Regular Article

3β-Angeloyloxy-8β,10β-dihydroxyeremophila-7(11)-en-12,8α-lactone Inhibits Lipopolysaccharide-Induced Nitric Oxide Production in RAW264.7 Cells

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Farfugium japonicum (L.) Kitam., named “Lian-Peng-Cao” in China, has been traditionally used in Chinese folk medicine to treat sore throat, cold, and cough due to its anti-inflammatory properties. In this study, the anti-inflammatory action of 3β-angeloyloxy-8β,10β-dihydroxyeremophila-7(11)-en-12,8α-lactone (FJ1) isolated from Farfugium japonicum and its molecular mechanism in RAW264.7 cells were investigated. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that FJ1 with or without 3μg/mL lipopolysaccharide (LPS) had no significant cytotoxicity in RAW264.7 cells. The production of nitric oxide (NO) was identified with a Griess reagent kit. The mRNA expression of inducible nitric oxide synthase (iNOS) and cytokines, including tumor necrosis factor α (TNF-α) and cyclooxygenase-2 (COX-2), was measured by real-time polymerase chain reaction (PCR). Reactive oxygen species (ROS) production was detected by flow cytometry analysis. Western blot was used to examine the protein expression of nuclear factor-kappa B (NF-κB), inhibitor of kappa B (IκB)-α, phosphorylated IκB-α (p-IκB-α), phosphorylated 1kB-α (p-IkB-α), and mitogen-activated protein kinase (MAPK) molecules, iNOS, and TNF-α. We discovered that FJ1 possesses anti-inflammatory effects that inhibit the release of LPS-stimulated pro-inflammatory cytokines, including NO and ROS. The molecular mechanism of FJ1-mediated anti-inflammation is associated with decreasing phosphorylation of MAPK molecules, including extracellular signal-related kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun NH2-terminal kinase (JNK). FJ1 also reverses IκB degradation and attenuates the mRNA and protein expression of NF-κB-related downstream inducible enzymes and cytokines, such as iNOS, TNF-α in RAW264.7 cells. The results suggest that FJ1 has anti-inflammatory properties, which indicates that F. japonicum can be utilized to treat inflammatory diseases. The potential mechanism is associated with the NF-κB and MAPK activation pathways in LPS-stimulated macrophages.

Key words inflammation; nitric oxide; nuclear factor-kappa B (NF-κB); reactive oxygen species (ROS); Farfugium japonicum

Macrophages are present in almost all body tissues. Macrophages as important effector cells of the immune system provide defense against microbial invaders and malignancies owing to their phagocytic, cytotoxic, and intracellular killing capacities. Macrophages play important roles in innate and adaptive immune response to pathogens, including phagocytosis, antigen presentation, and cytokine secretion. Macrophages release a number of factors, such as nitric oxide (NO), prostaglandin, and cytokines, during immune response. Overproduction of inflammatory mediators released by activated macrophages is associated with many inflammatory diseases.

Natural products provide a great chemical structural diversity. Farfugium japonicum, known as da-wu-feng-cao in China, is mainly distributed in southeast and south China, Japan, and Korea. F. japonicum is utilized as folk medicine in China to treat sore throat, cold, cough, and pain owing to its anti-inflammatory properties. Besides, modern pharmacological studies have shown that the total extract of F. japonicum and its compounds have many pharmacological properties, including anti-tumor, anti-inflammatory, and analgesic properties. Moreover, the total extract of F. japonicum exhibited significant inhibition effect on lipopolysaccharide (LPS)-stimulated NO production in RAW264.7 cells in our present study (Fig. 1B). In our continuing program to search for anti-inflammatory agents from this plant, we chose four higher yields compounds (FJ1, FJ3, FJ5, FJ12) to test inhibition potency on NO production. The striking activity and the highest yields of FJ1 suggested that FJ1 may pay an important role in the anti-inflammatory diseases of F. japonicum. In the present study, FJ1 was employed further to investigate the effects and molecular mechanisms on LPS-stimulated pro-inflammatory properties in RAW264.7 cells.

MATERIALS AND METHODS

Isolation and Purification Tested compounds (FJ1, FJ3, FJ5, FJ12) have been prepared as described in our group previous papers. In brief, the air-dried rhizome of F. japonicum were extracted three times (seven days each time) with petroleum ether (bp 60–90°C)–Et2O–CH2OH at room temperature. The extract was concentrated under reduced pressure. After that, the residue was subjected to silica gel CC eluting with...
a gradient of n-hexane–acetone to give a few fractions which were subsequently isolated by silica gel CC eluted with suitable gradient n-hexane–acetone elution. Then, compound FJ1 was further purified by repeated silica gel CC eluted with n-hexane–acetone as eluent, FJ3 and FJ5 were purified by silica gel CC with n-hexane–EtOAc as eluent, and FJ12 was purified by low pressure C-18 CC with H2O–CH3OH elution. The yields of FJ1, FJ3, FJ5, FJ12 were 1.05%, 0.25%, 0.45%, 0.25%.
The cells grown in 6-well plates were treated with a kit (Beyotime Institute of Biotechnology, China) with fluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, U.S.A.) at 37°C in a humidified atmosphere containing 5% CO₂. All other chemicals utilized in the experiments were commercial products and of reagent grade.

Cell Culture The murine macrophage cell line RAW264.7, obtained from the Shanghai Institute for Biological Science (SIBS), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen, U.S.A.) at 37°C. The media were supplemented with 10% fetal bovine serum (TBD, Shanghai Biological Science (SIBS), was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Invitrogen, U.S.A.). Each 50 µL of DCFH-DA was added to each well for 4 h. The medium was removed, and the formazan precipitates were solubilized in 150 µL of DMSO. Optical density of each well was measured by a microtiter reader (Biorad 680) at 570 nm. Wells with DMSO but untreated with FJ1 were used for control cell viability and represented 100% cell survival, and wells with DMSO were considered the standard.

Nitrite Assay with a Griess Reagent The nitrite accumulation in the supernatant was assessed with a Griess reagent kit (Beyotime Inst., Haimen, China). According to the manufacturer’s instructions, each 50 µL of culture supernatant was mixed with 50 µL of Griess reagent I and 50 µL of Griess reagent II at room temperature. Absorbance was measured with a microplate reader (Bio-Rad 680) at 540 nm. A series of known concentrations of sodium nitrite was considered the standard. The difference between the values of the experimental and control samples represented the decrease in intracellular nitrite.

Cell Fractionation The cells were separated into two fractions consisting of a nucleus and cytosol with a nuclear and cytoplasmic protein extraction kit (Beyotime Inst., Haimen, China). The cells (2×10⁵) were washed with ice-cold phosphate buffered saline (PBS) and suspended in 200 µL of Reagent A containing 1 mM of phenylmethylsulfonylfluoride (PMSF). The cells were incubated in ice for 15 min after vortexing for 5 s. Then, 10 µL of Reagent B was added. The supernatant was the cytosol fraction. The nuclear pellets were resuspended in a nuclear protein reagent for 30 min at 4°C under agitation. The supernatants containing the nucleus proteins were collected for Western blot analysis after centrifugation (16000×g, 10 min at 4°C).

Western Blot Analysis The total cell lysates, cytosolic and nucleus proteins were analyzed by Western blot. The samples were subjected to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was washed in distilled water and then blocked with 5% nonfat milk in TBS-T (10 mM Tris–HCl at pH 7.8, 150 mM NaCl, and 0.05% [v/v] Tween-20 buffer, pH 7.8) for at least 1 h at room temperature. After a short washing in TBS-T buffer, the membrane was incubated overnight in a solution of monoclonal antibodies specific for extracellular signal-regulated kinase (ERK) 1/2, c-Jun terminal kinase (JNK), p38, p-ERK1/2, p-JNK, p-p38, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB/p65), inhibitor of nuclear factor-kappa B (IκB)-α, phosphorylated IκB-α (p-IκB-α), nitric oxide synthase (iNOS) and TNF-α (All 1:1000, Santa Cruz Biotechnology) at 4°C. Then the membrane was incubated with secondary horseradish peroxidase (HRP) goat anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (1:1000; Santa Cruz Biotechnology). Proteins on the membrane were detected with an enhanced chemiluminescence detection system (ECL®, Amersham Biosciences). Protein expression was quantified by densitometry with the Image J and Java software.

RNA Extraction and Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was extracted with an RNA easy kit (Biocon Bietec, China) according to the manufacturer’s instructions. The purity of the total RNA was evaluated by OD₂₆₀/₂₈₀ of the RNA samples (≥1.8). cDNA was synthesized through reverse transcription with an M-MLV reverse transcriptase and Oligo (dT) primer. The expression of iNOS, TNF-α, cyclooxygenase-2 (COX-2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes was detected through real-time PCR assay. PCR amplification was performed thrice in an 8-tube strip format (Axygen, CA, U.S.A.). Each reaction contained 1×SYBR Green PCR Master mix, 1 µL of forward primer and reverse primer and 1 µL of template cDNA in a final volume of 20 µL. A Mastercycler ep realplex apparatus (Eppendorf, Germany) was utilized. Primers were based on the iNOS gene (forward: 5’-CGC TAC AAC ATC CTC GTG GAG GAA GT-3’ and reverse: 5’-GTC TTT CCA GAG CGA GGC CAG-3’), TNF-α (forward 5’-CAT CTT CTC AAA ATT CGA GTG ACA A-3’ and reverse 5’-TGG GAG TAG ACA AGG TAG AAC CC-3’), and COX-2 (forward: 5’-TGA GTA CCG CAA ACG CTT CTC-3’ and reverse: 5’-TGG AGG AGG TTT TAC CAG-3’). The evaluation of the GAPDH expression utilized as a control for the RNA amount was conducted with a primer (forward: 5’-GAC GGC CGC ATC TTC TTG T-3’ and reverse: 5’-CAC ACC GAC...
NO production in a concentration-dependent manner. The inhibition rates were 28.61%, 43.42%, and 71.80% at the concentration of FJ1, FJ3, FJ5, FJ12 significantly inhibited LPS-stimulated NO production from 0.1 mg/mL to 100 mg/mL resulting in slight toxicity but exhibited a more significant NO production compared with the vehicle control. Figure 1D showed that FJ1 with and without LPS pretreatment did not display significant cytotoxicity up to 15 μM in RAW264.7 cells, the cell viability with LPS was 96.37%, 89.78%, 82.55%, 83.68% and without LPS was 100%, 96.07%, 92.19%, 94.44%, respectively. This finding suggests that the anti-inflammatory effect of FJ1 is not related to cytotoxicity.

**Effect of FJ1 on LPS-Induced ROS Production** Considering that ROS is important for LPS-induced inflammation, we investigated whether FJ1 inhibits LPS-induced intracellular accumulation of ROS. The results of the flow cytometric assay indicate that a greater ROS accumulation was occurred in the LPS-treated RAW264.7 cells compared to the cells that had not been treated with FJ1. The increase in LPS-stimulated ROS levels was significantly attenuated by FJ1 in a dose-dependent manner (Fig. 2).

**FJ1 Inhibits LPS-Induced Activation of Mitogen-Activated Protein Kinase (MAPK) and NF-κB** Many signaling pathways, such as MAPK and NF-κB pathways, are involved in the regulation of microglial activity and contribute to the production of inflammatory mediators. The phosphorylation of three MAPK molecules, ERK1/2, p38, and JNK, were examined in this study by Western blot analysis. LPS can stimulate the phosphorylation of ERK1/2, p38, and JNK in RAW264.7 cells. FJ1 suppressed the expression of p-ERK, p-JNK, and p-p38 in a concentration dependent manner in LPS-stimulated macrophages. These results indicate that signal transduction by the MAPK molecules was blocked by FJ1 in LPS-activated macrophages (Fig. 3).

NF-κB is also a well-known critical transcription factor in the induction of a wide variety of genes involved in the regulation of inflammation.
lation of immune and inflammatory responses. We determined NF-κB/p65, IκB-α, and phosphorylation of IκB-α by Western blot analysis to investigate the effect of FJ1 on the stimulation of NF-κB activity in RAW264.7 cells. Figure 4 shows that the FJ1-treated RAW264.7 cells reduced the expression of NF-κB/p65 in the nucleus and augmented the phosphorylation of IκB-α stimulated by LPS in the cytosol in a dose-dependent manner.

**Effect of FJ1 on Inflammation-Related Genes and Proteins in RAW264.7 Cells**

The above data demonstrated the inflammation-associated activities and NF-κB pathway activities of FJ1 induced in RAW264.7 cells. Further experiments show that FJ1 has effects on NF-κB-related downstream inflammation genes. The expressions of iNOS, TNF-α, and COX-2 genes in RAW264.7 cells were evaluated by quantitative real-time PCR after incubating FJ1 with and without pretreatment with LPS for 24 h. Besides, the expressions of iNOS and TNF-α proteins were checked using a Western blot analysis. Figures 5A, B, and C indicated that FJ1 significantly reduced the LPS-induced upregulation of iNOS, COX-2, and TNF-α mRNA levels, consisting with the expression of iNOS and TNF-α proteins (Figs. 5D, E), which further confirmed the distinct effects of FJ1 on the modulation of LPS-induced inflammation.

**DISCUSSION**

Inflammation is a complex biological process. Macrophages play a critical role in the initiation, maintenance and resolution as well as the overproduction of pro-inflammatory cytokines and inflammatory mediators such as NO, ROS, COX-2, and TNF-α. NO, the broadest screening inflammatory mediator, is the product of inducible iNOS. NO production triggers a rapid but transient release of ROS and activates the inflammatory pathways through redox reactions. Many reports have demonstrated LPS-stimulated inflammatory responses in mouse macrophage RAW264.7 cells. Inflammatory response stimulated by LPS is a suitable method to identify anti-inflammatory activity compounds in vitro. LPS stimulates macrophages and activates the expression of genes responsible for the synthesis of inflammatory mediators, such as reactive oxygen and nitrogen species. These free radicals are important mediators to provoke or sustain inflammatory processes, thereby protecting hosts from harmful stimuli. However, high levels of these free radicals can damage cells and tissues resulting in inflammation.

This study explores the anti-inflammatory components of the Chinese herb *F. japonicum*, which is used to treat various inflammatory diseases. We perform the drug screening
Fig. 4. Effect of FJ1 on the LPS-Induced Nucleus Translocation of NF-κB/p65 and Degradation and Phosphorylation of IκB-α

The cells were pretreated with FJ1 for 2h and then stimulated with LPS (3µg/mL) for 1h. (A) The decreased expression of NF-κB/p65 in the nucleus fraction was detected through Western blotting and its corresponding histogram quantified through densitometry. (B) The increased expression of IκB-α in the cytosolic extracts was determined through Western blot and its corresponding histogram-quantified through densitometry. Actin protein was utilized as an internal control, and experiments with similar results were repeated at least three times. *p<0.05 and **p<0.01 compared with the LPS only group.

Fig. 5. Effect of FJ1 on Inflammation-Related Genes and Proteins in LPS-Stimulated RAW264.7 Cells

The RAW264.7 cells were pre-incubated with different concentrations of FJ1 for 2h and then treated with LPS (3µg/mL) for another 24h. The mRNA expressions of iNOS (A), COX-2 (B), and TNF-α (C) were analyzed through real-time PCR (RT-PCR). The protein expressions of iNOS and TNF-α (D) were checked by Western blot. The corresponding histograms quantified through densitometry are presented on the right side (E). Data are expressed as means±S.D. with three independent experiments. **p<0.01 compared with the LPS only group.
process of compounds isolated from *F. japonicum* by LPS-stimulated RAW264.7 cells. We utilize a Griess reagent kit to screen the compounds. The compounds (FJ1, FJ3, FJ5, and FJ12) effectively inhibited the LPS-stimulated production of NO in RAW264.7 cells (Fig. 1C). Among these compounds, FJ1 exhibited the strongest anti-inflammatory ability with the highest NO inhibition rates of 28.61%, 43.42%, and 71.80% at concentrations of 5 \( \mu M \), 10 \( \mu M \), and 15 \( \mu M \), respectively. Further results by flow cytometry analysis demonstrate the capacity of FJ1 to decrease intracellular ROS production.

MAPK and NF-\( \kappa B \) are known to regulate the expression of inflammatory mediators. Primary inflammatory stimuli and cytokines come into work through the Toll receptors, IL-1 receptor (TIR) family, or the TNF receptor family. The activation of receptors triggers major intracellular signaling pathways: MAPK pathways and the pathway leading to the activation of the transcription factor NF-\( \kappa B \). There are three major groups of distinctly regulated MAPK cascades, ERK1/2, JNK, and p38 MAPK, regulating gene expression in humans. NF-\( \kappa B \) is a multi-protein complex that can activate a variety of genes involved in the early defense reactions of higher organisms. NF-\( \kappa B \) resides in the cytoplasm as an inactive complex with the inhibitor I\( \kappa B \) in non-stimulated cells. Various stimuli, including LPS, cytokines, and viruses, activate NF-\( \kappa B \) via several signal transduction pathways, leading to the phosphorylation of I\( \kappa B \). The phosphorylated I\( \kappa B \) becomes rapidly ubiquitinated and degraded by the proteasome complex. Following I\( \kappa B \) degradation, NF-\( \kappa B \) translocates to the nucleus, binds to DNA control elements, and induces the synthesis of various types of mRNA. The transcription factors present in the cytoplasm or nucleus are phosphorylated and activated upon the activation of MAPK and NF-\( \kappa B \), leading to the expression of target genes and resulting in a biological response.

Therefore, the two major kinase-mediated signaling pathways activated by LPS stimulation, namely, MAPK and NF-\( \kappa B \), are examined to explore the possible underlying mechanism of FJ1. Western blot analysis indicates that LPS enhanced the phosphorylation of the three MAPK molecules: ERK1/2, p38, and JNK. Treatment of FJ1 significantly inhibited the LPS-stimulated phosphorylation of ERK1/2, p38, and JNK, which are responsible for triggering over-reactive inflammatory responses. Next, we perform Western blot analysis of sub-cellular fractions to detect the NF-\( \kappa B \) signal. FJ1 significantly inhibited the LPS-induced phosphorylation and degradation of I\( \kappa B \)-\( \alpha \) as well as the translocation of activated NF-\( \kappa B \)/p65 to the nucleus in RAW264.7 cells.

The activation of MAPK and NF-\( \kappa B \) contributes to the expression of inflammatory genes in activated macrophages. Reports exhibited that MAPK pathway positively regulates the stability of several inflammatory mediator mRNAs, including TNF-\( \alpha \) and COX-2.\(^{20,22}\) Several anti-oxidant compounds are also known to reduce inflammation by inhibiting the expression of cytokines consistent with NF-\( \kappa B \).\(^{30}\) The suppressive effects of these anti-oxidant compounds on the production of associated inflammatory mediators confirm that ROS enhances the expression of pro-inflammatory cytokine genes by activating NF-\( \kappa B \).\(^{31}\) The upregulations of mRNA and protein levels involving LPS-induced TNF-\( \alpha \), iNOS, and COX-2, were strongly attenuated by FJ1 in RAW264.7 cells. FJ1 also inhibited the production of LPS-induced ROS and NO in the same cells. The potential mechanism is associated with MAPK and NF-\( \kappa B \) activity in RAW264.7 cells.

In conclusion, the results of this study suggest that FJ1 possesses anti-inflammatory effects that decrease NO and ROS. We identified the anti-inflammatory mechanisms of FJ1 by modulating MAPK and NF-\( \kappa B \) signaling pathways.

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**Conflict of Interest** The authors declare no conflict of interest.

### Supplementary Materials

The online version of this article contains supplementary materials.

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