Two Flavonoids from the Leaves of *Morus alba* Induce Differentiation of the Human Promyelocytic Leukemia (HL-60) Cell Line

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Two flavonoids, quercetin-3-O-β-D-glucopyranoside (1) and quercetin-3,7-di-O-β-D-glucopyranoside (2), were isolated from the leaves of *Morus alba* (Moraceae). These two flavonoids exerted a significant inhibitory effect on the growth of the human promyelocytic leukemia cell line (HL-60) at the concentration of 2×10^{-5} M.

Compound 2 also induced differentiation of the HL-60 cell line to express CD 66b and CD 14 antigens. These flavonoids exhibited significant free radical scavenging effects on 1,1-diphenyl-2-picryl-hydrazyl radical.

Key words mulberry leaf; flavonoid; differentiation

Mulberry (*Morus alba* L.) leaves containing many nutritional compounds are considered the best food for silkworms. It has been reported that the extracts from mulberry leaves demonstrate a potent antihyperglycemic activity in diabetic mice. 1,2 N-Containing sugars, 3 plant hormone 4 and moracitin 5 have also been isolated from mulberry leaves. Many phenolic compounds have been identified from the root bark of the mulberry tree. 6 We isolated nine flavonoids from the 85% methanol extracts of the leaves of *M. alba*. Also, antioxidative activities of the flavonoids of *M. alba* have been reported. 7–9 Hirano et al. have identified 28 naturally occurring and synthetic flavonoids as novel antileukemic compounds with potent cytostatic activity and low toxicity against normal cells. 8 Also, the antiproliferative action of flavonoids appears to be specific to leukemia cells rather than normal lymphocytes, because they are less suppressive on normal lymphocyte blastogenesis. Several reports have recently shown that leukemia cells are able to differentiate in response to various treatments. Also, these reports have suggested that oxygen free radicals might be involved in the mechanism of cell differentiation. 9,10

The purpose of the present study was to determine whether the two flavonoids isolated from mulberry leaves could induce the differentiation of leukemic cells. We have chosen the HL-60 cell line, derived from a patient with acute promyelocytic leukemia, because different studies have reported that these cells can be induced to terminally differentiate to morphologically mature granulocytes by incubation with a wide variety of compounds. 11–15 On the basis of these concepts, induction of the differentiation of HL-60 cells by two flavonoids isolated from mulberry leaves was examined in vitro assay systems, i.e., cytotoxic assay using a dye exclusion test and the induction of differentiation of HL-60 cells using analysis of a cell surface antigen using fluorescence-activated cell sorter (FACS). This study could lead to the better understanding of the relationship between oxygen free radical scavengers and cell differentiation.

MATERIALS AND METHODS

Instrumentation and General Techniques UV spectra were obtained on an Uncial UV/Visible (VIS) spectrophotometer. IR spectra were recorded with a Jasco FT/IR 5300 spectrometer, and 1H- and 13C-NMR spectra were run on a Bruker AMX 500 spectrometer. FABMS spectra were measured on a VG 70-VSEQ mass spectrometer with a direct inlet system using PEG600/glycerol as a matrix. TLC and column chromatography were carried out on Merck precoated silica gel F254 plates and Si gel 60 (Merck, 70–230 mesh) or Sephadex LH 20 (Sigma, 25–100 μm). All other chemicals and solvents were of analytical grade and used without further purification.

Plant Materials Mulberry (*Morus alba* L.) leaves were collected at Suwon, Kyunggi Province, Korea in June, 1997. The voucher specimen is deposited in the herbarium of the Department of National Sericulture and Entomology, National Institute Agriculture Science Technology (NIAST), Rural Development Administration (RDA). The leaves were harvested, cleaned, freeze-dried and ground into fine powder in a mill (Tecator Cemotec 1090 sample mill, Hoganas, Sweden). The material passed through an 80-mesh sieve was retained, sealed in a glass bottle and stored at 4°C until use.

Extraction and Isolation Dry powdered leaves of *M. alba* (2 kg) were extracted with a mixture of water and methanol (15:85) under a sonicator (5×5 l, 25°C) for 1 h and then concentrated under a vacuum. The resulted MeOH extract (287 g) was suspended in water (200 ml), and was partitioned with CH₂Cl₂ (500 ml×5) and BuOH (400 ml×5), successively to give CH₂Cl₂ (53 g), BuOH (43 g) and H₂O (166 g) fractions. The BuOH extract (43 g) was subjected to Dianion HP-20 column chromatography with H₂O (3500 ml), 50% EtOH (2000 ml) and EtOH (2000 ml), respectively. The 50% EtOH eluate (18 g) was chromatographed on a silica gel column (55×5 cm) and eluted with CH₂Cl₂-MeOH-H₂O [80:10:0 (2500 ml); 60:10:0 (2000 ml); 40:10:1 (1530 ml); 50:15:2 (2100 ml); 70:30:5 (3150 ml); 0:1:0 (3000 ml)] to give 12 fractions (A—L). Fraction F was subjected to Sephadex LH-20 chromatography with H₂O-MeOH (2:8, 400 ml) and produced 280 mg, a yield of 0.028%, of compound 1. Fraction K was subjected to Sephadex LH-20 chromatography with gradient H₂O-MeOH (1:0–9:0:1, each of 350 ml) and produced 25 mg, a yield of 0.001%, of compound 2.

Compound 1 (quercetin-3-O-β-D-glucopyranoside); yellow needles, mp 238—241°C. IR νmaxBr cm⁻¹: 3310 (OH), 1665 (C=O), 1607, 1507 (aromatic C=C). UV (MeOH) λmax: © 2000 Pharmaceutical Society of Japan
208, 257, 267 (sh), 359 nm. FABMS (m/z) 465 [MH]+. 1H-NMR (DMSO-d6, 500 MHz). δ: 6.19 (1H, d, J = 2.0 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8), 7.58 (1H, d, J = 2.0 Hz, H-2'), 6.84 (1H, d, J = 8.8 Hz, H-7), 7.57 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 5.45 (1H, d, J = 7.4 Hz, H-1'). 13C-NMR (DMSO-d6, 125 MHz). δ: 156.1 (C-2'), 133.2 (C-3'), 177.4 (C-4'), 161.2 (C-5'), 98.6 (C-6'), 164.1 (C-7'), 93.4 (C-8'), 156.3 (C-9'), 103.9 (C-10'), 121.5 (C-1'), 115.1 (C-2'), 148.4 (C-3'), 144.7 (C-4'), 116.1 (C-5'), 121.1 (C-6'), 100.8 (C-7'), 74.0 (C-2'), 76.4 (C-3'), 69.9 (C-4'), 77.5 (C-5'), 60.9 (C-6'). On acid hydrolysis, 1 gave quercetin and glucose.

Compound 2 (quercetin-3,7-di-O-β-D-glucopyranoside); yellow needles. IR νmax cm⁻¹: 3310 (OH), 1665 (C=O), UV (MeOH) λmax: 208, 256, 356 nm, UV (NaOAc) λmax: 259, 367 nm, UV (NaOAc+H3BO3) λmax: 261, 383 nm, UV (AcOH) λmax: 275, 434 nm, UV (AcOH+HCl) λmax: 270, 299 (sh) nm. FABMS (m/z) 627 [MH]+. 1H-NMR (DMSO-d6, 500 MHz). δ: 6.43 (1H, d, J = 2.0 Hz, H-6), 6.75 (1H, d, J = 2.0 Hz, H-8), 7.59 (1H, d, J = 2.0 Hz, H-2'), 6.65 (1H, d, J = 8.5 Hz, H-7), 5.78 (1H, d, J = 8.5, 2.0 Hz, H-6'), 5.48 (1H, d, J = 7.3 Hz, H-1'), 5.08 (1H, d, J = 7.3 Hz, H-1). 13C-NMR (DMSO-d6, 125 MHz). δ: 156.0 (C-2), 133.4 (C-3), 177.6 (C-4), 160.7 (C-5), 99.3 (C-6), 162.7 (C-7), 94.4 (C-8), 156.8 (C-9), 105.6 (C-10), 121.4 (C-1'), 115.2 (C-2'), 144.4 (C-3'), 148.8 (C-4'), 116.3 (C-5'), 120.4 (C-6'), 100.7 (C-7'), 74.2 (C-2'), 76.4 (C-3'), 69.9 (C-4'), 77.5 (C-5'), 60.8 (C-6'), 99.7 (C-1'), 73.0 (C-2'), 76.4 (C-3'), 69.6 (C-4'), 77.2 (C-5'), 60.6 (C-6'). On acid hydrolysis, 2 gave quercetin and glucose.

Acid Hydrolysis of Compounds Three milligrams of each compound was dissolved in 2 ml of 2 N HCl-MeOH (1:1) in a 10 ml round bottomed flask and heated at 100 °C for 1 h. Isolation of the sugar aglycones for further analysis, the reaction mixture was evaporated to half of the original volume to remove the MeOH, and were then extracted several times with EtOAc by vigorously shaking in a test tube. In each case, the aglycon was fractionated into the EtOAc layer and the sugar into the H2O layer. The aglycone portion was analyzed by analytical HPLC with an octadecyl silica (ODS) column (CH3CN-H2O, 1:1, 250×4.60 mm) and the sugar portion by analytical HPLC with a carbohydrate column (CH3CN-H2O, 4:1, 300×3.90 mm). The retention times were compared with those of authentic samples.

1,1-Diphenyl-2-pircylyhydrazyl (DPPH) Radical Scavenging Effect MeOH solutions (4 ml) of flavonoids at various concentrations (1—20 μg/ml) were added to a solution of DPPH in MeOH (1.5×10⁻⁴ m, 1 ml), and the reaction mixtures were shaken vigorously. After storing these mixtures for 30 min at room temperature, the remaining amounts of DPPH were determined by colorimetry at 520 nm.¹⁴ The radical scavenging activity of each flavonoid was expressed by the ratio of the lowering of the absorption of DPPH (%) relative to the absorption (100%) of the DPPH solution in the absence of flavonoids. Mean values were obtained from triplicate experiments.

In Vitro Lipid Peroxidation Test Rats were dissected, and the liver removed. The liver was homogenized in 5 ml of 0.9% NaCl and the homogenate was adjusted to a volume of 10 ml with 0.9% NaCl. Mixtures of liver homogenate (0.3 ml) and sample at various concentrations (0.1 ml) in an open test tube were incubated at 37 °C. 1.5 ml of 20% acetic acid and 1 ml of 1.2% thiobarbituric acid (TBA) solution were added. The test solution was heated for 30 min on a boiling water bath, then cooled at room temperature. The solution was centrifuged at 2500 rpm for 15 min, and the absorbance of the upper layer was measured at 532 nm.¹⁵

Cell Culture The human promyelocytic leukemia cell line, HL-60, was purchased from Korean Cell Line Bank (KCLB) and maintained in RPMI 1640 medium containing 10% fetal bovine serum (GibcoBRL) supplemented with 50 U/ml of penicillin G, 50 mg/ml of streptomycin and 125 μg/ml of amphotericin B (GibcoBRL). The leukemia cells were washed and resuspended with the above medium, and the resulting cell suspensions (2×10⁵ cells/ml) were placed in each well of a 24-well flat bottom plate. The cells were incubated for 96 h at 37 °C in 5% CO2/air. After the incubation, 5 μl of phosphate buffered (pH 7.4) solution was prepared by adding two flavonoids to give a final concentration of 2.0×10⁻⁵ M and 2.0×10⁻⁴ M. Five microliters of buffer was added into the control wells. The cells were incubated for another 96 h in the presence of each agent, then the cell number was evaluated by trypan blue exclusion with a haemocytometer.

Determination of Cell Viability The number or percentage of viable cells was determined by staining the cell populations with trypan blue.¹⁶ One part of 0.16% trypan blue dissolved in saline was added to one part of the cell suspension, and the numbers of unstained (viable) and stained (dead) cells were counted. After being stained with trypan blue, the cells were counted within 3 min.

Assays for Cellular Differentiation HL-60 cells (2×10⁵ cells/ml) were suspended with compounds at a final concentration of 2.0×10⁻⁴ M. After incubation for 6 d, cells were harvested and examined for the induction of differentiation by analysis of FACS. For analysis of cell surface antigens, cells were stained by direct immunofluorescent staining using fluorescein-isothiocyanate conjugated mouse antihuman CD 66b (Pharmingen) or CD 14 (Pharmingen), and R-phycocerythrin conjugated mouse antihuman CD 33 (Pharmingen). Control studies were performed with non-binding control mouse IgG1 isotype antibodies (Pharmingen). Analysis of fluorescence was performed on a fluorescence-activated cell sorter flow cytometer (Becton Dickinson : FACStarPLUS).

Statistical Analysis All data are expressed as the mean±S.D. The evaluation of statistical significance was determined by the one-way ANOVA test using a standard package (SAS) for microcomputers.

RESULTS AND DISCUSSION

The investigation of 85% methanol extracts from the leaves of M. alba yielded two flavonoids. The structures of compound 1 and 2 were determined by comparison of their mps, UV and NMR spectral data with those reported in the literature. Compound 1 was determined to be isoquercitrin, and 2 quercitin-3,7-di-O-β-D-glucopyranoside, respectively.¹⁷ The ortho-dihydroxyl group in the B-ring of compound 2 was confirmed on the basis of band I in the AlCl₃ and AlCl₃/HCl spectrum as well as the NaOAc/H₃BO₃ spectrum. The 1H-NMR spectrum of compound 2 was similar to that of isoquercitrin except for the presence of an additional glucose
CD 33/ 66b

CD 33/ 14

CD 66b - FTTC

CD 14 - FTTC

Fig. 1. FACS Analysis of Expression of CD66b and CD14 Antigens in HL-60 Cells by Compounds

unit $[H: 5.08 (d, J=7.3 \text{ Hz})$ for anomic proton signal]. This showed that compound 2 was a monoglucoside of isoquercitrin. Compared with isoquercitrin, the H-6 and H-8 signals of quercetin-3-$O$-(6'-$O$-acyetyl)-$\beta$-$d$-glucopyranoside were shifted downfield to 6.43 and 6.75 ppm, respectively. The glycosidation site was also deduced from $^{13}$C-NMR data. Only the C-3 and C-7 signals were shifted upfield, by 2.4 and 1.3 ppm, respectively, in comparison with quercetin. These changes confirmed that the quercetin was substituted at C-3 and C-7. The downfield shifts of C-2, C-4, C-6 and C-8 signals also supported this assignment. The coupling constant ($J=7.3 \text{ Hz}$) between H-1 glucose and H-2 glucose indicated that the linked sugars at both sites were $\beta$ configured. The pyranoside form of sugar was established on the basis of $^{13}$C-NMR spectral data. From the above data, compound 2 was determined to be quercetin-3,7-di-$O$-$\beta$-$d$-glucopyranoside.

Damage caused by the toxic effects of OH$^-$ radicals is often decreased by radical scavengers such as phenolic compounds. Flavonoids are also known to act as strong superoxide radical ($\cdot$O$_2^-$) scavengers and singlet oxygen ($^1$O$_2$) quenchers. To examine the radical scavenging effects of flavonoids, two flavonoids isolated from the leaves of Morus alba L. were tested by the DPPH radical (Table 1). The radical scavenging activities ($EC_{50}$) of the flavonoids isolated were 17.6 and 14.6 $\mu$g/ml, respectively. The antioxidative activities of the two compounds were not comparable to that of a strong antioxidant, $l$-ascorbic acid, but were better than other flavonoids. Also, the antioxidant activity of compounds was determined by measuring lipid peroxides using TBA.
TBA reacts with malondialdehyde (MDA), a product of lipid oxidation. Table 2 shows the effects of compounds isolated from the M. alba on lipid peroxidation of rat liver homogenate. Compound 2 inhibited the lipid peroxidation by 29.2% and 21.0% at concentrations of $10^{-4}$ and $10^{-2}$ mg/ml, respectively. However, compound 1 did not show any inhibitory activity of lipid peroxidation at a low concentration.

In previous sections, the inhibitory effects of flavonoids on free radical mediated processes based on their free radical scavenging properties have been discussed. In addition to direct radical scavenger activity, compound 2 inhibited the growth of HL-60, human acute promyelocytic leukemia cells. It seemed that the inhibitory effect of compound 2 on the growth of HL-60 was derived from the induction of differentiation. When several cell lines established from leukemic patients, such as HL-60, U937, and K562, are cultured with various compounds, they differentiate to phagocyte-like cells that can produce O$_2^-$. During differentiation, the protein components essential for O$_2^-$ generation are thought to be induced in these cells. There have been many reports on the induction of differentiation and apoptosis of HL-60 cells. HL-60 cells are induced to differentiate into granulocyte-like cells by dimethyl sulfoxide (DMSO) and retinoic acid (RA), or monocyte (macrophage)-like cells by 1, 25-dihydroxyvitamin D$_3$ and phorbol diesters. It has been reported that ascorbates, gallates or benzo(a)phenothiazines induce apoptosis.

To clarify the effects of the compounds on the proliferation of HL-60 cells in liquid and semi-solid cultures, HL-60 cells were cultured for 4 days in a medium containing two compounds. The effects of compounds on the growth of the HL-60 leukemic cells are summarized in Table 3. During the 96 h culture period, the cell numbers in the control increased an average of almost ten-fold. However, a treatment of 96 h with compound 1 ($2\times10^{-3}$ M) and 2 ($2\times10^{-4}$ M) produced a 51.4% and 57.1% inhibition of cell growth, respectively.

The induction of differentiation of HL-60 cells by compound 1 and 2 was assessed by the expression of cell surface antigens. Exposure of cells to $10^{-3}$ M of 2 for 6 days resulted in HL-60 cells expressing CD 66b and CD 14 antigens. The treatment of leukemic cells with compound 2 increased the percentage of HL-60 cells expressing CD 66b antigen from 1.7% in the control to 52.8%. Also, the portion of expressing CD 14 antigens was remarkably changed (Table 4). But compound 1 had no effect on HL-60 leukemic cell differentiation. These results showed HL-60 cells differentiating toward mature granulocytes and monocytes by compound 2. These results indicate that compound 2 is an interesting agent which has been shown to induce differentiation and inhibit the in vitro proliferation of human myeloid leukemic cells.

The present study indicates that the induction of differentiation by the flavonoid, quercetin, 3, 7-di-O-D-glucopyranoside, from isolated mulberry leaves, might be initiated by an oxygen free radical mediated reaction. To our knowledge, this is the first report to describe the induction of granulocytic and monocytic differentiation of HL-60 by flavonoids.

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REFERENCES