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# Loganin Inhibits α-MSH and IBMX-induced Melanogenesis by Suppressing the Expression of Tyrosinase in B16F10 Melanoma Cells

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Ultraviolet radiation exposure is a major cause of extrinsic skin aging, which leads to skin hyperpigmentation. Loganin, a major iridoid glycoside obtained from *Corni fructus,* has anti-inflammatory, anti-diabetic, and neuroprotective effects. In this study, we investigated the mechanisms underlying the anti-melanogenic effects of loganin in B16F10 melanocytes treated with α-melanocyte stimulating hormone (α-MSH) and 3-isobutyl-1-methylxanthine (IBMX). Anti-melanogenic activity was measured by treating cells with loganin at concentrations between 1 and 20 μM. Cell viability assays confirmed that doses of loganin up to 20 μM were not cytotoxic. Loganin significantly and dose-dependently decreased intracellular melanin production. We also investigated potential molecular signaling pathways for the anti-melanogenesis effects of loganin. Western blotting showed that treatment with α-MSH and IBMX increased the phosphorylation of cAMP response element-binding protein (CREB) and the gene expressions of microphthalmia-associated transcription factor (MITF) and tyrosinase. Addition of loganin suppressed these increases, while promoting the phosphorylation of extracellular signal regulated kinase (ERK) and the anti-melanogenesis response. Our data therefore indicated that loganin could attenuate the increased melanin synthesis induced by α-MSH and IBMX treatment of B16F10 melanocytes. This attenuation appears to occur by downregulation of CREB phosphorylation and MITF and tyrosinase gene expression and upregulation of ERK phosphorylation. These finding suggests that loganin could be a valuable candidate for treatment of skin diseases related to hyperpigmentation.

**Key words** : Anti-melanogenesis, iridoid glycoside, loganin, tyrosinase

## Introduction

Melanin is produced and formed by melanocytes in the layer of epidermis outermost in the skin, and protects against damage due to ultraviolet (UV) radiation [44]. However, dysregulated activation of melanin synthesis can induce skin diseases associated with hyperpigmentation, including melasma, melanoma, freckles, age spots, and senile lentigines [18]. Therefore, numerous studies have focused on the development of effective skin whitening agents.

Melanin synthesis is activated by several important molecules and environmental conditions, including α-melanocyte stimulating hormone (α-MSH), cyclic AMP (cAMP) elevating agents, such as forskolin, glycyrrhizin, and 3-isobutyl-1-methylxanthine (IBMX); and UV radiation, and cAMP-mediated pathway, which plays a pivotal role in regulating melanogenesis [8, 22, 23], binds and activates the melanocortin 1 receptor (MC1R) in the epidermis layer of the skin, which subsequently activates cAMP-dependent protein kinase A (PKA) and other regulatory protein molecules [8]. PKA subsequently activates the cAMP response element-binding protein (CREB), which is a transcriptional activator of the microphthalmia-associated transcription factor (MITF) gene [4, 5]. The increase of CREB phosphorylation induces the transcription of MITF, which is a core transcription factor that induces the expression of tyrosinase, consequently increasing melanin synthesis [47, 55]. Furthermore, inhibition of extracellular signal-regulated kinase (ERK) signaling mediates melanin biosynthesis by increasing the activity of tyrosinase [25, 28, 30].

Loganin, an iridoid glycoside, is a biologically active compound obtained from *Corni fructus* (*Cornus officinalis* et Zucc), and one of the most popular plant products used in the traditional medicines in Japan, China, and Korea [12, 40, 53]. Previous studies have demonstrated that loganin possesses

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several biological activities, including immune regulatory, anti-inflammatory properties related to pulmonary complications [32] anti-atherosclerotic [39], hepatoprotective [46, 59], anti-Alzheimer's [6, 24, 63], anti-Parkinson's [56], neuroprotective [15, 31, 33, 52, 61], and anti-diabetic [26, 35, 42, 62] properties. A previous study reported that hot aqueous extracts and ethanolic extracts of *C. officinalis* inhibit UVB-induced pigmentation *via* radical scavenging activity in B16 melanoma cells, and are responsible for the inhibitory effects of loganin on melanogenesis [43]. Recently, An et al. [1] reported that methanolic extract of *C. officinalis* stimulates melanogenesis in Melan-a cells by increasing the levels of tyrosinase-related protein (TRP)-1, TRP-2 and MITF.

Despite of numerous studies, the mechanism by which loganin suppresses melanin production induced by α-MSH and IBMX is known. Therefore, our aim was to investigate the anti-melanogenic property of loganin against in α-MSH and IBMX-induced hyper pigmentation and the underlying molecular mechanism.

## Materials and Methods

### Materials

Loganin was isolated from *C. officinalis* according to the procedure described by Yamabe and coworkers [57-59]. The structure of loganin is depicted in Fig. 1. Kojic acid, α-MSH, and IBMX were purchased from Sigma-Aldrich (St. Louis, MO, USA). RIPA buffer was obtained from Biosesang (Sungnam, Gyeonggi-do, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin, and amphotericin were purchased from Welgene Inc. (Gyeongsan-si, Korea). The CREB (9197s) antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against p-CREB (sc-81486), p-ERK (sc-7383), tyrosinase (sc-7833), MITF (sc-11002), ERK (sc-514302), and β-actin (sc-47778) were acquired from Santa Cruz Biotechnology (Dallas, TX, USA). All other reagents were purchased from Merck (Frankfurt Str., Darmstadt, Germany), Fluka (St. Louis, Mo, USA), or Sigma Aldrich Co., unless stated otherwise.

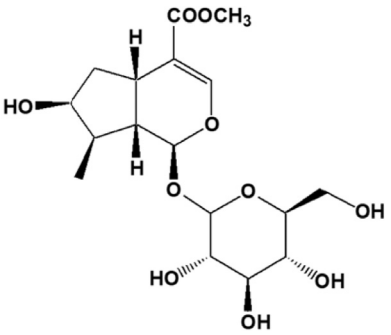


Fig. 1. Chemical structure of loganin.

### Cell culture

Murine melanoma B16F10 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% FBS and 1% streptomycin, and incubated at 37℃, in a humidified atmosphere with 5% $CO_2$.

### Cell viability assay

Cell viability assays were performed as previously described [27]. Briefly, the cells were seeded at a density of $1\times10^4$ cells/well in a 96-well plate for 24 hr. On the following day, the cells were exposed to different concentrations of loganin and incubated for 24 hr or 48 hr, respectively. Subsequently, 10 μl of EZ-Cytox solution was added to each well, and the cells were incubated for 2-4 hr at 37℃. Absorbance was determined using a spectrophotometric microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm. Each assay was performed in triplicate.

### Assay for melanin contents

Melanin content was determined as previously described [7]. B16F10 melanoma cells ($2\times10^5$ cells/well) were seeded in 6-well culture plates. In order to determine the inhibitory effect of loganin on melanogenesis, fresh medium was replaced with media supplemented with 1, 5 and 20 μM loganin or 20 μM kojic acid, which served as the positive control, and incubated for 2 hr. The cells were subsequently stimulated with 500 nM α-MSH and 200 μM IBMX for 48 hr. After washing twice with PBS, the cells were detached by incubating in trypsin/EDTA, and the pellets were dissolved in 100 μl of 1N NaOH incubated at 60℃ for 1 hr, and pipetting to solubilize the melanin. The melanin content was determined by measuring the absorbance at 405 nm by an ELISA reader (Tecan, Mannedorf, Switzerland). The melanin content was calculated using the following equation: (Δ sample/Δ control) ×100%. All measurements were performed in triplicate.

### Western blot analysis

Western blotting was performed as previously described [21]. B16F10 cells were treated with the samples for 2 hr, following which 500 nM α-MSH and 200 μM IBMX were added. After 6 hr, the cells were collected and lysed in a

RIPA cell lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.2% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The total protein-equivalents were separated by 8-10% SDS-PAGE and subsequently transferred onto PVDF membranes at 25 V for 10 min in a semi-dry transfer system (ATTO, Tokyo, Japan). The membranes were immediately placed in blocking buffer (5% non-fat milk) in 0.1% Tween 20 and were blocked at 25℃ for 1 hr. The membrane was incubated overnight at 4℃ with the appropriate specific primary antibodies, and then treated with horseradish peroxidase conjugated anti-mouse, anti-rabbit, or anti-goat antibodies at 25℃ for 1 hr. The immunoblots were visualized using Western Bright Peroxide solution (Advansta, CA, USA) and Davinch-Chemi CAS-400 (Davinch-K, Seoul, Korea) according to the manufacturer's instructions. Pre-stained protein markers were used for determining the molecular weight.

### Statistical analyses

Statistical significance was analyzed by one-way analysis of variance for determining the differences within treatments, and subsequently analyzed by the Bonferroni test (GraphPad Prism 5 software, La Jolla, CA, USA). Values of $^{*}p<0.05$ were considered to be statistically significant. All experiments were carried out in triplicate and represented as the mean ± standard errors of mean (S.E.M.) ($n$=3).

## Results

### Cell viability of loganin in the murine B16F10 melanocyte

To investigate the anti-melanogenic properties of loganin in the cell culture system, we investigated its cytotoxic effects on B16F10 cells. The cells were treated with several concentrations of loganin for 24 hr or 48 hr, respectively, and cell viability was determined using the EZ-Cytox assay. As shown in Fig. 2, loganin had no cytotoxic effect on B16F10 cells up to a concentration of 20 μM (Fig. 2A, Fig. 2B). Therefore, subsequent experiments were performed with loganin concentrations up to 20 μM.

### Inhibitory effect of loganin on melanin biosynthesis induced by α-MSH and IBMX in B16F10 melanoma cells

Melanogenesis can be reduced by inhibiting the formation of melanin precursors or by converting them into their reduced forms [54]. In order to investigate the inhibitory effects of loganin on the elevation of cAMP levels and subsequent hyperpigmentation, B16F10 cells were stimulated with α-MSH and IBMX prior to treatment with 1, 5, and 20 μM loganin or 20 μM kojic acid for 48 hr, and the melanin content was determined. Melanin content was found to be significantly lower in the cells that were treated with α-MSH, IBMX, and loganin than in the cells that were treated with α-MSH and IBMX. Additionally, loganin inhibited the production of melanin in a dose-dependent manner (Fig. 3).

### Effects of loganin on the CREB phosphorylation and expression levels of MITF/tyrosinase

The α-MSH-induced melanogenesis signaling pathway is mediated by MC1R, which triggers cAMP/PKA, subsequently resulting in CREB phosphorylation and regulation

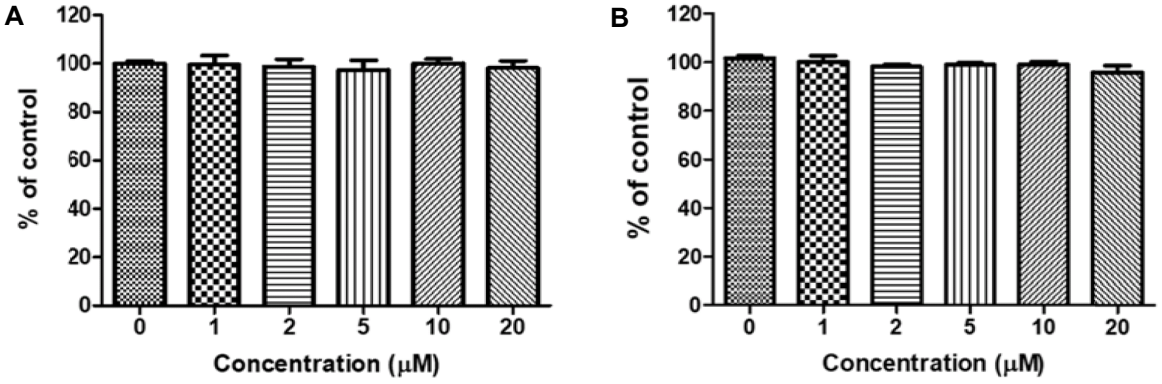


Fig. 2. Cell viability of loganin in B16F10 melanoma cells. Cells ($1\times10^4$ cells/well) were preincubated using various concentrations of loganin, up to 20 μM, for 24 hr (A) or 48 hr (B). Cell viability was determined by the EZ-Cytox assay and expressed as the percentage of absorbance values relative to those of the control group. Data are represented as the mean ± S.E.M. of experiments performed in triplicate.

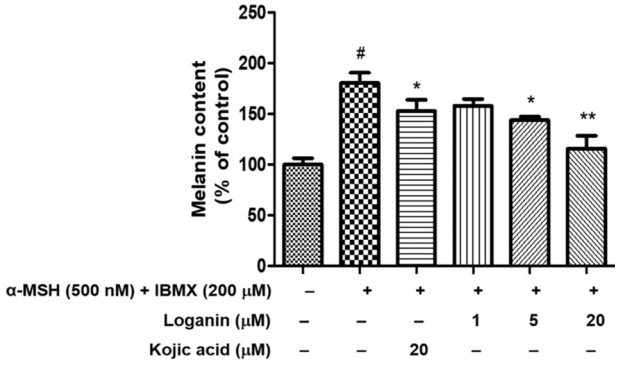


Fig. 3. Effects of loganin on the melanin content of B16F10 melanoma cells. Melanin content of the cells was determined following incubation with1, 5, or 20 μM loganin or 20 μM kojic acid for 48 hr. Each experiment was performed in triplicate, and the data are represented as the mean ± S.E.M. $^{\#}p<0.05$ compared with the control; $^{*}p<0.05$ and $^{**}p<0.01$, compared with the cells treated with α-MSH and IBMX.

of MITF [14, 49, 54]. The CREB phosphorylation and expression of MITF transcription factor and tyrosinase in B16F10 cells were evaluated by Western blotting. As exhibited in Fig. 4A, CREB phosphorylation (Ser133) increased after treatment with α-MSH and IBMX, and significantly decreased following treatment with loganin. As depicted in Fig. 4B, MITF expression increased following treatment with α-MSH and IBMX, and the significant changes in MITF expression were attenuated in the cells that were pretreated with 20 μM loganin. Tyrosinase is a rate-limiting enzyme for melanin synthesis, and inhibition of its activity or expression is frequently engaged to treat hyper-pigmentation [36]. As shown in Fig. 4C, tyrosinase expression slightly decreased following treatment with 20 μM loganin, compared to that in the control group. These results suggested that loganin has the potential to be developed into a skin depigmenting agent.

### Effects of loganin on the ERK expression levels

ERK phosphorylation has been reported to inhibit tyrosinase expression, which subsequently results in reduction in melanogenesis [17, 37, 38, 45]. We therefore proceeded to investigate the effects of loganin on ERK expression levels in B16F10 melanoma cells. The results demonstrated that treatment with loganin induced expression levels of ERK phosphorylation (Fig. 5). This indicated that the suppression of α-MSH and IBMX-induced melanogenesis could be related to expression of ERK phosphorylation.

## Discussion

In order to identify an effective whitening agent from a plants source, we evaluated the anti-melanogenic activity of loganin in cultured B16F10 cells, in which melanin synthesis was induced by α-MSH and IBMX, which are inducer of cAMP. Loganin had no significant cytotoxic effects up to a concentration of 20 μM, when treated for a period of 48 hr (Fig. 2). Therefore, we used this concentration of loganin for all experiments. There results of the melanin production assay revealed that loganin reduced the melanin content in a dose-dependent manner (Fig. 3). In order to understand the molecular mechanism underlying the anti-melanogenic activity of loganin, we examined the effects of loganin on the expression levels of proteins involved in the melanogenic

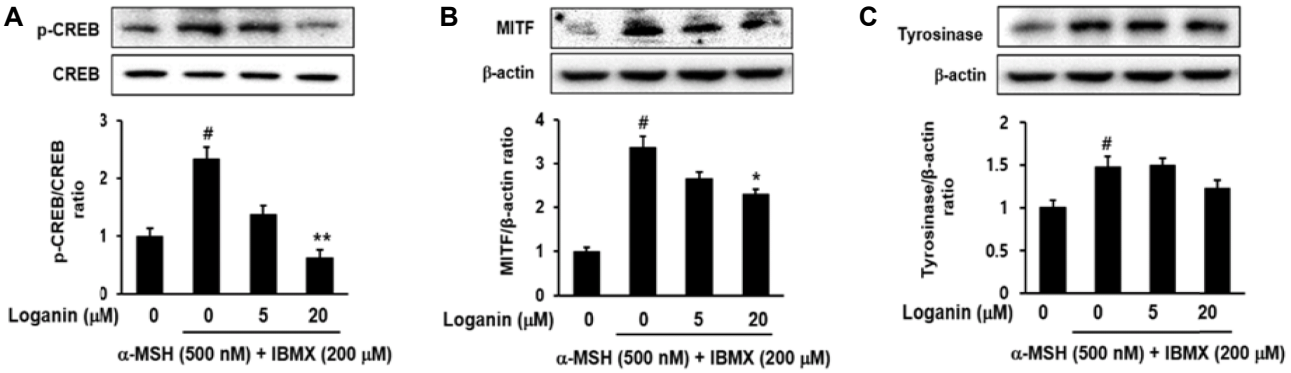


Fig. 4. Loganin decreased the levels of CREB phosphorylation (A), MITF (B) and tyrosinase (C) proteins in B16F10 cells. Equal protein loading was checked by reaction with β-actin and phosphorylation-independent CREB antibodies. The protein levels of p-CREB, MITF, and tyrosinase were quantified by CS analyzer software. A representation of three experimental replicates yielded similar results. One-factor ANOVA: $^{\#}p<0.05$ versus vehicle treated controls; $^{*}p<0.05$ and $^{**}p<0.01$ versus cells treated with 500 nM α-MSH and 200 μM IBMX. The bars indicate the S.E.M.

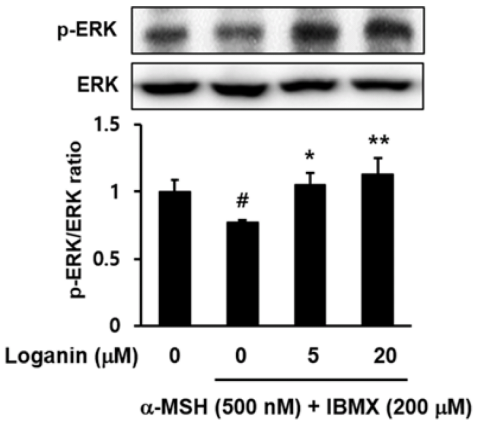


Fig. 5. Loganin increased the levels of ERK phosphorylation in B16F10 cells. Equal protein loading was checked by reaction with the phosphorylation-independent ERK antibody. The protein levels of p-ERK was quantified by CS analyzer software. A representation of three experiments yielded similar results. One-factor ANOVA: $^{\#}p<0.05$ versus vehicle treated controls; $^{*}p<0.05$ and $^{**}p<0.01$ versus cells treated with 500 nM α-MSH and 200 μM IBMX. The bars indicate the S.E.M.

signaling pathway, including CREB phosphorylation, MITF and tyrosinase, by western blotting.

Tyrosinase is a critical regulatory enzymes necessary for melanin biosynthesis because it acts as an initiating catalyst for the rate-limiting reaction in melanin synthesis [3]. Transcription of the tyrosinase enzyme is regulated by MIFT, a major transcription factor in the melanogenic signaling pathways [16, 19]. The results of this study demonstrated that loganin significantly suppressed the expression levels of MIFT and tyrosinase in a dose-dependent manner (Fig. 4). These results indicated that loganin exerts its inhibitory effect by regulating tyrosinase by inhibiting the gene expression of MITF. Interestingly, we observed that loganin had no inhibitory effect on the activity of mushroom tyrosinase in a cell-free system (data not shown). Based on these data, we supposed that the loganin-induced reduction in pigmentation should be attributed to the action of loganin on the signaling pathways that regulate the expression of tyrosinase.

MIFT plays a key role in accelerating hyperpigmentation by upregulating the cAMP-mediated melanogenic signaling pathway [50]. The activation of cAMP upregulates CREB phosphorylation by activating PKA, and CREB activation has been reported to induce the expression of MITF gene [13] However, several studies have reported that melanin synthesis is controlled via upregulation of ERK signaling

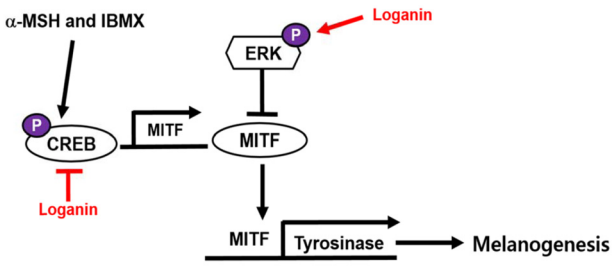


Fig. 6. Possible mechanism underlying the anti-melanogenic effect of loganin.

pathways in human melanocytes [60] and B16F10 melanoma cells [34, 48]. On the other hand, the results of this study demonstrated that the inhibition of melanogenesis by loganin was significantly related to the expression of ERK phosphorylation (Fig. 5). Generally, ERK phosphorylation has been reported to be a negative regulator of melanogenesis, which induces the double phosphorylation of MITF on Ser73 and Ser409 through proteasome-mediated MITF protein degradation, followed by a reduction in the tyrosinase activity of G361 melanoma and B16 melanoma cells [9, 29, 41, 51]. These findings indicated that ERK phosphorylation by loganin may contribute to the loganin-mediated melanogenesis by downregulating the CREB phosphorylation and expression of MITF and tyrosinase (Fig. 6). Although this study dose not explain the detailed underlying the mechanism of loganin, it is necessary to study the signaling pathways involved in loganin-mediated anti-melanogenic *in vivo* study.

Loganin, a major iridoid glycoside obtained from *Corni fructus*, is also distributed in the plants *Lonicerae japonica* Flos [10], *Neonauclea reticulata* (Havil.) Merr [11], and *Stychonos nux vomica* Linne [20]. In addition, Skihisa and coworkers [2] previously reported that iridoid-, hemiterpene-, and fatty acid-glycosides isolated from the fruits of *Morinda citrifolia* reduce the melanin content in α-MSH-induced B16 melanoma cells, and are potent inhibitors of melanin production. This finding is supported by previous studies on iridoid glycosides that reported their anti-melanogenic activity.

In conclusion, this study demonstrated the anti-melanogenic effect of loganin in B16F10 cells and the underlying mechanisms. These findings indicated that loganin could be an effective agent in treating hyperpigmentation disorders.

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## 초록 : 마우스 흑색종 B16F10세포에서 loganin의 티로시나아제 발현 억제를 통한 멜라닌 생성 억제에 대한 기전연구

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Loganin은 *Corni fructus*의 주요 iridoid glycoside이며 항염증, 항당뇨 그리고 뇌신경보호 효과 등이 보고되었다. 본 연구에서는 α-MSH와 IBMX처리된 B16F10세포에서 loganin의 melanogenesis억제효과의 신호전달 경로를 조사하였다. Loganin의 미백 활성을 확인하기 위해 B16F10세포에서 1 μM에서 20 μM사이의 농도를 처리하여 세포독성 실험을 수행한 결과 최대 20 μM농도에서 독성을 나타내지 않았다. 또한 loganin은 α-MSH와 IBMX처리된 B16F10세포에서 농도-의존적으로 멜라닌 생성을 감소시키는 것을 확인하였다. 또한 loganin의 멜라닌 생성을 억제하는 신호전달 경로를 Western blotting을 실시하여 조사하였다. Western blot결과에 따르면 loganin은 α-MSH와 IBMX 처리된 B16F10세포에서 증가된 CREB인산화(Ser133)와 MITF 발현 및 tyrosinase의 유전자 발현을 감소시켰고 ERK의 인산화를 증가시켜 melanin 생성을 억제하였다. 결론적으로 loganin은 α-MSH와 IBMX에 의해 유도된 과도한 멜라닌 합성을 CREB인산화와 MITF 및 tyrosinase의 유전자 발현을 억제하고 ERK의 활성화를 통해 멜라닌 합성을 감소됨을 확인하였다. 따라서 loganin은 과색소 침착과 관련된 피부질환의 보호제로서 활용될 가능성을 가지는 것으로 사료된다.