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Phytochemistry and Nematicidal Activity of the Essential Oils from 8 Greek Lamiaceae Aromatic Plants and 13 Terpene Components

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Eight essential oils (EOs) as well as 13 single terpenes were studied for their nematicidal activity against *Meloidogyne incognita*, for three immersion periods (24, 48, and 96 h). The EOs were isolated from eight Greek Lamiaceae species: *Melissa officinalis*, *Sideritis clandestina*, *Origanum dictamnus*, *Ocimum basilicum*, *Mentha pulegium*, *Origanum vulgare*, *Vitex agnus castus*, and *Salvia officinalis*. The EOs nematicidal activity was correlated to their chemical composition as well as to the pure terpenes' activity tested individually. Clear dose and time response relationships were established. The EOs of *O. vulgare*, *O. dictamnus*, *M. pulegium*, and *M. officinalis* exhibited high nematicidal activity against *M. incognita*, and the EC_{50} values (96 h) were calculated at 1.55, 1.72, 3.15, and 6.15 μL/mL, respectively. The activity of the nematicidal terpenes was found to decrease in the order L-carvone, pulegone, *trans*-anethole, geraniol, eugenol, carvacrol, thymol, terpinen-4-ol, and the respective EC_{50} values (24 h) were calculated in the range of 115−392 μg/mL. Terpenes tested individually were more active than as components in EO, implementing antagonistic action.

KEYWORDS: Essential oils; nematicidal activity; *Meloidogyne incognita*; *Origanum dictamnus*; *Origanum vulgare*; *Melissa officinalis*; *Mentha pulegium*; pulegone; *trans*-anethole; L-carvone; GC-MS

INTRODUCTION

Meloidogyne incognita (Kofoid and White) Chitwood is a root-knot nematode species, which is among the most notoriously difficult crop pests with a wide host range (*1*). Many synthetic pesticides such as carbamate, organophosphate, and organophthalide formulations have been used in the past as chemical nematicides. Nowadays the number of these pesticides has been dramatically restricted due to adverse environmental effects and health concerns. Phytonematode control is achieved mainly with cultural practices, crop rotation, and resistant cultivars, combined with a few available chemical nematicides that are still authorized. The need for discovering less toxic and environmentally acceptable substitutes for commercial nematicides is amplified, a fact that creates a significant market opportunity for alternative products such as biorationals (*2*). Plants are a source of phytochemicals that can be used as pesticides themselves, or they can serve as model compounds for the development of chemically synthesized, easily biodegradable derivatives, with low plant and human toxicity (*1*). Essential oils (EOs) are natural extracts of aromatic plants used in many fields, including agriculture, aromatherapy, and nutrition. The constituents of EOs contribute to the overall activity of each EO, against target organisms, individually as well as by interacting among them (*3*). Essential oils have been studied most from the aspect of their flavor and fragrance chemistry for flavoring foods, drinks, and other goods. They are relatively safe and acceptable by the consumers for multipurpose functional use (*4*, *5*). Moreover, their antimicrobial, antifungal, antioxidant (*6*, *7*), and antibacterial activities have been reported in the literature (*8*), as well as their efficacy against insects (*2*, *9*) and nematodes (*10*−*12*). Similarly to EOs, pure terpenes play a substantial role in medicinal therapy (*13*, *14*) and have been also shown to possess properties against insects (*1*, *2*, *9*). Components of EOs such as thymol, carvacrol, pulegone, limonene, anethole, geranial, and artemisia ketone have been identified with nematicidal activity (*15*). Presently, only a few commercial biopesticides containing EOs or artificial blends of constituent terpenes are available. Cinnamite and Valero (Mycotech Corp.), with cinnamon oil and cinnamaldehyde as active ingredients, are two examples of such biopesticides (*2*). The biological activity of EOs is related to their chemical composition, which in turn is influenced by the climatic, seasonal, and geographic conditions affecting the aromatic species from which EO are derived (*16*, *17*). For this reason it is important to test different batches of EOs against target species. As part of our ongoing effort toward the study of natural substances with nematicidal properties (*18*), we have now focused on various plant species of Greek flora as sources of biologically interesting

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compounds (*2*, *19*). In previous studies we reported on the chemical composition and activity of EOs of Lamiaceae species as plant protection agents against bacteria and insects (*8*, *9*). Herein we report the nematicidal activity of some EOs, which, to the best of our knowledge, have never been tested in motility bioassays against *M. incognita*.

The objective of this study was the evaluation of the nematicidal activities of the EOs obtained from eight Greek Lamiaceae species and their correlation to the chemical composition and individual activity of constituent terpenes.

MATERIALS AND METHODS

Plant Materials. The aromatic plants used were *Melissa officinalis*, *Sideritis clandestina*, *Origanum dictamnus*, *Ocimum basilicum*, *Mentha pulegium*, *Origanum vulgare*, *Vitex agnus castus*, and *Salvia officinalis*. The aerial parts of all aromatic plants were collected at the flowering stage from various locations in Greece, and they were dried in the absence of light at room temperature. Afterward, they were sealed in paper bags and stored at room temperature, in the dark, until use. Voucher specimens were deposited in the Department of Ecology, School of Biology, Aristotle University of Thessaloniki, Greece.

Chemicals. Analytical standards of *p*-cymene (99.5%; $d = 0.86$ g/mL at 25 °C), limonene (97%; $d = 0.842$ g/mL at 20 °C), 1,8-cineole (99%; $d = 0.921$ g/mL at 25 °C), linalool (97%; $d = 0.862$ g/mL at 20 °C), terpinen-4-ol (96%; $d = 0.934$ g/mL at 20 °C), L-carvone (98%; $d = 0.959$ g/mL at 25 °C), pulegone (98%; $d = 0.936$ g/mL at 20 °C), geraniol (99%; $d = 0.879$ g/mL at 20 °C), *trans*-anethole (99%; $d = 0.988$ g/mL at 25 °C), thymol (99.5%), carvacrol (98%; $d = 0.976$ g/mL at 20 °C), eugenol (98%; $d = 1.067$ g/mL at 25 °C), and β-caryophyllene (98.5%; $d = 0.902$ g/mL at 20 °C) were purchased from Sigma-Aldrich, Italy. All solvents and reagents were of pesticide grade.

Isolation of EO. The dried plant samples were subjected to water distillation using a Clevenger apparatus (Winzer) for 3 h according to the *European Pharmacopoeia* (*20*). One hundred grams of each aromatic plant was added in a glass 2000 mL flask together with 1000 mL of distilled water. The EOs obtained were dried over anhydrous Na_2SO_4 and stored in dark glass vials with Teflon-sealed caps at -20 ± 0.5 °C until use. The yield of each EO was determined on average over three replicates.

Essential Oil Analysis. The analysis of all EOs was performed using an Agilent 6890 series GC system, equipped with a Phenomenex Zebron ZB-5 capillary column (30 m, 0.25 mm i.d., 0.25 cm film thickness) and a single-quadrupole mass spectrometer 5973 Network as detector. The carrier gas was helium, at a flow rate of 1 mL/min. The column temperature was initially set at 60 °C for 5 min, and then it was gradually increased to 100, 200, 250, and 290 °C at rates of 3, 5, 10, and 20 °C/min, respectively. Finally, the temperature was held at the final level for 7 min. The EOs were diluted 1:1000 (v/v) with hexane, and 1.0 μL of the diluted samples was injected in a splitless mode. Injector, transfer line, heater, and detector temperatures were set at 270, 290, 270, and 250 °C, respectively. Identification was performed by comparison of mass spectra with the database library (NIST 98 and Wiley 275, comparison quality > 90%), as well as by comparison of retention indices for alkanes C_9−C_{24} with the ones reported by Adams (*21*) (**Table 2**). Additionally, the identity of certain analytes (Supporting Information) was confirmed by comparison of retention time and mass spectra by the method of external standard, with authentic compounds using the single-ion mass chromatogram.

Nematode Motility Bioassays. *Nematode Cultures*. A population of *M. incognita* originally obtained from tomato roots collected from a greenhouse in Vassilika, Thessaloniki, northern Greece, was reared on tomato (*Solanum lycopersicum* L.) cv. Belladonna, a cultivar that is very susceptible to root-knot nematodes. All plants were maintained in a growth chamber at 25−28 °C, 60% relative humidity, and 16 h photoperiod, in plastic pots (18 cm diameter) containing a 10:1 (v/v) mixture of peat and perlite. Plants used for inoculations were 7 weeks old, at the five-leaf stage. After 40 days, the plants were uprooted, and the roots were washed free of soil and cut into 2 cm pieces. Eggs were extracted according to the sodium hypochlorite procedure (*22*), and second-stage juveniles (J2) were allowed to hatch in modified Baermann funnels at 28 °C. All J2 hatching in the first 3 days were discarded, and thereafter J2 collected, after 24 h, were used in the experiments.

Table 1. Yield in Essential Oil of the Eight Lamiaceae Species

plant species	plant part used for water distillation	yield[a] (mL/100 g of dw)
Melissa officinalis	stalks and leaves	0.11 ± 0.03
Sideritis clandestina	stalks and leaves	0.10 ± 0.00
Origanum dictamnus	stalks and leaves	0.94 ± 0.01
Ocimum basilicum	stalks and leaves	0.71 ± 0.00
Mentha pulegium	stalks and leaves	1.61 ± 0.00
Origanum vulgare	stalks and leaves	3.05 ± 0.00
Vitex agnus castus	fruits	1.42 ± 0.02
Salvia officinalis	stalks and leaves	2.70 ± 0.00

[a] Expressed in dry weight; values represent means ± standard deviation of three replicates.

Nematicidal Activity of EO and Terpene Components. The nematicidal activity of the EOs as well as constituent terpenes, in terms of nematode juveniles motility block, was tested, and the EC_{50} values were calculated. Stock solutions were prepared by dilution with ethanol, whereas working solutions were obtained by dilution with distilled water containing the polysorbate surfactant 20 (Tween-20). Final concentrations of ethanol and Tween-20 in each well never exceeded 1 and 0.3% v/v, respectively, because preliminary trials showed that motility of nematodes exposed at those concentration levels was similar to motility of nematodes maintained in distilled water. Distilled water, as well as a mixture of water with ethanol and Tween-20 at concentrations equivalent to those in the treatment wells, served as control. Fifteen juveniles were used per treatment well in Cellstar 96-well plates (Greiner bio-one). The plates were covered to prevent evaporation and were maintained in the dark at 28 °C. Border wells containing plain water with nematodes were placed around the wells of each treatment to check the vapor drift among wells that could possibly interfere with the efficacy results. Juveniles were observed with the aid of an inverted microscope (Euromex, The Netherlands) at 40× after 24, 48, and 96 h and were ranked into two distinct categories: motile or immotile. After the last assessment (96 h), the nematodes were transferred into plain water, after washing in tap water through a 20 μm pore screen to remove the excess of EO, and they were assessed again after 24 h for the reobtainment of motility.

Statistical Analysis. Treatments of motility experiments were replicated six times, and each experiment was performed twice. The percentages of immotile J2 in the microwell assays were corrected by elimination of the natural death/immotility in the water control according to the Schneider Orelli formula (*23*), corrected % = [(mortality % in treatment − mortality % in control)/(100 − mortality % in control)}] × 100, and they were analyzed (ANOVA) combined over time. Because ANOVA indicated no significant treatment by time interaction, means were averaged over experiments. Corrected percentages of immotile J2 treated with EO were subjected to nonlinear regression analysis using the log−logistic equation proposed by Seefeldt et al. (*24*): $Y = C + (D - C)/\{1 + \exp[b\ (\log(x) - \log(EC_{50}))]\}$, where C = the lower limit, D = the upper limit, b = the slope at the EC_{50}, and EC_{50} = the essential oil or terpene concentration required for 50% death/immotility of nematodes after elimination of the control (natural death/immotility). In the regression equation, the essential oil concentration (% w/v) was the independent variable (x) and the immotile J2 (percentage increase over water control) was the dependent variable (y). The mean value of the six replicates per essential oil concentration and immersion period was used to calculate the EC_{50} value.

RESULTS AND DISCUSSION

EO Composition. The plant parts used for EO isolation as well as the average oil yields, expressed in milliliters per 100 g of dry weight, ranged from 0.10 to 3.05% and are presented in **Table 1**. Among the aromatic plants studied, the highest yields of EO (3.05−1.42%) were obtained from *O. vulgare*, *S. officinalis*, *M. pulegium*, and *V. a. castus* followed by *O. dictamnus* and *O. basilicum* (0.94 and 0.71%), whereas the lowest yields (0.11 and 0.10%) derived from *M. officinalis* and *S. clandestina* (**Table 1**). Similar results have been reported in the literature for the EO yields obtained from *O. basilicum*, 0.5−0.8% (*25*), *O. dictamnus*,

Table 2. Chemical Composition and Percent Content[a] of the Essential Oil Components of the Eight Lamiaceae Species

no.	compound name in order of elution[b]	RI[c]	*S. officinalis*	*O. vulgare*	*M. pulegium*	*O. basilicum*	*S. cladestina*	*O. dictamnus*	*M. officinalis*	*V. a. castus*
1	α-thujene	931	1.8	1.0	–	–	0.4	2.5	–	2.3
2	α-pinene	939	3.4	0.4	0.7	1.0	4.3	0.7	–	4.5
3	camphene	953	4.9	–	–	–	–	–	–	–
4	sabinene	975	–	–	–	1.1	0.3	0.7	1.3	19.8
5	β-pinene	980	4.6	–	0.7	1.2	8.5	–	0.7	0.9
6	myrcene	989	3.8	1.2	0.4	1.3	1.0	1.8	–	2.3
7	α-phellandrene	1003	–	0.4	–	–	0.1	0.5	–	0.2
8	δ-3-carene	1011	–	–	–	–	0.7	–	–	–
9	α-terpinene	1018	0.8	0.3	–	–	1.9	3.5	0.6	0.4
10	*p*-cymene[d]	1023	0.8	8.1	–	–	2.0	13.2	0.1	1.0
11	limonene[d]	1029	–	–	1.0	–	3.1	–	0.3	–
12	1,8-cineole[d]	1033	25.5	–	–	13.0	–	–	–	18.4
13	β-phellandrene	1030	–	0.6	–	–	–	0.7	–	–
14	(*E*)-β-ocimene	1040	–	–	–	0.7	1.7	–	–	1.2
15	γ-terpinene	1062	1.4	7.2	–	–	0.9	18.3	1.4	2.6
16	*cis*-sabinene hydrate	1068	0.2	0.5	–	–	–	0.5	0.5	0.6
17	*cis*-linalool oxide	1074	–	–	–	0.3	–	–	–	–
18	terpinolene	1088	0.6	0.2	–	0.3	0.5	0.2	0.4	–
19	naphthalene decahydro	1094	–	–	–	–	–	–	0.2	–
20	linalool[d]	1098	–	0.2	–	43.1	0.6	2.2	1.0	0.9
21	nonanal	1104	–	–	–	–	–	–	0.2	0.2
22	α-thujone	1102	0.8	–	–	–	–	–	–	–
23	β-thujone	1114	6.0	–	–	–	–	–	–	–
24	*cis*-*p*-menth-2-en-1-ol	1121	–	–	–	–	–	–	–	0.3
25	*allo*-ocimene	1129	–	0.2	–	–	3.0	–	–	0.1
26	1-terpineol	1133	–	–	–	–	–	–	–	0.3
27	*trans*-pinocarveol	1139	–	–	–	–	0.4	–	1.1	–
28	*trans*-epoxy-ocimene	1142	–	–	–	0.2	–	–	–	–
29	verbenol	1144	–	–	–	–	0.2	–	–	–
30	camphor	1146	16.8	–	–	1.0	–	–	–	–
31	myrcenone	1149	–	–	–	–	–	–	0.3	–
32	isopulegol	1145	–	–	–	–	–	–	0.3	–
33	citronellal	1153	–	–	–	–	–	–	1.5	–
34	*trans*-pinocamphone	1160	0.1	–	–	–	–	–	–	–
35	pinocarvone	1164	–	–	–	–	0.3	–	–	–
36	menthone <iso>	1165	–	–	28.3	–	–	–	–	–
37	1-borneol	1166	3.4	0.4	–	1.0	0.3	–	–	–
38	*cis*-isopulegone	1182	–	–	6.6	–	–	–	–	–
39	terpinen-4-ol[d]	1189	1.3	0.9	–	0.5	0.4	1.0	4.0	4.6
40	α-terpineol	1197	4.5	–	–	1.8	1.1	–	–	5.2
41	*cis*-dihydrocarvone	1193	–	0.2	–	–	–	–	–	–
42	myrtenol	1194	–	–	–	–	–	–	2.2	–
43	verbenone	1204	–	–	–	–	–	–	0.2	–
44	*cis*-piperitol	1212	–	–	–	–	–	–	–	0.1
45	*trans*-(+)-carveol	1217	–	–	–	–	–	–	0.2	–
46	nerol	1225	–	–	–	t	–	–	0.3	t
47	citronellol	1228	–	–	–	–	–	–	0.4	–
48	isogeraniol	1237	–	–	–	–	–	–	–	0.1
49	pulegone[d]	1242	–	–	51.5	–	0.3	–	–	–
50	neral	1242	–	–	–	–	–	–	3.2	–
51	L-carvone[d]	1242	–	–	–	–	–	–	1.5	–
52	carvacrol methyl ether	1239	–	0.6	–	–	–	0.3	–	–
53	geraniol[d]	1249	–	–	–	0.3	–	–	0.1	0.2
54	*cis*-piperitone-oxide	1250	–	–	–	–	–	–	1.0	–
55	piperitone	1254	–	–	0.7	–	0.3	–	0.2	–
56	diosphenol	1270	–	–	–	–	–	–	0.1	–
57	2-cyclohexen-1-one	1263	–	–	0.3	–	–	–	–	–
58	geranial	1273	–	–	–	–	–	–	3.0	t
59	(−)-bornyl acetate	1282	–	–	–	2.1	–	–	–	–
60	*trans*-anethole[d]	1283	–	–	0.9	–	1.3	–	13.2	–
61	thymol[d]	1290	–	–	–	–	0.2	0.5	0.7	–
62	carvacrol[d]	1299	0.3	68.5	1.2	0.6	0.6	44.3	4.1	–
63	bicycloelemene	1336	–	–	–	–	0.5	–	–	–
64	δ-elemene	1339	–	–	–	–	3.6	–	–	–
65	piperitenone	1342	–	–	2.2	–	–	–	–	–
66	a-terpinyl acetate	1346	1.5	–	–	–	–	–	–	–
67	eugenol[d]	1350	–	–	–	4.8	0.6	–	0.5	t

Table 2. Continued

no.	compound name in order of elution[b]	RI[c]	*S. officinalis*	*O. vulgare*	*M. pulegium*	*O. basilicum*	*S. cladestina*	*O. dictamnus*	*M. officinalis*	*V. a. castus*
68	piperitetone oxide	1354	–	–	–	–	–	–	0.3	–
69	carvacrol acetate	1360	–	0.1	–	–	–	t	t	–
70	α-copaene	1365	0.1	0.1	–	0.2	0.3	1.4	–	–
71	geranyl formate	1373	–	–	–	–	–	–	0.8	–
72	*trans*-β-damascenone	1381	–	–	–	–	0.2	–	0.2	–
73	8-quinolinol	1385	–	–	–	–	0.3	–	–	–
74	β-bourbonene	1384	–	0.1	–	0.3	–	–	0.4	–
75	β-elemene	1390	–	–	–	1.7	0.1	–	0.3	0.1
76	methyleugenol	1401	–	–	–	0.3	–	–	–	–
77	(−)-α-gurjunene	1405	–	–	–	–	–	–	–	0.7
78	β-caryophyllene[d]	1411	4.2	3.2	–	–	2.9	1.7	6.0	2.3
79	*cis*-α-bergamotene	1416	–	–	–	5.5	–	–	–	0.4
80	(+)-β-funebrene	1422	0.1	–	–	–	–	–	–	–
81	γ-elemene	1430	–	–	–	–	0.3	–	–	–
82	epi-bicyclosesquiphellandrene	1438	–	–	–	–	–	–	0.3	–
83	aromadendrene	1444	0.2	–	–	–	–	–	–	–
84	α-humulene	1454	–	0.5	–	–	–	–	–	–
85	geranyl acetone	1454	–	–	–	–	–	–	0.3	–
86	(*E*)-β-farnesene	1456	–	–	–	0.2	2.5	–	0.3	5.1
87	dodecenol	1469	–	–	–	–	0.3	–	–	–
88	β-selinene	1474	1.2	–	–	0.6	–	0.1	0.6	–
89	valencene	1481	0.2	–	–	–	–	–	–	–
90	*allo*-aromadendrene	1478	0.7	0.2	–	–	0.2	–	–	1.2
91	germacrene D	1483	–	–	–	0.6	–	0.4	1.5	0.4
92	α-amorphene	1485	0.2	0.2	–	1.9	–	–	0.2	–
93	β-ionone	1494	–	–	–	–	–	–	0.5	–
94	bicyclogermacrene	1503	–	–	–	0.3	2.8	–	–	2.7
95	(*E*,*E*)-α-farnesene	1509	–	0.1	–	–	–	–	–	–
96	+ calarene	1510	0.1	0.1	–	–	–	0.2	–	–
97	δ-guaiene	1508	–	–	–	0.9	–	–	–	–
98	β-bisabolene	1509	–	2.6	–	–	1.6	0.3	0.2	0.4
99	γ-cadinene	1514	0.1	0.4	–	–	–	–	–	–
100	2*H*-1,4a-ethanonaphthalen-1-ol	1516	–	–	–	–	–	–	0.3	–
101	β-curcumene	1518	–	–	–	0.1	–	–	–	–
102	β-sesquiphellandrene	1525	–	0.1	–	–	–	–	–	–
103	δ-cadinene	1523	0.3	–	–	–	0.3	0.8	0.6	0.2
104	*cis*-calamenene	1530	–	–	–	0.7	0.7	–	–	–
105	*trans*-α-bisabolene γ	1536	–	0.1	–	–	0.3	–	–	0.2
106	sesquisabinene hydrate	1545	–	–	–	–	–	–	–	0.1
107	α-calacorene	1547	–	–	–	–	0.1	t	–	–
108	β-calacorene	1560	–	–	–	–	–	–	–	0.1
109	nerolidol <e>	1561	–	–	–	–	0.3	–	–	–
110	epi-a-selinene	1563	–	–	–	0.6	–	–	–	–
111	palustrol	1565	–	–	–	–	–	–	–	0.4
112	(−)-spathulenol	1580	0.2	0.1	–	0.4	5.0	–	–	1.8
113	(−)-caryophyllene oxide	1581	0.3	0.5	t		1.0	0.6	12.4	0.2
114	α-farnesene	1584	–	–	–	–	–	–	–	0.7
115	globulol	1585	0.1	–	–	–	0.3	–	–	–
116	*cis*-copaene-4a-ol	1595	–	–	–	–	–	–	0.6	–
117	viridiflorol	1590	–	–	–	–	–	–	–	0.2
118	β-eudesmol	1630	–	–	–	–	0.4	–	–	–
119	isospathulenol	1634	–	–	–	0.1	0.6	–	–	0.3
120	α-cadinol	1640	–	–	–	–	0.4	–	0.2	0.4
121	T-muurolol	1642	–	–	–	0.5	–	–	2.0	–
122	t-cadinol	1643	–	–	–	–	–	0.2	–	0.2
123	bisabolol oxide	1658	–	–	–	–	0.6	–	–	–
124	caryophylla-4,8-dien-5-ol	1661	–	–	–	–	0.1	–	2.9	–
125	valeranone	1668	–	–	–	–	0.5	–	–	–
126	β-bisabolol	1674	–	–	–	0.2	5.7	–	–	0.2
127	tetradecanol	1675	–	–	–	–	–	–	–	–
128	2-pentadecanone	1697	–	–	–	–	0.8	–	–	–
129	(2*Z*,6*E*)-farnesol	1722	–	–	–	–	0.8	–	–	–
130	(2*E*,6*E*)-farnesol	1713	–	–	–	–	0.9	–	–	0.1
131	2(1*H*)-quinolinone	1752	–	–	–	–	–	–	–	0.9
132	9-octadecanal	1828	–	–	–	–	0.5	–	–	–
133	rimuene	1894	–	–	–	–	–	–	0.2	–
134	*R*-(−)-cembrene	1942	–	–	–	–	0.6	–	–	–

Table 2. Continued

no.	compound name in order of elution[b]	RI[c]	*S. officinalis*	*O. vulgare*	*M. pulegium*	*O. basilicum*	*S. cladestina*	*O. dictamnus*	*M. officinalis*	*V. a. castus*
135	phytol	2112	–	–	–	–	–	–	0.2	–
136	squalene	2790	–	–	–	–	0.2	–	–	–
137	nonacosane	2900	–	–	–	–	0.4	–	–	–
total identified compounds (%)			90.5	99.3	94.5	89.4	77.4	96.6	76.1	85.5
number of identified compounds			34	32	12	36	62	25	55	46
monoterpenes	hydrocarbons (%)		22.1	19.6	2.8	5.6	28.4	42.1	5.5	35.3
	oxygenated (%)		60.4	71.4	91.7	69.2	6.6	48.8	41.4	30.9
sesquiterpenes	hydrocarbons (%)		7.4	7.7	–	13.4	16.1	4.8	10.4	14.6
	oxygenated (%)		0.6	0.6	–	1.2	18.8	0.9	18.1	3.8
others (%)			–	–	–	–	1.3	–	0.9	0.9
total monoterpenes (%)			82.5	91	94.5	74.8	35	90.9	46.9	66.2
total sesquiterpenes (%)			8	8.3	–	14.6	34.9	5.7	28.5	18.4
total oxygenated (%)			61	72	91.7	70.4	25.4	49.7	59.5	34.7
total hydrocarbons (%)			29.5	27.3	2.8	19	44.5	46.9	15.9	49.9

[a] Mean value of three determinations (three replicates) calculated from GC-MS areas; t (trace), relative content <0.1%; (–), not detected. [b] Compounds are listed in order of elution from a Phenomenex Zebron ZB-5 capillary column. Identification by comparison of mass spectra with the respective data of NIST and Willey (30:70) libraries in total ion current (TIC) and the literature, as well as retention indices as calculated according to Kovats (1978) for alkanes C_9-C_{24} compared with the ones reported by Adams (*21*). [c] Retention indices on a Phenomenex Zebron ZB-5 capillary column. [d] Identification by comparison with a co-injected reference standard.

0.4% (*26*), *O. vulgare*, 0.2% (*27*), *S. officinalis*, 1.6% (*28*), *M. pulegium*, 1.5% (*29*), and *M. officinalis*, 0.2−0.3% (*26*−*30*).

Data in **Table 2** show the identified compounds in order of their elution from the Phenomenex Zebron ZB-5 capillary column, the retention indices (RI), and the compounds' relative percentages in the total composition of each EO. The terpenic constituents indentified were mainly monoterpenens and sesquiterpenes, hydrocarbons and oxygenated compounds. More specifically, in EO obtained from *M. pulegium* and *O. dictamnus*, 12 and 25 constituents were identified, amounting to 94.5 and 96.6% of the total oil composition, respectively. In *O. vulgare* and *S. officinalis* oils the numbers of identified constituents were 32 and 34, representing 99.3 and 90.5% of the total oil content. The oils of *O. basilicum* and *V. a. castus* contained 36 and 46 constituents, amounting to 89.4 and 85.5% of the total oil composition. Finally, the greatest number of components was found in the oils obtained from *M. officinalis* and *S. clandestina*, 55 and 62 constituents, respectively, but representing about 76.1 and 77.4% of the oils' total content. In the latter two oils a great number of unidentified compounds, each contributing $< 0.1\%$ of the total oil composition (traces) and with a low comparison quality, were detected in GC-MS chromatograms. Generally, in all of the EOs the predominant compounds were monoterpenes (hydrocarbons and oxygenated compounds) and the highest levels were identified in the EO of *M. pulegium*, *O. vulgare*, and *O. dictamnus* (94.5, 91.0, and 90.9%, respectively). Moreover, the main subgroup of the monoterpene constituents identified in these three EOs was shown to be oxygenated compounds comprising 91.7, 71.4, and 48.8% of the total synthesis, respectively. On the contrary, almost half of the compounds (34.9%) identified in the EO of *S. clandestina* were sesquiterpenes (hydrocarbons and oxygenated products). In general, the oxygenated compounds were predominant over hydrocarbons in the EO monoterpene constituents, with the exception of *S. clandestina* and *V. a. castus*.. Hydrocarbons predominated over oxygenated compounds in the sesquiterpene constituents, with the exception of *S. clandestina* and *M. officinalis*. In order of decreasing percentage, the EO constituents were oxygenated monoterpenes, followed by monoterpene hydrocarbons, sesquiterpene hydrocarbons, and finally oxygenated sesquiterpenes.

The EO of *M. pulegium* is characterized by the presence of the ketones, menthone and pulegone, found at 28.3 and 51.5% of total content, respectively (**Table 2**). Carvacrol was the major constituent of *O. vulgare* and *O. dictamnus* EO, constituting almost the bulk of the oil, 68.5 and 44.3% of the total composition, respectively, as previously reported by others (*27*, *31*). *O. dictamnus* oil was also characterized by high contents in the monoterpene hydrocarbons *p*-cymene (13.2%) and γ-terpinene (18.3%). The major components of *M. officinalis* EO were defined as *trans*-anethole and (−)-caryophyllene oxide, representing 13.2 and 12.4% of the total oil, respectively. 1,8-Cineole was a major component of the EO of *S. officinalis*, *O. basilicum*, and *V. a. castus* determined at 25.5, 13.0, and 18.4%, respectively, whereas camphor, linalool, and sabinene represented 16.8, 43.1, and 19.8% of the above-mentioned oils, respectively. Similarly to our findings, linalool was reported as the most abundant component of the oil obtained from aerial parts of Pakistani *Ocimum basilicum* (*25*). Likewise, Novak also reported 1,8-cineole and sabinene to be the main components of *V. a. castus* EO (*26*). Interestingly, no major constituent compounds were found in the EO of *S. clandestina* because all identified substances contributed $< 10\%$ in the oil total synthesis.

To further correlate the chemical composition of the oils to their nematicidal activity tested in this study, 13 terpenoids were quantitatively characterized using the external calibration method (Supporting Information). Among these compounds, *p*-cymene, 1,8-cineole, linalool, pulegone, *trans*-anethole, and carvacrol were found in the studied EOs as main components, amounting to $> 10\%$ of the total oil, whereas limonene, terpinen-4-ol, L-carvone, geraniol, thymol, eugenol, and β-caryophyllene were determined as minor constituents, amounting to $< 10\%$ of the oils' composition.

Nematicidal Activity of EO and Terpene Components. The dose response curves and the EC_{50} values only of the EOs exhibiting nematicidal activity in the tested dose range are presented in **Figure 1**. Clear dose and time response relationships were established. Volatiles from wells containing the EO solutions did not affect nematodes in the nearby wells, where J2 were immersed in tap water, indicating no cross-contamination among experimental treatments. In all cases juveniles' motility loss was irreversible after transfer into plain water. Essential oils containing high levels of oxygenated compounds were highly nematicidal. In fact, EOs obtained from *O. vulgare* and *O. dictamnus* were the most potent, with EC_{50} values of 2.11 or 1.55 and 2.47 or 1.72 μL/mL, respectively, after 24 or 96 h of larvae immersion in

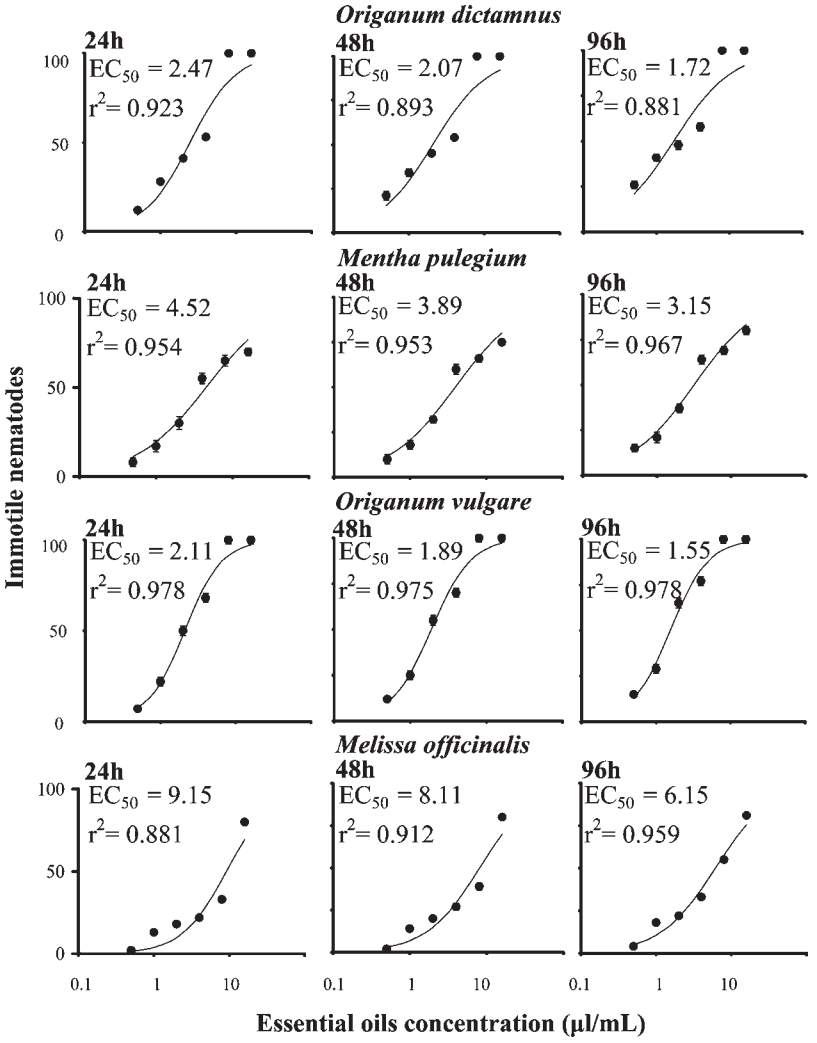

Figure 1. Dose response curves of *Meloidogyne incognita* immotility following immersion to essential oils solutions for 24, 48, and 96 h. Each point represents the average percent number of immotile nematodes of six replications per treatment (× two experiments replications) after elimination of natural death/mortality measured in the control.

the test solutions. Considerable activity was also found for the EOs of *M. pulegium* and *M. officinalis* and the EC_{50} values, after 24 or 96 h of exposure, were calculated at 4.52 or 3.15 μL/mL and 9.15 or 6.15 μL/mL, respectively. On the other hand, EOs of *O. basilicum*, *S. officinalis*, *V. a. castus*, and *S. clandestina* showed low nematicidal activity and the paralysis increase over control never exceeded 30% (data not shown). Similarly, Oka has reported paralysis of *M. javanica* larvae immersed at low concentration levels (1 μL/mL) of EOs obtained from two *Mentha* species (*M. rotundifolia* and *M. spicata*) and *O. vulgare*, but only limited activity of *O. basilicum* oil (*15*). Interestingly, Pandey has revealed higher nematicidal activity of *O. basilicum* oil compared to three *Mentha* species (*M. arvensis*, *M. piperita*, and *M. spicata*), at concentration levels as low as 0.125 μL/mL, against *M. incognita* (*32*). The activities of 13 terpene constituents of EOs against *M. incognita* are presented in **Table 3**. The oxygenated compounds were found to be more active than hydrocarbons *p*-cymene, limonene, and β-caryophyllene, which failed to paralyze half the number of J2 (**Table 3**). In some cases the oxygenated compounds were so active that the lowest test concentrations used provoked paralysis higher than 50%. In such cases, like L-carvone at 48 h, EC_{50} values are estimated to be lower than the lowest concentrations used for the experiment. Larvae immersion in test solutions of L-carvone, pulegone, and *trans*-anethole for 24 h at the concentrations of 239, 468, and 247 μg/mL achieved 100% paralysis and $EC_{50/24\,h}$ values were estimated at 115−170 μL/mL.

Table 3. EC_{50} and R^2 Values of Individual Terpenes against *Meloidogyne incognita* Calculated for Three Immersion Periods in Test Solutions[a]

		24 h		48 h		96 h	
no.	compound name	EC_{50} (μg/mL)	R^2	EC_{50} (μg/mL)	R^2	EC_{50} (μg/mL)	R^2
1	*p*-cymene	>1720	–	>1720	–	>1720	–
2	limonene	>1684	–	>1684	–	>1684	–
3	1,8-cineole	>1842	–	>1842	–	1603	0.80
4	linalool	862	0.95	707	0.89	284	0.98
5	terpinen-4-ol	392	0.99	290	0.97	168	0.88
6	L-carvone	115	0.98	<120	–	<120	–
7	pulegone	150	0.99	<117	–	<117	–
8	geraniol	237	0.99	158	0.99	105	0.98
9	*trans*-anethole	170	0.99	140	0.97	124	–
10	thymol	390	0.94	280	0.94	160	0.94
11	carvacrol	264	0.98	176	0.99	117	0.99
12	eugenol	256	0.94	192	0.85	<133	–
13	β-caryophyllene	>1804	–	433	0.97	307	0.95

[a] If R^2 values are not presented, the EC_{50} values have not been calculated because they were outside the test concentration range. In these cases > means that the estimated EC_{50} value is higher than the upper concentration used, achieving paralysis lower than 50%, whereas < means that the estimated EC_{50} value is lower than the lowest concentration used, achieving paralysis higher than 50%.

Eugenol and carvacrol followed, causing 100% paralysis of J2 exposed to 533 and 488 μL/mL for 24 h, whereas $EC_{50/24\ h}$ values were calculated at 256 and 264 μL/mL. Geraniol, thymol, and terpinen-4-ol were moderately nematicidal, causing 100% paralysis of J2 exposed to 439, 1000, and 939 μL/mL after exposure of J2 for 24 h, and the $EC_{50/24\ h}$ values were calculated at 237, 390, and 392 μL/mL respectively. The $EC_{50/24\ h}$ value of linalool was calculated at 862 μL/mL, whereas 1,8-cineole was the oxygenated terpene with the weakest nematicidal activity, achieving 50% paralysis at 1603 μg/mL after 96 h. Carvacrol, 1,8-cineole, eugenol, geraniol, and thymol have been previously reported to paralyze 91, 9, 10, 60, and 76%, respectively, of *M. incognita* larvae exposed for 24 h at concentrations as low as 0.5 μL/mL (*12*). To the best of our knowledge the present study's data, relevant to the nematicidal activity of all other terpenes tested against *M. incognita* for paralysis activity, are reported for the first time. Obviously, some of the constituent terpenes have been previously used against other nematode taxa or using other experimental protocols. Oka et al. reported strong activity of carvacrol and pulegone against *M. javanica*, and the EC_{50} values were calculated at 0.128 and 0.205 μL/mL after immersion of J2 in test solutions for 48 h, respectively (*11*). Sangwan and co-workers reported high activity of geraniol, eugenol, and linalool against *M. javanica* (*33*), whereas according to Walker and Merlin these terpenes did not reduce galling caused by *M. incognita* on tomato (*34*). Similarly to our results, carvacrol, thymol, *trans*-anethole, and L-carvone have been found to provoke already *M. javanica* larvae paralysis at a similar concentration range from 0.125 to 0.50 μL/mL (*11*, *15*).

The strong nematicidal activity of the EOs of *O. vulgare* and *O. dictamnus* could be correlated to the high contents of carvacrol determined at 68.5 and 44.3%, respectively. Likewise, the activities of *M. pulegium* and *M. officinalis* EO could be attributed to the high contents of pulegone and *trans*-anethole accounting for 51.5 and 13.2%, respectively. Nevertheless, the nematicidal activity of strongly nematicidal terpenes used individually as well as components of the EO was not found to be equal (pure terpenes $EC_{50/\text{terpene in EO}}$ values < $EC_{50/EO}$ values). According to the results, the $EC_{50/trans\text{-anethole}}$ value against J2 was found to be lower than 124 μL/mL, but when tested as a component of *M. officinalis* EO was found to be 988 μL/mL. The $EC_{50/carvacrol}$ value against J2 was calculated at 117 μg/mL, whereas the respective values tested as a component of *O. dictamnus* and *O. vulgare* EO were 751 and 1141 μL/mL, respectively. Finally, the $EC_{50/pulegone}$ value was lower than 117 μL/mL, whereas pulegone considered as a component of the *M. pulegium* EO had an $EC_{50/\text{pulegone in EO}}$ value of 1815 μL/mL. The lower activity of the EO compared to pure terpenes suggests antagonistic actions among terpenes in oils.

Finally, the EO containing a high number of constituents was not necessarily found to be active against nematodes. Characteristic was the case of the EO of *S. clandestina*, which, although comprising the highest number of constituents (62), showed poor activity, whereas the EO of *M. pulegium*, containing only 16 compounds, was highly nematicidal.

All of the above cohere to the fact that the contribution of each ingredient compound to the overall activity of an EO is a complicated pattern of interactions. Lahlou interestingly concluded that the relationship between composition and activity leads to the suggestion that the biological activity of the essences from the aromatic plants may be attributable both to their major components and to the minor ones present in the oils (*3*). It is possible that they may act together synergistically or antagonistically to contribute to the toxicity of the totality of the tested oil. Notably, it was confirmed that "inactive" constituents may have some synergic effect on the "active" constituents and that, although not active individually, their presence is necessary to achieve full toxicity (*3*, *35*). In this study a first attempt was made toward the comparative toxicity of EO and constituent compounds against *M. incognita*. To corroborate the role of individual constituents toward the synergic and antagonistic actions, among each other, artificial blends must be tested for their activity. In general, according to our findings the EC_{50} values corresponding to EO were higher than those of pure terpenes. On the other hand, blends are more effective than individual compounds in terms of avoiding resistance in a long-term use. The relatively low cost of terpenes resulting from their extensive use in fragrances and flavoring permits screening of terpenes and blends for efficacy on a large scale, and this is what future studies must be focused on.

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Supporting Information Available: Quantification of essential oils' terpene constituents using external standard calibration curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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