

Tyrosinase 및 TRP-2의 발현 억제를 통한 황칠나무 잎 추출물의 Melanin 생성 저해 효과

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Inhibitory Effects of *Dendropanax Morbifera* Leaf Extracts on Melanogenesis through Down-Regulation of Tyrosinase and TRP-2

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초 록

황칠나무는 한국의 남부 해안 및 제주도 등에서 자생하는 식물이다. 본 연구에서는 황칠나무 잎 추출물의 미백 활성의 원인을 규명하고자 연구를 수행하였다. α -MSH로 자극된 B16 melanoma 세포에 대한 추출물의 멜라닌 생합성 저해 활성을 측정하였다. 추출물은 25 및 50 $\mu\text{g/mL}$ 농도에서 유의적으로 멜라닌 함량을 감소시켰으며, 세포 내의 tyrosinase 활성뿐만 아니라 tyrosinase와 TRP-2의 단백질 발현도 상당히 저해하였다. 결과적으로, 황칠나무 잎 추출물은 세포 내의 tyrosinase활성과 멜라닌 생합성에 직접적으로 관련된 효소의 발현을 저해함으로써 미백효과를 나타내었다. 본 연구 결과는 황칠나무 잎 추출물이 새로운 미백 화장품의 원료로써 이용 가능성을 시사한다.

Abstract

Dendropanax morbifera (*D. morbifera*) grows in the southern coastal areas and on Jeju Island in Korea. In this study, *D. morbifera* leaf extract was investigated to determine the mechanism of its whitening effect. The inhibitory activities of the extract on melanogenesis were tested in B16 melanoma cells treated with the α -melanocyte stimulating hormone (α -MSH). *D. morbifera* leaf extracts remarkably decreased the melanin content at 25 and 50 $\mu\text{g/mL}$. The extracts significantly inhibited the intracellular tyrosinase activity and protein expression of tyrosinase and tyrosinase related protein-2 (TRP-2). In conclusion, *D. morbifera* leaf extracts would show a whitening effect by inhibiting intracellular tyrosinase activities and the expression of enzymes directly involved in the melanin biosynthesis. The results indicate that fractions of *D. morbifera* leaf extracts show potential for application as a whitening agent in the new whitening cosmetics.

Keywords: *Dendropanax morbifera* leaf, melanogenesis, tyrosinase, TRP-1, TRP-2

1. Introduction

Melanin is a natural biopolymer dye and one of the major factors determining the color of human skin. It is biosynthesized by melanocytes in the basal layer of the skin and translocated to keratinocytes via dendrites. This process results in skin pigmentation[1-3]. Three major enzymes are involved in melanogenesis : tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2[4]. Tyrosinase mediates the oxida-

tion of 3,4-dihydroxyphenylalanine (DOPA) into dopaquinone and plays the most important role in melanogenesis; it catalyzes an initial step and controls the speed of the reaction[5-6]. TRP-1 and TRP-2 function to regulate eumelanin synthesis. This process finally produces eumelanin for red pigment and pheomelanin for brown pigment. The synthesized melanin is translocated to keratinocytes where it protects the nucleus and suppresses aging of skin caused by ultraviolet rays or sunlight. However, excess generation of melanin causes dye deposition on the skin accompanied by formation of spots and freckles; such lesions often lead to skin cancer[7-11]. Therefore, new skin whitening agents are required to help maintain bright and healthy skin[12-14].

Dendropanax morbifera leveille is an evergreen tree belonging to the family araliaceae, class magnoliopsida, which grows in the southern coastal areas and on Jeju Island in Korea. Its leaves, stems, and roots

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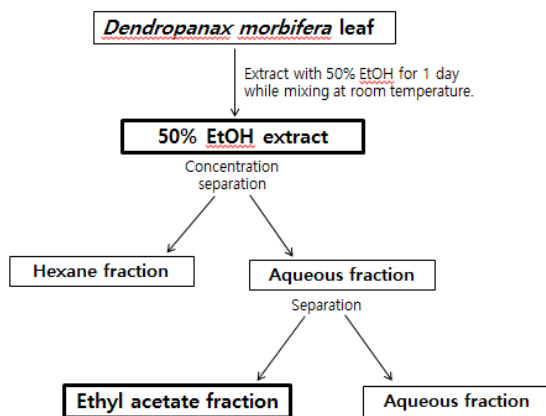


Figure 1. Scheme for preparation of extracts/fractions obtained from *D. moribifera* leaf.

have been reported to be effective in the treatment of migraine headaches, dysmenorrhea, and removal of wind dampness[15]. But, melanin biosynthesis inhibitory activities of the ethyl acetate and aglycone fractions of *D. moribifera* leaf extract have also been reported and the mechanisms underlying its whitening activity remain unknown.

In this study, we aimed to reveal the mechanism underlying the ability of *D. moribifera* leaf extracts to inhibit melanin biosynthesis and expression of melanin synthesis related proteins using B16 melanoma cells. We propose that *D. moribifera* leaf extracts show potential for application in whitening cosmetics.

2. Materials and Methods

2.1. Instruments and materials

An inverted phase-contrast microscope (Leica, Wetzlar, Germany) was used. ELISA results were determined using an ELISA reader (Tecan, Austria). Western blot analysis was performed using Thermo products. Ethanol, ethyl acetate, and all other chemicals were of analytical grade. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin, and bovine serum albumin (BSA) were purchased from PAA Co. (Pasching, Austria). Air-dried *D. moribifera* leaves were purchased from the Jecheon medicinal plant market in June 2012 (Hwasan-dong, Jecheon-si, Chungbuk, South Korea).

2.2. Extraction and isolation of *D. moribifera* leaves

Extraction and isolation methods for *D. moribifera* leaves are shown in Figure 1. Air-dried *D. moribifera* leaves (100 g) were extracted using 50% ethanol (1 L). To remove the non-polar components, a 50% ethanol extract was fractionated with n-hexane and ethyl acetate, and then dried.

Ethyl acetate fraction was processed by the acid hydrolysis method to obtain aglycone, which was then added to a 5% H₂SO₄ solution and heated in a boiling water bath under a reflux condenser for 4 h. The refluxed solution was neutralized with 5% KOH in methanol, washed

with distilled water, fractionated with ethyl acetate, and then dried. The obtained aglycone fraction was dissolved in 100% ethanol for use in subsequent experiments.

2.3. Cell Viability

B16 melanoma cells (provided by Kyunghee University Skin Biotechnology Center) were incubated in DMEM supplemented with 10% FBS (PAA, Austria) and 1% penicillin-streptomycin (PAA, Austria) at 37 °C in 5% CO₂ atmosphere.

Cell viability was determined using an MTT assay. B16 melanoma cells were seeded on a 96-well plate template at 37 °C in a 5% CO₂ atmosphere. *D. moribifera* leaf extract was added at various concentrations. After 72 min, MTT solution (2 µg/mL) was added to each well of a 96-well plate, and the samples were incubated for 3 h. The formazan crystals produced were dissolved in dimethyl sulfoxide and quantified by measuring their optical density at 570 nm using an ELISA reader (Tecan, Austria).

2.4. Determination of cell lysates

B16 melanoma cells were seeded on a 6-well plate template at a density of 1×10^5 cells/well at 37 °C in a 5% CO₂ atmosphere. After 24 h, cell adherence was confirmed and the medium was replaced, and then the cells were treated with the α -melanocyte stimulating hormone (α -MSH, Sigma, USA, 200 nM) and various concentrations of *D. moribifera* leaf extract for 72 h. After 72 h, the cells were harvested and the supernatant was removed. The cell pellet was solubilized in 1 N NaOH containing 10% DMSO, and the melanin concentration was observed with the unaided eye.

2.5. Fontana-Masson staining

Intracellular melanin was observed using Fontana-Masson staining. B16 melanoma cells were seeded on a 6-well plate template at a density of 1×10^5 cells/well at 37 °C in a 5% CO₂ atmosphere. The B16 melanoma cells were treated as described previously. After 72 h, cells were fixed in 4% p-formaldehyde (Sigma, USA) for 10 min and stained for melanin with an ammoniacal silver (Sigma, USA) solution overnight at room temperature, and incubated in 2% sodium thiosulfate (Sigma, USA) solution and then in nuclear fast red (Sigma, USA) solution. Following air drying, cells were mounted with manicure enamel, and then examined using an inverted phase-contrast microscope[16-17].

2.6. Tyrosinase activity assay

The inhibitory effects of *D. moribifera* leaf extract on tyrosinase activity were examined using a cell-free system. B16 melanoma cells were seeded on a 6-well plate template at a density of 1×10^5 cells/well at 37 °C in a 5% CO₂ atmosphere. After 72 h, cells were lysed in 50 µL RIPA buffer (Pierce, USA). Cell lysates were centrifuged at 4 °C, 1001 g for 30 min. The experiment was carried out in 90 µL of reaction mixture comprising the cell extract (40 µg), 1 M sodium phosphate buffer (pH 6.8, Biosesang Inc.) with various concentrations of *D. moribifera* leaf extract (3.125 ~ 50 µg/mL), and then 10

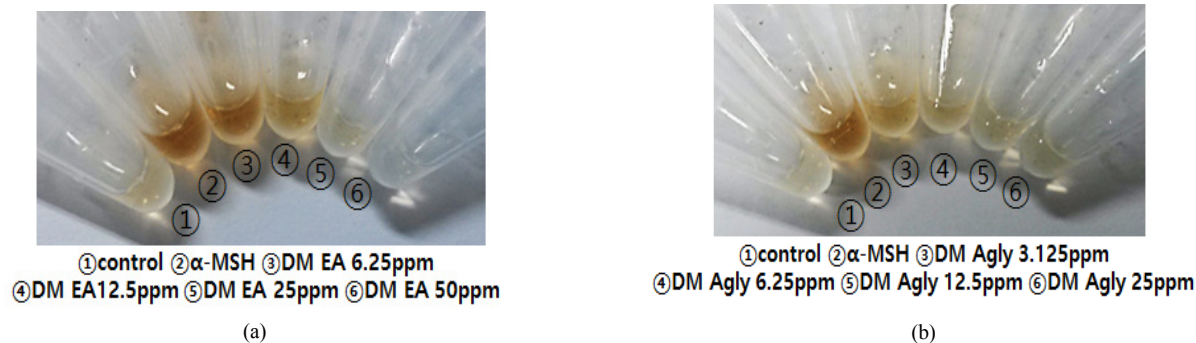


Figure 2. Photograph of lysates of B16 melanoma cells. B16 melanoma cells were treated with DM EA (A; Ethyl acetate fraction of *D. morbifera* leaf extract) and DM agly (B; Aglycone fraction of extract) in the presence of 200 nM α -MSH for 72 h and then ten thousand cells were collected and dissolved with 1 N NaOH containing 10% DMSO.

μ L L-DOPA was added. After incubating the reaction mixture at 37 °C in a 5% CO₂ atmosphere, DOPA chrome formation was estimated at 10-min intervals at 475 nm using an ELISA reader.

The inhibitory effects of *D. morbifera* leaf extract on tyrosinase activity in cells were examined by measuring the intracellular tyrosinase activity. B16 melanoma cells were seeded on a 6-well plate template at a density of 1×10^5 cells/well at 37 °C in a 5% CO₂ atmosphere. The B16 melanoma cells were treated as described previously. After 72 h, cells were lysed in 50 μ L RIPA buffer. Cell lysates were centrifuged at 4 °C, 1001 g for 30 min. The supernatant was used as a tyrosinase solution and the amount of protein was determined using a BCA protein assay kit (Pierce, USA). The experiment was carried out in 90 μ L of reaction mixture comprising the cell lysates as the tyrosinase enzyme source (40 μ g protein), 1 M sodium phosphate buffer (pH 6.8, Biosesang Inc.), and 10 μ L L-DOPA. After incubating the reaction mixture at 37 °C in a 5% CO₂ atmosphere, DOPA chrome formation was estimated at 10-min intervals at 475 nm using an ELISA reader[18-22].

2.7. Western blotting

B16 melanoma cells were seeded on a 6-well plate template at a density of 1×10^5 cells/well at 37 °C in a 5% CO₂ atmosphere. The B16 melanoma cells were treated as described previously. After 72 h, cells were lysed in 50 μ L RIPA buffer, and each cell lysate was centrifuged at 4 °C, 1001 g for 30 min. The amount of protein in the supernatant was determined using a BCA protein assay kit. Proteins were separated on a 4-20% Tris-glycine gel and the separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated in Tris buffer containing 5% skim milk for blocking, and then incubated with a primary antibody. A secondary antibody conjugated with horseradish peroxidase (HRP) was administered, and the bands were detected using an enhanced chemiluminescent (ECL, Pierce) kit. Following the reaction, the bands were quantified using X-ray film. GAPDH and β -actin were used as controls. The antibodies used for western blotting included tyrosinase (sc-7833), TRP-1 (sc-10443), TRP-2 (sc-10452), GAPDH (MA5-15738), and β -actin (sc-1616)[22-23].

2.8. Statistical Analysis

All reported data are presented as mean \pm S.E.M. Statistical significance was determined using the Student's *t*-test.

3. Results and Discussion

3.1. Visual confirmation of lysates of B16 melanoma cells

In order to analyze the depigmentation effect of the *D. morbifera* leaf extracts, B16 melanoma cells stimulated with α -MSH were treated with *D. morbifera* leaf extracts for 72 h. Ten thousand cells were collected per sample and dissolved with 1 N NaOH containing 10% DMSO. Cells treated with the α -melanocyte stimulating hormone (α -MSH) showed increased melanin contents and a dark brown color. However, in the treated groups with the extracts, the melanin content decreased in a concentration-dependent manner compared to group treated with α -MSH alone. The aglycone fraction of 50 μ g/mL was not used for experiment because of cytotoxicity of the fraction on B16 melanoma cells. In particular, the aglycone and ethyl acetate fractions of *D. morbifera* leaf extracts remarkably decreased the melanin content at 25 and 50 μ g/mL, respectively. In Figure 2 (A, B), photograph of lysate of B16 melanoma cells treated with α -MSH alone showed dark brown color of melanin(②). The ethyl acetate and the aglycone fractions of *D. morbifera* leaf extract decreased melanin production in the presence of 200 nM α -MSH in a concentration dependent manner. When the ethyl acetate fraction [③, ④ of Figure 2, (A)] was compared to the aglycone fraction [④, ⑤ of Figure 2, (B)] in 6.25 and 12.5ppm. The aglycone fraction exhibited higher inhibitory effect on melanogenesis than the ethyl acetate fraction.

3.2. Melanin dyeing by Fontana-Masson staining

In order to measure the inhibitory effect of *D. morbifera* leaf extracts on melanin production, B16 melanoma cells treated with varying concentrations of leaf extracts for 72 h were stained using a Fontana-Masson assay. In the α -MSH treated group, the melanin content was significantly increased compared to the untreated control. However, in cells treated with the ethyl acetate and aglycone fractions of *D. morbifera* leaf extract, a significant decrease in the intracellular melanin con-

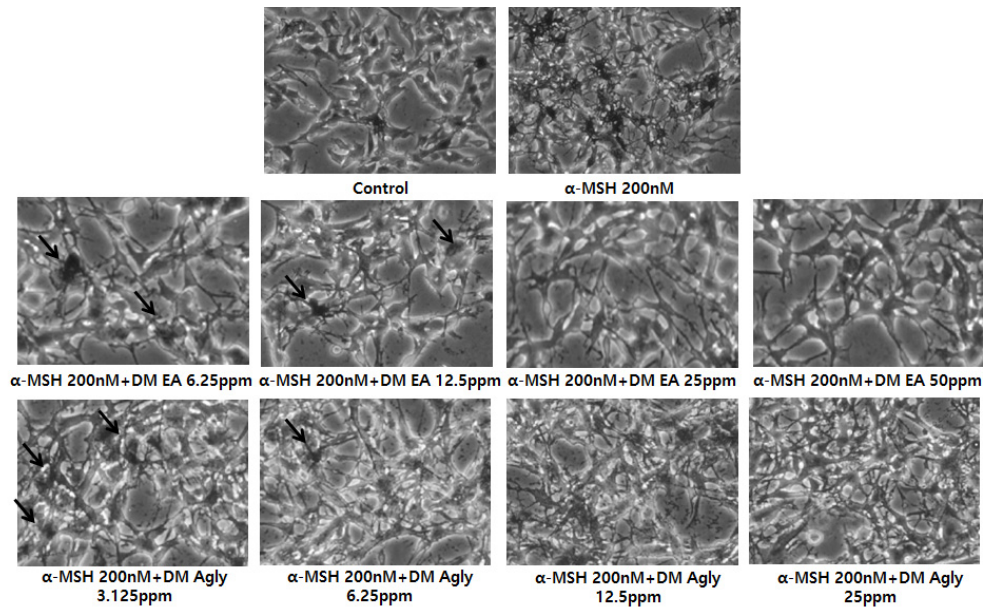


Figure 3. Typical images of B16 melanoma cells with Fontana-Masson staining using an inverted phase contrast microscope (20 \times). DM EA; Ethyl acetate fraction of *D. morbifera* leaf extract, DM agly; Aglycone fraction of *D. morbifera* leaf extract.

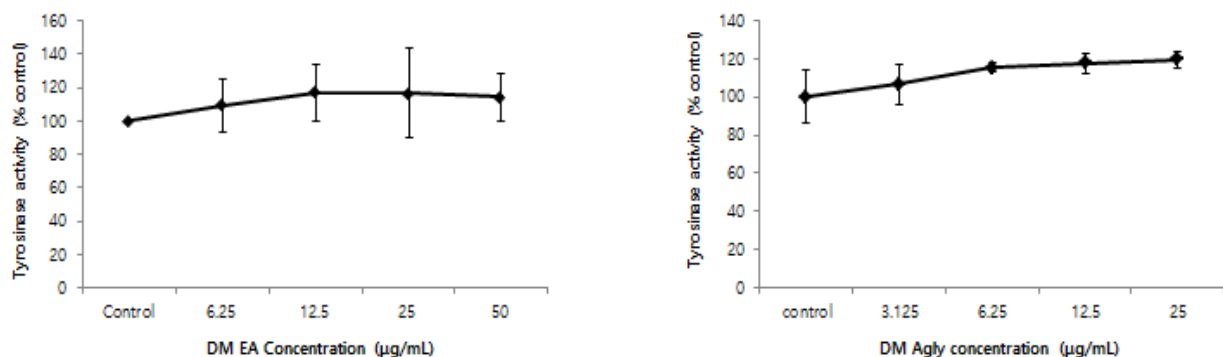


Figure 4. Inhibitory effects of *D. morbifera* leaf extracts on the activity of cell-extracted tyrosinase in B16 melanoma cells. DM EA; Ethyl acetate fraction of *D. morbifera* leaf extracts, DM agly; Aglycone fraction of *D. morbifera* leaf extracts.

tent was confirmed (Figure 3).

3.3. Inhibitory effect of leaf extract on cell-free tyrosinase

Mushroom tyrosinase was used to determine whether *D. morbifera* leaf extracts directly inhibit tyrosinase. *D. morbifera* leaf extracts were observed to have lower inhibitory activity than arbutin, a known whitening agent (data not shown). In addition, tyrosinase activity in the group treated with fractions of *D. morbifera* leaf extracts in a cell-free system was investigated to determine whether *D. morbifera* leaf extracts directly inhibit cellular tyrosinase. Tyrosinase activity of the untreated control was set at 100% activity. Tyrosinase activity of groups treated with the ethyl acetate and aglycone fractions of *D. morbifera* leaf extracts showed no significant differences from the control (Figure 4).

These results indicate that *D. morbifera* leaf extracts are not direct inhibitors, unlike kojic acid and arbutin[23-24], which directly inhibit tyrosinase.

3.4. Inhibition of intracellular tyrosinase activities

After treatment with various concentrations of *D. morbifera* leaf extracts for 3 days, their tyrosinase inhibitory activities were investigated in the cellular level. The ethyl acetate fraction of *D. morbifera* leaf extracts administered at 6.25, 12.5, 25, and 50 $\mu\text{g/mL}$ showed 2%, 13%, 26%, and 35% intracellular tyrosinase inhibitory activity, respectively, compared with the group treated with α -MSH alone (+ 85% of control). The aglycone fraction, administered at 3.125, 6.25, 12.5, and 25 $\mu\text{g/mL}$ reduced tyrosinase activity by 14, 18, 24, and 38%, respectively, compared with the group treated with α -MSH alone (+ 25% of control); at 25 $\mu\text{g/mL}$, in particular, tyrosinase activity was lower than that of the untreated control group (Figure 5). In contrast with *D. morbifera* leaf extracts, enhanced melanin synthesis by some other natural extracts has been reported with increase of tyrosinase activity in B16 melanoma cells. For example, compared to treatment with medium only, treatment with Kaliziri extract(*Vernonia anthelmintica*

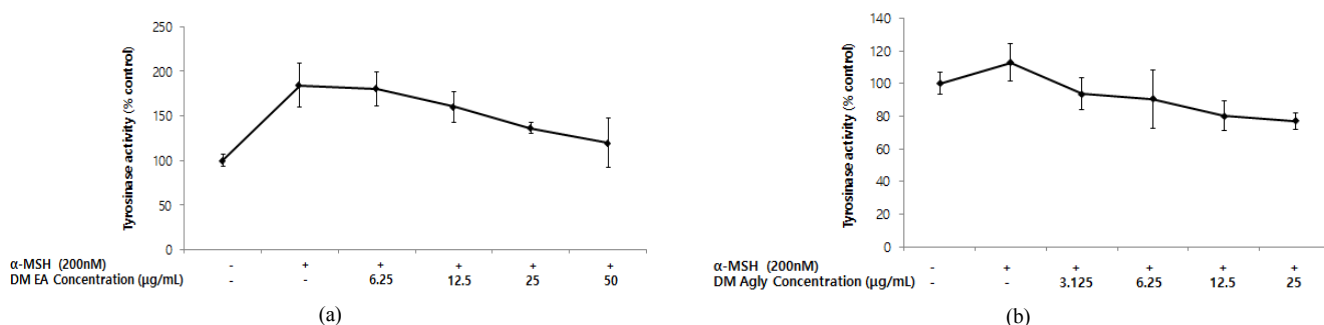


Figure 5. Inhibitory effects of *D. morbifera* leaf extracts on intracellular tyrosinase activity in B16 melanoma cells. After treatment with various concentrations of the ethyl acetate fraction (A; DM EA) and the aglycone fraction (B; DM Agly) of *D. morbifera* leaf extracts for 3 days, their tyrosinase inhibitory activities were investigated in the cellular level.



Figure 6. The inhibitory effects of *D. morbifera* leaf extracts on tyrosinase, TRP-1, and TRP-2 protein expression in B16 melanoma cells. DM EA; Ethyl acetate fraction of *D. morbifera* leaf extract, DM agly; Aglycone fraction of *D. morbifera* leaf extract.

(L.) Willd.) at 5–40 µg/ml resulted in a dose-dependent increase in tyrosinase activity in B16 melanoma cells[25].

These results indicate that the inhibition of melanin biosynthesis in B16 melanoma cells by *D. morbifera* leaf extracts is associated with inhibition of tyrosinase activity.

3.5. Inhibition of expression of melanin synthesis protein

The results showed that *D. morbifera* leaf extracts do not directly inhibit tyrosinase activity. However, the extracts showed an inhibitory effect on melanogenesis, and therefore must affect a process either before or after the tyrosinase step in B16 melanoma cells. We investigated whether *D. morbifera* leaf extracts could show a whitening effect through inhibition of protein expression. We measured tyrosinase protein expression, and also confirmed whether to be associated with protein expression of TRP-1 and TRP-2, two other enzymes involved in melanin synthesis. Thus, the expression levels of proteins involved in melanin synthesis were determined by western blot analysis, using antibodies to tyrosinase, TRP-1 and TRP-2. The ethyl acetate fraction of *D. morbifera* leaf extracts inhibited tyrosinase expression in a concentration-dependent manner and completely inhibited the expression of TRP-2; the band for TRP-2 was almost undetectable.

However, the ethyl acetate fraction of *D. morbifera* leaf extracts did not affect the expression levels of TRP-1. The aglycone fraction of *D. morbifera* leaf extracts also inhibited expression of tyrosinase and TRP-2 in a concentration-dependent manner in comparison with the group treated with α-MSH alone. Therefore, it appears that *D. morbifera* leaf extracts would produce a whitening effect by inhibiting the tyrosinase and TRP-2 enzymes directly involved in the melanin biosyn-

thesis induced by α-MSH (Figure 6).

4. Conclusion

1) To determine the degree of inhibition of melanin biosynthesis, we examined cell lysate color and Fontana–Masson stained melanin following treatment of α-MSH-stimulated B16 melanoma cells with *D. morbifera* leaf extracts. *D. morbifera* leaf extracts strongly inhibited melanin biosynthesis.

2) We attempted to determine whether the inhibitory activity of *D. morbifera* leaf extracts on melanogenesis was related to tyrosinase activity. First, we measured tyrosinase activity to confirm whether *D. morbifera* leaf extracts directly inhibit cellular tyrosinase in a cell-free system. The tyrosinase activity of the group treated with *D. morbifera* leaf extracts showed no significant difference compared to the control. These results indicate that *D. morbifera* leaf extracts do not directly inhibit tyrosinase.

We investigated the intracellular tyrosinase inhibitory activity to confirm whether *D. morbifera* leaf extracts inhibit tyrosinase activity at the cellular level. *D. morbifera* leaf extracts dose-dependently inhibited tyrosinase activity compared to the group treated with α-MSH alone. These results indicate that the inhibitory effect of melanin biosynthesis of *D. morbifera* leaf extracts is associated with inhibition of tyrosinase activity in B16 melanoma cells.

3) We intend to confirm that *D. morbifera* leaf extracts exert their whitening activity via inhibition of tyrosinase expression by observing the effect of *D. morbifera* leaf extracts on tyrosinase expression.

In addition, western blot analysis was used to determine whether the

inhibition of melanin synthesis is associated with the protein expression of TRP-1 and TRP-2. It appears that *D. morbifera* leaf extracts produce a whitening effect by inhibiting the expression of the tyrosinase and TRP-2 enzymes that are directly involved in α -MSH-induced melanin biosynthesis.

These results indicate that fractions of *D. morbifera* leaf extract show potential for application as a whitening agent in development of the new whitening cosmetics via the inhibition of α -MSH-induced melanin biosynthesis.

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