

## Original Research Article

# Protective Effect of Purple Sweet Potato (*Ipomoea batatas* Linn, Convolvulaceae) on Neuroinflammatory Responses in Lipopolysaccharide-Stimulated Microglial Cells

Hyun Kang<sup>1\*</sup>, Yeon-Gil Kwak<sup>2</sup> and Sushruta Koppula<sup>3</sup>

<sup>1</sup>Department of Medical Laboratory Science, College of Health Science, Dankook University, Cheonan-si, Chungnam, 330-714,

<sup>2</sup>Research Institute of Natural Food & Pharmaceutical Corp., O-Chang Science Industry Plaza 319-11 Songdaeri, O-changmyon, chongwongun Chungbuk, 363-883, <sup>3</sup>Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju-si, Chungbuk, 380-701, Republic of Korea

\*For correspondence: **Email:** [hkang@dankook.ac.kr](mailto:hkang@dankook.ac.kr); **Tel:** 82-41-550-1452; **Fax:** 82-41-559-7934

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### Abstract

**Purpose:** To evaluate the protective effects of purple sweet potato (*Ipomoea batatas* Linn, Convolvulaceae) extract (IBE) in stimulated BV-2 microglial cells and its anti-oxidant properties.

**Methods:** Cell viability assessment was performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. Lipopolysaccharide (LPS) was used to activate BV-2 microglia. Nitric oxide (NO) levels were measured using Griess assay. Inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 expressional levels were measured by Western blot analysis. Tumor necrosis factor-alpha (TNF- $\alpha$ ) production was evaluated by enzyme-linked immunosorbent assay (ELISA). Anti-oxidant properties were evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay.

**Results:** LPS-activated excessive release of NO in BV-2 cells was significantly inhibited by IBE ( $p < 0.001$  at 100  $\mu\text{g/ml}$ ). Increased production of inflammatory mediators such as iNOS, COX-2 and TNF- $\alpha$  ( $p < 0.01$  and  $p < 0.001$  at 100 and 200  $\mu\text{g/ml}$ , respectively) was attenuated by IBE concentration-dependently. IBE also scavenged DPPH radicals in a dose-dependent manner ( $p < 0.05$  at 10  $\mu\text{g/ml}$  and  $p < 0.001$  at 20 - 200  $\mu\text{g/ml}$ ).

**Conclusion:** These results indicate that IBE attenuated neuroinflammatory responses in LPS-activated BV-2 microglia by inhibiting excessive production of pro-inflammatory mediators such as NO, iNOS, COX-2 and TNF- $\alpha$ . The anti-neuroinflammatory potential of IBE may be related to its strong antioxidant properties.

**Keywords:** *Ipomoea batatas*, DPPH radicals, Anti-oxidant, Neuroinflammation, BV-2 microglia, Nitric oxide.

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## INTRODUCTION

Microglia cells are immune cells in the central nervous system (CNS) that able to produce several inflammatory mediators in response to stressors. Activated microglia play a critical role in the neuroinflammatory processes by releasing toxic mediators including nitric oxide (NO),

inducible NO synthase (iNOS), interleukins (IL), tumor necrosis factor-alpha (TNF- $\alpha$ ), and free radicals [1]. Activated microglia and the released inflammatory mediators may lead to several neurodegenerative diseases including multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease [2,3]. It is well known that microglia can

be activated by lipopolysaccharide (LPS) and is recognized to be a useful *in vitro* tool for studying neuroinflammatory mechanisms [4]. LPS-activated BV-2 microglia cells enhance the production of immune-related cytotoxic factors and pro-inflammatory cytokines [4,5]. Thus, the control of microglial activation has been suggested as a promising therapeutic target in combating neuroinflammatory-mediated neurodegenerative diseases.

Purple sweet potato scientifically known as *Ipomoea batatas* Linn. from the family Convolvulaceae is an herbaceous perennial vine that has white and purple flowers, large nutritious storage roots and heart-shaped, lobed leaves [6]. It is consumed as vegetables in tropical areas, especially Southeast Asia, used as a folk medicine in Brazil and commonly eaten as root crops in Japan, Korea and other Asian countries [6,7]. Nutritionists at the Center for Science in the Public Interest (CSPI) reported that *I. batatas* is the single most important dietary herb that would replace fatty foods [8]. Traditionally, the roots and leaves of *I. batatas* have been used in treating urinary infections, reducing fever, skin diseases, diabetes, curing boils and acne [9]. A review on pharmacological studies on *I. batatas* indicated that it possess anti-diabetic, hypoglycemic, neuroprotective, antiproliferative, antioxidant, antiulcer, antitumor, antiinflammatory, wound healing, antimutagenic and hepatoprotective properties [10].

However, till date the effect of *I. batatas* leaf extract on neuroinflammation and LPS-activated microglial neurotoxicity has not been documented. The aim of this study was to investigate whether *I. batatas* exhibits protective effects on LPS-activated neuroinflammatory processes in BV-2 microglial cells.

## EXPERIMENTAL

### Plant material and preparation of *I. batatas* leaf extract

The leaves of *I. batatas* were collected at the end of August to about the middle of September were obtained from a local market in Seoul, South Korea. The collected material was authenticated by Prof Jong-Bo Kim, a taxonomist at Konkuk University, Korea and a voucher specimen (IB-KU2013) has been kept in our laboratory Herbarium, Konkuk University, Korea for future reference. To obtain the *I. batatas* leaf extract, fresh leaves were washed to remove debris and air-dried for two days. The dried leaves were

then ground to powder using an electric blender (Model 4250, Braun Germany).

The powdered leaf (500 g) was extracted with three volumes of 80 % ethanol with mixing at room temperature for 1 h. The extract was filtered and lyophilized to obtain ethanol extract concentrate. The EtOH extract of *I. batatas* leaf extract obtained (175 g) was re-suspended in water:EtOH (9:1, v/v) and partitioned in turn via n-hexane, chloroform, ethyl acetate (EA) and n-butanol solvents to obtain a final yield of 4.8, 10, 47.5 and 37.36 %, respectively. Since EA fraction of *I. batatas* leaf (IBE) extract showed potent antioxidant effect in our preliminary evaluation, further studies on anti-neuroinflammatory effects in LPS-stimulated BV-2 microglial cells was investigated using IBE extract. The IBE extract was dissolved in sterile distilled water, filtered on 0.22  $\mu\text{m}$  filters and stored at  $-20^{\circ}\text{C}$ . All reagents used in this study were the highest grade commercially available.

### DPPH radical scavenging activity

The anti-oxidant activity of the IBE 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 IBE extract and a DPPH methanolic solution as described previously [11]. Briefly, a sample solution of 60  $\mu\text{l}$  of each IBE extract, was added to 60  $\mu\text{l}$  of DPPH (60  $\mu\text{M}$ ) in methanol. After mixing vigorously for 10 s, the mixture was then transferred into a 100  $\mu\text{l}$  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,  $6.3 \times 10^5$ , and temperature,  $298^{\circ}\text{K}$ .

### Cell culture and viability assay

BV-2 microglia cells were cultured at  $37^{\circ}\text{C}$  in 5 %  $\text{CO}_2$  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 IBE extract at indicated concentrations 10-100  $\mu\text{g/ml}$  for 1 h before the addition of LPS (5  $\mu\text{g/ml}$ , Sigma-Aldrich, St Louis, MO, USA) in serum free DMEM. An equal volume of sterile water was added to all control treatments.

For viability assay, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously [12]. Briefly, BV-2 cells were plated onto 96 well plates and exposed to various concentrations of IBE extract (10 - 200 µg/ml). MTT was added to each well and 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 %.

### Immunoblot analysis and antibodies

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<sub>3</sub>VO<sub>4</sub> 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. 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 and COX-2 followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Hercules, 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 and β-actin were purchased from Cell Signaling Technology INC. (Beverly, MA, USA).

### Nitric oxide assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [12]. Briefly, BV-2 cells (2 × 10<sup>5</sup> cells/ml) were seeded in 6-well plates in 500 µl complete culture medium and treated with the IBE extract at indicated concentrations for 1 h prior stimulation with LPS (5 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<sub>3</sub>PO<sub>4</sub>) 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).

### TNF-α assay

BV-2 microglia cells (1 × 10<sup>5</sup> cells/well) were cultured on 96 well plates and treated with the IBE extract at indicated concentrations for 1 h and stimulated with LPS (5 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 ± SEM of at least three independent experiments. Statistical analysis was performed with 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.

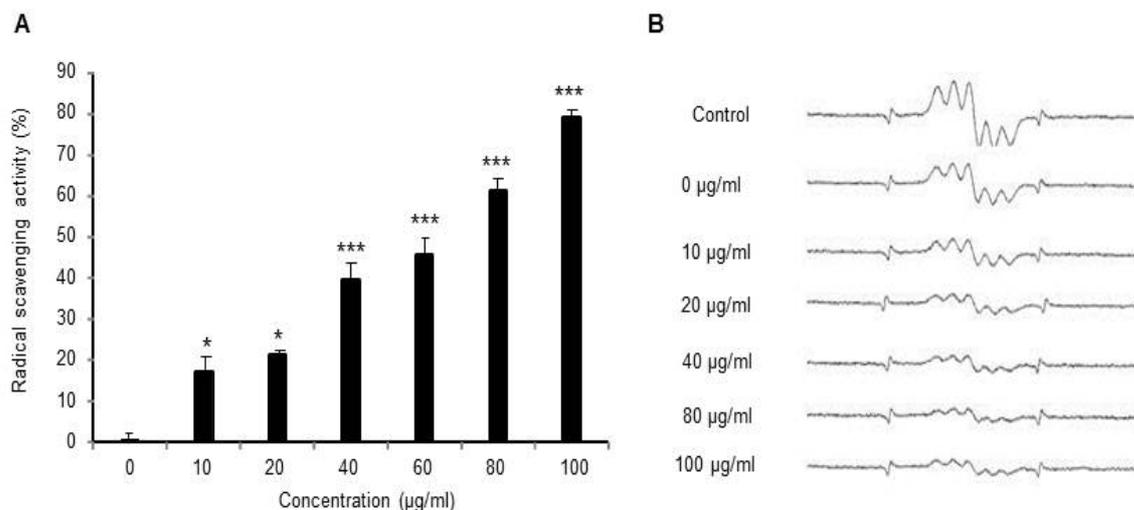
## RESULTS

### Effect of IBE extract on DPPH radical scavenging activity

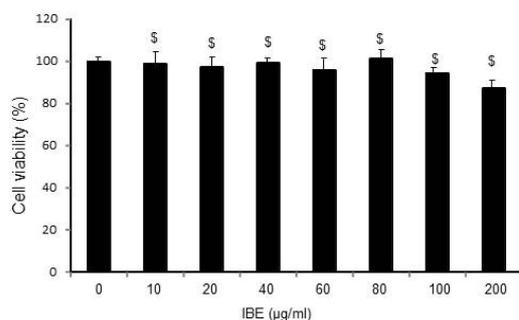
As shown in Fig. 1A, IBE exhibited significant DPPH radical scavenging activity in a concentration-dependent manner showing a maximum effect at 100 µg/ml (*p* < 0.001). The ESR spectroscopy data is represented in Fig 1B.

### Effect of IBE on BV-2 cell viability

Treatment with IBE extract at indicated concentrations (10 µg/ml - 100 µg/ml) did not affect the overall cell viability nor did they exhibit any cytotoxicity on BV2 microglia cells. Although not significant, IBE at 200 µg/ml concentration showed moderate signs of cytotoxicity. Therefore 100 µg/ml of IBE was utilized as maximum concentration for further experiments and activities.



**Fig 1:** Effect of IBE extract on DPPH radical scavenging activity. A = The capacities to scavenge DPPH radicals by different concentrations of IBE extract. B = ESR spectral data. BV-2 cells were treated with or without IBE extract 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  $\pm$  S.E.M. (n = 3) for three independent experiments; \* $p$  < 0.05 and \*\*\* $p$  < 0.001, compared with control group by Student t-test (IBE: *I. batatas*-ethylacetate)

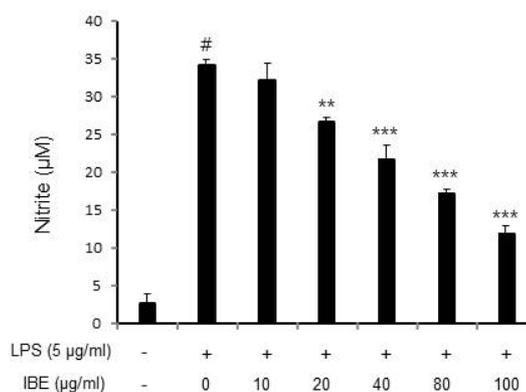


**Fig 2:** Effects of IBE extract on the viability of BV-2 microglial cells. Viability in IBE extract-treated cells was determined using MTT assay in the presence or absence of LPS (5 µg/ml). The results are depicted as percentage of control samples. Data are presented as the mean  $\pm$  S.E.M. (n = 3) for three independent experiments. <sup>§</sup>No significant when difference compared with control group (IBE: *I. batatas*-ethylacetate)

### IBE extract attenuates NO production in LPS-stimulated BV-2 cells

Cells treated with LPS (5 µg/ml) alone significantly increased in NO levels ( $p$  < 0.001). Pre-treatment with IBE extract at indicated concentrations significantly and dose-dependently suppressed the excessive release of NO in BV-2 cells in a (Fig. 3). Although IBE at 10 µg/ml concentration did not influence the NO

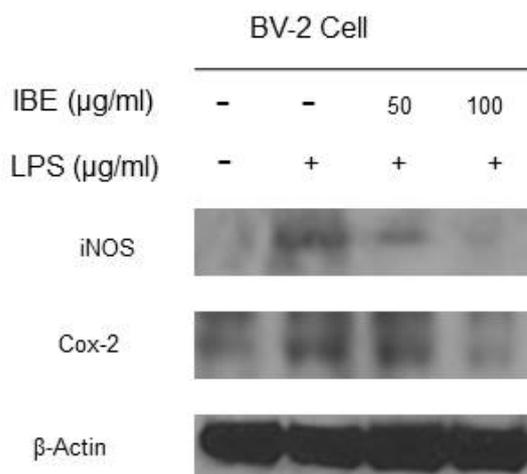
release, 20, 40, 60, 80 and 100 µg/ml concentrations showed significant effects. The maximum effect was observed at a concentration of 100 µg/ml ( $p$  < 0.001). (IBE: *I. batatas*-ethylacetate).



**Fig 3:** Effect of IBE extract on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with IBE extract at various concentrations (20, 40, 80 and 100 µg/ml) with or without LPS (5 µg/ml) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean  $\pm$  S.E.M. (n = 3) for three independent experiments. <sup>#</sup> $p$  < 0.001, when compared with control group. <sup>\*\*</sup> $p$  < 0.01 and <sup>\*\*\*</sup> $p$  < 0.001, when compared with LPS alone treated group by Student t-test (IBE: *I. batatas*-ethylacetate, LPS: Lipopolysaccharide)

### IBE extract attenuates iNOS and COX-2 expression in LPS-stimulated BV-2 cells

Western blot analysis showed that LPS-stimulation to BV-2 microglia increased the expression of iNOS and COX-2 levels. However, the increased expression of iNOS and COX-2 protein levels in LPS-stimulated BV-2 cells was suppressed when treated with IBE extract (50 and 100  $\mu\text{g/ml}$ ) in a concentration-dependent manner (Fig 4).



**Fig 4:** Effect of IBE 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 (5  $\mu\text{g/ml}$ )-stimulated BV-2 cells by various concentration of the IBE extract (50 and 100  $\mu\text{g/ml}$ ) was monitored by immunoblot analyses with the specific antibodies against iNOS and COX-2. The internal control used was  $\beta$ -actin. (IBE: *I. batatas*-ethylacetate, LPS: Lipopolysaccharide)

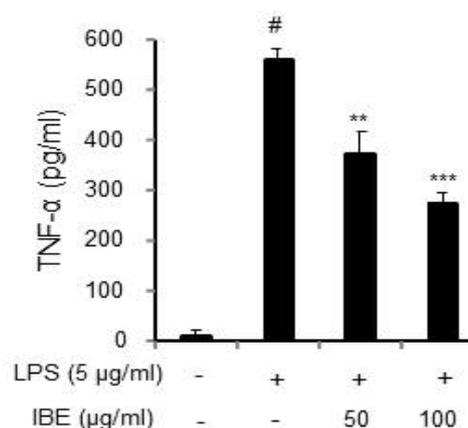
### Effect of IBE extract on TNF- $\alpha$ protein expression and production in LPS-stimulated BV-2 cells

As shown in Fig 5, TNF- $\alpha$  levels increased significantly after LPS treatment (5  $\mu\text{g/ml}$ ) when compared to those in untreated cells ( $p < 0.001$ ). However, IBE extract significantly inhibited TNF- $\alpha$  production in a concentration-dependent manner in LPS-stimulated BV-2 cells ( $p < 0.01$  at 50  $\mu\text{g/ml}$  and  $p < 0.001$  at 100  $\mu\text{g/ml}$ , respectively).

## DISCUSSION

Neuroinflammation is characterized by the activation of microglia and expression of major inflammatory mediators in the CNS. The importance of early therapeutic interference to inhibit microglial activation would be an effective

strategy to alleviate the progression of several neuroinflammatory diseases [2,3]. In the present study we report that IBE extract significantly inhibited production of NO, suppressed the expression of iNOS and COX-2 protein levels and attenuated the increased TNF- $\alpha$  production in LPS-stimulated BV-2 microglial cells. The IBE extract also exhibited significant antioxidant activity as evaluated by DPPH free radical scavenging assay.



**Fig 5:** Effect of IBE extract on TNF- $\alpha$  production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine TNF- $\alpha$  expression by IBE extract was measured with ELISA test. BV-2 cells were treated with IBE extract at 50 and 100  $\mu\text{g/ml}$  with or without LPS (5  $\mu\text{g/ml}$ ) for 4 h. The TNF- $\alpha$  in the culture supernatant was evaluated using a murine TNF- $\alpha$  ELISA kit. Data are presented as the mean  $\pm$  S.E.M. ( $n = 3$ ) for three independent experiments. <sup>#</sup> $p < 0.001$ , when compared with control group. <sup>\*\*</sup> $p < 0.01$  and <sup>\*\*\*</sup> $p < 0.001$ , when compared with LPS alone group by Student t-test. (IBE: *I. batatas*-ethylacetate, LPS: Lipopolysaccharide, TNF- $\alpha$ : Tumor necrosis factor-alpha)

Mounting evidence has implicated the pathogenesis of several human diseases including neurodegenerative disorders related to increased oxidative stress [13]. Increased reactive oxygen species (ROS) production can regulate the expression of diverse inflammatory mediators during brain injury. Elevated levels of several pro-inflammatory factors including toxic free radicals in the CNS have been detected in patients with neurodegenerative diseases [14]. Therefore inhibition 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 in a relatively short period of time [11]. Several studies have reported that antioxidants play important roles in the prevention of aging and age-related diseases [14]. Due to the safety concerns associated with

supplemental forms of antioxidants, consumers are paying more attention to fruits and vegetables as natural sources of antioxidants [7]. Reports also reveal that *I. batatas* possessed strong antioxidant compounds [15,16]. In our present study, the IBE extract also exhibited significant free radical scavenging effect indicating that IBE extract might contain potential antioxidant agents.

Previous studies on 3T3-L1 adipocytes in vitro revealed that *I. batatas* extract suppressed the inflammatory conditions [17]. The antioxidant glycosides and anthocyanin constituents present in *I. batatas* were also reported to exhibit anti-inflammatory effects [18-20]. Based on the published reports, in our study we evaluated IBE extract for its anti neuro-inflammatory activity in LPS-stimulated BV-2 microglial cells.

It was well documented that one of the major pro-inflammatory mediators released by activated microglia is NO, an uncharged lipophilic molecule that is toxic to neurons. Elevated levels of pro-inflammatory cytokines and mediators are also up-regulated in brain once microglial cells are activated [3,21]. Therefore inhibition of cytokine production in activated-microglia might serve as a key mechanism in the control of neuroinflammatory responses in neuro-degeneration. In the present study, IBE extract significantly inhibited the LPS-stimulated increase in NO production and suppressed the iNOS protein expression in LPS-stimulated BV-2 cells.

Neuroinflammatory processes involving an increased expression of COX-2 levels have been associated with several neurodegenerative diseases [22]. In agreement with this, LPS-activated BV-2 cells increased the expressional levels of COX-2. However, IBE extract reduced the increased protein expression of COX-2 in LPS-activated BV-2 cells. Activated microglial cells are known to release several proinflammatory cytokines including TNF- $\alpha$  which may not only amplify the inflammatory cascade, but also cause inflammatory injury [23,24]. Therefore, we investigated whether IBE extract had an effect on the production of TNF- $\alpha$  in LPS-activated BV-2 microglial cells. Our results indicate that IBE extract significantly suppressed the production of TNF- $\alpha$  in the LPS-activated BV-2 cells.

## CONCLUSION

This study revealed that purple sweet potato (IBE) extract attenuated the neuroinflammatory processes in LPS-induced BV-2 microglial cells. The anti-neuroinflammatory effects of IBE extract might be attributed to its regulatory actions on proinflammatory cytokine such as TNF- $\alpha$  and its strong antioxidant effects. Based on our results IBE extract might be developed as a promising candidate for the treatment of neuro-inflammation-mediated neurological disorders.

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