Tropical Journal of Pharmaceutical Research March 2015; 14 (3): 463-468
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v14i3.15

Original Research Article

Evaluation of Antioxidant and Anti-neuroinflammatory Activities of *Hizikia fusiformis* (*Harvey*) *Okamura* Extract

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Received: 18 December 2014 *Revised accepted: 27 February 2015*

Abstract

Purpose: *To evaluate the in vitro antioxidant and anti-neuroinflammatory activities of Hizikia fusiformis (Harvey) Okamura extract (HFE) in lipopolysaccharide (LPS)-stimulated murine BV-2 microglial cells.*
Methods: *The antioxidant activity of HFE was evaluated by measuring 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activity using an ESR spectrometer. Cell viability was estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay. LPS-stimulated BV-2 microglia were used to study the expression and production of inflammatory mediators such as nitric oxide (NO), inducible NO synthase (iNOS) and Interleukin 6 (IL-6).*
Results: *Treatment with Hizikia fusiformis extract (HFE) significantly scavenged the DPPH radicals with half-maximal concentration of (IC_{50}) of 9.64 ± 0.78 µg/ml ($p < 0.01$ at 10 µg/ml). The increased levels of NO (35.32 ± 3.62 µM) and protein expressions of iNOS were inhibited by HFE extract in LPS-stimulated BV-2 cells. Increased pro-inflammatory cytokines such as TNF-α and IL-6, were also significantly suppressed by HFE ($p < 0.001$ at 80 µg/ml). Further, HFE blocked the expression of NF-κB activation in LPS-stimulated BV-2 cells.*
Conclusion: *HFE can be considered as a useful therapeutic and preventive approach for the treatment of neurodegenerative diseases and oxidative stress-related diseases.*

Keywords: *Hizikia fusiforme, Antioxidant activity, Anti-neuroinflammation activity, Inducible nitric oxide synthase, Interleukin-6*

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INTRODUCTION

Hizikia fusiforme is important economic brown seaweed, used as a healthy foodstuff, medical herb and marine vegetable in Korea, Japan, and China [1]. Recent evidence suggests that *H. fusiforme* contains a number of compounds possessing anti-oxidant, anti-inflammatory, and anticoagulant proprieties [2]. Karawita *et al* [3] has reported that *H. fusiforme* has a variety of physiological activites. However, the beneficial effect of *Hizikia fusiforme* on neuroinflammation in activated microglial cells has not been studied.

Microglia are the resident macrophage-like cells of the central nervous system (CNS) with a broad role in the innate immunity and in inflammatory neuro pathologies [4]. 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) [5,6]. Also, 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) and interleukins (IL). Thus, modulation of activated microglia is an effective strategy for therapeutics in neuro inflammation.

The most common cause of inflammatory responses is exposure to lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria [8]. LPS-activation of BV-2 microglia cells results in a wide range of responses, including 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 [9].

In this study, we investigated the effects of *H. fusiforme* on NO production, expression of iNOS, and pro-inflammatory cytokines from LPS-stimulated BV-2 microglia cells. The findings demonstrate that *H. fusiforme* may be used as a source of drug candidate for the prevention or treatment of cerebral inflammatory diseases.

EXPERIMENTAL

Preparation of *Hizikia fusiforme* extract (HFE)

Hizikia fusiforme was washed with 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.

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 HFE. The radical scavenging capacity was evaluated by employing a reaction mixture constituted by aliquots of the HFE and a DPPH methanolic solution as described previously [10]. A sample solution of 60 µL of each *Hizikia fusiformis* 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 cultures and viabilities

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) at 37 °C in humidified atmosphere of 5 % CO_2. In all experiments, cells were pre-treated with the indicated concentrations of HFE 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 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 [11]. Cells were incubated with various concentrations of HFE 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 spectrophotometer (Tecan, USA). 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 [12]. 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 from a sodium nitrite standard curve.

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 Na_3VO_4 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 dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. After blocking with 5 % non-fat milk, the membranes were hybridized with 1:1000 dilutions 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 at 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 HFE 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 room temperature and optical absorbance was measured at 450 nm using ELISA reader.

Statistical analysis

All data are presented as mean ± S.E.M (at least n = 3). 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 HFE on DPPH radical scavenging activity

As shown in Fig 1A, HFE exhibited significant DPPH radical scavenging activity in a dose-dependent manner showing a maximum effect at 1 mg/mlL. ESR spectroscopy data at 0.1, 1, 10, 100 and 1000 μg/mL are represented in Fig 1B.

Effect of HFE on LPS-induced NO production

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

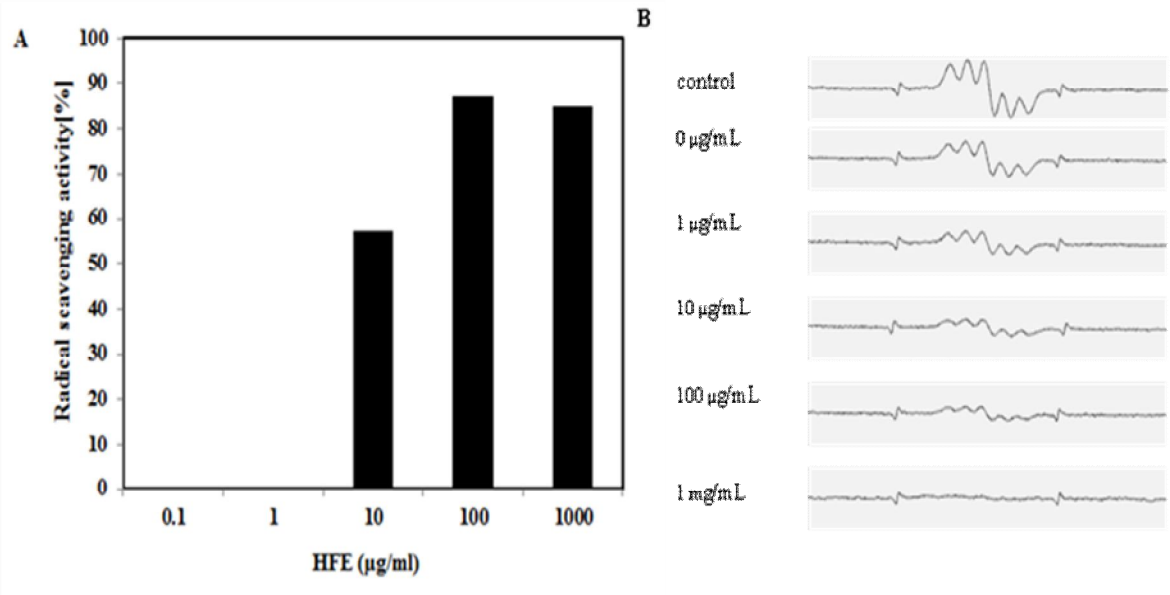

Fig 1: Effect of HFE on DPPH radical scavenging activity. The capacity to scavenge DPPH free radical by different concentrations HFE (A) and ESR spectra (B) was measured. BV-2 cells were treated with or without HFE at the various concentrations (0.1, 1, 10, 100, and 1000 μg/mL). The scavenging activity 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. HFE: *Hizikia fusiforme* ethanol extract

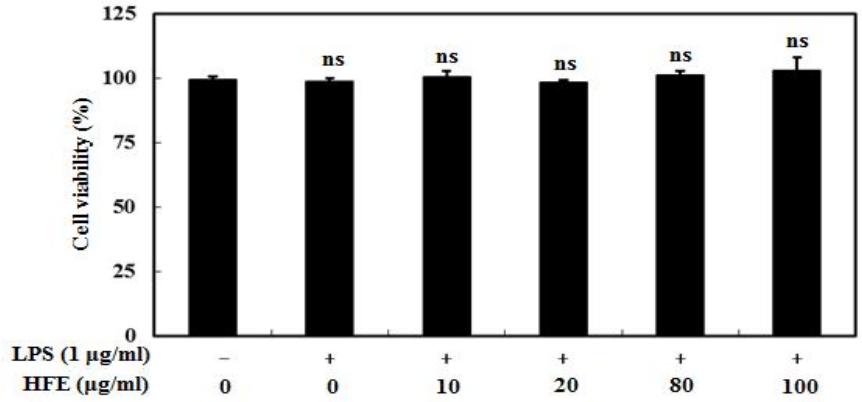

Fig 2: Effect of HFE on the viability of BV-2 microglial cells. Viability in HFE treated cells was determined using MTT assay. The results are displayed in percentage of control samples. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments; ^{NS} Not significant. HFE = *Hizikia fusiforme* ethanol extract

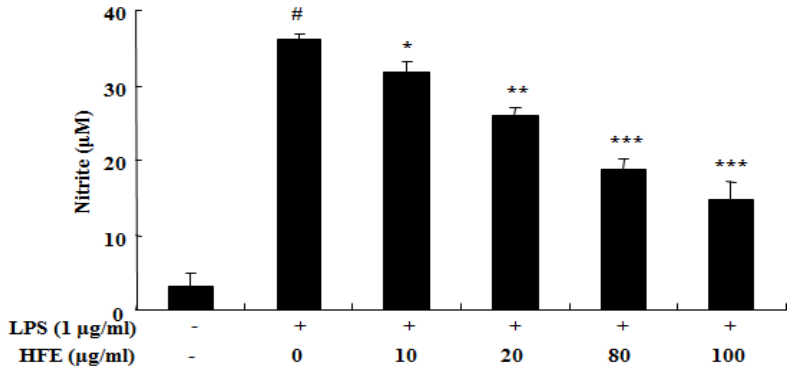

Fig. 3: Effect of HFE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with HFE at various concentrations (10, 20, 80, and 100 μg/ml) with or without LPS (1 μg/ml) for 24 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as 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. HFE: *Hizikia fusiforme* ethanol extract

Effect of HFE on LPS-induced expressional levels of iNOS

LPS strongly induced BV-2 cells to produce an increased expression of iNOS. However, treatment with HFE exhibited a broad spectrum of inhibitory effect on the expression of iNOS induced by LPS in BV-2 cells (Fig 4).

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

To determine the effect of HFE on the expression of pro-inflammatory cytokines such as IL-6, extracellular release of cytokines was examined using ELISA assay. 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, HFE significantly inhibited IL-6 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).

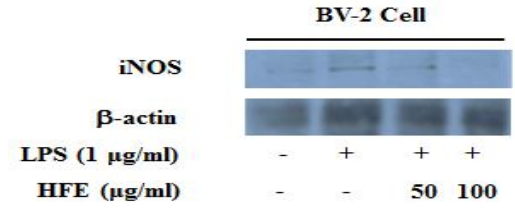

Fig 4: Effect of HFE on iNOS protein expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS production in the LPS-stimulated BV-2 cells by indicated concentrations (50 and 100 μg/ml) of the HFE was monitored by immunoblot analyses with the specific antibodies against iNOS. The internal control used was β-actin. HFE: *Hizikia fusiforme* ethanol extract; LPS= Lipopolysaccharide; iNOS: Inducible nitric oxide synthase

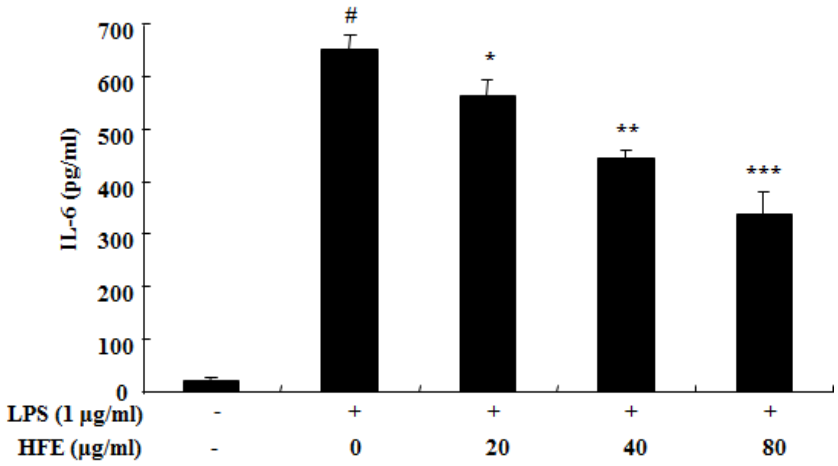

Fig 5: Effect of HFE on IL-6 production in LPS-stimulated BV-2 microglial cells. Suppression of pro-inflammatory cytokine IL-6 expression by HFE was measured with ELISA test. BV-2 cells were treated with HFE at 20, 40 and 80 µg/mL with or without LPS (1 µg/ml) for 24 h. The IL-6 in the culture supernatant was evaluated using a murine IL-6 ELISA kit. Data are presented as the mean ± S.E.M. (n = 3) for three independent experiments. $^{\#}p<$ 0.001, when compared with control group. * p <0.05, and ** p < 0.01, *** p < 0.001 when compared with LPS alone group by Student t-test. (HFE = *Hizikia fusiforme* ethanol extract; IL-6 = interleukin 6; LPS = lipopolysaccharide)

DISCUSSION

Hizikia fusiforme (HFE) is one of the major farmed alga species along the Korea, Japan and Chinese coast. The polysaccharides present in HFE have become the focus of intense interest due to their various bioactivities. HFE contains a number of compounds that exert anti-inflammatory, anti-oxidant and anticoagulant proprieties [2]. It also contains inorganic arsenic, which is carcinogenic to humans [3]. A polysaccharide with protective effects against ethanol-induced peptic injury has been isolated from HFE [19]. The present study demonstrated that HFE possesses an anti-neuroinflammatory activity in LPS-stimulated BV-2 microglia cells and antioxidant activity. Further HFE 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 neurodegenerative diseases [13]. 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 in a relatively short period of time [14]. In our present study, the HFE also exhibited significant free radical scavenging effect indicating that the HFE might contain potential antioxidant agent. Since antioxidants reduce neuro inflammation [13].

We used HFE to evaluate for its anti-neuroinflammatory activity in LPS-stimulated BV-2 microglial cells. In BV-2 microglia cells, NO is generated by the inducible isoform of NO synthase (iNOS) and has been described as a neurotoxic substance in the processes of CNS inflammation [15]. 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 [16]. Our results clearly showed that HFE attenuated LPS-induced iNOS expression and downstream NO production. This indicates that HFE 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 IL-6 are the initiators of the inflammatory response and the mediators of the development of chronic inflammatory diseases [17]. Therefore, the over production of pro-inflammatory cytokines, by activated microglia cell is a possible etiological factor of neurological disorders [18]. 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. Data from our study showed that HFE may act by suppressing the increased production of IL-6 thereby inhibiting NO production and iNOS expression levels in LPS-stimulated murine BV-2 cells indicating that HFE

may be an effective anti-neuroinflammatory agent.

CONCLUSION

The findings of this study demonstrate that HFE has anti-inflammatory properties in LPS-induced murine BV-2 microglial activation through the down-regulation of inflammation-related gene expression, including iNOS and pro inflammatory cytokine such as IL-6. The strong antioxidant effects exhibited by HFE may also be involved in delivering such actions. Therefore, HFE can be considered a useful therapeutic strategy for the natural treatment of neurological disorders and oxidative stress-related diseases in humans.

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