Anti-neuroinflammatory Effects of Ethanol Extract of *Inula helenium* L (Compositae)

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**Abstract**

**Purpose:** To evaluate the in vitro antioxidant and anti-neuroinflammatory activities of *Inula helenium* extract (IHE) against lipopolysaccharide (LPS)-induced production of nitric oxide (NO) by primary microglial cells.

**Methods:** Cell viability was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Antioxidant activity was evaluated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. LPS-stimulated BV-2 microglia were used to study the expression and production of inflammatory mediators, including NO, inducible NO synthase (iNOS) and Interukin-6 (IL-6).

**Results:** Pretreatment with IHE prior to LPS treatment significantly inhibited excessive production of NO (*p < 0.001* at 20, 40, 80 and 100 μg/mL) in a dose-dependent manner, and was associated with downregulation of expression of inducible nitric oxide synthase (iNOS). IHE also suppressed the LPS-induced increase in IL-6 level (*p < 0.01* at 40 and 80 μg/mL) in BV-2 cells. The antioxidant activity exhibited by IHE might play a critical role in ameliorating neuroinflammatory processes in LPS-stimulated BV-2 microglial cells.

**Conclusion:** IHE may be beneficial in preventing and treating neurodegenerative and oxidative stress-related diseases.

**Keywords:** Inula helenium, DPPH, Neurodegenerative diseases, Anti-inflammatory, Anti-oxidant, Microglial cell

INTRODUCTION

*Inula helenium*, also known as elecampane, is commonly found in China. In traditional medicine, it is extensively used primarily for treatment of abdominal pain, emesis, diarrhea, and threatened abortion [1]. In addition, the roots are effective in the treatment of diuretic, diaphoretic, expectorant, and anthelmintic remedy [2]. *I. helenium* has been identified that *I. helenium* contains sesquiterpene lactones and flavonoids [3–7], while other studies have reported that phenolic compounds exist in the Compositae [8].

However, till date, the effect of *I. helenium* extract on microglia-mediated neuroinflammatory diseases has not been reported.

Microglia are the resident macrophage-like cells of the central nervous system (CNS) with a broad role in the brains innate immunity and in inflammatory neuro-pathologies [9]. They are highly responsive to stress and injury and become immediately and focally activated in response to Alzheimer’s disease (AD), Parkinson’s disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS).
[10,11]. Microglia are involved in initiating inflammatory responses in the brain through secreting a variety of inflammatory mediators, including nitric oxide (NO), inducible NO synthase (iNOS), interleukins (IL) and tumor necrosis factor (TNF)-α [11]. Thus, modulation of activated microglia is an effective strategy for therapeutics in the neuro-inflammation.

The most common cause of inflammatory responses is an exposure to lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria [12]. LPS-activated BV-2 microglia cells responses induction and secretion of pro-inflammatory mediators. Therefore, suppression of aberrant activation of BV-2 microglia cells may have valuable therapeutic potential for the treatment of inflammatory diseases such as AD and PD [13].

In this study, we investigated the effects of *I. helenium* on the NO production, expression of iNOS, and pro-inflammatory cytokines from LPS-stimulated BV-2 microglia cells. The findings show that *I. helenium* may be used for the prevention or treatment of cerebral inflammatory diseases.

**EXPERIMENTAL**

**Preparation of *Inula helenium* extract (IHE)**

*Inula helenium* collected at the end of June to about the middle of August were obtained from the local market, Seoul, South Korea. *Inula helenium* was washed in running tap water, dried at 60 °C for 24 h and then ground to fine powder. Each powder was extracted with 10 volumes (v/w) of 70 % ethanol at a room temperature for 72 h and filtrated. The filtered extract was concentrated by a rotary evaporator (EYELA NVC-2000, Tokyo, Japan) under reduced pressure and lyophilized (Jeol Ltd, Tokyo, Japan).

**DPPH radical scavenging activity**

This assay was based on the scavenging of stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA) radicals by the radical scavenging antioxidants in IHE. The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the IHE and a DPPH methanolic solution as described previously [14]. Briefly, a sample solution of 60 µl of each IHE was added to 60 µl of DPPH (60 µM) in methanol. After mixing vigorously for 10 s, the mixture was 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 viability**

BV-2 microglia cells were grown in RPMI1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % FBS (Hyclone, Logan, UT, USA) containing 100 U/ml of penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen) in 37 °C in humidified atmosphere of 5 % CO₂. In all experiments, cells were pre-treated with the indicated concentrations of IHE for 1 h before the addition of LPS (1 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) in serum free RPMI1640. An equal volume of IHEriile 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 [15]. Cells were incubated with various concentrations of IHE 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 NO production in the medium was detected with the Griess reaction [16]. 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 Na₃VO₄ and 1 X protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany). The lysates were centrifuged at 15,000 x 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. 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).

IL-6 assay
BV-2 microglia cells (1 x 10^5 cells/well) were cultured on 96 well plates and treated with the IHE at indicated concentrations for 1 h and stimulated with LPS (1 µg/ml). At 24 h post-LPS treatment, the production of IL-6 was determined in cell supernatant using assay kits (BD Biosciences, San Jose, CA, USA) as per the manufacturer’s instructions. The IL-6 assay was performed at 20 °C and optical absorbance was measured at 450 nm using ELISA reader.

Statistical analysis
All data are presented as the mean ± SEM (n = 3). Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, 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 IHE on DPPH radical scavenging activity
As shown in Fig 1A, IHE exhibited significant DPPH radical scavenging activity in a dose-dependent manner showing a maximum effect at 1 mg/mL of concentration. The ESR spectroscopy data at 0.1, 1, 10, 100 and 1000 µg/mL is represented in Fig 1B.

Effect of IHE on LPS-induced NO production
IHE treatment did not exhibit any significant cytotoxicity in BV-2 microglial cells treated for 24 h at concentrations up to 200 µg/ml, and in all cases the viability was found above 96% by MTT assay (Fig 2). As shown in Fig 3, treatment of LPS resulted in the excessive production of IL-6 in the absence of IHE. The data are expressed as mean ± SEM (n = 3). Statistical analyses were performed using one-way analysis of variance, followed by Dunnett’s multiple range tests. P < 0.05 was considered statistically significant.

Figure 1: Effect of IHE on DPPH radical scavenging activity. The capacity to scavenge DPPH free radical by different concentrations IHE (A) and ESR spectra (B) was measured. BV-2 cells were treated with or without IHE at the various concentrations (0.1, 1, 10, 100, and 1000 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. IHE: Inula helenium ethanol extract.
Figure 2: Effects of IHE on the viability of BV-2 microglial cells. Viability in IHE-treated cells was determined using MTT assay. The results are displayed in percentage of control samples. Data are presented as mean ± SEM (n = 3) for three independent experiments. *ns Not significant; IHE = *Inula heleni*um ethanol extract

Figure 3: Effect of IHE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with IHE at various concentrations (10, 20, 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 ± SEM (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; IHE: *Inula heleni*um ethanol extract

NO. Pretreatment of IHE significantly suppressed the LPS-induced NO production in a concentration-dependent manner (Fig 2). The maximum effect was shown at 100 µg/mL (p < 0.001).

**Effect of IHE on LPS-induced expressional levels of iNOS**

Western blot analysis showed that the protein expression of iNOS in the LPS-stimulated BV-2 cells was also suppressed in a concentration dependent manner. However, treatment with IHE exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in BV-2 cells (Fig 4).

**Effect of IHE on IL-6 production in LPS-stimulated BV-2 cells**

As shown in Fig. 5, IL-6 levels increased significantly after LPS treatment (1 µg/ml) when compared to those in untreated cells (p < 0.001). However, IHE significantly inhibited IL-6
Figure 5: Effect of IHE on IL-6 production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine IL-6 expression by IHE was measured with ELISA test. BV-2 cells were treated with IHE at 20, 40 and 80 µg/mL with or without LPS (1 µg/ml) for 24 h. The IL-6 in the culture supernatant was evaluated using a murine IL-6 ELISA kit. Data are presented as mean ± SEM (n = 3) for three independent experiments. *p < 0.001, when compared with control group. *p < 0.05, and **p < 0.01, ***p < 0.001 when compared with LPS alone group by Student t-test. IHE = *Inula helenium* ethanol extract; IL-6: Interukin-6; LPS = lipopolysaccharide)

production in a concentration-dependent manner in LPS-stimulated BV-2 cells (p < 0.05 at 20 µg/mL and p < 0.01 at 40 and 80 µg/mL, respectively).

**DISCUSSION**

The present study demonstrated that IHE possesses an anti-neuroinflammatory activity in LPS-stimulated BV-2 microglia cells and antioxidant activity. IHE also exhibited significant antioxidant activity. Free radicals and reactive oxygen species (ROS) are important causative factors in the development of age-related neuroinflammatory and neurodegenerative diseases [14]. Thus neutralization by antioxidants and radical scavengers can reduce neuroinflammation. 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 [16]. In our present study, IHE also exhibited significant free radical scavenging effect indicating that it might contain potential antioxidant agents. In our present study, the IHE exhibited significant free radical scavenging effect indicating that the *Inula helenium* might contain potential antioxidant agents.

In BV-2 microglia cells, NO is generated by the inducible isoform of NO synthase (iNOS) and iNOS are responsible for the symptoms of many neuro-inflammatory diseases [16]. 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 [17]. Our results clearly showed that IHE attenuated LPS-induced iNOS expression and downstream NO production. This result indicates that IHE 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. Pro-inflammatory cytokines such as TNF-α are the initiators of the inflammatory response and the mediators of the development of chronic inflammatory diseases [18]. Therefore, the over-production of pro-inflammatory cytokines by activated microglia cell is a possible etiological factor of neurological disorders [19]. Microglial cell activation by LPS produces various cytokines including IL-6 leading to the attraction of neutrophils and the accumulation of neutrophil-secreted proteases and ROS at the site of inflammation. From our results, it appears that IHE may act by suppressing the expression of IL-6 protein levels and thereby inhibiting NO production and iNOS expression levels in LPS-stimulated BV-2 cells.

**CONCLUSION**

These data demonstrate that IHE has anti-inflammatory properties in LPS-induced BV-2 microglial activation through the down-regulation of inflammation-related gene expression, including iNOS and pro-inflammatory cytokine such as IL-6. Therefore, IHE can be considered as a useful therapeutic and preventive approach for the treatment of several neurodegenerative and oxidative stress-related diseases.
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REFERENCES

1. Chinese Pharmacopoeia Commission, Beijing, China. Pharmacopoeia of the People’s Republic of China. 2010.
2. Stojakowska A, Kędzia B, Kisiel W. Antimicrobial activity of 10-isobutyryloxy-8, 9-epoxythymol isobutyrate. Fitoterapia 2005; 76: 687–690.
3. Konishi T, Shimada Y, Nagao T, Okabe H, Konoshima T. Antiproliferative sesquiterpene lactones from the roots of Inula helenium. Biol Pharmaceut Bull 2002; 25: 1370–1372.
4. Nesterova YV, Zelenskaya KL, Vetoshkina TV, Aksinenko SG, Gorbacheva AV, Gorbatykh G. Some mechanisms of the antistressor activity of Inula helenium preparations. Eksp Klin Farmakol 2003; 66: 63–65.
5. Zhao YM, Zhang ML, Shi QW, and Kiyota H. Chemical constituents of plants from the Genus Inula. Chem Biodiver 2006; 3: 371–384.
6. Zhao YM, Wang YJ, Dong M. Two new eudesmanes from Inula helenium. Chem Nat Comp 2010; 46: 373–376.
7. Wang J, Zhao M, Guo CY. Ultrasound-assisted extraction of total flavonoids from Inula helenium, “Pharmacognosy Magazine, 2012; 8:166–170.
8. Samanidou V, Tsagiannidis A, and Sarakatsanou I. Simultaneous determination of polyphenols and major purine alkaloids in Greek Sideritis species, herbal extracts, green tea, black tea, and coffee by high-performance liquid chromatography-diode array detection. J. Separation Sci, 2012; 35: 608–615.
9. W irenfeldt M, Ladeby R, Dalmau I, Banati RB, Finsen B. Microglia-biology and relevance to disease. Ugeskr Laeger 2005; 167:3025-3030.
10. Garden GA, Møller. T Microglia biology in health and disease. J Neuroimmune Pharmacol 2006; 1: 127-137.
11. Perry VH, Gordon S. Macrophages and microglia in the nervous sym. Trends Neurosci 1988; 11:273-277.
12. Xie Q.W., Kashwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase J Biol Chem 1994; 269(7): 4705–4708.
13. Aubulo G, Gamet-Pyrastre L, Manenti S, Viala C, Rémesy C, Chap H, et al. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochim Pharmacol 1997; 53: 2087–2094.
14. Nanjo F, Ito K, Seto R, Suzuki H, Sakai M, Hara Y. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Rad Bio Med 1996; 21: 885-902.
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.
16. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite and (15N) nitrate in biological fluids. Anal Biochem 1982; 126: 131-138.
17. Murphy S. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. GLIA 2000; 29: 1-13.
18. Merrill JE, Benveniste EN. Cytokines in inflammatory brain lesions: helpful and harmful. Trends Neurosci. 1996; 19: 331–338.
19. Zindler E, Zipp F. Neuronal injury in chronic CNS inflammation. Best Pract Res Clin Anaesthesiol 2010; 24(4): 551-562.