Inhibitory Effect of *Farfarae Flos* Water Extract on COX-2, iNOS Expression and Nitric Oxide Production in lipopolysaccharide - activated RAW 264.7 cells

Tae Gyoung Yoon, Boo Hyeong Byun, Teag Kyu Kwon[1], Seong Il Suh[1], Sung Hui Byun, Young Kyu Kwon, Sang Chan Kim*

College of Oriental Medicine, Deagu Hanny University, Daegu, 1:College of Medicine, Keimyung University, Daegu

Farfrae Flos has been clinically used for the treatment of asthma in traditional oriental medicine. There is lack of studies regarding the effects of Farfrae Flos on the immunological activities. The present study was conducted to evaluate the effect of Farfrae Flos on the regulatory mechanism of cytokines and nitric oxide (NO) for the immunological activities in Raw 264.7 cells. In Raw 264.7 cells stimulated with lipopolysaccharide (LPS) to mimic inflammation, Farfrae Flos water extract inhibited nitric oxide production in a dose-dependent manner and abrogated inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2). Farfrae Flos water extract did not affect on cell viability. To investigate the mechanism by which Farfrae Flos water extract inhibits iNOS and COX-2 gene expression, we examined the on the phospholylation of inhibitor κBα and production of TNF-α, IL-1β and IL-6. Results provided evidence that Farfrae Flos inhibited the production of interleukin-1β (IL-1β) and the activation of phospholylation of inhibitor κBα in Raw 264.7 cells activated with LPS. These findings suggest that Farfrae Flos can produce anti-inflammatory effect, which may play a role in adjunctive therapy in Gram-negative bacterial infections.

Key words : *Farfrae Flos*, iNOS, COX-2, TNF-α, IL-1β, IL-6

Introduction

Coltsfoot was traditionally used as an anti-tussive cough medication, which explains its botanical name Tussilago (From *tussis, coughing; ago, to chase*) and its *common name*, cough-wort. The flower buds of Tussilago farfara L (Compositae), called " farfarae flos", have been widely used for the treatment of coughs, bronchitis and asthmatic disorders in traditional medicine[1].

According to the literature, the therapeutic effects of many oriental herbs are attributable to the phenolic substances[2]. Indeed, certain phenolic compound producing plants have often used as herbal anti-inflammatory remedies. Cyclooxygenase - 2(COX-2) inducible and nitric oxide synthase (iNOS) are important enzymes that mediate inflammatory process[3].

Improper up-regulation of COX-2 and/or iNOS has been associated with pathophysiology of certain types of inflammatory disorders. Since inflammation is closely linked to substances with potent anti-inflammatory activities are anticipated to exert chemopreventive effects on cacinogenesis, particularly in the promotion stage. Examples are resveratrol grapes, the green tea polyphenol epigallocathechin gallate (EGCG) that strongly suppress inflammation. Recent studies have demonstrated that nuclear factor-kappa B (NF-κB) is involved in regulation of COX-2 and iNOS expression. However, there is lack of studies regarding the effects of Farfrae Flos on the immunological activities. This experimental study was carried out to evaluate the effects of Farfrae Flos on the regulatory mechanism of cytokines and nitric oxide (NO) for the immunological activities in Raw 264.7 cells.

* To whom correspondence should be addressed at : Sang Chan Kim, College of Oriental Medicine, Daegu Hanny University, Daegu, 706-086 Korea.
· E-mail : sckim@dhu.ac.kr · Tel : 053-770-2247
· Received : 2004/03/19 · Revised : 2004/04/23 · Accepted : 2004/05/30

Experimental procedures

1. Extract of Farfrae Flos

Farfrae Flos extract (FFE) was prepared by boiling F. Flos (Wolsung, Daegu, Korea) in water (300 g in 9 L) for 3 h. The FFE was filtered through a 0.2μ m filter (Nalgene, New York, NY, USA) and stored at -20℃ until use. The amount of FFE was estimated by the dried weight of lyophilized water extract of F. Flos. The yield of lyophilized FFE from F. Flos was 13.6%.

2. Cell culture

Raw 264.7 cells, a murine macrophage cell line (KCLRF, Korean Cell Line Research Foundation, Seoul, Korea), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin. Raw 264.7 cells were plated at a density of $2\sim3\times10^6$/ml and preincubated for 24 h at 37℃. Cells were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. For all experiments, cells were grown to 80~ 90% confluency, and were subjected to no more than 20 cell passages. Raw264.7 cells were incubated in the medium without 10% FBS for 12h and then exposed to LPS or LPS+FFE for the indicated time periods (6-24 h). FFE as dissolved in medium (EMEM) was added to the incubation medium 1 h prior to the addition of LPS.

3. Reagents

LPS (Escherichia coli 026:B6; Difco, Detroit, MI, U.S.A.) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum (MTT) were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum(FBS) and antibiotics were purchased from Gibco/BRL (Eggenstein, Germany). Antibodies were obtained from BD Bioscience (USA), Cayman (USA) and Zymed (USA), NC paper was Schleicher & Schuell (USA). TNF-α, IL-1β and IL-6 ELISA Kits were purchased from Pierce endogen (Rockford, IL, USA).

4. Cell viability

MTT cell viability assay RAW 264.7 cells were plated at a density of 5×10^4 cells/well in a 96 well plate to determine cytotoxic concentrations of FFE. Cells were exposed to FFE at the concentrations of 0.1 through 3 mg/ml at 37℃ under 5% CO_2. After incubation of the cells in the presence of FFE, viable cells were stained with MTT (0.5 mg/ml) for 4 h. The media were then removed and produced formazan crystals in the wells were dissolved by addition of 200 μl of dimethylsulfoxide (DMSO). Absorbance was measured at 540 nm using a Titertek Multiskan Automatic ELISA microplate reader (Model MCC/340, Huntsville, AL). Cell viability was defined relative to untreated control cells [i.e. viability (% control) = 100×/(absorbance of treated sample)/(absorbance of control)].

5. Assay of nitrite production

NO production was monitored by measuring the nitrite content in culture medium. This was performed by mixing the samples with Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid). Absorbance was measured at 540 nM after incubation for 10 min.

6. Immunoblot analysis

Cells were lysed in the buffer containing 20mM Tris Cl (pH 7.5), 1% Triton X-100, 137mM sodium chloride, 10% glycerol, 2mM EDTA, 1mM sodium orthovanadate, 25mM b-glycerophosphate, 2mM sodium pyrophosphate, 1mM phenylmethylsulfonylfluoride and 1 mg/ml leupeptin. Cell lysates were centrifuged at 10,000×g for 10 min to remove debris. Expression of iNOS and COX-2 was immunochemically monitored in the lysate fraction of Raw264.7 cells using antimouse iNOS and COX-2 antibodies, respectively. Polyclonal anti p-I-kBα antibody was used to assess p-I-kBα protein in cytosol.

The secondary antibodies were alkaline phosphatase conjugated anti-mouse and anti-goat antibodies. The bands of iNOS and COX-2 proteins were visualized using 5-bromo-4-chloro-3-indolylphosphate and 4-nitroblue tetrazolium chloride, or ECL western blotting detection reagents (Amersham) according to the manufacturer's instruction.

7. Measurement of cytokine production

For cytokine immunoassays, the cells (1×106/ml) were pre-incubated 1 h with FFE and further cultured 6 h or 12 h with 1 μg/ml of LPS in 6-well plates. Supernatants were removed at the allotted times and TNF-α, IL-1β, and IL-6 productions were quantified by ELISA Kit (Pierce endogen) according to the manufacturer's instructions (Rockford, IL, USA), respectively. Each kit is specific for either TNFα, IL-1β, or IL-6 and does not measure other cytokines.

8. Statistical evaluations

Data are expressed as mean ± S.D. of results obtained from number (n) of experiments. One-way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman - Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $P<0.05$.

Results

1. Inhibition of LPS-stimulated NO production

To investigate the inhibition of NO production by FFE in Raw 264.7 cells, we measured NO production in Raw 264.7 cells treated with the concentration of 0.1-3.0 mg/ml FFE. As shown in Fig.1, in LPS plus FFE groups, NO production was decreased in a concentration dependent manner as compared with LPS group. In 0.3 mg/ml of FFE group, NO production was significantly inhibited at 18h and 24h. In 1.0 mg/ml or 3.0

mg/ml of FFE group, showed the significant inhibition of NO production at 12h, 18h and 24h (Fig. 1).

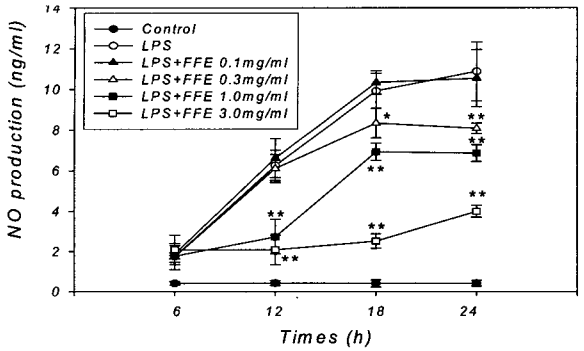

Fig. 1. Effects of FFE on the production of NO in LPS stimulated Raw264.7 cells. Raw264.7 cells were treated with various concentrations of FFE dissolved in EMEM for 1 h prior to the addition of LPS (1 μg/ml), and the cells were further incubated for 6-24 h. Control cells were incubated with vehicle alone. The concentrations of nitrite and nitrate in culture medium were monitored as described in the Experimental procedures. Data represent the mean ± S.D. with eight separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman - Keuls test (significant as compared to LPS alone, *P < 0.05, **P < 0.01).

2. Effects of FFE on cell viability in Raw264.7 cells.

To investigate whether the reduction of NO production is contributed to the decrease of cell population by FFE induced cytotoxicity, we measured cell viability by MTT assay at the degree of concentration and at the time manner. The results suggested that our used dosage of FFE did not exhibit any cell toxicity during 6-24h (Fig. 2).

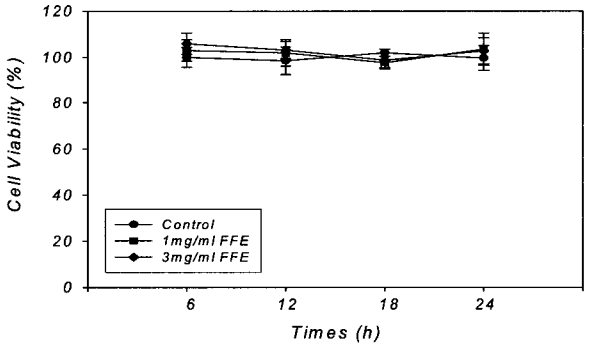

Fig. 2. The cell viability was measured after indicated time. Each bar shows the mean±S.D. of three independent experiments performed in triplicate.

3. Inhibition of LPS-stimulated iNOS expression

We examined iNOS protein expression in cytosol fraction using immunoblotting analysis. iNOS protein strongly induced by LPS. The groups of 1.0 and 3.0mg/ml of FFE with LPS showed the reduction of iNOS protein expression at the concentration dependent manner. In the group of 1.0mg/ml or 3.0mg/ml of FFE alone, iNOS protein expression was not monitored (Fig. 3).

4. Inhibition of LPS-stimulated COX-2 expression

COX-2 is a key enzyme catalyzing the production of prostaglandin in response to prooxidant and proinflammatory stimuli. COX-2 plays a main role in the development of inflammation[4,5]. We next investigated whether FFE might affect the levels of COX-2 and p-IκBα expression. As shown in this experiment, 1.0mg/ml of FFE with LPS slightly suppressed the induction of COX-2 and 3.0mg/ml of FFE with LPS strongly suppressed the induction of COX-2. However, In the group of 1.0mg/ml or 3.0mg/ml of FFE alone, COX-2 protein expression was not monitored (Fig. 4).

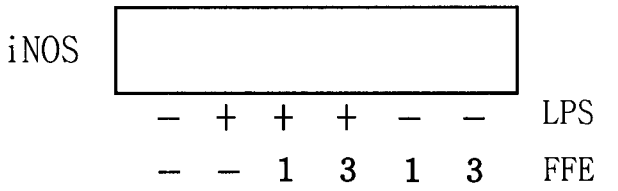

Fig. 3. Effect of FFE on the induction of iNOS by LPS. Inhibition of LPS-stimulated iNOS protein expression by FFE. The level of iNOS protein was monitored 18h after treatment of cells with LPS (1μg/ml) with or without FFE pretreatment (i.e. 1h before LPS)

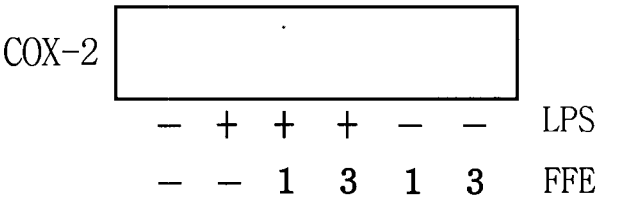

Fig. 4. Effect of FFE on the induction of COX-2 by LPS. Inhibition of LPS-stimulated COX-2 protein expression by FFE. The level of COX-2 protein was monitored 18h after treatment of cells with LPS (1μg/ml) with or without FFE pretreatment (i.e. 1h before LPS)

5. Effects of LPS-stimulated p-IκBα expression

We measured the phosphorylated form of IκBα. To assess whether FFE could directly affect p-IκBα expression in macrophage cell, the level of p-IκBα protein expression was immunochemically assessed in Raw264.7 cells incubated with or without FFE. LPS induced the p-IκBα level, however, 1mg/ml of FFE reduced LPS-inducible p-IκBα expression, and 3mg/ml of FFE markedly reduced the protein levels of p-IκBα expression in a dose dependent manner (Fig. 5).

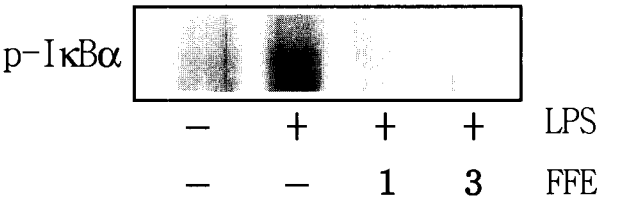

Fig. 5. Effect of FFE on the induction of p-IκBα by LPS. Inhibition of LPS-stimulated p-IκBα protein expression by FFE. The level of p-IκBα protein was monitored 15min after treatment of cells with LPS (1μg/ml) with or without FFE pretreatment (i.e. 1h before LPS)

6. Inhibitotory effects of FFE on LPS-stimulated TNF-α production

Tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1 β), interleukin-2(IL-2), and interleukin-6(IL-6) are frequently

encountered proinflammatory cytokines. These cytokines are involved in a variety of immunological functions as well as interaction with a variety of target cells[6]. As shown Fig.6, LPS affected the TNF-α production, however 1.0 or 3.0mg/ml of FFE did not affect the levels of TNF-α production (Fig. 6).

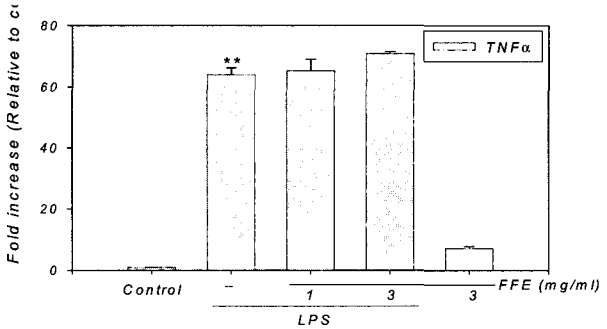

Fig. 6. The effect of FFE on LPS-stimulated TNF-α production. Production of TNF-α was measured in the medium of Raw264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of FFE for 6 h. The amount of TNFα was measured by immunoassay as described in Experimental procedures. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman - Keuls test (*: significant as compared to control, **P < 0.01)

7. Inhibitotory effects of FFE on LPS-stimulated IL-1β production

Interleukin-1 (IL-1) exhibits proinflammatory effects, especially by increasing synthesis of potent mediators and by up-regulating the expression of adhesion moleculeson leukocytes and endothelial cells[7]. IL-1β co-stimulates activation of T-cells, promotes maturation of B-cells, enhances NK activity, increases adhesion molecules expression, and acts as a chemotactic attractant. Cells that have been shown to secrete IL-1β are monocytes, macrophages, B-cells, dendritic cells, endothelial cells, neutrophils, and hepatocytes[6]. We determined whether FFE inhibited the production of IL-1β by LPS. This data suggested that LPS significantly increased the level of IL-1β production as compared to that of control. 1.0mg/ml and 3.0 mg/ml of FFE significantly inhibited LPS-inducible IL-1β production (Fig. 7).

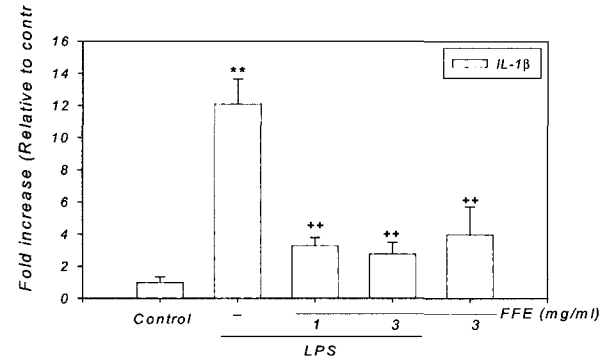

Fig. 7. The effect of FFE on LPS-stimulated IL-1β production. Production of IL-1β was measured in the medium of Raw264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of FFE for 12 h. The amount of IL-1β was measured by immunoassay as described in Experimental procedures. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman - Keuls test (*: significant as compared to control, **P < 0.01, +: significant as compared to LPS alone, + +P < 0.01).

8. Inhibitotory effects of FFE on LPS-stimulated IL-6 production

IL-6, cytokine secreted primarily by monocytes and macrophages, is always found in increased levels at the sites of inflammation6). Then we measured the effect of FFE on LPS-inducible IL-6 production. In this experiment, LPS significantly increased the secretion of IL-6, but 1.0 and 3.0mg/ml of FFE did not changed IL-6 production (Fig. 8).

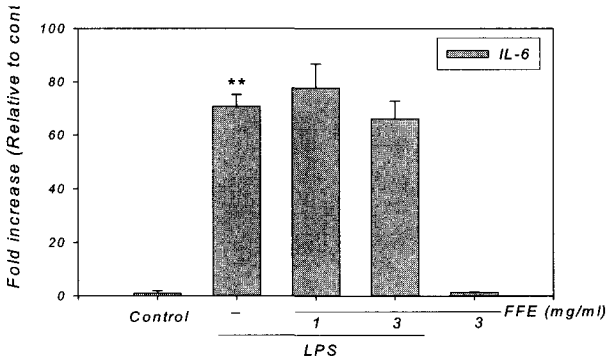

Fig. 8. The effect of FFE on LPS-stimulated IL-6 production. Production of IL-6 was measured in the medium of Raw264.7 cells cultured with LPS (1 μg/ml) in the presence or absence of FFE for 6 h. The amount of IL-6 was measured by immunoassay as described in Experimental procedures. Data represent the mean ± S.D. with three separate experiments. One-way ANOVA was used for comparisons of multiple group means followed by Newman - Keuls test (*: significant as compared to control, **P < 0.01)

Discussion

Coltsfoot was traditionally used as an anti-tussive cough medication, which explains its botanical name Tussilago (From coughing, coughing;ago, to chase) and its common name, cough-wort. It is similar to Petasites vulgaris, butterbur, which can be a dangerous toxic contaminant in coltsfoot products. Both the flowers and the leaves of coltsfoot are gathered for herbal use. For example, oral preparations were advocated for use in cough and various respiratory diseases, and the smoke from burning coltsfoot leaves or from herbal cigarettes was also used for treating asthma and bronchitis[1]. However, coltsfoot should be avoided by patients with liver disease and by those ingesting potentially hepatoxic drugs and/or alcohol. Occasional concerns arise about the allergenicity and carcinogenicity of coltsfoot, but these claims have not been substantiated in humans[8].

Nitric oxide (NO) is a radical produced from L-arginine via nitric oxide synthase (NOS), and also serves as an important cellular second messenger9). NO plays a dual role as a beneficial or detrimental molecule in the inflammatory process. Inducible NOS (iNOS) produces a high output of NO during inflammation, whereas constitutively expressed NOS (cNOS) generates a physiologically active low level of NO[10].

Farfrae Flos is known to have anti-asthma effects. Here, we demonstrate that Farfrae Flos extract inhibits production of

NO and IL-1β, expression of iNOS and COX-2 in LPS-stimulated cultured macrophages, and that these effects are mediated through the inhibition phosphorylation of p-IκBα. To investigate the relation of iNOS and NO production, we examined iNOS protein expression by using immunobloting analysis. iNOS protein strongly induced by LPS. The groups of 1.0 and 3.0mg/ml of FFE with LPS showed the reduction of iNOS protein expression at the concentration dependent manner.

COX-2 is a key enzyme catalyzing the production of prostaglandin in response to prooxidant and proimflammatory stimuli (i.e., TPA, LPS, TNF-, ROI, etc). COX-2 through NF-κB activation plays a main role in the development of inflammation[4,5]. We next investigated whether FFE might affect the levels of COX-2 and p-IκBα expression. As shown in this experiment, 1.0 and 3.0mg/ml of FFE suppressed the induction of COX-2 at the concentration dependent manner. NFκB, AP-1 and C/EBP have been well defined, associated with iNOS and COX-2 expression. Among these, the NFκB is a functional transcriptional factor[11]. NFκB is involved in the inhibition of cell apoptosis, cell cycle regulation and oncogenesis[12]. The NF-κB plays an important role in the regulation of immune response, inflammation. A wide stimuli including toxic materials, virus and bacterial infection activate NF-κB. Activated NF-κB translocates into nucleus where it modulates the expression of iNOS or TNF-α[13]. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form through its association with one of several inhibitory molecules, including IκBα, IκBβ, IκBε, p105, p100. Activation of the NF-κB signaling cascade results in a complete degradation of IκB or partial degradation of the carboxyl termini of p105 and p100 precursors, allowing the translocation of NF-κB to the nucleus, where it induces transcription including COX-2, iNOS, Bcl-xl, cIAPs. The kinds of IκB protein have been known to IκBα, IκBβ, IκBε, among which IκBα is the most abudant inhibitory protein for NF-κB[12]. In this study, LPS induced the p-IκBα level, however, 1mg/ml of FFE reduced LPS-inducible p-IκBα expression, and 3mg/ml of FFE markedly reduced the protein levels of p-IκBα expression in a dose dependent manner.

Tumor Necrosis Factor-alpha (TNF-α), Interleukin1-β (IL-1 β), Interleukin-2(IL-2), and Interleukin-6(IL-6) are frequently encountered proinflammatory cytokines. These cytokines are involved in a variety of immunological functions as well as interaction with a variety of target cells[6]. As shown Fig.6, LPS affect the TNF-α and IL-6 production, however 1 or 3mg/ml of FFE did not affect the levels of TNF-α and IL-6 production. Interleukin-1 (IL-1), a cytokine that is produced primarily by monocytes and macrophages, is considered to play important roles in the pathogenesis of a variety of surgically relevant conditions such as septic shock[14], burn[15], ischemia-reperfusion injury[16] in liver transplantation, and cardiopulmonary bypass[17].

IL-1β co-stimulates activation of T-cells, promotes maturation of B-cells, enhances NK activity, increases adhesion molecules expression, and acts as a chemotactic attractant. Cells that have been shown to secrete IL-1β are monocytes, macrophages, B-cells, dendritic cells, endothelial cells, neutrophils, and hepatocytes[6]. This data suggested that LPS significantly increased the level of IL-1β production as compared to control. 1.0mg/ml and 3.0 mg/ml of FFE significantly inhibited LPS-inducible IL-1β production.

These findings suggest that Farfrae Flos can produce anti-inflammatory effect, which may play a role in adjunctive therapy in Gram-negative bacterial infections.

Conclusion

Based on the above results, FFE was found to inhibit the production of nitrite and nitrate, interleukin-1β (IL-1β) and the activation of phospholylation of IκBα in Raw 264.7 cells activated with lipopolysaccharide (LPS). In addition to, FFE significantly suppressed iNOS and COX-2 expression. These results suggest that FFE could be an attractive candidate for adjunctive therapy associated with the production of proinflammatory cytokines.

Acknowledgments

This study was supported by a grant (02-PJ9-PG1-CO04-0009) of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea.

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