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Lemon Pepper Fruit Extract (*Zanthoxylum acanthopodium* DC.) Suppresses the Expression of Inflammatory Mediators in Lipopolysaccharide-Induced Macrophages *In Vitro*

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Abstract: Problem statement: Lemon pepper fruits (*Zanthoxylum acanthopodium* DC.; Rutaceae) have been used as a traditional source against stomach ache by Batak people in North Sumatera province, Indonesia. However, its scientific evidence for treatment of inflammatory disorders particularly gastritis has not been reported. **Approach:** Here, we investigated the inhibitory effects of Lemon Pepper Fruit Extract (LPFE) against inflammatory biomarkers by conducting cell culture experiments *in vitro*. The fruits of lemon pepper were dried and extracted twice in 70% ethanol, followed by evaporation and freeze-drying. The concentrated extract was further tested for its potential inhibition on the protein and gene expression of several inflammatory biomarkers, i.e., Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, inducible Nitric Oxyde Synthase (iNOS), Cyclooxygenase (COX)-2 and Matrix Metalloproteinase (MMP)-9, in lipopolysaccharide (LPS)-induced macrophages by performing Western blot, gelatin zymography and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). **Results:** LPFE (1-10 µg mL^{-1}) and LPS (2 µg mL^{-1}) had no cytotoxicity effects on macrophages. LPFE dose dependently decreased the expression of TNF-α and COX-2 proteins and MMP-9 activity in macrophages treated with LPS. At the gene level, LPFE were effectively found to block the mRNA expression of TNF-α, IL-6, iNOS, COX-2 and MMP-9. **Conclusion:** Our results suggest that LPFE significantly inhibits selected inflammatory biomarkers at the protein and gene levels in LPS-induced macrophages. Further *in vivo* study using animal models is needed to determine the exact anti-inflammatory potential of LPFE.

Key words: Lemon Pepper Fruit Extract (LPFE), *Zanthoxylum acanthopodium* DC., inflammatory mediators, lipopolysaccharide, macrophages

INTRODUCTION

Gastrointestinal inflammation or stomach ache may be caused by infection of pathogenic foodborne bacteria. Imbalance in gut microbiota population may lead to the production of toxin by pathogens. In normal condition, the population of beneficial bacteria suppresses the number of pathogen. However, the sudden increase in the number of pathogens leads to the colonization of intestine (Lakatos *et al*., 2006). The release of toxin by pathogen leads to response by immune system causing inflammation following the release of immune cells. Severe case of inflammation causes overexpression of proinflammatory proteins and cytokines leading to pain. During inflammation, macrophages play a key role in the immune response to infectious foodborne pathogens through the excessive production of proinflammatory cytokines (Interleukin (IL)-1β, IL-6 and Tumor Necrosis Factor (TNF)-α) and proteins (inducible Nitric Oxide Synthase (iNOS), Prostaglandin (PGE_2), cyclooxygenase (COX)-2 and matrix metalloproteinase (MMP)-9) (Gallin and Snyderman, 1999).

COX with its two is forms (COX-1 and COX-2) is known as the main enzyme responsible for the conversion of arachidonic acid to prostaglandins, which pleiotropic mediators of inflammatory responses. Nitrix Oxide (NO) is synthesized from arginine by iNOS. Similar to COX-2 and TNF-α, iNOS and MMP-9 are also induced by bacterial end toxin and inflammatory cytokines and the NO produced mediates killing of the invading foodborne pathogens (Park *et al*., 2009). In occasion whether the overexpression of iNOS occurred

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at high NO levels, it strongly promoted inflammatory disorders. Meanwhile, the 92 kDa MMP-9 degraded extracellular matrix proteins during inflammation, thus making them loose to facilitate the transport of leukocytes from blood vessels to the infected area (St-Pierre *et al*., 2003). Thus, downregulation of these proinflammatory molecules by specific therapeutic agents may exert anti-inflammatory properties.

Several therapeutic strategies for management of inflammation-related disorders particularly gastrointestinal inflammation have been widely reported, either by inhibiting the growth of pathogenic bacteria or suppressing the overexpression of immune cells. The use of antibiotics is common to treat bacterial infection, while synthetic drugs especially from the group of Non-Steroidal Anti-Inflammatory Drug (NSAID) are often used to treat inflammation. These methods however possess several negative side-effects such as antibiotic resistance and cardiovascular disease (Liang *et al*., 2007). This condition leads to the shift of focus in drug development moving from synthetic chemical compound to natural products. The use of natural fruits and herbs by native people to treat disease shows the potential of the discovery of novel potent yet safe bioactive compound to be processed into drug.

Zanthoxylum acanthopodium DC., known as andaliman or lemon pepper and belong to endemic plant in North Sumatera region, has been traditionally used as Bataknese culinary and folk medicine for treatment of diarrhea and stomach ache. Yang reported that the fruits of *Z. bungeanum* and *Z. schinifolium* have a high content of essential oils, such as lineally acetate, linalool, limonene and sabinene, which is decribed as having fresh, spicy, floral, cooling and green aroma notes (Yang, 2008). Other research groups found that the fruit, leaf and root of various *Zanthoxylum* species also contained neolignans, alkaloids, geranyl acetate, amides and benzenoids which were potential used for treatment of many chronic inflammatory disorders (Chen *et al*., 2008; 2009; Yang and Chen, 2008). However, there is no scientific evidence about the actual activity of Lemon Pepper Fruit Extract (LPFE) for treatment of inflammatory-related diseases including gastrointestinal inflammation. In this study, we investigated the efficacy of LPFE on blocking the expression of inflammatory cytokines and enzymes in macrophages induced by foodborne infection *in vitro*.

MATERIALS AND METHODS

Plant material and extraction: The lemon pepper fruits (*Zanthoxylum acanthopodium* DC.) were collected from traditional markets in the Central Tapanuli region, North Sumatera. The fruits were identified by Herbarium Bogoriense, Bogor Botanical Garden, Bogor, Indonesia and stored at Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta, Indonesia (Voucher specimen No. LY25). The dried fruits were extracted two times with 70% ethanol at room temperature for 3 days each and the combined extracts were concentrated in vacuo and freeze dried (yield: 10% w/w).

Cell culture and cell viability: RAW 264.7 cells (mouse macrophage-like cell line; ATCC TIB-71) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/mL of penicillin and 100 μg Ml^{-1} of streptomycin as previously described by Chi *et al*. (2001). Cells were incubated in the presence of 5% CO_2 at 37°C. The cells (passage 7-12) were seeded at a concentration of 2×10^5 cells mL^{-1} 75-cm^2 flask and cultured for 24 h. Cells were then activated with *Escherichia coli* lipolysaccharide (LPS) to enhance the production of inflammatory cytokines and proteins, such as TNF-α, IL-6, COX-2 and iNOS.

The effects of lipopolysaccharide and LPFE on cell viability were evaluated with the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-Tetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). LPFE was dissolved in 100% DMSO and the stock solution of the extract at a concentration of 1.000 μg mL^{-1} was prepared in 10% DMSO. The final concentrations of the extract ranged from 1-50 μg mL^{-1} in the culture media and all cells were treated with DMSO at a final concentration of 0.1%.

Sample treatment: Cells were seeded at a concentration of 2×10^5 cells mL^{-1} in 6-well plates and cultured for 24 h in DMEM-FBS. After washing with Dulbecco's Phosphate-Buffered Saline (DPBS), the cells were incubated in serum free-DMEM without LPS (negative control group), with 2 μg mL^{-1} LPS (positive control group), or with 2 μg mL^{-1} LPS plus treatment for 24 h. The treatment group is LPFE at various concentrations of 1-10 μg mL^{-1}. Cellular lysates were collected for further experiments.

Gelatin zymogram: Activity of MMP-2 and MMP-9 in the conditioned medium was measured by gelatin zymography. Briefly, the conditioned media from the negative control, positive control and treatment group (LPFE) were collected and subjected to electrophoresis with 10% SDS polyacrylamide gels containing 0.1% gelatin. Electrophoresis was run at 90 V for 1.5 h in an electrophoretic apparatus. After electrophoresis, gels

were washed twice with 25 mL of 2.5% Triton X-100 on a gyratory shaker for 1 h at room temperature to remove SDS. The gel was then incubated in 50 mL reaction buffer (50mM Tris-HCl, pH 7.5, 10mM CaCl2, 0.15 M NaCl) at 37°C for 18 h, stained with Coomassie brilliant blue R-250 and destained with methanol acetic acid in water. MMP-2 and MMP-9 were detected at 72 and 92 kDa as clear zones against the dark background.

Western blot: To determine the protein expression of TNF-α, COX-2 and iNOS, cellular lysates from the negative control, positive control and treatment groups were assayed by Western blot analysis. Protein concentrations were measured using Bradford reagent. Equal concentration of protein (30 µg) was resolved by 8-10% SDS-PAGE and transferred to nitrocellulose transfer membranes for Western blot analysis. The membranes were blocked with 5% skim milk for 1 h at room temperature and then probed with the primary anti-goat polyclonal antibodies to TNF-α and COX-2 (Santa Cruz, CA, USA) and anti-rabbit polyclonal antibody against iNOS (Santa Cruz, CA, USA) at a 1:1000 dilution overnight at 4°C. After 3 washes, the blots were subsequently incubated with the secondary antibody peroxidase-conjugated anti-goat IgG or anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) at a 1:5000 dilution for 2 h at room temperature. The blots were stained with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and visualized using an LAS 3000 Bio Imaging Analysis System (Lab Science, Fuji Film, Tokyo, Japan).

Reverse trancription-polymerase chain reaction (RT-PCR): The gene expression of TNF-α, IL-6, COX-2 and iNOS were determined by RT-PCR. Total RNA from the negative control, positive control and treatment groups was extracted using Trizol reagent according to the manufacturer's protocol and quantified spectrophotometrically at 260/280 nm.

Table 1: Mouse oligonucleotide primers used for RT-PCR

Gene	Sense (5-3') and antisense (5'-3') primers	Size (bp)	Annealing temp (°C)
TNF-α	cctgtagcccacgtcgtagc		
	ttgacctcagcgctgagttg	374	55
IL-6	gatgctaccaaactggatataatc		
	ggtccttagccactccttctgtg	269	55
COX-2	cctgtgttccaccaggagat		
	gtccctggctagtgcttcag	249	58
iNOS	ctgcagcacttggatcaggaacctg		
	gggagtagcctgtgtgcacctggaa	311	55
MMP-9	gagaccggtgagctggatag		
	tacacgcgagtgaaggtgag	236	58
Beta-actin	tggaatcctgtggcatccatgaaac		
	taaaacgcagctcagtaacagtccg	349	55

The oligonucleotide primers for TNF-α, IL-6, COX-2, iNOS, MMP-9 and beta-actin were designed according to a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database (Table 1).

PCR consisted of 27 amplification cycles and each cycle was carried out for 30 s at 94°C, 1 min at annealing temperature (55°C for TNF-α, IL-6, iNOS and beta-actin and 58°C for COX-2 and MMP-9) and 1 min at 72°C in a thermal cycler (Gene Amp PCR System 2700, Applied Biosystems, CA, USA). The beta-actin housekeeping gene was used as an internal control to standardize the relative expression levels for all biomarkers. PCR products were separated electrophoretically on a 1.5% agarose DNA gel and stained with ethidium bromide. The stained gel was visualized by using Gel-Doc Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis: Triplicate experiments were performed throughout this study. All data were presented as the mean ± Standard Deviation (SD). The significant difference between control and treated groups were analyzed by the paired Student's t-test ($p<0.05$).

RESULTS

Effects of LPFE on the protein and gene expression of inflammatory cytokines (TNF-α and IL-6) in LPS-induced macrophages: MTT colorimetric assay (Fig. 1) demonstrated that both LPFE (1-10 µg mL^{-1}) and LPS (2 µg mL^{-1}) did not show any cytotoxic effects in the macrophages *in vitro*. LPS at 2 µg mL^{-1} significantly resulted in the increased level of TNF-α protein and mRNA in the macrophages as compared with the untreated control (Fig. 2a). LPS also induced the expression of IL-6 mRNA in the cell system (Fig. 2b).

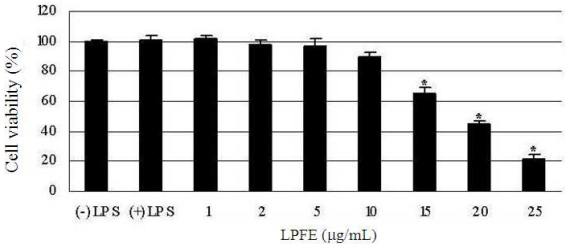

Fig. 1: Effects of LPS (2 µg mL^{-1}) and LPFE (1-25 µg mL^{-1}) on macrophage cell viability. Values represent the mean ± SD of triplicate experiments. * indicates $p<0.05$ against LPS-treated cells

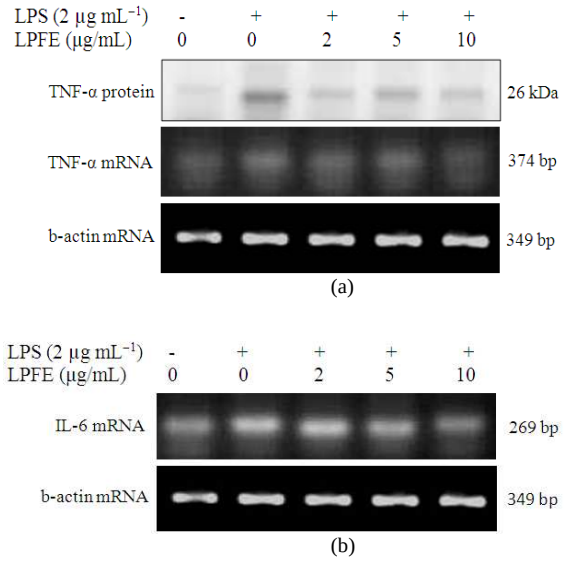

Fig. 2: Effects of LPFE on the protein and mRNA expression of TNF-α (a) and IL-6 (b) in LPS-induced macrophages assayed by Western blot and RT-PCR. Beta-actin was used as the internal control

Furthermore, we tested whether LPFE has anti-inflammatory potential in the expression of inflammatory cytokines in LPS-induced macrophages. As shown in Fig. 2a, treatment with LPFE (2, 5 and 10 µg mL^{-1}) dose-dependently inhibited the expression of TNF-α protein and mRNA as compared to the LPS treatment alone. In addition, LPFE also modulated the mRNA expression of IL-6 in macrophages treated with LPS (Fig. 2b).

Effects of LPFE on the protein and gene expression of inflammatory proteins (MMP-9, COX-2 and iNOS) in LPS-induced macrophages: Figure 3 showed that LPFE modulated the expression of inflammatory proteins in LPS-induced macrophages (Fig. 3). LPS significantly elevated the expression of inflammatory proteins (COX-2 and iNOS) and MMP-9 activity in macrophages. LPFE at various concentrations (2, 5 and 10 µg mL^{-1}) inhibited the expression of MMP-9 activity and mRNA compared to that of the untreated control (Fig. 3a). Cells treated with LPFE in presence of LPS were effectively found to reduce the protein and mRNA levels of COX-2 (Fig. 3b) and iNOS (Fig. 3c) as compared to LPS treatment alone.

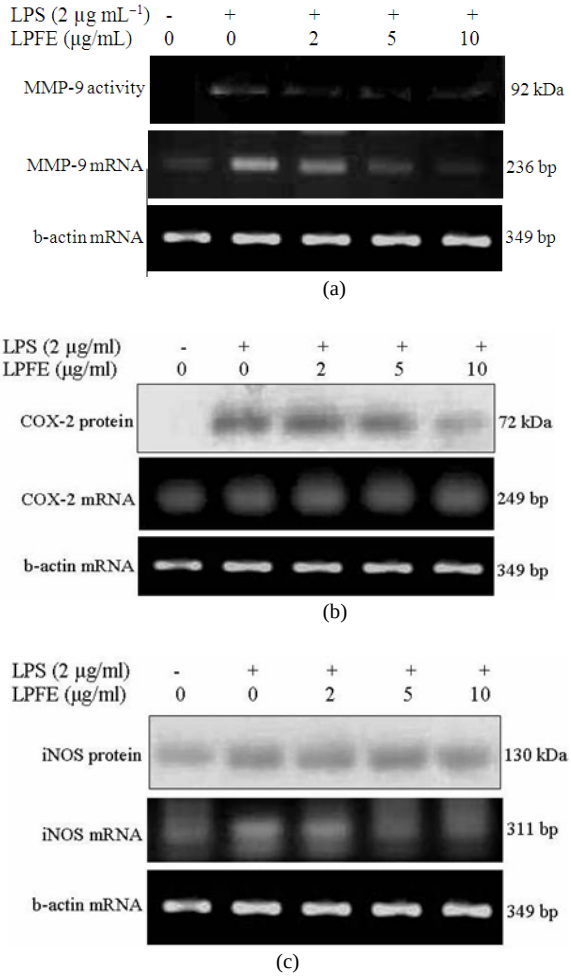

Fig. 3: Effects of LPFE on the protein and mRNA expression of MMP-9 (a), COX-2 (b) and iNOS (c) in LPS-induced macrophages assayed by gelatin zymogram, Western blot and RT-PCR. Beta-actin was used as the internal control

DISCUSSION

Most *Zanthoxylum* plants have been reported for their broad bioactivities including antioxidant, antimalarial, antimicrobial, anti-vascular inflammatory, antiproliferative, antinociceptive and anticaries potentials (Guo *et al*., 2010; Li *et al*., 2010; Pereira *et al*., 2010; Chou *et al*., 2011; Rodriguez-Guzman *et al*., 2011). Unfortunately, the scientific evidence of LPFE (*Z. acanthopodium* DC.) is rarely to be explored. In this present study, we demonstrated that LPFE exhibited anti-inflammatory activity on decreasing the production of various inflammatory mediators, i.e. TNF-α, IL-6, MMP-9, COX-2 and iNOS, in macrophages treated with LPS.

Macrophages are used as the *in vitro* culture cell model for gastric inflammation since they represent the similarity with the *in vivo* experimental models. Meanwhile, LPS is widely recognized as the major inducer for the production of inflammatory cytokines, including TNF-α and IL-6, which in turn stimulates iNOS induction during the inflammatory process in macrophages (Chang *et al*., 2009). Our results suggest that LPFE may reduce the production of TNF-α and IL-6, subsequently leading to the blockade of inflammatory enzyme induction (iNOS and COX-2).

We previously demonstrated that LPFE possesed antibacterial properties against food-borne pathogens, such as *Eschericia coli*, *Salmonella typhii*, *Vibrio parahaemolyticus* and *Staphylococcus aureus* (data not shown), indicating that foodborne infection may stimulate the inflammatory process that leads to the increase secretion of inflammatory cytokines and enzymes in the macrophages. Evidences showed that the methanol extracts of *Z. chalybeum* and *Z. usambarense*, medicinal plants from Kenya, possessed dual anti-bacterial and anti-inflammatory activities (Matu and Staden, 2003). Also, the ethyl acetate fraction of *Z. armatum* strongly inhibited xylene-induced ear swelling in mice *in vivo* (Guo *et al*., 2010). Some active constituents isolated from *Zanthoxylum* species, i.e., glycoprotein, phenylpropenoids, neolignan and alkaloids, were found to have anti-inflammatory properties through blocking the expression of inflammatory cytokines (TNF-α, IL-1β and IL-6) in various inflammatory cells *in vitro* (Chen *et al*., 2007; 2008; Hu *et al*., 2006; Lee and Lim, 2009a; 2009b). As *Z. acanthopodium* plant belong to the endemic lemon pepper in the Central Tapanuli region, North Sumatera province, Indonesia, we noticed that it is the first report about the potent inhibitory effects of LPFE in macrophages induced by LPS *in vitro*.

Furthermore, LPFE also affected the production of inflammatory proteins (COX-2 and iNOS) and MMP-9 activity in LPS-induced macrophages *in vitro*, indicating that LPFE possessed anti-inflammatory potential in macrophages in response to LPS *in vitro*. It has been recognized that the blockade of inflammatory cytokines and proteins by anti-inflammatory natural products is a potent strategy for management of various inflammatory diseases including gastrointestinal inflammation. In a decade, *Zanthoxylum* plants and their active constituents have been widely explored for their anti-inflammatory potentials using *in vitro* and in vivo experiments. For example, crude aqueous extract of the root bark of Z. xanthoxyloides demonstrated consistently anti-inflammatory activities via decreasing the production of PGE_2 subsequent to inhibition of COX-2 in inflammatory culture cells *in vitro* and carragenin-induced inflammation in rats (Oriowo, 1982; Prempeh and Mensah-Attipoe, 2008a; 2008b). Recently, a phytoglycoprotein with molecular weight of 115 kD isolated from *Z. piperitum* DC. has been reported for its inhibitory effects on the expression of pro- inflammatory proteins (COX-2 and iNOS) in primary cultured mouse thymocytes in response to 12-O-tetradecanoylphorbol 13-acetate (Park *et al*., 2009).

CONCLUSION

Based on the anti-inflammatory profile exposed through various assays, we summarized that lemon pepper fruit extract significantly ameliorated the protein and gene expression of TNF-α, IL-6, MMP-9, COX-2 and iNOS in LPS-induced macrophages *in vitro*. LPFE could be potentially used as a health food supplement and herbal medicine to heal inflammation particularly gastrointestinal inflammation. Further study on molecular mechanisms by which LPFE modulated the expression of inflammatory cytokines and proteins via signaling pathways in macrophages in response to LPS are still needed. Also, *in vivo* study using animal models is needed to determine the exact anti-inflammatory potential of LPFE.

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