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Evaluation of the effect of *Thymus vulgaris* oil on growth and fungal biomass of *Rhizoctonia solani*

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ABSTRACT

In the present study, we evaluated the antifungal activity essential oil of *Thymus vulgaris* against *Rhizoctonia solani* as important pathogen on tomato plants. The *T. vulgaris* EO was extracted using a Clevenger apparatus. A total of thirteen compounds, representing 98.6 % of the oil were identified by gas chromatography-mass spectrometry (GC-MS). The main components of thyme oil were included thymol (48.9 %), *p*-cymene (15.8 %), borneol (8.1 %), γ-terpinene (5.7 %), isoborneol (3.7 %) and 4-terpineol (3.2 %) that identified by gas chromatography-mass spectroscopy. The minimum inhibitory concentration (MIC) values obtained for thyme oil was considerably lower than the values obtained for synthetic fungicides such as Thiabendazole and Tebuconazole. The results of this evaluation of indicate that a compound found in thyme oil was effective in reducing growth and fungal biomass. These results indicate that thyme oil after suitable formulation could be used for the control of soil-borne fungal pathogens of tomato, especially *Rhizoctonia solani* are common in many parts of the world.

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Introduction

Tomato (Lycopersicon esculentum Mill.) is one of the most important crops in the world. In 2012, the global tomatoes acreage was 4.803 million hectares and tomatoes production was 161.793 million tons (Fao, 2013). R. solani is an important soil-borne necrotrophic pathogen, with a broad host range and little effective resistance in crop plants (Foley et al., 2013) and causes a wide range of diseases. This pathogen is the causal agent of crown rot, root rot and damping off in tomato producing areas. to date, R. solani has been characterized and grouped into 14 anastomosis groups (AG) that vary in pathogenicity, morphological characteristics and DNA sequence variations (Carling et al., 2002). Several AGs of R. solani such as AG 2-1 (Misawa and Kuninaga, 2010), AG 3 (Misawa and Kuninaga, 2010; Charlton et al., 2008), and AG 4 HG I (Kuramae et al., 2003) have been shown to be pathogenic on tomato, the most frequently reported being AG3.

Pathogenic fungi contaminate crops and foods and cause significant yield reduction and economic losses. Several diseases management strategies are available e.g. biological control, resistant cultivars (Takken and Rep, 2010), crop rotation (Gilligan et al., 1996; Kamal et al., 2009) and fungicides (Haggag and El-Gamal, 2012). The most common method for controlling these pathogens is the use of fungicides. Chemical

fungicides are known to be highly effective for diseases management in various plants. However, the alternative control methods are needed because of the negative public perceptions about the use of synthetic chemicals, increasing public concern regarding contamination of fruits and vegetables with fungicidal residues (Vitoratos et al., 2013), proliferation of resistance in the pathogen populations, and high development cost of new chemicals (Katooli et al., 2012). Research on plant-derived fungicides is now being intensified, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Lee et al., 2007). So, use of non-chemical ecofriendly means of control i.e. biocontrol agents and secondary metabolites secreted by medicinal plants have emerged as a viable alternative under such conditions (Singh, 2006). Medicinal plants are potential sources of antimicrobial compounds, which could be used in the management of plant diseases (Colpas et al., 2009). Essential oils as antimicrobial agents present two main characters: their natural origin generally means more safety to people and environment; and they can be considered at low risk for development of microbial resistance since they are mixture of compounds which may present different mechanisms of antimicrobial activity (Karbin et al., 2009). The use of plant extracts and essential oils could be a useful alternative to synthetic

fungicides in the management of various phytopathogenic fungi (Gatto et al., 2011). Essential oils have been widely used for bactericidal (Oussalah et al., 2007), antifungal (Silva et al., 2011; Tserennadmid et al., 2011), insecticidal (Essam, 2001; Kim et al., 2003). They contain a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components (Bakkali et al., 2008). Although several essential oils have been reported to have antifungal properties, few have been developed as commercial formulations for use in plant disease control. Application of essential oils is a very attractive method for controlling plant diseases. Essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and exploitation for potential multi-purpose functional uses (Ormancey et al., 2001).

The genus *Thymus* comprising of around 300 species of perennial, aromatic herbs and subshrubs is predominantly found in Mediterranean region, Asia, Southern Europe and North Africa (Maksimovic et al., 2008). Some studies have reported that thyme essential oil possesses a high level of the phenolic precursors, *p*-cymene and γ-terpinene, probably due to its early flowering time (Saez 1998). In previous studies, antimicrobial (Sienkiewicz et al., 2012), antifungal (Tullio et al., 2007) effects of this plant have been demonstrated.

although there still exist a little information in the literature about the possible mechanisms of action of thyme oil and its components Therefore, considering the important antimicrobial potential of the genus *Thymus*, together with evidence that the essential oil of *T. vulgaris* shows one of the best antifungal profiles. Therefore, the objectives of the present investigation were (i) elucidating antifungal mode of thyme oil action and (ii) identify the essential oil components of effective and (iii) evaluate activity of oil against pathogen *in vivo*.

Materials and Methods

Plant pathogenic fungus

The isolates of *Rhizoctonia solani* anastomosis group (AG) 3 (obtained from Phytopathology Laboratory in Ferdowsi University of Mashhad, Iran), the causing root rot and damping off on tomato was used. The fungus isolates were maintained on potato dextrose agar (PDA) medium slants at 4˚C, and sub-cultured at monthly intervals.

Plant material and extraction of essential oil

For the extraction of essential oil, leaves of *Thymus vulgaris* were collected September and October 2020 from Tehran province, Iran. The leaves were washed with water and finally with distilled water to remove dust and dried under shade at room temperature for 3 days. For isolation of the essential oil, 200 g of dried plant materials were subjected to hydro-distillation for about 3 h, using a Clevenger apparatus. The oils were dried over anhydrous Na_2SO_4 and preserved in sealed

glass bottles and protected from the light by wrapping in aluminum foil and stored at 4 °C until used.

Gas chromatography-mass spectrometry (GC-MS) analysis of *T. vulgaris* essential oil

GC analysis of the oil was done by a Shimadzu GC-MS model QP 5050 chromatograph DB-5 MS capillary column (30m × 0.2 mm, film thickness 0.32 µm). Helium was used as carrier gas at a flow rate 1.2 mL min^{-1} with injection volume of 0.1µL. injector and detector temperatures were both at 280°C. Oven temperature was kept at 60°C for 1 min, gradually raised to 200°C at 3°C min^{-1} and finally raised to 250 °C at 2°C min^{-1}. Retention indices were determined by using retention times of n-alkanes that had been injected after the oil under the same chromatographic conditions. The components of the essential oils were identified by comparison of their retention indices with those published in the literature (Nezhadali et al., 2010, 2012; Adams. 2009).

Growth inhibition assay in culture medium

The tests were performed using the agar medium assay described by Tatsadjieu et al., (2009) with some modifications. PDA medium with different concentrations of essential oil (0-2000 ppm) were prepared by adding appropriate quantity of essential oil to melted medium, followed by addition of Tween-20 (100µL to 100 mL of medium) to disperse the oil in the medium. Each Petri-dish was inoculated at the center with a mycelial

disc (10 mm diameter) taken at the periphery of pathogens colony grown on PDA for 120 h. Positive control (without essential oil) plates were inoculated following the same procedure. Plates were incubated at 27±1°C for 7 day and the colony diameter was recorded each day. The mycelial growth inhibition (MGI) percentage was calculated according to the following formula:

$$MGI = \frac{d_c - d_t}{d_c} \times 100$$

Where, D_c = mean diameter of colony in the control (mm) and D_t = mean diameter of colony in the treatment (mm). Three replicate plates were used per treatment and the experiment was repeated three times.

Nature of toxicity of essential oils

The nature of toxicity (fungistatic/fungicide) of the oils against fungi was determined as described by Thompson (1989). The inhibited fungal mycelia plugs of the oil treated sets were reinoculated into fresh medium and revival of their growth was investigated.

Determination of minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and inhibitory concentration 50 (IC50)

MIC and MFC of essential oil was determined as described by Plodpai et al., (2013) and Tyagi and Malik (2010) with few modifications. The PDA plates were amended with various concentrations of essential oil (0-2000 ppm). For enhancing the essential oil solubility, Tween-20, 0.5% (v/v) was added. Each plate was inoculated with a mycelial plug (10 mm diameter) of

pathogens. All plates were incubated in triplicate for each concentration at 25±1°C for 144 h. Plates with Tween-20 but without any essential oil were used as control. Observation of fungal growth was done at a time interval of 12 h up to 144 h after incubation. The MIC values were determined as the lowest concentration of essential oil that completely prevented the visible fungal growth.

To determine MFC, the mycelial plugs were obtained from each Petri dish treated with the oil concentrations lower than MIC, cultured on PDA and incubated at 25±1°C for 96 h. MFC was defined as the lowest concentration at which no colony growth was observed after subculturing into fresh PDA medium. IC50 (concentration that produces a 50% inhibitory effect) values were graphically calculated from the dose-response curves based on measurement at various concentrations.

Comparing the fungitoxicity of essential oil with some prevalent synthetic fungicides

The efficacy of the essential oil was compared with some common fungicides, such as Thiabendazole (Tecto) and Tebuconazole (Raxil) by the agar medium assay.

Effects of essential oil on biomass production

To determine the effect of essential oil on the fungi biomass production, various concentrations of essential oil in 50 mL of Potato Dextrose Broth (PDB) medium were prepared in conical flasks and inoculated with a mycelia disc (10 mm diameter) of *R. solani*. The flasks were incubated on a rotary shaker with 100 rev min^{-1}. The Dry

weight of mycelium was determined after 10 days of incubation on PDB medium. Flasks containing mycelia were autoclaved and subsequently filtered through filter papers (Whatman No.1). The mycelia were washed several times with distilled water and allowed to dry at 40±1°C overnight. The filter paper containing dry mycelia were weighed. Percent growth inhibition based on dry weight was calculated as (Siripornvisal 2010):

$$\%inhibiton = \frac{W_0 - W}{W_0} \times 100$$

Where, W_0 and W are dry weight of control and dry weight of sample, respectively.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) for a completely randomized design with four replicates using SPSS (version 21) software. The means were separated using Duncan's multiple range tests at $P < 0.05$, where the F-value was significant.

Results

Composition of the Essential oil

The chemical composition essential oil obtained from *T. vulgaris*, as determined by GC-MS analysis is shown in Table 1. Thirty compounds were identified in the oil which constitutes about 98.6% of this oil. The essential oil was characterized by the presence of major compounds such as thymol (48.9 %), *p*-cymene (15.8 %), borneol (8.1 %), γ-terpinene (5.7 %), isoborneol (3.7 %) and 4-terpineol (3.2 %). Nezhadali et al. (2012) analyzed the essential oil of *T. vulgaris* originating from Iran, and they detected 36 compounds, including thymol (45.4%), *p*-cymene (13.4%), γ-terpinene

(6.9%), borneol (6.6%), 4-terpineol (2.9 %) and isoborneol (4.2%).

Table 1- Chemical composition of *T. vulgaris* essential oil (%) determined by GC-MS

No.	Compound name	RI[a]	Composition (%)
1	α-Thujene	951	0.1
2	α-Pinene	957	0.1
3	Camphene	970	0.7
4	β-Pinene	992	0.1
5	β-Myrcene	1006	1.4
6	α-Phellandrene	1018	0.3
7	α-Terpinene	1032	0.2
8	*p*-Cymene	1053	15.8
9	γ-Terpinene	1090	5.7
10	*cis*-β-Terpineol	1092	0.6
11	Terpinolene	1103	0.2
12	Linalool	1117	2.1
13	Isopulegol	1135	0.5
14	Camphor	1162	1.7
15	Borneol	1187	8.1
16	4-Terpineol	1193	3.2
17	Isoborneol	1198	3.7
18	Thymol methyl ether	1254	1.3
19	Verbenone	1268	0.1
20	α-Terpineol	1279	0.2
21	Dihydro carvone	1293	0.1
22	Thymol	1380	48.9
23	Eugenoi	1400	0.3
24	Geranyl acetone	1453	0.4
25	GermacreneD	1509	0.8
26	γ-Elemene	1515	0.1
27	β-Bisabolene	1538	0.7
28	δ-Cadinene	1551	0.2
29	Caryophyllene oxide	1588	0.1
30	Spathulenol	1608	0.9
	Total	-	98.6

[a] RI: Retention index calculated on the basis of retention time of a mixture of n-alkanes (C8–C30).

Biological activity of essential oils depends on their chemical composition, which is determined by the plant genotype and is greatly influenced by several factors such as geographical origin and environmental and agronomic conditions (Rota et al., 2004). In addition antibacterial activity depends on the type, composition and concentration of the essential oils, the type and concentration of the target microorganism, the composition of the substrate, the processing and the storage conditions (Ceyhan et al., 2012).

Antifungal activity of essential oil on mycelial growth *in vitro*

The effect of different concentrations of thyme oil on mycelial growth of *R. solani* is shown in Figure 1. The thyme oil inhibited the growth of pathogens in a dose dependent manner. Our results revealed that the antifungal activity of this essential oil increased with increasing the concentration (Table 2).

Table 2. In vitro antifungal activity of the essential oil of *T. vulgaris* compared to synthetic fungicides against mycelial growth of *R. solani.*

Fungi	***R. solani***		
Treatments	IC50[a]	MFC[b]	MIC[c]
Essential oil			
T. vulgaris	500	1250	1050
Fungicides			
Thiabendazole	800	1500	1500
Tebuconazole	1750	IN	3000

[a] Inhibitory concentration with 50% inhibitory effect on the fungal growth (ppm)
[b] Minimum fungicidal concentration (ppm)
[c] Minimum inhibitory concentration (ppm)
IN: Ineffective

Minimum concentration of the thyme oil required to completely inhibit the mycelial growth of fungi was different (Figure 1). *R. solani* did not show any visible mycelial growth in presence of thyme oil at concentration of 1000 and 800 ppm, respectively. Investigating fungistatic and/or fungicide activity revealed that the thyme oil had fungicidal properties against *R. solani* at 1250 ppm concentration. The minimum inhibitory concentrations of synthetic fungicides including Thiabendazole and Tebuconazole against both fungi were found to be 2000 and 1500 ppm, respectively, which were higher than that of the essential oils tested in present study (Table 2).

Effects of essential oil on biomass production

The effects of essential oil on biomass production of fungi were evaluated in liquid cultures. Results showed that essential oil in culture medium caused considerable reduction of the fungal biomass (Table 3). In general, with increase concentration of thyme oil, the amount of fungi biomass declined. The maximum reduction in biomass of *R. solani* was related to the effect of thyme oil at concentration of 1400 ppm. It is thus reasonable that a little growth the fungus should be detected during longer incubation times. However, this phenomenon should be intensively studied in further research. Siripornvisal (2010) showed that the supplement of ajowan oil in

culture medium caused considerable reduction of the fungal biomass and It should be noted that supplement of ajowan oil at the MIC level (240 µg mL^{-1}) did not completely suppress the biomass production.

Table 3. Effects of thyme oil on biomass production *R. solani*.

Concentration of thyme oil (ppm)	***R. solani***			
	Dry weight[a] (mg)	% inhibition		
0	279	0^{g}		
200	251	11.2^{f}		
400	213	23.6^{e}		
600	185	33.7^{de}		
800	146	46.7^{d}		
1000	88	68.4^{c}		
1200	31	88.9^{b}		
1400	0	100^{a}		
1500	0	100^{a}		

[a] means of triplicate samples

The results are means ±standard errors of four replications. Means within a column indicated by the same letter were not significantly different according to Duncan's multiple range test at the level P <0.05.

Discussion

In the present study, the antifungal capability of essential oil obtained from *T. vulgaris* against *R. solani* was investigated using *in vitro* assays. The obtained data revealed that thyme oil used in this study had considerable inhibition effect on mycelial growth of *R. solani* in agar medium assay compared to the controls. The minimum concentration of the oil required to inhibit the mycelial growth of test fungi was difference. It is evident that the inhibitory effect of the oil on mycelial growth of fungi varied

among the fungal species. Thyme oil showed the best activity against *R. solani* exhibiting MIC value of 1050 ppm. The results showed that the antifungal activity of the essential oil increased with an increase in concentration. de Lira Mota et al., (2012) while reported that antifungal activity of *T. vulgaris* essential oil against *Rhizopus oryzae*, the MIC of essential oil and thymol varied 128–512 μg mL^{-1}, but the MFC of essential oil and thymol varied 512–1024 μg $mL^{-1}$and 128–1024 μg mL^{-1}, respectively. The results also showed that essential oil of *T. vulgaris* and thymol significantly inhibited mycelial development and germination of sporangiospores. Nzeako et al., (2006) show that thyme oil extract failed to kill *Staphylococcus aureus*, *Salmonella choleraesuis* or *Klebsilela pneumonia* but stopped the growth of *Pseudomonas aeruginosa* and *Candida albicans*.

Investigating fungistatic and/or fungicidal effects of the essential oil showed that the thyme oil at a concentration of 1250 had fungicidal activity against *R. solani*. The minimum inhibitory concentration values obtained for essential oil used in this assay was considerably lower than the values obtained for synthetic fungicides such as Thiabendazole and Tebuconazole. de Lima Houinsou et al., (2012) reported that the MFCs determined from this essential oil of *Ocimum gratissimum* against pathogenic fungi

isolated from tomato were respectively for *Fusarium oxysporum*, *F. graminearum*, *F. poae* and *Aspergillus niger* ; 200, 400, 800 and 1600 ppm, respectively, the last one which exhibited the highest resistance (de Lima Houinsou et al., 2012).

The present data also revealed significant decrease in fungal biomass of all treatments having various concentrations of essential oil compared to control. Antifungal activity of thyme oil had been previously reported against several phytopathogenic fungi, including *R. solani* (Lee et al., 2007) which are in accordance to our data. *T. vulgaris* of essential oil was rich in Thymol (48.9 %), o-cymene (15.8 %). Shabnum and Wagay (2011) analyzed the essential oil of *T. vulgaris* and they detected 30 compounds the major components were thymol (46.2%), γ-terpinene (14.1%), *p*-Cymene (9.9%), linalool (4.0%), myrcene (3.5%), α-Pinene (3%) and α-thujene (2.8%). According to Burt (2004), thyme oil consists of 10% - 64% thymol and 10% - 56% *p*-cymene. The terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms, with the most active monoterpenoids identified so far being carvacrol and thymol. The antimicrobial activity of carvacrol, thymol, linalool, and menthol were evaluated against *Listeria monocytogenes*, *Enterobacter aerogenes*, *E. coli*, and *Pseudomonas aeruginosa*. The most active compound was carvacrol

followed by thymol with their highest MIC being 300 and 800µg mL^{-1}, respectively (Bassole et al., 2010). These results confirm the high antimicrobial activity of a broad collection of terpenoids, and because their chemical structures are closely related to that of terpenes, the increased activity compared to terpenes can be attributed to the functional moieties (Hyldgaard et al., 2012). The mode of action of thymol, a phenolic monoterpenoid and one of the major constituents of thyme oil, has received much attention from researchers. Thymol is structurally very similar to carvacrol, having the hydroxyl group at a different position on the phenolic ring. The antimicrobial action of phenolic compounds, such as thymol and carvacrol, are expected to cause structural and functional damages to the cytoplasmic membrane (Sikkema et al., 1995).

In conclusion, thyme oil could be applied as an alternative to synthetic fungicides for the control of *R. solani*. According to the surveys conducted, thyme oil contains effective compounds antifungal against phytopathogenic fungi. These results indicate that thyme oil with suitable formulation could be used to control of tomato disease.

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