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ORIGINAL ARTICLE

Evaluation of Antioxidant and Antibacterial Activities of Morin Isolated from Mulberry Fruits (*Morus alba* L.)

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Abstract Antioxidant and antibacterial activities of materials isolated from the methanol extract of mulberry fruits were evaluated. The active constituent of the methanol extract derived from mulberry fruits was isolated by silica gel and LH-20 column chromatographies and was identified as morin by electron ionization mass spectrometer, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. Based on the IC_{50} values, the antioxidant activities of morin exhibited potent inhibition according to 1,1-diphenyl-2-picrylhydrazyl (30.0 μg/mL) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (71.0 μg/mL) radical scavenging assays. Furthermore, the antibacterial activity of morin showed moderate (++) inhibition against *Streptococcus mutans* at both 5 and 2 mg/disc, according to paper disc diffusion assays, indicating morin isolated from mulberry fruits could be a useful natural agent for the management of antioxidant and antibacterial treatments.

Keywords antibacterial activity, antioxidant activity, mulberry fruits, morin, *Morus alba* L.

Introduction

Reactive oxygen species (ROS) are involved with hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and superoxide anions ($O^{\bullet}_2^{-}$). There are increasing suggestions based on considerable evidence that free radicals induce oxidative damage to biomolecules such as lipids, nucleic acids, and proteins (Dreher and Junod, 1996). Therefore, the studies on antioxidants derived from natural sources have been reported and the efforts to identify active compounds have been attempted from new natural resources. In recent years, the interest in antioxidants derived from beverages, fruits, herbs and vegetables has increased. Plants contain a wide variety of free radical scavenging molecules, such as anthocyanins, carotenoids, dietary glutathione, endogenous metabolites, flavonoids, and vitamins. In addition, these naturally existing antioxidants can be formulated to give nutraceuticals which can help to prevent oxidative damage. Furthermore, plant compounds exhibiting target sites other than those of currently used antibiotics could be active against drug-resistant microbial pathogens. In addition, many plants have been screened as a viable source of natural antibacterial agents, which are responsible for maintenance of health by helping the human body to protect itself from coronary heart diseases and cancer (Yanga et al., 2002).

Plants play a significant role in improving the quality of human life (Lee et al., 2002). Mulberries (*Morus alba* L.) have long been used in traditional medicine to improve eyesight, lower blood pressure, prevent diabetes, protect the liver, strengthen joints, and treat fever (Zhishen et al., 1999). In particular, many studies on mulberry fruits have reported its biological activities such as antidiabetic, antioxidative (Park et al., 1995), and antiinflammatory (Kim et al., 2004) activities. Due to the functional quality characteristics, the demand for mulberry fruits by consumers is increasing. In the present study, antioxidant and antibacterial activities of various materials and isolate active constituent from mulberry fruits were evaluated.

Materials and Methods

Materials. The fruits (1 kg) of mulberry (*M. alba* L.) were purchased from a local market in Jeonju. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and caffeic acid, were purchased from Sigma (USA), and chlorhexidine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and potassium persulphate

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were from Aldrich Chemical Co. (USA). Folin-Ciocalteu reagent was purchased from Fluck Chemical Co. (Swizerland). All other chemicals used were of reagent grade.

Preparation of mulberry fruits extract. The mulberry fruits were extracted twice with a 100% methanol solvent system in a shaking incubator at room temperature for 24 h. The methanol extract of mulberry fruits was filtered through a filter paper (Tokyo filter paper NO. 2, Tokyo Roshi, Japan) *in vacuo*. The filtrate was concentrated *in vacuo* at 45°C using a rotary vacuum evaporator (EYELA auto jack NAJ-100, Japan). The methanol extract (220 g) was sequentially divided into hexane (11 g), chloroform (15 g), ethyl acetate (94 g), butanol (32 g), and water (68 g) fractions for bioassay. The organic solvent fractions were concentrated using rotary vacuum evaporation at 45°C, and the water fraction was freeze-dried.

Isolation and identification. The antioxidant activities of the five fractions obtained from the methanol extract of mulberry fruits were evaluated. The chloroform fraction had potent antioxidant activity. To isolate antioxidant constituent of mulberry fruits, various chromatography methods were implemented. First, the chloroform fraction (15 g) was loaded onto a silica gel column (Merck 70-230 mesh, 600 g, 5.5 cm i.d.×50 cm; USA) and continuously eluted with a step gradient of chloroform:methanol (5:1, 4:1, 3:1, and 1:1, v/v) which yielded four fractions (SB1 to SB4). The separated fractions were analyzed via thin layer chromatography (TLC), and those fractions showing similar patterns were pooled. In this step, the SB2 fraction (3.6 g) showed the antioxidant activity. Therefore, the active SB2 fraction was subjected to chromatography on a silica gel column and successively eluted with chloroform : methanol (5:1, v/v). The SB22 fraction (995 mg) exhibited the strongest antioxidant activity among the fractions. To determine the antioxidant constituent of the SB22 fraction, LH-20 column chromatography was performed with 100% methanol as a mobile phase. This step produced four fractions (SB221 to SB224). Finally, the potent active SB224 fraction (225 mg) was successfully isolated (Fig. 1). The chemical structure of the isolated active compound was determined using spectroscopic analysis methods. The ^{1}H- and ^{13}C-NMR spectra were recorded in deuterochloroform ($CDCl_3$) using a JNM-ECA 600 spectrometer at 600 and 150 MHz (with trimethylsilane as an internal standard), respectively, with chemical shifts expressed in δ (ppm). Additionally, EI-MS spectra were obtained with a JEOL JMS-DX 30 spectrometer (JEOL, Japan).

Measurement of scavenging activity on DPPH radicals. The scavenging activity of mulberry extracts on DPPH radical was measured according to the method of Blosis (1958) with some modifications. Ethanolic DPPH radical solution (0.2 mM) and samples with various concentrations were prepared. An aliquot of 1.0 mL of each sample was added to 1.0 mL of the ethanolic DPPH radical solution, and the reaction mixture was incubated in the dark at room temperature for 20 min. The absorbance of the mixture was measured at 525 nm using a UV-spectrophotometer. BHT was used as a positive control. The ability of scavenging

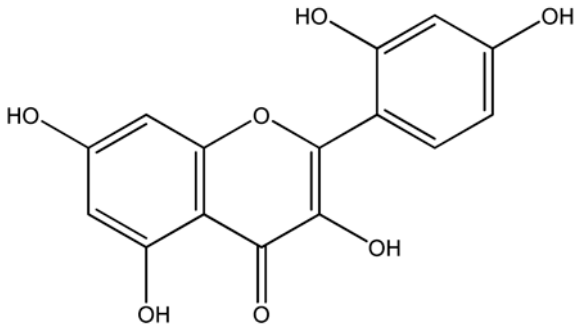

Fig. 1 Chemical structure of Morin isolated from Mulberry fruits (*Morus alba* L.)

DPPH radicals was calculated using the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = \{1-(A/B)\} \times 100$$

where A is the absorbance of the sample treated with the extract, and B is the absorbance of the untreated sample.

Measurement of scavenging activity on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt radicals. The ABTS assay was performed as described by Re et al. (1999). The stock solutions included 7 mM ABTS solution and 140 mM potassium persulfate (PPS) solution, and the two stock solutions were mixed in equal quantities for 12 h at room temperature in dark. The solution was diluted by mixing 1 mL $ABTS^{+}$ solution with 60 mL of 80% ethanol to obtain an absorbance of 0.7 units at 734 nm using a spectrophotometer. The mulberry extracts (1 mL) were allowed to react with 1 mL of the $ABTS^{+}$ solution, and after 7 min the absorbance was taken at 734 nm. The scavenging activity of ABTS radicals from the mulberry extracts was compared with that of BHT. The percentage inhibition was calculated as follows:

$$\text{ABTS radical-scavenging activity (\%)} = \{1-(A/B)\} \times 100$$

where A is the absorbance of the sample treated with the extract, and B is the absorbance of the untreated sample.

Determination of total polyphenol contents. Total polyphenol contents of the mulberry extracts were determined according to the Folin-Ciocalteu procedure (Singleton and Rossi, 1965) with slight modifications. Briefly, 1.0 mL mulberry extracts was mixed with 1.0 mL Folin-Ciocalteu reagent (10%). After 10 min, 1.0 mL of sodium carbonate solution (10%, w/v) was added to the samples and incubated at room temperature for 60 min. Measurements were calibrated to a standard curve of prepared caffeic acid solution (10–70 μg/mL). Determination of total polyphenol contents was then expressed as milligram of caffeic acid equivalents of the total polyphenol contents.

Preparation of test microorganisms. *Porphyromonas asaccharolytica* ATCC 25260, *Porphyromonas gingivalis* ATCC 33277, *Streptococcus mutans* ATCC 25175, and *Streptococcus sobrinus* ATCC 27607 were employed for determination of antibacterial activity. Stock cultures of these strains were routinely

stored on an Eggerth-Gagnon (EG) liver extract-Field's slant at –80°C. Microorganisms were used for antibacterial evaluation. These bacteria were maintained on EG agar (Eiken chemical, Japan) for 2 days at 37°C in an anaerobic jar (Hirayama, Japan) with an atmosphere comprising of 80% N_2, 15% CO_2, and 5% H_2. The bacteria were then grown in brain heart infusion (BHI) broth (pH 7.6). Only *P. gingivalis* ATCC 33277 was cultured in the *P. gingivalis* broth (PGB) medium (BHI 1.85%, L-cystein 0.05%, hemin solution 1%, menadione solution 0.1%, and yeast extract 0.5%).

Measurement of antibacterial activity. Antibacterial activities of test samples were evaluated by the paper disc diffusion method (Lee and Ahn, 1998) with minor modifications. To determine the effects of the chemicals on the growth inhibition of the evaluated bacteria, one loopful of bacteria was suspended in 1 mL of sterilized physiological saline, followed by standardization of the resultant microbial suspensions to a cell density of 1.5×10^5 CFU/mL. An aliquot (0.1 mL) of the bacterial suspension was seeded on the EG agar. The predetermined dose of the sample was then dissolved in 0.1 mL of methanol, which was subsequently applied to a paper disc (Advantec, diameter 8 mm and thickness 1 mm, Tokyo Roshi). After the solvent evaporated, the paper disc was then placed on the surface of the agar plates that had been inoculated with the test bacteria. All plates were then anaerobically incubated for 2 days at 37°C. The antibacterial activities were determined by assigning one of the following values, based on the estimated size (diameter) of the zone of inhibition produced by each test sample: potent response (++++), zone of inhibition diameter >30 mm; strong response (+++), zone of inhibition diameter 21–30 mm; moderate response (++), zone of inhibition diameter 16–20 mm; weak response (+), zone of inhibition diameter 10–15 mm; and little or no response (-), zone of inhibition diameter <10 mm.

Statistical analysis. All treatments were performed in triplicate, with each analysis replicated three times. According to SAS (version 6, SAS Institute Inc., USA), the IC_{50} values were calculated using ANOVA. The means of the treatments were compared and separated using a Scheffe's test at $p<0.05$.

Results and Discussion

In the present study, the yield of the methanol extract of mulberry fruits was 22%. The five fractions derived from the methanol extract of mulberry fruits varied. The highest yield was obtained from the ethyl acetate fraction, whereas the lowest yield was obtained from the hexane fraction, 42.73 and 5.00%, respectively.

The antioxidative activities of the methanol extract and the five fractions obtained from the methanol extract of mulberry fruits are shown in Table 1. Free radical scavenging is the accepted mechanism for antioxidants to inhibit lipid oxidation (Brand-Willams et al., 1995). The DPPH radical was used as a stable-free radical to determine the antioxidant capacity of natural compounds (Shimada et al., 1992). Based on the IC_{50} values of the DPPH radical, the scavenging activity on DPPH radicals of the methanol extract of mulberry fruits was 93.8 μg/mL. The chloroform fraction (158.7 μg/mL) had potent scavenging activity on DPPH radicals, followed by the ethyl acetate fraction (234.8 μg/mL), the hexane fraction (245.2 μg/mL), the butanol fraction (350.5 μg/mL), and the water fraction (725.4 μg/mL). Furthermore, Table 1 also demonstrated the scavenging activities on ABTS radicals. On the basis of the IC_{50} values of ABTS radicals, the scavenging activity on ABTS radicals of the methanol extract of mulberry fruits was 117.1 μg/mL. The chloroform fraction (104.5 μg/mL) revealed strong scavenging activity on ABTS radicals, followed by the ethyl acetate fraction (173.5 μg/mL), the hexane fraction (178.1 μg/mL), the butanol fraction (300.6 μg/mL), and the water fraction (925.5 μg/mL).

In the natural environment, the polyphenol compounds derived from plants are known as powerful antioxidants and may contribute to antioxidative action (Duh et al., 1999). The use of phenolics in the food industry is increasing, because they are known to retard oxidative degradation of lipids and, thereby, improve the quality and nutritional value of the food (Aneta et al., 2007). Total polyphenol contents in the methanol extract of mulberry fruits (45.6 mg/g) was similar to that of chloroform fraction (43.9 mg/g), followed by the ethyl acetate fraction (39.4 mg/g), the water fraction (35.9 mg/g), the hexane fraction (34.3 mg/g), and butanol fraction (29.8 mg/g). These results indicated that the antioxidant activities of methanol extract and its five fractions derived from mulberry fruits had similar patterns of activities to that of the scavenging activities on DPPH and ABTS radicals as well as the total polyphenol contents. Although the antioxidant capacities revealed by *in vitro* experiments are only indicative of the potential health benefits, these results serve as an essential step in screening for the antioxidant capacity of mulberry fruits.

Table 1 Antioxidant activities of five fractions obtained from the methanol extract of mulberry fruits (*M. alba* L.)

Samples	DPPH IC_{50} value (μg/mL)[a]	ABTS IC_{50} value (μg/mL)	Total polyphenol contents (mg/g)
Methanol extract	93.8±1.2	117.1±1.2	45.6±0.7
Hexane fraction	245.2±0.9	178.1±0.6	34.3±0.3
Chloroform fraction	158.7±1.0	104.5±0.7	43.9±0.4
Ethyl acetate fraction	234.8±0.8	173.2±0.5	39.4±0.9
Butanol fraction	350.5±0.3	300.6±0.5	29.8±0.5
Water fraction	725.4±0.6	925.5±1.2	35.9±0.2

[a]IC_{50} values calculated from regression lines, using five different concentrations in triplicate experiments.

Table 2 ^{1}H- and ^{13}C-NMR[a] spectral data of SB224 derived from Mulberry fruits (*M. alba* L.)

Carbon	Partial structure	δ_c (ppm)	δ_H (ppm)
2	C	149.6	-
3	C-OH	136.5	16.771 (*s*[b])
4	C=O	176.1	-
5	C-OH	161.8	4.661–4.675 (*d*, J=8.4 MHz)
6	C-H	98.3	5.453–5.541 (*d*, J=52.8 MHz)
7	C-OH	166.4	4.661–4.675 (*d*, J=8.4 MHz)
8	C-H	94.0	5.026–5.056 (*d*, J=18.0 MHz)
9	C	158.8	-
10	C	104.5	-
1'	C	111.1	-
2'	C-OH	157.8	4.661–4.675 (*d*, J=8.4 MHz)
3'	C-H	103.5	6.099–6.140 (*d*, J=24.6 MHz)
4'	C-OH	159.1	4.661–4.675 (*d*, J=8.4 MHz)
5'	C-H	108.4	6.621–6.659 (*t*, J=22.8 MHz)
6'	C-H	131.2	7.042–7.065 (*d*, J=13.8 MHz)

[a]^{1}H- and ^{13}C- spectra were measured in CD_3OD at 600 and 150 MHz, respectively.
[b]s: singlet, d: doublet, t: triplet.

Through the potent antioxidant activities of the chloroform fraction obtained from the methanol extract of mulberry fruits, the active compound of the chloroform fraction was isolated using various chromatographies, including silica gel and LH-20 column chromatography using a variety of mixed organic solvents. As a result, SB-224 was successfully isolated. The active compound was identified by various spectroscopic analyses including Electron ionization mass spectrometer (EI-MS), ^{1}H-NMR, and ^{13}C-NMR, which was compared with those of authentic reference compounds. The bioactive constituent was characterized as morin (2',3,4',5,7-pentahydroxyflavone) based on the following evidence. Morin (SB-224, $C_{15}H_{10}O_7$, MW: 302.24); EI-MS (70 eV) *m/z* 302 [M^+]. ^{1}H-NMR ($CDCl_3$, 600 MHz, δ ppm)=16.771 (1H, *s*), 7.042–7.065 (1H, *dd*), 6.621–6.659 (1H, *t*), 5.453–5.541 (1H, *d*), 5.026–5.056 (1H, *d*), and 4.661–4.675 (1H, *d*); ^{13}C-NMR ($CDCl_3$, 150 MHz, δ ppm)=176.1, 166.4, 161.8, 159.1, 158.8, 157.8, 149.6, 136.5, 131.2, 111.1, 108.4, 104.5, 103.5, 98.3, and 94.0 s ppm (Table 2, Fig. 1). The spectroscopic data of morin matched those of previously reported flavonoid compounds (Suganya et al., 2007). The present findings are similar to those of a study conducted by Lee et al. (2004). Mulberries (*M. alba* L.) have long been used for its antioxidant and antibacterial properties (Zhifeng et al., 2006).

The antioxidant activities of morin isolated from mulberry fruits were evaluated via DPPH and ABTS radical scavenging activity assays (Tables 3 and 4). The morin isolated from mulberry fruits exhibited 100% inhibition of DPPH and ABTS radical scavenging at 500 μg/mL; more specifically, based on the IC_{50} values, the IC_{50} values of morin isolated from mulberry fruits were 30.0 and 71.0 μg/mL, respectively. In a comparison of the IC_{50} value of butyl hydroxytoluene (BHT) as a positive control, the inhibition of DPPH and ABTS radical scavenging of morin was similar to those of BHT (IC_{50} 20.7 and 28.2 μg/mL). Overall, flavonoid compounds can potentially be employed as free radical scavengers owing to their competent hydrogen-donating potential (Orsolya et al., 2004).

The antibacterial activities of morin isolated from mulberry fruits were determined by the paper disc diffusion method against oral bacteria (Table 5). In particular, against *S. mutans*, morin exerted moderate (++) growth inhibition at 5 and 2 mg/disc and weak growth inhibition at 1 mg/disc. In addition, morin treatment at concentrations of 5 and 2 mg/disc produced weak (+) growth inhibition against *S. sobrinus*. However, morin had no antibacterial activity against *P. asaccharolytica* and *P. gingivalis*. In comparison with chlorhexidine as a positive control, morin was found to be useful for managing populations of oral bacteria although growth-inhibiting activity of morin was slightly lower than that of chlorhexidine.

Flavonoids, a group of phenolic compounds, are widely included in fruits and vegetables. Previous studies have demonstrated its numerous positive effects such as anticancer (Block, 1992), antiinflammatory (Middleton, 1998), and antiviral (Selway, 1986) effects in human health. In general, these activities are associated with free radical scavenging properties of flavonoids. In addition, flavonoid compounds may affect growth and metabolism of bacteria. They could have an activation or inhibition effect on microbial growth according to their constitution and concentration (Alberto et al., 2002). Some studies found that mulberries contain

Table 3 Inhibition of DPPH radical scavenging of morin isolated from mulberry fruits (*M. alba* L.)

Compounds	DPPH radical scavenging activity (%)					IC_{50} (μg/mL)
	1,000 (μg/mL)	500 (μg/mL)	100 (μg/mL)	50 (μg/mL)	25 (μg/mL)	
Morin	100	100	80.5±1.2	62.5±0.8	23.0±0.9	30.0±0.9
BHT	90.7±0.9	87.2±1.2	85.1±0.4	73.9±1.0	68.5±0.6	20.7±0.4

Table 4 Inhibition of ABTS radical scavenging of morin isolated from mulberry fruits (*M. alba* L.)

Compounds	ABTS radical scavenging activity (%)					IC_{50} (μg/mL)
	1,000 (μg/mL)	500 (μg/mL)	100 (μg/mL)	50 (μg/mL)	25 (μg/mL)	
Morin	100	100	63.1±0.5	40.0±0.3	36.0±0.9	71.0±0.4
BHT	100	90.3±0.6	79.2±1.0	61.3±0.8	45.3±0.3	28.2±0.6

Table 5 Antibacterial activities of morin isolated from mulberry fruits (*M. alba* L.) against oral bacteria

Samples[c]	Dose (mg/disc)	Test microorganisms[a]			
		P. asaccharolytica	*P. gingivalis*	*S. mutans*	*S. sobrinus*
Morin	5	-[b]	-	++	+
	2	-	-	++	+
	1	-	-	+	-
Chlorhexidine	2	+++	+++	++	+++
	1	++	++	++	+++
	0.5	+	+	+	++

[a]Cultured on Eggerth-Gagnon agar at 37°C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2.
[b]Inhibitory zone diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, -.
[c]Each assay was determined in triplicate.

bioactive phenolic chemicals such as β-carotene and α-tocopherol (Yen et al., 1996). In the present study, morin, a flavonoid compound isolated from mulberry fruits, was found to possess the greatest antioxidant capacity, based on the DPPH and ABTS radical scavenging activity assays. Furthermore, morin exerted moderate growth-inhibiting activity against *Streptococcus* spp. Jeong et al. (2010) suggested that the antioxidant and antimicrobial activities of phenolic compounds (ferulic acid and sinapic acid) derived from *Triticum aestivum* sprouts may have pharmacological values. These results indicated that phenolic compounds may produce high free radical scavenging and antibacterial activities. In conclusion, morin isolated from mulberry fruits could be useful as a natural agent for the management of antioxidant and antibacterial treatment.

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