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Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity

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Abstract

Oxidation of low-density lipoprotein (LDL) has been implicated in atherogenesis. Antioxidants that prevent LDL from oxidation may reduce atherosclerosis. We investigated LDL antioxidant activity and extracted compounds of mulberry (*Morus alba* L.) leaves. The LDL antioxidant activity of 60% ethanol extracted of mulberry leaves, which inhibits human LDL oxidation induced by copper ion, was determined on the basis of oxidation lag time and calculated as epigallocatechin 3-gallate equivalents (58.3 μmol of EGCG equivalent/g of dry weight). Three flavonol glycosides [quercetin 3-(6-malonylglucoside), rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside)] were identified as the major LDL antioxidant compounds by LC-MS and NMR. The amounts of these flavonol glycosides in mulberry leaves and mulberry-leaf tea were determined by HPLC. Our results showed that quercetin 3-(6-malonylglucoside) and rutin were the predominant flavonol glycosides in the mulberry leaves.
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Keywords: *Morus alba* L.; Mulberry; LDL; Antioxidant; Quercetin glycoside; Quercetin 3-(6-malonylglucoside)

1. Introduction

In recent years, there has been increasing interest in antioxidants derived from fruits, vegetables, herbs, and beverages. Epidemiological studies have indicated that dietary intake of antioxidants from plants is inversely associated with mortality from coronary heart disease (Giugliano, 2000). Oxidative modification of LDL is thought to play a key role in the pathogenesis of early atherosclerosis (Aviram, 1993; Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Vitamins C and E from plants protect LDL from oxidative modification in vitro (Babiy, Gebicki, & Sullivan, 1990; Jialal, Vega, & Grundy, 1990) and decrease the morbidity of coronary heart disease (Enstrom, Kanim, & Klein, 1992; Rimm et al., 1993; Stampfer et al., 1993; Stephens et al., 1996). Dietary supplementation in humans of nutrients rich in polyphenols, such as black tea, green tea (Serafini, Ghiselli, & Ferro-Luzzi, 1994), red wine (Aviram & Eias, 1993), and olive oil (Fuhrman, Lavy, & Aviram, 1995), have been shown to be associated with an increase in plasma antioxidant capacity, and a reduced risk of coronary heart disease.

Mulberry (*Morus alba* L.) leaves, bark and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure (Zhishen, Mengcheng, & Jianming, 1999). Leaves of mulberry species are consumed in Korea and Japan as antihyperglycemic nutracetical foods for patients with diabetes mellitus because the leaves contain

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1-deoxynojirimycin, known to be one of the most potent α-glycosidase inhibitors (Kim et al., 2003). In Japan, consumption of mulberry-leaf tea has been increasing.

The antioxidant activity of mulberry leaves has also been reported. Doi, Kojima, and Fujimoto (2000) reported that 1-butanol extract of mulberry leaves scavenged the DPPH radical and inhibited the oxidative modification of rabbit and human LDL. Five flavonol glycosides (rutin, isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin and kaempferol 3-(6-acetylglucoside)) have been reported in mulberry leaves (Matsuoka, Kimura, & Muraoka, 1994; Onogi et al., 1993). However, there are few reports on quantitative antioxidant activity or specifying amounts of antioxidants in mulberry leaves. We previously reported that edible plant antioxidant activity, which inhibits human LDL oxidation induced by copper ion, can be determined on the basis of oxidation lag time and represented as epigallocatechin 3-gallate (EGCG) equivalents (Katsube et al., 2004). It is possible to estimate LDL antioxidant activity as a numerical value and to compare antioxidant potentials among such plants. The purpose of the present study was to estimate LDL antioxidant activity of mulberry leaves, identify the antioxidant compounds, and investigate compound levels.

2. Materials and methods

2.1. Materials

Mulberry (*Morus alba* L.) leaves were harvested in Sakurae, Shimane Prefecture, Japan, in June, 2004. The mulberry leaves were lyophilized and ground to powder using a vibrating sample mill (Heiko Seisakusho, Ltd., Tokyo, Japan). Diaion HP20 (Mitsubishi Chemical Co., Tokyo, Japan) was used for column chromatography. EGCG, rutin, quercetin, ethanol, β-glucosidase, and $CuSO_4$ were obtained from Wako Chemicals Ltd. (Osaka, Japan). Isoquercitrin, quercetin 3-(6-acetylglucoside), and astragalin were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). All solvents used for chromatographic isolation were of analytical grade and purchased from Wako Chemicals.

2.2. Extraction solvent (crude extract)

Two grammes of dried powdered mulberry leaves was extracted with 20 ml of various concentrations of aqueous ethanol solution (0%, 20%, 40%, 60%, 70%, 80%, 100%, v/v) by incubation for 3 h. Each extract was separated by centrifugation (13,000*g*, 10 min), the solution removed, and the residue resuspended with 20 ml of the same solvent and again separated by centrifugation. The two resulting solutions were then combined and made up to 50 ml with the same solvent. Each of these samples was then filtered for HPLC analysis, using a disposable syringe and a 0.45 μm filter. Flavonol compounds of the extract were analyzed by a quantitative HPLC system (LaChrom, Hitachi, Ltd., Tokyo, Japan), using a C18 12μ ST column (4.6 × 250 mm) (Amersham Biosciences Co., Piscataway, USA); solvent, 0–30 min, acetonitrile/0.1% formic acid (18:82), 30–42 min, linear gradient of acetonitrile/0.1% formic acid (18:82)–acetonitrile/0.1% formic acid (50:50), 42–54 min, acetonitrile/0.1% formic acid (50:50). We found that different ethanol/water extraction solutions yielded varying results and that 60% ethanol was the most effective extraction solvent (Fig. 1). We thus chose the 60% ethanol aqueous solution as the extraction solvent for crude extract sample analysis.

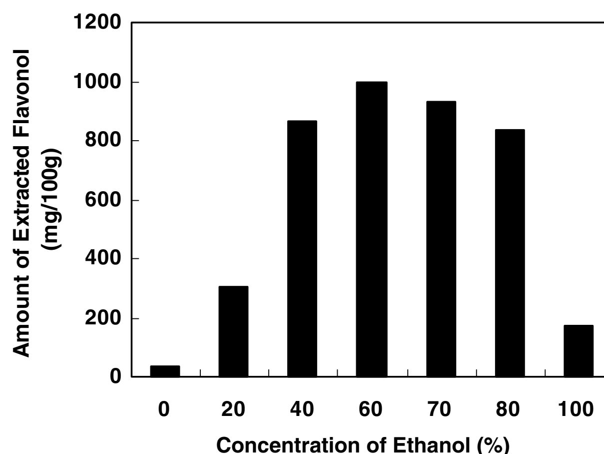

Fig. 1. Extraction solvent for HPLC analysis. Mulberry leaves were extracted with various concentrations of aqueous ethanol solution, and amounts of flavonol glycosides in each extract were measured by HPLC.

2.3. LDL oxidation assay

Venous blood was obtained from fasting, healthy adult human volunteers and dispersed into a tube containing EDTA (final concentration 0.1%) and plasma was immediately separated by centrifugation (1700*g*, 10 min, 4 °C). The plasma was then transferred to centrifuge tubes and centrifuged (20 h, 40,000*g*, 4 °C) by preparative ultracentrifugation using a Beckman ultracentrifuge L-60 (Beckman, Palo Alto, USA) equipped with an SW-40Ti rotor. After separation of the VLDL fraction on top and the next lower transparent part, the residual portion was transferred to a new tube and its volume was calculated by weighing. The density was then adjusted to 1.063 g/ml by adding solid potassium bromide and the resulting sample was centrifuged (20 h, 40,000*g*, 4 °C). The LDL fraction on top was then collected by aspiration and stored at −80 °C until used. The purity of this LDL fraction was verified by 2–16%

polyacrylamide gradient gel electrophoresis. The LDL was desalted using a Centricon-3 (Amicon, Inc, Beverly, USA) apparatus. Protein concentration in the LDL was determined using a Protein Assay Rapid kit (Wako Chemicals Ltd.). An LDL oxidation assay was performed as previously described (Katsube et al., 2004). Briefly, after preincubation with the water-diluted 60% ethanol crude extract from mulberry leaves for 5 min, reaction was initiated by adding 5 μM $CuSO_4$ to the LDL (20 μg of protein/ml) mixture in phosphate buffered saline (pH 7.4) at 37 °C; the total volume was 120 μl. Formation of conjugated dienes was monitored continuously at 234 nm for 7 h using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan) equipped with a 16-position automated sample changer. We analyzed oxidation kinetics on the basis of oxidation lag time, defined as the interval between initiation of oxidation and the intercept of the tangent for slope of the absorbance curve during the propagation phase. EGCG was used as a positive control for estimation of antioxidant activity of mulberry leaves. Antioxidant activity of 60% ethanol solution crude extract from mulberry leaves was calculated as EGCG-equivalents per 1 g of sample (μmol/g) by assuming that antioxidant elements in the samples were only EGCG. Three extractions of mulberry leaves were done, and antioxidant activity was measured for each extraction solution and represented as average ± SD.

2.4. Validation of assay

The kinetics of lipid oxidation in LDL monitored at 234 nm are characterized by three parameters: length of lag phase, maximum velocity of diene production, and maximum amount of oxidation products (Pinchuk & Lichtenberg, 2002). The mulberry-leaf extract prolonged oxidation lag time, but had no significant influences on the other two parameters, i.e., the maximum velocity of diene production and the maximum amount of oxidation products (data not shown). Lag time for the additional volume of the extracts occurred as a straight line in the moderate dilution range (Fig. 2). Lag time by concentration of copper ion was also observed as a straight line between 0.25 and 0.75 μM of EGCG, as we previously reported (Katsube et al., 2004). Thus, LDL antioxidant activity of the samples was measured quantitatively in the linear dilution range.

2.5. Isolation and identification of antioxidants

One hundred grammes of mulberry leaves was twice extracted with 1 l of 70% (v/v) ethanol solution. The resulting extract was concentrated to dryness under reduced pressure, and the residue was dissolved in water (100 ml) and partitioned with ethyl acetate (3 × 100 ml). The water layer was pumped into a Diaion HP20 column chromatograph (46 × 400 mm) and, after washing with water, methanol was added. Fractions of 50 ml each of eluate were collected and tested for LDL antioxidant activity (method described above). The greatest antioxidant activity was observed in the methanol-eluted fractions (Fig. 3). These methanol-eluted fractions were combined and concentrated to dryness under reduced pressure, and the residue was dissolved in water and filtered using a 0.45 μm filter. A portion of the water-dissolved fraction was loaded into a preparative

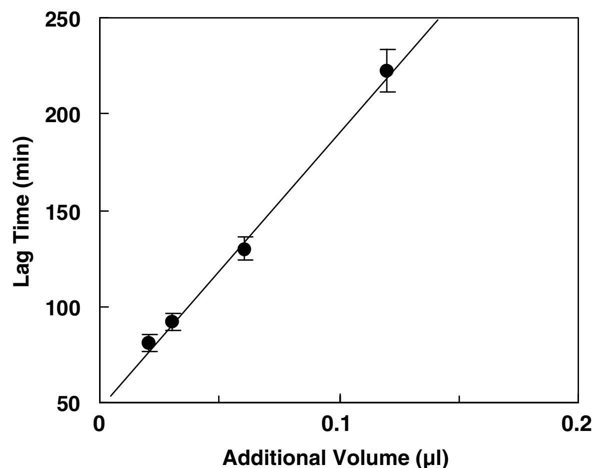

Fig. 2. Plot of lag time versus additional volume of extract from mulberry leaves. LDL (20 μg of protein/ml) was incubated with $CuSO_4$ (5.0 μM) with water-diluted 60% ethanol crude extract from mulberry leaves; lag time was determined by measurement of formation of conjugated dienes, and additional volume corresponded to the volume of prediluted extract; mean ± SD, $n = 3$.

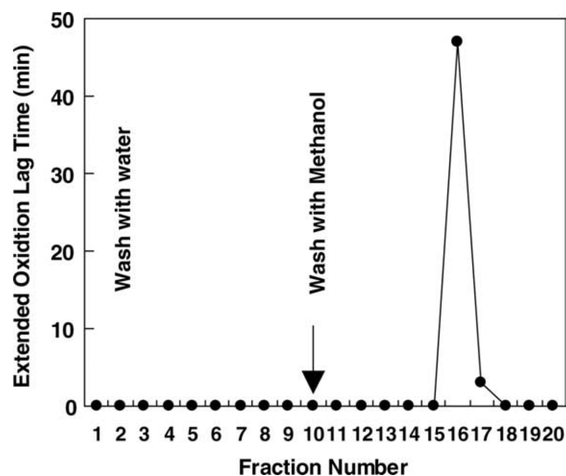

Fig. 3. Diaion HP20 column chromatography of antioxidants from mulberry leaves. Mulberry leaves were extracted with 70% aqueous ethanol solution. After partition between water and ethyl acetate, the water layer was loaded onto a Diaion HP20 column chromatograph. After eluting with water and methanol, LDL antioxidant activity of each fraction was measured. Activity was represented as extended oxidation lag time when LDL-added peak fractions compared to LDL only.

chromatography system (AKTA purifier, Amersham Biosciences Co.) using a C18 12μST column (4.6 × 250 mm); solvent, 0–8 min, acetonitrile/0.1% formic acid (12:88), 8–12 min, linear gradient of acetonitrile/0.1% formic acid (12:88)–acetonitrile/0.1% formic acid (20:80), 12–40 min, acetonitrile/0.1% formic acid (20:80); flow rate, 1.0 ml/min; UV detection, 370 nm. Another portion of the water-dissolved fraction was also loaded into an LC-MS (LCQ DECA XP, Thermo Electron Co., San Jose, USA), using an Inertsil ODS-80A column (4.6 × 150 mm) (GL Sciences Inc., Tokyo, Japan); solvent, acetonitrile/0.1% formic acid (20:80); flow rate, 1 ml/min; ion source, APCI. Each fraction recognized as a peak in the preparative chromatography was tested for LDL antioxidant activity; fraction numbers 17, 19 and 23 showed the greatest activity (Fig. 4). Fraction numbers 17 and 19 were identified as rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside), respectively, using LC-MS analysis and comparison with a commercially available reagent. The peak corresponding to fraction number 23 was further purified. This water-dissolved fraction was loaded serially into an AKTA purifier using a preparative ODS 80 Ts column (25 × 400 mm) (TOSOH Co., Tokyo, Japan); solvent, acetonitrile/0.1% formic acid (20:80); flow rate, 10 ml/min; UV detection, 370 nm. The peak fractions corresponding to fraction number 23 were pooled and concentrated to dryness under reduced pressure which produced 160 mg of a yellow powder, labelled as "Compound A".

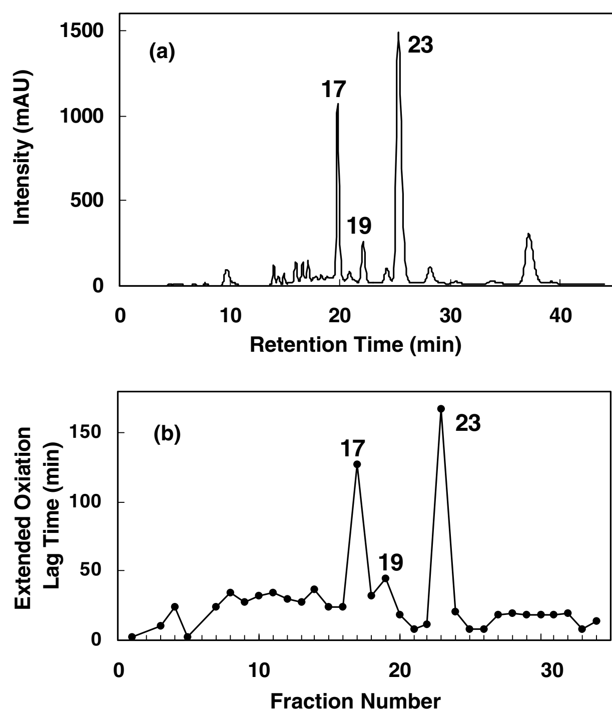

Fig. 4. Preparative HPLC of antioxidants from mulberry leaves. LDL antioxidant fractions obtained from Diaion HP20 column chromatography were loaded onto a preparative C18 column chromatograph. (a) Chromatogram monitoring at 370 nm. (b) LDL antioxidant activity of each fraction. Activities were represented as extended oxidation lag time when LDL-added peak fractions compared to LDL only. Numbered peaks indicate greatest antioxidant activity.

2.6. Hydrolysis reactions

Acid and enzymatic hydrolyses of Compound A were done. One hundred μg of Compound A was mixed with 2 N HCl in a total volume of 2 ml and incubated at 60 °C for 2 h. The reaction mixture was concentrated to dryness under reduced pressure, and the residue was dissolved in 50% (v/v) ethanol solution. One hundred μg of Compound A was also mixed with 0.1 mg of β-glucosidase in 100 mM acetate buffer (pH 4.1) in a total volume of 400 μl and incubated at 37 °C for 30 min. After the addition of 400 μl of 500 mM phosphate buffer (pH 2.5), 600 μl of ethyl acetate was added, and the sample was voltexed and centrifuged. The supernatant was concentrated to dryness under reduced pressure, and the residue was dissolved in 50% ethanol solution. The resulting flavonol was analyzed using TLC (RP-18 F, Merck, Darmstadt, Germany), and the sugar component was analyzed using a Carbo Pac PA1 column in a DX-500 liquid chromatograph (Dionex, Co., Sunnyvale, USA) as previously described (Katsube, Yamasaki, Iwamoto, & Oka, 2003).

2.7. APCI-MS and NMR

APCI-mass spectra were obtained using a LCQ DECA XP (Thermo Electron Co.): *m*/*z* 549 $[M-H]^-$.

^{1}H NMR (300 MHz, DMSO-d6): δ 7.52 (1H, d,$J = 2$ Hz, H-2′), 7.49 (1H, dd, $J = 2$ and 9 Hz, H-2′), 6.84 (1H, d, $J = 9$ Hz, H-5′), 6.40 (1H, d, $J = 2$ Hz, H-8), 6.20 (1H, d, $J = 2$ Hz, H-6), 5.38 (1H, d, $J = 7$ Hz, H-1 glucose), 4.21 (1H, d, $J = 11$ Hz, H-6A of glucose), 4.02 (1H, dd, $J = 11$ and 5 Hz, H-6B of glucose), 3.08 (2H, s, CH_2 malonyl).

^{13}C NMR (70 MHz, DMSO-d6): δ 177.2 (C4), 167.7 (CO malonyl), 166.6 (CO malonyl), 164.0 (C7), 161.1 (C5), 156.5 (C2), 156.2 (C9), 148.4 (C-4′), 144.7 (C-3′), 133.0 (C3), 121.3 (C-1′), 120.9 (C-6′), 116.1 (C-5′), 115.0 (C-2′), 103.8 (C-10), 101.0 (C-1 glucose), 98.6 (C-6), 93.4 (C-8), 76.1 (C-3 glucose), 73.8 (C-2 and C-5 glucose), 69.4 (C-4 glucose), 63.4 (C-6 glucose), 41.2 (CH_2 malonyl).

2.8. Quantitative determination of flavonols in mulberry leaves

Flavonol compounds from mulberry leaves were extracted by suspending 100 mg of mulberry leaves in a dry powder form in 10 ml of 60% (v/v) ethanol aqueous solution and stirring with a magnetic stir-bar for 3 h at room temperature. After centrifugation at 13,000*g* for

10 min, the extracted solution was filtered through a 0.45 μm filter. The amounts of flavonol compounds of the extract were analyzed by quantitative HPLC, as described above. The quercetin 3-(6-malonylglucoside) used as a standard was purified from mulberry leaves as described above.

3. Results

3.1. LDL antioxidant activity of mulberry leaves (crude extract)

The LDL antioxidant activity of mulberry leaves was assessed by using the 60%-ethanol extract and estimated at 58.3 ± 0.4 μmol of EGCG equivalent/g of dry weight, an average of the three separate extractions.

3.2. Antioxidant flavonols from mulberry leaves

Results of Diaion HP20 chromatography showed that the greatest LDL antioxidant activity occurred in the methanol-eluted fractions (Fig. 3). The antioxidant fractions were further evaluated by preparative ODS column chromatography, and three compounds showed the strongest antioxidant activities (Fig. 4). Two of these were identified as rutin (quercetin 3-rutinoside) (fraction 17) and isoquercitrin (quercetin 3-glucoside) (fraction 19) by LC-MS analysis and comparison with the authentic reagents (data not shown).

The third compound (Compound A), which showed the greatest LDL antioxidant activity (fraction 23), was reduced by evaporation to a yellow powder, and acid hydrolysis of Compound A detected quercetin and glucose, while treatment with β-glucosidase produced no hydrolyzed products, indicating that this was not a quercetin β-glucoside. ^{1}H NMR and ^{13}C NMR data of the compound were identical to that previously reported for quercetin 3-(6-malonyl)-glucoside (Ferreres, Gil, Castaner, & Tomas-Barberan, 1997). We confirmed this by APCI-MS in which a molecular ion at *m*/*z* 549 $[M-H]^{-}$ was observed, corresponding to quercetin monoglucoside acylated with malonic acid. In addition, fragments consistent with the sequential loss of the malonyl residue (*m*/*z* 463) and glucosyl residue (*m*/*z* 301) were also observed, confirming that this was quercetin 3-(6-malonyl)-glucoside.

3.3. HPLC analysis of antioxidant flavonols in mulberry leaves

Measurement of the amounts of LDL antioxidant flavonol glycosides in the mulberry leaves was done by HPLC analysis (Table 1). Quercetin 3-(6-malonylglucoside) was the most abundant flavonol (900 mg /100 g of dry weight). Rutin (573 mg/100 g of dry weight) was also abundant, followed by quercetin 3-(6-malonylglucoside). The amount of isoquercitrin (194 mg/100 g of dry weight) was one-fifth that of quercetin 3-(6-malonylglucoside). The amount of astragalin was low (31 mg/100 g of dry weight), and acetylated forms of isoquercitrin and astragalin were not found.

Table 1
Amounts of antioxidant flavonol glycosides in mulberry leaves

Compound	Amount[a] (mg/100 g of dry weight)
Rutin	573 ± 86
Isoquercitrin	194 ± 26
Quercetin 3-(6-malonylglucoside)	900 ± 146
Astragalin	31 ± 5

[a] Data represent means ± standard deviations for three separate measurements.

4. Discussion

In a prior study, we measured previously uninvestigated antioxidant characteristics of certain edible plants by LDL oxidation assay, and made a comparison analysis with DPPH radical scavenging assay and Folin–Ciocalteu assay results (Katsube et al., 2004). It has been suggested that measurement of LDL antioxidant activity is more physiopathologically important and informative for screening atherosclerosis-prevention antioxidant activity than other methods, such as Folin–Ciocalteu assay or DPPH radical scavenging assay; our prior study revealed high levels of LDL antioxidant activity in plant products for which activity levels have been underestimated by the latter two types of assays. Doi et al. (2000) reported that 1-butanol extract of mulberry leaves inhibits oxidative modification of rabbit and human LDL, but this extract's level of activity, as compared to that of other plants, is unknown. In our present study, the mulberry-leaf extract showed high LDL antioxidant activity (58.3 μmol of EGCG equivalents/g of dry weight) relative to that of other edible plants (Katsube et al., 2004). Here, we identified three quercetin glycosides as major LDL antioxidants in the mulberry-leaf extracts: quercetin 3-(6-malonylglucoside), rutin and isoquercitrin. Onogi et al. (1993) noted four flavonol glycosides in mulberry leaves: isoquercitrin and astragalin as major flavonoids, and their acetylated forms as minor flavonoids. In the present study, the most abundant flavonol glycoside was quercetin 3-(6-malonylglucoside) (900 mg/100 g of dried leaf), the greatest contributor to antioxidant activity in mulberry leaves (Fig. 4). Quercetin is one of the most abundant flavonoids in human diets. Quercetin has been recovered in rat plasma as sulfate, glucuronide, and sulfoglucuronide conjugates after intragastric administration of quercetin aglycone

(da Silva, Piskula, Yamamoto, Moon, & Terao, 1998) and these quercetin conjugates protect LDL from oxidation induced by copper ion (Yamamoto, Moon, Tsushida, Nagao, & Terao, 1999). Hayek et al. (1997) reported that dietary consumption of quercetin aglycone, by apolipoprotein E-deficient mice, attenuates the development of atherosclerotic lesions, and prolongs the lag phase for conjugated diene formation of LDL isolated from plasma, induced by copper ion. In plant foods, quercetin occurs mainly bound to various sugars via a β-glycosidic link. Quercetin glucosides are absorbed more easily than aglycone (Hollman, de Vries, van Leeuwen, Mengelers, & Katan, 1995) possibly with the participation in that mechanism of lactase phlorizen hydrolase (LPH) or sodium-dependent glucose transporter (SGLT1) (Sesink, Arts, Faassen-Peters, & Hollman, 2003). As the bioavailability of quercetin malonylglucoside is unknown, it is important to investigate its metabolism to clarify the effect of dietary consumption of mulberry leaves.

A reason for the higher levels of quercetin 3-(6-malonylglucoside) in our study may be due to the pre-treatment method of the mulberry leaves. Heat-treatment of flavonoid glycoside malonate solution (80 °C for 16 h) converts most of the flavonoid glycoside malonates into their glycoside form (Lin et al., 2000), an indication that the malonyl ester bond is easily broken by heat. Here, we used lyophilization for pre-treatment of the mulberry leaves. While quercetin 3-(6-malonylglucoside) has been isolated from plants such as lettuce (Ferreres et al., 1997), red clover (Lin et al., 2000), horseradish tree (Bennett et al., 2003), *Corchorus olitorius* L. (Azuma et al., 1999), ours is the first report of its existence in mulberry leaves.

The mulberry leaf is a promising dietary source of quercetin, one of the most powerful antioxidants (Vinson, Dabbagh, Serry, & Jang, 1995), due to its relatively high content of that compound (260 mg as aglycone/100 g of fresh weight in our results), compared to the white onion (48–56 mg/100 g of fresh weight) and red onion (40–100 mg/100 g of fresh weight), other known sources of quercetin (Arabbi, Genovese, & Lajolo, 2004).

Generally, ethanol or methanol solutions containing some water, particularly those ranging from 40% to 80% ethanol or methanol, are more efficient in the extraction of polyphenolic compounds than pure water, ethanol or methanol (Suzuki et al., 2002). In our previous study, 52 kinds of edible plants were extracted using 70% aqueous ethanol solution for mass screening to evaluate the LDL antioxidant activity of the extracts (Katsube et al., 2004). In the present study, a 60% ethanol solution proved to be the most efficient in extracting flavonol glycosides from the mulberry leaves (Fig. 1). As the polarities of antioxidant components from individual samples are likely to vary, the choice of extraction solvents is critical.

In conclusion, the mulberry-leaf extract showed relatively high LDL antioxidant activity, and quercetin 3-(6-malonylglucoside) and rutin were the predominant antioxidants in the mulberry leaf. We are presently conducting studies on the absorption, metabolism, and antioxidant functions in vivo of mulberry leaves and quercetin 3-(6-malonylglucoside) in our laboratory.

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