Published in final edited form as:

J Agric Food Chem. 2015 May 27; 63(20): 4999–5007. doi:10.1021/acs.jafc.5b01307.

Inhibition of Human Neutrophil Responses by Essential Oil of *Artemisia kotuchovii* and Its Constituents

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Abstract

Essential oils were obtained by hydrodistillation of the flowers+leaves and stems of *Artemisia kotuchovii* Kupr. (AKEO_{f+l} and AKEO_{stm}, respectively) and analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). The primary components of the oils were estragole, (*E*)- and (*Z*)- β -ocimenes, methyl eugenol, limonene, spathulenol, β -pinene, myrcene, and (*E*)-methyl cinnamate. Seventy four constituents were present at concentrations from 0.1 to 1.0%, and 34 compounds were identified in trace (<0.1%) amounts in one or both plant components. Screening of the essential oils for biological activity showed that AKEO_{stm}, but not AKEO_{f+l}, inhibited *N*-formyl-Met-Leu-Phe (*f*MLF)-stimulated Ca²⁺ flux and chemotaxis and phorbol-12-myristate-13-acetate (PMA)-induced reactive oxygen species (ROS) production in human neutrophils. Selected pure constituents, representing >96% of the AKEO_{stm} composition, were also tested in human neutrophils and HL-60 cells transfected with *N*-formyl peptide receptor 1 (FPR1). We found that one component, 6-methyl-3,5-heptadien-2-one (MHDO), inhibited *f*MLF- and interleukin 8 (IL-8)-stimulated Ca²⁺ flux, *f*MLF-induced chemotaxis, and PMA-induced ROS production in human neutrophils. MHDO also inhibited *f*MLF-induced Ca²⁺ flux in FPR1-HL60 cells. These results suggest that MHDO may be effective in modulating some innate immune responses, possibly by an inhibition of neutrophil migration and ROS production.

Keywords

Artemisia kotuchovii; calcium flux; chemotaxis; essential oil; neutrophil; *N*-formyl peptide receptor 1 (FPR1); reactive oxygen species

Introduction

Artemisia is one of largest genus in the Asteraceae family, with approximately 500 species. Many of these species produce essential oils used in folk and modern medicine and in the cosmetics and pharmaceutical industries.^{1,2} Extracts or compounds isolated from *Artemisia* have been shown to exhibit a wide range of biological properties, including anti-inflammatory activities that help to reduce inflammation.^{3,4} Essential oils from several species of this genus have also been reported to decrease the production of proinflammatory mediators.^{5,6} Extensive studies of the chemical components of these oils have demonstrated that they contain diverse bioactive secondary metabolites, including volatile monoterpenes and sesquiterpenes, with artemisinin as one of best studied.^{1,2,7,8} However, the chemical composition and biological properties of many endemic *Artemisia* spp. have not been evaluated.

Artemisia kotuchovii Kupr. is endemic in the Kazakhstan Altai. This perennial herb was described in 1999 and is closely related to *A. dracunculus* L. (tarragon).⁹ To date, the chemical composition and biological properties of *A. kotuchovii* have not been analyzed. Although ethnobotanical and ethnopharmacological data on medicinal usage of this herb are unknown, the related herb tarragon has been reported to have a wide range of applications in traditional medicine because of its therapeutic properties for a variety of ailments.¹ For example, pharmacological evaluation of tarragon extracts demonstrated anti-inflammatory activity.^{1,10} Although tarragon essential oil was inactive in modulating human neutrophil phagocytosis,¹¹ essential oils from other *Artemisia* spp. exhibited some immunomodulatory properties, such as inhibition of nuclear factor (NF)- κ B transcriptional activity and stimulation of nitric oxide and prostaglandin E₂ production by macrophages.^{5,6}

Neutrophils are a key cellular component of the immune response to infection or tissue injury.¹² These phagocytes are recruited to sites of injury or infection by a variety of factors, including formyl-Met-Leu-Phe (fMLF), a bacterial or mitochondria-derived peptide, and chemokines such as interleukin 8 (IL-8).¹³ IL-8 and fMLF via activate G-protein coupled receptors (GPCR) to induce neutrophil chemotaxis and the release of various mediators, such as reactive oxygen species (ROS), proteases, and cytokines.¹³ Growing evidence supporting the anti-inflammatory and tissue-protective effects of chemokine antagonists led to the design and screening of synthetic compounds and plant-derived constituents for novel small-molecule *N*-formyl peptide receptor (FPR) and α -chemokine receptor (CXCR) antagonists.^{14,15} Human neutrophil elastase (HNE) also plays an important role in regulation of inflammation, and some plant-derived compounds have been identified as elastase inhibitors.¹⁶

In the present work, we defined the composition of essential oils from *A. kotuchovii* and evaluated their biological activity.

Materials and Methods

Chemicals

The major and several minor constituents of essential oils were obtained from commercial sources. Sabinene, (–) linalool, hexanal, *p*-cymene-8-ol, 3,7-dimethyl-1,3,6-octatriene (β -ocimene), and MHDO were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Citronellol, (+)-limonene, *trans*-anethole, (–)-caryophyllene oxide, 4-allylanisole (estragole), 1,2-dimethoxy-4-(2-propenyl) (methyl eugenol), myrcene, isopropyl myristate, *p*-cymene, α -terpinene, and (–)-limonene were purchased from Acros Organics (Geel, Belgium). Myristic acid, α -pinene, and terpinolene were from Santa Cruz Biotechnology (Dallas, TX). Methyl cinnamate, 4-methoxystyrene (4-vinylanisole), palmitic (hexadecanoic) acid, and (1S)-(–)- β -pinene were from Alfa Aesar (Ward Hill, MA). For biological evaluation, compounds were dissolved in DMSO (20 mM stock solutions) and stored at –20°C.

Materials

8-Amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H)-dione (L-012) was obtained from Wako Chemicals (Richmond, VA). Dimethyl sulfoxide (DMSO), fMLF, HEPES, bacterial lipopolysaccharide (LPS) from *Escherichia coli* K-235, phorbol-12-myristate-13-acetate (PMA), and Histopaque 1077 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Roswell Park Memorial Institute (RPMI) 1640 medium and penicillin-streptomycin solution were purchased from Mediatech (Herdon, VA). Human neutrophil elastase, *N*-methylsuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin, ionomycin, and bovine serum albumin were purchased from EMD Biosciences (San Diego, CA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO). Human IL-8 was purchased from Peprotech Inc. (Rocky Hill, NJ). Tween-20 was from VWR (Radnor, PA). Tris was from J.T. Baker (Phillipsburg, NJ). Blastidicin S and zeocin were obtained from Invivogen (San Diego, CA). Hanks' balanced-salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 5.56 mM glucose, and 10 mM HEPES, pH 7.4), and G418 were from Life Technologies (Grand Island, NY). HBSS containing 1.3 mM CaCl₂ and 1.0 mM MgSO₄ is designated as HBSS⁺; HBSS without ions Ca²⁺ and Mg²⁺ is designated as HBSS[–].

Plant material

Aerial parts of *A. kotuchovii* were collected at the end of the blossoming stage in August 2013 in the southern Altai on the Tarbagatai range at altitude 1,709 m above sea level (latitude N 49°03'52", longitude W 85°59'35"). The plant was identified by Dr. Yuriy Kotukhov, and voucher specimens were deposited at the Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan). Plants components were air-dried for 7–10 days at room temperature away from direct sunlight. Weighed samples were cut under laboratory conditions before hydrodistillation.

Essential Oil Extraction

Two types of air dried material (inflorescence+leaves or stems) were separately subjected to hydrodistillation for 3 h using a Clevenger type apparatus to yield essential oils. Conventional hydrodistillation is considered the primary method for essential oil extraction.¹⁷ Although hydrodistillation could lead to artifacts at when performed at higher temperatures over long hydrodistillation times at low pH,¹⁸ we only applied conditions accepted by the European Pharmacopoeia (European Directorate for the Quality of Medicines, Council of Europe, Strasbourg, France, 2014) and thus avoided these artifacts. Solutions of the essential oils in DMSO (10 mg/ml stock solutions) for biological evaluation and in n-hexane (10% w/v) for gas-chromatographic analysis.

GC/MS analysis

Gas chromatography–mass spectrometry (GC/MS) analysis of the oils was carried out with an Agilent 5975 GC/MSD system, as reported previously.^{19,20} An Innowax FSC column (60 m × 0.25 mm, 0.25 µm film thickness) was used with He as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min, increased to 220°C at a rate of 4°C/min, kept constant at 220°C for 10 min, and then increased to 240°C at a rate of 1°C/min. The split ratio was adjusted to 40:1, and the injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450.

GC analysis was carried out using an Agilent 6890N GC system. In order to obtain the same elution order as with GC/MS, simultaneous injection was performed using the same column and appropriate operational conditions. Flame ionization detector (FID) temperature was 300°C. The components of essential oils were identified by coinjection with standards (whenever possible), which were purchased from commercial sources and/or isolated from natural sources. In addition, compound identities were confirmed by comparison of their mass spectra with those in Wiley GC/MS Library (Wiley, New York, NY, USA), MassFinder software 4.0 (Dr. Hochmuth Scientific Consulting, Hamburg, Germany), Adams Library, and NIST Library. A C₈–C₄₀ *n*-alkane standard solution (Fluka, Buchs, Switzerland) was used to spike the samples for the determination of relative retention indices (RRI). Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Cell Culture

Human MonoMac-6 monocytic cells (DSMZ, Germany) were grown in RPMI 1640 supplemented with 10% (v/v) endotoxin-free FBS, 10 µg/ml bovine insulin, 100 µg/ml streptomycin, and 100 U/ml penicillin. Human monocytic THP-1Blue cells obtained from InvivoGen (San Diego, CA) were cultured in RPMI 1640 medium supplemented with 10% (v/v) endotoxin-free FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, 100 µg/ml zeocin, and 10 µg/ml blasticidin S. These cells are stably transfected with a secreted embryonic alkaline phosphatase gene that is under the control of a promoter inducible by NF-κB and AP-1. Human promyelocytic leukemia HL-60 cells stably transfected with human FPR1 (FPR1-HL60 cells) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 100 µg/ml streptomycin, 100 U/ml penicillin, and G418 (1 mg/ml).¹⁵

Measurement of TNF production

MonoMac-6 cells were plated in culture medium supplemented with 3% (v/v) endotoxin-free FBS in 96-well plates at a density of 2×10^5 cells in 100 μ L per well. The cells were pre-treated with or without essential oil or DMSO (vehicle control) for 30 min, followed by treatment with LPS (200 ng/ml) and incubation for 24 h at 37°C and 5% CO₂. An enzyme-linked immunosorbent assay kit for human tumor necrosis factor (TNF) (Biolegend; San Diego, CA) was used to measure cytokine levels in the cell supernatants.

Analysis of NF- κ B/AP-1 activation

Activation of NF- κ B/AP-1 transcriptional activity was measured using an alkaline phosphatase reporter gene assay in THP1-Blue cells (2×10^5 cells in 100 μ L per well). The cells were pre-treated with or without essential oil or DMSO (vehicle control) for 30 min, followed by treatment with LPS (200 ng/ml) and incubation for 24 h at 37°C and 5% CO₂. Alkaline phosphatase activity was measured in cell supernatants using QUANTI-Blue mix (InvivoGen). Activation of NF- κ B is reported as absorbance at 655 nm and compared with positive control samples (LPS).

Human Neutrophil Elastase (HNE) Inhibition Assay

Essential oils and individual compounds were dissolved in 100% DMSO at 5 mM stock concentrations. The final concentration of DMSO in the reactions was 1%, and this level of DMSO had no effect on enzyme activity. One of our previously described HNE inhibitors (compound **5b**) was used as a positive control.²¹ The inhibition assay was performed, as described previously.²² Briefly, a buffer solution containing 200 mM Tris-HCl, pH 7.5, 0.01% bovine serum albumin, and 0.05% Tween-20 and 20 mU/mL of human neutrophil elastase was added to black, flat-bottom 96-well microtiter plates containing different concentrations of test compounds. Reactions were initiated by addition of 25 μ M elastase substrate *N*-methylsuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin in a final reaction volume of 100 μ L/well. Kinetic measurements were obtained every 30 s for 10 min at 25°C using a Fluoroskan Ascent FL fluorescence microplate reader (Thermo Electron, MA) with excitation and emission wavelengths at 355 and 460 nm, respectively. The concentration of inhibitor that caused 50% inhibition of the enzymatic reaction (IC₅₀) was calculated by plotting % inhibition versus logarithm of inhibitor concentration.

Neutrophil Isolation

For isolation of human neutrophils, blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Neutrophils were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as described previously.¹⁵ Isolated neutrophils were washed twice and resuspended in HBSS. Neutrophil preparations were routinely >95% pure, as determined by light microscopy, and >98% viable, as determined by trypan blue exclusion.

Ca²⁺ Mobilization Assay

Changes in intracellular Ca²⁺ were measured with a FlexStation II scanning fluorometer using fluorescent dye Fluo-4AM (Invitrogen). Neutrophils or FPR1-HL60 cells, suspended in HBSS, were loaded with Fluo-4AM dye (final concentration, 1.25 µg/ml) and incubated for 30 min in the dark at 37°C. After dye loading, the cells were washed with HBSS, resuspended in HBSS⁺, separated into aliquots, and deposited into the wells of flat-bottomed, half-area well black microtiter plates (2×10⁵ cells/well). The compound source plate contained dilutions of test essential oil or pure compound in HBSS⁺, and changes in fluorescence were monitored ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 538 \text{ nm}$) every 5 s for 240 s at room temperature after automated addition of compounds. Maximum change in fluorescence, expressed in arbitrary units over baseline, was used to determine agonist response. Responses were normalized to the response induced by 5 nM fMLF, which were assigned a value of 100%. Curve fitting (at least five to six points) and calculation of median effective concentration values (EC₅₀ or IC₅₀) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 5 (GraphPad Software, Inc., San Diego, CA).

Chemotaxis Assay

Human neutrophils were suspended in HBSS⁺ containing 2% (v/v) heat-inactivated fetal bovine serum (2×10⁶ cells/ml), and chemotaxis was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe, Gaithersburg, MD), as described previously.²³ In brief, neutrophils were preincubated with the indicated concentrations of the test sample (oil or pure compound) or DMSO for 30 min at room temperature and added to the upper wells of the ChemoTx chemotaxis chambers. The lower wells were loaded with 30 µl of HBSS⁺ containing 2% (v/v) fetal bovine serum and the indicated concentrations of test sample, DMSO (negative control), or 1 nM fMLF as a positive control. Neutrophils were added to the upper wells and allowed to migrate through the 5.0-µm pore polycarbonate membrane filter for 60 min at 37°C and 5% CO₂. The number of migrated cells was determined by measuring ATP in lysates of transmigrated cells using a luminescence-based assay (CellTiter-Glo; Promega, Madison, WI), and luminescence measurements were converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. Curve fitting (at least eight to nine points) and calculation of median effective concentration values (IC₅₀) were performed by nonlinear regression analysis of the dose-response curves generated using Prism 5.

ROS production

ROS production was determined by monitoring L-012-enhanced chemiluminescence, which represents a sensitive and reliable method for detecting superoxide anion (O₂^{•-}) production.²³ Human neutrophils were resuspended at 2×10⁵ cells/ml in HBSS⁺ supplemented with 40 µM L-012. Cells (100 µl) were aliquoted into wells of 96-well flat-bottomed microtiter plates containing essential oil or compounds at different concentrations (final DMSO concentration of 1%). Cells were preincubated for 30 min, and 200 nM PMA was added to each well to stimulate ROS production. Luminescence was monitored for 60 min (2-min intervals) at 37°C using a Fluroscan Ascent FL microtiter plate reader (Thermo Electron, Waltham, MA). The curve of light intensity (in relative luminescence units) was

plotted against time, and the area under the curve was calculated as total luminescence. The compound concentration that inhibited ROS production by 50% of the PMA-induced response (positive control) was determined by graphing the percentage inhibition of ROS production versus the logarithm of concentration of test sample (IC₅₀). Each curve was determined using five to seven concentrations.

Compound Cytotoxicity

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega), according to the manufacturer's protocol. Briefly, MonoMac-6 cells were cultured at a density of 1×10^5 cells/well with different concentrations of essential oil or compound for 4 or 24 h at 37°C and 5% CO₂. Following treatment, the cells were allowed to equilibrate to room temperature for 30 min, substrate was added, and the samples were analyzed with a Fluoroscan Ascent FL microplate reader.

Results

Composition of the essential oils from *A. kotuchovii*

Essential oils were obtained by conventional hydrodistillation of the dried stems or flowers +leaves of *A. kotuchovii* (designated as AKEO_{stm} and AKEO_{f/l}, respectively) and analyzed by GC and GC/MS techniques simultaneously to determine their chemical compositions. Hydrodistillation of the stems and flowers+leaves produced 0.096 and 1.010% (v/w on the basis of the weight of dried material) essential oils, respectively. The main constituents of AKEO_{f/l} and AKEO_{stm} were estragole (75.1 and 76.6%), (*E* and *Z*)- β -ocimene (9.2 and 8.2%), methyl eugenol (4.3 and 4.6%), limonene (1.0 and 1.0%), spathulenol (0.8 and 1.0%), β -pinene (0.9 and 1.0%), myrcene (1.5 and 0.7%), and (*E*)-methyl cinnamate (1.9 and 0.5%, respectively). Seventy four constituents were present at concentrations of 0.1 to <1.0% in one or both plant sources. The remaining 34 volatile compounds were identified in trace amounts, and their relative percentage amounts are indicated in Table 1. Thus, the major components of the AKEO_{f/l} and AKEO_{stm} are phenylpropanoids (81.5 and 79.6%, respectively) and monoterpene hydrocarbons (13.0 and 14.4%, respectively). Sesquiterpenes compose around 2–2.2% in both plant components. It should be noted that AKEO_{stm} had more oxygenated monoterpenes and fatty acids compared to AKEO_{f/l} (0.5 vs. 0.1% and 0.5 vs. 0.2%, respectively).

Evaluation of essential oil effect on phagocyte function

The essential oils from *A. kotuchovii* were screened for modulatory activity in various cell and enzymatic systems related to mechanisms of innate immunity (Table 2). Essential oils and their components have been reported previously to modulate intracellular Ca²⁺ levels²⁴ and inhibit of leukocyte migration.^{25,26} We found that AKEO_{stm}, but not AKEO_{f/l}, inhibited fMLF-stimulated Ca²⁺ flux and fMLF-induced chemotaxis in human neutrophils, with IC₅₀ values of 12.5 and 10.1 μ g/ml, respectively (Figure 1 and Table 2). Recently, several essential oils were reported to modulate ROS production in neutrophils.^{27,28} Thus, we evaluated the effect of AKEO_{stm} and AKEO_{f/l} on PMA-induced ROS production by human neutrophils and found that AKEO_{stm} inhibited ROS production with an IC₅₀ of 49.2 μ g/ml, whereas AKEO_{f/l} had no activity (Table 2). Although some essential oils and their

components were previously identified as HNE inhibitors,²⁹ evaluation of AKEO_{stm} and AKEO_{f/l} showed that they did not inhibit HNE, even at concentrations up to 50 µg/ml.

Because various essential oils have been reported to inhibit NF-κB/AP-1 transcriptional activity and production of pro-inflammatory cytokines,^{30,31} we also evaluated the effects of AKEO_{stm} and AKEO_{f/l} on these responses using cultures of monocytic cells. However, we found that AKEO_{stm} and AKEO_{f/l} did not alter NF-κB/AP-1 activity or TNF production in monocytic cells (Table 2).

Although our functional cell-based assays suggested that the essential oils were relatively nontoxic, we evaluated their potential cytotoxic effects to determine if the results might be influenced by background cytotoxicity. Using a cytotoxicity assay, we determined that neither of the essential oils significantly affected viability of MonoMac-6 cells over a concentration range up to 50 µg/ml, further demonstrating that AKEO_{stm} and AKEO_{f/l} were not cytotoxic (data not shown).

Effect of AKEO_{stm} components on neutrophil function

Because AKEO_{stm} inhibited neutrophil functional responses, we focused on analysis of the effects of AKEO_{stm} constituents to possibly identify the active compound(s). Twenty five commercially available components of AKEO_{stm}, including eight major (1%) and seventeen minor compounds, were tested. Note that among the minor constituents tested, hexanal, α-terpinene, p-cymene, MHDO, (*E*)-anethole, isopropyl myristate, and palmitic acid were all present and higher levels in AKEO_{stm} compared to AKEO_{f/l} (Table 1).

All selected compounds were evaluated for agonist and antagonist effects on Ca²⁺ flux in human neutrophils. We found that four compounds from different chemical classes, including β-pinene, sabinene, palmitic acid, and myristic fatty acid, had direct but weak agonist activity, as determined by monitoring Ca²⁺ flux (Table 3). Of all of the component compounds tested, only MHDO inhibited fMLF-stimulated Ca²⁺ mobilization in neutrophils (IC₅₀ = 8.2 µM), which is far below our common hit threshold of 50 µM,¹⁵ and this inhibition was concentration-dependent (Table 2 and Figure 2A). MHDO did not directly activate Ca²⁺ flux, suggesting it was not down-regulating the subsequent response to fMLF but was directly inhibiting fMLF-stimulated Ca²⁺ mobilization via a different mechanism. Furthermore, MHDO inhibited Ca²⁺ flux in fMLF-stimulated FPR1-HL60 cells (Table 2) and IL-8-induced Ca²⁺ mobilization in human neutrophils (IC₅₀ = 3.2 µM) (Figure 2B). On the other hand, MHDO did not inhibit non-specific Ca²⁺ flux induced by 10 µM ionomycin (data not shown). Thus, these data indicate that MHDO can modulate intracellular signaling pathways that are common to both FPR1 and CXCR1/2 chemokine receptors, but it is not an ion channel inhibitor or a calcium chelator.

Consistent with its effect on Ca²⁺ mobilization, MHDO also inhibited neutrophil chemotaxis with an IC₅₀ in the low micromolar range (Table 3). Among the other compounds tested, β-pinene, sabinene, β-citronellol, and elemicin were weak inhibitors of neutrophil chemotaxis (Table 3).

All 25 compounds were also evaluated for their effect on neutrophil ROS production. As with the other responses, only MHDO inhibited PMA-induced ROS production in human neutrophils ($IC_{50} = 2.8 \mu M$) (Table 3).

To ensure that the results on inhibition of Ca^{2+} flux and neutrophil chemotaxis were not influenced by possible compound toxicity, cytotoxicity of MHDO was evaluated at various concentrations (up to $100 \mu M$) in MonoMac-6 cells during a 4-h incubation, which is relevant for relatively short times used to measure Ca^{2+} mobilization (up to 35 min) and chemotaxis (up to 90 min). As shown in Figure 3, MHDO did not affect cell viability, even at the highest tested concentration, thereby verifying that this compound, like the parent essential oil, was also not cytotoxic, at least during the 4 hr incubation period.

Discussion

Essential oils are natural mixtures of terpenes, mainly monoterpenes and sesquiterpenes, which have a wide-range of pharmacological activities.^{11,32–34} Furthermore, essential oils from various plants, including *Artemisia* spp., have been reported to exhibit various biological effects *in vitro* and *in vivo*.^{2,35} In the present study, we defined the chemical profile of essential oils isolated from *A. kotuchovii* and evaluated their effects on phagocyte function.

Similar to the composition of essential oils from *A. dracunculus*,¹ we found that estragole, (*E*) and (*Z*) β -ocimenes, and methyl eugenol were the primary constituents of *A. kotuchovii* essential oils. Other primary constituents present at lower concentrations (1–1.9% total weight) were limonene, spathulenol, β -pinene, myrcene, and methyl cinnamate. These compounds, with the exception of methyl cinnamate, are also present in tarragon essential oils.^{1,36}

Although various essential oils have previously been found to inhibit NF- κ B/AP-1 transcription and production of pro-inflammatory cytokines^{5,30} and HNE activity,^{29,37} we did not find these activities in *A. kotuchovii* essential oils. Indeed, none of the major components of *A. kotuchovii* essential oils (>1% by total weight) have been reported previously to inhibit HNE, NF- κ B/AP-1 activity, or TNF production. Among the minor compounds (~ 1%), only limonene has been reported to inhibit NF- κ B activation *in vivo* during acute lung injury.³⁸

Evaluation of biological activities of *A. kotuchovii* essential oils revealed that only AKEO_{stm} inhibited Ca^{2+} flux and chemotaxis in human neutrophils. To date, there are no publications on modulation of Ca^{2+} mobilization in neutrophils by essential oils. Since intercellular Ca^{2+} flux is involved in chemotaxis, the inhibitory effect of AKEO_{stm} on neutrophil chemotaxis is likely due to a primary effect on Ca^{2+} flux. Previously, other essential oils or their constituents have been reported to inhibit cell migration. For example, rosemary and geranium essential oils inhibited leukocyte recruitment and chemotaxis.^{39,40} In addition, *Thymus vulgaris* L. and *Citrus latifolia* (Yu.Tanaka) Tanaka essential oils also inhibited leukocyte migration.^{25,41} Although we found that AKEO_{stm} also inhibited PMA-induced

ROS production in human neutrophils, the potency of this effect was relatively low compared to essential oils from *Sideritis italica* (Miller) Greuter et Burdet.²⁷

To further define the active component(s) in AKEO_{stm}, we evaluated 25 of its constituents and found that β -citronellol, elemicin, β -pinene, and sabinene had low activity, whereas MHDO was a relatively potent inhibitor of neutrophil chemotaxis (Table 3), suggesting that it could be one of the primary essential oil components responsible for the inhibitory effects of AKEO_{stm} on human neutrophils *in vitro* (Table 2). Indeed, only MHDO inhibited the other neutrophil responses tested (i.e., PMA-induced ROS production and *f*MLF- and IL-8-induced Ca²⁺ flux). Furthermore, MHDO was not present in AKEO_{f/l} (Table 1). Thus, these data provide a molecular basis to explain at least part of inhibitory activity of AKEO_{stm} on neutrophil chemotaxis. However, based on molecular weight of MHDO (124.18 Da), its relative percentage (~ 0.1%) in AKEO_{stm}, and the IC₅₀ values for biological activities of AKEO_{stm} (~ 10–50 μ g/ml) (see Tables 1 and 2), effective concentrations of this component would be in the range of 0.08–0.4 μ M, which is several fold lower than the IC₅₀ values for the biological activity profiles of MHDO (2.8–8.2 μ M, see Table 3). Thus, we could not exclude possible synergetic effects of other minor compounds, which are higher in AKEO_{stm} (for example dehydro-1,8-cineole, trans- α -bergamotol, etc.) versus AKEO_{f/l}. Since β -pinene and sabinene directly activated Ca²⁺ mobilization in neutrophils, albeit with low efficacy, it is possible that they could contribute to receptor desensitization and/or intracellular Ca²⁺ store depletion. Recently, Siani *et al.*⁴² reported that effective inhibition of eosinophil migration by essential oils from *Syzygium cumini* Skells and *Psidium guajava* L. correlated with the presence of β -pinene. Because α -pinene was completely inactive in all of our test-systems (Table 3), we suggest that human neutrophils can discriminate between α - and β - isomers of this bicyclic monoterpene. In support of this isomer preference, α -pinene inhibited inflammatory pathways in human chondrocytes, whereas β -pinene was inactive.⁴³ Thus, various cell and/or molecular targets may determinate potency of the biological activities of these isomers.

Although previous reports indicate estragole and limonene can inhibit neutrophil and eosinophil migration,^{25,26,44} we did not observe inhibition of neutrophil function by *R*- and *S*- enantiomers of limonene. Likewise, we did not observe any effects of anethole on neutrophil function, although it was reported to inhibit paw edema in mice in acute and persistent inflammation models.⁴⁵ These discrepancies may be explained by differences in methodologies and species specificity. For example, Kummer *et al.*²⁶ used murine neutrophils, which respond differently than human neutrophils to *f*MLF and other agonists.

Neutrophils express a large number of receptors for the recognition of pathogen invasion and communication, including chemosensory, chemoattractant, and chemokine GPCR, Fc-receptors, various cytokine receptors, adhesion receptors, as well as innate immune receptors, such as Toll-like receptors.^{46,47} Here, we found that MHDO inhibited both *f*MLF- and IL-8-induced Ca²⁺ fluxes in neutrophils and the *f*MLF-induced response in FPR1-HL60 cells. Thus, MHDO may interfere with intracellular signaling pathways common for both FPR1 and CXCR1/2. Nevertheless, further studies will be necessary in the future to determine the precise molecular targets for MHDO in human neutrophils. It should be noted that MHDO is found in paprika and tomato oleoresins⁴⁸ and can be formed by thermal

degradation of lycopene (ψ,ψ -carotene),⁴⁹ a red pigment of some fruits and vegetables. Although there are many publications describing the anti-inflammatory and antioxidant effects of lycopene,⁵⁰ our studies suggest that inhibition of leukocyte activation and/or migration could also contribute to the biological effects of paprika and tomato oleoresins, including lycopene metabolites and products of its thermal degradation.

In summary, we have defined the essential oil composition of *A. kotuchovii* and show that these essential oils and a primary component compound are able to inhibit human neutrophil responses, including Ca^{2+} mobilization, chemotaxis, and ROS production. Further studies are now needed to determine the molecular targets for MHDO, as well as the biological activity of other minor constituents of the oil and evaluation of therapeutic efficacy of AKEO_{stm} and MHDO in animal models of acute or chronic inflammatory diseases.

Acknowledgements

We would like to thank Professor Andrei Khlebnikov, Tomsk Polytechnic University, Tomsk, Russia for advice on compound structure interpretation. This research was supported in part by National Institutes of Health IDEA Program COBRE grant GM110732; grants 0504/GF3 and 2117/GF4 from The Ministry of Education and Science, Kazakhstan; a USDA National Institute of Food and Agriculture Hatch project; and the Montana State University Agricultural Experiment Station.

Abbreviations

AKEO	Essential oils of <i>A. kotuchovii</i>
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FID	flame ionization detector
FPR1	<i>N</i> -formyl peptide receptor 1
GC	gas chromatography
GPCR	G-protein coupled receptors
HBSS	Hanks' balanced-salt solution
HNE	human neutrophil elastase
IL	interleukin
LPS	lipopolysaccharide
MHDO	6-methyl-3,5-heptadien-2-one
MS	mass spectrometry
PMA	phorbol-12-myristate-13-acetate
ROS	reactive oxygen species
TNF	tumor necrosis factor

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Figure 1.

Effect of essential oils from *A. kotuchovii* on Ca^{2+} mobilization in human neutrophils. Neutrophils were preincubated with the indicated concentrations of essential oils isolated from stems (AKEO_{stm}) (closed circles) and flowers+leaves (AKEO_{f/l}) (open circles) of *A. kotuchovii* for 30 min at 25 °C and then stimulated with 5 nM of fMLF. The response induced by the peptide agonist alone was assigned a value of 100%. Values are the mean $\pm$ S.D. of triplicate samples from one experiment that is representative of three independent experiments.

Figure 2.

Effect of MHDO on fMLF-induced Ca^{2+} mobilization in human neutrophils. **Panel A.** Human neutrophils were preincubated for 30 min (at 25°C) with 15 μM MHDO or DMSO (vehicle control), followed by treatment with 5 nM fMLF or DMSO (vehicle control). **Panel B.** Effect of MHDO on IL-8-induced Ca^{2+} mobilization in human neutrophils. Human neutrophils were preincubated for 30 min (at 25°C) with 15 μM MHDO or DMSO (vehicle control), followed by treatment with 25 nM IL-8 or DMSO (vehicle control). Arrows

indicate time of treatment additions. The data shown in both panels are representative of three independent experiments.

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Figure 3.

Effect of MHDO on cell viability. MonoMac-6 cells were incubated for 4 hr with the indicated concentrations of MHDO, and cell viability was determined using a luminescent cell viability assay kit, as described. Values are the mean $\pm$ S.D. of triplicate samples from one experiment that is representative of two independent experiments. No statistically significant differences between untreated cells (DMSO alone) and cells treated with the compound were found.

Table 1

Composition of the volatile compounds identified in the essential oils from *A. kotuchovii*

Cmpd	RRI	Compound name	A (%)	B (%)	Cmpd	RRI	Compound name	A (%)	B (%)
1	1032	α -Pinene *	0.6	0.6	59	1783	β -Sesquiphellandrene **	0.2	0.2
2	1035	α -Thujene **	t	t	60	1786	<i>ar</i> -Curcumene **	0.1	0.1
3	1093	Hexanal *	t	0.1	61	1798	Methyl salicylate *	-	t
4	1118	β -Pinene *	0.9	1.0	62	1804	Myrtenol *	-	t
5	1132	Sabinene *	0.3	0.3	63	1815	2,6-Dimethyl-3(<i>E</i>),5(<i>E</i>),7-octatriene-2-ol ***	t	t
6	1174	Myrcene *	1.5	0.7	64	1827	(<i>E</i>),2,4-Decadienal	-	t
7	1188	α -Terpinene *	t	0.4	65	1830	2,6-Dimethyl-3(<i>E</i>),5(<i>E</i>),7-octatriene-2-ol ***	0.1	t
8	1195	Dehydro-1,8-cineole **	t	0.1	66	1845	(<i>E</i>)-Anethole *	t	0.1
9	1203	Limonene *	1.0	1.0	67	1857	Geraniol *	-	t
10	1218	β -Phellandrene **	0.2	0.1	68	1864	<i>p</i> -Cymen-8-ol *	0.1	t
11	1244	Amyl furan **	t	t	69	1868	(<i>E</i>)-Geranyl acetone *	t	t
12	1246	(<i>Z</i>)- β -Ocimene *	3.9	3.8	70	1885	1-Phenyl-3-methylpenta-1,2,4-triene **	0.1	-
13	1255	γ -Terpinene *	t	t	71	1893	(<i>Z</i>)- Methyl cinnamate **	t	t
14	1266	(<i>E</i>)- β -Ocimene *	5.3	4.4	72	1958	(<i>E</i>)- β -Ionone *	t	t
15	1280	<i>p</i> -Cymene *	0.3	0.5	73	1988	Isopropyl myristate *	-	0.1
16	1290	Terpinolene *	0.3	0.1	74	1990	Anisaldehyde *	-	t
17	1348	6-Methyl-5-hepten-2-one *	-	t	75	2001	Isocaryophyllene oxide **	t	t
18	1362	<i>cis</i> -Rose oxide ***	-	t	76	2008	Caryophyllene oxide *	0.4	0.2
19	1376	<i>trans</i> -Rose oxide ***	-	t	77	2014	(<i>E</i>)-Cinnamaldehyde *	t	-
20	1382	<i>cis</i> -Alloocimene **	t	t	78	2030	Methyl eugenol *	4.3	4.6
21	1413	Roze furan **	0.1	0.1	79	2037	Salvial-4(14)-en-1-one ***	t	0.1
22	1416	4,8-Dimethyl-1,3,7-nonatriene **	t	-	80	2041	Pentadecanal **	-	t

Cmpd	RRI	Compound name	A (%)	B (%)	Cmpd	RRI	Compound name	A (%)	B (%)
23	1429	Perillene **	t	t	81	2065	Salviadienol **	t	-
24	1446	2,6-Dimethyl-1,3(E),5(Z),7-octatetraene **	t	-	82	2071	Humulene epoxide-II **	t	-
25	1452	α , p -Dimethylstyrene *	t	-	83	2096	(E)-Methyl cinnamate *	1.9	0.5
26	1452	1-Octen-3-ol *	-	t	84	2097	(E)-Ethyl cinnamate **	-	t
27	1458	cis-1,2-Limonene epoxide *	t	-	85	2124	Zingiberenol **	t	t
28	1460	2,6-Dimethyl-1,3(E),5(E),7-octatetraene **	-	t	86	2131	Hexahydrofarnesyl acetone **	t	t
29	1468	trans-1,2-Limonene epoxide **	t	-	87	2139	Chavibetol **	t	-
30	1474	trans-Sabinene hydrate **	t	-	88	2144	Spathulenol **	0.8	1.0
31	1476	(Z)- β -Ocimene epoxide **	t	t	89	2179	3,4-Dimethyl-5-pentylidene-2(5H)-furanone **	-	t
32	1479	(E,Z)-2,4-Heptadienal **	t	-	90	2187	T-Cadinol **	t	t
33	1487	Isoneroloxide **	t	-	91	2204	Alismol **	0.1	0.2
34	1498	(E)- β -Ocimene epoxide **	t	t	92	2214	ar-Turmerol **	t	-
35	1520	3,5-Octadien-2-one **	t	t	93	2228	Isospathulenol **	t	-
36	1535	β -Bourbonene **	t	-	94	2232	α -Bisabolol *	t	-
37	1541	Benzaldehyde *	t	t	95	2245	Elemicin **	0.2	0.1
38	1542	α -Isocimene **	-	t	96	2247	trans- α -Bergamotol **	t	0.1
39	1553	Linalool ^a	-	0.2	97	2255	α -Cadinol **	t	-
40	1586	Pinocarvone **	-	t	98	2257	β -Eudesmol *	-	t
41	1602	6-Methyl-3,5-heptadien-2-one (MHDO) *	-	0.1	99	2259	Eudesma-4(15),7-dien-1-ol *	-	0.1
42	1611	Terpinen-4-ol *	t	t	100	2268	Torilenol **	t	t
43	1612	β -Caryophyllene *	0.1	t	101	2269	(6S,7R)-Bisabolone **	-	t
44	1639	trans-p-Mentha-2,8-dien-1-ol **	t	-	102	2281	Chavicol **	t	-
45	1670	Benzeneacetaldehyde *	t	-	103	2290	(2E,6Z)-Farnesol *	t	-
46	1672	trans-Pinocarveol *	t	-	104	2375	Eudesma-4(15),7-dien-1- β -ol **	0.1	0.1

Cmpd	RRI	Compound name	A (%)	B (%)	Cmpd	RRI	Compound name	A (%)	B (%)
47	1671	Acetophenone *	t	-	105	2300	Tricosane *	-	t
48	1687	α -Humulene *	t	t	106	2309	Farnesylacetone *	t	-
49	1687	Estragole *	75.1	76.6	107	2360	6-Dodecen-4-olide **	t	-
50	1694	<i>p</i> -Vinylanisole *	-	0.1	108	2369	(2 <i>E</i> ,6 <i>E</i>)-Farnesol *	-	t
51	1706	α -Terpineol *	t	0.1	109	2392	Caryophylla-2(12),6-dien-5 β -ol **	t	-
52	1726	α -Zingiberene **	-	t	110	2415	Demethoxyencecaline **	0.1	0.1
53	1726	Germacrene D **	0.2	0.1	111	2500	Pentacosane *	-	t
54	1733	Neryl acetate *	t	-	112	2528	(<i>E</i>)- <i>p</i> -Methoxy-cinnamaldehyde **	t	-
55	1755	Bicyclogermacrene **	-	t	113	2622	Phytol *	t	-
56	1765	Geranyl acetate *	t	t	114	2670	Myristic acid *	0.2	0.1
57	1772	Citronellol *	t	0.1	115	2700	Heptacosane *	-	t
58	1773	δ -Cadinene **	t	-	116	2931	Palmitic acid *	t	0.4

The data are presented as relative % for components of essential oil from flowers+leaves (A) and essential oil from stems (B). RRI: Relative retention indices calculated against *n*-alkanes; % calculated from flame ionization detector data. Trace amounts (tr) are present at <0.1 %.

* Identification based on comparison with co-injected with standards.

** Tentatively identified using Wiley, MassFinder mass spectra libraries and published RRI. Compounds that were selected for further biological screening are indicated in bold.

Table 2Biological screening of the essential oils from *A. kotuchovii*

Biological Activity	AKEO _{fl}	AKEO _{stm}
	IC ₅₀ (µg/ml)	
LPS-induced NF-κB/AP-1 activity in THP-1Blue cells	N.A.	N.A.
LPS-induced TNF production in MonoMac-6 cells	N.A.	N.A.
Neutrophil elastase enzymatic activity	N.A.	N.A.
fMLF-induced Ca ²⁺ flux in human neutrophils ^a	N.A.	12.5 ± 2.7
fMLF-induced chemotaxis in human neutrophils ^b	N.A.	10.1 ± 3.1
PMA-induced ROS production in human neutrophils ^c	N.A.	49.2 ± 5.4

^a Activity was evaluated as inhibition of Ca²⁺ flux induced by 5 nM fMLF in neutrophils.

^b Inhibition of chemotactic activity in neutrophils was evaluated in the presence of 0.5 nM fMLF.

^c Inhibition of ROS production in neutrophils was evaluated in the presence of 200 nM PMA and 40 µM L-012. N.A.: no activity was observed, even at the highest concentration tested (50 µM).

Table 3
Effect of selected volatile compounds on functional responses in human neutrophil and FPR1-HL60 cells

Compound Name		Ca ²⁺ flux			Inhibition of Chemotaxis ^b (IC ₅₀)	Inhibition of ROS Production ^c (IC ₅₀)
		Neutrophils		FPR1-HL60		
				cells		
		Activation (EC ₅₀)	Inhibition ^a (IC ₅₀)	Inhibition ^a (IC ₅₀)		
μM (μg/ml)						
(1R)-(+)-α-Pinene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Hexanal	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
β-Pinene	23.8 ± 3.1 (3.2 ± 0.4)	N.A.	N.A.	N.A.	22.7 ± 2.6 (3.1 ± 0.4)	N.A.
Sabinene (+/-)	49.4 ± 6.3 (6.7 ± 0.86)	N.A.	N.A.	N.A.	37.4 ± 4.3 (5.1 ± 0.6)	N.A.
Myrcene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
α-Terpinene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(R)-(+)-Limonene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(S)-(-)-Limonene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(E/Z)-β-OCimene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
p-Cymene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Terpinolene	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(-)-Linalool	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
MHDO	N.A.	8.2 ± 2.5 (1.02 ± 0.31)	18.0 ± 5.4 (2.2 ± 0.7)	3.6 ± 0.5 (0.45 ± 0.16)	2.8 ± 0.4 (0.35 ± 0.05)	
Estragol	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
4-Vinylanisole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(+/-)-β-Citronellol	N.A.	N.A.	N.A.	48.6 ± 3.7 (7.6 ± 0.6)	N.A.	N.A.
(E)-Anethole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
p-Cymen-8-ol	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Isopropyl myristate	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(-)-Caryophyllene oxide	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Compound Name	Ca ²⁺ flux			Inhibition of Chemotaxis ^b (IC ₅₀)	Inhibition of ROS Production ^c (IC ₅₀)
	Neutrophils		FPR1-HL60 cells		
	Activation (EC ₅₀)	Inhibition ^a (IC ₅₀)	Inhibition ^a (IC ₅₀)		
	μM (μg/ml)				
Methyl eugenol	N.A.	N.A.	N.A.	N.A.	N.A.
(<i>E</i>)-Methyl cinnamate	N.A.	N.A.	N.A.	N.A.	N.A.
Elemicine	N.A.	N.A.	N.A.	46.9 ± 4.2 (9.8 ± 0.9)	N.A.
Myristic acid	46.7 ± 5.3 (10.7 ± 1.2)	N.A.	12.9 ± 3.4 (3.0 ± 0.8)	N.A.	N.A.
Palmitic acid	40.7 ± 4.6 (10.4 ± 1.2)	N.A.	N.A.	N.A.	N.A.

^a Inhibition of Ca²⁺ flux induced by 5 nM fMLF.

^b Inhibition of chemotactic activity in neutrophils was evaluated in the presence of 0.5 nM fMLF.

^c Inhibition of ROS production in neutrophils was evaluated in the presence of 200 nM PMA and 40 μM L-012. N.A.: no activity was observed, even at the highest concentration tested (50 μM).