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Research Paper

Extract of *Cornus officinalis* Protects Keratinocytes from Particulate Matter-induced Oxidative Stress

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Abstract

The skin is one of the large organs in the human body and the most exposed to outdoor contaminants such as particulate matter < 2.5 μm ($PM_{2.5}$). Recently, we reported that $PM_{2.5}$ induced cellular macromolecule disruption of lipids, proteins, and DNA, via reactive oxygen species, eventually causing cellular apoptosis of human keratinocytes. In this study, the ethanol extract of *Cornus officinalis* fruit (EECF) showed anti-oxidant effect against $PM_{2.5}$-induced cellular oxidative stress. EECF protected cells against $PM_{2.5}$-induced DNA damage, lipid peroxidation, and protein carbonylation. $PM_{2.5}$ up-regulated intracellular and mitochondrial Ca^{2+} levels excessively, which led to mitochondrial depolarization and cellular apoptosis. However, EECF suppressed the $PM_{2.5}$-induced excessive Ca^{2+} accumulation and inhibited apoptosis. The data confirmed that EECF greatly protected human HaCaT keratinocytes from $PM_{2.5}$-induced oxidative stress.

Key words: ethanol extract of *Cornus officinalis* fruits (EECF); particulate matter 2.5 ($PM_{2.5}$); human HaCaT keratinocytes; oxidative stress

Introduction

Particulate matter (PM) is an air pollutant with harmful effects on the human skin that contribute to conditions such as skin cancers, alopecia, and skin aging [1,2]. In particular, the harmful effects of PM depend on the composition of deleterious contents such as heavy metals (Cu, Mn, Ni, Pb, and Ti) and polycyclic aromatic hydrocarbons [3]. PM < 2.5 μm ($PM_{2.5}$) is considered fine PM and its detrimental effects on the human skin are mediated by the generation of excessive intracellular reactive oxygen species (ROS), which creates oxidative stress [4-6]. $PM_{2.5}$-mediated excessive ROS generation could elicit lipid peroxidation, DNA damage, apoptotic protein expression, and mitochondria-dependent apoptosis, which eventually results in skin irritation and damage [7].

There are more than 65 species classified under the genus *Cornus* (family Cornaceae), but only two species, *Cornus mas* and *Cornus officinalis*, have been reported as medicinal plants used in traditional medicine [8]. These plants are mainly distributed in eastern Asia including Korea, Japan, and China. *C. officinalis* is commonly known as cornel dogwood or Asiatic dogwood [9]. *C. officinalis* grows up to 4-10 m high, has papery leaves that are 5.5-10 cm long, its flowers consist of four petals with a yellow lanceolate tongue that is 3.3 mm long [10]. *C. officinalis* fruit has been used to treat high blood pressure, kidney deficiency, dizziness, spermatorrhea, and waist and knee pain since ancient times [10,11]. Most related pharmacological studies have revealed that the ethanol extract of *C. officinalis* fruit (EECF) possesses anti-hyperglycemia, anti-aging, immune-regulatory, and renal and neuro-protective effects [12]. In addition, the neuro-protective, antioxidant, anti-inflammatory, cardiovascular, and anti-diabetic effects of the EECF have been revealed [13]. Furthermore, *C. officinalis* fruit contain high amounts

of volatile compounds, organic acids, carbohydrates, tannins, and iridoids. Particularly, iridoid glycosides are one of the active ingredients in the *C. officinalis* fruit [14]. However, there are few reports of the cytoprotective effect of EECF against $PM_{2.5}$-induced oxidative stress in human keratinocytes. Therefore, this study was conducted to investigate the potential of EECF to cure the $PM_{2.5}$-induced cell damage.

Materials and methods

Reagents and chemicals

The dried fruit of *C. officinalis* collected from an area around the city of Gurye (Jeollanam-do Province, Republic of Korea), were provided by Gurye Sansuyu Farming Association Corporation. For the preparation of EECF, the dried fruit (20 g) were cut into small pieces and extracted three times with 400 mL 70% ethanol at 4°C for 3 h. After filtering, the filtrate was concentrated using a vacuum rotary evaporator (EYELA SB-1000, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The residue was then freeze-dried using a freeze dryer and stored at -80°C. The powder (EECF) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) to obtain a final concentration of 100 mg/mL (extract stock solution), and was stored at 4°C. The stock solution was diluted with culture medium to the desired concentrations prior to use. EECF was dissolved in DMSO. Diesel $PM_{2.5}$ (NIST SRM 1650b, $PM_{2.5}$) was purchased from Sigma-Aldrich Chemical Co. and was dissolved in DMSO to prepare the stock solution (25 mg/mL). To avoid agglomeration of the suspended $PM_{2.5}$, the solution was sonicated for 30 min [15].

Cell culture

The human HaCaT keratinocytes (Cell Line Service, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corporation, Staley Rd, Grand Island, USA). The medium was supplemented with antibiotic solution consisting of 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Gibco, Life Technologies Co., Grand Island, NY, USA). In addition, the medium was supplemented with 10% fetal bovine serum. The cultured cells were incubated in a 100% humidified atmosphere at 37° C with 5% CO_2.

Cell viability

The cytotoxicity of the EECF on HaCaT cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were cultured in a 96-well plate at a density of 1.0×10^5 cells per well and specific wells were separately treated with EECF at final concentrations of 25, 50, 100, 200, 300, 400, and 500 µg/mL. The MTT stock solution (2 mg/mL) was incubated with the cells for 4 h until formazan crystals were formed. The crystals were then dissolved in DMSO and the absorbance of the reaction solution was detected using a multi-well spectrophotometer at a wavelength of 540 nm.

DPPH radical detection

EECF (25, 50, 100, 200, 300, 400, and 500 µg/mL) was mixed with 0.15 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH), shaken gently, and kept in the dark for 3 h. The residual DPPH was determined at 520 nm using a spectrophotometer.

ROS detection

Cells were seeded in 96 well palate at a 1.5×10^5 cell density and intracellular ROS levels those generated via 1 mM hydrogen peroxide (H_2O_2), were measured using the 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) assay. Cells were seeded on chamber slides at a 1.5×10^5 cell density and incubated with $PM_{2.5}$ (50 µg/mL) for 1 h. The cells were stained with DCF-DA for 30 min and the fluorescence emission was detected using confocal microscopy (Carl Zeiss, Oberkochen, Germany).

Detection of superoxide anion

Superoxide anion was generated though the reaction between 5,5-dimethylpyrroline-N-oxide (DMPO) and the xanthine/xanthine oxidase system. The generated DMPO/·OOH adduct was detected using electron spin resonance (ESR). Then, 20 µL xanthine oxidase (0.25 U/mL) was mixed with 20 µL each of xanthine (10 mM), EECF (200 µg/mL), and 3 M DMPO, and after 2.5 min, the ESR signaling was measured. ESR spectrophotometer settings were set as follows: power, 1.00 mW; central magnetic field, 336.8 mT; frequency, 9.4380 GHz; amplitude, 600; modulation width, 0.2 mT; sweep width, 10 mT; sweep time, 30 sec; gain, 500; time constant, 0.03 sec; temperature, 25°C [16].

Lipid peroxidation assay

A four-well chamber slide was used to plate the cells in the presence of 200 µg/mL EECF, followed by exposure to $PM_{2.5}$ (50 µg/mL) for 24 h and staining with diphenyl-1-pyrenylphosphine (DPPP) for 30 min in the dark. Images were analyzed using a confocal microscope [15].

Protein carbonylation assay

Cells were incubated with 200 µg/mL EECF for 1 h and treated with $PM_{2.5}$ (50 µg/mL) for 24 h. Protein oxidation was assessed using an OxiSelect™ protein carbonyl enzyme-linked immunosorbent assay kit

(Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

Single-cell gel electrophoresis

Cells were seeded in the medium with 200 μg/mL EECF in a 1 mL micro tube for 30 min and treated with $PM_{2.5}$ (50 μg/mL) for another 30 min. After coating with 110 μL 0.7% low-melting agarose, the cells were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% N-lauroyl-sarcossinate) for 1 h at 4°C. An electrical field (300 mA, 25 V) was used for electrophoresis. Slides were stained with 40 μL ethidium bromide (10 μg/mL) and analyzed using the comet 5.5 image analyzer (Andor Technology, Belfast, UK). The percentage total fluorescence and tail lengths were recorded (50 cells per slide).

Detection of 8-oxoguanine (8-oxoG) expression

ROS-induced DNA damage was assessed using the avidin-tetramethylrhodamine isothiocyanate (TRITC, 1:200) conjugate (Sigma-Aldrich) assay, based on fluorescence-binding activity. Initially, the cells were fixed on chamber slides at a 1.5×10^5 cell density and images of fluorescence-visualized cells were captured using a confocal microscope [17].

Detection of Ca^{2+} level

Treated cells were loaded with 10 μM fluoro-4-acetoxymethyl ester (Fluo-4-AM) or rhod-2-acetoxymethyl ester (Rhod-2-AM, Molecular Probes) for 30 min to detect intracellular Ca^{2+} level and mitochondrial Ca^{2+}, respectively. Fluorescence was measured using confocal microscopy [18].

Mitochondrial membrane potential ($\Delta\psi_m$) analysis

Cells were seeded on chamber slides at a density of 1.5×10^5 cells. After treatment with EECF (200 μg/mL), cells were exposed to $PM_{2.5}$ (50 μg/mL) for 24 h, stained with 5 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, USA), and analyzed using confocal microscopy.

Western blotting

Harvested cells were lysed using 150 μL of protein lysis buffer and the collected cell lysates were centrifuged at 13,000 rpm for 5 min. The resulting suspensions were collected and protein levels were analyzed as previously described [19]. Aliquots were electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the separated proteins were transferred onto the nitrocellulose membranes, which were sequentially incubated with the appropriate primary and secondary antibodies. Protein bands were detected using the Amersham ECL western blotting detection reagents and analysis system (GE healthcare, Amersham place, UK).

Hoechst 33342 staining

Cells were treated with 200 μg/mL EECF for 1 h, followed by $PM_{2.5}$ (50 μg/mL) for 18 h. The cells were stained with Hoechst 33342 (20 μM) and DNA-specific fluorescence was visualized using a fluorescence microscope. Nuclear condensation levels were evaluated and quantified for the apoptotic cells.

Statistical analysis

All experiments were performed in triplicate. Data are presented as the means ± standard error and were analyzed using the Sigma Stat 3.5 version software (Systat Software Inc., San Jose, CA, USA) using Tukey's test and analysis of variance (ANOVA). A $P < 0.05$ was considered statistically significant.

Results

EECF reduced ROS generation

Before commencing the experiment, we sought to determine if EECF had any cytotoxicity on human HaCaT keratinocytes using the MTT assay with different EECF concentrations (0, 25, 50, 100, 200, 300, 400, and 500 μg/mL, Figure 1A). The results confirmed that EECF was not cytotoxic against HaCaT cells at any of the tested concentrations. EECF showed DPPH radical scavenging activity at all the tested concentrations compared with N-acetylcysteine (NAC), a well-known antioxidant (Figure 1B). Next, the ROS scavenging ability of EECF was tested, and concentrations of 25-200 μg/mL showed rapidly increasing ROS (generated via 1 mM H_2O_2, respectively) scavenging activity and, therefore, 200 μg/mL was selected as the optimal concentration for further experiments (Figure 1C). To assess the ability of EECF (200 μg/mL) to scavenge superoxide anion, ESR spectrometry was performed. Superoxide anions produced by the xanthine/xanthine oxidase system were reduced by EECF, as shown in Figure 1D. The generated signal of 2,996 in the control was reduced to 1,505 in the presence of EECF. Intracellular ROS generation assessed using the DCF-DA assay revealed that 200 μg/mL EECF ameliorated the green color intensity caused by the $PM_{2.5}$, which was visualized using confocal microscopy (Figure 1E).

EECF significantly attenuated $PM_{2.5}$-induced lipid peroxidation, protein carbonylation, and DNA damage

The lipid peroxidation amount was assessed by

visualizing the fluorescent intensity of oxidized DPPP, which is an indicator of lipid peroxidation. The DPPP oxidase intensity was higher in $PM_{2.5}$-treated cells than it was in control cells. Pretreatment with EECF significantly reduced the florescent intensity of $PM_{2.5}$-containing cells (Figure 2A). The results indicated that EECF treatment has the potential to reduce ROS generation and further confirmed the ROS scavenging properties. Then, protein carbonylation was measured. Carbonyl groups are formed during the process of protein oxidation [16]. $PM_{2.5}$ significantly increased the expression of carbonyl moieties, whereas EECF-pretreated cells exhibited notably reduced formation of protein carbonyl when they were exposed to $PM_{2.5}$ (Figure 2B). Furthermore, $PM_{2.5}$-induced DNA damage was monitored using a comet assay. As shown in Figure 2C, treatment with the $PM_{2.5}$ distinctly elongated the comet tail and increased the damaged DNA around the nuclei. Pre-treatment of HaCaT cells with EECF before exposure to $PM_{2.5}$ obviously reduced the level of damaged DNA in comet tails. Finally, the level of 8-oxoG was analyzed using confocal microscopy, and $PM_{2.5}$-treated cells showed the highest 8-oxoG level. $PM_{2.5}$ exposure caused severe DNA lesions in cells, which were revealed by avidin-TRITC binding. Furthermore, EECF was shown to attenuate the $PM_{2.5}$-induced DNA lesions (Figure 2D).

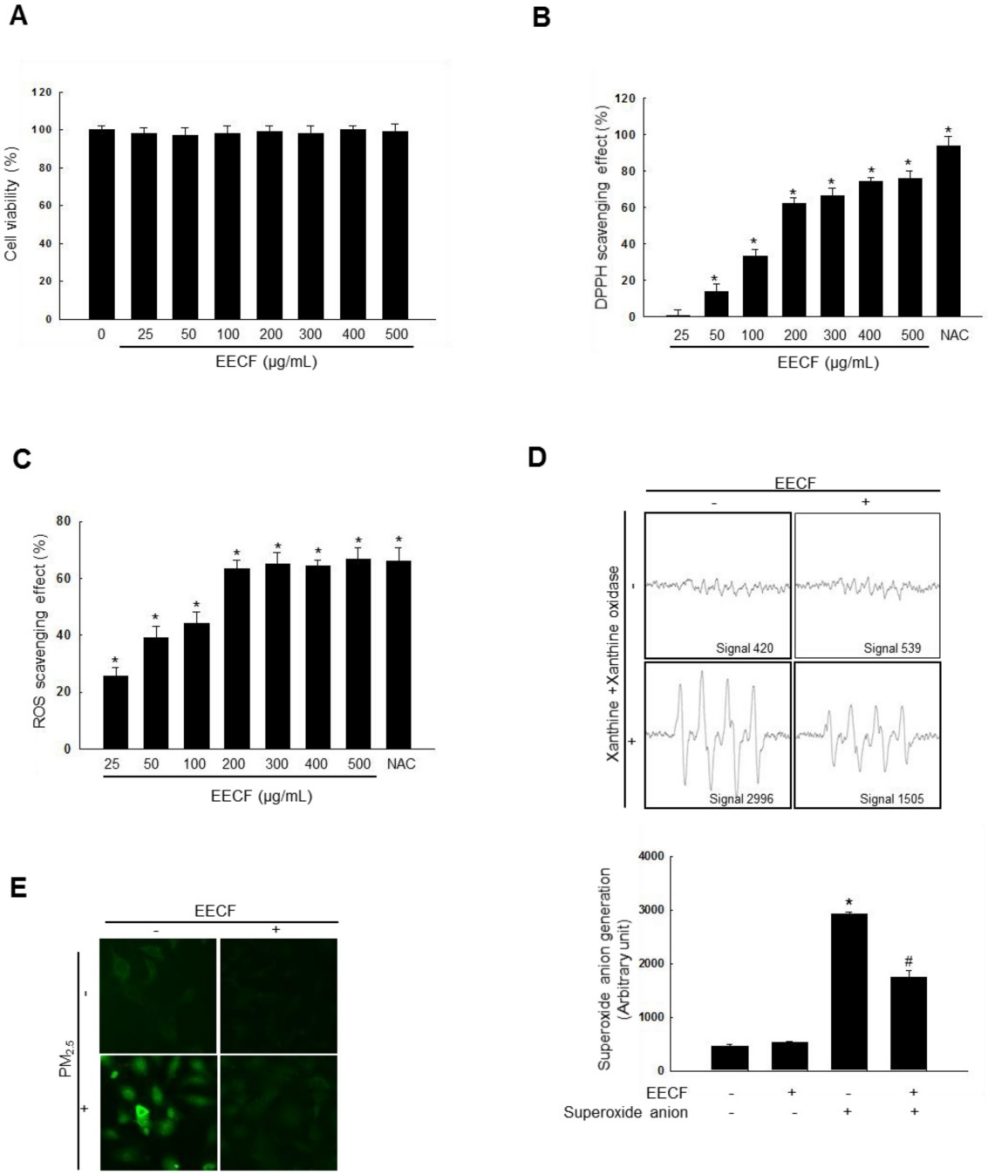

Figure 1. Ethanol extract of *C. officinalis* fruit **(**EECF) reduced ROS generation. (A) MTT assay was used to assess cell viability of EECF (0, 25, 50, 100, 200, 300, 400, and 500 μg/mL)-treated HaCaT cells for 24 h. (B) Radical-scavenging effects of EECF were investigated using DPPH assay. $^{*}p < 0.05$ compared with control. (C) Intracellular ROS level that generated by H_2O_2 (1mM), was detected using spectrophotometer after DCF-DA staining. NAC is the positive control. $^{*}p < 0.05$ compared with control. (D) Superoxide anion reducing ability of 200 μg/mL EECF was investigated using xanthine/xanthine oxidase system. $^{*}p < 0.05$ and $^{\#}p < 0.05$, compared with control and superoxide anion-treated group, respectively. (E) Effect of EECF on $PM_{2.5}$-induced intracellular ROS generation was assessed using DCF-DA staining by confocal microscopy.

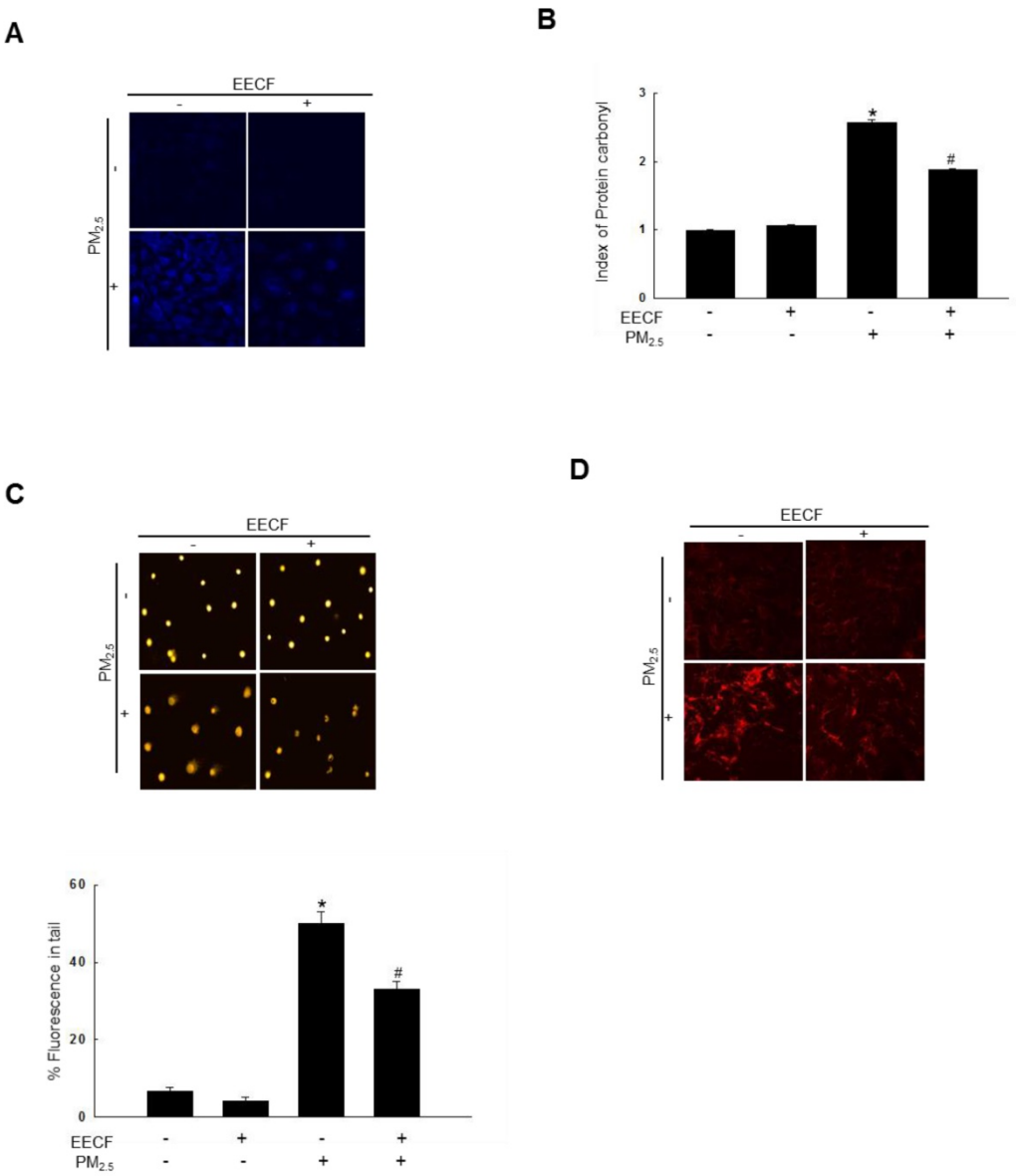

Figure 2. Ethanol extract of *C. officinalis* fruit **(**EECF) protected cells against $PM_{2.5}$-induced lipid peroxidation, protein carbonylation, and DNA damage. (A) EECF effect on $PM_{2.5}$-induced lipid peroxidation was assessed using confocal microscopy after DPPP staining. (B) Protein oxidation was assessed by measuring carbonyl formation. $^{*}p < 0.05$ and $^{\#}p < 0.05$, compared to control and $PM_{2.5}$-treated group, respectively. (C) DNA damage was assessed using comet assay. $^{*}p < 0.05$ and $^{\#}p < 0.05$, compared to control and $PM_{2.5}$-treated group, respectively. (D) Avidin-TRITC conjugate was examined to evaluate DNA oxidative adducts of 8-oxoG using confocal microscopy.

EECF attenuated $PM_{2.5}$-induced mitochondrial stress

Initially, we hypothesized that the oxidative ability of $PM_{2.5}$ was mediated by mitochondrial stress. Therefore, intracellular Ca^{2+} level were assessed, because previous studies have reported that disruption of Ca^{2+} homeostasis generates mitochondrial stress [15]. Cells were stained with Fluo-4-AM dye, and confocal microscopy analysis revealed that Ca^{2+} fluorescence was much higher in the $PM_{2.5}$-treated group than in the other cells. Pretreatment with EECF obviously reduced the intracellular Ca^{2+} level of $PM_{2.5}$-treated cells (Figure 3A). The mitochondrial Ca^{2+} level was assessed by staining cells with Rhod-2-AM dye and the confocal microscopy analysis revealed that treatment with EECF notably reduced the level (Figure 3B). The $\Delta\psi_m$ was assessed using JC-1 dye, where red and green fluorescence indicated the polarized and depolarized state of the mitochondria, respectively [20]. The results indicated that mitochondrial depolarization was enhanced by $PM_{2.5}$ but was notably reduced by EECF, as shown in the confocal microscopy analysis (Figure 3C).

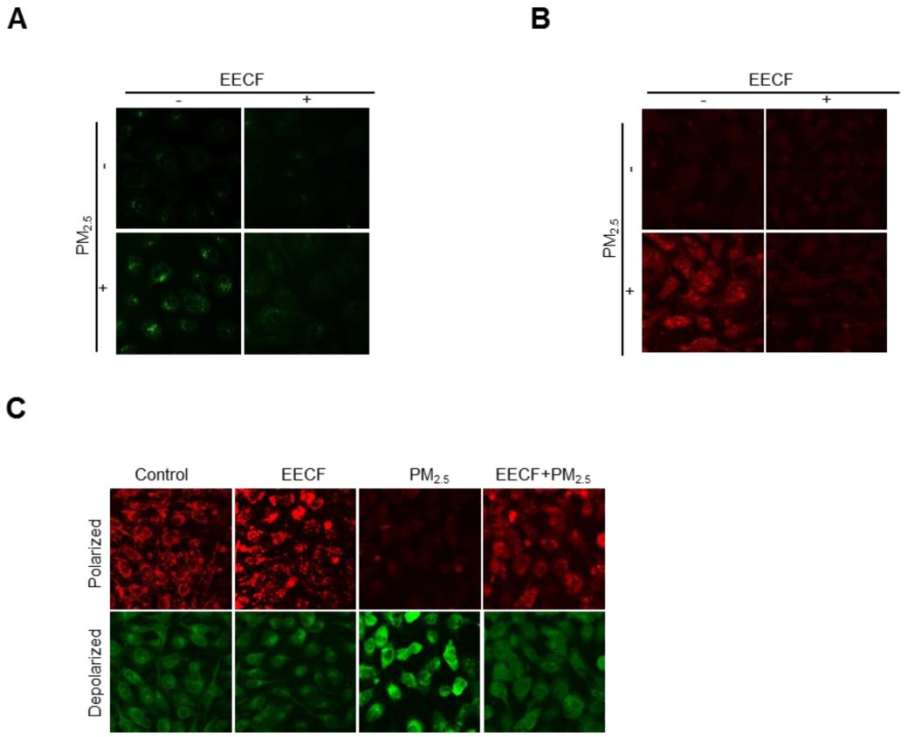

Figure 3. Ethanol extract of *C. officinalis* fruit **(**EECF) attenuated $PM_{2.5}$-induced mitochondrial stress. (A) Effect of EECF on intracellular Ca^{2+} was assessed using confocal microscopy after Fluo-4-AM staining. (B) EECF effect on the mitochondrial Ca^{2+} level was assessed using confocal microscopy after Rhod-2-AM staining. (C) Mitochondrial membrane potential ($\Delta\psi_m$) was detected using JC-1 staining.

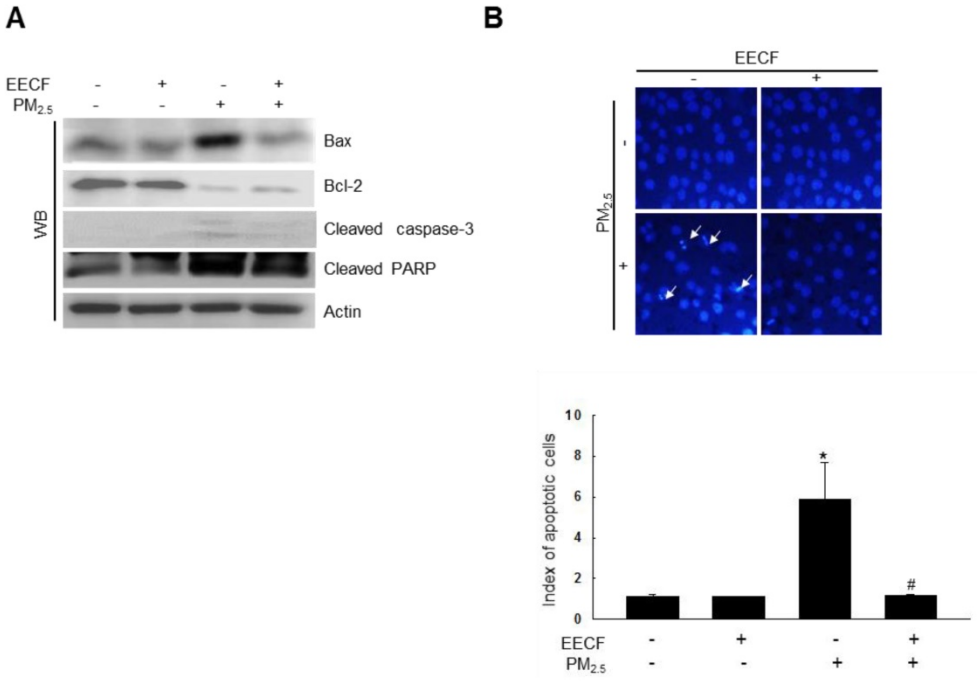

Figure 4. Ethanol extract of *C. officinalis* fruit (EECF) attenuated the $PM_{2.5}$-induced cellular apoptosis. (A) Cell lysates were analyzed for Bax, Bcl-2, cleaved caspase-3, and cleaved PARP protein expression using western blot (WB). (B) Cells were stained with Hoechst 33342 dye and apoptotic cells were observed and quantified. The arrow indicates apoptotic cells. $^{*}p < 0.05$ and $^{\#}p < 0.05$, compared to control and $PM_{2.5}$-treated group, respectively.

$PM_{2.5}$-induced cell apoptosis was attenuated by EECF

$PM_{2.5}$ enhanced the expression of cleaved caspase-3, cleaved poly-ADP ribose polymerase (PARP) and B-cell lymphoma-2-associated X protein (Bax) and decreased the expression of B-cell lymphoma-2 (Bcl-2) (Figure 4A). This finding suggests that caspase-3 was likely involved in the observed cell apoptosis. However, pretreatment with EECF

attenuated the cell apoptosis, and the cell nuclei stained with Hoechst 33342 were analyzed using microscopy, which showed significant nuclear condensation in $PM_{2.5}$-treated cells. Cells pretreated with EECF were observed to be normal (Figure 4B).

Discussion

The skin is the largest organ in the body and it protects the body by acting as a barrier to the external environment [21-23]. $PM_{2.5}$ is considered an air pollutant, which has harmful effects on the skin, such as skin aging and inflammatory skin diseases, mediated by the generation of intracellular ROS [24,25]. One of the most recent studies has reported that dried sarcocarp of *C. officinalis* consists of 11 highly polar compounds, particularly, iridoid isomers (7α-O-methylmorroniside, 7β-O-methylmorroniside, 7α-O-ethylmorroniside, and 7β- ethylmorroniside) [26]. Gallic acid, 5-hydroxymethylfurfural, morroniside, and loganin are the most abundant compounds in the *C. officinalis* fruit; however, their content could vary with the state of the fruit, depending on whether they are processed or crude. Particularly, loganin possesses immune-regulatory and anti-inflammatory activities, while morroniside is involved in the prevention of diabetic angiopathy [27]. In the present study, EECF did not show cytotoxicity at any of the tested concentrations as reported previously [28]. A previous study reported that EECF has relatively high DPPH radical scavenging activity, mediated by its antioxidant activity [29]. In agreement with previously reported findings, the results of this study indicate that EECF scavenged DPPH radical and superoxide anion (Figures 1B and 1D). It has been reported that EECF contains flavonoids, which are known to possess antioxidant activity via hydrogen donation [28,30]. Our results showed that EECF exhibited antioxidant activity by attenuating hydrogen peroxide-induced ROS generation, and ameliorated intracellular ROS generation, as revealed by DCF-DA staining (Figures 1C and 1E).

$PM_{2.5}$-induced ROS caused oxidative damage, which resulted in protein carbonylation, lipid peroxidation, and DNA damage [31]. ROS attack proteins by oxidation, which is the main mechanism of protein modification. Furthermore, protein modification can be reversible or irreversible. Protein modification leads to protein carbonylation, protein-protein cross linking, and adduct formation with lipid peroxidation products. Eventually, proteins become fragmented and degraded by ROS-mediated protein modification [32]. ROS affect lipids, mainly through hydroxyl radical and hydroperoxyl. Especially, polyunsaturated fatty acids are converted to lipid peroxyl radical and hydroperoxide as the result of oxygen insertion. Eventually, lipid peroxidation negatively affects cellular functions such as protein synthesis and alters biochemical properties [33]. It has been reported that $PM_{2.5}$ can arrest the cell cycle, resulting in DNA damage and the level of 8-OHdG (an oxidative DNA adduct) [34]. DPPP staining revealed that, EECF has an ability to reduce the $PM_{2.5}$-induced lipid peroxidation (Figure 2A). In addition, our results illustrated that EECF pretreatment significantly attenuated protein carbonyl formation in cells while the comet assay revealed the protective effect of EECF on $PM_{2.5}$-induced DNA damage. EECF significantly attenuated the DNA strand breaking and at 200 μg/mL, also reduced the elevated 8-oxoG level in $PM_{2.5}$-treated cells.

A previous study reported that cellular oxidative stress causes mitochondria stress, which eventually results in cell apoptosis [35]. Oxidative stress could be further enhanced by mitochondrial Ca^{2+} accumulation, while the endoplasmic reticulum releases Ca^{2+}. As previously reported, ROS degrade the $\Delta\psi_m$ [36], and our results revealed that EECF strongly ameliorated $PM_{2.5}$-induced excessive Ca^{2+} accumulation in the cell and mitochondria. This effect restored cellular Ca^{2+} homeostasis and EECF restored the $\Delta\psi_m$. In conclusion, our results confirmed that EECF has considerable antioxidant activity against $PM_{2.5}$-induced skin damage.

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Competing Interests

The authors have declared that no competing interest exists.

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