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# Antioxidant, Black Hair, and Hair Growth Effect of Mixed Extracts of *Nardostachys jatamansi*, *Ocimum basilicum* and *Crocus sativus*

Mi Jeong Choi* and Yu Ri Kim

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

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**The purpose of this study is to confirm the antioxidant, black hair, and hair growth effects of the *N. jatamansi*, *O. basilicum*, and *C. sativus* mixed extracts that pharmacological efficacy has been verified. Accordingly, four samples (NOC-1 to-4) produced under different extraction conditions were prepared and the results are as follows. First, all samples showed an increase in antioxidant content in a concentration-dependent manner from the results of antioxidant efficacy. In particular, NOC-4 extracted by steaming and ultrasonic methods showed the highest antioxidant effect among the four samples. As a result of analysis of the amount of melanin production in mouse melanoma cells, NOC-4 with concentration of 500 μg/ml showed higher melanin production compared to the control group, so the black hair efficacy was the best. Also, in the hair growth test results, it was found that the hair growth was the best at 0.94 ± 0.10 mm at experimental group orally administered with 500 mg/kg of NOC-4. In addition, as a result of cytotoxicity analysis in mouse melanoma cells, the safety of samples was demonstrated by maintaining cell viability of 95% or more at all concentrations. These results suggest that the steaming and ultrasonic extraction method increased the extraction yield of active ingredients for antioxidant, melanin, and hair generation, thereby affecting physiological activity. Based on these results, if the steaming and ultrasonic extraction methods are applied to the mixed extraction of *N. jatamansi*, *O. basilicum*, and *C. sativus*, it is judged that the practical potential as a natural material for black hair and hair growth agents will increase.**

**Keywords:** ***Nardostachys jatamansi, Ocimum basilicum, Crocus sativus,* black hair, hair growth**

## Introduction

As appearance is recognized as competitiveness and capital, and interest in sustainable beauty management increases among modern people, interest in hair is also increasing. The main component of hair is protein, and hair is composed of the hair cuticles, hair cortex, and hair medulla. The hair cortex, which surrounds the hair medulla, is the thickest part of the hair and contains melanin pigment, lipid membrane, and granules called melanosomes composed of tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), and other proteins [1].

The color of the hair is different depending on the types and amounts of melanin granules possessed by individuals [2]. Melanin granules that determine hair color include eumelanin and phaeomelanin and are formed from the amino acid tyrosine as a raw material [3]. Eumelanin is the most common and darkest pigment and produces brown and black hair, while phaeomelanin is of lighter shades of color that make blonde hair. These melanin granules are hardened, rigid proteins that are synthesized and released by melanocytes in hair follicles [4]. In human hair, in addition to the role of giving colors

***Corresponding author**
Phone: +82-31-811-9323, Fax: +82-31-965-5697
E-mail: choimijeong5@gmail.com

to the hair, melanin granules play the role for antioxidant properties to protect the scalp and photoprotection by absorbing or dispersing ultraviolet, visible, and near-infrared rays [5], thereby protecting the skin [6, 7].

White hair is a phenomenon of physiological aging and is caused by a decrease in the number of melanocytes in the hair and a decrease in melanin production due to hypofunction. Although many dyeing agents intended to hide white hair have been studied and are widely marketed, black hair dyeing is a temporary method and should be repeated as white hair grows, and the oxidative black hair dyeing, which is currently used widely, damages the skin as it uses an oxidizing agent [8]. Therefore, various studies have been conducted on anti-white hair agents that prevent the occurrence of white hair [9, 10]. However, existing anti-white hair agents generally contain a large number of chemicals harmful to the human body [11–13], and in the case of anti-white hair, agents made using natural materials, have a sufficient effect to prevent white hair and induce black hair has not been identified.

Hair loss is a phenomenon in which the amount of hair decreases due to aging or increased stress. Currently, drugs that promote hair growth include minoxidil and finasteride approved by the US FDA [14–16]. Although the hair growth effect of minoxidil appears after use for 6 to 12 months, it has been reported that hair loss progresses when the use of it has been stopped and long-term use of it causes itching, erythema, and dermatitis of the scalp [17]. Finasteride is an inhibitor of 5α-reductase, an enzyme that acts on the process through which testosterone is changed into dihydrotestosterone (DHT) [18]. Although its mechanism for hair growth is not clearly understood, it is thought that hair growth is induced to increase nutrient supply through vasodilation and to open potassium channels [14]. However, many tendencies to avoid drugs that promote hair growth appear because of the risk of side effects, and studies on natural substances such as herbal medicines and herbal extracts are in progress to minimize the side effects [19–21].

Meanwhile, *N. jatamansi* (*Nardostachys jatamansi*) is a perennial plant of the family *Apiaceae*, its roots contain ingredients such as *nardosinone*, *nardostachone*, and *valeranone* [22], and as its main effects, anti-inflammatory effects [22, 23], improvement of skin diseases [24] and nervous system related efficacies [25] have been reported. *O. basilicum* (*Ocimum basilicum*), which is used as a flavoring in cooking, has methysabicol as its main ingredient, is used for food flavoring, but is also used as an antineuralgic in the field of pharmacy because it contains saponin, etc. [26]. *C. sativus* (*Crocus sativus*) is reported to have efficacy for antioxidation [27] and the cardiovascular system [28].

Whereas many applied studies on phytotherapy, which uses plants as drugs without separating certain components, have been conducted in the past, recently, studies using scientific extraction methods such as steaming or ultrasound-assisted extraction to extract certain useful components from plants have been actively in progress [29–31]. Therefore, examining the efficacy appearing when the mixed extracts of *N. jatamansi* (*Nardostachys jatamansi*), *O. basilicum* (*Ocimum basilicum*), and *C. sativus* (*Crocus sativus*), of which the pharmacological effects have been individually verified act complexly seems to be very meaningful in the aspect of cosmetology. Therefore, in this study, the antioxidant, black hair, and hair growth effects of the mixed extracts of *N. jatamansi*, *O. basilicum*, and *C. sativus*, which have been proven pharmacologically effective, will be analyzed and their stability as materials of eco-friendly hair remedies will be reviewed.

## Materials and Methods

### Experimental sample preparation

The leaves of *N. jatamansi*, *O. basilicum*, and *C. sativus* were thoroughly washed, dried at 70℃ for 48 h, and pulverized to a size of 40 mesh or less. A NOC mixture was prepared by mixing the pulverized raw materials at a constant weight (100 g: 100 g: 100 g). This NOC mixture was prepared from NOC-1 to 4 samples according to the extraction conditions and the details are as follows (Table 1).

**Table 1. Sample preparation conditions.**

| | NOC-1 | NOC-2 | NOC-3 | NOC-4 |
|---|---|---|---|---|
| Extraction method | Ethanol | ethanol reflux | steaming ethanol reflux | steaming ultrasonic wave ethanol reflux |

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**NOC-1.** 70% ethanol was added to the NOC mixture in an amount 10 times the weight and extraction was repeated 3 times in total at 200 ×*g* at room temperature for 12 h. Then, the extract was filtered with a 0.45 μm membrane filter and then ethanol was removed with a vacuum concentrator to prepare NOC-1.

**NOC-2.** 70% ethanol was added to the NOC mixture in an amount 10 times the weight and reflux extraction was performed by repeating 3 times at 70℃ for 3 h. Then, the extract was filtered with a 0.45 μm membrane filter and then ethanol was removed with a vacuum concentrator to prepare NOC-2.

**NOC-3.** The NOC mixture was steamed at 100℃ for 12 h and then air-dried for 12 h. 10 times the weight of 70% ethanol was added to the dried NOC mixture. Thereafter, reflux extraction was performed three times at 70℃ for 3 h and the obtained extract was filtered with a 0.45 μm membrane filter and then ethanol was removed with a vacuum concentrator to prepare NOC-3.

**NOC-4.** The NOC mixture was steamed at 100℃ for 12 h and then air-dried for 12 h. Into the dried NOC mixture, 10 times the weight of 70% ethanol was added and treated with an ultrasonic extractor at a frequency of 50 kHz for 60 min. Then, reflux extraction was performed by repeating the ultrasonic extraction solution 3 times at 70℃ for 3 h. The extract was filtered with a 0.45 μm membrane filter and then ethanol was removed with a vacuum concentrator to prepare NOC-4.

### Antioxidant activity test

**ABTS radical scavenging activity.** To measure ABTS radical scavenging activity, samples NOC-1 to 4 were diluted to concentrations of 100, 250, 500, and 1000 μg/ml, respectively. 7 mM ABTS and 2.45 mM potassium persulfate are mixed and reacted at room temperature for 12 h in the dark to form ABTS cations. Thereafter, ethanol was added so that the absorbance value at 734 nm became 0.70 ± 0.02. 100 μl of the test solution and 100 μl of the prepared ABTS solution are added to a 96-well plate, reacted at room temperature for 7 min, and measured at 734 nm. Compared with the blank test solution, the ABTS 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)

**DPPH radical scavenging activity.** To measure DPPH radical scavenging activity, 100 μl of NOC-1 to 4 samples and 100 μl of 0.2 mM DPPH are put in a 96-well plate, and absorbance is measured at 517 nm using a microplate reader after 30 min. Compared with the blank test solution, the DPPH radical scavenging rate 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)

**SOD-like activity.** Dilute NOC-1 to 4 in water to obtain concentrations of 100, 250, 500, and 1000 μg/ml. To 0.2 ml of the diluted sample, 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 1 N 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 (%).

**Xanthine oxidase inhibitory activity.** Dilute NOC-1 to 4 in water to obtain concentrations of 100, 250, 500, and 1000 μg/ml. Add 0.6 ml of 0.1 M potassium phosphate buffer (pH 7.5) and 0.2 ml of 1 mM xanthine to 1.0 ml of the diluted sample. Then, 0.2 U/ml xanthine oxidase 0.1 ml was added to stop the reaction, and the resulting uric acid was obtained by measuring absorbance at 292 nm. Xanthine oxidase inhibitory activity was calculated as a percentage (%) compared to the blank test solution.

### Hair melanin synthesis test

Melanoma production was induced using L-DOPA in melanoma cells (B16F1): Mus musculus, mouse, and the melanogenesis efficacy of samples (NOC-1 to 4) was

compared. The murine melanoma cells (B16F10): a murine melanoma cell line from a C57BL/6J mouse and B16F1 (ATCC) were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. DMEM without phenol red was used for melanin measurements because phenol red, a pH indicator added to culture medium, can interfere with absorbance measurements. Cells were cultured in a humidified incubator in which 5% $CO_2$ was supplied and maintained at 37℃. Melanoma cells (B16F1) are aliquoted at $1 \times 10^4$ cells/ml in DMEM medium and incubated for 24 h at 37℃ under 5% $CO_2$ conditions. Afterward, samples (NOC-1~4) and L-DOPA at a concentration of 100–500 µg/ml were added to induce melanin production and incubated for 48 h. After incubation, the cells were collected and precipitated by centrifugation at 1,200 rpm for 5 min, and then dissolved with 1 ml homogenization buffer (50 mM sodium phosphate pH 6.5, 1% Triton X-100, 2 mM phenyl methyl sulfonyl fluoride) to obtain a pellet. After adding 200 µl of 1 N NaOH (10% DMSO) to the pellet and mixing, the absorbance was measured at 405 nm to determine the amount of melanin produced.

### Hair growth efficacy test

For 4 weeks, the experimental group (NOC-1~4) was orally administered at 250 and 500 mg/kg, and the hair growth efficacy was evaluated compared to the control group administered orally with water. First, a 6-week-old CL57BL/6 mouse (Orient Bio Co., Ltd.) was purchased and sufficiently supplied with water and solid feed to adapt to the laboratory environment (temperature 22 ± 2℃, humidity 55 ± 5%, 12-h light/dark cycle). The animals were bred in 5 groups, 5 in each group, and the experiments were carried out after the hair on the back of the mouse was primarily removed. For 4 weeks, the experimental group (NOC-1~4) was orally administered 250 and 500 mg/kg each, and the control group was administered the same amount of water. To check the degree of hair growth, the hair growth value during the growth phase was measured to 0.1 mm with vernier calipers, and then the experimental animal tissue was collected and fixed in 10% formalin for 24 h. After cutting the fixed tissue and embedding it in paraffin, the paraffin tissue section was prepared to a thickness of 4 µm using a microtome. Tissues were stained with Hematoxylin & Eosin, and changes in hair follicle tissue were observed and counted with an optical microscope (DP71, Olympus).

### Stability test

To evaluate the safety of the samples (NOC-1 to 4), MTS analysis was performed on mouse melanoma cells. First, mouse melanoma cells (B16F10, ATCC) were aliquoted at $1 \times 10^4$ cells/ml and incubated for 24 h at 37℃ under 5% $CO_2$ conditions. Thereafter, samples (NOC-1 to 4) and control (water) at a concentration of 100 to 500 µg/ml were added and further cultured 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 formula.

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

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

### Statistics and data processing

All experiments in this study were used for analysis based on the results of three or more independent runs under the same conditions, and all experimental results were expressed as Mean ± Standard Deviation. After calculating the mean and standard deviation of the experimental results, statistical significance was verified by the t-test.

## Results and Discussion

### Results of antioxidant activity tests

Radicals are active oxygen and cause aging phenomena such as wrinkles and pigmentation when they have accumulated in the body. ABTS and DPPH used in this experiment are used as substrates for the measurement of antioxidant activity and are known as indicators of antioxidant activity of phenolic substances such as phenol and flavonoids [32].

The measurement of antioxidant capacity using ABTS radicals is a method that uses the principle that ABTS radicals generated by reaction with potassium persulfate lose the blue-green color, which is their unique color, when they are in contact with antioxidants, and there

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are differences in radical scavenging activity among antioxidants [33]. It is known that the content of phenolic substances is proportionally and positively correlated with ABTS radical scavenging activity [34].

In the results of the analysis of ABTS radicals, ascorbic acid, a control group, was found to have the highest activity of 99.3% at the concentration of 500 μg/ml, and the activity of NOC-4 was shown to be 64.9% at 500 μg/ml, which was the highest scavenging activity of the sample group. NOC-3, NOC-2, and NOC-1 also showed scavenging activity levels exceeding 50%, which were 59.8%, 54.7%, and 51.1%, respectively, at 500 μg/ml. In particular, at 500 μg/ml, the ABTS radical scavenging activity of NOC-4 was not statistically different from that of NOC-3 but was significantly higher than that of NOC-2 ($p < 0.05$) and NOC-1 ($p < 0.05$) (Fig. 1). It was found that the antioxidant effect of NOC-4 was excellent.

Regarding the measurement of DPPH radical scavenging activity, since DPPH radicals are reduced by antioxidant active substances and the inherent indigo blue color of DPPH becomes pale so that antioxidant activity can be observed visually, DPPH radical scavenging activity is used as a measure to evaluate the antioxidant and antiaging activities of phenolic substances such as phenols and flavonoids against fat [35]. Kim *et al*. (2014) [36] reported that broccoli flower and stem ethanol extracts showed DPPH radical scavenging abilities of 17.18% and 15.15%, respectively, at a concentration of 500 μg/ml. When compared to the foregoing, NOC-4 showed a higher scavenging ability at 65.4%. Ascorbic acid, which is control was analyzed to have a scavenging ability of 98.3% at 500 μg/ml. Other samples also showed the same trend as the results of ABTS analysis, as the DPPH radical scavenging abilities of NOC-3, NOC-2, and NOC-1 were shown to be 63.8%, 60.7%, and 57.5%, respectively, at a concentration of 500 μg/ml. In particular, at 500 μg/ml, the DPPH radical scavenging activity of NOC-4 was significantly higher than that of NOC-3 ($p < 0.05$), NOC-2 ($p < 0.05$), and NOC-1 ($p < 0.01$). The antioxidant effect of NOC-4 was found to be excellent (Fig. 2).

As a result of SOD-like activity analysis, NOC-4 showed high scavenging activity at 34.3% at 500 μg/ml, followed by NOC-3, NOC-2, and NOC-1 at 32.4%, 31.2%, and 30.1%, respectively, at 500 μg/ml (Table 2). All experimental substances showed increases in the scavenging activity concentration dependently and showed lower scavenging abilities than the scavenging ability of ascorbic acid (97.9%), which is a control group. Likewise, in the results of xanthin oxidase inhibitory activity analysis, NOC-4 showed the highest inhibitory activity at 30.8% at 500 μg/ml, followed by NOC-3 (28.6%), NOC-2 (26.7%), and NOC-1 (24.4%) at 500 μg/ml, so that the same trend as the results of analysis of SOD-like activity could be identified (Table 2).

Free radicals that cause oxidative stress in the human body are generated by environmental pollution or drinking. In addition, reactive oxygen species and nitric oxide were generated by free radical reactions cause protein inactivation, tissue damage, genetic mutation, and the

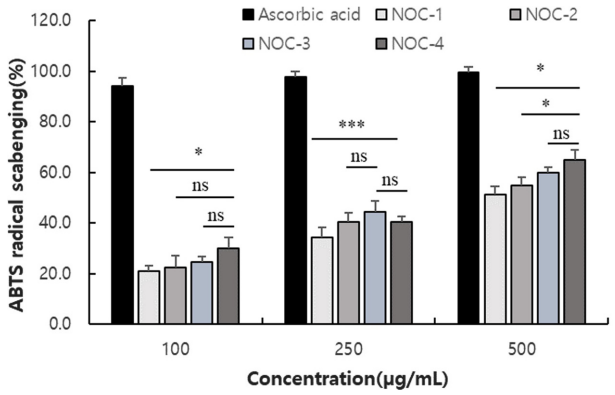


**Fig. 1. ABTS radical scavenging activity of ascorbic acid (positive control) and NOC group.** Values are the average of the triplicates ± SD. NS (No significant) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to NOC-4 by t-test)

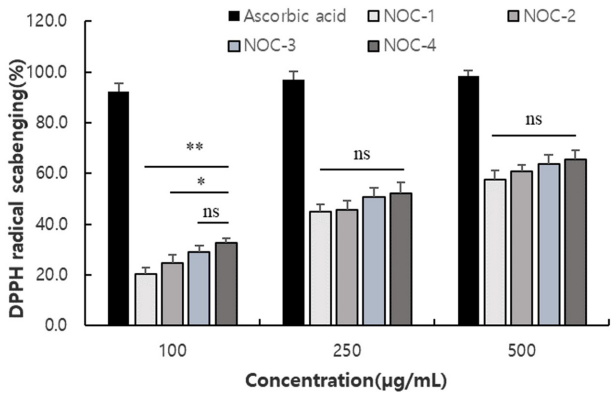


**Fig. 2. DPPH radical scavenging activity of ascorbic acid (positive control) and NOC group.** Values are the average of the triplicates ± SD. NS (No significant) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to NOC-4 by t-test)

**Table 2. SOD-like activity & Xanthin oxidase Inhibition.**

| Extract | SOD-like activity (%) | | | Xanthin oxidase Inhibition (%) | | |
|---|---|---|---|---|---|---|
| | 100 μg/ml | 250 μg/ml | 500 μg/ml | 100 μg/ml | 250 μg/ml | 500 μg/ml |
| Control* | 94.0 ± 3.7 | 96.1 ± 4.2 | 97.9 ± 4.2 | 95.2 ± 4.8 | 97.2 ± 3.5 | 98.2 ± 4.2 |
| NOC-1 | 11.2 ± 1.4 | 19.7 ± 2.8 | 30.1 ± 1.2 | 13.4 ± 3.1 | 19.1 ± 1.0 | 24.4 ± 3.3 |
| NOC-2 | 13.2 ± 2.1 | 20.8 ± 1.6 | 31.2 ± 2.3 | 15.4 ± 0.8 | 20.7 ± 2.3 | 26.7 ± 2.5 |
| NOC-3 | 18.2 ± 1.8 | 21.8 ± 2.5 | 32.4 ± 3.9 | 17.5 ± 2.8 | 21.7 ± 1.5 | 28.6 ± 2.1 |
| NOC-4 | 19.2 ± 2.4 | 23.9 ± 1.8 | 34.3 ± 2.4 | 19.6 ± 1.6 | 23.1 ± 2.5 | 30.8 ± 3.1 |

*Control: ascorbic acid.

like *in vivo*, and are reported as a major cause of aging and diseases such as degenerative diseases [37]. Therefore, it is important to search for natural plants that have fewer side effects and antioxidant components. As a result of the examination of the antioxidant efficacy of NOC-1~4, it was found that the antioxidant components of the samples increased concentration-dependently. In particular, the antioxidant effect of NOC-4 extracted by steaming and ultrasound-assisted extraction was found to be the best. In the results of ABTS and DPPH radical analysis, NOC-4 was shown to have a radical scavenging ability more excellent compared to NOC-1~3 although the radical scavenging ability was lower than that of the control group. Likewise, the results of SOD-like activity and Xanthin inhibitory activity analysis showed the same trend. Given the results as such, it seems that polyphenols present in natural plants were effectively extracted to enhance antioxidant activity, and the phenolic hydroxyl group of phenolic compounds is known to easily combine with other components to have various physiological activities such as anticancer and antioxidant activity [38, 39]. Therefore, each of NOC-1~4 is judged to be able to effectively protect the cell membranes and intracellular substances in the hair cortex from oxidative stress.

### Result of hair melanin synthesis test

Melanin is a phenolic polymer that is widely distributed in the natural world. It is a major factor that determines the color of human skin and acts to block UV rays and protect the skin from irritants [40]. However, excessively produced melanin pigment causes diseases such as melasma, freckle, pigmentation, apoptosis due to the toxicity of melanin precursors, and skin cancer [41]. As such, melanin production in the skin tissue can cause pigmentation or freckles, but the promotion of melanin production in the hair appears as a black hair effect [42]. As a result of analysis of melanin production in mouse melanoma cells, in the case of NOC-4, the highest production of 162.6% compared to the control group treated with distilled water was identified at 500 μg/ml, followed by NOC-3 (156.3%), NOC-2 (149.4%), and NOC-1 (147.1%). As such, all samples showed higher melanin production compared to the control group, indicating that they promoted melanin production more compared to the control group (Fig. 3). In particular, at 500 μg/ml, the melanin synthesis of NOC-4 was significantly higher than that of NOC-3 ($p < 0.05$), NOC-2 ($p < 0.001$), and NOC-1 ($p < 0.001$). Accordingly, it was found that the effect of black hair was excellent.

The promotion of melanogenesis is regulated by tyrosinase, TRP-1, and TRP-2 [43]. Tyrosinase promotes the hydroxylation reaction to convert tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and DOPA is converted

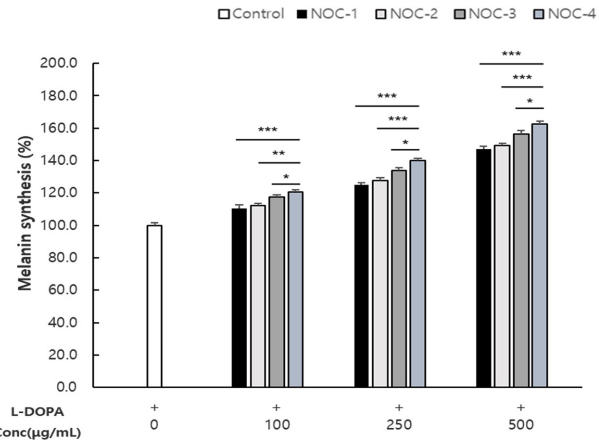


**Fig. 3. Hair melanin synthesis.** Values are the average of the triplicates ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to NOC-4 by t-test.)

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into DOPA-quinone [42]. TRP-1 oxidizes 5,6-dihydroxyindole-2-carboxylic acid (DHICA) into carboxylated indole-quinone [44, 45]. In addition, TRP-2 acts as a DOPA-chrome tautomerase to convert DOPA-chrome into DHICA [46]. These enzymes are known to play important roles in melanin biosynthesis. As a result of analysis of melanin production in mouse melanoma cells, NOC-4 at 500 μg/ml showed higher melanin production compared to the control group, and NOC-3, NOC-2, and NOC-1 showed melanin production of 156.3%, 149.4%, and 147.1% of that of the control group, respectively, at 500 μg/ml. It could be seen that all samples increased the amount of melanin production compared to the control group. These results indicate that NOC-4 increases melanin granule production in the hair cortex. Therefore, it seems that NOC-4 affected the activity to inhibit tyrosinase, TRP-1, and TRP-2, which are influencing factors for the promotion of melanin production.

### Result of hair growth efficacy test

As a result of the hair growth efficacy test, it was identified that hair growth was the most excellent at 0.94 ± 0.10 mm at 500 mg/kg in the case of NOC-4. In addition, the hair growth was shown to be 0.91 ± 0.09 mm at 500 mg/kg in the case of NOC-3, followed by the hair growth in the case of NOC-2 and NOC-1, at 0.85 ± 0.10 mm and 0.81 ± 0.09 mm at 500 mg/kg, respectively. On the other hand, it was identified that hair growth was the poorest at 0.65 ± 0.10 mm in the case of the control group (water) (Fig. 4A). In addition, in the results of the analysis of the number of hair follicles, the numbers of hair follicles in the cases of NOC-4, NOC-3, NOC-2, and NOC-1 were identified to be 11.9 ± 1.3, 10.5 ± 1.1, 9.5 ± 1.1, and 9.1 ± 1.2, respectively, at 500 mg/kg. Since the number of hair follicles in the control group was shown to be 4.5 ± 1.1, it could be seen that the experimental substances induce an increase in the number of hair follicles and that the efficacy of NOC-4 was the best (Fig. 4B). Given these results, it seems that NOC-4 induced increases in nutrient supply or potassium channel opening effects through vascular actions leading to the growth of hair and increases in the number of hair follicles.

### Result of the MTS experiment

As a result of cytotoxicity analysis in mouse melanoma cells, it was identified that the cell viability was at least 95% at all concentrations. Therefore, it was identified that NOC-4, NOC-3, NOC-2, and NOC-1 have no cytotoxicity at all concentrations (Fig. 5).

The results as such are judged to be attributable to the complex actions of various active ingredients involved in the anti-inflammatory and skin disease curing effect [24] and efficacy for the nervous system efficacy [26] of *N. jatamansi* and *O. basilicum* and the antioxidant efficacy of *C. sativus* [27]. In particular, NOC-4, which is extracted using the steaming and ultrasound-assisted extraction method, was shown to have excellent antioxidant, melanin, and hair production efficacies without

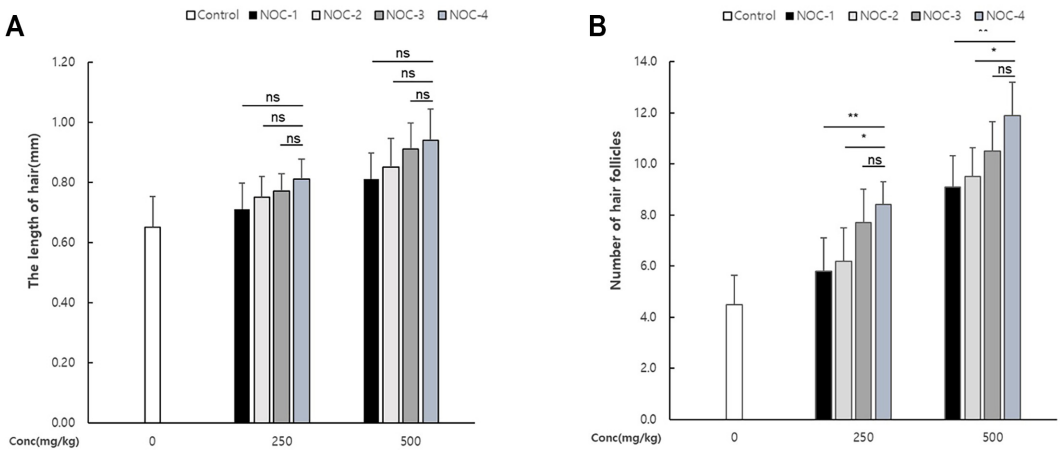


**Fig. 4. Hair growth efficacy.** Values are the average of the triplicates ± SD. (A) The length of hair, (B) Number of hair follicles. NS (No significant) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to NOC-4 by t-test.)

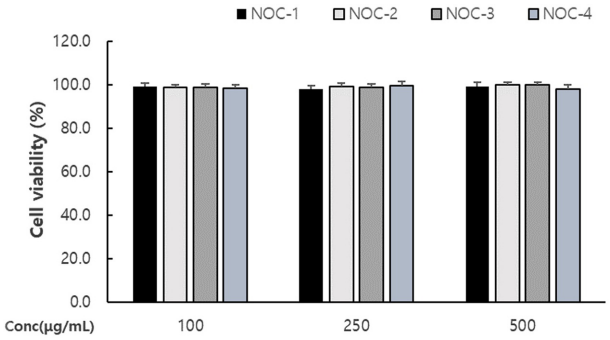


**Fig. 5. Cytotoxicity analysis in mouse melanoma cells.** Values are the average of the triplicates ± SD. NS (No significant).

toxicity to cells, indicating that steaming and ultrasound-assisted extraction methods effectively extract active ingredients involved in antioxidation or melanin production.

Ultrasound-assisted extraction has been used to improve the extraction efficiency of functional compounds from various plant materials, and this method has the advantage of simplifying the extraction process by increasing the extraction speed [47]. It is known that ultrasonic waves generate cavitation bubbles and that as the bubbles are collapse, plant cell walls are destroyed to increase the discharge of organic compounds inside plant cells. In addition, the ultrasonic energy impact effect induces pressure to rise so that high extraction efficiency can be obtained [48]. In this study, the efficacy of NOC-4 and NOC-3 applied with ultrasonic extraction was shown to be high. This is judged to be because the cavitation formed during ultrasonic irradiation created high pressure to effectively destroy the plant cell walls so that the travel range of the extracts was shortened, and diffusion occurred easily. Many previous studies [49–51] indicated that the extraction yield could be increased because ultrasonic energy destroyed plant tissues so that those components that could be hardly eluted with conventional extraction methods could be extracted. In addition, in a study of the antioxidation of *Oenothera odorata* extract, Kim & Lee (2016) [52] identified that active ingredients such as polyphenols and flavonoids were shown to be higher in the ultrasonic extraction method than in room temperature stirring extraction.

The experimental results of the samples are as follows. NOC-4 extracted by steaming and ultrasonic methods showed excellent efficacy in all of the antioxidant effects, melanin production in mouse melanoma cells, and hair growth experiments. In addition, in the cytotoxicity assay in mouse melanoma cells, the cell viability was more than 95%, confirming safety. Through the above results, it seems that as the steaming and ultrasonic extraction method improved the extraction yield of the active ingredients of *N. jatamansi*, *O. basilicum*, and *C. sativus*, the active ingredients for antioxidant, melanin, and hair production increased, which also affected the physiological activity. Based on the results as such, it is judged that if the steaming and ultrasonic extraction method is applied to the mixed extraction of *N. jatamansi*, *O. basilicum*, and *C. sativus*, the usability of the mixed extracts as a natural material for black hair and hair growth agents will increase.

## Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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