

Original Research Article

Inhibition of Lipopolysaccharide-Stimulated Neuro-Inflammatory Kuntze in BV-2 Microglial Cell Mediators by *Tetragonia tetragonoides* (Pall)

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Abstract

Purpose: To investigate the *in vitro* antioxidant and anti-neuroinflammatory effects of *Tetragonia tetragonoides* (Pall.) Kuntze extract (TKE) in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells.

Methods: To evaluate the effects of TKE, LPS-stimulated BV microglia were used and the expression and production of inflammatory mediators, namely, nitric oxide (NO), inducible NO synthase (iNOS) and tumor necrosis alpha (TNF- α) were evaluated. Antioxidant activity of TKE was measured using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Cell viabilities were estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay.

Results: TKE significantly suppressed LPS-induced production of NO ($p < 0.001$ at 20, 40, 80 and 100 $\mu\text{g/ml}$) and expression of iNOS in BV-2 cells. TKE also suppressed LPS-induced increase in TNF- α level ($p < 0.001$ at 100 $\mu\text{g/ml}$) in BV-2 cells. In addition, DPPH-generated free radicals were inhibited by TKE in a concentration-dependent manner.

Conclusion: The results suggest that TKE can be explored as a potential therapeutic agent for regulating microglia-mediated neuroinflammatory responses observed in several neurodegenerative diseases.

Keywords: *Tetragonia tetragonoides*, Anti-oxidant, Anti-inflammatory, Neurodegenerative diseases, Microglial cells, Lipopolysaccharide

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INTRODUCTION

Tetragonia tetragonoides (Pall.) Kuntze (*T. tetragonoides*) is a medicinal herb widely grown along the seashores of the Pacific region, South America, Japan, Southeast China (including Norfolk and Lord Howe Islands), New Zealand, Australia, Tasmania, the Kermadec Islands, New Caledonia, Hawaii, and other Pacific Islands [1]. Pharmacologically, *T. tetragonoides* was reported to be used for migraine, gastric ulcers, stomach cancer, gastritis, septicemia and asthma[2,3]. However, studies on its beneficial

effects on microglia-mediated neuroinflammatory diseases have not been reported.

Neuro-inflammation mediated by microglial activation appears to play an essential role in the pathogenesis of neurodegenerative diseases [4]. It is 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 indicate that microbial endotoxins like LPS can directly activate microglia triggering the production of pro-inflammatory mediators, such as nitric oxide (NO), inducible NO synthase (iNOS), interleukins (IL) and tumor necrosis factor (TNF)- α [6-8]. Therefore, LPS-induced stimulation to microglia might be used as an important tool for evaluating 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 against 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 *T. tetragonoides* extract (TKE) in LPS-stimulated BV-2 microglial cells.

EXPERIMENTAL

Materials

The antibodies against iNOS and β -actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

Preparation of TKE

T. tetragonoides 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 the institution's Biomedical and Health Sciences Department herbarium for future reference. To obtain the *S. maritima* extract, the dried plant material was ground in a mixer and defatted three times with three volumes of ethanol. The concentration of ethanol 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, the alcoholic extract (500 mg) was partitioned into hexane, chloroform and ethyl acetate (EA) to furnish 110.5, 16.6 and 174.6 mg, respectively. The EA fraction of TKE, 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 TKE was determined using the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA). Freshly prepared DPPH solution was

taken in test tubes and the indicated concentrations of TKE (0.01, 0.1 and 1 mg/mL) were added to the respective test tubes and made up to the final volume (5 ml). Thirty minutes later, the absorbance was read at 517 nm using JES-FA ESR UV spectrometer (Jeol Ltd, Tokyo, Japan). Control was a sample containing the same volume but without TKE. Methanol was served as blank. The activity (C) of the sample was calculated as in Eq 1.

$$C = \{(A1-A2)/A1\}100 \dots\dots\dots (1)$$

where A1 is the absorbance of the control, and A2 is the absorbance of the extract [9]. IC₅₀ values were extrapolated from inhibition plots.

Cell cultures and evaluation of cell viability

BV-2 microglia cells were cultured at 37 °C in 5 % CO₂ 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 1 μ g/mL LPS (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 TKE 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 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]. Culture supernatant (50 μ L) was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance at 540 nm was read in a Power Wavex 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 three times in cold PBS and lysed in 50 mM Tris-HCl buffer, pH 7.4, containing 1 % (v/v) NP-40, 0.25 % sodium

deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Na₃VO₄ and protease inhibitor cocktail (Complete Mini™, 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. Protein concentration was determined using BioRad DC protein assay kit. Equal amounts of protein were mixed 4:1 (v:v) with an SDS sample buffer and then boiled for 5 min. On pre-cast 10 % SDS-PAGE gels, 10 µL was run. The protein was transferred to 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 of iNOS and β-actin followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Hercules, 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.

Assay of TNF-α

BV-2 microglia cells (1 × 10⁵ cells/well) were cultured on 96 well plates and treated with the TKE 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 presented as 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 TKE on DPPH radical scavenging activity

As shown in Fig. 1A, TKE exhibited significant DPPH radical scavenging activity in a dose-dependent manner with a maximum effect at 1mg/ml (*p* < 0.001). The concentration needed for 50 % inhibition of DPPH radicals was found to be approximately at IC₅₀ was 0.42 mg/ml. The ESR spectroscopy data at 0.01, 0.1 and 1 mg/ml was represented in Fig 1B.

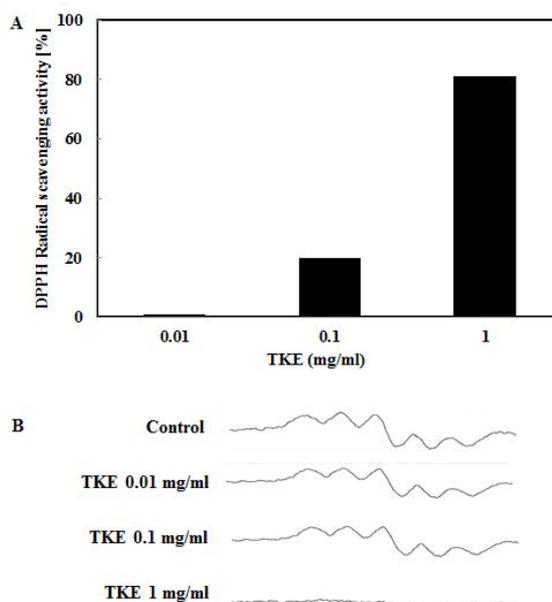


Fig 1: Effect of TKE on DPPH radical scavenging activity

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

NO production by LPS-activated cells was found to be significantly inhibited by TKE extract in a concentration-dependent manner (Fig 2). The maximum effect was observed at 80 and 100 µg/ml (*p* < 0.001). TKE extract treatment did not exhibit any significant cytotoxicity in BV-2 microglial cells 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 TKE extract on LPS-induced expressional levels of iNOS

LPS (1 µg/ml) strongly induced BV-2 cells showing an increased expression of iNOS. However, treatment with TKE at 50 and 100 µg/ml concentrations exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in BV-2 cells (Fig. 4). These results indicated that TKE may be potential inhibitor for iNOS.

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

As shown in Fig. 5, TNF-α levels increased significantly after LPS treatment (1 µg/ml) when compared to those in untreated cells (*p* < 0.001). However, TKE 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).

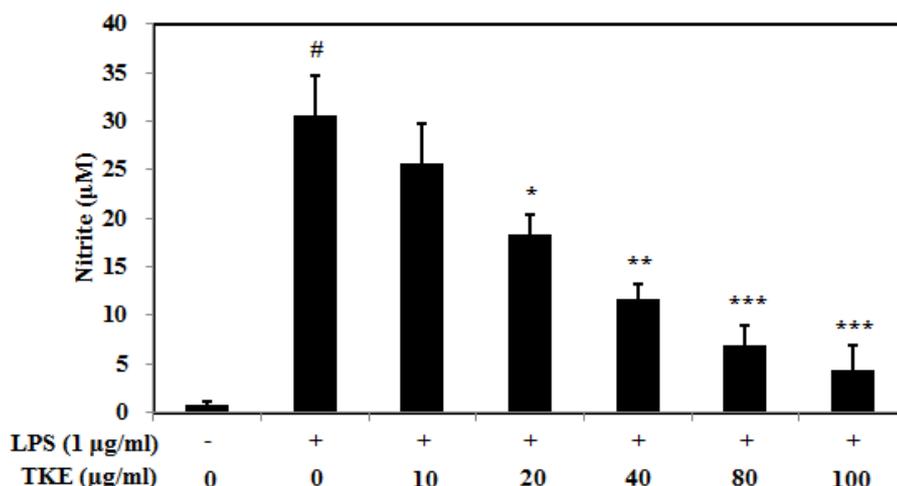


Fig 2: Effect of TKE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with SM-EA extract at various concentrations (10, 20, 40, 80, and 100 µg/ml) with or without LPS (1 µg/ml) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are 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, by one-way analysis of variance, followed by Dunnett's multiple range tests

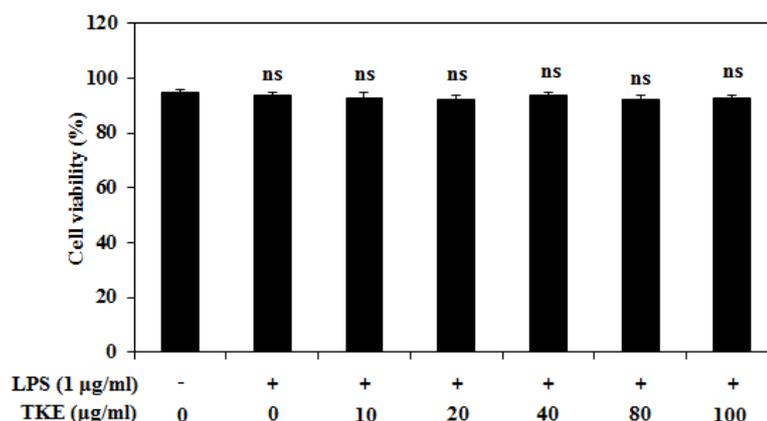


Fig 3: Effects of TKE on the viability of BV-2 microglial cells. Viability in TKE treated cells was determined using MTT assay. The results are displayed as percentage of control samples. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. NS Not significant

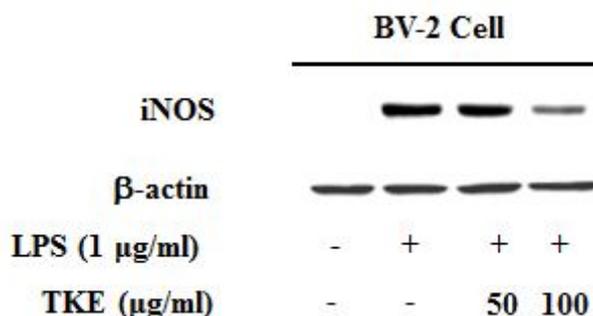


Fig 4: Effect of TKE on iNOS protein expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS production in the LPS-stimulated BV-2 cells by indicated concentrations (50 and 100 µg/ml) of the TKE was monitored by immunoblot analyses with the specific antibodies against iNOS. The internal control used was β-actin. TKE = *Tetragonia tetragonoides* (Pall.) Kuntze ethanol extract

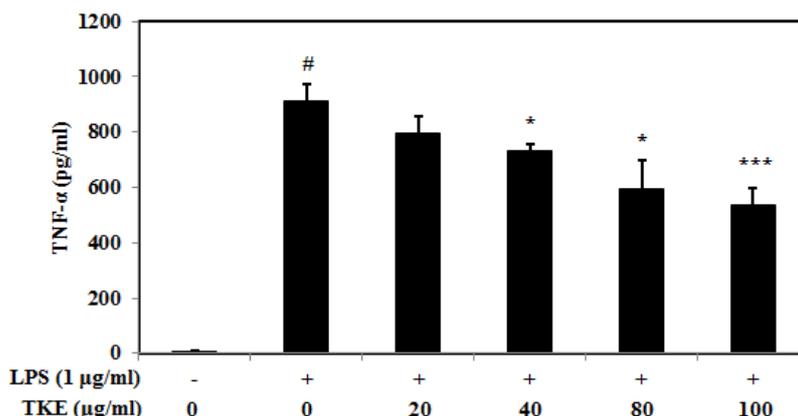


Fig 5: Effect of TKE on TNF- α production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF- α expression by TKE was measured with ELISA test. BV-2 cells were treated with TKE at 20, 40, 80 and 100 $\mu\text{g/ml}$ with or without LPS (1 $\mu\text{g/ml}$) for 4 h. The TNF- α in the culture supernatant was evaluated using a murine TNF- α ELISA kit. Data are presented as the mean \pm 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

DISCUSSION

In the present study, we report that TKE significantly inhibited production of NO, suppressed the expression of iNOS protein level and attenuated the increased levels of TNF- α production in LPS-stimulated BV-2 microglial cells. In addition, TKE 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 neuro-degenerative diseases [11]. Thus neutralization by antioxidants and radical scavengers can reduce neuroinflammation. It is well known that DPPH radical assay is one of the widely used methods for evaluating the free radical scavenging activities of several antioxidants [12]. Earlier studies revealed that *Tetragonia tetragonoides* (Pall) Kuntze possess strong antioxidant polyphenolic, vitamin C, and sterol compounds [2,3]. In our present study, the TKE also exhibited significant free radical scavenging effect indicating that the TKE might contain potential antioxidant agents. Since antioxidants reduce neuroinflammation [11], TKE extract was used to evaluate its anti-neuroinflammatory activity in LPS-stimulated BV-2 microglial cells.

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 and increase the iNOS expression in response to pro-inflammatory stimuli LPS leading to increased

inflammatory reaction [13]. Therefore, agents that decrease NO production and iNOS have appreciable therapeutic effect in the treatment of several neuroinflammatory diseases [13,14]. Our results clearly show that TKE attenuated LPS-induced iNOS expression and downstream NO production.

Pro-inflammatory cytokines such as TNF- α cause potent activation of iNOS gene expression in rodent glial cells and muscle cells [15]. 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. Data from our study show that TKE may act by suppressing the increased production of TNF- α thereby inhibiting NO production and iNOS expression levels in LPS-stimulated BV-2 cells indicating that TKE may be an effective anti-neuroinflammatory agent.

CONCLUSION

TKE plays an important role in attenuating neuroinflammatory responses in LPS-stimulated BV-2 microglial cells. The anti-neuroinflammatory effect of TKE may be due to its regulatory actions on proinflammatory cytokines such as TNF- α . The strong antioxidant effects exhibited by TKE may also be involved in exerting the effect. Based on these findings, TKE is a promising candidate for the treatment of neuro-inflammation-mediated neurodegenerative disorders.

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