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RESEARCH ARTICLE

Tissue Culture

Sonication-assisted water extract of *Dendronephthya gigantea* exhibits anti-fine dust effects; attenuation of MAPK phosphorylation in macrophages

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Abstract: Air pollution due to fine dust (FD) has become a global concern. Industrial effluents and traffic emissions combined with naturogenic sources influence this issue, which particularly impacts the East Asia region. Health concerns caused by this form of pollution include respiratory disorders. The development of anti-inflammatory agents requires an understanding of the underlying mechanism. This study investigated the impact of FD on RAW 264.7 macrophages and sonication-assisted water extract of soft coral *Dendronephthya gigantea* (DGE) as a potent anti-fine dust agent. Cytoprotective effects using MTT and NO production inhibition using the Griess assay were assessed. ELISA, western blotting, and RT-qPCR techniques were used to evaluate multiple molecular mediators involved in the inflammatory pathway. It was observed that iNOS, COX-2, and PGE_2, including pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), were downregulated in a dose-dependent manner by DGE treatment. DGE inhibited the inflammatory responses initiated through FD by suppressing MAPK signaling in RAW 264.7 macrophages. This study provides insight into the protective mechanisms against FD, and suggests that *D. gigantea* could be a valuable, low-cost source of protein for potential use in the cosmeceutical and pharmaceutical sectors.

Keywords: Anti-fine dust, *Dendronephthya gigantea*, inflammation, macrophages MAPK, RT-qPCR

INTRODUCTION

Air pollution is comprised of harmful suspended contaminants in the air and is referred to as fine dust (FD) (Kim *et al*., 2016). Concern regarding FD has increased due to its detrimental effects on humans and other organisms. Air pollution in the East Asia region, including China, Japan, and Korea, has become a major issue. This is mainly due to rapid industrialization, including coal burning, power generation, and traffic emissions consisting of hydrocarbons, oxides of nitrogen, and carbon monoxide (Pozzi *et al*., 2003). Naturogenic sources also contribute to fine dust levels. Lee *et al.* (2015) reported that emissions released by China due to its industries are less than those released by natural sources.

The association between fine dust and health issues, including lung function issues and conditions regarding the respiratory system, has been reported previously. Nel *et al.* (1998) described allergic conditions that arise due to diesel exhaust particles. The influence of FD on human monocytes and pro-inflammatory cytokine levels were described by Monn *et al.* (1999). The literature emphasizes two distinct sources of FD in the induction of inflammation. Several reports have suggested that the transition metal ion content influences inflammation via the oxidative stress pathway. According to some authors oxidative stress is caused by radicals that arise from the Fenton chemistry pathway. (Gilmour *et al*., 1996; Knaapen *et al*., 2002). The oxidative mechanism is most applicable to the smaller FD, which possess a higher surface area and a larger number of particles in

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a given volume of air. These are more toxic than their larger counterparts (Brown *et al*., 2001). Alternatively, the bacterial-derived endotoxin bound to the surface of the particle may stimulate inflammation (Becker *et al*., 1996; Schins *et al*., 2004).

The anti-inflammatory potential of soft coral species has been widely studied. Chao *et al.* (2008) reported the anti-inflammatory potential of cembranoids isolated from the soft coral *Lobophytum crissum*. Ethanol extracts of soft corals collected from Jeju Island were studied by Wang *et al.* (2016) for their potential anti-inflammatory properties against LPS-stimulated RAW 264.7 macrophages. The therapeutic ability of soft coral *D. gigantea* regarding anti-inflammation in *in vivo* and *in vitro* conditions has been extensively studied by Fernando *et al.* (2018b); the compounds of interest was steroidal congeners.

The present study aimed to investigate the inflammatory stimulation of FD using macrophages as a model. *D. gigantea* sonication-assisted water extract (DGE) was evaluated for its ability to reduce the inflammatory responses that occurred in the macrophages. Inhibition of NO production, cytoprotective effect, and pro-inflammatory cytokines, including PGE_2, were also examined, including their gene expression levels, to identify the regulatory mechanism. The effects of DGE treatment on the MAPK pathway were also investigated. To the best of our knowledge, this is the first study to demonstrate that the sonication-assisted water extract from *D. gigantea* exhibits anti-fine dust effects through anti-inflammatory responses in macrophages.

METERIALS AND METHODS

Materials

The fine dust used in the experiments was a certified reference material (CRM No. 28, National Institute for Environmental Studies, Ibaraki, Japan). RAW 264.7 macrophage cells purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) were used for experiments. Growth media (Dulbecco's modified Eagle's medium, penicillin, and streptomycin) were purchased from GIBCO Inc. (Grand Island, NY, USA). Western blotting was performed using antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Beverly, MA, USA). Cytokine levels were analyzed using kits purchased from eBioscience (San Diego, CA, USA), R&D Systems (Minneapolis, MN, USA), BD Opteia (San Diego, CA, USA), and Invitrogen (Carlsbad, CA, USA). All solvents used were of analytical grade and were purchased from Sigma-Aldrich.

Extraction of *Dendronephthya gigantea*

D. gigantea samples were collected from the sea area around Jeju Island, South Korea, in May 2018. The Jeju Biodiversity Research Institute assisted with the sample identification process. Salt, sand, and epiphyte interferences were removed by washing. A sample portion (sample:water, 1:9) was extracted by assisting the sonication power (40 kHz) in an ultrasonic bath (Kodo Technical Research Co. Ltd., Hwaseong, Korea), maintaining the temperature between 20–30 °C. This solution was lyophilized and extensively dialyzed to remove salt ions. The sample was then again lyophilized, resulting in a *D. gigantea* 5 h sonication water extract (DGE), which was used in the subsequent experiments.

Analysis of proximate composition and amino acids of DGE

The moisture, ash content, and protein and lipid approximations were conducted following the methods described by the Association of Official Analytical Chemists standard methods (AOAC International, 2005). The method described by Um *et al.* (2017) was used to determine amino acid composition. HCl (6.0 M, 2 mL) was used to hydrolyze a 50 mg sample portion of DGE. It was then pre-concentrated to remove the HCl, and sodium citrate buffer was used to dissolve it (0.2 M, pH 2.2, 10 mL). Thereafter, this was successfully separated using a cation exchange column (LCA K06/Na, 4.6 × 150 mm). An amino acid analyzer was used for compositional analysis (S433-H, Sykam GmbH, Germany).

Estimation of fine dust size and morphology using SEM

The particle size and morphology of the FD specimens were determined using a field-emission electron microscope (SEM) (JSM-670F, JEOL, Tokyo, Japan). Before SEM was introduced, the sample was sputter-coated with platinum (Q150R, Quorum Technologies, Lewes, UK) (Fernando *et al.,* 2017).

Maintenance of cell lines

Macrophage RAW 264.7 cells were cultured in DMEM medium (10 % FBS, 1 % P/S). Cell cultures were maintained in incubated conditions (5 % CO_2, 37 °C,

humidified). Periodical subcultures were conducted and used for experiments during the exponential growth phase.

Analysis of cell viability and NO production

The MTT assay was used to assess cytotoxicity in FD-induced RAW macrophages (Mosmann 1983). The cells were seeded in 24 well plates (1×10^5 cells/mL), incubated, and treated with the samples. After 30 min, FD stimulation was conducted and further incubated for 30 min. This was followed by rinsing and refilling with fresh medium. The culture plates were incubated for an additional 23 h. The FD sample was first dissolved in growth media (DMEM) to obtain a stock concentration of 2.5 mg/mL and was further diluted to achieve the treatment concentrations. The MTT assay was conducted by measuring the optical density at a wavelength of 540 nm. NO production was measured using the Griess assay (Wijesinghe *et al.,* 2014).

Inflammatory mediator level assessment via ELISA

To obtain the cell culture media for the assessment of cytokine experiments, the cells were seeded, and sample treatment was performed similarly. Tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and prostaglandin E2 (PGE_2) levels were measured in the collected cell culture supernatants. The process was assisted with commercially available cytokine assessment kits, and the test followed the manufacturer's instructions.

Western blot analysis

Macrophage cells were seeded for the western blot analysis, and cells were harvested after sample and FD treatment. The experiment was conducted following the method explained in Jayawardena *et al.,* (2019).

RNA extraction, cDNA synthesis, and qPCR analysis

RNA extraction was performed in macrophage cells to synthesize cDNA. Total RNA was extracted, followed by purity measurement and cDNA synthesis. Synthesized cDNA was stored at −80 °C, until use. qPCR analysis was performed using primers obtained from Bioneer (Seoul, Korea), following the method explained by Jayawardena *et al.* (2019). The method described by Livak *et al.* (2001) was used to analyze the relative expression levels.

Statistical analysis

A minimum of three trials were performed to express the data as the mean ± standard deviation. IBM SPSS statistics using one-way ANOVA was used for the statistical comparison of significant differences. p values less than 0.05 ($p < 0.05$) were considered significant.

RESULTS AND DISCUSSION

Fine dust

The scanning electron microscope image of the urban aerosol (CRM No. 28) is shown in Figure 1. The sample consisted of particles with an irregular morphology. The size distribution was between 2 and 14 μm in diameter, and most particles had diameters of < 2 μm. The CRM No. 28 certificate contains polycyclic aromatic hydrocarbons (PAHs) and inorganic components (Mg, Ca, Ba, Sr, Mn, and Pb).

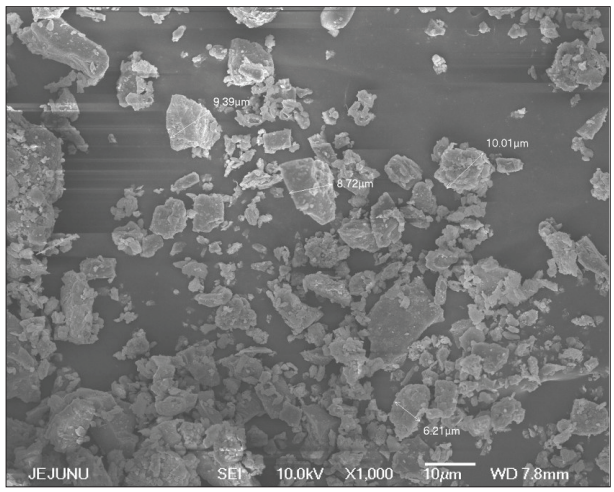

Figure 1: Fine dust subjected to scanning electron microscope (SEM) imaging (CRM No. 28 Urban Aerosols, National Institute for Environmental Studies (NIES), Ibaraki, Japan).

Fine dust air pollution has become a major issue in East Asia in recent years. Countries such as Korea, Japan, and China are the most affected. Recent publications have proposed that FD exposure is associated with respiratory complications, allergic reactions, and inflammatory skin conditions. The FD is a complex mixture of different components. It includes various types of dust, such as tobacco smoke, pollen, and exhaust gas from traffic emissions (Kim *et al.,* 2016). In the East Asia region, this is exacerbated by anthropogenic activities; the Asian desserts are also act as a source of dust, which is transported by the wind (Choi *et al.,* 2001). FD contains both organic and inorganic components. Asian dust is reported to contain water-soluble mineral particles, such as Mg^{2+} and Ca^{2+}. Furthermore, negative ions, such as

nitrate (NO_3^-) and sulfate (SO_4^{2-}), are associated with positive ions such as ammonium (NH_4^+) and potassium (K^+) (Maxwell-Meier 2004).

Proximate composition and amino acids of DGE

The 5 h sonication-assisted water extract yielded 4.3 ± 0.56 %. A higher amount of protein (68.38 ± 1.14 %) was identified compared to the lipid (5.82 ± 0.44 %) and carbohydrate (16.18 ± 0.38 %) content. The ash and moisture contents were reported as 5.14 ± 0.12 and 1.97 ± 0.16 respectively. DGE is an abundant source of protein and could potentially act as an important feature of the sample. The amino acid composition of DGE was rich in aspartic acid (11.97 %), glutamic acid (14.32 %), and proline (8.25 %). Branched-chain amino acids (BCAA; valine 6.39 %, isoleucine 4.00 %, and leucine 5.79 %), which are important in immune responses, also indicate an accountable portion of the DGE. The amino acid composition was as follows: Asp, 11.97; Glu, 14.32; Ser, 6.42; Gly, 5.54; His, 3.56; Arg, 5.37; Thr, 6.55; Ala, 5.14; Pro, 8.25, Tyr, 3.79; Val, 6.39; Met, 2.28; Cys, 2.20; Ile, 4.00; Leu, 5.79; Phe, 4.23; Lys, 4.12.

The present study evaluated the physical parameters of the FD via SEM, followed by its biological effect, using macrophages as a model. The relative effectiveness of time-dependent extraction methods in the marine soft coral *D. gigantea*, which could counter FD toxicity, was evaluated. Among them, the 5 h water extract (DGE) was found to be most effective (**p < 0.01).

Potential anti-fine dust effect of DGE in the inflammatory responses

The data presented in Figure 2a and 2b show that the viability of macrophages exposed to LPS and FD decreased while NO production increased. However, DGE treatment dose-dependently and substantially attenuated the cytotoxicity induced by LPS and FD. NO production, which was significantly upregulated by FD treatment, was successfully restored by treatment with DGE (Figure 2). This study demonstrates the significance of increasing levels of inflammatory regulators, including PGE_2, and hence the levels of cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines (IL-1β and IL-6) against lipopolysaccharide (LPS; 1 μg mL^{-1}) and FD stimulation (125 μg/mL). The use of LPS as an inflammatory stimulator provides a reference for the inflammatory induction that occurs via FD. DGE downregulates the expression of inflammatory mediators (Figure 3). TNF-α downregulation was notable but not significant, while IL-1β and IL-6 levels declined significantly. Western blot analysis showed that the levels of iNOS and COX-2 (Figure 4a and b) increased with the stimulation of FD, whereas co-treatment with DGE dose-dependently downregulated the protein levels.

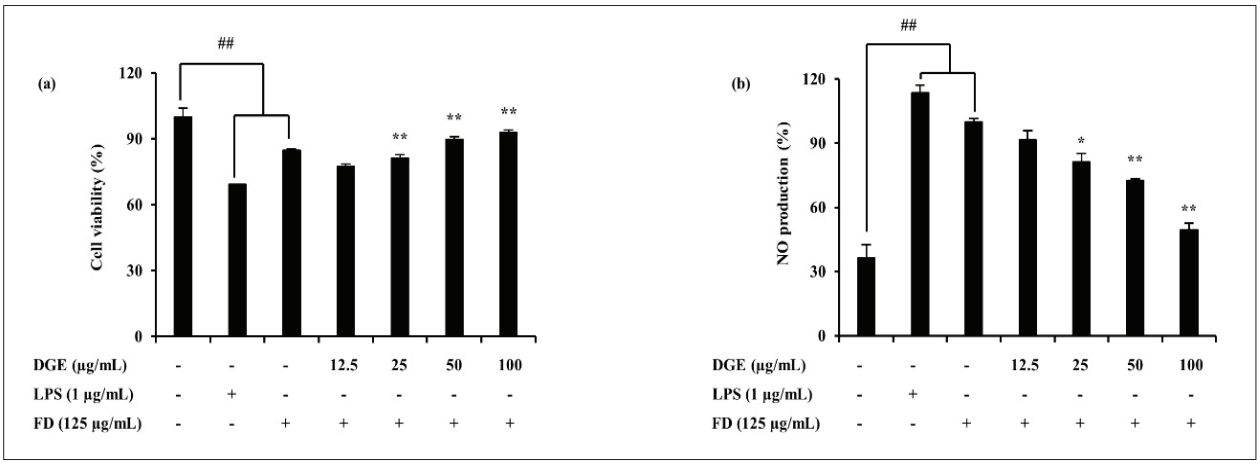

Figure 2: FD-induced RAW 264.7 macrophages, (a) cell viability and (b) NO production. LPS stimulation (1 μg/mL) or FD (125 μg/mL). Triplicated experiments; mean value is expressed with ± SD. *p < 0.05, **p < 0.01 vs. the FD treated group; #p < 0.05, ##p < 0.01 vs. the un-stimulated group.

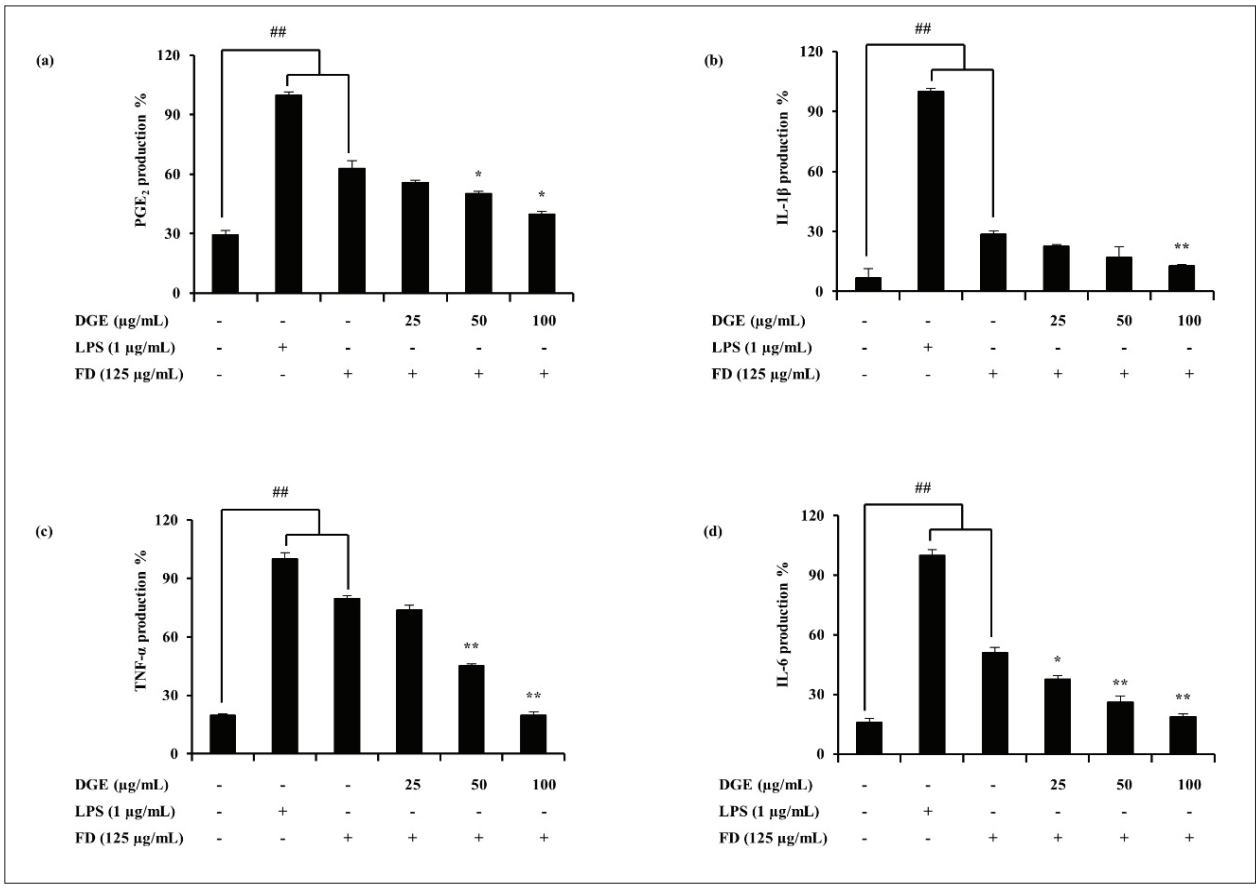

Figure 3: ELISA experiments performed using FD-induced RAW 264.7 cell supernatants. Potential of DGE to inhibit PGE2 and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) production. Triplicated experiments; mean value is expressed with ± SD. *p < 0.05, **p < 0.01 vs. the FD treated group; #p < 0.05, ##p < 0.01 vs. the un-stimulated group.

Inflammation is directly associated with macrophage cells and is regulated via different mechanisms, such as phagocytosis, antigen presentation, and the production of various cytokines and chemokines. Although the activation of macrophages is essential to counteract inflammation, their over-activation could lead to detrimental effects. Hence, the regulation of these cells is important for the treatment of inflammation. Important mediators in this process are NO, iNOS, COX-2, PGE_2, and pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Fernando *et al.,* 2017). The results indicate the upregulation of these mediators upon LPS and FD stimulation in macrophages. Their gradual downregulation is ensured via the dose-dependent treatment of DGE, verifying its ability to act as a potent anti-fine dust agent via anti-inflammatory mechanisms. Furthermore, the results indicated that DGE suppressed the mRNA expression of the above mediators, leading to a decline in the production of inflammatory mediators required for inflammation, thus inhibiting inflammation. NO plays a vital role as a vascular dilator, neurotransmitter, and a key component of the immunological system. It is synthesized from arginine via nitric oxide synthase (NOS) (Nakagawa *et al.,* 2002). Under obsessive conditions, NO production is inclined due to the activity of inducible NOS (iNOS), leading to cytotoxicity and tissue damage (Kim *et al.,* 1999). COX-2 is another protein closely related to this pathway. This helps in generating another inflammatory mediator, PGE_2, by catalyzing the process. Different cytokines and growth factors stimulate COX-2 expression (Park *et al.,* 2006). Hence, cytokine production may ultimately attenuate NO production in the inflammatory pathway.

Ability of DGE to reduce the FD induced inflammatory responses via the p38-MAPK pathway

In the process of evaluating the activity of DGE as an anti-fine dust agent, we subsequently assessed whether the inhibition of inflammatory responses is mediated via through p38-MAPK pathway. The FD-induced phosphorylation of p38, ERK1/2, and JNK MAPKs in macrophages was evaluated by western blotting. As shown in Figure 4, LPS encouraged phosphorylation, whereas FD stimulation lagged behind it. DGE treatment (25, 50, and 100 µg/mL) gradually downregulated the phosphorylation of p38, ERK1/2, and JNK. Among the MAPKs, p38 exhibited drastic down-regulation, whereas ERK1/2 remained the least affected by all three. Thus, the results indicate the potential of DGEs to inhibit MAP kinase phosphorylation against FD stimulation.

This study focused on MAP kinases, a highly conserved family of proteins that comprise serine/threonine. This includes three subfamilies: ERKs, JNKs, and p38 MAPKs (Ruland *et al*., 2003). The MAPK signaling pathway can be triggered by various extracellular signals, including cell proliferation, apoptosis, and inflammation. LPS is a potential activator of all three types of MAPKs (Hommes *et al*., 2003). Fernando *et al.* (2018a) described MAP kinase activation via stimulation of FD in keratinocytes and macrophages. Activated p38 is thought to exhibit significant activities in regulating inflammatory mediators, such as iNOS, COX-2, and TNF-α gene expression. Activated ERK is believed to increase the production of iNOS and other pro-inflammatory cytokines (Bhat *et al*., 1998; Ajizian *et al*., 1999). JNK is also involved in iNOS expression of the iNOS (Uto *et al*., 2005). It was observed that the

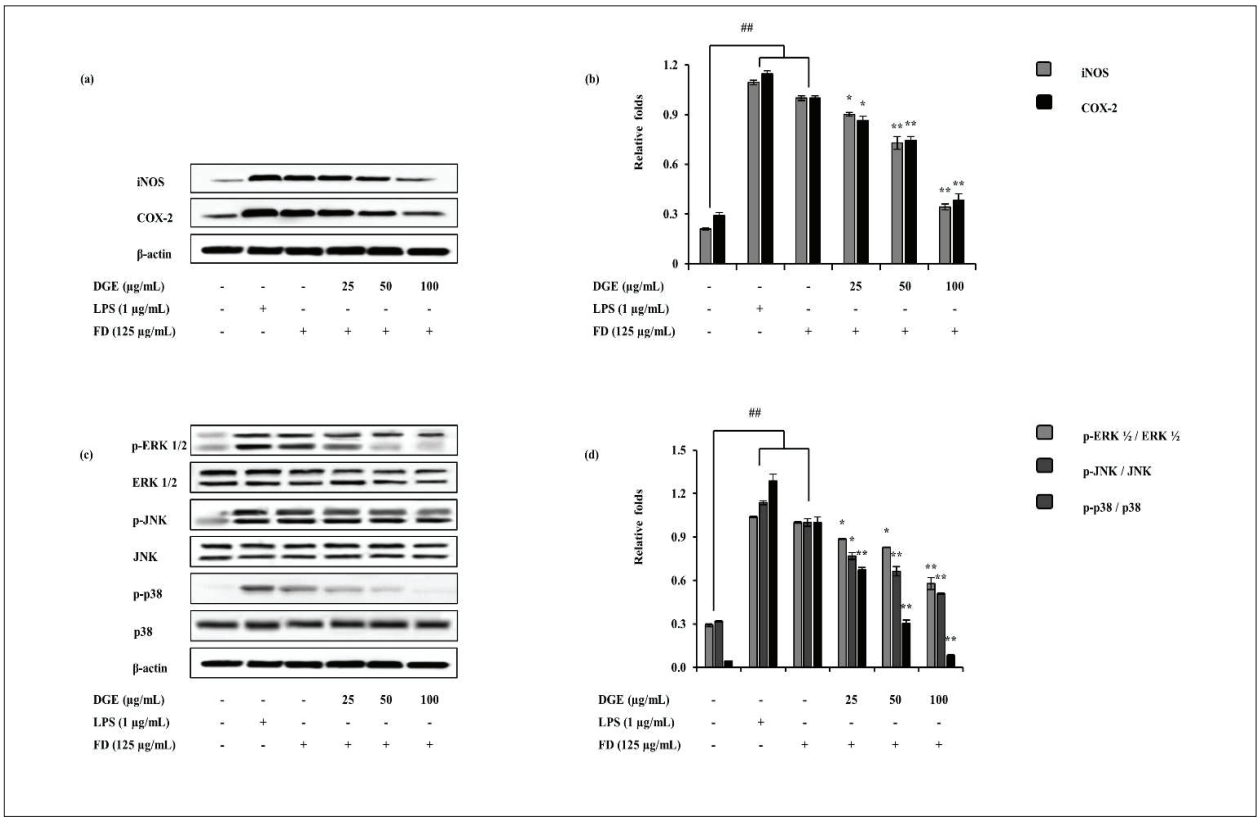

Figure 4: Potential of DGE to act upon inflammatory pathway mediators induced via FD. (a) iNOS and COX-2, (b) quantitative data, (c) ERK, JNK, and p-38 MAPKs, and relevant (d) quantitative data, determined using western blotting. β-actin was used as an internal control. Quantitative data were analyzed using ImageJ software. Results are expressed as the mean ± SD of three separate experiments. *p < 0.05, **p < 0.01 vs. the FD-treated group; #p < 0.05, ##p < 0.01 vs. the unstimulated group.

DGE dose-dependently inhibited the phosphorylation of MAPKs. This may lead to the anti-inflammatory activity of DGE in FD-stimulated macrophage cells.

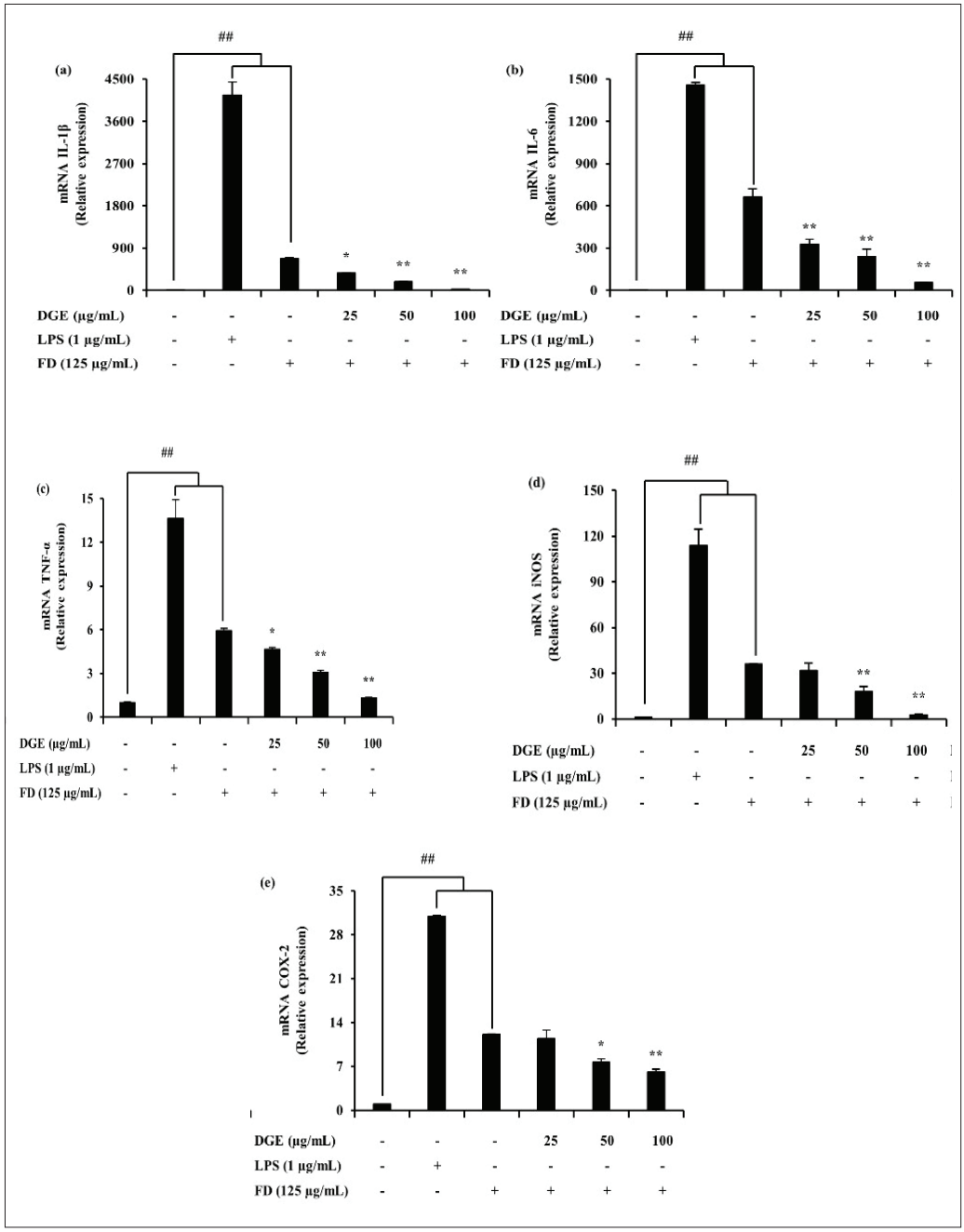

Figure 5: DGE inhibits inflammation-associated gene expression levels in FD-induced RAW 264.7 macrophages. (a) IL-1β, (b) IL-6, (c) TNF-α, (d) iNOS and (e) COX-2. The 2-ΔΔCt method was used to calculate the relative mRNA levels. GAPDH was used as an internal reference. Experiments were triplicated. mRNA significance relative to non-treated control was calculated using the Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$ vs. the FD treated group; #$p < 0.05$, ##$p < 0.01$ vs. the un-stimulated group.

Effect of DGE to attenuate FD-stimulated gene expression

DGE treatment downregulated the mRNA expression levels of iNOS and COX-2 and was observed with a similar trend to the western blot results. A significant decline in pro-inflammatory cytokine levels was also observed. The potential of DGE as an anti-fine dust agent in anti-inflammatory mechanisms was confirmed by the results.

It has been discovered that amino acids are involved in cell signaling and play an important role in gene expression regulation and protein phosphorylation cascades (Wu, 2009). It also involves the activation of natural killer cells (macrophages) and the production of cytokines. Arginine is a conditionally essential amino acid, and its major functions include regulation of cytokine production and acting as a mediator for autoimmune diseases. Glutamic acid and proline, which are abundant amino acids in DGE, are important for arginine synthesis (Li *et al.,* 2007). Similarly, the three BCAAs are vital for the regulation of immune responses. The anti-inflammatory potential of BCAAs in RAW 264.7 macrophages under LPS-stimulated conditions was evaluated recently by Lee *et al.* (2017). As DGE contains many amino acids involved in immune responses, it is potentially suitable as an anti-inflammatory agent.

CONCLUSIONS

This study reveals that DGEs have potential as anti-fine dust agents and their ability to regulate the production of important inflammatory mediators such as NO, PGE_2, and cytokines via the modulation of MAPK phosphorylation in macrophages. Hence, *D. gigantea* could be a valuable, low-cost source of protein for potential use in the cosmeceutical and pharmaceutical sectors. This study provides insight into the protective mechanisms against FD. As *D. gigantea* is a rapidly growing species due to the ocean temperature increment in the vicinity of Jeju Island, harvesting these soft coral species would not alter the diversity of its natural habitat.

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