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LPS로 자극한 RAW264.7 대식세포주에서 회향 추출물에 의한 염증성 매개물의 생성 억제

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Inhibition of lipopolysaccharide-stimulated inflammatory mediator production in RAW264.7 macrophages by *Foeniculum vulgare* fruit extract

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Abstract

이물질 침입에 대한 인식의 결과 NO, PGE_2, TNF-, IL-6와 같은 여러 신호전달물질들의 분비가 개시되며 이들을 억제하는 물질을 항염증제라고 볼 수 있다. 본 연구에서는 회향(*Foeniculum vulgare* Mill.) 열매 추출물이 mouse macrophages RAW264.7 세포에서 lipopolysaccharide(LPS)로 유도한 NO(iNOS 산물), PGE_2 (COX-2 산물) 및 cytokines (TNF-α, IL-6) 생성 억제에 미치는 영향을 살펴보았다. 회향 열매의 methanol 추출물 및 분획물(chloroform, butanol, and aqueous fractions)은 4~100 μg/mL 농도에서 LPS가 활성화한 대식세포에서 NO 생성을 억제하였으며 독성을 나타내지 않았다. LPS가 유도한 PGE_2 생성은 butanol 분획(100 μg/ mL)에 의해서만 유의적으로 감소하였다(P<0.05). 회향 열매 추출물 및 분획물은 TNF-α의 생성을 유의적으로 감소시켰으며 IL-6의 생성은 methanol extract(4～100 μg/mL), chloroform fraction(4 μg/mL), butanol fraction(4 and 100 μg/mL) 및 aqueous fraction(4～100 μg/mL)에 의해 감소되었다(P<0.05). 이는 회향 열매 추출물은 염증 상태에서 유용할 것이며 COX-2와 iNOS를 억제하는 butanol 분획은 새로운 항염증제 개발에 사용될 수 있음을 시사하여 주었다.

Key words: *Foeniculum vulgare*, nitric oxide, prostaglandin, cytokin

Ⅰ. Introduction

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages, mast cells, etc. Macrophages play a crucial role in modulating the initiation and perpetuation of the inflammatory response. Macrophages produce reactive oxygen intermediates and release various cytokines in response to lipopolysaccharide(LPS). These mediators include tumor necrosis factor(TNF-α), interleukin 6(IL-6), nitric oxide(NO) and prostaglandin E_2(PGE_2). The potential of macrophages to release cytokines such as TNF-α and IL-6, in response to an inflammatory stimuli, has proven a useful measure of the immune status of patients with e.g. rheumatoid arthritis(Zangerle et al., 1992), sepsis(Ertel et al., 1993), carcinoma (Elsasser-Beile et al., 1993), HIV infection(Hartung et al., 1998) and borreliosis(Diterich et al., 2001). After stimulation with LPS, many cells, including endothelial cells and macrophages, express the inducible isoforms of COX-2 and iNOS, which are responsible for the production of large amounts of PGE_2 and NO, respectively. As these inducible enzymes are essential components of the inflammatory response and are implicated in the pathogenesis of several inflammatory diseases, the selective modulation of these mediators overproduction might represent a therapeutic goal in

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different inflammatory pathologies. NO production catalyzed by iNOS, therefore, may reflect the degree of inflammation and provides a measure by which effects of drugs on the inflammatory process can be assessed. Also, COX-2 is considered to play a major role in the inflammatory process by catalyzing the production of PGE_2. Because of the role eicosanoids play in inflammation, pain and fever, their production is a target of pharmaceutical intervention.

Foeniculum vulgare Mill. has been known as a medicinal and aromatic herb and its fruit is commonly used as a natural remedy against digestive disorders (Yamini et al., 2002). It is also used to flavor foods, liqueurs and in the perfumery industry. Essential oils are mainly concentrated in the mericarps (fruits) and provide the unique aroma and taste. They are composed of several monoterpenes and phenylpropanoids, where *trans*-anethole, estragole, fenchone and limonene exist as main constituents. *Trans*-anethole, often the most prevalent constituents, counts for the anise taste, fenchone provides the bitterness, and estragole (methyl-chavicol) the sweetness (Guillen and Manzanos, 1994). In the course of our biological search for anti-inflammatory agents, we conducted a study using alcoholic extract from *Foeniculum vulgare* fruit and its subfractions (chloroform, butanol, and aqueous fractions) obtained bythe solvent extraction to separate the components according to their polarities. In this study, the ability of *Foeniculum vulgare* fruit extract and its fractions to modulate the production of some inflammatory mediators (NO, PGE_2, TNF-α and IL-6) were investigated in mouse macrophage cell line RAW264.7.

2. Materials and Methods

2.1. Extraction and fractionation

The fruits of *Foeniculum vulgare* Mill. were collected from the Biofarmaka Research Center of Bogor Agricultural University (Indonesia), identified by Dr I. Latifah, Dept. Pharmacy. The plant material was shade dried and ground to powder. The powdered material was soaked in 70% methanol for 24 hr. On the next day, the methanolic extract was filtered and dried on a rotary evaporator at temperature below 40℃ and the resulting product was designated as MeOH extract. For the purpose of fractionation, MeOH extract dissolved in distilled water was fractionated by successive solvent extraction with chloroform ($CHCl_3$) and n-butanol (BuOH). Each fraction was then evaporated to dryness under vacuo. The yields (%, w/w) of extract and fractions were as follows: MeOH extract; 6.4%, $CHCl_3$ fraction; 0.5%, BuOH fraction; 1.2%, aqueous (H_2O) fraction; 4.2%. All reagents were purchased from Sigma (USA), unless otherwise stated.

2.2. Cell culture

RAW264.7 cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) in a CO_2 incubator (5% CO_2 in air) at 37℃ and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin-0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

2.3. Cell viability

The cells were cultured in 24-well plate (5×10^5) containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with vehicle or samples in the presence of 10 μg/mL LPS for 24 hr. Cell viability was determined using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent (Tubaro et al., 1996). After culture on 96 well plate, the cells were washed twice with DPBS and incubated with 110 μl of 0.5mg/mL MTT for 2 h at 37℃. The medium was discarded and 100 μl dimethylsulfoxide (DMSO) was then added. After 30 min incubation, absorbance at 570 nm was read by using a microplate reader.

2.4. Measurement of nitric oxide formation by iNOS activity in cultured LPS-induced RAW274.7 cells

For evaluating the inhibitory activity of test materials on iNOS (Tsao et al., 2002), the cells in 10%FBS-DMEM without phenol red were plated in 24-well plates (5×10^5 cells per mL), and stimulated with 10 μg/mL LPS for 12 hr. Then the cells were

washed twice with PBS. Test samples were added for an additional 12 hr in the absence of LPS. The cell free culture medium was collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reagent (Ding et al., 1990). Briefly, 150 μl of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) were added to 100 μl of each supernatant from sample-treated cells. The plates were incubated for 5 min, and then were read at 570 nm against a standard curve of sodium nitrite. Percent inhibition was expressed as 100×[1−(NO release with sample−spontaneous release)/(NO release without sample−spontaneous release)].

2.5. Measurement of PGE_2 formation by COX-2 activity in cultured LPS-induced RAW274.7 cells

For evaluating the inhibitory activity of test materials on COX-2 (Hong et al., 2002), the cells were allowed to adhere for 4 hr in the presence of aspirin (500 μM) in culture plate, washed three times with media, and then incubated in the fresh medium with 10 μg/mL LPS. Test materials were simultaneously added to each well. After additional 16 h incubation, the media were removed and analyzed by PGE_2 enzyme immunometric assay kit (R&D System, USA) according to the manufacturer's instructions. The assay is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody. Percent inhibition was expressed as 100×[1−(PGE_2 release with sample−spontaneous release)/(PGE_2 release without sample−spontaneous release)].

2.6. Cytokine determination by ELISA

The cells were cultured in 24-well plate(5×10^5) containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with vehicle or samples in the presence of 10 μg/mL LPS for 48 hr. Cell supernatants were assayed for TNF-α and IL-6 using commercially available enzyme-linked immunosorbent assay(ELISA) kits(R&D System, USA) according to the manufacturer's instructions. Percent inhibition was expressed as 100×[1−(cytokine release with sample−spontaneous release)/(cytokine release without sample−spontaneous release)].

2.7. Statistical analysis

The results were expressed as mean ±SEM. Statistical analysis was performed using ANOVA followed by Dunnett's *t*-test ($p<0.05$). The analysis was performed using SAS statistical software.

3. Results

3.1. Effect of Foeniculum vulgare fruit extract on NO production

To assess the effect of *Foeniculum vulgare* fruitextract on LPS-induced NO production (for iNOS inhibitors) in RAW264.7 macrophages, cell culture medium was harvested, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method (Table 1). The addition of *Foeniculum vulgare* fruit extract and its fractions (chloroform, butanol and aqueous fractions) to cells that had been stimulated with LPS for 12 hr to induce iNOS activity inhibited the induced iNOS activity as evidenced by nitrite formation. Cell viability was more than 90% at the concentrations tested as assessed by the MTT assay (data not shown).

3.2. Effect of Foeniculum vulgare fruit extract on PGE_2 production

In this study, *Foeniculum vulgare* fruit extract and its fractions (chloroform, butanol and aqueous fractions) were evaluated with the LPS-induced PGE_2 accumulation (for COX-2 inhibitors) system in cultured RAW264.7 cells. As shown in Table 1, butanol fraction(100g/mL) showed significant inhibitory activity on COX-2, but other fractions did not inhibit COX-2 activity ($p<0.05$).

3.3. Effect of Foeniculum vulgare fruit extract on TNF-α and IL-6 production

To determine the effect of *Foeniculum vulgare* fruit extract on the production of TNF-α and IL-6, macrophages were treated with various concentrations (4∼100 μg/mL) of *Foeniculum vulgare* fruit extract

and its fractions (chloroform, butanol and aqueous fractions) and culture supernatants were assayed for cytokines by ELISA (Table 2). *Foeniculum vulgare* fruit extract and its fractions significantly decreased the production of TNF-α (p<0.05) and IL-6 production was reduced by methanol extract (4～100 μg/mL), chloroform fraction (4 μg/mL), butanol fraction (4 and 100 μg/mL) and aqueous fraction (4～100 μg/mL).

4. Discussion

Once activated by inflammatory stimulation, macrophages produce a large number of cytotoxic molecules. Several studies have suggested that overexpression of either inducible cyclooxygenase (COX-2) or nitric oxide synthase (iNOS) might be intimately involved in the pathogenesis of diseases including inflammation, cancer, multiple sclerosis, Parkinson's syndrome, and Alzheimer's disease (Hinz and Brune, 1999). Thus, there are many efforts to develop enzyme inhibitors or repressors of enzyme formation that are selective for theinducible forms of these enzymes and do not affect the desirable activity of their respective constitutive isoforms. Indeed, selective COX-2 inhibitors, such as celecoxib, NS-398, and sulindac, have been reported to exert for preventing cancer and treating inflammation (Bandaru et al., 1996). The production of NO, PGE_2, IL-6, and TNF-is an important part of the immune response to many inflammatory stimuli. However, excessive production of these mediators is seen in many acute and chronic human diseases, including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, and atherosclerosis (Bertolini et al. 2001). Thus, suppression of these mediators may be an effective therapeutic strategy for preventing inflammatory reaction and diseases.

In an effort of searching for novel anti-inflammatory agents from natural products, we fractionated *Foeniculum vulgare* fruit extract according to polarity and compared their effect on the production of NO, PGE_2 and cytokines (TNF-α and IL-6) released from cultured mouse macrophage cell line RAW264.7. Assay systems for determining of potential suppressors of COX-2 and iNOS expression were conducted in RAW264.7 cells. In the present study, we demonstrated that *Foeniculum vulgare* fruit extract and its fractions inhibitedNO generation. Inhibition of LPS-stimulated NO generation in RAW264.7 macrophages was not attributable to cytotoxicity as assessed by MTT assay. In addition, butanolfraction of *Foeniculum vulgare* fruit extract inhibited COX-2 activity. The physiologic or

Table 1. Inhibitory activity of iNOS and COX-2 by *Foeniculum vulgare* fruit extract and its fractions

Treatment (μg/mL)		NO production		PGE_2 production	
		μg/10^5 cells	% inh.[a)]	ng/10^5 cells	% inh.[b)]
Basal (cells alone)		1.92±0.16*	-	0.11±0.01*	-
Control		4.67±0.25	-	1.07±0.10	-
MeOH extract	20	2.99±0.38*	60.8	0.77±0.42	30.6
	100	3.07±0.36*	58.3	1.45±0.14	-40.1
$CHCl_3$ fraction	20	1.89±0.19*	101.2	1.57±0.56*	-52.5
	100	2.32±0.15*	85.4	1.31±0.21	-25.2
BuOH fraction	20	2.19±0.40*	90.3	0.61±0.18	47.5
	100	2.40±0.18*	82.7	0.61±0.03*	48.0
H_2O fraction	20	2.44±0.27*	81.0	1.41±0.23	-35.9
	100	2.59±0.48*	75.5	0.92±0.09	15.6
L-NMMAc)	50 M	2.75±0.23*	70.0		
Celecoxibd)	1 μg/mL			0.13±0.05*	98.0

RAW264.7 cells were treated with 10 μg/mL of LPS only (control) or LPS (10 μg/mL) plus samples. Data shown are mean ±SEM of the results of 6 cultures. Significant differences from control; *p<0.05.

[a)] Percent inhibition of iNOS activity: iNOS activity was determined by the production of NO in cultured LPS-stimulated RAW264.7 cells.

[b)] Percent inhibition of COX-2 activity: COX-2 activity was determined by the production of PGE_2 in cultured LPS-stimulated RAW264.7 cells.

[c)] L-NMMA (L-N^G-monomethyl arginine) was used as positive control for iNOS inhibitor.

[d)] Celecoxib was used as positive control for COX-2 inhibitor

normal production of NO from phagocytes is beneficial for the host defense against microorganisms, parasites, and tumor cells (Nathan, 1992). However, overproduction of NO can be harmful and result in inflammatory diseases (Evans, 1995). In most cases, the pathologies were significantly reduced through inhibiting NO synthesis *in vivo*by treating the animals with NO inhibitor derived from plants. It has been also known that plant-derived extracts and phytochemicals are potential alternatives to synthetic agents of NO inhibitors. Therefore, a therapeutic agent that inhibits the biosynthesis of NO may be useful for the relief of these inflammatory conditions.

We investigated whether *Foeniculum vulgare* fruit extract modulates the cytokines productions of RAW264.7 cells. As shown in Table 2, methanolic extract of *Foeniculum vulgare* exhibited remarkable inhibitory activity on the inflammatory cytokines. While the highest inhibitory ratios against TNF-α were obtained for methanol extract and chloroform fraction, potent inhibitory effect against IL-6 was exhibited by methanol extract and aqueous fraction. The macrophage-derived mediators, TNF-α and IL-6, which were used in the present study, were considered to play a key role in inflammatory and immune responses, based on their occurrence at inflammatory sites and their ability to inducemany of the hallmarks in the inflammatory response (Durum and Oppenheim, 1989). The observed inhibitory effect could be produced by a transcriptional activator, nuclear factor-κB (NF-κB), that controls the expression of several pro-inflammatory cytokines, as well as the expression of inducible enzymes such as iNOS and COX-2 (Bowie and O'Neil, 2000). Recently, NK-B has been characterized as a "sensor" of oxidative stress, a sensor that is activated by ROS and inhibited by antioxidants (Li and Karin, 1998). The suppressive effects of these antioxidant compounds on the production of these inflammatory mediators are associated with their antioxidant activities. It has been shown that *Foeniculum vulgare* fruit possess strong antioxidant activity (Toda, 2003).

In conclusion, the results of the present study demonstrated that the extract and its fractions from the fruits of *Foeniculum vulgare* inhibited the production of NO, TNF-α and IL-6 in LPS-activated RAW264.7 macrophages. Moreover, butanol fraction of *Foeniculum vulgare* fruit extract may be preferred, since higher inhibitory ratio was obtained against COX-2 activity than with the other solvent fractions. Our results suggest that the modulation of inflammatory mediators by *Foeniculum vulgare* fruit may be important in the prevention of inflammation and contribute to their anti-inflammatory and immunoregulatory effects. Further

Table 2. Effect of *Foeniculum vulgare* fruit extract and its fractions on the TNF-α and IL-6 production of RAW264.7 cells.

Treatment (μg/mL)		TNF-α production		IL-6 production	
		ng/10^5 cells	% inhibition	ng/10^5 cells	% inhibition
Basal (cells alone)		2.20±0.07*	-	4.35±0.04*	-
Control		2.70±0.04	-	5.32±0.04	-
	4	2.08±0.01*	124.5	3.66±0.01*	188.6
MeOH extract	20	2.19±0.01*	101.8	4.67±0.00*	73.9
	100	2.02±0.01*	136.1	4.85±0.08*	52.8
	4	2.19±0.01*	101.8	4.27±0.06*	119.3
$CHCl_3$ fraction	20	2.24±0.02*	93.4	5.52±0.04	-23.3
	100	2.08±0.04*	125.4	5.30±0.03	1.7
	4	2.54±0.01*	31.77	4.17±0.01*	130.1
BuOH fraction	20	2.38±0.03*	63.43	5.39±0.01	-8.5
	100	2.39±0.02*	61.3	4.96±0.03*	40.9
	4	2.63±0.02	13.7	3.71±0.04*	182.4
H_2O fraction	20	2.41±0.01*	57.4	4.08±0.04*	140.3
	100	2.51±0.02*	38.0	4.48±0.04*	94.9

RAW264.7 cells were treated with 10μg/mL of LPS only (control) or LPS (10μg/mL) plus samples. Cytokines concentration wasmeasured in the conditioned medium by an immunoassay. Data shown are mean ±SEM of the results of 6 cultures. Significant differences from control; *$p<0.05$.

studies are required to investigate the active constituents responsible for such activity.

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References

Bandaru, SR, Chinthalapally, VR and Karen, S : Evaluation of COX-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Research 56: 4566-4569, 1996

Bertolini, A, Ottani, A and Sandrini, M : Dual acting anti-inflammatory drugs: a reappraisal. Pharmacol. Res., 44: 437450, 2001

Bowie, A and O'Neill, LAJ : Oxidative stress and nuclear factor-kB activation: a reassessment of the evidence in the light of recent discoveries. Biochem. Pharmacol., 59: 13-23, 2000

Ding, A, Nathan, CF, Graycer, J, Derynck, R, Stuehr, DJ and Srimal, S : Macrophage deactivating factor and transforming growth factors-β1, -β2 and -β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. Journal of Immunology, 145: 940-944, 1990

Diterich, I, Harter, L, Hassler, D, Wendel, A and Hartung, T : Modulation of cytokine release in ex vivo-stimulated blood from borreliosis patients. Infect. Immun., 69: 687-694, 2001

Durum, SK and Oppenheim, JJ : Macrophage-derived mediator: interleukin 1, tumor necrosis factor, inteleukin 6, interferon, and related cytokines. In: Paul WE (Ed.), Fundamental Immunology, 2nd ed., Raven Press Ltd., New York. 1989

Elsasser-Beile, U, von Klerst, S, Fischer, R, Martin, M, Wetterauer, U, Gallati, H and Monting, JS : Impared cytokine production in whole blood cell cultures of patients with urological carcinomas. J. Cancer Res. Clin. Oncol., 119: 430-433, 1993

Ertel, W, Krombach, F, Kremer, JP, Jarrar, D, Thiele, V, Eymann, J, Muenzing, S, Faist, E, Messmer, K and Schildberg, FW : Mechanisms of cytokine transcription despite reduced CD14 receptor expression. Surgery, 114: 243-250, 1993

Evans, CH : Nitric oxide: what role does it play in inflammation and tissue destruction? Agents Actions Suppl, 47: 107-16, 1995

Guillen, MD and Manzanos, MJ : A contribution to study Spanish wild grown fennel (*Foeniculum vulgare* Mill.) as a source of flavor compounds. Chem. Mikrobiol. Technol. Lehensm., 16: 141-145, 1994

Hartung, T, Pitrak, DL, Foote, M, Shatzen, EM, Verral, SC and Wendel, A : Filgrastim restores interleukin-2 production in blood from patients with advanced human immunodeficiency virus infection. J. Infect. Dis., 178: 686-692, 1998

Hinz, B and Brune, K : Specific COX-2 inhibitors: prospects of therapy with new analgesic and anti-inflammatory substances. Wien. Klin. Wochenschr., 111: 103-112, 1999

Hong, CH, Hur, SK, Oh, OJ, Kim, SS, Nam, KA and Lee, SK : Evaluation of natural products on inhibition of inducible cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) in cultured mouse macrophage cells. Journal of Ethnopharmacology, 83: 153-159, 2002

Li, N and Karin, M: Is NF-kB the sensor of oxidative stress. FASEB J., 13: 1137-1143, 1998

Nathan, C : Nitric oxide as a secretory product of mammalian cells FAEB J., 6: 3051-64, 1992

Toda, S : Inhibitory effects of aromatic herbs on lipid peroxidation and protein oxidative modification by copper. Phytother. Res., 17(5): 546-8, 2003

Tsao, LT, Lee, CY, Huang, LJ, Kuo, SC and Wang, JP : Inhibition of lipopolysaccharide-stimulated nitric oxide production in RAW264.7 macrophages by asynthetic carbazole, LCY-2-CHO. Biochemical Pharmacology, 7254: 1-8, 2002

Tubaro, A, Florio, C, Luxich, E, Vertua, R, Loggia, RD and Yasumoto, T : Suitability of the MTT-based cytotoxicity assay to detect okadaic acid contamination of mussels. Toxicon, 34: 965-74, 1996

Yamini, Y, Sefidkon, F andPourmortazavi, SM : Comparison of essential oil composition of Iranian fennel (*Foeniculum vulgare*) obtained by supercritical carbon dioxide extraction and hydrodistillation methods. Flavour. Fragr. J., 17: 345-348, 2002

Zangerle, PF, De Groote, D, Lopez, M, Meuleman, RJ, Vrindts,Y, Fauchet, F, Dehart, I, Jadoul, M, Radoux, D and Franchimont, P : Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood: II. Application to rheumatoid arthritis and osteoarthritis. Cytokine, 4: 568-575, 1992

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