Anti-oxidative and anti-neuroinflammatory effects of ethyl acetate extract fractions of *Suaeda asparagoides* MIlq

Sung-Gyu Lee¹, Jong-Bo Kim² and Hyun Kang¹*

¹Department of Medical Laboratory Science, College of Health Science, Dankook University, Cheonan-si, Chungnam, 330-714,
²Department of Biotechnology, Research Institute (RIBHS) and College of Biomedical and Health Science, Konkuk University, Chungju, Chungbuk, 380-701, Republic of Korea

*For correspondence: Email: hyunbio@gmail.com, hkang@dankook.ac.kr

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**Abstract**

**Purpose:** To evaluate the in vitro antioxidant and anti-neuroinflammatory effects of *Suaeda asparagoides* ethylacetate extract (SAE) in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells.

**Methods:** The antioxidative activity of SAE was evaluated by measuring 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity spectrometrically. Cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, while LPS-stimulated BV-2 microglia were used to study the expression and production of inflammatory mediators, including nitric oxide (NO), inducible NO synthase (iNOS) and tumor necrosis alpha (TNF-α).

**Results:** Pretreatment with SAE prior to LPS treatment significantly inhibited excessive production of NO (p < 0.001 at concentrations of 40 and 80 μg/mL) in a dose-dependent manner, and was associated with down-regulation of expression of inducible nitric oxide synthase (iNOS). SAE also suppressed the LPS-induced increase in TNF-α level (p < 0.01 at concentrations of 40 and 80 μg/mL) in BV-2 cells. Furthermore, DPPH-generated free radicals were inhibited by SAE in a concentration-dependent manner.

**Conclusion:** These results indicate that SAE possesses strong anti-oxidant properties, and inhibits excessive production of pro-inflammatory mediators, including NO, iNOS and TNF-α, in LPS-stimulated BV-2 cells.

**Keywords:** *Suaeda asparagoides*, DPPH, Anti-inflammatory activity, Microglial cells, iNOS, TNF-α

**INTRODUCTION**

*Suaeda asparagoides* MIlq. (*S. asparagoides*) is a salt marsh plant which grows well in tide land due to land change [1,2]. Several halophytes are widely distributed along the estuary of Korea [3]. Among them, pharmacological and chemical characterization of Salicornia herbacea have recently been studied well [4]. *S. asparagoides* plant is traditionally folk medicine in Korea to treat hypertension and hepatitis [4]. Antioxidants are of interest to biologists and clinicians, because they help to protect the human body against damage by reactive free radicals [5,6].

The inflammatory response is mediated by the activated microglia, the resident immune cells of the central nervous system (CNS), which normally respond to neuronal damage [7]. They are activated in response to free radicals and exposure to lipopolysaccharide (LPS) [8]. Activation of microglia results in the release of various bioactive molecules, including nitric oxide (NO), inducible NO synthase (iNOS), interleukins...
(IL) and tumor necrosis factor (TNF)-α [9]. Overproduction of these inflammatory mediators causes severe neurodegenerative diseases, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD), multiple sclerosis (MS), and Huntington’s disease [9].

LPS is the main component of endotoxins, initiates a number of major cellular effects that play critical roles in the pathogenesis of inflammatory responses, and has been employed to induce microglial activation during infection by Gram-negative bacteria [9]. Therefore, LPS-induced stimulation to microglia might be used as an important tool for evaluating changes caused by activated microglia in vitro. Studies have shown that anti-oxidant and anti-inflammatory agents may inhibit microglial activation and thus protect neuronal cell death seen in various neurodegenerative disorders [10].

*Suaeda asparagoides* has long been used as a Korean folk herbal medicine for the treatment of functional gastrointestinal disorders [2]. However, studies on its beneficial effects on microglia-mediated neuroinflammatory diseases have not been reported. The aim of this study was to investigate whether *Suaeda asparagoides* ethylacetate extract (SAE) exhibits protective effects on LPS-activated neuroinflammatory processes in BV-2 microglial cells.

**EXPERIMENTAL**

**Preparation of *S. asparagoides* extract**

Dried *S. asparagoides* was purchased from traditional herb market, Seoul, South Korea and was authenticated by a taxonomist Dr. Kim Byung-Wook at Konkuk University, South Korea. A voucher specimen (KH-KU-2015) was kept in our department herbarium for future reference. To obtain the *S. asparagoides* extract, the dried plant material was ground in a mixer and defatted with three volumes of 70 % ethanol, three times. The residue was extracted with absolute ethanol at 1:10 ratio (w/v) for 2 h in heating mantle at 70 –80 °C. The supernatant was filtered and concentrated in vacuum heating mantle at 70 °C –80 °C.

Dried *S. asparagoides* was partitioned into hexane, chloroform and ethyl acetate (EA) fractions to furnish 0.67 g, 9.12 g and 10.35 g, respectively. The active EA fraction of *S. asparagoides* designated as SAE was re-dissolved in distilled water to evaluate for its anti-neuroinflammatory and antioxidant activities.

**Evaluation of DPPH radical scavenging activity**

The anti-oxidant activity of the SAE was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the SAE and a DPPH methanolic solution as described previously [10]. A sample solution of 60 µL of each SAE was added to 60 µL of DPPH (60 µM) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100 µL Teflon capillary tube and the scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer (Jeol Ltd., Tokyo, Japan). A spin adduct was measured on an ESR spectrometer exactly after 2 min. 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 x 10ö; and temperature, 298 °K. Depending on the sample concentration, ESR spectra were recorded as a function of time starting from the juice and radical solution contact. Recording intervals were 0.5 min and 1.0 min, depending on the sample activity. The signal intensities of radicals were calculated by the double integration of ESR spectra, using the EW (EPRWare) Scientific Software Service program and expressed in arbitrary units.

**Cell cultures and determination of cell viability**

BV-2 microglia cellswereground in RPMI1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS (Hyclone, Logan, UT, USA) containing 100 U/mL of penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen) at 37 °C in humidified atmosphere of 5 % CO2. In all experiments, cells were pre-treated with the indicated concentration of SAE for 1 h before the addition of LPS (1 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) in serum-free RPMI1640. An equal volume of sterile water was added to all control treatments. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [10,11]. Cells were incubated with various concentrations of SAE for 24 h followed by MTT for 4 h, and then 100 µL of isopropanol (in 0.04 N-hydrochloric acid) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using the Anthos 2010 spectrophotometer (Salzburg, Austria). Cell viability was calculated as relative absorbance compared to control.
Nitric oxide assay

The amount of NO production in the medium was detected with the Griess reaction [10]. Each supernatant was mixed with the same volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride in water). The absorbance of the mixture at 540 nm was determined using a spectrophotometer. The TNF-α (BD Biosciences, San Jose, CA, USA) concentration in the cell supernatant was determined using a cytokine assay kit according to the manufacturer's instructions. The antibody complexes were visualized with an ECL Western Blot Detection Kit (GE Healthcare) according to the manufacturer's instructions. The absorbance of the mixture at 450 nm was used to determine the concentration of NO produced.

Immunoblot analysis

BV-2 cells were lysed in a buffer containing 20 mMTris-HCl, pH 7.5, 150 mMNaCl, 1 mM EDTA, 50 mM β-glycerophosphate, 1 % NP-40, 1 mM Na3VO4 and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany). The lysates were centrifuged at 15,000 × g for 15 min and supernatants were collected. The concentrations of protein samples were measured using the BCA reagent (Pierce, Rockford, IL, USA). The protein was separated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. After blocking with 5 % non-fat milk, the membranes were hybridized with a 1:1000 dilution of primary antibodies for iNOS and β-actin for 12 h at 4 °C. The membranes were washed with Tris-buffered saline containing 0.1 % Tween-20 (TBST) three times with 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 β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

Statistical analysis

All data are presented as mean ± SEM of at least three independent experiments. Statistical analysis was performed using SAS statistical software (SAS Institute, Cray, NC, USA) by one-way analysis of variance, followed by Dunnett’s multiple range tests. P < 0.05 was considered statistically significant.

RESULTS

Effect of SAE on DPPH radical scavenging activity

As shown in Figure 1A, SAE exhibited significant DPPH radical scavenging activity in a dose-dependent manner showing a maximum effect at 200 µg/mL of concentration (p < 0.001). The ESR spectroscopy data at 10, 20, 40, 60, 80, 100 and 200 µg/mL are represented in Figure 1B.

Effect of SAE on LPS-induced NO production

SAE treatment did not exhibit any significant cytotoxicity in BV-2 microglial cells treated for 24 h at concentrations up to 80 µg/mL, and in all cases the viability was found above 95.8 % by MTT assay (Figure 2). As shown in Figure 3, treatment of LPS resulted in the excessive production of NO. Pretreatment on SAE significantly suppressed the LPS-induced NO production in a concentration-dependent manner (Figure 2). The maximum effect was shown at 80 µg/mL (p < 0.001).

Effect of SAE on LPS-induced expressional levels of iNOS

To know the effect of SAE extract on iNOS levels, BV-2 cells were stimulated with LPS (1 µg/ml) in the presence or absence of SM-EA extract (40, 80 and 100 µg/ml). LPS strongly induced BV-2 cells showing an increased expression of iNOS. However, treatment with SAE exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in BV-2 cells (Figure 4).

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

As shown in Figure 5, TNF-α levels increased significantly after LPS treatment (1 µg/mL) when compared to those in untreated cells (p < 0.001). However, SAE significantly inhibited TNF-α production in a concentration-dependent manner in LPS-stimulated BV-2 cells (p < 0.05 at 20 µg/mL).
µg/mL and $p < 0.01$ at 40 and 80 µg/mL, respectively).

**DISCUSSION**

Brown seaweed is a marine organism with various biological activities including anti-inflammatory and anti-oxidative effects [1]. *Suaeda asparagoides* distributed along the shallow marine coast of Korea and is perennially growing on rocks near low tide-level on calm open and sheltered coasts [2]. The present study demonstrated that SAE possesses an antineuro-inflammatory activity in LPS-stimulated BV-2 microglia cells and antioxidant activity.

![Figure 1: Effect of SAE on DPPH radical scavenging activity.](image1)

The capacity to scavenge DPPH free radical by different concentrations SAE (A) and ESR spectra (B) was measured. BV-2 cells were treated with or without SAE at the various concentrations (10, 20, 40, 60, 80, 100 and 200 µg/mL). The scavenging activity of the test samples on DPPH radical was measured using a JES-FA ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. SAE = *Suaeda asparagoides* ethylacetate extract

![Figure 2: Effects of SAE on the viability of BV-2 microglial cells.](image2)

Viability in SAE treated cells was determined using MTT assay. The results are displayed the values in percentages of control samples. Data are presented as the mean ± SEM (n = 3); ns = non-significant, SAE = *Suaeda asparagoides* ethylacetate extract

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Figure 3: Effect of SAE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with SAE at various concentrations (10, 20, 40, 80, and 100 µg/mL) with or without LPS (1 µg/mL) for 24 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as mean ± S.E.M. (n = 3) for three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001, when compared with control group. *p < 0.05, **p < 0.01 and ***p < 0.001, when compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. SAE = Suaeda asparagoides ethylacetate extract.

Figure 4: Effect of SAE on iNOS protein expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS production in the LPS-stimulated BV-2 cells by indicated concentrations (20, 40, 80, and 100 µg/mL) of the SAE was monitored by immunoblot analyses with the specific antibodies against iNOS. The internal control used was β-actin. SAE = Suaeda asparagoides Ethylacetate extract; LPS = lipopolysaccharide; iNOS: Inducible nitric oxide synthase.

Figure 5: Effect of SAE on TNF-α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF-α expression by SAE was measured with ELISA test. BV-2 cells were treated with SAE at 20, 40and 80 µg/mL with or without LPS (1 µg/mL) for 24 h. The TNF-α in the culture supernatant was evaluated using a murine TNF-α ELISA kit. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments; *p < 0.001, when compared with control group; *p < 0.05, and **p < 0.01, when compared with LPS alone group by Student t-test. (SAE = Suaeda asparagoides Ethylacetate extract; TNF-α: Tumor necrosis factor-alpha; LPS: Lipopolysaccharide)
Furthermore, the SAE exhibited significant antioxidant activity evaluated by DPPH free radical scavenging method. Free radicals and reactive oxygen species (ROS) are important causative factors in the development of age-related neuro-inflammatory and neurodegenerative diseases [12]. Thus, neutralization by antioxidants and radical scavengers can reduce neuro-inflammation. It is well known that DPPH radical assay as one of the widely used methods for evaluating the free radical scavenging activities of several antioxidants in a relatively short period of time [13]. In our present study, the SAE also exhibited significant free radical scavenging effect indicating that the SAE might contain antioxidant agents. Since antioxidants might reduce neuro-inflammation [12], we used SAE to evaluate for its anti-neuro-inflammatory activity in LPS-stimulated BV-2 microglial cells.

In BV-2 microglia cells, NO is generated by the inducible isoform of NO synthase (iNOS) and has been described as a neurotoxic substance including nitric oxide [13]. Thus, high levels of NO are produced from L-arginine by iNOS in the brain by prolonged activation of microglial cells, and this response is associated with the progression of various neurodegenerative diseases [14]. Our results clearly show that SAE attenuated LPS-induced iNOS expression and downstream NO production. This result indicates that SAE acts principally by regulating NO generation at the post-transcriptional level and could be beneficial for preventing the progression of neuro-inflammation by BV-2 microglial activation.

Proinflammatory cytokines such as TNF-α are the initiators of the inflammatory response and the mediators of the development of chronic inflammatory diseases [15]. Therefore, the over production of pro-inflammatory cytokines, by activated microglia cell is a possible etiological factor neurological disorders [16]. Microglial cell activation by LPS produces various cytokines including TNF-α leading to the attraction of neutrophils and the accumulation of neutrophil-secreted proteases and ROS at the site of inflammation [13]. SAE may act by suppressing the increased production of TNF-α thereby inhibiting NO production and iNOS expression levels in LPS-stimulated BV-2 cells.

**CONCLUSION**

The data demonstrate that SAE has anti-inflammatory properties in LPS-induced BV-2 microglial activation through the down-regulation of inflammation-related gene expression, including iNOS and proinflammatory cytokine such as TNF-α. The strong antioxidant effects exhibited by SAE might also be involved for delivering such potent antineuroinflammatory actions. Therefore, SAE can be considered a useful therapeutic and preventive approach for the treatment of several neurodegenerative diseases and oxidative stress-related diseases.

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**DECLARATIONS**

**Conflict of Interest**

No conflict of interest associated with this work.

**Contribution of Authors**

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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