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In vitro and *in vivo* anti-inflammatory activity of *Phyllanthus acidus* methanolic extract

Muhammad Jahangir Hossen [a,b,1], Sung Ho Jeon [c,1], Seung Cheol Kim [d,1], Ji Hye Kim [a], Deok Jeong [a], Nak Yoon Sung [a], Sungjae Yang [a], Kwang-Soo Baek [a], Jun Ho Kim [a], Deok Hyo Yoon [e], Won O. Song [f], Kee Dong Yoon [g], Sang-Ho Cho [a], Sukchan Lee [a], Jong-Hoon Kim [h,**], Jae Youl Cho [a,*]

[a] *Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[b] *Department of Animal Science, Patuakhali Science and Technology University, Bangladesh*
[c] *Department of Life Science Hallym University, Chuncheon 200-702, Republic of Korea*
[d] *Division of Gynecologic Oncology Department of Obstetrics and Gynecology, Ewha Womans University Mokdong Hospital College of Medicine, Ewha Womans University, Seoul 158-710, Republic of Korea*
[e] *Department of Biochemistry, Kangwon National University, Chuncheon 220-700, Republic of Korea*
[f] *Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824, USA*
[g] *College of Pharmacy, The Catholic University of Korea, Bucheon 420-743, Republic of Korea*
[h] *Department of Veterinary Physiology, College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, Jeonju 561-756, Republic of Korea*

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ABSTRACT

Ethnopharmacological relevance: Phyllanthus acidus (L.) Skeels (Phyllanthaceae) has traditionally been used to treat gastric trouble, rheumatism, bronchitis, asthma, respiratory disorders, and hepatitis. Despite this widespread use, the pharmacological activities of this plant and their molecular mechanisms are poorly understood. Therefore, we evaluated the immunopharmacological activities of the methanolic extract of the aerial parts of this plant (Pa-ME) and validated its pharmacological targets.

Materials and methods: Lipopolysaccharide (LPS)-treated macrophages, an HCl/EtOH-induced gastritis model, and an acetic acid-injected capillary permeability mouse model were employed to evaluate the anti-inflammatory activity of Pa-ME. Potentially active anti-inflammatory components of this extract were identified by HPLC. The molecular mechanisms of the anti-inflammatory activity were studied by kinase assays, reporter gene assays, immunoprecipitation analysis, and overexpression of target enzymes.

Results: Pa-ME suppressed the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) and prevented morphological changes in LPS-treated RAW264.7 cells. Moreover, both HCl/EtOH-induced gastric damage and acetic acid-triggered vascular permeability were restored by orally administered Pa-ME. Furthermore, this extract downregulated the expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 and reduced the nuclear levels of NF-κB. Signalling events upstream of NF-κB translocation, such as phosphorylation of Src and Syk and formation of Src/Syk signalling complexes, were also inhibited by Pa-ME. The enzymatic activities of Src and Syk were also suppressed by Pa-ME. Moreover, Src-induced and Syk-induced luciferase activity and p85/Akt phosphorylation were also inhibited by Pa-ME. Of the identified flavonoids, kaempferol and quercetin were revealed as partially active anti-inflammatory components in Pa-ME.

Conclusion: Pa-ME exerts anti-inflammatory activity *in vitro* and *in vivo* by suppressing Src, Syk, and their downstream transcription factor, NF-κB.

Abbreviations: Pa-ME, methanolic extract of *Phyllanthus acidus*; HPLC, high performance liquid chromatography; PGE_2, prostaglandin E_2; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; TLR, Toll-like receptor (TLR); NF-κB, nuclear factor-κB; Akt, protein kinase B; IKK, IκBα kinase; MyD88, myeloid differentiation primary response protein-88; Syk, spleen tyrosine kinase; EIA, enzyme immunoassay; MTT, (3–4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction

* Corresponding author. Tel.: +82 31 290 7868; fax: +82 31 290 7870.
** Corresponding author. Tel.: +82 63 270 2563; fax: +82 63 270 3780.
E-mail addresses: jhkim1@chonbuk.ac.kr (J.-H. Kim), jaecho@skku.edu (J.Y. Cho).
[1] These authors contributed equally to this work.

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1. Introduction

Since prolonged inflammation is known to cause serious diseases such as cancer, diabetes, and atherosclerosis (Lyman et al., 2014; Momi et al., 2012), studies on inflammation have focused on the systemic, cellular, and molecular mechanisms of inflammatory responses. As an important barrier in the innate immune system, inflammation is a normal mechanism. However, the products of inflammation such as reactive oxygen/nitrogen species [e.g. nitric oxide (NO)], hydrolytic enzymes, inflammatory mediators [e.g. prostaglandin E_2 (PGE_2)], and necrotic cytokines such as tumour necrosis factor (TNF)-α can cause damage to normal tissues and organs (Rosetti et al., 2012), thereby contributing to human disease. Moreover, various proinflammatory cytokines and chemokines produced from macrophages and neutrophils stimulate the chemotactic movement of other immune cells into inflamed sites, resulting in continuous damage of the inflammatory organs (Zhang et al., 2014). Recent trials of anti-inflammatory drugs and remedies have concentrated on blocking the production of inflammatory gene products by suppressing the transcriptional activation of inflammatory genes (Ayele et al., 2013; Kim et al., 2013). For instance, targeting the signalling cascade that activates nuclear factor (NF)-κB and activator protein (AP)-1 has been shown to effectively inhibit the release of NO, PGE_2, TNF-α, interleukin (IL)-1, MCP-1, and certain hydrolytic enzymes (Eo et al., 2013; Hyun et al., 2013). Much research has focused on identifying candidate materials that are safe and that can prevent inflammation-based diseases by inhibiting upstream signalling events involved in inflammatory gene expression.

Phyllanthus acidus (L.) Skeels, which belongs to the family Phyllanthaceae, is also known as the Otaheite gooseberry, Malay gooseberry, Tahitian gooseberry, country gooseberry, star gooseberry, West India gooseberry, damsel, grosella (in Puerto Rico), jimbilin (in Jamaica), karamay (in the Northern Philippines), or simply the gooseberry tree. The berries of this tree are often used by ethnic peoples of northeast India in folk medicine, and are also utilised in Asia, the Caribbean region, and Central and South America (Andel et al., 2007; Anjaria et al., 2002). Traditionally, *P. acidus* has been used in the treatment of several ailments including inflammatory and oxidative stress-related disorders such as gastric trouble (Jules and Paull, 2008), rheumatism, bronchitis (Chakraborty et al., 2012), respiratory disorders such as asthma (Sousa et al., 2007), hepatic disease (Jain et al., 2011; Lv et al., 2014; Srirama et al., 2012), diabetes (Banik et al., 2010), and gonorrhoea (Jagessar et al., 2008). This plant has also been reported to improve eyesight and memory; to cure cough, psoriasis, and skin disorders; and to be sudorific (Devi and Paul, 2011). The leaves and roots of this plant are also used as antidotes to viper venom (Anjaria et al., 2002), to treat fever (Hadi and Bremner, 2001), and to ameliorate hypertension (Leeya et al., 2010). The methanolic extract of its fruit has also been reported to have antimicrobial activity (Melendez and Capriles, 2006), as well as hepatoprotective activity (Lee et al., 2006). Sousa et al. (2007) also reported that the leaves of *P. acidus* contained adenosine, kaempferol, and hypogallic acid; moreover, these substances stimulated airway chloride secretion, which could be a potential treatment for cystic fibrosis (Sousa et al., 2007).

Despite this substantial body of work, the anti-inflammatory actions of this plant remain poorly characterised on the cellular and molecular levels. Therefore, in the present study, we explored the anti-inflammatory role of the methanolic extract (Pa-ME) of this plant through both *in vitro* [macrophage-like (RAW264.7) cells] and *in vivo* [HCl/EtOH-induced acute gastritis mouse model] approaches. To validate the target proteins upon which components of Pa-ME act, we employed a kinase assay, an overexpression strategy, immunoprecipitation analysis, and a luciferase reporter gene assay. To identify the active components of Pa-ME, HPLC analysis was also carried out using several anti-inflammatory standard compounds.

2. Materials and methods

2.1. Materials

The 95% methanol extract (code no: PBVN 11214) of the leaves of *P. acidus* (Pa-ME) was purchased from the Plant Extract Bank in the Plant Diversity Research Centre (http://extract.kribb.re.kr/extract/f.htm, E-mail: plantext@kribb.re.kr, Daejeon, Korea). Caffeic acid, kaempferol (KF), luteolin, quercetin (QC), (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), pam3CSK, fibronectin (FN), Evans blue, phorbol-12-myristate-13-acetate (PMA), an annexin V-FITC apoptosis detection kit, and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PP2 and piceatannol (Picea) were obtained from Calbiochem (La Jolla, CA, USA). Foetal bovine serum (FBS) and RPMI 1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a BALB/c-derived murine macrophage cell line (TIB-71); U937, a human promonocytic cell line (CRL-1593.2); and HEK293 cells, a human embryonic kidney cell line (CRL-1573) were purchased from ATCC (Rockville, MD, USA). Luciferase constructs containing binding sites for NF-κB and epitope-tagged signalling expression constructs (FLAG-MyD88, HA-Src, and Myc-Syk) were used as previously reported (Byeon et al., 2013). All other chemicals were purchased from Sigma. Phospho-specific and total antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

RAW264.7 and U937 cells were cultured in RPMI 1640; HEK293 cells were cultured in DMEM. All media were supplemented with 10% heat-inactivated FBS, glutamine, and antibiotics (penicillin and streptomycin). Cells were maintained at 37 °C under 5% CO_2.

2.3. Drug treatment

For *in vitro* experiments, a stock solution of Pa-ME (100 mg/ml) dissolved in 100% DMSO (1.10 g/ml) was further diluted with culture medium to prepare below 300 μg/ml (0.3% as final DMSO amount). For *in vivo* treatment, Pa-ME (50, 100, and 200 mg/kg) was resuspended in 1% Na CMC, as previously reported (Yang et al., 2012).

2.4. NO and PGE_2 production

RAW264.7 macrophage cells (1×10^6 cells/ml) were cultured for 18 h, pretreated with Pa-ME (0 to 300 μg/ml) for 30 min, and further incubated with LPS (1 μg/ml) for 24 h. The effect of Pa-ME on NO and PGE_2 production was determined by the Griess assay and enzyme immunoassays (EIAs), respectively, as previously described (Green et al., 1982; Ryoo et al., 2013).

2.5. Morphological examination

Pa-ME-treated RAW264.7 cells, cultured in either the presence or absence of LPS, were incubated for 6 h. Images of the cultured cells at the designated time points were obtained using an inverted phase contrast microscope equipped with a video camera. Images were captured using NIH imaging software as reported previously (Kim et al., 2013; Kim and Cho, 2013). A cell counter was used to determine the numbers of morphologically changed cells in each condition.