Immune-enhancing Effects of *Echinacea purpurea* Extracts on RAW264.7 Cells via TLR4-mediated NF-κB and MAPKs Pathways

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Echinacea purpurea (L.) Moench is a medicinal plant from North America, Europe, and Australia that has been traditionally used to treat the common cold, bronchitis, coughs, and inflammation of the pharynx and mouth. Furthermore, extracts of *Echinacea purpurea* (EP) exert various biological effects, such as antioxidant, antibacterial, and anti-inflammatory activities. However, the possible mechanisms of action of the immune-enhancing effects are yet to be elucidated. Therefore, this study investigated the role of EP extracts in the immune-enhancing effects of RAW264.7 cells and the underlying mechanisms of action. It was found that EP extracts considerably increased the protein expression of iNOS, COX-2, and mPGES-1 in RAW264.7 cells. Also, EP extracts increased NO production, phagocytic activity, and the expression of cytokines. Consistent with these results, phosphorylation of MAPKs (ERK, JNK, and p38) and NF-κB (IKKα/β, IκBα, and NF-κB p65) were induced after treatment with EP extracts. Finally, EP extracts caused a marked increase in activation of the TLR4-TRAF6-TAK1 pathway. These results suggest that the immune-enhancing effects of EP extracts are mediated through the TLR4-associated activation of the NF-κB and MAPK pathways in RAW264.7 cells. Thus, it is suggested that EP extracts could be considered as a potential immunostimulatory agent or functional food.

keywords : *Echinacea purpurea* extracts, Immune-enhancing effects, MAPK, NF-κB, RAW264.7 cells, TLR4 pathway

Introduction

The immune system is a necessary physiological element of living organisms and it is well established that a normal immune response plays a crucial role in protecting the human body from various diseases caused by pathogenic microorganisms.[1,2]

Immunity is classified into innate and adaptive immunity. Among them, innate immunity is considered a non-specific response, whereas adaptive immunity induces an antigen-specific immune response. Generally, innate immunity includes immune cells such as macrophages, dendritic cells, and natural killer (NK) cells, etc.[3] Macrophages, among the immune cells related to immunity enhancement, play the first role in defense against infection with pathogenic microorganisms and are known to affect innate and adaptive immunity.[4] Macrophages have also been reported to affect adaptive immunity by secreting immunomodulators such as interleukins (ILs), tumor necrosis factors (TNFs), and interferons (IFNs), and producing many mediators such as inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2).[5,6] These immunomodulators secreted by macrophages play an important role in removing dead cells and harmful factors through phagocytosis induction.[7]

The synthesis of these immunomodulators is primarily caused by activation of the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways.[5] The MAPK pathway, composed of extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38, responds to extra- and intra-cellular stimuli and regulates immune responses, including pro-inflammatory cytokine production, cell proliferation, and differentiation, etc.[8,9] The NF-κB pathway is one of the most considered pathways in inflammatory responses and modulates the immune response by regulating gene expression in relation to inflammatory and immune responses.[10] NF-κB exists in the cytoplasm in an inactive form and is bound to the protein inhibitor kappa B (IκB). Upon cell stimulation, IκBα is phosphorylated and degraded by IκB kinase (IKK), followed by the release of NF-κB which is translocated to the nucleus and binds to the κB-binding site in the promoter region of the target genes and induces the transcription of

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inflammatory mediators.[11,12]

Echinacea purpurea (L.) Moench, also known as purple coneflower, is an herbaceous perennial plant belonging to the Asteraceae family, which has a long history and is well-established in the traditional medicine used in North America, Europe, and Australia.[13,14] *Echinacea purpurea* (EP) extracts have been traditionally used to treat the common cold, bronchitis, coughs, and inflammation of the pharynx and mouth.[15] It has been demonstrated that EP extracts exert various biological effects, such as antioxidant, antibacterial, and anti-inflammatory activities.[16-18] In a previous study, EP extracts especially demonstrated immunomodulatory effects in a cyclophosphamide-induced immunosuppression mouse model.[19] However, the underlying molecular mechanisms are not well understood. Therefore, the present study aimed to demonstrate the immune-enhancing effects and the underlying molecular mechanisms of EP extracts in RAW264.7 cells.

Materials and Methods

1. Sample preparation

Echinacea purpurea (L.) Moench aerial parts were extracted using 60% ethanol (EtOH) at a temperature of 50 ℃. The resulting solution was then concentrated and dried to obtain *Echinacea purpurea* (EP) extracts. The yield of EP extracts under these conditions was approximately 36%. HPLC analysis of EP extracts was carried out on a Shimadzu LC-20AD system (Shimadzu, Kyoto, Japan). The HPLC mobile phase consisted of 0.1% phosphoric acid in water (solvent A) and acetonitrile (solvent B) at 1.5 mL/min flow through Waters Sunfire C18 column (4.6 × 250 mm, 5 μm). The column temperature was kept at 35 ℃, the UV detection wavelength was 330 nm, and the injection amount was 10 μL. The finished formulation was standardized to approximately 2% using chicoric acid and more than 4% using total polyphenols[19], obtained from Evear Extraction (Féline, France). The EP extracts were then dissolved in dimethyl sulfoxide (DMSO) for in vitro studies.

2. Chemicals and reagents

Mouse RAW264.7 cells were provided by the American Type Culture Collection (ATCC; Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were provided by Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS; Escherichia coli O111:B4) was provided by Sigma-Aldrich Co (St. Louis, MO, USA). DMSO was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Nitric oxide synthase (iNOS) was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Cyclooxygenase-2 (COX-2), p-extracellular signal-regulated kinase (p-ERK), ERK, p-c-Jun N-terminal protein kinase (p-JNK), JNK, p-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, toll-like receptor 4 (TLR4), tumor necrosis factor receptor-associated factor 6 (TRAF6), p-transforming growth factor-activated kinase 1 (p-TAK1), TAK1, p-inhibitor kappa B (IκB) kinase α/β (p-IKKα/β), IKKα, p-IκBα, IκBα, p-nuclear factor-κB (p-NF-κB) p65, NF-κB p65, and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Microsomal prostaglandin E synthase-1 (mPGES-1), interleukin-6 (IL-6), IL-1β, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were purchased from Abcam (Cambridge, UK). IL-2 and IL-10 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from GenDEPOT (Barker, TX, USA).

3. Cell culture

RAW264.7 cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 ℃ in a 5% CO_2 humidified atmosphere.

4. Cell viability assay

Cell viability was assessed by the 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. RAW264.7 cells were treated with EP extracts at concentrations of 12.5, 25, 50, or 100 μg/mL for 24 h. MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 3 h at 37 ℃. The supernatants were removed from each well, and formazan crystals were resolved with DMSO. Cell viability was determined from absorbance readings obtained using a microplate reader (Tecan, Männedorf, Switzerland) at a wavelength of 570 nm.

5. Measurement of nitric oxide (NO) production

NO concentrations in cell culture supernatants were measured using Griess reagent according to the manufacturer's instructions (Promega, Madison, WI, USA). RAW264.7 cells were treated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h, then the cell culture supernatants were harvested and briefly centrifuged. Next, 50 μL of the cell culture supernatants were mixed with 100 μL of Griess reagent and reacted for 10 min at room temperature in the dark. Absorbance was determined

using a microplate reader at a wavelength of 520 nm.

6. Cell morphology observation

RAW264.7 cells were treated with various concentrations of EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. The morphology of the RAW264.7 cells was observed using an ECLIPSE Ts2 microscope (Nikon Corporation, Tokyo, Japan)

7. Phagocytosis assay

The phagocytosis activity of RAW264.7 cells was evaluated using a neutral red uptake method. The cells were treated with EP extracts at concentrations of 12.5, 25, 50, or 100 μg/mL, or with LPS at a concentrations of 100 ng/mL. After 24 h of incubation, the culture medium was removed, and the cells were washed with 1 × phosphate-buffered saline (PBS). Neutral red solution (0.075%; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and the plate was incubated at room temperature for 2 h. Subsequently, the cells were washed five times with PBS to remove excess dye and dried. Cell images were captured using an ECLIPSE Ts2 microscope (Nikon Instech, Tokyo, Japan). Following this, a lysis solution [50% EtOH:glacial acetic acid (1:1)] was added to each well. After 2 h of lysis, the absorbance was measured at 540 nm using a microplate reader (Tecan, Mannedorf, Switzerland).

8. Protein extraction and western blot analysis

RAW264.7 cells were lysed with CelLytic buffer (Sigma-Aldrich, MO, USA). The cell lysate was centrifuged at 13,000 rpm for 15 min at 4 ℃. Cell supernatants were collected and the protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). These measurements were then used to quantify the cell supernatants to ensure that each sample contained the same protein concentration in the same volume. Protein was electrophoresed using SDS-PAGE and transferred onto membranes (Millipore Corp., Bedford, MA, USA). For blocking, the membranes were kept for 1 h at 23 ℃ and then reacted with the primary antibodies (1:1000) overnight at 4 ℃. The incubated membranes were washed and further reacted with secondary antibodies (1:10000) at 23 ℃ for 1 h. The intensity of each band, detected using ECL solution (GenDEPOT, Barker, TX, USA), was measured with a LuminoGraph (Atto, Tokyo, Japan). β-actin was used as a control for normalization. Bands on the membranes were quantified using the ImageJ program (developed at the NIH).

9. Statistical analysis

All data are presented as the mean ± standard deviation. Groups were compared using the Student's t-test and one-way analysis of variance, as applicable. Statistical analyses were performed using Origin 7 software (Microcal Software, Northampton, MA, USA). $P < 0.05$ and $P < 0.01$ were considered to be statistically significant.

Results

1. Composition of *Echinacea purpurea* extracts

The marker compound within *Echinacea purpurea* (EP) extracts was identified by high-performance liquid chromatography (HPLC) analysis. As a result of quantitative analysis, the content of chicoric acid approximately 2% in EP extracts was confirmed (Fig. 1). In addition, we confirmed that the total polyphenols content was more than 4% (data not shown). Optimized EP extracts was utilized for the following in vitro study.

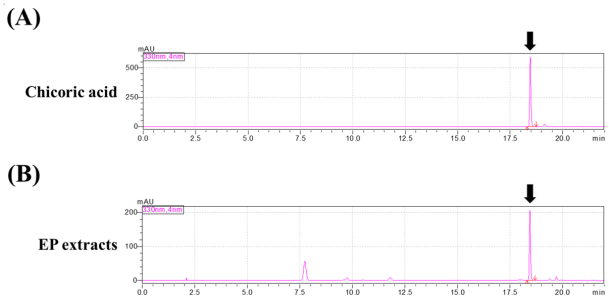

Fig. 1. Composition of *Echinacea purpurea* (EP) extracts. High-performance liquid chromatography (HPLC) chromatogram of chicoric acid and EP extracts. HPLC chromatogram of (A) Chicoric acid (standard) and (B) EP extracts. The arrow indicates the peak for chicoric acid.

2. Effects of *Echinacea purpurea* extracts on RAW264.7 cell viability

To confirm the cytotoxicity effects of EP extracts on RAW264.7 cells, the cells were exposed to various concentrations of EP extracts (12.5, 25, 50, or 100 μg/mL) for 24 h. As shown in Fig. 2A, EP extracts were not cytotoxic up to 100 μg/mL. Therefore, 12.5, 25, 50, or 100 μg/mL concentrations of EP extracts were selected for subsequent in vitro experiments.

3. Effects of *Echinacea purpurea* extracts on NO production in RAW264.7 cells

The study examined whether EP extracts could increase NO production. When RAW264.7 cells were treated with EP extracts at 12.5, 25, 50, or 100 μg/mL, NO production was significantly increased by 13.37% and 70.33%, at concentrations of 50 μg/mL and 100 μg/mL,

respectively (Fig. 2B).

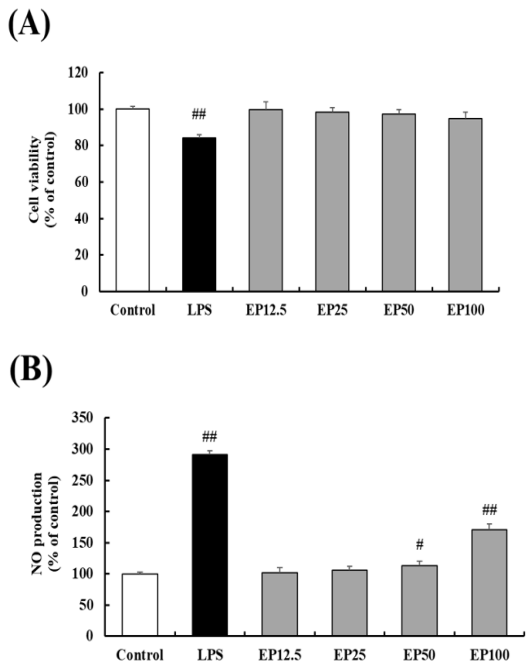

Fig. 2. Effects of *Echinacea purpurea* extracts on cell viability and NO production in RAW264.7 cells. (A) RAW264.7 cells were treated with various concentrations of EP extracts (12.5, 25, 50, or 100 μg/mL) for 24 h. Cell viability was evaluated using the MTT assay. (B) RAW264.7 cells were stimulated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. NO production levels in the culture supernatants were measured using Griess reagent. The data presented are the mean ± standard deviation of three independent experiments and differences between the mean values. #P < 0.05, ##P < 0.01 vs. control group.

4. Effects of *Echinacea purpurea* extracts on RAW264.7 cell morphology

Macrophage like cells usually exhibit three major morphologies: 1) rounded, 2) elongated, and 3) highly branched extensions.20) In the study results, the RAW264.7 cells in the control group had a round or oval-like normal morphology. However, the morphology of RAW264.7 cells was altered after treatment with EP extracts or LPS, as seen in the larger volume and presence of branches around the cells. Especially, at the concentration of 100 μg/mL, RAW264.7 cells exhibited hypertrophic cell bodies with ramified branches, and the number of branches increased markedly (Fig. 3A). This morphology could increase the contact area with outer substances and be helpful in phagocytic uptake.

5. Effects of *Echinacea purpurea* extracts on phagocytic activity in RAW264.7 cells

The phagocytic activity of macrophages was evaluated by the neutral red uptake method. When RAW264.7 cells were treated with EP extracts at 12.5, 25, 50, or 100 μg/mL, phagocytic activity was significantly increased by 28.36% and 36.13%, at a concentration of 50 μg/mL and 100 μg/mL, respectively (Fig. 3B). This indicated that EP extracts could activate RAW264.7 cells and enhance their phagocytic activity.

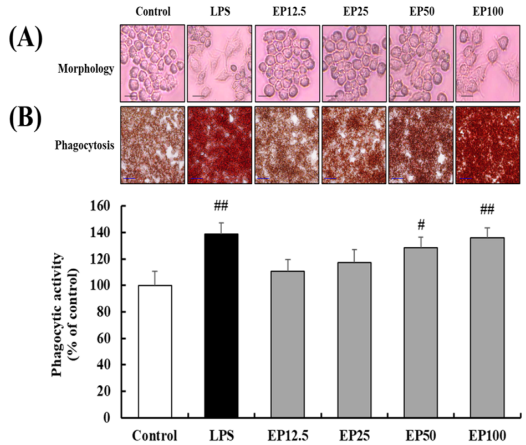

Fig. 3. Effects of *Echinacea purpurea* extracts on cell morphology and phagocytic activity in RAW264.7 cells. RAW264.7 cells were stimulated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. (A) Effects of EP extracts on cell morphology in RAW264.7 cells. (B) Effects of EP extracts on phagocytic activity in RAW264.7 cells. The data presented are the mean ± standard deviation of three independent experiments and differences between the mean values. #P < 0.05, ##P < 0.01 vs. control group.

6. Effects of *Echinacea purpurea* extracts on inflammatory cytokines in RAW264.7 cells

iNOS plays a critical role in the synthesis of NO. Cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) is associated with the conversion of arachidonic acid to prostaglandin E_2 (PGE_2).[21] Thus, this study investigated whether the EP extracts influenced the expression of iNOS, COX-2, and mPGES-1 in RAW264.7 cells. Treatment with EP extracts increased the expression of iNOS (33.40-51.48%), COX-2 (182.88-696.64%), and mPGES-1 (96.60-123.44%) in RAW264.7 cells (Fig. 4).

Activated macrophages can generate various cytokines to fight against pathogens. These cytokines play an important role in regulating innate immunity and adaptive immunity, and can be classified into interleukins (ILs), tumor necrosis factors (TNFs), and interferons (IFNs).[22] Therefore, cytokines were confirmed as indicators of the immunomodulatory effect of EP extracts, and their expression levels were measured. The results showed that EP extracts increased IL-2 (93.28-323.88%), IL-6 (145.39-449.17%), IL-10 (76.85-236.63%), IL-1β (149.93-415.93%), IFN-γ (84.93-127.61%), and TNF-α (48.11-225.02%) protein

expression in RAW264.7 cells (Fig. 4). These results demonstrated that EP extracts could activate RAW264.7 cells and exhibit immunomodulatory activity by stimulating the expression of cytokines.

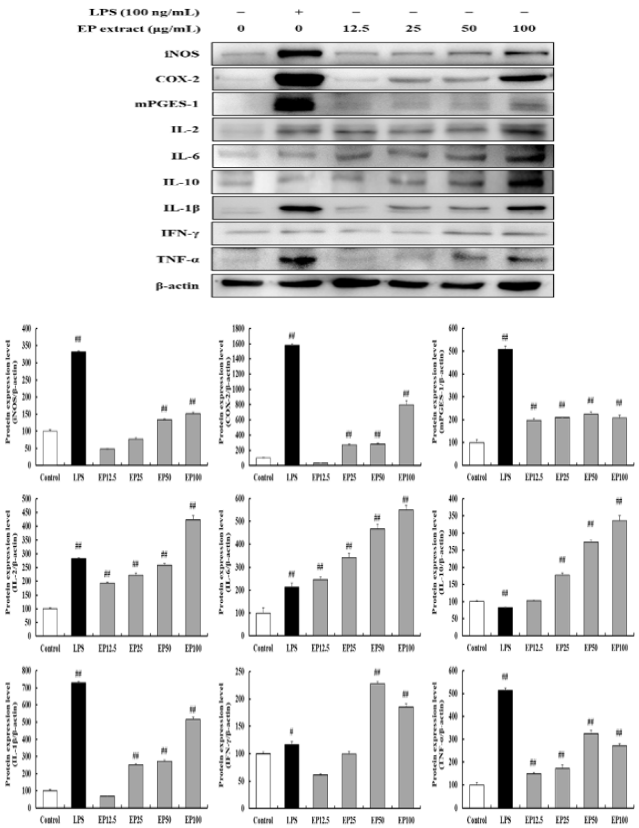

Fig. 4. Effects of *Echinacea purpurea* extracts on inflammatory cytokines in RAW264.7 cells. RAW264.7 cells were treated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. Protein expression was analyzed by western blot using specific antibodies. β-actin was used as controls. The data presented are the mean ± standard deviation of three independent experiments and differences between the mean values. #P < 0.05, ##P < 0.01 vs. control group

7. Effects of *Echinacea purpurea* extracts on the MAPK and NF-κB pathways in RAW264.7 cells

The mitogen-activated protein kinase (MAPK) cascade and nuclear factor-κB (NF-κB) are two important signaling pathways related to the innate immune response.[2,23] MAPKs can regulate cellular components of the immune system through modulating transcription factors. through the modulation of transcription factors, and they consist of the extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38.[24] This study thus investigated the effects of EP extracts on MAPKs phosphorylation by western blot. The results showed that EP extracts increased phosphorylation of ERK (11.27-26.02%), JNK (203.05-336.65%), and p38 (343.71-678.79%) in RAW264.7 cells (Fig. 5A).

NF-κB is inactive and retained in the cytoplasm by specific inhibitor IκBα, and IKKα/β is an important regulatory protein for the phosphorylation of IκB. When IKK α/β is activated, phosphorylation of IKK leads to the phosphorylation and degradation of IκBα. NF-κB is then released, translocated to the nucleus, and phosphorylated by nuclear kinases to induce transcription of immune-related genes.[25] To confirm whether the NF-κB pathway also participates in macrophage activation by EP extracts, phosphorylation of key proteins was investigated. The results showed that EP extracts significantly upregulated phosphorylation of IKKα/β, IκBα, and NF-κB p65 in the range of 61.44-911.86%, 394.36-2453.27%, and 17.52-60.07%, respectively (Fig. 5B).

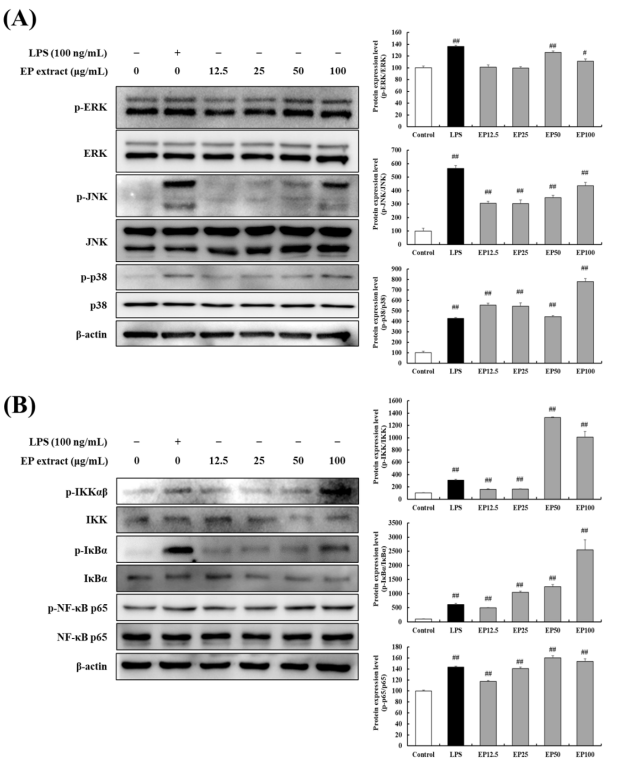

Fig. 5. Effects of *Echinacea purpurea* extracts on the MAPK and NF-κB pathway in RAW264.7 cells. RAW264.7 cells were treated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. Protein expression was analyzed by western blot using specific antibodies. (A) Effects of EP extracts on the MAPK pathway in RAW264.7 cells. ERK, JNK, or p38 were used as controls. (B) Effects of EP extracts on the NF-κB pathway in RAW264.7 cells. IKK, IκBα, or NF-κB p65 were used as controls. The data presented are the mean ± standard deviation of three independent experiments and differences between mean values. #P < 0.05, ##P < 0.01 vs. control group.

8. Effects of *Echinacea purpurea* extracts on the TLR4 pathway in RAW264.7 cells

To understand the mechanism through which the EP extracts-mediated inflammatory response is upregulated, the expression of TLR4 in RAW264.7 cells treated with EP extracts was investigated using western blots. The

expression of TLR4, TRAF6, and the phosphorylation of TAK1 in RAW264.7 cells was significantly increased by EP extracts in the range of 83.24-212.94%, 52.55-419.16%, and 28.32-66.53%, respectively (Fig. 6).

These results suggest that EP extracts treatment could activate the immune response in RAW264.7 cells via the TLR4-TRAF6-TAK1-MAPK/NF-κB pathway.

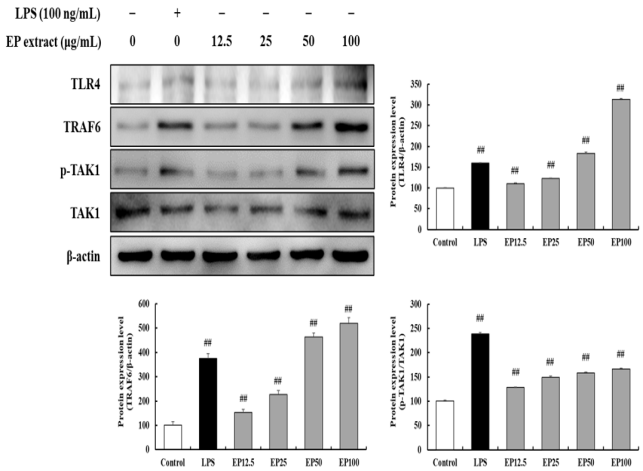

Fig. 6. Effects of *Echinacea purpurea* extracts on the TLR4 pathway in RAW264.7 cells. RAW264.7 cells were stimulated with EP extracts (12.5, 25, 50, or 100 μg/mL) or LPS (100 ng/mL) for 24 h. Protein expression was analyzed by western blot using specific antibodies. TAK1 or β-actin were used as controls. The data presented are the mean ± standard deviation of three independent experiments and differences between the mean values. #P < 0.05, ##P < 0.01 vs. control group

Discussion

The innate immune system is the body's first line of defense and a protection system that plays a critical role in destroying foreign substances and pathogens that invade the body. Numerous immune cells, such as macrophages and NK cells, play critical roles in the innate immune system. These cells play an essential role in immune enhancement and the inflammatory response and macrophages in particular are known to play an important role in the host immune system through phagocytosis.[26)]

Echinacea purpurea (L.) Moench is widely used as an herbal medicine in North America, Europe, and Australia.[13,14,27)] *Echinacea purpurea* (EP) is used as a medicinal preparation by most American Indians for the treatment of many diseases, such as toothaches, headaches, wound infections, fever, colds, etc.[28)] It also has been reported that EP extracts exert various biological functions, such as antioxidant, antibacterial, anti-inflammatory activities, and immunoenhancing effects.[16-19,29)] Especially, common symptoms of upper respiratory infections, such as sore throat, cough, and inflammation, are often caused by bacteria, sometimes as a complication of virus infection. Echinacea exerts a dual action against several important respiratory bacteria: a killing effect and an anti-inflammatory effect.[30)] These reports support the concept of using an Echinacea to control symptoms associated with bacterial respiratory infections. However, despite its long history of use for health-promoting and therapeutic effects, there are very few laboratory studies on the mechanism of its actions.

Macrophages are known to perform biological functions such as regulating inflammation and enhancing immunity.[31)] In terms of immune-enhancement, activation of macrophages produces various immunomodulators such as inducible nitric oxide synthase (iNOS), nitric oxide (NO), cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1), interleukins (ILs), tumor necrosis factors (TNFs), and interferons (IFNs) to attack foreign compounds in the body.[5,6,32,33)] Therefore, these immunomodulators, produced by activated macrophages, play a crucial role against noxious factors in the host defense system.[34)] However, no studies exist on the immune-enhancing activity and mechanism of action of EP extracts in RAW264.7 cells. Therefore, this study aimed to identify the immune-enhancing activity and mechanisms of action of EP extracts using RAW264.7 cells.

Among the immunomodulators, NO is a key biomolecule that is synthesized by iNOS to defend against infection and inhibit pathogen growth.[35)] In addition, COX-2 and mPGES-1 promote the production of prostaglandin E_2 (PGE_2) and play a role in activating the immune response in stimulated cells.[36)] Furthermore, inflammatory cytokines such as IL-2, IL-6, IL-10, IL-1β, IFN-γ, and TNF-α are known to be involved in the destruction of pathogens and the immune response.[37)] This study demonstrated that EP extracts increase the production of NO and the expression of immunomodulators such as iNOS, COX-2, mPGES-1, IL-2, IL-6, IL-10, IL-1β, IFN-γ, and TNF-α and promote phagocytosis activity in RAW264.7 cells. The results show that EP extracts can induce immune-enhancing activity through macrophage activation.

Mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways are representative signaling mechanisms related to the production of immunomodulators through the activation of macrophages.[38)] The MAPK pathway regulates immune responses, such as pro-inflammatory cytokine production, cell proliferation, and differentiation etc.[8,9)] The NF-κB pathway also stimulates the

production of many cytokines and has been implicated in the regulation of the innate and adaptive immune responses.[39] This study confirmed that EP extracts significantly stimulate the phosphorylation of MAPKs, such as extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 in RAW264.7 cells. Furthermore, the study identified that EP extracts effectively induced the phosphorylation of IκB kinase α/β (IKKα/β), inhibitor kappa B (IκBα), and NF-κB p65. These results suggest that EP extracts increased the activation of the MAPK and NF-κB pathways and consequently induced the production of immunomodulators.

Macrophages possess a variety of toll-like receptors (TLRs) on the cell membrane that recognize external noxious factors and activate intracellular signaling pathways.[40] In particular, TLR4 is a protein family that plays an important role in the immune system, and the TLR-mediated signaling pathway activates the MAPK and NF-κB pathways to produce immunomodulators.[41-46] The TLR4 pathway induces downstream activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), to form a complex composed of transforming growth factor-activated kinase 1 (TAK1) and TAB proteins, which activates TAK1 by autophosphorylation. TAK1 is also a key upstream factor for the MAPK and NF-κB pathways.[47-49] The study results proved that activation of the TLR4/TRAF6/TAK1 pathway was involved in the immune responses of RAW264.7 cells and induced by EP extracts.

The proposed mechanism is summarized: EP extracts-induced activation of NO production, phagocytosis activity, and proinflammatory cytokine expression via TLR4-TRAF6-TAK1-NF-κB/MAPK, which may act as one mechanism involved in immune responses (Fig. 7).

This study demonstrated the immune-enhancing effects of EP extracts in RAW264.7 cells. EP extracts induced macrophage activation through TLR4-associated activation of the MAPK and NF-κB pathways in RAW264.7 cells. These activations led to a significant increase in NO production, phagocytosis activity, and proinflammatory cytokine expression in RAW264.7 cells treated with EP extracts compared with the control group. The novelty of this study lies in the evaluation of the immune-enhancing effects induced by treatment with EP extracts in RAW264.7 cells. Particularly, noteworthy is the fact that this is the first report to provide further insight into the mechanism of action underlying the immune-enhancing effect of EP extracts in RAW264.7 cells. However, further studies are warranted to elucidate the immune-enhancing effect of EP extracts in various immune cell types, including natural killer cells, dendritic cells, T cells, and B cells etc. These additional studies in immune cells will provide direct evidence of the immune-enhancing effect of EP extracts. Collectively, EP extracts exerted immune-enhancing effects, suggesting their potential as an immunostimulatory agent or functional food.

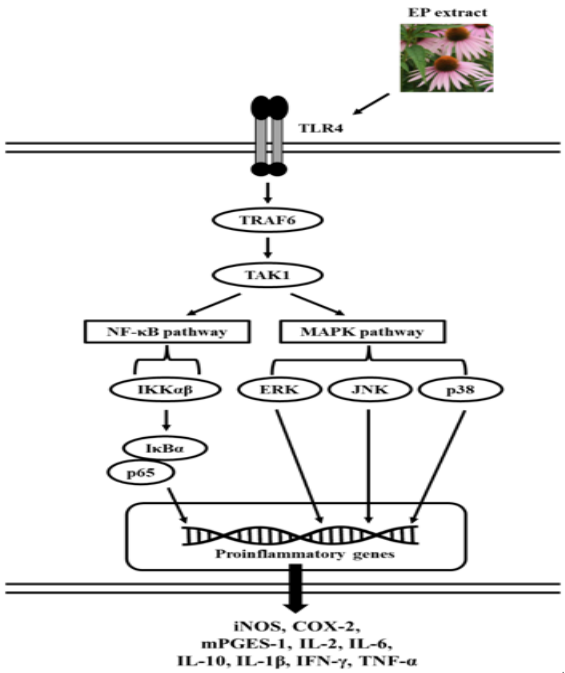

Fig. 7. Road map of the immune-enhancing function of *Echinacea purpurea* extracts via the TLR4-mediated pathway.

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References

1. Hong SH, Ku JM, Kim HI, Ahn CW, Park S-H, Seo HS, et al. The immune-enhancing activity of Cervus nippon mantchuricus extract (NGE) in RAW264.7 macrophage cells and immunosuppressed mice. Food Res Int. 2017;99:623-9.
2. Yang F, Li X, Yang Y, Ayivi-Tosuh SM, Wang F, Li H, et al. A polysaccharide isolated from the fruits of Physalis alkekengi L. induces RAW264.7 macrophages activation via TLR2 and TLR4-mediated MAPK and NF-κB signaling pathways. Int J Biol Macromol. 2019;140:895-906.
3. Lee J, Kim S, Kang CH. Immunostimulatory Activity of

Lactic Acid Bacteria Cell-Free Supernatants through the Activation of NF-κB and MAPK Signaling Pathways in RAW 264.7 Cells. Microorganisms. 2022;10:2247.
4. Geng L, Hu W, Liu Y, Wang J, Zhang Q. A heteropolysaccharide from Saccharina japonica with immunomodulatory effect on RAW 264.7 cells. Carbohydr Polym. 2018;201:557-65.
5. Geum NG, Eo HJ, Kim HJ, Park GH, Son HJ, Jeong JB. Immune-enhancing activity of Hydrangea macrophylla subsp. serrata leaves through TLR4/ROS-dependent activation of JNK and NF-κB in RAW264.7 cells and immunosuppressed mice. J Funct Foods. 2020;73:104139.
6. Kang CH, Kim JS, Kim H, Park HM, Paek NS. Heat-killed lactic acid bacteria inhibit nitric oxide production via inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 cells. Probiotics Antimicrob Proteins. 2021;13:1530-8.
7. Hirayama D, Iida T, Nakase H. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. Int J Mol Sci. 2017;19:92.
8. Rao KMK. MAP kinase activation in macrophages. J Leukoc Biol. 2001;69:3-10.
9. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta - Mol Basis Dis. 2010;1802:396-405.
10. Miettinen M, Lehtonen A, Julkunen I, Matikainen S. Lactobacilli and streptococci activate NF-κB and STAT signaling pathways in human macrophages. J Immunol. 2000;164:3733-40.
11. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev. 1995;9:2723-35.
12. Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 1998;16:225-60.
13. Wills RBH, Stuart DL. Alkylamide and cichoric acid levels in Echinacea purpurea grown in Australia. Food Chem. 1999;67:385-8.
14. Barnes J, Anderson LA, Gibbons S, Phillipson JD. Echinacea species (Echinacea angustifolia (DC.) Hell., Echinacea pallida (Nutt.) Nutt., Echinacea purpurea (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol. 2005;57:929-54.
15. Hohmann J, Rédei D, Forgo P, Szabó P, Freund TF, Haller J, et al. Alkamides and a neolignan from Echinacea purpurea roots and the interaction of alkamides with G-protein-coupled cannabinoid receptors. Phytochem. 2011;72:1848-53.
16. Woelkart K, Bauer R. The role of alkamides as an active principle of Echinacea. Planta Med. 2007;73:615-23.
17. Sharma M, Anderson SA, Schoop R, Hudson JB. Induction of multiple pro-inflammatory cytokines by respiratory viruses and reversal by standardized Echinacea, a potent antiviral herbal extract. Antiviral Res. 2009;83:165-70.
18. Todd DA, Gulledge TV, Britton ER, Oberhofer M, Leyte-Lugo M, Moody AN, et al. Ethanolic Echinacea purpurea extracts contain a mixture of cytokine-suppressive and cytokine-inducing compounds, including some that originate from endophytic bacteria. PloS one. 2015;10:e0124276.
19. Kim HR, Kim YS, Lee DR, Choi BK, Kwon KB, Bae GS. Echinacea purpurea Alleviates Cyclophosphamide-Induced Immunosuppression in Mice. Appl Sci. 2022;12:105.
20. Li ZP, Liu HB, Zhang QW, Li LF, Bao WR, Ma DL, et al. Interference of quercetin on Astragalus polysaccharide-induced macrophage activation. Molecules. 2018;23:1563.
21. Wang L, Shi J, van Ginkel FW, Lan L, Niemeyer G, Martin DR, et al. Neural stem/progenitor cells modulate immune responses by suppressing T lymphocytes with nitric oxide and prostaglandin E2. Exp Neurol. 2009;216:177-83.
22. Patra MC, Shah M, Choi S. Toll-like receptor-induced cytokines as immunotherapeutic targets in cancers and autoimmune diseases. Semin Cancer Biol. 2020;64:61-82.
23. Xu Z, Lin R, Hou X, Wu J, Zhao W, Ma H, et al. Immunomodulatory mechanism of a purified polysaccharide isolated from Isaria cicadae Miquel on RAW264. 7 cells via activating TLR4-MAPK-NF-κB signaling pathway. Int J Biol Macromol. 2020;164:4329-38.
24. Ji C, Zhang Z, Chen J, Song D, Liu B, Li J, et al. Immune-enhancing effects of a novel glucan from purple sweet potato Ipomoea batatas (L.) Lam on RAW264. 7 macrophage cells via TLR2-and TLR4-mediated pathways. J Agric Food Chem. 2021;69:9313-25.
25. Zhang Y, Zhang A, Zhao Y, Feng X, Sheng Y, Zhang H, et al. Expressions of TLR4, MyD88, IRAK4 and NF-ÎB in the oviduct of Chinese brown frog (Rana dybowskii). Eur J Histochem. 2019;63:3050.
26. Billack B. Macrophage activation: role of toll-like receptors, nitric oxide, and nuclear factor kappa B. Am J Pharm Educ. 2006;70:102.
27. Kumar KM, Ramaiah S. Pharmacological importance of

Echinacea purpurea. Int J Pharma Bio Sci. 2011;2:304-14.
28. Pullaiah T. Encyclopedia of world medicinal plants. Volume 1. Daya Books; 2006. p. 841-2.
29. Mishima S, Saito K, Maruyama H, Inoue M, Yamashita T, Ishida T, et al. Antioxidant and immuno-enhancing effects of Echinacea purpurea. Biol Pharm Bull. 2004;27:1004-9.
30. Sharma SM, Anderson M, Schoop SR, Hudson JB. Bactericidal and anti-inflammatory properties of a standardized Echinacea extract (Echinaforce®): dual actions against respiratory bacteria. Phytomedicine 2010;17:563-8.
31. Kasimu R, Chen C, Xie X, Li X. Water-soluble polysaccharide from Erythronium sibiricum bulb: Structural characterisation and immunomodulating activity. Int J Biol Macromol. 2017;105:452-62.
32. Siveen KS, Kuttan G. Role of macrophages in tumour progression. Immunol Lett 2009;123:97-102.
33. Ma X, Meng M, Han L, Cheng D, Cao X, Wang C. Structural characterization and immunomodulatory activity of Grifola frondosa polysaccharide via toll-like receptor 4-mitogen-activated protein kinases-nuclear factor κB pathways. Food Funct. 2016;7:2763-72.
34. Zhang S, Nie S, Huang D, Huang J, Wang Y, Xie M. Polysaccharide from Ganoderma atrum evokes antitumor activity via Toll-like receptor 4-mediated NF-κB and mitogen-activated protein kinase signaling pathways. J Agric Food Chem. 2013;61:3676-82.
35. Shreshtha S, Sharma P, Kumar P, Sharma R, Singh SP. Nitric Oxide: It's Role in Immunity. J Clin Diagn Res. 2018;12:BE01-BE05.
36. Lee HW, Choi IW, Ha SK. Immunostimulatory activities of theobromine on macrophages via the activation of MAPK and NF-κB signaling pathways. Curr Issues Mol Biol. 2022;44:4216-28.
37. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 2014;5:491.
38. Shen CY, Zhang WL, Jiang JG. Immune-enhancing activity of polysaccharides from Hibiscus sabdariffa Linn. via MAPK and NF-kB signaling pathways in RAW264. 7 cells. J Funct Foods. 2017;34:118-29.
39. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017;2:1-9.
40. Wang Y-Q, Mao J-B, Zhou M-Q, Jin Y-W, Lou C-H, Dong Y, et al. Polysaccharide from Phellinus Igniarius activates TLR4-mediated signaling pathways in macrophages and shows immune adjuvant activity in mice. Int J Biol Macromol. 2019;123:157-66.
41. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine. 2008;42:145-51.
42. Poltorak A, He X, Smirnova I, Liu M-Y, Huffel CV, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282:2085-8.
43. Qian Y, Commane M, Ninomiya-Tsuji J, Matsumoto K, Li X. IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFκB. J Biol Chem. 2001;276:41661-7.
44. Baud V, Liu ZG, Bennett B, Suzuki N, Xia Y, Karin M. Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. Genes Dev. 1999;13:1297-1308.
45. Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature. 1996;383:443-6.
46. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. 1999;13:1015-24.
47. Jadhav T, Geetha T, Jiang J, Wooten MW. Identification of a consensus site for TRAF6/p62 polyubiquitination. Biochem Biophys Res Commun. 2008;371:521-4.
48. Yang K, Zhu J, Sun S, Tang Y, Zhang B, Diao L, et al. The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination. Biochem Biophys Res Commun. 2004;324:432-9.
49. Chiu YH, Zhao M, Chen ZJ. Ubiquitin in NF-κB signaling. Chem Rev. 2009;109:1549-60.