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

Chang-Suk Kong

Department of Food and Nutrition, College of Medical and Life Science, Silla University, Busan 617-736, Korea

**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 H2O2-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., H2O, *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 H2O2-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 underlie chronic diseases (3-5). Reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxy 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-
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 CH2Cl2 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 fractionated in a mixture of n-buthanol (n-BuOH) and H2O. 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% CO2 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.

This process yielded the following solvent fractions of *S. marina* Griseb: 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.

RAW 264.7 cells were grown in fluorescence microtitre 96-well plates (final concentration) of lipopolysacc-haride (LPS). LPS-stimulated cells were incubated for 24 h and 48 h. After incubation, Griess reagent [1% sulfali-namide, 2% phosphoric acid, and 0.1% N-(1-naph-thyl)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'-TTCTCAG-AAT-CCC-TGG-ACA-AG-3' and reverse 5'-TGG-TCA-ACC-TCT-TGG-GGT-TC-3' for inducible nitric oxide synthase (iNOS); forward 5'-AGT-TGC-CTT-CTT-GGG-AC-3' and reverse 5'-CAG-AAT-TGCA-CAT-TGC-ACA-AC-3' for interleukin (IL)-6; and forward 5'-CCA-TCA-AAC-TCT-TGG-GGT-TC-3' for cyclooxygenase (COX)-2; forward 5'-AGC-CCC-CAG-TCT-GTC-TCC-TT-3' and reverse 5'-AGA-GGG-AAA-TGG-CTG-CAG-AA-3' and reverse 5'-TTCT-CAG-CCC-CAT-GAA-GG-3' for tumor necrosis factor (TNF)-β. 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 toDCF in H2O2-exposed RAW 264.7 cells. For all fractions tested, the intracellular oxidative stress of H2O2-induced RAW 264.7 cells treated with 0.5 mg/mL of S. marina was lower than the intracellular oxidative stress of untreated, H2O2-induced control cells (Fig. 1B). DCFH oxidation was lowest in cells treated with the 85% aq. MeOH fraction, indicating that this fraction significantly decreased H2O2-induced free radical scavenging. The oxidative stress level of H2O2-stimulated cells (i.e., control cells) was approximately 80% greater than the oxidative stress level of cells that were not exposed to H2O2 (i.e., blank cells). The free radical scavenging effects of the H2O2, 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). Halo-phytes have also been reported to contain such com-
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.

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 anti-oxidant effect, the 85% aq. MeOH fraction lowered the
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 H2O fraction did not decrease NO production by LPS-stimulated cells. However, after 48 h of incubation, the H2O, 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 H2O, 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 H2O 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 H2O 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.
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 H2O, 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.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Berlett BS, Stadtman ER. 1997. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 272: 20313-20316.
2. Mayne ST. 2003. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. J Nutr 133: 933S-940S.
3. Holvoet P. 2008. Relations between metabolic syndrome, oxidative stress and inflammation and cardiovascular disease. Verh K Acad Geneeskd Belg 70: 193-219.
4. Elmarakby AA, Sullivan JC. 2012. Relationship between oxidative stress and inflammatory cytokines in diabetic nephropathy. Cardiov Diath Ther 30: 49-59.
5. Khansari N, Shakhla Y, Mahmoudi M. 2009. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. Recent Pat Inflamm Allergy Drug Discov 3: 73-80.
6. Cadenas E, Davies KJ. 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med 29: 222-230.
7. Conner EM, Grisham MB. 1996. Inflammation, free radicals, and antioxidants. Nutrition 12: 274-277.
8. Geronikaki AA, Gavalas AM. 2006. Antioxidants and inflammatory disease: synthetic and natural antioxidants with anti-inflammatory activity. Comb Chem High Throughput Screen 9: 425-442.
9. Nichols JA, Katiyar SK. 2010. Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. Arch Dermatol Res 302: 71-83.
10. Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nikolaides DN. 2004. Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities. Curr Pharm Des 10: 3813-3833.
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11. de las Heras B, Slowing K, Benedí J, Carretero E, Ortega T, Toledo C, Bermejo P, Iglesias I, Abad MJ, Gómez-Serranillos P, Liso PA, Villar A, Chiriboga X. 1998. Antiinflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. *J Ethnopharmacol* 61: 161-166.

12. Schinella GR, Tournier HA, Prieto JM, Mordujovich de Buschiazzo P, Ríos JL. 2002. Antioxidant activity of anti-inflammatory plant extracts. *Life Sci* 70: 1023-1033.

13. Talhouk RS, Karam C, Fostok S, El-Jouni W, Barbour EK. 2007. Anti-inflammatory bioactivities in plant extracts. *J Med Food* 10: 1-10.

14. Flowers TJ, Colmer TD. 2008. Salinity tolerance in halophytes. *New Phytol* 179: 945-963.

15. Amor NB, Jiménez A, Megdiche W, Lundqvist M, Sevilla F, Abdelly C. 2006. Response of antioxidant systems to NaCl stress in the halophyte *Cakile maritima*. *Physiol Plant* 126: 446-457.

16. Ksouri R, Megdiche W, Falleh H, Trabelsi N, Boulaaba M, Smaoui A, Abdelly C. 2008. Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes. *C R Biol* 331: 865-873.

17. Kim YA, Kong CS, Um YR, Lim SY, Yea SS, Seo Y. 2009. Evaluation of *Salicornia herbacea* as a potential antioxidant and anti-inflammatory agent. *J Med Food* 12: 661-668.

18. De Tommasi N, Piacente S, Gacs-Baitz E, De Simone F, Pizza C, Aquino R. 1998. Triterpenoid saponins from *Spergularia ramosa*. *J Nat Prod* 61: 323-327.

19. Jouad H, Lacaille-Dubois MA, Eddouks M. 2001. Chronic diuretic effect of the water extract of *Spergularia purpurea* in normal rats. *J Ethnopharmacol* 75: 219-223.

20. Jouad H, Lembhard A, Maghrani M, Zeggwagh NA, Eddouks M. 2003. Cholesterol-lowering activity of the aqueous extract of *Spergularia purpurea* in normal and recent-onset diabetic rats. *J Ethnopharmacol* 87: 43-49.

21. Škerget M, Kotnik P, Hadolin M, Hraš AR, Simončič M, Knez Ž. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem* 89: 191-198.

22. Bertin RL, Gonzaga LV, Borges GSC, Azevedo MS, Maltez HF, Heller M, Micke GA, Tavares LBB, Fett R. 2014. Nutrient composition and, identification/quantification of major phenolic compounds in *Sarcocornia ambiguus* (Amaranthaceae) using HPLC-ESI-MS/MS. *Food Res Int* 55: 404-411.

23. Meng F, Lowell CA. 1997. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. *J Exp Med* 185: 1661-1670.

24. Pahl HL. 1999. Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 18: 6853-6866.

25. Yangamoto Y, Gaynor RB. 2001. Role of the NF-κB pathway in the pathogenesis of human disease states. *Curr Mol Med* 1: 287-296.

26. Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N, Smith PT, Bartlett J, Shannugam K, Münch G, Wu MJ. 2011. Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. *J Agric Food Chem* 59: 12361-12367.