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Inhibitory effects of Dianthi Herba ethanolic extract on inflammatory and nociceptive responses in murine macrophages and mouse models of abdominal writhing and ear edema

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ABSTRACT

Ethnopharmacological relevance: Dianthi Herba is a traditional herbal medicine used to treat inflammatory-related diseases including acute pyelonephritis, cystitis, laryngopharyngitis, and urethritis.

Aim of the study: We investigated the effects of Dianthi Herba ethanolic extract (DH) on lipopolysaccharide (LPS)-mediated inflammatory responses in murine macrophages including RAW 264.7 cell line and mouse peritoneal macrophages as well as nociceptive and edema mouse models.

Materials and methods: The biological effects of DH on inflammatory cytokine, mediator, and related protein production were assessed using enzyme-linked immunosorbent assay (ELISA), Western blotting, and real-time reverse transcription-polymerase chain reaction (real-time RT-PCR). Additionally, Western blotting was performed to investigate intracellular signaling pathways, and the anti-nociceptive activity of three doses of DH (100, 200, and 300 mg/kg) against acetic acid-induced writhing responses and its inhibitory effects on xylene-induced ear edema were researched in mice through oral administration.

Results: DH treatment significantly inhibited nitric oxide (NO) secretion and inflammatory cytokine production in RAW 264.7 cells and mouse peritoneal macrophages and induced heme oxygenase (HO)−1 expression. DH strongly inhibited the transcriptional activity of nuclear factor (NF)-κB and phosphorylation of mitogen-activated protein kinases (MAPK) in LPS-stimulated macrophages. Meanwhile, DH exerted anti-nociceptive effects on writhing responses and anti-edema effects in mice.

Conclusion: We confirmed the anti-inflammatory activities and inhibitory mechanism of DH in macrophages and clarified its inhibitory effects in vivo. These findings illustrate the therapeutic potential of DH as a natural anti-inflammatory agent.

1. Introduction

Dianthus superbus var. *longicalycinus* Williams and *Dianthus chinensis* Linné (Rainbow pink, Caryophyllaceae) are called “Paeraengeekot” (herbal name: Gumaek) in Korea. Dianthi Herba, consisting of the aerial parts of both species, is frequently used as a diuretic and anti-inflammatory therapy in traditional East Asian medicine (Jiangsu New Medical College, 1977; López-Expósito et al., 2011; The State Pharmacopoeia Commission of The People’s Republic of China, 2005). Specifically, Dianthi Herba is frequently used to treat urinary inflammation-related diseases including urinary infection, gonorrhea, carbuncles, and carcinoma (Oshima et al., 1984; Raman and Park, 2015; Wang et al., 1998; WHO, 1998). Previous studies illustrated that it exerts apoptotic effects in human oral cancer and HepG2 human hepatocellular carcinoma cells (Nho et al., 2012; Shin et al., 2013). However, its anti-inflammatory properties and mechanism of action remain unknown.

Inflammation, a host-induced protective mechanism in response to tissue injury, infection, or other stimuli, is typically accompanied by the activation of immune cells such as lymphocytes, macrophages, and neutrophils. Macrophages are important in the inflammatory response and serve a critical role in this process. Macrophages activated by LPS or other stimuli produce and release many inflammatory mediators including cyclooxygenase (COX)−2, inducible nitric oxide synthase (iNOS), NO, and inflammatory cytokines such as interleukin (IL)−6, IL-1β, and tumor necrosis factor (TNF)-α (Borges et al., 2013; Wang et al., 2011b). Among them, NO, which is synthesized by iNOS, regulates inflammatory cytokine and mediator expression, making it an important mediator in various inflammatory diseases (Sacco et al., 2006). Additionally, iNOS expression is closely related to HO-1 induction.

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Increasing HO-1 production thus weakens iNOS expression and suppresses free radical generation (Ashino et al., 2008).

LPS-induced inflammatory cytokine secretion is regulated by transcription factor NF-κB activation. The MAPK pathway represents a typical inflammatory signaling pathway in macrophages. Various natural substances and chemicals isolated from plants exhibit anti-inflammatory activities through inhibiting NF-κB and MAPK signaling (Oh et al., 2012; Yoon et al., 2010). NF-κB controls the expression of several immune-related cytotoxic factors including COX-2, iNOS, pro-inflammatory cytokines, and chemokines such as TNF-α, IL-6, IL-8, and IL-1β (Cortez et al., 2013; Sohn et al., 2012). When unstimulated, NF-κB is sequestered in the cytoplasm by inhibitor of NF-κB alpha (IκBα) inhibitor. Stimulation by agents such as LPS occurs through IκB degradation and phosphorylation, resulting in NF-κB nuclear translocation to promote pro-inflammatory mediator production (Barnes and Karin, 1997). The activated MAPK pathway also induces the production of various immune-related factors (Kyriakis and Avruch, 2012; Xie and Duan, 2012). Consequently, many drugs used to treat inflammatory diseases target NF-κB and MAPK signaling (Gilroy et al., 2004).

Here we examined the anti-inflammatory effects of DH on LPS-induced inflammation in macrophages. Moreover, we clarified whether DH regulates HO-1, NF-κB, and MAPK signaling to elucidate its anti-inflammatory mechanisms. We investigated the analgesic activities and anti-inflammatory effects of oral DH in mice.

2. Materials and methods

2.1. Materials and reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and antibiotics were purchased from Lonza (Basel, Switzerland). LPS, dexamethasone (Dex, ≥ 98%), bovine serum albumin (BSA), and 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenylthiazolium bromide (MTT) were acquired from Sigma (St. Louis, MO, USA), cell counting kit (CCK) from Dojindo (Kumamoto, Japan), MTS solution from Promega (Madison, WI, USA), ELISA antibody sets from eBioscience (San Diego, CA, USA), various primary and horseradish peroxidase (HRP)-conjugated secondary antibodies from Cell Signaling Technology, Inc. (Boston, MA, USA), and RNA extraction kit from iNtRON Biotech (Daejeon, Korea). Oligonucleotide primers for TNF-α, IL-6, IL-1β, iNOS, COX-2, and β-actin were synthesized by Bioneer (Daejeon, Korea). Acetic acid and xylene were obtained from Merck (Darmstadt, Germany). Triple-distilled water (DW) was filtered through a 0.45-μm membrane filter (ADVANTEC, Tokyo, Japan) before analysis.

2.2. Preparation of DH

Dianthi Herba was acquired as a dried herb from the Yeongcheonhyundai Herbal Market (Yeongcheon, Korea) and identified by Prof. KiHwan Bae, Chungnam National University, Korea. All voucher specimens were deposited in an herbal bank at the KM-Application Center, Korea Institute of Oriental Medicine (voucher number: E200). To prepare DH, dried, fragmentized Dianthi Herba (50.0 g) was extracted using 300 mL of 70% ethanol for 24 h in a 40 °C shaking incubator. Then, the solution was filtered using 150-mm filter paper (Whatman, Piscataway, NJ, USA) and concentrated using rotary vacuum evaporator (Buchi, Tokyo, Japan). The sample was freeze-dried and stored in desiccators at 4 °C until use (acquisition, 6.29 g; yield, 12.58%). The freeze-dried extract powder was then dissolved in DW and centrifuged at 14,000 rpm for 10 min, and the resulting supernatant was filtered (0.2-μm pore size) and stored at 4 °C until analysis.

2.3. Phytochemical analysis of DH

Gas chromatography mass spectrometry (GC-MS) was performed using a previously reported method (Jürgens et al., 2003) with some modification. A GC-MS system (Agilent Technologies, Atlanta, GA, USA) and DB-1 ms capillary columns (30 m × 0.25 mm × 0.25 μm) were used. Briefly, DH solution (1 μL) was injected in the split mode at a ratio of 1:10 at 280 °C. The oven temperature was programmed as follows: initial temperature of 50 °C for 3 min and increased at 3 °C/min to 280 °C and held for 5 min. The mass analyzer scanned from 50 to 600 amu. All compounds were identified using NIST and Wiley GC-MS libraries. DH included one alcohol (54.01%), one amide (9.59%), two amino acids (0.75%), one carbohydrate (8.33%), esters (3.18%), fatty acid (15.04%), heterocyclic aromatics (1.18%), two ketones (0.87%), and one lactone (1.64%) (Table 1).

Table 1
The composition of volatile phytochemical in DH.

No.	Retention Time (min)	Compounds	Molecular formula	Molecular weight	Relative area percent (%)
1	7.43	α-Angelica lactone	$C_5H_6O_2$	98.09	1.64
2	10.99	2-Norcaranone	$C_7H_{10}O_2$	110.15	0.42
3	16.40	Pyranone	$C_6H_8O_4$	144.12	0.45
4	17.25	Thiofuran	C_4H_4S	84.14	1.18
5	19.79	D-Glutamic acid	$C_5H_9NO_4$	147.13	0.36
6	20.00	Acetylcysteine	$C_5H_{10}O_4$	134.13	0.39
7	35.98	Caprylic acid	$C_8H_{16}O_2$	144.21	7.53
8	36.87	Lactose	$C_{12}H_{22}O_{11}$	342.29	8.33
9	37.20	Lauric acid	$C_{12}H_{24}O_2$	200.32	1.53
10	39.39	Mome inositol	$C_7H_{14}O_6$	180.16	54.01
11	48.85	Palmitic acid	$C_{16}H_{32}O_2$	256.42	2.32
12	50.00	Ethyl palmitate	$C_{18}H_{36}O_2$	284.48	0.91
13	53.89	Linoleic aicd	$C_{18}H_{32}O_2$	280.45	3.66
14	54.90	Ethyl linoleate	$C_{20}H_{36}O_2$	308.49	1.88
15	55.06	Ethyl Oleate	$C_{20}H_{38}O_2$	310.51	0.39
16	69.99	Oleic acid amide	$C_{18}H_{35}NO$	281.48	9.59
Total relative area percentage of identified compounds					94.58

2.4. RAW 264.7 cell culture and drug treatment

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in complete RPMI 1640 medium. Cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C. To stimulate cells, LPS (200 ng/mL) (Ma et al., 2017; Oh et al., 2007) was added with or without DH (10, 30, 50, or 100 μg/mL) for indicated periods.

2.5. Peritoneal macrophage isolation and cell culture

Male BALB/c mice (25 ± 3 g) were obtained from Samtako BioKorea (Osan, Korea) And inoculated with 300 μL of sterile 3% sodium thioglycollate (Sigma, St. Louis, MO, USA) and housed (five per cage) at room temperature under a 12 h:12 h light/dark cycle with food and water access (SCF Co., Ltd., Korea) ad libitum. After 3 days, the animals were sacrificed, and macrophages were harvested by washing peritoneal cavities with 10 mL of ice-cold phosphate-buffered saline (PBS). The cell suspension was centrifuged at 500 × *g* for 5 min at 4 °C, and supernatant was discarded. The cell pellet was suspended in complete RPMI 1640 medium and incubated for 18 h to allow anchoring to the culture plate. To stimulate cells, fresh RPMI 1640 medium was added, and LPS (200 ng/mL) (Fu et al., 2012) was added with or without DH for 24 h. All animal studies were performed as per the Guide for the Animal Care and Use Committee of Korea Institute of Oriental Medicine (reference numbers #14–079).

2.6. CCK, MTT, and MTS assay for cell viability

DH-induced cytotoxicity was analyzed via the CCK, MTT, and MTS assay. RAW 264.7 cells were seeded in 96-well plates (SPL Life Sciences,