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Antioxidant and Whitening Effects of the Mixed Extract of *Ipomoea nil, Arctostaphylos uva-ursi,* and *Angelica gigas* Nakai

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The growing demand for functional cosmetics, driven by modern individuals' quest for healthy and aesthetically pleasing skin, is being challenged by reports of side effects and toxicity associated with these products. This underscores the importance of exploring natural plant-based materials for functional cosmetics. This research focuses on the assessment of antioxidant and whitening properties of mixed extracts from *Ipomoea nil, Arctostaphylos uva-ursi*, and *Angelica gigas Nakai*, all of which have proven pharmacological benefits. The study evaluated the extracts' total polyphenol and flavonoid contents, ABTS and DPPH radical scavenging activities, SOD-like activity, and xanthine oxidase inhibition to determine their antioxidant capabilities. Moreover, the whitening potential was investigated through tyrosinase activity and melanin production assays, alongside a cytotoxicity evaluation via a cell viability test. The findings revealed that the extracts, IAA-1 to IAA-4, demonstrated both antioxidative and whitening capabilities without exhibiting cytotoxic effects. Notably, the extract IAA-4, processed through ultrasonic and ultra-high pressure extraction, exhibited superior effectiveness. These results indicate that the cavitation formed during ultrasonic irradiation effectively destroys the plant cell wall by creating high pressure, and as a result, it is judged that useful components are easily extracted. As a result of the study, it was confirmed that the IAA-4 extract could be applied as a material for functional cosmetics.

Keywords: ***Ipomoea nil, Arctostaphylos uva-ursi, Angelica gigas Nakai,* antioxidant, whitening**

Introduction

Although industrialization has brought about affluence and convenience, it has also led to various side effects. Environmental hormones and fine dust are typical examples of these side effects stemming from industrialization [1]. These factors adversely affect the skin, which serves as the primary barrier protecting the human body [2].

Exposure of the skin to environmental contaminants or ultraviolet rays results in the depletion of its antioxidant defenses, including enzymes like Vitamin C and E, as well as compounds such as glutathione. This decrease in antioxidants gives rise to the formation of reactive oxygen species (ROS), which are highly reactive entities [3]. ROS are known to cause photoaging as they promote melanin production and lead to the formation of wrinkles [4]. Furthermore, they damage the skin's enzymatic and non-enzymatic antioxidant defense systems, resulting in DNA and protein oxidation, reduced skin immunity, and inducing inflammatory responses [5]. Consequently, antioxidants are now being added to cosmetics. However, reports suggest that administering 50 mg/kg or more per day of synthetic antioxidants like butylated hydroxy anisole (BHA) and butylated hydroxytoluene (BHT) can lead to toxicity [6, 7]. Additionally, exposure of the skin to environmental pollutants or ultraviolet rays can induce pigmentation. This is

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because melanocytes in the basal layer of the skin's epidermis synthesize the melanin pigment, which acts as a filter to protect the skin from ultraviolet rays [8]. The biosynthesis of melanin involves the conversion of tyrosine into dopa and dopaquinone by tyrosinase, followed by continuous oxidation [9]. The produced melanin then migrates to epidermal cells known as keratinocytes, undergoes keratinization over approximately 28 days, and eventually fades away [10]. Since the melanin as such is deposited and causes various pigmentation such as melasma and freckles in cases where it has been synthesized excessively by ultraviolet rays or when the physiological function of the skin has declined due to aging [11], to prevent the overproduction of melanin and the consequent pigmentation, tyrosinase action or production should be inhibited or an intermediate stage involved in the production of melanin should be inhibited. Therefore, tyrosinase activity inhibitors such as hydroquinone, kojic acid, arbutin, ascorbic acid (Vitamin C), azelaic acid, and retinoid have been used [12], but it has been reported that such tyrosinase activity inhibitors can cause side effects such as skin damage [13, 14], dermatitis and cancer [15, 16], and genotoxicity [17]. Therefore, in recent years, studies on extracts, which are natural substances extracted from nature with few side effects while having various physiologically active functions, such as antioxidant, anti-aging, anti-wrinkle, and whitening functions, have been in progress [3].

Meanwhile, *Ipomoea nil* is a single plant sensitive to light belonging to the genus *Pharbitis nilchoisy* of the family *Convolvulaceae*. *I. nil* is a gamopetalous flower in the shape of a light blue funnel deeply divided into three parts. *I. nil* has also been widely used as genetic material, and the antioxidant effect of anthocyanins contained in flowers [18] or an effect to inhibit ROS, which causes skin diseases [19], have been reported. In addition, *Arctostaphylos uva-ursi* is a plant in the family *Ericaceae*, which is 8–10 cm tall. Its fruits and leaves are used as medicines for diuresis and to protect of the stomach [20, 21], and it contains a lot of arbutin, which is effective for whitening [22, 23]. In the case of *Angelica gigas* Nakai, effects for skin allergy [24], antioxidant activity, tyrosinase inhibitory activity [25], UV blocking effect, and whitening effect [28, 29] have been reported.

Previous studies have reported on various natural plants known for their whitening and antioxidant properties. Research on plant extracts such as *Hydrangea petiolaris* Leaves [30], Cheongyang Pepper [31], *Brassica juncea* L. *Czern*. [32], and Boseong *Camellia sinensis* [33] has documented their effects. However, these studies have focused on the efficacy of individual plants, with limitations in examining the combined effects of mixed extracts. Additionally, there has been a lack of research comparing pharmacological efficacies based on different extraction methods of these natural plants. Therefore, it appears highly meaningful for the development of eco-friendly, functional cosmetic materials to validate the combined effects of mixed extracts from pharmacologically proven ingredients like *I. nil*, *A. uva-ursi*, and *A. gigas* Nakai, which have been individually verified for their antioxidant and whitening properties, according to different extraction methods.

This study distinguishes itself from previous studies on individual plant extracts by investigating the combined effects of mixed extracts from plants such as *I. nil*, *A. uva-ursi*, and *A. gigas* Nakai, thereby proposing new advancements in the development of functional cosmetic materials. Additionally, by comparing various extraction methods to identify the most effective technique, this study deepens the understanding of pharmacological effects, such as antioxidant and whitening benefits, and offers an innovative approach to the development of eco-friendly functional cosmetic ingredients.

In this study, we aim to analyze the antioxidant and whitening efficacy of the mixed extracts of *I. nil, A. uva-ursi,* and *A. gigas* Nakai, based on the extraction methods, and to assess their stability as ingredients for functional cosmetics.

Materials and Methods

Experimental sample preparation

First, the seeds of *I. nil*, the fruits of *A. uva-ursi*, and the roots of *A. gigas* Nakai were thoroughly washed, dried at 70℃ for 48 h, and then ground to a particle size of 40 mesh or finer. An IAA mixture was prepared by mixing these ground materials in equal weights (100 g: 100 g: 100 g). This IAA mixture was used to prepare IAA-1 to IAA-4 samples, under different extraction conditions as follows (refer to Table 1).

There has been no prior research on the mix ratio of *I. nil*, the fruits of *A. uva-ursi*, and the roots of *A. gigas*

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Table 1. Sample preparation conditions.

	IAA-1	IAA-2	IAA-3	IAA-4
Extraction method	ethanol agitation	ethanol reflux	ethanol ultra high pressure reflux	ethanol ultrasonic wave ultra high pressure reflux

* IAA : *Ipomoea nil, Arctostaphylos uva-ursi,* and *Angelica gigas* Nakai

Nakai, deciding to adopt this ratio a significant step in pioneering a new research area. This study chose a 1:1:1 mix ratio to evenly mix the active ingredients extracted from each plant, thereby providing a balanced delivery of the unique antioxidant and whitening effects of each plant. Such a ratio is expected to maximize the synergistic action of each component, offering a more potent effect than when used individually, and serving as a balance point for effective action of each component while minimizing the risk of side effects. Future research should explore the optimal ratio that demonstrates the best efficacy and safety.

IAA-1. The IAA mixture was mixed with 10 times its weight in 70% ethanol, and extraction was carried out three times for 12 h each at 200 rpm at room temperature. The extract was then filtered through a 0.45 μm membrane filter, and ethanol was removed using a vacuum concentrator to prepare IAA-1.

IAA-2. The IAA mixture was mixed with 10 times its weight in 70% ethanol, and reflux extraction was conducted at 70℃ for 3 h, repeated multiple times. This extract was filtered through a 0.45 μm membrane filter, and ethanol was removed with a vacuum concentrator to prepare IAA-2.

IAA-3. The IAA mixture was mixed with 10 times its weight in 70% ethanol, and ultra-high pressure extraction was performed for 15 min at a pressure of 5,000 bar using an ultra-high pressure extractor (CIP-L2-100-300, Ilshin Autoclave Co., Ltd., Republic of Korea). This was followed by reflux extraction at 70℃ for 3 h, repeated multiple times. The extract was filtered through a 0.45 μm membrane filter, and ethanol was removed using a vacuum concentrator to prepare IAA-3.

IAA-4. The IAA mixture was mixed with 10 times its weight in 70% ethanol, and ultrasonic waves were applied using an ultrasonic device at a frequency of 50 kHz for 60 min. This was followed by ultra-high-pressure extraction at a pressure of 5,000 bar for 15 min using the same extractor, and then reflux extraction at 70℃ for 3 h, repeated three times. The extract was then filtered through a 0.45 μm membrane filter, and ethanol was removed with a vacuum concentrator to prepare IAA-4.

Antioxidant activity test

To confirm the antioxidant activity, total polyphenol and flavonoid content, ABTS and DPPH radical scavenging activity, SOD-like activity, and xanthine oxidase inhibitory activity experiments were performed.

Total polyphenol and flavonoid content. First, for total polyphenol measurement, 100 mg of each sample (IAA-1~4) was taken and diluted to 100 ml using 80% ethanol. The standard solution was prepared by taking 100 mg of gallic acid and making 100 ml using 80% ethanol, and then taking 0.1, 0.2, 0.5, and 1.0 ml of this solution and diluting it to 5 ml. Samples (IAA-1~4) and standard solutions were put into an e-tube by 100 μl, and 100 μl of 10% sodium carbonate was added thereto. Then, 100 μl of Folin-Ciocalteu reagent was added, mixed using a vortex for 30 sec, and left in the dark for 30 min. Then, the absorbance value of the reaction solution was measured at 750 nm and expressed as mg GAE/g. For the total flavonoid standard solution, 100 mg of quercetin was taken, 80% ethanol was added to make 100 ml, and 0.1, 0.2, 0.5, and 1.0 ml of this solution was taken and diluted to 5 ml. Samples (IAA-1~4) and standard solution were put into an e-tube by 500 μl, and 10% aluminum nitrate 100 μl and 1 M potassium acetate 100 μl were added and mixed. After 40 min of mixing, the absorbance was measured at 415 nm using a UV-vis spectrophotometer, and the absorbance was measured and expressed as mg QE/g.

ABTS and DPPH radical scavenging activity. To evaluate the ABTS radical scavenging activity of the samples designated IAA-1 to IAA-4, they were each diluted to concentrations of 100, 250, and 500 μg/ml. The generation of ABTS cation radicals was achieved by mixing 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand at room temperature for 12 h in darkness. Subsequent to this reaction, ethanol was added to adjust the absorbance to a target value of 0.70 ± 0.02 at 734 nm wavelength. For the assay, 100 μl of each diluted sample was mixed with 100 μl of the ABTS solution in a 96-well plate. This mixture was then incubated at room temperature for 7 min before the absorbance was measured at 734 nm. The radical scavenging activity was quantified by calculating the inhibition 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)

To measure the DPPH radical scavenging activity, an aliquot of 100 μl from the sample prepared for the ABTS assay was combined with 100 μl of 0.2 mM DPPH solution in a 96-well plate. The mixture was allowed to react for 30 min, post which the absorbance was quantified at a wavelength of 517 nm utilizing a microplate reader. The scavenging efficiency was then expressed as a percentage (%), calculated based on the absorbance measurements 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)

SOD-like activity, xanthine oxidase inhibitory activity. For the measurement of SOD-like activity, samples (IAA-1~4) were diluted to concentrations of 100, 250, and 500 μg/ml, respectively. To 0.2 ml of the sample, 2.6 ml of tris-HCl buffer corrected to pH 8.5 and 0.2 ml of 7.2 mM pyrogallol were added and reacted at 25℃ for 10 min. After stopping the reaction by adding 0.1 ml of 1 N HCl, the amount of oxidized pyrogallol was measured for absorbance at 420 nm and expressed as a percentage (%). To measure xanthine oxidase inhibitory activity, 0.6 ml of 0.1 M potassium phosphate buffer (pH 7.5) and 0.2 ml of 1 mM xanthine were added to 1.0 ml of the sample prepared in the SOD-like activity experiment. Then, 0.2 U/ml xanthine oxidase 0.1 ml was added to stop the reaction. The absorbance of the produced uric acid was measured at 292 nm and expressed as a percentage (%).

Melamine test

Tyrosinase activity test. Dilute samples (IAA-1 to 4) to concentrations of 100, 250, and 500 μg/ml. Thereafter, malignant melanoma cells (B16F10, ATCC) were aliquoted at 1×10^4 cells/ml and cultured for 24 h at 37℃ under 5% CO_2 conditions, and then exchanged with a medium containing 100 nM α-MSH. Thereafter, samples and control (water) were added and cultured for 3 days. After incubation, washed 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 reacting at 37℃ for 1 h, absorbance was measured at 405 nm using an ELISA reader.

Melanin production test. Samples (IAA-1 to 4) were diluted to concentrations of 100, 250, and 500 μg/ml. Malignant melanoma cells (B16F10, ATCC) were aliquoted at 1×10^4 cells/ml and replaced with a medium containing 100 nM α-MSH after 24 h pre-culture at 37℃ and 5% CO_2 conditions. Thereafter, samples and control (water) were added and cultured for 3 days. After incubation, it was washed with 10 mM PBS, suspended in 10 mM PBS containing 1% Triton X-100, and centrifuged for 5 min to remove the supernatant. Then, 200 μl of 1 N NaOH was added and reacted at 55℃ for 2 h to dissolve melanin, and absorbance was measured at 405 nm.

Stability test

MTS analysis was performed to confirm the cytotoxicity of the samples (IAA-1 to 4) for melanoma cells treated with melanocyte-stimulating hormone (α-MSH).

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Mouse melanoma cells (B16F10, ATCC) were aliquoted at 1×10^4 cells/ml, and primary cultured for 24 h at 37℃ and 5% CO_2 conditions. Thereafter, 100 nM melanocyte-stimulating hormone (α-MSH), samples, and control (water) were added, and secondary culture was performed for 24 h. Then, 20 μl of MTS reagent was added, and after 2 h, absorbance was measured at 570 nm with an ELISA reader (EpochTM 2, BioTek, USA). The formula for calculating cell viability is as follows.

Cell viability (%) = [(Exp. – Blank)/Control] × 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 test

Free radicals that cause oxidative stress in the human body are generated by environmental pollution or drinking. In addition, the reactive oxygen species and nitric oxide generated by free radical reactions in vivo are reported as major causes of aging and diseases such as degenerative diseases because they cause protein inactivation, tissue damage, genetic mutation, etc. Antioxidant mechanisms exist in the body to protect cell membranes and intracellular substances from such oxidative stress. One of them is created by antioxidant enzymes in the body, and the rest are created by various antioxidants in vivo, antioxidant nutrients supplied through diet, and antioxidants such as polyphenols. Phenolic compounds contained in vegetable foods include chemically heterogeneous substances, such as fat-soluble substances, which are dissolved only in organic solvents, water-soluble carboxylic acids or glycosides, and large-sized polymers. Therefore, their functions are as diverse as their diversity. In addition, it is known that the phenolic hydroxyl group of phenolic compounds is easily combined with other components to have various physiological activities such as anticancer and antioxidant activities [28]. As shown in Fig. 1, the total polyphenol content analysis revealed that IAA-4 had the highest content at 3.53 mgGAE/g, followed by IAA-3 at 3.42 mgGAE/g, IAA-2 at 3.02 mgGAE/g, and IAA-1 at 2.24 mgGAE/g. In other words, the total polyphenol content was highest in the ultrasonically extracted IAA-4, followed by IAA-3, IAA-2, and IAA-1 in that order. Notably, IAA-4 showed a significant increase in total polyphenol content compared to IAA-1 ($p < 0.01$), but no significant difference was found compared to IAA-2 and IAA-3.

In the total flavonoid content analysis, IAA-4 had the highest flavonoid content at 3.14 mgGAE/g, followed by IAA-3 at 2.91 mgGAE/g, IAA-2 at 2.11 mgGAE/g, and IAA-1 at 1.71 mgGAE/g (Fig. 1). IAA-4 showed the highest flavonoid content, followed by IAA-3, IAA-2, and IAA-1 in that order. Particularly, IAA-4 exhibited a significant increase in total flavonoid content compared to IAA-1 ($p < 0.001$) and IAA-2 ($p < 0.01$), but no significant difference was found compared to IAA-3.

Radicals, manifesting as active oxygen species, precipitate aging manifestations, including wrinkling and pigmentation, upon their accumulation in the body. In this investigation, ABTS and DPPH assays served as substrates to gauge antioxidant activity. These assays are acknowledged as benchmarks for evaluating the antioxidant capacity of phenolic compounds, inclusive of phenol

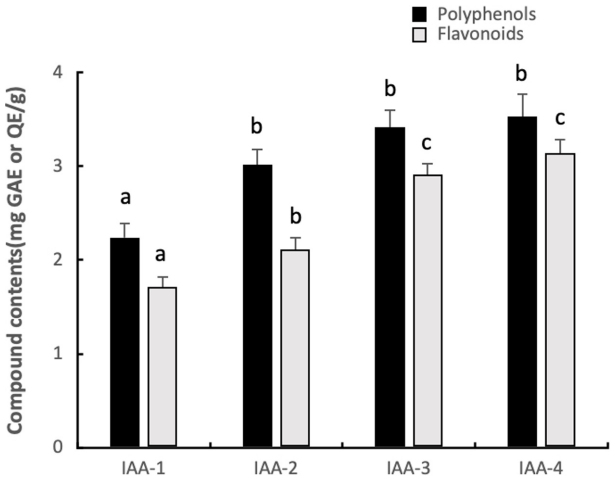

Fig. 1. Total polyphenol and flavonoid content analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

and flavonoids [29]. According to the results of ABTS radical analysis (Fig. 2), ultrasonically extracted IAA-4 showed a significantly higher ABTS radical scavenging ability compared to IAA-1 and IAA-2. In detail, IAA-4 showed a significantly higher ABTS radical scavenging ability than IAA-1 at concentrations of 100, 250, and 500 μg/ml ($p < 0.05$), and showed a significantly higher ABTS radical scavenging ability than IAA-2 at concentrations of 100, 250 and 500 μg/ml ($p < 0.05$). However, IAA-4 was significantly different from IAA-3 only at a concentration of 500 μg/ml ($p < 0.05$).

Also, in the results of the DPPH radical analysis (Fig. 2), IAA-4 showed a significantly higher ABTS radical scavenging ability compared to IAA-1 and IAA-2. In detail, IAA-4 had a significantly higher ABTS radical scavenging ability than IAA-1 at concentrations of 100, 250, and 500 μg/ml ($p < 0.05$) and had significantly more excellent ABTS radical scavenging ability than IAA-2 at a concentration of 100 μg/ml ($p < 0.05$). However, IAA-4 was significantly different from IAA-3 only at a concentration of 100 μg/ml ($p < 0.05$).

SOD protects cells by inhibiting the intracellular accumulation of hydrogen peroxide by removing superoxide radicals generated as a byproduct of intracellular respiration through enzymatic reactions [34]. Under normal conditions, the antioxidant defense mechanism of cells minimizes damage due to oxidative agents, and as part of the antioxidant system, glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase, etc. act as major protective enzymes against oxidative stress [35].

As a result of SOD-like activity analysis (Fig. 3), ultrasonically extracted IAA-4 and IAA-3 showed significantly increased SOD-like activity compared to IAA-1 and IAA-2. In detail, IAA-4 had significantly increased SOD-like activity than IAA-1, IAA-2, and IAA-3 at a concentration of 500 μh/ml ($p < 0.05$). It was found that

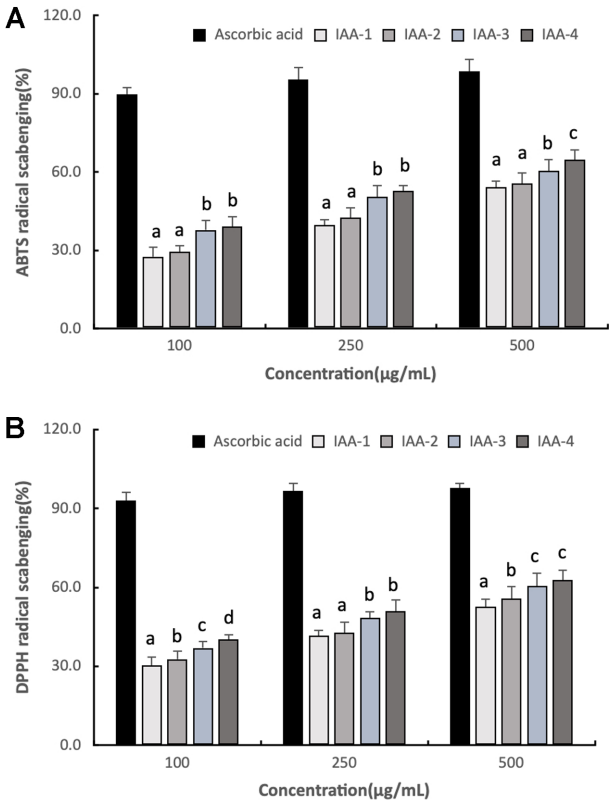

Fig. 2. ABTS(a) and DPPH(b) analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

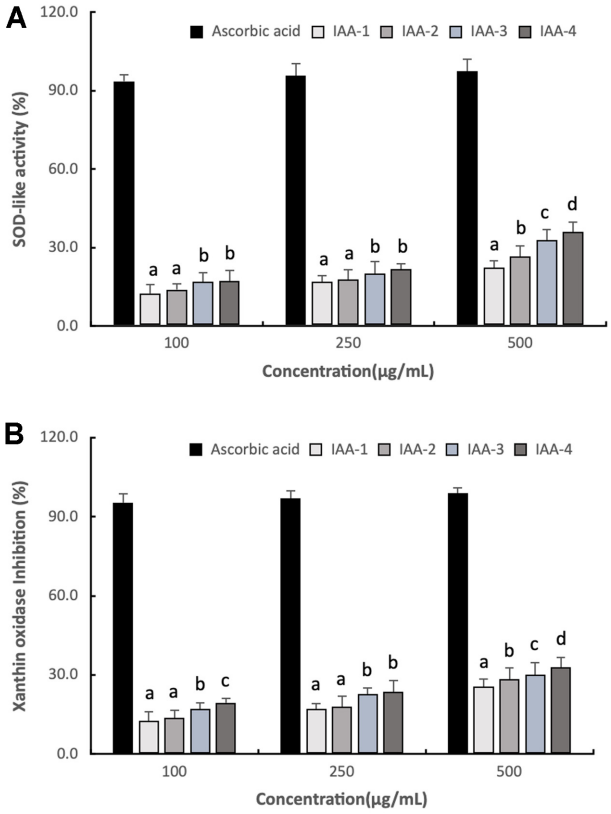

Fig. 3. SOD(a) and Xanthin(b) analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

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IAA-3 also showed significantly increased SOD-like activity compared to IAA-1 and IAA-2 at all concentrations ($p < 0.05$). Additionally, the analysis of xanthine oxidase inhibitory activity showed that IAA-4, extracted using ultrasonication, significantly increased xanthine oxidase inhibitory activity compared to both IAA-1 and IAA-2 (Fig. 3). Specifically, IAA-4 exhibited a significantly higher inhibitory activity against xanthine oxidase than IAA-1 and IAA-2 at all concentrations ($p < 0.05$). Additionally, IAA-4 was found to be significantly different from IAA-3 at 100 μg/ml ($p < 0.05$) and 500 μg/ml ($p < 0.05$).

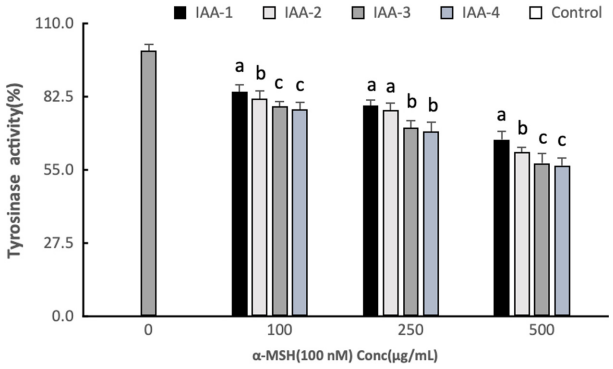

Fig. 4. Tyrosinase activity analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

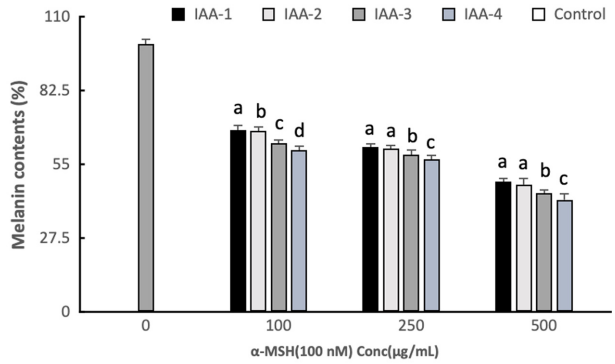

Fig. 5. Melanin analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

Result of melanin production test

Melanin, a phenolic polymer prevalent in nature, primarily determines human skin color and serves as a protective barrier against ultraviolet radiation and external irritants [36]. However, hyperproduction of melanin can lead to dermatological issues such as melasma, freckles, pigmentation, and even cell necrosis due to the toxicity of melanogenic precursors. It can also contribute to serious conditions like skin cancer [37]. To mitigate such effects, it is crucial to regulate melanin synthesis by inhibiting tyrosinase activity, which is responsible for the aging-induced pigmentation in the skin [38]. Additionally, the melanogenesis process is modulated by tyrosinase along with related proteins TRP-1 and TRP-2. Tyrosinase catalyzes the hydroxylation of tyrosine to produce 3,4-dihydroxyphenylalanine (DOPA), which subsequently forms DOPA-quinone. TRP-1 facilitates the oxidation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to its carboxylated indole-quinone derivative [39]. Furthermore, TRP-2 functions as a DOPA-chrome tautomerase, converting DOPA-chrome into DHICA [40]. These enzymes are critical in the enzymatic pathway of melanin production. Therefore, the inhibitory effect of tyrosinase, TRP-1, and TRP-2 plays an important role in inhibiting melanin production and enhancing the whitening effect [13]. The analysis of tyrosinase activity revealed that IAA-4, extracted using ultrasound, showed a significant decrease in tyrosinase activity compared to IAA-1 and IAA-2. Specifically, IAA-4 exhibited a significantly lower tyrosinase activity at concentrations of 100, 250, and 500 μg/ml compared to IAA-1 ($p < 0.05$) (Fig. 4). Also, IAA-4 showed a significant reduction in tyrosinase activity at concentrations compared to IAA-2 ($p < 0.05$). However, there was no significant difference between IAA-4 and IAA-3.

The analysis results of melanin production showed that IAA-4, extracted by ultrasound, had a significantly reduced melanin content compared to IAA-1, IAA-2, and IAA-3 (Fig. 5). Specifically, IAA-4 showed a significant reduction in melanin content at all concentrations, compared to IAA-1 ($p < 0.05$). Additionally, IAA-4 also exhibited a significant decrease in melanin content at all concentrations compared to IAA-2 ($p < 0.05$). Similarly, compared to IAA-3, IAA-4 showed a significant reduction in melanin content at all tested concentrations.

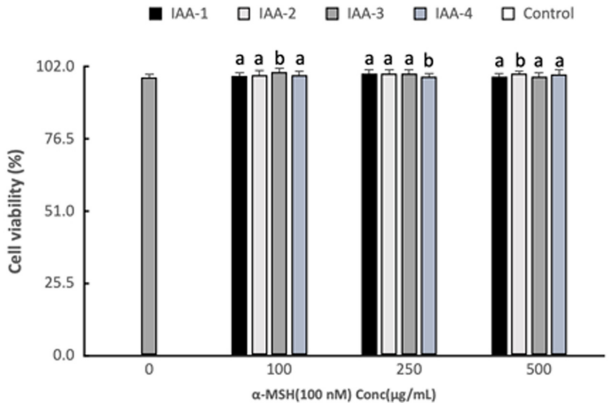

Fig. 6. Cell viability analysis results. Each value is means ± standard deviation of three replicate tests. The data were statistically analyzed using Tukey's honest significant difference (HSD), and differences were assessed to be significant at 5% level of probability ($p < 0.05$).

Results of the stability test

The cytotoxicity assessment conducted on mouse melanoma cells (B16F10, ATCC) revealed that cell viability remained above 95% across all tested concentrations, as detailed in Fig. 6. Consequently, stability was established for all samples, from IAA-1 to IAA-4, demonstrating no cytotoxic effects within the concentration range of 100 to 500 μg/ml.

Discussion

In this study, IAA-4, produced via ultrasonic extraction, exhibited the highest antioxidant efficacy. It also had higher total polyphenol and flavonoid contents, and showed the greatest radical scavenging ability, SOD-like activity, and xanthine oxidase inhibitory activity. Furthermore, it demonstrated the lowest tyrosinase activity and melanin production, while cell toxicity tests in melanoma cells (B16F10, ATCC) revealed a cell survival rate of over 95%, confirming its potential as a functional cosmetic ingredient. From the above experimental results, the mixed extract showed excellent antioxidant and whitening effects. The results as such are judged to be attributable to the complex actions of various active ingredients involved in the antioxidant effect [18] and ROS inhibitory effect [19] of the anthocyanins contained in *I. nil*, the whitening effect [23] of the arbutin contained in *A. uva-ursi*, and the tyrosinase inhibitory activity [25] and whitening effect [27] of *A. gigas* Nakai. In particular, IAA-4 extracted using the ultrasonic extraction method showed excellent antioxidant efficacy and effectively inhibited melanin production, indicating that ultrasonic extraction effectively extracts active ingredients involved in antioxidant and whitening effects.

The use of ultrasonic extraction method for IAA-4, which shows excellent antioxidant efficacy and effectively inhibits melanin production, can have multiple positive effects on skin health. Materials with outstanding antioxidant properties protect skin cells from free radicals, thus slowing down skin aging, reducing wrinkle formation, and maintaining skin elasticity [46]. These are essential elements in preserving the health and youthfulness of the skin. Effective inhibition of melanin production can prevent excessive pigmentation and improve existing pigmentation issues. This is particularly beneficial for individuals with pigment-related skin problems such as melasma, freckles, and sun-induced skin damage [47]. Maintaining an even skin tone and a brighter complexion is critically important from an aesthetic perspective, as it can enhance confidence and promote a positive self-image. Therefore, IAA-4, obtained through ultrasonic extraction and characterized by its superior antioxidant and whitening effects, can contribute to improving overall skin appearance by enhancing skin health, preventing skin aging, and improving pigmentation. These characteristics elevate its value as a material for functional cosmetics and demonstrate its potential as a new material that can positively affect skin health and beauty. The extraction of physiologically active substances from natural plants with conventional high-temperature and high-pressure treatment became a cause of functional deterioration because the oxidation promoted at high temperatures led to rapid changes in the physical and chemical properties of the physiologically active substance. The ultrasonic waves created by ultrasonic vibration is a process of transferring waves by compression and dissociation of the medium [41] and the cavitation generated in the process enables the delivery of large energy in a short time, the kinetic energy of the reactants located in the vicinity can be increased thanks to the rise of the local temperature by wobble friction, and the ultrasonic energy shock effect can induce pressure rise so that heating and mixing effects can be increased in parallel. Therefore, there is an advantage

that high extraction efficiency can be obtained in a short time [42]. The enhanced antioxidant and skin-lightening effects of the IAA-4 extract, obtained via ultrasonic extraction, can be attributed to the cavitation effects of ultrasonic waves. These waves generate high pressure that efficiently breaks down the plant cell walls, thus facilitating the release and diffusion of beneficial compounds. Kim & Choi (2009) [43] have observed that ultrasonic energy can disrupt plant tissues, allowing for the extraction of components that are less accessible with traditional methods, thereby improving yield [44]. Furthermore, Kim & Lee (2016) [45] reported an increase in the concentration of active compounds, such as polyphenols and flavonoids, with ultrasonic extraction compared to room-temperature stirring methods. These findings indicate that the ultrasonic process enhances the extraction of active ingredients from *I. nil, A. uva-ursi*, and *A. gigas* Nakai, leading to a higher content of compounds with antioxidant and skin-whitening properties, which in turn contributes to their physiological efficacy.

Conclusion

Upon evaluating the antioxidant capabilities of the IAA-1 to IAA-4 extracts developed in this study, a concentration-dependent augmentation in antioxidant efficacy was observed. Notably, the IAA-4 extract, obtained via ultrasonic extraction, exhibited superior antioxidant activity. Analysis of total polyphenols and flavonoids revealed a significant enrichment in the IAA-4 extract compared to its counterparts, IAA-1 to IAA-3. In assays evaluating ABTS and DPPH radical scavenging, IAA-4 demonstrated a markedly enhanced scavenging capacity, though it was still less than that observed in the control. Similarly, assessments of SOD-like activity and xanthine oxidase inhibition also positioned IAA-4 as the most potent, aligning with the aforementioned results. Moreover, at a concentration of 500 μg/ml, IAA-4 showed a significant reduction in tyrosinase activity, measured at 56.7% relative to the control, and melanin production was also at its lowest at 41.8% compared to the control. Cell viability assays conducted on B16F10 mouse melanoma cells (ATCC) indicated high stability, with viability remaining above 95% across all extract concentrations. These findings suggest the viability of these mixed extracts as ingredients for functional cosmetics. The data imply that ultrasonic extraction is an effective method to isolate active constituents without their degradation. Therefore, employing ultrasonic extraction for the co-extraction of *I. nil, A. uva-ursi*, and *A. gigas* Nakai could enhance the utility of these extracts as functional cosmetic materials with both antioxidant and whitening properties.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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