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ERK1- and TBK1-targeted anti-inflammatory activity of an ethanol extract of *Dryopteris crassirhizoma*

Yanyan Yang [a,1], Gang Jun Lee [a,1], Deok Hyo Yoon [b], Tao Yu [a], Jueun Oh [a], Deok Jeong [a], Jongsung Lee [c], Seong Hwan Kim [d,**], Tae Woong Kim [b], Jae Youl Cho [a,*]

[a] Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Department of Biochemistry, Kangwon National University, Chuncheon 200-701, Republic of Korea
[c] Department of Dermatological Health Management, Eulji University, Seongnam 461-713, Republic of Korea
[d] Laboratory of Chemical Genomics, Pharmacology Research Center, Korea Research Institute of Chemical Technology, PO Box 107, Yuseong-gu, Daejeon 305-600, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: Dryopteris crassirhizoma Nakai (Aspiadaceae) has been traditionally used as an herbal medicine for treating various inflammatory and infectious diseases such as tapeworm infestation, colds, and viral diseases. However, no systematic studies on the anti-inflammatory actions of *Dryopteris crassirhizoma* and its inhibitory mechanisms have been reported. We therefore aimed at exploring the anti-inflammatory effects of 95% ethanol extracts (Dc-EE) of this plant.

Materials and methods: The anti-inflammatory effect of Dc-EE on the production of inflammatory mediators in RAW264.7 cells and HCl/EtOH-induced gastritis was examined. Inhibitory mechanisms were also evaluated by exploring activation of transcription factors, their upstream signalling, and target enzyme activities. Finally, the active components from this extract were also identified using HPLC system.

Results: Dc-EE diminished the production of nitric oxide (NO) and prostaglandin (PG)E_2 in lipopolysaccharide (LPS)-stimulated RAW264.7 cells in a dose-dependent manner. Dc-EE also downregulated the levels of mRNA expression of pro-inflammatory genes such as inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and TNF-α by inhibiting the activation of activator protein (AP-1) and IRF3. Indeed, the extract strongly blocked the activities of their upstream kinases ERK1 and TBK1. This extract also strongly ameliorated gastritis symptoms stimulated by HCl/EtOH in mice. According to HPLC fingerprinting, resveratrol, quercetin, and kampferol were identified from Dc-EE.

Conclusion: Dc-EE displays strong anti-inflammatory activity by suppressing ERK/AP-1 and TBK1/IRF3 pathways, which contribute to its major ethno-pharmacological role as an anti-inflammatory and anti-infectious disease remedy.

1. Introduction

Inflammation is an innate immune response that is mainly regulated by tissue macrophages. The production of cytokines, chemokines, and inflammatory mediators [including nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumour necrosis factor-alpha (TNF)-α (Kinne et al., 2000; Owens et al., 2005)] initially appear and help other cells such as T lymphocytes, neutrophils, and NK cells to become activated and to infiltrate into inflamed tissues or sites (Deban et al., 2009). Even though this response is a natural defensive mechanism in our body, severe acute inflammation or chronic inflammation causes serious diseases such as septic shock, cancer, diabetes, rheumatoid arthritis, gastritis, and atherosclerosis (Gracie et al., 1999; Michaelsson et al., 1995; Stuhlmuller et al., 2000). This notion led us to develop potent and safe anti-inflammatory drugs to prevent or cure such diseases.

Initial approaches to screen for anti-inflammatory drug candidates employed various in vitro experimental models with macrophages. Toll like receptor (TLR) ligands such as lipopolysaccharides (LPS) are being used for stimulation of macrophages. Under the conditions, it is well-known that the cells release

Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; TNF-α, tumour necrosis factor-α; ERK, extracellular signal-regulated 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; MTT, 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; LPS, lipopolysaccharide; ATF2, activating transcription factor 2; CREB, cAMP response element-binding; TRIF, TIR-domain-containing adapter-inducing interferon; IFN-β, interferon beta; IRF3, interferon regulatory factor 3; TBK1, TANK-binding kinase 1

* Corresponding author. Tel.: +82 31 290 7868; fax: +82 31 290 7870.
** Corresponding author. Tel.: +82 42 860 7687; fax: +82 42 861 4246.
E-mail addresses: hwan@krict.re.kr (S.H. Kim), jaecho@skku.edu, jaecho@kangwon.ac.kr (J.Y. Cho).

[1] These authors equally contributed to this work.

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various inflammatory mediators such as NO and PGE_2 and express inflammatory genes such as inducible NO synthase (iNOS), pro-TNF-α, and cyclooxygenase (COX)-2 (Tsan and Gao, 2004). It is also important to identify which intracellular signalling enzyme(s) activated by TLR ligands are targets of candidate drugs. Targets of these inflammatory signaling include non-receptor type protein tyrosine kinases, phosphoinositide 3-kinases, and mitogen activated protein kinases (MAPKs) containing extracellular signal-regulated kinase (ERK), p38, and JNK (C-Jun *N*-terminal kinase) as well as the subsequent stimulation of transcription factors (e.g., nuclear factor [NF]-κB and activator protein [AP]-1) (Sekine et al., 2006; Takeda and Akira, 2001).

Dryopteris crassirhizoma Nakai (Aspiadaceae) is a plant used for traditional herbal medicine in Korea, Japan, and China (Yang, 2003). The root of this plant has been prescribed for the treatment of tapeworm infestation, epidemic flu, epidemic cerebrospinal meningitis, colds, and cancer (Chang et al., 2010; Lu et al., 2012; Magalhaes et al., 2010; Mazzio and Soliman, 2009). This plant also displays various pharmacological actions such as anti-oxidative, anti-obesity, anti-parasite, anti-bacterial, anti-viral and anti-cancer effects (Da Silva et al., 2010; Gao et al., 2008a; Kang et al., 2010; Lee et al., 2008; Min et al., 2003). These activities are mediated by active components such as phloroglucinol derivatives (albaspidin, aspidin, flavaspidic acids, and dryocrassin), triterpenes such as acylphloroglucinols, and dimethylflavanones such as desmethoxymatteucinol, matteucinol, and methoxymatteucin (Lee et al., 2009a, 2008; Nikaido et al., 2006; Ye et al., 2010).

Even though *Dryopteris crassirhizoma* has been used for a long time and investigated to systematically evaluate its pharmacological activities, detailed evidence explaining its anti-inflammatory activities has been limited. In this study, we aimed to demonstrate its ethnomedicinal benefits against numerous inflammatory diseases under several in vitro inflammatory conditions using an ethanolic extract (Dc-EE) of the plant. In particular, because no study has reported the exact molecular target for the anti-inflammatory effect of *Dryopteris crassirhizoma*, we explored the inhibitory targets of Dc-EE.

2. Materials and methods

2.1. Materials

A 95% ethanol extract (Code No.: FBM053-089) from the roots of *Dryopteris crassirhizoma* Nakai (Aspiadaceae) was purchased from the Plant Extract Bank in the Plant Diversity Research Center (http://extract.pdrc.re.kr/extract/f.htm, Daejeon, Korea). Forskolin, (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), and lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). U0126 and BX795 were obtained from Calbiochem (La Jolla, CA). Luciferase constructs containing binding promoters for NF-κB, CREB, IRF-3, and AP-1 were used as reported previously (Cho et al., 2009; Lee et al., 2011b). Enzyme immunoassay (EIA) kits for determining PGE_2 were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Foetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from ATCC (Rockville, MD). All other chemicals were of Sigma grade. Phospho-specific or total antibodies to c-fos, c-Jun, ATF2, ERK, c-Jun *N*-terminal kinase (JNK), p38, TBK1, γ-tubulin, and β-actin were obtained from Cell Signaling (Beverly, MA).

2.2. Preparation of the crude extract of Dryopteris crassirhizoma

To prepare the extract, the sample of *Dryopteris crassirhizoma* (30–40 g) was treated with 200 ml of 95% ethanol at 50 °C, 1500 psi for 3 days using an ultrasonic cleaner (Branson Ultrasonics Corp., CT, USA); the extracted materials were concentrated with a speed bag (Modul spin 40; Biotron Corporation, Gangwondo, Korea) at 40 °C for 24 h. The ethanol extract was then dissolved in 100% dimethyl sulfoxide (DMSO) to 350 mg/ml and diluted with the medium for in vitro assays with cell lines (0.33% DMSO at 150 μg/ml of Dc-EE).

2.3. Cell culture

RAW264.7 and HEK293 cells were cultured with RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), 2.05 mM L-glutamine and 1% 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.

2.4. NO and PGE_2 production

After preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, cells were pre-treated with Dc-EE (0 to 150 μg/ml) for 30 min and were further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effect of Dc-EE on NO and PGE_2 production was determined by analyzing NO and PGE_2 levels with the Griess reagent and enzyme linked immunoassay (EIA) kits, as described previously (Cho et al., 2000; Kim et al., 2012).

2.5. Cell viability test

After preincubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, Dc-EE (0 to 150 μg/ml) was added to the cells and incubated for 24 h. The cytotoxic effect of the Dc-EE was then evaluated by a conventional MTT assay, as reported previously (Mosmann, 1983; Son 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 continuously cultured until termination of the experiment. The incubation was halted by the addition of 15% sodium dodecyl sulphate into each well, solubilising the formazan (Kim et al., 2008a). The absorbance at 570 nm ($OD_{570-630}$) was measured using a Spectramax 250 microplate reader.

2.6. mRNA detection by quantitative real-time reverse transcription-PCR

Total RNA from LPS-treated RAW264.7 cells (5×10^6 cells/ml) was prepared by using TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol. The total RNA was dissolved in DEPC-treated water, and stored at −70 °C until used. Real-time PCR has been performed by previous method (Jo et al., 2011; Kim et al., 2010). Briefly, one microgram of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen). After first strand cDNA was synthesized, it was used as template to perform real-time PCR analysis. The real-time PCR was conducted with SYBR green real-time PCR Master mix system (Toyobo, Osaka, Japan), according to the manufacturer's instructions. For each sample, 25 μl of PCR master mix, 2 μl of forward primer (10 μM), 2 μl of reverse primer (10 μM), 5 μl of cDNA template, and PCR grade water were mixed to make 50 μl. The PCR cycling condition included (1) pre-denaturation for 1 min at 95 °C, (2) 40 cycles of denaturation for 15 s at 95 °C, (3) annealing for 15 s at 55 °C, and (4) extension for 45 s at 72 °C. The results were analyzed by Bio-Rad CFX manager (Bio-Rad, Hercules, CA) and