Kuromoji (*Lindera umbellata*) Essential Oil Inhibits LPS-Induced Inflammation in RAW 264.7 Cells

Hayato Maeda, Mao Yamazaki, and Yohtaro Katagata

Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan

Received September 5, 2012; Accepted December 10, 2012; Online Publication, March 7, 2013

[doi:10.1271/bbb.120692]

Kuromoji (*Lindera umbellata*) essential oil (KEO) has long been used in Japan as a traditional medicine. It contains linalool (C\(_{10}\)H\(_{18}\)O), a naturally occurring small terpenoid. For this study, we investigated the anti-inflammatory effect of KEO in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Mouse macrophage-like RAW 264.7 cells were stimulated with LPS. Then they were treated with 25 or 50 \(\mu\)g/mL of KEO for 24 h. KEO suppressed LPS-induced pro-inflammatory cytokine production such as that of nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) in a dose-dependent manner. In addition, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression and protein levels were suppressed by treatment with KEO cells. In addition, by treatment with 25 or 50 \(\mu\)g/mL of linalool showed the same anti-inflammatory effect. The results suggest that KEO and linalool can be regarded as a natural resource for use in anti-inflammatory therapeutic products.

**Key words:** essential oil; Kuromoji (*Lindera umbellata*); RAW 264.7 cells; linalool

Inflammation is a complex process mediated by the activation of various immune cells. Macrophages play a central role in mediating various immunopathological phenomena during inflammation, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as IL-6, TNF-\(\alpha\), and NO.\(^1\) NO has been identified as an important molecule involved in regulating biological activities in vascular, neural, and immune systems. NO produced by activated macrophages has been found to mediate host defense functions, including antimicrobial and antitumor activities, but excess production of it causes tissue damage associated with acute and chronic inflammation.\(^5\) NO synthase (NOS) is key enzyme producing large amounts of NO through macrophages. Increased NO production has been implicated as a cause of various inflammatory diseases, including rheumatoid arthritis and ulcerative colitis. Moreover, COX-2 promotes prostaglandin E\(_2\) (PGE\(_2\)) synthesis from arachidonic acid. Cyclooxygenase (COX) exists in two major isoforms, COX-1 and COX-2. COX-1 is expressed constitutively in many tissues and COX-2 is an inducible enzyme expressed in inflammation-related cells such as macrophages, producing large amounts of prostaglandins. Excess production of prostaglandins causes inflammatory disorders. Therefore, inhibition of iNOS and COX-2 under inflammatory disorder condition by macrophages is a useful strategy to screen anti-inflammatory drugs.\(^6\)

Essential oils diluted from several plants have been used for a long time in perfumery, aromatherapy, food, and flavors. Many essential oils are known to be potent antibacterial and antifungal agents.\(^7\) Some essential oil components reportedly have anti-inflammatory activity.\(^8\) The advantage of these components in anti-inflammatory therapy is their low or negligible toxicity. Therefore, such components are useful in the long-term chemoprevention and chemotherapy of inflammation.

Kuromoji, a deciduous shrub native to cool and warm temperate areas of Japan, is known as a spicebush because its twigs and leaves contain aromatic components. Consequently, kuromoji branches are used as indoor decorations and to produce shavings for aromatic baths. Furthermore, kuromoji essential oil (KEO) obtained through steam distillation is used traditionally as a medicine for neuralgia, stiff neck, and back pain. The main KEO components are terpenes and alcohol esters, of which linalool is the most abundant component. Geranyl acetate, an ester in KEO, has a relaxing and calming effect on humans. Other terpenes (limonene and \(\alpha\)-pinene) and alcohols (geraniol and cineol) in essential oils have beneficial activities in various diseases, supporting anti-inflammatory and anti-obesity effects.\(^8,9\) These terpenoid components become ligands in nuclear receptors, regulate gene expression, and ameliorate conditions related to these diseases.\(^10\)

Linalool, a monoterpine compound that is reportedly a major volatile component of the essential oils of several aromatic species, is used in traditional medicine systems to relieve symptoms and to cure various ailments, both acute and chronic. It was evaluated recently for its psychopharmacological activity in mice, revealing marked dose-dependent sedative effects on the central nervous system,\(^11,12\) including protection against pentylenetetrazol, picrotoxin, and transcorneal electroshock-induced convulsion, and its hypnotic and hypothermic properties.\(^13\) Furthermore, several recent reports have stated that linalool shows anti-proliferative activity against some solid tumor cells such as melanoma cells and renal cell adenocarcinoma cells\(^14\) and HepG2.\(^15\) It induces apoptosis in human leukemia cells.\(^16\) Our previous study indicated that KEO mainly
containing linalool showed antitumor activity against several human tumor cell lines.17) In addition, Peana et al. have reported that linalool and the corresponding ester (linalyl acetate) exhibited anti-inflammatory activity in rat inflammation model experiments.18) Therefore, KEO containing linalool and other terpenoids is necessary to elucidate anti-inflammatory principles.

In murine macrophage RAW 264.7 cells, LPS stimulation alone can induce the production of pro-inflammatory cytokines and inflammatory mediators. Thus, this experimental model is useful for drug screening and for the evaluation of potential inhibitors of the inflammatory response. The present study was undertaken to investigate the anti-inflammatory activity of KEO by adjusting the production of pro-inflammatory cytokines and inflammatory mediators by LPS-stimulated RAW 264.7 cells. In addition, the effect was compared to that of linalool of which is a major component of KEO.

Materials and Methods

Chemical reagents and cells. For use in this study, Mouse macrophage-like cell line RAW 264.7 cells were purchased from DS Pharma Biomedical (Osaka, Japan). Linalool was from Tokyo Chemi-Cial Industry (Tokyo). Fetal bovine serum (FBS) was from Biological Industries (Kibbutz Beit, Israel). Dulbecco’s Modified Eagle’s Medium (DMEM) was from Nissui Pharmaceutical (Tokyo). ELISA kits for TNF-α and IL-6 were from Immuno-Biological Laboratories (Gunma, Japan) and Shibayagi (Gunma, Japan). Anti-iNOS, anti-COX-2, and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); Cayman Chemical (Ann Arbor, MI), and AbD Serotec (Raleigh, NC) respectively. All other chemicals, guaranteed to be of reagent or tissue-culture grade, were from Sigma-Aldrich Japan (Tokyo) or Wako Pure Chemical Industries (Osaka, Japan).

Isolation of Kuromoji essential oil (KEO). KEO was donated by En'iyakudo (Aomori, Japan). Fresh kuromoji leaves and branches were collected in the mountains near the city of Aomori, and were heated to boiling. Then the hydro-distilled volatile fraction was collected and separated (upper layer) from the aqueous portion. Analysis of KEO constituents was conducted by gas chromatography-MS at the Aomori Prefectural Industrial Technology Research Center, as described in an earlier report.17) The GC-MS system (GC-17A/QP-5000; Shimadzu, Kyoto, Japan) was equipped with a DB-1701 column (30 mm × 0.25 mm × 0.25 μm; J&W Scientific). The carrier gas was helium. The program was operated with the following oven temperature program: 50 °C held for 5 min, rising at 5 °C/min to 100 °C, rising at 2 °C/min to 150 °C, rising at 10 °C/min to 250 °C, and the injection temperature and volume were 270 °C and 1.0 μL respectively. Constituents were identified by computer matching of mass spectral data with data from the NIST62 mass spectral database (Shimadzu). The major constituents of KEO were linalool (65.78%), geranyl acetate (17.59%), geranial (5.29%), cineol (2.34%), limonene (2.11%), 3-carene (1.78%), α-pinene (1.43%), and carvone (1.18%).

Cell culture. RAW 264.7 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 μg/mL of streptomycin, and 100 IU/mL of penicillin at 37 °C in a 5% CO2 humidified incubator. After 24 h of pre-incubation, KEO or linalool was added to the medium as ethanol (EtOH) solution. The final concentration of ethanol was less than 0.1% (v/v). Then LPS was added to the culture medium at 0.1 μg/mL in the presence of KEO or linalool, and RAW264.7 cell were stimulated for an additional 24 h.

Measurement of cell viability by WST-1 assay. RAW 264.7 cells (1.0 × 105 cells/mL) were cultured in a 96-well culture plate with 100 μL medium and incubated as described previously. Cell viability was expressed as percentage of control by measuring the density of the color produced by WST-1 dye at 450 nm. The WST-1 assay is based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase of viable cells.

Quantification of NO production. RAW 264.7 cells (5 × 105 cells/mL) were cultured in a 24-well culture plate with 500 μL medium and incubated as described previously. The nitrite-containing culture medium was measured as an indication of NO production based on the Griess reaction. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (Cayman Chemical, Ann Arbor, MI) in a 96-well culture plate. After incubation of 10 min at room temperature, the optical density was determined at 550 nm using a microplate reader. Fresh culture medium was used as the blank in all experiments. The nitrite concentration in the samples was measured by serial dilution the standard curve of NaNO2.

Extraction of total RNA and quantitative RT-PCR analysis. The total RNA of the cultured cells was extracted (Quick Gene mini 80; Fujifilm, Tokyo) following to the manufacturer’s instructions. Total RNA was reverse-transcribed by High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan, Tokyo) for cDNA synthesis. IL-6, TNF-α, COX-2, and iNOS mRNA expression was measured using a real-time PCR detection system (Opticon 2; Bio-Rad Laboratories, Hercules, CA). Thunderbird SYBER qPCR Mix (Toyobo, Osaka, Japan) was used in the PCR reaction. PCR cycling was done at 95 °C for 1 min at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The primer sequences used for RT-PCR were the following: 5'-CCA CGG CCT TCT CTT C-3' (forward) and 5'-TTG GGA GTG TTA TCC TTC A-3' (reverse) for mouse IL-6 (NM_031168.1); 5'-CAC AAG ATG CTG GGA CAG TGA-3' (forward) and 5'-TTG TCC ATG GTG CGT CAT GA-3' (reverse) for mouse TNF-α (NM_013693.2); 5'-TGC CTC CCA CCA CCT CAG ACT AGA-3' (forward) and 5'-CAT CCT AGT CTT ATC ACC TTT G-3' (reverse) for mouse COX-2 (NM_011983.3); 5'-GGA TCT TCC CAG GCA ACC A-3' (forward) and 5'-CAA TCA ACA ACT GCC CCC AA-3' (reverse) for mouse INOS (NM_010927.3); 5'-CAT GGC CTT CCG TGT TTC TA-3' (forward) and 5'-GCC GCA CGT CAG ATC CA-3' (reverse) for mouse GAPDH (NM_008084.2). PCR reactions were normalized to GAPDH.

Detection of IL-6 and TNF-α in the supernatants. The concentrations of IL-6 and TNF-α in the culture supernatants were determined by ELISA, conducted using a Mouse IL-6 ELISA Kit (Immuno-Biological Laboratories, Gunma, Japan) and a Mouse TNF-α ELISA Kit (Shibayagi, Gunma, Japan) in accordance with the manufacturers’ instructions.

Western blot analysis. Cells were lysed with cold RIPA buffer (pH 7.4) containing 20 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/mL of phenylmethylsulfonyl fluoride, 50 μg/mL of aprotinin and 1 mM Na3VO4 for 1 h on ice. Cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentrations were measured by D.C. Protein assay (Bio-Rad Laboratories, Tokyo, Japan). The supernatant (20 μg protein/lane) was separated 10% SDS-polyacrylamide gels electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). The membranes were incubated with blocking solution for 2 h at room temperature with subsequent incubation for 1 h with specific primary antibodies. The membranes were further incubated 1 h at room temperature with secondary antibodies. They were treated with reagents (Chem-Lumi One L; Cosmobio, Tokyo) following to the manufacturer’s instructions. Band intensities were quantified using Scion image software (Scion; Scion, Frederick, MD). β-Actin was used as control.

Peroxisome proliferator-activated receptor γ (PPARY) ligand activity. Agonistic activity for PPARγ was examined using a nuclear receptor cofactor assay system (EnBio RCAS for PPARγ; Cosmohbio, Tokyo). This is a cell-free assay system using a nuclear receptor and cofactor to screen chemicals. Changes in absorbance (450 nm) at KEO (25, 50, 100 μg/mL) and troglitazone (10 μM) were measured.

Statistical analysis. Results were expressed as mean ± SE. Statistical analysis between multiple groups were done by ANOVA.
Results

Inhibitory effects of Kuromoji essential oil (KEO) and linalool on LPS-induced NO production

The cytotoxicity of KEO or linalool on RAW 264.7 cells stimulated by LPS was investigated by WST-1 assay. Treatment with sample cells showed no cell toxicity on LPS-stimulated RAW 264.7 cells (LPS–Control cells: 100 ± 1.8%, LPS+Control cells: 100 ± 1.1%, LPS+25 µg/mL KEO cells: 106.6 ± 1.8%, LPS+50 µg/mL KEO cells: 99.5 ± 2.2%, LPS+25 µg/mL linalool cells: 98.5 ± 0.6%, LPS+50 µg/mL linalool cells: 107.0 ± 1.9%).

To evaluate anti-inflammatory effects on LPS-induced NO production, RAW 264.7 cells were stimulated with LPS (0.1 µg/mL) in the presence of KEO or linalool for 24 h. Control cells treated with LPS showed increased NO production as compared with the non-treated LPS Control cells. The cells treated with 10 µM troglitazone, chemosynthetic ligands of PPARγ, showed suppressed NO production. Similarly, the cells treated with KEO and linalool (25, 50 µg/mL) exhibited significantly (p < 0.01) suppressed NO production, in a dose-dependent manner (Fig. 1).

Inhibitory effects of KEO and linalool on LPS-induced IL-6 and TNF-α expression

To investigate the anti-inflammatory effects of KEO or linalool on IL-6 and TNF-α, which are pro-inflammatory cytokines mRNA, were quantified in RAW 264.7 cells stimulated by LPS. Control cells stimulated with 0.1 µg/mL of LPS showed markedly increased IL-6 and TNF-α mRNA expression as compared with non-treated control cells. However, those cells treated with KEO (25, 50 µg/mL) exhibited significantly (p < 0.01) attenuated IL-6 mRNA expression of dose-dependently (Fig. 2A). TNF-α mRNA expression did not change significantly, but it tended to be down regulated treated with KEO (25, 50 µg/mL). On the other hand, treatment with linalool (50 µg/mL) significantly (p < 0.05) suppressed TNF-α mRNA expression (Fig. 2B). IL-6 and TNF-α production in the culture supernatant was measured by ELISA. IL-6 and TNF-α concentrations in the media were suppressed significantly (p < 0.01) and dose-dependently in the cells treated with KEO and linalool (Fig. 2C and D).

Inhibitory effects of KEO and linalool on LPS-induced iNOS and COX-2 expression

iNOS and COX-2 expression in the cells treated with KEO and with linalool was determined by real-time RT-PCR and Western blotting. iNOS and COX-2 mRNA expression was markedly downregulated in the cells treated with KEO as compared to control cells treated with LPS (Fig. 3A and B). In addition, protein expression in the cells were determined Western blot. The cells treated with KEO (25, 50 µg/mL) suppressed COX-2 and iNOS protein expression as compared with the LPS-stimulated control cells. (Fig. 4A, B and C). Linalool treated with the same concentration of KEO showed similar effects on iNOS and COX-2 mRNA and protein expression.

Fig. 1. Inhibitory Effects of KEO and Linalool on LPS-Induced Nitrite Production in RAW264.7 Cells.

RAW264.7 cells were maintained in the culture medium (Control), 10 µM troglitazone (TR), 25 µg/mL KEO (KEO25), 50 µg/mL KEO (KEO50), 25 µg/mL linalool (Linalool 25), 50 µg/mL linalool (Linalool 50) stimulated with 0.1 µg/mL of LPS for 24 h. **p < 0.01 vs. control treated with LPS.

Fig. 2. Inhibitory Effects of KEO and Linalool on LPS-Induced IL-6 and TNF-α mRNA Expression, and Protein Secretion into the Culture Medium.

RAW 264.7 cells were stimulated with 0.1 µg/mL of LPS for 24 h in the presence of 25 µg/mL KEO (KEO25), 50 µg/mL KEO (KEO50), 25 µg/mL linalool (Linalool 25), 50 µg/mL linalool (Linalool 50): (A) IL-6 mRNA expression, (B) TNF-α mRNA expression, (C) IL-6 concentration in the supernatant, and (D) TNF-α concentration in supernatant. **p < 0.01 vs. control treated with LPS. *p < 0.05 vs. control treated with LPS.
**PPARγ ligand activity of KEO**

The PPARγ agonistic activity of KEO was examined using a nuclear receptor cofactor assay system. The absorbance of the non-treated well (control) was estimated to have a 100% change of absorbance percentage. The absorbance the well treated with Troglitazone (10 µM) increased by 286.7 ± 14.5% compared with the non-treated well (control: 100.0 ± 2.9%).

In addition, the PPARγ agonistic activities of the wells treated with KEO (25, 50 µg/mL) were significantly higher ($p < 0.05$) than those of the non-treated wells (KEO 25 µg/mL: 126.6 ± 9.3%, KEO 50 µg/mL: 132.5 ± 5.9%), but activity was considerably lower than with troglitazone.

**Discussion**

Macrophages play important roles in the initiation and amplification of various inflammatory diseases. Therefore, the development of methods to reduce the number of activated macrophages, to inhibit activation signals, and to prevent the production of inflammatory mediators is a promising therapeutic approach to the treatment of various inflammatory diseases.

This study demonstrated the anti-inflammatory effects of KEO in RAW 264.7 cells. Treatment with 25 or 50 µg/mL of KEO cells reduced NO production in LPS-stimulated RAW 264.7 cells (Fig. 1). In addition, pro-inflammatory cytokines such as IL-6 and TNF-α were suppressed by treatment with KEO cells (Fig. 2). NO, derived from arginine after activation of iNOS, is an important effector molecule involved in immune regulation and defense. COX-2 catalyzes the production of prostaglandins, which play important roles in the inflammatory process. Prostaglandin production in LPS-treated macrophages is primarily attributable to transcriptional activation of the COX-2 gene. Hence iNOS and COX-2 inhibitors are regarded as potential anti-inflammatory agents.

In this study, KEO suppressed iNOS and COX-2 mRNA expression. Protein expression was also suppressed. The results suggest that KEO is a natural product that can prevent the production of pro-inflammatory cytokines in RAW264.7 macrophages. A major constituent of KEO is linalool a monoterpene alcohol. Linalool similarly reduced NO, IL-6, and TNF-α production. Peana et al. reported that linalool inhibited NO production, but this was not associated with iNOS expression. In this study, KEO and linalool suppressed iNOS and COX-2 expression. It was assumed that difference cell types and LPS stimulation levels lead to different results.

NO produced by activated macrophages is associated with acute and chronic inflammation. Toll-like receptor 4 (TLR4) is regarded as a cell-surface receptor necessary for the recognition of LPS macrophages. TLR4 regulates several transcription factors encoding inflammatory mediators, such as NF-κB, an important transcription factor adjusting pro-inflammatory mediator production, including iNOS, IL-6, TNF-α and NO production in activated macrophages.

Recently, PPARγ ligands have been found to suppress the intranuclear NF-κB pathway, engendering suppres-
sion of the release of pro-inflammatory mediators. PPARγ are members of the nuclear receptor superfamily activated by 15-deoxy-D,12,14-prostaglandin J2 converted from arachidonic acid. Troglitazone, a PPARγ ligand, has been to suppress the intranuclear NF-κB pathway by inhibiting IκB degradation in LPS-stimulated macrophages, engendering suppression of the release of pro-inflammatory mediators. In this study, troglitazone showed PPARγ ligand activity, but KEO showed slightly low activity, although showing the same suppressive effect on NO production. Thus it is possible that there is another anti-inflammatory pathway, other than NF-κB signaling. It has been observed that LPS stimulated NO production is related to the MAPK (mitogen-activated protein kinase) pathways, including p38 MAPK, JNK 1/2 (c-jun NH2-terminal kinase), and the NF-κB pathway. NF-κB has a central role in regulating inflammatory mediator synthesis, and KEO might be related to regulation of the MAPK pathways.

KEO contains linalool, a monoterpene compound that is commonly found to be a major volatile component of essential oils in several aromatic plant species. Previous reports suggest that linalool and some terpenoids can enhance the permeability of numerous drugs through biological tissues such as skin and mucus membranes. Various terpenoids show PPARγ ligand activity. They regulate inflammatory processes such as diabetes mellitus, hyperlipidemia, and cardiovascular disease, which are associated with low-grade chronic inflammation.

Mueller et al. reported that various herbs and spices showed PPARγ ligand activity, including linalool. Linalool did not show PPARγ binding activity. A combination of herbs and spice components can show the synergistic effects of drugs. KEO showed an anti-inflammatory effect, but further studies are necessary to clarify the mechanism of this effect and the active components.

In conclusion, the results of this study indicate that an essential oil, KEO, and its major component, linalool, induced anti-inflammatory effects in LPS stimulated RAW264.7 cells. Therefore, KEO is inferred to be useful as a therapeutic product for controlling inflammation-related disorders.

Acknowledgments

This work was supported by a Grant-in-Aid for Young Scientists (21780119) from the Japan Society for the Promotion of Science (JSPS) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We are grateful to the Aomori Prefectural Industrial Technology Research Center and Enyakudo for preparing KEO.

References