

## Original Research Article

# Neuroprotective Effect of *Sargassum thunbergii* (Mertens ex Roth) Kuntze in Activated Murine Microglial Cells

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### Abstract

**Purpose:** To evaluate the anti-oxidant and anti-neuroinflammatory effects of the *Sargassum thunbergii* extract (Mertens ex Roth) Kuntze (STE) in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells in vitro.

**Methods:** STE antioxidative activity was evaluated with an Electron Spin Resonance (ESR) spectrometer, which measured 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity. Cell viabilities were estimated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assays. LPS-stimulated BV-2 microglia were used to study the expression and production of inflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS) and tumor necrosis alpha (TNF- $\alpha$ ).

**Results:** LPS treatment, following STE pretreatment, decreased NO production by 13 ~ 65% in a dose-dependent manner ( $p < 0.001$  at 20, 40, 80 and 100  $\mu\text{g/mL}$ ), and was associated with the down-regulation of inducible nitric oxide synthase (iNOS) expression. STE also attenuated the TNF- $\alpha$  soluble protein by 16 ~ 47% ( $p < 0.01$  at 20, 40 and 80  $\mu\text{g/mL}$ ) in activated murine microglia. Furthermore, the DPPH-generated free radicals were inhibited by STE concentration-dependently.

**Conclusion:** STE has therapeutic potential in the prevention or treatment of neurodegenerative and oxidative stress-related disorders.

**Keywords:** *Sargassum thunbergii*, Neurodegenerative diseases, Anti-inflammatory, Microglial cells, Inducible nitric oxide synthase (iNOS), Tumor necrosis factor (TNF)- $\alpha$

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## INTRODUCTION

Microglia, as immune cells of the central nervous system (CNS), produce a variety of inflammatory mediators in response to immunological stressors, and thus play a critical role in neuro-inflammatory processes [1]. Upon activation by exposure to free radicals and lipopolysaccharides (LPS) [2], microglia secrete various bioactive molecules such as nitric oxide (NO), inducible NO synthase (iNOS), interleukins (IL) and tumor necrosis factor (TNF)- $\alpha$  [3]. Over-

production of these inflammatory mediators can cause a number of severe neuro-degenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and Huntington's disease [4,5].

LPS, as the main component of endotoxins, initiates numerous cellular effects that play critical roles in the pathogenesis of inflammatory responses. LPS has been proven to induce microglial activation in response to Gram-negative bacterial infections [6]. These prior

experiments indicate that LPS-induced stimulation of microglia may be an potent mechanism to study *in vitro*. Studies have shown that antioxidant and anti-inflammatory agents may inhibit microglial activation, and protect neurons from cell death, a prominent symptom of various neurodegenerative disorders [6,7].

Brown seaweed is a marine organism with anti-inflammatory and anti-oxidative effects [8]. *Sargassum thunbergii* is found along shallow marine coastlines of Korea, and perennially grows on low tide-level rocks of open and sheltered coasts [9]. *Sargassum thunbergii*, also known as Hede, is also used as a food additive, an anti-helminthic treatment for lumps, dropsy, or swollen and painful scrotums [10].

Studies on *Sargassum thunbergii* for its beneficial effects on microglia-mediated neuro-inflammatory diseases have not yet been reported. The aim of this study was to investigate whether the ethanol extract of *Sargassum thunbergii* (STE) exhibits protective effects on LPS-activated neuro-inflammatory processes in murine microglial BV-2 cells.

## EXPERIMENTAL

### Preparation of *Sargassum thunbergii* extract (STE)

Dried plant materials of *S. thunbergii* were purchased from a traditional herb market in Seoul, South Korea and authenticated by taxonomist, Professor Jong-Bo Kim at Konkuk University, South Korea. The *S. thunbergii* was washed in running tap water, dried at 60 °C for 24 h and then ground to a fine powder. The STE was extracted from the fine powder by mixing with 70 % ethanol (v/w) for 2 h in a heating mantle at 70 ~ 80 °C. The filtered extract was concentrated by a rotary evaporator (EYELA NVC-2000, Tokyo, Japan) under reduced pressure and lyophilized to produce the supernatant.

### Measurement of DPPH radical scavenging activity

STE antioxidative activity was measured using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). Samples composed of a reaction mixture of STE aliquots and a DPPH methanolic solution (described previously) [9], each with 60 µL of STE and 60 µL of DPPH (60 µM) in methanol. After mixing vigorously for 10 s, the mixture was transferred to a 100 µL Teflon capillary tube. The

scavenging activity on the DPPH radical was measured in each sample using a JES-FA ESR spectrometer (Jeol Ltd, Tokyo, Japan). Spin adducts were measured by an ESR spectrometer. Experimental conditions were as follows: central field, 3,475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain,  $6.3 \times 10^5$ ; and temperature, 298 K.

### Cell cultures and viabilities

Murine microglia BV-2 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS (Hyclone, Logan, UT, USA), 100 U/mL of penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 5 % CO<sub>2</sub>. In all experiments, cells were pre-treated with indicated concentrations of STE for 1 h before the addition of LPS (1 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in serum-free RPMI-1640 medium. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays, described previously [10]. Cells were incubated with various concentrations of STE for 24 h, followed by MTT for 4 h. 100 µL of isopropanol (in 0.04 N-hydrochloric acid) was then added to the samples to dissolve formazan crystals. The absorbance was read at 570 nm using the Biochrom Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was compared at relative absorbances to the control.

### Nitric oxide assay

NO production assays indicated varying nitrite levels in the STE supernatant using the colorimetric assay with Griess reagent [10]. BV-2 cells were seeded in 6-well plates in 500 µl of the 10 % RPMI-1640 medium, and then stimulated with LPS (1 µg/ml) for 2 h. Fifty microliters of the STE-contained supernatant were combined with the equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride in water). ELISA reader (Bio-Tek Instrument, Winooski, VT, USA) indicated nitrite concentrations (absorbance of 540 nm) in comparison to sodium nitrite as control.

### Immunoblot analysis

Murine microglial BV-2 cells were lysed using a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM β-glycerophosphate, 1 % NP-40 detergent, 1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 X protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim,

Germany). Supernatants were collected upon centrifugation of the lysates at 15,000 x g for 15 min. The concentrations of protein samples were measured using the BCA reagent (Pierce, Rockford, IL, USA). STE-treated cell proteins were separated by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. After blocking with 5 % non-fat milk, the membranes were hybridized at 1:1000 dilutions of primary antibodies for iNOS and  $\beta$ -actin for 12 h at 4 °C. Membranes were washed with Tris-Buffered Saline containing 0.1 % Tween-20 (TBST) three times at 5 min interval and hybridized with horseradish peroxidase-conjugated mouse or rabbit secondary antibodies for 1 h at room temperature. After washing three times with TBST, antigen-antibody complexes were visualized with an ECL Western Blot Detection Kit (GE Healthcare) according to the manufacturer's instructions. The antibodies against iNOS and  $\beta$ -actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

### TNF- $\alpha$ assay

Murine microglial BV-2 cells ( $1 \times 10^5$  cells/well) were cultured on 96-well plates treated with the STE at indicated concentrations for 1 h and stimulated with LPS (1  $\mu$ g/mL). 24 h post-LPS treatment, TNF- $\alpha$  production was determined with cell supernatant using assay kits (BD Biosciences, San Jose, CA, USA) per manufacturer's instructions at room temperature. The optical absorbance was measured at 450 nm with the ELISA reader.

### Statistical analysis

All data is presented as the mean  $\pm$  S.E.M of at least three independent experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests.  $P < 0.05$  was considered statistically significant.

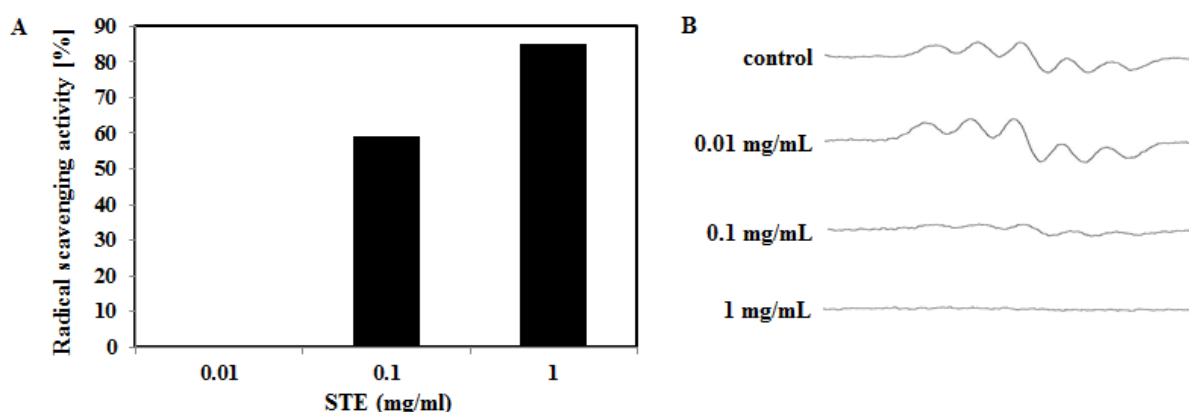
## RESULTS

### STE's effect on DPPH radical-scavenging activity

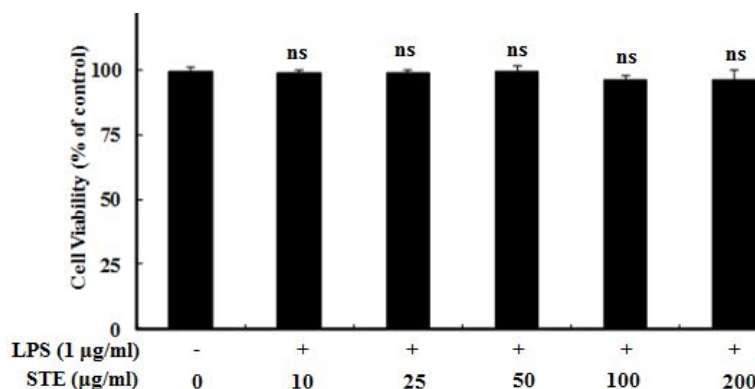
As shown in Fig 1A, STE exhibited significant DPPH radical scavenging activity in a dose-dependent manner, showing a maximum effect at a concentration of 1 mg/mL. The ESR spectroscopy data at 0.01, 0.1 and 1 mg/ml was represented in Fig 1B.

### STE's Effect on LPS-induced NO production

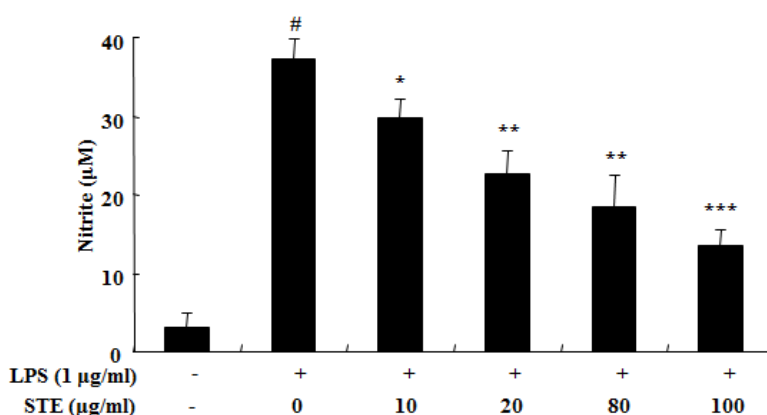
STE treatment did not result in cytotoxic over-production of NO in BV-2 microglial cells treated for 24 h at concentrations up to 200  $\mu$ g/mL. In all cases of STE treatment, cell viability was above 96 % (Fig 2). In contrast, LPS treatment resulted in excessive production of NO. Pretreatment with STE prior to LPS treatment, however, significantly decreased (by 13 ~ 65 %) the production of NO in comparison to LPS-only treatment in a dose-dependent manner (Fig 3). The maximum effect was observed at 100  $\mu$ g/mL ( $p < 0.001$ ).



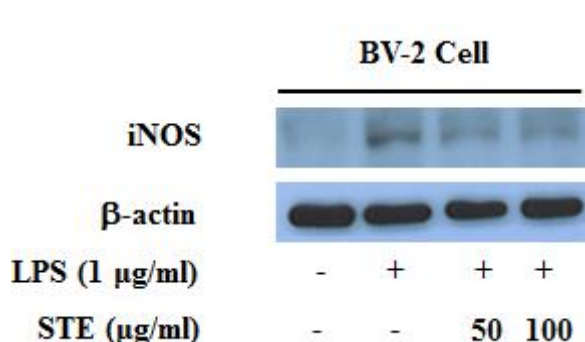
**Fig 1:** Effect of STE on DPPH radical scavenging activity. The capacity to scavenge DPPH free radicals at different concentrations STE (A), shown to vary through ESR spectra (B) measurements. BV-2 cells were treated at various concentrations (0, 0.01, 0.1 and 1 mg/ml). The scavenging activity on DPPH radical measured using JES-FA ESR spectrometer



**Fig 2:** STE's effect on murine microglial BV-2 cells using MTT assay. The results are displayed in percentages relative to control sample without LPS/STE treatment. Data presented is the mean ± SEM (n = 3) for three independent experiments. NS indicates statistical non-significance



**Fig 3:** Effect of STE on NO Production in LPS-stimulated murine microglial BV-2 cells. Cells treated with STE at various concentrations (10, 20, 40, 80, and 100 µg/ml) with or without LPS (1 µg/mL) for 24 hr showed varying cell viabilities. Data presented as the mean ± S.E.M. (n = 3) for three independent experiments. #*p* < 0.001, when compared with control group. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001, when compared with the LPS-only treated control, was assessed by one-way analysis of variance, and then with Dunnett's multiple range tests



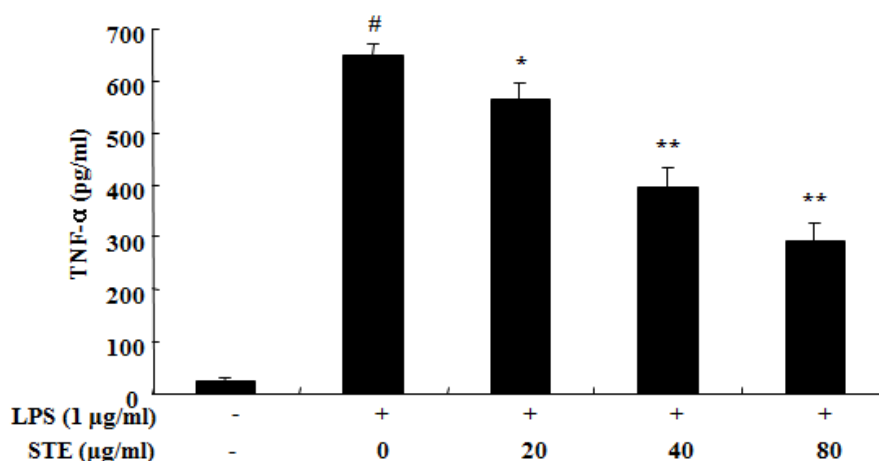
**Fig 4:** Effect of STE on level of iNOS protein expression in LPS-stimulated BV-2 microglial cells. iNOS production levels in the cells at indicated STE concentrations (50 and 100 µg/mL) monitored by immunoblot analyses with iNOS antibodies. The internal control used was β-actin protein expression levels

**Effect of STE on LPS-induced expressional levels of iNOS**

STE treatment of murine microglial BV-2 cells exhibited a broad spectrum of inhibitory effects on the expression of iNOS, in contrast to LPS treatment, which enhanced iNOS expression (Fig 4).

**Effect of STE on TNF-α production in LPS-stimulated BV-2 cells**

STE significantly inhibited the production of the pro-inflammatory cytokine, TNF-α, in a concentration-dependent manner in LPS-stimulated BV-2 cells (*p* < 0.05 at 20 µg/mL and *p* < 0.01 at 40 and 80 µg/mL, respectively). In the LPS-only treatment (1 µg/mL), TNF-α levels were significantly higher than those of the untreated cell control sample (*p* < 0.001) (Fig 5).



**Fig 5:** Effect of STE on TNF- $\alpha$  production in LPS-stimulated BV-2 microglial cells. STE's suppression of TNF- $\alpha$  expression at 20, 40 and 80  $\mu$ g/mL with or without LPS (1  $\mu$ g/mL) for 24 h. TNF- $\alpha$  levels measured using a murine TNF- $\alpha$  ELISA kit. Data presented are means  $\pm$  S.E.M. ( $n = 3$ ) for three independent experiments; # $p < 0.001$ , when compared with control group; \* $p < 0.05$ , and \*\* $p < 0.01$

## DISCUSSION

The present study has shown a few anti-neuroinflammatory effects of STE in murine microglial BV-2 cells, in tandem with LPS (neuroinflammatory) treatment. Although LPS activates TLR4 receptors of microglia to secrete various cytokines, STE treatment affects NO, iNOS and TNF- $\alpha$  production and expression in dose-dependent manner. These results show that STE extract has anti-neuroinflammatory and antioxidative effects.

STE exhibited significant antioxidant activity, as evidenced by the DPPH free radical scavenging method. The DPPH radical assay is a widely used method for evaluating the free radical scavenging activities of several antioxidants in a relatively short period of time [12]. Free radicals and reactive oxygen species (ROS) are important causative factors in the development of age-related neuro-inflammatory and neuro-degenerative diseases, [11] so neutralization of free radicals by antioxidants and radical scavengers reduces neuro-inflammation. In our present study, STE significantly affected free radical scavengers, indicating its potential as an antioxidative agent. Many common antioxidants have been effective in reducing neuroinflammation [11], but STE has not studied in activated murine microglial BV-2 cells. Our results present STE's strong potential in reducing neuroinflammatory activity in various neurodegenerative disorders.

In BV-2 microglia cells, NO is generated by the inducible isoform of NO synthase (iNOS), and has been identified as a neurotoxic substance contributing to central nervous system

inflammation [13]. High levels of NO are produced from L-arginine by iNOS activation in the brain, which prolongs microglial cell activation, and this mechanism is associated with the progression of various neuro-degenerative diseases [14]. Our results clearly show that STE's effect on two elements of this mechanism, as STE attenuates LPS-induced iNOS expression and decreases NO production. STE acts principally on NO generation by down-regulating iNOS gene expression at the post-transcriptional level, and can thus prevent the progression of neuro-inflammation.

STE treatment's significant effects on TNF- $\alpha$  expression also indicate STE's therapeutic potential to treat chronic neuroinflammatory diseases. TNF- $\alpha$  is a pro-inflammatory cytokine that initiates the inflammatory response, and its over-production is a possible etiological factor of most neurological disorders [16]. Microglial cell activation by LPS produces various cytokines, including TNF- $\alpha$ , which in turn attracts neutrophils and causes the accumulation of neutrophil-secreted proteases and ROS at sites of inflammation. Data from our study shows that STE attenuates production of TNF- $\alpha$ , an initiator of the inflammatory response, thereby inhibiting NO production and iNOS expression levels.

## CONCLUSION

These results demonstrate that STE has anti-inflammatory properties in LPS-induced BV-2 microglial activation through the down-regulation of inflammation-related gene expression, including iNOS and the proinflammatory cytokine, TNF- $\alpha$ . STE's ability to down-regulate key proteins involved in the neuroinflammatory

response, even combined with LPS, shows its strong potential as a therapeutic agent. STE can be considered as an effective therapeutic and preventative herbal extract for the treatment of several neurodegenerative and oxidative stress-related diseases.

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