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Anti-Melanogenic and Anti-Oxidative Effects of *Nostoc verrucosum* (*ashitsuki*) Extracts

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Abstract: *Nostoc verrucosum*, an edible microalgal species, forms colonies in streams. Here, we investigated the antimelanogenic and anti-oxidative effects of *N. verrucosum* extracts. We collected *N. verrucosum* from Toyama Prefecture, Japan, and successfully cultured it in indoor cultivation systems. Aqueous, methanol, and hexane extracts of *N. verrucosum* were prepared for various experiments. To elucidate the antimelanogenic effects of *N. verrucosum*, we performed tyrosinase assay, melanin content assay, western blotting, and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Anti-oxidative effects were evaluated using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. The methanol and hexane extracts significantly inhibited melanin synthesis in B16F1 melanoma cells. Western blotting showed that 12.5 and 25.0 μg/mL *N. verrucosum* hexane extract suppressed tyrosinase activity. The qRT-PCR analysis revealed that *N. verrucosum* hexane extract inhibited α-melanocyte stimulating hormone-enhanced *tyrosinase*, tyrosinase-related protein (*TRP*)-1, *TRP-2*, and melanocortin 1 receptor (*MC1R*) mRNA expression. On the contrary, *N. verrucosum* hexane extract did not alter microphthalmia-associated transcription factor (*Mitf*) transcription. The ABTS assay showed that all extracts had radical scavenging activities, and the IC_{50} values of the aqueous and methanol extracts were 294.6 and 172.8 μg/mL, respectively. Our findings demonstrate that *N. verrucosum* is a suitable candidate for the development of antimelanogenic agents, cosmetics, or functional food ingredients.

Keywords: *Nostoc verrucosum* (*ashitsuki*); microalga; melanin synthesis; melanoma; tyrosinase; antioxidant; MC1R; Mitf

Citation: Sato, K.; Hiraga, Y.; Yamaguchi, Y.; Sakaki, S.; Takenaka, H. Anti-Melanogenic and Anti-Oxidative Effects of *Nostoc verrucosum* (*ashitsuki*) Extracts. *Cosmetics* **2023**, *10*, 30. https://doi.org/10.3390/cosmetics10010030

Academic Editors: Vasil Georgiev, Christophe Hano and Duangjai Tungmunnithum

Received: 11 January 2023
Revised: 1 February 2023
Accepted: 7 February 2023
Published: 9 February 2023

1. Introduction

Nostoc verrucosum, "*ashitsuki*" in Japanese, is a cyanobacterium that forms macroscopic colonies comprising trichomes of cells and an extracellular matrix in shallow streams [1]. The eighth century Japanese *waka* poetry anthology "*Man-yo-shu*" describes that *N. verrucosum* was consumed as an edible alga in ancient Japan. *Nostoc commune* is also consumed as food in Indonesia, China, Peru, and Japan [2]. Although *N. commune* is highly tolerant to frost, high temperatures, and extreme desiccation, its colonies always occur in streams [3]. Oku et al. reported that the ethanolic extract of *N. verrucosum* inhibited the growth of bacteria owing to the presence of n-1 fatty acids, demonstrating its antibacterial activity [1]. Korteerakul et al. reported that *N. commune* aqueous solution has anti-oxidative and antiglycative properties [4].

Exposure to solar ultraviolet radiation results in skin pigmentation. The overproduction of melanin in the skin leads to hyperpigmentation disorders, such as freckles, melasma, and solar lentigo, as well as melanoma [5]. Melanin biosynthesis involves three key enzymes that catalyze several reactions. For example, tyrosinase catalyzes two different reactions: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the

oxidation of DOPA to DOPAquinone [6]. The tyrosinase-related proteins TRP-1 and TRP-2 produce other types of eumelanin [7,8].

Melanin production is triggered by an elevation in the intracellular cAMP level initiated by α-melanocyte-stimulating hormone (α-MSH), isobutylmethylxanthine (IBMX), forskolin, and adrenocorticotropic hormone. The increased cAMP levels lead to the activation of protein kinase A and cAMP-response element binding protein (CREB). CREB binds to the promoter of the microphthalmia-associated transcription factor (*Mitf*) gene, and Mitf activates tyrosinase (*TRP-1* and *TRP-2*) gene transcription [9].

In our previous study, we found that the aqueous extract of the coccolithophore *Pleurochrysis carterae* inhibits melanin synthesis by downregulating Mitf expression [10]. However, there is a lack of information on the application prospects of microalgae in cosmetic, food additive, and fine chemical industries. Moreover, there are only a few reports on the biological activities of *N. verrucosum*. In this study, wild colonies of *N. verrucosum* were collected and used to prepare aqueous, methanol, and hexane extracts (hereinafter referred to as AE, ME, and HE, respectively). Subsequently, the effect of *N. verrucosum* extracts on melanin synthesis was investigated.

2. Materials and Methods

2.1. Preparation of N. verrucosum Extracts

2.1.1. Collection of *N. verrucosum* Colonies

We collected *N. verrucosum* from Nanto, Toyama Prefecture (Japan), and named the strain MAC1606TGR (Figure 1). After isolation, *N. verrucosum* was incubated in a KNO_3-free BG11 medium at room temperature under a fluorescent light for 10 d (Figure 2). Thereafter, the cells were collected and freeze-dried.

Figure 1. *Nostoc verrucosum* (*ashitsuki*) colony in a shallow stream.

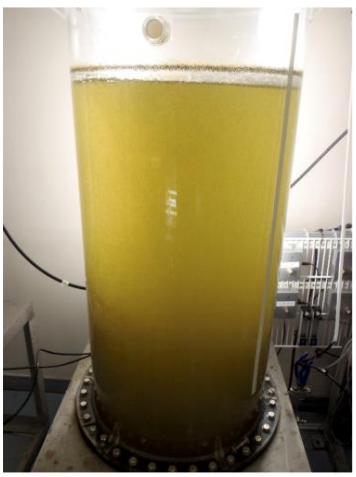

Figure 2. *Nostoc verrucosum* cultured in a cylindrical tank under a fluorescent light.

2.1.2. Preparation of *N. verrucosum* HE

To prepare the HE, *N. verrucosum* dried powder (10 g) was dissolved in 2 L of hexane, stirred for 1 h at room temperature, and passed through GA100 Glass Fiber Filters (Advantec, Tokyo, Japan). The filtrate was then centrifuged (10,000 rpm, 10 min) and dried in a vacuum; the resulting vacuum-dried product was used as HE.

2.1.3. Preparation of *N. verrucosum* ME

To prepare *N. verrucosum* ME, the hexane extraction residue was added to 2 L of methanol and stirred for 1 h at room temperature. The mixture was then centrifuged (10,000 rpm, 10 min), filtered, and dried in a vacuum.

2.1.4. Preparation of *N. verrucosum* AE

To prepare AE, the methanol extraction residue was added to 2 L of water and stirred for 1 h at 90–95 °C. The AE of *N. verrucosum* was then filtered and freeze-dried.

The HE and ME were dissolved in dimethyl sulfoxide, whereas the aqueous extract was dissolved in distilled water; the three extracts were then stored at −20 °C until use.

2.2. Cell Culture

B16F1 melanoma cells (Riken BioResource Center, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 5% fetal bovine serum and penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Assay of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Radical Scavenging Activity

The assay of ABTS radical scavenging activity was performed as described by Re et al. [11]. To determine the radical scavenging activity, 180 µL of ABTS radical solution was mixed with 20 µL of *N. verrucosum* extracts and incubated at room temperature in the dark for 30 min. The absorbance of the samples was measured at 732 nm, and ABTS radical scavenging activity was calculated as follows:

$$\text{ABTS radical scavenging activity (\%)} = (1 - A_{sample}/A_{control}) \times 100.$$

2.4. Assay of Tyrosinase Activity

To determine the effects of *N. verrucosum* extracts on tyrosinase activity, we performed a cell-free assay using mushroom tyrosinase as reported previously [12].

2.5. Assay of Cell Proliferation

To evaluate the effects of *N. verrucosum* extracts on cell proliferation, we performed the trypan blue exclusion test. In this test, the cells were treated with *N. verrucosum* extracts in the presence of α-MSH (10 nM) for 3 d. After treatment, the cells were rinsed once with phosphate-buffered saline (PBS) and resuspended in trypsin/EDTA (Life Technologies, Carlsbad, CA). The number of viable cells was determined using a Fuchs-Rosenthal cytometer under a light microscope.

2.6. Determination of Melanin Content

Melanin content was measured as described previously [13]. B16F1 melanoma cells were treated with *N. verrucosum* extracts for 3 d in the presence of α-MSH (10 nM). After treatment, the cells were collected and lysed by boiling in 2 M NaOH. Melanin content was spectrophotometrically determined at an absorbance wavelength of 405 nm.

2.7. DOPA Staining

DOPA staining was performed as described previously [13]. B16F1 melanoma cells were treated with *N. verrucosum* extracts for 3 d in the presence of α-MSH (10 nM). After

treatment, the cells were collected and lysed in 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg leupeptin, without mercaptoethanol, or by heating. Following electrophoresis, SDS-PAGE gels were stained with 0.5 mg/mL L-DOPA (Sigma) solution for 2 h at 37 °C. Gel images were obtained using WSE-6100 LuminoGraph 1 (ATTO Corporation, Tokyo, Japan).

2.8. Western Blotting

To elucidate the effect of *N. verrucosum* extracts on tyrosinase and Mitf levels, we performed western blotting analysis. *Nostoc verrucosum* HE-treated cells were lysed using RIPA lysis buffer, and whole-cell lysates were separated via SDS-PAGE; the resolved proteins were then transferred onto PVDF membranes (Merck Millipore, Burlington, MA, USA). After blocking with 5% skimmed milk in PBS containing 0.1% Tween 20, the membranes were probed overnight with tyrosinase and Mitf antibodies (Santa Cruz Biotechnology, TX, USA) at 4 °C and further incubated with horseradish peroxidase-conjugated secondary antibodies. The bound antibodies were detected via chemiluminescence using an ImmunoStar zeta (Fujifilm Wako Pure Chemical, Osaka, Japan) following the manufacturer's instructions.

2.9. Quantitative Reverse-Transcription Polymerase Chain Reaction

To investigate the effects of *N. verrucosum* extracts on melanin synthesis-related gene expression, we performed real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR). B16F1 melanoma cells were treated with *N. verrucosum* HE in DMSO for the indicated period. After treatment, qRT-PCR was performed as described in our previous report [14].

2.10. Statistical Analysis

The data were analyzed with GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA). All numerical data are presented as the mean ± SEM of experiments done in at least triplicate. Results with $p < 0.05$ and $p < 0.01$ were considered statistically significant.

3. Results

3.1. Antioxidant Activity

First, we evaluated the anti-oxidant activity of *N. verrucosum* extracts using the assay of ABTS radical scavenging activity. All extracts exhibited anti-oxidant activity, particularly the AE and ME (Figure 3). The HE did not show radical scavenging activity up to 25.0 μg/mL. The IC_{50} values of the AE and ME were 294.6 and 172.8 μg/mL, respectively.

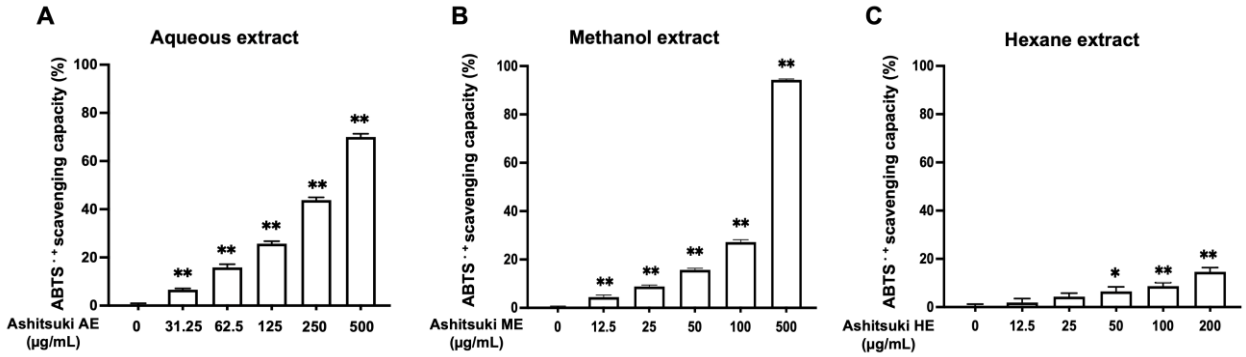

Figure 3. Anti-oxidant activity of *N. verrucosum* (*ashitsuki*) extracts. Anti-oxidant activity of *N. verrucosum* aqueous (**A**), methanol (**B**), and hexane (**C**) extracts were measured using the assay of ABTS radical scavenging activity. Results are represented as ABTS radical scavenging activity (%); the data are shown as mean ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$ versus the control group.

3.2. Mushroom Tyrosinase Activity

To clarify the mechanism underlying the antimelanogenic effect of *N. verrucosum* extracts, we performed a tyrosinase activity assay using mushroom tyrosinase. *Nostoc verrucosum* extracts did not inhibit mushroom tyrosinase activity (Figure 4). Unexpectedly, the ME and AE slightly enhanced mushroom tyrosinase activity at 12.5 and 25.0 μg/mL.

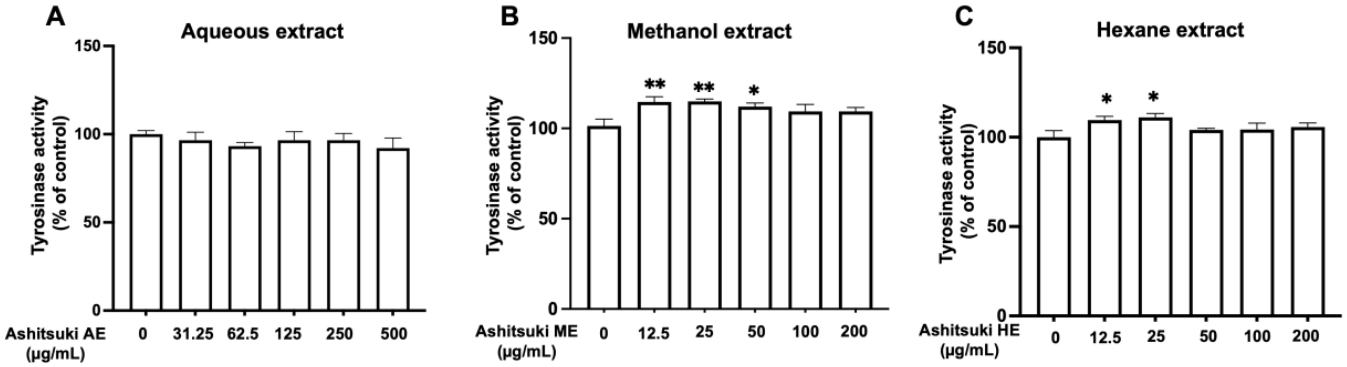

Figure 4. Effect of *N. verrucosum* (*ashitsuki*) extracts on tyrosinase activity. Effect of *N. verrucosum* aqueous (**A**), methanol (**B**), and hexane (**C**) extracts on monophenolase activity was measured. Results are represented as percentages of the control; the data are shown as mean ± SEM of three separate experiments. * $p < 0.05$, ** $p < 0.01$ versus the control group.

3.3. Cell Proliferation

The effects of *N. verrucosum* extracts on cell proliferation were assessed (Figure 5), and the number of α-MSH-treated cells was lower than that in the non-stimulated control. cAMP inducers, such as α-MSH and IBMX, inhibit cell proliferation [14]. Cell proliferation was not affected by *N. verrucosum* AE (Figure 5A). The ME inhibited cell proliferation at 20 μg/mL (73% of α-MSH treated control) (Figure 5B). The HE also inhibited cell proliferation at 25 μg/mL (49.4% of α-MSH treated control) (Figure 5C). These findings revealed that the lipophilic compounds in *N. verrucosum* might inhibit the growth of malignant melanoma cells. Additionally, none of the extracts induced cell death in the concentration range used in this study (data not shown).

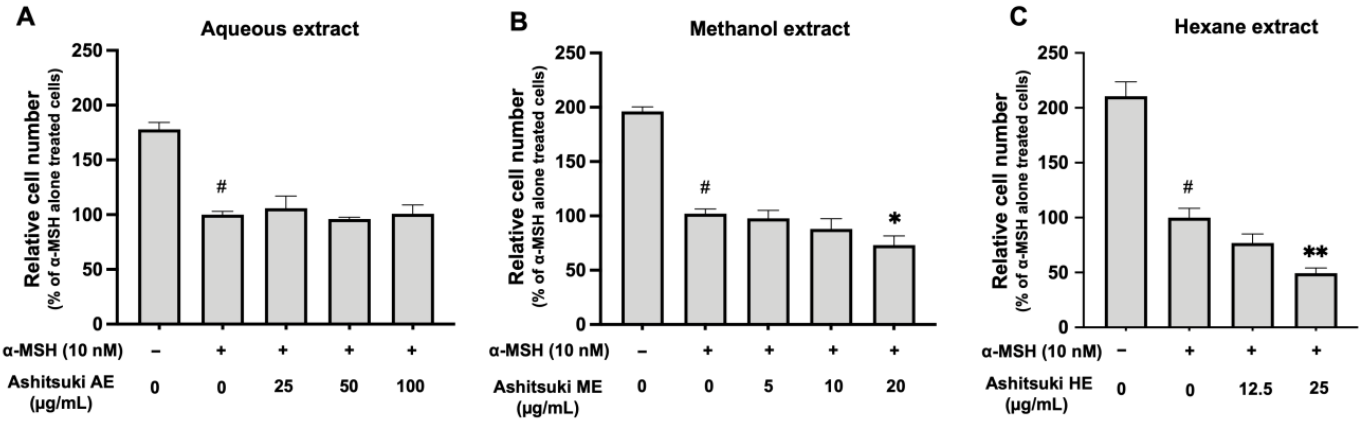

Figure 5. Effect of *N. verrucosum* (*ashitsuki*) extracts on the proliferation of B16F1 melanoma cells. B16F1 melanoma cells were treated with *N. verrucosum* aqueous (**A**), methanol (**B**), and hexane (**C**) extract for 3 d, in the presence of 10 nM α-melanocyte-stimulating hormone (α-MSH). After treatment, the trypan blue exclusion test was performed, and the cell proliferation rate was assessed. Results are represented as a percentage of the α-MSH-alone-treated control group; the data are shown as mean ± SEM of three separate experiments. # $p < 0.05$ versus the control group. * $p < 0.05$, ** $p < 0.01$ versus the α-MSH-alone-treated control group.

3.4. Melanin Production and Intracellular Tyrosinase Activity

To confirm the effect of *N. verrucosum* extracts on melanin synthesis, we determined the melanin content. In the presence of α-MSH, the melanin content increased (Figure 6A). However, *N. verrucosum* AE did not significantly decrease the melanin content. In contrast, the ME inhibited melanin synthesis in B16F1 melanoma cells (approximately 61% at 10 μg/mL and 52% at 20 μg/mL) (Figure 6B). Moreover, the HE inhibited melanin synthesis (approximately 46% at 12.5 μg/mL and 23% at 25.0 μg/mL) (Figure 6C).

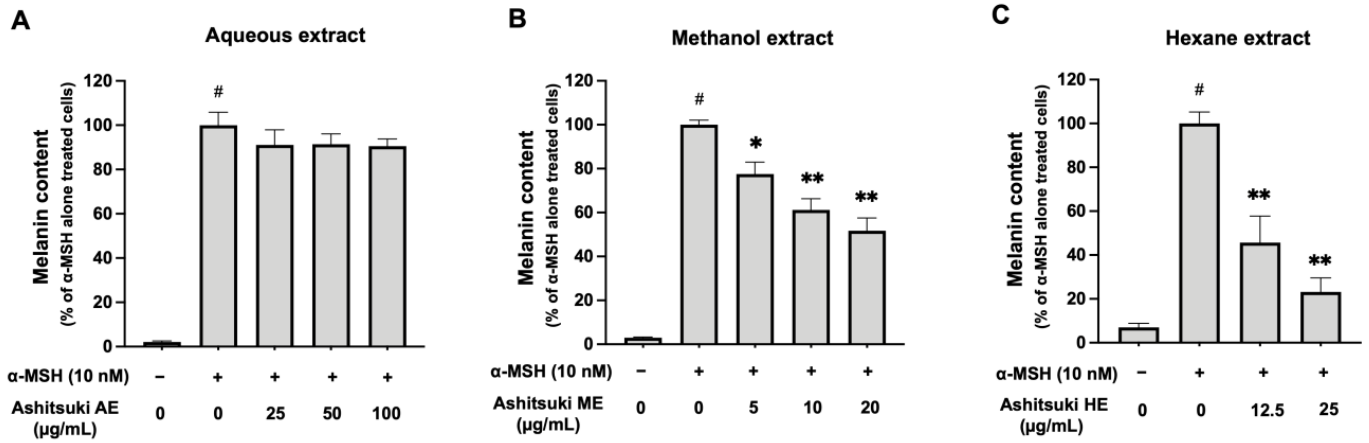

Figure 6. Effect of *N. verrucosum* (*ashitsuki*) extracts on melanin synthesis in B16F1 melanoma cells. B16F1 melanoma cells were treated with *N. verrucosum* aqueous (**A**), methanol (**B**), and hexane (**C**) extracts for 3 d in the presence of 10 nM α-MSH. After treatment, intracellular melanin content was measured as described in the materials and methods section. Results are represented as percentages of the α-MSH-alone-treated control group, the data are shown as mean ± SEM of three separate experiments. # $p < 0.05$ versus the control group. * $p < 0.05$, and ** $p < 0.01$ versus the α-MSH-alone-treated control group.

Intracellular tyrosinase activity was assessed using DOPA staining. The ME inhibited the tyrosinase activity in B16F1 melanoma cells (Figure 7). The HE also inhibited tyrosinase activity in a dose-dependent manner. However, the AE-treated cells did not exhibit any change in intracellular tyrosinase activity. These results imply that the ME and HE inhibited melanin synthesis by inhibiting melanogenic protein expression and gene transcription. Therefore, we attempted to elucidate the antimelanogenic mechanism of *N. verrucosum* HE.

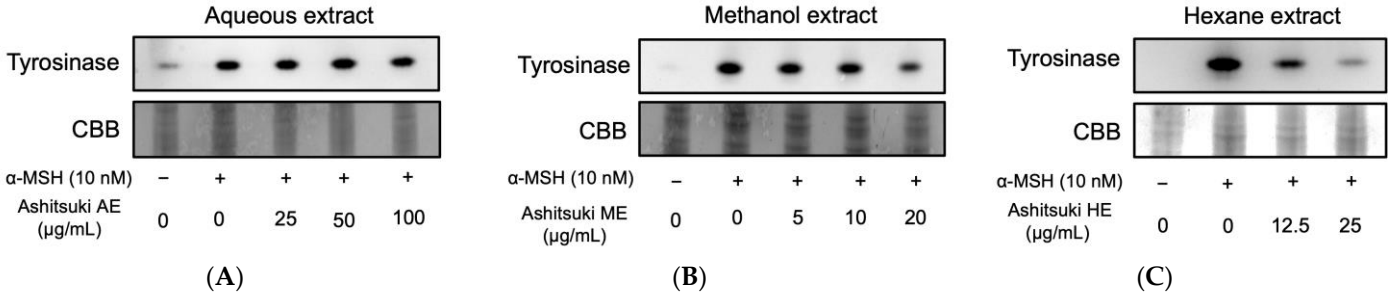

Figure 7. Effect of *N. verrucosum* aqueous (**A**), methanol (**B**), and hexane (**C**) extracts on intracellular active tyrosinase. B16F1 melanoma cells were treated with *N. verrucosum* extracts for 3 d in the presence of 10 nM α-MSH. After treatment, cell lysates were subjected to SDS-PAGE and DOPA staining was performed.

3.5. Effect of N. verrucosum HE on Tyrosinase and Mitf Protein Levels

Based on the results of DOPA staining, *N. verrucosum* extracts were found to inhibit tyrosinase expression. Therefore, we performed western blotting to determine tyrosinase

protein levels. The HE decreased the tyrosinase protein levels (Figure 8). Unexpectedly, the Mitf protein level was upregulated by the *N. verrucosum* extract treatment.

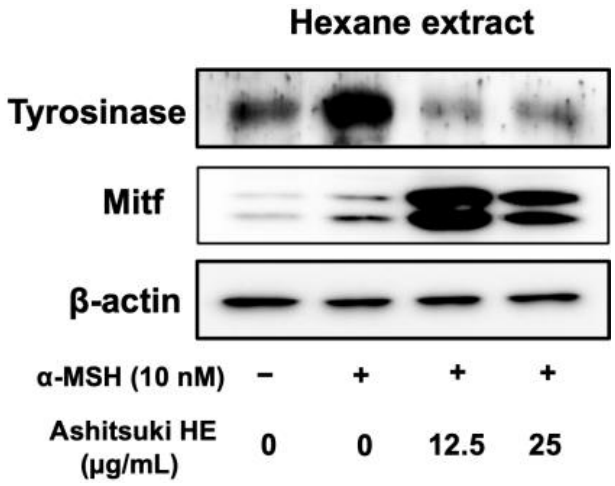

Figure 8. Effect of *N. verrucosum* (*ashitsuki*) hexane extract on tyrosinase and Mitf protein levels. B16F1 melanoma cells were treated with *N. verrucosum* hexane extract for 72 h in the presence of 10 nM α-MSH. After treatment, total cell lysates were prepared, and western blot analysis was performed.

3.6. Influence of N. verrucosum HE on Melanogenic Genes Transcription

Real-time RT-PCR analysis was performed to elucidate the effect of *N. verrucosum* on melanogenic gene transcription. Figure 9A shows the mRNA levels of genes in B16F1 melanoma cells after 72 h of treatment with the HE. In the absence of α-MSH, the *tyrosinase* mRNA level was slightly increased after *N. verrucosum* extract treatment. In contrast, the *MC1R* mRNA level was significantly decreased after treatment (approximately 40% of the untreated control). In the presence of α-MSH, the HE suppressed the α-MSH-induced increase in *tyrosinase* mRNA level. The *TRP-1* and *TRP-2* mRNA levels were suppressed by treatment with *N. verrucosum* extract at 6.25 and 12.5 μg/mL. The *MC1R* mRNA level was decreased in cells treated with α-MSH alone compared with that in the non-treated control. The *MC1R* mRNA levels in *N. verrucosum* extract and α-MSH co-treated cells were reduced (approximately 24% at 6.25 μg/mL and 30% at 12.5 μg/mL of the α-MSH-alone-treated control). Unexpectedly, the *Mitf* mRNA level did not show a significant difference after 3 d of treatment. The transcription levels of genes in the cells treated with *N. verrucosum* extract for 24 h were also assessed (Figure 9B); the *tyrosinase* and *MC1R* mRNA levels significantly decreased the mRNA level after *N. verrucosum* treatment, regardless of the presence of α-MSH. In the absence of α-MSH, *Mitf* mRNA transcription was inhibited after *N. verrucosum* extract treatment; conversely, the mRNA level increased after *N. verrucosum* extract and α-MSH co-treatment. Additionally, we assessed the *Mitf* mRNA levels after a short period of α-MSH treatment in the presence of *N. verrucosum* extract. As shown in Figure 9C, the upregulation of *Mitf* mRNA expression started shortly after α-MSH stimulation. Compared with that in *N. verrucosum*-pretreated groups, the *Mitf* mRNA level did not decrease, but rather increased.

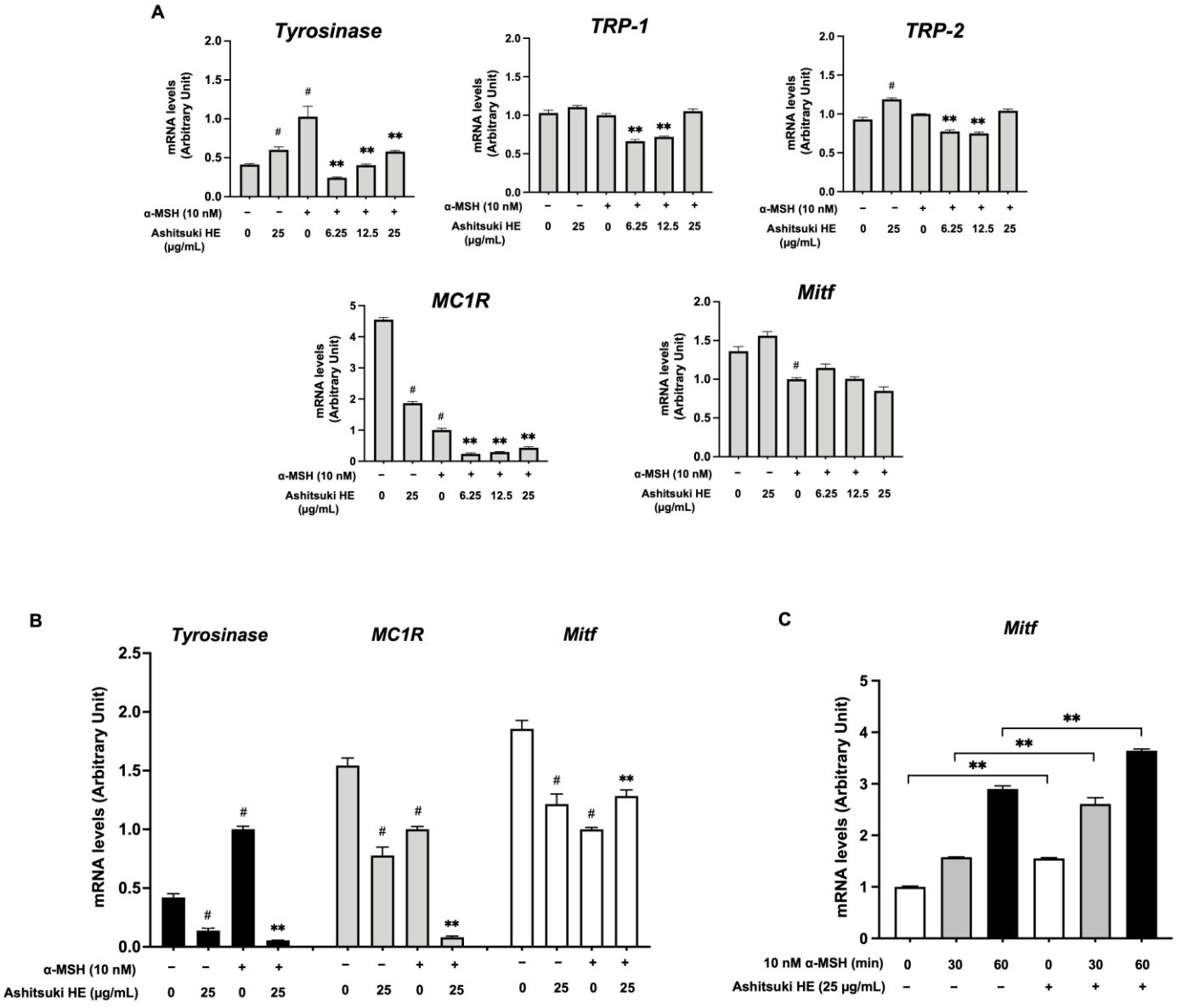

Figure 9. Effect of *N. verrucosum* (*ashitsuki*) hexane extract on melanogenic gene transcription. B16F1 melanoma cells were treated with *N. verrucosum* hexane extract and stimulated with 10 nM α-MSH for 72 h (**A**) and 6 h (**B**). (**C**) The cells were pre-treated with *N. verrucosum* hexane extract for 1 h; thereafter, α-MSH (10 nM) was added to the cells, which were then incubated for 30 or 60 min in the presence of α-MSH. Total RNA was extracted and cDNA was prepared. An equivalent amount of cDNA was amplified using primers specific for *tyrosinase*, *TRP-1*, *TRP-2*, *Mitf*, and *MC1R*. Results are represented as arbitrary unit of the α-MSH-alone-treated control group; the data are mean ± SEM of three separate experiments. # $p < 0.05$ versus the control group. ** $p < 0.01$ versus the α-MSH-alone-treated control group.

4. Discussion

In this study, we focused on assessing the anti-oxidative and antimelanogenic effects of *N. verrucosum* extracts. First, we isolated wild *N. verrucosum* from a stream and cultured it in artificial cultivation systems. Unlike *N. commune*, *N. verrucosum* is sensitive to desiccation [3]. Additionally, there is a possibility that the components of *N. verrucosum* vary between habitats and aquatic environments. Therefore, it is important to develop a robust in vitro culture system for a reliable supply of *N. verrucosum*.

To evaluate the radical scavenging activity of *N. verrucosum* extracts, we performed an ABTS assay. The results indicated that all of the extracts possessed radical scavenging capacities. Plant-derived extracts exert both radical scavenging activity and tyrosinase inhibitory activity [15–17]. Therefore, we also assessed the effect of *N. verrucosum* extracts on mushroom tyrosinase activity. Although *N. verrucosum* has a radical scavenging capacity, we did not observe any inhibitory effect of *N. verrucosum* on the monophenolase activity of mushroom tyrosinase.

Subsequently, we performed cell culture-based assays. First, we assessed the cell proliferation rate in the presence of *N. verrucosum* extracts. The AE did not inhibit cell proliferation, whereas ME and HE inhibited the rate of cell growth. The melanin content assay showed that the ME and HE significantly inhibited melanin synthesis in B16F1 melanoma cells. Additionally, the ME and HE decreased the level of the active form of tyrosinase in melanoma cells. These findings suggest that *N. verrucosum* extracts inhibit melanin synthesis via the downregulation of tyrosinase expression.

To clarify the antimelanogenic effect of *N. verrucosum* HE, we performed western blotting to determine the protein levels of tyrosinase and Mitf. The results indicated that *N. verrucosum* extracts significantly inhibited the α-MSH-induced increase in the tyrosinase protein level. In contrast, Mitf expression was upregulated by α-MSH and cotreatment with α-MSH and HE. Hence, we performed real-time RT-PCR to assess the transcription of the melanogenic genes. Tyrosinase mRNA expression was significantly inhibited by *N. verrucosum* HE treatment. However, The *Mitf* mRNA level was enhanced within a shorter duration of α-MSH stimulation. Interestingly, *MC1R* mRNA expression was strongly inhibited in the presence of *N. verrucosum* HE. These results indicate that *N. verrucosum* HE primarily inhibits tyrosinase transcription, subsequently downregulating melanin synthesis. MC1R, which is activated by α-MSH, is a receptor expressed on melanocytes and melanoma cells that triggers the melanin synthesis pathway [18]. Hasegawa et al. reported that *Elephantopus mollis* extract inhibits melanin synthesis via the downregulation of MC1R and Mitf expression, and the subsequent decrease in their mRNA levels [19]. Although *N. verrucosum* strongly inhibited *MC1R* transcription, there was no significant change in *Mitf* mRNA levels. This inconsistency may be attributed to the duration of the treatment. Importantly, downregulating MC1R expression leads to the inhibition of melanoma cell migration [20,21]. Moreover, we performed qRT-PCR analysis to elucidate the effect of *N. verrucosum* HE on matrix metalloproteinase 1 (*MMP-1*) transcription using human keratinocyte HaCaT. MMP-1 is involved in collagen degradation, and its enhancement by exposure to ultraviolet light results in the breakdown of type I procollagen level [22,23]. MMP-1 also acts as a metastasis promoter [24–26]. Our findings revealed that *N. verrucosum* HE strongly inhibits *MMP-1* mRNA expression (approximately 7% of untreated control) (data not shown). These findings suggest that *N. verrucosum* can prevent cancer metastasis.

With regard to extracts of other microalgae, Lee et al. reported that *Chlamydomonas reinhardtii* ethanolic extract can inhibit melanin synthesis via the downregulation of phosphorylated CREB, which leads to Mitf transcription [27]. However, *C. reinhardtii* did not inhibit tyrosinase activity directly. Kim et al. also investigated the functional effects of *Nannochloropsis sp.* on skin and reported that it had protective effects [28]. The authors indicated that *Nannochloropsis sp.* inhibits mushroom tyrosinase activity and exerts an anti-oxidative effect. Moreover, *Nannochloropsis sp.* suppressed the *MMP-1* mRNA level. Additionally, Oliva et al. demonstrated that *Botryococcus terribilis* ethanolic extract upregulates the expression of hair growth-related genes in human dermal papilla cells [29]. These reports suggest that microalgae have potential to be developed as therapeutic agents or functional food ingredients.

5. Conclusions

In conclusion, our results indicate that *N. verrucosum* HE may inhibit melanin synthesis by inhibiting tyrosinase gene transcription. We also demonstrated that *N. verrucosum* possesses anti-oxidant activity. However, the HE did not show strong anti-oxidant activity

at the concentration that we used in the melanin content assay. These findings show that the anti-oxidant effect of *N. verrucosum* HE does not significantly contribute to antimelanogenesis. Additionally, we established a steady cultivation system for *N. verrucosum*.

However, we believe that additional research on improving the system is required to enhance the beneficial effects of *N. verrucosum* and further identify compounds with antimelanogenic and anti-oxidative properties.

Author Contributions: Conceptualization, K.S. and H.T.; formal analysis, K.S. and Y.H.; investigation, K.S. and Y.H.; developed the indoor cultivation systems for *N. verrucosum*, Y.Y., S.S. and H.T.; preparation of *N. verrucosum* extracts, Y.Y., S.S. and H.T.; writing—original draft preparation, K.S.; writing—review and editing, K.S.; project administration, K.S. and H.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We are grateful to Professor Keiko Ohashi-Kaneko of Tamagawa University for her technical advice in this study.

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

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