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Original Article

Korean Red Ginseng and *Portulaca oleracea* Extracts Attenuate Lipopolysaccharide-induced Inflammation via Downregulation of Nuclear Factor Kappa-B and the Mitogen-activated Protein Kinase Signaling Pathway in Macrophage Cell Line RAW 264.7

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Korean red ginseng (*Panax ginseng* Meyer) is a well-known traditional medicine, with numerous biological functions in the body. *Portulaca oleracea* (*P. ole*) belongs to the Portulacaceae family and has bioactive potential as a traditional medicine. This study aimed to determine the anti-inflammatory effects of Korean red ginseng extract (RGE) and *P. ole* extract on lipopolysaccharide (LPS)-treated RAW 264.7 cells. The combination of RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extracts significantly suppressed LPS-induced nitric oxide synthesis. The expression of proinflammatory mediators, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and proinflammatory cytokines, including interleukin-1β, interleukin-6, and tumor necrosis factor-α, were markedly decreased by the combined treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL). Moreover, iNOS and COX-2 protein expression levels were also significantly reduced in the combined treatment compared to the LPS-stimulated group. In addition, the nuclear translocation of phosphorylated nuclear factor kappa-B was suppressed by the treatment with RGE and *P. ole*. Moreover, the mitogen-activated protein kinase pathway was also partially inhibited by the combination treatment with RGE and *P. ole*. Our results demonstrate that the treatment mixture with RGE and *P. ole* could be used as functional food and therapeutic herbal medicine in various inflammatory diseases.

Key Words: Korean red ginseng, *Portulaca oleracea*, Functional food, Anti-inflammatory effects, RAW 264.7 cells

INTRODUCTION

Ginseng (*Panax ginseng* Meyer) is an ancient herbal remedy that is used widely for its promising pharmacological properties on overall health (Saba et al., 2018; Saba et al., 2020b). As a traditional and alternative therapy, red ginseng extract (RGE) has been used to treat many diseases for over two thousand years (Wee et al., 2011). Ginsenosides are the major bioactive ginseng constituents which are essen-

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tial for its therapeutic effects (Farzaei et al., 2015; Saba et al., 2020a).

Portulaca oleracea (*P. ole*), belonging to the Portulacaceae family, is distributed worldwide. *P. ole* is considered as a medicinal food, especially in China (Yen et al., 2001; Li et al., 2016). Therefore, *P. ole* is widely used as a vegetable crop due to its many medicinal properties, including antibacterial, antioxidant, anti-inflammatory, and antiaging effects (Chan et al., 2000; Chen et al., 2012; E Abdel Moneim, 2013; Zhou et al., 2015; Baradaran Rahimi et al., 2019).

Inflammation is a critical part of the immune system that protects the host from harmful stimuli (Pyee et al., 2014; Ullah et al., 2014). However, dysregulated inflammation is closely related with many chronic diseases. Hence, inflammatory response regulation remains a challenge. Macrophages play key role in the operation of inflammatory processes, mainly through the production of various inflammatory factors, such as nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF)-α (Choi et al., 2014). Thus, the inhibition of macrophage activation is considered a vital approach to treat inflammatory diseases.

In this study, we selected the two extracts because combination of RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) showed higher effect than separate effect. This combined treatment exhibited synergistic effect in RAW cells without cytotoxicity. In order to show synergism, weak inhibitory of both extract (Korean red ginseng and *Portulaca oleracea*) was used. We investigated the anti-inflammatory effects of Korean RGE and *P. ole* extract on lipopolysaccharide (LPS)-induced RAW 264.7 cells, using NO and cell viability assays and mRNA and protein expression of proinflammatory mediators and cytokines. Thus, our study demonstrated that RGE and *P. ole* extracts have potential anti-inflammatory properties in the regulation of inflammation and may be used as adjuvant therapy in the treatment of various inflammatory diseases.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), streptomycin, and penicillin were purchased from WelGene, Daegu, Republic of Korea. LPS and MTT were obtained (Sigma, St. Louis MO, USA). TRIzol was obtained from Invitrogen (Carsbad, CA, USA). Polymerase chain reaction (PCR) primers were obtained from Bioneer (Daejeon, Republic of Korea). Antibodies for iNOS, COX2, IRAK1, phospho (p)-TAK1, p-IKKα/β, p-IκB/α, p-NF-κB, p-JNK, total (T)-JNK, p-P38, T-P38, p-ERK, T-ERK, and β-actin were purchased (Cell Signaling Technology, USA).

Preparation of the Korean red ginseng and *Portulaca oleracea* extracts

We purchased Korean red ginseng and *Portulaca oleracea* from the company and ground them separately into a fine coarse powder. We then extracted separately in 70% ethanol using a heating mantle at 80℃ for 2 hours, followed by extract condensation with the rotary evaporator system. The crude extract was frozen at -70℃ overnight and lyophilized using a freeze dryer to make a dry fine powder. Finally, the powder was dissolved in DMSO at the time of the experiment.

Cell culture and treatment

The macrophage cell line RAW 264.7 was cultured in DMEM supplemented with heat-inactivated 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL, 1% antibiotics). Then, cells were incubated in a humidified incubator at 37℃ with 5% CO_2.

Nitric oxide and cell viability assay

RAW 264.7 cells were seeded (2×10^5) per mL in 24-well plates. Cells were treated after 24 h with different concentrations of RGE (50 μg/mL) and/or *P. ole* (6.25 μg /mL), followed by 0.1 μg/mL of LPS after 30 min. Cells were incubated for 18 h for NO induction. Supernatant was collected for NO analysis using the Griess reagent and read at 540 nm using a microplate reader. For the measurement of cell viability, the MTT reagent was added to the cells and incubated for 2~3 h, and dimethyl sulfoxide was used to dissolve the crystal. The dissolved solution was analyzed at 560 nm using a microplate reader.

Polymerase chain reaction

RAW 264.7 cells were seeded (5×10^5) per mL in 6-well plates. After 24 h, the cells were treated with different concentrations of RGE (50 μg/mL) and/or *P. ole* (6.25 μg /mL), followed by LPS (0.1 μg/mL) after 30 min. Then, cells were incubated for another 18 h. Using the TRIzol reagent, RNA was extracted and measured using a nano-photometer. Kits were used according to the manufacturer's protocol (Bioneer, Daejeon, Republic of Korea) for reverse transcription. The cDNA product was subjected to PCR using gel electrophoresis, and gene expression was normalized using GAPDH.

Western blot analysis

The western blot analysis was done as described previously (Ullah et al., 2021). Cells were seeded (5×10^5 per mL) in 6-well plates. After 24 h, the cells were treated with RGE (50 μg/mL) and/or *P. ole* (6.25 μg/mL), followed by LPS (0.1 μg/mL) after 30 min. The cells were then incubated for another 18 h. Using Pro-Prep solution, protein was extracted, and the protein concentration was measured using the Bradford method. Proteins were separated in 10% SDS-PAGE and transferred into PVDF membrane. A 5% skim milk solution was used as a blocking buffer for 1 h. Primary antibodies (1:1,000) were incubated overnight, and a secondary antibody (1:3,000) was used for 1 h. The membrane was developed in a developer, and protein bands were analyzed using ImageJ.

Statistical analysis

Data were represented as the mean ± standard error of the mean. Significant differences were analyzed by one-way analysis of variance. Statistical significance was set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared to the LPS-treated group.

RESULTS

Effects of RGE and *P. ole* extracts on LPS-induced NO production and cell viability

First, to confirm LPS-induced NO production in the raw macrophage cell line, we measured the NO assay. Our results showed that the LPS-stimulated group significantly produced more NO compared to the basal group, but RGE and *P. ole* extract individually inhibited NO production. Of note, the combined use of RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) significantly suppressed NO production (Fig. 1A). Altogether, this result suggests that combination treatment with RGE and *P. ole* has anti-inflammatory properties.

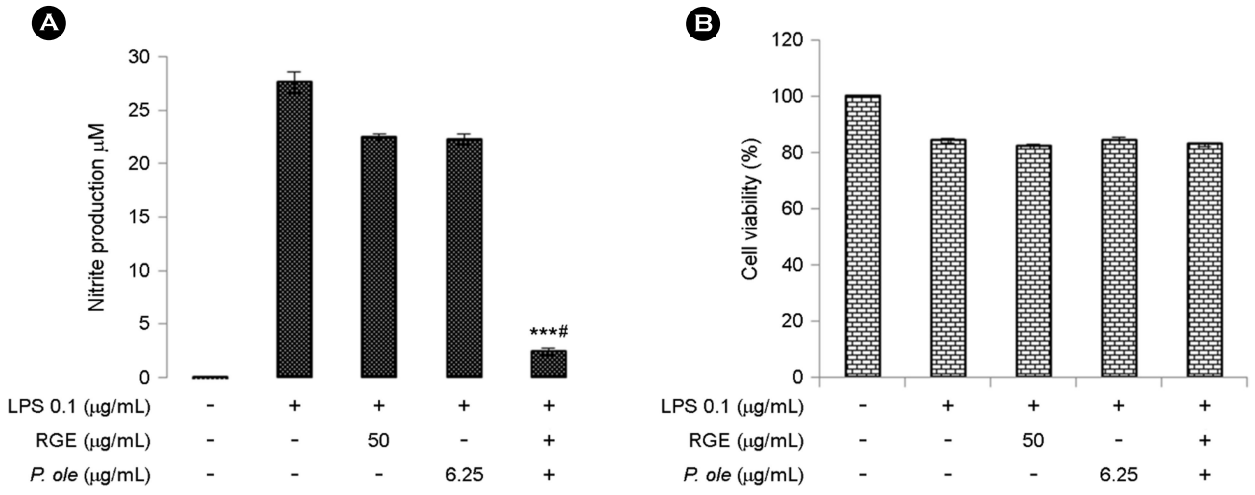

Fig. 1. Effects of Korean red ginseng extract (RGE) and *Portulaca oleracea* (*P. ole*) extract on nitric oxide (NO) production and cell viability in macrophage RAW 264.7 cells. (A) Suppression of NO production in the RAW 264.7 cells pretreated with the RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extract for 30 min, followed by cotreatment with LPS (0.1 μg/mL) for 18 h. (B) Cell viability of RAW 264.7 cells treated with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL). $***^{\#}P < 0.001$ combination of RGE and *P. ole* against individual treatment.

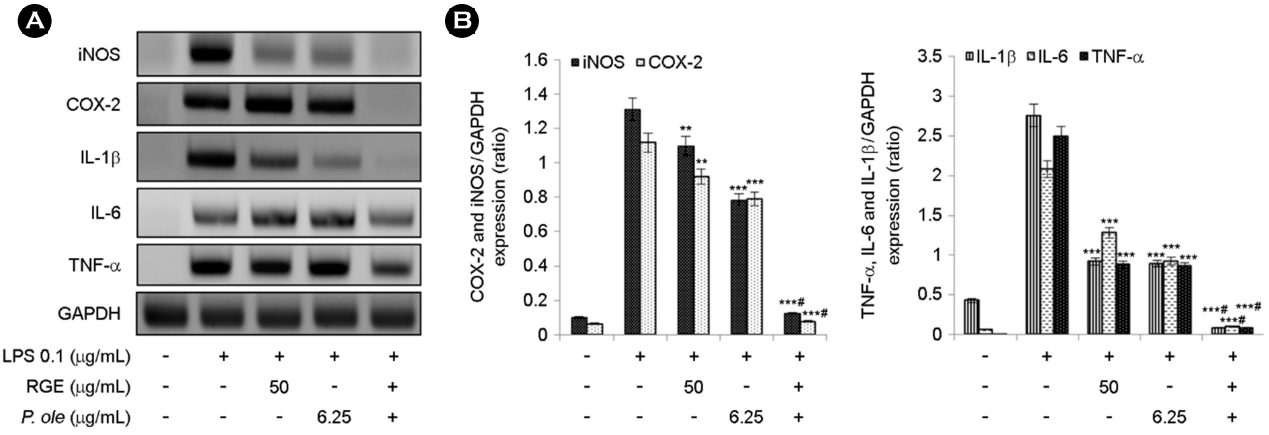

Fig. 2. Effects of Korean red ginseng extract (RGE) and *Portulaca oleracea* (*P. ole*) extracts on lipopolysaccharide (LPS)-induced proinflammatory mediators and cytokines in macrophage RAW 264.7 cells. (A) mRNA expression of iNOS, COX2, IL-1β, IL-6, and TNF-α. GAPDH was used as a housekeeping gene. (B) Quantitation of polymerase chain reaction (PCR) analysis of iNOS, COX2, IL-1β, IL-6, and TNF-α. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{***\#}P < 0.001$ combination of RGE and *P. ole* against individual treatment.

The cell viability assay determined the absence of cytotoxic effects of the RGE and *P. ole* extracts on the raw macrophage cells. We chose the concentration of RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) based on the cytotoxic effects (Fig. 1B). Thus, the cell viability assay suggests that RGE and *P. ole* have no cytotoxic effects on the concentrations used (50 and 6.25 μg/mL, respectively).

Inhibitory effects of RGE and *P. ole* extracts on LPS-induced proinflammatory mediators and cytokines in macrophages

PCR analysis was used to determine the effects of RGE and *P. ole* extracts on mRNA expression of proinflammatory mediators and cytokines in the LPS-treated macrophage cell line RAW 264.7. PCR results indicated that iNOS, COX2, IL-1β, IL-6, and TNF-α, were significantly upregulated in the LPS-stimulated treated group compared with the basal group. However, treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extracts markedly suppressed the release of inflammatory mediators and cytokines (Fig. 2A and B). Overall, these results indicate that LPS-induced inflammatory mediator secretion was inhibited by RGE and *P. ole*.

Effects of RGE and *P. ole* extracts on the protein expression of iNOS and COX2

Western blot analysis was used to confirm the secretion of proinflammatory mediators suppressed by RGE and *P. ole*. The Western blot results revealed that inflammatory mediators, including iNOS and COX2, were significantly upregulated in the LPS-stimulated group compared to control group, suggesting that LPS successfully induced inflammation. However, treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extracts significantly decreased the expression of iNOS and COX2 levels compared to the LPS-treated group (Fig. 3A and B). Thus, the immunoblot results suggest that RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extracts have significant anti-inflammatory activities in protein expression levels.

RGE and *P. ole* prevent the activation of NF-κB signaling in LPS-induced cells

The NF-κB-signaling pathway was investigated to clarify the anti-inflammatory mechanism of RGE and *P. ole*. The protein expression levels of IRAK-1, p-TAK-1, p-IKKα/β, p-IkB/α, and p-NF-κB were analyzed by Western blotting, whereas β-actin was used as an internal standard. Phosphorylation of NF-κB was increased in the LPS-stimulated group, whereas p-NF-κB was significantly downregulated by the combination of RGE (50 μg/mL) and *P. ole* (6.25 μg /mL) extracts (Fig. 4A and B). Overall, these results indicate that RGE and *P. ole* exhibited anti-inflammatory effects by blocking the activation of the NF-κB pathway.

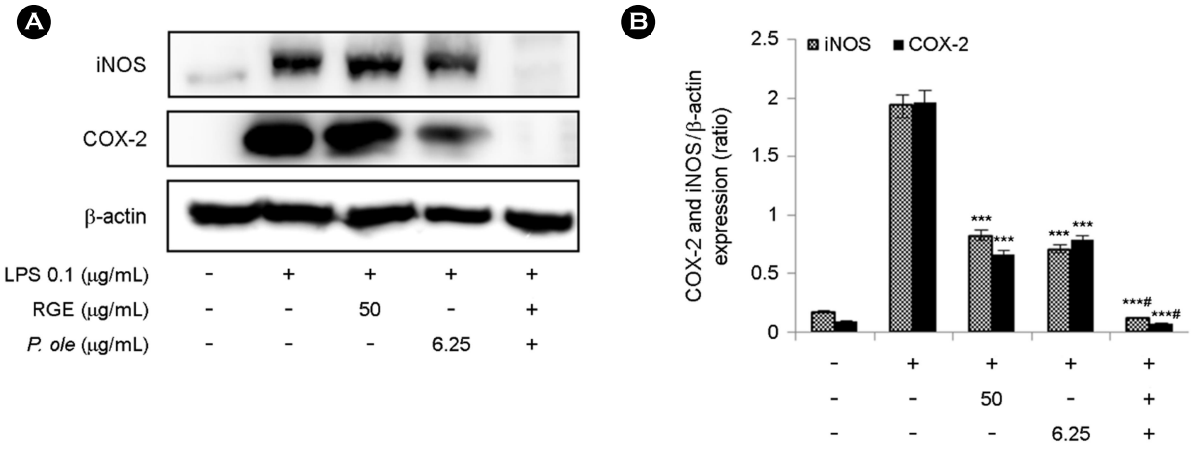

Fig. 3. Effects of Korean red ginseng extract (RGE) and *Portulaca oleracea* (*P. ole*) extracts on inflammatory mediators related protein such as iNOS and COX2 in macrophage RAW 264.7 cells. (A) Western blot analysis of iNOS and COX2. RGE, *P. ole*, and lipopolysaccharide (LPS) were used (50 μg/mL, 6.25 μg/mL, and 0.1 μg/mL, respectively). β-actin was used as a normalizing protein. (B) Quantitation of western blot protein band of iNOS and COX2. ***$P < 0.001$, and ***$^{\#}P < 0.001$ combination of RGE and *P. ole* against individual treatment.

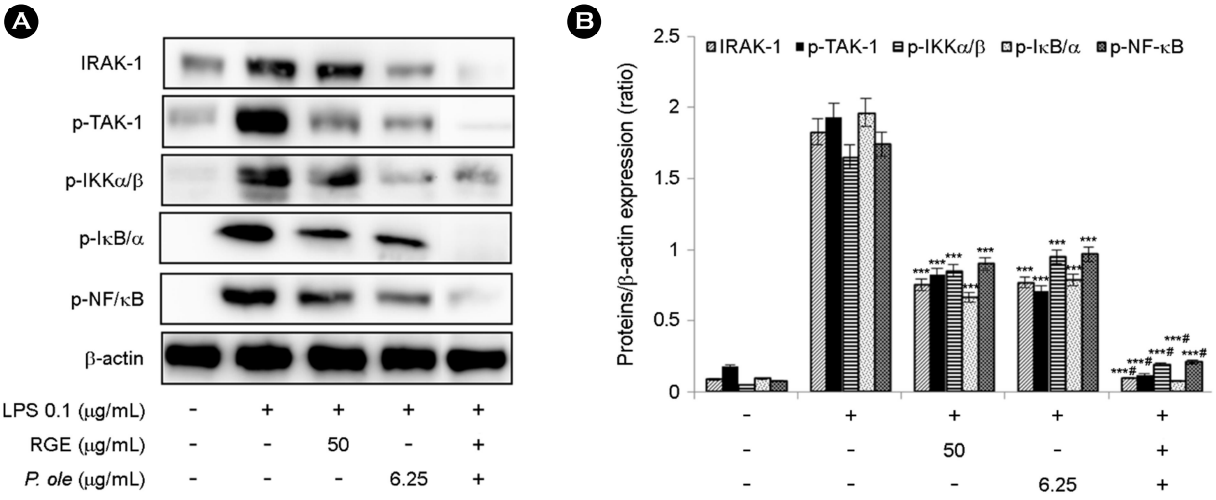

Fig. 4. Effects of Korean red ginseng extract (RGE) and *Portulaca oleracea* (*P. ole*) on the activation of the NF-κB signaling pathway in macrophage RAW 264.7 cells. (A) Western blot analysis of IRAK-1, p-TAK-1, p-IKKα/β, p-IκB/α, and p-NF-κB. β-actin was used as an internal standard. (B) Quantitation of western blot protein band. ***$P < 0.001$, and ***$^{\#}P < 0.001$ combination of RGE and *P. ole* against individual treatment.

RGE and *P. ole* prevent the activation of MAPK signaling in LPS-induced cells

The mitogen-activated protein kinase (MAPK) pathway was investigated to understand the other anti-inflammatory mechanisms of RGE and *P. ole*. The expression levels of p-P38, T-P38, p-JNK, T-JNK, p-ERK, and T-ERK proteins were analyzed by Western blotting, with the β-actin antibody used as an internal standard. The production of phosphorylated (p)-JNK, p-P38, and p-ERK were markedly increased in the LPS-stimulated group, whereas treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) extracts significantly inhibited the MAPK pathway-related protein expressions (Fig. 5A and B). Collectively, Western blot results suggest that RGE and *P. ole* showed anti-inflammatory effects by inhibiting the MAPK pathway.

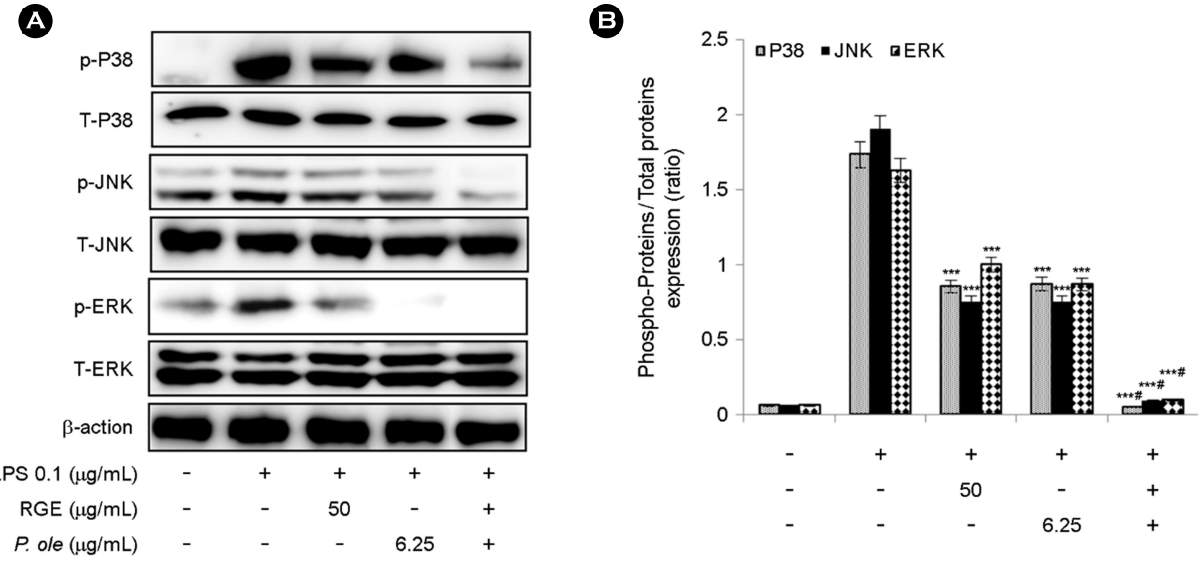

Fig. 5. Korean red ginseng extract (RGE) and *Portulaca oleracea* (*P. ole*) prevent the activation of MAPK signaling pathway in macrophage RAW 264.7 cells. (A) Western blot analysis of MAPK pathways including p-P38, T-P38, p-JNK. T-JNK, p-ERK, and T-ERK. β-actin was used as an internal standard. (B) Quantitation of western blot protein band. ***$P < 0.001$, and ***$^{\#}P < 0.001$ combination of RGE and *P. ole* against individual treatment.

DISCUSSION

Inflammation is an important biological immune response initiated by harmful stimuli and is closely associated with a number of inflammatory diseases, such as atherosclerosis, Alzheimer's disease, cancers, diabetes mellitus, and rheumatoid arthritis (Bag-Ozbek and Giles, 2015; Bessueille and Magne, 2015; Ullah et al., 2021). In this study, lipopolysaccharide (LPS)-treated RAW 264.7 macrophage cells were used as the experimental model. Macrophages easily respond to LPS, the bacterial endotoxin, which is the key stimulant of inflammation (Miao et al., 2019). Various proinflammatory mediators and cytokines, including NO, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF)-α, are released by activated macrophages to regulate the immune system against patho- gens.

The extreme production of NO related to iNOS synthesis is involved in the inflammatory process. iNOS plays a vital role in releasing NO during the pathophysiology of inflammatory diseases (Vodovotz et al., 1993; Zamora et al., 2000; Li et al., 2016). Additionally, COX2 is also stimulated by inflammatory stimuli during the inflammatory response. In our study, only the LPS-stimulated group showed overexpression of NO production compared to the basal group, whereas pretreatment with RGE and *P. ole* extracts reduced NO production. In particular, the combined treatment of RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) significantly decreased NO induction (Fig. 1A).

The mRNA expression levels of proinflammatory mediators, such as iNOS and COX2, and inflammatory cytokines, including IL-1β, IL-6, and TNF-α, were upregulated in the LPS-treated group, whereas the combination treatment of RGE and *P. ole* significantly downregulated mRNA levels of proinflammatory factors (Fig. 2). These results suggested that the mixture of RGE and *P. ole* has the potential to inhibit proinflammatory factors. Previously, it was found that protein expression levels of iNOS and COX2 were increased in LPS-induced RAW 264.7 cells (Li et al., 2016; Miao et al., 2019). Our results also support the previous findings that iNOS and COX2 were upregulated in the LPS-stimulated group and that treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) significantly reduced the protein expression of iNOS and COX2 (Fig. 3).

It was reported that NF-κB and MAPK signaling pathways are the key pathways for the inflammatory process

(Chen et al., 2003; Miao et al., 2019). Therefore, we explored the antiinflammatory mechanism of the RGE and *P. ole* extracts in the macrophage cell line. Protein expression levels of IRAK1, p-TAK1, p-IKKα/β, p-IκB/α, p-NF-κB, p-JNK, T-JNK, p-P38, T-P38, p-ERK, and T-ERK were investigated using Western blot. For the macrophage RAW 264.7 cell line treated with LPS, the NF-κB and MAPK signaling pathways were increased by LPS treatment, whereas treatment with RGE (50 μg/mL) and *P. ole* (6.25 μg/mL) significantly suppressed the NF-κB and MAPK signaling proteins (Fig. 4 and 5). These findings suggest that RGE and *P. ole* inhibited the LPS-stimulated inflammation by blocking the NF-κB and MAPK signaling pathways.

In conclusion, this study demonstrated that RGE and *P. ole* extract have potential anti-inflammatory effects. The combination of RGE and *P. ole* could be used as a functional food along with modern treatments in inflammatory diseases.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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