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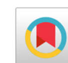

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Inhibition of inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells by *Pinus densiflora* root extract

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Abstract *Pinus densiflora* root (PDR) is used as a medicinal plant. In this study, we investigated whether the PDR extract has anti-inflammatory activities. Cell viability assays showed that the extract was not toxic toward RAW 264.7 cells at concentrations up to 10 μg/mL. At 10 μg/mL, the extract decreased nitric oxide (NO) content to 40% of the control level. The protein expression of inducible nitric oxide synthase (iNOS), which generates NO, decreased with increasing concentrations of the extract. Prostaglandin E_2 (PGE_2) levels were significantly inhibited by over 50% in the presence of 10 μg/mL of the extract. The protein expression of cyclooxygenase-2 (COX-2), which generates PGE_2, decreased with increasing concentrations of the extract. Proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1β, were detected in RAW 264.7 cells after lipopolysaccharide (LPS) treatment. The extract did not affect the levels of TNF-α and IL-6, but it significantly inhibited the level of IL-1β. It also completely inhibited the transcription of nuclear factor-kappaB (NF-κB). These results indicate that the PDR extract reduces inflammatory response-related proteins, such as NO, PGE_2, iNOS, and COX-2, in LPS-induced RAW 264.7 cells via the regulation of NF-κB. Consequently, we have provided a mechanism to explain the anti-inflammatory effect of the PDR extract; that is, it exerts such an effect by regulating NF-κB. The PDR extract can therefore be considered as an effective anti-inflammatory agent.

Keywords Inflammation · Inhibition · Lipopolysaccharide · *Pinus densiflora* root · RAW 264.7 cells

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Introduction

In general, inflammatory response is considered as a typical defense against infection or cell damage. It is followed by a systemic reaction and plays an important role in health and disease [1]. Macrophages, one of the cell types involved in the inflammatory response, have a significant effect on immune and inflammatory reactions. Macrophages are stimulated by lipopolysaccharide (LPS; a well-known lipoglycan and endotoxin) and cytokines such as interferon-gamma, interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) [2]. The activated macrophages then secrete diverse types of inflammatory substances, including nitric oxide (NO), prostaglandin E_2 (PGE_2), and various cytokines. A suitable amount of these inflammatory substances mediates the essential immune reaction to fight viruses and bacteria [3]. However, if the inflammatory response is excessive or prolonged, it can cause fatal chronic diseases, including arthritis, cardiovascular diseases, sepsis, cancers, and autoimmune disorders [4]. NO, a reactive radical molecule in cells, is generated from L-arginine through its oxidation by nitric oxide synthase (NOS) [5]. The two types of NOS are constitutive NOS and inducible NOS (iNOS), the latter being well known in the defense response against contagious pathogens, including viruses associated with various inflammatory diseases, circulatory disorders, and cancers [6]. PGE_2, also known as dinoprostone, is a principal

inflammatory mediator that is generated from arachidonic acid, which is converted by cyclooxygenase (COX) enzymes such as COX-1 and COX-2 [7]. COX-1, which is expressed in all cells of an organism, is involved in regulating blood platelet aggregation, controlling blood flow in the kidney, and protecting the cell lining in the stomach. COX-2 is produced during an inflammatory reaction [8]. The protein expression of iNOS and COX-2 is regulated by nuclear factor-kappaB (NF-κB), which comprises a complex of transcription factors, including p50 and p65 subunits. NF-κB usually exists in an inactivate form combined with the NF-κB inhibitory protein (IκB) in the cytoplasm of resting cells. Once IκB kinases (IKKs) are activated by stimuli, they phosphorylate IκB to initiate its degradation, thereby activating NF-κB to translocate from the cytoplasm to the nucleus where it regulates the transcription of target genes [9]. Although synthetic anti-inflammatory drugs have been developed, they may have side effects on the heart, stomach, and kidney [10]. Therefore, there is a need to develop safer anti-inflammatory drugs from natural resources.

Pinus densiflora, known as the pine tree, is a type of conifer that has been cultivated throughout Korea, Japan, and Manchuria [11,12]. From ancient times, all parts of the pine tree (viz., the needle, bark, cone, pollen, and roots) have been used as a hardy plant resource [13]. The majority of previous studies on *P. densiflora* were conducted on the pine needle, which is the part that is used the most and contains flavonoids, anthocyanin, and carotene as well as oil components (viz., alpha-pinene, beta-pinene, and camphene) [14]. These compounds have positive effects on gastroenteropathy, arteriosclerosis, hypertension, diabetes, and nervous system diseases [15-17]. However, despite it being a well-known traditional herbal medicine, the anti-inflammatory effects of the *P. densiflora* root (PDR) have not yet been investigated.

In this study, we aimed to determine whether the inhibitory effects of the PDR extract on LPS-induced inflammatory responses in RAW 264.7 macrophages is through repression of the NF-κB signaling pathway. Additionally, we hypothesized that the PDR extract could be used as a functional ingredient for the development of anti-inflammatory treatments.

Materials and Methods

Preparation of the *Pinus densiflora* root extract

The PDR was purchased from Yangnyeongsi Herbal Medicine Market (Daegu, Republic of Korea). The root sample was dried in an oven at 50 ^{o}C and pulverized to a 40-mesh size. Then, 5 g of the PDR powder was added to 100 mL of 70% ethanol and extraction was carried out at room temperature for 24 h. Thereafter, filtration of the PDR extract was performed using Whatman No. 1 filter paper (Whatman, Maidstone, Kent, UK). The ethanol used as the extraction solvent was evaporated with a vacuum rotary evaporator (R-200; Büchi Labortechnik AG, Flawil, Switzerland) and the PDR extract was freeze-dried in a freeze dryer (ilShin, Ede, the Netherlands), yielding 0.65 g of dried powder (yield ratio 13%).

Cell culture

RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells, which were used in all experiments, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were maintained in a 5% CO_2 incubator at 37 ^{o}C.

Cell viability assay

The cytotoxicity of the PDR extract was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were plated at a density of 1×10^4 cells/well in a 48-well plate for 24 h, following which various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract were added. After 16 h of incubation, 50 μg/mL of MTT solution was added to each well and the plate was allowed to stand for 4 h. Then, the supernatant was discarded, and 300 μg/mL of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance at 540 nm was determined using a UV-visible microplate reader (SPECTROstar Nano; BGM Labtech, Ortenberg, Germany).

Measurement of NO levels

The NO levels, measured as the nitrite concentration, were determined using the Griess reagent system. RAW 264.7 cells were seeded at a density of 5×10^4 cells/well in a 96-well plate for 24 h. Then, the cells were pretreated with 1 μg/mL of LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract, and incubation was carried out for 16 h. The supernatant fluid was used to measure the NO levels using the Griess reagent system protocol. The absorbance at 540 nm was then determined using the SPECTROstar Nano UV-visible microplate reader.

Measurement of PGE_2 levels

The PGE_2 levels in the supernatant fluid were determined by enzyme-linked immunosorbent assay (ELISA). RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate for 24 h. Then, the cells were pretreated with 1 μg/mL of LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract, and incubation was carried out for 16 h. The PGE_2 levels were measured using the protocol of R&D Systems (Minneapolis, MN, USA). The absorbance at 540 nm was then determined using the SPECTROstar Nano UV-visible microplate reader.

Western blot analysis

The protein expression levels of iNOS and COX-2 were analyzed

by western blot assay. RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in a 6-well plate for 24 h. Then, the cells were pretreated with 1 μg/mL of LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract, and incubation was carried out for 16 h. Activation of NF-κB was also analyzed by western blot assay. In brief, the cells were seeded at a density of 2×10^6 cells/well in a 100-mm culture dish for 24 h. Then, the cells were pretreated with 1 μg/mL of LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract, and incubation was carried out for 4 h.

All culture media were extracted and the cells were washed with cold phosphate-buffered saline. After deep-freezing, the cells were lysed. The proteins obtained were centrifuged (4 °C, 13,000 rpm, 20 min) and the supernatant fluid was collected. The protein concentration in the supernatant fluid was evaluated using a bicinchoninic acid protein assay kit. Thereafter, 20 μL of the protein samples was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene difluoride membrane, which was then blocked with blocking buffer (5% skim milk in Tris-buffered saline with Tween 20 (TBST)) in an incubator for 1 h. Then, the membrane was incubated overnight with primary antibodies (1:1,000) against iNOS, COX-2, and NF-κB at 4 °C. Following this, the membrane was washed for 30 min with TBST and then incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG1; 1:1,000) for 2 h at room temperature. Finally, the membrane was washed for 30 min with TBST and then reacted with an enhanced chemiluminescence solution in the dark. After exposure to X-ray film, the intensity of each protein band on the membrane was detected using an image analyzer (C300; Azure Biosystems, Dublin, CA, USA).

Measurement of proinflammatory cytokine levels

The levels of IL-1β, IL-6, and TNF-α in the culture supernatant fluid were determined by ELISA. RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate for 24 h. Then, the cells were pretreated with 1 μg/mL of LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract, and incubation was carried out for 16 h. The IL-1β, IL-6, and TNF-α levels were measured according to the protocols of R&D Systems. The absorbance was determined at 540 nm using the SPECTROstar Nano UV-visible microplate reader.

Statistical analysis

All of the tests were replicated 3 times (n =3). Statistical analysis was conducted using one-way analysis of variance in the SPSS v23 program (Statistical Package for Social Science, Chicago, IL, USA). Duncan's multiple-range test was performed to analyze differences between groups. A p-value of less than 0.05 was considered to be statistically significant.

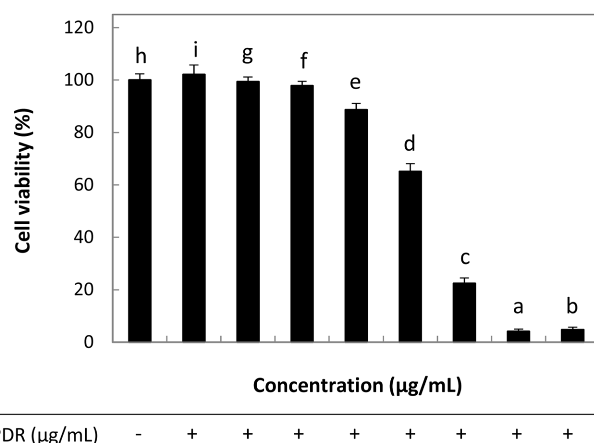

Fig. 1 MTT assay of the effect of the *Pinus densiflora* root (PDR) extract on the viability of RAW 264.7 cells. The cells were treated with various concentrations (3, 5, 7, 10, 15, 20, 30, and 50 μg/mL) of the PDR extract. Data are expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. Data are the mean ± SD (n =3). Means with different letters (a-i) above the bars are significantly different at p <0.05 by Duncan's multiple-range test

Results and Discussion

Effect of the PDR extract on the viability of LPS-induced RAW 264.7 cells

To evaluate the toxicity of the PDR extract toward RAW 264.7 cells, the MTT assay was performed. As shown in Fig. 1, the effect of the PDR extract on cell viability was observed to be dose-dependent. When the concentration of the extract was below 10 μg/mL, the cell viability was more than 90%. However, when the concentration of the extract was 15 μg/mL, the cell viability decreased to 65%. The cell viability significantly decreased at PDR extract concentrations above 15 μg/mL. Therefore, all of the subsequent experiments were performed using 3, 5, 7, and 10 μg/mL of the PDR extract, which were non-toxic toward the cells.

Effect of the PDR extract on LPS-induced NF-κB activation

LPS activates NF-κB, which leads to the induction of many inflammatory responses. NF-κB, which exists as a complex with p50 and p65, is kept in an inactive form in the cytoplasm by IκB-α. Upon activation by a stimulus, IκB-α is phosphorylated and subsequently degraded, and subunits of NF-κB are released to translocate to the nucleus [18]. To identify the mechanism by which the PDR extract reduces LPS-induced inflammatory responses through the NF-κB signaling pathway, we investigated whether the extract suppresses NF-κB activation. Data were expressed as a percentage of activation or inhibition, with non-LPS-treated cells (Normal group) considered as 100%. GAPDH protein expression was used as a loading control [19]. As shown in Fig. 2, activation of NF-κB increased in a dose-dependent

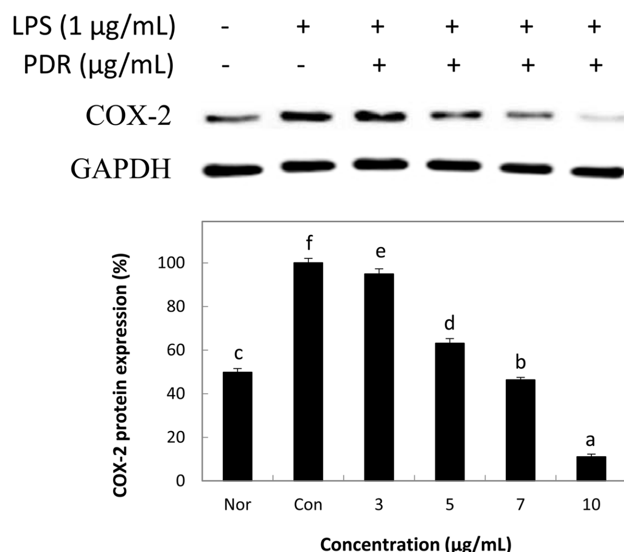

Fig. 2 Effect of the *Pinus densiflora* root (PDR) extract on NF-κB activation in RAW 264.7 cells. The activation of NF-κB was analyzed by western blot assay. The cells were treated with or without LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract for 4 h. Data are expressed as a percentage of activation or inhibition, with non-LPS-treated cells (Normal) considered as 100%. Data are the mean ± SD (n=3). Means with different letters (a-f) above the bars are significantly different at p<0.05 by Duncan's multiple-range test

manner with increasing concentrations of the PDR extract (3, 5, 7, and 10 μg/mL). Similar to the Normal group, 10 μg/mL of the PDR extract retained almost all of the protein expression of NF-κB. Yun et al. [20] reported that asiatic acid suppressed the protein expression of iNOS and COX-2 via an NF-κB-dependent mechanism. Hence, these results suggest that the PDR extract's anti-inflammatory effect is potentially through the NF-κB signaling pathway and it thus might have a beneficial effect on the treatment of inflammatory diseases.

Effect of the PDR extract on NO and PGE_2 levels

NO is synthesized from L-arginine by NOS. Large amounts of NO are associated with a wide range of diseases and inflammation [21]. PGE_2, which is synthesized from arachidonic acid by COX, is a proinflammatory factor that can cause erythema, edema, and pain [22]. The levels of NO and PGE_2 were expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. As shown in Fig. 3, the NO and PGE_2 levels decreased in a dose-dependent manner as the PDR extract concentration (3, 5, 7, and 10 μg/mL) was increased. At 10 μg/mL of the extract, the NO and PGE_2 levels were inhibited by over 60 and 45%, respectively. Yoon et al. [23] reported that NO levels were 60% of the Control level after treatment with 25 μg/mL *Scutellariae radix* extract. Appel et al. [24] reported significant inhibition of PGE_2 to 40% of the Control level after treatment with a combination of 0.1% chokeberry and 50 μg/mL Na_2SeO_3. In comparison with these studies, the PDR extract at low concentrations was more effective at inhibiting NO and PGE_2 levels than the extracts of *S. radix* and chokeberry were.

Effect of the PDR extract on iNOS and COX-2 protein expression levels

iNOS is not constitutively expressed. However, it produces NO, which upon induction can cause inflammation through vasorelaxation, cytotoxicity, and organ damage [25]. COX-2 is induced during inflammation and produces inflammatory mediators, such as PGE_2 [8]. To identify the mechanism by which the PDR extract reduces LPS-induced NO and PGE_2 levels, we were interested in whether the extract influences the LPS-induced protein expression of iNOS and COX-2. Data from the western blot assay were expressed as a percentage of activation or inhibition, with LPS

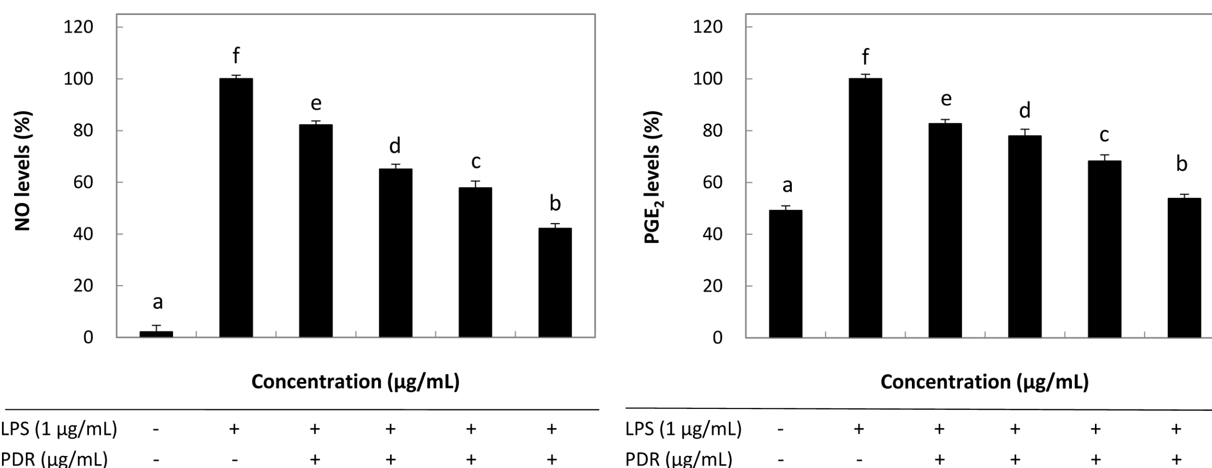

Fig. 3 Effect of the *Pinus densiflora* root (PDR) extract on NO and PGE_2 levels in RAW 264.7 cells. The amounts of NO and PGE_2 in the culture supernatant were measured using ELISA kits. The cells were treated with or without LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract for 16 h. Data are expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. Data are the mean ± SD (n=3). Means with different letters (a-f) above the bars are significantly different at p<0.05 by Duncan's multiple-range test

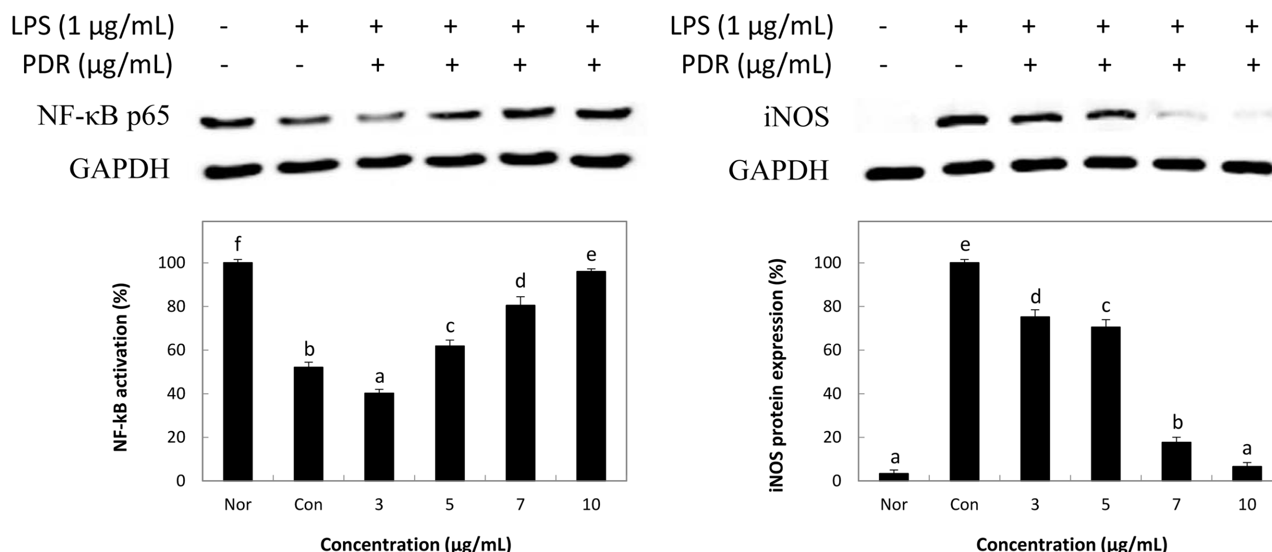

Fig. 4 Effect of the *Pinus densiflora* root (PDR) extract on iNOS and COX-2 protein expression in RAW 264.7 cells. The protein expression levels of iNOS and COX-2 were analyzed by western blot assay. The cells were treated with or without LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract for 16 h. Data are expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. Data are the mean ± SD (n =3). Means with different letters (a-f) above the bars are significantly different at p <0.05 by Duncan's multiple-range test

only-treated cells (Control) considered as 100%. GAPDH protein expression was used as a loading control [22]. As shown in Fig. 4, the PDR extract (3, 5, 7, and 10 μg/mL) inhibited the protein expression of iNOS and COX-2 in a dose-dependent manner. Compared with the Control, 10 μg/mL of the PDR extract completely repressed the protein expression of iNOS and COX-2 relative to GAPDH protein expression. Thus, we found that the PDR extract could reduce NO and PGE_2 levels by inhibiting iNOS and COX-2 protein expression. According to Hong [26], the protein expression of iNOS was inhibited to 32% of the Control level after treatment with 20 μg/mL *Hypericum ascyron* extract. Moreover, Kim [27] reported that the protein expression of COX-2 was inhibited to 60% of the Control level after addition of 10 μg/mL ferulic acid isolated from a *Tetragonia tetragonoides* extract. In comparison with these studies, our PDR extract at low concentration had an excellent effect on inhibiting the protein expression of iNOS and COX-2. These results suggest that the root extract has anti-inflammatory properties in LPS-induced RAW 264.7 cells.

Effect of the PDR extract on proinflammatory cytokine levels

TNF-α, IL-6, and IL-1β are well known as proinflammatory cytokines that regulate immune responses. An increase in the secretion of these cytokines will easily lead to inflammatory conditions, such as edema, erythema, osteoporosis, and rheumatoid arthritis [28,29]. IL-1β, which plays an important role in infection and inflammation [30], is generated in 2 stages. In the first stage, inflammatory stimuli activate NF-κB to promote the synthesis of pro-IL-1β. In the second stage, the pro-IL-1β protein is cleaved by caspase-1 to generate mature IL-1β [31]. We investigated whether the PDR extract could inhibit the levels of TNF-α, IL-6, and IL-1β in RAW 264.7 cells. Data were expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. As shown in Fig. 5, changes in the TNF-α and IL-6 levels were not detected as the PDR extract concentration (3, 5, 7, and 10 μg/mL) was increased. Kang et al. [32] reported that 100 μM of diphlorethohydroxycarmalol had no effect on TNF-α production, which was similar to the PDR extract. Likewise, Wong et al. [33] reported no significant difference in the levels of IL-6 by vomitoxin treatment in RAW 264.7 cells, similar to the PDR extract effect. From these results, we assume that the PDR extract does not affect the secretion of TNF-α and IL-6.

However, the IL-1β levels decreased in a dose-dependent manner with increasing concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract. At 10 μg/mL, the extract decreased the IL-1β levels to 45% of the Control level. Chuang et al. [34] reported that knockdown of death-associated protein kinase (DAPK), which generates caspase-1, significantly inhibited IL-1β levels, whereas it did not affect TNF-α levels. We assume that the PDR extract may also interfere with the activities of DAPK and caspase-1; however, further experiments should be conducted to verify if this is indeed the case.

In conclusion, we have provided a mechanism to explain the anti-inflammatory effects of the PDR extract; that is, it exerts such an effect by regulating NF-κB. The PDR extract can therefore be considered as an effective anti-inflammatory agent.

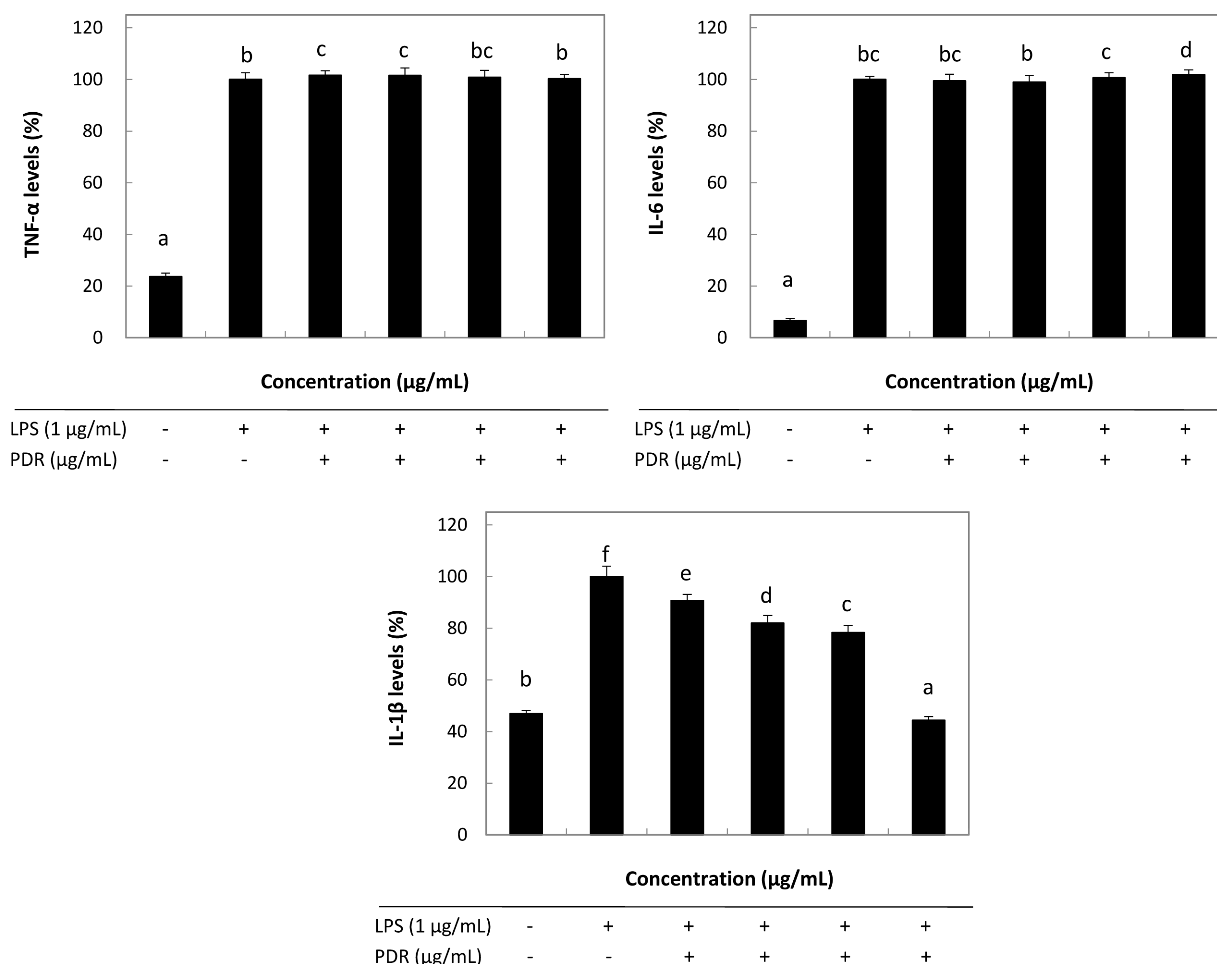

Fig. 5 Effect of the *Pinus densiflora* root (PDR) extract on TNF-α, IL-6, and IL-1β levels in RAW 264.7 cells. The amounts of TNF-α, IL-6, and IL-1β in the culture supernatant were measured using ELISA kits. The cells were treated with or without LPS, followed by various concentrations (3, 5, 7, and 10 μg/mL) of the PDR extract for 16 h. Data are expressed as a percentage of activation or inhibition, with LPS only-treated cells (Control) considered as 100%. Data are the mean ± SD (n =3). Means with different letters (a-f) above the bars are significantly different at p <0.05 by Duncan's multiple-range test

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