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Rapid communication

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.

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.

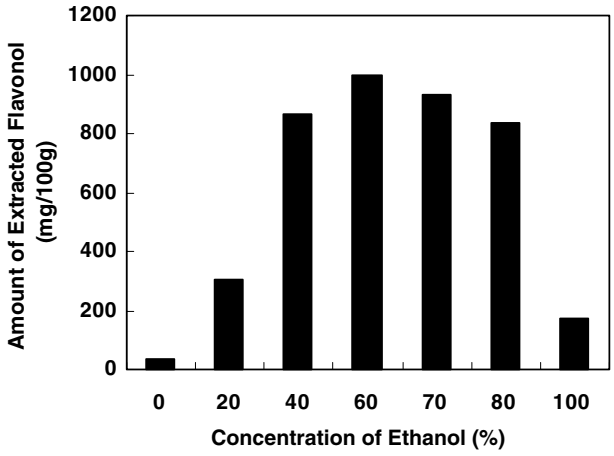

Fig. 1. Extraction solvent for HPLC analysis. Mulberry leaves were extracted with various concentrations of aqueous ethanol solution, and amounts of flavonol glycosides in each extract were measured by HPLC.

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%