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RESEARCH NOTE

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Phenolic Antioxidants Isolated from Mulberry Leaves

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Abstract In this study, the crude methanol extract of mulberry leaves was fractionated with chloroform, ethyl acetate, *n*-butanol, and water, successively. The antioxidant activities of the fractions were examined with the 2-deoxyribose oxidation and linoleic acid peroxidation methods. The ethyl acetate fraction showed the strongest antioxidant activity. From it we isolated chlorogenic acid, caffeic acid, quercetin 3-*O*-β-D-glucopyranoside, and kaempferol 3-*O*-β-D-glucopyranoside with preparatory octadecyl silane-high performance liquid chromatography (ODS-HPLC), and identified the compounds by nuclear magnetic resonance (NMR) and fast atom bombardment mass (FAB-MS) analyses. Overall, quercetin 3-*O*-β-D-glucopyranoside showed the strongest antioxidant activity by both the 2-deoxyribose oxidation and rat liver microsome peroxidation methods.

Keywords: quercetin 3-*O*-β-D-glucopyranoside, chlorogenic acid, mulberry leave, antioxidant activity

Introduction

Since the sericulture industry has been gradually decreasing in Korea, the cultivation of mulberry (*Morus alba* L.) leaves is being encouraged for their use as a health material rather than for silkworm feed. Presently, mulberry leaves are used as a healthful supplement in certain food products like noodles, cakes, and tea. Mulberry leaves, containing numerous nutritional and functional compounds, have exhibited antihyperglycemic activity in diabetic mice (1). In addition, some active compounds, including flavonoids (2-4) and 1-deoxy-nojirimycin (5), are reported to be associated with pharmacological effects. Doi *et al.* (6) isolated two novel prenyl flavanes from the butanol fraction of mulberry leaves that have strong activities related to atherosclerosis prevention. Recently, Lee *et al.* (7) also reported that Korean mulberry fruits contain anthocyanins and flavonoids. Meanwhile, lipid peroxidation by reactive oxygen species (ROS) is regarded as a main cause of food quality deterioration, in addition to diverse degenerative diseases such as diabetes, atherosclerosis, inflammation, and cancer (8, 9). Therefore, natural antioxidants and scavengers of ROS from various plant resources are being continuously studied for developing both functional health materials and therapeutic drug materials. In the course of searching for active antioxidants from Korean agricultural plant resources, we discovered the strong antioxidant effects of mulberry leaves. Hence, we have isolated and identified some of the phenolic antioxidants occurring in mulberry leaves.

Materials and Methods

Materials and chemicals The mulberry leaves for this study were collected at the farm of Kyungpook National University in Daegu, Korea, and were then dried in the shade. 2-Deoxyribose, ferric chloride, ammonium thiocyanate, linoleic acid, nordihydroguaiaretic acid (NDGA), butylated hydroxylated anisole (BHA), and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents were of analytical grade (Duksan Pure Chemical Co., Ltd., Ansan, Korea), with the exception of some that were HPLC grade (E. Merck Co., Darmstadt, Germany). The nuclear magnetic resonance (NMR) solvents were purchased from Sigma Chemical Co.

Isolation of phenolic antioxidants The dried mulberry leaves (250 g) were extracted with methanol for 12 hr, filtered, and concentrated *in vacuo*. The 10% methanol slurry of crude extract (57.5 g) was further successively extracted with chloroform, ethyl acetate, *n*-butanol, and water. The ethyl acetate fraction (6.9 g) was isolated and purified with preparative high performance liquid chromatography (HPLC, LC-10A; Shimadzu Co., Kyoto, Japan) using a Develosil octadecyl silane (ODS)-5 column (250×10 mm i.d., Normura Chemical Co., Ltd., Seito, Japan), with a gradient elution from water to 100% methanol at a flow rate of 2.0 mL/min, and monitored at 254 and 280 nm.

Instrumental analyses for identification UV-VIS absorption spectra were obtained with a spectrophotometer (UV 1601PC; Schimadzu Co.). ^{1}H NMR and ^{13}C NMR spectra were obtained by a NMR spectrometer (Unity Plus 500; Varian, Palo Alto, CA, USA) in CD_3OD containing TMS as an internal standard. Fast atom bombardment mass (FAB-MS) spectra were obtained using a mass spectrometer (JMS-DX 705L; Jeol Co., Tokyo, Japan) with glycerol as the mounting matrix.

Hydroxyl radical scavenging activity The hydroxyl radical scavenging activities of the solvent fractions and isolated compounds were examined by the 2-deoxyribose oxidation method (10).

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Linoleic acid peroxidation After a flask containing test solution and 2.88 mL of 2.51% linoleic acid in 80% EtOH was incubated at 40°C, the degree of hydroperoxide production was measured by the ferric thiocyanate method (11).

Rat liver microsome peroxidation test The microsomes were prepared from the livers of male Sprague-Dawly rats (12), and lipid peroxidation was induced by H_2O_2-$FeSO_4$ and measured by the TBA method (13, 14).

Statistical treatments All of the tests were examined in triplicate, and the values are exhibited as mean ± standard deviation; both of the values were obtained using Microsoft Excel (Microsoft Office XP Professional; Korea Microsoft Co., Seoul, Korea).

Results and Discussion

The antioxidant activities of the solvent fractions We examined the antioxidant activities of each solvent fraction by the 2-deoxyribose method and the linoleic acid peroxidation method. The results are shown in Fig. 1. The antioxidant activity of the ethyl acetate fraction by the 2-deoxyribose method was above 80%, comparable to that of BHA at the test solution concentration of 10 ppm. Moreover, the linoleic acid peroxidation method also showed that the ethyl acetate fraction's antioxidant activity was stronger than that of the other fractions. These results suggest that the ethyl acetate fraction from mulberry leaves might have a large quantity of diverse natural antioxidants such as polyphenolic compounds.

The isolation and identification of the active compounds The active compounds of the ethyl acetate fraction were obtained by preparatory ODS-HPLC, and their chemical structures were identified by ^1H, ^{13}C NMR, and FAB-MS analyses. The instrumental data are described as follows:

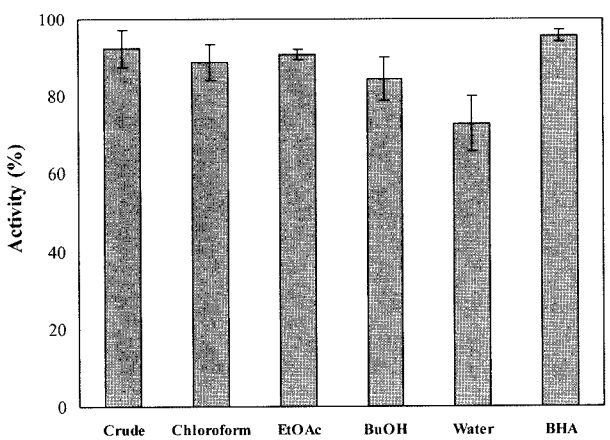

Fig. 1. The antioxidant activities of mulberry leaf solvent fractions by the 2-deoxyribose oxidation method. The test concentration was 10 ppm.

Compound 1 (5-Caffeoyl quinic acid, 22.4 mg). FAB-MS, *m/z* 355 (M+H); ^1H NMR (in CD_3OD) δ 7.55 (1H, d, 15.6 Hz, H7'), 7.04 (1H, d, 1.8 Hz, H2'), 6.94 (1H, dd, 8.2 Hz, 2.2 Hz, H6'), 6.75 (1H, d, 8.3 Hz, H5'), 6.25 (1H, d, 15.6 Hz, H8'), 5.34 (1H, m, H-5), 4.15 (1H, m, H-3), 3.70 (1H, m, H-2), 2.23 (1H, m, H-2ax), 2.15 (2H, m, H-2eq, H-6eq), and 2.01 (1H, m, H-6ax); ^{13}C NMR (in CD_3OD) δ176.99 (C7), 168.65 (C9'), 149.55 (C4'), 147.06 (C7'), 146.78 (C3'), 126.77 (C1'), 122.96 (C6'), 116.46 (C8'), 115.24 (C5'), 115.17 (C2'), 76.08 (C1), 71.87 (C2), 70.09 (C3), 65.7 (C5), 39.78 (C6), and 38.99 (C4).

Compound 2 (3,4-Dihydroxy cinnamic acid, 20.9 mg). FAB-MS, *m/z* 181 (M+H); ^1H NMR (in D_2O) δ 7.51 (1H, d, 15.6 Hz, H7), 7.03 (1H, d, 1.8 Hz, H2), 6.92 (1H, dd, 8.2 Hz, 2.2 Hz, H6), 6.78 (1H, d, 8.3 Hz, H5), and 6.21 (1H, d, 15.6Hz, H8); ^{13}C NMR (in CD_3OD) δ171.06 (C9), 149.51 (C4), 146.86 (C3), 147.09 (C7), 127.85 (C1), 122.89 (C6), 115.56 (C8), 116.53 (C5), and 115.13 (C2).

Compound 3 (Quercetin 3-*O*-β-D-glucopyranose, 75.6 mg). FAB-MS, *m/z* 465 (M+H); ^1H NMR (in CD_3OD) 7.71 (1H, s, H-2'), 7.57 (1H, d, 8.1 Hz, H-6'), 6.86 (1H, d, 8.1 Hz, H-5'), 6.37 (1H, d, 2.0 Hz, H-8), 6.18 (1H, d, 2.0 Hz, H-6), 5.24 (1H, d, 6.9 Hz, H-1"), and 3.74-3.30 (glucose H); ^{13}C NMR (in CD_3OD) δ179.40 (C4), 166.52 (C7), 162.98 (C5), 158.94 (C2), 158.48 (C9), 149.87 (C4'), 145.90 (C3'), 135.59 (C3), 123.18 (C6'), 123.03 (C1'), 117.52 (C2'), 115.99 (C5'), 105.52 (C10), 104.34 (C1"), 100.05 (C6), 94.82 (C8), 78.37 (C5"), 78.08 (C3"), 75.69 (C2"), 71.18 (C4"), and 62.51 (C6").

Compound 4 (Kaempferol 3-*O*-β-D-glucopyranose, astragalin, 42.3 mg). FAB-MS, *m/z* 449 (M+H); ^1H NMR (in CD_3OD) 8.06 (2H, d, 8.5 Hz, H-2',6'), 6.85 (2H, d, 8.5 Hz, H-3',5'), 6.42 (1H, d, 2.0 Hz, H-8), 6.19 (1H, d, 2.0 Hz, H-6), 5.45 (1H, d, 8.0 Hz, H-1"), and 3.71-3.20 (glucose H); ^{13}C NMR (in CD_3OD) δ179.42 (C4), 166.01 (C7), 164.02 (C5), 161.63 (C4'), 159.11 (C9), 158.55 (C2), 135.41 (C3), 132.20 (C2', 6'), 122.82 (C1'), 115.13 (C3', 5'), 105.71 (C10), 103.33 (C1"), 99.91 (C6), 94.83 (C8), 78.32 (C5"), 77.12 (C3"), 75.80 (C2"), 71.33 (C4"), and 62.61 (C6").

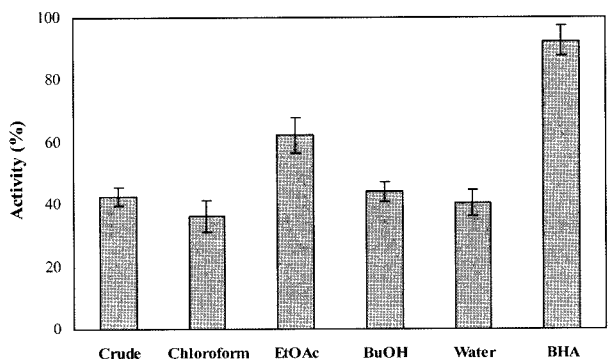

Fig. 2. The antioxidant activities of mulberry leaf solvent fractions by the linoleic acid peroxidation method. The test concentration was 10 ppm.

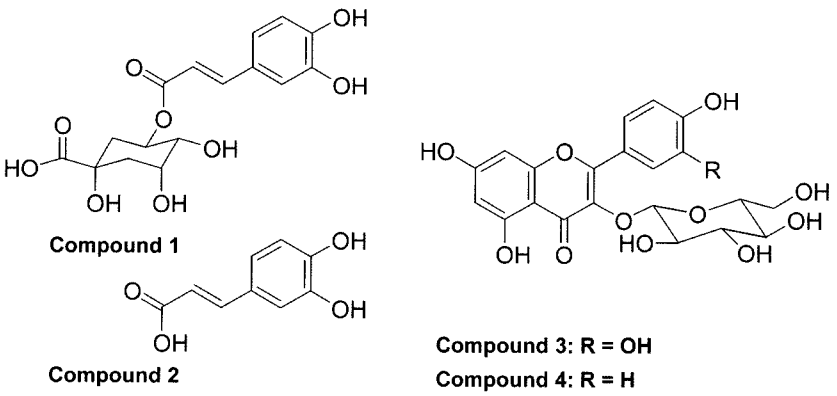

Fig. 3. The chemical structures of the phenolic compounds from mulberry leaves.

The chemical structures of the compounds are shown in Fig. 3. Compound 2 showed 3 chemical shift values, at δ 7.03, 6.92, and 6.78, respectively, signifying a benzene ring proton. Two doublets at δ7.51 and 6.21 with a coupling constant of 15.6 Hz were assumed to be the *trans*-olefinic protons (10) of the cinnamic acid moiety. The ^{13}C NMR spectra revealed that this compound might be composed of 9 carbons containing one carbonyl group at δ171.06. In the FAB-MS spectrum this compound exhibited major ion peaks at *m/z* 181 $(MH)^+$, 164 $(M\text{-}OH)^+$, and 147 $(M\text{-}COO)^+$. Therefore, we identified compound 2 as 3,4-dihydroxy cinnamic acid. The ^1H-NMR spectrum of compound 1 was very similar to compound 2, except for upfield chemical shifts under δ5.34. Furthermore, the molecular weight of 354 in the FAB-MS analysis implied this compound might contain a quinic acid moiety. Therefore, it was identified as 5-caffeoyl quinic acid, comparing with the data of the references (4, 15). The ^{13}C NMR chemical shift values of compounds 3 and 4, with molecular weights of 464 and 448, respectively, were compared with those of Markham *et al.* (16) and identified as quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) and kaempferol 3-*O*-β-D-glucopyranoside (astragalin), respectively. Kurioka and Yamazaki (17) isolated several cocoon flavonol glycosides and ascertained that isoquercetin and astragalin are major flavonol glycosides in the leaves of mullberry. On the other hand, Katsube *et al.* (18) reported that quercetin 3-(6-malonylglucoside) is a major flavonoid in mulberry leaves. In addition, 5 flavonoids, including quercetin and isoquercitin, have been isolated from mulberry fruits (7).

The antioxidant activities of the isolated compounds The antioxidant activities of the 4 isolated compounds by the 2-deoxyribose oxidation and rat liver microsome peroxidation methods are exhibited in Table 1. With the exception of compound 4 (kaempferol 3-*O*-β-D-glucopyranoside), the other 3 isolated compounds exhibited slightly higher antioxidant activities than that of the ethyl acetate fraction in the 2-deoxyribose oxidation test. On the other hand, compound 1 and 2 exhibited lower activities than compound 4 in the rat liver microsome peroxidation test. The discrepancy between these assays may be due to differences in the experimental conditions between the two methods, such as the reaction rate to the substrate, solubility, and reaction temperature. Compound 3 (quercetin 3-*O*-β-D-glucopyranoside) showed the highest activity for both the lipid peroxidation inhibition and 2-deoxyribose oxidation methods. Doi *et al.* (19) reported that isoquercitrin, the major compound in the butanol fraction of mulberry leaves, showed strong inhibition activity toward low density lipoprotein (LDL) oxidation, and the dietary consumption of mulberry leaves containing quercetin and quercetin 3-(6-malonylglucoside) decreased LDL's susceptibility to oxidative modification and atheromatous lesions (20). Overall, it is suggested that mulberry leaves, having phenolic antioxidants and strong antioxidant activities, could be used as a natural antioxidant material.

Table 1. Antioxidant activities of the phenolic compounds isolated from mulberry leaves

Compounds	Inhibition (%)	
	2-Deoxyribose oxidation	Rat liver microsome peroxidation
Compound **1**	92.0±3.0[1)]	27.1±1.9
Compound **2**	90.3±4.0	4.6±3.5
Compound **3**	96.7±2.6	44.1±2.7
Compound **4**	78.4±3.9	36.1±3.9
EtOAc fraction	90.7±1.3	31.7±4.3
BHA	95.3±1.7	62.5±2.7

[1)]Values are the mean±SD of triplicate tests; The test concentration was 10 ppm.

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