

Boiled-Water Extract from *Hizikia fusiformis* Showing Antioxidant Effects

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Boiled-water extract (BWE) of *Hizikia fusiformis* has antioxidant effects. To investigate antioxidant effects of BWE, 1,1-dephenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid (TBA) methods were used. BWE exhibited strong radical-scavenging activity (87.3% at final conc. 2000 µg BWE/ml in an assay mixture) on DPPH and good inhibitory activity (64% at final conc. 1000 µg BWE/ml in an assay mixture) on lipid peroxidation. Raw 264.7 cells treated with lipopolysaccharide (LPS) showed 26.7-fold increase in nitric oxide (NO) (48.9 µM) compared with control group (1.83 µM). When treated with BWE and LPS, NO production was inhibited by BWE dose-dependently.

Key words: *Hizikia fusiformis*, DPPH, TBA, NO, antioxidant

Oxidative stress is related to physiological and pathological conditions such as inflammation, diabetes, shock, arthritis, carcinogenesis, and aging.¹⁻³⁾ Antioxidants can markedly defer or inhibit the oxidation of molecules by inhibiting the initiation or multiplication of oxidative chain reactions.^{4,5)} Synthetic antioxidants, such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), are very effective but they may possess mutagenic activity.⁶⁾ For this reason, many studies have been carried out and some antioxidative substance have been found from natural sources.^{7,9)} Long studies on the natural antioxidants using a variety of seaweeds and plants as dietary and medicinal sources revealed they have antioxidant, antimutagenic, antitumor, and anticoagulant activities and play very important roles in lipid metabolism.¹⁰⁾ In particular, *Hizikia fusiformis*, distributed in the southern and western coastal areas of Korea including Jeju Island, has been extensively studied as a resource of dietary fiber.¹⁰⁻¹⁴⁾ Organic solvent extracts of *H. fusiformis* have been reported to possess strong antioxidant and antitumor activities.¹²⁾ In addition, its hot water extract have strong anticoagulant and antitumor activities.^{13,14)}

Nitric oxide (NO) is a multifunctional biomolecule involved in many physiological and pathological processes. In the immune system, NO is generated by inducible NO synthase (iNOS) in response to inflammatory stimuli such as lipopoly-

saccharide (LPS) and interferon- γ (IFN- γ) in macrophages^{15,16)} and acts as a cytotoxic agent against invading microorganisms and tumor cells; however, excess NO is related to inflammation, septic shock, rheumatoid arthritis, and autoimmune diseases.¹⁷⁾

In this study, antioxidant effects of boiled-water extract of *Hizikia fusiformis* (BWE), which have not been fully investigated were examined.

Materials and Methods

Materials. L-Ascorbic acid, BHA, DPPH, Griess reagent, linoleic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and TBA were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) was obtained from Lancaster (Eastgate, UK). Raw 264.7, a mouse macrophage cell line, was purchased from Korean Cell Line Bank. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Korea). *H. fusiformis* was collected from Wando, Chonnam.

Preparation of *H. fusiformis* extract. *H. fusiformis* was rinsed carefully in freshwater and steam-heated to 120°C at 2.5 kg/cm² for 50 min. One liter of the obtained layer was mixed with 3 l of 100% ethanol. After centrifugation at 5,000 rpm for 20 min, the supernatant was separated, dissolved with 75% ethanol, and filtered through Whatman No 2. filter paper. The filtrate was dried on a rotary evaporator (EYELA, Japan) under reduced pressure at 40°C. The residue was re-extracted with 75% ethanol evaporated *in vacuo* freeze-dried to give powered extract, and stored in a desiccator at room temperature until use.

DPPH free radical-scavenging method. DPPH free radical-scavenging assay measures the ability of antioxidants to scavenging free radicals. This assay was performed according to the method of Blois¹⁸⁾ with some modification. Namely, 100 µl of various concentrations (final conc. 0.1~

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Abbreviations: BWE, boiled-water extract from *Hizikia fusiformis*; DPPH, 1,1-dephenyl-2-picrylhydrazyl; TBA, thiobarbituric acid; LPS, lipopolysaccharide; NO, nitric oxide; BHA, butylated hydroxy anisole; BHT, butylated hydroxy toluene; iNOS, inducible nitric oxide synthase; IFN- γ , interferon- γ ; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; TCA, trichloroacetic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

2000 μg each sample/ml in an assay mixture) of sample were added to 900 μl of DPPH solution. DPPH solution was prepared as 39 $\mu\text{g} \cdot \text{mL}^{-1}$ in ethanol. The reaction mixture was shaken vigorously for 20 sec and left at room temperature for 10 min. Subsequently, the amount of DPPH remaining was determined using a UV-visible spectrophotometer (Mecasys, Korea) at 518 nm. DPPH free radical-scavenging activity (electron-donating activity) was calculated using the following formula:

$$\text{Electron donating ability (EDA) (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

TBA method. The level of lipid peroxidation, in terms of thiobarbituric acid reactive substances (TBARS), was estimated by TBA reaction. A mixture consisting of 80 mg sample in 5 ml ethanol or distilled water, 5 ml of 0.2 M phosphate buffer (pH 7.0), and 10 ml of 20% linoleic acid in ethanol was incubated in a vial at 40°C for 96 h. The mixture (2 ml) was combined with 1 ml of 35% TCA and 1.5 ml of 0.75% TBA, adjusted with distilled water to a final volume of 5 ml, and heated to 95°C for 40 min. After cooling to room temperature, 1 ml acetic acid and 2 ml chloroform were added to each sample, and the mixture (final conc. 1000 μg each sample/ml in a mixture) was shaken. After centrifugation at 3,000 rpm for 5 min, the supernatant was isolated, and the absorbance was measured spectrophotometrically at 532 nm. Lipid peroxidation inhibitory activity was calculated using the following formula:

$$\text{Inhibitory activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Cell culture. Raw 264.7 cells were cultured in DMEM with 10% FBS at 37°C in a CO₂ incubator (SANYO, Japan). BWE was dissolved in the culture medium. LPS was dissolved in distilled water and diluted in the culture medium.

Cell viability assay. Cell viability was determined by the MTT method. The cells were plated at 1×10^5 cells/well in 96-well plates and incubated for 24 h. One hour after BWE (final conc. 1~100 μg BWE/ml in a well) treatment, the cells were treated with LPS (final conc. 1 μg LPS/ml in a well) and incubated for 24 h. Subsequently, 10 μl MTT stock solution (5 mg \cdot mL⁻¹ in PBS) was added to the cells, which were then incubated at 37°C and 5% CO₂ for 4 h. The culture supernatant was removed, and the formazan crystals, formed from MTT by NADH-generating dehydrogenases in metabolically active cells, were dissolved in 150 μl DMSO : EtOH (1 : 1). Cell viability was evaluated in comparison to the control group (taken as 100%) by measuring the intensity of the blue color using a microplate reader (Bio-Tek, USA).

Measurement of NO production. Nitrite as the end product of NO was measured by the Griess reagent (1% sulfanilamine, 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), as an indirect assay procedure for NO production.¹⁹⁾ The cells were seeded at 1×10^5 cells/well in 96-well plates treated with LPS (final conc. 1 μg LPS/ml in

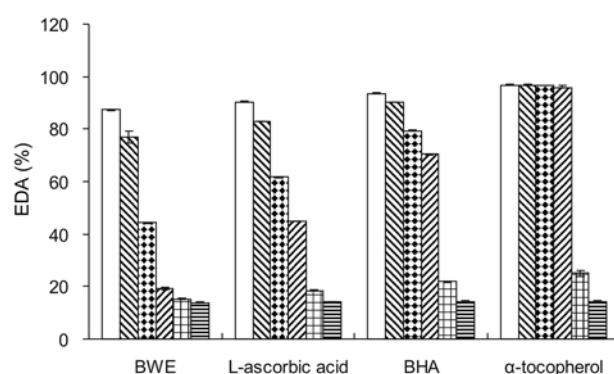


Fig. 1. Effects of BWE on DPPH free radical-scavenging activity. Values indicate means \pm S.E.M. (n = 3). \square , with final conc. 2000 μg each sample/ml in an assay mixture; ▨ , with final conc. 1000 μg each sample/ml in an assay mixture; ▩ , with final conc. 100 μg each sample/ml in an assay mixture; ▮ , with final conc. 10 μg each sample/ml in an assay mixture; ▭ , with final conc. 1 μg each sample/ml in an assay mixture; ▬ , with final conc. 0.1 μg each sample/ml in an assay mixture.

a well) for 24 h, and added with various concentrations of BWE (final conc. 1~100 μg BWE/ml in a well) 1 h before LPS treatment. The cells were then placed in a growth medium lacking phenol red and 50 μl culture medium was mixed with an equal volume of the Griess reagent and incubated at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 560 nm in a microplate reader (Bio-Tek, USA). The calibration curve was prepared using sodium nitrite (Sigma Chemicals Co.) as a standard.

Statistics. All experiments were repeated at least three times. Statistical analysis was performed using a one-way ANOVA, followed by LSD in SPSS version 10 (SPSS Inc., USA). p-Values less than 0.05 were considered significant.

Results and Discussion

Effect of BWE on DPPH free radical-scavenging activity. DPPH, a free radical, was used to investigate the effect of BWE on the radical-scavenging activity. DPPH directly abstracted hydrogen atoms from the phenolic compounds at a rapid rate. This reaction, which can be observed directly though the change in color from violet to yellow, can also be measured by the decrease in absorbance at 518 nm.²⁰⁾

Changes in DPPH free radical-scavenging activity with varying contents of BWE added were compared against the reference samples added with BHA, L-ascorbic acid, and α -tocopherol (Fig. 1). BWE caused a dose-dependant DPPH free radical-scavenging activity exhibiting the strongest scavenging activity (87.3%) at final conc. 2000 μg BWE/ml in an assay mixture, and 77.07 and 44.23% at final conc. 1000 and 100 μg /ml in an assay mixture, respectively. BHA and α -tocopherol showed stronger activity than BWE. Kang *et al.*²¹⁾ reported that the hot water extract of *Angelica gigas* showed 66.3% at final conc. 1000 $\mu\text{g} \cdot \text{mL}^{-1}$ and 27.1% inhibition at

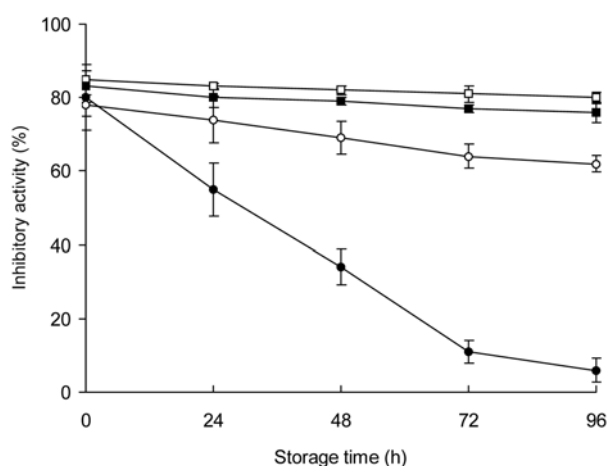


Fig. 2. Effects of BWE on inhibition lipid peroxidation during incubation at 40°C. Values indicate means \pm S.E.M. ($n = 3$). ○, BWE; ●, L-ascorbic acid; □, BHA; ■, α -tocopherol. All samples were measured at final conc. 1000 $\mu\text{g}/\text{m}^3$ in a mixture.

final conc. 100 $\mu\text{g} \cdot \text{m}^{-3}$ in the same assay system. These results demonstrate that even though BWE was lower than BHA or α -tocopherol in radical-scavenging activity, BWE also have strong DPPH free radical-scavenging activity.

Effect of BWE on lipid peroxidation. Effect of BWE on lipid peroxidation during incubation at 40°C are shown in Fig. 2. The inhibitory activity of BWE on lipid peroxidation increased time-dependently, 64% at final conc. 1000 $\mu\text{g}/\text{m}^3$ in a mixture after 72 h incubation. BHA and α -tocopherol also significantly inhibited lipid peroxidation, 81.66 and 77.54% at final conc. 1000 $\mu\text{g}/\text{m}^3$ in a mixture, respectively. However, L-ascorbic acid, a known antioxidant, showed higher lipid peroxidation than that of the positive control. Similar results were obtained by Franker *et al.*²²⁾ and Jang *et al.*²³⁾, who reported that hydrophilic antioxidants such as L-ascorbic acid and catechin were more effective in bulk oil than in oil-in-water emulsion system. In the oil-in-water system, the hydrophilic antioxidants are dissolved and become diluted during the water phase.²²⁾ These results suggest that, although the inhibitory activities on lipid peroxidation of BWE were lower than those of BHA or α -tocopherol, BWE nevertheless significantly inhibited lipid peroxidation.

Effect of BWE on cell viability. To confirm the effect of BWE on cell viability, the cells were initially treated with BWE (final conc. 1~100 μg BWE/ m^3 in a well) followed by stimulation with LPS (final conc. 1 μg LPS/ m^3 in a well). After 24 h incubation, cell viability was quantified. The control group showed 100% cell viability, whereas that of LPS-treated group was 78%. BWE and LPS-treated group showed 72%, 70%, and 69% cell viability at final conc. 1, 10, and 100 μg BWE/ m^3 in a well, respectively (Fig. 3). Results of this experiment revealed that BWE had a considerable effect on cytotoxic effects. BWE is still crude extract therefore, further research will be necessary for the identification of the effective compound.

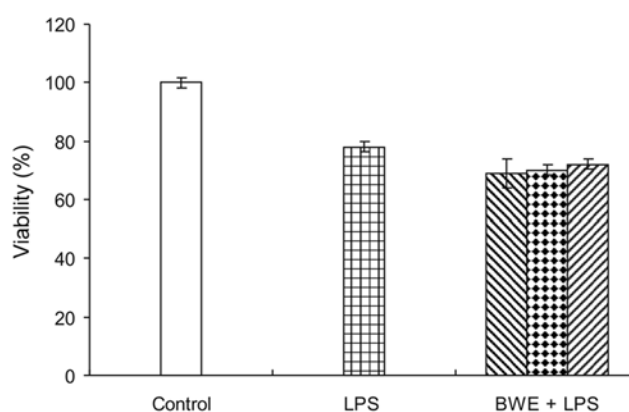


Fig. 3. Effects of BWE on cell viability in LPS-treated Raw 264.7 cells. Cell viability was determined by MTT method. The cells (1×10^5 cells/well) were plated on 96-well plates for 24 h, treated with LPS (final conc. 1 μg LPS/ m^3 in a well) for 24 h. BWE (final conc. 1~100 μg BWE/ m^3 in a well) was added 1 h before LPS treatment. Values indicate means \pm S.E.M. ($n = 3$). control (□), without BWE and LPS; LPS (▤), with final conc. 1 μg LPS/ m^3 in a well; BWE + LPS (▨), with final conc. 100 μg BWE and 1 μg LPS/ m^3 in a well; HWE + LPS (▧), with final conc. 10 μg BWE and 1 μg LPS/ m^3 in a well; HWE + LPS (▩), with final conc. 1 μg BWE and 1 μg LPS/ m^3 in a well.

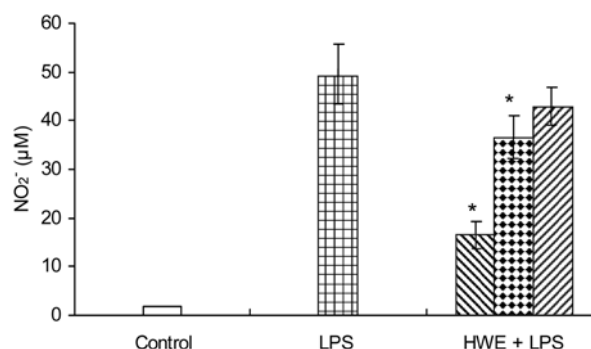


Fig. 4. Effects of BWE on NO production in LPS-treated Raw 264.7 cells. NO production was assessed using Griess reagent. The cells (1×10^5 cells/well) were plated and treated with LPS (final conc. 1 μg LPS/ m^3 in a well) for 24 h. BWE (final conc. 1~100 μg BWE/ m^3 in a well) was added 1 h before LPS treatment. Values indicate means \pm S.E.M. ($n = 3$). control (□), without BWE and LPS; LPS (▤), with final conc. 1 μg LPS/ m^3 in a well; BWE + LPS (▨), with final conc. 100 μg BWE and 1 μg LPS/ m^3 in a well; HWE + LPS (▧), with final conc. 10 μg BWE and 1 μg LPS/ m^3 in a well; HWE + LPS (▩), with final conc. 1 μg BWE and 1 μg LPS/ m^3 in a well. Significant differences from the control are $p < 0.05$ (*).

Effect of BWE on LPS-induced NO production. Excess NO reacts with superoxide to form spontaneously the potent oxidant peroxynitrite. Thus, inhibitory effects of NO production are related to antioxidant effects. To clarify whether BWE could inhibit LPS-induced NO production, BWE (final conc. 1~100 μg BWE/ m^3 in a well) was added to the cells 1 h prior to treatment with LPS (final conc. 1 μg LPS/ m^3 in a well) for 24 h and nitrite concentration was analyzed (Fig. 4). LPS treatment significantly increased NO concentration by

approximately 26.7-fold compared with the control group (1.83 μ M). The cells treated with BWE showed a reduction in LPS-induced NO production in a concentration manner. In particular, the cells treated with BWE (containing final conc. 100 μ g BWE/ml in a well) showed significant inhibitory effects. These results might indicate that BWE could have anti-inflammatory effect by reducing NO production. However, as NO production is regulated by iNOS in various cell types including macrophage, further investigation is needed to confirm whether the decreased NO production is correlated with anti-inflammatory effect. In addition to NO, many proinflammatory mediators such as tumor necrosis factor- α , interleukins, and prostaglandin E₂ have been suggested to be involved in various pathophysiological processes of inflammation.²⁴⁻²⁶ It is known that the anti-inflammatory action of drugs is achieved by inhibiting other pro-inflammatory mediators as well as NO.²⁷ In addition, further study is needed to determine anti-inflammatory effects in vivo, and to examine whether BWE could suppress pro-inflammatory mediators.

This study examined whether BWE can scavenge free radical, inhibit lipid peroxidation, and suppress NO production in LPS-treated macrophages. It was demonstrated that BWE has antioxidant effects by showing the activities of free radical-scavenging and lipid peroxidation inhibiting. It is also likely that BWE can exert anti-inflammatory effect through the suppression of NO production.

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