GC-MS Analysis of Essential Oils from Some Greek Aromatic Plants and Their Fungitoxicity on *Penicillium digitatum*

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The isolated essential oils from seven air-dried plant species were analyzed by gas chromatography−mass spectrometry (GC-MS). *Thymus vulgaris* (thyme), *Origanum vulgare* (oregano), and *Origanum dictamus* (dictamus) essential oils were found to be rich in phenolic compounds representing 65.8, 71.1, and 78.0% of the total oil, respectively. *Origanum majorana* (marjoram) oil was constituted of hydrocarbons (42.1%), alcohols (24.3%), and phenols (14.2%). The essential oil from *Lavandula angustifolia* Mill. (lavender) was characterized by the presence of alcohols (58.8%) and esters (32.7%). Ethers predominated in *Rosmarinus officinalis* (rosemary) and *Salvia fruticosa* (sage) essential oils, constituting 88.9 and 78.0%, respectively. The radial growth, conidial germination, and production of *Penicillium digitatum* were inhibited completely by oregano, thyme, dictamus, and marjoram essential oils at relatively low concentrations (250−400 μg/mL). Lavender, rosemary, and sage essential oils presented less inhibitory effect on the radial growth and conidial germination of *P. digitatum*. Conidial production of *P. digitatum* was not affected by the above oils at concentrations up to 1000 μg/mL. Apart from oregano oil, all essential oils were more effective in the inhibition of conidial germination than of radial growth. The monoterpene components, which participate in essential oils in different compositions, seem to have more than an additive effect in fungal inhibition.

Keywords: *GC-MS analysis; essential oils; fungitoxicity; antimicrobial; Penicillium digitatum*

INTRODUCTION

Aromatic plants produce volatile C_{10} and C_{15} terpenes that are derived from the isoprene unit. These substances, which are known as essential oils, can be isolated from various parts of plants by steam distillation or other modified methods. A wide variety of terpene hydrocarbons, cyclic or noncyclic, and their oxygenated isoprenoid compounds are present in essential oils as mixtures.

The chemical composition of a plant essential oil depends on a number of parameters, such as the enviromental conditions, the season that the aromatic plants have been collected, the dehydration procedure, the storage conditions under which the collected plants were kept until their essential oil extraction, the applied method for the isolation of the essential oil, and the analysis conditions (column, programmed temperature), which are used for the identification of the compounds (Hawthorne et al., 1993; Kokkini et al., 1997; Tarantilis and Polissiou, 1997; Russo et al., 1998).

Pharmacology, pharmaceutical botany, medical and clinical microbiology, phytopathology, and food preservation are some fields in which the essential oils can be applied. The antimicrobial activity of essential oils and their pure compounds from plant species of the Lamiaceae family has been reported by several researchers, although few of them have studied the chemical composition of the applied essential oils. A gas chromatography−mass spectrometry (GC-MS) (qualitative and quantitative) analysis is indispensable for the evaluation of the biological activity of the essential oil.

The antimicrobial activity of essential oils against important human pathogenic microorganisms has been examined in detail (Farag et al., 1989; Paster et al., 1990; Adam et al., 1998; Smith-Palmer et al., 1998; Hammer et al., 1999; Marino et al., 1999; Cosentino et al., 1999). The inhibitory effects of the main essential oil components or the total oil on microorganisms that cause food spoilage have been also studied (Thompson, 1989; Ismaiel and Pierson, 1990; Mahmoud, 1994; Basilico and Basilico, 1999). Recently, interest in the application of essential oils to control plant pathogens has increased (Gorris et al., 1994; Thanassoulopoulos and Laidou, 1997; Reddy et al., 1998; Arras et al., 1993, 1995).

Penicillium digitatum is one of the most common postharvest pathogens, causing green mold rots in *Citrus* species. A number of fungicides such as benzimidazoles, aromatic hydrocarbons, and sterol biosynthesis inhibitors are in use as postharvest treatments to control the pathogen. A serious problem in the effective use of these chemicals is the development of resistance by *P. digitatum*. The application of higher concentrations of chemicals to control the resistant strains, if possible, increases the risk of high levels of toxic residues in the products. In that case, the problem is particularly serious because fruits are often consumed in a relatively short time after harvest.

Our objectives in the present work were, first, to determine the chemical composition of the essential oils from *Thymus vulgaris* (thyme), *Origanum vulgare* (oregano), *Origanum dictamus* (dictamus), *Origanum majorana* (marjoram), *Lavandula angustifolia* Mill.

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(lavender), *Rosmarinus officinalis* (rosemary), and *Salvia fruticosa* (sage) by GC-MS analysis and, second, to evaluate the efficacy of the above oils and their pure major components on the radial growth, conidial germination and production of *P. digitatum*.

EXPERIMENTAL PROCEDURES

Materials. All of the dried aromatic plants, apart from marjoram, were collected from Crete (Greece) and stored at room temperature in darkness. Marjoram was collected from Attiki (Greece), air-dried, and stored under the same conditions.

Pure commercial essential oil components were purchased from the Sigma-Aldrich Co.

The test organism, *P. digitatum*, was provided by the Laboratory of Phytopathology of the Agricultural University of Athens (AUA).

Methods. *Isolation of the Essential Oils.* The essential oils were isolated according to the Lickens−Nickerson method, using a microsteam distillation−extraction apparatus for organic solvents lighter than water.

The apparatus consisted of a main body, a coldfinger, a 100 mL water flask, and a 5 mL solvent flask. Four milliliters of the extracting solvent (diethyl ether) was heated in the solvent flask and condensed on the coldfinger above the place where the water condensed. The sample (10 g) to be analyzed was heated in the water flask with water. The vapor, which also contained the volatile organic compounds, condensed on the coldfinger. Both phases returned via the connecting tubes into their flasks, and the procedure was started again. Refluxing was continued for ~2 h. In that way, all of the aroma constituents concentrated in the extracting solvent. Inert gas (N_2) was introduced into the main body of the apparatus to avoid the oxidation of molecules during the procedure. All diethyl ether extracts were stored at 4 °C until their analysis by GC-MS or their usage in bioassays.

Analysis Conditions. Apart from marjoram oil, the analysis of all essential oils was performed using a Hewlett-Packard 5890 II GC, equipped with a HP-5 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) and a mass spectrometer 5971 A as detector (method A). The carrier gas was helium, at a flow rate of 1 mL/min. Column temperature was initially 60 °C for 5 min, then gradually increased to 160 °C at 4 °C/min, and finally increased to 240 °C at 15 °C/min. For GC-MS detection an electron ionization system was used with an ionization energy of 70 eV. The extracts were diluted 1:100 (v/v) with diethyl ether, and 1.0 μL of the diluted samples was injected automatically in splitless mode. Injector and detector temperatures were set at 250 and 280 °C, respectively. Marjoram essential oil analysis was performed using a Fison 8000 GC, equipped with a CP-Sil 8 (30 m, 0.32 mm i.d.) capillary column and a mass spectrometer 800 as detector (method B). Column temperature was initially 60 °C for 5 min, then gradually increased to 240 °C at 4 °C/min, and kept there for 5 min. In this case, 1.0 μL of the diluted sample was injected manually in splitless mode.

Measurement of Fungitoxicity. A stock of pure essential oils of the aromatic plants was prepared to be used in all bioassays. The diethyl ether extracts of each plant were combined, and the solvent was evaporated by a flow of nitrogen gas at room temperature.

Inhibition of mycelial growth of *P. digitatum* was determined by daily measuring of the radial growth on PDA plates containing the respective essential oil at a range of concentrations, for 10 days at 25 °C (Ziogas and Girgis, 1993). Plates were inoculated with 2 mm disks from PDA on which conidia had been allowed to germinate.

For spore germination assays, conidia were plated on PDA medium with and without the essential oil. The proportion of conidia capable of producing germ tubes was counted after 12 h of incubation at 25 °C.

To determine conidial production in the absence and presence of the examined essential oil, PDA plates were inoculated with a conidial suspension and incubated for 7 days in an incubation cabinet at 25 °C. The total mycelial mass that was produced in each dish was transferred to a flask with 50 mL of sterile water. The flasks were agitated vigorously, and the concentration of conidia in the resulting spore suspension was evaluated by counting with a hemocytometer and converted to spores per square centimeter of plate culture (Ziogas and Girgis, 1993).

RESULTS AND DISCUSSION

Oil Composition. The compositions of essential oils from *T. vulgaris* (thyme), *O. vulgare* (oregano), *O. dictamus* (dictamus), *S. fruticosa* (sage), *O. majorana* (marjoram), *L. angustifolia* Mill. (lavender), and *R. officinalis* (rosemary) were determined by comparing the relative retention times and the mass spectra of oil components with those of authentic samples and mass spectra from data library. Most of the essential oils were characterized by the dominant presence of one or two substances. Only in marjoram essential oil have a large number of substances been found.

T. vulgaris, *O. vulgare*, and *O. dictamus* are a group of plants having their essential oils characterized by the predominant presence of thymol (Table 1).

Thyme essential oil was characterized by the presence of γ-terpinene (4.3%), *p*-cymene (23.5%), carvacrol (2.2%), and thymol (63.6%), which composed 93.6% of the total oil. In oregano essential oil the most abundant compounds were also γ-terpinene (12.7%), *p*-cymene (9.9%), carvacrol (7.8%), and thymol (63.3%), which participated in the mixture at 93.7%. Along with thymol (78%), *p*-cymene (10.1%) and γ-terpinene (7.9%) constituted 96% of dictamus oil. Oregano oil also has been characterized as a thymol chemotype by Russel et al. (1998). In contrast to our results, carvacrol instead of thymol was determined as the main compound in oregano and dictamus oils by other researchers (Sivropoulou et al., 1996; Baser et al., 1993). In some other cases the percentages of carvacrol and thymol in the total oil were almost equal (Adam et al., 1998; Russo et al., 1998).

The analysis of the marjoram oil gave a large number of constituents. Among them were detected 3-thujene (2.8%), β-myrcene (3.8%), 2-carene (7.8%), 2-ethyl-*m*-xylene (5.2%), 3-carene (10.4%), terpinen-4-ol (7.8%), sabinene hydrate (6.0%), α-terpineol (4.2%), and thymol (14%). Two chemotypes of *O. majorana* were found in the literature, the *cis*-sabinene hydrate/tepinen-4-ol chemotype and the carvacrol/thymol chemotype (Komaitis et al., 1992; Bellomaria et al., 1993; Baser et al., 1993). High amounts of carvacrol (78.3−79.5%) and thymol (11.55%) have been reported by Baser et al. (1993) and Pino et al. (1997) for *O. majorana* from Turkey and Cuba, respectively. Generally, the *Origanum* species is characterized by the presence of two major biochemically related groups of compounds (Skoula et al., 1999). The first group includes the aromatic monoterpenes such as *p*-cymene, thymol, carvacrol, their precursor γ-terpinene, and their derivatives. The second group includes the thujanes, such as sabinene, sabinene hydrate, and their derivatives. According to Skoula et al. (1999) there is a clear division into sabinyl-rich and carvacrol-rich plants with no intermediates observed.

From our results, the specified sample from Greek *O. majorana* included also a high amount of thymol (14%), and this is reported for the first time about Greek marjoram. It seems that this sample of *O. majorana* plants is characterized by an intermediate situation due

Table 1. Quantitative Composition of the Essential Oils from Thyme (T), Oregano (O), Dictamus (D), and Marjoram (M)

$t_{R-T,O,D}$[a] (min) by method A	t_{R-M}[b](min) by method B	component	certainty of identification	composition (%) T	O	D	M
		hydrocarbons					
5.67	4.87	3-thujene	**[c]	–[e]	0.3	0.3	2.8
	5.07	3,6,6-trimethyl-2-norpinene	**	–	–	–	1.9
5.79		α-pinene	***[d]	0.3	0.6	0.3	–
	7.71	*β*-myrcene	***	–	–	–	3.8
7.84		*β*-pinene	**	–	0.5	0.5	–
	8.07	2-carene	**	–	–	–	7.8
	8.32	2-ethyl *m*-xylene	**	–	–	–	5.2
	8.50	*m*-mentha-6,8-diene	**	–	–	–	4.2
9.01		α-terpinene	***	1.0	1.0	0.9	–
9.15		*p*-cymene	***	23.5	9.9	10.1	–
	9.72	3-carene	**	–	–	–	10.4
10.74		*γ*-terpinene	***	4.3	12.7	7.9	–
	12.14	sabinene	**	–	–	–	1.3
26.88	23.14	*β*-caryophyllene	***	1.3	0.5	0.4	2.6
31.77	26.15	*β*-bisabolene	**	–	0.4	–	2.1
		alcohols					
	11.32	sabinene hydrate	**	–	–	–	6.0
12.49	11.45	linalool	***	–	0.6	0.4	3.8
14.85	13.87	borneol	***	1.4	0.5	–	2.5
15.52	14.44	terpinen-4-ol	**	0.6	0.3	0.3	7.8
	14.92	α-terpineol	***	–	–	–	4.2
		phenols					
20.87	18.84	carvacrol	***	2.2	7.8	–	0.2
21.58	19.60	thymol	***	63.6	63.3	78.0	14.0
		esters					
	17.49	linalyl acetate	**	–	–	–	3.4
		others		1.8	1.6	0.9	16
		total		100	100	100	100

[a] $t_{R-T,O,D}$ is the retention time for the compounds of T, O, and D, according to method A of GC-MS analysis. [b] t_{R-M} is the retention time for the compounds of M, according to method B of GC-MS analysis. [c] **, tentative identification from mass spectra data. [d] ***, positive identification from mass spectrum and retention time which agree with authentic compound. [e] Not determined.

to the environmental conditions and the agricultural treatments (irrigation) which developed. The essential oils' chemical composition varies and depends of locality, the climatic conditions, and the season that the plants were collected (Kokkini et al., 1997; Russo et al., 1998).

The chemical composition of rosemary and sage essential oils was characterized by the predominant presence of 1,8-cineole, which composed 88.9 and 78% of the total oils, respectively (Table 2). Except for 1,8-cineole, α-pinene (2.7%), *p*-cymene (0.7%), ocimene (0.7%), borneol (1.5%), α-terpineol (1.3%), and camphor (2.4%) were detected in rosemary essential oil. The monoterpenes α-pinene (2.3%), *β*-pinene (3.7%), *β*-caryophyllene (1.0%), *β*-myrcene (1.4%), linalool (0.7%), α-terpineol (2.7%), thujone (4.2%), and camphor (1.2%) also participated in the case of sage oil, except for 1,8-cineole. The same terpenes have been detected in the Greek sage essential oil by Sivropoulou et al. (1997). In the case of lavender, linalool (44.5%), linalyl acetate (32.7%), and 1,8-cineole (4.8%) were dominant in the mixture, representing 82% of the total oil (Table 2). The same terpenes were also found to be the main components of lavender oil by Adam et al. (1998).

Effect of Essential Oils and Their Main Components on Radial Growth of *P. digitatum*. Pure commercial thymol, the basic compound of thyme, oregano, dictamus, and marjoram oils, and carvacrol, which differs from thymol only with regard to the position of the hydroxyl group at the aromatic ring, were tested independently for their effectiveness in the inhibition of the radial growth of *P. digitatum*. A dose-dependent inhibition of mycelial growth was observed in both cases. The ED_{50} value, the concentration causing

Table 2. Quantitative Composition of the Essential Oil from Lavender (L), Rosemary (R), and Sage (S)

$t_{R-L,R,S}$[a] (min) by method A	component	certainty of identification	composition % L	R	S
	hydrocarbons				
5.79	α-pinene	***[b]	–[d]	2.7	2.3
5.92	ocimene	**[c]	–	0.7	–
7.84	*β*-pinene	**	–	–	3.7
8.11	*β*-myrcene	***	–	–	1.4
9.15	*p*-cymene	***	–	0.7	–
23.44	2-carene	**	–	–	0.3
26.88	*β*-caryophyllene	***	0.3	–	1.0
28.77	α-caryophyllene	**	–	–	0.2
	alcohols				
12.49	linalool	***	44.5	–	0.7
14.85	borneol	***	3.9	1.5	–
15.19	myrcenol	**	–	–	1.0
15.52	terpinen-4-ol	**	6.9	–	0.4
15.85	α-terpineol	***	3.5	1.3	2.7
	ethers and ketones				
9.46	1,8-cineole	***	4.8	88.9	78.0
12.73	thujone	***	–	–	4.2
13.76	camphor	***	–	2.4	1.2
	esters				
18.95	borneol acetate	**	–	–	0.2
19.01	linalyl acetate	**	32.7	–	–
	others		3.4	1.8	2.7
	total		100	100	100

[a] $t_{R-L,R,S}$ is the retention time for the compounds of L, R, and S, according to method A of GC-MS analysis. [b] ***, positive identification from mass spectrum and retention time which agree with authentic compound. [c] **, tentative identification from mass spectra data. [d] Not determined.

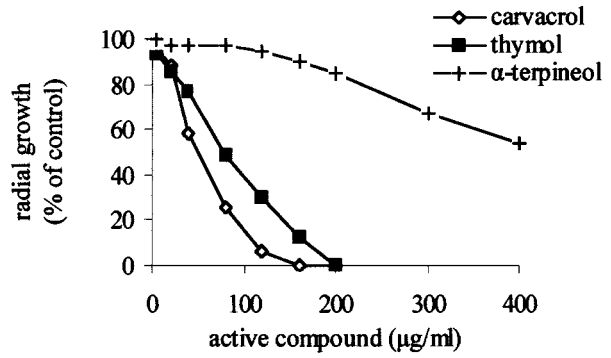

Figure 1. Effect of carvacrol, thymol, and α-terpineol on radial growth of *P. digitatum*.

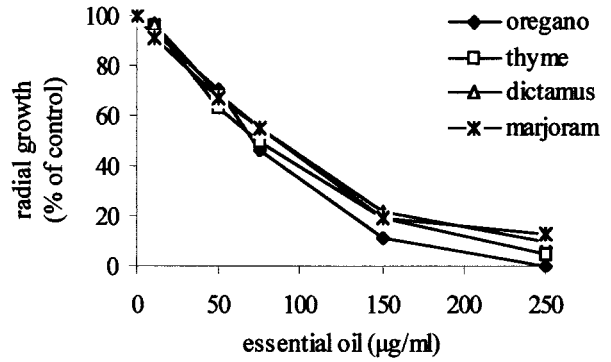

Figure 2. Effect of oregano, thyme, dictamus, and marjoram essential oils on radial growth of *P. digitatum*.

50% inhibition of radial growth, and the minimal inhibitory concentration (MIC) were approximately 47 and 160 μg/mL, respectively, for carvacrol and 79 and 200 μg/mL, respectively, for thymol (Figure 1). A comparable MIC value (125 μg/mL) was also found for carvacrol fungitoxic action by Caccioni and Guizzardi (1994).

Pure commercial cymene, cineole, linalool, β-myrcene, α-terpineol, and α-pinene were also tested for their fungitoxicity to the radial growth of *P. digitatum* at concentrations up to 400 μg/mL. Only α-terpineol was effective, reducing by 46% the radial growth of *P. digitatum* at a concentration of 400 μg/mL (Figure 1).

A dose-dependent inhibition of *P. digitatum* mycelial growth was also caused by thyme, oregano, dictamus, and marjoram essential oils (Figure 2). Mycelial growth was totally inhibited by these oils at 300, 250, 300, and 400 μg/mL, respectively.

Lavender, rosemary, and sage essential oils were slightly fungitoxic on the mycelial growth of *P. digitatum* (Figure 3). The radial growth was inhibited 29.5, 24.0, and 9.0% at 1000 μg/mL by lavender, rosemary, and sage essential oils, respectively.

Carvacrol and thymol seem to have more than an additive effect on fungal inhibition. This conclusion is drawn by the fungitoxicity of oregano, thyme, and dictamus essential oils in relation to their composition in thymol and carvacrol. Thus, oregano oil [carvacrol (7.8%) plus thymol (63.3%), total = 71%) and thyme oil [carvacrol (2.2%) plus thymol (63.6%), total = 66%) were more fungitoxic to *P. digitatum* than dictamus oil [thymol (78%)] at 250 μg/mL oil concentration (Figure 2).

Effect of Essential Oils on Conidial Germination and Production of *P. digitatum*. The oregano, thyme, dictamus, and marjoram essential oils inhibited ef-

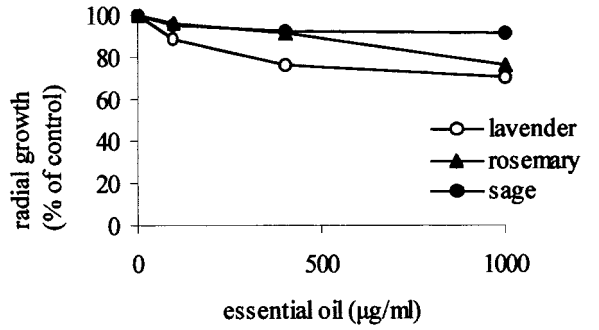

Figure 3. Effect of lavender, rosemary, and sage essential oils on radial growth of *P. digitatum*.

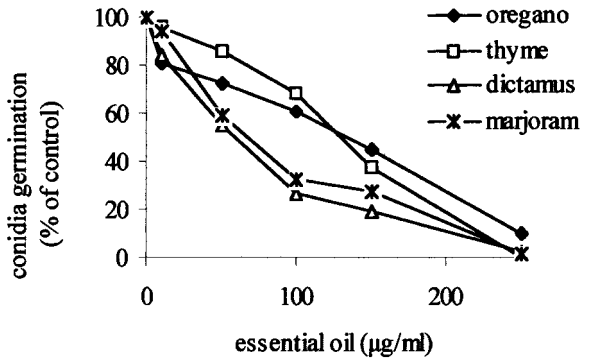

Figure 4. Inhibition of conidial germination in *P. digitatum* by oregano, thyme, dictamus, and marjoram essential oils.

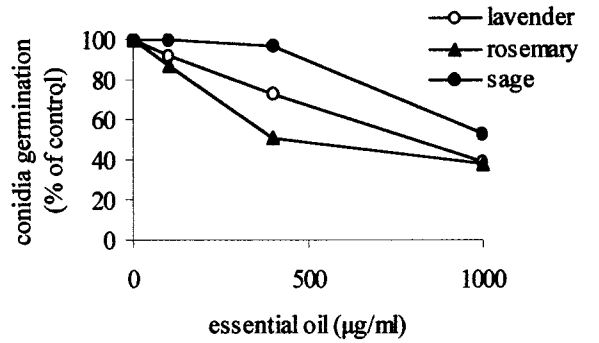

Figure 5. Inhibition of conidial germination in *P. digitatum* by lavender, rosemary, and sage essential oils.

fectively the conidial germination of *P. digitatum* (Figure 4). A complete inhibition of germination was observed at the concentration of ~250 μg/mL. A similar fungitoxitity on spore germination was obtained with pure carvacrol. The MIC of carvacrol on spore germination of *P. digitatum* was also determined at the level of 250 μg/mL by Caccioni and Guizzardi (1994).

Conidial germination was also found to be more sensitive than the radial growth to lavender, rosemary, and sage essential oils (Figure 5). Thus, at 1000 μg/mL of the above oils conidial germination was inhibited 2.0, 2.7, and 5.0 times more than the radial growth. Conidial production was also found to be sensitive to thyme, oregano, dictamus, and marjoram essential oils. A complete inhibition of conidial production was observed at the concentration of 250 μg/mL of thyme, oregano, and dictamus essential oils. At the same concentration, marjoram essential oil inhibited the conidial production by 84% (Figure 6). Lavender, rosemary, and sage essential oils were found to be nonfungitoxic in conidial production of *P. digitatum* at concentrations up to 1000 μg/mL.

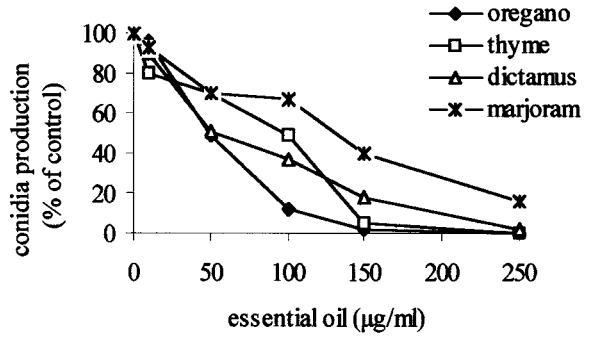

Figure 6. Inhibition of conidial production in *P. digitatum* by oregano, thyme, dictamus, and marjoram essential oils.

The presence of an aromatic ring with a phenolic hydroxylic group that forms hydrogen bonds with active sites of target enzymes was suggested by Farag et al. (1989) to be responsible for the fungitoxitity of the essential oils. Although this hypothesis fits well for the activity of essential oils containing high amounts of phenolic compounds such as the thyme, oregano, and dictamus oils, it is difficult to explain the fungitoxicity of marjoram oil, which was found by the present work to contain mainly hydrocarbons. The antifungal activity of the marjoram oil cannot be explained only by the presence of thymol (14%) in the total oil. In addition, alcohols and esters contribute 91.5% of the total mixture in *L. angustifolia* Mill. essential oil; ethers were predominant in *R. officinalis* and *S. fruticosa* essential oils, representing 88.9 and 78.0% of the total oils, respectively. These oils also showed a degree of fungitoxicity. It seems that in addition to phenolic compounds, many other active monoterpenes contribute to the fungitoxicity of the essential oils. This hypothesis is also supported by the following evidence: A nonphenolic thyme essential oil was found by Ruiz et al. (1993) to have strong antimicrobial activity, with MIC ranging from 15 to 175 μg/mL. Furthermore, two chemotypes of *Thymus longicaulis* subsp. *chaoubardii* with high contents of nonphenolic substances (geraniol and geranyl acetate for the first, linalool and α-terpinyl acetate for the second) possessed stronger antibacterial activities than the corresponding chemotype that contained a high amount of thymol (Tzakou et al., 1998). The antimicrobial activity of major oil compounds according to Faid et al. (1996) has the following order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. However, our study on *P. digitatum* linear growth, conidial germination, and production showed that the fungitoxicity order of essential oil components was also affected by the inhibited fungal physiological activity.

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