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Inhibitory effects of *Ramulus mori* extracts on melanogenesis

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Synopsis

To develop an active agent for skin whitening, the inhibitory effects of 285 plant extracts on tyrosinase activity were examined, and one plant extract having tyrosinase inhibition activity was chosen. *Ramulus mori* (young twigs of *Morus alba* L.) extracts showed inhibition activity in tyrosinase and melanin synthesis in B-16 melanoma cells.

To clarify the mechanism of its inhibition on melanogenesis, the effect of *R. mori* extracts on tyrosinase activity, synthesis, and gene expression was evaluated. *R. mori* extracts showed tyrosinase inhibition activity by competitive method, and there was no suppression of tyrosinase synthesis and gene expression. Further, to evaluate the inhibitory activity of *R. mori in vivo,* its effect on melanin production in UV-induced brown guinea pigs was examined, where a decrease of melanin production in the guinea pig model was observed. Also, *R. mori* extracts showed no toxicity in animal tests such as the acute toxicity test, the skin irritation test, the eye irritation test, the skin sensitization test, and the acute oral toxicity test, and no toxicity in the human skin irritation test.

A single compound from *R. mori* extracts was purified using various column chromatography and recrystallization, and its chemical structure was identifed using mass chromatography, IR spectroscopy, and NMR analysis. The chemical structure was that of 2,3′,4,5′-tetrahydroxystilbene(2-oxyresveratrol) and showed inhibition activity on tyrosinase (IC_{50} = 0.23 μg/ml). Also, *R. mori* extracts inhibited tyrosinase activity in a competitive manner (Ki = 1.5×10^{-6} M) when L-tyrosine was used as a substrate.

INTRODUCTION

It has been observed that the increase of melanin synthesis or uneven distribution can cause local pigmentation in the skin. Pigmentary disorders are caused by various factors, including UV radiation, inflammation, estrogens, and genetic disorders. Recently, the harmfulness of ultraviolet rays has increased due to the destruction of the ozone layer. Excessive exposure to UV radiation may cause post-inflammatory pigmentation (1). In East Asia, most women want to avoid uneven skin pigmentation. To satisfy this desire many cosmetic companies have been developing melanogenesis inhibitors and discov-

ering skin-whitening cosmetic preparations. In cosmetic preparations, inhibitors such as kojic acid, arbutin, ascorbic acid, and licorice extracts have been used as whitening ingredients (2).

Plant extracts having an inhibitory effect on melanogenesis may be a good choice for cosmetic purposes because of their relatively few side effects. Therefore, we screened 285 plant extracts for their inhibitory activity on tyrosinase (3). Among the plant extracts, *R. mori* extracts showed potent inhibition activity on tyrosinase and melanin synthesis but did not inhibit tyrosinase synthesis and gene expression by zymography and RT-PCR, respectively. *R. mori* extracts showed inhibition of pigmentation and no toxicity in animal tests.

Morus alba L. and other plants of the same genus have been used as antiphlogistics, diuretics, and expectorants in Chinese herbal medicine. *R. mori* (young twigs of *Morus alba* L.) is harvested in the early spring and used in folk remedies in Korea. Although the constituents of *Morus alba* L., such as flavonoids, coumarines, and stilbenes (4), have been studied by many investigators and isolated, few reports on the usage of cosmetic whitening ingredients have been published.

In this study, we purified and identified an active compound from the *R. mori* extract, 2,3′,4,5′-tetrahydroxystilbene(2-oxyresveratrol). A naturally occurring compound particularly found in *Morus alba,* it showed inhibition activity on tyrosinase (IC_{50} = 0.23 μg/ml). Also, it inhibited tyrosinase activity in a competitive manner (Ki = 1.5×10^{-6} M) when L-tyrosine was used as a substrate.

MATERIALS AND METHODS

PREPARATION OF PLANT EXTRACTS

We prepared the *R. mori* extracts for anti-melanogenic ingredients. *R. mori* was extracted by a mixture of ethyl alcohol and water (EtOH:H_2O = 70:30) and dried to powder. This powder was dissolved in 1,3 butylene glycol and used for this study.

TYROSINASE INHIBITION

For the assay, the test reaction mixture was prepared by adding 0.5 ml of *R. mori* extracts, to which 250 units of mushroom tyrosinase (Sigma, Saint Louis, MO) had been added, to 0.5 ml of L-tyrosine (0.1 mg/ml) or 0.5 ml of 50 mM sodium phosphate buffer (pH 6.8). After incubation for ten minutes at 37°C, we measured tyrosinase activity by absorbency at 475 nm. We determined the effect of the test sample on tyrosinase inhibition by IC_{50}, the concentration at which half the original tyrosinase activity is inhibited. We calculated the percent inhibition of tyrosinase activity as follows:

$$\% \text{ inhibition} = [(A - B)/A] \times 100$$

where A = absorbency at 475 nm without the test sample, and B = absorbency at 475 nm with the test sample.

INHIBITION OF MELANOGENESIS

B-16 melanoma cells were placed in a 25-ml T-flask at a density of 1×10^5 cells/flask and cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) containing 4.5 g/l of glucose, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) antibiotic-antimycotic (Gibco, Auckland, N.Z.). After 24 hours of cultivation, we replaced the medium with new DMEM medium containing *R. mori* extracts of various concentrations. After five days, we washed the cells with phosphate-buffered saline (PBS) and collected the cells by trypsinization and centrifugation. We separated melanin from the pellet of the cells using 5% (w/v) trichloroacetic acid, dissolved the melanin in 1N NaOH solution, and checked the melanin content by absorbency at 475 nm.

EVALUATION OF *R. MORI* EXTRACTS ON TYROSINE SYNTESIS IN B-16 MELANOMA CELLS (TYROSINASE ZYMOGRAPHY)

Analysis of tyrosinase synthesis was performed by the modified method of Imokawa and Mishima (5). Detergent-solubilized cell extracts were subjected to SDS gel electrophoresis as follows: Cell extracts (2 mg/ml protein) were electrophoresed on 10% (w/v) polyacrylamide gels. Total protein was measured by protein assay kit (Bio-Rad Laboratories, California). After electrophoresis, the gel was placed in renaturation buffer [50 mM Tris-HCl (pH 8.0) and 2.5% (v/v) triton X-100] at room temperature for one hour. The gel was then incubated in developing buffer [0.1 M sodium phosphate (pH 6.8), 0.2% (w/v) L-DOPA] at 37°C for four hours. Upon visualization of the tyrosinase bands, the gel was removed and dried, and the relative amount of tyrosinase band in each lane was quantified.

EVALUATION OF *R. MORI* EXTRACTS ON TYROSINASE GENE EXPRESSION IN B-16 MELANOMA CELLS (RT-PCR)

Total RNA was prepared using RNA PLUS™ (Quantum, Quevec, MW) from B-16 melanoma cells. Five micrograms per milliliter of total RNA was reverse transcribed by incubating the sample for one hour at 42°C in 25 µl of reaction mixture containing 200 U of MMLV (Moloney murine leukemia virus reverse transcriptase, Promega, Madison, WI); 1 µl of 100 pmol sequence specific primer dNTP (dATP, dCTP, dGTP, dTTP, Promega); and 1X buffer (50 mM Tris-Hcl, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DDT). Ten milliliters of RT reaction mixture was added to 40 µl of PCR mixture containing 1X PCR buffer (50 mM KCl, 10 mM, Tris-HCl (pH 9.0), 1.5 mM, $MgCl_2$, and 0.1% Triton X-100); 1 µl of 100 pmol forward and reverse primer; 5 µl of 2.5 mM each dNTP; 2 µl of 25 mM $MgCl_2$; and five units of Tag DNA polymerase (Promega). Amplification was performed at 33 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 50 sec with the Gene AMP PCR system 2400 (Perkin Elmer, Oak Brook, IL). Two microliters of loading dye were added to 10 µl of amplification products, and the mixture was analyzed by 2% Agarose (Sigma) gel electrophoresis.

EVALUATION OF *R. MORI* EXTRACTS ACTIVITY ON MELANOGENESIS IN ANIMAL TEST

R. mori extracts were dissolved at a final concentration, 1%, 5% (v/v) in dissolving solution (butylene glycol: H_2O = 50:50). This solution was topically applied to sepa-

rated areas on the back skin of guinea pigs (n = 9) for two days (200 μl application, twice/day) before UVB radiation, while the dissolving solution alone was applied to the other area as a control. These areas were then irradiated once with 1,350 mJ/cm^2 using a UVB lamp (Vilber Lourmat, Marine La Vallee, France). After two weeks, the UV-irradiated site was stained by the Fontana-Masson staining method.

SEPARATION AND IDENTIFICATION OF AN ACTIVE COMPOUND

Separation. We extracted dried young twigs with 70% ethanol solution, using a vacuum rotary evaporator to concentrate the extract to dryness. To isolate the tyrosinase inhibitor from ethanol extract, we purified the extract through solvent fractionation, silica column chromatography, and Prep-LC. The ethanol extracts were dissolved in ethyl acetate, and then the residue was crystallized from $CHCl_3$. We purified the solid using silica chromatography (Merck 200–400) and finally isolated the tyrosinase inhibitor.

Identification. We crystallized the isolated tyrosinase inhibitor from ether benzene to yield pale yellowish prisms. The compound was identified by infrared (IR) spectroscopy, mass chromatography, and nuclear magnetic resonance (NMR).

RESULTS

INHIBITION OF TYROSINASE ACTIVITY AND MELANIN SYNTHESIS

R. mori extracts were selected as potent tyrosinase inhibitors through our screening methods. We checked the extracts' inhibition activity on tyrosinase and melanin synthesis by changing the concentration (10, 20, 50, and 100 μg/ml). The extracts showed high tyrosinase inhibition activity. Melanin synthesis was also inhibited by *R. mori* extracts at a concentration of 50 μg/ml. The extracts showed no cytotoxicity. See Table I.

EFFECT OF *R. MORI* EXTRACTS ON TYROSINE SYNTHESIS AND GENE EXPRESSION

To examine the inhibitory mechanism of *R. mori* extracts on melanogenesis, we did the zymography for tyrosinase content in B-16 melanoma cells and RT-PCR for tyrosinase gene expression. Tyrosinase zymography showed that *R. mori* extracts at a concentration of 50–100 μg/ml did not lessen the tyrosinase synthesis (the band intensity does not change) (Figure 1). We also checked whether *R. mori* extracts inhibit the tyrosinase gene level by using RT-PCR. Figure 2 shows that the tyrosinase gene level was not changed by the treatment of *R. mori* extracts. These two results mean that *R. mori* extracts inhibit only tyrosinase activity, not tyrosinase synthesis and tyrosinase gene expression.

Table I
Inhibitory Effects of *R. mori* Extracts on Tyrosinase and Melanin Synthesis

R. mori extracts (μg/ml)	Tyrosinase inhibition (%)	Inhibition of melanin synthesis (%)
10	56.0	13.5
20	65.8	18.5
50	89.5	36.5
100	97.8	65.3

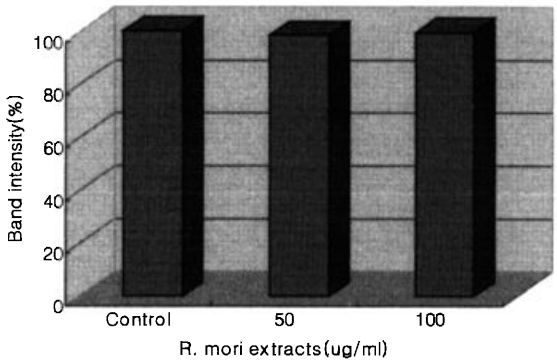

Figure 1. Effect of *R. mori* extracts on tyrosinase synthesis (band intensity).

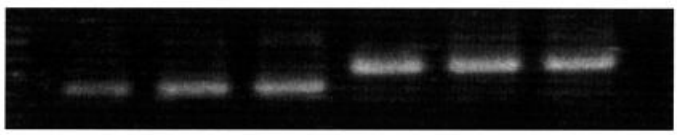

Figure 2. Effects of *R. mori* extracts on tyrosinase gene expression (RT-PCR). Lane 1: Tyr—control. Lane 2: Tyr—*R. mori* 50 μg/ml. Lane 3: Tyr—*R. mori* 100 μg/ml. Lane 4: Actin—control. Lane 5: Actin—*R. mori* 50 μg/ml. Lane 6: Actin—*R. mori* 100 μg/ml.

INHIBITORY EFFECT OF *R. MORI* EXTRACTS ON UVB-INDUCED PIGMENTATION OF BROWNISH GUINEA PIGS

R. mori extracts inhibited the pigmentation induced by UVB in brownish guinea pigs (Figure 3). In the histological comparisons, the melanin content produced by UV radiation in the basal layer of the epidermis (control) was increased as compared to the skin treated with *R. mori* extracts. These results showed that *R. mori* extracts had an inhibitory activity on UV-induced pigmentation in the brownish guinea pig model. *R. mori* extract induced no irritant signs such as redness in this study.

IDENTIFICATION OF AN ACTIVE COMPOUND IN *R. MORI* EXTRACTS AND EVALUATION OF A SINGLE COMPOUND ON MELANOGENESIS

We purified a single compound from *R. mori* extracts using column chromatography and Prep LC. (Figure 4). It was found that four compounds showed high inhibitory effects on tyrosinase activity (data not shown). From these compounds we isolated a single compound by recrystallization and identified its structure using mass chromatography, IR spectroscopy, and NMR analysis. The NMR data is shown in Figure 5. The compound was identified as 2,3′,4,5′-tetrahydroxystilbene(2-oxyresveratrol).

INHIBITION MECHANISM OF 2-OXYRESVERATROL ON TYROSINASE ACTIVITY

We checked the inhibitory effect of 2-oxyresveratrol on tyrosinase activity. It showed very high activity in tyrosinase inhibition (IC_{50} = 0.23 μg/ml). Significant inhibition in enzyme activity was shown by this compound in concentrations of more than 0.1 μg/ml. When L-tyrosine was used as a substrate, 2-oxyresveratrol decreased the Km value of tyrosinase but did not change the Vmax, and thus was a competitive inhibitor with a Ki value of 5×10^{-6} M (Figure 6).

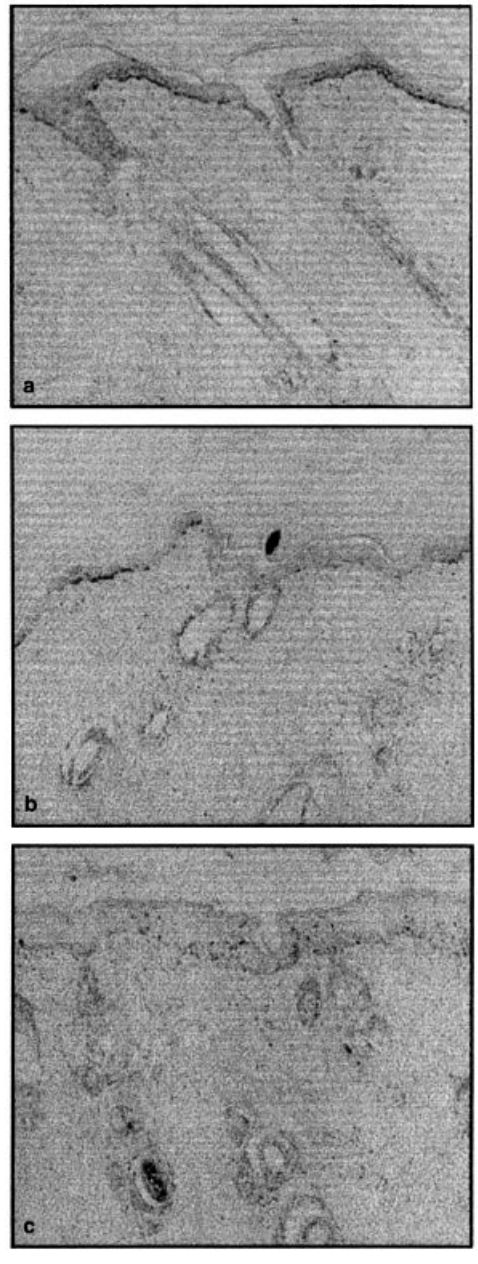

Figure 3. Effects of *R. mori* extracts on the inhibition of UV-induced pigmentation. (a) Placebo. (b) 1% (v/v) *R. mori*-extracts-treated skin. (c) 5% (v/v) *R. mori*-extracts-treated skin.

SAFETY TESTS OF *R. MORI* EXTRACTS

Acute toxicity test. We investigated the potential toxicity of the *R. mori* extracts. According to the CTFA guidelines, we assessed acute oral toxicity and acute dermal toxicity of the compound in 60 rats and 24 rabbits, respectively. We examined acute toxicity for 14 days after treatment. No death occurred, and abnormality was not detected at clinical findings in rats and rabbits administered orally with the compound. We did not observe

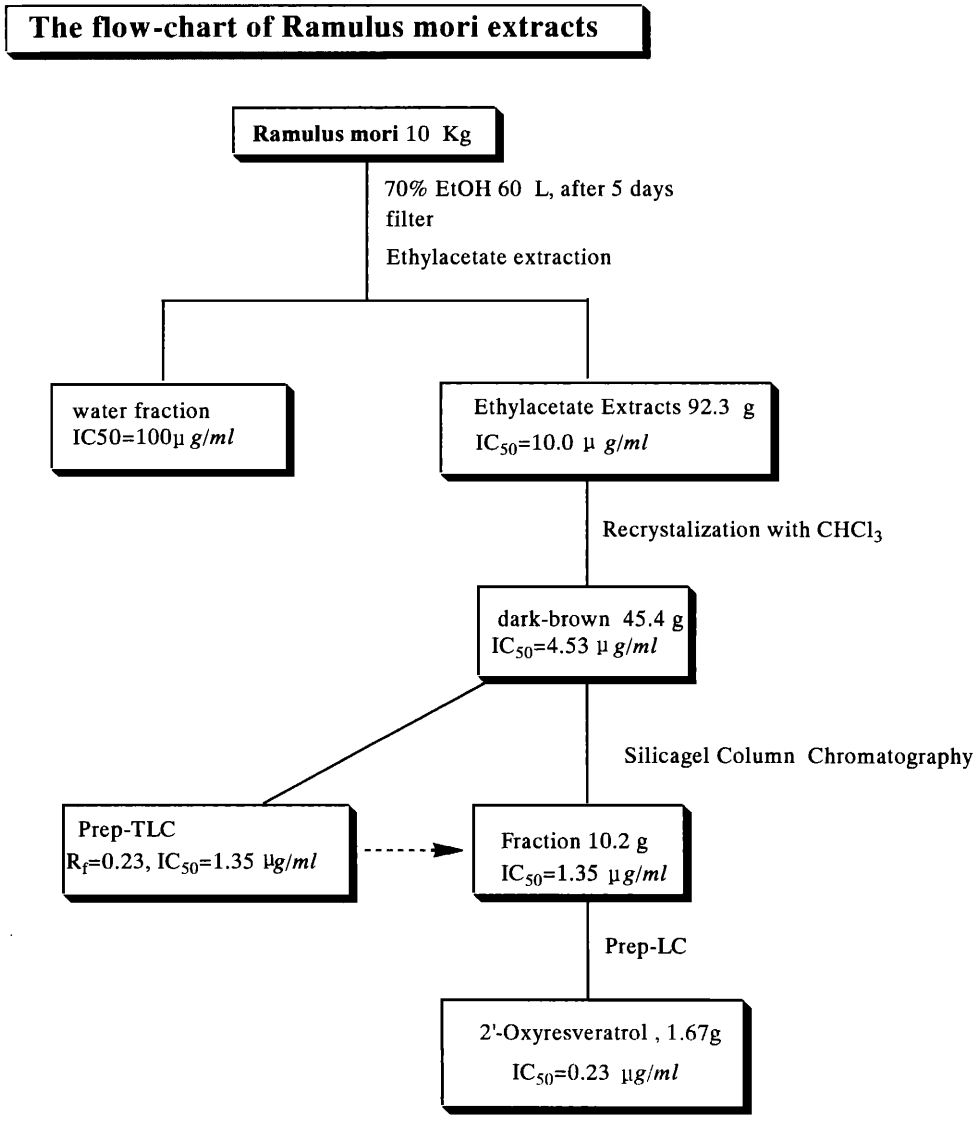

Figure 4. Flow chart of purification of single compound from *R. mori* extracts.

changes of body weight in the test animals or abnormalities of organs from gross findings of necropsy (data not shown).

Skin irritation test. The primary skin irritation from *R. mori* extracts was investigated in six rabbits. We examined skin irritation and clinical signs for 24~72 hours after a seven-day treatment using Draiz's P.I.I. (primary irritation index). We did not observe side effects such as erythema or edema.

Eye irritation test. The potential toxicity of the compound was determined according to AFNOR (Association Francaise de Normalization) guidelines in an eye irritation test on nine rabbits. We examined both eyes at one hour, and one, two, three, four, and seven days after treatment. We did not see any clinical signs.

A ^{1}H NMR(500 MHz, acetone-d_6): δ 6.05(s, 1H), 6.22(d, *J*=8.4 Hz, 1H), 6.31(d, *J*=2.3 Hz, 1H), 6.33(s, 2H), 6.76(d, *J*=16.5 Hz, 1H), 7.14(d, *J*=16.5 Hz, 1H), 7.33(d, *J*=8.5 Hz, 1H)

^{13}C NMR (125 MHz, acetone-d_6): δ 102.19, 103.39, 104.82, 108.06, 116.09, 124.00, 125.44, 127.99, 140.87, 156.87, 158.97, 159.21, 159.35

Mass(rel intensity): 245(M+, 15.8), 244(100), 243(12.6), 227(15.2), 226(19.4), 198(16.3), 197(10.5), 69(8.0)

B

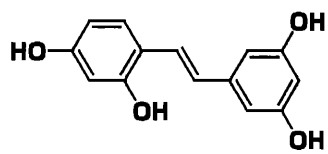

Figure 5. The spectrum data (A) and structure (B) of 2-oxyresveratrol(2,3′,4,5′-tetrahydroxystilbene).

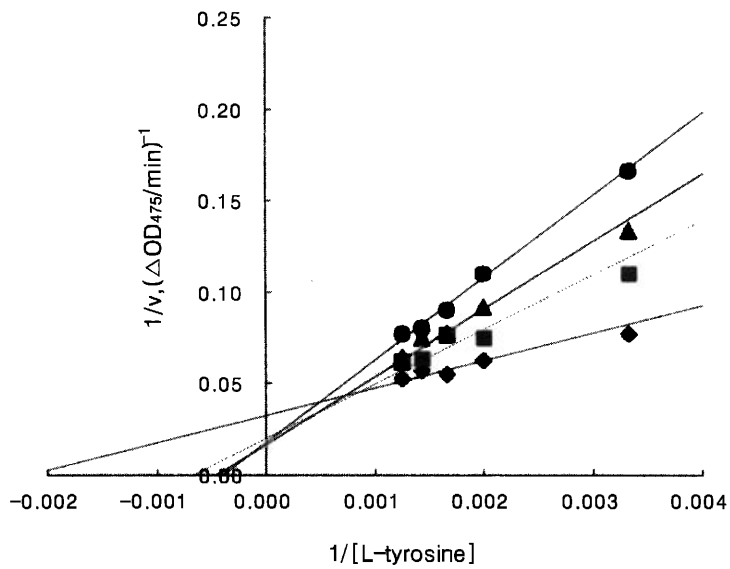

Figure 6. Kinetics of mushroom tyrosinase by 2-oxyresveratrol. Lineweaver-Burk plot of enzyme activity. Circle: 1.5 μg/ml. Triangle: 1.0 μg/ml. Rectangle: 0.5 μg/ml. Diamond: 0 μg/ml.

Skin sensitization test. The sensitization potential of the compound was assessed in 33 guinea pigs by the methods of the Magmusson and Kligman maximization test. We did not observe any skin responses in the test animals.

Acute oral toxicity. The acute oral toxicity of the compound was assessed in 60 rats and 24 rabbits by the "Toxicity Guideline of Drug" proposed by KFDA (Korea Food & Drug Administration). We did not observe any oral toxicity in the test animals.

Human skin irritation test. We studied the potential of the compound to irritate human skin in 50 healthy female volunteers using a 24 hour closed patch test. No skin irritation occurred after application in 50 volunteers.

DISCUSSION

In human skin, pigmentation results from the synthesis and distribution of melanins. Melanins are heterogenous biopolymers and the major determinants of skin color. They play an important role in the absorption of UV radiation, in free radical scavenging, and in protection against carcinogenesis and aging induced by UV rays. Melanin is formed through the progressive oxidation of the amino acid tyrosine. In these steps, tyrosinase, a copper-containing monophenol monooxygenase, plays a critical role (6,7). Several chemicals, including arbutin, kojic acid, and ascorbic acid, have been used as whitening ingredients for their anti-tyrosinase activity. In this study, we selected *R. mori* extracts as a melanogenic inhibitor and checked their activity on the inhibition of tyrosinase activity and melanin synthesis. *R. mori* extracts showed high tyrosinase inhibition activity and melanin synthesis inhibition. We examined the inhibition mechanism on melanogenesis using zymography and RT-PCR and found that *R. mori* extracts showed anti-tyrosinase activity *in vitro,* but no inhibitory activity in tyrosinase synthesis.

Also, we purified a single compound from *R. mori* extracts. It was identified as 2,3′,4,5′-tetrahydroxystilbene(2-oxyresveratrol) and showed inhibition in tyrosinase activity by competitive manner. In the animal tests, *R. mori* extracts showed inhibition activity on UVB-induced pigmentation in brownish guinea pigs. In the histological examination, the remarkable reduction of melanin granules in the epidermis was observed in comparison with the control skin. We did a clinical test for 79 volunteers having skin pigmentation disorders. The clinical test showed that the cream containing *R. mori* extracts [5% (v/v)] improved the condition of volunteers' pigmented faces. The visual assessment was evaluated and graded on a 0–9-point scale and demonstrated a statistically significant improvement in pigmented faces, with the *R. mori* extracts-containing cream providing better results than the placebo ($p < 0.05$). The melanin index for objective data by Mexameter (Courage+Khazaka, Germany) was also evaluated, and it demonstrated an improvement in pigmented faces ($p < 0.05$). The results of safety tests showed that the extracts have no irritation and sensitization potential.

CONCLUSION

We selected *R. mori* extracts as a whitening ingredient and found that the extracts have a strong inhibitory activity against melanogenesis and are strongly expected to be an effective ingredient for the improvement of skin darkness.

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