Introduction

An inflammatory response is one of the body defense mechanisms that recover damaged areas when physical and chemical stimuli such as wound or bacterial infections occur [29]. Chronic inflammatory responses facilitate mucosal damage to cause pain, swelling, redness, fever, and other symptoms [18]. They induce dysfunctions and are deeply related to the occurrence of arthritis and cancer. The main cells involved in inflammatory responses are known to be macrophages. They are activated by several stimuli and cytokines secreted by immune cells to induce inflammatory responses such as pain, swelling, and fever, and the facilitate the movement of immune cells to inflamed areas [12]. Macrophages such as RAW 264.7 or monocytes produce the inflammation-mediated cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 by the stimulation of the lipopolysaccharides (LPS), an endotoxin that exists in the extracellular membrane of gram-negative bacteria [27]. It is known that the formation of these inflammatory mediators plays a key role in inflammation mediation and causes fatal consequences to the host, as it is involved in the process where arachidonic acid is changed into leukotrienes, thromboxanes, and prostaglandins through the action of cyclooxygenase (COX) and mass generation of nitric oxide (NO) [8, 21]. NO is generated from L-arginine by NO synthase (NOS), as a highly reactive substance. NOS is divided into constitutive NOS and inducible NOS (iNOS). In particular, it is reported that iNOS is revealed from many different cells such as hepatocytes, smooth muscle cells, bone marrow cells, monocytes, and macrophages, and produces large amounts of NO when it is stimulated by external stimuli or pro-inflammatory cytokines [13]. COX-2 is plentifully revealed only in cells such as macrophages or monocytes by many different stimuli such as growth factors, cytokines, and LPS [2, 24]. Moreover, nuclear factor-kappa B (NF-κB), which plays an important role in inflammatory responses, is a transcription factor that controls the synthesis of many different cytokines, chemokines, and growth factors [3]. As it enters a nucleus to serve as a transcription factor, it synthesizes iNOS, COX-2, and inflammation-related cytokines [19].
Synthetic anti-inflammatory drugs developed up to now can largely be divided into steroids (hydrocortisone, prednisolone, and betamethasone) and non-steroids (aspirin, indomethacin, and ibuprofen). As they mostly cause side effects such as gastrointestinal disorders, nephritis, and cardiac diseases, the use of these drugs is limited [3, 20]. Therefore, it is necessary to develop nature-derived drugs that are safer and more effective than the existing drugs. Many attempts have actively been made to develop drugs for the treatment of various inflammatory diseases using food with anti-inflammatory activities and herbal extracts in recent years [11, 14].

Sargassum micracanthum is a representative seaweed that can easily be collected near the waters of Korea as brown algae. The study on S. micracanthum related to anti-obesity [17] and anti-oxidation [5, 10] has currently been conducted. In addition, Yang et al. [17] and anti-oxidation [5, 10] has currently been conducted. In addition, Yang et al. [28] reported that sargachromenol isolated from S. micracanthum showed anti-inflammatory activity by suppressing the activation of the NF-κB pathway in vitro, but they did not evaluate in vivo the anti-inflammatory properties. In this study, the inhibitory effects of the ethanolic extract of S. micracanthum on inflammatory mediators in LPS-induced RAW 264.7 macrophages were evaluated in vitro and in vivo.

**Materials and Methods**

**Materials**

S. micracanthum collected in Yeonhwa-ri, Busan, Korea was used in this study. It was cleanly washed with fresh water, freeze-dried, powdered, vacuum-packed, and stored at −20°C.

**Animals**

Eight-week-old male ICR mice were purchased from Orientbio (Seongnam, Korea) to be used for an ear edema test. Ten-week-old female Balb/c mice were used for the acute toxicity test. Mice were preliminarily bred in an animal room where the temperature of 20 ± 2°C, the humidity of 50 ± 10%, and a 12/12 h light-dark cycle were maintained for a week to be used in tests.

**Reagents**

Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone (Logan, Utah, USA), whereas TNF-α, IL-6, and IL-1β ELISA kits were purchased from BD Science (San Diego, CA, USA). Dimethylsulfoxide (DMSO), lipopolysaccharides (LPS), and 3-(4,5-dimethyl thiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were purchased from Sigma Co. (St. Louis, MO, USA). The antibodies of iNOS, COX-2, and NF-κB, β-actin, and anti-mouse IgG conjugated to horseradish peroxidase were purchased from Santa Cruz (San Diego, CA, USA). A BCA protein assay kit and an enhanced chemiluminescence kit (ECL kit) made by Pierce (Rockford, IL, USA) were used in tests.

**Extraction**

Powdered S. micracanthum was extracted with 80% ethanol for 24 h at room temperature with an agitator (H-0820; Dongwon Science Co., Busan, Korea). Then, the extract was centrifuged at 3,000 rpm for 10 min and the supernatant was filtered and concentrated using a rotary evaporator (RE200; Yamato Co., Tokyo, Japan). The residue was repeatedly extracted twice in the same way. The concentrate was dried at 37°C and stored at −20°C.

**Cell Culture**

RAW 264.7 cells, murine macrophages, were purchased from Korean Cell Line Bank (KCLB 40071). The cells were cultured in plastic dishes, containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin, in a CO₂ incubator (5% CO₂ in air) at 37°C. All cells were subcultured when they grew up to the density of about 80–90% in the experimental process. Only the cells that did not exceed 20 passages were used.

**Cell Viability**

RAW 264.7 cells (1 × 10⁵ cells/ml) were cultured in 96-well plates for 20 h. Then, the cells were cultured with LPS (1 µg/ml) and the ethanolic extract of S. micracanthum (0.1, 1, 10, 50, and 100 µg/ml) for 22 h at 37°C and 5% CO₂. Then 5 µg/ml MTT reagent was added and incubated for 2 h. The medium was then discarded and DMSO was added to each well and the absorbance was measured at 540 nm with a microplate reader (Model 550; Bio-Rad, Richmond, VA, USA). The cell proliferation ability was calculated according to the following formula:

\[
\text{Proliferation index (%) = optical density of the sample/optical density of the control \times 100.}
\]

**Measurement of Nitric Oxide**

NO production in cultured media was measured using the Griess reaction [15]. RAW 264.7 cells were plated in 24-well plates (2.5 × 10⁵ cells/ml) and incubated in a 5% CO₂ incubator (MCO-15AC; Sanyo, Osaka, Japan) for 20 h. After pre-incubation, LPS (1 µg/ml) and the ethanolic extract of S. micracanthum (0.1, 1, 10, 50, and 100 µg/ml) were added and incubated for 24 h. Then, 100 µl of supernatant was mixed with the same volume of Griess reagent (1% sulphanilamide + 0.1% naphthylendiamine dihydrochloride in 5% phosphoric acid, 1:1) and incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (Model 550; Bio-Rad) and the nitrite concentration was calculated with standard curves of sodium nitrite (NaNO₂).

**Enzyme-Linked Immunosorbent Assay**

The secretion of TNF-α, IL-6, and IL-1β cytokines was measured with an ELISA kit according to the instructions of the manufacturer. TNF-α, IL-6, and IL-1β were determined from a standard curve. The concentrations were expressed as pg/ml.
Western Blot Analysis

RAW 264.7 cells treated with various concentration of *S. micracanthum* extracts (0.1, 1, 10, 50, and 100 µg/ml) followed by LPS treatment (1 µg/ml) for 24 h were lysed with buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 5 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1% Triton X-100, and 0.1% NP-40. Cell lysates were centrifuged at 12,000 rpm for 20 min to remove cell membrane components. The protein concentration was quantified with a BCA protein assay kit (Pierce, IL, USA). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to PVDF membranes (Bio-Rad) at 200 mA for 1 h. The membranes were blocked with 5% skim milk (Fluka, Switzerland) in 0.1% Tris-buffered saline (TBST) for 2 h. The membranes were incubated with anti-mouse iNOS, COX-2, and NF-κB p65 antibodies in TBST (1:500) for 2 h and washed three times with TBST, then incubated for 1 h with anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase (1:2,000). The membranes were washed three times with TBST and the immunoreactive proteins were detected using an ECL detector and exposed to X-ray film (Kodak X-Omat blue film; Perkin-Elmer, Waltham, MA, USA). The signal intensity of each protein band was measured by densitometry, employing the ImageJ software [1].

Ear Edema Measurement and Histopathology Analysis

The ethanolic extract (200 µl) of *S. micracanthum* was orally administered to ICR mice at concentrations of 10, 50, and 200 mg/kg·body weight. In 1 h, 2.5% croton oil (20 µl/ear) was spread to the inner and outer surfaces of the right ear, and ear thickness was measured in 5 h. An increase in the ear thickness after croton oil treatment was regarded as edema formation.

For histopathology analysis, 20 µl of ethanolic extract of *S. micracanthum* was spread over the right ear of an ICR mouse at a concentration of 100 mg/ml and 20 µl of 5% croton oil was spread in 15 min. In 6 h, the ear tissue was dissected and fixed in 10% formaldehyde for 72 h. After it was fixed, the tissue slices were embedded in paraffin. Sections were deparaffinized and stained with hematoxylin-eosin stain and toluidine-blue for observation of the tissue.

Acute Toxicity

Balb/c mice (*n* = 5) were fasted for about 4 to 6 h and the ethanolic extract of *S. micracanthum* was orally administered to ICR mice at concentrations of 300, 2,000, and 5,000 mg/kg·body weight. Then, the animals were observed for any abnormal behaviors for 6 h, and mortality was noted up to 2 weeks. A group of animals treated with 5% Tween-80 served as the control.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean. Statistical evaluation was carried out by a one-way analysis of variance with SAS software (SAS Institute, Inc., Cary, NC, USA) and the differences between means were determined using Duncan’s multiple range test at *p* < 0.05.

Results and Discussion

Cell Viability

The MTT method was used to measure the viability of RAW 264.7 cells cultured with the ethanolic extract of *S. micracanthum* (Fig. 1). The ethanolic extract of *S. micracanthum* at the concentrations 0.1, 1, 10, 50, and 100 µg/ml did not induce any adverse reactions. Cell viability was significantly increased at all treatment concentrations in comparison with a negative control, and was similar to that of LPS treatment alone. It was considered that the ethanolic extract of *S. micracanthum* has no cell cytotoxicity. Thus, the inhibitory activity on the LPS-induced inflammatory effect of the ethanolic extract of *S. micracanthum* was deemed not to be attributable to cell cytotoxicity.

Inhibitory Effect on Nitric Oxide

Overproduction of NO is secreted by inflammation and synergistically acts with other inflammatory mediators. Effects of the ethanolic extract of *S. micracanthum* on LPS-induced NO production in RAW 264.7 cells were investigated (Fig. 2). After LPS treatment, NO production significantly increased compared with normal cells. When cells were treated with different concentrations of the ethanolic extract of *S. micracanthum* with LPS, NO production decreased significantly in a concentration-dependent manner: 54%, 62%, 83%, 89%, and 91% at 0.1, 1, 10, 50, and 100 µg/ml.
This result is similar to the result of Lee et al. [15], where NO production decreased concentration-dependently according to treatment with the ethanolic extract of *Sargassum honeri*. Thus, the ethanolic extract of *S. micracanthum* can be used as an anti-inflammatory agent via suppression of NO production.

**Inhibitory Effect on Production of LPS-Induced IL-6, TNF-α, and IL-1β**

It is known that inflammatory cytokines such as IL-6, TNF-α, and IL-1β are involved in the early inflammatory response as substances that mediate inflammatory responses [26]. LPS is well-known as a toxin that facilitates the expression of inflammatory cytokines such as TNF-α and IL-6 [22]. Fig. 3 shows the inhibitory effect of the ethanolic extract of *S. micracanthum* on IL-6 production by RAW 246.7 cells induced by LPS. The IL-6 production was significantly decreased in a concentration-dependent manner. In particular, it showed high inhibition at the concentrations of 50 and 100 µg/ml, 63% and 73%, respectively. As a result of investigating the inhibitory effect of the ethanolic extract of *S. micracanthum* on the production of TNF-α (Fig. 3), a concentration-dependent reduction was shown and the
highest inhibition by the extracts at a concentration of 100 µg/ml was found (73%). The inhibition effect of the extract on IL-1β production by LPS-induced RAW 264.7 was measured (Fig. 3). As a result, the extracts exhibited potent inhibitions on IL-1β production. The reductions in IL-1β were by 44%, 70%, 80%, 83%, and 94% at 0.1, 1, 10, 50, and 100 µg/ml, respectively. Thus, we observed that the ethanolic extract of S. micracanthum inhibited the LPS-induced IL-6, TNF-α, and IL-1β production in RAW 264.7 cells, which are important inflammatory mediators involved in inflammatory diseases [30].

Inhibitory Effect on iNOS, COX-2, and NF-κB Expression

Inducible NOS generates a large amount of NO, which facilitates not only inflammatory responses such as vascular permeability and swelling but also the biosynthesis of inflammatory mediators [9, 25]. COX is an enzyme that has all of the cyclooxygenase and peroxidase activities. It changes arachidonic acid into prostaglandin as a cyclooxygenase function, and endoperoxide into prostaglandin as a peroxidase function. It also uses prostaglandin as a precursor of thromboxane and prostacyclins. As it exists in all cells, COX-1 maintains normal cellular homeostasis. On the other hand, COX-2 is involved in the synthesis of prostaglandins in acute inflammatory responses, and its expression is induced by LPS and cytokines [6, 16]. The effects of the ethanolic extracts on the expression of iNOS and COX-2 were evaluated (Fig. 4). As a result, the expressions of iNOS and COX-2 were inhibited by the ethanolic extracts in a dose-dependent manner, especially at 50 µg/ml.

NF-κB, which plays an important role in inflammatory responses, exists in the cytosol in a state that it is combined with IκB as a transcription factor that controls cytokine synthesis [4]. It is phosphorylated by external stimuli, moved into the nucleus, and synthesizes COX-2, iNOS, and inflammation-related cytokines as a transcription factor. Therefore, a test was conducted to identify whether the inhibitory effects of iNOS and COX-2 are caused by inhibition of the NF-κB signal pathway. As a result, the expression of NF-κB caused by LPS treatment certainly increased, whereas the expression of NF-κB was inhibited considerably when the ethanolic extract of S. micracanthum was treated (Fig. 4). This is the result that the protein expression of inflammation-related substances reduces because the ethanolic extract of S. micracanthum inhibits the movement of NF-κB into the nucleus.

Inhibitory Effect on Ear Edema and Histological Changes

Inflammation is caused by physical, chemical and immunologic factors and accompanied with redness, fever, swelling, and pain as the primary protection action against injury and infection of the human body. When inflammation
is induced, the blood flow increases to cause hot flush and redness. Neutrophils and other factors in the blood are charged to the extravascular tissue to induce swelling and pain through an increase in prostaglandins and cytokine activation on the inflamed area [7]. After ethanolic extract (200 µl) of *S. micracanthum* was orally administered at the concentrations of 10, 50, and 250 mg/kg, inflammation was induced with croton oil and the ear thickness was measured (Fig. 5). It was identified that the ear thickness was significantly reduced at all concentration levels in comparison with control. In particular, it was identified that it was considerably reduced compared with the treatment of prednisolone 50 mg/kg when the extract treatment was at a concentration of 250 mg/kg. This shows the tendency matches the result of histopathology. Results from the changes for all groups of mice are shown in Fig. 6. The croton-oil application resulted in a marked increase in ear thickness with dermal hyperplasia (Fig. 6a-A) and mast cell numbers (Fig. 6b-A). By histological comparison, the ethanolic extract of *S. micracanthum* reduced ear thickness (Fig. 6a-C) and mast cell numbers (Fig. 6b-C), and showed the similar results of prednisolone, an anti-inflammatory agent. Non-steroidal analgesics are mainly used for pain and inflammation reduction. However, side effects had been reported. Therefore, there have been many active studies to find the possibility of developing new analgesic and anti-inflammatory medicines from natural substances [23]. From the result of ear edema, it is considered that the ethanolic extract of *S. micracanthum* has excellent effects on edema reduction.

**Evaluation of Acute Toxicity**

The ethanolic extract of *S. micracanthum*, a marine plant resource, was orally administered (200 µl) to mice at the concentrations of 300, 2,000, and 5,000 mg/kg and behavioral changes and mortality were observed for 2 weeks in order to evaluate its toxicity (Table 1). When behavioral changes had been observed up to 4 h after oral administration, one group showed the sleep state for about 30 min, but recovered in 2 h at 5,000 mg/kg body weight. This behavior...
was temporarily observed right after administration. It is considered that it did not result from the toxicity caused by the administration of the ethanolic extract of *S. micracanthum* because other general or death symptoms were not shown. According to the results of this study, it is thought that *S. micracanthum* can definitely be used and processed as an anti-inflammatory functional food with its biological activity previously identified.

## Acknowledgments

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## References


**Table 1.** Mortality of mice treated orally with *Sargassum micracanthum* ethanol extract.

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