

Anti-inflammatory effects of *Fritillaria thunbergii* Miquel extracts in LPS-stimulated murine macrophage RAW 264.7 cells

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Abstract. The aim of the present study was to demonstrate that *Fritillaria thunbergii* Miquel extract exerts anti-inflammatory and antioxidant effects on lipopolysaccharide-stimulated RAW 264.7 cells. To confirm the inhibitory effect of ethyl acetate fraction of FTM (EAFM) on inflammation, the expression of nitric oxide (NO) and inflammatory cytokines was assessed by performing ELISA. Expression of intracellular mRNA and protein was confirmed by reverse transcription PCR and western blotting. In addition, the anti-inflammatory and anti-oxidant mechanisms of NF- κ B, MAPK and heme oxygenase-1 (HO-1) were also investigated. EAFM significantly inhibited the expression of inflammatory factors including NO, IL-6 and TNF- α at non-toxic concentrations. EAFM also inhibited the mRNA and protein expression of inducible nitric oxide synthase in a concentration-dependent manner, but did not alter the expression of cyclooxygenase-2. Pre-treatment with EAFM inhibited the nuclear translocation of NF- κ B, and suppressed the phosphorylation of ERK and JNK. In addition, EAFM induced 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity and an increase in the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1. The results indicated that EAFM inhibited the expression of pro-inflammatory cytokines by inhibiting ERK/JNK phosphorylation and NF- κ B translocation. EAFM also exerted antioxidant effects via Nrf2/HO-1 stimulation. Collectively, the results of the present study indicated that EAFM may be a valuable alternative for the treatment of a variety of inflammatory diseases.

Introduction

Inflammation is an immune response to infections and tissue injury. An appropriate inflammatory response is indispensable in protecting the body from internal and external factors (1). However, excessive and irreversible inflammation causes destruction of normal tissues, which may result in diseases such as cancer, sepsis, atherosclerosis and autoimmunity (2).

Lipopolysaccharide (LPS) is a naturally-derived glycolipid compound that is commonly used to evaluate the anti-inflammatory effects of macrophages *in vitro*. As one of the cell membrane components of gram-negative bacteria (3), LPS is recognized by toll-like receptor 4 and stimulates mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF- κ B) signaling, key pathways of the inflammatory response (4). As a result, LPS signaling promotes the secretion of inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and nitric oxide (NO) and induces the expression of inflammatory cytokines such as interleukin (IL)-6, IL-1 β and tumor necrosis factor-alpha (TNF- α) (5). Additionally, the antioxidant effects of upregulating nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) are known to suppress the expression of various LPS-induced inflammatory factors. HO-1 is a protein associated with stress, ischemia and inflammatory conditions, and primarily exhibits protective, anti-inflammatory, antioxidant and anti-proliferative effects (6). Therefore, the anti-inflammatory mechanisms of MAPKs and NF- κ B, and the antioxidant effects of HO-1 expression, are common research targets for the treatment of inflammation.

A variety of diseases share the same underlying pathophysiological mechanisms of inflammation, and research into natural medicines to treat inflammation has gained considerable popularity. *Fritillaria thunbergii* Miquel (FTM) is located in the stem of *Fritillaria thunbergii*, a herbal medicine that is commonly used in East Asian regions including Korea, China and Japan. FTM (or 'Jeol-pae-mo' in Korean) has traditionally been used to effectively reduce heat, remove phlegm and prevent coughing (7). In a recent study, FTM was shown to inhibit the expression of cytokines following PMA/A23187 stimulation of HMC-1 cells, and to inhibit cytokine expression in an acute inflammatory BALB/c mouse model (8). In addition, peimine and peiminic, the main active ingredients

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of FTM, were found to improve DNCB-induced atopy (9,10). However, to the best of our knowledge, there are no studies of the LPS-induced inflammatory factors and antioxidant effects of the extract and fractions of FTM in macrophages.

In the present study, the potential anti-inflammatory effects of FTM extract and fractions were investigated through the verification of NO and prostaglandin E₂ (PGE₂) expression. To improve our understanding of the anti-inflammatory properties of FTM, its effects on the expression of MAPKs, NF- κ B, pro-inflammatory cytokines and HO-1 were also investigated.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) was obtained from Welgene, Inc., Gyeongsan, Korea. LPS, dimethylsulfoxide (DMSO), fetal bovine serum (FBS) and penicillin/streptomycin (P/S) were procured from Gibco; Thermo Thermo Fisher Scientific, Inc. C₂₇H₄₅NO₃: (3 β ,5 α ,6 α ,22 β)-Cevane-3,6,20-triol (peimine) and C₂₇H₄₃NO₃ (peiminine) were supplied by Abcam. The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. Griess reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay kit (cat. no. CS0790), the bicinchoninic acid assay (BCA) kit, protease inhibitor, and phosphatase inhibitor 2 and 3 cocktails were supplied by Sigma-Aldrich (Merck KGaA). IL-1 β , IL-6 and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences and the PGE₂ ELISA kit was supplied by R&D Systems, Inc. The reverse transcriptase and SYBR[®] Green reagents were purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and Taq polymerase was purchased from Kapa Biosystems (Roche Diagnostics). PCR primers were designed by GenoTech Corp. The anti-iNOS (cat. no. sc-651), anti-COX-2 (cat. no. sc-1746), anti-Nrf2 (cat. no. sc-722), anti-actin (cat. no. sc-8432) and anti-lamin B (cat. no. sc6216) primary antibodies, and the enhanced chemiluminescence (ECL) reagent were supplied by Santa Cruz Biotechnology, Inc. The anti-HO-1 (cat. no. GTX61906) antibody was obtained from GeneTex, Inc. Anti-NF- κ B (cat. no. 8242S), anti-extracellular signal-regulated kinase 1/2 (ERK; cat. no. 4695S), anti-c-Jun N-terminal kinase (JNK; cat. no. 9258S), anti-p38 (cat. no. 9212L), anti-phosphorylated (p)-NF- κ B (cat. no. 3033S), anti-p-ERK (cat. no. 4370S), anti-p-JNK (cat. no. 4668S) and anti-p-p38 (cat. no. 4511S) antibodies were supplied by Cell Signaling Technology, Inc. Horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. nos. 115-035-062 and 111-035-045) were obtained from Jackson ImmunoResearch Laboratories, Inc. All chemicals and reagents were of cell culture grade.

FTM preparation. FTM was obtained from Omniherb and verified by Professor Yungmin Bu of the Herbology Laboratory, College of Korean Medicine, Kyung Hee University (Seoul, Korea); FTM voucher specimens are stored in the medicine storage room of the Anatomy Laboratory, College of Korean Medicine, Kyung Hee University. For extraction with methanol (MtOH), 100 g FTM was stored in 1 l MtOH (80%) for 1 week. The extract was concentrated and lyophilized to obtain 10.1 g of powder (yield ratio, 10.1%). For fractionation, 800 g

FTM was immersed in 3 l MtOH (80%) for another week. The extract was filtered using filter paper and subsequently concentrated. The concentrate was sequentially fractionated using hexane, chloroform and ethyl acetate according to the polarity of the solvent. Briefly, 250 ml hexane was added to 500 ml of the water fraction and sufficiently mixed; the water and hexane fractions were separated, and 250 ml hexane was added to the water fraction once again; this sequence was repeated three times. Chloroform and ethyl acetate were then sequentially added to separate and collect each fraction, which was then concentrated, dried and used to assess activity. The hexane fraction was not extracted. Finally, 2.23 g chloroform (yield ratio, 0.28%) and 0.41 g ethyl acetate (yield ratio, 0.05%) were extracted, resulting in a final aqueous solution fraction of 11 g (yield). To extract FTM with ethanol, 100 g FTM was extracted by immersion in 1 L Et-OH (80%) for 1 week. The extract was concentrated and lyophilized to obtain 5.3 g powder (yield ratio, 5.3%). All extracts were stored at -4°C and dissolved in DMSO prior to use.

High-performance liquid chromatography (HPLC) analysis. To qualitatively evaluate the FTM, HPLC (Waters[®] 2695/UV detector 2487 system) was performed using peimine and peiminine, constituent compounds of FTM. The XBridge[™] C18 column (250x6 mm, 5 μ m) was used for analysis, with a flow rate of 1 ml/min and a sample injection volume of 10 μ l. H₂O and acetonitrile (pH 10) were used as the binary mobile phase, and the detection wavelength of the chromatogram was set to 220 nm.

Cell culture and viability assay. The RAW 264.7 murine macrophage cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in DMEM containing 10% FBS and 1% P/S at 37°C (5% CO₂, 95% humidity). To determine the effects of FTM on cellular viability, RAW 264.7 cells (5x10⁴ cells/well) were seeded into 96-well culture plates, and cultured in serum-free medium for 24 h in the presence or absence of FTM (12.5, 25, 50 and 100 μ g/ml). Then, 10 μ l CCK-8 reagent was added to each well and the plates were incubated for an additional 2 h. Cell viability was determined by absorbance measurement at a wavelength of 450 nm (VersaMax microplate reader; Molecular Devices, LLC). The absorbance values are expressed as a percentage of the non-treated cells, and a value of <90% was considered to indicate cytotoxicity. Serum-free medium was used to eliminate serum-associated interference.

Nitric oxide (NO) production. To determine the effects of FTM on the production of NO, RAW 264.7 the cells (5x10⁴ cells/well) were seeded into 96-well culture plates and cultured for 24 h in the presence or absence of LPS (1 μ g/ml) and/or FTM (12.5, 25 and 50 μ g/ml). To evaluate the expression of NO, 100 μ l Griess reagent was added to 50 μ l cell culture supernatant and allowed to react at room temperature for 30 min. The absorbance was measured using an ELISA reader at a wavelength of 540 nm, and NO levels were calculated using a sodium nitrite standard curve.

ELISA. To investigate the effects of FTM on the release of inflammatory cytokines and PGE₂, RAW 264.7 cells

Table I. Primer sequences used for reverse transcription PCR.

Gene name	Sequence (5'-3')	Accession no.	Annealing temperature, °C	Cycles	Base pairs
Nos2 (iNOS)	F: CACCTTGGAGTTCACCCAGT R: ACCACTCGTACTTGGGATGC	NM_010927.4 NM_001313921.1 NM_001313922.1	58	30	170
Ptgs2 (COX-2)	F: AGAAGGAAATGGCTGCAGAA R: GCTCGGCTTCCAGTATTGAG	NM_011198.4	55	35	194
Il6 (IL-6)	F: AGTTGCCTTCTTGGGACTGA R: TTCTGCAAGTGCATCATCGT	NM_001314054.1	58	30	223
Tnf (TNF- α)	F: GCAGAAGAGGCACTCCCCCA R: GATCCATGCCGTTGGCCAGG	NM_001278601.1	58	30	280
Il1b (IL-1 β)	F: GGCTGTGGAGAAGCTGTGGC R: GGGTGGGTGTGCCGTCTTTC	NM_008361.4	55	30	386
Hmox1 (HO-1)	F: GAGAATGCTGAGTTCAT R: ATGTTGAGCAGGAAGGC	NM_010442.2	58	25	548
Actb (β -actin)	F: TTCTACAATGAGCTGCGTGT R: CTCATAGCTCTTCTCCAGGG	NM_007393	58	30	456

iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; HO-1, heme oxygenase 1; F, forward; R, reverse.

(2×10^5 cells/well) were seeded into 24-well culture plates and incubated with or without LPS (1 $\mu\text{g/ml}$) and/or FTM (12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h. Inflammatory cytokine (IL-6; cat no. 555240; IL-1 β , cat. no. 559603; and TNF- α , cat. no. 555268) and PGE₂ (cat. no. 555268; KGE004B) expression levels were measured using the corresponding ELISA kit per the manufacturers' instructions.

Western blot analysis. To investigate the effects of ethyl acetate fraction of FTM (EAFM) on the expression of NF- κ B and MAPKs, RAW 264.7 cells (2×10^6 cells/well) were seeded into 60 π dishes and incubated with or without LPS (1 $\mu\text{g/ml}$) and/or EAFM (12.5, 25 and 50 $\mu\text{g/ml}$) for 30 min. To confirm the effect of EAFM on the expression of iNOS and COX-2, RAW 264.7 cells (2×10^6 cells/well) were seeded into 6-well culture plates and incubated with or without LPS (1 $\mu\text{g/ml}$) and/or EAFM (12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h. The cytoplasmic and nuclear proteins of RAW 264.7 cells were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were lysed in RIPA buffer (composition: 50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS), and protease inhibitor and phosphatase inhibitor 2 and 3 cocktails were added to the extraction solutions. The protein concentration was quantified (30 μg) using a BCA assay kit with a bovine serum albumin standard. Equal amounts of protein was separated by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C for 24 h (iNOS, dilution ratio: 1:1,000, 130 kDa; COX-2, dilution ratio: 1:200, 70 kDa; β -actin, dilution ratio: 1:1,000, 42 kDa; p-ERK, dilution ratio: 1:1,000, 44, 42 kDa;

ERK, dilution ratio: 1:1,000, 44, 42 kDa; p-JNK, dilution ratio: 1:1,000, 54, 46 kDa; JNK, dilution ratio: 1:1,000, 54, 46 kDa; p-p38, dilution ratio: 1:1,000, 40 kDa; p38, dilution ratio: 1:1,000, 40 kDa; NF- κ B, dilution ratio: 1:500, 65 kDa; p-NF- κ B, dilution ratio: 1:500, 65 kDa; Lamin B, dilution ratio: 1:1,000, 67 kDa; Nrf2, dilution ratio: 1:400, 120 kDa; HO-1, dilution ratio: 1:2,000, 33 kDa). HRP-conjugated secondary antibodies were then added and the membranes were incubated for 1 h at room temperature. Protein expression was visualized with an ECL reagent. The density of the bands was evaluated using the ImageJ software (version 1.51j8; National Institutes of Health) and the expression level of each protein was normalized to that of β -actin.

Reverse transcription PCR. To investigate the effects of EAFM on the expression of iNOS, COX-2 and pro-inflammatory cytokines, RAW 264.7 cells (2×10^6 cells/well) were seeded into 6-well culture plates and incubated with or without LPS (1 $\mu\text{g/ml}$) and/or EAFM (12.5, 25, 50 $\mu\text{g/ml}$) for 6 h. The expression of HO-1 was treated with EAFM (12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h. Total cellular RNA was extracted using RNAiso (Total RNA extraction reagent; Takara Bio, Inc.) according to the manufacturers' protocol, and cDNA was synthesized using reverse transcriptase. PCR was conducted using a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc.). The PCR cycles were as follows: 35-40 cycles of 1 min at 94°C (denaturation), 30 sec at 55-58°C (annealing) and a 1 min 72°C (extension), using Taq polymerase. The sequences of the primers are displayed in Table I. PCR products were electrophoresed on a SYBR® Green stained agarose gel; the band density was evaluated using ImageJ software and mRNA expression was normalized to that of β -actin.

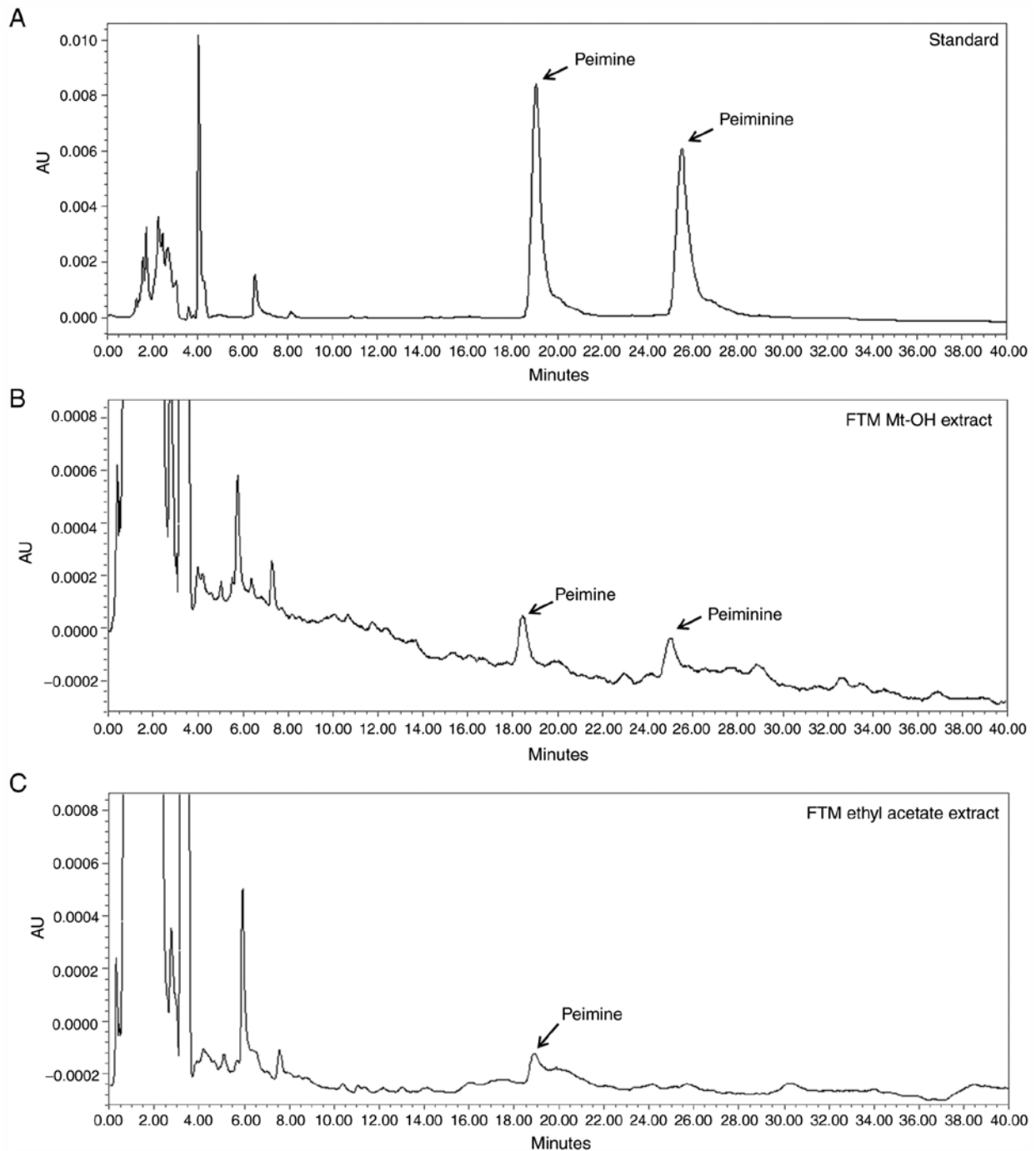


Figure 1. Quantitative analysis results of FTM and EAFM using HPLC. Chromatogram peak of (A) peimine, peiminine, (B) FTM and (C) EAFM. Standard peaks for peimine and peiminine were detected at 220 nm. FTM, *Fritillaria thunbergii* Miquel; EAFM, ethyl acetate fraction of *Fritillaria thunbergii* Miquel; AU, arbitrary units.

ABTS radical scavenging activity. To investigate the effects of EAFM on ABTS radical scavenging activity, RAW 264.7 cells (2×10^6 cells/well) were seeded into 6-well culture plates and incubated with or without FTM (12.5, 25 and 50 $\mu\text{g/ml}$) for 24 h. The experiment was carried out according to the manufacturer's protocol. The ABTS radical scavenging ability was analyzed compared to RAW 264.7 cells without treatment.

Statistical analysis. All experiments were independently conducted at least three times. All data are expressed as the mean \pm standard error of the mean (SEM) and were analyzed

using GraphPad prism software (version 5.01; GraphPad Software, Inc.). The significance of the experimental results was analyzed using one-way ANOVA test, followed by Tukey's multiple comparison post hoc analysis.

Results

Qualitative analysis of FTM. Peimine and peiminine are bioactive marker compounds of FTM (11,12). As shown in Fig. 1A and B, FTM Mt-OH extract revealed a number of chromatogram peaks for 0-30 min, and peimine and peiminine

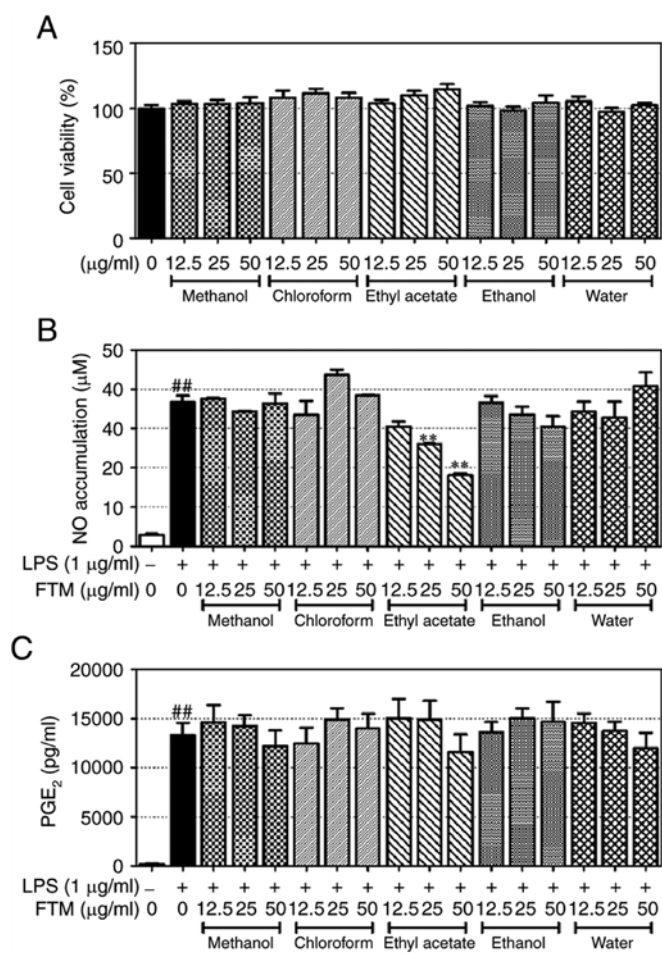


Figure 2. Effects of FTM extract and fractions on cytotoxicity and NO expression. (A) RAW 264.7 cells were treated with FTM extract at different fractions for 24 h, and the effects on cell survival were verified using a Cell Counting Kit-8 assay. (B) Inhibitory effects of FTM on NO expression following LPS treatment were verified using Griess reagent. (C) Inhibitory effects of FTM on prostaglandin E₂ were assessed using an ELISA kit. All experiments were repeated at least three times. ^{##}P<0.01 vs. the non-treated group. ^{**}P<0.01 vs. the LPS-treated control. FTM, *Fritillaria thunbergii* Miquel; LPS, lipopolysaccharides; NO, nitric oxide; PGE₂, prostaglandin E₂.

were identified at the same time points as for the standard; 19.607 and 26.316 min, respectively. As a result of examining the chromatogram peak of EAFM, peimine was detected at 19.353 min, but peiminine was not. (Fig. 1C).

EAFM significantly inhibits NO production. Prior to experimentation, the cytotoxicity of FTM was verified using a CCK-8 kit. FTM extract and all fractions showed no significant cytotoxicity at concentrations of 12.5, 25 and 50 µg/ml (Fig. 2A). As a result, concerns regarding FTM cytotoxicity were eliminated. The inhibitory effects of the FTM extract and fractions on NO expression were then assessed using Griess reagent and ELISA. EAFM was found to inhibit the generation of NO in a concentration-dependent manner (Fig. 2B); none of the other extracts and fractions had a significant effect on NO expression. Furthermore, none of the extracts and fractions (including those of FTM) significantly altered the expression of PGE₂ (Fig. 2C).

EAFM inhibits the expression of iNOS and inflammatory cytokines. To investigate the anti-inflammatory effects of

EAFM, iNOS and COX-2 expression were verified at the mRNA and protein levels. LPS stimulation of RAW 264.7 cells induced the expression of iNOS and COX-2. EAFM suppressed iNOS expression in a concentration-dependent manner, but increased the expression of COX-2 (Fig. 3A and B). IL-6, TNF-α and IL-1β are representative pro-inflammatory cytokines expressed in macrophages. To confirm the inflammatory potential of EAFM, the protein levels of these cytokines in the RAW 264.7 cell culture medium, as well as the mRNA expression levels, were investigated. As shown in Fig. 3C-E, LPS stimulation upregulated the expression of IL-6, TNF-α and IL-1β in RAW 264.7 cells. IL-6 and TNF-α mRNA expression was subsequently suppressed by EAFM in a concentration-dependent manner (Fig. 3C and D). However, EAFM did not significantly affect the mRNA expression levels of IL-1β. Furthermore, LPS induced the expression of pro-inflammatory cytokines in the culture medium of RAW 264.7 cells, of which IL-6 and TNF-α, but not IL-1β, were significantly inhibited by EAFM administration (Fig. 3E).

EAFM inhibits the phosphorylation of ERK and JNK, and inhibits the nuclear translocation of NF-κB. To investigate the anti-inflammatory signaling mechanisms of EAFM, the expression of MAPKs (ERK, JNK and p38) and NF-κB was investigated by western blotting. LPS stimulation induced the phosphorylation of ERK, JNK and p38; furthermore, treatment with EAFM significantly inhibited ERK and JNK phosphorylation at a concentration of 50 µg/ml (Fig. 4A and B). LPS stimulation initiates various inflammatory responses by promoting the nuclear translocation of NF-κB. In the present study, LPS induced the expression and phosphorylation of NF-κB in nuclear proteins. Furthermore, EAFM was confirmed to markedly inhibit the expression and phosphorylation of NF-κB at a concentration of 50 µg/ml (Fig. 4C and D).

EAFM exerts antioxidant effects by activating ABTS radical scavenging and inducing Nrf2 and HO-1 expression. Next, The ABTS radical scavenging ability of EAFM was confirmed. As shown in Fig. 5A, EAFM increased the ABTS radical scavenging ability in a concentration-dependent manner. In particular, the EAFM of 100 µg/ml was significantly increased compared to the non-treated cells. The mRNA expression levels of HO-1 were determined to confirm that the anti-inflammatory properties of EAFM resulted from an antioxidant effect. As shown in Fig. 5B and C, EAFM induces HO-1 expression at the mRNA level. The effect of EAFM on the expression of Nrf2 (a transcription factor of HO-1) was verified by western blotting. EAFM was also found to increase the expression of Nrf2 and HO-1 at the protein level. These results indicate that EAFM prevents oxidative damage by promoting HO-1 expression in macrophages.

Discussion

The aim of the present study was to reveal the mechanisms by which EAFM regulates inflammation and oxidation. The RAW 264.7 murine macrophage cell line is commonly used to verify the anti-inflammatory effects of drugs, and to evaluate the associated signaling pathways. Inflammatory mechanisms

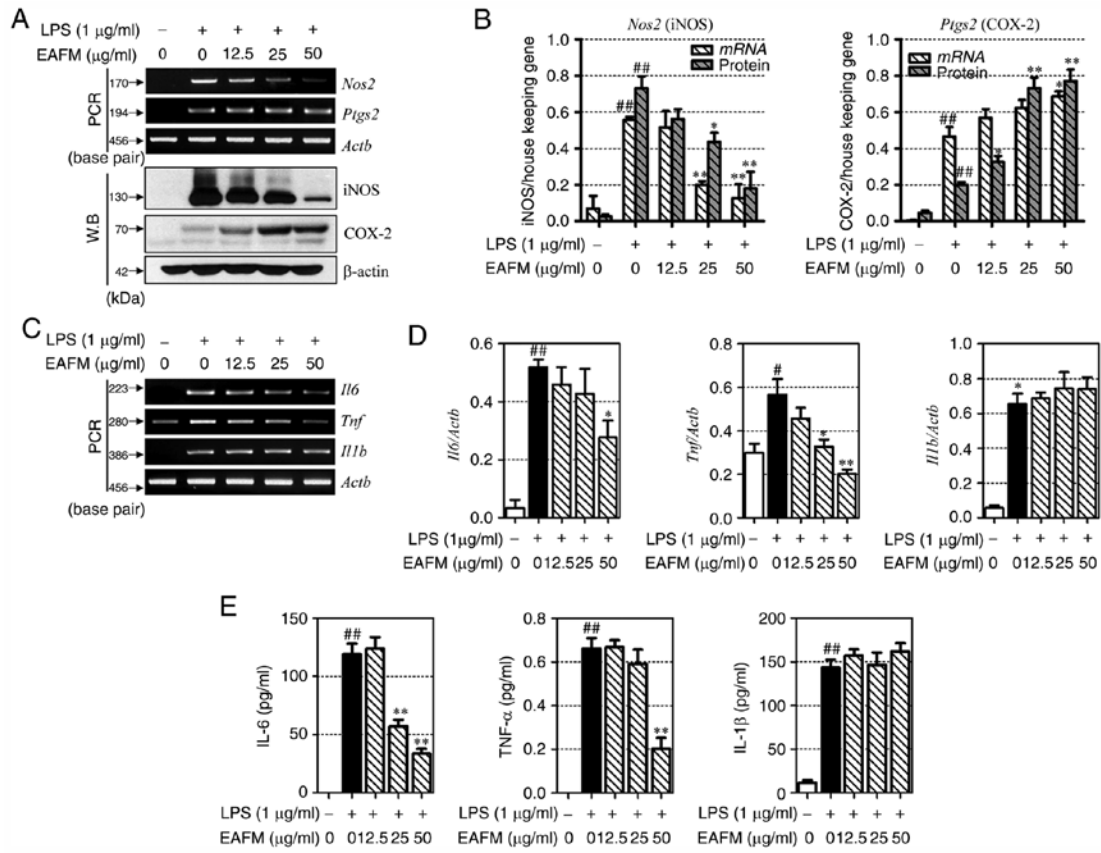


Figure 3. Effects of EAFM on iNOS, COX-2 and pro-inflammatory cytokine expression. (A) Effects of EAFM on the expression of LPS-induced iNOS and COX in RAW 264.7 cells were verified at the mRNA and protein levels by RT-PCR and western blotting. (B) Expression of iNOS and COX-2 was normalized to that of β -actin (*Actb*). (C) Effect of EAFM on the mRNA expression of pro-inflammatory cytokines in RAW 264.7 cells, demonstrated using RT-PCR. (D) mRNA expression was normalized to that of β -actin. (E) Expression of pro-inflammatory cytokines in RAW 264.7. Cell culture medium was assessed by ELISA. All experiments were repeated at least three times. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the non-treated group, $^*P<0.05$ and $^{***}P<0.01$ vs. the LPS-treated control. EAFM, ethyl acetate fraction of *Fritillaria thunbergii* Miquel; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; RT, reverse transcription; LPS, lipopolysaccharide.

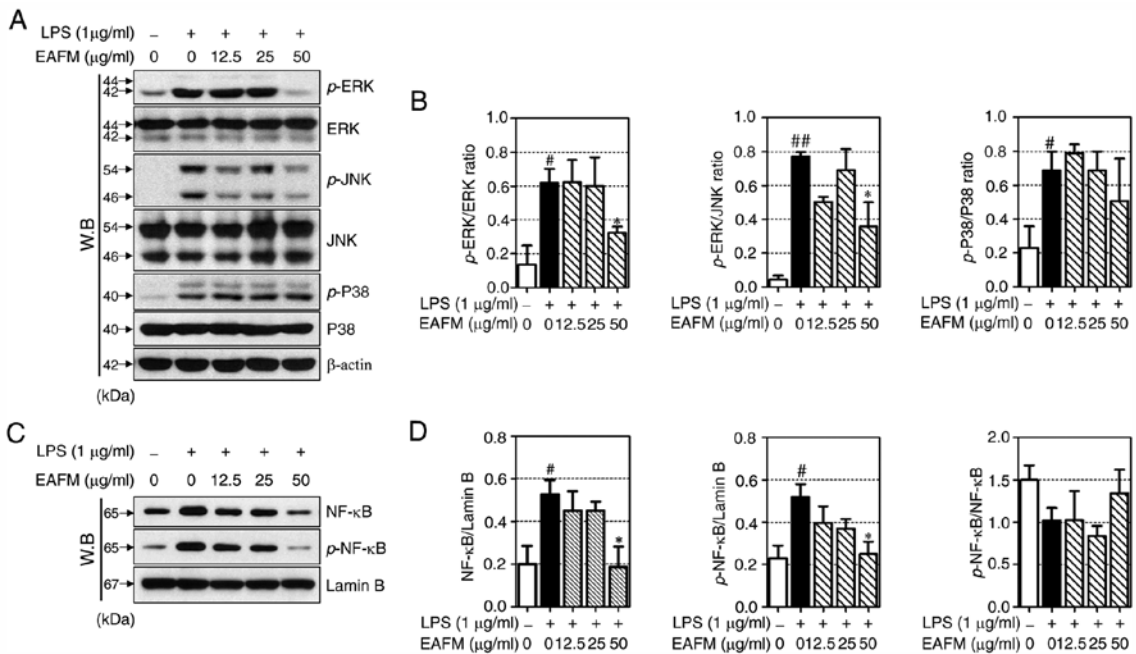


Figure 4. Effects of EAFM on MAPK phosphorylation and NF- κ B translocation. (A) Effect of EAFM on MAPK in RAW 264.7 cells was demonstrated by western blotting. (B) Phosphorylated ERK, JNK and p38 were quantified in each total form. (C) Effects of EAFM on NF- κ B expression and phosphorylation in the nucleus. (D) Expression levels of NF- κ B and p-NF- κ B were quantified using lamin B as an internal reference and the expression of p-NF- κ B was quantified as NF- κ B. All experiments were repeated at least three times. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the non-treated group. $^*P<0.05$ vs. LPS-treated control. EAFM, ethyl acetate fraction of *Fritillaria thunbergii* Miquel; LPS, lipopolysaccharides; W.B, western blot; p, phosphorylated.

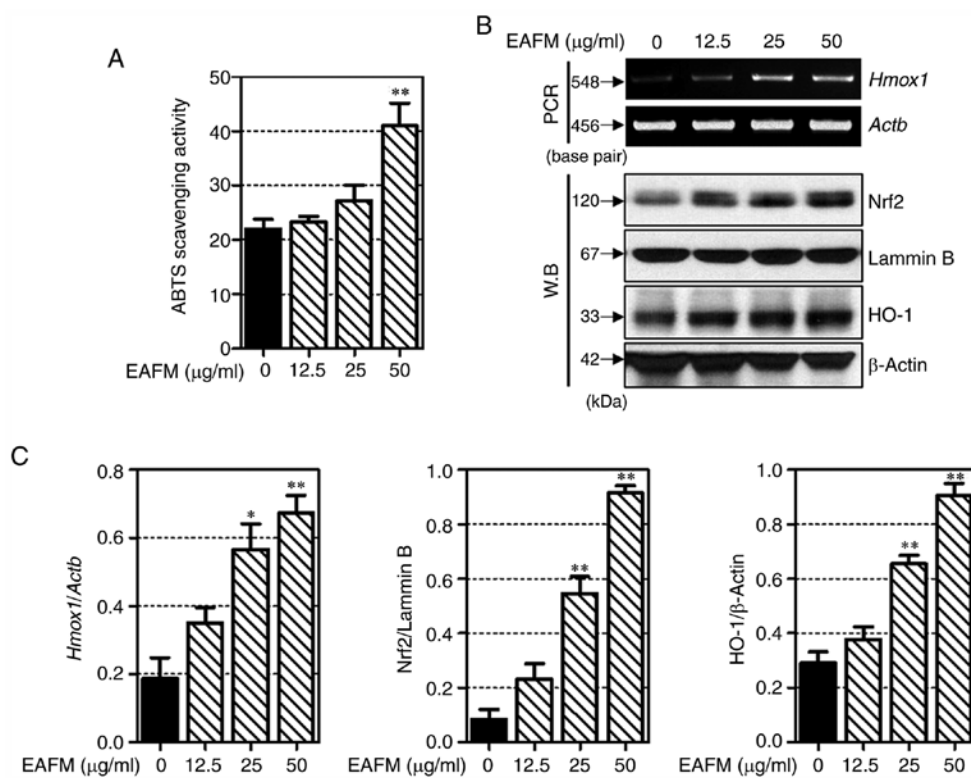


Figure 5. Effects of EAFM on anti-oxidative mechanisms in RAW 264.7 cells. (A) ABTS radical scavenging activity of EAFM. (B) Effects of EAFM on heme oxygenase 1 and nuclear factor erythroid 2-related factor 2 were demonstrated by reverse transcription PCR and western blotting. (C) mRNA and protein expression was quantified using β -actin and Lamin B as the internal reference. All experiments were repeated at least three times. * $P < 0.05$ and ** $P < 0.01$ vs. the non-treated group. EAFM, ethyl acetate fraction of *Fritillaria thunbergii* Miquel; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HO-1, heme oxygenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; W.B, western blot; Actb, β -actin.

are complex processes regulated by cytokine interactions and the induction of various pro-inflammatory genes (13). Macrophages are distributed throughout the human body and serve an important role in these inflammatory processes. Macrophages provide immediate defense against pathogens prior to leukocyte migration, and LPS stimulation induces the secretion of macrophage inflammatory mediators such as interleukins, TNF- α , iNOS and COX-2 (3). The pharmacological reduction of LPS-induced inflammatory mediators is considered to effectively alleviate various symptoms, including macrophage-associated inflammatory reactions. Therefore, an LPS-induced RAW 264.7 cell inflammation model was used in the present study.

NO is a well-known modulator of the inflammatory response, and is important for the defense against infectious organisms. However, it can also detrimentally affect host tissues. NO reacts with molecular oxygen to generate reactive nitrogen species, which can result in various modifications to cellular function (14). As a result, NO is considered to be an important factor of the inflammatory response. The expression of NO is regulated by iNOS, which is not expressed under normal conditions, but generated following the release of inflammatory cytokines such as TNF- α and IL-1 β . In the present study, EAFM suppressed the expression of NO as well as iNOS mRNA and protein (15,16). This indicates that the NO-suppressive effect of EAFM is the result of iNOS regulation.

PGE₂ is produced in macrophages and is involved in vasodilation, pain and fever in the early stages of the inflammatory

response and COX-2 plays a major role in the expression of PGE₂ (17). In previous studies, COX-2 inhibitors ablated PGE₂ synthesis, and consequently suppressed inflammation. In addition, LPS-induced hepatotoxicity was alleviated in COX-2 gene-positive mice. Therefore, PGE₂ and COX-2 have been used as anti-inflammatory indicators in numerous studies. However, in the present study, in contrast to the NO and iNOS results, EAFM had no effect on the expression of PGE₂ and increased COX-2 expression. The reason for the existence of the difference in expression between COX-2 and PGE₂ is assumed to be as follows. PGE₂ metabolism is first catalyzed by hydroxyprostaglandin dehydrogenase (15-PGDH) (18). Previous studies have shown that limited degradation of PGE₂ through decreased expression of 15-PGDH can lead to tumor growth (19), whereas elevation of 15-PGDH in lung cancer cells inhibited the expression of PGE₂ (20). Probably, EAFM seems to control the expression of PGE₂ through upregulation of 15-PGDH without affecting COX-2. However, additional research will have to be conducted to test this hypothesis.

In addition, the reason why EAFM suppresses iNOS, but not COX-2 expression is presumed to be as follows: The intracellular expression pathways of iNOS and COX-2 are complex, and the expression of these indicators depends on the degree of promoter dependence between them. The iNOS promoter contains cis-acting elements such as NF- κ B, activator protein 1 (AP-1), CCAAT/enhancer-binding protein beta (C/EBP β) and signal transducer and activator of transcription (Stat), whereas the COX-2 promoter includes cis-acting components such as NF- κ B, C/EBP β and cis-acting replication elements (CRE)

elements (21,22). The activities of different promoters vary depending on the cell type and stimulant. In the present study, the COX-2 promoter was confirmed to be less dependent on NF- κ B than the iNOS promoter. As the results of pro-inflammatory cytokine expression indicate, EAFM inhibited IL-6 and TNF- α by suppressing NF- κ B. These results suggest that EAFM may not inhibit COX-2 expression by complementing other promoters of COX-2, highlighting the need for further investigation.

Cytokines are small secretory proteins that facilitate intercellular communication. Pro-inflammatory cytokines are produced by activated macrophages, and representative cytokines such as IL-6, TNF- α and IL-1 β , are involved in both acute and chronic inflammatory signaling (13,23). IL-6 is rapidly produced in response to infection and tissue damage. In previous studies, IL-6-knockout animals displayed reduced susceptibility to disease symptoms in a variety of conditions, including Castleman's disease, rheumatoid arthritis and inflammatory myopathies (24). TNF- α is considered to be a master regulator of inflammatory cytokine expression and the abnormal production of TNF- α is associated with the development of various diseases, including rheumatoid arthritis, Crohn's disease and atherosclerosis. Therefore, TNF- α is a proposed target for the treatment of numerous inflammatory diseases (25). IL-1 β is induced at the site of inflammation and generally promotes the expression of PGE₂ and COX-2 (26). IL-1 β injection induces fever, headache, muscle and joint pain in humans by initiating inflammatory reactions (27). In the present study, EAFM significantly inhibited the expression of IL-6 and TNF- α , indicating its ability to regulate acute LPS-induced inflammatory responses. However, EAFM did not significantly affect IL-1 β expression, which supports previous results suggesting that EAFM does not inhibit the subsequent expression of COX-2 and PGE₂.

The NF- κ B pathway is one of the primary mechanisms by which macrophages express pro-inflammatory factors (28). In its inactive state, NF- κ B is bound to inhibitor of NF- κ B subunit α (I κ B) in the cytoplasm; following stimulation, I κ B is degraded and NF- κ B translocates to the nucleus to initiate various inflammatory responses. MAPKs serve regulatory roles in cellular proliferation and differentiation, and the regulation of cellular responses to cytokines and stress. There are three MAPK signaling pathways that involve ERK, JNK or p38, which transmit information from the extracellular environment to the nucleus. In addition, published studies indicate that MAPKs are involved in the regulation of COX-2 and iNOS expression (29). In the present study, EAFM inhibited both the nuclear transfer and phosphorylation of NF- κ B, as well as ERK and JNK phosphorylation. This indicates that the potential for EAFM to inhibit NO and inflammatory cytokine production is mediated by NF- κ B, ERK and JNK. However, although EAFM inhibits the nuclear potential and phosphorylation of NF- κ B, there is a limitation that it is not clear why it does not affect IL-1 β .

There are several ways to measure the antioxidant effect, and the results are slightly different. ABTS is a relatively stable free radical and is widely used for measuring antioxidant activity along with DPPH. This method can measure both lipophilic or hydrophilic substances (30). In this study, EAFM improved the ABTS radical scavenging ability. These

findings mean that EAFM improves the ability to remove free radicals, which means that it has antioxidant effects. The Nrf2/HO-1 axis is a major defense mechanism against oxidative stress, where HO-1 expression converts heme to biliverdin/bilirubin, carbon monoxide (CO) and free iron (6). The generation of biliverdin/bilirubin has a strong antioxidant effect, and CO also has anti-inflammatory properties. In activated macrophages, HO-1 induction reduces the production of IL-6, TNF- α and IL-1 β and HO-1 overexpression decreases the expression of iNOS, which is produced following COX-2 initiation. NO and PGE₂ production is also suppressed. HO-1 is dependently regulated by Nrf2; following nuclear translocation, Nrf2 binds to an antioxidant response element, regulating antioxidant mechanisms and serving an important role in the defense against oxidative stress. In the present study, EAFM notably increased the expression of both Nrf2 and HO-1. These results indicate that the antioxidant effect of EAFM, at least in part, influences preceding anti-inflammatory mechanisms.

In conclusion, the results of the present study confirm that EAFM suppresses the expression of NO and pro-inflammatory cytokines by inhibiting MAPK/NF- κ B signaling pathways. Additionally, EAFM also exerts its antioxidant effects by inducing HO-1 expression. The results of these experiments highlight the potential of EAFM as a future treatment for inflammatory diseases. The following limitations exist in this study. i) Peimine and peiminine, known as the main components of FTM, have been demonstrated in previous studies to have anti-inflammatory effects (9,31). However, referring to the results of the HPLC experiment, it was confirmed that peiminine was not present in the constituents of EAFM, and a very small amount of peimine was included. Therefore, further studies are needed to determine which chemicals of EAFM exhibit anti-inflammatory effects. ii) In this study, the expression of COX-2 increased with EAFM treatment. Referring to the anti-inflammatory effect of *Xanthii fructus* before (32), it showed anti-inflammatory effect similar to the result of this study, but the expression of COX-2 increased as the drug was treated. It can be inferred that there is some similarity between EAFM and *Xanthii fructus*, and it is suggested that further research is needed in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YS conceptualized the study. JHK, MK and EYK performed the experiments. SH, BK and HSJ statistically analyzed the data. HSJ, KS and MWS interpreted the results. JHK drafted

the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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