



Original Article

Asian Pacific Journal of Tropical Biomedicine



apjtb.org

doi: 10.4103/apjtb.apjtb_568_24

Ligustroside derived from *Ligustrum japonicum* inhibits melanogenesis *via* blocking the MAPK and PKA/CREB signaling pathwaysXian-Rong Zhou^{1,2}, Fatih Karadeniz³, Jung Hwan Oh^{3,4}, Youngwan Seo⁵, Chang-Suk Kong^{1,3,6}✉¹Department of Bioscience, Silla University, Busan 46958, Korea²Collaborative Innovation Center for Child Nutrition and Health Development, Chongqing University of Education, Chongqing 400067, China³Marine Biotechnology Center for Pharmaceuticals and Foods, Silla University, Busan 46958, Korea⁴Nutritional Education, Graduate School of Education, Silla University, Busan 46958, Korea⁵Division of Convergence on Marine Science, Korea Maritime & Ocean University, Busan, 49112, Korea⁶Department of Food and Nutrition, Silla University, Busan 46958, Korea

ABSTRACT

Objective: To investigate the anti-melanogenic potential of ligustroside isolated from *Ligustrum japonicum*.**Methods:** The cytotoxicity of ligustroside was tested *via* MTT assay. Furthermore, the effects of ligustroside on the expression of critical melanogenic markers such as tyrosinase, tyrosinase related proteins (TRPs), and microphthalmia-associated transcription factor (MITF) were analyzed at both mRNA and protein levels *via* RT-qPCR and Western blot, respectively, in α -melanocyte stimulating hormone-induced B16F10 cells. In addition, phosphorylation of p38, ERK and JNK proteins was investigated. Immunofluorescence analysis of MITF was also conducted.**Results:** Ligustroside significantly reduced intracellular tyrosinase activity and melanin content by 37.11% and 29.12%, respectively, compared to untreated cells. Moreover, it downregulated the expression of MITF, tyrosinase, TRP-1, and TRP-2 at the mRNA and protein levels by regulating both the mitogen-activated protein kinase (MAPK) and protein kinase A (PKA)/cAMP response element-binding protein (CREB) signaling pathways. Ligustroside also suppressed the nuclear protein expression of MITF, β -catenin, and p-CREB, and decreased immunofluorescence intensity of nuclear MITF.**Conclusions:** Ligustroside derived from *Ligustrum japonicum* shows a significant anti-melanogenesis effect *via* suppression of the MAPK and PKA/CREB signaling pathways.**KEYWORDS:** *Ligustrum japonicum*; Ligustroside; MAPK; Melanogenesis; CREB; PKA

1. Introduction

Melanin is a naturally occurring pigment synthesized from tyrosine, and it plays a crucial role in the skin. The primary function of melanin is to absorb ultraviolet (UV) radiation and protect the skin from UV damage. It also imparts color to the skin, hair, and eyes[1]. However, excessive production of melanin in the skin can lead to hyperpigmentation disorders such as freckles, melasma,

Summary

Question: How does ligustroside, a bioactive compound isolated from *Ligustrum japonicum*, affect α -melanin stimulating hormone-induced melanogenesis in B16F10 murine melanoma cells?**Findings:** Ligustroside suppressed α -melanin stimulating hormone-induced elevation in melanin production and inhibited the expression of melanogenic proteins *via* possible regulation of mitogen-activated protein kinase and protein kinase A/CREB signaling pathways.**Meaning:** Ligustroside may be utilized as a natural ingredient in cosmeceuticals that target hyperpigmentation. Future *in vivo* studies are required to verify its efficacy.

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How to cite this article: Zhou XR, Karadeniz F, Oh JH, Seo Y, Kong CS. Ligustroside derived from *Ligustrum japonicum* inhibits melanogenesis *via* blocking the MAPK and PKA/CREB signaling pathways. Asian Pac J Trop Biomed 2025; 15(2): 65-74.**Article history:** Received 23 September 2024; Revision 9 October 2024; Accepted 30 December 2024; Available online 17 January 2025

and lentigines. The excessive activity of melanocytes primarily causes these disorders and may be associated with factors such as sun exposure, genetic influences, and hormonal changes[2]. Additionally, excessive melanin production is also associated with the development of melanoma, a malignant skin tumor that can metastasize to other sites if not diagnosed and treated early, posing a significant threat to the health and life of affected individuals[3].

The generation of skin pigmentation is a complex and crucial biological process, in which tyrosinase, an enzyme, and transcription factors involved in regulating melanin synthesis play key regulatory roles. Microphthalmia-associated transcription factor (MITF) is widely considered the primary and crucial regulator of melanin production in dark skin[4]. The expression of MITF is closely linked to the development and differentiation of melanocytes, and it controls a series of gene expressions, such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2), which participate in the regulation of melanin synthesis[5]. Tyrosinase is a critical enzyme catalyzing the conversion of tyrosine to dihydroxyphenylalanine (DOPA) in melanin synthesis. TRP-1 and TRP-2, on the other hand, are responsible for further converting DOPA to melanin granules, thus forming the final pigment melanin[6].

Except for the regulation effect on tyrosinase, TRP-1, and TRP-2, MITF also acts as a downstream target of various signaling pathways, including protein kinase A (PKA)/cAMP response element binding protein (CREB), glycogen synthase kinase 3 β (GSK3 β)/ β -catenin, and MAPKs[7,8]. Activation of these pathways occurs in melanocytes upon stimulation of melanocortin 1 receptor and receptor tyrosine kinase by their respective ligands α -melanocyte stimulating hormone (α -MSH) and stem cell factor[9]. PKA and CREB activation, triggered by α -MSH, leads to an increase in cAMP levels, resulting in upregulated *MITF* gene expression. Elevated MITF protein production positively influences tyrosinase and TRP expressions, promoting melanin synthesis[10]. MAPK family proteins, like p38 and JNK, enhance MITF expression, while phosphorylation of ERK MAPKs inhibits melanogenesis[11]. Additionally, Wnt signaling pathways are critical for melanocyte development and melanogenesis, as β -catenin accumulation in the nucleus promotes MITF expression, cell proliferation, and melanin production. These signaling pathways show potential as novel therapeutic targets for modulating melanin synthesis[12].

Ligustrum japonicum (*L. japonicum*) belongs to the Oleaceae family under the genus *Ligustrum*. *L. japonicum* is primarily distributed in China, Japan, and Korea, holding a longstanding historical application in traditional herbal medicine and traditional Chinese medicine[13]. *L. japonicum* is known to contain active compounds such as triterpenoids, cyclohexene oxides, phenylethanol derivatives, and flavonoids[13–15]. *L. japonicum* contains nearly a hundred cyclohexene oxide terpenoids, which are primarily divided into two categories: cyclohexene oxide terpenes and cleavage-ring cyclohexene oxide terpenes. Among these, the cyclohexene oxide terpene glycosides are the most abundant and representative

compounds found in this plant, such as ligustroside, neo-ligustroside, and specnuezhenide[16]. Ligustroside is a derivative of secoiridoid compounds, and studies have shown that this active compound has antiviral, mitochondrial dysfunction improving, and antioxidant effects[17,18], but the effect of ligustroside on melanogenesis remains unknown. Reports showed that several secoiridoids such as isoorientin exert anti-melanogenic and/or tyrosinase inhibitory activities[19–21]. Therefore, the present study aimed to evaluate the anti-melanogenesis effect of ligustroside on α -MSH-induced B16F10 murine melanoma cells.

2. Materials and methods

2.1. Experimental materials

The fruit samples of *L. japonicum* were procured from Jeju Island and generously supplied by the Division of Marine Bioscience at Korea Maritime and Ocean University (Busan, Korea). A voucher specimen (No. 17Y-1) was deposited in the Division of Convergence on Marine Science, Korea Maritime & Ocean University (Busan, Korea). The cell culture medium, penicillin/streptomycin, fetal bovine serum, and other cell culture materials were obtained from Gibco BRL, Life Technology (Grand Island, NY, USA) except the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) which were bought from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for immunoblot were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against proteins TRP-1 (sc-166857), tyrosinase (sc-20035), TRP-2 (sc-166716), JNK (sc-293136) and β -actin (sc-47778) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and MITF (#97800S), PKA (#4781), p-PKA (#9196), CREB (#9104), p-CREB (#9198), p38 (#8690), p-p38 (#4511), p-JNK (#9255), ERK (#4695), p-ERK (#4370), β -catenin (#9562), goat anti-mouse IgG, HRP (#7074), and goat anti-rabbit IgG, HRP (#7076) were purchased from Cell Signaling Technology (Danvers, MA, USA). Kojic acid (K3125, Sigma Chemical Co.) was employed as a positive control in this study due to its known effect to inhibit tyrosinase and melanogenesis[22].

2.2. Extraction and structure identification methods of ligustroside

The extraction and structure identification methods of ligustroside from *L. japonicum* can be found in the previously published article[23]. Briefly, the crude extract was initially obtained from dried *L. japonicum* fruit using CH₂Cl₂ as the solvent. Subsequently, fractionation of the crude extract was performed using a distillation technique by utilizing solvents with different polarities to isolate the *n*-BuOH fraction. *n*-BuOH fraction (10.0 g) was chromatographed

on C18 silica gel using a MeOH-H₂O solvent gradient of decreasing polarity (50%, 60%, 70%, 80%, 90% MeOH in water and 100% MeOH). Fraction 2 (60% MeOH aqueous solution, 2.93 g) was further separated by HP20 column chromatography to obtain 5 fractions: 100% H₂O, 50% MeOH aqueous solution, 50% acetone aqueous solution, 100% MeOH and 100% acetone, which were named HP1 to HP5, respectively. The HP2 (50% MeOH aqueous solution, 0.532 g) fraction was separated by reverse-phase HPLC (ODS-A, 20% MeOH aqueous solution) to obtain compound ligustroside (5.6 mg). The chemical structure of ligustroside was elucidated and verified by 2D NMR spectroscopy and comparison with previously published reports.

2.3. MTT assay

B16F10 cells (Korea Cell Line Bank, Seoul, Korea) were seeded at a density of 1×10^5 cells per well in a 96-well plate and incubated at 37 °C with 5% CO₂ for 24 h. After incubation, different concentrations of ligustroside (1, 5, 10, 15 and 20 µM) were added to the plate and incubated under the same conditions for another 24 h. Following this, the culture medium (DMEM) was removed, and the cells were washed twice with 1×phosphate buffered saline (PBS). Subsequently, 100 µL of MTT solution was added to each well of the 96-well plate, which was then incubated at 37 °C for 4 h. After the incubation period, the MTT solution was removed, and 100 µL of 100% DMSO was added to each well. The plate was then further incubated at 37 °C for 10 min. Finally, the absorbance of each well at 560 nm was measured using a microplate reader (Multiskan GO, Tecan Austria GmbH, Grodig, Austria).

2.4. Measurement of intracellular tyrosinase activity

B16F10 cells were seeded in 6-well plates at a density of 5×10^4 cells/well in phenol red-free DMEM medium and incubated at 37 °C for 24 h. In one set of wells, 100 nM α-MSH was included in the DMEM medium, while the other set served as controls without α-MSH. After the initial 24-hour incubation period, various concentrations of samples and kojic acid (10 µM) were added to the plates, followed by another 24-hour incubation. The DMEM medium was then removed, and the cells were washed twice with 1×PBS. Subsequently, 200 µL of lysis buffer was added to each well, and the plates were placed on ice for 30 min. The cells were then scraped using a cell scraper, and the lysate was collected into tubes. The tubes were centrifuged at $13\,000 \times g$, 4 °C for 30 min, and the supernatant was collected to determine the tyrosinase activity. Additionally, at this step, the cell pellet obtained after centrifugation was later used to measure the intracellular melanin content. For the tyrosinase activity assay, the supernatant containing 50 µg protein was mixed with sodium phosphate buffer (0.1 mol/L, pH 7.0) in a 96-well plate. Subsequently, 50 µL of 0.1% (w/v) L-DOPA was added to each well, and the plate was incubated at 37 °C for 1 h. The

tyrosinase activity was determined by measuring the absorbance at 490 nm.

2.5. Measurement of melanin content

The phenol red-free DMEM medium that was removed in section 2.4 at the end of the ligustroside treatment was used to determine the extracellular melanin content. The absorbance of the culture medium was measured at 405 nm with three replicates for each treatment group. The extracellular melanin content was calculated as a relative percentage value compared to the cells that were not treated but only stimulated with α-MSH. The cell pellet obtained after centrifugation in section 2.4. was used to measure the intracellular melanin content. The cell pellet was washed with pre-cooled 75% ethanol, and the ethanol was subsequently removed. The pellet was then dried at room temperature for 10 min. Next, 200 µL of 1 mol/L NaOH dissolved in 10% DMSO solution was added to the cell pellet. The mixture was lysed at 90 °C for approximately 10 min, ensuring that the melanin was dissolved in the solution. The resulting melanin solution was transferred to a 96-well plate, and the absorbances were measured at 405 nm. The intracellular melanin content was then calculated using a melanin standard curve.

2.6. Measurement of melanogenesis-related genes using RT-qPCR

QIAzol reagent was used to extract the total RNA, and the cDNA was synthesized from total RNA using a premixed cDNA kit (CellSafe, Yongin, Korea). The qPCR amplification system included master mix (10 µL), forward primer (0.5 µL), reverse primer (0.5 µL), cDNA (0.5 µL), and nuclease-free water (8.5 µL). The qPCR amplification was performed using a TP800 Thermal Cycler Dice™ Real-Time System (Takara Bio, Otsu, Japan) with the following conditions: initial heating at 95 °C for 60 s, denaturation at 95 °C for 15 s, extension at 60 °C for 30 s, and the denaturation and extension steps were carried out for 40 cycles. The relative mRNA expression levels of genes were calculated using $2^{-\Delta\Delta CT}$ method and given as fold change compared to the control group, and β-actin was selected as an internal control gene. The primer sequences that were used in the master mix are given in Table 1.

Table 1. Sequences of primers used in RT-PCR analysis.

Gene	Sequence
<i>MITF</i>	Forward: 5'-AAC-CGA-CAG-AAG-AAG-CTG-GA-3' Reverse: 5'-ACA-AGT-TCC-TGG-CTG-CAC-TT-3'
<i>Tyrosinase</i>	Forward: 5'-TTA-TGC-GAT-GGA-ACA-CCT-GA-3' Reverse: 5'-ACT-GGC-AAA-TCC-TTC-CAG-TG-3'
<i>TRP-1</i>	Forward: 5'-AGG-AAT-CTG-GCT-TGG-GAT-TT-3' Reverse: 5'-AGA-AGA-CAG-GGG-TGC-TCA-GA-3'
<i>TRP-2</i>	Forward: 5'-AGC-AGA-CGG-AAC-ACT-GGA-CT-3' Reverse: 5'-GCA-TCT-GTG-GAA-GGG-TTG-TT-3'
<i>β-actin</i>	Forward: 5'-CCA-CAG-CTG-AGA-GGG-AAA-TC-3' Reverse: 5'-AAG-GAA-GGC-TGG-AAA-AGA-GC-3'

2.7. Western blot analysis

The cells were lysed using RIPA reagent, and the total protein concentration was determined using a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein samples (20 µg) were mixed with protein loading buffer and boiled before loading onto 10% SDS-PAGE gels. The nuclear fraction of the cells were extracted using NE-PER™ Nuclear Extraction Kit (#78835; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Both total protein and nuclear fractions were subjected to same protocols for immunoblotting. After SDS-PAGE, the proteins were transferred onto PVDF membranes (Bio-Rad Laboratories Inc., California, USA) and blocked with 5% skim milk. Primary antibodies were incubated with the membranes overnight at 4 °C, followed by washing and incubation with secondary antibodies for 1 h at room temperature. Both primary and secondary antibodies were diluted in specific buffers and concentrations according to the suppliers' instructions. Protein bands were visualized using a chemiluminescent substrate (Biomax, Seoul, Korea) and imaged using CAS-400SM Davinch-Chemi Imager (Davinch-K, Seoul, Korea). The expression levels of proteins were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), with β -actin serving as the internal control protein for normalization.

2.8. Immunofluorescence staining

The expression of MITF in α -MSH-induced B16F10 cells was analyzed by immunofluorescence staining. Cells were cultured and induced as described earlier but on glass coverslips placed in 6-well plates and stimulated with α -MSH in the presence and absence of ligustroside. After 24 h treatment, cells were hybridized with primary MITF antibody at 4 °C overnight, followed by treatment with the Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (A-11008, Invitrogen, Carlsbad, CA, USA). Next, the

cells were stained with ProLong Gold Antifade Reagent with DAPI (#8961; Cell Signaling Technology) for 1 h after which the cells were fixed on glass coverslips and observed *via* commercial Immunofluorescence Application Solutions Kit (#12727; Cell Signaling Technology), according to the manufacturer's instructions.

2.9. Statistical analysis

The experimental data are reported as mean $\pm$ standard deviation (SD), and each experiment was performed with a minimum of three replicates. Statistical analysis was conducted using SPSS 21.0 software (IBM Corp., Armonk, NY, USA) to determine the significance between groups. A one-way ANOVA test followed by Duncan's multiple range test was employed, and a *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxic effect of ligustroside on B16F10 cells

The cytotoxic effect of ligustroside was evaluated on B16F10 cells at concentrations of 1, 5, 10, 15 and 20 µM (Figure 1A). When the concentrations ranged from 1-10 µM, the cell viability was 100.96%, 100.60%, and 99.20%, respectively, which presented no significant difference compared to the control group (*P*>0.05). But at 15 and 20 µM, the cell viability decreased to 71.78% and 56.78%, respectively, indicating that these concentrations had a markedly cytotoxic effect on B16F10 cells. Based on these results, the nontoxic concentrations of 1, 5, and 10 µM were selected to perform further experiments.

3.2. Effect of ligustroside on intracellular tyrosinase activity

As shown in Figure 1B, the intracellular tyrosinase activity was lowest in the blank group and highest in the α -MSH-stimulated

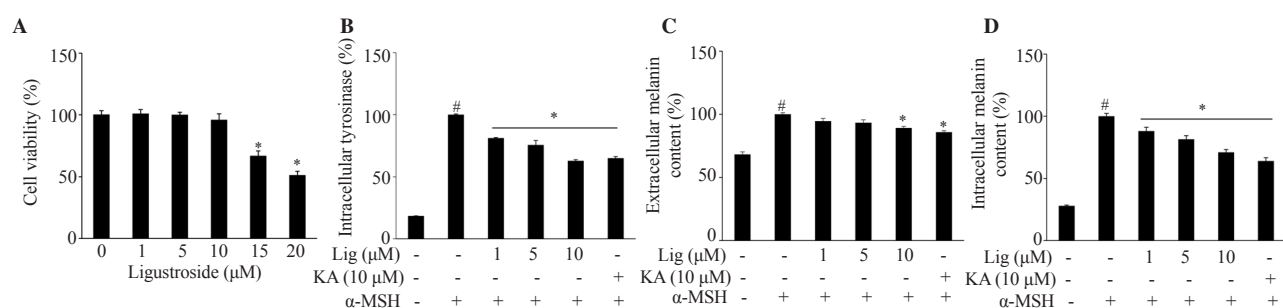


Figure 1. Effect of ligustroside on cell viability and tyrosinase and melanin content in α -MSH (100 nM)-induced B16F10 cells. (A) Cytotoxicity of ligustroside: Cells were treated with ligustroside at the indicated concentrations for 24 h and cell viabilities were measured by MTT assay; (B) intracellular tyrosinase activity was measured after 24 h treatment using *L*-DOPA as a substrate; (C) extracellular melanin content was measured using the colorless cell culture medium following 24 h treatment; (D) intracellular melanin content was determined after 24 h treatment. ^{*}*P*<0.05 vs. control cells. [#]*P*<0.05 vs. the blank group. α -MSH: alpha-melanocyte stimulating hormone; KA: kojic acid; Lig: ligustroside.

group ($P<0.05$). However, it was decreased significantly after the α -MSH-stimulated B16F10 cells were treated with ligustroside at 1, 5, and 10 μ M ($P<0.05$), in which 10 μ M of ligustroside exhibited the most significantly inhibitory effect on intracellular tyrosinase.

3.3. Effect of ligustroside on extracellular and intracellular melanin content

As presented in Figure 1C, the extracellular melanin content was

substantially increased in the α -MSH-stimulated group compared with the blank group ($P<0.05$). Treatment with 1, 5, and 10 μ M of ligustroside lowered extracellular melanin content (94.57%, 93.21%, and 89.13%, respectively) in α -MSH-stimulated B16F10 cells, with only 10 μ M showing a significant effect ($P<0.05$).

Figure 1D demonstrates the intracellular melanin content in the blank group (28.04%) was significantly lower than that in the α -MSH-stimulated group (100%) ($P<0.05$). Treatment with 1, 5, and 10 μ M of ligustroside markedly decreased α -MSH-induced increase

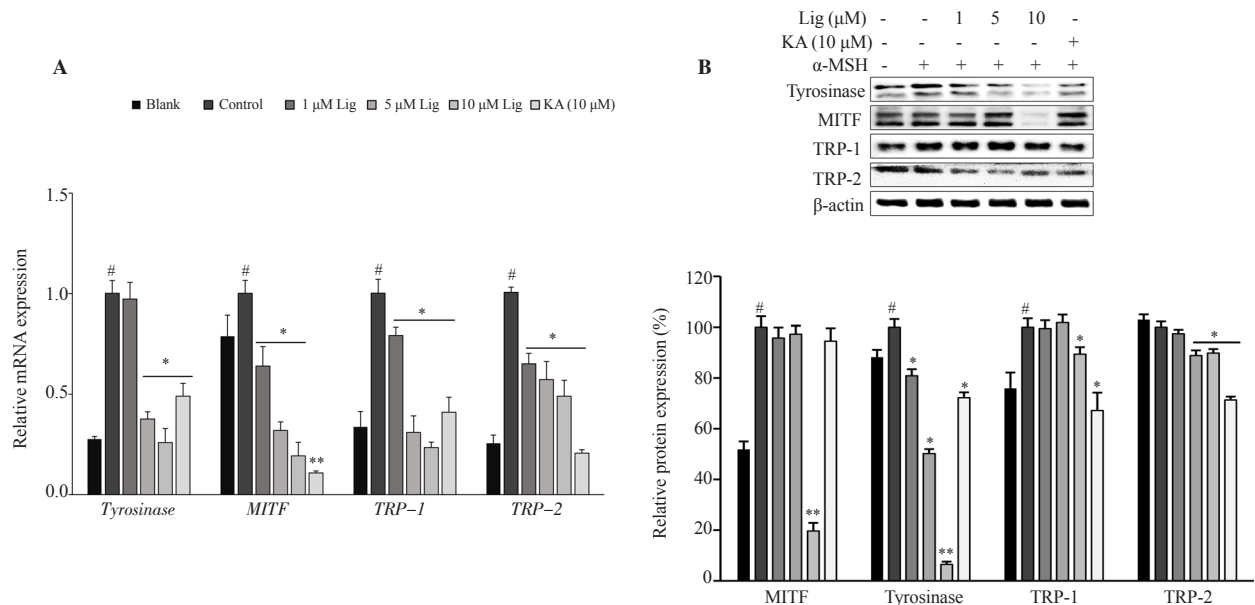


Figure 2. Effect of ligustroside on mRNA and protein expression levels of tyrosinase, MITF, TRP-1, and TRP-2 in α -MSH-stimulated B16F10 cells. Cells were treated with ligustroside for 24 h and the changes in mRNA and protein expressions were quantified by RT-qPCR and Western blot, respectively. Values were given as relative changes compared to the control group. β -actin was used as a housekeeping gene. KA: kojic acid. * $P<0.05$, ** $P<0.001$ vs. control cells, # $P<0.05$ vs. the blank group.

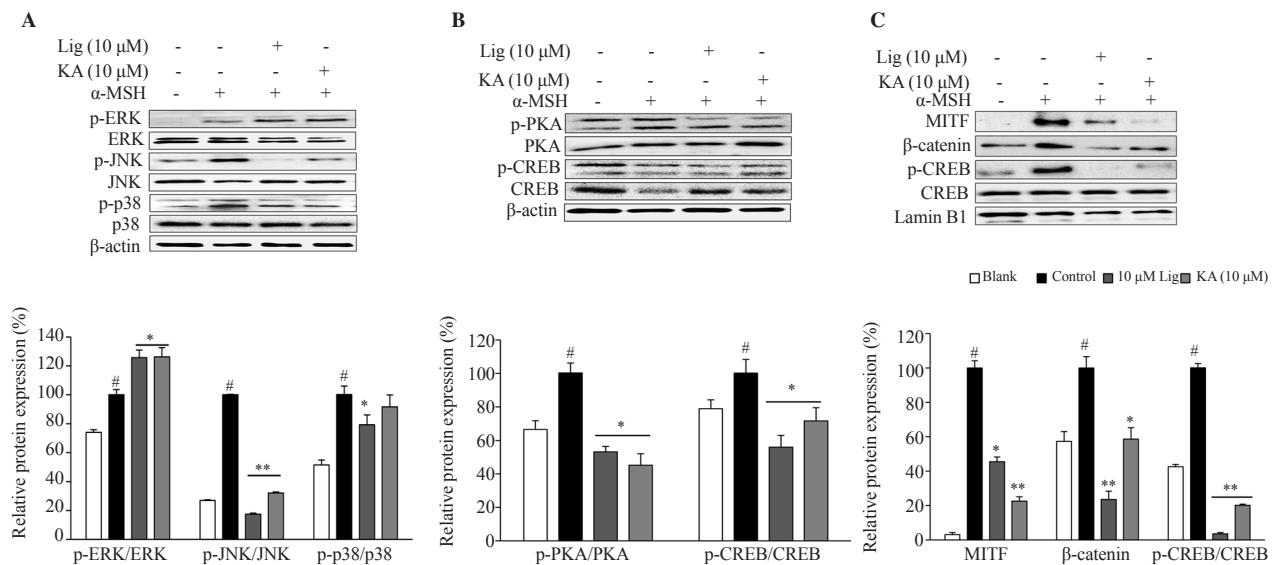


Figure 3. Effect of ligustroside on (A-B) the MAPK and PKA/CREB signaling pathways as well as (C) nuclear protein levels of MITF, β -catenin, and p-CREB/CREB in α -MSH-stimulated B16F10 murine melanoma cells. Cells were treated with ligustroside for 24 h and the changes in protein expression were quantified by Western blot. Values were given as relative percentage of the control group. β -actin or Lamin B1 was used as a housekeeping protein. * $P<0.05$, ** $P<0.001$ vs. control cells, # $P<0.05$ vs. the blank group.

($P < 0.05$) in intracellular melanin content (87.15%, 81.38%, and 70.88%, respectively).

3.4. Effect of ligustroside on the mRNA and protein expression levels of melanin synthesis-related factors

Figure 2A shows that α -MSH treatment could significantly upregulate the mRNA expression levels of *tyrosinase*, *MITF*, *TRP-1*, and *TRP-2* ($P < 0.05$). The expressions of these genes were downregulated by treatment with ligustroside in a dose-dependent manner ($P < 0.05$). In addition, Western blot results showed that ligustroside downregulated α -MSH-induced upregulation in the protein expression levels of MITF, tyrosinase, TRP-1, and TRP-2, especially at 10 μ M (Figure 2B).

3.5. Effect of ligustroside on the MAPK signaling in α -MSH-stimulated B16F10 cells

Figure 3A exhibits that the phosphorylation levels of JNK and p38 were significantly downregulated, while the phosphorylation of ERK was obviously upregulated by treatment of ligustroside at 10 μ M in α -MSH-stimulated B16F10 cells. These results indicate that ligustroside could inhibit melanin synthesis by regulating the MAPK signaling pathway.

3.6. Effect of ligustroside on the PKA/CREB signaling in α -MSH-stimulated B16F10 cells

The effect of ligustroside on the PKA/CREB signaling pathway was also detected. As shown in Figure 3B, α -MSH treatment markedly increased the phosphorylation levels of PKA and CREB compared to those in the blank group. In contrast, the phosphorylation levels

of PKA and CREB were significantly downregulated after the cells were treated with 10 μ M ligustroside. These results reveal that ligustroside could inhibit melanogenesis by suppressing the activation of the PKA/CREB signaling pathway.

3.7. Effect of ligustroside on melanogenesis-related nuclear proteins

To verify the anti-melanogenesis effect of ligustroside, the nuclear expression of MITF, β -catenin, and p-CREB/CREB was detected. As shown in Figure 3C, the relative expression values of MITF, β -catenin, and p-CREB/CREB were noticeably upregulated in α -MSH-stimulated B16F10 cells, while they were significantly downregulated after the cells were treated with 10 μ M ligustroside ($P < 0.05$).

3.8. Immunofluorescence staining results

Based on the protein expression results in Western blot, the immunofluorescence experiment was further carried out to verify the inhibitory effect of ligustroside on melanin synthesis in α -MSH-induced B16F10 cells. As demonstrated in Figure 4, the immunofluorescence intensity of MITF obviously enhanced in α -MSH-induced B16F10 cells. In contrast, treatment with 10 μ M ligustroside reduced the immunofluorescence intensity of MITF.

4. Discussion

As a traditional Chinese herbal medicine, researchers have conducted comprehensive analysis and identification of the chemical components in *L. japonicum*. Ligustroside, a compound found in *L. japonicum*, has gained attention for its potential bioactive properties,

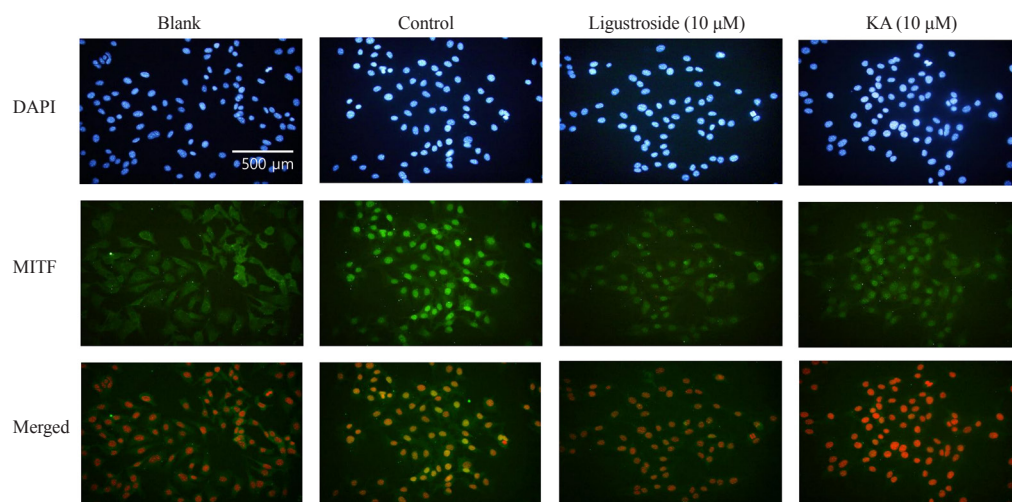


Figure 4. Representative images of immunofluorescence staining of MITF in α -MSH-induced B16F10 cells. Cells were stained with DAPI for viability and MITF was stained with green Alexa Fluor 488-conjugated antibody.

such as antioxidant, anti-cancer, and neuroprotective effects[18,24,25]. In this study, we investigated the anti-melanogenesis effects of ligustroside on α -MSH-induced B16F10 cells. Our findings suggest that ligustroside has the potential to modulate melanogenesis through its effects on tyrosinase activity, melanin content, melanogenesis-related gene and protein expression, MAPK signaling pathway, and PKA/CREB signaling pathway in α -MSH-induced B16F10 cells.

The MTT assay is a commonly used colorimetric assay to measure cell viability and assess cytotoxicity in various cell-based experiments[26]. The cytotoxicity of ligustroside on B16F10 cells is an important consideration to ensure the safety of its application. The MTT assay was used to evaluate the cytotoxic effect of different concentrations of ligustroside on B16F10 cells. Our results showed that ligustroside did not exhibit significant cytotoxic effects when the concentrations were no more than 10 μ M, but cell viability decreased obviously when the concentration increased to 15 and 20 μ M. Consequently, the non-cytotoxic concentrations of 1, 5, and 10 μ M of ligustroside were used to perform the subsequent experiments. B16F10 cells are commonly used in melanogenesis studies and screening of anti-melanoma drugs due to their ability to secrete melanin and tyrosinase[27]. α -MSH, a peptide hormone, regulates melanin synthesis and secretion by activating signaling pathways through the melanin-1 receptor on B16F10 cell surfaces[28]. In the present study, we investigated the potential of ligustroside to inhibit tyrosinase activity and melanin secretion in α -MSH-induced B16F10 cells. Our results revealed that α -MSH significantly increased intracellular tyrosinase activity and extra/intracellular melanin content, which was consistent with a previous study[29]. Many studies have shown that natural active compounds extracted from plants can inhibit tyrosinase activity or inhibit melanin production. The chemically modified curcumin exhibited a skin depigmentation effect by inhibiting intracellular tyrosinase activity[30]. Hinokitiol was isolated from the woody oils in Cupressaceae heartwood, a previous study revealed that this compound could effectively inhibit tyrosinase activity and melanin secretion in B16 melanoma cells[31]. Similarly, treatment with different concentrations of ligustroside resulted in a reduction in both tyrosinase activity and melanin content in α -MSH-induced B16F10 cells. These findings suggest that *L. japonicum* possesses the ability to directly inhibit tyrosinase activity and melanin secretion in α -MSH-stimulated B16F10 cells.

MITF, a basic helix-loop-helix leucine zipper transcription factor, is a central regulator in most signaling pathways, particularly in melanogenesis. It plays a pivotal role in melanocyte development, survival, and proliferation, and the regulation of melanin synthesis process[32]. By binding to the conserved consensus elements in the promoter regions, MITF induces the transcription of key melanogenic genes such as tyrosinase, TRP-1, and TRP-2[33]. Therefore, evaluating the expression levels of these genes and proteins is an important indicator to reflect the effect of tested samples on melanogenesis. Studies verified that α -MSH treatment could stimulate the mRNA and protein expression of MITF,

tyrosinase, TRP-1, and TRP-2[34]. Our results are consistent with the previous findings. However, these gene and protein expression levels were downregulated significantly after treatment with different concentrations (1, 5 and 10 μ M) of ligustroside. These results indicated ligustroside possesses the ability to regulate the gene and protein expressions of melanogenesis-related factors.

The generation of melanin is strongly associated with the MAPK signaling pathway. This pathway is an important cellular signaling pathway that includes multiple family members, with the most common ones ERK, JNK, and p38[35,36]. These members can be activated by various stimulating factors such as growth factors and UV radiation, which in turn regulate the expression and activity of MITF and ultimately lead to the transcription of genes related to melanin synthesis[37]. The phosphorylation of ERK inhibits the expression and activity of MITF, thus inhibiting melanin synthesis. Besides ERK, JNK and p38 also play important roles in melanocytes. The activation of JNK and p38 signaling pathways is crucial for processes such as melanocyte proliferation, survival, and differentiation[38,39]. The current findings elucidated that α -MSH treatment substantially elevated levels of p-p38/p38, p-JNK/JNK and p-ERK/ERK. Conversely, treatment with ligustroside reversed α -MSH-induced upregulation of p-p38/p38 and p-JNK/JNK levels, while further elevating the p-ERK/ERK ratio. These results suggest that ligustroside inhibits melanogenesis *via* its modulation of the MAPK signaling pathway, except the ERK activation.

On the other hand, the PKA/CREB signaling pathway is another important signaling pathway involved in melanin synthesis. This pathway is mainly mediated through the activation of adenylyl cyclase to trigger PKA. It plays an important role in the regulation of tyrosinase, an enzyme involved in melanin regulation[40]. CREB is one of the downstream targets of PKA, and its phosphorylation is mediated by activated PKA, thereby promoting the expression of tyrosinase, MITF, TRP-1, and TRP-2[41]. Nuclear translocation is the first step and most crucial protein expression process, which is finished with the steps of gene transcription, mRNA cutting and maturation, *etc.* In the present study, α -MSH treatment significantly increased the nuclear levels of phosphorylated PKA and CREB in B16F10 cells, while ligustroside treatment could downregulate the phosphorylation levels of the above two proteins, indicating ligustroside could inhibit melanin synthesis *via* blocking the PKA/CREB signaling pathway.

The cell nucleus serves as the central hub for many crucial biological processes within the cell, including gene expression, DNA repair, and cell cycle control. Determining the protein expression within the cell nucleus allows for a more accurate understanding of the specific protein expression dynamics within this cell[42]. β -Catenin plays a crucial role in regulating the differentiation of melanocyte stem cells through the Wnt/ β -catenin signaling pathway. Activation of this pathway leads to overexpression of β -catenin which translocates into the nucleus and forms a complex with LEF/

TCF transcription factors. This complex induces the expression of MITF and stimulates melanin synthesis[43,44]. To gain a deeper understanding of the regulatory processes and mechanisms involved in melanin production within the cells, Western blot was utilized to determine the expression levels of MITF, CREB, and β -catenin within the nucleus of B16F10 cells. In addition, immunofluorescence experiments were conducted to analyze the fluorescence intensity of MITF within the cell nucleus. The results demonstrated that 10 μ M ligustroside inhibited the protein expression of MITF, p-CREB, and β -catenin, and also decreased the fluorescence intensity of MITF within the nucleus of α -MSH-induced B17F10 cells. The findings of the current study are consistent with other reports of similar compounds that showed melanogenesis inhibitory effects. Other similar compounds such as 3-*O*-ethyl ascorbic acid, a stable form of vitamin C, and mulberroside A, a flavonoid glycoside from *Morus alba*, also inhibit melanogenesis *in vitro* by modulating the same molecular targets involved in melanin biosynthesis[45,46]. Although current study lacks the necessary data to suggest a structure-activity relationship, in the light of previous reports, it might be suggested that the glycosidic nature of the ligustroside plays a significant role in its anti-melanogenic properties.

The present study demonstrated that ligustroside could inhibit melanogenesis *via* regulation of key proteins involved in the MAPK, PKA/CREB, and Wnt/ β -catenin signaling pathways. While the study provides compelling *in vitro* evidence of ligustroside's anti-melanogenic effects, further research is needed to confirm these findings *in vivo* as well as a direct enzyme inhibitory assay on tyrosinase to find out whether ligustroside could inhibit tyrosinase enzymatic activity along with its expression. Additionally, the current study lacks a detailed structure-activity relationship analysis, which would help clarify how specific chemical features of ligustroside contribute to its melanogenesis inhibitory effects which would enable derivatization of ligustroside into a more stable and bioavailable form to be utilized in pharmaceutical approaches as well as cosmetics.

In summary, the current results demonstrated that ligustroside could inhibit melanogenesis by blocking the expression levels of MITF, tyrosinase, TRP-1, and TRP-2. This inhibition effect is due to the downregulation of p-JNK, p-p38, p-PKA, p-CREB, and β -catenin and the upregulation of p-ERK (Supplementary Figure). These results suggest that ligustroside may be a promising candidate for the development of nutraceuticals and cosmeceuticals targeting melanin synthesis. Further research should be conducted to thoroughly verify the melanin inhibitory effects in both animal models and clinical trials.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

This research was supported by the BB21plus funded by Busan Metropolitan City and Busan Techno Park, and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. NRF-2023R1A2C1006268 and RS-2023-00212560).

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

XRZ and CSK conceived the idea and designed the study. XRZ, and JHO performed the experiments, acquired the data and validated the results. XRZ and FK drafted the original manuscript and XRZ, FK and YS contributed to the revisions. JHO and YS performed the extraction and fractionation. JHO, FK, and CSK performed the statistical analysis, data curation, and validation and visualization of the results. YS and CSK supervised the project.

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Edited by Liang Q, Tan BJ

**Ligustroside derived from *Ligustrum japonicum* inhibits melanogenesis via
blocking the MAPK and PKA/CREB signaling pathways**

Running title: Anti-melanogenic effect of ligustroside

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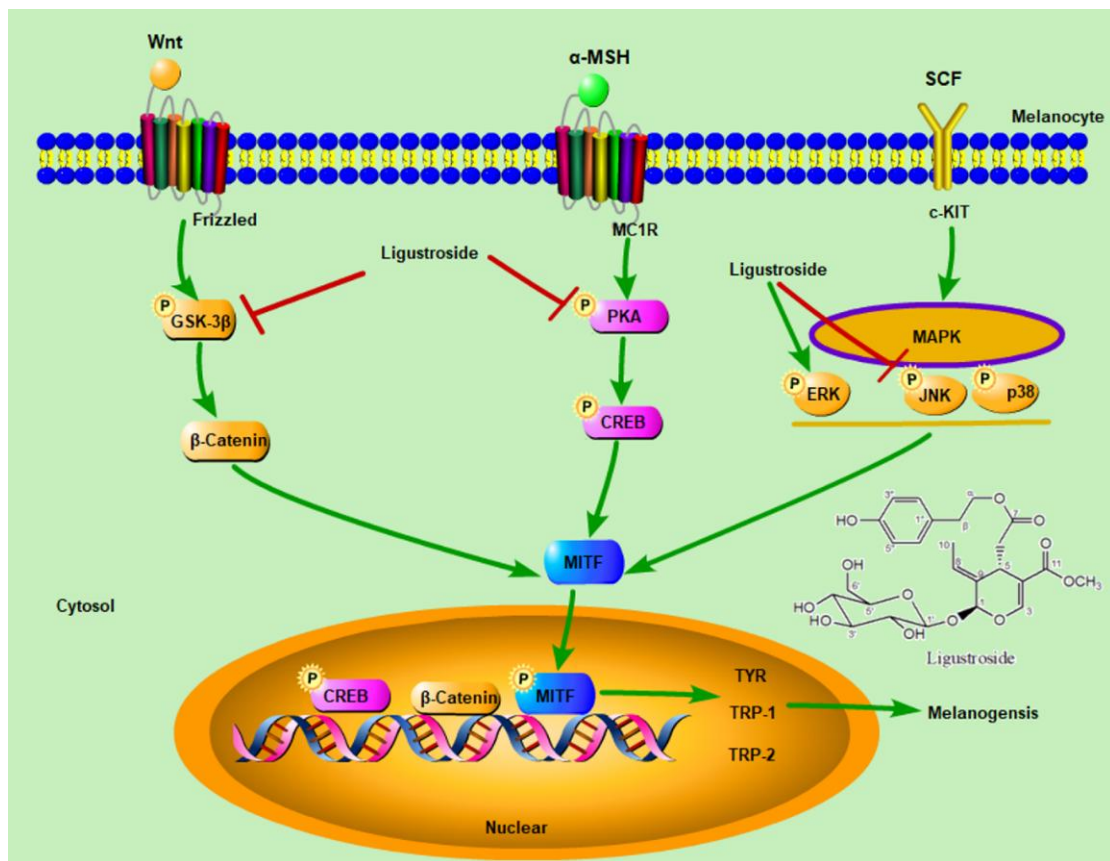
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Supplementary Figure. The proposed mechanism of the inhibitory effects of ligustroside on melanogenesis. Green arrows indicate increase or activation while red lines indicate decrease or inhibition.