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A STUDY OF THE ANTI-INFLAMMATORY EFFECTS OF THE ETHYL ACETATE FRACTION OF THE METHANOL EXTRACT OF FORSYTHIAE FRUCTUS

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Abstract

Background: The dried fruit of *Forsythia suspensa* (Thunb.) Vahl. (Oleaceae) are better known by their herbal name *Forsythiae Fructus*, and have a bitter taste, slightly pungent smell, and cold habit. FF has been widely used to treat symptoms associated with the lung, heart, and small intestine. Recently, bioactive compounds isolated from hydrophobic solvent fractions of FF have been reported to have anti-oxidant, anti-bacterial, and anti-cancer effects. Traditionally, almost all herbal medicines are water extracts, and thus, extraction methods should be developed to optimize the practical efficacies of herbal medicines.
Materials and Methods: In this study, the anti-inflammatory effects of the ethyl acetate fraction of the methanol extract of FF (FFE) were assessed by measuring NO and PGE_2 production by and intracellular ROS and protein levels of iNOS and COX-2 in RAW 264.7 cells.
Results: FFE inhibited COX-2 expression in LPS-stimulated RAW 264.7 cells.
Conclusion: In summary, FFE effectively reduced intracellular ROS and NO levels and inhibited PGE_2 production by down-regulating COX-2 levels.

Key words: Forsythiae Fructus, herb, inflammation, efficacy.

Abbreviations: FF, of Forsythiae Fructus; NO, nitric oxide; iNOS, inducible NO synthase; COX-2, cyclooxygenase-2; ROS, reactive oxygen species; PGE_2, prostaglandin E_2.

Introduction

Forsythiae Fructus (FF), the fruit of *Forsythia suspensa* (Thunb.) Vahl. (Oleaceae) is mainly produced in Korea (Uiseong-gun) and China (Shaanxi Henan Province). Many compounds including flavonoids, lignans, terpenes, glycosides and saponins have been isolated from FF (Guo et al. 2007, Li et al. 2005). Furthermore, FF and its constituent compounds have been shown to have anti-oxidant (Schinella et al. 2002), anti-inflammatory (Kang et al. 2008, Ozaki et al. 1997), anti-bacterial (Nishibe et al. 1982, Qu et al. 2008), anti-tumor (Hausott et al. 2003, Sunet al. 2007) and anti-cancer effects (Sun et al. 2007, Zhao et al. 2015). Recently, FF and its active compounds, arctigenin, arctiin, forsythiaside, and phylligenin have been reported to have anti-inflammatory effects (Cheng et al. 2015, Kang et al. 2008, Lee et al. 2011, Lim et al. 2008). Lim *et al*. reported that phylligenin effectively lowered NO, iNOS, and PGE_2 levels (Lim et al. 2008), and Kang *et al*. reported arctigenin showed significant anti-inflammatory effects by inhibiting leukocyte infiltration into inflamed issues (Kang et al. 2008). Interestingly, both of these compounds were isolated from ethyl acetate fractions of FF methanolic extract. Furthermore, in a preliminary study, we found the ethyl acetate fraction of the methanol extract of Forsythiae Fructus (FFE) showed greater anti-inflammatory activity than other fractions.

Pro-oxidants give rise to oxidative stress, which causes inflammation and the overproductions of pro-inflammatory mediators by stimulated immune cells, such as, T cells and macrophages (Boje et al. 1992, Storck et al. 1994). RAW 264.7 cells are a mouse-derived macrophage cell line, which continues to be commonly used in studies of anti-inflammatory agents. Stimulated

macrophages release many pro-inflammatory mediators, such as, interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, PGE_2 and NO by overexpressing iNOS and COX-2 (Bonfield et al. 1995, Feghali et al. 1997, Sprague et al. 2009). Activated macrophages secrete nitric oxide (NO) synthesized from L-arginine by inducible nitric oxide synthase (iNOS), and this NO provides a non-specific host defense mechanism. However, the inhibition of NO production is viewed as a useful therapeutic strategy for inflammatory diseases (Sharma et al. 2007). Furthermore, prostaglandin E_2 (PGE_2) is produced from arachidonic acid metabolites by cyclooxygenase-2 (COX-2) (Bae et al. 2012, Nakanishi et al. 2013), and these various cytokines and pro-inflammatory molecules may cause metabolic disorders (Hotamisligil, 2006).

Many studies have been conducted on the biological mechanisms of anti-oxidants and anti-inflammatories (Chohan et al. 2012, Gacche et al. 2008, Laughton et al. 1991). After being stimulated by various factors, macrophages can cause pathologic injuries via a variety of inflammatory mechanisms. For these reasons, we investigated the anti-inflammatory effects of FFE by measuring NO, PGE_2 and ROS, and the protein levels of iNOS and COX-2 in RAW 264.7 cells.

Materials and Methods

Materials

Medicinal herb

FF was purchased from Hwalim Natural Herb Company (Busan, South Korea). Korea), and was authenticated by one of the authors (Cho, an experienced pharmacognocist). Specimens were deposited in the School of Korean Medicine, Pusan National University.

Reagents

n-Hexane was purchased from Merck Millipore (KGaA, Darmstadt, Germany). Chloroform, ethyl acetate, butanol and dimethyl sulfoxide (DMSO) were purchased from Junsei Chemical Co., (Tokyo). All solvents were of analytical grade.
Thiazolyl blue tetrazolium bromide (MTT), lipopolysaccharide (LPS), 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) and other reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO). Tris was purchased from Duchefa Biochemie (BH Haarlem, Netherlands), dimethyl sulfoxide (DMSO) from Junsei Chemical Co., (Tokyo). Fetal bovine serum (FBS) and penicillin-streptomycin (P/S) were purchased from Gibco (Los Angeles, CA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Welgene Inc., (Gyeongsangbuk-do, Korea). BCA reagent and albumin were purchased from Thermo Scientific (Waltham, MA, USA). Primary iNOS antibodies were obtained from Calbiochem, β-actin from Santa Cruz. COX-2, and secondary antibodies (Goat anti-rabbit IgG, pAb and Goat anti-mouse IgG, pAb were obtained from Enzo Life Science). West-Q chemiluminescent substrate was purchased from GenDEPOT. All solvents used were of HPLC grade, unless stated otherwise.

Cell Line

RAW 264.7 cells (a mouse macrophage cell line) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea).

Solvent Fractionation

Crude drugs (FF, 458.5 g) were extracted three times by maceration in methanol (5L × 3) for 48 hours at room temperature. The methanolic extract obtained was filtered and solvent was evaporated from filtrate using a rotary evaporator and speed vacuum concentrator.

The total yield of methanol extract was 88.83 g (19.37%). Extract (37.57 g) was suspended in water, and partitioned sequentially to produce the following fractions; hexane (FFH, 5.10 g), chloroform (FFC, 1.91 g), ethyl acetate (FFE, 0.76 g), butanol (FFB, 4.60 g), and an aqueous residue (FFW, 14.01 g). These materials were kept in sterile micro tubes and stored in a refrigerator at -20°C (Fig. 1).

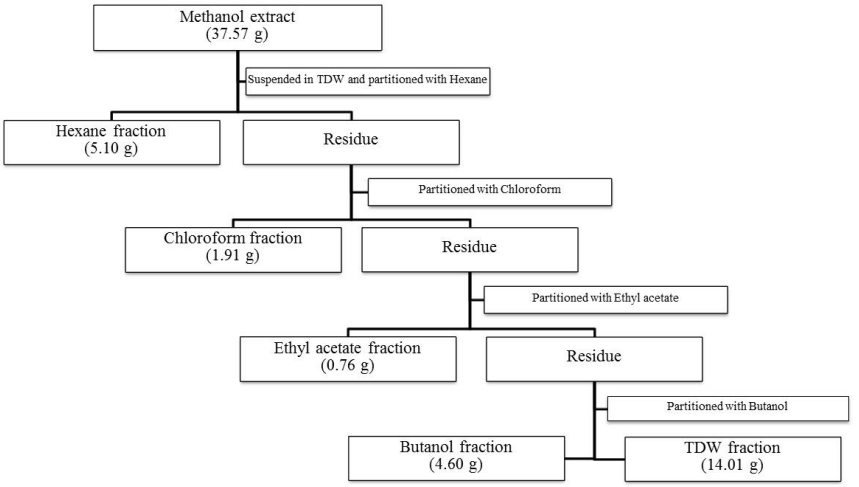

Figure 1: Preparation of *Forsythiae Fructus* subfractions.
458g of dried herb was extracted using methanol, then filtered and dried. The methanol extract was again fractionated sequentially using different solvents and the fractions so obtained were tested anti-inflammatory effects.

TLC Profile

The methanolic extract and fractions of FF were analyzed by high performance thin layer chromatography (HPTLC; Merck, Darmstadt, Germany). Samples were spotted on HPTLC plates and developed using chloroform: methanol (50: 1 (v/v)). After drying, plates sprayed with a chromogenic reagent (acetic acid: sulfuric acid: p-anisaldehyde = 100: 2: 1) and scanned using a TLC visualizer documentation system (CAMAG, Switzerland) using white light.

Cell Culture

RAW 264.7 cells were cultured in DMEM media supplemented with 10% FBS and 1% P/S and maintained in a 5% CO_2/95% air atmosphere at 37°C.

Cell Viability Assay and Cell Morphology

Cell viabilities were measured using a modified version of as previously described 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) method (Carmichael et al. 1987). Briefly, 2×10^5 per well of RAW 264.7 cells were incubated at 37°C in a 5% CO_2 atmosphere for 18 hours. Cells were then incubated with various concentrations of FFE for 24 hours, and then stimulated with 50 ng/ml of LPS for 23 hours. MTT solution (0.5 mg/ml) was then added for 2 hours and then culture medium was removed. Absorbance was measured at 570 nm using a spectrophotometer (Tecan, Infinite® M200, Switzerland).

The effects of FFE on Raw 264.7 cell morphology and density were investigated as follows. Cells (5×10^4 per well) were incubated at 37°C in 5% CO_2 for 18 hours using the procedure described above. Morphological changes were examined under an inverted microscope at 100x (Nikon Eclipse, TS100F, Japan).

Intracellular Reactive Oxygen Species (ROS) Generation

The production levels of intracellular reactive oxygen species (ROS) were measured using DCFH-DA fluorescent dye. Briefly, 2×10^5 per well of RAW 264.7 cells were incubated at 37°C in 5% CO_2 for 18 hours. Cells were then incubated with various concentrations of FFE for 24 hours, and then stimulated with 50 ng/ml of LPS for 23 hours. DCFH-DA was added to a final

concentration of 5 μM and incubated for 30 minutes. Cells were then washed three times in PBS, and fluorescence was measured using a fluorescence reader (Tecan, Infinite® M200, Switzerland) at excitation and emission wavelengths of 485 and 530 nm, respectively.

Concentrations of intracellular reactive oxygen species were assessed by flow cytometry (BD FACSCanto Ⅱ, USA)

Nitric Oxide (NO) Production

Nitrite oxide production by cells was measured using Griess reagent. Briefly, 2×10^5 RAW 264.7 cells per well were incubated at 37°C in a 5% CO_2 atmosphere for 18 hours, and then incubated with various concentrations of FFE for 24 hours. Cells were then stimulated with 50 ng/ml of LPS for 23 hours. Supernatants were collected and mixed with Griess reagent (1%(w/v) sulfanilamide, 0.1%(w/v) naphthylethylenediamine in 30% acetic acid). Absorbance was measured at 540 nm using a spectrophotometer (Tecan, Infinite® M200, Switzerland).

Prostaglandin E_2 (PGE_2) Production

Concentrations of PGE_2 in culture media was measured using an enzyme-linked immunosorbent assay (ELISA). Briefly, 2×10^5 RAW 264.7 cells per well were incubated for 18 hours at 37°C in 5% CO_2, and then with various concentrations of FFE for 24 hours. After incubation, cells were stimulated with 50 ng/ml of LPS for 23 hours. Supernatants (100 μl) were added to a Goat anti-mouse IgG coated 96-well plate and then incubated with 50 μl of PGE_2 conjugated solution and 50 μl of primary antibody solution for 2 hours at room temperature. After incubation, supernatants were discarded and wells were washed 3 times. Cells were then treated with 200 μl of pNpp substrate solution for 45 minutes at room temperature when the reaction was terminated by adding 50 μl of stop solution. Absorbance was measured at 405 nm using a spectrophotometer (Tecan, Infinite® M200, Switzerland).

Western Blot Analysis

The expression levels of iNOS and COX-2 were assessed by western blotting. Briefly, RAW 264.7 cells (1×10^6 per well) were incubated for 18 hours at 37°C in 5% CO_2, and then with various concentrations of FFE for 24 hours. Cells were then stimulated with 50 ng/ml of LPS for 23 hours when attached cells were washed twice with ice-cold PBS. Total proteins were isolated using protein extraction solution (pro-prep, iNtRON, Gyeonggi-do, Korea). Cell lysates were obtained by centrifugation at 4°C at 13,250×*g* for 10 minutes. Proteins were separated in sodium-dodecyl sulfate polyacrylamide gels, and transferred to PVDF membranes (Millipore, Darmstadt, Germany), which were blocked using 5% skim milk in TBST buffer for 1 hour at room temperature and then incubated overnight at 4°C with specific antibodies of iNOS (1:500), COX-2 (1:1000) and β-actin (1:500). After overnight incubation, Horse Radish Peroxidase (HRP) conjugated Goat anti-rabbit IgG, pAb (1:5000) and Horse Radish Peroxidase (HRP) conjugated Goat anti-mouse IgG pAb (1:3000) were added for 2 hours. Membranes were then treated with ECL solution (GenDEPOT, Houston, TX, USA) and protein bands were detected by a photosensitive luminescent analyzer system (Amersham™ Imager 600, UK). Protein relative quantities were analyzed using the Image J program (NIH, Maryland, USA) versus β-actin.

Statistical Analysis

One-way ANOVA was used to determine the statistical significance of differences. Data are expressed as means ± standard deviations (STDEVs). SIGMAPLOT 12.0 version was used to statistical analysis, and *p*-values of ≤0.05 were considered statistically significant.

Results and Discussion

Yields of Fractions from the Methanolic Extract of Forsythiae Fructus

Yield percentages were 0.47% (hexane), 0.18% (chloroform), 0.07% (ethyl acetate), 0.42% (butanol), and 1.29% (aqueous residue) (Table 1).

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Table 1: Yields of fractions from the methanolic extract of Forsythiae Fructus.

Samples	Yield (%)
FFM	19.37
FFH	2.63
FFC	0.98
FFE	0.39
FFB	2.37
FFW	7.22

Confirmation of Components

Components were confirmed and visualized using a TLC method (Fig. 2). The fractions were provided from the FFM, and depending on the efficacies of fractionated ingredients, the next experiments were conducted by using each of potential samples.

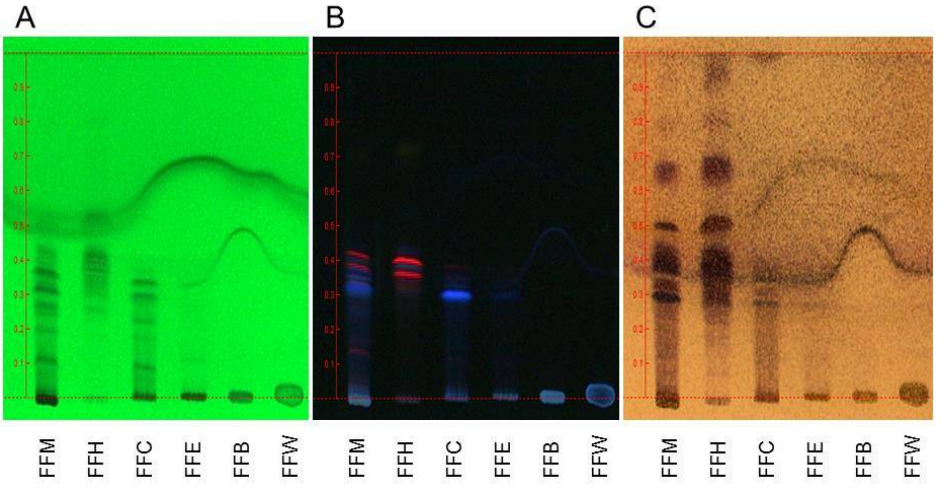

Figure 2: TLC analysis of Forsythiae Fructus extract and fractions.
Images were obtained using a Visualizer (Camag, Swiss). A, under 254 nm UV detected; B, under 366 nm UV detected; C, under white light detected after spraying with p-anisaldehyde.

Cell Viability and Morphological Changes in RAW 264.7 Cells

The cell viability of RAW 264.7 in the presence of 12.5 – 200 μg/ml FFM or FFE for 24 hours were assessed using an MTT assay. At concentrations of less than 100 μg/ml, FFM treated did not affect RAW 264.7 cell viability, and concentrations of less than 200 μg/ml FFE did not affect cell viability. Therefore, to study anti-inflammatory properties, FFE was administered at 12.5 – 200 μg/ml (Fig. 3(A)).

As shown in Fig. 3(B), no abnormal changes in morphology were observed after treating cell with FFE. These results imply that FFE is less cytotoxic than FFM, and for this reason, FFE was used in subsequent experiments.

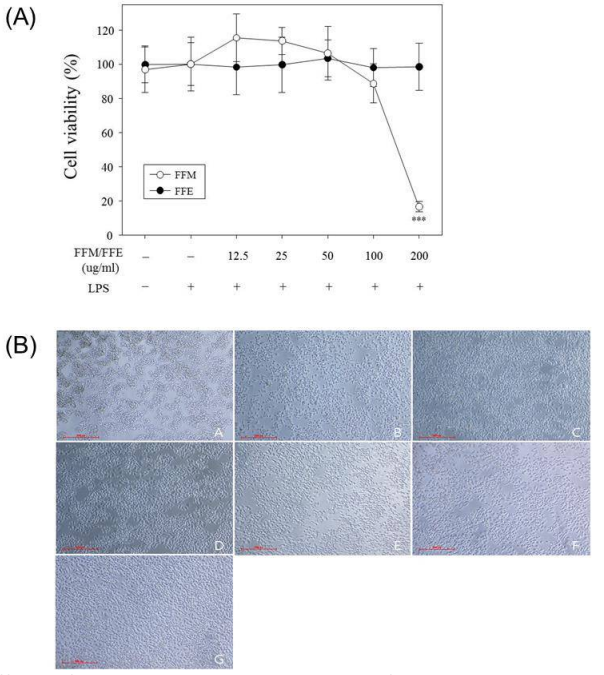

Figure 3: Effects of FFM and FFE on the viability of LPS-induced RAW 264.7 cells.

Cell viabilities were measured using a MTT assay as described in Material and Methods. (A) Values were presented as the means ± STDEVs of three independent experiments. $^{***}p < 0.001$ vs. LPS-treated controls. (B) The effects of FFE on cell density and morphological changes in RAW 264.7 cells were also observed under an inverted microscope (magnification, 100x; scale bar, 500px). A, neither LPS nor FFE treated control; B, LPS treated; C, LPS and 12.5 μg/ml of FFE; D, LPS and 25 μg/ml of FFE; E, LPS and 50 μg/ml of FFE; F, LPS and 100 μg/ml of FFE; G, LPS and 200 μg/ml of FFE.

Measurements of Intracellular ROS

Many authors' studies have found correlations between oxidative stress and DNA damage. Reactive oxygen species (ROS) causes DNA damage and pathological cellular senescence of the cells (Ghanta et al. 2007, Kalim et al. 2010, Poljsak et al. 2013, Wei 1998). Therefore, we examined the effects of FFE on intracellular ROS generation.

The level of intracellular ROS in 50 ng/ml LPS treated RAW 264.7 cells was elevated by 200% versus non-treated controls. FFE pretreatment effectively inhibited this ROS elevation in a dose-dependent manner (Fig. 4). In addition, intracellular ROS was detected by flow cytometric analysis in FFE pretreated RAW 264.7 cells. As shown in Fig 5, the effects of FFE on the production of intracellular ROS were in accord with the results shown in Fig 4. Our preliminary studies have shown FFE has higher flavonoid and phenolic contents that the other fractions. In addition, FFE effectively scavenged free radicals as shown in Tables 2 and 3 of PART 1. These results are in accordance with its effects on intracellular ROS levels (Figs. 4 and 5).

These results imply that the anti-oxidative properties of FFE in RAW 264.7 cells are closely related in its anti-oxidant contents and free radical scavenging activity.

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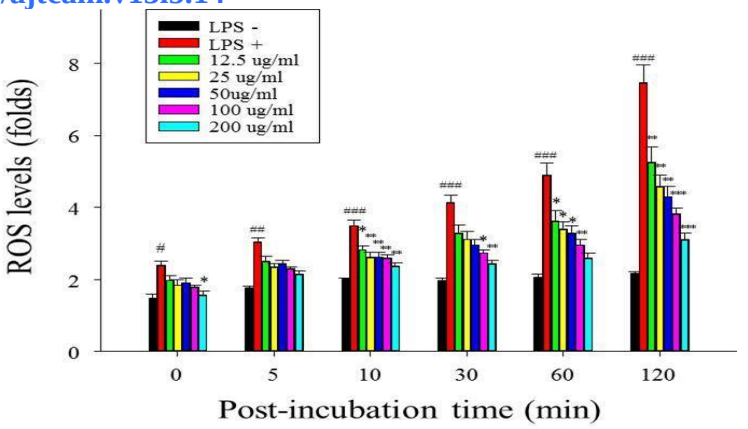

Figure 4: Effects of FFE on ROS levels in LPS-induced RAW 264.7 cells (kinetic analysis).
Amounts of reactive oxygen species were determined using DCFH-DA. All values were presented as the means ± STDEVs of three independent experiments. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. the treatment naïve control, and $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the LPS treated control.

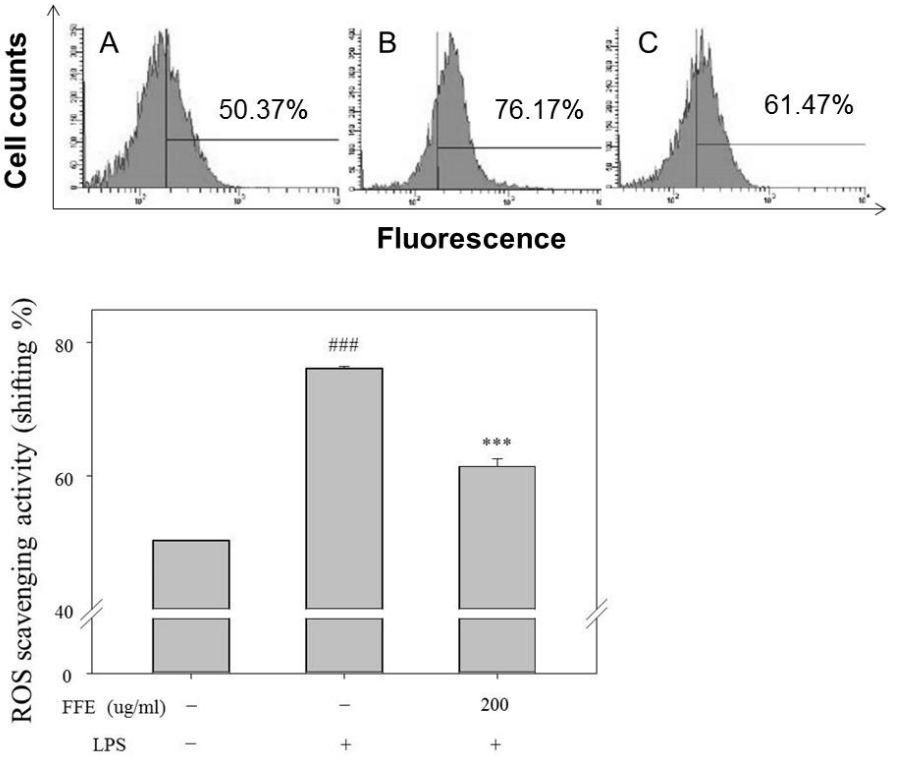

Figure 5: ROS scavenging activity by FFE on LPS-induced RAW 264.7 cells as determined by flow cytometric analysis.
Percentages of ROS positive cells in histograms were evaluated by flow cytometry. A, treatment naïve control; B, LPS treated; C, LPS and 200 μg/ml FFE treated. All values are the means ± STDEVs of three independent experiments. $^{###}p < 0.001$ vs. the treatment naïve control $^{***}p < 0.001$ vs. the LPS treated control.

Nitric Oxide (NO) Production and iNos Protein Levels

The biological roles of nitric oxide (NO) as an intracellular messenger are of considerable interest to those investigating anti-inflammatory effects. NO mediators can have physiological and pathological effects on the cardiovascular, endocrine, immune, and nervous systems (Bone 1996, Esposito et al. 2010), and it is well known that LPS-stimulated RAW 264.7 cells can release pro-inflammatory mediators, such as, cytokines, prostaglandins, and NO. Therefore, ability to inhibit NO is frequently used as a yardstick for estimating anti-inflammatory effects.

For these reasons, we investigated the inhibitory effects of FFE on NO production induced by LPS in RAW 264.7 cells, which were found to have NO levels almost 3 times that of non-treated cells. Furthermore, this elevation was effectively and dose-dependently inhibited by FFE (Fig. 6).

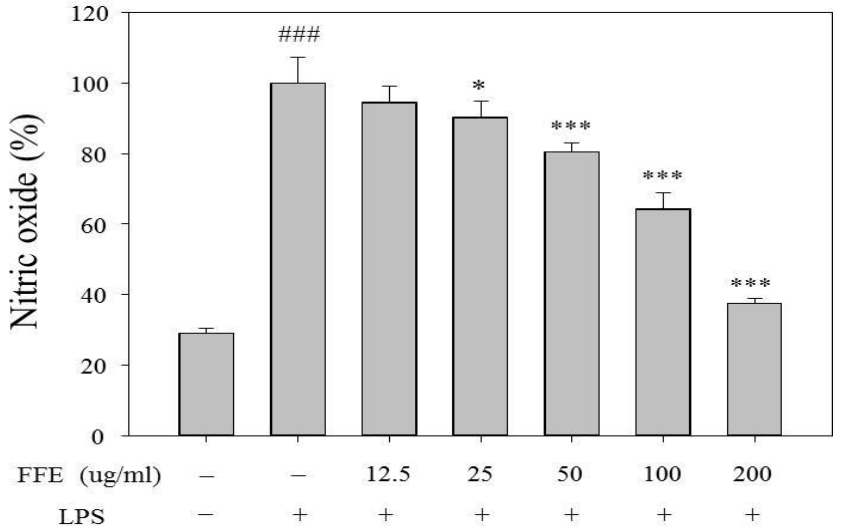

Figure 6: Inhibitory effects of FFE on LPS-induced NO production.
Amounts of nitric oxide in RAW 264.7 cells were determined using Griess reagent. Values are presented as the mean ± STDEVs of three independent experiments. $^{###}p < 0.001$ vs. the treatment naïve control $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. the LPS treated control.

In addition, the expression levels of iNOS protein in RAW 264.7 cells were evaluated. As shown in Fig. 7, FFE effectively inhibited iNOS expression, which was in-line with its observed effects on NO production.

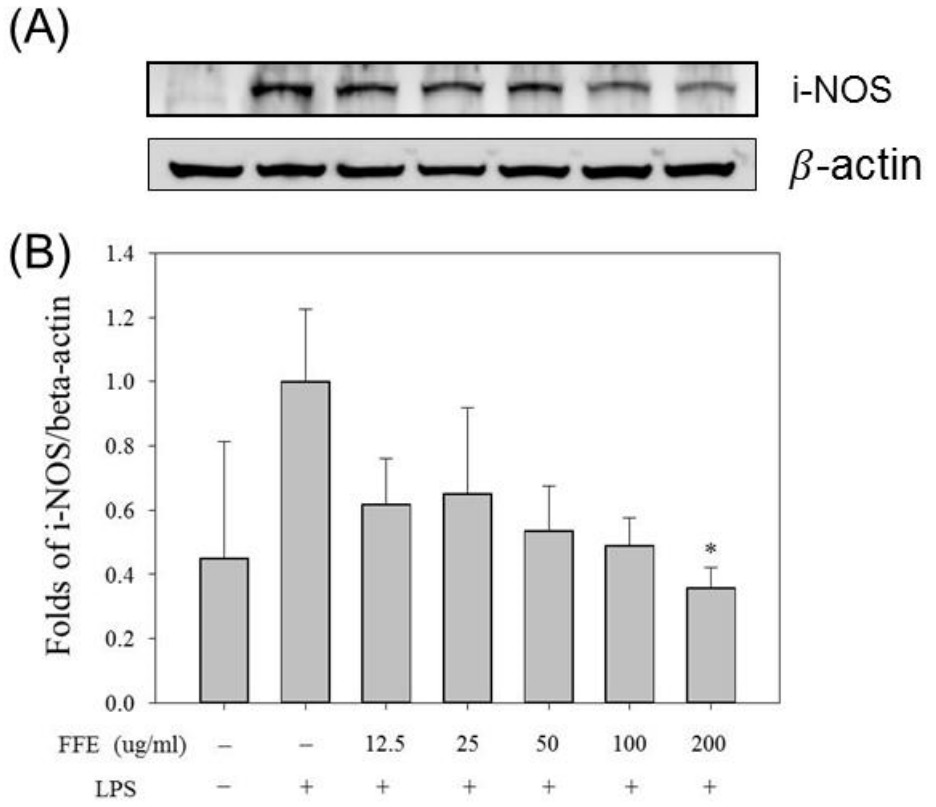

Figure 7: Inhibitory effects of FFE on LPS-induced iNOS expression.
(A) iNOS levels were evaluated by western blotting using 30 μg of RAW 264.7 cell lysates. (B) Protein expression were normalized versus β-actin. Values are presented as the means ± STDEVs of three independent experiments. $^{*}p < 0.05$ vs. LPS treated control.

In view of the observed inhibitory effects of FFE on intracellular ROS production, these results imply FFE removes ROS and thus inhibits NO production and iNOS expression.

Prostaglandin E_2 (PGE_2) Production and COX-2 Protein Levels

COX enzymes play central roles in the biosynthesis of prostaglandins from arachidonic acid and catalyze the formation of prostaglandin G_2 from arachidonic acid, which is then converted into prostaglandin H_2 that is the precursor of several structurally related prostaglandins, such as, prostaglandin D_2 and prostaglandin E_2 (PGE_2) (Jung 1988).

COX enzymes, especially COX-2, can be activated by several types of pro-inflammatory mediators, such as, cytokines, ultraviolet radiation, and bacterial endotoxins (Chun et al. 2004). As a result, PGE_2, which also signals inflammatory response is synthesized and released by activated COX enzymes during inflammatory processes. Accordingly, we examined the effects of FFE on COX-2 expression and the PGE_2 production. As shown in Fig. 8, FFE treatment effectively prevented PGE_2 production induced by LPS in a dose-dependent manner. FFE treatment at >50 μ0/ml lowered PGE_2 production significantly as compared with LPS treated RAW 264.7 cells.

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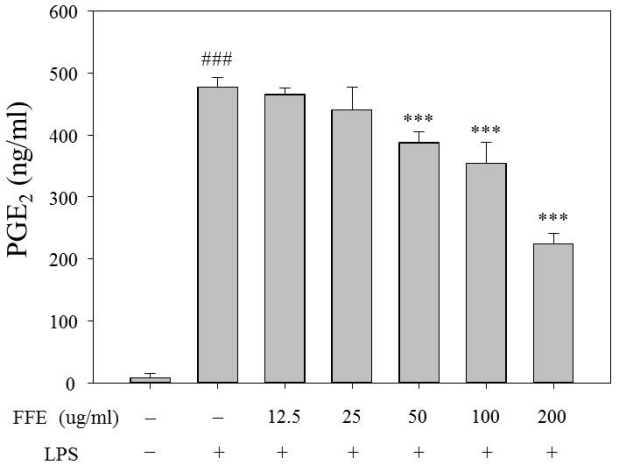

Figure 8: Inhibitory effects of FFE on LPS-induced PGE_2 production.
Prostaglandin E_2 levels in culture media for RAW 264.7 cells were determined by ELISA. Values represent as the means ± STDEVs of three independent experiments. $^{###}p < 0.001$ vs. the treatment naïve control, $^{***}p < 0.001$ vs. the LPS treated control.

In addition, pretreatment with >200 μg/ml of FFE inhibited COX-2 protein up-regulation in LPS-stimulated RAW 264.7 cells (Fig. 9).

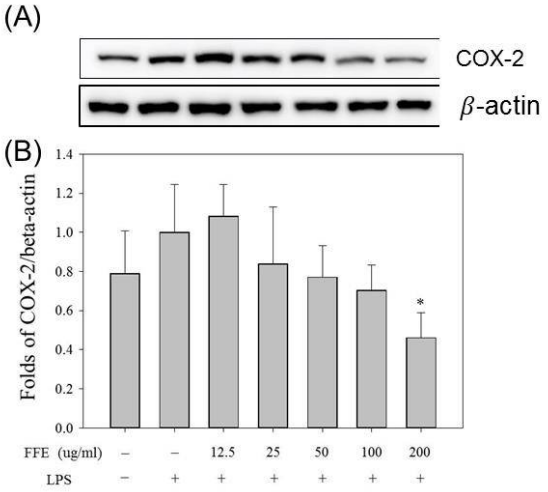

Figure 9: Inhibitory effects of FFE on LPS-induced COX-2 expression in RAW 264.7 cells.
(A) The COX-2 protein levels were assessed by western blotting using 30 μg of cell lysates. (B) Proteins expressions were normalized versus β-actin. Values are presented as the means ± STDEVs of three independent experiments. $^{*}p < 0.05$ vs. the LPS treated control.

These results imply that FFE regulates PGE_2 production by inhibiting the up-regulation of COX-2 by LPS in RAW 264.7 cells.

Conclusion

In this study, we investigated the anti-inflammatory effects of FFE in RAW 264.7 cells. FFE was found to inhibit intracellular ROS and NO production in a dose-dependent manner, and to inhibit iNOS and COX-2 expression, and PGE_2

production. These results indicate FFE acts as an effective anti-oxidant, and inhibits PGE_2 production by reducing COX-2 expression. We suggest FFE be considered an anti-inflammatory agent and hope the results of the present study will be found useful by those studying the anti-inflammatory actions of natural products.

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Disclosure of Potential Conflicts of Interests

The authors have no conflict of interest to declare.

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