

Full Length Research Paper

Evaluation of the antifungal activity of the Iranian thyme essential oils on the postharvest pathogens of Strawberry fruits

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Postharvest diseases cause considerable losses to harvested fruits and vegetables during transportation and storage. The aim of this study was to evaluate the antifungal potential of *Thymus danensis* and *Thymus carmanicus* against four postharvest pathogenic fungi (*Rhizopus stolonifer*, *Penicillium digitatum*, *Aspergillus niger* and *Botrytis cinerea*) which can reduce the shelf life of strawberry fruit. The chemical composition of plant oils was determined by capillary gas chromatography and mass spectrometry. Antifungal assays were carried out *in vitro* using PDA plates. Antifungal potential was found for 2 analysed essential oils. *T. carmanicus* oils have chemical compositions characterized by carvacrol (70%), p-cymene (12.4%) and γ -terpinene (2.5%) as the major components while the major constituents of the *T. danensis* were thymol (64.8%), α -terpinene (11.3%) and p-cymene (7.9%). *Thymus* sp. oils showed inhibitory effect even at low concentration (300 μ l/L) against *B. cinerea* and *R. stolonifer* and showed inhibitory effect against *A. niger* and *P. digitatum* at 600 μ l/L. Both essential oils tested *in vivo* at the preliminary concentration exhibited inhibitory activity against the four pathogens.

Key words: *Thymus danensis*, *Thymus carmanicus*, antifungal activity.

INTRODUCTION

Postharvest diseases cause heavy losses of fruits during storage. The species reported to damage strawberries during this period include *Rhizopus stolonifer*, *Penicillium digitatum*, *Aspergillus niger*, and *Botrytis cinerea*. There have been some studies on the effects of essential oils on postharvest pathogens (Bishop and Thornton, 1997). These essential oils are thought to play a role in plant defence mechanisms against phytopathogenic microorganisms (Mihaliak et al., 1991). Most of the essential oils have been reported to inhibit postharvest fungi in *in vitro* conditions (Bellerbeck et al., 2001; Hidalgo et al., 2002). However, the *in vivo* efficacy and practical activity of only a few of the essential oils have been studied. There are also some reports on essential oils in

enhancing the storage life of fruit and vegetables by controlling their fungal rotting. Dubey and Kishore (1988) found that the essential oils from leaves of *Melaleuca leucadendron*, *Ocimum canum* and *Citrus medica* were able to protect several stored food commodities from biodeterioration caused by *Aspergillus flavus* and *Aspergillus versicolor*.

Thymol is an essential oil component from thyme and has been used as medicinal drug, food preservative, and beverage ingredient (Jain, 1985; Mansour et al., 1986). Fumigation of sweet cherries with thymol was effective in controlling postharvest grey mold rot caused by *B. cinerea* (Chu et al., 1999), and brown rot caused by *M. fructicola* (Chu et al., 2001). Fumigation with thymol at 30 mg/l reduced the incidence of grey mold rot from 35% in untreated fruit to 0.5%. Liu et al. (2002) also found that thymol was more effective for controlling brown rot symptoms on apricots, and fumigation of plums with relatively low concentrations such as 2 or 4 mg/l can

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greatly reduce postharvest decay without causing any phytotoxicity.

The main purpose of this study was to evaluate the antifungal potential of *Thymus danensis* and *Thymus carmanicus* oils against four important pathogenic fungi (*R. stolonifer*, *P. digitatum*, *A. niger*, and *B. cinerea*) which can reduce the shelf life of strawberry fruit.

MATERIALS AND METHODS

Plant material

Plant materials of *T. danensis* and *T. carmanicus* were collected from Karaj in Tehran province (Iran) in August 2007. A voucher specimen was deposited at the herbarium of College of Agriculture and Natural Resource, University of Tehran, Iran.

Essential oils extraction and GC and GC-MS analysis

About 200 g dried material (plant material was dried at ambient temperature and shade condition) of each species were cut into pieces, ground and then submitted to hydrodistillation for 3 h using a Clevenger type apparatus. The obtained essential oils were dried with anhydrous sodium sulphate and stored at 4°C before analysis and use for antifungal study.

The composition of the oils was analysed by GC and GC-MS. The GC apparatus (Shimadzu model GC-7A, Tokyo, Japan) was combined with a Flame Ionization Detectors (FID, Shimadzu, Tokyo, Japan) equipped with DB-1 column (60 m x 0.25 mm ID, film thickness 0.25 µm). The oven temperature was programmed to increase from 60 to 250°C at a rate of 3°C/min and finally held isothermal for 4 min. The carrier gas was helium at a flow rate of 1.1 ml/min. Detector temperature was adjusted to 280°C and injector temperature to 250°C (Adams, 2001).

After optimization of GC conditions, each sample was injected to the GC-MS (Shimadzu GC-MS model QP-1100 EX, Tokyo, Japan). GC-MS was equipped with the column of DB-1 (30 m x 0.25 mm ID, film thickness of 0.25 mm). The GC temperature program was also used for GC-MS, for ionizing a voltage of 70 eV, which was used in MS analysis. The components were identified by comparison of their mass spectra with those of NIST98 library data of the GC-MS system and Adams libraries spectra (Adams, 2001), as well as by comparison with the compounds' elution order with their retention indices reported in the literature (Adams, 2001). Retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation. Major compounds of both oils were obtained from Merck and Sigma-Aldrich companies.

Fungal strains

B. cinerea UTBC113, *A. niger* UTAN118, *P. digitatum* UTPD111 and *R. stolonifer* UTRH110 were obtained in mycological collection of department of plant protection, University of Tehran.

In vitro antifungal assay

The antifungal tests were carried out *in vitro* according to the method described by Pitarokili et al. (2003) using Petri dishes 8 cm in diameter containing potato dextrose agar (PDA). The essential

oils were dispersed as an emulsion in water using Tween 20 (0.05%) and added to PDA immediately before it was filled into the Petri dishes at a temperature of 45-50°C. The concentrations tested were 150, 300, 600 and 1200 µl/L. The controls included the same quantity of Tween 20 mixed with PDA. The phytopathogenic fungi were inoculated immediately after preparation of the Petri dishes by placing in the centre of each plate a 6 mm diameter disk of the test species, cut with a sterile cork borer from the periphery of actively growing cultures on PDA plates. The Petri dishes were incubated in the dark at a temperature of 24°C. Mean growth rates were calculated from five replicates of each fungal species every 24 h until fungi in the control filled the Petri dishes completely. The measurement of the fourth day was used to determine the minimum inhibitory concentration (MIC) and the EC₅₀ values (concentration causing 50% inhibition of mycelial growth on control media). In addition, the antifungal activity of major compounds of both oil were determined. Fungi toxicity was expressed as the percentage of mycelial growth inhibition. EC₅₀ values were calculated from the data subjected to Probit analysis (statistical software SPSS 10.0 Inc., Chicago, IL). To check the fungicidal or fungistatic properties, parts of the media from plates without mycotic growth were transferred into new PDA plates; no fungal growth after an incubation of 10 days was indicative of fungicidal activity.

In vivo assay

Strawberries (*Fragaria ananassa* Duch., cv Selva) were obtained from a commercial greenhouse, washed in 1% sodium hypochlorite and distilled water twice and after drying were dipped in each fungi conidial suspension (105 conidia per ml) for 1 min. Essential oils were prepared as emulsion in water using Tween 20 (0.05%) and then fruit treated with these emulsions and same quantity of Tween 20 as control. Spraying, dipping and fumigation were the methods of treatment. Fruits were placed in plastic trays and sealed with PVC stored in 25°C until fungi in control covered their surface completely. Each fruit divided to 8 pieces and observation of disease symptoms in each pieces estimated equal 12.5% of disease incidence. The data were collected from 4 replicates each of 8 fruits.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

By hydrodistillation, the dried materials of *T. danensis* and *T. carmanicus* yielded 5.1 and 6.3% (v/w) of essential oils respectively. The chromatographic analyses resulted in the identification of 30 components, representing 98.1–99.6% of the oils. Quantitative and qualitative analytical results by GC-MS are shown in Table 1. About 30 compounds of the oils were determined. *T. carmanicus* oil has chemical compositions characterized by carvacrol (70%), p-cymene (12.4%) and γ-terpinene (2.5%) as the major components while the major constituents of the *T. danensis* were thymol (64.8%), α-terpinene (11.3%) and p-cymene (7.9%). There are considerable differences between the oils compositions obtained in this study and those previously reported (Nickavar et al., 2005). This may be attributable that the amounts of the essential oil differ by geographic region, collection time, altitude and climate.

Table 1. Percentage composition of the essential oils of *T. carmanicus*, *T. danensis*.

Compound	RI	<i>T. carmanicus</i>	<i>T. danensis</i>
alpha-pinene	933	2.5	1.1
Camphene	947	0.2	0.1
beta-pinene	974	1.6	0.7
Myrcene	981	2.3	1.2
alpha-phelandrene	999	0.4	0.2
delta-3-carene	1007	0.1	-
p-cymene	1014	12.4	7.9
1.8-cineole	1023	0.9	-
Z-beta-ocimene	1036	0.1	-
gama-terpinene	1053	2.5	-
cis-sabinene hydrate	1056	0.2	0.3
alpha-terpinene	1080	-	11.3
Linalool	1085	0.2	-
t-sabinene hydrate	1055	-	0.1
trans-2-carene-4-ol	1145	0.1	-
4.5-epoxy-carane	1151	-	0.1
terpin-4-ol	1163	0.5	0.3
alpha-terpineol	1175	0.2	0.1
thymyl methyl ether	1225	0.1	0.1
Thymol	1266	0.3	64.8
Carvacrol	1282	70	0.9
4-terpinyl acetate	1296	-	0.2
carvacryl acetate	1345	1.4	2.8
beta-caryophyllene	1345	0.8	3.5
alpha-humulene	1427	-	0.1
beta-bisabolene	1501	0.6	1.2
Spathulenol	1576	0.1	0.1
caryophyllene oxide	1960	0.2	-
Total		99.6	98.1

RI, Retention indices relative to n-alkanes C6–C24 on the DB-1 column.

Antifungal activity

Incorporation of different essential oil concentrations (150, 300, 600 and 1200 µL/L) in PDA showed a considerable reduction on the growth of four common pathogens of strawberry fruits (Figure 1). The inhibition of the fungi depends on the concentration of the essential oils. The essential oils presented noticeable antifungal activity. Complete minimum inhibitory concentration (inhibition 100%; MIC) of both oils against *B. cinerea*, and *R. stolonifer* were 300 µL/L. The MIC of *T. danensis* and *T.*

carmanicus against *P. digitatum* and *A. niger* was 600 µL/L (Table 3). The minimum fungicidal concentration (MFC) of the oils of *T. Danensis* and *T. carmanicus* against *B. cinerea*, and *R. stolonifer* was 1200 µL/L whereas a fungicidal effect was not observed on *P. digitatum* and *A. niger* even at high concentration (Table 2). The major compounds of both oils (p-cymene, α-terpinene, Thymol and carvacrol) also exhibit antifungal activity (Figure 2 and Table 3). The MIC of p-cymene and α-terpinene was 600 µL/L on *B. cinerea* and *R. stolonifer* and 1200 µL/L on *A. niger*, whereas the MFC of the both compounds on all pathogens was more than 1200 µL/L. The MIC of Thymol and carvacrol against *R. stolonifer* was 300 and 150 µL/L respectively. In addition, the MFC of thymol and carvacrol was 600 µL/L against *B. Cinerea* and 1200 and 600 µL/L against *R. stolonifer* respectively, whereas it was more than 1200 µL/L on *A. niger* (Table 3). However, thymol and carvacrol exhibited fungicidal activity at the highest concentration (1200 µL/L) whereas both oils didn't show fungicidal activity at this concentration.

Both essential oils tested *in vivo* at the preliminary concentration exhibited inhibitory activity against four pathogens (Figure 3). *T. carmanicus* oil had better result and significantly decrease the disease incidence in strawberry fruits. These essential oils were more effective against *A. niger* on strawberry fruits. In the case of *A. niger*, dipping of fruits in *T. carmanicus* and *T. danensis* and essential oils causes less disease incidence with 18 and 30%, respectively. In all cases, dipping and spraying the essential oils were better than fumigation. Biological activity of essential oils depends on their chemical composition which is determined by the genotype and influenced by environmental and agronomic conditions (Marotti et al., 1993). The antifungal and antibacterial activity exhibited by *Thymus* genus essential oil has been demonstrated by several researchers (Rasooli and Mirmostafa, 2003). Antifungal and antibacterial activity of carvacrol and thymol as main constituents of *T. danensis* were reported in different studies (Consentino et al., 1999). Therefore, considerable antifungal activity of these oils observed in this study can be attributed to the presence of these compounds in the oils and their synergistic effects. Sensitivity of fungal species to plant essences is different and depends on essence type and dose of application. Difference in antifungal activity of essences depends on the ingredients. It is possible that one compound results in antifungal activity alone or in synergism with other compounds (Plotto et al., 2003). In the study, *B. cinerea*, and *R. stolonifer* were the most sensitive fungus that were completely inhibited at a concentration of 300 µL/L whereas *P. digitatum* and *A. niger* were the most resistant fungus which cannot be inhibited completely even at 1200 µL/L.

Recently, the exploitation of natural products to control decay and prolong storage life of perishable commodity

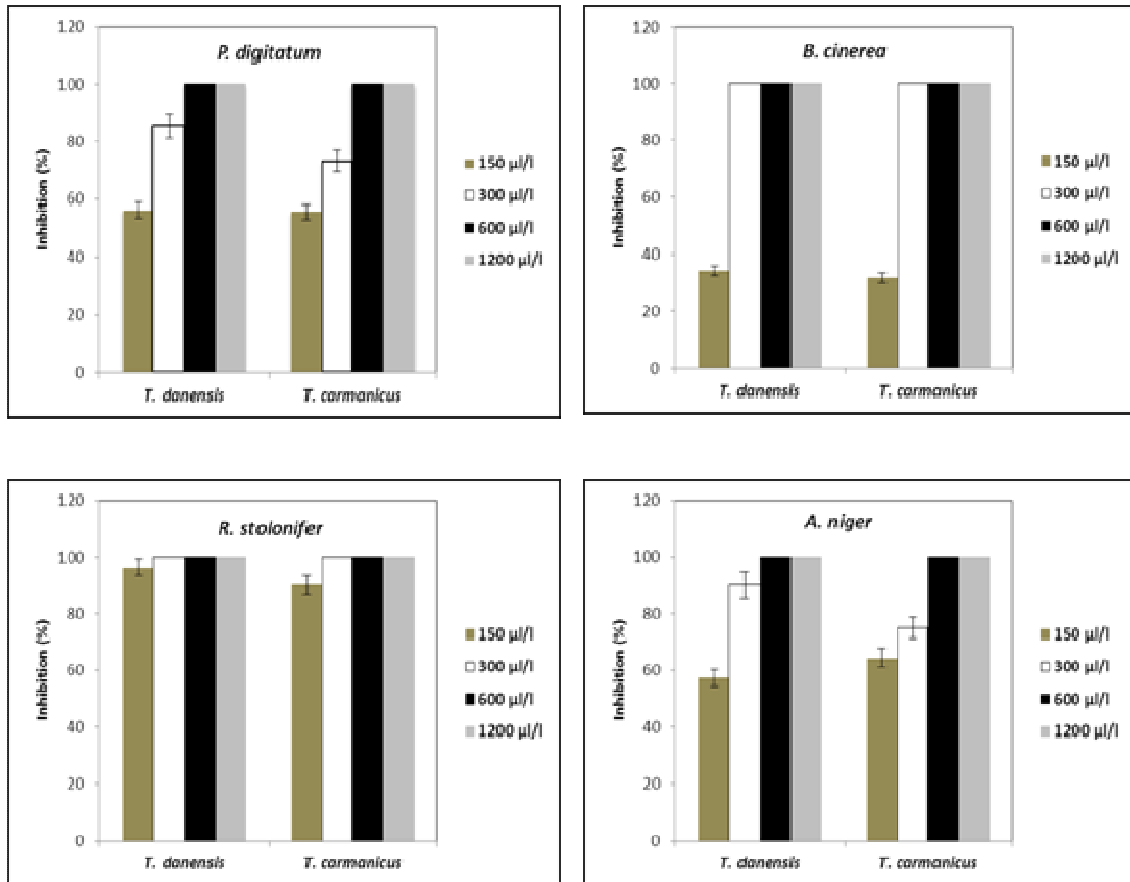


Figure 1. Inhibition of examined essential oils at different concentrations on the radial growth of four postharvest fungi on PDA medium. Each value represented mean value of four replications.

Table 3. MIC and MFC value of major compounds against four postharvest fungi.

Fungal species	p-cymene		alpha-terpinene		Thymol		Carvacrol	
	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^a	MFC ^a
<i>P. digitatum</i>	1200	>1200	600	>1200	300	>1200	300	1200
<i>B. cinerea</i>	600	>1200	600	>1200	300	600	300	600
<i>R. stolonifer</i>	600	>1200	600	>1200	300	1200	150	600
<i>A. niger</i>	1200	>1200	1200	>1200	600	>1200	600	>1200

^a Value given as µl/L.

Table 3. MIC and MFC value of major compounds against four postharvest fungi.

Fungal species	p-cymene		alpha-terpinene		Thymol		Carvacrol	
	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^a	MFC ^a	MIC ^a	MFC ^a
<i>P. digitatum</i>	1200	>1200	600	>1200	300	>1200	300	1200
<i>B. cinerea</i>	600	>1200	600	>1200	300	600	300	600
<i>R. stolonifer</i>	600	>1200	600	>1200	300	1200	150	600
<i>A. niger</i>	1200	>1200	1200	>1200	600	>1200	600	>1200

^a Value given as µl/L.

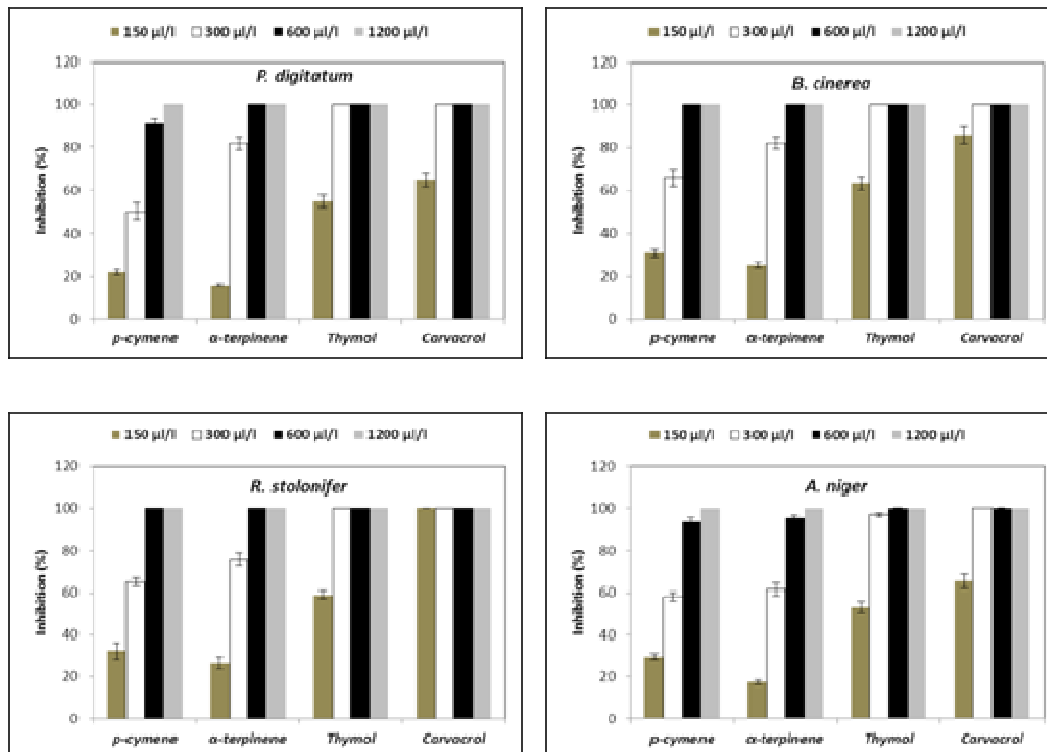


Figure 2. Inhibition of major compounds at different concentrations on the radial growth of four postharvest fungi on PDA medium. Each value represented mean value of four replications.

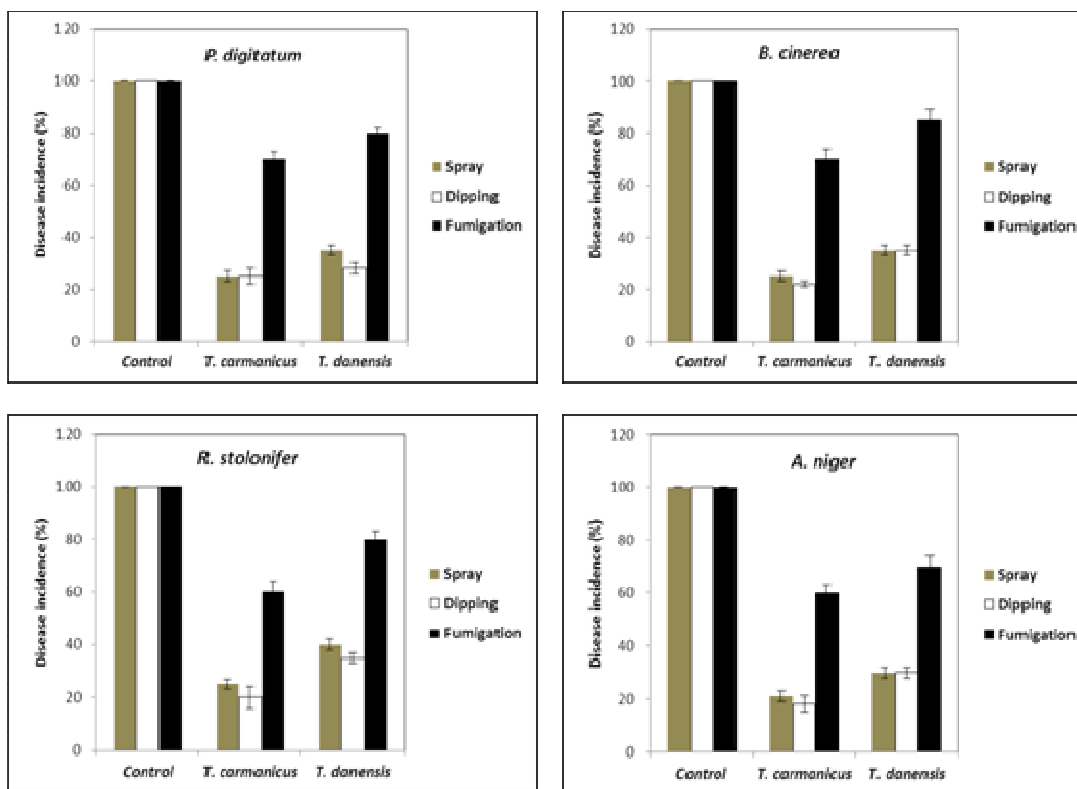


Figure 3. Antifungal efficacy of three essential oils on decay of strawberry fruits caused by *P. Digitatum*, *B. cinerea*, *R. Stolonifer* and *A. niger*. Each value represented mean value of four replications.

has received more attention. Biologically, active natural products have the potential to replace synthetic fungicides (Tripathi and Dubey, 2004). The essential oil is one of the plant extracts applicable for the management of fungal rotting of fruit and vegetables, thereby prolonging shelf life (Meepagala et al., 2002). Most of the essential oils have been reported to inhibit postharvest fungi under *in vitro* conditions (Bellerbeck et al., 2001). However, the *in vivo* efficacy and practical activity of only some essential oils have been studied.

In conclusion, examination with various concentrations of these oils exhibited promising prospects for the utilization of essential oils against a wide spectrum of microorganisms without leaving detectable residues. So essential oils can be used as a source of sustainable eco-friendly fungicides.

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