

1-1-2010

Brown alga *Sargassum muticum* inhibits proinflammatory cytokines, iNOS, and COX-2 expression in macrophage RAW 264.7 cells

WEON-JONG YOON

WOOK JAE LEE

NAM HO LEE

CHANG-GU HYUN

Follow this and additional works at: <https://journals.tubitak.gov.tr/biology>

 Part of the [Biology Commons](#)

Recommended Citation

YOON, WEON-JONG; LEE, WOOK JAE; LEE, NAM HO; and HYUN, CHANG-GU (2010) "Brown alga *Sargassum muticum* inhibits proinflammatory cytokines, iNOS, and COX-2 expression in macrophage RAW 264.7 cells," *Turkish Journal of Biology*: Vol. 34: No. 1, Article 4. <https://doi.org/10.3906/biy-0810-14>
Available at: <https://journals.tubitak.gov.tr/biology/vol34/iss1/4>

This Article is brought to you for free and open access by TÜBİTAK Academic Journals. It has been accepted for inclusion in Turkish Journal of Biology by an authorized editor of TÜBİTAK Academic Journals. For more information, please contact academic.publications@tubitak.gov.tr.

Brown alga *Sargassum muticum* inhibits proinflammatory cytokines, iNOS, and COX-2 expression in macrophage RAW 264.7 cells

Weon-Jong YOON^{1,2}, Young Min HAM^{1,2}, Wook Jae LEE^{1,2},
Nam Ho LEE^{2,3}, Chang-Gu HYUN^{1,2,*}

¹Research Group for Cosmetic Materials, Jeju Biodiversity Research Institute (JBRI),
Jeju High-Tech Development Institute (HiDI), Jeju 699-943 - KOREA

²Jeju Seaweed Industry Development Agency and

³Department of Chemistry, Cheju National University, Jeju 690-756 - KOREA

Received: 16.10.2008

Abstract: The present study was designed to elucidate the pharmacological and biological effects of *Sargassum muticum* extracts on the production of inflammatory mediators in macrophages. *S. muticum* was extracted with 80% EtOH. The extract was then successively partitioned with *n*-hexane, CH₂Cl₂, EtOAc, BuOH, and water. The results indicate that the CH₂Cl₂ fraction of *S. muticum* extract was an effective inhibitor of LPS-induced NO and PGE₂ production in RAW 264.7 cells. These inhibitory effects of the CH₂Cl₂ fraction of *S. muticum* included dose-dependent decreases in the expression of iNOS and COX-2 proteins and iNOS and COX-2 mRNA. To test the inhibitory effects of *S. muticum* fractions on other cytokines, we also measured IL-1 β and IL-6 mRNA expression by RT-PCR in LPS-stimulated RAW 264.7 macrophage cells. In these assays, the CH₂Cl₂ fraction of *S. muticum* decreased the expression of IL-1 β and IL-6 mRNA in a dose-dependent manner. Based on these results, we suggest that *S. muticum* extracts may be considered possible anti-inflammatory candidates for human health.

Key words: Algae, extract, inflammation, *Sargassum muticum*

Kahverengi alg *Sargassum muticum* RAW 264,7 makrofaj hücrelerindeki iltihaba sebep olan sitokinler, iNOS ve COX-2 ekspresyonunu engeller

Özet: Bu çalışma makrofajlardaki iltihap iyileştiricilerin üretimi üzerine *Sargassum muticum* özütünün farmakolojik ve biyolojik etkilerini aydınlatmak için tasarlanmıştır. *S. muticum* % 80 EtOH ile özütlenmiştir. Özüt daha sonra *n*-hekzan, CH₂Cl₂, EtOAc, BuOH ile başarılı bir şekilde bölünmüştür. Sonuçlar *S. muticum* özütünün CH₂Cl₂ kısmının RAW 264,7 hücrelerindeki PGE₂ üretimi ve NO indükleyici LPS'nin etkili bir inhibitör olduğunu göstermiştir. *S. muticum*'ün CH₂Cl₂ kısmının bu inhibitör etkisi iNOS ve COX-2 proteinlerinin ifadesinde ve iNOS ve COX-2 mRNA'larında doza bağlı düşüş göstermiştir. *S. muticum*'ün diğer sitokinlere olan inhibitör etkisini test etmek için, LPS ile uyarılmış RAW 264,7 makrofaj hücrelerinde IL-1 β ve IL-6 mRNA ifadesi de RT-PCR ile ölçülmüştür. Bu denemelerde, *S. muticum*'ün CH₂Cl₂ kısmı doza bağlı bir şekilde IL-1 β ve IL-6 mRNA ifadesini azalttı. Bu sonuçlara dayanarak, *S. muticum* özütünün insan sağlığı için olası bir enfeksiyon giderici bir aday olarak düşünülebileceğini önermekteyiz.

Anahtar sözcükler: Alg, özüt, iltihap, *Sargassum muticum*

Introduction

During the inflammatory process, large amounts of the pro-inflammatory mediators nitric oxide (NO) and prostaglandin E₂ (PGE₂) are generated by the inducible isoforms of NO synthase (iNOS) and COX-2 (1-3). In mammals, NO is synthesized by 3 different isoforms of NO synthase (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). While nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon- γ (IFN- γ), lipopolysaccharide (LPS), and a variety of pro-inflammatory cytokines (4-6). COX converts arachidonic acid to prostaglandins (PGs) and, like NOS, COX exists in 2 isoforms, i.e. COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is only detected in certain types of tissues and is transiently induced by growth factors, pro-inflammatory cytokines, tumor promoters, and bacterial toxins. Moreover, elevated COX levels have been detected in various tumor types, which may account for the excessive production of inflammatory PGs (7-12).

NF- κ B functions in the expression of many pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-6, IL-8, and IFN- β ; these cytokines are regulated by the signal transduction pathway of I κ B/NF- κ B activation (12,13). IL-1 β is one of the most important inflammatory cytokines secreted by macrophages and is induced by LPS in macrophages. During inflammation, increased release of IL-1 β leads to cell or tissue damage; thus reduction in IL-1 β release from macrophages may retard inflammatory responses to LPS stimulation (14,15). In addition, the production of IL-6 is induced by several factors, such as TNF- α , IL-1 β , and the bacterial endotoxin, LPS. IL-6, a pro-inflammatory cytokine, acts as an endogenous pyrogen and has multiple effects on the immune system and on hematopoiesis (16,17).

S. muticum is widely distributed on the seashore of southern and eastern Korea. In general, *S. muticum* has not been considered a medicinal seaweed. However, its extracts have various biological activities, including antioxidant activity and antibacterial activities (18). Nevertheless, the anti-inflammatory

activity of *S. muticum* has remained uncharacterized. This study is designed to estimate the anti-inflammatory effect of solvent fraction constituents from this species by measuring the production of pro-inflammatory factors (IL-1 β , IL-6, iNOS, COX-2, and PGE₂) in murine macrophage RAW 264.7 cells.

Materials and methods

Materials and solvent extraction

S. muticum were collected in March 2006 from Jeju Island, Korea. Voucher specimen number JBR-253 was deposited at the herbarium of Jeju Biodiversity Research Institute (JBRI). The materials for extraction were cleaned, dried at room temperature for 2 weeks, and ground into a fine powder. The dried powder (62 g) was extracted with 80% ethanol (EtOH; 2 L) at room temperature for 24 h and then evaporated under vacuum. The evaporated EtOH extract (13 g) was suspended in water (1 L) and partitioned with 4 solvents: *n*-hexane (1 L), dichloromethane (CH₂Cl₂; 1 L), ethyl acetate (EtOAc; 1 L), and butanol (BuOH; 1 L); this partition was repeated 3 times. These 4 solvent partitions yielded *n*-hexane (1.7 g, 13.1%), CH₂Cl₂ (0.7 g, 5.4%), EtOAc (0.1 g, 0.8%), BuOH (1.2 g, 9.2%), and H₂O (8.2 g, 63.1%) fractions.

Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Inc., NY, USA) supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% fetal bovine serum (FBS; GIBCO Inc., NY, USA). The cells were incubated in an atmosphere of 5% CO₂ at 37 °C and were subcultured every 3 days.

LDH cytotoxicity assay

RAW 264.7 cells (1.5×10^5 cells/mL) plated in 24-well plates were pre-incubated for 18 h and then treated with LPS (1 μ g/mL) plus aliquots of the 80% EtOH extract or each solvent fraction (25 μ g/mL) at 37 °C for 24 h. The release of lactate dehydrogenase (LDH) from RAW 264.7 cells was used to detect cytotoxicity and was measured at the end of each proliferation experiment. LDH leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol

into the medium. LDH activity was determined from the production of NADH during the conversion of lactate to pyruvate (19,20) and was measured using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, culture medium was centrifuged at 12,000 rpm for 3 min at room temperature. The cell-free culture medium (50 μ L) was then collected and incubated with 50 μ L of the reaction mixture from the cytotoxicity detection kit for 30 min at room temperature in the dark. Subsequently, 1N HCl (50 μ L) was added to each well to stop the enzymatic reaction. The optical density of the solution at a wavelength of 490 nm was then measured using an ELISA plate reader. Percent cytotoxicity was determined relative to the control group. All experiments were performed in triplicate.

Determination of NO production

After the pre-incubation of RAW 264.7 cells (1.5×10^5 cells/mL) with LPS (1 μ g/mL) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production (21). Briefly, 100 μ L of cell culture medium was mixed with 100 μ L of Griess reagent [1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid]; the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. A fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve. All experiments were performed in triplicate.

Measurement of pro-inflammatory cytokine (IL-1 β and IL-6) production

The 80% EtOH extract and each solvent fraction from *S. muticum* solubilized with EtOH were diluted with DMEM before treatment. The inhibitory effect of the 80% EtOH extract and each solvent fraction (25 μ g/mL) on pro-inflammatory cytokine (IL-1 β and IL-6) production in LPS-treated RAW 264.7 cells was determined as described by Cho et al. (22). Supernatants were used for pro-inflammatory cytokine assays with mouse ELISA kits (R & D Systems Inc., MN, USA). All experiments were performed in triplicate.

Determination of PGE₂ production

Each fraction (25 μ g/mL) from *S. muticum* was diluted with DMEM before treatment. Cells were

treated with LPS (1 μ g/mL) to allow cytokine production for 24 h. The PGE₂ concentration in the culture medium was quantified using a competitive enzyme immunoassay kit (R&D Systems, MN, USA) according to the manufacturer's instructions. The production of PGE₂ was measured relative to the following control treatment. All experiments were performed in triplicate.

RNA isolation and RT-PCR analysis

Total RNA from LPS-treated RAW 264.7 cells was prepared with Tri-Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. RNA was stored at -70 °C until used. One microgram of RNA was reverse transcribed with M-MuLV reverse transcriptase (Promega, WI, USA), oligo dT-18 primer, dNTP (0.5 μ M) and 1 U RNase inhibitor. The reaction cocktail was sequentially incubated at 70 °C for 5 min, 25 °C for 5 min, and 37 °C for 60 min, and M-MuLV reverse transcriptase was then inactivated by heating at 70 °C for 10 min. A polymerase chain reaction (PCR) was performed in reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3'- and 5'-primer (50 μ M each) and 200 mM dNTP in 200 mM Tris-HCl buffer (pH 8.4) containing 500 mM KCl and 1-4 mM MgCl₂]. The PCR was performed in a DNA gene cycler (BIO-RAD, HC, USA) with amplification for 30 cycles of 94 °C for 45 s (denaturing), 55-60 °C for 45 s (annealing), and 72 °C for 1 min (primer extension). The primers (Bioneer, Seoul, Korea) used in this experiment are indicated in the Table (F: forward, R: reverse). The PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Immunoblotting

RAW 264.7 cells were pre-incubated for 18 h and then stimulated with LPS (1 μ g/mL) in the presence of test materials for 24 h. After incubation, the cells were collected and washed twice with cold PBS (phosphate-buffered saline). Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO₃, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μ g/mL aprotinin, 25 μ g/mL leupeptin] and kept on ice for 30 min. The cell lysates were centrifuged at 15,000 \times g at 4 °C for 15 min and the supernatants were stored at -70 °C until use. Protein concentration was measured by the

Table. Primer sequences and fragment sizes of the investigated genes in RT-PCR analysis.

Gene		Primer Sequences	Size (bp)
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	496
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'	
COX-2	F	5'-CACTACATCCTGACCCACTT-3'	696
	R	5'-ATGCTCCTGCTTGAGTATGT-3'	
IL-1 β	F	5'-CAGGATGAGGACATGAGCACC-3'	447
	R	5'-CTCTGCAGACTCAAACCTCCAC-3'	
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'	308
	R	5'-TGCTGGTGACAACCACGGCC-3'	
β -Actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

Bradford method (23). Aliquots of the lysates (30~50 μ g of protein) were separated on 8%~12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes (BIO-RAD, HC, USA) with glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking nonspecific sites with 5% nonfat dried milk, the membrane was incubated with specific primary mouse monoclonal anti-iNOS Ab (1:1000, Calbiochem, CA, USA) or rabbit polyclonal anti-COX-2 Ab (1:1000, BD Biosciences Pharmingen, CA, USA) at 4 °C overnight. Each membrane was further incubated for 30 min with a secondary peroxidase-conjugated goat IgG (1:5000, Amersham Pharmacia Biotech, Little Chalfont, UK) to mouse or rabbit, respectively. Immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham Biosciences, NJ, USA).

Statistical Analysis

Student's t-test and one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data are expressed as means \pm standard errors (SEM) and the results were taken from at least 3 independent experiments performed in triplicate. P values of 0.05 or less were considered statistically significant.

Results and discussion

To investigate the effect of *S. muticum* on NO production, we measured the accumulation of nitrite, a stable oxidized product of NO, in culture media. NO production was examined in RAW 264.7 cells stimulated by LPS in the presence or absence of *S. muticum* extracts for 24 h. Nitrite levels in LPS-stimulated cells increased significantly compared to the levels in control cells. To evaluate whether extracts of *S. muticum* could modulate NO production in activated macrophages, we examined the effects of the hexane, CH₂Cl₂, EtOAc, BuOH, and water fractions of *S. muticum* on NO production in the murine macrophage cell line, RAW 264.7. As shown in Figure 1, among the 5 fractions, the CH₂Cl₂ extract (25 μ g/mL) markedly inhibited LPS-induced NO production by RAW 264.7 cells by 86%. The hexane fraction also inhibited LPS-induced production of NO by 52%. There was no basal NO production in the incubation with the crude extract from *S. muticum* without LPS (data not shown). As determined by LDH assays, the numbers of viable activated macrophages were not altered by the solvent fractions, indicating that the inhibition of NO synthesis by the CH₂Cl₂ fraction was not simply due to cytotoxic effects. Although the hexane extract also significantly inhibited NO synthesis at 25 μ g/mL, this activity may have been caused by cytotoxicity.

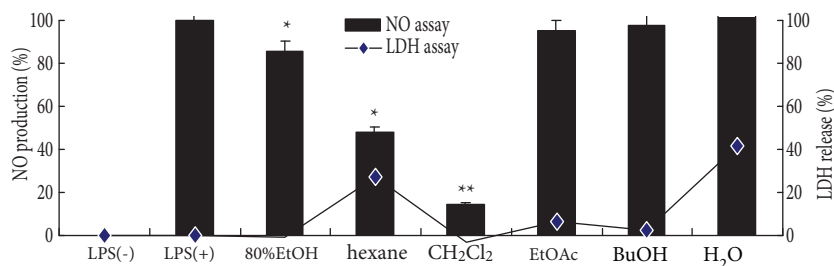


Figure 1. Effect of *S. muticum* extracts and solvent fractions on cell viability and nitric oxide production in RAW 264.7 cells.

Nitric oxide production was assayed in the culture media of cells stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 24 h in the presence of the *S. muticum* extracts and solvent fractions (25 $\mu\text{g}/\text{mL}$). Cytotoxicity was determined using the LDH method. Values are the mean \pm SEM of triplicate experiments.

*, $P < 0.05$; **, $P < 0.01$

PGE_2 is an inflammatory mediator that is produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount of PGE_2 at inflammatory sites. Therefore, we examined the effects of *S. muticum* on PGE_2 production in LPS-stimulated RAW 264.7 macrophages. When macrophages were stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 24 h, the levels of PGE_2 increased in the culture medium. As shown in Figure 2, the CH_2Cl_2 , EtOAc, and hexane fractions (25 $\mu\text{g}/\text{mL}$) suppressed LPS-induced PGE_2 production.

To further evaluate whether reduced NO and PGE_2 syntheses correlated with iNOS and COX-2 protein expression, we examined iNOS and COX-2 protein levels by Western blot analysis. Although no iNOS or COX-2 protein was detected in unstimulated RAW 264.7 cells, 130-kDa iNOS- and 72-kDa COX-2-specific bands appeared when cells were stimulated with LPS for 24 h. As shown in Figure 3, the 5 fractions and the EtOH extract of *S. muticum* each reduced the expression of iNOS protein. Although the hexane and water fractions at 25 $\mu\text{g}/\text{mL}$ significantly inhibited the expression of iNOS protein, their activities may have been caused by cytotoxicities. Of the 5 fractions, the CH_2Cl_2 fraction produced the best inhibitory effect on iNOS production; at concentrations of 25 $\mu\text{g}/\text{mL}$ or greater, iNOS protein

could not be detected. The correlation of the amount of iNOS protein with the accumulation of NO suggests that the CH_2Cl_2 fraction inhibited NO production by reducing iNOS protein expression. We also found that the amounts of the 72-kDa COX-2 protein were increased by LPS, and these increases were antagonized by 25 $\mu\text{g}/\text{mL}$ of the CH_2Cl_2 fraction of *S. muticum*. The inhibitory activity of the hexane fraction may be caused by cytotoxicity.

To determine whether the inhibition of LPS-stimulated NO and PGE_2 production by *S. muticum* was mediated by the regulation of iNOS and COX-2 expression, RT-PCR analyses were performed. As shown in Figure 4, the expressions of iNOS and COX-2 mRNA were significantly elevated in macrophages treated with LPS (1 $\mu\text{g}/\text{mL}$) compared to those in unstimulated cells (control). RT-PCR analyses indicated that *S. muticum* reduced iNOS and COX-2 mRNA without affecting the mRNA of β -actin, a house-keeping protein. Among the 5 fractions, the CH_2Cl_2 fraction of *S. muticum* reduced the expression of iNOS and COX-2 protein. Therefore, the inhibitory effect of *S. muticum* on iNOS and COX-2 gene expression is one of the possible mechanisms responsible for the anti-inflammatory action of *S. muticum*. In conclusion, *S. muticum* actively suppressed the expression of genes implicated in inflammation.

IL-1 β and IL-6 are produced mainly by activated monocytes or macrophages (24). Therefore, we

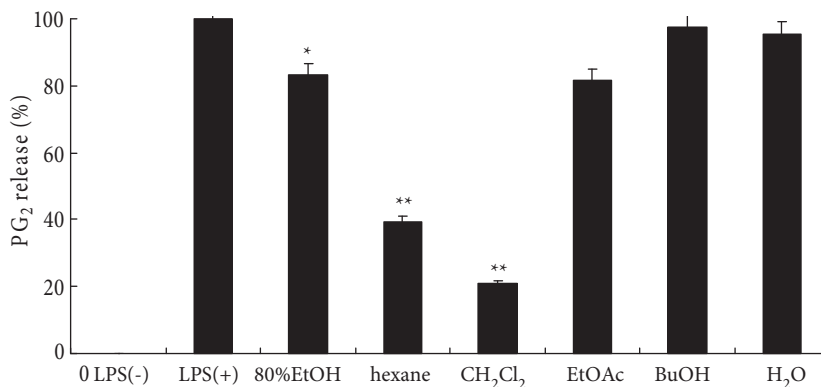


Figure 2. Inhibitory effects of *S. muticum* extracts and solvent fractions on PGE₂ production in RAW 264.7 cells.

Cells (1.5×10^5 cells/mL) were stimulated with LPS (1 μ g/mL) for 24 h in the presence of the 80% EtOH extract or the solvent fractions (25 μ g/mL) of *S. muticum*. Supernatants were collected, and the PGE₂ concentration in the supernatants was determined by ELISA. Values are the mean \pm SEM of triplicate experiments.

*, P < 0.05; **, P < 0.01

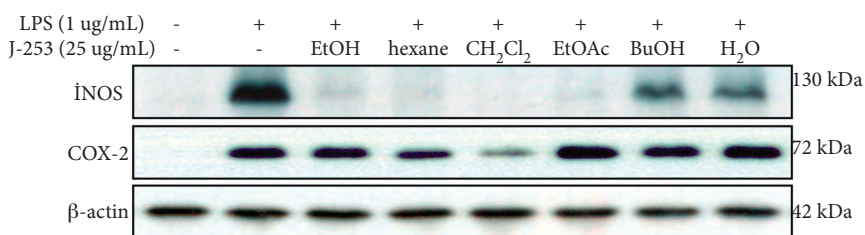


Figure 3. Inhibitory effect of *S. muticum* extracts and solvent fractions on iNOS and COX-2 protein levels in RAW 264.7 cells.

RAW 264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 μ g/mL) in the presence of the 80% EtOH extract or the solvent fractions (25 μ g/mL) of *S. muticum* for 24 h. The iNOS protein level was determined by immunoblotting.

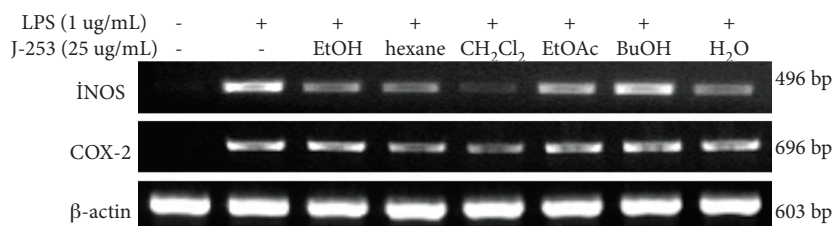


Figure 4. Inhibitory effects of *S. muticum* extracts and solvent fractions on iNOS and COX-2 mRNA expression in RAW 264.7 cells.

RAW 264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and the iNOS mRNA expression was determined from cells stimulated with LPS (1 μ g/mL) for 24 h in the presence of 80% EtOH extract and solvent fractions (25 μ g/mL) of *S. muticum*.

determined the effect of the solvent fractions from *S. muticum* on pro-inflammatory cytokines in murine macrophage RAW 264.7 cells through RT-PCR analyses. After 18 h of pre-incubation, mRNA expression of pro-inflammatory cytokines was determined from a culture stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 8 h in the presence of *S. muticum* solvent fractions (25 $\mu\text{g}/\text{mL}$). There was no basal change in IL-1 β and IL-6 expression when cells were incubated with only the crude extract from *S. muticum* without LPS (data not shown). After 24 h of incubation with both LPS (1 $\mu\text{g}/\text{mL}$) and the solvent fractions of *S. muticum* (25 $\mu\text{g}/\text{mL}$), there was remarkable inhibition

of IL-1 β and IL-6 production and mRNA expression in RAW264.7 cells, especially in cells treated with the *n*-hexane and CH_2Cl_2 fractions (Figure 5). Furthermore, the CH_2Cl_2 fraction (25 $\mu\text{g}/\text{mL}$) strikingly decreased the levels of IL-1 β mRNA and protein by up to 100%.

To assess the effects of the CH_2Cl_2 fraction of *S. muticum* extract on LPS-induced protein and mRNA expression of iNOS and COX-2 in RAW 264.7 cells, culture media were collected from the cells that were treated with or without LPS and various concentrations of the CH_2Cl_2 fraction. As our results indicate, 6.25, 12.5, and 25 $\mu\text{g}/\text{mL}$ of the CH_2Cl_2

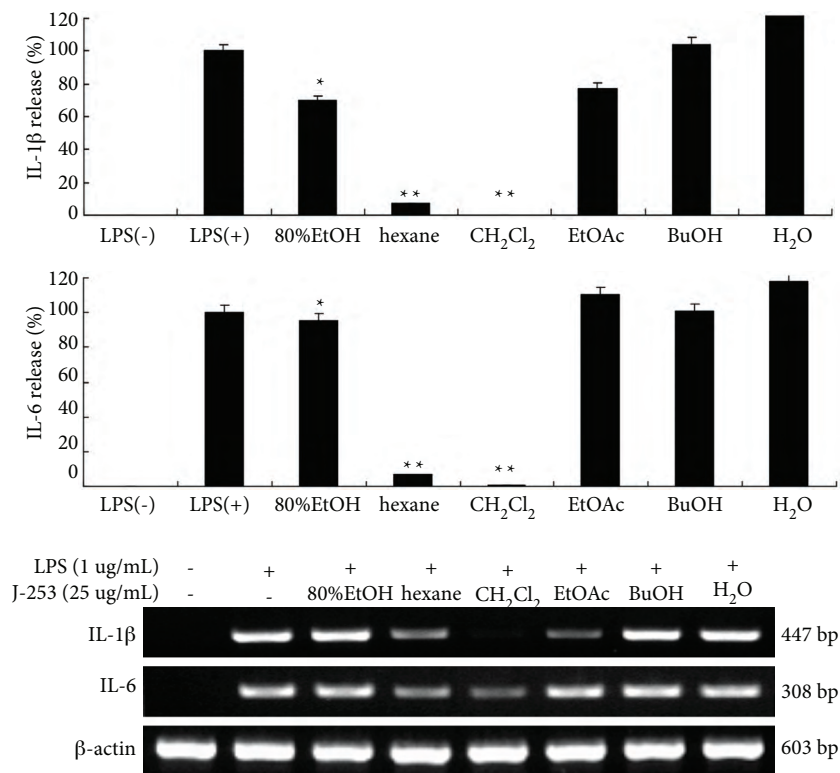


Figure 5. Inhibitory effects of the 80% EtOH extract and solvent fractions of *S. muticum* on IL-1 β and IL-6 production and mRNA expression in RAW 264.7 cells.

Cells (1.5×10^5 cells/mL) were stimulated by LPS (1 $\mu\text{g}/\text{mL}$) for 24 h in the presence of the 80% EtOH extract and the solvent fractions from *S. muticum* (25 $\mu\text{g}/\text{mL}$). Supernatants were collected, and the IL-1 β and IL-6 concentrations in the supernatants were determined by ELISA. Values are the mean \pm SEM of triplicate experiments.

*, $P < 0.05$; **, $P < 0.01$

fraction of *S. muticum* extract significantly attenuated LPS-induced protein and mRNA expressions of iNOS and COX-2 in a dose-dependent manner (Figure 6).

Since IL-6 and IL-1 β are also inflammatory mediators, the effects of the CH₂Cl₂ fraction of *S. muticum* extract on IL-6 and IL-1 β production in LPS-stimulated RAW 264.7 cells were measured using the culture media. Consistent with the attenuated LPS-induced protein and mRNA expressions of iNOS and COX-2, co-incubation of cells with 6.25, 12.5, and 25 μ g/mL of the CH₂Cl₂ fraction of *S. muticum* extract prior to LPS treatment caused a significant ($P < 0.05$ or $P < 0.01$) and dose-dependent decrease in the release of IL-6 and IL-1 β (Figure 7).

In conclusion, in our search for inhibitors of COX-2 and iNOS, we found that extracts of *S. muticum* inhibited NO and PGE₂ generation in LPS-stimulated

RAW 264.7 macrophage cells, as well as levels of iNOS and COX-2 mRNA and protein. The CH₂Cl₂ fractions exhibited strong inhibitory activity against NO and PGE₂ production. The CH₂Cl₂ fraction of *S. muticum* also decreased the expression of IL-1 β and IL-6 mRNA in a dose-dependent manner. Considering these results, we suggest that *S. muticum* extracts may be considered possible anti-inflammatory candidates for topical application. Further investigations will focus on the in vivo assessment of the biological activity of *S. muticum* extracts and on the chemical identification of the major active components responsible for anti-inflammatory activity in the efficacious extracts. To the best of our knowledge, this is the first report demonstrating the in vitro anti-inflammatory activity of *S. muticum* extracts and providing a scientific basis for human health.

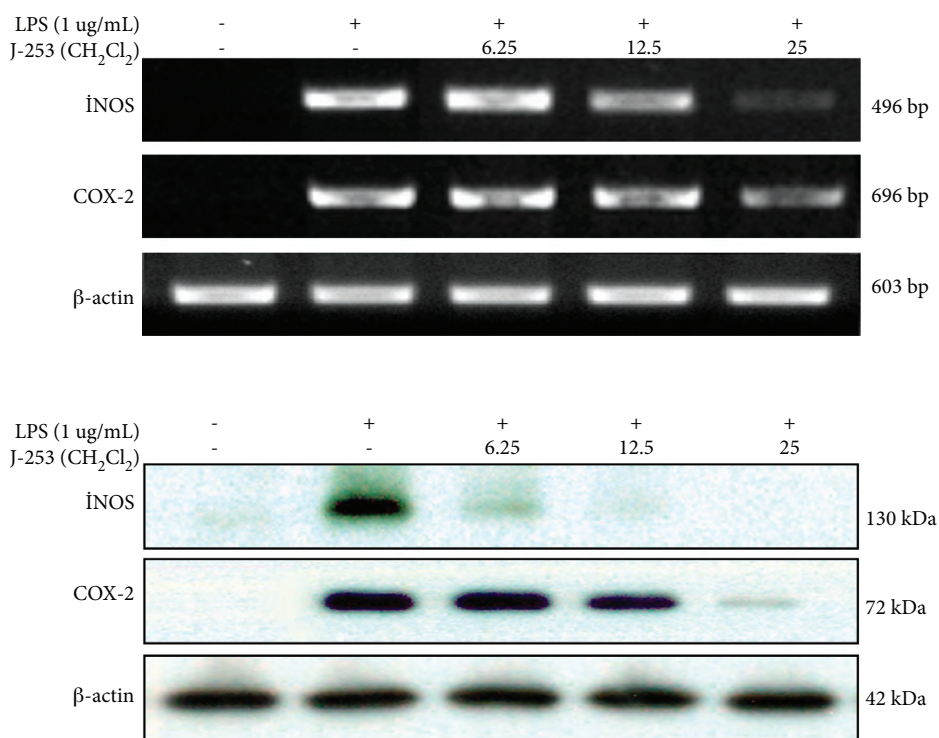


Figure 6. Inhibitory effect of CH₂Cl₂ fraction of *S. muticum* on the mRNA expression protein level of iNOS and COX-2 in RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and pro-inflammatory cytokine mRNA expression was determined from 24-h culture of cells stimulated with LPS (1 μ g/mL) in the presence of 80% EtOH extract and solvent fractions of *S. muticum* (25 μ g/mL).

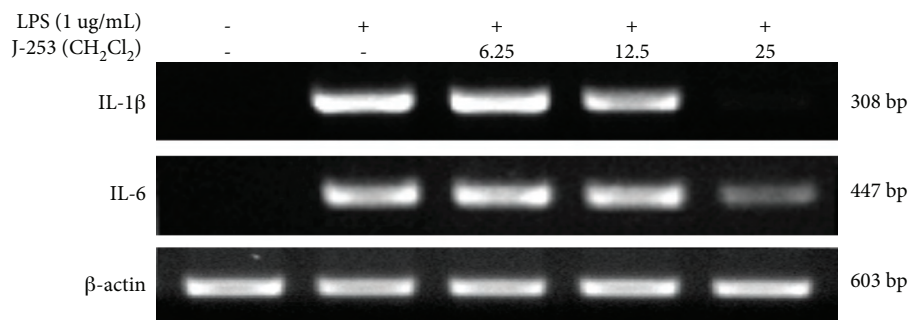


Figure 7. Inhibitory effect of CH_2Cl_2 fraction of *S. muticum* on the pro-inflammatory cytokine mRNA expression in RAW 264.7 cells.

RAW 264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 18 h, and the pro-inflammatory cytokine mRNA expression was determined from 24-h culture of cells stimulated with LPS (1 $\mu\text{g/mL}$) in the presence of *S. muticum* fraction (CH_2Cl_2 - 6.25, 12.5, 25 $\mu\text{g/mL}$).

Acknowledgements

This research was partially supported by the Program for the RIS (Regional Innovation System; JEJU SEA-GREEN PROJECT) and the Regional Technology Innovation Program (70004243), which are managed by the Ministry of Knowledge and Economy, KOREA.

Corresponding author:

Chang-Gu HYUN

Research Group for Cosmetic Materials

Jeju Biodiversity Research Institute (JBRI)

Jeju High-Tech Development Institute (HiDI)

Jeju 699-943-KOREA

E-mail: cghyun@jejuhidi.or.kr

References

- Murakami A, Ohigashi H. Targeting NOX, iNOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. *Int J Cancer* 121: 2357-2363, 2007.
- Vane JR, Mitchell JA, Appleton I et al. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Pharmacology* 91: 2046-2050, 1994.
- Won JH, Im HT, Kim YH et al. Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via the NF- κ B inactivation. *Br J Pharmacol* 148: 216-225, 2006.
- MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 15: 323-350, 1997.
- Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-142, 1991.
- Nathan C, Xie QW. Nitric oxide synthases: roles, tolls and controls. *Cell* 78: 915-918, 1994.
- Hinz B, Brune K. Cyclooxygenase-2, 10 years later. *J Pharmacol Exp Ther* 300: 367-375, 2002.
- Mitchell JA, Larkin S, Williams TJ. Cyclooxygenase-2: regulation and relevance in inflammation. *Biochem Pharmacol* 50: 1535-1542, 1995.
- Needleman P, Isakson PC. Selective inhibition of cyclooxygenase 2. *Sci Med* 1: 35-36, 1998.
- Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* 1470: 69-78, 2000.
- Tsatsanis C, Androulidaki A, Venihaki M et al. Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol* 38: 1654-1661, 2006.
- Baeuerle PA, Henkel T. Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 12: 141-179, 1994.
- Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF- κ B. *J Clin Invest* 107: 241-246, 2001.

14. Molloy RG, Mannick JA, Rodrick ML. Cytokines, sepsis and immunomodulation. *Br J Surg* 80: 289-297, 1993.
15. West MA, Li MH, Seatter SC et al. Pre-exposure to hypoxia or septic stimuli differentially regulates endotoxin release of tumor necrosis factor, interleukin-6, interleukin-1, prostaglandin E2, nitric oxide, and superoxide by macrophages. *J Trauma* 37: 82-89, 1994.
16. Stein B, Sutherland MSK. IL-6 as a drug discovery target. *Drug Discov Today* 3: 202-213, 1998.
17. Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol* 8: 253-278, 1990.
18. Kim JY, Lee JA, Kim KN et al. Antioxidative and antimicrobial activities of *Sargassum muticum* extracts. *J Korean Soc Food Sci Nutr* 36: 663-669, 2007.
19. Fernández M, Ríos JC, Jos A et al. Comparative cytotoxicity of alachlor on RTG-2 trout and SH-SY5Y human cells. *Arch Environ Contam Toxicol* 51: 515-520, 2006
20. Kor JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Meth* 20: 83-89, 2005.
21. Hevel JM, Marletta MA. Nitric-oxide synthase assay. *Method Enzymol* 233: 250-258, 1997.
22. Cho JY, Baik KU, Jung JH et al. In vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. *Eur J Pharmacol* 398: 399-407, 2000.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
24. Dinarello CA. Pro-inflammatory cytokines. *Chest* 118: 503-508, 2000.