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Anti-inflammatory Effects of the Fruits of *Foeniculum vulgare* in Lipopolysaccharide-stimulated Macrophages

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Foeniculum vulgare has long been prescribed in traditional medicine for the treatment of inflammation diseases. In this study, we aimed to investigate the inhibitory effects of the fruits of *F. vulgare* on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells under non-cytotoxic (100 μg/ml) conditions. The 80% methanol extract was subsequently partitioned successively with hexane, methylene chloride, ethyl acetate, and n-butanol, and the fractions so obtained were also examined for their anti-inflammatory effects. Among them, the hexane, methylene chloride, and ethyl acetate fractions inhibited nitric oxide (NO) and prostaglandin E2 (PGE2) production in LPS stimulated macrophages. The methylene chloride and ethyl acetate fractions also suppressed the productions of interleukin (IL)-1β and IL-6 by down-regulating their mRNA levels in LPS stimulated RAW 264.7 cells. Furthermore, the ethyl acetate fraction strongly suppressed tumor necrosis factor (TNF)-α at the protein and mRNA levels in LPS stimulated RAW 264.7 cells. These observations suggest that the anti-inflammatory actions of *F. vulgare* are due to inhibitions of the productions of NO, PGE2, and pro-inflammatory cytokines.

Key words : Anti-inflammation, apiaceae, *Foeniculum vulgare*, RAW 264.7 cell

Introduction

Inflammatory reactions are physiological defense responses against invading pathogens [1, 2, 3, 4]. In deregulated inflammation reactions, massive cytokine release, by immune cells like monocytes and macrophages, contributes to the pathogenesis of inflammatory reactions [5, 6, 7, 8]. Therefore, compounds that suppress cytokines release may have therapeutic potential for the treatment of inflammatory diseases [9, 10, 11].

Lipopolysaccharide (LPS)-activated macrophages have usually been used to evaluate anti-inflammatory effects [12, 13, 14]. LPS is a principle component of the outer membranes of Gram-negative bacteria and is an endotoxin that induces septic shock syndrome and stimulates the productions of inflammatory mediators, such as, nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukins, and prostaglandin E2 (PGE2), which can cause tissue injury and multiple organ failure [15, 16, 17].

Medicinal plants are considered important sources of new drugs for the prevention and treatment of inflammatory diseases via the suppressions of pro-inflammatory mediators [18, 19, 20, 21]. *Foeniculum vulgare* (Apiaceae) is a highly flavored herb and has long been used as spice and culinary material, and is also prescribed by traditional medical practices in Korea and China for the treatment of digestive disorders [22]. Furthermore, its fruits have been reported to have anti-inflammatory, antioxidant, and antimicrobial effects [23, 24]. Many compounds have been isolated from this plant, such as, monoterpenes, phenylpropanoids, aromatics, and coumarins [25]. Although *F. vulgare* is used as an anti-inflammatory drug in traditional medicine, its biological activity has not been fully explored. The methanol extract and *n*-butanol fraction of this medicinal herb has been reported to have anti-inflammatory effects [23, 26], but the effects on inflammatory cytokine mRNA translation are unknown and no studies have been performed on other fractions.

In view of the small amount known about the anti-inflammatory activity of *F. vulgare*, we evaluated the effects of its methanolic extract and of other fractions on NO levels and PGE2 cytokine levels at the protein and mRNA levels in lipopolysaccharide-stimulated RAW 264.7 macrophages.

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Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and streptomycin-penicillin were purchased from GIBCO (Grand Island, NY, USA). LPS (E. coli 026:B6) was obtained from Sigma (St Louis, MO, USA). 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) and NO detection kits were purchased from Roche Applied Science (Mannheim, Germany) and iNtRON Biotechnology (Seongnam-si, Korea), respectively. IL-1β, IL-6, TNF-α, and PGE2 enzyme linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). First Strand cDNA Synthesis Kit were purchased from Thermo Scientific (Rockford, IL, USA). All PCR primers were designed using Primer 3 (Whitehead Institute/MIT Center for Genome Research) and purchased from Bioneer (Daejeon, Korea) (Table 1). All other chemicals and reagents were of the highest analytical grades commercially available.

Extraction and fractionation

Dried fruits of *F. vulgare* (1.0 kg) were chopped and extracted with hot 80% methanol for 3 hr to obtain crude extract (92.5 g). A portion (90 g) of this extract was then successively partitioned with *n*-hexane, methylene chloride, ethylacetate, and *n*-butanol to afford corresponding fractions at yields of 47.8 g, 1.4 g, 1.6 g, and 5.5 g, respectively.

Cell culture

RAW 264.7 mouse macrophages were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and used for all studies. Cells were cultured in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 ng/ml streptomycin at 37℃ in a 5% CO_2 humidified atmosphere. Cells were made quiescent by starvation after being allowed to grow to 70-80% confluence, and were then treated with experimental reagents. Cells were pretreated with or without 100 μg/ml of each extract for 1 hr, and then stimulated with or without 1 μg/ml of LPS for the indicated times.

Table. 1. Sequences of PCR primers of the genes investigated

Primer name		Sequence (5′ to 3′)
IL-1β	F.	ccgtggaccttccaggatga
	R.	gatccacactctccagctgc
IL-6	F.	agaggagacttcacagagga
	R.	atctctctgaaggactctgg
TNF-α	F.	tggaactggcagaagaggca
	R.	tttgagatccatgccgttgg
iNOS	F.	gacaagctgcatgtgacatc
	R.	gctggtaggttcctgttgtt
COX-2	F.	tgggtgaagtgctgggcaaa
	R.	tgagcccaccccaaacaca
GAPDH	F.	ccatggagaaggctgggg
	R.	caaagttgtcatggatgacc

Cell viability assay

Cytotoxicity was assessed using a colorimetric assay (XTT Cell Proliferation Kit), which was based on the metabolization of XTT to formazan by mitochondrial dehydrogenase in living cells. Briefly, attached cells were treated with each extract for 18 hr, 50 μl of XTT solution was then added, and cells were incubated for an additional 4 hr. Amounts of formazan were determined by measuring absorbance at 450 nm (using a 650 nm reference filter) on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of nitric oxide (NO) production

After pretreating RAW 264.7 cells with each fraction (100 μg/ml) for 1 hr, they were treated with LPS for 12 hr. Nitrite levels were measured in culture media using a NO detection kit. Briefly, samples (100 μl) were mixed with N1 buffer (50 μl) and incubated at room temperature for 10 min. N2 buffer (50 μl) was then added and incubated at room temperature for another 10 min. Absorbances were measured at 520 nm. Nitrite levels were determined using an absorbance versus nitrite concentration plot, which was obtained using dilutions of a 1mM standard nitrite solution.

Determination of Prostaglandin E2 (PGE2) production

After pretreating RAW 264.7 cells with each fraction (100 μg/ml) for 1 hr, cells were treated with LPS for 12 hr, and PGE2 levels in culture media were measured using a PGE2 detection kit, according to the manufacturer's instructions.

Determination of pro-inflammatory cytokine production

The inhibitory effects of each fraction on cytokine (IL-1β, IL-6, and TNF-α) production by LPS-stimulated RAW 264.7 cells were determined using ELISA assay kits. After pre-incubation of RAW 264.7 cells with each fraction (100 μg/ml) for 1 hr, cells were treated with LPS for an additional 12 hr. Supernatants were removed and levels of IL-1β, IL-6, and TNF-α were quantified using an ELISA kit. Concentrations

of target cytokines were determined using standard curves.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from RAW 264.7 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), and 5 μg aliquots of total RNA were used for cDNA synthesis. PCR was carried out using selective primers over 27 cycles consisting of; denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min. PCR products were electrophoresed on 1% agarose gels at 100 V, and photographed using an ultraviolet transilluminator and a digital capture system (DNR Bio-Imaging Systems, Jerusalem). The band intensities of specific genes were quantified using Gelquant 2.7 software (DNR Bio-Imaging Systems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

Statistical analysis

All results are presented as the means ± SDs of experiments conducted at least in triplicate. Data were analyzed by one-way ANOVA followed by Duncan's multiple range test using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted for p values of <0.05.

Results

Effect on cell viability

Prior to studying the anti-inflammatory effects of the fruits of *F. vulgare*, we examined their methanol extract or fractions on RAW 264.7 cell viability. Cells were treated for 18 hr with the samples at 100 μg/ml and viabilities were determined using the XTT assay. RAW 264.7 cells showed more than 90% viability (Fig. 1). Therefore, samples were administered at 100 μg/ml during subsequent experiments.

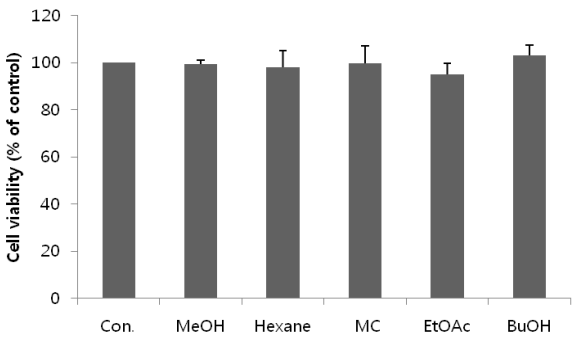

Fig. 1. Effects of *F. vulgare* methanol extract and of its fractions on RAW 264.7 cell viability. Raw 264.7 cells were treated with each sample (100 μg/ml) for 18 hr. Cell viabilities were evaluated using an XTT colorimetric assay. Results are expressed as the means ± SDs of three independent experiments. MeOH, 80% methanol extract; Hexane, *n*-hexane fraction; MC, methylene chloride fraction; EtOAc, ethylacetate fraction; BuOH, *n*-butanol fraction.

Effect on the productions of NO and PGE2

Since NO is known to be a pro-inflammatory mediator, we first evaluated whether the methanol extract or its fractions inhibited NO production in RAW 264.7 cells activated by LPS. As shown in Fig. 2A, NO production was reduced by the hexane, methylene chloride, ethylacetate, and *n*-butanol fractions versus LPS only treated RAW 264.7 cells. Analyses of the iNOS mRNA expressions by RT-PCR showed that the hexane, methylene chloride, and ethylacetate fractions (100 μg/ml) suppressed iNOS mRNA levels (Fig. 2C). We next investigated their inhibitory effects on the production of PGE2. As shown in Fig. 2B, treatment of RAW 264.7 cells with the hexane, methylene chloride, or ethylacetate fractions reduced LPS-induced PGE2 production. The stimulation of Raw 264.7 cells with LPS increased PGE2 production, but treatment with either of these fractions strongly inhibited LPS induced PGE2 production. In addition, these fractions strongly blocked LPS-induced COX-2 mRNA production. Overall, levels of NO and PGE2 released from LPS-stimulated RAW 264.7 cells were reduced in the presence of the hexane, methylene chloride, or ethylacetate fractions. Since more than 90% of RAW 264.7 cells survived at extract concentrations of 100 μg/ml, it is unlikely that pro-inflammatory mediator inhibition was due to a cytotoxic effect.

Effects on the productions of pro-inflammatory cytokines

Inflammation is the result of the responses of immune cells to infection or injury. It is caused by cytokines, such as, IL-1β, IL-6, and TNF-α. Thus, inhibitors of these cytokines have been considered candidate anti-inflammatory drugs. The effects of the methanolic extract of the fractions of *F. vulgare* in LPS stimulated RAW 264.7 cells are shown in Fig. 3. According to enzyme-linked immunosorbent assays (ELISA) using culture supernatants, methanolic extract and all four fractions significantly inhibited IL-1β production in LPS stimulated RAW 264.7 cells. LPS-activated IL-6 production was reduced decreased by the hexane, meth-

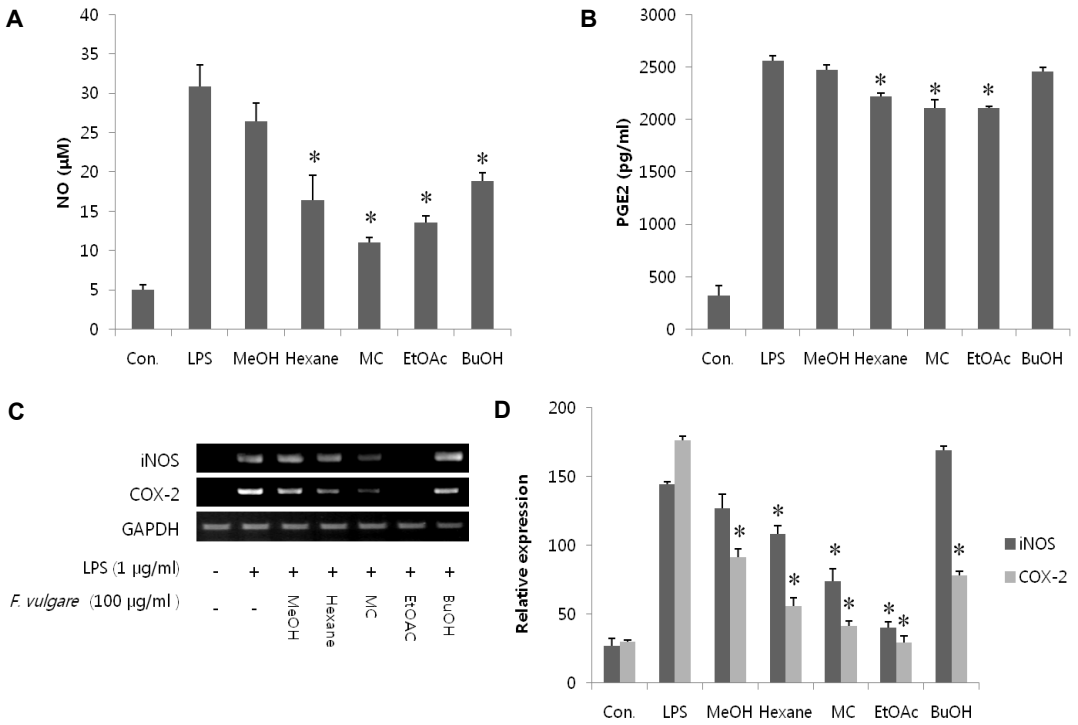

Fig. 2. Effect of *F. vulgare* methanol extract and of its fractions on the productions of NO and PGE2 in LPS-stimulated RAW 264.7 cells. Raw 264.7 cells were pre-incubated with each sample (100 μg/ml; 1 hr) and then stimulated with or without LPS (1 μg/ml) for 12 hr. NO (A) and PGE2 (B) levels were determined in culture supernatants using commercial detection kits (C) The mRNA expressions of iNOS and COX-2 were determined by RT-PCR using GAPDH as an internal control. Results are the means ± SDs of three independent experiments. *p<0.05 versus LPS alone. MeOH, 80% methanol extract; Hexane, *n*-hexane fraction; MC, methylene chloride fraction; EtOAc, ethylacetate fraction; BuOH, *n*-butanol fraction.

ylene chloride, and ethylacetate fractions versus LPS treated controls (Fig. 3B). TNF-α levels were also highly induced by LPS, but the pretreatment of LPS-stimulated RAW 264.7 cells with the ethylacetate extract prevented LPS-induced TNF-α production.

Effect on the mRNA levels of pro-inflammatory cytokines

F. vulgare methanol extract and its fractions were further investigated to determine their affects on the mRNA levels of pro-inflammatory cytokines. RT-PCR showed IL-1β, IL-6, and TNF-α mRNA levels were strongly induced by LPS. The methylene chloride and ethylacetate fractions reduced the

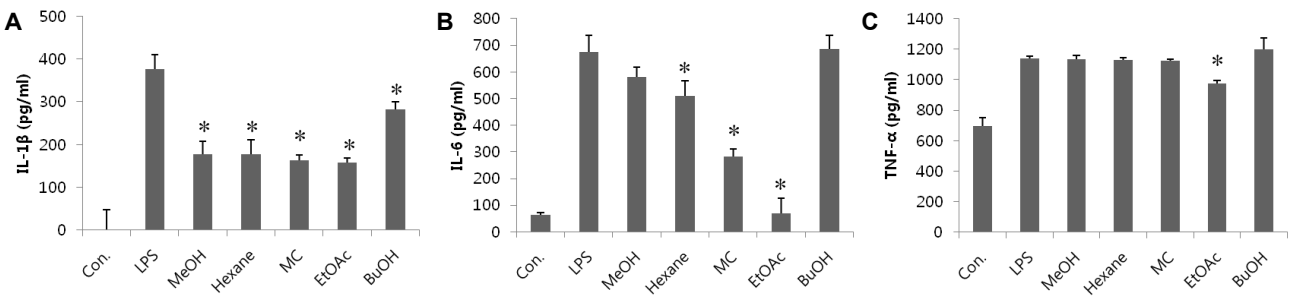

Fig. 3. Effects of *F. vulgare* methanol extract and of its fractions on the expression of pro-inflammatory cytokines. Raw 264.7 cells were pre-incubated with each sample (100 μg/ml; 1 hr) and then stimulated with or without LPS (1 μg/ml) for 12 hr. IL-1β (A), IL-6 (B), and TNF-α (C) were determined in culture supernatants using commercial detection kits. Results are the means ± SDs of three independent experiments. *p<0.05 versus LPS treatment alone. MeOH, 80% methanol extract; Hexane, *n*-hexane fraction; MC, methylene chloride fraction; EtOAc, ethylacetate fraction; BuOH, *n*-butanol fraction.

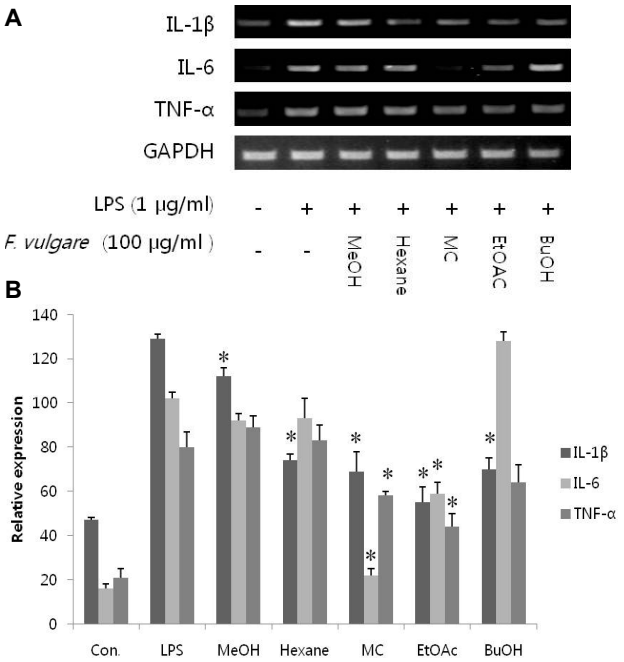

Fig. 4. Effects of *F. vulgare* methanol extract and of its fractions on the mRNA expressions of pro-inflammatory cytokines. Raw 264.7 cells were pre-incubated with each sample (100 μg/ml; 1 hr) and then stimulated with or without LPS (1 μg/ml) for 12 hr. The mRNA levels of IL-1β, IL-6, and TNF-α were determined by RT-PCR (A). The band intensities were quantified by densitometry and the relative gene expression, normalised with GAPDH. Results are the means ± SDs of three independent experiments. *$p<0.05$ versus LPS alone (B). MeOH, 80% methanol extraction; Hexane, *n*-hexane fraction; MC, methylene chloride fraction; EtOAc, ethylacetate fraction; BuOH, *n*-butanol fraction.

mRNA levels of IL-1β, IL-6, and TNF-α mRNA, but the methanol extract and hexane and *n*-butanol fractions only inhibited the mRNA expression of IL-1β (Fig. 4A, B). This observation suggests the ethylacetate fraction inhibits pro-inflammatory cytokine production at the transcriptional and translational levels.

Discussion

LPS, the major component of the outer membrane of Gram-negative bacteria, is recognised by toll-like receptor 4 (TLR4) in macrophages and causes a secretion of pro-inflammatory mediators [15, 16, 17]. Thus, inhibitions of the release of these inflammatory mediators have been considered as a promising anti-inflammatory candidate [9, 10]. The present study demonstrates the effects of crude methanolic extract of *F. vulgare* and its partitioned fractions (hexane, methylene chloride, ethyl acetate, and n-butanol) on LPS-induced RAW 264.7 macrophages. Our data revealed that hexane, methylene chloride, and ethyl acetate fractions inhibited LPS-induced NO and PGE2 production in RAW 264.7 macrophages. The methylene chloride and ethyl acetate fractions also decreased the IL-1β and IL-6 production at the protein and mRNA levels. Furthermore, the ethyl acetate fraction strongly suppressed TNF-α production in LPS stimulated RAW 264.7 cells.

The reactive free radical, NO, produced from L-arginine by nitric oxide syntheses, is an important mediator involved in various pathophysiological processes including inflammation and septic shock. Over-production of NO can promote inflammation and induce cell and tissue destruction directly by the formation of free radicals [27, 28]. PGE2 is a bioactive lipid generated by the sequential metabolism of arachidonic acid by cyclooxygenase and it is also a key mediator of immunological reaction during infections. PGE2 secretion can promote inflammation responses by increasing synthesis and migration of pro-inflammatory cytokines into the site of inflammation [29, 30]. Therefore, a compound capable of preventing the production of pro-inflammatory mediators in macrophage could potentially possess anti-inflammatory activities [10, 11]. In this study, we found that hexane, methylene chloride, and ethyl acetate fractions inhibited the induction of iNOS, COX-2 by LPS, and consequently reduced the production of NO and PGE2.

IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines that lead to tissue injury and organ dysfunction. They are produced by a variety of cell types, but the most important sources are macrophages at inflammatory sites [10, 12, 13]. IL-1β is secreted in response to inflammatory stimuli following an infection and subsequently promotes inflammation. The overproduction of IL-1β plays a critical role in the pathogenesis of inflammatory diseases due to amplified inflammation by recruiting macrophages [4, 10, 31]. Another pro-inflammatory cytokines, IL-6 is a pleiotropic cytokine that regulates immunological reactions in host defence. At adequate concentrations, IL-6 plays important roles in the host immune system, dysregulated, however its uncontrolled release can play a major role in the pathogenesis of many inflammatory diseases, including rheumatoid arthritis, rheumatoid arthritis, and inflammatory bowel disease [5, 32]. TNF-α was originally described as a circulating factor that can cause necrosis of tumours. But now it has been identified as a key regulator of diverse range of inflammatory

condition, such as septic shock and cachexia. TNF-α antagonists, however, may be effective in treating inflammatory disorders [33, 34]. In the present study, we demonstrate that methylene chloride and ethyl acetate fractions inhibited the productions of IL-1β and IL-6 by down-regulating their mRNA levels in LPS stimulated RAW 264.7 cells. In addition, the ethyl acetate fraction inhibited TNF-α at the protein and mRNA levels. On the basis of these results, we suggest that *F. vulgare* possesses potential anti-inflammatory activity via down-regulation of inflammatory mediators in macrophages.

In summary, the present study describes the anti-inflammatory effects of F. *vulgare* methanol extract and of its fractions on inflammatory responses in LPS-stimulated RAW 264.7 cells. According to the results obtained treatment with the hexane, methylene chloride, or ethylacetate fractions inhibited NO and PGE2 production in LPS stimulated Raw 264.7 cells. In addition, the methylene chloride and ethylacetate fractions also suppressed the IL-1β and IL-6 production by suppressing their mRNA levels. Furthermore, the ethylacetate fraction strongly suppressed TNF-α protein and mRNA levels. These findings suggest that of the tested fractions, the ethylacetate fraction be considered the more attractive potential source of anti-inflammatory compounds.

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초록 : 대식세포에서 LPS로 유도된 염증에 대한 회향 열매의 항염 효과

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회향(*Foeniculum vulgare*) 열매의 메탄올 추출물과 분획물(hexane, methylene chloride, ethylacetate, *n*-butanol)을 cell culture model system을 이용하여 항염증 활성을 평가하였다. 이를 위해, LPS처리로 염증이 유도된 RAW 264.7 macrophage에서 시료가 세포독성을 나타나지 않는 100 μg/ml의 농도를 처리한 후 염증매개물질인 NO, PGE2, 염증성 사이토카인(IL-1β, IL-6 및TNF-α) 생성 및 mRNA 발현을 측정하였다. 그 결과 hexane, methylene chloride 및 ethylacetate 분획물은 대조군에 비해 NO, PGE2 생성을 억제하였으며, 이는 iNOS, COX-2 mRNA 발현 저해에서 기인함을 확인하였다. Methylene chloride와 ethylacetate 분획물은 100 μg/ml 의 농도에서 IL-1β, IL-6 생성 및 mRNA 발현을 효과적으로 억제하였으며, 특히 ethyl acetate 분획물은 TNF-α 의 생성과 mRNA발현을 유의적으로 저해하였다. 본 연구 결과를 통해 회향 및 회향 분획물의 항염증 활성을 확인하였으며, 이는 염증매개물질인 NO, PGE2 그리고 염증성 사이토카인 mRNA 활성 및 분비 억제에 의한 것으로 나타났다.