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Antioxidant and Melanin-Reduction Effect of Mixed Extracts of *Bixa orellana*, *Ammi majus*, and *Glycyrrhiza glabra*

Mi Jeong Choi* and Yu Ri Kim

Biomedical Biotechnology Research Institute Co., Ltd., Goyang 10326, Republic of Korea

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The desire of modern people to maintain a healthy and beautiful appearance is increasing day by day along with the increasing interest in skin health and the demand for functional cosmetics. Accordingly, research on functional cosmetic materials with few side effects and excellent efficacy is being actively conducted. Therefore, this study tried to verify the antioxidant and whitening effects of the mixed extracts of *Bixa orellana, Ammi majus,* and *Glycyrrhiza glabra*, whose efficacy has been individually verified. Extracts (BAG-1~4) with different extraction methods such as steaming, fermentation, and ultrasonication were prepared for 3 types of natural plants, and antioxidant and whitening effects of these extracts were confirmed. For this purpose, antioxidant, tyrosinase activity, melanin production and stability experiments were conducted. Extracts (BAG-1~4) had no cytotoxicity, and antioxidant and whitening effects were confirmed. BAG-4 extracted by steaming and fermentation showed the best efficacy. It seems that enzymes such as lipase, protease, and amylase increase phenol components by various yeasts involved in the fermentation process, thereby improving antioxidant and melanin production inhibitory effects. It was confirmed that the three types of natural plant extracts could be used as safe and functional cosmetic materials.

Keywords: ***Bixa Orellana, Ammi majus, Glycyrrhiza glabra,* antioxidant, melanin**

Introduction

Various skin aging phenomena such as wrinkle formation, skin sagging, and pigmentation occur in modern people due to increased exposure to ultraviolet (UV) rays due to the increase in outdoor activities following the improvement of the quality of life [1, 2]. In addition, modern people's desire to maintain a healthy and beautiful appearance leads to increased demand for functional cosmetics daily along with increased interest in skin health. Therefore, the development of natural extract materials is actively progressing in various fields such as functional cosmetics using physiologically active substances, which are secondary metabolites of natural extracts [3]. Melanin, which is present in the epidermis of the skin, is one of the important factors determining skin color, and external skin color is determined by the amount and distribution of melanin pigment. A small number of melanin results in light skin color, while a large amount of melanin results in dark skin color. In addition, excessive pigmentation of the skin is caused by overexpression or accumulation of melanin in the skin.

When the skin is exposed to excessive UV rays, the production of melanin is promoted in melanosomes in the melanocytes present in the basal layer of the epidermis to protect the skin [4]. However, when the melanin as such has been produced excessively, hyperpigmentation such as melasma and freckle is induced [5]. Melanin production is induced by enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2 [6].

***Corresponding author**
Phone: ***-****-**** Fax: +82-31-356-5656
E-mail: choimijeong5@gmail.com

Since tyrosinase is a copper-containing enzyme that controls the rate of melanin production [7], if the activity of tyrosinase can be inhibited, melanin production and the induction of melasma, freckle, etc. can be suppressed [8]. Representative tyrosinase activity inhibitors include hydroquinone, kojic acid, arbutin, ascorbic acid (Vitamin C), azelaic acid, and retinoid [9]. However, hydroquinone that has strong efficacy is used only as a pharmaceutical due to its cytotoxicity, nephrotoxicity, carcinogenic potential, and skin damage such as ochonosis [10, 11]. Although kojic acid has an excellent whitening effect, it is known that it can cause contact dermatitis or cancer [12, 13]. Arbutin has genotoxicity [14], and it has been reported that azelaic acid can cause erythema and skin irritation [15]. Since cosmetics with whitening function are generally highly likely to cause of side effects due to long-term use, whitening materials with fewer side effects should be developed, and recently, active studies have been conducted with natural plants that have fewer side effects. Meanwhile, *Bixa orellana* is a shrub belonging to the genus *Bixa* of the family *Bixaceae*, and recently, the whitening or moisturizing effect of the extract of this plant has been reported [16, 17]. In addition, *Ammi majus* is a plant belonging to the genus *Ammi* of the family *Asteraceae*, and its leaves and fruits are known to be effective in treating skin diseases [18], and in the case of *Glycyrrhiza glabra*, which is a medical herb, pharmacological efficacies such as the antioxidative activity of chemical components existing in various parts have been reported [19, 20]. Although many applied studies have been conducted on phytotherapy methods, which use certain components of plant extracts directly as drugs without isolating them, studies conducted through a scientific approach to steam, fermentation, and separate certain components of plant extracts, and study the functions of the components are very rare. The steaming process uses steam for heat treatment to prevent the destruction of active ingredients and induces changes in the constituents of crops, creating new compounds or breaking down tissues to enhance the extraction of useful components [21]. The fermentation process typically uses microorganisms or fungi to obtain substances beneficial for humans, involves a sugar breakdown process that doesn't require oxygen for energy, and improves the aroma, flavor, texture, and storage stability of food. Fermentation of food also destroys toxic substances, produces bioactive materials, and enhances digestibility [22, 23].

In this study, for the antioxidant and melanin reduction efficacy of the *B. orellana*, *A. majus, G. glabra* mixture extract, strains such as *Bifidobacterium longum* (KACC 20597), *Lactobacillus acidophilus* (KACC 12419), *Leuconostoc mesenteroides* (KACC 12312) were used. *B. longum* is well known for its antioxidant abilities, including scavenging reactive oxygen species and having reducing activity, thereby contributing to the antioxidative characteristics of the fermented mixture [24]. The probiotic *Lac. acidophilus* is known to enhance the antioxidative capacity of fermented products. According to research, *Lac. acidophilus* stimulates the growth of probiotics through antioxidants and is used in the development of functional foods with antioxidant properties [25]. It also increases the inhibition of ascorbic acid autoxidation and the scavenging effect of superoxide anion radicals during the fermentation process [26]. The microorganism *Leu. mesenteroides* affects the color properties, phenolic profile, and antioxidant activities of fermented products. *Leu. mesenteroides* fermentation impacts the phenolic profile and increases antioxidant activities, making it suitable for fermentation processes aiming to enhance these properties [27].

While numerous studies have focused on the efficacy of single plant extracts, this research examines the synergistic effects of mixed extracts from several plants, including *B. orellana*, *A. majus,* and *G. glabra*. This study explores whether mixed extracts can exert more potent effects compared to individual extracts, thereby presenting a new direction for the development of functional cosmetic materials. Additionally, this research contrasts with previous studies by comparing the efficacy of mixed extracts obtained through various extraction methods, highlighting its originality in suggesting the most effective extraction techniques. Such an approach could offer a novel methodology for the development of eco-friendly functional cosmetic materials.

Individually verified medicinal effects of *B. orellana*, *A. majus,* and *G. glabra* in a mixed extract may synergistically produce significant therapeutic effects. Therefore, it is meaningful to verify the medicinal effects that manifest from the combination of these ingredients, depending on the extraction method. In this study, the *B. orellana, A. majus*, and *G. glabra* mixture extract was

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prepared using various methods, and the antioxidant and melanin reduction efficacy of these extracts were examined.

Materials and Methods

Experimental Sample Preparation

First, *B. orellana* leaves, *A. majus* leaves, and *G. glabra* leaves (BAG) were washed and dried at 70℃. for 48 h. After the dried mixture was pulverized to a size of 40 mesh or less, a BAG mixture was prepared with a constant weight (100 g: 100 g: 100 g). This BAG mixture was prepared from 4 samples (BAG-1 to BAG-4) according to the extraction conditions, and the extraction conditions are as follows (see Table 1).

a) BAG-1

The leaves of *B. orellana, A. majus,* and *G. glabra* were thoroughly cleaned and dried at 70℃ for 48 h. The dried raw materials were crushed to a size below 40 mesh. The crushed raw materials were mixed in equal weights (100 g: 100 g: 100 g). Ten times the weight of 70% ethanol, relative to the total weight of the dried solid, was added to the mixture. Extraction was carried out by stirring at room temperature at 200 rpm for 12 h, repeated a total of 3 times. The obtained extract was filtered through a 0.45 μm membrane filter, followed by concentration under vacuum and atmospheric pressure to prepare the room-temperature agitate extract (sample BAG-1).

(b) BAG-2

The leaves of *B. orellana, A. majus,* and *G. glabra* were thoroughly cleaned and then dried at 70℃ for 48 h. The dried raw materials were pulverized to a size below 40 mesh. The pulverized raw materials were mixed in equal weights (100 g: 100 g: 100 g). Ten times the weight of 70% ethanol, relative to the total weight of the dried solid, was added to the mixture, and ultrasonic extraction was performed for 2 h at 40 kHz. The obtained ultrasonic extract was filtered through a 0.45 μm membrane filter, followed by concentration under vacuum and at room temperature to prepare the ultrasonic extract (sample BAG-2).

(c) BAG-3

The leaves of *B. orellana, A. majus,* and *G. glabra* were thoroughly cleaned and dried at 70℃ for 48 h, followed by steaming at 100℃ for 6 h. They were then naturally dried for 12 h at 50℃. The dried raw materials were ground to a size below 40 mesh. The ground materials were mixed in equal weights (100 g: 100 g: 100 g). A total of 10 times the weight of 70% ethanol was added to the steamed and dried mixture, and reflux extraction was performed three times at 70℃ for 3 h each time. The obtained extract was filtered through a 0.45 μm membrane filter, followed by concentration under vacuum and at room temperature to prepare the steamed extract (sample BAG-3).

(d) BAG-4

The leaves of *B. orellana, A. majus,* and *G. glabra* were thoroughly cleaned, dried at 70℃ for 48 h, and then steamed at 100℃ for 6 h. They were subsequently naturally dried for 12 h at 50℃. The dried materials were crushed to a size below 40 mesh. The crushed materials were mixed in equal weights (100 g: 100 g: 100 g). To the mixed and steamed dry materials, *B. longum* (KACC 20597), *Lac. acidophilus* (KACC 12419), and *Leu. mesenteroides* (KACC 12312) were inoculated at a concentration of 10^6 CFU/g each, and fermented at 37℃ for 3 days. After fermentation, the supernatant was obtained by centrifugation. Subsequently, 10 times the weight of 70% ethanol, relative to the total weight of the fermented product, was added, and reflux extraction was performed three times at 90℃ for 8 h each. The obtained extract was filtered through a 0.45 μm membrane filter, and then ethanol was removed using a vacuum concentrator to prepare the fermented extract (sample BAG-4).

Table 1. Sample preparation conditions.

Step	BAG-1	BAG-2	BAG-3	BAG-4
Raw material mixing	-	-	Steaming	Steaming + Fermentation
Extraction	Ethanol	Ethanol + Ultrasound	Ethanol	Ethanol

Antioxidant Activity Test

For the antioxidant activity test, ABTS and DPPH radical scavenging activity, SOD-like activity, and xanthine oxidase inhibitory activity, which can be evaluated for radical scavenging activity, were performed.

(a) ABTS radical scavenging activity

Dilute each of the samples (BAG-1 to 4) in water to prepare sample solutions with concentrations of 100 μg/ml, 250 μg/ml, and 500 μg/ml. 7 mM ABTS and 2.45 mM potassium persulfate were mixed and reacted in the dark for 12 h at room temperature to form ABTS cations, and then ethanol was added so that the absorbance value at 734 nm was 0.70 ± 0.02. 100 μl of the sample and 100 μl of the prepared ABTS solution were added to a 96-well plate, reacted at room temperature for 7 min, and absorbance was measured at 734 nm. Compared with the blank test solution, the ABTS radical scavenging ability was calculated as a percentage (%) as follows.

ABTS radical scavenging ability (%) =
[Control – (Sample – Blank)]/Control × 100

(Control: Absorbance of ABTS reagent, Sample: Absorbance of Sample + ABTS reagent, Blank: Absorbance of Sample + Blank)

(b) DPPH radical scavenging activity

Put 100 μl of the sample solution prepared in (a) and 100 μl of 0.2 mM DPPH into a 96-well plate, and measure the absorbance at 517 nm using a microplate reader after 30 min. Compared with the blank test solution, the DPPH radical scavenging ability was calculated as a percentage (%) as follows.

DPPH radical scavenging ability (%) =
[Control – (Sample – Blank)]/Control × 100

(Control: Absorbance of DPPH reagent, Sample: Absorbance of Sample + DPPH reagent, Blank: Absorbance of Sample + Blank)

(c) SOD-like activity

To 0.2 ml of the sample solution prepared in (a), 2.6 ml of tris-HCl buffer corrected to pH 8.5 and 0.2 ml of 7.2 mM pyrogallol are added and reacted at 25℃ for 10 min. After adding 0.1 ml of 1N HCl to the reaction solution, measure the absorbance at 420 nm to determine the amount of oxidized pyrogallol. Compared with the blank test solution, the SOD-like activity was calculated as a percentage (%).

(d) xanthine oxidase inhibitory activity

Add 0.6 ml of 0.1M potassium phosphate buffer (pH 7.5) and 0.2 ml of 1 mM xanthine to 1.0 ml of the test solution prepared in (a). Then, 0.2 U/ml xanthine oxidase 0.1 ml was added to stop the reaction, and the absorbance was measured at 292 nm to calculate the uric acid produced. Xanthine oxidase inhibitory activity was calculated as a percentage (%) compared to the blank test solution.

Melamine test

(a) Tyrosinase activity test

Tyrosinase is an enzyme involved in melanin biosynthesis, and whitening ingredients have a mechanism of action that inhibits this enzyme. Malignant melanoma cells (B16F10, ATCC) were aliquoted at 1×10^4 cells/ml, and after 24 h pre-culturing at 37℃ under 5% CO_2 conditions, the medium was exchanged with a medium containing 100 nM α-MSH. Afterward, samples (BAG-1 to 4) and control (water) were added and cultured for 3 days. After washing with 10 mM PBS, suspended in 10 mM PBS containing 1% Triton X-100, centrifuged for 5 min, and the supernatant was used as an enzyme solution for measuring activity. 40 μl of this enzyme solution was placed in a 96-well plate, and 100 μl of 3,4-Dihydroxyphenylalanine (L-DOPA) at a concentration of 2 mg/ml as a substrate was added. After the reaction was allowed to proceed at 37℃ for 1 h, absorbance was measured at 405 nm using an ELISA reader, and the activity of tyrosinase was calculated as a percentage of the absorbance of the control group.

(b) Melanin production test

Melanin is produced by exposure to ultraviolet rays or external stimulation and causes skin disorders such as flakiness, freckles, age spots, and aging. Melanin is produced through the biosynthesis of the tyrosinase enzyme, and by inhibiting the activity of tyrosinase, melanin production can be inhibited. Samples (BAG-1 to 4) are diluted in water to prepare sample solutions with concentrations of 100 μg/ml, 250 μg/ml, and 500 μg/ml. Malignant melanoma cells (B16F10, ATCC) were ali-

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quoted at 1×10^4 cells/ml and cultured for 24 h before 37℃ under 5% CO_2 conditions and then exchanged with a medium containing 100 nM α-MSH. After that, the sample and the control (water) were added, incubated for 3 days, and washed with 10 mM PBS. After suspension in 10 mM PBS containing 1% Triton X-100, centrifugation was performed for 5 min to remove the supernatant. Then, 200 μl of 1N NaOH was added and left at 55℃. for 2 h to dissolve melanin, and absorbance was measured at 405 nm to calculate the amount of melanin production (%).

Stability Test

MTS analysis was performed to evaluate the safety of the samples (BAG-1 to 4). Mouse melanoma cells (B16F10, ATCC) were aliquoted at 1×10^4 cells/ml each, and cultured for 24 h at 37℃ under 5% CO_2 conditions, 100 nM melanocyte-stimulating hormone (α-MSH) and samples (BAG-1~4), Control (water) was added and incubated for 24 h. Then, 20 μl of MTS reagent was added, and after incubation for 2 h, absorbance was measured at 570 nm with an ELISA reader (EpochTM 2, BioTek, USA). Cell viability was calculated by the following equation.

$$\text{Cell viability (\%)} = [(\text{Exp.} - \text{Blank})/\text{Control}] \times 100$$

(Exp: Absorbance of the extract containing cells, Blank: Absorbance of the extract without cells, Control: Absorbance of distilled water containing cells)

Statistical Analysis Method Addition

The experimental results were presented as the mean and standard error after being replicated three times. The significance was tested using a One-way ANOVA test with post hoc Tukey's Test. Results with a p-value of less than 0.05 were considered statistically significant.

Results and Discussion

Results of Antioxidant Activity Tests

Oxidative stress in the human body, caused by free radicals, can be induced by environmental pollution, alcohol consumption, and similar factors. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), produced by free radical reactions in the body, can lead to protein inactivation, tissue damage, and genetic mutations, thereby contributing to aging and the development of degenerative diseases. The body has antioxidant mechanisms to protect cell membranes and intracellular substances from oxidative stress. One such mechanism involves endogenous antioxidant enzymes, while others rely on various antioxidants and nutrients, including polyphenols, supplied through diet. Radicals, as reactive oxygen species, can lead to aging phenomena such as wrinkles and pigmentation when accumulated in the body. Research evaluating antioxidant functions to suppress the production of these reactive oxygen species is prevalent. Common experimental methods, such as DPPH and ABTS assays, involve using these reagents to generate reactive oxygen species, and the extent to

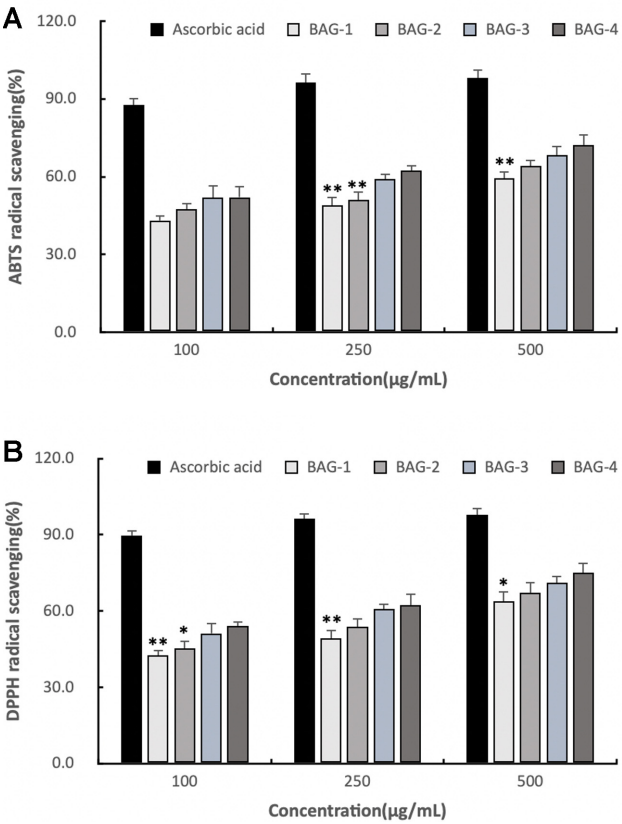

Fig. 1. ABTS & DPPH radical scavenging analysis results. BAG-1 (Ethanol), BAG-2 (Ethanol + Ultrasound), BAG-3 (Steaming + Ethanol), BAG-4 (Steaming + Fermentation + Ethanol). Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and values with different letters represent statistically significant differences. *$p < 0.05$, **$p < 0.01$, as compared to only BAG-4.

which these are scavenged is measured to assess antioxidant activity [28]. In the ABTS radical analysis, the steamed and fermented BAG-4 showed significant ABTS radical scavenging ability compared to BAG-1 and BAG-2 (Fig. 1). Specifically, at concentrations of 250 μg/ml and 500 μg/ml, BAG-4 exhibited significantly greater ABTS radical scavenging ability than BAG-1 ($p < 0.01$). In the case of BAG-2, a significant difference was observed at a concentration of 250 μg/ml ($p < 0.01$). However, no significant difference was found between BAG-3 and BAG-4.

Similarly, in the DPPH radical analysis, BAG-4 also showed significant ABTS radical scavenging ability compared to BAG-1 and BAG-2. Specifically, BAG-4 demonstrated significantly greater ABTS radical scavenging ability than BAG-1 at all concentrations ($p < 0.05$). Additionally, BAG-4 showed a significant difference compared to BAG-2 at concentrations of 100 and 500 μg/ml ($p < 0.05$). However, similar to the ABTS results, there was no significant difference between BAG-4 and BAG-3. Therefore, it is inferred that the steaming process during the material mixing stage influenced the extraction of antioxidant substances.

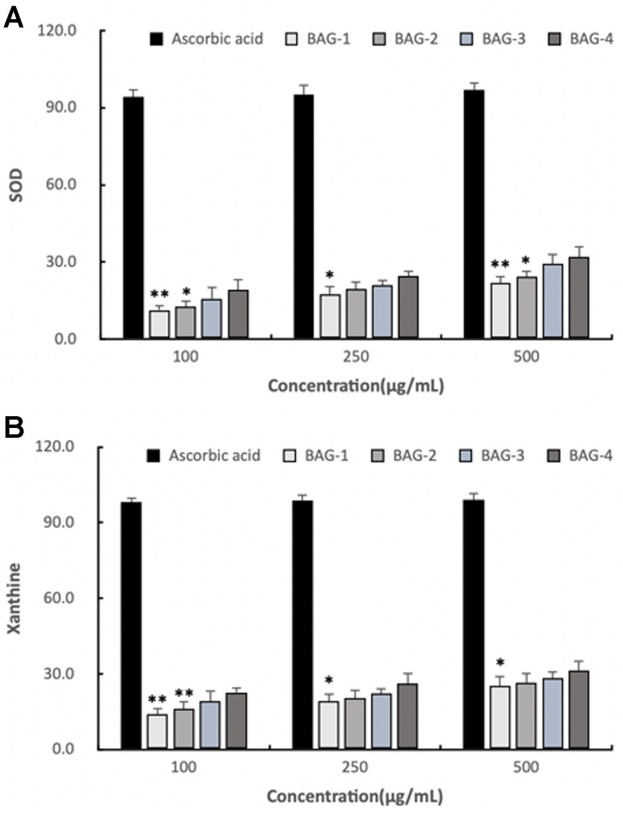

Fig. 2. SOD-like activity & xanthine oxidase inhibitory activity analysis results. BAG-1 (Ethanol), BAG-2 (Ethanol + Ultrasound), BAG-3 (Steaming + Ethanol), BAG-4 (Steaming + Fermentation + Ethanol). Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and values with different letters represent statistically significant differences. *$p < 0.05$, **$p < 0.01$, as compared to only BAG-4.

In the analysis of SOD-like activity, the steamed and fermented BAG-4 exhibited significant ABTS radical scavenging ability compared to BAG-1 and BAG-2 (Fig. 2). Specifically, BAG-4 showed significantly higher SOD-like activity than BAG-1 at all concentrations ($p < 0.05$). Additionally, BAG-4 demonstrated a significant difference compared to BAG-2 at concentrations of 100 and 500 μg/ml ($p < 0.05$).

In the analysis of xanthine oxidase inhibitory activity, the steamed and fermented BAG-4 also showed significant ABTS radical scavenging ability compared to BAG-1 and BAG-2. Specifically, BAG-4 exhibited significantly greater xanthine oxidase inhibitory activity than BAG-1 at all concentrations ($p < 0.05$). Moreover, BAG-4 had a significant difference compared to BAG-2 at concentrations of 100 and 500 μg/ml ($p < 0.05$). However, as with the ABTS and DPPH experiments, there was no significant difference between BAG-4 and BAG-3, suggesting that the steaming process influenced both SOD-like activity and xanthine oxidase inhibitory activity.

Results of Melanin Production Test

Melanin, a widely distributed polymeric phenolic substance in nature, is a key determinant of human skin color and acts to protect the skin from ultraviolet radiation and irritants [29]. However, excessive production of melanin can lead to hyperpigmentation disorders such as melasma, freckles, and pigmentation, as well as cell death and skin cancer due to the toxicity of melanin precursors [6]. Therefore, inhibiting the activity of tyrosinase, which induces melanin pigment deposition and accelerates skin aging, can reduce melanin production [30].

Tyrosinase is an enzyme involved in melanin biosynthesis, and ingredients effective for skin whitening have mechanisms that inhibit this enzyme. Tyrosinase catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which is then converted to DOPA-quinone. TRP-1 oxidizes 5,6-dihydroxyindole-2-carboxylic

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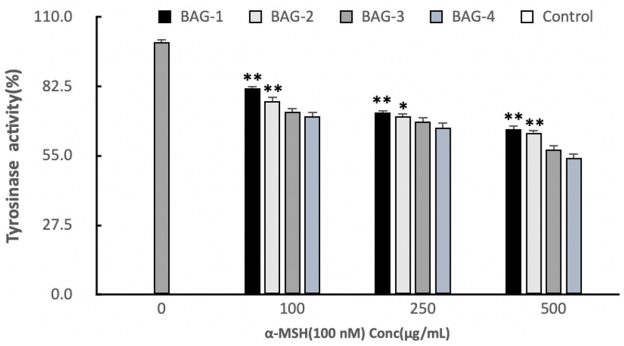

Fig. 3. Tyrosinase activity analysis results. BAG-1 (Ethanol), BAG-2 (Ethanol + Ultrasound), BAG-3 (Steaming + Ethanol), BAG-4 (Steaming + Fermentation + Ethanol). Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and values with different letters represent statistically significant differences. *$p < 0.05$, **$p < 0.01$, as compared to only BAG-4.

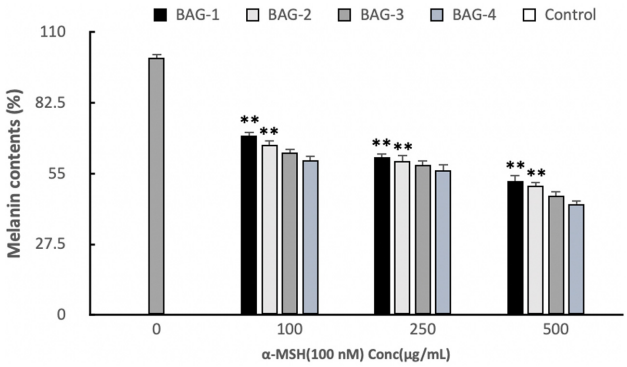

Fig. 4. Melanin contents analysis results. BAG-1 (Ethanol), BAG-2 (Ethanol + Ultrasound), BAG-3 (Steaming + Ethanol), BAG-4 (Steaming + Fermentation + Ethanol). Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and values with different letters represent statistically significant differences. *$p < 0.05$, **$p < 0.01$, as compared to only BAG-4.

acid (DHICA) to carboxylated indole-quinone [31], while TRP-2 acts as a DOPA-chrome tautomerase, converting DOPA-chrome to DHICA [32]. These enzymes play a crucial role in melanin biosynthesis, hence inhibiting the activity of tyrosinase is vital in reducing melanin production and enhancing the whitening effect [10]. In the tyrosinase inhibition assay, the steamed and fermented BAG-4 significantly inhibited tyrosinase activity at all concentrations compared to BAG-1 and BAG-2, but there was no significant difference when compared to BAG-3 (Fig. 3). Specifically, BAG-4 showed significantly higher tyrosinase activity inhibition than BAG-1 at concentrations of 100, 250, and 500 μg/ml ($p < 0.01$). Additionally, BAG-4 exhibited a significant difference compared to BAG-2 at all concentrations ($p < 0.01$). The lack of a significant difference between BAG-4 and BAG-3 suggests that the active ingredients extracted during the steaming process contributed to the inhibition of melanin production.

In the analysis of melanin production (Fig. 4), the steamed and fermented BAG-4 showed a significant decrease in melanin production compared to BAG-1 and BAG-2, but there was no significant difference when compared to BAG-3. Specifically, BAG-4 exhibited a significant difference in melanin production compared to BAG-1 and BAG-2 at all concentrations ($p < 0.01$). The lack of a significant difference between BAG-4 and BAG-3 suggests that the active ingredients extracted during the steaming process contributed to the inhibition of melanin production. These results appear to be the result of inhibition of the activities of tyrosinase, TRP-1, and TRP-2, which play important roles in melanin biosynthesis, by the ingredients produced by various yeasts or enzymes involved in the fermentation process. Most of the studies that examined the inhibition of tyrosinase and melanin by natural plant extracts [33–35] report that polyphenols, including tannins, present in plants affect the melanin biosynthesis process but those studies have not revealed the exact mechanism of the foregoing.

Results of Stability Test

According to the results of cytotoxicity analysis in mouse melanoma cells (B16F10, ATCC), the cell viability was at least 95% at all concentrations, indicating no significant difference (Fig. 5). Therefore, stability was confirmed because all samples (BAG-4~1) showed no cytotoxicity at all concentrations of 100–500 μg/ml.

Based on the above results, it was found that the efficacy of the fermented mixed extract (BAG-4) was the best. Fermentation means the process through which sugar is decomposed without oxygen to generate energy in a narrow sense, and means the process through which organic acids, gases, or alcohols are produced using bacteria or microorganisms in a broad sense [36]. According to a study conducted by Park *et al.* (2012), in the case of

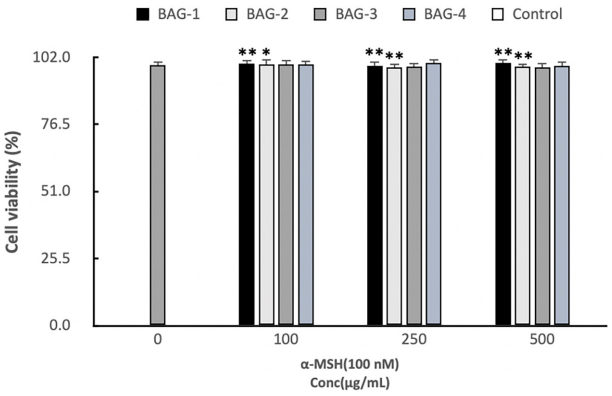

Fig. 5. Cell viability analysis results. BAG-1 (Ethanol), BAG-2 (Ethanol + Ultrasound), BAG-3 (Steaming + Ethanol), BAG-4 (Steaming + Fermentation + Ethanol). Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and values with different letters represent statistically significant differences. *$p < 0.05$, **$p < 0.01$, as compared to only BAG-4.

the yeast (*S. cerevisiae*, KCCM 50583) fermented *Gastrodia elata* Blume extract, the total polyphenol content increased by at least three times and the DPPH radical scavenging activity increased significantly [37]. In addition, Choi *et al*. (2011) reported that the fermented *Mori Cortex* extract had excellent melanin production inhibitory activity [38]. The results of previous studies as such support the results of this study. The excellent efficacy of BAG-4 that appeared in this study is considered attributable to the fact that enzymes such as lipase, protease, and amylase increased phenolic components thanks to various yeasts involved in the fermentation process leading to the improvement of the antioxidant and melanin production inhibitory efficacies. In addition, it is judged that more polyphenol components including tannins were extracted through the low-temperature fermentation reaction so that high antioxidant efficacy was shown. Recently, there have been studied findings indicating that biochemical conversion by microbial fermentation induces phenol production of natural extracts, and through this conversion, skin functional efficacies such as the antioxidant efficacy of the extracts are enhanced [39, 40].

Conclusion

The antioxidant efficacies of the samples prepared in this study were examined and the result indicated that the antioxidant efficacies increased concentration-dependently. In particular, BAG-4, which underwent steaming and fermentation processes, showed the highest antioxidant efficacy, followed by BAG-3, BAG-2, and BAG-1 in order of precedence. These results are judged to be different according to the extraction methods. That is, BAG-4 and BAG-3, which underwent the steaming or fermentation stage during the extraction process, showed significantly higher antioxidant efficacies, but BAG-1 and BAG-2 extracted by ethanol or ultrasonic method instead of steaming or fermentation extraction were found to have relatively low antioxidant efficacies. These results mean that steaming and fermentation extraction is a very useful method for extracting active ingredients with antioxidant efficacies.

As a result of the tyrosinase experiment, the mixed extracts were found to inhibit tyrosinase activity concentration dependently. In particular, the activity-inhibiting effect of BAG-4, which underwent steaming and fermentation, was the highest, followed by that of BAG-3, BAG-2, and BAG-1, in order of precedence. These differences are judged to be due to the different extraction methods. That is, BAG-4 and BAG-3, which underwent steaming or fermentation stage during the extraction process, showed a significantly higher ability to inhibit tyrosinase activity, but BAG-1 and BAG-2 extracted by ethanol or ultrasonic method instead of steaming or fermentation extraction. showed relatively low inhibitory efficacy. In addition, the cytotoxicity of the mixed extracts was checked in mouse melanoma cells (B16F10, ATCC) to check the stability and the results showed that that the cell viability was at least 95% at all concentrations of all samples, indicating that the raw materials that constitute cosmetics have no toxicity to skin cells. The superior antioxidative and tyrosinase inhibitory efficacy observed in BAG-4 in this study can be largely attributed to the bioconversion effects manifested during the fermentation process involving probiotic strains such as *Lac. acidophilus*. *Lac. acidophilus* (KACC 12419), a type of lactic acid bacteria found in various fermented foods, is known to offer several health benefits as a probiotic, including gut health improvement, immune system enhancement, and antimicrobial effects. The bioconversion effect of *Lac. acidophilus* stems from its ability to transform the chemical structure of natural plant extracts during the fermentation process through

specific enzymes and metabolites it produces. This process can enhance the effects of antioxidative components or lead to the generation of new antioxidative and melanin synthesis inhibiting compounds. Notably, *Lac. acidophilus* can improve the bioavailability of polyphenolic components [41] and produce specific metabolites that can increase the antioxidative and tyrosinase inhibitory activity of these components [42]. The activity of *Lac. acidophilus* during the fermentation process can induce structural transformations in effective components such as polyphenols present in the plant extract, potentially leading to increased antioxidative efficacy or tyrosinase inhibition [43]. For example, polyphenolic components can be converted into more activated forms through the fermentation process or transformed into new antioxidative or melanin synthesis inhibiting compounds. This is possible through the involvement of low molecular weight metabolites produced during fermentation, which can directly affect the activity of enzymes within cells or intervene in the mechanisms of antioxidation and melanin synthesis inhibition. Based on the result of this study, mixed fermented extracts of *B. orellana*, *A. majus*, and *G. glabra* were identified to have antioxidant and melanin production inhibitory efficacies, and are considered to be effective as materials of functional cosmetics that can inhibit pigmentation due to aging.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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