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Anti-Inflammatory Activity of the Solvent-Partitioned Fractions from *Spergularia marina* in LPS-Stimulated RAW 264.7 Cells

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ABSTRACT: As a part of ongoing research to elucidate and characterize antioxidant and anti-inflammatory nutraceuticals, solvent-partitioned fractions from *Spergularia marina* were tested for their ability to scavenge radicals and suppress inflammation. The results of the 2',7'-dichlorofluorescein diacetate assay indicate that solvent-partitioned fractions from *S. marina* scavenged intracellular radicals in H_2O_2-stimulated mouse macrophages. The tested fractions decreased the generation of nitric oxide (NO) and the expression of inflammation mediators, namely, inducible nitric oxide synthase (iNOS) and interleukin (IL)-6, by lipopolysaccharide (LPS)-induced mouse macrophages, indicating that *S. marina* decreases inflammation. Among all tested fractions [i.e., H_2O, *n*-buthanol (*n*-BuOH), 85% aqueous methanol (aq. MeOH), and *n*-hexane], the 85% aq. MeOH fraction showed the strongest antioxidant and anti-inflammatory response. The 85% aq. MeOH fraction scavenged 80% of the free radicals produced by H_2O_2-induced control cells. In addition, NO production was 98% lower in 85% aq. MeOH fraction-treated cells compared to LPS-induced control cells. The mRNA expression of iNOS and IL-6 was also suppressed in 85% aq. MeOH fraction-treated cells. The results of the current study suggest that the phenolic compound components of *S. marina* are responsible for its antioxidant and anti-inflammatory effects.

Keywords: antioxidant, anti-inflammatory, solvent fraction, halophyte, *Spergularia marina*

INTRODUCTION

Currently, elevated oxidative stress is thought to be responsible for various chronic diseases and complications such as aging, diabetes, and cancer (1,2). Recent studies have shown that there is a close interaction between the oxidative stress and inflammation pathways that underline chronic diseases (3-5). Reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxyl radicals are generated in situations of oxidative stress. Although the body has its own cellular mechanisms to pacify these radicals (i.e., antioxidant enzymes and antioxidants), excessive oxidative stimulation can overpower these inherent defense mechanisms (6). Untreated exposure to free radicals causes vital cell damage that can lead to inflammation and clinical disease (7). Therefore, functional treatments should be developed to treat this underlying cause of clinical disease. In this regard, researchers have turned their attention to attenuating severe inflammation instead of suppressing its symptoms. Likewise, several recent studies have explored the use of natural antioxidant and anti-inflammatory agents as biochemical mechanisms to counter common oxidative stress and inflammation pathways (8-10).

Modern medicine and folk medicine-derived treatments, especially plant materials are being used worldwide to treat oxidative stress and inflammation (11-13). Among the bioactive material sources that have been studied, halophytes have garnered increasing attention throughout the past decade. Halophytes are known for their resistance against the harsh environmental conditions of high salinity waters, mangrove swamps, and marshes (14). Halophytes are praised by researchers for their ability to resist and suppress excessive ROS because of their strong antioxidant capacities (15,16). One such halophyte, *Salicornia herbacea*, is reported to have antioxidant and anti-inflammatory effects (17).

Spergularia, a halophyte, is a common plant that is widely distributed throughout subtropical areas. *Spergularia* sp. are a source of therapeutic agents such as flavonoids and saponins (18,19). Several species of *Spergularia* have been experimented with and appear to have beneficial effects on human health. These beneficial effects include anti-diabetic (20), hypoglycemic (21), diu-

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retic (22), and cholesterol-lowering (23) effects. A prominent research trend to develop novel nutraceutical substances from natural plants has spurred interest in *Spergularia* sp., but the mechanism of action of *Spergularia* sp. remains unknown. For decades, *Spergularia marina*, a local food preference in South Korea, has been regarded as a nutritious source of amino acids, vitamins, and minerals, but the bioactive nutraceuticals present in *S. marina* Griseb have remained unknown. In the present study, the antioxidant and anti-inflammatory activities of the solvent fractions of *S. marina* Griseb extract were measured in cell-based *in vitro* models.

MATERIALS AND METHODS

Plant materials and fractionation

The *S. marina* Griseb was purchased from Yaerak village greenhouse, Munnaemyeon, Haenam in Jeollanam-do, Korea in February, 2012. The sample was air-dried under shade, ground to a powder, and extracted three times with EtOH. The extracts were concentrated under reduced pressure.

The crude extracts (25 g) were suspended in a mixture of CH_2Cl_2 and water. The organic layer was further partitioned in a mixture of 85% aqueous methanol (aq. MeOH) and *n*-hexane and the aqueous layer was fractioned in a mixture of *n*-buthanol (*n*-BuOH) and H_2O. This process yielded the following solvent fractions of *S. marina* Griseb extract: 85% aq. MeOH (1.6 g) and *n*-hexane (3.5 g) fractions from the organic layer and *n*-BuOH (1.2 g) and water (18.9 g) fractions from the aqueous layer.

Cell culture and cytotoxicity

Murine RAW 264.7 cells were grown as monolayers in a 5% CO_2 and 37°C humidified atmosphere using Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 μg/mL penicillin-streptomycin (Gibco-BRL). The medium was changed two or three times per week.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes, was used to determine the cytotoxic effects of the solvent-partitioned fractions from *S. marina* on cultured cells. The cells were plated in 96-well plates at a density of 5×10^3 cells/well. After 24 h, the cells were washed with fresh medium and were treated with control medium or medium supplemented with *S. marina* Griseb. After incubation for 24 h, cells were rewashed, 100 μL of MTT solution (1 mg/mL) was added, and the cells were incubated for an additional 4 h. Finally, 100 μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The amount of formazan present in each well was determined by measuring the absorbance of each well at 540 nm with a GENios® microplate reader (Tecan Austria GmbH, Grödig, Austria). Relative cell viability was determined by the measuring the amount of MTT converted into formazan crystals. The viability of the RAW 264.7 cells and dose response curves are shown as a percentage of the viability of the control-treated RAW 264.7 cells.

Determination of intracellular formation of ROS using 2',7'-dichlorofluorescin diacetate (DCF-DA) labeling

The intracellular formation of ROS was assessed using DCF-DA, an oxidation sensitive dye, as a substrate. RAW 264.7 cells growing in fluorescence microtiter 96-well plates were loaded with 20 μM DCF-DA in Hank's buffered salt solution (HBSS) and incubated for 20 min in the dark. The nonfluorescent DCF-DA dye freely penetrated into cells, where it was hydrolyzed by intracellular esterases to 2',7'-dichlorodihydrofluororescein (DCFH), which was trapped inside the cells. The cells were then treated with different concentrations of each test compound and incubated for 1 h. The cells were washed with PBS three times, and then 500 μM H_2O_2 in HBSS was added to the cells. Every 30 min, a GENios® microplate reader (Tecan Austria GmbH) was used to measure the formation of 2',7'-dichlorofluorescein (DCF) due to the oxidation of DCFH in the presence of various ROS at an excitation wavelength (Ex) of 490 nm and an emission wavelength (Em) of 620 nm. The dose-dependent and time-dependent effects of each treatment were plotted and compared with the intensity of the fluorescence of the control and blank groups.

Measurement of nitric oxide production

RAW 264.7 cells (2×10^5 cells/well) were seeded onto 96-well plates with DMEM without phenol red. The cells were allowed to adhere overnight and then were pre-treated with the *S. marina* Griseb extracts for 1 h. Cellular nitric oxide (NO) production was stimulated by adding 1 μg/mL (final concentration) of lipopolysaccharide (LPS). LPS-stimulated cells were incubated for 24 h and 48 h. After incubation, Griess reagent [1% sulfanilamide, 2% phosphoric acid, and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride] was used to determine NO production. Briefly, 50 mL of culture supernatant was mixed with an equal volume of Griess reagent. After 15 min of incubation at room temperature, the absorbance was measured at 550 nm with a GENios® microplate reader (Tecan Austria GmbH). Nitrite concentrations were calculated by regression analysis. Serial dilutions of sodium nitrite were used as a standard.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analyses

TRIzol® reagent (Invitrogen Co., Carlsbad, CA, USA) was used to isolate the total RNA from RAW 264.7 macrophages treated with/without the solvent-partitioned fractions from *S. marina*. To synthesize cDNA, the total RNA (2 μg) was added to RNase-free water and oligo(dT). The mixture was denatured at 70°C for 5 min and then immediately cooled. RNA was reverse transcribed in a master mix containing 1× RT buffer, 1 mM dNTPs, 500 ng oligo(dT), 140 U M-MLV reserve transcriptase, and 40 U RNase inhibitor at 42°C for 60 min and then at 72°C for 5 min using an automatic T100 Thermal Cycler (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The target cDNA was amplified using the following sense and antisense primers: forward 5'-TTC-CAG-AAT-CCC-TGG-ACA-AG-3' and reverse 5'-TGG-TCA-AAC-TCT-TGG-GGT-TC-3' for inducible nitric oxide synthase (iNOS); forward 5'-AGT-TGC-CTT-CTT-GGG-ACT-GA-3' and reverse 5'-CAG-AAT-TGC-CAT-TGC-ACA-AC-3' for interleukin (IL)-6; and forward 5'-CCA-CAG-CTG-AGA-GGG-AAA-TC-3' and reverse 5'-AAG-GAA-GGC-TGG-AAA-AGA-GC-3' for β-actin. The cDNA was amplified with 30 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 45 s. After amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel for 30 min at 100 V. The gels were then stained with 1 mg/mL ethidium bromide and visualized by UV light using a Davinch-Chemi imager™ (CAS-400SM, Wako Co., Osaka, Japan).

Gene expression was measured by real-time PCR in a Thermal Cycler Dice® Real Time System TP800 (Takara Bio Inc., Ohtsu, Japan) following the manufacturer's protocol. Briefly, 1.0 μL of DNA sample was mixed with 12.5 μL of Maxima® SYBR Green qPCR Master Mix (Thermo Scientific, Inc., Waltham, MA, USA), which contained Taq DNA polymerase, dNTPs, and reaction buffer. The target cDNA was amplified using the same primers described for the RT-PCR analysis plus the following sense and antisense primers: forward 5'-AGA-AGG-AAA-TGG-CTG-CAG-AA-3' and reverse 5'-GCT-CGG-CTT-CCA-GTA-TTG-AG-3' for cyclooxygenase (COX)-2; forward 5'-AGC-CCC-CAG-TCT-GTC-TCC-TT-3' and reverse 5'-CAT-TCG-AGG-CTC-CAG-TGA-AT-3' for tumor necrosis factor (TNF)-α; and forward 5'-GGG-CCT-CAA-AGG-AAA-GAA-TC-3' and reverse 5'-TAC-CAG-TTG-GGG-AAC-TCT-GC-3' for IL-1β. The mixture was denatured at 95°C for 10 min, and then underwent 40 PCR cycles of 95°C for 15 s and 60°C for 60 s. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. β-actin was used as the internal control.

Determination of total polyphenol content

The total polyphenol content of *S. marina* fractions was determined by the Folin-Ciocalteu method. In brief, 20 μL of each extract was mixed with 100 μL of 1:10 Folin-Ciocalteu reagent in a microplate. Then, 80 μL of 7.5% Na_2CO_3 was added to each well and the microplate was incubated in the dark at room temperature for 2 h. After incubation, the absorbance at 600 nm was recorded for each well. Gallic acid was used as the standard reference. Polyphenol content was expressed as mg gallic acid equivalents per gram of sample (mg GAE/g).

Statistical analysis

The data are presented as mean±SD. Differences between the means of the individual groups were analyzed with the analysis of variance (ANOVA) procedure of Statistical Analysis System SAS v9.1 (SAS Institute, Cary, NC, USA) with Duncan's multiple range tests. The significance of differences was determined at the $P<0.05$ level.

RESULTS AND DISCUSSION

Effect of solvent-partitioned fractions from *S. marina* on free radical scavenging activity

Prior to performing the RAW 264.7 mouse macrophage-based *in vitro* oxidative stress assay, the cytotoxicity of the solvent-partitioned fractions from *S. marina* was evaluated by MTT assay. For all solvent fractions tested, an *S. marina* concentration of 0.5 mg/mL did not affect the viability of RAW 264.7 mouse macrophages (Fig. 1A). Hence, this *S. marina* concentration was used for further *in vitro* assays.

The DCF-DA assay was used to determine the antioxidant potential of *S. marina* solvent fractions in RAW 264.7 cells. ROS scavenging ability was determined by measuring the change in fluorescent intensity accompanied by the oxidation of DCFH to DCF in H_2O_2-exposed RAW 264.7 cells. For all fractions tested, the intracellular oxidative stress of H_2O_2-induced RAW 264.7 cells treated with 0.5 mg/mL of *S. marina* was lower than the intracellular oxidative stress of untreated, H_2O_2-induced control cells (Fig. 1B). DCFH oxidation was lowest in cells treated with the 85% aq. MeOH fraction, indicating that this fraction significantly decreased H_2O_2-induced free radical scavenging. The oxidative stress level of H_2O_2-stimulated cells (i.e., control cells) was approximately 80% greater than the oxidative stress level of cells that were not exposed to H_2O_2 (i.e., blank cells). The free radical scavenging effects of the H_2O, *n*-BuOH, and *n*-hexane fractions were 41%, 40%, and 63%, respectively. Several published reports have indicated that MeOH fractions contain phenolic substances, especially flavonoids that can act as strong antioxidants (24). Halophytes have also been reported to contain such com-

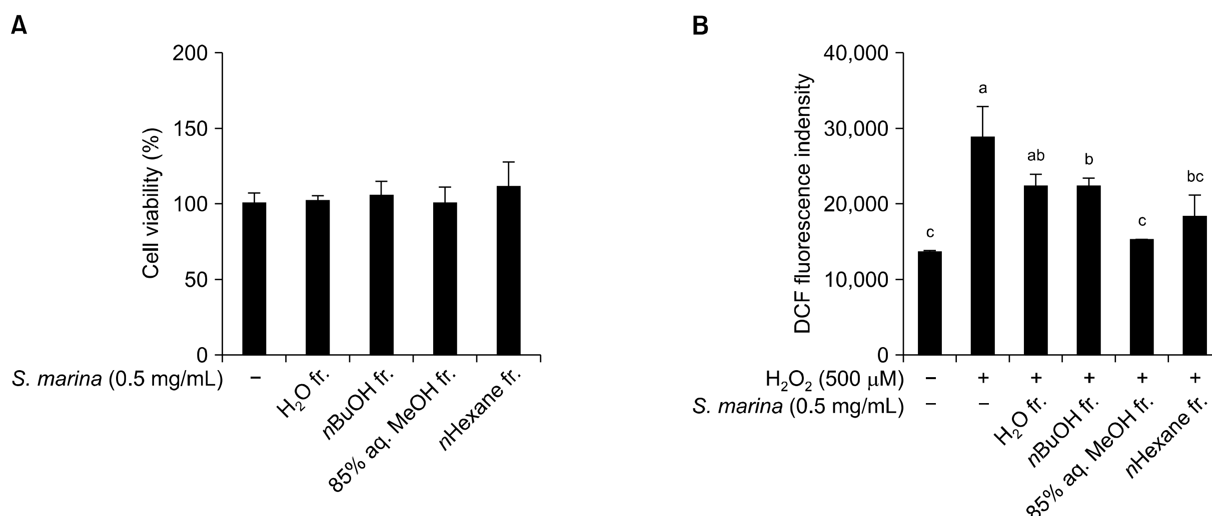

Fig. 1. Cytotoxicity (A) and cellular radical scavenging activity (B) of the solvent-partitioned fractions from *S. marina* in RAW 264.7 cells. (A) Cytotoxicity was determined by MTT assay. (B) After preincubation in 20 μM DCF-DA, RAW 264.7 cells were treated with the solvent fractions for 120 min. Following the addition of 500 μM H_2O_2, DCF fluorescence was measured at $\lambda_{excitation}$ 490 nm and $\lambda_{emission}$ 620 nm. Means with different letters (a-c) are significantly different (P<0.05) by Duncan's multiple range test.

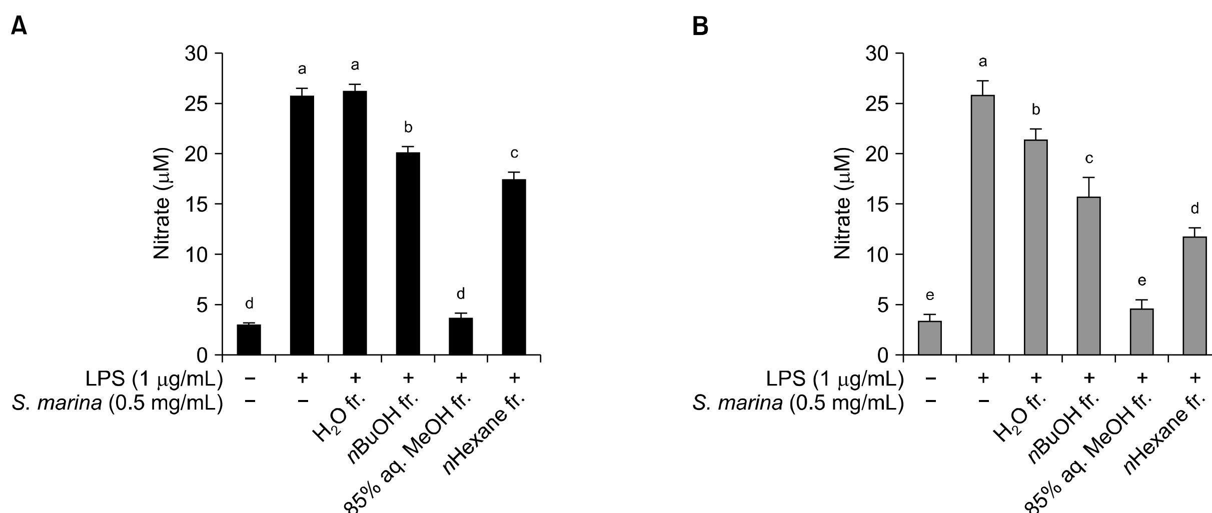

Fig. 2. Effect of the solvent-partitioned fractions from *S. marina* on intracellular NO levels in LPS-stimulated RAW 264.7 cells. The cells were pretreated with LPS (1 μg/mL), followed by treatment with the solvent fractions for 24 h (A) and 48 h (B). The nitrite content of the culture media was analyzed. Means with different letters (a-e) are significantly different (P<0.05) by Duncan's multiple range test.

pounds (25). This suggests that the phenolic and/or flavonoid content of *S. marina* may be responsible for its strong antioxidant properties.

Anti-inflammatory effects of solvent-partitioned fractions from *S. marina*

Strong antioxidant molecules are considered to show anti-inflammatory effect due to close relation between inflammatory and oxidative stress pathways (8). Therefore, the anti-inflammatory activity of solvent-partitioned fractions from *S. marina* on LPS-stimulated RAW 264.7 mouse macrophages was evaluated in this study. When activated with pathogenic substances (e.g., LPS), macrophages initiate and regulate inflammatory responses through a broad range of inflammatory mediators. LPS-stimulated macrophages produce inflammatory mediators such as free radicals, NO, iNOS, and IL-6 (26).

After confirming the anti-oxidative effect of the *S. marina* solvent fractions, the anti-inflammatory potential of these fractions was evaluated by assessing NO production by LPS-stimulated RAW 264.7 cells in the presence and absence of the solvent-partitioned *S. marina* fractions. The presence of LPS activated the RAW 264.7 mouse macrophages, leading to an elevation in NO production from 3.4 μM to 26 μM after a 24 h-incubation (Fig. 2A) and from 3.6 μM to 26 μM after a 48 h-incubation (Fig. 2B). In accordance with its strong antioxidant effect, the 85% aq. MeOH fraction lowered the

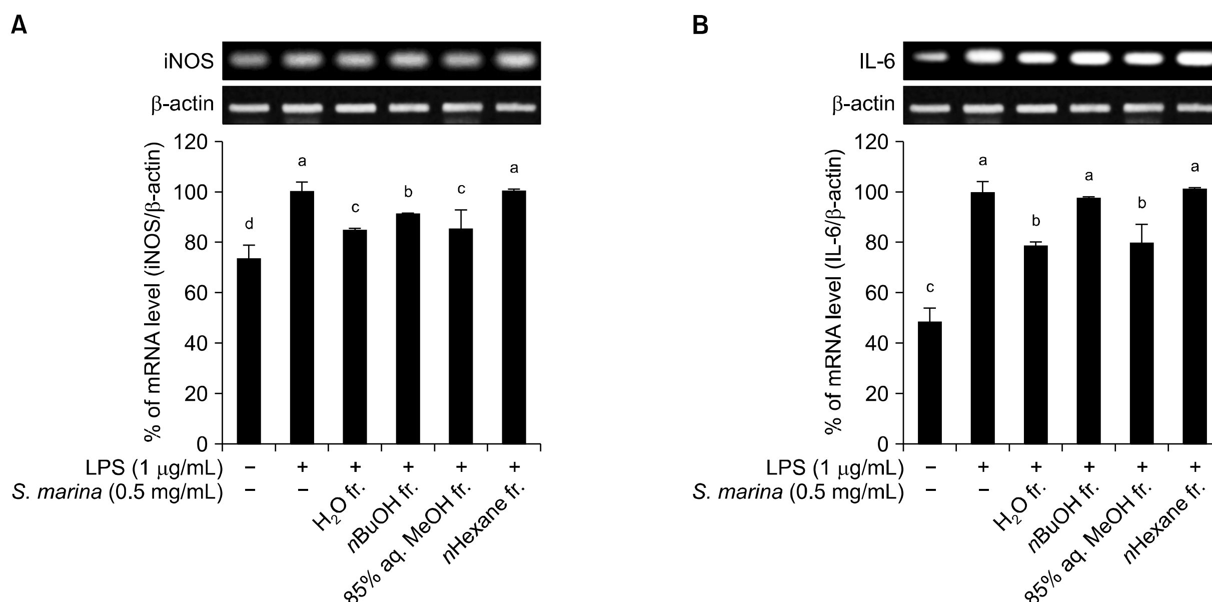

Fig. 3. Effect of the solvent-partitioned fractions from *S. marina* on iNOS (A) and IL-6 (B) gene expression in LPS-stimulated RAW 264.7 cells. The cells were treated with various concentrations of the solvent fractions for 1 h prior to the addition of LPS. Following LPS-stimulation, the cells were further incubated for 24 h. Means with different letters (a-d) are significantly different ($P<0.05$) by Duncan's multiple range test.

NO production the most. Treatment with the 0.5 mg/mL concentration of the 85% aq. MeOH fraction decreased the amount of NO produced under LPS-stimulation to approximately 3.9 μM and 4.6 μM for the 24 h and 48 h incubation periods, respectively. The other *S. marina* fractions were not as effective as the 85% aq. MeOH fraction at decreasing NO production by LPS-stimulated RAW 264.7 cells, especially during the 24 h incubation trial. After 24 h of incubation, the *n*-BuOH and *n*-hexane fractions decreased NO production to 20 μM and 17 μM, respectively, while the H_2O fraction did not decrease NO production by LPS-stimulated cells. However, after 48 h of incubation, the H_2O, *n*-BuOH, and *n*-hexane fractions decreased NO production to 23 μM, 16 μM, and 12 μM, respectively.

In order to evaluate the anti-inflammatory effect of solvent-partitioned fractions from *S. marina* in detail, the expression of key indicators of the inflammatory response were measured by RT-PCR and quantitative real-time PCR. The inflammatory response of macrophages is accompanied by the induction of inflammatory gene expression. This induction of the inflammatory response is mediated by the generation of transcriptional factors by inducible iNOS and cytokines such as IL-6 (27).

Various reports have suggested that elevated iNOS expression is closely related to the pathogenesis of inflammation and diseases such as cancer and Alzheimer's disease (28,29). Therefore, the effect of *S. marina* fractions on the gene expression of the inflammation mediator enzyme iNOS was observed in LPS-stimulated mouse macrophages. As shown in Fig. 3A, iNOS mRNA expression was elevated by the inflammatory response to LPS and slightly suppressed by treatment with the H_2O, *n*-BuOH, and 85% aq. MeOH fractions of *S. marina*. However, treatment with n-hexane fraction did not affect LPS-stimulated iNOS expression. Among all fractions tested, the 85% aq. MeOH and H_2O fractions were the most effective at suppressing iNOS mRNA expression.

The effect of the *S. marina* solvent fractions on the gene expression of IL-6, an inflammatory cytokine, was also evaluated. As expected, treatment with the H_2O and 85% aq. MeOH fractions of *S. marina* decreased the expression of IL-6, while the *n*-BuOH and *n*-hexane fractions did not significantly change the expression levels (Fig. 3B).

To further confirm the fractions' anti-inflammatory effects, the expression of key markers related to inflammation was evaluated by quantitative real-time PCR. The data obtained were in accordance with our previous results. The MeOH fraction lowered the expression of key markers, namely iNOS, IL-6, COX-2, IL-1β, and TNF-α (Fig. 4). While all fractions were effective to an extent, the MeOH fraction was the most effective inhibitor of anti-inflammatory marker expression. In the presence of the MeOH fraction of *S. marina*, the LPS-stimulated expression of inflammation markers was significantly lowered to the level of unstimulated, blank cells.

Collectively, *S. marina* was shown to possess antioxidant and anti-inflammatory effects *in vitro*. Comparison of the solvent-partitioned fractions from *S. marina* also presented valuable insights for future activity-based compound isolation. The strong effect of the 85% aq.

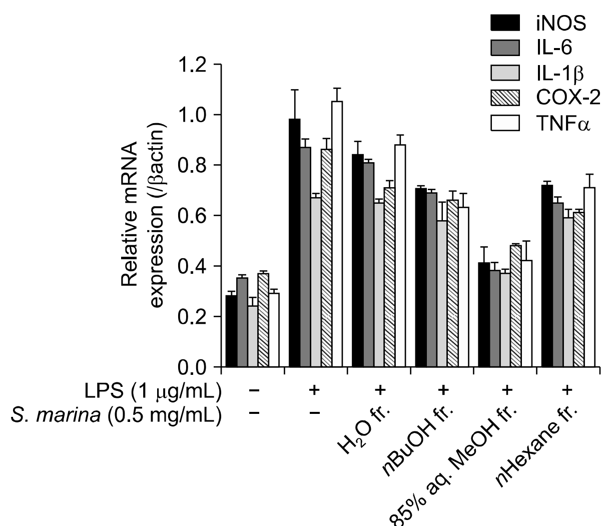

Fig. 4. Effect of the solvent-partitioned fractions from *S. marina* on the mRNA expression of key inflammation markers in LPS-stimulated RAW 264.7 cells. mRNA expression was measured by real-time PCR assay.

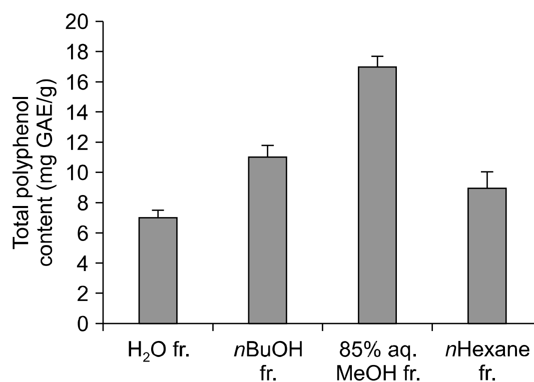

Fig. 5. Total polyphenol content of the solvent-partitioned fractions from *S. marina*. Polyphenol content is expressed as mg gallic acid equivalents per gram of sample (mg GAE/g).

MeOH fraction suggested that this fraction contained more bioactive phenolic compounds with notable antioxidant and anti-inflammatory effects than the other fractions tested. Reports have already described that the MeOH fractions of marine plants contain various bioactive substances, including tannins and flavonoids (24). Because previous works indicate that polyphenols may be one class of bioactive materials present in *S. marina*, we also measured the polyphenol content of the *S. marina* fractions. A notably greater polyphenol content was observed in the MeOH fraction of *S. marina* than in the H_2O, *n*-BuOH, or *n*-hexane fractions of *S. marina* (Fig. 5). Previous work also indicates that antioxidant tannins and flavonoids possess anti-inflammatory activities (30). *S. marina* fractions were shown to contain antioxidant and anti-inflammatory substances, 85% aq. MeOH being the most active fraction among others. Coupled with the high phenolic content of 85% aq. MeOH fraction, antioxidant and anti-inflammatory materials of *S. marina* were suggested to be phenol derivatives.

Taken together, the results of the present study suggest that *S. marina* could be a significant source of therapeutic agents. Further studies focused on the isolation and elucidation of active ingredients from the 85% aq. MeOH fraction of *S. marina* would pave the way for the efficient utilization of *S. marina* as a nutraceutical source. Nonetheless, the MeOH fraction of *S. marina* extract was exhibited to possess antioxidant and anti-inflammatory activities.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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