Leaves in Relation to Varieties, Harvest Period, and Heat Processing

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Abstract

Six polyphenolic compounds, such as chlorogenic acid (CA), rutin (RT), isoquercitrin (IQT), quercetin-3-*O*-(6-*O*-malonyl)-β-D-glucoside (QMG), astragalin (AG), kaempferol-3-*O*-(6-*O*-malonyl)-β-D-glucoside (KMG), were isolated from mulberry leaves by a series of isolation procedures, such as Diaion HP-20, silica-gel, Sephadex LH-20, and ODS-A column chromatographies. The chemical structures of the phenolic compounds were identified by UV and NMR spectral analyses. Levels of polyphenols in mulberry leaves from six different mulberry cultivars ranged from 1,042.16 to 1,871.97 mg% per dry weight; *Guksang* cultivar showed the highest levels of polyphenols, whereas *Gaeryangdaehwa* contained the least polyphenol contents. Generally, levels of polyphenols in mulberry leaves decreased with increasing harvest time, except for *Yoolmok*, but increased with heat processing time, except QMG and KMG. These results suggest that the heat processed mulberry leaves of *Guksang* cultivar harvested in early May can be potentially useful sources for production of high quality mulberry leaf teas.

Key words: mulberry (*Morus alba*) leaf, polyphenols, cultivars, harvest time, heat processing

INTRODUCTION

Naturally occurring plant polyphenols, such as phenolic acids, cinnamic acids, flavonoids, lignans, and tannins, have been reported to possess a variety of biological effects, such as anticancer, anti-diabetic, anti-hypertensive, anti-inflammatory, anti-aging, and antioxidant activities (1,2). Epidemiological studies demonstrated that the intake of dietary phenolic and flavonoids in fruits and vegetables have been linked to reducing the risk of chronic diseases including cancer and coronary heart disease (3-5). Thus, polyphenolic compounds have recently received much attention as dietary supplements for prevention of degenerative diseases.

Mulberry (*Morus alba* L.) leaves have widely been used in traditional Korean medicine to treat diabetes and fevers, reduce high blood pressure, and to promote urination (6). Mulberry leaf extracts are known to possess anti-hyperglycemic, anti-hypertensive, anti-hyperlipidemic, anti-aging, and antioxidant activities (7-9). Several functional phytochemicals, such as 1-deoxynojirimycin (DNJ), γ-aminobutyric acid (GABA), flavonoids, and 2-arylbenzofurans, have been isolated as major active components with biological activities from mulberry leaves (10,11). In particular, several different flavonol glycosides and chlorogenic acid have been reported as antioxidant constituents in mulberry leaves (12,13). Moreover, the quercetin 3-(6-malonyl)glucoside in mulberry leaves was identified as the predominant active component with anti-atherogenic, anti-hyperglycemic, and antioxidant activities (14-16). Thus, mulberry leaves are taking much interest as promising dietary sources of functional foods with health benefits.

Polyphenolic compounds are most frequently present as glycosides, and their types and contents vary with varieties, cultivation, maturation, and processing (17-19). To date, several researches have been undertaken on quantification of polyphenols in mulberry leaves according to cultivars, harvest time, and processing (20-23). However, characterization and quantitation of phenolic composition and content of mulberry leaves cultivated in Korea are still very limited.

The objective of this study was to isolate and identify major polyphenolic compounds from mulberry leaves, and further determine their contents in relation to mulberry varieties, harvest period, and heat processing.

MATERIALS AND METHODS

Materials and reagents

Mulberry (*Morus alba*) leaves of six different varieties, *Yoolmok*, *Suhoik 20*, *Busa*, *Guksang*, *Gaeryangdaehwa*, and *Gaeryangiljiroi*, grown in the fields of Sericulture and Entomology Experimental Station, Sangju, Korea, were harvested in late May, July, and September 2011, and freeze-dried before use. All solvents for HPLC anal-

ysis were of Merck HPLC grade (Darmstadt, Germany). All other reagents used in this study were of analytical grade.

Heat processing

Freshly harvested mulberry leaves were steamed in a domestic stainless steel steamer (Kitchen-Art, Incheon, Korea) for 10, 20, and 30 min, and cooled. Fresh mulberry leaves were roasted in an electric roaster (Dongkwang Oil Machine Co., Seoul, Korea) with constant stirring at 200°C for 1, 3, and 5 min. Finally, fresh mulberry leaves were placed in a rotating glass container (dimensions 290 mm i.d.) in the center of a domestic microwave oven (Samsung RE-C200T, Suwon, Korea) and heated for 1, 3, and 5 min. Finally, three heat pretreated mulberry leaves were dried for 12 hr in a drying oven (JISICO J-300M, Seoul, Korea) at 45 ± 5°C and milled to 20 mesh by a mechanical coffee mixer. All samples in each treatment were stored into plastic bags at -40°C until use.

Isolation and identification of polyphenols

The freeze-dried mulberry leaves (1.1 kg) were extracted twice with 60% aqueous EtOH (20 L) at 40°C under an ultrasonic cleaner (Power Sonic 420, Hwashintech, Incheon, Korea) for 2 hr, filtered and evaporated under reduced pressure. The concentrated EtOH extract (257.5 g) was solubilized in 20% aq. EtOH and successively loaded into a Diaion HP-20 (Mitsubishi Chem. Co., Tokyo, Japan) column (5.5 × 50 cm). The column was eluted successively with 10%, 30%, and 50% aq EtOH, and each fraction was then evaporated to yield 10% EtOH fraction (fr.) (31.2 g), 30% EtOH fr. (12.0 g), and 50% EtOH fr. (10.1 g), respectively. All fractions were monitored by UV-vis spectrophotometer and analytical HPLC to ascertain polyphenols. Among three fractions, the 10% EtOH fr. was chromatographed on a silica gel (70 ～ 230 mesh, Merck) column (10.5 × 70 cm) with CHCl$_3$-MeOH-H$_2$O (65:35:10, v/v) as an eluent and obtained four fractions; Fr. 1 (1.23 g), Fr. 2 (3.58 g), Fr. 3 (0.82 g), and Fr. 4 (1.82 g). Fr. 2 was successively chromatographed on a Sephadex LH-20 (Parmacia Biotech., Uppsala, Sweden) column (2.5 × 80 cm) with 50% aq. EtOH to obtain composition (Comp.) 1 (1.59 g). The 30% EtOH fr. (12.0 g) was chromatographed on a silica gel column with CHCl$_3$-MeOH-H$_2$O (15:10:2, v/v) as an eluent and obtained five fractions; Fr. 1 (0.84 g), Fr. 2 (3.57 g), Fr. 3 (2.16 g), Fr. 4 (1.98 g) and Fr. 5 (3.28 g). Frs. 2 ～ 5 were further successively chromatographed on a ODS-A (YMC Inc., Milford, MA, USA) column (4.5 × 60 cm) with 40% aq. EtOH, and a Sephadex LH-20 column (2.5 × 80 cm) with 60% aq. EtOH and yielded Comp. 2 (553 mg) from fr. 2, Comp. 3 (837

mg) from fr. 3, Comp. 4 (114 mg) from fr. 4, and Comp. 5 (474 mg) from fr. 5, respectively.

Finally, the 50% EtOH fr. (10.1 g) was also subjected to the same purification procedure using ODS-A (eluted with 50% EtOH) and Sephadex LH-20 columns, and thereby isolating pure Comp. 6 (720 mg).

Identification of polyphenols

UV absorption spectra of six isolated polyphenols (in MeOH) were obtained with a photodiode array UV-vis spectrophotometer (S-1100, Sinco, Seoul, Korea). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra of polyphenols were measured in CD$_3$OD (Comp. 1) and DMSO-d_6 (Comp. 2 ～ 6) on a spectrometer (Unity Plus 500, Varian, Palo Alto, CA, USA), and chemical shifts are given as δ value with tetramethylsilane (TMS) as an internal standard.

Quantification of polyphenols by HPLC

Dried mulberry leaf powders (2 g) were extracted twice with 200 mL of 60% aq. EtOH in an ultrasonic cleaner (5210R-DTH, Bransonic, Danbury, CT, USA) for 30 min, filtered and evaporated under reduced pressure. The EtOH extract was further redissolved in 20 mL of 60% aq. EtOH and left to stand overnight in a refrigerator at 4°C. The upper layer was taken and filled up 100 mL with the same solvent. The aliquot was properly diluted, passed through 0.45 μm membrane filter (Whatman, Maidstone, England) and finally injected into an analytical HPLC. HPLC was performed on a Waters e2690/5 HPLC system (Milford, MA, USA) equipped with 2998 photodiode array detector at 310 (chlorogenic acid) and 370 nm (five flavonoids) and autosampler. HPLC analysis was carried out using a YMC-Pack Pro C$_{18}$ column (46 mm i.d. × 250 mm, YMC Inc.) with a Guard-Pak C$_{18}$ precolumn insert. The separation was conducted using a linear gradient of two solvent systems; solvent A, 0.05% H$_3$PO4 in H$_2$O, and solvent B, CH$_3$CN, at a flow rate of 0.8 mL/min. Individual polyphenols were identified by a comparison of their retention time with those of the six standard flavonoids isolated previously. Linear correlation coefficients were superior to 0.999 for each polyphenol. Levels of polyphenols were determined by calibration curves of six standard polyphenols (chlorogenic acid, $y = 3.398x - 1.297$; rutin, $y = 1.922x + 0.121$; isoquercitrin, $y = 2.068x - 0.830$; quercetin-3-O-(6-O-malonyl)-β-D-glucoside, $y = 0.621x + 0.034$; astragalin, $y = 1.602x - 0.900$; kaempferol-3-O-(6-O-malonyl)-β-D-glucoside, $y = 4.983x - 0.781$) and expressed as mg per 100 g of dried weight of mulberry leaves. Recovery rates of six polyphenols were above 95%. The typical HPLC profiles of the six standard polyphenols and the 60% aq. EtOH extract of mulberry
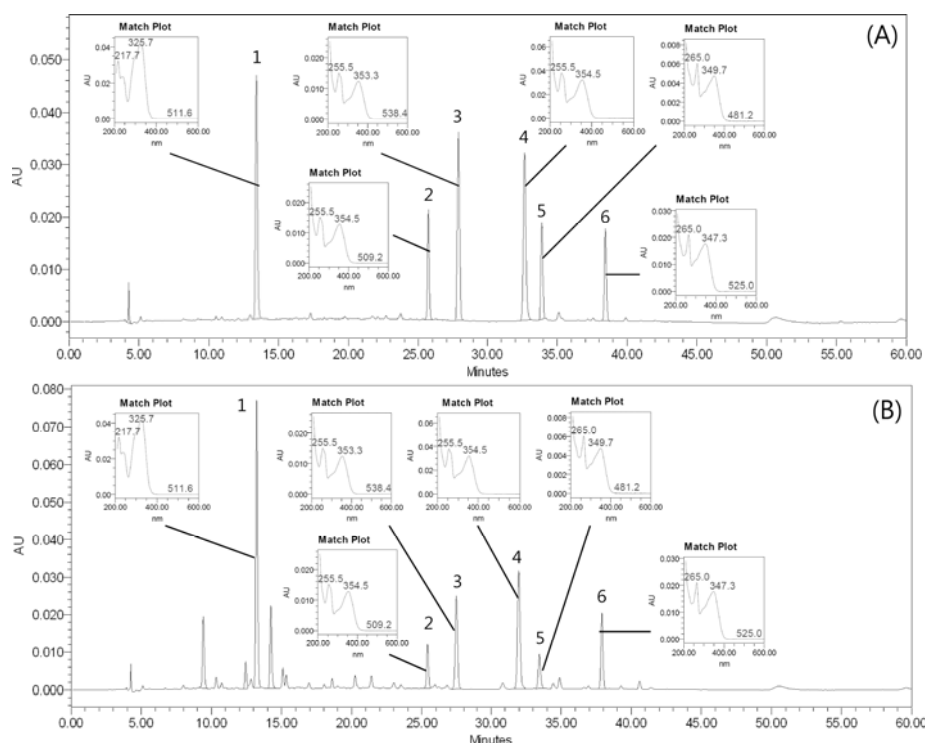
Fig. 1. HPLC chromatograms of six standard phenolic compounds (A) and the ethanolic extract (B) of the mulberry leaves. 1: chlorogenic acid, 2: rutin, 3: isoquercitrin, 4: quercetin-3-O-(6-O-malonyl)-β-D-glucoside, 5: astragalin, 6: kaempferol-3-O-(6-O-malonyl)-β-D-glucoside. HPLC chromatograms were detected at 310 and 370 nm.

leaves are shown in Fig. 1.

Statistical analysis

Data are expressed as mean of two determinations. Standard deviation and statistical analysis are omitted for simplicity.

RESULTS AND DISCUSSION

Isolation and identification of six polyphenols from mulberry leaf

As described previously, six phenolic compounds were isolated from mulberry leaves by a series of separation procedures using Diaion HP-20, silica gel, ODS-A and Sephadex LH-20 column chromatograhies. The six polyphenols, Comp. 1~6, were easily characterized as chlorogenic acid (CA), rutin (RT), isoquercitrin (IQT), quercetin-3-O-(6-O-malonyl)-β-D-glucoside (QMG), astragalin (AG), and kaempferol-3-O-(6-O-malonyl)-β-D-glucoside (KMG), respectively, by UV and NMR spectral analysis, and by comparison to spectral data of published reports (10,12,15). Four phenolic compounds, CA, RT, IQT, AG, were previously isolated and characterized from mulberry fruits (24), however, two flavonol glycosides, QMG and KMG, were not previously found in mulberry fruits. Katsube et al. (13,15) isolated and identified QMG and KMG, from mulberry leaves, of which QMG was found more abundantly. In contrast, Onogi et al. (25) and Kim et al. (12) confirmed the existence of quercetin-3-O-(6-O-acetyl)-β-D-glucoside (QAG) in mulberry leaves in-

stead of QMG. Thus, no information on the isolation and identification of the two flavonoids, QMG and KMG, from mulberry leaves produced in Korea is available, although they were already found in mulberry leaves cultivated in Japan (15). The detailed NMR spectral data of QMG and KMG are given in Table 1.

Quantification of polyphenols by HPLC analysis

To determine the polyphenol content in mulberry leaves according to variety, harvest time, and processing, HPLC was carried out using six polyphenols isolated previously as standards. As shown in Fig. 1, the six polyphenols in mulberry leaf extracts were detected using the 60% aq. EtOH extract by comparing retention times of each standard polyphenol. First, levels of the six polyphenols of mulberry leaves harvested in Sangju according to the varieties and harvest times presented in Table 2. Among the six polyphenols, CA (570.65~1,259.67 mg/100 g of dry weight of mulberry leaf harvested in late May) was a predominant phenolic compound in mulberry leaves, and QMG (237.30~530.55 mg/100 g) was the most abundant flavonol glycoside, followed by RT (100.86~250.85 mg/100 g)＞ IQT (52.38~109.45 mg/100 g)＞ KMG (28.89~71.62 mg/100 g＞ AG (trace~21.70 mg/100 g). In comparing the six different mulberry cultivars, *Guksang* (1,871.97 mg/100 g) had the highest amounts of polyphenols in the leaves, followed by, in descending order, *Gaeryangiljiroi* (1,696.47 mg/100 g), *Busa* (1,663.89 mg/100 g), *Suhoik 20* (1,602.33 mg/100 g), *Yoolmok* (1,221.14 mg/100 g) and *Gaeryangdaehwa*

Table 1. ^{1}H- and ^{13}C-NMR spectral data of QMG and KMG, two flavonol malonylglucosides, isolated from mulberry leaves

Position	QMG	KMG
^{1}H-NMR		
6	6.17 (H, d, J=2.0 Hz)	5.98 (H, d, J=2.0 Hz)
8	6.38 (H, d, J=2.0 Hz)	6.19 (H, d, J=2.0 Hz)
2'	7.45 (H, d, J=2.0 Hz)	7.92 (H, d, J=8.8 Hz)
3'		6.84 (H, d, J=8.8 Hz)
5'	6.80 (H, d, J=8.0 Hz)	6.84 (H, d, J=8.8 Hz)
6'	7.27 (H, dd, J=2.0 & 8.0 Hz)	7.92 (H, d, J=8.8 Hz)
1"	5.26 (H, d, J=7.2 Hz)	5.12 (H, d, J=6.4 Hz)
2"~5"	3.20~3.43	3.21~3.56
6" A	3.94 (H, m)	3.98 (2H, m)
B	4.10 (H, m)	
Malonyl 2H	2.85	2.89
^{13}C-NMR		
2	156.57	155.82
3	133.02	133.13
4	177.10	176.26
5	161.07	160.35
6	101.82	98.71
7	164.13	165.92
8	93.84	95.29
9	157.44	156.95
10	103.80	101.41
1'	120.68	120.31
2'	114.89	133.13
3'	145.58	115.26
4'	149.08	160.49
5'	116.58	115.26
6'	120.87	133.13
1"	101.66	100.49
2"	74.16	75.61
3"	76.12	76.34
4"	69.15	68.53
5"	73.86	75.60
6"	62.34	62.89
Malonate(CO$_2$R)	168.51	168.74
Malonate(COOH)	169.07	169.21
Malonate(CH$_2$)	45.25	45.18

Chemical shifts in δ ppm, coupling constant (J) expressed in Hz in parenthesis and measured in the solvent DMSO-d_6, taking TMS as an internal standard.

(1,042.16 mg/100 g). In addition, *Suhoik 20* possessed the highest levels of flavonol glycosides (829.01 mg/100 g). Meanwhile, the content of polyphenols in most of the mulberry cultivars, except for *Yoolmok*, decreased sharply with increasing harvest times from May to September, similar to a previously published report (20). Interestingly however, *Yoolmok* (774.86 mg/100 g) cultivar showed the greatest content of flavonoids in mulberry leaves harvested in late September, supporting a previous report that the antioxidant capacity of mulberry leaves increased gradually just before the falling leaves stage (21). Thus, considerable differences in polyphenol contents of mulberry leaves exist among mulberry varieties.

Meanwhile, quantitative changes of the six polyphenols in *Cheongil*, a mulberry cultivar widely used as a material in mulberry leaf tea, were investigated according to three different heat pre-treatments: steaming, roasting, and microwaving. As shown in Table 3, most of the heat processing greatly increased the levels of polyphenols except QMG and KMG. More notably, the steaming process significantly increased the levels of polyphenols by about 1.5~4.0 times as compared to control (untreated). In contrast, the microwaving process moderately increased levels of polyphenols, and especially increased levels of QMG and KMG, contrary to the roasting process. Thus, it is noteworthy that the heat processes are effective for enhancing the levels of antioxidant polyphenols. These results supported earlier reports in that the thermal processing increased some functional constituents in plants (26,27). To explain the heat-induced increase in the levels of polyphenols in sweet corn, Dewanto et al. (28) suggested that thermal processing releases bound phenolic acids within cell walls.

Table 2. Quantitative changes of polyphenols in six different mulberry leaves according to cultivars and harvest periods

Cultivar[1]	Harvest period (month)	Polyphenol (mg/100 g, dry weight)						
		CA[2]	RT[3]	IQT[4]	QMG[5]	AG[6]	KMG[7]	Total
YM	May	621.39	100.86	53.68	398.14	Tr[8]	47.07	1,221.14
	July	623.43	106.76	66.26	481.54	25.18	71.56	1,374.73
	Sep	557.71	130.22	73.17	451.43	37.08	82.96	1,332.57
SH 20	May	773.32	139.82	78.16	530.55	21.70	58.78	1,602.33
	Jul	771.03	115.16	66.81	588.12	24.41	82.58	1,648.11
	Sep	373.02	51.56	49.36	294.28	28.55	61.59	858.36
BS	May	940.46	250.85	109.45	315.27	18.97	28.89	1,663.89
	Jul	965.71	161.05	63.90	317.59	Tr	44.09	1,552.34
	Sep	549.22	80.40	77.86	301.14	33.04	51.72	1,093.38
GS	May	1,259.67	160.07	69.39	353.49	Tr	29.35	1,871.97
	Jul	709.61	84.05	33.40	245.29	Tr	29.09	1,101.44
	Sep	460.06	74.75	50.89	289.05	22.72	46.29	943.76
GD	May	570.65	138.73	52.38	237.30	13.49	29.61	1,042.16
	Jul	639.92	70.91	48.10	219.79	Tr	35.19	1,013.91
	Sep	396.51	32.68	37.88	166.43	Tr	30.82	664.32
GI	May	885.75	134.20	63.14	523.17	18.59	71.62	1,696.47
	Jul	495.52	46.46	30.40	213.38	Tr	48.32	834.08
	Sep	328.19	20.38	27.38	110.61	Tr	33.56	520.12

[1]YM: *Yoolmok*, SH 20: *Suhoik 20*, BS: *Busa*, GS: *Guksang*, GD: *Gaeryangdaehwa*, GI: *Gaeryangiljiroi*.
[2]CA: chlorogenic acid. [3]RT: rutin. [4]IQT: isoquercitrin. [5]QMG: quercetin-3-*O*-(6-*O*-malonyl)-β-D-glucoside.
[6]AG: astragalin. [7]KMG: kaempferol-3-*O*-(6-*O*-malonyl)-β-D-glucoside.
[8]Tr: Trace ($<$1 mg/g).
All data are mean of two determinations.
Standard deviation and statistical analysis are omitted for simplicity.

Table 3. Quantitative changes of polyphenols in mulberry leaves of *Cheongil* cultivar by three different heat processes

Heat process (min)		Polyphenol (mg/100 g, dry weight)						
		CA[1]	RT[2]	IQT[3]	QMG[4]	AG[5]	KMG[6]	Total
Control		386.76	89.18	109.45	348.92	25.44	45.27	1,005.02
Steaming	10	614.71	126.69	200.80	416.73	77.90	65.88	1,502.71
	20	553.74	113.90	213.45	491.15	87.58	53.11	1,512.93
	30	820.30	120.96	242.27	560.13	96.81	49.07	1,889.54
Roasting	1	497.40	112.16	149.09	415.16	65.37	66.54	1,305.72
	3	604.51	129.63	175.66	336.08	71.09	51.55	1,368.52
	5	656.88	139.36	190.73	318.48	78.12	49.46	1,433.03
Microwaving	1	414.63	95.99	126.49	384.49	54.29	62.15	1,138.04
	3	460.77	106.02	137.76	420.55	62.91	68.04	1,256.05
	5	510.17	117.31	154.15	445.15	69.23	71.75	1,367.76

[1]CA: chlorogenic acid. [2]RT: rutin. [3]IQT: isoquercitrin. [4]QMG: quercetin-3-*O*-(6-*O*-malonyl)-β-D-glucoside.
[5]AG: astragalin. [6]KMG: kaempferol-3-*O*-(6-*O*-malonyl)-β-D-glucoside.
All data are mean of two determinations.
Standard deviation and statistical analysis are omitted for simplicity.

Meanwhile, Katsube et al. (15) previously demonstrated that heat treatment at high temperatures induce the release of CO_2 from the malonyl group of QMG, resulting in the formation of QAG. However, QAG and KAG were not found in the heated mulberry leaves in the present study. Also, Lim et al. (22) reported that levels of flavonoids in mulberry leaves decreased during the roasting process at $350 \pm 50^{\circ}C$. Thus, the quantitative changes of flavonoids in mulberry leaves are greatly affected by heating temperatures. More recently, we also found a

big difference in the quantity of polyphenols in several mulberry leaf teas available in Korean markets, which were prepared by steaming, roasting, and fermenting the mulberry leaves (data not shown).

In conclusion, six polyphenols were isolated and identified from mulberry leaves. Their contents and compositions were considerably influenced by cultivars, harvest time and processing. In particular, appropriately heat-processed *Guksang* and *Suhoek-20* mulberry leaves harvested in May could be useful as potential materials for

high quality mulberry leaf teas and powders. The quantitative analysis of functional polyphenols in mulberry leaves from this study is helpful for standardization and physiological evaluation of mulberry leaf powders and teas. Further studies on quantitative changes of other functional constituents, such as DNJ (1-deoxynojirimycin), GABA (γ-aminobutyric acid), and moracin, having anticancer, anti-hypertentive, and anti-diabetic activities in mulberry leaves according to varieties, cultivation, processing, and storage are now in progress.

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