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Control of nectarine fruits postharvest fungal rots caused by *Botrytis Cinerea* and *Rhizopus Stolonifer* via some essential oils

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Abstract Nectarines (*Prunus persica* L. Bath) are very sensitive fruit to fungal infection. Today, the control of postharvest fruit diseases with essential oils (EOs) has been significantly noticed as a novel trend in biological preservation. In this study, volatile compounds of *Cinnamom zeylanicum* (CEO), *Zataria multiflora* (ZEO), and *Satureja khuzestanica* (SEO) were analyzed by Gas Chromatography–Mass spectroscopy. Also, the in vitro antifungal activities of EOs against *Botrytis cinerea* and *Rhizopus stolonifer* were evaluated at different concentrations. The in vivo antifungal activity of these EOs on artificially infected nectarine fruits was also considered. The major components were *Thymol* (32.68%) and *Carvacrol* (30.57%) for ZEO, *cinnamaldehyde* (80.82%) for CEO, and *carvacrol* (38.43%) for SEO. The application of different concentrations showed a decreasing trend in the fungus radial growth in all EOs. In the in vitro experiments, ZEO and CEO exhibited more significant mycelial inhibition results and reduction of the IC₅₀, MIC and MFC values against *Botrytis cinerea* and *Rhizopus stolonifer*, respectively. However, in the in vitro experiments, none of the treatments were capable of completely inhibiting the

growth of the fungi. According to the results of this study, ZEO and CEO could reduce the damage caused by these fungi.

Keywords Essential oil · In vivo and in vitro · Postharvest rot · *Botrytis cinerea*, *Rhizopus stolonifer* · Nectarine fruits

Introduction

Nectarines (*Prunus persica* L. Bath), as mutated peaches, are climacteric fruits and have a similar physiological ripening pattern to peaches (Serrano et al. 2004). They are highly spoilable due to their high moisture content and respiration rates (Lurie and Crisosto 2005). Moreover, nectarine fruits are very sensitive to fungal infection appear during postharvest, packaging, storage, and transportation and these diseases continue their development even under room temperature or cold storage. *Rhizopus stolonifer*, *Botrytis cinerea*, *Monilinia* spp., *Penicillium* spp., and *Aspergillus* spp. are the most devastating postharvest fungal pathogens in nectarines (Navarro et al. 2011). These fungi even in regions with high technology stores are damaging, and it is estimated that about 50% and 25% of the product will be lost in the developing and developed countries, respectively (Eckert and Ogawa 1985; Spadaro and Gullino 2004). In addition, the promotion of fungal infections may result in the contamination of products with mycotoxins (Wu et al. 2014).

Spraying fruits before harvest with Benomyl and immediately after harvest with Thiabendazole, washing fruits with sodium orthophenylphenol or imazalil as soluble or wax treatments in the packaging and packing lines are the most common post-harvest disease control methods (Narayanasamy and Narayanasamy 2006). The use of these

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chemical pesticides results in acne or chronic toxicity to non-target organisms, including humans, which are associated with cumulative properties of living organisms or carcinogenic properties. On the other hand, increasing the resistance to fungi used in postharvest patients is a serious problem (Barkai-Golan 2001). Today, incentives to find alternatives to pesticides have become much more prominent, because of special attention to human health and the environment. Therefore, much attention is being paid to strengthen the natural methods and the use of herbal products that have antifungal properties (Gyawali and Ibrahim 2012).

Recently, the control of postharvest fruit diseases with essential oils (EOs) has been significantly noticed as a novel trend in biological preservation (Chen et al. 2013). Several studies have shown that EOs of Thyme (Nikkhah et al. 2017), Cinnamomum (Bassolé and Juliani 2012), savory (Farzaneh et al. 2015), Peppermint (Desam et al. 2017) Zhumeria, Heracleum, and Eucalyptus (Davari and Ezazi 2017) have antifungal activity against phytopathogenic fungi and postharvest fungal diseases in fruits such as apple (Yilmaz et al. 2016), banana (Bhutia et al. 2016), citrus (Boubaker et al. 2016), strawberry (Etemadi et al. 2012), grapes (dos Santos et al. 2012), avocado, mango, and papaya (SarkhoSh et al. 2018). Nikkhah et al. (2017) investigated the inhibitory effect of thyme, cinnamon, rosemary, and marjoram EOs separately and simultaneously against *Botrytis cinerea* and *Penicillium expansum* on pear fruits. In their study, cinnamon at concentration of 625 $\mu\text{L/L}$ could inhibit the lesion diameter to less than the half of the control fruits. But EOs combination (cinnamon, thyme, and rosemary) showed the highest inhibitory effect with average lesion diameter of 6 and 8 mm on *Botrytis cinerea* and *Penicillium expansum*, respectively Farzaneh et al. (2015). investigated chemical composition and antifungal effects of three species of savory essential oils against the *Botrytis cinerea*, *Rhizopus stolonifer*, *Penicillium digitatum*, and *Aspergillus niger* and reported that at the maximum concentration (1200 $\mu\text{L/L}$), all of the savory species EOs do not possess fungicidal effects on *Aspergillus niger* but they exhibit fungicidal activities against *Botrytis cinerea*, *Rhizopus stolonifer*, and *Penicillium digitatum*. Also, *S. khuzistanica* was the strongest oil in fungicidal activity. *S. hortensis* oil was more effective than *S. spicigera* against *Botrytis cinerea* whereas *S. spicigera* oil showed stronger fungicidal activity against *Rhizopus stolonifer*. Alizadeh et al. (2015) applied emulsion and nanoemulsion CEO on strawberry fruits and reported that EO emulsion at concentration of 2000 $\mu\text{L/L}$ possesses the most effective antifungal activity even in comparison with thiabendazole fungicide at the same dose, after 10 days. However, no doses of EO emulsion, nanoemulsion, and thiabendazole (500, 1000,

and 2000 $\mu\text{L/L}$) showed full inhibitory effect against *Botrytis cinerea*. Also, in a study conducted by Yilmaz et al. (2016), none of the EOs (Oregano, Fennel, Sage, Rosemary, and Eucalyptus) showed complete inhibitory on strawberry fruits against *Botrytis cinerea* even up to concentration of 5000 $\mu\text{L/L}$ after 6 days.

The aim of the present study was to evaluate the in vitro antifungal activities of *Zataria multiflora* (ZEO), *Cinnamomum zeylanicum* (CEO), and *Satureja khuzestanica* (SEO) EOs against *Botrytis cinerea* and *Rhizopus stolonifer*. The in vivo antifungal activity of these EOs on artificially infected nectarine fruits was also considered.

Materials and methods

Essential oils

ZEO was a generous gift from Tabib daru Co (Kashan, Iran), CEO and SEO were purchased from Zardband Co (Tehran, Iran).

Essential oil analysis

Gas Chromatography–Mass spectroscopy (GC–MS; Agilent, 7890 B series, USA) equipped with an HP-5MS capillary column (30 m Length, 0.25 mm Film and 0.25 mm Diam) and helium carrier gas (99.999% purity) at a flow of 1 mL/min connected to a mass spectrometer (Agilent-MSD5975C) was used to analyze the EOs volatile components. The injector temperature was set at 250 °C and 1 μL of essential oil was injected. Column temperature was kept at 50 °C for 3 min, then increased to 180 °C and held for 2 min (Davari and Ezazi 2017). Identification of the components was carried out based on studying their patterns of fragmentation and also their coincidence with Adams libraries spectra (Adams 2007). For the percentage of each component the relative area for each compound was calculated based on the area under its chromatogram.

Phytopathogenic fungi

The fungi used in this study were *Botrytis cinerea* (IRAN 1304C) that was a generous gift from University of Tabriz and *Rhizopus stolonifer* was isolated from infected Peach fruit on Potato Dextrose Agar (PDA: Merck Company, Germany), purified by single spore method and identified based on morphological characteristics (Machado et al. 2007). The fungal isolates were cultured and maintained on PDA in a growth chamber at 25 ± 2 °C under 12 h light and 12 h dark mode conditions.

Conidia of fungus were recovered from tree day (for *Rhizopus stolonifer*) and one week (for *Botrytis cinerea*) old

cultures. For this purpose, 10 mL of sterile distilled water contained 0.1% v/v Tween 80 as an emulsifier poured on each plate. Then Conidia were released from fungal mycelium by scratching surface of the medium with a scalpel. Afterward, the conidia were transferred to falcon tubes and mixed with a vortex for 2 min to be separated from mycelium and suspended. The final solution was filtered to remove fungal mycelium and obtain a pure conidia suspension. The conidia concentration was evaluated using a Hemocytometer. The suspension was diluted with sterile water to the final concentration of approximately 1×10^6 conidia/mL.

The in vitro assay

The in vitro assays of ZEO, CEO, and SEO were performed with the method as described by Mohammadi et al. (2015). Briefly, 75, 150, 300, 600, and 1200 $\mu\text{L/L}$ (v/v) of each EOs were mixed with sterilized (under conditions of 1.5 atm pressure and 120 °C PDA in 90 mm diameter petri dishes. Tween-80 (0.05% v/v) was added to each treatment as an emulsifier. After inoculating of 5 mm disks of the fungi on the center of plates, the plates were sealed with parafilm and incubated at 25 ± 2 °C under 12 h light and 12 h dark period until the control plates were fully occupied with the fungi cultures. Then, the antifungal index (AI) was calculated by the following equation:

$$AI = \frac{C - T}{C} \times 100 \quad (1)$$

where C and T are the average diameter of fungi in the control and treated plates, respectively. MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentrations) were also calculated according to the method described by Pinto et al. (2013). When the control plates were fully occupied with the fungi cultures, the lowest concentration that completely inhibited the mycelium growth was defined as MIC and a cultured fungal disc from the MIC plate was transfer to a non-EOs medium. After 4 days, lowest concentration with no recovery of fungi considered as MFC (Pinto et al. 2013). Probit analysis was used to determine the IC_{50} (concentration inhibited 50% of the mycelium growth).

The in vivo assay

The in vivo assay was conducted on nectarines fruits that were harvested from commercial maturity in Meshghin Shahr county, Ardabil province, Iran. The fruits were absence of fungal infection and damage. They were selected for uniformity of size, maturity, and color. They were sterilized with sodium hypochlorite solution (2.5% v/v) for 2 min and then washed with sterile distilled water under laminar air flow hood and left for 1 h to dry.

Afterwards, artificial injury (wounds) made on both sides of the fruits (4×4 mm of diameter and depth); then 10 μL of the EOs were dropped into each injury. For comparison, instead of EOs, sterile distilled water dropped onto the artificial injury of positive and negative control fruits and left under laminar air flow hood for 1 h to absorb the EOs or water. Then 10 μL of spore suspensions with concentration around of 1×10^6 were applied to each wound. Nectarine fruits were inoculated and dried under the laminar air flow hood for 1 h, placed in plastic sealed pack and stored for 4 days at 25 °C and 85% RH. There were 5 fruits in each treatment and the assay was repeated three times.

Statistical analysis

The resulting data were investigated with factorial design on a completely randomized design (CRD) base. Data was analyzed by one-way ANOVA and the Tukey test (at $p < 0.05$ level) was used for comparison of means. The statistical analysis was performed using Minitab 18 statistical software (Minitab software, Cleocom, Birmingham, UK).

Results

Volatile components of EOs

The results of the chemical analysis of the studied EOs by GC-MS are summarized in Table 1. Twenty six components were identified in the ZEO, representing 98.46% of the total detected components, with *thymol* (32.68%), *carvacrol* (30.57%) and *p-cymene* (8.94%) and *γ -terpinene* (5.96%) as the major constituents. Also, 16 components were identified in the CEO (97.66%), *cinnamaldehyde* was the major component (80.82%) and the other detected components were lower than 5%. In addition, 12 components were identified in SEO representing 96.99% of the total essential oil. The major components for this EOs were *carvacrol* (38.43%), *γ -terpinene* (21.89%), *p-cymene* (16.55%), and *α -terpinene* (5.76%).

In vitro assays

According to results given in Table 2 and Fig. 1 a significant decreasing trend in the fungus radial growth of all treatments was observed ($p < 0.05$). However, the antifungal activity of these compounds depends on the type of EO plant and fungus. Also, concentration of the EOs had a significant effect on growth of colony and with increasing the concentration, significant effect on growth prohibition of fungi was observed in the all three EOs ($p < 0.05$). In general, among the EOs, SEO showed the lowest antifungal

Table 1 Chemical composition of ZEO, CEO, and SEO

Relative area (%)	ZEO (%)	CEO (%)	SEO (%)	Retention index
α -Thujene	3.63	1.53	2.32	933
α -pinene	0.43	–	2.64	938
Benzaldehyde	–	0.37	–	944
Camphene	0.15	–	–	946
3-Octanone	0.14	–	–	966
β -pinene	0.39	–	1.83	973
β -Myrcene	0.71	–	2.47	981
α -Phellandrene	0.13	–	–	990
3-Carene	–	0.65	–	998
α -Terpinene	0.52	–	5.76	1008
p-Cymene	8.94	1.54	16.55	1009
Limonene	0.56	–	1.45	1018
β -Phellandrene	–	0.37	–	1024
Eucalyptol	0.43	1.15	–	1034
γ -Terpinene	5.96	0.08	21.89	1048
Linalool	1.13	–	–	1098
β -Terpineol, cis-	0.1	–	–	1135
α -Terpineol	1.54	0.08	–	1189
Thymol methyl ether	0.67	–	–	1216
Carvacrol methyl ether	0.98	–	–	1225
Carvone	–	–	1.21	1243
Linalool acetate	–	2.65	–	1248
Cinnamaldehyde	–	80.82	–	1263
Thymol	32.68	–	1.94	1281
Carvacrol	30.57	–	38.43	1294
Thymol acetate	1.45	–	–	1332
Carvacrol acetate	1.78	–	–	1348
Eugenol	–	4.47	–	1376
Caryophyllene, (Z)-	2.38	–	–	1404
Aromadendrene	1.21	–	–	1441
<i>Components</i>				
Caryophyllene	–	3.57	–	1456
(Z)-Cinnamic acid	–	0.12	–	1471
γ -Bisabolene, (E)-	0.8	–	2.32	1523
Spathulenol	0.83	–	–	1563
2H-1-Benzopyran-2-one	–	0.1	–	1581
Cinnamyl acetate	–	0.14	–	1635
Caryophyllene oxide	0.78	0.03	–	1961
Total	98.46	97.66	98.81	

Bold values indicates the major components of Essential oils

activity against both fungi. MICs and MFCs values for this EO were 600 μ L/L for *Botrytis cinerea*, and 600 and 1200 μ L/L for *Rhizopus stolonifer*, respectively (Table 3). While, ZEO showed the highest antifungal activity against *Botrytis cinerea* (MIC = 150 and MFC = 300 μ L/L), CEO was more effective EOs in only *Rhizopus stolonifer* growth inhibition with MIC = 300 and MFC = 600 μ L/L.

The in vivo assay

Although in vitro experiments of EOs is an important factor for selecting plants with potential antifungal activity against phytopathogenic fruits, in first step, in vivo experiments are needed to check their effectiveness in actual state (Askarne et al. 2012). Therefore, the in vivo experiments conducted on nectarines fruits. According to

Table 2 Antifungal activity (AI) of ZEO, CEO, and SEO against *Botrytis cinerea* and *Rhizopus stolonifer* and the radial growth (RG) of fungal pathogens

Components	Concentration ($\mu\text{L/L}$)	<i>Botrytis cinerea</i>		<i>Rhizopus stolonifer</i>	
		RG ^a (mm)	AI ^b (%)	RG (mm)	AI (%)
Control	0	90 \pm 0.00a	0.00h	90 \pm 0.00a	0.00g
ZEO	75	79.33 \pm 1.53d	11.85e	74.33 \pm 0.58ab	17.41ef
	150	0h	100a	61.33 \pm 4.16d	31.85d
	300	0h	100a	21.33 \pm 1.80f	76.29b
	600	0h	100a	0g	100a
	1200	0h	100a	0g	100a
CEO	75	86.90 \pm 0.46b	3.50g	65.67 \pm 3.03cd	27.04de
	150	9.33 \pm 1.15g	89.63b	39.67 \pm 1.51e	55.92c
	300	0h	100a	0g	100a
	600	0h	100a	0g	100a
	1200	0h	100a	0g	100a
SEO	75	83.32 \pm 0.35c	7.43f	78.60 \pm 0.23b	12.67f
	150	46.33 \pm 3.51e	48.52d	67.20 \pm 0.69cd	25.33de
	300	16.83 \pm 1.04f	81.25c	31.33 \pm 1.53e	65.19c
	600	0h	100a	0g	100a
	1200	0h	100a	0g	100a

Mean values followed by the same letters (i.e. a, b, c, etc.) are not significantly different according to Tukey test ($p < 0.05$)

^aRadial growth of fungus pathogen

^bAntifungal index

the obtained results (Fig. 2), there was a significant difference between different EOs at different concentrations ($p < 0.01$) on lesion diameter. Generally, in all EOs with increasing the concentration, lesion diameter Caused by *Botrytis cinerea* and *Rhizopus stolonifer* (Fig. 3) fungi was decreased. Like the in vitro experiments, SEO showed the lowest antifungal activity against both fungi, ZEO had the best antifungal activity against *Botrytis cinerea* and CEO was more effective EO in fungus growth inhibition of *Rhizopus stolonifer*. However, none of the treatments were capable of completely inhibiting the growth of fungi.

Discussion

In this study, the EOs components which identified by GC-MS were in accordance with those reported by other researchers (Farzaneh et al. 2015; Mahmoudvand et al. 2017; Saharkhiz et al. 2016). However, the slight differences between the percentages of EOs components can be attributed to the differences in climate, region, elevation, and geographical location of the growth place, phonological stages, and techniques that are applied for extraction of EOs (Hadipanah et al. 2015; Saharkhiz et al. 2016; Valero and Salmeron 2003).

A decreasing trend in the fungus radial growth of the all treatments was observed as well. Due to the high number of

chemical components identified in the EOs, a single mechanism for their antifungal effects cannot be considered as the components might have several roles in fungus cells. One of the important properties of essential oils and their constituents is their hydrophobic properties, which results in the penetration of these materials into fungus cell membranes, disruption of cellular structure, and increasing the permeability. This causes exhausted ion leakage and other cellular contents, resulting in cell death (dos Santos et al. 2012). In general, more phenolic materials with functionalized loop structures lead to higher antifungal and antibacterial properties (Burt 2004). It has been accepted that the *thymol* and *Carvacrol* in ZEO (Mohammadi et al. 2015), *cinnamaldehyde* (Bassolé and Juliani 2012), and *eugenol* (Burt 2004) in CEO, and *carvacrol* (Farzaneh et al. 2015) in SEO are responsible for antifungal activity.

Consistent with the literature, we found that the initial concentrations have decreasing effect on the fungus radial growth in all of the EOs. However, there are minor differences in terms of MIC and MFC in different studies Nikkhah et al. (2017). reported the MIC and MFC of CEO against *Botrytis cinerea* at 625 and 1250 $\mu\text{L/L}$, respectively, which were more than that in our study. This difference can be related to various amounts of the involved components especially cinnamaldehyde in CEO in two studies, which was 88.82% in our study and 44.25% in Nikkhah's study. In other studies, Behdani et al. (2012)

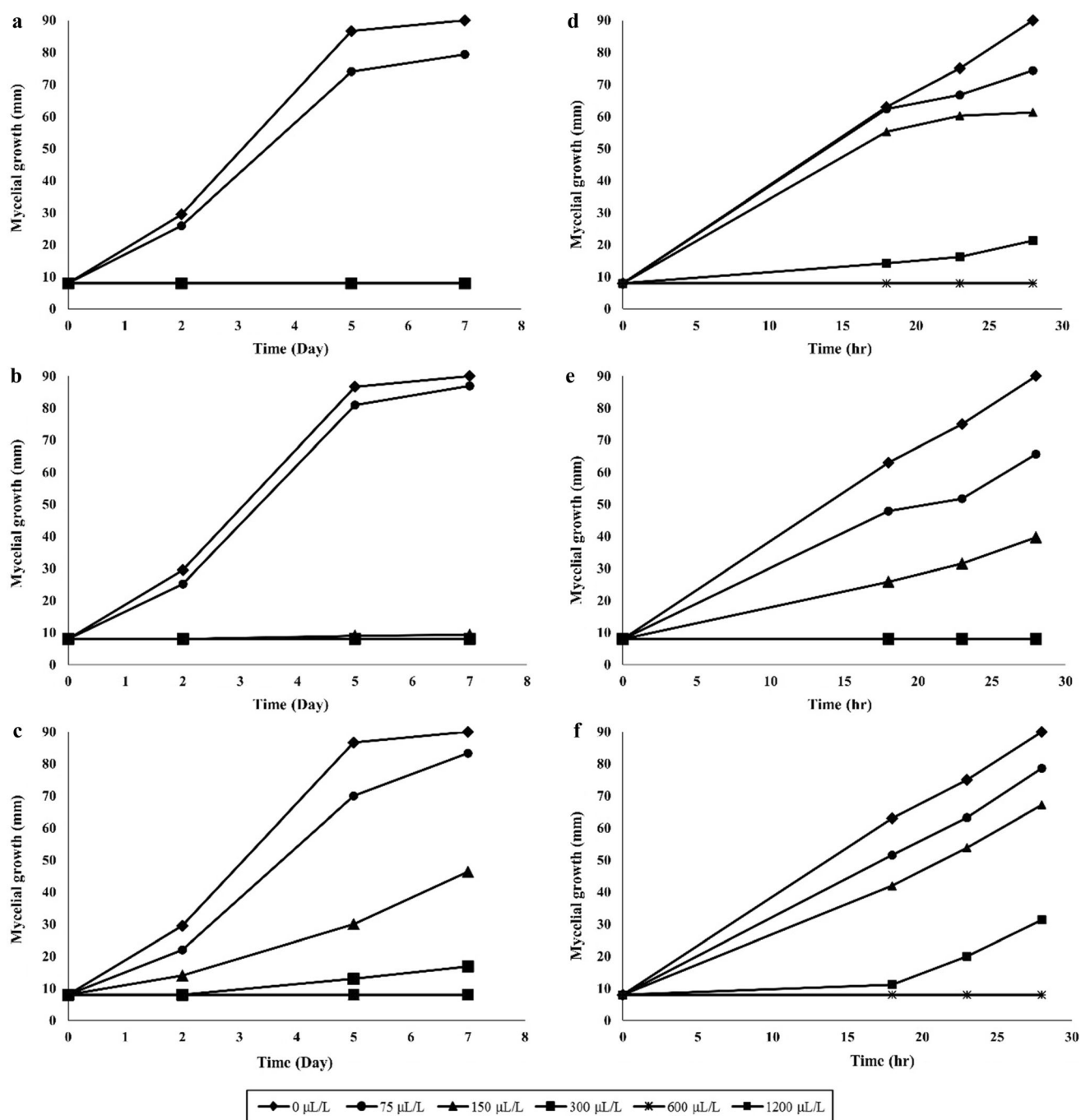


Fig. 1 Growth of the fungi *Botrytis Cinerea* and *Rhizopus Stolonifer* incubated at various concentrations of the ZEO (a, d), CEO (b, e) and SEO (c, f) for 7 day and 28 h, respectively

results showed that CEO has strongly antifungal effect against *Botrytis cinerea* and it possesses fungus growth inhibitory effect at 500 μL/L. Fathi et al. (2013) reported that CEO has the MIC of 400 μL/L. In a study conducted by Etemadi et al. (2012) on *Botrytis cinerea*, MIC of ZEO was reported at 200 μL/L which was the minimum effective concentration and approximately in accordance with

value that was reported in our study. In a study performed by Farzaneh et al. (2015) on *Botrytis cinerea* and *Rhizopus stolonifer* the MICs value of SEO was 300 μL/L, which was half of the amount we obtained in this study. This difference can be attributed to the amount of carvacrol in SEO that we used in agreement with that used by Safari et al. Because they used 57.4% amount of carvacrol as the

Table 3 IC50, MIC and MFC values (in $\mu\text{L/L}$) of EOs against *Botrytis cinerea* and *Rhizopus stolonifer*

Compounds	<i>Botrytis cinerea</i>			<i>Rhizopus stolonifer</i>		
	IC50	MIC	MFC	IC50	MIC	MFC
ZEO	97.937c	150c	300b	204.253b	600a	600b
CEO	120.152b	300b	300b	128.357c	300b	600b
SEO	185.286a	600a	600a	238.596a	600a	1200a

Mean values followed by the same letters (i.e. a, b, c, etc.) are not significantly different according to Tukey test ($p < 0.05$)

main component which was higher than that in our study (i.e. 30.57%). Presumably, the amount of the compounds is involved as well.

The results of in vitro assays proved that the EOs in the same concentrations have less antifungal activity when tested as in vivo on fruits. There is no clear explanation for the cause of this incident in the literature, although in the host/antifungal/pathogen complex system, several factors can lead to divergent results with respect to that observed in the in vitro experiments. It is reported that this

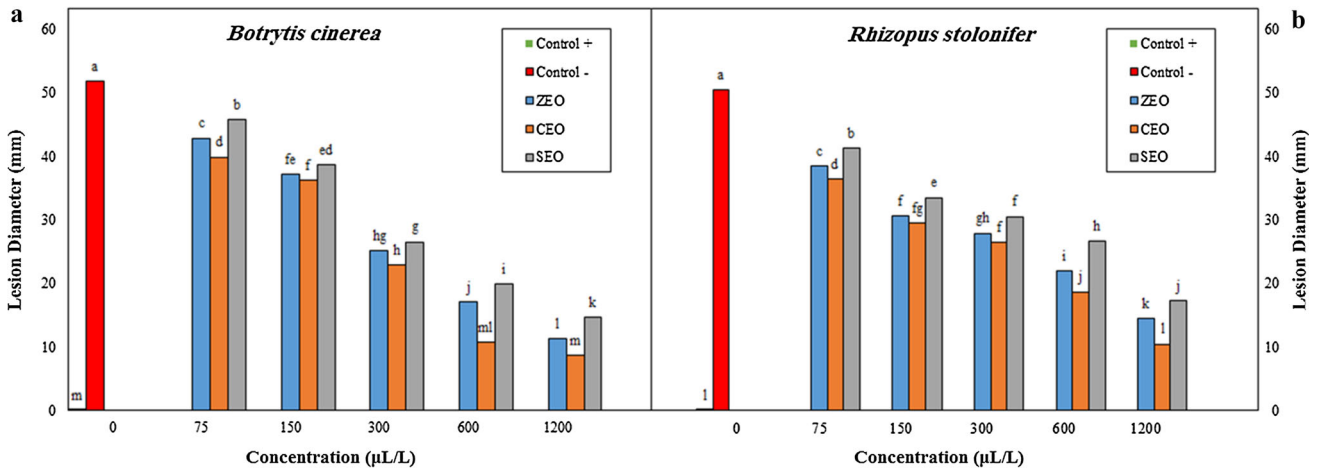
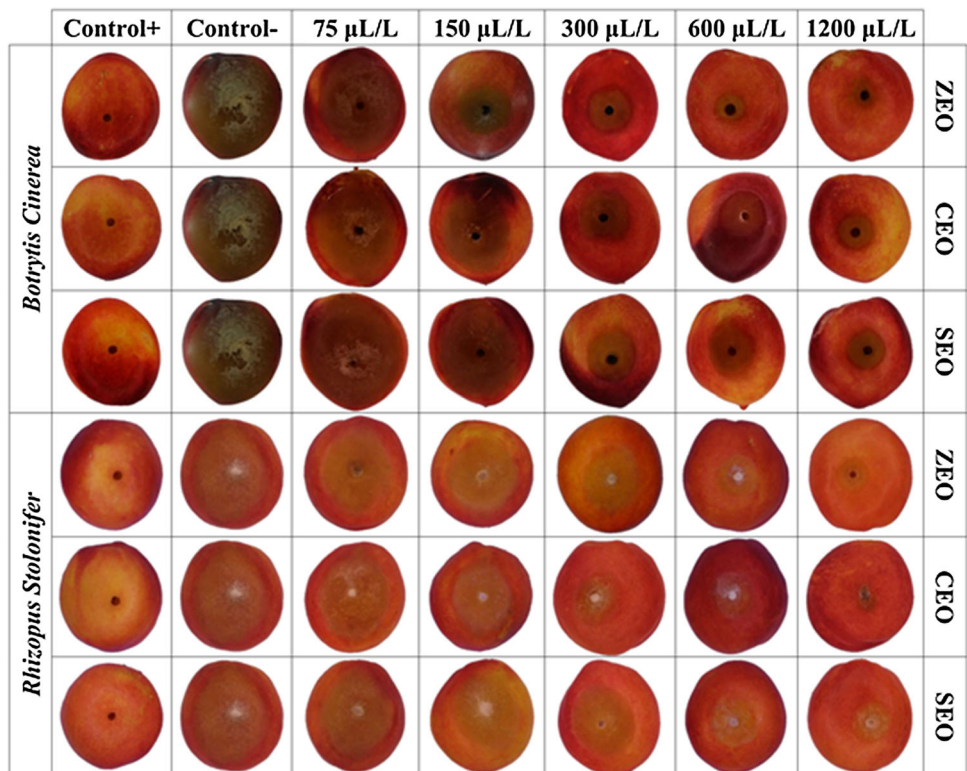


Fig. 2 Effect of EOs at different concentrations against *Botrytis cinerea* (a) and *Rhizopus stolonifer* (b) on Nectarine fruits (each data represents average value of 15 replications). [In each fungus, bars that

have the same letters (i.e. a, b, c, etc.) are not significantly different according to Tukey test ($p < 0.05$)]

Fig. 3 Effect of different EOs on the control of *Botrytis cinerea* and *Rhizopus stolonifer* in artificially inoculated infected Nectarines fruits after 4 days storage at 25 °C



contradiction may be due to the alteration of site action (Abdolahi et al. 2010) or structural changes such as hydrolysis, degradation, and polymerization, etc. (Gatto et al. 2011) of fruits under in vivo condition. Similar results have been reported in other studies (Mohammadi et al. 2015; Nikkhah et al. 2017).

Conclusion

To the best of our knowledge, this is the first report on the ZEO, CEO, and SEO, which were tested to control postharvest fungal rots on nectarines fruits caused by *Botrytis cinerea* and *Rhizopus stolonifer*. According to the obtained results, ZEO and CEO could be suitable to reduce the damage caused by these fungi. As well as, since these EOs were obtained from edible plants, we believe on their safety for humans and environment. However, in order to be used as organic alternative to chemical fungicides, deeper investigations about the absence of whatever form of toxicity are needed.

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