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Anti-inflammatory activities of ethanol extract and its various solvent fractions from edible freshwater green algae, *Prasiola japonica*, in LPS-induced macrophages and carrageenan-induced paw edema in AP-1 pathway

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Abstract: *Prasiola japonica* has been described to possess several biological activities. However, the anti-inflammatory activities and its molecular mechanisms using different solvent fractions remain limited. In this study, we investigated the potential anti-inflammatory activities of *P. japonica* ethanol extract (Pj-EE) and four solvent fractions (hexane (Pj-EE-HF), chloroform (Pj-EE-CF), butanol (Pj-EE-BF), and water (Pj-EE-WF)) *in vitro* (LPS-induced macrophage-like RAW264.7 cells) and *in vivo* (carrageenan-induced acute paw edema mouse models). The active fraction was selected to determine the further activities. Various *in vitro* and *in vivo* assessments, including nitric oxide (NO) and cytokines assays, luciferase assay, real-time PCR, and immunoblotting analyses were performed to evaluate its underlying mechanisms. In addition, the phytochemical constituents were characterized by LC-MS/MS. In *in vitro* studies, the highest inhibition of NO production was observed in the chloroform fraction of Pj-EE. Eventually, further examination revealed that Pj-EE-CF decreased the expression of inflammation-related cytokines on LPS-induced RAW264.7 cells, and suppressed AP-1-luciferase activity following by inhibition of the phosphorylation events in the AP-1 signaling pathway. Pj-EE-CF treatment also demonstrated the strongest reducing thickness and volume of carrageenan-induced paw edema, while butanol fraction showed the lowest activity. Furthermore, Pj-EE-CF also reduced gene expression and cytokines production in tissue lysates of carrageenan-induced paw edema. These findings support and validate the evidence that Pj-EE could be a good natural source as an anti-inflammatory agent especially the chloroform fraction by targetting the AP1-pathway.

Keywords: *Prasiola japonica*; anti-inflammatory; paw edema; AP-1 pathway

1. Introduction

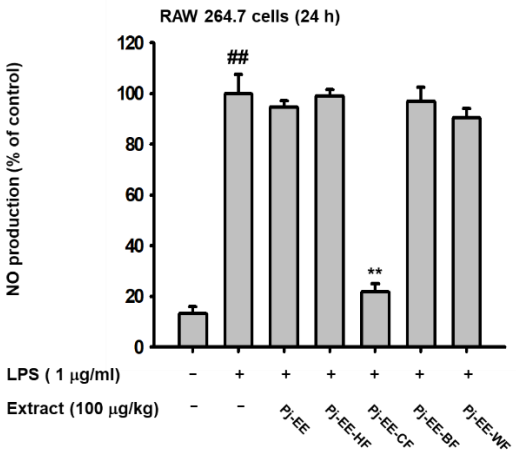
Inflammation is the protective response of the immune system against various pathogens and cellular danger signals, mediated primarily by immune cells such as macrophages. Acute inflammation is a process that involved the activation of complex enzymes, secretion of free radicals, and release of several inflammatory and pro-inflammatory mediators, which are commonly characterized by redness, swelling, pain, heat, and loss of tissue function [1-3]. The inflammatory response happens through the interaction of either pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) with pattern recognition receptors (PRRs) [1,4]. For instance, engagement of PAMPs such as gram-negative bacteria-derived lipopolysaccharides (LPS) or carrageenan into TLR-4 will induce recruitment of adaptor proteins, MyD88 and TRIF, into the cytoplasm that eventually triggers inflammatory signaling cascades, including activator protein-1 (AP-1) pathway [2,5,6]. The activation of the inflammatory signaling pathway is mediated by the initiation of signal transduction cascades activating a variety of intracellular signaling molecules. Three major mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) mediate nuclear translocation and activate transcription factors in the AP-1 pathway [3,7]. Activation of this transcription factor induces the expression of numerous inflammatory genes, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-6, IL-1β, and tumor necrosis factor-alpha (TNF-α), which will also stimulate matrix metalloproteinases (MMPs) expression. Subsequently releasing the production of the inflammatory mediator, nitric oxide (NO) and various cytokines [4,8-11]. Even though inflammation is a protective immune response, uncontrolled acute inflammation may become chronic, contributing to a variety of chronic inflammatory diseases. Several studies have provided evidence that inflammation is involved in the pathogenesis of many diseases including autoimmune, cancer, and other life-threatening disorders [2,3,5,12]. Therefore, various efforts including developing anti-inflammatory agents to regulate inflammatory responses and authenticating to specifically target the molecular signaling could potentially ameliorate various inflammatory diseases.

Some green algae (phylum *Chlorophyta*), are considered as one of the representative natural sources to be applied such as pharmaceutical, nutraceutical, and cosmeceutical products [13,14]. The green algae, especially from the genus *Prasiola*, show a diverse distribution, with at least 14 of 36 species are freshwater organisms, and others growing in terrestrial habitats. *Prasiola japonica* belongs to the family *Prasiolaceae* and is found growing in the freshwater ecosystem [15]. *P. japonica* has been reported in east Asia, especially Japan and the Republic of Korea [16,17]. This alga is known to obtain various components including mannitol, loliolide, glucitol, alverine, diisopropylamine, and methyl pyrazine [18]. In recent years, the freshwater green algae, *Prasiola japonica*, has been described to possess medicinal benefits including anti-oxidant, anti-apoptotic, anti-melanogenic, and anti-inflammatory. However, most of those studies were limited to identify such effects of the crude ethanolic extract of *P. japonica* in the NF-κB pathway and mainly focused on the skin cell lines [13,18-20]. To the best of our knowledge, the present study is the first to describe the potential anti-inflammatory activities of *P. japonica* ethanol extract (Pj-EE) and four solvent fractions (hexane (Pj-EE-HF), chloroform (Pj-EE-CF), butanol (Pj-EE-BF), and water (Pj-EE-WF)) *in vitro* (LPS-induced macrophage-like RAW264.7 cells) thereby the active fraction, Pj-EE-CF, was selected to determine the further activities, and *in vivo* (carrageenan-induced acute paw edema mouse models). Its molecular targets and its phytochemical constituents were also evaluated.

2. Results

2.1. Effect of Pj-EE and its various solvent fractions on the NO production of LPS-induced macrophages

To initially determine whether Pj-EE and the four solvent fractions have anti-inflammatory potential, we tested the production level of an inflammatory mediator such as NO on LPS-induced macrophage with the same concentrations of Pj-EE and its fractions (100 μg/ml). As demonstrated in Figure 1a, LPS significantly increased ($p = 0.0021$) the secretion of NO in macrophage-like RAW264.7 cells. Meanwhile, 100 μg/ml of Pj-EE-CF had the strongest effect ($p = 0.0071$) of the secretion of NO up to 80% inhibition on LPS-induced RAW264.7 cells. Whereas, treatment with Pj-EE, Pj-EE-HF, Pj-EE-BF, and Pj-EE-WF did not exert significant inhibition. Furthermore, using a conventional MTT assay, we checked the cell viability on macrophage-like RAW264.7 cells upon treatment with Pj-EE and the four solvent fractions. However, treatment with Pj-EE-CF at a concentration that significantly reduced NO production indicated an interference of cell viability up to 25% (Figure 1b). Consequently, we sought to identify the potential effect of Pj-EE-CF at a lower concentration than 100 μg/ml that did not exert cytotoxicity. As shown in Figure 1c, treatment with Pj-EE-CF did not suppress cell viability up to 50 μg/ml. Conversely, 100 μg/ml and 200 μg/ml of Pj-EE-CF treatment significantly decreased cell viability up to 80%. Further to check whether a lower concentration than 100 μg/ml of Pj-EE-CF also has anti-inflammatory activity, we re-evaluate the secretion of NO on LPS-induced in RAW264.7 cells. The result showed that pre-treatment with Pj-EE-CF concentration-dependently and significantly suppressed secretion of NO production at concentration 25 and 50 μg/ml ($p = 0.0134$ and $p = 0.0044$, respectively) (Figure 1d). These suggest that treatment with Pj-EE-CF up to 50 μg/ml affects NO production and has anti-inflammatory potential were not due to cell death. Therefore, Pj-EE-CF was chosen to complete further experiments. Ultimately, we performed LC/MS-MS analysis to determine the phytochemical constituents of chloroform fraction of Pj-EE, especially parameter assayed for flavonoids. Around 23 components from various flavonoid classes were observed, including the presence of maltol, at the retention time (RT) of 0.84, bavachinin at RT 6.11, flavonol at RT 6.42, 3'-deoxysappanone B at RT 8.20, kushenol N, X, at RT 10.61, 11.62, respectively, nobiletin at RT 12.64, and phellochinin A at RT 14.53 as characterized in Figure 1e and Supplementary Table S1.



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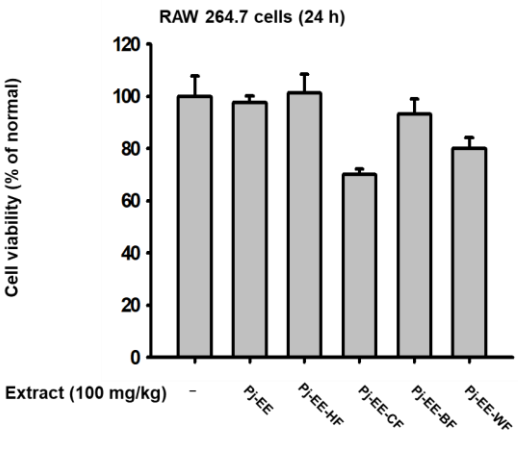

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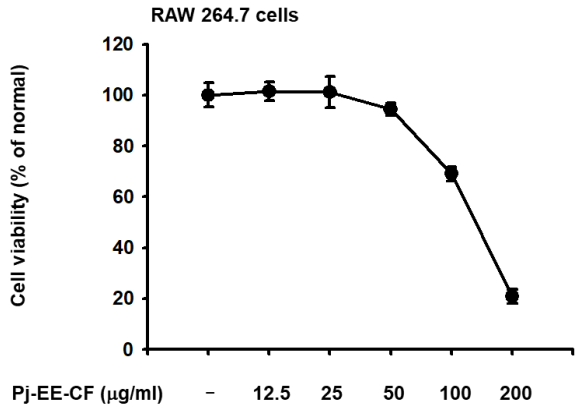

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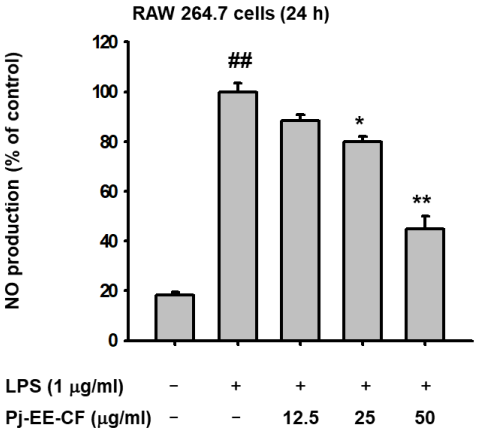

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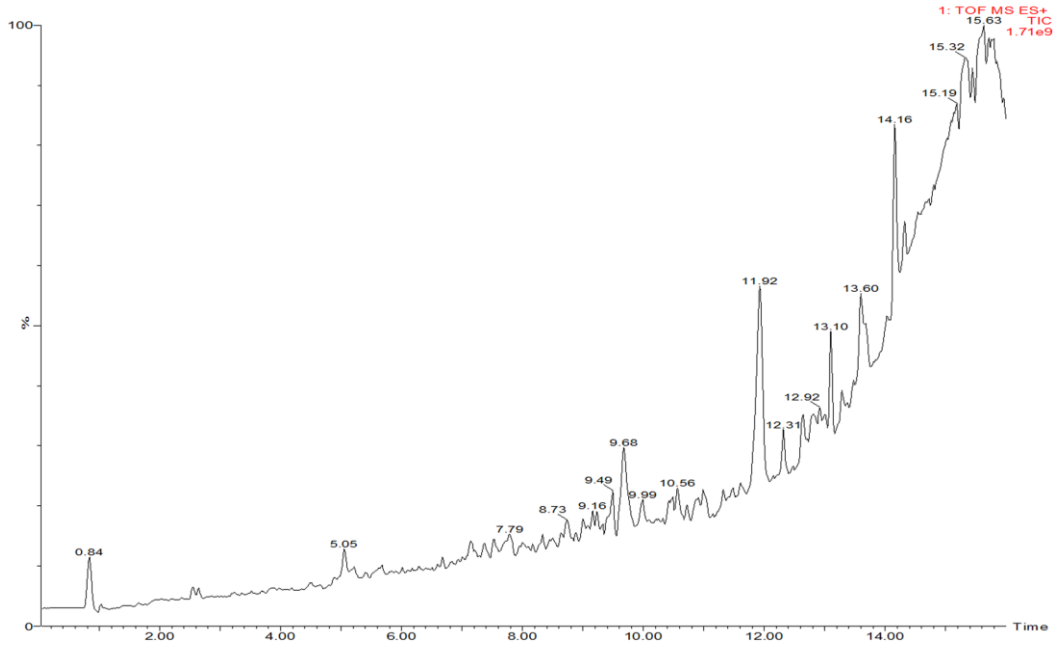


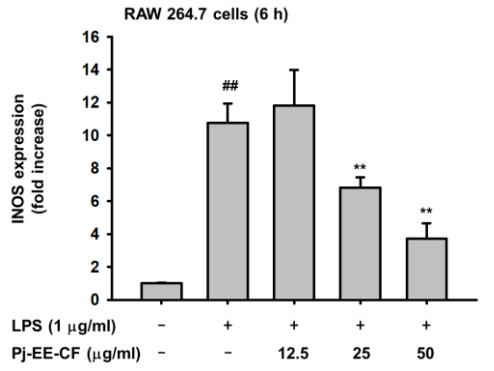
(**e**)

Figure 1. Effect of Pj-EE and its fractions on the production of NO, cell viability profile, and phytochemical constituents of Pj-EE-CF. (a and d) Supernatant NO levels on LPS (1 µg/ml)- induced RAW264.7 cells pre-treated with 100 µg/ml of Pj-EE, Pj-EE-HF, Pj-EE-CF, Pj-EE-BF, or Pj-EE-WF (a) and with indicated concentrations of PJ-EE-CF (d) were analyzed using the Griess assay. (b and c) Cell viability of RAW264.7 cells upon treatment with Pj-EE, Pj-EE-HF, Pj-EE-CF, Pj-EE-BF, or Pj-EE-WF (b) and PJ-EE-CF (c) at the same concentration on NO assay were analyzed using the MTT assay. (e) The phytochemical screening performed on Pj-EE-CF using LC/MS-MS chromatogram. Results (a-d) are expressed as mean ± SD. $^{\#\#}p < 0.01$ compared to normal group (no treatment), and $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control group (LPS alone) by one-way ANOVA.

2.3. Effects of Pj-EE-CF on the expression of pro-inflammatory genes on LPS-induced macrophages

We initially have evaluated that Pj-EE-CF treatment on LPS-stimulated RAW264.7 cells can reduce inflammatory mediators such as NO, for further steps, we examined whether Pj-EE-CF also can regulate the expression of pro-inflammatory at the transcriptional level. Using quantitative real-time PCR (qPCR), we assessed mRNA expression of iNOS, COX-

2, and other pro-inflammatory cytokines such TNF-α, IL-1β, IL-6, and IL-10. As shown in Figure 2, LPS alone upregulated the level of inflammatory genes ($p < 0.001$), and treatment with Pj-EE-CF downregulated the expression of pro-inflammatory genes. In line with the result of NO assay, LPS-stimulated RAW264.7 cells pre-treated with Pj-EE-CF at concentration 25 and 50 μg/ml significantly inhibited the mRNA levels of iNOS ($p = 0.0094$ and $p = 0.0068$, respectively), the enzyme which responsible to catalyze the secretion of NO, in a concentration-dependent manner (Figure 2a). Moreover, other pro-inflammatory and cytokine genes such as COX-2 (Figure 2b), TNF-α (Figure 2c), IL-1β (Figure 2d), IL-6 (Figure 2e), and IL-10 (Figure 2f) also displayed in a concentration-dependent manner reduce by pre-treatment with Pj-EE-CF up to 50 μg/ml ($p = 0.0026$, $p = 0.0211$, $p = 0.0005$, $p = 0.0022$, and $p = 0.0009$, respectively).



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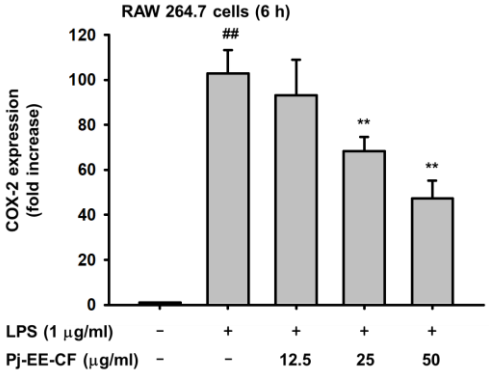

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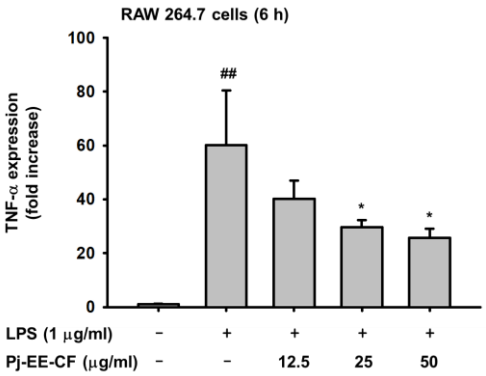

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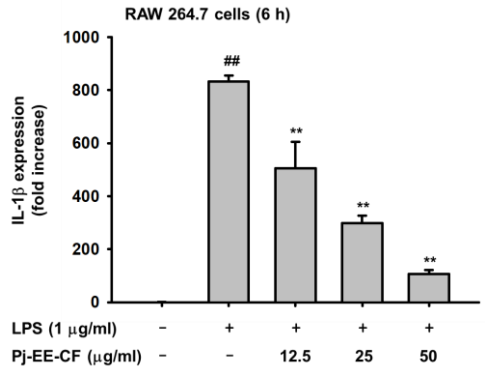

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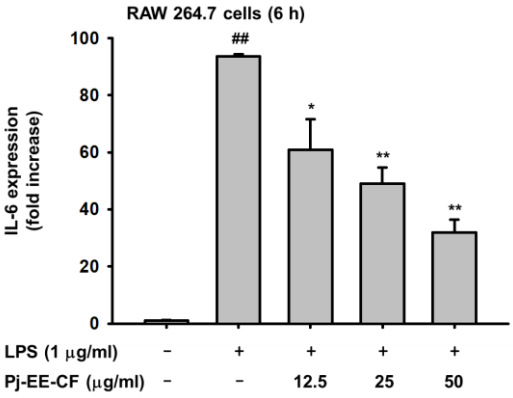

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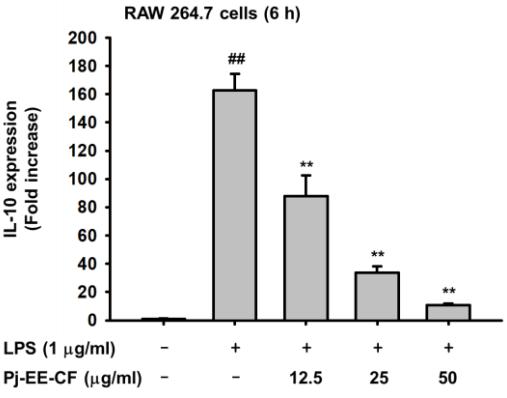
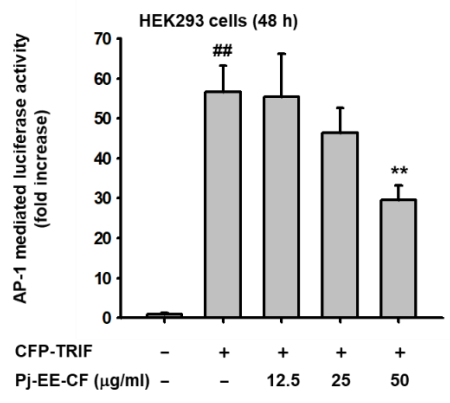


(f)

Figure 2. Effect of Pj-EE-CF on the expression of pro-inflammatory genes. The mRNA levels of iNOS (a), COX-2 (b), TNF-α (c), IL-1β (d), IL-6 (e), and IL-10 (f) were evaluated by qPCR on LPS-stimulated RAW264.7 cells. Results are expressed as mean ± SD. $^{\#\#}p < 0.01$ compared to normal group, and $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control group (LPS alone). A p-value was performed using Bio-Rad CFX software.

2.3. Effects of Pj-EE-CF on the transcriptional activation of AP-1 signalling and the upstream signaling molecules of AP-1 activation

Next, due to the significant inhibitory effect of pro-inflammatory gene regulation which was analyzed by qPCR, we sought to explore the transcriptional level using other assessments, luciferase assay, in HEK293 cells co-transfected with either TRIF or MyD88-mediated AP-1 activity. The results indicated that LPS alone significantly enhanced TRIF or MyD88-mediated AP-1 activity ($p = 0.0078$, $p = 0.0035$), on other hand, treatment with Pj-EE-CF up to 50 μg/ml concentration-dependently suppressed AP-1 luciferase activity induced by both TRIF and MyD88 conditions (Figure 2a and b). In addition, we also examine the protein level of AP-1 subunits activation, the phosphorylation of c-Jun and c-Fos, from whole lysates in LPS-induced RAW264.7 cells at different concentrations (12.5, 25, and 50 μg/ml). This finding showed that Pj-EE-CF decreased the level of p-c-Jun and p-c-Fos at all indicated concentrations (Figure 2c), which indicated that Pj-EE-CF could regulate the activity of AP-1 by inhibiting the dimerization of AP-1 via the reduction of activation of c-Jun and c-Fos. Afterward, in order to determine the target molecules of Pj-EE in the AP-1 pathway, we deeply identify the effect of Pj-EE-CF at different concentrations in the upstream signaling molecules of AP-1 activation by immunoblot analysis. MAPKs activation was generated by LPS-induced RAW264.7 cells. Among MAPKs, extracellular signal-regulated kinase (ERK) phosphorylation and phosphorylation levels of p38 were clearly decreased in the presence of Pj-EE-CF treatment at 25 and 50 μg/ml, however, the phosphorylation levels of c-Jun N-terminal kinase (JNK) was not affected, despite the presence of Pj-EE-CF treatment (Figure 2d). Taken together, these results suggest that Pj-EE-CF exerts anti-inflammatory responses through suppression of ERK and p38 in the AP-1 pathway.



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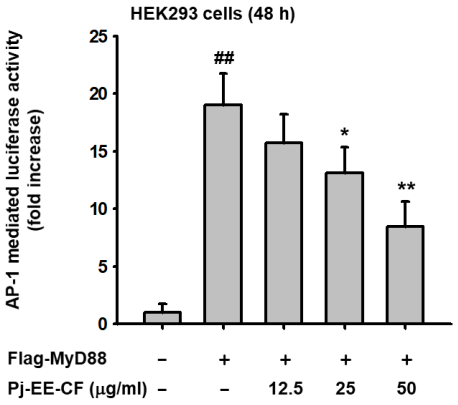

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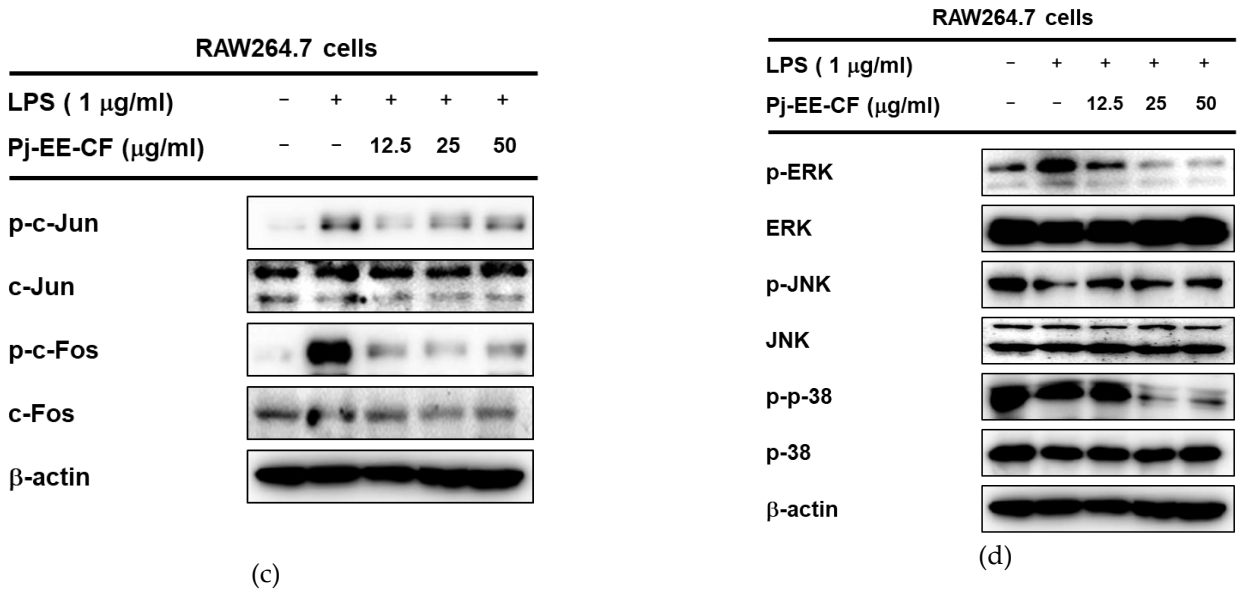
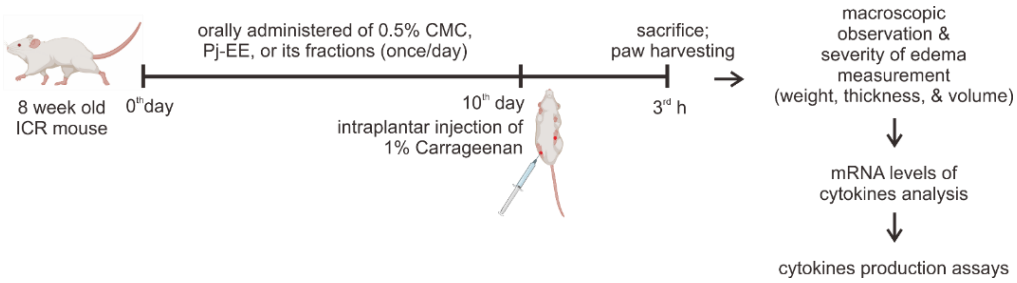


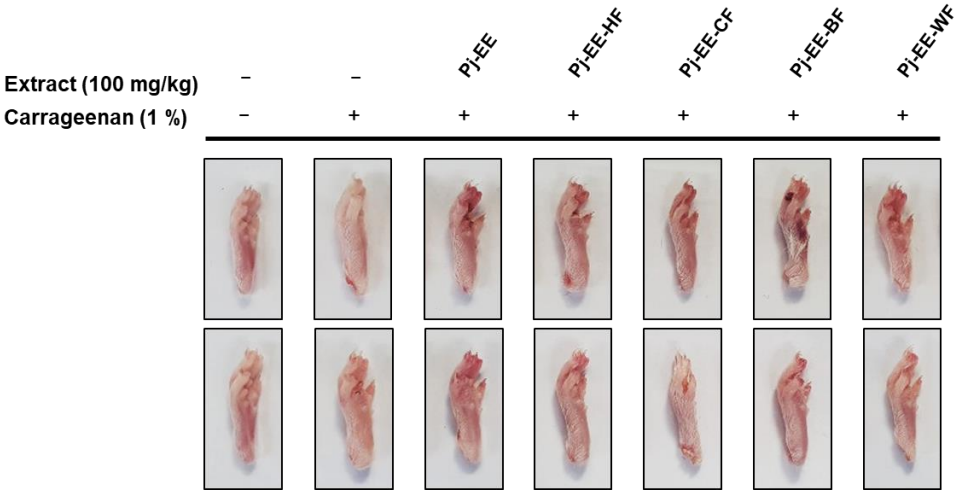
Figure 3. Effect of Pj-EE-CF on the transcriptional activation of AP-1 signalling and the upstream signaling molecules of AP-1 activation. (a and b) HEK293 cells were co-transfected with AP-1-Luc and β-gal (0.8 μg), as well as TRIF and MyD88 for 48 h in the presence or absence of Pj-EE-CF (12.5, 25, and 50 μg/ml) which then were examined using a luminometer. Results are expressed as mean ± SD (n= 4). $^{\#\#}p < 0.01$ compared to normal group, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control group (LPS alone) by one-way ANOVA. (c) The phospho- and total forms of AP-1 subunits, c-Jun and c-Fos, from whole-cell lysates from LPS-treated RAW264.7 cells in the presence or absence of Pj-EE-CF (12.5, 25, and 50 μg/ml) were determined by immunoblot analysis. (d) RAW264.7 cells were pre-treated with Pj-EE-CF (12.5, 25, and 50 μg/ml) for 30 min, followed by the presence or absence of LPS. The phosphorylated and total protein levels of ERK, JNK, and p38 were assessed by immunoblot analysis. β-actin was utilized for control protein.

2.4. Effects of Pj-EE and its solvent fractions on carrageenan-induced acute paw edema model

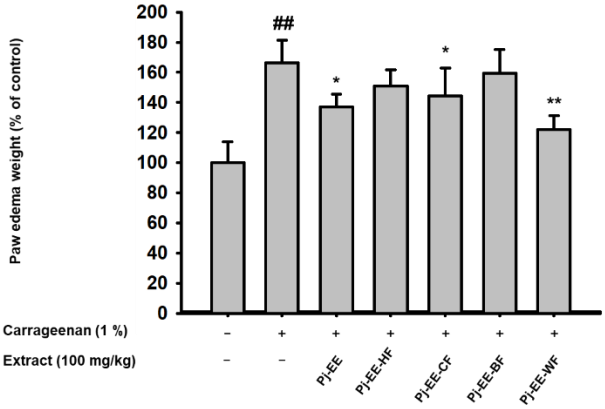
To determine the anti-inflammatory efficacy of Pj-EE and its solvent fractions in vivo, the widely performed edema models were utilized, the carrageenan-induced acute paw edema mouse model. Inflammation in both hind paws of each mouse was triggered by intraplantar injection of 1% newly prepared solution of carrageenan in PBS after pre-treated with 100 mg/kg Pj-EE and four different solvent fractions once a day for 10 days, as illustrated in Figure 4a. As expected, carrageenan-induced paw edema showed prominent inflammatory symptoms such as redness and swelling of the paws (Figure 4a). On the contrary, the 100 mg/kg Pj-EE and its fractions groups decreased the symptoms compare to the control group (carrageenan alone). In addition, we quantitatively measured the weight (Figure 4b), thickness (Figure 4c), and volume (Figure 4d) of each paw mouse to compare the severity of edema in each group. Water fraction followed by Pj-EE and Pj-EE-CF showed significantly reduced of the weight of paw compare to control group ($p = 0.0098$, $p = 0.0477$, and $p = 0.0354$, respectively). However, Pj-EE-CF had the lowest calculation both of thickness and volume compared to 100 mg/kg of Pj-EE or the other fraction groups which suggest more potent inhibitory effects (Figure 4c and d, respectively), followed by Pj-EE group, hexane fraction, and water fraction. Meanwhile, the butanol fraction of Pj-EE did not show inhibitory symptoms on the carrageenan-induced paw edema model.


8 week old
ICR mouse
0th day
orally administered of 0.5% CMC,
Pj-EE, or its fractions (once/day)
10th day
intraplantar injection of
1% Carrageenan
3rd h
sacrifice;
paw harvesting
macroscopic
observation &
severity of edema
measurement
(weight, thickness, & volume)
mRNA levels of
cytokines analysis
cytokines production assays

(a)


Extract (100 mg/kg)
Carrageenan (1 %)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(b)


Paw edema weight (% of control)
200
180
160
140
120
100
80
60
40
20
0
##
*
*
**
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(c)

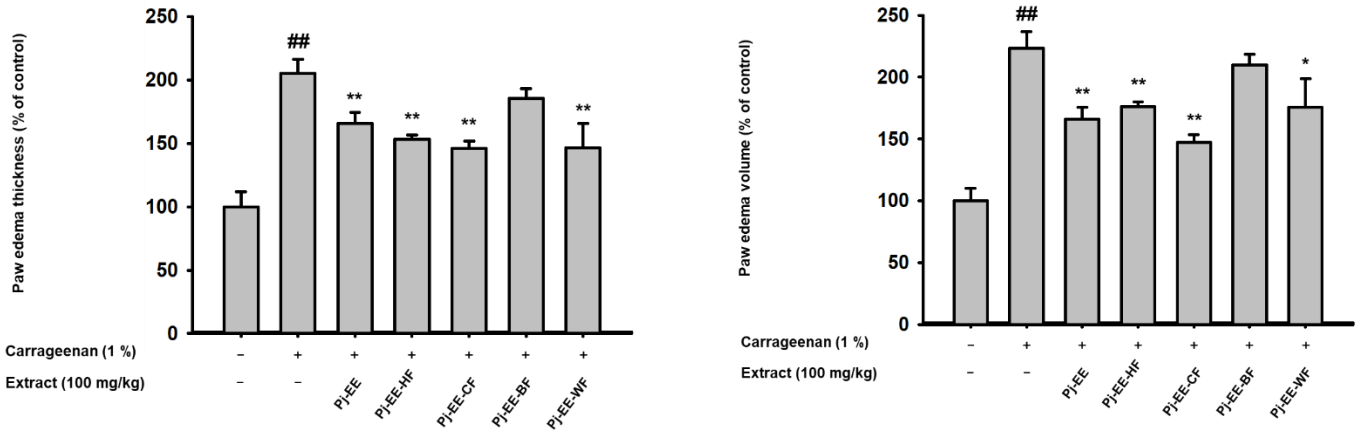
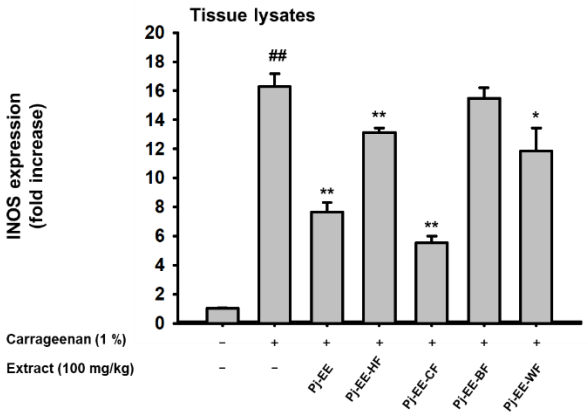


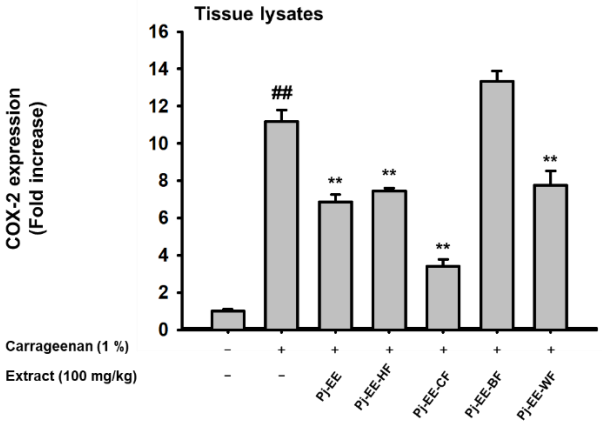
Figure 4. Effects of Pj-EE and four different fractions on carrageenan-induced acute paw edema model (a) Schematic of carrageenan-induced acute paw edema experiment. The ICR mice were orally treated with 100 μl different solutions based on different groups for 10 days: normal group (0.5% CMC), carrageenan group or as control group (0.5% CMC), treatment groups (100 mg/kg Pj-EE, Pj-EE-HF, Pj-EE-CF, Pj-EE-BF, or Pj-EE-WF). Acute paw edema was triggered by intraplantar injection of 1% carrageenan (100 μl μl/mouse) and mice were sacrificed after 3 h. (b) The representative photograph of paw inflammatory symptoms. (c-e) The severity of paw edema was evaluated by measuring the weight (c), thickness (d), and volume (e) of each paw of mouse. Results are expressed as mean ± SD (n = 12). $^{\#\#}p < 0.01$ compared to normal group, and $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control group (carrageenan alone). A p-value was analyzed using one-way ANOVA.

2.5. Effects of Pj-EE and its solvent fractions on the pro-inflammatory genes and cytokines production in carrageenan-induced acute paw edema models

Furthermore, to evaluate the effects of Pj-EE and its fractions at the molecular level, using tissue lysate of paw samples, the level of inflammatory genes was examined by quantitative real-time PCR (Figure 5 a-g). In agreement with in vitro experiments, Pj-EE-CF possessed the highest inhibition of inflammatory genes such as iNOS (Figure 5a), COX-2 (Figure 5b), and MMP-9 (Figure 5g), whereas Pj-EE-BF had the lowest or barely activities. Additionally, on the other pro-inflammatory genes such as TNF (Figure 5c), Pj-EE-HF showed more significantly reduced expression of TNF-α, followed by Pj-EE and Pj-EE-CF groups. Also, on the expression of IL-6 (Figure 5d), IL-1β (Figure 5e), and MMP-2 (Figure 5f), the crude Pj-EE showed more prominent inhibition of the inflammatory genes, despite Pj-EE-CF still significantly demonstrated inhibition activities of these pro-inflammatory genes. To confirm the efficacy of Pj-EE and its fraction inhibited the secretion of various cytokines, we further look into the production of IL1-β, IL-4, and TNF-α using enzyme immunoassay (EIA). Carrageenan-induced paw edema significantly generated the production of these cytokines, on the other hand, treatment with 100 mg/kg PJ-EE and its four solvent fractions showed a reduction of these enzymes, with Pj-EE-CF displayed the highest attenuation of IL1-β (Figure 5h), IL-4 (Figure 5i), and TNF-α (Figure 5j) secretions. These data indicated that Pj-EE alleviated the inflammatory symptoms on carrageenan-induced paw edema, notably, the chloroform fraction confers more potent inhibition of almost all of the assessments.


Tissue lysates
INOS expression (fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(a)


Tissue lysates
COX-2 expression (Fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
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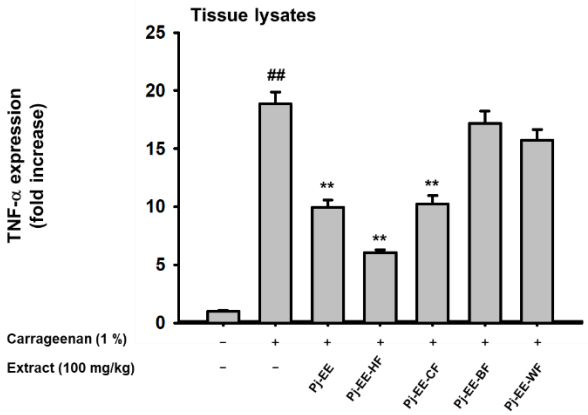
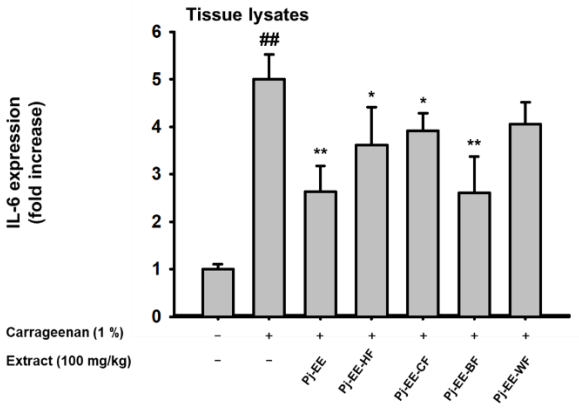
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

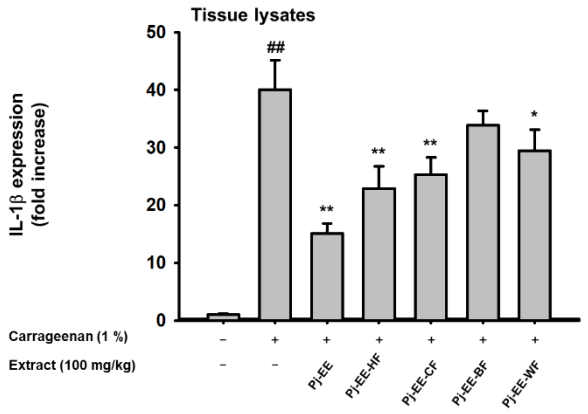
(b)


Tissue lysates
TNF-α expression (fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

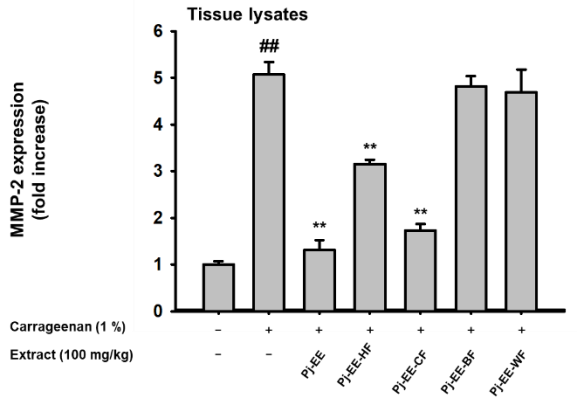
(c)


Tissue lysates
IL-6 expression (fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(d)


Tissue lysates
IL-1β expression (fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(e)


Tissue lysates
MMP-2 expression (fold increase)
Carrageenan (1 %)
Extract (100 mg/kg)
Pj-EE
Pj-EE-HF
Pj-EE-CF
Pj-EE-BF
Pj-EE-WF

(f)

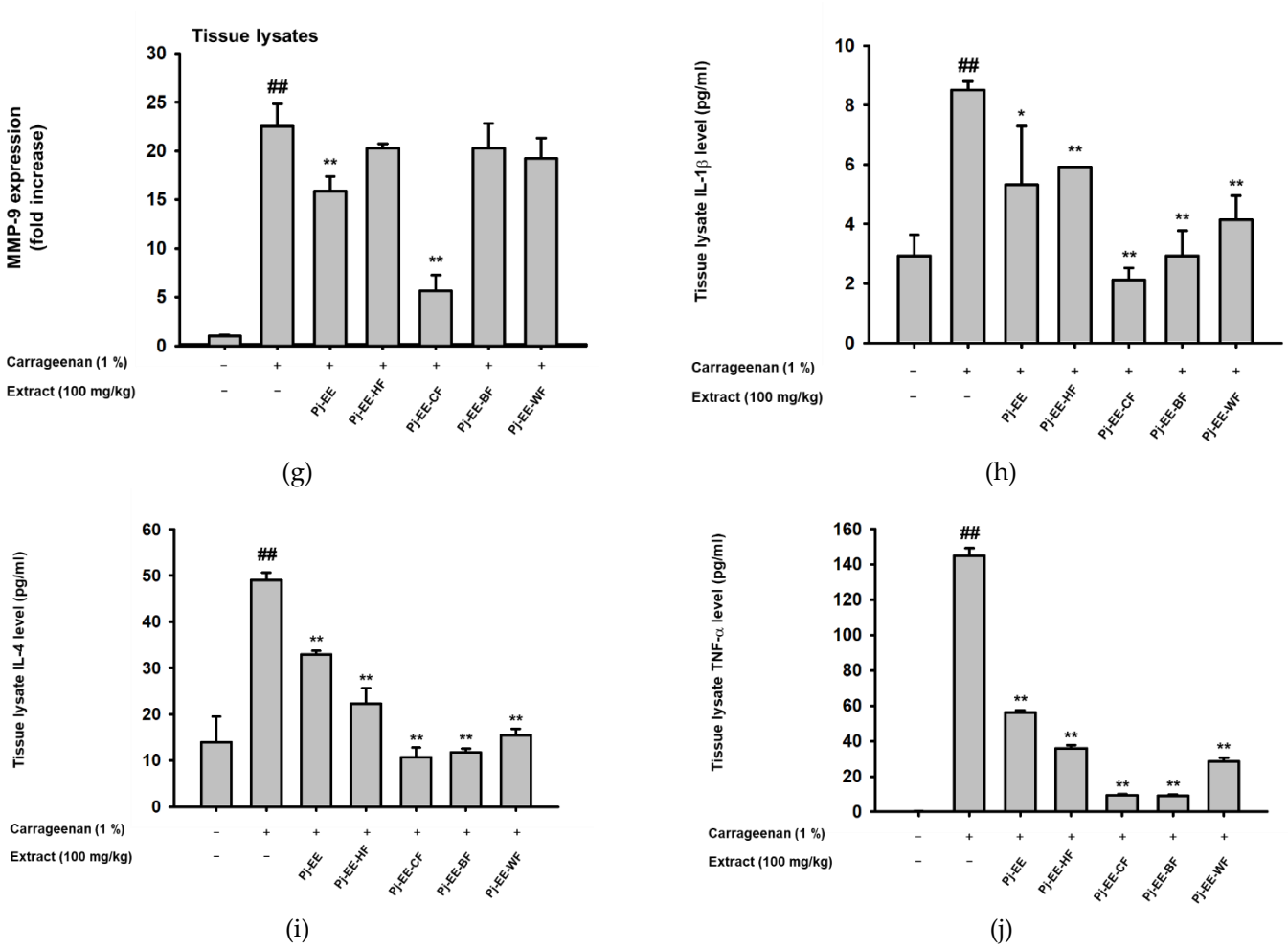


Figure 5. Effects of Pj-EE and four solvent fractions (HF, CF, BF, and WF) on the pro-inflammatory genes and cytokines production in carrageenan-induced acute paw edema models. (a-g) The expression of inflammatory genes, including iNOS (a), COX-2 (b), TNF-α (c), IL-6 (d), IL-1β (e), MMP-2 (f), and MMP-9 (g) from paw lysates was measured by quantitative RT-PCR. (h-j) Supernatant IL-1β (h), IL-6 (i), and TNF-α (i) levels of tissue lysates on carrageenan-trigerred paw edema were examined by EIA. All of the data are expressed as mean ± SD (n = 6). $^{\#\#}p < 0.01$ compared to normal group, and $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control group (inducer alone) by one-way ANOVA.

3. Discussion

Medicinal plants with natural sources still offer promising options and are recognized as therapeutic agents. In addition, understanding the mechanisms of therapeutic action could lead to the discovery of new drugs from natural sources with reduced or no side effects [21-23]. The edible freshwater green algae, *Prasiola japonica*, has been described to possess pharmacological benefits. In the latest year, our research group has reported that the ethanol extract of *Prasiola japonica* exerts anti-apoptotic, anti-melanogenic, and anti-oxidant on skin cell lines [13,19]. Also, recently has shown in vivo anti-inflammatory effects via NF-κB signaling pathway [20]. Still, further studies with respect to which fractions have potential activity remain unclear. Therefore, in this study, using Pj-EE extract and four different solvent fractions, we aimed to investigate their anti-inflammatory potential against LPS-induced macrophage-like RAW264.7 cells and carrageenan-induced paw edema mouse model, and then focus to explore which solvent fraction performed more potent activities. Its molecular mechanisms concerning in AP-1 pathway, and phytochemical constituent also were determined.

As initially evaluated, nitric oxide (NO) has been described to play an important role in biological activities such as neurotransmission and immune defenses. However, while the production of NO is uncontrolled in the cells, it acts as one of the pro-inflammatory mediators and a key molecule during inflammatory responses [8]. In this study, NO production was stimulated by LPS. LPS is a major component of the outer membrane of gram-negative bacteria. Aggregation of the TLR4-adaptor proteins complex after binding LPS leads to activation of multiple signaling molecules, and the subsequent production of pro-inflammatory cytokines and mediators [3,24]. Our first finding revealed that Pj-EE-CF showed the highest inhibition of the secretion of NO (Figure 1). Based on this finding, we focus to explore its molecular mechanisms, then its phytochemical constituent also was described. Pj-EE-CF inhibited the expression of iNOS, an enzyme that responsible to mediate NO production, in LPS-induced macrophages [8]. Other inflammatory cytokines and mediators which broadly involved in inflammation via activation of its transcription, including COX-2, TNF-α, interleukins, as well as MMPs were also evaluated. The mRNA levels of those related genes were analyzed by quantitative real-time PCR which was downregulated in a concentration-dependent manner by Pj-EE-CF in vitro and in vivo (Figure 2 and 5), suggesting that Pj-EE-CF could regulate inflammatory responses at the transcriptional level. Importantly, in indicated concentrations of Pj-EE-CF produces these effects without affecting cell viability, which suggests that the inhibitory effects are not due to non-specific toxicity.

In addition, to deeply identify the molecular target and mechanism of Pj-EE-CF, other experimental approaches including luciferase assays and immunoblot analysis were performed. The results of the luciferase reporter genes indicated that Pj-EE-CF regulates the mRNA level of cytokines by regulating the transcriptional activity of the AP-1. Strengthening these findings, immunoblot analysis of the level of phosphorylation of AP-1 subunits, c-jun and c-Fos, revealed that Pj-EE-CF modulates the transcriptional activation of AP-1. Previous studies reported the essential role and mechanism of MAPKs in activating AP-1 pathways [7,23,25]. Consequently, we checked signaling molecules upstream of this AP-1 activation, and Pj-EE-CF specifically inhibited ERK and p38 activation. These results indicated how effective the chloroform fractions of Pj-EE can be in attenuating anti-inflammatory responses, resulting in a reduction of ERK and p38 of AP-1 pathway (Figure 3).

Several studies with respect to the discovery and development of anti-inflammatory agents were based on the use of carrageenan-induced inflammation. The carrageenan-induced paw edema is an established experimental model of acute inflammatory diseases that is widely performed for studying novel analgesic and anti-inflammatory agents [6,20,26-28]. Carrageenan injection triggered an innate immune response characterized by edema, redness, and the continuing of neutrophil infiltration, thereby pro-inflammatory mediators and cytokines involved in this process through TLR4-MyD88 or TRIF complex [6]. Accordingly, to further evaluate and screen the anti-inflammatory efficacy of Pj-EE and its fractions in vivo, we performed a carrageenan-induced paw edema mouse model. In agreement with other reported studies, intraplantar injection of 1% carrageenan into the hind paw performed inflammatory symptoms of paws and generated the expression and production of pro-inflammatory cytokines. Also, consistent with our in vitro results, Pj-EE treatment especially PJ-EE-CF decreased inflammatory symptoms and exhibited protective effects in carrageenan-induced paw edema (Figure 4). Furthermore, since carrageenan has been reported to induce pro-inflammatory cytokines production, such as TNF-α, IL-1β, and IL-4, we also determined these cytokines production by enzyme immunoassay. The results indicated that Pj-EE and four solvent fractions decreased the cytokines production, with Pj-EE-CF showed the highest and significant inhibition (Figure 5). Taken together, the results support that orally administered Pj-EE and its fractions, especially chloroform fraction were able to alleviate inflammatory responses and may be clinically beneficial for treating inflammatory symptoms.

Phytochemical screening, especially pertaining to the flavonoid, for the possible presence of the chloroform fraction was characterized using LC-MS/MS. Numerous flavonoid molecules have been described to obtain anti-inflammatory activity through various mechanisms, including inhibition of the expression of inflammation-related enzymes via suppression of transcription factors activation, including AP-1 [29-31]. Among the various flavonoid types that present, maltol, bavachinin, nobiletin, flavonol, and 3′-deoxysappanone B have been widely studied to possess bioactivity such as anticancer, antimicrobial, and anti-inflammatory properties. The major flavonols such as quercetin, kaempferol, isorhamnetin, and galangin were found to exhibit anti-inflammatory activity [29,31]. Maltol exerts a significant liver protection effect, anti-inflammatory and anti-apoptotic action [32]. Some studies have demonstrated that bavachinin, nobiletin as well as 3′-deoxysappanone B regulate the production of several cytokines as well as inflammatory mediators in activated macrophages and various other cell types [32-38]. Consequently, we considered that these components of Pj-EE-CF could be responsible for its anti-inflammatory effect.

4. Materials and Methods

4.1. Materials

Macrophage-like RAW264.7 cells (ATCC number TIB-71) and HEK293 cells (ATCC number CRL-1573) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). TRIzol reagent was purchased from MRCgene (Cincinnati, OH, USA). Carrageenan, dimethyl sulfoxide (DMSO), carboxymethylcellulose (CMC), lipopolysaccharide (LPS, E. coli 0111:B4), polyethylenimine (PEI), sodium dodecyl sulfate (SDS), and 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was acquired from Biotechnics Research, Inc. (Irvine, CA, USA). RPMI 1640, DMEM, trypsin, PBS, and penicillin-streptomycin were obtained from HyClone (Logan, UT, USA). Primers used for quantitative real-time PCR were obtained from Macrogen Inc. (Seoul, Korea). Phospho-specific or total-protein antibodies against c-Fos, c-Jun, JNK, ERK, p38, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). CATX124 balance was purchased from CAS Co. (Yangju, Korea), mitutoyo thickness gauge 547 was received from Mitutoyo (Kanagawa, Japan), and plethysmometer 37,140 was obtained from UgoBasile (Comerio, VA, Italy).

4.2. Pj-EE and its fractions preparation

Prasiola japonica was acquired from the Prasiola japonica Research Center (Samcheok City, Gangwon-do, Republic of Korea) and was extracted as previous studies [13,19,20]. Briefly, the pieces of cut samples were extracted with 70% ethanol for 24 h at a ratio of 1:20 (w/v). Subsequently, the filtrate was filtered using 120-nm filter paper (No. 2, Advantec, Toyo Co., Tokyo, Japan) and concentrated using a vacuum concentrator (Eyela New Rotary Vacuum Evaporator, Rikakikai Co., Tokyo, Japan), which then were dried using a vacuum freeze dryer (Eyela FD1, Rikakikai Co.), and the yield of the dried samples was measured. The final weight of the extract was 29.974 g (original sample: 210.41 g) with a yield of 14.24%. In addition, the ethanolic extract of *P. japonica* (crude extract) was then fractionated by various polarity solvents including n-hexane (Pj-EE-HF), chloroform (Pj-EE-CF), n-butanol (Pj-EE-BF), and water (Pj-EE-WF) as illustrated in Figure 6. The dried samples were kept at a − 20◦C freezer for future use. For the in vitro studies, the Pj-EE and its fractions stock solution were prepared by dissolving with DMSO at a concentration of 100 mg/ml. When each experiment was performed, the stock solution was diluted to the desired final concentration of 12.5, 25, 50, 100, or 200 μg/ml using the suitable culture medium. For the paw edema mouse model experiments, the stock was made in 0.5% CMC at doses of 100 mg/kg.

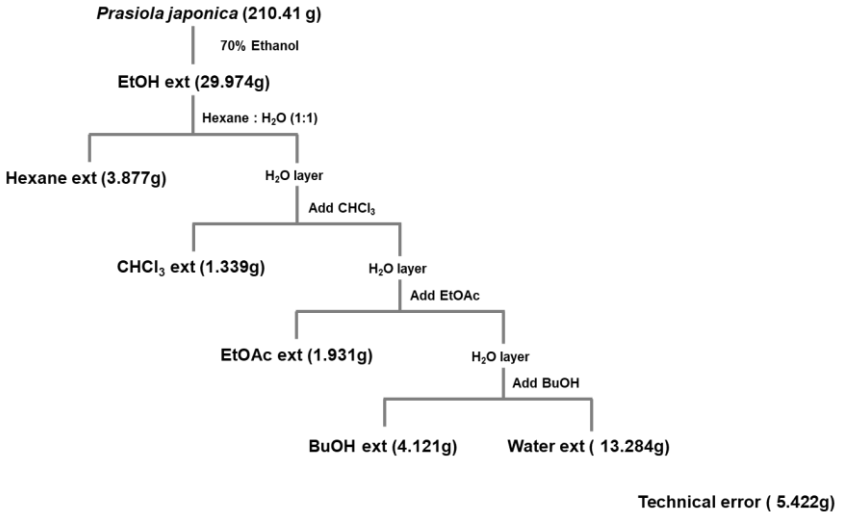


Figure 6. Schematic: preparation of ethanolic extract and four solvent fractions of *Prasiola japonica*.

4.3. Cell culture and treatment

The murine macrophage cell line (RAW264.7) and the human embryonic kidney cell line (HEK293) and were cultured as previously reported [39]. For preliminary study, LPS-induced RAW264.7 cells were pre-treated at concentration 100 μg/ml of Pj-EE and its fractions, whereas for the next experiments, Pj-EE-CF group was pre-treated with Pj-EE-CF at concentration 12.5, 25, and 50 μg/ml. The control (LPS alone) and normal groups were pre-treated with diluted DMSO in culture medium for 30 min. The final concentration of DMSO in cellular experimental conditions was <0.5%.

4.4. Determination of NO and cytokines production

RAW264.7 cells ($1x10^6$ cells/ml) was seeded in 96-well-plate and then pre-treated with indicated concentrations of Pj-EE and its fractions for 30 min. LPS (1 μg/ml) was then treated for the next 24 h. The suppression effect of Pj-EE and its fractions on the secretion of NO was analyzed using Griess reagents, as previously described [40]. Furthermore, The suppression effects of Pj-EE and its fractions on the secretion of IL-1β, IL-4, and TNF-α were detected using an EIA kit according to the manufacturer's instructions (R&D systems, catalog no. MLB00C, M4000B, and MTA00B, respectively). Briefly described, after diluting the supernatant in indicated assay diluent 1:1, add 100 μl to each well in a 96 well plate coated with anti-mouse IgG. After washing 5 times with wash buffer (400 μl), 100 μl of Mouse IL-1β, IL-4, or TNF-α conjugate was added to each well and incubated for 2 h. After the reaction and washing, 100 μl of substrate solution was added to each well and incubated for further 30 min, which then was stopped with 100 μl of the substrate solution and was measured at 450 nm.

4.5. Cell viability assay

The cytotoxic effects of Pj-EE and its fractions were determined by a conventional MTT assay as previously reported [41]. We seeded RAW264.7 cells into 96-well plates and pre-incubated for overnight (16 hours), afterwards, we treated with indicated concetrations of Pj-EE and its fractions or DMSO (for normal group) for further 24 h.

4.6. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Phytochemical screening performed on the chloroform fraction was characterized by LC-MS/MS. LC-MS/MS analyses were performed as previously described [23].

4.7. mRNA analysis by quantitative real-time (q) polymerase chain reaction (PCR)

Total RNA was obtained from macrophage-like RAW264.7 cells (1x10^6 cells/mL) that were pre-treated with Pj-EE-CF (0, 12.5, 25, and 50 μg/ml) for 30 min followed by induction with LPS (1 μg/mL) for 6 h. Paw tissues of mice were grinded, and stored at -70°C until further use. Total RNA was prepared using TRIzol as described by the manufacturer's instructions, and then 1 μg of total RNA was immediately made for cDNA synthesis using a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA USA) as described by the manufacturer's instructions. The qPCR was performed as previously reported [21]. The primer sequences used in this study are listed in **Table 1**.

Table 1. Primer sequences used for quantitative real-time PCR.

PCR Type	Genes name		Sequence (5′-3′)
q PCR	GAPDH	Forward	GAAGGTCGGTGTGAACGGAT
		Reverse	AGTGATGGCATGGACTGTGG
	iNOS	Forward	CAAGAGAACGGAGAACGGAGA
		Reverse	GATGGACCCCAAGCAAGACT
	COX-2	Forward	TGAGTACCGCAAACGCTTCT
		Reverse	TGGGAGGCACTTGCATTGAT
	TNF-α	Forward	TTGACCTCAGCGCTGAGTTG
		Reverse	CCTGTAGCCCACGTCGTAGC
	IL-1β	Forward	CAGGATGAGGACATGAGCACC
		Reverse	CTCTGCAGACTCAAACTCCAC
	IL-6	Forward	GCCTTCTTGGGACTGATGCT
		Reverse	TGGAAATTGGGGTAGGAAGGAC
	IL-4	Forward	
		Reverse	
	MMP-2	Forward	GTCCCTACCGAGTCTCTTCT
		Reverse	TTTTTAAGTTTCCGCTTCTG
	MMP-9	Forward	GCCACTTGTCGGCGATAAGG
		Reverse	CACTGTCCACCCCTCAGAGC

4.8. Luciferase reporter gene assay

HEK293 cells (2x10^5 cells/ml) were seeded in 24-well plates and pre-incubated overnight before transfection of plasmids encoding AP-1-Luc under co-transfection conditions with MyD88 and TRIF, by polyethylenimine (PEI) methods as previously reported [23]. After 24 h transfection, 12.5, 25, and 50 μg/ml Pj-EE-CF were treated for further 24 h.

4.9. Cell lysate extraction and immunoblotting analysis

RAW264.7 cells (1 x10^6 cells/ml) were seeded in a 6-well-plate. After pre-treated with different concentrations of PJ-EE-CF for 30 min, LPS was treated and then incubated for the next 24 h. After harvesting and washing with PBS, RAW264.7 cells were lysed in lysis buffer as previously described [42]. Around 20 μg of protein were subjected to western blot analysis as previously described [23].

4.10. Animal

ICR mice (8 weeks old, 20-22 g, male) were purchased from Daehan Biolink (Chungcheonbuk, Korea). The mice (n= 6 per group) obtained water and a pelleted diet

(Samyang, Daejeon, Korea) ad libitum in separate cages under a 12-h light/dark cycle. The in vivo experiments were conducted in agreement with the guidelines of the Institutional Animal Care and Use Committee Sungkyunkwan University (Suwon, Korea; approval ID: SKKUIACUC2020-06-39-1).

4.11. Carrageenan-induced acute paw edema mouse model

Using ICR mice (6 mice/group), paw edema was stimulated by 100 μl subplantar injection of 1% newly made solution of carrageenan in PBS into the both hind paws, as previously published method [27]. ICR mice were orally administrated with 100 μl different solutions based on different groups: normal group (0.5% CMC), carrageenan alone as control group (0.5% CMC), and treatment groups (100 mg/kg Pj-EE and its fractions) once a day for 10 days. After that, carrageenan-induced paw edema was performed to each group, except normal group (subplantar injection with PBS). At 3 h later, mice were sacrificed by CO_2. Subsequently, the redness and swelling of both paws were observed. The weight, thickness, and volume of the edema were measured as previously described method [20].

4.12. Statistical analysis

All data in this study represent the mean ± SD of four samples (*in vitro* experiments*)* which similar experimental data were obtained from an additional independent experiments performed under same conditions, and six mice per group (*in vivo* experiments*)*. Statistical analyses was performed using the computer program SPSS (version 26, SPSS Inc., Chicago, IL, USA), which comparison of statistical differences of all measured data was subjected to one-way ANOVA followed by Holm-Sidak or the Kruskal-Wallis/Mann-Whitney test. A p-value of $p < 0.05$ was considered to be a statistically significant difference.

5. Conclusions

The present study, using *in vitro* and *in vivo* experiments, clearly indicated and supported the evidance of the anti-inflammatory effects of Pj-EE. Particularly, its chloroform fraction showed the potent inhibition of inflammatory responses by targeting ERK and p38, thereby suppressed the activity of AP-1 that could result in attenuation of various inflammatory mediator and cytokines, as summarized in Figure 7. The anti-inflammatory activity of chloroform fraction of Pj-EE is provided of the presence of bioactive molecules that have demonstrated this function in various studies. In the future, Pj-EE could be a good natural source as an anti-inflammatory agent especially its chloroform fraction. Further investigations are necessary to evaluate and isolate the bioactive molecules present in Pj-EE-CF.

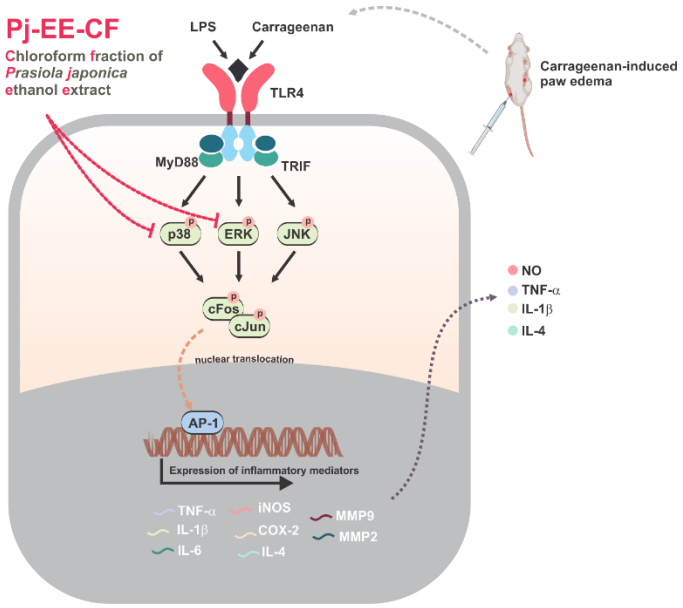


Figure 7. Schematic: anti-inflammatory mechanisms of Pj-EE-CF in the suppression of AP-1 signaling pathway.

Abbreviations

LPS	Lipopolysaccharide
MyD88	Myeloid differentiation factor 88
AP-1	Activator protein-1
MAPKs	Mitogen-activated protein kinases
JNK	c-Jun N-terminal kinase
RPMI 1640	Roswell Park Memorial Institute 1640
DMEM	Dulbecco's modified Eagle's medium
COX-2	Cyclooxygenase-2
MMP	Matrix Metalloproteinase
IL-1β	Interleukin-1β
IL-6	Interleukin 6
TNF-α	Tumor necrosis factor alpha
MKK	Mitogen-activated protein kinase
iNOS	Inducible nitric oxide synthase

Author Contributions: S.H.P., D.S.K., S.J. and J.Y.C. conceived and designed the experiments; L.R., S.H.P., D.S.K., H.P.L, C.Y.L, S.A.K. and J.Y.C. analyzed the data; S.H.P., D.S.K.,and J.Y.C. performed the experiments; L.R. and N.A. visualized the data; L.R., D.S.K., and J.Y.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors have no conflicts of interest to declare.

Institutional Review Board Statement: The in vivo experiments were performed in agreement with the guidelines of the Institutional Animal Care and Use Committee Sungkyunkwan University (Suwon, Korea; approval ID: SKKUIACUC2020-06-39-1).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

488 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Supplementary Table S1: LC-MS/MS of Pj-
489 EE-CF.

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