Original Article

Asian Pacific Journal of Tropical Biomedicine

apjtb.org

doi: 10.4103/2221-1691.385571

Impact Factor® 1.7

Inhibitory effect of *Cyrtomium falcatum* on melanogenesis in α-MSH-stimulated B16F10 murine melanoma cells

Xian-Rong Zhou[1,2], Jung Hwan Oh[3,4], Fatih Karadeniz[3], Hyunjung Lee[5], Hyo Eun Kim[5], Migeon Jo[5], Youngwan Seo[6], Chang-Suk Kong[3,5]✉

[1]*Department of Bioscience, Silla University, Busan 46958, Korea*

[2]*Collaborative Innovation Center for Child Nutrition and Health Development, Chongqing University of Education, Chongqing 400067, China*

[3]*Marine Biotechnology Center for Pharmaceuticals and Foods, Silla University, Busan 46958, Korea*

[4]*Nutritional Education, Graduate School of Education, Silla University, Busan 46958, Korea*

[5]*Department of Food and Nutrition, Silla University, Busan 46958, Korea*

[6]*Division of Convergence on Marine Science, Korea Maritime and Ocean University, Busan 49112, Korea*

ABSTRACT

Objective: To explore the anti-melanogenic potential of *Cyrtomium falcatum*.

Methods: The effects of *Cyrtomium falcatum* crude extract and its solvent fractions on tyrosinase activity, melanin content, and the expressions of melanogenesis-related genes and proteins were analyzed in α-melanocyte-stimulating hormone (α-MSH)-stimulated B16F10 cells.

Results: α-MSH treatment significantly increased tyrosinase activity, and extracellular and intracellular melanin content, as well as the expression levels of tyrosinase, microphthalmia-associated transcription factor (MITF), tyrosinase-related protein (TRP)-1, and TRP-2 in B16F10 cells. Treatment with *Cyrtomium falcatum* crude extract and its solvent fractions reduced tyrosinase activity and extracellular and intracellular melanin content and downregulated the expression levels of tyrosinase, MITF, TRP-1, and TRP-2 in a dose-dependent manner.

Conclusions: *Cyrtomium falcatum* has potential anti-melanogenesis effects and can be used as a potential source material in cosmeceutical industry for the research and development of novel lead molecules with whitening properties.

KEYWORDS: *Cyrtomium falcatum*; Melanogenesis; α-MSH; B16F10 melanoma cells

1. Introduction

Melanocytes that are located in the deepest part of the epidermis, produce melanin to protect the skin tissue from harmful effects that may arise from prolonged UV exposure[1]. Despite its necessity, the excessive production and consequent accumulation of melanin leads to unwanted skin complications such as sunburn, wrinkles, and freckles on the epidermis, which overall affect the appearance and health of the skin. In severe cases, it may evolve into skin melanoma and other diseases, endangering human health[2]. Melanin has a complex structure and can be found in organisms from all kingdoms. In humans, it defines the colors of human skin, eyes, and hair[3].

Tyrosinase, tyrosinase-related protein (TRP)-1, and TRP-2 are crucial members of the biosynthesis of melanin, catalyzing

Significance

Cyrtomium falcatum is a plant with reported bioactivities. The current study showed that *Cyrtomium falcatum* extracts decreased tyrosinase activity and extracellular and intracellular melanin content, as well as downregulated the expression levels of tyrosinase, MITF, TRP-1, and TRP-2 in α-melanocyte-stimulating hormone-stimulated murine melanoma cells.

✉To whom correspondence may be addressed. E-mail: cskong@silla.ac.kr

How to cite this article: Zhou XR, Oh JH, Karadeniz F, Lee H, Kim HE, Jo M, et al. Inhibitory effect of *Cyrtomium falcatum* on melanogenesis in α-MSH-stimulated B16F10 murine melanoma cells. Asian Pac J Trop Biomed 2023; 13(9): 403-410.

Article history: Received 26 June 2023; Revision 27 July 2023; Accepted 1 September 2023; Available online 21 September 2023

the process[4]. Tyrosinase first exerts its mycophenolate activity, catalyzing the hydroxylation of tyrosine to *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) in melanocytes. Subsequently, tyrosinase uses *L*-DOPA as a substrate to exert diphenol activity and oxidize DOPA to DOPAquinone. After DOPAquinone undergoes a polymerization reaction to generate DOPAchrome, most of it generates eumelanin intermediate 5, 6-dihydroxy indole (DHI) and 5,6-dihydroxy indole carboxylic acid (DHICA) under the action of TRP-2[5]. DHI and DHICA generate 5, 6-indolequinone and 5, 6-indolequinone carboxylic acid, respectively, as a result of tyrosinase and TRP-1 activity. Interaction between them and other intermediates finally produces eumelanin[6]. Some part of DOPAquinone is converted into cysteinyl-DOPA in the presence of cysteine or glutathione which then turns into pheomelanin[7]. On the other hand, the transcriptional activation of tyrosinase, TRP-1, and TRP-2 genes is controlled by the activities of the microphthalmia transcription factor (MITF), activation of which can be stimulated by α-melanocyte-stimulating hormone (α-MSH)[8]. Therefore, it is usually employed as a melanogenesis-inducing agent to provide a stimulated melanogenesis model in murine B16F10 melanoma cells[9].

Increasing research focuses on the prevention and treatment of abnormal melanogenesis, in which natural-origin ingredients from plants draw much attention[10]. Plants are rich in active compounds that exert antioxidative, anti-inflammatory, and anti-cancer activities in cell, animal, and even clinical experiments[11–13]. Various studies verified the extracts and compounds derived from plants had an anti-melanogenesis effect, such as ethanol extract derived from the flowers of *Gaillardia aristata*, water extract derived from the fruits of *Phyllanthus emblica*, and leukodin derived from the whole plant of *Artemisia capillaris*[14]. *Cyrtomium falcatum* (*C. falcatum*), commonly known as the Japanese holly Fern, is a species of fern in the family Dryopteridaceae and is mainly distributed in Japan, Korea, and China[15]. A previous study revealed that *C. falcatum* extract exhibited antioxidant and anti-inflammatory effects on lipopolysaccharide-induced RAW264.7 macrophages[16]. Another research found that the compounds in the species of *Cyrtomium* included kaempferol, quercetin, and myricetin[17]. However, to the best of our knowledge, the effect of *C. falcatum* on melanogenesis is yet to be investigated.

Based on these reasons, the present study investigated the melanogenesis-suppressing effect of *C. falcatum* crude extract and its solvent fractions by determining the active tyrosinase levels, melanin production, and expression levels of melanogenesis-related genes and proteins.

2. Materials and methods

2.1. Reagents

The Division of Marine Bioscience, Korea Maritime and Ocean University (Busan, Korea) kindly provided the *C. falcatum* crude extract and its solvent fractions. Fetal bovine serum (FBS), RIPA reagent, Dulbecco's Modified Eagle Medium (DMEM), and QIAzol lysis reagent were obtained from Thermo Fisher Scientific (Waltham, MA, USA). α-MSH, 5-hydroxy-2-hydroxymethyl-4H-pyranone (kojic acid), *L*-DOPA, and 3-(4-5-dimethylthiazol-2-yl)-2-5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific primers for polymerase chain reaction were procured from Bioneer (Daejeon, Korea). Antibodies recognizing TRP-1 (sc-166857), tyrosinase (sc-20035), TRP-2 (sc-166716), and β-actin (sc-47778) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and MITF (#97800S), Goat anti-Mouse IgG, HRP (#7074), and Goat anti-Rabbit IgG, HRP (#7076) were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Extraction method for C. falcatum extracts

The extracts derived from *C. falcatum* were obtained according to the method described previously with slight modifications[18]. At room temperature, the dried *C. falcatum* powder was immersed in methylene chloride (CH_2Cl_2) for 24 h, and then the solvent was transferred to an empty container to be filtered and yield crude solvent extract. Next, CH_2Cl_2 was added to the remaining residue and kept at room temperature for another 24 h, after which the solvent was again collected and added to the previously collected sample after filtration. The same steps were followed by only swapping the extraction solvent, CH_2Cl_2, with methanol. The two types of collected and filtered solvents (CH_2Cl_2 and MeOH) were combined, and the combined solvent extract was concentrated in a 40℃ water bath with a rotary vacuum evaporator to obtain the crude extract. A part of the crude extract was dissolved in 10% DMSO to be used in experiments while some parts of the crude extract were dissolved in CH_2Cl_2/H_2O (*v:v*, 1:1) solute suspension with vigorous shaking and the mixture was laid to rest until two solvents separated fully. The CH_2Cl_2 layer and H_2O layer were separately collected using a separatory funnel. Continually, the CH_2Cl_2 layer was subjected to evaporation as described earlier and the obtained extract was redissolved in 85% aqueous (aq.) MeOH and *n*-hexane (*v:v*, 1:1) solute suspension to obtain these solvent fractions named after the solvent from which they are obtained. Similarly, the H_2O layer was directly mixed with the same volume of *n*-BuOH without any evaporation to obtain the H_2O and *n*-BuOH solvent fraction extract.

2.3. Cell culture

The murine melanoma cell line, B16F10, was procured from Korea Cell Line Bank (KCLB, Seoul, Korea). Unless otherwise noted or the experiment required otherwise, the cells were provided with DMEM that contains 10% FBS and 1% *L*-glutamine penicillin-

streptomycin solution and maintained at a constant temperature (37℃) and atmosphere (5% CO_2).

2.4. MTT assay

The viability of B16F10 cells treated with increasing concentrations of *C. falcatum* crude extract and solvent fractions was assessed by traditional MTT assay method as previously reported with necessary modifications[18]. Briefly, the B16F10 cells were transferred to 96-well plates (1×10^4 cells/well), and the cells were kept in incubators at 37℃, 5% CO_2 for 24 h. Then the culture medium was swapped with a fresh medium containing different concentrations of crude extract and fractions (0-100 μg/mL) followed by incubation for a further 24 h. After the treatment, wells were aspirated and were added with 100 μL of 100 μg/mL MTT solution each. Plates were placed in the incubator after the addition of MTT solution. After 4 h, MTT solution was removed from the wells, and the wells were washed with PBS. One hundred microliters of DMSO (100%) were added to the wells after washing. Finally, the absorbance values were measured at 560 nm using a microplate reader (Multiskan GO, Tecan Austria GmbH, Grodig, Austria).

2.5. Detection of intracellular active tyrosinase levels and melanin content

The B16F10 cells were seeded in 6-well plates at a density of 3.4×10^4 cells/well. The groups were as follows: the blank group, the control group (α-MSH-stimulated and untreated), the kojic acid group (α-MSH-stimulated and 10 μM kojic acid-treated), the crude extract group (α-MSH-stimulated and treated with 5, 10 and 20 μg/mL *C. falcatum* crude extract, separately), and the solvent fraction group (same with the crude extract group but treated with H_2O, *n*-BuOH, 85% aq. MeOH, *n*-hexane fractions instead of crude extract). For future analysis, the same groups were used. Briefly, the cells in plates were grown to confluency and exposed to different concentrations of samples for 24 h. Following the treatment, the cell culture medium was removed, and the cells were washed twice with fresh PBS each time. Subsequently, cells in wells were lysed by QIAzol lysis buffer (200 μL/well) with vigorous pipetting, and the lysates were collected and centrifuged at 13 000 rpm for 10 min. To assess the intracellular active tyrosinase levels, the supernatant was used whereas the pellet was kept separately for melanin analysis. Specifically, the protein content of each sample was determined using BioRad Protein Assay Reagent (Bio-Rad Laboratories, California, USA), after which 0.1% (*w/v*) *L*-DOPA was added to each supernatant containing the same amount of protein along with 0.1 M sodium phosphate buffer in a 96-well plate. The plate was then kept in an incubator for 1 h. After 1 h, the absorbance values of the wells were read at 490 nm with a microplate reader (Multiskan GO).

For the quantification of intracellularly accumulated melanin amount, cell pellets were used. The pellets were first washed with ice-cold ethanol (75%) and then dissolved in 200 μL of 1 N NaOH. The resuspended cell pellets were heated to 90℃ and kept at this temperature for 10 min. After cooling down to room temperature, the absorbance values were recorded at 405 nm using a microplate reader. Intracellular melanin content was calculated based on the absorbance values fitted into the standard curve for melanin content.

2.6. Extracellular melanin content assay

The melanin secretion by stimulated B16F10 cells was measured *via* the quantification of the melanin in the cell culture medium. The cells were grown to confluency under the conditions given in section 2.4 only changing DMEM to phenol red-free DMEM. The cell groups and treatment were consistent with those described in section 2.5. Following a 24-hour incubation with samples, the absorbance values of the cell culture medium were measured at 405 nm to determine extracellular melanin content.

2.7. RT–PCR experiment

The expression levels of the genes related to melanogenesis were determined *via* RT-PCR assay with a modified method described previously[19]. In brief, cells were grown and exposed to samples under the conditions given in section 2.5. After the treatment, cells were washed with PBS, and total RNA was extracted from the cells using QIAzol reagent. Subsequently, total RNA was reverse transcribed into cDNA using the CellScript cDNA Master Mix kit (Yongin, Korea), according to the manufacturer's protocol. Amplification of the cDNA was carried out by 33 cycles of 95℃ for 30 s, 60℃ for 45 s, and 72℃ for 1 min using the primers given in Table 1. The end products were loaded and visualized on a 1.5% (*w/v*) agarose gel. To evaluate the target gene expression levels, images of the bands were taken and were subsequently quantified with densitometric quantification using ImageJ software, with *β–actin* serving as the internal control gene.

Table 1. Gene-specific primers used in RT-PCR analysis.

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

MITF: microphthalmia transcription factor, TRP: tyrosinase-related protein.

2.8. Immunoblotting

The cells were grown and exposed to samples in groups described in section 2.5 in 6-well plates. After the treatment, the whole intracellular protein was extracted from each well using RIPA buffer. The amount of protein isolated was quantified using a BCA kit from Thermo Fisher Scientific (Waltham, MA, USA). From each treatment group, 20 μg of total protein were transferred onto 10% SDS-PAGE gels to be separated (100 V, 90 min). The separated proteins were then transferred from the SDS-PAGE gels onto membranes, and the membranes were kept in 5% skim milk (in TBST) for 1 h at room temperature for blocking. After blocking was ended, skim milk was removed, and the membranes were washed 3 times with 1×PBST. Next, the blocked membranes were introduced to the primary antibodies. The membranes were kept with primary antibodies overnight at 4℃. All primary antibody solutions were prepared and diluted according to the suggested buffers and dilution levels of the manufacturer for immunoblotting. Hybridization of the primary antibodies was then carried out for 1 h at room temperature by removing the primary antibody solution, washing the membranes with PBST, and the addition of secondary antibody solution. Finally, the pictures of the PDVF membranes were taken with CAS-400SM Davinch-Chemi Imager (Davinch-K) following the addition of WestGlowTM FENTO Chemuliluminescent Substrate (Biomax, Seoul, Korea). The related expression levels of proteins were analyzed with densitometric quantification of the bands using ImageJ software.

2.9. Statistical analysis

All data were given as mean ± standard deviation (SD) (n=3). Statistical differences between groups were analyzed using the one-way ANOVA test followed by the Duncan's test. Statistical analysis was performed using SPSS 21.0 software (IBM Corp., NY, USA).

3. Results

3.1. Effect of *C. falcatum* crude extract on melanogenesis in B16F10 cells

After exposure to various concentrations of *C. falcatum* crude extract for 24 h, the cytotoxicity was assessed by the MTT assay. As demonstrated in Figure 1A, the cells exhibited high viability and minimal cytotoxicity at concentrations of 5, 10, 15, and 20 μg/mL. Accordingly, these concentrations were employed in subsequent experiments. Notably, Figures 1B, C, and D indicate that *C. falcatum* crude extract exhibited an inhibitory effect on active tyrosinase levels, extracellular melanin content, and the accumulated melanin amount, respectively, in α-MSH-stimulated B16F10 cells.

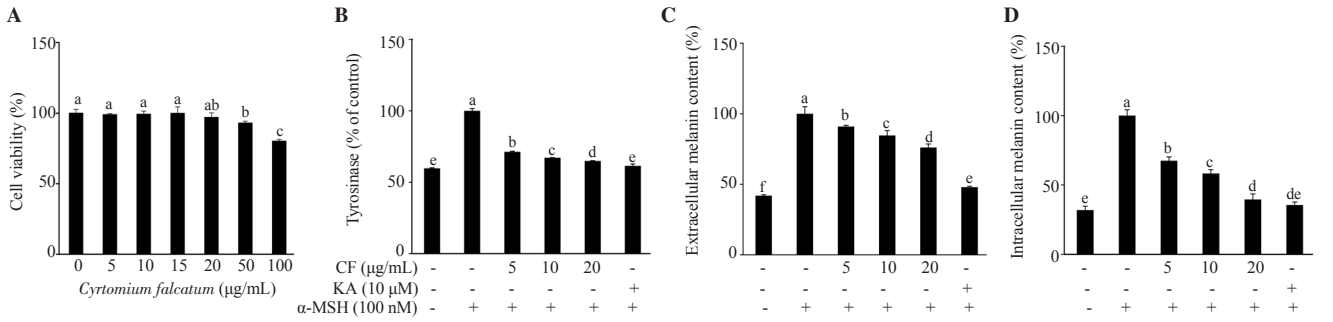

Figure 1. Effect of *Cyrtomium falcatum* crude extract on (A) the viability and (B-D) melanogenesis in B16F10 cells. [a-f]Different letters indicate statistical significance ($P < 0.05$) using a one-way ANOVA test followed by Duncan's test. CF: *Cyrtomium falcatum*; KA: kojic acid.

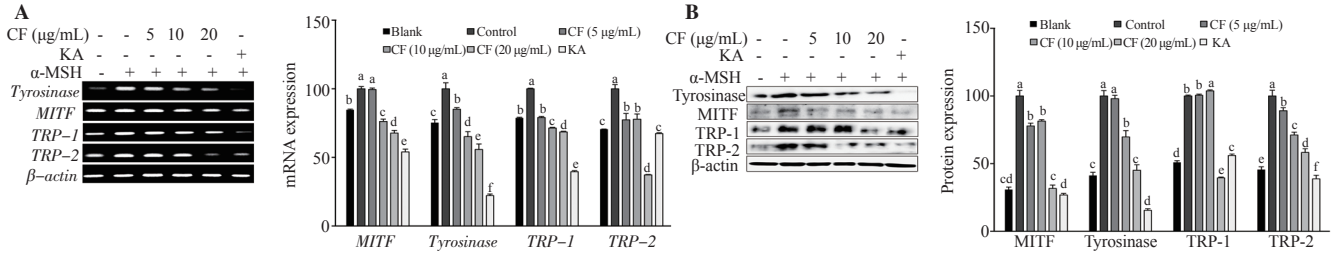

Figure 2. Effect of *Cyrtomium falcatum* crude extract on the expression levels of melanogenesis-related genes and proteins. (A) The mRNA and (B) protein expression levels of tyrosinase, MITF, TRP-1, and TRP-2 were analyzed by RT-PCR and immunoblotting analyses, respectively, and normalized using β-actin levels. [a-f]Different letters indicate statistical significance ($P < 0.05$) using a one-way ANOVA test followed by Duncan's test.

3.2. Effect of C. falcatum crude extract on related gene and protein expressions

The RT-PCR and immunoblotting assays were used to observe the expressions of genes and proteins related to melanogenesis in α-MSH-induced B16F10 cells. Figure 2A shows that α-MSH induced higher mRNA expression levels of *tyrosinase*, *MITF*, *TRP–1*, and *TRP–2* compared with the blank group. Compared with the control group, *C. falcatum* crude extract treatment dose-dependently inhibited the expression of the aforementioned genes. Consistent with the RT-PCR results, *C. falcatum* crude extract suppressed the levels of tyrosinase, MITF, TRP-1, and TRP-2 proteins (Figure 2B).

3.3. Cytotoxic effect of C. falcatum crude extract–derived solvent fractions

MTT assay was carried out to evaluate the non-toxic concentrations of H_2O, *n*-BuOH, 85% aq. MeOH, and *n*-hexane fractions derived from *C. falcatum* crude extract. As demonstrated in Figure 3A, all these four solvent fractions exhibited no significant toxicity to B16F10 cells at concentrations of 5-20 μg/mL, while the cell viability decreased by more than 10% at the concentrations of 50 and 100 μg/mL. Therefore, *C. falcatum* crude extracts at 5-20 μg/mL were used for the follow-up experiments.

3.4. Effect of C. falcatum crude extract–derived solvent fractions on tyrosinase activity and melanin production

Melanin content and active tyrosinase levels are two critical indicators for analyzing melanogenesis. Figures 3B and 3C indicate that α-MSH stimulation substantially elevated both melanin secretion and intracellular melanin accumulation compared to the unstimulated group. However, the melanin content was decreased after treatment with different concentrations of H_2O, *n*-BuOH, 85% aq. MeOH, and *n*-hexane solvent fractions. The highest concentration of 20 μg/mL resulted in the highest decrease. As seen in Figure 3D, α-MSH

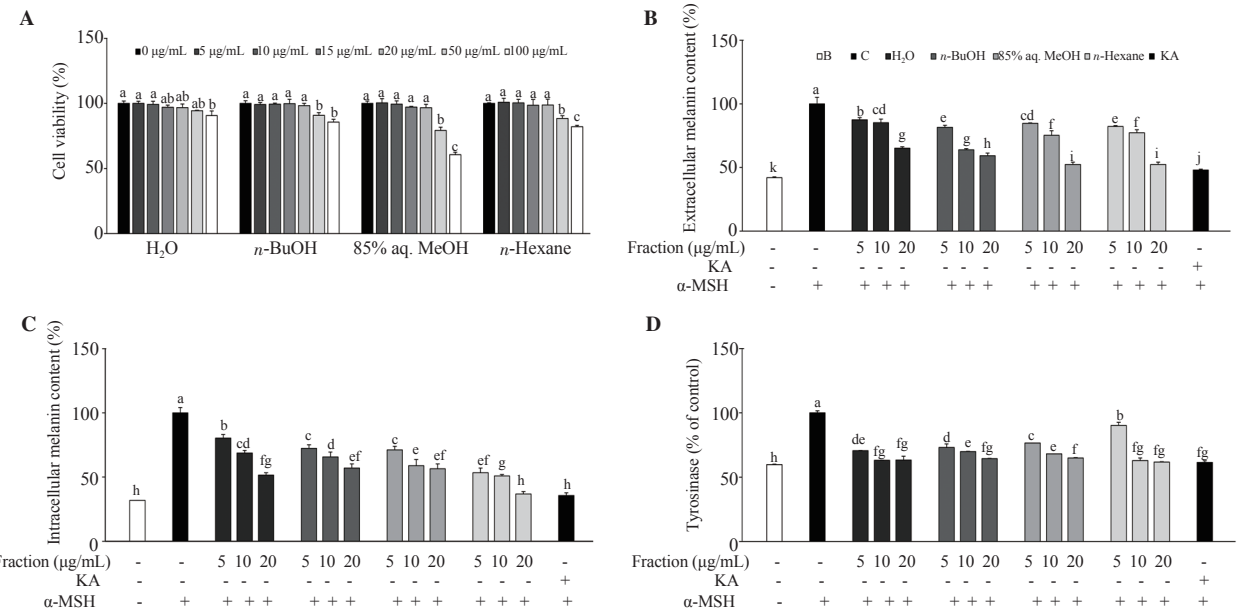

Figure 3. Effect of *Cyrtomium falcatum* crude extract-derived fractions on extracellular and intracellular melanin production as well as tyrosinase activity. (A) The viability of B16F10 cells treated with H_2O, *n*-BuOH, 85% aqueous MeOH, and *n*-hexane solvent fractions derived from *Cyrtomium falcatum*; (B) Melanin levels were detected in DMEM medium; (C) Melanin content inside the cells; (D) Intracellular active tyrosinase levels. [a-k]Different letters indicate statistical significance ($P < 0.05$) using a one-way ANOVA test followed by Duncan's test. B: blank; C: control.

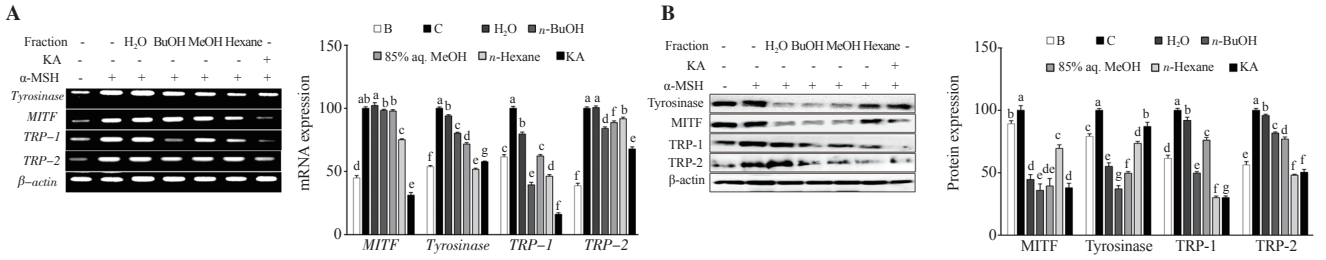

Figure 4. Effect of *Cyrtomium falcatum* crude extract-derived fractions on the mRNA and protein expressions of melanogenic markers. (A) The mRNA and (B) protein expression levels of tyrosinase, MITF, TRP-1, and TRP-2 were analyzed by RT-PCR and immunoblotting analyses, respectively, and normalized using β-actin levels. [a-g]Different letters indicate statistical significance ($P < 0.05$) using a one-way ANOVA test followed by Duncan's test.

significantly elevated the active tyrosinase levels. All concentrations of solvent fractions decreased the tyrosinase levels.

3.5. Melanogenesis-related mRNA and protein expression levels in solvent fraction-treated B16F10 cells

RT-PCR and immunoblotting experiments were used to determine mRNA and protein expression levels of tyrosinase, MITF, TRP-1, and TRP-2 in solvent fraction-treated B16F10 cells. As presented in Figure 4, the mRNA expression levels of *tyrosinase*, *MITF*, *TRP-1*, and *TRP-2* were substantially upregulated under α-MSH stimulation ($P<0.05$). However, the aforementioned genes were downregulated after the cells were exposed to 20 μg/mL of *n*-BuOH, 85% aq. MeOH, and *n*-hexane solvent fractions, but the H_2O fraction was not able to induce any significant decrease in terms of the expression levels of *MITF* and *TRP-2* mRNA. In addition, the suppressive effect of the fractions was not as strong as kojic acid, except for the effect of the *n*-hexane fraction on tyrosinase expression. Moreover, the immunoblotting results demonstrated a similar effect. All four fractions effectively suppressed the tyrosinase, MITF, TRP-1, and TRP-2 protein levels at the concentration of 20 μg/mL.

4. Discussion

C. falcatum is a widely cultivated plant in Japan and Korea, and reports indicate that it possesses antioxidant and anti-inflammatory activities[16]. The results of the current study indicated that *C. falcatum* crude extract and its fractions reduced the tyrosinase and extra/intracellular melanin content and suppressed the protein and mRNA expressions of MITF, tyrosinase, TRP-1, and TRP-2 in α-MSH-stimulated B16F10 cells.

The MTT compound is a commonly employed agent that has been used in experiments to detect cell viability *via* the ability of the alive cells to convert it into formazan crystals which give a distinctive color. Therefore, it is widely employed to assess viable cell levels in order to observe the potential cytotoxicity of reagents[20]. Before testing *C. falcatum* for its anti-melanogenic properties, MTT was conducted to determine the non-toxic concentrations in order to eliminate any toxic effect of samples that could be interpreted as an inhibitory effect.

Tyrosinase is an important rate-limiting enzyme that controls melanin synthesis[21]. The production, activation, and enzymatic activity of tyrosinase are directly linked with the production rate of melanin. Although necessary and beneficial in normal conditions, abnormal accumulation of melanin in skin tissue causes undesired and mostly harmful complications such as age spots, actinic damage, melanoma, and dermatological disorders. Considering the control effect of tyrosinase on melanin production, searching for tyrosinase inhibitors to prevent excessive melanin production has drawn much attention in food processing and storage, medical, and cosmetic industries[22]. A previous study claimed that four chemically modified curcumin derivatives showed very significant inhibitory effects on tyrosinase activity, greater than kojic acid, in stimulated murine melanoma cells[23]. Another study also revealed that several flavonoids from *Dalbergia parviflora* decreased melanin content by inhibiting tyrosinase activity in the same type of cells under similar conditions[24]. Consistent with these reports, the results of the present study also revealed the anti-melanogenic effects of *C. falcatum* crude extract and its H_2O, *n*-BuOH, 85% aq. MeOH and *n*-hexane fractions. They could decrease melanin content as well as significantly lower the active tyrosinase levels in α-MSH-stimulated B16F10 cells at non-toxic concentrations. This data suggested that *C. falcatum* might contain bioactive ingredients that inhibit tyrosinase activation and as a result limit melanin production.

In addition, the expression levels of melanogenesis-related genes and proteins including MITF, tyrosinase, TRP-1, and TRP-2 were evaluated. MITF is a vital transcription factor for melanogenesis by controlling the transcription of tyrosinase, TRP-1, and TRP-2[25]. The MC1R receptor on melanocytes is stimulated by α-MSH, which then activates cAMP and upregulates the activation of cAMP-response element binding protein through phosphorylation and consequently promotes the expression of MITF[26]. Expectedly, the current results also showed that α-MSH stimulation significantly elevated the expression of MITF, tyrosinase, TRP-1, and TRP-2 in both mRNA and protein levels, which suggested the successful establishment of melanogenesis *in vitro* in B16F10 cells.

Various research revealed that extracts or active compounds derived from natural plants exhibit effective bioactivities against a variety of symptoms and diseases. The methanolic extracts of *Cuscuta pedicellata* and *Tribulus terrestris* showed strong antioxidant, anti-inflammation, and antimicrobial potential[27]. Other studies also demonstrated that several endemic plants from Mauritius Island contained flavonoids, terpenes, fatty acids, terpenoids, and phenolic acids that exhibited immunomodulatory effects on primary T and B cells, and anti-cancer effects on T cell lymphoma and B16F10 melanoma cells[28]. Likewise, numerous studies focus on exploring the whitening effect of natural plants and natural-origin bioactive compounds. In this context, the ethanolic extract of *Sorghum bicolor* was reported to contain compounds that exhibited the whitening effect on 3-isobutyl-1-methylxanthine-stimulated B16F10 cells by downregulating the transcriptional pathways of MITF, tyrosinase, TRP-1, and TRP-2[29]. Another study suggested that a 3% Korean red ginseng extract cream could improve skin moisture and resilience of human skin, and suppress collagen degradation which in turn decreased wrinkle formation in UVB-irradiated HRM-2 hairless mice[30]. Consistent with these results, the present study demonstrated that *C. falcatum* extracts suppressed the mRNA and protein expression levels of tyrosinase, TRP-1, and TRP-2 by inhibiting the expression of MITF.

Ferns are a group of spore plants that are diverse in both germplasm

and reported to contain unique secondary metabolites that might not be found in other types of plants. There are more than ten thousand species of ferns and expectedly several of them are mentioned in folk medicine records for their medicinal value[31]. Medicinal ferns mostly have antipyretic, detoxification, muscle relaxer, antimicrobial and anti-inflammatory effects[32–34]. *C. falcatum* belongs to a fern family that originated in Asia and to date it is usually cultivated as an ornamental plant in other countries[35]. Up to now, most studies paid attention to researching the distribution, diversity, and phylogenetic relationships of *C. falcatum* in relation to its decorative and environmental value, but rarely evaluated its health-beneficial properties through the analysis of the bioactive function of its metabolites[36]. In the current study, *C. falcatum* extracts were shown to possess anti-melanogenesis potential in α-MSH-stimulated B16F10 melanoma cells. The suggested melanogenesis inhibitory effect may be related to its phenolic acid and flavone content[37]. However, further research should be carried out to analyze the active compounds and the possible anti-melanogenesis mechanism of *C. falcatum*. On the other hand, there are certain limitations to the interpretation of the current results. The results only show the ability of *C. falcatum in vitro* and using one type of cell line. Studies carried out with more skin-like models and *in vivo* studies would provide more constructive results toward the safe utilization of *C. falcatum*. Also, in-depth mechanism analyses using specific pathways and their inhibitors will strengthen the present study's hypothesis. Nonetheless, the present study provided insight into the anti-melanogenic effect of *C. falcatum* extract and fractions which would pave the way for isolation and characterization of bioactive ingredients with melanogenesis inhibitory effects.

In summary, the results of the current study revealed that *C. falcatum* crude extract and its solvent fractions exhibited an inhibitory effect on α-MSH-induced melanogenesis in B16F10 cells. Both crude extract and solvent fractions suppressed melanin secretion and tyrosinase activity. In addition, they suppressed the mRNA and protein expression levels of tyrosinase, MITF, TRP-1, and TRP-2 at the non-toxic concentrations of 5, 10, and 20 μg/mL. These results indicate that *C. falcatum* crude extract and its solvent fractions might contain anti-melanogenic substances and that it can be utilized as a potential source of lead molecules for the research and development of whitening agents.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Funding

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No.2023R1A2C1006268 and RS-2023-00212560).

Authors' contributions

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

References

[1] Slominski RM, Sarna T, Płonka PM, Raman C, Brożyna AA, Slominski AT. Melanoma, melanin, and melanogenesis: The yin and yang relationship. *Front Oncol* 2022; **12**: 842496.

[2] Cherdchom S, Sereemaspun A, Aramwit P. Urea-extracted sericin is potentially better than kojic acid in the inhibition of melanogenesis through increased reactive oxygen species generation. *J Tradit Complement Med* 2021; **11**(6): 570-580.

[3] Li Z, Heng H, Qin Q, Chen L, Wang Y, Zhou Z. Physicochemical properties, molecular structure, antioxidant activity, and biological function of extracellular melanin from *Ascosphaera apis*. *J Zhejiang Univ B* 2022; **23**(5): 365-381.

[4] Avogadri F, Gnjatic S, Tassello J, Frosina D, Hanson N, Laudenbach M, et al. Protein expression analysis of melanocyte differentiation antigen TRP-2. *Am J Dermatopathol* 2016; **38**(3): 201-207.

[5] Kim M, Mohamed E, Kim D, Park MJ, Ahn BJ, Jeung EB, et al. Inhibitory effects and underlying mechanisms of *Artemisia capillaris* essential oil on melanogenesis in the B16F10 cell line. *Mol Med Rep* 2022; **25**(4): 113.

[6] Soliman SSM, Hamdy R, Elseginy SA, Gebremariam T, Hamoda AM, Madkour M, et al. Selective inhibition of *Rhizopus eumelanin* biosynthesis by novel natural product scaffold-based designs caused significant inhibition of fungal pathogenesis. *Biochem J* 2020; **477**(13): 2489-2507.

[7] Mariano A, Bigioni I, Scotto d'Abusco A, Baseggio Conrado A, Maina S, Francioso A, et al. Pheomelanin effect on UVB radiation-induced oxidation/nitration of l-tyrosine. *Int J Mol Sci* 2021; **23**(1): 267.

[8] Liu X, Li H, Cong X, Huo D, Cong L, Wu G. α-MSH-PE38KDEL kills melanoma cells *via* modulating Erk1/2/MITF/TYR signaling in an MC1R-dependent manner. *Onco Targets Ther* 2020; **13**: 12457-12469.

[9] Zhou S, Riadh D, Sakamoto K. Grape extract promoted α-MSH-induced melanogenesis in B16F10 melanoma cells, which was inverse to resveratrol. *Molecules* 2021; **26**(19): 5959.

[10]Qian W, Liu W, Zhu D, Cao Y, Tang A, Gong G, et al. Natural skin whitening compounds for the treatment of melanogenesis (Review). *Exp Ther Med* 2020; **20**(1): 173-185.

[11]Li HY, Gan RY, Shang A, Mao QQ, Sun QC, Wu DT, et al. Plant-based

foods and their bioactive compounds on fatty liver disease: Effects, mechanisms, and clinical application. *Oxid Med Cell Longev* 2021; **2021**: 6621644.

[12]Ahmed SR, Rabbee MF, Roy A, Chowdhury R, Banik A, Kubra K, et al. Therapeutic promises of medicinal plants in Bangladesh and their bioactive compounds against ulcers and inflammatory diseases. *Plants* 2021; **10**(7): 1348.

[13]Sioud F, Maatouk M, Bzeouich IM, Ghedira LC, Kilani-Jaziri S. *In vitro* anti-melanoma effect of polyphenolic compounds. *Asian Pac J Trop Biomed* 2022; **12**(10): 446-452.

[14]Merecz-Sadowska A, Sitarek P, Kowalczyk T, Zajdel K, Kucharska E, Zajdel R. The modulation of melanogenesis in B16 cells upon treatment with plant extracts and isolated plant compounds. *Molecules* 2022; **27**(14): 4360.

[15]Raman G, Choi K, Park S. Phylogenetic relationships of the fern *Cyrtomium falcatum* (Dryopteridaceae) from Dokdo island based on chloroplast genome sequencing. *Genes (Basel)* 2016; **7**(12): 115.

[16]Kim H, Kim H, Kong CS, Lee BH, Sim HB, Seo Y. Antioxidant and anti-inflammatory activities of the halophyte *Cyrtomium falcatum*. *Ocean Polar Res* 2021; **43**(3): 113-126.

[17]Iwashina T, Kitajima J, Matsumoto S. Flavonoids in the species of *Cyrtomium* (Dryopteridaceae) and related genera. *Biochem Syst Ecol* 2006; **34**(1): 14-24.

[18]Oh JH, Karadeniz F, Lee JI, Seo Y, Kong CS. *Ligustrum japonicum* Thunb. fruits exert antiosteoporotic properties in bone marrow-derived mesenchymal stromal cells *via* regulation of adipocyte and osteoblast differentiation. *Stem Cells Int* 2021; **2021**: 8851884.

[19]Chaiprasongsuk A, Onkoksoong T, Pluemsamran T, Limsaengurai S, Panich U. Photoprotection by dietary phenolics against melanogenesis induced by UVA through Nrf2-dependent antioxidant responses. *Redox Biol* 2016; **8**: 79-90.

[20]Huang HC, Wang SS, Tsai TC, Ko WP, Chang TM. *Phoenix dactylifera* L. seed extract exhibits antioxidant effects and attenuates melanogenesis in B16F10 murine melanoma cells by downregulating PKA signaling. *Antioxidants* 2020; **9**(12): 1270.

[21]Yu F, Lu Y, Zhong Z, Qu B, Wang M, Yu X, et al. Mitf involved in innate immunity by activating tyrosinase-mediated melanin synthesis in *Pteria penguin*. *Front Immunol* 2021; **12**: 626493.

[22]D'Mello S, Finlay G, Baguley B, Askarian-Amiri M. Signaling pathways in melanogenesis. *Int J Mol Sci* 2016; **17**(7): 1144.

[23]Goenka S, Johnson F, Simon SR. Novel chemically modified curcumin (CMC) derivatives inhibit tyrosinase activity and melanin synthesis in B16F10 mouse melanoma cells. *Biomolecules* 2021; **11**(5): 674.

[24]Promden W, Viriyabancha W, Monthakantirat O, Umehara K, Noguchi H, De-Eknamkul W. Correlation between the potency of flavonoids on mushroom tyrosinase inhibitory activity and melanin synthesis in melanocytes. *Molecules* 2018; **23**(6): 1403.

[25]Kim DH, Shin DW, Lim BO. Fermented *Aronia melanocarpa* inhibits melanogenesis through dual mechanisms of the PI3K/AKT/GSK-3β and PKA/CREB pathways. *Molecules* 2023; **28**(7): 2981.

[26]Hseu YC, Vudhya Gowrisankar Y, Wang LW, Zhang YZ, Chen XZ, Huang PJ, et al. The *in vitro* and *in vivo* depigmenting activity of pterostilbene through induction of autophagy in melanocytes and inhibition of UVA-irradiated α-MSH in keratinocytes *via* Nrf2-mediated antioxidant pathways. *Redox Biol* 2021; **44**: 102007.

[27]Naz R, Ayub H, Nawaz S, Islam ZU, Yasmin T, Bano A, et al. Antimicrobial activity, toxicity and anti-inflammatory potential of methanolic extracts of four ethnomedicinal plant species from Punjab, Pakistan. *BMC Complement Altern Med* 2017; **17**(1): 302.

[28]Kauroo S, Govinden-Soulange J, Ranghoo-Sanmukhiya VM, Miranda K, Cotham WE, Walla MD, et al. Extracts of select endemic plants from the Republic of Mauritius exhibiting anti-cancer and immunomodulatory properties. *Sci Rep* 2021; **11**(1): 4272.

[29]Han HJ, Park SK, Kang JY, Kim JM, Yoo SK, Heo HJ. Anti-melanogenic effect of ethanolic extract of *Sorghum bicolor* on IBMX-induced melanogenesis in B16/F10 melanoma cells. *Nutrients* 2020; **12**(3): 832.

[30]Saba E, Kim SH, Lee YY, Park CK, Oh JW, Kim TH, et al. Korean Red Ginseng extract ameliorates melanogenesis in humans and induces antiphotoaging effects in ultraviolet B-irradiated hairless mice. *J Ginseng Res* 2020; **44**(3): 496-505.

[31]Xu L, Xing Y, Wang B, Liu C, Wang W, Kang T. Plastid genome and composition analysis of two medical ferns: *Dryopteris crassirhizoma* Nakai and *Osmunda japonica* Thunb. *Chin Med* 2019; **14**(1): 9.

[32]Hashemzehi M, Yavari N, Rahmani F, Asgharzadeh F, Soleimani A, Shakour N, et al. Inhibition of transforming growth factor-beta by Tranilast reduces tumor growth and ameliorates fibrosis in colorectal cancer. *EXCLI J* 2021; **20**: 601-613.

[33]Farràs A, Mitjans M, Maggi F, Caprioli G, Vinardell MP, López V. Exploring wild Aspleniaceae ferns as safety sources of polyphenols: The case of *Asplenium trichomanes* L. and *Ceterach officinarum* Willd. *Front Nutr* 2022; **9**: 994215.

[34]Su Y, He Z, Wang Z, Hong Y, Wang T. Characterization of the complete chloroplast genome of *Leptochilus decurrens* (Polypodiaceae), a least concern folk medicinal fern. *Mitochondrial DNA Part B* 2019; **4**(2): 3346-3347.

[35]Chung MY, Chung MG. Significant spatial aggregation and fine-scale genetic structure in the homosporous fern *Cyrtomium falcatum* (Dryopteridaceae). *New Phytol* 2013; **199**(3): 663-672.

[36]Imai R, Tsuda Y, Matsumoto S, Ebihara A, Watano Y. The relationship between mating system and genetic diversity in diploid sexual populations of *Cyrtomium falcatum* in Japan. *PLoS One* 2016; **11**(10): e0163683.

[37]Cao H, Chai TT, Wang X, Morais-Braga MFB, Yang JH, Wong FC, et al. Phytochemicals from fern species: Potential for medicine applications. *Phytochem Rev* 2017; **16**(3): 379-440.

Publisher's note