Journal of Ethnopharmacology 146 (2013) 495–504

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

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

Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophages RAW 264.7 and its action in animal models of inflammation

Doungnapa Buapool [a,b], Nadtaya Mongkol [c], Jirapa Chantimal [a,b], Sittiruk Roytrakul [d], Ekaruth Srisook [b,e], Kloakwan Srisook [b,f,*]

[a] Biological Science Program, Faculty of Science, Burapha University, Saensook, Muang, Chonburi 20131, Thailand
[b] Center of Excellence for Innovation in Chemistry, Burapha University, Saensook, Muang, Chonburi 20131, Thailand
[c] Department of Biochemistry, Faculty of Science, Mahidol University, Payathai, Bangkok 10400, Thailand
[d] Genome Institute, National Center for Genetic Engineering and Biotechnology, Thailand Science Park, Pathumthani 12120, Thailand
[e] Department of Chemistry, Faculty of Science, Burapha University, Saensook, Muang, Chonburi 20131, Thailand
[f] Department of Biochemistry, Faculty of Science, Burapha University, Saensook, Muang, Chonburi 20131, Thailand

ARTICLE INFO

Article history:
Received 24 July 2012
Received in revised form
17 December 2012
Accepted 10 January 2013
Available online 23 January 2013

Keywords:
Pluchea indica
Asteraceae
Nitric oxide
PGE_2
Anti-inflammatory
Carrageenan
Inducible nitric oxide synthase

ABSTRACT

Ethnopharmacological relevance: Pluchea indica Less.: (Asteraceae) is a Thai medicinal plant used in traditional medicine for the treatment of hemorrhoids, lumbago, leucorrhoea and inflammation. This study investigated the molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaf extract in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages and also determined its action in acute inflammation animal models.

Materials and methods: The inhibitory effect of *Pluchea indica* leaf extract on LPS-induced nitric oxide (NO) production was evaluated by Griess reaction. Protein and mRNA expressions were determined by real time RT-PCR and Western blotting analysis, respectively. Inducible nitric oxide synthase (iNOS) promoter activity was evaluated by iNOS promoter based reporter gene assay. *In vivo* anti-inflammatory effect was examined in ethylphenylpropiolate (EPP)-induced ear edema and carrageenan-induced paw edema in rat models.

Results: Ethyl acetate fraction of ethanol extract of *Pluchea indica* leaves (EFPI) exhibited the potent inhibitory effect on NO production in LPS-induced macrophages and also inhibited PGE_2 release. EFPI reduced iNOS mRNA and protein expression through suppressed iNOS promoter activity and nuclear translocation of subunit p65 of nuclear factor-κB, but did not inhibit phosphorylation of the mitogen-activated protein kinases (MAPKs). Moreover, EFPI possessed anti-inflammatory activities on acute phase of inflammation as seen in EPP-induced ear edema and carrageenan-induced paw edema in rats.

Conclusions: These data support the pharmacological basis of *Pluchea indica* plant as a traditional herbal medicine for treatment of inflammation.

1. Introduction

Inflammation is a host defense mechanism in response to tissue injury, noxious chemicals or pathogens to eradicate invading pathogens and to remove irritants (Kumar et al., 2007). Inflammations may be acute or chronic. Its cardinal signs, pain, swelling, redness and heat, develop as an acute response to a local inflammatory insult (Mequanint et al., 2011). In the inflammatory process, infiltrated cells are activated to secrete mediators, such as bradykinin, serotonin, histamine, prostaglandins (such as PGE_2), leukotrienes and nitric oxide (NO) (Mequanint et al., 2011). NO is a short-lived free radical produced from L-arginine by nitric oxide synthase (NOS; 1.14.13.39). There are three distinct isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Alderton et al., 2001). Of these, iNOS is an important enzyme involved in the regulation of inflammation. High-output NO by iNOS can provoke deleterious consequences such as rheumatoid arthritis, septic shock, Alzheimer's diseases and inflammatory diseases (Wright et al., 1992; Dorheim et al., 1994; Guzik et al., 2003 and Latham et al. 2005).

Macrophages play an important role in the inflammatory response. LPS activates multiple signal pathways in macrophages such as the nuclear factor-κB (NF-κB) pathway leading to the transcription and expression of iNOS gene (Chen et al., 1999; Yang et al., 2000; Alderton et al., 2001; Kim et al. 2004). In unstimulated cells, NF-κB is present in the cytoplasm as an inactive heterodimer, and binds to inhibitory κB-α (IκB-α) subunits. Stimulation with specific inducers, most notably LPS, causes the degradation of IκB-α leading to NF-κB translocation to nuclei

* Corresponding author at: Department of Biochemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand. Tel.: +66 38 103 058; fax: +66 38 393 495.
E-mail address: klaokwan@buu.ac.th (K. Srisook).

0378-8741/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2013.01.014

where it binds to a specific sequence in promoter of target genes (Chen et al., 1999). Furthermore, mitogen-activated protein kinases (MAPKs) signal pathways (*e.g.*, p38 MAPK, extracellular-regulated kinase [ERK] 1/2, and c-Jun N-terminal kinase [JNK]) are involved in lipopolysaccharide (LPS)-induced iNOS expression in activated macrophage (Chen et al., 1999; Kim et al. 2004). Thus, inactivation of MAPKs might inhibit inflammatory mediators (NO and PGE_2), making them potential targets for anti-inflammatory therapeutics (Nakano et al., 1998; Carter et al., 1999; Kim and Shin, 2009).

Pluchea indica Less. is a shrub of the family Asteraceae and is widespread in Southeast Asia. In Thailand, various parts of *Pluchea indica* have been used in traditional medicine. Bark in decoction form, has been used for the treatment of hemorrhoids and leaves as a nerve tonic and treatment of inflammation, lumbago and leucorrhoea (Sen et al., 2002; Office of Mangrove Resources Conservation, 2009). Previous studies have reported extracts of *Pluchea indica* have anti-oxidant, anti-ulcer, antinociceptive, anti-inflammatory and anti-microbial activities (Sen et al., 1993; 2002; Choi and Hwang, 2005; Biswas et al., 2007; Roslida et al., 2008; Noridayu et al., 2011; Srisook et al., 2012). Leaf extract of *Pluchea indica* has been shown *in vitro* and *in vivo* to have anti-inflammatory properties (Choi and Hwang, 2005; Roslida et al., 2008; Srisook et al., 2012), however, the molecular mechanisms of this process have not been described.

The purpose of this study was to investigate an anti-inflammatory effect of *Pluchea indica* leaf extract on acute inflammation of animal ear and paw edema in LPS-induced RAW 264.7 macrophages to explain the anti-inflammatory effect proven *in vivo*.

2. Materials and methods

2.1. Drugs and chemicals

Fetal bovine serum (FBS), penicillin-streptomycin and Dulbecco's modified Eagle's medium (DMEM) were bought from Invitrogen/Gibco (Grans Island, NY, USA). Antibodies for iNOS were obtained from BD Bioscience (San Jose, CA, USA.). Antibodies for Lamin A, p44/42 MAPK (ERK1/2), p38 MAPK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A. Antibodies for NF-κBp65, phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), phospho-p38 MAP kinase (Thr180/Tyr182), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Danvers, MA, USA). Avian myeloblastosis virus reverse transcriptase, pGEM-T easy vector, pGL3 Basic vector, pRL-TK plasmid, horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG (H+L) were bought from Promega (Madison,WI, USA). Oligonucleotide primers were obtained from Operon Biotechnologies (Huntsville, AL, USA). Lipopolysaccharide or LPS (*Escherichia coli* serotype O111:B4), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anti-mouse β-actin antibody, diclofenac sodium salt, lambda carrageenan, quercetin and phenylbutazone were all purchased from Sigma Chemical (St. Louis, MO, USA). Super Signal West Pico Chemiluminescent substrate was bought from Pierce (Rockford, IL, USA). TRI reagent was bought from Molecular Research Center (Cincinnati, OH, USA). PGE_2 enzyme immunoassay kit was bought from R&D Systems (Minneapolis, MN, USA.). Ethyl phenylpropiolate (EPP) was purchased from Fluka Chemicals (St. Gallen, Switzerland). Protease inhibitor cocktail tablets (complete mini) were obtained from Roche (Germany).

2.2. Preparation of plant extracts

Pluchea indica leaves were obtained from community enterprise in Boa sub-district, Khlung district, Chantaburi Province, Thailand and authenticated by B. Chewprecha, a Plant Taxonomist at Department of Biology, Faculty of Science, Burapha University. A voucher specimen (KS-SCBUU-0014) was preserved at Faculty of Science, Burapha University. Fresh leaves (8.23 kg) were washed in tap water, dried, finely powdered, soaked (1 kg) in10 L absolute ethanol for 5 days with occasional shaking and filtered through Whatman No.1 filter paper at room temperature. Remaining residues were re-extracted twice with ethanol (1:10). The filtrate was pooled and evaporated by rotary evaporator and vacuum pump until dry. The ethanol extract (44.57 g) was sequentially extracted with *n*-hexane and ethyl acetate, filtered, evaporated until dry and ultimately yielded hexane (HFPI), ethyl acetate (EFPI) and water (WFPI) fraction, as 17.21 g (2.56% w/w), 4.27 g (0.63% w/w), 6.7 g (0.99% w/w), respectively. The dried powder of HFPI, EFPI and WFPI were dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.22 μM sterile filter.

2.3. Cell culture and reagents

Murine macrophage cell line RAW 264.7 (a kind gift from C. Kim, Inha University College of Medicine, Republic of Korea) was grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin. Cells were plated and incubated in humidified air containing 5% CO_2 at 37 °C. Cells were subcultured by scraping and those used in all experiments were from passage 10th to 11th.

2.4. Measurement of nitrite production

Nitrite, the stable end product of NO, was used as an indicator of NO production in the culture medium. Nitrite released in the culture medium was measured according to Griess reaction (Srisook and Cha, 2005). In brief, RAW 264.7 cells (1.5×10^5 cells/well) were plated into 24-well plates for 18 h. Cells were treated with LPS (1 μg/mL) for 20 h. in the presence or absence of plant extracts. Cell culture supernatants were measured for nitrite concentration and 100 μL of each was mixed with an equal volume of Griess reagent [0.1% (w/v), *N*-(1-naphthyl) ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid] for 10 min at room temperature. Absorbance (546 nm) of mixture was measured using a microplate reader and nitrite concentration determined from a standard curve of sodium nitrite made up in DMEM free of phenol red.

2.5. Cell viability assay

Cell viability was evaluated on the basis of their ability to reduce tetrazolium salt (MTT) to formazan, which is proportional to the number of functional mitochondria in living cells (Srisook and Cha, 2005).

2.6. Determination of PGE_2

PGE_2 is also a key inflammatory mediator and an increased level of PGE_2 mediates the cardinal features of inflammation such as pain, edema and fever. To determine whether EFPI inhibits LPS-induced PGE_2 production in RAW 264.7 macrophage, cells (1.5×10^5 cells/well) were subcultured in 24-well plates and incubated with LPS (1 μg/mL) in the presence or absence of EFPI for 24 h. Thereafter PGE_2 concentration in culture medium was measured with a PGE_2 enzyme immunoassay kit (R&D Systems).

2.7. Quantitative real time-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from mouse macrophage RAW 264.7 cells (1×10^6 cells/plate) using Trizol reagent according to the

manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using 3U AMV reverse transcriptase, 1x RT buffer (50 mM Tris–HCl pH 8.8, 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM spermidine and 1 mM DTT), 0.083 mM oligo $(dT)_{15}$ primer, 0.67 mM dNTPs, 20 U RNase inhibitor and RNase, DNase-free water to make up volume to 30 μL. The reaction mixture was incubated at 42 °C for 45 min and at 99 °C for 5 min. Complementary DNA (cDNA) from the mixture was used in real-time RT-PCR. Real-time quantitative PCR was conducted in a iCycler real-time PCR detection system (Bio-Rad) using SYBR Green I as the detection dye. The total 20 μL reaction mixture contained 2x Prime Q-Master with SYBR Green I, DNase-free water and 1 μL of cDNA. The PCR cycling parameters were set as follows: 94 °C for 5 min followed by 40 cycles of PCR reactions at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s. The sequences of the PCR primers were as follows: iNOS, 5′ GCACAGCACAGGAAATGTT TCAGCAC 3′ and 5′ AGCCAGCATACCGGATGAGC 3′; EF-2, 5′ CTGAAGCGGCTG-GCCA AG 3′ and 5′ GGGTCAGATTTCTTGATGGG 3′. EF-2, a housekeeping gene, was chosen as an internal standard to control for variability in amplication because of differences on starting mRNA concentrations. Gene expression was analyzed with the iCycler software program and the copy number of RNA for iNOS and EF-2 genes calculated by using standard calibration curve of plasmid which contains iNOS or EF-2 gene. The data was shown as a ratio of copy number for iNOS and EF-2 gene.

2.8. Western blot analysis

Cells for analyses of iNOS expression were lysed in RIPA lysis buffer [150 mM Tris–HCl, 150 mM NaCl, 5 mM EGTA, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40)] containing 1 mM DTT and protease inhibitor cocktail. Cell lysates were centrifuged at 12,000 × g for 10 min at 4 °C. Protein concentrations in supernatant were quantified by using Quick Start Bradford protein assay kit (Bio-Rad). Equal amounts of the soluble protein were resolved using 10% SDS-polyacrylamide gels and transferred to PVDF membranes. Non-specific bindings were blocked with 5% (w/v) non-fat dried milk solution dissolved in TBS-T buffer [10 mM Tris–HCl, pH 7.4, 100 mM NaCl and 1% (v/v) Tween 20] for 1 h at room temperature. Membranes were incubated with antibodies specific for β-actin, iNOS, NF-κB p65, lamin A, ERK1/2, p-ERK1/2, p38, p-p38, JNK or p-JNK at room temperature. Membranes were then incubated with horseradish peroxidase-conjugated secondary goat anti-mouse or goat anti-rabbit antibodies again for 1 h at room temperature. Specific protein bands on the PVDF membranes were visualized on X-ray film activated by chemiluminescence using Super Signal West Pico Chemiluminescent substrate. Intensities of each band signal were determined by densitometry using BIOPROFIL Bio-1D version 11.9 (Villber Lourmat, France).

For determination of MAPKs, phosphorylation was determined on cells scraped in the presence of 150 μL of ice-cold RIPA lysis buffer containing phosphatase inhibitor (50 mM β-glycerophosphate, 40 mM NaF, 2 mM Na_3VO_4) and a mixture of protease inhibitors cocktail. Nuclear protein extraction for analyses of NF-κB level was performed according to Srisook et al. (2011).

2.9. iNOS promoter based reporter gene assay

The plasmid containing murine iNOS promoter was constructed. The promoter sequence from −1595 to +169 (Lowenstein et al., 1993), was amplified from mouse genomic DNA using forward primer (sequence 5′–TAAGGTACCGAGGTTGACTTTGATATG CTG-3′) and reverse primer (sequence 5′–TAAAAGCTTTTGCAGTTGAC-TAGGCTACTC-3′). PCR product of iNOS promoter (1778 bp) was, then, cloned into pGEM-T easy vector and sequenced. After that, the clone with the correct sequence was digested with restriction enzymes at KpnI and HindIII sites and ligated into the KpnI and HindIII-digested pGL3-Basic vector containing luciferase reporter genes (piNOS luciferase report plasmid) before verified by DNA sequencing. The promoterless pGL3-Basic vector was used as a negative control plasmid for luciferase assays. RAW 264.7 cells were seeded in 24-well plates at 2×10^5 cells/well and grown in complete DMEM medium with antibiotic at 37 °C for 24 h. Cells were co-transfected with 0.5 μg of piNOS-luciferase reporter plasmids and 50 ng of pRL-TK plasmids (kindly provided by S. Jitrapakdee, Faculty of Science, Mahidol University) in Opti-MEM I using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. After 6 h of transfection, the cell culture media were replaced with complete DMEM and further incubated for 24 h. Macrophage cells were treated with 1 μg/mL of LPS in the presence or absence of EFPI for 6 h. Treated cells were lysed in 1 × *Renilla* luciferase assay lysis buffer. Equal amounts of total protein were subjected to luciferase reporter assay in a luminometer using luciferase reaction buffer (2 × luciferase buffer, 10 mM bettle luciferin, 10 mM adenosine-5′ -triphosphate (ATP), 10 mM Coenzyme A) while Renilla luciferase assay was evaluated using Renilla luciferase assay systems (Promega). An internal control plasmid, pRL-TK, was used to normalize reporter gene activity. iNOS promoter activity was obtained from normalization of iNOS-promotor-luciferase activity with Renilla luciferase activity. Level of induced luciferase activity was measured relative to that of promotorless pGL3-Basic vector treated cells without LPS.

2.10. Experimental animals

Male Sprague-Dawley rats weighing 40–60 g and 100–120 g were purchased from the National Laboratory Animal Center, Nakorn Pathom, Thailand. Animals were treated in accordance with the best practice ethical standards procedures approved by Animal Ethics Committees, Burapha University. All animals were given food and water *ad libitum* and kept in a room at 24 ± 2 °C, 40–60% relative humidity and a photoperiod of 12 h light–12 h dark for at least 7 days before starting experiments.

2.11. Drug administration

All test drugs and EFPI were dissolved in 5% (v/v) Tween 80, except in the ear edema model where they were dissolved in acetone. In carrageenan-induced hind paw edema model, the rats were orally administered with an equivalent volume of 0.5 mL/100 g body weight. For the ear edema model, a local application of test drugs and EFPI was administered to the outer and inner surfaces of the ear. Control animals received only the vehicle in an equivalent volume and the same route of administration as did the experimental group.

2.12. Ethyl phenylpropiolate (EPP)-induced ear edema in rats

Topical anti-inflammatory activity of EFPI was assessed as described by Brattsand et al. (1982) and slightly modified by Panthong et al. (2007). Male rats (40–60 g) were used and divided into three groups, each of six animals. Ear edema was induced by the topical application of EPP solution to the inner and outer surfaces of both ears using an automatic microliter pipette. Each ear received EPP 1 mg/20 μL, immediately after application of the vehicle, phenylbutazone or the EFPI. Ear thickness was measured with a Vernier caliper before and at 15, 30, 60 and 120 min after EPP application. The effect of EFPI on the ear edema was compared with that of the vehicle-control group, and expressed as relative inhibition.

2.13. Carrageenan-induced hind paw edema in rats

Carrageenan-induced paw edema was produced in rats by the method of Winter et al. (1962) and slightly modified by Panthong et al. (2007). Male rats (100–120 g) were used and divided into five groups (six per group). Carrageenan, 50 μL of 1%, was injected intradermally into the plantar of the right hind paw. Test drugs and vehicle were given orally 1 h before carrageenan injection. Paw volume was measured with a plethysmometer before as well as 1, 3 and 5 h after carrageenan injection. Results were compared with those from their control groups, which received only vehicle.

2.14. High performance liquid chromatography (HPLC) analysis of EFPI extract

Phytochemicals of EFPI were characterized by HPLC analysis. The system was equipped with a model Waters 600 HPLC pump and controller in-line degasser coupled to Waters 2487 dual lambda absorbance detector (all from Waters, USA), utilizing a LiChrocart 250-4 Purospher Star RP-18 endcapped column (250×4.6 nm I.D., particle size 5 μm, Merck, Germany). EFPI was dissolved in acetronitrile and filtered the solutions through 0.45 μm membrane filter before HPLC analysis. The compounds were eluted with 60% methanol/water for 15 min and then 100% methanol for 35 min at a flow rate of 0.7 mL/min. Column temperature was 25 °C and detection wavelength, 370 nm. For each experiment, 20 μL of standard or extract solution was injected. Standard compound quercetin was also analyzed by HPLC with the same conditions, and the retention time (*Rt*) was used to identify its presence in the extract.

2.15. Statistical analysis

Data are presented as mean ± S.D. Statistical significance was determined via two-tailed Student's *t*-test. Statistical comparison between groups was analyzed by one-way analysis of variance and *post hoc* least-significant difference test. Significance was accepted at $p < 0.05$.

3. Results

3.1. Effect of Pluchea indica leaf extracts on NO production in RAW 264.7 cells

The effect of each fraction of the ethanol *Pluchea.indica* leaf extracts (HFPI, EFPI and WFPI) on NO production in LPS-treated RAW 264.7 macrophage cells were examined by Griess reaction. Nitrite concentration in culture media of resting RAW 264.7 cells during incubation was 0.75 ± 0.28 μM, whereas that of cells treated with LPS (1 μg/mL) increased to 36.89 ± 5.46 μM. HFPI, EFPI and WFPI decreased LPS-stimulated NO production in a dose-dependent manner (Fig. 1) without significantly altering cell viability (Fig. 2). IC_{50} values of HFPI, EFPI and WFPI were 26.08 ± 4.90, 19.11 ± 2.58 and 33.17 ± 3.22 μg/mL, respectively. As a positive control, aminoguanidine (AG), a specific inhibitor of iNOS activity, also inhibited LPS-stimulated NO production in a concentration-dependent manner with an IC_{50} value of 20.66 ± 2.80 μM. In addition, nitrite concentration in cells incubated with all extracts alone did not increase significantly ($p < 0.05$) when compared to control cells. Among each fraction, EFPI exhibited the highest inhibitory effect on NO production comparable to that of aminoguanidine. Based on these results, EFPI was selected to determine the molecular mechanism underlying its anti-inflammatory effect in LPS-stimulated RAW 264.7 cells.

3.2. Inhibitory effect of EFPI on LPS-induced iNOS protein and mRNA expression

LPS treatment significantly increased iNOS protein expression whereas with EFPI it decreased significantly in a concentration-dependent manner (Fig. 3A). iNOS protein expression was undetected

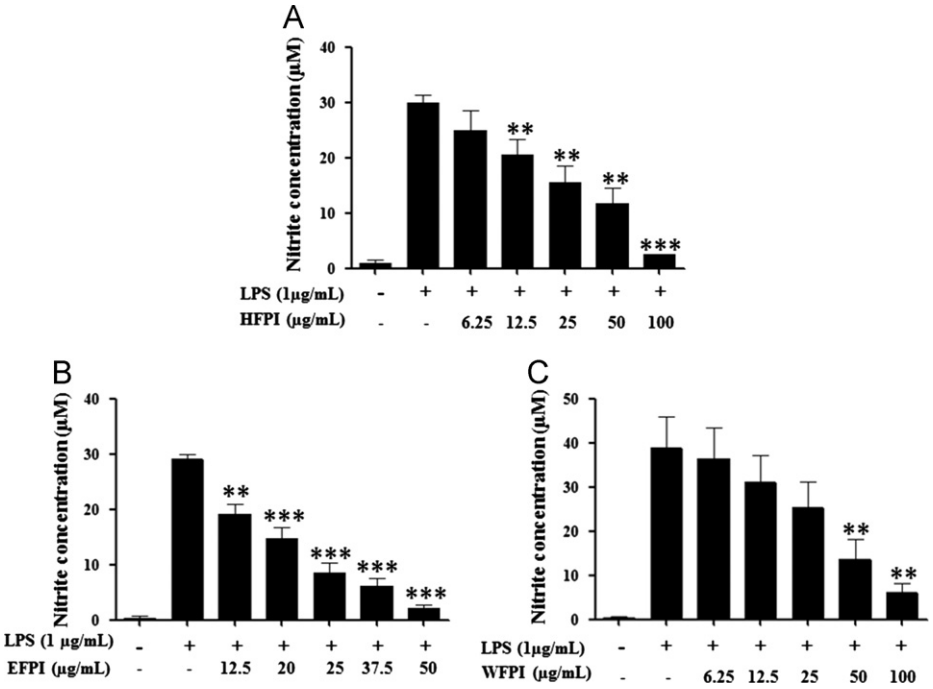

Fig. 1. Effect of *Pluchea indica* leaf extracts on nitrite production, (A) HFPI, (B) EFPI and (C) WFPI. Cells were co-incubated with the indicated concentrations of *Pluchea indica* leaf extracts and LPS (1 μg/mL) for 20 h. The nitrite concentration of culture media was analyzed by Griess reaction. Each column shows the mean ± SD of three independent experiments with triplicate samples. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs* LPS alone.

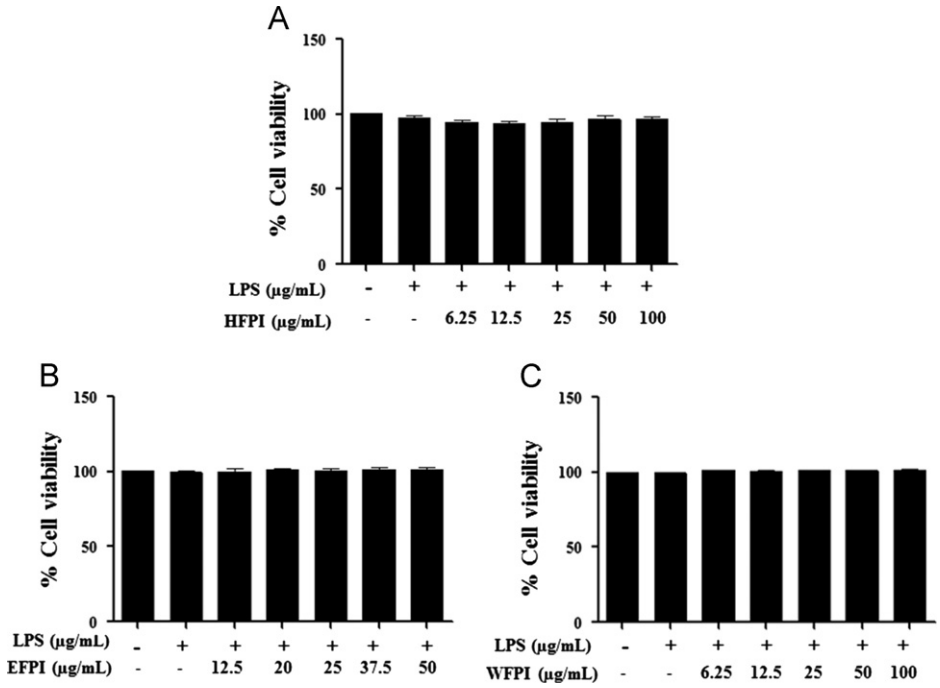

Fig. 2. Cell viability of *Pluchea indica* leaf extracts in LPS-stimulated RAW 264.7 cells was determined by MTT assay, (A) HFPI, (B) EFPI and (C) WFPI. Cells were treated with indicated concentrations of HFPI, EFPI and WFPI for 20 h. Each column shows the mean ± SD of three independent experiments, each with triplicate samples.

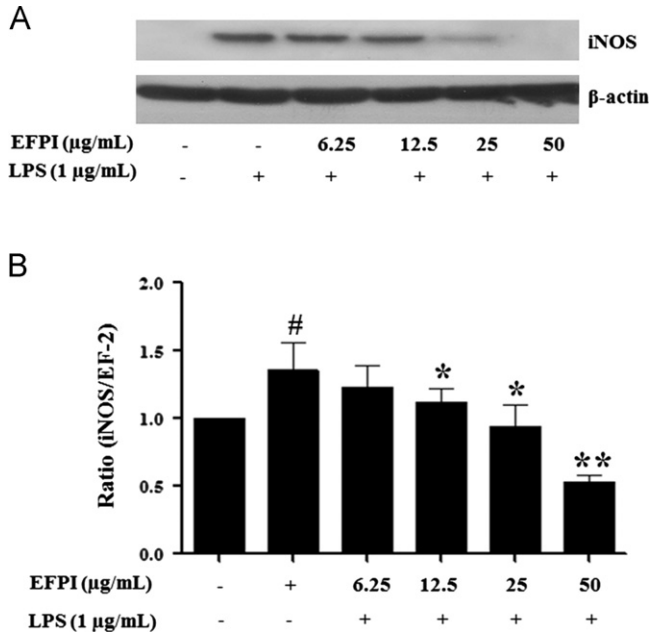

Fig. 3. Effect of EFPI on iNOS expression in LPS-induced RAW 264.7 cells. (A) Cells were stimulated with LPS (1 μg/mL) and indicated concentrations of EFPI for 24 h. Cells were lyzed, and iNOS protein was detected by Western blot analysis as described in Section 2. (B) Cells were treated with LPS (1 μg/mL) and EFPI (6.25 to 50 μg/mL) for 9 h. Total RNA was isolated, iNOS mRNA levels were determined by real-time RT-PCR. Each column shows the mean ± SD of three independent experiments with triplicate samples. $^{\#}p < 0.05$, significantly different from unstimulated macrophage cells. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* LPS alone.

in unstimulated cells and those treated only with EFPI. Expression of β-actin, an internal control, was not significantly affected by treatment with LPS or EFPI. mRNA level of iNOS in RAW 264.7 cells increased precipitously after stimulation with LPS. In the presence of EFPI, iNOS mRNA levels declined in a dose-dependent manner that was significant at concentration of 12.5–50 μg/mL (Fig. 3B). Content of EF-2 mRNA in each sample was used as an internal standard to compare with that of iNOS mRNA.

3.3. Effect of EFPI on nuclear translocation of NF-κB p65 subunit and iNOS promoter activity

The effects of EFPI on LPS-induced nuclear translocation of NF-κB p65 subunit was demonstrated by Western blot analysis of nuclear proteins. LPS (1 μg/mL) increased dramatically the level of NF-κB p65 subunit in the nucleus. NF-κB p65 subunit varied inversely with EFPI concentration. Lamin A, a nuclear protein was used as nuclear loading control. (Fig. 4A). Promoter activity of iNOS was low in untransfected cells and those treated with pGL3-Basic vector (Fig. 4B). The ratio of piNOS-LUC/pRL-TK increased significantly with LPS treatment, whereas iNOS promoter activity decreased with EFPI addition in a dose-dependent manner.

3.4. Effect of EFPI on LPS-induced phosphorylation of MAPKs

The effects of EFPI on LPS-induced phosphorylation of MAP kinase, ERK 1/2, p38 MAPK and JNK were examined by Western blot analysis as shown in Fig. 4. Phosphorylation of ERK 1/2, p38 MAPK and JNK occurred when RAW 264.7 cells were exposed to LPS and was not reduced in the presence of EFPI and LPS regardless of assessment concentration (Fig. 5). Therefore, decreased activity of EFPI on LPS-induced iNOS gene expression might not involve the MAPKs pathway.

3.5. Inhibitory effect of EFPI on LPS-induced PGE_2 production

PGE_2 production (5,220 ± 2,191 pg/mL) increased in LPS-treated cells but decreased with EFPI treatment in a concentration-dependent manner at 6.25–50 μg/mL ($p < 0.05$; Fig. 6). Culture media of unstimulated macrophages and cells treated only with EFPI contained low levels of PGE_2 (270 ± 122 and 59 ± 4 pg/mL, respectively). A non-selective COX inhibitor, indomethacin at 10 μM significantly reduced LPS-induced PGE_2 production in RAW 264.7 cells (70 ± 7 pg/mL). Treatment of RAW 264.7 cells with

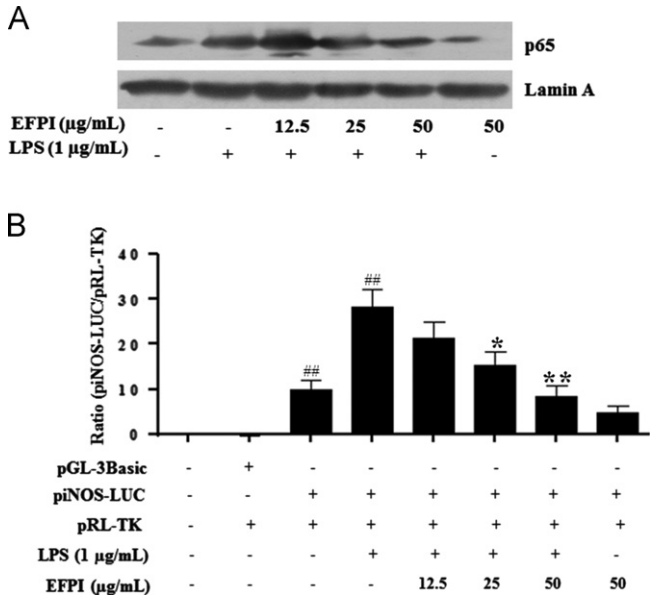

Fig. 4. Effect of EFPI on the activation of LPS-induced NF-κB and iNOS promotor. (A) The cells were stimulated with LPS (1 μg/mL) and EFPI for 2 h. Nuclear extracts were prepared and analyzed the levels of NF-κB p65 subunit by Western blot analysis. (B) The piNOS-LUC and pRL-TK-co-transfected cells were treated with LPS with or without EFPI at concentrations of 12.5, 25 and 50 μg/mL for 6 h. Cell were lyzed and iNOS promoter activities were determined by iNOS promoter based reporter gene assay. Each column shows the mean ± SD of four independent experiments with triplicate samples. $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from transfected RAW 264.7 macrophage cells treated with LPS. $^{\#\#}p < 0.05$, significantly different from untransfected macrophage cells.

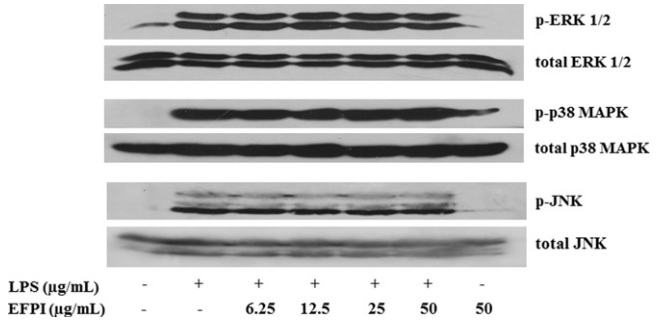

Fig. 5. Effects of EFPI on LPS-stimulated phosphorylation of MAPKs in RAW 264.7 macrophages. Cells were pre-treated with EFPI (6.25, 12.5, 25 and 50 μg/mL) for 1 h, and stimulated with LPS (1 μg/mL) for 30 min. Cells were lysed and determined phosphorylated ERK1/2, JNK and p38 MAPK by Western blot analysis.

indomethacin alone had no effect on basal levels of PGE_2 production (113 ± 16 pg/mL).

3.6. Inhibitory effect of EFPI on EPP-induced ear edema in rats

Ear edema thickness of control rats increased gradually during EPP treatment reaching a maximum at 30–60 min (Table 1). The edema was reduced with phenylbutazone at 1 mg/ear. EFPI at 3 mg/ear significantly inhibited EPP-induced ear thickness with the same intensity as phenylbutazone except 120 min after EPP application when its effect was weaker. However, the inhibitory effects of both EFPI and phenylbutazone persisted for the full assessment period.

3.7. Inhibitory effect of EFPI on carrageenan-induced paw edema in rats

The inhibitory activities of oral administration of EFPI and diclofenac on carrageenan-induced rat hind paw edema were assessed for 5 h. after treatment (Table 2). Subplantar injections of carrageenan in control rats produced a local edema that reached a maximal intensity 5 h after the injection of the phlogistic agent. The COX–inhibitor, diclofenac (10 mg/kg) significantly inhibited the carrageenan-induced paw edemas at all observation times. EFPI extract (150 mg/kg) slightly but significantly inhibited edema formation at the 3rd hour. Moreover, EFPI (300 mg/kg) significantly inhibited edema formation at the 1st and 3rd hours after carrageenan injection. In contrast, EFPI at 600 mg/kg markedly reduced edema formation induced by carrageenan at all assessment times. However, the inhibition decreases over the time with concentration of 600 mg/kg.

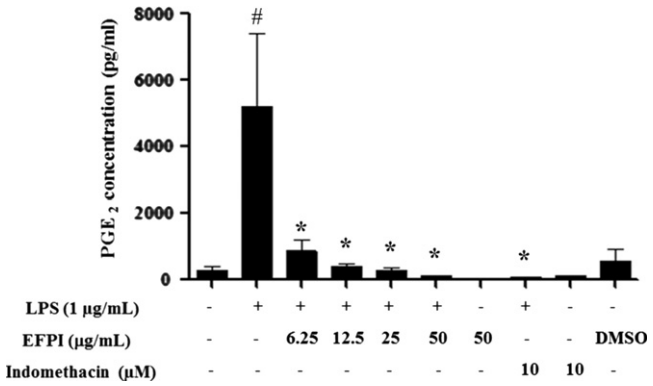

Fig. 6. Effects of EFPI on LPS-induced PGE_2 production in RAW 264.7 macrophages. Cells were treated with LPS (1 μg/mL) in the presence or absence of EFPI (6.25, 12.5, 25, 50 μg/mL) or indomethacin (COX-2 inhibitor), as the positive control at 10 μM for 20 h. After that the culture medium was collected to measure PGE_2 concentration. Each column shows the mean ± SD of two independent experiments with triplicate samples. $^{\#}p < 0.05$, significantly different from unstimulated macrophage cells. $^{*}p < 0.05$, significantly different from LPS-treated RAW 264.7 macrophage cells.

3.8. HPLC profile

A simple HPLC fingerprint was developed in this work. HPLC chromatograms at 370 nm showed that quercetin was present in EFPI (Fig. 7).

4. Discussion

Many plants used in traditional medicine exhibit pharmacological properties and may offer significant potential as therapeutic agents. However, some still lack scientific evidences of their mechanism of action. The leaves of *Pluchea.indica* have been used as a traditional medicine for the treatment of various diseases (Sen et al., 2002; Office of Mangrove Resources Conservation, 2009). A previous study reported that the methanol leaf extract of *Pluchea indica* inhibited LPS-induced NO production on RAW 264.7 macrophage cells (Choi and Hwang, 2005). In addition, the ethanol extract of *Pluchea indica* leaf exhibited anti-inflammatory activity in carrageenan-induced rat hind paw edema (Roslida et al., 2008). However, the mechanism underlying the anti-inflammatory action of the *Pluchea indica* leaf extract has not been described. In this study, the anti-inflammatory activity of the ethanol extract of *Pluchea indica* leaf proven on the carrageenan-induced rat hind paw edema can be explained by the involvement of the NF-κB pathway, whereas MAPKs are apparently not targeted.

Macrophages are the primary host defense against invading pathogens. RAW 264.7 is a murine macrophage cell line used to model macrophage-mediated inflammatory events *in vitro* (Hartley et al., 2008; Srisook et al., 2011) and, in the present study

Table 1
Effects of EFPI on EPP-induced ear edema in rats.

Group	Dose (mg/ear)	Time after topical application of EPP							
		15 min		30 min		60 min		120 min	
		ED (μm)	% EI	ED (μm)	% EI	ED (μm)	% EI	ED (μm)	% EI
Control	–	123.30 ± 5.16	–	166.7 ± 10.33	–	166.7 ± 15.06	–	143.3 ± 12.11	–
Phynylbutazone	1.0	3.30 ± 5.16**	97.30	25.00 ± 8.37**	85.00	43.30 ± 5.16**	74.00	48.30 ± 9.83**	66.28
EFPI	3.0	8.30 ± 7.53**	93.24	18.30 ± 7.53**	89.00	45.00 ± 5.48**	73.00	65.00 ± 5.48**	54.65

Values are expressed as mean ± SD (n=6). ED=edema thickness (μm) at time. % EI=percent edema inhibition of test substance at time.
Significantly different from the control group; **$p < 0.01$.

Table 2
Effects of EFPI on carrageenan-induced hind paw edema in rats.

Group	Dose (mg/kg)	Time after 1% carrageenan inject					
		1 h		3 h		5 h	
		EV (mL)	% EI	EV (mL)	% EI	EV (mL)	% EI
Control		0.36 ± 0.10	–	0.47 ± 0.07		0.50 ± 0.01	
Diclofenac	10	0.16 ± 0.05*	55.09	0.21 ± 0.10**	54.61	0.25 ± 0.10**	51.32
EFPI	150	0.23 ± 0.08	37.50	0.30 ± 0.05*	36.52	0.39 ± 0.10	23.51
EFPI	300	0.17 ± 0.05*	52.78	0.21 ± 0.10**	56.03	0.32 ± 0.10	36.09
EFPI	600	0.12 ± 0.08**	66.20	0.20 ± 0.05**	56.74	0.27 ± 0.10**	47.35

Values are expressed as mean ± SD (n=6). EV=edema volume (mL). % EI=percent edema inhibition of test substance at time.
Significantly different from control group; *$p < 0.05$, **$p < 0.01$.

to investigate the molecular mechanisms of macrophages involved in regulating immunity. LPS stimulation has been demonstrated to induce iNOS expression as well as to increase NO production, and prostaglandin synthesis in RAW 264.7 cells (Hseu et al., 2005; Israf et al., 2007; Cheon et al., 2009; Srisook et al., 2011). Our data show that HFPI, EFPI and WFPI suppress the production of NO in RAW 264.7 macrophage (Fig. 1). When macrophage cells were treated with *Pluchea.indica* leaf extracts, cell viabilities were more than 90%. This indicated that reduction of nitrite concentration was not a cytotoxic effect (Fig. 2). Moreover, changes in iNOS mRNA levels correlated directly with those of protein expression levels (Fig. 3). Thus, the inhibition of NO production by EFPI could be attributed to the inhibition of iNOS mRNA transcription level followed by protein expression. These results are in agreement with the finding of *Chrysanthemum indicum* Linné and *Chrysanthemum zawadskii* var. latilobum, (Asteraceae). The extract of *Chrysanthemum indicum* and *Chrysanthemum zawadskii* exhibited inhibitory effect on NO production through suppression of iNOS mRNA and protein expression in LPS-induced RAW 264.7 cells (Cheon et al., 2009; Kim et al., 2012).

NF-κB is a ubiquitous transcription factor that regulates the expressions of genes involved in inflammatory responses such as iNOS and COX-2 expression in LPS- stimulated cells (Hseu et al., 2005; Israf et al., 2007; Cheon et al., 2009). Therefore, blocking the NF-κB transcriptional activity in the macrophage nucleus can suppress the expression of inflammatory mediators. This study indicates that EFPI suppressed nuclear translocations of NF-κB, p65 subunit and iNOS promoter activity as shown in Fig. 4. These suggest that the target of EFPI inhibition was transcription of iNOS gene, possibly due to the suppression of the nuclear translocation of NF-κB p65 protein. Findings from this study indicated that EFPI inhibited the production of inflammatory mediators, NO and PGE_2, and the iNOS expression via reduction of NF-κB p65 subunit translocation. This is related with reports that demonstrated the various plant extracts that produce an anti-inflammatory effect by inhibiting NO formation through inactivation of NF-κB translocation in LPS-induced RAW264.7 cells (Cheon et al., 2009; Lee et al., 2011; Srisook et al., 2011). MAPKs are a highly conserved family of protein serine/threonine kinases and include the p38, ERK1/2 and JNK subgroups. Several studies have shown that the activation of MAPKs is involved in LPS-induced iNOS expression and NF-κB activation (Nakano et al., 1998; Carter et al., 1999). The failure of EFPI to inhibit phosphorylation of ERK1/2, p38 MAPK and JNK (Fig. 5) suggests the inhibition of its iNOS gene expression is not mediated through the regulation of MAPKs signaling pathways.

EFPI exhibited effective anti-inflammatory action on EPP-induced ear and carrageenan-induced paw edema. EPP-induced ear edema in rat model is a useful screening model to investigate the anti-inflammatory activity of the test substance on the acute phase of inflammation. Carrageenan-induced paw edema, a classical model of acute inflammation, has been widely used in the discovery and evaluation of anti-inflammatory agents, since the relative potency estimates obtained from most drugs tend to reflect clinical experience (Panthong et al., 2007; Sae-Wong et al., 2009). The edema or swelling, one of the cardinal signs of acute inflammation, is an important parameter to be considered when evaluating agents with potential anti-inflammatory activity (Morris, 2003). Carrageenan-induced edema causes the release of several inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins. The development of an edema induced by carrageenan is a three phase event; the first occurring between 0 and 1.5 h after injection, is caused by the release of histamine and serotonin, the second is mediated by bradykinin after 1.5–2.5 h and finally, the third phase, after 2.5–6 h, has been correlated with an increased production of prostaglandins (Sae-Wong et al., 2009). Moreover, NO is also released in carrageenan-induced inflammation (Salvemini et al., 1996). The result of our study found EFPI at higher doses of 300 and 600 mg/kg has an inhibitory effect on edema formation in all three phases of the carrageenan-induced rat paw edema (Table 2), similar to diclofenac, a reference drug. This is consistent with a previous

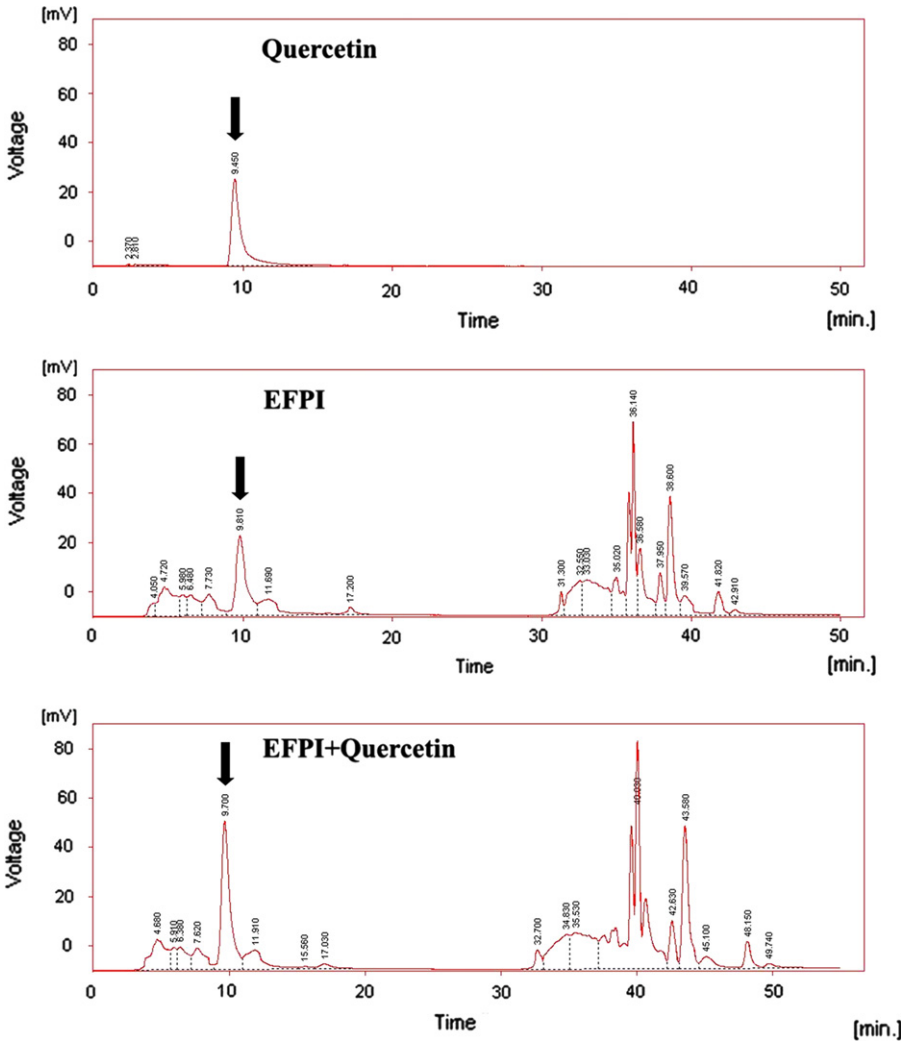

Fig. 7. High performance liquid chromatography chromatogram of the standard quercetin, EFPI and EFPI+ quercetin detected at 370 nm.

study in which *Pluchea indica* ethanol leaf extract at a maximum dose, 300 mg/kg, exhibited anti-inflammatory activity when administered orally on carrageenan-induced rat hind paw edema during the whole experiment (6 h) (Roslida et al., 2008). This action of EFPI might be due to its inhibition and/or release of inflammatory mediators. However, the results from LPS-induced PGE_2 and NO release in RAW 264.7 macrophages indicate that the anti-inflammatory mechanism of EFPI on acute inflammation is attributed to the inhibition of PGE_2 and NO. Oral administration of all test doses of the plant extract caused significant decrease in edema volume at 3 h after carrageenan injection except the dose of 600 mg/kg continued to possess anti-edematous effect after 3 h. The onset of an anti-inflammatory effect at a low dose (150 mg/kg) was delayed (3 h), whereas that at high dose (300 and 600 mg/kg) was faster (1 h). This is probably associated with low systemic bioavailability of EFPI after oral administration due to slow absorption of the extract from intestinal tract and some degree of gastrointestinal inactivating metabolism. However, the anti-inflammatory activity of the *Pluchea indica* leaf extract found in the present study seems to support the use of this plant in traditional medicine.

The chemical composition of *Pluchea indica* indicates the presence of eudesmane derivatives, terpene glycosides, thiophene derivatives and lignan glycosides in aerial parts and roots (Mukhopadhyay et al., 1983; Uchiyama et al., 1989, 1991; Chakravarty and Mukhopadhyay, 1994; Biswas et al.,2007) and quercetin and chlorogenic acid in leaves (Ohtsuki et al., 2008; Shukri et al. 2011). Many sesquiterpenes can be found in the numerous genera of the family Asteraceae including the genus *Pluchea* (Mukhopadhyay et al., 1983; Uchiyama et al., 1991; Wu et al., 2006). They display diverse biological activities such as anti-inflammatory, antibacterial, antimalarial, antifungal and antitumor activities (Valério et al., 2007; Chaturvedi, 2011; Cheng et al., 2011). Thus, anti-inflammatory activity of *Pluchea indica* might be attributed to its contents of sesquiterpenes. Quercetin has been demonstrated to possess anti-inflammatory activities (Lin et al., 2003; Garcia-Mediavilla et al., 2007). From HPLC analysis, quercetin was found in the extract and could be, at least in part, responsible for the anti-inflammatory effect of EFPI. However, further investigations are required to determine the active compounds other than quercetin which responsible for the potent anti-inflammatory activity of EFPI.

5. Conclusion

This study showed that EFPI exhibited anti-inflammatory effects not only in LPS-induced RAW 264.7 macrophages but also in acute inflammation rat models. Moreover, we provided the mechanism underlying the anti-inflammatory activity of EFPI by inhibition of NO production and iNOS suppression. These activities appeared to be mediated via the suppression of NF-κB activation but not

phosphorylation of MAPKs. Nevertheless, these results supported the effectiveness of traditional use of *Pluchea indica* for treatment of inflammatory diseases.

Acknowledgements

The work was financially supported by Faculty of Science, Burapha University and the Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education, Thailand. Authors are grateful to Professor Frederick W.H. Beamish, Faculty of Science, Burapha University, for his comments and English correction. We acknowledge for valuable technical advices in animal models of inflammation of Associate Professor Ampai Panthong and Dr. Natthakarn Chiranthanut, Department of Pharmacology, Faculty of Medicine, Chiang Mai University.

References

Alderton, W.K., Cooper, C.E., Knowles, R.G., 2001. Nitric oxide synthases: structure, function and inhibition. Biochemical Journal 357, 593–615.

Biswas, R., Dutta, P.K., Achari, B., Bandyopadhyay, D., Mishra, M., Pramanik, K.C., Chatterjee, T.K., 2007. Isolation of pure compound R/J/3 from *Pluchea indica* (L.) Less. and its anti-amoebic activities against *Entamoeba histolytica*. Phytomedicine 143, 534–537.

Brattsand, R., Thalen, A., Roempke, K., Kallstrom, L., Gruvstad, E., 1982. Influence of 16α, 17α-acetal substitution and steroid nucleus fluorination on the topical to systemic activity ratio of glucocorticoids. Journal of Steroid Biochemistry 16, 779–786.

Carter, A.B., Knudtson, K.L., Monick, M.M., Hunninghake, G.W., 1999. The p38 mitogen activated protein kinase is required for NF-κB-dependent gene expression. The role of TATA-binding protein (TBP). Journal of Biological Chemistry 274, 30858–30863.

Chaturvedi, D., 2011. Sesquiterpene lactones: structural diversity and their biological activities. In: Tiwari, V.K. (Ed.), Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry. Research Signpost, Kerala, pp. 313–334.

Chakravarty, A.K., Mukhopadhyay, S., 1994. New thiophene derivatives from *Pluchea indica*. Indian Journal of Chemistry 33, 978–980.

Chen, B.C., Chen, Y.H., Lin, W.W., 1999. Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophage. Immunology 97, 124–129.

Cheng, X., Zeng, Q., Ren, J., Qin, J., Zhang, S., Shen, Y., Zhu, J., Zhang, F., Chang, R., Zhu, Y., Zhang, W., Jin, H., 2011. Sesquiterpene lactones from *Inula falconeri*, a plant endemic to the Himalayas, as potential anti-inflammatory agents. European Journal of Medicinal Chemistry 46, 5408–5415.

Cheon, M.S., Yoon, T., Lee, D.Y., Choi, G., Moon, B.C., Lee, A.Y., Choo, B.K., Kim, H.K., 2009. *Chrysanthemum indicum* Linne extract inhibits the inflammatory response by suppressing NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. Journal of Ethnopharmacology 122, 473–477.

Choi, M.E., Hwang, K.J., 2005. Screening of Indonesian medicinal plants for inhibitor activity on nitric oxide production of RAW 264.7 cells and antioxidant activity. Fitoterapia 76, 194–203.

Dorheim, M.A., Tracey, W.R., Pollock, J.S., Grammas, P., 1994. Nitric oxide synthase activity is elevated in brain microvessels in Alzheimer's disease. Biochemical and Biophysical Research Communication 205, 659–665.

Garcia-Mediavilla, V., Crespo, I., Collado, P.S., Esteller, A., Campos, S.S., Tunon, M.J., Gallego, J.G., 2007. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of indicible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. European Journal of Pharmacology 557, 221–229.

Guzik, T.J., Korbut, R., Adamek-Guzik, T., 2003. Nitric oxide and superoxide in inflammation and immune regulation. Journal of Physiology and Pharmacology 54, 469–487.

Hartley, J.W., Evans, L.H., Green, K.Y., Naghashfar, Z., Macias, A.R., Zerfas, P.M., Ward, J.M., 2008. Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. Retrovirology 5, 1–6.

Hseu, Y.C., Wu, F.Y., Wu, J.J., Chen, J.Y., Chang, W.H., Lu, F.J., Lai, Y.C., Yang, H.L., 2005. Anti-inflammatory potential of *Antrodia camphorata* through inhibition of iNOS, COX-2 and cytokines via the NF-κB pathway. International Immunology 5, 1914–1925.

Israf, D.A., Khaizurin, T.A., Syahida, A., Lajis, N.H., Khozirah, S., 2007. Cardamonin inhibits COX and iNOS expression via inhibition of p65NF-κB nuclear translocation and I-κB phosphorylation in RAW264.7 macrophage cells. Molecular Immunology 44, 673–679.

Kim, S.H., Kim, J., Sharma, R.P., 2004. Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. Pharmacological Research 49, 433–439.

Kim, S.H., Shin, T.Y., 2009. Anti-inflammatory effect of leaves of *Eriobotrya japonica* correlating with attenuation of p38 MAPK, ERK, and NF-κB activation in mast cells. Toxicology *In Vitro* 23, 1215–1219.

Kim, Y.H., Han, J.Y., Sung, J.H., Sung, M.S., Choi, Y.M., Jeong, H.S., Lee, J.S., 2012. Anti-inflammatory activity of *Chrysanthemum zawadskii* var. latilobum leaf extract through haem oxygenase-1 induction. Journal of Functional Foods 4, 474–479.

Kumar, V., Abbas, A.K., Fausto, N., Mitchell, R.N., 2007. Robbins Basic Pathology, eight ed. Elsevier Saunders, United States of America.

Latham, K.A., Whittington, K.B., Zhou, R., Qian, Z., Rosloniec, E.F., 2005. *Ex vivo* characterization of the autoimmune T cell response in the HLA-DR1 mouse model of collagen-induced arthritis reveals long-term activation of type II collagen-specific cells and their presence in arthritic joints. Journal of Immunology 174, 3978–3985.

Lee, M.Y., Lee, J.A., Seo, C.S., Ha, H., Lee, H., Son, J.K., Shin, H.K., 2011. Anti-inflammatory activity of *Angelica dahurica* ethanolic extract on RAW24.7 cells via upregulation of heme oxygenase-1. Food and Chemical Toxicology 49, 1047–1055.

Lin, H.Y., Juan, S.H., Shen, S.C., Hsu, F.L., Chen, Y.C., 2003. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. Biochemical Pharmacology 66, 1821–1832.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., Murphy, W.J., 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proceedings of the National Academy of Sciences of the United States of America. 90, 9730-9734.

Mequanint, W., Makonnen, E., Urga, K., 2011. *In vivo* anti-inflammatory activities of leaf extracts of *Ocimum lamiifolium* in mice model. Journal of Ethnopharmacology 134, 32–36.

Morris, C.J., 2003. Carrageenan-induced paw edema in the rat and mouse. Method in Molecular Biology 225, 115–121.

Mukhopadhyay, S., Cordell, A.G., Ruangrungsi, N., Rodkird, S., Tantivatana, P., Hylands, P.J., 1983. Traditional medicanal plants of Thailand. IV. 3-(2′,3′-Diacetoxy-2′-methyl butyryl)-cuauhtehmone from *Pluchea indica*. Journal of Natural Products 46, 671–674.

Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., Okumura, K., 1998. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-κB inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proceedings of the National Academy of Sciences of the United States of America 95, 3537–3542.

Noridayu, A.R., Hii, H.F., Faridah, A., Khozirah, S., Lajis, N., 2011. Antioxidant and antiacetylcholinesterase activities of *Pluchea indica* Less. International Food Research Journal 18, 925–929.

Office of Mangrove Resources Conservation, 2009. Department of Marine and Coastal Resources, Ministry of Natural Resources and Environment. Plants in the Mangrove forest of Thailand. Printing House of the Agricultural Co-operative Federation of Thailand Limited, Nonthaburi, (in Thai).

Ohtsuki, T., Yokosawa, E., Koyano, T., Preeprame, S., Kowithayakorn, T., Sakai, S., Toida, T., Ishibashi, M., 2008. Quinic acid esters from *Pluchea indica* with collagenase, MMP-2 and MMP-9 inhibitory activities. Phytotherapy Research 22, 264–266.

Panthong, A., Supraditaporn, W., Kanjanapothi, D., Taesotikul, T., Reutrakul, V., 2007. Analgesic, anti- inflammatory and venotonic effects of *Cissus quadrangularis* Linn. Journal of Ethnopharmacology 110, 264–270.

Roslida, A.H., Erazuliana, A.K., Zuraini, A., 2008. Anti-inflammatory and antinociceptive activities of the ethanolic extract of *Pluchea indica* (L) Less leaf. Pharmacologyonline 2, 349–360.

Sae-Wong, C., Tansakul, P., Tewtrakul, S., 2009. Anti-inflammatory mechanism of *Kaempferia parviflora* in murine macrophage cell (RAW264.7) and in experimental animals. Journal of Ethnopharmacology 124, 576–580.

Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., Currie, M.G., 1996. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. British Journal of Pharmacology 118, 829–838.

Shukri, M.A.M., Alan, C., Noorzuraini, A.R.S., 2011. Polyphenols and antioxidant activities of selected traditional vegetables. Journal of Tropical Agriculture and Food Science 39, 1–15.

Sen, T., Dhara, A.K., Bhattacharjee, S., Pal, S., Chaudhuri, A.K., 2002. Antioxidant activity of the methanol fraction of *Pluchea indica* root extract. Phytotherapy Research 16, 331–335.

Sen, T., Ghosh, T.K., Nag Chaudhuri, A.K., 1993. Studies on the mechanism of anti-inflammatory and anti-ulcer activity of *Pluchea indica*-probable involvement of 5-lipoxygenase pathway. Life Sciences 52, 737–743.

Srisook, K., Buapool, D., Boonbai, R., Simmasut, P., Charoensuk, Y., Srisook, E., 2012. Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less herbal tea. Journal of Medicinal Plants Research 6, 4077–4081.

Srisook, K., Cha, Y.N., 2005. Superinduction of heme oxygenase-1 in macrophages stimulated with lipopolysaccharide by prior depletion of glutathione decreases iNOS expression and NO production. Nitric Oxide: Biology and Chemistry 12, 70–79.

Srisook, K., Palachot, M., Mongkol, N., Srisook, E., Sarapusit, S., 2011. Anti-inflammatory effect of ethyl acetate extract from *Cissus quadrangularis* Linn may be involved with induction of heme oxygenase-1 and suppression of NF-κB activation. Journal of Ethnopharmacology 133, 1008–1014.

Uchiyama, T., Miyase, T., Ueno, A., Usmanghani, K., 1989. Terpenic glycosides from *Pluchea indica*. Phytochemistry 28, 3369–3372.

Uchiyama, Y., Miyase, T., Ueno, A., Usmanghani, K., 1991. Terpene and lignan glycosides from *Pluchea indica*. Phytochemistry 30, 655–657.

Valério, D.A., Cunha, T.M., Arakawa, N.S., Lemos, H.P., Da Costa, F.B., Parada, C.A., Ferreira, S.H., Cunha, F.Q., Verri Jr., W.A., 2007. Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: inhibition of cytokine production-dependent mechanism. European Journal Pharmacology 562, 155–163.

Winter, C.A., Risley, E.A., Nuss, G.W., 1962. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drug. Proceedings of the Society for Experimental Biology and Medicine 111, 544–547.

Wright, C.E., Rees, D.D., Moncada, S., 1992. Protective and pathological roles of nitric oxide in endotoxin shock. Cardiovascular Research 26, 48–57.

Wu, Q.X., Shi, Y.P., Jia, Z.J., 2006. Eudesmane sesquiterpenoids from the Asteraceae family. Natural Product Reports 23, 699–734.

Yang, H., Young, D.W., Gusovsky, F., Chow, J.C., 2000. Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. Journal of Biological Chemistry 27, 20861–20866.