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Anti-inflammatory activities and mechanisms of *Artemisia asiatica* ethanol extract

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ABSTRACT

Ethnopharmacological relevance: *Artemisia asiatica* Nakai (Compositae) is a representative herbal plant used to treat infection and inflammatory diseases. Although *Artemisia asiatica* is reported to have immunopharmacological activities, the mechanisms of these activities and the effectiveness of *Artemisia asiatica* preparations in use are not known.

Materials and methods: To evaluate the anti-inflammatory activities of *Artemisia asiatica* ethanol extract (Aa-EE), we assayed nitric oxide (NO), tumor necrosis factor (TNF)-α, and prostaglandin E_2 (PGE_2) in macrophages and measured the extent of tissue injury in a model of gastric ulcer induced in mice by treatment with HCl in EtOH. Putative enzymatic mediators of Aa-EE activities were identified by nuclear fractionation, reporter gene assay, immuoprecipitation, immunoblotting, and kinase assay. Active compound in Aa-EE was identified using HPLC.

Results: Treatment of RAW264.7 cells and peritoneal macrophages with Aa-EE suppressed the production of NO, PGE_2, and TNF-α in response to lipopolysaccharide (LPS) and induced heme oxygenase-1 expression. The Aa-EE also ameliorated symptoms of gastric ulcer in HCl/EtOH-treated mice. These effects were associated with the inhibition of nuclear translocation of nuclear factor (NF)-κB and activator protein (AP)-1, implying that the anti-inflammatory action of the Aa-EE occurred through transcriptional inhibition. The upstream regulatory signals Syk and Src for translocation of NF-κB and TRAF6 for AP-1 were identified as targets of this effect. Analysis of Aa-EE by HPLC revealed the presence of luteolin, known to inhibit NO and PGE_2 activity.

Conclusion: The anti-inflammatory activities attributed to *Artemisia asiatica* Nakai in traditional medicine may be mediated by luteolin through inhibition of Src/Syk/NF-κB and TRAF6/JNK/AP-1 signaling pathways.

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Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; (TNF)-α, tumor necrosis factor; ERK, extracellular signal-related kinase; TLR, toll-like receptors (TLRs); MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; JNK, c-Jun N-terminal kinase; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; PI3K, phosphoinositide 3-kinases; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; IκBα, inhibitor of kappa B alpha; Iκκ, IκB kinase; Syk, spleen tyrosine kinase; CMC, sodium carboxymethylcellulose; PEI, polyethylenimine

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1. Introduction

Activation of pro-inflammatory signaling proteins such as the toll-like receptors (TLRs) by tissue damage and infection has been studied intensively in the human and the mouse. Mutation or deletion of genes encoding these proteins may be lethal in the event of a bacterial, viral, or fungal infection. Stimulation of the TLRs in the presence of infection induces phagocytic activities in the macrophages and neutrophils, thereby supporting the immunological defense. On activation, these cells generate cytokines and chemokines such as monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α, and the reactive oxygen species (ROS), prostaglandin (PG)E_2, and nitric oxide (NO) to sustain and amplify the response (Toltl et al., 2008). Underlying inflammation and immunity, is a network of transcriptional and translational regulation (Sohn et al., 2007). Transcriptional mediators include nuclear factor kappa beta (NF-κB) and activator protein 1 (AP-1), downstream from protein tyrosine kinases such as Syk and Src, serine/threonine kinases such as Akt, IKK, and TBK1, and mitogen-activated protein kinases [MAPK: ERK (extracellular signal-related kinase), p38, and JNK (c-Jun N-terminal kinase)] (Byeon et al., 2012; Yu et al., 2012b). Inappropriate expression of these mechanisms may contribute to a cycle of cell injury, death and regeneration, thereby setting the stage for cancer, diabetes, atherosclerosis, and arthritis (McGeer and McGeer, 2008; Bae, 2012; Kim and Joh, 2012). Therefore, the control of abnormal inflammation presents a potentially important strategy for prevention of degenerative disease (Massarotti, 2008; Murakami et al., 2013). Traditional herbal remedies present a resource for anti-inflammatory drug development, pending analysis and testing (Lukhoba et al., 2006; Wang et al., 2013).

Various preparations from *Artemisia asiatica* Nakai have long been used to treat bacterial infection, inflammation, and cancer in Korea, China, and Japan (Huh et al., 2003; Song et al., 2008). The protective effects of these preparations in the liver and gastric mucosa, and their anti-allergic, and anti-inflammatory activities have been confirmed (Ryu et al., 1998; Choi et al., 2011b), and based on pharmacological effects in gastric ulcer, an ethanol extract of *Artemisia asiatica*, DA-9601 (Stillen™), is now used clinically to treat gastric mucosal ulcers and inflammation in South Korea. Continuous research has explored that the gastroprotective activity of *Artemisia asiatica* ethanol extract was due to diminishing lipid peroxidation, suppressing cytochrome 2E1 ethanol-metabolizing enzyme activity, increasing cellular level of depleted glutathione, downregulating the expression of interleukin-1β and interferon-γ, and suppressing the overexpression of intercellular adhesion molecule-1 (Park et al., 2008). Phytochemical analysis characterizes active components in *Artemisia asiatica* as flavonoids, sesquiterpenoids, and essential oils such eupatilin, artemisolide, 1,8-cineole, and terpinen-4-ol (Kalemba et al., 2002; Kim et al., 2004; Reddy et al., 2006).

Previous studies describe the anti-inflammatory and anti-gastritis activities of *Artemisia asiatica* and confirm the suppressive effects on the production of inflammatory mediators such as PGE_2 and NO (Oh et al., 2005; Reddy et al., 2006; Park et al., 2008); however, molecular mechanisms for the effects in terms of direct target enzymes are not determined. In this study, we investigated the molecular targets of bioactive component(s) in an ethanol extract of *Artemisia asiatica* (At-EE), using cultured cells and a mouse model of gastric mucosal inflammation. Using HPLC analysis we identified luteolin as one of bioactive components of the extract showing the same activities.

2. Materials and methods

2.1. Materials

A 95% ethanol extract (Code no.: CA02-070) of *Artemisia asiatica* was purchased from the Plant Extract Bank of the Plant Diversity Research Center (⟨http://extract.pdrc.re.kr/extract/f.htm⟩, Daejeon, Korea). Luteolin, (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, tetrazole (MTT), indomethacine, N^G-nitro-L-arginine methyl ester (L-NAME), and lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). U0126, SP600125, PP2, and piceatannol were obtained from Calbiochem (La Jolla, CA, USA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a BALB/c-derived murine macrophage cell line (ATCC No. TIB-71), and HEK293 cells, a human embryonic kidney cell line (ATCC No. CRL-1573), were purchased from ATCC (Rockville, MD, USA). All other chemicals were purchased from Sigma. The phospho-specific and total antibodies to p65, c-Fos, c-Jun, HO-1, IκBα, Src, Syk, p85/PI3K, ERK, JNK, p38, MKK4/7, TAK1, IRAK1, IRAK4, TRAF6, lamin A/C, and β-actin were purchased from Cell Signaling (Beverly, MA, USA) or Santa Cruz Biotech (Santa Cruz, CA, USA).

2.2. Animal experiments

Male ICR and C57BL/6 mice (6–8 weeks old, 17–21 g) were obtained from Daehan Biolink (Chungbuk, Korea) and maintained in plastic cages under standard conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied *ad libitum*. Studies were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee at Kangwon National University (Chuncheon, Korea).

2.3. Preparation of peritoneal macrophages

Peritoneal exudates were obtained from C57BL/6 male mice by lavage 4 days after the intraperitoneal injection of 1 ml of sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI, USA). After washing with RPMI 1640 medium containing 2% FBS, peritoneal macrophages (1×10^6 cells/ml) were plated in 100-mm tissue culture dishes for 4 h at 37 °C in a 5% CO_2 humidified atmosphere.

2.4. Cell culture

Primary macrophages, and RAW264.7, and HEK293 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin) at 37 °C under 5% CO_2. For each experiment, the cells were detached with a cell scraper. At the experimental cell density applied here (2×10^6 cells/ml), the proportion of dead cells was less than 1% based on Trypan blue dye exclusion.

Table 1
Real-time PCR primers used in this study.

Gene		Primer sequences	Fragment size (bp)
TNF-α	F	5′-TTGACCTCAGCGCTGAGTTG-3′	364
	R	5′-CCTGTAGCCCACGTCGTAGC-3′	
iNOS	F	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′	496
	R	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′	
COX-2	F	5′-CACTACATCCTGACCCACTT-3′	696
	R	5′-ATGCTCCTGCTTGAGTATGT-3′	
β-Actin	F	5′-GTGGGCCGCCCTAGGCACCAG-3′	603
	R	5′-GGAGGAAGAGGATGCGGCAGT-3′	

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