Research Article

Whitening effects of fermented *Trigonotis radicans* var. *sericea* with *Lactobacillus brevis* in α-MSH-stimulated B16F10 melanoma cells

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Abstract This study was designed to compare the whitening effects of 60% ethanol extracts of *Trigonotis radicans* var. *sericea* (TR) and *Lactobacillus brevis*-fermented *T. radicans* var. *sericea* (FTR). Measurement of cytotoxicity in B16-F10 melanoma cells to confirm the whitening effect, FTR showed higher cell viability than TR. FTR showed inhibitory activity on melanin contents similar to the normal group at concentrations of 50 and 100 μg/mL. MITF expression was used to confirm the effect on melanogenesis-related protein expression. TR and FTR showed significant concentration-dependent decrease, and FTR showed lower expressions than the normal group at concentrations of 25, 50, and 100 μg/mL. Additionally, the mRNA expression of melanogenesis-related genes (MC1R, Rab27a, TGF-β1 and Myo5a) were measured by RT-qPCR to confirm the whitening effect. In MC1R expression at a concentration of 100 μg/mL in FTR showed effective inhibitory activities, and in TGF-β1 expression, TR and FTR both showed effective activities compared to normal groups even at low concentrations. Results of myo5a and Rab27a, a similar pattern was shown, and FTR showed effective inhibitory activities at a concentration of 100 μg/mL. As a result, FTR had higher whitening effects through bioconversion and is expected to be a good material for whitening functional cosmetics.

Keywords *Trigonotis radicans* var. *sericea*, fermentation, whitening effects, bioconversion

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1. Introduction

Recent improvements in income levels and increased social activity have led to a steady growth in the global cosmetics industry, forming a market worth approximately 351.6 billion dollars as of 2015 (Lee, 2017). With the enforcement of the "Cosmetics Act", the cosmetics-related industry is increasingly demanding advanced and functional technologies, as well as new ingredient development. Markets related to functional cosmetics with skin-whitening and anti-aging are expected to grow rapidly. According to the recent trend of being environmentally friendly and nature-oriented, the active ingredients in cosmetics are mixed and used in various forms based on animal or plant-derived natural products. This development trend is expected to continue in the future, with a preference for natural and organic products (Sohn et al., 2004).

Melanin is a natural polymeric pigment widely found in animals and plants, and its biosynthesis is promoted as a mechanism to protect against skin damage caused by ultraviolet radiation (UVR), such as the synthesis of melanin to absorb more than a certain amount of UVR to block and protect (Park, 2015; Yoneta et al., 2004). With increasing levels of exposure to UVR, melanin can be excessively synthesized, and the physiological function of the skin decreases owing to aging,

causing damage to skin connective tissue and diseases such as pigmentation in various forms such as freckles (Hill et al., 1997). The outermost layer of the human skin consists of the epidermis, dermis, and hypodermis, and the basal layer of the epidermis is directly exposed to the external environment, containing melanocytes. The production of melanin takes place in melanosomes which are produces in melanocytes. There are two major types of melanin: pheomelanins (complexes of lighter yellow and red pigments) and eumelanins (darker brown and black pigments). Generally, all types of skin have a higher proportion of eumelanin than pheomelanin (Brenner et al., 2008). The binding of α-melanocyte stimulating hormone (α-MSH) to melanocytes increases by UVR, and α-MSH is produced with several other peptides, by the proteolytic cleavage of the large precursor protein pro-opiomelanocortin (POMC) (Tsatmali et al., 2002). Melanocortin 1 receptor (MC1R) involved in regulating melanin synthesis and, is one of the key receptors. α-MSH binds to MC1R which positively regulates melanogenesis. α-MSH stimulates the production of intracellular concentration of cyclic adenosine monophosphate (cAMP) by activating adenylyl cyclase (AC). cAMP production protein kinase A (PKA) activation in turn activates microphthalmia-associated transcription factor (MITF) by phosphorylating cAMP response element-binding protein (CREB). MITF plays a fundamental role in the transcriptional regulation of melanogenesis. In this process transforming growth factor-β (TGF-$\beta 1$) inhibits the activity of PKA activated by MC1R to downregulate the activity of CREB-MITF transcription factor. MITF regulates the expression of TYR, TRP-1, and TRP-2. In melanin synthesis, biochemical-catalyzed and complex enzymatic reactions are involved. Enzymatically, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), and tyrosinase (TYR) are key regulators of melanogenesis. Synthesis of melanin begins by the oxidation of L-dihydroxyphe-nylalanine (L-DOPA) and/or L-tyrosine to dopaquinone (DQ), which play a role as a substrate for the synthesis of eumelanin and pheomelanin (Pillaiyar et al., 2018; Slominski et al., 2004). As a result of the reactions of these enzymes and factors, the synthesized melanin accumulates in melanosomes and is transported to keratinocytes. The transportation of melanosomes to the melanocyte dendrite primarily involves three components of proteins: rab27a, melanophilin, and myosin Va (myo5a). When these three components form a tripartite protein complex, it is possible to transfer melanosomes to the dendrite tips, then melanin is transferred to keratinocytes (Fukuda et al., 2002).

Bioconversion refers to the technology of producing and manufacturing various metabolites through fermentation using microorganisms and enzymes. Unlike traditional fermentation processes, bioconversion utilizes the selectivity of microorganisms or enzymes to produce products from precursors. Bioconversion processes, which have enhanced fermentation technology, are particularly used in various ways in the fields of functional foods, modern pharmaceuticals, and cosmetics (Fuller, 1989).

Fermented cosmetics enhance the rapid absorption of active ingredients into the skin during the microbial fermentation process and improve the efficacy of existing enzymes. Fermented cosmetics are emerging as a trend in the domestic cosmetics market and several domestic major companies introducing cosmetics that utilize fermentation techniques, resulting in high sales and rapid growth (Lee et al., 2010). Various studies have been conducted to increase the biological activity and active ingredients of natural products through probiotic fermentation (Li et al., 2023).

Trigonotis radicans var. *sericea*, commonly known as "Chamkkotmari," belongs to the Boraginaceae family and is a perennial herb found in various fields and moist areas in Korea. It typically grows in sunny or semi-shaded environments, reaching a height of 10-15 cm and its leaves are 1.5-4 cm long, pointed at the end, and oval. The flowering time is from May to July, with light navy flowers that are approximately diameter of 0.7-1 cm. The fruits appear around September, and the young sprouts of this plant are used as food (Jung et al., 2010).

To date, there have been no studies on the bioconversion of *T. radicans* through microbial fermentation. Therefore, this study induced the bioconversion of *T. radicans* using Lactobacillus brevis isolated from the traditional Korean fermented food, *kkakdugi*. Additionally, this study aimed to provide fundamental data for the potential use of fermented *T. radicans* extracts as natural product-based functional cosmetic ingredients by confirming the differences in whitening improvement between the extracts of non-fermented and fermented *T. radicans*.

2. Materials and methods

2.1. Sample

Trigonotis radicans was purchased from the Korea Native

Wild Grass Laboratory (Busan, Korea). The roots were removed, whole plants were washed and dried in a dry oven (FO600M, Jeiotech, Daejeon, Korea) at 50℃. The dried plants were pulverized into 40 mesh size and stored at 4℃ for use as a sample.

2.2. Lactobacillus brevis culture

Lactobacillus brevis used for the fermentation of *T. radicans* was isolated from *kkakdugi* and stored at the School of Food Science and Biotechnology at Kyungpook National University. *Lactobacillus brevis* was preserved by mixing with a 40% glycerol (Duksan, Seoul, Korea) and culture medium ratio of 1:1 and stored at -80℃. *Lactobacillus brevis* was inoculated into MRS broth, subcultured twice, and 2% (v/v) of *L. brevis* culture medium was inoculated into 200 mL of MRS broth in a triangular flask and incubated at 37℃ for 24 h.

2.3. Fermentation of Trigonotis radicans

The fermentation of *T. radicans* was performed by adding 40 g of *T. radicans* powder to 200 mL of *L. brevis* culture medium in an Erlenmeyer flask and incubated in a 37℃ shaking incubator for 72 hours at 120 rpm. The fermented samples were freeze-dried and used as fermented *T. radicans* samples. Sixty percent ethanol was selected as the optimal extraction solvent for *T. radicans* and fermented *T. radicans*, as reported by Cho et al. (2022).

2.4. Cell culture

B16-F10 cells isolated from the skin tissue of a mouse with melanoma were purchased from ATCC and cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). The cells were cultured in a 5% CO_2 incubator at 37℃ and subcultured at a density of approximately 80% growth.

2.5. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was measured as described by Carmichael et al. (1987). B16-F10 cells were seeded at a density of 5×10^3 cells per well in a 48-well plate and cultured in a 5% CO_2 incubator at 37℃ for 24 h. Each freeze- dried powder of 60% ethanol extracts of *T. radicans* and fermented *T. radicans* was

prepared for sample making, sample was added 500 µL per well in different concentrations, and the cells were cultured under the same conditions for 18 h. After adding 50 µL of 5 mg/mL MTT solution, the plate was incubated in a 5% CO_2 incubator for 4 h at 37℃, the culture medium was removed, and 500 µL of DMSO was added. The absorbance was measured at 540 nm using a SPECTROstar Nano Microplate Reader (BMG Labtech). Cell viability(%) was calculated as (1 - Absorbance of sample / Absorbance of control) × 100.

2.6. Measurement of cellular melanin contents

The melanin contents were measured using the method described by Hosoi et al. (1985). B16-F10 cells were seeded at a density of 5×10^5 cells per well in 100-mm dishes for cell cultures and cultured in a 5% CO_2 incubator at 37℃ for 24 h. Subsequently, control and sample groups were stimulated with 1 µM α-MSH for 1 h, each freeze-dried sample of *T. radicans* and fermented *T. radicans* was treated by concentration, and incubated for 48 h in a 5% CO_2 incubator at 37℃. After treatment, the cells were washed with phosphate buffer saline (PBS) and stored at -80℃. Mixture of mammalian protein extraction reagent M-PER (Thermo Fisher Scientific) and protease was added 100 µL per well and lysis at 4℃. The harvested cells were centrifuged at 12,000 rpm for 20 minutes at 4℃. The lysate was stored at -80℃ until western blot analysis and collected pellet was dissolved with 1 N sodium hydroxide 10% DMSO at 70℃ for 1 h using a heating block (Daihan Scientific Co., Wonju, Korea) The melanin contents were measured at 405 nm using a SPECTROstar Nano microplate reader (BMG Labtech.). Melanin content was calculated as (1 - Absorbance of sample / Absorbance of control) × 100.

2.7. Western blot analysis for protein expression

The lysate obtained by centrifugation was quantified using a Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific™), and 20 µL of protein was loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and separated by electrophoresis at 100 V for 1.5 h. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes for 2.5 h at 60 V using a transfer cell device, and incubated in 5% bovine serum albumin blocking buffer in Tris-Buffered Saline with Tween 20 (TBST) for 1 h. Then membranes were incubated

overnight at 4℃ using MITF and GAPDH antibody. The membranes were washed three times with TBST and incubated for 1 h using secondary antibodies, rabbit and mouse. Protein bands were detected using a Chemiluminescent Western Blot Imager AZURE 300 (AZURE Biosystems, Dublin, CA, USA) using West Pico PLUS Chemiluminescent Substrate (Thermo Scientific™, Rockford, USA). Images of the detected bands were analyzed and quantified using ImageJ software version 1.51j8 (Wayne Rasband, National Institutes of Health, USA).

2.8. Reverse transcription quantitative polymerase chain reaction (RT−qPCR) for gene expression analysis

B16-F10 cells were seeded at a density of 5×10^5 cells per well in 100-mm dishes for cell cultures and cultured in a 5% CO_2 incubator at 37℃ for 24 h. Subsequently, control and sample groups were stimulated with 1 μM α-MSH for 1 h, each freeze-dried sample of *T. radicans* and fermented *T. radicans* was treated by concentration, and incubated for 48 h in a 5% CO_2 incubator at 37℃. The cells were washed with phosphate buffer saline (PBS) and stored at -80℃. Total RNA was extracted using TRI- Solution™ (BSK-Bio, Daegu, Korea) to compare and analyze the mRNA expression of melanogenesis-related genes. The extracted total RNA samples were stored at -80℃, and cDNA was synthesized using an UltraScript 2.0 cDNA Synthesis Kit (PCR Biosystems Ltd.,

London, UK). 1 μL of synthesized cDNA was diluted with 5 μL of qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems Ltd.), 0.3 μL of forward and reverse primer, and 3.4 μL of nuclease-free water. The PCRmax Eco 48 Real-Time qPCR system (PCRmax, Staffordshire, UK) was used to analyze mRNA expression, RT-qPCR was performed using the primer sequences listed in Table 1 and the PCR conditions were listed in Table 2.

2.9. Statistical analysis

The experiments were repeated three times for each measurement, and the results were presented as the mean± standard deviation. Significant differences ($p < 0.05$) were compared and analyzed using Duncan's multiple range test and one-way analysis of variance using IBM SPSS Statistics 25 (Statistical Package for Social Science, Chicago, IL, USA).

3. Results and discussions

3.1. Changes in the cell viability of B16−F10 cells through fermentation

The results of cell viability of treatment with concentrations of 12.5, 25, 50, and 100 μg/mL using each freeze-dried powder from *T. radicans* and fermented *T. radicans* are shown in Fig. 1. As a result, *T. radicans* showed cell viability of 83.74, 83.54, 84.75, and 82.47%, while fermented *T.*

Table 1. Primer sequences and accession numbers of melanogenesis−related genes analysed by RT−qPCR

Gene	Accession no.	Primer	Sequence (5'−3')	Amplicon size (bp)
MC1R[1]	NM_008559.2	Forward	AGAAGCTGGGGGCTGATAC	60
		Reverse	AGGAAGTAGGAAGCGGTCTGT	
TGF−β1[2]	NM_011577.2	Forward	CTCCCGTGGCTTCTAGTGC	133
		Reverse	GCCTTAGTTTGGACAGGATCTG	
Myo5a	NM_010864.2	Forward	GCGCCATCACCCTAAACA	90
		Reverse	CCAGTTGACTGACATTGTACCTG	
Rab27a	NM_023635.5	Forward	GAAGACCAGAGGGCAGTGAA	78
		Reverse	ACTGGTTTCAAAATAGGGGATTC	
β−Actin	NM_007393.4	Forward	CGTGCGTGACATCAAAGAGAA	137
		Reverse	GCTCGTTGCCAATAGTGATGA	

[1]MC1R, melanocortin 1 receptor.
[2]TGF−β1, transforming growth factor−β.

Table 2. RT-qPCR conditions for melanogenesis-related genes

Gene	Real-time qPCR condition
MC1R[1]	95℃ for 5 min, followed by undergoing 40 cycles of 95℃ for 10 s, 60℃ for 20 s, followed by each 95℃, 60℃, 95℃ for 15 s
TGF-β1[2]	95℃ for 5 min, followed by undergoing 40 cycles of 95℃ for 10 s, 60℃ for 20 s, followed by each 95℃, 60℃, 95℃ for 15 s
Myo5a	95℃ for 5 min, followed by undergoing 40 cycles of 95℃ for 10 s, 60℃ for 20 s, followed by each 95℃, 60℃, 95℃ for 15 s
Rab27a	95℃ for 5 min, followed by undergoing 40 cycles of 95℃ for 10 s, 60℃ for 20 s, followed by each 95℃, 60℃, 95℃ for 15 s
β-Actin	95℃ for 5 min, followed by undergoing 40 cycles of 95℃ for 10 s, 60℃ for 20 s, followed by each 95℃, 60℃, 95℃ for 15 s

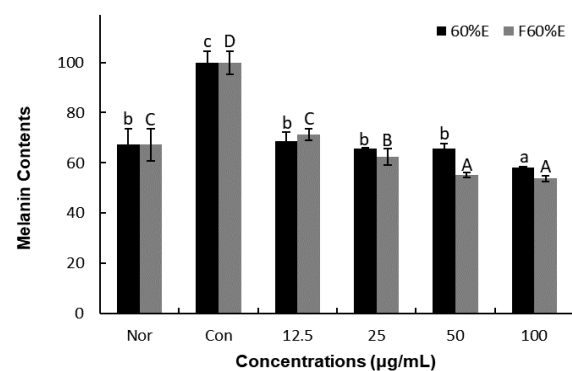[1]MC1R, melanocortin 1 receptor.
[2]TGF-β1, transforming growth factor-β.
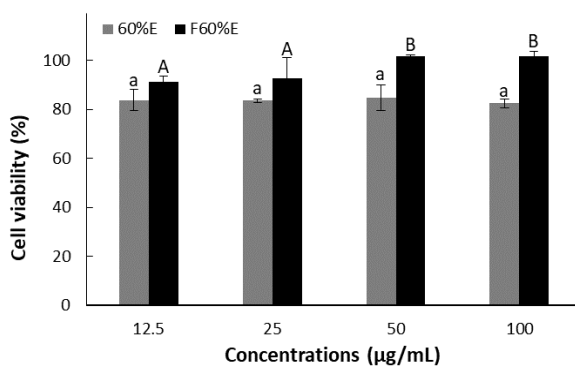
Fig. 1. Cell viability on B16-F10 of 60% ethanol extracts of *T. radicans* and fermented *T. radicans.* Values are mean±SD (n=3). Different letters ([A-B]) on the bars indicate significant differences (p⟨0.05) by Duncan's multiple range test.

Fig. 2. Melanin contents on B16-F10 of 60% ethanol extracts of *T. radicans* and fermented *T. radicans.* Values are mean±SD (n=3). Different letters ([a-c]) ([A-D]) on the bars indicate significant differences (p⟨0.05) by Duncan's multiple range test.

radicans showed higher cell viability of 91.31, 92.59, 101.83, and 101.63% at 12.5, 25, 50, and 100 μg/mL. Fermented *T. radicans* showed significantly lower cell toxicity, confirming that bioconversion through fermentation reduced the toxicity of the extracts.

3.2. Cellular melanin contents

To confirm the effects on melanin contents, freeze-dried samples of *T. radicans* and fermented *T. radicans* were treated at 12.5, 25, 50, and 100 μg/mL. As shown in Fig. 2., *T. radicans* showed melanin contents of 68.61, 65.54, 65.65, and 58.27%, and fermented *T. radicans* showed 71.41, 62.36, 55.21, and 53.71% at 12.5, 25, 50, and 100 μg/mL. *T. radicans* and fermented *T. radicans* showed a concentration-dependent decrease in melanin contents. Fermented *T. radicans* showed similar melanin contents compared to normal group at 12.5 and 25 μg/mL and showed lower melanin contents than the normal group at 50 and 100 μg/mL. Fermented *T.*

radicans showed lower melanin contents than *T. radicans* at 25, 50, and 100 μg/mL. It is confirmed that fermented *T. radicans* effectively reduced melanin content and showed potential as a good material for whitening functional cosmetics.

3.3. Inhibitory effect of MITF protein expression

All signaling pathways are associated with a master regulator of melanogenesis MITF, which controls melanogenesis gene expression (Pillaiyar et al., 2018). After confirming that *T. radicans* and fermented *T. radicans* decreased melanin production stimulated by α-MSH, expression of MITF, a transcription factor, was analyzed by western blot to confirm the effect on melanogenesis-related protein expression. As shown in Fig. 3, *T. radicans* and fermented *T. radicans* both showed significant concentration-dependent decrease in MITF expression, *T. radicans* showed MITF expression of 74.49, 64.14, 48.86, and 17.42% at 12.5, 25, 50, and 100 μg/mL, and showed lower expression at 50 and 100 μg/mL
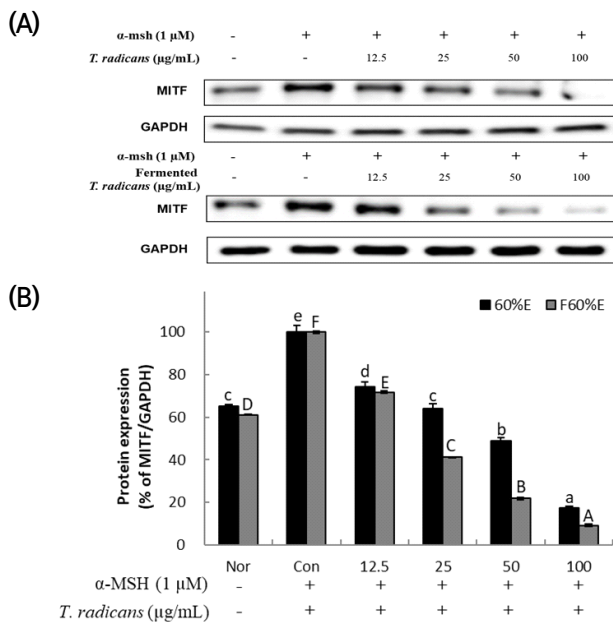
Fig. 3. Effects of *T. radicans* (A) and fermented *T. radicans* (B) on protein expression of microphthalmia‒associated transcription factor (MITF) in *α*‒melanocyte stimulating hormone (*α*‒MSH)‒stimulated B16F10 melanoma cells. Values are mean±SD (n=3). Different letters ([a‒e]) ([A‒F]) on the bars indicate significant differences (p<0.05) by Duncan's multiple range test.

than normal group. Fermented *T. radicans* showed 71.78, 41.20, 21.84, and 9.21% at 12.5, 25, 50, and 100 μg/mL, confirmed significant decreases in MITF expression, and showed lower expressions than normal group at 25, 50, and 100 μg/mL. Fermented *T. radicans* showed a significantly higher inhibitory effect of MITF protein expression than *T. radicans*. As a result, fermented *T. radicans* is expected to be used as a good material of whitening functional cosmetics.
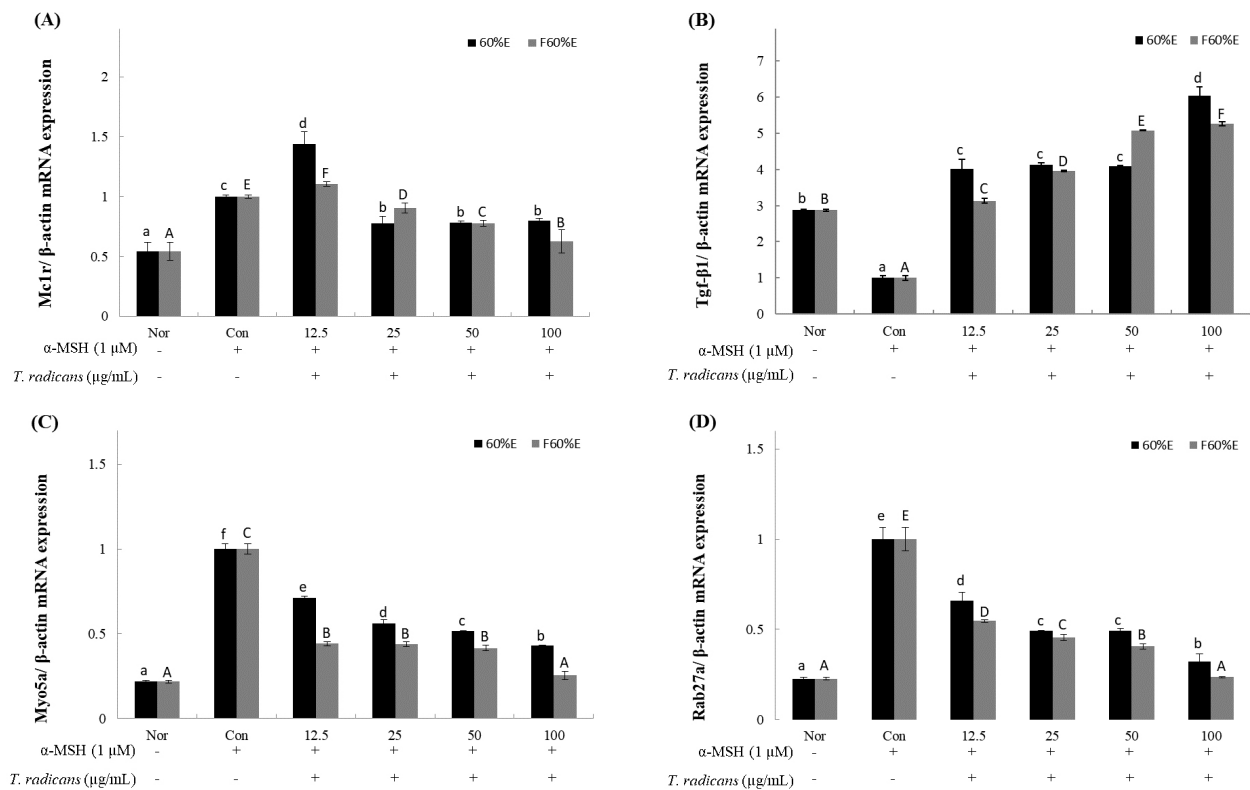
3.4. Changes in gene expression of melanogenesis‒related genes

After confirming that *T. radicans* and fermented *T. radicans* inhibit the expression of MITF protein, to confirm the changes by RT-qPCR in gene expression of melanogenesis-related genes (MC1R, TGF-β1, Myo5a, Rab27a), freeze-dried samples of *T. radicans* and fermented *T. radicans* were treated at 12.5, 25, 50, and 100 μg/mL. MC1R, one of the key receptors involved in regulating the melanin synthesis, shown in Fig. 4A, *T. radicans* showed mRNA expression of 1.44, 0.77. 0.78, and 0.80, and fermented *T. radicans* showed expression of 1.10, 0.91, 0.78,

and 0.63 at 12.5, 25, 50, and 100 μg/mL. Fermented *T. radicans* showed concentration-dependent decrease and showed effective inhibitory activities at 100 μg/mL. TGF-β1, activated by MC1R, is a potent inhibitor of melanin synthesis (Martínez-Esparza et al., 1997). As shown in Fig. 4B, *T. radicans* and fermented *T. radicans* both showed concentration-dependent increase in TGF-β1 expression, *T. radicans* showed mRNA expression of 4.02, 4.13, 4.10, and 6.05, and fermented *T. radicans* showed expression of 3.13, 3.96, 5.08, and 5.27 at 12.5, 25, 50, and 100 μg/mL. *T. radicans* and fermented *T. radicans* both showed effective activities compared to normal groups even at low concentrations, and results of TGF-β1 expression showed similar patterns with melanin contents. Myo5a and Rab27a, which regulates skin pigmentation during the melanosome transport process, showed in Fig. 4C and 4D. As shown in Fig. 4C, *T. radicans* showed Myo5a expression of 0.71, 0.56, 0.52, and 0.43, and fermented *T. radicans* showed expression of 0.44, 0.44, 0.42 and 0.25 at 12.5, 25, 50, and 100 μg/mL. As shown in Fig. 4D, *T. radicans* showed Rab27a expression of 0.66, 0.49, 0.49, and 0.32, and fermented *T. radicans* showed expression of 0.55, 0.45, 0.41, and 0.24 at 12.5, 25, 50, and 100 μg/mL. Results of Myo5a and Rab27a expression, *T. radicans* and fermented *T. radicans* both showed concentration-dependent decrease and showed similar patterns. Myo5a and Rab27a expressions of fermented *T. radicans* showed similar expression rates compared to normal groups at 100 μg/mL. As a result, fermented *T. radicans* showed higher inhibitory effects of expression of melanogenesis-related genes, confirming that bioconversion through fermentation inhibits the expression of melanogenesis- related gene. Fermented *T. radicans* expected to be used as a good material of whitening functional cosmetics.

4. Conclusions

In this study, *Trigonotis radicans* var. *sericea* was used to extract with 60% ethanol of *T. radicans* and *T. radicans* fermented with *L. brevis* and compare their whitening effects through bioconversion. The MTT assay to measure cell viability in B16-F10 cells, fermented *T. radicans* treated group showed higher cell viability than *T. radicans*, and confirmed significantly lower cell toxicity. And fermented *T. radicans* treated group showed lower melanin contents than *T. radicans* treated group at 25, 50, and 100 μg/mL. After

Fig. 4. Effects of *T. radicans* and fermented *T. radicans* on mRNA expression of melanogenesis−related gene melanocortin 1 receptor (MC1R) (A), transforming growth factor−β (TGF−β1) (B), Myo5a (C), and Rab27a (D) in α−melanocyte stimulating hormone (α−MSH)−stimulated B16F10 melanoma cells. Values are mean±SD (n=3). Different letters ([a−e]) ([A−E]) on the bars indicate significant differences (p<0.05) by Duncan's multiple range test.

confirming melanin production stimulated by α-MSH, expression of MITF was analyzed by western blot to confirm the effect on melanogenesis-related protein expression, and fermented *T. radicans* treated group showed lower expressions at 25, 50, and 100 μg/mL. RT-qPCR was performed to confirm the changes in gene expression of melanogenesis-related genes (MC1R, TGF-β1, Myo5a, Rab27a) by RT-qPCR. In MC1R expression, Fermented *T. radicans* treated group showed effective inhibitory activities at 100 μg/mL. In TGF-β1, *T. radicans* and fermented *T. radicans* treated groups showed effective activities compared to normal groups even at low concentrations. Results of Myo5a and Rab27a expression showed similar patterns, and Myo5a and Rab27a expressions of fermented *T. radicans* treated group showed effective inhibitory activities at 100 μg/mL. Western blot and RT-qPCR confirmed that fermented *T. radicans* has higher whitening effects through bioconversion. As a result, fermented *T. radicans* is expected to be a good material for whitening functional cosmetics.

Conflict of interests

The authors declare no potential conflicts of interest.

Author contributions

Conceptualization: Jeong DE, Kim BO, Cho YJ. Methodology: Jeong DE, Kim BO, Cho YJ. Formal analysis: Jeong DE, Cho YJ. Validation: Jeong DE, Kim BO, Cho YJ. Writing - original draft: Jeong DE. Writing - review & editing: Jeong DE, Cho YJ

Ethics approval

This article does not require IRB/IACUC approval because

there are no human and animal participants.

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