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Anti-inflammatory Effect of *Ribes fasciculatum* in IFN-γ/LPS-stimulated Mouse Peritoneal Macrophage

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Abstract – *Ribes fasciculatum* which belongs to Saxifragaceae has been widely used as a traditional medicine for the treatment of symptoms associated with lacquer poison. However, pharmacological studies on the *R. fasciculatum* are extremely limited until now. Thus, in this study, we evaluated the possible anti-inflammatory effects of ethyl acetate fraction of *R. fasciculatum* (ERF) using IFN-γ/LPS-stimulated peritoneal macrophage model. We investigated the change in nitrite level in the absence or presence of ERF after LPS stimulation, and we found that ERF effectively attenuates the NO production in a dose dependent manner without notable toxicity. To determine the mechanism of the inhibitory action of ERF on NO production, we performed iNOS enzyme activity assay and Western blotting. Here we showed that both of iNOS enzyme activities and iNOS expressions were significantly down-regulated by ERF, indicating that these dual activities of ERF are responsible for ERF-mediated NO suppression. In addition, ERF inhibitied the expression of cyclooxygenase-2 (COX-2), an another key enzyme in inflammation through suppression of NF-κB activation. We also tested anti-inflammatory properties of ERF not only *in vitro*, but *in vivo* using trypsin-induced paw edema model in mice. Our results revealed that the increased paw volume in response to trypsin injection was recovered by ERF supplement dose dependently.

Keywords – *Ribes fasciculatum*, Inflammation, Nitric oxide, Edema

Introduction

Inflammation is defined as a response of living tissue to outer stimuli or local injury.[1] Although physiological activity of inflammation may provide a distinct benefit to the organism by minimizing the damage by infection or irritation, excessive inflammatory response has been implicated in the pathogenesis of many disease including rheumatoid arthritis, asthma and cancer.[2] Macrophages play a central role in innate and adaptive immunity and act as a mediator of the inflammatory responses.[3] Macrophages are known to play a central role in inflammation as a major immune cell. These cells can be activated by different inflammatory stimuli such as inflammatory cytokines and bacterial endotoxic lipopolysaccharide (LPS) results in providing inflammatory mediators such as nitric oxide (NO), prostagladins (PGs) and cytokines.

NO participates in various physiological functions such as vasodilation, neurotransmission, and inflammation.[4,5] NO can be synthesized by one of three different kind of NO synthetases (NOS) including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS).[6] Among them, iNOS is expressed in response to several inflammatory mediators and produces large amount of NO which can cause tissue damage during inflammatory response.[7,8] Similar to NOS, cyclooxygenase (COX), an another pro-inflammatory mediator has two isoforms, that is, COX-1 and COX-2. While COX-1 is expressed at a constant level and involved in normal physiologic functions, the inducible COX-2 is responsible for the production of pro-inflammatory prostaglandins (PGs) at the inflammatory site.[9] In addition, it is well known that expressions of pro-inflammatory mediators such as iNOS and COX-2 is predominantly regulated by the ubiquitously expressed

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nuclear factor kappa B (NF-κB) which is required for the inducible expression of genes associated with inflammatory responses. Thus, suppression of NF-κB activation may be a reasonable target for anti-inflammatory drugs.

Ribes fasciculatum (Saxifragaceae) is widely distributed throughout area, japan, and china. This plant has been used as folk medicine to treat cough, antidote, cold, lacquer poison, sore throat. However, pharmacological studies on this plant are extremly limited. Thus, here in this study we validate the anti-inflammatory action of ERF by checking the production and expression of pro-inflammatory mediators in the IFN-γ/LPS-stimulated murine peritoneal macrophages In addition, we also confirmed ERF's anti-inflammatory activities *in vivo* with trypsin-induced paw edema test in mice.

Experimental

Reagents – Murine recombinant IFN-γ was purchased from Pharmingen (Munchen, Germany). LPS, sodium nitrite and Nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO, USA). Thioglycollate (TG), Phosphate buffered saline (PBS) and Bovine serum albumin (BSA) was purchased from Gibco Laboratories (Detroit, MI, USA). 0.4 μm syringe filter and tissue culture plates of 96 wells, 24 wells and 60-mm, 100-mm diameter dishes were purchased from Nunc (Naperville, IL, USA). DMEM containing L-arginine (84 mg/l), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY, USA).

Animals – Male C57BL/6 (6 weeks old) and ICR (6 weeks old) mice were purchased from Damul Science Co. (Daejeon, South Korea). All animals were housed at 22 ± 1 °C with a 12 h light/dark cycle maintaining humidity at 50% and fed a standard pellet diet with tap water *ad libitum.*

Plant material and sample preparation – The plant materials were purchased from Hainyakupsa (Jeonbuk, South Korea) in March 2012. A voucher specimen (WH083) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried sample (3 kg) with 12,000 mL of MeOH under sonification for 2 h. The resultant methanolic extract was concentrated into 100.64 g (Yield : 3.021%) using a rotary evaporator. The sample was then subjected to successive solvent partitioning to give *n*-hexane (10.4 g), CH_2Cl_2 (8.5 g), EtOAc (5.4 g) and *n*-BuOH (24.1 g) soluble fractions. Each fractions were lyophilized and then stored at –20 °C until use. The preliminary experiments showed that among the four fractions, the EtOAc fraction (ERF) has the most potent pharmacological potential, and therefore, further studies were conducted using ERF (Fig. 2A).

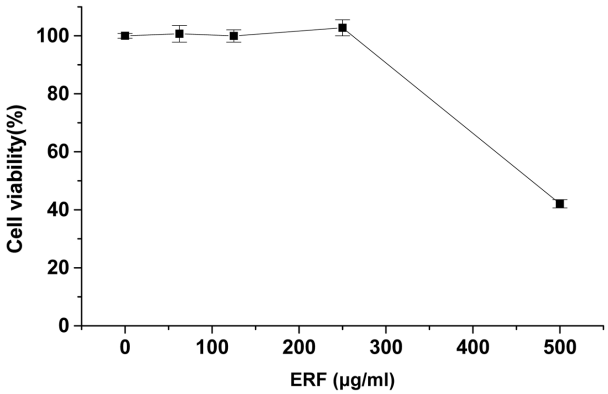

Fig. 1. Effects of ERF on the viability in IFN-γ/LPS-treated peritoneal macrophages. Various concentrations of ERF-treated peritoneal macrophages (3×10^5 cells/well) were primed for 6h with IFN-γ (20 U/mL). The peritoneal macrophages were then stimulated with LPS (10 μg/mL) for 24h. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means ± S.D. of three independent experiments duplicate in each run.

Peritoneal macrophage culture – TG-elicited macrophages were harvested 3 days after i.p. injection of 2.5 mL TG to the mice and isolated. Using 8 mL of PBS containing 10 U/mL heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 24-well tissue culture plates (3×10^5 cells/well) incubated for 3 h at 37 °C in an atmosphere of 5% CO_2, washed three times with PBS to remove non-adherent cells and equilibrated with DMEM that contained 10% FBS before treatment.

MTT assay – Cell respiration, an indicator of cell viability, was performed by the mitochondrial dependent reduction of 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, as described by Mosmann.[10] The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density (OD) at 570 nm using an automated microplate reader (GENios, Tecan, Austria).

Assay of nitrite concentration – Peritoneal macrophages (3×10^5 cells/well) were cultured with various concentrations of ERF (0.1, 1, 10, and 100 μg/mL). The cells were then stimulated with rIFN-γ (20 U/mL). After 6 h, the cells were finally treated with LPS (10 μg/mL). NO synthesis in cell cultures was measured by a microplate assay method. To measure nitrite, 100 μL aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent at room temperature for

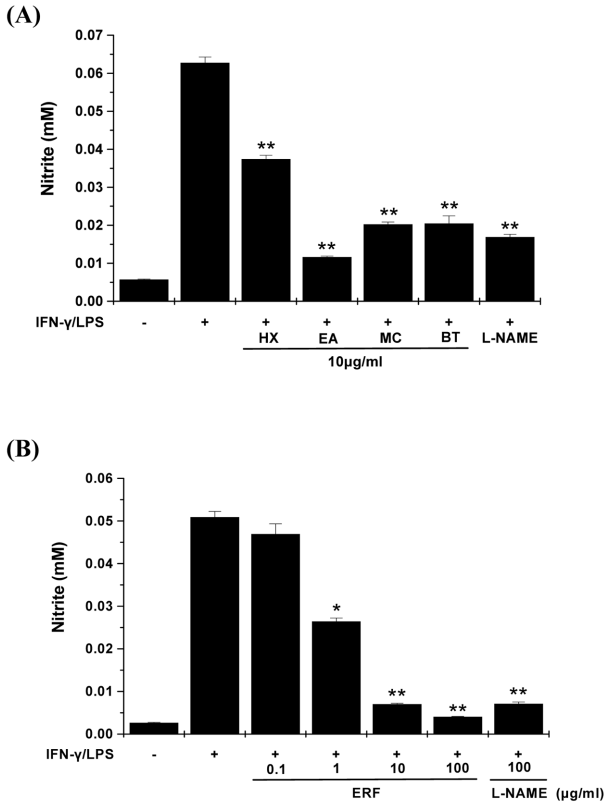

Fig. 2. Effects of the fractions of *R. fasciculatum* and ERF on NO production in IFN-γ/LPS-treated peritoneal macrophages. Peritoenal macrophages (3×10^5 cells/well) were cultured with various concentration of ERF. The peritoneal macrophages were then stimulated with IFN-γ (20 U/mL) and LPS (10 μg/mL). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.D. of three independent experiments duplicate in each run; $*p < 0.01$ and $**p < 0.001$ compared to IFN-γ + LPS.

10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2^- was determined by using sodium nitrite as a standard. And Nitro-L-arginine methyl ester (L-NAME) was used as a reference drug.

Measurement of iNOS enzyme activity – To determine whether ERF affected the iNOS enzyme activity, iNOS enzyme activity was conducted as described by Israf with minor modification.[11] Cells were induced to produce iNOS over a 12 h period with rIFN-γ (20 U/mL) and LPS (10 μg/mL). After 12 h the medium was discarded and changed. Then, cells were treated with various concentrations of ERF, and incubated another 12 h. Supernatants were removed and levels of nitrite were determined using the Griess reagent as describe previously.

Western blot analysis – Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% BSA solution for 3 h at room temperature and then incubated with anti-iNOS, COX-2 (SantaCruz, CA, USA). After washing in with phosphate buffered saline (PBS) containing 0.05% tween 20 three times, the blot was incubated with secondary antibody (anti-mouse, anti-rabbit) for 1 h and the antibody specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, Germany).

Trypsin-induced paw edema – Edema was induced in the right-hind paw by a 30 μL intraplantar (i.pl.) injection of trypsin (30 μg/paw, prepared in saline). The left paw received 30 μL of saline and it was used as the control. Edema was measured with a plethysmometer (LE7500, Panlab, Spain), at 1 h after injection of trypsin. Edema was expressed as the difference between the right and left paws.

Densitometric and statistical analysis – All measurement are expressed as the mean ± S.D. of independent experiments. Data between groups were analyzed by a paired Student's *t*-test and *p*-values less than 0.01 were considered significant. Intensity of the bands obtained from Western blotting was estimated with Image Quant TL (GE Healthcare, Sweden).

Results and Discussion

Here in this work, we evaluated ERF's anti-inflammatory properties *in vitro* and *in vivo* using IFN-γ/ LPS-stimulated murine peritoneal macrophage model and trypsin-induced paw edema model, respectively. In macrophage, NO is synthesized from L-arginine and contributes to the immune defense against viruses, bacteria and other parasites.[6,12] However, large amount of NO act as a toxic radical and can cause tissue and cell damage results in rheumatoid arthritis, gastritis, autoimmune diseases.[13-15] Therefore, down-regulation of NO production can be one of the desirable ways to avoid excess inflammatory responses. In this work, we pretreated the macrophages with various concentration of ERF (0.1, 1, 10, and 100 μg/mL), and then, stimulated with IFN-γ (20 U/mL) and LPS (10 μg/mL). After 48 h incubation, the cell supernatants were collected and added to Griess Reagent to determine nitrite concentration. As shown in Fig. 2, LPS-induced NO production was inhibited dose-dependently by ERF. Interestingly, high concentration of ERF (100 μg/mL) perfectly suppressed NO production compared to positive

control, L-NAME (Fig. 2B). At the treatment concentration, no notable cytotoxicity was detected, and thus, we could exclude the possibility that ERF's NO inhibitory action was due to cytotoxicity (Fig. 1).

Since excess production of NO is achieved by iNOS in activated macrophages, suppression of iNOS expression levels might be an attractive therapeutic target for the treatment of NO-mediated inflammatory condition. To verify whether ERF might suppress the iNOS enzyme activities, we performed iNOS enzyme activity assay. Fig. 3A shows that the iNOS enzyme activity was inhibited by L-NAME, a well-known iNOS inhibitor. Interestingly, ERF also significantly attenuated the iNOS enzyme activity at the maximum concentration (Fig. 3A). Next, we checked whether ERF might shift the iNOS expression by Western blotting. As can be seen in Fig. 3B, the expression of iNOS protein were markedly increased after rIFN-γ plus LPS challenge and this enhanced expression of iNOS protein was significantly reduced by ERF exposure. Taken together, we could conclude that a dual mechanism that both of direct scavenging of NO radical and attenuation of iNOS protein mediates the ERF's NO inhibitory effects.

Cyclooxygenase-2 (COX-2), another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. Since, COX-2-meidated high levels of PGE_2 take an important part in inflammatory conditions, COX-2 inhibitors might be candidates for the new type of nonsteroidal anti-inflammatory drugs (NSAIDs). In this regards, the possibility that ERF might inhibit COX-2 expression was examined and ERF suppressed the expression of COX-2 in a dose dependent manner (Fig. 3B). However, further studies are required to determine whether ERF is selective COX-2 inhibitor.

Then, we also evaluated the influence of ERF on the NF-κB activation. Because many previous studies have demonstrated that NF-κB is an important transcriptional factor for the expression of various inflammatory mediators. In unstimulated cells, inactive NF-κB is sequestered in the cytoplasm and is linked to the inhibitory IκB protein. However, in active state it translocate to the nucleus with phosphorylation, ubiquitation and degradation of IêBá and also acts upon the pro-inflammatory gene promoter to activate transcription. As shown in Fig. 4, translocation of NF-κB to nucleus was blocked significantly in the presence of ERF. These findings strongly suggest that ERF inhibited the pro-inflammatory mediators in the activated macrophage by suppressing transcriptional activity of NF-κB.

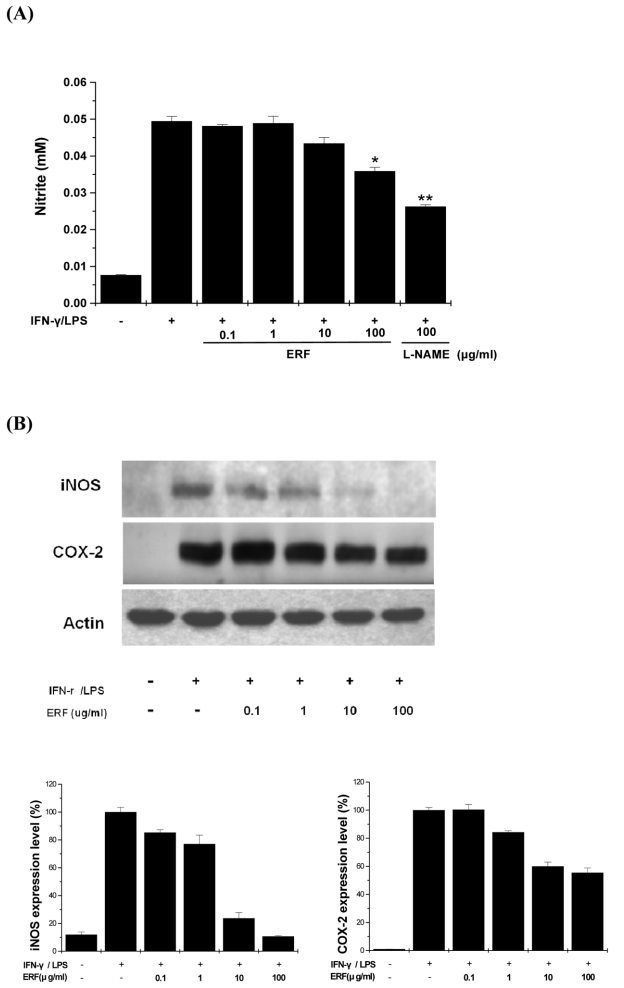

Fig. 3. Effects of ERF on iNOS enzyme activity and expressions of iNOS and COX-2. iNOS enzyme activity was measured by stimulating peritoneal macrophages (3×10^5 cells/well) with IFN-γ (20 U/mL) for 6 h, then stimulated with LPS (10 μg/mL). After 12 h, various concentrations of ERF were treated and incubated for another 12 h. The supernatant were obtained and measured by the Griess method as described previously. iNOS and COX-2 expression were measured as follows. Peritoneal macrophages (5×10^6 cells/well) were pretreated with ERF and then stimulated for 6 h with IFN-γ (20 U/mL). The peritoneal macrophages were then stimulated with LPS (10 μg/mL) for 24 h. The protein extracts were prepared and samples were analyzed for iNOS and COX-2 expression by Western blotting as described in the method. Determined expression of iNOS and COX-2 was subsequently quantified by densitometric analysis with that of IFN-γ/LPS treated control being 100% as shown just below the gel data.

Next, we further confirmed ERF's anti-inflammatory activity *in vivo* with trypsin-induced paw edema model in mice. Subplantar injection of trypsin, a proteinase-activated receptor2 (PAR2) agonist, induces an increase in vascular permeability as well as in the marked infiltration of granulocytes.[16] In addition, trypsin is able to evoke the classic signals of inflammation, mainly by the secondary

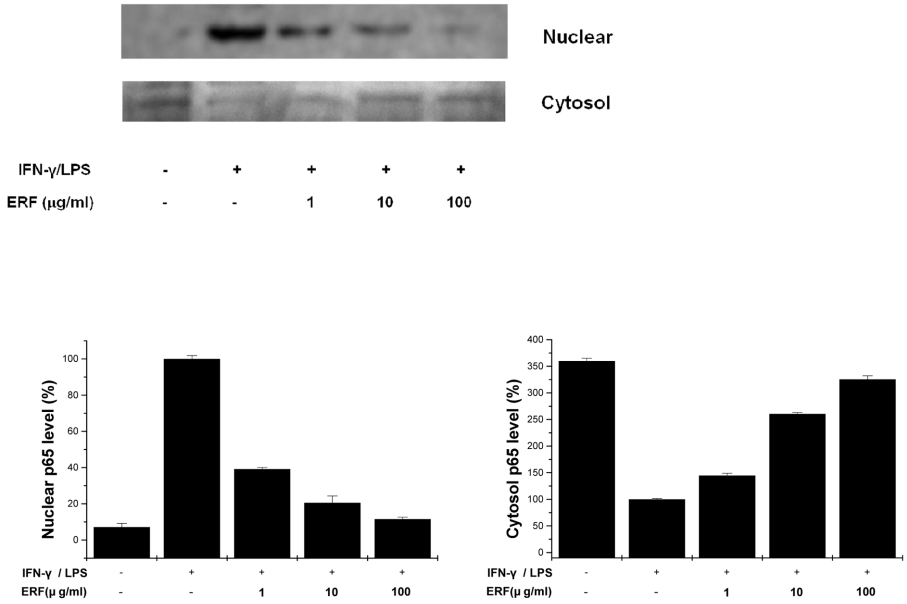

Fig. 4. Inhibitory effect of ERF on NF-κB translocation by LPS-stimulated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with ERF. After 30 min, the cells were stimulated with rIFN-γ (20 U/ml) for 30 min. The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 2 h. The nuclear extracts were prepared; samples were analyzed by western blotting as described in the method and quantified by densitometry.

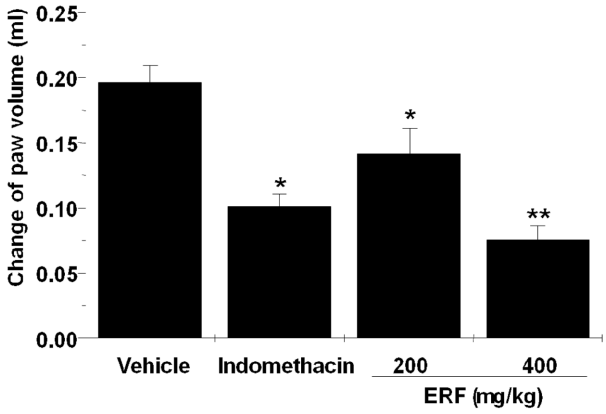

Fig. 5. Effects of ERF on trypsin-induced paw edema in mice. Mice were orally pre-treated with distilled water, Indomethacin (10 mg/kg), ERF (200, 400 mg/kg), respectively. After 1 h, the trypsin solution was injected in a volume of 30 μL subplantarly. Trypsin was dissolved in sterile 0.9% saline. The size of edema was assessed by measuring the volume of the hindpaw immediately before and 1 h after the agonist injection. Data show the mean ± S.E.M. (n = 8 – 12). *$p < 0.01$ and **$p < 0.001$ compared to the control group.

production of several inflammatory mediators. Herein, we showed that ERF reduced the paw volume about 20% at 200 mg/kg and 50% at 400 mg/kg concentration (Fig. 4). Paszcuk et al. reported that the edematogenic response is likely related to the production of COX-2,[17] and thus, we speculated that the ERF might block trypsin-induced paw edema through inhibition of COX-2 expression, at least in part.

Previously, several compounds were isolated from *R. fasciculatum* including threo-(7S,8R)-1-(4-hydroxyphenyl)-2-[4-(E)-propenylphenoxy]-propan-1-ol, octadecanyl 3-(4-hydroxy-3-methoxy-phenyl)-acrylate ester, catechin, and their inhibitory activities on the nuclear factor of activated T cells (NFAT) activation were demonstrated.[18] Since excessive activation of NFAT is related with immunopathological conditions including autoimmunity and inflammation, our result that shows the anti-inflammatory properties of *R. fasciculatum* is consistent with the findings of Dat's group.

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