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Original Contribution

ANTIFUNGAL ACTIVITY OF THREE MEDICINAL PLANT ESSENTIAL OILS AGAINST SOME PHYTOPATHOGENIC FUNGI

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ABSTRACT

In this study we surveyed the effectiveness, Minimum inhibitory concentration (MIC) and Minimum fungicide concentration (MFC) of three medicinal plant essential oils of *Zataria multiflora, Thymus vulgaris* and *Thymus kotschyanus* on the mycelial growth of four pathogenic fungi including *Pythium aphanidermatum, Rhizoctonia solani* (AG4), *Fusarium graminearum* and *Sclerotinia sclerotiorum.* The rates of growth inhibition were measured after placing active mycelial plugs of each fungus on Petri dishes containing PDA amended with specific concentrations of essential oils and incubated at 28 ± 1 ºC. The data were analyzed using MSTATC and SAS (version 9.1.3) software. The results showed that these essential oils were very effective on the four studied plant pathogenic fungi with growth inhibition average of 100% at 200µl/l concentration. Nevertheless, MIC and MFC of the essential oils were variable depending to species of fungi. *P. aphanidermatum* and *S. sclerotiorum* were the most sensitive and most resistant to the studied essential oils with average growth inhibition 89.54% and 75.35%, respectively. Since growth inhibition of studied essential oils was evident in this study, they have potential to control of some plant pathogenic fungi and could be considered for developing new fungicides.

Key words: Fungistatic activity, Iranian medicinal plant, Lamiaceae, Natural fungicide, Plant diseases.

INTRODUCTION

Nowadays, application of chemical compounds is considered as the most inexpensive and common method in plant disease control. However, their adverse affects on human health and the environment, promoted man to produce natural pesticides (1). Biologically active compounds found in plants appear to be more adaptable, acceptable and safer than synthetic compounds and display a wealthy source of potential pathogens control agents (2). Extracts and essential oils of medicinal plants are effective against fungal and bacterial pathogens; meanwhile they are biodegradable compounds which have high potential for using in integrated pest management programs (3). The use of biological compounds extracted from plants may be an alternative to conventionally used fungicides to control phytopathogenic fungi, due to their being bioactive chemicals such as flavonoids, phenols, tannins, alkaloids, quinons, saponins and sterols (4). During recent years, some commercial companies used the results of research findings about the pesticide properties of plant essential oil and presented new pesticide compounds to the market. For example Mycotech company making Cinnamite ™ as a fungicide for use in the greenhouse and Valero ™ as a fungicide and acaricide for use on crops such as grapes, citrus and nuts (5). There are many reports about the impact of plant essential oils and plant extracts against food and grain storage fungi, foliar pathogens, soil-born fungi and nematodes (6). The response to the different plant essential oils and plant extracts generally depends on the fungal species tested and may include ranges from resistant (inhibition of growth) to various degree of susceptibility (7).

Results of several researches have shown that some of these compounds are able to control plant pathogens or at least used as a model for construction of new pesticide compounds (8-11). Despite their potentialy great importance,

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there are not many studies on the effects of Iranian medicinal plants essential oils on plant pathogenic fungi. The objectives of this study were: 1) to test the inhibitory effects of three essential oils derived from three Iranian medicinal plants namely, *Zataria multiflora* Boiss., *Thymus vulgaris* L. and *Thymus kotschyanus* Boiss. & Hohen. on the mycelial growth of four pathogens including *Pythium aphanidermatum* (Edson) Fitzp., *Rhizoctonia solani* Kühn. (AG4), *Fusarium graminearum* Schwabe (anamorph of *Gibberella zeae* (Schwein) Petch.) and *Sclerotinia sclerotiorum* (Lib.) de Bary. that attack and cause heavy losses on important crops; 2) to determine the closer Minimum inhibitory concentration (MIC) and Minimum fungicide concentration (MFC) of these plant essential oils that reduced mycelial growth.

MATERIAL AND METHODS

Plant materials:

Aerial parts of *Z. multiflora, T. vulgaris* and *T. kotschyanus* were collected from growing fields in their natural habitats in Iran. The plant materials were dried under the shade condition with proper ventilation and then maintained at -24 °C until required and then hydrodistillated to extract their essential oils.

Extraction of essential oil:

Extraction of essential oil from subjected plants was carried out using a modified Clevenger-type apparatus (12) at the following condition: 40 g of air-dried plant material, 500 ml distilled water and 4 h distillation. Anhydrous sodium sulfate was used to remove water after extraction. The resulting oil placed into sealed plastic tubes (13) and was stored in refrigerator at 4 °C.

Fungal cultures:

Phytopathogenic fungi including *P. aphanidermatum, R. solani, F. graminearum* and *S. sclerotiorum* were supplied from the Department of Plant pathology of Tarbiat Modares University (TMU). The fungi cultures were maintained and grown on Potato Dextrose Agar (PDA).

In-vitro antifungal activity test:

The antifungal assay was carried out in Petri dishes (9 cm in diameter) containing PDA. When temperature of the medium (PDA) reached about 40 °C, specific initial concentrations (0 and 200 µl/l) of 50% stocks of plant essential oils (diluted in ethanol 96% and sterilized using 0.2 µm filters (Orange scientific) were added to PDA and mixed thoroughly. The rate of mycelial growth inhibition was measured after placing an active mycelial plug of fungi on Petri dishes containing PDA with specific initial concentrations of essential oils and incubated at 28±1 °C. The observations were recorded every 12 hours after first 10 hours for acclimation period to complete growth of control treatments. The rates of mycelial growth inhibition (GI%) was calculated by the following formula:

GI% = dc-dt/dc*100

Where dc is mean colony diameter of control sets and dt is mean colony diameter of treatment sets (2). The initial experiments were conducted in a completely randomized design (CRD) with two concentrations and two replications.

A factorial experiment in completely randomized design (FE using CRD) was designed for those plant essential oils which showed 100% mycelial growth inhibition in 200 µl/l concentration. These experiments were conducted with three factors and three replications to determine the closed MIC and MFC of essential oils on fungi. Three factors were: fungi with four levels, plant essential oils with three levels and concentration with three levels (50, 100, 150 µl/l). Inoculations of fungi and data collection were based on above instruction.

Statistical analysis:

Analysis of variance (ANOVA) was used to determine the effects of plant essential oils on mycelial growth inhibition of fungi. Statistical analysis was performed with MSTAT C and SAS (version 9.1.3) statistic softwares.

RESULTS

The results showed that three plant essential oils caused 100% growth inhibition on all species of fungi at 200 µl/l concentration (data not shown). These plant essential oils exhibited a broad fungitoxic spectrum by inhibiting the mycelial growth of fungi.

Therefore, we used lower concentrations (50, 100 and 150 µl/l) to determine MIC and MFC of each plant essential oils on these fungi (FE using CRD). The results of the statistical analysis are presented in **Tables 1 and 2.**

Table 1. *Analysis of variance for model*

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	35	25288.37	722.52**	34.09	<.0001
Error	72	1526.05	21.19		
Corrected Total	107	26814.42			

CV= 5.41; Mean Growth Inhibition Rates = 84.98; ** significant at 0.01 probability level

Table 2. *Analysis of variance for the effects of plant essential oils and concentrations on growth inhibition of fungi*

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Fungi	3	3445.34	1148.44**	54.18	<.0001
Plant Essential Oils	2	1406.35	703.17**	33.18	<.0001
Concentrations	2	15396.80	7698.40**	363.22	<.0001
Fungi * Plant Essential Oils	6	2800.53	466.75**	22.02	<.0001
Fungi * Concentrations	6	947.27	157.87**	7.45	<.0001
Plant Essential Oils * Concentrations	4	512.77	128.19**	6.05	0.0003
Fungi * Plant Essential Oils* Concentrations	12	779.29	64.94**	3.06	0.0016

** significant at 0.01 probability level

The results showed that the essential oil from *T. vulgaris* was the most effective essential oil on the *P. aphanidermatum, R. solani, F. graminearum* with growth inhibition average of 91.83%, whereas the essential oil from *T. kotschyanus* showed the major effect on *S. sclerotiorum* with growth inhibition average 91%.

P. aphanidermatum and S. sclerotiorum were the most sensitive and most resistant to the studied essential oils with average growth inhibition of 89.54% and 75.35%, respectively.

The results showed that the plant essential oils have same MIC and MFC on the *P. aphanidermatum* and *F. graminearum.* The MIC and MFC of these essential oils was approximately the same on the *R. solani* and *S. sclerotiorum* **(Table 3)**. *P. aphanidermatum* and *F. graminearum* were more sensitive than *R. solani* and *S. sclerotiorum* to the studied plant essential oils.

The results indicated significant differences between factors (P<0.01), hence done slicing between any of factors and other factors levels (data not shown). Slicing data showed that in all studied fungi, the plant essential oils caused significant differences (p<0.01) on growth inhibition rates. In other words, the effects of growth inhibitory of plant essential oils depend on species of fungi. For example, using of any type of plant essential oils does not make a significant difference on growth inhibition of *R. solani.*

According to the slicing data and regardless type of plant essential oils, using of any concentrations caused significant differences (p<0.01) on growth inhibition rates. Whereas increase in concentrations, the susceptibility of fungi increases as well **(Figures 5 and 6).**

According to the slicing data and regardless species of fungi, using of any concentrations caused significant differences (p<0.01) on inhibitory effect of plant essential oils. It could be seen that as plant essential oils concentrations increases the inhibitory effect increases **(Figures 1, 2, 3 and 4)**. In other words, the inhibitory effect of the plant essential oils was proportional to its concentration.

Table 3. *Growth inhibition rates (%) with MIC and MFC concentrations of plant essential oils*

Essential oils (µl/l)	Fungus											
	P. aphanidermatum			R. solani			F. graminearum			S. sclerotiorum		
	GI%	MIC	MFC	GI%	MIC	MFC	GI%	MIC	MFC	GI%	MIC	MFC
Z. multiflora												
50	61.33			70.52			67.91			40.48		
100	93.44			84.18			89.36			70.14		
150	100	*	*	97.27			100	*	*	86.34		
200	100			100	*	*	100			100	*	*
T. vulgaris												
50	78.48			78.96			82.96			48.2		
100	97.46			90.56			98.04			66.18		
150	100	*	*	100	*	*	100	*	*	93.81		
200	100			100			100			100	*	*
T. kotschyanus												
50	76.72			77.6			68.4			77.52		
100	98.43			85.03			87.76			95.49		
150	100	*	*	96.97			100	*	*	100	*	
200	100			100	*	*	100			100		*

GI% = growth inhibition rate (%), MIC = Minimum Inhibition Concentration, MFC = Minimum Fungicide Concentration

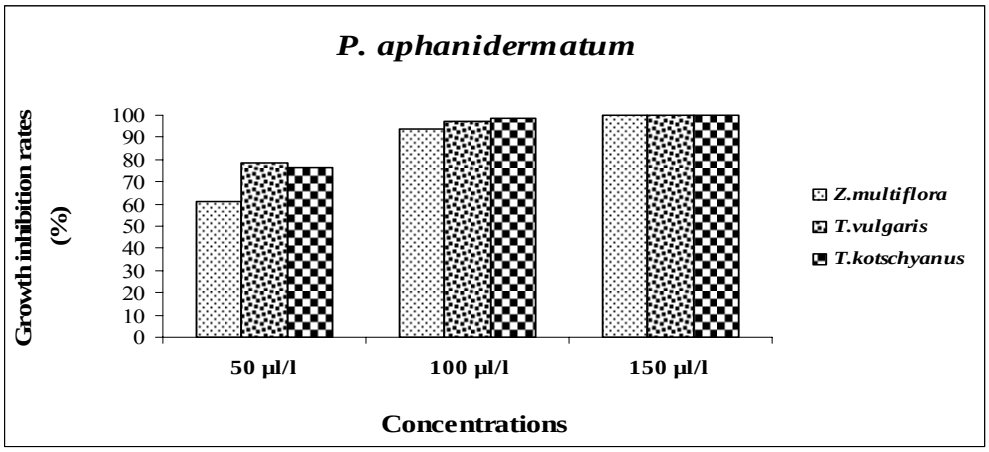

Fig. 1. Average growth inhibition of plant essential oils at different concentrations on *P. aphanidermatum.*

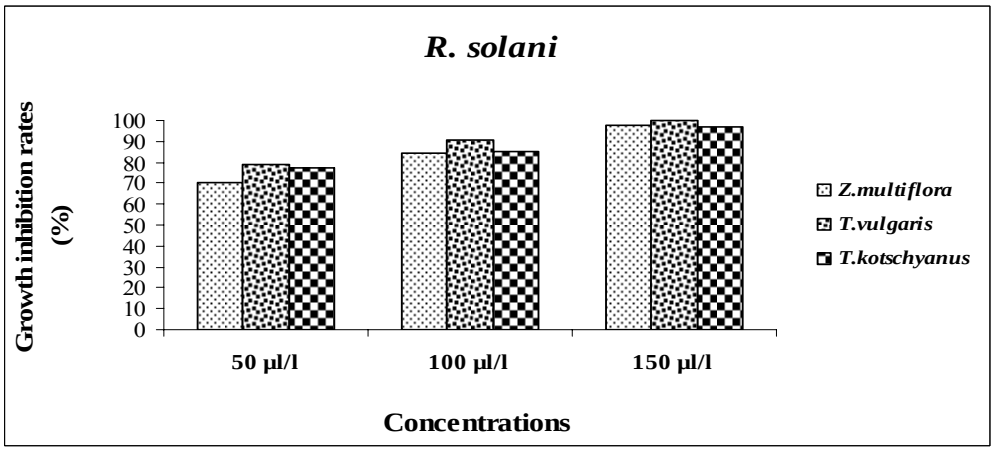

Fig. 2. Average growth inhibition of plant essential oils at different concentrations on *R. solani.*

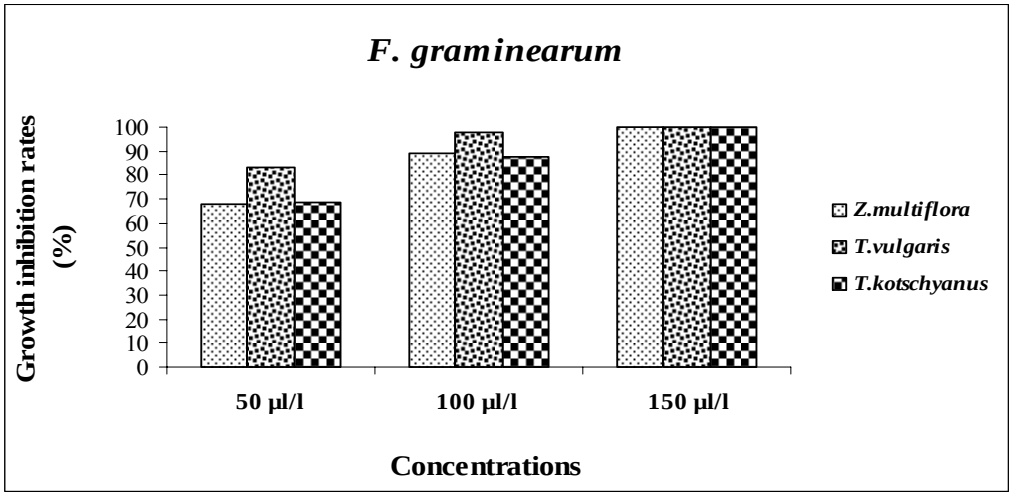

Fig. 3. Average growth inhibition of plant essential oils at different concentrations on *F. graminearum.*

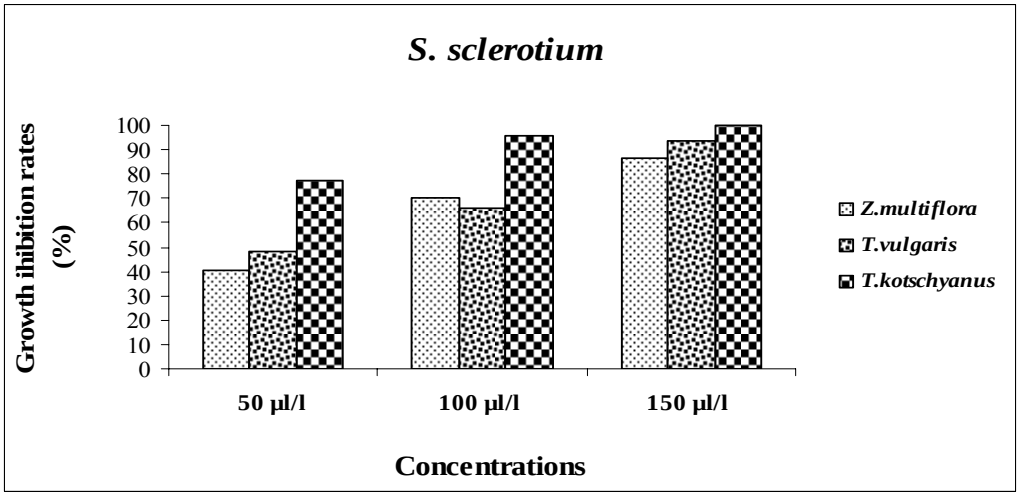

Fig. 4. Average growth inhibition of plant essential oils at different concentrations on *S. sclerotium.*

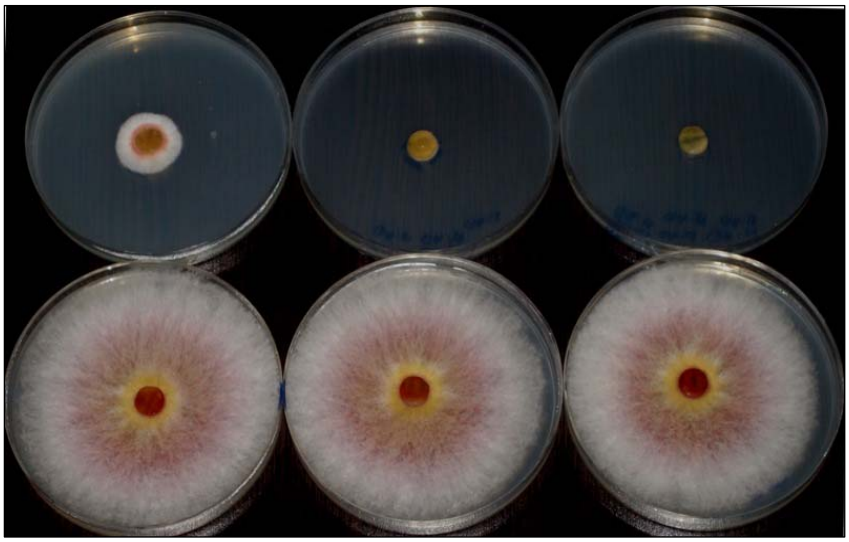

Fig. 5. Mycelial growth inhibition of *T. vulgaris* on *F. graminearum* at 50, 100 and 150 µl/l concentrations, respectively (from left to right) compared with control.

Fig. 6. Mycelial growth inhibition of *T. vulgaris* on *R. solani* at 50, 100 and 150 µl/l concentrations, respectively (from left to right) compared with control.

DISCUSSION

The essential oils extracted from *Z. multiflora*, *T. vulgaris* and *T. kotschyanus* showed 100% growth inhibition on four species of fungi – *Pythium aphanidermatum, Rhizoctonia solani* (AG4), *Fusarium graminearum* and *Sclerotinia sclerotiorum* at 200 µl/l concentration. Antimicrobial effects of *Z. multiflora* essential oils were previously documented on foodborne pathogens and food spoilage microorganisms (14-17).

Until now, there were no reports of the effects of *Z. multiflora* and *T. kotschyanus* on phytopathogenic fungi that can outbreak simultaneously. Based on our findings, *Z. multiflora* and *T. kotschyanus* strongly affected the growth inhibition of the studied fungi, especially on *P. aphanidermatum* and *F. graminearum*.

The most important compounds of *Z. multiflora* and *T. kotschyanus* essential oils are carvacrol and thymol (Terpenoidic phenols) (9, 14, 18), although the level of carvacrol is more than that of thymol. Thymol is the most important compound of the essential oil of *T. vulgaris*. Other biologically active compounds such as limonene, myrcene, γ-terpinen, α-pinene, β-pinene, ρ-cymene (Monoterpene hydrocarbons), linalool, borneol (Monoterpene alcohols), camphor (Monoterpene ketone) are available in these plant essential oils (14, 15, 18-22). All of the studied medicinal plants belonging to Lamiacae family. Previous studies have shown this fact that the essential oils which belonging to a same family, may contain analogous and similar active compounds (23).

Transmission electron microscopy (TEM) of *Aspergillus niger* van Tieghem. treated with MIC levels of *Thymus eriocalyx* (Ronniger.) Jalas. and *Thymus* × *porlock* [Add the names of the authors of this hybrid] has shown severe damage to cell wall, cell membrane and cellular organelles. Thyme essential oils caused morphological changes in the hyphae, plasma membrane disruption and mitochondrial destruction (24). According to our finding, the increase in concentrations of plant essential oils increases the growth inhibition rates of fungi. This is in accordance with Rasooli *et al.* (24), whose results proved that the increase in the concentrations of *Thymus* spp. essential oils caused lack of cytoplasm, folding of the nuclear membrane and thickened cell wall. Such performance of *Thymus* species can be related to lipophilic properties and the ability to penetrate the plasma membrane (25). Such disturbances of thyme essential oils can be caused through interference of essential oil components with enzymatic reactions of wall synthesis that produce changes in the morphology of fungi and inhibition of their growth (24).

The antifungal activity of thyme essential oils has well proved against fungi such as *Botrytis cinerea* (De Bary.) Whetzel. (26), *Rhizopus stolonifer* (Ehrenb. Fr.) Vuill. (27), *Aspergillus* spp. (28), *Rhizoctonia solani*, *Pythium ultimum*

Trow., *Fusarium solani* (anamorph of *Haematonectria haematococca* (Berk. & Broome.) Samuels. & Rossman.), *and Colletotrichum lindemuthianum* (Sacc. & Magnus.) Briosi. & Cavara. (29). Thyme essential oils in addition to complete inhibition growth of grain storage fungi, prevents toxin production (19).

Carvacrol is a phenolic compound. Its antimicrobial activity may be attributed to the presence of an aromatic nucleus and a phenolic OH group that is active and forming hydrogen bonds with active sites of target enzymes (30). Antifungal effect of carvacrol is slightly stronger than that of thymol (31). Carvacrol react with membrane cell through the changes in permeability of H^+/K^+ channels. Changes in ion gradient lead to impairment of fundamental cell functions and cell death (32). Interaction of essential components with each other play an important role on plant essential oils antimicrobial effects. Thymol and carvacrol have synergistic effects (33). High antifungal effects of studied plant essential oils can be attributed to the presence of phenolic compounds such as thymol and carvacrol. Phenolic compounds cause disturbance on the cell wall enzymes such as chitinase, chitin synthase and α- & β- glucanase (34). Soković *et al.* (31) explained that the commercial fungicide "Bifonazole" showed lower antifungal potential than thyme species (*T. vulgaris* and *Thymus tosevii* Velen.). Thymol has no specific effect on growth parameters of plant tissue (35).

In the study of Soliman and Badeaa (19), MFC of thyme essential oils are twice of MIC that is inconsistent to our findings. This difference could be due to the different strains of fungi used or to the chemical compounds of plant essential oils.

Essential oils have two prominent features; low toxicity for people and environment due to their natural properties and low risk for resistance development by pathogenic microorganisms (36). For these reasons and considering the results, we recommend the use of those Iranian medicinal plant essential oils for development of new and safe fungicides. Further formulation and field experiments are necessary to achieve this target.

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