RESEARCH COMMUNICATION

Eryngium foetidum Suppresses Inflammatory Mediators Produced by Macrophages

Chusana Mekhora[1], Channarong Muangnoi[1], Pimjai Chingsuwanrote[1], Suwitcha Dawilai[1], Saovaros Svasti[2], Kaimuk Chasri[3], Siriporn Tuntipopipat[1]*

Abstract

Objective: This study assessed anti-inflammatory and antioxidant activities of *E. foetidum* leaf extract on LPS-activated murine macrophages. Methods: RAW264.7 cells were pretreated with or without *E. foetidum* extract for 1 h prior to incubation with LPS for 24 h. Anti-inflammatory activity was evaluated with reference to iNOS, COX-2, TNF-α and IL-6 gene expression. In addition, NO and intracellular ROS generation were determined by Griess method and fluorescence intensity and activation of MAPKs and IκB by Western blotting. Results: Prior treatment with *E. foetidum* leaf extract inhibited elevation of IL-6, TNF-α, iNOS and COX-2, together with their cognate mRNAs in a dose-dependent manner. NO and intracellular ROS contents were similarly reduced. These effects were due to inhibition of LPS-induced phosphorylation of JNK and p38 as well as IκB. *E. foetidum* ethanol extract were shown to contain lutein, β-carotene, chlorogenic acid, kaempferol and caffeic acid, compounds known to exert these bioactive properties. Conclusions: *E. foetidum* leaf extract possesses suppressive effects against pro-inflammatory mediators. Thus, *E. foetidum* has a high potential to be used as a food supplement to reduce risk of cancer associated with inflammation.

Keywords: *E. foetidum* - iNOS - COX 2 - TNF α - IL 6 - inflammatory mediators

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Introduction

Inflammation is a physiological response that protects host against external and internal stimuli. It is a quick response and self-limiting process. However, if this protective response dysregulates or fails, inflammation becomes chronic. Several microbial infections, autoimmune disease, and some unknown causes of inflammation are well-defined triggers of chronic inflammation associated with cancer development (Mantovani et al., 2008). During chronic inflammation, some main transcription factors e.g., NF-κB, HIF-α, and STAT3 regulating expression of key pro-inflammatory mediators (e.g., cytokines, chemokines, COX-2 and iNOS) are activated (Mantovani et al., 2008). Over-production of pro-inflammatory mediators produced during chronic inflammatory processes contributes to tumor development via several mechanisms (Mantovani et al., 2008). Mouse models have been demonstrated that inflammatory response by innate immunity plays a role in the activation of the adaptive immune immunity to eliminate nascent tumors (Dunn et al., 2002). However, tumor cells can modify their genes. This characteristic of neoplastic cells allows them to evade the immune surveillance, resulting in tumor progression (Dunn et al., 2002). Macrophages are major population of inflammatory cells infiltrating in tumors microenvironmant. The tumor- associated macrophage (TAM) was an indication of the poor prognosis for many different human cancers (Bingle et al., 2002). Macrophage plays a pivotal role in host immune defense mechanisms, particularly in inflammatory response against internal and external stimuli including lipolysaccharide (LPS). The binding of LPS to endotoxin receptor complex (MD2/TLR-4) on cell membrane results in the activation of downstream signal pathway of inflammation including mitogen-activated protein kinases (MAPK), NF-κB, and activator protein-1 (AP-1) (Guha et al., 2001). LPS-stimulated macrophage induces NF-κB activation and phosphorylation of MAPKs, resulting in production of reactive oxygen species (ROS) via activation of NADPH oxidase, and inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Guha et al., 2001; Chen et al., 2007). These inflammatory mediators are principle players linking between inflammation and cancers (Kundu et al., 2008; Mantovani et al., 2008).

Bioactive substances from dietary plants such as vegetables, fruits, spices, and herbs have chemopreventive effects against various cancers (Aggarwal et al., 2006; 2008; 2009; Pan et al., 2008). The molecular targets of several dietary agents, in particular their potential

*[1]Institute of Nutrition, [2]Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, [3]Faculty of Allied Health Science, Thammasart University, Pathumthani, Thailand *For correspondence: nustt@mahidol.ac.th*

role in suppression of inflammation and tumorigenesis, have been defined in many studies. Many vegetables are used not only in various habitual diets but also in traditional medicine. Vegetables are rich not only in essential nutrients but also in non-nutrients, including phytochemicals. Phytochemicals play a role in several biological functions beyond their basic nutritive value. A number of studies have demonstrated that some Thai vegetables and herbs exhibit anti-inflammation and antioxidant activities *in vivo* and *in vitro* (Punturee et al., 2004; Nanasombat et al., 2009; Tuntipopipat et al., 2009).

Eryngium foetidum or Fitweed or Mexican coriander is a medicinal plant known as cilantro or spiny coriander. It is in Apiaceae family and originates from tropical America and West India (Simon et al., 1986). *E. foetidum* has long been used to treat fever, vomiting, diarrhea, hypertension, arthritic pain, and convulsions (Saenz et al., 1997). *E. foetidum* leaves are consumed as a dressing and side dish in Southeast Asia, Latin America, and the Caribbean. Its extract contains several bioactive compounds and exhibits a number of biological functions *in vitro* and *in vivo* (Saenz et al., 1997; Jiwajinda et al., 2002; Chanwitheesuk et al., 2005). However, its underlying mechanism of anti-inflammation has not been clearly defined. Thus, the present study demonstrates a chemopreventive potential of *E. foetidum* leaf extract via suppression of LPS-induced inflammatory mediator generated by RAW264.7 murine macrophage cell line.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), lipopolysaccharide (LPS) of Escherichia coli O11:B4, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), protease inhibitor cocktail, anti-β-actin-HRP, and species-specific HRP-conjugated secondary antibodies were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories (Haidmannweg, Austria), and penicillin and streptomycin from Invitrogen (Grand Island, NY, USA). Primary antibodies against iNOS, COX-2, phospho-p38, total p38, phospho-SAPK/JNK, total SAPK/JNK, phospho-ERK1/2, and total ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA).

Preparation of E. foetidum dry powder

Fresh *E. foetidum* plants were purchased from four distributors in Thailand (Bangkok, Nakhon Pathom, Chang Mai, and Ubon Ratchathani provinces). Leaves were washed two times with tap water, rinsed with deionized water and air dried. Roots were removed, and the leaves were chopped into small pieces (2-3 cm.) and frozen in liquid nitrogen. Frozen samples were dried by lyophilization prior to homogenization by a kitchen electric blender. The dry powder samples were vacuum-packed in aluminum foil and kept at -20 °C until used.

Preparation of E. foetidum leaf extract

Equal quantities of the dry powder from the four sources were pooled. Sample (0.1 g) was extracted twice by vigorously mixing with 3 ml of 90% ethanol for 2 min each and sonicating in ultrasonic bath (Mettler Electronics Corp, Anaheim, CA, USA) for 2 min each. The mixture was centrifuged at 4,500 g for 10 min, and the supernatant was evaporated at 45-50 °C under vacuum to dryness. The extract was dissolved in a mixture of dimethyl sulfoxide (DMSO) and ethanol (1:1) to give 0.2% (w/v) concentration and diluted to desired concentrations with serum-free, phenol red-free medium and passed through 0.2 μm sterile membrane filter (Millipore, Carrigtwohill, Ireland) prior to adding to cell culture.

HPLC analysis of carotenoids and phenolic compounds

The extraction protocol and HPLC analysis of carotenoid were described previously (Tuntipopipat et al., 2011). In brief, dry extract was dissolved with 1 ml of mobile phase solution and analyzed by HPLC using Waters model 600 (Milford, MA,USA) controller module with photodiode array detector (Agilent 1100 series, Santa Clara, CA, USA). Separation was conducted in VYDAC 201TP54 5-μm (particle size) C18 reversed-phase column (250 mm x 4.6 mm internal diameter; GRACE, Southborough, MA, USA) equipped with a guard cartridge (Phenomenex KJO-4282, Southborough, MA, USA). Carotenoids were eluted isocratically with mobile phase containing acetonitrile: methanol: dichloromethane (80:11:9 v/v/v) with 0.1% (v/v) triethylamine and 0.1% (w/v) ammonium acetate at a flow rate of 0.7 ml/min. Absorbance was monitored at 450 nm, and carotenoids were identified and quantified by retention time, spectral profile and peak area compared with carotenoid standards. The carotenoid contents of *E. foetidum* leaf extract are composed of 129.5 ± 2.5 μg lutein/g dry weight and 66.6 ± 1.1 μg β-carotene /g dry weight.

For the analysis of phenolic compounds, dry extract was dissolved in 2 ml of 62.5% methanol: 6M HCl (4:1) mixture and shaken at 70 °C for 2 h. Phenolic compounds were separated by HPLC coupled with photodiode array detector (Agilent Technologies 1100 series, Santa Clara, CA, U.S.A.), with Zorbax Eclipase XDB-C18 column (4.6 mm x 150 mm, 5 μm beads) (Agilent Technologies, USA) protected with a cartridge guard column. Column temperature was controlled at 30 °C. Phenolic compounds were eluted by a gradient program as previously described (Merken et al., 2000) with 100% water containing 0.5% (w/w) trifluoroacetic acid (TFA) (solvent A), 100% methanol containing 0.5% (w/w) TFA (solvent B), and 100% acetonitrile containing 0.5% (w/w) TFA (solvent C). Phenolic compounds were identified and quantified by comparison of retention time, spectral profile, and peak area with pure standards. Phenolic content of *E. foetidum* extract composed of 38.5 ± 0.6 μg chlorogenic acid/g, 36.5 ± 1.2 μg kaempferol/g, and 30.6 ± 0.8 μg caffeic acid/g.

Culture of murine macrophage

RAW 264.7 murine macrophage (ATCC, Bethesda, MD, USA) were cultured in DMEM with 10% FBS, 15 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used at 80% confluency between passages 8 and 20. Cells were seeded at 7.5 x 10^5 cells/ml for 24 h and subsequently incubated with 35-140 μg/ml

extract for 1 h prior to co-incubation with 5 ng/ml LPS for a further 24 h.

Cytotoxicity

Viability of treated cells was assessed using sulforhodamine B (SRB) assay (Vichai et al., 2006). In brief, cells with or without extract treatment and co-cultured with LPS were washed with phosphate-buffered saline (PBS) and fixed with 50% cold trichloroacetic acid (TCA) at 4 °C for 2 h. Cells were stained with 0.1% w/v sulforhodamine B in 1% acetic acid for 20 min and solubilized with 10 mM Tris-hydromethylaminomethane. Absorbance at 500 nm was measured with microplate reader (TECAN, GmbH, Austria), and value of cells treated with vehicle was considered 100% viable.

Nitric oxide production

Nitric oxide generated was indirectly determined by measuring nitrite content in culture medium using Greiss reagent (Green et al., 1982) by adding 100 µl of 0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated phosphoric acid to 100 µl of culture medium. Absorbance was monitored at 530 nm and nitrite concentration was estimated from a calibration curve from sodium nitrite.

Reactive scavenging capacity

Intracellular reactive oxygen species (ROS) levels were assessed by monitoring fluorescent signal generated from oxidized 2',7'-dichlorofluorescein diacetate (DCFH-DA)(Sigma). Nonfluorescent DCFH-DA diffuses into cells, which contain esterase that cleaves DA to form DCFH, which can be oxidized by ROS to the fluorescent DCF (Wolfe et al., 2007). In brief, LPS-treated cells were washed with warm basal medium and incubated with 5 μM DCFH-DA in basal medium at 37 °C for 30 min. Cells were washed 3 times with cold PBS and lyzed with 0.5% TritonX-100 in cold PBS. Fluorescence signal in supernatant obtained by sedimenting at 14,000 g for 5 min was measured in Luminescence Spectrometer LS55 (Perkin Elmer Instruments LCC, Shelton, CT, USA) with excitation at 485 nm and emission at 530 nm.

ELISA for cytokines

IL-6 and TNF-α levels in treated cell culture medium were determined by sandwich ELISA using paired antibodies (eBioscience Inc., San Diego, CA, USA) as previously described (Tuntipopipat et al., 2011). In brief, plates were coated with capture antibody for mouse IL-6 and TNF-α and incubated overnight at room temperature. Wells were washed once with PBS containing 0.05% Tween 20 (PBST) and incubated with 1% bovine serum albumin (BSA) in PBS. Culture medium or recombinant mouse TNF-α and IL-6 proteins were diluted in 1% BSA in PBST and incubated at 4 °C overnight. After washing with PBST, biotinylated antibodies were added, and the plate incubated at room temperature for 2 hr prior to adding streptavidin horseradish peroxidase (HRP)-tetra methyl benzidine detection system (Pierce, Rockford, IL, USA). Sulfuric acid was added to terminate the reaction, and absorbance at 450 nm was determined in a microtiter plate reader (TECAN, GmbH, Austria). Concentrations of TNF-α and IL-6 in samples were estimated by comparing with standard curves.

Western blot analysis

Treated cells were resuspended with ice cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 1% phosphatase inhibitor cocktail (Bio Basic Inc, Ontario, Canada) and 0.5% protease inhibitor cocktail (Sigma). Supernatant was obtained after the centrifugation at 13,500 g at 4 °C for 5 min. Protein content was determined using bicinchoninic acid method (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane (Whatman, GmbH, Germany), and incubated with specific primary antibody overnight at 4 °C., followed by incubation with horseradish peroxidase-conjugated secondary antibody for 2 h. Membranes were incubated with Super Signal solution (Endogen Inc, Rockford, IL, USA) for 5 min and exposed to X-ray film. Bound antibodies were then stripped off membrane and reprobed with either anti-β actin or anti-total MAPK proteins for normalization. Density of target bands was quantified by Image J program (freeware download from http://rsb.info.nih.gov/ij/). Results are expressed as relative ratio of band density between protein of interest and β-actin or between phosphorylated MAPK proteins and corresponding total MAPK proteins.

RNA extraction and RT-PCR

RNA extraction and RT-PCR condition were conducted as previously described (Tuntipopipat et al., 2011). In brief, RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA (1 µg) was reversed transcribed at 50 °C by 200 U reverse transcriptase (SuperScriptTM III Reverse Transcriptase, Invitrogen, USA) with oligo-dT18 primer and terminated by heating at 70 °C for 15 min. Primer pairs (Bio Basic, Ontario, Canada) used for PCR amplification were as follows: COX-2,sense:5'-AGGTCATTGGTGGAGAGGTG-3'; antisense:5'-GAGTCCATGTTCCA GGAGGA-3'; iNOS,sense:5'-CACCTTGGAGTTCACCCAGT-3'; antisense:5'-TGGTCA CATTCTG CTTCTGG-3'; TNF-α,sense: 5'-TCGTAGCAAACCACCAAGTG-3'; antisense: 5'-CGGACTCCGCAAAGTCTAAG-3'; IL-6,sense: 5'-GCAAGAGACTTCCATCCAGTTG-3'; antisense:5'-ACTCCAGGTAGCTATGGTACTCCA-3'; β-actin, sense: 5'-GGCACCACACCTTCTACAATG-3'; antisense:5'-GGTCTCAAACATGATCTGGGTC-3'. PCR was performed in MyCycler thermal cycler (BioRad, Hercules, CA, USA) using duplex PCR of target gene together with β-actin as an internal control. PCR products were separated in 1.5% agarose gel and stained with ethidium bromide. Density of target bands was quantified by Image J program and results are expressed as relative ratio of band intensity to β-actin.

Construction of iNOS and TNF-α promoter-luciferase plasmid

The iNOS and TNF-α promoter sequences were

prepared by PCR amplification using forward primer for iNOS (5'-GCGCCTCGAGCACGAGGCTGAGCT-GACTTT-3') and TNF-α (5'-GCGCCTCGAGGCTGTT CTCTATTTTAGT-3') containing a *Xho*I restriction site and reverse primer for iNOS (5'-CCGGAAGCTT A C C A A G G T G G C T G A G A A G T T - 3 ') a n d T N F - α (5 ' - C C G G A A G C T T TTGCAGAGAGGAACTTCATA-3') containing *Hind*III restriction site were used to amplify fragments of iNOS and TNF-α from *Mus musculus* DNA. The PCR was conducted by Phusion® High-Fidelity DNA Polymerase (Finnzyme, Vantaa, Finland). The amplified DNA was digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI/*Xho*I-digested pMetLuc-Reporter vector (Clontech, Mountain View, CA) to generate pMetLuc-iNOS and pMetLuc-TNF-α. The nucleotide sequences of the constructs were confirmed by automated DNA sequencing (ABI PRISM330xl, Applied Biosystems, Carlsbad, California) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), prior to transform into *Escherichia coli* JM109.

Transient transfection and luciferase assay

RAW264.7 cells were seeded at a density of 7.4×10^6 cells in T-25 Flasks for 24 h. Cells were cotransfected in Opti-MEM® I Reduced Serum Medium (Invitrogen) containing 3 μg of iNOS or TNF-α plasmid construct and 1 μg of pCMV-β-galactosidase in lipofectamine (Invitrogen) for 6 h and aspirated medium and cultured in complete medium for another 24 h. The $1x10^6$ cells/ml transfected cells were plated in 12-well plate for 24 h and incubated with or without extract for 1 h prior to activating with LPS. Luciferase was measured with the Ready-to-Glow™ secreted luciferase reporter system (Clontech, Mountain View, CA, USA) at 405 nm in a luminometer (Multimode detector DTX880, Beckman Coulter), and β-galactosidase activities (Stratagene, La Jolla, CA, USA) were assayed according to manufacturer's protocol. The luciferase activity was assayed and normalized with β-galactosidase activity in cell lysate and expressed as relative luciferase activity.

Statistical Analysis

Statistical analysis was performed using SPSS (version 14.0, SPSS Inc., Chicago, Illinois). Data are presented as mean ± SD from at least three separate experiments conducting on different days. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for multiple comparison of group means. Significance is set at $p < 0.05$

Results

E. foetidum leaf extract inhibits LPS-induced iNOS gene expression and NO production

Exposure of macrophages with LPS activates NO generation from L-arginine through iNOS activity. Treated RAW264.7 cells with *E. foetidum* leaf extract (35-140 μg/ml) for 1 h before stimulating with LPS (5ng /ml) for 24 h significantly inhibited nitrite generation in

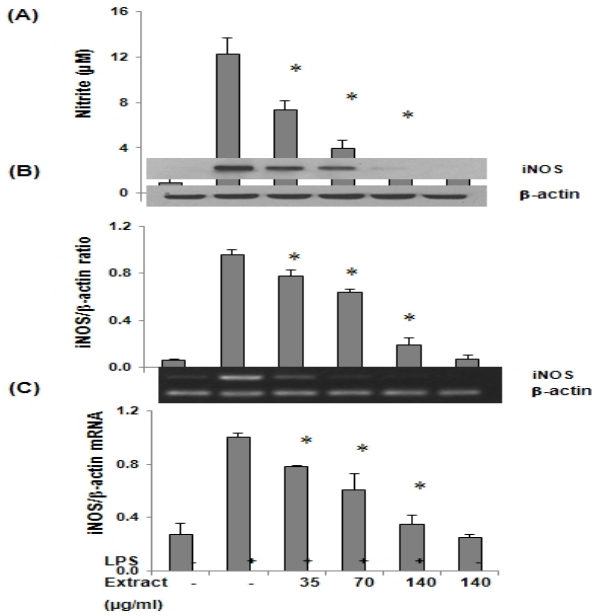

Figure 1. Inhibition by E. *foetidum* Leaf Extract of LPS-induced NO, iNOS and iNOS mRNA Expression. RAW264.7 cells were treated as described in methods. Cell culture medium was assayed for NO using Griess assay of nitrite (a). Cell lysate iNOS was measured by western blot, with β-actin as loading control and results are expressed as relative ratio of iNOS to β-actin (b). The iNOS mRNA content was quantified by semi-quantitative RT-PCR using β-actin cDNA as an internal control. Results are expressed as relative ratio of band intensity of iNOS to β-actin cDNA (c). Results are presented as mean ±SD in three independent experiments. *P < 0.05 compared with LPS treatment alone.

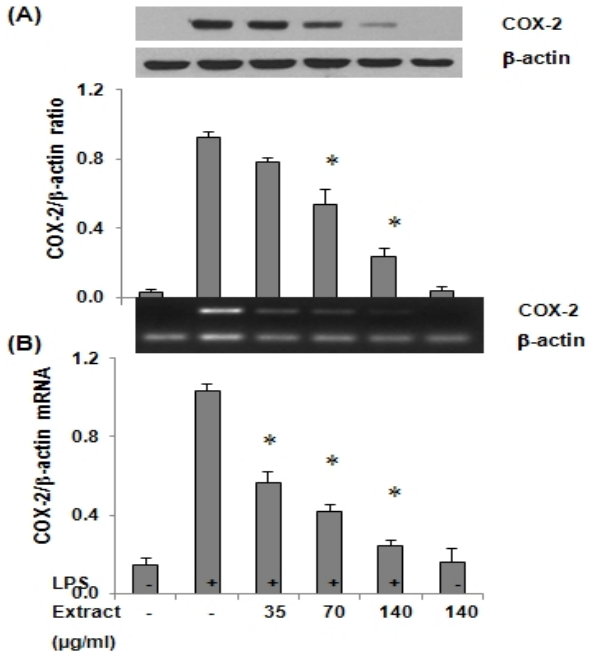

Figure 2. Suppression of LPS-induced COX-2 and COX-2 mRNA Expresion by E. *foetidum* Leaf Extract. Cells were treated as described in methods. (a) COX-2 protein was measured by western blotting and results are expressed as relative β-actin protein. (b) COX-2 mRNA was quantified by semi-quantitative RT-PCR using β-actin cDNA as internal control. Results are expressed as relative ratio of band intensity of COX-2 to β-actin cDNA. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

a dose-dependent manner (Figure 1A) without affecting the cell viability (data not shown). To investigate the underlying cause of reduction in NO production, iNOS protein and mRNA levels were determined by western blot and RT-PCR respectively. Pretreatment with *E. foetidum* leaf extract significantly suppressed LPS-induced iNOS protein and mRNA levels in a dose-dependent manner (Figure 1B and 1C).

E. foetidum leaf extract suppresses LPS-activated COX-2, TNF-α and IL-6 gene expression

In macrophage, COX-2, a pro-inflammatory enzyme, is induced by various stimuli including LPS through activation of NF-ϰB and MAPK (Guha et al., 2001). Pretreatment of RAW264.7 cells with *E. foetidum* leaf extract significantly suppressed LPS-induced COX-2 protein and mRNA levels in a dose-dependent manner (Figure 2A and 2B). Challenging RAW264.7 cells with LPS significantly resulted in secretion of TNF-α and IL-6 (Figure 3A and 4A). *E. foetidum* leaf extract-treated cells significantly attenuated LPS-induced TNF-α (Figure 3A and 3B) and IL-6 (Figure 4A and 4B) protein levels, together with their cognate mRNA expression in a dose dependent manner.

Suppression of iNOS and TNF-α promoter activity by E. foetidum leaf extract

We examined whether the extract suppressed iNOS and TNF-α expression via attenuation LPS-induced iNOS and TNF-α promoter activities by transient tranfected iNOS (pMetLuc-iNOS) and TNF-α promoter construct (pMetLuc- TNF-α) in RAW264.7 cells with lipofection technique. Transfected cells increased iNOS and TNF-α

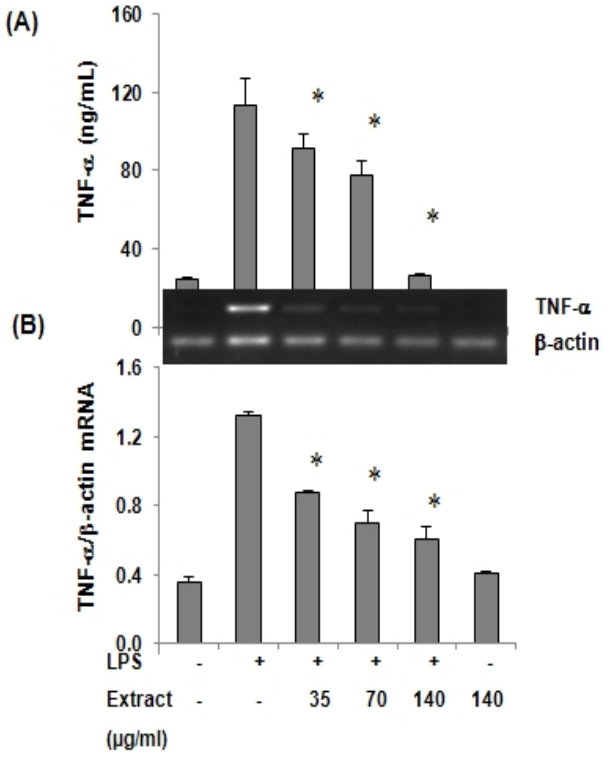

Figure 3. Suppression of LPS-induced TNF-α gene expression by E. *foetidum* leaf extract. Cells were treated as described in methods. TNF-α level was measured in cell culture medium (a). TNF-α mRNA was measured by semi-quantitative RT-PCR using β-actin cDNA as internal control (b). Results are expressed as relative ratio of band intensity of TNF-α to β-actin cDNA. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

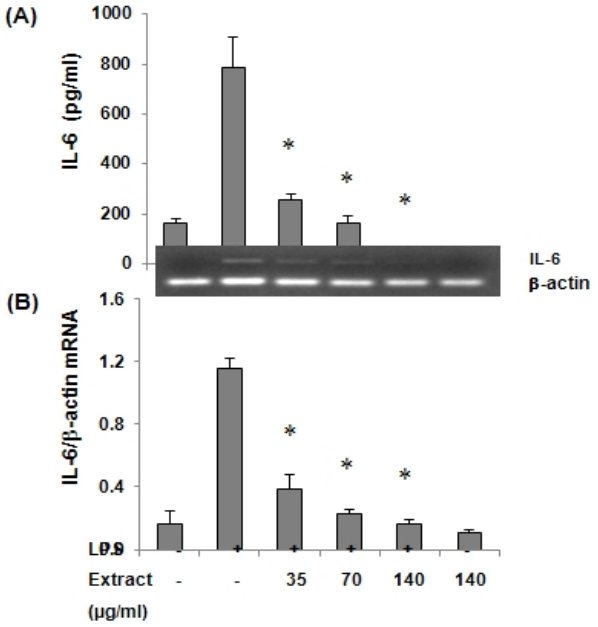

Figure 4. Suppression of LPS-induced IL-6 gene Expression by E. *foetidum* Leaf Extract. Cells were treated as described in methods . IL-6 was measured in cell culture medium (a). IL-6 mRNA content was quantified by semi-quantitative RT-PCR using β-actin cDNA as internal control (b). Results are expressed as relative ratio of band intensity of Il-6 to β-actin cDNA. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

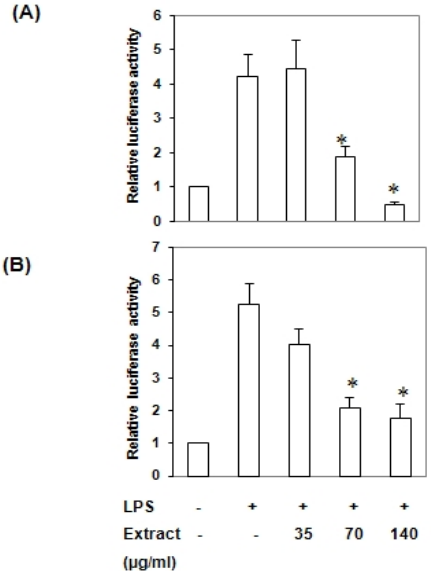

Figure 5. Suppression of LPS-induced iNOS and TNF-α promoter activity by E. *foetidum*. RAW 264.7 cells transfected with iNOS and TNF-α promoter luciferase reporter plasmid and treated with E. *foetidum* extract for 1 h prior to incubation with LPS for 24 h. Luciferase activites were determined. Results are expressed as luciferase activity relative to β-galactosidase activity. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

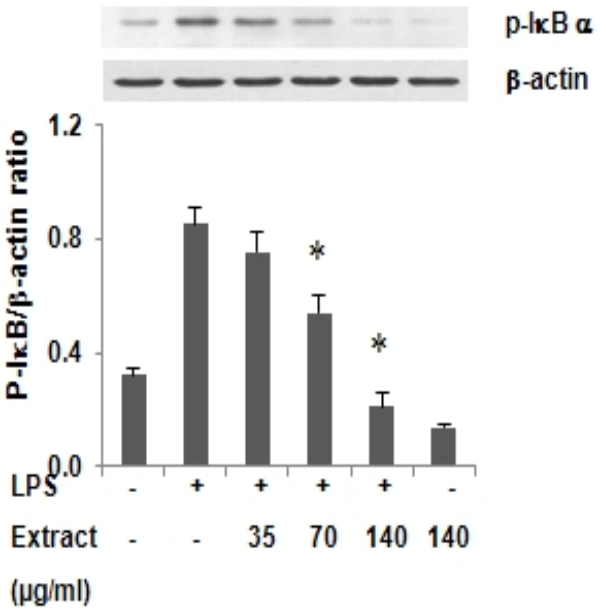

Figure 6. Inhibition of LPS-induced phosphorylation of IκB-α by E. foetidum. Cells were treated as described in methods. Phospho-IκB-α was determined by western blotting and used β-actin as a loading control. Results are expressed as ratio of band intensity of phospho-IκB-α to β-actin. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

promoter activities around 3.8 (Figure 5A) and 5.3 fold (Figure 5B). The extract attenuated the enhanced luciferase activity of iNOS promoter by 70% - 92% at the extract concentration of 70 - 140 μg/ml respectively with IC50 at 49 μg/ml. In contrast, it decreased the luciferase activity of TNF-α promoter by 60%-67% at the extract concentration of 70 - 140 μg/ml respectively with IC50 at 58 μg/ml.

E. foetidum leaf extract attenuates LPS-induced phosphorylation of IκB

Retention of NF-κB in cytosol is due to binding to IκB-α, which undergoes degradation upon phosphorylation. IκB-α in RAW264.7 cells became phosphorylated after exposure to LPS, and the pretreatment of cells with E. foetidium leaf extract (70-140 μg/ml) effectively inhibited IκB-α phosphorylation (Figure 6).This indicates that *E. foetidum* extract mediates its inhibitory effect on LPS-induction of iNOS, COX-2, TNF-α, and IL-6 levels by partially inhibiting IκB-α phosphorylation.

E. foetidum leaf extract inhibits LPS-induced MAPK signaling pathway

Besides causing NF-κB nuclear translocation through phosphorylation of IκB-α, LPS activates MAPK cascade, viz. phosphorylation of ERK1/2, p38, and JNK. Exposure of RAW 264.7 cell to LPS significantly enhanced phosphorylation of all MAPKs without alteration of total forms, while pretreatment with *E. foetidum* leaf extract (70-140 μg/ml) significantly attenuated LPS-mediated JNK and p38 phosphorylation in a dose-dependent manner (Figure 7A and 7B).However, phosphorylation of ERK1/2 was not affected (Figure 7C).

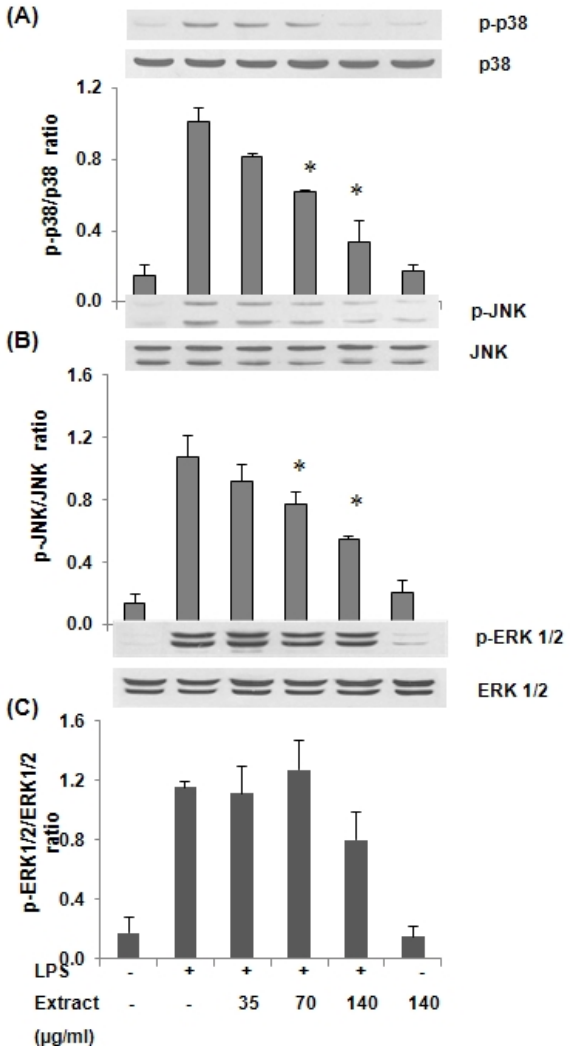

Figure 7. Inhibition of LPS-induced MAPK signaling Pathway by E. *foetidum* Leaf Extract. Cells were treated as described in methods. MAPK proteins were determined by western blot using antibodies against (a) phospho-p38 and total p38, (b) phospho-JNK and total JNK, and (c) phospho-ERK1/2 and total ERK1/2. Results are expressed as ratio of band intensity of phospho-JNK, phospho-38 and phospho-ERK1/2 relative to total forms of the proteins. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone.

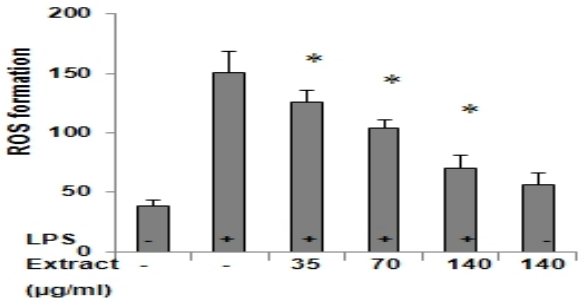

Figure 8. Reduction in LPS-induced intracellular ROS level by E. *foetidum* leaf extract. Cells were treated as described in methods. After treatment with LPS, 2',7'-dichlorofluorescein diacetate was added to cells for 30 min. After washing, 0.5% TritonX-100 was added to lyse cells and fluorescence in supernatant was measured. Results are presented as mean ±SD in three independent experiments. *P < 0.05 relative to LPS treatment alone

E. foetidum leaf extract possesses ROS scavenging capacity

Upon LPS activation, macrophages produce ROS via activation of NADPH oxidase activity, leading to active NF-κB and MAPK cascade thereby generating pro-inflammatory mediators. Pre-treatment of RAW 264.7 cells for 1 h with *E. foetidum* leaf extract (35-140 μg/ml) significantly decreased intracellular ROS induced by exposure to LPS for 24 h in dose-dependent manner (Figure 8).

Discussion

E. foetidum is a common vegetable used for dressing and side dish of several recipes in tropical regions. The serving size (at 97.5 percentile of eating only population) in the Thai population is 0.48 g/kg body weight (The Agricultural Co-operative Federation of Thailand, 2006). The present study clearly demonstrated that pretreatment with *E. foetidum* leaf extract (35-140 μg/ml) for 1 h effectively inhibited LPS-induced pro-inflammatory mediators gene expression, including NO, iNOS, COX-2, TNF-α, and IL-6 in murine macrophage cell lines. The anti-inflammatory activity was mediated through blocking the upstream intracellular signaling ERK1/2 and p38 cascade by inhibition of IκB phosphorylation, which probably via scavenged LPS-induced intracellular ROS accumulation. The *E. foetidum* leaf extract contains kaempferol, chlorogenic acid, caffeic acid, lutein, and β-carotene. All components have been previously reported to have anti-inflammatory activities, and some also have antioxidant activities (Ching et al., 2002; Lee et al., 2004; Shin et al., 2004; Bai et al., 2005; Kowalski et al., 2005; dos Santos et al., 2006; Hamalainen et al., 2007; Kim et al., 2008; Chao et al., 2009; Shan et al., 2009; Zhang et al., 2010). Several bioactive compounds in *E. foetidum* leaves were recently reported (Paul et al., 2010) to play a role in anti-inflammatory and antioxidant activities.

Nitric oxide (NO), a soluble free radical gas, is synthesized from L-arginine through the activity of various NOS isoform enzymes (Singh et al., 2011). Lipopolysaccharide-induced macrophage activates iNOS isoform expression and NO production through the activation of NF-κB and MAPKs signaling pathway (Kim et al., 2006). This inducible isoform expresses in several human malignant tumors (Hajri et al., 1998; Klotz et al., 1998; Weninger et al., 1998; Lagares-Garcia et al., 2001; Masri et al., 2005). Nitric oxide plays physiological role in modulation of vascular tone, inhibits platelet aggregation, and induces cell apoptosis (Boscá et al., 2005). However, the increasing expression of iNOS enzyme generates excess amounts of NO leading to tissue damage, inducing septic shock, and contributing to develop pathological complication during ongoing chronic inflammatory response and cancer development (Jaiswal et al., 2000; Surh et al., 2001). The iNOS-derived NO induced inflammation-related carcinogenesis occurs through the interaction between NO and ROS to form toxic agents including peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), induction of other pro-inflammatory mediators such as COX-2 and activates vascular endothelial growth factor (VEGF). Patients with iNOS-expressing melanomas have significant lower survival rates than their iNOS-negative peers (Ekmekcioglu et al., 2000). An iNOS inhibitor showed potential to suppress tumor growth and reduce VEGF level in gastric cancer cell induced mice (Wang et al., 2005). The *E. foetidum* leaf extract significantly suppresses NO production and iNOS expression.Therefore, consumption of *E. foetidum* might suppress or halt cancer development.

COX-2 is an inducible enzyme playing role in the synthesis of prostaglandins, prostacyclines, and thromboxane A2 from arachidonic acid during inflammatory process (Vane et al., 1998). Many studies have demonstrated that transgenic mice that overexpressed COX-2 in mammary glands, skin, and stomach are prone to develop tumours in these organs (Neufang et al., 2001; Muller-Decker et al., 2002; Oshima et al., 2004). In contrast, COX-2 knock-out mice are more resistant to tumorigenesis in various organs (Oshima et al., 1996; Tiano et al., 2002; Howe et al., 2005). A number of clinical studies were performed for colorectal cancer prevention by administration of Nonsteroidal anti-inflammatory drugs (NSAIDs) or COX-2 inhibitors including sulindac, aspirin, celecoxib, rofecoxib (Giardiello et al., 1996; Steinbach et al., 2000; Giardiello et al., 2002; Baron et al., 2003; Higuchi et al., 2003; Sandler et al., 2003; Bertagnolli et al., 2006). These COX-2 inhibitors reduced adenoma only at a high dose but significantly increased mortality from cardiovascular complication. Due to the adverse effects, combination of NSAIDs with other chemopreventive agents should be considered for general population. For therapeutic interventions targeting COX-2 inhibitor, recent evidence found a positive correlation between COX-2 expression in primary breast cancer and bone marrow micrometastasis. Thus, COX-2 inhibitors can be used to halt breast cancer progression and dissemination (Lucci et al., 2009). The extract of *E. foetidum* significantly suppresses LPS-activated COX-2 expression in a concentration-dependent manner. Therefore, *E. foetidum* extract would be a safe chemopreventive agent that can be used in combination with NSAIDs against tumorigenesis by downregulating COX-2 expression.

TNF-α is a primary pro-inflammatory cytokine acting as a tumor promoter in inflammation-associated cancer- There were several pieces of evidence supporting role s in several steps of carcinogenesis. TNF-α was highly expressed in various tumor tissues (Ahmed et al., 2001; Ferrajoli et al., 2002; Szlosarek et al., 2006). The elevated expression of TNF-α in pre-cancerous and tumor cells was related to the progression of malignant diseases, such as prostate cancer, breast cancer, and cervical carcinoma.(Ahmed et al., 2001; Michalaki et al., 2004; Garcia-Tunon et al., 2006). Serum TNF-α level also correlated with the extent of therapy responses. These evidences indicate that TNF-α may play role in tumor development and could be used to follow up the prognosis and therapeutic responses or used as a predictor of cancer risk. Many animal studies also showed the role of TNF-α in tumor growth and promotion. The knockout of TNFR-1 or neutralization of TNF-α activity, with

antibody in mice inducing colitis with azoxymethane and dextran sulfate sodium, attenuated tumor formation comparing with their peers (Popivanova et al., 2008). Mice lacking TNF-α or its receptor are resistant to skin carcinogenesis (Moore et al., 1999; Arnott et al., 2004). It also initiates cancer development via inducing genetic mutation and DNA instability by enhancing intracellular ROS formation (Babbar et al., 2006). TNF also involves in tumor angiogenesis, invasive, and metastasis. It enhances cytokines, chemokines, and angiogenic factor expression which promotesneovascularization to support tumor growth (Nabors et al., 2003; Kulbe et al., 2007). TNF increases the invasiveness of tumor cells by inducing expression of matrix metalloproteinase (MMP) (Esteve et al., 2002; Cheng et al., 2007). In a study with a co-culture model between macrophages and tumor cells demonstrated that TNF-α secreted by macrophages promotes invasiveness of tumour cells by induction of matrix metalloproteinase (Pollard 2004). Because TNF-α involves on several steps of carcinogenesis, consumption of *E. foetidum* may prevent or interfere cancer development via the inhibition of TNF-α expression.

IL-6 is another cytokine that is produced by LPS-activated macrophage through the activation of NF-ϰB. IL-6 is a strong activator of acute phase response contributing both systemic and local inflammatory response. It activates vascular endothelial cells leading to a release of chemokines e.g., IL-8, and monocyte chemoattractant protein-1, which facilitates leukocyte recruitment to inflammatory sites (Kaplanski et al., 2003). Elevated serum IL-6 in cancer patients or in tumor biopsy indicated that this cytokine plays role in cancer development (Chung et al., 2003; Kai et al., 2005). IL-6 induction by ras gene is a crucial factor for tumor growth in DMBA-TPA-induced skin carcinogenesis model (Ancrile et al., 2007). It also promotes proliferation and survival of pre-malignant intestinal epithelial cells and subsequently enhances the initiation and progression of colitis-associated cancer (Bollrath et al., 2009; Grivennikov et al., 2009). Drachenberg et al. (1999) suggested that serum IL-6 level can be used as a maker in patients with cancer metastatic state (Drachenberg et al., 1999). The *E. foetidum* leaf extract from the present study effectively suppressed LPS-induced IL-6 expression. Thus, *E. foetidum* leaf may be an alternative chemopreventive food supplement for cancer risk reduction.

NF-ϰB is a transcription factor that plays an important role in several physiological functions. It regulates expression of more than 200 genes by modulation of e transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, and inflammation, and by suppression of apoptosis (Burstein et al., 2003; Luo et al., 2005; Dutta et al., 2006; Cilloni et al., 2007; Jost et al., 2007; Melisi et al., 2007). Deregulation of NF-ϰB and upstream signaling pathways involve in cancer development and progression. Blocking activation of NF-ϰB cascade can cease tumor proliferation or make tumor cells more sensitive to the action of antitumor agents (Karin 2006). In pre-neoplastic, malignant cells and inflammatory cells, NF-ϰB up regulates the expression of gene encoding inflammatory cytokines, adhesion molecules, angiogenic factors and COX-2 and iNOS, which are important for the synthesis of inflammatory mediators (PGE2 and NO)(Aggarwal 2004; Aggarwal et al., 2006). Thus, suppression of NF-ϰB activation should be an alternative and effective approach to prevention or treatment of cancer. The present results showed that *E. foetidum* leaf extract suppressed phosphorylation of IϰB, which is a part of the NF-ϰB activation. Thus, suppression of aforementioned proinflammatory cytokines may be modulated by suppression of IϰB phosphorylation.

Mitogen-activated protein kinases (MAPKs) compose of at least three kinds of signal transduction protein kinases including ERK, JNK, and p38. These protein kinases response against exogenous and endogenous stimuli by converting the sensing signal to a wide range of cellular responses, such as proliferation, survival, differentiation, and migration. Because of their essential roles in these cellular functions, perturbation in cascade activation of MAPKs may cause cancer development (Arnoldussen et al., 2009; Min et al., 2011). Hyperactivation of JNK has been recently shown to involve on human renal cell carcinomas (Liu et al., 2009). Phosphorylation of JNK has been shown to account for human prostate tumor cell proliferation and angiogenesis (Vivanco et al., 2007). Upregulated JNK phosphorylation was observed in biopsy of hepatocellular carcinoma patients (Guo et al., 2005). Dramatically suppressive activation of the JNK-c-Jun pathway is attributable for the attenuated proliferation of Fas deficient liver tumor cells in diethylnitrosamine (DEN)-induced mouse hepatocellular carcinoma development (Chen et al., 2010). The p38 was found to play role in regulating production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α (Kumar et al., 2003). Recent experiment showed that dominant negative p38α MAPK (p38DN) in the epidermis of SKH-1 hairless mice significantly inhibited UVB-induced activation protein-1(AP-1) and COX-2 expression leading to a significant reduction in UVB-induced tumor number and growth compared with wild-type littermates in a chronic UVB skin carcinogenesis model. These results indicate that p38 MAPK is a good target for pharmacological intervention for UV-induced skin cancer in patients with sun damaged skin and suggest that inhibition of p38 signaling reduces skin carcinogenesis by inhibiting COX-2 expression and proliferation of UVB-irradiated cells (Dickinson et al., 2011). In addition, p38 inhibitor agent significantly increased the sensitivity of colorectal cancer cell to chemotherapeutic agent 5-Fluorouracil (FU). Thus, p38 MAPK activation may involve in cancer cell survival, and the inhibition of p38 MAPK can enhance 5-FU activity against colorectal cancer cells (Yang et al., 2011). Therefore, *E. foetidum* may exert chemoprevention via suppression of p38 and JNK activation.

Oxidative stress or excess ROS generation alters DNA base structure leading to damage and also contributes to epigenetic alteration. Many cancer cells have high accumulation of ROS causing direct DNA strand break, base modifications, and DNA-protein cross linkages which are involved in the initiation step of carcinogenesis (Kim et al., 2008). ROS such as hydroxy radicals can

attack DNA molecule causing strand break and purine/pyrimidine lesion, which affect genomic stability. High levels of DNA damage lesion were reported in many tumor types (Tudek et al., 2010). High level of ROS is associated with modification of DNA methylation patterns which play a role in human cancer development (Donkena et al., 2010; Ziech et al., 2010). Due to hydroxy radical-induced DNA damage lesions involve in decreasing DNA methylation by reducing the capacity of DNA as a substrate for enzyme DNA methyltransferases (DNMTs) and result in global hypomethylation (Franco et al., 2008). In addition, oxidative stress can involve gene silencing by hypermethylation of tumor suppressor gene promoter region and lead to malignant progression (Lim et al., 2008). Thus, both DNA hypo- and hypermethylation ROS-induced epigenetic on promoter of genes involve in malignant transformation and progression of several tumors (Campos et al., 2007). The *E. foetidum* leaf extract may reduce risk factors of carcinogenesis by reducing intracellular ROS accumulation.

In summary, *E. foetidum* leaf extract has potential to reduce risk factors of carcinogenesis associated with inflammatory mediator activities. These effects should partially stem from the presence of kaempferol, chlorogenic acid, caffeic acid, lutein, and β-carotene, which have been previously reported to have anti-inflammatory and antioxidant activities. However, the other compounds in the leaves such as phytosterol were also reported to possess anti-inflammatory activity (García et al., 1999). The efficacy studies in animals or humans suffering from inflammation-related carcinogenesis need to be explored.

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