Thesis for the Degree of Master of Science

Anti-inflammatory effect of hexane fraction from *Eisenia bicyclis* on RAW 264.7 cells and mouse ear edema

By

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Anti-inflammatory effect of hexane fraction from *Eisenia bicyclis* on RAW 264.7 cells and mouse ear edema

(대황 헥산 분획물의 RAW 264.7 세포와 쥐 귀 부종에 대한 항 염증 효과)

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by

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김보운

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요 약

우리 몸에서 일어나는 염증반응은 외부 항원의 침입에 대한 중요한 면역 체계이다. 하지만 과도한 염증성 반응이 발생할 경우 여러 가지 염증성 질병을 유발하게 된다. 다년생 갈조류인 대황은 항균 활성과 항산화 능력이 밝혀진 바 있지만, 대식세포에서 가지는 항염증 활성은 현재까지 자세하게 연구된 바가 없다. 이 논문에서 대황의 헥산 분획물(EHF)이 염증성 반응을 조절하여 염증인자의 생성을 억제하고, 항산화 능력과 세포 보호 기능을 가지는 천연 항염증성 물질임이 밝혀졌다. 대황의 항염증 활성 확인을 위하여, 대황 분획물중 NO 생성 억제 능력이 가장 뛰어난 헥산 분획물 (EHF)을 이용 하였다. LPS로 염증을 유도시킨 RAW 264.7 세포에서 EHF (10, 25, 50 μg/mL)는 염증성 매개체인 NO, PGE_2, 그리고 ROS의 생성을 감소시켰다. NO와 PGE_2를 생성하는 iNOS와 COX-2 효소 발현에 영향을 미치는 EHF의 효과를 확인하기 위해 Western blot을 이용하였으며, EHF의 처리에 따라 농도 의존적으로 염증성 단백질의 발현이 감소하였다. RT-PCR을 통하여 이 단백질들의 mRNA들을 확인함으로써, 염증성 단백질과 매개물의 조절이 유전자 전사 단계에서부터 발생한다는 것을 추가로 확인하였다. 그리고 EHF의 처리를 통해 항산화 효소 hemeoxygenase (HO)-1가 mRNA 전사 단계에서 발현이 증가함을 알 수 있었다.

염증성 cytokine인 tumor necrosis factor (TNF)-α와 interleukin (IL)-6, interleukin (IL)-1β의 생성 또한 EHF의 처리에 의하여 전사적 단계에서부터 감소하는 것을 확인 하였다. Western blot과 Confocal 촬영, 그리고 면역 형광 분석법을 이용한 실험 결과를 통해 EHF의 처리에 의해서 NF-κB의 핵으로의 이동과 promoter활성이 현저히 줄어 들었음을 알

수 있었다. LPS를 이용한 염증 반응 유도와 NF-κB의 활성 과정이 어떠한 신호전달 체계를 통해 조절 되었는지 확인하기 위하여 Akt와 extracellular-signal regulated kinase (ERK), c-Jun NH2-ternimal kinase (JNK), p38의 인산화 정도를 Western blot을 통해서 확인한 결과, EHF의 처리에 따라 MAPKinase와 Akt의 인산화가 농도 의존적으로 감소하였다.

in vitro 실험에 이어, 쥐의 귀에 부종을 일으킨 후 EHF를 처리하여 EHF의 *in vivo*에서의 효과도 확인하였다. EHF의 처리는 부종을 상당히 억제 시켰으며 이와 같은 실험 결과들은 EHF가 *in vivo*와 *in vitro*에서 효과적으로 염증성 반응을 감소시키는 기능성 물질로 이용 될 수 있음을 시사한다.

1. Introduction

Inflammatory system is an important mechanisms keeping our body in a healthy state via modulating a number of related proteins and cytokines against extracellular stimuli (Lawrene et al., 2002). However, over-activated or prolonged inflammation can be another harmful factor on our health that should be regulated since it causes further damage in other cells and tissue (MacMicking et al., 1997) and develops inflammation related diseases such as a cancer in various tissues (Maccio & Madeddu, 2012), bronchitis (Vernooy et al., 2002), chronic renal disease (Sean & Cockwell, 2005), and arthritis (Yamamoto et al., 2014). Thus, regulating inflammatory response could be an important approach to suppress diseases related on inflammation.

Murine macrophage RAW 264.7, playing a critical role in the process of inflammation, is a representative immune cell of mammal that immortalized to be used for various *in vitro* experiments. The macrophage induced by lipopolysaccharide (LPS) stimulation produce nitric oxide (NO), prostaglandin E2 (PGE2), reactive oxygen species, and cytokines including TNF-a, IL-6, and IL-1b (Dey et al., 2006). LPS form gram negative bacteria induces inflammatory responses by activating many inflammatory signaling pathways (Guha & Machman, 2001). Once an inflammation is induced in RAW 264.7 cells, large amount of NO is produced. NO is very important mediator of inflammatory process made by inducible isoforms of NOS (Kleinert et al., 2004). In addition, PGE_2 is also generated during the inflammation. PGE2 plays an important role in inflammation (Kalinski, 2012). Over produced NO and PGE2, due to severe inflammation, could have negative effects such as leading to cell death on neighboring cells and tissues.

Health beneficial effects of diverse marine algae on modulating inflammation in RAW 264.7 (Jeoung et at., 2012), microglia (Kim et al., 2012), and human hepatocarcinoma cell (Lee et al., 2012) were revealed. For this reason, marine algae have been attracted scientists' attentions as a potential functional source for the treatment of inflammation related disease. Marine algae is expected to provide a source of diverse compounds that could be possibly used to develop new drug or functional food having anti-inflammatory effect (Nisizawa et al., 1987). *Eisenia bicyclis* is an edible brown algae distributed on the coast of Korea and Japan, belonging to family of laminariaceae (NFR&D, 2009). Diverse biological activities including anti-oxidative cytoprotective activities have been reported previously (Choi JS et al., 2014, Chowdhury MT et al., 2014, Jung Ha et al., 2013). However, its anti-inflammatory effect in murine macrophage has not been studied yet. Therefore, in this study, *E. bicyclis* was subjected to potential anti-inflammatory marine plant source.

Through the screening test, hexane fraction of *E. bicyclis* (EHF) has the strongest inhibitory action on nitrite generation in LPS-stimulated RAW 264.7 cells among organic solvent fractions. To confirm the anti-inflammatory action EHF, we investigated the molecular mechanism of the anti-inflammatory activity of EHF using LPS-stimulated RAW 264.7 cells. This result suggests that dietary supplements of EHF could help to prevent or treat inflammatory diseases.

2. Materials and Methods

2-1. Materials and reagents

LPS (Escherichia coli O55:B5), bovine serum albumin (BSA), indomethacin, phenylmethylsulfonyl fluoride (PMSF), 2',7'-dichlorofluorescin diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay, and reverse transcriptase were obtained from Promega (Madison, WI, USA). Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies and enhanced chemiluminescence (ECL) detection kit were obtained from GE Healthcare Bio-Science (Piscataway, NJ, USA). Enzyme-linked immunesorbent assay (ELISA) kits for cytokines, 4′,6-diamidino-2-phenylindole (DAPI), Lipofectamine/Plus, TRIzol, Alexa Fluor® 488-conjugated secondary antibody, and dual luciferase assay kit were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin mixture, 0.25% trypsin-ethylenediamine tetra-acetic acid (EDTA), and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA).

2-2. Preparation of the extraction and fractionation of EFH

E. bicyclis was collected from the coast of Dok-do, South Korea, in 2013. The seaweed was dried in an air-drier for 3 days and powdered. Dried powder of *E. bicyclis* was extracted three times with 96% (v/v) ethanol (EtOH) for 3 h at 70℃. The combined extracted were concentrated under reduced pressure to obtain the EtOH

extract. For further fractionation of the EtOH extract, the extract was re-suspended in water:EtOH (9:1, v/v) and partitioned successively with *n*-hexane and ethyl acetate. The *n*-hexane fraction, showed the highest anti-inflammatory activities on LPS-stimulated RAW 264.7 cells, was kept at -20℃ and used for further analysis of this study.

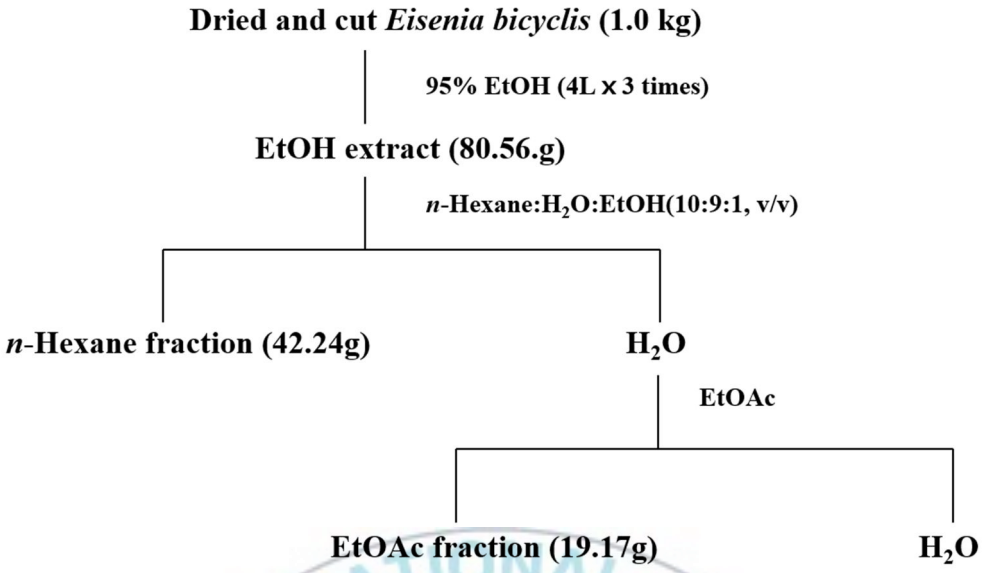


Fig. 1. Extraction and Fractionation procedure of *Eisenia bicyclis*

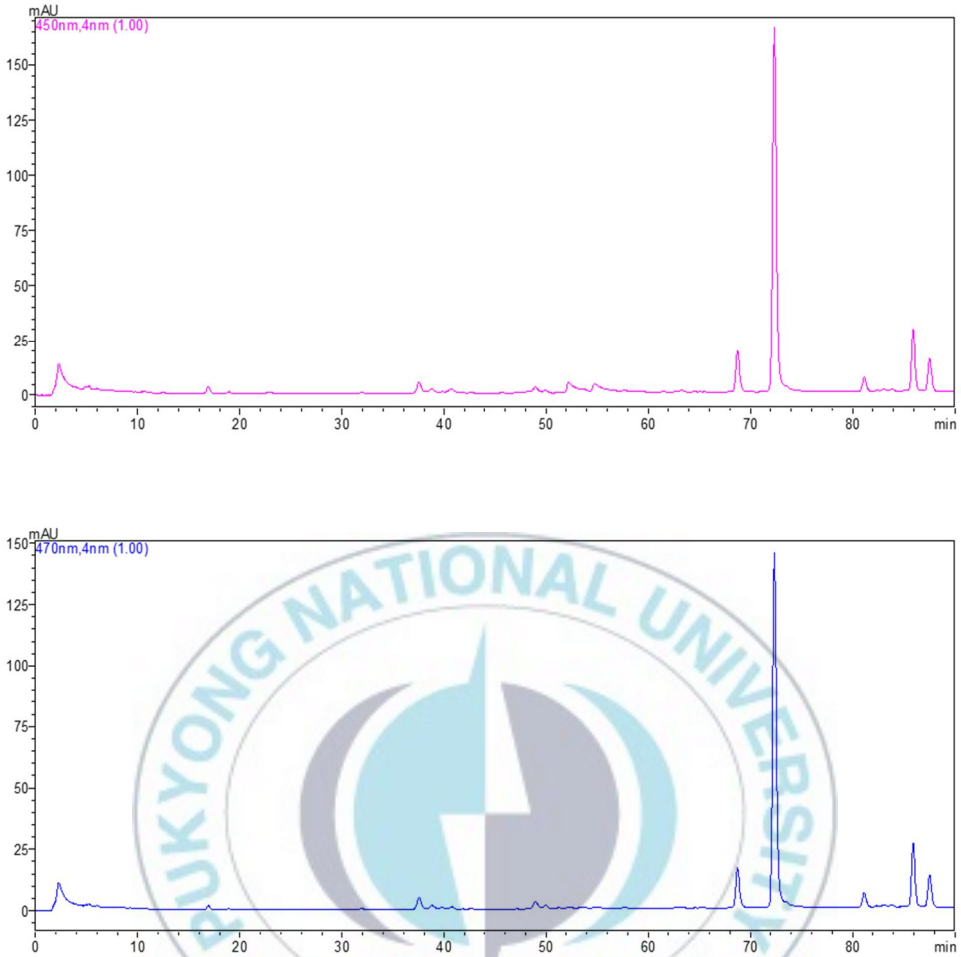


Fig. 2. HPLC chromatogram of EHF

2-3. Cell Culture and Treatment

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in humidified incubator containing 5% CO2 at 37℃ in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 μg/mL).

2-4. Cell viability

Viability of RAW 264.7 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using the CellTiter$^{96®}$ AQueous One Solution Proliferation Assay (Promega, Madison, WI, USA) in the presence or absence of EFH in different concentrations according to the manufacturer's instruction. Briefly, cells were inoculated at a density of 5×10^5 cells/well into 96-well plate and cultured at 37℃ for 24 h. The cells were co-treated with EFH (0-50 μg/mL) and LPS (1 μg/mL) for 24 h. The medium was replaced with 95 μL fresh medium and 5 μL MTS solution. After one hour, the absorbance was measured at 490 nm using a microplate reader (Glomax Multi Detection System, Promega, Madison, WI, USA).

2-5. Measurements of NO, PGE_2, and pro-inflammatory cytokines

RAW 264.7 cells were plated in a 96-well plate at a density of 1.2 x 10^5 cells/well and incubated for 24 h. Incubated RAW 264.7 cells were co-treated with EFH (0, 10, 25, and 50 μg/mL) and LPS (1 μg/mL) for 24 h or 16 h. After the treatment, the cultured media of the 24 h treated RAW264.7 cells were collected to measure the nitrite concentrations which is an indicator of NO production according to the Griess reaction. To measure the concentrations of PGE_2, IL-1β, IL-6, and TNF-α, cell cultured media were quantified by enzyme-linked immunosorbent assay (Carlsbad, CA, USA) according to the manufacturer's instructions.

2-6. Preparation of cytosolic and nuclear extracts

RAW264.7 cells (10×10^5 cells/well) were co-treated with EFH (0, 10, 25, and 50 μg/mL) and LPS (1 μg/mL). Cells were washed two times with ice-cold PBS, and harvested cells were centrifuged at 12000×g for 5 min at 4 °C. Pellets were suspended in 180 μL of hypotonic buffer (10 mM Tris–HCl, 10 mM NaCl, 3 mM MgCl2, 0.02% NaN3, 0.5mM DTT and 1 mM PMSF, pH 7.4), and incubated on ice for 5 min after adding 20 μL of 5% Nonidet P-40. The mixture was centrifuged at 1800×g for 5 min. Supernatant was collected as cytosolic extract. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mMEDTA, 0.02% NaN3, 0.5 mM DTT, and 1 mM PMSF, pH 7.4), remained for 1 hour on ice and centrifuged at 14000×g for 10 min. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of the protein concentration.

2-7. Western blot analysis

RAW 264.7 cells (10 x 10^5 cells/well) were plated in a 6-well plate and incubated for 24 h. Cells were treated with EHF (0, 10, 25, and 50 μg/mL) was treated with LPS (1μg/mL) for 30 min and 16 h. The cells were washed twice with cold PBS and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 1% Tween 20, 0.1% SDS, 1 mM Na3VO4, 10 μg/mL leupeptin, 50 mM NaF, and 1 mM PMSF) on ice. After centrifugation at 14,000g for 20 min, the protein concentrations in the supernatants were determined, and aliquots of the protein (30 μg) were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Trisbuffered saline with 0.1% Tween 20 (TBST) for 2 h, followed by the incubation for 10 h with primary antibody in TBST. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST for 2 h, and immune complexes were detected using an ECL detection kit. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software.

2-8. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells placed in a 6-well plate were co-treated with EFH (0, 10, 25, and 50 μg/mL) and LPS (1 μg/mL) for 6 h. Total RNA from each group was isolated with the TRIzol reagent. 5 microgram of total RNA was used for reverse transcription using oligo-dT-adaptor primer and superscript reverse transcriptase. PCR was carried out with the gene-specific primers arranged on Table. 1. GAPDH

and Actin were used as an internal standard to evaluate relative expression of COX-2, iNOS, and pro-inflammatory cytokines. Densitometric analysis of the data obtained from at least three independent experiments was performed using cooled CCD camera system EZ-Capture II and CS analyzer ver. 3.00 software (ATTO & Rise Co., Tokyo, Japan).

Primers		Sequences
iNOS	Forward	5'-TCT TTG ACG CTC GGA ACT GT-3‘
	Reverse	5'-CCA TGA TGG TCA CAT TCT GC-3‘
COX-2	Forward	5'-TGG GCA AAG AAT GCA AAC AT-3‘
	Reverse	5'-CAG CAA ATC CTT GCT GTT CC-3’
HO-1	Froward	5'-AAG ATT GCC CAG AAA GCC CTG GAC-3'
	Reverse	5'-AAC TGT CGC CAC CAG AAA GCT GAG-3‘
TNF-α	Forward	5'-CAA GGG ACA AGG CTG CCC CG-3'
	Reverse	5'-GGT CAG AGT GGG GGC TGG GT-3‘
IL-1β	Forward	5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3'
	Reverse	5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'
IL-6	Forward	5'-GTA TGA ACA ACG ATG ATG CAC TTC CAG-3'
	Reverse	5'-GCA TTG GAA ATT GGG TAG GAA GG-3'
B-Actin	Forward	5'-CCT CAT GAA GAT CCT GAC CG-3'
	Reverse	5'-TCC ACA TCT GCT GGA AGG TG-3'

Table 1. Primer sequences used in this study

2-9. Measurement of Intracellular ROS

The intracellular ROS scavenging activities of EHF were measured using the oxidant-sensitive fluorescent probe DCF-DA. RAW 264.7 cells (5 × 10^4 cells/well) were incubated with EFH (0, 10, 25, and 50 μg/mL) of in the absence or presence of LPS (100 ng/ml) for 2 h. After the media was removed, their fluorescence intensities were measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using fluorescence microplate reader (Dual Scanning SPECTRAmax,Molecular Devices Corporation, Sunnyvale, CA).

2-10. Immunofluorescence analysis

RAW 264.7 cells (10 × 10^4 cells/well) cultured on glass coverslips (SPL Lifesciences Co., Gyeonggi-do, Korea) were co-treated with EFH (50 μg/mL) and LPS (1 μg/mL) for 1 h. The cells were fixed in cold, 4% paraformaldehyde (PFA) in PBS for 15 min, and then permeated in 0.5% Triton X-100 in PBS for 10 min. Thereafter, the cells were incubated with 0.5% bovine serum albumin (BSA) to minimize non-specific adsorption of the antibodies for 2 h before incubation with the anti-NF-κB polyclonal antibody diluted in 3% BSA/PBS for 3 h, and it is followed by incubation with Alexa FluorW 488-conjugated secondary antibody diluted in 3% BSA/PBS for 1 h. The cells were stained with 2 μg/mL DAPI and images were captured using an LSM700 laser scanning confocal microscope
(Carl Zeiss, Oberkochen, Germany).

2-11. Transient transfection and luciferase assay

Murine NF-κB promoter/luciferase DNA (1 μg) along with 20 ng control pRL-TK DNA was transiently transfected into 2 × 10^5 RAW 264.7 cells/well in a 96-well plate using Lipofectamine/Plus reagents for 40 h. The Cells were co-treated with EHF (0, 10, 25, and 50 μg/mL) and LPS (100 ng/ml) for 6 h. Each well was washed twice with cold phosphate-buffered saline (PBS), harvested in 100 μL of lysis buffer (0.5 mM HEPES, pH 7.8, 1% Triton N-101, 1 mM CaCl2, and 1 mM MgCl2) and used for the measurement of luciferase activity using a luciferase assay kit. Luminescence was measured on a TopCount microplate scintillation and luminescence counter (PerkinElmer, Santa Clara, CA, USA) in single photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. Luciferase activity was normalized to the expression of control pRL-TK.

2-12. PMA-induced mouse ear edema

6-week old ICR mice (male, 25-30 g) were purchase from Samtako Bio Korea Co. (Gyeonggido, Korea). Animal study was carried out in accordance with the University Animal Care and Use Committee guidelines at Pukyong National University. PMA was treated only on the right ears of the mice to induce ear edema. Briefly, the mice in the control group was treated with normal saline on the right ears, and the test group was treated with EFH (0.5 and 1 mg/ear) on the right ears. After 1 h, PMA (6 μg/ear) was treated on the right ears except for the control group. 6 h after PMA application, mice were anesthetized and killed. Their ears were removed and ear edema weight was calculated by subtracting the weight of the left ear from the right ear. Inhibition percentage (IP) was expressed as a reduction in weight compared

to the PMA-treated group.

2-13. Statistical analysis

Data was expressed as the means ± standard deviations (SDs) of at least three independent experiments unless otherwise indicated. Statistical analysis was performed by Student's t-test (two-sample assuming equal variance) for multiple comparisons.

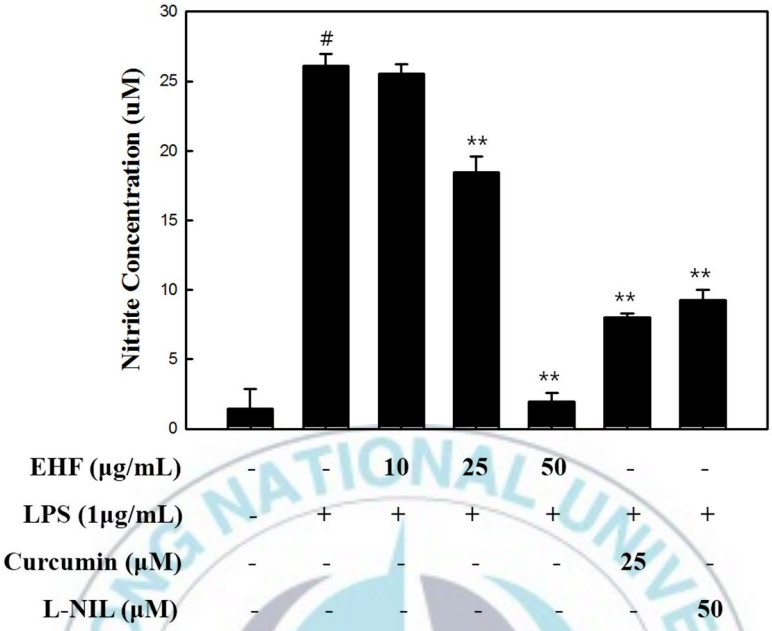
3. Results

3-1. EHF reduced the secretion of NO and PGE_2 without cytotoxicity in LPS-stimulated RAW 264.7 cells.

To study the anti-inflammatory effect of EHF on the production of NO, the nitrite concentrations of murine macrophage RAW 264.7 cells in the culture supernatant, as an index of NO, were determined by Griess reagent. RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) and LPS (1μg/mL) for 24 h. According to the result of NO assay, the nitrite concentration of the culture supernatant of LPS-only treated cells was increased to around 13 times of non LPS-treated cells. However, EHF significantly reduced the nitrite concentrations in the culture supernatants in a dose-dependent manner (Fig. 3A). Curcumin (25 μg/mL), known as anti-inflammatory substance, and N6-(1-iminoethyl)-L-lysine (L-NIL) (50 μg/mL), which is an iNOS inhibitor, were used as positive controls of reducing NO production. The NO reducing activity of EHF (50 μg/mL) was proved to be a more effective anti-inflammatory agent than curcumin and L-NIL (Fig. 3A). The concentrations of EHF used in this study did not show any cell cytotoxicity in RAW 264.7 cells (Fig 3B), which means the anti-inflammatory effects of EHF were not from its cytotoxicity.

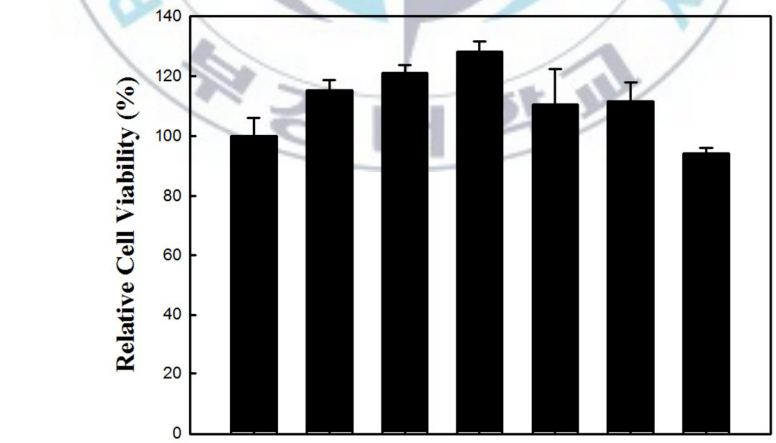
PGE_2 is one of the most important inflammatory mediators, made by COX-2. To assess whether EHF could reduce the generation of PGE_2, the RAW 264.7 cells were treated with indicated EHF concentrations (0, 10, 25, and 50 μg/mL) and LPS (1 μg/mL) for 16 h. The generation of PGE_2 in the culture supernatants was measured using PGE_2 detection kit. Similar to the results of the nitrite production, generation of the PGE_2 decreased by EHF in a concentration dependent manner (Fig. 3C).

(A)



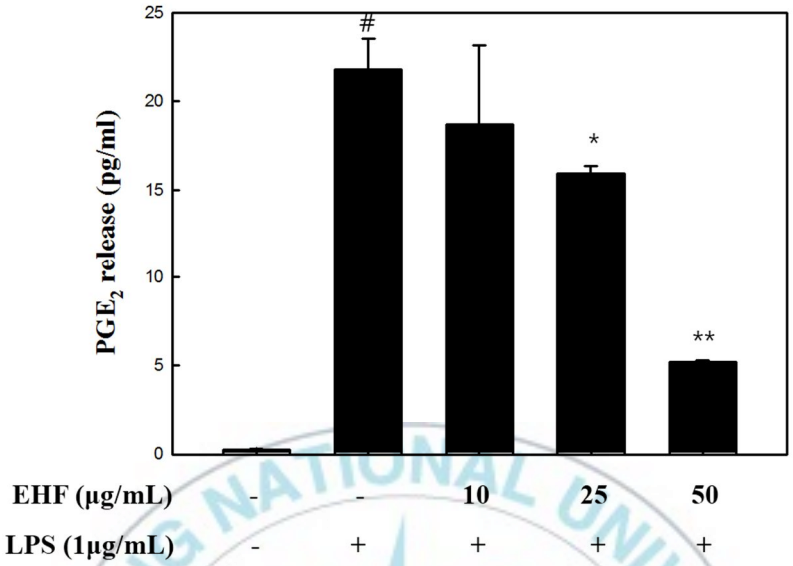
EHF (μg/mL)	-	-	10	25	50	-	-
LPS (1μg/mL)	-	+	+	+	+	+	+
Curcumin (μM)	-	-	-	-	-	25	-
L-NIL (μM)	-	-	-	-	-	-	50

(B)



EHF (μg/mL)	-	-	10	25	50	-	-
LPS (1μg/mL)	-	+	+	+	+	+	+
Curcumin (μM)	-	-	-	-	-	25	-
L-NIL (μM)	-	-	-	-	-	-	50

(C)



(D)

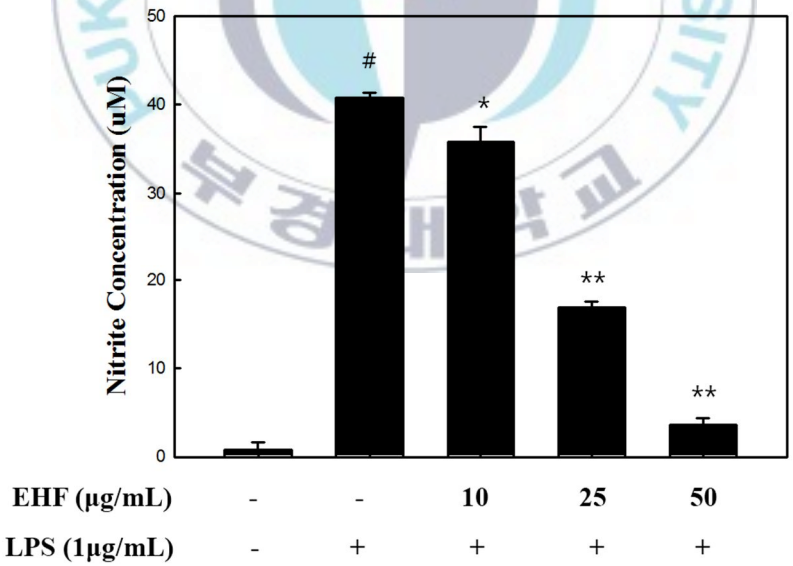


Fig. 3. Anti-inflammatory effect of EHF on cell viability and the NO and PGE_2 production in LPS-stimulated RAW 264.7 cells. Cells were co-treated with indicated concentrations of EHF with LPS for 24 h or 16 h. The treated culture media for 24 h was used to measure the amount of NO production (A) and the treated culture media for 16 h was used to measure PGE_2 production (B). The culture media of the cells post-treated with different concentrations of EHF after 1 h LPS treatment was used to measure the amount of NO production (C). Cell viability was measured by MTS assay (D). Curcumin and N6-(1-iminoethyl)-L-lysine (L-NIL) were used as positive controls for the comparison. The results presented are representative of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

3-2. EHF reduced ROS production in RAW 264.7 cells

Many studies have suggested that LPS-induced reactive oxygen species (ROS) is one of the major causes of NF-κB activation and stimulators of NF-κB gene expression (Cho et al., 2000).

To assess if EHF inhibits ROS production in LPS-induced RAW 264.7 cells, the cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) and LPS (1μg/mL) for 2 h. As shown in Fig. 4, it is monitored by using DCFH-DA florescence method that the ROS production of LPS-stimulated cells significantly increased compared to of the non LPS-stimulated cells. The ROS levels of EHF treated cells decreased in a dose dependent manner (Fig. 4). 50 μg/mL of EHF showed the meaningful inhibitory effect on ROS production.

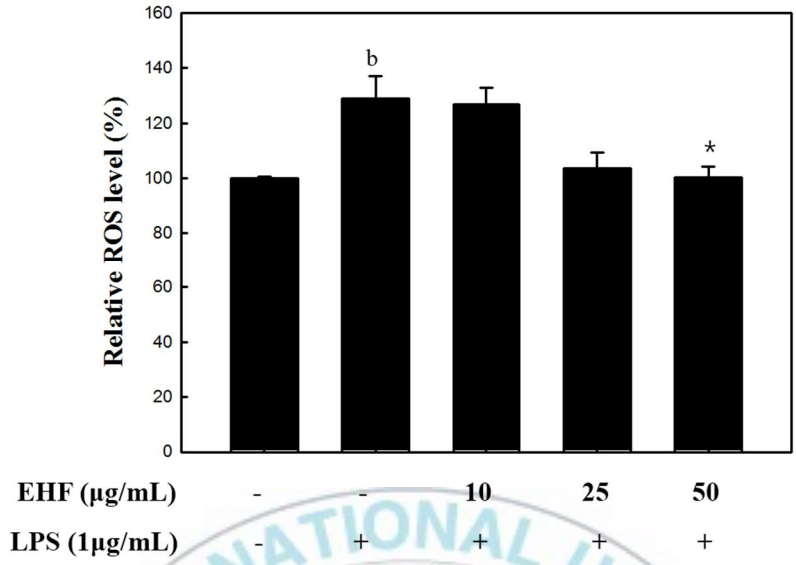


Fig. 4. Anti-inflammatory effect of EHF on LPS-induced ROS production in RAW 264.7 cells. Cells were co-treated with indicated concentrations of EHF, LPS, and DCFH-DA (0.02μM) for 2 h. ROS accumulation and the ROS-scavenging activity of EHF were monitored by using DCFH-DA florescence method. The results presented are representative of three independent experiments. *$p < 0.05$ indicates significant differences compared to the LPS-only treated group. $^{b}p < 0.05$ indicates significant differences compared to the control group.

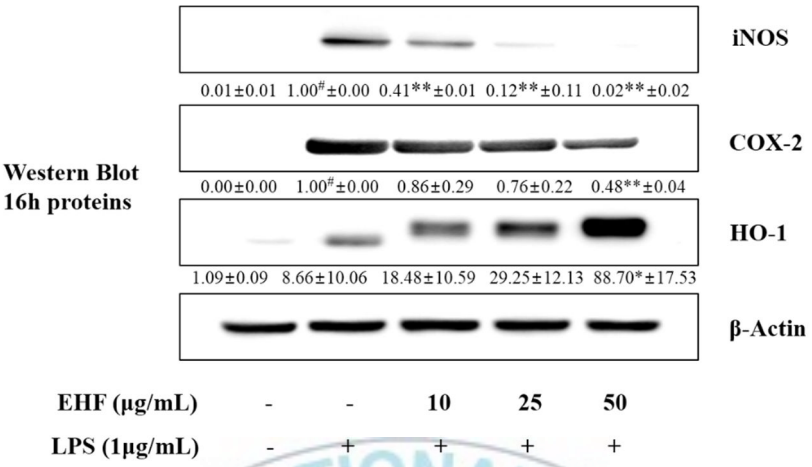
3-3. EHF reduced the expression of the pro-inflammatory proteins in LPS-stimulated RAW 264.7 cells.

To verify whether the decreased nitrite concentration and PGE_2 are due to the down-regulated of iNOS and COX-2 proteins and, by extension, their related mRNAs, Western blot and Real-time PCR (RT PCR) were conducted. In addition to it, to fine other anti-inflammatory effects of EHF, which turned out to be the ability to increase anti-oxidative protein, Heme oxygenase (HO)-1, other potential inflammation-related proteins were also measured by the same procedures.

RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) with LPS (1μg/mL) for 16 h. Equal amount of whole cell proteins were subjected to 10% SDS-PAGE and the iNOS, COX-2, HO-1, and β–actin expressions were detected. We could barely find the expressions of iNOS and COX-2 proteins in unstimulated RAW 264.7 cells. However, in the LPS-induced cells, the expressions of the proteins were obvious. With the treatment of different concentrations of EHF, the expressions of iNOS and COX-2 were markedly attenuated in a dose-dependent manner (Fig. 5A). As we mentioned, the expression of HO-1 was also measured and it was showed that EHF up-regulated the expression of HO-1 dose-dependently in LPS-induced RAW 264.7 cells (Fig. 5A).

To conduct RT-PCR, RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) with LPS (1μg/mL) for 6 h and the cells were collected with Trizol. In the same tendency with the expressions of the proteins (Fig. 5A), the mRNA expressions of iNOS and COX-2 were down-regulated and of HO-1 was up-regulated by the treatment of EHF (Fig. 5B). For both protein and mRNA quantitative analysis, β–actin was used as a housekeeping standard (Fig. 5A, 5B).

(A)



(B)

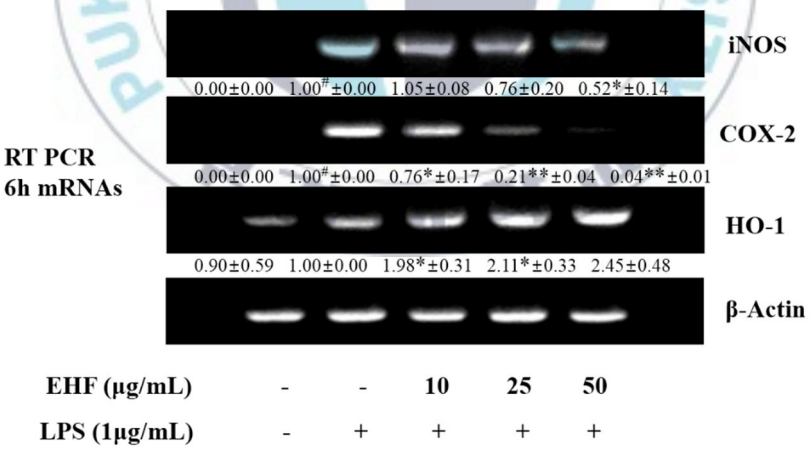


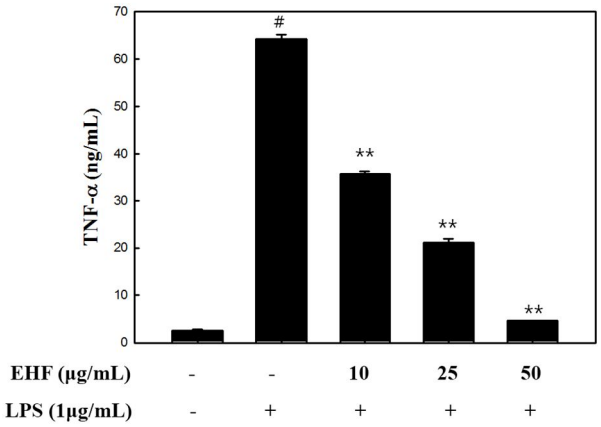
Fig. 5. Anti-inflammatory effect of EHF on expressions of iNOS, COX-2, and HO-1 proteins and mRNA expressions in LPS-stimulated RAW 264.7 cells. Cells were co-treated with indicated concentrations of EHF with LPS for 16 h. Equal amount of total proteins were subjected to 10% SDS-PAGE. iNOS, COX-2, HO-1, and β –actin protein expressions were detected by Western blot using corresponding antibodies (A). Cells were co-treated with indicated concentrations of EHF and LPS for 6 h and then collected with TRIzol. mRNA levels of iNOS, COX-2, HO-1 and β-actin were determined by RT-PCR analysis using respective gene-specific primers. The results presented are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

3-4. EHF reduced the production of pro-inflammatory cytokine in LPS-stimulated RAW 264.7 cells.

Release of the inflammatory cytokine release is one of the features of inflammation. TNF-α, IL-6, and IL-1β are the major pro-inflammatory signaling cytokines in LPS-induced macrophages (Uto et al., 2010). RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) with LPS (1μg/mL) until enough to detect the secreted cytokines in culture media (for 16 h). ELISA, quantitative detection method, was used to analyze the release of TNF-α, IL-6, and IL-1β. LPS stimulation extremely increased the releases of the cytokines to at least more than 30 times of the cytokines in the culture supernatant of non LPS-induced. When the cells were treated with EHF, however, the releases of TNF-α (Fig. 6A), IL-6 (Fig. 6B), and IL-1β (Fig. 6C) were clearly abolished dose dependently.

By the same procedure of mRNAs in Fig. 5, the expressions of the pro-inflammatory cytokine mRNAs were analyzed. In accordance with cytokine releases, the mRNA expressions of TNF-α, IL-6, and IL-1β were decreased (Fig. 6D). mRNA expression of GAPDH was used as a housekeeping standard (Fig. 6D).

(A)



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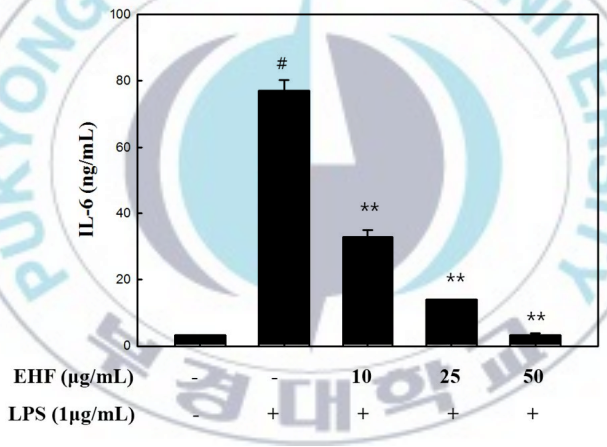
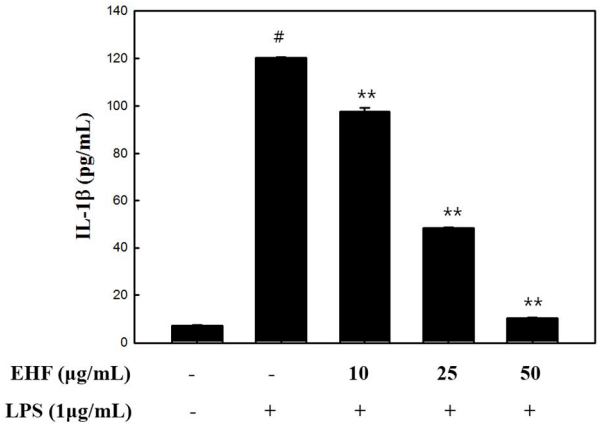


(C)



(D)

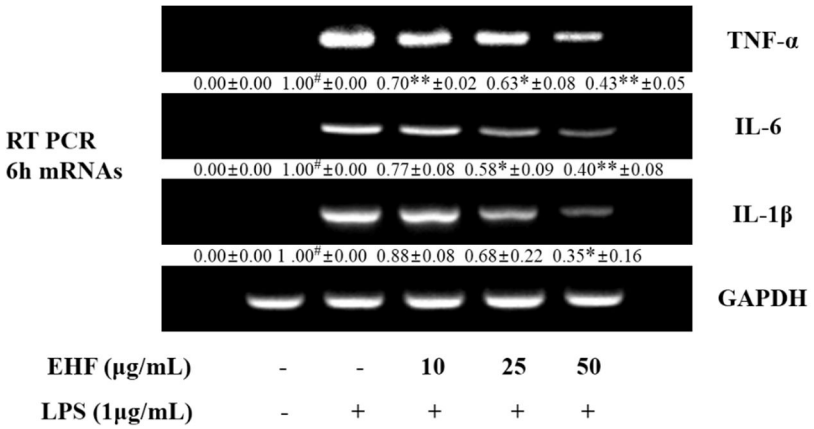


Fig. 6. Anti-inflammatory effect of EHF on generation of pro-inflammatory cytokines such as TNF-a, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells. Cells were co-treated with indicated concentrations of EHF with LPS for 16 h. Inflammatory-cytokine TNF-α (A), IL-6 (B), and IL-1β (C) in the cultured media were measured by ELISA kit. mRNA levels of TNF-α, IL-6, and IL-1β were determined by RT-PCR analysis using respective gene-specific primers. The results presented are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

3-5. EHF inhibited NF-κB activation in LPS-stimulated RAW 264.7 cells.

NF-κB translocation into the nucleus is a crucial factor for iNOS and COX-2 expression, which is induced by LPS or pro-inflammatory cytokine stimulation (Chapman & Perkins, 2000). NF-κB translocation and DNA binding of this transcriptional factor are modulated by phosphorylation and degradation of inhibitory factor kappa B (IκB)-α and phosphorylation of Ikk-β.

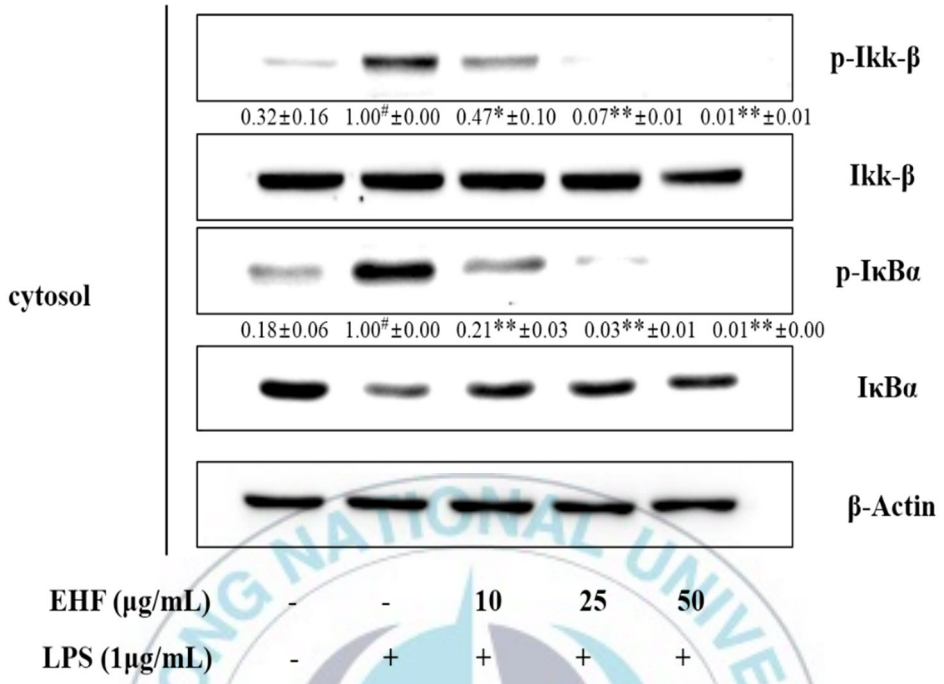
We analyzed the degree of the phosphorylation of IκB-α and Ikk-β to assess weather EHF affects on NF-κB translocation into the nucleus. RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) with LPS (1μg/mL) for 30 min and the phosphorylation of cytosolic IκB-α and Ikk-β were anaylzed. Furthermore, the levels of IκB-α in cytoplasm was analyzed to confirm the recovery of IκB-α. As shown in the Fig. 7A, the phosphorylation of IκB-α and Ikk-β were increased by LPS stimulation. Co-treatment of EHF with LPS on RAW 264.7 cells inhibited not only LPS-mediated IκB-α degradation but also phosphorylation of Ikk-β (Fig. 7A). Along with this result, the recovery of cytosolic IκB-α was also sighted (Fig. 7A).

To see the definite effect of EHF on NF-κB translocation, the level of p65, a major component of NF-κB, in nucleus was detected. The translocation of NF-κB in LPS-induced cells was markedly attenuated by EHF treatment (Fig. 7B).

In addition, to have more precise influence of EHF on NF-κB/p65 translocation into nucleus found, we fulfilled immunofluorescence analysis and luciferase assay. According to the taken confocal images, NF-κB/p65 nuclear translocation was strongly induced by LPS in RAW 264.7 cells (Fig. 7C). It was

revealed that when EHF were co-treated with LPS, NF-κB/p65 in nuclear was sequestered again in the cytoplasmic compartment as it is supposed to be in non-stimulated cells and as we can see on the images of control cell group (Fig. 7C). Since the inhibitory effect of EHF on NF-κB/p65 translocation into nucleus was proved, as the next step, we decided to determine if EHF has an effect on the promoter activity of NF-κB in LPS-induced RAW 264.7 cells. The result suggests that EHF strongly suppressed the expression of promoter activity of NF-κB marked by NF-κB promoter/luciferase DNA in LPS-induced RAW 264.7 cells (Fig. 7D).

(A)



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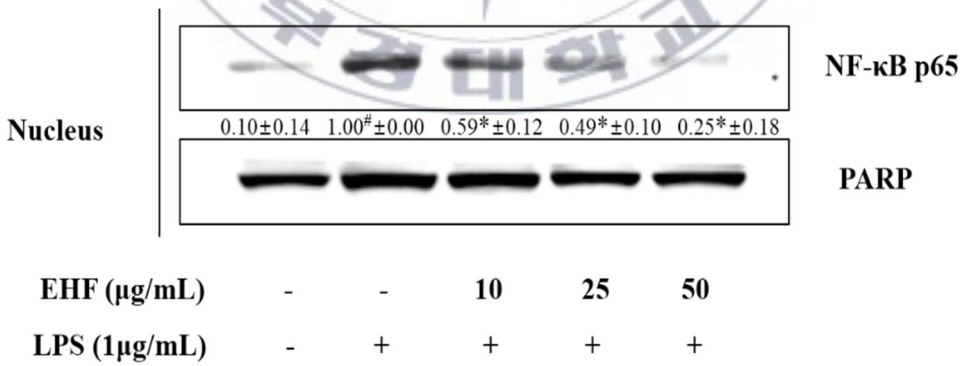

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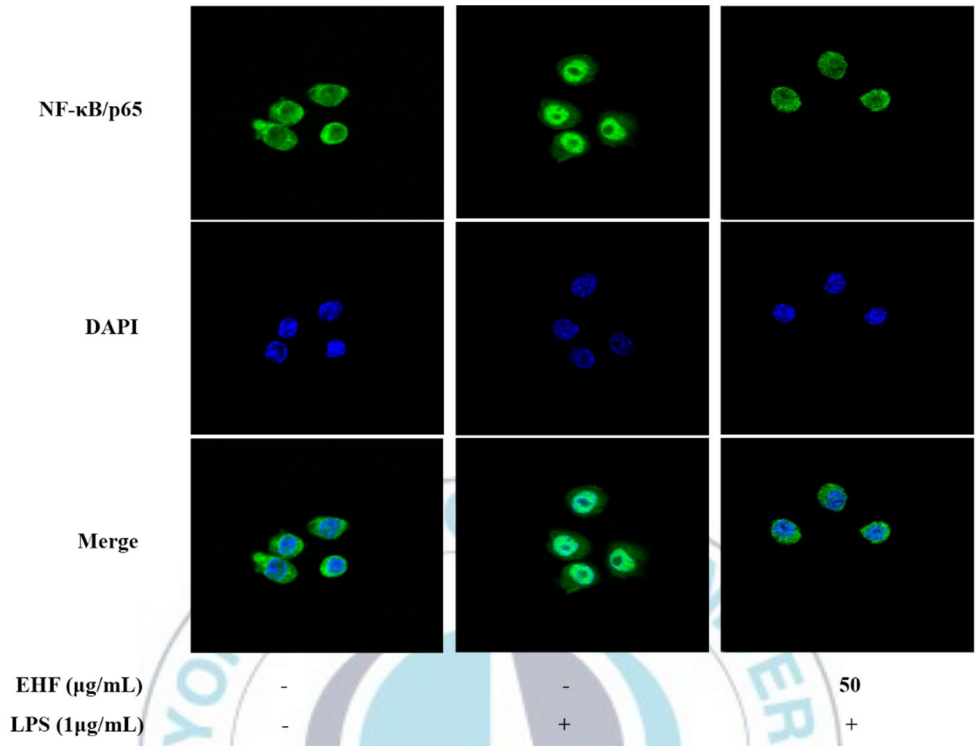

(D)

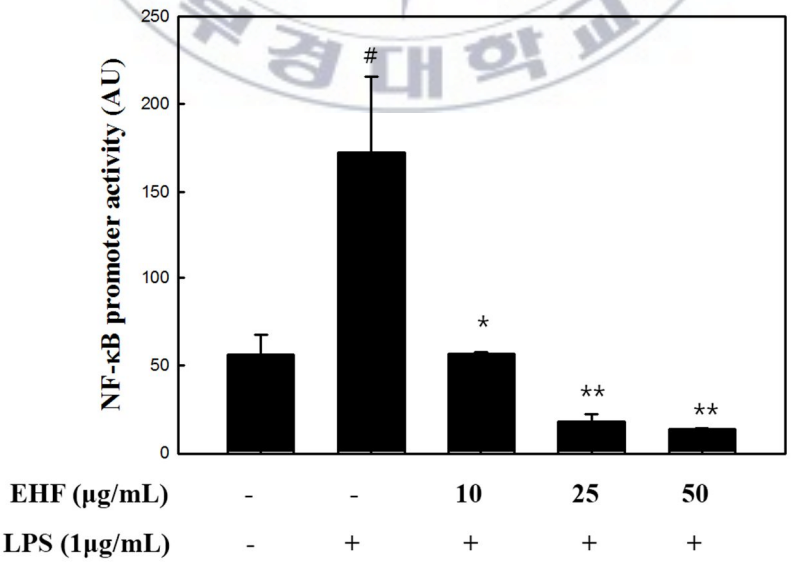

Fig. 7. Inhibitory effect of EHF on the degradation of IκB-α, the activation and translocation of NF-κB, and promoter activity in LPS-stimulated RAW 264.7 cells. The regulation of IκB-α phosphorylation, p-Ikk-β Phosphorylation (A), and Nuclear localization of NF-κB (B) were analyzed by Western blot using corresponding antibodies. Cells were co-treated with indicated concentrations of EHF with LPS for 30 min. Cells were co-treated with indicated concentrations of EHF with LPS for 1 h. NF-κB/p65 subunits were probed by anti-NF-κB polyclonal primary antibody and Alexa Fluor® 488-conjugated secondary antibody. The nuclei were stained with DAPI and the images were captured by confocal microscopy analysis (C). Cells were co-transfected with 1 μg of NF-κB promoter-containing luciferase DNA along with 40 ng of control pRL-TK DNA for 40 h. Transfected cells were co-treated with indicated concentrations of EHF with LPS for 6 h (D). The results presented are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

3-6. EHF inhibited the phosphorylation of MAPKinase and Akt in LPS-induced RAW 264.7 cells.

It is well known that MAPKinase and Akt signaling pathways play a key role in the regulation of inflammatory mediator production. MAPKinase and Akt transduce extracellular signals to intracellular response, leading to inflammation response (Cho et al., 2003). For that reason, we investigated the phosphorylation of MAPKinase and Akt by EHF treatment in RAW 264.7 cells.

RAW 264.7 cells were treated with different concentrations of EHF (0, 10, 25, and 50 μg/mL) with LPS (1μg/mL) for 30 min. Western blot was conducted on the equal amount of whole cell proteins. ERK, JNK, p38, and Akt in LPS-induced cells were highly phosphorylated. The co-treatment of different concentrations of EHF, however, reduced the level of the phosphorylation with effect (Fig. 8). This result suggests that EHF has a strong anti-inflammatory effect which down-regulates the NF-κB translocation into the nucleus through the modulation on MAPKinase and Akt.

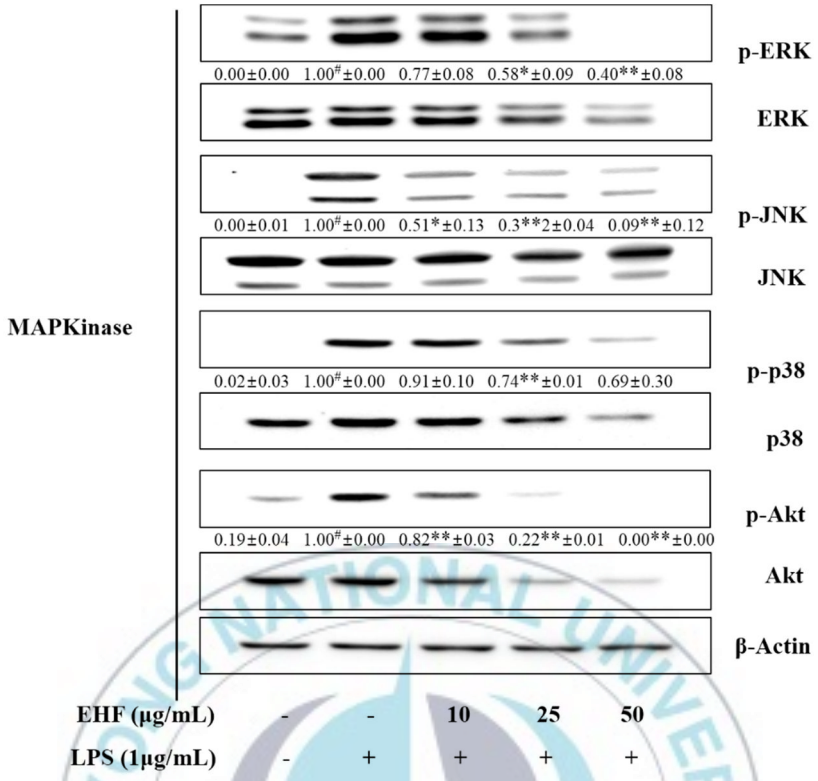

Fig. 8. Inhibitory effect of EHF on the phosphorylation of MAPKinase and Akt in LPS-stimulated RAW 264.7 cells. Western blot analysis on phosphorylation of MAPK and Akt was conducted. Cells were incubated with indicated concentrations of EHF and LPS (1 μg/ml) for 30 min. Whole cell lysates were prepared and analyzed by Western blot for total and phosphorylated proteins of Akt, ERK, JNK, or p38 MAPK using respective primary antibodies. The results presented are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

3-7. EHF suppressed the PMA- induced ear edema of mouse.

To confirm the anti-inflammatory effect of EHF *in vivo*, we carried out edema test on mouse ears. 6-week old mice were used for this experiment. 6-h application of the PMA on the right ear surfaces generated severe edema. The treatment of EHF could mitigate the edema occurred on the right ears (Fig. 9). A common clinical non-steroidal anti-inflammatory drug, Indomethacin, was used as a positive control of EHF (Fig. 9).

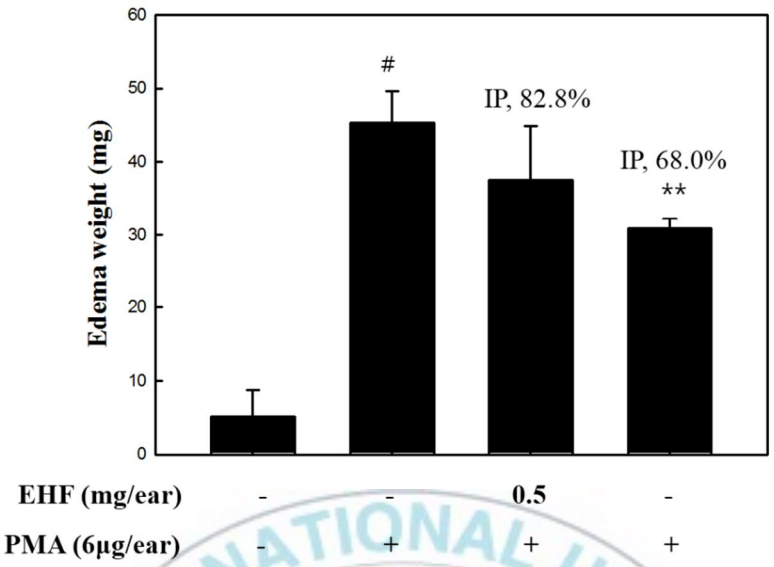

Fig. 9. Effect of EHF on ear edema induced by PMA in mouse. PMA induced ear edema was set to confirm the effect of EHF on edema. EHF (0.5 and 1 mg/ear) was treated 1 h before the application of PMA (6 μg/ear) on the right ears. 6 h after the PMA application, ear edema and inhibition percentage of the treatment were calculated. The results presented are representative of three independent experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences compared to the LPS-only treated group. $^{\#}p < 0.01$ indicates significant differences compared to the control group.

4. Discussion

Inflammation response, the crucial immune system of our body, should be modulated to maintain healthy conditions. To find if EHF is helpful to reduce over-activated inflammation, its anti-inflammatory activity was examined in this study. To mimic the inflammatory condition of our body, murine macrophage RAW 264.7 was stimulated with LPS which is the component of outer membrane of Gram-negative bacteria. In LPS-induced RAW 264.7 cells, EHF proved its strong anti-inflammatory effect by showing decreased the levels of NO, PGE_2, ROS, and pro-inflammatory cytokines and increased HO-1 during the immune response.

Endotoxin, pathogen derived pro-inflammatory factors, and microbial attacks are recognized by the family of Toll-like receptors (TLRs) (Takeda & Asira, 2004). TLR4 is expressed on many immune cells and LPS is also recognized by TLR4 receptor working with its co-receptors, CD14 and MD2 (Shimazu et al., 1999). LPS-medicated TLR4 signaling initiates inflammatory response in RAW 264.7 cells (Brandl et al., 2005), and leads to increased NO and PGE_2 generation. NO is one of the most important biological messenger molecule in host-defense and inflammation (MacMicking et al., 1997). In LPS-stimulated RAW 264.7 cells, NO is over produced by enzymatic oxidation of L-arginine by inducible nitric oxide synthase (iNOS) (Moncada & Higgs, 1993; Bredt et al., 1992). Over-produced inflammatory mediators including NO cause severe inflammatory condition leading various inflammatory diseases such as pulmonary fibrosis (Coker & Laurent, 1998), chronic hepatitis (Tilg et al., 1992), rheumatoid arthritis (Isomaki & Punnonen, 1997). Moreover, inflammation mediated up-regulations of NO generation are the factors causing cellular injury and autoimmune diseases (Singh et al., 2000). Thus, NO generation is

an appropriate therapeutic strategy for treatment of inflammation-related diseases. In this study, EHF showed the strong inhibitory effect on NO generation, proving good anti-inflammatory agent (Fig. 3A). In addition, PGE_2 generation was also inhibited by EHF (Fig. 2B). PGE_2 plays a povital role in inflammation of immune response (Kalinski, 2012). Decreased PGE_2 by EHF treatment suggests its potential to be developed as an anti-inflammatory substitute.

Free radicals are produced in normal respiration unavoidably and by exogenous factors including smoking, ultraviolet, and metabolism of foreign materials (Pryor, 1986). Aerobic respiration produces reactive oxygen species (ROS) such as hydrogen peroxide (H2O2), singlet oxygen, hydroxyl radical, superoxide anion radical (Sawa et al., 2000). Excess ROS causes not only cell damage but also inflammatory responses finally leading to cell death (Black, 2003), and several studies have shown that these reactive species are also responsible for aging (Piotrowska & Bartnik, 2014), rheumatoid arthritis (Griffths & Lunec,. 1996), and cancer (Dreher & Junod, 1996). Increased ROS by LPS stimulation are decreased when EHF is co-treated on the cells (Fig. 4). This result supported that EHF has strong anti-oxidant effects.

It has been reported that the selective inhibition of iNOS alone could have a direct inhibitory effect on inflammatory diseases such as arthritis (Cuzzocrea et al., 2002). While the pro-inflammatory protein expressions were decreased, heme oxygenase (HO)-1 protein was increased by EHF treatment (Fig. 5A). HO-1 is known to have cyto-protective and anti-inflammatory abilities (Weis et al., 2009). HO-1 is the rate-limiting enzyme induced by various inducers including inflammatory cytokines, ultraviolet irradiation, oxidative stress, and heavy metals to scavenge

produced ROS (Naito et al., 2014). HO-1 catalyzes the degradation of heme, producing iron, carbon monoxide, and biliverdin. This biliverdin is converted to bilirubin which is byproduct of HO-1 and regarded as anti-oxidative molecules (Tenhunen., 1969). Bilirubin is oxidized instead of other cells and tissues, so it has an anti-oxidative effect and thus gives the meaning of an expanded anti-oxidant ability to increased HO-1. By the mechanism above, HO-1 plays a significant role in scavenging ROS in cells. Furthermore, EHF suppressed the expression of iNOS, COX-2, and HO-1 mRNAs, which suggests that iNOS, COX-2, and HO-1 are regulated at transcriptional level.

NO, PGE_2, and ROS levels were not the only factors influencing inflammation. Cytokines also play a crucial role in this system. As the TLR4-mediated MyD88-dependent signaling pathway is induced by LPS, the generation of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and 1β arise (Palsson-McDermott EM & O'Neill LA, 2004). These inflammatory cytokines are found in a variety of acute and chronic inflammatory condition. EHF treatment significantly reduced the generation of the cytokines (Fig. 6A, B, and C). Furthermore, EHF inhibited the production of cytokines at transcriptional level (Fig. 6D). Modulating cytokine production at transcriptional level seems to be a better way to control the cytokines, and it suggests that the reduced cytokine mRNAs are possibly affected by EHF treatment at its prior modulation step.

NF-κB is well known as a regulatory factor of a number of gene expressions in inflammatory responses. Pro-inflammatory cytokines, microbial infections, oxidant stress, growth factor, and endotoxin are NF-κB inducers (Sha, 1998), and it has been proved in many studies that some natural antioxidants or functional compounds

inhibit the production of NF-κB dependent proteins such as iNOS and COX-2 and cytokines, and, lead to inhibit inflammation. NF-κB plays an important role in up regulation of the iNOS, COX-2, and pro-inflammatory cytokines in LPS-induced cells (Lee et al., 2003). Inactive NF-κB distributed in the cytoplasm as a combined form with an inhibitory molecule IκB-α (Chapmam & Perkins, 2000). By exogenous stimuli, IκB kinase (Ikk) is phosphorylated leading to the phosphorylation of IκB-α. Once IκB-α is phosphorylated, its ubiquitination takes place and finally degrade, leading to free NF-κB translocation into the nucleus (Karin & Ben-Neriah, 2000). EHF treatment inhibited the phosphorylation of Ikk and IκB-α in the cytoplasm, thus, ultimately inhibits the translocation of NF-κB into the nucleus (Fig. 7A, B). Inhibited NF-κB translocation into the nucleus was found not only by western blotting but also by immunofluorescence analysis (Fig. 7C). Furthermore, NF-κB promoter activity was inhibited by EHF. The result demonstrated that EHF is highly potential and competitive candidate of a therapeutic substrate for inflammation treatment.

Up to date, a great number of studies asserted MAPK signaling, activated by a variety of environmental factors, induces inflammatory factor activation as well as NF-κB activation (Kaminska, 2005). More specifically, it has been implicated ERK, JNK, and p38 modulates LPS-induced NO generation, affects expression of the iNOS and COX-2 via NF-κB pathway (Sung et al., 2014). Besides MAPKs, Akt plays a role in integrating signals and phosphorylation IκB-α resulting in NF-κB translocation into the nucleus (Hsing et al., 2011). In this study, phosphorylation of MAPKs and Akt is significantly reduced by EHF treatment, proving that EHF has an anti-inflammatory effect on LPS-stimulated RAW 264.7 cells through the inhibition of NF-κB via the regulation of MAPKs and Akt phosphorylation (Fig. 8).

PMA is widely used as an inducer of acute inflammation, leading to epidermal tissue swelling and infiltration of cells (Wershil et al., 1988). To examine the effect of EFH *in vivo*, a well- established in vivo mouse edema model was set. As the EHF was treated on the ears, the PMA-induced ear swelling was suppressed (Fig. 9). EHF is helpful for inhibiting inflammation *in vivo* as well as in vitro.

Conclusions

According to the results, it is demonstrated that EHF suppressed the pro-inflammatory proteins such as iNOS and COX-2 and cytokines while it up-regulated HO-1 and boosts ROS scavenging activity in LPS-induced RAW 264.7 cells. EHF down-regulated NF-κB translocation to the nucleus, NF-κB promoter activity, and phosphorylation of MAPKs and Akt. To sum up, based on the results, EHF could be a highly potential therapeutic agent, dietary supplement, and medicine for a treatment of inflammatory diseases.

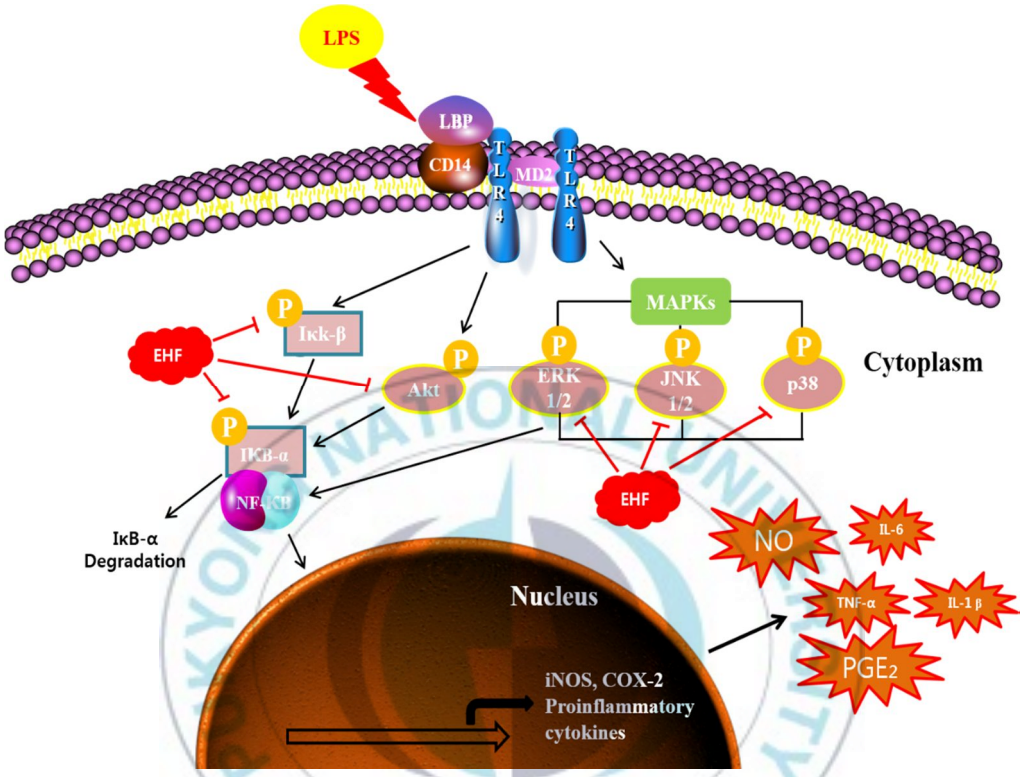

Fig. 10. Putative inhibitory mechanisms of EHF in LPS-induced RAW 264.7 cells.

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