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Methanol extract of *Hopea odorata* suppresses inflammatory responses via the direct inhibition of multiple kinases

Yanyan Yang [a,1], Tao Yu [a,1], Yong Gyu Lee [b,1], Woo Seok Yang [a], Jueun Oh [a], Deok Jeong [a], Sukchan Lee [a], Tae Woong Kim [c], Yung Chul Park [d], Gi-Ho Sung [e], Jae Youl Cho [a,*]

[a] Department of Genetic Engineering, Sungkyunkwan University, Suwon 440–746, Republic of Korea
[b] College of Biomedical Sciences, Kangwon National University, Chuncheon 200–701, Republic of Korea
[c] Department of Biochemistry, Kangwon National University, Chuncheon 200–701, Republic of Korea
[d] Department of Forest Environment Protection, Kangwon National University, Chuncheon 200–701, Republic of Korea
[e] Department of Herbal Crop Research, National Institutes of Horticultural & Herbal Science, Rural Development Administration, Suwon 441–707, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: Hopea odorata Roxb. (Dipterocarpaceae) is a representative Thai ethnopharmacological herbal plant used in the treatment of various inflammation-related diseases. In spite of its traditional use, systematic studies of its anti-inflammatory action have not been performed.

Materials and methods: The inhibitory activities of a *Hopea odorata* methanol extract (Ho-ME) on the production of nitric oxide (NO), tumour necrosis factor (TNF)-α, and prostaglandin E_2 (PGE_2) in RAW264.7 cells and peritoneal macrophages were investigated. The effects of Ho-ME on the gastritis symptoms induced by HCl/EtOH and on ear oedemas induced by arachidonic acid were also examined. Furthermore, to identify the immunopharmacological targets of this extract, nuclear fractionation, a reporter gene assay, immunoprecipitation, immunoblot analysis, and a kinase assay were employed.

Results: Ho-ME strongly inhibited the release of NO, PGE_2, and TNF-α in RAW264.7 cells and peritoneal macrophages stimulated by lipopolysaccharide (LPS). Ho-ME also clearly suppressed the gene expression of pro-inflammatory cytokines and chemokines, such as interferon (IFN)-β, interleukin (IL)-12, and monocyte chemotactic protein-1 (MCP-1). By analysing the inhibited target molecules, Syk and Src were found to be suppressed in the inhibition of nuclear factor (NF)-κB pathway. In addition, the observed downregulation of activator protein (AP)-1 and cAMP response element-binding (CREB) was due to the direct inhibition of interleukin-1 receptor-associated kinase (IRAK)1 and IRAK4, which was also linked to the suppression of c-Jun N-terminal kinase (JNK) and p38. In agreement with the *in vitro* observations, this extract also ameliorated the inflammatory symptoms in EtOH/HCl-induced gastritis and arachidonic acid-induced ear oedemas in mice.

Conclusion: Ho-ME has potential as a functional herbal remedy targeting Syk- and Src-mediated anti-inflammatory mechanisms. Future pre-clinical studies will be needed to investigate this possibility.

1. Introduction

When bacteria or fungi infect the body, phagocytic inflammatory cells such as macrophages and neutrophils participate in removing the pathogens. During this response, various chemokines and cytokines, such as monocyte chemoattractant protein (MCP)-1 and tumour necrosis factor (TNF)-α, and various inflammatory mediators, such as reactive oxygen species (ROS), prostaglandin (PG)E_2, and nitric oxide (NO), mediate the activation of inflammatory cells (Toltl et al., 2008). If inflammatory inducers are not fully neutralised, chronic inflammation is generated and sustained without a strong inflammatory response. Tissues and organs can be damaged by the inflammatory mediators released during chronic inflammation, leading to the loss of normal

Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; (TNF)-α, tumour necrosis factor; ERK, extracellular signal-related kinase; TLR, Toll-like receptor; 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-kinase; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; DSS, dextran sulphate sodium; PMA, phorbol 12-myristate 13-acetate; MyD88, myeloid differentiation primary response gene (88); IκBα, inhibitor of kappa B alpha; IκK, IκB kinase; Syk, spleen tyrosine kinase; CREB, cAMP response element-binding; CMC, sodium carboxymethylcellulose; PEI, Polyethylenimine

* Corresponding author. Tel.: +82 31 290 7868; fax: +82 31 290 7870.
E-mail addresses: jaecho@skku.edu, jaecho@kangwon.ac.kr (J. Youl Cho).
[1] These authors equally contributed to this work.

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functions. It is generally accepted that chronic inflammation is a silent killer, causing serious diseases such as cancer, diabetes, atherosclerosis, and arthritis (McGeer and McGeer, 2008). Therefore, there have been recent efforts to develop safe, potent anti-inflammatory drugs for the treatment of chronic inflammation (Massarotti, 2008). One of the best sources of safe anti-inflammatory remedies is ethnomedicinal herbal plants, which have been in use for many years (Lukhoba et al., 2006).

Hopea odorata Roxb. (known as Tkhian Thong, Dipterocarpaceae) is a representative medicinal plant used in the Khok Pho District (Pattani Province) of Thailand (Mahady, 2002; Wiyakrutta et al., 2004) to treat various inflammation-related diseases. The tannin-rich bark and leaves of this plant have been used for treating paralysis, haemorrhoids, diarrhoea, gum inflammation, and urinary incontinence (Chuakul, 2005; Gardner et al., 2000). The stem bark of *Hopea odorata* has also been traditionally used to treat neck pains in the North Andaman Islands, India (Prasad et al., 2008). This plant contains various resveratrol derivatives, including hopeaphenol, vaticanol B, hemsleyanol B, stemonoporal A, e-viniferin, and laevifonol, which show radical scavenging activity as well as other important biological properties, such as anti-inflammatory, antibacterial, and anti-cancer activity (Hasan et al., 2009; Zain et al., 2010, 2011). Although *Hopea odorata* has been used in traditional herbal medicine, detailed studies on its anti-inflammatory actions have not yet been performed. Therefore, in this study, we aimed to investigate the anti-inflammatory potential of *Hopea odorata* under both *in vitro* and *in vivo* inflammatory conditions and to examine the molecular targets involved in its anti-inflammatory pharmacology.

2. Materials and methods

2.1. Materials

A 99% methanol extract (Code No.: FBM018-051) of whole parts (leaves and bark) of *Hopea odorata* Roxb. (Dipterocarpaceae) was purchased from the Plant Extract Bank of the Plant Diversity Research Center (http://extract.pdrc.re.kr/extract/f.htm, Daejeon, Korea). Resveratrol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, tetrazole (MTT), and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SB203580, SP600125, PP2, and piceatannol were obtained from Calbiochem (La Jolla, *CA*, USA). The luciferase constructs containing binding promoters for NF-κB, CREB, and AP-1 were gifts from Prof. Chung, Hae Young (Pusan National University, Pusan, Korea) and Man Hee Rhee (Kyungpook National University, Daegu, Korea). Foetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). The 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 or total antibodies for p65, p50, c-Fos, ATF-2, c-Jun, PDK1, Src, Syk, IκBα, lamin A/C, and β-actin were obtained from Cell Signaling (Beverly, MA, 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 food pellets (Samyang, Daejeon, Korea) were supplied *ad libitum*. All studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Sungkyunkwan University (Suwon, 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) as reported previously (Lee et al., 2010). After washing with RPMI 1640 medium containing 2% FBS, the 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

The primary macrophages, RAW 264.7, 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. Under our experimental cell density (2×10^6 cells/ml), the proportion of dead cells was less than 1% according to Trypan blue dye exclusion tests.

2.5. NO, TNF-α, and PGE_2 production

A stock solution (350 mg/ml) of Ho-ME was prepared in 100% dimethyl sulfoxide (DMSO) and diluted to 0–200 μg/ml with media for the *in vitro* assays with cell lines or suspended with 1.5% sodium carboxymethylcellulose (CMC) for the *in vivo* experiments.

After the pre-incubation of the RAW264.7 cells or peritoneal macrophages (1×10^6 cells/ml) for 18 h, the cells were pre-treated with Ho-ME (0 to 40 μg/ml) for 30 min and then further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effect of Ho-ME on NO, TNF-α, and PGE_2 production was determined by analysing the levels of NO, TNF-α, and PGE_2 using the Griess reagent, an enzyme immunoassay kit (Amersham, Little Chalfont, Buckinghamshire, UK), and an enzyme-linked immunosorbent assay kit (Amersham), as described previously (Cho et al., 2000; Green et al., 1982).

2.6. Cell viability test

After the pre-incubation of the RAW264.7 cells and peritoneal macrophages (1×10^6 cells/ml) for 18 h, the cells were incubated with Ho-ME (0 to 40 μg/ml) for 24 h. The cytotoxic effects of Ho-ME were then evaluated using a conventional MTT assay, as reported previously (Gerlier and Thomasset, 1986; Yoo et al., 2011). At 3 h prior to culture termination, 10 μl of an MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4) was added, and the cells were cultured until the termination of the experiment. Incubation was halted by the addition of 15% sodium dodecyl sulphate to each well, solubilising the formazan (Do Kim et al., 2010). The absorbance at 570 nm ($OD_{570-630}$) was measured using a Spectramax 250 microplate reader.

2.7. mRNA analysis by real-time polymerase chain reaction (PCR)

To evaluate the cytokine mRNA expression levels, RAW264.7 cells pre-treated with Ho-ME (200 μg/ml) for 30 min were incubated with LPS (1 μg/ml) for 6 h. The total RNA from the cells was then isolated using the TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. The total RNA was stored at −70 °C until use. The mRNA was quantified with real-time RT-PCR using SYBR Premix Ex Taq according to the manufacturer's instructions (Takara, Japan) and a real-time thermal cycler (Bio-Rad, USA), as reported previously (Khorolragchaa et al., 2010). The results are expressed as the ratio of the optimal