Tyrosinase 및 TRP-2의 발현 억제를 통한 황칠나무 잎 추출물의 Melanin 생성 저해 효과
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(2014년 6월 6일 접수, 2014년 7월 24일 심사, 2014년 8월 14일 채택)

Inhibitory Effects of Dendropanax Morbifera Leaf Extracts on Melanogenesis through Down-Regulation of Tyrosinase and TRP-2
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(Received June 6, 2014; Revised July 24, 2014; Accepted August 14, 2014)

초 록
황칠나무는 한국의 남부 해안 및 제주도 등에서 자생하는 식물이다. 본 연구에서는 황칠나무 잎 추출물의 미백 성과의 원인을 규명하고자 연구를 수행하였다. α-MSH로 자극된 B16 melanoma 세포에 대한 추출물의 멜라닌 생성 저해 활성을 측정하였다. 추출물은 25 및 50 µg/mL 농도에서 유의적으로 멜라닌 함량을 감소시켰으며, 세포 내의 tyrosinase 활성뿐만 아니라 tyrosinase와 TRP-2의 단백질 발현도 상당히 저해하였다. 결과적으로, 황칠나무 잎 추출물은 세포 내의 tyrosinase 활성과 멜라닌 생성에 직접적으로 관련된 효소의 발현을 저해함으로써 미백 효과를 나타내었다. 본 연구 결과는 황칠나무 잎 추출물이 새로운 미백 화장품의 원료로 이용 가능함을 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 µg/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 oxidation 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].
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. morbifera 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. morbifera leaf extracts to inhibit melanin biosynthesis and expression of melanin synthesis related proteins using B16 melanoma cells. We propose that D. morbifera 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. morbifera 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. morbifera leaves

Extraction and isolation methods for D. morbifera leaves are shown in Figure 1. Air-dried D. morbifera 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. morbifera 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. morbifera 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. morbifera 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. morbifera leaf extract (3.125 – 50 µg/mL), and then 10

![Figure 1. Scheme for preparation of extracts/fractions obtained from D. morbifera leaf.](image)