Anti-photoaging Effects of Steam-exploded Pine (*Pinus densiflora*) Extract in Ultraviolet B Irradiation-damaged HaCaT Keratinocytes

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Ultraviolet B (UVB) irradiation causes photoaging, such as wrinkles, roughness and dryness of the skin, and it activates the production of reactive oxygen species (ROS) and inflammatory cytokines. In this study, the anti-photoaging activity of SEP-E (steam-exploded pine extract) was evaluated in HaCaT keratinocytes damaged by UVB irradiation. SEP-E treatment showed cytoprotective effects in HaCaT keratinocytes irradiated with UVB (40 mJ/cm^2). SEP-E treatment reduced ROS overproduction and promoted the expression of antioxidant enzymes, such as catalase, superoxide dismutase 1, and superoxide dismutase 2. Additionally, SEP-E treatment suppressed the expression of inflammatory cytokines, including interleukin 6, interleukin 8, and monocyte chemoattractant protein-1. Consequently, SEP-E shows potential as a natural material for photoaging treatment.

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Keywords: Pinus densiflora; Steam-explosion; UVB; HaCaT keratinocytes; Anti-photoaging

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INTRODUCTION

Ultraviolet (UV) radiation is naturally emitted by the sun, but it can also be created by artificial sources used in industry, commerce, and recreation (Zaid *et al.* 2007). UV is divided into UVA (315 to 400 nm), UVB (280 to 315 nm), and UVC (100 to 280 nm), depending on the wavelength. UVB is absorbed into the epidermis, and its chronic exposure is a major cause of skin aging, reduced immunity, DNA damage, and skin cancer. In addition, UVB exposure stimulates excessive reactive oxygen species (ROS) generation, which overwhelms cellular antioxidant defense systems and leads to oxidative stress in the skin (Park *et al.* 2018; Petruk *et al.* 2018). An excessive accumulation of ROS can cause oxidative damage to cellular macromolecules, including DNA modification, lipid peroxidation, and apoptotic signal transduction, which alters cellular function (Fernando *et al.* 2016). The human skin possesses a range of mechanisms that modulate the damaging effects of UV radiations. These defense systems comprise an intimately interlinked network of antioxidant processes involving enzymatic, hydrophilic, and lipophilic scavengers (Anderson 1996). Therefore, to protect skin cells from environmental stress and inhibit photoaging, it is necessary to understand the antioxidant defense mechanism that can remove free radicals from oxidative stress, and it is important to study natural antioxidants that are safe for humans.

Natural antioxidants have been reported to have good potential as photoprotective agents (Berson 2008). Reports have indicated that phenolic compounds could protect the skin from UVR effects when ingested or applied topically (Hu *et al.* 2017). Phenolic compounds derived from plants have a wide range of functional properties such as anti-inflammatory and antioxidant properties. Many studies have attempted to isolate natural phenolic compounds effective for anti-aging from plants (Tsoyi *et al.* 2008; Bae *et al.* 2009; Mitani *et al.* 2007). *Pinus densiflora* is the most common tree species found in South Korea (Shin 2008). The leaves, pollen, and bark of *P. densiflora* are known to contain bioactive phytochemicals and have been used in traditional medicine. However, pine chips are rarely used as natural raw materials for extracting phenolic compounds or bioactive phytochemicals. Phenolic compounds in pine chips are frequently present in a covalently bound form with an insoluble polymer (Niwa and Miyachi 1986). Therefore, it is necessary to find an effective processing method to release the compounds.

Steam explosion treatment of *P. densiflora* breakdown the cell wall and is effective in decomposing major components, such as cellulose, hemicellulose, and lignin. Depending on the steam explosion treatment conditions, phenolic compounds linked to the cell wall are converted and produced in various types. These compounds are known to exhibit pharmacological properties, such as antioxidant, anticancer, and anti-inflammatory properties (Boussaid *et al.* 2001; Garrote *et al.* 2008). However, there are few detailed reports on the protective effects of steam-exploded pine extract (SEP-E) on UVB irradiation-induced damage in human keratinocytes. Thus, this study aimed at evaluating the anti-photoaging effect of SEP-E on UVB-induced skin damage in human keratinocytes. The cytotoxicity and protective effects of pine extracts were confirmed by applying SEP-E to HaCaT keratinocytes exposed to UVB irradiation. In addition, the antioxidant and anti-inflammatory effects were analyzed by gene expression analysis.

EXPERIMENTAL

Materials

Pine (*Pinus densiflora*) wood was harvested from a forest in the city of Hongcheon, Republic of Korea. The pine wood was cut into 2×1×0.5 -cm^3 chips for the steam explosion treatment, and stored at 20 °C, at a moisture level below 10 to 15%.

Preparation of Extract

The pine chips were subjected to steam-explosion treatment in a batch pilot unit (Jung *et al*. 2022). The pine chips were steam-exploded in a reactor at 225 °C (25 kg/cm^2) with saturated steam for 10 min. The steam-exploded pine chips were air-dried and milled to a particle size to pass 20 mesh and be retained on a 80 mesh screen. Ethanol extraction was performed on a steam-exploded pine chip using 75% ethanol for 24 h at 30 °C (solid-to-liquid ratio of 1:20). The ethanol extract was freeze dried to make the steam-exploded pine extract (SEP-E) powder, which was then used for cytotoxicity, antioxidant enzyme, and inflammation-related gene analyses.

HaCaT Keratinocyte Culture

Spontaneously transformed human skin HaCaT keratinocytes were purchased from the Korea Cell Line Bank (Republic of Korea). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine

serum, 4 mM L-glutamine, 1% penicillin solution, 1% streptomycin solution in a humidified atmosphere with 5% CO_2 at 37 °C.

UVB Irradiation

HaCaT cells were seeded in a 96-well plate and a 60-mm culture dish at a density of 3×10^3 cells/well, the day prior to UVB irradiation. The medium was removed immediately prior to UVB irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells.

Cytotoxicity

The cytotoxicity of SEP-E on cells was assessed using the MTT assay (Mosmann 1983). For the cytotoxicity evaluation of SEP-E, HaCaT cells were seeded in 96-well plates at a density of 3×10^3 cells/well. The cells were exposed to the SEP-E (10 to 1,000 µg/mL) for 2 h at 37 °C. Cytotoxicity of SEP-E was studied immediately after the end of exposure and 24 h later by the MTT assay. The cells were exposed to MTT solution and incubated for 4 h at 37 °C and measured spectrophotometrically on a microplate reader at 490 nm (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA). The number of viable cells was calculated as the percentage of the negative control cells set at 100%. The MTT assay was also used to assess the lower intensity of UVB exposure in HaCaT cells. HaCaT cells were pretreated and irradiated with different doses of UVB (1 to 100 mJ/cm^2). Cytotoxicity was assessed immediately and 24 h after irradiation, using the MTT assay described above.

Evaluation of Oxidative Stress

Generation of ROS was assessed with use of a reactive oxygen species assay kit according to the manufacturer's instructions. HaCaT cells were pretreated with 20, 40, and 80 µg/mL of SEP-E for 2 h at 37 °C and then irradiated with UVB (40 mJ/cm^2). HaCaT cells were cultured for another 4 h, and the medium was discarded. The cells were cultured in serum-free DMEM containing 10 µM DCFH-DA at 37 °C for 30 min. Cells were washed three times with PBS and detected on a microplate reader at an 488 nm and an 525 nm (SpectraMax 190, Molecular Devices LLC, San Jose, CA, USA).

Table 1. Nucleotide Sequences of Primers used in RT-PCR Experiments

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
β-actine	GGATTCCTATGTGGGCGACGA	CGCTCGGTGAGGATCTTCATG
CAT	ATGGTCCATGCTCTCAAACC	CAGGTCATCCAATAGGAAGG
SOD1	GGGAGATGGCCCAACTACTG	CCAGTTGACATCGAACCGTT
SOD2	TAGGGCTGAGGTTTGTCCAG	GGAGAAGTACCAGGAGGCGT
IL6	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
IL7	AGTTTCAGAAGGCACAACAA	ATGCAGCTAAAGTTCGTGTT
IL8	CTCTCTTGGCAGCCTTCC	CTCAATCACTCTCAGTTCTTTG
MIP-1β	CCCTGGGTCACTGAGTACATGA	CTCTCTCCCTTCTGAGCAGCTG
MCP-1	CTCACCTGCTGCTACTCATTC	GCTTGAGGTGGRRGRGGAAAA

RT-PCR, reverse transcription-polymerase chain reaction; β-actin, beta-actin; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; IL-6, interleukin 6; IL-7, interleukin 7; IL-8, interleukin 8; MIP-1, macrophage inflammatory protein-1 β; MCP-1, monocyte chemoattractant protein 1

Investigation of Antioxidant Enzymes and Inflammation-Related Genes

Total RNA extraction and cDNA synthesis were conducted using the TRIzol Reagent and AccuPower® CycleScript RT PreMix (Bioneer, Daejeon, Korea), respectively (Tang *et al*. 2017). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA expression of CAT (catalase), SOD1 (superoxide dismutase 1), SOD2 (superoxide dismutase 2), IL-6 (interleukin 6), IL-7 (interleukin 7), IL-8 (interleukin 8), MIP-1β (macrophage inflammatory protein-1 β), and MCP-1 (monocyte chemoattractant protein 1) as the house-keeping gene were performed using primers in Table1. Amplification and analysis were performed using the QuantStudio 6 Flex Real-time PCR System (Thermo Fisher, Waltham, Massachusetts, USA).

Statistical Analysis

Data are presented as mean ± standard deviation. One-way analysis of variance using GraphPad Prism ver. 5.03 (GraphPad Software Inc., San Diego, CA, USA) was performed for multi-group comparisons. T tests were applied to analyze the significance of the variations among the groups. Statistical significance was set at $p<0.05$ and $p<0.001$.

RESULTS AND DISCUSSION

Effects of SEP-E on UVB-induced Oxidative Damage in HaCaT Cells

First, the cytotoxicity was evaluated depending on the concentration of SEP-E. HaCaT cells were treated with increasing doses of SEP-E (10, 20, 40, 60, 80, 100, 200, 400, 600, 800, or 1,000 μg/mL) for 24 h, and the MTT assay was used to determine the results. As Fig. 1 shows, low doses (up to 80 μg/mL) of SEP-E had no significant cytotoxic effect on HaCaT cells, whereas higher doses (100 and 1,000 μg/mL) were more cytotoxic.

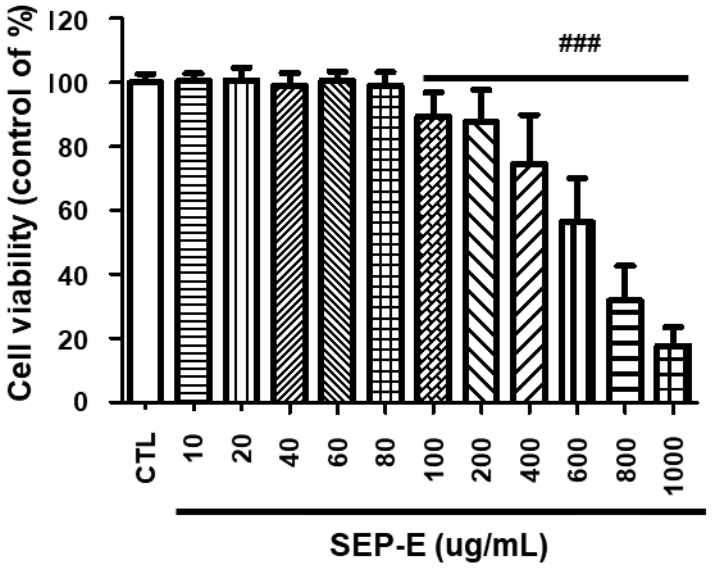

Fig. 1. Effects of steam-exploded pine extract (SEP-E) on cell viability in HaCaT keratinocytes. HaCaT cells were inoculated into 96-well plate at a 3×10^3 cells/well and incubated for 24 h. The results show the mean and standard deviation for three independent experiments. $^{###}p<0.001$ compared with the CTL. CTL is untreated control cell.

Next, the protective effect of SEP-E on HaCaT cells damaged by UVB was determined. Human skin keratinocytes are essential cells in the skin and connective tissue, and are continuously exposed to UV irradiation (Fusenig and Boukamp 1998). HaCaT cells pretreated with SEP-E were exposed to 40 mJ/cm^2 of UVB without any protective cover. After UVB irradiation, cells were incubated with the indicated doses of SEP-E for 6 h. As shown in Fig. 2, UVB (40 mJ/cm^2) significantly induced cell death in the HaCaT cells. The MTT assay showed that the UVB irradiated cells without SEP-E displayed 56.3% cell viability, compared to non-irradiated control cells, and SEP-E treatment (80 μg/mL) markedly improved the cell viability to 85.5%. These results suggest that SEP-E exhibited a significant protective effect, which may be due to its free-radical scavenging activity.

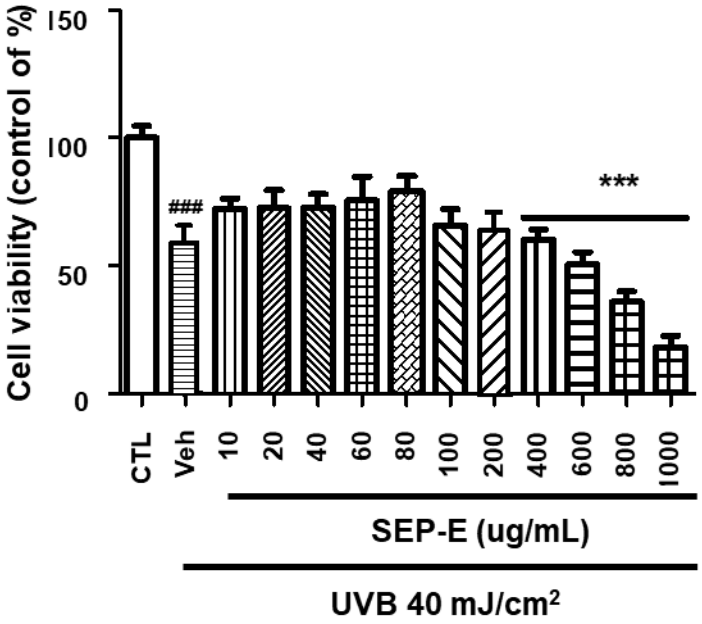

Fig. 2. Effects of steam-exploded pine extract (SEP-E) on cell viability in UVB (40 mJ/cm^2) irradiated HaCaT keratinocytes. The results show the mean and standard deviation for three independent experiments. ###p<0.001 compared with the CTL. ***p<0.001 compared with the Veh. CTL is untreated control cell. Veh is UVB-irradiated control cells.

Effects of SEP-E *vs.* UVB-induced intracellular ROS levels in HaCaT Cells

UVB irradiation induces the generation of ROS near or within the cell surface membrane of human skin (Jurkiewicz and Buettner 1994). Oxidative stress occurs when UV-induced generation of ROS exceeds the capacity of cells to chemically reduce ROS *via* both enzymatic and non-enzymatic mechanisms. Severe oxidative stress can result in apoptotic or necrotic cell death (Bertling *et al*. 1996). To investigate the protective effects of SEP-E on UVB-irradiated HaCaT cells, the intracellular ROS levels were evaluated using DCF-DA, a fluorescent probe. As shown in Fig. 3, ROS generation in UVB-irradiated cells (6.77) was significantly increased as compared to that in normal cells (0.99). In UVB-irradiated cells, SEP-E significantly reduced ROS generation at concentrations between 40 and 80 μg/mL (3.12 and 3.35, respectively). In addition, when compared with 10 mM (2.82) NAC, showed similarly reduced values. In UVB-irradiated cells, SEP-E reduced ROS generation and NAC serving as a ROS scavenger, confirming that there was as much antioxidant effect as the drug control (Fig. 3). These results suggest that SEP-E is a free-radical scavenger.

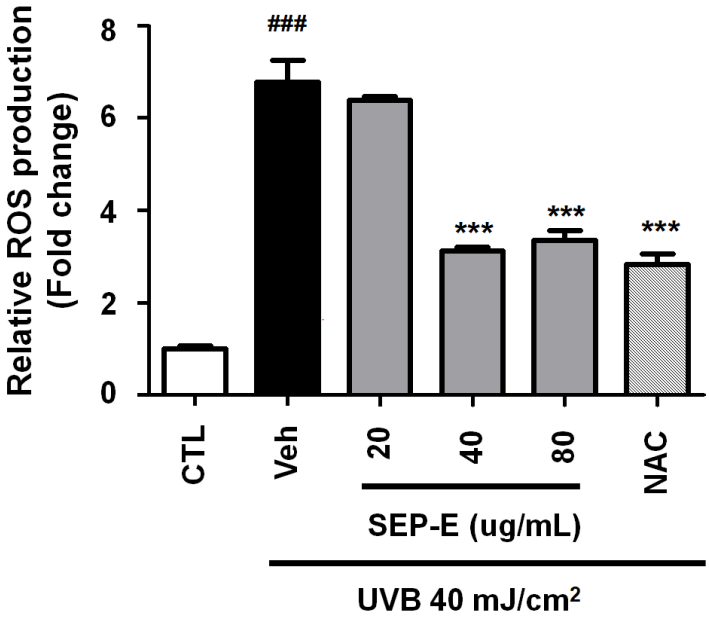

Fig. 3. Effects of steam-exploded pine extract (SEP-E) on intracellular reactive oxygen species (ROS) levels in UVB-irradiated (40 mJ/cm^2) HaCaT keratinocytes. The results show the mean and standard deviation for three independent experiments. $^{###}p<0.001$ compared with the CTL. ***$p<0.001$ compared with the Veh. CTL is untreated control cell. Veh is UVB-irradiated control cells. NAC(10 mM N-acetyl-L-cysteine) is positive control group.

Effects of SEP-E on the Antioxidant Gene Expression in UVB-irradiated HaCaT Cells

When irradiated with UVB, skin cells secrete ROS products (Ito *et al.* 2003). The high expression level of ROS can effectively oxidize DNA, cellular proteins, and lipids, thus activating various signaling pathways and the release of many kinds of inflammatory mediators (Fischer *et al.* 2006). Antioxidant enzymes protect the skin from UVB-induced thickening and wrinkle formation, and the activity of these enzymes may need enhancement to prevent photoaging (Draelos 2007). Figure 4 shows the intracellular antioxidant enzyme activity of SEP-E in UVB-irradiated HaCaT cells. In cells treated with SEP-E, the mRNA expression of the following antioxidant enzymes was evaluated: catalase (CAT), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2). The activity of CAT was reduced in UVB-irradiated cells (0.29) compared to normal cells (1.08). This reduction was attenuated by various concentrations of SEP-E (20, 40, and 80 μg/mL) and the CAT activity at these concentrations was 0.36, 0.78, and 0.91, respectively. The cellular SOD1 activity in UVB-irradiated cells was reduced (0.51) as compared to that in normal cells (1.11). However, the reduction in CAT activity was significantly attenuated ($p<0.001$) by treatment with SEP-E. The SOD1 activities at 20, 40, and 80 μg/mL SEP-E were 0.72, 0.95, and 1.21, respectively. SEP-E attenuated the UVB-induced reduction in SOD2 activity in HaCaT cells. Following SEP-E treatment, the SOD2 activity in UVB-irradiated cells significantly increased, with levels ranging from 0.78 to 0.91. Results of the RT-PCR assay revealed that UVB significantly reduced the mRNA expressions of CAT, SOD1, and SOD2 in HaCaT cells. Following treatment with various concentrations of SEP-E in UVB-irradiated HaCaT cells, the mRNA levels of these antioxidant enzymes increased. These results suggest that SEP-E treatment increases the activity of antioxidant enzymes in UVB-irradiated HaCaT cells, thus reducing UVB-induced oxidative stress.

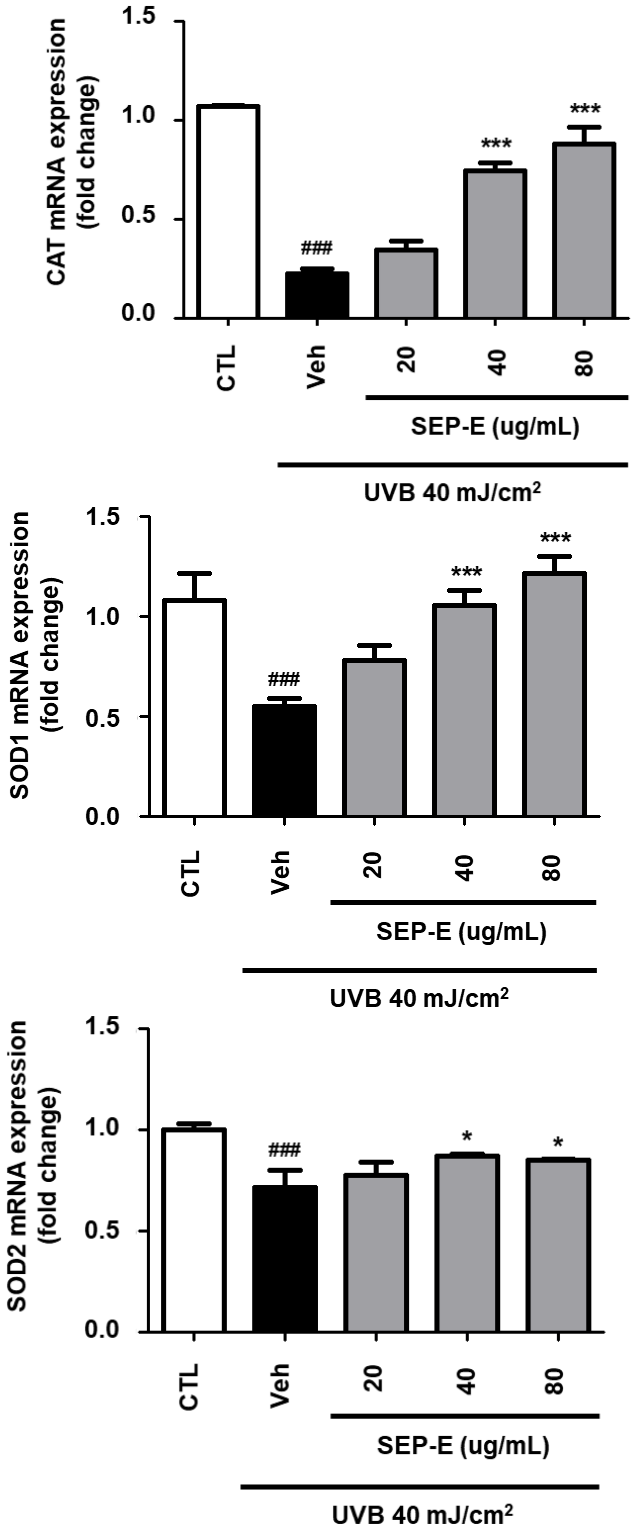

Fig. 4. Antioxidant effect of steam-exploded pine extract (SEP-E). Reverse transcription-polymerase chain reaction analysis of the expression of catalase (CAT), superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2), after SEP-E (20, 40 and 80 μg/mL) treatment. The results show the mean and standard deviation for three independent experiments. ###p<0.001 compared with the CTL. ***p<0.001 compared with the Veh. CTL is untreated control cell. Veh is UVB-irradiated control cells.

Effects of SEP-E on the Inflammatory Gene Expression in UVB-irradiated HaCaT Cells

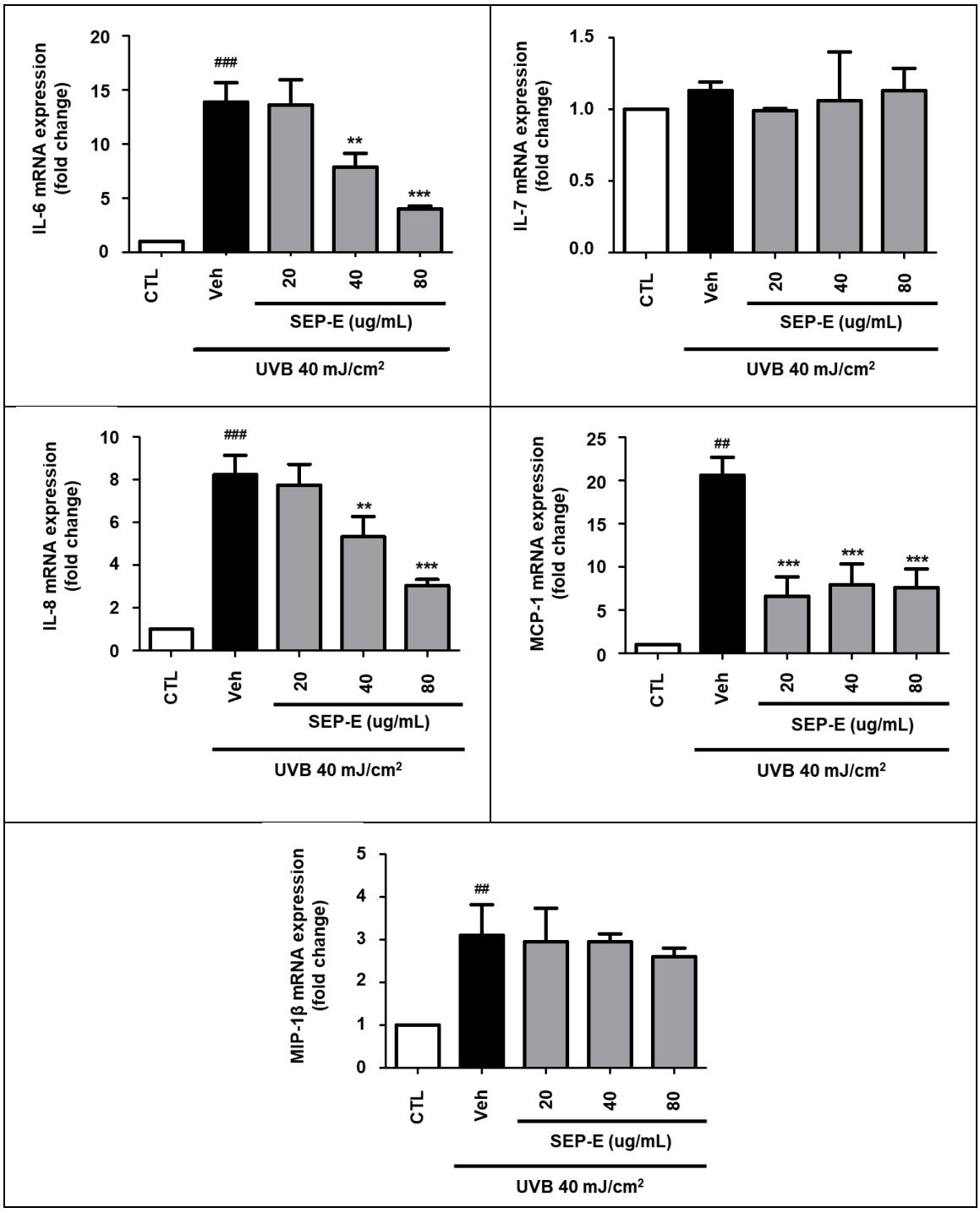

Fig. 5. Inflammatory effect of steam-exploded pine extract (SEP-E). Reverse transcription-polymerase chain reaction analysis of the expression of interleukin (IL)-6, IL-7, IL-8, macrophage inflammatory protein-1β (MIP-1β) and monocyte chemoattractant protein-1 (MCP-1), after SEP-E (20, 40, and 80 μg/mL) treatment. The results show the mean and standard deviation for three independent experiments. ##p<0.01 and ###p<0.001 compared with the CTL. ***p<0.001 compared with the Veh. CTL is untreated control cell. Veh is UVB-irradiated control cells.

UVB irradiation enhances the inflammatory response by activating NF-κB and stimulating the expression of NF-κB-mediated inflammatory cytokines (Bradley 2008). To analyze whether SEP-E exerts anti-inflammatory effects on UVB-damaged HaCaT cells, mRNA expression levels of interleukin (IL)-6, IL-7, IL-8, macrophage inflammatory protein-1β (MIP-1β), and monocyte chemoattractant protein-1 (MCP-1) were measured (Fig. 5). Expressions of IL-6 ($p<0.001$), IL-8 ($p<0.001$), MCP-1 ($p<0.01$), and MIP-1β ($p<0.01$) related genes in HaCaT cells after UVB irradiation significantly increased. The activities of IL-6 and IL-8 showed a concentration-dependent decrease with SEP-E, and showed a significant decrease at 40 and 80 μg/mL SEP-E 6 ($p<0.001$). The UVB-induced expression of MCP-1 showed the strongest decrease after treatment with 20 to 80 μg/mL SEP-E. SEP-E treatment did not induce a decrease in IL-7 and MIP-1β expressions. A reduced amount of subcutaneous fat is a representative phenomenon of the photoaging process (Kim *et al.* 2010). UVB-induced cytokines, such as IL-6 and IL-8 in skin fibroblasts, have been reported as regulators of subcutaneous fat metabolism (Kim *et al.* 2011). Our results suggest that the administration of SEP-E reduces subcutaneous fat by preventing UVB-induced expression of cytokines in HaCaT keratinocytes.

CONCLUSIONS

1. Steam-exploded pine extract (SEP-E) has the potential to attenuate photoaging.
2. SEP-E treatment (80 μg/mL) significantly improved the viability of ultraviolet B (UVB)-irradiated skin cells (HaCaT), indicating that SEP-E had a significant protective effect. In addition, SEP-E effectively attenuated reactive oxygen species (ROS) levels.
3. The messenger ribonucleic acid (mRNA) levels of antioxidant enzymes (CAT, SOD1, and SOD2) were increased and UVB-induced expressions of HaCaT-derived cytokines such as MCP-1, IL-6, and IL-8 were effectively attenuated by the administration of SEP-E.
4. Taken together, SEP-E was shown to have a strong potential to attenuate photoaging by inhibiting UV-induced expression of cytokines.

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