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Suppressive effects of extracts from the aerial part of *Coriandrum sativum* L. on LPS-induced inflammatory responses in murine RAW 264.7 macrophages

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Abstract

BACKGROUND: *Coriandrum sativum* is used not only as a spice to aid flavour and taste values in food, but also as a folk medicine in many countries. Since little is known about the anti-inflammatory ability of the aerial parts (stem and leaf) of *C. sativum*, the present study investigated the effect of aerial parts of *C. sativum* on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. We further explored the molecular mechanism underlying these pharmacological properties of *C. sativum*.

RESULTS: Ethanolic extracts from both stem and leaf of *C. sativum* (CSEE) significantly decreased LPS-induced nitric oxide and prostaglandin E_2 production as well as inducible nitric oxide synthase, cyclooxygenase-2, and pro-interleukin-1β expression. Moreover, LPS-induced IκB-α phosphorylation and nuclear p65 protein expression as well as nuclear factor-κB (NF-κB) nuclear protein–DNA binding affinity and reporter gene activity were dramatically inhibited by aerial parts of CSEE. Exogenous addition of CSEE stem and leaf significantly reduced LPS-induced expression of phosphorylated mitogen-activated protein kinases (MAPKs).

CONCLUSION: Our data demonstrated that aerial parts of CSEE have a strong anti-inflammatory property which inhibits pro-inflammatory mediator expression by suppressing NF-κB activation and MAPK signal transduction pathway in LPS-induced macrophages.
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Keywords: *Coriandrum sativum*; inflammation; lipopolysaccharides; mouse RAW 264.7 macrophages; NF-κB

INTRODUCTION

Macrophages, the major immune cells in the innate immune system, are essential for host defence and inflammation against intracellular parasitic bacteria, pathogenic protozoa and fungi by producing a variety of cytokines and inflammatory mediators, such as interleukin-1β (IL-1β), nitric oxide (NO) and prostaglandin E_2 (PGE_2).[1] IL-1β from cleavage of an inactive pro-IL-1β protein is yielded by stimulated leukocytes and is a fundamental contributor to local and systemic inflammatory responses.[2] Large amounts of NO and PGE_2, produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), are described not only at inflammatory sites but also in process of carcinogenesis.[3–5] Although inflammation plays an important role in the host defence system, inappropriate activation of macrophages results in an overproduction of the inflammatory mediators, which is involved in the pathologies of many chronic diseases of modern society, such as rheumatoid arthritis, atherosclerosis, diabetes and cancer.[5–7] Current clinical approaches to treatments of chronic inflammation involve the inhibition of pro-inflammatory mediator production and the suppression of mechanisms responsible for the initiation of inflammatory responses.

Activation of the nuclear factor-κB (NF-κB) pathway plays a key role in the transcriptional regulation of pro-inflammatory response gene expression, including iNOS, COX-2, cytokines, chemokines, growth factors, cell adhesion molecules, and several acute phase proteins.[8] The transcription factor NF-κB exists in

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the cytoplasm of most eukaryotes by forming homodimers or heterodimers with proteins of the NF-κB family, including p65 (RelA), p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB, and c-Rel. In unstimulated cells, NF-κB is sequestered in the cytoplasm by noncovalently binding to an inhibitor protein termed IκB (α, β or ε). Activation of NF-κB occurs in response to inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins, which induce phosphorylation, ubiquitination and degradation of IκBα. Then the activated NF-κB migrates into the nucleus and induces transcriptional expression of its target genes. Constitutive activation of the NF-κB pathway leads to persistent increases in the expression of pro-inflammatory mediators and exerts pathogenic effects on chronic inflammatory related diseases. Regulation and control of NF-κB activation may be a key molecular target for anti-inflammatory therapy.[8,9]

Spices are used not only to aid flavour, colour and nutritional values in food, but also to treat various physical problems in traditional medicines.[10] *Coriandrum sativum,* commonly known as coriander or Chinese parsley, belongs to the family *Apiaceae*, which is widely cultivated all over the world. The seeds and aerial parts (stem and leaf) of *C. sativum* are commonly used as spices in Middle Eastern, Mediterranean, Indian, Latin American, African, Southeast Asian and Taiwanese cuisines. Data from numerous researchers have shown the therapeutic values of the seeds and seed oil of *C. sativum* due to its hypoglycaemic, hypolipidaemic, hepatoprotective, antimutagenic, antihypertensive, antioxidant, anxiolytic, antimicrobial and post-coital antifertility activity.[11–19] The aerial parts of *C. sativum* have antioxidant and free radical scavenging activities, suppressive activity on lead and mercury deposition and bactericidal and anti-adhesive effects on *Helicobacter pylori*.[20–23] Recent studies reported that *C. sativum* seed oil reduced UV-induced erythema test of human skin and leaves of *C. sativum* water extract decreased LPS-induced NO production and had scavenging effects on NO.[24,25] Based on previous studies of *C. sativum* presented herein, it is worth conducting a detailed investigation on the anti-inflammatory property of aerial parts of *C. sativum*. The objective of the present study is to assess the regulatory efficacy of aerial parts of *C. sativum* on LPS-induced inflammatory responses in RAW 264.7 macrophages as well as to explore the possible molecular mechanism behind these actions.

MATERIALS AND METHODS

Materials

The mouse macrophage-like cell line RAW 264.7 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan), and fetal bovine serum was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). RPMI 1640 medium and media supplements for cell culture were obtained from Invitrogen Corporation (Carlsbad, CA, USA). LPS, ferulic acid, gallic acid, 4-hydroxycoumarin, hesperidin, luteolin, dihydroquercetin, quercetin and Folin–Ciocalteu phenol reagent were from Sigma Chemical Company (St Louis, MO, USA). The specific antibodies for iNOS, p65, c-Jun NH_2-terminal kinase (JNK), and phosphorylated JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against p38, extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated p38, ERK1/2 and IκB-α, as well as p65 were from Cell Signaling Technology Inc. (Beverly, MA, USA). The specific antibodies for COX-2, pro-IL-1β, and β-actin were obtained form Cayman Chemical Co. (Ann Arbor, MI, USA), CytoLab Ltd. (Rehovot, Israel), and Sigma Chemical Company, respectively. Nucleotides and RNase inhibitor were obtained from Promega Co. (Madison, WI, USA) and M-MMLV reverse transcriptase was from Gibco BRL (Gaithersburg, MD). Real-time polymerase chain reaction (PCR) primers and TaqMan® Universal PCR Master Mix were from Applied Biosystems (Foster City, CA, USA). The biotin-labelled and unlabelled double-stranded NF-κB consensus oligonucleotides and a mutant double-stranded NF-κB oligonucleotide for electrophoretic mobility shift assay (EMSA) were synthesised by MDBio Inc. (Taipei, Taiwan). The pNF-kB-Luc plasmid was from Stratagene Inc. (La Jolla, CA, USA) and the Luciferase Assay System, β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, and pSV-β-galactosidase control vector were from Promega. All other chemicals were of the highest quality available.

Preparation of extractions

C. sativum was purchased from local markets at Taichung, Taiwan, and was identified by Dr Lee-Yan Sheen (Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan). A voucher specimen was kept in our laboratory, at the Department of Nutrition, Chung Shan Medical University, Taichung, Taiwan, for further reference.

C. sativum was washed and its leaves and stems were separately cut into small pieces, placed in a $-70\,^{\circ}C$ freezer for 12 h and then freeze-dried (FD4; Heto Lab Equipment, Birkerød, Denmark) for at least 12 h. The dried *C. sativum* leaves and stems were ground to a fine power using an electric food grinder and were then stored at $-20\,^{\circ}C$ for further use.

The powdered freeze-dried food material was extracted by 95% ethanol (25 mL g^{-1} powder) for 24 h and then centrifuged at 3200 $\times$ *g* for 10 min. The supernatants were concentrated at 37 $^{\circ}C$ under reduced pressure and then dissolved in ether. The ether in the ethanolic extract was evaporated by nitrogen. The ethanolic extracts were weighed to measure the extraction yield and then dissolved in dimethyl sulfoxide (DMSO) for cell treatments.

Determination of total phenolics and flavonoids

The amount of total phenolics in the leaf or stem of *C. sativum* ethanolic extract (CSEE) was measured according to a modification of the Folin–Ciocalteu method.[26] A 300 µL aliquot of each extract or standard solution was mixed with 300 µL of Folin–Ciocalteu reagent. The mixture was allowed to stand for 5 min followed by the addition of 600 µL of 20% Na_2CO_3. After a 10 min incubation at room temperature, the absorbance of the reaction mixture was measured at 730 nm and total phenolics were calibrated by using a standard curve of gallic acid. The total phenolic content of stem and leaf of CSEE was expressed as the gallic acid equivalent (GAE) in mg g^{-1} dry material.

A 250 µL aliquot of each extract or standard solution was mixed with 1.25 mL of distilled water and 75 µL of 5% $NaNO_2$ solution. After 6 min, 150 µL of 10% $AlCl_3$-H_2O solution was added. After 5 min, 0.5 mL of 1 mol L^{-1} NaOH solution was added and the total volume was brought up to 2.5 mL with ddH_2O. Following thorough mixing of the solution, absorbance against the blank was determined at 510 nm and the total flavonoids were calibrated by a standard curve of quercetin. The total flavonoid content of the stem and leaf of CSEE was expressed as the quercetin equivalent (QE) in mg g^{-1} dry material.[27]

Cell culture

The RAW 264.7 macrophages of passages 10 to 15 were maintained in RPMI-1640 medium supplemented with 2 mmol L^{-1}

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L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. Cells were plated at a density of 8×10^5 per 30 mm culture dish and were incubated until 90% confluence was reached.

Cells were treated with 25–150 μg mL^{-1} leaf or stem of CSEE in the presence of 1 μg mL^{-1} LPS for 8–24 h as indicated or treated with the leaf or stem of CSEE for 1 h or 14 h prior to addition of LPS (1 μg mL^{-1}). The final DMSO concentration in the medium was 0.1%.

Cell viability assay

The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability.[28] After incubation with leaf or stem of CSEE with or without LPS for 24 h, cells were incubated in RPMI medium containing 0.5 mg mL^{-1} MTT for an additional 3 h. The medium was then removed and isopropanol was added to dissolve the formazan. After centrifugation at 5000 × *g* for 5 min, 100 μL of supernatant from each sample was transferred to 96-well plates, and the absorbance was read at 570 nm in a VersaMaxTM Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

Nitrite and PGE_2 determination

The nitrite in the medium was measured by use of the Griess assay and used as an indicator of NO synthesis in cells.[29] Briefly, equal volumes of the culture supernatants and Griess solution [1 : 1 mixture of 1% sulfanilamide and 0.1% *N*-(naphthyl)ethylenediamine dihydrochloride in 5% H_3PO_4] were added to 96-well plates at room temperature for 10 min. Absorbance was measured at 550 nm and nitrite concentration was determined by using a standard curve of sodium nitrite prepared in the culture medium.

Cells were treated with leaf or stem of CSEE in the presence of LPS for 12 h. The diluted culture supernatants were used to quantify PGE_2 by use of an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the protocol provided by the manufacturer.

Western blot analysis

Cells were washed twice with cold phosphate-buffered saline (PBS) and were harvested in 150 μL lysis buffer containing 10 mmol L^{-1} Tris-HCl, 5 mmol L^{-1} EDTA, 0.2 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF), and 20 μg mL^{-1} aprotinin, pH 7.4. The protein content in each sample was quantified by use of the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, USA). Equal amounts of proteins were denatured and separated on SDS-polyacrylamide gels and were then transferred to polyvinylidene difluoride membranes (NewTM Life Science Product, Inc., Boston, MA, USA). Nonspecific binding sites on the membranes were blocked with 5% nonfat dry milk in a buffer containing 10 mmol L^{-1} Tris-HCl and 100 mmol L^{-1} NaCl, pH 7.5, at 4 °C overnight. The blots were then incubated sequentially with primary antibody and horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). Immunoreactive protein bands were developed by enhanced chemiluminescence kits (Amersham Life Sciences, Arlington Heights, IL, USA) and then were quantified through densitometric analysis by Zero-Dscan (Scanalytics Inc., Fairfax, VA, USA).

Isolation of RNA and real-time quantitative reverse transcriptase-PCR

Total RNA was isolated from cells by using Tri-Reagent TM (Molecular Research Center Inc., Cincinnati, OH, USA) as described by the manufacturer and RNA extracts were suspended in nuclease-free water. Total RNA (0.1–0.25 μg) was reverse transcribed with M-MMLV reverse transcriptase in a 20 μL final volume of the reaction buffer consisting of 1 mmol L^{-1} of each deoxynucleotide triphosphate, 2.5 units RNase inhibitor and 2.5 m mol L^{-1} oligo(dT)$_{16}$. For the synthesis of complementary DNA, reaction mixtures were incubated for 15 min at 45 °C and stopped by denaturing the reverse transcriptase at 99 °C for 5 min. Complementary DNA was amplified with TaqMan® Universal PCR Master Mix primers and probes and the reactions were measured in ABI 7000 Real Time PCR System (Applied Biosystems). The primers and probes were obtained from Applied Biosystems: iNOS (Mm00440502_m1), COX-2 (Mm00478374_m1), IL-1β (Mm01336189_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm00484668_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard gene and the threshold cycles (Ct) of a test sample to a control sample ($\Delta\Delta$Ct method) was used for relative quantification of target gene expressions.[30]

Preparation of nuclear protein and EMSA

At the time of harvest, cells were scraped with cold PBS and centrifuged. The pellets were resuspended in the hypotonic extraction buffer (10 mmol L^{-1} HEPES, 10 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EDTA, 0.5 mmol L^{-1} dithiothreitol, 0.2 mmol L^{-1} PMSF, 4 μg mL^{-1} leupeptin, 20 μg mL^{-1} aprotinin, and 0.5% NP-40) for 15 min on ice and were then centrifuged at 6000 × *g* for 15 min. The pelleted nuclei were resuspended in 50 μL hypertonic extraction buffer (10 mmol L^{-1} HEPES, 400 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EDTA, 0.5 mmol L^{-1} dithiothreitol, 0.2 mmol L^{-1} PMSF, 4 μg mL^{-1} leupeptin, 20 μg mL^{-1} aprotinin, and 10% glycerol), were constantly shaken at 4 °C for 30 min, and were then centrifuged at 10 000 × *g* for 15 min. The resultant supernatants containing nuclear proteins were collected and stored at −70 °C until the EMSA was performed.

EMSA was performed according to our previous study.[31] The LightShiftTM Chemiluminescent EMSA Kit from Pierce Chemical Co. and synthetic biotin-labelled double-stranded NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used to measure the effect of the CSEE leaf or stem on NF-κB nuclear protein-DNA binding activity. Nuclear proteins (2 μg), poly(dI-dC), and biotin-labelled double-stranded NF-κB oligonucleotide were mixed with the binding buffer to a final volume of 20 μL and were incubated at room temperature for 30 min. In addition, the excess amount (100-fold molar excess) of unlabelled and a mutant double-stranded NF-κB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were used for the competition assay to confirm specificity of binding. The nuclear protein–DNA complex was separated by electrophoresis on a 6% Tris/boric acid/EDTA–polyacrylamide gel and was then electrotransferred to a nylon membrane (HybondTM-N+, Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). The membrane was treated with streptavidin–horseradish peroxidase, and the nuclear protein–DNA bands were developed by using a SuperSignal West Pico kit (Pierce Chemical Co.).

Reporter gene assay

Reporter enzyme activity was evaluated by a cell-based analysis method for assaying NF-κB activity. The pNF-κB-Luc reporter

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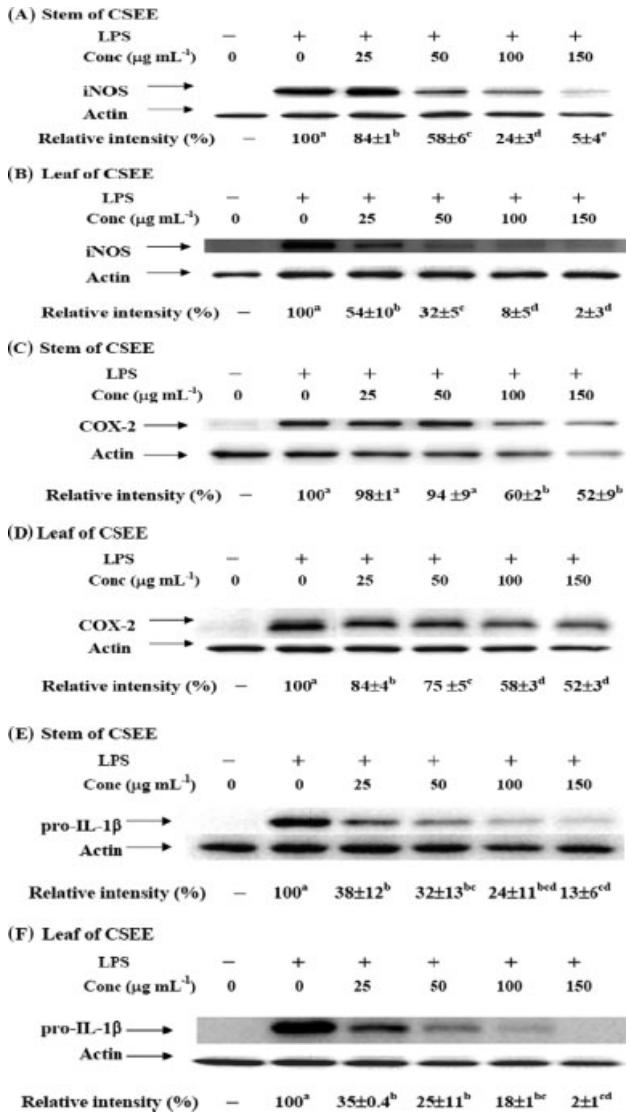

Figure 1. Effects of stem and leaf of CSEE on LPS-induced iNO, COX-2 and Pro-IL-1β protein expressions in RAW 264.7 macrophages. (A) iNOS protein expression was measured by RAW 264.7 macrophages treated with or without LPS (10 ng mL^{-1}) plus DMSO vehicle control and 25–150 μg mL^{-1} stem or leaf of CSEE for 24 h. (B) COX-2 and (C) Pro-IL-1β protein expression were measured in cells preincubated with 25–150 μg mL^{-1} stem or leaf of CSEE for 1 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 6 h. Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone. Values not sharing the same letter are significantly different ($P < 0.05$).

plasmid contains five tandem copies of the NF-κB consensus sequences, and it permits luciferase expression in response to NF-κB activity. When RAW264.7 cells reached confluence, transient transfection of the reporter plasmid and the pSV-β-galactosidase control vector was performed using the Lipofectamine™ transfection reagent for 6 h. Cells were then placed in fresh culture media for 18 h before treating with DMSO vehicle control, or LPS plus stem or leaf of CSEE for 8 h. Supernatants of the cell lysates were applied to measure the luciferase and β-galactosidase activities by the Luciferase Assay System and β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer from Promega Co., respectively.

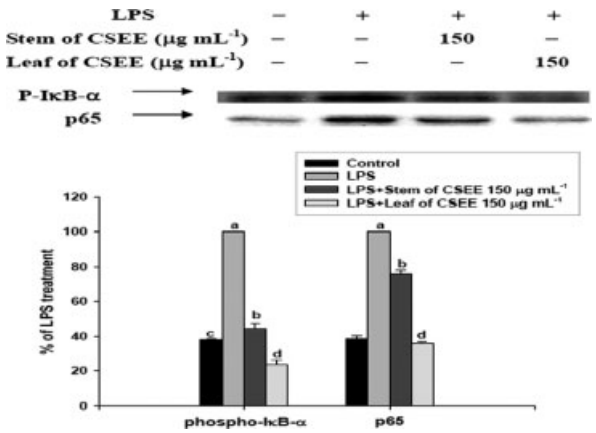

Figure 2. Effects of stem and leaf of CSEE on LPS-induced of expressions of cytoplasmic phosphorylated IκBα and nuclear p65. RAW 264.7 macrophages were preincubated with 150 μg mL^{-1} stem or leaf of CSEE for 14 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 30 min. Western blot analysis was used to measure the protein content of phosphorylated IκB-α in the cytosolic factions and to measure p65 protein content in the nuclear protein fractions of RAW 264.7 macrophages. Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone. Values not having the same letter are significantly different ($P < 0.05$).

Liquid chromatography/mass spectrometry analysis

The stem and leaf of CSEE were dissolved in DMSO, diluted with an appropriate volume of 50% methanol (in deionised water) and injected into the liquid chromatography–mass spectroscopy (LC-MS) system. LC-MS was carried out with an Agilent 1100 Series LC System equipped with a UV detector (Agilent Technologies, Palo Alto, CA, USA). An Alltima C18 reverse-phase column (5 μm, 250 × 4.6 mm) was used. The mobile phase consisted of 10 mmol L^{-1} ammonium acetate containing 0.5% formic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.6 mL min^{-1}. The total running time was 90 min. The gradient system used was 5% B (0–10 min), 5% B to 40% B (10–40 min), 40–55% B (40–55 min), 55% B to 80% B (55–65 min), 80–100% B (65–70 min), 100% B to 5% B (75–80 min), and 5% B (80–90 min). The column temperature was 25 °C. The effluent was monitored using a UV detector set at 254 nm and a mass spectrometer operating in the negative ion mode over the *m/z* range 100 to 700. Identification of constituents was carried out by LC-UV and LC-MS analyses by comparing their retention times, UV and mass spectra of the peaks with those of authentic standards based on the literature.[32] For quantitative analysis of rutin, calibration curves were constructed from working standard solutions of rutin at final concentrations of 1–1000 ng mL^{-1} and applied the same way for the stem and leaf of CSEE. Ions representing the [M-H]$^-$ species at *m/z* 609 were selected and the peak area was measured.

Statistical analysis

Data are expressed as the means ± SD from at least three independent experiments. Differences among treatments were analysed by ANOVA and Tukey's multiple-range test by using the Statistical Analysis System (Cary, NC, USA). *P* values less than 0.05 were considered to be significant.

RESULTS

The extraction yields of leaf and stem of CSEE were 11.76% and 11.97%, respectively. In addition, the phenolic contents,

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Table 1. Effects of an ethanolic extract from the stem of *Coriandrum sativum* (CSEE) on the MTT assay and LPS-induced NO and PGE_2 production in RAW 264.7 macrophages

Treatment*	MTT†	NO	PGE_2
Control	102.9 ± 2.0^{b}	ND	6.7 ± 0.2^{d}
LPS	100.0 ± 0.0^{b}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
LPS + Stem of CSEE 25 μg mL^{-1}	98.9 ± 4.6^{b}	94.4 ± 4.6^{b}	53.6 ± 8.9^{b}
LPS + Stem of CSEE 50 μg mL^{-1}	100.3 ± 3.8^{b}	60.0 ± 7.6^{c}	43.2 ± 2.1^{b}
LPS + Stem of CSEE 100 μg mL^{-1}	117.7 ± 7.9^{a}	32.7 ± 7.5^{d}	44.7 ± 9.7^{b}
LPS + Stem of CSEE 150 μg mL^{-1}	110.6 ± 5.0^{a}	19.7 ± 6.8^{e}	25.2 ± 2.4^{c}

* RAW 264.7 macrophages were treated with or without LPS (10 ng mL^{-1}) plus DMSO vehicle control and 25 to 150 μg mL^{-1} stem of CSEE for 24 h (MTT assay and NO production) or for 12 h (PGE_2 production).
† Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone.
[a,b,c,d] Means with different letters within the same column are significantly different ($P < 0.05$).

Table 2. Effects of leaf of CSEE on MTT assay and LPS-induced NO and PGE_2 production in RAW 264.7 macrophages

Treatment*	MTT†	NO	PGE_2
Control	102.9 ± 2.0^{b}	ND	6.7 ± 0.2^{d}
LPS	100.0 ± 0.0^{b}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
LPS + Leaf of CSEE 25 μg mL^{-1}	90.9 ± 0.7^{c}	82.8 ± 6.1^{a}	96.6 ± 6.2^{a}
LPS + Leaf of CSEE 50 μg mL^{-1}	102.9 ± 6.6^{b}	62.0 ± 7.2^{b}	80.7 ± 2.6^{b}
LPS + Leaf of CSEE 100 μg mL^{-1}	93.1 ± 1.4^{c}	46.9 ± 9.0^{c}	82.8 ± 3.8^{b}
LPS + Leaf of CSEE 150 μg mL^{-1}	116.0 ± 3.2^{a}	24.7 ± 7.8^{d}	52.5 ± 7.7^{c}

* RAW 264.7 macrophages were treated with or without LPS (10 ng mL^{-1}) plus DMSO vehicle control and 25 to 150 μg mL^{-1} leaf of CSEE for 24 h (MTT assay and NO production) or for 12 h (PGE_2 production).
† Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone.
[a,b,c,d] Means with different letters within the same column are significantly different ($P < 0.05$).

Table 3. Effects of stem of CSEE on LPS-induced iNO, COX-2, and IL-1β mRNA expressions in RAW 264.7 macrophages

Treatment*	iNOS†	COX-2	IL-1β
Control	0.7 ± 0.7^{e}	0.8 ± 0.7^{d}	0.1 ± 0.2^{d}
LPS	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
LPS + Stem of CSEE 25 μg mL^{-1}	59.1 ± 6.7^{b}	66.0 ± 4.7^{b}	78.8 ± 8.8^{ab}
LPS + Stem of CSEE 50 μg mL^{-1}	59.8 ± 5.1^{b}	63.1 ± 9.0^{b}	71.2 ± 6.4^{b}
LPS + Stem of CSEE 100 μg mL^{-1}	42.1 ± 7.2^{c}	54.6 ± 11.6^{c}	51.2 ± 10.2^{c}
LPS + Stem of CSEE 150 μg mL^{-1}	28.0 ± 4.4^{d}	45.3 ± 2.7^{c}	47.8 ± 9.0^{c}

* iNOS mRNA expression was measured by RAW 264.7 macrophages treated with or without LPS (10 ng mL^{-1}) plus DMSO vehicle control and 25 to 150 μg mL^{-1} stem of CSEE for 8 h. COX-2 and IL-1β mRNA expression were measured in cells preincubated with 25 to 150 μg mL^{-1} stem or leaf of CSEE for 1 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 6 h.
† Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone.
[a,b,c,d] Means with different letters within the same column are significantly different ($P < 0.05$).

Table 4. Effects of leaf of CSEE on LPS-induced iNO, COX-2, and IL-1β mRNA expressions in RAW 264.7 macrophages

Treatment*	iNOS†	COX-2	IL-1β
Control	0.7 ± 0.7^{e}	0.8 ± 0.7^{e}	0.1 ± 0.2^{d}
LPS	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
LPS + Leaf of CSEE 25 μg mL^{-1}	79.5 ± 8.8^{b}	47.8 ± 1.2^{b}	94.5 ± 5.4^{a}
LPS + Leaf of CSEE 50 μg mL^{-1}	42.6 ± 6.2^{c}	46.7 ± 4.1^{bc}	71.1 ± 8.7^{b}
LPS + leaf of CSEE 100 μg mL^{-1}	23.1 ± 5.8^{d}	26.6 ± 4.2^{cd}	62.6 ± 17.0^{b}
LPS + Leaf of CSEE 150 μg mL^{-1}	14.1 ± 3.8^{d}	21.6 ± 2.8^{d}	42.0 ± 6.2^{c}

* iNOS mRNA expression was measured by RAW 264.7 macrophages treated with or without LPS (10 ng mL^{-1}) plus DMSO vehicle control and 25 to 150 μg mL^{-1} leaf of CSEE for 8 h. COX-2 and IL-1β mRNA expression were measured in cells preincubated with 25 to 150 μg mL^{-1} stem or leaf of CSEE for 1 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 6 h.
† Data are the mean ± SD of at least four separate experiments and are expressed as the percentage of the culture treated with LPS alone.
[a,b,c,d] Means with different letters within the same column are significantly different ($P < 0.05$).

expressed as GAE, of leaf and stem extracts were 15.5 ± 1.9 and 17±3.8 mg g^{-1} dry extract, respectively. The amount of flavonoids, expressed as QE, of the leaf extract was 16.14 ± 1.17 mg g^{-1} dry extract which was 5.7 times higher than that of stem extract.

At the test concentrations, cell viability of the LPS-activated cells treated with leaf or stem of CSEE was more than 90% of that of cells treated with LPS alone, as assessed by mitochondrial reduction of MTT after 18 h challenge (Tables 1 and 2).

As shown in Tables 1 and 2, stimulation of macrophages with LPS resulted in a strong increase in NO and PGE_2 production. A dose-dependent decrease in NO and PGE_2 production was noted in cells treated with leaf or stem of CSEE in the presence of LPS. At a concentration of 150 μg mL^{-1}, 80% and 75% reduction in nitrite production were noted in cells treated with leaf and stem of CSEE, respectively. The PGE_2 levels in cells treated with 150 μg mL^{-1} of the leaf and stem of CSEE were 25.2 ± 2.4% and 52.5 ± 7.7%, respectively, of the level of cells treated with LPS alone.

The immunoblot assay showed that the protein expression of iNOS, COX-2 and proIL-1β was undetectable in resting RAW 264.7 macrophages and was highly induced in the presence of LPS. The addition of exogenous leaf or stem of CSEE significantly reduced LPS-induced protein expression of iNOS, COX-2 and pro-IL-1β ($P < 0.05$, Fig. 1). As noted for the changes in protein expression, real-time RT-PCR further showed that LPS-induced mRNA expression of iNOS, COX-2 and pro-IL-1β was significantly decreased by leaf and stem of CSEE (Tables 3 and 4).

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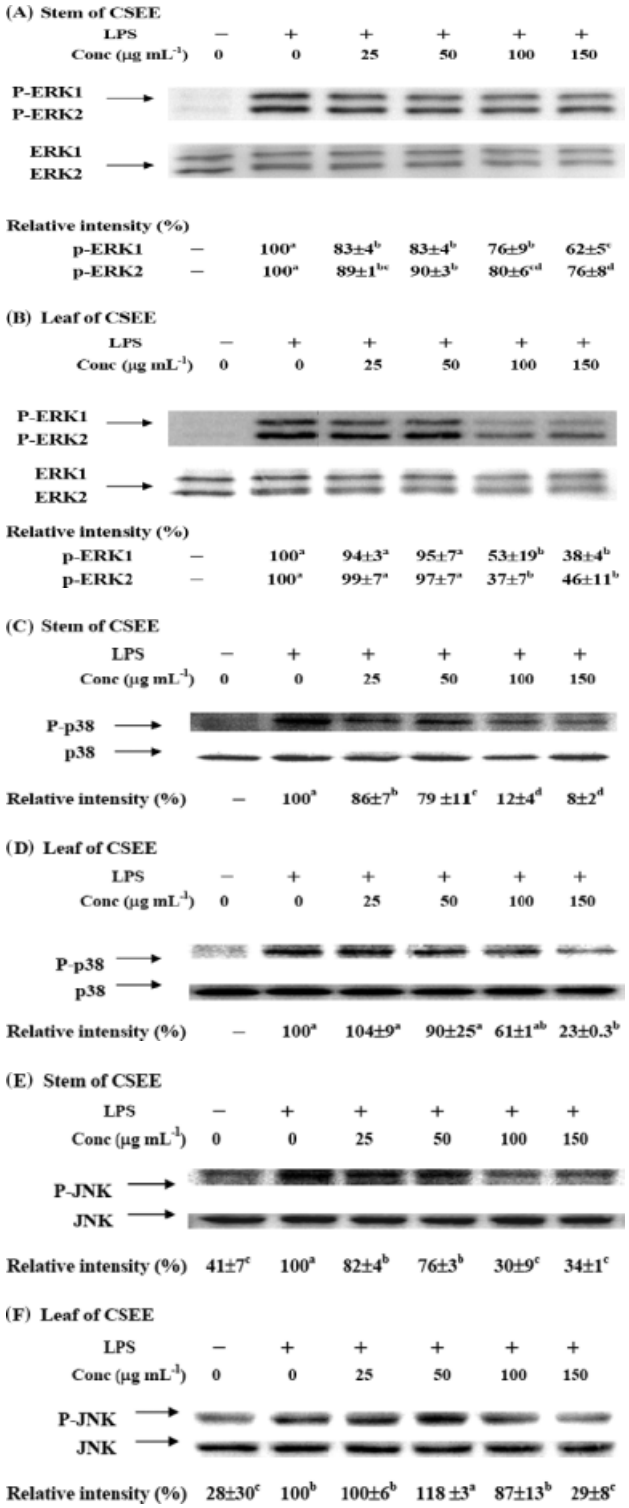

Figure 3. Effects of stem and leaf of CSEE on LPS-induced of activation of MAPKs. RAW 264.7 macrophages were preincubated with 25–150 μg mL^{-1} stem or leaf of CSEE for 1 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 30 min. Cells were lysed and western blotting was performed with the antibodies for phosphorylated (A) ERK 1/2, (B) p38 and (C) JNK and the cells were then reprobed with antibodies against the corresponding MAPKs. The ratios of immunointensity between the MAPKs and the phosphorylated MAPKs are shown and are expressed as the percentage of the culture treated with LPS alone. Data are the mean ± SD of at least four separate experiments and values not sharing the same letter are significantly different ($P < 0.05$).

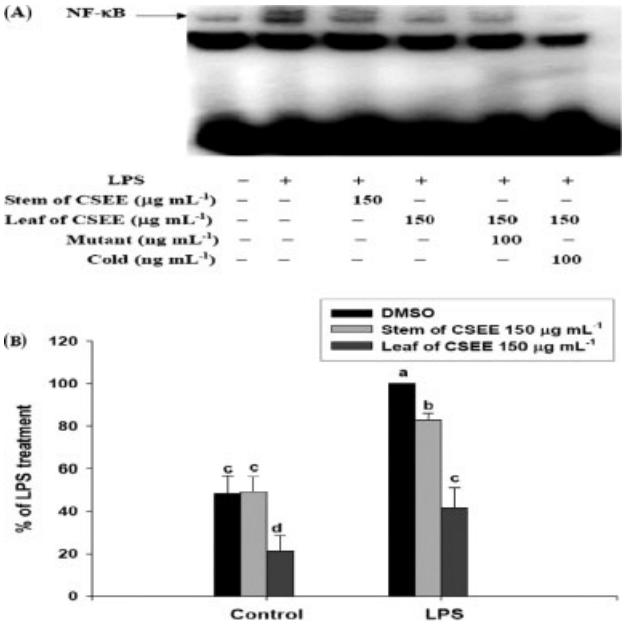

Figure 4. Effects of stem and leaf of CSEE on activation of NF-κB. (A) RAW 264.7 macrophages were preincubated with 150 μg mL^{-1} stem or leaf of CSEE for 14 h and then treated with either DMSO vehicle control or 10 ng mL^{-1} LPS for 30 min. EMSA experiments were carried out by using the LightShift Chemiluminescent EMSA Kit from Pierce Chemical Co. The unlabelled double-stranded oligonucleotides of NF-κB and the unlabelled double-stranded mutant NF-κB oligonucleotide were added for the competition assay and specificity assay, respectively. Bands were detected by using streptavidin–horseradish peroxidase and were developed by using a SuperSignal West Pico kit from Pierce Chemical Co. (B) Cells were transiently transfected with pSV-β-galactosidase and pNF-κB-Luc reporter gene for 6 h and cells were treated with either vehicle control or 10 ng mL^{-1} LPS plus 150 μg mL^{-1} stem or leaf of CSEE for 8 h. Cells were harvested and the level of luciferase and β-galactosidase activity were measured by the Luciferase Assay System and β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer from Promega Co., respectively. Data are the mean ± SD of at least three separate experiments and are expressed as the percentage of the culture treated with LPS alone. Values not having the same letter are significantly different ($P < 0.05$).

Upon LPS treatment, the amounts of cytoplasmic phosphorylated IκB-α protein and nuclear p65 protein were greatly increased compared with those of the control (Fig. 2). Addition of 150 μg mL^{-1} leaf or stem of CSEE significantly abolished the level of LPS-induced protein expression of phosphorylated IκB-α and nuclear p65 ($P < 0.05$).

To test whether the mitogen-activated protein kinase (MAPK) signalling pathway was involved in the anti-inflammatory property of *C. sativum*, we examined the effect of leaf and stem of CSEE on LPS-induced MAPK activation. LPS treatment resulted in strong increases in the amounts of phosphorylated ERK1/2, p38 and JNK-1 expression ($P < 0.05$, Fig. 3). Addition of stem extracts significantly reduced LPS-induced phosphorylated JNK and p38. However, LPS-induced activation of ERK1/2 was diminished only by high doses of CSEE stem. Addition of high doses of leaf extracts significantly inhibited LPS-induced activation of MAPKs. The amount of the unphosphorylated form of MAPKs was not influenced by the LPS treatment or LPS plus stem or leaf of CSEE.

EMSA experiments were used to evaluate the effect of *C. sativum* on activation of NF-κB. As shown in Fig. 4A, the nuclear extract from LPS-stimulated macrophages showed a marked increase in NF-κB nuclear protein DNA-binding activity compared with that

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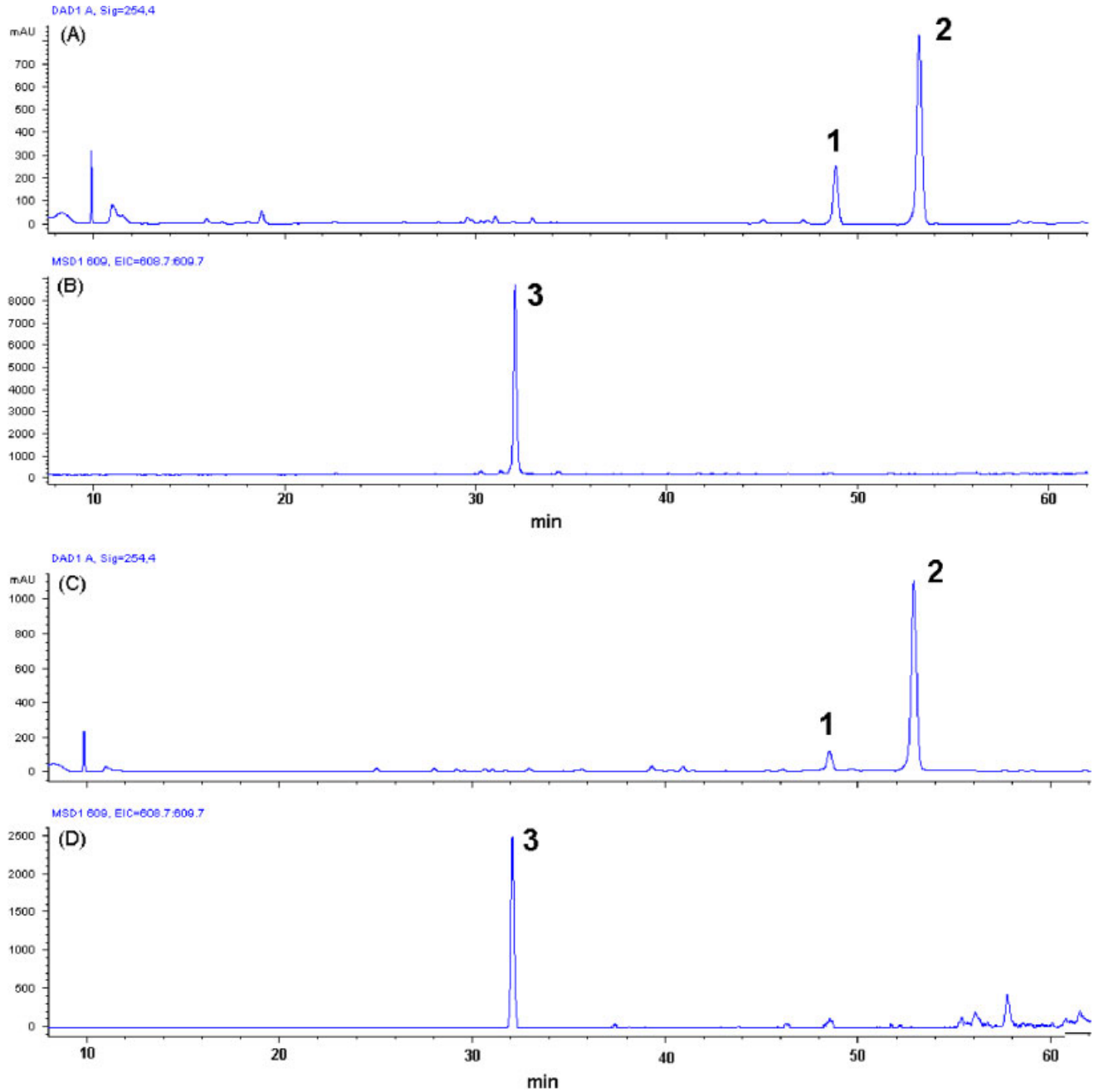

Figure 5. The HPLC-UV (UV 254 nm) and selectively extracted ion (rutin: $[M\text{-}H]^-$, *m/z* 609.0) chromatograms of CSEE of stem (A, B) and leaf (C, D). 1 and 2: unknown compounds; 3: rutin.

in unstimulated macrophages. Pretreatment of cells with leaf or stem of CSEE suppressed the activation of NF-κB binding to its consensus DNA sequences. The specificity of the NF-κB nuclear protein–DNA binding was verified by competition assay with a 50-fold excess of unlabelled NF-κB probe and unlabelled mutant NF-κB probe.

To investigate the transcriptional activity of NF-κB, the expression of reporter genes in cells transfected with pNF-κB-Luc and the internal control pSV-β-galactosidase were analysed. Consistent with the EMSA assay result, the expression of LPS-induced NF-κB-Luc activity was significantly inhibited in cultures treated with 150 μg mL^{-1} leaf or stem of CSEE (Fig. 4B, $P < 0.05$).

LC-MS analysis of the testing samples and several authentic standards revealed that only rutin is identified in both the stem and leaf of CSEE. Rutin showed the $[M\text{-}H]^-$ ion at *m/z* 609 and its retention time was 31.9 min. Based on the peak area of mass spectral peak, the rutin concentrations in stem and leaf extracts were 130.5 and 42.0 μg g^{-1}, respectively. Two unknown large UV peaks presented at 48.3 min ($[M\text{-}H]^-$ ion at *m/z* 253) and 52.6 min ($[M\text{-}H]^-$ ion at *m/z* 221) were not identified (Fig. 5).

DISCUSSION

Numerous studies have focused on herbal remedies and botanicals because they offer much promise in health benefits and disease

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treatments without excessive side effects and cytotoxicity.[33] A wide variety of plant-derived products, especially spices, have shown an anti-inflammatory effect but only a few have been examined to determine the molecular mechanism of this inhibitory action.[34] In this study, we investigated the anti-inflammatory properties of aerial parts of CSEE and dissected the possible molecular mechanism of action of *C. sativum*. The data presented herein showed that both the leaf and stem of CSEE significantly decreased NO and PGE_2 production. The inhibition of NO and PGE_2 was due to the inhibition of iNOS and COX-2 expression respectively, at mRNA and protein levels as shown by real-time RT-PCR and western blot. Moreover, we demonstrated that the aerial part of CSEE suppressed iNOS, COX-2 and IL-1β expression, acting at the transcriptional level possibly via inhibition of the LPS-induced MAPK pathway and transcription factor NF-κB activation.

Considerable interest in immunomodulation therapy is now focused on blocking the activation of NF-κB in macrophages, which results in suppressing a range of inflammatory mediator expression such as iNOS, COX-2 and IL-1β.[2,35,36] Our data clearly showed that CSEE effectively inhibited the NF-κB pathway by blocking LPS-induced IκB-α phosphorylation, nuclear p65 expression and subsequent DNA binding affinity and transcriptional activation. These results suggested that stem and leaf of CSEE decreased the expression of pro-inflammatory mediators via down-regulating the NF-κB pathway in stimulated macrophages.

MAPKs, one of the most important intracellular signalling pathways, are a family of serine/threonine protein kinases, which include JNK, ERK and p38 kinase subgroups at least in mammalian cells. MAPK pathways are involved in a battery of cellular events, including cell proliferation and growth, cell differentiation, cell movement, cellular senescence and apoptosis.[37] Although the exact signal pathways of MAPKs are still unclear, LPS-induced phosphorylation and activation of MAPKs in macrophages lead to the production of pro-inflammatory mediators as a result of the activation of transcription factors including NF-κB.[38] In the present study, the aerial part of CSEE significantly decreased LPS-induced phosphorylation of the three MAPKs, which implies that the inflammatory signal transduction by the MAPK pathways could be impeded by *C. sativum* in LPS-induced macrophages. Numerous studies have demonstrated that phenolic compounds in spices contribute to the health benefits of spices.[39] A previous study indicated that luteolin, vicenin, ferulic acid and arbutin were the main components in the aerial part of CSEE.[32] However, in this study, only rutin was identified in the stem and leaf of CSEE and the rutin concentration in stem extracts (130.5 $\mu g\ g^{-1}$) was higher than that in the leaf extracts (42.0 $\mu g\ g^{-1}$). It was interesting to note that the total amount of flavonoids in the stem extracts was also lower than that in the leaf extracts, although there was no significant difference in the amount of total phenolics between stem and leaf of CSEE. Furthermore, the amount of rutin in the IC_{50} values of the stem and leaf of CSEE against LPS-induced NO production in RAW264.7 macrophage was 8.2 $\mu g\ mL^{-1}$ and 2.7 $\mu g\ mL^{-1}$, much lower than the reported IC_{50} value of rutin on LPS-induced NO production (25.3 $\mu g\ mL^{-1}$).[40] These results indicated that it is not only rutin which provides contributions to the anti-inflammatory properties of *C. sativum*, but other yet unknown compounds in the stem and leaf of CSEE may present further contributions as well. It is noteworthy that the antimutagenicity of *C. sativum* was dependent on the chlorophyll content in *C. sativum* juice.[14] Moreover, the chlorophyllin, a water-soluble derivative of chlorophyll, inhibited NO production and iNOS expression by modulating LPS-induced NF-κB activation in RAW264.7 macrophages.[41] Therefore, the amount of chlorophyll in CSEE is likely to play an important in its anti-inflammatory property.

In conclusion, we have demonstrated that both the leaf and the stem of CSEE modulate LPS-induced inflammatory events in RAW 264.7 macrophages. This inhibitory activity of *C. sativum*, at least in part, occurs through *C. sativum* modulating the NF-κB activation and MAPK pathway. According to these experimental results supporting the anti-inflammatory property of the leaf and stem of CSEE, it would be worthwhile to explore the biomedical importance of the aerial parts of CSEE in the treatment and prevention of chronic inflammatory related diseases.

ACKNOWLEDGEMENTS

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無衍生研發成果推廣資料

96 年度專題研究計畫研究成果彙整表

計畫主持人：劉凱莉	計畫編號：96-2320-B-040-027-MY3
計畫名稱：評估常見植物性食材萃出物調控與細胞發炎、抗氧化/解毒作用相關物質表現及轉錄因子活化之功效	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	2	2	100%	人次	
		博士生	2	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

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□實驗失敗

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說明：

2. 研究成果在學術期刊發表或申請專利等情形：

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本實驗室研究結果證實 95%酒精之香菜莖及葉萃出物可經由抑制 LPS 誘發轉錄因子 NF-κB 活

化及 MPAKs 磷酸化之作用抑制 LPS 誘發吞噬細胞 RAW264.7 發炎反應。由液相層析質譜儀分析

(Liquid Chromatography /Mass Spectrometry analysis)結果證實 95%酒精之香菜莖及葉萃出物分別含有

多酚類 rutin 130.5 及 42.0 μg/ g 萃出物。為更了解香菜酒精萃出物保健功效，本實驗室 99 至 102 年之

研究計畫目的在評估香菜酒精萃出物對 adipogenesis 及 RAW264.7 macrophage conditional medium 誘發

脂肪細胞發炎反應之影響。此研究計劃實驗結果有助於了解香菜酒精萃出物對抗肥胖及預防第二型糖

尿病、心血管疾病及代謝症候群等與肥胖有關的病症發生之功效。此外，由於 rutin 為香菜酒精萃出物中含量最多的天然植物化合物，故實驗室 99 至 102 年之研究計畫亦加入 rutin 處理組，以評估 rutin 是否為香菜酒精萃出物具保健功效之主要天然植物化合物.