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The CH_2Cl_2 Extract Fraction from *Ficus erecta* var. *sieboldii* (Miq.) King Suppresses Lipopolysaccharide-mediated Inflammatory Responses in Raw264.7 Cells

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Abstract A phytochemical application of leaves from *Ficus erecta* var. *sieboldii* (Miq.) King has not been widely investigated. We determined an anti-inflammatory effect of *F. erecta* extracts on lipopolysaccharide (LPS)-mediated production through modulation of several pro-inflammatory mediators and prostaglandin E2 (PGE_2). Among the *F. erecta* extracts, the CH_2Cl_2 fraction (CFE) most effectively suppressed the LPS-mediated production of nitric oxide (NO) in Raw264.7 cells. As determined by immunoblotting and PCR, CFE was shown to have an inhibitory effect on LPS-induced mRNA and protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). In addition, CFE showed significant inhibitory effects on LPS-mediated production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and PGE_2 ($P<0.05$), demonstrating its effects on inflammation. The main active compounds that suppressed PGE_2 production were syringaresinol (**C1**) and 6,7-furano-5-methoxy hydrocoumaric acid (**C2**). In conclusion, CFE showed an inhibitory effect on LPS-mediated inflammatory responses by suppressing iNOS, COX-2, TNF-α, IL-1β, and IL-6 production. The compounds **C1** and **C2** showed strong inhibitory effects on LPS-mediated production of PGE_2 and may be applicable as starter compounds for developing anti-inflammatory and anti-nociceptive drugs.

Keywords: *Ficus erecta var. sieboldii (Miq.) King, Inflammation, Cyclooxygenase-2, Prostaglandin E2, Pro-inflammatory cytokine, Syringaresinol, 6,7-Furano-5-methoxy Hydrocoumaric acid*

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1. Introduction

Inflammation is the body's attempt at protecting itself against infections or tissue injury. Systemic diseases such as obesity, hypertension, and coronary artery disease may be linked to inflammatory responses, which are initiated through the production of cytokines such as interleukins (IL), tumor necrosis factor (TNF)-α, and other types of inflammatory mediators [1,2,3]. Depending on duration, inflammation can be classified as acute or chronic. Generally, the first stage of the inflammatory process lasts only 3-14 days and plays an important role in normal wound healing. By contrast, chronic inflammation has been shown to predispose an individual to cardiovascular disease and neurodegeneration and has been linked to tumorigenesis, tumor progression, and metastasis in many different cancers [4]. Thus, by reducing chronic inflammation, which might be suppress connected to pathological progression, there will be benefits to maintaining good health.

Lipopolysaccharide (LPS) is a component of Gram-negative bacteria cell walls and acts as a causal factor in many serious infectious diseases such as sepsis and arthritis [5,6]. However, since gut microbiota-derived LPS has recently been recognized as a factor involved in the onset and progression of inflammation and metabolic diseases, exposure to LPS is not just limited to infections [7]. Experimentally, LPS is among the most potent and

well-studied inducers of inflammation, interacting with specific receptors on host effector cells. The overproduction of pro-inflammatory cytokines, for example TNF-α, interleukin (IL)-1β, and IL-6, leads to an uncontrolled inflammatory reaction, which can lead to serious physiological disorders. In inflammatory reactions, the over-production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is harmful, and iNOS level is considered to be an important determinant of inflammatory damage [5,6,8]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an important transcription factor involved in gene regulation and plays a pivotal role in the cellular response to stress, cytokines, free radicals, ultraviolet radiation, and bacterial LPS or viral antigens [1,9]. Protein level of COX-2, which is involved in biosynthesis of prostaglandins from arachidonic acid, is increased primarily during inflammatory processes. Numerous pro-inflammatory genes that encode cytokines (IL-6, IL-1β, TNF-α, IL-4, IL-5, etc.), chemokines, adhesion molecules, and enzymes including iNOS, cyclooxygenase-2 (COX-2), and phospholipase 2 are under control of NF-κB activity [10]. In contrast, increased production of IL-1β and TNF-α amplifies NF-κB signaling [11]. Strategies for inhibiting NF-κB signaling and/or suppressing pro-inflammatory signals have potential therapeutic application in inflammatory diseases. Anti-inflammatory drugs are frequently used to alleviate pain caused by inflammation, but long-term usage of high doses of anti-inflammatory drugs can lead to side effects such as stomach ulcers, hemorrhage, or cardiovascular complications. Therefore, we attempted to identify naturally-occurring, anti-inflammatory components that are harmless to humans even after long-term use.

Ficus erecta (*F. erecta*) var. *sieboldii* (Miq.) King is a 2-7 m tall deciduous or semi-deciduous tree (or shrub). The bark fiber of *F. erecta* is used for making paper, but using its leaves in a phytochemical application has not been widely investigated. It has been shown that extracts of *F. erecta* show anti-osteoporotic [12] and anti-tyrosinase activities, which are applicable in cosmetics formulations [13]. It is speculated that the anti-osteoporotic activity of *F. erecta* may be associated with an anti-inflammatory effect on bone cells. Therefore, the anti-inflammatory effects of *F. erecta* extracts in different solvents, including 80% EtOH, *n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, and distilled water, were tested on Raw264.7 cells. We determined that the CH_2Cl_2 fraction of the *F. erecta* extract (CFE) had the most potent anti-inflammatory activity. We identified syringaresinol (**C1**) and 6,7-furano-5-methoxy hydrocoumaric acid (**C2**) as the main active compounds. Therefore, we propose that these compounds are potential factors for anti-inflammatory drug development.

2. Materials and Methods

2.1. Chemicals and Reagents

Dulbecco's modified eagle's medium (DMEM) was obtained from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS from Escherichia coli O111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). PGE_2, TNF-α, IL-1β, and IL-6 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies against iNOS, COX-2, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were purchased from Sigma-Aldrich.

2.2. Isolation and Fractionation of *F. erecta* Leaf Extracts

The leaves of *F. erecta* (1 kg) were collected from Jeju (Jeju-do, Korea) in August 2015, and were identified by Prof. Kang Se Chan, Kyung Hee University (Gyeonggi-do, Korea). The voucher specimen (JBR419) was deposited in the Laboratory of Natural Medicine Resources in BioMedical Research Institute, Kyung Hee University. Dried *F. erecta* leaves were extracted with 80% ethanol (EtOH) by stirring for 24 h at room temperature with vacuum filtration. *F. erecta* EtOH extracts were prepared in different solvents, including *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-BuOH, and distilled water (H_2O) (Figure 1). The CH_2Cl_2 fraction was further subfractionated on a column and identified as F_1-F_{20}. Compound 1 and compound 2 were isolated from F_{12} and F_{16} subfractions and defined as **C1** and **C2**, respectively (Figure 6).

2.3. Cell Culture

Raw264.7 murine macrophage cell lines were obtained from the American Type Culture Collection (ATCC TIB-71; Rockville, MD, USA). The cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated FBS. The cells were grown at 37°C in fully humidified air with 5% CO_2 and subcultured twice weekly.

2.4. Measurement of Nitric Oxide (NO) Production

After Raw264.7 cells were subjected to a 2 h pretreatment with various *F. erecta* fractions of different doses (0-100 μg/mL), 1 μg/mL of LPS was added, and NO was measured as the amount of nitrite released, as described previously [14]. Briefly, 100 μL of supernatant was combined with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalenediamine dihydrochloride, 2.5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was determined with an E MAX precise microplate reader (Molecular Devices, Eugene, OR, USA). Nitrite concentrations were calculated from a nitrite standard curve.

2.5. Measurement of Prostaglandin E2 (PGE_2) Production

Raw264.7 cells (1.0×10^6 cells/mL) cultured in 96-well plate were pretreated for 2 h with the CH_2Cl_2 fraction of the *F. erecta* leaf extract (CFE), F_{12}, F_{16}, **C1,** or **C2** and then stimulated with 1 μg/mL of LPS for 24 h. PGE_2 level was determined using an ELISA kit (R&D Systems) according to the manufacturer's instructions. Absorbance was measured at 450 nm [15].

2.6. Determination of Pro-inflammatory Cytokine Levels

Raw264.7 cells (1.0×10^6 cells/mL) were pretreated with CFE for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. The concentrations of TNF-α, IL-1β, and IL-6 in the culture supernatants were determined using a DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions. Experiments were assessed in triplicate and compared to the standards supplied by the manufacturer [15].

2.7. Quantitative RT-PCR Analysis

CFE (5, 10, and 20 μg/mL) was pretreated for 2 h, and then treated with 1 μg/mL LPS for 24 h. Total RNA was extracted using the PureLink™ RNA Mini Kit (Ambion, CA, USA), and 1 μg of total RNA was reverse transcribed in a 20 μL volume using oligo (dT) primers with enzyme and buffer supplied in the PrimeScript II 1st strand cDNA Synthesis kit (Takara, Tokyo, Japan). Quantitative real-time RT-PCR reactions were performed in an MX3005P instrument (Stratagene, CA, USA). The primers used in the experiments are shown in Table 1. For quantitative real-time PCR, SYBR Premix Ex Taq II (Takara) was used. The final volume of the reaction was 25 μL and contained 2 μL of cDNA template, 12.5 μL Master Mix, 1 μL of each primer (10 μM stock solution), and 8.5 μL sterile distilled water. The thermal cycling program consisted of a pre-incubation step at 95°C for 10 min, followed by 40 cycles of 95°C (15 s) and 60°C (60 s). Relative quantitative evaluation of adipocyte differentiation and lipogenesis gene levels was performed by the comparative CT (cycle threshold) method [16].

2.8. Protein Extraction and Immunoblotting

Protein extraction and immunoblotting were performed as previously described [14]. Briefly, the cells were washed twice with cold Dulbecco's Phosphate-Buffered Saline (DPBS) and then homogenized in the presence of 0.025 $mol\cdot L^{-1}$ of radioimmunoprecipitation assay (RIPA) buffer (Tris-HCl pH 7.6, 0.15 $mol\cdot L^{-1}$ NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease/phosphatase inhibitor cocktails; Sigma-Aldrich). Equal amounts of protein (50 μg) were electrophoresed on 10% or 12% SDS-polyacrylamide gels and transferred to an Immobilon®-P polyvinylidene difluoride membrane. The binding of each specific antibody was visualized using the enhanced chemiluminescence method (Amersham Biosciences, Pittsburgh, PA, USA). Equal loading of samples was confirmed by re-probing the membranes with an anti-β-actin antibody. The band density was analyzed using the Multi Gauge Ver. 3.0 software (Fujifilm, Tokyo, Japan).

2.9. Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Student's *t*-test or one-way ANOVA/Dunnett's *t*-test was used to assess significance between control and treated groups. Statistical analysis was performed using SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Inhibitory Effects of Different *F. erecta* Extract Fractions on LPS-mediated Production of NO in Raw264.7 Cells

In this study, the ability of *F. erecta* extracts to inhibit the production of inflammatory mediators in LPS-induced Raw264.7 murine macrophages was determined. NO plays an important role in mediating inflammatory responses, and an increased level of NO is linked to pain in osteoarthritis [17]. Therefore, it is important to suppress the expression of NO in various inflammatory responses.

After extraction with 80% EtOH, we obtained fractions from *n*-hexane, CH_2Cl_2, EtoAc, *n*-BuOH, and H_2O solvents (Figure 1). The inhibitory effects of the fractions on 1 μg/mL LPS-mediated production of NO in Raw264.7 cells are summarized in Table 2. Both CH_2Cl_2 and EtOAc fractions showed higher inhibitory effects against LPS-mediated production of NO compared to other fractions. Both CH_2Cl_2 fraction (CFE) and EtOAc fraction were significant at 100 μg/mL ($P<0.01$), however, the inhibition of LPS-mediated NO production was stronger in the CH_2Cl_2 fraction. Therefore, we used the CFE in further experiments.

Table 1. Lists of PCR primers

Gene name	Primer sequences
Interleukin -6 (IL-6)	5'-GAGGATACCACTCCCAACAGACC-3' (sense)
	5'-AAGTGCATCATCGTTGTTCATACA-3' (antisense)
Interleukin -1β (IL-1β)	5'-TGCAGAGTTCCCCAACTGGTACATC-3' (sense)
	5'-GTGCTGCCTAATGTCCCCTTGAATC-3' (antisense)
Tumor Necrosis Factor-α (TNF-α)	5'-ATGAGCACAGAAAGCATGATC-3' (sense)
	5'-TACAGGCTTGTCACTCGAATT-3' (antisense)
Inducible Nitric Oxide Synthase (iNOS)	5'-CAGCTGGGCTGTACAAACCTT-3' (sense)
	5'-TGAATGTGATGTTTGCTTCGG-3' (antisense)
Cyclooxygenase-2 (COX-2)	5'-CCAGCACTTCACCCATCAGTT-3' (sense)
	5'-ACCCAGGTCCTCGCTTATGA-3' (antisense)
β-actin	5'-TCATGAAGTGTGACGTTGACATCCGT-3' (sense)
	5'-CCTAGAAGCATTTGCGGTGCACGATG-3' (antisense)

Table 2. Inhibitory effect of *F. erecta* var. *sieboldii* (Miq.) King extracts on LPS-mediated production of nitric oxide in Raw264.7 cells

Extract / dose (μg/mL)	LPS (1 μg/mL)			
	0 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
80% EtOH	100 ± 2.16	91.6 ± 2.37	74.9 ± 1.88*	67.8 ± 1.45*
n-hexane fraction	100 ± 2.31	73.4 ± 2.24*	61.4 ± 2.08*	52.8 ± 2.18**
CH_2Cl_2 fraction	100 ± 2.19	71.7 ± 2.26*	49.2 ± 2.12**	16.5 ± 1.12**
EtOAc fraction	100 ± 2.46	76.4 ± 2.33*	55.1 ± 1.76*	27.0 ± 1.26**
n-BuOH fraction	100 ± 2.09	97.7 ± 1.97	92.8 ± 2.13	91.2 ± 1.87
H_2O fraction	100 ± 1.78	99.1 ± 2.41	98.6 ± 2.29	95.7 ± 2.01

Various doses of extracts of *F. erecta* were pretreated for 2 h, and then LPS was applied for 24 h. NO production was assayed in the culture medium via a Griess reaction. Data are mean ± standard deviation (SD). The value of the LPS alone group was set at 100, and results were expressed as a percentage of that value. *P<0.05 and **P<0.01 (test fraction vs. the LPS alone group). LPS, lipopolysaccharide; NO, nitric oxide.

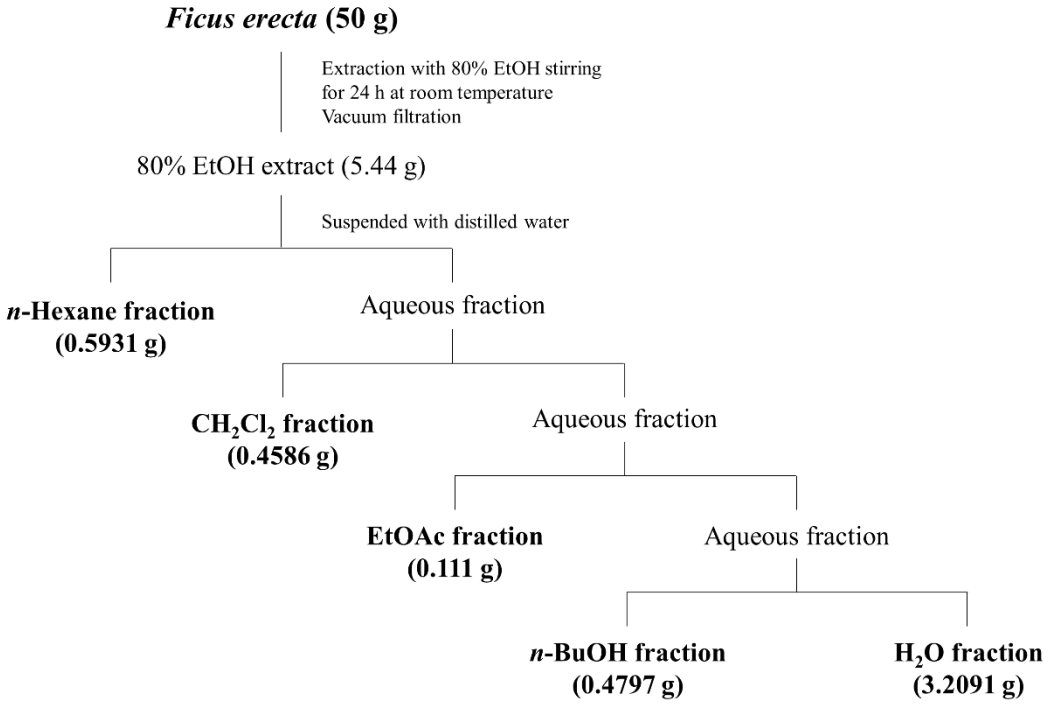

Figure 1. Extraction, fractionation, and isolation from leaves of *Ficus erecta*. var. *sieboldii* (Miq.) King

3.2. Inhibitory Effects of *F. erecta* Extracts on LPS-mediated Expression of Pro-inflammatory Mediators in Raw264.7 Cells

As shown in Table 2, CFE suppressed the production of NO. LPS-mediated NO production is highly associated with up-regulation of iNOS, and the reduction in NO production might be related to suppression of iNOS [7]. Immunoblotting was used to determine protein expression level of iNOS. Treatment with 25 μg/mL of CFE led to an 80% reduction of iNOS expression, and 50 μg/mL of *F. erecta* extract completely blocked iNOS expression (Figure 2). In addition, CFE also inhibited an increase of LPS-mediated mRNA expression of iNOS in a dose-dependent manner (Figure 3). Inhibition of iNOS expression by CFE may be connected to the decrease in LPS-mediated NO production. However, iNOS mRNA expression was more strongly suppressed at a lower dose (10 μg/mL) of CFE than observed by immunoblotting (Figure 2 and Figure 3). These results indicate that the decrease of LPS-mediated overproduction of NO by CFE is mediated by suppressing iNOS mRNA expression, which results in a lower abundance of iNOS on the immunoblot.

Inducible COX-2 is augmented by LPS stimulation [18]. As shown in Figure 2, LPS stimulation strongly increased the expression level of COX-2 protein, but this increase was highly suppressed in the presence of CFE in a dose-dependent manner. The expression of COX-2 was not completely blocked at the given dose of CFE (even at 100 μg/mL), whereas COX-2 mRNA expression was completely blocked at 20 μg/mL of CFE (Figure 3). This discrepancy is not clear, but it is possible that the COX-2 protein expression is more resistant than iNOS, or that their translational efficiency differs [19]. In the present study, expression of iNOS and COX-2 was dramatically suppressed in the presence of CFE. This may be associated with the inhibitory effect of CFE in LPS-mediated activation of NF-κB signaling [11,20,21] since iNOS and COX-2 mRNA expression was blocked (Figure 3). We did not examine the inhibitory effect of CFE in LPS-mediated activation of NF-κB signaling. However, suppression of iNOS and COX-2 might be related to suppression of NF-κB activation possibly via CFE.

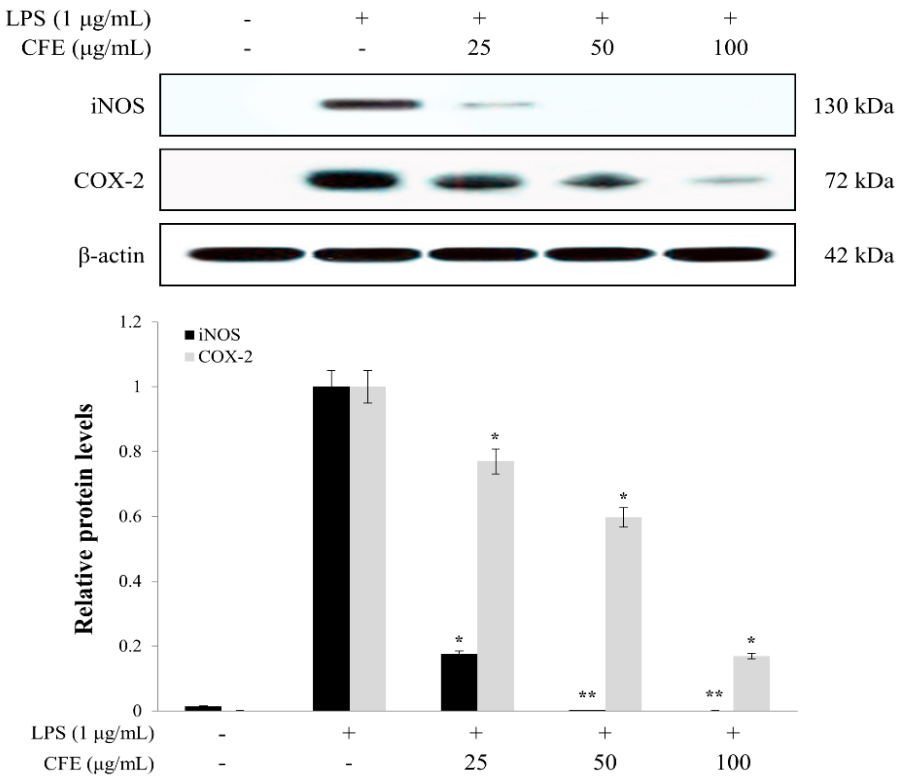

Figure 2. **Inhibitory effects of CFE administration on protein expression of iNOS and COX-2.** Raw264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 2 h with *F. erecta* CH_2Cl_2 fractions (CFE; 0, 25, 50, and 100 μg/mL). Cells were then stimulated with LPS (1 μg/mL) for 24 h. LPS-induced changes in the protein levels of iNOS and COX-2 were determined by Western blot analysis. β-actin was used as a loading control. Data were expressed as a ratio relative to the LPS alone group. $^{*}P<0.05$ and $^{**}P<0.01$ (test fraction vs. the LPS alone group). LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2

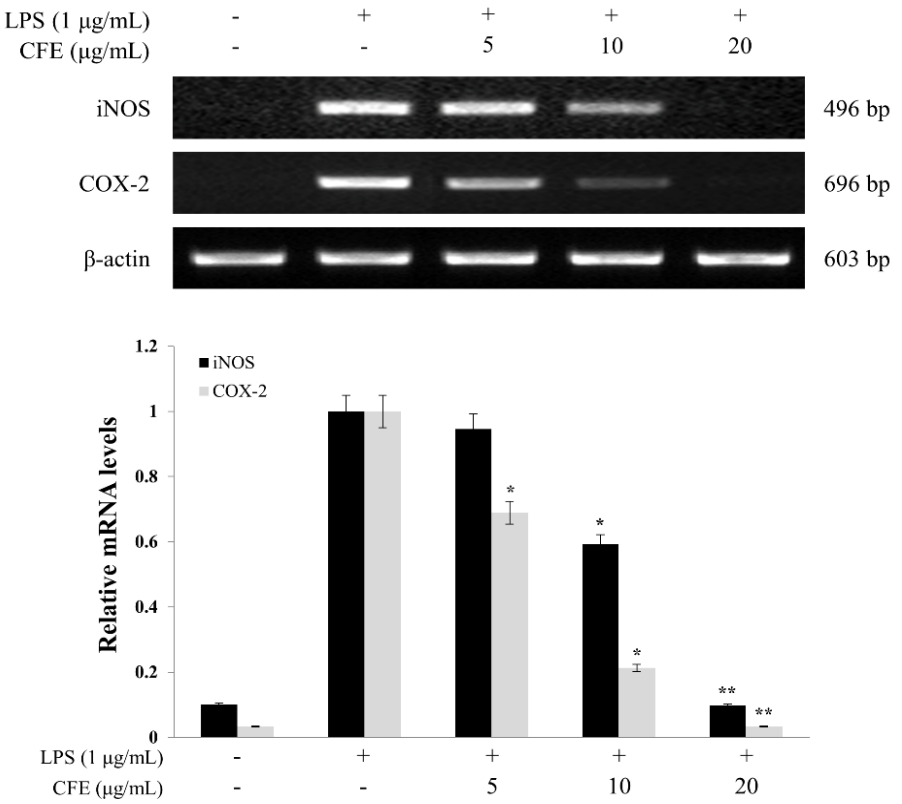

Figure 3. Inhibitory effects of CFE administration on mRNA expression of iNOS and COX-2. Raw264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 2 h with *F. erecta* CH_2Cl_2 fractions (CFE; 0, 5, 10 and 20 μg/mL). Cells were then stimulated with LPS (1 μg/mL) for 24 h. The LPS-induced changes in the mRNA levels of iNOS and COX-2 were determined by PCR. β-actin was used as a loading control. Data are mean ± standard deviation (SD). The value of the LPS alone group was set at 1, and results were expressed as a ratio relative to the LPS alone group. $^{*}P<0.05$ and $^{**}P<0.01$ (test fraction vs. the LPS alone group). LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2

PGE_2, the main product of COX-2, has an established role in the sensitization of nociceptors, and its production is regulated by COX-2. As shown in Figure 4A, LPS stimulation led to an increase in PGE_2 production and release. However, this increase of PGE_2 production was significantly inhibited in the presence of 50 μg/mL and 100 μg/mL of CFE. This result may be associated with the inhibitory effect of CFE on COX-2 expression (Figure 2 and Figure 3).

3.3. Inhibitory Effects of *F. erecta* Extracts on LPS-mediated Production of Pro-inflammatory Cytokines in Raw264.7 Cells

Inflammation and pain, which are signs of inflammation, are associated with various pathophysiological conditions, such as arthritis, cancer, and cardiovascular disease. Activated macrophages secrete TNF-α, IL-1β, IL-6, as well as macrophage-derived NO and PGE_2, near the sites of injury [18,22] and thereby amplify the inflammatory response. Therefore, inhibition of inflammatory mediators may be a useful strategy in not only the treatment of inflammatory diseases, for instance septic shock, but also for pain that is sustained in the absence of any peripheral noxious stimuli after inflammation [8]. TNF-α, IL-1β, and IL-6 are well known pro-inflammatory cytokines [18]. During LPS stimulation, pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were highly expressed. As shown in Figure 4, LPS stimulation significantly increased production and release of TNF-α, IL-1β, and IL-6; and these increases were mediated by upregulation of TNF-α, IL-1β, and IL-6 mRNA expression (Figure 5). Although the addition of CFE (50 μg/mL and 100 μg/mL) strongly suppressed the protein levels of TNF-α, IL-1β, and IL-6 in a dose-dependent manner (Figure 4); mRNA expression levels of TNF-α, IL-1β, and IL-6 were dramatically suppressed at even lower doses of CFE (10 μg/mL and 20 μg/mL) (Figure 5). This discrepancy may be related to the sensitivity of the applied methods or to some other unknown reason.

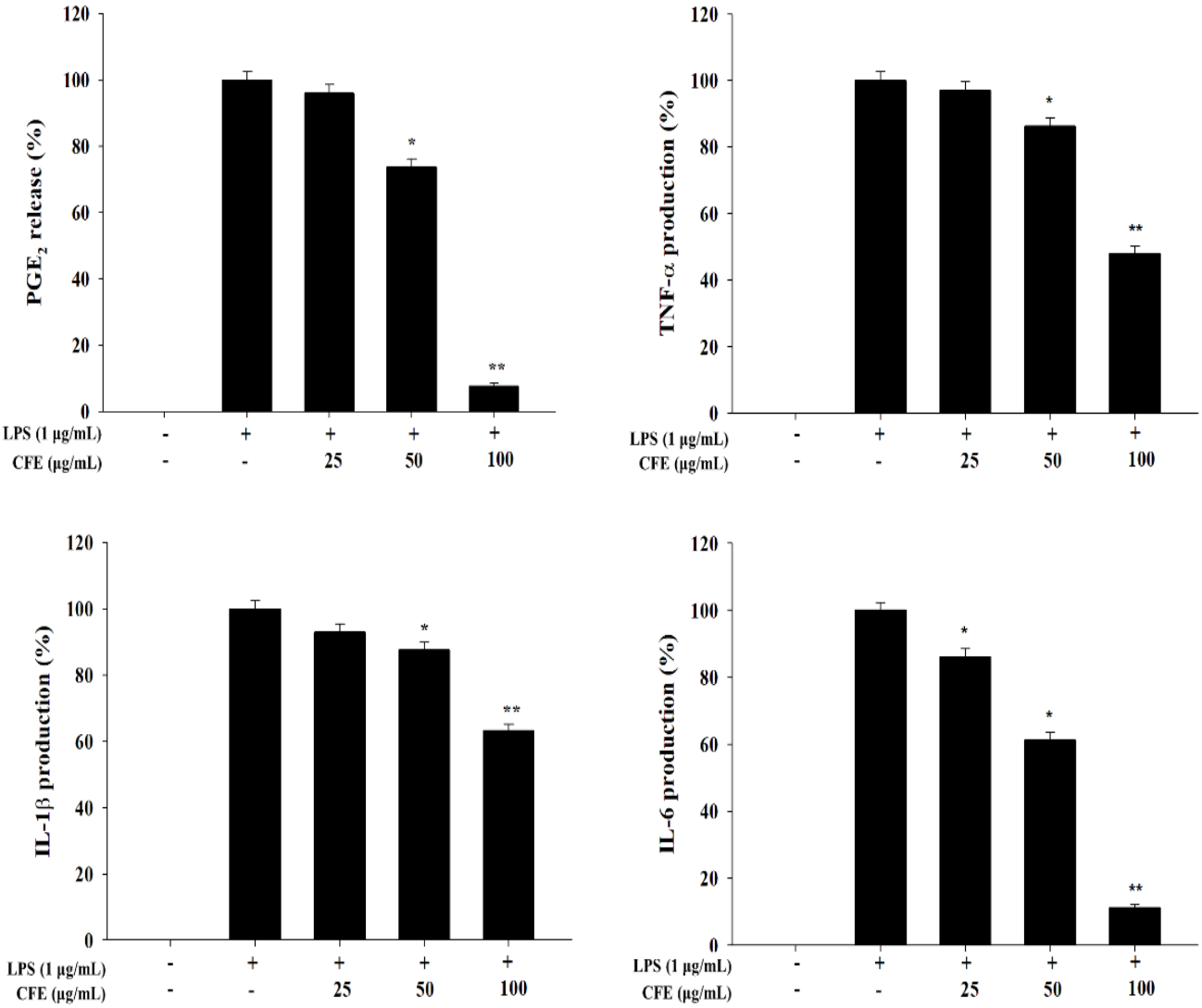

Figure 4. Inhibitory effects of CFE administration on production of PGE_2, TNF-α, IL-1β, and IL-6 in Raw264.7 cells. Raw264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 2 h with *F. erecta* CH_2Cl_2 fractions (CFE; 0, 25, 50, and 100 μg/mL). Cells were stimulated with LPS (1 μg/mL) for 24 h. LPS-induced production/release of PGE_2 (A), TNF-α (B), IL-1β (C), and IL-6 (D) were determined by ELISA. Data are mean ± standard deviation (SD). The value of the LPS alone group was set at 1, and results were expressed as a ratio relative to the LPS alone group. $^*P<0.05$ and $^{**}P<0.01$ (test fraction vs. the LPS alone group). LPS, lipopolysaccharide; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6

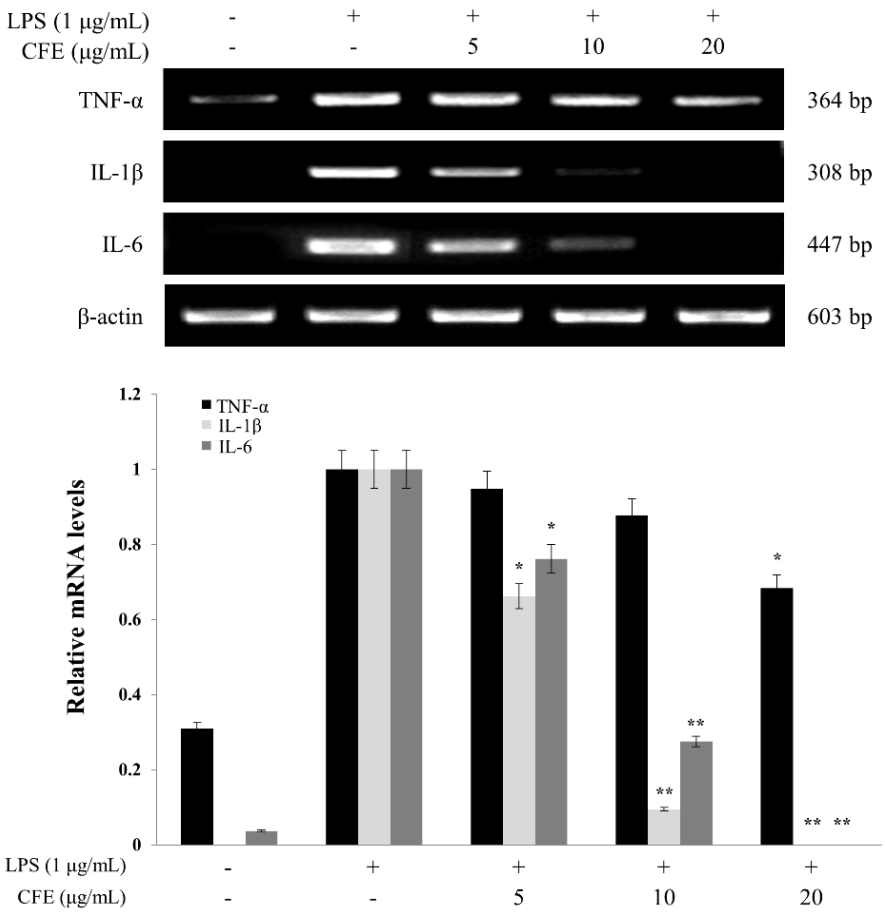

Figure 5. Inhibitory effects of CFE administration on mRNA expression of TNF-α, IL-1β, and IL-6 in Raw264.7 cells. Raw264.7 cells (1.0×10^6 cells/mL) were pre-incubated for 2 h with *F. erecta* CH_2Cl_2 fractions (CFE; 0, 5, 10, and 20 μg/mL). Cells were then stimulated with LPS (1 μg/mL) for 24 h. The LPS-mediated changes in mRNA levels of TNF-α, IL-1β, and IL-6 were determined by PCR. Data are mean ± standard deviation (SD). The value of the LPS alone group was set at 1, and results were expressed as a ratio relative to the LPS alone group. $^{*}P<0.05$ and $^{**}P<0.01$ (test fraction vs. the LPS alone group). LPS, lipopolysaccharide; TNF-α, tumor necrosis factor- α; IL-1β, interleukin-1β; IL-6, interleukin-6

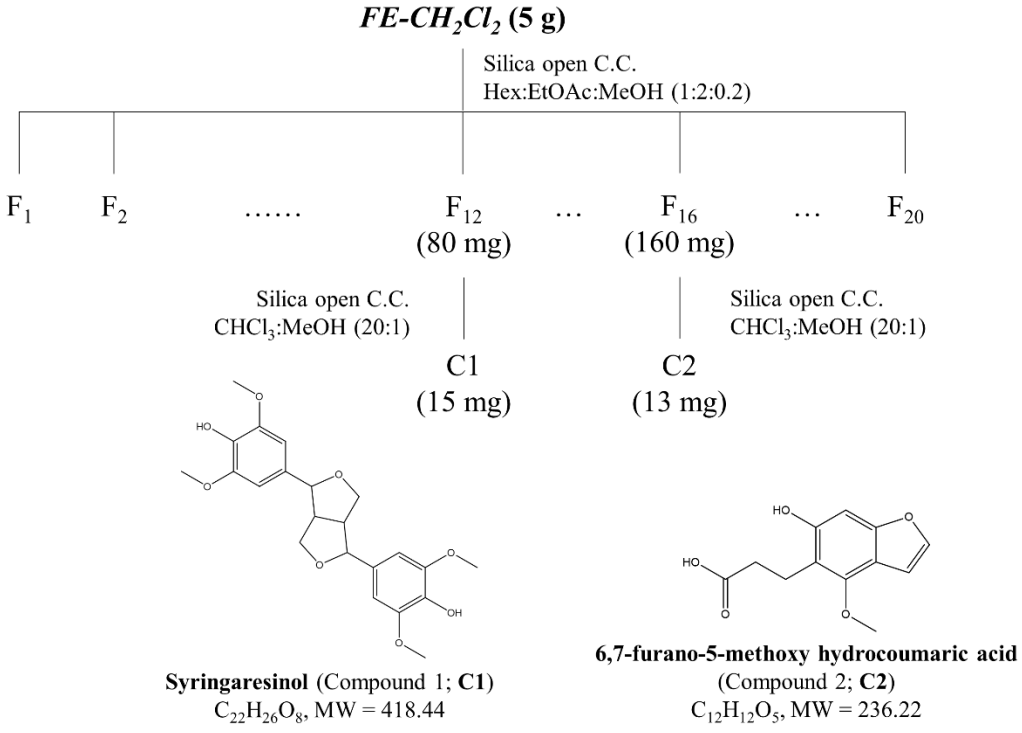

Figure 6. Outline of the isolation scheme for the *F. erecta* CH_2Cl_2 fraction. C1 and **C2** were identified as syringaresinol (molecular weight = 418.44) and 6, 7-furano-5-methoxy hydrocoumaric acid (molecular weight = 236.22), respectively

Table 3. Inhibitory effect of the *F. erecta* CH_2Cl_2 fraction and sub-fractions on LPS-mediated production of PGE_2 in Raw264.7 cells

	LPS (1 μg/mL)			
Extract / dose (μg/mL)	0	25	50	100
CFE	100 ± 1.98	70.4 ± 2.15*	49.8 ± 2.05**	15.4 ± 1.34**
F_{12}	100 ± 2.34	64.8 ± 1.78*	43.8 ± 2.31**	12.8 ± 1.97**
F_{16}	100 ± 2.14	63.0 ± 1.39*	41.3 ± 1.79**	11.9 ± 1.38**
C**1**	100 ± 1.67	73.9 ± 1.57*	46.4 ± 1.63**	16.8 ± 2.07**
C**2**	100 ± 2.45	77.4 ± 2.04*	57.1 ± 1.36*	20.7 ± 2.25**

Raw264.7 cells were pretreated with extracts, **C1** (syringaresinol) or **C2** (6,7-furano-5-methoxy hydrocoumaric acid) for 2 h and then treated with LPS for 24 h. The production of PGE_2 was determined by enzyme-linked immunosorbent assay (ELISA). Data are mean ± standard deviation (SD). The value of the LPS alone group was set at 100, and results were expressed as a percentage of that value. *P<0.05 and **P<0.01 (test fraction vs. the LPS alone group). LPS; lipopolysaccharide.

3.4. Inhibitory Effects of Compounds in *F. erecta* Extracts on LPS-mediated Production of Inflammatory Mediators in Raw264.7 Cells

We have determined the anti-inflammatory efficacy of CFE through previous experiments. In order to identify the presence of a key compound with anti-inflammatory activity within the extract, the CFE was subfractionated with Silica Open C.C. and obtained from F_1 to F_{20} (Figure 6). The F_{12} and F_{16} subfractions were able to inhibit the production of PGE_2. The structures of the two compounds isolated were identified using ^{1}H-NMR and ^{13}C-NMR spectroscopy. The isolated compounds that make up these subfractions were identified as syningaresinol (compound 1; **C1**, MW=148.44, from F_{12}) and 6,7-Furano-5-methoxy Hydrocoumaric acid (compound 2; **C2**, MW=236.22, from F_{16}).

Syringaresinol (**C1**): ^{1}H-NMR (CDCl3, 500 MHz) δH: 6.57 (4H, s, H-2,6,2′,6′), 5.53 (2H, br, OH-4,4′), 4.72 (2H, d, J = 5.15 Hz, H-7,7′), 4.27 (2H, m, H-9e,9′e), 3.90 (2H, m, H-9a,9′a), 3.89 (3H, s, MeO-4), 3.10 (2H, m, H-8,8′). ^{13}C-NMR (CDCl3, 125 MHz) δC: 147.04 (C-3,5,3′,5′), 134.09 (C-4,4′), 131.95 (C-1,1′), 102.49(C-2,6,2′,6′), 86.01 (C-7,7′), 71.73 (C-9,9′), 56.47 (OMe), 54.26 (C-8,8′).

6,7-Furano-5-methoxy Hydrocoumaric acid (**C2**): ^{1}H-NMR (DMSO, 500 MHz) δH : 7.69 (1H, s, H-9), 7.00 (1H, s, H-10), 6.69 (1H, s, H-8), 3.99 (3H, s, 5-OCH3), 2.83-2.80 (2H, m, H-4), 2.34-2.31 (2H, m, H-3) ^{13}C-NMR (DMSO, 125 MHz) δC: 174.3 (C-2) 154.9 (C-7), 153.8 (C-8a), 151.1 (C-5), 142.5 (C-9), 112.9 (C-4a), 109.6 (C-6), 104.9 (C-10), 92.1 (C-8), 59.7(OMe), 33.9(C-3), 19.1(C-4).

As shown in Table 3, F_{12} and F_{16} subfractions strongly suppressed LPS-mediated PGE_2 production. It is unknown why purified **C1** and **C2** showed slightly less inhibitory activity against LPS-mediated PGE_2; however, we suspect that it may be due to the synergistic effect of the substances contained in the solvent. In a previous study, **C1** from EtOAc extracts of Acanthopanax senticosus stem bark was shown to have *in vivo* anti-inflammatory and anti-nociceptive effects [23]. **C1** isolated from *F. erecta* also had inhibitory effects on LPS-mediated PGE_2 production. Thus, **C1** is proposed to be a natural inhibitory compound of PGE_2, which is possibly mediated by induction of COX-2 expression. Furthermore, it was found that **C2**, which is one of the naturally occurring coumarins, also showed an inhibitory effect on LPS-mediated production of PGE_2.

4. Conclusion

In conclusion, LPS potently induced pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 and inflammatory mediators NO (including iNOS) and PGE_2. However, administration of CFE suppressed LPS-mediated inflammatory responses and might be helpful in treating inflammatory complications or serving as an anti-nociceptive agent. Especially, subfractions **C1** and **C2** of CFE were potent inhibitors of LPS-mediated overproduction of PGE_2 and thus could serve as possible starter compounds in the development of anti-inflammatory and anti-nociceptive drugs.

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References

[1] Lawrence, T. – Fong, C: The resolution of inflammation: anti-inflammatory roles for NF-κB. International Journal of Biochemistry & Cell Biology, *42*, 2010, pp. 519-523.

[2] Bellik, Y. – Hmmoudi, S. M. – Abdellah, F. – Iguer-Ouada, M. – Boukraa, L.: Phytochemicals to prevent inflammation and allergy. Recent Patents on Inflammation & Allergy Drug Discovery, *6*, 2012, pp. 147-158.

[3] Hsing, C. H. – Wang, J. J.: Clinical implication of perioperative inflammatory cytokine alteration. Acta Anaesthesiol Taiwanica, *53*, 2015, pp. 23-28.

[4] Arnold, K. M. – Opdenaker, L. M. – Flynn, D. – Sims-Mourtada, J.: Wound healing and cancer stem cells: inflammation as a driver of treatment resistance in breast cancer. Cancer Growth & Metastasis, *8*, 2015, pp. 1-13.

[5] Solov'eva, T. – Davydova, V. – Lrasikova, I. – Yermak, I.: Marine compounds with therapeutic potential in gram-negative sepsis. Marine Drugs, *11*, 2013, pp. 2216-2229.

[6] Martich, G. D. – Boujoukos, A. J. – Suffredini, A. F.: Response of man to endotoxin. Immunology, *187*, 1993, pp. 403-146.

[7] Cani, P. D. – Osto, M. – Geurts, L. – Everard, A.: Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut Microbes, *3*, 2012, pp. 279-288.

[8] Shih, M. F. – Chen, L. Y. – Tsai, P. J. – Cherng J. Y.: In vitro and in vivo therapeutics of beta-thujaplicin on LPS-induced

inflammation in macrophages and septic shock in mice. International Journal of Immunopathology & Pharmacology, *25*, 2012, pp. 39-48.

[9] Lawrence, T.: The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harbor Perspectives in Biology [online] , *1*(6), 7 October 2009, [cit. 2 May 2018].

[10] Sen, R. – Smale, S. T.: Selectivity of the NF-{kappa}B response. Cold Spring Harbor Perspeictives in Biology [online], *2*(4), 14 October 2009. [cit. 1 May 2018].

[11] Bonizzi, G. – Karin, M.: The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trands in Immunology, *25*, 2004, pp. 280-288.

[12] Yoon, W. J. – Lee, H. J. – Kang, G. J. – Kang, H. K. – Yoo, E. S.: Inhibitory effects of Ficus erecta leaves on osteoporotic factors in vitro. Archieves of Pharmacal Research, *30*, 2007, pp. 43-49.

[13] Park, S.H. – Oh, T. H. – Kim, S. S. – Kim, J. E. – Lee, S. J. – Lee, N.H.: Constituents with tyrosinase inhibitory activities from branches of Ficus erecta var, sieboldii King. Journal of Enzyme Inhibition & Medicinal Chemistry, *27*, 2012, pp. 390-394.

[14] Lim, J. D. – Lee, S. R. – Kim, T. – Jang, S. A. – Kang, S. C. – Koo, H. J. – Sohn, E. – Bak, J. P. – Namkoong, S. – Kim, H.K. – Song, I. S. – Kim, N. – Sohn, E. H. – Han, J.: Fucoidan from Fucus vesiculosus protects against alcohol-induced liver damage by modulating inflammatory mediators in mice and HepG2 cells. Marine Drugs, *13*, 2015, pp. 1051-1067.

[15] Koo, H. J. – Yoon, W. J. – Sohn, E. H. – Ham, Y. M. – Jang, S. A. – Kwon, J. E. – Jeong, Y. J. – Kwak, J. H. – Sohn, E. – Park, S. Y. – Jang, K. H. – Namkoong, S. – Han, H. S. – Jung, Y. H. – Kang, S. C.: The analgesic and anti-inflammatory effects of Litsea japonica fruit are mediated via suppression of NF-kappaB and JNK/p38 MAPK activation. International Immunopharmacology, *22*, 2014, pp. 84-97.

[16] Livak, K. J. – Schmittgen, T. D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, *25*, 2001, pp. 402-408.

[17] Li, X. – Ellman, M. – Muddasani, P. – Wang, J. H. C. – Cs-Szabo, G. – van Wijnen, A. J. – Im, H. -J.: Prostaglandin E2 and it cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis, Arthritis & Rheumatism, *60*, 2009, pp. 513-523.

[18] Blatteis, C. M. – Li, S. – Li, Z – Feleder, C. – Perlik, V.: Cytokines. PGE2 and endotoxic fever: a re-assessment. Prostaglandins and Other Lipid Mediators, *76*, 2005, pp. 1-18.

[19] Swierkosz, T. A. – Mitchell, J. A. – Warner, T. D. – Botting, R. M. – Vane, J. R.: Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. British Journal of Pharmacology, *114*, 1995, pp. 1335-1342.

[20] Kim, J. B. – Han, A. R. – Park, E. Y. – Kim, J. Y. – Cho, W. – Lee, J. – Seo, E. K. – Lee, T. K.: Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. Biological & Pharmaceutical bulletin, *30*, 2007, pp. 2345-2351.

[21] Chen, C. C.: Signal transduction pathways of inflammatory gene expressions and therapeutic implications. Current Pharmaceutical Design, *12*, 2006, pp. 3497-3508.

[22] Lawrence, T. – Willoughby, D. A. – Gilroy, D. W.: Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nature Reviews. Immunology. *2*, 2002, pp. 787-795.

[23] Jung, H. J. – Park, H. J. – Kim, R. G. – Shin, K. M. – Ha, J. – Choi, J. W. – Kim, H. J. – Lee, Y. S. – Lee, K. T.: In vivo anti-inflammatory and antinociceptive effects of liriodendrin isolated from the stem bark of Acanthopanax senticosus. Planta Medica, *69*, 2003, pp. 610-616.