JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Inhibition of Lipopolysaccharide (LPS)-Induced Inflammatory Responses by *Sargassum hemiphyllum* Sulfated Polysaccharide Extract in RAW 264.7 Macrophage Cells

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**ABSTRACT:** *Sargassum hemiphyllum*, a kind of brown seaweed generally found along coastlines in East Asia, has long served as a traditional Chinese medicine. *S. hemiphyllum* has shown an anti-inflammatory effect; however, its mechanism has not been elucidated clearly. This study explored *S. hemiphyllum* for its biomedical effects. *S. hemiphyllum* sulfated polysaccharide extract (SHSP) was first prepared; the mouse macrophage cell line (RAW 264.7) activated by lipopolysaccharide (LPS) was used as a model system. The secretion profiles of pro-inflammatory cytokines, including IL-1$\beta$, IL-6, TNF-$\alpha$, and NO, were found significantly to be reduced in 1−5 mg/mL dose ranges of SHSP treatments. RT-PCR analysis suggested SHSP inhibits the LPS-induced mRNA expressions of IL-$\beta$, iNOS, and COX-2 in a dose-dependent manner. At protein levels, Western blot analysis demonstrated a similar result for NF-$\kappa$B (p65) in cytosol/nuclear. Taken together, the anti-inflammatory properties of SHSP may be attributed to the down-regulation of NF-$\kappa$B in nucleus.

**KEYWORDS:** brown seaweed, *Sargassum hemiphyllum*, sulfated polysaccharide extract, anti-inflammation, macrophage, cytokine, NF-$\kappa$B

## INTRODUCTION

Inflammation, a complicated physiological phenomenon, is a response to injury, infection, and stress. Cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-$\alpha$, have been known to play important roles in pro-inflammatory responses. Cytokines are released from activated macrophages, which result in the expressions of adhesion molecules out of vascular endothelial cells to recruit neutrophils, monocytes, and lymphocytes, and then move out of the vessel to an injured tissue.[1] Nitric oxide (NO), a product of inflammation, is synthesized by nitric oxide synthases (NOS), which is found in various isoforms, such as neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). iNOS is expressed in response to activation of nuclear factor $\kappa$B (NF-$\kappa$B) that is otherwise induced by various effectors, such as lipopolysaccharide (LPS).[2,3] Cyclooxygenase-2 (COX-2) synthesized in macrophages is inducible in response to infections, injuries, and stresses, which are considered to relate to inflammatory states.[4] NF-$\kappa$B exists as an inactive heterotrimer including p50, p65 (Rel A), and I$\kappa$B in cytoplasm. Once I$\kappa$B is phosphorylated, NF-$\kappa$B is activated and then translocated to the nucleus, where it initiates transcriptions of proinflammatory cytokines.[5] NF-$\kappa$B dysrhythmia results in constitutive pro-inflammatory disorders, including rheumatoid arthritis and Crohn's syndrome.[6,7] Persistent NF-$\kappa$B activation also causes chronic inflammation, which has long been related to certain types of cancers.[8,9]

On the other hand, natural substances, such as orange *Poncirus trifoliate*,[10] curcumin, and capsaicin,[11] have been reported to possess some biological effects to inactivate NF-$\kappa$B. Such substances may have potential in treating inflammatory disorders. *Sargassum hemiphyllum* is a kind of brown seaweed, which grows around seashores of the Far East, including Taiwan, Korea, Japan, Hong Kong, and East China. The nonstarch polysaccharide of *S. hemiphyllum* is known to consist of xylose and glucose,[12] which are soluble in water.[13] The structure of sulfoglycolipid from *S. hemiphyllum* has also been determined to be 1-*O*-acyl-3-*O*-(6′-sulfo-$\alpha$-D-quinovopyrannosyl) glycerol.[14] *S. hemiphyllum* was reported to be able to affect protein bioavailability in growing rats[15] as well as inhibit an atopic allergic reaction.[16] Other seaweed extracts, such as *Sargassum fulvellum* and *Sargassum thunbergii*, have been known to have antipyretic, analgesic, and anti-inflammatory activities.[17] They suppress TNF-$\alpha$-induced NF-$\kappa$B expression in human embryonic kidney cells (HEK-293). Methanol extracts of *S. hemiphyllum* have been reported to inhibit the releases of IL-8 and TNF-$\alpha$;[16] however, the detailed mechanisms in anti-inflammation are still unknown.

In this study, we profiled cytokine contents, inflammatory products, and mRNA expressions of inflammatory mediators in an activated mouse macrophage cell line (RAW 264.7). We evaluated the anti-inflammatory effects of the sulfated polysaccharide extract of *S. hemiphyllum* by determining the inhibitory levels of cytosol/nuclear NF-$\kappa$B (p65).

**Received:** November 25, 2010
**Accepted:** January 16, 2011
**Revised:** January 13, 2011
**Published:** February 15, 2011





dx.doi.org/10.1021/jf1043647 | *J. Agric. Food Chem.* 2011, 59, 2062–2068

## MATERIALS AND METHODS

**Materials and Reagents.** Fresh *S. hemiphyllum* was collected from the coast of Penghu county, Taiwan, from November 2006 to January 2007. *S. hemiphyllum* was washed and dried in the dryer with current air (RISEN Co., Taiwan) at 40 °C for 90 min. The dried sample was ground to flour with a miniblender (YOUQI, Taiwan) and then dried with a dryer (RISEN Co.) at 50 °C for 10 min. The dried seaweed (100 g) was treated with 5 L of distilled water and boiled at 100 °C for 30 min. The extract was centrifuged at 10000*g* for 20 min, and the supernatant was lyophilized under the reduced pressure of 2 mmHg at −20 °C (EYELA, Tokyo Rikakikai Co., Japan). Once the hot water extract was obtained, it was kept at −20 °C for further tests. All of the reagents employed were purchased from Sigma Chemical Co. (St. Louis, MO).

**Chemical Composition Analysis.** The hot water extract was homogenized by using a blender and analyzed in triplicate to determine the moisture, protein, fat, and ash according to official methods of analysis of the Association of Official Analytical Chemists.[18] The percentage of carbohydrate was determined by subtracting the sum percentage of protein, fat, and ash on a dry basis from 100.

**Heavy Metal Analysis.** Five milligrams of hot water extract was digested in 5 mL of concentrated $HNO_3$, and the solution was filtered through filter paper. The solution was analyzed for heavy metal concentrations by ICP-MS.[19] The detection limit value of the analysis is 0.01 ppm.

**Isolation of Polysaccharide Extract.** The lyophilized hot water extract and 4 volumes of 95% ethanol were added and then allowed to precipitate overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude polysaccharide extract sample.

**Hydrolysis of Polysaccharide Extract.** The hydrolysis was preformed as described.[20] Briefly, 1 mg of the crude polysaccharide extract sample with 6 N HCl was heated in a heating block at 80 °C for 6−8 h. The sample was cooled and the acid evaporated. The hydrolyzed polysaccharides were resuspended in Milli-Q water and filtered through a Millipore-GX nylon membrane prior to analysis.

**Monosaccharide Composition Analysis.** The monosaccharide fractions of the polysaccharide extract hydrolysates were separated by HPAEC (Diones BioLC) with an anion-exchange column (Carbopac PA-10, 4.6 × 250 mm). The analysis of monosaccharide was carried out at an isocratic NaOH concentration of 18 mM at ambient temperature.[20]

**Cell Culture.** RAW 264.7 cells (ATCC no. TIB-71), the mouse macrophage cell line, was obtained from the American Type Culture Collection. RAW 264.7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 $\mu$g/mL) and maintained at 37 °C in an atmosphere of 5% $CO_2$.

RAW 264.7 cells ($2 \times 10^5$) were treated with 1 $\mu$g/mL lipopolysaccharide (LPS; Sigma, St. Louis, MO) with or without different concentrations of polysaccharide extract samples (1, 2.5, and 5 mg/mL) for 24 h. Cells treated without LPS and polysaccharide extract samples were utilized as the negative control, and cells treated with LPS alone were utilized as the positive control. After 24 h of treatment, the cell culture supernatants were collected for cytokine assay and nitrite assay. Cell lysates were collected for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot assay.

**Cell Proliferation Assay.** RAW 264.7 cells were treated with polysaccharide extract samples in concentrations of 1, 2.5, or 5 mg/mL and without samples under normal cell culture condition. Proliferation of cells was determined by streptomycin and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[21] RAW 264.7 cells were reacted with MTT (1 mg/mL) for 4 h, and absorbance readouts were recorded at 570 nm by an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Tokyo, Japan).[22] The percent relative activity was determined as $(A_1/A_0) \times 100\%$, where $A_0$ and $A_1$ are the absorbances in the absence of samples and presence of samples, respectively.

**Lactate Dehydrogenase (LDH) Assay.** RAW 264.7 cells were treated with polysaccharide extract samples in concentrations of 1, 2.5, or 5 mg/mL and without samples under normal cell culture condition. After culture, the cell culture supernatants were collected for LDH assay. The concentrations of LDH were determined by using an LDH cytotoxicity assay kit (Cayman Chemical Co.) according to the manufacturer's instructions.

**Cytokine Assay.** To determine the amount of secreted IL-1$\beta$, IL-6, and TNF-$\alpha$, RAW 264.7 cells were treated with various concentrations of polysaccharide extract samples in the presence of LPS (1 $\mu$g/mL). Twenty-four hours post-treatment, cell culture supernatants were collected and stored at −80 °C for further analysis. The concentrations of IL-1$\beta$, IL-6, and TNF-$\alpha$ were determined by using ELISA kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions.

**Nitrite Assay.** To determine the NO secretion, the concentration of nitrite in the supernatants was measured by the Griess reaction. Each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 1% naphthylethylenediamine dihydrochloride in water) (Promega Co., Madison, WI). The absorbance of the mixture at 530 nm was determined with an ELISA reader (Molecular Devices, Tokyo, Japan). Nitrite concentration was determined by using sodium nitrite as a standard.[22]

**Reverse Transcriptase-Polymerase Chain Reaction.** Total RNA was isolated by RNAzol B (Amersham Pharmacia Biotech, Sweden), and the concentration of total RNA was detected by spectrophotometer (Hitachi, Japan). RNA was reverse transcribed using Improm-II TM Reverse Transcriptase (Promega), 10 mM dNTP (Promega), 1 $\mu$g/mL oligo d(T) primer (Promega), and $dH_2O$/diethylpyrocarbonate (DEPC) to a final volume of 30 $\mu$L. The RT reaction was achieved at 25 °C for 5 min, at 42 °C for 60 min, and at 70 °C for 15 min to perform cDNA (cDNA) and then kept at −20 °C.

PCR was performed on the reverse transcribed cDNA product to determine the expression of IL-1, iNOS, COX-2, and $\beta$-actin (as an internal control) using a thermal cycler (biometra, UNO-Thermoblock, U.K.). The reactions were carried out in a volume of 25 $\mu$L containing 1 unit of *Taq* DNA polymerase (Dong-Sheng Biotech Co., Taiwan), 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of sense and antisense primers.

After the initial 1 min of 95 °C denaturation, the amplification sequence protocol of IL-1 was 1 min of 94 °C annealing and 1 min of 72 °C extension); that of iNOS, 1 min of 60 °C annealing and 1.5 min of 72 °C extension; that of COX-2, 1 min of 60 °C annealing and 1 min of 72 °C extension; and that of $\beta$-actin, 30 cycles. Sense and antisense PCR primers used in this study were as follows: IL-1, 5′-TTGACGGACCCCAAAGAGTG-3′ and 5′-CACGATTTCCCAGAGAACATGTG-3′; iNOS, 5′-TCATTGTACTCTGAGGGCTGACACA-3′ and 5′-GCCTTCAACACCAAGGTTGTCTGCA-3′; COX-2, 5′-CCGTGGTGAATGTATGAGCA-3′ and 5′-CCTCGCTTCTGATCTGTCTT-3′; and $\beta$-actin, 5′-GACTACCTCATGAAGATCCT-3′ and 5′-CCACATCTGCTGGAAGGTGG-3′; the above primers were purchased from Mission Biotech Co., Ltd. (Taipei, Taiwan). The PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining under UV irradiation.[10] The image of the resulting gel was captured and analyzed by ImageMaster VDS and ImageMaster 1D Elite software (Amersham Pharmacia Biotech, Sweden).

**Western Blot Assay.** After incubation, RAW 264.7 cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested in 0.1 mL of ice-cold hypotonic lysis buffer [10 mM of Tris-HCl ,pH 7.8, 5 mM $MgCl_2$, 0.3 mM ethylene glycol tetraacetic acid (EGTA), 10 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitors]. After 15 min of incubation,

25 μL of Nonidet P-40 was added, and the mixture was centrifuged at 10000*g* for 1 min. The supernatant was the cytosolic extract. The nuclear pellets were reacted with hypertonic extraction buffer and were centrifuged at 10000*g* for 10 min. The supernatant was the nuclear extract. The protein contents of cytosol and nuclear extract were determined by the Bio-Rad detergent compatible protein assay (Bio-Rad, Richmond, CA). Ten micrograms of cytosol and nuclear extract was separated on an SDS−15% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The immunoblot was incubated overnight with blocking solution (5% skim milk in pH 7.5, 20 mM Tris−150 mM NaCl−0.2% Tween 20) and followed by incubation at 4 °C overnight with primary antibody. The secondary antibody was incubated with the membrane at room temperature for 1 h. Expression of protein was detected by staining with NBT/BCIP (Pierce, Rockford, IL) and quantitated by densitometric analysis (Pharmacia, Imagemaster VDS, Sweden). Primary antibodies, anti-NF-κB (p65)-polyclonal antibodies and β-actin polyclonal antibody (BioVision, USA), were applied at 1:1000 dilution. Secondary antibody, anti-mouse IgG-peroxidase from goat (Sigma, St. Louis, MO), was applied at 1:5000 dilution.[23] Images of resulting gels were captured and analyzed by ImageMaster VDS and ImageMaster 1D Elite software (Amersham Pharmacia Biotech, Sweden). difluoride

**Statistical Analysis.** All data obtained from experiments were analyzed to determine the significance of different treatments by one-way ANOVA. Significant differences were reported at a $p$ value of $<0.05$.

## RESULTS AND DISCUSSION

*Sargassum* sp. and some other brown seaweeds are used as traditional Chinese medicines in treating cervical lymphadenitis, edema, allay, urinary diseases, etc., in Taiwan.[24] These materials also have other valuable effects on antioxidative,[25] antibacterial,[26] antiviral,[27] antitumor [28] and immunomodulating activities.[17,29−31] To understand the anti-inflammatory mechanism of *S. hemiphyllum*, we in vitro examined the sulfated polysaccharide extract of *S. hemiphyllum* modulation for LPS-induced inflammatory responses in this study.

**Compositions and in Vitro Toxicity of *S. hemiphyllum* Sulfated Polysaccharide Extract (SHSP).** The yield of *S. hemiphyllum* water extract is about 9.7 ± 0.3% of total *S. hemiphyllum*, and the contents of carbohydrate, protein, fat, and ash in the water extract are 90.6 ± 0.87, 3.9 ± 0.18, 0.8 ± 0.03, and 4.7 ± 0.07%, respectively; carbohydrate is the major component of the water extract. Then, the precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude polysaccharide extract. The monosaccharide composition analysis of the polysaccharide extract was next determined. Seven monosaccharides were observed; *myo*-inositol, sorbitol, fucose, galactosamine, galactose, glucose, and mannose; fucose (234.48 ± 0.08 μmol/g) is the highest one (Table 1). In addition, our previous paper showed that the content of sulfates is 51.30 ± 4.10%.[32] Thus, it is demonstracted that SHSP could be extracted by hot water from *S. hemiphyllum*.

In the literature, fucoidan is identified as a class of fucose-enriched sulfated polysaccharide found in the extracellular matrix of brown seaweed, and the structures of it from different brown seaweeds vary from species to species.[33] The fucoidan from *Sargassum stenophyllum* was composed of fucose, galactose, mannose, glucuronic acid, glucose, and xylose.[34] Fucoidans are mainly composed of fucose and sulfate, but the chemical compositions of most fucoidans are complex. Besides fucose and sulfate, they also contain other monosaccharides (mannose, galactose, glucose, xylose, etc.) and uronic acids, even acetyl groups and protein. Fucoidan extracted with hot water[35] has been the basis of the commercial preparation in recent years, so we suggested that SHSP might consist of fucoidan and that the hot water process is an effective way to obtain fucose-enriched sulfated polysaccharide in *S. hemiphyllum*.

**Table 1. Characteristics of *S. hemiphyllum* Sulfated Polysaccharide**

| | content[a] |
|---|---|
| yield[b] (%) | 9.7 ± 0.3 |
| carbohydrate (%) | 90.6 ± 0.87 |
| protein (%) | 3.9 ± 0.18 |
| fat (%) | 0.8 ± 0.03 |
| ash (%) | 4.7 ± 0.07 |
| sulfates (%) | 51.30 ± 4.10[32] |
| monosaccharide (μmol/g) | |
| *myo*-inositol | 1.04 ± 0.02 |
| sorbitol | 0.32 ± 0.03 |
| fucose | 234.48 ± 0.08 |
| galactosamine | 0.65 ± 0.48 |
| galactose | 49.14 ± 0.07 |
| glucose | 7.12 ± 0.01 |
| mannose | 133.74 ± 0.18 |

[a] Each value is the mean ± SEM of three replicates. [b] Each value is determined on the basis of the dry weight.

It was known that brown seaweeds can absorb heavy metals from their environment,[36] and these natural toxins may cause cytotoxicity. Thus, we used an in vitro toxicity assay to assess the cytotoxicity of SHSP. The in vitro toxicity assay assessed heavy metal concentration, cell proliferation, and LDH secretion. LDH is a stable enzyme, present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane; therefore, it is the most widely used marker in cytotoxicity study.[37] Our results demonstrated that cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), and zinc (Zn) were not detected in 5 mg of SHSP. The cytotoxic study of *S. hemiphyllum* with six human cell lines (HDL, WI38, HL60, MG63, MOLT4, and WIL2NS) indicated that cytotoxicity was not observed.[38] Furthermore, SHSP at concentrations of 1, 2.5, and 5 mg/mL did not affect cell proliferation and LDH secretion for 24 h incubations (Figure 1), so that the concentrations of SHSP used in this study were considered to be noncytotoxic.

**Effect of SHSP on Cytokine Secretion.** In an attempt to know how SHSP acts as an anti-inflammatory substance, we analyzed cytokine secretions of IL-1β, IL-6, and TNF-α. The secretions of IL-1β, IL-6, and TNF-α from RAW 264.7 cells were determined using a commercially available ELISA kit. The secretion levels of IL-1β, IL-6, and TNF-α from a negative control were measured to be 25.37 ± 3.17, 11.34 ± 2.38, and 35.32 ± 4.10 μg/mL, respectively. They were 45.37 ± 3.69, 29.34 ± 2.48, and 65.32 ± 6.22 μg/mL, respectively, when cells were stimulated by LPS. The secretion levels of IL-1β, IL-6, and TNF-α were found decreased in a dose-dependent manner, when SHSP was added to the LPS-stimulated RAW 264.7 cells. In a 5 mg/mL addition of SHSP, the secretion levels of IL-1β, IL-6, and TNF-α were determined to be 27.65 ± 4.58, 14.84 ± 3.41, and 31.14 ± 2.67 μg/mL, respectively (Figure 2). SHSP thus was considered to inhibit the initial phase of an LPS-stimulated inflammatory reaction, likely by interfering degranulation.[39] This result agrees with that using methanol extracts of *S. hemiphyllum*,

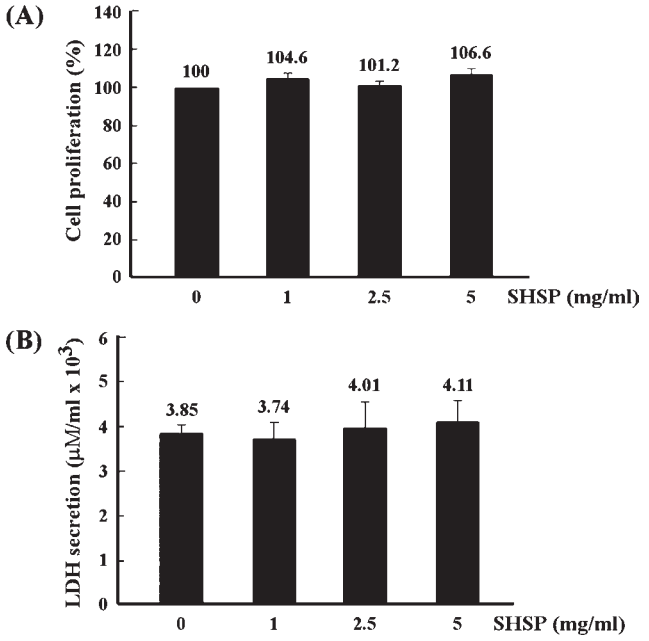


**Figure 1.** Effects of SHSP on the cell proliferation (A) and LDH secretion (B) by RAW 264.7 cells ($n = 3$).

in which IL-8 and TNF-α secretions decreased in phorbol 12-myristate 13-acetate (PMA) and A23187-induced HMC-1 mast cells,[16] and that using *Cladosiphon okamuranus* Tokida extract, which improved chronic colitis via down-regulation of IL-6.[30]

Moreover, the extracts of *S. fulvellum* and *S. thunbergii* obtained from boiling water showed the inhibition of edema, erythema, and inflammation.[17] These biological properties of *S. fulvellum* and *S. thunbergii* water extracts were quite similar to those of SHSP. On the basis of these previous findings and our current data, it was suggested that fucose-enriched sulfated polysaccharide was the active substance in SHSP.

**Effects of SHSP on NO Secretion.** Previous studies reported that fucoidan significantly inhibited the release of NO in LPS-stimulated RAW 264.7 cells[40] and brown seaweed *Ecklonia cava* also had anti-inflammatory potential through inhibiting another inflammatory product, prostaglandin $E_2$ ($PGE_2$), during LPS treatment.[41] Nakamura et al. also reported that fucoidan significantly induced iNOS promoter activation.[42] To assess whether SHSP is able to reduce NO production, we measured nitrite contents, a stable end-product of NO, using the Griess reaction. The secretion levels of NO in RAW 264.7 cells were measured to be 16.72 ± 2.43 μM/mL in a negative control and 36.72 ± 2.43 μM/mL in the LPS-induced cells. The NO secretion levels were 25.70 ± 2.53, 24.31 ± 4.13, and 20.44 ± 5.46 in the LPS-induced RAW 264.7 cells treated with SHSP of 1, 2.5, or 5 mg/mL, respectively (Figure 3). Thus, SHSP can decrease NO secretion. Brown seaweed polyphenols, phlorotannins, also significantly inhibit the LPS-induced production of NO through the down-regulation of iNOS.[46] The SHSP contained detectable polyphenols (Table 1), which suggested anti-inflammation activity also resulted from the sulfated polysaccharide.

**Effect of SHSP on the mRNA Expression of IL-β, iNOS, and COX-2.** Lowenstein et al. reported that NO played a role in inflammatory responses and that the amount of NO secretion was correlated with the gene expression level of iNOS.[43] Recently, Yang et al. have shown that fucoidan significantly inhibited the release of NO and suppressed iNOS mRNA in RAW 264.7 cells

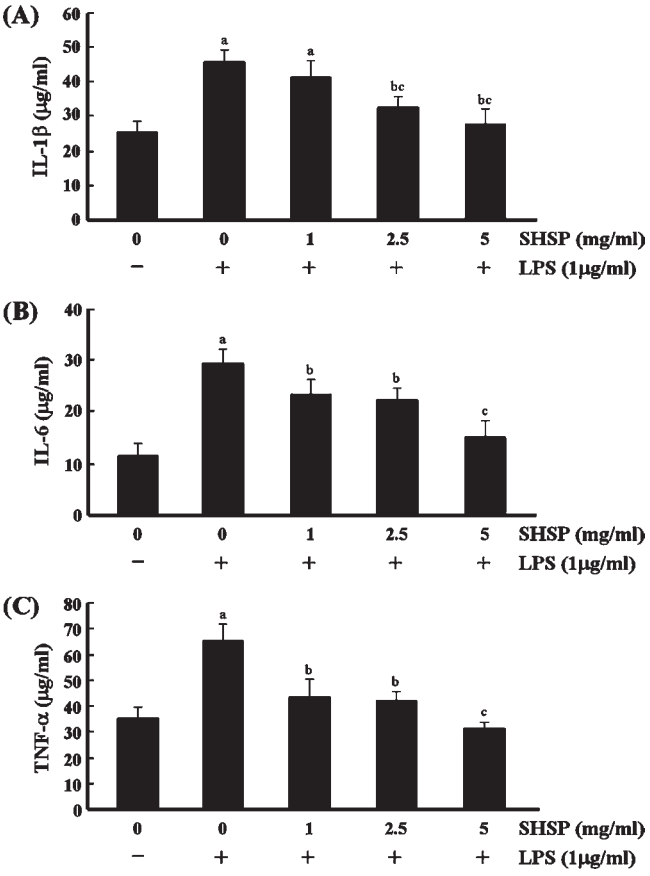


**Figure 2.** Effects of SHSP on the secretion of IL-1β (A), IL-6 (B), and TNF-α (C) by LPS-stimulated RAW 264.7 cells ($n = 3$). Means with different letters were significantly different from the positive control ($P < 0.05$).

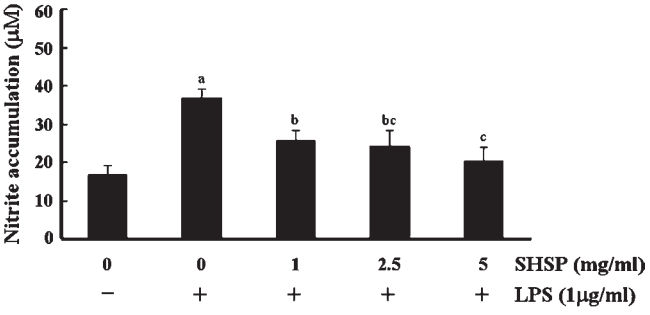


**Figure 3.** Effects of SHSP on the secretion of NO by LPS-stimulated RAW 264.7 cells ($n = 3$). Means with different letters were significantly different from the positive control ($p < 0.05$).

in the presence of LPS.[38] The brown seaweed *Dictyota dichotoma* could also decrease the mRNA levels of iNOS and COX-2 in a concentration-dependent manner,[44] and *Capsosiphon fulvescens* treatment reduced the expressions of iNOS and COX-2 proteins, which related to the status of ulcers.[45] In this study, the expression levels of IL-β, iNOS, and COX-2 mRNA were measured by using RT-PCR. The increase for a given mRNA was semiquantitatively determined by calculating the ratio of densitometric values from the given gene to an internal reference (the increasing fold of a given mRNA expression = the given mRNA expression/β-actin expression). The increasing fold for the negative

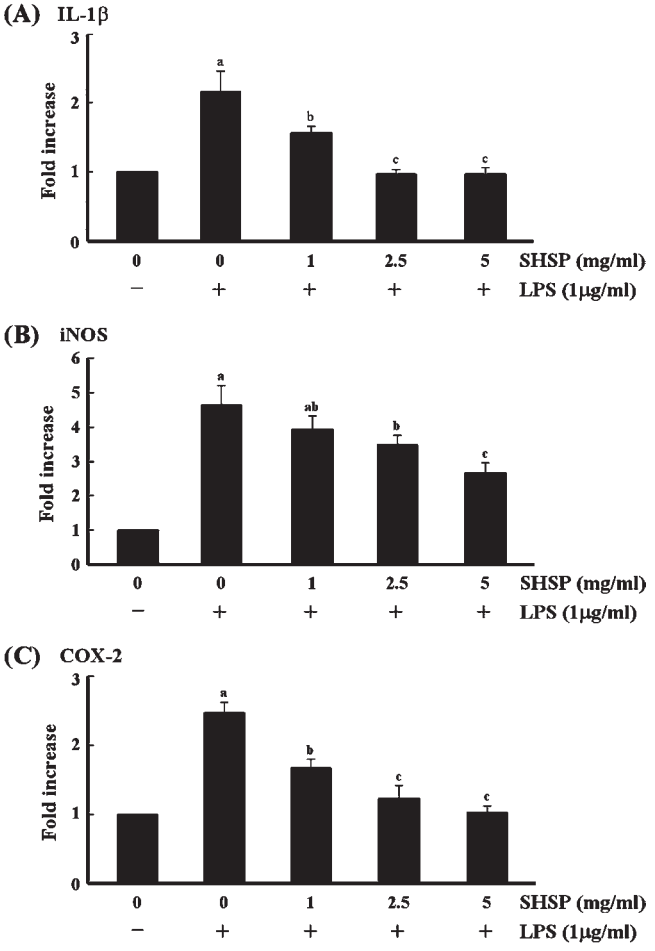


**Figure 4.** Effects of SHSP on the mRNA expression of IL-$\beta$ (A), iNOS (B), and COX-2 (C) by LPS-stimulated RAW 264.7 cells ($n = 3$). Means with different letters were significantly different from the positive control ($p < 0.05$).

control was regarded as 1. The increasing folds of IL-1$\beta$, iNOS, and COX-2 in the stimulation of LPS were determined to be 2.16 ± 0.29, 4.64 ± 0.58, and 2.48 ± 0.14, respectively. When 5 mg/mL of SHSP was added, the mRNA expression folds of IL-1$\beta$ and COX-2 were determined to be 0.97 ± 0.08 and 1.03 ± 0.09 $\mu$g/mL, respectively (Figure 4A,C). With the addition of 5 mg/mL, the increasing fold of iNOS mRNA expression was 2.66 ± 0.28, which was still significantly decreased when compared to the positive control (Figure 4B). Thus, SHSP can inhibit the expressions of IL-1$\beta$ and COX-2 as low as 1 mg/mL, whereas it requires at least 2.5 mg/mL to inhibit the expression of iNOS (Figure 4). The reduction of NO secretion is in line with the decrease of iNOS mRNA expression in the SHSP-treated RAW 264.7 cells activated by LPS. We thereby concluded SHSP is able to inhibit NO secretion through suppressing the iNOS gene expression.

**Effect of SHSP on the Expression of NF-$\kappa$B (p65).** When cells are stimulated by LPS, NF-$\kappa$B is translocated into the nucleus and modulates gene expressions.[47] NF-$\kappa$B (p65) protein expression was analyzed using Western blotting. The cytosol/nuclear NF-$\kappa$B (p65) increasing fold in a negative control was regarded as 1. The cytosol/nuclear NF-$\kappa$B (p65) increasing folds were determined to be 0.68 ± 0.06 and 0.80 ± 0.08 for the SHSP additions of 2.5 and 5 mg/mL, respectively, which were

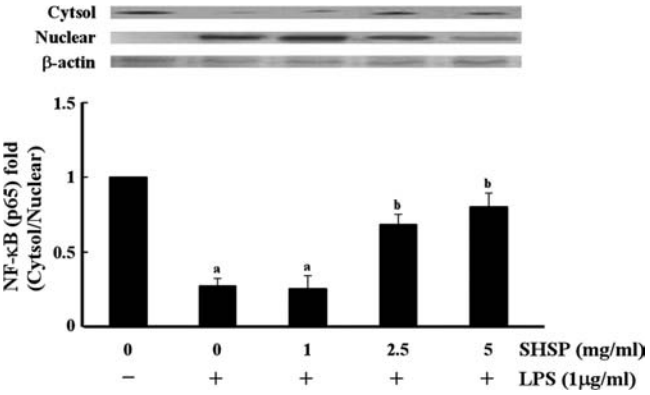


**Figure 5.** Effects of SHSP on the cytosol/nuclear NF-$\kappa$B (p65) expression level by LPS-stimulated RAW 264.7 cells ($n = 3$). Means with different letters were significantly different from the positive control ($p < 0.05$).

significantly increased compared with that in a positive control (0.27 ± 0.05) (Figure 5). Thus, SHSP can reduce the cytosol/nuclear NF-$\kappa$B p65 expression so as to inhibit cytokine secretions of IL-1$\beta$, IL-6, TNF-$\alpha$, and NO and mRNA expressions of IL-$\beta$, iNOS, and COX-2. The inhibitory effects of SHSP may be via the NF-$\kappa$B signaling pathway. This agrees with some published results, in which *S. hemiphyllum* methanol extracts reduced inflammation through the inhibition of NF-$\kappa$B,[16] but fucoidan may suppress the NF-$\kappa$B receptor activity.[40] Therefore, we suggested that *S. hemiphyllum* polysaccharide extract, SHSP, and methanol extract may be via the similar pathway to reduce inflammation. In addition, SHSP is a crude extract without purification; the effective doses (effective dose range = 1−5 mg/mL) are obviously higher than fucoidan from *Fucus vesiculosus* (effective dose range = 2.5−10 $\mu$g/mL).[48] We estimate that the effective dose of SHSP will be significantly lower after purification. In conclusion, SHSP was confirmed to be a potent inhibitor, effective in reducing the secretions of IL-1$\beta$, IL-6, and TNF-$\alpha$ as well as mRNA expressions of NO, IL-$\beta$, iNOS, COX-2, and cytosol/nuclear NF-$\kappa$B (p65).

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### Funding Sources

This work was supported financially by the Council of Agriculture, Taiwan.

## ACKNOWLEDGMENT

We express our gratitude to Dr. Tsung-Lin Li for help with and critical evaluation of the English.

## ABBREVIATIONS USED

cDNA, cDNA; COX-2, cyclooxygenenase-2; DEPC, diethylpyrocarbonate; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial NOS;

FBS, fetal bovine serum; HEK, human embryonic kidney cells; IL, interleukin; iNOS, inducible NOS; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-$\kappa$B, nuclear factor kappa-B; nNOS, neuronal NOS; NO, nitric oxide; NOS, nitric oxide synthases; PBS, phosphate-buffered saline; $PGE_2$, prostaglandin $E_2$; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcriptase-polymerase chain reaction; SHSP, *S. hemiphyllum* sulfated polysaccharide extract; TNF, tumor necrosis factor.;

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