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The leaf extract of *Mallotus japonicus* and its major active constituent, rutin, suppressed on melanin production in murine B16F1 melanoma

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ABSTRACT

Objective: To find anti-melanogenesis materials used in whitening cosmetics.
Methods: The ethanolic leaf extract of *Mallotus japonicus* (*M. japonicus*) having an anti-melanogenesis activity was separated by a sephadex LH-20 chromatography. Each fraction was measured for its tyrosinase inhibitory activity together with its polyphenol content using the Folin–Ciocalteu method. The anti-melanogenesis activity of the active fractions was determined by the melanin content in the murine B16F1 melanoma. The active fractions were put together due to similar constituents, and then separated by high performance liquid chromatography using a C-18 ODS column. The major anti-melanogenesis compound was identified using ^{1}H and ^{13}C-NMR and liquid chromatography-mass spectrometry.
Results: The ethanolic leaf extract of *M. japonicus* showed an anti-tyrosinase activity with a high polyphenol content, resulting in suppression of melanin production in the B16F1 melanoma. The extract was separated and the active compound was identical as rutin based on the ^{1}H, ^{13}C-NMR and liquid chromatography-mass spectrometry analysis data. In addition, the rutin treatment with cells reduced the melanin content in a concentration dependent manner without any cell toxicity. The leaf extract of *M. japonicus* containing rutin would be useful in whitening cosmetics for protection from UV-light exposure to be limiting the accumulation of melanin in skin.
Conclusions: The leaf extract of *M. japonicus* and/or rutin isolated from the extract as a key whitening agent would be useful as a whitening cosmetic material for protecting against disorder skin due to melanin accumulation.

1. Introduction

Melanin is the major pigment of skin and hair color in mammals, which is synthesized by tyrosinase through tyrosine converted to 3,4-dihydroxyphenylalanine (DOPA) and catalyzed the oxidation of DOPA into DOPA quinones through a radical coupling pathway in melanocytes. Melanin plays a significant role in the protection against UV-irradiated skin damage, but excess exposure of the skin to sun-light causes to macula and pigmentation in the skin and leads to esthetic problems with depression. Free radicals, such as lipid radicals, hydroxyl radicals and lipid peroxidation, are produced in sun-light exposed skin with DOPA-oxidation [1–3]. Therefore, tropical-subtropical plants contain abundant antioxidants that are expected to protect the skin against the accumulation of melanin. Also, phenolic compounds, such as arbutin and kojic acid, have been developed as anti-melanogenesis agents, to inhibit the tyrosinase activity *in vitro* [4–6] and citrus extract containing certain flavonoids suppressed the down-regulation of tyrosinase, tyrosinase related protein (TRP)-1 and TRP-2 in melanocytes [7]. In this study, a preliminary evaluation was carried out as an index of the tyrosinase inhibitory activity and polyphenol content in botanical extracts, and the anti-mutagenesis activity in melanoma was continuously assessed. As a result, the ethanolic leaf extract of *Mallotus japonicus* (*M. japonicus*) was a candidate for whitening cosmetic materials. *M. japonicus* is widely distributed

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in the tropical and temperate areas of Asia and it has been used as a folk medicine, such as the bark for ulcers and cancer, and also its leaves for the treatment of boils. Several articles demonstrated scientific evidence that the constituent in *M. japonicus* showed a protective activity against the oxidative modification of proteins and lipids [8], anti-hepatotoxic protective effect on carbon tetrachloride-intoxicated hepatocytes [9] and the anti-inflammatory effect of the lipopolysaccharide-induced macrophage-like cell line due to suppression of the NO production with pro-inflammatory cytokine expression through inhibition of the nuclear factor-κB activation, and induction of prostaglandin E_2 [10]. This study shows the anti-melanogenesis activity of the leaf extract of *M. japonicus* and its main contributing compound will be potential whitening cosmetic materials for UV exposed skin disorders.

2. Materials and methods

2.1. Reagents

Gallic acid and L-3,4-dihydroxyphenylalanine (L-DOPA) were obtained from the Wako Pure Chemical Co. (Osaka, Japan). L-tyrosine and tyrosinase (2.5×10^5 units/mg-lyophilized from mushroom) was from the Sigma–Aldrich Co. LLC. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All others were of the highest analysis grade reagents.

2.2. Sample preparation

The leaf of *M. japonicus* was collected from Okinawa Island, Japan. The leaf was freeze-dried (EYELA TDU-1000, Tokyo Rikakiki Co., Ltd., Tokyo, Japan) and powdered using a mixer. The leaf powder (1 g) dissolved in 20 mL of ethanol was incubated for 24 h using a shaker (RECIPRO SHAKER SR-2S, Titec Co., Ltd., Aich, Japan) at room temperature. The leaf powder extract was filtered, then it was concentrated using a centrifugal thickener (Eyela Uni trap UT-1000, Tokyo Rikakiki Co., Ltd., Tokyo, Japan). The sample for an individual assay was dissolved in methanol (100 mg/mL), then filtered using a disk filter (0.45 μmol/L, Millipore, MA, USA).

2.3. Cell culture

B16F1 melanocytes (American Type Culture Collection) were cultured in Dulbecco's modified eagle medium (Gibco-BRL, Life Technologies, CA) medium (including 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO_2 atmosphere.

2.4. Cell viability

The cell viability due to treatment with a test sample was examined by an MTT assay as previously reported [11]. Briefly, the cells were seeded at a density of 5.0×10^5 cells/mL and cultured for 24 h with or without the test sample. After the culture, MTT (0.05%) was added to each well and incubated for 3 h, then the suspension was carefully removed. Extraction with dimethylsulfoxide (DMSO) (50 μL) was measured at 540 nm with a reference at 655 nm using a microplate reader (BIO-RAD Model 550, BIO-RAD, CA).

2.5. Melanin content in cells

The cells for precultured 24 h were treated with the extract or various concentrations of the test samples. The cells were cultured for 48 h and then exchanged with fresh medium. The cells were treated with 20 mmol/L of L-DOPA (100 μL) and incubated at 37 °C for 4 h. After the medium was removed, the melanin pigment was extracted with 1 mol/L NaOH (150 μL) and 1% triton X-100 (150 μL). The melanin content was determined at 450 nm using a microplate reader. Inhibition of the melanin production was indicated using the following equation:

Inhibition (%) = [1 − untreated sample cell/treated sample cell (control)] × 100

2.6. Tyrosinase inhibitory activity

The tyrosinase inhibitory activity was determined by reducing the melanin content in the presence of the test samples. The reaction mixture containing 0.3 mg/mL L-tyrosine (100 μL) in McIlvaine buffer (pH 6.8, 188 μL), with or without the test sample (2 μL), was incubated at 37 °C for 10 min. Tyrosinase (1 530 IU/mL, 10 μL) was then added to the reaction mixture and incubated at 37 °C for 30 min. The solution was measured at 450 nm and the tyrosinase inhibitory activity was determined by the following equation:

$$\text{Tyrosinase inhibitory activity (\%)} = [1 - (A_1 - A_2)/(B_1 - B_2)] \times 100$$

where A_1 is the complete system and A_2, B_1 and B_2 are for without the tyrosine, without the test sample, and without the test sample and tyrosine, respectively.

2.7. Polyphenol content

The total polyphenol content was measured using a microplate reader as previously reported using the Folin–Ciocalteu method [12]. Briefly, the solution containing the Folin–Ciocalteu phenol reagent (4.0%) (Merck Millipore, Darmstadt, Germany) and sodium carbonate (2.3%) with or without the test sample (0.1%) was incubated at 37 °C for 60 min, then the reaction mixture was measured at the absorbance of 655 nm using a microplate reader. The total polyphenol contents were expressed as μmol of gallic acid equivalent per g-leaf dry-weight.

2.8. Isolation of active compound

The ethanol extract (2 mL) was isolated by methanol using a Sephadex chromatograph (LH-20, 20–100 μm particle size, GE Healthcare Japan). Each fraction (40 mL) was collected. They were prepared at 100 mg/mL-MeOH, then their tyrosinase activity and polyphenol content were measured. The active fraction was separated using a C-18 ODS column (TSK-gel ODS-80TM, 300 mm × 21.5 mm inside diameter, Tosoh CO., Ltd.) by a high performance liquid chromatography (LC-20 AP, Shimazu Corporation) and monitored at 326 nm with the flow rate of 6.0 mL/min. The mobile phase consisting of a formic acid aqueous solution (0.5%) and acetonitrile was carried out by a

linear gradient to 2%, 45% and 100% for 15, 50 and 60 min, respectively.

2.9. Liquid chromatography-mass spectrometry (LC-MS) analysis

The active compound in the leaf extract of *M. japonicus* was determined by an LC-MS analysis. The LC-MS (Agilent 1200, Agilent Technologies) was carried out using a photodiode array detector and monitored in the operating wavelength range from 210 to 700 nm at the flow rate of 0.80 mL/min on a reversed-phase chromatographic column, YMC-Pack Pro C18 (100 × 4.6 mm inner diameter, 5 μm particle size, YMC Co., Ltd., Kyoto, Japan) at 40 °C. The mobile phase consisting of a formic acid aqueous solution (0.5%) and acetonitrile was used with a linear gradient. The initial isocratic step, with 0.5% formic acid, was a linear gradient to 2% acetonitrile for 15 min, 45% acetonitrile for 50 min, and the final gradient to 100% acetonitrile was carried out for 10 min. The mass spectra were measured under the following conditions: ESI positive ion mode, desolvation temperature (350 °C), desolvation pressure (35 psi), and desolvation gas flow (12.01 mL/min) (6120 Quadrupole LC-MS spectrometer, Agilent Technologies).

2.10. Nuclear magnetic resonance (NMR) analysis

The proton and carbon-13 spectra were recorded by a JNM-LA400 spectrometer (JEOL) and the chemical shifts were referenced to the solvent peaks (dH 2.49 and dC 39.5 in DMSO-*d*6).

2.11. Statistical analysis

Data were expressed as mean ± SD. Statistical significance was determined by a *t*-test. Significance at $P < 0.05$ was considered statistically significant.

3. Results

Various subtropical-tropical plants have been evaluated for anti-melanogenesis activity as an index of the relationship between the polyphenol content and inhibitory tyrosinase activity. As a result of the screening test, the ethanolic leaf extract of *M. japonicus* showed the high polyphenol content of (455.57 ± 25.15) μmol-gallic acid equivalent/g-dry weight with the high anti-tyrosinase activity (%) of 76.26 ± 4.45 (Table 1). Also, the treatment of the murine B16F1 melanoma by the extract reduced the melanin content of ca. 40%. The ethanol extract was eluted by 100% methanol using a sephadex LH-20 column and obtained 11 fractions. For each fraction, the polyphenol content and tyrosinase inhibitory activity were determined. As shown in Table 1, the fourth and fifth fractions indicated a high polyphenol content (μmol of gallic acid equivalent per g-leaf dry-weight) of 270.71 ± 25.01 and 254.79 ± 21.42, and the tyrosinase inhibitory activity (%) of 89.24 ± 4.29 and 83.29 ± 3.57, respectively. The LC-MS spectrum obtained from these fractions showed similar components (data not shown), thus both fractions were combined and its anti-melanogenesis activity was determined. The melanin content in cells treated with the active fraction was reduced in a dose dependent manner (Figure 1). The fraction was separated using a C-18 ODS column by high performance liquid chromatography, and the major compound was isolated from the active fraction. The ^{1}H and ^{13}C-NMR data were obtained from the compound as follows:

^{1}H-NMR (400 M Hz, DMSO-d_6, δ: 2.49), δ: 7.53 (1H, dd, J = 2.2, 8.7 Hz, H-6′), 7.52 (1H, brs, H-2′), 6.86 (1H, d, J = 8.1 Hz, H-5′), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.18 (1H, d, J = 1.7 Hz, H-6), 5.33 (1H, d, J = 7.6 Hz, H-1″), 4.37 (1H, H-1‴), 3.70 (1H, d, J = 10.5 Hz, H-6″), 3.2–3.4 (overlap, H-4″, H-2‴, H-3‴, 3.27 (H-6″), 3.26 (H-5″), 3.25 (H-5‴), 3.22 (H-3″), 3.21 (H-2″), 3.06 (1H, t, J = 18.8 Hz, H-4‴), 0.98 (3H, d, J = 6.1 Hz, H-6‴). ^{13}C-NMR (400 M Hz, DMSO-d_6, δ: 39.5), δ: 177.3 (C-4), 164.0 (C-7), 161.2 (C-9), 156.5 (C-5), 156.4 (C-2), 148.3 (C-4′), 144.7 (C-3′), 133.3 (C-3), 121.5 (C-6′), 121.1 (C-1′), 116.2 (C-2′), 115.2 (C-5′), 103.9 (C-10), 101.1 (C-1″), 100.7 (C-1‴), 98.6 (C-6), 93.5 (C-8), 76.4 (C-3″), 75.9 (C-5″), 74.0 (C-2″), 71.8 (C-4‴), 70.5 (C-3‴), 70.3 (C-2‴), 70.0 (C-4″), 68.2 (C-5‴), 66.9 (C-6″), 17.7 (C-6‴).

Table 1
Tyrosinase inhibitory activity and polyphenol content of each fraction obtained from *M. japonicus*.

Test sample	Polyphenol (μmol-gallic acid equivalent/g-dry weight)	Inhibition of tyrosinase activity (%)
Ethanol extract	455.57 ± 25.15	76.26 ± 4.45
Fraction number		
1	16.29 ± 1.58	19.93 ± 9.95
2	10.79 ± 2.10	31.68 ± 15.09
3	57.23 ± 1.92	43.08 ± 11.67
4	270.71 ± 25.01	89.24 ± 4.29
5	254.79 ± 21.42	83.29 ± 3.57
6	273.68 ± 32.50	38.96 ± 8.25
7	293.70 ± 16.49	45.10 ± 6.18
8	295.10 ± 24.29	37.50 ± 3.86
9	432.82 ± 54.40	23.12 ± 2.23
10	177.54 ± 11.91	35.27 ± 3.65
11	23.86 ± 1.54	0.56 ± 3.31

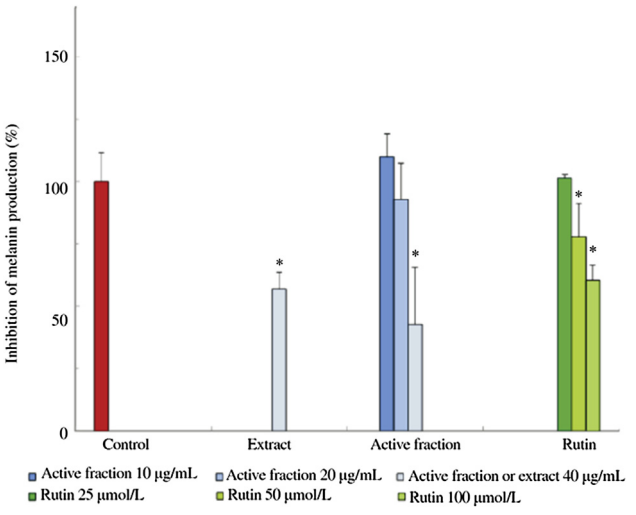

Figure 1. Anti-melanogenesis activity of the ethanolic leaf extract, active fraction and isolated compound from *M. japonicus*.
Cells treated with the extract or various concentrations of the test samples were cultured in B16F1 melanoma and the melanin content was determined as shown in the text. Data were expressed as mean ± SD and the significant difference for the control cells indicated at $^{*}P < 0.05$.

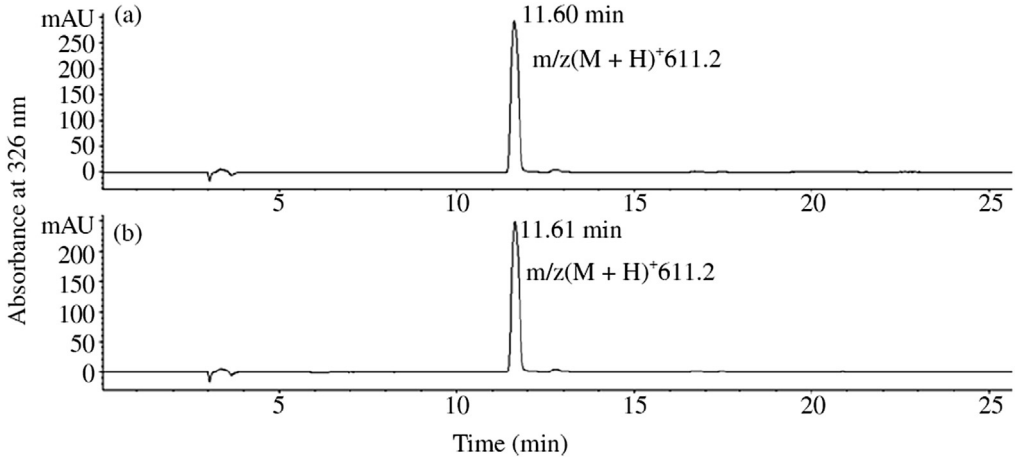

Figure 2. LC-MS chromatogram of rutin obtained from the ethanolic leaf extract of *M. japonicus*.
The detailed isolation procedures of rutin from the extract are described in the text. a: LC-MS chromatogram of purified rutin in the leaf extract of *M. japonicus*; b: LC-MS chromatogram of authentic rutin.

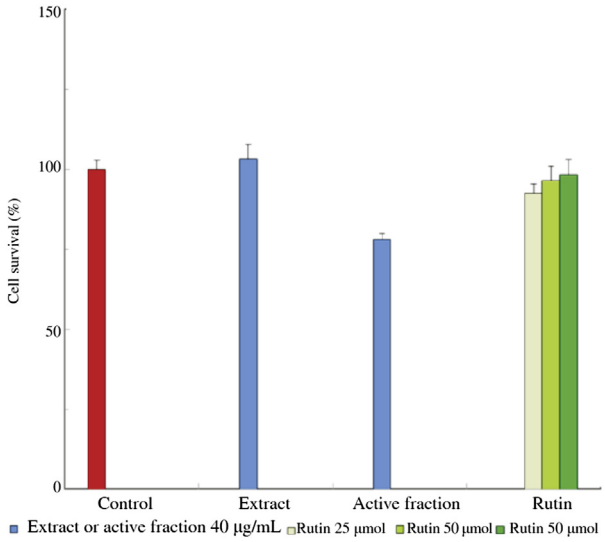

Figure 3. Cell survival of the ethanolic leaf extract, active fraction and isolated compound from the *M. japonicus* in B16F1 melanoma.
The cell viability due to treatment with a test sample was determined by an MTT assay as described in the text and the data were expressed as mean ± SD.

The NMR data were similar to that of the chemical structure of rutin. The LC-MS chromatogram of the compound is indicated in Figure 2. The molecular weight [m/z 611.2 $(M + H)^+$] and its retention time (11.60 min) were similar to the authentic rutin [611.2 $(M + H)^+$, retention time (11.61 min)] (Figures 2a and 2b). The compound was identical to rutin based on the NMR and LC-MS analytical data. The anti-melanogenesis activity of the compound was assessed, which significantly reduced the melanin content in the concentration range of 50–100 µmol/L (Figure 1). In addition, the cell cytotoxicity of the compound was not detected when used in the test concentrations, indicating that the anti-melanogenesis activity is due to the effect of the compound (Figure 3).

4. Discussion

Melanin is synthesized from tyrosine with tyrosinase and autoxidation in melanocytes. The excess accumulation of melanin in the skin often causes skin problems, such as freckles, chloasma and melasma. Therefore, pharmaceutical and cosmetic companies are exploring useful materials including whitening agents. However, the whitening agents used in cosmetics are prone to have some adverse reactions that may aggravate the appearance of the skin spots and damage the heart of the user. In fact, the whitening agent used by a Japanese cosmetic company induced vulgaris leukoderma on the skin of many users [13]. Therefore, safe cosmetics are required as well as its functions. Anti-melanogenesis as whitening agents are being developed by the cosmetic company. The anti-melanogenesis compounds used in cosmetics are generally phenolic compounds, such as kojic acid, arbutin and magnolignan which have been used by some Japanese cosmetic companies [4]. This study assessed the content of polyphenol in various Asian plants with an anti-tyrosinase activity, and the ethanolic leaf extract of *M. japonicus* was found to contain the highest polyphenol content with a tyrosinase inhibitory activity. Additionally, the extract showed an anti-melanogenesis activity with no cytotoxicity in melanoma cells, suggesting that the materials would allow their use in safe cosmetics. Through isolation experiments for determining the active compound, rutin was identified as the major active component in the leaf extract fraction. In a previous article, rutin in *M. japonicus* leaves was reported to contribute to the antioxidants for scavenging hydroxyl radicals and superoxide anion radicals [14]. Skin exposed to UV produces active oxygen species during the melanogenesis process, thus rutin could contribute to protecting against oxidative stress due to the UV irradiated skin by reducing the excess accumulation of melanin. The anti-melanogenesis activity of the quercetin derivatives including rutin from *Allium cepa* was reported in a previous report [14]. Many flavonols have been isolated from plants, and some flavonoids, such as dihydromorin, streppogenin and quercetin, were identified as tyrosinase inhibitors. The inhibitory mode of the flavonol inhibitors is typically competitive inhibition for the oxidation of L-DOPA by tyrosinase, and the 3-hydroxy-4-keto moiety of the flavonol structure acts as the key factor in copper chelation [15,16]. A structure similar to rutin would also reduce the melanin content due to the copper chelation of tyrosinase.

Tyrosinase is regulated by the microphthalmia-associated transcription factor (MITF), a master transcription factor in melanogenesis. TRP-1 and TRP-2 are major melanogenesis enzymes induced by MITF. In a previous study, it was shown

that citrus inhibited the tyrosinase, TRP-2 and MITF expressions. The authors determined flavonoids, such as hesperidin, rutin and narirutin, in the citrus extract and suggested that these compounds may lead to the downregulation of the TRP-2 and MITF expressions [7]. The leaf extract of *M. japonicus* containing rutin may also inhibit by a similar mechanism involving the downregulation of the tyrosinase-related proteins, TRP-1 and TRP-2, and/or their transcription factor, MITF.

Conflict of interest statement

We declare that we have no conflict of interest.

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