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3-Bromo-5-(ethoxymethyl)-1,2-benzenediol inhibits LPS-induced pro-inflammatory responses by preventing ROS production and downregulating NF-κB *in vitro* and in a zebrafish model

Eun-Yi Ko[a,b,1], Soo-Jin Heo[c,1], Su-Hyeon Cho[a,d], WonWoo Lee[d,h], Seo-Young Kim[d], Hye-Won Yang[d], Ginnae Ahn[e], Seon-Heui Cha[f], Seung-Hae Kwon[a], Myeong Seon Jeong[a], Kang Pa Lee[g], You-Jin Jeon[d,*], Kil-Nam Kim[a,*]

[a] Chuncheon Center, Korea Basic Science Institute (KBSI), Chuncheon 24341, Republic of Korea
[b] Bio research Center, Dermapro, Jeju 63309, Republic of Korea
[c] Jeju International Marine Science Center for Research & Education, Korea Institute of Ocean Science and Technology, Jeju 63349, Republic of Korea
[d] Department of Marine Life Science, Jeju National University, Jeju 63243, Republic of Korea
[e] Department of Marine Bio-food Science, College of Fisheries and Ocean Sciences, Chonnam National University, 59626, Republic of Korea
[f] College of Pharmacy, Gachon University, Incheon 21936, Republic of Korea
[g] Department of Physiology, School of Medicine, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea
[h] Freshwater Bioresources Utilization Division, Nakdonggang National Institute of Biological Resourecs, Sangju 37242, Republic of Korea

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ABSTRACT

The anti-inflammatory effects of 3-bromo-5-(ethoxymethyl)-1,2-benzenediol (BEMB) from *Polysiphonia morrowii* were evaluated in lipopolysaccharide (LPS)-induced RAW264.7 cells and zebrafish embryo. BEMB showed anti-inflammatory effects by inhibiting the production of nitric oxide (NO) and reactive oxygen species (ROS), and the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in the LPS-activated RAW264.7 cells and zebrafish embryo without cytotoxicity. Moreover, BEMB suppressed the protein and mRNA expression levels of nuclear factor (NF)-κB (p65 and inhibitor of NF-κB [IκB]-A) in RAW264.7 cells and zebrafish embryo, respectively. Collectively, the results of this study indicate that BEMB suppressed the production of pro-inflammatory mediators such as NO, iNOS, and COX-2 as well as their regulation in LPS-induced RAW264.7 cells and zebrafish embryos by inhibiting ROS production and NF-κB expression. Therefore, this study suggests that BEMB could be a potential anti-inflammatory agent for the treatment of inflammatory diseases.

1. Introduction

Inflammatory reaction, which is typically characterized by redness, swelling, heat, and pain, is a common physiological response that defends the host from injurious stimuli such as pathogens, toxins, and local injuries. However, excessive or aberrant inflammation induces overactivity of the body and is harmful. Indeed, inflammation contributes to the pathogenesis of several diseases that affect a significant portion of the human population, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis, pulmonary fibrosis, and inflammatory brain diseases [1,2]. Lipopolysaccharide (LPS), the major component of the cell wall of gram-negative bacteria, increases the production of pro-inflammatory cytokines, nitric oxide (NO), and prostaglandin E_2 (PGE_2), which are the main cytotoxic and pro-apoptotic mediators involved in the innate response in many mammals [3–5]. Therefore, LPS is associated with a wide variety of inflammatory diseases and has been widely used to mimic the features of inflammatory diseases [6,7]. Thus, inhibition of the production of these inflammatory cytokines and mediators is an important target in the development of anti-inflammatory agents.

Zebrafish share a high degree of sequence and functional homology with mammals, including humans [8]. Since the nervous system and various organogenesis processes of zebrafish are very similar to those of humans, research is actively being conducted using it as an animal model of various human diseases [9,10]. In particular, LPS-induced zebrafish exhibit an inflammatory response similar to that of mammals [11]. Therefore, zebrafish has been used as an *in vivo* model of inflammation to confirm the anti-inflammatory effects of numerous

* Corresponding authors.
E-mail addresses: youjinj@jejunu.ac.kr (Y.-J. Jeon), knkim@kbsi.re.kr (K.-N. Kim).
[1] These authors contributed equally to this study.

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natural products.

Marine algae are currently attracting attention as a material with multiple pharmacological functions such as whitening, anti-inflammatory, anticancer, and anti-obesity effects [12,13]. *Polysiphonia morrowii* is a species of red algae native to Korea, Japan, China, and Russia [14]. A number of studies have been reported on the biological activities of *P. morrowii* [15,16]. However, the anti-inflammatory activity of 3-bromo-5-(ethoxymethyl)-1,2-benzenediol (BEMB) from *P. morrowii* has not been studied and, therefore, in this study, we examined this phenomenon *in vitro* and in an *in vivo* zebrafish model, for the first time.

2. Material and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal vobine serum (FBS), penicillin-streptomycin, trypsin-EDTA were purchased from Gibco. (Grand Island, NY). Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS; from *Escherichia coli* strain) and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The ELISA kits for PGE_2, IL-1β, and IL-6 were purchased from R& D Systems, Inc. (St. Louis, MO, USA) and BD Biosciences (San Diego, CA, USA). Antibodies were from the following sources: iNOS, COX-2, IκB-α, P-P65, P-P105 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The secondary antibodies were obtained from Cell Signaling Technology. 2,7-dichlorofluorescein diacetate (DCF-DA), diaminofluorophore4-amino-5-methylamino-2′7′-difluorofluorescein diacetate (DAF-FM DA), acridine orange were purchased from Sigma (St. Louis, MO, USA). All other reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Isolation of BEMB

The red alga *P. morrowii* was collected along the coast of Jeju Island, Korea. The sample was washed thrice with tap water to remove the salt, sand, and epiphytes attached to its surface, followed by careful rinsing with fresh water and then it was frozen in a medical refrigerator at −20 °C. The freeze-dried *P. morrowii* powder was extracted thrice with 80% aqueous methanol at room temperature. The liquid layer was obtained by filtration, and the filtrate was concentrated using an evaporator under reduced pressure. The extract was suspended in water, and the aqueous layer was partitioned with chloroform. Then, the chloroform fraction was fractionated using silica column chromatography with stepwise elution using a chloroform-methanol mixture (30:1 → 1:1) to separate the active fractions of the chloroform extract. The combined active fraction was further separated using a Sephadex LH-20 column saturated with 100% methanol, and then purified using reversed phase high-performance liquid chromatography (HPLC) using a Waters HPLC system (Alliance 2690, Waters Corp., Milford, MA, USA) equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 250 × 4.6 mm, 4 μm; YMC Co., Kyoto, Japan) by stepwise elution with a methanol-water gradient (UV range, 290 nm; flow rate, 1 ml/min). Finally, the purified compounds were identified by comparing their proton (^1H) and ^{13}C nuclear magnetic resonance (NMR) data with those published in the literature [17]. The chemical structure of the purified compound is shown in Fig. 1A. The compound was dissolved in dimethylsulfoxide (DMSO) prior to use in the experiments, and the final concentration of DMSO in culture medium was adjusted to < 0.01%.

2.3. Cell culture

Raw 264.7 murine macrophage cells were purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). The cells were cultured in DMEM supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and 10% FBS at 37 °C in a 5% CO_2 atmosphere.

2.4. Cell viability assay

RAW 264.7 cells (2×10^5/well) were plated in 96-well plates and incubated overnight, and subsequently treated with LPS (1 μg/ml) in the absence or presence of various concentrations (12.5, 25, and 50 μM) of BEMB at 37 °C for 24 h. After incubation, MTT solution (5 mg/ml) was added to each well and plates were incubated for 4 h in a CO_2 incubator at 37 °C. Subsequently, supernatant was removed and DMSO was added to dissolve formazan crystals. The absorbance was measured at 540 nm.

2.5. Measurement of NO production

RAW 264.7 cells (2×10^5/well) were plated in 24-well plates and subsequently treated with LPS (1 μg/ml) in the absence or presence of various concentrations (12.5, 25, and 50 μM) of BEMB at 37 °C for 24 h. Culture supernatants were mixed with Criess reagent (1:1 mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated at room temperature for 10 min. Subsequently, the NO concentration in supernatant was determined by measuring absorbance at 540 nm.

2.6. Measurement of intracellular ROS production

RAW 264.7 cells (2×10^5/well) were plated in 96-well plates and subsequently treated with LPS (1 μg/ml) in the absence or presence of various concentrations (12.5, 25, and 50 μM) of BEMB at 37 °C for 24 h. Then, the cells were treated with DCHF-DA (20 μM) for 30 min at 37 °C in the dark. DCHF-DA was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrophotometer.

2.7. Measurement of cytokines (IL-1β and IL-6) and PGE_2 by ELISA

RAW 264.7 cells (2×10^5/well) were plate in 24-well plates and subsequently treated with LPS (1 μg/ml) in the absence or presence of various concentrations (12.5, 25, and 50 μM) of BEMB at 37 °C for 24 h. Culture supernatants were collected for determination of IL-1β, IL-6, and PGE_2 concentrations by ELISA according to the manufacturer's instructions.

2.8. Western blot analysis

RAW 264.7 macrophages were cultured at 2×10^5 cells per 35 mm dish and incubated at 37 °C for 24 h. The cells were then treated with different concentrations of BEMB and incubated for 24 h or 15 min. The cells were lysed using RIPA lysis buffer. Protein concentrations were determined using a Bio-Rad protein assay kit; bovine serum albumin (BSA) was used as the calibration standard. Cell lysates were electrophoresed on SDS polyacrylamide gels (8–12%) and the separated proteins were transferred to PVDF membranes. The membranes were incubated in the blocking solution (Tris-buffer/Tween 20, TBST) containing 3% BSA (w/v) for 2 h under gentle shaking at room temperature. Subsequently, the membranes were incubated with primary antibodies (iNOS, COX-2, p-p65, and p-p105 diluted 1:1000 in 3% BSA in TBST) for 24 h at 4 °C. After incubation, the membranes were washed three times with TBST buffer at room temperature, and then incubated with secondary antibodies (1:3000) in 3% BSA in TBST for 2 h at room temperature. Signals were developed using ECL western blotting detection kit and visualized on a Bio-Rad ChemiDoc system.

2.9. Confocal microscopy analysis

Cells were fixed in 4% formaldehyde for 15 min at room

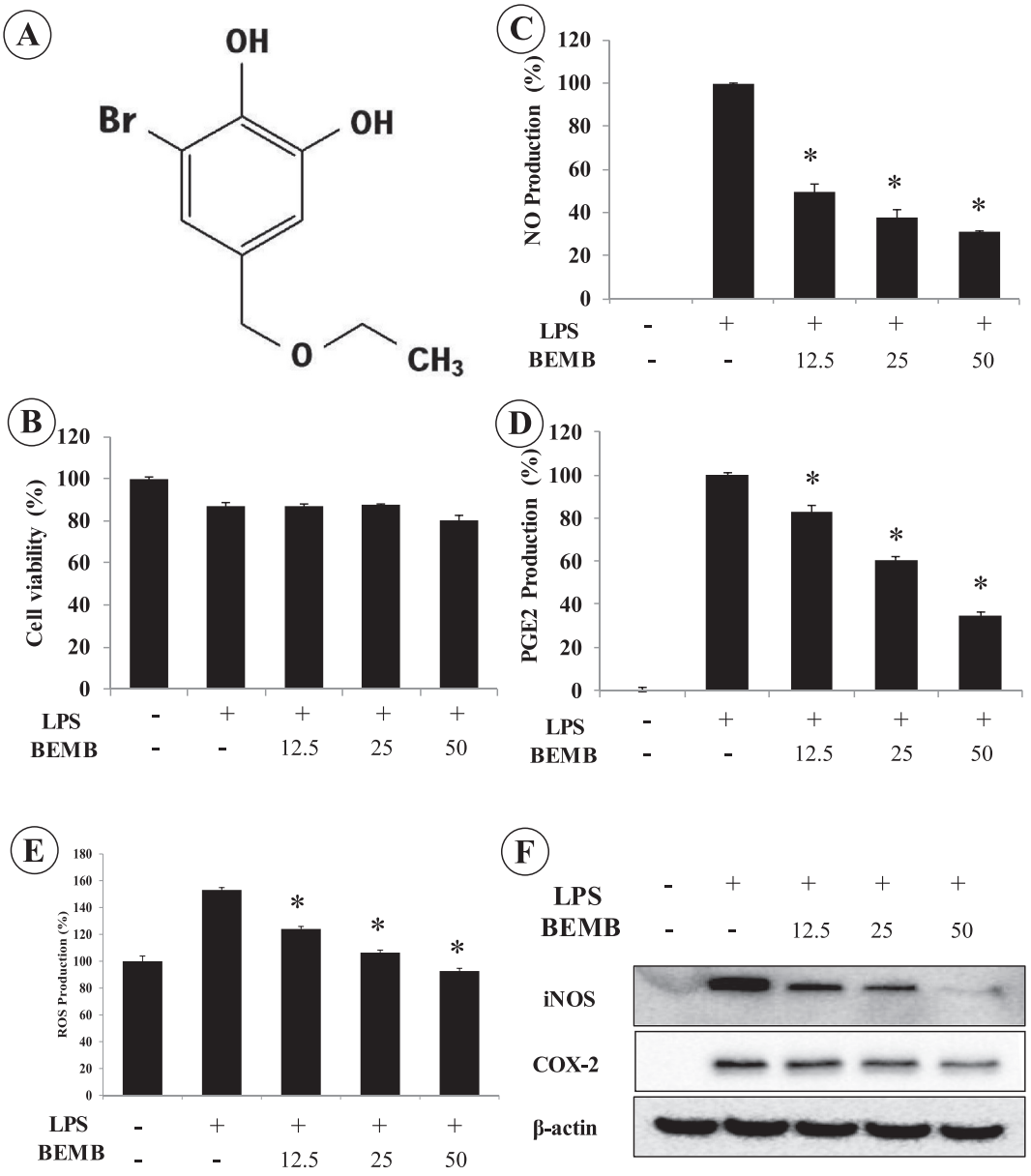

Fig. 1. (A) Chemical structure of 3-bromo-5-(ethoxymethyl)-1,2-benzenediol (BEMB) and its effects on cell (B) viability and. Inhibition of (C) nitric oxide (NO), (D) Prostaglandin E_2 (PGE_2) and (E) reactive oxygen species (ROS) production in lipopolysaccharide (LPS)-induced RAW264.7 cells. Cells were pretreated for 1 h with different concentration (12.5, 25, and 50 μM) of BEMB and then LPS (1 μg/ml) was added and incubated for 24 h. Cell viability was determined using the MTT assay. Values are expressed as means ± standard deviation (S.D.) of triplicate experiments. *P < 0.05 indicate significant differences compared with the LPS-stimulated group. (F) The cells were sampled and lysed following a 24 h treatment, and equal amounts of protein were separated by on SDS–polyacrylamide gel electrophoresis. Western blotting was performed using anti-iNOS and anti-COX-2 antibodies.

temperature and permeated with methanol at −20 °C for 10 min. Cells were then blocked for 1 h with 1% BSA in PBS and permeabilized by incubation with 0.4% Triton X-100 for 30 min. Subsequently, cells were washed with PBS and incubated with anti-p65 primary antibody (1:100) at 4 °C overnight. After washing with PBS, cells were incubated with a secondary antibody (1:100) at 4 °C for 1.5 h. Cells were then washed with PBS and incubated with Alexa Fluor 488 goat anti-rabbit antibody (1:800) for 1.5 h at RT in the dark. After washing with PBS, slides were mounted and counter-stained using vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slides were visualized under a laser scanning microscope (LSM 700; ZEISS, Jena, Germany).

2.10. Maintenance of zebrafish

Zebrafish were maintained in a temperature-controlled room at 28 °C with a 13:11 h day/night cycle. Zebrafish were fed two times per day. The day before the experiment began, zebrafish were randomly selected for interbreeding in a male-to-female ratio of 2:1. The embryos were collected from the breeding case and washed to remove any debris lying at the bottom of the tank. The embryos obtained post-spawning were staged and dispensed into embryo media, which contained deionized water with 60 mg/l Instant Ocean red salts (Spectrum Brands, Mentor, OH, USA). All animal experiments were approved by the Jeju National University Animal Care and Use Committee (2016-0052).

2.11. Measurement of the toxicity of BEMB

Sample toxicity was determined by means of survival rate of zebrafish embryos. The embryos were transferred to individual wells of 12-well plates containing 950 μL embryo media from approximately 7 to 8 h post-fertilization (7–8 hpf), BEMB was exposed to the embryos up to 7 day post-fertilization (dpf). The survival rate was measured every day.

2.12. Measurement of ROS and NO production by image analysis

Generation of NO in the zebrafish embryos was estimated using a fluorescent probe dye, DAF-FMDA, and production of intracellular ROS was detected using an oxidation-sensitive fluorescent probe dye, DCF-DA. Embryos were treated with 12.5, 25, and 50 μM BEMB, then, 2 h later, 10 μg/ml LPS was added to the plate. Embryos were selected at 3 dpf, transferred into a 24-well plate and treated with embryo medium containing 10 μM DAF-FMDA or 20 μg/ml DCF-DA. They were incubated for 1 h for ROS and for 2 h for NO, both in the dark at 28 °C. After incubation, the embryos were washed with embryo media and anesthetized before visualization. The images of stained embryos were observed using a fluorescent microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan).

2.13. RNA extraction and quantitative RT-PCR (qRT-PCR)

Zebrafish and isolated gonads were homogenized and RNA was isolated using an Invitrogen RNA elution kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using a qScript cDNA synthesis kit (Quantabio, Beverly, MA, USA). Primers were designed for genes listed in Table 1. SYBR Green (Takara Bio Inc., Japan) was used to determine the expression levels of all genes. PCR conditions for SYBR Green consisted of a denaturation step for 5 min at 95 °C followed by 40 cycles of 95 °C for 2 s and 60 °C for 30 s. Data analysis was performed using the standard curve and ΔΔCt methods.

2.14. Statistical analysis

All data in this study are expressed as means ± S.D. Significant differences among the groups were determined using unpaired Student's *t*-tests. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of BEMB on production of NO, PGE2, and ROS in LPS-stimulated RAW 264.7 cells

To identify the potential anti-inflammatory activity of BEMB, its effects on LPS-induced production of NO and PGE_2 were measured using Griess reagent and ELISA, respectively. BEMB markedly reduced LPS-induced NO, PGE, and ROS production in a dose-dependent manner (Fig. 1C, D, and E). BEMB did not have any cytotoxic effect on RAW264.7 cells at the concentrations tested (12.5, 25, and 50 μM, Fig. 1B). Therefore, the anti-inflammatory effect of BEMB was deemed to not be attributable to cytotoxicity.

Table 1
Quantitative PCR primer sequences used for the analysis of genes involved in zebrafish NF-κB.

Gene symbol	Oligo	Primer sequence	Annealing temperature (°C)	Amplification product (bp)
p65	Forward	tccctggagagaagagcaac	60	200
	Reverse	cagtcttttcccaccagctc	60	
ikbA	Forward	tttcggaggagatggagaga	60	184
	Reverse	ctgttcaggtacgggtcgtt	60	
b-Actin	Forward	ggttttgctggagatgatgc	60	171
	Reverse	cacaataccgtgctcaatgg	60	

3.2. Effects of BEMB on expression of iNOS and COX-2 protein in LPS-stimulated RAW264.7 cells

Western blot analysis was carried out to investigate whether the inhibitory effects of BEMB on LPS-induced NO and PGE_2 production were related to modulation of iNOS and COX-2 expression. In these experiments, LPS stimulation significantly increased iNOS and COX-2 protein expression; however, BEMB inhibited the increase in a dose-dependent manner. (Fig. 1F). Therefore, inhibition of NO and PGE_2 production in LPS-induced RAW 264.7 cells was related to the inhibition of iNOS and COX-2 protein expression.

3.3. Effects of BEMB on pro-inflammatory cytokines production in LPS-stimulated RAW 264.7 cells

In this study, we further evaluated the inhibitory activity of BEMB against pro-inflammatory cytokine (IL-1β and IL-6) production in LPS-stimulated RAW 264.7 cells. The production of these cytokines was significantly increased by LPS. In contrast, pre-treatment with BEMB decreased the LPS-induced IL-1β and IL-6 production in a concentration-dependent manner (Fig. 2).

3.4. Effect of BEMB on LPS-induced NF-kB activity in RAW264.7 cells

NF-κB is an important transcriptional factor that regulates a variety of inflammatory and immune responses [18]. Accordingly, we performed western blot analysis to determine the effect of BEMB on the phosphorylation of NF-κB in LPS-induced RAW264.7 cells. BEMB significantly blocked the phosphorylation of P65 and p105 NF-κB activation induced by LPS. (Fig. 3A). In addition, confocal microscopy analysis revealed the induction of NF-kB translocation in RAW264.7 nuclei following a 30-min LPS treatment. Consequently, as shown in Fig. 4B, untreated cells expressed low levels of p65 and exhibited fluorescence and LPS-induced cells mainly showed nuclear accumulation of p65. Nuclear translocation of NF-κB p65 induced by LPS was markedly reduced in cultures treated with BEMB. (Fig. 3B). Thus, these results proved that the anti-inflammatory effect of BEMB on LPS-induced RAW 264.7 cells involved the NF-κB pathway.

3.5. Effect of BEMB on LPS-induced NO and ROS production in zebrafish embryos

First, to determine the toxicity of BEMB, we examined the survival rate of treated zebrafish embryos. The results showed no significant change in the survival rate of the control compared to that of the treated group, indicating there was no toxicity at the tested concentrations (data not shown).

The production of NO and ROS in the LPS-induced inflammatory zebrafish model was analyzed using 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM DA) and dichloro-dihydro-fluorescein diacetate (DCFH-DA), respectively. The NO and ROS levels in LPS-stimulated zebrafish increased to 143% and 153%, respectively, compared with the levels in the control group (Fig. 4A and B, respectively). However, the NO and ROS production in zebrafish treated with BEMB decreased in a concentration-dependent manner Fig. 4. In particular, treatment of LPS-induced zebrafish embryos with 50 μM BEMB led to the generation of NO (108%) and ROS (102%) at levels similar to those of the control group without LPS treatment.

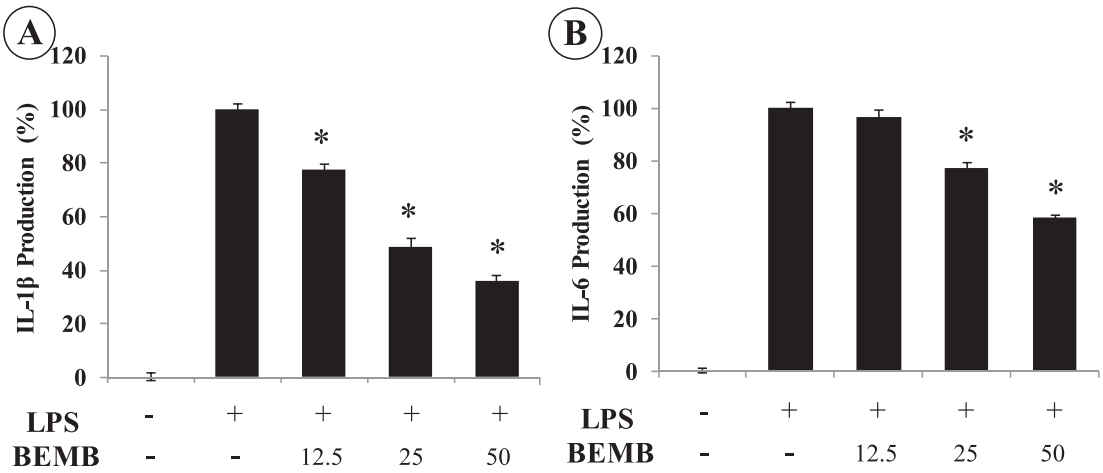

Fig. 2. Effect of BEMB on the pro-inflammatory cytokine production in LPS-induced RAW264.7 cells. The production of (A) IL-6 and (B) IL-1β were assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of BEMB (12.5, 25, and 50 μM). Supernatants were collected, and the IL-1β and IL-6 concentration in the supernatants were determined by ELISA. Values are expressed as means ± S.D. of triplicate experiments. *P < 0.05.

3.6. Effect of BEMB on LPS-induced iNOS and COX-2 expression in zebrafish embryos

To determine the mechanism by which BEMB reduced LPS-induced NO production, we investigated the effect of BEMB on the LPS-induced expression of iNOS and COX-2 using western blot analysis. As shown Fig. 5A, the protein expression levels of iNOS and COX-2 more significantly increased in LPS-treated zebrafish embryos than in the

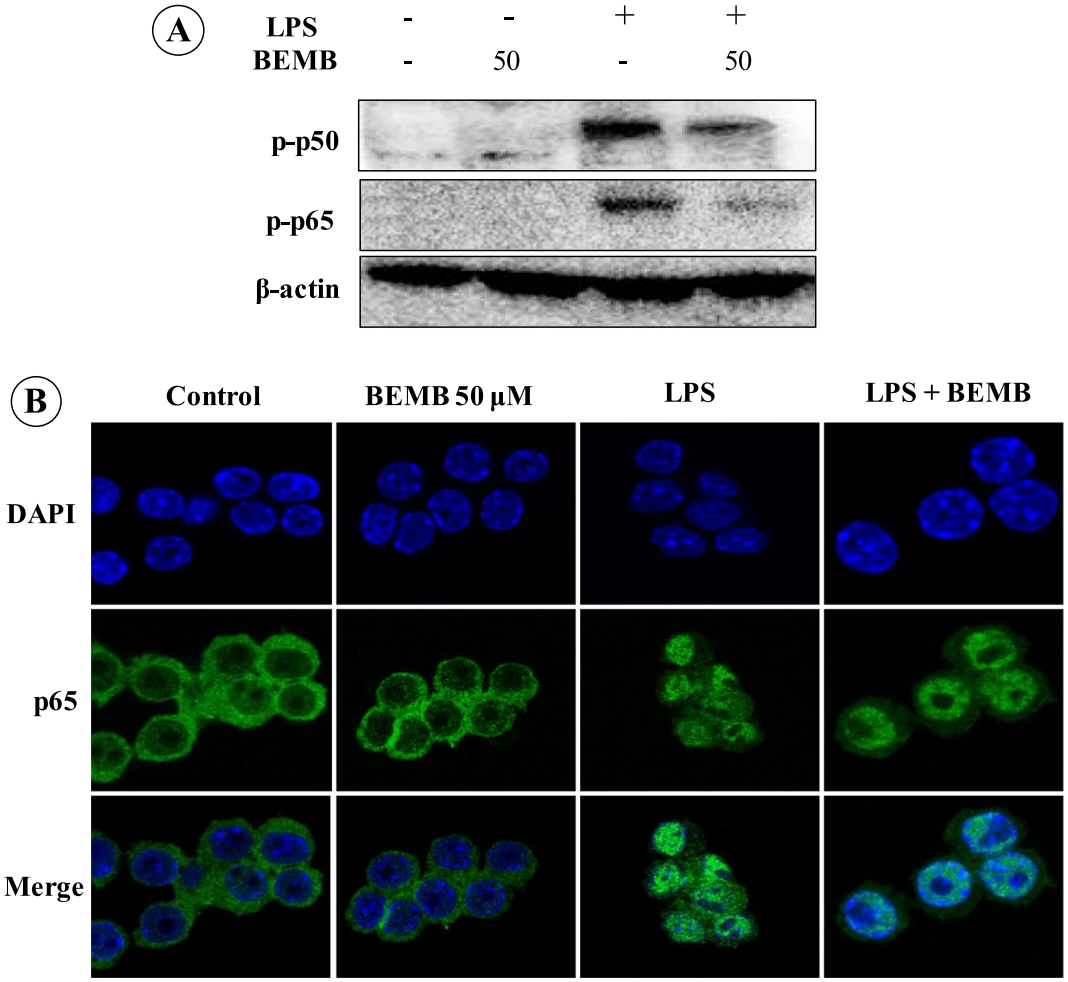

Fig. 3. Inhibitory effect of BEMB on NF-κB activation in LPS-induced RAW264.7 cells. Cells were treated for 15 min with LPS (1 μg/ml) alone or with LPS (1 μg/ml) coupled with 50 μM BEMB. The expression levels of (A) NF-κB protein were determined *via* Western blotting. (B) The p65 protein localization in cells was determined with an anti-p65 antibody and Alexa Fluor 488 goat anti-rabbit antibody by laser confocal scanning microscopy.

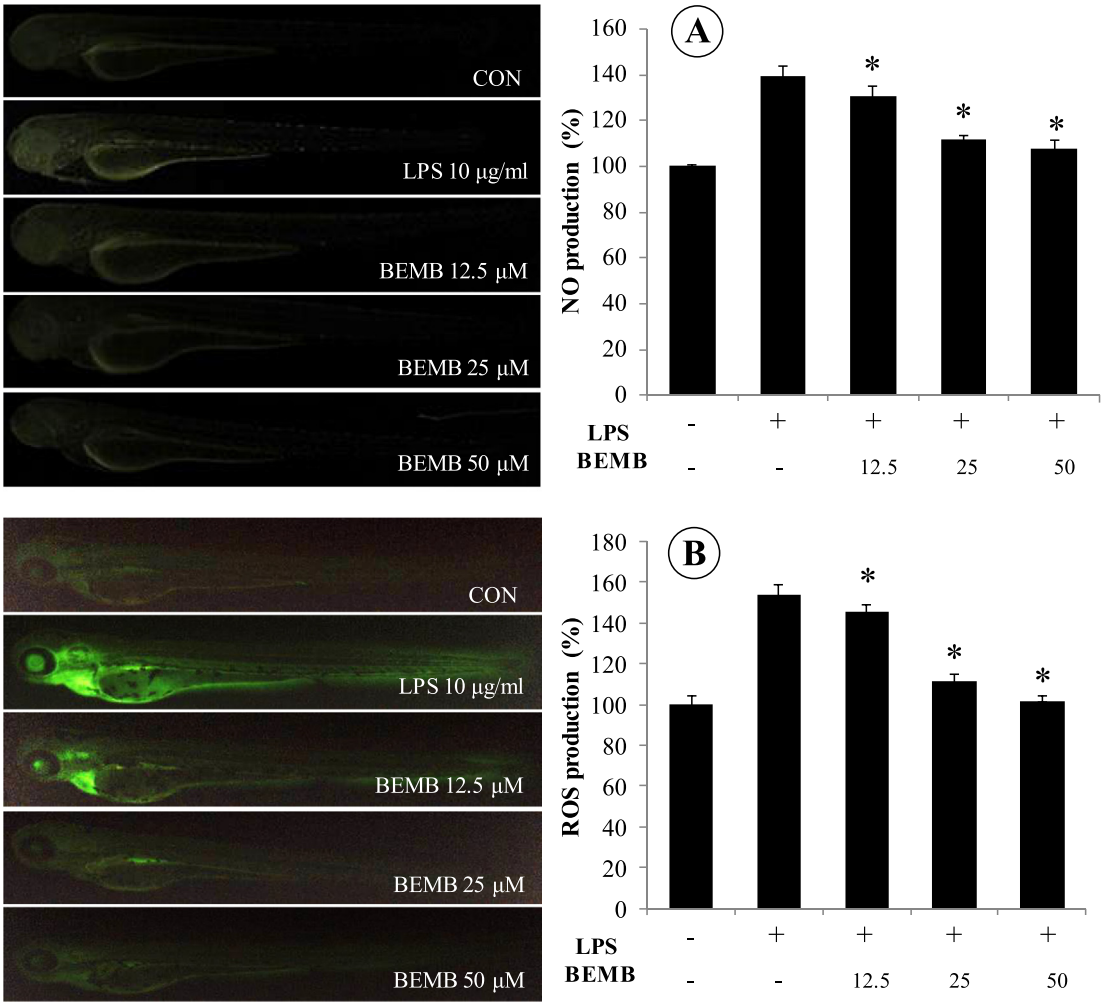

Fig. 4. Effects of 3-bromo-5-(ethoxymethyl)-1,2-benzenediol (BEMB) on production of (A) nitric oxide (NO) and (B) reactive oxygen species (ROS) in lipopolysaccharide (LPS)-stimulated zebrafish embryos. Zebrafish embryos were stimulated with LPS (10 μg/ml) in the presence of BEMB (12.5, 25, and 50 μM) for 3 days post fertilization (dpf). NO and ROS levels were measured using image analysis and fluorescence microscopy. Fluorescence intensity was quantified using the ImageJ software program. Results are means ± standard error (S.E.) of three individual experiments. *P < 0.05 indicates significant differences compared with LPS-stimulated group.

control group. However, BEMB significantly suppressed the protein expression of iNOS and COX-2 in a concentration-dependent manner.

3.7. Effect of BEMB on LPS-induced NF-κB activation in zebrafish embryos

NF-κB is known to play an important role in the induction of inflammation and, therefore, the mRNA expression of NF-κB in LPS-stimulated zebrafish embryos was investigated using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). As shown in Fig. 5B, p65NF-κB expression level of LPS-treated zebrafish embryos increased 9-fold compared to that of the unstimulated control group. However, LPS-stimulated zebrafish embryos treated with BEMB showed a significant reduction in *p65NF-κB* gene expression levels. The inhibitory effect of *IκB-A* levels was slightly but not significantly reduced by BEMB in LPS-induced zebrafish models.

4. Discussions

ROS and NF-κB are known to be important inflammatory regulators, and we also confirmed their critical role in controlling inflammatory factors in LPS-stimulated zebrafish embryos [11]. Discovering substances that modulate ROS and NF-κB in LPS-induced zebrafish embryos could be an important strategy for identifying anti-inflammatory agents. Therefore, in this study, we demonstrated that BEMB isolated from *P. morrowii* inhibited pro-inflammatory mediators by regulating ROS and NF-κB.

NF-κB is a nuclear protein that is critical in controlling the expression of inflammation-associated factors [19]. Under normal conditions, it exists as a heterodimer of the p65 and p50 subunits in the cytoplasm by binding to the inhibitory subunit, IκB. Activation of NF-κB by stimulants such as LPS results in a series of events that lead to the phosphorylation and degradation of IκB-α and, then, the NF-κB subunit freely translocates to the nucleus where it acts as a transcription factor in the synthesis of iNOS and COX-2 [20].

Increased expression of iNOS produces large amounts of NO, which in excess induces tissue damage, gene mutation, nerve damage, and increases vascular permeability to promote inflammatory responses such as edema [21]. In addition, COX-2 is involved in the production of PGE_2, an inflammatory mediator [22]. Therefore, the anti-inflammatory effect of BEMB was confirmed by evaluating its potential inhibition of the production of iNOS, COX-2, and NF-κB in the LPS-stimulated RAW264.7 cells and zebrafish inflammatory reaction. The results revealed that iNOS, COX-2, and NF-κB expression levels were increased by LPS compared with levels of the control groups, but significantly decreased following treatment with BEMB. Additionally, we observed that BEMB inhibited the production of NO in LPS-stimulated

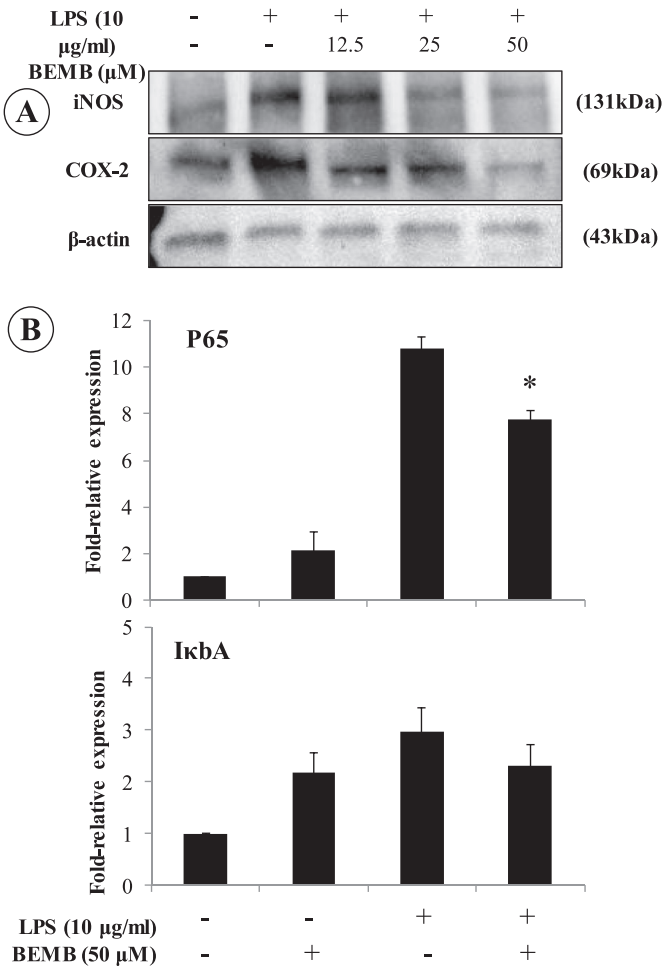

Fig. 5. Effects of 3-bromo-5-(ethoxymethyl)-1,2-benzenediol (BEMB) on expression levels of inducible nitric oxide synthetase (iNOS), cyclooxygenase-2 (COX-2), and nuclear factor (NF)-κB in lipopolysaccharide (LPS)-stimulated zebrafish embryos. Zebrafish embryos were stimulated with LPS (10 μg/ml) in the presence of BEMB (12.5, 25, and 50 μM) for 3 days post fertilization (dpf). (A) iNOS and COX-2 protein levels were determined using western blotting. (B) mRNA expression of P65 and IκB-A were evaluated using reverse transcription-polymerase chain reaction (RT-PCR). Results are expressed as means ± standard error (S.E.) of three individual experiments. *P < 0.05 indicate significant differences compared with LPS-stimulated group.

RAW264.7 cells and zebrafish embryos and BEMB also inhibited the production of PGE2 in LPS-stimulated RAW264.7 cells.

ROS are products of normal cellular metabolism and play vital roles in stimulating signaling pathways in animal cells in response to changes in intra- and extracellular environmental conditions [23]. High ROS levels have been implicated in a variety of pathological conditions including cardiovascular disease, cancer, and aging [24–27]. In addition, ROS are known to be crucial inflammatory mediators, and excessive levels lead to the upregulation of iNOS and COX-2 expression, as well as the induction of pro-inflammatory cytokines [28–30].

ROS can activate NF-κB both directly [31] and *via* other stimuli such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β [32,33]. TNF-α is an endogenous pyrogen that can cause fever and stimulate endotheliocytes and leukocytes to release a series of inflammatory mediators (such as NO and oxyradicals) that may further promote TNF-α production [34]. IL-6 is a multifunctional cytokine with pro-/anti-inflammatory properties and plays a major role in immune and inflammatory responses. In addition, over-expression of IL-6 is involved in pathological conditions such as rheumatoid arthritis and fever [35]. IL-β is an important component in the initiation and enhancement of inflammatory response to microbial infection [36]. In this study, we confirmed the effect of BEMB on the generation of ROS in LPS-stimulated RAW264.7 cells and zebrafish embryos. LPS induced the generation of ROS in RAW264.7 cells and zebrafish embryos; however, BEMB markedly inhibited this production. Taken together, these results demonstrate the anti-inflammatory effects of BEMB, which were mediated by the regulation of NF-kB expression and ROS production in LPS-stimulated RAW264.7 cells and zebrafish embryos.

In conclusion, our results demonstrate that treatment with BEMB decreased the production of NO and expression of iNOS and COX-2 in LPS-induced RAW264.7 cells and zebrafish embryos. In addition, BEMB significantly inhibited the production of ROS and attenuated protein and mRNA expression levels of NF-κB in RAW264.7 cells and zebrafish embryos, respectively. Taken together, these results suggest that BEMB inhibited pro-inflammatory mediators by regulating ROS and NF-κB in LPS-induced RAW264.7 cells and a zebrafish model.

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References

[1] R. Medzhitov, C.A. Janeway Jr., Innate immunity: the virtues of a nonclonal system of recognition, Cell 91 (3) (1997) 295–298.
[2] C.N. Serhan, J. Savill, Resolution of inflammation: the beginning programs the end, Nat. Immunol. 6 (12) (2005) 1191–1197.
[3] J. Shi, Y. Zhao, Y. Wang, W. Gao, J. Ding, P. Li, L. Hu, F. Shao, Inflammatory caspases are innate immune receptors for intracellular LPS, Nature 514 (7521) (2014) 187–192.
[4] E.M. Palsson-McDermott, L.A. O'Neill, Signal transduction by the lipopolysaccharide receptor, toll-like receptor-4, Immunology 113 (2) (2004) 153–162.
[5] S.-M. Song, Y.-M. Ham, Y.-J. Ko, E.-Y. Ko, D.-J. Oh, C.-S. Kim, D. Kim, K.-N. Kim, W.-J. Yoon, Anti-inflammatory activities of the products of supercritical fluid extraction from Litsea japonica fruit in RAW 264.7 cells, J. Funct. Foods 22 (2016) 44–51.
[6] D. Laveti, M. Kumar, R. Hemalatha, R. Sistla, V.G. Naidu, V. Talla, V. Verma, N. Kaur, R. Nagpal, Anti-inflammatory treatments for chronic diseases: a review, Inflamm. Allergy Drug Targets 12 (5) (2013) 349–361.
[7] Y.-M. Ham, S.-H. Cho, S.-M. Song, S.-A. Yoon, Y.-B. Lee, C.-S. Kim, S.-H. Kwon, M.S. Jeong, W.-J. Yoon, K.-N. Kim, Litsenolide A2: the major anti-inflammatory activity compound in Litsea japonica fruit, J. Funct. Foods 39 (2017) 168–174.
[8] J.H. Postlethwait, I.G. Woods, P. Ngo-Hazelett, Y.L. Yan, P.D. Kelly, F. Chu, H. Huang, A. Hill-Force, W.S. Talbot, Zebrafish comparative genomics and the origins of vertebrate chromosomes, Genome Res. 10 (12) (2000) 1890–1902.
[9] N.S. Trede, D.M. Langenau, D. Traver, A.T. Look, L.I. Zon, The use of zebrafish to understand immunity, Immunity 20 (4) (2004) 367–379.
[10] K. Howe, M.D. Clark, C.F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J.E. Collins, S. Humphray, K. McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J.C. Barrett, R. Koch, G.J. Rauch, S. White, W. Chow, B. Kilian, L.T. Quintais, J.A. Guerra-Assuncao, Y. Zhou, Y. Gu, J. Yen, J.H. Vogel, T. Eyre, S. Redmond, R. Banerjee, J. Chi, B. Fu, E. Langley, S.F. Maguire, G.K. Laird, D. Lloyd, E. Kenyon, S. Donaldson, H. Sehra, J. Almeida-King, J. Loveland, S. Trevanion, M. Jones, M. Quail, D. Willey, A. Hunt, J. Burton, S. Sims, K. McLay, B. Plumb, J. Davis, C. Clee, K. Oliver, R. Clark, C. Riddle, D. Elliot, G. Threadgold, G. Harden, D. Ware, S. Begum, B. Mortimore, G. Kerry, P. Heath, B. Phillimore, A. Tracey, N. Corby, M. Dunn, C. Johnson, J. Wood, S. Clark, S. Pelan, G. Griffiths, M. Smith, R. Glithero, P. Howden, N. Barker, C. Lloyd, C. Stevens, J. Harley, K. Holt, G. Panagiotidis, J. Lovell, H. Beasley, C. Henderson, D. Gordon, K. Auger, D. Wright, J. Collins, C. Raisen, L. Dyer, K. Leung, L. Robertson, K. Ambridge, D. Leongamornlert, S. McGuire, R. Gilderthorp, C. Griffiths, D. Manthravadi, S. Nichol, G. Barker, S. Whitehead, M. Kay, J. Brown, C. Murnane, E. Gray, M. Humphries, N. Sycamore, D. Barker, D. Saunders, J. Wallis, A. Babbage, S. Hammond, M. Mashreghi-Mohammadi, L. Barr, S. Martin, P. Wray, A. Ellington, N. Matthews, M. Ellwood, R. Woodmansey, G. Clark, J. Cooper, A. Tromans, D. Grafham, C. Skuce, R. Pandian, R. Andrews, E. Harrison, A. Kimberley, J. Garnett, N. Fosker, R. Hall, P. Garner, D. Kelly, C. Bird, S. Palmer, I. Gehring, A. Berger, C.M. Dooley, Z. Ersan-Urun, C. Eser, H. Geiger, M. Geisler, L. Karotki, A. Kirn, J. Konantz, M. Konantz, M. Oberlander, S. Rudolph-Geiger, M. Teucke, C. Lanz, G. Raddatz, K. Osoegawa, B. Zhu, A. Rapp, S. Widaa, C. Langford, F. Yang, S.C. Schuster, N.P. Carter, J. Harrow, Z. Ning, J. Herrero, S.M. Searle, A. Enright,

R. Geisler, R.H. Plasterk, C. Lee, M. Westerfield, P.J. de Jong, L.I. Zon, J.H. Postlethwait, C. Nusslein-Volhard, T.J. Hubbard, H. Roest Crollius, J. Rogers, D.L. Stemple, The zebrafish reference genome sequence and its relationship to the human genome, Nature 496 (7446) (2013) 498–503.

[11] E.Y. Ko, S.H. Cho, S.H. Kwon, C.Y. Eom, M.S. Jeong, W. Lee, S.Y. Kim, S.J. Heo, G. Ahn, K.P. Lee, Y.J. Jeon, K.N. Kim, The roles of NF-kappaB and ROS in regulation of pro-inflammatory mediators of inflammation induction in LPS-stimulated zebrafish embryos, Fish Shellfish Immunol. 68 (2017) 525–529.

[12] Y.-X. Li, I. Wijesekara, Y. Li, S.-K. Kim, Phlorotannins as bioactive agents from brown algae, Process Biochem. 46 (12) (2011) 2219–2224.

[13] A.A. El Gamal, Biological importance of marine algae, Saudi Pharm. J. 18 (1) (2010) 1–25.

[14] D. Curiel, G. Bellemo, B.L. Rocca, M. Scattolin, M. Marzocchi, First report of *Polysiphonia morrowii* Harvey (Ceramiales, Rhodophyta) in the Mediterranean Sea, Bot. Mar. (2002) 66.

[15] S.Y. Kim, S.R. Kim, M.J. Oh, S.J. Jung, S.Y. Kang, In vitro antiviral activity of red alga, *Polysiphonia morrowii* extract and its bromophenols against fish pathogenic infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus, J. Microbiol. 49 (1) (2011) 102–106.

[16] N.J. Kang, S.C. Han, H.J. Kang, G. Ko, W.J. Yoon, H.K. Kang, E.S. Yoo, Anti-inflammatory effect of 3-bromo-4,5-dihydroxybenzaldehyde, a component of polysiphonia morrowii, in vivo and in vitro, Toxicol. Res. 33 (4) (2017) 325–332.

[17] D. Mikami, H. Kurihara, S.M. Kim, K. Takahashi, Red algal bromophenols as glucose 6-phosphate dehydrogenase inhibitors, Mar. Drugs 11 (10) (2013) 4050–4057.

[18] J. Caamano, C.A. Hunter, NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions, Clin. Microbiol. Rev. 15 (3) (2002) 414–429.

[19] P.A. Baeuerle, D. Baltimore, NF-kappa B: ten years after, Cell 87 (1) (1996) 13–20.

[20] Y. Ben-Neriah, Regulatory functions of ubiquitination in the immune system, Nat. Immunol. 3 (1) (2002) 20–26.

[21] H.Y. Yun, V.L. Dawson, T.M. Dawson, Neurobiology of nitric oxide, Crit. Rev. Neurobiol. 10 (3–4) (1996) 291–316.

[22] I. Posadas, M.C. Terencio, I. Guillen, M.L. Ferrandiz, J. Coloma, M. Paya, M.J. Alcaraz, Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation, Naunyn Schmiedeberg's Arch. Pharmacol. 361 (1) (2000) 98–106.

[23] T. Jabs, Reactive oxygen intermediates as mediators of programmed cell death in plants and animals, Biochem. Pharmacol. 57 (3) (1999) 231–245.

[24] J.T. Hancock, R. Desikan, S.J. Neill, Role of reactive oxygen species in cell signalling pathways, Biochem. Soc. Trans. 29 (Pt 2) (2001) 345–350.

[25] T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing, Nature 408 (6809) (2000) 239–247.

[26] D.L. Cox, D.R. Akins, K.W. Bourell, P. Lahdenne, M.V. Norgard, J.D. Radolf, Limited surface exposure of *Borrelia burgdorferi* outer surface lipoproteins, Proc. Natl. Acad. Sci. U. S. A. 93 (15) (1996) 7973–7978.

[27] K.-N. Kim, S.-J. Heo, C.B. Song, J. Lee, M.-S. Heo, I.-K. Yeo, K.A. Kang, J.W. Hyun, Y.-J. Jeon, Protective effect of Ecklonia cava enzymatic extracts on hydrogen peroxide-induced cell damage, Process Biochem. 41 (12) (2006) 2393–2401.

[28] N. Bryan, H. Ahswin, N. Smart, Y. Bayon, S. Wohlert, J.A. Hunt, Reactive oxygen species (ROS)–a family of fate deciding molecules pivotal in constructive inflammation and wound healing, Eur. Cell. Mater. 24 (2012) 249–265.

[29] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr. Biol. 24 (10) (2014) R453–R462.

[30] A.M. Sadowska, Y.K.B. Manuel, W.A. De Backer, Antioxidant and anti-inflammatory efficacy of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review, Pulm. Pharmacol. Ther. 20 (1) (2007) 9–22.

[31] R. Schreck, P. Rieber, P.A. Baeuerle, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1, EMBO J. 10 (8) (1991) 2247–2258.

[32] R. Schreck, R. Grassmann, B. Fleckenstein, P.A. Baeuerle, Antioxidants selectively suppress activation of NF-kappa B by human T-cell leukemia virus type I tax protein, J. Virol. 66 (11) (1992) 6288–6293.

[33] A.C. Chen, P.R. Arany, Y.Y. Huang, E.M. Tomkinson, S.K. Sharma, G.B. Kharkwal, T. Saleem, D. Mooney, F.E. Yull, T.S. Blackwell, M.R. Hamblin, Low-level laser therapy activates NF-kB via generation of reactive oxygen species in mouse embryonic fibroblasts, PLoS One 6 (7) (2011) e22453.

[34] J.S. Murray, C. Pfeiffer, J. Madri, K. Bottomly, Major histocompatibility complex (MHC) control of CD4 T cell subset activation. II. A single peptide induces either humoral or cell-mediated responses in mice of distinct MHC genotype, Eur. J. Immunol. 22 (2) (1992) 559–565.

[35] Q. Van, B.N. Nayak, M. Reimer, P.J. Jones, R.G. Fulcher, C.B. Rempel, Anti-inflammatory effect of *Inonotus obliquus*, *Polygala senega* L., and *Viburnum trilobum* in a cell screening assay, J. Ethnopharmacol. 125 (3) (2009) 487–493.

[36] E.Y. Kim, K.D. Moudgil, Regulation of autoimmune inflammation by pro-inflammatory cytokines, Immunol. Lett. 120 (1–2) (2008) 1–5.