

Original Research Article

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

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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-diphenyl-tetrazolium 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 20, 40, 80 and 100 $\mu\text{g}/\text{mL}$) 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 $\mu\text{g}/\text{mL}$) 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

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INTRODUCTION

Suaeda asparagoides Miq. (*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 evaporator system at 50 °C. For further fractionation, alcoholic extract (50 g) 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 redissolved 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 μ L of each SAE, was added to 60 μ L of DPPH (60 μ M) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100 μ L 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, 6.3×10^5 , 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 cells were ground in RPMI1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS (Hyclone, Logan, UT, USA) containing 100 U/mL of penicillin (Invitrogen), and 100 μ g/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 μ g/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-diphenyl-tetrazolium 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 μ L 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 with an ELISA reader (Bio-Tek Instrument, Winooski, VT, USA), nitrite concentration was determined using sodium nitrite as a standard.

Immunoblot analysis

BV-2 cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM β -glycerophosphate, 1 % NP-40, 1 mM Na_3VO_4 and protease inhibitor cocktail (Complete Mini™, Roche, Mannheim, Germany). The lysates were centrifuged at 15,000 $\times g$ 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 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).

TNF- α assay

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

Statistical analysis

All data are presented as mean \pm 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 $\mu\text{g}/\text{mL}$ of concentration ($p < 0.001$). The ESR spectroscopy data at 10, 20, 40, 60, 80, 100 and 200 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$, 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 $\mu\text{g}/\text{mL}$ ($p < 0.001$).

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 $\mu\text{g}/\text{mL}$) in the presence or absence of SM-EA extract (40, 80 and 100 $\mu\text{g}/\text{mL}$). 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 $\mu\text{g}/\text{mL}$) 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

$\mu\text{g/mL}$ and $p < 0.01$ at 40 and 80 $\mu\text{g/mL}$, 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.

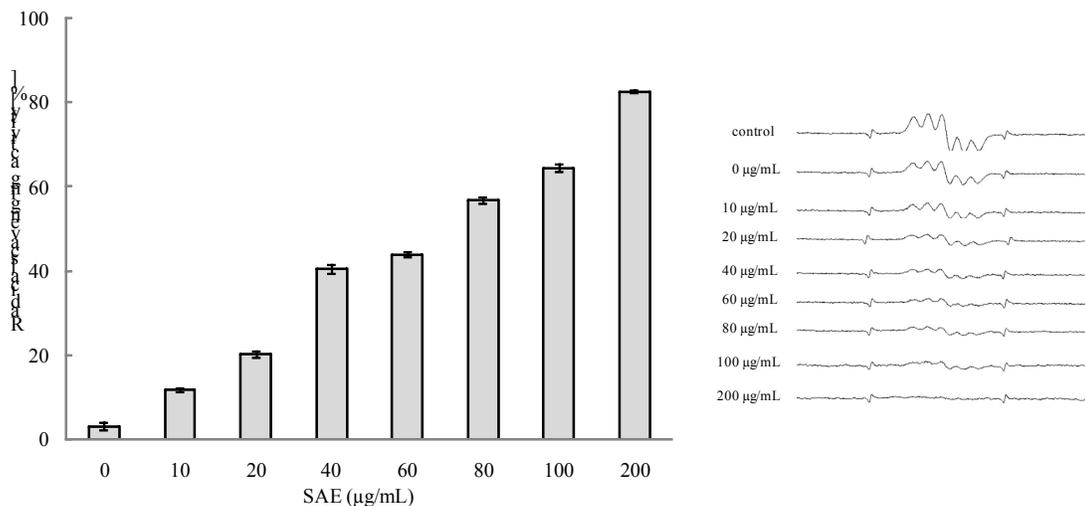


Figure 1: Effect of SAE on DPPH radical scavenging activity. The capacity to scavenge DPPH free radical by different concentrations SAE (A) and ESR spectra (B) was measured. BV-2 cells were treated with or without SAE at the various concentrations (10, 20, 40, 60, 80, 100 and 200 $\mu\text{g/mL}$). The 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

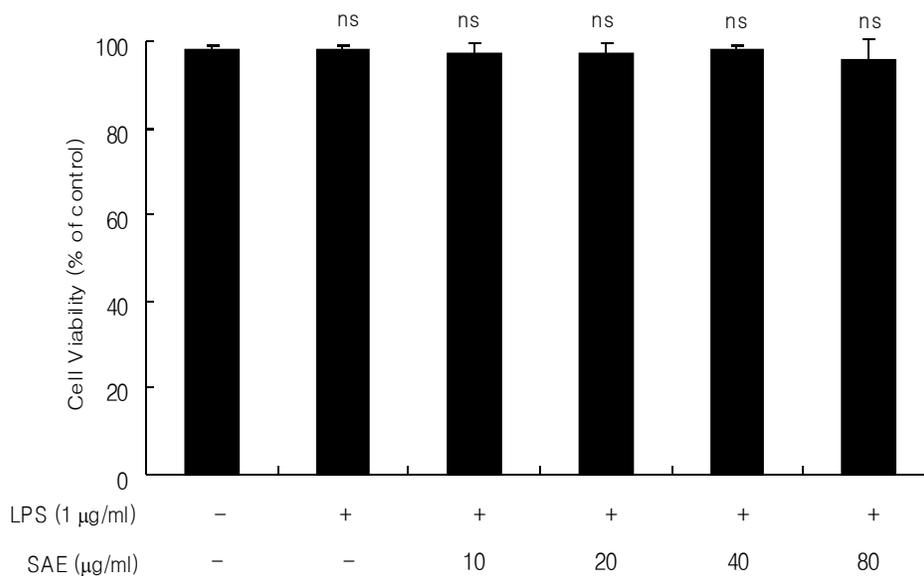


Figure 2: Effects of SAE on the viability of BV-2 microglial cells. Viability in SAE treated cells was determined using MTT assay. The results are displayed the values in percentages of control samples. Data are presented as the mean \pm SEM ($n = 3$); ns = non-significant, SAE = *Suaeda asparagoides* ethylacetate extract

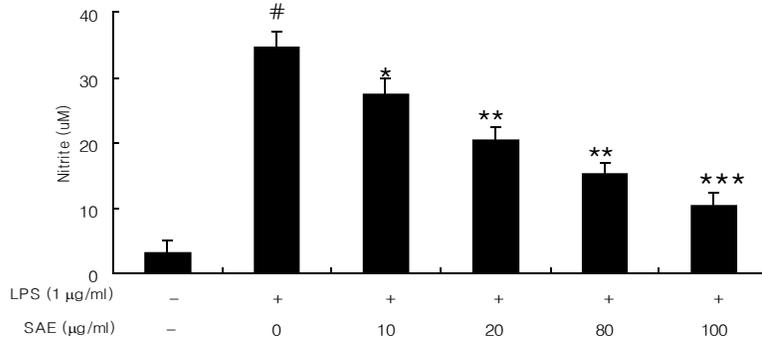


Figure 3: Effect of SAE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with SAE at various concentrations (10, 20, 40, 80, and 100 µg/mL) with or without LPS (1 µg/mL) for 24 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as mean ± S.E.M. (n = 3) for three independent experiments. #p < 0.001, 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

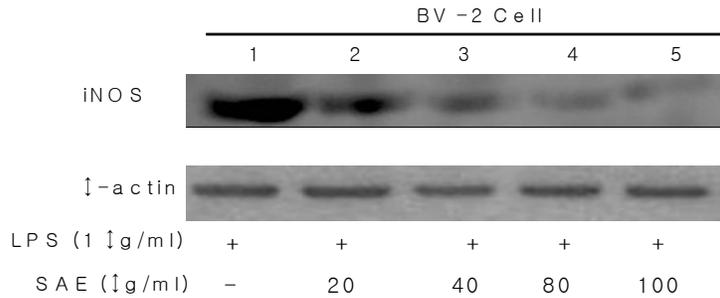


Figure 4: Effect of SAE on iNOS protein expression levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS production in the LPS-stimulated BV-2 cells by indicated concentrations (20, 40, 80, and 100 µg/mL) of the SAE was monitored by immunoblot analyses with the specific antibodies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

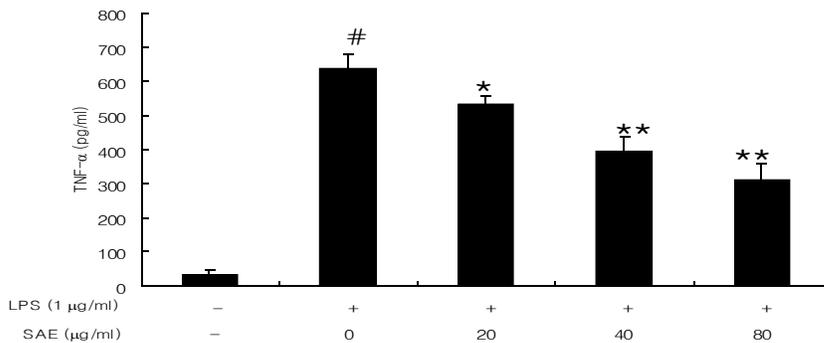


Figure 5: Effect of SAE on TNF-α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF-α expression by SAE was measured with ELISA test. BV-2 cells were treated with SAE at 20, 40 and 80 µg/mL with or without LPS (1 µg/mL) for 24 h. The TNF-α in the culture 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.

ACKNOWLEDGEMENT

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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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