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Original Article

The Anti-inflammatory Mechanism of *Protaetia brevitarsis* Lewis via Suppression the Activation of NF-κB and Caspase-1 in LPS-stimulated RAW264.7 Cells

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The larva of *Protaetia brevitarsis* Lewis (*P. brevitarsis*), edible insect, is traditionally consumed as alternative source of nutrients and has various health benefits. However, the exact pharmaceutical effects of *P. brevitarsis* on inflammatory response are still not well understood. Thus, we investigated the anti-inflammatory effects and mechanisms of *P. brevitarsis* in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. We investigated the effects of *P. brevitarsis* on the expression levels of inflammatory-related genes, including inflammatory cytokines, prostaglandin E_2 (PGE_2), cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in LPS-stimulated RAW264.7 cells. To understand the anti-inflammatory mechanism of *P. brevitarsis*, we explored the regulatory effect of *P. brevitarsis* on nuclear factor (NF)-κB and caspase-1 activation. The findings of this study demonstrated that *P. brevitarsis* inhibits the LPS-induced inflammatory cytokine and PGE_2 levels, as well as COX-2 and iNOS expression. Moreover, we confirmed that the anti-inflammatory effect of *P. brevitarsis* occurs via suppression of the activation of NF-κB and caspase-1. Conclusively, these findings provide experimental evidence that *P. brevitarsis* may be useful candidate for the treatment of inflammatory-related diseases.

Key Words: *Protaetia brevitarsis* Lewis; Inflammation; Nuclear factor-kappa B; Caspase-1

INTRODUCTION

Edible insects have received a revived interest as healthy nutritious source or traditional remedies for various diseases, including anemia, hypertension and asthma (Van Itterbeeck and van Huis, 2012; Van Huis, 2016). Although edible insects have long been employed in effective treatment of diseases, their precise pharmaceutical mechanisms are still not well understood.

Inflammatory diseases, including allergic rhinitis, asthma Crohn's disease and rheumatoid arthritis, have become a global health problem. Generally, inflammatory processes are involved in the action of multiple factors within a complex network (Guo et al., 2015). Although inflammatory reactions are considered an important response to host defense against pathogens, prolonged inflammation can lead to various chronic diseases. Activated macrophages induce variety of inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), which play crucial role in the process of inflammation (Liu et al., 2014). The increase in inflammatory mediators may be important in the development of chronic inflammatory diseases (Wicks

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and Roberts, 2016). Therefore, the suppression of inflammatory mediator release by macrophage is a useful therapeutic strategy for inflammation-related diseases.

Nuclear factor-kappa B (NF-κB) performs an important function in the expression of genes associated with the inflammatory responses (Lee et al., 2020). In response to inflammatory stimuli, the IκB kinase (IKK) complex is phosphorylated and the IκB is degraded, allowing free NF-κB to translocate into the nucleus, modulate gene transcription, and activate various inflammatory mediators. An increased NF-κB activity has been reported in macrophages and epithelial cells of patients with inflammatory diseases (Wong et al., 2001). Moreover, inhibition of NF-κB activation diminishes the influx of inflammatory cells and reduces the allergic inflammation (Birrell et al., 2005). Therefore, NF-κB is currently considerd to be a target for the treatment of inflammatory diseases.

Protaetia brevitarsis Lewis (*P. brevitarsis*) is a species of Coleoptera, and the larvae stage of *P. brevitarsis* has been used as a traditional medicine for various diseases (Yoo et al., 2007). Our previous study showed that *P. brevitarsis* has the anti-obesity activity through the inhibition of body weight gain, serum lipid levels, peritoneal fat and obesity-related gene expression (Ahn et al., 2019). However, the exact anti-inflammatory mechanism of *P. brevitarsis* is still not well understood. To provide experimental evidence that *P. brevitarsis* might be a useful therapeutic drug for inflammatory diseases, we investigated the effects of *P. brevitarsis* on the expression of inflammatory mediators, as well as activation of NF-κB and caspase-1 in lipopolysaccharide (LPS)-induced RAW264.7 cells.

MATERIALS AND METHODS

Reagents

LPS, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium-bromide (MTT), avidin peroxidase (AP), and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) kits were obtained from Thermo Fisher Scientific Inc. (Somerset, NJ, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Difco Laboratories (Detroit, MI, USA). The assay kits for mouse TNF-α/ IL-6/ IL-1β were procured from BD Biosciences (San Diego, CA, USA). Caspase-1 assay kit were obtained from Biovision (Milpitas, CA, USA). The specific antibodies (Ab) against COX-2, iNOS, NF-κB, and histone were procured from Santa Cruz Biotechnology (CA, USA).

Preparation of *P. brevitarsis*

P. brevitarsis (100 g) was pulverized into fine powder and 500 mL of 70% aqueous ethanol solution was used for extraction for 24 h and then concentrated under vacuum. The ethanol extract was filtered, concentrated, and lyophilized (yield: 8.42%). The samples were dissolved in PBS, and then filtered through 0.22-μm syringe filter.

Cell culture

RAW264.7 cells were cultured in DMEM containing with penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% FBS at 37℃ in in a humidified atmosphere of 5% CO_2.

MTT assay

To investigate the cell viability by *P. brevitarsis*, the MTT assay was conducted. Briefly, cells were incubated with *P. brevitarsis* (0.01, 0.5, and 1 mg/mL) for 24 h, and 50 μL of MTT solution was added and the mixture was incubated for 4 h. Then, the crystallized formazan was dissolved in dimethyl sulfoxide and the absorbance of plate was read at 540 nm using a microplate reader (Molecular Devices, CA, USA).

Cytokine assay

The levels of TNF-α/ IL-6/ IL-1β were assayed by using a modification of an enzyme-linked immunosorbent assay (ELISA), as previously described (Kim et al., 2010). Briefly, plates (96-well) were coated with TNF-α/ IL-6/ IL-1β monoclonal Abs and then incubated overnight at 4℃. After washes, standard solution of TNF-α/ IL-6/ IL-1β or sample were added and incubated for 2 h. The plate was exposed to biotinylated TNF-α/ IL-6/ IL-1β Abs followed by incubation for 2 h. After washing the plates, AP and ABTS substrate was sequentially added. The optical density was read at 405 nm.

PGE_2 assay

The PGE_2 concentration in cell culture supernatant was measured using PGE_2 colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocols.

Measurement of nitrite (NO) concentration

RAW264.7 cells (3×10^5 cells/well) were treated with *P. brevitarsis* for 1 h, and then stimulated with LPS (1 μg/ mL) for 24 h. The NO content was determined using Griess reagent. The supernatant of cell culture was mixed with an equal volume of griess reagent. The optical density was measured at 540 nm. The NO concentration was measured by a standard curve generated using sodium nitrite.

Western blot analysis

To investigate the protein levels, cells were lysed using ice-cold lysis buffer. Nuclear extracts were isolated by Nuclear Extraction Reagents. After protein quantification using bicinchoninic acid (BCA), the lysed protein was mixed with a sample buffer, separated using gel electrophoresis, and transferred to membrane. The membrane was then blocked with 5% skimmed milk and reacted with primary Abs. After washing with 0.1% PBST, membrane was incubated with secondary Abs for 2 h. After washing with PBST, the protein bands were visualized by an ECL detection system.

Luciferase reporter gene assay

RAW264.7 cells were transfected with NF-κB-luc DNA and the medium was refreshed. The transfected cells were seeded and treated with *P. brevitarsis* for 1 h and stimulated with LPS for 2 h. Luciferase activity was measured using a Dual-Glo luciferase assay kit following the manufacturer's protocols (Promega, Madison, Wisconsin, USA).

Caspase-1 activity assay

The caspase-1 activity was measured using a caspase-1 colorimetric assay kit according to the manufacturer's instructions. The lysed cells were centrifuged at 12,000 rpm for 10 min. After protein quantification by BCA, the protein was incubated with 50 μL reaction buffer and 5 μL caspase-1 substrates at 37℃ for 2 h. The absorbance was measured at 405 nm.

Statistical analysis

Results are shown as the mean ± SD, and each experiment was performed at least-three times. The statistical results were performed using an independent *t*-test and ANOVA with a Tukey post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of *P. brevitarsis* on cell viability and production of inflammatory cytokines in LPS-stimulated RAW264.7 cells

We evaluated the cytotoxic effects of *P. brevitarsis* after treatment with various concentrations of *P. brevitarsis* (0.01, 0.5, and 1 mg/mL) for 24 h using an MTT assay. In present study, no cell cytotoxicity of *P. brevitarsis* was observed (Fig. 1A). Next, to investigate the anti-inflammatory activity of *P. brevitarsis*, we evaluated the effects of *P. brevitarsis* on TNF-α, IL-6 and IL-1β production from LPS-stimulated RAW 264.7 cells. The cells were treated with or without *P. brevitarsis* (0.01, 0.5 and 1 mg/mL) for 1 h prior to stimulation with LPS for 24 h. As shown in Fig. 1B and D, LPS alone increased TNF-α, IL-6, and IL-1β production compared to the LPS-untreated control. However, treatment with *P. brevitarsis* significantly inhibited LPS-induced TNF-α, IL-6 and IL-1β production in a dose-dependent manner. The maximal inhibition rates of TNF-α, IL-6, and IL-1β production by *P. brevitarsis* (1 mg/mL) were approximately 40.1% ($P < 0.05$), 33.7% ($P < 0.05$) and 29.5% ($P < 0.05$), respectively.

Effects of *P. brevitarsis* on COX-2 expression and PGE_2 production in LPS- stimulated RAW264.7 cells

Increase of COX-2 levels are associated with physiological processes of inflammation (Liu et al., 2018). Western blot analysis was conducted to measure the effects of *P. brevitarsis* on LPS-induced COX-2 expression. The cells were treated for 1 h with *P. brevitarsis* (0.01, 0.5 and 1 mg/mL) and stimulated for 24 h with LPS. As shown in Fig. 2A,

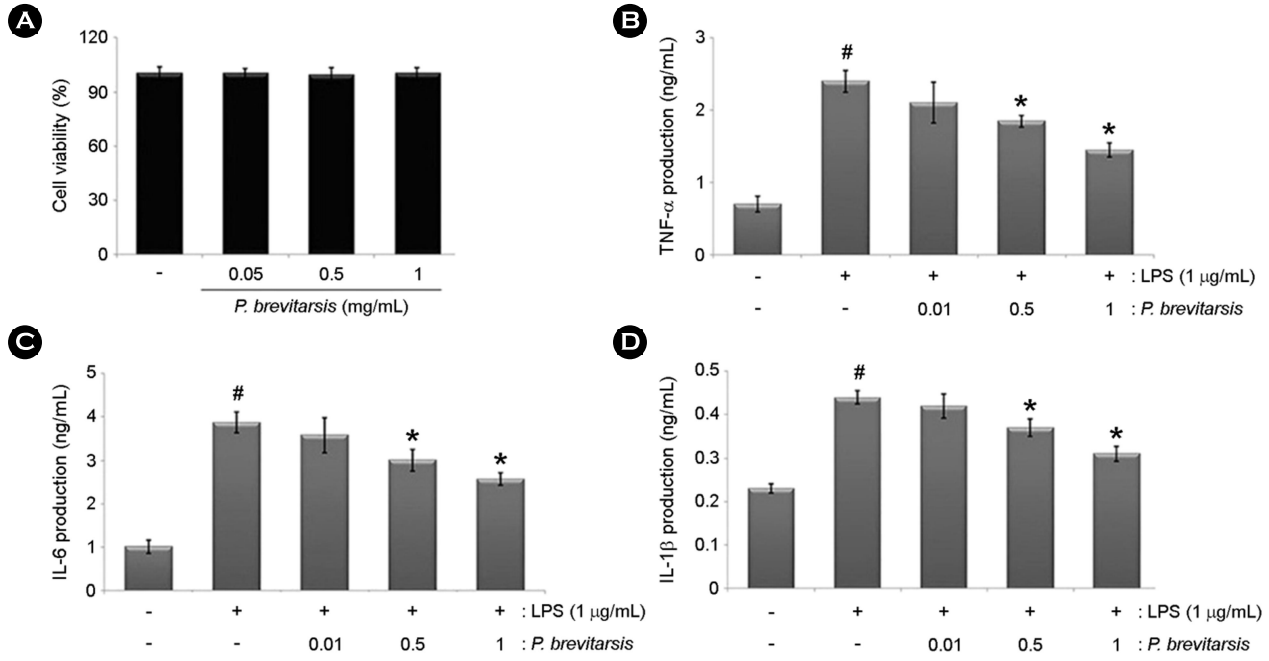

Fig. 1. Effects of *P. brevitarsis* on cell viability and production of inflammatory cytokines in LPS-induced RAW264.7 cells. Cells (3×10^5 cells/mL) were treated for 1 h with *P. brevitarsis* (0.1, 0.5 and 1 mg/mL), and stimulated with LPS (1 μg/mL) for 24 h. (A) Cell viability was analyzed by MTT assay. (B-D) The levels of TNF-α, IL-6 and IL-1β in the supernatant were measured using ELISA. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

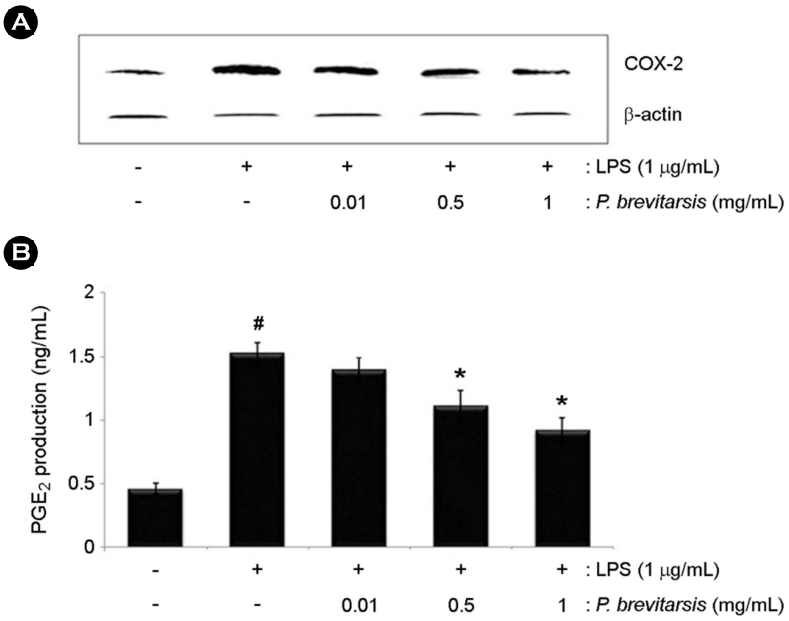

Fig. 2. Effects of *P. brevitarsis* on COX-2 and PGE_2 levels in LPS-induced RAW264.7 cells. (A) Cells (1×10^6 cells/mL) treated for 1 h with *P. brevitarsis* (0.1, 0.5, and 1 mg/mL) and stimulated with LPS (1 μg/mL) for 24 h. The protein extracts were assayed via Western blot analysis for COX-2. (B) Cells were pretreated for 1 h with *P. brevitarsis* (0.1, 0.5 and 1 mg/mL) and stimulated for 24 h with LPS (1 μg/mL). The levels of PGE_2 production was evaluated with assay kit. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

LPS induced the expression of COX-2, but *P. brevitarsis* inhibited the LPS-induced the COX-2 levels.

COX-2 catalyzes the biosynthesis of PGE_2, which contributes to the pain and swelling during inflammatory process (Rumzhum and Ammit, 2016). Thus, we examined the regulatory effect of *P. brevitarsis* on PGE_2 production in LPS-stimulated RAW264.7 cells. As shown in Fig. 2B, PGE_2 production was enhanced in response to LPS treatment;

however, this increase was significantly suppressed by *P. brevitarsis* treatment. The maximal inhibition rate of PGE_2 production by *P. brevitarsis* (1 mg/mL) was approximately 40.4% ($P < 0.05$).

Effects of *P. brevitarsis* on the NO and iNOS levels in LPS- stimulated RAW264.7 cells

As a NO increase is associated with inflammatory process, we investigated the effects of *P. brevitarsis* on NO production using Griess reagent. *P. brevitarsis* was shown to induce a reduction in NO production in a dose-dependent manner (Fig. 3A), with a maximal inhibition rate of NO production by *P. brevitarsis* (1 mg/mL) being measured as 38.9% ($P < 0.05$). Additionally, Western blot analysis was conducted to measure the effect of *P. brevitarsis* on LPS-induced iNOS expression. As shown in Fig. 3B, LPS induced a increase in iNOS expression, but the level of iNOS decreased, in response to treatment with *P. brevitarsis*.

Effects of *P. brevitarsis* on NF-κB activation in LPS-stimulated RAW264.7 cells

As suppression of NF-κB activation was identified as an anti-inflammatory strategy, we theorized that the anti-inflammatory mechanism of *P. brevitarsis* may be mediated via the attenuation of NF-κB activation. Because NF-κB activation generally requires the translocation of NF-κB into nuclear, we elucidated the effects of *P. brevitarsis* on the nuclear and cytosolic pools of NF-κB using Western blot

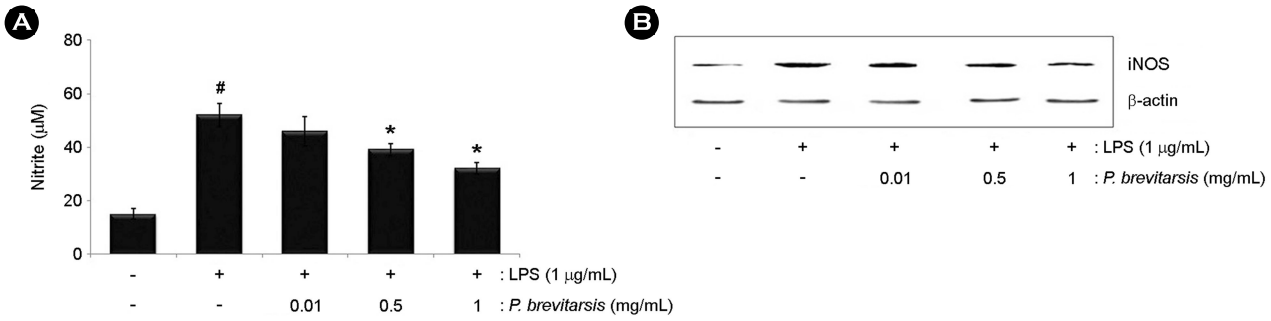

Fig. 3. Effects of *P. brevitarsis* on NO and iNOS levels in LPS-induced RAW264.7 cells. Cells were treated for 1 h with *P. brevitarsis* (0.1, 0.5 and 1 mg/mL) followed by LPS (1 μg/mL) for 24 h. (A) NO production in supernatant was measured via the Griess reagent. (B) The protein extract was assayed via Western blot analysis for iNOS. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

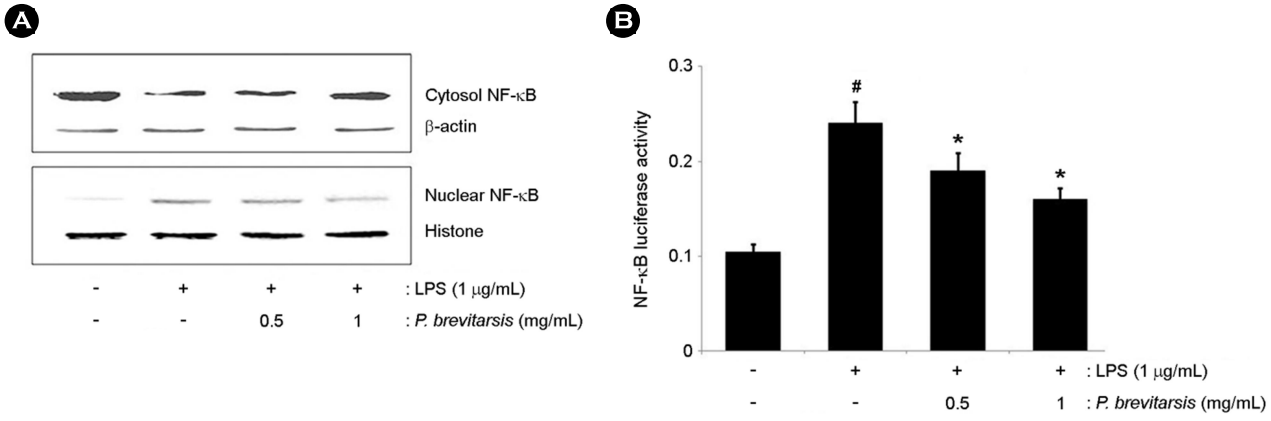

Fig. 4. Effect of *P. brevitarsis* on NF-κB activation in LPS-stimulated RAW264.7 cells. (A) Cells (1×10^6 cells/mL) were treated for 1 h with *P. brevitarsis* and stimulated for 2 h with LPS. Nuclear extracts were prepared by Nuclear Extraction Reagents and evaluated for NF-κB via Western blot analysis. (B) The activity was measured by a luciferase assay kit following the manufacturer's protocols. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

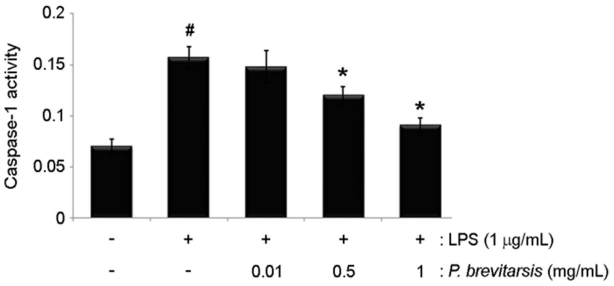

Fig. 5. Effect of *P. brevitarsis* on caspase-1 activity in LPS-induced RAW264.7 cells. The cells were treated with *P. brevitarsis* (0.1, 0.5 and 1 mg/mL) for 1 h and stimulated with LPS for 12 h. Caspase-1 activity was determined using a caspase-1 colorimetric assay. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

analysis. The levels of NF-κB (Rel/p65) in the nuclear fraction were increased in LPS-stimulated cells, but *P. brevitarsis* reduced the enhanced nuclear levels of Rel/p65 (Fig. 4A). Moreover, to measure the effect of *P. brevitarsis* on the promoter activity of NF-κB, a luciferase reporter assay was conducted. As illustrated in Fig. 4B, *P. brevitarsis* attenuated the NF-κB-driven luciferase activity in LPS-induced RAW 264.7 cells.

Effect of *P. brevitarsis* on caspase-1 activation in LPS-stimulated RAW264.7 cells

Caspase-1 activation is associated with inflammatory reaction by causing an increase in inflammatory cytokines and in the recruitment of inflammatory cells (Han et al., 2017). Therefore, to identify the regulatory mechanism of *P. brevitarsis* in inflammation, we assessed whether *P. brevitarsis* inhibits the caspase-1 activity in LPS-stimulated cells. The results showed that LPS treatment induced the caspase-1 activation but, the increase in caspase-1 activity was down-regulated by *P. brevitarsis* in a dose-dependent manner (Fig. 5). The maximal inhibition rate of caspase-1 activity by *P. brevitarsis* (1 mg/mL) was approximately 45.8% ($P < 0.05$).

DISCUSSION

Edible insects have been widely used, since they are healthy nutritious source and can be used in traditional medicine. Although *P. brevitarsis* demonstrated various biological activities, the precise anti-inflammatory mechanisms of *P. brevitarsis* have yet to be thoroughly elucidated. The findings of this study show that *P. brevitarsis* inhibited the levels of TNF-α, IL-1β, NO, and PGE_2 as well as the expression of COX-2 and iNOS. Moreover, we confirmed that the anti-inflammatory effect of *P. brevitarsis* is mediated via the suppression the activation of NF-κB and caspase-1 in LPS-stimulated RAW264.7 cells. These results suggest a molecular mechanism via which *P. brevitarsis* ameliorates the inflammatory reaction.

Inflammatory processes are biological response of the immune system that can be induced by a variety of factors including pathogens, injury, and toxic compounds (Edwards et al., 2017). Macrophages actively contribute to inflammatory responses by releasing inflammatory mediators (Beutler, 2000). In response to stimuli, macrophages generate a variety of inflammatory cytokines that contribute to the infiltration of immune cells to inflammation sites (Trefzer et al., 2003). It has also been reported that TNF-α and IL-6 levels are increased in patients with inflammatory diseases (Fedenko et al., 2011). PGE_2, produced by COX-2 at inflammatory sites, contributes to the pain and swelling, which are associated with inflammation (Liu et al., 2018). These results suggest that suppression of inflammatory mediators from activated macrophages is a useful therapeutic strategy for treatment of inflammatory diseases. Therefore, we investigated whether the anti-inflammatory activity of *P. brevitarsis* is mediated by the attenuation of inflammatory mediators, such as inflammatory cytokines, COX-2, PGE_2 and NO in LPS-simulated RAW 264.7 cells. In this study, we showed that *P. brevitarsis* significantly suppressed the LPS-induced production of inflammatory cytokines (TNF-α, IL-6, and IL-1β) and mediators (PGE_2, COX-2, NO and iNOS). Based on these results, we propose that the anti-inflammatory activity of *P. brevitarsis* may be associated with the suppression of inflammatory-related gene expression.

Accumulating evidence suggests that the NF-κB performs a crucial function by regulation the transcription of numerous genes involved in inflammation (Shin et al., 2019). An increased NF-κB activity associated with the secretion of IL-6 and TNF-α was shown to be involved in inflammatory

diseases (Sultuybek et al., 2019). It was reported that inhibition of NF-κB activation reduced the influx of inflammatory cells and diminished the inflammation (Birrell et al., 2005). Caspase-1 plays an important role in apoptosis and inflammation (Siegmund et al., 2001). Caspase-1 activation is associated with an increased number of inflammatory mediators. It was been reported that caspase-1- deficiency mice have a reduced cytokine production (Kuida et al., 1995). Additionally, it was confirmed that activation of caspasse-1 induced NF-κB and MAPK-signaling pathways. Based on these results, the suppression of NF-κB/caspase-1 activation was identified as an anti-inflammatory strategy. Therefore, to investigate the anti-inflammatory mechanism of *P. brevitarsis*, we tested whether *P. brevitarsis* could ameliorate the NF-κB/caspase-1 activation. The results demonstrated that *P. brevitarsis* inhibits NF-κB translocation into the nucleus and caspase-1 activation. We hypothesized that inhibitory effects of *P. brevitarsis* on inflammation might derive from the blockage of NF-κB/caspase-1 activity in LPS-stimulated RAW 264.7 cells.

In conclusion, our results suggest that the anti-inflammatory activities of *P. brevitarsis* could be attributed to the suppression of inflammatory mediators. Moreover, the anti-inflammatory mechanism of *P. brevitarsis* is caused by the attenuation of LPS-induced NF-κB/caspase-1 activation. Our results provide experimental evidences that *P. brevitarsis* may be a potential candidate for the treatment of inflammatory-related diseases.

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CONFLICT OF INTEREST

There is no conflict of interest.

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