Radical Scavenging and Anti-Inflammatory Effects of the Halophyte *Spergularia marina* Griseb.

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Z. Naturforsch. **69c**, 425 – 433 (2014) / DOI: 10.5560/ZNC.2014-0098
Received May 20 / December 5, 2014 / published online January 14, 2015

As a part of ongoing research to develop antioxidant and anti-inflammatory nutraceuticals, an ethanolic extract of *Spergularia marina* Griseb. was tested for its ability to scavenge radicals and suppress inflammation. The extract was able to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and superoxide radicals, respectively, in cell-free environments as well as intracellular radicals in H_2O_2-stimulated mouse macrophages. Inflammation in mouse macrophages induced by lipopolysaccharides was suppressed by *S. marina* according to the measurement of nitric oxide generation and expression of inflammatory cytokines, *i.e.* tumour necrosis factor-α (TNFα), interleukin (IL)-1β, and IL-6. The anti-inflammatory effect of *S. marina* was substantiated by the finding that the expression of the inflammatory modulators, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), was significantly decreased. The chemical composition of *S. marina* was evaluated by FT-IR analysis of the extract indicating the presence of polyphenols and flavonoids. In conclusion, *S. marina* is suggested as a novel source for antioxidant and anti-inflammatory agents.

Key words: Antioxidant, Anti-Inflammatory, Free Radical Scavenger, *Spergularia marina* Griseb.

Introduction

Several chronic diseases and phenomena, such as aging, diabetes, and cancer, are reported to be accompanied by elevated oxidative stress (Berlett and Stadtman, 1997; Mayne, 2003). Recent studies clearly indicated a set of distinctive interactions including well-studied pathways that relate oxidative stress to inflammation (Elmarakby and Sullivan, 2012; Holvoet, 2008; Khansari *et al*., 2009). It is generally accepted that oxidative stress is a consequence of the formation of reactive oxygen species (ROS) such as superoxide, hydroxyl, and peroxyl radicals, respectively. Although the body has cellular mechanisms to detoxify these radicals through antioxidant enzymes and antioxidants, defence mechanisms can be overpowered by excessive oxidative stimulation (Cadenas and Davies, 2000). Free radicals cause cell damage that can eventually lead to inflammation and, lastly, clinical diseases (Conner and Grisham, 1996). Functional treatment of severe inflammation and other diseases related to ROS should be directed towards elimination of their causes rather than suppression of their symptoms. In this regard, intervention with the biochemical mechanisms of common oxidative stress and inflammation pathways is the aim of several recent studies trying to identify natural antioxidant and anti-inflammatory agents (Fylaktakidou *et al*., 2004; Geronikaki and Gavalas, 2006; Nichols and Katiyar, 2010). Therapeutic agents acting against both oxidative stress and inflammation may be beneficial to human health by allowing prevention and treatment of pathologic complications caused by ROS.

Worldwide, plants are the major sources of natural antioxidant and anti-inflammatory agents, and many of them are used in folk medicine (de las Heras *et al*., 1998; Schinella *et al*., 2002; Talhouk *et al*., 2007). Among them, halophytes have been receiving increasing attention. Halophytes are known for their resistance to harsh environmental conditions of high salinity waters, mangrove swamps, and marshes (Flowers and Colmer, 2008). Halophytes are credited for their ability to resist and suppress excessive ROS due to their arsenal of strong antioxidants (Amor *et al*., 2006; Ksouri *et al*., 2008), among which polyphenols play a major role and have potential uses for therapeutic purposes (Ksouri *et al*., 2009; Meot-Duros *et al*., 2008). Kim *et al*. (2009) promoted the halophyte *Salicornia herbacea* as a source of strong antioxidant and anti-inflammatory agents. Species of another plant genus, *Spergularia*, contain quite common halophytic species that are widely distributed in subtropical areas, and *Spergularia* species have been shown to be the source of biologically active compounds with reputed medicinal properties such as flavonoids and saponins (De Tommasi *et al*., 1998; Jouad *et al*., 2001a, b). Several species of *Spergularia* have been investigated for biochemical and pharmacological aspects and found to have antidiabetic (Vinholes *et al*., 2011), hypoglycemic (Eddouks *et al*., 2003), diuretic (Jouad *et al*., 2001a), and cholesterol-lowering (Jouad *et al*., 2003) bioactivities, respectively, yet few mechanistic studies are available. *S. marina* Griseb., which is used locally as a food in South Korea, is regarded as a nutritious source of amino acids, vitamins, and minerals, but there are no reports on potential health beneficial effects of this plant, apart from its nutritious value, while other *Spergularia* species have been reported to be important sources of several therapeutic agents. Therefore in the present study, as part of our continuous search for bioactive nutraceuticals, we investigated the ethanolic extract of *S. marina* for its antioxidant and anti-inflammatory activities both in cell-free and cell-based *in vitro* models.

Materials and Methods

Materials

Spergularia marina Griseb. was purchased from a greenhouse in the village Yaerak, Haenam district of Jeollanam-do province, Korea, in February 2012. The sample was authenticated in the Marine Biotechnology Department of Silla University (Busan, Korea), and a voucher sample was deposited in the collection of the same department. The sample was air-dried under shade and ground to a powder. To 100 g of powdered *S. marina* in a conical flask, 500 ml of ethanol were added, and the resulting suspension was shaken at 150 rpm for 24 h. This process was repeated twice with the respective residues. The combined extracts were filtered through Whatman no. 1 filter paper, and the solvent was removed by rotary evaporation yielding 34 g of crude extract. The crude extract was dissolved in 10% (v/v) dimethylsulfoxide (DMSO) for further use.

Determination of DPPH radical scavenging activity

The free radical scavenging activity was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method (Blois, 1958; Hasan *et al*., 2006). The DPPH (Sigma-Aldrich, St. Louis, MO, USA) solution (150 μM) was prepared in 95% ethanol. One hundred μl of sample were added to 100 μl of DPPH solution and kept for 30 min in the dark at room temperature. Finally, the discoloration of the mixture was measured at 520 nm using a GENios® microplate reader (Tecan Austria, Grödig, Austria). The control (vitamine C) was prepared identically, but without sample, and 95% ethanol was used as the blank. Percent scavenging of the DPPH free radicals was quantified relative to the control.

Determination of free radial scavenging activity by ESR spectroscopy

Different radicals were generated, and spin adducts were recorded using a JES-FA electron spin resonance (ESR) spectrometer (JEOL, Tokyo, Japan) at 25 °C (Kong *et al*., 2009). Instrument settings were as follows: magnetic field, (336 ± 5) mT; sweep time, 30 s; sweep width, 10 mT; modulation width, 0.1 mT; and modulation frequency, 100 kHz.

DPPH radical assay

DPPH radicals were measured using the method described by Nanjo *et al*. (1996). A solution (60 μl) of sample in ethanol (or pure ethanol as a control) was added to a solution (60 μl) of 60 μM DPPH in ethanol. The mixture was vortexed for 10 s and then transferred to a capillary tube and sealed. After 2 min, the DPPH radical spin resonance was recorded at 1 mW microwave power and 1000 amplitude.

Hydroxyl radical assay

The Fenton reaction was performed by reacting 50 μl of 10 mM $FeSO_4$ and 50 μl of 10 mM H_2O_2 to generate hydroxyl radicals. Generated radicals were trapped by 50 μl of 0.3 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in the presence of sample solution (50 μl) or the same volume of 0.1 M sodium phosphate buffer (pH 7.4) as a control. After 2.5 min, the reaction mixture was transferred to a capillary tube and, after sealing, the DMPO/$^{\bullet}$OH spin adduct was recorded at 1 mW microwave power and 400 amplitude.

Superoxide radical assay

Superoxide anion radicals ($O_2^{\bullet -}$) were generated in the riboflavin/EDTA system by UV irradiation (Zhao *et al.*, 1989). Reaction mixtures containing 0.8 mM riboflavin, 1.6 mM EDTA, 0.8 M DMPO, and sample solutions of different concentrations were irradiated for 1 min under a UV lamp at 365 nm (power, 100 W), then sealed in a capillary tube, and the DMPO/$O_2^{\bullet -}$ spin adduct was recorded at 10 mW microwave powder and 1000 amplitude.

Cell culture and cytotoxicity determination by the MTT assay

Murine RAW 264.7 cells (ATCC TIB-71™) were grown at 5% CO_2, 37 °C, and 95% humidity as monolayers in Dulbecco's modified Eagle 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 twice or three times each week. Cytotoxicity of the *S. marina* extract on the cells was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983), which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes. The cells were grown in 96-well plates at a density of $5 \cdot 10^3$ cells/well. After 24 h, the cells were washed with fresh medium and then treated with control medium or the medium supplemented with *S. marina* extract at various concentrations. After incubation for 24 h, cells were rewashed and 100 μl of MTT solution in phosphate-buffered saline (PBS; 1 mg/ml) were added, and the mixture was incubated for 4 h. Finally, 100 μl of DMSO were added to solubilize the formed formazan crystals, and the absorbance was measured at 540 nm using a GENios® microplate reader (Tecan Austria). Relative cell viability was determined by the amount of MTT converted to formazan and quantified as the percentage compared to the control.

Determination of intracellular formation of ROS

Intracellular formation of ROS was assessed using the oxidation-sensitive dye 2′,7′-dichlorofluorescein diacetate (DCF-DA) as the substrate (Kong *et al.*, 2009). RAW 264.7 cells growing in black polystyrene 96-well microplates were loaded with 20 μM DCF-DA in Hanks' balanced salt solution (HBSS), and the mixture was incubated for 20 min in the dark. Cells were then treated with different concentrations of *S. marina* extract and incubated for 1 h. After washing the cells with PBS three times, 500 μM H_2O_2 dissolved in HBSS was added to the cells. The formation of 2′,7′-dichlorofluorescein (DCF), due to oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) in the presence of various ROS, was followed by determining the fluorescence every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 528 nm using a GENios® microplate reader (Tecan Austria). Values of dose- and time-dependent fluorescence were plotted and related to the fluorescence intensity of control and blank groups.

Measurement of nitric oxide production

RAW 264.7 cells were seeded onto 96-well plates ($2 \cdot 10^5$ cells/well) using DMEM without phenol red and allowed to adhere overnight. Cells were pretreated with the *S. marina* extract at various concentrations for 1 h, and cellular nitric oxide (NO) production was stimulated by adding 1 μg/ml (final concentration) of lipopolysaccharides (LPS) (Sigma-Aldrich), and the mixture was further incubated for 24 h. Thereafter, the NO concentration was determined based on the Griess reaction in the cultured medium (Verdon *et al.*, 1995). Briefly, 40 μl of 5 mM sulfanilamide, 10 μl of 2 M HCl, and 20 μl of 40 mM naphthylethylenediamine were added to 50 μl of culture medium. After incubation for 15 min at room temperature, the absorbance was measured at 550 nm using a GENios® microplate reader (Tecan Austria). The concentrations of nitrite were calculated by regression analysis, using serial dilutions of sodium nitrite as a standard.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from RAW 264.7 macrophages treated with/without *S. marina* extracts using Trizol reagent (Invitrogen, Carlsbad, CA, USA). For synthesis of cDNA, 2 μg RNA were added to RNase-free water containing oligo (dT), denaturated at 70 °C for 5 min, and cooled immediately. RNA was reverse transcribed in a master mix containing 1X RT buffer, 1 mM dNTPs, 500 ng oligo (dT), 140 U M-MLV reserve transcriptase, and 40 U RNase inhibitor (Promega, Madison, WI, USA) in a total volume of 20 μl at 42 °C for 60 min and at 72 °C for 5 min using an automatic T100 Thermo Cycler (Bio-Rad, Hercules, CA, USA). 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′-AGA-AGG-AAA-TGG-CTG-CAG-AA-3′, and reverse, 5′-GCT-CGG-CTT-CCA-GTA-TTG-AG-3′, for cyclooxygenase-2 (COX-2); forward, 5′-AGC-CCC-CAG-TCT-GTC-TCC-TT-3′, and reverse, 5′-CAT-TCG-AGG-CTC-CAG-TGA-AT-3′, for tumour necrosis factor-α (TNFα); forward, 5′-GGG-CCT-CAA-AGG-AAA-GAA-TC-3′, and reverse, 5′-TAC-CAG-TTG-GGG-AAC-TCT-GC-3′, for interleukin-1β (IL-1β); forward, 5′-AGT-TGC-CTT-CTT-GGG-ACT-GA-3′, and reverse, 5′-CAG-AAT-TGC-CAT-TGC-ACA-AC-3′, for interleukin-6 (IL-6); 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 amplification cycles were carried out at 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 45 s. After 30 cycles, the PCR products were separated by electrophoresis on 1.5% agarose gel for 30 min at 100 V. Gels were then stained with 1 mg/ml ethidium bromide and bands visualized by UV light using a Davinch-Chemi imager™ (CAS-400SM; Davinch-K, Seoul, South Korea).

Spectral measurements

Classes of compounds in the extract were identified by Fourier transform infrared spectroscopy (FT-IR) (IRAffinity-1; Shimadzu, Kyoto, Japan) at room temperature using a KBr disc containing 1.0 mg of dry *S. marina* extract and 0.1 mg of fine-grade KBr (Sigma-Aldrich). According to the absorbance characteristics, a cluster analysis of the data was performed.

Statistical analysis

The data are presented as mean ± SD. Differences between the means of the individual groups were analysed using the analysis of variance (ANOVA) procedure of Statistical Analysis System, SAS v9.1 (SAS Institute, Cary, NC, USA), followed by post-hoc analysis of Duncan's multiple range tests.

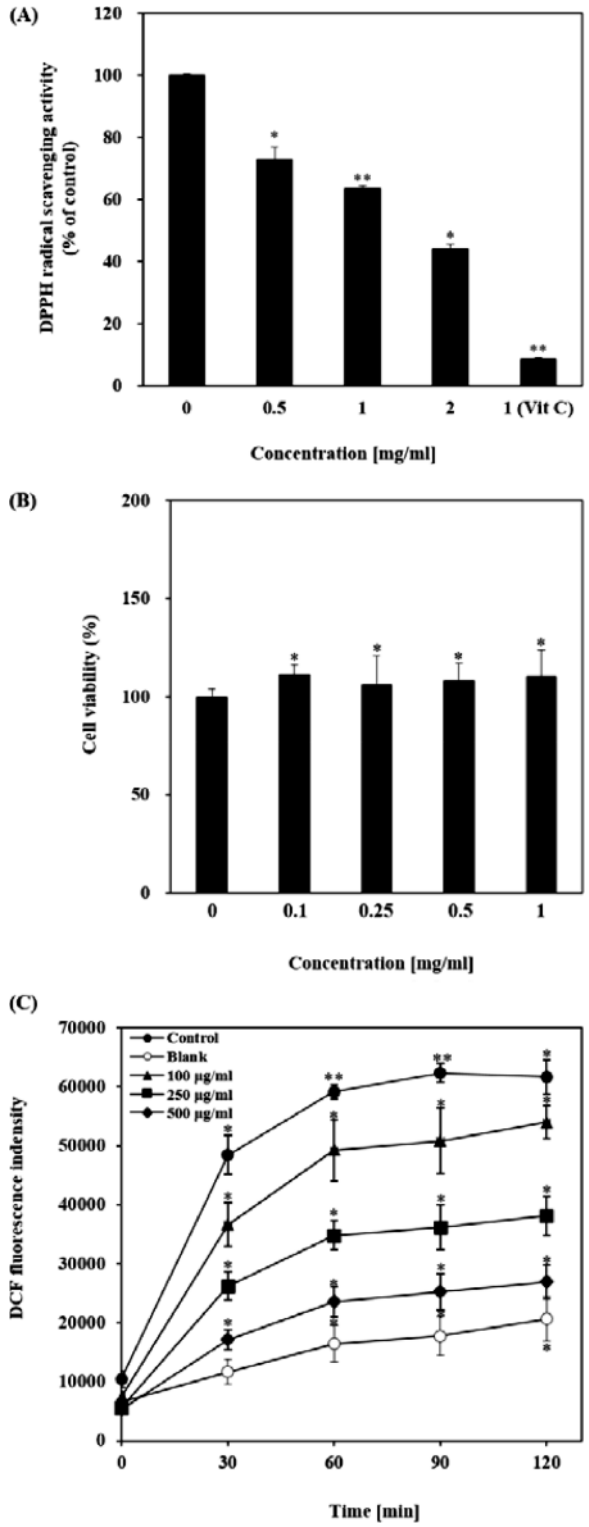
Results and Discussion

In vitro antioxidant activity

In order to test the antioxidant potential of *S. marina*, firstly, the ability of the ethanolic extract to scavenge free radicals was assessed in the cell-free DPPH assay. A distinct, dose-dependent DPPH radical scavenging effect of the extract was observed (Fig. 1A). At an extract concentration of 2 mg/ml, 57% of the DPPH radicals were scavenged, while the positive standard, vitamin C, scavenged 91% at 1 mg/ml.

At concentrations of 0.1 to 1 mg/ml, the *S. marina* extract had no cytotoxic effect on RAW 264.7 mouse macrophages, as evident from the results of the MTT assay (Fig. 1B). The antioxidant potential of the *S. marina* extract in these cells was subsequently determined by DCF-DA labeling of intracellular ROS generated by exposure of the cells to H_2O_2. The non-fluorescent 2′,7′-dichlorofluorescein diacetate (DCF-DA) dye that freely penetrates into cells, is hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescein (DCFH), which is oxidized by ROS to fluorescent 2′,7′-dichlorofluorescein (DCF). From 0.1 mg/ml to 0.5 mg/ml, the *S. marina* extract lowered the level of ROS, and hence the intracellular oxidative stress, in the cells in a dose-dependent manner, from 20% to 95%, respectively (Fig. 1C), and the protective effect increased linearly over time. Based on the results obtained in the DPPH and DCF-DA assays, we can conclude that the *S. marina* extract has potent antioxidant activity both *in vitro* and intracellularly.

Furthermore, the ability of the *S. marina* extract to scavenge DPPH, the hydroxyl, and the superoxide radicals, respectively, was examined by ESR spectroscopy (Fig. 2). At 500 μg/ml, the extract reduced the DPPH, hydroxyl, and superoxide radicals by 74%, 85%, and 43%, respectively. Thus, the hydroxyl radicals were most effectively scavenged. It is well known that the metal-binding properties of phenolic compounds are responsible for their efficient scavenging of hydroxyl radicals generated in the Fenton reaction (Guo *et al.*,



2007). As polyphenols with notable antioxidant potential are known to occur in several halophytes (Gargouri *et al*., 2013; Trabelsi *et al*., 2013), we assume that the higher scavenging effect of the *S. marina* extract on hydroxyl radicals is due to its high phenolic content.

The saline environment of halophytes is a cause of permanent oxidative stress (Flowers and Colmer, 2008) to which they respond with enhanced antioxidative systems which was confirmed here for *S. marina*.

Anti-inflammatory activity

Several antioxidant agents are effective against inflammation, which is related to ROS production in inflammation (Geronikaki and Gavalas, 2006). Therefore, we examined the potential anti-inflammatory activity of the *S. marina* extract on LPS-stimulated RAW 264.7 mouse macrophages. Upon activation by pathogenic substances, macrophages initiate and regulate inflammatory responses through a broad range of inflammatory mediators. LPS are among the most effective activators of macrophages and stimulate the production of oxidative stress-related free radicals and of NO, in addition to cytokines such as tumour necrosis factor-α (TNFα), interlenkin-1β (IL-1β), and -6 (IL-6) (Meng and Lowell, 1997). The anti-inflammatory potential of the *S. marina* extract was first evaluated by assessing the NO production in LPS-stimulated RAW 264.7 cells (Fig. 3A). LPS treatment raised the NO level of the macrophages from 3.5 μM to 9 μM, and the latter level was reduced to 5 μM by the *S. marina* extract. However, unlike the antioxidant effect, the effect of the extract on the NO level did not appear to be dose-dependent in the range from 100 to 500 μg/ml.

In order to evaluate the anti-inflammatory effect of the extract in more detail, expression of key indicators of the inflammatory response was assessed by RT-PCR. In addition to the induction of the aforementioned cytokines, the expression of the genes encoding inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) is enhanced during inflammation (Hori *et al*., 2001), which is supposed to be related to the

Fig. 1. (A) DPPH radical scavenging effect of *Spergularia marina* extract, (B) effect of the extract on the viability of RAW 264.7 mouse macrophages and (C) on intracellular ROS scavenging in RAW 264.7 cells. Cells in (B) were exposed to hydrogen peroxide. Values are means $\pm$ SD ($n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the untreated control).

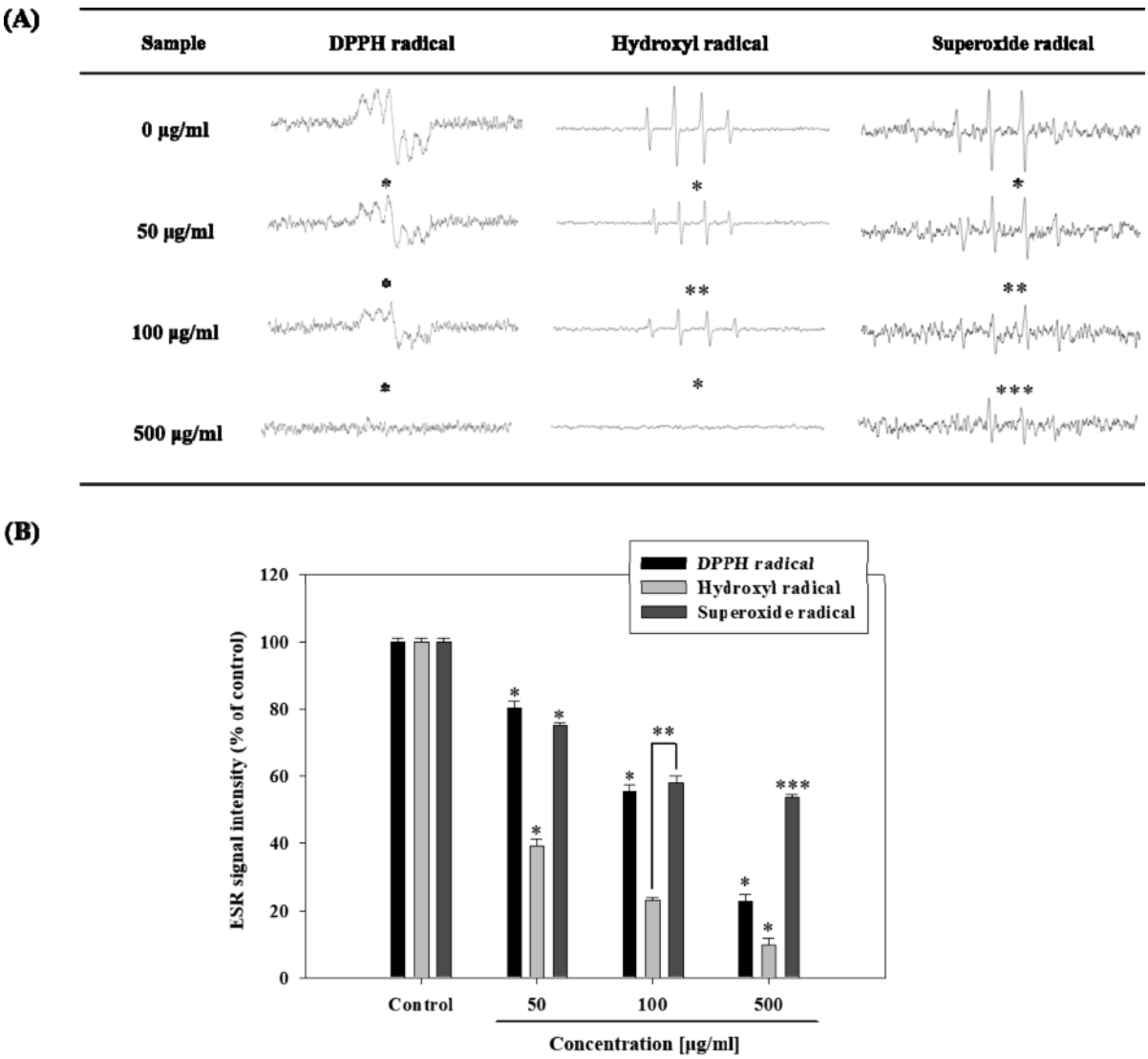


Fig. 2. Scavenging of DPPH, hydroxyl, and superoxide radicals, respectively, by *Spergularia marina* extract *in vitro* monitored by electron spin resonance spectroscopy. (A) Electron spin resonance absorbance spectrum, (B) relative signal intensity compared to the untreated control. Values are means ± SD ($n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the untreated control).

pathogenesis of inflammation and several other diseases including cancer and Alzheimer's disease (Pahl, 1999; Yamamoto and Gaynor, 2001). As seen in Fig. 3B, the *S. marina* extract suppressed the expression of the inflammatory mediators iNOS and COX-2 as well as of the cytokines TNFα, IL-1β, and IL-6 in a concentration-dependent fashion thus relieving inflammation.

Taken together, the *S. marina* extract might be a useful source of therapeutic agents having both antioxidative and anti-inflammatory effects. While other halophytes have been suggested for this purpose (Bandaranayake, 2002; Gargouri *et al*., 2013; Ksouri *et al*., 2009), to the best of our knowledge, an anti-inflammatory effect of *S. marina* has not been described previously.

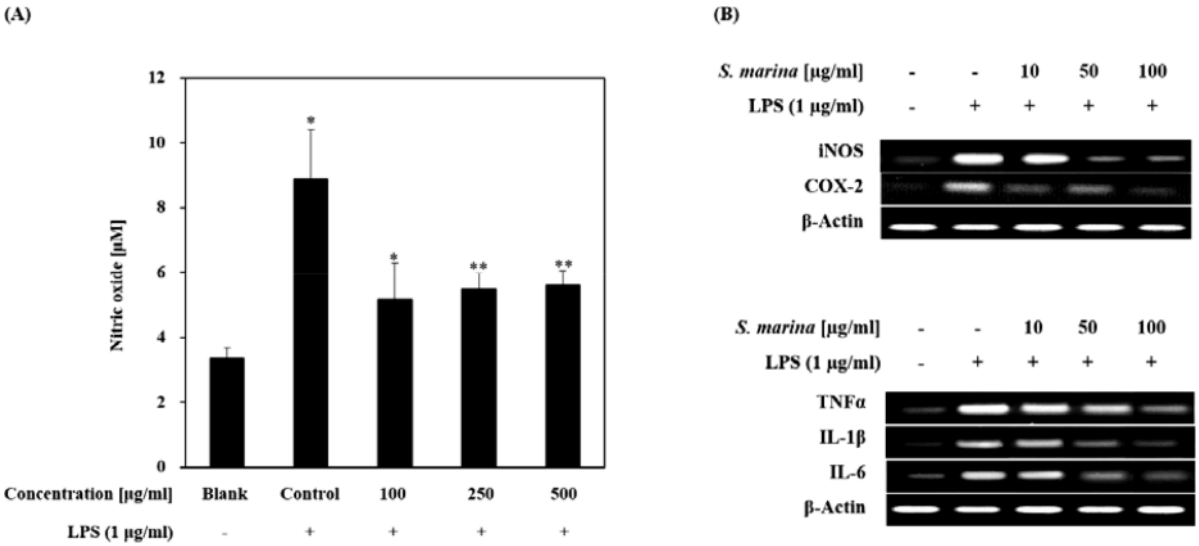


Fig. 3. Effect of *Spergularia marina* extract on (A) NO production and (B) the expression of inflammation-related genes in LPS-stimulated RAW 264.7 mouse macrophages. Values are means ± SD ($n = 3$; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the untreated control).

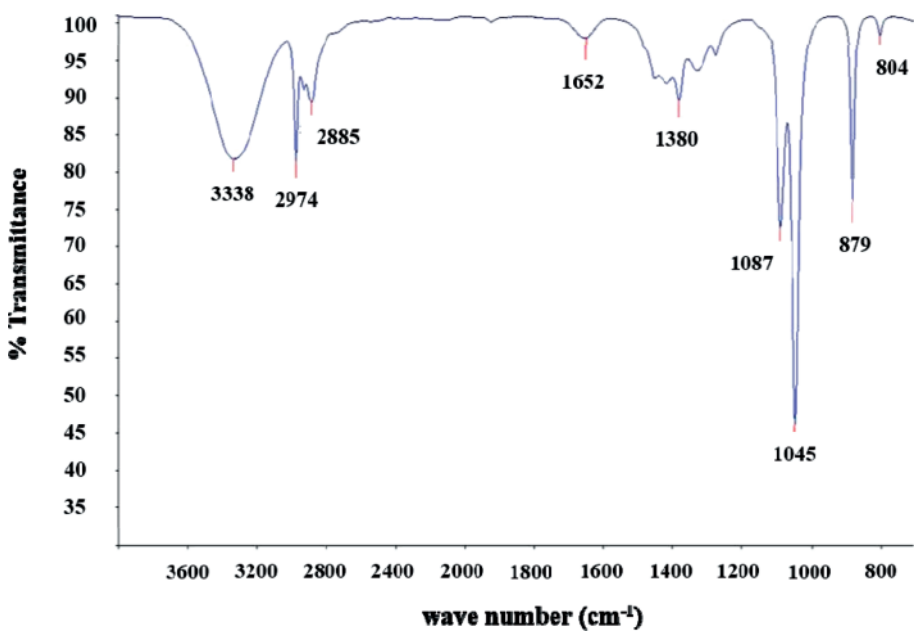


Fig. 4. FT-IR spectrum of *Spergularia marina* extract.

In order to evaluate the bioactive material content of *S. marina* in regard to its antioxidant and anti-inflammatory effects, an FT-IR spectrum of the *S. marina* extract was recorded (Fig. 4). FT-IR analysis of the *S. marina* extract showed the distinctive intensities for water stretching (3338 cm^{-1}), C–H bond of aldehydes (2974, 2885 cm^{-1}), C=O bond of amides (1652 cm^{-1}), alkane C–H bond (1380 cm^{-1}),

C–N stretching (1087 cm^{-1}), C–X haloalkane bond (1045 cm^{-1}), and C–H peak of aromatic ring compounds (879, 804 cm^{-1}).

In conclusion, *S. marina* is suggested as a novel source for therapeutic agents against oxidative stress and inflammation. Future studies are required to elucidate and characterize the bioactive compounds.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2012R1A1A3014642).

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