

Original Research Article

Anti-Neuroinflammatory Effects of *Houttuynia cordata* Extract on LPS-Stimulated BV-2 Microglia

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Abstract

Purpose: To evaluate the anti-neuroinflammatory effects of *Houttuynia cordata* extract (*H. cordata*) in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells, and its anti-oxidant properties.

Methods: Anti-oxidant properties were evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. LPS was used to stimulate BV-2 cells. Nitric oxide (NO) levels were measured using Griess assay. Inducible NO synthase (iNOS) expression, interleukin (IL)-6 expression level were determined by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

Results: Ethyl acetate (HC-EA) extract of *H. cordata* significantly scavenged DPPH free radicals in a concentration-dependent fashion. The increased levels of NO, iNOS and IL-6 in LPS-stimulated BV-2 microglial cells were also suppressed by HC-EA extract in a concentration-dependent manner.

Conclusion: The result indicate that the HC-EA extract exhibited strong anti-oxidant properties and inhibited the excessive production of pro-inflammatory mediators, including NO, iNOS and IL-6, in LPS-stimulated BV-2 cells. The anti-oxidant phenolic compounds present in HC-EA extract might play an important role in ameliorating neuroinflammatory processes in LPS-stimulated BV-2 microglial cells.

Keywords: *Houttuynia cordata*, DPPH radicals, antioxidant, neuroinflammation, BV-2 cells, iNOS, COX-2, IL-6.

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INTRODUCTION

Inflammation in brain disorders is characterized by activation of glial cells (mainly microglia and astrocytes) further expressing key inflammatory mediators as well as neurotoxic free radicals. Microglia are the resident immune cells of the central nervous system which show increased inflammatory activity (i.e. neuroinflammation) by infections, cerebral ischemia, traumatic brain injury, or neuronal/parenchyma damage [1]. Activation of microglia and subsequent release of pro-inflammatory mediators including nitric oxide

(NO), inducible NO synthase (iNOS), IL-1 β , IL-6 and TNF- α , progressively increase during normal aging and are also commonly present in neurodegenerative diseases [2].

It is well known that lipopolysaccharide (LPS)-stimulated microglia releases various pro-inflammatory and neurotoxic factors and is recognized to be a useful *in vitro* model for the studying mechanisms related to neuronal injury-mediated inflammatory processes [3]. Therefore, regulation of microglial activation may have therapeutic benefits that lead to alleviating neuroinflammatory diseases. *Houttuynia cordata*

(*H. cordata*), a grass from the Saururaceae family is a perennial herb native to Southeast Asia and has immense medicinal values. Traditionally, *H. cordata* is used in folk medicine for diuresis and detoxification, as well as an anti-viral, anti-bacterial and anti-leukemic agent [4]. It is also known to be promisingly effective drug for allergic inflammation [5] and anaphylaxis [6]. Recently, it was used to tackle severe acute respiratory syndrome (SARS) as it is conventionally used to treat pneumonia [7]. However, the anti-neuroinflammatory activity of *H. cordata* has not been studied in activated BV-2 microglial cells. In this study, we sought to determine whether *H. cordata* extract possesses anti-neuroinflammatory activity in LPS-stimulated BV-2 microglial cells. In addition, its anti-oxidant potential was evaluated to see if it is consistent with its anti-neuroinflammatory effects.

EXPERIMENTAL

Preparation of the *H. cordata* extract

Dried *H. cordata* was purchased from the traditional herb market and was authenticated by a taxonomist at Konkuk University, South Korea. A voucher specimen (K-KU-2013) was kept in our department herbarium for future reference. To obtain the *H. cordata* extract, the dried plant material was ground in a mixer and defatted three times with three volumes of 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 (50 g) was partitioned into hexane, ethyl acetate (EA), and n-butanol fractions to yield 0.53, 8.72 and 38.25 g, respectively. The EA fraction of *H. cordata* (HC-EA) with potent antioxidant properties was re-dissolved in distilled water and used for evaluating its anti-neuroinflammatory and anti-oxidant properties.

DPPH radical scavenging activity

The anti-oxidant property of the HC-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 HC-EA extract and a DPPH methanolic solution as described previously [8]. 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 % 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 HC-EA for 1 hr 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 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 [9]. Briefly, BV-2 cells were plated onto 96 well plates and exposed to EA-OFP extract. MTT was added to each well then incubated for additional 2 hr 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 (absorbance of extract treated sample/absorbance of non-treated sample) x 100 %.

Assay of NO

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [10]. Briefly, BV-2 cells (2×10^5 cells/ml) were seeded in 6-well plates in 500 µl complete culture medium and then were stimulated with LPS (1 µg/ml) for 2 h. Fifty microliters of culture supernatant reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1 % sulfanilamide in 5 % H₃PO₄) in 96 well plates at room temperature in the dark. Nitrite concentrations in the culture supernatant were determined by using standard solutions of sodium nitrite. The absorbance was read at 540 nm using a microplate reader (Tecan).

Immunoblot analysis and antibodies

Cells were washed in cold PBS and lysed in buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) NP-40, 0.25 % sodium deoxycholate, 150mM NaCl, 1mM EDTA, 25mM 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. 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 method. 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, 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 as 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 and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA).

IL-6 assay

BV-2 microglia cells (1 × 10⁵ cells/well) were cultured on 96 well plates and treated with HC-EA extract at indicated dosages and LPS (1 μg/ml). At 4 hr post-LPS treatment, the cells were collected and the supernatants were used 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.

Statistical analysis

All data are represented as the mean ± SEM 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

Effect of HC-EA extract on DPPH radical scavenging activity

As shown in Fig. 1, HC-EA exhibited significant DPPH radical scavenging activity in a

concentration-dependent manner showing a maximum effect at 100 μg/ml of ($p < 0.001$).

Effect of HC-EA extract on BV-2 cell viability

The cytotoxicity of HC-EA extract was evaluated based on its effects on cell growth using MTT assay (>95% cell viability). Treatment with HC-EA at various concentrations (0.1 μg/ml to 100 μg/ml) did not affect the overall cell viability nor did they exhibit any significant cytotoxicity in BV-2 microglia (Fig 2).

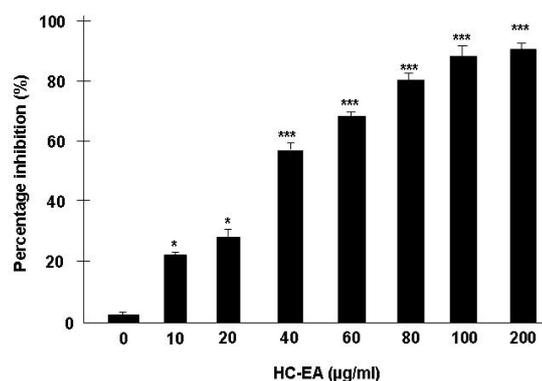


Fig 1: Effect of HC-EA extract on DPPH radical scavenging activity. Data are presented as mean ± SEM (n = 3) for three independent experiments; * $p < 0.05$ and *** $p < 0.001$, compared with control group by Student t-test. HC-EA= *Houttuynia cordata* ethyl acetate extract

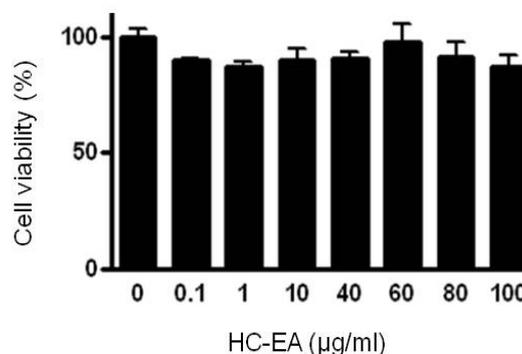


Fig 2: Effect of HC-EA extract on the viability of BV-2 microglial cells. Data are presented as mean ± SEM (n = 3); HC-EA = *Houttuynia cordata* ethyl acetate extract

HC-EA extract attenuates NO production in LPS-stimulated BV-2 cells

As shown in Fig. 3, cells treated with LPS alone significantly increased NO levels ($p < 0.001$). However, pre-treatment with HC-EA extract significantly suppressed the LPS-stimulated increased NO release in BV-2 cells in a concentration-dependent manner compared to LPS cells. The maximum effect was observed at 80 μg/ml ($p < 0.001$). The other concentrations of HC-EA (10, 20 and 40 μg/ml) also significantly ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively) and

concentration-dependently inhibited the release of NO in LPS-stimulated BV-2 cells.

HC-EA extract attenuates iNOS expressional levels in LPS-stimulated BV-2 cells

Western blot analysis showed that the increased protein expression of iNOS in the LPS-stimulated BV-2 cells was also suppressed in a concentration-dependent manner (Fig 4). Although LPS-stimulated BV-2 cells are associated with the increased iNOS expression, pre-treatment with HC-EA extract suppressed increased iNOS protein expressional levels with maximum effect at 80 $\mu\text{g/ml}$ concentration.

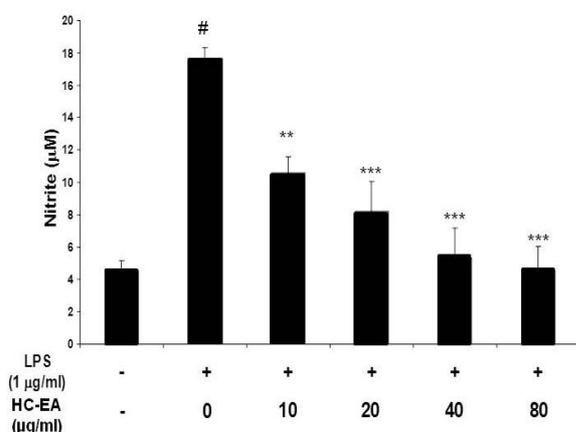


Fig 3: Effect of HC-EA extract on NO Production in LPS-stimulated BV-2 microglial cells. Data are presented as mean \pm S.E.M. (n = 3) for three independent experiments; [#] $p < 0.001$, when compared with control group. ^{**} $p < 0.01$ and ^{***} $p < 0.001$, when compared with LPS-treated group; HC-EA = *Houttuynia cordata* - ethyl acetate.

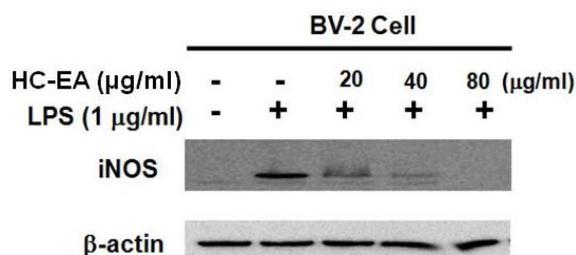


Fig 4: Effect of HC-EA extract on iNOS expressional levels in LPS-stimulated BV-2 microglial cells. HC-EA = *Houttuynia cordata* ethyl acetate extract

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

The level of IL-6 increased significantly after LPS (1 $\mu\text{g/ml}$) treatment when compared to those in the control group. However, HC-EA extract significantly reduced IL-6 production in a concentration-dependent manner (Fig 5).

DISCUSSION

Activation of microglia and the subsequent release of inflammatory cytokines and toxic free radicals are the hallmarks of neuroinflammation observed in several neurodegenerative diseases [1,2]. Microglial activation in response to LPS is well documented and it generates numerous pro- and anti-inflammatory mediators [11,12]. Recent studies have shown that anti-oxidant and anti-inflammatory agents may suppress microglial activation and thus protect neuronal cell death [13,14].

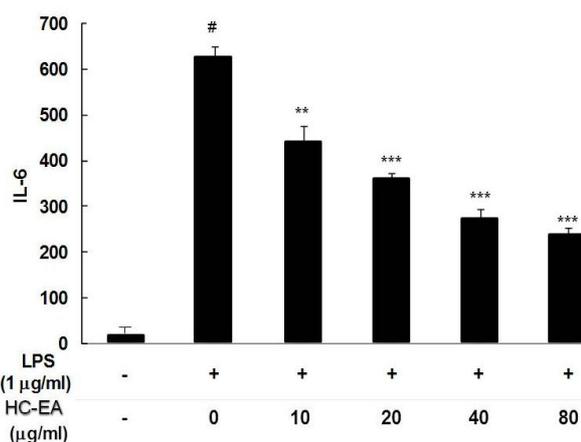


Fig 5: Effect of HC-EA extract on pro-inflammatory cytokine IL-6 expression in LPS-stimulated BV-2 cells. Data are presented as mean \pm SEM (n = 3); [#] $p < 0.001$, compared with control group; ^{**} $p < 0.01$, ^{***} $p < 0.001$, compared with LPS alone treated group; HC-EA = *Houttuynia cordata* ethyl acetate extract

H. cordata, a traditional plant is used as folk medicine for treating several diseases including allergic inflammation and anaphylaxis [4-6]. However, not much is known about the anti-neuroinflammatory activities of *H. cordata* extract in activated BV-2 microglial cells *in vitro*. The results of this study show that HC-EA extract suppressed the inflammatory mediators such as NO, iNOS and IL-6 in LPS-stimulated BV-2 microglial cells and exhibited strong anti-oxidant effects.

It is well documented that activated microglia can produce several potentially neurotoxic substances, including NO synthesized by iNOS. iNOS, an important mediator of inflammation speeds up the production of NO. NO which is a neurotransmitter in the CNS has protective functions in anti-inflammatory pathways but at high concentrations produces nitrite free radicals which are neurotoxic [15]. In our study, it was observed that HC-EA extract significantly suppressed nitrite production in LPS-stimulated BV-2 cells in concentration-dependent manner.

Further, HC-EA extract attenuated the increased iNOS protein levels in LPS-stimulated BV-2 cells, suggesting that HC-EA extract has potent anti-neuroinflammatory benefits through the suppression of increased NO production and iNOS expression.

Pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 cause potent activation of iNOS gene expression in rodent glial cells and muscle cells [16]. In particular, the involvement of IL-6 in inflammatory process has been highly recognized as targets of therapeutic intervention as IL-6 not only amplifies the inflammatory cascade, but also causes inflammatory injury [16,17]. The likelihood of the involvement of HC-EA extract in attenuating such factor is supported by our observations that pro-inflammatory mediator IL-6 produced by LPS stimulation was significantly suppressed by HC-EA in BV-2 microglial cells indicating that HC-EA may be an effective anti-neuroinflammatory agent.

Polyphenolic compounds are powerful anti-oxidants and are widely known to have protective effect against several human diseases including neurodegenerative diseases [18]. Therefore to identify whether the polyphenolic compounds present in HC-EA extract possess anti-oxidant actions, we estimated using the DPPH free radical scavenging assay. DPPH radical assay is one of the widely used methods for screening the free radical scavenging activities of several anti-oxidants in a relatively short period of time [8]. In our study HC-EA significantly scavenged the DPPH free radicals, indicating that the extract has anti-neuroinflammatory effects in LPS-stimulated BV-2 cells. Previous reports indicated that essential oils from *H. cordata* contain several agents such as β -myrcene, β -pinene, α -pinene, α -terpineol and *n*-decanoic acid [19]. Reports also exists that *H. cordata* contains polyphenolic compounds such as chlorogenic acid, quercetin-3-O- β -D-galactopyranosyl-7-O- β -D-glucopyranoside, quercetin 3-O- α -L-rhamnopyranosyl-7-O- β -D-glucopyranoside, rutin, hyperin, isoquercitrin, quercitrin, afzelin, piperolactam A, and aristolactam B [19]. These antioxidant polyphenolic compounds present in HC-EA extract might be responsible for its potent anti-neuroinflammatory actions in activated microglial cells.

CONCLUSION

Our findings suggest that HC-EA extract acts by suppressing the expression of inflammatory mediators such as NO, iNOS and IL-6 in LPS-stimulated BV-2 cells and also inhibits free radicals which are involved in the inflammatory

events. Therefore, HC-EA extract may be developed as a potential therapeutic agent in treating microglia-mediated neuroinflammatory diseases.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this work.

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