

## 지하철 미세먼지에 의하여 유발되는 피부염증에 대한 달맞이꽃 뿌리 추출물의 완화 효과

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### Relaxing Effect of Evening Primrose Root on Skin Irritation Caused by Particulate Matter in Subway Tunnel

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**요약:** 인간의 피부가 지하철 터널과 같은 외부환경에서 고농도의 입자 먼지(PM<sub>2.5</sub>, PM<sub>10</sub>)에 장시간 노출되면 피부에 나쁜 영향을 받게 된다. 특히, 미세입자 먼지는 피부를 손상시켜 염증과 알러지 반응을 일으킨다. 본 연구에서는 달맞이꽃뿌리 추출물이 피부에 미세입자 먼지가 반응하여 피부손상을 유발할 때 피부염증 저해능력을 조사하였다. 입자형태의 먼지는 지하철에서 하루에 가장 높은 농도로 존재할 때 수집하였다. 달맞이꽃뿌리 추출물은 대조군에 비하여 강한 항산화능을 보였다 (62.6%). 미세입자 형태와 달맞이꽃뿌리 추출물의 혼합물은 일산화질소 생성을 억제하여 달맞이꽃뿌리 추출물이 미세입자 먼지에 의하여 유발되는 피부염증을 완화하는 효과가 확인되었다. 달맞이꽃뿌리 추출물은 세포독성이 대조군에 비하여 낮았다. 입자형태의 먼지(PM<sub>10</sub>)를 세포에 노출시켰을 때 달맞이꽃뿌리 추출물의 농도를 증가시킬수록(5, 10, 20  $\mu\text{g}/\text{mL}$ ) 활성산소 수준이 감소함과 동시에 양성 대조군에 비하여 더욱 효과적이었다. 따라서 본 연구결과는 달맞이꽃뿌리 추출물이 미세입자 형태의 먼지에 의하여 유발되는 피부 손상을 완화시킬 수 있는 효능을 제공하여 피부용 화장품 소재로 활용이 가능함을 입증하였다.

**Abstract:** If human skin is exposed to high concentrations of particulate matter (PM<sub>2.5</sub> and PM<sub>10</sub>) for a long time in the outdoor environment such as subway tunnel, it will be adversely affected. In particular, fine particles can damage the skin, causing inflammation and allergic reactions. This study investigated the ability of evening primrose root (EEPR) extract to suppress the skin damages caused by the fine particles. PM was collected from a subway tunnel, where high concentrations have been reported per day over the course of a study. The EEPR had higher antioxidant activity than that of control group (62.6%). The mixture of EEPR and PM inhibited the production of nitric oxide (NO), thereby alleviating skin inflammation caused by fine particle dust. EEPR had weaker cytotoxic activity than the positive control. When cells were exposed to particulate-type dust (PM<sub>10</sub>), the levels of free radicals were decreased with the increased concentrations of the extract (5, 10, 20  $\mu\text{g}/\text{mL}$ ). While at the same time more effective than positive controls. Therefore, this study proved that the Moonlight flower root extract can be used as a cosmetic material for skin by providing an effect to alleviate skin damage caused by fine particle-type dust.

**Keywords:** evening primrose, skin irritation, particulate matter, inflammation, cosmetic

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## 1. Introduction

In South Korea, the average time spent travelling on weekdays is 1.75 h, while on weekends the figure is 1.68 h[1]. Commuters are susceptible to exposure to different pollutants according to transportation mode during travelling. The skin acts as a barrier that protects humans against microbes and other harmful elements; hence, it is one of the first and major targets of various air pollutants, including ambient particulate matter[2]. However, the protective barrier constituted by the skin is limited, and problems can arise when environmental exposure exceeds the skin's normal defensive capability[3]. Coarse particles are able to overcome the initial defense provided by the nose and throat, and penetrate beyond the larynx for deposition along the airways in the thorax. Ambient PM is associated with cell damage and oxidative stress in the respiratory system[3]. House and road PM ( $PM_{2.5}$  and  $PM_{10}$ ) has been shown to induce oxidative stress, posing a risk of eye problems such as allergic conjunctivitis[4].

Evening primrose (EP) (*Oenothera biennis*), a biennial plant, is a species of *Oenothera* native to eastern and central North America, and widely naturalized elsewhere in temperate and subtropical regions, such as the northeast of Korea, China and Japan[5]. EP is good sources of the essential fatty acid  $\gamma$ -linolenic acid (GLA), which contain from 10 to 17% oil in seed, and GLA may constitute up to 10% of the fatty acid. Use of GLA in clinical and pharmaceutical applications is increasing, since it is a very active essential fatty acid and a precursor of prostaglandin E1 and its derivatives[6]. Moreover, the phenolic compound of EP extract provide a high antioxidative activity[7]. EP oil has an abundance of sterols, which confer significant protection against pro-inflammatory mediators[8]. Previous studies reported significant results of evening primrose oil application to treat various dermatological problems, such as atopic dermatitis[9], as well as the uremic symptoms of pruritus, erythema, and dryness[10]. The evening primrose roots (REP) have high phytotoxicity due to the presence of gallic acid, in addition to moderate antifungal activity[11]. Active anti-inflammatory compounds have also been found in the EEPR, and support its traditional use for

managing arthritis problems, while EEPR has also shown free radical scavenging activity[12].

A lot of study reported high concentration (2 - 4 times higher than outdoor) and chemical composition (mainly Fe) of particulate matter in subway tunnel and the it is harmful than other sources (e.g. wood combustion, vehicles, roadways) due to redox active iron on the surface of the subway particles[13-21]. Also, these can expose to passenger or citizen using the subway via heating, ventilation, and air-conditioning and ventilation system[22]. Prolonged exposure to high concentrations of PM can adversely affect the skin. In particular, fine particles in micro environment such as subway or subway tunnel can damage the skin, causing inflammation and allergic reactions. Therefore, expose of PM must be reduced for health. In this study, we investigated the effects of EEPR on relaxation of skin irritation caused by  $PM_{10}$  which was contented  $PM_{2.5}$  particle samples in subway tunnel.

## 2. Materials and Methods

### 2.1. Materials

Evening primrose (*Oenothera biennis*) and *Portulaca oleracea* were obtained by Wonkwang Herb Inc. (Korea). Filter paper and microfiber filter were purchased from Whatman Inter. Ltd. (UK), L-ascorbic acid, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), dimethyl sulfoxide (DMSO),  $K_2S_2O_8$  solution and 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). RAW 264.7 and HaCaT cellline were purchased from Korea cell line bank (Korea). Griess reagent kit and cytotoxicity assay kit were purchased from Promega Corp. (USA), Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (USA), TNF- $\alpha$  ELISA kit was purchased from BD Biosciences (USA), OxeSelect™ Intracellular ROS Assay Kit was purchased from Cell Biolabs, Inc. (USA).

### 2.2. Preparation of Extract of Evening Primrose Root (EEPR)

The root of Evening primrose (REP) was washed and

ground by using a mill having a particle size of 100 mesh. Added ground REP into 200 mL of 70% ethanol, extracted at 70 °C for 72 h with stirring. The extract of evening primrose root (EPR) was filtered with filter paper (NO. 4) and concentrated with vacuum evaporator. Finally, the EPR concentrate was dissolved in DMSO.

### 2.3. Sample Collection and Preprocessing

PM was collected from a subway tunnel, which is part of an urban railway where high concentrations of PM have been reported over the course of a day. A quartz microfiber filter (QM-A) was used to collect the PM samples. The filter paper was made from SiO<sub>2</sub>, which can withstand temperatures up to about 500 °C and generates few artifacts, such as SO<sub>4</sub><sup>2-</sup> and NO<sub>3</sub><sup>-</sup>. Such filters are suitable for collecting suspended particles, and the SO<sub>2</sub> or NO<sub>2</sub> that is deposited on the surface during sample collection[23]. The 38 mm filter paper was cut into pieces, which were then placed into a 100 mL extraction cartridge. Next, 30 mL of deionized water was added and the PM was extracted using an ultrasonic extractor for 30 min[24]. In order to compare the effect of EPR on skin, *Portulaca oleracea* extract (POE) and ascorbic acid were used as the positive control respectively. POE has anti-inflammatory and analgesic properties such as sedatives, and extract process was approximately equal to EPR process, skin moisturizers to reduce skin irritation and soothe allergic reactions. Ascorbic acid is an organic compound with antioxidant properties and one of the vitamins C. And also L-ascorbic acid (L-AA) is effective for skin aging, wrinkle reduction, and whitening.

### 2.4. Analysis of Inorganic Elements, Ions and Carbon in Particulate Matter

Inductively coupled plasma atomic emission spectroscopy (Icap 7000, Thermo Fisher Scientific, USA) was used for the analysis of inorganic elements, ion chromatography (Dionex™ ICS-6000, Thermo Fisher Scientific, USA) was used for the analysis of ions, and the carbon in particulate matter determined by using a carbon analyzer (OCEC Lab Instrument Model 5, Sunset Laboratory Inc., Netherlands).

### 2.5. Total Flavonoid Content

The total flavonoid content of EPR was determined by the aluminium chloride colorimetric method[25]. In brief, 50 µL of EPR (1 mg/mL DMSO) were made up to 1 mL with ethanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% NaNO<sub>2</sub> solution; 0.3 mL of 10% AlCl<sub>3</sub> solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm using a microplate Reader (Synergy™ HT, BioTek, USA). The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg catechin equivalent per g weight.

### 2.6. Antioxidant Activity

ABTS assay was performed to determine the antioxidant activity, by measuring the radical scavenging ability. In this study, modified method was used as described by Tan *et al.*[26]. 7 mM of ABTS solution and 2.6 mM of K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> solution were mixed to obtain the working solution, and left in the dark for 12 h to enable the reaction to complete. Then, the ABTS radical solution was diluted with phosphate-buffered saline to achieve a spectrophotometric absorbance of 0.700 ± 0.001 units at 734 nm. Next, 180 µL of ABTS solution was added to 20 µL of different concentrations (2.5, 5, and 10 µg/mL) of EPR and mixed thoroughly. Ascorbic acid was used as a positive control. The reactive mixture was incubated at room temperature for 10 min and the absorbance was immediately recorded at 734 nm.

### 2.7. Cell Viability

RAW 264.7 and HaCaT cells were seeded at a density of 1 × 10<sup>4</sup> cells/well in a 96 well plate respectively. The cells were treated with various concentrations (1, 2, 5 and 10 µg/mL) of PM<sub>10</sub>, and various concentrations (5, 10 and 20 µg/mL) of EPR and POE, respectively, and incubated for 24 h after which MTT solution was added. Formazan was dissolved by DMSO after removal of the MTT solution. The absorbance was measured at 540 nm.

## 2.8. Cytotoxicity Assay

A cytotoxicity assay kit (Cytotoxic 96<sup>®</sup>) was used to quantitatively measure the lactate dehydrogenase (LDH). A total of  $1 \times 10^4$  HaCaT cells/well were seeded on 96 well plates for 24 h. The cells were then incubated with various concentrations (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) of EEPR; L-ascorbic acid was used as a positive control, with  $\text{PM}_{10}$  (5  $\mu\text{g}/\text{mL}$ ) exposure for 24 h. Cell-free supernatants were collected to determine the amount of LDH released as a result of cytotoxicity. The amount of LDH was determined at an absorbance of 490 nm as previously reported method[28].

## 2.9. Nitric Oxide Production Quantitative Analysis

Nitrite production was determined using  $1 \times 10^4$  RAW 264.7 cells/well, grown in 96 well plates overnight. The cells were then incubated with various concentrations (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) of EEPR and POE was used as a positive control, with  $\text{PM}_{10}$  (2  $\mu\text{g}/\text{mL}$ ) exposure for 24 h. Then, 50  $\mu\text{L}$  of cell-free supernatant was well-mixed with an equal volume of the Griess reagent, and the NO concentration was determined as per a previously reported method, with a slightly modification[27].

## 2.10. TNF- $\alpha$ Production Quantitative Analysis

HaCaT ( $1 \times 10^4$  cells/well) were pretreated with various concentrations (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) of EEPR, with the  $\text{PM}_{10}$  (2  $\mu\text{g}/\text{mL}$ ) for 24 h. TNF- $\alpha$  level in the supernatant were measured according to the commercial instructions using the TNF- $\alpha$  ELISA kit. The ELISA plates were coated overnight at 4 °C with anti-TNF- $\alpha$  monoclonal antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5). The plates were washed with PBS containing 0.05% Tween 20 and nonspecific protein binding sites were blocked with assay diluent (PBS with 10% FBS) for 1 h. Each supernatant sample was added to the wells and incubated for 2 h. One-hundred microliters of working detector (biotinylated anti-TNF- $\alpha$  monoclonal antibody and avidin-HRP reagent) was applied to the sample wells for 1 h. The substrate solution (tetramethylbenzidine (TMB) + hydrogen peroxidase, 100  $\mu\text{L}$ ) was added to each well and incubated for 30 min at room temperature in the dark. After addition of 2 N- $\text{H}_2\text{SO}_4$  (50  $\mu\text{L}$ ), the optical density

was measured at 450 nm using a microplate reader. POE was used as a positive control.

## 2.11. Intracellular ROS Measurement

Intracellular ROS was quantified by using a OxeSelect<sup>™</sup> Intracellular ROS Assay Kit. A density of  $1 \times 10^4$  HaCaT Cells were pre-incubated in 96 well black plate for 24 h. Following the cells were washed with PBS. 10  $\mu\text{M}$  dichlorofluorescein diacetate (DCFH-DA) was added to the cells at 37 °C and left for 1 h in the dark room. Removing supernatant and washed with PBS again, then the DCFH-DA loaded cells were treated with various concentration (5, 10, and 20  $\mu\text{g}/\text{mL}$ ) of EEPR and L-ascorbic acid (L-AA) as a positive control with the  $\text{PM}_{10}$  (2  $\mu\text{g}/\text{mL}$ ) at 37 °C for 4 h in the dark. Non-fluorescent DCFH-DA is converted to fluorescent dichlorofluorescein in proportion to the amount of ROS generation in cells. The fluorescence signal was quantified at excitation and emission wavelengths of 485 and 530 nm respectively.

## 2.12. Statistical Analysis

Data are presented as the mean  $\pm$  SD. Comparison between groups was analyzed using Student's *t*-test.  $P < 0.05$  was considered statistically significant.

# 3. Results

## 3.1. Total Flavonoid Content

Total flavonoid content of EEPR and POE were  $65.80 \pm 1.59$  and  $50.26 \pm 0.52$  mg catechin equivalents/g, respectively (Table 1).

**Table 1.** Total Flavonoid Content of Ethanolic Extract of EEPR and POE

Samples	Total flavonoid (mg CAE <sup>*</sup> /g)
EEPR	$65.80 \pm 1.59$
POE	$50.26 \pm 0.52$

\*CAE, Catechin equivalents

3.2. Characteristics of Particulate Matter

In this study, PM<sub>10</sub> being the dominant fraction of the particulate matter, that containing ranged 200 ~ 300  $\mu\text{g}/\text{m}^3$  compared to 100 ~ 150  $\mu\text{g}/\text{m}^3$  of PM<sub>2.5</sub>, PM<sub>2.5</sub> and PM<sub>10</sub> contained metal, cation, anion, organic carbon, elemental carbon and other compounds (Table 2). Some of these compounds, such as metal and organic carbon were major compounds in both of PM<sub>2.5</sub> and PM<sub>10</sub>. Ioncontent of PM<sub>2.5</sub> and PM<sub>10</sub>, such as SO<sub>4</sub>, Na, NH<sub>4</sub>, NO<sub>3</sub>, Ca and Cl showed 32, 34, 16, 11, 3 and 3%, and 30, 17, 10, 20, 16 and 4%, respectively (Table 3). Total concentration of carbon for PM<sub>2.5</sub> and PM<sub>10</sub> were 18 and 36  $\mu\text{g}/\text{m}^3$ , respectively (Figure 1). Carbon concentration was consists of organic carbon (OC), carbonate carbon (CC), pyrolysis carbon (PC) and elemental carbon (EC). The ratio of EC to OC showed a higher value approximately four times for PM<sub>10</sub>, however PM<sub>2.5</sub> exhibited much higher value approximately three times. Therefore, we considered that EC would be dominant carbon in PM<sub>10</sub> of particulate matter, and the particle size would be larger than 2.5  $\mu\text{m}$ .

**Table 2.** Composition of PM<sub>2.5</sub> and PM<sub>10</sub> in Particulate Matter

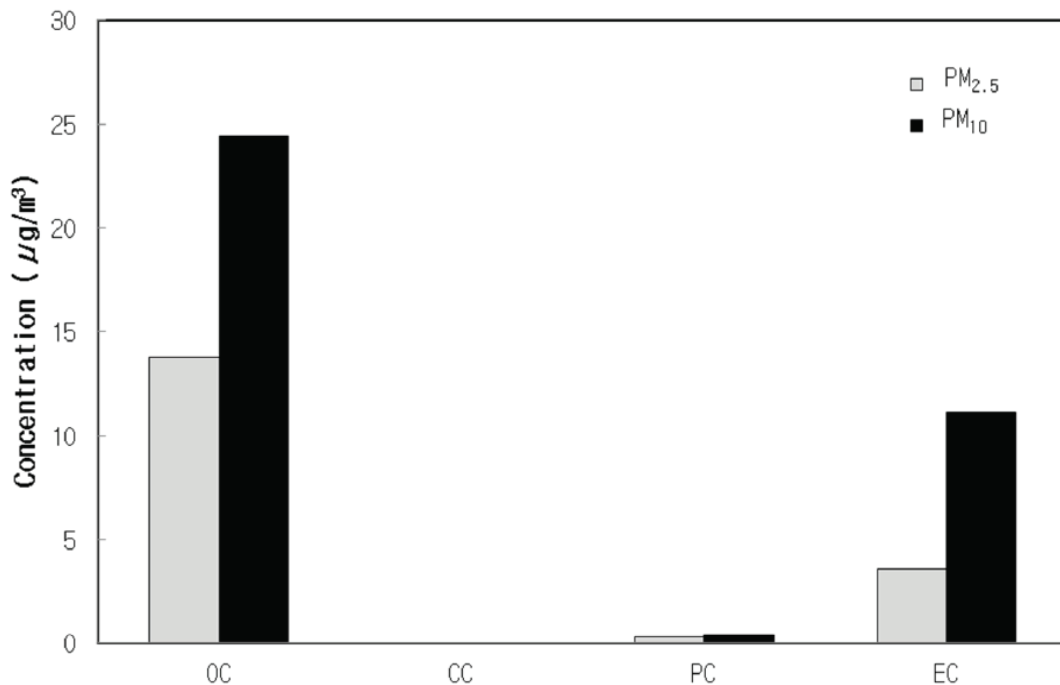
	PM <sub>2.5</sub>	PM <sub>10</sub>
Total metal	63	65
Cation	3	2
Anion	3	2
OC	17	10
EC	4	5
Unknown	10	16

OC: Organic carbon, EC: Elemental carbon. Unit : %

**Table 3.** Ion Content of PM<sub>2.5</sub> and PM<sub>10</sub> in Particulate Matter

	PM <sub>2.5</sub>	PM <sub>10</sub>
SO <sub>4</sub> <sup>2-</sup>	32	30
Na <sup>+</sup>	34	17
NH <sub>4</sub> <sup>+</sup>	16	10
NO <sub>3</sub> <sup>-</sup>	11	20
Ca <sup>+</sup>	3	16
Cl <sup>-</sup>	3	4
K <sup>+</sup>	1	1

Unit : %



**Figure 1.** Carbon concentration of PM<sub>2.5</sub> and PM<sub>10</sub> in particulate matter. OC: Organic carbon, CC: Carbonate carbon, PC: Pyrolysis carbon, EC: Elementalcarbon.

### 3.3. Effect of EEPR on Antioxidant Activity

Antioxidant activity of EEPR and ascorbic acid as a positive control were shown in Figure 2. The highest activity ( $62.6 \pm 1.55\%$ ) was obtained from the mixture of ABTS with  $10 \mu\text{g/mL}$  of EEPR, followed by treatments of  $33.0 \pm 3.37\%$  and  $17.2 \pm 1.68\%$  from 5 and  $2.5 \mu\text{g/mL}$  of EEPR, respectively. As a positive control of L-ascorbic acid ( $10 \mu\text{g/mL}$ ) showed the greatest activity, with a value of  $99.2 \pm 1.11\%$ ; the treatment of  $5 \mu\text{g/mL}$  had a value of  $80.2 \pm 5.59\%$ , while by treatment of  $2.5 \mu\text{g/mL}$  had a value of  $43.9 \pm 2.14\%$ .

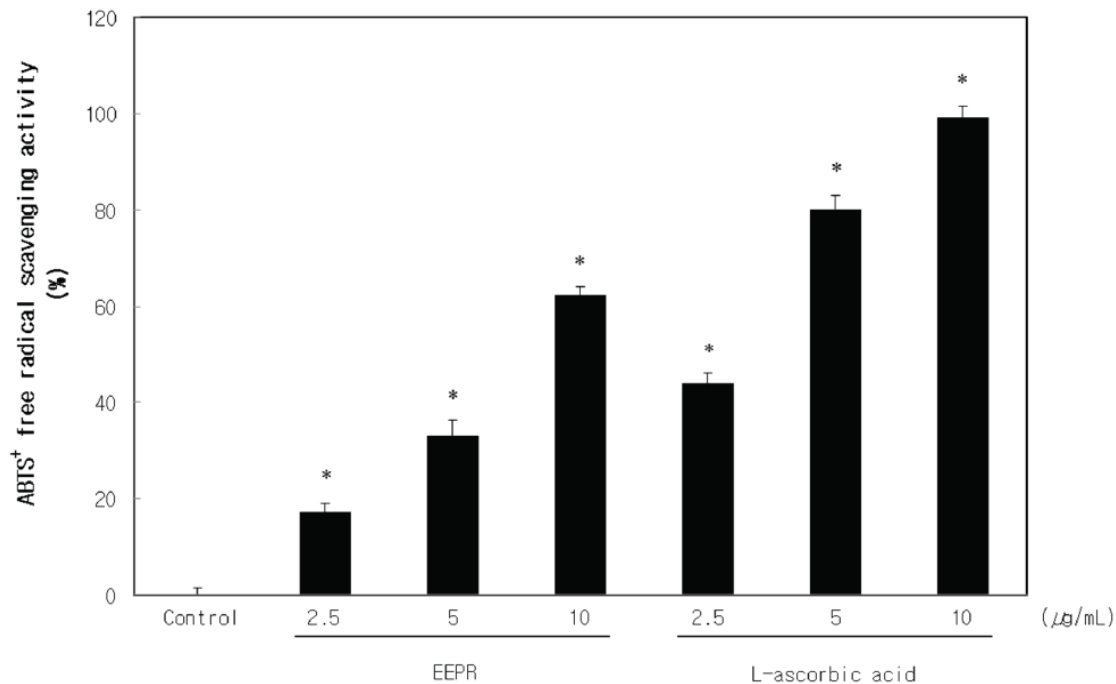
### 3.4. Cell Viability in RAW 264.7 and HaCaT Cells treated with $\text{PM}_{10}$ , EEPR and POE

Cell viability was measured by using MTT assay. Figure 3 (A, B) showed that  $\text{PM}_{10}$  stimulation at low (1,  $2 \mu\text{g/mL}$ ) concentration had no effect on cell viability, but high (5,  $10 \mu\text{g/mL}$ ) concentration was toxic for both of RAW 264.7 and HaCaT cells. Figure 3 (C, D) showed that all the different

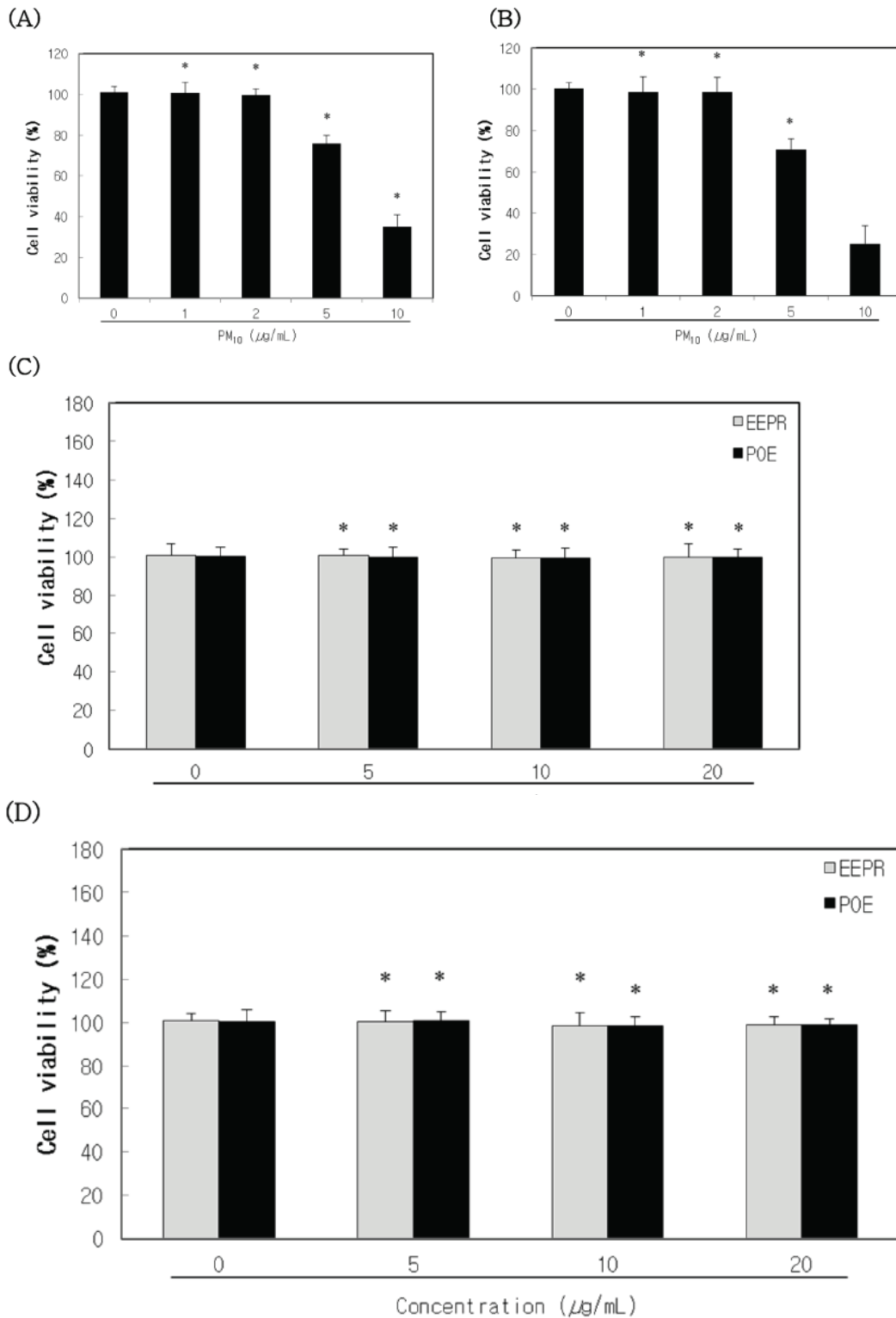
concentrations (5, 10 and  $20 \mu\text{g/mL}$ ) of EEPR and POE had no toxicity in RAW 264.7 (C) and HaCaT cells (D), respectively.

### 3.5. Inhibitory Effect of EEPR on Nitric Oxide (NO) Production

The anti-inflammatory activity of negative control, the mixture of EEPR and POE are shown in Figure 4. The NO value for the control was  $31.5 \pm 37.6 \text{ nM}$ , while for  $\text{PM}_{10}$  it was  $357.2 \pm 67.8 \text{ nM}$ . Thus, NO was readily apparent and, with the assay added to EEPR and the positive control, the highest NO concentrations obtained were from the  $5 \mu\text{g/mL}$  of EEPR and POE treatments were  $161.8 \pm 18.8$  and  $281.2 \pm 37.6 \text{ nM}$ , respectively. For the  $10 \mu\text{g/mL}$  treatments, the respective values were  $96.9 \pm 18.8$  and  $161.8 \pm 18.8 \text{ nM}$ . The results showed that  $20 \mu\text{g/mL}$  of EEPR and POE treatments had the lowest NO concentrations with mean value of  $42.4 \pm 32.6$  and  $118.4 \pm 18.8 \text{ nM}$ , respectively.

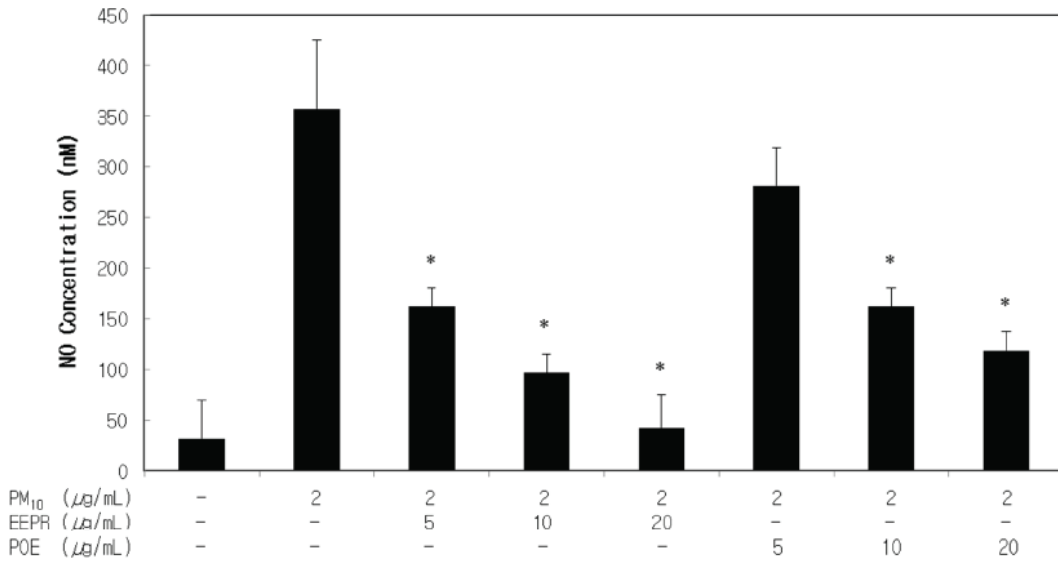


**Figure 2.** Effect of EEPR on antioxidant activity. The amount of ABTS<sup>+</sup> radicals was determined at various concentrations of EEPR and L-ascorbic acid by a microplate reader at 723 nm. All data are expressed as the mean  $\pm$  SD of three separate experiments performed in triplicate ( $^*p < 0.05$  vs Control).



**Figure 3.** Cell viability in RAW 264.7 (A, C) and HaCaT (B, D) cells treated with PM<sub>10</sub>, EEPR and POE, respectively. Viability was determined in the MTT assay by measuring the absorbance at 450 nm. The results are expressed as the percentage of viability control ( $p < 0.05$ ).



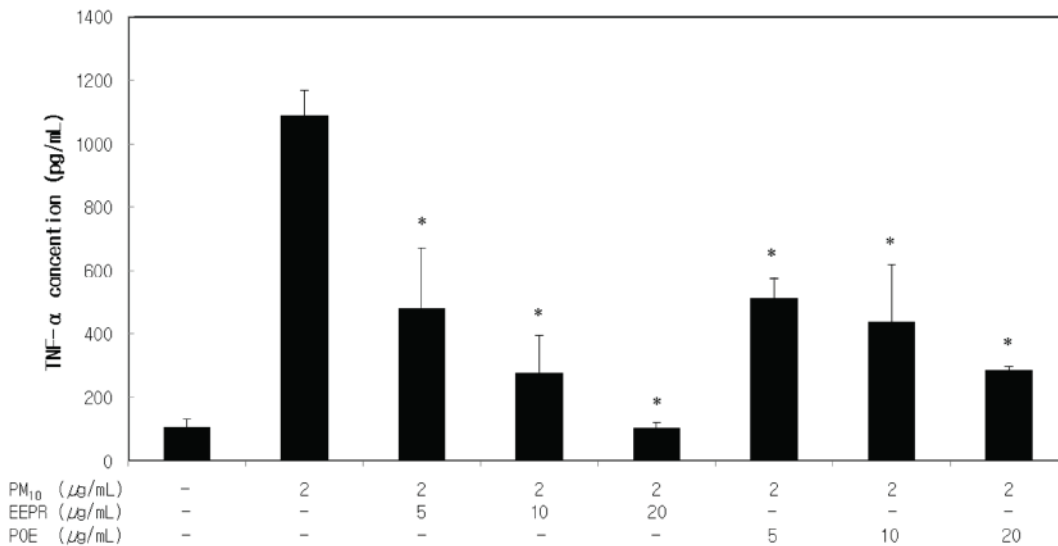


**Figure 4.** Effect of EEPR on the PM<sub>10</sub>-induced NO production in RAW 264.7 cells. The PM<sub>10</sub>-induced cells were exposed for 24 h to various concentrations of EEPR and POE. The NO amount was determined by a microplate reader at 540 nm and data are expressed as the mean ± SD of three separate experiments performed in triplicate (\**p* < 0.05).

3.6. Inhibitory Effect of EEPR on TNF-α Production

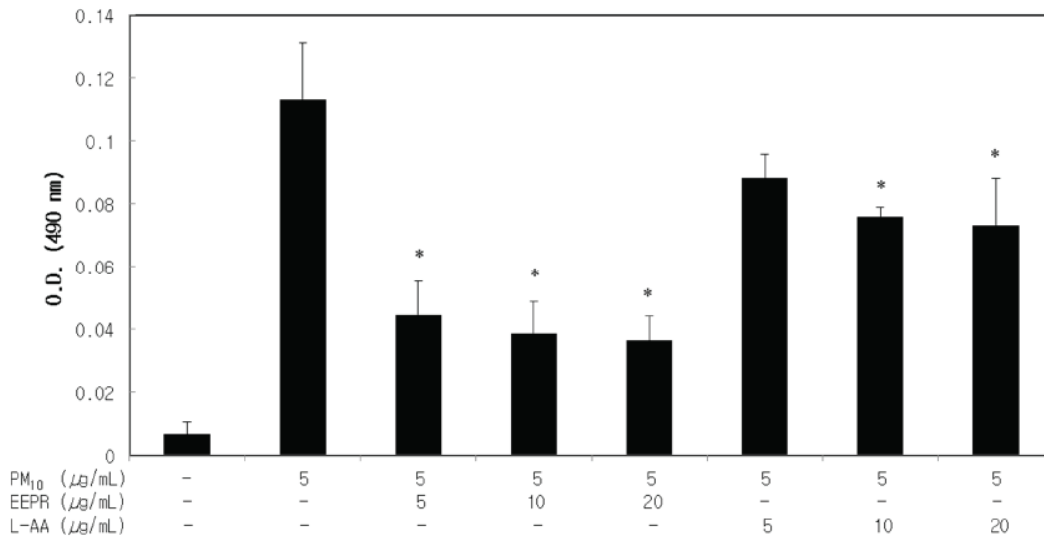
As shown in Figure 5, EEPR inhibited the production of TNF-α from PM<sub>10</sub>-induced HaCaT cells in a dose-dependent manner. The level of TNF-α in 3 different concentration (5, 10

and 20 μg/mL) of EEPR were recorded as 482.73 ± 187.35, 279.40 ± 117.19 and 102.73 ± 17.32 pg/mL, respectively. For a positive control, POE were recorded as 512.73 ± 60.00, 439.40 ± 177.86 and 286.70 ± 11.55 pg/mL, respectively.

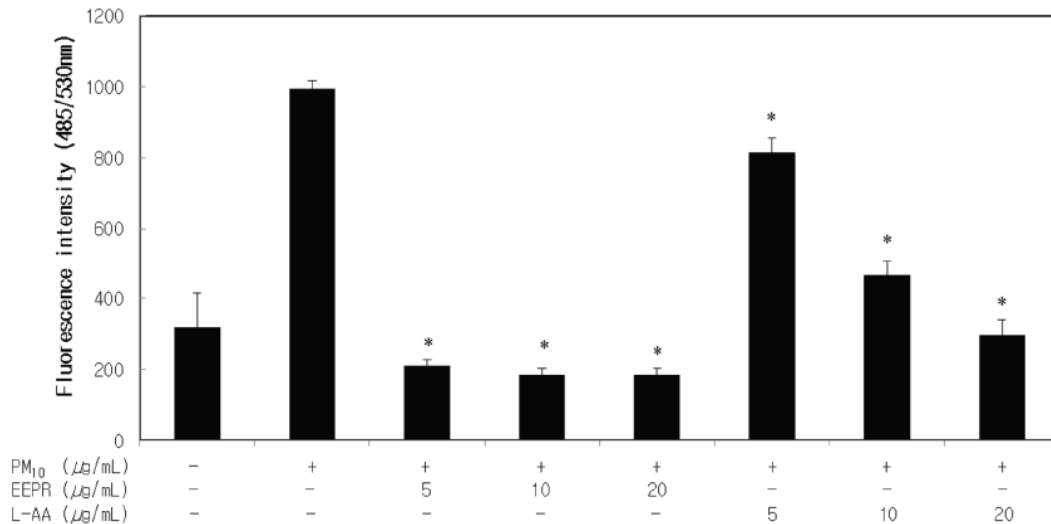


**Figure 5.** Effects of EEPR on the PM<sub>10</sub>-induced TNF-α production in HaCaT cells. The PM<sub>10</sub>-induced cells were exposed for 24 h to various concentrations of EEPR and POE. The TNF-α amount was determined using a TNF-α ELISA kit by a micro-plate reader at 540 nm and data are expressed as the mean ± SD of three separate experiments performed in triplicate (\**p* < 0.05).





**Figure 6.** Protection effect of EEPR in PM<sub>10</sub>-induced HaCaT cells. The PM<sub>10</sub>pre-treated cells were exposure for 24 h to various concentrations of EEPR, and L-ascorbic acid (L-AA) was used as a positive control. Then, Cell cytotoxicity was measured by LDH assay and values represent means ± SD of three independent experiments performed in triplicate (\**p* < 0.05).



**Figure 7.** Inhibitory effect of EEPR on ROS production in PM<sub>10</sub>-stimulated HaCaT cells. L-ascorbic acid (L-AA) was used as a positive control. Result are expressed as mean ± SD of three independent experiments performed in triplicate (\**p* < 0.05).

### 3.7. Effect of EEPR on Cytotoxicity

To examine when the decrease of cell viability was triggered with EEPR, the effect of concentration of the mixture of PM and EEPR was examined (Figure 6). The toxicity of the mixture of PM and EEPR on the skin cell was determined by exposure of cells to PM<sub>10</sub> and obtained an OD

of 0.113 ± 0.018. Figure 6 shows that EEPR had weaker cytotoxic activity than the positive control. The lowest OD was obtained with 20 μg/mL of EEPR and L-AA treatments, which had values of 0.036 ± 0.008 and 0.073 ± 0.009 nM, respectively. 10 μg/mL treatments had similar results, with values of 0.039 ± 0.010 nM for the EEPR and 0.076 ± 0.009

nM for the positive control. The highest mean OD was obtained for 5  $\mu\text{g}/\text{mL}$  treatments, with values of  $0.045 \pm 0.011$  and  $0.088 \pm 0.019$  nM for EEPR and L-AA, respectively. OD values for EEPR decreased by about 39%, 34% and 32% for 5, 10 and 20  $\mu\text{g}/\text{mL}$  treatments, respectively, whereas for L-AA, the values were about 78%, 67% and 65% for the 5, 10 and 20  $\mu\text{g}/\text{mL}$  treatments, respectively.

### 3.8. Effect of EEPR on ROS

Figure 7 shows that ROS after the cells were exposed to  $\text{PM}_{10}$ , with 5  $\mu\text{g}/\text{mL}$  of EEPR treatment decreased the ROS content by  $211.0 \pm 14.73$  (FI), while with 10  $\mu\text{g}/\text{mL}$  of EEPR treatment decreased it by  $184.3 \pm 17.10$  (FI), with an  $183.7 \pm 18.34$  (FI) reduction observed following with 20  $\mu\text{g}/\text{mL}$  of EEPR treatment. The positive control only decreased the ROS content by  $814.7 \pm 14.22$  (FI),  $465.0 \pm 13.08$  (FI), and  $297.7 \pm 23.44$  (FI) for 2, 10 and 20  $\mu\text{g}/\text{mL}$  of L-AA treatments, respectively.

## 4. Discussion and Conclusion

EP has been used in folk medicine as an oil type which can be available from its sources such as root, whole stem and leaves. EEPR was obtained from by the extraction of 70% ethyl alcohol. We conducted an *in vitro* test to evaluate the relaxation effect of EEPR on skin irritation caused by particulate matter. EEPR showed a relatively strong antioxidant activity compared to negative and positive control. The antioxidant activity of EP extracts from seed using ethanol or ethyl acetate also reported, in which active fractions contained phenolic acids and their esters; gallic acid, methyl and ethyl gallates, protocatechuic acid and its methyl ester[29]. In anti-inflammatory activity, EEPR modulate anti-inflammatory properties with mean value of NO concentration ( $42.4 \pm 32.6$  nM), which was close to negative control and much higher (8.5 times) than that of positive control. The emulsions with seedcake extracts from EP can protect skin against oxidative stress and can play an important role in preventing the skin aging[30]. The application of emulsions with EP seedcake extracts can be suggested as a

possible strategy to prevent and modulate oxidative skin damages and anti-inflammatory properties[30]. A mixture of long chain fatty alcohols such as hexacosanol, tetracosanol, docosanol and octocosanol, which significantly and dose-dependently decreased nitric oxide production induced by lipopolysaccharide and the inhibitory effect seems to be consequence of an action at the level of the inducible nitric-oxide synthetase gene enzyme expression rather than to a direct inhibitory action on enzyme activity[31]. Cytotoxic effect of EEPR on skin cell, EEPR had weaker cytotoxic activity than the positive control. The lowest OD was obtained with 20  $\mu\text{g}/\text{mL}$  of EEPR and ascorbic acid treatments, which had values of  $0.036 \pm 0.008$  and  $0.073 \pm 0.009$  nM, respectively. Among cellular processes, a large body of evidences has accumulated to suggest that ROS play a key role as a common mediator of apoptosis. EEPR significantly decreased the ROS level by  $184.3 \pm 17.10$  (FI) and  $183.7 \pm 18.34$  (FI) with 10.0  $\mu\text{g}/\text{mL}$  and 20.0  $\mu\text{g}/\text{mL}$  treatment of EEPR, respectively. ROS include free radicals such as the superoxide anion ( $\text{O}_2^-$ ), hydroxyl radicals (OH) and the non-radical hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). It is well documented that hydrogen peroxide serves as a mediator of apoptosis and that hydrogen peroxide itself induces apoptosis in many types of cells[32-35]. The seed of EP-induced apoptosis in Ehrlich ascites tumor cells as evidenced by morphological changes. Furthermore, the results demonstrated rapid increase of intracellular peroxides levels, loss of mitochondrial membrane potential and the release of cytochrome c from mitochondria to cytosol. These results suggest that the rapid increase of intracellular peroxides levels after addition of EP extract triggers off induction of apoptosis[35]. EEPR did not affect the cell viability of HaCaT cells. Cell viability of EEPR was  $82.8 \pm 1.05\%$  with 20  $\mu\text{g}/\text{mL}$  treatment of mixture of EEPR and  $\text{PM}_{10}$ . In cell viability of mouse embryo fibroblast cells (NIH/3T3) treated with EP extract, it did not affect cell viability of NIH/3T3 cells[36]. The EP extract had little influence on Caco-2 proliferation, but effectively in a time- and dose-dependent manner inhibited MMP-7, MMP-9 and MMP-14 mRNA synthesis induced by TNF- and TPA. Additionally, zymographic analysis revealed that after 24 h, the EP extract

caused a 10-fold reduction in MMP-9 synthesis. Moreover, this EP extract might be a potent inhibitor of MMP activity [37]. Results from our study, EEPR must have a significant impact on the effectiveness of an antioxidant and anti-inflammatory activity, cytotoxic effect and reducing ROS. Consequently, our results suggest that EEPR could be useful natural ingredient for cosmetics.

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