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Anti-Inflammatory Activity of Ethanolic Extract from Skipjack Tuna (*Katsuwonus pelamis*) Heart in LPS-Induced RAW 264.7 Cells and Mouse Ear Edema Model

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Abstract This study investigated the anti-inflammatory activity of the ethanolic extract (THEE) obtained from the heart of skipjack tuna using lipopolysaccharide (LPS)-induced RAW 264.7 cells. THEE markedly suppressed the production of nitric oxide (NO), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and IL-1β in a dose-dependent manner. In addition, THEE decreased the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-kappa B p65 (NF-κB p65), and mitogen-activated protein kinases (MAPKs) including phosphorylated c-Jun NH_2-terminal kinase (p-JNK), phosphorylated extracellular signal-related kinase (p-ERK), and p-p38 proteins. Moreover, THEE orally treated at doses of 50, 100, and 250 mg/kg inhibited the croton oil-induced edema formation and the reduction of the epidermal/dermal thickness and the mast cell numbers was observed in histological analysis. There were no mortalities occurred in mice administered THEE at 5,000 mg/kg body weight. Taken together, these results indicate that THEE exerts the anti-inflammatory activities via inhibition of NF-κB and MAPKs activation.

Keywords: tuna heart, anti-inflammatory activity, NF-κB, MAPKs, croton oil-induced edema

Introduction

Inflammation is a host defense mechanism against infection and pathogenic injury (1), and its symptoms include redness, swelling, pain, fever, and functional disturbances (2). When toll-like receptor 4 (TLR-4) recognizes lipopolysaccharide (LPS) of bacteria, inflammatory and immune responses are activated, thereby activating the intracellular signaling pathways such as the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathway. Subsequently, the production of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX), nitric oxide (NO), and pro-inflammatory cytokines is induced, and these pro-inflammatory mediators activate the macrophages and immune cells (3,4). However, overexpression of these mediators results in tissue injuries and chronic inflammatory diseases such as rheumatoid arthritis and asthma (5). Therefore, studies on regulating the pro-inflammatory mediators for treating or preventing inflammatory diseases are required.

Katsuwonus pelamis belongs to the family Scombridae, and since 2,000, its collection has ranged from 23-27 million tons (6). *K. pelamis* is mostly used to prepare canned food; however, various kinds of tuna by-products such as skin, tail, fin, eyeball, and heart are produced during the canning process. Up to 45% of the by-products of the tuna are disposed or used as feed, and this causes serious environmental pollution. To date, only few studies have examined biological activities of the tuna by-products; specifically, the gelatin extract from the skin (7), anti-oxidant (8,9), and anti-cancer (9) activity of the steaming liquid, biomaterials for implant (10), and lecithin isolated from the viscera oil (11). The biological activities of tuna heart and its applications have not been examined thus far.

Therefore, this study investigated the *in vitro* and *in vivo* anti-inflammatory effects of ethanolic extract of tuna heart (THEE) by measuring the levels of pro-inflammatory mediators and formation of ear edema.

Materials and Methods

Chemicals Specific antibodies against β-actin, COX-2, iNOS, NF-κB p65, phosphorylated c-Jun NH_2-terminal kinase (p-JNK), p-ERK, p-p38, and anti-mouse immunoglobulin G (IgG)-conjugated horseradish peroxidase were obtained from Santa Cruz (San Diego, CA, USA). LPS, dimethylsulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay

(ELISA) kits for tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-1β were purchased from BD Biosciences (San Diego, CA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) was from GIBCO (Grand Island, NE, USA). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Hyclone (Logan, UT, USA), and BCA protein assay kit and enhanced chemiluminescence kit (ECL kit) were from Pierce (Rockford, IL, USA).

Materials Tuna heart powder was provided from Dongwon F&B Co. (Seongnam, Korea). Heart from tuna was collected, washed, grinded, and lyophilized.

Animals Eight-week-old male ICR mice were purchased from Orient Bio (Seongnam, Korea) for an ear edema test. Ten-week-old female Balb/c mice were used for the acute toxicity test. Mice were preliminarily bred in an animal room at a temperature of 20±2°C, 50±10% humidity, and under a 12-h light/12-h dark cycle for one week. The study was approved by the Ethics Committee of Pukyong National University and all the procedures were in strict accordance with "guidelines for the care and use of laboratory animals" of Pukyong National University (No. 2014-01).

Ethanol extraction Tuna heart powder was extracted with 95% ethanol for 24 h at room temperature with an agitator (H-0820; Dongwon Science Co., Busan, Korea). Then, the extract was centrifuged at 1,977×*g* and the supernatant was filtered and concentrated using a rotary evaporator (RE200; Yamato Co., Tokyo, Japan). The residue was extracted twice in the same way. The concentrate was dried at 37°C and stored at −20°C before use.

Cell culture The murine macrophage RAW 264.7 cells were purchased from Korean Cell Line Bank (KCLB 40071). The cells were cultured in plastic dishes containing DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a CO_2 incubator (5% CO_2 in air, MCO-15AC; Sanyo, Osaka, Japan) at 37°C. Cells were subcultured when they had reached about 80-90% confluence and the total number of passages did not exceed 20.

Cell proliferation assay RAW 264.7 cells (1×10^6 cells/mL) were cultured in a 96-well plate for 20 h. Then, the cells were cultured with LPS (1 μg/mL) and THEE (0.1, 1, 10, 50, and 100 μg/mL) for 22 h at 37°C and 5% CO_2. Subsequently, 5 mg/mL of MTT was added, and the cells were incubated for 2 h. After incubation, the medium was then discarded and DMSO was added to each well and the absorbance was measured at 540 nm by using a microplate reader (Model 550; Bio-rad, Richmond, VA, USA). The ability of cell proliferation was calculated according to the following formula:

Proliferation index (%)=Absorbance of sample group/Absorbance of control (PBS-treated) group×100

Determination of NO levels RAW 264.7 cells were plated in 24-well plates (2.5×10^5 cells/mL) and incubated in a 5% CO_2 incubator for 20 h. After pre-incubation, the cells were treated with LPS (1 μg/mL) and THEE (0.1, 1, 10, 50, and 100 μg/mL) and incubated for 24 h. Then, 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide+0.1% naphthalenediamine dihydrochloride in 5% phosphoric acid, 1:1) and the mixture was incubated at room temperature for 10 min. The absorbance was measured at 540 nm by using a microplate reader, and nitrite concentration was calculated using the standard curves of sodium nitrite.

ELISA The levels of TNF-α, IL-6, and IL-1β were determined using an ELISA kit. Briefly, RAW 264.7 cells (2.5×10^5 cells/mL) were stimulated with LPS (1 μg/mL) and indicated concentrations of THEE in 24-well plates for 24 h. Then, the levels of TNF-α, IL-6, and IL-1β in the culture medium were measured by ELISA using anti-mouse TNF-α, IL-6, and IL-1β antibodies and biotinylated secondary antibodies according to the manufacturer's instructions.

Western blot analysis RAW 264.7 cells treated with various concentrations of THEE (0.1, 1, 10, 50, and 100 μg/mL) followed by treatment with LPS (1 μg/mL) for 24 h were lysed with buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 5 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1% Triton X-100, and 0.1% NP-40. Cell lysates were centrifuged at 14,240×*g* to remove the cell membrane components. Protein concentration was quantified using a BCA protein assay kit (Pierce). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then were transferred to PVDF membranes (Bio-rad) at 200 mA for 1 h. The membranes were blocked with tris-buffered saline containing 5% skim milk (Fluka, Buchs, Switzerland) (TBSS) for 2 h. The membranes were incubated with anti-mouse iNOS, COX-2, p-NF-κB p65, NF-κB p65, JNK, p-JNK, ERK, p-ERK, p38, and p-p38 antibodies (1:500) in TBS for 2 h and washed three times with TBSS and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (1:2,000). The membranes were washed three times with TBSS and the immunoreactive proteins were detected using an ECL detector. The signal intensity of each protein band was measured by densitometry by using the Gene Tools (GeneGnome5; Syngene, Cambridge, UK) from the Syngene software.

Measurement of ear edema and histopathological analysis THEE (200 μL) was orally administered to ICR mice (n=5) at concentrations of 10, 50, and 250 mg/kg body weight. In 1 h, 2.5% croton oil (20 μL) was spread on the inner and outer surfaces of the right ear, and the ear thickness was measured in 5 h. An increase in the ear thickness after croton oil treatment was considered as development of edema. For histopathological analysis, 20 μL of THEE was spread on the

surface of the right ear of an ICR mouse at a concentration of 100 mg/mL and 20 μL of the 5% croton oil was spread for 15 min. After 6 h, the ear tissue was dissected and fixed in 10% formaldehyde for 72 h. Subsequently, the tissue slices were embedded in paraffin. Sections were deparaffinized and stained with hematoxylin-eosin and toluidine-blue stain for observation of the tissue and mast cells. Edema formation (%)=Ear thickness of sample group/Ear thickness of control group×100

Acute oral toxicity test Balb/c mice (*n*=5) were fasted for about 4 to 6 h right before the acute toxicity test, and THEE was orally administered at concentrations of 300, 2,000, and 5,000 mg/kg body weight. Then, the animals were observed for any abnormal behaviors for 6 h, and mortality was noted up to 2 weeks. A group of animals treated with 5% tween-80 served as control.

Statistical analysis Data are expressed as mean±standard error of the mean. Statistical evaluation was performed by determining the analysis of variance by using the SAS software (SAS Institute, Inc., Cary, NC, USA) according to Duncan's multiple range test (p<0.05).

Results and Discussion

Cell proliferation Treatment with THEE at doses of 0.1, 1, 10, 50, and 100 μg/mL had no significant effect on the proliferation of RAW 264.7 cells (Fig. 1).

Effects of THEE on LPS-stimulated production of NO NO is a free radical produced by iNOS, which catalyzes the oxidative deamination of L-arginine, and it plays an important role in the regulation of physiological function (12). However, prolonged production of NO in inflammatory conditions leads to increased vascular permeability and swelling, and induces chronic inflammation and autoimmune

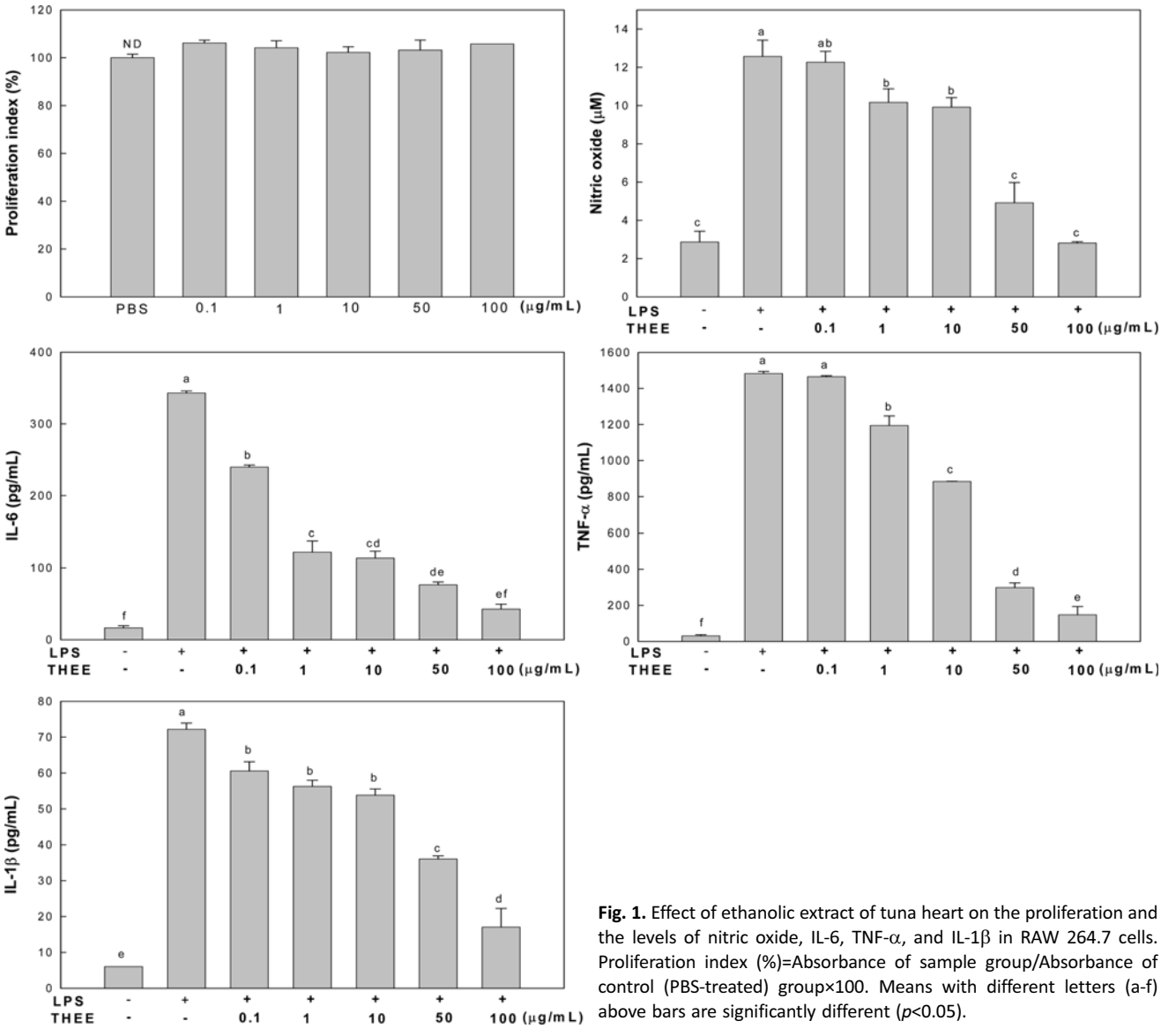

Fig. 1. Effect of ethanolic extract of tuna heart on the proliferation and the levels of nitric oxide, IL-6, TNF-α, and IL-1β in RAW 264.7 cells. Proliferation index (%)=Absorbance of sample group/Absorbance of control (PBS-treated) group×100. Means with different letters (a-f) above bars are significantly different (p<0.05).

diseases (13). In addition, NO is associated with the activation of COX-2, and high levels of prostaglandin E_2 (PGE_2) produced by COX-2 intensify the inflammatory reaction (14). Therefore, inhibition of NO production is the main target to reduce inflammatory response. The production of NO in the negative control group was 2.87±0.54 μM. After LPS treatment, the NO production significantly increased (12.57±0.85 μM) compared with the negative control (Fig. 1). THEE decreased the NO production increased by LPS in a dose-dependent manner; in particular, the NO production of THEE treatment group at 50-100 μg/mL decreased by about 77% compared to the LPS only treatment group. Previous studies have reported the anti-inflammatory activity of eicosapentaenoic acid (C20:5, EPA) and docosahexaenoic acid (C22:6, DHA) isolated from tuna (15). The tuna used in this study contain low levels of lipids such as EPA and DHA (Table 1). Generally, fish have various kinds of bioactive peptides (16) that are related to the regulation of biological immune system in our body (17). Cheong (18) reported that the peptides L-anserine and L-carnosine isolated

Table 1. Proximate compositions and salinity of tuna heart

	Contents (%)
Moisture	65.63
Crude protein	29.86
Crude fat	1.06
Carbohydrate	2.3
Crude ash	1.15
Salinity	0.4

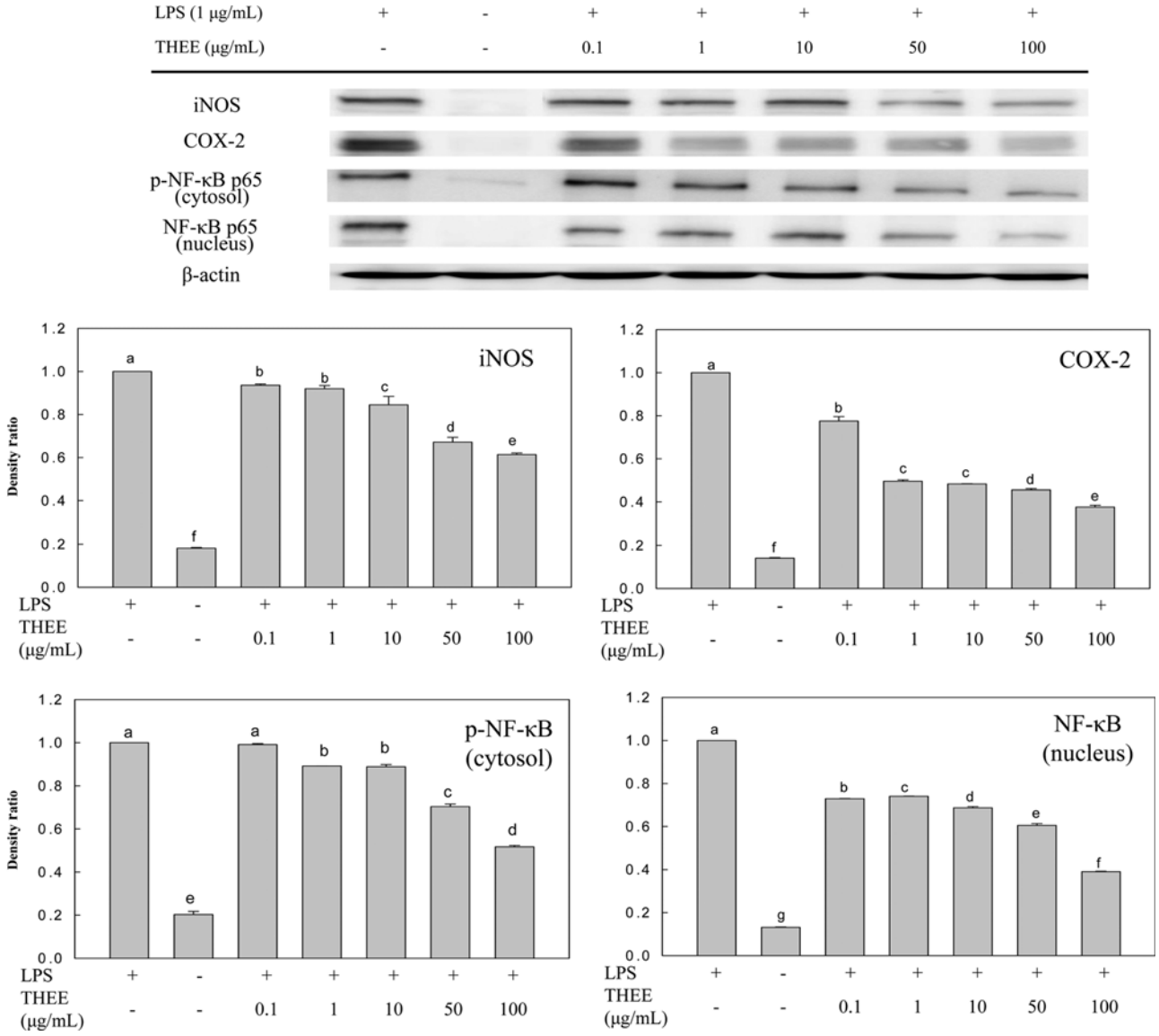

Fig. 2. Effect of ethanolic extract of tuna heart on LPS-induced expression of iNOS, COX-2, p-NF-κB p65, and NF-κB p65 in RAW 264.7 cells. The levels of iNOS, COX-2, and p-NF-κB p65 in the cytosolic protein and the p65 subunit of NF-κB in nuclear protein were determined by a western blot analysis. RAW 264.7 cells were treated with the indicated concentrations of THEE (0.1, 1, 10, 50, and 100 μg/mL) and LPS (1 μg/mL) for 18 h or 30 min and the proteins were detected using specific antibodies. Means with different letters (a-g) above bars are significantly different (p<0.05).

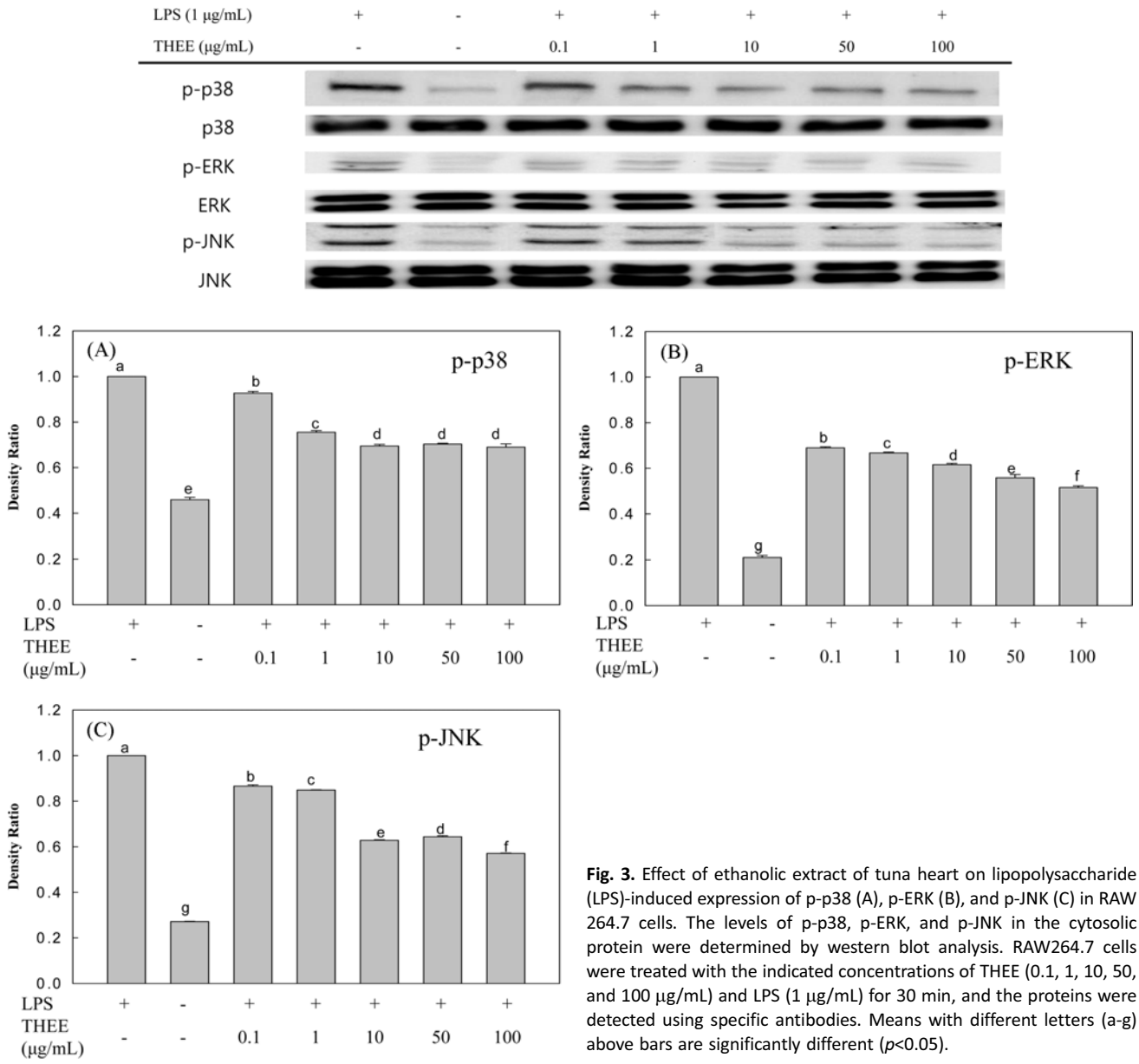

Fig. 3. Effect of ethanolic extract of tuna heart on lipopolysaccharide (LPS)-induced expression of p-p38 (A), p-ERK (B), and p-JNK (C) in RAW 264.7 cells. The levels of p-p38, p-ERK, and p-JNK in the cytosolic protein were determined by western blot analysis. RAW264.7 cells were treated with the indicated concentrations of THEE (0.1, 1, 10, 50, and 100 μg/mL) and LPS (1 μg/mL) for 30 min, and the proteins were detected using specific antibodies. Means with different letters (a-g) above bars are significantly different (p<0.05).

(20) reported that peptides derived from manila clam and oysters inhibited the NO production in LPS-induced RAW 264.7 cells. Thus, the anti-inflammatory effects of THEE in our study may be attributed to the high levels of bioactive peptides present in the tuna heart.

Effects of THEE on LPS-stimulated production of pro-inflammatory cytokines Pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β are involved in the early stage of inflammatory response (1). In particular, IL-6 contributes to chronic inflammation by accelerating the differentiation of B cells and production of antibodies (21). Over production of IL-6 is related to autoimmune diseases and malignant tumors (22). TNF-α is secreted by macrophages, NK cells, and CD4+ T cells through the endocrine and external system. This cytokine is associated with the expression of pro-inflammatory mediators such as iNOS, COX-2, IL-6, and IL-1β by inducing the activation of NF-κB (7). Another crucial pro-inflammatory cytokine, IL-1β, is produced mainly in activated macrophages, and its active form induces the production of NO. The inhibitory effect of THEE on these pro-inflammatory cytokines is examined using an ELISA kit. Our results showed that THEE effectively reduced the production of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β in a dose-dependent manner (Fig. 1). LPS induced high levels of production of IL-6 and TNF-α; however, treatment with 100 μg/mL of THEE decreased the levels of IL-6 and TNF-α by about 87 and 90% from 343.06±2.92 to 42.62±6.82 pg/mL and 1,482.85±11.59 to 147.33±46.35 pg/mL, respectively. Compared to the LPS only treatment group, cells treated with 50 and 100 μg/mL of THEE inhibited the production of IL-1β by about 50 and 70%, respectively. Thus, these results indicated that THEE exerts anti-inflammatory effects by suppressing the secretion of pro-inflammatory cytokines.

Effects of THEE on LPS-induced expression of iNOS, COX-2, and NF-κB p65 Typically, three isoforms of NOS, including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), are present in cells. nNOS and eNOS are classified as constitutive NOS because they are consistently present in cells under normal physiological conditions. iNOS is an enzyme that plays a key role in immune response against pathogens through the production of peroxynitrite in macrophages. Under pathological conditions, LPS and interferon-γ (IFN-γ) induce NO production via iNOS (23). The high levels of NO cause cytotoxicity and tissue damage (24). Two isozymes of COX, COX-1 and COX-2, are present in cells. COX-1 is constitutively expressed in many organs or tissues under normal conditions and performs cellular housekeeping functions of prostaglandins. In contrast, COX-2 is the inducible isoenzyme of cyclooxygenases, and overexpression of COX-2 is associated with a number of inflammatory or inflammation-associated processes because of the catalysis of PGE_2 from arachidonic acid (25). Therefore, methods to decrease the production of NO induced by iNOS and PGE_2 by COX-2 have received much attention. These inflammatory mediators are regulated primarily through the involvement of transcription factors such as NF-κB, which control the synthesis of various cytokines, chemokines, and growth factors (26). NF-κB consists of p50 and p65 subunits and binds to IκB in normal conditions. However, under stimulation by LPS, NF-κB forms a complex with TLR4/myeloid differentiation primary response gene 88 (TLR4/MyD88), and thus, the upstream signaling molecule IKK induces the phosphorylation and degradation of IκB, which releases the free NF-κB. This free NF-κB translocates into the nucleus and leads to the production of various inflammatory cytokines and mediators (27). In this study, we examined the anti-inflammatory effects of THEE on the expression of mediators, including iNOS, COX-2, and NF-κB p65 in LPS-induced RAW 264.7 cells. Our results showed that the LPS only treatment group showed a marked increase in the expression of iNOS, COX-2, p-NF-κB p65, and NF-κB p65. whereas THEE treatment decreased the expression of iNOS, COX-2, p-NF-κB p65, and NF-κB p65 (Fig. 2). THEE at 100 μg/mL inhibited the expression of iNOS, COX-2, and NF-κB p65 by 38, 61, and 60%, respectively. A study similar to the present study showed that melittin exerts anti-inflammatory effects by inhibiting the translocation of NF-κB subunit into the nucleus and decreasing the secretion of iNOS, COX-2, cytosolic phospholipase A2, PGE_2, and NO (28). In addition, tripeptide, a protein hydrolysate from a pectoral of salmon, inhibits the production of NO, COX-2, PGE_2, and pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β (29). These results indicate that THEE suppressed the LPS-induced expression of iNOS, COX-2, NO, and pro-inflammatory cytokines by inhibiting the activation of NF-κB p65.

Effects of THEE on LPS-induced expression of p-JNK, p-ERK, and p-38 The MAPK is another signaling pathway involved in the modulation of inflammatory responses. MAPKs include JNK, ERK, and p38 (30). These are regulated by the phosphorylation of serine and threonine and lead to inflammatory responses by activating transcription factors such as activator protein-1 (AP-1), cAMP responsive element binding protein, activating transcription factor-2, and NF-κB (31). In this study, we examined the inhibitory effect of THEE on the phosphorylation of MAPKs using an *in vitro* model of

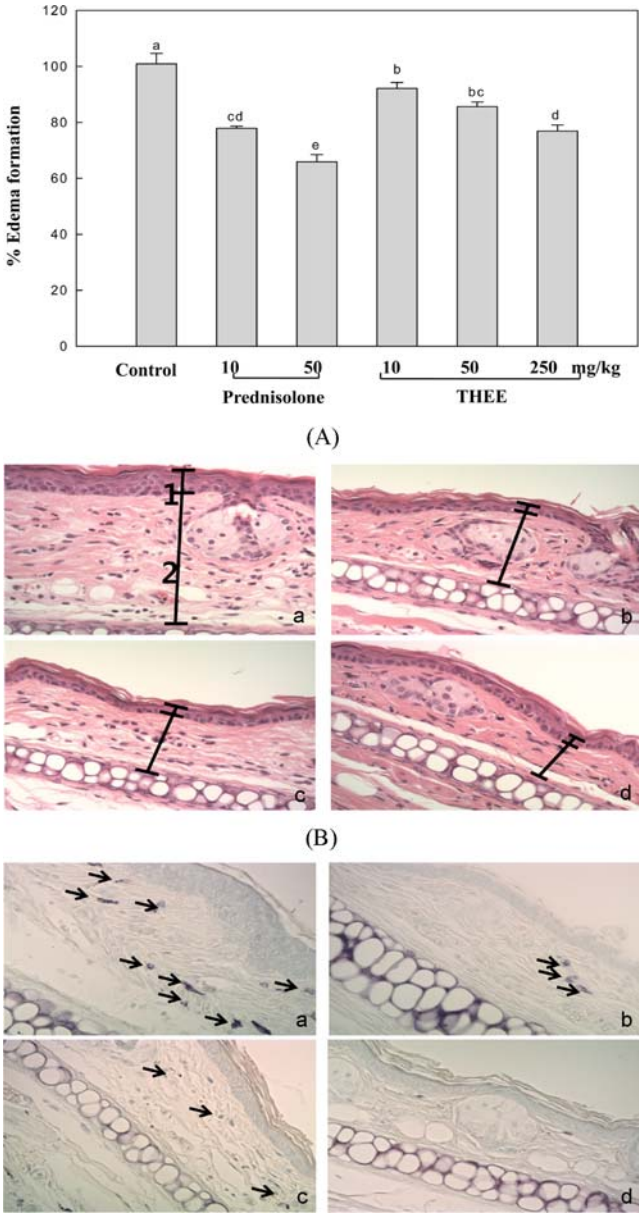

Fig. 4. Inhibitory effect of ethanolic extract of tuna heart on croton oil-induced mouse ear edema and photomicrograph of transverse sections of mice ears sensitized with topical application of 5% croton oil (v/v) in acetone (a-c) or vehicle acetone (d, non-inflamed), stained with hematoxylin-eosin (A) and toluidine-blue (B), and examined under light microscopy (magnification: 200×). Treatments: vehicle 2% Tween 80 (a), prednisolone 0.08 mg/ear (b) and ethanolic extract of tuna heart 100 mg/mL (20 μL/ear) (c). The numbers 1 and 2 indicate epidermis and dermis, respectively, and the arrow in (B) means infiltration of mast cells. % Edema formation=Ear thickness of sample group/Ear thickness of control group×100. Means with different letters (a-c) above bars are significantly different (p<0.05).

Table 2. Mortality of mice treated orally with ethanolic extract of tuna heart

	Days after treatment							
	0	2	4	6	8	10	12	14
Control	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
300 mg/kg body weight	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
2,000 mg/kg body weight	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
5,000 mg/kg body weight	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

LPS-induced inflammation. The expression of p-p38, p-ERK, and p-JNK in the cytoplasm decreased by 31, 49, and 43% after treatment with 100 μg/mL of THEE (Fig. 3). Kim *et al.* (32) showed that the protein tat-biliverdin reductase A suppressed the expression of COX-2, iNOS, and pro-inflammatory cytokines by inhibiting the phosphorylation of p38, JNK, and ERK in LPS-induced RAW 264.7 cells. Therefore, our results indicated that THEE exerted significant anti-inflammatory activity by inhibiting the MAPK pathway.

***In vivo* anti-inflammatory activity of THEE** Inflammation is a response to recover a damaged area in the immune system; however, failure in the regulation of response leads to vasodilatation, which induces pyrexia and redness through an increase in blood flow. This also causes edema, production of prostaglandins, and activities of immune cells via an infiltration of neutrophils and mast cells in blood vessels into the inflammatory area (33). To examine the effect of THEE on acute inflammation, we used a mouse model of croton oil-induced ear edema, which is a typical model for assessing inflammatory responses (34). Thus, the change in ear thickness by oral administration of THEE (10, 50, and 200 mg/kg) in croton oil-induced mice was measured. Ear edema decreased significantly after treatment with THEE (Fig. 4). In particular, ear edema decreased by 24% at a dose of 250 mg/kg body weight of THEE, suggesting that it was more effective than 10 mg/kg body weight of prednisolone. These results were consistent with those obtained from the photo-micrographs (Fig. 4). The significant decrease in ear thickness and the number of infiltrated mast cells observed with 100 mg/mL of THEE was similar to that observed with 0.08 mg/ear of prednisolone. Nonsteroidal anti-inflammatory drugs are normally used to treat inflammatory diseases; however, they are associated with various side effects such as gastric ulcers and cardiovascular risk (35). Therefore, results from our *in vivo* study indicate that THEE can be used as a potent therapeutic agent for inflammatory disorders.

Acute oral toxicity test The acute oral toxicity of THEE was evaluated in mice to support the results that indicate that the extract was not toxic. No mice died after administration of 300, 2,000, and 5,000 mg/kg body weight of THEE over the 2-week observation periods. After treatment with THEE, the mice slept or wandered for 1 h and then resumed normal behavior (Table 2). According to the World Health Organization, a herbal medicine with median lethal dose (LD_{50}) of less than 5,000 mg/kg body weight is considered toxic. Thus, any adverse effects by THEE (up to 5,000 mg/kg) did not occur and THEE can be safely used as a natural anti-inflammatory agent.

In conclusion, The THEE exerts anti-inflammatory effects by reducing the levels of NO and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β without any cytotoxicity. Moreover, these results showed that the levels of pro-inflammatory mediators such as NO, pro-inflammatory cytokines, iNOS, and COX-2 were decreased by downregulating the NK-κB and MAPKs signaling pathways in LPS-induced RAW 264.7 cells. The increase in the ear thickness induced by croton oil decreased significantly by 24% after treatment with 250 mg/kg body weight of THEE. Results from photomicrographs, showed that ear thickness and the number of infiltrated mast cells decreased significantly after THEE treatment. Finally, in an acute oral toxicity test, no mice died after administration of the THEE, which indicates the safety of the extract. Therefore, the THEE could be used as a therapeutic agent for the treatment of inflammatory disorders.

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Disclosure The authors declare no conflict of interest.

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