Anti-inflammatory effects of *Passiflora foetida* L. in LPS-stimulated RAW264.7 macrophages

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Abstract. *Passiflora foetida* L. (*Passifloraceae*), a perennial climber in general, is used for treating many ailments in conventional medicine. In this study, the anti-inflammatory effect of methanolic extracts of *P. foetida* L. (PFME) and the involvement of nuclear factor-κB (NF-κB) signalling in the regulation of inflammation were investigated. PFME prevented the production of prostaglandin E_2 (PGE_2) and the expression of inducible cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced macrophage cells. Additionally, PFME reduced the release of pro-inflammatory cytokines. Moreover, in LPS-induced RAW264.7 cells, the phosphorylation of MAPKs (ERK1/2, p38 and JNK) was suppressed by PFME. Furthermore, PFME inhibited the NF-κB activation induced by LPS, which was associated with nuclear p65 levels with the abrogation of IκBα degradation and subsequent decreases. These results indicated that the PFME inhibited the LPS-induced inflammatory and oxidative responses. Therefore, we propose that the PFME may be therapeutic for treating inflammatory diseases.

Introduction

Inflammation is a crucial physiological response of the immune system to infection, and this complex biological symptom is frequently caused by the assault of pathogens during destructive injury of human tissues (1-4). Inflammation is an acute phase that is the initial response, which involves the increased motion of plasma and inflammatory cells from the bronchial tubes to the tissue. Inflammation is described in the pathogenesis of several diseases, including metabolic disease, infection, pulmonary disease and neoplasm (5-7). Then, the lipopolysaccharides (LPS) of gram-negative bacteria activate macrophages, as indicated by the production of tumour necrosis factor-α (TNF-α), interleukins (ILs) and leukotrienes (8,9).

MAPKs and nuclear factor-κB (NF-κB) signalling pathways are major adjusters in the expression of inflammatory mediators involving cyclooxygenase-2 (COX-2) (10,11). The dimeric form of NF-κB is taken into the cytoplasm by the physical combination in unstimulated cells with an inhibitory protein, IκBα (7,12-14). With degradation of IκBα, NF-κB is translocated to the nucleus, and specific NF-κB inhibitors check COX-2 expression (15). Activation of MAPKs leads to the production of inflammatory mediators such as COX-2 in activated macrophage cells (16). In this study, as part of a continuing search to establish the anti-inflammatory mechanism of methanolic extracts of *P. foetida* L. (PFME), we demonstrated that PFME is a strong inhibitor of COX-2 expression in LPS-stimulated RAW264.7 macrophage cells. Moreover, the anti-inflammatory mechanism by which PFME blocks LPS-induced inflammation via NF-κB and MAPKs signalling pathways is novel.

Passiflora foetida L. (*Passifloraceae*) is better known as 'Wild maracuja', 'Bush Passion fruit' or 'Buah tikus' in Indonesia. The 'foetid' stinking passionflower is a species of passionflower with a stinky smell that is indigenous to tropical America in Mexico, the Southwestern United States (Arizona, Southern Texas) the Caribbean and much of South America. During the Age of Exploration, the species was introduced to Europe, India and Southeast Asia for cultivation and gardening, which was followed by naturalization in other tropical countries (17,18). The plant is a climbing herb with tendrils, and the flowers are white to pale cream and ~5-6 cm

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in diameter with 2-4-fold pinnatifid bracts at the base. Leaves are ovate to obovate, with 3 shallow lobes, 6-9 cm in length, with a heart-shaped base and frequently a winding and ciliate and pointed tip. Black seeds are embedded in the pulp. The woody and wiry stems are sheer, covered by yellow tacky hairs, and the 2-3 cm diameter fruit is yellowish-orange to red (when ripe). The plant is generally scattered, growing in wayside thickets and riverbeds and dried-up forest floors and is similarly found close to human settlements (18). Extracts from the leaves and fruit of *P. foetida* (PF) have been used to treat biliousness and asthma (19), and leaf and root extracts are used to treat hysteria, and in the case of headache, a paste of the leaf is rubbed on the head. The herb is used in lotions or poultices for skin diseases with erysipelas and inflammation in Brazil (20). Present study has focused on the pain reliever, anti-diarrhoeal and cytotoxic activities of PF (17), in addition to the antiulcer effects, antioxidant activity (21), analgesic activities, biological and pharmacological activities (17,22,23) and anti-inflammatory effects (22,23). However, the anti-inflammatory activity of PFME and the underlying mechanisms have not been closely examined. In the present study, we investigate the anti-inflammatory effects of PFME and the fundamental mechanisms in RAW264.7 macrophage cells.

Materials and methods

Preparation of Passiflora foetida L. (PF) extract. Plants were collected from the 'Pirangrung, Ujung Kulon National Park' West Java of Indonesia in 2008. The Center for Pharmaceutical and Medical Technology (PTFM, BPPT, Jawa Timur, Indonesia) collected and identified the plants, with the identifications verified by Herbarium Bogoriense (LIPI, Jakarta, Indonesia). Voucher specimens are deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) and in the PTFM and Herbarium Bogoriense. *Passiflora foetida* was treated with methanol and sonicated repeatedly for 3 days at room temperature to produce the extract.

Cell culture and reagents. Murine macrophage RAW264.7 cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (v/v) penicillin (100 U/ml)/streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS). The RAW264.7 cells were cultured three times a week, and the RAW264.7 cells were used for experimentation at 70-80% confluence. After pre-incubation of the cells for 4 h, 5, 10, 20 and 30 μg/ml of the extract was added. The cell lines were incubated under a humidified condition of 5% CO_2 at 37˚C. *Escherichia coli* LPS and the Griess reagent were purchased from Sigma (St. Louis, MO, USA). DMEM (Welgene, Gyeongsan-si, Korea), FBS (HyClone, Logan, UT, USA), and the 1% (v/v) penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen, Grand Island, NY, USA) were also purchased. In another set of cultures, the cells were co-incubated with the p42/44 inhibitor, PD98059 (10 μM), the p38 inhibitor, SB203580 (10 μM), the c-Jun N-terminal kinase (JNK) inhibitor, SP600125 (10 μM), and the IκBα inhibitor (BAY11-7082) (all from Merck Millipore, Darmstadt, Germany).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, the RAW264.7 cells were seeded in a 96-well plate ($1x10^5$ cells/ml) and treated with 5, 10, 20 and 30 μg/ml PFME for 24 h. The cell proliferation was analysed by MTT (Amnesco, Solon, OH, USA), using the analysis expressed previously (24). To calculate actual observance, the background levels of PFME when incubated only with MTT solution were subtracted. The optical density (OD) was measured using a multimode microplate reader (Tecan Trading AG, Mannedorf, Switzerland) at 570 nm.

Prostaglandin E_2 (PGE_2) assay. In the supernatant, the PGE_2 concentration was verified using a commonly available PGE_2 enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's protocols as described previously (25).

ELISA. The levels of pro-inflammatory cytokines were determined with commercial ELISA kits. IL-6, IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The assay procedure for each item was conducted according to manufacturer's instructions. The concentrations of mediators were determined at 450 nm using a multimode microplate reader (Tecan Trading AG).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed to detect the mRNA expression of COX-2 and β-actin. Briefly, after LPS (0.5 μg/ml) stimulation of the RAW264.7 cells for 6 h, the total RNA was isolated using TRIzol™ reagent (Invitrogen) as recommended by the producer, and a reverse transcription reaction was accomplished using an AMPIGENE® cDNA synthesis kit (Enzo Life Sciences, Farmingdale, NY, USA). The PCR was conducted using a premix and specific sense and antisense primers in conformity with the manufacturer's instructions (Bioneer, Daejeon, Korea). The amplification course consisted of denaturing at 94˚C for 5 min, followed by 94˚C for 20 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 45 sec, with a final extension performed at 72˚C for 1 min. The primer sequences were as follow: murine COX-2, 5'-GAA GTC TTT GGT CTG GTG CCT G-3' (sense) and 5'-GTC TGC TGG TTT GGA ATA GTT GC-3' (antisense); and murine β-actin, 5'-TGT TTG AGA CCT TCA ACA CC-3' (sense) and 5'-CGC TCA TTG CCG ATA GTG AT-3' (antisense). β-actin was used as the housekeeping gene when indicated. PCR products were resolved on 1.5% agarose gels and stained with ethidium bromide. The images were captured by an Olympus C4000 Zoom Camera system (Olympus Corp. America, Inc., Center Valley, PA, USA).

Immunoblot analysis. Equal amounts of extracted protein (30 μg) were divided by 11% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Each membrane was blocked for 1 h with 5% skim milk in TBS/T buffer (0.1% Tween-20, 0.1 M Tris-HCl, 0.9% NaCl, pH 7.4) to block non-specific binding and was then incubated with primary antibodies that recognized COX-2 (1:1,000), the total forms of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and JNK1/3 (1:1,000), the phosphorylated forms of p38 MAPK (1:1,000)

(all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the phosphorylated forms of ERK1/2 and JNK1/2 (1:1,000), in addition to those for β-actin (1:4,000) and PARP (1:1,000) (all from Cell Signaling Technology, Beverly, MA, USA). Secondary antibodies were goat anti-rabbit or anti-mouse antibodies (1:5,000; Santa Cruz Biotechnology, Inc.). The enhanced chemiluminescence reaction was performed using a Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA), and the positive bands were detected on radiographic film.

Statistical analyses. From the statistical analyses, the values are expressed as the mean ± SEM of the sample determinations. The statistical significance was determined using a two-tailed Student's t-test for independent means, with P-values of <0.05 statistically significant.

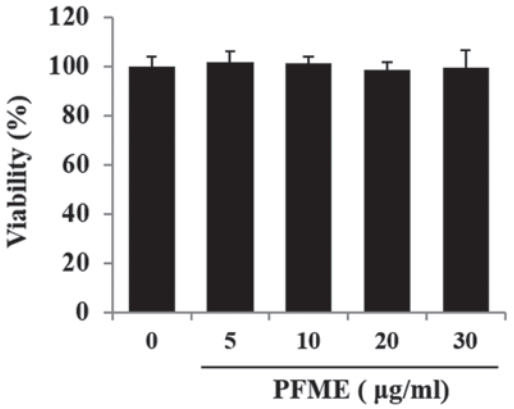

Figure 1. Effect of *Passiflora foetida* L. (PFME) on cell viability. RAW264.7 cells were incubated in the presence or absence of 0-30 μg/ml of PFME, and cell viability was determined using the MTT assay. The data are presented as the mean ± SEM of three independent experiments.

Results

Effects of PFME on cell viability in RAW264.7 macrophages. Fig. 1 shows the effect of PFME on cell viability as determined by the MTT assay, with morphology of the cells confirmed with microscopic pictures. Cell viability did not decrease significantly at concentrations of PFME up to 30 μg/ml. Accordingly, for all ensuing experiments, the concentration range used was 0-30 μg/ml PFME.

PFME decreases the production of PGE_2 by suppressing COX-2 and pro-inflammatory cytokine expression in LPS-stimulated RAW264.7 cells. Unstimulated RAW264.7 cells secreted basal levels of PGE_2, whereas PGE_2 production increased with LPS stimulation. We determined that PFME decreased the LPS-stimulated production of PGE_2 and inhibited the expression of COX-2 in the macrophage cells. Moreover, PFME substantially decreased the LPS-stimulated PGE_2 production in a concentration-dependent manner (Fig. 2A). The LPS-stimulated RAW264.7 cells overexpressed COX-2, whereas the PFME-treated cells stimulated with LPS suppressed expression of COX-2 protein (Fig. 2B). The expression of COX-2 protein was consistent with the mRNA results, and the PFME suppressed the expression of COX-2 mRNA in the LPS-stimulated RAW264.7 cells (Fig. 2C). Cytokines and pro-inflammatory mediators play significant roles in an immune reaction (26,27). Therefore, we estimated the influence of PFME on the production of cytokines, such as IL-6, TNF-α and IL-1β, and pro-inflammatory mediators in RAW264.7 macrophage cells (Fig. 2D-F). As shown in Fig. 2D-F as measured by ELISA, PFME suppressed production of IL-6, TNF-α and IL-1β in a concentration-dependent manner.

PFME inhibits the phosphorylation of the MAPKs in LPS-stimulated RAW264.7 cells. MAPKs play a vital role in the regulation of differentiation and cell growth and also control cellular responses to cytokines and stress (28). To determine the suppression of PFME on MAPKs signalling of inflammation in RAW264.7 cells, the regulation of the phosphorylation of the mediators JNK, p38 and ERK1/2 was investigated. The whole cell lysates were then examined with phospho-specific antibodies for JNK, p38 and ERK. The RAW264.7 cells treated with LPS alone showed increased phosphorylation of JNK, p38 and ERK1/2; however, PFME treatment decreased levels of phosphorylation of JNK, p38 and ERK1/2 in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (Fig. 3). The level of expression of non-phosphorylated JNK, p38 and ERK1/2 did not change in cells treated with LPS or LPS and PFME. These experiments indicated that suppression of the phosphorylation of p38 and ERK1/2 kinases may explain the dramatic inhibitory effect of PFME on LPS-stimulated inflammatory mediator activation in RAW264.7 cells, although the phosphorylation of JNK was only slightly inhibited by PFME in the macrophage cells.

PFME and an inhibitor of MAPK phosphorylation inhibit the phosphorylation of the activation of MAPKs and the expression of NO and pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. To investigate the molecular mechanism of inflammatory mediator inhibition by PFME and MAPK inhibitors in LPS-stimulated RAW264.7 cells, we studied the inhibition of the phosphorylation of JNK, p38 and ERK1/2. In cells treated with LPS alone, phosphorylation of JNK, p38 and ERK1/2 increased. However, PFME, SP600125 (JNK), SB203580 (p38), and PD98059 (ERK) treatment decreased levels of phosphorylated JNK, p38 and ERK1/2 in LPS-stimulated RAW264.7 cells (Fig. 4). We further investigated the effect of the PFME, SP600125, SB203580, and PD98059 on the inhibition of the NO assay and ELISA (IL-6 and TNF-α) (Fig. 5). These results suggested that suppression of phosphorylation of JNK, p38 and ERK1/2 may be involved in the inhibitory effect of PFME, SP600125, SB203580 and PD98059 on LPS-stimulated inflammatory mediator activation in RAW264.7 cells.

PFME and an inhibitor of IκBα phosphorylation inhibits the phosphorylation of the activation of NF-κB in LPS-stimulated RAW264.7 cells. The levels of IκBα, a molecular marker in the NF-κB mechanism, were examined by immunoblot analysis. As shown in Fig. 6A, the basal level of IκBα and p65 in resting cells was high, but LPS treatment led to reduction in the translocation of p65 and IκBα degradation through the ubiquitin-proteasome pathway, with decreases in levels of p65 and IκBα. IκBα sequestered NF-κB in unstimulated RAW264.7

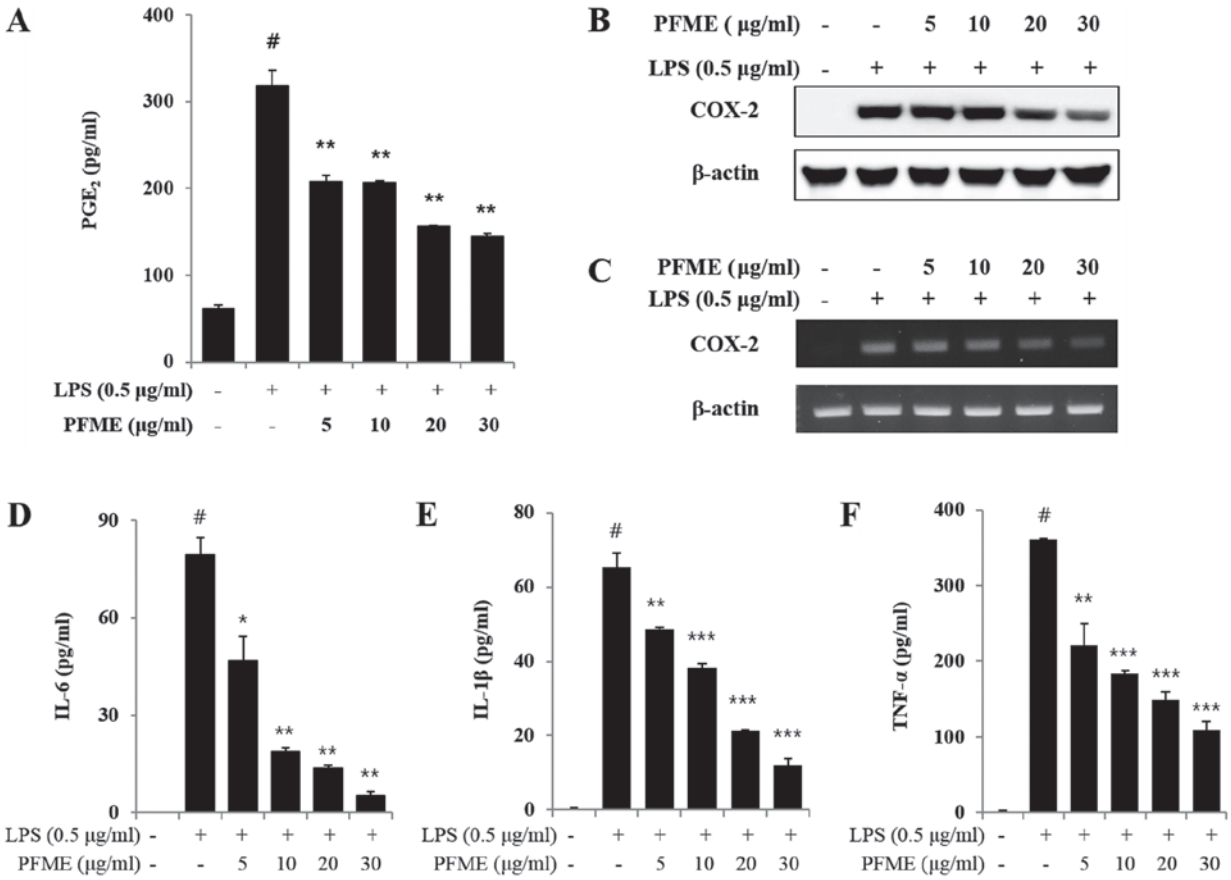

Figure 2. *Passiflora foetida* L. (PFME) inhibits lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) production and cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines expression in RAW264.7 cells. (A) RAW264.7 cells were pretreated with PFME (5, 10, 20 or 30 μg/ml) for 1 h and then stimulated with LPS (0.5 μg/ml). The culture media were collected at 24 h, and the PGE_2 concentrations were measured using an enzyme immunoassay. (B) RAW264.7 cells were pretreated with PFME for 1 h and stimulated with LPS for 6 h. β-actin expression was used as the internal control for RT-PCR. (C) RAW264.7 cells were pretreated with PFME for 1 h and stimulated with LPS for 24 h. β-actin expression was used as the internal control for the western blotting. The effect of PFME on the production of (D) TNF-α, (E) IL-6 and (F) IL-1β in LPS-stimulated RAW264.7 macrophages. The data are presented as the mean ± SEM of three samples. Statistically significant differences ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$) between the treatment groups are indicated with different letters.

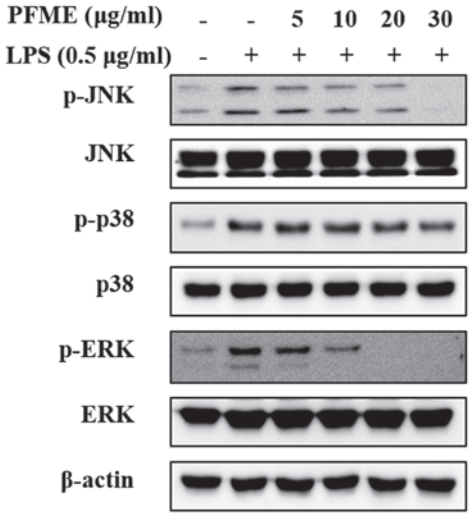

Figure 3. *Passiflora foetida* L. (PFME) suppresses phosphorylation of MAPK molecules in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The effect of PFME on the activities of signalling enzymes upstream from MAPK translocation. Levels of phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 proteins [upstream activators of nuclear factor-κB (NF-κB) translocation] were determined in RAW264.7 cells using antibodies to the phosphorylated and total proteins. The representative results of three independent experiments are shown.

cells, but in LPS-induced cells, degradation of IκBα allowed NF-κB to translocate to the nucleus, with subsequent activation of gene expression resulting from the interaction of NF-κB and the corresponding *cis*-acting element. Therefore, we examined the levels of p65, the major subunit of NF-κB, in nuclear and cytoplasmic fractions, using PARP as a nuclear loading control. In this study, the inhibitor of cytokine-induced IκBα phosphorylation was BAY11-7082 (IC_{50}, 10 μM), which was used to inhibit the NF-κB pathway as an irreversible inhibitor of IκBα phosphorylation that increases stabilization of IκBα and specifically blocks NF-κB signalling. As shown in Fig. 6B, the NF-κB p65 subunit decreased in the cytoplasm and increased in nuclear extracts after treatment with LPS. Treatment with PFME and BAY11-7082 reversed these trends in a concentration-dependent manner. We further investigated the effect of PFME and BAY11-7082 on the inhibition of NO. The LPS-stimulated translocation of the NF-κB p65 subunit from the cytosol to the nucleus in RAW264.7 cells (Fig. 6C) was inhibited by PFME. The NF-κB p65 and IκBα protein levels were significantly correlated with the reduced nuclear accumulations. Thus, we suggest that the PFME inhibited the translocation of the NF-κB p65 subunit and IκBα via the regulation of a signal transduction mechanism related to NF-κB activation.

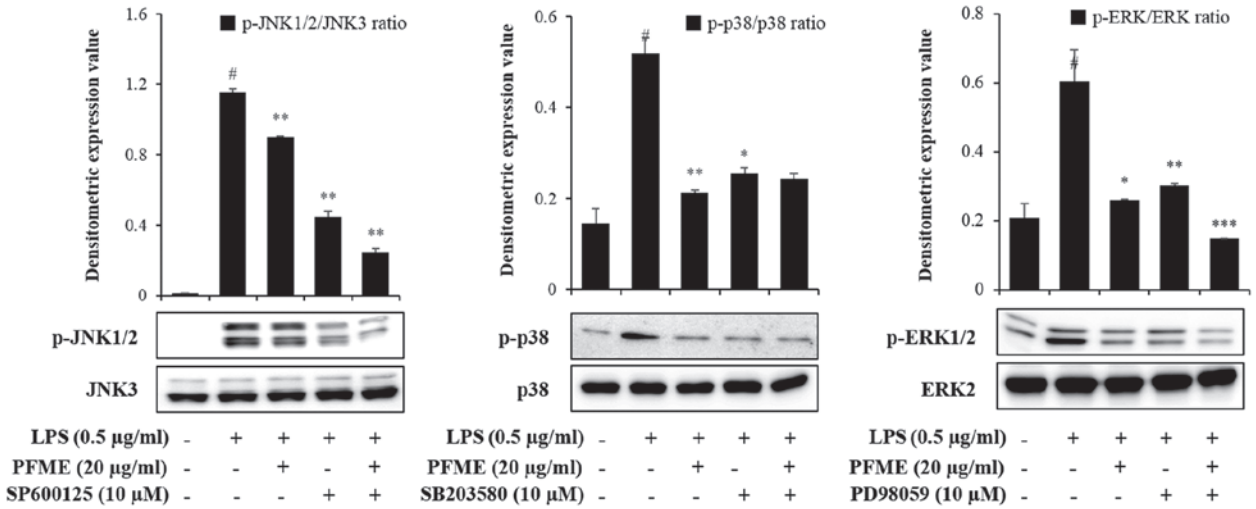

Figure 4. *Passiflora foetida* L. (PFME) and MAPK inhibitors suppress phosphorylation of MAPK molecules in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. The effect of PFME, SP600125 [c-Jun N-terminal kinase (JNK)], SB203580 (p38) and PD98059 [extracellular signal-regulated kinase (ERK)] on the activities of signalling enzymes upstream from MAPK translocation. Levels of phosphorylated and total ERK, JNK and p38 proteins were determined in RAW264.7 cells using antibodies to the phosphorylated and total proteins. The representative results of three independent experiments are shown.

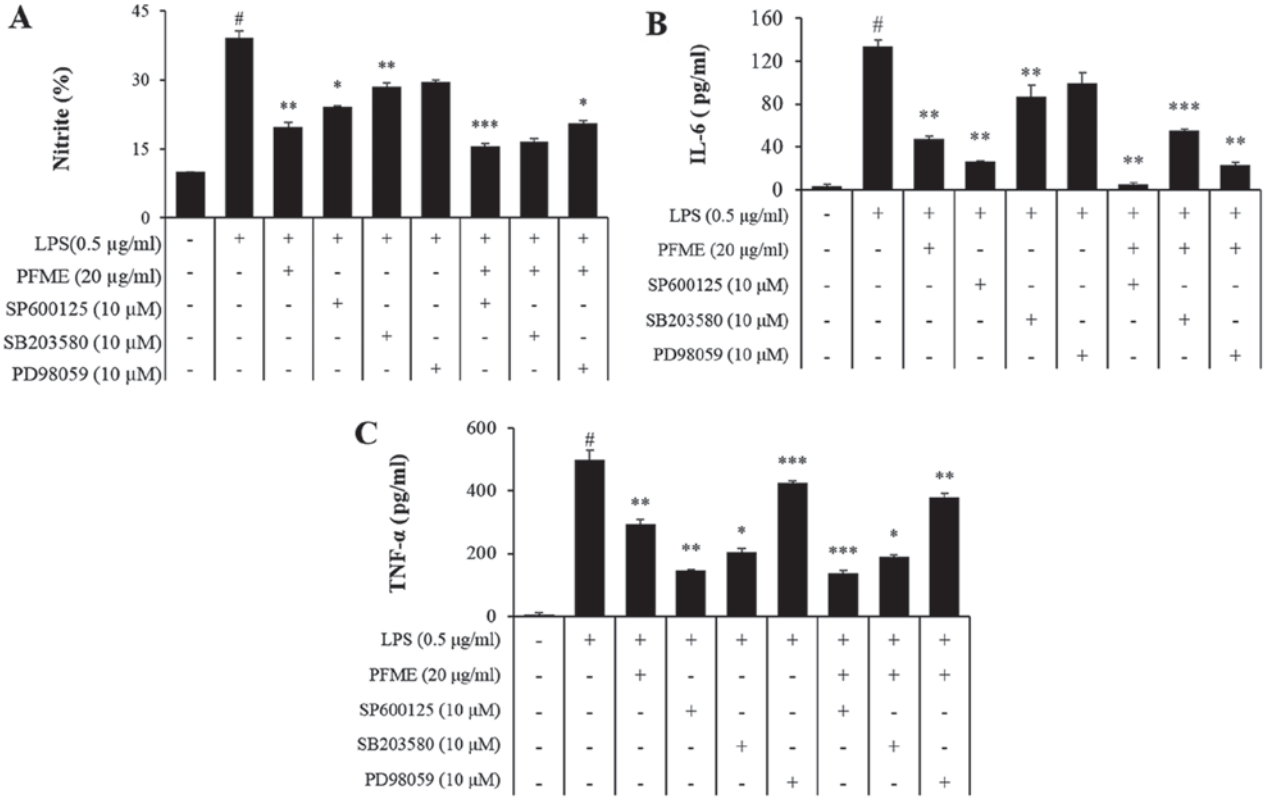

Figure 5. *Passiflora foetida* L. (PFME) and MAPK inhibitors suppress NO and pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. (A) RAW264.7 cells were pretreated with increasing concentrations of PFME (30 μg/ml), SP600125, SB203580 and PD98059 for 1 h and then stimulated with LPS (0.5 μg/ml) for 24 h. The effect of PFME, SP600125, SB203580 and PD98059 on the production of (A) NO, (B) IL-6 and (C) TNF-α in LPS-stimulated RAW264.7 macrophages. The values are expressed as the mean ± SEM of three independent experiments, each performed in triplicate. $^{\$}$P<0.05, significant difference from unstimulated cells; *P<0.05 and **P<0.01, significant difference from LPS-treated cells. Control, DMSO (0.1%); LPS, LPS only (0.5 μg/ml), LPS+PFME: PFME (30 μg/ml) and LPS treatment.

Discussion

Natural products play an important role in both drug discovery and chemical biology (29). PFME is traditionally used as an herbal medicine in several countries (21,30), although the anti-inflammatory effect of PFME is not fully understood. Therefore, we investigated the molecular mechanism and anti-inflammatory effect of PFME in LPS-stimulated RAW264.7 macrophage cells. We found that PFME decreased the release of the inflammatory mediators NO (data not shown) and

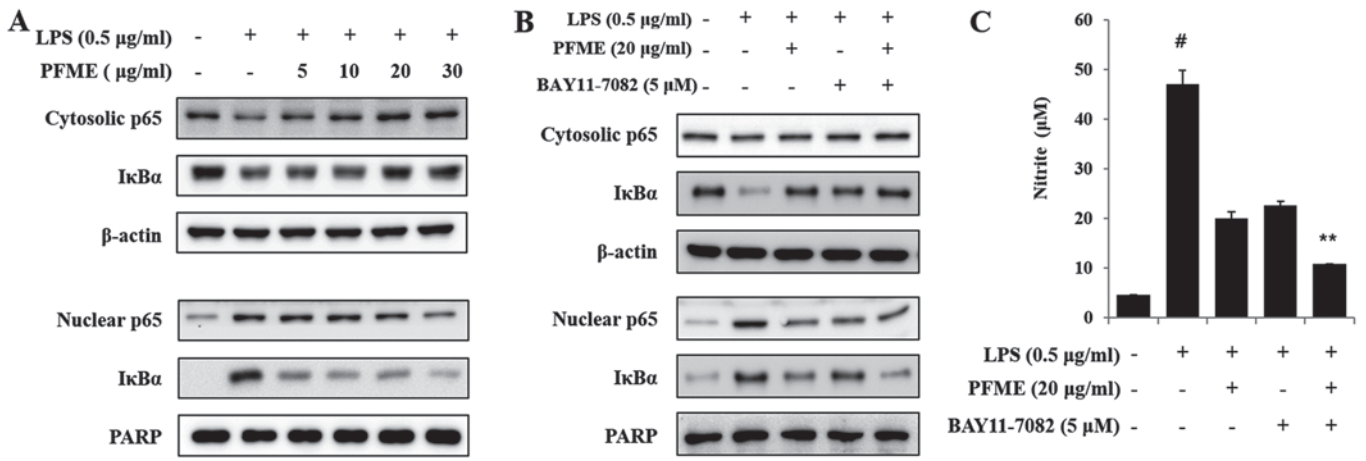

Figure 6. The inhibitory effects of *Passiflora foetida* L. (PFME) and BAY11-7082 on the nuclear translocation of nuclear factor-κB (NF-κB) and the inhibition of nitric oxide expression. (A) Cells were treated with PFME for 1 h, followed by stimulation with lipopolysaccharide (LPS) for 30 min. Equal amounts of protein were analysed using specific antibodies for IκBα and p65. (B) RAW264.7 cells were pretreated with increasing concentrations of PFME (30 μg/ml) and BAY11-7082 for 1 h and then stimulated with LPS (0.5 μg/ml) for 30 min. The nuclear extracts were prepared for western blotting of NF-κB p65 in the nuclear fractions, as described in the Materials and Methods. PARP was used as the internal control. (C) RAW264.7 cells were pretreated with PFME (20 μg/ml) and BAY11-7082 for 1 h, and then stimulated with LPS (0.5 μg/ml). The NO concentrations were measured using the Griess reaction. The values are expressed as the mean ± SEM of three independent experiments, each performed in triplicate. *P<0.05 and **P<0.01, significant difference from LPS-treated cells. Control, DMSO (0.1%); LPS, LPS only (0.5 μg/ml), LPS+PFME: PFME (30 μg/ml) and LPS treatment.

PGE_2 and suppressed pro-inflammatory cytokine production, including that of IL-1β, IL-6 and TNF-α (Fig. 2C-2). Additionally, iNOS (data not shown) and COX-2 protein expression in LPS-stimulated RAW264.7 cells was downregulated by PFME. We also discovered that PFME considerably inhibited inflammatory process signalling pathways, including NF-κB nuclear translocation and phosphorylation of MAPKs. Therefore, we suggest that PFME increased the anti-inflammatory effect in LPS-stimulated macrophages.

During inflammation, excess levels of PGE_2, NO, and pro-inflammatory cytokines are caused by activated inflammatory cells, which result in damaging effects on tissues and cells and the activation in inflammation-associated sickness (31). Through the inflammatory pathways, iNOS and COX-2 expression remarkably increases the generation of NO and PGE_2, respectively, and accordingly, the inhibition of PGE_2 and NO production can be a critical marker for anti-inflammatory responses (32). The fundamental role of the NF-κB signalling pathway is regulation of the transcription factors that control expression of iNOS and COX-2. In LPS-unstimulated macrophages, NF-κB was suppressed by IκB in the cytoplasm. Then, NF-κB can freely translocate from the cytosol to the nucleus in which it promotes transcription of target genes and further induces the transcription of pro-inflammatory mediators, such as IL-6, IL-1β, TNF-α and COX-2 (9,33,34). Many anti-inflammatory agents express their potencies by suppressing NF-κB signalling (35). The molecular signalling of the extract-mediated emaciation in macrophage cells had a close relationship with the inhibition of p65 subunit translocation into the nucleus. Our results indicated that PFME downregulated the expression of COX-2 and also the production of PGE_2 in LPS-stimulated cells (Fig. 2). Additionally, PFME reduced the secretions of IL-6, IL-1β and TNF-α (Fig. 2). The decrement of PFME-mediated molecular signalling in the macrophage cells was closely associated with the oppression of IκB kinase activation followed by inhibition of the translocation of NF-κB p65 subunits into the nucleus (Fig. 6). These results suggest that the PFME inhibited the expression of pro-inflammatory mediators by downregulating the NF-κB pathway in stimulated macrophage cells.

MAPKs, including p38, ERK and JNK, are a syndicate of signalling molecules that likely have an important function in inflammatory mechanisms (36). Zhang *et al* (37) suggested that LPSs induced MAPK cascades and the pathways leading to activation of NF-κB p65. Additionally, MAPKs are complicated by the expression of iNOS in the LPS-induced signalling pathway (38). In this study, LPS induced the phosphorylation of MAPKs. The treatment with PFME significantly inhibited the phosphorylation of LPS-induced ERK, p38 and JNK at 1 h. Consequently, these results suggest that ERK, p38 and JNK were complicated in the inhibition by PFME of LPS-stimulated NF-κB p65 binding in RAW264.7 macrophage cells. In this study, the treatment with PFME blocked the activation of ERK, p38 and JNK, which suggested that PFME suppressed LPS-induced NF-κB translocation by inhibiting the activation of these intracellular signalling cascades, leading to decreased protein levels of iNOS and COX-2 (Fig. 4).

The inhibitory activity of PFME on the MAPKs (JNK, ERK and p38) is shown in Fig. 3. The PFME showed excellent inhibitory activity of the MAPK pathway. When the major MAPK signal in the primary inflammatory response is suppressed, related diseases are affected. Regarding these effects, a comparative analysis was performed using inhibitors known to be actual inhibitors of MAPK. The importance of this experiment was to show that PFME had some influence on the inhibitory activity of each inhibitor. As confirmed in Fig. 4, the MAPK was inhibited with an inhibitor and PFME for each target. As shown in Fig. 5, the inhibitory activity of MAPK against NO, TNF-α and IL-6 was confirmed, which was the result of treating with inhibitors of MAPK and PFME, indicating that the signal-affected targets were inhibited. As a result, the activity of PFME was confirmed to be better than that

of the actual MAPK inhibitors. As with the inhibitors, PFME affected the target and was more effective when using two bells simultaneously. Additionally, the NF-κB inhibitor BAY11-7082 decreased translocation of NF-κB p65 and IκBα and iNOS production in LPS-stimulated macrophages. BAY11-7082 with PFME also inhibited p65 and IκBα expression in this result. The results suggested that blocking of the MAPKs pathway was involved in the suppression of LPS-stimulated NF-κB bound by PFME in RAW264.7 cells (Fig. 6).

To conclude, PFME exhibited anti-inflammatory activities that were related to the suppression of NO, PGE_2, IL-1β, IL-6 and TNF-α and reduced expression of COX-2 and iNOS via activation of the NF-κB p65 pathway in macrophage cells. The anti-inflammatory effect of PFME was particularly maximized when macrophages were pre-treated with PFME and an extract containing a mixture of ten plant-based food groups. Combined, these results suggest that PFME can help diminish the inflammation mediated by activated macrophages, which could potentially improve the response of a host to inflammation-mediated illnesses.

In conclusion, the results of this study indicated that PFME inhibited the LPS-induced inflammatory and oxidative responses, with these effects likely closely related with the suppression of NF-κB activation. Therefore, we propose PFME for possible use as a therapeutic for treating inflammatory diseases.

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Availability of data and material

The analyzed datasets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

JWP analyzed the data and wrote the manuscript. OKK, HWR, JHP, IP and PY prepared the *Passiflora foetida* L., and analyzed and edited the manuscript. SC, SRO and KSA designed the study and edited the manuscript. All authors critically revised the article and have approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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