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Anti-inflammatory activity of methyl ferulate isolated from *Stemona tuberosa* Lour

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ABSTRACT

Objective: To evaluate the anti-inflammatory activity of methyl ferulate (MF) isolated from the roots of *Stemona tuberosa* (*S. tuberosa*) Lour (Stemonaceae) in lipopolysaccharide activated macrophage cells.

Methods: Methanol extracts of a root powder of *S. tuberosa* were prepared for isolation of a potential anti-inflammatory agent using ultrasound extraction combined with repeated chromatography on silica gel. After the quantitative analyses, anti-inflammatory activity of the isolated compound was evaluated by measurement of cytokine release, NO generation, expression of cyclooxygenase-2 and phosphorylation of mitogen activated protein kinases including p38 and c-Jun NH2-terminal kinase using quantitative kits and Western blotting with specific antibodies.

Results: The isolation process yielded a potential anti-inflammatory compound with a purity level of 99% determined by high performance liquid chromatography. The compound was identified as MF by using nuclear magnetic resonance. MF strongly inhibited the release of pro-inflammatory cytokines from macrophages, including IL-6, TNFα, IFNγ, yet it did not affect the anti-inflammatory cytokine IL-10. Phosphorylation of p38 and c-Jun NH2-terminal kinase were clearly reduced in MF-treated macrophages stimulated with lipopolysaccharide. cyclooxygenase-2 expression and NO generation by macrophages were also suppressed when the cells were treated with MF.

Conclusions: The data suggested that MF is a possible inhibitor of the mitogen activated phosphor kinase pathway and could be a potential anti-inflammatory agent isolated for the first time in medicinal plant *S. tuberosa*.

1. Introduction

Stemona tuberosa (*S. tuberosa*) Lour (Stemonaceae), known as Bach Bo in Vietnam, is a hairless, perennial, herbaceous twiner which can grow up to 4-10 m long. Its roots form a fascicle of many thick, fleshy shoots. The root of *S. tuberosa* has been used in Vietnamese traditional medicine for its antitussive and anti-ectoparasitic activities moisten the lungs and stop cough, as well as kill parasites[1]. The chemical constituents of the plant are alkaloids, stilbenoides and tocopherols[2,3]. Recently, *S. tuberosa* extracts have been attracting new interest for their multi-biological functions including anti-tuberculotic, antifungal, demulcent, and anti-cancer activities[1,2]. However, the anti-inflammatory activity of this plant required further investigation. In this study, we aimed to examine the anti-inflammatory activity and potential mechanisms of action in lipopolysaccharide (LPS)-activated macrophage cells for methyl ferulate (MF) isolated from roots of *S. tuberosa*.

2. Materials and methods

2.1. Plant material

The roots of *S. tuberosa* and eleven other traditional medicinal plants for screening test were collected in Quang Binh, Thanh Hoa, Phu Tho, Hoa Binh provinces and identified by the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi.

2.2. Chemicals

LPS was purchased from Sigma-Aldrich (St. Louis, MO).

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ELISA kits for IL-6, IL-10, TNFα, IFNγ were purchased from BD Pharmingen (Franklin Lakes, NJ). Antibodies p38 and phospho-(Thr180/Tyr182)-p38, c-Jun NH2-terminal kinase (JNK) and p-JNK were purchased from Cell Signalling Technology (Beverly, MA). The anti-cyclooxygenase-2 (COX-2) antibody was purchased from Abfrontier (Korea). The NO detection kit was purchased from iNtRON Biotechnology (Korea). All chemicals used were of analytical grade.

2.3. Extraction and isolation

The air-dried and powdered roots of *S. tuberosa* (2.0 kg) were extracted five times with 75% methanol (5×10 L) in ultrasound extracting system (Elma, Japan) at room temperature and filtered. The combined extract was concentrated to dryness by evaporating in vacuo to give a residue (18 g), which was used for antiflammatory screening assays. The residue was chromatographed on silica gel (200-300 mesh) column chromatography, eluting with an ether:acetone gradient (9:1) to give 10 fractions E 1-10. The further separation of fraction E4 (0.82 g) by silica gel column chromatography eluted with a hexane:ethyl acetate gradient (2:1), yielded ten fractions (H1-10). Compound 1 (85 mg) was obtained from fraction H5 by preparative high performance liquid chromatography (HPLC) (hexane-EtOAc, 2/1; flow rate 1 mL/min).

2.4. Cell culture

Primary bone marrow derived-macrophages (BMDMs) were isolated from the bone marrow of mice, which were reviewed and approved by the animal care unit of National Institute of Hygiene and Epidemiology, Vietnam. The cells were differentiated for 5-7 d in macrophage colony-stimulating factor (M-CSF)-containing media. The medium contains Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) with 10% L929 cell-conditioned medium (as a source of M-CSF), 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL), 1 mmol/L sodium pyruvate, 50 IU/mL penicillin, 50 μg/mL streptomycin and 5×10^5 mol/L β-mercaptoethanol, sodium pyruvate, non-essential amino acids, penicillin G (100 IU/mL), and streptomycin (100 μg/mL). Mouse macrophage cell line, RAW264.7 (American Type Culture Collection; ATCC) was maintained in complete medium (DMEM with 10% FBS), sodium pyruvate, non-essential amino acids, penicillin G (100 IU/mL), and streptomycin (100 μg/mL).

2.5. Cell viability assay

Cell viability assessment was performed using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) as per the manufacturer's instructions. Briefly, 10 μL of CCK-8 solution was added and incubated with treated cells for 60 min in Corning Costar 48 well plate and then absorbance was measured at 450 nm. Values from each treatment were calculated as a percent relative to the untreated control (100% survival).

2.6. ELISA

BMDMs were treated in Corning Costar 48 well plate as indicated in the Figures 3 and 4. The cell-culture supernatants were analyzed for levels of cytokines TNFα, IL6, IL10, and IFN-γ secreted by cell culture were measured by ELISA reagents (BD Pharmingen).

2.7. Western blotting

The treated BMDMs were processed for analysis by Western blotting[4]. Briefly, BMDMs were treated with the test agent for 60 min in Corning Costar 48 well plate before stimulation with LPS (1 μg/mL). The total cell lysates obtained by using lysis buffer NP 40% (Life Technologies) were then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred overnight to nitrocellulose membranes, (AmershamTM HybondTM-ECL) and the nonspecific binding of antibodies was blocked with 3% nonfat dried milk in phosphate-buffered saline. Membranes were probed with p38, p-p38, JNK, and p-JNK antibodies (1:1 000 dilution in Tris-buffered saline, pH 7.4) for 2 h at room temperature. After three washes with phosphate-buffered saline, blots were developed by a chemiluminescence assay (ECL; Amersham-Pharmacia).

2.8. Measurement of NO content

Raw 246.7 cells at density of 10^6 were seeded in Corning Costar 48 well plate for 16 h before treatment. The cells were treated with the test agent for 60 min and activated with 1 μg/mL of LPS. The culture supernatants were obtained by centrifuge and concentrations of NO were assessed by Griess reaction using a NO detection kit (iNtRON Biotechnology, Korea).

2.9. Determination of COX-2 expression

RAW 246.7 cells were treated with test agent in Corning Costar 48 well plate for 60 min before stimulation with LPS (1 μg/mL). The total cell lysates were then analyzed with Western blot using anti-COX-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The antibodies were diluted at ratio of 1:1 000. The membranes were developed by a chemiluminescence assay (ECL; Amersham-Pharmacia).

2.10. Statistical analyses

For statistical analysis, data obtained from independent experiments are presented as mean±SD and were analyzed by the Student's *t*-test with ANOVA for multiple comparisons. Differences were considered significant at $P<0.05$.

3. Results

3.1. Isolation of potential anti-inflammatory agent(s) from S. tuberosa extract

Macrophages and lymphocytes produce powerful pro-inflammatory mediators, including IL6, IL1, IL8, and TNFα, one of the most important mediators that regulates biochemical changes and symptomatic pathophysiological responses in the human body. In later stages of sepsis, anti-inflammatory mediators are produced (*e.g.* IL10), leading to an abatement in the production of many of the pro-inflammatory mediators[5]. Macrophages activated by bacterial LPS induce antibody production and local inflammation, which includes the release of pro-

inflammatory cytokines, chemokines, and inflammatory mediators[5]. Therefore, macrophage cell lines are routinely used to evaluate the anti-inflammatory activities of crude extracts.

In this study, assaying LPS-activated macrophages *in vitro*, we screened for a potential anti-inflammatory extract from 12 traditional medicinal plants of Vietnam by measurement of IL6 and TNFα production when LPS-induced macrophage cells were treated with these plant extracts. We found that a methanolic *S. tuberose* extract strongly inhibited pro-inflammatory IL6 and TNFα release while it did not affect the release of the anti-inflammatory cytokine IL10, suggesting that the extract possesses potential anti-inflammatory properties (data not shown). Based on this result, *S. tuberosa* was selected for isolation of the desired compound. The isolation process was based on the bioassay-guided chromatographic separations. Compound 1 (85 mg) in the form of a yellow-brown oil was received by a preparative chromatography system after two repeated silica gel column chromatography steps showed a purity of up to 99% by HPLC. The obtained nuclear magnetic resonance (NMR) spectroscopic data indicated that compound 1 is MF with a molecular fomular of $C_{11}H_{13}O_4$ (Figure 1), which displays the following spectral data: UV (MeOH)_max nm: 236, 322; IR (KBr) _max cm^{-1}: 3424, 2903, 2649, 2549, 1744, 1670; EIMS *m/z*: 208 [M]+, 177, 145, 137, 131, 89, 77, 51; ^1H and ^{13}C NMR (Table 1).

Ferulic acid and some derivatives, especially ethyl ferulate, have been reported to be effective antioxidant, anti-microbial, anti-inflammatory, hepatoprotective, neuroprotective, anticarcinogenic, anti-diabetic, anti-cholesterolemic, UV-protective and radioprotective compounds[6,7]. However, anti-inflammatory activity of MF derivative has not been well documented.

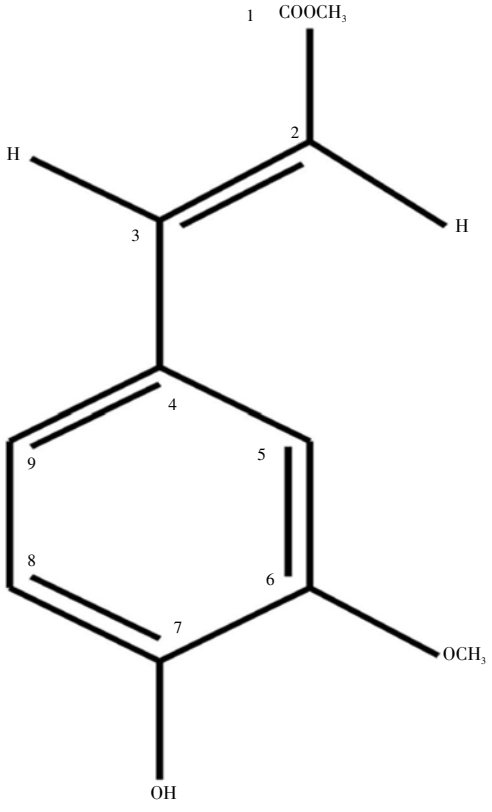

Figure 1. Chemical structure of methyl ferulate isolated from *S. tuberosa*.

Table 1

The spectroscopic data of MF ($CDCl_3$) about compound of ^1H NMR and ^{13}C NMR.

Position	Compound 1	
	^1H NMR (J, Hz)	^{13}C NMR
1		167.7
2	6.28 d, J=16.0	115.2
3	7.62 d, J=16.0	145.0
4		126.9
5	7.03 brd s	109.4
6		148.0
7		146.8
8	6.92 d, J=8.5	114.7
9	7.07 dd, J=1.0, 8.0	123.0
1-OMe	3.80 s	51.6
6-OMe	3.93 s	55.9
7-OH	5.90 s	

3.2. Effect of MF on cell viability

To evaluate the anti-inflammatory activity of MF, we examined the cytotoxic effects of MF and a dexamethasone (Dex) control on BMDMs. BMDMs were treated with MF (25 μg/mL) or Dex (100 nmol/L) for various times, and the percentage of surviving cells was counted using CCK-8 cell viability assay. As shown in Figure 2, no significant difference was observed for MF or Dex-induced cell viability at the indicated times, up to 48 h (P>0.05).

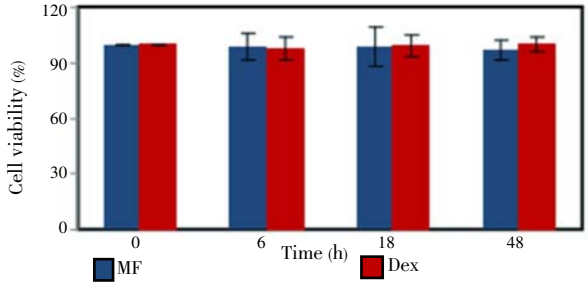

Figure 2. No effect on cell viability after treatment of BMDMs with MF or Dex. BMDMs were seeded in Corning Costar 48 well plate at a density of 1×10^6 cells/well. After 3-4 d, cells were treated with MF (25 μg/mL) for the times indicated. Cell viability was assessed after incubation for indicated times in the presence of CCK-8. Data are presented as the mean±SD of three independent experiments.

3.3. Inhibition of MF against cytokine production by LPS activated macrophages

Anti-inflammatory properties of MF concentrations 0, 5, 10, and 25 μg/mL were confirmed by examination of their effect on pro-inflammatory cytokine production of IL-6, IFNγ, TNFα, and post-anti-inflammatory cytokine IL-10. The results (Figure 3) indicated that the release of IL6, INFγ, and TNFα was strongly inhibited in BMDMs while IL10 was unaffected compared to untreated control cells (P<0.05). These data indicate that MF could be a good candidate for use as an anti-inflammatory agent. At a concentration of 10 μg/mL (45 μmol/L), release of all the tested cytokines in the treated BMDMs were suppressed by at least 50%.

3.4. MF inhibits phosphorylation by p38 and JNK in MAPK pathway

Mitogen activated protein kinases (MAPK) are a group

of signaling molecules that also appear to play important roles in inflammatory processes. At least three MAP kinase cascades are well described including, extracellular signal and regulated kinase, JNK and p38[8,9]. These have also been reported to differentially activate depending on the stimuli present and cell type affected[9]. As shown in Figure 4, the activation of p38 and JNK was significantly reduced in the MF-treated RAW 264.7 cells at a concentration of 25 μg/mL (compared to the control).

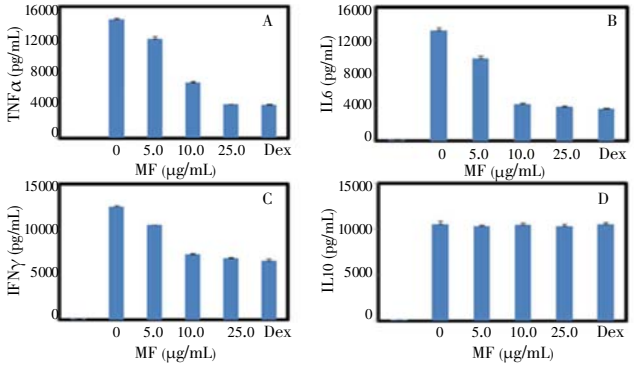

Figure 3. Effect of MF on cytokines TNFα, IL6, IFNγ and IL10 produced by macrophages.

BMDMs were treated with MF at concentrations of 0, 5, 10, and 25 μg/mL in vehicle DMSO 0.1% in 60 min before stimulation with LPS. Supernatants were harvested 18 h after stimulation with 1 μg/mL LPS to induce inflammation. Concentrations of IL-10, IFNγ, IL-6, and TNFα in the culture supernatants were determined by ELISA. Dexamethasone (Dex) was used as positive control. Data are presented as the mean±SD of three independent experiments.

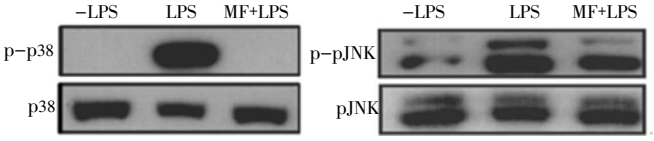

Figure 4. Inhibition of MF against p38 and JNK phosphorylation in BMDMs.

BMDMs were treated with MF at concentrations of 25 μg/mL in vehicle DMSO 0.1% for 60 min before stimulation with LPS (1 μg/mL). The cells then were then analyzed with Western blot using p38, p-p38, JNK, and p-JNK antibodies.

3.5. Inhibition of MF against NO formation in BMDMs

Reactive oxygen species (ROS), such as H_2O_2, NO, superoxide anions, and hydroxyl radicals (OH) are often important intracellular signaling molecules in pathways involved in cell proliferation, stress responses and apoptosis[5,9]. The inhibitory effects of MF on activation of MAPK (phosphorylation of p38 and JNK), which was mentioned in section 3.4, suggested that p38 and JNK could be the important signals involved in the MF-induced inhibition of NO generation. Therefore, we investigated whether MF inhibits LPS-induced NO generation in BMDMs. The RAW264.7 cells were treated with MF and inflammation was induced by using LPS. The data (Figure 5) indicated that LPS-induced NO generation was inhibited in a dose dependent manner with about 20% and 50% inhibition at concentrations 10 and 25 μg/mL MF, respectively.

3.6. Inhibition of MF against COX-2 expression

COX-2 is primarily present at sites of inflammation and plays a role in the conversion of arachidonic acid to prostaglandin, resulting in pain and inflammation[10]. Inhibitors of COX-2 have profound anti-inflammatory effects[10]. We also examined COX-2 expression in MF-treated and LPS-stimulated BMDMs, as COX-2 is an important inflammatory mediator that is induced by various stimuli, including LPS and cytokines[9]. As shown in Figure 6, MF clearly suppressed COX-2 expression at a concentration of 25 μg/mL while the inhibitory effect seems to not be very profound at a concentration of 10 μg/mL.

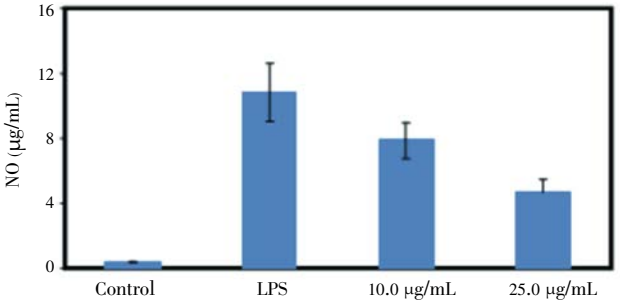

Figure 5. Inhibition of MF against NO generation in BMDMs.

Raw 246.7 cells at density of 10^6 were seeded in Corning Costar 48 well plate for 16 h before treatment. The cells were treated with MF at concentrations of 10 and 25 ug/mL for 60 min and activated with 1 ug/mL of LPS for another 1.5 h. The culture supernatants were harvested by centrifuge and concentrations of NO were determined using a NO detection kit. Data are presented as the mean±SD of three independent experiments.

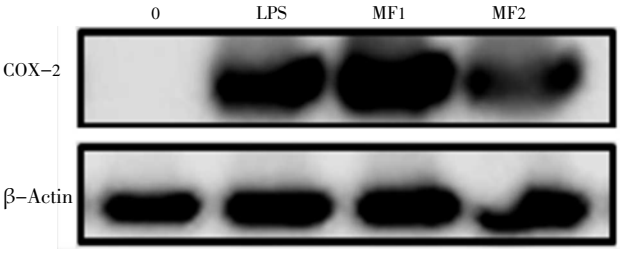

Figure 6. Inhibition of MF against COX-2 expression in BMDMs.

RAW 246.7 cells were treated with MF at concentrations of 10 μg/mL (MF1) and 25 μg/mL (MF2) in vehicle DMSO 0.1% in Corning Costar 48 well plate for 60 min before stimulation with LPS (1 μg/mL). The cells were then analyzed with Western blot using anti COX-2 antibody.

4. Discussion

In the treatment of inflammation, non-steroid anti-rheumatic drugs, such as acetylsalicylic acid, more commonly known as aspirin or ibuprofen, have been popular choices. However, many of these drugs cause potential risks and side effects, such as stomach and cardiovascular problems[11,12]. Therefore, novel anti-inflammatory, especially natural agents are badly needed. Plants are rich in naturally-occurring bioactive compounds. This is an ideal bio-resource to develop new therapeutic agents[13-15]. In an attempt to find novel anti-inflammatory agents from plants of Vietnam, we were screened and selected *S. tuberosa*, a traditional medicinal plant. We found the MF compound, and even though it is not a new compound, this is the first time that it has been isolated from this plant and shown to possess anti-inflammatory activity when tested *in vitro*.

First, MF strongly inhibited MAPK phosphorylation induced by LPS, which affected two important signal pathways p38 and JNK. The most well-known pathway involved in LPS-

induced pro-inflammatory responses in macrophages is the MAPK pathway, which is involved intracellular signaling cascades[8,16]. Intense efforts have been made to develop and evaluate compounds that target components of these pathways[9,16]. The most extensive activity in MAPK inhibitor development has found with pJNK and p38, which regulate the production of TNFα and IL1[17]. p38 inhibitors are expected to inhibit not only the production of pro-inflammatory cytokines, but also their actions, thereby interrupting the vicious cycle that often occurs in inflammatory and immune-responsive diseases[5,9]. Our data suggest that MF is a potential inhibitor of the MAPK pathway.

Second, several studies have demonstrated that activation of MAPK plays a role in the regulation of inflammation by affecting the activation of NF-κB[8,17]. Moreover, NF-κB plays an important role in the regulation of inflammatory genes, such as, inducible nitric oxide synthetase (iNOS), COX-2, and TNF-α[9,10,17]. In our study, NF-κB expression was also evaluated in BMDMs using specific antibodies to the p50/105 subunit of NF-κB. We found an only minor suppression of NF-κB expression by MF in the Western blot image at a concentration of 25 μg/mL (data not shown). However, the inhibitory effect of MF on NO generation and COX-2 at the same concentration suggests that MF could still be affecting the NF-κB pathway. Islam *et al*. demonstrated that anti-inflammatory activity of ethyl ferulate, another derivative of ferulate acid comes from the inhibition of ROS production and the significant suppression of the NF-κB activity when the translocation of NF-κB p65 in LPS-stimulated RAW 264.7 macrophages was measured[18].This suggests that an additional study would be warranted to further investigate this, perhaps by employing the use of an NF-κB reporter. Thus, it is possible that there could be cross-talk between the MAPK and the NF-κB signals that may be important for relaying the biological effect of MF. Moreover, Minutoli *et al*. demonstrated that during the development of testicular ischemia-reperfusion injury, the JNK and p38 signals were abolished when NF-κB was disrupted[19]. Sethi *et al*. also found that TNF-induced NF-κB activation was stopped in cells deleted of the MKK4 gene encoding activates both JNK and p38 MAPK[20].

In summary, the present study suggests MF is a potential inflammatory compound isolated for the first time from medicinal plant *S. tuberosa* grown in Vietnam.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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