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Ethyl acetate fraction of *Sargassum pallidum* extract attenuates particulate matter-induced oxidative stress and inflammation in keratinocytes and zebrafishWook Chul Kim¹, Ji-Won Park², Bohyun Yun², WonWoo Lee², Kyung-Min Choi², Seung-Hong Lee^{1,3}✉¹Department of Medical Science, Soonchunhyang University, Asan 31538, Republic of Korea²Honam National Institute of Biological Resources, 99, Gohadoan-gil, Mokpo-si, Jeollanam-do 58762, Republic of Korea³Department of Pharmaceutical Engineering, Soonchunhyang University, Asan 31538, Republic of Korea

ABSTRACT

Objective: To evaluate the effect of the ethyl acetate fraction derived from *Sargassum pallidum* extract against particulate matter (PM)-induced oxidative stress and inflammation in HaCaT cells and zebrafish.

Methods: HaCaT cells and zebrafish were used to evaluate the protective effects of the ethyl acetate fraction of *Sargassum pallidum* extract against PM-induced oxidative stress and inflammation. The production of nitric oxide (NO), intracellular ROS, prostaglandin E₂ (PGE₂), and pro-inflammatory cytokines, and the expression levels of COX-2, iNOS, and NF-κB were evaluated in PM-induced HaCaT cells. Furthermore, the levels of ROS, NO, and lipid peroxidation were assessed in the PM-exposed zebrafish model.

Results: The ethyl acetate fraction of *Sargassum pallidum* extract significantly decreased the production of NO, intracellular ROS, and PGE₂ in PM-induced HaCaT cells. In addition, the fraction markedly suppressed the levels of pro-inflammatory cytokines and inhibited the expression levels of COX-2, iNOS, and NF-κB. Furthermore, it displayed remarkable protective effects against PM-induced inflammatory response and oxidative stress, represented by the reduction of NO, ROS, and lipid peroxidation in zebrafish.

Conclusions: The ethyl acetate fraction of *Sargassum pallidum* extract exhibits a protective effect against PM-induced oxidative stress and inflammation both *in vitro* and *in vivo* and has the potential as a candidate for the development of pharmaceutical and cosmeceutical products.

KEYWORDS: Particulate matter; Inflammation; Oxidative stress; *Sargassum pallidum*; Ethyl acetate fraction; Zebrafish

1. Introduction

Rapid industrialization and urbanization have accelerated the release of fine dust and subsequent air pollution, which has become a serious public health threat. Especially, particulate matter (PM), one of the air pollutants, contains components that are harmful to human health such as carbon, ions, and metallic or non-metallic elements[1,2]. Previous studies showed that PM is the major cause of inflammation and oxidative stress which result in a variety of diseases such as respiratory, cardiovascular, and brain diseases[3]. In addition, a recent study reported that PM is harmful to skin health[4]. The skin plays an important role in protecting the internal organs

Significance

A previous study suggested that an ethyl acetate fraction derived from *Sargassum pallidum* extract possesses strong antioxidant activities *in vitro*. However, the protective effect of this fraction against particulate matter-induced inflammation and oxidative stress has not been reported. Our study demonstrates that the ethyl acetate fraction of *Sargassum pallidum* extract alleviated particulate matter-stimulated oxidative stress and inflammation in HaCaT cells and zebrafish by reducing ROS production and the expression levels of pro-inflammatory mediators and cytokines.

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from external harmful substances. External factors, especially PM, can penetrate the skin and evoke toxicity and inflammation leading to diverse skin dysfunctions such as psoriasis, atopic dermatitis, and acne[5–7].

Previous studies have reported that PM causes inflammation by promoting overproduction of reactive oxygen species (ROS)[8]. ROS increases the expression level of nuclear factor kappa B (NF- κ B) and pro-inflammatory proteins and cytokines such as cyclooxygenase-2 (COX-2), prostaglandin E₂ (PGE₂), nitric oxide (NO), tumor necrosis factor (TNF), and interleukin (IL)[9]. Therefore, many studies have been conducted on therapeutic agents and cosmeceuticals that could prevent or improve PM-induced skin diseases. Especially, natural products without toxicity or side effects have attracted great interest worldwide.

Natural products that are natural compounds or substances produced by living organisms can produce a wide range of secondary metabolites. The metabolites are classified according to their chemical structure and composition, and the representative examples include phenolic, terpenoid, and alkaloid compounds[10,11]. Phenolic compounds possess a phenolic hydroxyl group that can readily bind to proteins and macromolecules and have reported anti-cancer and antioxidant effects in various cellular and animal models[12]. Other metabolites also show a variety of bioactivities such as anti-allergic, anti-cancer, and antibacterial effects[13,14].

Owing to their habitat, marine seaweed is rich in bioactive compounds[15,16]. Hence, there have been many previous studies using marine seaweed as a potential ingredient to develop pharmaceutical and cosmeceuticals, responsible for blocking air pollution-induced skin damage[17]. *Sargassum pallidum* (*S. pallidum*), a type of seaweed, inhabits Korea (southern coast, Jeju Island), China, Japan, and Indonesia, and is known to have anti-obesity and anti-cancer effects[18,19]. In particular, the ethyl acetate fraction of *S. pallidum* has been shown to have high antioxidant effects[20]. Recently, numerous studies have reported that antioxidants derived from natural sources protect against PM-induced skin damage[21,22]. Hence, potent natural antioxidants are considered potential pharmacological and cosmeceutical agents for the prevention of PM-induced skin diseases. Therefore, it was hypothesized that the ethyl acetate fraction of *S. pallidum*, which has high antioxidant activity, would suppress PM-induced ROS generation and subsequent skin inflammation. Notably, so far, the protective effects of the ethyl acetate fraction of *S. pallidum* against PM-induced inflammation and oxidative stress have not been reported. Therefore, our study investigated the protective effects of the ethyl acetate fraction of *S. pallidum* on ROS generation and skin inflammation in PM-induced HaCaT cells and a zebrafish model.

2. Materials and methods

2.1. Materials and reagents

2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA), and DAF-

FM Diacetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acridine orange, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Thermo Fisher Scientific. Cytokine kits were purchased from R&D Systems and Abcam. Antibodies for Western blot analysis were purchased from Cell Signaling Technology (Danvers, MA, USA), Santa Cruz Biotechnology (Dallas, TX, USA), and Invitrogen (Carlsbad, CA, USA).

2.2. Preparation of PM

Urban aerosol PM collected in Beijing, China over 10 years was purchased from the National Institute for Environmental Studies, Ibaraki, Japan. PM was sonicated for 30 min and used within 1 h to avoid variability.

2.3. Preparation of the ethyl acetate fraction of *S. pallidum* and analysis of the total phlorotannin content

S. pallidum collected from Jeju Island, Korea in August 2020 was purchased in Para Jeju (Jeju, Korea). The preparation of the ethyl acetate fraction of *S. pallidum* extract was done according to the methods described in a previous study[23]. In brief, powdered *S. pallidum* was extracted with 70% ethanol using a shaking incubator for 24 h at room temperature. The mixture was filtered with a filter paper to remove any residues and the filtrate was obtained. Evaporation was then performed using a rotary evaporator to obtain the 70% ethanol extract, which was dissolved in water. Next, using the solvent-solvent partition technique, the ethyl acetate fraction was isolated from the *S. pallidum* extract and stored in the dark at 4 °C until further use.

The total phlorotannin content in the ethyl acetate fraction of *S. pallidum* extract was determined according to the Folin-Ciocalteu method. In this method, 1 mL of the ethyl acetate fraction of *S. pallidum* extract, 1 mL of 95% EtOH, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent were mixed. The mixtures were allowed to react for 5 min, and then 1 mL of 5% Na₂CO₃ was added, and the mixture was thoroughly mixed and placed in the dark for 1 h. The absorbance was measured at 725 nm. The total phlorotannin content was expressed based on a phloroglucinol (PG) standard curve. According to measurements of the quantity of total phlorotannin, the ethyl acetate fraction of *S. pallidum* extract contained (42.16 ± 4.22) μg PG/mg.

2.4. Cell culture

Human keratinocyte (HaCaT) cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in DMEM (Welgene, Republic of Korea) containing 10% FBS (Welgene, Republic of Korea), 100 U/mL of penicillin, and 100 μg/mL of streptomycin in an incubator with 5% CO₂ at 37 °C.

2.5. Evaluation of cell viability and ROS generation

The experimental procedures were conducted in accordance with the previous method[23]. MTT colorimetric assay and DCFH-DA assay were performed to assess cell viability and intracellular ROS levels, respectively. Following sample treatment and a 24-hour incubation period post-seeding, MTT was introduced into the wells and incubated for 3 h. Subsequently, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO), and absorbance was measured at 540 nm. For ROS measurement, DCFH-DA was added to the wells after a 30-minute incubation period, and the fluorescence intensity was measured using a microplate reader (Synergy HTX Multi-Mode Reader) at excitation/emission wavelengths of 485 nm and 528 nm.

2.6. Measurement of NO production

NO production measurement was performed using a previously described method with some modifications[21]. HaCaT cells were seeded in a 24-well plate for 24 h. Cells were then treated with the ethyl acetate fraction of *S. pallidum* extract. After 1 h, PM (50 µg/mL) was added to the cells and further incubated for 24 h. After incubation, NO production was measured by a Griess assay.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Following 1-hour treatment with the ethyl acetate fraction of *S. pallidum* extract, HaCaT cells were incubated with PM for 24 h. The cell culture media were then collected to evaluate the expression levels of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and PGE₂ using commercial ELISA kits in accordance with the manufacturer's instructions.

2.8. Western blot analysis

The effect of the ethyl acetate fraction of *S. pallidum* extract on NF- κ B, COX-2, and iNOS expression in PM-stimulated HaCaT cells was analyzed using a Western blot assay. The cells were harvested and lysed using the NE-PER[®] nuclear and cytoplasmic extraction kit. Total protein concentrations were determined. Equal proteins were subjected to 15% SDS-PAGE gel. After separation by electrophoresis, the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in TBST (a blocking buffer) for 1.5 h at room temperature and then incubated with each of the following primary antibodies: NF- κ B (CAT NO. 8242), COX-2 (CAT NO. 4842), iNOS (CAT NO. 2977), nucleolin (CAT NO. 14574), and GAPDH (CAT NO. sc-365062) diluted in a blocking buffer at 1:1 000. Thereafter, they were incubated with secondary antibodies: goat anti-rabbit IgG (Cat No. G-21234), and goat anti-mouse IgG (Cat No. G-21040) diluted in a blocking buffer at 1:3 000 for 2 h at room temperature and the membranes were washed with TBST. Protein bands were captured with a chemiluminescence detection

system (FUSION SOLO Vilber Lourmat system, Paris, France). Quantitative analysis of protein bands was performed using a ImageJ software. Data were expressed as fold of control. Nucleolin served as a loading control for nuclear extracts, and GAPDH as a loading control for iNOS and COX-2.

2.9. Zebrafish maintenance

Zebrafish were maintained according to a previously described method[24]. They were obtained from a commercial dealer (Sin-yong Aquarium, Cheonan, Korea) and housed in a 3 L acrylic tank under controlled conditions at 28.5°C with a 14 h:10 h light-dark cycle. The zebrafish were fed three times daily. Embryos were obtained through natural spawning induced by light exposure. Embryo collection was completed within a 30-minute in Petri dishes.

2.10. Evaluation of the morphological changes of zebrafish treated with the ethyl acetate fraction of *S. pallidum* extract

The survival rate of zebrafish was calculated for 3 d by counting the surviving zebrafish daily. Morphological observation in zebrafish embryos exposed to different concentrations of the ethyl acetate fraction of *S. pallidum* extract from 0 day post-fertilization (dpf) to 3 dpf was performed.

2.11. Evaluation of PM-stimulated inflammation and oxidative stress in zebrafish treated with the ethyl acetate fraction of *S. pallidum* extract

Zebrafish embryos at the 7-9 hour post-fertilization (hpf) stage were transferred into a 6-well plate (15 embryos per well, 3 wells per group) and treated with the ethyl acetate fraction of *S. pallidum* extract (25, 50, and 100 µg/mL) for 1 h. Subsequently, the embryos were exposed to PM at a concentration of 50 µg/mL. The survival rate of PM-treated zebrafish was determined over three days by counting the surviving zebrafish daily.

At 3 dpf, intracellular ROS, cell death, lipid peroxidation, and NO levels were assessed using DCFH₂-DA, acridine orange, 1,3-bis(diphenylphosphino) propane (DPPP), and DAF-FM-DA, respectively. The fluorescence intensities were measured using a fluorescence microscope (EVOS, Thermo Fischer Scientific, USA) [25,26].

2.12. Statistical analysis

All the experiments were conducted in triplicate. The data are expressed as mean \pm standard deviation (SD), and analyzed by one-way analysis of variance. Analysis of the results was performed using the SPSS statistical program (Version 28, IBM, Armonk, NY, USA). Significant differences ($P < 0.05$) between the groups were identified using the Tukey *post hoc* test.

2.13. Ethical statement

Animal studies were conducted in accordance with the University Guidelines for Animal Experimentation and approved by the Laboratory Animal Administration Committee of Soonchunhyang University (Approval number: SCH23-0032).

3. Results

3.1. Cytotoxicity of PM on HaCaT cells

We first examined the effects of PM on HaCaT cells at the indicated concentrations via DCFH-DA and MTT assay. As shown in Figure 1, PM treatment reduced cell viability and increased intracellular ROS level in a dose-dependent manner. In particular, PM at the concentration of 50 µg/mL significantly reduced cell survival rate about 30% and promoted intracellular ROS production by about 200% in human keratinocytes. Taken together, the optimal concentration of PM treatment on HaCaT cells was determined to be 50 µg/mL.

3.2. Effect of the ethyl acetate fraction of *S. pallidum* extract on PM-induced oxidative stress in HaCaT cells

Before evaluating the protective effect of the ethyl acetate fraction of *S. pallidum* extract against PM-induced oxidative stress, the cytotoxicity of varying concentrations of the ethyl acetate fraction of *S. pallidum* extract on HaCaT cells was determined. The cytotoxicity results revealed that the ethyl acetate fraction at 200 µg/mL showed significant cytotoxicity on HaCaT cells (Figure 2A). In contrast, no cytotoxicity was observed at concentrations of up to 100 µg/mL. Thus, the ethyl acetate fraction of *S. pallidum* extract at the non-toxic concentrations was used for further investigations. When HaCaT cells were treated with PM, cell viability significantly decreased, whereas the ethyl acetate fraction of *S. pallidum* extract protected against PM-induced cellular damage in a dose-dependent manner (Figure 2B). In addition, the intracellular ROS level was significantly elevated in PM-treated cells, while treatment with the ethyl acetate fraction of *S. pallidum* extract remarkably reduced the intracellular ROS level in a dose-dependent manner (Figure 2C and 2D). These findings suggest that the ethyl acetate fraction of *S. pallidum* extract protects against cell damage from oxidative stress by suppressing PM-induced overproduction of ROS.

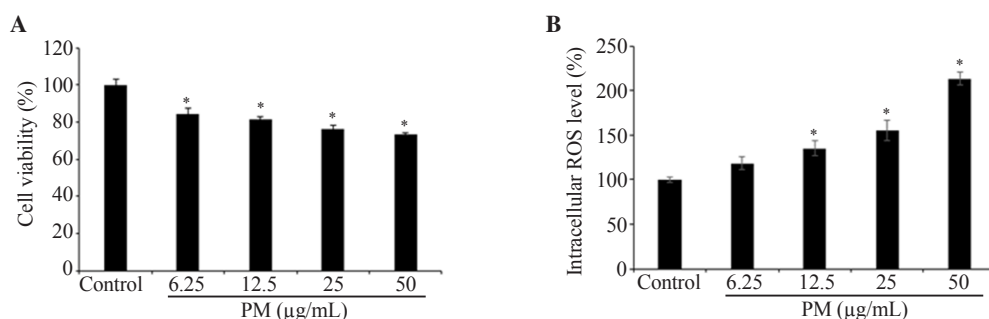


Figure 1. Effects of particulate matter on cell viability (A) and intracellular ROS level (B) in HaCaT cells. Values are expressed as mean ± SD of triplicate experiments. *P < 0.01 compared to the control group. PM: particulate matter; ROS: reactive oxygen species.

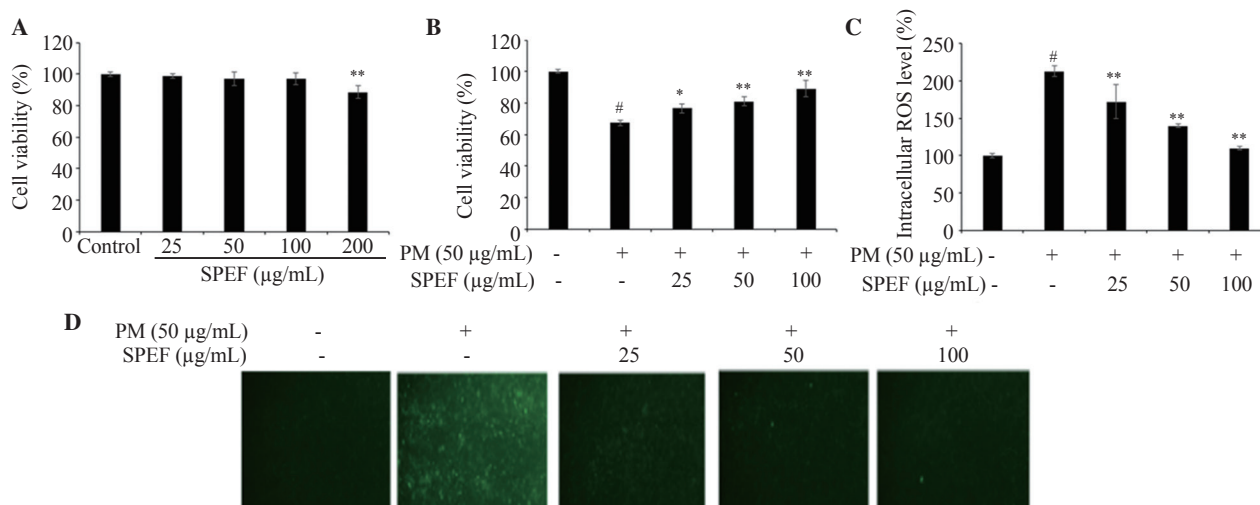


Figure 2. Effects of the ethyl acetate fraction of *Sargassum pallidum* (SPEF) on PM-treated HaCaT cells. (A-B) Effect of SPEF on the viability of HaCaT cells with or without PM exposure. (C) Intracellular ROS levels were detected by DCFH-DA assay. A fluorescence spectrophotometer was used for the quantitative analysis of ROS generation. (D) Fluorescence images of PM-induced ROS generation. Values are expressed as mean ± SD of triplicate experiments. #P < 0.01 compared to the control group, and *P < 0.05 and **P < 0.01 compared to the PM-treated group.

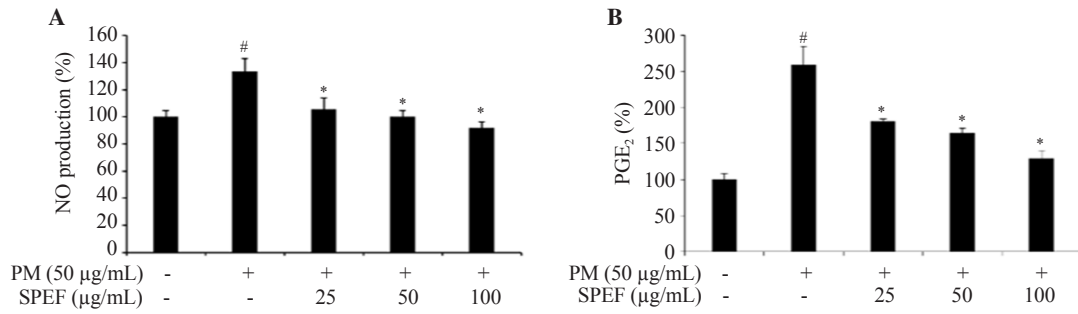


Figure 3. Effects of SPEF on the production of pro-inflammatory mediators NO (A) and PGE₂ (B) in PM-treated HaCaT cells. Values are expressed as mean ± SD of triplicate experiments. [#]*P* < 0.01 compared to the control group and ^{*}*P* < 0.01 compared to the PM-treated group. NO: nitric oxide; PGE₂: prostaglandin E₂.

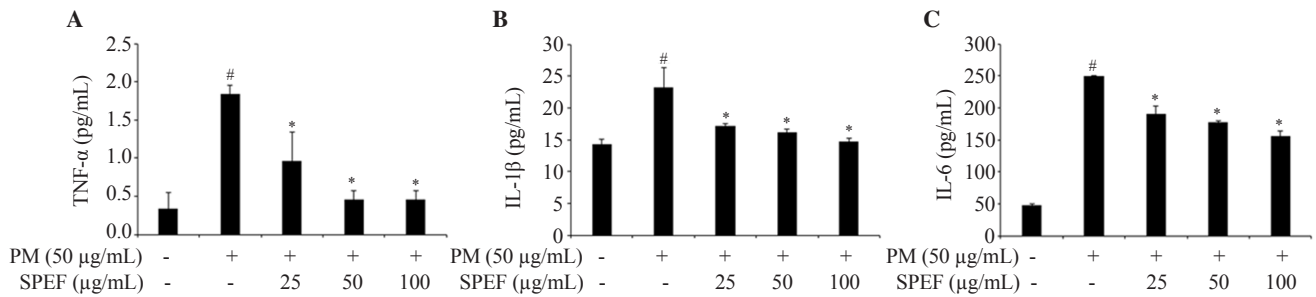


Figure 4. Effects of SPEF on the expression of pro-inflammatory cytokines TNF-α (A), IL-1β (B), and IL-6 (C) in PM-treated HaCaT cells. Values are expressed as mean ± SD of triplicate experiments. [#]*P* < 0.01 compared to the control group and ^{*}*P* < 0.01 compared to the PM-treated group.

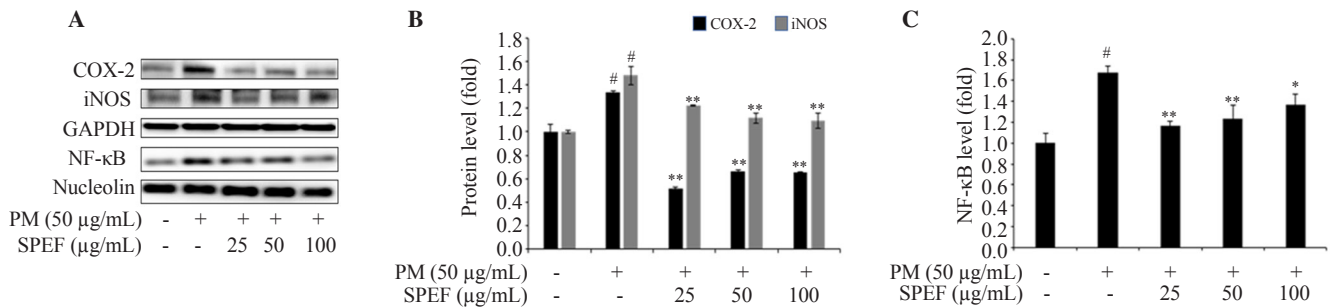


Figure 5. Effects of SPEF on the protein expression of iNOS, COX-2, and NF-κB in PM-treated HaCaT cells. (A) Protein bands. (B-C) Quantitative results. Values are expressed as mean ± SD of triplicate experiments. [#]*P* < 0.01 compared to the control group, ^{*}*P* < 0.05 and ^{**}*P* < 0.01 compared to the PM-treated group.

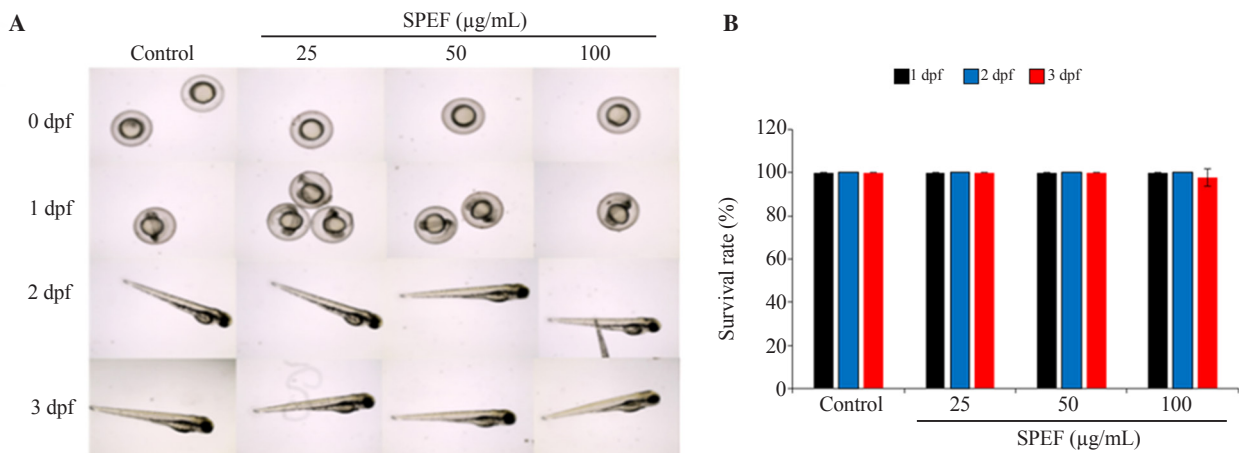


Figure 6. Developmental toxicity of SPEF in zebrafish. (A) Representative images of developmental stages of zebrafish embryos exposed to the indicated concentrations of SPEF from 0 to 3 days post fertilization (dpf). (B) Variations of survival rates in zebrafish embryos exposed to SPEF during 1-3 dpf. Values are expressed as mean ± SD of triplicate experiments.

3.3. Effect of the ethyl acetate fraction of *S. pallidum* extract on NO and PGE₂ production in PM-treated HaCaT cells

PM-induced ROS production increases inflammatory response by promoting the levels of inflammatory mediators such as NO and PGE₂. Therefore, we investigated whether the ethyl acetate fraction of *S. pallidum* extract can inhibit the production of PM-induced NO and PGE₂. As shown in Figure 3A and 3B, PM significantly elevated the production of NO and PGE₂, while the ethyl acetate fraction of *S. pallidum* extract significantly decreased the production of NO and PGE₂ dose-dependently. These results indicate that the ethyl acetate fraction of *S. pallidum* extract protects HaCaT cells from an excessive inflammatory response by suppressing the PM-induced NO and PGE₂ production.

3.4. Effect of the ethyl acetate fraction of *S. pallidum* extract on pro-inflammatory cytokine levels in PM-treated HaCaT cells

Pro-inflammatory cytokines like TNF- α , IL-1 β , and IL-6 play an important role in abnormal inflammatory response and related skin disorders. Therefore, the expression levels of these pro-inflammatory cytokines in PM-treated HaCaT cells were investigated. The effects of the ethyl acetate fraction of *S. pallidum* extract on the expression levels of PM-induced pro-inflammatory cytokines are shown in Figure 4. The expression levels of TNF- α , IL-1 β , and IL-6 were significantly increased in PM-treated HaCaT cells. In contrast, the ethyl acetate fraction of *S. pallidum* extract reduced their levels in PM-treated HaCaT cells in a dose-dependent manner.

3.5. Effect of the ethyl acetate fraction of *S. pallidum* extract on the expression of iNOS, COX-2, and NF- κ B in PM-treated HaCaT cells

NF- κ B is a key transcriptional factor in skin inflammation by regulating pro-inflammatory enzymes including iNOS and COX-2. Therefore, we investigated the effect of the ethyl acetate fraction of *S. pallidum* extract on iNOS, COX-2, and NF- κ B expression. The expressions of iNOS, COX-2, and NF- κ B were significantly increased in PM-treated HaCaT cells (Figure 5). In contrast, the ethyl acetate fraction of *S. pallidum* extract inhibited PM-induced iNOS, COX-2, and nuclear NF- κ B expressions, indicating that the ethyl acetate fraction of *S. pallidum* extract inhibits the production of pro-inflammatory mediators and cytokines through downregulation of iNOS, COX-2, and nuclear NF- κ B.

3.6. Effect of the ethyl acetate fraction of *S. pallidum* extract on the survival rate and morphological changes in zebrafish

To determine the toxicity of the ethyl acetate fraction of *S. pallidum* extract, we calculated the survival rate and evaluated the morphological changes in zebrafish embryos following exposure to varying concentrations of the ethyl acetate fraction. No morphological abnormalities were observed in zebrafish embryos at indicated concentrations of the ethyl acetate fraction at 0, 1, 2, and 3 dpf (Figure 6A). In addition, 25, 50, and 100 μ g/mL of the ethyl acetate fraction of *S. pallidum* extract did not result in a significant death rate in zebrafish embryos at 1-3 dpf (Figure 6B). These results indicate that the ethyl acetate fraction of *S. pallidum* extract did not cause any toxic effects on the developmental stages of zebrafish embryos.

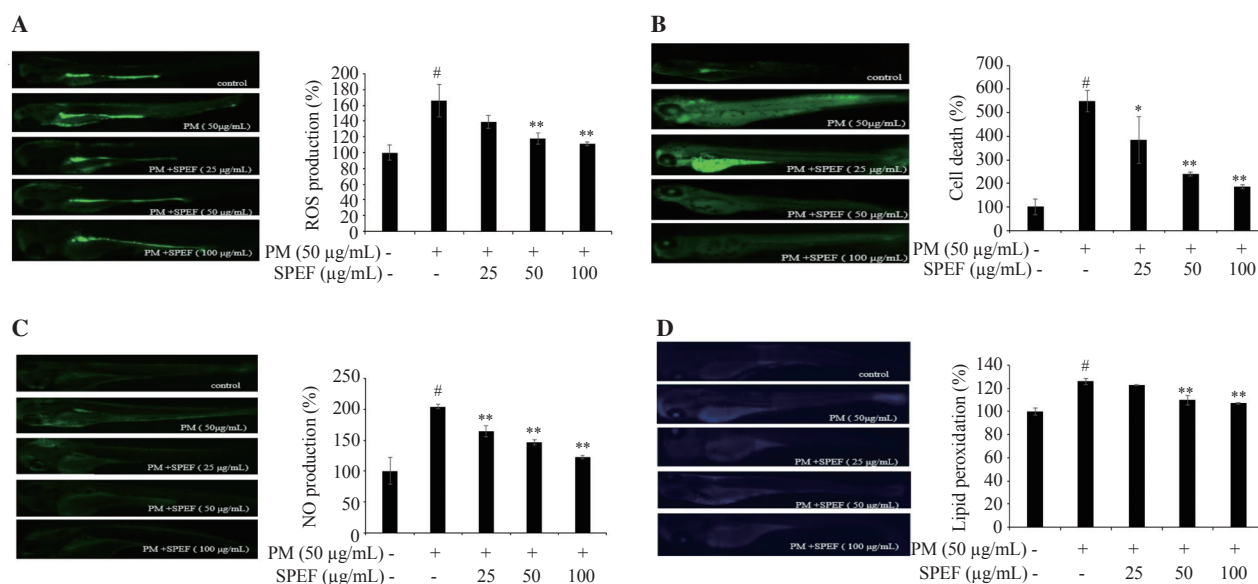


Figure 7. Protective effects of SPEF against PM-induced ROS production (A), cell death (B), NO production (C), and lipid peroxidation (D) in zebrafish. The fluorescence intensity of zebrafish embryos was quantified using the Image J program. Values are expressed as mean \pm SD of triplicate experiments. [#] $P < 0.01$ compared to the control group, and ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared to the PM-treated group.

3.7. Protective effects of the ethyl acetate fraction of *S. pallidum* extract against PM–induced oxidative stress and inflammation in zebrafish

The protective effects of the ethyl acetate fraction of *S. pallidum* extract against PM-induced ROS generation, cell death, NO production, and lipid peroxidation in the *in vivo* zebrafish model were investigated in the present study. As shown in Figure 7, ROS generation, cell death, NO production, and lipid peroxidation were assessed by measuring the relative fluorescence intensity after staining PM-treated zebrafish embryos with DCF-DA, acridine orange, DAF-FM-DA, and DPPP. After PM exposure, the fluorescence intensity was significantly increased, suggesting that ROS generation, cell death, NO production, and lipid peroxidation had taken place in the PM-treated zebrafish embryos compared to that in the control group. In contrast, when zebrafish embryos were treated with the ethyl acetate fraction of *S. pallidum* extract before PM exposure, dose-dependent reductions in ROS generation, cell death, NO production, and lipid peroxidation were observed. These findings suggest that the ethyl acetate fraction of *S. pallidum* extract might have a protective effect against PM-induced inflammation and oxidative stress *in vivo*.

4. Discussion

Recently, several studies have demonstrated that skin exposure to PM-induced oxidative stress and inflammation can lead to skin damage and the development of disease[7,27]. Thus, developing therapeutic agents is consistently needed to improve skin health against PM-induced ROS production and inflammation. Several studies have suggested that natural antioxidants can prevent pathological skin damage caused by PM-induced inflammation and oxidative stress[21,28]. Therefore, there is a great interest in natural antioxidants that can prevent PM-induced skin damage, without showing toxicity or side effects.

Seaweed is rich in polyphenolic compounds which have biological activities including antioxidant activity. *S. pallidum*, a type of brown seaweed, has been shown to have potent anti-obesity and anti-cancer effects[18,19]. In particular, the ethyl acetate fraction of *S. pallidum* exhibited strong antioxidant activity[20]. However, the effects of the ethyl acetate fraction of *S. pallidum* on PM-induced oxidative stress and inflammation have not been reported. Thus, in this study, we investigated whether the ethyl acetate fraction of *S. pallidum* attenuates PM-induced oxidative stress and inflammation in HaCaT cells and zebrafish.

Keratinocytes are main skin cells located in the outer part of the skin epidermis and contribute to maintaining skin barrier function and protecting inner skin cells against environmental irritants[29].

In recent years, several studies have demonstrated that the repeated exposure of keratinocytes to PM can induce the overproduction of ROS, which consequently results in oxidative stress and skin damage[27,30]. In this study, our results also confirmed that treatment with PM significantly increased ROS levels and decreased cell viability in HaCaT cells in a dose-dependent manner. Hence, to reduce the risk of PM-induced skin damage, attenuating oxidative stress induced by the excessive production of ROS is an effective approach. To investigate the anti-oxidative stress potential of the ethyl acetate fraction of *S. pallidum*, this study determined the inhibitory effects of this fraction on PM-induced ROS generation. We found that the fraction significantly inhibited excessive production of ROS in PM-exposed HaCaT cells. Treatment with the ethyl acetate fraction of *S. pallidum* also inhibited cell damage, suggesting the protective effects of the fraction against PM-induced cytotoxicity. These results suggest that the ethyl acetate fraction of *S. pallidum* could have a protective effect against PM-induced oxidative stress, leading to reduced cellular injuries and skin aging. Polyphenolic compounds such as phlorotannins from marine brown algae are one of the most bioactive compounds[31,32]. It possesses strong antioxidant activity. A previous study demonstrated that the ethyl acetate fraction of *S. pallidum* that is rich in phlorotannins has strong antioxidative activities[20]. The present study also confirmed that the fraction contained a considerable amount of phlorotannins. Recent studies have shown that the fraction is rich in phlorotannin content, and UHPLC-MS analysis revealed that the major constituent of these phlorotannins is the phlorotannin dimers having a main structure as phenyl-furan-phenyl with four hydroxyl groups and one methyl group on the two phenyls[20]. The results showed that phlorotannin dimers in the ethyl acetate fraction of *S. pallidum* may prove useful as a potential natural antioxidant. Therefore, these results indicate that the protective effects of the ethyl acetate fraction of *S. pallidum* against PM-induced oxidative stress can be attributed to the antioxidant effects of phlorotannin dimers contained in the fraction.

Generally, pro-inflammatory mediators such as NO and PGE₂ are representative derivatives of upregulated skin inflammation and are associated with various skin diseases[33,34]. It has previously been demonstrated that PM exposure induced inflammatory responses through the overproduction of NO and PGE₂ in PM-treated HaCaT cells[18,35]. Therefore, the present study investigated whether the ethyl acetate fraction of *S. pallidum* can inhibit the production of NO and PGE₂ in PM-treated HaCaT cells. The results showed that the levels of NO and PGE₂ increased in the PM-treated HaCaT cells, but the fraction significantly inhibited their production. These findings imply that the ethyl acetate fraction of *S. pallidum* suppresses the inflammatory response by inhibiting the production of pro-inflammatory mediators, such as NO and PGE₂. Activation of pro-inflammatory enzymes including iNOS and COX-2 plays an

important role in NO and PGE₂ production[36,37]. Thus, the inhibition of the expression levels of iNOS and COX-2 can be an effective method to alleviate PM-induced skin inflammation and related disorders. In the present study, treatment with the ethyl acetate fraction of *S. pallidum* significantly reduced iNOS and COX-2 expression levels in PM-treated HaCaT cells, indicating the fraction could inhibit the production of PM-induced pro-inflammatory mediators, which may contribute to the downregulation of the inflammatory response.

The inhibition of pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) is a key anti-inflammatory mechanism[38,39]. The results showed that the ethyl acetate fraction of *S. pallidum* significantly reduced TNF- α , IL-1 β , and IL-6 production in PM-treated HaCaT cells. These results suggest that the ethyl acetate fraction of *S. pallidum* could inhibit PM-mediated inflammatory responses in keratinocytes. NF- κ B, an oxidative stress-responsive transcription factor, plays an important role in cell injury. In particular, activated NF- κ B has important roles in the induction of a variety of pro-inflammatory enzymes and cytokines involved in inflammatory responses[40,41]. Therefore, we further examined the effects of the ethyl acetate fraction of *S. pallidum* on the activation of NF- κ B in the PM-treated HaCaT cells to identify the mechanism of the fraction-induced suppressive effect on the expression of pro-inflammatory enzymes and cytokines. We observed that the fraction inhibited PM-induced NF- κ B activation in HaCaT cells, suggesting that it reduced the levels of pro-inflammatory enzymes and cytokines by regulating NF- κ B pathway. Studies have revealed that NF- κ B activation is inhibited by antioxidant agents[42,43]. As previously mentioned, the ethyl acetate fraction of *S. pallidum* contains phlorotannins, which are potential antioxidants. Therefore, the inhibitory action of this fraction against NF- κ B activation demonstrated in this study may be associated with the antioxidant effect of phlorotannins.

Zebrafish models are useful and popular animal models for a variety of human disease research because they have most of the organs that humans have and their nervous system and various organ formation processes are very similar to those of humans[44]. Studies have reported that zebrafish can be used as a rapid and simple *in vivo* model to assess the protective effect against PM-induced inflammation and oxidative stress[21]. This study investigated the protective effects of the ethyl acetate fraction of *S. pallidum* against PM-induced inflammation and oxidative stress in a zebrafish model. The results showed that ROS generation, NO production, cell death, and lipid peroxidation were significantly increased by PM treatment in zebrafish embryos. In contrast, the ethyl acetate fraction of *S. pallidum* significantly inhibited PM-stimulated ROS generation, cell death, NO production, and lipid peroxidation. These results confirmed that the fraction has a protective effect against PM-induced inflammation and oxidative stress in zebrafish embryos.

However, our study did not demonstrate the protective effects of

the fraction against PM-induced inflammation and oxidative stress in mice. Therefore, further studies are needed to verify its efficacy in mice.

In conclusion, our current study demonstrated the protective effect of the ethyl acetate fraction of *S. pallidum* against PM-induced skin damage by inhibiting ROS generation, pro-inflammatory enzymes, and the expression of pro-inflammatory cytokines in HaCaT cells and a zebrafish model. Altogether, the ethyl acetate fraction of *S. pallidum* can be a potential candidate for the treatment and management of fine dust-induced skin inflammation and related disorders.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

WCK and SHL designed the study. WCK performed experimental analysis. WCK, JWP, BY, WWL and KMC performed the analytic calculations and numerical simulations. WCK and SHL contributed to the final version of the manuscript. SHL supervised the project.

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