Short Communication

Anti-Inflammatory Effects of *Sargassum muticum* Ethanol Extract in Lipopolysaccharide-Induced RAW 264.7 Cells and Mouse Ear Edema Models

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This study investigated the effects and the anti-inflammatory activity of *Sargassum muticum* ethanol extract in lipopolysaccharide-induced RAW 264.7 murine macrophage cells and in a croton oil-induced mouse ear edema model. Pretreatment of lipopolysaccharide-induced RAW 264.7 cells with *Sargassum muticum* ethanol extract (0.1-100 µg/ml) inhibited lipopolysaccharide-induced production of nitric oxide, interleukin-6 and tumour necrosis factor alpha in a dose-dependent manner. The expression of lipopolysaccharide-induced, inducible nitric oxide synthase and cyclooxygenase-2 in the *Sargassum muticum* ethanol extract-treated group was also suppressed in a dose-dependent manner. Furthermore, we found that *Sargassum muticum* ethanol extract induced anti-inflammatory effects by inhibiting Mitogen-activated protein kinases (extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38) and nuclear factor kappa B, p65 phosphorylation in lipopolysaccharide-stimulated RAW 264.7 cells. The anti-inflammatory activity of *Sargassum muticum* ethanol extract *in vivo* was evaluated in the ear edema model of a croton oil-treated mouse. Compared to the untreated control, croton oil-induced ear edema was found to be reduced by about 33 % upon treatment with 250 mg/kg *Sargassum muticum* ethanol extract.

Key words: Anti-inflammation, *Sargassum muticum*, nuclear factor kappa B, mitogen-activated protein kinases, ear edema

Inflammation is a complex biological process caused by activated immune cells and is an innate immune response to protect the body from infection and foreign antigens[1,2]. Persistent inflammation, however, promotes mucosal injury causing pain, edema, fever and other dysfunctions. Chronic inflammation can cause diseases such as cancer and arthritis[3]. Macrophages play a central role in the activation of inflammatory mediators such as pro-inflammatory cytokines and control their overproduction under inflammatory conditions[1,4]. In the early stages of external infection, Nitric Oxide (NO) and cytokines are secreted, which play an important role in biological defense. Macrophages are reactivated by these secretions and are involved in inflammatory reactions[5].

Lipopolysaccharide (LPS) is an extracellular membrane component of gram-negative bacteria that activates macrophages[6]. LPS stimulates Toll-Like Receptor 4 (TLR4) on the macrophage surface, leading to the activation of Mitogen-Activated Protein Kinase (MAPK), a subcellular signaling pathway. These signaling pathways in turn activate Nuclear Factor kappa B (NF-κB), a nuclear transcription factor, which induces transcription of inflammation regulators such as various pro-inflammatory cytokines, inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) and increases secretion of Interleukin (IL)-6, IL-1 beta (β), Tumor Necrosis Factor alpha (TNF-α), NO and Prostaglandin E_2 (PGE_2)[7,8]. Therefore, inhibition of NF-κB and MAPK activities is important for the treatment of inflammatory responses.

Seaweed has been widely consumed and used in Asia as a functional food and medicinal herb[9]. Seaweeds are rich in lipids, minerals, dietary fiber and vitamins.

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They are considered as functional food as they contain various bioactive substances such as proteins, polysaccharides and polyphenols that have been shown to have beneficial effects on oxidative stress, cancer, diabetes, inflammation[10], thrombosis[11], allergies[12] and obesity[13].

Sargassum muticum (*S. muticum*) is a brown algae belonging to the genus *Sargassum*[14] and is widely distributed in the southern and eastern coasts of Korea. *S. muticum* is a polyphenol-rich seaweed that has been shown to have biological activities such as antioxidant, antiproliferative and anti-angiogenesis activities[15]. Therefore, the present study further examined the anti-inflammatory activity of *S. muticum* Ethanol Extract (SMEE) in a croton oil-induced ear edema mouse model and the impact of not only NF-κB but also MAPKs in inflammatory pathways.

S. muticum was collected at Song-Jung, Busan, Korea in 2016. It was washed with tap water to remove salt and sand. After air-dry at room temperature for 1 d, it was lyophilized, powdered, packed in vacuum and stored at -20°. Powdered *S. muticum* was extracted with 95 % ethanol at room temperature for 24 h with an agitator (H-0820, Dongwon Science Co., Busan, Korea). The extraction process was carried out twice. The extract was then centrifuged at 2090×g for 10 min and the supernatant was filtered. The filtrate was concentrated using a rotary evaporator (RE200, Yamato Co., Tokyo, Japan).

Male Institute of Cancer Research (ICR) mice (8 w old) and female Bagg and Albino (BALB)/c mice (10 w old) were purchased from Orient Bio (Seongnam, Korea). Mice were raised in an animal facility maintained at a temperature of 20°±2°, humidity of 50 %±10 % and 12/12 h light-dark cycles for a week before the experiment. All experimental protocols for animal care were performed in accordance with the rules and regulations of the Animal Ethics Committee, Pukyong National University, Busan, Korea (Approval No. 201504).

The murine macrophage cell line RAW 264.7 was purchased from Korean Cell Line Bank (KCLB 40071). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, New York, United States of America (USA)) supplemented with 10 % inactivated Fetal Bovine Serum (FBS; Hyclone, Logan, Utah, USA) and 1 % penicillin-streptomycin (Hyclone, Logan, Utah, USA) in a 5 % Carbon Dioxide (CO_2) incubator (MCO15AC, Sanyo, Osaka, Japan) at 37°. Cells were sub-cultured every 3 d and counted with a hemocytometer. The number of viable cells was determined by trypan blue dye exclusion staining.

The cytotoxicity of SMEE was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagents (Sigma-Aldrich Co., St. Louis, Missouri, USA). RAW 264.7 cells (1×10^6 cells/ml) were seeded on 96-well plates and pre-incubated for 20 h. The cells were then cultured with different concentrations of SMEE (0.1, 1, 10, 50 and 100 μg/ml) for 22 h at 37° and 5 % CO_2. MTT reagent (5 mg/ml) was added and the cells were incubated for 2 h. The medium was then discarded and Dimethylsulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, Missouri, USA) was added to each well and absorbance was measured at 540 nm with a microplate reader (Model 550, Bio-Rad, Richmond, Virginia, USA). The cell proliferation ability was calculated according to the following formula: Proliferation index (%)=(Absorbance of the sample/Absorbance of the control)×100.

RAW 264.7 cells (2.5×10^5 cells/ml) were pre-cultured for 20 h and then incubated for 24 h in presence of 1 μg/ml LPS (Sigma-Aldrich Co., St. Louis, Missouri, USA) and SMEE (0.1, 1, 10, 50 and 100 μg/ml). After that, 100 μl of cell culture medium and the same amount of Griess reagent (1 % sulfanilamide and 0.1 % naphthalene diamine dihydrochloride in 5 % phosphoric acid) were mixed and incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (Model 550) and the amount of nitrite was calculated using a standard curve of Sodium Nitrite ($NaNO_2$).

RAW 264.7 cells (2.5×10^5 cells/ml) were incubated for 24 h in presence of LPS (1 μg/ml) and different concentrations of SMEE (0.1, 1, 10, 50 and 100 μg/ml). The levels of TNF-α and IL-6 in culture medium were determined using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Mouse ELISA set; BD Biosciences, San Diego, California, USA) according to the manufacturer's instructions.

RAW 264.7 cells were incubated for 24 h with SMEE (0.1, 1, 10, 50 and 100 μg/ml) and LPS (1 μg/ml) and cells were collected. The cells were washed three times with cold Phosphate Buffered Saline (PBS) and lysed in lysis buffer (50 mM 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES) (pH 7.4), 150 mM Sodium Chloride (NaCl), 5 mM Ethylenediamine Tetraacetic Acid (EDTA), 1 %

deoxycholate, 5 mM Phenylmethylsulfonyl Fluoride (PMSF), 1 µg/ml aprotinin, 1 % Triton X-100 and 0.1 % NP-40) and kept on ice for 30 min. In the case of analyses involving NF-κB p65, nucleus lysis buffer (10 mM HEPES, 100 mM NaCl, 1.5 mM Magnesium Chloride ($MgCl_2$), 0.1 mM EDTA, 0.1 mM dithiothreitol) was added and the cells were lysed on ice for 30 min. The cell lysates were centrifuged at 15 520×g for 20 min and the protein concentration in the supernatant was measured by Pierce Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific, NY, USA). Protein samples were separated by 10 % Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and then transferred on to Polyvinylidene Fluoride (PVDF) membranes (Bio-rad, USA) with transfer buffer (25 mM Tris-HCl (pH 8.8), 192 mM glycine, 20 % methanol). After blocking nonspecific sites with 5 % skim milk (Fluka, Switzerland) in 0.1 % Tris-Buffered Saline (TBS)-Tween 20 for 2 h, the membranes were incubated with specific primary anti-mouse iNOS, COX-2, NF-κB p65, phosphorylated c-Jun N-Terminal Kinase (p-JNK), phosphorylated Extracellular Signal-Regulated Kinase (p-ERK) and p-p38 antibodies in TBS (1:500) at 4°, overnight. Each membrane was further incubated for 1 h with secondary antibody, Horseradish Peroxidase (HRP)-conjugated anti-mouse Immunoglobulin G (IgG) and anti-rabbit IgG (1:2000). The immune-active proteins were detected using an Enhanced Chemiluminescence (ECL) detector (Pierce, USA) and the signal intensity of each protein band was measured by densitometry, employing the Gene Tools from Syngene software (Synoptics Ltd., Cambridge, United Kingdom (UK).

To evaluate the anti-inflammatory effects of SMEE *in vivo*, ear edema measurements were performed[16]. SMEE (200 µl) was orally administered to 8 w old male ICR mice at concentrations of 10, 50 and 250 mg/kg body weight. After 1 h, 2.5 % croton oil was applied at a concentration of 20 µl/ear on the inner and outer surfaces of the right ear. Ear thickness was measured 5 h after application and an increase in ear thickness after croton oil treatment was considered to be edema formation.

For histopathology analysis, 20 µl/ear of 100 mg/ml SMEE was applied to the right ear of the ICR mouse and was followed by application of 20 µl of 5 % croton oil 15 min later. Mice were sacrificed after 6 h, following which ear tissues were dissected and fixed in 10 % formaldehyde for 72 h. The tissue slices were embedded in paraffin and sections were deparaffinized and stained with hematoxylin-eosin and toluidine-blue stains to allow visualization of the tissue and mast cells.

10 w old female BALB/c mice (n=5) were used for the experiment. The mice were fasted for about 4 to 6 h immediately before the acute toxicity test. SMEE was orally administered at concentrations of 300, 2000 and 5000 mg/kg body weight and 5 % Tween 80 was orally administered for the control group. All mice were observed for general symptoms at least once daily. On the day of administration, observations were made every hour for 30 min after administration and up to 6 h thereafter and general symptoms were observed at least once a day until 14 d after administration.

Data are expressed as mean±standard error of the mean (n=3). Statistical evaluation was carried out using analysis of variance with Statistical Analysis System (SAS) software (SAS Institute, Inc., Cary, North Carolina, USA), according to Duncan's multiple range test ($p<0.05$).

The cytotoxicity of SMEE in RAW 264.7 cells was determined based on MTT assay. Treatment with 0.1 to 100 µg/ml SMEE had no significant effect on MTT-based cell viability (fig. 1). Therefore, SMEE was used at a concentration of 0.1 to 100 µg/ml in subsequent experiments.

In order to investigate whether SMEE could control NO and pro-inflammatory cytokine production, NO and pro-inflammatory cytokine (IL-6 and TNF-α) secretion was measured in LPS-induced RAW 264.7 cells. As shown in fig. 2A, SMEE suppressed NO production in a dose-dependent manner. According to a previous

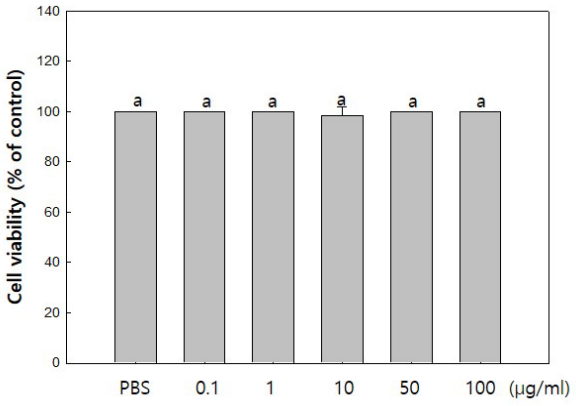

Fig. 1: Effect of SMEE on proliferation of RAW 264.7 cells. Proliferation index (%)=(Absorbance of the sample/Absorbance of the control)×100. The results are expressed as mean±standard error of the mean (n=3). Means with different superscripts (a) above the bars are significantly different by Duncan's multiple range test ($p<0.05$)

study that polyphenols extracted from *S. muticum* significantly inhibited NO release of LPS-induced RAW 264.7 cells[17], it can be inferred that the inhibition of NO is due to polyphenols, a bioactive component of *S. muticum*.

The secretion of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) in the SMEE treated group decreased in a dose-dependent manner compared to that in the LPS-treated group. In particular, IL-6 secretion showed a significant decrease at SMEE concentrations of 0.1~100 μg/ml and decreased about 82.3 % at a concentration of 100 μg/ml (fig. 2B). In case of TNF-α, secretion decreased about 27.5 % at SMEE concentration of 100 μg/ml (fig. 2C). In addition, IL-1β secretion was decreased by 80.90 % and 84.64 % at SMEE concentrations of 50 and 100 μg/ml, respectively (fig. 2D).

The macrophage RAW 264.7 cells are activated by the stimulation of antigens such as LPS to promote the secretion of cytokines such as IL-6, TNF-α and IL-1β to regulate the immune response[18]. The secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β plays an important role in the development of early inflammatory responses[19]. TNF-α produced by LPS-stimulated macrophages and monocytes is cytotoxic to tumor cells and is associated with chronic inflammatory diseases such as autoimmune diseases, rheumatoid arthritis and inflammatory bowel disease[20]. In addition, TNF-α sustains the inflammatory response by inducing the production of other cytokines[21]. Therefore, it is important to reduce pro-inflammatory cytokine secretion in anti-inflammatory treatment.

The effect of SMEE on the expression of iNOS and COX-2 in the cytoplasm and NF-κB p65 in the nucleus was investigated. In the control group, expression of each protein was increased on LPS stimulation, but in the SMEE-treated group, the expression of LPS-stimulated iNOS, COX-2 and NF-κB p65 was inhibited in a dose-dependent manner by SMEE (fig. 3). In particular, it was confirmed that the expression level of each protein significantly decreased at SMEE of 100 μg/ml. These results suggest that the inhibition of NO production in fig. 2A may be related to the regulation of iNOS expression. This suggests that inhibition of iNOS, COX-2 and pro-inflammatory cytokines (fig. 2B) may be due to inhibition of NF-κB p65 activation.

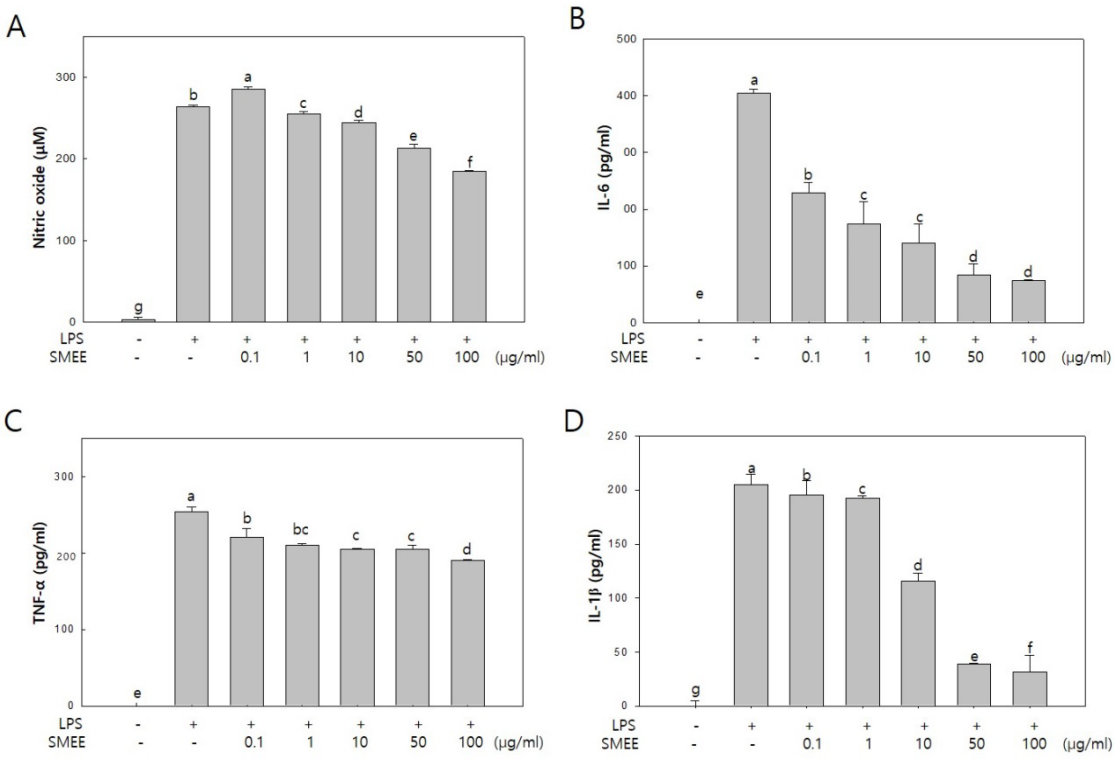

Fig. 2: Inhibitory effect of SMEE on the production of (A) NO; (B) IL-6; (C) TNF-α and (D) IL-1β in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of SMEE (0.1, 1, 10, 50 and 100 μg/ml) in the presence or absence of LPS (1 μg/ml) for 24 h. Culture supernatants were then isolated and analyzed using Griess reagent for nitric oxidizers and ELISA kit for cytokines. The results are expressed as mean±standard error of the mean (n=3). Means with different superscripts (a-g) above the bars are significantly different by Duncan's multiple range test ($p<0.05$)

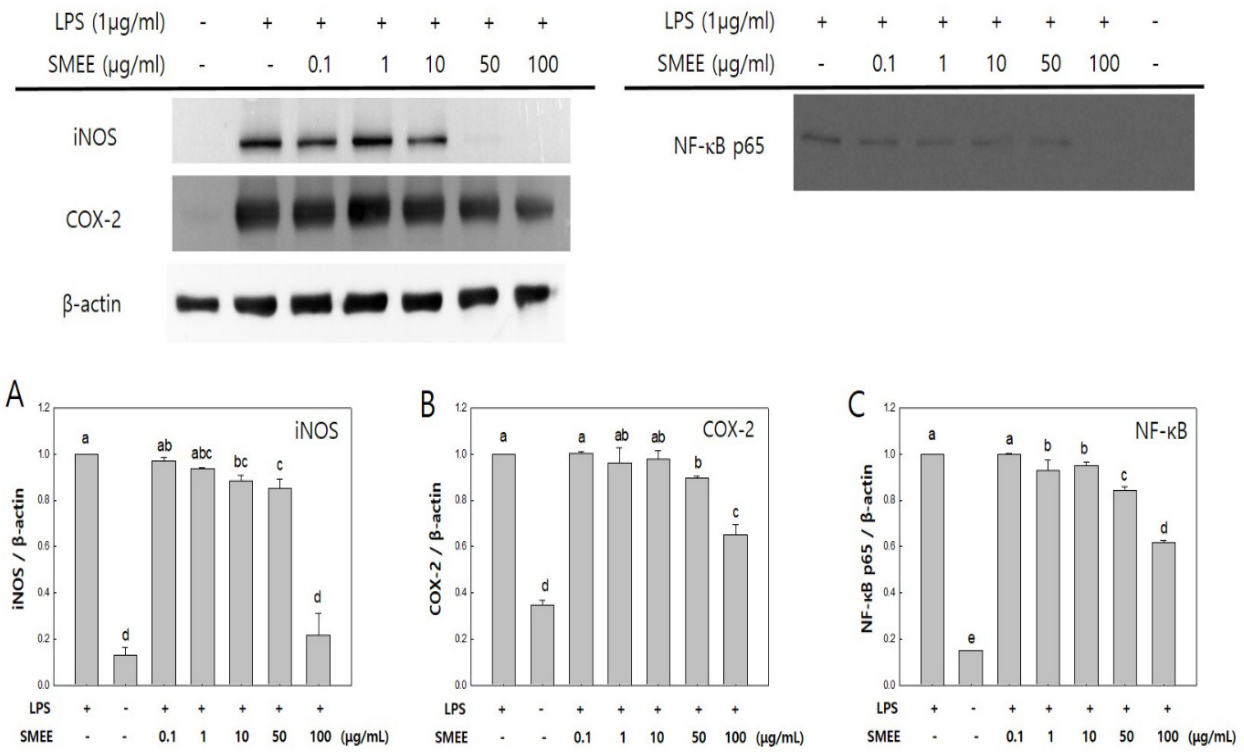

Fig. 3: Effect of SMEE on LPS-stimulated (A) iNOS; (B) COX-2 and (C) NF-κB p65 expression in RAW 264.7 cells. The levels of iNOS, COX-2 in the cytosolic protein fraction and the p65 subunit of NF-κB in nuclear protein fraction were determined by a western blot analysis. RAW 264.7 cells were treated with indicated concentrations of SMEE (0.1, 1, 10, 50 and 100 μg/ml) and LPS (1 μg/ml) for 24 h and the proteins were detected using specific antibodies. For quantification, the expression data were normalized to the beta (β)-actin signal. The results are expressed as mean±standard error of the mean (n=3). Means with different superscripts (a-e) above the bars are significantly different by Duncan's multiple range test ($p<0.05$)

NF-κB binds to promoters of genes related to the inflammatory response and activated NF-κB is known to be primarily involved in the expression of COX-2 and iNOS[22]. iNOS produces NO when activated by stimuli such as LPS, cytokines and bacterial toxins. Excessive NO production is known to cause inflammation[8,23]. COX-2 is involved in the biosynthesis of PGE_2 and causes inflammation by increasing the vascular permeability of free PGE_2[24].

The effect of SMEE on phosphorylation of MAPKs, such as p38, ERK1/2 and JNK subfamilies in LPS-induced RAW 264.7 cells was investigated by western blot analysis. The SMEE treatment was found to reduce the expression levels of p-ERK and p-JNK in LPS-induced RAW 264.7 cells (fig. 4). Especially, the expression level of p-ERK was significantly decreased at SMEE concentrations of 10 μg/ml and higher (fig. 4B).

MAPKs (ERK, JNK, p-38) are important signaling molecules that mediate macrophage activity. MAPKs can localize to the nucleus and induce the production of other immune activators to further activate the inflammatory response[25]. MAPKs are activated by phosphorylation and are known to play an important role in the expression of iNOS, COX-2 and pro-inflammatory mediators by activating NF-κB[26].

In this study, SMEE inhibited the phosphorylation of NF-κB and MAPKs that were activated by LPS and resulted in reduced production of iNOS, COX-2, NO and pro-inflammatory cytokines (fig. 2-fig. 4). These results are similar to the reports on *S. miyabei* Yendo extract and *S. patens* Carl Adolph Agardh extract, known as brown algae in *Sargassum*, which exhibit anti-inflammatory effects by inhibiting the expression of NF-κB and MAPKs[27,28].

To investigate the anti-inflammatory effects of SMEE on acute inflammation, we performed a croton oil-induced ear edema test using a mouse model. It was confirmed that ear thickness decreased significantly at all concentrations of SMEE when compared to the untreated control group. In particular, it was reduced by about 33 % at 250 mg/kg of SMEE and slightly decreased compared with 50 mg/kg treatment of prednisolone, a steroidal anti-inflammatory drug (fig. 5A). The effect of SMEE on ear edema relaxation was also observed in tissues. In the tissues of mouse

ear edema induced by croton oil, treatment with 100 mg/ml SMEE was effective in reducing the dermis and epidermis thickness of tissues to a degree similar to that observed with prednisolone treatment (fig. 5B). In addition, the degree of mast cell infiltration was confirmed by toluidine-blue staining and treatment with SMEE significantly inhibited mast cell infiltration in the tissues (fig. 5C).

Inflammation is a protective reaction against physical and chemical damage of the human body. Representative

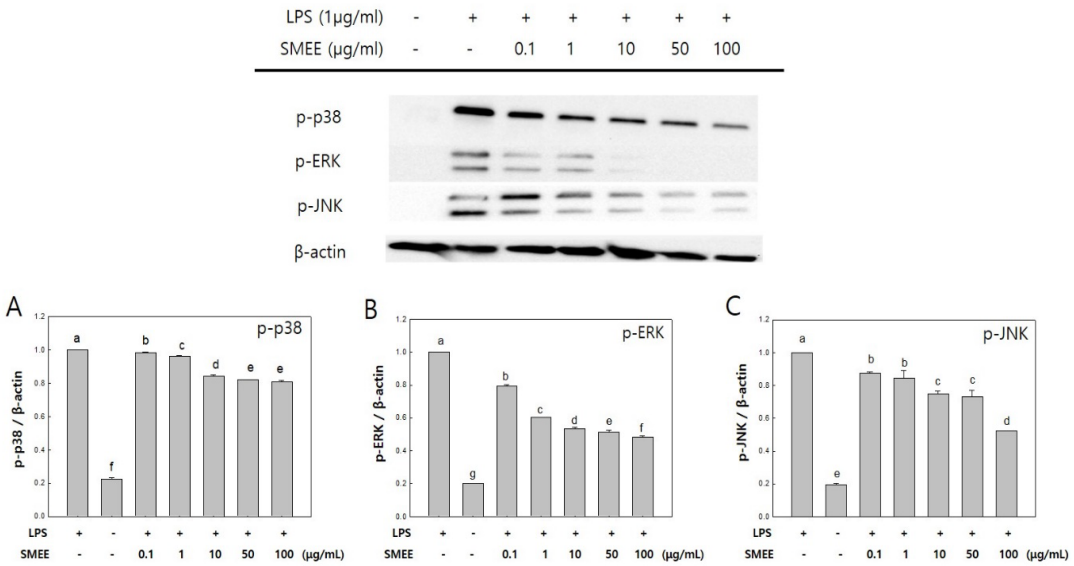

Fig. 4: Effect of SMEE on the MAPK pathway in LPS-stimulated RAW 264.7 cells. (A) The levels of p-p38; (B) p-ERK and (C) p-JNK in the cytosolic protein fraction were determined by western blot analysis. RAW 264.7 cells were treated with indicated concentrations of SMEE (0.1, 1, 10, 50 and 100 μg/ml) and LPS (1 μg/ml) for 30 min and the proteins were detected using specific antibodies. For quantification, the expression data were normalized to the total MAPKs signal. The results are expressed as mean±standard error of the mean (n=3). Means with different superscripts (a-f) above the bars are significantly different by Duncan's multiple range test ($p<0.05$)

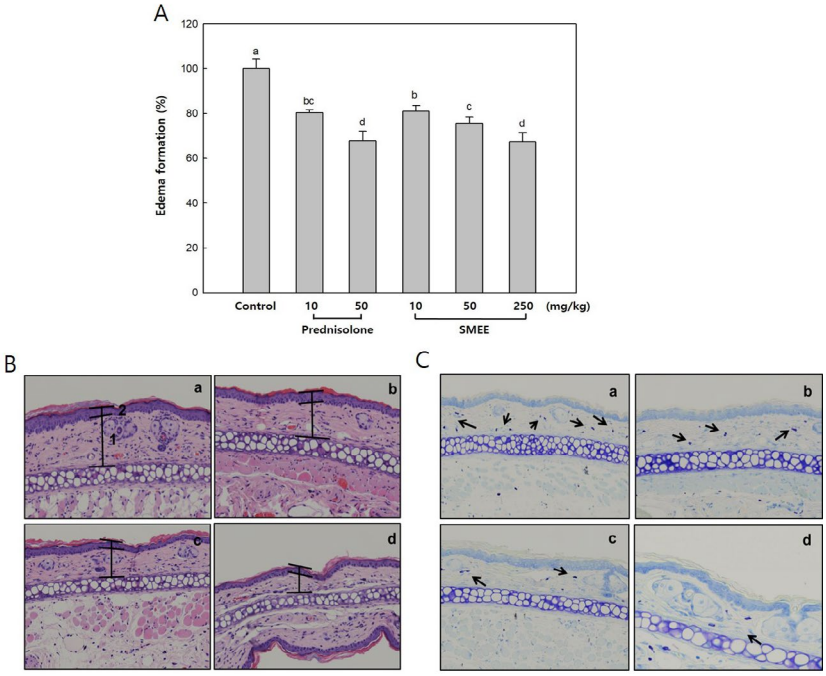

Fig. 5: (A) SMEE-mediated inhibition of croton oil-induced mouse ear edema; (B) Photomicrographs of transverse sections of mouse ears sensitized with topical application of croton oil 5 % (v/v) in acetone (a-c) or acetone alone (d, non-inflamed), stained with hematoxylin-eosin or (C) Toluidine-blue. Photomicrographs recorded under light microscopy (magnification: 200×). Treatments for histopathology analysis: (a) Vehicle 2 % Tween 80; (b) Prednisolone 0.08 mg/ear; (c) SMEE 20 μl/ear and (d) Acetone. The numbers 1 and 2 indicate dermis and epidermis, respectively and arrows indicate mast cell infiltration in the tissue. The results are expressed as mean±standard error of the mean (n=3). Means with different superscripts (a-d) above the bars are significantly different by Duncan's multiple range test ($p<0.05$)

TABLE 1: MORTALITY AND CLINICAL SIGNS OF BALB/c MICE TREATED ORALLY WITH SMEE

		Groups (mg/kg body weight)			
		0	300	2000	5000
Mortalities	Number of dead animals	0/5	0/5	0/5	0/5
	Percentage (%)	0	0	0	0
Clinical signs	No abnormalities	5/5	5/5	5/5	5/5

responses are vasodilation, increased cell membrane fluidity and edema[29]. Mast cells activated by external stimuli secrete vasodilators such as various proteases and histamines. Due to the increase in blood flow, neutrophils in the blood migrate to tissues outside the blood vessels, causing edema and pain due to an increase in prostaglandin at the site of inflammation[30]. Therefore, it is possible to prove the efficacy of anti-inflammatory substances by experimentally verifying the effects of inhibiting these responses.

In this study, we evaluated the anti-inflammatory activity of SMEE *in vivo* using the croton oil treated-ear edema model. SMEE (250 mg/kg) relieved ear edema caused by croton oil by about 33 % compared to the control group and significantly inhibited mast cell infiltration in the tissues (fig. 5). According to the study by Jeon *et al.*[31], treatment of *S. muticum* extract reduced the expression of inflammatory cytokines IL-6 and TNF-α, contributing to the relief of edema and symptoms in Collagen-Induced Arthritis (CIA) DBA/1J mice model. Therefore, SMEE treatment seems to cause edema relief by reducing the expression of inflammatory cytokines and inhibiting mast cell infiltration in damaged tissues.

The acute oral toxicity of SMEE was assessed by administering 300, 2000 and 5000 mg/kg body weight of SMEE to mice over 2 w. No death and abnormal behaviors were observed among the treated mice (Table 1). According to the World Health Organization (WHO), a medicinal herb is considered toxic if its median Lethal Dose (LD_{50}) is less than 5 g/kg body weight[32]. This indicates that the concentration of SMEE used in this study is nontoxic and safe for human use.

In summary, SMEE significantly inhibited the secretion of pro-inflammatory cytokines, iNOS and COX-2 expression and phosphorylation of NF-κB and MAPK in LPS-stimulated RAW 264.7 cells ($p<0.05$). In addition, SMEE treatment showed edema inhibition *in vivo*, similar to that of prednisolone, an anti-inflammatory agent. Thus, these results suggest the possibility of developing therapeutics using the remarkable anti-inflammatory effects of SMEE.

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Conflict of interests:

The authors declare that there are no conflicts of interest.

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