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Chemical Composition and Antifungal Activity of *Arnica longifolia*, *Aster hesperius*, and *Chrysothamnus nauseosus* Essential Oils

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Essential oils from three different Asteraceae obtained by hydrodistillation of aerial parts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS). Main compounds obtained from each taxon were found as follows: *Arnica longifolia* carvacrol 37.3%, α-bisabolol 8.2%; *Aster hesperius* hexadecanoic acid 29.6%, carvacrol 15.2%; and *Chrysothamnus nauseosus* var. *nauseosus* β-phellandrene 22.8% and β-pinene 19.8%. Essential oils were also evaluated for their antimalarial and antimicrobial activity against human pathogens, and antifungal activities against plant pathogens. No antimalarial and antimicrobial activities against human pathogens were observed. Direct bioautography demonstrated antifungal activity of the essential oils obtained from three Asteraceae taxa and two pure compounds, carvacrol and β-bisabolol, to the plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides*. Subsequent evaluation of antifungal compounds using a 96-well micro-dilution broth assay indicated that α-bisabolol showed weak growth inhibition of the plant pathogen *Botrytis cinerea* after 72 h.

KEYWORDS: ***Arnica longifolia*; *Aster hesperius*; *Chrysothamnus nauseosus*; hexadecanoic acid; carvacrol; *β*-phellandrene; *β*-pinene; α-bisabolol; *Colletotrichum acutatum*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides***

INTRODUCTION

The continuing development of fungicidal resistance in plant and human pathogens necessitates the discovery and development of new fungicides. Because biologically derived chemicals are perceived by consumers as having less environmental toxicity and lower mammalian toxicity, chemical companies currently have a greater desire to discover and develop natural product-based plant protectans (*1*). In a program aimed at identifying natural fungicides as alternatives to conventional synthetic agrochemicals, the biological activities of the essential oils from three Asteraceae were investigated in the present study.

Asteraceae plants have a long history of use in folk medicine in many cultures (*2*). In this study, we focused our investigation on the essential oils and their biological activity of three members of the Asteraceae known for their medicinal use collected from the Pacific Northwest region of the United States: *Arnica longifolia* D.C. Eaton, *Aster hesperius* A. Gray, and *Chrysothamnus nauseosus* (Pall.) Britt. var. *nauseosus*.

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Arnica extracts and salves have been used to treat bruises, sprains, rheumatism, and joint problems (*3, 4*). The genus *Arnica* is best known for the European medicinal species *Arnica montana* where extracts and tinctures are manufactured for the German herbal market (*3*). In the Pacific Northwest region of the United States, 14 species of *Arnica* including *A. longifolia* are distributed from Canada south to California and east to Colorado. Previous phytochemical studies of *A. longifolia* demonstrated several major volatile compounds: T-muurolol (from rhizomes) and 4-hydroxythymoldimethylether, T-muurolol, thymol, and thymolmethylether (from flowers) (*5*).

Aster also known as willow or marsh aster has been used in Chinese folk medicine to treat fever, cold, tonsillitis, snake bites, and bee stings (*7*). Triterpene glycosides were reported as the main compounds in *Aster subspicatus* and *As. ageratoides* (*6*). *Aster hesperius* has worldwide distribution but grows in North America stretching from the Canadian mountains south into Idaho and Nevada and east to Wisconsin and Missouri (*8*). There is no report on the essential oil composition of *Aster hesperius*.

Chrysothamnus nauseosus, known as rabbit brush, has a history of ethnobotanical uses (*9*). Wood bark was used by the American Indians as chewing gum (*10*), and plant extracts were used as teas or syrups to treat coughs and chest pain (*10*).

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Chrysothamnus nauseosus is a complex and variable species in the United States within which six taxonomic varieties have been identified (*11*). *Chrysothamnus nauseosus* var. *nauseosus* has been studied phytochemically by Hegerhost et al. (*11*) and several varieties of *C. nauseosus* contained β-pinene, myrcene, and β-phellandrene as major compounds.

We report for the first time the essential oil composition, biological activity, and GC and GC/MS fingerprints for *Aster hesperius*, *Chrysothamnus nauseosus* var. *nauseosus*, and *Arnica longifolia*.

MATERIALS AND METHODS

General. Pure essential oil compounds (carvacrol, β-eudesmol, α-bisabolol, hexadecanoic acid, and β-pinene) (>95%, Aldrich-Sigma, St., Louis, MO); fungicide technical grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc. West Chester, PA); antimalarial standards chloroquine (Aldrich-Sigma, St., Louis, MO) and artemisinin (Aldrich-Sigma, St., Louis, MO); and antimicrobial standards ciprofloxacin (ICN Biomedicals, Aurora, OH) for bacteria and amphotericin B (ICN Biomedicals, Aurora, OH) for fungi were purchased from commercial sources. In order to isolate β-phellandrene, *Schinus molle* oil was obtained from Erdoğmuş Parfüm Sanayi, Istanbul.

Plant Material. *Arnica longifolia* D.C. Eaton samples were collected from Lemhi Co., ID (Crockett NW28), *Aster hesparius* A. Gray samples were collected from Idaho Co. ID (Crockett NW6), and *Chrysothamnus nauseosus* (Pall.) Britt. var. *nauseosus* samples were collected from Blaine Co., ID (Crockett NW16). All samples were collected in August 2002. Authenticated voucher specimens were deposited at the University of Mississippi (UMISS) herbarium. Aerial portions of the plants, consisting of the flowers, inflorescence and bracts, and upper stems with leaves were collected while in full flower. Plant materials were dried to a moisture content of less than 2% in ventilated cabinets in the dark and then used for subsequent phytochemical analysis.

Isolation of the Essential Oils and β-Phellandrene. Essential oils were hydrodistilled from dried aerial parts for 3 h using a Clevenger apparatus to obtain essential oils (*12*). The yields were calculated on a dry weight basis (**Table 1**).

Since β-phellandrene is not available commercially, we prepared a β-phellandrene standard from *Schinus molle* oil. *Schinus molle* oil (100 mg) was subjected to a high performance flash chromatography system (HPFC, Biotage, Inc., A Dynax Corp. Company) using a Biotage SI 12M column (150 mm × 12 mm i.d.; 9 g of KP-Sil silica; 40–63 μm particle size; flow rate 5.0 mL/min) and eluted with *n*-hexane 100%, *n*-hexane–Et_2O mixtures 5%, 20%, 50%, and 80% (120 mL and 3 mL each eluent), and 100% EtOAc. Similar fractions according to TLC profiles (*n*-hexane/diethyl ether (95:5, 90:10, 85:15, 80:20, 70:30 v/v) were combined to give 15 pooled samples (fractions A1–A15, each 3 mL). Fraction A1 gave β-phellandrene (3.0 mg). β-Phellandrene was reanalyzed by GC/MS to confirm its identity.

Gas Chromatography Analysis Conditions. Essential oils were analyzed by GC using a Hewlett Packard 6890 system (SEM Ltd., Istanbul, Turkey), and an HP Innowax FSC column (60 m × 0.25 mm ∅, with 0.25 μm film thickness) was used with nitrogen at 1 mL/min. Initial oven temperature was 60 °C for 10 min, and it was increased at 4 °C/min to 220 °C, then kept constant at 220 °C for 10 min and increased at 1 °C/min to 240 °C. Injector temperature was set at 250 °C. Percentage compositions of the individual components were obtained from electronic integration using flame ionization detection (FID, 250 °C). *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI) (*13–15*). Relative percentages of the separated compounds were calculated from FID chromatograms as cited in **Table 1**.

Gas Chromatography–Mass Spectrometry Analysis Conditions. GC/MS analysis was performed with a Hewlett-Packard GCD, system (SEM Ltd., Istanbul, Turkey), and Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium. GC oven temperature conditions were as described above, split flow was adjusted at 50 mL/min, and the injector temperature was at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 425.

Identification of the essential oil components were carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of *n*-alkanes (*13–15*). Computer matching against commercial (Wiley and MassFinder 2.1) (*16*, *17*) and in-house ("Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils), as well as MS literature data (*18–20*), was also used for the identification.

Assay for Antimalarial Activity. The *in vitro* antimalarial activity was determined against the D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) clones of *Plasmodium falciparum*. The assay was based on the determination of parasite lactic acid dehydrogenase (LDH) activity using Malstat reagent and was performed as described earlier (*21*). Chloroquine (Aldrich-Sigma, ST, Louis, MO) and artemisinin (Aldrich-Sigma, ST, Louis, MO) were included as standards in each assay.

Assay for Antimicrobial Activity. Antimicrobial activity was determined against *Candida albicans* (ATCC 90028), *Cryptococcus neoformans* (ATCC 90113), *Aspergillus fumigatus* (ATCC 90906), *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *S. aureus* (ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), and *Mycobacterium intracellulare* (ATCC 23068) using a modified version of the Clinical and Laboratory Standards Institute (CLSI), formerly National Committe for Clinical Laboratory Standards (NCCLS), methods (*22–25*) as reported previously (*21*). Antimicrobial standards ciprofloxacin (ICN Biomedicals) for bacteria and amphotericin B (ICN Biomedicals) for fungi were included as positive control in each assay.

TLC Analysis. Thin layer chromatography (TLC) analysis was conducted by using precoated silica gel 60 F_{254} (Merck, Suwanee, GA). Four microliters of 20 mg/mL samples in *n*-hexane were applied to plate using capillary pipettes (Fisher Science, Norcross, GA). The TLC plate was developed in a presaturated solvent chamber (*n*-hexane–Et_2O, 9:1; 8:2 7:3, v/v). TLC plate was air-dried, inspected under UV light (254 nm), and visualization was achieved by vanillin–sulfuric acid reagent (1 g of vanillin in 100 mL of 20% H_2SO_4 in EtOH) and heat. This plate was used as a reference plate. Multiple plates for direct bioautography were developed sequentially using the protocol described.

Direct Bioautography Assay for Activity against Plant Pathogenic Fungi. Pathogen production and bioautography procedures of Tabanca et al. (*21*), Meazza et al. (*26*), and Fokialakis et al. (*27*) were used to evaluate antifungal activity against fungal plant pathogens. Sensitivity of each fungal species to each test compound was determined 4 days after treatment by comparing size of inhibitory zones, affording means, and standard deviations of inhibitory zone size were used to evaluate antifungal activity of test compounds. Bioautography experiments were performed multiple times using both dose– and non-dose–response formats. Fungicide technical grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc. West Chester, PA) were used as controls at 2 mM in 2 μL of 95% ethanol. Pure essential oil compounds were applied at 2–32 μL of 2 mM concentrations in *n*-hexane. *Arnica longifolia*, *Aster hesperius*, and *Chrysothamnus nauseosus* var. *nauseosus* essential oils were applied as a 20 mg/mL in 4 μL of *n*-hexane onto TLC plates. Antifungal activity can be visualized directly on the TLC plate as "clear zones" where no fungal mycelia, stroma, or condia grow (*28*). Zones with "diffuse inhibition" are "growth suppressive" in nature and mycelia, stroma, or condia grow at a reduced level. Fungal growth inhibition means for compounds and essential oils were analyzed separately by ANOVA using SAS software, version 8. Mean separations were performed based on Fisher's protected least significant difference (LSD) ($P = 0.05$). Statistical comparisons were made for fungal growth across compounds and of compound across fungal growth.

Microdilution Broth Assay against Plant Pathogenic Fungi. A standardized microdilution broth assay in a 96-well microtiter format was developed by Wedge and Kuhajek (*29*) and subsequently used by Tabanca et al. (*21*) to evaluate antifungal activity of isolated compounds against *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*,

Table 1. The Composition of the Essential Oils[a] of *A. longifolia* (AL), *As. hesparius* (AH), and *C. nauseosus* var. *nauseosus* (CN)

RRI[b]	compound	AL	AH	CN	characterization method[f]	RRI[b]	compound	AL	AH	CN	characterization method[f]
1032	α-pinene			1.1	a,b	1868	(*E*)-geranyl acetone		0.2		a
1035	α-thujene			0.8	a	1871	1-undecanol		0.2		a
1118	β-pinene		*c*	19.8	a,b	1871	neryl isovalerate	0.4			a
1132	sabinene			5.7	a,b	1878	2,5-dimethoxy-*p*-cymene	0.2			a
1174	myrcene			0.9	a,b	1893	italicene			0.1	a
1176	α-phellandrene			*c*	a,b	1893	geranyl isovalerate	*c*			a
1188	α-terpinene			1.0	a,b	1895	italicene ether		0.2		a
1195	dehydro-1,8-cineole			0.6	a	1900	*epi*-cubebol	0.4	0.1	0.1	a
1203	limonene			4.7	a,b	1904	geranyl 2-methyl butyrate	0.1			a
1213	1,8-cineole	0.1	0.1		a,b	1912	*cis*-dihydrocarveol			0.1	a
1218	β-phellandrene			22.8	a,b	1933	neryl valerate	0.1			a
1246	(*Z*)-β-ocimene			*c*	a	1933	tetradecanal		0.3		a
1255	γ-terpinene			2.6	a,b	1941	α-calacorene	0.1	0.1	0.2	a
1266	(*E*)-β-ocimene			0.3	a	1945	1,5-epoxy-salvial(4)14-ene	0.4	0.4		a
1280	*p*-cymene	0.1	0.1	0.6	a,b	1957	cubebol	0.5			a
1290	terpinolene			0.4	a,b	1958	(*E*)-β-ionone	0.1	0.7		a
1358	artemisia ketone			0.1	a	1973	dodecanol	0.1	0.2		a
1400	tetradecane		*c*		a,b	2001	Isocaryophyllene oxide	0.2	0.2	0.2	a,b
1403	yomogi alcohol	0.1			a	2008	caryophyllene oxide	4.0	3.9	0.2	a,b
1460	2,6-dimethyl-1,3(*E*),5(*E*),7-octatetraene			0.1	a	2029	perilla alcohol			0.1	a
1466	α-cubebene			0.1	a	2037	salvial-4(14)-en-1-one	1.1	0.5		a
1474	*trans*-Sabinene hydrate	*c*		0.6	a	2045	carotol		1.9		a
1476	(*Z*)-β-ocimene epoxide	0.1			a	2050	(*E*)-Nerolidol	1.0	0.1		a,b
1497	α-copaene	*c*		0.2	a	2053	germacrene D 1,10-epoxide	0.1			a
1506	decanal	0.1			a	2071	humulene epoxide-II	0.5	0.7	0.1	a
1510	artemisia alcohol	*c*			a	2073	*p*-mentha-1,4-dien-7-ol			0.1	a
1532	camphor	0.3	0.1	0.3	a,b	2080	cubenol	0.3		0.1	a
1541	benzaldehyde	*c*	0.1		a,b	2088	1-*epi*-cubenol		0.1	0.2	a
1545	*cis*-α-bergamotene			0.1	a	2092	β-oplopenone	0.2			a
1553	linalool	0.1	0.1		a,b	2096	elemol			1.3	a
1556	*cis*-sabinene hydrate	0.1		0.5	a	2104	viridiflorol			0.3	a
1562	octanol	0.1			a,b	2113	cumin alcohol			0.4	a,b
1571	*trans*-*p*-menth-2-en-1-ol	*c*		0.8	a	2131	hexahydrofarnesyl acetone		1.7	0.1	a
1586	pinocarvone	0.2	*c*	0.2	a,b	2144	spathulenol	3.3	2.0	0.2	a,b
1590	bornyl acetate	0.2	0.3		a,b	2148	(*Z*)-3-hexen-1-yl benzoate	0.5			a
1594	*trans*-β-bergamotene			0.2	a	2156	α-bisabolol oxide B	0.5			a
1600	hexadecane		0.1		a,b	2174	fokienol	0.2			a
1601	nopinone			0.1	a	2179	tetradecanol		0.4		a,b
1604	thymol methyl ether	0.3			a,b	2183	γ-decalactone			0.3	a
1611	terpinen-4-ol	0.3	0.1	2.4	a,b	2185	γ-eudesmol	0.1			a
1612	β-caryophyllene	0.5	0.1	0.1	a,b	2187	T-cadinol			0.4	a
1638	*cis*-*p*-menth-2-en-1-ol			0.6	a	2192	nonanoic acid		0.2		a,b
1648	myrtenal	0.1	0.1	0.2	a	2204	eremoligenol			0.3	a
1651	sabinaketone			0.1	a	2198	thymol	1.4	0.3		a,b
1662	pulegone	0.1	0.1	0.2	a,b	2209	T-muurolol	2.7		0.3	a
1664	nonanol	0.1			a,b	2214	ar-turmerol			0.1	a
1670	*trans*-pinocarveol	0.4	0.3	0.5	a	2219	δ-cadinol			0.1	a
1683	*trans*-verbenol	0.3			a	2232	α-bisabolol	8.2	0.8		a,b
1687	α-humulene	0.3	*c*		a,b	2239	carvacrol	37.3	15.2	2.5	a,b
1689	*trans*-piperitol			0.2	a	2247	*trans*-α-bergamotol	0.1			a
1690	cryptone		0.1	2.6	a	2250	α-eudesmol			0.4	a
1700	*p*-mentha-1,8-dien-4-ol (limonen-4-ol)	*c*			a	2251	1-methylethyl hexadecanoate [d]		0.6		a
1704	γ-muurolene	0.2	0.1	0.3	a	2257	β-eudesmol	0.2	2.5	7.7	a,b
1706	α-terpineol	0.2	0.3	0.1	a,b	2298	decanoic acid		0.5		a,b
1719	borneol	0.3	0.2		a,b	2300	tricosane	1.2	0.4		a,b
1726	germacrene D	0.9			a,b	2300	γ-undecalactone				a
1740	α-muurolene	0.2	0.5	0.5	a	2324	caryophylla-2(12),6(13)-dien-5α-ol (caryophylladienol II)			0.1	a
1744	phellandral			1.2	a,b	2369	(2*E*,6*E*)-farnesol		0.8		a
1748	piperitone			0.1	a,b	2369	eudesma-4(15),7-dien-1β-ol	3.4	1.2		a
1751	carvone	0.1		0.1	a,b	2392	caryophylla-2(12),6-dien-5β-ol (caryophyllenol II)	1.3	1.7		a
1758	*cis*-piperitol			0.2	a	2396	γ-dodecalactone			0.3	a
1773	δ-cadinene	0.4		0.8	a	2500	pentacosane	0.4	1.2	0.1	a,b
1776	γ-cadinene	0.1	0.2	0.2	a	2503	dodecanoic acid	0.2	1.7		a,b
1779	(*E*,*Z*)-2,4-decadienal		0.1		a	2533	γ-costol	0.4			a
1786	neryl propionate	0.2			a	2600	hexacosane		0.1		a,b
1786	*ar*-curcumene		0.6	1.1	a	2617	tridecanoic acid		0.3		a,b
1799	cadina-1,4-diene (cubenene)			0.1	a	2622	phytol		1.8		a
1802	cumin aldehyde			0.5	a,b	2670	tetradecanoic acid	1.2	4.8		a,b
1804	myrtenol	*c*	0.4	0.3	a	2822	pentadecanoic acid	0.4	1.7		a,b
1808	nerol	0.5			a,b	2857	palmito-γ-lactone		0.3		a
1811	*p*-mentha-1,3-dien-7-al			0.2	a	2900	nonacosane		0.2	0.1	a,b
1819	geranyl isobutyrate	0.4			a	2931	hexadecanoic acid	1.4	29.6	*c*	a,b
1823	*p*-mentha-1(7),5-dien-2-ol			*c*	a		monoterpene hydrocarbons	0.1	0.1	60.7	
1827	(*E*,*E*)-2,4-decadienal	*c*	*c*		a		oxygenated monoterpenes	44.3	17.6	15.9	
1829	octyl hexanoate		0.1		a		sesquiterpene hydrocarbons	3.0	1.9	4.2	
1838	(*E*)-β-damascenone	0.1			a		oxygenated sesquiterpenes	29.1	17.1	12.2	
1849	calamenene		0.1	0.2	a		others	5.9	47.5	1.0	
1857	geraniol	0.7			a,b		identified compounds (%)	82.4	84.2	94.0	
1864	*p*-cymen-8-ol		0.1		a,b		oil yield[e] (%)	*c*	*c*	0.77	
1868	10-*epi*-italicene ether			0.1	a						

[a] % calculated from FID data. [b] Relative retention indices calculated against *n*-alkanes. [c] Trace (< 0.1 %). [d] Syn: isopropyl palmitate. [e] Essential oil yields are given on moisture free basis (v/w). [f] Method a, comparison of mass spectra with the Wiley and Mass Finder libraries and retention times; method b, comparison with genuine compounds on the HP Innowax column.

Fusarium oxysporum, *Botrytis cinerea*, and *Phomopsis obscurans*. Azoxystrobin was used as a commercial fungicide standard. Each fungus was challenged in a dose–response format using test compounds where the final treatment concentrations were 0.3, 3.0, and 30.0 μM. Microtiter plates (untreated, Nunc MicroWell, Roskilde, Denmark) were covered with a plastic lid and incubated in a growth chamber as described previously. Fungal growth was then evaluated by measuring absorbance of each well at 620 nm using a microplate photometer (Packard Spectra Count, Packard Instrument Co., Downers Grove, IL). Mean growth inhibition and standard errors for each fungus at each dose of test compound were used to evaluate fungal growth. Each test fungicide and standard was run in duplicate at each concentration, and the experiment was repeated independently three times.

RESULTS AND DISCUSSION

Essential oils were obtained by hydrodistillation from the aerial parts of *A. longifolia*, *As. hesperius*, and *C. nauseosus* var. *nauseosus*. Each essential oil was subsequently analyzed using both GC and GC/MS systems in which the individual components were identified according their relative retention indices with their relative percentages (see **Table 1**).

A. longifolia oil was found to be rich in carvacrol (37.3%) and α-bisabolol (8.2%). Seventy-nine compounds were characterized and represented 82.4% of the total oil. Although T-muurolol was not identified in the total oil as a major volatile constituent, as it had been in previous studies (*5*), it was found as a minor constituent (2.7%). The volatile extract was dominated by oxygenated monoterpenes (44.3%) where oxygenated sesquiterpenes also showed a high proportion with 29.1% and contained 0.1% monoterpene hydrocarbons.

Sixty-nine individual compounds were characterized in *As. hesperius* oil and represented 84.2% of the total essential oil. This oil was characterized by a high content of hexadecanoic acid (29.6%) and carvacrol (15.2%). Hydrocarbons such as alkanes and fatty acids classified as "others" constituted 47.5% of the essential oil, followed by oxygenated monoterpenes and oxygenated sesquiterpenes, with 17.6% and 17.1%, respectively (**Table 1**). *As. hesperius* essential oil also contained a low amount of monoterpene hydrocarbons (0.1%). To the best of our knowledge, this is the first report of the essential oil composition of *As. hesperius*.

Oil obtained from *C. nauseosus* var. *nauseosus* contained 83 compounds and represented 94% of the total oil. This oil was characterized by the major compounds β-phellandrene (22.8%) and β-pinene (19.8%), and these amounts are in agreement with previous studies of Hegerhost et al. (*11*). Monoterpene hydrocarbons constituted 60.7% of the *Chrysothamnus* oil, followed by 15.9% oxygenated monoterpenes, and 12.2% oxygenated sesquiterpenes. Our phytochemical data on major compounds in *C. nauseosus* var. *nauseosus* are consistent with those reported for *C. nauseosus* var. *albicaulis* and showed the presence of β-pinene, limonene, and β-phellandrene; *C. nauseosus* var. *consimilus* contained limonene, β-phellandrene, and β-ocimene; and *C. nauseosus* var. *glabratus* contained β-pinene, myrcene, limonene, and β-phellandrene (*30*).

As part of our effort to investigate the biological activity of essential oils, we evaluated *A. longifolia*, *As. hesperius*, and *Chrysothamnus nauseosus* var. *nauseosus* for their antimalarial and antimicrobial activities against human pathogenic microorganisms, as well as their antifungal activity against selected plant pathogenic fungi. No antimicrobial activity was observed at the highest test concentration of 200 μg/mL against *Candida albicans*, *Ca. glabrata*, *Ca. krusei*, *Cryptococcus neoformans*, methicillin-resistant *Staphylococcus aureus*, *Mycobacterium intracellulare*, and *Aspergillus fumigatus* for these three oils using the method previously described (*21*). *A. longifolia*, *As. hesperius*, and *C. nauseosus* var. *nauseosus* oils were inactive up to 4.76 μg/mL against *Plasmodium falciparum* D6 (chloroquine sensitive) and W2 (chloroquine resistant) clones.

Table 2. Antifungal Activity of *Arnica longifolia* (AL), *Aster hesparius* (AH), and *Chrysothamnus nauseosus* var. *nauseosus* (CN) Essential Oils and Pure Compounds Using Direct Bioautography against Three *Colletotrichum* Species[a]

	Mean fungal growth inhibition (mm)			
sample	*C. acutatum*	*C. fragariae*	*C. gloeosporioides*	$LSD_{0.05}$
AL essential oil	10.3 cd, A	8.7 f, A	9.0 c, A	2.3
AH essential oil	15.7 a, A	12.3 d, A	15.7 b, A	4.4
CN essential oil	13.3 ab, A	11.0 e, A	9.3 c, A	7.7
β-pinene[b]	0e, A	0h, A	0e, A	0
β-phellandrene[b]	0 e, A	0 h, A	0 e, A	0
β-eudesmol[b]	0 e, A	0 h, A	0 e, A	0
α-bisabolol[b]	8.0 a, A	6.5 g, AB	5.5 d, B	1.8
Hexadecanoic acid[b]	0 e, A	0 h, A	0 e, A	0
$LSD_{0.05}$	3.0	1.2	2.1	
Benomyl[c]	*d*	21.3 b	*d*	
Captan[c]	11.5 bc, C	15.0 c, B	18.9 a, A	1.6
Cyprodinil[c]	*d*	*d*	*d*	
Azoxystrobin[c]	*d*	26.0 a	*d*	

[a] Mean values followed by different letters within column (lower case, by fungus) and within the row (upper case, by chemical) are significantly ($P = 0.05$) different as determined by LSD. AL, AH, and CN essential oils were applied as a 20 mg/mL in 4 μL sample onto a silica TLC plate. Mean inhibitory zones were used to determine the level of antifungal activity against each fungal species. [b] Pure compounds were applied 4 μL of 2 mM in acetone. [c] Technical grade agrochemical fungicides (without formulation) with different modes of action were used as internal standards at 2 μL of 2 mM in EtOH. [d] Diffuse inhibitory zones indicate compounds that appear to be suppressive in nature.

Samples were also evaluated against three plant fungal pathogens of *Colletotrichum* species using a direct-bioautography assay. Essential oils showed comparable activity at 20 mg/mL, using a 4 μL test volume, against *Colletotrichum acutatum*, *Co. fragariae*, and *Co. gloesporides* as compared with the commercial fungicide standards (**Table 2**). Since all three essential oils showed antifungal activity, it was of interest to identify their major constituents using TLC bioautography. Pure standards of β-phellandrene, β-pinene, carvacrol, β-eudesmol, α-bisabolol, and hexadecanoic acid were subsequently spotted onto the TLC plate in a 2 mM concentration using the direct bioautography assay against three *Colletotrichum* species. Only α-bisabolol and carvacrol demonstrated nonselective activity against all three *Colletotrichum* species. Demirci et al. (*31*) and Altintaş et al. (*32*) have previously reported that carvacrol possesses antifungal activity against all three *Colletotrichum* species. Altintaş et al. (*32*) recently demonstrated that carvacrol at 30 μM showed 36.9% growth inhibition of *B. cinerea* in a 96-well microdilution broth assay. Carvacrol at 30 μM did not have antifungal activity against any of the three *Colletotrichum* species, *Phomopsis obscurans*, and *Fusarium oxysporum* in the microdilution assay in that study (*32*). Therefore, in the present study, carvacrol has not been tested against these fungal species. Direct bioautography (**Table 3**) demonstrated nonselective activity of α-bisabolol in a dose-dependent manner with inhibitory zones from 5 to 11.5 mm against the three *Colletotrichum* species. This is probably due to diffusion effects on the TLC plate. The inhibitory activity of 12 μL of 2 mM α-bisabolol was close to the inhibitory activity observed for 2 μL of 2 mM captan. Captan is a well known multisite inhibitor fungicide with no systemic activity and is used as a protectant fungicide in commercial strawberry production to prevent anthracnose of fruits and plants caused by *Colletotrichum*

Table 3. Antifungal Activity of α-Bisabolol in a Dose–Response from 2 to 12 μL of 2 mM Concentration Using Direct Bioautography against Three *Colletotrichum* Species[a]

samples	Mean fungal growth inhibition (mm)		
	C. acutatum	*C. fragariae*	*C. gloeosporoides*
2 μL α-bisabolol	5.5 d	5.0 e	5.0 e
4 μL α-bisabolol	8.0 cd	6.5 de	5.5 de
6 μL α-bisabolol	7.5 cd	7.0 de	7.5 cde
8 μL α-bisabolol	7.5 cd	8.0 cde	8.0 cd
10 μL α-bisabolol	9.5 bc	9.5 cd	9.0 bc
12 μL α-bisabolol	11.0 b	11.5 bc	11.5 ab
benomyl	*b*	19.5 a	*b*
captan	14.0 a	13.5 b	13.0 a
cyprodinil	*b*	23.0 a	*b*
azoxystrobin	*b*	23.0 a	*b*
LSD	2.83	3.63	2.75

[a] Mean values followed by different letters within column by fungus are significantly ($P = 0.05$) different as determined by LSD. [b] Diffuse inhibitory zones indicate compounds that appear to be suppressive in nature.

species (*29*). α-Bisabolol was subsequently evaluated in a 96-well microdilution broth assay against six fungal species. The microdilution broth assay indicated that 0.3 and 3.0 μM α-bisabolol caused 46.5% and 47.3% growth inhibition of *Botrytis cinerea* at 72 h. Because α-bisabolol appeared to precipitate at 30 μM in the aqueous based microdilution assay, we were unable to further evaluate this lipophillic compound in the microdilution broth assay. α-Bisabolol showed no antifungal activity at micromolar concentrations in the microtiter assay against any of the three *Colletotrichum* species or *Phomopsis obscurans* or *Fusarium oxysporum*. Essential oils are hydrophobic and insoluble in aqueous-based antimicrobial disc diffusion and microtiter bioassays. Direct bioautography on silica gel is our preferred primary screening bioassay for lipophillic compounds as fungicides for agricultural use and more closely mimics a leaf surface than most aqueous-based bioassays (*33*). TLC bioautography of essential oil from *As. hesperius* and *A. longifolia* indicated the presence of two clear zones where antifungal compounds matched the retention factor of the carvacrol and α-bisabolol standards (**Table 2**). TLC bioautography of essential oil from *C. nauseosus* var. *nauseosus* indicated the presence of a single zone where the antifungal compound matched the retention factor of the carvacrol standard. Essential oils are complex mixtures obtain by distillation of a large number of volatile constituents from the plant. Even though carvacrol is twice the concentration (37.3%, **Table 1**) in *A. longifolia* as in *As. hesperius* (15.2%, **Table 1**), the antifungal activity is lower in *A. longifolia* than in *As. hesperius* across all three *Colletotrichum* species (**Table 2**). From past studies, we know that monoterpene hydrocarbons such as β-pinene and β-phellandrene in *C. nauseosus* do not show antifungal activity. This confirms the lack of inhibion of these compounds and β-eudesmol to *Colletotrichum* in this study (**Table 2**). Carvacrol was 2.5% in *C. nauseosus* oil, and antifungal activity of *C. nauseosus* essential oil is intermediate between those of *As. hesperius* and *A. longifolia*. We believe that unraveling biological activity is complex, but there appears to be synergy between components of the crude essential oil that increases the antifungal activity when compared with pure standards tested at the same concentration. Plants compartmentalize many toxic substances, and upon pathogen attack, mechanical or insect damage, or stress, these compounds once released from their cellular compartments mix and become more toxic making the combined components defensive in nature. While essential oils were thought to assist plants in surviving and adapting to arid environments, our studies indicate that essential oils from numerous plant species contain considerable levels of antifungal substances (*31*, *33*, *34*).

In conclusion, this study demonstrates *in vitro* antifungal activity of essential oils from three traditional medicinal plants from the Asteraceae family against important plant pathogens for the first time. The successful discovery and development of new natural product fungicides are totally dependent upon the availability of high-quality miniaturized antifungal assays. Bioautography provides a simple technique to visually follow antifungal components through the separation process. The 96-well microbioassay allows researchers to effectively test extracts and compounds in microgram quantities, to determine dose–response relationships, and to compare observed antifungal activities with those of fungicides with known modes of action.

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