



*Letter*

# **Leaf extracts from *Camellia sinensis* and *Argania spinosa* suppress oxidative stress and chemokine release in human 3-dimensional cultured epidermis exposed to PM2.5 collected with cyclonic separation**

**Maori Kono<sup>1,2</sup>, Tomoaki Okuda<sup>3</sup>, Masayuki Takaishi<sup>1,2</sup>, Hidefumi Ikeda<sup>2</sup>,  
Nami Ishihara<sup>4</sup> and Yasuhiro Ishihara<sup>4</sup>**

<sup>1</sup>Laboratory of advanced cosmetic science, Graduate School of Pharmaceutical Sciences, Osaka University,  
Osaka, 565-0871, Japan

<sup>2</sup>Product Assurance Division, Mandom Corporation, Osaka, 540-8530, Japan

<sup>3</sup>Faculty of Science and Technology, Keio University, Kanagawa, 223-8522, Japan

<sup>4</sup>Program of Biomedical Science, Graduate School of Integrated Sciences for Life, Hiroshima University,  
Hiroshima, 739-8521, Japan

(Received July 22, 2022; Accepted August 2, 2022)

**ABSTRACT** — Skin is the primary tissue exposed to ambient air pollution because it acts as an interface between the body and the surrounding atmosphere. We previously reported that particulate matter 2.5 (PM2.5) induced oxidative stress and subsequent chemokine release in the human epidermis, followed by neutrophil chemotaxis. We identified in this study that the leaf extracts from *Camellia sinensis* and *Argania spinosa* showed high radical scavenging activity as evaluated by 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazinyl and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid assays. PM2.5 exposure induced lipid peroxidation, IL-8 release and neutrophil migration in human 3-dimensional cultured epidermis. Pre-treatment with leaf extracts from *Camellia sinensis* or *Argania spinosa* significantly suppressed the above harmful effects elicited by PM2.5. Taken together, both extracts can protect the epidermis from PM2.5 exposure. *Camellia sinensis* and *Argania spinosa* extracts could be added to a novel cosmetic that protects skin from air pollution.

**Key words:** Leaf extracts, 3D epidermis culture, Chemokines, PM2.5, Oxidative stress

## **INTRODUCTION**

Particulate matter 2.5 (PM2.5) is a type of air pollutant with a diameter of less than 2.5  $\mu\text{m}$ , characterized by its small particle size, large surface area and toxin absorption ability. PM2.5 comprises a mixture of solid and liquid particles, including black carbon, metals, nitrate, sulfate, polycyclic aromatic hydrocarbons and endotoxins (Bell *et al.*, 2007). The impacts of ambient PM2.5 on public

health have become great concerns worldwide, and the respiratory system is a major target of PM2.5. In fact, the Harvard Six Cities study showed that air pollution was positively associated with death from lung cancer and cardiopulmonary disease but not with death from other causes considered together (Dockery *et al.*, 1993). Japan and other countries regulate the air pollution/concentration of PM2.5 in the air to minimize the adverse health effects of PM2.5, primarily because of the interaction

between PM2.5 exposure and cardiopulmonary diseases.

Among the organs, skin is the primary tissue exposed to ambient air pollution because it acts as an interface between the body and the surrounding atmosphere. PM2.5 is suggested to be associated with several skin diseases, such as atopic dermatitis (Park *et al.*, 2022). The generation of reactive oxygen species (ROS) is an important mechanism for PM2.5-induced skin damage. When HaCaT cells (immortalized nontumorigenic human keratinocytes) were exposed to PM2.5, intracellular ROS were increased, and subsequently, the increased ROS induced endoplasmic reticulum stress and mitochondrial damage (Piao *et al.*, 2018). Dong *et al.* also reported that treatment of HaCaT cells with PM2.5 increased intracellular ROS levels and subsequent upregulation of NLRP1 and IL-1 $\beta$  via NF- $\kappa$ B activation (Dong *et al.*, 2020).

We previously reported that chemokine expression and subsequent neutrophil chemotaxis occurred after PM2.5 exposure in a human 3-dimensional cultured epidermis and that oxidative stress can be involved in this chemokine expression (Kono *et al.*, 2022). Therefore, antioxidants could preserve skin health from PM2.5 exposure via suppression of oxidative stress and subsequent neutrophilic inflammation. In this study, we screened 42 plant extracts using radical scavenging ability as an index, and then we selected 2 extracts, leaf extracts from *Camellia sinensis* and *Argania spinosa*. We investigated the effects of these 2 extracts on epidermal damage induced by PM2.5 exposure in this study.

## MATERIALS AND METHODS

### Leaf extracts

*Camellia sinensis* and *Argania spinosa* leaf extracts were obtained from BASF Japan Ltd. (Tokyo, Japan) and Ichimaru Pharcos Co., Ltd. (Gifu, Japan).

### Determination of radical elimination activity

The 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazinyl (DPPH) radical scavenging activity of leaf extracts was determined according to a previous report (Zheng *et al.*, 2022). Each leaf extract was diluted 20-fold with ethanol, and 10  $\mu$ L of the resulting diluent was mixed with 1 mL of DPPH solution (0.1 mM in 50% ethanol). After 1 min of incubation, the absorbance at 517 nm was monitored using an Ultrospec 3300 pro UV/Visible Spectrophotometer (GE Healthcare, Chicago, IL, USA). The DPPH radical scavenging activity was calculated as an ascorbic acid equivalent value.

The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was also deter-

mined according to a previous report (Zheng *et al.*, 2022). A stock solution of ABTS radicals containing ABTS (7 mM) and ammonium peroxodisulfate (2.45 mM) was incubated overnight in the dark at room temperature. The working solution was prepared by diluting the stock solution to an absorbance of approximately 0.7 at 734 nm. One milliliter of ABTS working solution and 10  $\mu$ L of diluted leaf extracts were incubated for 1 min at room temperature. The absorbance at 734 nm was recorded using an Ultrospec 3300 pro UV/Visible Spectrophotometer. The ABTS radical scavenging activity was calculated as an ascorbic acid equivalent value.

### Collection of PM2.5

The PM2.5 used in this study was collected in Yokohama, Japan, in winter 2018 as previously described (Ishihara *et al.*, 2022; Okuda *et al.*, 2015). Briefly, PM2.5 with an aerodynamic diameter of 2.5  $\mu$ m was separated using an impactor prior to entry into the cyclone device. Subsequently, the cyclone imparted a centrifugal force on the gas stream within a conical-shaped chamber and created a vortex inside the cyclone body.

### Human epidermis model

A 3D reconstructed human epidermis model, Lab-Cyte EPI-MODEL 24, composed of normal human epidermal keratinocytes forming a multilayered structure, was purchased from Japan Tissue Engineering Co., Ltd. (Aichi, Japan) and maintained according to the manufacturer's instruction manuals. PMs were suspended in distilled water, and 50  $\mu$ L of the suspension after sonication was exposed from the upper side (stratum corneum) of the epidermis model cultured in a 24-well plate with an inset cup. The leaf extracts were suspended in the culture media at 100-fold dilution (1%) and then added to the upper side (stratum corneum) of the epidermis model 30 min before PM treatment.

### Measurement of cellular viability

Cellular viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

### Determination of IL-8 concentrations in media

The collected conditioned media was stored at  $-80^{\circ}\text{C}$  before use. The IL-8 concentration in the media was determined using the LEGEND MAX Human IL-8 ELISA Kit (BioLegend, San Diego, CA, USA) according to the manufacturers' instructions.

### Neutrophil migration assay

Neutrophil migration was evaluated using a CytoSelect 96-well Cell Migration Assay (3  $\mu$ m), Fluorometric (Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturers' instructions. Briefly, human neutrophils (STEMCELL Technologies, Vancouver, BC, Canada) were seeded into 96-well membrane chambers at a concentration of  $1 \times 10^5$  cells/well. Conditioned media from the epidermis model was added to the feeder tray. The membrane chamber was placed onto the feeder tray, and then the cells were cultured for 1 hr in a CO<sub>2</sub> incubator. The cells were detached from the bottom side of the membrane chamber and then lysed and fluorescently labeled. The fluorescence from migrated cells was measured with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

### Evaluation of lipid peroxidation

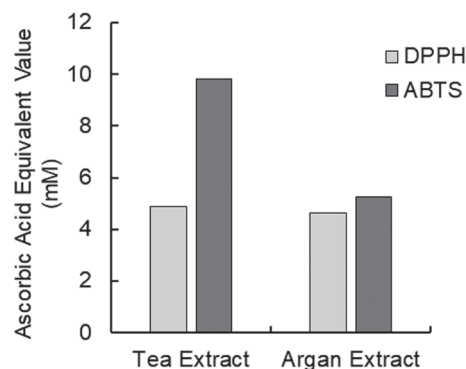
The content of thiobarbituric acid-reactive substance (TBARS) was estimated by the method described in our previous report (Ishihara *et al.*, 2012) and was used as an index of lipid peroxidation. Briefly, cultured epidermis was gently sonicated in 1.15% KCl solution. The tissue suspension was mixed with 7 mM sodium dodecyl sulfate, 16 mM thiobarbituric acid, and 340  $\mu$ M dibutylhydroxytoluene in acetic acid buffer at pH 3.5. The mixture was incubated at 100°C for 60 min, followed by rapid cooling and extraction with a 1-butanol-pyridine (15:1) solution. The absorbance of this extract at 540 nm was measured using 1,1,3,3-tetraethoxypropane as the standard.

### Statistical analyses

All data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Student's t test or ANOVA with Dunnett's corrected multiple comparison test was used to determine significant differences between the means of two or more independent groups. Significance was considered when p values were less than 0.05. Error bars were calculated using S.D.

## RESULTS AND DISCUSSION

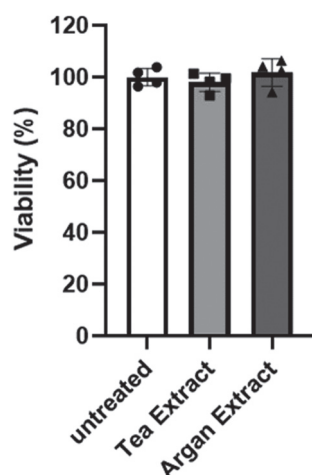
We previously reported that exposure to PM2.5, which was collected by a cyclonic separation method directly from the air, increased the expression of chemokines CXCL1 and IL-8 in human 3-dimensional cultured epidermis and that these chemokines were released from the epidermis and induced neutrophil chemotaxis (Kono *et al.*, 2022). Because chemokine release occurs downstream of oxidative stress induced by PM2.5 exposure (Kono *et al.*, 2022), antioxidants could suppress



**Fig. 1.** Radical scavenging action of *Camellia sinensis* and *Argania spinosa* extracts. *Camellia sinensis* and *Argania spinosa* extracts (labeled as Tea Extract and Argan Extract, respectively) were mixed with DPPH or ABTS working solution. Radical elimination was monitored by the changes in absorbance. Radical scavenging activity was represented as an ascorbic acid equivalent value. The values are the average of 3 experiments.

chemokine release and subsequent neutrophilic inflammation. We screened 42 kinds of plant extracts by DPPH and ABTS radical scavenging activities, and we determined that leaf extracts from *Camellia sinensis* showed the highest radical scavenging activity and that the *Argania spinosa* leaf extract showed the second highest radical scavenging activity (Fig. 1). The *Camellia sinensis* and *Argania spinosa* leaf extracts were diluted 100-fold with culture media and then treated from the upper side (stratum corneum) of the epidermis model for 30 min (final 1% concentration of extracts). Because both extracts showed no toxicity to human 3D cultured epidermis for no longer than 24 hr of culture at a concentration of 1% (Fig. 2), we examined the protective action of the extracts on PM2.5-exposed epidermis.

The *Camellia sinensis* and *Argania spinosa* leaf extracts were treated from the upper side of the epidermis model for 30 min (final 1% concentration of extracts). A PM2.5 suspension (100  $\mu$ g/mL) was added onto the same side as the extract treatment, and then the epidermis was cultured for 24 hr. Treatment with PM2.5 increased TBARS levels in the epidermis, indicating the induction of lipid peroxidation by PM2.5 (Fig. 3A). Pretreatment with both extracts significantly suppressed TBARS contents (Fig. 3A), showing that the extracts have strong antioxidative capacity in the epidermis. Exposure to PM2.5 elicited IL-8 release from the epidermis (Fig. 3B), and the conditioned media from PM2.5-treated cultured epidermis potentiated neutrophil chemotaxis (Fig. 3C). Pretreatment with leaf extracts of *Camellia sinensis* and *Argania*

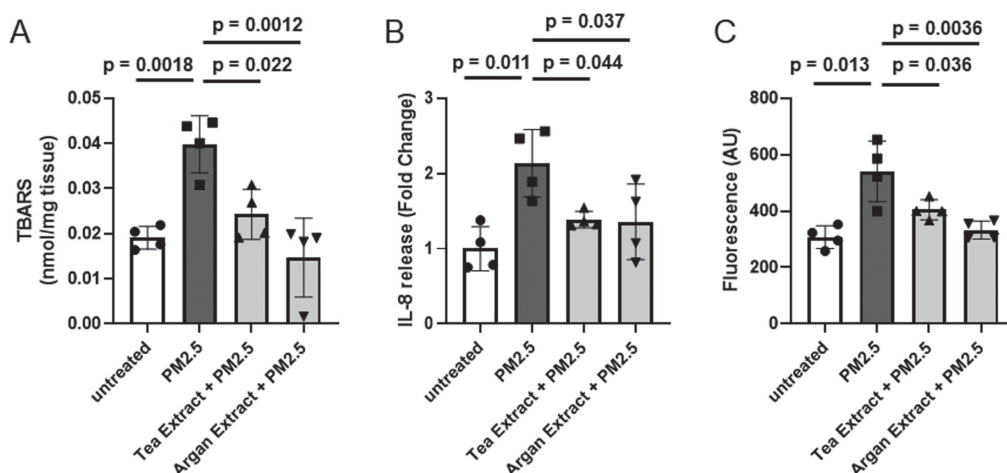


**Fig. 2.** Toxicity assay of *Camellia sinensis* and *Argania spinosa* extracts for human 3D cultured epidermis. The human epidermis model was pretreated with 1% *Camellia sinensis* and *Argania spinosa* extracts (labeled as Tea Extract and Argan Extract, respectively) for 48 hr. Cell viability was measured by Cell Counting Kit-8. The values are presented as the mean  $\pm$  S.D. ( $n = 4$ ).

*spinosa* significantly attenuated IL-8 release and neutrophil migration induced by PM2.5 (Fig. 3B and C). The suppressive effects were almost the same for both extracts (Fig. 3A, B and C). Collectively, leaf extracts from *Camellia*

*sinensis* and *Argania spinosa* suppressed oxidative stress, chemokine induction and subsequent neutrophil activation induced by PM2.5 exposure in human 3D epidermis culture.

Tea extract is believed to be effective against various pathological conditions based on evidence using cultured cells and experimental animals or from human epidemiological studies. Xiang *et al.* (2016) reported the suppressive effects of *Camellia sinensis* on breast cancer. *Camellia sinensis* extract was reported to attenuate cardiac remodeling accompanied by acute doxorubicin toxicity (Modesto *et al.*, 2021) and suppress brain ischemic injury induced by middle cerebral artery occlusion (Machin *et al.*, 2021). In the field of cosmetology, *Camellia sinensis* extract protects hair from damage induced by ultraviolet irradiation (Davis *et al.*, 2022). Among many components in *Camellia sinensis*, one of the major polyphenol compounds, green tea catechins, shows strong antioxidative and anti-inflammatory actions (Braicu *et al.*, 2013), suggesting that catechins are effective components to suppress PM2.5-induced epidermal injury. In addition, the effects of tea polysaccharides have recently been under debate. Tea polysaccharides are a group of heteropolysaccharides bound to proteins. There is evidence suggesting that tea polysaccharides not only improve immunity but also have various bioactivities, such as antioxidant, antitumor, anti-hyperglycemia, and anti-inflammation activities (Du *et al.*, 2016). Scientific evidence about the protective action of *Argania spinosa* extract is less than that of *Camellia*



**Fig. 3.** Protective effects of *Camellia sinensis* and *Argania spinosa* extracts on epidermal damage induced by PM2.5 exposure. The human epidermis model was pretreated with 1% *Camellia sinensis* and *Argania spinosa* extracts (labeled as Tea Extract and Argan Extract, respectively) for 30 min and then exposed to 100  $\mu$ g/mL PM2.5 collected in Yokohama, Japan, in winter 2018 for 24 hr. (A) Tissue was collected, and TBARS levels were measured. (B) The culture medium was collected, and the IL-8 concentration was determined by ELISA. (C) Human neutrophils in the transwell plate were treated with the conditioned media for 1 hr to evaluate their migration. The values are presented as the mean  $\pm$  S.D. ( $n = 4$ ). The data were analyzed using Student's *t* test and Dunnett's test. Holm's correction was performed for multiple comparisons.



sinensis extract, but several studies suggest that *Argania spinosa* extract can prevent oxidative stress and inflammation (Bejaoui *et al.*, 2021) and that these antioxidative and anti-inflammatory effects could depend on polyphenolic compounds included in *Argania spinosa* (Kamal *et al.*, 2019; Berrougui *et al.*, 2006). Further study is needed to uncover which component in the extracts is protective against epidermal damage induced by PM2.5 exposure.

PM2.5 contains polycyclic aromatic hydrocarbons (PAHs) and PAH quinones. Quinones are electrophilic compounds and thus easily react with nucleophilic compounds or nucleophilic groups such as thiols in proteins. Quinones also generate superoxide anion radicals via redox cycling (Ishihara *et al.*, 2006). In addition, one of the representative PAHs, benzo[a]pyrene, does not have electrophilicity but can be converted to an electrophile by metabolic activation (Boysen and Hecht, 2003). *Camellia sinensis* and *Argania spinosa* extracts contain many kinds of polyphenols, and phenolic compounds are highly nucleophilic and react with the above electrophilic substances. Among them, a compound having a catechol structure is considered to have high radical scavenging activity because it is converted into a stable o-quinone form when oxidized. In this regard, the protective effects of leaf extracts from *Camellia sinensis* and *Argania spinosa* on epidermal damage caused by PM2.5 might be related to the removal of harmful electrophilic substances by polyphenols.

In conclusion, leaf extracts from *Camellia sinensis* and *Argania spinosa* protect the epidermis from PM2.5 exposure-induced oxidative stress and subsequent chemokine release. These extracts are considered to be safe in the present practices of use and concentration, although continuous safety evaluation is needed (Becker *et al.*, 2019; Leeyaphan *et al.*, 2022). Therefore, *Camellia sinensis* and *Argania spinosa* extracts could be added to a novel cosmetic that protects skin from air pollution.

## ACKNOWLEDGMENTS

The authors thank Dr. Miki Tanaka and Ms. Kaede Namba for technical assistance during the course of this study. This work was supported by KAKENHI grant from the Japan Society for the Promotion of Science (grant number 20H04341 to YI) and the Environment Research and Technology Development Funds of the Environmental Restoration and Conservation Agency of Japan (JPMEERF20165051 to TO and JPMEERF20205007 to YI).

**Conflict of interest----** The authors declare that there is no conflict of interest.

## REFERENCES

- Becker, L.C., Bergfeld, W.F., Belsito, D.V., *et al.* (2019): Safety Assessment of *Camellia sinensis*-Derived Ingredients As Used in Cosmetics. *Int. J. Toxicol.*, **38**, 48S-70S.
- Bejaoui, M., Taarji, N., Saito, M., Nakajima, M. and Isoda, H. (2021): Argan (*Argania spinosa*) press cake extract enhances cell proliferation and prevents oxidative stress and inflammation of human dermal papilla cells. *J. Dermatol. Sci.*, **103**, 33-40.
- Bell, M.L., Dominici, F., Ebisu, K., Zeger, S.L. and Samet, J.M. (2007): Spatial and temporal variation in PM(2.5) chemical composition in the United States for health effects studies. *Environ. Health Perspect.*, **115**, 989-995.
- Berrougui, H., Cloutier, M., Isabelle, M. and Khalil, A. (2006): Phenolic-extract from argan oil (*Argania spinosa* L.) inhibits human low-density lipoprotein (LDL) oxidation and enhances cholesterol efflux from human THP-1 macrophages. *Atherosclerosis*, **184**, 389-396.
- Boysen, G. and Hecht, S.S. (2003): Analysis of DNA and protein adducts of benzo[a]pyrene in human tissues using structure-specific methods. *Mutat. Res.*, **543**, 17-30.
- Braicu, C., Ladamery, M.R., Chedea, V.S., Irimie, A. and Berindan-Neagoe, I. (2013): The relationship between the structure and biological actions of green tea catechins. *Food Chem.*, **141**, 3282-3289.
- Davis, S.L., Marsh, J.M., Kelly, C.P., *et al.* (2022): Protection of hair from damage induced by ultraviolet irradiation using tea (*Camellia sinensis*) extracts. *J. Cosmet. Dermatol.*, **21**, 2246-2254.
- Dockery, D.W., Pope, C.A. 3rd, Xu, X., *et al.* (1993): An association between air pollution and mortality in six U.S. cities. *N. Engl. J. Med.*, **329**, 1753-1759.
- Dong, L., Hu, R., Yang, D., *et al.* (2020): Fine Particulate Matter (PM2.5) upregulates expression of Inflammasome NLRP1 via ROS/NF-kappaB signaling in HaCaT Cells. *Int. J. Med. Sci.*, **17**, 2200-2206.
- Du, L.L., Fu, Q.Y., Xiang, L.P., *et al.* (2016): Tea Polysaccharides and Their Bioactivities. *Molecules*, **21**.
- Ishihara, N., Okuda, T., Hagino, H., *et al.* (2022): Involvement of polycyclic aromatic hydrocarbons and endotoxin in macrophage expression of interleukin-33 induced by exposure to particulate matter. *J. Toxicol. Sci.*, **47**, 201-210.
- Ishihara, Y., Kawami, T., Ishida, A. and Yamazaki, T. (2012): Tributyltin induces oxidative stress and neuronal injury by inhibiting glutathione S-transferase in rat organotypic hippocampal slice cultures. *Neurochem. Int.*, **60**, 782-790.
- Ishihara, Y., Shiba, D. and Shimamoto, N. (2006): Enhancement of DMNQ-induced hepatocyte toxicity by cytochrome P450 inhibition. *Toxicol. Appl. Pharmacol.*, **214**, 109-117.
- Kamal, R., Kharbach, M., Vander Heyden, Y., *et al.* (2019): In vivo anti-inflammatory response and bioactive compounds' profile of polyphenolic extracts from edible Argan oil (*Argania spinosa* L.), obtained by two extraction methods. *J. Food Biochem.*, **43**, e13066.
- Kono, M., Okuda, T., Ishihara, N., Hagino, H., Tani, Y., Okochi, H., Tokoro, C., Takaishi, M., Ikeda, H. and Ishihara, Y. (2022): Chemokine expression in human 3-dimensional cultured epidermis exposed to PM2.5 collected by cyclonic separation. *Toxicol. Res.* (in press).

- Leeyaphan, C., Varothai, S., Trakanwittayarak, S., *et al.* (2022): A randomized controlled trial to compare the effectiveness and safety of adsorbent lotion containing tapioca starch, spent grain wax, *Butyrospermum parkii* extract, *argania spinosa* kernel oil, aloe barbadensis, rosehip oil, and allantoin with a low-potency topical corticosteroid in the treatment of intertrigo. *J. Cosmet. Dermatol.*, **21**, 679-688.
- Machin, A., Syaharani, R., Susilo, I., Hamdan, M., Fauziah, D. and Purwanto, D.A. (2021): The effect of *Camellia sinensis* (green tea) with its active compound EGCG on neuronal cell necroptosis in *Rattus norvegicus* middle cerebral artery occlusion (MCAO) model. *J. Basic Clin. Physiol. Pharmacol.*, **32**, 527-531.
- Modesto, P.N., Polegato, B.F., Dos Santos, P.P., *et al.* (2021): Green Tea (*Camellia sinensis*) Extract Increased Topoisomerase II $\beta$ , Improved Antioxidant Defense, and Attenuated Cardiac Remodeling in an Acute Doxorubicin Toxicity Model. *Oxid. Med. Cell. Longev.*, **2021**, 8898919.
- Okuda, T., Isobe, R., Nagai, Y., Okahisa, S., Funato, K. and Inoue, K. (2015): Development of a high-volume PM<sub>2.5</sub> particle sampler using impactor and cyclone techniques. *Aerosol Air Qual. Res.*, **15**, 759-767.
- Park, T.H., Park, S., Cho, M.K. and Kim, S. (2022): Associations of particulate matter with atopic dermatitis and chronic inflammatory skin diseases in South Korea. *Clin. Exp. Dermatol.*, **47**, 325-334.
- Piao, M.J., Ahn, M.J., Kang, K.A., *et al.* (2018): Particulate matter 2.5 damages skin cells by inducing oxidative stress, subcellular organelle dysfunction, and apoptosis. *Arch. Toxicol.*, **92**, 2077-2091.
- Xiang, L.P., Wang, A., Ye, J.H., *et al.* (2016): Suppressive Effects of Tea Catechins on Breast Cancer. *Nutrients*, **8**.
- Zheng, Z., Li, J., Chen, Y., Sun, H. and Liu, Y. (2022): Preparation and characterization of lipophilic antioxidative peptides derived from mung bean protein. *Food Chem.*, **395**, 133535.