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Article

Inonotus Obliquus extracts as an inhibitor of α-MSH-induced melanogenesis in B16F10 mouse melanoma cells

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Abstract: Melanogenesis is a biosynthetic pathway for producing of the pigment melanin in human skin. Tyrosinase, a key enzyme, catalyzing is the first step in melanogenesis and the downregulation of the tyrosinase enzyme activity is the most reported method for anti-melanogenesis. According to the hyperpigmentation as an important issue in cosmetic industry, there is a big demand for melanogenesis inhibitors. In the present study, we identified the anti-melanogenic effect of *Inonotus Obliquus* in α-MSH-induced B16F10 mouse melanoma cells as a new inhibitor. Comparing with control and *Inonotus Obliquus* extracts treated B16F10, we identified melanin contents, tyrosinase activity, tyrosinase mRNA and protein expression, MITF activity using a constructed plasmid. As shown in these results, we demonstrated that *Inonotus Obliquus* extracts down-regulated melanin synthesis using down-regulating activity and expression of tyrosinase which is key enzyme to produce melanin. In addition, we revealed expression of tyrosinase is regulated by MITF through repressing MITF transcriptional activity. *Inonotus Obliquus* extracts has potential to repress melanogenesis and decreased of hyperpigmentation and to use as cosmetic ingredient.

Keywords: *Inonotus Obliquus*, Melanogenesis, B16F10, Tyrosinase, MITF

1. Introduction

The skin has epidermal units that are composed of keratinocytes and melanocytes. The melanocytes are responsible for melanin production and distribution which process called melanogenesis [15]. These units are composed of a melanocytes surrounded by keratinocytes and regulated by a closed paracrine system [18]. Melanin is the primary determinant of skin, hair and eye color [16]. Melanogenesis is a complex process with different stages [14]. When the melanin is abnormal deposited, it may cause different types of pigmentation disorders, which are classified as hypo or hyperpigmentation [17]. Melanocytes produce melanin via melanogenesis and controlled by tyrosinase [20]. The reason that tyrosinase activity is important on skin-pigmentation. Since this enzyme has an important impact on skin color or pigmentation defects treatment, cosmetic and pharmaceutical industries have been continuously seeking new candidates of ingredient regulating tyrosinase activity [5,6]. New tyrosinase inhibitors are continually required to deal with melanogenesis caused problems effectively with a few side effects. Therefore, the effects of various natural source extracts on human skin whitening and anti-melanogenesis have drawn significant attention, and numerous studies have been conducted to verify their benefits in cosmetic formulations [8,9].

In this study, we found that the *Inonotus Obliquus* extracts as a potent melanogenesis inhibitors by decreasing the activity of tyrosinase, a key enzyme in the synthetic pathway of melanin. *Inonotus Obliquus* are commonly known as Chaga mushroom, which used to natural medicine in many other countries such as Japan, China, Russia and Baltics [1]. *Inonotus Obliquus* extracts have been evaluated

as a traditional and natural source of bioactive compounds over many centuries and have recently been used for potential components in the cosmetic and pharmaceutical industries. *Inonotus Obliquus* extracts have been reported to have numerous physiological functions such effects on anticancer, homeostasis, anti-virus and antioxidant actions in *in vitro* and *in vivo* models [2,3,7,10,11]. We paid attention to melanogenesis as one of the new physiological functions of *Inonotus Obliquus* extracts.

In the present study, *Inonotus Obliquus* extracts evaluated its anti-melanogenesis effects by monitoring the melanin contents, intracellular tyrosinase activity and MITF expression in α-MSH-induced melanogenesis in B16F10 mouse melanoma cells.

2. Materials and Methods

2.1. B16F10 mouse melanoma cell culture

B16F10 mouse melanoma cells from Neonatal Tissue were obtained from Korea cell line bank (Korea) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 10% penicillin (Gibco, 100 units/mL) and 10% streptomycin (Gibco, 100ug/Ml) at 37 ℃ in 5% CO_2 of humidified incubator.

2.2. Preparation of Inonotus Obliquus extract

Inonotus Obliquus were washed and dried entirely at 60 ℃ dryer (ON-50; Daehan Science, Korea). The dried *Inonotus Obliquus* were powdered by a Grinder (SMX-5800LM; Shinil, Korea) and extracted in 70% ethanol at 60 ℃ during the half an hour. To increased efficiency, we used ultrasonic waves over 20 kHz (Ultrasonic cleaner 8891; Cole-Parmer, USA). After the extract process, the residue was separated from the extract by filter paper (Whatman No.2; GE Healthcare Life Science, USA). The ethanolic extract was evaporated using a rotary evaporator (EYELA N-3010; Tokyo Rikakikai, Japan), a freeze-drier (LP 10-30; Ilshin, Korea). The lyophilized extracts were dissolved in dimethyl sulfoxide (DMSO) to yield a stock solution concentration of 100 μg/mL.

2.3. Cell viability assay

To determined cell viability, we performed 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B16F10 mouse melanoma cells were seeded in 96 well plate at a density of $3X10^3$ cells per well to 80% confluence. B16F10 cells were treated with *Inonotus Obliquus* extracts range of 0-200 μg/mL concentrations. After 48 h incubation, cells were rinsed with PBS and treated with 0.5 mg/mL MTT (Sigma-Aldrich) and incubated additional 1 h. MTT formazan was placed in DMSO and measured by a Microplate Reader (SpectraMax® i3x; Molecular Deviced, USA) with absorbance at 595 nm.

2.4. Measurement of melanin content

B16F10 cells were harvested 48 h after treatment, washed with PBS and lysed in 1 N sodium hydroxide (NaOH; Sigma-Aldrich) at 95 ℃ for 15 min. Melanin content was determined by absorbance at 450 nm using a Microplate Reader (SpectraMax® i3x; Molecular Deviced, USA). The amount of melanin was calculated using a melanin standard curve. The protein concentration of each lysate was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The cellular melanin content was normalized to total protein concentration.

2.5. Intracellular tyrosinase activity assay

The B16F10 cells were washed with PBS and lysed with lysis buffer containing 1% Triton X-100 (Biopure), 150 mM NaCl (Biopure), 50 mM HEPES (pH 7.5, Biopure) and 5 mM EDTA (Biopure). The supernatant was separated from the cell lysate by centrifugation and mixed with 2 mM L-DOPA(Sigma-Aldrich) in 0.1 M sodium phosphate buffer (pH 7.4) solution. After 30 min incubation at 37℃, the absorbance at 450 nm was read using a Microplate Reader (SpectraMax® i3x; Molecular Deviced, USA). The protein concentration of each lysate was determined by Bradford protein assay (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The activity of intracellular tyrosinase was normalized to total protein concentration.

2.6. Expression of tyrosinase mRNA

Total RNA was isolated using TRIzol™ (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. The purity and concentration of the RNA was evaluated based on using MaestroNano®, a micro volume spectrophotometer (Maestrogen Inc., USA). The cDNAs were synthesized from total RNAs by using the miScript Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Tyrosinase mRNA expression was performed using the following Tyrosinase specific primers: Tyrosinase forward 5′-CAAGTACAGGGATCGGCCAAC-3′; Tyrosinase reverse 5′-GGTGCATTGGCTTCTGGGTAA-3′. PCR was performed using the HOT FIREPol EvaGreen® qPCR Mix Plus (ROX). The expression of mRNA was analyzed by $2^{-\Delta\Delta CT}$ calculate method and nomalized with β-actin.

2.7. Determination of tyrosinase protein

Cells were washed with cold phosphate-buffer saline (PBS) and lysed at 1% SDS lysis buffer (Promega, USA) at 95 ℃ for 20 min in rotator and added 5X sample buffer [10% SDS, 1 M Tris-HCl (pH 6.8), 50% glycerol, 25% β-mercaptoethanol, 1% bromophenol blue] (Sigma-Aldrich). The total proteins were normalized by using Bradford protein Assay kit. The same amounts of protein extraction were loaded on 12 % Tris-polyacrylamide gels (SDS-PAGE). Anti-tyrosinase and anti-β-actin primary antibody was purchased from Santacruz (CA, USA). The detection system was used Clarity™ Western ECL Substrate (Bio-Rad, USA). The chemiluminescence signals were visualized under Fusion FX7 Imaging System (Vilber Lourmat, France).

2.8 MITF transcription activity

To determined transcription activity of MITF when B16F10 cells were treated with *Inonotus Obliquus* extracts. B16F10 melanoma cells were transfected with pGL3 (Invitrogen) and pGL3-MITF plasmids using Lipofectamine 2000 (Invitrogen) and co-transfected with the pCMV-β-galactosidase plasmid to normalize the transfection efficiency. After incubation for 24h, the transfected cells were treated with *Inonotus Obliquus* extracts. After treatment, the cells were lysed at Passive Lysis Buffer (Promega Corp., USA). The luciferase activity was determined by Veritas™ Microplate Luminometer (Veritas, USA). The results are the averages of three independent experiments.

2.9. Statistical analysis.

All data were presented as means ± standard deviation of different measurements obtained by three independent experiments at least. Significance of the data was estimated by student's *t*-test. p value < 0.05 was considered statistically significant. (p values: * < 0.05).

3. Results

3.1. Cell viability of Inonotus Obliquus extracts in B16F10 mouse melanoma cells

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We first examined whether *Inonotus Obliquus* is cytotoxic to B16F10 melanoma cells using MTT assay. B16F10 cells were treated with *Inonotus Obliquus* extracts at indicated concentration respectively and measured 48 h later. Treatment with *Inonotus Obliquus* extracts caused no significant change in cell viability at final concentrations of 100 μg/mL, but markedly reduced it at 200 μg/mL as revealed by short-term (48 h) cell viability assay (Figure 1). Therefore, the following experiments about *Inonotus Obliquus* extracts were performed within the concentration that have no cytotoxicity.

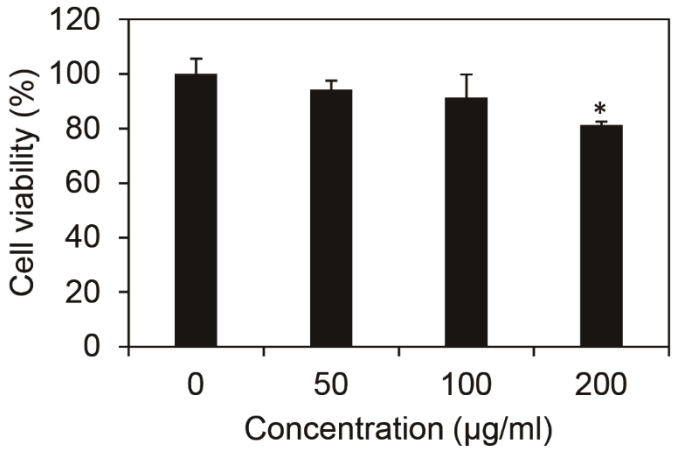

Figure 1. Cytotoxicity of extracts of *Inonotus Obliquus* in B16F10 mouse melanoma cells. Effects of *Inonotus Obliquus* extracts on the cell viability. There is no significant difference between control and *Inonotus Obliquus* extracts treated cells. Cytotoxicity was exhibited as a percentage of control at the indicated concentrations. Values are M±S.D. from triplicate experiments. M±S.D., mean±standard deviation.

3.2. Inonotus Obliquus extracts decreased melanin contents in the B16F10 mouse melanoma cells

To measure of melanin contents, after the treatment with the α-MSH (200 ng/ml) and *Inonotus Obliquus* extracts simultaneously, and thereafter compared to α-MSH as a negative control (Figure 2). The *Inonotus Obliquus* extracts dose-dependently reduced the melanin contents of the B16F10 cells at the concentrations that did not influence the cell viability. The *Inonotus Obliquus* extracts (100 μg/mL) was found to have a greater inhibitory effect on melanin contents in the cells.

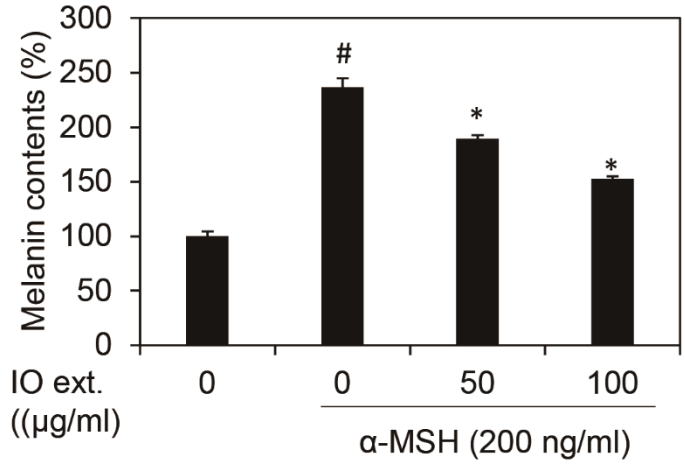

Figure 2. Effects of *Inonotus Obliquus* extracts on melanin contents in B16F10 mouse melanoma cells. *Inonotus Obliquus* extracts decreased melanin contents in B16F10 mouse melanoma cells. Melanin contents were exhibited as a percentage of control at the indicated concentrations. Values are M±S.D. from triplicate experiments. #p <0.05 compared with non-treated cells; *p <0.05 compared with α-MSH-treated cells; IO

ext., *Inonotus Obliquus* extracts; α-MSH, melanocyte-stimulating hormone; M±S.D., mean±standard deviation.

3.3. Inonotus Obliquus extracts directly inhibit the tyrosinase activity

Tyrosinase is a rate-limiting enzyme in melanin synthesis. Many melanin synthesis inhibitors reduce melanogenesis by directly inhibiting the tyrosinase activity. We examined the direct effect of *Inonotus Obliquus* extracts on tyrosinase activity by using mushroom tyrosinase, because the inhibitory effect of the *Inonotus Obliquus* extracts on melanin synthesis was remarkable. Figure 3A shows the change in cellular tyrosinase activity when the *Inonotus Obliquus* extracts treated in the range of 0-100 μg/mL on the α-MSH treated B16F10 mouse melanoma cells. The tyrosinase activity was dose-dependently decreased when adding the *Inonotus Obliquus* extracts on the B16F10 mouse melanoma cells compared with the control. The mushroom tyrosinase also decreased at concentration of 100 μg/mL *Inonotus Obliquus* extracts. These results indicate that the inhibitory effect of the *Inonotus Obliquus* extracts on melanin synthesis was related to the direct inhibitory effect on tyrosinase activity.

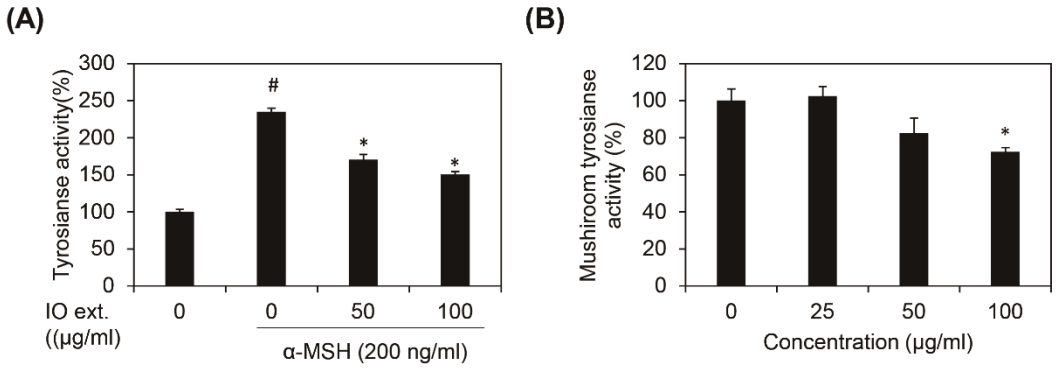

Figure 3. Effects of *Inonotus Obliquus* extracts on tyrosinase activity. *Inonotus Obliquus* extracts decreased tyrosinase activity in B16F10 mouse melanoma cells. (A) Cellular tyrosinase activity, (B) mushroom tyrosinase activity. Melanin contents were exhibited as a percentage of control at the indicated concentrations. Values are M±S.D. from triplicate experiments. #$p<0.05$ compared with non-treated cells; *$p<0.05$ compared with α-MSH-treated cells; IO ext., *Inonotus Obliquus* extracts; α-MSH, melanocyte-stimulating hormone; M±S.D., mean±standard deviation.

3.4. Inonotus Obliquus extracts downregulate α-MSH-induced tyrosinase expression

To evaluate the effect of *Inonotus Obliquus* extracts on tyrosinase expression, B16F10 cells were pre-treated with *Inonotus Obliquus* extracts before stimulation with α-MSH and tyrosinase protein levels were examined using western blot analysis. Pre-treatment with *Inonotus Obliquus* extracts at the 100 and 200 μg/mL concentrations dose-dependently inhibited the α-MSH-induced accumulation of tyrosinase proteins (Figure 4A). RT-PCR analysis shows similar results in 50 and 100 μg/mL concentrations (Figure 4B). These data demonstrated that *Inonotus Obliquus* extracts significantly decreased α-MSH-induced tyrosinase expression at the mRNA level without eliciting cytotoxic effect in B16F10 melanoma cells.

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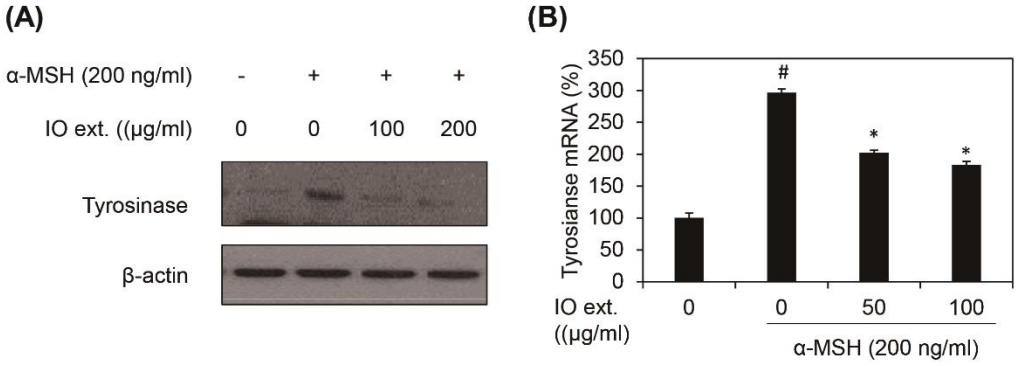

Figure 4. Effects of *Inonotus Obliquus* extracts on tyrosinase expression. *Inonotus Obliquus* extracts decreased tyrosinase expression in B16F10 mouse melanoma cells. (A) Protein level of tyrosinase, (B) mRNA level of tyrosinase. mRNA level of tyrosinase were exhibited as a percentage of control at the indicated concentrations. Values are M±S.D. from triplicate experiments. #p<0.05 compared with non-treated cells; *p<0.05 compared with α-MSH-treated cells; IO ext., *Inonotus Obliquus* extract extracts; α-MSH, melanocyte-stimulating hormone; M±S.D., mean±standard deviation.

3.5. Inonotus Obliquus extracts

To determine whether *Inonotus Obliquus* extracts affect the MITF promoter activity and if so, which regulatory region is required for *Inonotus Obliquus* extracts mediated suppression of the MITF promoter expression, we constructed a series of reporter plasmid containing a MITF gene promoter. Luciferase reporter activities of the MITF region constructs were increased by α-MSH stimulation, which was respectably reduced by treated to *Inonotus Obliquus* extracts (Figure 5). These data suggest that *Inonotus Obliquus* extracts downregulate MITF expression at the promoter level and that the *Inonotus Obliquus* extracts response element responsible for the suppression of the MITF transcriptional level.

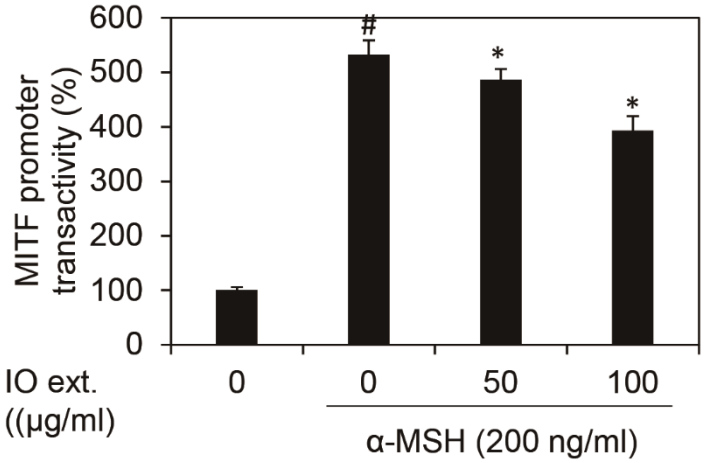

Figure 5. Effects of *Inonotus Obliquus* extracts on transcriptional activity of MITF. *Inonotus Obliquus* extracts decreased MITF transcriptional activity in B16F10 mouse melanoma cells. Transcriptional activity of MITF was exhibited as a percentage of control at the indicated concentrations. Values are M±S. D. from triplicate experiments. #p<0.05 compared with non- treated cells; *p<0.05 compared with α-MSH-treated cells; MITF, microphthalmia-associated transcription factor; IO ext., *Inonotus Obliquus* extracts; α-MSH, melanocyte-stimulating hormone; M±S.D., mean±standard deviation.

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4. Discussion

Melanogenesis is a biosynthetic pathway for producing the pigment melanin in for the most of colors human has such as eyes, hair, skin [15]. Melanogenesis is a complex process with different steps. A key enzyme, tyrosinase, catalyzes the first step of melanogenesis and the down-regulation of the enzyme activity is the most investigated method for the inhibition of melanogenesis [16]. According to the hyperpigmentation as an important issue in cosmetic industry, there is a big demand for melanogenesis inhibitors. [14].

There are many requirements for cosmetics products, but the most important that they should be use safety with no side effects and have positive effects on the skin. Recently, natural products have attracted extensive attention in cosmetic industry. There are numerous products from natural source such as foods, fruits, plants that are being exploited in pharmaceutical or cosmetics, and many potential products are yet to be used [8-9, 12-13]. *Inonotus Obliquus* is one of the medicinal mushrooms and also use in traditional oriental therapy and several nutrient functional foods [1,13]. *Inonotus Obliquus* extracts have been reported to have physiological functions such effects on anticancer, homeostasis, anti-virus and antioxidant actions [2-3,7,10-11]. In this study, we paid attention to anti-melanogenesis as one of the new physiological functions of *Inonotus Obliquus* extracts. Over the last years, understanding of melanocyte biology and the melanin synthesis processes have made remarkable progress as increased interest in whitening and hyperpigmentation, opening paths in the discovery of new melanogenesis inhibitors [5]. Many other approaches to inhibition of melanogenesis include accelerating the tyrosinase degradation and inhibiting tyrosinase mRNA transcription through a reduction of MITF activity in addition to the direct inhibition of tyrosinase activity.

We investigated the effects of *Inonotus Obliquus* extracts on melanogenesis by using mushroom tyrosinase in a cell-free system and cellular tyrosinase in cultured B16F10 melanoma cells with α-MSH as a negative control. In advance of following experiments, we measured melanin contents. The melanin contents in the *Inonotus Obliquus* extracts treated cells was significantly reduced, and then we examined the effect of the *Inonotus Obliquus* extracts on the cellular tyrosinase level. B16F10 cells treated with the *Inonotus Obliquus* extracts showed the cellular tyrosinase activity to be significantly decreased as well. When using mushroom tyrosinase, the inhibition effect of the *Inonotus Obliquus* extracts was also considerable. These results indicate that the *Inonotus Obliquus* extracts significantly inhibited not only melanin production but also intracellular tyrosinase activity in α-MSH-induced B16F10 mouse melanoma cells. The results of the western blot and the real-time RT-PCR analysis indicated that the tyrosinase protein and mRNA levels decreased by the *Inonotus Obliquus* extracts. We suggest that the *Inonotus Obliquus* extracts inhibited melanogenesis at the transcriptional level and also directly inhibited tyrosinase activity. Tyrosinase gene expression is regulated by the MITF [20]. MITF (microphthalmia-associated transcription factor) is a melanocyte-specific transcription factor and can regulate biological process in melanoma and melanocytes cells including pigmentation, proliferation and survival [21]. MITF binds and activated melanogenic gene, tyrosinase, promoters, thereby increasing their expression, which results in increased melanin synthesis. To determine whether *Inonotus Obliquus* effects on MITF activity, we constructed plasmid that has MITF promoter region and performed Luciferase assay using the plasmid. In this experiment, *Inonotus Obliquus* extracts also decreased MITF expression in a concentration-dependent manner. Thus, we expected that anti-melanogenesis effect of *Inonotus Obliquus* extracts was contributed by a mechanism related to MITF expression.

In conclusion, it is suggested that the *Inonotus Obliquus* extracts was a melanogenesis inhibitor in B16F10 mouse melanoma cells which stimulated α-MSH and that the mechanism might include decreasing of intracellular tyrosinase at the mRNA level by inhibiting MITF transcriptional activity. Taken together, *Inonotus Obliquus* extracts may be useful in anti-melanogenic ingredient for therapeutic treatment of hyperpigmentation and whitening cosmetic products as a natural source.

5. Conclusions

In this study, we determined the anti-melanogenic capability of *Inonotus Obliquus* extracts in B16F10 melanoma cells *in vitro*, the several experiments provide evidence supporting a key role for *Inonotus Obliquus* extracts in whitening by regulating melanogenesis. All these data led us to believe that the *Inonotus Obliquus* can represent a promising candidate as anti-melanogenesis ingredient in cosmetic products.

Author Contributions: Data curation, Eunji Lee; Formal analysis, Eunji Lee; Project administration, Hwa Jun Cha; Writing – original draft, Hwa Jun Cha.

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Conflicts of Interest: The authors declare no conflict of interest.

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