

Anti-inflammatory Activities of *Chopi* (*Zanthoxylum piperitum* A.P. DC) Essential Oil: Suppression of the Inducible Nitric Oxide Synthase and Cellular Adhesion

Je-Hyuk Lee¹, Kyung-Mi Chang¹, and Gun-Hee Kim^{1,2*}

¹Plant Resource Research Institute, Duksung Women's University, Seoul 132-714, Korea

²Department of Food and Nutrition, Duksung Women's University, Seoul 132-714, Korea

Abstract The aim of this study is to elucidate the anti-inflammatory activities of *chopi* (*Zanthoxylum piperitum* A.P. DC.) essential oil. Essential oil (EO) of *chopi* was extracted by steam distillation method, and its major constituents were limonene and geranyl acetate. *Chopi*-EO decreased approximately 38% of nitrite production, as compared to the lipopolysaccharide (LPS)-induced nitrite production. However, *chopi*-EO and its components did not quench nitric oxide (NO) chemically in cell-free system, and markedly inhibited approximately 40.4% of inducible nitric oxide synthase (iNOS) mRNA transcription. In addition, the inhibition of E-selectin gene transcription by *chopi*-EO caused the suppression of cellular adhesion. These results suggest that *chopi*-EO may exert potential anti-immunological inflammatory activity.

Keywords: *chopi*, *Zanthoxylum piperitum* A.P. DC, essential oil, inflammation, interleukin, E-selectin

Introduction

Nitric oxide (NO) and inducible nitric oxide synthase (iNOS) have a significant pathophysiological role, being involved in the development and/or exacerbation of inflammation in many cells, including murine macrophages, hepatocytes, endothelial cells, and human monocytes/macrophages (1). Expression of iNOS, which catalyzes the production of large amounts of NO from L-arginine and molecular oxygen, is triggered by inflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and bacterial lipopolysaccharide (LPS) in murine macrophages (2,3). NO, in turn, participates in the inflammatory response of macrophages (4). Therefore, inhibiting high-output NO production by blocking iNOS production or activity may be a useful strategy for treatment of inflammatory disorders.

Chopi (*Zanthoxylum piperitum* A.P. DC), belonging to Rutaceae family, has been mainly used for seasoning a food and medicines for strengthening of stomach, antiseptic, diuresis, and neuralgia in Korea, Japan, and China (5). *Chopi* has been investigated mainly for the composition of volatile aroma, the stimulant effect, and the antimicrobial activity for *Escherichia coli* and *Staphylococcus aureus*.

Recently, the essential oils (EOs), which are volatile plant secondary metabolites, have been attracted attention for the usages in pharmacy, medicine, food, and beverages, cosmetics, perfumery, and aromatherapy. EOs have been shown to have the bactericidal, antioxidant (6), and anti-inflammatory effects on suppression of pro-inflammatory mediator production (7). Additionally, it has been reported that pro-inflammatory cytokines, such as IL-1 β , modulate

the expression of cyclooxygenase (COX)-2 through nuclear factor (NF)- κ B, which is implicated in gene expression, in human neuroblastoma cell and tracheal smooth muscle (8). Considering these findings, it can be speculated that a spinal cytokine/NF- κ B/COX-2 pathway may play an important role in the development of chronic pain following peripheral tissue inflammation. IL-6 is a multifunctional cytokine that play a central role in both innate and acquired immune responses, which is triggered by infection and inflammation.

In early stage of an immunological inflammatory disease, the adherence of monocyte to human umbilical vein endothelial cell (HUVEC) monolayer is elevated. Cellular adhesion molecules (CAMs) are stimulated by inflammatory mediators, such as TNF- α and ILs. Up-regulated expression of CAMs increases the adhesion of monocyte to HUVEC monolayer, and is involved in rheumatoid arthritis (RA) and atherosclerotic responses (9).

So far, relatively little research has been carried out on anti-inflammatory activities of *chopi*-EO. To elucidate the mechanism for potential anti-inflammatory activities of *chopi*-EO, it was investigated the EO composition of *chopi* and the inhibitory effects on the production of NO, the cytokines, and E-selectin transcription level for cellular adhesion in this report.

Materials and Methods

Plant material and chemicals *Chopi* was purchased from Kyungdong herbal market (Seoul, Korea) in March 2007, and was authenticated by Prof. Gun-Hee Kim, Plant Resources Research Institute, Duksung Women's University. The voucher specimen (No. LDU2007-052) was deposited at the Plant Resources Research Institute, Duksung Women's University, Korea.

Dimethyl sulfoxide (DMSO), calcein *O,O'*-diacetate tetrakis (acetoxymethyl) ester (calcein-AM), 3-(4,5-

*Corresponding author: Tel: +82-2-901-8663; Fax: +82-2-901-8661

E-mail: ghkim@duksung.ac.kr

Received April 14, 2009; Revised August 3, 2009;

Accepted August 8, 2009

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), gelatin, and heparin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin, endothelial cell growth supplement (ECGS), and trypsin-ethylenediamine tetraacetic acid (EDTA) were obtained from Gibco (Invitrogen Inc., Grand Island, NY, USA). TNF- α was purchased from BD Science (San Jose, CA, USA). All other chemicals and solvents were of highest commercial grade.

Cell culture RAW 264.7 murine macrophage cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μ g/mL) in a 5% CO₂ humidified incubator at 37°C. RAW 264.7 cells were used at passage numbers 10-20 for LPS-induced nitrite production assay. Monocytic cell line, THP-1, was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Monocytes were cultured in RPMI-1640 medium containing 10% FBS and 100 units/mL of penicillin/streptomycin at 37°C in a 5% CO₂ incubator under controlled moisture. THP-1, the floating cell line, was subcultured after being collected by centrifugation at 2,090 \times g for 2 min, and were used for cell adhesion assay at passage numbers 60-70. HUVECs (CRL-2480; ATCC) were cultured with F-12K nutrient mixture (Kaighn's modification, Gibco, Invitrogen Inc.) containing 10% FBS, 100 units/mL of penicillin/streptomycin, 0.1 mg/mL of heparin, and 0.03 mg/mL of ECGS in a 5% CO₂ humidified incubator at 37°C. For subculture, RAW 264.7 murine macrophage cells and HUVECs rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum (which can inhibit trypsin), and were subdivided using 0.05% trypsin with 0.53 mM EDTA. HUVECs were used at passage numbers 20-30 for cellular adhesion assay.

Extraction and separation of EO EO of *chopi* was extracted by steam distillation method (10). Briefly, EO was collected from the air-dried and ground *chopi* for 3 hr using a Clevenger-type apparatus (Hanil Labtech Ltd., Incheon, Korea), and was dried over anhydrous sodium sulfate for 24 hr.

Gas chromatography-mass spectrometry (GC-MS) analysis and identification of EO The constituents of essential oil were analyzed using an Agilent 6890 gas chromatography/5973 mass selective detector (Agilent Co., Palo Alto, CA, USA) equipped with a HP-5MS capillary column (30 m length \times 0.25 mm i.d. \times 0.25 μ m film thickness; Agilent Co.). Helium was used as the carrier gas at the constant flow of 1.1 mL/min. The oven temperature was held at 40°C for 5 min, then programmed from 40 to 150°C at the rate of 3°C/min, then held isothermal at 150°C for 5 min, raised to 220°C at the rate of 7°C/min, and held finally at 220°C for 5 min. The injector and detector (flame-ionization detector, FID) temperatures were kept at 250°C at a rate of 4°C/min, and then hold for 10 min. The constituents of the volatile flavor were identified by comparison of the mass spectra with those in an on-line

computer library, Wiley 275 (Agilent Co.). Alkanes were used as reference points in the calculation of relative retention indices (RI). The RIs of compounds, determined using C₈-C₂₂ as external references (11), were compared with the published data (12). The quantification of each volatile component was carried out based on the ratio of the peaks obtained from a mass total ion chromatogram. Standard EOs (limonene, citronellal, and geranyl acetate) were purchased from Sigma-Aldrich and used for the test of potential anti-inflammatory activity as major constituents of *chopi*-EO.

Antioxidant assay For 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, a 0.2 mL of methanolic solution containing 500 μ g/mL of *chopi*-EO was mixed with 4 mL of methanol, and a methanolic solution of DPPH (1 mM, 0.5 mL) was added. The mixture was vortexed for 15 sec, left to stand at room temperature for 30 min, and the absorbance was read at 517 nm.

The reducing power of *chopi*-EO was determined by Fe³⁺ reduction. *Chopi*-EO (500 μ g/mL) in distilled water were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% K₃Fe(CN)₆. The mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2,090 \times g for 10 min. A 2.5 mL of supernatant layer was added to 2.5 mL of distilled water and 0.5 mL of 0.1% FeCl₃. The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry (Agilent Technologies Inc., Santa Clara, CA, USA).

Cell viability assay RAW 264.7 cells and HUVEC monolayers were plated at a density of 1 \times 10⁵ cells/well in 96-well tissue culture plate (Corning Inc., Corning, NY, USA), and were incubated at 37°C for 3-4 hr. Plated cells were treated with indicated concentrations of *chopi*-EO. After 24 hr incubation, MTT was added to all well at 0.5 mg/mL of concentration, and was incubated for 4 hr at 37°C. After discarding all medium from the plates, 100 μ L of DMSO was added to the all well. The plates were placed for 5 min at room temperature with a shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax, Molecular Devices Inc., Sunnyvale, CA, USA).

Nitrite assay RAW 264.7 cells were plated at a density of 2 \times 10⁵ cells/well in a 96-well culture plate and incubated for 3-4 hr in a 5% CO₂ humidified incubator at 37°C. Plated cells were treated with (1 μ g/mL) for stimulation of nitrite-production and an indicated concentrations of *chopi*-EO for 24 hr incubation. LPS-stimulated nitrite-production from RAW 264.7 cells was measured by the Griess reaction (13). Briefly, 100 μ L of each supernatant was mixed with 100 μ L of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture was determined with a microplate reader (Emax) at 540 nm. In this experiment, 10 μ M of L-NMMA, an iNOS inhibitor, was used as a positive control.

Extracellular NO scavenging assay Extracellular NO radical scavenging activity was measured by the modified protocol (14). In aqueous solution at physiological pH, NO generated from sodium nitroprusside interacts with oxygen to produce nitrite ions which were measured by Griess reaction. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM in PBS) and *chopi*-EO or major components (50 μ M) were incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture and 0.5 mL of Griess reagent were mixed. The absorbance of the chromophore formed was evaluated at 540 nm. Hemoglobin (Hb, 5 μ M) was used as a NO chemical scavenger (15).

Measurement of cytokines RAW 264.7 macrophage cells were cultured in 24-well culture plates. After reaching confluence, LPS-induced cells were treated with *chopi*-EO, and then incubated in a humidified incubator at 37°C. After 48 hr incubation, the supernatant were collected, subdivided into portions and frozen at -74°C. Productions of IL-1 β and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction on a monoclonal antibody based mouse cytokines microplate strip (Quantikine®M, R&D System, Minneapolis, MN, USA).

Cellular adhesion assay Prior to adhesion assay, monocytic THP-1 cells were fluorescent-labeled by the incubation with 5 μ M of calcein-AM in PBS (pH 7.4) for 30 min at 37°C. After loading calcein-AM, cells were washed 3 times with PBS to remove excess calcein-AM. Then THP-1 cells were resuspended in RPMI-1640 medium for adhesion assay.

HUVECs were seeded at 1×10^5 cells/well in 96-well tissue culture plate (Corning 3603; Corning Inc.). After 24 hr incubation at 37°C, HUVEC monolayers were treated with 50 μ g/mL of *chopi*-EO and 3 major constituents, and then were stimulated with 10 ng/mL of TNF- α for 24 hr. HUVEC monolayers were washed 3 times with PBS before cell adhesion assay (16). Calcein-AM labeled monocytes, THP-1, were cocultured at a density of 5×10^5 cells/well with HUVEC monolayers for 1 hr in a 5% CO₂ humidified incubator at 37°C. Non-adherent monocytes were removed by 4 time-wash with PBS. Adherence of calcein-AM labeled monocytes was determined by fluorescent intensity, measured using a fluorescent plate reader (FL600; Bio-Tek Instruments Inc., Winooski, VT, USA). The excitation and emission wavelengths for the calcein-AM molecule were 485 and 530 nm, respectively.

For standardization of cell adhesion assay data, the amount of plated monolayer was measured by BCA protein assay (Pierce Inc., Rockford, IL, USA) using bovine serum albumin (BSA) as a standard, following solubilizing cells using 0.1 N NaOH and 1% of 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis Total RNA was isolated from RAW 264.7 cells and HUVEC monolayers using RNeasy kit (Qiagen Inc. Valencia, CA, USA) after treated with *chopi*-EO. RT-PCR was performed using One-Step RT-PCR kit (Qiagen Inc.) and primers at a final concentration of 1 μ M. For PCR of iNOS and E-selectin, the primers were used as

followed: mouse iNOS forward primer: 5'-CCCTTCC GAAGTTTCTGGCAGC-3', mouse iNOS reverse primer: 5'-GGCTGTCAGAGCCTCGTGGCTT-3', human E-selectin forward primer: 5'-ATCATCCTGCAACTTCA CC-3', and human E-selectin reverse primer: 5'-ACACCT CACCAAACCCTTC-3'. GAPDH primers were used for the PCR efficiency and the quantitation, as followed; mouse GAPDH forward primers, 5'-TGAAGGTCGGTGT GAACGGATTTGGC-3'; mouse GAPDH reverse primers, 5'-CATGTAGGCCATGAGGTCCACCAC-3', human GAPDH forward primer: 5'-ATGACAACAGCCTCAAG ATCATCAG-3', and human GAPDH reverse primer: 5'-CTGGTGGTCCAGGGGTCTTACTCCT-3'. For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. The PCR reaction of iNOS gene (494 bp-PCR product) was cycled 25 times between 94°C (denaturation) for 45 sec; 60°C (annealing), and GAPDH for 1 min; and 72°C (extension) for 2 min (17,18). And PCR amplification of E-selectin gene (345 bp-PCR product) was performed by thermocycling 30 times between 95°C for a 1 min denaturation, 55°C for 2 min annealing, and 72°C for a 3 min extension (19,20). And the last 1 cycle was performed for the final extension at 72°C for a 10 min using Bio-Rad thermal cycler (MJ Mini, Bio-Rad Inc., Hercules, CA, USA). And RT-PCR product was stored at 4°C until agarose gel separation. Transcriptional changes were calculated using an electrophoresis image quantify program (Bio-Rad Inc.).

Statistical analysis All results were expressed as mean \pm standard deviation (SD), and were analyzed using one way analysis of variance (ANOVA) and Dunnett's multiple comparison test for individual comparisons. Results were considered statistically significant when *p*-values were **p*<0.05.

Results and Discussion

Composition of EO from *chopi* A colorless EO was extracted from *chopi*. Among detected chemical compounds of *chopi*-EO, the components (more than 0.5% amount, 91.92%) were listed in Table 1. Limonene (18.04) and geranyl acetate (15.33%) were the major constituents of *chopi*-EO. Also, the considerable amounts of cryptone (8.51), citronellal (7.08), phellandral (5.21), and piperitone (4.43%) were detected. This result was consistent with several reports on the analysis of *chopi*-EO (5,21,22). However its composition varied with reports, due to the different plant part and place of origin. Although it is reported that 1,8-cineol, limonene, geranyl acetate, and myrcene were detected from *chopi*-EO (24), 1,8-cineol was not detected in this report. *Chopi*-EO and citronellal, geranyl acetate, and limonene, as major components, were investigated for a further study.

Antioxidant activities of *chopi*-EO Antioxidant activities of *chopi*-EO were showed in Table 2. *Chopi*-EO had the hydrogen donating activity to DPPH radical partly, and 3 EO constituents had the similar scavenging activities. DPPH radical scavenging of the reference antioxidant ascorbic acid (10 μ M) was found experimentally to be 92.21%. Although

Table 1. Chemical composition of *chopi*-EO

Constituents ¹⁾	R.T.	R.I. ²⁾	Composition (%) ³⁾
Limonene	15.39	1,025	18.04
Geranyl acetate	32.52	1,386	15.33
Cryptone	23.23	1,170	8.51
Citronellal	22.15	1,143	7.08
Cuminal	25.34	1,230	6.21
Phellandral	27.24	1,263	5.21
Piperitone	26.09	1,250	4.43
Citronellyl acetate	30.74	1,345	3.18
<i>p</i> -Cymen-7-ol	28.74	1,280	3.18
β -Myrcene	13.28	988	2.87
Geraniol	26.65	1,258	2.36
Chrysanthenone	27.52	1,265	1.81
Cuminol	28.75	1,282	1.62
Isopuegol	21.08	1,130	1.58
Rose oxide	19.14	1,103	1.50
Linalool	18.79	1,095	1.44
Citronellol	25.51	1,235	1.17
2-Nonadecene	54.52	1,190	1.10
(<i>E</i>)-Dehydrocarvone	23.78	1,180	0.83
Geranial	28.38	1,275	0.83
α -Terpinyl acetate	30.22	1,336	0.81
β -Fenchyl alcohol	23.35	1,172	0.74
Hexadecanoic acid	54.16	1,898	0.54
Dehydro <i>p</i> -cymene	20.56	1,118	0.53
Terpinen-4-ol	22.62	1,168	0.52
Methyl palmitate	53.36	1,881	0.50
Total			91.92

¹⁾Constituents having over 0.5% of peak area were showed in this report.

²⁾Retention index were calculated against *n*-alkanes (C₈-C₂₂) as external references on HP-5MS capillary column.

³⁾Area composition is the average of the relative percentage of the peak area in the MS total ion chromatogram (*n*=3).

Table 2. Antioxidant activity of *chopi*-EO and its constituents

	DPPH radical scavenging (% control)	Reducing power (Abs _{700 nm})
<i>Chopi</i> -EO	18.3±2.65* ¹⁾	0.02±0.01
Citronellal	10.67±0.37*	0.01±0.001
Geranyl acetate	10.23±0.37*	0.01±0.001
Limonene	10.13±0.46*	0.01±0.001
Positive control	92.21±0.52 ²⁾ *	2.24±0.01 ³⁾ *

¹⁾All samples were used in 500 μ g/mL for antioxidant assay; Significant difference at **p*<0.05, as compared to the control.

²⁾Ascorbic acid (10 μ M).

³⁾Pyrogallol (100 μ g/mL).

all constituents in *chopi*-EO showed antioxidant activities in significant difference, it was much lower than that of ascorbic acid, a reference antioxidant. Therefore, *chopi*-EO was expected to be partly electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reactions. Also, the reducing power of *chopi*-EO was not detected at all.

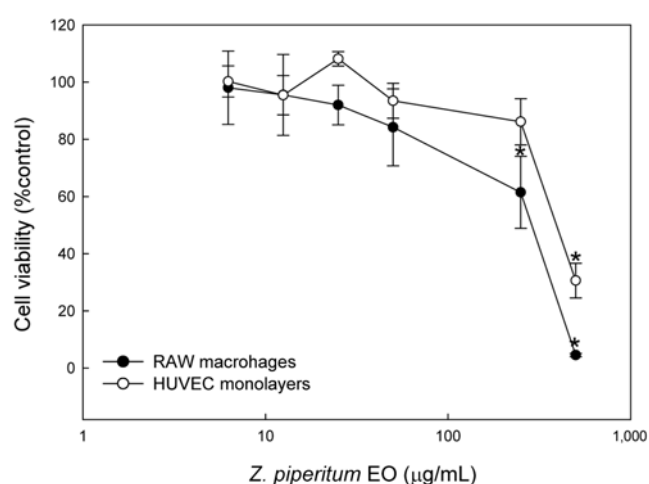


Fig. 1. Effect of *chopi*-EO on cell viability. The control value for cell viability was 99.98±5.56 μ g/mL. Significant difference at **p*<0.05 as compared to the control (untreated group by *chopi*-EO)

Sometimes the cell and tissue injuries are due to the toxicity of reactive oxygen species (ROS) generated and released by activated phagocytes. NADPH oxidase in phagocytes is responsible for the production of superoxide anion and represents a major host defense mechanism of phagocytes against invading microorganisms. Superoxide initiates the formation of other ROS, such as hydroxyl radical and oxygen singlet, which are known as strong oxidant products, and hydrogen peroxide and hypochlorous acid (23). Free radical scavenging activity plays the critical role for quenching superoxide anions and maintaining the cellular redox homeostasis against harmful oxidants and free radicals in cells. In healthy blood, several enzymes, such as glutaredoxin (GRX) and glutathione reductase (GRD), and chemical compounds, such as ascorbic acid, vitamin E, and glutathione, play as antioxidant and reducing enzyme system for scavenging harmful superoxides and free radical compounds. From this result, the antioxidant activity of *chopi*-EO will help reduce the oxidative risk, caused by harmful radical compounds.

Cytotoxicity of *chopi*-EO For murine macrophage cells and HUVEC monolayers, *chopi*-EO had the significant cytotoxicity over 50 μ g/mL concentration (IC₅₀=236.1 and 411.5 μ g/mL, respectively) (Fig. 1). Limonene and geranyl acetate did not show any cytotoxic effect in tested concentration (0-500 μ g/mL) for both cell lines, whereas citronellal affected a partly inhibition on cell viability (data not shown). However, it is reported that limonene has cytotoxicity on gastric cancer cells and leukemia cells, causing the apoptosis through the decrease of bcl-2 protein and the increase of p53 protein (24). In addition, limonene has been shown to have the carcinogenesis activity when bound to rat-specific α_{2u} -globulin (25). From the above, *chopi*-EO containing limonene and geranyl acetate is presumed to exert the cell-specific cytotoxicity for human cancers and leukemia cells without any harmful effects on normal cells, such as macrophage cells and HUVEC monolayers.

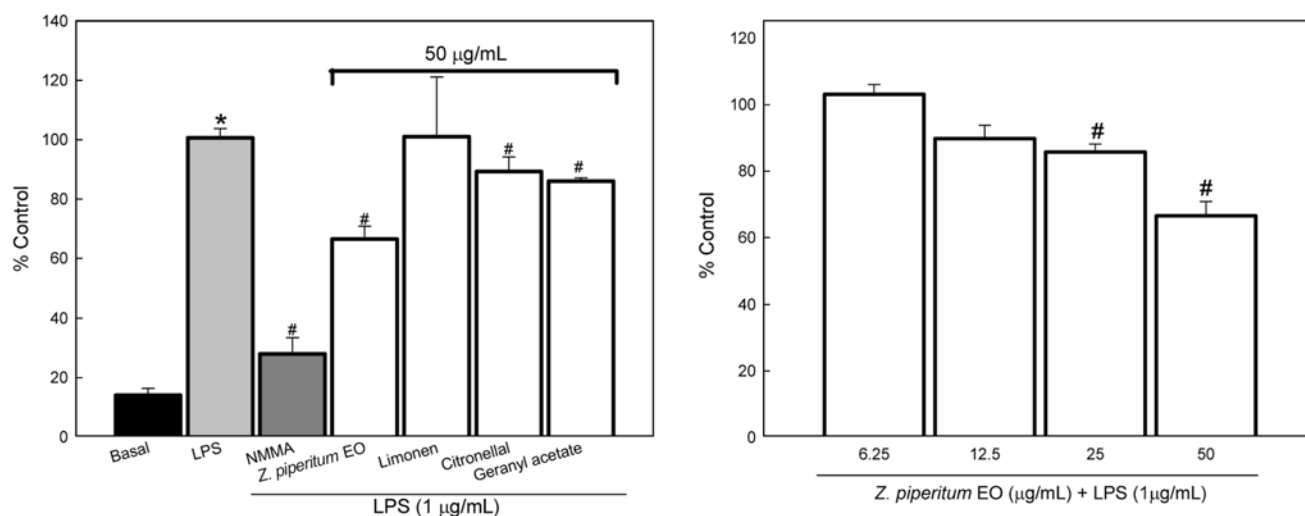


Fig. 2. Effects of *chopi*-EO and its constituents on NO production from LPS-stimulated RAW 264.7 macrophage cells. Significant difference at $p < 0.05$ as compared to *control and to #LPS-treated group.

Inhibition of *chopi*-EO on nitrite production *Chopi*-EO inhibited partly LPS-induced nitrite production in a dose-dependent manner, as shown in Fig. 2. LPS stimulated nitrite production from macrophage cells, as compared to the basal. Also, L-NMMA (100 μ M), which is one of selective NOS blockers, inhibited LPS-induced nitrite production significantly as a positive control. L-NMMA had suppressed approximately 82% of nitrite production of LPS-induced nitrite production to the basal. A 50 μ g/mL of *chopi*-EO showed approximately 38% decrease in nitrite production, as compared to the LPS-induced nitrite production at a significant difference ($p < 0.05$). Citronellal and geranyl acetate (50 μ g/mL), except of limonene, showed a partly inhibitory effect (approximately 11-14%), on nitrite production in macrophage cells to the basal. Little is known of the original copy of the inhibition of nitrite production by citronellal and geranyl acetate.

NO is related to the pathophysiology of inflammation including inflammatory joint disease (26,27). In addition, NO released from endothelial cells via the endothelial nitric oxide synthase (eNOS) is a pivotal vasoprotective molecule (28) and a potent vasodilator, and possesses many antiatherogenic properties. NO decreases platelet aggregation and adhesion limits vascular smooth muscle proliferation, inhibits neointima formation, prevents monocyte chemotaxis, and inhibits leukocyte adhesion to the endothelium (29). Our results suggest that *chopi*-EO might have the dual-functions for NOs biosynthesized in different cell lines. In macrophage cells, *chopi*-EO is expected to reduce the production of NO, which is an inflammatory mediator, derived from iNOS reactions. Simultaneously, it is presumed that *chopi*-EO has no chemical quenching effect on NO, a potent vasodilator in atherosclerosis, derived from eNOS reaction in HUVEC. As a whole, this finding show that *chopi*-EO, containing limonene, geranyl acetate, and citronellal, raise the possibility that *chopi*-EO may exacerbate partly the inflammatory process, giving no chemical quenching effect on eNOS-derived NO in HUVECs.

Effect of *chopi*-EO on chemical scavenging of NO and suppression of iNOS gene transcription Partly inhibition of nitrite production by *chopi*-EO can be explained by two ways. One explanation is by extracellularly chemical NO quenching activity of *chopi*-EO. Sodium nitroprusside, which is a chemical NO donor, produces nitrite ions in aqueous solution at physiological pH. Chemical quenching of NO by *chopi*-EO was investigated. However, *chopi*-EO and its major components showed no chemical NO quenching, whereas Hb, used as a NO scavenger (15), showed significant NO scavenging (Fig. 3A). The inhibition of nitrite production by *chopi*-EO was expected to be due to suppression of iNOS in LPS-induced RAW 264.7 macrophage cells. For inhibition of nitrite production from LPS-stimulated RAW 264.7 cells, the other explanation is to suppress iNOS mRNA transcription by *chopi*-EO. LPS stimulated significantly to up-regulate mRNA transcription of iNOS in RAW 264.7 cells (Fig. 3B). NMMA suppressed approximately 49% of upregulated iNOS gene transcription. *Chopi*-EO markedly inhibited approximately 40.4% of iNOS mRNA transcription as compared to LPS-induced group. However geranyl acetate and limonene showed upregulated-iNOS mRNA transcription on the contrary, showing over-range values (approximately 0.130 and 0.166 iNOS/GAPDH density for transcription), except of citronellal showing approximately 34% suppressive effect. NO production and iNOS expression are considered to be related to the inflammation and carcinogenesis (30, 31), and NO is involved in the pathophysiology of joint disease and plays a key role in the cartilage catabolism (26).

Inhibitory effect of *chopi*-EO on the cellular adhesion

The adhesion of the monocytic THP-1 cells to HUVEC monolayers acts as one of the initial steps for immunological inflammatory responses. The treatment of *chopi*-EO reduced the TNF- α -stimulated adhesion of THP-1 to HUVEC monolayers (Fig. 4). The analysis by fluorescent plate reader showed that *chopi*-EO (50 μ g/mL) reduced the

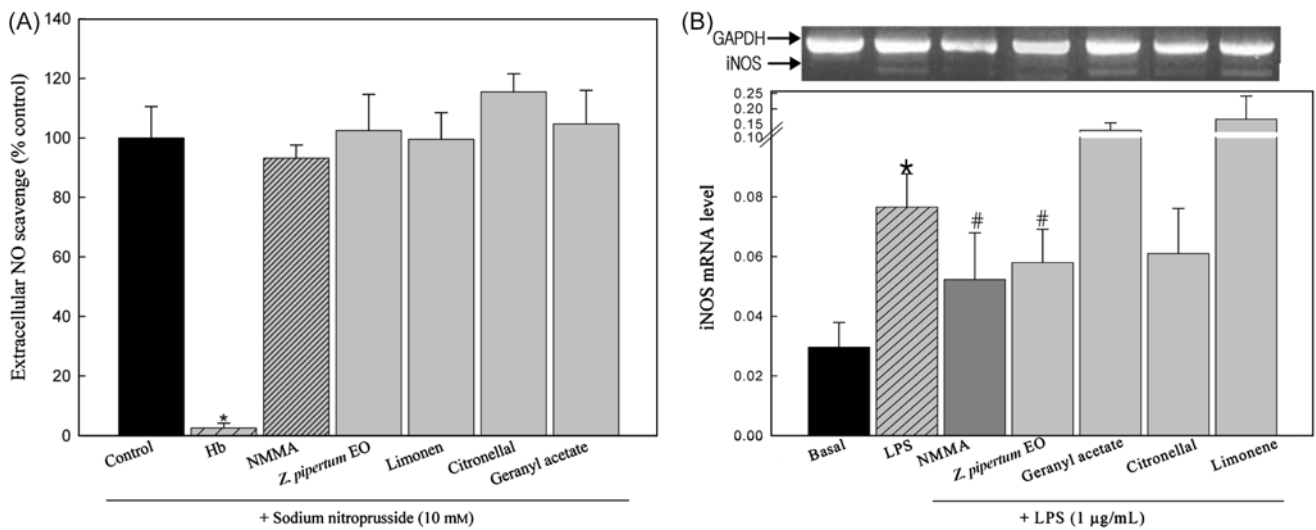


Fig. 3. Effects of *chopi*-EO and its constituents on chemical NO quenching and the suppression of iNOS mRNA transcription. A, Chemical NO scavenging of *chopi*-EO in the cell-free system; B, suppression of iNOS mRNA transcription by *chopi*-EO in LPS-induced RAW 264.7 macrophage cells. The intensity of PCR product bands was quantitated by scanning densitometry and standardized to equivalent GAPDH mRNA levels. Significant difference at $p < 0.05$ as compared to *control and to #LPS-treated group.

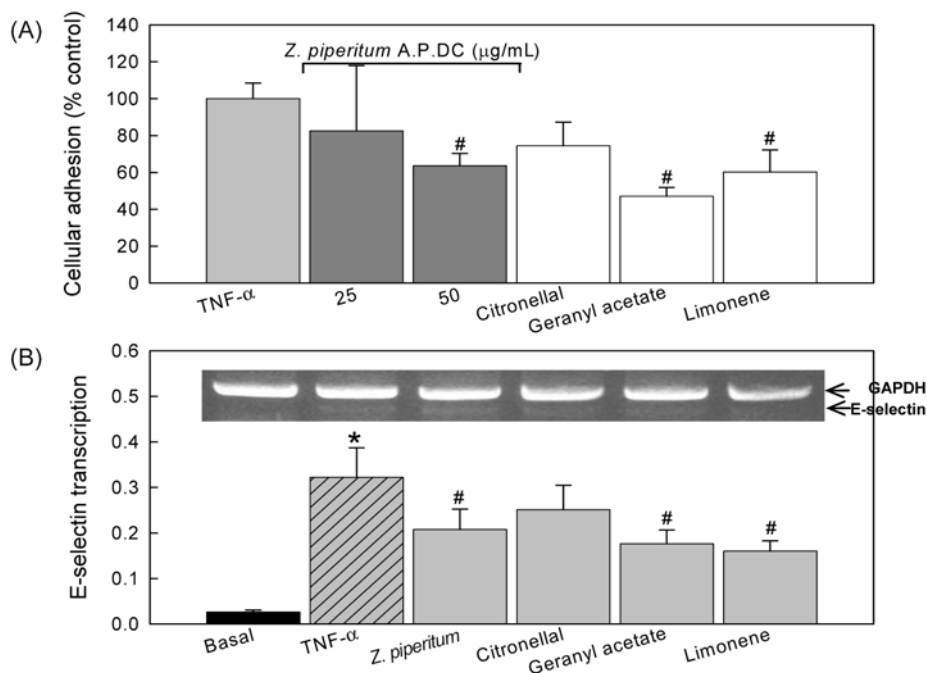


Fig. 4. Effects of *chopi*-EO and its constituents on cellular adhesion. A, Inhibition of *chopi*-EO on monocytic THP-1 cells to HUVEC monolayer; B, suppression of E-selectin mRNA transcription level by *chopi*-EO in TNF- α -stimulated HUVEC. The intensity of PCR product bands was quantitated by scanning densitometry and standardized to equivalent GAPDH mRNA levels. Significant difference at $p < 0.05$ as compared to *basal and to #TNF- α -treated group.

adhesion of calcein-AM labeled THP-1 to HUVEC monolayer to approximately 35.7%. It was expected that *chopi*-EO might affect the expression of TNF- α -upregulated E-selectin in HUVEC. In resting HUVEC monolayers, there was no signal of the basal mRNA transcription of E-selectin gene, as shown in Fig. 4. However, the transcription of E-selectin gene was significantly increased by stimulation of cytokine, TNF- α .

TNF- α -stimulated E-selectin mRNA transcriptional level by *chopi*-EO (50 $\mu\text{g/mL}$) was reduced to 34.8% remarkably, compared to the control. These results were consistent with inhibitory data of cellular adhesion. In addition, geranyl acetate and limonene had approximately 53.7 and 58% of suppressive activities for E-selectin transcription. In immunological inflammatory responses, TNF- α and interleukin family activate neutrophils and up-regulate

Table 3. Effect of chopi-EO on the biosynthesis of cytokines

	IL-1 β (pg/mL)	IL-6 (pg/mL)
Basal	1.20 \pm 0.69	20.49 \pm 1.65
LPS (1 μ g/mL)	26.53 \pm 1.25*	839.69 \pm 0.55*
Chopi-EO (50 μ g/mL) +LPS (1 μ g/mL)	25.68 \pm 4.70*	836.59 \pm 1.35*

*Significant difference at $p < 0.05$ as compared to basal.

CAMs expression in HUVEC monolayer. Upregulated expression of CAMs protein causes the adherence of monocytes and neutrophils to blood vessel wall, especially HUVEC monolayer. These responses are closely related to immunological inflammatory diseases and atherosclerosis (16).

Inhibition of chopi-EO on the biosynthesis of inflammatory mediators Additionally, chopi-EO did not show the effect of the biosynthesis of inflammatory cytokines in LPS-induced RAW 264.7 cells (Table 3). E-selectin is constitutive, and was expressed by immunological inflammatory agent-modulation. Also, the expression of E-selectin protein is regulated by mitogen-activated protein kinases (MAPKs) and NF- κ B pathways (32).

As mentioned above, the inhibitory effect of chopi-EO on NO production was not due to cell viability or chemical NO scavenging effect. These results suggest that chopi-EO may inhibit iNOS gene transcriptional level through it exerts its anti-inflammatory activity. Most of reports regarding the inhibition of NO production have elucidated only iNOS protein suppression without investigation of chemical NO scavenge. However the chemical quenching should be elucidated for inhibition of NO production in RAW 264.7 cells. And chopi-EO inhibited the cellular adhesion through the suppression of E-selectin transcription level in HUVEC monolayers. Taken together, chopi-EO may be useful as a functional food component or an alternative medicine for the relief and retardation of immunological inflammatory responses, and its action may occur through the reduction of inflammatory mediators.

Acknowledgments

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0094018).

References

- Marletta MA. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* 268: 12231-12234 (1993)
- Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664-666 (1988)
- Xie Q-W, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan CF. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 256: 225-228 (1992)
- Hibbs JB, Taintor RR, Vavrin Z. Macrophage cytotoxicity: Role for L-arginine deiminase and imino nitrogen oxidation in nitrite. *Science* 235: 473-476 (1987)
- Chung MS. Volatile compounds of *Zanthoxylum piperitum* A.P. DC. *Food Sci. Biotechnol.* 14: 529-532 (2005)
- Caldefite-Chezet F, Guerry M, Chalchat JC, Fusillier C, Vasson MP, Guillot J. Anti-inflammatory effects of *Melaleuca alternifolia* essential oil on human polymorphonuclear neutrophils and monocytes. *Free Radical Res.* 38: 805-811 (2004)
- Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm. Res.* 49: 619-626 (2000)
- Fiebich BL, Mueksch B, Boehringer M, Hull M. Interleukin-1 β induces cyclooxygenase-2 and prostaglandin E2 synthesis in human neuroblastoma cells: Involvement of p38 mitogen-activated protein kinase and nuclear factor- κ B. *J. Neurochem.* 75: 2020-2028 (2000)
- Silverman MD, Zamora DO, Pan Y, Teixeira PV, Planck SR, Rosenbaum JT. Cell adhesion molecule expression in cultured human iris endothelial cells. *Invest. Ophthalm. Vis. Sci.* 42: 2861-2866 (2001)
- Gómez NE, Witte L. A simple method to extract essential oils from tissue samples by using microwave radiation. *J. Chem. Ecol.* 27: 2351-2359 (2001)
- Van den Dool H, Kratz PD. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatogram. *J. Chromatogr.* 11: 463-471 (1963)
- Kondjoyan N, Berdague JL. A Compilation of Relative Retention for the Analysis of Aromatic Compounds. Laboratoire flaveur, de Recherches sur la Viande. Clermont-Ferrand, France. pp. 15-138 (1996)
- Chattoopadhyay S, Bhaumik S, Purkayastha M, Basu S, Chaudhuri AN, Gupta SD. Apoptosis and necrosis in developing brain cells due to arsenic toxicity and protection with antioxidants. *Toxicol. Lett.* 136: 65-76 (2002)
- Babu BH, Shylesh BS, Padikkala J. Antioxidant and hepatoprotective effect of *Acanthus ilicifolius*. *Fitoterapia* 72: 272-277 (2001)
- Wei T, Chen C, Hau J, Zhao B, Xin W, Mori A. The antioxidant EPC-K1 attenuates NO-induced mitochondrial dysfunction, lipid peroxidation, and apoptosis in cerebellar granule cells. *Toxicology* 134: 117-126 (1999)
- Lee JH, Choi SI, Lee YS, Kim GH. Antioxidant and anti-inflammatory activities of *Allium victorialis* subsp. *platyphyllum* extracts. *Food Sci. Biotechnol.* 16: 796-801 (2007)
- Ahn KS, Noh EJ, Zhao HL, Jung SH, Kang SS, Kim YS. Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor- κ B activation in RAW 264.7 cells. *Life Sci.* 76: 231-2328 (2005)
- Sasaki M, Ohara-Nemoto Y, Tajika S, Kobayashi M, Yamaura C, Kimura S. Antigenic characterization of a novel *Streptococcus anginosus* antigen that induces nitric oxide synthesis by murine peritoneal exudates cells. *J. Med. Microbiol.* 50: 952-958 (2001)
- Choi JS, Choi YJ, Park SH, Kang JS, Kang YH. Flavones migrate tumor necrosis factor- α -induced adhesion molecule upregulation in cultured human endothelial cells: Role of nuclear factor- κ B. *J. Nutr.* 134: 1013-1019 (2004)
- Rasmussen LM, Hansen PT, Nabipour MT, Olesen P, Kristiansen MT, Ledet T. Diverse effects of inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase on the expression of VCAM-1 and E-selectin in endothelial cells. *Biochem. J.* 360: 363-370 (2001)
- Kim JH, Lee KS, Oh WT, Kim KR. Flavor components of the fruit peel and leaf oil from *Zanthoxylum piperitum* DC. *Korean J. Food Sci. Technol.* 21: 562-568 (1989)
- Chung MS. Compositional changes in essential oil of *Zanthoxylum piperitum* A.P. DC during storage. *Korean J. Food Culture* 21: 433-438 (2006)
- Babior BM. The respiratory burst oxidase. *Adv. Enzymol. RMB* 65: 49-95 (1992)
- Lu XG, Feng BA, Zhan LB, Yu ZH. D-Limonene induces apoptosis of gastric cancer cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 25: 325-327 (2003)
- Whysner J, Williams GM. D-Limonene mechanistic data and risk assessment: Absolute species-specific cytotoxicity, enhanced cell proliferation, and tumor promotion. *Pharmacol. Therapeut.* 71: 127-136 (1996)

26. Belmont HM, Levartovsky D, Goel A, Amin A, Giorno R, Rediske J, Skovron ML, Abramson SB. Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. *Arthritis Rheum.* 40: 1810-1816 (1997)
27. Amin AR, Attur M, Abramson SB. Nitric oxide synthase and cyclooxygenases: Distribution, regulation, and intervention in arthritis. *Curr. Opin. Rheumatol.* 11: 202-209 (1999)
28. Leikert JF, Räthel TR, Wohlfart P, Cheynier V, Vollmar AM, Dirsch VM. Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. *Circulation* 106: 1614-1617 (2002)
29. Cauin-Glaser T, Garcia-Cardena G, Sarrel P, Sessa WC, Bender JR. 17β -Estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ. Res.* 81: 885-892 (1997)
30. Mordan LJ, Burnett TS, Zhang LX. Inhibitors of endogenous nitric oxide formation block the promotion of neoplastic transformation in C3H10T1/2 fibroblasts. *Carcinogenesis* 14: 1555-1559 (1993)
31. Chan MM, Huang HI, Fenton MR, Fong D. *In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem. Pharmacol.* 55: 1955-1962 (1998)
32. Lee CW, Lin WN, Lin CC, Luo SF, Wang JS, Pouyssegur J, Yang CM. Transcriptional regulation of VCAM-1 expression by tumor necrosis factor- α in human tracheal smooth muscle cells: Involvement of MAPKs, NF- κ B, p300, and histone acetylation. *J. Cell. Physiol.* 207: 174-186 (2006)