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Original Research Article

Attenuation of Neuroinflammatory Responses in Lipopolysaccharide-Induced BV-2 Microglia by *Suaeda asparagoides* Miq. (Chenopodiaceae)

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Abstract

Purpose: *To investigate the protective effect of Suaeda asparagoides (Chenopodiaceae) extract on neuroinflammatory responses induced by lipopolysaccharide (LPS) in BV-2 microglial cells and its antioxidant effects.*
Methods: *Biochemical studies carried out include 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay for cell viability and radical scavenging activities, respectively. To evaluate the anti-neuroinflammatory effects of S. asparagoides (SAE) extract, LPS (1μg/ml)-stimulated BV-2 microglial cells were used and pro-inflammatory mediators and cytokines such as nitric oxide (NO), inducible NO (iNOS), cyclooxygenase (COX)-2, tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB) were measured using Western blotting and enzyme-linked immunosorbent assay (ELISA).*
Results: *LPS-stimulation of BV-2 cells increased the levels of NO (25.2 ± 2.15, $p < 0.001$) and pro-inflammatory mediators such as iNOS, COX-2 and TNF- α. However, treatment with SAE extract (20, 40 and 80 μg/ml) to LPS-stimulated BV-2 cells significantly inhibited the excessive release of NO ($p < 0.05$ at 20 μg/ml and $p < 0.001$ at 40 and 80 μg/ml, respectively) and suppressed the increased levels of iNOS, COX-2 and TNF-α. SAE also concentration dependently inhibited the NF-κB activation in LPS-stimulated BV-2 microglia. Further, SAE significantly and concentration-dependently ($p < 0.001$ at 20 - 200 μg/ml, respectively) scavenged DPPH radicals with IC_{50} of 36.33 ± 2.12 μg/ml.*
Conclusion: *The results strongly suggest that SAE exhibits protective activity against LPS-stimulated neuroinflammatory responses. Mechanistic study reveals that SAE might by regulating NF-κB signaling. The antioxidant activity exhibited by SAE extract might also play a role in the plant's significant anti-neuroinflammatory effect.*

Keywords: *Suaeda asparagoides, Chenopodiaceae, Microglia, Lipopolysaccharide, Neuroinflammation, Cytokines, Antioxidant*

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INTRODUCTION

Biological studies on the halophilic vegetation of arid and humid areas have been attracted great attention [1]. *Suaeda* species one of the important halophyte plants has gained a lot of importance because of its various pharmacological and medicinal properties. In particular, *Suaeda asparagoides* (*S. asparagoides*), a salt-marsh plant from the family *Chenopodiaceae* has been used as a traditional folk herbal medicine for the treatment of functional gastrointestinal disorders, hypertension and hepatitis [2,3]. Earlier

pharmacological studies revealed that *S. asparagoides* possess anti-inflammatory [2] and as an anti-aging agent in cosmeceuticals [4]. However, its pharmacological actions on neuroinflammation have not been elucidated.

Microglial cells, the immune resident cells in central nervous system are activated in response to various stressors [5]. It was well documented that activated microglia plays a crucial role in neuroinflammation by releasing several pro-inflammatory mediators and toxic free radicals seen in several neurodegenerative diseases [5,6]. Mounting evidence suggest that reduction of pro-inflammatory mediators such as nitric oxide (NO), inducible NO synthase (iNOS), tumor necrosis factor-alpha (TNF-α) and other inflammatory cytokines in activated microglia could attenuate the severity of these disorders [7,8]. Lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria can stimulate microglia both *in vitro* and *in vivo* to release various pro-inflammatory and neurotoxic factors [6]. Therefore, LPS-stimulated microglia is a useful *in vitro* model for rapid screening of anti-neuroinflammatory agents [9].

In the light of such reports, in the present investigation we evaluated the effect of *S. asparagoides* on neuroinflammatory responses in LPS-stimulated BV-2 microglia and explored the possible mechanism. Further the antioxidant effect of *S. asparagoides* was also performed using the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay to substantiate the anti-neuroinflammatory properties.

EXPERIMENTAL

Preparation of the S. asparagoides extract

The whole aerial parts of *S. asparagoides* collected at the end of July to beginning of September were obtained from the local herb market, Seoul, South Korea. The collected fruit material was authenticated by Prof. Jong-Bo Kim, a taxonomist at Konkuk University, Korea and a voucher specimen (SA-KU2013) has been kept in our laboratory herbarium, Konkuk University, Korea for future reference. To obtain the *S. asparagoides* extract, the dried plant material was ground in a mixer and defatted three times with three volumes of 80 % ethanol. 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 a rotary evaporator at 50 °C. For further fractionation, the alcoholic extract (100 g) was partitioned into hexane, ethyl acetate (EA) and n-butanol fractions to yield 0.94, 18.84 and 75.62 g, respectively. The EA fraction of *S. asparagoides* (SAE) extract with potent antioxidant properties in our preliminary evaluation was re-dissolved in distilled water and used for evaluating its anti-neuroinflammatory and antioxidant activities. The extract was dissolved in sterile distilled water and filtered on 0.22 µm filters before use. All reagents used in this study were of highest grade available commercially.

Evaluation of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity

The antioxidant property of the SAE extract was determined using the stable radical DPPH (Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the SAE extract and a DPPH methanolic solution as described previously [10]. Briefly, a sample solution of 60 µl of each concentration of HC-EA extract, 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, 6.3×10^5, and temperature, 298 °K.

Cell culture and viability assay

BV-2 microglia cells were cultured at 37 °C in 5 % CO_2 in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5 % FBS (Hyclone, Logan, UT, USA) and antibiotics (Invitrogen). BV-2 cells were pre-treated with SAE at various concentrations ranging from 10-100 µg/ml for 1 h before the addition of LPS (1 µg/ml, Sigma-Aldrich, St Louis, MO, USA) in serum free DMEM. An equal volume of sterile water was added to all control treatments.

For cell viability, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously [11]. Briefly, BV-2 cells were plated onto 96 well plates and exposed to SAE (10, 20, 40, 80 and 100 µg/ml). MTT was added to each well then incubated for additional 2 h in dark at 37 °C. The medium was then

aspirated from the wells and the blue formazan product obtained was dissolved in DMSO. The plates were analyzed at 570 nm using a microplate reader (Tecan Trading AG, Switzerland). Each experiment was conducted in triplicate. Percentage of the cell viability was calculated as (O.D. of extract treated sample/O.D. of non-treated sample) x 100 %.

NO assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [11]. Briefly, BV-2 cells (2 x 10^5 cells/ml) were seeded in 6-well plates in 500 µl complete culture medium and treated with the OFP-EA extract at indicated concentrations for 1 h prior stimulation with LPS (1 µg/ml) for 2 h. Culture supernatant (50 µl) was reacted with an equal volume of Griess reagent (0.1 % naphthylethylenediamine and 1 % sulfanilamide in 5 % H_3PO_4) in 96-well plates at room temperature in the dark. Nitrite concentrations were determined by using standard solutions of sodium nitrite prepared in the culture medium. The absorbance was determined at 540 nm using a microplate reader (Tecan).

Immunoblot analysis and antibodies

BV-2 cell were washed in cold PBS three times and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) NP-40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Na_3VO_4 and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) at 4 °C. The lysate was clarified by centrifugation at 10,000 g for 20 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using BCA reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10 % PAGE gels and separated by standard SDS-PAGE procedure. Proteins were transferred to an NC membrane (S&S, Dassel, Germany) and blocked with 5 % non-fat dry milk in TBS. To detect protein expression, the blots were probed with the specific antibodies against iNOS, COX-2 and NF-κB followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Herculus, CA, USA). The detection of β-actin with a specific antibody was used for an internal control. The immunoreactive proteins on the membrane were detected by chemiluminescence using the West-Save substrate (Lab-Frontier, Seoul, Korea) on X-ray film. The antibodies against iNOS, COX-2, NF-κB and β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

TNF-α assay

BV-2 microglia cells (1 x 10^5 cells/well) were cultured on 96 well plates and treated with the SAE at indicated concentrations (20, 40 and 80 µg/ml) for 1 h and stimulated with LPS (1 µg/ml). At 4 h post LPS treatment, the cells were collected and the supernatants were evaluated for TNF-α level using a murine TNF-α ELISA kit from BD Biosciences (San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis

All data are represented as the mean ± S.E.M. of at least three independent experiments. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA) using student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

SAE extract scavenges DPPH free radicals

SAE at indicated concentrations (10 - 100 µg/ml) exhibited significant DPPH free radical scavenging activity in a concentration dependent manner (Fig 1). The maximum effect was observed at 60 µg/ml ($p < 0.001$). The concentration needed for 50 % inhibition of DPPH radicals was at 36.33 ± 2.12 µg/ml ($p < 0.001$; Fig 1A). The ESR spectroscopy data was represented in Fig 1B.

Effect of SAE extract on BV-2 microglial cell viability

As shown in Fig. 2, treatment with LPS (1 µg/ml) with or without SAE extract at various concentrations (10 - 100 µg/ml) did not affect the overall cell viability nor did they exhibit any cytotoxicity on BV-2 microglia cells.

However, pre-treatment with SAE extract (20, 40 and 80 µg/ml) significantly suppressed the LPS-stimulated increased NO production in BV-2 cells in a concentration dependently fashion compared to LPS-treated cells. The maximum effect was observed at a concentration of 80 µg/ml ($p < 0.001$). SAE extract at 20 and 40 µg/ml also significantly and concentration-dependently inhibited the release of NO in LPS-stimulated BV-2 cells ($p < 0.05$ at 20 µg/ml and $p < 0.001$ at 40 µg/ml).

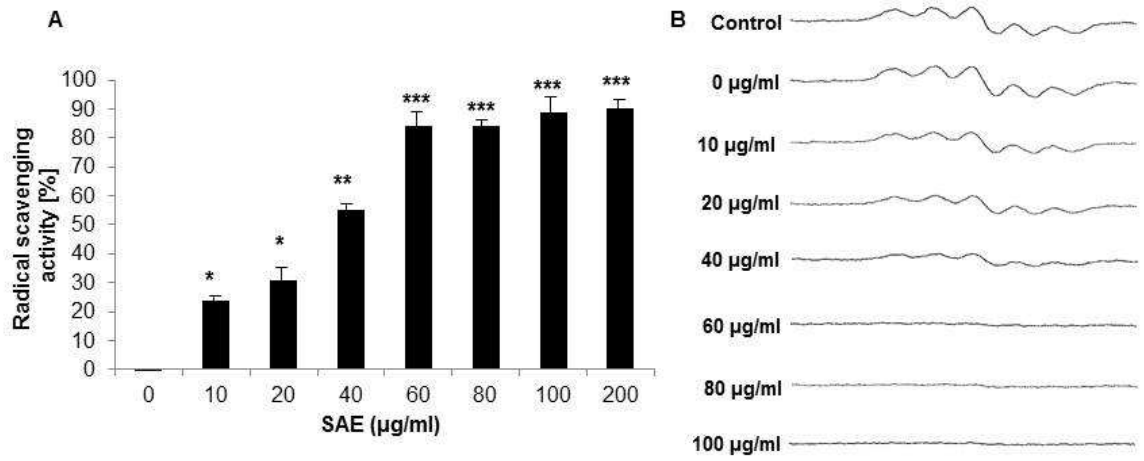

Fig 1: Effect of SAE on DPPH radical scavenging activity. A: The capacities to scavenge DPPH radicals by different concentrations of SAE extract. B: ESR spectral data. BV-2 cells were treated with or without SAE at the various concentrations (10, 20, 40, 60, 80 and 100 µg/ml). The scavenging activity of each sample on DPPH radical was measured using a JES-FA ESR spectrometer. A spin adduct was measured on an ESR spectrometer exactly 2 min later. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001, compared with control group by Student t-test. SAE: *Suaeda asparagoides* ethylacetate extract

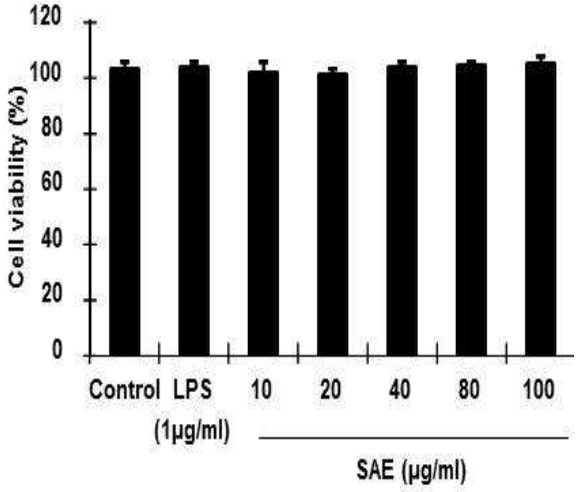

Fig 2: Effect of SAE extract on the BV-2 microglial cell viability. Viability in SAE extract-treated cells was determined using MTT assay in the presence or absence of LPS (1 µg/ml). The results are depicted as percentage of control samples. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. SEA: *Suaeda asparagoides* ethylacetate extract; LPS: Lipopolysaccharide

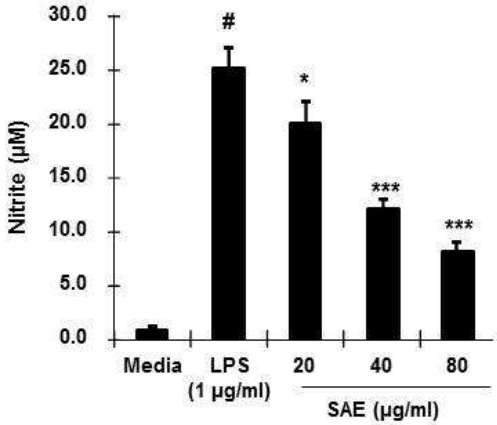

Fig 3: Effect of SAE extract on NO Production in LPS-stimulated BV-2 microglial cells**.** BV-2 cells were treated with SAE extract at indicated concentrations (20, 40 and 80 µg/ml) with or without LPS (1 µg/ml) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data 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 < 0.001, when compared with LPS alone treated group by Student t-test. SEA: *Suaeda asparagoides* ethylacetate extract; LPS: Lipopolysaccharide

SAE extract inhibited LPS-stimulated NO production in BV-2 microglial cells

As shown in Fig. 3, LPS (1 µg/ml) treated BV-2 cells significantly increased the production of NO when compared to the control group (p < 0.001).

SAE extract attenuates iNOS and COX-2 protein expression in LPS-stimulated BV-2 cells

As shown in Fig. 4, LPS-stimulation to BV-2 cells increased the protein expressions of iNOS and

COX-2. However, pre-treatment with SAE extract (20, 40 and 80 µg/ml) suppressed the increased iNOS and COX-2 protein expression in a concentration dependent manner (Fig. 4).

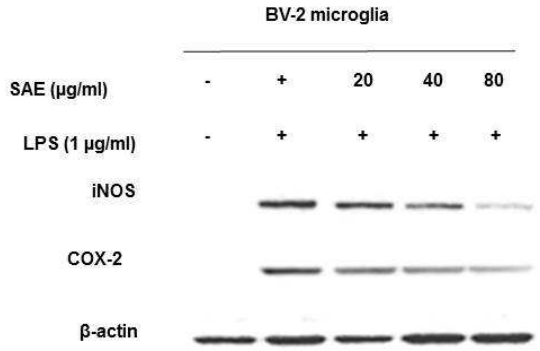

Fig 4: Effect of SAE extract on iNOS and COX-2 expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS and COX-2 production in the LPS (1 µg/ml)-stimulated BV-2 cells by various concentration of the SAE extract (20, 40 and 80 µg/ml) was estimated by immunoblot analyses with the specific antibodies against iNOS and COX-2. The internal control used was β-actin. SAE: Suaeda asparagoides ethylacetate extract, LPS: Lipopolysaccharide, iNOS: Inducible nitric oxide, COX-2: Cyclooxygenase-2

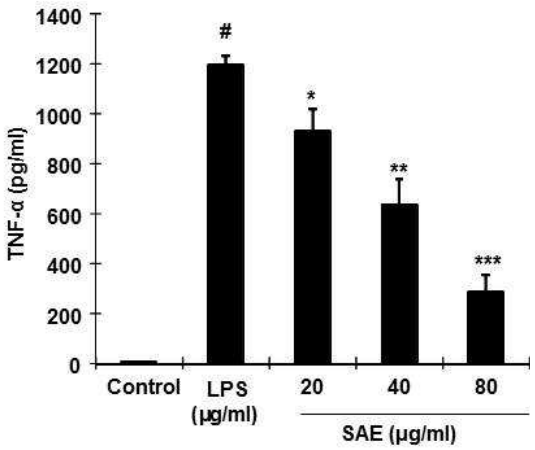

Fig 5: Effect of SAE extract on TNF-α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF-α level by SAE extract was measured with ELISA test. BV-2 cells were treated with SAE extract at indicated concentrations (20, 40 and 80 µg/ml with or without LPS (1 µg/ml) for 4 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. $^{*}p < 0.05$ at 20 µg/ml, $^{**}p < 0.01$ at 40 µg/ml and $^{***}p < 0.001$ at 80 µg/ml, when compared with LPS alone group by Student t-test. SAE: Suaeda asparagoides ethylacetate, TNF-α: Tumor necrosis factor-alpha, LPS: Lipopolysaccharide

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

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

SAE extract inhibited the NF-κB activation in LPS-stimulated BV-2 cells

Stimulation of cells with LPS (1 µg/ml) significantly induced NF-κB activation in BV-2 microglia. However, treatment with SAE inhibited the LPS-induced activation of NF-κB in a concentration-dependent manner (Fig. 6).

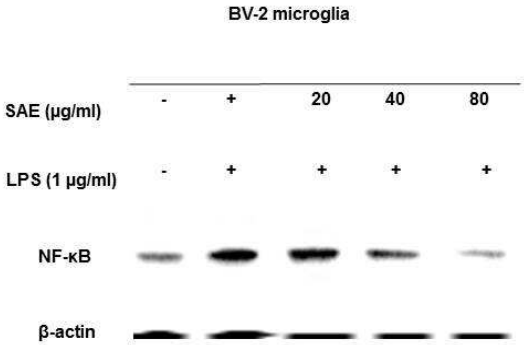

Fig 6: Effect of SAE on NF-κB expression in LPS-stimulated BV2 microglia. BV-2 microglia cells were stimulated with 1 µg/ml of LPS in the absence or presence of the SAE (20, 40 and 80 µg/ml) that had been added 1 h before the stimulation. LPS-induced NF-κB activation was assayed by Western blotting. SAE: Suaeda asparagoides ethylacetate extract, LPS: Lipopolysaccharide, NF-κB: Nuclear factor-kappa B

DISCUSSION

Brain inflammation is characterized by chronic activation of microglia which may in turn cause neuronal damage through the release of potentially cytotoxic molecules such as pro-inflammatory cytokines and reactive oxygen species [12,13]. Therefore agents that inhibit microglial activation by suppressing the excessive release of inflammatory mediators and cytokines might be beneficial for preventing and delaying the progression of neuroinflammatory disease. It is well documented that LPS-stimulated microglia produces elevated levels of NO and other pro-inflammatory mediators such as iNOS, COX-2 and TNF-α which were known to contribute to neuroinflammatory conditions

[14,15]. Our current data clearly showed that SAE attenuated LPS-induced NO production and suppressed iNOS expression.

It was well documented that increased expression of the COX-2 enzyme plays an important role in the inflammatory neurodegenerative process and LPS strongly activates microglia thereby inducing increased COX-2 expression [14]. Our present study showed that the expression of COX-2 protein was gradually suppressed by SAE extract treatment in LPS-stimulated BV- cells in a concentration dependent manner. Since SAE inhibited the increased NO production, iNOS and COX-2 expressions, the possibility remains that SAE extract might further act upon key inflammatory signaling mediators. Pro-inflammatory cytokines such as TNF-α, and interleukins cause potent activation of iNOS gene expression in microglial cells and increased levels of TNF-α has been associated with neuroinflammation [15]. In our present study, LPS-stimulation increased the levels of TNF-α and pre-treatment with SAE extract significantly inhibited this increase in BV-2 microglial cells indicating that SAE extract may convincingly be an effective anti-neuroinflammatory agent.

Earlier studies revealed that NF-κB activation plays an important role in regulating the expression of iNOS, COX-2 and is the key event for the induction of all major inflammatory mediators [16]. Therefore, we determined whether the inhibitory effects of the SAE extract occurred through suppression of NF-κB activation in BV-2 microglia cells. Our result showed that SAE inhibited the LPS-induced activation of NF-κB in a concentration-dependent fashion concluding that SAE might regulate NF-κB signaling pathway as a major target.

It was well reported that activated microglia release toxic free radicals that may participate in the neuroinflammatory processes [17]. DPPH radical scavenging assay is a widely used method for evaluating the antioxidant effect of agents in a relatively short period of time [18]. In the present study, SAE extract exhibited significant DPPH radical scavenging effect, indicating that SAE might contain potential antioxidant agents. Earlier reports indicated that *Suaeda* species possess several antioxidant constituents such as triterpenoids and anthocyanins [19,20].

Considering the overall data, our study provided evidence for the first time that SAE extract exhibited anti-neuroinflammatory properties through the inhibition of pro-inflammatory responses via regulating NF-κB signaling in LPS-stimulated BV-2 microglia. The antioxidant activity shown by SAE extract might also be involved for exhibiting such actions. In conclusion, SAE might be developed as a potential therapeutic target for treating neuroinflammatory-mediated neurodegenerative diseases.

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