Original Research Article

Korean J. Plant Res. 23(3) : 207～213 (2010)

Antioxidant Activities of Various Extracts of *Hovenia dulcis* Thunb Fruits

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Abstract - *Hovenia dulcis* Thunb fruits were successively extracted with hot water, water, methanol, ethyl acetate, and chloroform. The crude extracts were investigated for potential antioxidant by measuring scavenging against DPPH free radicals, reducing power, superoxide radicals, and protection of protein damage and cultured cells from a lethal dose of hydrogen peroxide (H_2O_2). In all chemical assays used, the hot water extract of *H. dulcis* fruits, which contained 61.14 ± 2.57 (Tannic acid mg/g extract, n=3) of total phenolic compounds contents exhibited highest activity in *in vitro* models of DPPH free radical scavenging activity, reducing power assay, superoxide radical scavenging activity and protection of protein damage. In addition, the hot water extract protected cultured RAW 264.7 macrophages from a lethal dose of H_2O_2 and reduced reactive oxygen species level in RAW 264.7 cells.

Key words - *Hovenia dulcis* fruit, Antioxidant, Reactive oxygen species, Protein damage

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) including free radicals such as superoxide radical anion ($\bullet O2^-$), hydroxyl radicals ($\bullet OH$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) are constantly produced continuously in the cells of the human body (Fang *et al.*, 2002). Oxidation is essential in living organisms to obtain the energy needed for biological processes. However, excessively high levels of free radicals or ROS create oxidative stress, which leads to produce some detrimental effects, including lipid peroxidation of cellular membranes, alteration of lipid–protein interaction, enzyme inactivation and DNA breakage, and in the end, to cause cell injury, necrosis or apoptosis (Lefebvre *et al.*, 2002; Agarwal *et al*., 2003). Although human body has multiple antioxidant systems to protect the cellular molecules against the oxygen radicals induced damage. These defense mechanisms include antioxidative enzymes, such as superoxide dismutase, catalase and glutathione peroxidase. The innate defense is not enough for severe oxidative stress, and therefore overproduction of oxidative radicals may cause tissue damages (Blokhina *et al*., 2003; Lassen *et al.,* 2008). Consequently, certain amounts of exogenous antioxidants are constantly required to retain an adequate level of antioxidants in order to balance the ROS in human body (Reiter *et al.,* 1997). Therefore, much attention has been focused on natural antioxidants. Plants contain a variety of free radical scavenging molecules such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and other endogenous metabolites that are rich in antioxidant activity (Su *et al.*, 2009; Huang *et al.*, 2010).

Hovenia dulcis Thunb, belongs to Rhamnaceae family, is wildly distributed in East Asia. It has been used in traditional folk remedies for the treatment of liver diseases and detoxification of alcoholic poisoning. *H. dulcis* can remarkably reduce alcohol concentration in blood; thereby alleviate alcoholic liver tissue (Hase *et al.*, 1997; Kim, 2001). Some pervious literatures showed that *H. dulcis* possessed antioxidative, antimicrobial, anti-diabetic, and anticancer activities (Wang *et al*., 1994; Lee *et al.*, 1999; Cho *et al.*, 2000; Ji *et al.*, 2002). The fruit of *H. dulcis* is edible raw or cooked. Some researchers reported that the fruits of *H. dulcis* own hepatoprotective activity (Ko *et al.*, 2006). Nevertheless, research on the directly free radical scavenging activity the fruit of *H.*

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dulcis has not been reported.

To investigate the potential antioxidant properties of fruits of *H. dulcis*, we utilized a number of *in vitro* assay systems, including DPPH free radical scavenging activity, reducing power assay, superoxide radical scavenging assay, and protective ability on protein oxidation and H_2O_2-injured RAW 264.7 cell death.

Material and Methods

Chemicals and reagents

L-Ascorbic acid, DPPH (1,1-diphenyll-2-2-pricylhydrazyl), ferrous chloride, 2N folin-ciocalteu's phenol reagent, tannic acid, α-tocopherol, and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide, gallic acid, and sodium carbonate were purchased from Junsei (Junsei Chemical Co., Ltd., Tokyo, Japan). Iron (III) chloride hexahydrate was purchased from Cicareagent (Kanto Chemical Co., Ltd., Tokyo, Japan). RPMI medium 1640, fetal bovine serum (FBS), and trypsin-EDTA were acquired from Gibco BRL (Grand Island, NY, USA). The culture supplies (e.g., 96-well plates) were obtained from SPL Brand Products (SPL, Korea). All other unlabelled chemicals and reagents were of analytical grade.

Sample and sample preparation

H. dulcis fruits were harvested in October 2008 from 11-years old plant by Korea forest service. Fruits were dried in the shade at room temperature and then powdered. One hundred grams of the fruit powder were extracted separately with distill water, absolute methanol, ethyl acetate, chloroform at 50°C and with hot water for 3 hours. The hot water (BWE), water (WTE), methanolic (MTE), ethyl acetate (ETE) and chloroform extracts (CTE) were filtered (110-mm Whatman filter paper; Maidstone, UK) and evaporated under reduced pressure using a vacuum rotary evaporator (CCA-1110; Eyela, Tokyo, Japan). The dried samples were weighed and kept in a refrigerator until further analysis.

Determination of the total phenolic compounds content

The total phenolic compounds contents were determined using the Folin-Ciocalteu reagent. An aliquot of each sample (1 ml) was mixed with 1.8 ml of Folin-Ciocalteu reagent, and the solution was allowed to stand at 25°C for 5 min before the addition of 1.2 ml of a 15% sodium carbonate solution. Tannic acid (Tan) was used as the standard to create a calibration curve. The total phenolic content is expressed as mg Tan/g extract.

DPPH free radical scavenging activity

The free radical scavenging activity of text sample was evaluated by the DPPH test. Briefly, 0.5 ml of 0.1 mM DPPH (in methanol) was added to a test tube containing 0.5 ml of the sample. The mixture was then shaken vigorously for 1 min and kept at room temperature for 30 min in the dark. The absorbance of each sample solution was measured at 515 nm using a multiplate spectrophotometer (ELx800TM, BioTek, Winooski, VT, USA)

Reducing power assay

The reducing power was determined as described previously by Hu *et al.* (2009). One milliliter of sample was mixed with 2.5 ml of sodium phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.2 M, pH 6.6) and 2.5 ml of 0.1% potassium ferricyanide, and the mixture was incubated at 50°C for 30 min. After the addition of 2.5 ml of 10% TCA, the mixture was centrifuged at 3,000 ×g for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm.

Superoxide radical scavenging assay

The superoxide radical scavenging activity was determined by the PMS-NADH generating system which was described by Singh and Rajini. (2004) with minor modifications. Briefly, 100 μl of the extract solution was mixed with 150 μl of NADH (166 μM), 450 μl of NBT (86 μM) and 150 μl of PMS (16.2 μM). After 5 min of incubation at room temperature, the absorbance at 560 nm was measured. Gallic acid at the concentration of 0.5 mg/ml was used as a positive control.

Protein damage protection assay

The effects of sample on protein oxidation were carried out according the method of Kızıl *et al.* (2009). BSA was oxidized by a H_2O_2/Fe^{3+}/ascorbic acid system. The reaction mix-

ture (1.0 ml), containing sample (1 mg/ml), phosphate buffer (20 mM pH 7.4), BSA (1 mg/ml), $FeCl_3$ (50 μM), H_2O_2 (1 mM) and ascorbic acid (100 μM) were incubated for 3 hours at 37°C. After incubation, the reaction mixture was analyzed by electrophoresis in 10% SDS polyacrylamide gel. The gel was stained with brilliant blue R staining solution for 2 hours and then destained and digitally photographed. To determine the amount of protein damage, band intensity was estimated using the image analysis software (Quantity One, Bio-Rad, Hercules, CA).

Cytotoxicty and cytoprotective activity of DIM

The cytotoxicity of sample was investigated. RAW 264.7 cells were exposed to 1 mg/ml sample for 24 hours. Then, a total of 20 μl of MTT solution [2 mg/ml in phosphate-buffered saline (PBS)] was added to each well at the time of incubation. After 4 hours of incubation, the supernatant was discarded and 200 μl of DMSO was added to each well to terminate the reaction. Absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, Winooski, VT, USA). Cytoprotective activity of sample on H_2O_2 induced cell injury was investigated by MTT assay (Hu and Wang, 2009). In total, 2×10^4 cells were plated per well in 96-well plates with culture medium for 16 hours and then exposed to test sample for 30 min before exposure to 4 mM H_2O_2 for 3 hours. The cell viability was measured by MTT method as described previously.

Determination of the ROS level

RAW 264.7 cells (1×10^5) were treated with 4 mM H_2O_2 in the presence of BWE for 3 hours at 37°C. The cells were detached and washed with PBS. Cells were treated with 20 μM DHR 123 for 30 min in the dark. Intracellular ROS were measured via flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For each sample, 10,000 cells were analyzed using three replicates.

Data analysis

All tests were carried out independently in triplicate (n = 3). The data are expressed as the mean ± standard derivation (SD). All analyses were performed using SPSS 16 (SPSS Institute, Cary, NC, USA); individual comparisons were made using Tukey's multiple-range test, which was used to determine the differences between the means.

Results and Discussion

Total phenolic compounds content

Yields of *H. dulcis* fruits obtained from various solvents were as follows: hot water gave the highest yield (21.6%), followed by water (18.7%), methanol (14.3%), ethyl acetate (4.3%), and chloroform (3.5%). Furthermore, the data of extraction yields revealed that water and methanol could solubilise more materials from *H. dulcis* fruits. This indicated that most of the soluble compounds in the fruit were high in polarity. The total phenolic content in each *H. dulcis* fruits extract was presented in Table 1. The total phenolic content in BWE, WTE, MTE, ETE, and CTE was 61.14, 44.55, 33.79, 32.96 and 42.20 mg Tan equivalent/g, respectively. Notably, BWE had significantly higher total phenolic compounds contents than the other extracts. It is reported that intense heat from hot water was able to release cell wall phenolics or bound phenolics due to the breakdown of cellular constituents, thus causing more polyphenols to be extracted (Lim and Murtijaya, 2007).

Table 1. Total phenolic contents of obtained from *H. dulcis* fruits

Extracts	Yield (%)*	Phenolic content (Tan mg/g extract)**
BWE	21.6	61.14 ± 2.57^a
WTE	18.7	44.55 ± 3.54^b
MTE	14.3	33.79 ± 0.54^c
ETE	4.3	32.96 ± 2.14^c
CTE	3.5	42.20 ± 1.93^b

*Extraction yield (%) is expressed as: (sample extract weight / sample weight) × 100.

**Tan means tannic acid equivalent. Values with different letters differ significantly (p<0.05).

DPPH free radical scavenging activity

Free radicals are known as the major cause of oxidative damage of biological molecules in human body, including coronary heart disease, aging, cancer and dementia (Chen *et al.*, 2006). DPPH is a stable free radical compound, which has been widely used as a substrate to evaluate the antioxidative action of the antioxidants (Leong and Shui, 2002). The radical-scavenging activity of *H. dulcis* fruits was shown in Table 1.The values for the DPPH radical scavenging activity of BWE, WTE, MTE, ETE, and CTE were 25.72, 9.56, 12.72, 14.11 and 14.21% at 100 μg/ml, respectively. Based on the DPPH free radical scavenging activity results, BWE showed the highest DPPH scavenging activity. However, scavenging activity of α-tocopherol, known antioxidant, used as positive control, was relatively more pronounced than that of samples. Alcohol-induced liver injury model was made by Ji and Li (2000) to investigate hepaprotective effect of *H. dulcis* fruits on alcohol-induced liver injury. The biochemical indexes such as AST and ALT in blood serum and MDA, GSH, cholesterol, and TG from mice liver remarkably reduced after treatment with *H. dulcis* fruits.

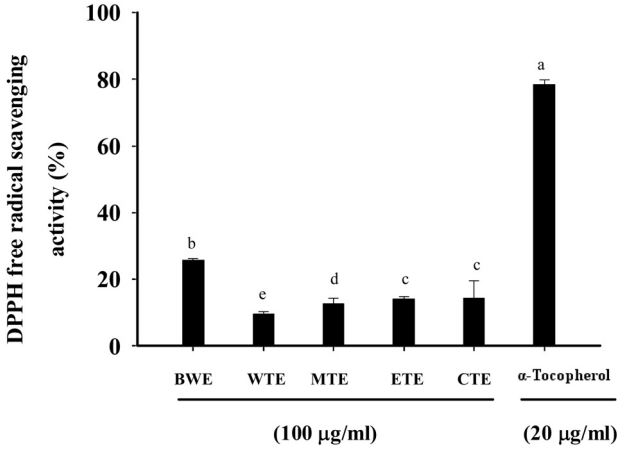

Fig. 1. DPPH free radical scavenging activity of extracts obtained from *H. dulcis* fruits and α-tocopherol. Concentration of extracts was 100 μg/ml. α-Tocopherol (20 μg/ml) was used as the positive control. Each value is the mean ± SD of triplicate measurements. Values with different letters differ significantly ($p<0.05$).

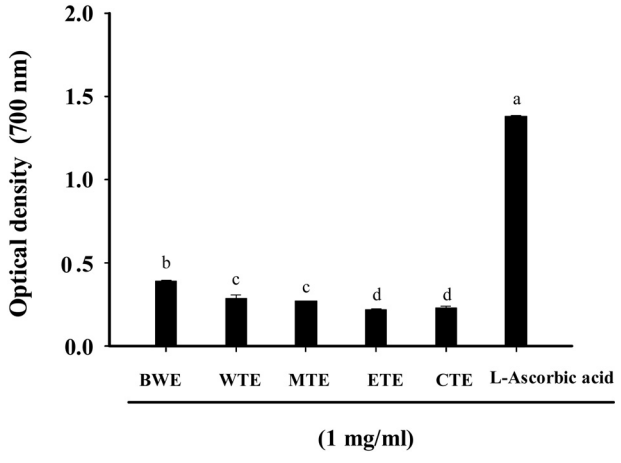

Fig. 2. Reducing power activity of extracts obtained from *H. dulcis* fruits and L-ascorbic acid. Concentration of extracts was 1 mg/ml. α-Tocopherol (1 mg/ml) was used as the positive control. Each value is the mean ± SD of triplicate measurements. Values with different letters differ significantly ($p<0.05$).

Reducing power assay

There's a positive correlation between antioxidant activity and reducing power. The reducing activity is associated with the presence of reductones, which act as primary and secondary antioxidants; break the free radical chain by donating a hydrogen atom (Duh, 1998; Tanaka *et al.*, 1998). For the measurement of the reductive activity, we investigated the Fe^{3+}-Fe^{2+} transformation in presence of sample. As shown in Fig. 2, the extracts *H. dulcis* fruits can react with free radials to covert them to stable products, leading to termination of radical chain reaction. The higher reducing ability of BWE may be due to the high phenolic content which may act as reductones by donating electors to free radicals.

Superoxide radical scavenging activity

Superoxide radical is very harmful to cellular components as a precursor of ROS, such as single oxygen and hydroxyl radicals. In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents. Scavenging of superoxide radicals is one of the most important ways of clarifying the mechanism of antioxidant activity (Chang *et al.,* 2001). Hence, a NBT assay was carried out to test studying the scavenging effects of samples on superoxide radicals. As shown in Fig. 3, the scavenging effects of test samples (1 mg/ml) on superoxide radical were found to be 74.17, 55.28, 46.97, 17.16 and

14.38% for BWE, WTE, MTE, ETE and CTE, respectively. Gallic acid was used as the positive control showed 78.68% superoxide scavenging activity at the concentration of 0.5 mg/ml. These results indicated that BWE had a notable effect on scavenging of superoxide radicals, but a bit weaker than that of gallic acid.

Protein damage assay

Because of their abundance in biological systems, proteins are a major target for oxidants. The protein damages induced by free radicals have been demonstrated to play a significant role in aging and other pathological events (Du and Gebicki, 2004). Radical-mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions, and autoxidation of lipids and sugars (Bahramikia *et al*., 2009). Electrophoretic patterns of BSA after incubation with Fe^{3+}/H_2O_2/ascorbic acid system in the presence of the samples was shown in Fig. 4. The density of BSA band decreased to 31.2% of control after incubated with Fe^{3+}/H_2O_2/ascorbic acid. A 1 mg/ml of BWE, WTE, MTE, ETE and CTE restored BSA band intensity to 81.6, 69.6, 55.9, 11.1 and 17.6% of the control levels, respectively. These results suggest BWE is more potent in Fe^{3+}/H_2O_2/ascorbic acid induced protein damage.

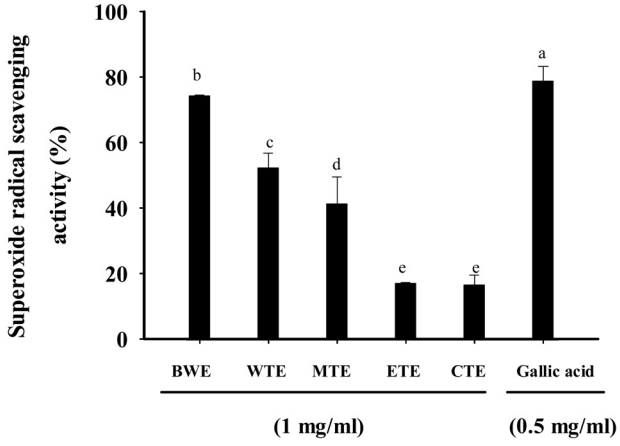

Fig. 3. Superoxide radical scavenging activity of extracts obtained from *H. dulcis* fruits and gallic acid. Concentration of extracts was 1 mg/ml. Gallic acid (0.5 mg/ml) was used as the positive control. Each value is the mean ± SD of triplicate measurements. Values with different letters differ significantly (p<0.05).

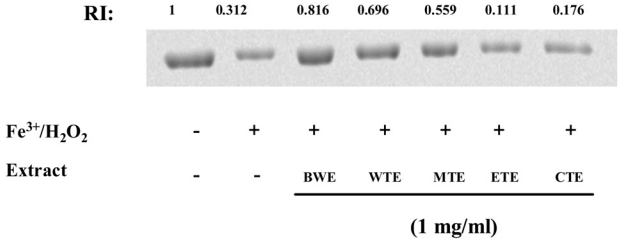

Fig.4. Visualization of the damage induced by oxidative stress on protein in the presence of extracts obtained from *H. dulcis* fruits by SDS-PAGE. Line 1, protein incubated without free radicals; Line 2, protein incubated with free radicals; Lines 3-7, protein incubated with free radicals in the presence of extracts obtained from *H. dulcis* fruits.

Cytotoxicity and cytoprotective assay

H_2O_2, one of the main ROS, is generated during the redox process and is reported to function as messenger in cell signaling pathway (Rhee, 1999). In particular, it is well known that H_2O_2 could be produced from nearly all sources of oxidative stress and can enhance apoptosis in many different cell types through lipid peroxidation and DNA damage (Li *et al.*, 2003). We examined the effect of sample on the viability of RAW 264.7 cells. H_2O_2-induced cytotoxic injury to RAW 264.7 cells were quantified by MTT assay. As shown in Fig. 5A, there was no apparent cytotoxic or inhibitory effect on the growth of RAW 264.7 cells incubated with BWE, WTE, or MTE. As illustrated in Fig. 5B, exposure to 4 mM $H2O_2$ for 3 hours decreased cell viability to 34.23% of the control group, while pretreatment of BWE, WTE, and MTE prevented cells from H_2O_2- induced damage, restoring cell survival to 89.32, 76.24, and 56.60%, respectively.

ROS level

As it has been stated that ROS in moderate concentrations exerts some functions is necessary for cell homeostasis maintenance. However, when produced in excess they readily damage biological moleculars and ultimately lead to apoptotic or necrotic cell death (Jang *et al.,* 2009). Due to BWE showed higher cytoprotecitve activity on H_2O_2 induced RAW 264.7 cell death; we determine whether BWE could prevent H_2O_2-induced ROS generation. RAW 264.7 cells exposed to H_2O_2 for 3 hours displayed a significant increase in the intracellular level of ROS compared with untreated cells (more

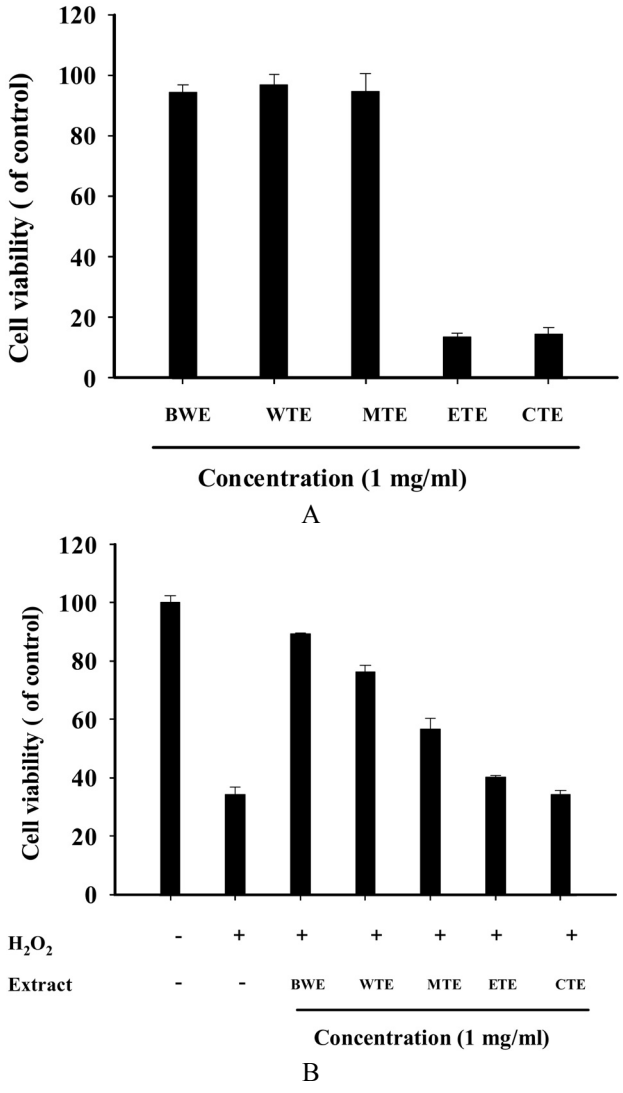

Fig. 5. Cytotoxicty and cytoprotective activity of extracts obtained from *H. dulcis* fruits. RAW 264.7 cells was incubated with different extracts for 24 hours (Fig. 5A). RAW 264.7 cells were pretreated with different extracts before exposure to 4 mM H_2O_2 for 3 hours (Fig. 5B). Then, cell viability was measured by MTT assay.

than 2 fold). RAW 264.7 cells pretreated with 1 mg/ml BWE before H_2O_2 exposure markedly reduced the ROS levels in RAW 264.7 cells (Fig. 6). However, 1 mg/ml of BWE has no significant effect on the ROS levels of the RAW 264.7 cells (data not shown).

The present study clearly demonstrated that hot water extract had good effect on DPPH free radical scavenging activity, reducing power assay, superoxide radical scavenging assay,

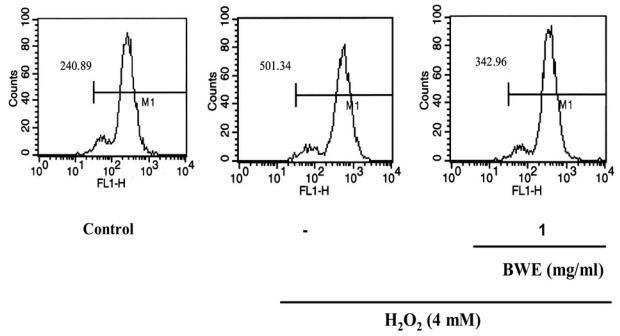

Fig. 6. Effect of BWE on H_2O_2-induced intracellular accumulation of ROS. Intracellular ROS levels were measured based on the hydrogen peroxide-sensitive DHR 123 fluorescence. RAW 264.7 cells were pretreated with 1 mg/ml of BWE before exposure to 4 mM H_2O_2 for 3 hours. Data were expressed as the mean fluorescent intensity (MFI).

and protective ability on protein oxidation and H_2O_2-injured RAW 264.7 cell death. An activity-directed methods leads to identification of hot water extract as the most potent radical scavenging extraction way. Additional studies are currently underway to assess the *in vivo* antioxidant and to identify the active compounds in this plant.

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(Received 2 March 2010 ; Accepted 16 June 2010)