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Effect of *Cordyceps militaris* mycelia containing improved cordycepin on expression gene in the melanin biosynthesis pathway

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ABSTRACT: This study aimed to verify the whitening effect of *Cordyceps militaris*, which is distributed in several countries worldwide, including Korea, Japan, and China, and has various medical effects. To screen the efficacy of *C. militaris*, the inhibitory activity of tyrosinase, which was 66% at a concentration of 1 mg/mL, was measured. Thereafter, the survival rate of melanoma cells was measured, and cell experiments were conducted at a concentration of 90% or more in which *C. militaris* was not toxic to cells. After measuring the inhibitory effect of TRP-1, TRP-2, tyrosinase protein, and mRNA expression, which are factors influencing melanin synthesis, *C. militaris* was found to decrease in all factors, with an expression level that was significantly lower compared to quercetin. This confirmed that *C. militaris* stimulated with LED has excellent whitening activity and can be used as a functional whitening cosmetics material.

KEYWORDS: *Cordyceps militaris*, Gene expression, LED, Melanin, Tyrosinase

INTRODUCTION

Skin color is determined by melanin pigmentation in epidermal keratinocytes (Naik and Farrukh, 2022). Melanin pigments are produced by melanocytes present in the base layer of the epidermis and are transferred to neighboring keratinocytes through mechanisms including phagocytosis (Ando *et al*., 2012). The microphthalmia-associated transcription factor, a key transcription factor related to melanin production, moves to the nucleus and activates the promoters of TRP-1 and TRP-2 (Aoki and Moro, 2002). Thereby regulating the expression of tyrosinase, an enzyme in the melanin production rate control stage, and promotes melanin production (Busca and Ballotti, 2000). In addition, the skin is a tissue that can be easily contacted by the external environment and is one of the tissues that perform physical and biochemical defense functions of the human body (Kohen, 1999; Dobrynin *et al*., 2009). From a dermatological and cosmetic point of view, the development of bleaching and whitening agents is still an important issue not only as a basic study but also as a commercial application study (Blay, 2011).

Cordyceps sp. is very small in size, and difficult to find due to its specific environment, and scarcity (Cui, 2015). *Cordyceps* sp. is rich in moisture, fat, and protein, and has excellent anti-cancer effects, immunity enhancement, anti-aging, immune response suppression after kidney transplantation, and blood sugar lowering (Shashidhar *et al*., 2013). *Cordyceps* sp. is very expensive compared to other mushrooms, and it is a type of medicinal mushroom that is mostly consumed in medicinal prescriptions or in water (Zhou *et al*., 2009).

Cordycepin (3'-deoxyadenosine) was first isolated from *Cordyceps militaris* (*C. militaris*) in 1950 (Yoshikawa *et al*., 2008). The efficacy of Cordycepin has actively progressed as it was known about the inhibitory effect

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of hexane synthesis in the 1970s, and it shows the possibility of discovery as a preventive and treatment for various human diseases including cancer (Kim *et al*., 2011; Verma, 2022). Cordycepin showed immune activity, inhibition of cancer cell growth, and metastasis inhibition, and efficacy in inducing apoptosis and overcoming drug resistance in various cancer cells has been reported (Lu *et al*., 2014; Chaicharoenaudomrung *et al*., 2018).

What we note here is a previous study that cordycepin can act as a powerful whitening agent. Cordycepin inhibited melanin synthesis-related enzymes, such as tyrosinase, tyrosinase-related protein-1 (TRP1) and tyrosinase-related protein-2 (TRP2) (Jin *et al*., 2011). Zaidi *et al*. (2019) have reported the inhibitory potential of cordycepin on melanogenesis. These results indicate the role of cordycepin as a potent depigmenting agent for cosmetics. Our previous study reported that a light emitting diode (LED) improved the cordycepin content of *C. militaris* mycelium (Ha et al., 2020).

So far, many studies have been reported on the various efficacy of *C. militaris*, but few studies have been reported on the activity and efficacy of *C. militaris* stimulated by LEDs. We assumed that *C. militaris* with enhanced cordycepin, which has a strong whitening effect, will stimulate various genes associated with the whitening effect. Therefore, this study aims to test the whitening activity of mycelium stimulated with LED and to confirm the effect on expression gene in the melanin biosynthesis pathway.

MATERIALS AND METHODS

Fungal strains

Cordyceps militaris (KCCM 60304) was purchased from Korean Culture Center of Microorganisms (Seoul, Republic of Korea). Stock cultures were maintained on potato dextrose agar plates at 25°C.

Submerged culture conditions

The 3 mycelial discs (5 mm) were transferred 100 mL flask containing 50 mL sabouraud dextrose broth medium (pH 4.5). The samples were illuminated by a combination of blue LED (467-472 nm) for 12 h per day (Ha *et al*., 2020). The medium was cultured at 24°C and 100 rpm for 3 days in a shaking incubator. The culture was centrifuged at 5,000 rpm for 10 min. The obtained mycelium was washed with distilled water, and it was lyophilized in a freeze dryer to further analysis.

Cell culture

B16F10 melanoma cells (Korean Cell Line Bank, Seoul, Republic of Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were stored in a 5% CO_2 incubator at 37°C.

Cell viability assay

B16F10 melanoma cells (1.5×10^5 cells/mL) were seeded into 96 well plates, and it was incubated 24h in the 5% CO_2 incubator at 37°C. They were treated with dried *C. militaris* mycelia at a concentration of 0.0625 mg/mL to 1 mg/mL (dissolved with dimethyl sulfoxide (DMSO)) for overnight. Cell viability was measured using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution based on the manufacturer's instructions. The absorbance was read using an absorbance microplate reader (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA) at 490 nm.

Tyrosinase activity assay

Tyrosinase inhibitory activity was measured according to the method of Yagi *et al*. (1986). The substrate solution was prepared by mixing 80 μL of 67 mM sodium phosphate buffer (pH 6.8) with 10 mM L-DOPA (Sigma, USA). For absorbance measurement, 40 μL of the prepared substrate solution, 40 μL of the sample, and 40 μL of mushroom tyrosinase (Sigma) were mixed, and the mixture was reacted at 37°C for 10 min. The generated DOPA chrome was measured at 492 nm. The tyrosinase inhibitory activity of the sample was shown compared to the control cell (sample not treated). We used arbutin for the positive control.

Melanin content assay

B16F10 cells were inoculated into a 6 well plate with 2×10^5 cells/well and then cultured for 24 h. The medium was removed, 50 nM α-melanocyte stimulating hormone, which is a melanin biosynthesis inducing substance, and a sample by concentration were added at the same time, and cultured for 48 h. The medium was removed, washed three times with phosphate buffered saline, and 100 μL of 100 mM sodium phosphate (pH 6.8) containing 1% (w/v) triton X-100 was added and left at –80°C for 30 min. The reaction solution was centrifuged at 12,000 rpm for 30 min and the supernatant was collected. To measure the

amount of melanin produced, the supernatant was mixed with 1 N NaOH containing 10% DMSO. Melanin was dissolved while leaving the mixture at 65°C for 1 h. Absorbance was measured at 405 nm and expressed as relative melanin production.

Investigation of genes

B16F10 melanoma cells were inoculated in a 100 mm dish at a density of 1×10^6 cells in DMEM. They were incubated in 5% CO_2 at 37°C. After changing to a fresh 10% DMEM medium, *C. militaris* mycelia was added to a culture plate. The polymerase chain reaction (PCR) was performed to determine the mRNA expression level of TRP-1, TRP-2, and tyrosinase. The primer sequences used in the experiment are shown in Table 1. GoFlexi DNA polymerase, primer, and synthesized cDNA were added to PCR tube, mixed well, and PCR was performed. TRP-1 and TRP-2 were reacted at 94°C for 30 seconds, at 58°C for 45 seconds, and at 72°C for 45 seconds (40 cycles) using PCR. Tyrosinase was reacted at 94°C for 30 seconds, at 60°C for 45 seconds, and at 72°C for 45 seconds (40 cycles). Under the condition of 100 V, electrophoresis was reacted for 40 min using 1.5% agarose gel with 0.002% ethidium bromide added. The amount of gene expression was confirmed using a UV transilluminator.

Investigation of protein expression

Cell line (B16F10) was seeded in 100 mm dish and then cultured for 24 hours for stabilize cells. The medium was replaced with a medium in which *C. militaris* mycelium was mixed by concentration. The cells were cultured in a 5% CO_2 incubator for 24 hours. A 10 mL radioimmunoprecipitation assay (RIPA) buffer containing a complete mini 1 tab was added to the culture medium and centrifuged at 4°C for 20 min (12,000 rpm). The supernatant obtained by centrifugation was quantified using a BCA protein assay kit and electrophoresis was performed on 10% SDS-PAGE. The isolated protein was transferred to a polyvinylidene fluoride (PVDF) membrane and reacted in a blocking buffer (5% skim milk in tris buffered saline and twin 20, TBST) at room temperature for 1 hour. Diluted primary antibodies were cultured at 4°C for overnight. It was washed three times at 10 min intervals using TBST. The secondary antibody diluted to 1:2,000 was reacted at room temperature for 2 hours.

Table 1. Sequence of the primers used for PCR

Gene	Primer	Sequence (5'→3')
TRP-1	Forward	GCT GCA GGA GCC TTC TTT CTC
	Reverse	AAG ACG CTG CAC TGC TGG TCT
TRP-2	Forward	TGA CCG TGA GCA ATG GCC
	Reverse	CGG TTG TGA CCA ATG GGT GCC
Tyrosinase	Forward	GGC CAG CTT TCA GGC AGA GGT
	Reverse	TGG TGC TTC ATG GGC AAA ATC

RESULTS AND DISCUSSION

Cell viability of melanoma cells induced by *C. militaris* mycelia

Our results showed that murine melanoma B16F10 cells treated with a concentration of 0.0625 to 1 mg/mL of *C. militaris* mycelia for 24 h did not induce any changes in cell viability. At a concentration of 0.0625 to 1 mg/mL, the cell viability was 90% to 100%, which indicated low cytotoxicity (Fig. 1). Therefore, all concentrations were suitable for further evaluating the effects of *C. militaris* mycelia on tyrosinase activity and melanin synthesis in B16F10 cells. Although not included in the study results, we also performed toxicity assessment for DMSO used to dissolve mycelium. As a result, cytotoxicity to DMSO was also not observed.

Inhibition mushroom tyrosinase activity of *C. militaris* mycelia

The effect of *C. militaris* mycelia on the oxidation of L-DOPA catalyzed by tyrosinase, as well as that of arbutin, a well-known tyrosinase inhibitor, was investigated. As shown in Fig. 2, *C. militaris* mycelia and arbutin

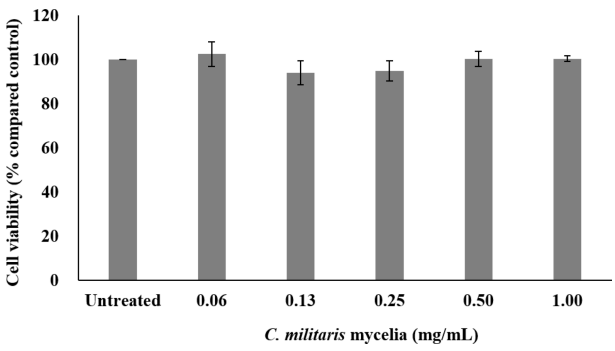

Fig. 1. The effects of *C. militaris* mycelia stimulated LED on the cell viability of B16F10 cells. The data are representative of three independent experiments. *<0.05, **<0.01 and ***<0.001 compared 'Untreated'; However, no significance was found in this result.

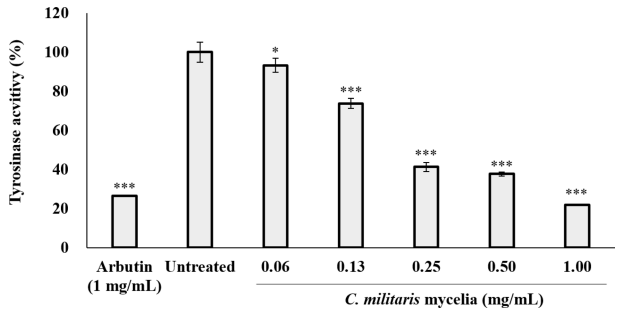

Fig. 2. The tyrosinase inhibitory activities of *C. militaris* mycelia stimulated LED. The data are representative of three independent experiments. *: $p < 0.05$, ***<0.001 compared untreated group.

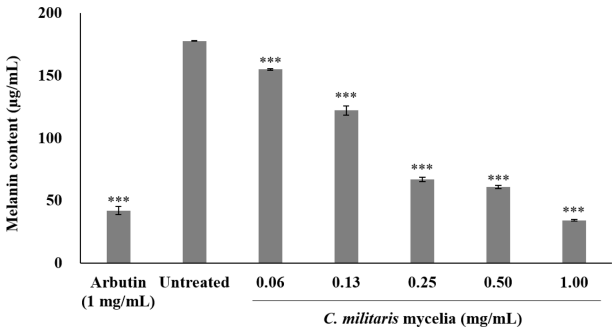

Fig. 3. The effects of *C. militaris* mycelia stimulated LED on melanin content in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. ***<0.001 compared untreated group.

exhibited potent inhibitory effects on L-DOPA oxidase activity in a dose-dependent manner. The results show that *C. militaris* mycelia and arbutin exhibited similar tyrosinase inhibitory activities. In particular, *C. militaris* mycelium showed tyrosinase activity inhibition similar to arbutin, a positive control, at a concentration of 0.125 mg/mL or more. Tyrosinase plays a very important role in skin melanin production (Pillaiyar *et al*., 2017). Tyrosinase acts as a DOPA oxidase that oxidizes tyrosine in melanosome to make DOPA and oxidizes DOPA to make DOPA chrome (Krainc *et al*., 2023). That is, tyrosinase plays an important role in synthesizing melanin polymer. Sun *et al*. (2017) reported that phenolic extracts extracted from lap bee pollen inhibit mushroom tyrosinase by about 80%. Extracts obtained from *Asphodelus microcarpus* inhibited mushroom tyrosinase by about 6-40% (Di Petrilo *et al*., 2016). Sułkowska-Ziaja *et al*. (2022) reported the strongest inhibition of tyrosinase was observed for *Ganoderma lucidum* extract, which inhibited the activity of the enzyme by 50.53% at 5 mg/mL concentration. This suggests that *C. militaris* mycelium has strong whitening activity. Interestingly, Chien *et al*. (2008) studied tyrosinase activity by extracts of different mushrooms and reported low (about 20%) inhibitory power of *C. militaris*. It is known that there is a difference in activity in the culture environment of *C. militaris* or in the case of different bacteria first collected before subculture.

Effect of *C. militaris* mycelia on melanin content in B16F10 cells

To confirm whether *C. militaris* mycelia contributed to the inhibition of melanin production, the difference in melanin secretion after treatment with each concentration of *C. militaris* mycelia was analyzed under an environment promoting melanin production by α-MSH stimulation. *C. militaris* mycelia was treated at concentrations of 0.0625 to 1 mg/mL, in the same manner as for tyrosinase activity. *C. militaris* mycelia significantly inhibited melanogenesis in the α-MSH-treated group, and a significant amount of melanin was suppressed (Fig. 3). This was similar to the observation in the positive control, arbutin. In particular, at the lowest concentration of 0.0625 mg/mL in the treated group, the melanin content reduced significantly as compared with the untreated group. Therefore, it was concluded that *C. militaris* mycelia effectively inhibited the synthesis or secretion of melanin in B16F10 cells. In the 0.0625 to 1 mg/mL *C. militaris* mycelia-treated groups, the melanin content decreased by maximum 5 times, compared with the untreated group, indicating a prominent melanin secretion inhibitory effect. The cytotoxicity test, tyrosinase inhibition test, and melanin content test results indicate that *C. militaris* mycelia is extremely valuable as a natural material in whitening cosmetics. Melanin production may contribute to some of the histopathological features exclusive to malignant cancer (Casalou *et al*., 2022). Chen *et al*. (2020) confirmed that *Antrodia cinnamomea* mycelium decreased maximum 58% melanin cells. Therefore, it is suggested that *C. militaris* mycelium is medically available as well as for simple skin beauty.

Genetic investigation

To determine the gene in *C. militaris* mycelia that inhibits melanin biosynthesis by affecting the expression of genes involved in the melanin biosynthesis pathway,

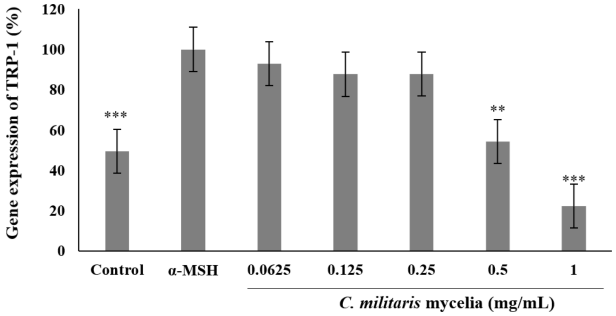

Fig. 4. The effects of *C. militaris* mycelia stimulated LED on TRP-1 gene expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

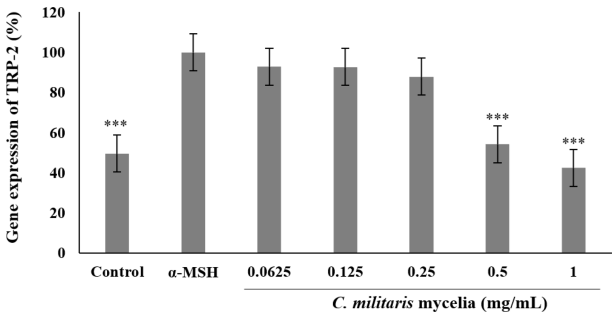

Fig. 5. The effects of *C. militaris* mycelia stimulated LED on TRP-2 gene expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

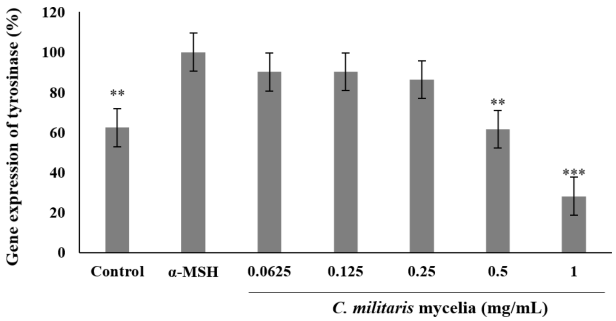

Fig. 6. The effects of *C. militaris* mycelia stimulated LED on tyrosinase gene expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

an experiment was performed using the PCR method, and the results are shown in Figs. 4 – 6. α-MSH was used as a negative control, and *C. militaris* mycelia showed inhibitory effects on the expression of TRP-1, TRP-2 and tyrosinase at all concentrations. In particular, *C. militaris* mycelia inhibited the expression of tyrosinase by more than 50% at 0.5 mg/mL or higher. We discovered that the inhibitory efficacy of TRP (tyrosinase related protein)-1, TRP-2 and tyrosinase expression decreased as the concentration of *C. militaris* mycelia increased. The results of this experiment suggest that the melanogenesis inhibitory effect of *C. militaris* mycelia contributed to the inhibition of the expression of TRP-1, TRP-2 and tyrosinase. Melanin biosynthesis is catalyzed by melanocyte-specific enzymes such as TRP-1 and TRP-2 (Liu *et al*., 2021). In our study, *C. militaris* mycelia decreased melanin synthesis, slightly affected tyrosinase activity, and yielded a significant difference as compared with the untreated group; therefore, we concluded that the promotion of pigmentation by *C. militaris* mycelia may be associated with other enzymes (TRP-1 and TRP-2). Zaidi *et al*. (2019) reported the cordycepin exerts its effect by inhibiting melanogenic enzymes like TYR, TRP1 and TRP2. Therefore, we speculated that enhanced cordycepin with stimulation of LEDs may have helped suppress genes involved in whitening.

Investigation of protein expression

The effect of *C. militaris* mycelia on the expression of proteins involved in melanin biosynthesis was confirmed using western blotting, and the results are shown in Fig. 7 - 9. When *C. militaris* mycelia was compared with the α-MSH-treated group, which was a negative control group, it was confirmed that *C. militaris* mycelia exhibited an inhibitory effect. The low concentration of *C. militaris* mycelia exhibited the most prominent inhibitory effect on TRP-1 and TRP-2. The percent of inhibition was 46.6%, 59.4% and 40.71% at 1 mg/mL of TRP-1, TRP-2 and tyrosinase, respectively, compared with the α-MSH-treated group. Therefore, the protein analysis showed the same trend as the gene analysis, and it was confirmed that *C. militaris* mycelia stimulated tyrosinase, TRP-1, and TRP-2 to induce melanin reduction. Based on our results, we tentatively conclude that *C. militaris* mycelia can inhibit melanocytes from harmful factors such as TRP-1. Therefore, we assumed that improved cordycepin was the major biologically active compound in *C. militaris* mycelia. However, further research is needed later to prove this hypothesis. Existing studies regarding the whitening property of *C. militaris* mycelia is insufficient; therefore, it can only be used as basic data in future studies pertaining to main ingredients that exhibit whitening efficacy.

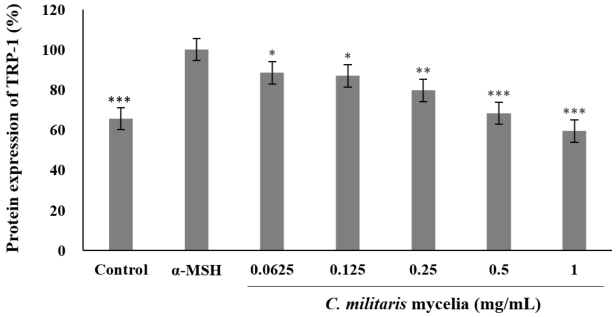

Fig. 7. The effects of *C. militaris* mycelia stimulated LED on TRP-1 protein expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

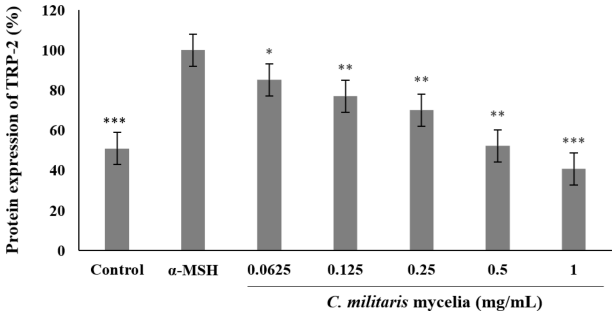

Fig. 8. The effects of *C. militaris* mycelia stimulated LED on TRP-2 protein expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

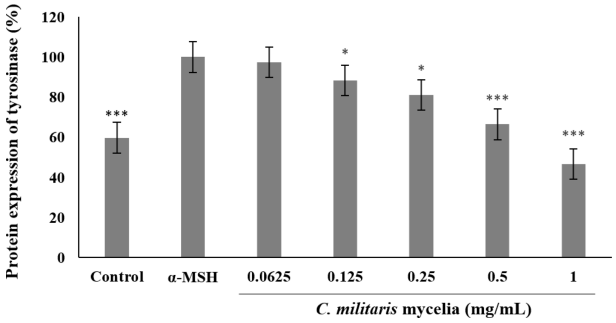

Fig. 9. The effects of *C. militaris* mycelia stimulated LED on tyrosinase protein expression in α-MSH-treated B16F10 cells. The data are representative of three independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared α-MSH.

CONCLUSION

To the best of our knowledge, this is the first study to report the efficacy of *C. militaris* mycelia stimulated LED in inhibiting melanin production in B16F10 melanoma cells. Our observations indicated that *C. militaris* mycelia inhibited α-MSH-induced melanogenesis through tyrosinase inactivation and the simultaneous suppression of the expression of proteins involved in melanin biosynthesis in B16F10 melanoma cells. *C. militaris* mycelia is known to be safe, and we confirmed that it is non-cytotoxic in this study. Therefore, *C. militaris* mycelia can potentially be employed as an effective skin-whitening agent and as an antioxidant for the future development of complementary and alternative medicine-based aromatherapy.

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Data Availability

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors Contributions

Si Young Ha, Ji Young Jung and Jae-Kyung Yang contributed equally to this work.

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