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Alisma canaliculatum ethanol extract suppresses inflammatory responses in LPS-stimulated macrophages, HCl/EtOH-induced gastritis, and DSS-triggered colitis by targeting Src/Syk and TAK1 activities

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Ethnopharmacological relevance: *Alisma canaliculatum* A.Braun & C.D.Bouché, distributed in Korea, Japan, China, and Taiwan, is a traditional medicine. In particular, the stem and root of *Alisma canaliculatum* A.Braun & C.D.Bouché are prescribed to relieve various inflammatory symptoms resulting from nephritis, cystitis, urethritis, and dropsy.

Aim of study: However, the curative mechanism of *Alisma canaliculatum* A.Braun & C.D.Bouché with respect to inflammatory symptoms is poorly understood. In this study, the curative roles of this plant in various inflammatory conditions as well as its inhibitory mechanism were aimed to examine using an ethanol extract (Ac-EE).

Materials and methods: Anti-inflammatory effects of Ac-EE were evaluated in lipopolysaccharide (LPS)-induced macrophages in vitro and HCl/EtOH-stimulated mouse model of gastritis and DSS-treated mouse model of colitis. To determine the potentially active anti-inflammatory components in this extracts, we employed HPLC. We also used kinase assays, reporter gene assay, immunoprecipitation analysis and target enzyme overexpressing cell analysis to analyze the molecular mechanisms and the target molecules.

Results: This extract dose-dependently inhibited the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) from RAW264.7 cells and peritoneal macrophages activated by lipopolysaccharide (LPS). Additionally, Ac-EE ameliorated inflammatory symptoms resulting from gastritis and colitis. Ac-EE down-regulated the mRNA levels of inducible NO synthase (iNOS), tumor necrosis factor (TNF)-α, and cyclooxygenase-2 (COX-2). Ac-EE also blocked the nuclear translocation of nuclear factor (NF)-κB and activator protein (AP) − 1 in LPS-stimulated RAW264.7 cells. By analyzing the target signaling molecules activating these transcription factors, we found that Src and Syk, as well as molecular association between TAK1 and mitogen-activated protein kinase kinase 4/7 (MKK4/7), were targeted by Ac-EE.

Conclusions: Our data suggest that the Ac-EE NF-κB/AP-1-targeted anti-inflammatory potential is mediated by suppression of Src and Syk as well as the complex formation between TAK1 and its substrate proteins MKK4/7.

1. Introduction

Inflammation is a complicated biological defense system used to protect our body against various infectious agents, such as viruses, fungi, bacteria, and other microorganisms (Hossen et al., 2015). While inflammation is a defense mechanism against various dangers, uncontrolled inflammation can lead to serious diseases such as rheumatoid arthritis, sepsis, diabetes, and cancer (Chen et al., 2016a; Dung et al., 2016; Kaur et al., 2013). Many studies have focused on understanding inflammation and developing new drugs to regulate the inflammatory response (Crawford, 2014).

Although various types of immune cells are involved in inflammatory responses, macrophages play critical roles in the inflammatory process. Macrophages are activated by interactions between pathogen-associated molecular patterns (PAMPs) and several pattern recognition receptors (PRRs), including toll-like receptors (TLRs), c-type lectin receptors (CLRs), and mannose receptors (MRs) (Mahla et al., 2013; Yoon et al., 2015). Following the recognition of PAMPs, an inflammatory signaling pathway is initiated by adapter molecules such as myeloid differentiation factor 88 (MyD88) and toll-receptor associated activator of interferon (TRIF). Activation of MyD88 and/or TRIF induces downstream signaling molecules, such as Src,

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spleen tyrosine kinase (Syk), transforming growth factor beta-activated kinase 1 (TAK1), and serine/threonine kinases (AKT and IKK); and mitogen-activated protein kinase kinases (MAPKKs), such as MKK3/6, MKK4/7, and extracellular signal regulated kinase (ERK) kinases (MEK) 1/2, as well as their substrate proteins ERK, c-Jun N-terminal kinase (JNK), and p38. These signaling molecules are known to regulate transcription factors such as nuclear factor (NF)-κB, cAMP response element binding protein (CREB), and activator protein (AP) − 1 (Akira et al., 2004; Arthur et al., 2013; Beutler, 2004; Yang et al., 2015c). Activation of transcription factors allows their translocation into the nucleus, where they play important roles in the expression of inflammatory genes [e.g., inducible nitric oxide synthase (iNOS), cyclooxygenase (COX) − 2, and prostaglandin E_2 (PGE_2)] (Byeon et al., 2012; Yu et al., 2012). Since each of the intracellular protein molecules is known to play critical roles in inflammatory signaling cascades, it is well accepted that these proteins can be targeted to develop new anti-inflammatory drugs or remedies. Even though many drugs, such as indomethacin, aspirin, and ibuprofen, are prescribed to treat various inflammatory diseases, many side effects, including hepatotoxicity and gastric trouble, are reported (Blumenthal et al., 2018; Varga et al., 2017). Therefore, the development of safer and stronger anti-inflammatory drugs that do not produce such side effects is essential.

Alisma canaliculatum A.Braun & C.D.Bouché, commonly known as channeled water plantain, is a species of plant in the Alismataceae family (Blumenthal et al., 2017). The roots and tubers of *Alisma canaliculatum* A.Braun & C.D.Bouché have traditionally been used in Korea as a medicinal herb in the forms of infusions to cure liver, kidney, and stomach inflammatory diseases, according to literature on Korean traditional medicine, Dongeui Bogam (Treasured Mirror of Eastern Medicine), written by Heo, Jun (Heo, 1613). This plant was also used to reduce diarrhea and acute intestinal infection (Hossain et al., 2012; Jung, 1994). It was also recently demonstrated that this plant is able to suppress hepatitis symptoms induced by CCl_4 in mice (Lee et al., 2016). Moreover, macrophage-derived osteoclast differentiation was found to be suppressed by this plant through inhibition of NFATc1 (Kim et al., 2015b). Although *Alisma canaliculatum* A.Braun & C.D.Bouché has traditionally been used to treat different inflammatory diseases, previous studies on inflammatory responses and inhibitory mechanism have not yet provided a full understanding of anti-inflammatory activity of this plant and its action mechanism. In this study, we aimed to demonstrate the therapeutic activity of *Alisma canaliculatum* A.Braun & C.D.Bouché and its molecular mechanism using an ethanol extract of *Alisma canaliculatum* A.Braun & C.D.Bouché and by employing in vitro models [lipopolysaccharide (LPS)-stimulated macrophages (RAW264.7 cells and peritoneal macrophages)] and in vivo models [HCl/ethanol (EtOH)-induced gastritis model and 3% dextran sulfate sodium (DSS)-induced colitis] as well as through the use of molecular analysis approaches (immunoblotting and immunoprecipitation analyses, luciferase reporter gene assay, overexpression strategy, and kinase assay).

2. Materials and methods

2.1. Materials and reagents

A 70% ethanol extract of *Alisma canaliculatum* A.Braun & C.D.Bouché (Ac-EE) was purchased from the Plant Extract Bank in the Plant Diversity Research Center (Daejeon, Korea; http://extract.kribb.re.kr, e-mail: mplantext@kribb.re.kr). RAW264.7 cells, a BALB/c-derived murine macrophage cell line (No. TIB-71), and HEK293 cells, a human embryonic kidney cell line (No. CRL-1573) were acquired from ATCC (Rockville, MD, USA). Roswell Park Memorial Institute 1640 (RPMI1640) and Dulbecco's Modified Eagle's medium (DMEM) cell culture media, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from HyClone (Logan, UT, USA). Polyethylenimine (PEI), forskolin, phorbol 12-myristate 13-acetate (PMA), N-(1-Naphthyl)ethylene-diamine dihydrochloride (NEDHC), ranitidine, sulfanilamide, lipopolysaccharide (LPS, Escherichia coli 0111:B4), L-N^G-nitroarginine methyl ester (L-NAME), indomethacin, and 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co.(St. Louis, MO, USA). The enzyme immune assay (EIA) kit for detecting the expression of PGE_2 level was obtained from Amersham (Little Chalfont, Buckinghamshire, UK). NF-κB and AP-1 luciferase constructs were obtained from Prof. Hae Young Chung (Busan National University, Busan, Korea). Antibodies against the phospho-specific or total forms of p65, p50, c-Fos, c-Jun, Lamin A/C, IκBα, IKKα/β, AKT, Src, Syk, PI3K, JNK, p38, ERK, MKK4/7, MKK3/6, MEK1/2, TAK1, HA, Myc, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). TRI reagent was acquired from Molecular Research Center Inc. (Cincinnati, OH, USA). Hot start PCR primers specific for iNOS, COX-2, TNF-α, and GAPDH were attained from Macrogen (Seoul, Korea).

2.2. Cell culture

RAW264.7 cells were maintained in RPMI 1640 medium, and HEK293 cells were culture in DMEM medium supplemented with 10% heat-inactivated FBS and antibiotics (penicillin and streptomycin) at 37 ℃ in a humid 5% CO_2 incubator. The cells were maintained by passaging every two days. For experiments, we detached the cells using a cell scraper for RAW264.7 cells and trypsin for HEK293 cells.

2.3. Treatment of Ac-EE

A stock solution (100 mg/ml) of Ac-EE was prepared in 100% dimethyl sulfoxide (DMSO) and diluted in 0–400 μg/ml. Ac-EE was diluted with medium for the in vitro assay and with 1% sodium carboxymethylcellulose for the in vivo experiments.

2.4. Animals

Male ICR mice (5 weeks old, 15–20 g) were purchased from DAEHAN BIOLINK (Chungbuk, Korea) and cared for in plastic cages housing 4–5 mice under a 12 h light/dark cycle (lights on at 8 A.M.). Water and a pelleted diet (Samyang, Daejeon, Korea) were supplied ad libitum. Animals were maintained in accordance with guidelines issued by the Institutional Animal Care and Use Committee at Sungkyunkwan University. All animal experiments were carried out by guidelines of the National Institute of Health for the Care and Use of Laboratory Animals (NIH Publication 80–23, revised in 1996) and with approval of the Institutional Animal Care and Use Committee at Sungkyunkwan University (Suwon, Korea; approval ID: SKKUBBI 12–6-1).

2.5. NO and PGE_2 production

After RAW264.7 cells and peritoneal macrophages (1×10^6 cells/ml) were pre-incubated for 18 h, Ac-EE (0–400 μg/ml) was added to cells for 30 min. Following the incubation, LPS (1 μg/ml) was added for 24 h, as reported previously (Jeong et al., 2014). The inhibitory effects of Ac-EE on NO and PGE_2 were evaluated by analyzing NO level using Griess reagent (Misko et al., 1993), and the PGE_2 level was evaluated using an enzyme immunoassay (EIA) kit (Nelson et al., 1992).

2.6. Cell viability test

After preincubation for 18 h, Ac-EE (0–400 μg/ml) was added to RAW264.7 cells and peritoneal macrophages (1×10^6 cells/ml), and cells were incubated for 24 h. The cytotoxic effects of Ac-EE were determined using an MTT assay, as previously described (Yoon et al., 2013).