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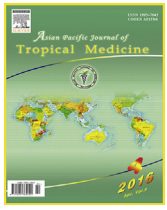

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Fruits extracts of *Hovenia dulcis* Thunb. suppresses lipopolysaccharide-stimulated inflammatory responses through nuclear factor-kappaB pathway in Raw 264.7 cells

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ABSTRACT

Objective: To investigate the anti-inflammatory effects and the action mechanism of the fruits of *Hovenia dulcis* (*H. dulcis*) in lipopolysaccharide (LPS)-induced mouse macrophage Raw 264.7 cells.

Methods: The extract of *H. dulcis* fruits (EHDF) were extracted with 70% ethanol. Mouse macrophages were treated with different concentrations of EHDF in the presence and absence of LPS (1 μg/mL). To demonstrate the inflammatory mediators including nitric oxide, inducible nitric oxide synthase and cyclooxygenase (COX)-2 expression levels were analyzed by using *in vitro* assay systems. COX-derived pro-inflammatory cytokines including interleukin-1β, tumor necrosis factor-α and prostaglandin E_2 were determined using ELISA kits. Cell viability, heme oxygenase-1 expression, nuclear factor-kappaB and nuclear factor E_2-related factors 2 translocation were also investigated.

Results: EHDF potently inhibited the LPS-stimulated nitric oxide, inducible nitric oxide synthase, COX-2, interleukin-1β and tumor necrosis factor-α expression in a dose-dependent manner. EHDF suppressed the phosphorylation of inhibited kappaB-alpha and p65 nuclear translocation. Treatment of macrophage cells with EHDF alone induced the heme oxygenase-1 and nuclear translocation of nuclear factor E2-related factor 2.

Conclusions: These results suggest that the ethanol extract of *H. dulcis* fruit exerts its anti-inflammatory effects by inhibiting inhibited kappaB-alpha phorylation and nuclear translocation of nuclear factor-kappaB.

1. Introduction

Inflammation is the first response of body immune system which is triggered by exogenous pathogens, external stimuli such as harmful chemicals and bacterial infection [1]. The inflammatory processes which are characterized by leukocyte recruitment and macrophages are considered to play a critical role in the initiation and development of inflammation [2–5]. In the presence of an endotoxin such as lipopolysaccharide (LPS), activated macrophages produce numerous pro-inflammatory cytokines such as tumor necrosis-α (TNF-α), interleukin-1β (IL-1β) and interleukin-10, as well as macrophage-derived inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) [6,7]. Inducible nitric oxide synthase (iNOS) expression is involved during the NO production during inflammation and cyclooxygenase-2 (COX-2) enzyme is considered to be responsible for the expression of PGE_2 in inflammatory progress [8].

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Nuclear factor-kappaB (NF-κB), a protein complex, is one of the most ubiquitous transcription factors. The phosphorylation of NF-κB plays a key role in regulating the inflammatory responses [9]. NF-κB is constitutively localized in the cytoplasm as a homodimer or heterodimer in normal cells, which is activated by a family of inhibitory factors called inhibitory-factor kappaB (IκB) [10]. When cells are under inflammatory condition, NF-κB is activated and subsequently translocated into the nucleus, followed by amplification of the inflammatory response by overexpression of several inflammation related genes including *iNOS*, *COX-2* and *TNF-α* [11,12].

Oxidative stress is potently associated with the process of inflammation not only through injurious effects, but also by the host defense against invading microbes [13]. There are many enzymatic anti-oxidants and anti-inflammatory systems in the host defense including phase II detoxifying enzymes and the expression of stress response proteins such as heme oxygenase-1 (HO-1) [14,15]. HO-1, which belongs to the heat shock protein family, is catalyzed by the oxidation of heme to the biologically active products such as carbon monoxide, biliverdin, and ferrous iron (Fe^{2+}) [16,17]. Hence, HO-1 is considered to contribute in diverse immune responses through anti-oxidation and anti-inflammation activities in the human body [18]. Nuclear factor E2-related factors 2 (Nrf2), as well known HO-1 promotor, is a basic leucine zipper transcription factor that regulates phase II enzyme by induction of detoxifying genes through anti-oxidant response elements [19]. An increasing number of reports have also suggested that Nrf2 play a predominant role by interacting with cognate DNA-binding domains [20,21].

Hovenia dulcis (*H. dulcis*) Thunb. (Rhamnaceae), mainly found in China, Japan and Korea, has been widely used as a valuable crude drug to treat various diseases such as hepatitis and diabetes [22]. Recently, several studies indicated that the *H. dulcis* extract contains an extensive of pharmacological compounds, such as alkaloids, flavonoids, and triterpenoids [23]. However, the precise mechanisms responsible for the suppression of LPS-stimulated inflammatory response by the ethanol extract of *H. dulcis* fruits (EHDF) remains unclear. This study demonstrated the bio-pharmacological mechanisms of EHDF, and functions for these anti-inflammatory effects. Furthermore, the study investigated that EHDF have anti-inflammatory effects in LPS-induced Raw 264.7 macrophage cells through the Nrf2-dependent HO-1 induction. In addition, EHDF strongly inhibited LPS-induced NO production via the direct down-regulation of iNOS, and the study also showed the Nrf-dependent HO-1 up-regulation. These results suggest that EHDF is a potential Nrf-dependent HO-1 regulator for anti-inflammatory activities.

2. Methods and materials

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium was purchased from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum from Gibco BRL (Gaithersburg, MD). ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Tin protoporphyrin IX (SnPP) was from Porphyrin Products (Logan, UT, USA). Primary antibodies, including anti-HO-1, anti-COX-2, anti-iNOS, anti-IκB, anti-p-IκB, anti-NF-κB (p65), anti-Nrf2 and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DAPI (4′,6-diamidino-2phenylindole) was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Other chemicals for this study were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless indicated otherwise.

2.2. Plant material and preparation of ethanol extracts

The fruits of *H. dulcis* Thunb. (Rhamnaceae) were purchased from Daehak Hanyakguk, Iksan, Korea in 2014. A voucher specimen (no. 14-MRC-220AP) was authenticated by Dr. Sun-Dong Park, college of Korean Medicine, Dongguk University (Korea). The specimens were deposited at the College of Korean Medicine, Dongguk University. Dried fruits of *H. dulcis* (50 g) was extracted with 70% ethanol (300 mL) at Soxhlet extraction for 3 h, and the extract was concentrated *in vacuo* to obtain a 70% ethanol extract (2.31 g). Ethanol extract was suspended in H_2O and centrifuged at 2 500 rpm for 30 min. Precipitate was dissolved in ethanol, and evaporated *in vacuo* to obtain an extraction (696 mg). For each experiment, EHDF was dissolved in dimethylsulfoxide (final culture concentration, 0.05%). Preliminary studies indicated that the solvent had no effect on the cell viability at the concentration used.

2.3. High-performance liquid chromatography (HPLC)

Chromatographic experiments were performed on YL-9100 series HPLC instrument equipped with a sample injector and a photodiode array UV/Vis detector (PDA) (YoungLin, Korea). For all experiments, an SHISEIDO CAPCELL PAK C-18 column (4.6 nm × 250 nm, 5 μm, SHISEIDO CO., Tokyo, Japan) was used as the stationary phase, and the injection volume was 20 μL. The mobile phase was composed of water (contain 0.1% formic acid) and methanol, with an applied gradient of 10% B increasing to 100% B in 40 min. The column was cleaned with 100% B for 10 min. Flow rate was 0.7 mL/min, and the detection wavelength was adjusted to 254 nm.

2.4. Cell culture and viability assay

RAW 264.7 macrophages were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin and maintained in a humidified incubator at 37 °C, 5% CO_2. To assess the cell viability caused by EHDF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used. The cells were suspended in 96 well plates at 4×10^5 cells/mL, and treated with varying EHDF. After pre-treatment of EHDF, cells were incubated for 24 h, and the supernatant was removed. Ten μL of MTT solution (5 mg/mL) in 90 μL of serum-free medium was treated for additional 4 h. The formazan was dissolved in dimethylsulfoxide, and the absorbance was measured at 517 nm.

2.5. Nitrite assay

The cells were pre-treated with EHDF for 30 min, with or without LPS (1 μg/mL). 100 μL of each supernatant from EHDF

treated well were responded with an equal volume of the Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethyle-nediamine dihydrochloride/2.5% H_3PO_4] at room temperature for 10 min. The nitrite concentration was determined by measuring the absorbance at 540 nm with a microplate reader, and calculated using a standard curve of sodium nitrite.

2.6. TNF-α, IL-1β and PGE_2 ELISA assay

RAW 264.7 cells were pre-incubated with various concentrations of EHDF for 12 h, and then stimulated for 18 h with LPS. The supernatants of each well were harvested, and contents of cytokines including TNF-α, IL-1β and PGE_2 were evaluated using ELISA kit as per the manufacturer's instructions (R&D Systems).

2.7. Preparation of nuclear and cytosolic fractions

The cells treated with or without EHDF were harvested and washed with PBS. The pellets were resuspended using NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Scientific, IL, USA) according to the manufacturer's instructions. Briefly, the cytosolic fraction of cells were prepared by centrifugation at 15 000 *g* for 5 min at 4 °C, and the supernatant was transferred to a clean tube. Subsequently, the nuclear fractions were prepared by centrifugation at 15 000 *g* for 10 min at 4 °C. The nuclear and cytoplasmic extracts were then stored at −80 °C, until further use. The amounts of proteins in each extract were quantified using the bicinchoninic acid protein assay kit (Pierce, Thermo Scientific).

2.8. Western blot analysis

Cells were harvested by centrifugation at 3 000 *g* for 5 min, and the cell pellet was collected and washed twice with ice-cold PBS. The cell pellet was dissolved in PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Inc.), and the concentrations of proteins were measured by bicinchoninic acid protein assay kit (Pierce). The cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis with an equal volume, and then transferred onto polyvinylidene fluoride membrane. After blocking at room temperature for 1 h, with 5% skimmed milk powder in Tris-Buffered Saline Tween-20 (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20), the blots were incubated with iNOS, COX-2, Nrf2, NF-κB (p65), lamin B, β-actin, phospho-IκB-α, IκB-α, HO-1 polyclonal antibodies at 4 °C for overnight. After incubation with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h, the bands were visualized with enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). The intensity of the bands was quantified with Fusion FX7 chemiluminescence imaging system (Vilber Lourmat, Marne-la-vallée, France).

2.9. Immunofluorescence microscopy

For localization of NF-κB and Nrf2, Raw 264.7 macrophages were grown on Lab-Tek II chamber slides (Nunc, Thermo Scientific). The day after plating macrophages on coverslips with or without of EHDF at a different concentrations in the presence and absence of LPS (1 μg/mL), the cells were fixed in methanol for 10 min at −20 °C. The cells were then permeabilized in PBS containing 1% Triton X-100 for 10 min Nrf2 antibody followed by fluorescein isothiocyanate-labeled secondary antibody (Santa Cruz Biotechnology). And the coverslips were mounted on glass slides using Dako Fluorescent mounting medium. And observed using a Zeiss fluorescence microscope (ProvisAX70, Olympus Optical Co., Tokyo, Japan).

2.10. Statistical analysis

The results are expressed as mean ± sd. Experimental data were analyzed using Graph Pad Prism version 5.0 software. The significance was assessed with a one-way ANOVA test with tukey's multiple comparison, and $P < 0.05$ was considered significant. Each experiment was performed at least 3 times using samples from different preparations.

3. Result

3.1. HPLC analysis of EHDF

Data from the HPLC analysis of EHDF were recorded in the form of chromatograms by monitoring detector responses at 254 nm. As shown in Figure 1, the results indicated that two bioactive components of *H. dulcis* observed were kaempferol and quercetin, which were mainly detected upon UV absorption.

3.2. Effect of EHDF on cell viability in Raw 264.7 macrophage

MTT assay was used to first investigate the effect of EHDF on cell viability. As shown in Figure 2, exposures to the EHDF at five different concentrations for 24 h caused little effect on the macrophages. This result clearly revealed that the EHDF at the highest test concentration (120 μg/mL) produced 25% reduction of cell viability, whereas the extract at the lower concentrations had no effects on the cell viability, proving that EHDF was safe for the mouse macrophage Raw 264.7 cells. For all further experiments, the macrophages were treated with EHDF in the range of 5–100 μg/mL.

3.3. Effect of EHDF on iNOS and COX-2 expression in Raw 264.7 macrophage

As shown Figure 3A, LPS stimulation significantly increased the iNOS expression. However, pretreatment with the EHDF up to 100 μg/mL had potently reduced the protein expressions of iNOS in a dose-dependent manner. Next, study found that pretreated with non-cytotoxic concentrations (5–100 μg/mL) of EHDF for 1 h strongly reduced the COX-2 enzyme levels on LPS-stimulated macrophage cells in a dose-dependently manner (Figure 3B).

3.4. Effect of EHDF on LPS-induced production of NO and pro-inflammatory cytokines

Pretreatment of cells with 5 and 100 μg/mL of EHDF dramatically reduced the NO production in LPS-stimulated Raw

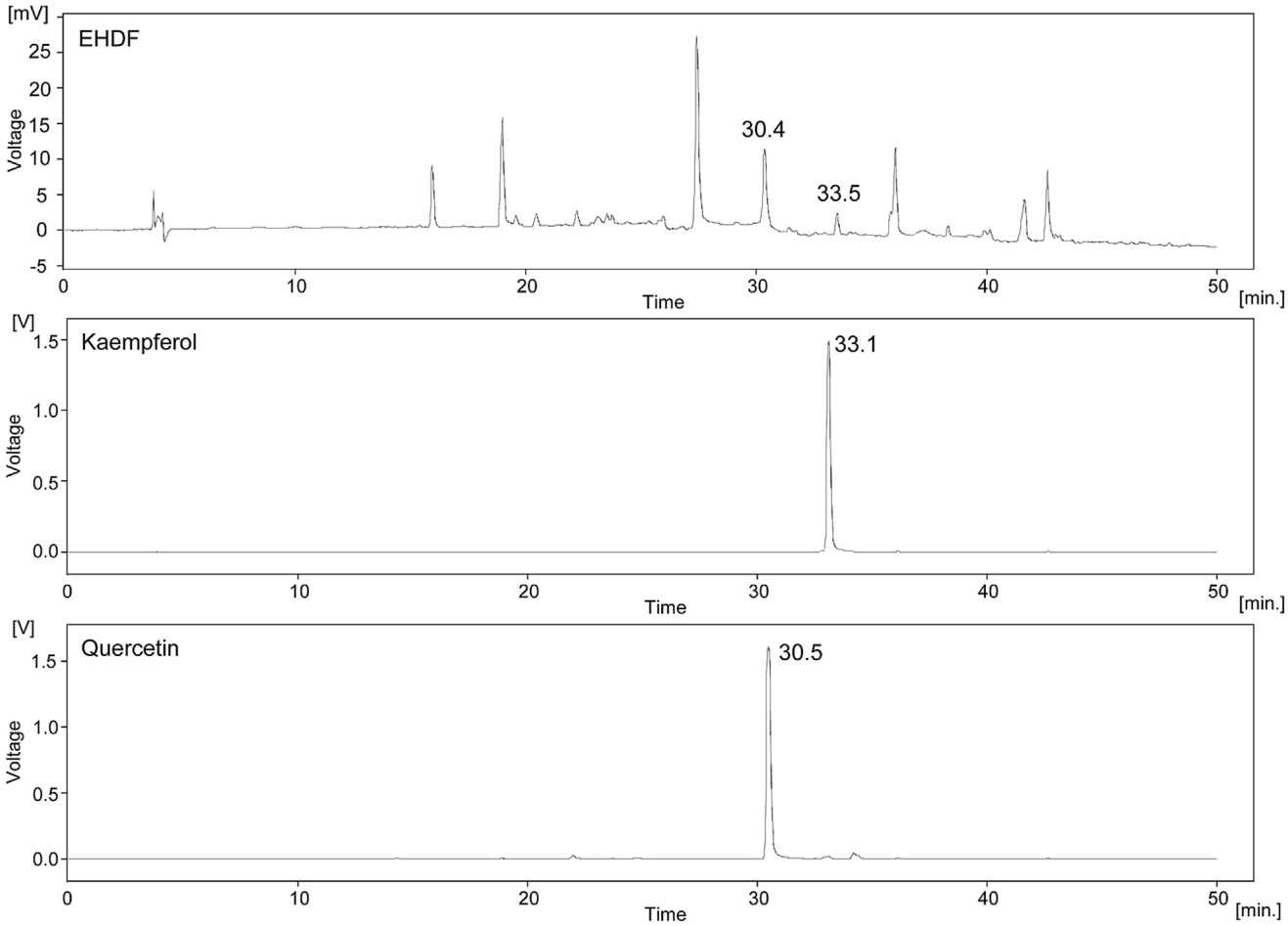

Figure 1. HPLC chromatogram of EHDF.
Kaempferol and quercetin were identified in the extract.

264.7 cells (Figure 4A). As shown in Figure 4B, the pre-treatment of macrophages with EHDF for 18 h results in the inhibition of IL-1β production. Under the same conditions, EHDF also caused a decrease in TNF-α production in macrophage cells (Figure 4C). In addition, PGE_2 level in the supernatants were determined by ELISA. As shown in Figure 4D, EHDF a significant reduction of PGE_2 level was observed in the medium.

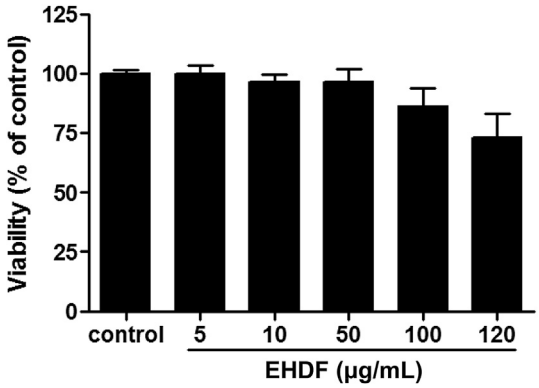

Figure 2. Effects of EHDF on cell viability.
Raw 264.7 cell viability was determined by the MTT assay. Cells were treated with various concentrations of the ethanol extract of EHDF (5, 10, 50, 100 and 120 μg/mL) for 24 h. Values are expressed as percentage of the control. All data represent the mean ± SD of three different experiments.

3.5. Effect of the EHDF on LPS-induced NF-κB nuclear translocation and IκB phosphorylation

As shown in Figure 5A, IκB-α was degraded after treatment with LPS at 1 μg/mL for 1 h, and this degradation was markedly reduced by pre-treatment of the macrophage cells with various concentrations of EHDF ranging from 5 to 100 μg/mL for 18 h. On the other hand, pretreatment of the EHDF reduced the nuclear translocation of NF-κB and phosphorylation of IκB in the cytoplasm. In addition, Figure 5B shown that LPS alone significantly increased the translocation of NF-κB p65 subunits to the nucleus in the cytoplasm. However, pre-treatment of macrophages with 100 μg/mL of EHDF for 18 h results in the reduction of p65 translocation.

3.6. Effects of EHDF on HO-1 expression and nuclear translocation of Nrf2

As shown in Figure 6A, in cells treated for 18 h with various concentrations of EHDF ranging from 5 to 100 μg/mL, we found a concentration-dependent increase of HO-1 protein expression. We also determined a time course-dependent HO-1 induction

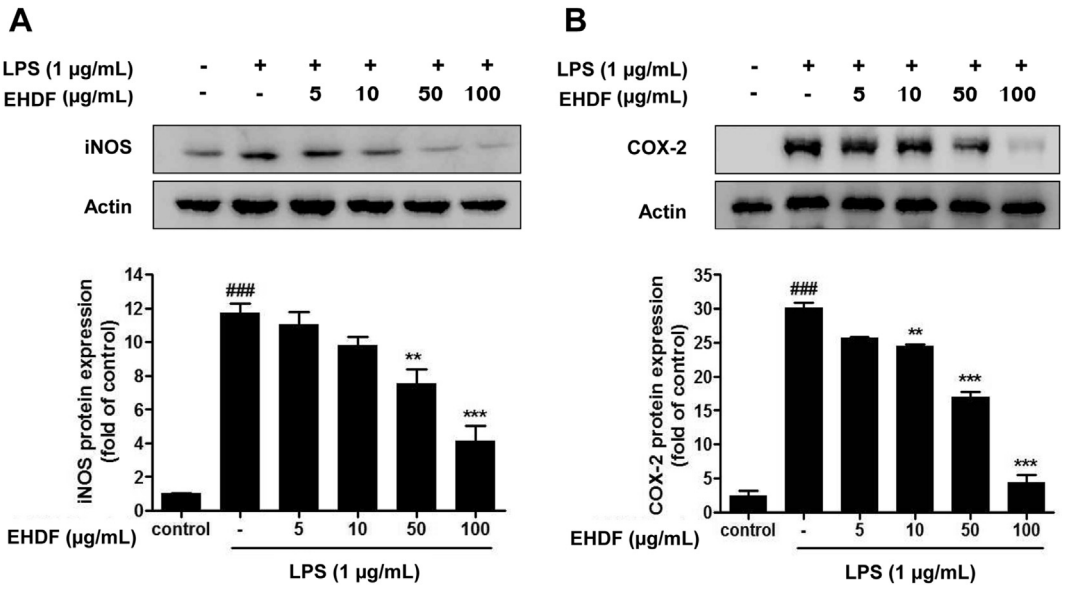

Figure 3. Effects of EHDF on LPS-induced expression of iNOS and COX-2 protein in Raw 264.7 macrophages.
Protein expression of iNOS and COX-2 in Raw 264.7 cells incubated with different concentrations of EHDF and with or without LPS (1 μg/mL) for 24 h was examined by Western blot analysis. The graphs show a change in protein levels of iNOS and COX-2 (A and B). Values are expressed as a percentage of the LPS-treated cells. All data represent the mean ± SD of three different experiments. Asterisk indicates statistically significant difference between LPS-treatment group (*$P < 0.05$; **$P < 0.01$).

was determined in the protein expressions level (Figure 6B). Nrf2 is a well-known HO-1 regulator and coordinates the induction of phase II enzyme genes. Therefore, we investigated whether treating macrophage cells with EHDF induced nuclear translocation of Nrf2. As shown in Figure 7A, at 100 μg/mL of EHDF, the nuclear fractions of time course-treated macrophage cells showed a gradual increase in Nrf2 levels at 0.5, 1.0, 1.5, and 3.0 h. In addition, for localization of Nrf2, similar results were also observed using immunofluorescence microscopy (Figure 7B).

3.7. Effects of EHDF-induced up-regulation of HO-1 on the inhibition of pro-inflammatory mediators

As the pre-incubation of macrophages with EHDF resulted in markedly inhibited LPS-stimulated production of pro-inflammatory mediators (Figure 4), and EHDF was able to induce the expression of HO-1 in a time and dose-dependent manner (Figure 6). Therefore, to confirm the involvement of HO-1 in the inhibitory effects of LPS-induced inflammation, pro-inflammatory mediators were examined using SnPP.

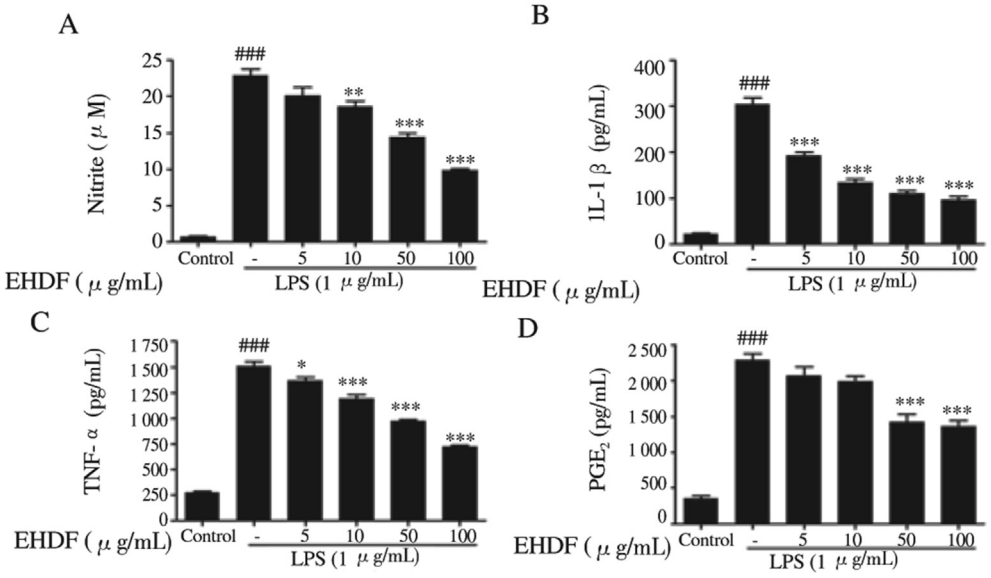

Figure 4. Effects of EHDF on LPS-induced NO and pro-inflammatory mediators levels in Raw 264.7 macrophages.
Raw 264.7 were pre-treated with different concentrations of EHDF for 12 h and stimulated with LPS (1 μg/mL) for 18 h. A, NO production was determined using Griess reagent. B, C, and D, The production of the pro-inflammatory mediators including IL-1β, TNF-α and PGE_2 were determined using by ELISA kits. All data represent the means ± SD of three different experiments. Asterisk indicates statistically significant difference between LPS-treatment group (*$P < 0.05$; **$P < 0.01$, ***$P < 0.005$).

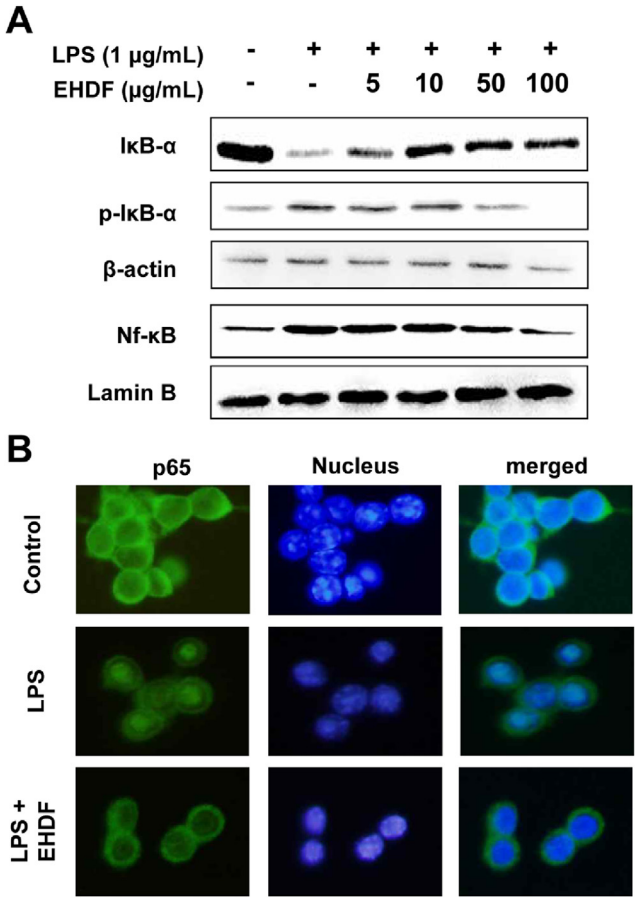

Figure 5. Effects of EHDF on LPS-induced activation of NF-κB nuclear translocation and I-κB phosphorylation.
Macrophages were pre-treated with indicated concentrations of EHDF for 12 h, and stimulated with LPS (1 μg/mL) for 1 h. A, Western blot analysis of p65 subunit of NF-κB in nuclear fractions of Raw 264.7 cells treated with LPS (1 μg/mL) in the presence or absence of different concentrations of EHDF. B, Immunofluorescence microscopy analysis was determined as described in the Materials and methods sections.

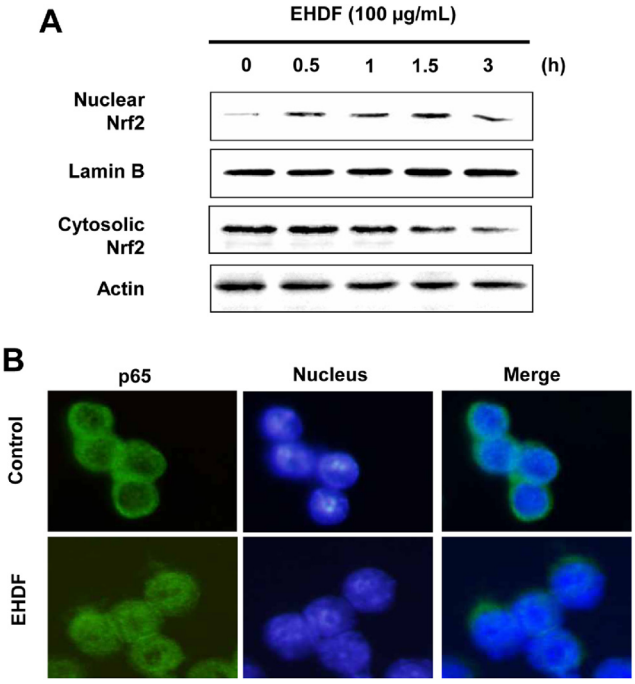

Figure 7. Effects of EHDF on nuclear translocation of Nrf2.
A, Macrophages were incubated for indicated periods with 100 μg/mL of EHDF. Western blot analysis for nuclear translocation Nrf2 expression was performed as described in the Materials and methods sections. B, Immunofluorescence microscopy analysis was determined as described in the Materials and methods sections.

Macrophage cells were treated with SnPP (50 μM) for 1 h, followed by treatment with LPS (1 μg/mL) in the presence or absence of EHDF at 100 μg/mL. As shown in Figure 8, SnPP partially abolished EHDF suppression of LPS-induced production of pro-inflammatory mediators including NO, IL-1β, TNF-α and PGE_2.

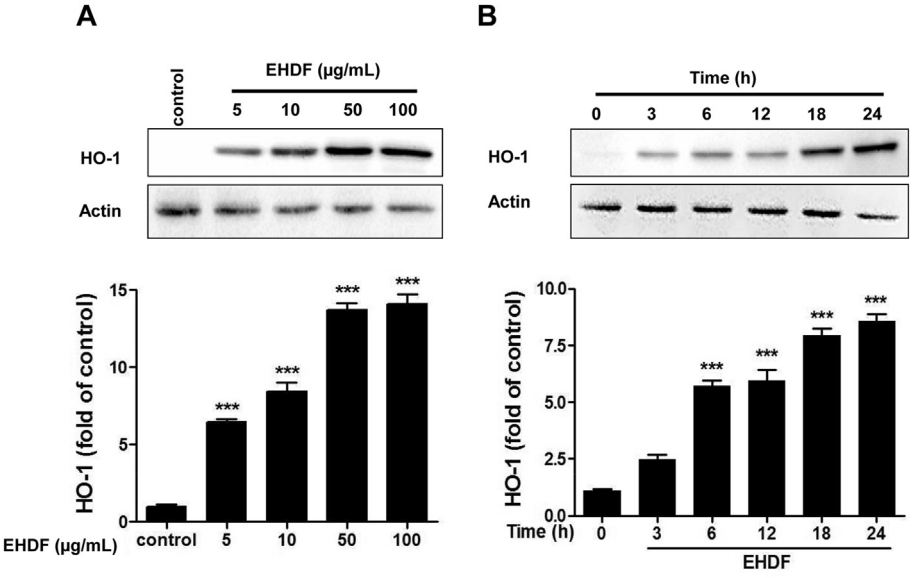

Figure 6. Effects of EHDF on HO-1 expression in Raw 264.7 macrophages.
A, Macrophages were incubated for 18 h with different concentrations of EHDF. B, Macrophages were incubated for indicated periods with 100 μg/mL of EHDF. Western blot analysis for HO-1 expression was performed as described in the Materials and methods sections. Data representative blots of three independent experiments are shown. All data represent the mean ± SD, and asterisk indicates statistically significant difference between control group (***$P < 0.005$).

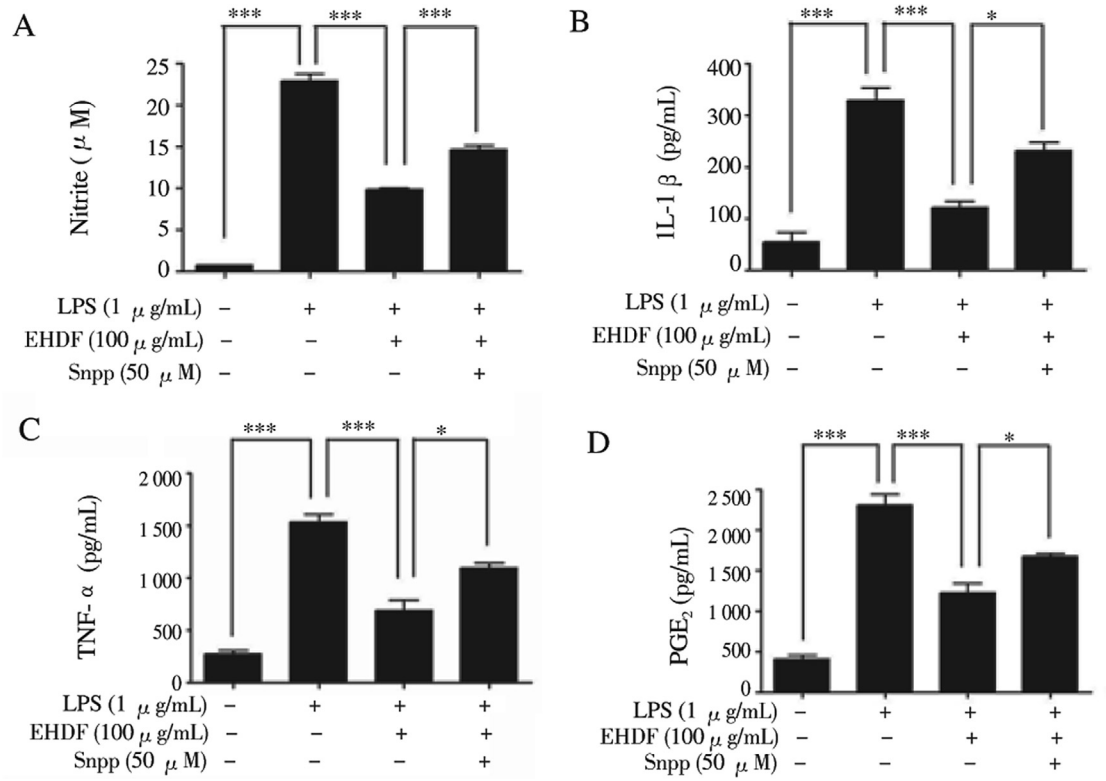

Figure 8. HO-1 mediates the suppressive effect of EHDF on LPS-stimulated production of pro-inflammatory mediators.
Macrophages were pre-treated with EHDF (100 μg/mL) for 12 h in the presence or absence of SnPP (50 μM), and stimulated with LPS (1 μg/mL) for 12 h. SnPP was pre-treated with macrophages for 3 h in this experiment. NO, IL-1β, TNF-α and PGE_2 are represented as mean ± SD, and asterisk indicates statistically significant difference between indicated group (*$P < 0.05$, ***$P < 0.005$).

4. Discussion

H. dulcis Thunberg (Rhamnaceae) is mainly found in China, Japan, and Korea, and is commonly used as a folk remedy in the form of tea and dietary supplements to promote health. Several reports suggested that the fruit of *H. dulcis* has detoxification properties on alcohol poisoning, hepatoprotective effect, antioxidant and antidiabetic activities [24–27]. Recently, the major biologically active components identified in the fruits of *H. dulcis* were myricetin and quercetin [23,27]. However, underlying mechanisms to explain the anti-inflammatory effects of *H. dulcis* fruits remain unknown. In this study, we investigated the anti-inflammatory effects of the ethanol EHDF in LPS-induced mouse macrophage Raw 264.7 cells. Inflammation is a defense mechanism that living organisms use to protect themselves from various external infections [1]. The inflammatory response is well known to be regulated by central inflammatory responses proteins such as iNOS and COX-2 [28,29]. Furthermore, macrophages play a critical role in both the inflammatory responses and overproduction of pro-inflammatory mediators such as NO, PGE_2, and cytokines [30–32]. It has been implicated in several inflammation-related diseases including atherosclerosis, cancer, arthritis and autoimmune diseases [33,34]. Our major finding is that the ethanol EHDF suppresses NO production. NO is up-regulated in activated LPS-treated macrophage cells, and is secreted during extracellular space gas formation. Also, we demonstrated that the pre-treatment of different concentrations of EHDF (5–100 μg/mL) results in the suppressed expression of iNOS and COX-2 enzymes. In this study, EHDF also potently inhibited various LPS-induced pro-inflammatory mediators, such as IL-1β, PGE_2 and TNF-α in a dose-dependent manner. These results suggest that EHDF exerts its anti-inflammatory effects by down-regulating pro-inflammatory enzymes, and by the suppression of inflammatory mediators in the macrophages, in the absence of LPS stimulation.

Pro-inflammatory cytokines are significant transcription factors for inflammatory response associated enzymes including NF-κB [35]. NF-κB is also a transcription factor controlling the gene expressions involving immune responses and inflammation [9,36]. Therefore, regulation by NF-κB translocation might be considered a major therapeutic target for the treatment of inflammatory diseases [2,37]. This study evaluated the effects of EHDF on phosphorylation and degradation of IκB-α and nuclear translocation of NF-κB p65 induced by LPS, and observed that they were significantly reduced after the pre-treatment of macrophages. One of possible explanations for the inhibitory role of EHDF in pro-inflammatory mediator production in LPS-induced macrophage cells is that EHDF may interrupt the interaction of LPS to receptors. These findings were similar to those shown in the previously studies [32].

HO-1 participates in maintaining cellular homeostasis and plays an important role in reducing oxidative injury and attenuating the inflammation in macrophage and tissues [18]. A growing body of evidence shows that HO-1 directly represses inflammatory responses by down regulation of inflammatory mediators such as NO, PGE_2, IL-β, and TNF-α [14,38]. The HO-1 gene also can be transcriptionally activated by Nrf2 and NF-κB [39]. When Nrf2 is activated by oxidative stress such LPS, electrophiles and harmful chemicals, it translocates to the nucleus and bind to its cis-acting response [40]. Other previous

studies have demonstrated that the Nrf2-dependent activation of antioxidant systems reduces inflammation [30,41]. In addition, our results suggest that EHDF can activate Nrf2 by promoting the dissociation of the Nrf-2 and subunit complex, resulting in the induction of HO-1 activity. Furthermore, our study clearly demonstrates that treatment with SnPP reverses the inhibitory effects of EHDF on NO, PGE_2, IL-1β, and TNF-α secretion.

In conclusion, EHDF, the ethanol EHDF, showed potent anti-inflammatory functions on LPS-stimulated macrophage activation via the Nrf2-mediated up-regulation of HO-1 and inhibition of NF-κB signaling. These finding suggest that EHDF might be a potent agent for further investigation in the treatment of inflammation related diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008; **454**(7023): 428-435.

[2] Kim HY, Hwang KW, Park SY. Extracts of *Actinidia arguta* stems inhibited LPS-induced inflammatory responses through nuclear factor-κB pathway in Raw 264.7 cells. *Nutr Res* 2014; **34**(11): 1008-1016.

[3] Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat Rev Immunol* 2002; **2**(10): 787-795.

[4] Koo HJ, Yoon WJ, Sohn EH, Han YM, Jang SA, Kown JE, et al. The analgesic and anti-inflammatory effects of *Litsea japonica* fruit are mediated via suppression of NF-κB and JNK/p38 MAPK activation. *Int Immunopharmacol* 2014; **22**(1): 84-97.

[5] Yang J, Li S, Xie C, Ye H, Tang H, Chen L, et al. Anti-inflammatory activity of ethyl acetate fraction of the seeds of *Brucea Javanica*. *J Ethnopharmacol* 2013; **147**(2): 442-446.

[6] Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on I-kappaB alpha phosphorylation, C/EBP and AP-1 activation. *Br J Pharmacol* 2003; **139**(1): 11-20.

[7] Ying X, Yu K, Chen X, Chen H, Hong J, Cheng S, et al. Piperine inhibits LPS induced expression of inflammatory mediators in Raw 264.7 cells. *Cell Immunol* 2013; **285**(1–2): 49-54.

[8] Goodwin JS, Ceuppens J. Regulation of the immune response by prostaglandins. *J Clin Immunol* 1983; **3**(4): 295-315.

[9] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 2000; **18**: 621-663.

[10] Nam NH. Naturally occurring NF-kappaB inhibitors. *Mini Rev Med Chem* 2006; **6**(8): 945-951.

[11] Ben-Neriah Y. Regulatory functions of ubiquitination in the immune system. *Nat Immunol* 2002; **3**(1): 20-26.

[12] Aggarwal BB. Nuclear factor-kappaB: the enemy within. *Cancer Cell* 2004; **6**(3): 203-208.

[13] Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med* 2010; **49**(11): 1603-1616.

[14] Choi S, Nguyen VT, Tae N, Lee S, Ryoo S, Min BS, et al. Anti-inflammatory and heme oxygenase-1 inducing activities of lanostane triterpenes isolated from mushroom *Ganoderma lucidum* in Raw 264.7 cells. *Toxicol Appl Pharmacol* 2014; **280**(3): 434-442.

[15] Xu Z, Zhou J, Cai J, Zhu Z, Sun X, Jiang C. Anti-inflammation effects of hydrogen saline in LPS activated macrophages and carrageenan induced paw oedema. *J Inflamm (Lond)* 2012; **9**(1): 2.

[16] Choi HG, Lee DS, Li B, Choi YH, Lee SH, Kim YC. Santamarin, a sesquiterpene lactone isolated from *Saussurea lappa*, represses LPS-induced inflammatory responses via expression of heme oxygenase-1 in murine macrophage cells. *Int Immunopharmacol* 2012; **13**(3): 271-279.

[17] Maines MD, Panahian N. The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions? *Adv Exp Med Biol* 2001; **502**: 249-272.

[18] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000; **6**(4): 422-428.

[19] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997; **236**(2): 313-322.

[20] Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007; **47**: 89-116.

[21] Morse D, Lin L, Choi AM, Ryter SW. Heme oxygenase-1, a critical arbitrator of cell death pathways in lung injury and disease. *Free Radic Biol Med* 2009; **47**(1): 1-12.

[22] Yang J, Wu S, Li C. High efficiency secondary somatic embryogenesis in *Hovenia dulcis* Thunb. through solid and liquid cultures. *Sci World J* 2013; **2013**: 1-6.

[23] Park JS, Kim IS, Rehman SU, Na CS, Yoo HH. HPLC determination of bioactive flavonoids in *Hovenia dulcis* fruit extracts. *J Chromatogr Sci* 2015; http://dx.doi.org/10.1093/chromsci/bmv114.

[24] Chen SH, Zhong GS, Li AL, Li SH, Wu LK. Influence of *Hovenia dulcis* on alcohol concentration in blood and activity of alcohol dehydrogenase (ADH) of animals after drinking. *Zhongguo Zhong Yao Za Zhi* 2006; **31**(13): 1094-1096.

[25] Hyun TK, Eom SH, Yu CY, Roitsch T. *Hovenia dulcis*-an Asian traditional herb. *Planta Med* 2010; **76**(10): 943-949.

[26] Wang M, Zhu P, Jiang C, Ma L, Zhang Z, Zeng X. Preliminary characterization, antioxidant activity in vitro and hepatoprotective effect on acute alcohol-induced liver injury in mice of polysaccharides from the peduncles of *Hovenia dulcis*. *Food Chem Toxicol* 2012; **50**(9): 2964-2970.

[27] Kim HL, Sim JE, Choi HM, Choi IY, Jeong MY, Park J, et al. The AMPK pathway mediates an anti-adipogenic effect of fruits of *Hovenia dulcis* Thunb. *Food Funct* 2014; **5**(11): 2961-2968.

[28] Becker S, Mundandhara S, Devlin RB, Madden M. Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: further mechanistic studies. *Toxicol Appl Pharmacol* 2005; **207**(Suppl 2): 269-275.

[29] Oh YC, Cho WK, Im GY, Jeong YH, Hwang YH, Ma JY. Anti-inflammatory effect of *Lycium* fruit water extract in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. *Int Immunopharmacol* 2012; **13**(2): 181-189.

[30] Choi HJ, Choi HJ, Park MJ, Lee JY, Jeong SI, Lee S, et al. The inhibitory effects of *Geranium thunbergii* on interferon-γ- and LPS-induced inflammatory responses are mediated by Nrf2 activation. *Int J Mol Med* 2015; **35**(5): 1237-1245.

[31] Park EJ, Kim YM, Park SW, Kim HJ, Lee JH, Lee DW. Induction of HO-1 through p38 MAPK/Nrf2 signaling pathway by ethanol extract of *Inula helenium* L. reduces inflammation in LPS-activated RAW 264.7 cells and CLP-induced septic mice. *Food Chem Toxicol* 2013; **55**: 386-395.

[32] Li B, Choi HJ, Lee DS, Oh H, Kim YC, Moon JY, et al. Amomum tsao-ko suppresses lipopolysaccharide-induced inflammatory responses in RAW 264.7 macrophages via Nrf2-dependent heme oxygenase-1 expression. *Am J Chin Med* 2014; **42**(5): 1229-1244.

[33] Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008; **454**(7203): 436-444.

[34] Drexler SK, Kong PL, Wales J, Foxwell BM. Cell signaling in macrophages, the principal innate immune effector cells of rheumatoid arthritis. *Arthritis Res Ther* 2008; **10**(5): 216.

[35] Didonato JA, Mercurio F, Karin M. NF-κB and the link between inflammation and cancer. *Immunol Rev* 2012; **246**(1): 379-400.

[36] Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004; **25**(6): 280-288.

[37] Lee AS, Jung YJ, Kim D, Nguyen-Thanh T, Kang KP, Lee S, et al. Sirt2 ameliorates lipopolysaccharide-induced inflammation in macrophages. *Biochem Biophys Res Commun* 2014; **450**(4): 1363-1369.

[38] Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol Rev* 2008; **60**(1): 79-127.

[39] Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* 2010; **80**(12): 1895-1903.

[40] Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. *Arch Toxicol* 2011; **85**(4): 241-272.

[41] Manandhar S, You A, Lee ES, Kim JA, Kwak MK. Activation of the Nrf2-antioxidant system by a novel cyclooxygenase-2 inhibitor furan-2-yl-3-pyridin-2-yl-propeone: implication in anti-inflamma tory function by Nrf2 activator. *J Pharm Pharmacol* 2008; **60**(7): 879-887.