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Original Research Article

Suppression of Lipopolysaccharide-Stimulated Neuroinflammatory Mediators by Chenopodiacea Mangrove, *Suaedea maritima* (L) Dumort, in BV-2 Microglial Cells

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Abstract

Purpose: To investigate the in-vitro antioxidant and anti-neuroinflammatory effects of Suaeda maritime (L) Dumort ethyl acetate (SM-EA) extract in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells.
Methods: LPS-stimulated BV- microglia were used to study the expression and production of inflammatory mediators, viz, nitric oxide (NO), inducible NO synthase (iNOS, interleukin (IL)-6) and tumor necrosis alpha (TNF-α). Antioxidant activity was measured using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Cell viability was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay.
Results: SM-EA extract significantly suppressed LPS-induced production of NO ($p < 0.001$ at 80 and 100 µg/ml) and expression of iNOS in BV-2 cells. SM-EA also suppressed LPS-induced increase in IL-6 and TNF-α levels ($p < 0.001$ at 100 µg/ml) in BV- cells. Further, DPPH-generated free radicals were inhibited by SM-EA extract in a concentration-dependent manner ($p < 0.01$ at 0.1 mg/ml and $p < 0.001$ at 1 mg/ml) with half maximal inhibitory concentration (IC50) of 0.42 mg/ml.
Conclusion: The findings imply that SM-EA extract can be developed as a potential therapeutic agent in regulating microglia-mediated neuroinflammatory responses observed in several neurodegenerative diseases.

Keywords: Suaedea maritime, Chenopodiaceae, Anti-oxidant, Anti-inflammatory, Microglial cells, Inducible nitric oxide synthase, Interleukin-6

INTRODUCTION

Suaeda maritima (L) *Dumort*, from the family Chenopodiaceae, is an annual salt-tolerant plant species seen abundantly on muddy seashores in Korea, Europe, Japan and Iran. The leaves are used as vegetable to reduce saltiness and also pickled in vinegar for consumption [1 Tanaka]. Traditionally, *S. maritima* has been used as herbal remedy for treating hepatitis and reported to possess anti-viral activities [2]. Pharmacologically, *S. maritima* was reported to possess antimicrobial, antioxidant and hepatoprotective activities due to the presence of triterpinoids and sterols [2,3]. However, studies on its beneficial effects on microglia- mediated neuroinflammatory diseases have not been reported.

Neuroinflammation mediated by microglial activation appears to play an essential role in the pathogenesis of neurodegenerative diseases [4]. It was well documented that activated microglia releases proinflammatory mediators and free radicals which may participate progressive neurodegeneration. Therefore early attenuation of activated microglia and the neuroinflammatory processes can attenuate the severity of neurodegeneration [5].

Mounting evidence indicated that microbial endotoxins like LPS can directly activate microglia triggering the production of proinflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS), interleukins (IL) and tumor necrosis factor (TNF)-α [6]. Therefore, LPS-induced stimulation to microglia might be used as an important tool to evaluate the 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 [7,8]. In this study we evaluated the *in vitro* antioxidant potential and anti-neuroinflammatory properties of *S. maritima* ethyl accrete (SM-EA) extract in LPS-stimulated BV-2 microglial cells.

EXPERIMENTAL

Preparation of *S. maritima* extract

S. maritima whole plant was collected from the western coast region in South Korea. The plant was authenticated by a taxonomist, Dr Kim Jong Bo at Konkuk University, South Korea and a voucher specimen (SM-KU2013) was stored in our department herbarium for future reference. To obtain the *S. maritima* extract, the dried plant material was ground in a mixer and defatted with three volumes of 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 (500 mg) was partitioned into hexane, chloroform and ethyl acetate (EA) fractions to furnish 110.5 mg, 16.6 mg and 174.6 mg, respectively. The active EA fraction of *S. maritima* designated as SM-EA was re-dissolved in distilled water to evaluate for its anti-neuroinflammatory and anti-oxidant activities.

DPPH radical scavenging activity

The anti-oxidant activity of the SM-EA EXTRACT 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 SM-EA extract and a DPPH methanolic solution as described previously [9]. Briefly, a sample solution of 60 µl of each SM-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 x 10^5, and temperature, 298 °K.

Cell cultures and viabilities

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). In all experiments, cells were pre-treated with the indicated concentrations of SM-EA extract 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. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [10]. Cells were incubated with various concentrations of SM-EA extract 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 stable nitrite, the end product of NO generation, by activated microglia was determined by a colorimetric assay as previously described [10]. Briefly, 50 µl of culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance at 540 nm was read using a PowerWavex Microplate Scanning spectrophotometer (Bio-Tek Instrument, Winooski, VT, USA). Nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Western blot analysis

Cells 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 x 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 followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Herculus, CA, USA) with β-actin as 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 and β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

IL-6 assay

BV-2 microglia cells (1 x 10^5 cells/well) were cultured on 96 well plates and treated with SM-EA extract at indicated dosages with or without LPS (1 μg/ml). At 4 h of post LPS treatment, the cells were collected and the supernatants were subjected to estimate IL-6 contents using a murine IL-6 ELISA kit from BD Biosciences (San Jose, CA, USA) according to the manufacturer's instruction.

TNF-α assay

BV-2 microglia cells (1 x 10^5 cells/well) were cultured on 96 well plates and treated with the SM-EA extract at indicated concentrations 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 one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of SM-EA extract on DPPH radical scavenging activity

DPPH is a stable free radical, and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which has been used to evaluate free radical scavenging activity of natural anti-oxidants. The capacity of SM-EA extract to scavenge DPPH was measured by ESR spectrometry. As shown in Fig 1A, SM-EA extract exhibited significant DPPH radical scavenging activity in a dose-dependent manner showing a maximum effect at 1mg/ml of concentration ($p < 0.001$). The concentration needed for 50 % inhibition of DPPH radicals was found to be approximately at 0.42 mg/ml. The ESR spectroscopy data at 0.01, 0.1 and 1 mg/ml was represented in Fig 1B.
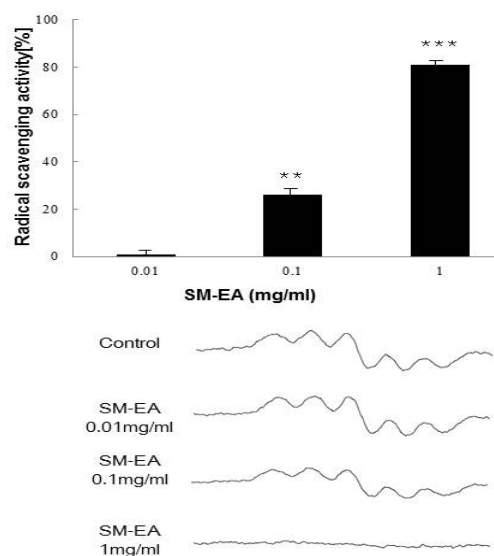

Fig 1: Effect of SM-EA extract on DPPH radical scavenging activity. The capacity to scavenge DPPH free radical by different concentrations of SM-EA extract (A) and ESR spectra (B) was measured. BV-2 cells were treated with or without SM-EA extract at the various concentrations (0.01, 0.1 and 1 mg/ml). 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. **$p < 0.01$ and ***$p < 0.001$, when compared with control group by one-way analysis of variance, followed by Dunnett's multiple range tests. SM-EA extract = *Suaeda maritima* ethyl acetate extract

Effect of SM-EA extract on LPS-induced NO production

BV-2 microglia were treated with various concentrations of SM-EA EXTRACT (10, 20, 40, 80 and 100 µg/ml) for 30 min prior to LPS (1 µg/ml) stimulation for 4 h. As shown in Fig. 2, NO production by LPS-activated cells was found to be significantly inhibited by SM-EA extract in a concentration-dependent manner (Fig. 2). The maximum effect was shown at 80 and 100 µg/ml ($p < 0.001$). Further, SM-EA extract treatment did not exhibit any significant cytotoxicity in BV-2 microglial cells treated for 24 h at concentrations up to 100 µg/ml, and in all cases the viability was found above 93 % by MTT assay (Fig 3).

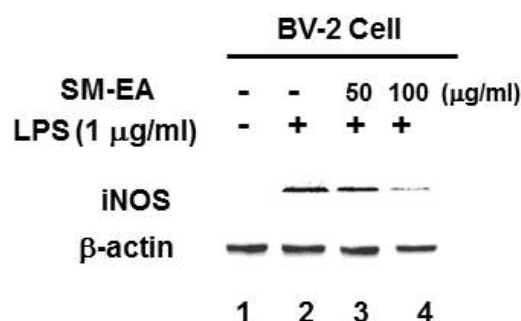## Effect of SM-EA extract on LPS-induced expressional levels of iNOS

To know the effect of SM-EA extract on iNOS levels, BV-2 cells were stimulated with LPS (1 µg/ml) in the presence or absence of SM-EA extract (50 and 100 µg/ml). LPS strongly induced BV-2 cells showing an increased expression of iNOS. However, treatment with SM-EA extract exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in BV-2 cells (Fig 4).
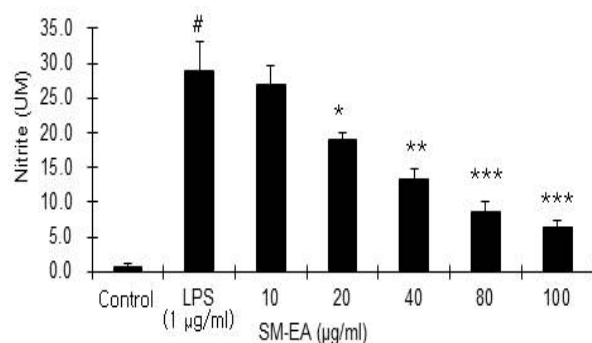
Fig 2: Effect of SM-EA extract on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with SM-EA extract at various concentrations (10, 20, 40, 80, and 100 µg/ml) with or without LPS (1 µg/ml) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean ± S.E.M. (n=3) for three independent experiments. $^{\#}p < 0.001$, when compared with control group. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, when compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. SM-EA extract = *Suaeda maritima* ethyl acetate extract; LPS: lipopolysaccharide
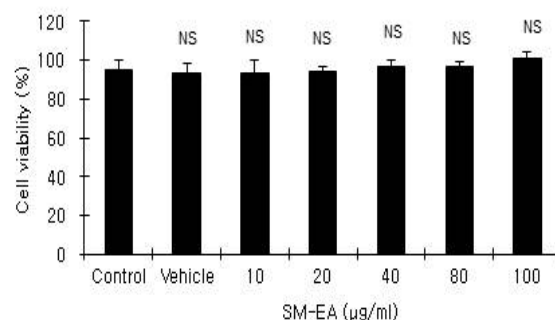
Fig 3: Effects of SM-EA extract on the viability of BV-2 microglial cells. Viability in SM-EA extract treated cells was determined using MTT assay. The results are displayed in percentage of control samples. Data are presented as the mean ± S.E.M. (n=3) for three independent experiments. ^{NS} Not significant. SM-EA extract = *Suaeda maritima* ethyl acetate extract

Fig 4: Effect of SM-EA extract 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 (50 and 100 µg/ml) of the SM-EA extract was monitored by immunoblot analyses with the specific antibodies against iNOS. The internal control used was β-actin. SM-EA extract = *Suaeda maritima* ethyl acetate extract; LPS= Lipopolysaccharide; iNOS: Inducible nitric oxide synthase

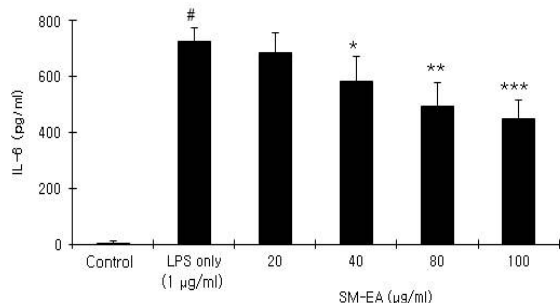## Effect of SM-EA extract on IL-6 production in LPS-stimulated BV-2 cells

BV-2 microglia was pretreated with SM-EA extract for 30 min (10, 20, 40 and 80 µg/ml) with or without LPS (1 µg/ml). As shown in Fig. 6, LPS stimulation increased the IL-6 expression in BV- cells. However, SM-EA extract pretreatment decreased the LPS-induced IL-6 levels in BV-2 microglia (Fig. 5).

Effect of SM-EA extract on TNF-α production in LPS-stimulated BV-2 cells

As shown in Fig. 6, TNF-α levels increased significantly after LPS treatment (1 µg/ml) when compared to those in untreated cells ($p < 0.001$).

However, SM-EA extract significantly inhibited TNF-α production in a concentration-dependent manner in LPS-stimulated BV-2 cells ($p < 0.05$ at 40 µg/ml and $p < 0.001$ at 100 µg/ml, respectively).

Fig 5: Effect of SM-EA extract on pro-inflammatory cytokine IL-6 expression in LPS-stimulated BV-2 cells. BV-2 cells were treated with SM-EA extract at indicated concentrations (20, 40, 80 and 100 µg/ml) with or without LPS (1 µg/ml) for 4 h. The IL-6 in the culture supernatant was evaluated using a murine IL-6 ELISA kit from BD Sciences according to the manufacturer's instruction. Data are presented as the mean ± S.E.M. (n=3) for three independent experiments. [#]$p < 0.001$, when compared with control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. SM-EA extract = *Suaeda maritima* ethyl acetate extract; LPS= lipopolysaccharide; IL-6= Interleukin-6

DISCUSSION

In the present study we report that SM-EA extract significantly inhibited production of NO, suppressed the expression of iNOS protein level and attenuated the increased levels of IL-6 and TNF- α production in LPS-simulated BV-2 microglial cells. Further the SM-EA extract 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 neuroinflammatory and neuro-degenerative diseases [11]. Thus neutralization by antioxidants and radical scavengers can reduce neuroinflammation. It was 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 [12]. Earlier studies revealed that *S. maritima* possessed strong antioxidant polyphenolic and sterol compounds [2,3]. In our present study, the ethyl acetate fraction obtained from the SM-EA extract also exhibited significant free radical scavenging effect indicating that the SM-EA might contain potential antioxidant agents. Since antioxidants might reduce neuroinflammation [11], we used SM-EA extract to evaluate for its anti-neuroinflammatory activity in LPS-stimulated BV-2 microglial cells.
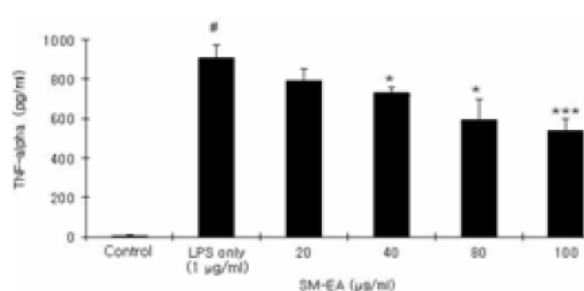
Fig 6: Effect of SM-EA extract on TNF-α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF-α expression by SM-EA extract was measured with ELISA test. BV-2 cells were treated with SM-EA extract at 20, 40, 80 and 100 µg/ml with or without LPS (1 µg/ml) for 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. *$p < 0.05$, and ***$p < 0.001$, when compared with LPS alone group by Student t-test. (SM-EA- *Suaeda maritima* ethyl acetate extract; TNF-α: Tumor necrosis factor-alpha; LPS: Lipopolysaccharide)

A significant number of reports have established that inflammatory mediators, including NO and iNOS are responsible for the symptoms of many neuroinflammatory diseases It is well documented that microglia produces NO in response to pro-inflammatory stimuli LPS. The induced NO by the induction of iNOS will lead to increased inflammatory reaction. Therefore, agents that decrease NO production and iNOS have appreciable therapeutic effect in the treatment of several neuroinflammatory diseases [13,14]. Our results clearly showed that SM-EA extract attenuated LPS-induced iNOS expression and downstream NO production.

Proinflammatory cytokines such as TNF-α and IL-6 cause potent activation of iNOS gene expression in rodent glial cells and muscle cells [15]. Microglial cell activation by LPS produces various cytokines including IL-6 and TNF-α leading to the attraction of neutrophils and the accumulation of neutrophil-secreted proteases and ROS at the site of inflammation. Data from our study showed that SM-EA extract may act by suppressing the increased production of IL-6 and TNF-α thereby inhibiting NO production and iNOS expression levels in LPS-stimulated BV-2 cells indicating that SM-EA extract may convincingly be an effective antineuroinflammatory agent.

CONCLUSION

SM-EA plays an important role in attenuating neuroinflammatory responses in LPS-stimulated BV-2 microglial cells. The anti-neuroinflammatory

effects of SM-EA extract may be attributed to its regulatory actions on proinflammatory cytokine such as TNF-α and IL-6. The strong antioxidant effects exhibited by SM-EA extract may also be involved in delivering such actions. Thus, SM-EA extract may be developed as a promising candidate for the treatment of neuroinflammation-mediated neurodegenerative disorders.

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