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Antifungal activity of some essential oils and their major chemical constituents against some phytopathogenic fungi

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ABSTRACT

In Vitro studies were carried out to determine the antifungal activity of fifteen essential oils, caraway, clove, fennel, thyme, chenopodium, cinnamon, geranium, rosemary, matrecary, rose, dill, garlic, eucalyptus, lemon and peppermint and their monoterpenoidal constituents against six plant pathogenic fungi (*Rhizoctonia solani, Macrofomina phaseolina, Fusarium oxysporum, Helminthosporium sp, Alternaria alternate and Diplodia sp).* The results revealed that caraway, clove, fennel, and thyme oils exhibited potencies to inhibit the growth of the tested fungi. The joint action for the most promising essential oils with piperonyl butoxide, tween 20 and triton X-100 was studied. The antifungal activity was dramatically enhanced against all the tested fungi, particularly in case of clove oil with triton X-100 mixture which proved to be the most effective one. Among the monoterpenoidal constituents, thymol, chlorothymol and carvacrol showed the highest fungicidal activity compared with the other essential oil components used.

Keywords: Fungicidal effect, Radial growth, Natural plant products, Essential oils, Monoterpenoids, *Rhizoctonia solani, Macrofomina phaseolina, Fusarium oxysporum, Helminthosporium sp. Aternaria alternate, Diplodia spp.*

INTRODUCTION

Pathogenic fungi alone cause nearly 20 % reduction in the yield of major food and cash crops (Agrios, 2000). In the last two decades, scientists all over the world tried to minimize the use of synthetic chemicals for the management of plant pathogens, insects and weeds to avoid the environmental pollution hazards. In addition to the target pathogen, pesticides may also kill various beneficial organisms. The increasing incidence of resistance among pathogen towards synthetic chemicals is also a cause of serious concern.

Because of these problems, there is a need to find alternatives, natural plant products that are biodegradable and ecofriendly are catching the attention of scientists worldwide. Various attempts have been carried out to search extensively for new biologically active terpenoids as a potential source for agrochemicals. In recent years, essential oils have received renewed attention due to their wide spectrum of biological activities against several pests such as micro-organisms (Toxopeus and Bouwmeester, 1992; Isman, 2000; Karaman *et al.*, 2001; Soliman and Badeaa, 2002; Rasooli and Mirmostafa, 2002; Rasooli and Abyaneh, 2004; Tripathi and Dubey, 2004; Gayoso *et al.*, 2005; Maksimovic *et al.*, 2005 and Rasooli *et al.*, 2005), insects (Rice and Coats, 1994; Ellis and Baxendale, 1997; Traboulsi *et al.*, 2002 and El-Zemity *et al.*, 2002), mollusca (El-Zamity, 2001 and El-Zamity *et al.*, 2001a,b) and weeds (Duke *et al.*, 2000). Among these essential oils are the antimicrobial properties of eugenol (Gayoso *et al.*, 2005), thyme (Rasooli and Abyaneh, 2004) and cinnamon (Soliman and Badeaa, 2002) which gave results that are difficult to reconcile. Yegen *et al.* (1992) reported that the essential oils of several aromatic plants exhibited fungicidal toxicity against soil-borne phytopathogenic fungi *in vitro*. The essential oils of *Thymbra spicata* and *Satureja thymbra* were most effective in inhibiting mycelial growth of the test fungi *Fusarium moniliforme*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Phytophthora capsici* with minimum inhibitory concentrations (MIC) between 400 and 800 $\mu g\ ml^{-1}$.

In this paper, we report a comparative study of the antifungal properties of some representative essential oils and their main chemical constituents in order to determine which component(s) is responsible for the fungicidal activity. Furthermore, the synergistic effects of tween-20, triton X-100 and piperonyl butoxide with the most promising essential oils were studied.

MATERIALS AND METHODS

1- Test fungi

The six plant pathogens which are chosen for the study are: 1) *Rhizoctonia solani;* causes damping off in many plants such as pre- and post-emergence damping off of potatoes, 2) *Fusarium oxysporum;* Causes damping off in tomato and cotton plants, 3) *Alternaria alternate;* causes alternaria spot, and brown spot in both potato and tomato as well as leaf spot in squash and apple, Also, it has a role in post-harvest disease in orange, lemon and potato, 4) *Diplodia sp.;* causes Diplodia spot in sweet potato and banana, 5) *Macrofomina phaseolina*; causes charcoal rot of bean, winter rape, sesamine, saffron, squash, cotton, potato, sorghum, cucumber and recently okra was reported as a new host and 6) *Helminthosporium sp.*, causes leaf blight of maize. Cultures of test fungi were provided by the Fungicide Bioassay Laboratory, Department of Pesticide Chemistry, Faculty of Agriculture, Alexandria University and maintained during the course of experiments on Czapic Dox Agar (CDA) medium at 25 °C.

2- Test chemicals

Fifteen commercially available essential oils were used in this study namely: Caraway, Clove, Fennel, Thyme, Chenopodium, Cinnamon, Geranium, Rosemary, Matrecary, Rose, Dill, Garlic, Eucalyptus, Lemon and Peppermint. The respective monoterpenoidal constituents of these oils, namely: Thymol, Chlorothymol, Carvacrol, Carveol, Carvone (R), Carvone (S), Gerniol, Citronellol, Eugenol, Menthol, Vanilline, Benzyl alcohol, Camphor, Cinnamaldehyde, Borneol and Cineol were supplied by Aldrich Chemical Company except Camphor which was provided by BDH. Tween-20, Triton X-100 and Piperonyl butoxide were purchased from Aldrich Chemical Company. Captan (Orthocide, 50 % WP, BASF) was included in this study for comparison.

3-Measurement of antifungal activity

The radial growth method of Zambonelli *et al.*, (1996) was used for the evaluation. An appropriate volume of the stock solutions of the essential oil or the pure monoterpenoid dissolved in dimethyl sulfoxide (DMSO) was added to molten medium (CDA: 15 ml) to obtain the desired concentration. Seven different concentrations, ranged from 50 to 1000 $\mu g\ ml^{-1}$ for each essential oil and from 10 to 500 $\mu g\ ml^{-1}$ for monoterpenoid were prepared. Each treatment was replicated five times. The fungal media which contains the test essential oil or the monoterpenoid was poured into each sterile Petri dish (90 mm diameter) at 40-45 °C under aseptic conditions and left to settle. Addition of dimethyl sulfoxide alone to the medium was served as control. Mycelial discs (5 mm diameter) of the plant pathogenic fungi, taken from 8-day-old culture on CDA plates and were transferred aseptically to the center of Petri dishes after solidification of the medium. The treatments were incubated at 25 °C in the dark. The antifungal activity was determined by measured the radial growth in terms of diameter (mm) in all treatments at different intervals till the end of experiment (for the control reach to full growth). Percentage of mycelial growth inhibition was calculated from the formula: Mycelial growth inhibition = {((DC-

DT)/DC)*100} (Pandey *et al.*, 1982), where DC and DT are average diameters of fungal colony of control and treatment, respectively. The concentration that inhibiting the fungi mycelial growth by 50 % (EC_{50}) was determined by a linear regression method (Finney, 1971).

The most potent essential oils were mixed with tween-20, triton X-100 or piperonyl butoxide (P.B) in 1:2 ratio. Seven different concentrations ranged from 10 to 500 μgml^{-1} were prepared and five replicates were used per each treatment. Control assay were carried out using tween-20, triton X-100 or P.B.

RESULTS AND DISCUSSION

The *in vitro* antifungal properties of fifteen essential oils against *Rhizoctonia solani*, *Macrofomina phaseolina*, *Fusarium oxysporum*, *Helminthosporium sp*, *Alternaria alternate*, *Diplodia sp*. by radial growth technique are shown in Table 1. Caraway, clove, thyme oils exhibited high fungicidal activity against all the tested fungi. Clove proved to be very effective oil and gave the most promising antifungal effects against *Diplodia sp* (EC_{50} = 34 $\mu g\ ml^{-1}$). The EC_{50} of caraway was 75 $\mu g\ ml^{-1}$ against R. *solani*. Similarly, clove oil gave high effect, where the EC_{50} was 84 and 77 $\mu g\ ml^{-1}$ against *R. solani*, and *Helminthosporium spp*, respectively. Furthermore, thyme oil gave very interesting results particularly against *A. alternate* and the EC_{50} value of 30 $\mu g\ ml^{-1}$ which exceeding the value of the standard fungicide (EC_{50} for captan was 41.88 $\mu g\ ml^{-1}$). In addition, thyme oil gave very important results against *R. solani* (EC_{50} = 90 $\mu g\ ml^{-1}$) and *Diplodia spp* (EC_{50} = 175 $\mu g\ ml^{-1}$). The results of thyme are in agreement with that reported by Soliman and Badeaa (2002) and Rasooli and Abyaneh (2004). The fennel oil was quite effective against *Diplodia sp* with EC_{50} value of 54 $\mu g\ ml^{-1}$. The results indicated that cinnamon oil was found to be more effective against *Diplodia spp* and *R. solani* than the other tested fungi, while Soliman and Badeaa, (2002) found that cinnamon oil with concentrations less than 500 ppm completely inhibited the growth of *Fusarium spp*. The EC_{50} values for the rest of essential oils including geranium, rosemary, matrecary, rose, dill, garlic, eucalyptus, lemon and peppermint were over 500 $\mu g\ ml^{-1}$ against all the tested fungi.

Table (1): Antifungal activity of some essential oils against six phytopathogenic fungi by radial growth technique.

Fungi / Essential oil	R.S.	M.P	F.O.	H.S	A.A.	D.S
			EC$_{50}$ ($\mu g\ ml^{-1}$)*			
Caraway	75	190	352	182	221	115
Clove	84	266	186	77	245	34
Fennel	417	435	339	315	316	54
Thyme	91	> 500	304	231	30	175
Chenopodium	188	> 500	> 500	307	222	308
Cinnamon	356	> 500	> 500	387	> 500	244
Geranium	290	> 500	> 500	> 500	> 500	> 500
Rosemary	> 500	> 500	> 500	> 500	> 500	> 500
Matrecary	> 500	> 500	> 500	> 500	> 500	> 500
Rose	> 500	> 500	> 500	> 500	> 500	> 500
Dill	> 500	> 500	> 500	> 500	> 500	> 500
Garlic	> 500	> 500	> 500	> 500	> 500	> 500
Eucalyptus	> 500	> 500	> 500	> 500	> 500	> 500
Lemon	> 500	> 500	> 500	> 500	> 500	> 500
Peppermint	> 500	> 500	> 500	> 500	> 500	> 500

R.S. = Rhizoctonia solani — *H.S. = Helminthosporium sp.*
M.P. = Macrofomina phaseolina — *A.A. = Aternaria alternata*
F.O. = Fusarium oxysporum, — *D.S. = Diplodia sp.*

* The concentration of essential oil ($\mu g\ ml^{-1}$) causing 50% reduction in linear growth of the fungi on CDA.

The effect of piperonyl butoxide, tween-20 and/or triton X-100 on potentiating the fungicidal activity of the studied essential oils at 1:2 ratio is presented in Table 2. Piperonyl butoxide is known to exert its synergistic action on synthetic pesticides and / or natural products by inhibiting the mixed function oxidase activity which detoxifies xenobiotics (Metcalf, 1967 and Matsumura, 1985). To evaluate the synergistic effect of piperony butoxide , tween-20 and/or triton X-100 with the essential oils, at first we run an experiment to determine their EC$_{50}$ values (Table 2). Piperonyl butoxide did not affect the growth of *R. solani* or *M. phaseolina* up to 1000 $\mu g\ ml^{-1}$. By fifty percent mycelial growth of *Helminthosporium spp, F. oxysporum* and *A. alternate* was inhibited by concentration reached to 99 $\mu g\ ml^{-1}$ in case of *Helminthosporium spp.* It suggests that piperonyl butoxide could be used to potentiate some liquid formula of the traditional used fungicides.

Table (2): Synergstic effect of piperonyl butoxide (PB), tween-20 and triton X-100 with some essential oils

Materials \ Fungi	R.S.	M.P	F.O.	H.S	A.A.
	EC_{50} ($\mu g\ ml^{-1}$)*				
P.B	>1000	>1000	625	99	992
Tween-20	>1000	>1000	>1000	>1000	>1000
Triton X-100	367	166	198	106	256
Clove + P.B	23	47	54	48	78
Clove + Tween-20	54	79	90	86	64
Clove + Triton X-100	24	21	47	52	14
Caraway + P.B	56	174	194	50	133
Caraway + Tween-20	96	89	262	446	22
Caraway+Triton X-100	127	26	78	60	55
Chenopodium + P.B	861	49	>1000	81	246
Chenopodium+Tween-20	>1000	342	>1000	399	>1000
Chenopodium+TritonX100	212	54	78	33	181
Thyme + P.B	102	>1000	476	>1000	51
Thyme + Tween-20	>1000	>1000	374	374	>1000
Thyme + TritonX-100	161	51	92	27	62
Fennel + P.B	>1000	>1000	>1000	929	65
Fennel + Tween-20	>1000	>1000	>1000	767	984
Fennel + Triton X-100	83	65	107	75	85

Triton x-100 also exhibited antifungal activity against all of the tested fungi when used alone. These results indicated that mixing triton X-100 with other fungicide may give a significant reduction on the active ingredient of the commonly used fungicides and consequently in the chemical impact to the environment. However, tween-20 did not affect on the hyphal growth of all tested fungi up to concentration of 1000 $\mu g\ ml^{-1}$.

Synergistic effect of piperonyl butoxide, tween-20 and / or triton X-100 with the most effective essential oils gave increasing effect in the fungicidal activity. Mixing synergists e.g. triton X-100 or piperonyl butoxide with clove oil increased the fungicidal activity against all the tested fungi. However, the mixture with triton X-100 gave a high reduction in the EC_{50}

clove oil increased the fungicidal activity against all the tested fungi. However, the mixture with triton X-100 gave a high reduction in the EC_{50} values more than the mixture with piperonyl butoxide except in the case of *R. solani* and *Helminthosporium spp.* Also, the mixture with tween 20 enhanced the fungicidal activity sharply although the effect of tween-20 alone against the test fungi was inactive up to 1000 $\mu g\ ml^{-1}$.

In addition to the obtained results of the synergists with clove oil, mixing those compounds with caraway oil also enhanced the fungicidal activity. Triton X-100 gave the highest fungicidal activity except in the case of *R. solani* and *Helminthosporium spp.* These results are similar to the effect which was obtained by mixing triton X-100 with clove oil, indicating that the two types of fungi were less affected by triton X-100. The EC_{50} values of triton X-100 and caraway oil mixture were reduced seven times in case of *M. phaseolina* and four times in case of *A. alternate* respectively. However, *R. solani* overcome the effect of triton X-100 with caraway oil and the EC 50 was increased.

The fungicidal activity of triton X-100 and chenopodium oils was highly improved against all the tested fungi except *R. solani* particularly *M. phaseolina* (EC 50 value was 54 $\mu g\ ml^{-1}$ versus >500 for chenopodium alone). The mixture of chenopodium oil with piperonyl butoxide gave an increase in the fungicidal activity especially in the case of *M. phaseolina* and *Helminthosporium spp* (EC_{50} 49 *versus* >500 and 81 *versus* 307 respectively), but was reduced with the rest of the tested fungi. However, mixing tween-20 with chenopodium oil reduced the fungicidal activity except in the case of *M. phaseolina.*

Thyme oil was effective against all the tested fungi particularly against *A. alternate* and was inactive in the case of *M. phaseolina.* Mixing thyme oil with triton X-100 highly improved the fungicidal activity except in the case of *R. solani* and *A. alternate.* However, the use of either piperonyl butoxide or tween-20 reduced the fungicidal activity. Likewise, the mixture of triton X-100 with fennel oil enhanced the fungicidal effects against all the tested fungi but the effect was reduced with both piperonyl butoxide and tween-20 except against *A. alternata.*

The fungicidal activity of essential oils constituents is shown in Table 3. One of the most important constituents is thymol and its structure mimic chlorothymol. Both compounds exhibited very strong fungicidal activity

Table (3): Antifungal activity of some essential oils constituents against six phytopathogenic fungi by radial growth technique.

Monoterpenoid \ Fungi	R.S.	M.P	F.O.	H.S	A.A.	D.S
			EC_{50} ($\mu g\ ml^{-1}$)			
Thymol	4	32	28	16	31	26
Chlorothymol	11	6	4	2	12	NT
Carvacrol	41	52	8	31	99	NT
Carveol	92	139	14	122	137	NT
Carvone (R)	22	111	393	185	174	NT
Carvone (S)	11	745	244	110	102	NT
Geraniol	30	223	90	89	135	42
Citronellol	9	379	177	136	207	5
Eugenol	54	97	137	71	37	112
Menthol	99	183	263	228	211	112
Vanilline	152	> 500	113	219	> 500	NT
Benzyl alchol	> 500	54	> 500	> 500	> 500	NT
Camphor	488	> 500	> 500	> 500	> 500	> 500
Cinnamaldehyde	169	439	406	94	> 500	> 500
Borneol	447	> 500	410	> 500	> 500	NT
Cineol	> 500	288	> 500	> 500	> 500	NT
Captan	42	19	196	51	52	4.00

NT = not tested

against all the tested fungi. Thymol gave an excellent fungicidal activity superior to the standard fungicide (captan). The EC_{50} of thymol was less than the standard fungicide ten and half times against *R. solani*. In addition, thymol strongly inhibited the growth of *F. oxysporium* and *A. alternate*. The thymol chemically structure mimic chlorothymol affected twenty five and half more than the standard fungicide against *Helminthosporium spp.* Also, its effect exceeded the standard by forty nine times against *F. oxysporum*. Those two promising compounds are under investigation and structure modification for improving their fungicidal activity. Likewise, carvacrol which is structure isomer of thymol was considerably effective and exceeded the standard particularly against *F. oxysporum*, our results concerning to carvacrol were similar to those results which obtained by Karaman *et al.*, (2001). However, Menthol is unsaturated analogue of thymol, exerted lower fungicidal activity against all the tested fungi. Also, the activity was less than thymol and carvacrol. These important monoterpenoids. Phenols (containing three carbon-carbon double bonds), carvacrol, thymol and eugenol were the most effective relative to the monocyclic alcohols, our results which belong eugenol were similar to the results of Gayoso *et al.*, (2005). Also, the bicyclic ketones were less toxic than the monocyclic ketones (camphor versus carvones). However, the activity was diminished in bicyclic ether, cineol, which was turned out to be almost inactive.

Finally, the major constituents of these biologically active essential oils e.g. thymol, chlorothymol and carvacrol could be used as a suitable lead to design an effective and specific new fungicides. Some structural modifications of these compounds are now under investigation that will be described in a future publication.

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النشاط الأبادى الفطرى لبعض الزيوت النباتية والمكونات الكيماوية الأساسية بها ضد بعض الفطريات الممرضة للنبات

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فى دراسة معملية تم تقييم التأثير الأبادى الفطرى لخمسة عشر من الزيوت الطبيعية ألا وهى الكراوية، القرنفل، الشمر، الزعتر، الشينوبوديم، القرفة، الجيرانيوم، حصى لبان، ماتيركارى، الورد، الشبت، الثوم، اوكالبتوس، الليمون والنعناع الفلفلى وكذلك المكونات الأساسية لتلك الزيوت: المونوتيربينويد (ثيمول، كلوروثيمول، كارفاكرول، كارفيول، كارفون، جيرانيول، سيترنيول، ايجينول، مينتول، فانيلين، كحول البنزيل، كامفور، السينامالداهيد، بورنيول وسينيول) ضد ست انواع من الفطريات الممرضة للنبات (ريزوكتونيا سولانى، ماكروفومينا فاصيولينا، فيوزاريوم اوكسى سبورم، هلمينثوسبوريم سبيشز، الترناريا الترناتا وديبلوديا سبيشز). وكانت اكثر الزيوت تأثيرا الكراوية، القرنفل، الشمر والزعتر ضد كل الفطريات المستخدمة. ايضا تم دراسة تأثير خلط تلك الزيوت ببعض المواد النشطة سطحيا مثل ببرونيل بيوتكسيد، توبين 20 وترايتون اكس 100 وقد زادت الفعالية نتيجة الخلط زيادة ملحوظة وخاصة فى حالة القرنفل مع ترايتون اكس-100 .وبالنسبة للمكونات الأساسية للزيوت وجد ان اكثر المكونات فعالية كان الثيمول، الكلوروثيمول والكارفاكرول.