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Anti-melanogenic Effects of *Cnidium japonicum* in B16F10 Murine Melanoma Cells

Hyun Jin Jo[1], Fatih Karadeniz[2], Jung Hwan Oh[2], Youngwan Seo[3] and Chang-Suk Kong[1,2]*

[1]*Department of Food and Nutrition, College of Medical and Life Sciences, Silla University, Baegyang-daero 700 beon-gil 140, Sasang-gu, Busan 46958, Korea*
[2]*Marine Biotechnology Center for Pharmaceuticals and Foods, Silla University, Baegyang-daero 700 beon-gil 140, Sasang-gu, Busan 46958, Korea*
[3]*Division of Convergence on Marine Science, Bioscience, Korea Maritime and Ocean University, Busan 49112, Korea*

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Melanin is a pigment produced by melanocytes to protect the skin from external stimuli, mainly ultraviolet (UV) rays. However, abnormal and excessive production of melanin causes hyperpigmentation disorders, such as freckles, age spots, and discoloration. Natural cosmeceuticals are a new trend for treating or preventing hyperpigmentation due to fewer side effects and biocompatibility. In this context, the current study focused on *Cnidium japonicum*, a halophyte with several uses in folk medicine, to evaluate its potential as a skin-whitening agent. The effect of *C. japonicum* extract (CJE) on melanin production was analyzed in melanogenesis-stimulated B16F10 melanoma cells. The results showed that CJE successfully inhibited the oxidation of tyrosine and L-DOPA by tyrosinase and subsequently decreased the production of the key enzymes responsible for melanin production: tyrosinase, tyrosinase-related protein-1, and protein-2. This effect was confirmed by decreased intracellular and extracellular melanin levels in B16F10 melanoma cells after CJE treatment. Further experiments to elucidate the action mechanism revealed that CJE treatment suppressed melanin production by inhibiting the activation of glycogen synthase kinase 3 β (GSKβ)/β-catenin and protein kinase A (PKA)/cAMP-response element binding protein (CREB) pathways, which are the upstream activators of melanogenesis. In conclusion, the present study suggests that *C. japonicum* is a potential natural source of bioactive substances for the development of novel cosmeceuticals that can act against hyperpigmentation.

Key words : B16F10, *Cnidium japonicum*, melanin, skin whitening, tyrosinase

Introduction

Interest in functional cosmetics is on the rise along with increased living standards and exposure to numerous environmental factors. As the life expectancy increases each year, the need and demand of functional cosmetics, also known as cosmeceuticals, increase. New cosmeceuticals with skin whitening, anti-wrinkle, UV protection and anti-aging properties are being developed continuously [14].

The human skin is exposed to several harmful irritants during the day, among which the UV rays are considered to be the most abundant and detrimental. As a defense mechanism against UV-induced harmful effects, skin protects itself by producing UV absorbent pigments called melanin [4]. Melanin is produced in the melanosomes of the skin cells called melanocytes through a complex process named melanogenesis. Although the reason behind melanin production is to absorb UV rays to protect the skin, excessive production of melanin and its subsequent accumulation in the skin result in unwanted complications [7]. Excessive melanin formation is a known cause for pigmentation disorders such as freckles, discoloration, and age spots [9].

Melanogenesis is stimulated by several factors including UV rays, cytokines, growth factors, and some other hormones. Among the hormones that induces melanogenesis, α-melanocyte stimulating hormone (α-MSH) secreted from the middle pituitary, plays an important role in melanin synthesis [1]. The intracellular signaling pathway for melanogenesis includes α-MSH/melanocortin 1 receptor (MC1R) signaling cas-

***Corresponding author**
Tel : +82-51-999-5429, Fax : +82-51-999-5657
E-mail : cskong@silla.ac.kr

cade which is also regulated by canonical Wnt/β-catenin signaling pathway which directly affects the activity of microphtalmia-associated transcription factor (MITF) [2, 3]. Briefly, α-MSH binds to MC1R on the surface of melanocytes and increases cAMP levels in cells, leading to activation of protein kinase (PKA) while activated PKA further activates the cAMP responsive element binding protein (CREB). MITF expression is directly increased as a result of CREB activation. The expression of MITF which is a stimulatory factor for melanin synthesis is negatively regulated by the degradation of β-catenin via canonical Wnt/β-catenin signaling pathway [19]. MITF acts as a transcription factor for tyrosinase, tyrosinase-related protein -1 (TRP-1), and TRP-2, which are key enzymes in the melanin synthesis process [15]. Tyrosinase catalyzes the oxidation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and further oxidation of L-DOPA to dopaquinone to produce melanin and other pigments. On the other hand, TRP-1 and TRP-2 regulates the activity of tyrosinase in the further oxidation of dopaquinone to form red eumelanin and brown pheomelanin that constitute melanocytes [16].

Considering its importance in the melanin production, tyrosinase inhibitors attract interest in order to develop anti-melanogenic substances. Up to date several tyrosinase inhibitors have been reported including but not limited to hydroquinone, 4-hydro-xyanisole, ascorbic acid derivatives, retinoids, arbutin, catechin, and Kojic acid [20]. However, use of most of these inhibitors are limited or hindered due to biosafety and economic issues [12, 13, 20]. Parallel to raise of natural product utilization in other fields, cosmeceutical research also focused on natural origin molecules with skin-whitening effects minus the problems former tyrosinase inhibitors faced [11].

In this context, current study focused on the anti-melanogenic effect of *Cnidium japonicum* as a potential natural product source to be utilized. *C. japonicum* Miq. is a halophyte and a biennial herb of the umbel family, widely distributed along the coasts of Hwanghae and Gangwon-do in Korea. It mainly grows on sandy soils in coastal wetlands. Although the reports on the bioactivities of *C. japonicum* is limited to anti-tumor [6] and anti-inflammatory [18] effects, there is no report on the cosmeceutical potential of the plant to the best of our knowledge.

Therefore, in this study, anti-melanogenic effect of *C. japonicum* crude extract was analyzed in α-MSH-stimulated B16F10 murine melanoma cells to confirm its skin-whitening effects via inhibition of melanogenesis and its related factors.

Materials and Methods

Plant material and extraction

C. japonicum Miq. plants used in this study was collected from the coast of Donggeom-ri, Gilsang-myeon, Ganghwa-gun (Gyeonggi-do, Korea) and was kindly provided by Korea Maritime University (Yeongdo, Busan, Korea).

Air-dried whole plants were ground to powder and extracted by immersing in methylene chloride (CH_2Cl_2) for 24 hr at room temperature with constant stirring. Following 24 hr, the extract was filtered and put aside while the remaining residue was extracted with same method once more. Remaining residue was then extracted by immersing in methanol under same conditions with previous extraction. The final crude extract was obtained by mixing methanol and CH_2Cl_2 extracts and concentrating using a rotary vacuum evaporator.

Tyrosinase inhibitory activity assay

In vitro tyrosinase inhibitory activity of the *C. japonicum* crude extract (CJE) was first tested using mushroom tyrosinase and L-tyrosine as its substrate. Each well of a 96-well plate was filled with 110 μl of 0.1 M phosphate buffered saline (PBS, pH 6.5) and added with 10 μl of different concentrations of CJE (final conc. 10, 20, 30, 40, 50 μg/ml) dissolved in 10% dimethyl sulfoxide (DMSO). Next, 10 μl mushroom tyrosinase (1,800 U/ml) was dispensed to each well. The reaction was started by the addition of 20 μl tyrosinase (1.5 mM) and the plate was incubated at 37℃ for 15 min. After 15 min. the absorbance of each well was measured at 490 nm wavelength using Tecan Infinite F200 microplate reader (Mannedorf, Switzerland).

L-DOPA oxidation inhibitory activity assay

The effect of CJE on the inhibition of L-DOPA oxidation, a crucial step in melanogenesis, was analyzed *in vitro* using mushroom tyrosinase and L-DOPA as its substrate. Each well of 96-well plate was filled with 170 μl of PBS (pH 7.0) and added with 10 μl of mushroom tyrosinase (1,800 U/ml) and CJE (final conc. 10, 20, 30, 40, and 50 μg/ml). Reaction was started by adding 10 μl of 10 mM L-DOPA and the plate was incubated at 37℃ for 10 min. Absorbance values was measured at 475 nm with Tecan Infinite F200 microplate reader. For positive control 1 μM Kojic acid were used.

Cell culture and cytotoxicity assay

B16F10 murine melanoma cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea). Cells were fed

Dulbecco's modified Eagle's medium (DMEM, Welgene, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% L-Glutamine penicillin streptomycin solution (Welgene). Cells were kept at 37℃ incubators with 5% CO_2.

Any cytotoxic presence of CJE in B16F10 cells were assessed by traditional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates with 5×10^3 cells/well density and incubated for 24 hr. Each well was then treated with different concentration of CJE and the plate was further incubated for 24 hr. Next, the wells were aspirated and added with 100 μl of MTT reagent (0.05%, m/v). The viability of the cells were measured by exchanging MTT reagent in wells with same amount of 100% DMSO and subsequent absorbance measurement at 540 nm using Tecan Infinite F200 microplate reader.

Tyrosinase production inhibitory activity assay

Inhibitory effects of CJE on the cellular tyrosinase production was tested using α-MSH-stimulated B16F10 murine melanoma cells. Cells were seeded at 6-well plates at 5×10^4 cells/well density and incubated for 24 hr. Following incubation melanogenesis in B16F10 cells were stimulated by addition of 100 nM α-MSH. Different concentrations of CJE were then added to each well and the cells were incubated for further 72 hr. After 72 hr., cells were washed with PBS and lysed for 30 min. by adding 200 μl lysis buffer to each well. Cell lysates were harvested and centrifuged at 13,000 rpm at 4℃. Supernatant was collected and assayed for total protein content via commercial protein assay kit (Bio-Rad, Hercules, USA). Supernatant from each sample with same amount of protein were then mixed with 0.1 M sodium phosphate buffer (pH 6.8) to give the final volume of 150 μl and added to 96-well plates. Reaction was started by adding 50 μl of 0.1% (m/w) L-DOPA. Plate was incubated at 37℃ for 1 hr for tyrosinase in the supernatant oxidize L-DOPA. After 1 hr, the absorbance was measured at 490 nm, and the tyrosinase activity was measured as a relative percentage of α-MSH-stimulated but not sample-treated control group.

Measurement of intracellular melanin content

The intracellular melanin content in α-MSH-stimulated B16F10 murine melanoma cells was measured using the cell lysates from tyrosinase production inhibitory activity assay. Lysates were washed with ice-cold 75% ethanol and air-dried. Two hundred microliters of 1 N NaOH containing 1% DMSO was added to each lysate and the intracellular melanin was dissolved by keeping lysates at 80-90℃ for 1 hr. Then, 50 μl of each lysate was dispensed into the 96-well plate wells. Absorbance was then measured at 405 nm using Tecan Infinite F200 microplate reader. A standard calibration curve was prepared using purified melanin (Sigma-Aldrich, St. Louis, MO, USA) and measuring absorbance of different purified melanin concentrations at 405 nm. The intracellular melanin content was then calculated using this standard curve.

Measurement of melanin secretion

Extracellular melanin content was measured in cultured α-MSH-stimulated B16F10 murine melanoma cells. Cells were seeded at 6-well plates at 5×10^4 cells/well density and incubated for 24 hr. Prior to α-MSH-stimulation and sample treatment, culture medium was swapped with DMEM without phenol red. Cells were incubated for further 72 hr after100 nM α-MSH addition and treatment with different concentrations of CJE. After 72 hr, cell culture medium was harvested and used for melanin content measurement. Briefly, 50 μl of culture medium from each well was dispensed into 96-well plate wells and absorbance was measured at 405 nm. The melanin content in the culture medium was calculated using the standard curve prepared previously.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The mRNA expression of melanogenesis-related factors in B16F10 melanoma cells were analyzed by RT-PCR. Cells were seeded, α-MSH-stimulated and treated with different concentration of CJE as previously described in intracellular melanin content measurement assay. After 72 hr treatment, total RNA was isolated from cells with an AccuPrep Universal RNA extraction kit (Bioneer, Daejeon, Korea) following the enclosed protocol. Reverse transcription of same amount of RNA (2 μg) from each sample was carried out with CellScript All-in-One cDNA synthesis kit (CellSafe, Yongin, Korea) according to manufacturer's protocol. PCR reaction followed the cDNA synthesis with the gene-specific sense and antisense primers [8] and Luna Universal PCR mix following manufacturer's protocol. After PCR, samples were run on 1.5% agarose gel and stained with ethidium bromide to obtain bands.

Western blotting

The protein expression of melanogenesis-related factors in α-MSH-stimulated B16F10 melanoma cells were analyzed by common Western blotting assay. Briefly, cells were seeded,

α-MSH-stimulated and treated with different concentration of CJE as previously described. After 72 hr treatment, cells were lysed with RIPA buffer (Sigma-Aldrich). Lysates were centrifuged at 13,000 rpm at 4℃ and the supernatant was used for the assay. Supernatant from each well that contains same amount protein (20 μg) according to protein content assay was used for Western blotting. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose blotting membrane (Amersham, Freiburg, Germany). Then membranes were first blocked with 5% skim milk for 1 hr, washed with TBS-T buffer for 30 min, and hybridized with primary antibodies overnight at 4℃. Next, the membranes were reacted with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature after washing with TBS-T for 30 min. Protein bands on membranes were visualized using an ECL solution kit (Amersham) following manufacturer's directions. Images of the bands were taken with a CAS400M Imager (Davinch-K, Seoul, Korea).

Statistical analysis

All data is given as an average of three independent experiments (n=3) ± standard deviation. Statistical differences were defined after one way analysis of variance coupled with Duncan's multiple range post-hoc test.

Results and Discussion

Inhibition of tyrosinase activity

Tyrosinase is the most crucial enzyme in the melanin synthesis pathway and the oxidation of tyrosinase by the tyrosinase is the rate-determining step in melanogenesis. Inhibition of the tyrosinase activity directly results in decrease of melanin production. Treatment with CJE showed a dose-dependent inhibitory activity on the mushroom tyrosinase (Fig. 1A). The IC_{50} of the CJE was calculated as 123.6 μg/ml for the inhibition of tyrosine oxidation by the tyrosinase.

Inhibition of L-DOPA oxidation

L-DOPA is the product of tyrosine oxidation. The further oxidation of L-DOPA by the tyrosinase enzyme catalyzes the further steps of melanogenesis and thereby the melanin production. The effect of CJE on L-DOPA oxidation was analyzed with a similar method to tyrosine oxidation. Results again showed that CJE treatment decreased the L-DOPA oxidation in a dose-dependent manner (Fig. 1B). One millimolar Kojic acid was used as a positive control tyrosinase inhibitor. At the highest concentration treated (50 μg/ml), the L-DOPA oxidation was decreased to the 64% of the untreated control. However, Kojic acid treatment showed 55% decrease, higher inhibition rates compared to CJE treatment.

Inhibition of intracellular tyrosine production

Prior to any *in vitro* experiments involve B16F10 melanoma cells, cytotoxicity of CJE was established. MTT assay results showed that concentrations higher than 50 μg/ml CJE caused a decrease in cell viability more than 10% (Fig. 2A).

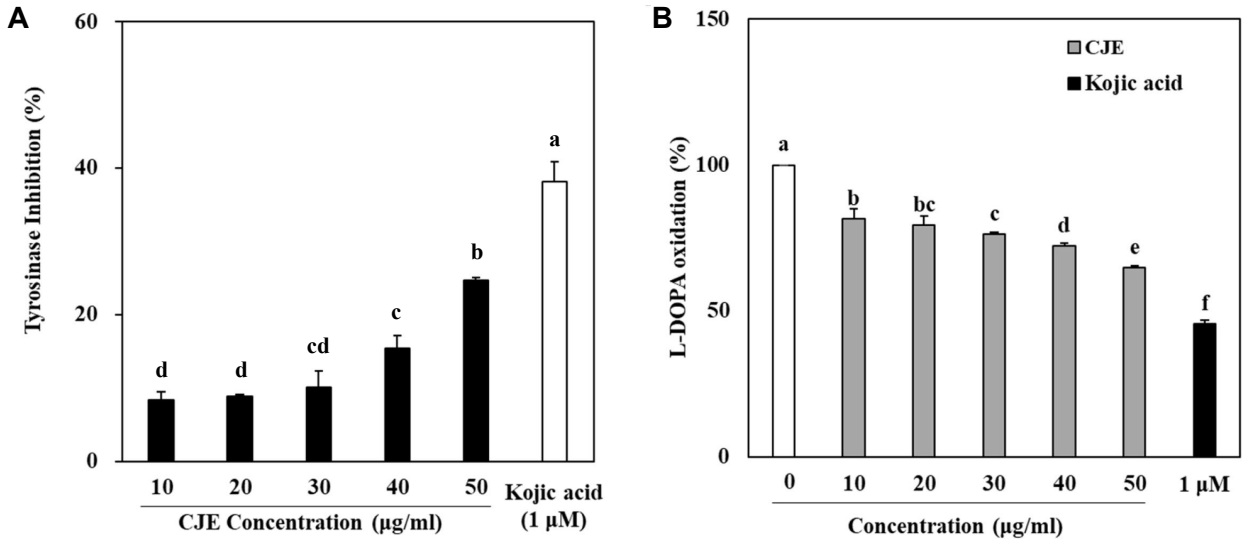

Fig. 1. Effect of CJE on the *in vitro* tyrosinase activity (A) and L-DOPA oxidation (B). Kojic acid, a tyrosinase inhibitor, was used a positive control. [a-f]Means with different letters are significantly different (p<0.05) by Duncan's multiples range test.

Therefore, all experiments with cells were conducted using CJE concentrations not higher than 50 μg/ml.

B16F10 melanoma cells were stimulated by α-MSH in order to mimic the UV-induced melanin overexpression. Studies showed that α-MSH presence in the culture medium stimulates the melanin production in same way with UV exposure [10]. The tyrosinase inhibitory activity of CJE was previously assessed using mushroom tyrosinase. This time, the activity of tyrosinase in CJE treated cells were tested to evaluate the effect of CJE on intracellular active tyrosinase amounts. As seen in Fig. 2B, α-MSH-stimulated cells expressed significantly increased tyrosinase activity. The activities of tyrosinase from the cells treated with 5, 10, and 50 μg/ml CJE were measured to be 58.5%, 55.0% and 51.3% of the untreated α-MSH-stimulated control group. In addition, all treated groups showed less tyrosinase activity than that of 1 μM Kojic acid treatment. Overall, the results indicated that CJE treatment decreased the amount of active tyrosinase in melanoma cells which was significantly increased by α-MSH stimulation.

Inhibition of intracellular and extracellular melanin content

The final product of the melanogenesis is the melanin pigment, of which accumulation is the main reason behind pigmentation disorders and complications [17]. The higher

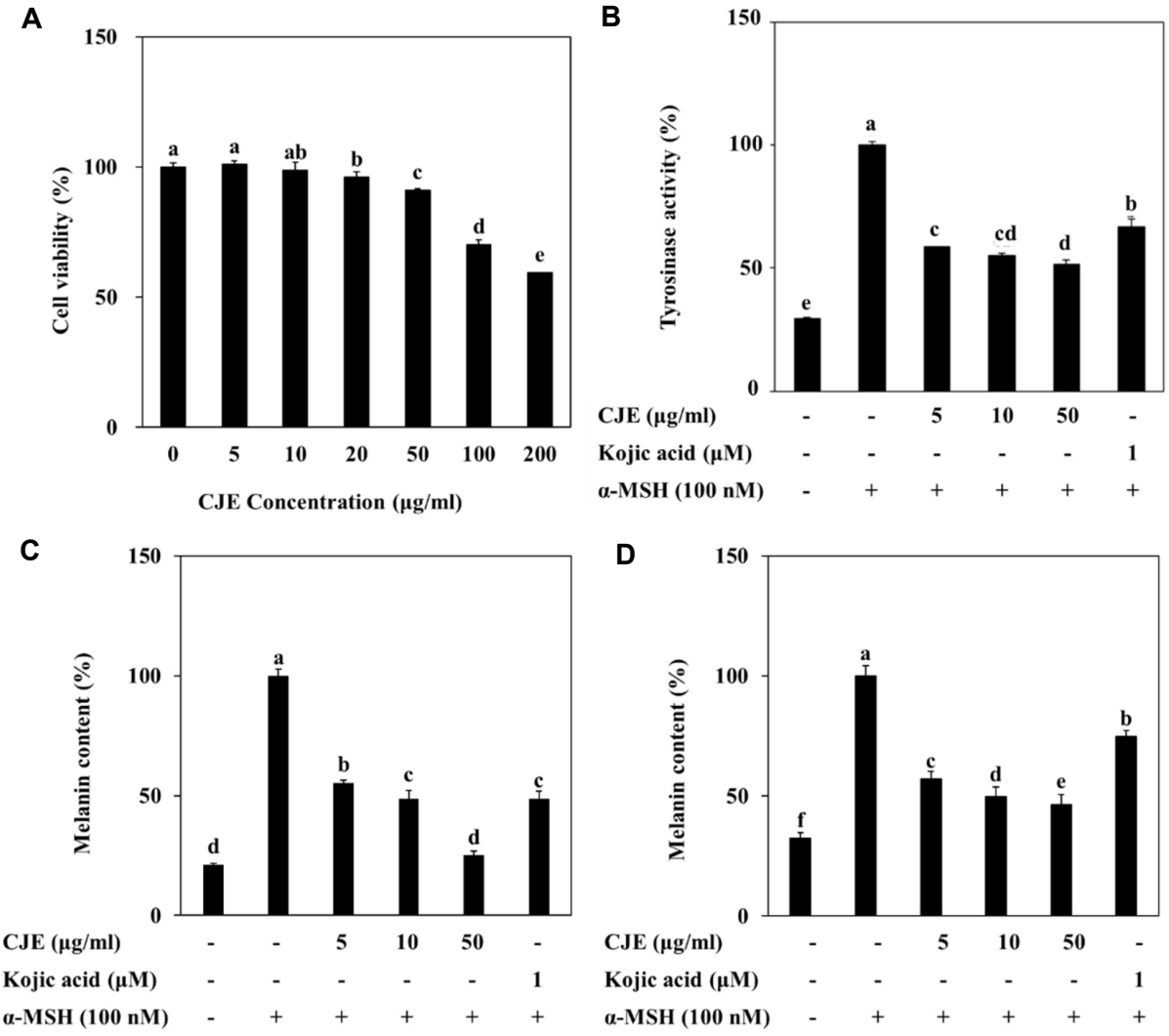

Fig. 2. Effect of CJE on the viability (A), intracellular tyrosinase content (B), and extracellular (C) and intracellular (D) melanin content of α-MSH-stimulated B16F10 murine melanoma cells. Cells were treated with or without CJE at given concentrations for 72 hr. Tyrosinase content was measured as a relative percentage of the activity of the intracellular tyrosinase obtained from untreated control group. [a-f]Means with different letters are significantly different (p<0.05) by Duncan's multiples range test.

amount of melanin stored in cells or secreted to extracellular matrix means darker complexion, freckles and age spots. Therefore, tyrosinase inhibitory effect of CJE was further analyzed via amount of melanin content in α-MSH-stimulated B16F10 melanoma cells.

As seen in Fig. 2C and 2D, α-MSH presence significantly increased both intracellular and extracellular melanin content, therefore indicating stimulated melanogenesis. On the other hand, cells treated with CJE contained less melanin. Treatment with 5, 10, and 50 μg/ml CJE resulted in 43%, 50.4%, and 53.8% less intracellular melanin amount, respectively (Fig. 2C). Similar trend was observed for extracellular melanin content as the treatment with 5, 10, and 50 μg/ml CJE decreased the secreted melanin amount to 55.4%, 48.8%, and 25.0% of the untreated control group (Fig. 2D). Treatment with 1 μM Kojic acid as positive control resulted in 51.3% extracellular and 25.3% intracellular melanin content decrease, both of which was under the level of decrease observed by the CJE treatment. These results confirmed the previous tyrosinase inhibitory effect of CJE as the inhibition of tyrosinase was translated into halted melanin production in melanoma cells.

Effect of CJE on the mRNA and protein expression of melanogenesis-related factors

Following the confirmation of the effect of CJE on tyrosinase activity and melanin production, the expressional changes in melanogenesis role players were evaluated in order to elucidate the action mechanism of CJE. The mRNA and protein expressions of tyrosinase enzyme as well as MITF, TRP-1 and TRP-2 were analyzed for this purpose. As previously noted, tyrosinase the key enzyme responsible for the oxidation of tyrosine towards the end product melanin. While MITF is responsible for the transcriptional regulation of TRP-1 and TRP-2 which are regulatory proteins take role in the downstream of conversion of tyrosine to melanin along with tyrosinase. In this context, B16F10 melanoma cells were stimulated with α-MSH to stimulate the expression of MITF and subsequent factors that carry out melanin production. As seen in Fig. 3, α-MSH-stimulation lead to significant increase in expression of mRNA (Fig. 3A) levels of tyrosinase, MITF, TRP-1 and TRP-2 as well as protein (Fig. 3B) levels of tyrosinase and MITF. However, treatment with CJE showed a dose-dependent suppression on both mRNA and protein expression of these factors. These results showed that CJE treat-

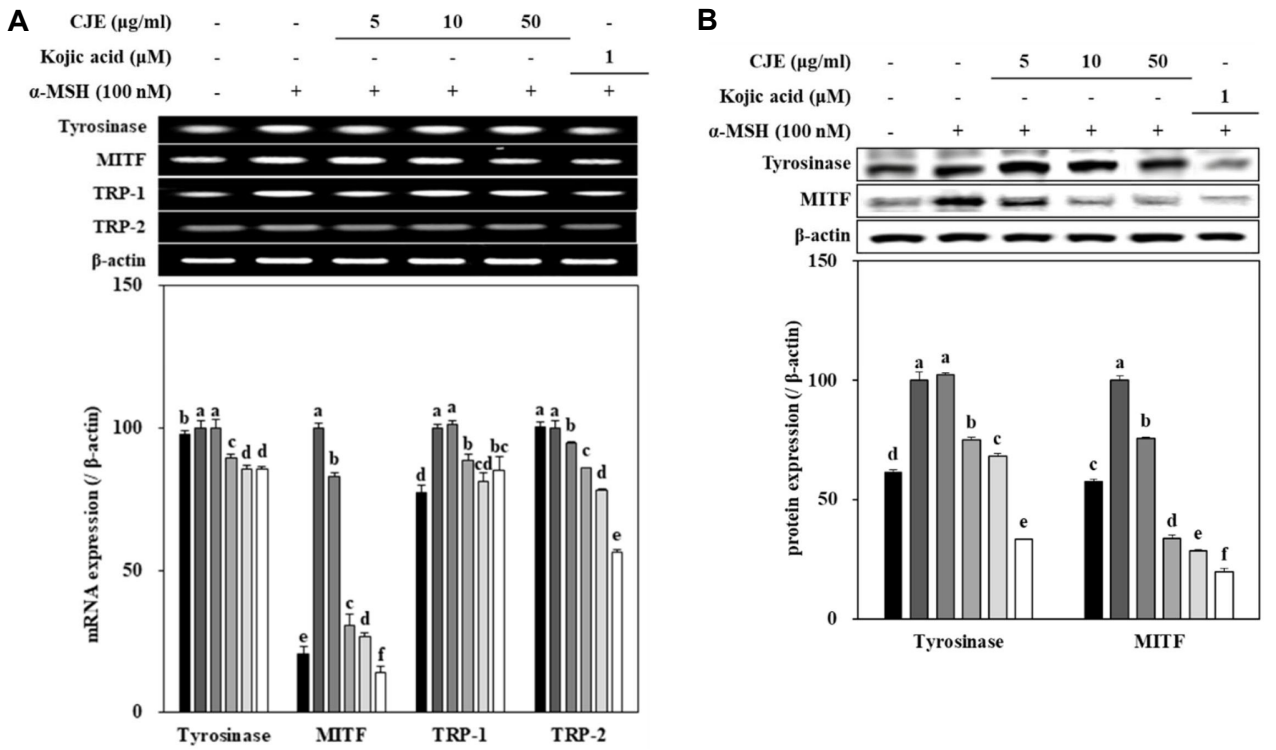

Fig. 3. Effect of CJE on the mRNA (A) and protein (B) expression levels of melanogenesis-related factors in α-MSH-stimulated B16F10 murine melanoma cells. Cells were treated with or without CJE at given concentrations for 72 hr. Bands were densiometrically quantified, normalized against internal loading control β-actin and given as relative percentage of untreated control group. [a-f]Means with different letters are significantly different (p<0.05) by Duncan's multiples range test.

ment not only inhibits the tyrosinase activity, but it also suppresses the melanogenesis pathway by regulating the expression of related factors in both mRNA and protein levels.

Effect of CJE on GSK3β/β-catenin signaling pathway

Activation of GSK3β via cAMP plays a key role in the melanogenesis by facilitating the intrinsic transcriptional activity of MITF to stimulate tyrosinase, TRP-1 and TRP-2 expressions [2, 3]. On the other hand, increased activation of GSK3β via upstream activation of pKA and cAMP induces nuclear translocation of β-catenin which further stimulates the transcriptional activities of MITF [19]. As seen in Fig. 4A, α-MSH-induced melanin production stimulation in B16F10 melanoma cells exhibited a significant increase in GSK3β phosphorylation and β-catenin levels. Treatment with 50 μg/ml CJE was able to decrease the both phosphorylation of GSK3β and the total β-catenin levels. These results indicated that the effect of CJE on melanogenesis might stemmed from its suppressive properties on PKA-dependent activation of MITF.

Effect of CJE on the activation of PKA and CREB

The UV-induced activation of melanogenesis via downstream effectors of α-MSH receptor MC1R is initially started with the phosphorylation of PKA and CREB [5]. The cAMP/PKA-dependent activation of MITF is the key for melanin production. In the previous assay, it was shown that the CJE treatment suppressed the phosphorylation of GSK3β and therefore the activation of MITF. Next, the effect of CJE on the upstream activators of melanogenesis, PKA and CREB was analyzed. As expected, stimulation by α-MSH significantly increased the activation of PKA and CREB in B16F10 melanoma cells (Fig. 4B). Treatment with 50 μg/ml CJE suppressed the PKA phosphorylation significantly. Although it was statistically significant, the decrease in the activation of CREB by CJE treatment was not as notable as PKA. Overall, results suggested that CJE inhibited the α-MSH-induced melanogenesis in B16F10 melanoma cells via suppression of PKA-dependent activation of MITF signaling (Fig. 5).

In conclusion, current study confirmed the anti-melanogenic potential of CJE. It was shown that CJE was able to inhibit the tyrosinase activity on both tyrosine and L-DOPA. In addition, CJE decreased the α-MSH-induced melano-

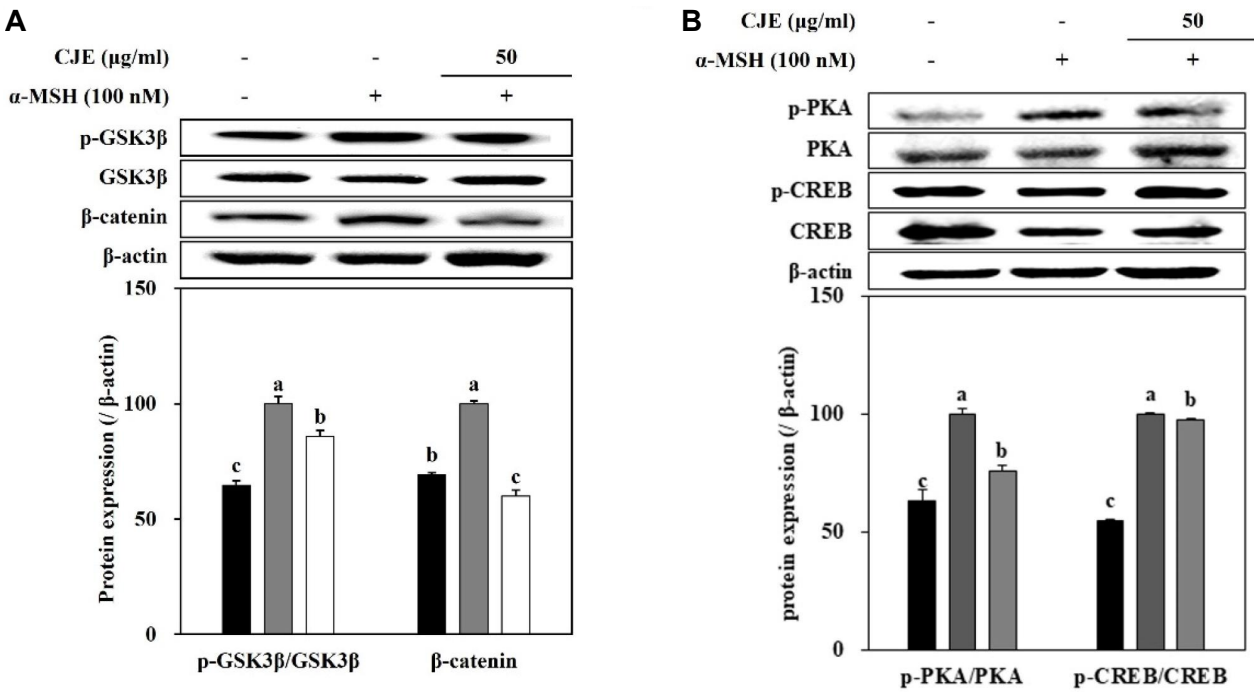

Fig. 4. Effect of CJE on the activation of GSK3β (A) and PKA (B) signaling cascades given as protein expression levels in α-MSH-stimulated B16F10 murine melanoma cells. Cells were treated with or without CJE at given concentrations for 72 hr. Bands were densiometrically quantified, normalized against internal loading control β-actin and given as relative percentage of untreated control group. [a-f]Means with different letters are significantly different (p<0.05) by Duncan's multiples range test.

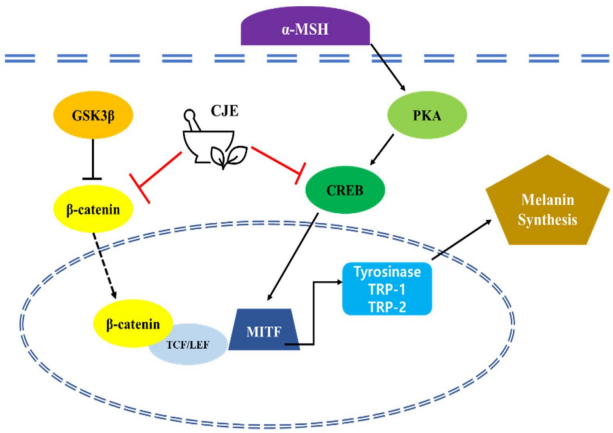

Fig. 5. Suggested intracellular anti-melanogenic action mechanism of *C. japonicum* extract (CJE).

genesis in B16F10 melanoma cells shown as decreased melanin and tyrosinase content via suggested mechanism of suppressing the PKA-dependent activation of MITF. Overall, it was suggested that *C. japonicum* might contain novel natural products to be utilized as cosmeceuticals that have tyrosinase inhibitory and anti-melanogenic properties.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : B16F10 피부 흑색종세포에서 갯사상자 추출물의 멜라닌 합성 저해 효과

조현진[1] · 카라데니즈 파티[2] · 오정환[2] · 서영완[3] · 공창숙[1,2*]

([1]신라대학교 의생명과학대학 식품영양학과, [2]신라대학교 해양식의약소재융합기술연구소, [3]한국해양대학교 해양과학융합학부)

멜라닌은 자외선과 같은 외부자극이 가해지면 피부 기저층에 존재하는 멜라닌 세포에서 피부를 보호하기 위한 방어기전으로써 생성이 된다. 하지만 과도한 자외선 노출로 멜라닌이 필요이상으로 생성이 되면 기미, 주근깨, 검버섯과 같은 색소침착 및 색소성 피부장애를 유발할 수 있다. 최근에는 부작용이 적은 식물 추출물을 대상으로 미백소재를 찾기 위한 연구들이 활기를 띠고 있다. 이에 본 연구에서는 국내 서식 염생식물인 갯사상자 추출물을 이용하여 B16F10 흑색종 세포에서 피부 색소 멜라닌 생성 억제에 미치는 효과를 확인하였다. 갯사상자 추출물 처리시 tyrosine 및 L-DOPA 산화를 농도 의존적으로 저해하였으며 세포 내의 멜라닌 생성을 담당하는 tyrosinase, tyrosinase-related protein-1, -2 발현을 억제하였다. 이는 갯사상자 추출물이 α-MSH에 의한 세포신호 전달 경로인 GSKβ/β-catenin 및 PKA/CREB 조절에 의한 것으로 밝혀졌다. 따라서 갯사상자 추출물은 GSKβ/β-catenin 및 PKA/CREB 기전을 통해 멜라닌 합성을 억제하여 미백 효능 지닌 천연물 유래 기능성 화장품 소재로서 활용 가능할 것으로 사료된다.