Mulberroside F Isolated from the Leaves of *Morus alba* Inhibits Melanin Biosynthesis

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The current study was carried out to investigate the *in vitro* effects of an 85% methanol extract of dried *Morus alba* leaves on melanin biosynthesis, which is closely related to hyperpigmentation. These extracts inhibited the tyrosinase activity that converts dopa to dopachrome in the biosynthetic process of melanin. Mulberroside F (moracin M-6, 3′-di-*O*-β-D-glucopyranoside), which was obtained after the bioactivity-guided fractionation of the extracts, showed inhibitory effects on tyrosinase activity and on the melanin formation of melan-a cells. This compound also exhibited superoxide scavenging activity that is involved in the protection against autooxidation. But its activity was low and was weaker than of kojic acid. These results suggest that mulberroside F isolated from mulberry leaves might be used as a skin whitening agent.

Key words *Morus alba*; Moraceae; mulberroside F; whitening agent

Recently, much attention has focused on the application of naturally occurring crude drugs in cosmetics. From this point of view, we examined crude drugs that originated from the Moraceae family. Mulberry (*Morus alba* L.) leaves containing many nutritional components are the best food for silkworms. Chen *et al.* reported that extracts from mulberry leaves have a potent antihyperglycemic activity in diabetic mice.[1] N-containing sugars,[2,3] plant hormone[4] and moracetin[5] have been isolated from mulberry leaves. Many phenolic compounds have been identified from the root bark of mulberry tree.[6] *Morus alba* L. also contains rutin,[7] isoquercitrin and astragalin.[8] Recently, the root bark of *Morus alba* has been shown to have a skin whitening effect. Oxyresveratrol was suggested to be an active ingredient of the plant for this effect. However, components with anti-hyperpigmentation activity from mulberry leaves have not been reported so far. In the present study, the inhibitory effects of compounds obtained from mulberry leaves on melanin biosynthesis were investigated.

MATERIALS AND METHODS

Instrumentation and General Techniques UV spectra were obtained with an Uncial UV/VIS spectrophotometer. IR spectra were recorded with a Jasco FT/IR 5300 spectrometer and ^{1}H- and ^{13}C-NMR spectra were run on a Bruker AMX 500 spectrometer. FAB-MS 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 was carried out on Merck precoated silica gel F_{254} plates and Si gel 60 (Merck, 70—230 mesh) or Sephadex LH-20 (Sigma, 25—100 μm). All other chemicals and solvents were 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 was deposited in the herbarium of the Department of National Sericulture and Entomology, NIAST, 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 that 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 *Morus alba* L. (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 resulting MeOH extract (287 g) was suspended in water (200 ml), and was partitioned with CH_2Cl_2 (500 ml×5) and BuOH (400 ml×5), successively to give CH_2Cl_2 (53 g), BuOH (43 g) and H_2O (166 g) fractions. The BuOH extract (43 g) was subjected to Diaion HP-20 column chromatography with H_2O (3500 ml), 50% EtOH (2000 ml) and EtOH (2000 ml), respectively. The 50% EtOH elute (18 g) was chromatographed on a silica gel column (55×5 cm) eluting with CH_2Cl_2–MeOH–H_2O [80 : 10 : 0, (2500 ml); 60 : 10 : 0 (2100 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 K was subjected to Sephadex LH-20 chromatography with gradient H_2O–MeOH (1 : 0→0 : 1, each of 350 ml), and produced residues of mixed compounds K-a, K-b and K-c. The mixed compound K-c was further chromatographed on a Sephadex LH-20 column eluting with H_2O–MeOH (3 : 7, 150 ml) and produced 13 mg, a yield of 0.001%, of compound I.

Compound I (Mulberroside F): $[\alpha]_D^{22}$ 75°, yellowish powder. mp 231—234 °C; IR ν_{max}^{KBr} (cm^{-1}): 3439 (OH), 1604, 1498 (C=C), 1062, 1016 (glycosidic C–O); UV (MeOH) λ_{max}: 210, 271 (sh), 313 nm; FAB-MS (*m*/*z*) 589.1 $[M+Na]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz, ppm): δ 7.45 (1H, d, J=8.0 Hz, H-4), 7.30 (1H, br d, J=2.0 Hz, H-7), 7.03 (1H, dd, J=8.0, 2.0 Hz, H-5), 7.00 (1H, s, H-3), 6.95, 7.10 (each 1H, br s, H-2′, H-6′), 6.56 (1H, t, J=2.0 Hz, H-4′), 4.93, 4.95 (each 1H, d, J=7 Hz), 3.30, 3.95 (12H, m); ^{13}C-NMR (DMSO-d_6, 500 MHz, ppm): δ 159.0 (C), 158.7 (C), 155.7 (C), 154.7 (C), 154.6 (C), 131.3 (C), 123.0 (C), 121.0 (CH), 113.6 (CH), 104.9 (CH), 103.9 (CH), 103.6 (CH), 101.9 (CH), 101.1 (CH), 99.1 (CH), 77.0 (CH), 76.6 (CH), 76.5 (CH), 73.2 (CH), 73.0 (CH), 69.8 (CH), 69.7 (CH), 60.7 (CH_2). Acid hydrolysis of compound I gave glucose.

Acid Hydrolysis of the Compound Three mg of com-

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pound I 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. To isolate the sugar and aglycon for further analysis, the reaction mixture was evaporated to half of its original volume to remove MeOH, and then extracted several times with EtOAc by shaking vigorously in a test tube. In each case, the aglycon was fractionated into the EtOAc layer and the sugar into the H_2O layer. The aglycon portion was analyzed by analytical HPLC with an octadecyl silane (ODS) column (CH_3CN–H_2O, 1 : 1, 250×4.60 mm) and the sugar portion by analytical HPLC with a carbohydrate column (CH_3CN–H_2O, 4 : 1, 300×3.90 mm). The retention times (5.57 min) were compared with those of authentic samples.

Assay of Mushroom Tyrosinase One mM of test substance was dissolved in MeOH : H_2O = 1 : 1.5 μl of *l*-tyrosine (1.5 mM), 40 μl of *l*-dopa (25 mM), 80 μl of 67 mM phosphate buffer (pH 6.8) and 40 μl of either the same buffer or test sample were added to a 96-well microplate, followed by mixing with 80 μl of mushroom tyrosinase (60 U). After incubation at 37 °C for 30 min, the amount of dopachrome in the reaction mixture was determined. Based on the optical density at 490 nm (OD_{490}), the inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity (IC_{50}). Kojic acid was used as a reference.

Assay of Mammalian Tyrosinase in Cultured HM3KO Human Melanoma Cell Line HM3KO cell lines were cultured in modified Eagles medium supplemented with 10% fetal calf serum. Tyrosinase activity using cultured HM3KO human melanoma cell homogenates was assayed according to the method of Pomerantz[9] with a slight modification. Briefly, cell pellets were sonicated in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% Triton X-100. After incubation in ice for 1 h, 25 μg of solubilizing protein was used for an enzyme assay. Each concentration of compounds was incubated in 0.5 ml of a reaction mixture consisting of 0.25 μM tyrosine, 5 μCi[^{3}H]tyrosine, 0.25 mM *l*-3,4-dihydroxyphenylalanine (DOPA) and 0.1 mM PMSF in 0.1 M sodium phosphate buffer (pH 6.8) at 37 °C for 1 h. After removing non-reacted [^{3}H] tyrosine through absorption into an activated charcoal, aliquots of supernatants were assayed for the release of ^3H_2O. The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity (IC_{50}). Kojic acid was used as a reference.

Super Oxide Scavenging Activity Super oxide scavenging was measured according to the methods of Oyanagui.[10] A 0.2 ml portion of reagent A solution (0.5 mM hypoxanthine, 10 mM hydroxylamine hydrochloride) and 0.8 ml of EDTA–phosphate buffered saline (PBS) (pH 8.2) solution containing the test substance were combined and preincubated at 37 °C for 10 min. Five mU/ml xanthine oxidase solution, 0.2 ml, was added to the above solution, and the mixture was incubated for 30 min. Reagent B (2 ml coloring reagent to a final concentration of 20 μM *N*-1-naphthyl ethylene diamine, 2 mM sulfanilic acid and 16.7% acetic acid) was added to the reaction mixture. The resulting mixture was allowed to stand for 30 min at room temperature, then the optical density (OD) was measured at 550 nm. It was expressed as an inhibitory percentage against the production of a superoxide anion from hypoxanthine oxidase. Kojic acid was used as a positive control.

Cell Line and Culture Procedures Melan-a cells were kindly donated by Dr. Byeonggon Lee at the Skin Research Institute, Pacific Co, Korea. Melan-a cells were grown in 10 ml of RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum (FBS), and 20 nM TPA. The cells were subcultured every 6 d. The cell suspension was poured into a 24-well plate (10^5 cells/ml) and the cells were allowed to completely adhere to the plate overnight. Then, 10 μl of each test sample and phenylthiourea (PTU) as a reference dissolved in mixture solvents (propylene glycol : ethyl alcohol : H_2O (50 : 30 : 20)) were added to the plate and incubated at 37 °C for 3 d in a CO_2 incubator with an atmosphere containing the same solution. The medium was renewed everyday.

Cell Viability Determination The percentage of viable cells was determined by staining the cell population with crystal violet. 200 μl of 0.1% crystal violet solution dissolved in 10% ethyl alcohol was added to the culture plates. After incubation for 5 min at room temperature, the plates were washed with PBS. After being shaken with 1 ml of ethyl alcohol for 10 min, the optical density at 590 nm of the resulting supernatant was measured by an ELISA reader (Molecular Devices 09090, U.S.A.).

Determination of Melanin Content in Melan-a cells After 3 d of incubation, the adherent cells were washed with PBS. In order to extract melanin from the adherent melan-a cells, 1 N NaOH solution was added to the cells and incubated at room temperature for 10 min. After centrifugation at 3000 rpm for 10 min, the optical density at 450 nm of the resulting supernatant was measured by an ELISA reader (Molecular Devices 09090, U.S.A.).

Statistical Analysis All data were expressed as the mean±S.D. The evaluation of statistical significance was performed by the one-way analysis of variance (ANOVA) test using a standard software package.

RESULTS

85% of the methanol extract of mulberry leaves inhibited tyrosinase activity. Bioassay-guided fractionation of the extract from the leaves was carried out. The extract was partitioned with CH_2Cl_2, BuOH and H_2O in succession. The active fraction, BuOH layer was further separated by silica gel column chromatography to afford 12 fractions (fr. A—fr. L). Fraction K retained the inhibitory activity (data not shown). Thus, fraction K was then subjected to column chromatography on Sephadex LH-20 to afford the compound I (13 mg). The structure of compound I was determined by comparison of its mps, UV, and NMR spectral data with those reported in the literature; Compound I was identified as a diglucoside of a 2-arylbenzofuran derivative. The molecular formula was determined by FAB-MS {(M+Na) *m/z* 589.15}. The ^{1}H-NMR spectrum of compound I disclosed the presence of a 2-arylbenzofuran moiety [7.45 (1H, d, J=8.0 Hz, H-4), 7.03 (1H. dd, J=8.0, 2.0 Hz, H-5), 7.00 (1H, s, H-3), 6.95. 7.10 (each 1H, br s, H-2′, H-6′), 6.56 (1H, t, J=2.0 Hz, H-4′)] and two glycosyl moieties [4.93, 4.95 (each 1H, d, J=7.0 Hz), 3.30—3.95 (12H, m)]. The 2D nuclear overhauser effect spectroscopy (NOESY) spectrum demonstrated a correlation between one of two anomeric protons at δ 4.93 ppm (Gl-H) and two aromatic protons at δ 6.56 (H-4′) and 7.10 ppm (H-

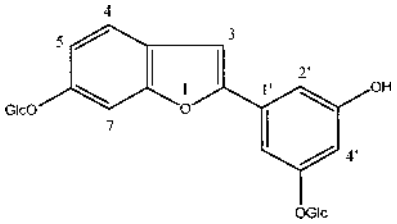

Fig. 1. Moracin M-6, 3′-di-*O*-*β*-D-glucopyranoside (Compound I)

Table 1. Inhibitory Effects on Tyrosinase Activity of Compound I (Mulberroside F) from Mulberry Leaves and Kojic Acid

Sample	Concentration (μg/ml)	Inhibition[a] (%)	IC_{50}[b] (μg/ml)
Compound I	0.01	35.5±1.1	0.29
	0.1	41.9±1.9	
	1	51.6±2.0	
Kojic acid	1	42.1±4.3	1.30
	3	70.0±3.8	
	10	98.6±0.8	

a) Tyrosinase was preincubated with test substances at 25 °C for 10 min prior to incubation with dopa for 30 min, and the absorbance was read at 490 nm. Each value represents the mean±S.D. of three experiments. *b*) 50% inhibitory concentration.

Table 2. Inhibitory Effects on Mammalian Tyrosinase Activity of Compound I (Mulberroside F) from Mulberry Leaves and Kojic Acid

Sample	Concentration (μg/ml)	Inhibition[a] (%)	IC_{50}[b] (μg/ml)
Compound I	10	43.4±0.1	68.3
	100	81.0±0.9	
	1000	94.9±2.2	
Kojic acid	10	21.8±0.4	58.5
	100	65.9±0.8	
	1000	96.0±8.0	

a) HM3KO cell lines were cultured in modified Eagles medium supplemented with 10% fetal calf serum. Each concentration of compounds was incubated in 0.5 ml of a reaction mixture consisting of 0.25 μM tyrosine, 5 μCi [^{3}H]tyrosine, 0.25 mM *l*-DOPA and 0.1 mM PMSF in 0.1 M sodium phosphate buffer (pH 6.8) at 37 °C for 1 h. After removing non-reacted [^{3}H] tyrosine through absorption into an activated charcoal, aliquots of supernatants were assayed for the release of [^3H_2O]. *b*) The inhibitory activity of the sample was expressed as the concentration which inhibits 50% of the enzyme activity (IC_{50}).

2′) as well as another anomeric proton at δ 4.95 ppm (Gl-H) and two aromatic protons at δ 7.03 (H-5) and 7.30 ppm (H-7) indicating that the two glycosyl residues are linked to different aromatic rings. Comparison of the ^{13}C-NMR spectrum of compound I with that of moracin M-3′-*O*-*β*-D-glucopyranoside[11] revealed that compound I is moracin M-6, 3′-di-*O*-*β*-D-glucopyranoside (Fig. 1). Thus, the structures of the glucosides in mulberry leaves were established by spectroscopic evidence.[12]

Compound I and Kojic acid as a positive control were examined for their tyrosinase inhibitory activity. As shown in Table 1, compound I (IC_{50}=0.29 μg/ml) and kojic acid (IC_{50}=1.30 μg/ml) exhibited strong inhibitory activity against tyrosinase. Tyrosinase activities in the HM3KO cell line treated with compound I also decreased in a manner correlating with mushroom tyrosinase. Mammalian tyrosinase activity for compound I was suppressed to 50% at a concentration of 68.3 μg/ml. Through additional experiments using a mammalian tyrosinase, we have provided more convincing evidence of the potential cosmetic relevance. Tyrosinase is the key enzyme involved in melanin biosynthesis, playing a role in tyrosinase converts tyrosine directly into dopaquinine, and *l*-dopa is only produced from the recycling of dopaquinone.[13] This process is a determinant of animal skin color, and is involved in local hyperpigmentations such as melanoma, ephelide and lentigo.[14—19]

Table 3. Superoxide Scavenging Activity of Compound I (Mulberroside F) Isolated from the Mulberry Leaves and Kojic Acid

Sample	Concentration (μg/ml)	Inhibition[a] (%)
Compound I	1	5.6±0.8
	10	7.2±0.8
	100	8.3±0.5
Kojic acid	1	8.6±0.9
	10	10.2±0.1
	100	12.3±0.7

a) Superoxide scavenging activity was expressed as percent inhibition against the production of superoxide anion from hypoxanthin–xanthin oxidase. Each value represents the mean±S.D. of three experiments. IC_{50} for authentic SOD as a positive control is 0.02 (U/ml).

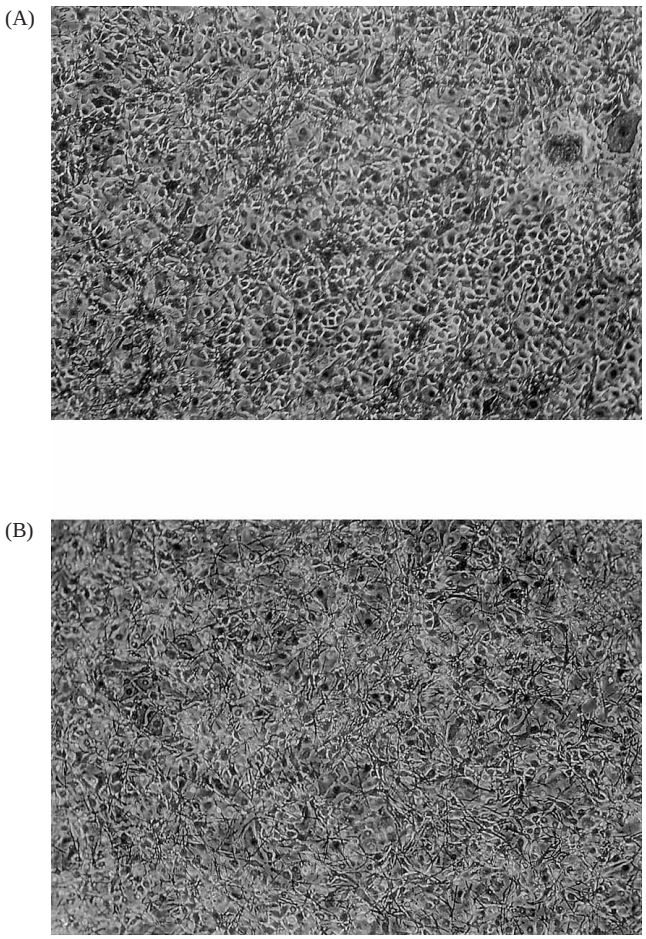

Fig. 2. Morphological Changes Induced by Compound I in Melan-a Cells

The cells were treated with or without compound I for 3 d. A: untreated control B: treatment with compound I at 10 μg/ml (magnification ×200).

Superoxide scavenging activity was expressed as the percentage inhibition of the production of superoxide anion from hypoxanthin–xanthine oxidase. The experiments in Table 3 denote superoxide-scavenging activity. As shown in Table 3, compound I showed superoxide-scavenging activity.

Table 4. Effects of Compound I (Mulberroside F), PTU and Koiic Acid on Cell Growth and Melanin Production of Melan-a Cells

Sample	Concentration (μg/ml)	Melanin production (%)	Cell viability (%)
PTU	0.1	80.4	95.1
	1	53.3	87.3
Kojic acid	0.1	96.7	94
	1	95.7	96.5
Compound I	0.1	80.5	89.1
	1	69.4	84.6

Melan-a cells were grown to confluence in 24-well culture plates overnight. Test samples were added to the plates and incubated for 3 d. The medium was renewed every day. After 3 d of incubation, cell viability and melanin content in melan-a cells were determined by ELISA. The results were reproduced with three different cultures.

At 100 μg/ml, inhibition was about 8.3%.

A typical morphology of melan-a cells is shown in Fig. 2. The pigmentation of a confluent monolayer is visible microscopically. All the cells contain black melanin pigment. Reduced pigmentation was seen in the cells treated with the highest concentrations of both compound I and PTU. The reduced production of melanin in melan-a cells was about 30.6% by compound I and 46.7% by PTU, respectively, at 1 μg/ml (Table 4). Compound I, and PTU, a positive control, inhibited the production of melanin in melan-a cells. However, Kojic acid had no effect on melanin production of melan-a cells at 1 μg/ml the highest concentration used here. Thus, PTU is more effective than compound I in inhibiting melanin production.

DISCUSSION

In this work, we demonstrated that the inhibitory effects of the crude extract of mulberry leaves is attributable to mulberroside F, moracin M-6, 3′-di-*O*-β-D-glucopyranoside. The superoxide scavenging activity and inhibitory effects on dopa oxidase activity of tyrosinase by compound were evaluated in order to screening of depigmenting agents. A compound with a concentration of 100 μg/ml exhibited 51.6% inhibition on the dopa oxidase activity of mushroom tyrosinase, where 50% inhibition (IC_{50}) was shown at the concentration of 0.29 μg/ml. This, compound exhibited about 4.5-fold more potent than kojic acid in inhibitory effect on the enzyme activity. Also, the compound showed a similar inhibitory effect on mammalian tyrosinase. We did not compare the effects of moracin (aglycon), monoglucoside, or diglucoside (mulberroside F, compound) on tyrosinase activity and melanin synthesis. Throughout the reference, moracin (aglycon) derived from the hydrolysis of the compound had a good inhibitory effect due to minimal steric hindrance by a bulky glucosyl moiety. The glucosyl moiety would not allow it to reach the target site of the enzyme.[20] Compared with kojic acid, the compound was more effective in inhibiting melanin production in melan-a cells. Therefore, it has been suggested to play a role in the depigmenting effect of skin. Due to the small quantity of the compound, We were unable to determine whether the compound was a noncompetitive or competitive inhibitor. Until now, chemicals such as tocopherol ferulate and kojic acid have been used as strong inhibitors of melanin formation in pigmented human melanoma cells.[21] Here we report that the inhibitory effect of the tyrosinase activity of mulberroside F isolated from natural products was stronger than that of kojic acid. Most importantly, the compound not only inhibited tyrosinase activity, but also showed superoxide scavenging activity and inhibited melanin production in cultured melan-a cells. As such, the compound might hold significant therapeutic value in the prevention or treatment of hyperpigmentation. Currently, hypopigmentative effects of this compound are being tested *in vivo* using animals exposed to UV radiation in our laboratory. Our efforts are also directed towards the synthesis of new compound I derivatives with stronger skin-whitening activity.

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