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Chlorogenic Acid, a Component of *Oenanthe javanica* (Blume) DC., Attenuates Oxidative Damage and Prostaglandin E_2 Production Due to Particulate Matter 10 in HaCaT Keratinocytes

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Abstract: *Oenanthe javanica* (OJ) is a perennial herb that grows wildly or is cultivated in Asia, and it is used as food or in traditional medicine. The antioxidant and anti-inflammatory effects of OJ-derived materials have been extensively explored previously, but their effects on the cytotoxicity of air pollution are currently unknown. Therefore, the present study aimed to evaluate the effect of the hot water extract of OJ on atmospheric particulate matter 10 (PM_{10})-induced cytotoxicity and oxidative damage in human HaCaT keratinocytes, and to identify its active ingredient and mechanism of action. When the hot water extract of OJ was divided into methylene chloride, ethyl acetate (EA), n-butanol (BA), and water fractions, caffeic acid was enriched in the EA fraction and chlorogenic acid was enriched in the BA fraction. PM_{10} increased reactive oxygen species (ROS) production, lipid peroxidation, protein carbonylation, and inflammatory prostaglandin (PG) E_2 production in cells. The BA fraction reduced the PM_{10}-induced ROS production in cells more effectively than the total extract and other solvent fractions. Chlorogenic acid was more effective in reducing ROS levels than caffeic acid and N-acetyl cysteine (NAC). Chlorogenic acid attenuated the increase in lipid peroxidation and the PG E_2 production of cells due to PM_{10} exposure. Of the genes involved in PG E_2 production, phospholipase A2 group IVA (*PLA2G4A*), Prostaglandin-endoperoxide synthase 1 (*PTGS1*), and 2 (*PTGS2*) were transcriptionally up-regulated by PM_{10}, whereas phospholipase A2 group IIA (*PLA2G2A*) was down-regulated and prostaglandin E synthetase 1 (*PTGES1*) and 2 (*PTGES2*) were a little altered. The PM_{10}-induced increase in *PLA2G4A* mRNA was alleviated by chlorogenic acid and NAC. Accordingly, PM_{10} increased the expression levels of cytosolic phospholipase A2 (cPLA2) protein and its phosphorylated form, which were attenuated by chlorogenic acid and NAC. Thus, chlorogenic acid may attenuate the PM_{10}-induced PG E_2 production through the suppression of *PLA2G4A* mRNA and cPLA2 protein expressions. This study suggests that chlorogenic acid contained in OJ extract may help alleviate the oxidative damage to and inflammatory responses of the skin cells due to exposure to air pollutants.

Keywords: *Oenanthe javanica* (Blume) DC.; water parsley; water dropwort; chlorogenic acid; particulate matter; keratinocytes; lipid peroxidation; protein carbonylation; prostaglandin E_2; phospholipase A2; cPLA2

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1. Introduction

Air pollution has a detrimental effect on skin health. The particulate matter (PM) that is suspended in the atmosphere with an approximate diameter of less than 10 and 2.5 μm

is called PM_{10} and $PM_{2.5}$, respectively [1]. PM is a mixture of various organic compounds, heavy metals, and biological constituents [1,2]. PM is capable of penetrating the skin through pores and weak skin barriers [3,4]. PM can exacerbate various skin diseases, such as atopic dermatitis, acne, and psoriasis [5,6]. PM can also cause premature skin aging [7] and hyperpigmentation [8]. Combined exposure to PM and ultraviolet (UV) rays exerts a synergistic harmful effect, accelerating skin photo-aging and cancer development [9,10].

PM exposure stimulates Ca^{2+} signaling and increases the production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), which mediate the oxidative damage to and inflammatory responses of cells [11,12]. ROS can be generated in the aryl hydrocarbon receptor-mediated metabolism of organic components of PM and the chemical reactions catalyzed by its transition metal components [13,14]. PM induces the activation of the NADPH oxidase family, including dual oxidase 2, which plays a critical role in the production of ROS and the maintenance of redox balance in cells [12,15].

PM-derived ROS can cause the activation of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase, and the stimulation of the nuclear factor-kappa B (NF-κB) signaling pathway, leading to the activation of redox-sensitive transcription factors [16]. This increases the gene expression and secretion of the inflammatory eicosanoid mediators, such as prostaglandin (PG) E_2 [17,18], and the inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 [19]. PM stimulates the expression of matrix metalloproteinases (MMPs), causing the loss of the extracellular matrix, including collagen [20], and decreases the expression of filaggrin, causing the loss of the skin barrier function [21,22]. Thus, there is a need for antioxidants that alleviate the adverse effects of PM.

Plant-derived phenolic compounds may function as antioxidants in cells that alleviate oxidative stress due to PM_{10} [23]. Phenolic antioxidants can prevent ROS production, scavenge exiting ROS, or enhance the antioxidant capacity of cells by regulating gene expression [24]. We have shown that extracts derived from various plants, such as green tea, pomegranate, Siegesbeckiae herba, propolis, and Ecklonia cava, and their phenolic compounds, such as (−)-epigallocatechin-3-gallate, punicalagin, chlorogenic acid, ferulic acid, and dieckol, reduce ROS production, lipid peroxidation, and inflammatory responses in HaCaT cells exposed to PM_{10} [18,19,25,26].

Oenanthe javanica (Blume) DC, abbreviated to OJ herein, is generally called water dropwort, water parsley, water celery, or 'minari' in Korean. It is a dicotyledonous perennial plant that grows in wetlands or watersides and is cultivated for food or medicinal purposes [27,28]. OJ contains a substantial amount of phenolic compounds, with chlorogenic acid being in the highest concentration, followed by caffeic acid [29–31]. The extract of OJ alleviates the hepatic steatosis and oxidative damage induced by ethanol poisoning [32] via acting as an antioxidant with free radical scavenging capabilities and reducing power [29], enhancing cellular antioxidant enzymes, such as superoxide dismutase (SOD)-1, SOD-2, catalase, and glutathione peroxidase [33], or accelerating the metabolic removal of ethanol [34]. Its anti-inflammatory effects have also been demonstrated in RAW 264.7 macrophages, which were stimulated by lipopolysaccharide [35,36] in the skin of mice irradiated with UV rays [37], and in mice with sodium dextran sulfate-induced colitis [31].

Although the antioxidant and anti-inflammatory effects of OJ-derived materials have been explored in various experimental models, no previous studies have reported their protective effects against the cytotoxicity of air pollution. Therefore, the present study aimed to evaluate the effect of the hot water extract of OJ on PM_{10}-induced cytotoxicity and oxidative damage in human HaCaT keratinocytes, and to identify its active ingredient and mechanism of action. The results of this study demonstrated that chlorogenic acid, a main component of OJ, attenuated ROS production, lipid peroxidation, and protein carbonylation in PM_{10}-stimulated cells. It also attenuated PM_{10}-induced PG E_2 production in cells, which was associated with the suppressed expression of cytosolic phospholipase

A2 (cPLA2). Thus, the chlorogenic acid contained in OJ extract is suggested to help alleviate the oxidative damage to and inflammatory responses of the skin cells caused by exposure to air pollutants.

2. Materials and Methods

2.1. Reagents

Chlorogenic acid, caffeic acid, standardized fine dust (PM_{10}-like, European standard ERM-CZ120), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2-thiobarbituric acid, and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of the Hot Water Extract and Fractions of OJ

Dried Leaves and stems of OJ *(Oenanthe javanica* (Blume) DC.) purchased from Cheongmyeongyakcho (Chungbuk, Republic of Korea) (http://www.good1075.com, accessed on 31 March 2023) were used for extraction. The plant material (100 g) was extracted with water (600 mL) at 90 °C for 2 h. After filtering, the filtrate was concentrated to dryness using a rotary evaporator (Eyela, Bohemia, NY, USA) under reduced pressure, and the total extract of OJ (3.702 g) was obtained. The total extract was suspended in water (100 mL) and transferred to a separating funnel where the aqueous suspension was partitioned with an equal volume of methylene chloride (MC), ethyl acetate (EA), and n-butyl alcohol (BA), sequentially [34]. The evaporation of each solvent fraction under reduced pressure yielded an MC fraction (0.168 g), EA fraction (0.181 g), BA fraction (0.555 g), water (WT) fraction (13.781 g), and insoluble materials (8.096 g). Each fraction was re-dissolved in 30% (v/v) aqueous ethanol at a 10% concentration and kept at −20 °C until use.

2.3. High-Performance Liquid Chromatography with Photodiode Array Detection (HPLC-DAD)

The HPLC-DAD profiles of the total extract of OJ and its solvent fractions were compared using a Waters Alliance HPLC system (Waters, Milford, MA, USA) equipped with an e2695 separation module and a 2996 photodiode array detector [30]. Chromatographic separation was performed using a Hector-M C_{18} column (4.6 mm × 250 mm, 5 μm) (RS Tech Co. Daejeon, Republic of Korea) as the stationary phase, and a mixture of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B) as the mobile phase. The mobile phase composition was changed as follows: 0–30 min, a linear gradient from 0 to 100% B; 30–40 min, 100% B; 40–45 min, a linear gradient from 100 to 0% B. The flow rate of the mobile phase was set at 0.6 mL min^{-1}. Samples were diluted with water, filtered, and injected into HPLC in 10 μL aliquots.

2.4. Cell Culture and Treatments

An immortalized human keratinocyte HaCaT cell line (CLS Cell Lines Service GmbH, Eppelheim, Germany) was cultured as previously described [26]. The growth medium consisted of DMEM/F-12 medium (GIBCO-BRL, Grand Island, NY, USA), 10% fetal bovine serum, antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B), and 10 μg mL^{-1} of hydrocortisone. In each experiment, cells were cultured in 96-well, 12-well, or 6-well culture plates (SPL Life Sciences, Pocheon, Republic of Korea) for 24 h and then subjected to various treatments. PM_{10} was suspended in phosphate-buffered saline (PBS) at 100 times the final treatment concentrations. Other test materials were diluted or dissolved in 30% (v/v) aqueous ethanol at 100 times the final treatment concentrations. Cells were treated with the total extract of OJ, its fractions, compounds, and PM_{10} individually, in combination with each other at the specified concentrations, or with vehicles for the indicated time.

2.5. Cell Viability Assay

Cell viability was assessed by measuring the reduction of MTT to water-insoluble formazan in the viable cells [38,39]. Cells were seeded on 96-well culture plates (4×10^3 cells/well) and cultured in a growth medium (200 µL) for 24 h. After various treatments with the total extract of OJ, its fractions, compounds, and PM_{10} for 48 h, the cells were washed with PBS and incubated in 100 µL of growth medium containing 1 mg mL^{-1} of MTT at 37 °C for 2 h. After discarding the medium, cells were washed with PBS, and the formazan dye that had accumulated inside the cells was extracted with 100 µL of dimethyl sulfoxide per well. Then, the absorbance was measured at 570 nm using a Spectrostar Nano microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

2.6. Cellular Reactive Oxygen Species (ROS) Production Assay

Cellular ROS production was assessed by measuring the oxidation of DCFH-DA to a fluorescent compound inside the cells [40]. The cells were seeded on 12-well culture plates (1.4×10^5 cells/well) and cultured in a growth medium (1 mL) for 24 h. Cells were treated with 10 µM DCFH-DA for 30 min. The spent medium was replaced with the growth medium containing various test materials, which was followed by the incubation of cells for 60 min. Cells were then washed with PBS and the fluorescing cells were observed under a LEICA DMI3000 B microscope (Leica Microsystems GmbH, Wetzlar, Germany). For a quantitative analysis of the fluorescing compound formed inside the cells, the washed cells were extracted with 150 µL of the cell lysis buffer (1% sodium dodecyl sulfate, 20 mM of Tris-Cl, 2.5 mM of EDTA, pH of 7.5), followed by centrifugation at 14,500× *g* for 15 min. After 200 µL aliquots of the supernatants were transferred to black 96-well plates (SPL Life Sciences, Pocheon, Republic of Korea), the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm with a Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Cellular Lipid Peroxidation Assay

Cellular lipid peroxidation was assessed by measuring the production of malondialdehyde (MDA) [41]. Cells were seeded on 6-well culture plates (2×10^5 cells per well) and cultured in the growth medium (2 mL) for 24 h. Cells were then treated with various test materials, such as the total extract of OJ, its fractions, compounds, and $PM_{10,}$ for 48 h. Then, cells were washed with PBS and harvested using 150 µL of the cell lysis buffer (1% sodium dodecyl sulfate, 20 mM of Tris-Cl, 2.5 mM of EDTA, pH of 7.5). The reaction mixture (500 µL) consisting of 100 µL of cell lysate (ca. 200 µg protein), 50 µL of 1.0% *m*-phosphoric acid, and 350 µL of 0.9% 2-thiobarbituric acid was heated at 95 °C in a water bath for 45 min. The reaction was also run with 100 to 400 nM of TMP as a donor of MDA to construct a standard curve. After the reaction mixture was cooled to room temperature, 500 µL of BA was added to the reaction mixture, mixed vigorously, and centrifuged at 14,500× *g* for 15 min to separate the mixture into a BA layer and an aqueous layer. After 200 µL of the BA layer was transferred to black 96-well plates, the fluorescence intensity was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a Gemini EM fluorescence microplate reader. Data are presented as MDA levels normalized to protein content.

2.8. Protein Carbonylation Assay

Protein carbonylation was measured using a fluorometric assay kit (ab235631) from Abcam (Cambridge, MA, USA). The whole cell lysate (75 µg of protein in 50 µL) was mixed with 50 µL of 0.2 mM fluorescein-5-thiosemicarbazide (FTC) fluorophore in an assay buffer in microcentrifuge tubes, followed by incubation overnight at 25 °C in the dark. Then, proteins were precipitated by adding 200 µL of ice-cold 20% trichloroacetic acid solution, keeping the tubes on ice for 10 min. The tubes were centrifuged at 14,500× *g* for 15 min and the supernatant was removed by suction aspiration. The pellet was washed with 200 µL of ice-cold isopropanol 3 times and then dissolved in 50 µL of 6 M guanidine solution by

heating it at 50 °C for 1 h. The tubes were cooled to 50 °C and the samples were diluted with 70 µL of sample dilution buffer. Aliquots of the diluted samples (100 µL) were transferred to a 96-well plate and the fluorescence was measured at a 485 nm excitation and at a 535 nm emission using a Gemini EM fluorescence microplate reader. Protein carbonyl contents were estimated by comparing a standard curve that was prepared using FTC fluorophore. The protein carbonyl contents were normalized to the protein content.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA) for PG E_2

The secreted level of PG E_2 protein in the culture medium was measured using a PG E_2 ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA). In this colorimetric competitive enzyme immunoassay, a fixed amount of PG E_2-phosphatase conjugate is used as a PG E_2 tracer, whose binding to the PG E_2 monoclonal antibody is inversely proportional to the amount of PG E_2 derived from the sample. Cells were seeded on 6-well culture plates (8×10^4 cells per well) and cultured in a growth medium (1 mL) for 24 h. Cells were then treated with various test materials for another 48 h. The conditioned culture medium was centrifuged at 14,500× *g* for 15 min, and the supernatants were used as samples in the assay. Briefly, 100 µL samples of the culture medium or a standard PG E_2 solution were transferred to microtiter plates coated with a goat antibody specific to mouse IgG. To each well, 50 µL of assay buffer, 50 µL of PG E_2 tracer solution, and 50 µL of PG E_2 monoclonal antibody solution were added, and the mixtures were incubated at 25 °C for 2 h. The well was rinsed 3 times with wash buffer and 200 µL of p-nitrophenyl phosphate substrate solution was added to initiate the phosphatase reaction. After 45 min of incubation at 25 °C, absorbances were measured at 405 nm with a SPECTROstar Nano microplate reader (BMG LABTECH GmbH). The amount of PG E_2 in the samples was estimated from a standard curve.

2.10. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

The mRNA level was analyzed by qRT-PCR, as described in a previous study [18]. Cells were treated with various test materials for 24 h and the total cellular RNA from the treated cells was used in the preparation of complementary DNA. The qRT-PCR was run using the gene-specific primers, whose nucleotide sequences are shown in Table 1. The mRNA level of each target gene was compared to that of the internal reference, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), using the comparative Ct method [42]. Data are presented as a percentage of the control group.

Table 1. Forward (F) and reverse (R) primers for the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Gene Name	GenBank Accession Number	Primer Sequences	Ref.
Phospholipase A2 group IIA (*PLA2G2A*)	NM_000300.4	F: 5′-GAAAGGAAGCCGCACTCAGTT-3′ R: 5′- CAGACGTTTGTAGCAACAGTCA-3′	[43]
Phospholipase A2 group IVA (*PLA2G4A*)	NM_024420.3	F: 5′-GACGTGCTGGGAAGGTACAC-3′ R: 5′-AGCCCACTGTCCACTACA-3′	[44]
Prostaglandin-endoperoxide synthase 1 (*PTGS1*)	NM_000962.4	F: 5′-CAGAGCCAGATGGCTGTGGG-3′ R: 5′-AAGCTGCTCATCGCCCCAGG-3′	[45]
Prostaglandin-endoperoxide synthase 2 (*PTGS2*)	NM_000963.3	F: 5′-CTGCGCCTTTTCAAGGATGG-3′ R: 5′-CCCCACAGCAAACCGTAGAT-3′	[46]
Prostaglandin E synthase 1 (*PTGES1*)	NM_004878.5	F: 5′-AACCCTTTTGTCGCCTG-3′ R: 5′-GTAGGCCACGGTGTGT-3′	[47]
Prostaglandin E synthase 2 (*PTGES2*)	NM_025072.7	F: 5′-GAAAGCTCGCAACAACTAAAT-3′ R: 5′-CTTCATGGCTGGGTAGTAG-3′	[47]
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	NM_002046.3	F: 5′-ATGGGGAAGGTGAAGGTCG-3′ R: 5′-GGGGTCATTGATGGCAACAA-3′	[41]

2.11. Western Blotting

The primary antibodies for cPLA2 (#2832) and phospho-cPLA2 (Ser505) (#2831) were purchased from Cell Signaling Technology (Danvers, MA, USA), and β-actin (#47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit IgG (#7074) secondary antibody was purchased from Cell Signaling Technology. Protein samples were denatured by mixing them with a Laemmli sample buffer and heating them at 95 °C for 5 min. Equal amounts of proteins (20 μg) were resolved using 10% SDS-polyacrylamide gel electrophoresis at 80 V and were then electrically transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia, Little Chalfont, UK) at 4 °C overnight. After blocking the incubation of the membrane with TBST (137 mM of sodium chloride, 20 mM of Tris, 0.1% Tween 20, pH of 7.6.) containing 5% skim milk, it was incubated with the primary antibody in TBST containing 5% skim milk at 4 °C overnight; this was followed by incubation with the secondary antibody in TBST containing 5% skim milk at room temperature (25 °C) for 1 h. The target protein bands were visualized with a chemiluminescence method using the picoEPD Western Reagent kit (ELPIS-Biotech, Daejeon, Republic of Korea). The captured blot images were analyzed using the Image J program provided by the U.S. National Institute of Health (Bethesda, MD, USA).

2.12. Statistical Analysis

Experimental data were analyzed using SigmaStat v.3.11 software (Systat Software Inc., San Jose, CA, USA) and presented as mean ± standard deviation (SD). The presence of group means that were significantly different from other groups was determined using a one-way analysis of variance (ANOVA) at the $p < 0.05$ level. Then, Duncan's multiple range test was subsequently run to compare all groups to each other.

3. Results

The dried leaves and stems of OJ were used in the preparation of the total hot water extract of OJ and its MC, EA, BA, and WT fractions. HPLC-DAD analysis was performed to compare the overall composition of the total OJ extract and its solvent fractions. Chlorogenic acid and caffeic acid were used as the standards, as these compounds are known to be the main components of OJ [29–31]. As shown in Figure 1, two of the main peaks observed in the total OJ extract and its solvent fractions were identified as chlorogenic acid and caffeic acid by comparing their retention times and absorption spectra with those of the standards. The total OJ extract contained a substantial level of chlorogenic acid, while its caffeic acid content was relatively lower. Chlorogenic acid was highly enriched in the BA fraction and caffeic acid was enriched in the EA fraction, and their contents were relatively lower in the MC fraction and the WT fraction.

As shown in Figure 2, in quantities up to 300 μg mL^{-1}, PM_{10} decreased the viability of HaCaT cells in a concentration-dependent manner. The effects of the total OJ extract and each fraction on cell viability were evaluated at 10–1000 μg mL^{-1} in the absence and presence of 200 μg mL^{-1} PM_{10}. The total extract of OJ significantly reduced cell viability at concentrations above 300 μg mL^{-1} in the absence of PM_{10}, but had no additional effect on cell viability in the presence of PM_{10}. None of the solvent fractions showed significant cytotoxicity up to 100 μg mL^{-1}, but they did show reduced cell viability at 300 μg mL^{-1} in the order of the EA fraction, MC fraction, BA fraction, and WT fraction. In the presence of 200 μg mL^{-1} of PM_{10}, the water fraction had no additional effect on cell viability, but the other fractions enhanced the cytotoxic effects of PM_{10} at their high concentrations (300 or 1000 μg mL^{-1}). In the following experiments, cells were treated with the total OJ extract or each fraction at 30 and/or 100 μg mL^{-1} within non-cytotoxic ranges.

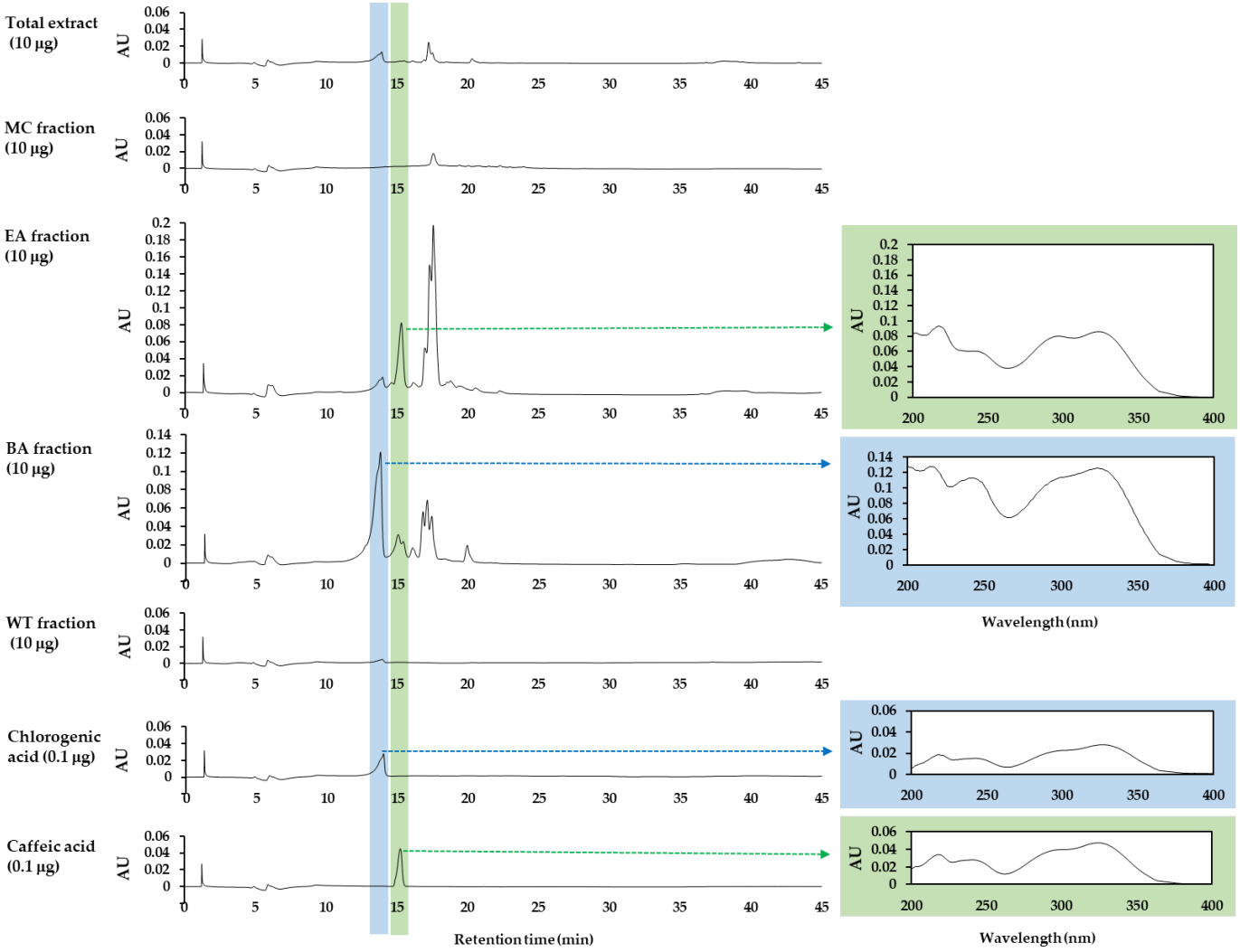

Figure 1. High-performance liquid chromatography with photodiode array detection (HPLC-DAD) analysis of the total extract *Oenanthe javanica* (Blume) DC, abbreviated OJ, and its MC, EA, BA, and WT fractions. Authentic chlorogenic acid and caffeic acid were used as the standards for comparing the retention times and absorption spectra. Chromatograms at 330 nm and UV absorption spectra of the indicated peaks are shown.

The effects of chlorogenic acid and caffeic acid, identified as major components of OJ, on cell viability were examined at 10–1000 μM in the absence and presence of 200 μg mL^{-1} of PM_{10}, and were compared with that of NAC, a positive control. As shown in Figure 3, caffeic acid decreased cell viability at concentrations above 100 μM, and chlorogenic acid and NAC decreased cell viability at concentrations above 300 μM. Chlorogenic acid and caffeic acid at 1000 μM enhanced the cytotoxic effects of PM_{10}, but NAC did not. In the following experiments, cells were treated with each of these compounds at 30 and/or 100 μM.

The effects of the total OJ extract, solvent fractions, and compounds on PM_{10}-induced ROS production were examined using a fluorescent probe. Cells were treated with the total extract and each fraction at 30 and 100 μg mL^{-1}, or with chlorogenic acid, caffeic acid, and NAC at 30 and 100 μM in the absence and presence of 200 μg mL^{-1} PM_{10}. As shown in Figure 4, PM_{10} at 100–300 μg mL^{-1} increased ROS production in a concentration-dependent manner. The total extract, MC fraction, EA fraction, BA fraction, and WT fraction at 30–100 μg mL^{-1} did not affect ROS production in the absence of PM_{10}. However, the BA fraction (30–100 μg mL^{-1}), EA fraction (30–100 μg mL^{-1}), MC fraction (30–100 μg mL^{-1}),

and WT fraction (100 μg mL^{-1}) attenuated the PM_{10}-induced ROS production, in that order. Chlorogenic acid, caffeic acid, and NAC at 30–100 μM did not affect the basal level of ROS production, but reduced the PM_{10}-induced ROS production, in that order. The images of cells fluorescing due to ROS confirmed that the basal ROS production was not affected by the total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM), but that the PM_{10}-induced ROS production was reduced by chlorogenic acid (100 μM), BA fraction (100 μg mL^{-1}), and NAC (100 μM), in that order.

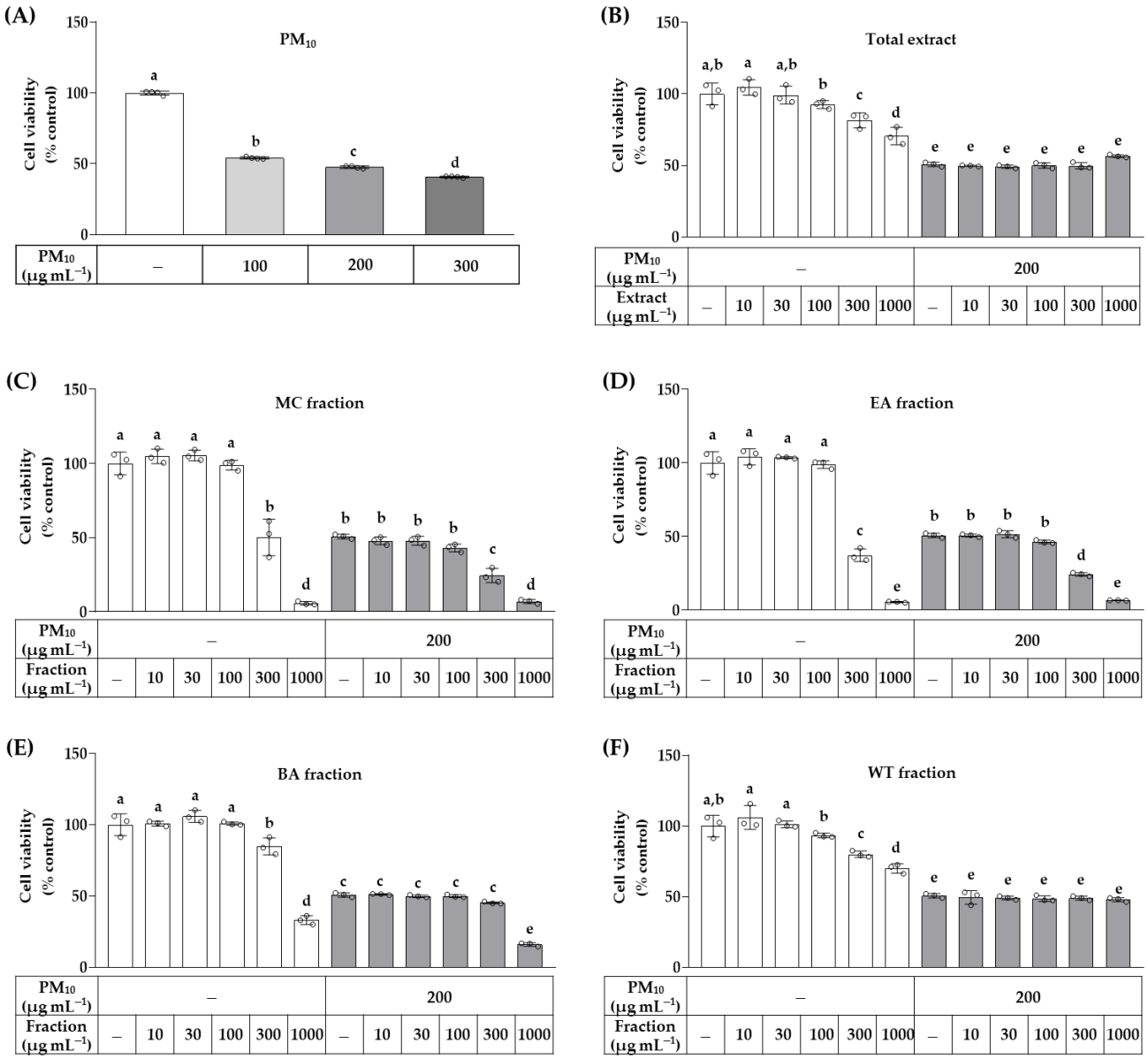

Figure 2. Effects of the total OJ extract and its solvent fractions on the viability of HaCaT keratinocytes exposed to particulate matter 10 (PM_{10}). In (**A**), cells were treated with PM_{10} at different concentrations for 48 h. In (**B**–**F**), cells were treated with the total OJ extract (**B**), MC fraction (**C**), EA fraction (**D**), BA fraction €, or WT fraction (**F**) at the specified concentrations, alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Data are presented as mean ± SD (n = 4 for A, n = 3 for B–F). Duncan's multiple range test was performed to compare all group means to each other. Groups that do not share the same lowercase alphabet letters (a–e) are considered to have significantly different means at the $p < 0.05$ level.

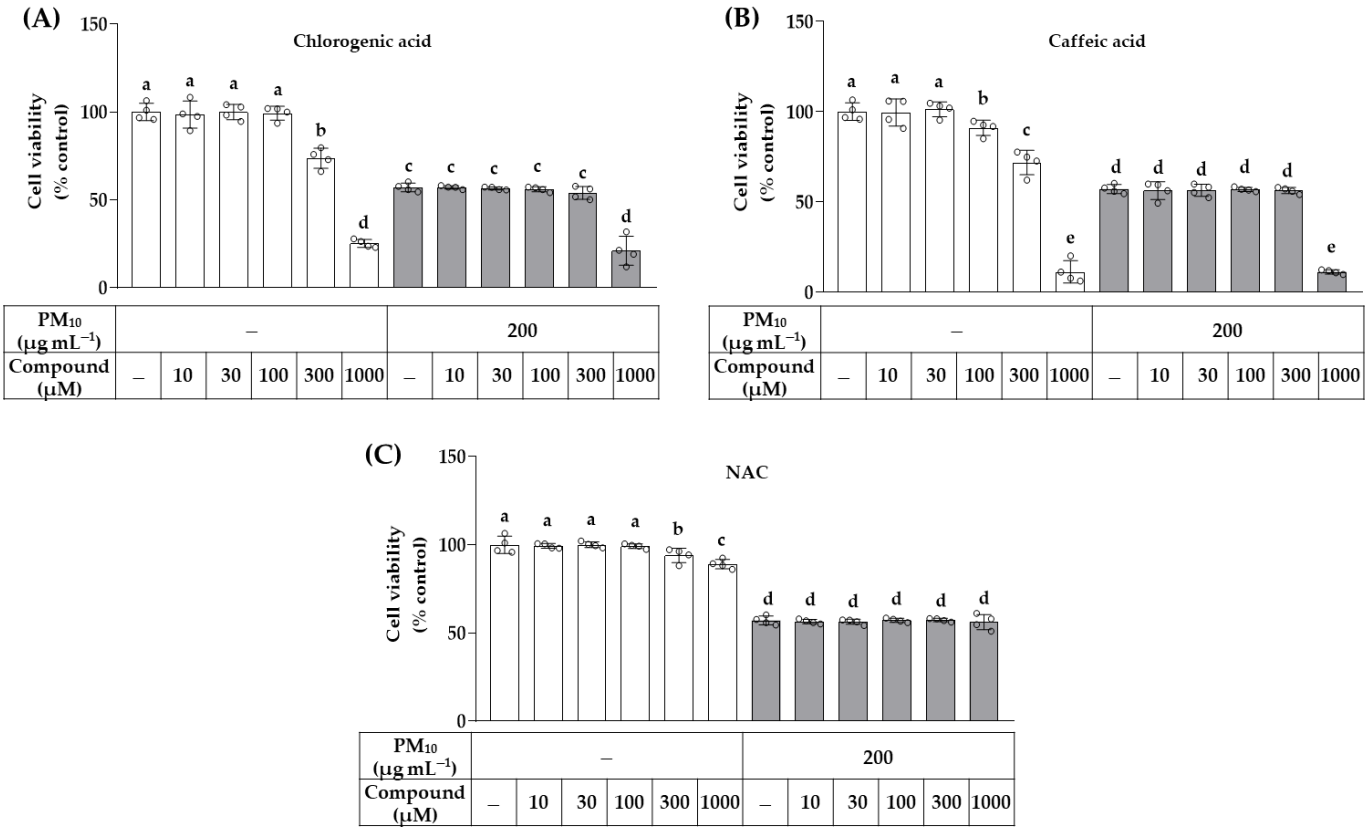

Figure 3. Effects of chlorogenic acid, caffeic acid, and N-acetyl cysteine (NAC) on the viability of HaCaT keratinocytes exposed to PM_{10}. Cells were treated with chlorogenic acid (**A**), caffeic acid (**B**), or NAC (**C**) at the specified concentrations, alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. Data are presented as mean ± SD (n = 3). Groups that do not share the same lowercase alphabet letters (a–e) are considered to have significantly different means at the $p < 0.05$ level.

The effects of the total OJ extract, BA fraction, chlorogenic acid, and NAC on PM_{10}-induced lipid peroxidation were examined by measuring MDA production. As shown in Figure 5, PM_{10} at 100–300 μg mL^{-1} increased lipid peroxidation in a concentration-dependent manner. The total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM) did not affect the basal level of lipid peroxidation in the absence of PM_{10}. The BA fraction (100 μg mL^{-1}) and chlorogenic acid (100 μM) suppressed the lipid peroxidation induced by PM_{10} (200 μg mL^{-1}), whereas the total OJ extract (100 μg mL^{-1}) and NAC (100 μM) had no significant effects. The increases in the MDA levels due to PM_{10} exposure were smaller in groups treated with the BA fraction (100 μg mL^{-1}) or chlorogenic acid (100 μM) compared to the control group.

The effects of the total OJ extract, BA fraction, chlorogenic acid, and NAC on protein carbonylation were examined in HaCaT cells exposed to PM_{10}. As shown in Figure 6, PM_{10} at 100–300 μg mL^{-1} increased the protein carbonyl content in a concentration-dependent manner. The total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM) slightly increased the protein carbonyl content of cells in the absence of PM_{10}, but attenuated an increase in the protein carbonyl contents due to the exposure of cells to PM_{10} (200 μg mL^{-1}).

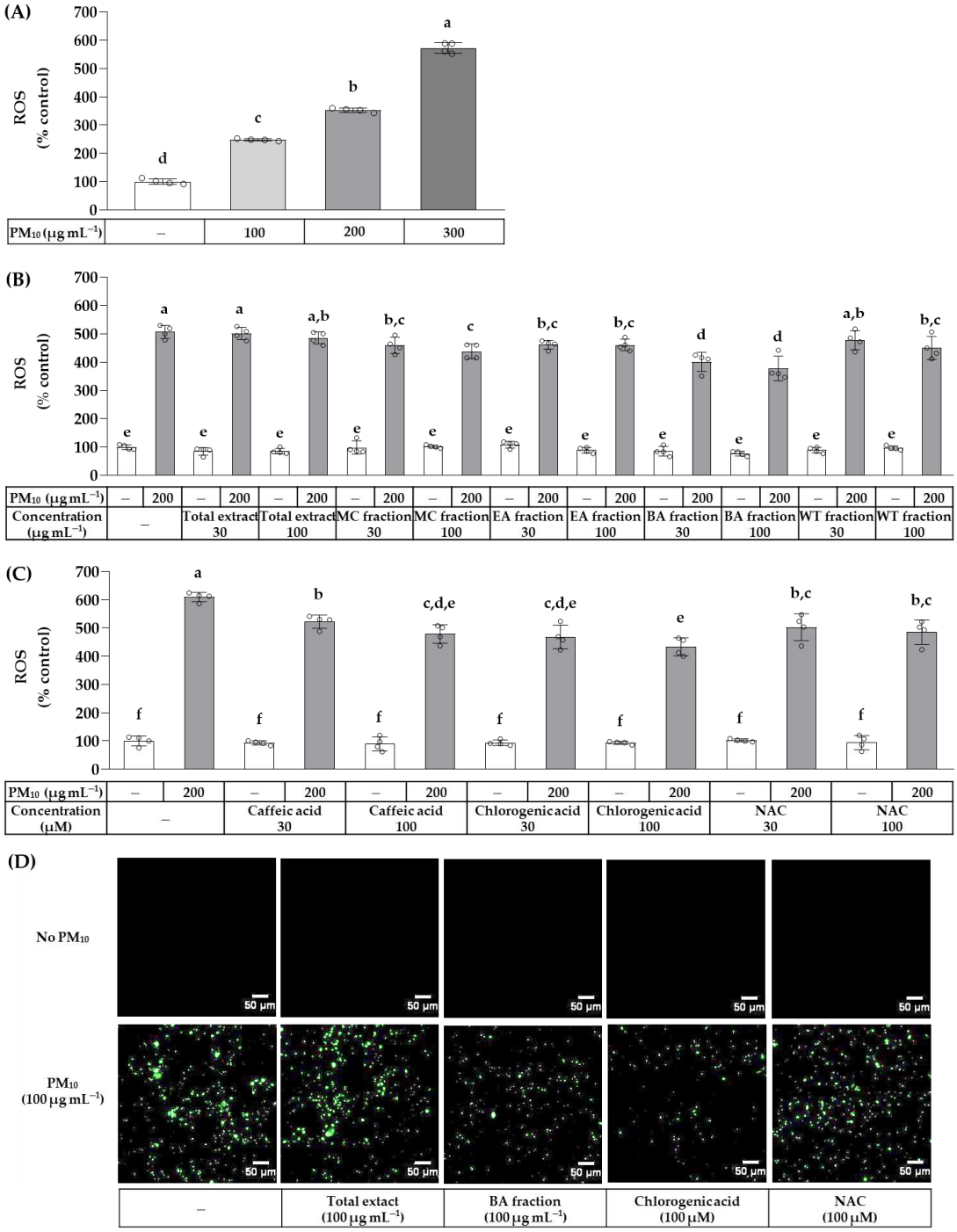

Figure 4. Effects of the total OJ extract, its solvent fractions, chlorogenic acid, caffeic acid, and NAC on the production of reactive oxygen species (ROS) in HaCaT keratinocytes exposed to PM_{10}. In (**A**), after pre-labeling with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min, cells were treated with PM_{10} at different concentrations for 60 min. In (**B**,**C**), the pre-labeled cells were treated with the total OJ extract, its solvent fractions, or compounds at the specified concentrations, alone or in combination with PM_{10} (200 μg mL^{-1}), for 60 min. Typical images of cells fluorescing due to ROS production are shown in (**D**). The change in the fluorescence intensity due to cellular ROS production was determined. Data are presented as mean ± SD (n = 4). Groups that do not have the same lowercase alphabet letters (a–f) are considered to have significantly different means at the $p < 0.05$ level.

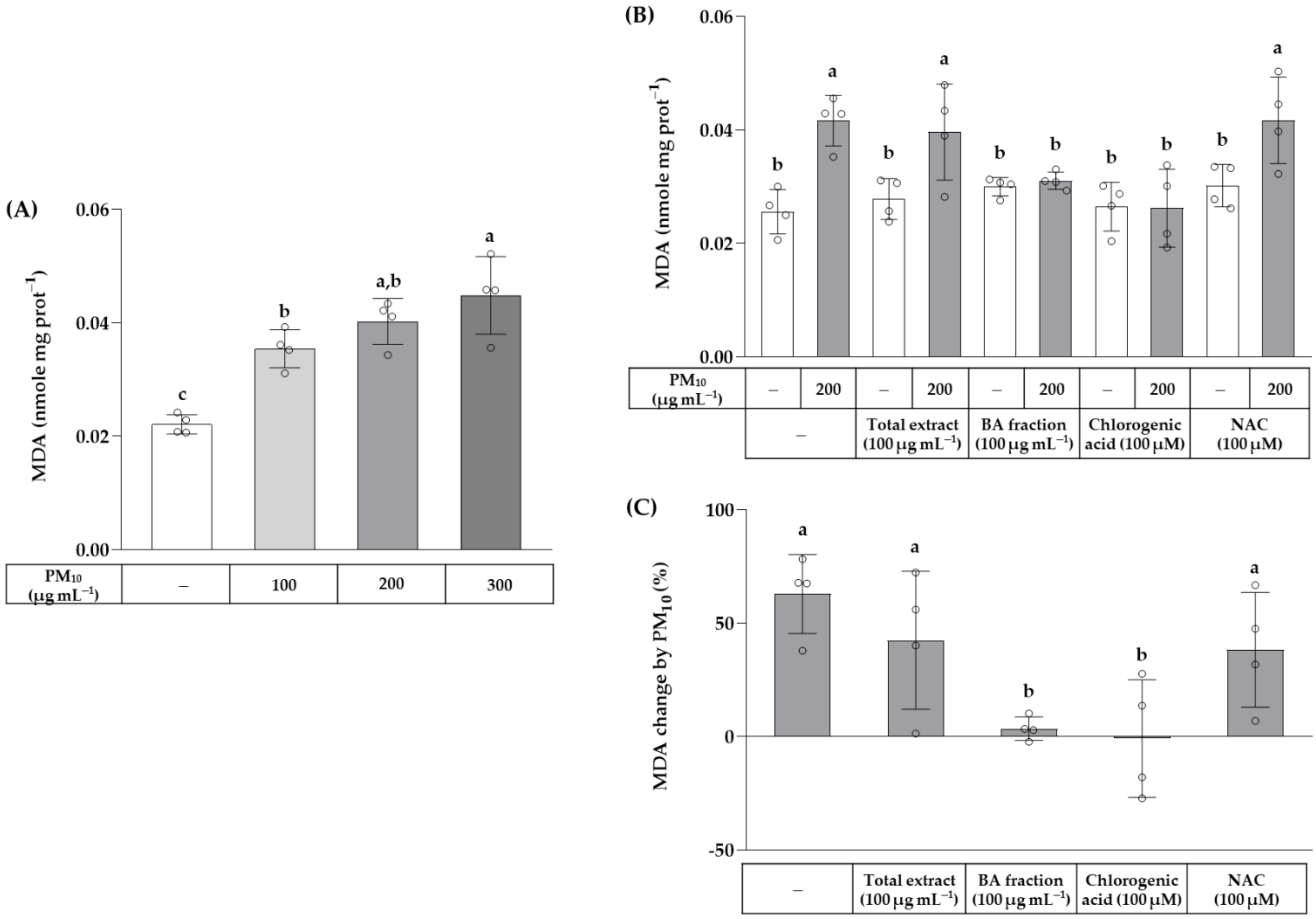

Figure 5. Effects of the total OJ extract, its BA fraction, chlorogenic acid, and NAC on lipid peroxidation in HaCaT keratinocytes exposed to PM_{10}. In (**A**), cells were treated with PM_{10} at different concentrations for 48 h. In (**B**,**C**), cells were treated with the total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), or NAC (100 μM), alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. Lipid peroxidation was determined by measuring malondialdehyde (MDA) levels in whole-cell lysates and the values were normalized to the protein contents (nmole mg protein^{-1}) (**B**). The % change in the MDA values due to PM_{10} exposure in each group was calculated using the following equation: change (%) = $(\alpha - M)/M \times 100$, where α is a value in the presence of PM_{10} and M is the mean value of each group in the absence of PM_{10} (**C**). Data are presented as mean ± SD (n = 4). Groups that do not share the same lowercase alphabet letters (a–c) are considered to have significantly different means at the $p < 0.05$ level.

The effects of the total OJ extract, BA fraction, chlorogenic acid, and NAC on the secreted level of PG E_2 in HaCaT cells exposed to PM_{10} were examined using ELISA. As shown in Figure 7, PM_{10} at 100–300 μg mL^{-1} increased the secreted level of PG E_2 up to 14-fold in a concentration-dependent manner. The total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM) did not affect the secreted level of PG E_2 in the absence of PM_{10}. The BA fraction (100 μg mL^{-1}) and chlorogenic acid (100 μM) inhibited the increase in the secreted level of PG E_2 in cells stimulated by PM_{10} (200 μg mL^{-1}), whereas the total OJ extract (100 μg mL^{-1}) and NAC (100 μM) had no significant effects.

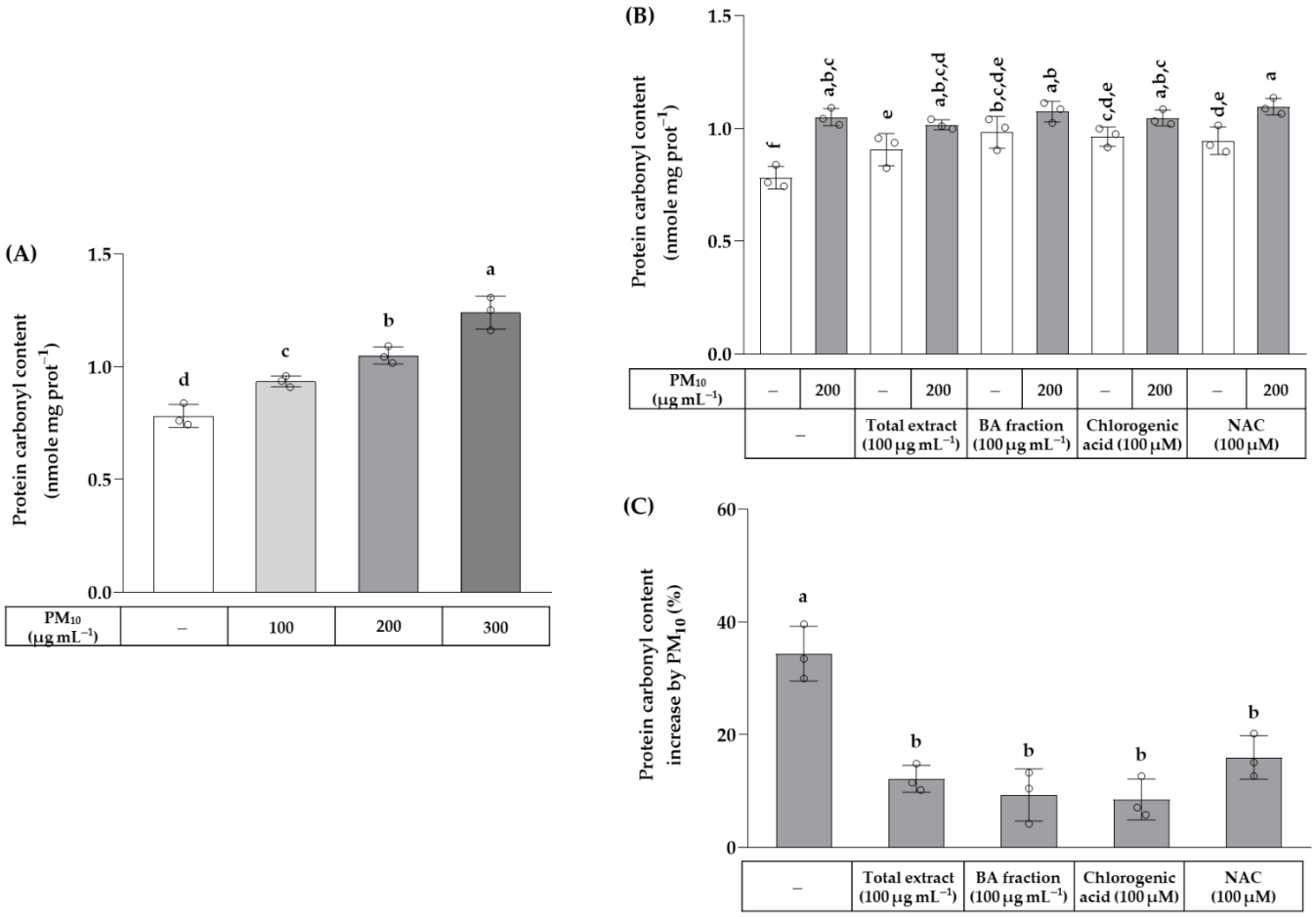

Figure 6. Effects of the total OJ extract, its BA fraction, chlorogenic acid, and NAC on the protein carbonyl content in HaCaT keratinocytes exposed to PM_{10}. In (**A**), cells were treated with different concentrations of PM_{10}. In (**B**,**C**), cells were treated with the total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM), alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. Protein carbonyl contents were normalized to the protein contents and presented in nmole mg protein^{-1} (**B**). The % change in the protein carbonyl contents due to PM_{10} exposure in each group was calculated using the following equation: change (%) = (α − M)/M × 100, where α is the value in the presence of PM_{10} and M is the mean value of each group in the absence of PM_{10} (**C**). Data are presented as mean ± SD (n = 3). Groups that do not share the same lowercase alphabet letters (a–f) are considered to have significantly different means at the $p < 0.05$ level.

The effects of the total OJ extract, BA fraction, chlorogenic acid, and NAC on the mRNA expression levels of the genes involved in PG E_2 production were examined. The qRT-PCR was run to analyze the mRNA expression levels of phospholipase A2 group IIA (*PLA2G2A*), phospholipase A2 group IVA (*PLA2G4A*), prostaglandin-endoperoxide synthase 1 (*PTGS1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), prostaglandin E synthetase 1 (*PTGES1*), and prostaglandin E synthetase 2 (*PTGES2*). As shown in Figure 8, PM_{10} at 200 μg mL^{-1} decreased the mRNA expression level of *PLA2G2A*, but increased that of *PLA2G4A*. The PM_{10}-induced increase in *PLA2G4A* mRNA was reduced by chlorogenic acid (100 μM) and NAC (100 μM), whereas the total OJ extract (100 μg mL^{-1}) and BA fraction (100 μg mL^{-1}) had no effects. PM_{10} increased the mRNA expression levels of *PTGS1* and *PTGS2*; these changes were not affected by being treated with the total OJ extract, BA fraction, chlorogenic acid, or NAC. PM_{10} and other test materials had little effect on the mRNA expression levels of *PTGES1* and *PTGES2*.

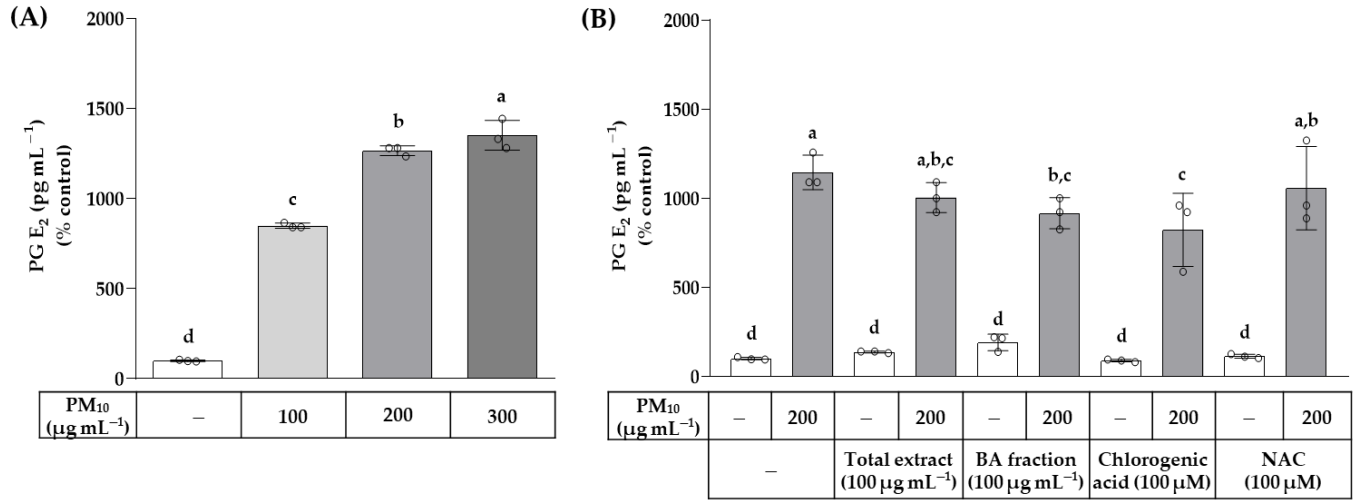

Figure 7. Effects of the total OJ extract, its BA fraction, chlorogenic acid, and NAC on the secreted level of prostaglandin (PG) E_2 in HaCaT keratinocytes exposed to PM_{10}. In (**A**), cells were treated with different concentrations of PM_{10}. In (**B**), cells were treated with the total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM), alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. The secreted level of PG E_2 was determined using a PG E_2 enzyme-linked immunosorbent assay (ELISA). Data are presented as mean ± SD (n = 3). Groups that do not share the same lowercase alphabet letters (a–d) are considered to have significantly different means at the $p < 0.05$ level.

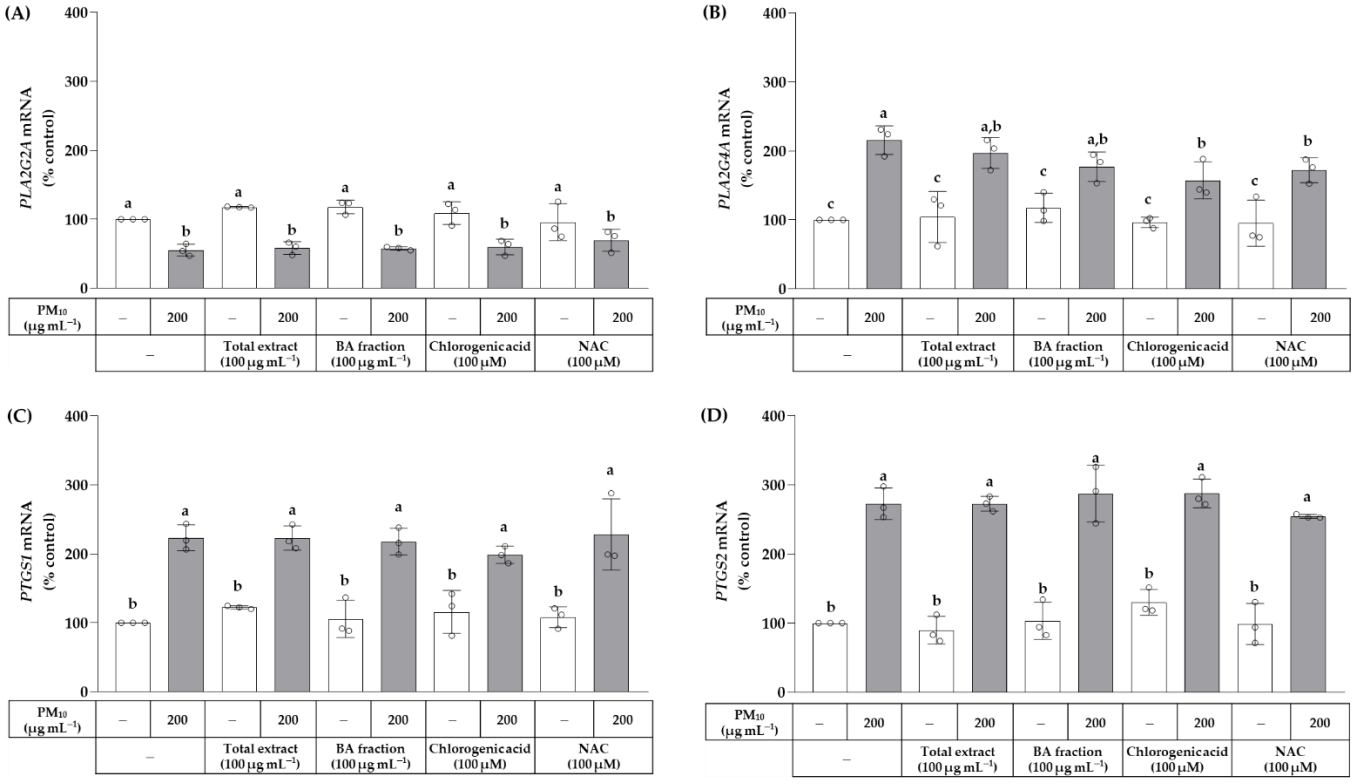

Figure 8. *Cont.*

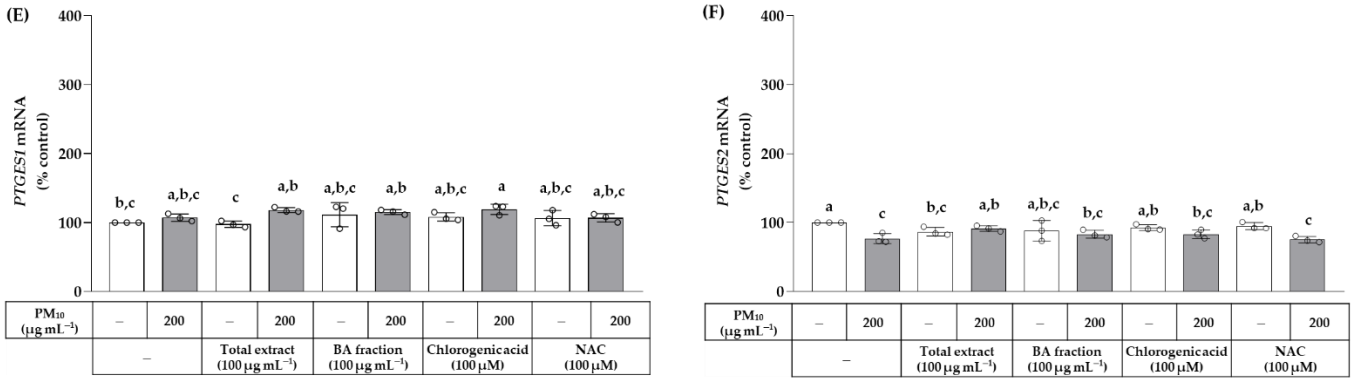

Figure 8. Effects of the total OJ extract, its BA fraction, chlorogenic acid, and NAC on the mRNA expression levels of phospholipase A2 group IIA (*PLA2G2A*) (**A**), phospholipase A2 group IVA (*PLA2G4A*) (**B**), prostaglandin-endoperoxide synthase 1 (*PTGS1*) (**C**), prostaglandin-endoperoxide synthase 2 (*PTGS2*) (**D**), prostaglandin E synthetase 1 (*PTGES1*) (**E**), and prostaglandin E synthetase 2 (*PTGES2*) (**F**) in HaCaT keratinocytes exposed to PM_{10}. Cells were treated with the total extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM), alone or in combination with PM_{10} (200 μg mL^{-1}), for 24 h. The mRNA expression level of each gene was analyzed by the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Data are presented as % of control (means ± SD, $n = 3$). Groups that do not share the same lowercase alphabet letters (a–c) have statistically different means at the $p < 0.05$ level.

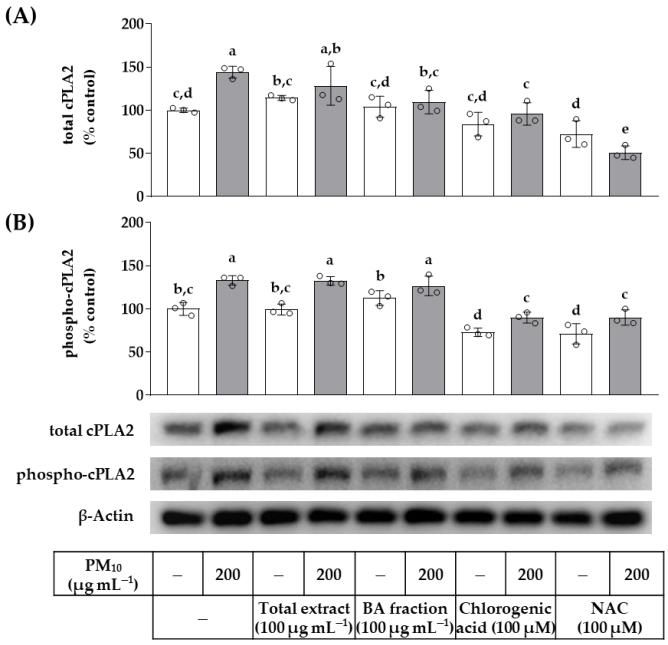

Figure 9. Effects of the total OJ extract, its BA fraction, chlorogenic acid, and NAC on the protein levels of cytosolic phospholipase A2 (cPLA2) and phospho-cPLA2 in HaCaT keratinocytes under basal and PM_{10}-exposed conditions. Cells were treated with the total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM), alone or in combination with PM_{10} (200 μg mL^{-1}), for 48 h. The protein levels of total cPLA2 (**A**) and phospho-cPLA2 (**B**) were determined by Western blotting and compared to that of β-actin. Representative blots are shown. Data are presented as percentages of the control (mean ± SD, $n = 3$). Duncan's multiple range test was performed to compare all group means to each other. Groups that do not share the same lowercase alphabet letters (a–e) have significantly different means at the $p < 0.05$ level.

The effects of the total OJ extract, BA fraction, chlorogenic acid, and NAC on the protein expression levels of cPLA2 and its phosphorylated active form (phospho-cPLA2) were examined by Western blotting. As shown in Figure 9, PM_{10} increased the protein levels of cPLA2 and phospho-cPLA2. The total OJ extract (100 μg mL^{-1}), BA fraction (100 μg mL^{-1}), chlorogenic acid (100 μM), and NAC (100 μM) did not affect the basal levels of the cPLA2 protein. However, the BA fraction, chlorogenic acid, and NAC lowered the levels of cPLA2 protein under PM_{10}-exposed conditions. In addition, chlorogenic acid and NAC lowered the levels of phospho-cPLA2 protein under basal and PM_{10}-exposed conditions.

4. Discussion

This study, for the first time, has examined the antioxidant and anti-inflammatory effects of the total OJ extract and its solvent fractions on skin cells exposed to PM_{10}. Neither the total extract nor the solvent fraction of OJ significantly reversed HaCaT cell death due to a high level of PM_{10} (200 μg mL^{-1}), but some solvent fractions within a non-cytotoxic range significantly reduced the PM_{10}-induced cellular ROS production. When the effect of reducing ROS production was compared between the solvent fractions, the BA fraction was more effective than the EA fraction and other fractions.

The BA fraction contained chlorogenic acid (49.0 mg g^{-1}), whereas the EA fraction contained caffeic acid (18.7 mg g^{-1}) as a major phenolic component. These two phenolic compounds did not significantly alleviate the cell death caused by PM_{10}, but effectively reduced the cellular ROS production, and their effects were comparable to that of NAC, a positive control antioxidant. Thus, we speculated that chlorogenic acid and caffeic acid might have been the active components of OJ that reduced ROS levels in cells exposed to PM_{10}. Chlorogenic acid showed a stronger effect than the same molar concentration of caffeic acid in the inhibition of cellular ROS production. These results explain why the BA fraction that was rich in chlorogenic acid was more effective than the EA fraction, which was rich in caffeic acid, in reducing cellular ROS levels under PM_{10} exposure.

Because plant-derived substances may have various effects on cell physiology, it is important to optimize preparations for targeting biological activity. In a previous study comparing the phytochemical composition and antioxidant activity of a 70% ethanol extract of OJ and its solvent fractions, the EA fraction showed a higher content of total phenolic compounds and flavonoids, and a higher free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical, and hydroxyl radical in vitro, compared to the BA fraction [29]. Another study showed that the BA fraction of the hot water extract of OJ was more effective than other fractions in reducing the blood ethanol concentration after the oral administration of ethanol in ICR mice [34]. In the current study, the BA fraction would be more effective and potentially useful in attenuating oxidative stress in human keratinocytes under PM_{10}-exposed conditions.

Therefore, additional experiments were performed focusing on the antioxidant and anti-inflammatory effects of the BA fraction and its major component, chlorogenic acid. The results showed that the BA fraction (100 μg mL^{-1}) and chlorogenic acid (100 μM) were effective in inhibiting lipid peroxidation and PG E_2 secretion in cells exposed to PM_{10}. The results suggest the potential utility of the BA fraction of OJ in attenuating the PM_{10}-induced oxidative damage to and inflammatory responses of cells. It is also suggested that chlorogenic acid may be an active component that is responsible for the antioxidant and anti-inflammatory effects of the BA fraction of the OJ extract.

The arachidonic acid that is released from membrane phospholipids via the enzymatic action of phospholipases A2 (PLA2) is converted to PG G_2 and then to PG H_2 in a reaction that is catalyzed by prostaglandin-endoperoxide synthases (PTGS), also known as cyclooxygenase (COX); further, the conversion of PG H_2 to PG E_2 is catalyzed by prostaglandin E synthetases (PEGS) [48,49]. The *PLA2G4A* gene encodes a cytosolic phospholipase A2 (cPLA2), while the *PLA2G2A* gene encodes a secretory phospholipase A2 (sPLA2) [43,44]. Interestingly, PM_{10} decreased the mRNA expression of *PLA2G2A* and increased that of

PLA2G4A. It also increased the mRNA expression of *PTGS1* and *PTGS2*, which encode COX1 and COX2, respectively, but had no significant effects on the mRNA expression of *PTGES1* and *PTGES2*, which encode microsomal prostaglandin E synthetase 1 (mPEGS1) and mPEGS2. This suggests that the increase in PG E_2 production by PM_{10} could be mediated by cPLA2, COX1, and COX2 proteins. Since chlorogenic acid attenuated the expressions of *PLA2G4A* mRNA and cPLA2 protein induced by PM_{10}, this is considered to be one of chlorogenic acid's main mechanisms of action regarding its ability to reduce PM_{10}-induced PG E_2 production, as depicted in Figure 10.

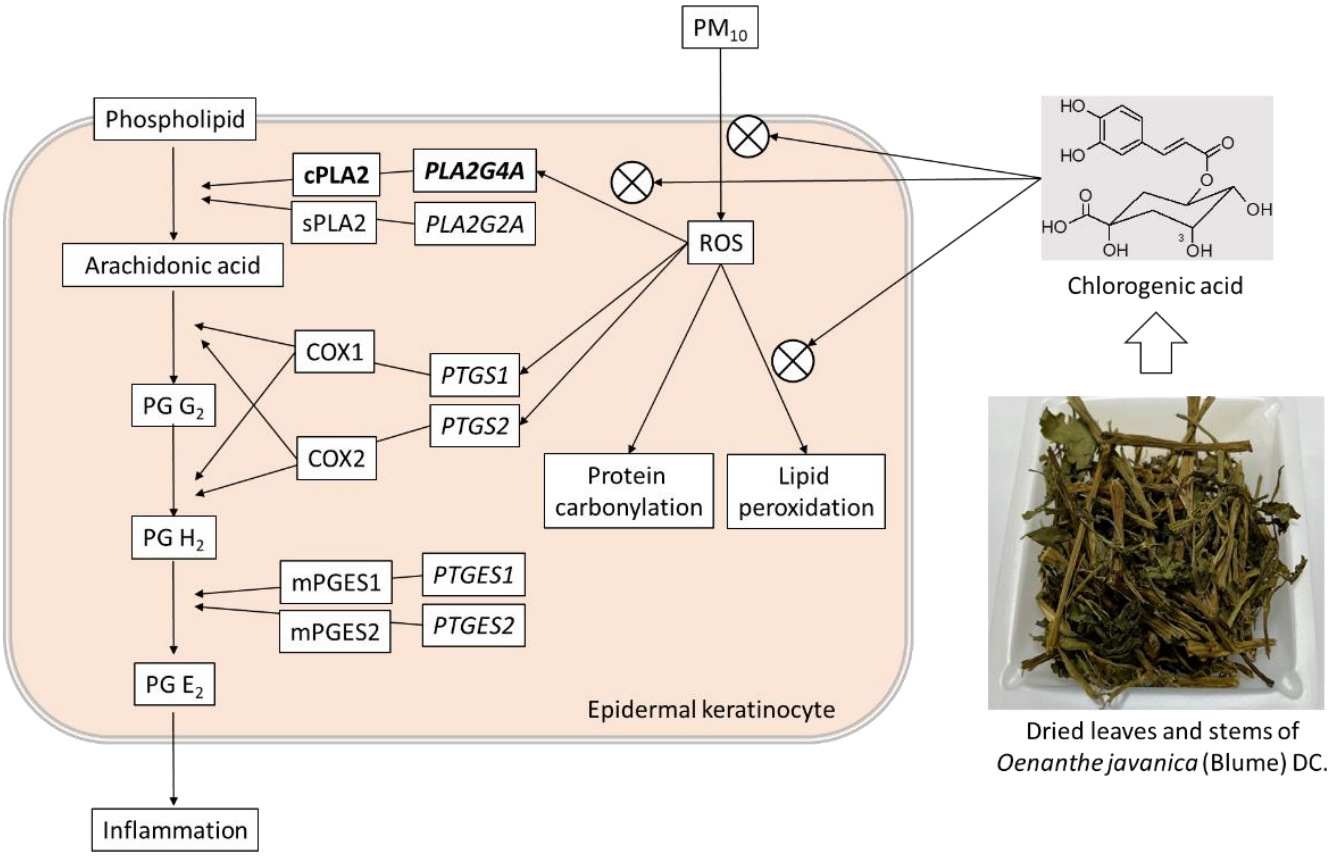

Figure 10. Antioxidant and anti-inflammatory effects of chlorogenic acid in HaCaT keratinocytes exposed to PM_{10}. PM_{10} increased ROS production and lipid peroxidation. It also increased PG E_2 production through the enhanced expressions of *PLA2G4A* mRNA and cPLA2 protein. These changes were attenuated (⊗) by chlorogenic acid, a main component of OJ.

Although the ability of chlorogenic acid to reduce the PM_{10}-induced mRNA expression of *PTGS1* and *PTGS2*, which encode COX1 and COX2, respectively, was not observed in the present study, it is worth recalling that chlorogenic acid is a potent inhibitor of COX2 enzyme activity (IC_{50}, 8.1 μM) [50]. Chlorogenic acid could reduce PG E_2 production through the direct inhibition of COX-2 enzymatic activity as an additional mechanism of action.

The cPLA2 is activated by airborne agricultural particulate matter and mediates inflammatory responses in human lung epithelial A549 cells [51]. The differential regulation of the gene expression of *PLA2G4A* versus *PLA2G2A* by PM_{10} observed in the present study suggests that each isozyme may play a distinct role under different cellular contexts. The cPLA2 is a calcium-dependent enzyme that is additionally activated by phosphorylation at Ser505 and other sites [52]. In the present study, PM_{10} increased the total cPLA2 protein and phospho-cPLA2 (Ser505), and these changes were reduced by chlorogenic acid and

NAC. A more expanded study is needed to elucidate the differential role of phospholipase A2 isoenzymes in association with the PM_{10}-induced inflammatory reaction.

Previous studies demonstrated the anti-inflammatory effects of OJ extracts on the attenuation of the NF-kB-mediated expression of COX2, and on the secretion of TNF-α and PG E_2 in lipopolysaccharide-stimulated RAW 264.7 macrophages [35,36]. The topical application of OJ extracts alleviated the decrease in skin collagen caused by UV irradiation in male ICR mice, and this effect was associated with the restoration of the expression of collagen types I and III, MMP1, MMP3, TNF-α, and COX2 [37]. The present study additionally showed that the BA fraction purified from the OJ extract has anti-inflammatory effects, by reducing the secretion of PG E_2 in keratinocytes stimulated by PM_{10}. Taken together, data from these previous studies and the current study suggest that OJ extract is a useful natural product to alleviate skin inflammation caused by UV rays, atmospheric fine dust, or a combination thereof. In addition, it is suggested that the efficacy can be further improved by purifying the OJ extract and increasing the content of chlorogenic acid, one of its active ingredients.

Chlorogenic acid has been previously reported to attenuate oxidative stress and inflammatory responses in various in vitro and in vivo models [53,54]. In our recent study, chlorogenic acid was identified as a major component of the *Siegesbeckiae herba* extract, which has now been shown to mitigate PM_{10}-induced cytotoxicity by activating the NRF_2 pathway [25]. It has also been shown to suppress the production of nitric oxide (NO) and PG E_2 by inhibiting the expression of inducible nitric oxide synthase (iNOS) and COX2 in lipopolysaccharide-stimulated RAW 264.7 macrophages [55], and in human chondrocytes stimulated by IL-1β [56]. In the present study, chlorogenic acid was shown to reduce the production of PG E_2 in human keratinocytes induced by PM_{10}. Thus, there is consolidating evidence that chlorogenic acid exhibits antioxidant and anti-inflammatory activities in cells.

OJ contains a variety of compounds that exhibit anti-inflammatory activity [27]. In previous studies, ten biphenyl derivatives were isolated from the cyclohexane fraction of an 85% ethanol extract of the aerial part of OJ, and some significantly inhibited COX2 activity in vitro [57]. Thirteen kinds of phenylpropanoids were isolated from the EA fraction of the extract, and some of these compounds exhibited anti-inflammatory activities that inhibited NO production in RAW 264.7 macrophages [58]. Therefore, it should not be overlooked that OJ has various components that exhibit anti-inflammatory capabilities via various mechanisms, in addition to chlorogenic acid and caffeic acid.

In this study, OJ extract and its fractions showed different degrees of cytotoxicity at high concentrations in the absence of fine dust. Currently, the identity and mechanism of action of these toxic components are unknown. Nonetheless, it is desirable to remove toxic components as much as possible, while leaving active components, when using OJ extract to protect cells. Since even active components can be toxic at high concentrations, it is important to find the optimal concentration range, considering both toxicity and efficacy.

5. Conclusions

This study demonstrated that the BA fraction of OJ extract reduces PM_{10}-induced ROS production in HaCaT cells most effectively among the solvent fractions. Of its major phenolic components, chlorogenic acid was more effective than caffeic acid in reducing ROS levels. The BA fraction and chlorogenic acid attenuated lipid peroxidation, protein carbonylation, and PG E_2 production due to the exposure of cells to PM_{10}. The PM_{10}-induced expressions of *PLA2G4A* mRNA and cPLA2 protein were alleviated by chlorogenic acid, and this action of chlorogenic acid is suggested to be the main mechanism that enables a reduction in PM_{10}-induced PG E_2 production. This study suggests that the chlorogenic acid contained in OJ extract may help to alleviate the oxidative damage to and inflammatory responses produced by the skin cells when exposed to air pollutants.

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Abbreviations

BA	n-butanol
COX	cyclooxygenase
cPLA2	cytosolic phospholipase A2
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
EA	ethyl acetate
ELISA	enzyme-linked immunosorbent assay
FTC	fluorescein-5-thiosemicarbazide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPLC-DAD	high-performance liquid chromatography with photodiode array detection
IL	interleukin
iNOS	inducible nitric oxide synthase
MC	methylene chloride
MDA	malondialdehyde
MMP	matrix metalloproteinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAC	N-acetyl cysteine
NF-κB	nuclear factor-κB
NO	nitric oxide
OJ	*Oenanthe javanica* (Blume) DC.
PBS	phosphate-buffered saline
PG	prostaglandin
PEGS	prostaglandin E synthetase
PLA2	phospholipases A2
PM	particulate matter
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
ROS	reactive oxygen species
sPLA2	secretory phospholipase A2
SOD	superoxide dismutase
TNF-α	tumor necrosis factor-α
TMP	1,1,3,3-tetramethoxypropane
UV	ultraviolet
WT	water

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