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Development of HPLC method for differentiation of three parts of mulberry tree

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Abstract: The leaves (Mori Folium; MF), branches (Mori Ramulus; MR), and root bark (Mori Cortex Radicis; MCR) of the mulberry tree have been used as therapeutic herbs for centuries. Existing analytical methods were developed specifically for different parts of the tree and cannot be applied to samples containing a mixture of tree parts. Such method specialization is time-consuming and requires separate identification and quality control of each tree part. This report describes an HPLC method for the simultaneous quality control and discrimination of MF, MR, and MCR using four marker compounds: rutin, kuwanon G, oxyresveratrol, and morusin. An Optimapak C_{18} column (4.6 × 250 mm, 5 μm) was used with a gradient elution of 0.1 % formic acid in water and acetonitrile. The flow rate was 1.0 mL/min and the detection wavelength was 270 nm. In quantitative analyses of the three parts, rutin (0.11 % w/w) was detected only in MF. The oxyresveratrol content (0.12 % w/w) was highest in MR. Kuwanon G (0.33 % w/w) and morusin (0.18 % w/w) were higher in MCR than in other parts. The HPLC method given herein can be used to simultaneously classify and quantify three herbal medicines from the mulberry tree.

Key words: HPLC, mori folium, mori ramulus, mori cortex radicis, simultaneous analytical method

1. Introduction

The mulberry tree (*Morus alba* L.) grows in Korea, Japan, and China. Another name of this tree is sacred tree because all of its parts, particularly the leaves, branches, and root bark, are used in herbal medicines to treat ailments such as diabetes, edema, and cough.[1,2] The galenical names for these particular parts are Mori Folium (MF), Mori Ramulus (MR), and Mori Cortex Radicis (MCR), respectively. In pharmacological studies, each part of the mulberry tree has exhibited various bioactivities and medicinal properties. MF

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and MCR have shown *in vivo* activities in animals and humans including hypolipidemic,[3] antihypoglycemic,[4] antihypertensive,[5] anti-inflammatory,[6] antiatherosclerotic,[7] antitumor,[8] anticonvulsant,[9] and vasodilator effects.[10] MR extracts contain relatively high amounts of oxyresveratrol, which has anti-inflammatory properties.[11]

The primary bioactive constituents of the mulberry tree are flavonoids, cumarins, phenols, and terperols.[12] Some flavonol glycosides, such as rutin, isoquercitrin, astragalin, and quercetin-3-(6-malonyl) glucoside, are antioxidants.[13] Rutin is effective for the development of brain capillary blood vessels, activation of glucose metabolism, and reduction of blood pressure.[14] Recent studies have reported that oxyresveratrol possesses a potent inhibitory activity against tyrosinase. Further investigations have revealed the antiherpetic, anti-HIV, anti-inflammatory, antioxidant, and neuroprotective activities of oxyresveratrol.[15] Kuwanon G has shown antibacterial effects against cariogenic and periodontal bacteria.[16] Morusin can inhibit arachidonic acid and platelet activating factor (PAF), affect collagen-induced platelet aggregation, suppress superoxide anion formation, and reduce bacterial activity.[17]

At present, several analytical methods have been developed for the identification and quality control of MF, MR, and MCR individually using HPLC-UV,[18] ELSD,[19] and LC-MS.[20] The Korean, Chinese, and Japanese Pharmacopoeias also treat the various parts of the mulberry tree individually. Recently, complex herbal products using a mixture of MF, MR, and MCR have been developed to prevent adult-onset diabetes and inflammation.[21] However, there is no HPLC method that can be used for quality control of a mixture of mulberry tree components.

To develop new herbal remedies and quality control parameters, an efficient way to identify each of the three primary herbal medicines found in mulberry tree, and to determine their relative amounts in a mixture of different tree parts, is required. This study describes an HPLC method employing marker compounds for the simultaneous identification and quantification of MF, MR, and MCR.

2. Materials and Methods

2.1. Plant materials

A total of 30 samples of mulberry tree were collected: 10 MF samples (MF1 to MF10), 10 MR samples (MR1 to MR10), and 10 MCR samples (MCR1 to MCR10) as shown in *Table* 1. The collected samples were dried and stored in sealed packages at room temperature. All of the collected samples were identified by Professor Young Ho Kim (College of Pharmacy, Chungnam National University).

2.2. Standards and chemical reagents

Four standards (rutin, kwanon G, oxyresveratrol, and morusin) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and their chemical structures are shown in *Fig.* 1. The purity of purchased standards was higher than 95 %. Ethanol, methanol, and

Table 1. Sample list of three medicinal herbs of mulberry tree

Mori Folim		Mori Ramulus		Mori Cortex Radicis	
Code No.	Purchased place	Code No.	Purchased place	Code No.	Purchased place
MF1	K. Kyungnam	MR1	K.Kyungnam	MCR1	K.chungsong
MF2	Korea	MR2	Korea	MCR2	China
MF3	Korea	MR3	Korea	MCR3	Korea
MF4	Korea	MR4	Korea	MCR4	K.Jaechun
MF5	K.Imsil	MR5	K.Jaechun	MCR5	China
MF6	Korea	MR6	K.Youngchun	MCR6	China
MF7	K.Jaechun	MR7	K.Chunchun	MCR7	China
MF8	K.Sanchung	MR8	Korea	MCR8	K.Kyungbook
MF9	K.Imsil	MR9	Korea	MCR9	K.Youngwol
MF10	K.Hoengseong	MR10	Korea	MCR10	Korea

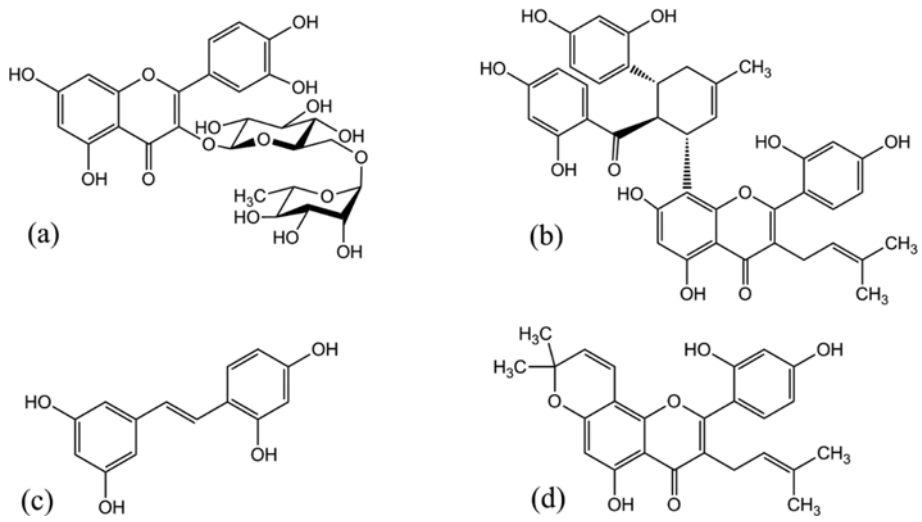

Fig. 1. Chemical structure of marker compounds; (a) rutin, (b) kuwanon G, (c) oxyresveratrol and (d) morusin.

acetonitrile were all HPLC-grade and purchased from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were analytical grade unless otherwise noted. Distilled water was prepared with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.3. Sample preparation procedure

Stock standard solutions of individual marker compounds were prepared by dissolving each standard (0.1 mg) in methanol (1.0 mL). The working standard solution for calibration was prepared by dilution of the stock standard solution. All dried samples were finely ground and passed through a 20-mesh sieve. Each powdered sample (0.1 g) was extracted with 70 % ethanol (10 mL) by sonication for 60 min. The extracted sample was centrifuged at 3500 rpm for 5 min. The supernatant was filtered through a 0.22 μm syringe filter and injected (10 μL) into the HPLC system.

2.4. HPLC-UV and LC-MS conditions

HPLC-UV and LC-MS were used to analyze the mulberry extracts. The HPLC system consisted of an SPD-20A UV-Vis detector, an SIL-20A auto-sampler, a CTO-20A column oven, and a DGU-20A3 degasser (Shimadzu, Japan). Peaks with the same retention time as the standard were identified using an LC-MS-2010-EV spectrometer (Shimadzu, Japan) with an electrospray ionization (ESI) source operating in both positive and negative modes. The stationary phase was an Optimapak C18 column (250 × 4.6 mm, 5 μm; RStech Corp., Korea). The mobile phase was composed of water and acetonitrile at a flow rate of 1.0 mL/min. The temperature of the column oven was set at 30 °C. The chromatogram was monitored at 270 nm considering the UV-λ_{max} of the marker compounds. LC-MS analyses were performed at an interface voltage of 4.5 kV and -3 kV. The conditions also consisted of a drying gas flow at 15 L/min, a nebulizing gas flow at 3 L/min, a desolvation line (DL) temperature of 250 °C, and a heat block temperature of 400 °C at a flow rate of 0.5 mL/min. Other analytical conditions were the same as those used for HPLC-UV analyses.

2.5. Optimization of sample preparation

Sample preparation conditions were optimized for a combination of MF, MR, and MCR. Four solvents (water, methanol, ethanol, and acetonitrile) were evaluated for the extraction of marker compounds, with ethanol giving the highest extraction efficiency. Then ethanol was evaluated at different concentrations (30 %, 50 %, 70 % in water and 100 % ethanol) and extraction times (10, 30, 60, 90, and 120 min).

2.6. Validation of simultaneous measurements

The specificity, linearity, precision, and accuracy of intra- and inter-day analyses were verified in accordance with the method validation guidelines of the Ministry of Food and Drug Safety (MFDS).[22] Stock standard solutions were prepared at five different concentrations and injected in triplicate to generate calibration curves based on the peak area of each standard as a function of concentration. Linearity was verified by the square of the correlation coefficient (r^2) of the calibration curve. The limit of detection (LOD) and limit of quantification (LOQ) were estimated to be the concentration of standard yielding signal-to-noise ratios of 3 and 10, respectively. Repeatability was estimated based on the relative standard deviation (RSD) of the relative retention time (RRT) and relative retention area (RRA) by six replicate injections of standard solution. Accuracy and precision were performed at low, medium, and high standard concentrations. Analyses were performed in triplicate within a single day for intra-day values and over three consecutive days for inter-day values. Recovery was verified in triplicate by spiking three different concentrations of standard solution (50%, 100%, and 200% of the sample concentration) into the sample and calculated as

Recovery (%) = (observed conc.) /
(theoretical spiked conc.) × 100.

3. Results and Discussion

3.1. Selection of marker compounds

Using pattern analyses to screen for marker compounds, rutin, isoquercitrin, and astragalin were detected in MF. Rutin was chosen as the marker compound as per the Chinese Pharmacopeia (CP), which states a criterion of 1.0% rutin for MF.[23] In the current study, rutin was unique to MF. Furthermore, no peaks were present in the chromatograms of MR and MCR at the retention time of rutin. Therefore, rutin makes an excellent marker for identifying MF. Oxyresveratrol, kuwanon G, and morusin were identified in both MR and MCR extracts, but with different relative chromatographic peak intensities. Mulerronside A and mulberrin were also detected in MCR but with low peak intensities. Given these results, rutin, oxyresveratrol, kuwanon G, and morusin were selected as marker compounds for simultaneous analyses of MF, MR, and MCR.

3.2. Identification of marker compounds by LC-MS

Target compounds were qualitatively identified by their retention times in HPLC analyses. However, it is possible for multiple compounds to have the same retention time. Therefore, chromatographic peaks corresponding to marker compounds were definitively identified using LC-MS analyses. Four peaks were confirmed as corresponding to rutin, oxyresveratrol, kuwanon G, and morusin, respectively, in both positive $[M+H]^+$ and negative $[M-H]^-$ ion modes (*Table* 2).

3.3. Optimization of HPLC conditions

A mixture of water (mobile phase A) and acetonitrile (mobile phase B) was chosen as the eluent because it yielded better separation than a water–methanol eluent. To reduce peak tailing, 0.1 % formic acid was added to the water making up mobile phase A. Four different gradient elutions were evaluated: (M1) 0 % B to 100 % B over 70 min, (M2) 15 % B to 100 % B over 60 min, (M3) 15 % B to 90 % B over 40 min, and (M4) 15 % B to 90 % over 25 min. M4 was selected as the optimal gradient based on the peak resolution of marker compounds and the total analytical runtime for a mixture of MF, MR, and MCR extracts. Detection wavelengths were evaluated from 190 nm to 400 nm. Each of the four marker compounds showed a strong absorbance at 270 nm.

Overall, optimal HPLC-UV separation occurred

Table 2. Identification of marker compounds by HPLC-MS

Compounds	Molecular weight (Da)	Positive mode	Negative mode
Rutin	610	611 $[M+H]^+$	609 $[M-H]^-$
Oxyresveratrol	224	225 $[M+H]^+$	223 $[M-H]^-$
Kwanon G	692	693 $[M+H]^+$	691 $[M-H]^-$
Morusin	420	421 $[M+H]^+$	419 $[M-H]^-$

with a mobile phase of 0.1 % formic acid in water and acetonitrile under gradient conditions (0 min at 15 % B, 25 min at 90 % B) with detection at 270 nm.

3.4. Method Validation

The four marker compounds for MF, MR, and MCR were separated without interference among themselves or with other peaks originating from the sample extracts (*Fig.* 2). LC-MS data confirmed that each HPLC peak located at the appropriate retention time corresponded to a single marker compound. This demonstrates the specificity of the developed method for the marker compounds. The linearity of peak intensity as a function of marker concentration was verified by a correlation coefficient (r^2) of > 0.9990 for all four markers (*Table* 3). LODs of the four marker compounds ranged from 81 ng/mL to 138 ng/mL. LOQs ranged from 229 ng/mL to 459 ng/mL (*Table* 3). The degree of repeatability was also high, as indicated by RSDs of RRTs that were less than 0.21 %. Those of RPAs were lower than 0.81 % (*Table* 4). The quantitative accuracy for all marker compounds ranged from 98.98 % to 107.56 % in intra-day experiments and from 98.48 % to 108.40 % in inter-day experiments. Analytical precision, as indicated by RSD in *Table* 5, ranged from 0.31 % to 2.10 % (intra-day) and from 0.98 % to 2.96 % (inter-day). Percent recoveries ranged from 96.42 % to 103.68 % with RSDs from 0.80 % to 2.97 % (*Table* 6). The repeatability, recovery, and intra-/inter-day accuracy and precision indicate the high reproducibility of the developed method.

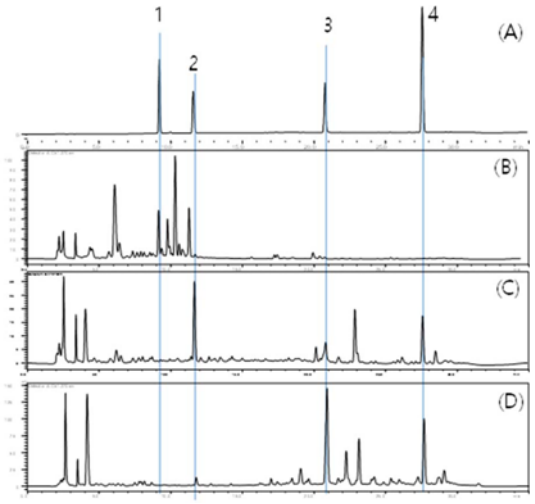

Fig. 2. HPLC Chromatograms of (A) standard mixture and (B) Mori Folium, (C) Mori Ramulus,(D) Mori cortex radicis : 1, rutin (9.4 min); 2, oxyresveratrol (11.7 min); 3, kuwanon G (20.9 min) and 4, morusin (27.7 min).

3.5. Optimization of extraction methods

The effects of extraction solvent and extraction time were evaluated to optimize the sample preparation method used for mixed samples of MF, MR, and MCR. Samples (0.1 g) were extracted via sonication in various solvents (10 mL): water, methanol, ethanol, and acetonitrile. The number of peaks in the HPLC chromatograms of MF samples extracted with water was higher than with other solvents, but peaks corresponding to marker compounds, as well as other major peaks, were not evident in MR and MCR chromatograms. Considering the peak intensities

Table 4. Repeatability of marker compounds (*n=6*)

Compounds	RRT (min)	RSD (%)	RPA (area, × 10^4)	RSD (%)
Rutin	9.78±0.02	0.21	181.51±1.46	0.81
Oxyresveratrol	12.31±0.01	0.08	46.33±0.12	0.26
Kuwanon G	21.83±0.01	0.05	175.63±0.34	0.19
Morusin	28.53±0.01	0.04	166.96±0.36	0.22

Mean±SD

Table 3. Linearity, LOD and LOQ of marker compounds

Compounds	Range (μg/mL)	Calibration equation	Correlation factor (r^2)	LOD (ng/mL)	LOQ (ng/mL)
Rutin	10-200	y = 17740x + 11741	0.9999	81	285
Oxyresveratrol	10-200	y = 13678x + 35248	0.9992	118	372
Kuwanon G	10-200	y = 16541x – 2005	0.9999	138	459
Morusin	5-100	y = 103988x + 134548	0.9995	69	229

Table 5. Intra-/inter-day accuracy and precision of marker compounds

Compounds	Conc. (μg/mL)	Intra-day (n=3)			Inter-day (n=3)		
		Mean±SD	Accuracy (%)	RSD (%)	Mean±SD	Accuracy (%)	RSD (%)
Rutin	25	26.89±0.55	107.56	2.05	27.10±0.80	108.40	2.95
	50	49.49±0.54	98.98	1.09	49.24±0.95	98.48	1.93
	100	100.45±0.57	100.45	0.57	102.3±1.55	102.30	1.52
Oxyresveratrol	25	24.98±0.43	99.92	1.72	25.93±0.69	103.72	2.66
	50	49.68 ±0.84	99.36	1.69	50.74±1.10	101.48	2.17
	100	100.63±0.70	100.63	0.70	101.53±0.99	101.53	0.98
Kuwanon G	25	26.60±0.52	106.40	1.95	26.82±0.42	107.28	1.60
	50	50.97±0.16	101.94	0.31	49.24±0.82	98.48	1.67
	100	103.21±1.42	103.21	1.38	101.02±1.89	101.02	1.87
Morusin	25	25.73±0.54	102.92	2.10	25.42±0.64	101.68	2.52
	50	50.97±0.16	101.94	0.31	51.97±0.65	103.94	1.25
	100	103.21±1.42	103.21	1.38	101.53±1.61	101.53	1.59

Table 6. Recovery of marker compounds (n=3)

Compounds	Sample conc. (μg/mL)	Spiked conc. (μg/mL)	Observed conc. (μg/mL)	Mean Recovery (%)	RSD (%)
Rutin	20.23	10	31.36±0.25	103.74	0.80
		20	39.10±0.31	97.19	0.80
		40	60.86±0.27	101.05	0.44
Oxyresveratrol	39.52	20	60.88±0.58	102.28	0.96
		40	76.67±1.55	96.42	2.02
		80	115.32±3.43	96.49	2.97
Kuwanon G	30.97	15	47.66±0.57	103.68	1.20
		30	61.93±0.42	101.57	0.68
		60	88.57±1.51	97.36	1.70
Morusin	5.11	2.5	7.42±0.01	97.50	0.14
		5	10.34±0.02	102.27	0.16
		10	14.84±0.03	98.21	0.19

of marker compounds in mixed MF, MR, and MCR extracts, methanol was selected as an optimal extraction solvent. Separation efficiency was further improved by diluting the solution to 70 % methanol with water (*Fig.* 3). An extraction time of 60 min resulted in higher concentrations of marker compounds than other evaluated extraction times (10, 30, 90 min) (*Fig.* 4).

3.6. Discrimination of MF, MR, and MCR

In quantitative analyses of 30 medicinal extracts of mulberry tree, characteristic patterns of marker compounds were observed, corresponding to MF, MR, and MCR. Rutin was detected only in MF at 0.11 ± 0.0 2 %. Oxyresveratrol, kuwanon G, and morusin were detected in MR and MCR but not in MF. The levels of these three marker compounds differed between MR and MCR samples. The concentrations of oxyresveratrol, kuwanon G, and morusin were 0.12 ± 0.08 %, 0.04 ± 0.01 %, and 0.03 ± 0.01 % in MR, and 0.05 ± 0.08 %, 0.33 ± 0.16 %, and 0.18 ± 0.06 % in MCR. MR had twice the amount of oxyresveratrol as MCR, while MCR contained approximately eight-fold higher levels of kuwanon G and six-fold higher levels of morusin. Based on

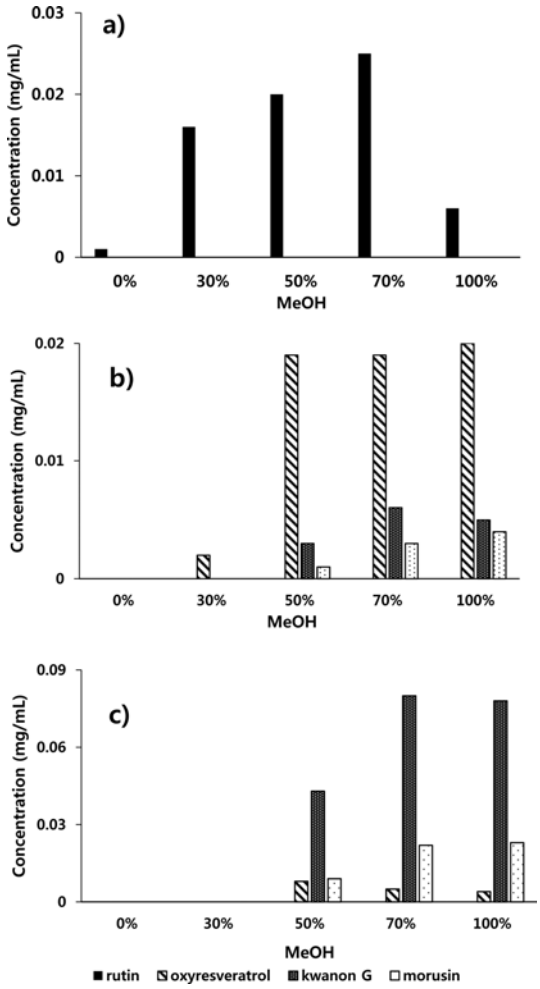

Fig. 3. Effect of extraction time; (a) Mori Folium, (b) Mori Ramulus, (c) Mori Cortex Ramulus.

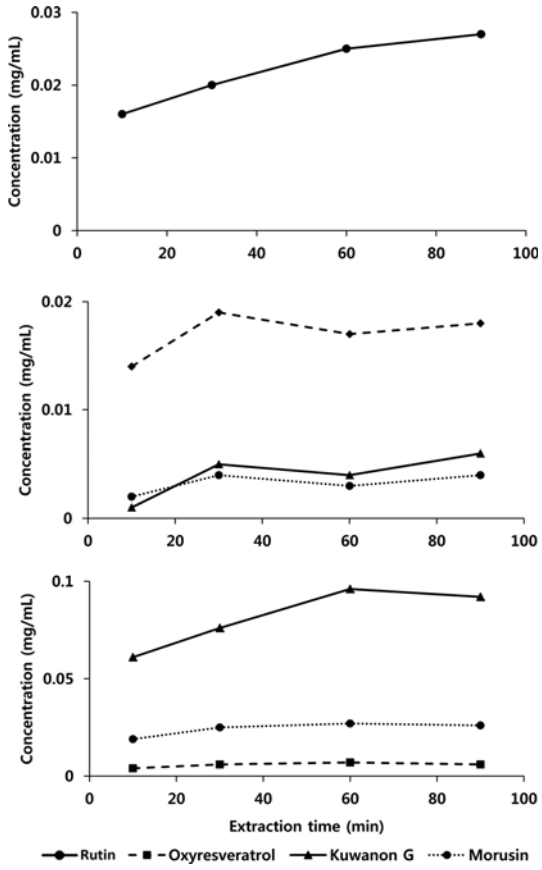

Fig. 4. Effect of extraction time; (a) Mori Folium, (b) Mori Ramulus, (c) Mori Cortex Ramulus, (●) rutin, (■) oxyresveratrol, (▲) kuwanon G, (✕) morusin.

these quantitative results, MF, MR, and MCR extracts could be discriminated according to their differential contents of rutin, oxyresveratrol, kuwanon G, and morusin.

4. Conclusions

This study describes a simple HPLC method to simultaneously identify and discriminate MF, MR, and MCR extracts by detecting and quantifying characteristic marker compounds. Using pattern analyses, rutin, oxyresveratrol, kuwanon G, and morusin were selected as marker compounds representing three herbal medicines obtained from the mulberry tree. Among the three herbal medicines, MF was the only one that contained rutin (0.11 %) and an absence of the other marker compounds. Oxyresveratrol, kuwanon G, and morusin were found in both MR and MCR, but at concentrations that differed according to the part of the mulberry tree from which they were obtained. MR had the highest oxyresveratrol content, while MCR contained greater amounts of kuwanon G and morusin. The developed HPLC method simultaneously identifies and differentiates the three primary herbal medicines extracted from the mulberry tree (MF, MR, and MCR). In addition, the ratio of MF, MR, and MCR in mixed samples can be determined according to the type and quantity of marker compounds.

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