Original Article
Ficus carica leaf extract decreases melanogenesis in B16F10 mouse melanoma cells

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Abstract: In the epidermis, pigmentation is a protective mechanism to ultraviolet (UV) radiation mediated damage. However, excessive melanin synthesis induces hyperpigmentation diseases and aged skin. Thus, development of depigmentation agents, such as whitening agents, are required in the cosmetics industry. In our study, we show the whitening effects of *Ficus carica* leaf extracts in B16F10 cells. Additionally, we revealed synergetic whitening effects of *Ficus carica* leaf extracts with arbutin. First, we show that *Ficus carica* leaf extracts decreased melanin content in B16F10 cells. Tyrosinase activity and expression are also decreased by *Ficus carica* leaf extracts in B16F10 cells. Therefore, our results suggest that *Ficus carica* leaf extract is a novel whitening agent. Additionally, we show synergetic depigmentation effects. Co-treatment with *Ficus carica* leaf extracts and arbutin decreased melanin content rather than arbutin alone. Also co-treatment with *Ficus carica* leaf extracts and arbutin decreased tyrosinase activity and expression in B16F10 cells. Overall, we suggest that *Ficus carica* leaf extract is a new whitening agent and a supplemental whitening agent with arbutin in the cosmetic industry.

Keywords: *Ficus carica* leaf extracts, melanogenesis, B16F10, tyrosinase

Introduction

The epidermis is the outermost layer of the skin which is exposed to various external stimuli [1]. Skin is aged by endogenous factors which induce structural changes of the skin naturally with age; and photo-aging, which is a type of exogenous skin aging that is mainly caused by exposure of ultraviolet radiation [2, 3]. Exposure to ultraviolet radiation causes burns, inflammation, inhibition of the immune response, and damage to skin and connective tissues, and persistent ultraviolet exposure destroys skin structure and ultimately causes photo-aging and skin cancer [4]. Thus, the epidermis protects itself using melanin that absorbs ultraviolet radiation which penetrates into human body [5, 6].

Melanin is synthesized in the melanosome of melanocytes and is induced by various factors, such as ultraviolet light, cytokines, growth factors and hormones, through various mechanisms [7, 8]. When melanogenesis is initiated by various factors, tyrosinase is expressed and sequentially catalyzes the oxidation of L-ty-rosine, one of the amino acids, to melanin in the melanosome [9, 10]. Then the synthesized melanin is distributed to keratinocytes, which make a skin color and a protective layer from ultraviolet radiation in skin [11, 12]. However, abnormal hyper-melanogenesis caused by skin aging induces skin diseases, such as a melasma and freckles, and nonuniform pigmentation [13]. Therefore, in the cosmetics industry, pigmentation regulation is required [16, 17].

Currently, in the cosmetics field, whitening agents are represented by arbutin and kojic acid, which inhibit tyrosiase activity [14-16]. However, since these whitening agents have a problem of stability and safety, they are used only in a limited amount. Thus, development of new whitening agents using natural materials is required [17].

Material and method

Cell culture

Melanoma (B16F10) cells were purchased from the American Type Culture Collection (ATCC,
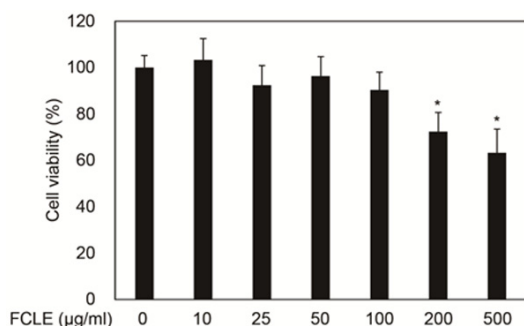
Figure 1. Cell viability of FCLE in B16F10 cells. B16F10 cells were treated with FCLE for the indicated concentrations for 48 h. The cells were collected, and cell viability was analyzed using MTT assay. Result are means ± SD of three independent experiments. Significance was determined using T-test (*P<0.05 compared with non-treated B16F10 cells).

USA). B16F10 cells were cultured in Dulbecco's modified eagles medium (DMEM; Gibco, USA) containing 1% penicillin/streptomycin (P/S; Sigma, USA) and 10% fetal bovine serum (FBS; Sigma).

Preparation of Ficus carica leaf extracts

Ficus carica leaves were washed by water, and dried in a 60°C drying oven (ON-50; Deahan, Korea). Completely dried leaves are pulverized by blend mill (SMX-5800LM; Shinil, Korea). The powder of *Ficus carica* leaves is extracted by 70% ethanol for 30 min at 60°C. During extraction, extraction efficiency was increased by sonication (Ultrasonic Cleaner 8891; Cole-Parmer, USA) at or above 20 kHz. After extraction, the solids were separated by Whatman No.2 filter paper (GE Health Care Life Sciences, USA). The filtrate extracts were dried with EYELA N-3010; Tokyo Rikakikai (Japan) and Freeze Dryer (LP 10-30; Shinil). Dried *Ficus carica* leaf extract was dissolved again with dimethyl sulfoxide (DMSO; Biopure, Canada).

Cell viability

B16F10 cells were plated in 96-well plates at 1 × 10⁴ cells/well and cultured for 24 h. *Ficus carica* leaf extracts (FCLE) were treated at a concentration of 0-500 μg/ml and after 1 h, α-melanocytestimulating hormone (alpha-MSH; Sigma) at a concentration of 200 nM for 48 h. Then 0.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) was treated for 1 hr, and then measured using a multi-plate reader (SpectraMax® i3x; Molecular Devices, USA) at 595 nm.
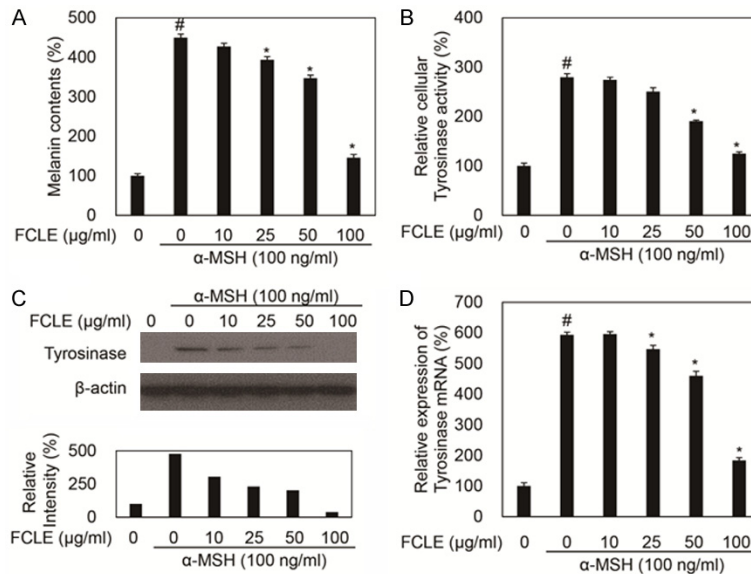
Melanin content

Melanin content was measured by using the Hosoi method. B16F10 cells were seeded at a density of $1.0 × 10^5$ cells/well in a 6-well plate, cultured for 24 h, treated with FCLE at a concentration of 200 μg/mL, and treated with α-MSH at a concentration of 200 nM after 1 h. After 48 h, the cells were recovered with Dulbecco's phosphate-buffered saline (DPBS; Gibco) and lysed with 1 N sodium hydroxide (NaOH) at 90°C for 1 h. Absorbance was measured at 405 nm with a multi-plate reader.

Western blot

B16F10 cells were seeded at a density of $1.0 × 10^5$ cells/well in a 6-well plate, cultured for 24 h, treated with FCLE at 200 μg/mL, and treated with α-MSH at 200 nM for 1 h. After 48 h of sample treatment, the cells were washed twice with PBS, and then dissolved in 1% SDS lysis buffer (Promega, USA). The cell lysis solvent was centrifuged at 13,000 rpm for 20 min at 4°C, and the supernatant was quantified using Bradford reagent (Bio-Rad, USA). The cell lysis solution was mixed with 5X sample buffer (iNtRON, Korea), heated at 95°C for 5 min, and separated into 20 μg protein by electrophoresis on 10% SDS-PAGE. The electrophoresed proteins were transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Germany) and blocked for 30 min at room temperature (5% skim milk in TBST). The primary antibodies (tyrosinase; Santa Cruz, USA) were diluted 1:1,000 and treated overnight at 4%. The cells were then washed three times with TBST for 10 min, diluted 1:2,000 with secondary antibody (cell signaling technology), and reacted for 1 h. The cells were washed three times with TBST at intervals of 10 min and reacted with enhanced chemiluminescence (ECL) western blot detection kit (Bio-Rad). Protein expression was confirmed by chemiluminescence imaging systems (CAS-400MF; Davinch-K, Korea). Band density was measured by Image J (NIH, USA).

Quantitative real time polymerase chain reaction (qRT-PCR)

To reveal the expression of tyrosinase mRNA, total RNA was extracted using TRIzol™ (In-

Figure 2. FCLE decreased melanogenesis in B16F10 cells. B16F10 cells were treated with FCLE for the indicated concentrations for 48 h with 100 ng/ml α-MSH. The cells were harvested to investigate the (A) melanin content, (B) cellular tyrosianse activity, and the expression of (C) Tyrosinase protein and (D) mRNA. Result are means ± SD of three independent experiments. Significance was determined using T-test (#P<0.05 compared with non-treated B16F10 cells, *P<0.05 compared with α-MSH only treated B16F10 cells).
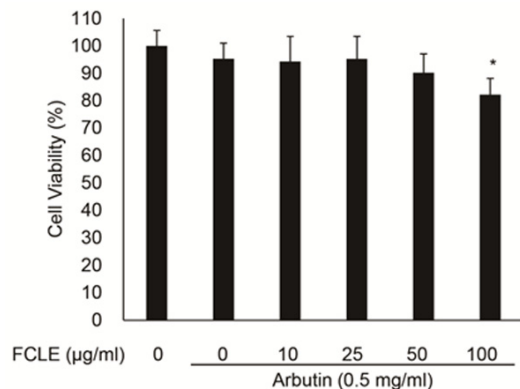
Figure 3. Cell viability of FCLE in 0.5 mg/ml arbutin treated B16F10 cells. 0.5 mg/ml arbutin treated B16F10 cells were treated with FCLE for the indicated concentrations for 48 h. The cells were collected, and cell viability was analyzed using MTT assay. Result are means ± SD of three independent experiments. Significance was determined using T-test (*P<0.05 compared 0.5 mg/ml arbutin only treated B16F10 cells).

vitrogen, USA). Total RNA was quantified using MaestroNano Pro Micro-Volume Spectrophotometer (Maestro Inc., USA) and cDNA was synthesized with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) and same amount total RNA. qRT-PCR was performed at StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) to recognize the expression of tyrosinase using synthesized cDNA. Mixture of PCR was composed with HOT FIREPol EvaGreen® qPCR Mix Plus (ROX) (Solis BioDynne, Estonia), 0.5 pmoles/µL forward primer, 0.5 pmoles/µL reverse primer, and cDNA. The qRT-PCR results were quantified using the calculation method of 2-ΔΔCT by measuring Ct value, and all values were normalized for differences between samples using β-actin.

Statistically analysis

All experiments were repeated three times and were expressed as mean ± SD. A two-tailed t-test was used for statistical analysis using Excel 2013 (Microsoft, USA), and statistical significance was set at *P*<0.05.

Results

Cytotoxicity of FCLE in B16F10 mouse melanoma cells

To confirm the toxicity of FCLE in B16F10 cells, they were treated with extract at concentrations of 0-500 µg/ml for 48 h, and then MTT assay is performed. The cell viability was 63-103% in FCLE-treated B16F10 cells. FCLE was found to be non-toxic (>90% viability) even at 100 µg/ml (**Figure 1**). Based on these results, further studies were conducted using 100 µg/ml, the concentration that does not affect cell viability.

FCLE decreased melanin content in B16F10 mouse melanoma cells

To verify the reduction in melanin production by FCLE in B16F10 cells, B16F10 cells were treated with 100 µg/ml FCLE and 200 ng/ml α-MSH for 48 h, and then melanin content assay was
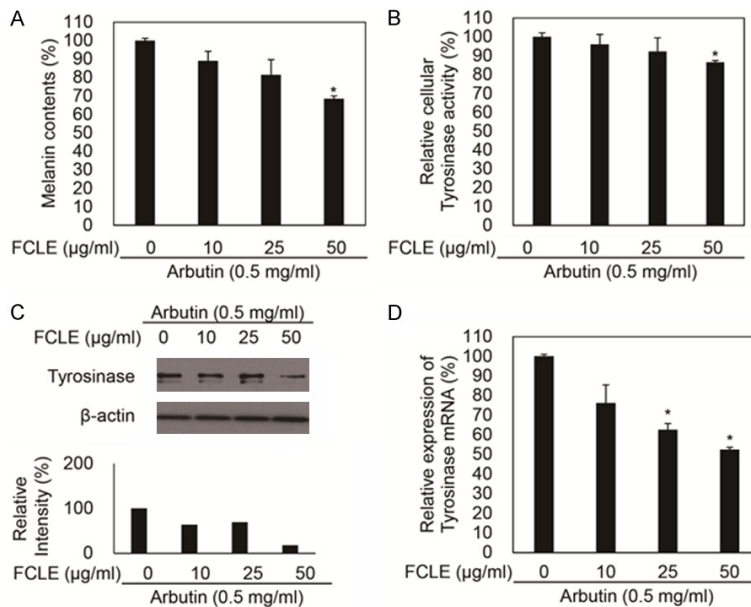
Figure 4. FCLE additionally decreased melanogenesis in 0.5 mg/ml arbutin treated B16F10 cells. 0.5 mg/ml arbutin treated B16F10 cells were treated with FCLE for the indicated concentrations for 48 h with 100 ng/ml α-MSH. The cells were harvested to investigate the (A) melanin content, (B) cellular tyrosianse activity, and the expression of (C) Tyrosinase protein and (D) mRNA. Result are means ± SD of three independent experiments. Significance was determined using T-test (*P<0.05 compared with 0.5 mg/ml arbutin and 100 ng/ml α-MSH-treated B16F10 cells).

performed. FCLE showed inhibitory activity of melanin synthesis in B16F10 cells. In the α-MSH treatment group, the melanin content increased to 449.64%, and again decreased to 145.82% by treatment with 100 µg/ml FCLE (**Figure 2A**). Since melanin synthesis is produced by tyrosinase in the melanosome, the tyrosinase activity was measured in subsequent experiments [18].

FCLE decreased tyrosinase activity and expression in B16F10 mouse melanoma cells

To verify that FCLE decreased tyrosinase activity in B16F10 cells, B16F10 cells were treated with 100 µg/mL FCLE and 200 ng/ml α-MSH for 48 h, and then tyrosinase activity was measured. As shown **Figure 2B**, tyrosinase activity was decreased from 279.34% to 124.98 by 100 µg/mL FCLE compared to 200 ng/ml α-MSH treated B16F10 cells. Additionally, we showed that expression of tyrosinase was decreased by FCLE. As shown **Figure 2C** and **2D**, expression level of tyrosinase protein and mRNA was decreased from 476.16% and 593.24% to 38.68% and 183.64% respective-

ly, by 100 µg/mL FCLE in 200 ng/ml α-MSH treated B16F10 cells.

FCLE decreased melanogenesis along with arbutin in B16F10 mouse melanoma cells

To verify synergistic effects between FCLE and arbutin, we first measured the cytotoxicity of FCLE in 0.5 mg/ml arbutin treated B16F10 cells. As shown **Figure 3**, FCLE was found to be non-toxic (>90% viability) even at 50 µg/ml in 0.5 mg/ml arbutin treated B16F10 cells. Based on these results, further studies were conducted using 50 µg/ml FLCE and 0.5 mg/ml arbutin, a concentration that does not affect cell viability.

Arbutin is a very well-known whitening agent, however, it induces apoptosis when high concentrations are used. Therefore, the concentration of arbutin in which the whitening effect occurs was verified for additional whitening effect by FCLE. Melanin synthesis was additionally decreased to 64.48% by 50 µg/mL FCLE in 0.5 mg/ml arbutin treated B16F10 cells (**Figure 4A**). Additionally, tyrosinase activity was also synergistically decreased to 86.47% by 50 µg/mL FCLE in 0.5 mg/ml arbutin treated B16F10 cells (**Figure 4B**). As shown **Figure 4C** and **4D**, co-treatment of 50 µg/mL FCLE and 0.5 mg/ml arbutin showed 82.11% and 47.51% more decrease of tyrosinase protein and mRNA level than that of arbutin only treatment.

Discussion

Melanin synthesis is regulated by the expression of melanogenesis related enzymes (19). In the early stage, tyrosinase converts L-tyrosine to L-DOPA, and then melanin is finally synthesized by tyrosinase, TRP-1 and TRP-2 from L-DOPA [19]. Therefore, in this study, we first revealed the expression level of tyrosinase, which is a key regulator in melanin biosynthesis, using western blot. At the concentration of

200 μg/mL, expression of tyrosinase, an essential factor for the production of melanin, was inhibited by FCLE (**Figure 2C**, **2D**). In particular, 100 μg/ml FCLE showed 14.9% inhibitory activity compared to α-MSH treatment group (**Figure 2B**). Sequentially, melanin content is decreased by 100 μg/ml FCLE to 145.82% compared to α-MSH treatment group (449.64%; **Figure 2A**).

In addition, we investigated whether FCLE increases the whitening effect of arbutin, a representative whitening agent used in cosmetics [20]. Since most whitening agents, such as abutin, kojic acid, etc., have a cytotoxicity at a high-concentrations, it is necessary to show synergestic whitening effects between arbutin and FCLE. As shown in **Figure 4**, co-treatment with 100 μg/ml FCLE and 0.5 mg/ml arbutin decreased melanin synthesis in non-toxic concentrations compared to when treated with 0.5 mg/ml arbutin only (**Figure 4B**). Moreover, levels of tyrosinase mRNA and protein were decreased by co-treatment with 100 μg/ml FCLE and 0.5 mg/ml arbutin than when treated with 0.5 mg/ml arbutin only (**Figure 4C**, **4D**).

Overall, as our results, we suggest that FCLE inhibit the formation of melanin as well as the expression of melanin-related enzymes and thus has a whitening effect, which can be used as an ingredient for whitening in functional cosmetics. FCLE can also be used as auxiliary agent of arbutin without cytotoxicity.

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Disclosure of conflict of interest

None.

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