

In vitro activity of some essential oils against Penicillium digitatum

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

Received for publication 25 January 2018 Accepted for publication 21 May 2018 Abstract: Natural plant essential oils (EOs) can be used instead of synthetic fungicides because of human health concerns and environmental protection. In this study, the in vitro activity of some plants EOs against Penicillium digitatum, the cause of citrus green mold was evaluated during 8 days of incubation at 25°C. The EOs extracted from sweet orange (Citrus sinensis), lemon (Citrus limon), lime (Citrus aurantifolia), and sour orange (Citrus aurantium) fruit peel (500, 1000 and 2000 µl l⁻¹ concentrations), cinnamon (Cinnamomum cassia) bark and summer savory (Satureia hortensis) aerial parts (400, 500 and 600 µl l-1 concentrations) were used on Penicillium digitatum mycelium. None of the EOs extracted from tested citrus in this study could inhibit mycelial growth completely even at concentration of 2000 µl l-1. The best results were obtained with cinnamon and summer savory EOs at concentration of 500 and 600 µl l⁻¹. So, based on the results, cinnamon and summer savory EOs can be ideal candidates to replace the synthetic fungicides to control postharvest green mold of citrus fruit. GC-MS analysis showed that the most abundant of all constituents in EO extracts were carvacrol and y-terpinene in summer savory and (E)-cinnamaldehyde in cinnamon.

1. Introduction

Citrus spp. are the most important produced fruits in the world (Sharma and Saxena, 2004), due to their good taste, useful nutrients, and widespread availability (Liu et al., 2012). Nevertheless, the high water content and nutrient composition make them also very susceptible to decay by pathogens after harvest (Tripathi and Dubey, 2004). One of the most common diseases that infects citrus fruit is green mold caused by Penicillium digitatum (Zheng et al., 2005). The yield losses and the worsening of the quality caused by the fungus are economically important. This pathogen infects the fruit through wounds on the peel inflicted during harvest, transportation, handling or commercialization. Penicillium digitatum is one of the most important pathogen in citrus industry, because one generation of green mold complete during 7-10 days in rotten fruit at 20-25°C, and the large amounts of spores are disseminated easily by air currents (Palou, 2014).

Currently, the use of synthetic fungicides is the primary and most sim-

ple method for the control of postharvest diseases of citrus fruit (Palou *et al.*, 2008). However, fungicides consumption is strongly becoming restricted because of residual toxicity, carcinogenicity, long degradation period and increasing human health concerns (Tripathi and Dubey, 2004; Palou *et al.*, 2008).

Recently, researchers have been interested in development of alternative methods to manage postharvest decay. The essential oils (EOs) are one of non-chemical and useful control options for the management of fungal postharvest diseases (Sassi *et al.*, 2008). Essential oils are complex compounds that are natural and environmentally friendly, having antioxidant, antimicrobial and medicinal properties (Bakkali *et al.*, 2008). So, they can be ideal candidates to replace synthetic antimicrobials for maintenance of harvested horticultural crops (Tripathi and Dubey, 2004).

Many studies reported the beneficial effects of EO treatments for the control of postharvest decay caused by *P. digitatum*, such as *Thymus vulgaris* at concentration of 1000 ppm (Fatemi *et al.*, 2012), *Mentha spicata* and *Lippia scaberrima* at concentrations of 1000 and 3000 µl l⁻¹, respectively (Du Plooy *et al.*, 2009), *Bubonium imbricatum* at concentration of 1000 ppm (Alilou *et al.*, 2008), *Citrus* spp. at concentration of 10% (Badawy *et al.*, 2011), and *Cinnamomum zeylanicum* at concentration of 0.5% (Kouassi *et al.*, 2012), thereby enhancing shelf life of fruits and vegetables.

The purpose of this study was to investigate the *in vitro* activity of EOs obtained from sweet orange (*Citrus sinensis*), lemon (*Citrus limon*), lime (*Citrus aurantifolia*), and sour orange (*Citrus aurantium*) fruit peel, cinnamon (*Cinnamomum cassia*) bark and summer savory (*Satureja hortensis*) aerial parts for the control of green mold caused by *P. digitatum* as a preliminary study to find a suitable and effective EO as alternative to synthetic fungicides to control green mold in citrus postharvest management.

2. Materials and Methods

Extraction of essential oils

Plant materials used in this study are shown in Table 1. The air-dried plants material (300 gr) were cut into pieces, grounded into powder by blender, then the EOs extracted through hydro-distillation for 3-4 hours using a clevenger apparatus (Miquel *et al.*, 1976). Then the EOs were dehydrated with anhydrous sodium sulfate and stored in dark bottles at -20°C before using for antifungal study.

Isolation of fungus

The fungus used throughout this study was *P. digitatum*, the cause of citrus green mold. For isolation of fungus colony, *P. digitatum* spores were isolated from a decayed orange and cultured on potato dextrose agar (PDA) by the single spore procedure at 25°C. The isolates were maintained on PDA until needed.

In vitro antifungal assay

The antifungal assay was performed on PDA plates amended with three concentrations (500, 1000 and 2000 µl l-1) of sweet orange, lemon, lime and sour orange EOs and three concentrations (400, 500 and 600 μl l-1) of cinnamon and summer savory EOs. Tween 80 (Merck-KGaA, Germany) as an emulsifier was mixed with 80 ml of sterilized and molten PDA media, cooled to about 45°C, and then enriched with EOs. There were four 80 mm plates/replicates per treatment. After one day, the mycelia of P. digitatum from 4-days-old cultures were put in the center of amended PDA petri plates with a cork borer. All of the plates were sealed with parafilm. Inoculated plates were kept at 25°C for 8 days. Colony diameter was determined daily by measuring the average radial growth (Obagwu and Korsten, 2003). In order to evaluate its effect on fungal growth, tween 80 (emulsifier) was also considered as a treatment in the experiment.

Table 1 - Plant materials used for EOs extraction

Name	Family	Used part	Origin
Sweet orange (Citrus sinensis cv. Thomson navel)	Rutaceae	Fruit rind tissue (flavedo and albedo)	Fars-Iran
Lemon (Citrus limon cv. Lisbon)	Rutaceae	Fruit rind tissue (flavedo and albedo)	Fars-Iran
Lime (Citrus aurantifolia cv. Mexican lime)	Rutaceae	Fruit rind tissue (flavedo and albedo)	Fars-Iran
Sour orange (Citrus aurantium cv. amara)	Rutaceae	Fruit rind tissue (flavedo and albedo)	Fars-Iran
Cinnamon (Cinnamomum cassia)	Lauraceae	Tree bark	China
Summer savory (Satureja hortensis)	Lamiaceae	Aerial parts	Fars-Iran

Inhibition percentage (IP) of fungal growth was calculated as the radial growth of treated fungus (T) relative to the growth in control (C) treatment (plates without EO and Tween 80) according to the following formula:

IP (%) =
$$(\frac{C-T}{C}) \times 100$$

Essential oils analysis

At the end of the study, the main components of the most effective EOs on *P. digitatum* were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The GC analysis was carried out by the use of Agilent GC (7890-A, PerkinElmer, USA) and a flame ionization detector. It was done on fused silica capillary HP-5 column. The temperatures of injector and detector were held at 250°C and 280°C, respectively. Nitrogen was selected as carrier gas; oven temperature was 60-210°C at a rate of 4°C/min, which was then increased to 240°C at a rate of 20°C/min, and finally, kept for 8.5 min.

The GC-MS analysis was performed using an Agilent GC series 7890-A (PerkinElmer, USA) with a fused silica capillary HP-5MS column and 5975-C mass spectrometer (UNICO, USA). Carrier gas was helium. Ion source and interface temperatures were set at 230°C and 280°C, respectively. Mass range was programmed from 45 to 550 amu. Oven temperature

was 60-210°C at a rate of 4°C/min. N-alkanes was used as a standard to determine the retention indices for all constituents. The constituents were recognized by comparing their retention indices with literature reports, and their mass spectra comparison with the Wiley, Adams and Mass Finder 2.1 Library data (Adams, 1997).

Statistical analysis

The experiment was distributed according to a split plot in time design. The analysis of variance (ANOVA) was performed. Mean comparisons were conducted by LSD (least significant difference) at $P \le 0.01$. Data were analyzed by SAS software (v. 9.1).

3. Results and Discussion

Inhibitory effects of different treatments on Penicillium digitatum growth

The *in vitro* activity of the tested EOs on colony diameter of *P. digitatum* during 8 days of incubation is summarized in Table 2.

Our results indicated that colony radial growth of *P. digitatum* was inhibited completely (100%) under *in vitro* condition by both cinnamon and summer savory EOs at 500 and 600 μ l l⁻¹ concentrations during 8 days of incubation. Also, the mycelial growth

Table 2 - Inhibition percentage (%) of plant essential oils on in vitro radial growth of Penicillium digitatum

Tanaharan	EO	Time (day)							
Treatment	Concentration - (µl l ⁻¹)	1	2	3	4	5	6	7	8
Control	-	0.00 g * s^	0.00 g s	0.00 ^h s	0.00 g s	0.00 e s	0.00 g s	0.00 ^e _s	0.00 ^e s
Tween 80	-	0.80 ^g _{Q-S}	0.43 g RS	0.38 h RS	1.29 g _{P-S}	1.24 e _{P-S}	5.09 fg _{N-S}	7.08 ^e _{L-S}	8.13 d $^{\rm e}$ $_{\text{K-S}}$
Sweet orange	500	22.42 ^f _{w-i}	5.81 ^{fg} _{L-S}	10.34 gh I-S	12.73 fg _{D-Q}	6.59 e _{L-S}	9.37^{fg}_{J-S}	6.55 e _{L-S}	0.00 ^e s
Sweet orange	1000	23.81 f W-g	5.75 fg _{M-S}	18.02^{e-g} B-K	23.07 ^{ef} _{W-h}	12.56 e _{D-R}	16.26^{e-g} C-N	11.55 ^e _{G-S}	0.00 ^e _s
Sweet orange	2000	75.80 ^b _B	54.74 b _{E-I}	48.24 c	50.72 ° _{G-K}	32.09 ^{cd} _{R-b}	38.42 ^{cd} _{1-S}	41.76 ° _{J-S}	36.56 bc _{0-V}
Lemon	500	32.81 ^{ef} _{R-a}	17.41 d-g C-M	20.79 e-g Z-j	23.18 $^{\rm ef}$ $_{\rm W-h}$	13.03 e _{D-Q}	12.79 fg _{D-Q}	12.19 e _{F-S}	0.00 ^e S
Lemon	1000	46.91 ^{c-e} _{L-P}	24.46 c-f	38.18 ^{cd} _{M-S}	41.15 cd _{J-S}	17.42 ^{c-e} _{C-M}	22.27 ^{d-f} _{X-i}	20.14 de _{B-K}	12.50 ^{c-e} _{E-R}
Lemon	2000	61.38 b-d D-H	23.54 ^{c-f} _{W-H}	45.84 ^c _{I-Q}	50.87 ^c _{G-K}	34.39 ° _{Q-X}	44.26 ^c	34.66 ^{cd} _{P-W}	25.31 ^{b-e} _{T-c}
Lime	500	36.98 ef _{M-U}	20.71 d-f	21.95 e-g _{Y-i}	17.98 ^f _{с-м}	15.74 ^{de} _{C-N}	16.98^{e-g}_{C-N}	17.25 de _{C-N}	0.00 ^e s
Lime	1000	54.54 b-e E-I	30.49 ^{c-e} _{s-h}	44.03 ^c	48.19 ° _{I-O}	$30.72^{\text{ cd}}_{\text{ S-B}}$	33.27 ^{c-e} _{R-Y}	33.22 ^{cd} _{R-Y}	30.85 b-d _{S-b}
Lime	2000	70.43 bc _{B-D}	42.48 bc I-S	49.22 ^c _{н-м}	50.62 ° _{G-L}	34.11 ° _{Q-Y}	41.72 ^c _{J-S}	40.39 ° _{K-S}	32.50 ^{b-d} _{R-a}
Sour orange	500	48.92 ^{c-e} _{I-N}	36.82 b-d	14.64 fg _{c-o}	12.61 fg D-R	11.36 ^e _{H-S}	16.16 e-g c-n	17.89 de _{C-M}	0.00 ^e s
Sour orange	1000	50.94 ^{c-e} _{G-K}	24.00 c-f w-f	30.77 ^{de} _{S-b}	33.67 ^{de} _{Q-Y}	13.36 ^e _{C-P}	15.97 e-g _{C-N}	14.85 ^{de} _{c-o}	9.37 ^{de} _{J-S}
Sour orange	2000	61.44 b-d D-H	33.01 ^{c-e} _{R-Z}	44.12 c	53.39 ° _{F-J}	33.12 ^c _{R-Y}	37.28 ^{cd} _{M-T}	41.08 ° _{J-S}	40.94 b K-S
Cinnamon	400	40.52 d-f K-S	12.63 e-g D-R	24.77 ef _{U-d}	22.22 ef x-I	3.34 ^e _{o-s}	6.48 fg L-S	9.40 e _{J-S}	3.44 ^e _{o-s}
Cinnamon	500	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A
Cinnamon	600	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A
Savory	400	100.00 a A	89.61 ^a _A	74.01 b BC	70.91 b _{B-D}	66.01 b B-E	65.25 ^b _{в-F}	61.83 b _{c-G}	51.25 ^b _{G-К}
Savory	500	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.0 0 a A	100.00 a A	100.00 a A	100.00 a A
Savory	600	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A	100.00 a A

^{*} For each column, similar letters (lower case, superscript) are not significantly different according to LSD (P≤0.01) test.

[^] Means followed by similar letters (subscript), are not significantly different according to LSD (P≤0.01) test.

was decreased by cinnamon and summer savory EOs at concentrations lower than 500 μ l l⁻¹, but it was not suppressed completely.

The main activity of the EOs in the postharvest fruit are derived from their ability to inhibit pathogen growth (Periago et al., 2004). Cinnamon EO has the potential to be employed as a natural antifungal agent for fruit disinfectation, as cinnamaldehyde is its main constituent (Xing et al., 2010). Furthermore, it has been reported that summer savory contains some substances with antibacterial properties (Deans and Svoboda, 1989). In this research, cinnamon and summer savory EOs have the strongest effect on P. digitatum growth (Table 2). It has been reported that eucalyptus and cinnamon (Cinnamomum zeylanicum, Blume) oil vapour (500 ppm) reduced decay almost by 50% in tomatoes after 10 days of storage (Tzortzakis, 2007). Moreover, Win et al. (2007) presented that EOs from cinnamon at the concentration of 5.0 g l⁻¹ completely inhibited conidial germination and mycelial growth of all fungi on banana (Colletotrichum musae, Fusarium spp. and Lasiodiplodia theobromae). In addition, Lopez-Reyes et al. (2010) showed that summer savory, oregano and thyme EOs at 10% showed significant inhibitory effect (similar to chemical control) against P. expansum and Botrytis cinerea on four cultivars of apples.

The mechanism by which EOs suppress the microbial growth is not fully understood, but a number of possible explanations have been postulated. Essential oils are lipophilic and this property enables them to preferentially move from an aqueous phase into fungi membrane. This action leads to membrane expansion, increasing in membrane fluidity and permeability, membrane proteins disorder, respiration rate control, change of ion transportation in fungi and induced cellular contents leakage (Burt, 2004; Oonmetta-Aree et al., 2006; Khan et al., 2010; Fadli et al., 2012).

In the present study, the lowest inhibition was observed in control plates that contained only PDA (0%); however, this was not significantly different from plates containing PDA and the tween 80 without the EOs during 8 days. So, results indicated that the tween 80 used as an emulsifier had no effect on the mycelial growth (Table 2).

We observed an increase of antifungal effects of the tested citrus fruits peel EOs such as sweet orange, lemon, lime and sour orange as the EOs concentration increased, but the fungi growth was not inhibited completely even at concentration of 2000 µl l⁻¹. So, as the results showed, none of the tested concentrations of citrus fruits EOs in this study could inhibit radial growth completely (Table 2).

Essential oils are present in great quantities in the flavedo of citrus fruit (Caccioni *et al.*, 1998). The citrus fruits EO consists a mixture of components such as terpenes, hydrocarbons, ketones, aldehydes, alcohols, acids, and esters. The amount of them depends on the citrus cultivar, the extraction and separation techniques (Fisher and Phillips, 2008).

The positive effect of the volatile components of citrus fruit essential oils on P. digitatum and italicum growth has been reported (Caccioni et al., 1998). The spore germination and mycelium growth of P. italicum and digitatum were stimulated by the essential oil of Citrus reticulata Blanco at concentration of more than 2.5 μ l ml $^{-1}$ (Wang et al., 2012). Moreover, Badawy et al. (2011) reported that Citrus aurantifolia EOs had the antifungal effects against P. digitatum pathogens at concentration of 10% (v/v). However, in our study the application of Citrus spp. could not provide acceptable control of green mold disease.

Analysis of the summer savory and cinnamon EOs

The analysis of the volatile profiles in summer savory and cinnamon EOs are listed in Table 3 and 4,

Table 3 - Chemical composition of the summer savory essential oil

Number	Component	RI*	(%)
1	α- Thujene	924	1.15
2	α-Pinene	932	0.64
3	Camphene	946	0.06
4	Hepten-1-ol	958	0.05
5	Sabinene	969	0.01
6	β-Pinene	974	0.21
7	3- Myrcene	988	1.15
8	Phellandrene	1002	0.23
9	α-Terpinene	1014	3.75
10	p-Cymene	1020	2.19
11	Sylvestrene	1025	0.37
12	E-β- Ocimene	1044	0.07
13	γ-Terpinene	1054	31.98
14	Terpinolene	1086	0.05
15	trans-α Sabinene hydrate	1098	0.07
16	Isoborneol	1155	0.06
17	Terpinene-4-ol	1174	0.2
18	α-Terpineol	1186	0.1
19	carvacrol methyl ether	1241	0.09
20	Thymol	1289	0.8
21	Carvacrol	1298	55.66
22	Thymol acetate	1349	0.03
23	Carvacrol acetate	1370	0.07
24	Caryophyllene	1417	0.36
25	Aromadendrene	1439	0.08
26	α–Humulene	1454	0.01
27	Bicyclogermacrene	1500	0.18
28	Bisabolene	1505	0.21
29	Unknown	-	0.01
30	Spathulenol	1577	0.04

^{*} Retention index

Table 4 - Chemical composition of the cinnamon essential oil

Number	Component	RI*	(%)
1	α-Pinene	932	0.57
2	Camphene	946	0.34
3	Benzaldehyde	958	0.49
4	β -Pinene	975	0.16
5	α-Phellandrene	1004	0.02
6	p-Cymene	1023	0.07
7	Limonene	1026	0.13
8	1,8-Cineole	1029	0.07
9	γ-Terpinene	1056	0.04
10	Benzenepropanal	1160	0.26
11	Borneol	1163	0.19
12	α-Terpineol	1189	0.03
13	(Z)-Cinnamaldehyde	1217	0.73
14	(E)-Cinnamaldehyde	1271	70.04
15	Unknown	1333	0.06
16	Cyclosativene	1367	0.59
17	α-Copaene	1373	10.82
18	Unknown	1387	0.18
19	β-Elemene	1389	0.14
20	Sativene	1393	0.44
21	(E)-Caryophyllene	1416	0.20
22	β-Gurjunene	1426	0.06
23	lpha -Humulene	1450	0.20
24	γ-Muurolene	1474	1.25
25	ar-Curcumene	1480	0.15
26	Viridiflorene	1492	0.28
27	α -Muurolene	1497	4.23
28	β-Bisabolene	1506	0.15
29	γ-Cadinene	1511	0.26
30	δ-Cadinene	1521	5.35
31	(E)-ortho-Methoxy cinnamaldehyde	1528	0.27
32	trans-Cadina-1(2),4-diene	1529	0.96
33	lpha -Calacorene	1540	0.50
34	epi-a-Muurolol	1639	0.40
35	α -Muurolol	1643	0.20
36	lpha -Cadinol	1651	0.03
37	Cadalene	1671	0.10

^{*} Retention index

respectively. A total of 30 different components of summer savory, and 37 components of cinnamon were identified and isolated by GC and GC-MS from the EOs. The principal components of the summer savory EO were carvacrol (55.66%), γ -terpinene (31.98%), α -terpinene (3.75%), p-cymene (2.19%), 3-myrcene (1.15%), and α -thujene (1.15%). The major components of the cinnamon EO were (E)-cinnamaldehyde (70.04%), α -copaene (10.82%), δ -cadinene (5.35%), α -muurolene (4.23%), and γ -muurolene (1.25%). Other constituents which were less than 1% have been shown in Table 3 and 4.

As shown in Table 2, both summer savory and cinnamon EOs were equally effective in inhibiting the growth of *P. digitatum*. This is in accord with the reported in vitro inhibitory effect of carvacrol against

pathogens (Periago et al., 2004). In fact, the main component of summer savory EO is a phenol (Sacchetti et al., 2005), and its most important mechanism of antimicrobial activity is connected with the phenolic ring in its chemical structure (Ultee et al., 2002). Furthermore, it has been reported that the toxicity rate of the phenol ring is due to the site (s) and number of hydroxyl groups (Cowan, 1999). Concerning cinnamon EOs, its major volatile compound is cinnamaldehyde. Moreover, it has been reported that cinnamon EO had potent anti-bacterial and anti-fungal activities due to cinnamaldehyde (Ooi et al., 2006), because it acts as membrane irritants (Nabavi et al., 2015).

4. Conclusions

In this study, the *in vitro* activity of plants EOs against *P. digitatum* were tested at different concentrations during 8 days of incubation at 25°C. As showed by the results, the stronger inhibitions were obtained by cinnamon and summer savory EOs at concentration of 500 and 600 μ l l⁻¹. None of the citrus EOs could inhibit fungus radial growth completely compared with cinnamon and savory EOs. GC-MS analysis showed that the most abundant of all constituents in EO extracts were carvacrol and γ -terpinene in summer savory and (E)-cinnamaldehyde in cinnamon.

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