

# Antioxidant and Anti-inflammatory Responses of Pycnogenol, Hibiscus Flower Extract, and Niacin in Skin Epithelial Cells

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**Abstract:** Currently, much research is being conducted on the potential of natural materials. Although many studies use plants to treat disease, studies on the effects of natural ingredients as cosmetics on the skin are limited. This study aimed to evaluate the anti-inflammatory effects of pycnogenol (PG), hibiscus flower extract (HE), and niacin (NA) to determine whether they are safe and effective as a cosmetic material with a soothing effect on the skin. Two concentrations (1 µg/mL and 10 µg/mL) of PG, HE, and NA were added to  $1 \times 10^4$  cells/well of skin epithelial cells to determine cell viability (MTT), antioxidant capacity (DPPH) and interleukin-6(IL-6) was measured. The inflammatory response triggered by each material was compared using absorbance. Cell viability in two concentrations of material (1 µg/mL and 10 µg/mL) was  $103 \pm 0.03\%$  and  $102 \pm 0.05\%$  for PG,  $98 \pm 0.04\%$  and  $158 \pm 0.07\%$  for HE, and  $72 \pm 0.09\%$  and  $106 \pm 0.17\%$  for NA, respectively. DPPH results were  $54 \pm 0.01\%$  and  $82 \pm 0.01\%$  for PG,  $45 \pm 0.01\%$  and  $47 \pm 0.01\%$  for HE, and  $48 \pm 0.01\%$  and  $46\%$  for NA. The results above show that natural substances such as PG, HE, and NA do not exhibit cytotoxicity. The IL-6 levels in those following material-free group treatments were 17.6 pg/mL, in those following PG treatments were 9.5 pg/mL and 3.9 pg/mL, and in those following HE treatments were 9 pg/mL and 4.1 pg/mL. IL-6 levels, which are involved in inflammation, were also reduced. Through this study, it was revealed that PG and HE, which are mainly used as raw materials for health functional foods aimed at improving diseases, are not toxic to cells and have the effect of lowering the concentration of IL-6 that causes inflammation. This means that PG and HE natural materials can be used as raw materials for cosmetics. In particular, since it can be expected to be effective for sensitive skin and skin troubles related to inflammation, it is judged that it can affect cosmeceutical cosmetics by using the functional effect of the ingredient.

**Keywords:** Anti-inflammatory, IL-6, Hibiscus Flower Extract, Pycnogenol, Soothing

## 1. Introduction

Research on the potential of natural ingredients as cosmetic ingredients has been conducted for a long time. Especially for Oriental medicine material and herb natural products, research on cell viability, antioxidant levels, and inflammation is still actively underway[1]. Among these, inflammation affects

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the skin a lot.

Many factors such as pollution, alcohol consumption, smoking, and stress trigger the production of cytokines, which in turn cause IL-1, IL-6, and TNF- $\alpha$  of inflammation in the body. Some cytokines benefits cells but induced inflammation, which is highly correlated with disease[2]. As this inflammation also affects the skin, the development of ingredients that suppress inflammatory cytokine production is also crucial for having a beautiful and healthy skin. Anti-inflammatory action is an important factor for resolving skin problems, such as acne and atopic dermatitis, and it also plays a major role in soothing sensitive skin[3].

Currently, several studies are being conducted on the various antioxidant substances contained in plants[4][5], and health supplements and disease-related supplements using plant ingredients are being sold. Although most of these studies focus on developing health supplements aimed at improving vascular diseases, diabetes, cancer, etc. diseases, there is a need to actively develop plant-based raw materials for use in cosmetics. This is because cosmetics also contain various botanical ingredients, refraining from using animal and synthetic ingredients[6]. In addition, due to the safety concerns of synthetic raw materials, development research is being conducted on natural materials[7].

Pycnogenol (PG) contains 2–12 units of polymers including procyanidins, catechins, epicatechins, flavonoids such as taxifolin, and phenolic compounds. PG is a standardized substance containing  $70 \pm 5\%$  (w/w) procyanidins[8][9]. These ingredients have high anti-inflammatory and antioxidant properties [2]. Therefore, it has been shown to exert physiological and alleviate disease effects and is widely used as a health supplement[10]. Hibiscus flower extract (HE) also contains many compounds with excellent antioxidant capacities, such as flavonoids, anthocyanins, terpenoids, sesquiterpenes, and phenolic acids. It has long been used in medicine to treat ailments since ancient times. Also widely used in the food and beverage industry[11]. Niacin (NA) is a skin-lightening ingredient that inhibits the activity of tyrosinase enzyme[12]. It is called vitamin B3, nicotinic acid, or vitamin PP, and has been extensively studied in disease management[13][14].

Interleukin 6 (IL-6) is a proinflammatory cytokine and has been extensively addressed in inflammation-related research[10][15]. Ingredients used in cosmetics must not irritate and inflame the skin, as this is an important part of the safety of cosmetics. Therefore, research on inflammatory cytokines is necessary.

In this study used PG, HE, and NA materials extracted from natural plants to compare the expression of IL-6 along with cell viability and antioxidant levels affecting skin cells. By analyzing the characteristics of each material and evaluating its potential as a cosmetic ingredient, it would like to confirm the possibility of its functional action to soothe irritated skin.

## 2. Materials and Methods

### 2.1 Materials and Equipment

#### 2.1.1 Cell Culture

The B16F10 Cell was purchased from the Korean Cell Line Bank (Seoul, KR). To this, Fetal Bovine Serum (FBS) 10% (Equitech-bio, Inc., Texas, US) and penicillin 1% (Invitrogen, Massachusetts, US) in Dulbecco's Modified Eagle's Medium (DMEM; WELGENE Inc., Gyeongsangbuk-do, KR) were added and used for cell culture. Cells were cultured at 3-day intervals in an incubator at 37°C under 5% CO<sub>2</sub> conditions and then page numbers 4 and 5 were used for experiments.

#### 2.1.2 Experiment Material

The materials used are summarized in [Table 1].

[Table 1] List of Materials

Material	Origin	Concentration	Abbreviation
Pycnogenol	Switzerland	1µg/mL 10µg/mL	PG
Hibiscus Flower Extract	France	1µg/mL 10µg/mL	HE
Niacin	Korea	1µg/mL 10µg/mL	NA
Vitamin C	Spain	10µg/mL	Vit C
Fine dust	Belgium	10µg/mL	FD
Influenza virus	Korea	MOI 0.1	InF

PG (Horphag Research Ltd., Geneva, CH) was made from extracts of pine bark obtained from the southwest coast of France, and HE (Naturex, Vaucluse, FR) was made from extracts of hibiscus flowers. NA was purchased from Innotech, Daejeon, Korea.

A standard sample of ambient dissolved fine dust (PM10-LIKE; certificate of analysis: ERM-CZ120) obtained from the European Commission's Joint Research Centre (JRC; Geel, BE) was used. Vit C (PH FORMULA, Barcelona, ES) and influenza A (subtype A (H1N1) NCCP 43228) (National Culture Collection for Pathogens, Chungcheongbuk-do, KR) were also used.

### 2.1.3 Equipment

Absorbance was measured utilizing a FlexStation 3 Multimode Microplate Reader (Molecular Devices, LLC., CA, US).

## 2.2 Methods

The experiment conducted in this study is shown in the schematic diagram of [Fig. 1].

B16F10 cells were used for in vitro experiments.

### 2.2.1 MTT Assay

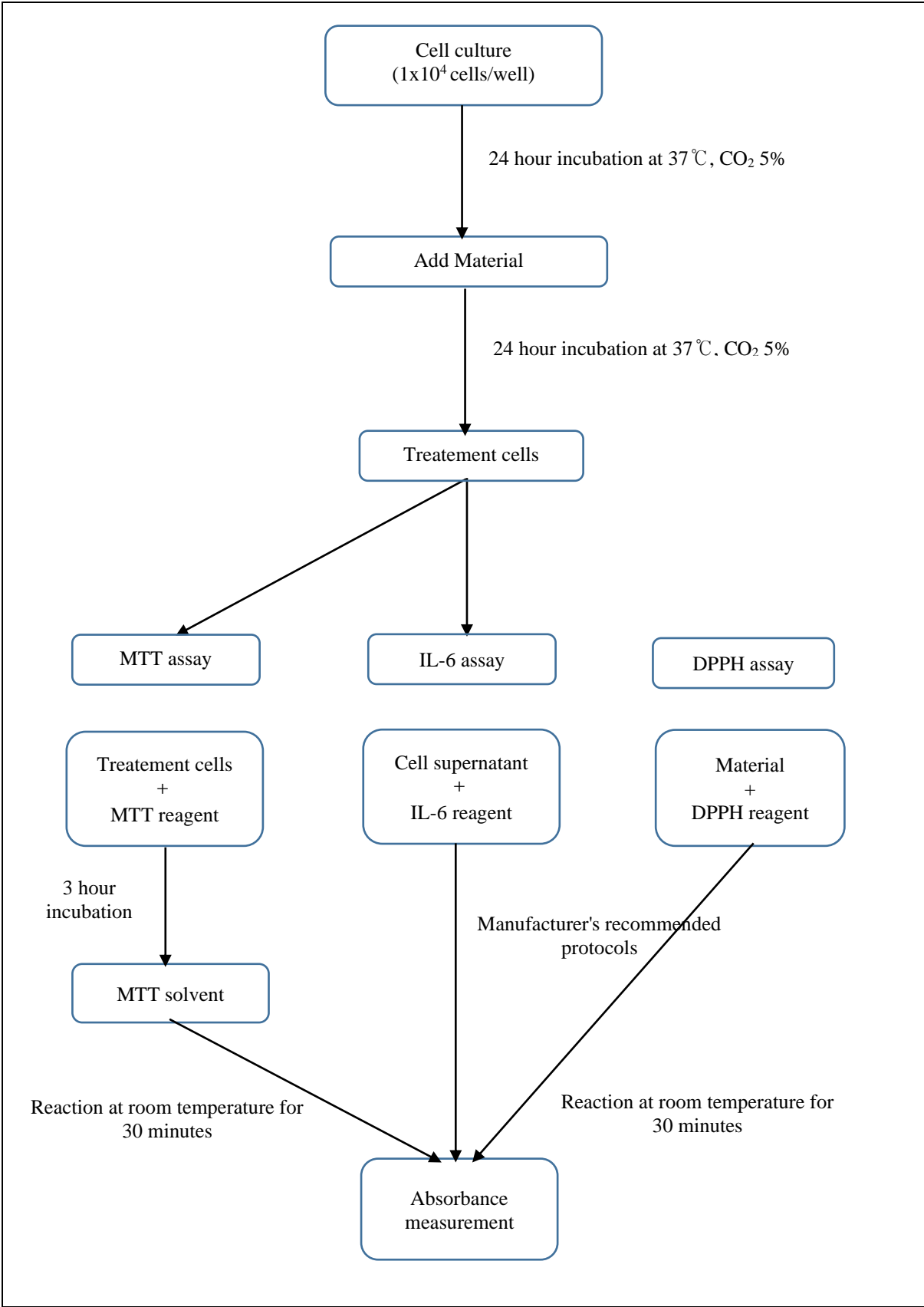
To determine the cell viability of B16F10 cells, the MTT Assay Kit (Abcam, Cambridge, UK) was used. First, the cultured cells were dispensed into a 96-well plate so that the final count of cells was  $1 \times 10^4$  cells/well. They were cultured at 37°C in an incubator under 5% CO<sub>2</sub> for 24 h. After adding 100 µL of PG, HE, and NA to the cultured cells, they were cultured for another 24 h. The culture plate was treated with MTT reagent and reacted for 3 h in an incubator; thereafter, the MTT solvent was added and allowed to react at 21–23°C, and a microplate reader was used to measure the absorbance at 590 nm.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of material additive group}}{\text{Absorbance of material-free group}} \times 100$$

### 2.2.2 DPPH Assay

To evaluate the antioxidant ability of the material, a 0.2 mM DPPH Solution (Merck, Darmstadt, Germany) was used. PG, HE, NA, and DPPH solutions were added in a ratio of 1:1. They reacted at room temperature (21–23°C) for 30 min while shielded from light. A microplate reader was used to measure the absorbance at 517 nm.

$$\text{Inhibition Ratio of Sample (\%)} = 1 - \left( \frac{\text{Absorbance of material additive group}}{\text{Absorbance of material-free group}} \times 100 \right)$$



[Fig. 1] Flow Chart of the Study. Cell: B16F10; Material: Material by concentration (PG, HE, NA, VIT C, FD, InF, G55)

### 2.2.3 IL-6 Assay

IL-6 ELISA Kit (BioLegend, Inc., California, US) was used to assess the anti-inflammatory ability of the materials. Substance treated cell supernatants were tested following the instructions in the kit's recommended protocol. A microplate reader was used to measure the absorbance at 517 nm and 570 nm. For IL-6 calculations, a standard curve was generated using the analyte concentrations and absorbance values for the standard reagents in the kit. The IL-6 level was then calculated using the absorbance value of the material.

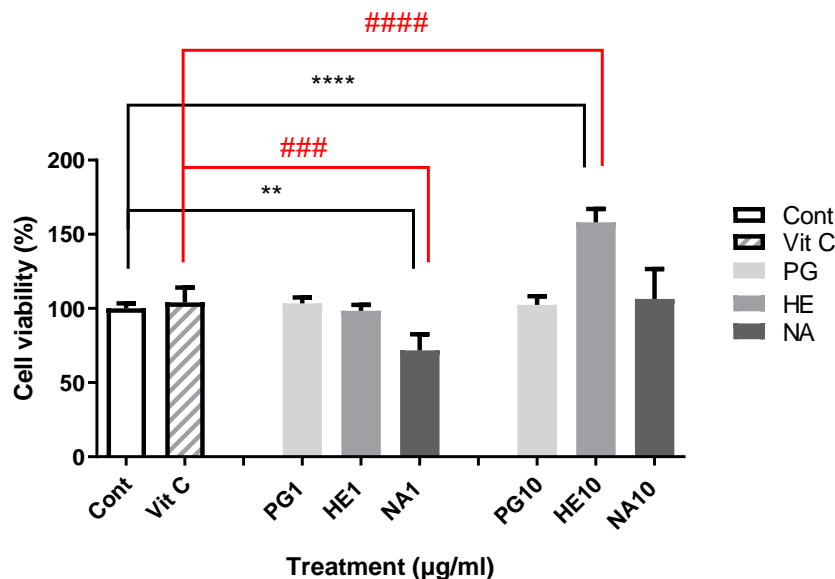
### 2.3 Statistical Analysis

IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, US) was used for statistical analyses, and a one-way ANOVA test was performed to verify significance. Additionally, values below the significance level ( $p$ -value) < 0.05 were considered statistically significant.

## 3. Results

### 3.1 Cell Viability

Cell viability results when B16F10 cells were treated with two concentrations of PG, HE, and NA (1  $\mu$ g/mL, 10  $\mu$ g/mL) were shown in [Fig. 2]. At 1  $\mu$ g/mL of NA, cell viability was significantly lower than that in the material-free and Vit C groups (\*\* $p$ <0.01). At 10  $\mu$ g/mL, the cell viability with HE was significantly higher than that in the material-free and Vit C groups (\*\*\*\* $p$ <0.0001).

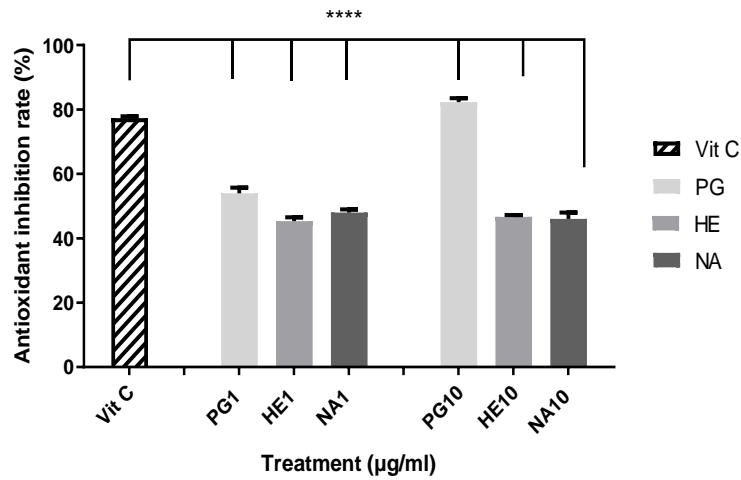


[Fig. 2] MTT Assay according to PG, HE, NA Concentrations. The cell viability of each material was compared. Cont: material-free group; Vit C: Vitamin C (10  $\mu$ g/mL); PG: Pycnogenol ( $\mu$ g/mL); HE: Hibiscus flower extract ( $\mu$ g/mL); NA: Niacin ( $\mu$ g/mL); \* is a significant result for Cont and the material groups (\*\* $p$  > 0.01, \*\*\*\* $p$  > 0.0001). # is a significant result for Vit C and the material groups (### $p$  > 0.001, #### $p$  > 0.0001).

### 3.2 DPPH

The antioxidant ability for each material at different concentrations (1  $\mu$ g/mL, 10  $\mu$ g/mL) shown in [Fig. 3].

1µg/mL for concentration, the antioxidant capacity of PG was higher than the other substances, but the difference was not significant. At a concentration of 10µg/mL, PG had a higher antioxidant capacity than that of HE, NA, and Vit C.

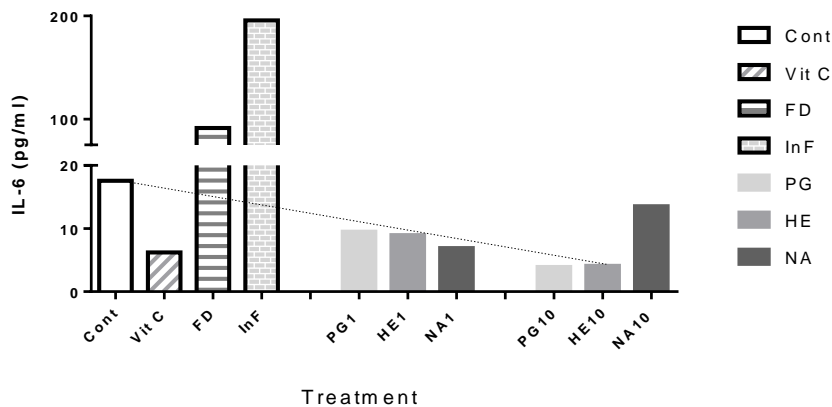


[Fig. 3] Comparison of the DPPH of PG, HE, NA, and Vit C. The antioxidant ability of Vit C and the other materials was compared. The concentration of Vit C was 10µg/mL, and that of the other materials was 1µg/mL and 10µg/mL. Vit C: Vitamin C (10µg/mL); PG: Pycnogenol (µg/mL); HE: Hibiscus Flower Extract (µg/mL); NA: Niacin (µg/mL). There was a significant difference in antioxidant capacity when comparing Vit C and other materials (\*\*\*\*p<0.0001).

### 3.3 IL-6

The standard curve of IL-6 levels for the materials is shown in [Fig. 4].

IL-6 levels were lower with PG, HE, and NA than in the material-free group. At 10 µg/mL, cells with PG had the lowest IL-6 expression level, but there was no significant difference compared with HE. IL-6 levels with PG and HE were lower than those with Vit C. The IL-6 level was significantly lower with PG, HE, and NA than with fine dust and influenza virus. As the concentration of PG and HE increased, the amount of inflammation decreased.



[Fig. 4] Comparison of PG, HE, NA, and Vit C Mouse IL-6. The levels of IL-6 were compared after material treatment. Control is Cont, negative control is InF. Cont: material-free group; Vit C: Vitamin C (10 µg/mL); FD: Fine dust (10 µg/mL); InF: Influenza virus (MOI 0.1); PG: Pycnogenol (µg/mL); HE: Hibiscus Flower Extract (µg/mL); NA: Niacin (µg/mL).

## 4. Discussion

We studied the skin soothing effect of PG and HE, which are natural products, exerted via the anti-inflammatory effect of IL-6; IL-6 plays an important role in skin inflammatory response.

First, the cell viability of PG, HE, and NA was investigated. Each substance did not adversely affect cell viability and showed more vigorous viability[10]. These results indicate that each material was not toxic to cells[11]. Especially for HE and NA, cell viability was greatly increased at 10  $\mu\text{g}/\text{mL}$ [16]. PG has been reported to be a safe material; PG at 700 mg per day is the safe dose recommended by the European Food Safety Authority, and 30–200 mg is commonly used in common oral dosing studies[17].

PG exerted inflammation-related antioxidant properties at 10  $\mu\text{g}/\text{mL}$ , which was higher than that of the other materials and Vit C at similar concentrations[18]. This is supported by the fact that, owing to its excellent antioxidant properties, it is used in several products[19]. The antioxidant capacity of HE also showed similar values to those in a previous study[20]. Choi et al.[15] reported a higher antioxidant level for HE than that observed in this study (10  $\mu\text{g}/\text{mL}$  in this study). In other previous studies, antioxidant research was conducted at high concentrations[21]; thus, experiments at various concentrations should be performed in the future.

IL-6 levels were the lowest with PG; they were also low with HE. All the materials showed lower IL-6 levels than that of the material-free group. As a result, natural materials can reduce inflammation and ultimately have an anti-inflammatory effect on the skin [14]. When comparing materials with fine dust and the influenza virus, the very low IL-6 levels with the materials in this study resulted from their calming effect on the skin. This is consistent with previous studies in which PG did not induce inflammation-associated IL- $\beta$ , IL-6, and TNF- $\alpha$  secretion[22][23]. In previous studies, it also inhibited the NF- $\kappa\text{B}$  transcription factor that causes dysmenorrhea[24]. As NF- $\kappa\text{B}$  transcription factors influence the induction of IL-1, IL-6, and IL-8, the inhibitory effects of NF- $\kappa\text{B}$  influence anti-inflammatory function[25]. In particular, a substance having an anti-inflammatory action[3] has a soothing effect on the skin. This has also been shown in studies of compounds with anti-inflammatory effects in acne and atopic dermatitis[26].

In previous studies, RT-qPCR was performed on tyrosinase and tyrosinase-related proteins, which are transcription factors related to melanin production, for whitening efficacy. Cell viability was measured with MTT. As a result, it was confirmed that it does not affect cells, has no toxicity, and has a whitening effect[27][28]. Therefore, in this study, IL-6 production and DPPH assessments were performed to evaluate different efficiencies of the materials, and cytotoxicity testing with MTT revealed the functional aspects and non-cytotoxic properties of these materials. Thus, PG and HE can be used as cosmetic raw materials.

In this experiment, we used B16F10 cells; however, studying other cells related to the skin and evaluate synergistic effects when blending materials at various concentrations are necessary. Moreover, additional studies involving different cytokines that induce inflammation in the skin should be performed to study the anti-inflammatory effects on the skin.

## 5. Conclusions

This study is significant in that it examined the effect of PG, HE on skin soothing by using plant-derived materials and assessing cell viability, antioxidant ability, and inhibition of IL-6 production.

Cell viability at 1 $\mu\text{g}/\text{mL}$  and 10 $\mu\text{g}/\text{mL}$  was  $103 \pm 0.03\%$  and  $102 \pm 0.05\%$  for PG.  $98 \pm 0.04\%$  and  $158 \pm 0.07\%$  for HE. For NA, it was  $72 \pm 0.09\%$  and  $106 \pm 0.17\%$ . The experiment results show that natural substances such as PG, HE, and NA do not exhibit cytotoxicity. This is because the cell viability of the material-free group was similar or higher at the concentration of the material except for NA 1 $\mu\text{g}/\text{mL}$ . NA 1 $\mu\text{g}/\text{mL}$  also had a lower cell viability than the material-free group, but was not toxic. IL-6

levels, which are involved in inflammation, were also reduced. The IL-6 levels in those following material-free group treatments were 17.6 pg/mL, in those following PG treatments were 9.5 pg/mL and 3.9 pg/mL, and in those following HE treatments were 9 pg/mL and 4.1 pg/mL. In NA, the level of IL-6 was lower than that of the material-free group at both concentrations, but the level of inflammation increased as the concentration increased. This indicates that the anti-inflammatory effect is not as great as that of PG and HE. The fact that PG at the concentration of 10 µg/ml, which has the highest antioxidant effect, has the lowest inflammation level indicates that the antioxidant activity is closely related to the expression level of inflammation.

This means that PG and HE are safe as cosmetic materials and have an IL-6 inhibitory effect. Therefore, PG and HE materials can be safely used as raw materials for skin cosmetics, and skin problems caused by inflammation can be suppressed. This is expected to provide a skin soothing effect. In the future, research on the development of cosmetics containing bioactive compounds, which are effective in protecting against oxidative stress, improving skin elasticity, reducing wrinkles, and reducing pigment concentration, should be conducted.

This research presents data on the applicability of cosmetics as a functional ingredient and provides basic data for its use for future research purposes.

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