International Journal of Food Microbiology 257 (2017) 285–294

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Synergistic effects of some essential oils against fungal spoilage on pear fruit

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ARTICLE INFO

Keywords:
Essential oils
Fractional inhibitory concentration
Two and three dimensional checker board
Fungal growth inhibition kinetics
Pear fruit

ABSTRACT

The development of natural protective agents as alternatives to chemical fungicides is currently in the spotlight. In the present investigation, chemical composition and antifungal activities of thyme, cinnamon, rosemary and marjoram essential oils (EO), as well as synergism of their possible double and triple combinations were investigated. The compositions of the oils were determined by GC/MS. For determination of antifungal activity against *Penicillium expansum* and *Botrytis cinerea*, a broth microdilution method was used. The possible interactions of some essential oil combinations were performed by the two and three-dimensional checkerboard assay and isobologram construction. An *in vivo* antifungal assay was performed by artificial wounding of pear fruits. The maximum antifungal activity was demonstrated by thyme and cinnamon oils which displayed lower MIC values whereas rosemary and marjoram oils with MIC range between 2500 and 10,000 μg/mL exhibited weak antifungal activities against tested fungi. In synergy testing, some double combinations (thyme/cinnamon, thyme/rosemary, cinnamon/rosemary) were found to be synergistic (FICi ≤ 0.5). The triple combination of thyme, cinnamon and rosemary was synergistic for *B. cinerea* and *P. expansum* (FICi values of 0.5 and 0.375, respectively); while combination of cinnamon, marjoram and thyme exhibited additive and synergistic effect against *P. expansum* (FIC = 0.625) and *B. cinerea* (FIC = 0.375) respectively. The usage of a mathematical Gompertz model in relation to fungal kinetics, showed that the model could be used to predict growth curves (R^2 = 0.993 ± 0.05). For *B. cinerea*, Gompertz parameters for double and triple combination treatments showed significant increase in lag phase (1.92 and 2.92 days, respectively) compared to single treatments. Increase lag time up to 2.82 days ($P < 0.05$) also observed in *P. expansum* treated by triple combination of EOs. Base on the results, the lowest maximum growth rate (0.37 mm/day) was observed in *B. cinerea* treated by triple combination of thyme, cinnamon and rosemary. The *in vivo* test also demonstrated considerable inhibitory effects of EO combination treatments. Average lesion diameter of pears treated with triple combination of cinnamon/rosemary/thyme (78, 1250, 39 μg/mL) was 6 mm and 8 mm against *B. cinerea* and *P. expansum* respectively, in 10 days at 25 °C. Results also showed that double combination of thyme/cinnamon (78, 156 μg/mL) has more inhibitory effect than single EO treatments.

1. Introduction

Filamentous fungi are widely dispersed in nature and are a significant agent in deterioration and spoilage of food and agricultural crops. The majority of fresh fruits are susceptible to infection by some pathogenic fungi in postharvest period. Contamination by some filamentous fungi is the main cause of rapid spoilage of fresh fruits, which affects their quality and decreases the shelf life (Tejeswini et al., 2014). Pathogenic fungi, including *Botrytis cinerea* and *Penicillium expansum* are main infectious agents of apples, pears, and a number of other pectin-rich fruits (Miedes and Lorences, 2004; van Kan, 2006). *B. cinerea* is a particularly opportunistic and selective plant pathogen that contains cutinases and lipases that break down pectin in fruits (van Kan, 2006). Natural antifungal agents have been used since ancient times as an effective method for controlling food spoilage. Because of the health and environmental risks of chemical fungicides, biological or integrated approaches are becoming increasingly important for controlling crop losses (Lima et al., 2008). In the past decade, due to concerns regarding safety of the chemical control measures, particular attention has been given to the potential applications of essential oils (EOs) as alternative. They have a wide range of antifungal properties (Carmo et al., 2008; Koul et al., 2008; Mohammadi et al., 2015) and they are

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.06.021
Received 14 March 2017; Received in revised form 4 June 2017; Accepted 21 June 2017
Available online 24 June 2017

environmentally friendly (biodegradable and without toxic residues). The complex structure of the essential oils and the variety of chemical nature of their components are responsible of a broad range of biological interactions many of which are of increasing interest in the subject of food preservation. Considering that greater amount of EOs are required for preservation in food systems, their using can have a negative impact on sensory properties. To avoid this adverse side effect, various EOs can be mixed (Rentsenkhand et al., 2010). The possible synergistic effect produced by the combination of plant essential oils was referred as an efficient strategy to combat microbial development (Wagner, 2011). There are several studies on the synergistic effect of combination of EOs on food spoilage and pathogenic bacteria (De Rapper et al., 2013; Lv et al., 2011; Magi et al., 2015; Matan et al., 2006). While the antifungal effectiveness of the EOs have been reviewed entirely (Elshafie et al., 2015; Esper et al., 2014; Kedia et al., 2016; Kohiyama et al., 2015; Vitoratos et al., 2013), few studies on the synergistic effects of combination of EOs have been reported (Nguefack et al., 2012; Pooja et al., 2013; Sharma and Sharma, 2011). In particular, no comprehensive study has been done to evaluate antifungal properties of triple combination of EOs. The purpose of present study was to determine the antifungal effects of four essential oils (thyme, cinnamon, rosemary and marjoram) against *B. cinerea* and *P. expansum* by evaluating (i) fungal growth inhibitory and fungicidal properties of the EOs, (ii), the interaction and possible synergistic impacts of double and triple combinations of the EOs, (iii) determination of inhibition kinetics induced by EOs on fungal strains, and finally (iv) evaluation of antifungal properties of selected treatments on pear fruits.

2. Material and methods

2.1. Essential oils and GC/MS analysis

The essential oils of thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum zeylanicum*), rosemary (*Rosmarinus officinalis*) and marjoram (*Origanum majorana*) were used in this study. They were obtained from Cultivation and Industry Golkaran (Kashan, Iran), except for the essential oil of marjoram, which was derived from the Giah Essence Phytopharm Co (Gorgan, Iran).

The volatile constituents of essential oils were analyzed using an Agilent 6890 GC equipped with BPX5 capillary columns (30 m × 0.25 mm i.d. 0.25 μm film thicknesses) and a mass detector (Agilent 5973). Helium was selected as the carrier gas at a steady flow of 1 mL/min and an injection volume of 1 μL was used. The injector temperature was 220 °C, and detector temperature was 290 °C, while column temperature was linearly programmed from 60 to 220 °C (at rate of 2°/min) for polar column and from 60 to 240 °C (at rate of 3°/min) for non-polar column. The mass spectrometer was operated with a high ionization voltage (70 eV). Identification of components was based on a comparison of their relative retention time and mass spectra with Willey7n, NIST98 (National Institute of Standards) and Adams libraries spectra (Adams, 2007).

2.2. Inoculum preparation

The fungal strains used in this study were *Botrytis cinerea* ATCC 12481, purchased from Iranian Research Organization for Science and Technology (IROST) and *Penicillium expansum*, obtained from Agriculture Biotechnology Research Institute of Iran (ABRII). The fungal strains were cultured on Potato Dextrose Agar (PDA) and grown for 5 days (for *P. expansum*) and 10 days (for *B. cinerea*) at 28 ± 2 °C. The conidial suspension was prepared from fresh culture by adding 0.1% Tween 80 to the culture plates and scratching surface of the medium with the wire loop to release the conidia from fungal mycelium. The inoculum size was assessed using a Neubauer chamber cell counting (Heamocytometer) and adjusted to approximately 2×10^4 conidia/mL for each strain.

2.3. Determination of antifungal activity using broth microdilution method

Broth microdilution protocols based on the CLSI reference documents M38-A2 (Clinical and Laboratory Standards Institute, 2008) with some modifications, were used to determine minimum inhibitory concentration (MIC) for filamentous fungi. A stock solution of each essential oil in ethanol (40% w/v) was diluted in RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with L-glutamine, without sodium bicarbonate (Sigma Chemical Co.), buffered with 0.165 M MOPS [3-(*N*-morpholino) propane sulfonic acid] (Sigma-Aldrich) at pH 7, to acquire 2% w/v solution (20,000 μg/mL). Two-fold serial dilutions of the EOs (100 μL) were performed on a 96-wellplate containing RPMI-1640. After the adding 0.1 mL of conidial suspension, the microplates were incubated at 28 ± 2 °C for 7 days. Positive controls (a series of essential oils-free wells) and negative controls (a series of wells containing RPMI-1640 with ethanol) were considered for each treatment. The MIC of essential oil was defined as the minimum concentration required to completely inhibit visible growth after 7 days of incubation (Tullio et al., 2007). The determination of MFC (minimum fungicidal concentration) was performed by culturing 10 μL culture broth from wells with no visible turbidity on Sabouraud dextrose agar plates that were incubated for 3 days at 28 °C (Hammer et al., 2002). The MFC was defined as minimum concentration completely inhibiting the growth of the fungi. Each test was performed in duplicate.

2.4. Assessing synergistic interaction between essential oils

Interactions of EOs were determined using a checkerboard microdilution test. The evaluated concentrations were in the range of 5 dilutions below the MIC to twice the MIC. The final concentrations of the EOs were 19 to 2500 μg/mL for thyme oil, 39 to 5000 μg/mL for cinnamon oil, 156 to 20,000 μg/mL for rosemary oil and 312 to 40,000 μg/mL for marjoram oil. For the double combinations, a two-dimensional checkerboard with dual dilutions of each EO was used. A checkerboard with twofold dilutions of six treatments comprising thyme/rosemary, thyme/marjoram, cinnamon/rosemary, cinnamon/marjoram, rosemary/marjoram and thyme/cinnamon was set up for the dual combinations. The triple combinations were examined by a three-dimensional checkerboard method in the following manner. A checkerboard with double dilutions of cinnamon and either rosemary or marjoram was set up as mentioned above for the dual combinations. The third component of the combination (Thyme) was then distributed all over the wells at sub inhibitory concentrations ranging from 1/2 to 1/32 of the MIC (19–312 μg/mL). Growth control wells (medium with inocula but without essential oils) were included in each microplate (Bhusal et al., 2005; Turgis et al., 2012).

Each test was done in duplicate. For the first clear well in each row of the microtiter plate containing all EOs, the fractional inhibitory concentration (FIC) was calculated as follows: FIC of compound A (FIC_A) = MIC (A) in combination/MIC (A) alone, FIC of compound B (FIC_B) = MIC (B) in combination/MIC (B) alone, FIC of compound C (FIC_C) = MIC (C) in combination/MIC of (C) alone. Where A, B and C were the three respective tested EOs. FIC Index (FICI), calculated as the sum of each FIC (FICA + FICB for double combinations and FICA + FICB + FICC for triple combinations). The obtained results were interpreted as follows: synergistic effect (FICI ≤ 0.5); additive effect (0.5 < FICI ≤ 1); no interactive effect (1 < FICI ≤ 4); antagonistic effect (FICI > 4) (Gutierrez and Bourke, 2008; Krisch et al., 2011). The test results were graphically represented as isobolograms using Minitab 17 software (Minitab, Inc., State College, PA, USA). The isobolograms were used to define the type of interaction between EO combinations. Isobolograms were performed by mixing 2 or 3 EOs to determine what antifungal interactions could be observed if different concentrations of essential oils (different proportions at MICs) were combined. The isobologram curves can be constructed by plotting the data points of the different ratios where the MIC for each concentration

is determined in relation to the independent MICs. A concave curve indicates synergy, whereas convex curve and straight line represents antagonism and additive effect respectively (Van Vuuren et al., 2009).

2.5. Agar diffusion assay

To evaluate the inhibitory effect of essential oils on the kinetics of fungal growth, agar diffusion assays were done according to the method explained by Inouye et al. (2006). One milliliter of each fungal conidial suspension (1×10^8 conidia/mL) was added to 100 mL of agar medium formulated with 1% glucose, 1% peptone and 1% agarose at a temperature of about 50 °C. A quantity of 3 mL of the prepared media was poured into the surface of solid PDA medium (20 mL) in a plate (8 cm) to provide a double layered agar medium. Sterile blank disk (10 mm diameter) was transferred to the center of the Petri dish. Finally, 10 μL of each EO was added to blank disk. Because double combination of thyme/cinnamon and triple combination of cinnamon/rosemary/thyme demonstrated a synergistic effect (FICI ≤ 0.5) against both fungal strains, their antifungal activity was assessed by adding 10 μL of them on paper disk. The plates were then incubated at 28 ± 2 °C for 10 days. The diameter of inhibition zone (mm) was measured using calipers. The assessment of fungal growth (colony diameter) was done by subtracting zone of inhibition from the inner diameter of the plate (Inouye et al., 2006).

2.6. Modeling of fungal growth and statistical analysis

Since nonlinear behavior of growth rate was found in the majority of the studied cases, data from fungal growth on pure culture media were modeled using the modified Gompertz equation as reported by Avila-Sosa et al. (2012) and Char et al. (2007):

$$\ln \frac{D_t}{D_0} = A \cdot \exp\left\{-\exp\left[\frac{\upsilon_m \cdot e}{A}(\lambda - t) + 1\right]\right\}$$

where: D_t (cm) is the average colony diameter at time t (day), and D_0 (cm) is the average colony diameter at initial time; A is the maximum growth achieved during the stationary phase, υ_m is the maximum specific growth rate (1/day), λ is the lag phase (day) and e = exp (1).

Statistical analysis was done with ANOVA and Duncan test at α = 0.05 (SPSS 17.0, Chicago, IL, USA). Data were determined by triplicate independent experiments.

2.7. In vivo antifungal assay

Pear (*Pyrus communis* L. cv. Natanz) fruits were obtained from Tehran central fruit market and sorted for uniformity in size, form and the absence of physical defects. The fruits were immersed in the solution of 2% sodium hypochlorite for 2 min, rinsed under sterile tap water, and dried at room temperature. The skin of each pear was wounded (approximately 4 mm in depth) by the sterile cork border close to fruits' equatorial region, 10 μL of the selected essential oils (single, double and triple treatments) were dropped into each wound prior to inoculation with fungal spores. After 1 h from the treatments, the pathogen suspensions (10 μL, 5×10^5 conidia/mL) were applied on wounded fruits. Following inoculation, the treated pears were stored in polyethylene-lined plastic boxes under moist conditions at room temperature and lesion diameter of pears was measured after 5 and 10 days. The fruits inoculated with the pathogens were considered as controls. Each treatment was replicated three times, and the experiment repeated twice (Zhang et al., 2014).

Table 1
Main constituents (%) of the essential oils of thyme, cinnamon, rosemary and marjoram as identified by GC/MS analysis.

Compounds	*Thymus vulgaris* thyme	*Cinnamon zeylanicum* cinnamon	*Rosmarinus officinalis* rosemary	*Origanum majorana* marjoram
α–Pinene	3.34	–	28.04	–
Para-cymen	7.88	–	–	2.45
γ-Terpinene	6.57	–	–	16.76
Thymol	39.14	–	–	–
Carvacrol	26.61	–	–	–
Caryophyllene	2.11	–	–	2.38
Cinnamaldehyde	–	44.25	–	–
Cinnamaldehyde propylene glycol acetal	–	25.07	–	–
Phensuximide	–	17.37	–	–
Camphene	–	–	7.01	–
Myrcene	–	–	4.20	–
Limonene	–	–	4.10	–
1.8-Cineole	–	–	13.27	–
Linalool	–	–	2.72	2.42
Camphor	–	–	8.06	–
Borneol	–	–	7.18	–
α–Terpineol	–	–	2.06	4.44
Verbenone	–	–	6.76	–
Isobornyl acetate	–	–	2.87	–
Sabinene	–	–	–	3.89
α-Terpinene	–	–	–	10.08
Terpinolene	–	–	–	3.76
cis-Sabinene hydrate	–	–	–	2.31
trans-Sabinene hydrate	–	–	–	3.37
Terpinene-4-ol	–	–	–	33.84

Table 2
Antifungal activity[a] of tested essential oils against *B. cinerea* and *P. expansum*.

Fungal strain	Essential oils							
	Thyme		Cinnamon		Rosemary		Marjoram	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
B. cinerea	312	625	625	1250	2500	2500	5000	5000
P. expansum	625	625	1250	1250	5000	5000	10,000	10,000

MIC: minimum inhibitory concentration; MFC: minimum fungicide concentration.
[a] Results expressed as μg/mL.

3. Results and discussion

3.1. Chemical compounds of the essential oils

The results of chemical analysis of the essential oils are shown in Table 1. The main components of thyme essential oil were thymol (39.14%), carvacrol (26.61%) and para-cymene (7.88%). Cinnamon oil was comprised mainly of cinnamaldehyde (44.25%), cinnamaldehyde propylene glycol acetal (25.07%) and phensuximide (17.37%) while rosemary constituted mainly α-pinene (28.04%), 1, 8-cineole (13.27%) and camphor (8.06%). In marjoram essential oil, the main components were terpinene-4-ol (33.84%), γ-terpinene (13.27%) and α-terpinene (10.08%).

3.2. Determination of MIC and MFC of essential oils

The inhibition rate of the thyme, cinnamon, rosemary and marjoram essential oils against *B. cinerea* and *P. expansum* studied using broth microdilution method is shown in Table 2. Thyme and cinnamon showed MIC activity at lower concentrations (312 μg/mL, 625 μg/mL for *B. cinerea*, and 625 μg/mL, 1250 μg/mL for *P. expansum*;

Table 3
FIC[a] and interaction effects of double combinations of essential oils on *Botrytis cinerea*.

Combination of two essential oils (A/B)	MIC A (alone)	MIC B (alone)	MIC A (in the presence of B)	MIC B (in the presence of A)	Checkerboard FIC Index
Thyme/rosemary	312	2500	78	625	0.5
Thyme/marjoram	312	5000	156	2500	1
Cinnamon/rosemary	625	2500	625	625	1.25
Cinnamon/marjoram	625	5000	312	2500	1
Thyme/cinnamon	312	625	78	156	0.5
Rosemary/marjoram	2500	5000	2500	1250	1.25

*FICI ≤ 0.5: synergic effect; 0.5 < FICI ≤ 1: additive effect; 1 < FICI ≤ 4: no interactive effect; FICI > 4: antagonistic effect. *Results expressed as μg/mL.
[a] Abbreviations: FIC, fractional inhibitory concentration; and MIC, minimum inhibitory concentrations.

Table 4
FIC[a] and interaction effect of double combinations of essential oils on *P. expansum*.

Combination of two essential oils (A/B)	MIC A (alone)	MIC B (alone)	MIC A (in the presence of B)	MIC B (in the presence of A)	Checkerboard FIC Index
Thyme/rosemary	625	5000	625	5000	2
Thyme/marjoram	625	10,000	312	5000	1
Cinnamon/rosemary	1250	5000	625	1250	0.75
Cinnamon/marjoram	1250	10,000	312	2500	0.5
Thyme/cinnamon	625	1250	78	156	0.25
Rosemary/marjoram	5000	10,000	5000	10,000	2

*FICI ≤ 0.5: synergic effect; 0.5 < FICI ≤ 1: additive effect; 1 < FICI ≤ 4: no interactive effect; FICI > 4: antagonistic effect.
*Results expressed as μg/mL.
[a] Abbreviations: FIC, fractional inhibitory concentration; and MIC, minimum inhibitory concentrations.

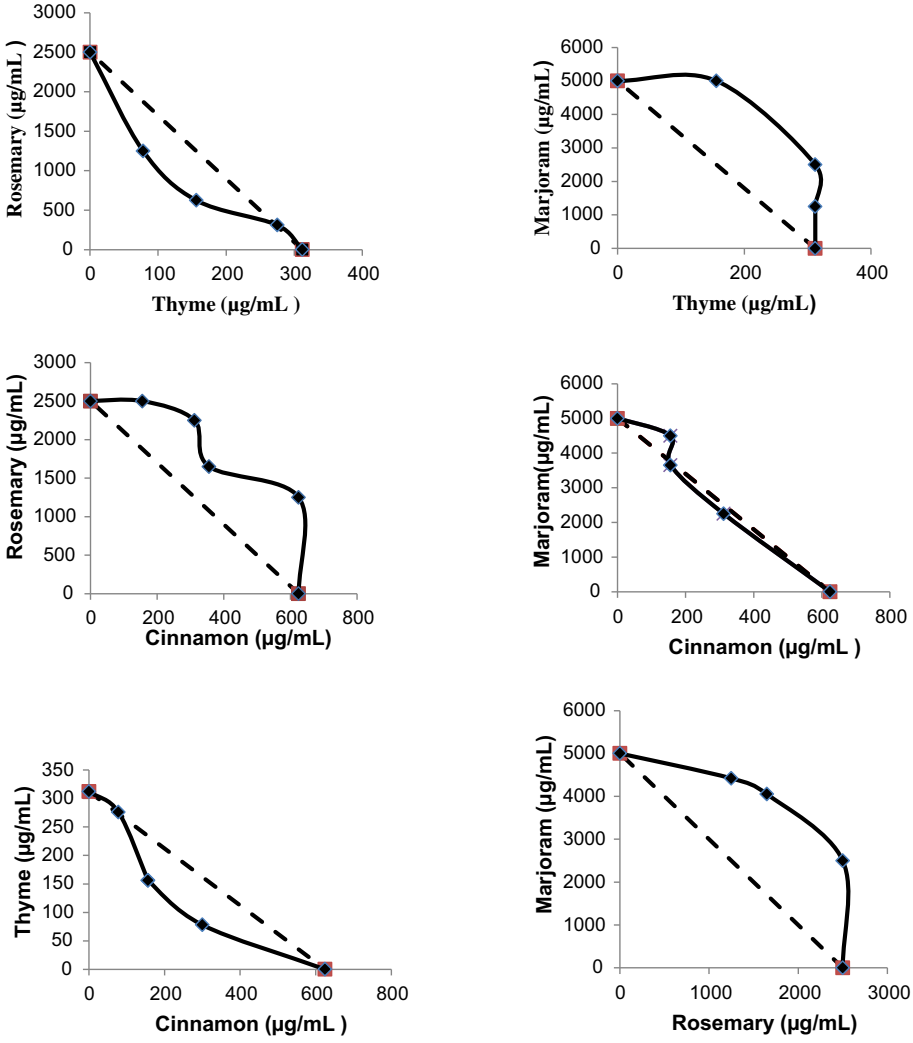

Fig. 1. The isobologram curve of double combinations of essential oils against *B. cinerea*, the dotted line indicates the theoretical additive line (line of additive effect).

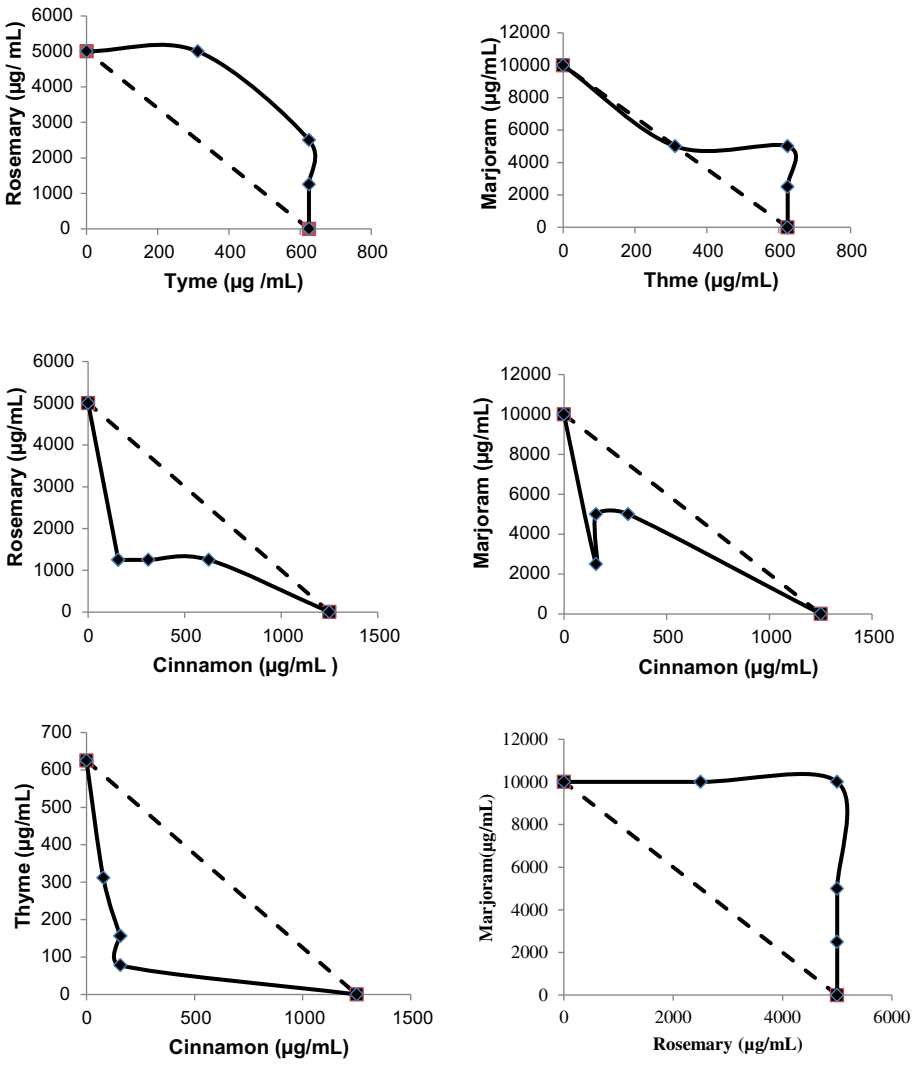

Fig. 2. The isobologram curve of double combinations of essential oils against *P. expansum*, the dotted line indicates the theoretical additive line (line of additive effect).

Table 5
FIC[a] and interaction effect of triple combinations of essential oils on *B. cinerea*.

Combination of tree essential oils (A/B/C)	MIC A (alone)	MIC B (alone)	MIC C (alone)	MIC A (in the presence of B & C)	MIC B (in the presence of A & C)	MIC C (in the presence of A & B)	Checkerboard FIC Index
Cinnamon/rosemary/thyme	625	2500	312	78	625	39	0.5
Cinnamon/marjoram/thyme	625	5000	312	78	625	39	0.375

*FICI ≤ 0.5: synergic effect; 0.5 < FICI ≤ 1: additive effect; 1 < FICI ≤ 4: no interactive effect; FICI > 4: antagonistic effect.
*Results expressed as µg/mL.
[a] Abbreviations: FIC, fractional inhibitory concentration; and MIC, minimum inhibitory concentrations.

Table 6
FIC[a] and interaction effect of triple combinations of essential oils on *P. expansum*.

Combination of tree essential oils (A/B/C)	MIC A (alone)	MIC B (alone)	MIC C (alone)	MIC A (in the presence of B & C)	MIC B (in the presence of A & C)	MIC C (in the presence of A & B)	Checkerboard FIC Index
Cinnamon/rosemary/thyme	1250	5000	625	78	1250	39	0.375
Cinnamon/marjoram/thyme	1250	10,000	625	78	5000	39	0.625

*FICI ≤ 0.5: synergic effect; 0.5 < FICI ≤ 1: additive effect; 1 < FICI ≤ 4: no interactive effect; FICI > 4: antagonistic effect.
*Results expressed as µg/mL.
[a] Abbreviations: FIC, fractional inhibitory concentration; and MIC, minimum inhibitory concentrations.

respectively); whereas for rosemary and marjoram, the MIC was determined at higher concentrations. The MFC was as follows: thyme (625 µg/mL), cinnamon (1250 µg/mL), rosemary (2500 µg/mL for *B. cinerea* and 5000 µg/mL for *P. expansum*) and marjoram (5000 µg/mL for *B. cinerea* and 10,000 µg/mL for *P. expansum*). Based on these results the inhibitory effect of essential oils in defined concentrations was more efficient on *Botrytis cinerea* than on *Penicillium expansum*. Some previous studies evaluated the inhibitory activity of essential oils, especially

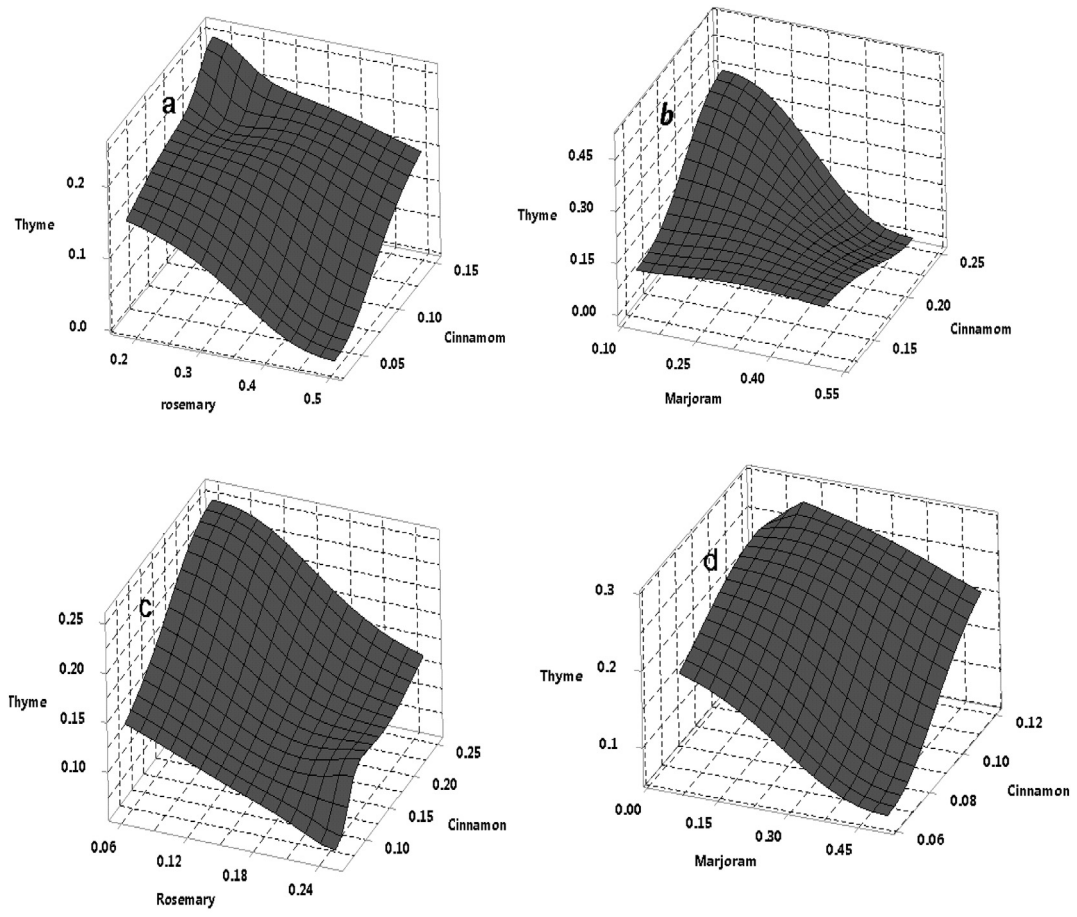

Fig. 3. Three-dimensional isobologram of triple combination of cinnamon/rosemary/thyme and cinnamon/marjoram/thyme against *B. cinerea* (a, b) and *P. expansum* (c, d).

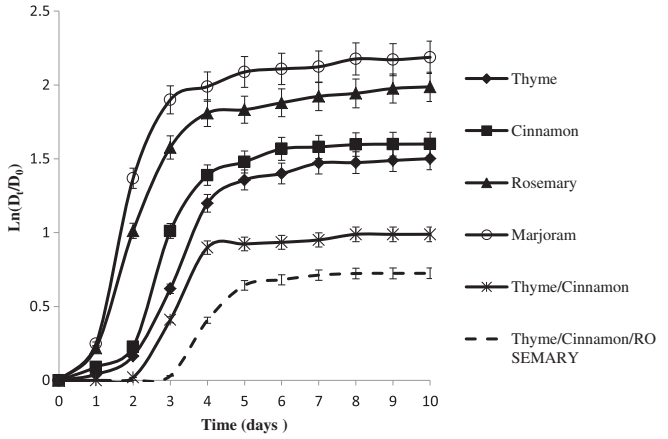

Fig. 4. Effect of different EOs on the colony diameter, Ln (D_t/D_0), of *B. cinerea*; means ± SD.

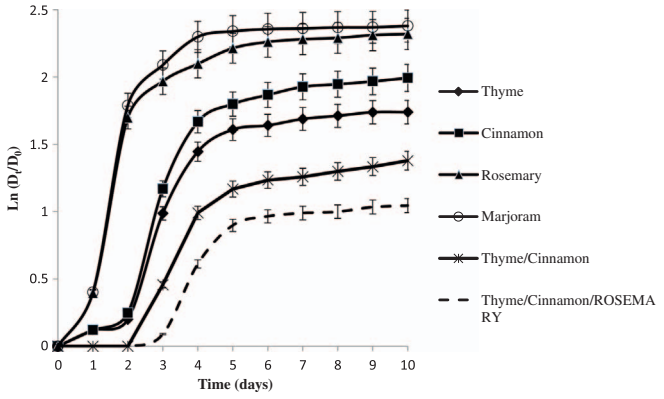

Fig. 5. Effect of different EOs on the colony diameter, Ln (D_t/D_0), of *P. expansum*; means ± SD.

thyme and cinnamon against fungi (Daferera et al., 2003; López et al., 2005; Razzaghi-Abyaneh et al., 2006; Viuda-Martos et al., 2007; Xing et al., 2010; Mohammadi et al., 2016). It has been emphasized that EOs containing phenols or aldehydes specially thymol, carvacrol and cinnamaldehyde, as major components displayed the highest antimicrobial activity, followed by EOs containing terpene alcohols. A number of EOs containing esters or ketones as main components had weaker activity, whereas other essential oils containing terpene hydrocarbons were generally ineffective (Ait-Ouazzou et al., 2011; de Barros et al., 2009; Nostro et al., 2002; Sacchetti et al., 2005; Tajkarimi et al., 2010). In this study, as expected, the monoterpene phenols in thyme (thymol and carvacrol), and aldehyde derivatives (cinnamaldehyde and cinnamaldehyde propylene glycol acetal) were found to be the most active constituents, although other main components in rosemary and marjoram include α–pinene, 1,8-cineole, terpinene-4-ol and γ-terpinene were generally found to have moderate or weak antifungal activity.

3.3. FIC of double combinations using two-dimensional checkerboard method

The FICs of the dual combinations of essential oils examined in this study are presented in Tables 3 and 4. The more effective combinations including thyme/rosemary (FICI = 0.5) and thyme/cinnamon

Table 7
Modified Gompertz model parameters (means ± standard error) for *B. cinerea* and *P. expansum* subjected to four EOs and two selected mixtures of EOs by direct contact assay.

EOs	*B. cinerea*				*P. expansum*			
	A (cm)	υ_m $(day)^{1-}$	λ (day)	R^2	A (cm)	υ_m $(day)^{1-}$	λ (day)	R^2
Thyme	1.51 ± 0.1^c	0.51 ± 0.1^c	1.71 ± 0.0^c	0.996	1.74 ± 0.0^c	0.79 ± 0.1^b	1.73 ± 0.0^c	0.994
Cinnamon	1.62 ± 0.0^d	0.78 ± 0.0^d	1.70 ± 0.2^c	0.996	1.96 ± 0.1^d	0.91 ± 0.0^c	1.70 ± 0.1^b	0.994
Rosemary	1.98 ± 0.0^e	0.78 ± 0.0^d	0.72 ± 0.1^a	0.996	2.32 ± 0.1^e	1.30 ± 0.0^d	0.70 ± 0.1^a	0.983
Marjoram	2.16 ± 0.0^f	1.13 ± 0.0^e	0.79 ± 0.0^b	0.994	2.37 ± 0.1^f	1.38 ± 0.0^e	0.71 ± 0.1^a	0.993
Thyme/cinnamon	1.08 ± 0.1^b	0.42 ± 0.1^b	1.92 ± 0.1^d	0.997	1.37 ± 0.0^b	0.53 ± 0.0^a	1.87 ± 0.0^d	0.978
Thyme/cinnamon/marjoram	0.73 ± 0.1^a	0.37 ± 0.1^a	2.92 ± 0.1^e	0.999	1.03 ± 0.1^a	0.52 ± 0.0^a	2.82 ± 0.0^e	0.999

A: maximum colony diameter during stationary phase; υ_m: maximum growth rate; λ: lag time; R^2: coefficient of determination.
Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different ($P > 0.05$).
*The concentration of single EO and total concentration of combination of EOs was 20,000 μg/mL.
**The concentration ratio of thyme/cinnamon and thyme/cinnamon/marjoram was 1:2 and 1:2:16 respectively.

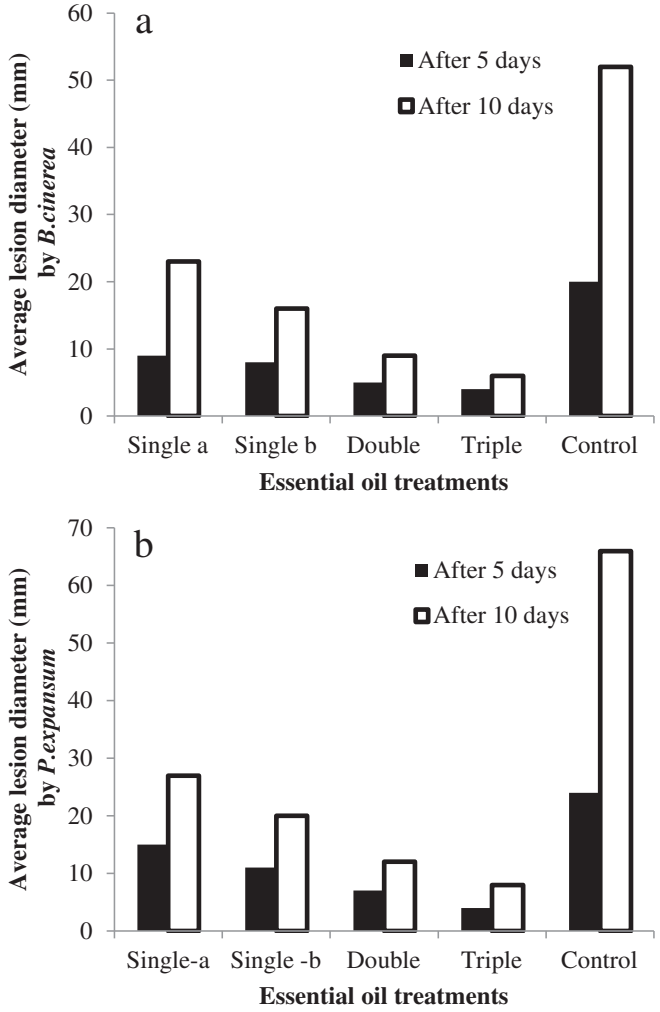

Fig. 6. Inhibitory activity of essential oil treatments expressed as average lesion diameter (in millimeters) caused by *B. cinerea* (a) and *P. expansum* (b) on pear fruits after 5 and 10 days at 25 °C; means ± SD. EO treatments including single-a: cinnamon (625 μg/mL), single-b: thyme (1250 μg/mL), double: thyme/cinnamon (78, 156 μg/mL) and triple: cinnamon/rosemary/thyme (78, 1250, 39 μg/mL).

(FICI = 0.5) displayed a synergistic effect against *B. cinerea*, whereas combinations of thyme/marjoram and cinnamon/marjoram exhibited additive effects. Combination of cinnamon with thyme and rosemary (FICI = 0.25 and 0.5 respectively) resulted in synergistic effects against *P. expansum*. Marjoram mixed with cinnamon and thyme showed additive effects against *P. expansum*. In the case of synergistic inhibitory effects of EO combinations, most studies involved pathogenic and food-borne bacteria (Bassolé and Juliani, 2012; Gutierrez and Bourke, 2008; Karatzas et al., 2001; Santiesteban-López et al., 2007). There are a few studies that have been conducted to assess the antifungal activities of EO combinations (Cruz-Vega et al., 2009; Dilokkunanant et al., 2008; Nguefack et al., 2012). Stević et al. (2014) described a synergism between carvacrol and thymol in oregano and thyme EOs on *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata*, and *Fusarium* species (Stević et al., 2014). Similar synergistic activity, mainly between thymol and carvacrol has been reported against the selected fungal strains (*A. niger*, *A. flavus*, *A. parasiticus* and *Penicillium chrysogenum*) where a combined treatment caused a more significant decrease of the fungal growth than when used alone (Hossain et al., 2016). In our study, similar results were obtained against *P. expansum* and *B. cinerea*. A number of studies have explained that whole EOs generally have greater antimicrobial activity than the blend of their major constituents, indicating that the minor components are important to the synergistic properties of EOs, although additive and antagonistic activity have also been reported (Bassolé and Juliani, 2012; Mourey and Canillac, 2002).

A majority of the antimicrobial properties of EOs is due to the presence of oxygenated terpenoids, especially phenolic terpenes, phenyl propanoids and alcohols. Other components such as hydrocarbons that in general show low antimicrobial activities can be applied in combinations to enhance their effectiveness (Bassolé and Juliani, 2012; Hossain et al., 2016). It is noteworthy to observe that most of these distinct synergisms are between compounds, which exhibited strong and weak antimicrobial activity when tested alone. Based on obtained results, it is obviously that of all the synergistic interactions, 60% occur between the compounds with weak and strong antimicrobial properties when tested alone. Only thyme and cinnamon, both in a single mode and dual mode showed significant antifungal effect on *B. cinerea* and *P. expansum*. Among other double combinations, the most synergistic effects were seen between the weakly active components of rosemary and the strongly active thymol and carvacrol against *B. cinerea*. Also a noticeable synergism was seen between rosemary/cinnamon and marjoram/cinnamon against *P. expansum*. Although the major components of thyme and cinnamon (especially thymol, carvacrol and cinnamaldehyde) are very important for their antifungal activity, other less-active compounds in rosemary and marjoram (such as α–pinene, 1,8-cineole, terpinene-4-ol and γ-terpinene) play a considerable role, as they can enhance the effects of major constituents, though synergistic activities have also been observed. The synergistic effect was demonstrated graphically by applying the isobologram method (Figs. 1 and 2).

3.4. Evaluation of synergy by three-dimensional checkerboard assay

Most of the studies reporting the evaluation of combination treatments have focused on two antifungal combinations, and triple combinations synergistic effects have been studied mainly in the medical

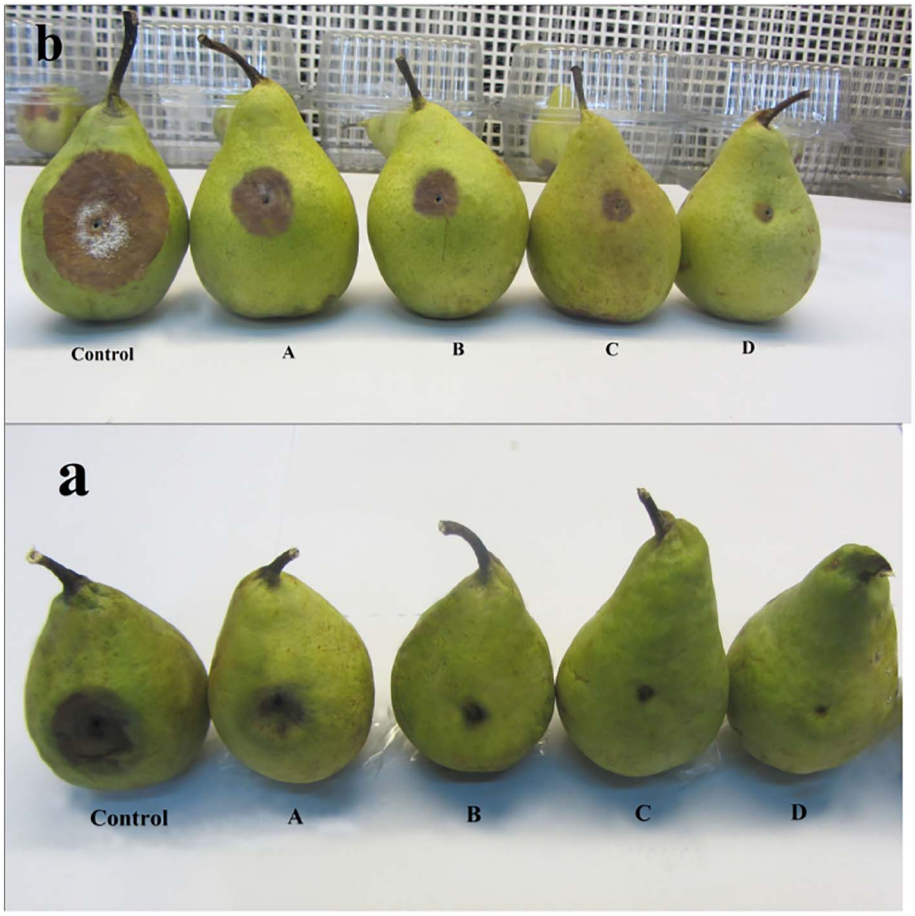

Fig. 7. Effect of EO treatments on the control of decay caused by *B. cinerea* (a) and *P. expansum* (b) in artificially wounded pear fruits after at 25 °C. A: cinnamon (625 μg/mL), B: thyme (1250 μg/mL), C: thyme/cinnamon (78, 156 μg/mL) and D: cinnamon/rosemary/thyme (78, 1250, 39 μg/mL).

field such as in combination antimicrobial therapy (Dannaoui et al., 2004; Diamond et al., 1998; Mukherjee et al., 2005). A few studies on antimicrobial properties of essential oils combined with antibiotics have been reported (El-Ahmady et al., 2013; Ilić et al., 2014; Turgis et al., 2012; Van Vuuren et al., 2009), however, the antifungal effects of triple combination of essential oils have so far not been studied. This study aimed to investigate whether combinations of two or more EOs were synergistic against *B. cinerea* and *P. expansum*. As shown in Tables 5 and 6, the two triple combinations showed synergistic or additive effects in the checkerboard assays. Triple combinations of cinnamon, rosemary and thyme had a synergistic effect on *B. cinerea* and *P. expansum* (FIC ≤ 0.5), whereas combination of cinnamon, marjoram and thyme exhibited additive and synergistic effect against *P. expansum* (FIC = 0.625) and *B. cinerea* (FIC = 0.375) respectively. The interpretation of FICs index for triple combinations has been well defined and a value of ≤ 0.5, exhibiting a six-to eight-fold reduction in MICs, is considered to be more synergistic compared to single and double-agent combination. These results are also shown as three-dimensional isobolograms for essential oil combinations (Fig. 3).

3.5. Gompertz model of antifungal activity and growth kinetics

The change in colony size of the two fungal strains under various EO treatments (individually and in combination) is presented in Figs. 4 and 5. Based on initial results, four essential oils and most effective double and triple combinations of EOs were used. The modified Gompertz model proposed by some authors (Avila-Sosa et al., 2012; Char et al., 2007; Hossain et al., 2016) was applied to evaluate the fungal growth rate in presence of the EOs, and the predictions of the model variables were compared with the experimental growth data. The Gompertz model can be used to describe mold growth kinetics, despite the fact that this model was originally suggested for bacterial growth (Char et al., 2007). The determined growth parameters were helpful to evaluate antifungal activities of the tested EOs. Velázquez-Nuñez et al. (2013) evaluated the antifungal activity of orange peel essential oils, performed either by direct contact or vapor exposure against *Aspergillus flavus* and the lag phase and radial growth rate were computed by the modified Gompertz equation. Significant differences in modified Gompertz model parameters were observed in both the methods. Model results explaining the maximum specific growth rate, lag time and maximum mold growth in the stationary phase, are shown in Table 7. The modified Gompertz model satisfactorily fitted the observed data (average coefficient of determination 0.993 ± 0.05). Thyme and cinnamon had the highest antifungal effect on the two fungal strains by reducing their maximum growth rates, whereas rosemary and marjoram appeared to be the least active EOs in limiting growth rate of the tested fungi. As shown in Figs. 4 and 5, double combination of thyme and cinnamon and triple combination of thyme, cinnamon and rosemary were very effective against *B. cinerea* and *P. expansum*; these combinations significantly limited the colony growth and extended the lag times. In the case of *B. cinerea*, Gompertz parameters (Table 7) for double and triple combination treatments showed significant increase in lag phase (1.92 and 2.92 days, respectively) compared to single treatments. There was also an increased lag time of up to 2.82 days ($P < 0.05$) in *P. expansum* treated by triple EOs combination. Bases on the results, the lowest maximum growth rate (0.37/day) was observed in *B. cinerea* treated by triple combination of thyme, cinnamon and rosemary. These findings provide reliable evidence that EO combinations applied their synergistic antifungal effects by changing the growth kinetics of fungal strains. Portillo-Ruiz et al. (2012) assessed the antifungal properties of Mexican oregano essential oil constituents against *Penicillium*, *Aspergillus* and *Rhizopus* sp., and the growth curves were

fitted by the modified Gompertz model. They reported a noticeable increase in the lag time and linear decrease in specific growth rate as the concentration of the tested essential oils increased. The current study extends previous literature by evaluating the growth kinetics of other fungal strains for a greater range of EOs as single or double and triple combinations.

3.6. Antifungal effect of EOs treatments on pear fruits

According to the *in vitro* results, four EO treatments including thyme (625 μg/mL), cinnamon (1250 μg/mL), thyme/cinnamon (78, 156 μg/mL) and cinnamon/rosemary/thyme (78, 1250, 39 μg/mL) were selected for antifungal *in vivo* study. As shown in Fig. 6, EO combinations, especially the triple treatment, effectively reduced the lesion diameter caused by *B. cinerea* and *P. expansum*. Cinnamon/rosemary/thyme treatment demonstrated the strongest inhibitory activity, with average lesion diameter (ald) 6 and 8 mm against *B. cinerea* and *P. expansum* respectively, in 10 days at 25 °C. There was no visible growth of two fungal strains in pears treated with triple EO combination after 5 days. Results also showed that double combination of EOs (thyme/cinnamon) with ald 9 mm for *B. cinerea* and 12 mm for *P. expansum* was more inhibitory than single EO treatments. Between the single EO treatments, thyme showed greater activity than cinnamon against fungal species. As can be seen in Fig. 7, all treatments exhibited inhibitory effects compared to control treatment, and as observed with *in vitro* experiments, antifungal activity of EOs (single, double and triple) against *B. cinerea* was greater than for *P. expansum*.

4. Conclusions

In vitro antifungal synergistic effects of four essential oils alone and in combination, against *B. cinerea* and *P. expansum* were studied. Based on MIC values, thyme and cinnamon exhibited the highest antifungal activity. Checkerboard assays for the double and triple combinations of essential oils showed significant synergy in some EO combinations. A noticeable synergistic interaction in double combinations was observed with thyme/cinnamon against both mold species. The FICi analysis indicated that triple combination of cinnamon/marjoram/thyme exhibited the most synergistic antifungal effect on *B. cinerea* and *P. expansum*. Assessing the effectiveness of the EOs against fungal spoilage of crops by the modified Gompertz model resulted in extensive and informative view on their growth kinetics. Essential oils especially in combination were highly effective in limiting growth rate while prolonging the lag phase of the fungi. Most of the studies to date have been done evaluating the effect of EOs on the growth of fungi in the laboratory under controlled conditions. The difficulty may be to apply the oils effectively under *in vivo* conditions. In our study the synergistic inhibitory activity of EO combinations was demonstrated by performing *in vivo* test on pear fruits. It is important to develop new ways of preserving fresh commodities, that are efficient, safe and cost-effective, as well as easy to obtain. This work highlights the potential for using essential oils for postharvest disease control of fresh fruit and vegetables. This field of synergistic studies is encouraged on a broader scope involving other post-harvest spoilage organisms and food systems.

Acknowledgments

The financial support of Agricultural Biotechnology Research Institute of Iran (ABRII, Karaj, Iran) is gratefully acknowledged.

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