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Anti-inflammatory activity of ethanol extract derived from *Phaseolus angularis* beans

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ABSTRACT

Ethnopharmacological significance: Phaseolus angularis Wight (adzuki bean) is an ethnopharmacologically well-known folk medicine that is prescribed for infection, edema, and inflammation of the joints, appendix, kidney and bladder in Korea, China and Japan.
Aim of study: The anti-inflammatory effect of this plant and its associated molecular mechanisms will be investigated.
Materials and methods: The immunomodulatory activity of *Phaseolus angularis* ethanol extract (Pa-EE) in toll like receptor (TLR)-activated macrophages induced by ligands such as lipopolysaccharide (LPS), Poly (I:C), and pam3CSK was investigated by assessing nitric oxide (NO) and prostaglandin (PG)E_2 levels. To identify which transcription factors such as nuclear factor (NF)-κB and their signaling enzymes can be targeted to Pa-EE, biochemical approaches including reporter gene assays, immunoprecipitation, kinase assays, and immunoblot analyses were also employed. Finally, whether Pa-EE was orally available, ethanol (EtOH)/hydrochloric acid (HCl)-induced gastritis model in mice was used.
Results: Pa-EE dose-dependently suppressed the release of PGE_2 and NO in LPS-, Poly(I:C)-, and pam3CSK-activated macrophages. Pa-EE strongly down-regulated LPS-induced mRNA expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2. Interestingly, Pa-EE markedly inhibited NF-κB, activator protein (AP)-1, and cAMP response element binding protein (CREB) activation; further, according to direct kinase assays and immunoblot analyses, Pa-EE blocked the activation of the upstream signaling molecules spleen tyrosine kinase (Syk), p38, and transforming growth factor β–activated kinase 1 (TAK1). Finally, orally administered Pa-EE clearly ameliorated EtOH/HCl-induced gastritis in mice.
Conclusion: Our results suggest that Pa-EE can be further developed as a promising anti-inflammatory remedy because it targets multiple inflammatory signaling enzymes and transcription factors.

Abbreviations: Pa-EE, ethanol extract of *Phaseolus angularis*; LPS, lipopolysaccharide; NO, nitric oxide; PG, prostaglandin; iNOS, inducible NO synthase; COX, cyclooxygenase; IL, interleukin; IKK, I (B) kinase; Akt, protein kinase B; PDK, phosphoinositide-dependent kinase 1; PI3K, p85/phosphoinositide-3-kinase; TLR, toll-like receptor; MYD88, myeloid differentiation primary response gene (88); NF-κB, nuclear factor-B; AP-1, activator protein-1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; JNK, c-jun N-terminal kinase; PTP, protein tyrosine phosphatase; PMA, phorbal-12-myristate-13-acetate; ELISA, enzyme linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide a tetrazole; RT-PCR, reverse transcriptase polymerase chain reaction; Syk, spleen tyrosine kinase.

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1. Introduction

Acute or chronic inflammatory responses play important pathological roles in the generation of serious diseases such as cancer, diabetes, atherosclerosis, and arthritis (Aggarwal et al., 2006). In particular, the pathophysiological roles of macrophages have been highlighted; macrophage overproduction of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, and inflammatory molecules, such as nitric oxide (NO) and prostaglandins (PG)s, is critical to the onset of inflammation (Allam and Anders, 2008). Inflammatory macrophages are generally induced by bacterial or viral products interacting with pattern recognition receptors and their adaptor molecules. For Toll-like receptors, this most commonly involves TRIF and MyD88. In turn, these molecules activate protein kinases, such as Src, spleen tyrosine kinase (Syk), phosphoinositide 3-kinase (PI3K), and Akt (protein kinase B), as well as mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. After the completion of the signaling cascades, inflammation-regulatory transcription factors, such as nuclear factor (NF)-κB and activator protein (AP)-1, are activated (O'Neill, 2003; O'Neill et al., 2003). Targeting specific events or steps in the inflammatory process is important for the development of anti-inflammatory drugs and the treatment of inflammation-mediated diseases (Garcia-Lafuente et al., 2009).

Like other beans such as mung bean, black bean, and rice bean, adzuki bean (*Phaseolus angularis* Wight) is a well-known food and folk medicine that is ethnopharmacologically prescribed in Korea, China, Taiwan, and Japan. Two earlist literatures "Shennong Bencao Jing" and "Beiji Qianjin Yao Fang" in China have mentioned the ethnopharmacological uses of this plant and its seeds. Thus, "Shennong Bencao Jing" reported that Adzuki bean can relax the bowels, promote urination, evacuate pus, cure the beriberi, dispel the effects of alcohol, and function lactogenesis. "Beiji Qianjin Yao Fang" written at A.D.652 by sun simiao also described that Adzuki bean can cure edema and beriberi of pregnant woman. In addition, this plant is used to control infections or inflammation of the kidney or bladder and to improve reproductive functions (Hori et al., 2006). This plant is also incorporated as part of an anti-inflammation diet to prevent and reduce risk factors for heart disease (Sato et al., 2008). Water, methanolic or ethanolic extracts of this plant have been demonstrated to up-regulate inducible NO synthase (iNOS) and endothelial NOS activities and to reduce blood pressure (Mukai and Sato, 2009); decrease serum cholesterol levels (Itoh and Furuichi, 2009); ameliorate diabetes progression (Itoh et al., 2009); stimulate melanogenesis and pigmentation (Itoh and Furuichi, 2005); inhibit tumor cell adhesion, invasion, and metastasis (Itoh et al., 2005); and induce hepatoprotection against acetaminophen-induced liver damage (Han et al., 2004).

Thus far, only one study has shown that *Phaseolus angularis* suppressed vascular oxidative stress and inflammation; in spontaneously hypertensive rats, polyphenol-containing azuki bean-treated rats showed reduced levels of p47phox, iNOS, and COX-2 in the aorta compared to control animals (Mukai and Sato, 2011). Thus, the anti-inflammatory effects of *Phaseolus angularis* on macrophages and its inhibitory mechanisms remain to be elucidated. To address these issues, we investigated the immunomodulatory activity of *Phaseolus angularis* ethanol extract (Pa-EE) on activated macrophages induced by TLR ligands such as LPS (a TLR4 ligand), Poly (I:C) (a TLR3 ligand), and pam3CSK (a TLR2 ligand); in addition to NO and PGE_2 assays, biochemical studies including reporter gene assays, immunoprecipitation, kinase assays, and immunoblot analyses were utilized.

2. Materials and methods

2.1. Materials

The 95% ethanol extract (Code No.: CA04-038) from seeds (whole bean) of *Phaseolus angularis* Wight (Fabaceae) was purchased from the Plant Extract Bank of the Plant Diversity Research Center (Daejeon, Korea). LPS, quercetin, tumor necrosis factor (TNF)-α, sodioum carboxymethylcellulose (CMC), and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). SB203580 and piceatannol were purchased from Calbiochem (La Jolla, CA). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals were of reagent grade. Anti-phospho or total antibodies against Src, Syk, ERK, p38, JNK, TAK1, MKK3/6, p65 (NF-κB), TLR4, MyD88, Akt, phosphoinositide-dependent kinase 1 (PDK1), p85/PI3K, inhibitors of κBα (IκBα), IκBα kinase (IKK), c-Jun, c-fos, β-actin, and histone H4 were obtained from Cell Signaling (Beverly, MA).

2.2. Cell culture

RAW 264.7 and HEK293 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), glutamine and antibiotics (penicillin and streptomycin) at 37 °C with 5% CO_2. For each experiment, cells were detached with a cell scraper. Experiments were performed at a cell density of 2×10^6 cells/ml; at this density, more than 99% of cells were viable according to Trypan blue staining.

2.3. Mice

Six-week old, male ICR mice (6–8 weeks old, 17–21 g) were obtained from Daehan Biolink (Osung, Korea) and maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied ad libitum. Studies were performed in accordance with guidelines established by the Kangwon University Institutional Animal Care and Use Committee.

2.4. Treatment of Pa-EE

The stock solution (100 mg/ml) of Pa-EE was dissolved in 100% DMSO. Based on previous papers, non-cytotoxic concentrations (0–200 μg/ml) of Pa-EE were prepared by dilution with RPMI1640 medium for *in vitro* conditions. For gastritis experiments, 100–200 mg/kg prepared with 1% CMC solution were chosen to be orally administered.

2.5. NO, PGE_2 and TNF-α production

After RAW264.7 cells or peritoneal macrophages (1×10^6 cells/ml) were incubated for 18 h, cells were pretreated with Pa-EE (0–200 μg/ml) for 30 min. Next, cells were stimulated with LPS, Poly (I:C), and pam3CSK, and incubated for 24 h. The inhibitory effect of Pa-EE on NO, PGE_2, and TNF-α production was determined by analyzing NO, PGE_2, and TNF-α levels with the Griess reagent, enzyme immunoassay (EIA), and enzyme linked immunosorbent assay (ELISA) kits, as described previously (Cho et al., 2000; Kang et al., 2011).

2.6. MTT assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Shen et al., 2011).