Anti-inflammatory and Anti-nociceptive Effects of the Methanol Extract of *Fomes fomentarius*

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In an attempt to find bioactive natural products with an anti-inflammatory activity, we evaluated the effects of the methanol extract of *Fomes fomentarius* (MEFF) on *in vivo* anti-inflammatory and anti-nociceptive activities. MEFF (50, 100 mg/kg/d, *p.o.*) reduced acute paw edema induced by carrageenin in rats, and showed MEFF analgesic activity, as determined by an acetic acid-induced writhing test and a hot plate test in mice. To investigate the mechanism of the anti-inflammatory action of MEFF, we examined the effect of MEFF on lipopolysaccharide (LPS)-induced responses in murine macrophages cell line RAW 264.7. MEFF potently inhibited the production of nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor-α (TNF-α) in LPS-stimulated RAW 264.7 macrophages. Consistent with these observations, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) levels were reduced by MEFF in a dose-dependent manner. Furthermore, MEFF suppressed nuclear factor-κB (NF-κB) activation in LPS-stimulated RAW 264.7 macrophages. These findings suggest that the anti-inflammatory and anti-nociceptive properties of the methanol extract of MEFF may result from the inhibition of iNOS and COX-2 expression through the down-regulation of NF-κB binding activity.

Key words *Fomes fomentarius*; anti-inflammatiory; anti-nociceptive; nitric oxide; nuclear factor-κB

Fomes fomentarius is a fungus of the polyporaceae family, and is parasitic on broadleaf trees.[1)] It is also used to make a popular drink, which is believed in Japan to be a tonic and to have an anticancer effect.[2)] It has also been reported that *Fomes fomentarius* has an inhibitory effect of virus infection on plants.[3)]

In terms of the pathogenesis of inflammatory disease, proinflammatory enzymes, including the inducible forms of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for elevated levels of NO and prostaglandins (PGs), respectively, are well-known pro-inflammatory key mediators.[4)] There are three major types of NOS isoenzymes, neural (nNOS), endotherial (eNOS), and inducible (iNOS) NOS. In contrast to the constitutive isoforms of NOS (eNOS and nNOS) that generate low levels of NO, iNOS produces high NO levels.[5)] COX is the enzyme that converts arachidonic acid to PGs. Like NOS, COX has been found in two isoforms (COX-1 and COX-2), and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site.[6,7)] Furthermore, tumor necrosis factor (TNF)-α is a potent proinflammatory cytokine that plays an important role in immunity and inflammation.[8)]

One of the most ubiquitous transcription factors that regulate gene expressions involved in cellular proliferation, inflammatory responses, and cell adhesion is nuclear transcription factor kappa-B (NF-κB). NF-κB is activated in response to various inflammatory stimuli, including bacterial LPS, cytokines, and viral proteins.[9)] Activated NF-κB results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of IκB proteins, followed by the translocation of NF-κB to the nucleus, and induction of gene transcription through the binding to the *cis*-acting κB element.[10,11)]

In this study, we evaluated the *in vivo* anti-inflammatory and anti-nociceptive activities of the methanol extract of *Fomes fomentarius* after oral administration and cell based anti-inflammatory activity to clarify the mechanism.

MATERIALS AND METHODS

Plant Materials and Preparation of the Methanol Fraction The sporophores of *Fomes fomentarius* were purchased from the Chun-Il Oriental Herbal Store in Wonju, Korea, and the plant origin was identified by J. D. Lee, one of the authors. A voucher specimen (#NATCHEM-51) has been deposited with the Department of Food and Nutrition, Pusan National University, Korea. To positively identify the specimen certificated *Fomes fomentarius* (NBRC 8246) was obtained from Japan. To prepare total genomic DNA from *Fomes fomentarius*, we used a benzyl chloride DNA extraction method.[12,13)] Oligonucleotide sense and anti-sense universal primers based on Internal Transcribed Spacers (ITS) were used for PCR amplification (ITS 5F; 5′-GGAAGTAAAAGTCGTAACAAGG-3′. ITS 4R; 5′-TCCTCCGCTTATTGATATGC-3′). The results of the phylogenetical analysis of ITS 1, 5.8S and ITS2 ribosomal RNA identified it as *Fomes fomentarius* with similarity of over 97% with NBRC 8246. Crushed plant material (1.00 kg) was extracted under reflux with hot MeOH three times, filtered, evaporated on a rotatory evaporator under reduced pressure, and freeze-dried to give a powdered extract (340 g).

Chemicals Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). COX-1, COX-2

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and iNOS monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enzyme immunoassay (EIA) kit for determination of prostaglandin E_2 and TNF-α was obtained from R&D Systems (Minneapolis, MN, U.S.A.). NS-398, a COX-2 enzyme inhibitor, was obtained from Calbiochem (CA, U.S.A.). Morphine sulphate was supplied from Kuju Pharmaceutical Co. (Seoul, Korea). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, caffeic acid, L-N^6-(1-iminoethyl)lysine (L-NIL), *Escherichia coli* LPS, acetylsalicylic acid (Aspirin), carrageenin, α-methyl-4-(isobutyl)phenylacetic acid (ibuprofen) and all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals ICR male mice weighing 20—25 g and Sprague-Dawley male rats weighing 100—120 g were purchased from the Daehan Biolink (Eumsung-Gun, Chungbuk) and maintained under constant conditions (temperature: 20±2 °C, humidity: 40—60%, 12 h light/dark cycle) and acclimatized for two weeks or more. Twenty-four hours before the experiment, only water was offered to the animals. In view of daily enzyme activity variations, animals were sacrificed at 10 : 00 a.m.—12 : 00 a.m. All animal experiments were approved by the University of Kyungsung Animal Care and Use Committee, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the Korea National Institute of Health.

Carrageenin-Induced Edema in Rats The initial hind paw volumes of the Sprague-Dawley rats was determined volumetrically. A 1% solution of carrageenin in saline (0.1 ml/rat) was injected subcutaneously into the right hind paw 1 h after the test samples had been administered orally. The test samples were first dissolved in 10% Tween 80 and diluted with saline. The same volume of solvent only was administered in the control group. A test solution (50 or 100 mg/kg) was administered orally for 7 d prior to injecting carrageenin. The control group received vehicle only. Paw volumes were measured up to 5 h after carrageenin administration at 60 min intervals, and the volume of the edema was measured with a plethysmometer.[14] α-methyl-4-(isobutyl)phenylacetic acid (ibuprofen), an anti-inflammatory drug, was used as a positive control.[15]

Writhing Test in Mice The acetic acid abdominal constriction test used was as described by Whittle.[16] Vehicle, aspirin (100 mg/kg) and test solution (50, 100 mg/kg) were orally administered 30 min before the experiment, and 0.1 ml/10 g of 0.7% acetic acid-saline was then injected i.p. 10 min after injection, the frequency of writhing in mice was the counted for 10 min.

Hot Plate Test The hot plate test was used to measure response latencies according to the method described previously by Eddy and Leimback,[15] with minor modifications. In these experiments, the hot plate (Ugo Basile, model-DS 37) was maintained at 56±1 °C. Reaction times were noted by observing either the licking of the hind paws or jumping movements before and after drug administration. The cut-off time was 10 s and morphine sulphate 10 mg/kg (Kuju Pharmaceutical Co.), administrated intraperitoneally was used as a positive control.[17]

Cell Culture and Sample Treatment The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown at 37 °C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a humidified 5% CO_2 atmosphere. Cells were incubated with MEFF at different concentrations (12.5, 25, or 50 μg/ml) or L-NIL (10 μM) or NS-398 (10 μM) and stimulated with 1 μg/ml LPS for 24 h.

MTT Assay for Cell Viability Cell viability studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped and plated at 1×10^5/well in 96-well plates containing 100 μl of DMEM medium containing 10% FBS and incubated overnight. MEFF was dissolved in DMSO (the DMSO concentration in all assays was <0.1%). After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl of FBS-free medium containing MTT 5 mg/ml. After 4 h of incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 μl. The optical density was measured at 540 nm.

Determination of Nitrite, PGE_2, and TNF-α Nitrite accumulation in culture medium was measured as an indicator of NO production based on the Griess reaction.[18] Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid with 0.1% (w/v) naphtylethylenediamine–HCl], and incubated at room temperature for 10 min. Absorbance at 550 nm was then measured using a microplate reader. Fresh culture medium was used as a blank in all experiments. The amount of nitrite in the samples was determined *versus* a sodium nitrite standard curve. PGE_2 and TNF-α levels in macrophage culture media were quantified using EIA kits according to the manufacture's instructions.

Western Blot Analysis Cellular proteins were extracted from control and MEFF-treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride (NaF), 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin, and then incubated for 15 min at 4 °C. Cell debris was removed by microcentrifugation, and supernatants were quickly frozen. Protein concentrations were determined using Bio-Rad protein assay reagent according to the manufacture's instruction. Forty micrograms of cellular proteins from treated or untreated cell extracts were electroblotted onto a nitrocellulose membrane after 10% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, and then incubated for 4 h with a 1 : 500 dilution of monoclonal anti-iNOS and COX-2 antibody (Santa Cruz Biotechnology Inc.). Blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and then incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Nuclear Extraction and Electrophoresis Mobility Shift

Table 1. Inhibitory Effect of MEFF and Ibuprofen on Carrageenin-Induced Edema of the Hind Paw in Rats

Treatment	Dose (mg/kg)	Swelling (ml)				
		1	2	3	4	5 (h)
Control		$1.3\pm0.06^{q,r)}$	$2.4\pm0.08^{d)}$	$2.9\pm0.07^{a)}$	$2.5\pm0.05^{c,d)}$	$1.9\pm0.06^{i,j)}$
MEFF	50	$1.4\pm0.04^{n,p,r)}$	$2.3\pm0.05^{e)}$	$2.7\pm0.05^{b)}$	$2.2\pm0.07^{f,h)}$	$1.8\pm0.03^{i,k,l)}$
	100	$1.3\pm0.07^{o,p,r)}$	$2.1\pm0.06^{g,h)}$	$2.5\pm0.03^{c)}$	$2.1\pm0.02^{f,g)}$	$1.7\pm0.05^{l)}$
Ibuprofen	100	$0.8\pm0.08^{s)}$	$1.8\pm0.09^{j,k)}$	$1.6\pm0.05^{m)}$	$1.4\pm0.07^{n,o)}$	$1.0\pm0.04^{s)}$

The assay procedure was described in the experimental methods. Values are expressed mean±S.D. The number of animal used for each group was 10. $^{a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s)}$ Values sharing the same superscript letter are not significantly different ($p<0.05$) each other by ANOVA followed by Dunnett's multiple range test.

Assay (EMSA) RAW 264.7 macrophages were plated in 100-mm dishes (1×10^6 cells). The cells were treated with various concentrations of MEFF (12.5, 25, 50 μg/ml), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously with slight modification.[19] Briefly, cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. The cells were then lysed by adding 0.1% Noidet P-40 and vigorously vortexing for 10 s. Nuclei were pelleted by centrifugation at $12000\times g$ for 1 min at 4 °C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extract 10 μg was mixed with the double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACTTTCCCAGGC-3′ end-labeled with [γ-^{32}P] dATP (the underline indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). Binding reactions were performed at 37 °C for 30 min in 30 μl of reaction buffer containing 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with 80-fold unlabeled oligonucleotide. DNA-protein complexes were separated from the unbound DNA probe in native 5% polyacrylamide gels at 100 V in 0.5×Tris–borate–EDTA (TBE) buffer. The gels were vacuum dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

Statistical Analysis Statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Data are reported as means±S.D. The numbers of independent experiments assessed are provided in the figure legends.

RESULTS

***In Vivo* Anti-inflammatory and Anti-nociceptive Effects** When we examined the anti-inflammatory effect of MEFF (50, 100 mg/kg, *p.o.*) using the carrageenin-induced edema model, it exhibited an inhibitory effect on carrageenin-induced edema for 2—4 h, as shown in Table 1. A standard drug, ibuprofen (100 mg/kg, *p.o.*), showed significant inhibition than MEFF. In addition, the anti-nociceptive effect of test samples was assayed using two different models, *i.e.*, by the acetic acid-induced writhing-test and the hot plate test in mice. MEFF showed anti-nociceptive activity by oral administration (50, 100 mg/kg), and aspirin (100 mg/kg) also exerted a significant protective effect (Table 2). Although acid-induced writhing-test is non-specific (*e.g.* anticholinergic, antihistaminic, and other agents show activity), it is widely used for analgesic screening and involves local peritoneal receptors (cholinergic and histamine receptor) and the mediators of acetylcholine and histamine. The hot plate test was used to determine whether MEFF has any central analgesic effect, and as expected MEFF showed significant activity (Table 2). The results obtained for morphine (10 mg/kg) were highly significant by the hot plate test. These results show that MEFF has anti-inflammatory and analgesic effects.

Table 2. Anti-nociceptive Effect of MEFF, Aspirin and Morphine by Acetic Acid-Induced Writhing and Hot-Plate Method in Mice

Treatment	Dose (mg/kg)	Stretching episodes (count/10 min)	Action time (s)
Control		$35.0\pm2.7^{a)}$	$14.3\pm1.9^{h)}$
MEFF	50	$31.7\pm3.2^{b)}$	$17.1\pm1.6^{g)}$
	100	$27.0\pm2.0^{c)}$	$20.1\pm1.2^{f)}$
Aspirin	100	$9.7\pm0.9^{d)}$	NT
Morphine	10	NT	$35.8\pm3.7^{e)}$

The assay procedure was described in the experimental methods. Values are expressed mean±S.D. The number of animal used for each group was 10. $^{a,b,c,d,e,f,g,h)}$ Values sharing the same superscript letter are not significantly different ($p<0.05$) each other by ANOVA followed by Dunnett's multiple range test.

Inhibition by MEFF of LPS-Induced NO, PGE_2 and TNF-α Production To determine the effects of MEFF on NO production in RAW 264.7 cells, the cells were treated with LPS (1 μg/ml) in the presence or absence of MEFF for 24 h. MEFF showed an inhibitory effect on LPS-induced NO production in a dose-dependent manner with an IC_{50} of 25 μg/ml based on three separate experiments (Fig. 1). L-N^6-(1-Iminoethyl)lysine (10 μM) was used as a positive inhibitor. This effect was more evident at high concentrations (25, 50 μg/ml). In addition, when treated with LPS, RAW 264.7 cells produced PGE_2 and pro-inflammatory cytokine such as TNF-α. These PGE_2 and TNF-α increases were inhibited in the presence of various concentrations of MEFF in a dose-dependent manner (Figs. 2, 3). Under the experimental conditions described above, cell viability was determined at MEFF concentrations of 12.5, 25 and 50 μg/ml, but no effects were observed by MTT assay (data not shown).

Effect of MEFF on LPS-Induced iNOS, COX-2 and COX-1 Protein Expression To determine if the inhibitory effect of MEFF on these inflammatory mediators (NO and PGE_2) is related to the modulation of iNOS and COX-2 enzymes, we examined these expression levels by Western blot analysis. The level of iNOS was significantly increased in RAW 264.7 cells after LPS treatment and this increase was suppressed in a dose-dependent manner by MEFF (Fig.

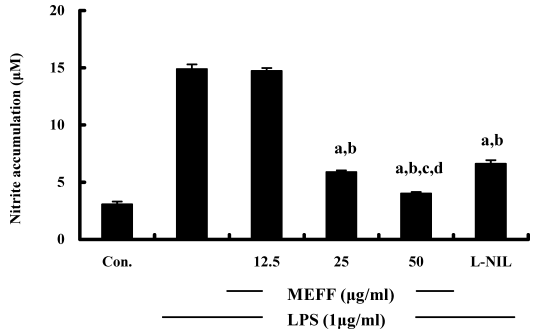

Fig. 1. Effect of MEFF and L-N^6-(1-Iminoethyl)lysine on Nitrite Accumulation (μM) by LPS-Induced RAW 264.7 Cells

The cells were treated with LPS 1 μg/ml alone or plus various concentrations (12.5, 25, 50 μg/ml) of MEFF for 24 h. Control (Con) values were obtained in the absence of LPS or MEFF. L-N^6-(1-iminoethyl)lysine (L-NIL) 10 μM was used as a positive control. The values are mean±S.D. from three independent experiments. [a] $p<0.05$ *vs.* LPS-treated group; [b] $p<0.05$ *vs.* 12.5 μg/ml MEFF-treated group; [c] $p<0.05$ *vs.* 25 μg/ml MEFF-treated group; [d] $p<0.05$ *vs.* L-NIL-treated group; significance of differences between the treated groups by ANOVA followed by Dunnett's test.

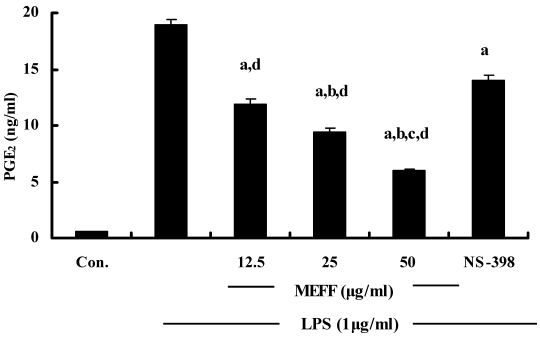

Fig. 2. Effect of MEFF and NS-398 on LPS-Induced PGE_2 Production in RAW 264.7 Cells

The cells were treated with LPS 1 μg/ml alone or plus various concentrations (12.5, 25, 50 μg/ml) of MEFF for 24 h. Control (Con) values were obtained in the absence of LPS or MEFF. NS-398 10 μM was used as a positive control in the assay. The values are presented as the means±S.D. from three independent experiments. [a] $p<0.05$ *vs.* LPS-treated group; [b] $p<0.05$ *vs.* 12.5 μg/ml MEFF-treated group; [c] $p<0.05$ *vs.* 25 μg/ml MEFF-treated group; [d] $p<0.05$ *vs.* NS-398-treated group; significance of differences between the treated groups by ANOVA followed by Dunnett's test.

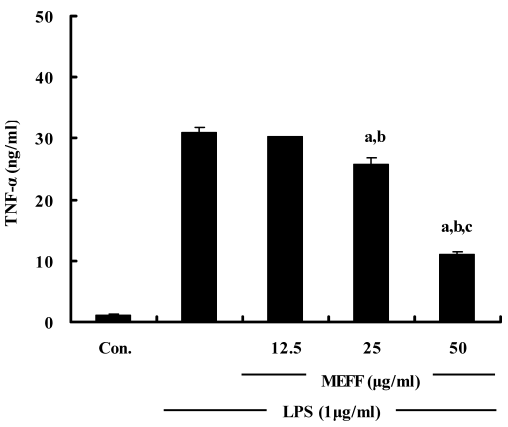

Fig. 3. Effect of MEFF on LPS-Induced TNF-α Release in RAW 264.7 Cells

Cells were treated with LPS 1 μg/ml alone or plus various concentrations (12.5, 25, 50 μg/ml) of MEFF for 24 h. Control (Con) values were obtained in the absence of LPS or MEFF. The values represented as the means±S.D. from three independent experiments. [a] $p<0.05$ *vs.* LPS-treated group; [b] $p<0.05$ *vs.* 12.5 μg/ml MEFF-treated group; [c] $p<0.05$ *vs.* 25 μg/ml MEFF-treated group; significance of differences between the treated groups by ANOVA followed by Dunnett's test.

4). A similar pattern was observed when the effect of MEFF was examined on LPS-induced COX-2 expression (Fig. 4). In unstimulated RAW 264.7 cells, iNOS and COX-2 proteins were undetectable. In general, these results are consistent with the profile of the inhibitory effect of MEFF on NO (Fig. 1) and PGE_2 release (Fig. 2). However, COX-1 protein expression level on LPS-induced RAW 264.7 cells was not influenced by MEFF (Fig. 4).

Inhibition of LPS-Induced NF-κB Activation by MEFF To investigate further the mechanism of the MEFF-mediated inhibition of iNOS and COX-2 expression, we focused on NF-κB, which is known to transactivate iNOS, COX-2, and TNF-α.[20] EMSA analysis demonstrated a reduction in NF-κB DNA binding activity in nuclear extracts obtained from LPS-activated RAW 264.7 macrophages treated with MEFF, and this binding inhibition increased in a dose-dependent manner (Fig. 5). These results suggest that the suppression of NF-κB activation and of TNF-α, iNOS, and COX-2 gene expression by MEFF occurred in a dose-dependent manner, and thus these gene expression may be due to the attenuation of NF-κB activation.

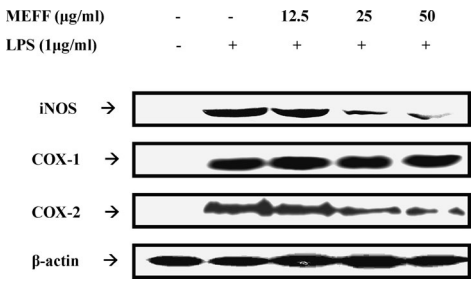

Fig. 4. Effect of MEFF on LPS-Induced iNOS, COX-1 and COX-2 Protein Expression in RAW 264.7 Cells

Lysates were prepared from control or 24 h LPS (1 μg/ml)-stimulated cells alone or in combination with increasing concentrations (12.5, 25, 50 μg/ml) of MEFF. All lanes contained 50 μg of total proteins. Western blot analysis using a specific antibody was performed in triplicate with similar results.

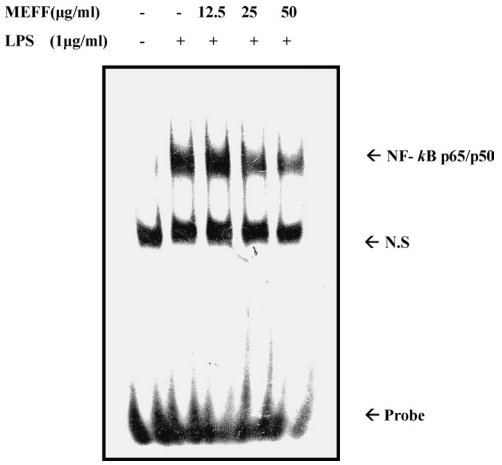

Fig. 5. Inhibition of NF-κB DNA Binding by MEFF

Nuclear extract, prepared from control or 1 h LPS (1 μg/ml)-stimulated cells in the absence or presence of different concentrations (12.5, 25, 50 μg/ml) of MEFF, were prepared and analyzed for NF-κB binding to DNA by EMSA. The arrow indicates the position of the NF-κB and nonspecific (N.S) bands. The data shown are representative of three experiments.

DISCUSSION

Since *F. formentarius* has not been investigated previously in terms of its pharmacological effects, we investigated its anti-inflammatory and anti-nociceptive effects by examining its methanol extract (MEFF) *in vivo* and *in vitro*. Whilst investigating the anti-inflammatory and anti-nociceptive effects of MEFF *in vivo*, we found that MEFF mildly decreased the edema induced by carrageenin, in which peak edema is characterized by the presence of PGs.[21] The anti-nociceptive effects of test samples were assayed using two different models, *i.e.*, the acetic acid-induced writhing test and the hot plate test in mice. From the results obtained from the acetic acid writhing test, it was observed that MEFF showed a dose–response correlation at doses of 50 and 100 mg/kg. A significant reduction of these abdominal constriction model reflected that it is also related to the sensitization of nociceptive receptors to prostaglandins (PGs). The results of the hot plate test in mice shows that MEFF significantly increased the latency of the jumping response when treated at 50 and 100 mg/kg without affecting the animals' abilities to detect the thermal pain threshold (licking response), suggesting that these samples had possible central analgesic properties. The anti-nociceptive activities shown by MEFF in these models indicate that MEFF may possess peripherally and centrally mediated anti-nociceptive properties.

To investigate the modes of action of MEFF as an anti-inflammatory agent, the effect of MEFF on LPS-induced inflammatory response was investigated in the RAW 264.7 murine macrophage cell line. It is well known that macrophages play a crucial role in both non-specific and acquired immune responses, and that macrophage activation by LPS leads to a functionally diverse series of responses, including the production of pro-inflammatory cytokines (IL-1β and TNF-α),[22] the activation of phospholipase A_2, which produces lipid metabolites of arachidonic acid such as PGs, and NO production.[23] In the present study, we found that LPS-induced NO production is inhibited by MEFF in a concentration-dependent manner without notable cytotoxicity. Results from western blot analysis further indicated that LPS-induced iNOS expression in RAW 264.7 macrophages was significantly blocked by MEFF. In addition to inhibiting NO release and iNOS induction, MEFF also potentially inhibited PGE_2 production and COX-2 protein expression in LPS-treated RAW 264.7 macrophages. Therefore, these results suggest that the inhibition of LPS-induced NO and PGE_2 production by MEFF occurs mainly through the regulation of iNOS and COX-2 gene expression. Moreover, the development of hyperalgesic states during inflammation is thought to be mediated by pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6.[24] Therefore, we investigated whether MEFF could influence the formation of TNF-α in an *vitro* model, and we found that MEFF significantly inhibits TNF-α production in a concentration-dependent manner.

The expressions of iNOS, COX-2, and TNF-α in murine macrophages requires the activation of NF-κB,[25,26] which is critical mechanism of LPS and cytokine induced inflammatory mediator.[9] Our results indicate that MEFF suppress the DNA binding activity of NF-κB as assessed in nuclear extracts by EMSA. The inhibitions of the expression of iNOS and COX-2 proteins and the production of NO, PGE_2, and TNF-α are probably due to the suppression of NF-κB activation. This is consistent with reports that NF-κB response elements are present on the promoters of the iNOS and COX-2 genes.[25,26]

Recent advance in molecular neurobiology have allowed for the discovery that the pathogenesis of pain involved not only neuropeptides, receptors, and ion channels, but also cytokine and transcription factor.[27,28] In addition, non-steroidal anti-inflammatory drugs (NSAIDS) are known to induce analgesia mainly *via* inhibition of COX. Although the inhibition of COX in the periphery is commonly accepted as the primary mechanism, experimental and clinical data suggest a potential role of spinal COX-inhibition to produce antinociception and reduce hypersensitivity.[29] The result of presented here suggest that inhibition of NF-κB and COX-2 activity may, therefore, involved in the noniceptive responses.

In summary, our results demonstrate that the MEFF has anti-inflammatory and anti-nociceptive effects in animals. In addition, we found that MEFF is a potent inhibitor of the LPS-induced NO, PGE_2 and TNF-α production *via* gene expression, and this inhibition was found to be caused by the blocking of NF-κB activation in RAW 264.7 macrophages. We conclude that MEFF appears to have the potential to prevent inflammatory and pain diseases.

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