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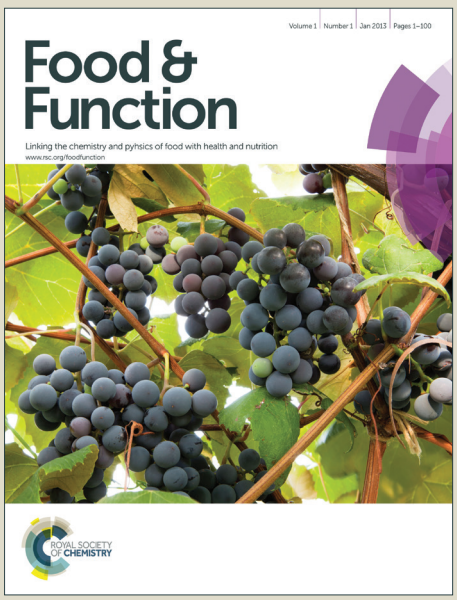

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Running title: Hepatoprotective effect of *N. nucifera* leaves

Nelumbo nucifera leaves protects hydrogen peroxide-induced hepatic damage via antioxidant enzyme and HO-1/Nrf2 activation

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ABSTRACT

Naturally occurring phenolic compounds are widely found in plants. Here, phenolic composition and hepatoprotective effect of butanolic extract (BE) from *Nelumbo nucifera* leaves against H_2O_2-induced hepatic damage in cultured hepatocytes were investigated. BE showed high total phenol and flavonoid contents, and major phenolic compounds are quercetin, catechin, ferulic acid, rutin, and protocatechuic acid by HPLC analysis. BE effectively scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) cation radical (IC_{50} values of 5.24 μg/mL for DPPH and 6.22 μg/mL for $ABTS^{+}$) and showed strong reducing power. Pretreatment of BE prior to 650 μM H_2O_2 exposure markedly increased cell viability and suppressed H_2O_2-induced intracellular reactive oxygen species generation and AAPH-induced cell membrane lipid peroxidation. Additionally, BE up-regulated intracellular glutathione levels under normal and oxidative stress conditions. Notably, the hepatoprotective effect of BE was directly correlated with the increased expression of superoxide dismutase-1 (SOD-1) by 0.62-fold, catalase (CAT) by 0.42-fold, and heme oxygenase-1 (HO-1) by 2.4-fold. Pretreatment of BE also increased nuclear accumulation of Nrf2 by 8.1-fold indicating that increased SOD-1, CAT, and HO-1 expressions are Nrf2-mediated.

***Keywords*: *Nelumbo nucifera*, phenolic composition, hepatoprotection, HO-1, Nrf2**

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Introduction

In the recent years, there has been an immense research with regard to the use of natural antioxidants from fruits, vegetables, and herbs to suppress oxidative stress. *Nelumbo nucifera* leaves had been used widely for food and folk medicine and are becoming popular as an ingredient of antioxidant beverages and tea bags in China and Korea. The major components of *N. nucifera* leaves are flavonoid and other phenolic compounds such as heparin, isoquercetin, catechin, quercetin, and kaempferol and are reported to exhibit antioxidant, antiphototoxicity, antifungal and antiobesity.[1-5] *N. nucifera* leaves exerted the antioxidant activity through scavenging hydroxyl radical, reducing power, inhibiting low-density lipoprotein oxidation, and protected H_2O_2-mediated cell cytotoxicity via antioxidant action.[1,2,6] In addition, hepatoprotective effects have been associated with plant extracts rich in antioxidants.[3,7] However, to the best our knowledge, the hepatoprotective effect of *N. nucifera* leaves in H_2O_2-mediated hepatocyte damage has not been exactly elucidated against the expressions of antioxidant and phase II detoxifying enzymes via transcriptional activation of NF-E2-related factor-2 (Nrf2).

Nrf2 is a transcriptional regulator of antioxidant response element (ARE)-driven antioxidant gene expression and is generally inactivated by binding of Kelch-like ECH-associated protein 1(Keap 1), which facilitates the degradation of Nrf2 through ubiquitinated proteasomal degradation, under normal condition.[8] Upon stimulation by stimuli or antioxidants, Nrf2 dissociates from Keap 1, and then free Nrf2 translocates to the nucleus to form heterodimer with small Maf protein. Heterodimer finally binds to ARE to activate the promoter region of many genes encoding phase II detoxifying enzymes and antioxidants, such as hemoxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1, and glutamate-cysteine ligase as well as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH).[8,9] These enzymes and antioxidants are playing an important role in cell protection against oxidative stress caused by ROS that are produced during normal oxygen metabolism. Thus, the induction of phase II detoxifying or antioxidant enzymes is one of the most important components of cellular defense mechanism.[10] It has been demonstrated that plant-derived polyphenols can stimulate

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the Nrf2/ARE-mediated expression of phase II detoxifying or antioxidant enzymes.[8,10-12] Thereby, the present study was carried out in order to understand the molecular mechanism underlying hepatoprotective activity of polyphenol-rich butanolic extract from *N. nucifera* leaves against H_2O_2-induced hepatocyte damage via measuring cellular antioxidant activity and Nrf2/ARE-driven antioxidant gene expressions.

Materials and methods

Materials

Powdered Nelumbo nucifera leaves were purchased from local farm in August 2012 (Muan, Korea). Folin-Ciocalteu's phenol reagent, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), potassium ferricyanide, DPPH, ABTS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standard phenolic compounds (gallic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, sinapic acid, catechin, rutin, and quercetin) for HPLC analysis were purchased from Sigma Chemical Co. Monobromobimane (mBBr), diphenyl-1-pyrenylphosphine (DPPP), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Molecular Probes Inc. (Eugene, OR, USA). The other materials required for cell culture were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

Preparation of *N. nucifera* leaves extract

The dried powders from lotus leaves (20 g) were extracted three times with 2 L of 80% ethanol at 80°C for 3 h. The extracts were combined and concentrated to dryness under reduced pressure. The ethanolic extract was obtained by soxhlet extraction from powdered *N. nucifera* leaves, and solvent was eliminated by reduced-pressure evaporation in rotary evaporator. The butanolic extract was obtained by the partition of ethanolic extract in *n*-butanol-water mixture. The butanol phase was

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evaporated by reduced-pressure evaporation and finally freeze-dried to yield of 4.44%. The dried extract was kept in airtight bottles at 4°C in a refrigerator until use.

Determination of total phenolic content and total flavonoid content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method.[13] Gallic acid (G7384, purity >98.5%, Sigma Co.) was used as a standard and results were expressed as μg gallic acid equivalents (GAE)/g butanolic extract (BE). Total flavonoid content (TFC) was determined using the method described by Meda et al.[14] Quercetin (337951, purity >95%, Sigma-Aldrich) was used as a standard and results were expressed as μg quercetin equivalents (QUE)/g BE.

Analysis of phenolic compounds by HPLC

Butanolic extract (BE) was hydrolyzed with 1 N HCl and heated at 85°C for 1 h. Samples were allowed to cool down to room temperature and then centrifuged at 3000*g* for 5 min. The supernatant was filtered using Millipore membrane (0.22 μm) and subjected to HPLC analysis as described in our previous report.[15] After filtration, 20 μL of the solution was injected into the HPLC system. Chromatographic separation was performed using a reverse phase column (Luna C18(2), 150 × 3.0 mm, 3 μm, Phenomenex, Torrance, CA). The mobile phase consisted of methanol (solvent A) and 0.1% formic acid (solvent B). The non-linear gradient elution used was as follow: A/B(10:90) to (15:85) at 5 min, (23:77) at 25 min, (50:50) at 30 min, and then hold for 5 min. The flow rate was 0.34 mL/min and peaks are detected at 270 nm. Bioactive components were identified by the retention time and the UV spectra of standards.

Measurement of DPPH scavenging activity

Antioxidant activity was evaluated by DPPH scavenging assay modified from that of Blois.[16] A 100 μL of DPPH solution (150 μM in methanol) was incubated with 100 μL of BE, and the mixtures were then kept in the dark for 30 min. The absorbance was measured at 517 nm on microplate reader

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(SpectraMax M2/M2e; Molecular Devices, Sunnyvale, CA, USA). IC_{50} value, which is the concentration required to scavenge DPPH by 50%, was determined by non-linear regression method.

Measurement of $ABTS^+$ radical scavenging activity

$ABTS^+$ radical stock solution was prepared by incubation of 7 mM ABTS with 2.4 mM potassium persulfate for 16 h in the dark.[17] The stock solution was diluted to working solution with absorbance of 1.50 ± 0.05 at 414 nm. A 50 µL of BE and 150 µL of $ABTS^+$ radical working solution were mixed, incubated for 10 min, and absorbance was measured on microplate reader (SpectraMax M2/M2e). IC_{50} value, which is the concentration required to scavenge $ABTS^+$ radical by 50%, was determined by non-linear regression method.

Determination of reducing power

The reducing power was determined using the method described by Oyaizu.[18] Briefly, BE were mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%, w/v). The mixture was incubated at 50°C for 20 min. Next, 0.5 mL TCA (10%, w/v) was added to the mixture, which was centrifuged at 1036 × *g* for 10 min. Finally, 0.5 mL of the supernatant was mixed with 0.5 mL distilled water and 0.1 mL $FeCl_3$ (0.1%, w/v), and absorbance was measured at 700 nm.

Cell culture

The hepatocytes (Chang liver cells) were obtained from the American Type Culture Collection (ATCC CCL-13, Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin, and 5% CO2 humidified atmosphere at 37°C.

Cell viability and hepatoprotective effect of butanolic extract under oxidative stress

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Cell viability was measured through blue formazan by the mitochondrial respiration-dependent reduction of MTT. Cells (1×10^4 cells/well) in 96-well plates were treated with various concentrations of BE (10, 50, 100 and 200 μg/mL) at 37°C for 24 h. After aspiration of medium, 100 μL of MTT solution (1 mg/mL) was added to each well, followed by incubation for 4 h at 37°C. The medium was discarded and the formazan crystals in viable cells were dissolved in 100 μL DMSO. The optical density of each well was measured at 540 nm using a microplate reader (SpectraMax M2/M2e).

To examine hepatoprotective effect of butanolic extract, the cells were pretreated with BE (0.01-0.1 μg/mL) for 2 h and then washed thrice with phosphate buffered saline (PBS). The cells were then exposed to 650 μM H_2O_2 to give oxidative stress, followed by incubation for 24 h at 37°C. After 24 h incubation, MTT assay was performed as described above method.

Measurement of ROS generation

Intracellular ROS in H_2O_2-stimulated the hepatocytes was analyzed by staining using DCFH-DA. Cells were stained with 20 μM of DCFH-DA for 20 min, followed by addition of BE (0.01-0.1 μg/mL) for 2 h. Cells were rinsed with PBS, then 650 μM H_2O_2 was added to each well. Fluorescence due to oxidative formation of 2',7'-dichlorogluorescin (DCF) by ROS was measured after 30, 60, and 90 min at excitation and emission wavelengths of 485 and 528 nm, respectively.[19]

Measurement of lipid peroxidation

DPPP fluorescence probe was used to estimate the amount of lipid peroxidation in the hepatocyte induced by AAPH. Cells were grown in 100-mm diameter dishes and washed with PBS, followed by staining of 13 μM DPPP in DMSO and incubation for 30 min at 37°C in the dark. Cells were seeded into a 96-well plate at 4×10^5 cells/mL using serum-free media after washing with PBS and incubated for complete attachment. Cells were pretreated with BE (0.01-0.1 μg/mL) for 2 h and then challenged with 3 mM AAPH in PBS to initiate cell membrane lipid peroxidation. DPPP oxide fluorescence

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intensity after 6 h was measured at excitation and emission wavelengths of 361 and 380 nm, respectively.[19]

Measurement of glutathione (GSH) level

Intracellular GSH level was measured by using a thiol-staining agent, mBBr. Cells were plated in a 96-well plate at 4×10^5 cells/mL, followed by incubation for confluence and then pretreated with BE (0.01-0.1 μg/mL) for 2 h. Cells were rinsed with PBS thrice, and then 650 μM H_2O_2 was added to each well for 2 h to give oxidative stress, followed by staining of 40 μM mBBr for 30 min. Fluorescence due to mBBr-GSH was measured excitation and emission wavelengths of 360 and 465 nm, respectively.[19]

Western blot analysis

Whole cell protein lysates were extracted in RIPA buffer (Sigma Chemical CO.) and nuclear extract were extracted by using nuclear protein extraction kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Equal amounts of protein (30 μg) were loaded and separated on 8-12% SDS-PAGE and then transferred to PVDF membrane and blocked with 5% skim milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.02% Tween-20) for 1 h. PVDF membranes were incubated with primary antibodies against SOD-1, CAT, HO-1 or Nrf2 overnight at 4°C. After the membrane was washed four times for 15 min each with TBST buffer, it was incubated in the appropriate HRP-conjugated secondary antibody for 2 h. Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Pierce Biotechnology, Rockford, IL, USA). The bands were imaged on Davinch-Chemi™ imaging system (Core Bio, Seoul, Korea). The basal levels of the proteins were normalized by analyze the level of β-actin protein.

Statistical analysis

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The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). Differences between means of each group were assessed by one-way analysis of variance followed by Duncan's test using PASW Statistics 19.0 software (SPSS, Chicago, IL, USA). A *P*-value < 0.05 was considered statistically significant.

Results and discussion

Determination of TPC, TFC, and phenolic composition of BE

Polyphenol-rich BE from *N. nucifera* leaves was prepared, and TPC, TFC, and compositions of phenolic compounds in BE were determined by HPLC. As summarized in Table 1, BE has high TPC and TFC as 406.12±13.56 mg GAE/g BE and 216.40±4.44 QUE/g BE, respectively. HPLC analysis was further conducted to determine phenolic compounds, and five benzoic acid derivatives (gallic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid, and syringic acid), six cinnamic acid and/or derivatives (caffeic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid and sinapic acid), and three flavonoids (catechin, rutin, and quercetin) were detected by using the fourteen standard phenolic compounds (Table 1). Among the selected phenolics detected in BE the major component was quercetin (6289.1±0.4 mg/100 g BE) followed by catechin (428.4±0.5 mg/100 g BE), ferulic acid (317.2±0.3 mg/100 g BE), rutin (241.2±0.3 mg/100 g BE), and protocatechuic acid (224.9±0.1 mg/100 g BE). Sinapic acid, *p*-coumaric acid, and syringic acid were also detected as plentiful phenolic acids in BE.

Phenolic compounds occur broadly in plants and their central role in plants defense against herbivory. The benefits of phenolic compounds are antioxidant actions through scavenging ROS and free radicals, thus these compounds are considered to be one of the most potent and therapeutically useful biocompounds since they have a wide range of important biological and therapeutic properties including antiinflammatory, antibacterial, antidiabetic, anticarcinogenic, antiaging, and neuroprotective effects.[20] Thus, in the present study, we carried out to determine hepatoprotective effect of polyphenol-rich BE from *N. nucifera* leaves against H_2O_2-induced hepatocyte damage.

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Antioxidant activities of BE from *N. nucifera* leaves

Antioxidant activities of BE from *N. nucifera* leaves were investigated before evaluation of hepatoprotective effect against H_2O_2-induced hepatic damage. DPPH, $ABTS^+$ radical scavenging activity and reducing power of BE from *N. nucifera* leaves were summarized in Table 2. BE showed a strong antioxidant activity by effectively scavenging the DPPH and $ABTS^+$ radicals and possessed the IC_{50} values of 5.21 μg/mL for DPPH and 6.22 μg/mL for $ABTS^+$ radicals. Ascorbic acid was used as a positive control (3.04 and 3.45 μg/mL). BE also showed a strong reducing power and the optical density at 700 nm was 0.234, whereas ascorbic acid was 0.218.

Antioxidant assays employed in the present study were strongly related with hydrogen and/or electron donating ability of antioxidant compounds. Plants are widely used as primary source of antioxidants and possessed various phenolic compounds. Kaur et al.[21] reported that methanolic extract from *Pteris vittata* L. showed a strong antioxidant activity against DPPH with IC_{50} value of 103.37 μg/mL and $ABTS^+$ radical with IC_{50} value of 64.42 μg/mL and possessed ellagic acid, rutin, caffeic acid and epicatechin in appreciable amount. In the present study, our results also suggested that BE from *N. nucifera* leaves are potent antioxidant source.

BE from *N. nucifera* leaves attenuated H_2O_2-induced hepatocyte damage

To examine the cytotoxic potential of BE, its effect on the cell viability of cultured hepatocytes was measured by MTT assay. BE had no cytotoxic effect at the tested concentrations and increased cell proliferation in a dose-dependent manner (Figure 1A). Next, the protective effect of BE against H_2O_2-induced hepatotoxicity in cultured hepatocytes was examined. As shown in Figure 1B, addition of 650 μM H_2O_2 to cultured hepatocytes resulted in a dramatic ($p < 0.05$) decrease in the cell viability up to 56%, whereas pretreatment with BE of 0.01-0.1 μg/mL prior to H_2O_2 protected cell death in a dose-dependent manner ($p < 0.05$). The cell viability was restored up to 93.76% by BE pretreatment (0.1

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μg/mL). This result clearly indicate that the exposure of cultured hepatocytes to polyphenol-rich BE from *N. nucifera* leaves confers to significant protective effect against H_2O_2.

H_2O_2 is one of the main ROS that cause lipid peroxidation and DNA damage, thus inducing apoptosis in many different cell types.[22] Thus, preventing cell injury by ROS might be promising strategy for the treatment of ROS-mediated human diseases. Several phenolic compounds isolated from plants and/or fruits have been reported to possess cytoprotective effects against ROS-mediated oxidative stress.[23,24] In the present study, our result clearly demonstrated that polyphenol-rich BE from *N. nucifera* leaves could protect H_2O_2-induced hepatotoxicity in cultured hepatocytes.

Effects of BE against oxidative stress-induced ROS generation, lipid peroxidation and GSH levels in cultured hepatocytes

Further, we utilized the well-characterized DCFH-DA fluorescence assay to investigate BE effect on intracellular ROS generation in cultured hepatocytes. Time-dependent fluorescence increase by treatment of H_2O_2 (control) compared to un-stimulated control hepatocytes (blank) was observed and this fluorescence increase was 5.4-fold as an indicator of intracellular ROS generation after 90 min incubation (Figure 2A). Pretreatment of BE in cultured hepatocytes significantly ($p < 0.05$) decreased intracellular ROS generation in a time- and dose-dependent manner. Intracellular ROS generation was attenuated by 2.1-fold compared to H_2O_2 treatment hepatocytes at 0.1 μg/mL BE after 90 min incubation.

Next, to investigate the effect of BE on cell membrane lipid peroxidation in cultured hepatocytes, AAPH, a hydrophilic peroxyl radical generator, was challenged for cellular lipid peroxidation, which can be detected by DPPP fluorescent probe that is oxidized by lipid hydroperoxides to give a strongly fluorescent product.[25] As shown in Figure 2B, the cell membrane lipid peroxidation was dramatically ($p < 0.05$) increased in response to AAPH compared to the blank group, whereas pretreatment of BE significantly ($p < 0.05$) inhibited 43% lipid peroxidation at 0.1 μg/mL BE.

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GSH is tripeptide acting as a scavenger of free radicals, detoxifies electrophiles as a part of phase II detoxification reactions and plays a role in a number of important cellular processes.[26] Thus endogenous GSH was consumed during the oxidative stress, resulting in depletion of cellular GSH in hepatocytes. The effect of BE on intracellular GSH levels under normal and oxidative stress conditions was measured using an mBBr fluorescent probe. As shown in Figure 2C, treatment of BE without oxidative stress significantly ($p < 0.05$) increased intracellular GSH level in a dose-dependent manner. As expected, treatment of H_2O_2 caused the depletion of intracellular GSH, however pretreatment of BE completely ($p < 0.05$) restored intracellular GSH level under oxidative stress (Figure 2D).

Imbalance between ROS production and antioxidant defense systems causes oxidative stress and ROS damage cell through interaction with biomolecules such as DNA, protein and cell membranes. This process has been implicated in a number of human diseases, especially the peroxidation of the fatty acids in the phospholipid cell membrane, producing lipid peroxides that are major underlying contributor to the development of severe hepatic damage in liver tissue. In the present study, the inhibitions of H_2O_2-induced ROS generation and AAPH-induced cell membrane lipid peroxidation by the pretreatment of BE may be due to direct antioxidant mechanism through free radical scavenging activity since polyphenolic compounds exert a wide range of antioxidant effects, acting as ROS scavengers and free radical reaction terminator.[27,28] Moreover, the pretreatment of BE in cultured hepatocytes significantly increased and restored intracellular GSH levels, which may be also affected hepatoprotective action of BE against H_2O_2-induced hepatic damage in cultured hepatocytes.

Effects of BE on SOD-1, CAT and HO-1 gene expression

To understand exact underlying mechanism for hepatoprotective action, the expression of antioxidant and phase II detoxifying enzymes such as SOD-1, CAT, and HO-1 were analyzed by western blotting. As shown in Figure 3A, the treatment with H_2O_2 resulted in down-regulated expressions of CAT, SOD-1 and HO-1 compared to blank group, whereas the pretreatment with BE prior to the treatment

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with H_2O_2 resulted in a dose-dependent increases in CAT, SOD-1 and HO-1 protein expression, and the pretreatment of 0.1 μg/mL BE up-regulated 0.42-fold CAT, 0.62-fold SOD-1 and 2.4-fold HO-1 protein expression compared to non-treatment group (Figure 3B, C and D).

To protect against harmful effects of ROS, our body has developed an antioxidant defense system and antioxidant enzymes such as CAT, SOD, GPx are the most important enzymes detoxifying ROS to safe molecules. SOD averts oxidative stress by catalyzing superoxide anions to H_2O_2 and CAT further reduces redox damage by catalyzing the reduction of H_2O_2. Thus the increase in the expression of SOD and CAT may be able to protect against oxidative stress. HO-1, an inducible cytoprotective enzyme, catalyzed the rate-limiting step in conversion of heme into biliverdin, free ion, and carbon monoxide. In the presence of biliverdin reductase, biliverdin is further converted to bilirubin, which is potent antioxidant.[29] HO-1 induction is crucial in the cellular adaptive response to oxidative injury and plays a key role in maintaining antioxidant homeostasis during cellular stress.[10,29] Several papers have demonstrated that phenolic compounds could be induced HO-1 expression and its induction exhibited various cytoprotective effects.[11,12] In the present study, our findings demonstrated that polyphenol-rich BE up-regulated antioxidant and phase II detoxifying enzymes and this increase of SOD-1, CAT, and HO-1 expression may be conferred hepatoprotection against H_2O_2-induced oxidative stress. Moreover, HO-1 induction is higher than that of SOD-1 and CAT, which may be major factor affecting hepatoprotective effect by polyphenol-rich BE. These results suggest that polyphenol-rich BE-induced SOD-1, CAT, and HO-1 gene expression might serve as an important mechanism for the hepatoprotective effects of polyphenol-rich BE.

Effect of BE on Nrf2 nuclear translocation

To understand whether polyphenol-rich BE is able to activate Nrf2 in association with antioxidant and phase II detoxifying enzyme expression observed in the present study, the activation of transcriptional factor Nrf2 by pretreatment with polyphenol-rich BE was assessed by western blot analysis of hepatocytes nuclear fraction. As shown in Figure 4A, H_2O_2 treatment slightly increased nuclear

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translocation of Nrf2, whereas pretreatment with polyphenol-rich BE in cultured hepatocytes for 2 h significantly ($p < 0.05$) increased nuclear translocation of Nrf2 compared to blank group. The nuclear translocation of Nrf2 by pretreatment with polyphenol-rich BE was 8.1-fold increase compared to blank group (Figure 4B).

Nrf2 plays a key role in the adaptive response to oxidative stress and regulates ARE-driven antioxidant and phase II detoxifying enzyme expression. In recent years, several researches have reported that plant-derived phenolic compounds activate Nrf2-dependent ARE activity and induce HO-1, SOD and CAT that exhibited cytoprotective effects against oxidative stress in various cells.[11,12,23,30] In the present study, our results clearly shown that pretreatment with polyphenol-rich BE activated both nuclear translocation of Nrf2 and expression of HO-1, SOD and CAT in cultured hepatocytes under oxidative stress. Most studies of lotus leaves had focused on antioxidant activities such as *in vitro* free radical scavenging and did not reported exact antioxidant mechanism.[4,31,32] However, for the first time, we demonstrated underlying mechanism how *N. nucifera* leaves could protect cultured hepatocyte under oxidative stress condition. Thus, polyphenol-rich BE from *N. nucifera* leaves protects hepatocytes from H_2O_2-induced hepatic damage by elevating intracellular antioxidant and phase II detoxifying enzymes via activating nuclear translocation of Nrf2, particularly inducing the expression of phase II detoxifying gene HO-1 following H_2O_2 exposure.

Conclusions

In the present study, hepatoprotective effect of polyphenol-rich BE from *N. nucifera* leaves was assessed. Polyphenol-rich BE exerted hepatoprotective effect through inhibiting intracellular ROS generation and lipid peroxidation and up-regulated intracellular GSH levels under oxidative stress. In addition, polyphenol-rich BE enhanced antioxidant and phase II detoxifying enzyme expression through activating nuclear translocation of Nrf2 that may provide a pivotal mechanism for its hepatoprotective action against H_2O_2-induced hepatic damage.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. Polyphenol-rich BE from *N. nucifera* leaves protects H_2O_2-induced hepatic damage in cultured hepatocytes. (A) Hepatocytes were incubated with Polyphenol-rich BE for 24 h and the cell viability was determined by MTT assay. (B) Hepatocytes were pretreated with Polyphenol-rich BE for 2 h and then washed with PBS, before being challenged with 650 μM H_2O_2 for an additional 24 h. The hepatoprotective effect was measured by MTT assay. [a-e]The bars with different letters represent significant difference (p<0.05). Values are expressed as means ± S.D. (n=3).

Figure 2. Polyphenol-rich BE from *N. nucifera* leaves inhibited (A) intracellular ROS generation, (B) membrane lipid peroxidation and up-regulated (C) intracellular GSH levels under normal and (D) oxidative stress. [a-e]The bars with different letters represent significant difference (p<0.05). Values are expressed as means ± S.D. (n=3).

Figure 3. Effects of Polyphenol-rich BE from *N. nucifera* leaves on SOD-1, CAT and HO-1 protein expression. (A) Hepatocytes were exposed to various concentrations of Polyphenol-rich BE for 2 h and then washed with PBS, followed by addition of 650 μM H_2O_2 for an additional 24 h. Protein expression was analyzed by western blotting. (B) Quantification of HO-1 protein expression. (C) Quantification of CAT protein expression. (D) Quantification of SOD-1 protein expression. Data are expressed as fold change. [a-d]The bars with different letters represent significant difference (p<0.05). Values are expressed as means ± S.D. (n=3).

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418 **Figure 4.** Effect of polyphenol-rich BE from *N. nucifera* leaves on nuclear translocation of Nrf2. (A)
419 Hepatocytes were pretreated with polyphenol-rich BE for 2 h and then washed PBS, followed by
420 addition of 650 μM H_2O_2 for an additional 2 h. Nuclear extracts were prepared and analyzed by
421 western blotting. (B) Quantification of Nrf2 protein expression. Data are expressed as fold change. [a-
422 e]The bars with different letters represent significant difference (p<0.05). Values are expressed as
423 means ± S.D. (n=3).

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424 Table 1. TPC, TFC and phenolic composition of BE from *N. nucifera* leaves.

	Retention time (min)	mg/100 g BE
Gallic acid	5.1	0.0±0.0
Protocatechuic acid	9.5	224.9±0.1
Hydroxybenzoic acid	16.2	100.4±0.1
Catechin	18.1	428.4±0.5
Vanillic acid	25.8	71.4±0.3
Caffeic acid	27	49.3±0.1
Chlorogenic acid	29.5	0.0±0.0
Syringic acid	35.0	142.2±0.1
p-Coumaric acid	37.9	172.6±0.2
Ferulic acid	41.1	317.2±0.3
Sinapic acid	42.5	173.6±0.1
Rutin	47.7	241.2±0.3
Cinnamic acid	54.5	5.6±0.7
Quercetin	56.5	6289.1±0.4
TPC (mg GAE/g BE)		406.12±13.56
TFC (mg QUE/g BE)		216.40±4.44
Yield (%)		4.44

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427 Table 2. Antioxidant activities of BE from *N. nucifera* leaves.

Assays	BE	Ascorbic acid
DPPH (IC_{50}, μg/mL)[#]	5.21±0.03[a]	3.04±0.01[b]
$ABTS^+$(IC_{50}, μg/mL)	6.22±0.03[a]	3.45±0.01[b]
Reducing power (A_{700})*	0.234±0.002[a]	0.218±0.001[b]

428 [a-b]Different letters indicated a significant difference at the same assay ($p < 0.05$).

429 [#] The IC_{50} value was defined as the concentration required to scavenge 50% of DPPH or $ABTS^+$

430 radical.

431 * Reducing power was evaluated at 100 μg/mL.

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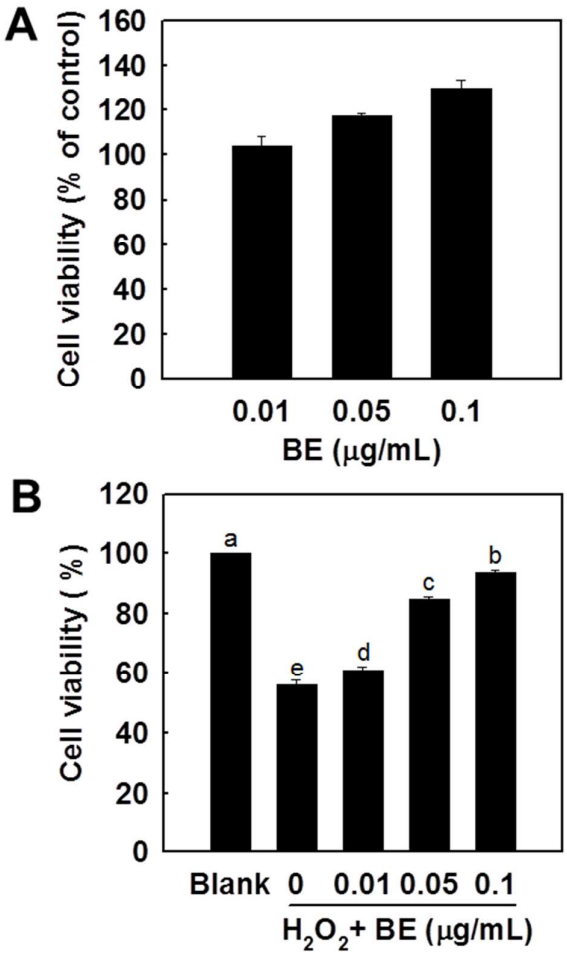

434

435 **Fig. 1.**

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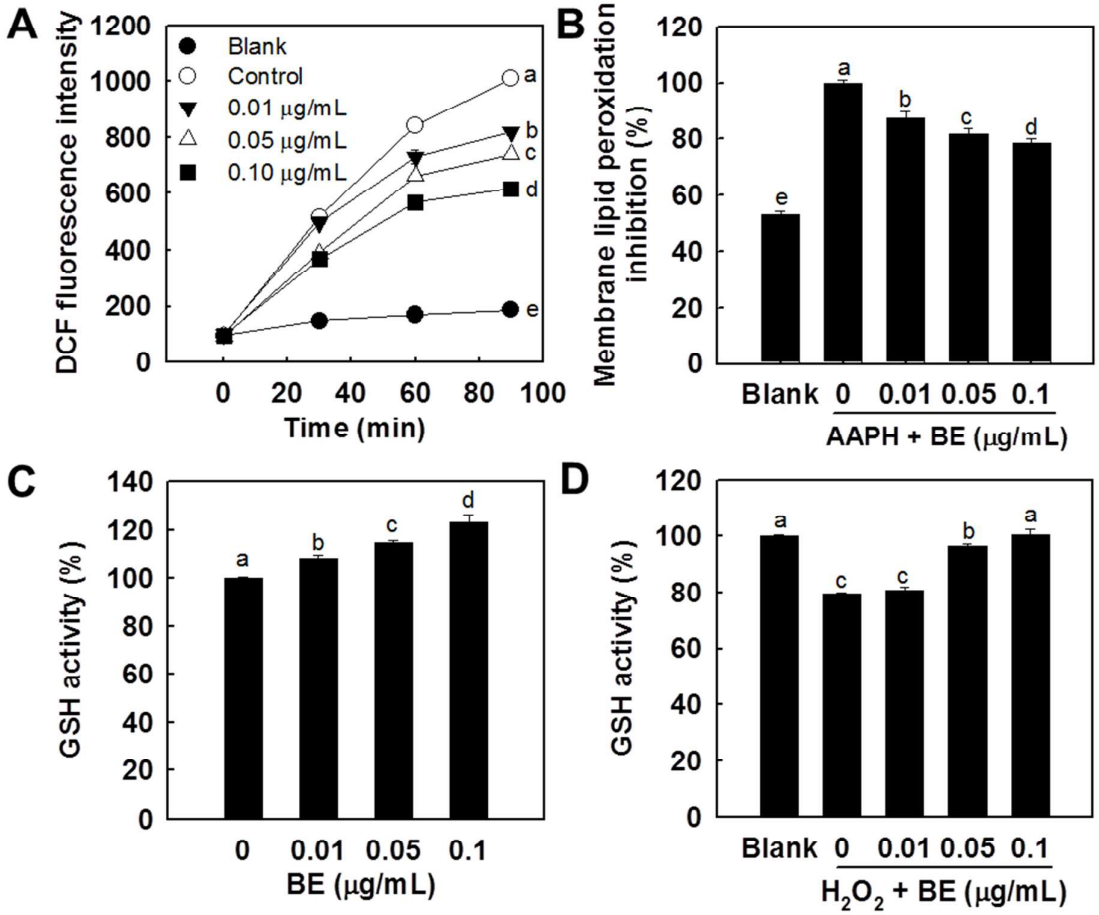

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438 **Fig. 2.**

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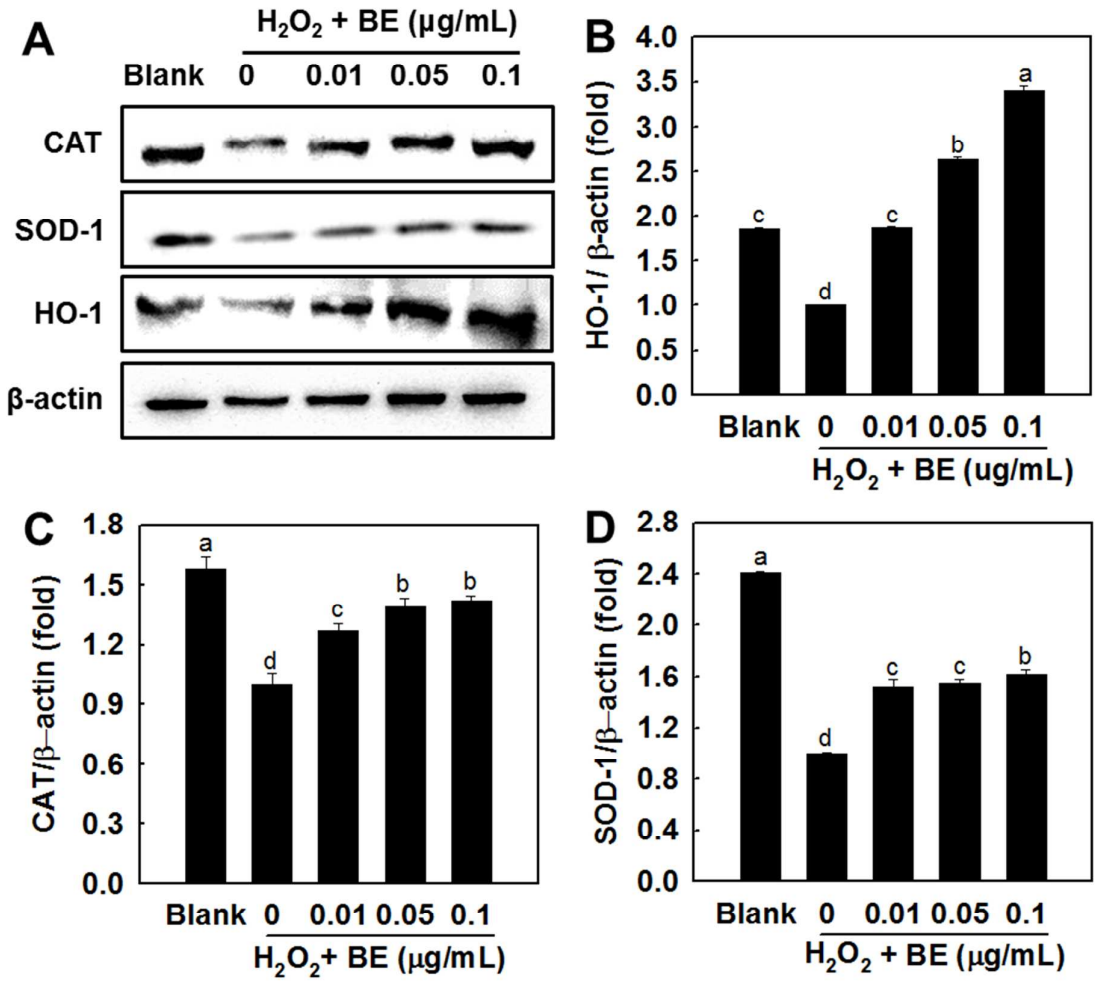

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441 **Fig. 3.**

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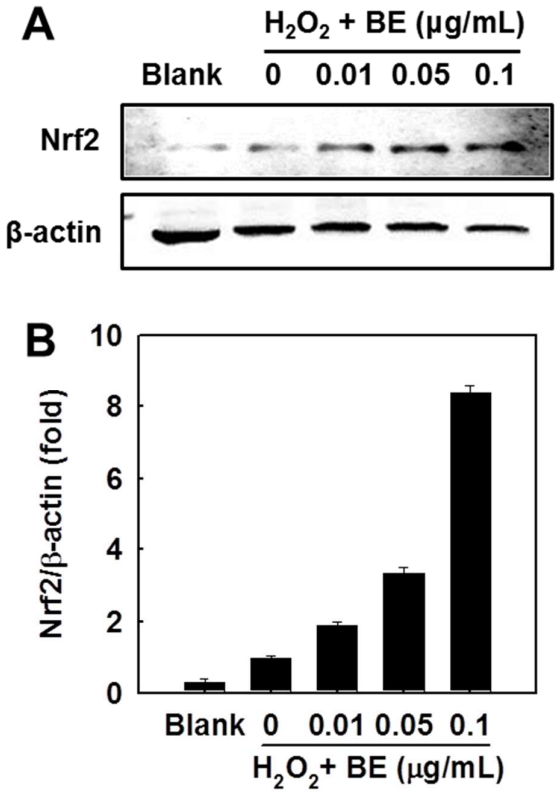

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443 **Fig. 4.**

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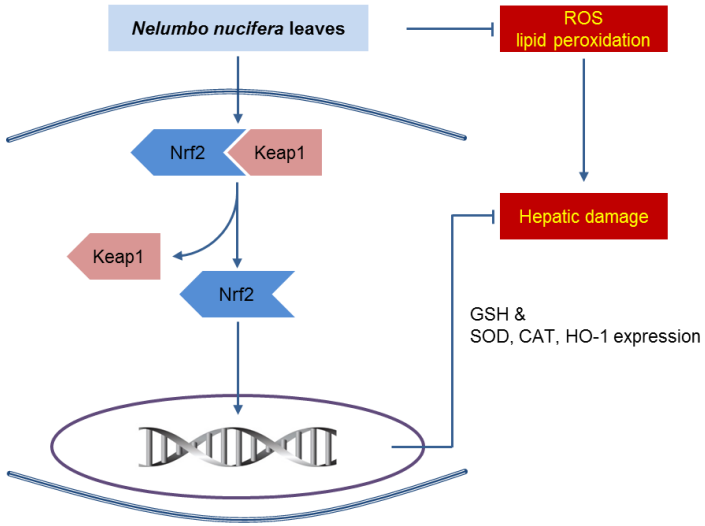
TOC Graphic

Nelumbo nucifera leaves protects hydrogen peroxide-induced hepatic damage via antioxidant enzyme and HO-1/Nrf2 activation



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