Journal of Ethnopharmacology 165 (2015) 94–102

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Bioassay-guided isolation and mechanistic action of anti-inflammatory agents from *Clerodendrum inerme* leaves

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ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form
16 February 2015
Accepted 17 February 2015
Available online 26 February 2015

Chemical compounds studied in this article:
Acacetin (PubChem CID: 5280442)
Hispidulin (PubChem CID: 5281628)
Diosmetin (PubChem CID: 5281612)

Keywords:
Clerodendrum inerme
Nitric oxide
Prostaglandin E_2
Anti-inflammatory
Flavone

ABSTRACT

Ethnopharmacological relevance: The leaves of *Clerodendrum inerme* (L.) Gaertn. have commonly been used in Thai traditional medicine for treatment of inflammatory diseases. However, the bioactive compounds responsible for the anti-inflammatory effect of leaves have not been yet determined. The objective of the present study was to isolate these bioactive compounds by bioassay-guided isolation technique and to determine the mode of action of isolated compounds in LPS-induced macrophages.
Materials and methods: Anti-inflammatory effect of various fractions (hexane, ethyl acetate and water) of ethanol extract of *C. inerme* leaves was determined from the production of nitric oxide (NO) in RAW 264.7 macrophage stimulated with LPS. The mRNA and protein levels were determined also by real-time reverse transcription-polymerase chain reaction and western blot analysis, respectively. Leaf bioactive compounds were isolated by bioassay-guided fractionation technique using column chromatography.
Results: The ethyl acetate fraction (EA) among solvent extracts provided the most potent inhibitory activity on NO production. Also, EA reduced the mRNA and protein expressions of inducible nitric oxide synthase (iNOS) in LPS-stimulated macrophages. Three known flavones, acacetin (**1**), hispidulin (**2**) and diosmetin (**3**), were isolated based on inhibition of NO production. Furthermore, hispidulin also inhibited PGE_2 production as well as iNOS and cyclooxygenase-2 expressions via the blockade of NF-κB DNA-binding activity and JNKway.
Conclusions: Our results found acacetin (**1**), hispidulin (**2**) and diosmetin (**3**), were responsible for the anti-inflammatory properties of *C. inerme* leaves. We provide scientific evidence to support the usefulness of *C. inerme* leaves in traditional medicine for the treatment of inflammation-related diseases.

1. Introduction

Inflammation is a response of the body to harmful stimuli such as injury and infection (Kumar et al., 2007). Macrophages are principally involved in acute and chronic inflammation. Upon activation, they secrete a series of pro-inflammatory mediators including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). An overproduction of these mediators causes a harmful effect to tissues and organisms as well as has been associated with the pathogenesis of various inflammatory-related diseases, including rheumatoid arthritis, diabetes, inflammatory bowel disease, atherosclerosis, and cancer (Yang et al., 2013).

Clerodendrum inerme (L.) Gaertn. (Verbenaceae family) known also as Sam-Ma-Nga in Thai or, seaside *clerodendrum* in English, occurs widely in coastal mangrove forests of Thailand (Office of Mangrove Resources Conservation, 2009) and other South Asian countries and is used in traditional medicine for the treatment of skin diseases, rheumatic pain and arthritis, fever, cough, hepatitis, and other inflammatory diseases (Shrivastava and Patel, 2007a; Chethana et al., 2013). The major chemical constituents of *C. inerme* are flavonoids, terpenes, steroids and phenolic compounds (Shrivastava and Patel, 2007b; Parveen et al., 2010; Shahabuddin et al., 2013). *C. inerme* possesses a number of biological activities including antimicrobial, anti-hepatotoxic, anti-oxidant, analgesic and anti-inflammatory activities (Gopal

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http://dx.doi.org/10.1016/j.jep.2015.02.043
0378-8741/

and Sengottuvelu, 2008; Gurudeeban et al., 2010; Yankanchi and Koli, 2010; Sangeetha et al., 2011; Chethana et al., 2013). Methanol extract of *C. inerme* leaves is reported to inhibit sub-chronic inflammation in cotton pellet-induced granuloma in mice (Yankanchi and Koli, 2010). Recently, ethanol extracts of *C. inerme* exhibited anti-inflammatory in carrageenan-induced paw edema and xylene-induced ear edema (Kalavathi and Sagayagiri, 2014; Khanam et al., 2014). Such in vivo anti-inflammatory activities of *C. inerme* indicated the presence of anti-inflammatory agents. However, the bioactive compounds responsible for the anti-inflammatory effect of *C. inerme* are not explored. To find out scientific evidence for therapeutic uses of *C. inerme* leaves, the objective of the present study was to identify the compounds responsible for the anti-inflammatory activity based on inhibition of NO production of *C. inerme* leaves through bioassay-guided isolation. We investigated the mechanism behind their anti-inflammatory activity on LPS-induced RAW 264.7 macrophages to assess the effectiveness of *C. inerme* leaves for various inflammatory diseases.

2. Material and methods

2.1. Reagents

Antibodies for p44/42 MAPK (ERK1/2), p38 MAPK and lamin A were bought from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). Antibodies for phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-p38 MAP kinase (Thr180/Tyr182), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), NF-κBp65 and goat anti-mouse IgG conjugated horseradish peroxidase secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse β-actin antibody, lipopolysaccharide or LPS (*Escherichia coli* serotype O111:B4), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM) free of phenol red were all obtained from Sigma Chemical (St. Louis, MO, USA). DMEM, fetal bovine serum (FBS), penicillin–streptomycin were purchased from Invitrogen/Gibco (Grans Island, NY, USA). Antibodies for iNOS and COX-2 were obtained from BD Bioscience (San Jose, CA, USA.). iScript™ Reverse Transcription Supermix for RT-qPCR and iTaq™ Universal SYBR®Green Supermix were bought from BIO-RAD (Hercules, CA, USA). Oligonucleotide primers were obtained from Sigma-Aldrich (Singapore). PGE_2 enzyme immunoassay kit was bought from R&D Systems (Minneapolis, MN, USA.). Protease inhibitor cocktail tablets and Phosphatase inhibitor cocktail tablets (PhosStop) were purchased from Roche (Germany). Super Signal West Pico Chemiluminescent substrate was obtained from Pierce (Rockford, IL, USA). TRI reagent was bought from Molecular Research Center (Cincinnati, OH, USA). TransAm NF-κB kit was purchased from Active Motif (Tokyo, Japan). Silica gel 60, used for chromatographic technique, was purchased from Merck (Germany).

2.2. Plant material

C. inerme (L.) Gaertn. (The Plant List record 42703) was collected from Welu wetland, Chantaburi Province, Thailand, GPS coordinates: 12° 22′ 05.82″ N to12° 22′ 15.42″ N, 102° 20′ 26.35″ E to 102° 20′ 27.22″ E in May 2010. The plant was identified by Dr. B. Chewprecha, Department of Biology, Faculty of Science, Burapha University. A voucher specimen (KS-SCBUU-0016) is kept at Faculty of Science, Burapha University.

2.3. Plant extraction, isolation and elucidation

Leaves of *C. inerme* were washed in tap water, dried at 50 °C, and finally ground in a blender. Ground leaves (1.2 kg) were macerated in ethanol (12 L) at room temperature for 5 days with occasionally shaking and filtered through Whatman filter paper no. 3. The plant residue was then re-extracted with ethanol twice as described above. Filtrates were pooled and evaporated in vacuo. The ethanol extract (EE, 195 g,16.2% w/w) was then partitionally separated to yield the residues of hexane (HF, 36.7 g, 21.7% w/w), ethyl acetate (EF, 26.7 g, 15.8% w/w), and water (WF, 98.5 g, 58.3% w/w) fractions, respectively. Fractions were kept at −20 °C and protected from light until investigated for their anti-inflammatory effects.

Ethyl acetate fraction was chosen for further studies due to its high potent inhibitory effect on NO production in LPS-induced macrophages. The ethyl acetate fraction (EF, 17 g) was separated by column chromatography using silica gel as stationary phase. Solvent system (dichloromethane: methanol) gradient from 100:0 to 50:50 v/v was used to isolate subfraction 1–8 (F1–F8) from EF. Based on anti-inflammatory activity, the subfraction F5 (1.3549 g, 0.11% w/w) was then subjected to silica gel column with step gradient of ethyl acetate and hexane (30:70, 50:50 and 70:30 v/v) to obtain 8 subfractions (SF5.1–5.8). Based on anti-inflammatory activity, the subfraction SF5.1 and 5.2 were pooled (0.810 g, 0.07% w/w) and loaded on to silica gel column chromatography and eluted to obtain compound **1** (119 mg), **2** (129.7 mg) and **3** (6 mg).

Subfraction F8 (2.930 g, 0.24% w/w) was loaded onto a silica gel column and eluted with a gradient of dichloromethane: methanol (99:1–80:20, v/v) to obtain 4 subfractions (SF8.1–8.4). SF8.1 (0.1662 g, 0.01% w/w) was, based on its anti-inflammatory activity, further submitted to silica gel column using a gradient of dichloromethane: methanol (97:3–92:8, v/v) as the mobile phase obtaining compound **1** (4.1 mg). SF 8.2 (0.2648 g, 0.02% w/w) was consequently separated in a silica gel column with a gradient of hexane-ethyl acetate (70:30–95:5, v/v) to obtain compound **2** (12.6 mg). Yields of compound **1**, **2** and **3** were 0.0103%, 0.0118% and 0.0005%, respectively. The scheme showing fractionation procedure of *C. inerme* leaves is shown in Fig. 1.

Proton NMR and Carbon NMR spectra were recorded on a Bruker AVANC 400 at 400 and 100 MHz, respectively. All spectra were measured in $CDCl_3$, DMSO-d_6 and methanol-d_4 solvents and chemical shifts are reported as δ values in parts per million (ppm) relative to solvent peak as internal standard.

2.4. Cell culture

RAW 264.7 murine macrophage cell line was a gift from Prof. C. Kim, Inha University College of Medicine, Republic of Korea. Cells were cultured in DMEM containing 25 mM D-glucose, 100 U/mL of penicillin,100 μg/mL of streptomycin and 10% heat-inactivated FBS. Cells were incubated at 37 °C in 5% CO_2 atmosphere.

2.5. Cell viability test by MTT assay

RAW 264.7 macrophages were plated into a 24-well plate (1.5×10^5 cells/well). After an overnight growth, media containing various concentrations of the test compounds were added to wells for an indicated time. 10 μL of MTT solution (5 mg/mL in PBS) was put into each well for 2–3 h before aspiration of the solution. 500 μL of dimethyl sulfoxide (DMSO) was added into each well to solubilize the blue formazan crystal product. Subsequently, the formazan solution was measured the absorbance at 550 nm using a microplate reader (Molecular Device, USA.). The amount of formazan was proportional to the number of functional mitochondria in viable cells. Percentage of cell viability was expressed as: (absorbance of treated well/absorbance of control well) × 100.

2.6. Determination of nitrite and PGE_2 concentration

Nitrite is a stable oxidation product of NO, a major pro-inflammatory mediator involved in various inflammation-related