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In vitro and *in vivo* immunostimulatory effects of hot water extracts from the leaves of *Artemisia princeps* Pampanini cv. Sajabal

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ABSTRACT

Ethnopharmacological relevance: Artemisia princeps Pampanini (Asteraceae) is used as a traditional medicine to immune function-related diseases, such as dysmenorrhea, inflammation, cancer, and ulcers.
Aim of the study: The purpose of this study is to evaluate the immunostimulatory effects of the hot water extract from the leaves of *Artemisia princeps* Pampanini (WAPP) in recombinant interferon-γ (rIFN-γ)-primed RAW 264.7 macrophages and in cyclophosphamide (20 mg/kg, i.p.)-induced immunesuppressed Sprague-Dawley rats.
Materials and methods: RAW 264.7 macrophages were treated with WAPP and production and expressions of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) via nuclear factor-κB (NF-κB) were detected by immunoassay, western blot, qRT-PCR and reporter gene assay. In addition, *in vivo* immunomodulatory activity was studied by cyclophosphamide-induced myelosuppression in rats.
Results: In rIFN-γ-primed RAW 264.7 macrophages, pretreatment with WAPP increased the productions of nitric oxide (NO) and tumor necrosis factor-α (TNF-α),and increased the expressions of inducible nitric oxide synthase (iNOS) at the protein level and of iNOS and TNF-α at the mRNA level. Molecular data revealed that WAPP upregulated the transcriptional activity and translocation of nuclear factor-κB (NF-κB) by activating inhibitory kappa B-α (IκB-α) degradation and phosphorylation. Furthermore, WAPP upregulated the phosphorylations of p38 MAP kinase, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase1/2 (ERK1/2). In cycloheximide-induced immunosuppressed rats, pretreatment with WAPP (100, 200, or 400 mg/kg, p.o.) increased the serum levels of albumin and globulin, and reduced immobility times.
Conclusion: Our results suggest that upregulations of the expressions of iNOS and TNF-α via the activations of NF-κB and MAPK are responsible for the immunostimulatory effects of WAPP.
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1. Introduction

Immunostimulation is regarded an important strategy for enhancing defense mechanisms in the elderly and in cancer patients (Borchers et al., 2008). Furthermore, a substantial amount of experimental evidence suggests that various plants upregulate the immune system by stimulating natural killer (NK) cells, B cells, T cells, and macrophages-dependent immune responses (Kim et al., 2006).

Macrophages have long been regarded as important immune effector cells due to their regulations of innate and adaptive immunity in vertebrates. Their role is phagocytose, engulf, and then digest cellular debris and pathogens, either as stationary or as mobile cells. They also stimulate lymphocytes and other immune cells to respond to pathogens (Luft, 2008).When the body is stimulated by pathologic stimuli or injury, phagocytes are in the first line of macrophage response. In addition, macrophages can defend against pathogen invasion by secreting pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and

Abbreviations: ERK1/2, extracellular signal-regulated kinase1/2; iNOS, inducible nitric oxide synthase; IκB-α, inhibitory kappa B-α; JNK, c-Jun NH2-terminal kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; rIFN-γ, recombinant interferon-γ; TNF-α, tumor necrosis factor-α; WAPP, hot water extract from the leaves of *Artemisia princeps* Pampanini.

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interleukine-1 (IL-1) (Balkwill, 2009), and by releasing cytotoxic and inflammatory molecules, such as nitric oxide (NO) and reactive oxygen species (ROS) (Carini et al., 2004; Goossens et al., 1995). NO is synthesized by inducible nitric oxide synthase (iNOS) and mediates diverse functions, including vasodilatation, neurotransmission, immunoresponses, and the inhibitions of platelet aggregation and extracellular matrix production (Moncada et al., 1991). On the other hand, TNF-α is a cytokine involved inflammatory and immune responses, and has been implicated in host defense against pathogenic bacteria and parasites (Smirnova et al., 2002).

Certain proteins protect the body against various infections and ensure a healthy immune system. The two main proteins in the blood are albumin and globulin which is produced by the liver, while others are made by the immune system. Albumin is not only a major contributor to "colloidal osmotic pressure," which is responsible for fluid retention within vessels, but also an important host defense against microbial pathogens. Albumin reversibly binds a variety of diverse ligands, such as lipoteichoic acid (*Streptococcus pyogenes*), several streptococcal proteins, and component of viruses, and then interferes with host–pathogen interactions (Giles and Czuprynski, 2003). On the other hand, the globulins are the main building blocks for several substances, such as antibodies, glycoproteins, lipoproteins, clotting factors, and other components of the immune system. Thus, albumins and globulins play important immunologic roles, and several studies have reported that their serum levels are depressed by malnutrition and immune deficiency (An et al., 2010; Obatolu et al., 2003).

The genus *Artemisia* is grown worldwide and includes several well-known medicinal herbs, and one of these, *Artemisia princeps* Pampanini (Asteraceae), is a herbaceous plant that is widely used in Korean, Chinese, and Japanese traditional medicine for the treatment of various disorders including colic pain, diarrhea, vomiting, ulcers, dysmenorrhea, microbial infections, and cancer (Trinh et al., 2011; Zhao et al., 1994). Since these diseases are related to immune system dysfunction, we presumed that *Artemisia princeps* Pampanini has immunomodulatory properties. In recent scientific literature, *Artemisia princeps* Pampanini has been reported to have anti-atherosclerotic (Han et al., 2009), anti-inflammatory (Joh et al., 2010), anti-oxidative (Toda, 2005), anti-scratching behavioral (Ryu et al., 2011), anti-bacterial (Trinh et al., 2011), radical scavenging, and anti-obesity effects (Kim et al., 2010). However, no previous study has examined the effect of the hot water extract from the leaves of *Artemisia princeps* Pampanini (WAPP) on immunomodulatory functions and on the molecular mechanisms involved. We hypothesized that the immunostimulatory effects of WAPP are due to the enhancement of macrophage functions, and thus, we investigated NO and TNF-α secretions and their expressions on recombinant interferon (rIFN)-γ-primed RAW 264.7 macrophages *in vitro*, and then evaluated the effects of WAPP on a cyclophosphamide-treated immunosuppressed rat model.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). Inhibitory κB-α kinase α/β (IKKα/β), Inhibitory κB-α (IκB-α), iNOS, p65, poly [ADP-ribose] polymerase-1(PARP-1), extracellular-signal-related kinase (ERK) and β-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p-IKKα/β, p-IκB-α, c-Jun N-terminal kinase (JNK) and p38 monoclonal antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Enzyme linked immunosorbent assay (ELISA) kit for TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). Dimethyl sulfoxide (DMSO), sulfanilamide, phenylmethylsulfonylfluride (PMSF), dithiothreitol (DTT), murine recombinant IFN-γ, lipopolysaccharide (LPS), sodium bicarbonate, HEPES, sodium dodecyl sulfate (SDS), PD98059, SP600125, SB203580, cyclophosphamide and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of WAPP

The aerial parts of *Artemisia princeps* Pampanini (Asteraceae) cv. Sajabalwere provided from Ganghwa Agricultural R&D Center (Inchon, Republic of Korea) and identified by Prof. Nam-In Back (Kyung Hee University, Suwon, Korea). A voucher specimen (KHU-051020) was deposited at the herbarium of the Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University (Suwon, Korea). The aerial parts of *Artemisia princeps* Pampanini were dried (5.4 kg), cut, and extracted in 20 volumes of distilled water at 100 °C for 4 h. The extracted solutions were filtered and the filtrates were evaporated at 65 °C and freeze-dried to afford a powder (WAPP, 1000 g). WAPP was analyzed for major flavonoids, and eupatilin and jaceosidin were found to be present at concentrations of 189.4 and 104.6 μg/g, respectively, by HPLC (data not shown).

2.3. Cell culture and MTT assay

RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). Cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin sulfate at 37 °C in a 5% CO_2 atmosphere.

RAW 264.7 macrophages were seeded in 96-well plates and pretreated with rIFN-γ (100 ng/ml) and then stimulated with various concentrations of WAPP (200, 400, or 800 g/ml) or LPS (1 g/ml) for the indicated time. On the day of collection, cells were incubated with a MTT solution for 4 h at 37 °C under 5% CO_2. The MTT-containing medium was removed and the cells were solubilized in DMSO. Absorbance of each well at 540 nm was measured using an automatic microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.4. Nitrite assay

RAW 264.7 macrophages (1×10^5 cells/ml) were cultured in 24-well plates and stimulated with the WAPP. After 24 h, culture supernatants were collected and nitrite, the stable reaction product of NO with molecular oxygen, was measured using Griess reagent. Equal volumes of Griess reagent (1:1 of 0.1% *N*-1 naphthylethylenediamine in 5% phosphoric acid and 1% sulfanilamide in 5% phosphoric acid) and sample were incubated together at room temperature for 10 min. Absorbance of each well at 540 nm was measured using an automatic microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

2.5. Determination of TNF-α production

TNF-α level in cell culture media were quantified using ELISA kits, according to the manufacturer's instructions.

2.6. Western blot analysis

The cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). Washed cell pellets were resuspended in protein extraction solution PRO-PREP (Intron

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