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 E2 (PGE2) 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
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 (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 purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The RAW264.7 cells were 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% CO₂ 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).

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).

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 GCT GTG CCT GCT G-3' (sense) and 5'-GTC TGC TGG TTT GGA ATA GTC GTG CCT G-3' (antisense); and murine β-actin, 5'-TGA TGT TCT AGA CCT TCA ACA CC-3' (sense) and 5'-CGC TCA TTG CCG ATG 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).
such as IL-6, TNF-α, and IL-1β, and pro-inflammatory mediators in RAW264.7 macrophage cells. Therefore, we investigated the effect of the PFME, SP600125, SB203580, and PD98059 on LPS-stimulated inflammatory mediator activation in RAW264.7 cells. We further investigated the effect of the PFME, SP600125, SB203580, and PD98059 on the inhibition of the phosphorylation of 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 phosphorylation of 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 phosphorylation of 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 phosphorylation of 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 phosphorylation of JNK, p38 and ERK1/2 in LPS-stimulated RAW264.7 cells (Fig. 4).
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 (IC50, 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.
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...
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 and the activation in inflammation-associated sickness (31). Therefore, we suggest that PFME increased the anti-inflammatory effect in LPS-stimulated macrophages.

During inflammation, excess levels of PGE₂, 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 PGE₂, respectively, and accordingly, the inhibition of PGE₂ 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 IkB 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 extracellular emaciation in macrophage cells had a close relationship with the inhibition of pro-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 implicated 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 PFME-mediated emaciation in macrophage cells.
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, PGE₂, IL-β, 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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