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Characterization of Volatile Constituents of *Haplopappus greenei* and Studies on the Antifungal Activity against Phytopathogens

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Essential oil of *Haplopappus greenei* A. Gray was obtained by hydrodistillation of aerial parts, which were subsequently analyzed by gas chromatography and gas chromatography−mass spectrometry. Major components were identified as carvacrol (8.7%), β-pinene (7.6%), *trans*-pinocarveol (6.2%), and caryophyllene oxide (5.8%), respectively. In total, 104 components representing 84.9% of the investigated essential oil were characterized. Furthermore, the essential oil was evaluated for antimalarial, antimicrobial, and antifungal activities. However, only antifungal activity was observed against the strawberry anthracnose-causing fungal plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides* using the direct overlay bioautography assay. Major essential oil components were also evaluated for antifungal activity; the carvacrol standard demonstrated nonselective activity against the three *Colletotrichum* species and the other compounds were inactive.

KEYWORDS: ***Haplopappus greenei*; Asteraceae; essential oil; GC-MS; carvacrol; β-pinene; strawberry pathogenic fungi; biological activity; antifungal activity**

INTRODUCTION

The genus *Haplopappus* Cass, which belongs to the family of Asteraceae, is characterized by perennial herbs or shrubs with yellow flowers and is distributed in North and South America. Some *Haplopappus* species are used in Chilean folk medicine as choleretics and cholagogues (*1*, *2*). Furthermore, the exudates of the various *Haplopappus* species are used to treat infected wounds and gastrointestinal infections, to promote wound healing, and as digestive stimulants (*3*).

Haplopappus greenei A. Gray [= *Ericameria greenei* (A. Gray) G.L. Nesom, *H. bloomeri* (Gray) var. *greenei*, *Macronema greenei* (Gray)] is commonly known as "Greene's goldenweed". This species produced a significant amount of resinous exudates from the twigs and leaves (*1*, *2*). Previous phytochemical investigations of *Haplopappus* species have revealed the presence of triterpenes (*4*, *5*), diterpenes (*3*, *6*−*14*), flavonoids (*5*, *15*−*20*), coumarins (*1*, *12*, *16*, *20*, *21*), monoterpenes (*22*, *23*), and sesquiterpenes (*22*−*26*).

In the published literature only a few volatile compounds from *Haplopappus* species have been reported (*23*−*25*). For example, α-pinene, β-phellandrene, and phellandral were found as major compounds in the steam-distilled oil of the leaves and stems of *H. laricifolius* (*24*). Isocomene, modhelphene, 1,2,3,4-tetrahydro-1,1,5,6-tetramethylnaphthalene, β-caryophyllene, caryophyllene oxide, limonene, borneol, bornyl acetate, and carvone were reported in the *H. heterophyllus* volatile oil (*25*). Urzua et al. described the chemical composition of the resinous exudates from *H. foliosus* and *H. uncinatus* and identified a number of monoterpenes, sesquiterpenes, hydrocarbons, and phenyl propanoids (*23*). Urzua et al. recently reported the antibacterial diterpenoids of the resinous exudate (*3*). Several groups investigating the biological activities of *Haplopappus* species have reported antioxidant, antimicrobial, and antibacterial activities (*2*, *3*, *13*, *21*, *27*, *28*).

The aim of this study was to evaluate the antimalarial, antimicrobial, and antifungal activities of *H. greenei* essential oil and its major constituents for activity against various plant and human pathogenic microorganisms. Furthermore, to the best of our knowledge we are reporting for the first time the volatile constituents of *H. greenei* characterized by gas chromatography (GC) and gas chromatography−mass spectrometry (GC-MS).

MATERIALS AND METHODS

General. Pure essential oil compounds (β-pinene, carvacrol, *trans*-pinocarveol, and caryophyllene oxide) (>95%, Aldrich-Sigma, St. Louis, MO) and fungicide technical grade standards benomyl, cyprodi-

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Table 1. Composition of the Essential Oil of *H. greenei*

no.	compound	RRI[a]	%[b]	ID method[c]	no.	compound	RRI[a]	%[b]	ID method[c]
1	α-pinene	1032	0.3	GC-MS	**55**	perilla aldehyde	1807	0.1	GC-MS
2	β-pinene	1118	7.6	GC-MS	**56**	*p*-mentha-1,5-dien-7-ol	1814	0.3	MS
3	sabinene	1132	1.3	GC-MS	**57**	*trans*-carveol	1845	0.4	GC-MS
4	myrcene	1174	0.2	GC-MS	**58**	*p*-cymen-8-ol	1864	0.1	GC-MS
5	α-terpinene	1188	0.3	GC-MS	**59**	(*E*)-geranyl acetone	1868	0.1	MS
6	limonene	1203	0.8	GC-MS	**60**	*epi*-cubebol	1900	0.3	MS
7	1,8-cineole	1213	1.0	GC-MS	**61**	α-calacorene	1941	0.3	MS
8	β-phellandrene	1218	tr[d]	MS	**62**	cubebol	1957	0.2	MS
9	(*Z*)-β-ocimene	1246	0.1	MS	**63**	(*E*)-β-ionone	1958	0.2	MS
10	γ-terpinene	1255	0.8	GC-MS	**64**	γ-calacorene	1984	0.1	MS
11	(*E*)-β-ocimene	1266	0.1	MS	**65**	isocaryophyllene oxide	2001	0.2	GC-MS
12	*p*-cymene	1280	0.5	GC-MS	**66**	caryophyllene oxide	2008	5.8	GC-MS
13	terpinolene	1290	0.3	GC-MS	**67**	perilla alcohol	2029	0.6	GC-MS
14	artemisia ketone	1358	0.1	MS	**68**	presilphiperfolan-9α-ol	2030	0.2	MS
15	perillen	1429	0.1	MS	**69**	salvial-4(14)-en-1-one	2037	0.4	MS
16	γ-campholene aldehyde	1439	0.1	MS	**70**	(*E*)-nerolidol	2050	0.1	GC-MS
17	α,*p*-dimethylstyrene	1452	0.1	MS	**71**	humulene epoxide II	2071	0.7	MS
18	7β-(*H*)-silphiperfol-5-ene	1454	0.1	MS	**72**	*p*-mentha-1,4-dien-7-ol	2073	0.2	MS
19	α-cubebene	1466	0.3	MS	**73**	caryophylla-2(12),6(13)-dien-5-one	2074	0.3	MS
20	*trans*-sabinene hydrate	1474	0.1	GC-MS	**74**	1-*epi*-cubenol	2088	0.2	MS
21	(*Z*)-3-hexenyl 2-methylbutyrate	1482	tr	MS	**75**	hexyl benzoate	2095	0.3	MS
22	cyclosativene	1492	tr	MS	**76**	viridiflorol	2104	0.1	MS
23	α-campholene aldehyde	1499	0.5	MS	**77**	cumin alcohol	2113	0.3	GC-MS
24	(*E*)-theaspirane	1516	0.1	MS	**78**	hexahydrofarnesyl acetone	2131	1.2	MS
25	camphor	1532	0.4	GC-MS	**79**	spathulenol	2144	3.4	MS
26	dihydroachillene	1547	0.2	MS	**80**	T-cadinol	2187	1.0	MS
27	linalool	1553	0.4	GC-MS	**81**	nonanoic acid	2192	0.2	GC-MS
28	*cis*-sabinene hydrate	1556	0.1	MS	**82**	thymol	2198	0.4	GC-MS
29	*trans*-*p*-menth-2-en-1-ol	1571	0.2	MS	**83**	*ar*-turmerol	2214	0.2	MS
30	pinocarvone	1586	4.2	GC-MS	**84**	carvacrol	2239	8.7	GC-MS
31	nopinone	1601	0.7	MS	**85**	1-methylethyl hexadecanoate[e]	2251	0.2	MS
32	terpinen-4-ol	1611	2.0	GC-MS	**86**	α-cadinol	2255	1.0	MS
33	β-caryophyllene	1612	0.4	GC-MS	**87**	*epi*-α-bisabolol	2256	0.6	MS
34	hotrienol	1616	0.7	MS	**88**	selin-11-en-4α-ol	2273	0.5	MS
35	*cis*-*p*-menth-2-en-1-ol	1638	0.2	GC-MS	**89**	decanoic acid	2298	0.3	GC-MS
36	myrtenal	1648	4.9	MS	**90**	caryophylla-2(12),6(13)-dien-5β-ol (= caryophylladienol I)	2316	0.5	MS
37	pulegone	1662	0.2	GC-MS					
38	*trans*-pinocarveol	1670	6.2	GC-MS	**91**	caryophylla-2(12),6(13)-dien-5α-ol (= caryophylladienol II)	2324	1.1	MS
39	*trans*-verbenol	1683	0.9	MS					
40	cryptone	1690	0.8	MS	**92**	eudesma-4(15),7-dien-4β-ol	2369	1.1	MS
41	α-terpineol	1706	0.9	GC-MS	**93**	caryophylla-2(12),6-dien-5α-ol (= caryophyllenol I)	2389	0.7	MS
42	*trans*-sabinol	1720	0.3	GC-MS					
43	verbenone	1725	0.5	GC-MS	**94**	caryophylla-2(12),6-dien-5β-ol (= caryophyllenol II)	2392	1.7	MS
44	thujol	1729	0.1	MS					
45	*p*-mentha-1,5-dien-8-ol	1738	0.7	MS	**95**	kaur-16-ene	2438	0.1	MS
46	valencene	1740	0.2	MS	**96**	pentacosane	2500	0.5	GC-MS
47	phellandral	1744	0.6	MS	**97**	dodecanoic acid	2503	0.4	GC-MS
48	carvone	1751	0.3	GC-MS	**98**	phytol	2622	0.1	MS
49	*cis*-piperitol	1758	0.1	MS	**99**	benzyl benzoate	2655	0.2	MS
50	δ-cadinene	1773	0.4	MS	**100**	tetradecanoic acid	2670	0.8	GC-MS
51	γ-cadinene	1776	0.1	MS	**101**	heptacosane	2700	0.2	GC-MS
52	*ar*-curcumene	1786	0.6	MS	**102**	pentadecanoic acid	2822	0.2	GC-MS
53	cumin aldehyde	1802	0.6	GC-MS	**103**	nonacosane	2900	0.5	GC-MS
54	myrtenol	1804	4.1	MS	**104**	hexadecanoic acid	2931	0.8	GC-MS
			monoterpene hydrocarbons					12.3	
			oxygenated monoterpenes					41.5	
			sesquiterpene hydrocarbons					3.0	
			oxygenated sesquiterpenes					20.9	
			others					7.2	
			total					84.9	

[a] RRI, relative retention indices calculated against *n*-alkanes on the HP Innowax column. [b] Percent calculated from flame ionization detector (FID) data. [c] Method of identification: GC, identification based on retention times of genuine compounds on the HP Innowax column; MS, tentatively identified on the basis of computer matching of the mass spectra of peaks with the Wiley (*29*) and MassFinder (*30*) libraries. [d] tr, trace (<0.1%). [e] Syn, isopropyl palmitate, CAS Registry No. 142-91-6.

nil, azoxystrobin, and captan (Chem Service, Inc., West Chester, PA) were purchased from commercial sources.

Plant Material. Aerial plant parts were collected on August 2002 from Lemhi County, Idaho. Voucher specimens (UMISS 70433) were deposited at The University of Mississippi Herbarium.

Isolation of the Essential Oil. Dried aerial parts were hydrodistilled for 3 h using a Clevenger apparatus to obtain essential oils in 0.29% dry weight yield.

GC and GC-MS Analysis of the Essential Oil. *Haplopappus* essential oil was 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 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 composition 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). Relative percentages of the characterized components were as cited in **Table 1**.

GC-MS analysis was performed with a Hewlett-Packard GCD system (SEM Ltd.), 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 was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to those of a series of *n*-alkanes. Computer matching against commercial (Wiley and MassFinder 2.1) (*29*, *30*) and in-house "Baser Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (*31*−*34*), was also used for the identification.

Antimalarial Assay. The in vitro antimalarial activity was determined against D6 (chloroquine sensitive) and W2 (chloroquine resistant) strains of *Plasmodium falciparum*. The assay was based on the determination of parasite LDH activity using Malstat reagent (*35*). The IC_{50} was calculated from dose−response curves of *Plasmodium* growth inhibition. Chloroquine (Aldrich-Sigma) and artemisinin (Aldrich-Sigma) were included as control drugs in each assay (*36*).

Antimicrobial Assay. All organisms were obtained from the American Type Culture Collection (ATTC, Manassas, VA) and include *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, *Aspergillus fumigatus* ATCC 90906, *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the NCCLS methods (*37*−*40*) as reported previously (*36*).

Bioautography. Bioautography procedures of Meeaza et al. (*41*) and Tabanca et al. (*42*) for detection of naturally occurring antifungal agents were used to evaluate antifungal activity of *Haplopappus* essential oil and pure components. Conidia of *Colletotrichum fragariae*, *Colletotrichum acutatum*, and *Colletotrichum gloeosporioides* suspensions were each adjusted to 3.0×10^5 conidia/mL with liquid potato−dextrose broth (PDB; Difco, Detroit, MI) and 0.1% Tween-80. Each glass silica gel thin-layer chromatography (TLC) plate with fluorescent indicator (250 mm, Silica Gel GF Uniplate, Analtech, Inc., Newark, DE) was sprayed lightly three times with the conidial suspension. Inoculated plates were placed in a 30 × 13 × 7.5 cm moisture chamber (100% relative humidity, 398-C; Pioneer Plastics, Inc., Dixon, KY) and incubated in a growth chamber at 24 ± 1 °C for a 12-h photoperiod under 60 ± 5 μmol m^{-2} s^{-1} of light. The sensitivity of each fungal species to each test compound was determined 4 days after treatment by comparing sizes of inhibitory zones. Means and standard deviations (SD) of inhibitory zone size were used to evaluate antifungal activity of essential oil and pure 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.) were used as controls at 2 mM in 2 μL of EtOH (*42*).

RESULTS AND DISCUSSION

In this present work, the hydrodistilled essential oil from aerial parts of *H. greenei* (HG) was analyzed by both GC and GC-MS. The compounds characterized and reported with their relative percentages are listed in **Table 1**. A total of 104 compounds were identified, representing 84.9% of the total oil, with oxygenated monoterpenes (41.5%) dominating. Oxygenated sesquiterpenes (20.9%) represented the second largest group, followed by monoterpenes (12.3%), sesquiterpenes (3%), and other compounds (7.2%). The main constituents were found to be carvacrol (8.7%), β-pinene (7.6%), *trans*-pinocarveol (6.2%), and caryophyllene oxide (5.8%).

Table 2. Antifungal Activity of *H. greenei* Essential Oil Using Direct Bioautography with Three *Colletotrichum* Test Species[a]

	mean fungal growth inhibition (mm) ± SD		
	C. acutatum	*C. fragariae*	*C. gloeosporioides*
HG essential oil	10.7 ± 0.5	10.3 ± 2.1	10.7 ± 1.2
β-pinene[b]	0[c]	0	0
carvacrol[b]	19 ± 0.71	19 ± 0.71	19 ± 0.71
trans-pinocarveol[b]	0	0	0
caryophyllene oxide[b]	0	0	0
benomyl[d]	19.7 ± 0.71	19.7 ± 0.71	20.2 ± 0.01
captan[d]	14.7 ± 0.71	14.7 ± 0.71	9.61 ± 0.69
cyprodinil[d]	30.3 ± 0.02	30.8 ± 0.71	30.3 ± 0.01
azoxystobin[d]	24.8 ± 0.71	20.7 ± 0.72	30.3 ± 0

[a] *H. greenei* (HG) essential oil was applied as a 20 mg/mL in 4 μL sample onto a silica TLC plate. Mean inhibitory zones and standard deviations (SD) were used to determine the level of antifungal activity against each fungal species. [b] Commercial samples (Aldrich-Sigma, ST, Louis, MO) [c] 0 = no inhibition. [d] Technical grade agrochemical fungicides (without formulation) with different modes of action were used as internal standards.

HG essential oil showed no antimalarial activity against *P. falciparum* D6 and W2 clones. The essential oil was tested for antimicrobial activity using a previously described microdilution technique against *Ca. albicans*, *Co. glabrata*, *Co. krusei*, *Cr. neoformans*, methicillin-resistant *S. aureus*, *M. intracellulare*, and *A. fumigatus* (*36*). The investigated oil showed no antimicrobial activity at the highest concentration of 200 μg/mL when tested against microorganisms.

Direct bioautography on silica gel TLC revealed the antifungal activity of the essential oil of HG against *Co. acutatum*, *Co. fragariae*, and *Co. gloeosporioides*. Antifungal activity was indicated by the presence of clear inhibitory zones appearing against a dark background on the TLC plate. These clear zones represented regions where fungal mycelical or reproductive stroma was not present. The essential oil showed activity against all three *Colletotrichum* species at 20 mg/mL in 4 μL (**Table 2**).

To the best of our knowledge, this is also the first report of the antifungal activity of *Haplopappus* oil against *Co. acutatum*, *Co. fragariae*, and *Co. gloeosporioides*. HG oil showed the same level of activity against each of the *Colletotrichum* species. Whereas HG oil showed 68% of the activity of the standard antifungal captan against *Co. acutatum* and *Co. fragariae*, it showed 100% captan activity against *Co. gloeosporioides*. Captan is well-known as a multisite inhibitor fungicide with no systemic activity and is used as a protectant fungicide to prevent anthracnose diseases in fruits and ornamentals (*43*−*45*). HG oil (an unpurified mixture) showed ∼50% of the antifungal activity of standard commercial antifungals (pure compounds) such as benomyl, cyprodinil, and azoxystrobin. These commercial fungicides are all known to be systemic and have both protective and curative activity (*46*).

β-Pinene, carvacrol, *trans*-pinocarveol, and caryophyllene oxide were determined to possess insignificant activity against all three *Colletotrichum* species. One-dimensional TLC of HG oil and commercial standards of β-pinene, carvacrol, *trans*-pinocarveol, and caryophyllene oxide in *n*-hexane/diethyl ether (8:2) were subsequently tested against the three *Colletotrichum* species, and *C. fragariae* appeared to be the most sensitive target fungus to these essential oil components. β-Pinene, *trans*-pinocarveol, and caryophyllene oxide showed no activity. Carvacrol demonstrated nonselective activity with a 19 mm zone inhibition in each of the three *Colletotrichum* species. This level of activity is similar to that of benomyl and greater than that of

captan. TLC and bioautography of HG oil indicated the presence of four antifungal compounds: one major compound that matched the retention factor (R_f) of carvacrol standard and three minor polar unidentified compounds. Future studies should focus on bioassay-guided fractionation, identification, and confirmation of the four active antifungal components.

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