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Immunomodulatory effects of *Abelmoschus esculentus* water extract through MAPK and NF-κB signaling in RAW 264.7 cells

Min Nyeong Ko [a], Su Bin Hyun [a], Keun Jae Ahn [b], Chang-Gu Hyun [a,*]

[a] Jeju Inside Agency and Cosmetic Science Center, Department of Chemistry and Cosmetics, Jeju National University, Jeju, 63243, Republic of Korea
[b] Biology Education, College of Education, Jeju National University, Jeju, 63243, Republic of Korea

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ABSTRACT

During the current COVID-19 pandemic, the world is facing a new, highly contagious virus that suppresses innate immunity as one of its early virulence mechanisms. Therefore, finding new methods to enhance innate immunity is a promising strategy to attenuate the effects of this major global health problem. With the aim of characterizing bioactive ingredients as immune-enhancing agents, this study focuses on *Abelmoschus esculentus* (okra), which has several previously demonstrated bioactivities. Firstly, we investigated the immune-stimulatory effects of okra leaf ethanol extract (OLE) and okra leaf water extract (OLW) on nitric oxide (NO) production in macrophages. OLE significantly decreased nitrite accumulation in LPS-stimulated RAW 264.7 cells, indicating that it potentially inhibited NO production in a concentration-dependent manner. In contrast, OLW significantly enhanced the production of prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and NO in a dose-dependent manner. OLW also increased the expression levels of NO synthase (iNOS) and cyclooxygenase (COX)-2, potentially explaining the OLW-induced increase in NO and PGE_2 production. In addition, OLW stimulated the phosphorylation of mitogen-activated protein kinases (MAPKs; ERK, p38, and JNK) as well as the activation and subsequent nuclear translocation of nuclear factor κB (NF-κB). This indicated that OLW activates macrophages to secrete PGE_2, TNF-α, IL-1β, and NO, inducing iNOS and COX-2 expression via activation of the NF-κB and MAPK signaling pathways. In conclusion, our results demonstrate that OLW can effectively promote the activation of macrophages, suggesting that OLW may possess potent immunomodulatory effects and should be explored as a potential health-promoting materials to boost the immune system.

1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a major public health concern. Due to the significant number of deaths resulting from this epidemic, there is huge research effort attempting to further understand this new and dangerous virus. More than two years after the first identification of SARS-CoV-2, scientists around the world are trying to identify potential prophylaxes and effective therapies against the virus. As well as the development of inhibitors to slow viral replication, such as 3C-like proteases, papain-like protease, and angiotensin-converting enzyme II, immunosuppressive treatments may be useful in preventing the cytokine storm in the later stages of COVID-19, a major cause of patient death.[1–4] In this study, we focused on identifying potential prophylaxes for SARS-CoV-2 infection, specifically an immune-enhancing compound derived from natural products. Immune-enhancing compounds are generally classified as those capable of enhancing the non-specific immune system response by promoting the activity of phagocytes such as neutrophils and macrophages. Immune-enhancing compounds predominantly activate macrophage function after being specifically combined with cell surface acceptors.[5] Toll-like receptor 4 (TLR4) is a type 1 transmembrane glycoprotein with a molecular weight of 100 kDa which is known to be expressed in macrophages and other cells.[6] A number of downstream signaling pathways, including the nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, are triggered by various adapter complexes that interact with TLR4, resulting in the production of nitric oxide (NO) and inflammatory cytokines/chemokines.[7] Foreign antigens are phagocytosed by macrophages, while inflammatory factors, such as NO and the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are secreted, increasing phagocytosis and inducing innate immune activity via

* Corresponding author.
E-mail address: cghyun@jejunu.ac.kr (C.-G. Hyun).

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natural killer cells and dendritic cells.[8,9] Therefore, macrophages are known to contribute to the activation of adaptive immunity by recognizing foreign antigens and presenting them to T cells to regulate cell activation and differentiation. Conversely, a decline in the immune function of the human body can lead to a higher occurrence of infections, diseases, and tumor growth, as well as hindering patients' recovery. With this in mind, immune stimulation has emerged as an important strategy for improving the immune defense system. In particular, attention has recently been focused on the development of natural products and dietary components that can improve immune potential in response to these demands.

Abelmoschus esculentus (L.) Moench (okra), a subtropical vegetable belonging to the Amalvaceae family, is also known as "ladyfinger" due to its similarity in shape to a female finger. It is native to northeastern Africa and is widely cultivated in tropical and subtropical regions around the world, including India, Southeast Asia, Africa, and Latin America.[10,11] The seeds, pods, leaves, fruit, stem, and even buds of okra have been shown to convey numerous bioactivities, for example modulating immunity, promoting gastrointestinal motility, regulating blood sugar level, inhibiting cancer and bacterial growth, preventing cardiovascular disease, and eliminating reactive oxygen species. Owing to these effects, okra has also been referred to as "green ginseng".[12–15] According to reports on its nutritional composition and phytochemical structure, *A. esculentus* contains a number of antioxidant vitamins (e.g., folate, niacin, choline, A, K, E, and β-carotene) as well as lipids, minerals, carbohydrates, dietary fibers, and amino acids. Moreover, it also contains health-promoting phenolics and flavonoids such as epigallocatechin, myricetin, gossypetin-8-O-β-D-glucuronide, quercetin glycosides and other quercetin derivatives and its glycosides, and gossypetin-8-O-β-D-glucuronide.[16,17] Although there have been several studies on the alternative medicinal uses of okra leaves, few studies have investigated its potential mechanisms of action or immune-enhancing properties. Therefore, the focus of this investigation was to explore whether okra leaf extract could induce an immune response. First, we evaluated the immune-stimulatory capacity of okra leaf water extract and ethanol extract by quantifying NO release. In addition, we investigated the potential for the development of okra leaf extract as an immune-enhancing compound and its molecular mechanism of action by observing the effects of okra extract on the activation of MAPKs and NF-κB signaling pathways.

2. Materials and methods

2.1. Preparation of okra leaf extracts

The *A. esculentus* leaves used in this study were obtained from the National Institute of Horticultural and Herbal Science (Jeju, Korea), and leaf powder was produced by crushing the dried leaves. Water or ethanol *A. esculentus* leaf extracts were produced by mixing leaf powder with distilled water and extracting three times for 4 h at 65 °C, or mixing with 70% ethanol (10 L–1 kg leaf powder) and extracting three times over 4 h. The okra leaf water extract (OLW) and okra leaf ethanol extract (OLE) were filtered through filter paper (No. 4, Whatman, Kent UK), concentrated using a rotary vacuum evaporator (Heidolph Laborota 4000 Efficient, Heidolph Instruments GmbH & Co., KG, Schwabach, Germany), and freeze-dried. The obtained yields of OLW and OLE were 10.25% and 6.71%, respectively.

2.2. Cell culture

Mouse macrophage RAW 264.7 cells (KCLB, Seoul, Korea) were maintained in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Merck Millipore, Darmstadt, Germany) with 100 units/mL penicillin and streptomycin (P/S; Thermo Fisher Scientific). RAW 264.7 cells were cultured at 37 °C in a 5% CO_2 incubator (NB-203XL; N-BIOTEK, Inc., Bucheon, South Korea) and sub-cultured every 2 days.

2.3. Measurement of cell viability

To evaluate cell viability, RAW 264.7 cells (1.5×10^5/well) were dispensed into 24 well plates, and left to completely attach at 37 °C in a 5% CO_2 incubator for 24 h. Subsequently, OLE and OLW were dissolved at a concentration of 100 mg/mL in 70% ethanol or distilled water, respectively, and then used to treat macrophages at concentrations of 6.25, 12.5, 25, 50, 100, and 200 μg/mL for 24 h. Thiazolyl blue tetrazolium bromide (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Sigma Aldrich, St Louis, MO, USA) solution (20 μL/well at 0.4 mg/mL) was added for 4 h. To dissolve the formazan produced by the addition of MTT reagent, 500 μL of dimethyl sulfoxide (DMSO; Biosesang, Sungnam, Gyeonggi-do, Korea) was added to each well, and after 30 min absorbance was measured at 570 nm using a microplate reader (Epoch, BioTek, Winooski, VT, USA). Viability was assessed by comparing to the absorbance values of the control (medium only).

2.4. Measurement of NO production

RAW 264.7 cells were aliquoted into 24-well plates at 1.5×10^5 cells/well, incubated at 37 °C and 5% CO_2 for 24 h to allow complete attachment, and then treated with various concentrations (6.25, 12.5, 25, 50, 100, and 200 μg/mL) of OLE and OLW. The cells were also treated with lipopolysaccharide (LPS; Sigma Aldrich) at a concentration of 1 μg/mL as a positive control. Following 24 h incubation, the culture supernatant was separated, and 100 μL supernatant was treated with Griess reagent (Sigma Aldrich). After 10 min incubation in the dark, absorbance was measured at 540 nm using a microplate reader.

2.5. Measurement of PGE_2 and pro-inflammatory cytokine expression

RAW 264.7 cells were aliquoted into 24 well plates at 1.5×10^5 cells/well, incubated for 24 h at 37 °C and 5% CO_2, and then treated with various concentrations of OLE and OLW (6.25, 12.5, 25, 50, 100, and 200 μg/mL), or 1 μg/mL LPS as a positive control. Following 24 h incubation, the culture supernatant was separated, and the cytokine content in the supernatant was determined using enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-1β (R&D Systems, Minneapolis, MN, USA), mouse TNF-α (BD Biosciences, Franklin Lakes, NJ, USA), or mouse PGE_2 (Abcam, Cambridge, Cambridgeshire, UK). Cytokine concentrations were assessed according to the standard curves produced using the standard solutions provided in each kit.

2.6. Western blotting

Cells (6×10^5/well) were aliquoted into 60 mm culture dishes and incubated for 24 h at 37 °C with 5% CO_2. After washing with cold 1 × PBS (Biosesang), cell lysis was performed by addition of RIPA buffer (Biosesang) containing 1% protease/phosphatase inhibitor cocktail (Sigma Aldrich) at 4 °C for 20 min. The cell lysates were collected into 1.5 mL e-tubes, and then centrifuged at 15,000 rpm and −8 °C for 20 min to obtain the supernatants. Protein concentrations were quantified using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein concentrations were adjusted to 30 μg/mL, mixed at a 1:1 ratio with sample loading buffer, and heated at 100 °C for 5 min. Samples were stored at −20 °C until loading. The prepared protein samples were separated by electrophoresis on SDS-polyacrylamide gels, then transferred to a polyvinylidene (PVDF) membrane. After blocking in 5% skim milk (in 1X TBST) for 1.5 h, membranes were washed six times for 10 min each with 1X TBS-T. Membranes were incubated with primary antibodies diluted 1:2000 in 1X TBS-T at 4 °C for at least 18 h, followed by washing six times for 10 min each with 1X TBS-T. Membranes were then incubated with secondary antibodies diluted 1:2000 in 1X TBS-T at

room temperature for at least 2 h and then washed with 1X TBS-T for 10 min 6 times. Finally, ECL solution (Chemidoc, Fusion Solo 6S.WL, VILBER LOURMAT, France) was added to the membranes, and proteins were detected using ChemiDoc (WL, Vilber Lourmat, France).

2.7. Statistical analysis

All data are expressed as the mean ± standard deviation (n = 3). Each experiment was repeated at least four times. Statistical analyses were performed by one-way analysis of variance using SPSS (v. 22.0; SPSS, Chicago, IL, USA). Various levels of significance were considered as $p < 0.05$, <0.01, <0.001, and <0.001.

3. Results

3.1. Measurement of cell viability and NO production

First, we produced two different *A. esculentus* extracts, okra leaf water extract (OLW) and okra leaf ethanol extract (OLE), Next, in order to evaluate any cytotoxic effects of the *A. esculentus* extracts, RAW264.7 macrophages were treated with various concentrations of each extract, and viability was assessed after 24 h by MTT assay. As shown in Fig. 1A and B, when cell survival rates were compared to the untreated negative controls (set to 100% survival), OLW and OLE showed no change in cell survival rates up to concentrations of 200 μg/mL and 600 μg/mL, respectively. Therefore, it was concluded that OLW and OLE cause no significant cytotoxicity in RAW264.7 macrophages, and concentrations of 25, 50, 100, and 200 μg/mL were subsequently used to determine the effect of OLW and OLE on the NO production capacity of RAW264.7 macrophages. Following treatment, the degree of NO production of RAW264.7 macrophages was determined from cell supernatants using the Griess reaction. As shown in Fig. 1C and D, treatment with the positive control LPS significantly increased NO secretion, while OLE treatment inhibited NO production in a concentration-dependent manner. Surprisingly, treatment with OLW increased NO production, in contrast to the effect of OLE. Therefore, since many studies have reported anti-inflammatory effects of okra extracts in various cells, we continued to investigate whether OLW possesses immune-activating properties by promoting NO generation.

3.2. Measurement of prostaglandin E_2 (PGE_2) and pro-inflammatory cytokine expression

Activated macrophages not only produce NO and PGE_2, but also secrete pro-inflammatory cytokines that mediate interactions between immune cells and regulate the immune response. Therefore, we monitored the production of the pro-inflammatory cytokines PGE_2, TNF-α, IL-6, and IL-1β, which activate innate immunity, following treatment of RAW264.7 cells with OLW. When cells were stimulated with LPS (1 μg/mL), the production of PGE_2, TNF-α, and IL-1β increased by approximately 5.9-fold, 22.7-fold, and 6.7-fold, respectively, compared to the control group. In addition, when cells were treated with OLW, the cytokine expression levels increased in a concentration-dependent manner. When the cells were treated with the highest OLW concentration (200 μg/mL), the expression levels of PGE_2, TNF-α, and IL-1β increased by approximately 5.4-fold, 20.0-fold, and 3.6-fold, respectively, compared to the control group. However, OLW did not affect IL-6 production. These results show that OLW treatment not only increases NO production, but also enhances the immunomodulatory activity of macrophages by regulating the production of various cytokines involved in immune responses.

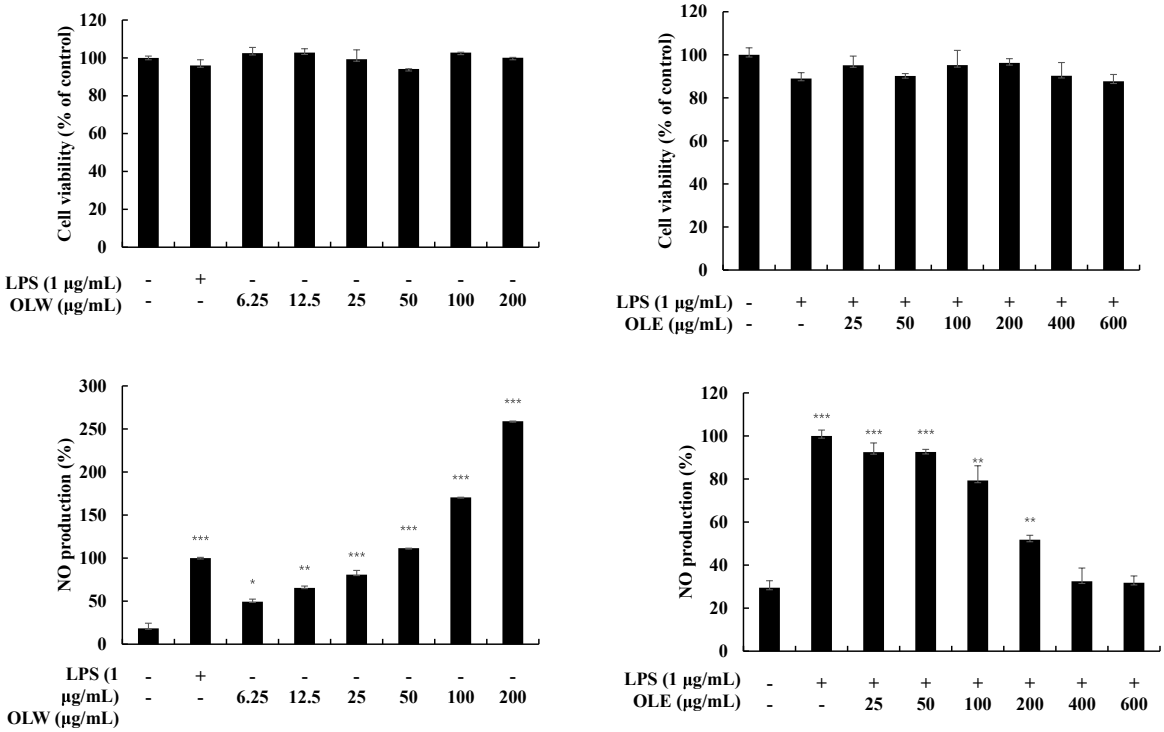

Fig. 1. Effect of OLW and OLE on cell viability and NO production in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of OLW (6.25, 12.5, 25, 50, 100, 200 μg/mL) and OLE (25, 50, 100, 200, 400, 600 μg/mL) for 24 h. (A, B) Cell viability was evaluated using MTT assay. Formed formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm using a microplate reader. (C, D) NO production was evaluated using Griess assay on the cell supernatant, followed by measurement of absorbance at 540 nm. The results shown are the average of three independent experiments (n = 3), and error bars represent standard deviation. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with the control group.

3.3. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in RAW 264.7 cells

To investigate the cause of the OLW-induced increase in NO and PGE_2 production, we next analyzed the expression of the immune-regulating proteins iNOS and COX-2, which regulate the production of NO and PGE_2 production, respectively. As shown in Fig. 3, the expression level of iNOS and COX-2 in LPS-treated cells (1 μg/mL) was 10.0-fold and 11.0-fold higher than that of the control group, respectively. However, when cells were treated with OLW, iNOS and COX-2 expression levels increased in a concentration-dependent manner. After treatment with the highest concentration of OLW (200 μg/mL), the levels of iNOS and COX-2 increased by 15.0-fold and 33.0-fold, respectively, compared to the control group. These results indicate that OLW treatment can enhance the immune response by increasing NO and PGE_2 production through upregulation of iNOS and COX-2 expression.

3.4. Activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in RAW 264.7 cells

MAPK signaling pathways containing proteins such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, play an important role in the macrophage activation by promoting the production of NO and cytokines such as PGE_2, TNF-α, and IL-1β. Therefore, in order to investigate the molecular mechanism of the OLW-induced immune activation, we investigated the effect of OLW treatment on MAPK signaling by western blotting. As shown in Fig. 4, OLW treatment increased the phosphorylation levels of p38, ERK1/2, and JNK in a concentration-dependent manner, and to a level similar to or higher than that of the LPS-treated group. In addition, since MAPKs are known to activate the NF-κB signaling pathway to upregulate the production of NO and cytokines in macrophages, we separated the nuclear and cytoplasmic protein fractions of macrophages to determine the expression of each NF-κB protein following OLW treatment. It was observed that with increasing concentration of OLW, the expression of the NF-κB p65 protein in the nuclear fraction increased, whereas the expression of the IκB protein in the cytoplasmic protein fraction decreased (Fig. 5). These results suggest that OLW induces the translocation of the p65 protein to the nucleus to induce NF-κB activation.

4. Discussion

The immune system protects against viruses and diseases and plays a crucial role in maintaining the health of the host.[18] The immune system recognizes, removes, and metabolizes substances or organisms from the external environment through innate and acquired immune responses.[19] However, the immune system can be considered as a double-edged sword, in that its primary defense against foreign pathogens needs to be balanced with the limits that can lead to hyperactivity and hyper-inflammatory conditions.[20]

The enormity of COVID-19 pandemic has led to considerable interest in the development of functional bioactive ingredients that act as immune modulators. Although it can be beneficial to target specific molecules that act at each stage of the immune system response, this strategy has the disadvantage of potentially causing persistent side effects. With this in mind, it is imperative to identify effective immunomodulating strategies that work ubiquitously in the absence of an optimal vaccination or cure for COVID-19. During the ongoing COVID-19 pandemic, which continually threatens a vulnerable population, there has been no more important time to optimize the function and efficiency of the human immune system in the defense against viral infection.[21]

Despite previous studies demonstrating that *A. esculentus* has anti-inflammatory, anti-diabetic, anti-tumor, anti-bacterial, and anti-oxidant activities, its immune-stimulatory activity has not thus far been studied.[12–15] This is the first study to describe the immune-stimulatory activity of *A. esculentus* water extract in RAW264.7

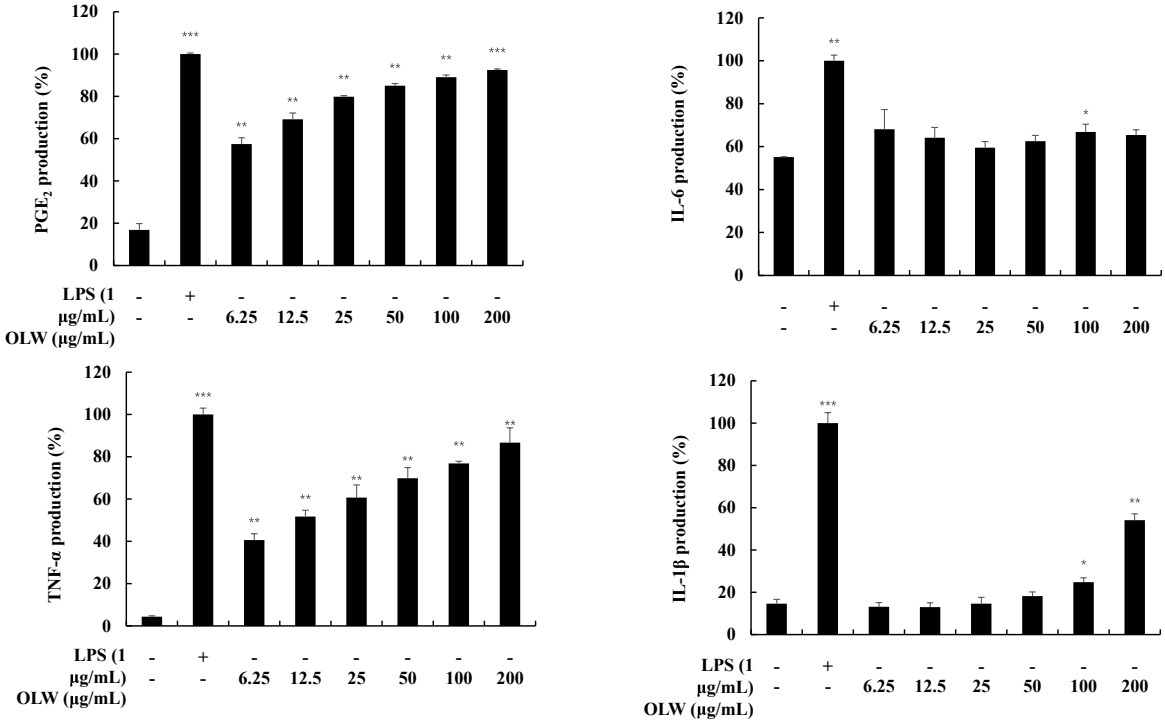

Fig. 2. Stimulatory effect of OLW on the production of PGE_2, IL-6, TNF-α, and IL-1β in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of OLW (6.25, 12.5, 25, 50, 100, 200 μg/mL) in the absence of lipopolysaccharide (LPS) for 24 h. The culture supernatant was collected and analyzed for production of (A) PGE_2, (B) IL-6, (C) TNF-α, and (D) IL-1β. The results shown are the average of three independent experiments (n = 3), and error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control group.

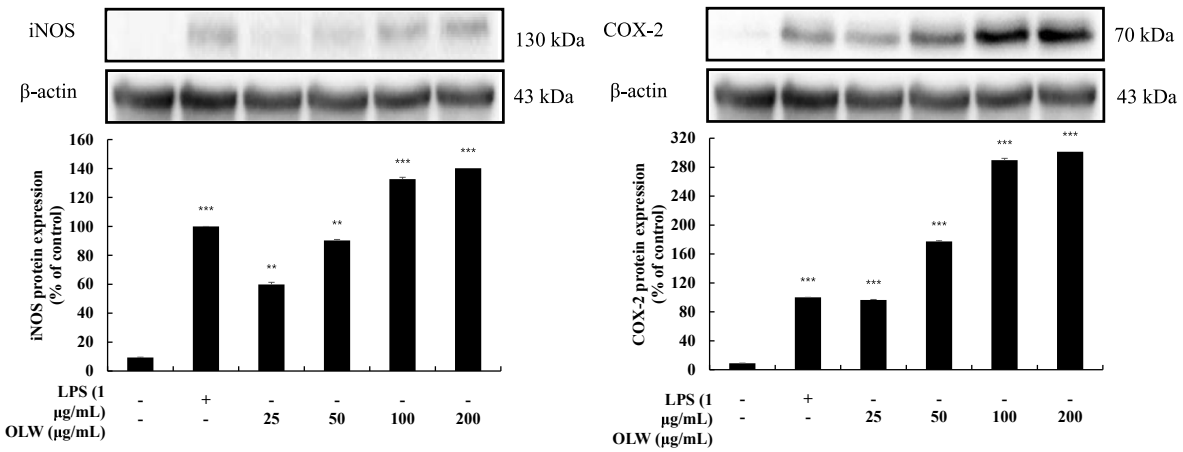

Fig. 3. Stimulatory effect of OLW on the protein expression of iNOS and COX-2 in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of OLW (25, 50, 100, 200 μg/mL) in the absence of lipopolysaccharide (LPS) for 24 h. Proteins (30 μg per well) were separated by SDS-PAGE, transferred to PVDF, and probed with specific antibodies for (A) iNOS, or (B) COX-2. β-actin was used as the loading control. The relative protein expression levels were calculated by normalizing the band intensity of the given protein to that of β-actin using ImageJ software. The results shown are the average of three independent experiments (n = 3), and error bars represent standard deviation. **p < 0.01, and ***p < 0.001 compared with control group.

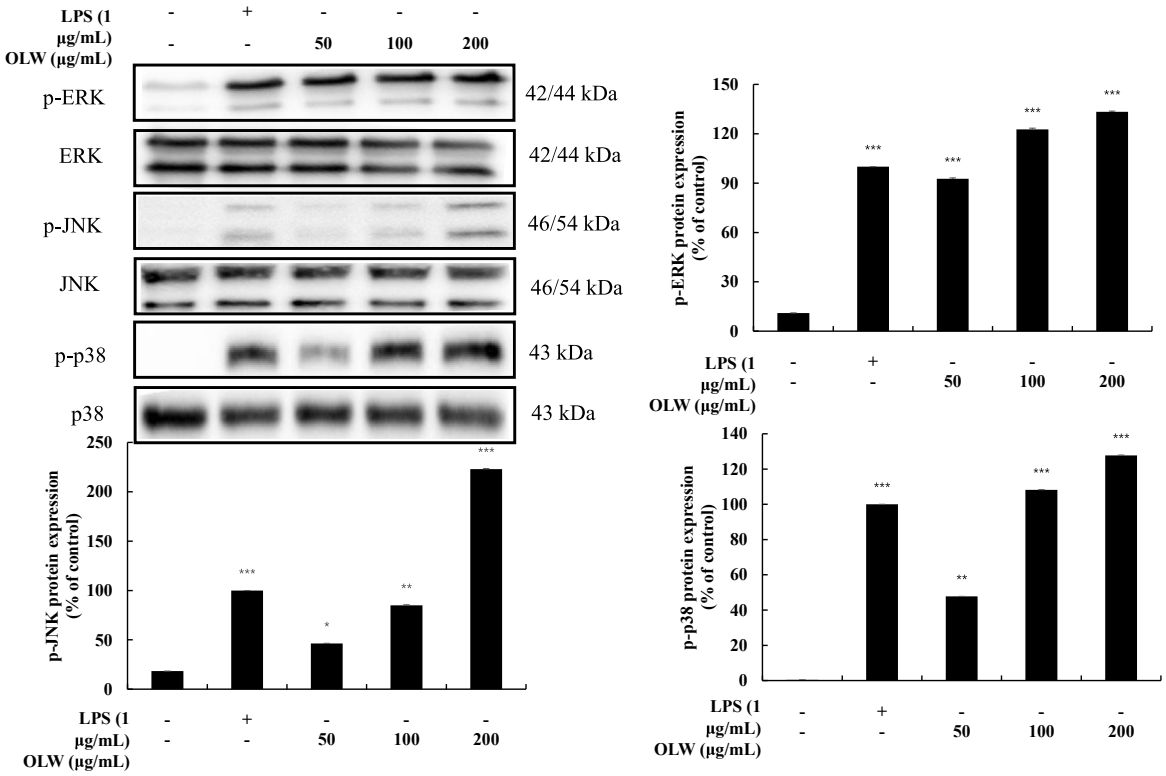

Fig. 4. Stimulatory effect of OLW on the protein expression of MAPKs in RAW 264.7 cells. (A) RAW 264.7 cells were treated with the indicated concentrations of OLW (50, 100, 200 μg/mL) in the absence of lipopolysaccharide (LPS) for 15 min. (A) Proteins (30 μg per well) were separated by SDS-PAGE, transferred to PVDF, and probed using specific antibodies for phospho- or total ERK, JNK, and p38. (B–D) The relative phospho-protein expression levels were calculated by normalizing the band intensity of the phospho-protein to that of the total protein using ImageJ software. The results shown are the average of three independent experiments (n = 3), and error bars represent standard deviation. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control group.

macrophages. Macrophages are important components of the host defense system, and are involved at all stages of the immune response. When macrophages are stimulated, they acquire specialized phenotypic properties and release numerous cellular factors, such as NO, PGE_2, and pro-inflammatory cytokines. In particular, NO secretion contributes to the physiology and pathophysiology of multiple systems.[22] NO, a multifunctional and diffusive gas molecule, is the most important mediator generated during the early innate immune response and has substantial microbicidal and tumor suppressive activity through direct attack on viruses, pathogens, and tumor cells.[23] It is therefore well established that NO production is a feasible target for immune stimulation in macrophages, and RAW 264.7 cells have been shown to produce significant amounts of NO upon external stimulation.

As shown in Figs. 2 and 3 of this study, OLW promoted NO production and iNOS expression in RAW 264.7 macrophages, even to levels comparable to those observed following treatment with the positive

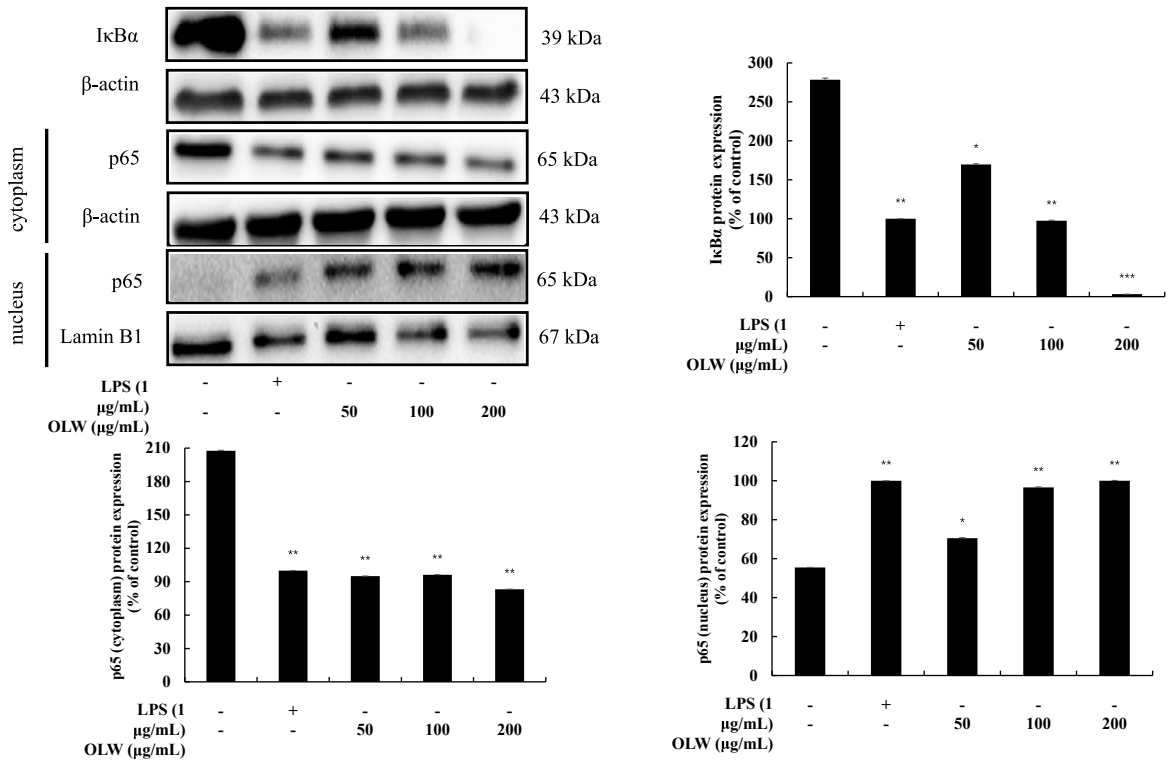

Fig. 5. Stimulatory effect of OLW on the nuclear translocation of NF-κB in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of OLW (50, 100, 200 μg/mL) in the absence of lipopolysaccharide (LPS) for 15 min. (A) Proteins (30 μg per well) were separated by SDS-PAGE, transferred to PVDF, and probed with specific antibodies for IκBα and p65. β-actin and Lamin B1 were used as the loading controls. (C–D) The relative protein expression levels were calculated by dividing the band intensity of the given protein by that of β-actin or Lamin B1. The results shown are the average of three independent experiments (n = 3), and error bars represent standard deviation. *p < 0.05, and **p < 0.01 compared with control group.

control LPS. In this study, NO production was estimated by measuring nitrite levels, which were shown to increase in a manner dependent on OLW concentration. We also demonstrated upregulation of iNOS protein expression in macrophages treated with 200 μg/mL OLW. The results suggest that the increased NO production in RAW 264.7 cells may be due to the upregulated expression of iNOS by OLW. The activation of macrophages is one of the main sources of prostaglandins, which are important mediators of inflammation and immune responses. Prostaglandins can not only induce local inflammation at the site of immune response, but can also inhibit the release of acute inflammatory mediators, thus controlling the side effects of immune modulation during infection.[23] As mentioned above, OLW treatment significantly increased PGE_2 production and COX-2 protein expression in RAW 264.7 macrophages in a concentration-dependent manner, even to levels comparable to those observed following treatment with the positive control, LPS. Taken together, these results suggest that the increased PGE_2 production in macrophages may be due to the upregulated expression of COX-2 by OLW. Consistent with the NO and PGE_2 results, OLW treatment of RAW 264.6 macrophages also markedly induced the production of pro-inflammatory cytokines such as TNF-α and IL-1β. Lee et al.[24] reported anti-inflammatory and antioxidant properties of ethanolic *A. esculentus* extracts in RAW264.7 cells co-treated with LPS or H_2O_2. However, in the present study we demonstrated that the *A. esculentus* leaf extract OLW alone stimulated the expression of NO, PGE_2, TNF-α, iNOS, and COX-2, in RAW 264.7 macrophages. It is interesting that, despite using the same biological resources, our *A. esculentus* leaf extract enhanced immune activity without co-stimulation, in contrast to the study of Lee et al.[24] We speculate that these conflicting results could be due to the differences between ethanol extraction, which simultaneously dissolves non-polar and polar substances, and water extraction, which dissolves only polar substances. In fact, various studies have reported that polysaccharides and hot water extracts, which are polar substances, have the ability to activate the immune system.[25–27] It is easy to overlook the fact that the method of extraction of substances from terrestrial and marine plants can have a significant impact on the effects on biological systems, giving variations in results. Indeed, decoctions of medicinal plants have played an important role in traditional oriental medicine for several centuries. In general, hot water extracts or decoctions of plants provide better pharmacokinetics and pharmacodynamics, and modern medical knowledge has revealed that their faster pharmacological action is due to their higher bioavailability and more potent biological activity when compared to whole plants.

Based on the remarkable effects of OLW on the activation of RAW264.7 cells mentioned above, we next focused our attention on the underlying molecular mechanisms and signaling pathways involved. It is well established that NF-κB is a transcription factor involved in a variety of cellular responses, including stimulation of the immune system through activation of pro-inflammatory genes such as TNF-α, IL-6, iNOS, and COX-2. Therefore, we evaluated the effect of OLW on the activation of NF-κB protein in RAW264.6 cells. As shown in Fig. 5, our results indicate that OLW is associated with a significant increase in NF-κB protein activation by inducing the ubiquitination and proteasomal degradation of the inhibitory protein IκBα, and subsequent translocation of the liberated NF-κB to the nucleus. MAPK is a protein Ser/Thr kinase that converts extracellular stimulation into a broad range of cellular responses, and the production of macrophage-related cytokines and chemokines is highly regulated by numerous MAPK signaling molecules such as MAPK, ERK, JNK, p38, and NF-κB.[26,27] Therefore, we evaluated the effect of OLW on the phosphorylation of MAPK in RAW 264.7 macrophages, and confirmed that OLW dramatically increased the phosphorylation of p38, JNK, and ERK in a concentration-dependent manner (Fig. 4). These results indicate that OLW exerts its immune-stimulatory effects on RAW 264.7 macrophages by activating MAPK. In conclusion, OLW activates macrophages to secrete PGE_2,

TNF-α, IL-1β, and NO, inducing iNOS and COX-2 expression via activation of the NF-κB and MAPK signaling pathways. These findings suggest that OLW may possess potent immunomodulatory effects and may be explored as a potential health-promoting compound to boost the immune system. However, the further research is needed on natural products that exhibit immunostimulatory effects. In recent years, numerous studies have shown that water-soluble polysaccharide fraction isolated from plants exhibit an immune-enhancing effects. In this study, it is presumed that the natural product responsible for the immunostimulatory effect of OLW are polysaccharides, and further research on this is needed in the future.[28–30]

Authors contribution

K.J.A and C.G.H. designed the research; M.N.K. and S.B.H. performed research; M.N.K. and S.B.H. analyzed data; C.G.H. wrote and revised the paper. All authors contributed to the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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