Artemisia asiatica Nakai Attenuates the Expression of Proinflammatory Mediators in Stimulated Macrophages Through Modulation of Nuclear Factor- κ B and Mitogen-Activated Protein Kinase Pathways

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ABSTRACT The present study aimed to examine the anti-inflammatory effects and potential mechanism of action of *Artemisia asiatica* Nakai (*A. asiatica* Nakai) extract in activated murine macrophages. *A. asiatica* Nakai extract showed dose-dependent suppression of lipopolysaccharide (LPS)-induced nitric oxide, inducible nitric oxide synthase, and cyclooxygenase-2 activity. It also showed dose-dependent inhibition of nuclear factor- κ B (NF- κ B) translocation from the cytosol to the nucleus and as an inhibitor of NF- κ B- α phosphorylation. The extract's inhibitory effects were found to be mediated through NF- κ B inhibition and phosphorylation of extracellular signal-regulated kinase 1/2 and p38 in LPS-stimulated J774A.1 murine macrophages, suggesting a potential mechanism for the anti-inflammatory activity of *A. asiatica* Nakai. To our knowledge, this is the first report of the anti-inflammatory effects of *A. asiatica* Nakai on J774A.1 murine macrophages; these results may help develop functional foods possessing an anti-inflammatory activity.

KEY WORDS: • *Artemisia asiatica* Nakai • ERK1/2 • p38 • NF- κ B

INTRODUCTION

HISTORICALLY, VARIOUS PLANTS or plant-derived compounds with medicinal properties have played important roles in disease prevention and treatment. Plant-derived compounds are increasingly used in health-promoting bioactive food components, as many new plant products are showing a wide range of activities such as anticancer, anti-inflammatory, and antiaging effects.^{1,2} About 25% of all prescribed drugs are derived from plants with or without

further alteration.^{3,4} *Artemisia asiatica* Nakai has been traditionally used for the treatment of inflammation, cancer, and microbial infections. A novel antipeptic phytochemical derived from *A. asiatica* Nakai has been suggested to have antioxidant and anti-inflammatory properties.^{5,6}

Inflammation can often result from the attack of pathogens or cell injury and represents a normal physiological immune response. Typically, it involves the activation of several different immune cells such as macrophages, neutrophils, and lymphocytes. In particular, macrophages play an important role in modulating inflammation and the immune response to maintain this self-protective reaction. During the inflammatory response, activated macrophages produce a large quantity of proinflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2) through inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2),

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respectively, as well as proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), IL-6, and monocyte chemoattractant protein (MCP-1).^{7,8}

MCP-1 is the most important chemotactic factor involved in this process and is produced in response to the action of lipopolysaccharide (LPS), TNF- α , IL-1 β , IL-6, COX-2, and iNOS.^{9,10} Overexpression of these proinflammatory mediators is involved in the pathogenesis of many diseases. Therefore, blockade of these mediators is a promising tactic to inhibit inflammation, carcinogenesis, and other disorders.

NF- κ B plays an important role in the expression of the genes involved in inflammation.¹¹ In unstimulated cells, NF- κ B remains in the cytoplasm as a heterodimer by corporal association with an inhibitory protein, inhibitor κ B (I κ B). The release of NF- κ B from I κ B can occur because of numerous external or internal stimuli. After activation, the NF- κ B heterodimer (p65/p50) is transferred rapidly from the cytoplasm to the nucleus. There, NF- κ B modulates the transcription of numerous target genes, including proinflammatory mediators such as iNOS and COX-2, various cytokines such as TNF- α , IL-6, and IL-1 β , chemokines such as MCP-1, and adhesion molecules.^{12–14}

Mitogen-activated protein kinases (MAPKs) belong to a large family of serine/threonine kinases. They are divided into three major subfamilies, including extracellular signal-regulated kinase 1/2 (ERK1/2), the c-jun N-terminal kinase or stress-activated protein kinase (JNK or SAPK), and p38 MAPK. MAPKs play an important role in the intervention of signals generated by cytokines, growth factors, and environmental stress, as well as in various cellular functions.^{15–17} Recently, it has been reported that MAPKs are involved in the expression of LPS-induced inflammatory mediators and NF- κ B activation.^{18–20}

To the best of our knowledge, there are no reports on the anti-inflammatory effects of *A. asiatica* Nakai through modulation of these signaling pathways in LPS-stimulated J774A.1 macrophages. Therefore, this study attempted to elucidate the mechanisms involved in its anti-inflammatory action on stimulated J774A.1 macrophages.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 026:B6) was purchased from Sigma (St. Louis, MO, USA). Pyrrolidine dithiocarbamate (PDTC), PD98059, and SB203580 were from Calbiochem (La Jolla, CA, USA). Dulbecco Modified Eagle Medium (DMEM) and TRIzol reagent were from Invitrogen (Carlsbad, CA, USA). Antibodies for western blot were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA) except for phospho-I κ B α (Cell Signaling Technology, Beverly, MA, USA). Rabbit polyclonal antibodies against phosphorylated or nonphosphorylated ERK, JNK, and p38 were from Cell Signaling Technology.

Preparation of *A. asiatica* Nakai extracts

The detailed method of extraction and preparation of *A. asiatica* Nakai has been previously reported.²⁰ Briefly, the

herb was macerated in 95% ethanol for 20 h; then, the extract was filtered and concentrated to 1/20 (w/w) of the initial herbal weight. The contents of jaceosidin and eupatilin, flavones responsible for its biological effect, were 1.42% and 0.78% (w/w), respectively (Fig. 1). These values were estimated using reversed-phase high-performance liquid chromatography (HPLC) on a C₁₈ column (4.6 $\times$ 150 mm) with acetic acid–ammonium acetate:acetonitrile (58:42) at a flow rate 1.0 mL/min. The UV-vis detector was monitored at 345 nm, and the injection volume for all samples and standards was 20 μ L. Quantitative HPLC analysis of each compound was calculated from its peak area.

Cell cultures

The BALB/c murine macrophage cell line J774A.1 (TIB-67; ATCC, Manassas, VA, USA) was grown in DMEM supplemented with 2 mM glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% nonheat-inactivated fetal bovine serum in 5% CO₂ at 37°C. Thioglycollate (TG)-elicited macrophages were isolated 3 days after intraperitoneal injection of 2.5 mL of TG to 8-week-old BALB/c mice according to a previously established method.²¹ Peritoneal lavage was performed by using 8 mL of Hank's balanced salt solution (HBSS) supplemented with heparin. J774A.1 macrophages were treated with *A. asiatica* Nakai extracts at various concentrations (0, 10, and 50 μ g/mL) with or without LPS for 24 h. In addition, the cells treated with *A. asiatica* Nakai extracts without LPS were examined for endotoxin contamination. After the contamination test, only the nonendotoxin cells were used for all of the experiments for this study.

Nitric oxide

The amount of stable nitrite, which is the end product of NO generation, was determined by a colorimetric assay as previously described.²² Briefly, 50 μ L of culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance at 540 nm was read using a Power Wavex Microplate Scanning spectrophotometer (Bio-Tek Instrument, Winooski, VT, USA). The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Analysis of mRNA expression

For the reverse transcription–polymerase chain reaction (RT-PCR), total cellular RNA was isolated from cells by using the TRIzol reagent according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Invitrogen). PCR was performed as previously described with the exception of the following primers: iNOS (s 5'-AAA GCC ACG AGG CTC TGA CA-3'; as 5'-GTG AGA GGC AAA GGA GGA GA-3'), TNF- α (s 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3'; as 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'), IL-1 β (s 5'-ATA ACC TGC TGG TGT GTG AC-3'; as 5'-AGG TGC TGA TGT ACC

FIG. 1. High-performance liquid chromatography (HPLC) images and the chemical structures. HPLC chromatograms: standard compounds (**A**); 95% ethanol extract of *Artemisia asiatica* Nakai (**B**); jaceosidin (peak 1) and eupatilin (peak 2); chemical structures: jaceosidin (**C**) and eupatilin (**D**).

AGT TG-3'), IL-6 (s 5'-CCG GAG AGG AGA CTT CAC AG-3'; as 5'-GGA AAT TGG GGT AGG AAG GA-3'), COX-2 (s 5'-GAA GGG ACA CCC TTT CAC AT-3'; as 5'-ACA CTC TAT CAC TGG CAT CC-3'), MCP-1 (s 5'-CAG CAG GTG TCC CAA AGA A-3'; as 5'-CTT GAG GTG GTT GTG GAA AA-3'), and MIP1- α (s 5'-TCT GCA ACC AAG TCT TCT CAG-3'; as 5'-GAA GAG TCC CTC GAT GTG GAT A-3'). β -Actin (s 5'-TAG ACT TCG AGC AGG AGA TG-3'; as 5'-TTG ATC TTC ATG GTG CTA GG-3') was used for internal control. RT-PCR was performed as previously described in related studies.²³ For quantitative PCR, real-time PCR was performed in triplicate using 12.5

μ L of SYBR Premix Ex Taq (Takara, Japan) and 2 μ L of cDNA as a template in a final volume of 25 μ L. PCR amplification was preceded by incubation of the mixture for 15 min at 95°C and 40 cycles of the amplification step. Denaturation was performed for 30 s at 95°C; annealing was performed in a transitional temperature range from 58°C to 62°C, with an increase of 0.5°C per cycle. Extension was performed for 30 s at 72°C with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses of all samples were performed within the range from 65°C to 95°C with continuous fluorescence detection. β -Actin was used for normalization.

Nuclear protein extraction and western blot

The nuclear extracts were prepared using previously reported methods.²⁴ Briefly, after cell activation for the times indicated, cells were washed in 1 mL of ice-cold phosphate-buffered saline, centrifuged at 125 *g* for 5 min, resuspended in 400 μ L of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 *g* for 30 s. Pelleted nuclei were gently resuspended in 50 μ L of ice-cold buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15,000 *g* for 5 min at 4°C. Aliquots of supernatants that contained nuclear proteins were frozen in liquid nitrogen and stored at -70°C. Cell extracts were prepared by using a previously described detergent lysis procedure.²⁵ Samples of protein (80 μ g) were electrophoresed using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes at 150 mA for 1 h in a semidry system. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Animal design

BALB/c mice manipulations were performed according to the European Community Guidelines for Animal Care (DL 116/92, application of the European Communities Council Directive of 24 November 1986; 86/609/EEC) and approved by the Committee for Animal Care and Experimental Use, Konkuk University. The ethical policy of Konkuk University conforms to the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996; Konkuk University assurance No. KU14011). The animals were housed with free access to food and water, and maintained on a 12-h light/12-h dark cycle at 22°C room temperature. All efforts were made to minimize animal suffering and the number of animals sacrificed.

Statistical analyses

The experiments shown are summaries of data from at least three experiments. All data are presented as mean $\pm$ standard error. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. All results showing $P < .05$ were taken to indicate statistical significance.

RESULTS

Cytotoxicity of *A. asiatica* Nakai on macrophages

In the present study, J774A.1 macrophages were treated with various concentrations of *A. asiatica* Nakai for 48 h, and cell viability was assessed by MTT assay. In *A. asiatica* Nakai, no significant cytotoxic effects were observed over 48 h of incubation, even at a high concentration (50 μ g/mL). Cell viability was found to be higher than 93% at all concentrations (data not shown).

Effect of *A. asiatica* Nakai on the expressions of iNOS and COX-2

Stress-induced NO production in macrophages is related to inflammation.²⁶ *A. asiatica* Nakai significantly inhibits LPS-induced NO production in J774A.1 macrophages in a dose-dependent manner (Fig. 2A). Stressed macrophages are known to generate excessive iNOS and COX-2 as part of the inflammatory response.²⁷ To investigate the effects of *A. asiatica* on the expression of iNOS and COX-2, J774A.1 macrophages were stimulated with 30 ng/mL LPS for 24 h in the presence of increasing concentrations of *A. asiatica* Nakai, and iNOS and COX-2 protein expression was determined by immunoblot analysis (Fig. 2B). *A. asiatica* Nakai showed dose-dependent inhibition of LPS-induced iNOS and COX-2 expression.

Effect of *A. asiatica* Nakai on the expression of proinflammatory mediators

We also examined the effect of *A. asiatica* Nakai on gene expression of proinflammatory mediators in LPS (30 ng/

FIG. 2. Reduction of lipopolysaccharide (LPS)-induced nitric oxide (NO) production and expression of inflammatory mediators. NO in the culture media was assayed using Griess reagent (A). The protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was reduced following *A. asiatica* Nakai treatment as detected by western blot assay using specific antibodies (B). Results are expressed as mean $\pm$ standard error ($n = 3$). *Significant difference from the LPS-alone group shown at $P < .05$.

mL)-stimulated J774A.1 macrophages using RT-PCR (Fig. 3A) and quantitative real-time PCR (Fig. 3B). Figure 3 shows that treatment with *A. asiatica* Nakai effectively decreased the LPS-induced mRNA expression of TNF- α , IL-1 β , IL-6, and MCP-1 in a dose-dependent manner. This indicates that *A. asiatica* Nakai (2, 10, and 50 μ g/mL) inhibits the production of a broad spectrum of proinflammatory cytokines in stimulated J774A.1 macrophages.

Effect of *A. asiatica* Nakai on the translocation of NF- κ B and phosphorylation of ERK1/2 and p38

NF- κ B activation is the key event for induction of all major inflammatory mediators. The production of proinflammatory cytokines is also regulated by NF- κ B. *A. asiatica* Nakai was able to inhibit NF- κ B activation in LPS-stimulated macrophages, as indicated by western blotting (Fig. 4A). *A. asiatica* Nakai treatment inhibited I κ B- α phosphorylation (Fig. 4A). J774A.1 macrophages were pretreated with *A. asiatica* Nakai at the indicated concentrations for 30 min and then stimulated with 30 ng/mL LPS for 15 min. The total cell lysates were then probed with phospho-specific antibodies against ERK1/2, JNK, and p38. Phosphorylation of ERK1/2, JNK, and p38 increased in the cells treated with LPS alone. *A. asiatica* Nakai treatment decreased phosphorylated ERK1/2 and p38 levels in the stimulated J774A.1 macrophages in a concentration-dependent manner (Fig. 4B, D); however, the phosphorylation of JNK was unaffected (Fig. 4C).

A. asiatica Nakai modulates anti-inflammatory mediators through suppression of NF- κ B and MAPKs

To determine whether the inhibition of NF- κ B and MAPKs is correlated with the anti-inflammatory effects of *A. asiatica*,

isolated primary mouse macrophages were pretreated with a specific NF- κ B inhibitor (PDTC), an ERK1/2 inhibitor (PD98059), and a p38 inhibitor (SB203580) for 30 min, and then cotreated with LPS (30 ng/mL) for the indicated times. As shown in Figure 4E, PDTC, PD98059, and SB203580 significantly inhibited the mRNA expression of iNOS, COX-2, TNF- α , IL-1 β , and IL-6 in LPS-activated primary macrophages. The results indicate that PDTC, PD98059, and SB202190 could downregulate the expression of proinflammatory mediators, and that the anti-inflammatory effect of *A. asiatica* may be mediated through inhibition of the NF- κ B and ERK/p38 MAPK pathways.

DISCUSSION

Recently, the interest in using herbal products to treat inflammatory disorders by blocking inflammatory mediators has increased. In our ongoing screening of *A. asiatica* Nakai for potential as an anti-inflammatory agent, it was found to exhibit a potent activity in *in vitro* anti-inflammatory experimental models, without any significant cytotoxicity. In addition, the mechanisms underlying this anti-inflammatory action were investigated in a murine macrophage cell line and primary murine macrophages.

The extract of *A. asiatica* Nakai has been used as herbal medicine for the treatment of various diseases.^{5,6} Jaceosidin (40,5,7-trihydroxy-30,6-dimethoxyflavone) and eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone) are pharmacologically active flavones derived from medicinal herbs of the genus *Artemisia*.²⁸ In fact, a novel pharmacologically active formulation prepared from the ethanol extracts of *A. asiatica* Nakai, namely, DA-9601 (StillenTM), is now on the market in South Korea and other Asian countries.²⁹ We determined the concentrations of the principal bioactive flavones,

FIG. 3. The expression of proinflammatory mediators using reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR. RT-PCR mRNA expression of tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), IL-6, and monocyte chemoattractant protein (MCP)-1 (A). TNF- α (B), IL-1 β (C), IL-6 (D), and MCP-1 (E) mRNA levels were quantified by real-time PCR. The results were similar in three independent experiments. * $P < .05$: significantly different from the LPS group.

FIG. 4. Nuclear translocation of nuclear factor- κ B (NF- κ B), phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (MAPKs), and proinflammatory mediators in activated J774A.1 macrophage cells. *A. asiatica* Nakai suppresses LPS-induced NF- κ B activation and phosphorylation of inhibitor κ B (I κ B) in J774A.1 macrophage cells (A). Phosphorylation of ERK1/2 (B) and p38 (D) was dose dependently inhibited by *A. asiatica* Nakai, but that of JNK was not (C). Primary cultured mouse macrophage cells were pretreated with *A. asiatica* Nakai with PDTC, PD98059, and SB203580, and then cotreated with LPS (E). The results were similar in three independent experiments.

jaceosidin and eupatilin, to be 1.42% and 0.78% (w/w), respectively (Fig. 1).

Macrophages play an important role in inflammation; activated macrophages secrete large amounts of proinflammatory mediators such as NO and PGE2 through iNOS and COX-2. The overproduction of these mediators can be harmful and is responsible for various inflammatory diseases.³⁰ Therefore, novel mechanisms to suppress the overproduction of these mediators can be a significant therapeutic target in the development of anti-inflammatory agents. *A. asiatica* Nakai inhibited LPS-induced NO production in J774A.1 macrophages in a dose-dependent manner (Fig. 2A).

To investigate whether the inhibitory effect of *A. asiatica* Nakai on NO and PGE2 gene expression corresponded with protein levels, the expression of iNOS and COX-2 were determined by western blot. *A. asiatica* Nakai treatment attenuated iNOS and COX-2 protein expression in macrophages in a dose-dependent manner (Fig. 2B). Inflammatory disorders can occur through the production of abnormal amounts of proinflammatory cytokines such as TNF- α , IL-1 β , and IL-6 and MCP-1 and macrophage inflammatory protein 1 α (MIP-1 α). All of these factors can be released by macrophages. TNF- α is identified as a critical cytokine that

can stimulate the production of IL-1 β and IL-6. Furthermore, TNF- α can cause the release of a series of inflammatory mediators such as NO and oxyradicals.³¹ Alternatively, NO also promotes TNF- α production by the activation of macrophages.³² At the beginning and throughout the development of the inflammatory response, IL-1 β plays a significant role in infection caused by *Helicobacter pylori*, and IL-6 is reflected as an endogenous mediator of fever induced by LPS.^{33,34} Many natural products show anti-inflammatory activity by increasing anti-inflammatory factors and decreasing proinflammatory cytokine/chemokine production. Our data demonstrate that *A. asiatica* Nakai significantly inhibits the mRNA expression of proinflammatory mediators such as TNF- α , IL-6, IL-1 β , and the chemokine MCP-1 (Fig. 3). These results were confirmed with quantitative real-time PCR of proinflammatory mediator expression. These data suggest that *A. asiatica* has anti-inflammatory properties owing to its ability to downregulate the induced proinflammatory mediators.

LPS can initiate numerous intracellular signaling events, such as the activation of NF- κ B, NF- κ B-inducing kinase (NIK), inhibitor of κ B kinase (IKK), and MAPK pathways.^{35–38} Inactivated NF- κ B is localized to the cytoplasm through interaction with a set of inhibitory proteins, namely

I κ Bs (I κ B α , I κ B β , and I κ B ϵ). Upon stimulation, it is translocated to the nucleus by the signal-induced degradation of I κ B proteins. NF- κ B stimulation plays an important role in innate and adaptive immune responses.^{35,39,40} Moreover, NF- κ B also has an important role in regulating the production of LPS-stimulated proinflammatory mediators released from macrophages.⁴¹ To determine the mechanisms by which *A. asiatica* Nakai inhibits inflammatory mediators, we examined the effects of *A. asiatica* Nakai on NF- κ B nuclear translocation and I κ B degradation. Our results demonstrate that *A. asiatica* Nakai dose dependently inhibited LPS-mediated NF- κ B transcriptional activity and decreased I κ B- α expression (Fig. 4A). Artemisolide, a sesquiterpene-monoterpene lactone isolated from *A. asiatica*, suppresses LPS-induced NF- κ B activation and NO production in macrophages.⁴² Recent investigations have highlighted the roles of NF- κ B and MAPK pathways in response to inflammatory reactions and infectious agents. NF- κ B activation can be regulated by cellular kinases such as MAPKs, including the ERK1/2, JNK, and p38 subgroups.⁴³ MAPKs are known to be involved in the regulation of cytokine responses.⁴⁴ Activated ERK1/2 has been shown to increase proinflammatory cytokines and iNOS in LPS-induced macrophages.^{45,46} The JNK pathway, also known as SAPK (SAPK1), is quickly activated in the LPS stimulation of RAW264.7 cells.⁴⁶

To investigate the molecular mechanism of NF- κ B inhibition by *A. asiatica* Nakai in LPS-stimulated J774A.1 macrophages, we studied the activity of MAPKs (ERK1/2, p38, and JNK). *A. asiatica* Nakai treatment reduced phosphorylated ERK1/2 and p38 levels in LPS-stimulated J774A.1 macrophages in a concentration-dependent manner (Fig. 4B, D). However, *A. asiatica* Nakai treatment had no significant effect on phosphorylated JNK levels under these conditions (Fig. 4C). Furthermore, to determine whether the inhibition of NF- κ B, ERK1/2, and p38 is correlated with the anti-inflammatory mechanism of *A. asiatica* Nakai, isolated primary mouse macrophages were pretreated with specific NF- κ B, ERK1/2, and p38 inhibitors and then costimulated with LPS. These inhibitors significantly reduced the mRNA expression of iNOS, COX-2, TNF- α , and IL-1 β in LPS-activated primary macrophages (Fig. 4E). Our results indicate that PDTC, PD98059, and SB203580 could downregulate proinflammatory mediator expression, and that the anti-inflammatory effect of *A. asiatica* Nakai may be mediated through inhibition of the NF- κ B and ERK1/2 pathways. The mechanism of *A. asiatica* Nakai is in accordance with that described in a previous study using a mouse skin model.⁴⁷

In the current study, *A. asiatica* Nakai showed potent anti-inflammatory effects by inhibiting multiple proinflammatory mediators in stimulated J774A.1 murine macrophages *in vitro*. This effect is mediated by the suppression of NF- κ B activation and reduced phosphorylation of ERK1/2 and p38. These data indicate that *A. asiatica* Nakai may downregulate the ERK/p38 signaling pathway, and the study provides a potential mechanism that explains the anti-inflammatory activity of *A. asiatica* Nakai.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Chua DT, Sham JS, Wei WI, Ho WK, Au GK: The predictive value of the 1997 American joint committee on cancer stage classification in determining failure patterns in nasopharyngeal carcinoma. *Cancer* 2001;92:2845–2855.
2. Chan KY, Yen LK, Chu NM, Tan TD, Tsou MH, Huang AT: Prognostic features and treatment outcome in locoregionally advanced nasopharyngeal carcinoma following concurrent chemotherapy and radiotherapy. *Int J Radiat Oncol Biol Phys* 1998;41:755–762.
3. Hoon DS, Ferris R, Tanaka R, Chong KK, Alix-Panabieres C, Pantel K: Molecular mechanisms of metastasis. *J Surg Oncol* 2011;103:508–517.
4. Westermarck J, Kahari VM: Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 1999;13:781–792.
5. Hahm KB, Kim JH, You BM, *et al.*: Induction of apoptosis with an extract of *Artemisia asiatica* attenuates the severity of cerulein-induced pancreatitis in rats. *Pancreas* 1998;17:153–157.
6. Ryu BK, Ahn BO, Oh TY, Kim SH, Kim WB, Lee EB: Studies on protective effect of DA-9601, *Artemisia asiatica* extract, on acetaminophen- and CCl₄-induced liver damage in rats. *Arch Pharm Res* 1998;21:508–513.
7. Hwang Y, Noh G, Kim S: Effect of *Inonotus obliquus* extracts on proliferation caspase-3 activity in human gastro-intestinal cancer cell lines. *Korean J Nutr* 2003;36:18–23.
8. Lim BO, Yamada K, Cho BG, *et al.*: Comparative study on the modulation of IgE and cytokine production by *Phellinus linteus* grown on germinated brown rice, *Phellinus linteus* and germinated brown rice in murine splenocytes. *Biosci Biotechnol Biochem* 2004;68:2391–2394.
9. Steinberg D, Lewis A: Conner memorial lecture. Oxidative modification of LDL and atherogenesis. *Circulation* 1997;95:1062–1071.
10. Takahashi M, Masuyama J, Ikeda U, *et al.*: Induction of monocyte chemoattractant protein-1 synthesis in human monocytes during transendothelial migration *in vitro*. *Circ Res* 1995;76:750–757.
11. Verma N, Tripathi SK, Sahu D, Das HR, Das RH: Evaluation of inhibitory activities of plant extracts on production of LPS-stimulated pro-inflammatory mediators in J774 murine macrophages. *Mol Cell Biochem* 2010;336:127–135.
12. Baeuerle PA, Baltimore D: I κ B: A specific inhibitor of the NF- κ B transcription factor. *Science* 1998;242:540–546.
13. Baldwin AS, Jr.: The NF- κ B and I κ B proteins: New discoveries and insights. *Annu Rev Immunol* 1996;14:649–683.

14. Jeong JH, Ryu DS, Suk DH, Lee DS: Anti-inflammatory effects of ethanol extract from *Orostachys japonicus* on modulation of signal pathways in LPS-stimulated RAW 264.7 cells. *BMB Rep* 2011;4:399–404.
15. Davis RJ: MAPKs: New JNK expands the group. *Trends Biochem Sci* 1994;19:470–473.
16. Han J, Lee JD, Bibbs L, Ulevitch RJ: A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 1994;265:808–811.
17. Nishida E, Gotoh Y: The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem Sci* 1993;18:128–131.
18. Carter AB, Knudtson KL, Monick MM, Hunninghake GW: The p38 mitogenactivated protein kinase is required for NF- κ B-dependent gene expression. The role of TATA-binding protein (TBP). *J Biol Chem* 1999;274:30858–30863.
19. Nakano H, Shindo M, Sakon S, *et al.*: Differential regulation of I κ B kinase α and β by two upstream kinases, NF- κ B-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc Natl Acad Sci USA* 1998;95:3537–3542.
20. Wei WI, Sham JS: Nasopharyngeal carcinoma. *Lancet* 2005;365:2041–2054.
21. Jun CD, Choi BM, Hoon R, *et al.*: Synergistic cooperation between phorbol ester and IFN- γ for induction of nitric oxide synthesis in murine peritoneal macrophages. *J Immunol* 1994;153:3684–3690.
22. Kim MS, Park SB, Suk K, *et al.*: Gallotannin isolated from *Euphorbia* species, 1,2,6-tri-O-galloyl- β -D-allose, decreases nitric oxide production through inhibition of nuclear factor- κ B and downstream inducible nitric oxide synthase expression in macrophages. *Biol Pharm Bull* 2009;32:1053–1056.
23. Choi EJ, Lee S, Chae JR, Lee HS, Jun CD, Kim SH: Eupatilin inhibits lipopolysaccharide-induced expression of inflammatory mediators in macrophages. *Life Sci* 2011;88:1121–1126.
24. Kim SH, Shin TY: *Amomum xanthiodes* inhibits mast cell-mediated allergic reactions through the inhibition of histamine release and inflammatory cytokine production. *Exp Biol Med* 2005;230:681–687.
25. Kim YD, Choi SC, Oh TY, Chun JS, Jun CD: Eupatilin inhibits T-cell activation by modulation of intracellular calcium flux and NF- κ B and NF-AT activity. *J Cell Biochem* 2009;108:225–236.
26. Gotoh T, Endo M, Oike Y: Endoplasmic reticulum stress-related inflammation and cardiovascular diseases. *Int J Inflam* 2011;259462:8.
27. Sittisart P, Chitsomboon B: Intracellular ROS scavenging activity and downregulation of inflammatory mediators in RAW264.7 macrophage by fresh leaf extracts of *Pseuderanthemum palatiferum*. *Evid Based Complement Alternat Med* 2014;309095:11.
28. Ji HY, Kim SY, Kim DK, Jeong JH, Lee HS: Effects of eupatilin and jaceosidin on cytochrome P450 enzyme activities in human liver microsomes. *Molecules* 2010;15:6466–6475.
29. Choi EJ, Oh HM, Na BR, *et al.*: Eupatilin protects gastric epithelial cells from oxidative damage and down-regulates genes responsible for the cellular oxidative stress. *Pharm Res* 2008;25:1355–1364.
30. Kim JB, Han AR, Park EY, *et al.*: Inhibition of LPS induced iNOS, COX-2 and cytokines expression by poncirin through the NF- κ B inactivation in RAW 264.7 macrophage cells. *Biol Pharm Bull* 2007;30:2345–2351.
31. Mazza J, Rossi A, Weinberg JM: Innovative uses of tumor necrosis factor alpha inhibitors. *Dermatol Clin* 2010;28:559–575.
32. Weber TJ, Smallwood HS, Kathmann LE, Markillie LM, Squier TC, Thrall BD: Functional link between TNF biosynthesis and CaM-dependent activation of inducible nitric oxide synthase in RAW 264.7 macrophages. *Am J Physiol Cell Physiol* 2006;290:C1512–C1520.
33. Molloy RG, Mannick JA, Rodrick ML: Cytokines, sepsis and immunomodulation. *Brit J Surg* 1993;80:289–297.
34. West MA, Li MH, Seatter SC, Bubrick MP: Pre-exposure to hypoxia or septic stimuli differentially regulates endotoxin release of tumor necrosis factor, interleukin-6, interleukin-1, prostaglandin E2, nitric oxide, and superoxide by macrophages. *J Trauma* 1994;3:82–89.
35. Li Q, Verma IM: NF- κ B regulation in the immune system. *Nat Rev Immunol* 2002;2:725–734.
36. Majumdar S, Aggarwal BB: Methotrexate suppresses NF- κ B activation through inhibition of I κ B α phosphorylation and degradation. *J Immunol* 2001;167:2911–2920.
37. Cavaillon JM, Haeflner-Cavaillon N: Signals involved in interleukin 1synthesis and release by lipopolysaccharide-stimulated monocytes/macrophages. *Cytokine* 1990;2:313–329.
38. Garrington TP, Johnson GL: Organization and regulation of mitogenactivated protein kinase signaling pathways. *Curr Opin Cell Biol* 1999;11:211–218.
39. Mancuso G, Midiri A, Beninati C, Teti G, *et al.*: Mitogen-activated protein kinases and NF κ B are involved in TNF- α responses to group B streptococci. *J Immunol* 2002;169:1401–1409.
40. Sha WC, Liou HC, Tuomanen EI, Baltimore D: Targeted disruption of the p50 subunit of NF- κ B leads to multifocal defects in immune responses. *Cell* 1995;80:321–330.
41. Bae IK, Min HY, Han AR, Seo EK, Lee SK: Suppression of lipopolysaccharide-induced expression of inducible nitric oxide synthase by brazilin in RAW 264.7 macrophage cells. *Eur J Pharmacol* 2005;513:237–242.
42. Reddy DB, Reddanna P: Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing NF- κ B and MAPK activation in RAW 264.7 macrophages. *Biochem Biophys Res Commun* 2009;381:112–117.
43. Guha M, Mackman N: LPS induction of gene expression in human monocytes. *Cell Signal* 2001;13:85–94.
44. Bhat NR, Zhang P, Lee JC, Hogan EL: Extracellular signal regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. *J Neurosci* 1998;18:1633–1641.
45. Ajizian SJ, English BK, Meals EA: Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J Infect Dis* 1999;179:939–944.
46. Hambleton J, Weinstein SL, Lem L, DeFranco AL: Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc Natl Acad Sci USA* 1996;93:2774–2778.
47. Seo HJ, Park KK, Han SS, *et al.*: Inhibitory effects of the standardized extract (DA-9601) of *Artemisia asiatica* Nakai on phorbol ester-induced ornithine decarboxylase activity, papilloma formation, cyclooxygenase-2 expression, inducible nitric oxide synthase expression and nuclear transcription factor- κ B activation in mouse skin. *Int J Cancer* 2002;100:456–462.