

Modulation of Human Neutrophil Responses by the Essential Oils from *Ferula akitschkensis* and Their Constituents

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ABSTRACT: Essential oils were obtained by hydrodistillation of the umbels+seeds and stems of *Ferula akitschkensis* (FAEO_{u/s} and FAEO_{stm}, respectively) and analyzed by gas chromatography and gas chromatography–mass spectrometry. Fifty-two compounds were identified in FAEO_{u/s}; the primary components were sabinene, α -pinene, β -pinene, terpinen-4-ol, eremophilene, and 2-himachalen-7-ol, whereas the primary components of FAEO_{stm} were myristicin and geranylacetone. FAEO_{u/s}, β -pinene, sabinene, γ -terpinene, geranylacetone, isobornyl acetate, and (*E*)-2-nonenal stimulated [Ca²⁺]_i mobilization in human neutrophils, with the most potent being geranylacetone (EC₅₀ = 7.6 ± 1.9 μ M) and isobornyl acetate 6.4 ± 1.7 (EC₅₀ = 7.6 ± 1.9 μ M). In addition, treatment of neutrophils with β -pinene, sabinene, γ -terpinene, geranylacetone, and isobornyl acetate desensitized the cells to *N*-formyl-Met-Leu-Phe (*f*MLF)- and interleukin-8 (IL-8)-induced [Ca²⁺]_i flux and inhibited *f*MLF-induced chemotaxis. The effects of β -pinene, sabinene, γ -terpinene, geranylacetone, and isobornyl acetate on neutrophil [Ca²⁺]_i flux were inhibited by transient receptor potential (TRP) channel blockers. Furthermore, the most potent compound, geranylacetone, activated Ca²⁺ influx in TRPV1-transfected HEK293 cells. In contrast, myristicin inhibited neutrophil [Ca²⁺]_i flux stimulated by *f*MLF and IL-8 and inhibited capsaicin-induced Ca²⁺ influx in TRPV1-transfected HEK293 cells. These findings, as well as pharmacophore modeling of TRP agonists, suggest that geranylacetone is a TRPV1 agonist, whereas myristicin is a TRPV1 antagonist. Thus, at least part of the medicinal properties of *Ferula* essential oils may be due to modulatory effects on TRP channels.

KEYWORDS: *Ferula akitschkensis*, calcium flux, essential oil, neutrophil, transient receptor potential channel

INTRODUCTION

The genus *Ferula* (Apiaceae) comprises ~185 species distributed throughout Central Asia, the Mediterranean, and northern Africa. Various parts of these plants (e.g., roots, leaves, seeds) and resin prepared from the plant have been used historically in traditional herbal medicine for a variety of ailments, including diarrhea, hysteria, whooping cough, asthma, bronchitis, and ulcers.^{1,2} Asafetida, an oleo-gum-resin obtained from the roots of various *Ferula* spp., including *F. assa-fetida*, *F. fetida*, *F. rubricaulis*, *F. rigidula*, *F. alliacea*, and *F. narthex*, contains up to 17% of the essential oil and is used as a traditional phytomedicine for the treatment of asthma, epilepsy, intestinal parasites, weak digestion, stomach ache, and influenza.¹ Asafetida and *Ferula* essential oils have also been used as food additives (spices) and natural food preservatives.¹ Secondary metabolites isolated from *Ferula* spp., such as terpenoids and sesquiterpenes, have been shown to exhibit a variety of pharmacological properties, including anti-inflammatory, estrogenic, antitumor, antibacterial, and antiviral activ-

ities.³ In addition, essential oils isolated from different parts of *Ferula* herbs, including stems, flowers, and seeds, have been reported as a rich source of monoterpenes, sesquiterpenes, and polysulfides,^{4,5} and biochemical studies of these chemical components have demonstrated desirable therapeutic properties, including antioxidant, antimutagenic, antimicrobial, and antifungal effects.^{4,6}

Despite the importance of *Ferula* spp. as a potential source of novel therapeutic compounds, the chemical composition and biological properties of many endemic *Ferula* spp. have not yet been evaluated. For example, *Ferula akitschkensis* B. Fedtschenko ex Koso-Poljansky grows on mountain slopes at an altitude of 900–2100 m and is endemic to northern China, Kazakhstan, Kyrgyzstan, and Russia. When mature, the plant produces

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numerous 2–4-in.-wide rounded yellow umbels on branched stems. Although several unique secondary metabolites have been isolated from *F. akitschkensis*, including akiferine, akiferidine, akiferidine, and akichenin,⁷ the chemical composition and biological properties of essential oils from *F. akitschkensis* have not yet been analyzed. Although previous studies demonstrated that essential oils from a number of plants can have direct antimicrobial activity, the effects of essential oils and their components on the innate immune system are not well understood.

Neutrophils are a key cellular component of the innate immune system and play a prominent role in inflammatory response. These leukocytes are recruited to sites of infection or injury by a variety of factors, including *N*-formyl-Met-Leu-Phe (*f*MLF), a bacterial or mitochondria-derived peptide, and chemokines such as interleukin 8 (IL-8).⁸ IL-8 and *f*MLF activate G-protein coupled receptors (GPCR) to induce neutrophil chemotaxis and the release of various mediators, such as reactive oxygen species (ROS), cytokines, and proteases. Recently, we found that some essential oil components of *Artemisia kotuchovii* were able to modulate some of these neutrophil functional responses.⁹ Thus, plant essential oils likely represent a source of novel therapeutics that could be developed to modulate innate immune responses and either enhance defense against microbial infection or control excessive inflammation.

In the present studies, we determined the composition of essential oils from umbels+seeds and stems of *F. akitschkensis* (designated FAEO_{u/s} and FAEO_{stm}, respectively) and evaluated their immunomodulatory activity in human neutrophils. We found that FAEO_{u/s} can activate Ca²⁺ mobilization and inhibit neutrophil migration. We also evaluated the main components of FAEO_{u/s} and FAEO_{stm} and found the primary bioactive constituents to be sabinene, β -pinene, γ -terpinene, geranylacetone, and isobornyl acetate, which were neutrophil agonists. We also found that one component, myristicin, inhibited neutrophil function. Using a set of pharmacological inhibitors and molecular modeling, the primary molecular targets for the agonists were determined to be transient receptor potential (TRP) channels. Indeed, the most potent compound, geranylacetone, directly activated Ca²⁺ flux in TRPV1-transfected HEK293 cells, whereas myristicin inhibited capsaicin-induced Ca²⁺ flux in TRPV1-transfected HEK293 cells. Thus, these data suggest that at least some of the medicinal properties of *Ferula* essential oils may be due to modulatory effects on TRP channels.

MATERIALS AND METHODS

Chemicals. The major and some minor compounds in the essential oils were obtained from commercial sources. Sabinene, *p*-cymen-8-ol, camphene, α -pinene oxide, α -terpineol, γ -terpinene, 4-carvomenthenol (terpinen-4-ol), myrtenol, and 3,7-dimethyl-1,3,6-octatriene (β -ocimene) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Myrcene, (+)-limonene, (–)-limonene, *p*-cymene, (–)-caryophyllene oxide, and α -terpinene were purchased from Acros Organics (Geel, Belgium). Elemicin was from Toronto Research Chemicals (Toronto, ON, Canada). Geraniol and (*E*)-2-nonenal were from AK Scientific Inc. (Union City, CA, USA). Terpinolene and α -pinene were from Santa Cruz Biotechnology (Dallas, TX, USA). (*E*/*Z*)-Geranylacetone and (1*S*)-(–)- β -pinene were from Alfa Aesar (Ward Hill, MA, USA). Thymol, (–)-menthol, (*R*)-(–)-carvone, isobornyl acetate, carvacrol, (–)-fenchone, and capsaicin were from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Myristicin was from Cayman Chemical Co. (Ann Arbor, MI, USA). The compounds

were dissolved in dimethyl sulfoxide (DMSO; 10 mM stock solutions) and stored at –20 °C.

Materials. DMSO, *N*-formyl-Met-Leu-Phe (*f*MLF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and Histopaque 1077 were purchased from Sigma-Aldrich Chemical Co. Trp-Lys-Tyr-Met-Val-Met (WKYMVM), pertussis toxin (PTX), and phospholipase C inhibitor U73122 were from Tocris Bioscience (Ellisville, MO, USA). Capsazepine, SKF 96365, and *N*-(*p*-amylcinnamoyl) anthranilic acid (ACA) were from Cayman Chemical Co. 2-Aminoethyl diphenyl borate (2-APB) was from BioVision Inc. (Milpitas, CA, USA). Trp-Arg-Trp-Trp-Trp (WRW₄) was from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Cyclosporin H was from Toronto Research Chemicals. Stock solutions for the inhibitors/antagonists (except PTX) were prepared in DMSO; PTX was dissolved in H₂O. Human IL-8 was purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). Fura-2 acetoxy methylester (fura-2/AM) was from TEFLabs (Austin, TX, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and penicillin–streptomycin solution were purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO, USA). 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, USA). HBSS without Ca²⁺ and Mg²⁺ is designated HBSS[–]; HBSS containing 1.3 mM CaCl₂ and 1.0 mM MgSO₄ is designated HBSS⁺.

Plant Material. *F. akitschkensis* was collected at the fruiting stage (July 2015) in the Almaty region of Kazakhstan at an altitude of 1525 m. Voucher specimens were deposited at the Institute of Plant Biology and Biotechnology (Almaty, Kazakhstan). Umbels with seeds and stems were air-dried for 7–10 days at room temperature away from direct sunlight before hydrodistillation.

Essential Oil Extraction. Essential oil was obtained by hydrodistillation using a Clevenger type apparatus, as described previously.⁹ For the hydrodistillation, we used conditions accepted by the European Pharmacopoeia (European Directorate for the Quality of Medicines, Council of Europe, Strasbourg, France, 2014) to avoid artifacts. Solutions of the essential oils were prepared in DMSO (10 mg/mL stock solutions) for biological evaluation and in *n*-hexane (10% w/v) for gas chromatographic analysis.

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. GC-MS analysis was performed with an Agilent 5975 GC-MSD system (Agilent Technologies, Santa Clara, CA, USA), as reported previously.⁹ An Agilent Innovax FSC column (60 m × 0.25 mm, 0.25 μ m film thickness) was used with He as the carrier gas (0.8 mL/min). The 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 250 °C. MS spectra were monitored at 70 eV with as mass range from *m/z* 35 to 450.

GC analysis was carried out using an Agilent 6890N GC system. 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 co-injection with standards (whenever possible), which were purchased from commercial sources or isolated from natural sources. In addition, compound identities were confirmed by comparison of their mass spectra with those in the Wiley GC/MS Library (Wiley, 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.

Chiral GC-MS Analysis. Chromatographic separation on a chiral column was performed for α -pinene, β -pinene, and sabinene. GC-MS analysis of the enantiomers in the oil was performed with an Agilent 7890 GC (Agilent; SEM Ltd.) equipped with a FID and 5975 MSD

with a triple-axis detector and an Agilent G 4513 autoinjector, integrated with a Gerstel CIS (Gerstel, Mülheim an der Ruhr, Germany; SEM Ltd., Istanbul, Turkey). Chiral separation was performed on a Lipodex G column (25 m × 0.25 mm × 0.125 μm film thickness; Macherey-Nagel, Düren, Germany) with He as the carrier gas (65 min at 5 mL/min, average velocity 77.985 cm/s). Injection quantity was 1 μL (10% in hexane). The temperature program for separation of α-pinene, β-pinene, and sabinene enantiomers was 50 min at 35 °C and then increased 40 °C/min to 200 °C for 10.875 min. Run time was 65 min. The split ratio was adjusted to 40:1, and the injector temperature was at 250 °C. FID temperature was 250 °C.

Cell Culture. Wild-type human promyelocytic leukemia HL-60 cells and HL-60 cells stably transfected with human N-formyl peptide receptor (FPR) 1 (FPR1-HL60 cells) or human FPR2 (FPR2-HL60 cells) (kind gift from Dr. Marie-Joséphine Rabiet, INSERM, Grenoble, France) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 100 μg/mL streptomycin, and 100 U/mL penicillin, as described.⁹ Transfected HL-60 cells were cultured in the presence of G418 (1 mg/mL).

Cultured HEK293 cells were plated in a 6-well plate for 24 h, after which the cells were transfected with human transient receptor potential vanilloid receptor type 1 (TRPV1) cDNA using TransIT-2020 (Mirus Bio LLC, Madison, WI, USA) according to the manufacturer's protocol. Following transfection, the cells were suspended in DMEM supplemented with 10% fetal bovine serum at 37 °C and used for intracellular Ca²⁺ measurements.

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.⁹ 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. Neutrophils were obtained from multiple different donors (*n* = 8); however, the cells from different donors were never pooled during experiments.

Ca²⁺ Mobilization Assay. Changes in intracellular Ca²⁺ ([Ca²⁺]_i) in neutrophils and HL-60 cells were measured with a FlexStation 3 scanning fluorometer (Molecular Devices, Sunnyvale, CA, USA) using fluorescent dye Fluo-4AM (Invitrogen, Carlsbad, CA, USA). Human neutrophils, wild-type HL-60 cells, or transfected HL-60 cells were suspended in HBSS, 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 aliquotted into the wells of flat-bottom, half-area well black microtiter plates (2 × 10⁵ cells/well). Essential oils or pure compounds diluted in DMSO were added to the wells (final concentration of DMSO was 1%), and changes in fluorescence were monitored (λ_{ex} = 485 nm, λ_{em} = 538 nm) every 5 s for 240 s at room temperature and normal atmospheric conditions immediately after an addition of the test compound/oil. The 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 was assigned a value of 100%. Curve fitting (at least five or 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, USA). To examine the inhibitory effects of PTX, FPR1-HL60 cells were treated with PTX (100 ng/mL) for 18 h.

Transient Ca²⁺ influx in HEK293 cells (transfected with TRPV1 and nontransfected cells) was evaluated as previously described.¹⁰ HEK293 cells were incubated in growth medium containing fura-2 (2 μM) for 30 min at 37 °C and 5% CO₂. Coverslips containing the fura-2-loaded HEK293 cells were mounted on the stage of an Olympus IX-81 inverted fluorescence microscope (Olympus America, Lake Success, NY, USA), and the cells were superfused continuously with serum-free

medium at a flow rate of 2 mL/min. Cells were exposed to compounds under investigation by switching from control medium to compound-containing medium for 10 s unless noted otherwise. Ca²⁺ influx measurements and data acquisition were simultaneously performed on multiple individual cells using a fluorescence imaging system (Easy Ratio Pro, Photon Technology International, Lawrenceville, NJ, USA) equipped with a multiwavelength spectrofluorometer (DeltaRAM X, Photon Technology International Inc.) and a QuantEM 512SC electron multiplying charge-coupled device camera (Photometrics, Tucson, AZ, USA), as previously described.¹⁰ Representative traces were acquired using an alternating excitation wavelength protocol (340, 380 nm/20 Hz) and emission wavelength of 510 nm.

Neutrophil Migration Assay. Human neutrophils were suspended in HBSS⁺ containing 2% (v/v) heat-inactivated FBS (2 × 10⁶ cells/mL), and cell migration was analyzed in 96-well ChemoTx chemotaxis chambers (Neuroprobe, Gaithersburg, MD, USA). In brief, neutrophils were pre-incubated with the indicated concentrations of the test sample (oil or pure compound) or 1% 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) FBS and the indicated concentrations of test sample, 1% DMSO as a 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, USA). Chemiluminescence was recorded using a Fluoroskan Ascent FL fluorescence microplate reader (Thermo Scientific, Rockford, IL, USA), and the signal was converted to absolute cell numbers by comparison of the values with standard curves obtained with known numbers of neutrophils. Curve fitting (at least six points) and calculation of median effective concentration values (IC₅₀) were performed by nonlinear regression analysis of the dose–response curves generated using Prism 5.

Compound Cytotoxicity. Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega), according to the manufacturer's protocol. Briefly, HL-60 cells were cultured at a density of 1 × 10⁵ cells/well with different concentrations of essential oil or compound (final concentration of DMSO was 1%) 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 Fluoroskan Ascent FL microplate reader.

Molecular Modeling. We used a ligand-based approach for molecular modeling employing field point methodology.¹¹ The structures of (–)-β-pinene, (–)-sabinene, and γ-terpinene in Tripos MOL2 format were imported into the FieldTemplater program (FieldTemplater version 2.0.1; Cresset Biomolecular Discovery Ltd., Hertfordshire, UK) for the construction of template A. Similarly, the structures of (–)-carveol, 6-*tert*-butyl-*m*-cresol, carvacrol, and thymol were imported to construct template B; and capsaicin, *N*-geranyl cyclopropylcarboxamide (NGCC), and (Z)-*N*-(2,7-dimethylocta-2,6-dienyl)-2-phenylacetamide (compound 2k)^{12,13} were imported to construct template C. Using built-in capabilities of the FieldTemplater software, representative sets of conformations for the compounds were generated corresponding to local energy minima calculated within the extended electron distribution force field.¹¹ To lower the number of rotatable bonds, “force amides trans” option was applied to the conformational search. For the generation of field point patterns, probe atoms having positive, negative, and zero charge were placed in the vicinity of a given conformation, and the energy of their interaction with the molecular field was calculated using the extended electron distribution parameter set. Positions of energy extrema for positive probes give “negative” field points, whereas energy extrema for negative and neutral probe atoms correspond to “positive” and steric field points, respectively. Hydrophobic field points were also generated with neutral probes capable of penetrating into the molecular core and reaching extrema in the centers of hydrophobic regions. A detailed description of the field point calculation procedure has been published

Table 1. Composition of the Volatile Compounds Identified in *F. akitschkensis* Essential Oils^a

compd no.	RRI	compd name	A (%)	B (%)	compd no.	RRI	compd name	A (%)	B (%)
1	1032	<i>α</i>-pinene^b	15.4	1.3	32	1661	<i>α</i> -himachalene ^c	0.3	0.7
2	1035	<i>α</i>-thujene^c	0.6		33	1662	<i>trans</i> -pinocarvyl acetate ^b		0.7
3	1076	camphene^b	0.1		34	1706	<i>α</i>-terpineol^b	0.1	
4	1118	<i>β</i>-pinene^b	8.5	1.6	35	1711	<i>γ</i> -himachalene ^c	0.3	0.9
5	1132	sabinene^b	58.7	1.4	36	1739	<i>β</i> -himachalene ^c	0.4	0.9
6	1174	myrcene^b	0.6		37	1743	eremophilene ^b	1.4	2.1
7	1188	<i>α</i>-terpinene^b	0.3		38	1755	dauca-8,11-diene ^c	0.1	
8	1203	limonene^b	0.4		39	1758	<i>cis</i> -piperitol ^b	0.1	
9	1218	<i>β</i> -phellandrene ^c	0.3		40	1771	(<i>E</i>)- <i>γ</i> -bisabolene ^b	tr	
10	1255	<i>γ</i>-terpinene^b	0.8	0.7	41	1783	<i>β</i> -sesquiphellandrene ^c	tr	
11	1266	(<i>E</i>)- <i>β</i>-ocimene^b	tr		42	1786	<i>ar</i> -curcumene ^c	tr	
12	1280	<i>p</i>-cymene^b	0.9		43	1792	<i>p</i> -mentha-1(7),5-dien-2-ol ^b	tr	
13	1290	terpinolene^b	0.2		44	1804	myrtenol^b	tr	
14	1384	<i>α</i>-pinene oxide^c	tr		45	1854	germacrene B ^b	0.1	
15	1474	<i>trans</i> -sabinene hydrate ^c	1.0	0.2	46	1864	<i>p</i>-cymen-8-ol^b	tr	
16	1482	longipinene ^b	0.1		47	1868	(<i>E</i>)- geranylacetone^b		4.0
17	1493	<i>α</i> -ylangene ^c	0.2		48	1888	<i>ar</i> -himachalene ^c	tr	
18	1504	daucene ^b	0.1		49	1941	<i>α</i> -calacorene ^b	tr	
19	1512	longicyclene ^b	tr		50	1985	<i>γ</i> -calacorene ^b	tr	
20	1548	(<i>E</i>)-2-nonenal ^c		0.3	51	2008	caryophyllene oxide^b		1.0
21	1549	<i>β</i> -cubebene ^b	0.2		52	2028	junenol ^c		0.4
22	1556	<i>cis</i> -sabinene hydrate ^c	0.6		53	2045	<i>β</i> -himachalene oxide ^c	tr	
23	1571	<i>trans</i> - <i>p</i> -menth-2-en-1-ol ^b	0.3		54	2066	<i>p</i> -mentha-1.4-dien-7-ol ^b	tr	
24	1586	pinocarvone ^c	tr		55	2180	6-epi-cubenol ^b	tr	
25	1594	<i>α</i> - <i>trans</i> - <i>β</i> -bergamotene ^c	tr		56	2245	elemicine^c		0.8
26	1604	isobornyl acetate^c		0.3	57	2254	2-himachalen-7-ol ^c	1.3	7.9
27	1611	terpinen-4-ol^b	3.9	1.6	58	2296	myristicin^b		67.9
28	1638	<i>cis</i> - <i>p</i> -menth-2-en-1-ol ^c	tr		59	2300	tricosane ^b		1.1
29	1645	(<i>Z</i>)- <i>β</i> -santalene ^b	0.1		60	2500	pentacosane ^b		0.5
30	1651	sabinaketone ^b	tr		61	2900	nonacosane ^b	0.1	
31	1653	<i>γ</i> -elemene ^b	tr		62	2931	hexadecanoic acid ^b	tr	

^aThe data are presented as relative % by weight for components of FAEO_{u/s} (A) and FAEO_{stm} (B). RRI, relative retention index calculated on the basis of retention of *n*-alkanes; %, calculated from flame ionization detector data. Trace amounts (tr) were present at <0.1%. ^bIdentification based on comparison with co-injected standards. ^cTentatively identified using Wiley and MassFinder mass spectra libraries and published RRI. Compounds selected for further biological screening are indicated in bold.

elsewhere.¹¹ A clique-matching algorithm with further simplex optimization was applied to obtain the conformations of three (templates A and C) or four molecules (for template B) giving good mutual overlays in terms of geometric and field similarity (*S*). The best overlays were taken as templates representative of the bioactive conformations.

The structures of geranylacetone and isobornyl acetate were imported into FieldAlign program (FieldAlign version 2.0.1; Cresset Biomolecular Discovery Ltd.) in Tripos MOL2 format. Conformational search and field point calculation were performed as described above for template building. Conformations with the best fit to the geometry and field points of template A (for geranylacetone and isobornyl acetate) and of template C (for geranylacetone) were identified, and their superimpositions were refined by the simplex optimization algorithm and visualized using instruments incorporated in FieldAlign.

Statistical Analysis. To calculate median effective concentration values (EC₅₀ or IC₅₀), curve fitting (at least five or six points) was performed by nonlinear regression analysis of the dose–response curves generated using Prism 7 (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) was performed on the data sets, followed by Tukey's pairwise comparisons. Pair-wise comparisons with differences at *P* < 0.05 were considered to be statistically significant.

RESULTS

Composition of the Essential Oils from *F. akitschkensis*. Essential oils were obtained by conventional hydrodistillation of the dried stems or umbels with seeds of *F. akitschkensis* (designated FAEO_{stm} and FAEO_{u/s}, respectively) and analyzed by GC and GC-MS simultaneously to determine their chemical compositions. Hydrodistillation of the umbels+seeds and stems produced 0.7 and 0.02% (v/w on the basis of the weight of dried material) essential oils, respectively. Table 1 summarizes the identified compounds, their retention times, and percentage composition.

Fifty-two compounds were identified in FAEO_{u/s}, representing around 98% of the total oil composition. The main components of the FAEO_{u/s} were sabinene (58.7%), *α*-pinene (15.4%), *β*-pinene (8.5%), terpinen-4-ol (3.9%), eremophilene (1.4%), 2-himachalen-7-ol (1.3%), and *trans*-sabinene hydrate (1.0%). Twenty-four compounds were present at concentrations from 0.1 to <0.1%. The remaining 21 volatile compounds were identified in trace amounts (<0.1%). Thus, monoterpene hydrocarbons dominated (85.7%) over all other compound groups. Among the other significant compounds, 14 oxygenated monoterpenes were present (6.0%), with terpinen-4-ol (3.9%) as the major representative. Small amounts of sesquiterpene hydrocarbons (20 compounds) and oxygenated

sesquiterpenes (3 compounds) were also present (3.3 and 1.3%, respectively), with eremophilene (1.4%) as the major sesquiterpene constituent. Only trace amounts of alkanes and fatty acids were detected in FAEO_{u/s}. Finally, we observed that FAEO_{u/s} contained a series of himachalene-type compounds (α -, β -, and γ -himachalene, *ar*-himachalene, β -himachalene oxide, and 2-himachalen-7-ol).

Gas chromatographic analysis of the volatiles on a Lipodex G chiral column revealed the existence of enantiomeric pairs of α -pinene, β -pinene, and sabinene in the FAEO_{u/s}. We found (1S)-(-)- α -pinene (95%), (1S)-(-)- β -pinene (94%), and (1R,5R)-(+)-sabinene (97%).

Twenty-one compounds were identified in FAEO_{stm}, representing 96.6% of the total oil composition. FAEO_{stm} was distinguished by a high percentage of phenylpropanoids, such as myristicin (67.9%) and elemicine (0.8%). Mono- and sesquiterpenoids were detected in the oil in small amounts (5.0 and 4.6%, respectively), although oxygenated sesquiterpenes were present in higher amounts (9.3%) than in FAEO_{u/s}. Interestingly, six himachalene-type sesquiterpenoids were present in relatively high amounts (10.4%).

Effect of the Essential Oils on Ca²⁺ Mobilization in Neutrophils. Essential oils and their components have been reported previously to modulate [Ca²⁺]_i levels in neutrophils and other cells.⁹ We found that FAEO_{stm} did not activate human neutrophil Ca²⁺ flux, whereas FAEO_{u/s} stimulated the cells with an EC₅₀ of 16.5 μ g/mL (Table 2). As shown in Figure 1, the kinetics of the [Ca²⁺]_i response activated by 50 μ g/mL of FAEO_{u/s} resembled the response stimulated by our positive control, fMLF, which is an agonist of FPR1, a chemotactic receptor expressed on neutrophils and other leukocytes.¹⁴ Treatment with fMLF or FAEO_{u/s} caused a rapid increase in [Ca²⁺]_i that peaked by ~40 s and gradually declined to basal levels, reflecting clearance of Ca²⁺ from the cytosol. Because essential oils and individual test compounds were suspended in DMSO, we also evaluated the effects of DMSO on our assay system and confirmed that 1% DMSO did not induce a [Ca²⁺]_i flux in human neutrophils (Figure 1).

Effect of Essential Oil Components on Ca²⁺ Mobilization in Neutrophils. Because FAEO_{u/s} activated Ca²⁺ flux in human neutrophils, we focused on analysis of the effects of its constituents to possibly identify the active compound(s). Sixteen commercially available compounds, including 4 of the major compounds (sabinene, α -pinene, β -pinene, and terpinen-4-ol) and 12 of the minor compounds, were tested. All tested compounds, representing >89.5% of the FAEO_{u/s} composition, were monoterpenes or their oxygenated forms. The terpenes were first evaluated for agonist effects on [Ca²⁺]_i in human neutrophils. Consistent with our previous studies,⁹ we found that β -pinene and sabinene can directly activate neutrophil Ca²⁺ flux. In addition, we found that γ -terpinene, a minor component of FAEO_{u/s}, also had agonist activity (Table 2). Thus, these three compounds, representing 68.0% of FAEO_{u/s}, directly activated a neutrophil [Ca²⁺]_i flux similarly to FAEO_{u/s} itself.

Although FAEO_{stm} did not stimulate Ca²⁺ flux in neutrophils, we also evaluated several of its components that were not present in FAEO_{u/s}. The commercially available compounds included myristicin, isobornyl acetate, elemicine, (*E*)-2-nonenal, (*E/Z*)-geranylacetone, and caryophyllene oxide. These compounds, together with the above tested compounds that were included in both oils, represented 80.9% of the FAEO_{stm} composition. In addition to the active compounds shared by FAEO_{stm} and FAEO_{u/s}, we found that 2-nonenal,

Table 2. Effect of *F. akitschkensis* Essential Oils and Selected Components on Ca²⁺ Flux in Human Neutrophils and HL-60 Cells

essential oil	EC ₅₀ ^a μ g/mL (efficacy, %)	
	neutrophils	HL-60 cells
FAEO _{u/s}	16.5 \pm 3.6 (90)	5.0 \pm 1.2
FAEO _{stm}	NA ^b	NA
compound	EC ₅₀ μ M (efficacy, %)	
camphene	NA	NA
caryophyllene oxide	NA	NA
<i>p</i> -cymen-8-ol	NA	NA
<i>p</i> -cymene	NA	NA
elemicine	NA	NA
(<i>E/Z</i>)-geranylacetone	7.6 \pm 1.9 (105)	6.5 \pm 1.9
isobornyl acetate	6.4 \pm 1.7 (100)	4.3 \pm 0.9
myrcene	NA	NA
α -terpinene	NA	NA
(<i>R</i>)-(+)-limonene	NA	NA
(<i>S</i>)-(-)-limonene	NA	NA
myristicin	NA	NA
myrtenol	NA	NA
(<i>E</i>)-2-nonenal	33.7 \pm 4.7 (70)	31.3 \pm 4.2
(<i>E/Z</i>)- β -ocimene	NA	NA
(\pm)- α -pinene	NA	NA
(1S)-(-)- β -pinene	24.6 \pm 3.4 (70)	16.4 \pm 2.4
α -pinene oxide	NA	NA
(\pm)-sabinene	49.4 \pm 6.3 (55)	22.7 \pm 3.5
terpinen-4-ol	NA	NA
γ -terpinene	23.2 \pm 3.5 (30)	9.3 \pm 1.6
α -terpineol	NA	NA
terpinolene	NA	NA

^aEC₅₀ values are presented as the mean \pm SD of three independent experiments, and efficacy (in parentheses) is expressed as % of the response induced by a positive control (5 nM fMLF). ^bNA, no activity was observed, even at the highest concentration tested (50 μ M).

isobornyl acetate, and geranylacetone also stimulated neutrophil Ca²⁺ flux (Table 2). Thus, the active constituents with agonist potential represent 8.3% of the FAEO_{stm} composition. Chemical structures of the active compounds identified in FAEO_{u/s} and FAEO_{stm} are shown in Figure 3. 2-Nonenal is a known product of lipid peroxidation with a broad spectrum of biological properties (e.g., see ref 15). Because this bioactive aldehyde can easily conjugate with proteins and was previously reported to activate phospholipase C and stimulate neutrophils,¹⁵ we did not further evaluate properties of this minor oil component.

Myristicin, a major component of FAEO_{stm}, was reported previously to inhibit [Ca²⁺]_i flux in murine RAW 264.7 macrophages.¹⁶ Therefore, we tested if myristicin also inhibited [Ca²⁺]_i flux in human neutrophils activated by some of the active components, including β -pinene, sabinene, isobornyl acetate, and geranylacetone, and found that myristicin dose-dependently inhibited [Ca²⁺]_i flux induced by these compounds, indicating that myristicin can suppress agonist effects of other components. As an example, Figure 2A shows myristicin inhibition of the neutrophil [Ca²⁺]_i flux induced by geranylacetone and isobornyl acetate.

Because it was apparent that FAEO_{stm} contained both agonist and antagonist compounds, we evaluated a mixture of the major agonist component of FAEO_{stm} (geranylacetone; 4% by weight of the oil, molecular weight 194.3 Da) with the major

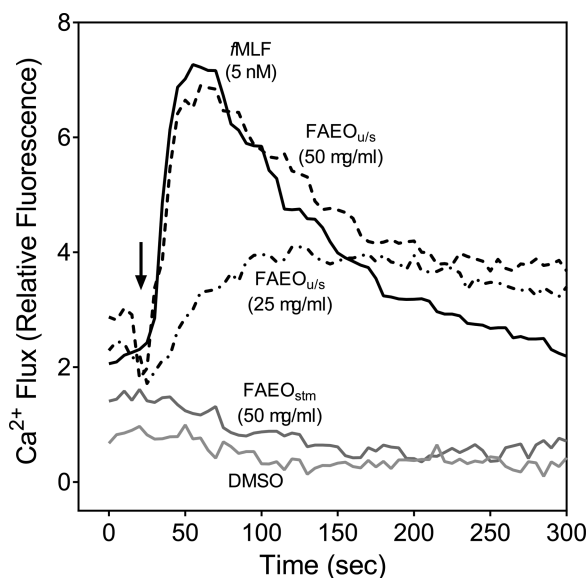


Figure 1. Effect of *Ferula* essential oils on Ca^{2+} mobilization in human neutrophils. Human neutrophils were treated with the indicated concentrations of FAEO_{u/s}, FAEO_{stm}, and *f*MLF (positive control) or 1% DMSO (negative control), and $[\text{Ca}^{2+}]_i$ flux was monitored for the indicated times (arrow indicates when treatment was added). The data are from one experiment that is representative of three independent experiments.

antagonist compound (myristicin, 67.9% by weight of the oil, molecular weight 192.2 Da) at a molar ratio of 1:17, which corresponds approximately to the percentage ratio of these constituents in the original oil. Treatment of human neutrophils with this mixture at a final concentration of geranylacetone equal to its EC₅₀ value (7.6 μM) did not activate $[\text{Ca}^{2+}]_i$ flux (data not shown). Thus, these findings explain why FAEO_{stm}, which contains neutrophil-activating compounds, appeared to be inactive. Indeed, myristicin also inhibited *f*MLF- and IL-8-induced $[\text{Ca}^{2+}]_i$ mobilization in neutrophils (Figure 2B) with IC₅₀ values of 5.4 and 17.3 μM , respectively. These data indicate that myristicin can modulate intracellular signaling pathways that are common to both FPR1 and CXCR1/2 chemokine receptors.

Effect of the *Ferula* Essential Oils and Components on Neutrophil Migration and Cell Viability. Various essential oils and their components have been reported previously to inhibit leukocyte migration (e.g., see ref 17). We found that pretreatment with FAEO_{u/s} for 30 min dose-dependently attenuated *f*MLF-induced chemotaxis in human neutrophils, with an IC₅₀ of 10.1 $\mu\text{g}/\text{mL}$, but FAEO_{stm} and myristicin had low activity in this assay. Even at the highest concentrations tested, FAEO_{stm} (100 $\mu\text{g}/\text{mL}$) and myristicin (100 μM) inhibited cell migration only by 20–30%. Other components of the oils, including β -pinene, sabinene, γ -terpinene, isobornyl acetate, and geranylacetone, were also evaluated for their effects on *f*MLF-induced cell migration. In agreement with our previous studies on *Artemisia* essential oils,⁹ we found that pretreatment with β -pinene and sabinene inhibited neutrophil migration with IC₅₀ values of 23.9 and 39.1 μM , respectively. Likewise, γ -terpinene, isobornyl acetate, and geranylacetone also inhibited *f*MLF-induced neutrophil migration with IC₅₀ values of 32.5, 47.5, and 37.2 μM , respectively. As examples, Figure 4 shows the effects of FAEO_{u/s}, γ -terpinene, and geranylacetone on *f*MLF-induced neutrophil migration.

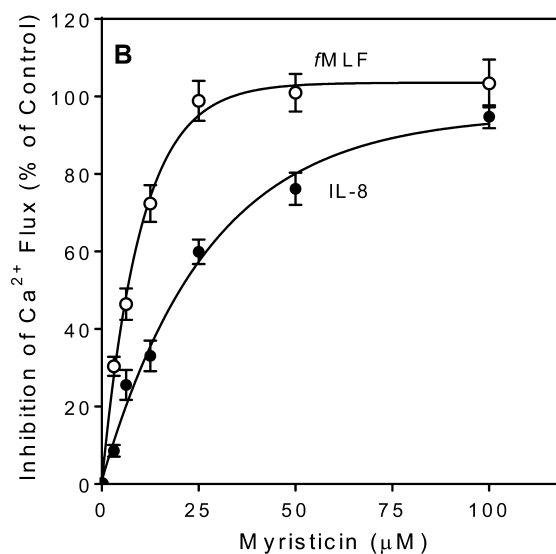
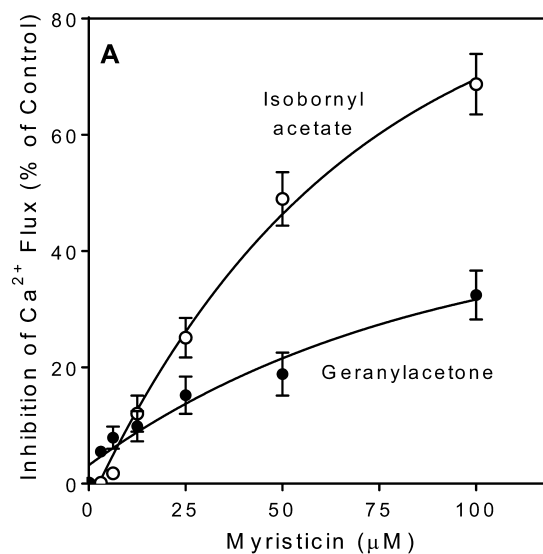


Figure 2. Effect of myristicin on Ca^{2+} mobilization in human neutrophils. (A) Human neutrophils were pre-incubated with the indicated concentrations of myristicin for 5 min and then stimulated with 50 μM isobornyl acetate (○) or geranylacetone (●). (B) Neutrophils were pre-incubated with the indicated concentrations of myristicin for 5 min and then stimulated with 5 nM *f*MLF (○) or 25 nM of IL-8 (●). For panels A and B, the response induced by an agonist alone in neutrophils that were pre-incubated for 5 min with control 1% DMSO was assigned a value of 100%. Values are the mean \pm SD of triplicate samples from one experiment that is representative of three independent experiments.

To ensure that the results on inhibition of neutrophil migration were not influenced by possible compound toxicity, we evaluated the cytotoxicity of sabinene, β -pinene, γ -terpinene, isobornyl acetate, geranylacetone, and myristicin at various concentrations (up to 50 μM) in undifferentiated HL-60 cells during a 4 h incubation. This incubation period is consistent with the relatively short time used to measure cell migration (up to 90 min). All compounds tested did not affect cell viability, thereby verifying that they are not cytotoxic, at least during the 4 h incubation period (data not shown).

Desensitization of Neutrophils by Essential Oil Active Components. Activation of Ca^{2+} channels, specific receptors, or other unidentified molecular targets by agonists can result in

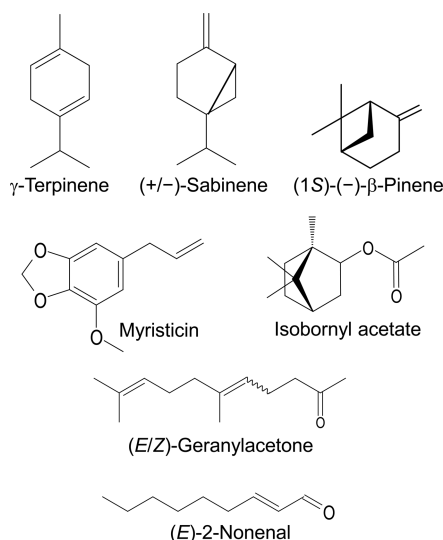


Figure 3. Chemical structures of the compounds evaluated for biological activity. For sabinene and geranylacetone, mixtures of stereoisomers were studied; general structures are shown for these compounds.

their desensitization and subsequent down-regulation of neutrophil responses.¹⁸ To evaluate this issue, we examined whether neutrophil pretreatment with the active components of *Ferula* essential oils could cross-desensitize the neutrophil response to *f*MLF and IL-8. After pretreatment with various concentrations of FAEO_{u/s}, β -pinene, sabinene, γ -terpinene, isobornyl acetate, or geranylacetone, neutrophils were treated with *f*MLF or IL-8, and $[Ca^{2+}]_i$ flux was evaluated. We found that FAEO_{u/s} and the individual compounds tested all attenuated *f*MLF- and IL-8-induced $[Ca^{2+}]_i$ flux in neutrophils. As examples, Figure 5 shows that pretreatment with FAEO_{u/s}, sabinene, isobornyl acetate, and geranylacetone markedly attenuated neutrophil $[Ca^{2+}]_i$ mobilization induced by 5 nM *f*MLF or 25 nM IL-8, indicating that these treatments did indeed cross-desensitize the cells to subsequent agonist activation.

Elucidation of Molecular Targets/Pathways for *Ferula* Essential Oil and Active Components. Human neutrophils and undifferentiated human HL-60 cells express a different spectrum of receptors and ion channels¹⁹ and can be evaluated in comparison to identify ligand targets. Thus, we tested if the active oil compounds could also stimulate $[Ca^{2+}]_i$ flux in HL-60 cells. Similar to their effects in neutrophils, the active essential oil (FAEO_{u/s}), β -pinene, sabinene, γ -terpinene, isobornyl acetate, and geranylacetone all stimulated a $[Ca^{2+}]_i$ flux in HL-60 cells, with EC₅₀ values in the low/intermediate micromolar range (Table 2).

On the basis of the similarity of the $[Ca^{2+}]_i$ flux kinetics in neutrophils treated with *f*MLF and FAEO_{u/s}, we considered whether FPR1 and/or FPR2 might be involved in the response to *Ferula* essential oils or its components because these receptors are known to be highly promiscuous.¹⁴ Although undifferentiated HL-60 cells do not express FPRs, these cells can be transfected to express either human FPR1 (FPR1-HL60 cells) or FPR2 (FPR2-HL60 cells), and receptor-specific antagonists can be used to assess their involvement in a given response.²⁰ Whereas $[Ca^{2+}]_i$ flux was induced in FPR1-HL60 and FPR2-HL60 cells treated with FAEO_{u/s}, β -pinene, sabinene, γ -terpinene, isobornyl acetate, or geranylacetone,

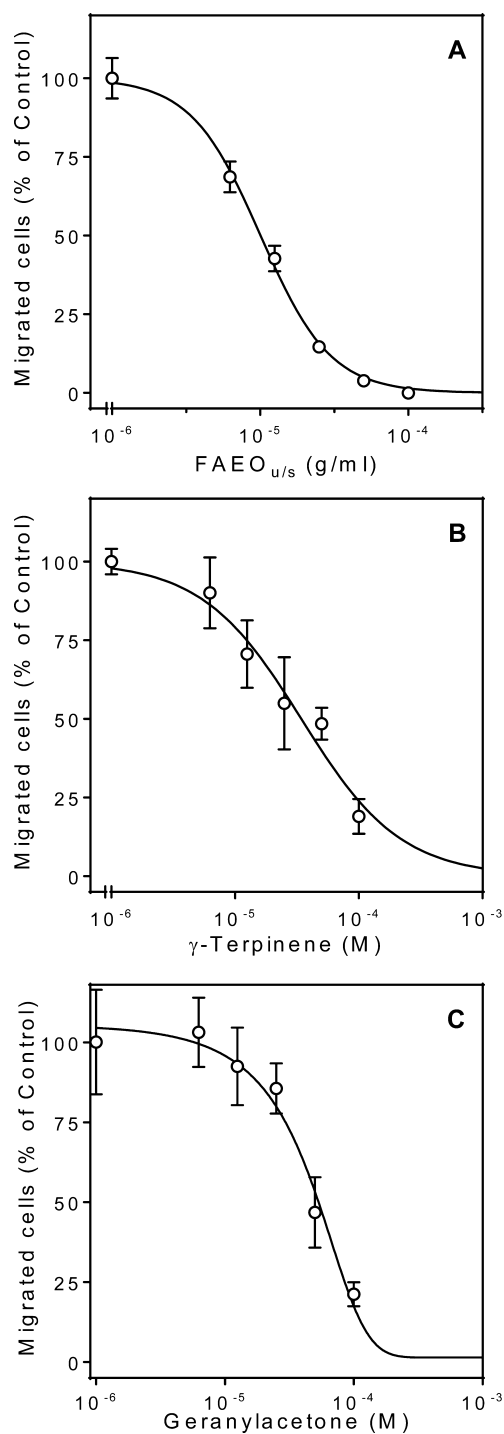


Figure 4. Inhibition of human neutrophil migration by FAEO_{u/s} and selected components. Human neutrophils were pre-incubated with the indicated concentrations of FAEO_{u/s} (A), γ -terpinene (B), and geranylacetone (C) for 30 min, and migration toward 1 nM *f*MLF was analyzed, as described. In each experiment, positive control cells were pretreated with 1% DMSO, and cell migration toward 1 nM *f*MLF was analyzed. The results are expressed as % of positive control (1 nM *f*MLF). The background level of cell migration was 3258 ± 1809 cells/well, and migration toward control *f*MLF resulted in 15,946 ± 1421 cells/well. Values are the mean ± SD of triplicate samples from one experiment that is representative of three independent experiments.

this response was not inhibited by pretreatment of the cells with the specific FPR1 and FPR2 antagonists, cyclosporin H

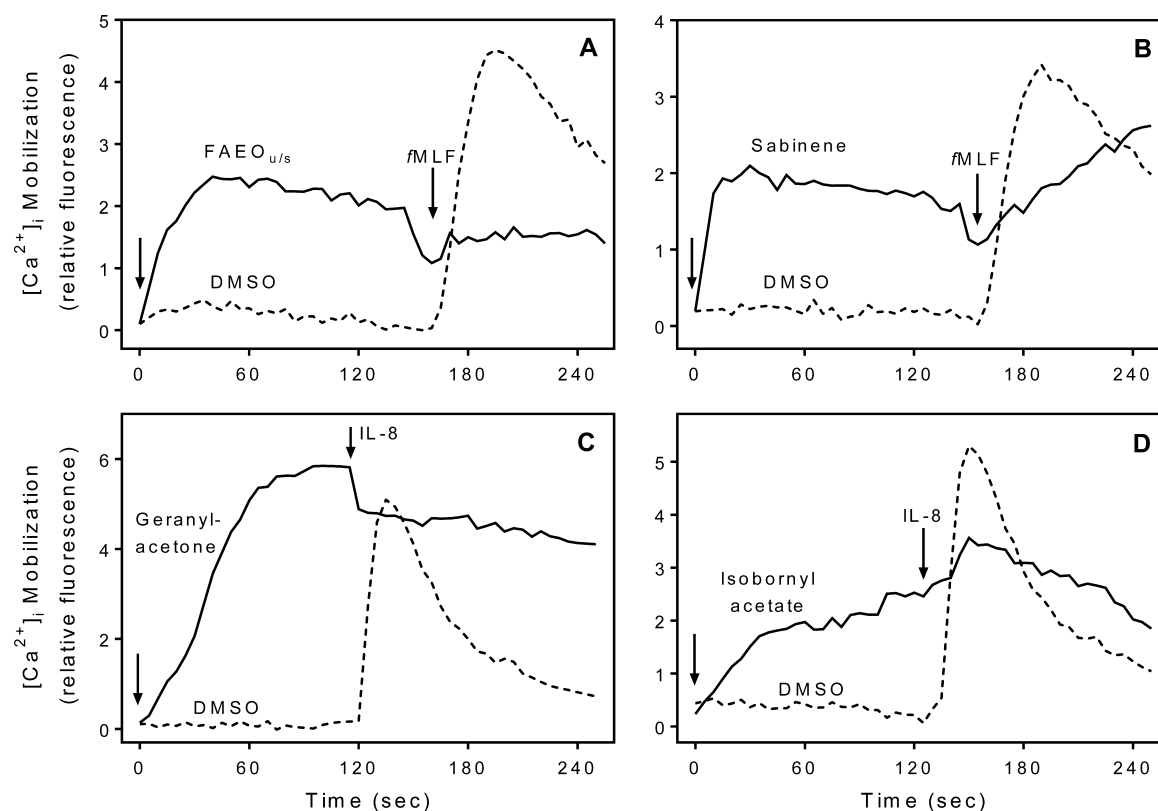


Figure 5. Effect of selected compounds on *f*MLF- and IL-8-induced $[Ca^{2+}]_i$ mobilization. Human neutrophils were loaded with calcium dye and pretreated with 50 $\mu\text{g}/\text{mL}$ FAEO_{u/s} (A), 50 μM sabinene (B), 50 μM geranylacetone (C), 50 μM isobornyl acetate (D), or 1% DMSO (A–D) followed by a second addition of 5 nM *f*MLF (A, B) or 25 nM IL-8 (C, D). $[Ca^{2+}]_i$ mobilization was monitored, as described, and the data are presented as relative fluorescence intensity. Data from two representative experiments are shown.

Table 3. Effect of TRP Channel Blockers and a Phospholipase C Inhibitor on $[Ca^{2+}]_i$ Flux in Human Neutrophils Treated with FAEO_{u/s} and Active Components^a

essential oil/compound	IC ₅₀ (μM)				
	2-APB	ACA	capsazepine	SKF 96365	U73122
FAEO _{u/s}	12.6 \pm 2.3	9.4 \pm 2.4	27.4 \pm 3.4	23.6 \pm 4.1	NA
β -pinene	13.7 \pm 2.5	8.0 \pm 1.6	27.2 \pm 3.1	17.8 \pm 2.1	NA
sabinene	24.4 \pm 2.6	17.0 \pm 3.1	32.7 \pm 3.5	25.4 \pm 3.8	NA
γ -terpinene	18.6 \pm 2.9	6.1 \pm 1.8	22.6 \pm 2.4	15.6 \pm 2.2	NA
isobornyl acetate	NA	8.9 \pm 1.1	NA	26.7 \pm 3.6	NA
geranylacetone	39.5 \pm 4.3	22.5 \pm 2.8	14.2 \pm 3.1	17.3 \pm 3.1	NA

^aNeutrophils were pre-incubated with various concentrations of the indicated TRP inhibitors (up to 50 μM) for 10 min. The cells were stimulated with 25 $\mu\text{g}/\text{mL}$ FAEO_{u/s} or the indicated compound (100 μM), and Ca^{2+} flux was measured, as described. NA, no activity was observed, even at the highest concentration tested (50 μM).

and WRW4, respectively (data not shown). In control experiments, cyclosporin H and WRW4 inhibited *f*MLF- and WKYMVM-induced Ca^{2+} flux in FPR1-HL60 and FPR2-HL60 cells, respectively (data not shown). Thus, these results indicate that FAEO_{u/s} and the active constituents of FAEO_{stm} and FAEO_{u/s} stimulated molecular targets other than FPR1/FPR2.

Various constituents of essential oils (e.g., monoterpenes) have been previously reported to be agonists/antagonists of temperature-sensitive transient receptor potential (TRP) channels TRPA1, TRPV1/3, and TRPM8.²¹ Thus, we evaluated the effects of TRP channel blockers, including 2-APB, ACA, capsazepine, and SKF 96365 (reviewed in ref 22), on neutrophil $[Ca^{2+}]_i$ flux stimulated by FAEO_{u/s} and the active *Ferula* essential oil components. As shown in Table 3, most of the TRP blockers tested dose-dependently inhibited neutrophil

$[Ca^{2+}]_i$ flux induced by these treatments, suggesting that TRPs may be at least one of the cellular targets for these compounds. Indeed, several essential oil-derived monoterpenes have been previously reported as TRP agonists, including thymol, menthol, carvacrol, geraniol, (\pm)-camphor, (–)-carvone, (–)-fenchone, and capsaicin (Table 4).^{23,24} Evaluation of these compounds in neutrophils showed that the mixed TRPA1/TRPV3/TRPM8 agonists thymol and menthol activated $[Ca^{2+}]_i$ flux with EC₅₀ values in the midmicromolar range, whereas the other tested monoterpenes were inactive. Furthermore, the TRPV1 agonist capsaicin also stimulated $[Ca^{2+}]_i$ flux in human neutrophils (Table 4), which is consistent with previous studies.²⁵ Together, these results suggest a role of TRP channels in neutrophil responses to essential oil-derived

Table 4. Reported Effects of Essential Oil Components and Capsaicin on TRP Channels^{23,24} and Evaluation of Their Effects on $[Ca^{2+}]_i$ Flux in Human Neutrophils

compound	previously reported effects on TRP channels	$[Ca^{2+}]_i$ flux in neutrophils ^a EC ₅₀ (μ M)
carvacrol	TRPA1, TRPV3, and TRPM8 agonist; TRPM7 antagonist	NA
thymol	TRPA1, TRPV3, and TRPM8 agonist	29.5 \pm 3.4
geraniol	TRPV3 and TRPM8 agonist	NA
(-)-menthol	TRPA1, TRPV3, and TRPM8 agonist	30.8 \pm 3.6
(\pm)-camphor	TRPV1/3 and TRPM8 agonist; TRPA1 antagonist	NA
(-)-carvone	TRPV1/3 agonist	NA
(-)-fenchone	TRPV3 agonist	NA
capsaicin	TRPV1 agonist	17.5 \pm 2.1

^aEvaluated as described under Materials and Methods.

monoterpenes, such as those found in essential oils from *Ferula* and other plant species.

On the basis of the results of our TRP channel inhibitor studies (Table 4) and the structural similarity of geranylacetone to capsaicin^{12,13} (see below), we evaluated the effects of the neutrophil-activating compounds (sabinene, β -pinene, γ -terpinene, geranylacetone, and isobornyl acetate) on transient Ca^{2+} influx in HEK293 cells transfected with TRPV1. Interestingly, only geranylacetone dose-dependently induced a transient Ca^{2+} influx in TRPV1-HEK293 cells (Figure 6A), whereas sabinene, γ -terpinene, and isobornyl acetate had no effect, and β -pinene induced only a small Ca^{2+} influx at the highest concentration tested (100 μ M) (data not shown). Importantly, geranylacetone did not induce a Ca^{2+} influx in nontransfected HEK293 cells at all tested concentrations (1, 10, and 100 μ M) (Figure 6B), demonstrating the specific requirement of TRPV1 for this response. Furthermore, repetitive application of 10 μ M geranylacetone desensitized TRPV1, and no response to capsaicin was observed post desensitization (Figure 6C).

We also evaluated the effect of myristicin on Ca^{2+} influx in TRPV1-HEK293 cells and found that it dose-dependently inhibited the capsaicin-induced a transient Ca^{2+} influx in TRPV1 expressing cells (Figure 7). Additionally, myristicin did not induce Ca^{2+} influx in TRPV1-HEK293 cells.

Although limonene, *p*-cymene, α -pinene, α -pinene oxide, and α -terpineol are components of FAEO_{u/s} and/or FAEO_{stm} (see Table 1) and have previously been reported to weakly activate TRPV3-transfected HEK293 cells, as determined by patch-clamp techniques,²³ these monoterpenes did not activate neutrophil $[Ca^{2+}]_i$ flux (Table 2). In addition, one of the most potent TRPV3 agonists identified, carvacrol (EC₅₀ = 490 nM in TRPV3-transfected HEK293 cells),²³ was also inactive in human neutrophils (Table 4), indicating that TRPV3 is likely not a target for *Ferula* essential oils/components in human neutrophils.

It should be noted that the TRP blocker 2-APB is also an inositol triphosphate (IP₃) receptor antagonist.²⁶ To evaluate the involvement of IP₃, the phospholipase C inhibitor U73122 was used to inhibit IP₃ production. In agreement with previous studies (e.g., ref 27), U73122 dose-dependently inhibited the fMLF-induced $[Ca^{2+}]_i$ flux. In contrast, the neutrophil $[Ca^{2+}]_i$ flux stimulated by FAEO_{u/s} or its active components was not affected by U73122 at concentrations up to 50 μ M (Table 3),

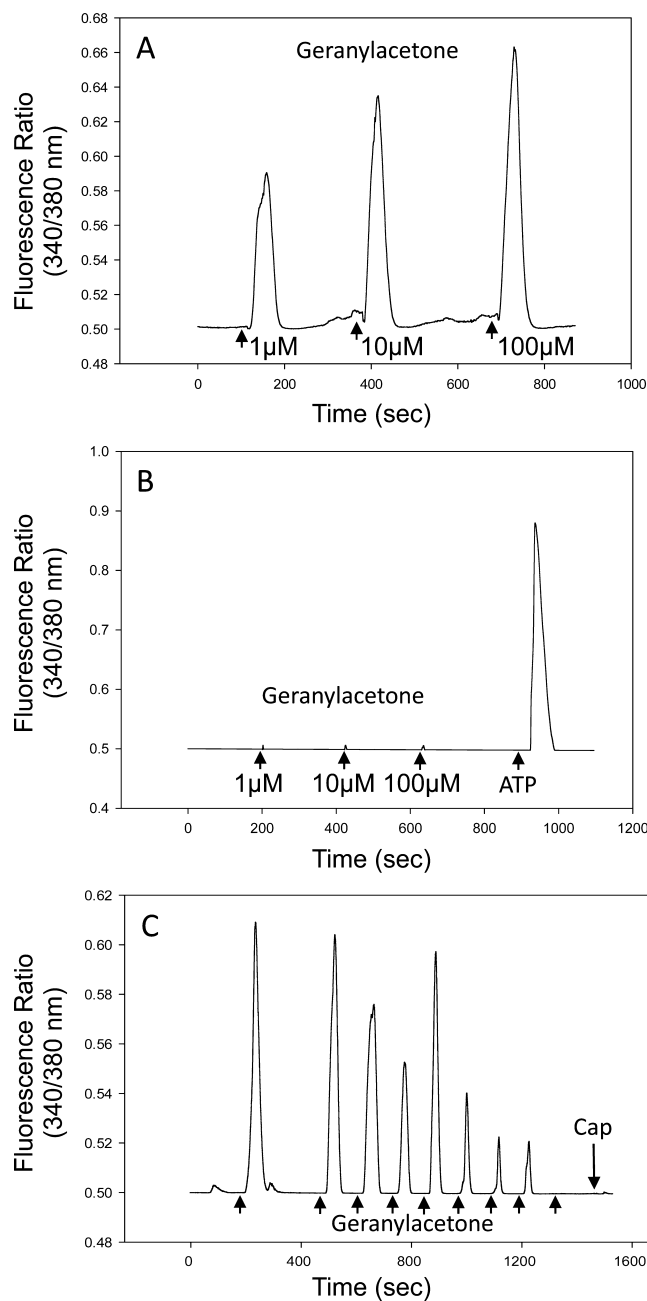


Figure 6. Activation of TRPV1-dependent Ca^{2+} influx by geranylacetone. TRPV1-HEK293 cells (A) and nontransfected HEK293 cells (B) were treated with the indicated concentrations of geranylacetone, and transient Ca^{2+} influx was monitored, as described. In panel B, cells were treated with 10 μ M ATP as a positive control to verify receptor activity. (C) TRPV1-HEK293 cells were treated with repetitive applications of 10 μ M geranylacetone at the indicated times (arrows) to evaluate response desensitization. After full desensitization by geranylacetone, the cells were treated with 100 nM capsaicin (Cap) to evaluate the post-desensitization response. Data from three representative experiments are shown.

indicating that the phospholipase C pathway is not involved in the neutrophil response to these compounds.

Activation of TRP channels can be mediated by $G_{i/o}$ proteins (e.g., ref 28). Although we did not find a direct involvement of FPR1/FPR2 in $[Ca^{2+}]_i$ flux stimulated by the active compounds, TRP channels could be activated by other $G_{i/o}$ -coupled GPCR. PTX, a G_i inhibitor, was used to assess whether

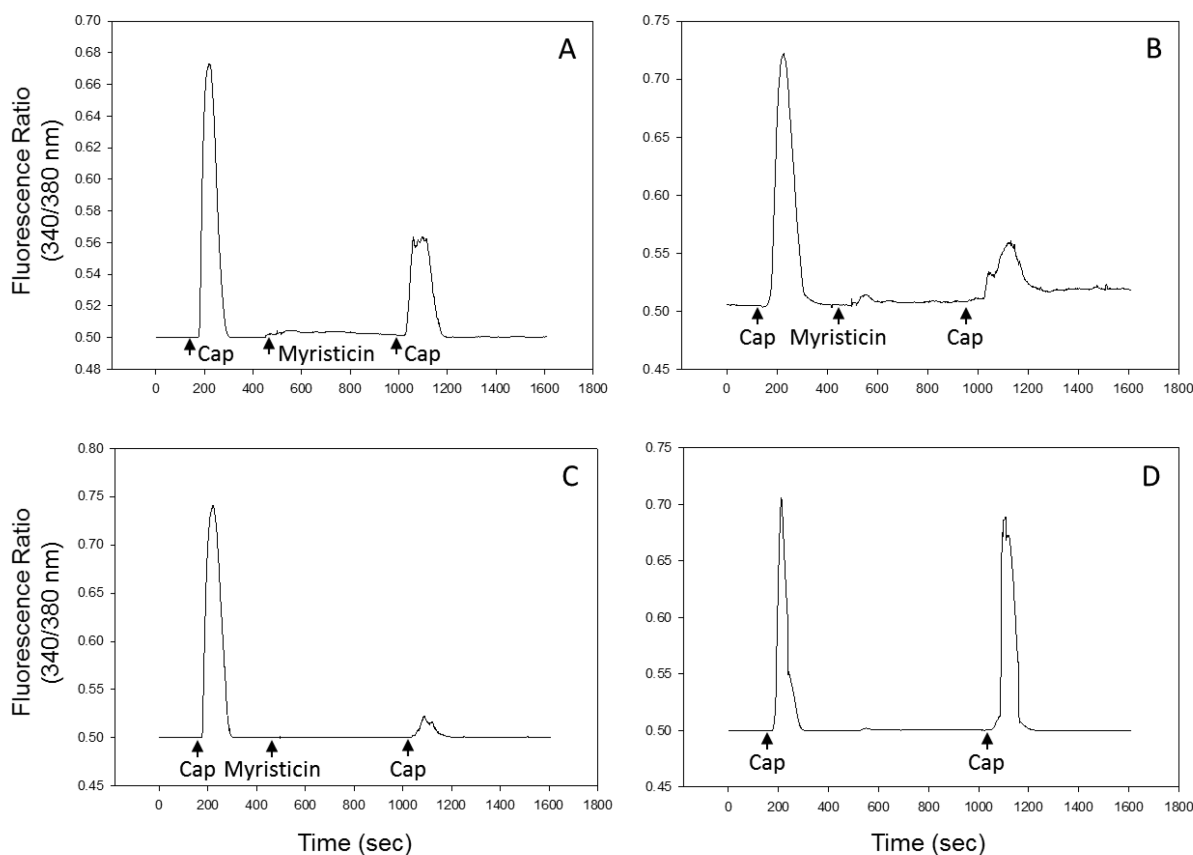


Figure 7. Inhibition of capsaicin induced Ca^{2+} influx in TRPV1-HEK293 cells by myristicin. TRPV1-HEK293 cells were treated with 100 nM capsaicin (Cap) to establish the basal transient Ca^{2+} influx response. The cells were then treated with 1 μM (A), 10 μM (B), and 100 μM (C) myristicin followed by 100 nM capsaicin (Cap) to evaluate response desensitization, as described. (D) To confirm that the initial application of capsaicin did not desensitize TRPV1, cells were treated with 100 nM capsaicin (Cap) followed by a second treatment of 100 nM capsaicin (Cap) at the same interval that was used in panels A–C. Data from three representative experiments are shown.

these G-proteins were involved in the $[\text{Ca}^{2+}]_i$ mobilization stimulated by $\text{FAEO}_{u/s}$ and essential oil components. Although the $[\text{Ca}^{2+}]_i$ flux induced by *fMLF* in FPR1-HL60 transfected cells was significantly inhibited by PTX, as expected, no inhibition was found in PTX-treated cells activated with $\text{FAEO}_{u/s}$, β -pinene, sabinene, γ -terpinene, isobornyl acetate, or geranylacetone (data not shown). Thus, neutrophil activation by *Ferula* essential oils also does not appear to involve G_i -coupled GPCRs.

Pharmacophore Modeling. Molecular docking experiments and virtual screening can be helpful in defining or confirming the molecular target of an active molecule. However, crystal structures for only 2 (TRPV1 and TRPA1) of the 28 known TRP members have been resolved.^{29,30} In the absence of a defined target structure, ligand-based methods for compound evaluation can be utilized. Here, we evaluated molecular conformations of the active components in *Ferula* essential oils [$(-)$ - β -pinene, $(-)$ -sabinene, γ -terpinene, geranylacetone, and isobornyl acetate] using the FieldTemplater program¹¹ to try to develop a pharmacophore template with optimal alignment of the molecules. This software is based on field point methodology and performs superimposition of compounds with the best volume overlap and coincidence of molecular fields. We found that only three of these compounds [$(-)$ - β -pinene, $(-)$ -sabinene, and γ -terpinene] could be overlaid simultaneously with good correspondence of their molecular fields (Figure 8A), suggesting that they likely have

cellular targets different from those of geranylacetone and isobornyl acetate.

Although molecular templates are usually represented as sets of field points, we also analyzed shapes of the molecular fields scanned by negative, positive, and neutral probe atoms around the template and in the vicinity of individual compounds. Template A has two small areas of negative field and a large central hydrophobic region, which is consistent with the hydrocarbon nature of the compounds involved in this model (Figure 8B). This result indicates that $(-)$ - β -pinene, $(-)$ -sabinene, and γ -terpinene are likely recognized by the same or a similar molecular target, such as one of the TRP channel members.

Although a comparative analysis of the pharmacophore features of template A and all 28 known TRP channel members lies out of scope of the present work, we created two pharmacophore models on the basis of previous studies showing that essential oil-derived compounds targeted TRPV3/TRPM8, as well as our biological results implicating TRPV1. The molecular conformations of $(-)$ -carveol, 6-*tert*-butyl-*m*-cresol, carvacrol, and thymol were used for the model of TRPV3/TRPM8 agonists,²⁵ whereas conformations of capsaicin, *N*-geranyl cyclopropylcarboxamide (NGCC),¹² and compound **2k**¹³ were used for the model of TRPV1 agonists.

In the four-molecule pharmacophore model of TRPV3/TRPM8 channel agonists (template B), the molecules overlap by four methyl groups and hydrophobic isopropyl, isopropenyl, and *tert*-butyl substituents. Their hydroxyl groups are all

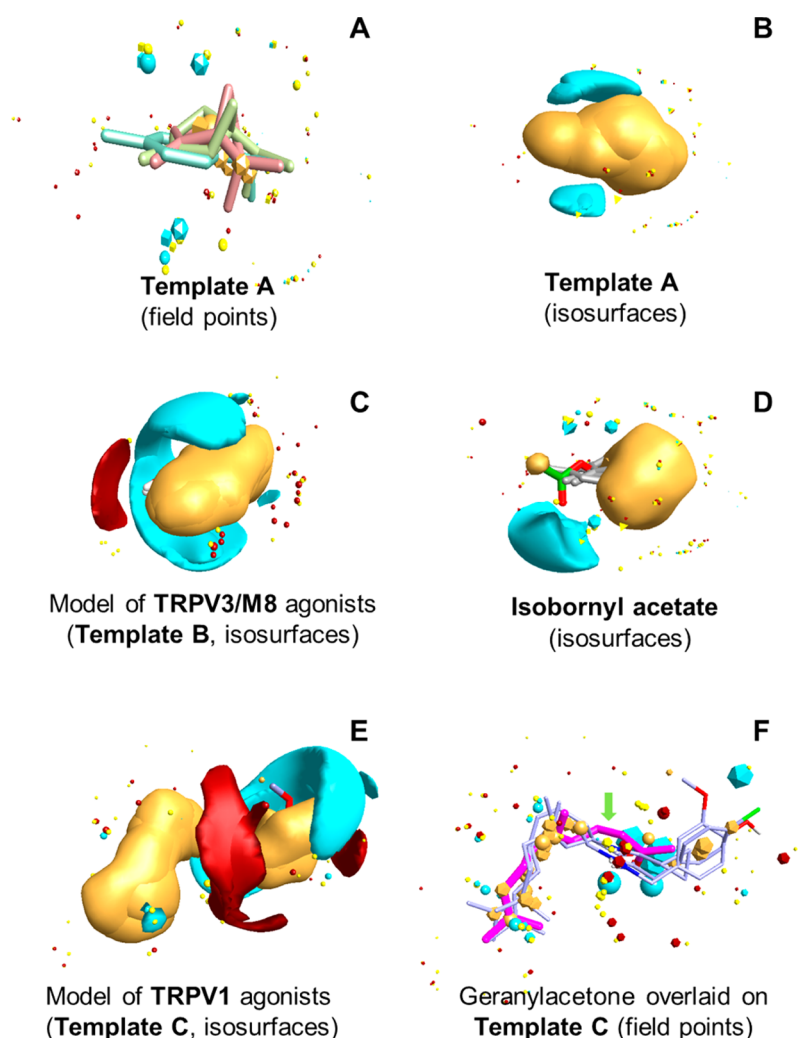


Figure 8. Pharmacophore modeling based on active constituents of essential oils and known agonists of TRP channels: (A) three-molecule template A created by alignment of (–)- β -pinene (magenta), (–)-sabinene (light-green), and γ -terpinene (light-blue); (B) field isosurfaces for template A; (C) field isosurfaces for the four-molecule pharmacophore model of TRPV3/TRPM8 agonists, which includes carveol, 6-*tert*-butyl-*m*-cresol, carvacrol, and thymol (template B); (D) field isosurfaces for the best alignment of isobornyl acetate onto template A; (E) field isosurfaces for the three-molecule pharmacophore model of TRPV1 agonists, which includes capsaicin, NGCC, and compound **2k** (template C); (F) geranylacetone (magenta) overlaid on the pharmacophore model of TRPV1 agonists. In all panels, negative, positive, hydrophobic, and van der Waals field points and corresponding isosurfaces are colored blue, red, orange, and yellow, respectively (van der Waals isosurfaces on the panels are not shown). The field isosurfaces were calculated at a threshold of 3 kcal/mol.

oriented in the same direction, forming a prolonged isosurface of negative field embracing the central hydrophobic region (Figure 8C). Template B also includes a small area of positive field near sterically nonhindered hydroxyl hydrogens. Its location, shown in red in Figure 8C, may correspond to H-bond acceptor(s) in TRPV3/TRPM8 channels. The substantial differences between templates A and B indicate that (–)- β -pinene, (–)-sabinene, and γ -terpinene target TRP channels other than TRPV3/TRPM8, which is consistent with our studies above suggesting that TRPV3 is likely not a relevant target for *Ferula* essential oils.

Consistent with the differences observed in biological activities, geranylacetone and isobornyl acetate had relatively low similarity with template A ($S = 0.591$ and 0.625 , respectively) and template B ($S = 0.621$ and 0.622 , respectively) in their best alignments determined by the FieldAlign program. Indeed, the shapes of their molecular fields are quite different from templates A and B (e.g., see Figure 8D), confirming that geranylacetone and isobornyl

acetate do not interact with the same molecular targets reflected by templates A and B. On the other hand, the molecular structure of geranylacetone resembles such TRPV1 agonists as capsaicin and its analogues.^{12,13} Indeed, the pharmacophore model of TRPV1 agonists based on capsaicin, NGCC, and compound **2k**^{12,13} (template C) differs substantially from pharmacophore templates A and B and contains two different hydrophobic regions. A large area of negative field (blue area in Figure 8E) surrounds the overlapping carbonyl groups and electron acceptor substituents in the aromatic rings. The blue field in the template corresponds to the protein's positively charged regions or to amino and hydroxyl groups in the TRPV1 binding site that are capable of forming H-bonds with electronegative atoms of the agonist. In addition, a positive field (red area in Figure 8E) is located near NH groups of the three molecules, where potential H-bond acceptors could be present in the protein. Superimposition of the geranylacetone molecule onto this template using the FieldAlign program shows a relatively high quality of alignment and similarity ($S =$

0.644). Thus, the proposed bioactive conformation of geranylacetone has a molecular field distribution close to that of other known TRPV1 agonists (Figure 6F) and provides support for our biological data showing that geranylacetone is indeed a TRPV1 agonist.

DISCUSSION

Essential oils from *Ferula* species are an important source of bioactive monoterpenes, phenylpropanoids, sesquiterpenes, and organosulfur compounds with a broad spectrum of pharmacological properties.^{4,6,31} In the present study, we defined the chemical profile of essential oils extracted from *F. akitschkensis* umbels+seeds and stems (FAEO_{u/s} and FAEO_{stm}), evaluated the effect of these oils and their pure constituents on human neutrophil functions, and investigated their cellular targets.

A total of 52 constituents were identified in FAEO_{u/s}, with sabinene, α -pinene, β -pinene, and terpinen-4-ol being the major components. Although α -pinene and β -pinene are common monoterpenes in *Ferula* oils,^{4–6,31} high levels of sabinene were previously found only in *F. behboundiana*.³¹ Other primary components present at lower concentrations (1–1.5% total weight) were *trans*-sabinene hydrate and the sesquiterpenoids eremophilene and 2-himachalen-7-ol. FAEO_{stm} had a high content of phenylpropanoids, with myristicin as the major component. Among *Ferula* spp., myristicin was previously found only in *F. glauca*.⁶

Neutrophils are principal effectors of the innate immune response to injury or infection and are essential for elimination of invading bacterial pathogens.⁸ Thus, it is significant that FAEO_{u/s} activated Ca²⁺ mobilization in human neutrophils. To the best of our knowledge, only a few studies have reported that essential oils can directly activate neutrophil functions. For example, essential oils from *Citrus aurantium* (bergamot) stimulated ROS production in human neutrophils.³² These oils have some common components with FAEO_{u/s}, including monoterpenes, such as α -pinene, β -pinene, γ -terpinene, and limonene. Given the critical role played by neutrophils in innate immunity against pathogens, our data support the possibility that at least some of the observed therapeutic effects of essential oils with similar primary constituents are due to enhancement of innate immune responses.

To further define the bioactive component(s) in *Ferula* essential oils, we evaluated 22 of their constituents and found that sabinene, β -pinene, γ -terpinene, isobornyl acetate, geranylacetone, and 2-nonenal directly activated [Ca²⁺]_i flux in human neutrophils. Although the agonist effects of β -pinene, sabinene, and 2-nonenal in neutrophils were previously reported,^{9,15} this is the first report that γ -terpinene, isobornyl acetate, and geranylacetone can directly modulate human neutrophil responses, including [Ca²⁺]_i mobilization and chemotaxis (in vitro). Note that γ -terpinene treatment reduced neutrophil migration in a carrageenan-induced peritonitis model (in vivo).³³ Whether this was due to direct effects on neutrophils is not known; however, our current studies support this possibility. Eosinophil migration was inhibited by essential oils from *Syzygium cumini* and *Psidium guajava*, which have relatively high levels of β -pinene.³⁴ The current studies are the first to report inhibitory effects of isobornyl acetate and geranylacetone on leukocyte migration.

TRP channels are functionally expressed in various cells of the immune system, including neutrophils, and elevation of intracellular Ca²⁺ through activation of these channels may regulate various aspects of the inflammatory response.³⁵ In

addition, it has been reported that TRPM2 channels can be activated by neutrophil-generated ROS.³⁶ Several essential oil-derived monoterpenes have been evaluated for their effects on TRP channels, including TRPA1, TRPV1/3, and TRPM8, which are molecular detectors of thermal and chemical stimuli.^{21,23,37} For example, TRPV3 is activated by camphor, menthol, 6-*tert*-butyl-*m*-cresol, carvacrol, dihydrocarveol, eucalyptol, thymol, carveol, and (+)-borneol.²³ Camphor and carvone, which is a major compound in spearmint essential oil, can also activate TRPV1 and TRPA1 channels.³⁷ Borneol, one of the major constituents of rosemary oil, inhibited TRPA1-mediated cationic currents,³⁸ whereas carvacrol, which is abundant in oregano oil, activated TRPA1 but not TRPV1 channels.³⁹ Finally, menthol, one of the major constituents of peppermint essential oil, is a ligand of TRPM8 channels.⁴⁰ Although α -pinene oxide, (–)- α -pinene, and *p*-cymene had weak agonist activity for TRPV3 channels,²³ we found that these monoterpenes did not activate human neutrophils (Table 2).

Evaluation of the effects of TRP channel blockers showed that they inhibited neutrophil [Ca²⁺]_i flux stimulated by FAEO_{u/s}, β -pinene, sabinene, γ -terpinene, and geranylacetone. However, 2-APB and capsazepine did not block [Ca²⁺]_i flux stimulated by isobornyl acetate (Table 3). Capsazepine is relatively specific for TRPV1 and TRPM8 channels.²⁴ It should be noted that capsazepine had the lowest inhibitory activity on [Ca²⁺]_i flux in human neutrophils activated by *Ferula* essential oil-derived agonists, with the exception of geranylacetone. Although geranylacetone does not possess a vanillyl group like capsaicin, its structure exhibits spatial similarity with capsaicin and other TRPV1 agonists, such as compound 2k and NGCC.^{12,13} Indeed, the proposed bioactive conformation of geranylacetone fits well with the TRPV1 pharmacophore template and has hydrophobic, negative, and positive molecular field distributions similar to those of the template (Figure 8F). This channel is expressed in neutrophils,³⁵ and capsaicin, a potent TRPV1 agonist, can activate neutrophil [Ca²⁺]_i flux (Table 4 and ref 25). We also found that geranylacetone activated a transient Ca²⁺ influx in transfected TRPV1-HEK293 cells, but not in nontransfected cells. Together, these data demonstrate that geranylacetone is a TRPV1 agonist and can activate TRPV1 channels in human neutrophils.

The other three TRP channel blockers, 2-APB, ACA, and SKF 96365, can inhibit a broad range of TRP channels (for a review see ref 22) and even other molecular targets. For example, 2-APB blocks TRPC3/6/7 and several nonclassical TRP channels (TRPM2/7 and TRPV6).⁴¹ ACA directly blocks TRPC6, TRPM2/8, and phospholipase A₂.⁴¹ SKF 96365 is believed to be a nonselective blocker of TRPC3/6/7 channels, but not TRPV1 channels.⁴¹ Thus, we propose that the SKF 96365-blockable [Ca²⁺]_i flux in neutrophils activated by FAEO_{u/s} and its active components (β -pinene, sabinene, γ -terpinene) is not mediated by TRPV1 but more likely via TRPM and/or TRPC channels; however, further studies will be necessary to address this issue. In support of this conclusion, TRPM2/7 and TRPC6 channels are expressed in neutrophils and implicated in various inflammatory responses (e.g., see ref 42). Moreover, neutrophils from *Trpc6*–/– knockout mice have modestly decreased Ca²⁺ flux in response to fMLF.⁴³ In addition, our molecular modeling showed that the ligand-based pharmacophore for β -pinene, sabinene, and γ -terpinene (template A) was substantially different than the pharmacophore models for TRPV3/TRPM8 (template B) and TRPV1

(template C) agonists, indicating that these compounds target TRP channels other than TRPV1/3 and TRPM8. Finally, sabinene, γ -terpinene, and β -pinene did not induce a significant Ca^{2+} influx in transfected TRPV1-HEK cells, confirming they are not TRPV1 agonists.

Monoterpenes are hydrophobic small-molecule compounds with relatively high Log *P* values, suggesting that they could allosterically interact with a hydrophobic pocket(s) within the TRP transmembrane bundle and regulate these gates to control channel opening and closing.⁴⁴ Moreover, TRP channels can have several sites for pharmacological intervention.⁴¹ Thus, the exact nature of TRP channel interactions and/or other additional targets for essential oil-derived constituents in human neutrophils remains unclear, and further studies will be required to elucidate the molecular mechanisms of how such target(s) could be involved in modulation of neutrophil functions by monoterpenes and other essential oil constituents.

On the basis of our neutrophil Ca^{2+} flux assays, sabinene, β -pinene, and γ -terpinene are the main bioactive components of FAEO_{u/s}. On the basis of the molecular weight of these compounds (136.2 Da), their relative levels in FAEO_{u/s} and the EC₅₀ value for this oil in Ca^{2+} flux activation assays (16.5 $\mu\text{g}/\text{mL}$), the effective concentration of these compounds would be in the range of 80 μM , which is close to the EC₅₀ value for sabinene (49.4 μM), the major component (58.7%) of this oil. It should be noted that natural essential oils are mixtures of monoterpenes, which could have opposing agonist and antagonist activities that could modify or even suppress agonist effects of the primary component(s).⁴⁵ This could explain some discrepancies between EC₅₀ values for pure compounds and their partial activity in a mixture. In addition, activation of TRP channels by agonists can result in their desensitization (e.g., ref 46) and inhibition of $[\text{Ca}^{2+}]_i$ flux induced by other neutrophil agonists. Although there are no reports on habitual intake and/or medicinal application of any essential oil preparations from *F. akitschkensis*, its active components are common constituents or even active principles of some essential oils with well-known therapeutic properties (e.g., refs 4, 33, and 47). Interaction of these constituents with various ion channels, including TRP channels in neutrophils, may be an additional mechanism-based explanation for the immunomodulatory and anti-inflammatory properties of these oils.⁴³

Myristicin is the major component in parsley, carrot, and the essential oils of mace and nutmeg.⁴⁸ This phenylpropanoid has anticholinergic, antibacterial, anti-inflammatory, and hepatoprotective effects.^{16,49} Here, we found that myristicin inhibited both fMLF- and IL-8-induced $[\text{Ca}^{2+}]_i$ flux in neutrophils. Moreover, myristicin dose-dependently inhibited capsaicin-induced transient Ca^{2+} influx in TRPV1-HEK293 cells, indicating that it is indeed a TRPV1 channel antagonist. Whether it can also inhibit other pathways will need to be investigated in future studies. In any case, our findings support the anti-inflammatory potential of myristicin. Note that myristicin had low activity in the neutrophil migration assay. The discrepancy in this effect could be explained by the relatively long incubation time in the cell migration assay (90 min) compared with the $[\text{Ca}^{2+}]_i$ flux assay (<5 min). Indeed, myristicin, like other allyl-benzenes, can form covalent conjugates with macromolecules during its enzymatic bioconversion.⁵⁰ In addition, the activation of neutrophil migration via FPR1 and CXCR1/2 could be independent of TRPV1 channels. Nevertheless, further studies will be necessary to determine the precise molecular mechanisms for the inhibitory

effects of this phenylpropanoid in human neutrophils and other cells, as well as its applicability as an anti-inflammatory agent in preventive or therapeutic animal models.

In conclusion, we showed that *Ferula* essential oils (FAEO_{u/s} and FAEO_{stm}) and several components are agonists of human neutrophils but can also attenuate neutrophil migration via the likely desensitization of TRP channels. Our experimental and molecular modeling studies suggest that geranylacetone can activate TRPV1 channels in human neutrophils. Overall, these results suggest a potential new strategy for developing novel medicines based on components of essential oils that could effectively modulate neutrophil functional responses. Further detailed studies are warranted to define the structure and pharmacological effects of *Ferula* essential oil-derived constituents to understand their potential as therapeutic remedies for various disorders.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACA, *N*-(*p*-amylcinnamoyl)anthranilic acid; 2-APB, 2-aminoethyl diphenyl borate; FAEO, essential oils of *F. akitschkensis*; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FID, flame ionization detector; FPR, *N*-formyl peptide receptor; GC, gas chromatography; GPCR, G-protein coupled receptors; HBSS, Hanks' balanced salt solution; IL, interleukin; IP3, inositol triphosphate; MS, mass spectrometry; NGCC, *N*-geranyl cyclopropylcarboxamide; PTX, pertussis toxin; ROS, reactive oxygen species; RRI, relative retention indices; TRP, transient receptor potential

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