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Dipterocarpus tuberculatus ethanol extract strongly suppresses *in vitro* macrophage-mediated inflammatory responses and *in vivo* acute gastritis

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ABSTRACT

Ethnopharmacological relevance: *Dipterocarpus tuberculatus* Roxb. (Dipterocarpaceae) has been traditionally used to treat various inflammatory symptoms. However, no mechanistic studies on the anti-inflammatory actions of *D. tuberculatus* have been reported. This study is therefore aimed at exploring the anti-inflammatory effects of 95% ethanol extracts (Dt-EE) of this plant.

Materials and methods: The regulatory activity of Dt-EE and its molecular mechanism on the release of nitric oxide (NO) and prostaglandin (PG)E_2 in lipopolysaccharide (LPS)-treated macrophage-like RAW264.7 cells were elucidated by evaluating the activation of transcription factors and their upstream signals and by analyzing the kinase activities of target enzymes. Furthermore, to confirm its availability for oral use, an EtOH/HCl-induced acute gastritis model was tested with this extract.

Results: Dt-EE effectively suppressed LPS-mediated inflammatory responses such as the production of NO and PGE_2 from macrophages in a dose-dependent manner. In particular, Dt-EE clearly blocked the activation of NF-κB by blocking the phosphorylation of its upstream enzymes IKK and Akt. Using a direct enzyme assay, Dt-EE was shown to block the enzyme activity of PDK1. Finally, this extract also remarkably ameliorated inflammatory lesions in the stomach induced by EtOH/HCl.

Conclusion: Our data strongly suggest that Dt-EE can be considered as a novel anti-inflammatory remedy with PDK1/NF-κB inhibitory properties and can also be used to treat gastritis symptoms. In addition, our findings can serve as a basis for further phytochemical and pharmacological studies in the future.

1. Introduction

The pathophysiological roles of tissue-associated macrophages have been well studied (Tsan and Gao, 2004). Activation of these cells can be induced by treatment with various ligands such as lipopolysaccharides (LPS), and subsequent activation of its receptor Toll-like receptor (TLR) 4 can induce similar cellular events seen in acute and chronic inflammatory conditions linked to certain diseases. A number of downstream signaling events involving non-receptor type protein tyrosine kinases such as Syk and Src, phosphoinositide 3-kinases (PI3K), mitogen activated protein kinases (MAPKs), and the activation of transcription factors (*e.g.*, nuclear factor [NF]-κB and activator protein [AP]-1) (Sekine et al., 2006; Takeda and Akira, 2001) leads to expression of pro-inflammatory genes including cytokines, inducible NO synthase (iNOS), and cyclooxygenase (COX)-2 (Bresnihan, 1999; Burmester et al., 1997;

Abbreviations: Dt-EE, ethanol extract of *Dipterocarpus tuberculatus* Roxb.; PGE_2, prostaglandin E_2; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; TNF-α, tumor necrosis factor-α; ERK, extracellular signal-related kinase; TLR, Toll-like receptors; NF-κB, nuclear factor-κB; AP-1, activator protein-1; Akt, protein kinase B; IKK, IκBα kinase; MyD88, myeloid differentiation primary-response protein 88; TRAF6, tumor necrosis factor receptor-associated factor 6; PDK1, phosphoinositide-dependent kinase-1; TRIF, TIR-domain-containing adapter-inducing interferon-β; EIA, enzyme immunoassay; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; PI3K, phosphoinositide 3-kinase; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction.

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Gracie et al., 1999). Each of the biochemical components in inflammatory signaling is now considered as an anti-inflammatory target for new drug development.

Dipterocarpus tuberculatus, a genus of flowering plants and the type genus of family Dipterocarpaceae, is a representative medicinal herb prescribed for various diseases in Southeast Asia (Devi and Yadava, 2007). These plants have been used for leishmanicidal , anti-septic and anti-inflammatory purposes (Takahashi et al., 2004) and also traditionally prescribed for curing skin inflammation, bronchial infections, colitis, and anxiety (Khare, 2007). Several pharmacologic properties of heartwood constituents that play a role in the defense of heartwood tissue have been reported. Many quinone analogs have been isolated from the heartwood of woody plants and they are thought to function as free radical scavengers of oxidants.

Although this plant is traditionally valuable, so far, systematic trials and scientific approaches to prove its ethnopharmacological roles have not been done. In this study, therefore, its ethnomedicinal benefits and direct pharmacological target enzymes against inflammatory symptoms or diseases were explored in *in vitro* and *in vivo* models of inflammation using an ethanolic extract (Dt-EE) of the plant.

2. Materials and methods

2.1. Materials

The leaves and twigs of *D. tuberculatus* were collected at the Popa Mountain National Park, Mandalay Prov., Myanmar in August 2011. This plant was identified by Prof. Yong Dong Kim (Hallym University, Chuncheon, Korea). A voucher specimen (number: Cho S.H. et al. MM208) was deposited in the herbariums of Hallym University (Chuncheon, Korea) and National Institute of Biological Resource (Incheon, Korea). Wortmannin, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, a tetrazole), and LPS (*E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Piceatannol, SP600125, U0126, and PP2 were obtained from Calbiochem (La Jolla, CA, USA). A luciferase construct containing the binding promoter for NF-κB was a gift from Prof. Chung, Hae Young (Pusan National University, Pusan, Korea). Enzyme immunoassay (EIA) kits for determining PGE_2 were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum and RPMI 1640 were obtained from Gibco (Grand Island, NY, USA). RAW264.7 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were Sigma grade. Phospho-specific or total antibodies to transcription factors (p65 and p50), IκBα, IKKβ, Akt, p85/PI3K, PDK1, γ-tubulin, and β-actin were obtained from Cell Signaling (Beverly, MA, USA).

2.2. Preparation of ethanol extract

The leaves and twigs of *D. tuberculatus* were dried at room temperature and then cut and pulverized. The pulverized plant tissues (125 g) were extracted with 95% ethanol (1,500 ml) in an ultrasonic bath and evaporated to dryness under reduced pressure to give a 95% ethanol extract of *D. tuberculatus* (3.2 g).

2.3. Mice

Six-week-old male ICR mice were purchased from DAEHAN BIOLINK (Chungbuk, Korea). Mice were given food pellets (Samyang, Daejeon, Korea) and water *ad libitum* under a 12-h light/dark cycle. Studies were performed in accordance with guidelines established by the Sungkyunkwan University Institutional Animal Care and Use Committee.

2.4. Cell culture

RAW264.7 (a mouse leukemic monocyte macrophage cell line) and HEK293 (a human embryonic kidney cell line) cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), glutamine, and antibiotics (penicillin and streptomycin) at 37 °C under 5% CO_2. For each experiment, 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 and PGE_2 production

After preincubation of RAW264.7 cells or peritoneal macrophages (1×10^6 cells/ml) for 18 h, cells were pre-treated with Dt-EE (0 to 400 μg/ml) for 30 min and were further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effect of Dt-EE on NO and PGE_2 production was determined by analyzing NO and PGE_2 levels with the Griess reagent and enzyme-linked immunosorbent assay (EIA) kits, as described previously (Jin et al., 2010).

2.6. Cell viability test

After preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, Dt-EE (0–400 μg/ml) was added to the cells and incubated for 24 h. The cytotoxic effect of Dt-EE was then evaluated by a conventional MTT assay, as reported previously (Park et al., 2011). At 3 h prior to culture termination, 10 μl of MTT solution (10 mg/ml in phosphate-buffered saline [PBS], pH 7.4) was added and the cells were continuously cultured until termination of the experiment. The incubation was halted by the addition of 15% sodium dodecyl sulfate into each well, solubilising the formazan (Kim et al., 2008). The absorbance at 570 nm ($OD_{570-630}$) was measured using a Spectramax 250 microplate reader.

2.7. mRNA analysis by quantitative real-time polymerase chain reaction (PCR) and semi-quantitative reverse transcriptase (RT)-PCR

To evaluate cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells with TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions (Shen et al., 2011). Total RNA was stored at −70 °C until use. Semi-quantitative RT reactions were conducted using MuLV reverse transcriptase. Total RNA (1 μg) was incubated with oligo-dT15 primers for 5 min at 70 °C and mixed with a 5× first-strand buffer, 10 mM of each dNTP, and 0.1 M dithiothreitol (DTT). The reaction was further incubated for 5 min at 37 °C, and then incubated at 37 °C for 60 min following the addition of MuLV reverse transcriptase (2 U). The reactions were terminated at 70 °C for 10 min, and total RNA was depleted with RNase H. PCR was performed with 2 μl cDNA, 4 μM 5′ and 3′ primers, 10× buffer (10 mM of Tris–HCl, pH 8.3, 50 mM of KCl, and 0.1% Triton X-100), 250 μM of each dNTP, 25 mM of $MgCl_2$, and 1 U of Taq polymerase (Promega, USA) under the following incubation conditions: 30 cycles of denaturation at 94 °C for 45 s, annealing between 55 and 60 °C for 45 s, and extension at 72 °C for 60 s, followed by 7 min final extension at 72 °C. The primers (Bioneer, Seoul, Korea) used are indicated in Table 1. To determine cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells with TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. Total RNA was stored at −70 °C until use. Quantification of mRNA was also performed using real-time RT-PCR with SYBR Premix Ex Taq (Takara, Japan) on a real-time thermal cycler (Bio-Rad, USA), as reported previously (Kim and Lee, 2011; Sun et al., 2010). Results are expressed as the ratio of