Antioxidant activities and polyphenol content of *Morus alba* leaf extracts collected from varying regions

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Abstract. *Morus alba* leaf (MAL), also known as Mori folium when used as a herbal medicine, has traditionally been used in Chinese medicine to treat diabetes, protect the liver and lower blood pressure. In the present study, MAL was collected from various regions in Korea and the antioxidant activity, total polyphenol contents and main flavonoid contents was investigated. MAL were collected from various areas in Korea and extracted with methanol. The total polyphenol contents were evaluated based on the Folin-Ciocalteu method using a spectrophotometer. The antioxidant activities were determined by a 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay method. The identification and quantification of three main polyphenol constituents was performed using high-performance liquid chromatography/diode array detection analysis. The total polyphenol contents of the MAL extracts varied between 23.2 and 55.4 mg gallic acid equivalent/g. The radical scavenging activity (SC_{50}) of the MAL extracts ranged between 584 and 139 μg/ml. Three flavonol compounds (rutin, isoquercitrin and astragalin) were identified as main polyphenol constituents. These contents varied from 0.68-12.7, 0.69-9.86 and 0.05-3.55 mg/g, respectively. The average of the total was 9.52 mg/g, which was similar to that of commercial MAL extracts (10.58 mg/g). Among the three flavonol compounds, isoquercitrin showed the highest content (5.68 mg/g) followed by rutin (3.1 mg/g) and astragalin (2.4 mg/g). In the present study, the radical scavenging activity, polyphenol content and flavonol content of MAL were significantly different according to growing area. These three flavonol compounds were identified as main constituents of MAL in this study, and are known to have various biological activities, as well as strong antioxidant activities. Therefore, the sum of these three flavonol compounds was indicated as a good marker for the quality control of Mori folium.

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Introduction

Morus alba is a fast-growing and small-medium sized mulberry tree. According to the Dong-eui-bo-gam, the oldest Korean medicinal book, *Morus alba* leaf (MAL), also known as Mori folium when used as a herbal medicine, alleviates the symptoms of beriberi, body swelling, dropsy and diabetes (1). Modern medical studies of the leaves of the white mulberry have reported anti-atherogenic (2), anti-hypertensive (3,4), anti-obesity (5), anti-diabetic (6) and liver protective (7,8) effects.

The main constituents of MAL are known to consist of antioxidative and anti-inflamatory flavonols, including quercetin, astragalin, isoquercitrin and rutin (9-11).

Polyphenols are alcohols containing ≥2 benzene rings, which each have ≥1 hydroxyl (OH) group attached and can range from simple molecules (phenolic acids, phenylpropanoids and flavonoids) to highly polymerized compounds (lignins, melanins and tannins), with flavonoids representing the most common and widely distributed subgroup (12). These compounds have been reported to have antioxidant activity, but flavonoids in particular exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic, anticarcinogenic, hepatoprotective and vasodilatory activities, in addition to their antioxidant activities (13,14). Indeed, a number of these biological functions have been attributed to their free radical scavenging and antioxidant activities (15). Oxidative damage appears to be associated with the etiology of cardiovascular disease, diabetes mellitus, gastric ulcers, arthritis, cancer and inflammation (16,17).

The antioxidant activity of MAL has been previously reported. A previous study has shown that the butanol extract of MAL and isoquercitrin is able to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and inhibited oxidation of rabbit and human low-density lipoprotein (18). The levels of the main antioxidative components of MAL,

namely rutin, isoquercitrin and astragalin, have also been reported (10). However, the antioxidant activity, the polyphenol content and the levels of the antioxidants in MAL have not been reported according to collection area.

The present study was designed to investigate the antioxidant activity and total polyphenol content, including the main polyphenol constituent content, of MAL according to growing region.

Materials and methods

Preparation of MAL extracts. MAL was collected from 7 provinces in Korea (Table I). Commercial MAL was purchased from Omniherb Co., Ltd., (Yeoungcheon, Korea), JungDo Co., (Seoul, Korea) and Dongkyung Corporation (Jeungpyung, Korea), and was authenticated, based on its microscopic and macroscopic characteristics, by the Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM). The committee consisted of nine experts in the fields of plant taxonomy, botany, pharmacognosy and herbology. The voucher specimens were deposited at the herbarium of Herbal Medicine Resources Group at the KIOM.

The dried and coarsely powdered leaves (each 100 g) were extracted with methanol (each 2 liters) for 4 h at 60˚C. The extracts were filtered and evaporated until dry under a reduced pressure at 40˚C.

Determination of total polyphenol content. The total polyphenol content of the samples was determined by the Folin-Ciocalteu method (19). Appropriate dilutions of samples (2 ml) were oxidized with Folin-Ciocalteu's reagent (2 ml; Sigma, St. Louis, MO, USA) for 3 min. The reaction was neutralized with 10% sodium carbonate solution (2 ml). The contents in the tubes were then thoroughly mixed and allowed to stand at ambient temperature for 1 h until the characteristic blue color developed. The absorbance of the clear supernatant was measured at 700 nm using a spectrophotometer (LAMBDA 25 UV/Vis Spectrophotometer, PerkinElmer Inc., Waltham, MA, USA). The total polyphenol content in each sample was calculated based on a standard curve, which was prepared using gallic acid (Sigma) and expressed as milligrams of gallic acid equivalent (GAE) per gram of sample.

Free radical scavenging assay. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined, as previously described (20). A DPPH solution in ethanol and dimethylsulfoxide (DMSO) was prepared and 900 μl of this solution was added to 100 μl of each sample dissolved in ethanol (500 μg/ml). The mixture was agitated and then allowed to stand at room temperature for 10 min. The absorbance was subsequently measured at 518 nm using a spectrophotometer (LAMBDA 25 UV/Vis Spectrophotometer, PerkinElmer Inc.). The percentage of scavenging activity at different concentrations was determined and compared with that of L-ascorbic acid (100 μg/ml), which was used as the standard. The inhibition of the DPPH radical scavenging effect was calculated as: DPPH radical scavenging effect (%) = (Ao-A)/Ao x 100, where Ao was the absorbance of the control solution (containing only DPPH) and A was the absorbance of DPPH in the sample solution. The determinations were performed in triplicate for each sample and the values were averaged.

Table I. Collection area and date of MAL.

Sample	Collection area	Collection date
MAL628A	Mountain in Daejeon	June 28
MAL628B	Street in Daejeon	June 28
MAL704	Mountain in Chungbuk Chungwon	July 4
MAL712	Mountain in Kyungbuk Youngchun	July 12
MAL718	Mountain in Kangwon Yanggu	July 18
MAL730	Street in Jeonnam Damyang	July 30
MAL801	Street in Kyungnam Sanchung	August 1
MAL805	Street in Chungbuk Chungju	August 5
MAL903	Mountain in Daejeon	September 3
MAL906	Mountain in Chungnam Cheonan	September 6

MAL, *Morus alba* leaf.

High-performance liquid chromatography (HPLC) analysis. HPLC-grade reagents, acetonitrile and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of reagent grade.

The samples were analyzed by reversed-phase HPLC using an Waters Alliance 2695 HPLC system (Waters Co., Milford, MA, USA), coupled with a 2996 photodiode array detector. A Phenomenex Luna C18 column (250x4.6 mm; particle size, 5 μm; Phenomenex, Torrance, CA, USA) was used, and the mobile phase was composed of 0.1% (v/v) trifluoroacetic aqueous solution (A) and acetonitrile (B).

The elution conditions for identification of the main polyphenol constituents were as follows: At 0 min, the mobile phase consisted of 90% A/10% B and was held for 10 min. From 10-40 min a gradient was applied to 60% A/40% B, which was followed by a wash with 100% B for 5 min and a 15-min equilibration period at 90% A/10% B. The elution conditions for simultaneous quantification of rutin, isoquercitrin and astragalin were as follows: At 0 min, the mobile phase consisted of 85% A/15% B and was held for 30, which was followed by a wash with 100% B for 5 min and a 15-min equilibration period at 85% A/15% B. The separation temperature was kept at a constant 40˚C throughout the analysis, with a flow rate of 1.0 ml/min and an injection volume of 20 μl.

Identification was based on retention time and UV spectra by comparison with commercial standards. The identified components were quantified based on peak areas at 260 nm. Calibration curves of the standards ranged between 12.5 and 200 μg/ml (5 levels), revealing good linearity, with R^2 values exceeding 0.99 (peak areas vs. concentration).

Statistical analysis. All data are presented as the mean ± standard deviation of at least triplicate measurements. The significance of differences among treatment means were calculated using the SPSS package for Windows (version

Table II. Polyphenol contents and free radical scavenging effects of MAL collected from various areas.

Sample	Extraction yield, %	Free radical scavenging activity (SC_{50}[a]), μg/ml	Polyphenol contents, mg/g
MAL628A	9.3	455±56	32.6±1.3
MBL628B	9.7	468±54	32.2±1.3
MAL704	9.3	301±27	45.2±1.5
MAL712	9.1	330±41	36.2±1.8
MAL718	10.1	139±15	55.4±2.1
MAL730	9.4	250±19	45.1±1.7
MAL801	9.2	461±31	33.3±1.3
MAL805	10.4	317±27	29.7±1.9
MAL903	9.2	584±71	28.2±1.7
MAL906	9.8	339±28	35.0±1.6

[a]Concentration for scavenging 50% of DPPH free radical. The standard compound for use in the total phenolics assay was gallic acid. Values are presented as the mean ± standard deviation. MAL, *Morus alba* leaf.

12.0; SPSS Inc., Chicago, IL, USA) with a significance level indicated by $P<0.05$.

Results

Extraction yields. Methanol was selected as the extraction solvent since it is commonly used for polyphenols and flavonoids. The yield of methanol extracts obtained from 10 different MAL samples that were collected from seven provinces in Korea is presented in Table II. The extraction yields did not differ greatly in terms of overall mass, and revealed values ranging between 9.1 (MAL712) and 10.4% (MAL805).

Polyphenol contents. The total polyphenol content of 10 MAL extracts are shown in Table II. Among the extracts tested, the highest total polyphenol level was observed at 55.4 mg GAE/g extract of MAL718 and the lowest at 28.2 mg GAE/g extract of MAL903.

The total polyphenol content of MAL628A and MAL628B, collected from a mountain and a street in Daejeon, respectively, were extremely similar, with values of 32.6 and 32.2 mg/g, respectively.

The lowest total polyphenol content was 23.2 mg/g (MAL903) and the highest total polyphenol content was 55.4 mg/g (MAL718), with large differences in their inhibition of DPPH radical scavenging. Meanwhile, MAL903 and MAL628A collected at varying times from the same tree had a total polyphenol content of 32.6 and 23.2 mg/g, respectively. This indicated certain variations within the area, but the change was not significantly different compared with samples from varying locations.

Free radical scavenging activity. DPPH radical scavenging activities of MAL628A and MAL628B collected from adjacent areas in Daejeon were similar, with an SC_{50} of 455 and 468 μg/ml, respectively (Table II). The lowest DPPH radical scavenging activity (MAL903) was an SC_{50} of 584 and the highest DPPH radical scavenging activity (MAL718) was an SC_{50} of 139 μg/ml. MAL628A and MAL903 were collected from the same tree, with a gap of 2 months, with no significant difference in their SC_{50} values of 455 and 584 μg/ml, respectively.

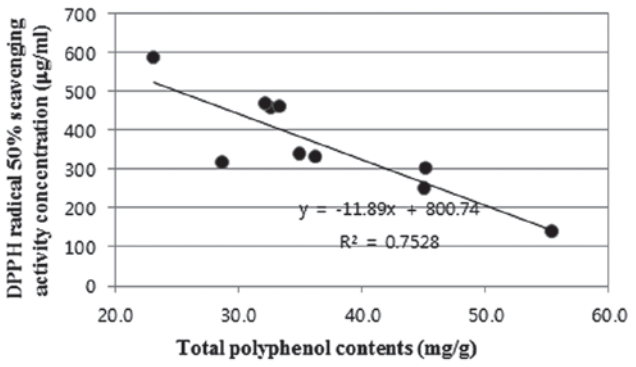

Figure 1. Association between total phenolic content and DPPH radical scavenging activity of MAL extracts. Correlation between total polyphenol content and concentration for scavenging 50% of DPPH free radical (R^2=0.7464, $P<0.01$). DPPH, 2,2-diphenyl-1-picrylhydrazyl; MAL, *Morus alba* leaf.

The correlation between total polyphenol contents and antioxidant activity has been widely studied in a variety of herbs. As previously reported (21), the present study also showed that antioxidant activity significantly increases with the presence of a high total polyphenol content. The present study also showed a good correlation between radical scavenging activity and the total polyphenol content of 10 MAL extracts. The polyphenol contents were correlated with DPPH radical scavenging activity (R^2=0.7528) (Fig. 1).

Identification and quantification of main polyphenol constituents of MAL extracts. The main polyphenol constituents were identified based on the ultraviolet (UV)-visible spectrum using a HPLC/diode array detection (DAD) chromatogram. At the gradient elution conditions for identification [retention times, ~17.5, 21.0 and 36.2 min; wavelengths, with maximum absorption from UV-visible spectrum (λmax_1 and λmax_2), 255.4 and 353.2, 255.4 and 353.2, and 254.2 and 348.4 nm, respectively], the constituents, rutin, isoquercitrin and astragalin, were an exact match to the commercial standards (Fig. 2).

Simultaneous quantitative analysis of these three components in MAL extracts was further developed with good separation. The flavonol contents were varied as the values for rutin ranged from 0.68 (MAL906)-12.7 mg/g (MAL718), isoquercitrin ranged from 0.69 (MAL903)-9.86 mg/g (MAL718), astragalin ranged from 0.05 (MAL903)-3.55 mg/g (MAL730) and the total ranged from 1.72 (MAL903)-25.82 mg/g (MAL718). The average total flavonol content was 9.52 mg/g. Of the three components, isoquercitrin showed the highest average content (5.68) followed by rutin (3.1) and then astragalin (2.4 mg/g).

Although MAL628A and MAL628B were located in the same area, within a short distance, the total flavo-

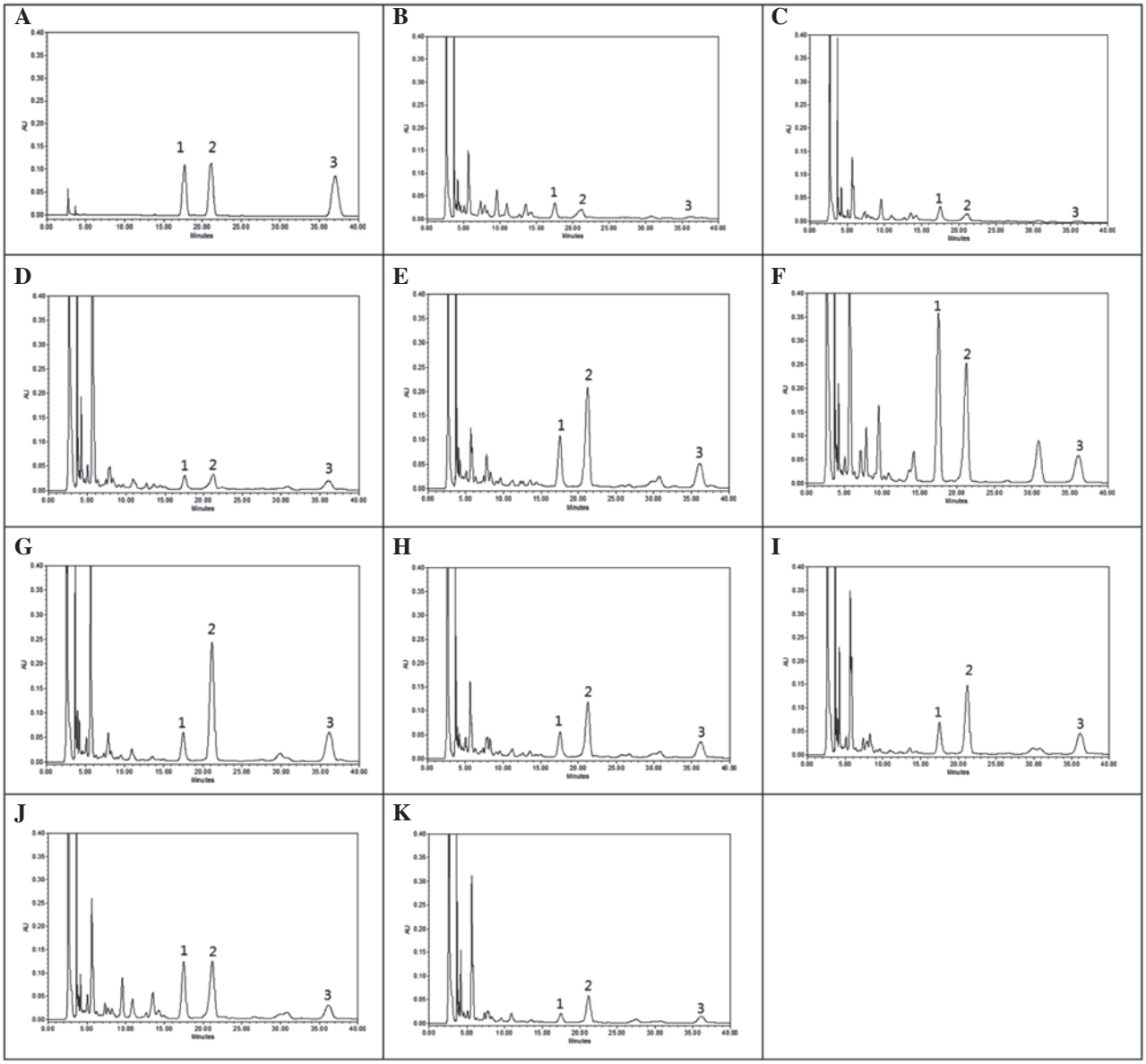

Figure 2. Analytical HPLC chromatograms of *Morus alba* leaf (MAL) extracts. (A) Three standard mixtures; (B) MAL628A; (C) MAL628B; (D) MAL704; (E) MAL712; (F) MAL718; (G) MAL730; (H) MAL801; (I) MAL805; (J) MAL903; (K) MAL906. 1, rutin; 2, isoquecitrin; 3, astagalin; HPLC, high-performance liquid chromatography.

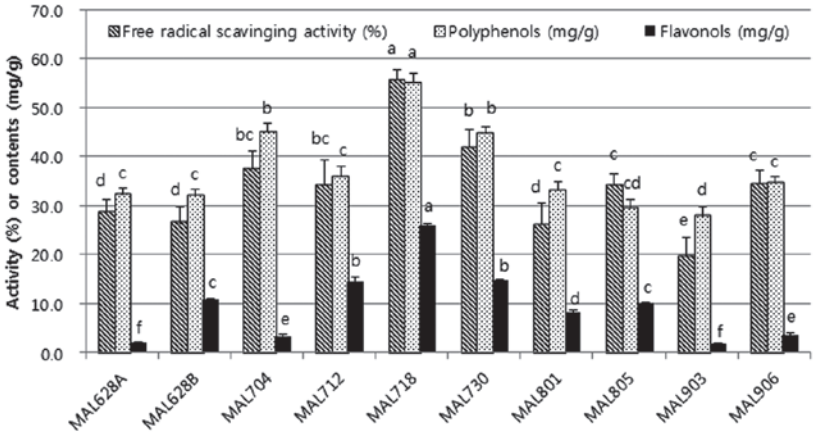

Figure 3. Free radical scavenging activities, polyphenol content and total flavonol content of *Morus alba* leaf (MAL) extracts. The vertical bars represent the mean ± standard deviation (n=3) and those with different superscript alphabetical letters are significantly different by Duncan's multiple range test ($P<0.05$). All the sample concentrations for the free radical scavenging activities were 100 μg/ml. The total polyphenol content in each sample was expressed as milligrams of gallic acid equivalent per gram of sample (GAE/g). Total flavonoid content was defined as the sum of rutin, isoquercitrin and astragalin.

Table III. Content of flavonols of MAL collected from various areas.

Extract	Content, mg/g			
	Rutin	Isoquercitrin	Astragalin	Total
MAL628A	0.98±0.04	0.84±0.05	0.17±0.01	1.99
MAL628B	4.24±0.16	4.97±0.18	1.68±0.07	10.89
MAL704	1.03±0.06	1.32±0.06	1.04±0.04	3.39
MAL712	4.01±0.13	7.75±0.23	2.85±0.11	14.62
MAL718	12.70±0.39	9.86±0.35	3.26±0.13	25.82
MAL730	2.08±0.08	9.09±0.31	3.55±0.14	14.71
MAL801	1.99±0.08	4.32±0.17	1.96±0.08	8.27
MAL805	2.16±0.09	5.42±0.19	2.62±0.12	10.20
MAL903	0.98±0.04	0.69±0.03	0.05±0.00	1.72
MAL906	0.68±0.03	2.10±0.07	0.78±0.04	3.56
Mean	3.10	5.68	2.41	9.52

MAL, *Morus alba* leaf.

Table IV. Content of flavonols of MAL collected from markets.

Extract	Content, mg/g			
	Rutin	Isoquercitrin	Astragalin	Total
Omni herb	1.27±0.05	4.73±0.14	2.57±0.13	8.57
JungDo herb	1.86±0.04	4.66±0.11	3.11±0.09	9.63
Dongkyung herb	2.12±0.07	7.53±0.19	3.90±0.08	13.55
Mean	1.75	5.64	3.56	10.58

MAL, *Morus alba* leaf.

noid contents of MAL628A (collected in the street) and MAL628B (collected in the mountain) were substantially different, recorded as 10.89 and 1.99 mg/g, respectively (Table III). Meanwhile, the total flavonols of MAL628A and MAL903 collected at various times from the same tree were not substantially different, recorded as 1.99 and 1.72 mg/g, respectively.

Quantification of three major flavonols in methanolic extract in commercial MAL. Using the established quantitative HPLC analysis method, the flavonol content of the methanolic extracts from three different commercial MAL extracts were analyzed. The flavonol content of the methanolic extracts from MAL purchased at a variety of markets were also analyzed for comparison with those MAL samples that were collected in the fields (Table IV). The flavonol contents were also varied as the values for rutin ranged from 1.27-2.12, isoquercitrin ranged from 4.66-7.53 and astragalin ranged from 2.57-3.90 mg/g. The flavonol content of the samples from the various collection areas were not markedly different.

Discussion

Flavonols are flavonoids that possess the 3-hydroxyflavone backbone (IUPAC name, 3-hydroxy-2-phenylchromen-4-one).

The flavonols are subclassified into kaempferol, quercitrin and myricetin, according to the number and position of the OH groups of the A and B rings. In the present study, rutin (quercetin-3-O-β-rutinoside), isoquercitrin (quercetin-3-O-β-D-glucoside) and astragalin (kaempferol-3-O-β-D-glucoside) were identified as the main polyphenol constituents in MAL.

Rutin has been reported to have anti-platelet aggregation (22), anti-inflammatory (23) and aldose reductase inhibitory (24) effects. Isoquercitrin has been reported to have antimicrobial (25) and antioxidant (26) effects. Astragalin has also been reported to have antioxidant (27) and anti-inflammatory (28) effects. The present study also identified quercetin from a minor peak (data not shown), but its contents in extract samples were far lower than those of the other compounds of interest, with a maximum concentration of 0.28 mg/g determined.

In the present study, the radical scavenging activity, polyphenol content and flavonol content of MAL were significantly different according to the growing area (Fig. 3). However, no association could be determined between the polyphenol content and the geographic location or altitude. Thus, changes in the polyphenol content and the scavenging activities may be soil-related conditions rather than, for example, temperature-, height- or weather-related conditions, as soil in forests is specific to the particular environment and has a direct bearing on the composition of the plants found there.

Significantly, samples collected in the mountain in Kangwon Yanggu (MAL718) had the highest radical scavenging activity, polyphenol content and flavonol content. If the factors affecting polyphenol content were to be identified, this would greatly advance cultivation technology for mulberry polyphenol production.

While there were was variation in the three flavonol compounds (rutin, isoquercitrin and astragalin) of several locations, the average total flavonol compound content in the collected samples was similar to the average in samples purchased on the market. The isoquercitrin content of samples collected from several locations and the samples purchased from the market were particularly similar, recorded as 5.68 and 5.64 mg/g, respectively. Therefore, it may be concluded that the sum of these three flavonol compounds or isoquercitrin alone is indicated as a good marker for the quality control of mulberry leaves. The present study also established a simultaneous analysis method of these three compounds using HPLC/DAD, which will be useful for quality control.

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