JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Molecular and Phytochemical Investigation of *Angelica dahurica* and *Angelica pubescentis* Essential Oils and Their Biological Activity against *Aedes aegypti*, *Stephanitis pyrioides*, and *Colletotrichum* Species

Nurhayat Tabanca,*[,†] Zengping Gao,[†,§] Betul Demirci,[⊥] Natascha Techen,[†] David E. Wedge,[‡] Abbas Ali,[†] Blair J. Sampson,[○] Chris Werle,[○] Ulrich R. Bernier,[⊗] Ikhlas A. Khan,[†,#,△] and Kemal Husnu Can Baser[⊥,▽]

[†]National Center for Natural Products Research, The University of Mississippi, University, Mississippi 38677, United States

[§]Department of Chinese Herbal Chemistry, School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing 100102, China

[⊥]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskisehir, Turkey

[‡]Natural Products Utilization Research Unit, ARS, U.S. Department of Agriculture, The University of Mississippi, University, Mississippi 38677, United States

[○]Southern Horticultural Laboratory, ARS, U.S. Department of Agriculture, Poplarville, Mississippi 39470, United States

[⊗]Center for Medical, Agricultural and Veterinary Entomology, ARS, U.S. Department of Agriculture, Gainesville, Florida 32608 United States

[#]Department of Pharmacognosy, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, United States

[△]Department of Pharmacognosy, College of Pharmacy and [▽]Botany and Microbiology Department, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia

ABSTRACT: In this study, *Angelica dahurica* and *Angelica pubescentis* root essential oils were investigated as pest management perspectives, and root samples were also analyzed genetically using the nuclear ribosomal internal transcribed spacer (ITS) region as a DNA barcode marker. *A. pubescentis* root essential oil demonstrated weak antifungal activity against *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*, whereas *A. dahurica* root essential oil did not show antifungal activity. Conversely, *A. dahurica* root essential oil demonstrated better biting deterrent and insecticidal activity against yellow fever mosquito, *Aedes aegypti*, and azalea lace bugs, *Stephanitis pyrioides*, than *A. pubescentis* root oil. The major compounds in the *A. dahurica* oil were found as α-pinene (46.3%), sabinene (9.3%), myrcene (5.5%), 1-dodecanol (5.2%), and terpinen-4-ol (4.9%). α-Pinene (37.6%), *p*-cymene (11.6%), limonene (8.7%), and cryptone (6.7%) were the major compounds found in the *A. pubescentis* oil. In mosquito bioassays, 1-dodecanol and 1-tridecanol showed antibiting deterrent activity similar to the positive control DEET (*N*,*N*-diethyl-3-methylbenzamide) at 25 nmol/cm^2 against *Ae. aegypti*, whereas only 1-tridecanol showed repellent activity in human-based cloth patch bioassay with minimum effective dosages (MED) of 0.086 ± 0.089 mg/cm^2 (DEET = 0.007 ± 0.003 mg/cm^2). In larval bioassays, 1-tridecanol was more toxic with an LC_{50} value of 2.1 ppm than 1-dodecanol having an LC_{50} value of 5.2 ppm against 1-day-old *Ae. aegypti* larvae. 1-Dodecanol and 1-tridecanol could be useful for the natural mosquito control agents.

KEYWORDS: *Angelica dahurica, Angelica pubescentis, internal transcribed spacer region, Colletotrichum species, Aedes aegypti, Stephanitis pyrioides, 1-dodecanol, 1-tridecanol, terpinen-4-ol, sabinene, myrcene*

INTRODUCTION

Traditional Chinese medicine (TCM) plays an important role in drug discovery and provides novel lead molecules useful for improving human health.[1−3] Although TCM plants are rich in chemical diversity and are popular sources of traditional herbal medicines, there has been little research carried out to evaluate them as potential sources of biopesticides, for example, fungicides and insecticides, rather than pharmaceutical research.[3−5] The excessive use of many synthetic chemical pesticides is causing problems such as harm to nontarget organisms, destruction of natural enemies, development of chemical resistance, and toxicological implications to human health.[6,7] Therefore, new integrated pest management (IPM) approaches have recently been adopted to replace synthetic chemicals with more selective botanical pesticides, which are much safer for humans and animals. Natural products including essential oils appear to have a possible role in the development of fungicides and insecticides.[6−11] Essential oils have recently

Received: May 23, 2014
Revised: August 13, 2014
Accepted: August 18, 2014
Published: August 18, 2014

dx.doi.org/10.1021/jf5024752 | J. Agric. Food Chem. 2014, 62, 8848−8857

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
18 AUG 2014		**00-00-2014 to 00-00-2014**

4. TITLE AND SUBTITLE
Molecular and Phytochemical Investigation of Angelica dahurica and Angelica pubescentis Essential Oils and Their Biological Activity against Aedes aegypti, Stephanitis pyrioides, and Colletotrichum Species

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Mississippi,National Center for Natural Products Research,University,MS,38677

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In this study, Angelica dahurica and Angelica pubescentis root essential oils were investigated as pest management perspectives, and root samples were also analyzed genetically using the nuclear ribosomal internal transcribed spacer (ITS) region as a DNA barcode marker. A. pubescentis root essential oil demonstrated weak antifungal activity against Colletotrichum acutatum, Colletotrichum fragariae, and Colletotrichum gloeosporioides, whereas A. dahurica root essential oil did not show antifungal activity. Conversely, A. dahurica root essential oil demonstrated better biting deterrent and insecticidal activity against yellow fever mosquito, Aedes aegypti, and azalea lace bugs, Stephanitis pyrioides, than A. pubescentis root oil. The major compounds in the A. dahurica oil were found as ??-pinene (46.3%), sabinene (9.3%), myrcene (5.5%), 1-dodecanol (5.2%), and terpinen-4-ol (4.9%). ??-Pinene (37.6%), p-cymene (11.6%), limonene (8.7%), and cryptone (6.7%) were the major compounds found in the A. pubescentis oil. In mosquito bioassays, 1-dodecanol and 1-tridecanol showed antibiting deterrent activity similar to the positive control DEET (N,N-diethyl-3-methylbenzamide) at 25 nmol/cm2 against Ae. aegypti, whereas only 1-tridecanol showed repellent activity in human-based cloth patch bioassay with minimum effective dosages (MED) of 0.086 ?? 0.089 mg/cm2 (DEET = 0.007 ?? 0.003 mg/cm2). In larval bioassays, 1-tridecanol was more toxic with an LC50 value of 2.1 ppm than 1-dodecanol having an LC50 value of 5.2 ppm against 1-day-old Ae. aegypti larvae. 1-Dodecanol and 1-tridecanol could be useful for the natural mosquito control agents.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**10**	

Table 1. Composition of the Root Essential Oils of *Angelica dahurica* (Ad) and *A. pubescentis* (Ap)

RRI[a]	compound	Ad (%)	Ap (%)	identification method[b]
1032	α-pinene	46.3	37.6	t_R, MS
1035	α-thujene	0.6	0.5	t_R, MS
1048	2-methyl-3-buten-2-ol	0.5		MS
1076	camphene	0.2	1.8	t_R, MS
1100	undecane		0.3	t_R, MS
1093	hexanal		0.1	t_R, MS
1118	β-pinene	2.5	2.2	t_R, MS
1132	sabinene	9.3	0.3	t_R, MS
1159	δ-3-carene		0.4	MS
1174	myrcene	5.5	0.7	t_R, MS
1194	heptanal	0.1		t_R, MS
1203	limonene	1.0	8.7	t_R, MS
1218	β-phellandrene	1.8	3.8	t_R, MS
1280	*p*-cymene	3.3	11.6	t_R, MS
1296	octanal	0.2		t_R, MS
1300	tridecane		0.3	t_R, MS
1384	α-pinene oxide		0.2	MS
1398	2-nonanone		0.2	MS
1429	perillen	0.1		MS
1455	*p*-cresyl methyl ether		0.7	MS
1471	2-nonylacetate		0.2	MS
1479	δ-elemene	0.3		MS
1492	cyclosativene		0.1	MS
1496	2-decanone		0.2	MS
1499	α-campholene aldehyde		0.2	MS
1504	daucene		0.1	MS
1521	2-nonanol		0.7	MS
1571	*trans*-*p*-menth-2-en-1-ol		0.1	MS
1586	pinocarvone	0.1	0.5	t_R, MS
1591	bornyl acetate		1.2	t_R, MS
1594	*trans*-β-bergamotene	0.1		MS
1600	β-elemene	1.6	0.2	t_R, MS
1604	thymol methyl ether		0.5	t_R, MS
1611	terpinen-4-ol	4.9		t_R, MS
1614	carvacrol methyl ether		1.0	t_R, MS
1620	selina-5,11-diene	0.2		MS
1638	*cis*-*p*-menth-2-en-1-ol	0.1	0.2	MS
1668	(*Z*)-β-farnesene	0.1	0.3	MS
1669	sesquisabinene		0.5	MS
1670	*trans*-pinocarveol	0.2	0.3	MS
1688	selina-4,11-diene	0.5		MS
1690	cryptone	2.5	6.7	MS
1725	verbenone	0.4	0.3	MS
1740	valencene		0.2	MS
1741	β-bisabolene	0.1		MS
1742	β-selinene	0.1	0.3	MS
1744	α-selinene	0.1		MS
1745	selina-4(15),7(11)-diene	tr[c]		MS
1758	*cis*-piperitol	tr		MS
1786	*ar*-curcumene		0.2	MS
1802	cumin aldehyde	0.2	0.8	t_R, MS
1804	myrtenol	0.1	0.2	MS
1811	*p*-mentha-1,3-dien-7-al		0.1	MS
1814	*p*-mentha-1,5-dien-7-ol	tr		MS
1823	*p*-mentha-1(7),5-dien-2-ol		0.1	MS
1845	*trans*-carveol		0.1	t_R, MS
1849	cuparene	0.2		MS
1854	germacrene-B	0.1		MS
1864	*p*-cymen-8-ol	tr		t_R, MS
1973	1-dodecanol	5.2		t_R, MS
2008	caryophyllene oxide	0.6		t_R, MS
2071	humulene epoxide II	tr	0.1	t_R, MS
2077	1-tridecanol	2.0		t_R, MS
2113	cumin alcohol	0.3	1.3	t_R, MS
2144	spathulenol	0.2		MS
2232	α-bisabolol		tr	MS
2232	4-isopropylphenol		0.1	MS
2269	guaia-6,10(14)-dien-4β-ol	0.5		MS
	total	92.1	86.2	

[a]RRI, relative retention indices calculated against *n*-alkanes; % calculated from FID data. [b]Identification method: t_R, identification based on the retention times (t_R) of genuine standard compounds on the HP Innowax column; MS, identification was performed on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data. [c]tr, <0.01.

received greater attention as natural sources of potentially useful biopesticides targeting a diversity of pests including plant pathogens, *Varroa* mites, cockroaches, mosquitoes, stored-product insects, and house flies.[4,6−16]

Angelica dahurica (Fisch. Ex Hoffm.) Benth. & Hook. and *Angelica pubescentis* Maxim. are well-known in TCM, and their roots are recorded as Baizhi and Duhuo, respectively, in the Chinese pharmacopoeia.[17] Both *Angelica* roots have a long history of use in Asian countries. *A. dahurica* roots have been used for the treatment of headache, rhinitis, nose problems, skin problems,[18−23] and toothache.[17,24,25] *A. pubescentis* roots have been used for the treatment of rheumatoid arthritis, headache, paralysis,[17,24] and insomnia.[17,26] The pharmacological activities associated with *A. dahurica* include antibacterial, antifebrile, analgesic, and antispasmodic,[17,27] and those for *A. pubescentis* include antibacterial, analgesic, antirheumatic, antispasmodic, antifungal, and antitumor.[17,28−30]

As part of our research program aimed at the identification of new natural-based insecticides and fungicides, we previously investigated two *Angelica* essential oils for their antifungal activity.[4] In our previous study, *A. sinensis* root oil showed good antifungal activity against three *Colletotrichum* species; however, *A. archangelica* root oil did not show any antifungal activity.[4] The bioassay-guided isolation was followed, and the active compound (*Z*)-ligustilide was isolated. This compound also demonstrated potent biting deterrent activity against *Aedes aegypti* and *Anopheles stephensi*.[4] In a continuing effort to investigate new alternative biopesticides from other *Angelica* species, *A. dahurica* and *A. pubescentis* root essential oils from China were explored in the current study for their pest management properties. Both *Angelica* essential oils were analyzed by gas chromatography (GC) with a flame ionization detector (FID) and gas chromatography−mass spectrometry (GC-MS) using an Agilent GC-mass selective detector (MSD) system. In addition, a nuclear molecular marker to distinguish between these two *Angelica* species was also investigated.

dx.doi.org/10.1021/jf5024752 | J. Agric. Food Chem. 2014, 62, 8848−8857

MATERIALS AND METHODS

Chemicals. Myrcene (CAS Registry No. 123-35-3), 1-tridecanol (CAS Registry No. 112-70-9), 1-dodecanol (CAS Registry No. 112-53-8), thymol methyl ether (CAS Registry No. 1076-56-8), carvacrol methyl ether (CAS Registry No. 6379-73-3), *p*-cymene (CAS Registry No. 99-87-6), (−)-terpinen-4-ol (CAS Registry No. 20126-76-5), and (+)-terpinen-4-ol (CAS Registry No. 2438-10-0) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sabinene (CAS Registry No. 3387-41-5) was purchased from ChromaDex (Irvine, CA, USA). Fungicide standards benomyl, cyprodinil, azoxystrobin, and captan were purchased from Chem Service, Inc. (West Chester, PA, USA).

Plant Material. *A. dahurica* and *A. pubescentis* roots were purchased from Beijing Heng Yu Hua Kang Pharmaceutical Co., Ltd. Species were identified by Professor Dr. Yu-Ning Yan, School of Chinese Materia Medica, Beijing University of Chinese Medicine, Beijing, China. Voucher specimens of these samples (No. 20 and 21) were deposited at the Herbarium of the Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey. For the molecular analysis, additional samples of *Angelica* species were chosen: *A. acutiloba* 2935; *A. gigas* 2944, and *A. sinensis* 14427. Vouchers 2935 and 2944 were from the repository at the National Center for Natural Products (NCNPR), The University of Mississippi, University, MS, USA. Voucher 14427 was deposited at the Herbarium of the Faculty of Pharmacy, Anadolu University.

Isolation of the Essential Oils. Air-dried roots of *A. dahurica* and *A. pubescentis* were water distilled for 3 h using a Clevenger-type apparatus to produce essential oils at yields of 0.45 and 0.65%, respectively.

Gas Chromatography (GC) and Gas Chromatography−Mass Spectrometry (GC-MS) Conditions. The *A. dahurica* and *A. pubescentis* root oils were analyzed by GC-FID and GC-MS using an Agilent GC-mass selective detector (MSD) system. The GC-MS analyses were carried out with an Agilent 5975 GC-MSD system. An Innowax fused silica capillary column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as the carrier gas (0.8 mL/min). The oven temperature was kept at 60 °C for 10 min, then programmed to 220 °C at a rate of 4 °C/min, then maintained constant at 220 °C for 10 min, and finally programmed to 240 °C at a rate of 1 °C/min. The injector temperature was set at 250 °C. The split flow was adjusted at 50:1. Mass spectra were recorded at 70 eV over the mass range m/z 35−450.

The GC analyses were performed using an Agilent 6890N GC system. The FID detector temperature was set to 300 °C, and the same operational conditions were used with a duplicate of the same column employed in GC-MS analyses. Simultaneous autoinjection was done to obtain equivalent retention times. Relative percentages of the separated compounds were calculated from integration of the peak areas in the GC-FID chromatograms (Table 1).

Identification of essential oil components was accomplished by comparison of retention times with authentic samples or by comparison of their relative retention index (RRI) to a series of *n*-alkanes.[31,32] Computer matching for identification was accomplished with commercial mass spectral libraries (Wiley GC/MS Library, MassFinder 3 Library) and with an in-house "Baser Library of Essential Oil Constituents", which includes over 3200 genuine compounds with MS and retention data from pure standard compounds and components of known oils as well as MS literature data.[33,34]

DNA Extraction and ITS Amplification from Plant Tissue. DNA from *Angelica* species (dried root/rhizome) was extracted with a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). The ITS region (internal transcribed spacer region, consisting of 18S rRNA gene, partial sequence; internal transcribed spacer 1, 5.8S rRNA gene; internal transcribed spacer 2, complete sequence; and 28S rRNA gene, partial sequence) was amplified from genomic DNA using the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).[35] PCR amplifications were carried out in a 50 μL reaction mixture containing 1× PCR reaction buffer, 0.2 mM dNTP mixture, 0.2 μM of each forward and reverse primer, 1.5 mM $MgCl_2$, and 2 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The PCR program consisted of one initial denaturation step at 96 °C for 3 min followed by 35 cycles at 96 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. PCR products were cut with one of the restriction endonucleases, *Cac*8I, *Hae*III, *Rsa*I, or *Sau*96I (New England Biolabs, Ipswich, MA, USA), without prior purification or precipitation.[36] In brief, 2 μL of the appropriate restriction buffer and 5−10 units of restriction enzyme *Cac*8I, *Hae*III, *Rsa*I, or *Sau*96I (New England Biolabs) were added to 20 μL of PCR product and incubated at 37 °C for 15 h, followed by 60 °C for 10 min to inactivate the enzyme. PCR and restriction reactions were run in an M&J Research Gradient Cycler PTC-225. After amplification and restriction, an aliquot was analyzed by electrophoresis on a 1−2% TAE agarose gel and visualized under UV light. The PCR products were compared to the molecular size standard 1 kb plus DNA ladder (Invitrogen).

Antifungal Bioassay against Plant Pathogens. The antifungal assay was carried out by using the direct bioautography bioassays against *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*, and the detailed assay procedures were described previously.[4,8−10,14,15] Fungal growth inhibition was evaluated 4−5 days after treatment by measuring zone diameters. Conidia were harvested from 7−10-day-old cultures by flooding plates with 5 mL of sterile distilled water, and conidia concentrations were determined photometrically from a standard curve based on the percent transmittance (%T) at 625 nm. Suspensions were then adjusted with sterile distilled water to a concentration of 1.0×10^5 conidia/mL.[4−8,14,15] The essential oils of *A. dahurica* and *A. pubescentis* were applied at 80 and 160 μg/spot in *n*-hexane onto a silica plate. To detect biological activity directly on the TLC plate, silica gel plates were sprayed with one of the three spore suspensions adjusted to a final concentration of 3.0×10^5 conidia/mL with liquid potato dextrose broth (PDB, Difco) and 0.1% Tween-80. Using a 50 mL chromatographic sprayer, each TLC plate with a fluorescent indicator (250 μm, silica gel GF Uniplate, Analtech, Inc., Newark, DE, USA) was sprayed lightly (to a damp appearance) three times with the conidial suspension. Inoculated plates were then placed in a 30 × 13 × 7.5 cm moisture chamber (398 °C, 100% relative humidity, Pioneer Plastics, Inc., Dixon, KY, USA) and incubated in a growth chamber at 24 °C and a 12 h photoperiod under 60 ± 5 μmols m^{-2} s^{-1} light. Inhibition of fungal growth was measured 4 days after treatment. The sensitivity of each fungal species to each test compound was determined by comparing sizes of inhibitory zones. Clear zones of fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents in each extract. Antifungal metabolites were readily located on the plates by visually observing clear zones where the active compounds inhibited fungal growth. Fungicide standards of benomyl, cyprodinil, azoxystrobin, and captan were used as positive controls at 1.16, 0.9, 1.61, and 1.2 μg, respectively, in 95% EtOH.

Insects. *Ae. aegypti* L. used in these studies were from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, FL, USA.[37] For biting deterrence bioassays, pupae were maintained in the laboratory at 27 ± 2 °C and 60 ± 10% relative humidity (RH), and 7−15 d-old adult females were used. For repellent activity, pupae were maintained in the laboratory at 28 ± 1 °C and 30−60% RH, and the resulting adult females aged 5−9 days were selected from the stock cages by a hand-draw box.[38] For larval bioassays, the eggs were hatched and the larvae were maintained at a temperature of 27 ± 2 °C and 60 ± 10% RH in a photoperiod regimen of 12/12 h (L/D). Adults of azalea lace bug, *Stephanitis pyrioides* (Scott), were collected by using electric aspirators (Hausherr's Machine Works, Tom's River, NJ, USA) from bouquets of azalea terminals (*Rhododendron* species). These plants were constantly maintained in plant growth chambers (Percival Scientific, Perry, IA, USA) at a temperature of 27 °C and 65% RH with a photoperiod of 14/10 L/D.

Mosquito Biting Bioassay. Experiments were conducted by using a six-celled in vitro Klun and Debboun (K&D) module bioassay system, developed by Klun et al.,[39] for quantitative evaluation of biting deterrent properties of candidate compounds. Briefly, the assay system

consists of a 6-well reservoir with each of the 3 × 4 cm wells containing 6 mL of blood. As described by Ali et al.,[8] a feeding solution consisting of CPDA-1 and ATP was used instead of blood. Green fluorescent tracer dye (www.blacklightworld.com) was used to determine the feeding by the females. *A. dahurica* and *A. pubescentis* root essential oils and some of their pure compounds were tested in this study. Treatments of essential oils were applied at 10 $\mu g/cm^2$, and DEET (97%, *N*, *N*-diethyl-*m*-toluamide) (Sigma-Aldrich, St. Louis, MO, USA) at 4.8 $\mu g/cm^2$ was used as positive control. All of the treatments were freshly prepared in molecular biology grade 100% ethanol (Fisher Scientific Chemical Co., Fair Lawn, NJ, USA) at the time of bioassay. The temperature of the solution in the reservoirs was maintained at 37.5 °C by continuously passing warm water through the reservoir using a circulatory bath. The reservoirs were covered with a layer of collagen membrane (Devro, Sandy Run, SC, USA). The test compounds were randomly applied to six 4 × 5 cm areas of organdy cloth and positioned over the membrane-covered CPDA-1 + ATP solution with a Teflon separator placed between the treated cloth and the 6-cell module to prevent contamination of the module. A 6-cell module containing five female mosquitoes per cell was positioned over cloth treatments covering the six CPDA-1 + ATP solution membrane wells, and trap doors were opened to expose the treatments to these females. The number of mosquitoes biting through cloth treatments in each cell was recorded after a 3 min exposure, and mosquitoes were prodded back into the cells to check the actual feeding. Mosquitoes were squashed, and the presence or absence of green fluorescent tracer dye in the gut was used as an indicator of feeding. A replicate consisted of six treatments: four test oils/compounds, DEET (a standard biting deterrent), and ethanol-treated organdy as solvent control. Two sets of five replications each with five females per treatment were conducted on two different days using a newly treated organdy and a new batch of females in each replication. Treatments were replicated 10 times.

Mosquito Repellent Bioassay. Repellency was determined as the minimum effective dosage (MED), which is the minimum threshold surface concentration necessary to prevent mosquitoes from biting through the treated surface.[40] Approximately 500 (±10%) mosquitoes were collected and loaded into a test cage (size of 45 cm × 37.5 cm × 35 cm) and held in the cage for 25 (±2.5) min before initiating repellency assays. Serial dilutions were then made such that the concentrations on the cloth for the remaining 1 mL of solution were 1.5, 0.75, 0.375, 0.094, 0.047, 0.023, and 0.011 mg/cm^2. Each concentration was tested to determine the point at which the repellent failed for each of the volunteers in the study; this concentration was averaged and reported. Each test was conducted by having a volunteer affix the treated cloth onto a plastic sleeve to cover a 32 cm^2 window previously cut into the sleeve. Each of the volunteers wore this sleeve/cloth assembly above a nylon stocking covering their arm, with their hands protected by a glove.[41] The arm with the sleeve/cloth assembly was inserted into a cage, where approximately 500 female *Ae. aegypti* mosquitoes (aged 6−10 days) had been preselected as host-seeking using a draw box.[38] Failure of the repellent treatment was 1% bite through; that is, the volunteer received five bites through the cloth over the sleeve window in the 1 min assay. There were three human volunteers in this study, and all three provided written informed consent to participate in this study as part of a protocol (636-2005) approved by the University of Florida Human Use Institutional Review Board (IRB-01).

Larval Bioassay. Bioassays were conducted to test *A. dahurica* and *A. pubescentis* root essential oils and some of their pure compounds for larvicidal activity against 1-day-old *Ae. aegypti* larvae by using the bioassay system described by Pridgeon et al.[42] Five 1-day-old *Ae. aegypti* larvae were added in a droplet of water to each well of 24-well plates (BD Labware, Franklin Lakes, NJ, USA) by use of a disposable 22.5 cm Pasteur pipet. Fifty microliters of larval diet [2% slurry of 3:2 beef liver powder (Now Foods, Bloomingdale, IL, USA) and brewer's yeast (Lewis Laboratories Ltd., Westport, CT, USA)] was added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). All chemicals tested were diluted in dimethyl sulfoxide (DMSO). Eleven microliters of the test chemical was added to the labeled wells, whereas 11 μL of DMSO was added to control treatments. After the treatment application, the plates were swirled in clockwise and counterclockwise motions, front to back, and side to side five times to ensure even mixing of the chemicals. Larval mortality was recorded 24 and 48 h post treatment. Larvae that showed no movement in the well after manual disturbance of water were recorded as dead. Permethrin (46.1% cis −53.2% trans, Chemical Service, West Chester, PA, USA) was used as positive control. Five concentrations ranging between 300 and 6.25 ppm were used in each treatment to obtain a range of mortality between 0 and 100. Treatments were replicated 10 times for each oil or pure compound.

Data Analyses. The parameter of proportion not biting (PNB) mosquitoes was calculated as described earlier by Ali et al.[43] The K&D module bioassay system can handle only four treatments along with negative and positive controls at the one setup experiment. To make direct comparisons among more than four test compounds and to compensate for variation in overall response among replicates, the biting deterrent activity was quantified as the biting deterrence index (BDI). BDIs were calculated using the formula

$$[\mathrm{BDI}_{i,j,k}] = \left[\frac{\mathrm{PNB}_{i,j,k} - \mathrm{PNB}_{c,j,k}}{\mathrm{PNB}_{d,j,k} - \mathrm{PNB}_{c,j,k}}\right]$$

where $\mathrm{PNB}_{i,j,k}$ denotes the proportion of females not biting when exposed to test compound i for replication j and day k (i = 1−4, j = 1−5, k = 1−2), $\mathrm{PNB}_{c,j,k}$ denotes the proportion of females not biting the solvent control c for replication j and day k (j = 1−5, k = 1−2), and $\mathrm{PNB}_{d,j,k}$ denotes the proportion of females not biting in response to DEET d (positive control) for replication j and day k (j = 1−5, k = 1−2). This formula makes an adjustment for interday variation in response and incorporates information from the solvent control as well as the positive control. A BDI value of 0 indicates an effect similar to that of ethanol, whereas a value significantly greater than 0 indicates biting deterrent effect relative to ethanol. BDI values not significantly different from 1 are statistically similar to DEET. BDI values were analyzed using SAS Proc ANOVA [single factor: test compound (fixed)],[44] and means were separated using the Ryan−Einot−Gabriel−Welsch multiple-range test. To determine whether confidence intervals include the values of 0 or 1 for treatments, Scheffe's multiple-comparison procedure with the option of CLM was used in SAS. LC_{50} values for larvicidal data were calculated by using SAS, Proc Probit. Control mortality was corrected by using Abbott's formula.[45] Toxicity was compared among treatments based on nonoverlapping 95% CI.[46]

Adulticidal Activity against Azalea Lace Bug. Bioassay and statistical methods follow those of Sampson et al.,[12,13] with some modifications. For these bioassays, *A. dahurica* and *A. pubescentis* essential oils as well as five chemical constituents [*p*-cymene, carvacrol methyl ether (CME), (+)-terpinen-4-ol, (−)-terpinen-4-ol, thymol methyl ether (TME)] were tested against adult azalea lace bug at a single dose of 10000 ppm (1% oil). In addition, *Angelica sinensis* and *Angelica archangelica* essential oils from our previous study[6] were also included to identify chemical diversity and activity relationship. Mortality data for lace bugs exposed to the two isomers of terpinen-4-ol were combined before analysis (total n = 16). Selection of 10000 ppm as our trial dose was based on standard evaluation methods used for essential oil-based insecticides such as Ecotrol and previous bioassay results with other essential oils.[12−15] Oil emulsions were freshly prepared using DMSO as a solvent and a 10% aqueous solution of DMSO as the solvent's control. Positive controls used for evaluating *Angelica* spp. included 1% emulsions of two essential oils that are currently commercialized as insecticides and acaricides (*Chenopodium ambrosioides* (Requiem EC, AgraQuest, Inc., Davis CA, USA) and azadirachtin (ChemService, Inc.). Twenty microliters of each treatment and control emulsions was pipetted into individual plastic wells of a standard 96-well microtiter plate in a randomized complete block design. To prevent bugs from drowning in residual fluid, an absorbent disk of Whatman no. 2 filter paper was placed at the bottom of each well. Three adult azalea lace bugs were transferred from their holding vials to treatments and control wells. Mortality data were recorded by observing lace bugs under a dissecting microscope at 1 h intervals for 5 h at 21 °C. Lace bugs that remained motionless in the well after

manual disturbance with a probe were recorded as dead. Between observations, bugs were kept at 23 °C in a separate growth chamber.

RESULTS AND DISCUSSION

Water-distilled essential oils from roots of *A. dahurica* and *A. pubescentis* were analyzed by GC-FID and GC-MS systems. The components identified are given in Table 1 with their relative percentages. Forty-three compounds were characterized in the oil of *A. dahurica*, making up 92.1% of the oil. Forty-six compounds were identified in the oil of *A. pubescentis*, representing 86.2% of the oil. The oil of *A. dahurica* was characterized with α-pinene (46.3%), sabinene (9.3%), myrcene (5.5%), and 1-dodecanol (5.2%) as major constituents. α-Pinene (37.6%), *p*-cymene (11.6%), limonene (8.7%), cryptone (6.7%), and β-phellandrene (3.8%) were characterized as main compounds in the oil of *A. pubescentis*. Both *Angelica* species were mainly rich in monoterpene hydrocarbons in our study. Tetradecanol (19.4%), limonene (15.3%), δ-3-carene (11.0%), 1-dodecanol (5.8%), and α-pinene (3.9%) were reported as major compounds of *A. dahurica* root oil.[47] δ-3-Carene (9.0%), β-phellandrene (8.4%), α-bisabolol (6.0%), and *m*-cymene (5.0%) were found as the main components in *A. pubescentis* radix oil.[48] The observed differences in the constituents of these two *Angelica* essential oils even collected inside the same country may be due to different environmental and harvesting season of the plants.

The coding ribosomal 18S, 5.8S, and 28S RNA genes are highly conserved, but the regions that separate them (internal spacer) are variable in length and nucleotide sequence. The diversity of the spacer region can be used as an identification basis.[49,50] In addition to the chemical profiling, a PCR–restriction fragment length polymorphism (PCR-RFLP) method was applied on the internal transcribed spacer region (ITS) using *Cac*8I, *Hae*III, *Rsa*I, or *Sau*96I restriction endonucleases to help distinguish between *A. dahurica* and *A. pubescentis*, *A. acutiloba*, *A. gigas*, and *A. sinensis*. Using the ITS primers, a single fragment of >600 bp was produced from all of the analyzed samples. PCR products were digested with restriction endonucleases *Cac*8I, *Hae*III, *Rsa*I, and *Sau*96I. A RFLP was observed from almost all of the samples analyzed (Figure 1). The digestion showed that *Cac*8I, *Rsa*I, or *Sau*96I produced polymorphic patterns that distinguished the *A. dahurica* from the *A. pubescentis* sample and from the other analyzed *Angelica* samples (*A. acutiloba*, *A. gigas*, and *A. sinensis*). A powerful tool for plant material identification, the PCR-RFLP approach was also successfully employed for the identification of plant materials such as *Bursaphelenchus* species[49] and for the discrimination of *Mitragyna* species[50] and the parasitic nematode *Anisakis* species.[51]

To discover natural product fungicides, essential oils of *A. dahurica* and *A. pubescentis* were evaluated for antifungal activity using direct bioautography assays leading to the discovery of promising antifungal compounds against three *Colletotrichum* species, which cause anthracnose diseases of strawberry.[4,8−10,14,15] The *A. pubescentis* essential oil demonstrated clear zones (2.83 ± 0.29 mm) against plant pathogens *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* at 160 μg/spot, whereas the *A. dahurica* essential oil did not show antifungal activity at the same concentration (Figure 2). Antifungal activity was evident by the presence of clear zones with a dark background where fungal mycelia or reproductive stroma were not present on the TLC plates. *A. pubescentis* essential oil demonstrated weaker antifungal activity compared to the positive control captan. The commercial fungicide captan, which is a well-known multisite inhibitor fungicide standard, showed clear inhibitory zones of 18.67 ± 1.53 mm at 1.2 μg concentration. The TLC profile of *A. pubescentis* in *n*-hexane/diethyl ether (8:2, v/v) was subsequently tested against three *Colletotrichum* species to identify the active inhibitory zones. The minor polar compounds appeared to be responsible for antifungal activity (Figure 3).

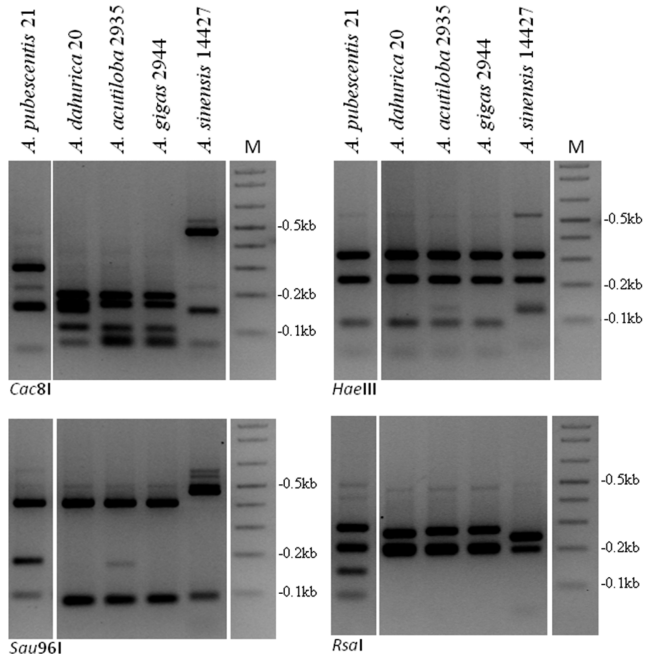

Figure 1. Agarose gel image of digested ITS PCR products with various restriction endonucleases (PCR-RFLP analysis). A different RFLP was observed from almost all of the samples analyzed. *A. dahurica* can be distinguished from *A. pubescentis* or any of the tested *Angelica* samples by cutting the amplified ITS region with *Cac*8I, *Rsa*I, or *Sau*96I. M = molecular size standard; fragment sizes are given in kilo base pairs (kb).

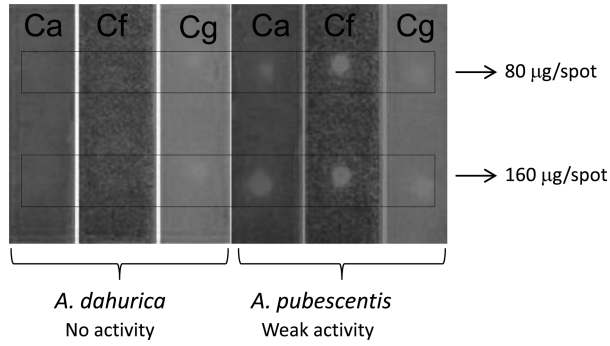

Figure 2. Bioautography of *A. dahurica* and *A. pubescentis* essential oils at 80 and 160 mg/spot against *C. acutatum* (Ca), *C. fragariae* (Cf), and *C. gloeosporioides* (Cg). Clear inhibitory zones indicate the antifungal activity.

BDI values representing the biting deterrent activity of *A. dahurica* and *A. pubescentis* root essential oils against *Ae. aegypti* are given in Figure 4. Both *Angelica* oils were tested at 10 μg/cm^2, and *A. dahurica* oil demonstrated slightly higher biting deterrent activity than *A. pubescentis*, but both were less active than DEET. Therefore, we selected possible active compounds that were present in *A. dahurica* oil but not present in the *A.*

dx.doi.org/10.1021/jf5024752 | J. Agric. Food Chem. 2014, 62, 8848−8857

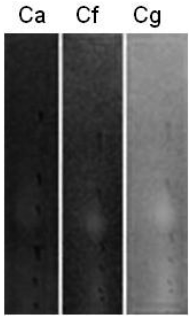

Figure 3. 1D-bioautography of *A. pubescentis* essential oil against *C. acutatum* (Ca), *C. fragariae* (Cf), and *C. gloeosporioides* (Cg) in *n*-hexane/diethyl ether (8:2, v/v) at 160 μg/spot concentration. The *A. pubescentis* essential oil showed weak antifungal activity with the diffuse zones against all three *Colletotrichum* species.

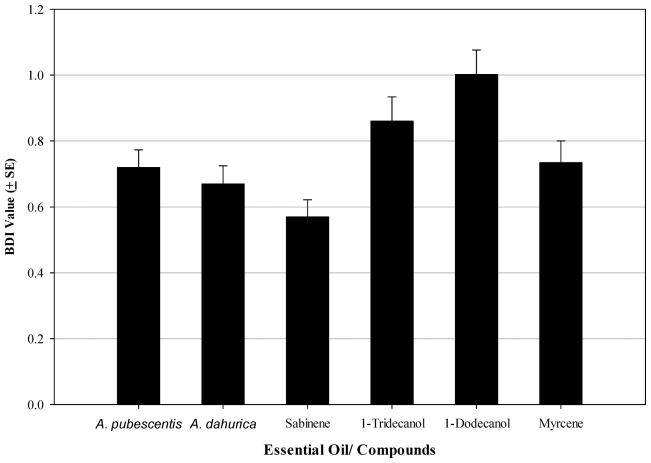

Figure 4. Mean biting deterrent index (BDI) value of *Angelica* species and active pure compounds. Essential oils were evaluated at 10 μg/cm^2 and the pure compounds were tested at 25 nmol/cm^2. DEET at 25 nmol/cm^2 was used as a positive control.

pubescentis oil. The major compound, α-pinene, and its β isomer, β-pinene, showed in our previous paper low biting deterrent activity at 25 nmol/cm^2.[52] The other monoterpene hydrocarbons sabinene and myrcene were present in higher concentration in *A. dahurica* oil than in *A. pubescentis* oil. Myrcene showed higher biting deterrence activity than sabinene, and the activity was similar to that of *A. dahurica* oil (Figure 4). We previously tested (+)- and (−)-terpinen-4-ol for biting deterrent activity, and both enantiomers showed biting deterrent activity greater than that of the solvent control.[53] Two aliphatic alcohols, 1-dodecanol and 1-tridecanol, that were present only in *A. dahurica* oil were individually tested for their biting deterrent activity. On the basis of 95% CI values, 1-dodecanol (C12:0) and 1-tridecanol (C13:0) at 25 nmol/cm^2 showed biting deterrent activity similar to that of DEET, whereas all the other compounds, such as sabinene and myrcene, were lower than 1-dodecanol and 1-tridecanol. In our research group it was previously reported that carboxylic acid forms of these alcohols, dodecanoic acid (C12:0) and tridecanoic acid (C13:0) showed higher BDI than other short- and long-chain fatty acids.[43] It appears that the number of carbons in the aliphatic compounds influences the biting deterrent activity. These two aliphatic alcohols (1-dodecanol and 1-tridecanol) may not be active at the concentrations present in the *A. dahurica* oil, and, although they are not also present in *A. pubescentis* essential oil, the activity of these oils may be due to the combined effect of all the compounds present in the complex oil. Active biting deterrent compounds 1-dodecanol and 1-tridecanol were also tested in cloth patch assays. 1-Tridecanol showed positive repellency in the cloth patch bioassay, with a minimum effective dosage (MED) of 0.086 ± 0.089 mg/cm^2 as compared to that of DEET, 0.007 ± 0.003 mg/cm^2, whereas 1-dodecanol did not show repellency at the maximum dose of 0.375 mg/cm^2.

Essential oils of *A. dahurica* and *A. pubescentis* did not show any larvicidal activity against 1-day-old *Ae. aegypti* larvae at the highest screening dose of 125 ppm. The pure compounds 1-dodecanol and 1-tridecanol demonstrated good larvicidal activity, with LC_{50} values of 2.1 (1.8−2.3) and 5.2 (4.7−5.7) ppm, respectively. Myrcene showed weak toxicity with a LC_{50} value of 120.3 (103.6−141.9) ppm, whereas sabinene killed only 40% of the larvae at the highest screening dose of 100 ppm (Table 2). Therefore, dose response bioassays were not necessary for sabinene. The LC_{50} value of permethrin, which was used as positive control, was 0.0034 ppm against *Ae. aegypti*. In a previous study, 1-dodecanol was reported to have larvicidal activity against *Ae. aegypti* and *Ae. scutellaris* at low doses,[54] and both dodecanol and tridecanol exhibited good toxicity against first instar *Culex tartalis* larvae.[55] Sinniah has suggested that aliphatic alcohols can act as irritants, so they can break down the cellular structure to cause the death of larvae.[54]

The azalea lace bug *S. pyrioides* is a major leaf pest of azalea plants in commercial nurseries and residential landscapes. Foliar injury inflicted by this insect is mostly cosmetic and appears as black ovipositional scabs, leaf stippling, and leaf chlorosis. However, if there is no early control for lace bug feeding, leaf chlorosis induced by unrestricted herbivory can lead to necrosis, leaf abscission, and, in severe cases, plant death. Azalea lace bugs are difficult to control. Adult females protect their offspring by laying eggs underneath leaves and

Table 2. Toxicity of Essential Oils of *A. dahurica* and *A. pubescentis* Essential Oils and Active Biting Deterrent Compounds from *A. dahurica* Oil against 1-Day-Old *Aedes aegypti* Larvae

compound/oil	LC_{50}	(95% CI)[a]	LC_{90}	(95% CI)[a]	χ^2	DF[b]
1-tridecanol	2.1	(1.8−2.3)	3.8	(3.2−4.7)	66.1	47
1-dodecanol	5.2	(4.7−5.7)	7.5	(6.7−8.9)	46.2	38
myrcene	120.3	(103.6−141.9)	273.5	(214.5−411.5)	46.1	28
sabinene	*c*					
A. dahurica oil	*d*					
A.pubescentis oil	*d*					

[a]LC_{50} and LC_{90} values are given in ppm (95% confidence interval). [b]DF refers to degree of freedom. [c]Killed 40% of the larvae at the highest dose of 100 ppm. [d]No larvicidal mortality at the maximum dose of 125 ppm.

dx.doi.org/10.1021/jf5024752 | J. Agric. Food Chem. 2014, 62, 8848−8857

Table 3. Insecticidal Bioassays for the Essential Oils (in a DMSO Emulsion) of Four *Angelica* Species, Five of Their Chemical Constituents, Three Positive Controls [*Chenopodium ambrosioides*, Malathion (Baseline), Azadirachtin], and a Negative Control (DMSO) Topically Applied to Adult Azalea Lace Bugs *Stephanitis pyrioides*

treatment	insect toxicity rating[a]	*n*	estimate ± SEM	*t*	*P*
azadirachtin[b]	1	5	0.427 ± 0.051	8.43	0.0001
terpinen-4-ol	1	16	0.390 ± 0.034	11.24	0.0001
A. dahurica oil	2	10	0.294 ± 0.040	7.41	0.0001
thymol methyl ether	2	8	0.227 ± 0.043	5.32	0.0001
C. ambrosioides[b]	2	10	0.167 ± 0.040	4.22	0.0001
A. sinensis	2	8	0.111 ± 0.043	2.59	0.0097
malathion[b]	3	17	0.000 ± 0.000		
A. pubescentis oil	4	10	−0.133 ± 0.040	−3.34	0.0009
carvacrol methyl ether	4	8	−0.172 ± 0.043	−4.04	0.0001
A. archangelica oil	4	10	−0.273 ± 0.040	−6.86	0.0001
p-cymene	5	8	−0.548 ± 0.043	−12.82	0.0001
DMSO	5	27	−0.544 ± 0.031	−17.62	0.0001
exposure time		669		28.43	0.0001

[a]Insect toxicity rankings based on *t* comparisons and Tukey's HSD test ($P < 0.05$; 1, active; 5, least active). [b]Malathion, azadirachtin, and *C. ambrosioides* extract were used as positive controls for testing the relative biological activity of the other materials. Malathion served as the baseline control.

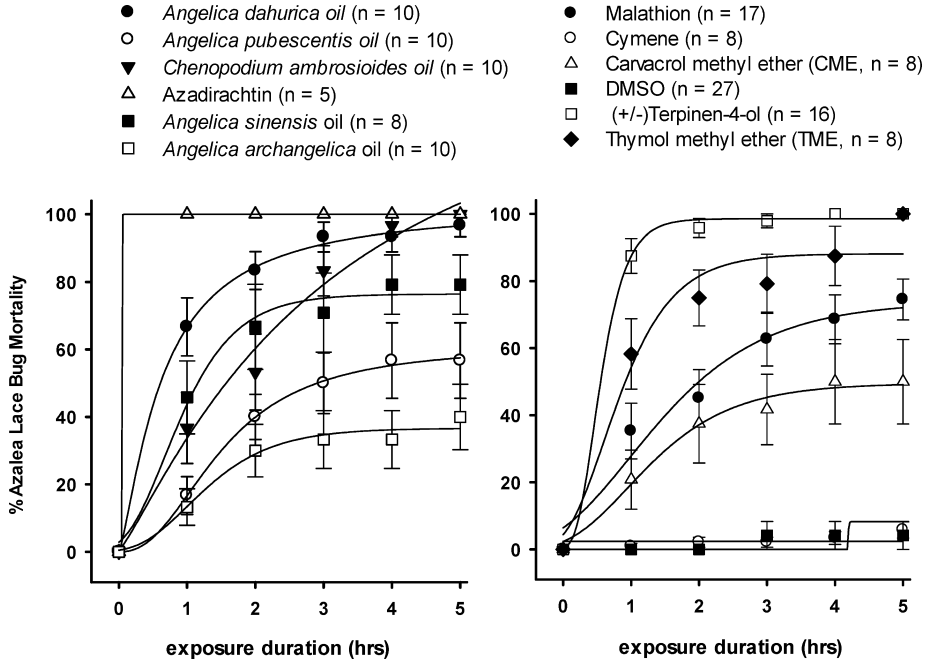

Figure 5. Azalea lace bug *S. pyrioides* mortality during 5 h of exposure to 1% emulsions of root essential oils from *Angelica dahurica*, *A. pubescentis*, *A. sinensis*, and *A. archangelica*, four constituent compounds of *A. dahurica* and *A. pubescentis* (*p*-cymene, carvarol methyl ether, thymol methyl ether, and terpinen-4-ol), and three insecticide standards (azadirachtin, *C. ambrosioides* oil, and malathion).

covering them with a hard fecal dome. Such egg-laying behaviors minimize an immature bug's exposure to natural enemies and water-soluble insecticides.[15] Natural oil-based insecticides, such as those based on extracts of the terpenoid-rich *Angelica* species and *Chenopodium ambrosioides*, show promise for development into commercial products for the control of highly gregarious insect herbivores such as *S. pyrioides*. Four *Angelica* essential oils (*A. dahurica*, *A. pubescentis*, *A. sinensis*, and *A. archangelica*) were then tested in insecticidal bioassays against *S. pyrioides* with *A. sinensis* and *A. archangelica* oils originating from the mosquito study[4] to compare their insecticidal activity. *A. sinensis* oil was composed of 81% phthalides, whereas *A. archangelica* included 72% monoterpene hydrocarbons.[4] There was good chemical diversity between these two species, which helped reveal their insecticidal activity results. Azalea lace bugs were most susceptible to emulsions of azadirachtin, in particular to the *Angelica* component terpinen-4-ol. Adult lace bugs exposed to these two compounds suffered 100% mortality within the first 4 h of exposure (Table 3; Figure 5). In fact, *A. dahurica* and *A. sinensis* essential oils compared favorably with the active ingredients of the two broad-spectrum commercial biopesticides azadirachtin and *Chenopodium* oil. *A. dahurica* oil was as bioactive as azadirachtin in killing nearly 100% of azalea lace bugs within 5 h (Table 3; Figure 5). Oils from *A. dahurica*, *A. sinensis*, and *C. ambrosioides* were more lethal to bugs than malathion. The other two TCM plant species tested (*A. pubescentis* and *A. archangelica*), which were high in monoterpene hydrocarbons (65 and 72%, respectively),

dx.doi.org/10.1021/jf5024752 | J. Agric. Food Chem. 2014, 62, 8848−8857

were less toxic than malathion and other essential oil extracts assessed, and they were only weakly active against lace bugs. *p*-Cymene was relatively nontoxic to lace bugs and can be associated with mortality rates no greater than those induced by the DMSO control. Although we did not test the bioactivity of *Angelica*'s major promising component, α-pinene, both species had roughly the same proportion of this molecule. Therefore, *A. dahurica*'s greater toxicity to lace bugs may originate from the presence of terpinen-4-ol, which at a concentration of 1% can kill >85% adult *S. pyrioides* within the first hour of exposure and all bugs within 4 h of exposure. Conversely, the weaker activity of *A. pubescentis* oil may stem from its higher concentration of the relatively nontoxic monoterpene hydrocarbons. It was also interesting to see that two phenolic compounds, thymol methyl ether and carvacrol methyl ether, showed different levels of toxicity against azalea lace bugs. The position of functional group plays an important role in this insecticidal activity. Myrcene and sabinene were present in higher concentration here than in *A. pubescentis* oil. Higher proportions of 1-dodecanol and 1-tridecanol in *A. dahurica* oil may also contribute to its potency; however, these compounds remain to be bioassayed against *S. pyrioides*.

As a conclusion, in this study, *A. dahurica* and *A. pubescentis* essential oils were evaluated for the first time for detailed antifungal and insecticidal activity. The diverse chemical characteristics between these two species play important roles for their biological activity. The 1D-TLC bioautography of *A. dahurica* and *A. pubescentis* oils proved that monoterpene hydrocarbons are not antifungal. Therefore, the antifungal activity of *A. pubescentis* observed in this study might be related to the presence of polar compounds such as cryptone and cumin alcohol or due to possible synergistic effects of the oil's minor compounds. Terpenoid substances, however, are naturally involved in antibiosis, many being potent antifeedents or insecticides. Monoterpene hydrocarbons are significantly less active in the insecticidal activity against *Ae. aegypti* or *S. pyrioides*. Multiple modes of action enhance pesticidal efficacy. With *A. dahurica* rich in alcohols and *A. sinensis* rich in phthalides, the chemical diversity within this genus offers promise as novel sources of broad-spectrum insecticides capable of quick knockdown of small arthropod pests. They compare favorably with the bioactivity of the active ingredients of commercially available botanical pesticide (*C. ambrosioides*). Terpinen-4-ol appears to be the leading active compound in *A. dahurica* root oil. Aliphatic alcohols play an important role in mosquito control, with the chain length and the degree of unsaturation important factors for bioactivity. Plants contain fatty alcohols with carbon numbers ranging from C6 to C16, which are important features for future tests for biting deterrent, repellent, and larvicidal activity against *Ae. aegypti*. Using the PCR-RFLP method, we were able to distinguish between *A. dahurica* and *A. pubescentis* samples, which can be a feasible method for the identification and authentication of *Angelica* samples.

AUTHOR INFORMATION

Corresponding Author

*(N.T.) E-mail: ntabanca@olemiss.edu.

Funding

This study was supported in part by USDA-ARS Grant 56-6402-1-612 and a Deployed War-Fighter Protection research program grant funded by the U.S. Department of Defense through the Armed Forces Pest Management Board and the Food and Drug Administration, Grant 300 212 193G.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank J. L. Robertson, R. Pace, M. Ruscoe, Dr. V. Raman, Dr. J. U. Rehman, Dr. V. Joshi, Dr. Maia Tsikolia, Natasha M. Agramonte, Nathan Newlon, and Greg Allen for performing bioassays and for their technical support and Dr. James J. Becnel, Mosquito and Fly Research Unit, Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, FL, USA, for supplying *Ae. aegypti* eggs. We thank Dr. Jon F. Parcher, National Center for Natural Products Research, The University of Mississippi, University, MS, USA, for English grammar corrections and suggestions.

REFERENCES

(1) Li, B.; Xu, X.; Wang, X.; Yu, H.; Li, X.; Tao, W.; Wang, Y.; Yang, L. A systems biology approach to understanding the mechanisms of action of Chinese herbs for treatment of cardiovascular disease. *Int. J. Mol. Sci.* **2012**, *13*, 13501−13520.

(2) Ganzera, M. Recent advancements and applications in the analysis of Traditional Chinese Medicines. *Planta Med.* **2009**, *75*, 776−783.

(3) Xie, P. S.; Leung, A. Y. Understanding the traditional aspect of Chinese medicine in order to achieve meaningful quality control of Chinese material medica. *J. Chromatogr., A* **2009**, *1216*, 1933−1940.

(4) Wedge, D. E.; Klun, J. A.; Tabanca, N.; Demirci, B.; Ozek, T.; Baser, K. H.C.; Liu, Z.; Zhan, S.; Cantrell, C. L.; Zhan, J. Bioactivity-guided fractionation and GC/MS fingerprinting *Angelica sinensis* and *A. archangelica* root components for antifungal and mosquito deterrent activity. *J. Agric. Food Chem.* **2009**, *57*, 464−470.

(5) Gao, F.; Hu, Y.; Fang, G.; Yang, G.; Xu, Z.; Dou, L.; Chen, Z.; Fan, G. Recent developments in the field of the determination of constituents of TCMs in body fluids of animals and human. *J. Pharm. Biomed.* **2014**, *87*, 241−260.

(6) Tabanca, N.; Wang, M.; Avonto, C.; Chittiboyina, A. G.; Parcher, J. F.; Carroll, J. F.; Kramer, M.; Khan, I. A. Bioactivity-guided investigation of geranium essential oils as natural tick repellents. *J. Agric. Food Chem.* **2012**, *61*, 4101−4107.

(7) Tabanca, N.; Bernier, U. R.; Ali, A.; Wang, M.; Demirci, B.; Blythe, E. K.; Khan, S. I.; Baser, K. H. C.; Khan, I. A. Bioactivity-guided investigation of two *Monarda* essential oils as repellents of yellow fever mosquito *Aedes aegypti*. *J. Agric. Food Chem.* **2013**, *61*, 8573−8580.

(8) Ali, A.; Tabanca, N.; Demirci, B.; Baser, K. H. C.; Ellis, J.; Gray, S.; Lackey, B. R.; Murphy, C.; Khan, I. A.; Wedge, D. E. Composition, mosquito larvicidal, biting deterrent and antifungal activity of essential oils of different plant parts of *Cupressus arizonica* var. *glabra* (Sudw.) Little ('Carolina Sapphire'). *Nat. Prod. Commun.* **2013**, *8*, 257−260.

(9) Tabanca, N.; Bernier, U. R.; Tsikola, M.; Becnel, J. J.; Sampson, B. J.; Werle, C.; Demirci, B.; Baser, K. H. C.; Blythe, E. K.; Pounders, C.; Wedge, D. E. *Eupatorium capillifolium* essential oil: chemical composition, antifungal activity, and insecticidal activity. *Nat. Prod. Commun.* **2010**, *5*, 1409−1415.

(10) Tabanca, N.; Wedge, D. E.; Wang, X.; Demirci, B.; Baser, K. H. C.; Zhou, L.; Cutler, S. J. Chemical composition and antifungal activity of *Angelica sinensis* essential oil against three *Colletotrichum* species. *Nat. Prod. Commun.* **2008**, *3*, 1073−1078.

(11) Carroll, J. F.; Tabanca, N.; Kramer, N.; Elejalde, N. M.; Wedge, D. E.; Bernier, U. R.; Coy, M.; Becnel, J. J.; Demirci, B.; Baser, K. H. C.; Zhang, J.; Zhang, S. Essential oils of *Cupressus funebris, Juniperus communis,* and *J. chinensis* (Cupressaceae) as repellents against ticks (Acari: Ixodidae) and mosquitoes (Diptera: Culicidae) and as toxicants against mosquitoes. *J. Vector Ecol.* **2011**, *36*, 258−268.

(12) Sampson, B. J.; McLaughlin, J. L.; Wedge, D. E. Evaluating the extract of the paw paw tree extract as a botanical insecticide. *Arthropod Manage. Tests* **2003**, *28*, L5.
(13) Sampson, B. J.; Tabanca, N.; Kirimer, N.; Demirci, B.; Baser, K. H. C.; Khan, I. A.; Spiers, J. M.; Wedge, D. E. Insecticidal activity of 23 essential oils and their major compounds against adult *Aphis pseudobrassicae* (Davis) (Aphididae: Homoptera). *Pest Manage. Sci.* **2005**, *61*, 1122−1128.
(14) Wedge, D. E.; Tabanca, N.; Sampson, B. J.; Werle, C. T.; Demirci, B.; Baser, K. H. C.; Nan, P.; Duan, J.; Liu, Z. Antifungal and insecticidal activity from two *Juniperus* essential oils. *Nat. Prod. Commun.* **2009**, *4*, 123−127.
(15) Sakhanokho, H. F.; Sampson, B. J.; Tabanca, N.; Wedge, D. E.; Demirci, B.; Baser, K. H. C.; Bernier, U. R.; Tsikolia, M.; Agramonte, N. M.; Becnel, J.; Chen, J.; Rajasekaran, K.; Spiers, J. M. Chemical composition, antifungal and insecticidal activities of *Hedychium* essential oils. *Molecules* **2013**, *18*, 4308−4327.
(16) Gupta, A.; Sharma, S.; Naik, S. N. Biopesticidal value of selected essential oils against pathogenic fungus, termites and nematodes. *Int. Biodeter. Biodegr.* **2011**, *65*, 703−707.
(17) *The Pharmacopoeia Commission of P.R. China*; Chemical Industry Press: Beijing, China, 2010; pp 97 and 246.
(18) Zhang, J. R.; Xu, D. S.; Liu, L.; Jin, P. G. Effect and clinical application of radix *Angelicae Duhuricae*. *Shanghai J. TCM* **2013**, *47*, 82−83.
(19) Qiu, M. S.; Xu, M.; Chen, J. C.; Zhang, Q.; Peng, H. C.; Zhang, Y. Y.; Yang, M. Y. To observe the effect of *Angelica dahurica* combined with conventional medicine on the treatment of rheumatoid anthritis. *Rheum. Arthrit.* **2012**, *2*, 15−18.
(20) Yang, Z. B. The syndrome differentiation and treatment of headache during spring. *Bei Fang Yao Xue* **2013**, *10*, 56.
(21) Ji, Y. X.; Yu, M. The treatment of Xin Fang Bai Zhi decoction on 52 cases of allergic sinusitis. *Shi Yong Zhong Yi Yao Za Zhi* **2013**, *29*, 38.
(22) Yang, P. F.; Wang, S. N. Review of TCM mask in treating chloasma. *Clin J. Chinese Med.* **2013**, *5*, 116−118.
(23) Li, G. X.; Fa, L. L. The treatment of drying up in hands and feet with a self-made plaster of Chinese herbs. *Zhong Guo Min Jian Liao Fa* **2013**, *21*, 56.
(24) Lee, M. Y.; Seo, C. S.; Lee, J. A.; Lee, N. H.; Kim, J. H.; Ha, H.; Zhen, M. S.; Son, J. K.; Shin, H. K. Anti-asthmatic effects of *Angelica dahurica* against ovalbumin-induced airway inflammation via upregulation of heme oxygenase-1. *Food Chem. Toxicol.* **2011**, *49*, 829−837.
(25) Seo, W. D.; Kim, J. Y.; Ryu, H. W.; Kim, J. H.; Han, S. I.; Ra, J. E.; Seo, K. H.; Jang, K. C.; Lee, J. H. Identification and characterization of coumarins from the roots of *Angelica dahurica* and their inhibitory effects against cholinesterase. *J. Funct. Foods* **2013**, *5*, 1421−1431.
(26) Zaugg, J.; Eickmeier, E.; Rueda, D. C.; Hering, S.; Hamburher, M. HPLC-based activity profiling of *Angelica pubescens* roots for new positive GABAA receptor modulators in *Xenopus oocytes*. *Fitoterapia* **2011**, *82*, 434−440.
(27) Lechner, D.; Stavri, M.; Oluwatuyi, M.; Pereda-Miranda, R.; Gibbons, S. The anti-staphylococcal activity of *Angelica dahurica* (Bai Zhi). *Phytochemistry* **2004**, *65*, 331−335.
(28) Qiu, M. S.; Chen, J. C.; Xu, Z. X.; Zhang, Y. Y.; Jiang, Y. F.; Peng, H. C. Effect of Du-Huo on collagen induced arthritis in rats. *Zhong Yi Zheng-Gu* **2012**, *24*, 6−8.
(29) Zhao, D.; Zhang, X. D.; Hao, H. G.; Hu, Y.; Sun, D.; Li, H. Y.; Yang, J. X. Study on neural protective function of duhuo experimental allergic encephalomyelitis. *Chin Med. J. Res. Prac.* **2013**, *27*, 31−33.
(30) Lin, L.; Qian, X. P.; Liu, B. R. Research progress on the chemical constituents and the anti-tumor activity of *Angelica pubescitis*. *Xian Dai Zhong Liu Yi Xue* **2011**, *19*, 373−376.
(31) McLafferty, F. W.; Stauffer, D. B *The Wiley/NBS Registry of Mass Spectral Data*; J. Wiley and Sons: New York, 1989.
(32) Joulain, D.; König, W. A.; Hochmuth, D. H. Terpenoids and related constituents of essential oils. *Library of MassFinder 3.0*; Hamburg Germany, 2004.
(33) Joulain, D.; König, W. A. *The Atlas of Spectra Data of Sesquiterpene Hydrocarbons*; E.B.-Verlag: Hamburg, Germany, 1998.
(34) ESO. *The Complete Database of Essential Oils*; Boelens Aroma Chemical Information Service: Huizen, The Netherlands, 2000.
(35) White, T. J.; Bruns, T.; Lee, S.; Taylor, J. W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J., Eds.; Academic Press: New York, 1990; pp 315−322.
(36) Turbett, G. R.; Sellner, L. N. Digestion of PCR and RT-PCR products with restriction endonucleases without prior purification or precipitation. *Promega Notes Magazine 60*; 1996; p 23, http://www.promega.com/pnotes/60/6079_23/6079_23_core.pdf.
(37) Pridgeon, J. W.; Meepagala, K. M.; Becnel, J. J.; Clark, G. G.; Pereira, R. M.; Linthicum, K. J. Structure-activity relationships of 33 piperidines as toxicants against female adults of *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **2007**, *44*, 263−269.
(38) Posey, K. H.; Schreck, C. E. An airflow apparatus for selecting female mosquitoes for use in repellent and attraction studies. *Mosquito News* **1981**, *41*, 566−568.
(39) Klun, J. A.; Kramer, M.; Debboun, M. A new *in vitro* bioassay system for discovery of novel human-use mosquito repellents. *J. Am. Mosq. Control Assoc.* **2005**, *21*, 64−70.
(40) U.S. Department of Agriculture. Repellent activity of compounds submitted by Walter Reed Army Institute of Research. Part 1. Protection time and minimum effective dosage against *Aedes aegypti* mosquitoes. *Technical Bulletin 1549*; 1977.
(41) Katritzky, A. R.; Wang, Z.; Slavov, S.; Dobchev, D.; Hall, C. D.; Tsikolia, M.; Bernier, U. R.; Elejalde, N. M.; Clark, G. G.; Linthicum, K. J. Novel carboxamides as potential mosquito repellents. *J. Med. Entomol.* **2010**, *47*, 924−938.
(42) Pridgeon, J. W.; Becnel, J. J.; Clark, G. G.; Linthicum, K. J. A high-throughput screening method to identify potential pesticides for mosquito control. *J. Med. Entomol.* **2009**, *46*, 335−341.
(43) Ali, A.; Cantrell, C. L.; Bernier, U. R.; Duke, S. O.; Schneider, J. C.; Agramonte, N. M.; Khan, I. *Aedes aegypti* (Diptera: Culicidae) biting deterrence: structure activity relationship of saturated and unsaturated fatty acids. *J. Med. Entomol.* **2013**, *49*, 1370−1378.
(44) SAS Institute. *SAS OnlineDoc*, version 9.2; SAS Institute: Cary, NC, USA, 2007.
(45) Abbott, W. S. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **1925**, *18*, 265−267.
(46) Savin, N. E.; Robertson, J. L.; Jacqueline, L.; Russell, R. M. A critical evaluation of bioassay in insecticide research: likelihood ratio tests of dose-mortality regression. *Bull. Entomol. Soc. Am.* **1977**, *23*, 257−266.
(47) Zhao, A.; Yang, X.; Yang, X.; Tao, H.; Yu, J.; Wang, W. GC-MS analysis of chemical components of volatile oil from root of *Angelica dahurica*. *Yaowu Fenxi Zazhi* **2012**, *32*, 763−768.
(48) Liu, Y. X.; TaoH, Y.; Yang, Z.; Shiying, Z. GC-MS analysis of essential oils from *Angelica pubescens*. *Zhongguo Zhongyao Zazhi* **2006**, *31*, 663−666.
(49) Han, H.; Han, B. Y.; Jin, Y.; Chung, J.; Shin, S. C. A Simple PCR-RFLP for identification of *Bursaphelenchus* species collected from Korea. *Plant Pathol. J.* **2008**, *24*, 159−163.
(50) Sukrong, S.; Zhu, S.; Ruangrungsi, N.; Phadungcharoen, T.; Palanuvej, C.; Komatsu, K. Molecular analysis of the genus *Mitragyna* existing in Thailand based on rDNA ITS sequences and its application to identify a narcotic species: *Mitragyna speciosa*. *Biol. Pharm. Bull.* **2007**, *30*, 1284−1288.
(51) D'Amelio, S.; Mathiopoulos, K. D.; Santos, C. P.; Pugachev, O. N.; Webb, S. C.; Picanco, M.; Paggi, L. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. *Int. J. Parasitol.* **2000**, *30*, 223−226.
(52) Abbas, A.; Tabanca, N.; Kurkcuoglu, M.; Duran, A.; Blythe, E. K.; Khan, I. A.; Baser, K. H. C. Chemical composition, larvicidal and biting deterrent activity of essential oils of two subspecies of

Tanacetum argenteum (Lam.) Willd and individual constituents against *Aedes aegypti* (L) (Diptera: Culicidae). *J. Med. Entomol.* **2014**, *51*, 824−830.

(53) Tabanca, N.; Avonto, C.; Wang, M.; Parcher, J. F.; Ali, A.; Demirci, B.; Khan, I. A. Comparison of chemical composition and mosquito activity of *Umbellularia californica* and *Laurus nobilis* leaf essential oils and the active compounds against *Aedes aegypti*. *J. Agric. Food Chem.* **2013**, *61*, 12283−12291.

(54) Sinniah, B. Insecticidal effect of aliphatic alcohols against aquatic stages of *Aedes* mosquitoes. *Trans. R. Soc. Trop. Med. Hyg.* **1983**, *77*, 35−38.

(55) Hammond, D. G.; Kubo, I. Structure-activity relationship of alkanols as mosquito larvicides with novel findings regarding their mode of action. *Bioorg. Med. Chem.* **1999**, *7*, 271−278.