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Carica papaya leaf water extract promotes innate immune response via MAPK signaling pathways

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Abstract The emergence and rapid spread of the potentially fatal coronavirus disease 2019, caused due to infection by severe acute respiratory syndrome coronavirus-2, has led to worldwide interest in developing functional bioactive ingredients that act as immunomodulatory agents. In this study, we aimed to characterize *Carica papaya* extract and explore its potential as an immunomodulator by performing in vitro cell screening. Papaya leaf water extract (PLW) was found to significantly increase the levels of nitric oxide (NO) and prostaglandin E_2 (PGE_2) by upregulating inducible nitric oxide synthase and cyclo-oxygenase-2 activity, respectively. Additionally, PLW increased the production of tumor necrosis factor-α and interleukin 1β in RAW 264.7 cells. Furthermore, PLW activated the expression of c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) but not that of p38 mitogen-activated protein kinase. These results indicate that PLW increased the production of NO, PGE_2, and pro-inflammatory cytokines by activating the JNK and ERK pathways in macrophages, thus demonstrating immunomodulatory properties. Finally, high-performance liquid chromatography fingerprint analysis indicated the presence of rutin, narirutin, and ρ-coumaric acid in PLW (6.30, 119.76, and 47.25 ppm, respectively). Treating cells with these compounds at non-toxic concentrations had no effect on NO production. Taken together, these results suggest that PLW may have potential as an immunity-enhancing supplement.

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Keywords *Carica papaya* · High-performance liquid chromatography fingerprint · Immunomodulatory activity · Mitogen-activated protein kinase signaling pathway

Introduction

Infectious diseases that affect the immune system, such as those caused by severe acute respiratory syndrome coronavirus 2 and Middle East respiratory syndrome coronavirus, which have recently spread worldwide, are a serious health concern [1-3]. The immune system, a biological defense against the external environment, is an important countermeasure for infectious diseases. The immune system recognizes, eliminates, and metabolizes substances or organisms from the external environment via both innate and acquired immune responses [4-5]. The immune system plays a crucial role in maintaining the health of the host.

Macrophages are associated with both the innate and acquired immune responses and are distributed throughout all tissues in the body. Macrophages primarily protect the body from invasion by bacteria, viruses, infectious pathogens, and cancer cells as well as from the effects of aging [6]. Moreover, activated macrophages act as important mediators that maximize the immune response by enhancing macrophage capacity and producing nitric oxide (NO), prostaglandin E_2 (PGE_2), and various proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Macrophages can eliminate target cells by secreting O_2 and H_2O_2, and activated macrophages are reported to secrete nitric oxide (NO) to attack parasitic or tumor cells that are resistant to oxygen intermediates. In addition, macrophages express antigens on the cell surface that stimulate T lymphocytes, thereby inducing antibody secretion [7-10].

Immune disorders, which can result from both underactive and overactive immune systems, increase the risk of developing allergies and diseases, such as type 1 diabetes mellitus, rheumatoid

arthritis, lupus, psoriasis, and inflammatory bowel disease [11-12]. Therefore, there is an urgent need to identify effective approaches for treating immune disorders. In this regard, immunomodulation is emerging as an important strategy for improving the immune defense system, and research is underway to advance the development of natural substances that activate macrophages. Several types of immunomodulators have been recently identified, including natural polysaccharides purified from botanical sources, marine phospholipids with omega-3 polyunsaturated fatty acids (docosahexaenoic acid and eicosapentaenoic acid), and polyphenols derived from thinned peaches [13-16].

Native to Mexico and northern South America, *Carica papaya* L. (papaya) is now one of the most cultivated plants worldwide, including in tropical and subtropical regions; it is the most popular and economically important species in the Caricaceae family [17]. Although only the fruits are commonly used as commercial products, the leaves are used in traditional medicines for various diseases in several Asia-Pacific countries [18]. Several in vivo and in vitro studies have reported that *C. papaya* leaves possess anti-inflammatory properties and that disorders with inflammatory components, such as asthma and arthritis, can be treated using *C. papaya* leaf extract [19]. In addition, papaya leaf extract has been reported to confer antibacterial, antioxidant, anti-thrombocytopenic, wound-healing, anti-dengue, anti-plasmodial, anti-cancer, and hepatoprotective effects [20-21]. These properties may be associated with the presence of abundant phytochemicals, such as β-carotene, carpaine, flavonols, and niacin as well as phenolic compounds and alkaloids [22]. Several studies have described the alternative pharmaceutical use of papaya leaves; however, few studies have investigated their possible mechanisms of action or immunity-enhancing properties [19-21]. Therefore, in this study, we evaluated the immunomodulatory effects and mechanism of action of papaya leaf water extract (PLW), using the mouse macrophage cell line RAW 264.7.

Materials and Methods

Preparation of papaya leaf water extract

The papaya leaves used in this study were obtained from the Agricultural Research Center for Climate Change (Jeju, Korea). After crushing the dried papaya leaves, distilled water was added to the papaya leaf powder, and hot water extraction was performed at 65 °C for 4 h. The extract was filtered using filter paper, concentrated using a rotary vacuum evaporator, and freeze-dried.

Cell culture

RAW 264.7 cells were used to verify the immunomodulatory effect. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Merck Millipore, Billerica, MA, USA) with penicillin and streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator and sub-cultured every 2 days.

Measurement of cell viability

To investigate the viability of RAW 264.7 cells treated with PLW, the number of living cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method [13-16]. The cells were then removed from the culture dish, seeded at a density of 1.5×10^5 cells/well in the medium, added to 24-well plates, incubated for 24 h, and then treated with the PLW samples. After the treated cells were incubated for 24 h, the medium was removed, MTT reagent (0.4 mg/mL) was added, and the culture was incubated for 4 h. After the remaining medium was removed, MTT was reduced, and formazan was dissolved in dimethyl sulfoxide (DMSO). The absorbance at 570 nm was measured using a microplate reader, and the cell viability was calculated based on the absorbance value of the control (pure medium).

Measurement of NO production

The amount of nitrite accumulated in the activated macrophage culture was measured after processing the sample in the same manner as in the MTT assay [15]. After the same amount of Griess reagent was mixed in 100 μL of the cell culture solution, the absorbance at 540 nm was measured using a microplate reader, and the amount of NO produced was calculated based on the control (cells treated with only lipopolysaccharide [LPS]).

Measurement of PGE_2 and pro-inflammatory cytokine expression

Cytokine levels were measured using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, Franklin Lakes, NJ, USA), mouse IL-1β ELISA kit (R&D Systems, Minneapolis, MN, USA), and a mouse PGE_2 ELISA kit (BD Biosciences). After the sample was processed in the same manner as in the MTT assay, the culture supernatant was separated. The separated supernatant was centrifuged at 15,000 rpm for 20 min, and the cytokine levels were measured following the manufacturer's instructions for each kit.

Western blotting

After the RAW 264.7 cells were removed from the culture dish, the cells were seeded at a density of 9×10^5 cells/dish in the medium, dispensed in a 60 mm culture dish, cultured for 24 h, and then treated with the PLW sample. The sample processing time ranged between 20 min and 24 h. Cells were washed once with cold phosphate-buffered saline, and then radioimmunoprecipitation lysis buffer (150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS], 50 mM Tris-HCl [pH 7.5], and 2 mM ethylenediaminetetraacetic acid, protease inhibitor cocktail) was added to obtain the cell lysate. Centrifugation was performed at 15,000 rpm for 20 min to obtain the supernatant. Protein quantification was performed using

the Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins (30 μg/well) were separated via 10% SDS-polyacrylamide gel electrophoresis, and the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Each membrane was blocked with 5% skim milk for 4 h to suppress non-specific binding of the antibody. The membrane was then washed six times with Tris-buffered saline Tween-20 (TBST) for 10 min, followed by overnight incubation with diluted primary antibody at room temperature. After six washes with TBST, the secondary antibody was diluted 1:5000 and allowed to react with the membrane for 2 h. After six washes with TBST, the protein was confirmed by X-ray using the enhanced chemiluminescence (Biosesang, Seongnam, Korea) method.

High-performance liquid chromatography (HPLC) fingerprint analysis

To analyze its standard components, PLW was prepared at a concentration of 10,000 ppm, filtered through a 0.22 μm PVDF filter (Merck Millipore, Billerica, MA, USA), and analyzed via HPLC (e2695 Separations Module; Waters, Milford, MA, USA). YMC-Triart C18 8 nm, S-5 μm, 250×4.6 mm columns and a UV/visible detector (Waters 2489) were used. The flow rate was maintained at 2 mL/min, and detection was performed at 280 nm. The mobile phase conditions for separating rutin, narirutin, narringin, hesperidin, and neohesperidin were acetonitrile: 20 mM phosphoric acid with 2:8 ratio. In the case of quercetin, naringenin, and hesperetin were acetonitrile: 20 mM phosphoric acid with 4:6 ratio, and nobiletin and tangeretin were acetonitrile: 20 mM phosphoric acid with 6:4 ratio. All reagents were of HPLC grade. The standard substances were dissolved in methanol and DMSO and then mixed for sequential dilution to 200, 100, 50, and 25 ppm. The Waters Empower System was used for data analysis.

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 via one-way analysis of variance using SPSS (v. 22.0; SPSS, Chicago, IL, USA). Values of p <0.05, <0.01, <0.001, and <0.001 were considered significant.

Results

Measurement of cell viability

An MTT assay was performed to determine the optimal concentration of PLW for use in cell experiments. RAW 264.7 cells treated with concentrations of PLW ranging from 12.5 μg/mL to 400 μg/mL exhibited no signs of toxicity after 24 h (Fig. 1). Therefore, subsequent experiments were conducted using this concentration range.

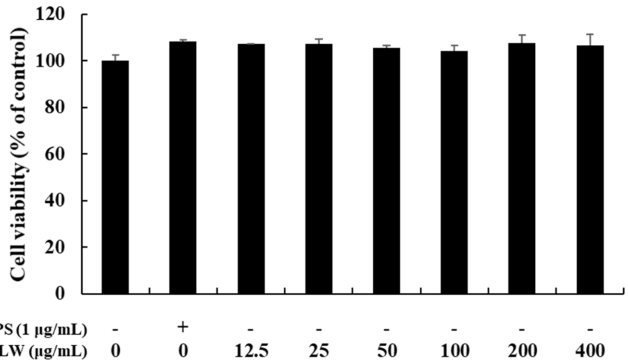

Fig. 1 Viability of RAW 264.7 cells treated with LPS and PLW (12.5, 25, 50, 100, 200, and 400 μg/mL). Cells were incubated with PLW for 24 h. Cell viability was determined via MTT assay. Data are presented as the mean ± standard deviation of at least three independent experiments (n =3). *p <0.05, **p <0.01, and ***p <0.001 versus control. LPS, lipopolysaccharide; PLW, *Carica papaya* leaf water extract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Measurement of NO and PGE_2 levels

Cells treated with LPS (1 μg/mL) produced 5.5 times more NO than cells in the control group. When cells were treated with PLW alone, the amount of produced NO increased in a concentration-dependent manner. Additionally, cells treated with the highest concentration of PLW (400 μg/mL) produced 26 times more NO than cells in the control group (Fig. 2). An increase in NO production at a concentration that does not induce cytotoxicity can be used as an index for identifying an increase in immune function.

RAW 264.7 cells were treated with PLW at concentrations of 12.5, 25, 50, 100, 200, and 400 μg/mL, and then the levels of cytokines in the cell culture supernatant were measured via ELISA (Fig. 2). The production of PGE_2 in cells stimulated with LPS (1 μg/mL) increased by approximately 9.3-fold compared to that in the control group. Western blotting was performed to confirm whether these results were associated with an increase in cyclo-oxygenase (COX)-2 protein expression, which is a mediator of PGE_2 expression.

iNOS and COX-2 expression in RAW 264.7 cells

To investigate the cause of the increase in NO production, we analyzed the intracellular expression of inducible nitric oxide synthase (iNOS), a protein involved in NO production. Western blotting was performed to determine the expression levels of iNOS and COX-2, which are proteins associated with immunomodulation (Fig. 3). The expression levels of iNOS and COX-2 in cells treated with LPS (1 μg/mL) were 20 and 50 times higher, respectively, than those in the control group. When cells were treated with PLW alone, iNOS and COX-2 expression levels increased in a concentration-dependent manner. In addition, when cells were treated with the highest concentration of PLW (400 μg/

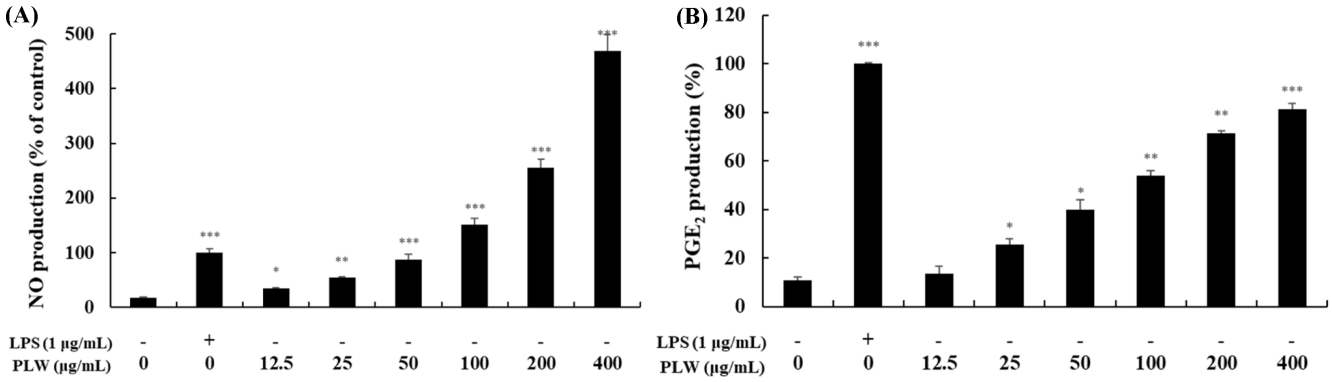

Fig. 2 (A) NO and (B) PGE_2 production in RAW 264.7 cells. Cells were incubated with different concentrations of PLW (12.5, 25, 50, 100, 200, and 400 μg/mL) for 24 h. Data are presented as the mean ± standard deviation of at least three independent experiments (n=3). *p <0.05, **p <0.01, and ***p <0.001 versus control. NO, nitric oxide; PGE_2, prostaglandin E_2; LPS, lipopolysaccharide; PLW, *Carica papaya* leaf water extract

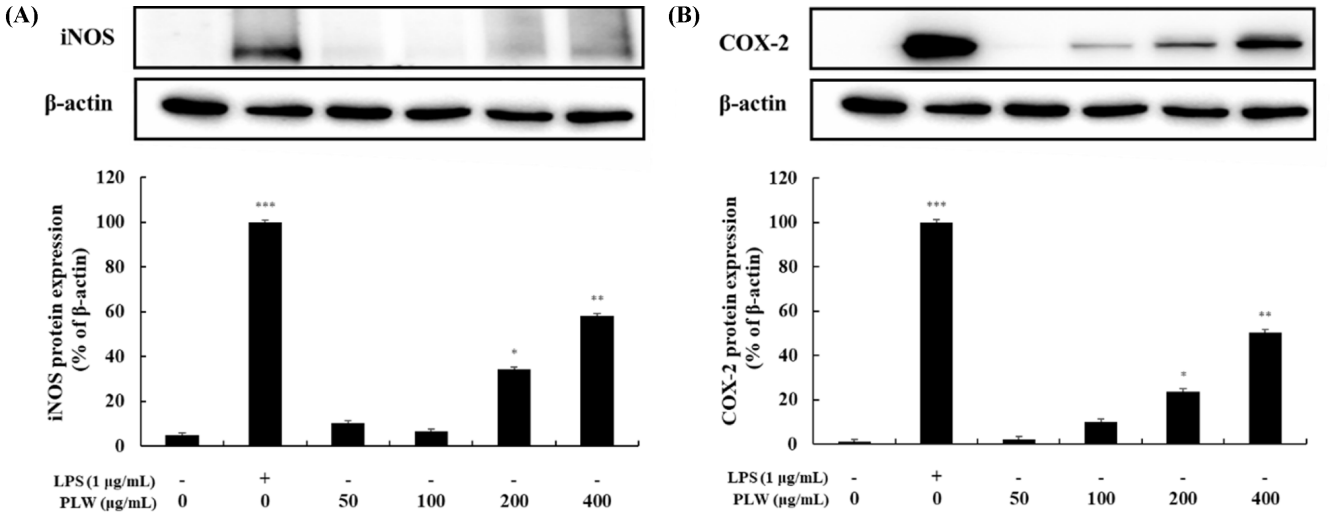

Fig. 3 Effect of PLW on the expression level of (a) iNOS and (b) COX-2 protein levels in LPS-induced RAW 264.7 cells. RAW 264.7 cells were incubated with different concentrations of PLW (50, 100, 200, and 400 μg/mL) for 24 h. β-actin was used as a loading control. Total cellular protein was separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and detected using specific antibodies against iNOS and COX-2. Data are presented as the mean ± standard deviation of at least three independent experiments (n =3). *p <0.05, **p <0.01, and ***p <0.001 versus control. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; PLW, *Carica papaya* leaf water extract

mL), the levels of iNOS and COX-2 increased by 12- and 25-fold, respectively, compared to those in the control group.

These results indicated that PLW treatment enhanced NO production by upregulating iNOS activity. PLW treatment might have also enhanced the immune response by increasing PGE_2 production via the upregulation of COX-2 expression.

Measurement of pro-inflammatory cytokine expression

RAW 264.7 cells were treated with PLW at concentrations of 12.5, 25, 50, 100, 200, and 400 μg/mL, and then the levels of cytokines in the cell culture supernatant were measured via ELISA (Fig. 4). When cells were stimulated with LPS (1 μg/mL), the production of TNF-α and IL-1β increased by approximately 14.0- and 6.7-fold, respectively, compared to that in the control group. In addition, when cells were treated with PLW alone, the expression levels increased in a concentration-dependent manner. When cells were treated with the highest PLW concentration (400 μg/mL), the expression levels of TNF-α and IL-1β increased by approximately 11.0- and 2.2-fold, respectively, compared to those in the control group. The results indicate that PLW treatment not only increased NO production in macrophages, but also enhanced macrophage immunomodulatory activity by regulating the production of various cytokines involved in the immune response.

Activation of mitogen-activated protein kinase (MAPK) signaling in RAW 264.7 cells

To investigate the molecular mechanisms of the immunomodulatory effects of PLW, the expression levels of extracellular

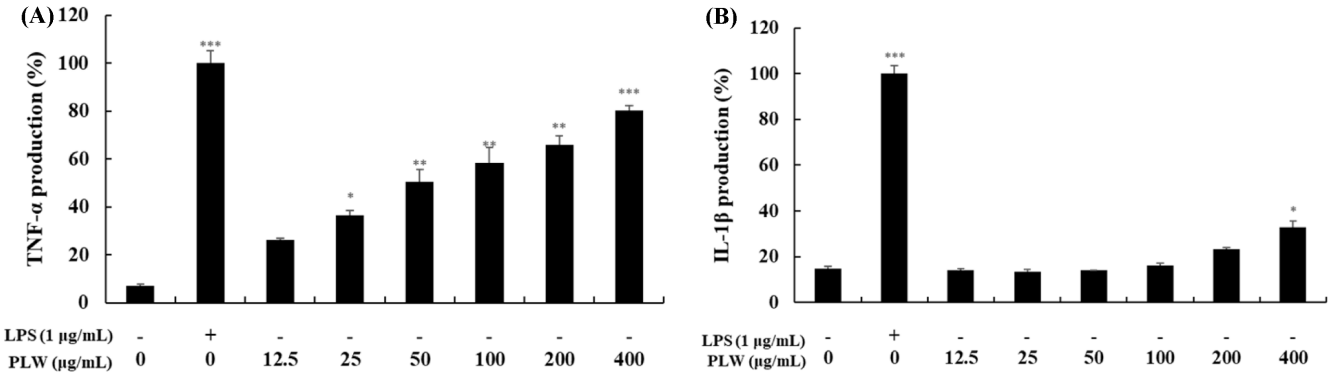

Fig. 4 (A) TNF-α and (B) IL-1β production in RAW 264.7 cells. Cells were incubated with different concentrations of PLW (12.5, 25, 50, 100, 200, and 400 μg/mL) for 24 h. Data are presented as the mean ± standard deviation of at least three independent experiments (n=3). *p <0.05, **p <0.01, and ***p <0.001 versus control. TNF-α, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; LPS, lipopolysaccharide; PLW, *Carica papaya* leaf water extract

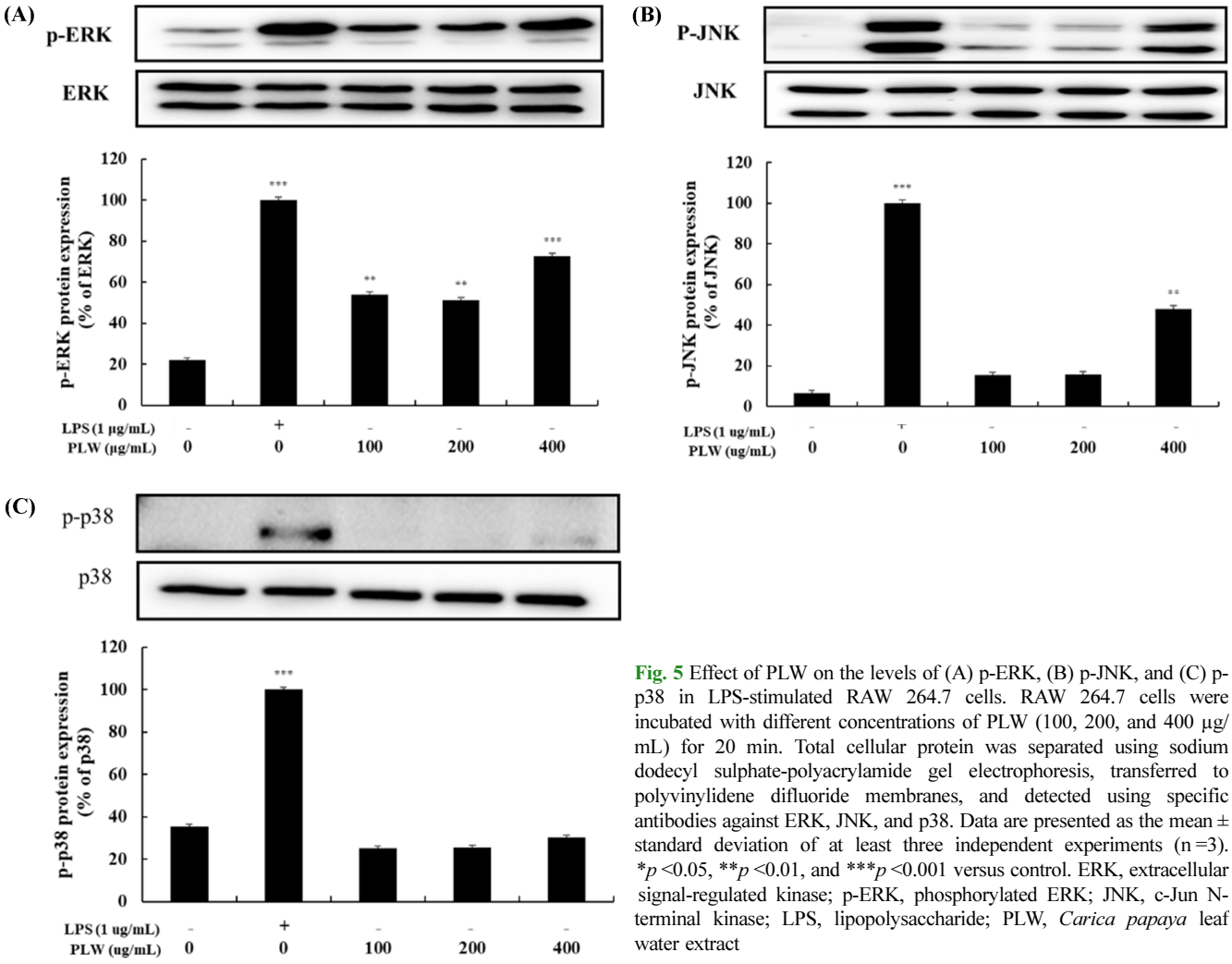

Fig. 5 Effect of PLW on the levels of (A) p-ERK, (B) p-JNK, and (C) p-p38 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were incubated with different concentrations of PLW (100, 200, and 400 μg/mL) for 20 min. Total cellular protein was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and detected using specific antibodies against ERK, JNK, and p38. Data are presented as the mean ± standard deviation of at least three independent experiments (n =3). *p <0.05, **p <0.01, and ***p <0.001 versus control. ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated ERK; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; PLW, *Carica papaya* leaf water extract

signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 proteins were examined (Fig. 5). The phosphorylation of ERK and JNK increased in response to LPS treatment. After PLW treatment at concentrations of 100, 200, and 400 μg/mL, the phosphorylation of ERK and JNK increased significantly. Previous studies have reported that when RAW 264.7 macrophages are

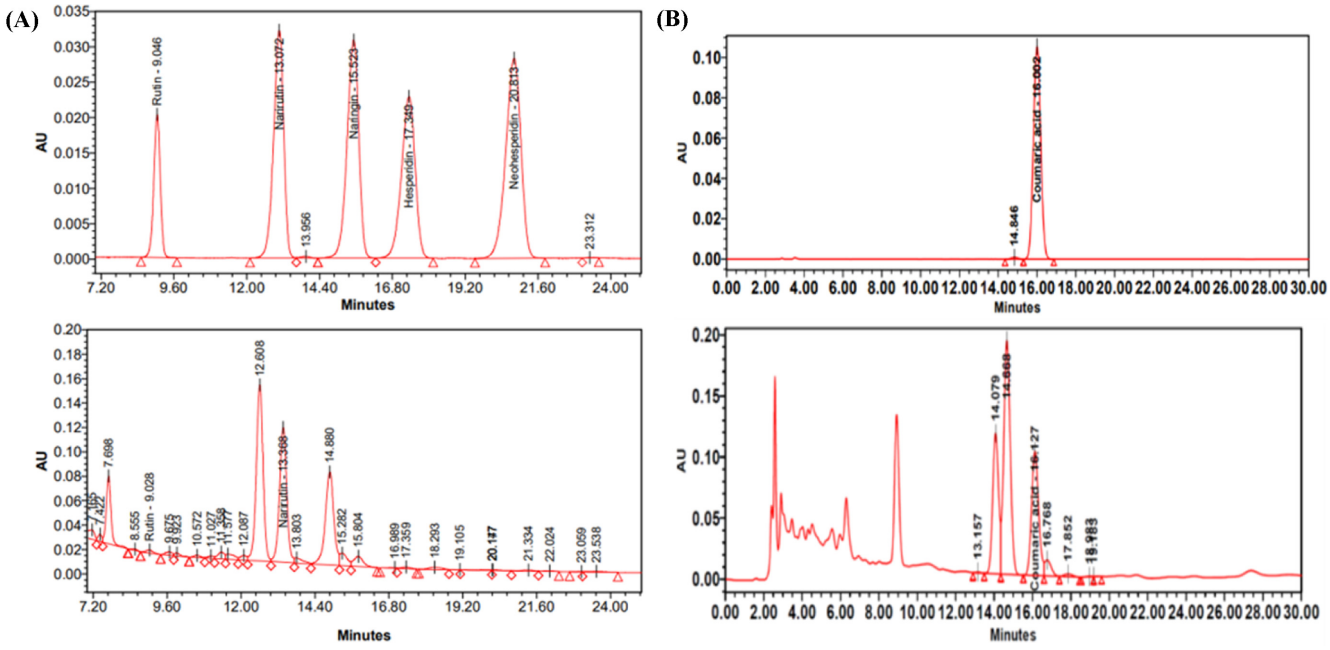

Fig. 6 HPLC fingerprint of PLW. The (A) flavonoids and (B) p-coumaric acid present in PLW were analyzed via HPLC. The upper charts represent the standard peaks of flavonoids and p-coumaric acid, respectively. The retention times (min) for quantified phenolic acids were as follows: 49.028 (rutin), 13.368 (narirutin), and 16.127 (p-coumaric acid). HPLC, high-performance liquid chromatography; PLW, *Carica papaya* leaf water extract; AU, arbitrary units

treated with papaya extracts, the phosphorylation of MAPKs decreases, subsequently inhibiting the production of cytokines and NO, and indicating an anti-inflammatory effect [23-24]. On the other hand, in our experiment, the immune response may have been enhanced by a PLW-induced increase in the phosphorylation of ERK and JNK.

HPLC fingerprint of PLW

Treatment with herbal medicine in traditional Eastern medicine is based on the complete interaction of various ingredients. Recent advances in analytical technology have enabled the use of chromatographic methods for identifying the components and functional compounds present in traditional medicine [25]. Consequently, global interest in HPLC fingerprint analysis has increased [26-27]. Therefore, a simple HPLC fingerprint was developed in this study. Flavonoids and coumaric acid were used as standard substances because they have been reported as functional ingredients present in plants. The analytical conditions described in the experimental section enable acquisition of an optimal resolution and well-defined peaks for flavonoids and coumaric acid in PLW [28]. For analysis of PLW-derived flavonoids, HPLC was used to target rutin, narirutin, naeringin, hesperidin, neohesperidin, quercetin, naeringenin, hespertin, nobiletin, and tangeretin. Rutin and narirutin were identified; their contents in PLW were found to be 6.30 mg and 119.76 mg per 100 g, respectively (Fig. 6). In addition, PLW contained a large amount of the primary metabolite ρ-coumaric acid (47.25 mg per 100 g).

Discussion

Innate immune cells such as macrophages initiate the inflammatory response by increasing the production of various inflammatory mediators, including proinflammatory cytokines, NO, adhesion molecules, and PGE_2. A limited inflammatory response is sufficient in a healthy system to prevent the development of a variety of diseases; however, an excessive or defective inflammatory response can lead to the development of serious inflammatory disorders, including autoimmune diseases [10-13]. Therefore, identifying candidates that can modulate the immune response is an important strategy for the development of drugs targeting various inflammatory diseases.

While the fruit and leaves of *C. papaya* have been actively studied for their various health benefits, including antioxidant, anti-cancer, and anti-inflammatory activities, extensive immunological studies of this species are scarce [16-22]. Therefore, in the present study, an extract of *C. papaya* leaf water was prepared via decoction of the dried leaves with distilled water using reflux extraction. The potential immunostimulatory effects of PLW were assessed using murine RAW 264.7 macrophages.

The first objective of this study was to characterize the immunostimulatory activity of PLW by measuring the production levels of NO and cytokines. We found that PLW treatment increased the production of cytokines, including PGE_2, IL-6, and TNF-α, and effector molecules, such as NO, in RAW 264.7 macrophages. PLW increased the protein expression levels of

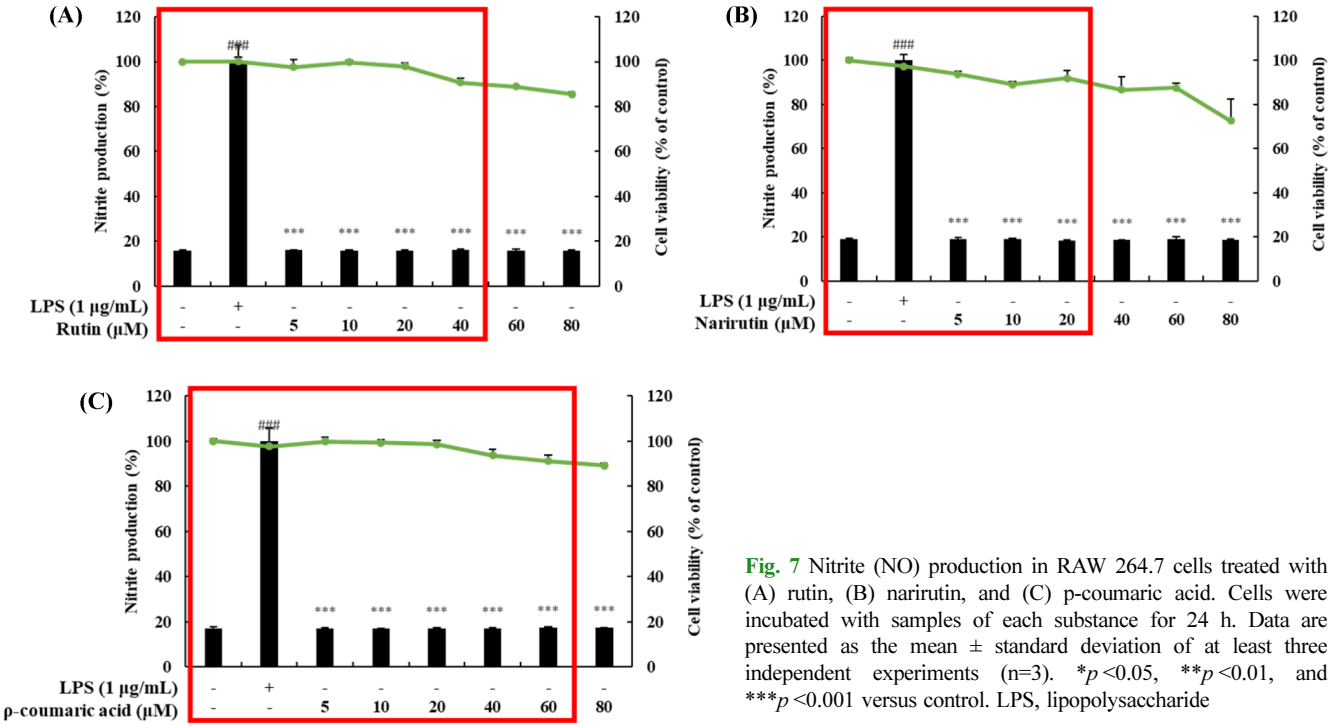

Fig. 7 Nitrite (NO) production in RAW 264.7 cells treated with (A) rutin, (B) narirutin, and (C) p-coumaric acid. Cells were incubated with samples of each substance for 24 h. Data are presented as the mean ± standard deviation of at least three independent experiments (n=3). *p <0.05, **p <0.01, and ***p <0.001 versus control. LPS, lipopolysaccharide

iNOS and COX-2 in a dose-dependent manner.

Lee et al. [29] reported that the anti-inflammatory activity of *C. papaya* leaves is associated with the significant inhibition of NO synthesis in LPS-treated RAW 264.7 cells. Similarly, in our study of *C. papaya* leaf water extract, we found that PLW itself might have stimulated the expression of cytokines including NO, PGE_2, IL-6, TNF-α, iNOS, and COX-2 in RAW 264.7 macrophages. Despite using the same biological resources, it is interesting to find that unlike the previously identified anti-inflammatory effects of *C. papaya* leaf, it improves immune activity. We believe that these conflicting findings are attributable to the ethanol extraction that dissolves nonpolar or polar and nonpolar substances at the same time, and the water extraction that dissolves only polar substances. In fact, various studies have been reported that polysaccharides, a polar substance, have immune-enhancing capabilities [14].

Toll-like receptor stimulation leads to the activation of several MAPK pathways and increases antibody and cytokine production in macrophages. Previous studies have shown that the MAPK pathway plays an important role in signaling the innate immune response. The three main families of MAPK pathways that mediate innate immune response signals include ERK 1/2, p38, and JNK [23-24]. Therefore, we evaluated the protein expression of MAPKs that are involved in the regulation of cytokine production. PLW treatment stimulated the concentration-dependent protein expression of phosphorylated (p)-JNK and p-ERK. This result suggested that PLW might have enhanced cytokine synthesis via the MAPK signaling pathway.

We performed HPLC fingerprint analysis to screen for bioactive components present in PLW. As shown in Fig. 6, we confirmed the presence and content of rutin, narirutin, and p-coumaric acid. Although it was reported that rutin, narirutin, and p-coumaric acid analyzed by HPLC fingerprint inhibit NO production in LPS-induced RAW 264.7 macrophage cells, we have analyzed the association between the above three substances and NO production in the absence of LPS induction. However, treating cells with rutin, narirutin, and p-coumaric acid at non-toxic concentrations had no effect on NO production (Fig. 7). These findings indicate that further research is needed on natural products that exhibit immunostimulatory effects.

In conclusion, we found that PLW treatment enhanced NO and cytokine production in macrophages. We also found that the immunomodulatory activity of PLW occurs via the MAPK pathway. Therefore, PLW has potential uses as a supplement for stimulating and treating disorders of the immune system.

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