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Antifungal and antimycotoxigenic activity of essential oils from *Eucalyptus globulus*, *Thymus capitatus* and *Schinus molle*

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Abstract

Essential oils (EO) of eucalyptus (*Eucalyptus globulus* L.), thymus (*Thymus capitatus* L.) pirul (*Schinus molle* L.) were evaluated for their efficacy to control *Aspergillus parasiticus* and *Fusarium moniliforme* growth and their ability to produce mycotoxins. Data from kinetics radial growth was used to obtain the half maximal inhibitory concentration (IC_{50}). The IC_{50} was used to evaluate spore germination kinetic and mycotoxin production. Also, spore viability was evaluated by the MTT assay. All EO had an effect on the radial growth of both species. After 96 h of incubation, thymus EO at concentrations of 1000 and 2500 μL L^{-1} totally inhibited the growth of *F. moniliforme* and *A. parasiticus*, respectively. Eucalyptus and thymus EO significantly reduced spore germination of *A. parasiticus*. Inhibition of spore germination of *F. moniliforme* was 84.6, 34.0, and 30.6% when exposed to eucalyptus, pirul, and thymus EO, respectively. Thymus and eucalyptus EO reduced aflatoxin (4%) and fumonisin (31%) production, respectively. Spore viability was affected when oils concentration increased, being the thymus EO the one that reduced proliferation of both fungi. Our findings suggest that EO affect *F. moniliforme* and *A. parasiticus* development and mycotoxin production.

Keywords: antifungal properties; essential oils; mycotoxin production; *Aspergillus parasiticus*; *Fusarium moniliforme*.

Practical Application: Possible application of natural products as antifungal in maize.

1 Introduction

The presence and growth of fungi may cause spoilage, which results in a reduction of quality and quantity of foods. Fungi are commonly encountered in grains in the field at harvest as well as during storage, making them inappropriate for human consumption since their nutritive value is diminished and sometimes due to mycotoxins contamination (Mishra & Dubey, 1994).

Aspergillus sp. and *Fusarium* sp. are the most common fungal species that are able to produce mycotoxins such as aflatoxins and fumonisins in food and feedstuffs (Montes-Belmont & Carvajal, 1998). Synthetic fungicides are currently used as the primary means for the control of phytopathogenic fungi, leading to both health and environmental problems, as well as to the development of microorganisms resistant to synthetic fungicide. Because of the increase of consumer demand for both safer and natural preservatives, natural fungicides have been intensively investigated (El-Ghaouth, 1997). Natural plant extracts may provide an alternative to chemical preservatives. Essential oils (EO) are mixtures of different volatile aromatic compounds obtained from plant material and have been used to control fungi due to the increase on the levels of pesticide residues in foods. EO are also less likely to be associated with the development of resistance by fungi, as observed with synthetic fungicides, and are less hazardous to the environment and human health (Daferera et al., 2003). Therefore, the present study was undertaken to investigate the antifungal activity of two commercial EO and fresh oil extracted from pirul against the growth and mycotoxin production of *F. moniliforme* and *A. parasiticus*.

2 Materials and methods

2.1 Plant material and essential oil extraction

Pirul (*Schinus molle*) leaves (5 kg) were collected near Ciudad Obregón, Sonora (México) (27° 34' 39 N and 109° 56' 19 W) in August 2013, and dried at room temperature for one week. One hundred grams of leaves were placed into a hydrodistillation system (a circulatory Clevenger-type apparatus) for 4 h. Pure EO oils from eucalyptus (*Eucalyptus globulus* Labillardiere) and thymus (*Thymus capitatus)* were purchased from Sigma Aldrich®.

2.2 Fungal strains

A. parasiticus (ATCC 16992) strain was activated in potato dextrose agar (PDA) and incubated at darkness at 27 ± 2 °C for 7 days. In addition, a *F. moniliforme* (ATCC 52539) strain was activated at 25 ± 2 °C for 10 days. The spores were harvested by pouring a sterile solution of 0.1% (v/v) Tween 20 into the flask and stirred with a magnetic bar for 5 min. The spore concentration of the resulting suspension was determined using a Neubauer chamber and adjusted at a final concentration of 1×10^5 spores per mL (Cota-Arriola et al., 2011).

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2.3 Antifungal assay

EO were individually tested against *A. parasiticus* and *F. moniliforme*. After autoclaving agar Czapek, it was combined with the study EO dissolved in Tween 80 (1.0% v/w), testing ten different concentrations (1, 10, 50, 100, 250, 500, 1000, 2500, 5000, and 10,000 μL L^{-1}), same which were poured into Petri dishes. Plates were centrally point-inoculated with 1×10^5 spores mL^{-1} from 7-day-old cultures of *A. parasiticus* or *F. moniliforme*. Three types of controls were prepared: one containing Czapek agar alone, another containing Tween (Czapek + Tween 80, 1.0%), and a control (Czapek plus 2.5 g L^{-1} of Terravax®, which is a commercial fungicide). Inoculated dishes were incubated in darkness at 27 °C for *A. parasiticus*, or at 25 °C for *F. moniliforme*. Radial extension growth of the colonies was measured daily and compared to controls. The radial inhibition percentages were calculated using Equation 1:

$$\text{Radial inhibition (\%)} = \frac{(\bar{R}_c - R_i)}{\bar{R}_c} \times 100 \quad (1)$$

where $\bar{R}_c$ was the mean value of radius of colonies grown in Tween control and R_i was the colony radius of the EO amended media (Plascencia-Jatomea et al., 2003). The radial extension rate of the colony, U (μm/h), was determined from the slope resulting from the radio versus time graph.

Growth data were fitted using the Gompertz model with the Equation 2.

$$Ln(D/D_o) = A\exp\{-\exp[(V_m \cdot e/A)(\lambda - t) + 1]\} \quad (2)$$

where Dt is the average colony diameter (mm) at time t (day); Do (cm) is the average colony diameter at initial time; A is the maximum fungus growth in the stationary phase; Vm is the maximum specific growth rate (1/0.5 days); λ is the lag phase (day); e = exp (1).

The minimum inhibitory concentration (MIC) and the half maximal inhibitory concentration that delayed 50% of spore germination (IC_{50}) were determined.

2.4 Kinetics of spore germination

Once the IC_{50} for each EO was determined, its effects on spore germination was evaluated. Microscope slides were placed at the bottom of plates; then, a solution containing Czapek broth, 1.0% Tween 80, and testing EO at IC_{50} was poured on each. These plates were inoculated with approximately 1×10^5 spores, and were incubated either at 27 or at 25 °C for *A. parasiticus* or *F. moniliforme*, respectively. The number of germinated spores per plate was determined every 3 h by counting 200 spores (germinated and non-germinated) using a light microscope. The same three types of controls described above were prepared. Each assay was made in quadruplicate.

The percentage of germinated spores of *F. moniliforme* and *A. parasiticus* were fitted to the logistic expression (Equation 3), where *S* was the percentage of germinated spores after time (t), S_0 was the initial percentage of germinated spores, S_{max} was the highest percentage of germinated spores (t→∞), and k was the spore germination rate. The S_0, S_{max} and k values were estimated using the NCSS 2001 program (NCSS Inc., U.S.A.).

$$S = \frac{S_{max}}{1 + \left(\frac{S_{max} - S_0}{S_0}\right) e^{-kt}} \quad (3)$$

Spore germination inhibition was represented as a percentage of spore germination in control (Equation 4), in which S_e is the percentage of spores germinated in EO amended media and S_c was the percentage of spores germinated in Tween control. The percentage of spore germination inhibition was calculated using data from 100 h old cultures.

$$\text{Inhibition (\%)} = \frac{(\bar{S}_c - S_e)}{\bar{S}_c} \text{x} 100 \quad (4)$$

2.5 Fumonisin and aflatoxin production

Fumonisin production was carried out using FB_1 free corn grain portions (50 g), which were placed in 500 mL flasks, adjusted to 40% humidity, and sterilized for two consecutive days in an autoclave for 15 min at 121 °C (Castellá et al., 1999). Autoclaved corn was separately treated with 10 mL of the IC_{50} of each EO. The control was prepared following the same procedure with no EO added just 10 mL of water instead. Another flask was prepared substituting EO by adding 10 mL of Tween 80 (1.0%); and a third control, now using 10 mL of the commercial fungicide Terravax, was also prepared. Each testing flask was inoculated with 1×10^5 spores/mL of *F. moniliforme* and were incubated for 30 days at 25 ± 2 °C with a 12 h light/dark cycle. Three replicates for each treatment were performed.

Grain from each flask was oven dried for 24 h and finely ground. Extraction procedure and fumonisin analysis were based on the Fumonitest method from VICAM as described by Cortez-Rocha et al. (2003). For aflatoxin production, the same procedure as described above was carried out. Autoclaved AFB_1 free corn samples were inoculated with 1×10^5 spores of *A. parasiticus*, and 10 mL of the IC_{50} of each EO was added. Flasks were incubated for 28 days at 27 °C. Similar controls to those mentioned above were prepared and tree replicates for each treatment were performed. Separation, purification, and quantification of AFB_1 were carried out according to the VICAM procedure (Cota-Arriola et al., 2011).

2.6 Spore viability

Spore viability was performed in a 96-well-flat-bottom microtitration plate. Fifty-μL of 3×10^6 spores/mL suspension of *A. parasiticus* or *F. moniliforme* were placed in a well and incubated during 4 h at 27 ± 2 °C or 24 ± 2 °C, respectively. Then, 50 μL of each testing EO was added at different concentrations (100, 250, 500, 1000, 2500, 5000, and 10,000 μL L^{-1}). Incubation continued at the aforementioned temperatures for 4 h, and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Sigma® were added to each well and incubated again for 42 h. After that, 100 μL of isopropanol containing 0.05 N HCl was added to each well. Controls consisted of non-inoculated plates (Czapek media alone, one with each testing EO, and one with Czapek + 0.1% DMSO). Another set of controls with inoculum was also prepared. The optical density (OD) of the plates was measured at 570 and 630 nm in a microtitration plate

spectrophotometer (BIO RAD Model iMark) (Meletiadis et al., 2000).

2.7 Statistical analysis

Analysis of data was carried out by ANOVA using a factorial design (type of EO, concentration, and fungus). Comparison of means in homogeneous subsets was performed using the Tukey multiple comparisons test at 95% confidence interval employing the JMP version 5.0, NCSS, SigmaPlot 10.0.

3 Results and discussion

3.1 Pirul essential oil yield

Pirul leaves (*Schinus molle*) EO presented a pale yellow color and light pepper odor. EO oil yield was 1.71% (dry basis). Zahed et al. (2010) reported 1.1% EO yield from pirul leaves and fruits, which also showed a pale yellowish color; meanwhile, Hayouni et al. (2008) only obtained a 0.58% yield from the same plant. The fact that oil yield depends on several factors such as the climate, season in which the plant was collected, location, and extraction technique, has been reported (Zahed et al., 2011). EO from eucalyptus, thymus and pirul had a density of 0.94, 0.91 and 0.83 g mL^{-1}, respectively.

3.2 Antifungal activity

Control cultures of *F. moniliforme* and *A. parasiticus* reached maximum growth after 144 and 168 h, respectively (Table 1). All the EO were found to significantly affect the growth of both fungi. *F. moniliforme* was more susceptible to EO than *A. parasiticus*. EO delayed the radial growth for the first 24 h compared to those in controls. Significant differences ($P < 0.05$) between doses, time, and EO were observed.

Table 1. Radial growth of fungal colonies exposed to different concentrations of essential oils (EO) from eucalyptus, pirul and thymus essential oils (EO) from eucalyptus, pirul and thymus.

EO (µL/L)	*A. parasiticus*				*F. moniliforme*		
	Time (h)						
	24	96	144	168	24	96	144
CONt	0.8 ± 0.1^{ac}	13.1 ± 0.3^{ac}	20.3 ± 0.1^{ad}	22 ± 0^{a}	1.2 ± 0.1^{a}	12.3 ± 0.1^{bj}	22 ± 0^{a}
CONc	1.0 ± 0^{a}	14.0 ± 0.2^{a}	20.6 ± 0.1^{a}	22 ± 0^{a}	1.5 ± 0.2^{a}	14.0 ± 0.3^{a}	22 ± 0^{a}
CONv	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
Eucalyptus							
1	0.5 ± 0^{cd}	12.8 ± 0.1^{ac}	19.6 ± 0.4^{ad}	22 ± 0^{a}	0.3 ± 0.1^{b}	11.7 ± 0.1^{bd}	20.3 ± 0.2^{bk}
10	0.5 ± 0^{cd}	12.8 ± 0.1^{ac}	19.5 ± 0^{ad}	22 ± 0^{a}	0.2 ± 0.1^{b}	11.2 ± 0.1^{bde}	19.6 ± 0.1^{cd}
50	0.5 ± 0^{cd}	12.5 ± 0.1^{ac}	19.5 ± 0^{ad}	22 ± 0^{a}	0.1 ± 0.1^{b}	10.3 ± 0.1^{def}	19.3 ± 0.3^{cd}
100	0 ± 0^{b}	12.4 ± 0.1^{ac}	19.5 ± 0^{ad}	22 ± 0^{a}	0 ± 0^{b}	10.2 ± 0.3^{def}	19.1 ± 0.1^{cd}
250	0 ± 0^{b}	12.1 ± 0.1^{ce}	19.3 ± 0.1^{ad}	22 ± 0^{a}	0 ± 0^{b}	9.3 ± 0.2^{fg}	18.8 ± 0.1^{d}
500	0 ± 0^{b}	12.3 ± 0.1^{ce}	19.5 ± 0^{ad}	22 ± 0^{a}	0 ± 0^{b}	10 ± 0^{ef}	18.6 ± 0.2^{d}
1000	0 ± 0^{b}	12.1 ± 0.1^{ce}	19.5 ± 0^{ad}	22 ± 0^{a}	0 ± 0^{b}	9.8 ± 0.1^{ef}	18.7 ± 0.1^{d}
2500	0 ± 0^{b}	10.6 ± 0.2^{ef}	18.4 ± 0.3^{dei}	22 ± 0^{a}	0 ± 0^{b}	9.5 ± 0^{fg}	$18. \pm 0.3^{dj}$
5000	0 ± 0^{b}	9.9 ± 0.1^{f}	17.3 ± 0.1^{egi}	21.6 ± 0.1^{a}	0 ± 0^{b}	8 ± 0.4^{g}	16.1 ± 0.5^{e}
10000	0 ± 0^{b}	6.5 ± 0.8^{dg}	13.3 ± 0.9^{c}	17.1 ± 1.6^{c}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
Pirul							
1	0.8 ± 0.1^{ac}	13.4 ± 0.2^{ac}	19.5 ± 0^{ad}	22 ± 0^{a}	0.4 ± 0.1^{b}	13.3 ± 0.9^{aj}	22.0 ± 0^{a}
10	0.6 ± 0.1^{cd}	13.1 ± 0.1^{aci}	19.4 ± 0.3^{ad}	22 ± 0^{a}	0.4 ± 0.1^{b}	13.1 ± 0.5^{aj}	22.0 ± 0^{a}
50	0.5 ± 0^{cd}	13.0 ± 0^{aci}	19.4 ± 0.1^{ad}	22 ± 0^{a}	0.1 ± 0.1^{b}	12.4 ± 0.2^{bj}	22.0 ± 0^{a}
100	0 ± 0^{b}	12.8 ± 0.1^{aci}	19.4 ± 0.1^{ad}	22 ± 0^{a}	0 ± 0^{b}	12.6 ± 0.4^{abj}	22.0 ± 0^{a}
250	0 ± 0^{b}	12.9 ± 0^{aci}	19.5 ± 0^{ad}	22 ± 0^{a}	0 ± 0^{b}	12.3 ± 0.1^{bj}	22.0 ± 0^{a}
500	0 ± 0^{b}	12.9 ± 0.1^{aci}	19.3 ± 0.1^{ad}	22 ± 0^{a}	0 ± 0^{b}	11.6 ± 0.2^{bd}	20.4 ± 0.1^{c}
1000	0 ± 0^{b}	12.3 ± 0.1^{cei}	18.8 ± 0.1^{ade}	22 ± 0^{a}	0 ± 0^{b}	9.6 ± 0.1^{f}	17.1 ± 0.4^{ej}
2500	0 ± 0^{b}	8.0 ± 0.3^{g}	13.8 ± 0.4^{c}	22 ± 0^{a}	0 ± 0^{b}	5 ± 0.2^{h}	8.8 ± 0.4^{g}
5000	0 ± 0^{b}	5.6 ± 0.2^{d}	9.5 ± 0.4^{f}	11.5 ± 0.3^{d}	0 ± 0^{b}	4.6 ± 0.3^{h}	7.6 ± 0.4^{fg}
10000	0 ± 0^{b}	5.0 ± 0.1^{d}	8.1 ± 0.3^{f}	9.8 ± 0.1^{d}	0 ± 0^{b}	3.8 ± 0.3^{hi}	6.5 ± 0.2^{fh}
Thymus							
1	0.4 ± 0.1^{d}	13.1 ± 0.4^{aci}	20.5 ± 0.2^{a}	22 ± 0^{a}	0.8 ± 0.1^{b}	12.1 ± 0.1^{bj}	21.8 ± 0.3^{ak}
10	0.4 ± 0.1^{d}	12.1 ± 0.6^{cei}	18.5 ± 0.4^{de}	21.3 ± 0.4^{a}	0.6 ± 0.1^{b}	12.0 ± 0^{bj}	21.4 ± 0.6^{ak}
50	0.3 ± 0.1^{b}	11.5 ± 0.3^{ei}	16.5 ± 0.6^{gi}	18.8 ± 0.7^{c}	0.3 ± 0.1^{bc}	11.8 ± 0.1^{bdj}	21.3 ± 0.4^{ak}
100	0 ± 0^{b}	9.8 ± 0.3^{f}	16 ± 0.5^{g}	18.5 ± 0.7^{c}	0 ± 0^{b}	9.9 ± 0.2^{ef}	19.3 ± 0.1^{bd}
250	0 ± 0^{b}	6.5 ± 0.7^{dg}	10 ± 0.5^{f}	11.6 ± 0.6^{d}	0 ± 0^{b}	2.6 ± 0.1^{i}	5.3 ± 0.3^{h}
500	0 ± 0^{b}	3.0 ± 0.1^{h}	5.1 ± 0.1^{h}	$5.9 \pm +0.2^{e}$	0 ± 0^{b}	1.3 ± 0.1^{c}	2.6 ± 0.3^{i}
1000	0 ± 0^{b}	0.3 ± 0.1^{b}	1.1 ± 0.1^{b}	1.6 ± 0.1^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
2500	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
5000	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
10000	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}

Values represent means of measurements made on four repetitions. In each column, values followed by the same letter do not differ significantly at $P = 0.05$ according to Tukey's test. CONt (control Tween); CONc (control Czapek); CONv (control Terravax).

Thymus EO was more effective in reducing radial growth at doses higher than 50 μL L^{-1}. After 168 h, plates containing eucalyptus and pirul EO at doses ranging from 1 to 2500 μL L^{-1}, were completely covered by colonies, similar to those observed in control plates. For *F. moniliforme*, the growth delaying effect lasted more than 144 h, a time at which colonies totally covered control plates. The radial growth of *F. moniliforme* was delayed when exposed to EO at doses higher than 250 μL L^{-1} and, since the beginning of the experiment, this fungi was totally affected by eucalyptus EO (10,000 μL L^{-1}). The effect of EO in both organisms was different due to their chemical composition and possibly due to the fungi membrane composition. Tabti et al. (2014) reported that terpene hydrocarbons and phenolic compounds in thymus EO were responsible for the effects observed in 14 food spoilage microorganisms and fungal strains such as *Aspergillus niger*, *A. flavus* and *Fusarium oxysporum*. On the other hand, the principal constituents of eucalyptus EO are 1.8 cineole and limonene (Tyagi & Malik, 2011), and α-pinene, β-pinene and L-limonene in pirul EO (Rocha et al., 2012; Gomes et al., 2013).

Thymus EO at 2500 μL L^{-1} caused total inhibition of *A. parasiticus* radial growth, while EO from eucalyptus and pirul, at the maximum concentration evaluated (10,000 μL L^{-1}), only caused 50.0 and 61.3%, respectively (Table 2). After 96 h of incubation the radial growth of *F. moniliforme* was totally inhibited by eucalyptus and thymus EO (10,000 and 1000 μL L^{-1}), respectively, and the pirul EO (10,000 μL L^{-1}) reduced radial growth only in 75.0%.

The radial extension rate of colonies in plates amended with EO is shown in Table 3. A decrease in the radial extension rate was observed as the EO concentration increased, compared to control plates. This observation confirms that radial growth in both fungi is delayed by EO in the medium during the adaptation and assimilation phases. With these obtained data, the IC_{50} was calculated by Probit analysis for *A. parasiticus* (4209, 3377 and 110 μL L^{-1}), and *F. moniliforme* (1105, 418 and 68 μL L^{-1}) for eucalyptus, pirul and thymus EO, respectively.

Vilela et al. (2009) report a 100% inhibition growth of *A. parasiticus* when exposed to 1000 μL L^{-1} of eucalyptus EO, a high resulting value compared to results from this study, whereas 9 mg mL^{-1} was enough for inhibiting 100% of *F. oxysporum* growth (Tyagi & Malik, 2011). Martins et al. (2014) required up to 1000 μg L^{-1} to achieved similar results. Also, Tabti et al. (2014) reported that 0.1 and 0.2 μg mL^{-1} of thymus EO completely inhibited *A. niger* and *F. solani* growth, respectively. Also, the results of Pagnussatt et al. (2013) indicates that *Spirulina* LEB-18

Table 2. Effect of EO from eucalyptus, pirul and thymus on radial growth (% of inhibition) and colony diameter of *A. parasiticus* and *F. moniliforme* after 96 h.

Essential oil(μL L^{-1})	Eucalyptus		Pirul		Thymus	
	% Inhibition	Colony diameter (mm)	% Inhibition	Colony diameter (mm)	% Inhibition	Colony diameter (mm)
A. parasiticus						
CONt	0 ± 0^{d}	13 ± 0.3^{a}	0 ± 0^{d}	13 ± 0.3^{a}	0 ± 0^{d}	13 ± 0.3^{a}
CONc	0 ± 0^{d}	14 ± 0.2^{a}	0 ± 0^{d}	14 ± 0.2^{a}	0 ± 0^{d}	14 ± 0.2^{a}
CONv	100 ± 0^{a}	0 ± 0^{f}	100 ± 0^{d}	0 ± 0^{d}	100 ± 0^{a}	0 ± 0^{e}
1	5.1 ± 1.1^{d}	12.8 ± 0.1^{a}	0 ± 0^{d}	13.4 ± 0.2^{a}	3.0 ± 1.11^{e}	13.1 ± 0.4^{a}
10	5.1 ± 1.1^{d}	12.8 ± 0.1^{a}	3.0 ± 0.9^{d}	13.1 ± 0.3^{ab}	10.3 ± 4.3^{e}	12.1 ± 0.6^{a}
50	7.4 ± 0^{d}	12.5 ± 0^{a}	3.7 ± 0^{d}	13.0 ± 0^{ab}	14.8 ± 2.2^{e}	11.5 ± 0.3^{a}
100	8.1 ± 0.9^{d}	12.4 ± 0.1^{a}	5.1 ± 1.1^{d}	12.8 ± 0.1^{ab}	27 ± 1.9^{d}	9.8 ± 0.3^{b}
250	10.3 ± 0.9^{d}	12.1 ± 0.1^{a}	4.4 ± 0.9^{d}	12.9 ± 0.1^{ab}	51.9 ± 5.4^{c}	6.5 ± 0.7^{c}
500	8.9 ± 1.1^{d}	12.3 ± 0.1^{a}	4.4 ± 0.9^{d}	12.9 ± 0.1^{ab}	77.7 ± 2.2^{b}	3.0 ± 0.3^{d}
1000	10.3 ± 0.9^{d}	12.1 ± 0.1^{a}	8.9 ± 1.1^{d}	12.3 ± 0.1^{b}	97.7 ± 1.1^{a}	0.3 ± 0.1^{e}
2500	21.4 ± 1.8^{c}	10.6 ± 0.2^{b}	40.7 ± 2.7^{c}	8.0 ± 0.3^{c}	100 ± 0^{a}	0 ± 0^{e}
5000	26.6 ± 0.9^{c}	9.9 ± 0.1^{b}	59.0 ± 1.8^{b}	5.6 ± 0.2^{d}	100 ± 0^{a}	0 ± 0^{e}
10000	51.8 ± 6.0^{b}	6.5 ± 0.7^{c}	63.0 ± 1.5^{b}	5.0 ± 0.2^{d}	100 ± 0^{a}	0 ± 0^{e}
F. moniliforme						
1	14.6 ± 1.2^{f}	11.7 ± 0.1^{a}	2.9 ± 6.3^{d}	13.3 ± 0.9^{a}	11.7 ± 0.9^{e}	12.1 ± 0.1^{a}
10	18.2 ± 1.2^{ef}	11.2 ± 0.1^{ab}	4.4 ± 3.4^{d}	13.1 ± 0.5^{a}	12.4 ± 1^{e}	12.0 ± 0^{a}
50	24.8 ± 1de	10.3 ± 0.1^{bc}	10.2 ± 1.6^{d}	12.3 ± 0.2^{a}	14.6 ± 1.0^{e}	11.7 ± 0.1^{a}
100	25.5 ± 2.6^{cd}	10.2 ± 0.3^{cd}	8.0 ± 2.5^{d}	12.6 ± 0.4^{a}	28.5 ± 1.7^{d}	9.8 ± 0.2^{b}
250	32.1 ± 1.9^{c}	9.3 ± 0.3^{d}	10.9 ± 0.9^{d}	12.2 ± 0.1^{a}	81.0 ± 0.9^{c}	2.6 ± 0.1^{c}
500	27.0 ± 0^{cd}	10.0 ± 0^{cd}	15.3 ± 1.6^{d}	11.6 ± 0.2^{a}	91.2 ± 1.0^{b}	1.2 ± 0.1^{d}
1000	28.5 ± 1.0^{cd}	9.8 ± 0.1^{cd}	29.9 ± 0.8^{c}	9.6 ± 0.1^{b}	100 ± 0^{a}	0 ± 0^{e}
2500	30.7 ± 0^{cd}	9.5 ± 0^{cd}	63.5 ± 1.4^{b}	5.0 ± 0.2^{c}	100 ± 0^{a}	0 ± 0^{e}
5000	41.6 ± 2.8^{b}	8.0 ± 0.3^{e}	66.4 ± 2.1^{b}	4.6 ± 0.3^{c}	100 ± 0^{a}	0 ± 0^{e}
10000	100 ± 0^{a}	0 ± 0^{f}	73.0 ± 1.6^{b}	3.7 ± 0.2^{c}	100 ± 0^{a}	0 ± 0^{e}

Values represent means of measurements made on four repetitions. In each column, values followed by the same letter do not differ significantly at P = 0.05 according to Tukey's test. CONc (control Czapek); CONt (control Tween); CONv (control Terravax).

phenolic extracts reduced the growth rate of 12 toxigenic strains of *Fusarium graminearum* isolated from barley and wheat. These studies were carried out in other fungi species of the same genera used in the present study; the effects of the EO are correlated.

3.3 Effect of essential oils on spore germination

When the IC_{50} of EO was evaluated on *A. parasiticus* and *F. moniliforme* spore germination in liquid Czapek medium, significant differences ($P \leq 0.05$) were found (Table 4). Spore germination was complete after 24 h in both controls, Tween and Czapek. EO of eucalyptus and thymus inhibited spore germination of *A. parasiticus* in more than 90%, while in *F. verticillioides*, EO of eucalyptus, thymus and pirul inhibited spore germination in 84.6, 30.6% and 34.0, respectively.

The logistic model was applied to data obtained to estimate the kinetic parameters of S_{max} and *k*. These values were lower in EO amended plates than those obtained in controls (Table 5), which suggests that EO are affecting the spore germination phase by delaying the germination rate. Carmo et al. (2008) reported that 40 and 80 μL mL^{-1} of origanum oil (*Origanum vulgare* L.) caused 100% inhibition of spore germination in *A. parasiticus*. Information on the mechanism(s) of action by the EO is not available. Velluti et al. (2003) mentioned that other authors have attributed this phenomenon, not only to the presence of terpenes, phenolic compounds, and other components, but also to the chemical structure, such as the presence of hydroxyl groups in their phenolic compounds. Ultee et al. (2002) mentioned that the antimicrobial activity of EO could be attributed to the presence of an aromatic nucleus, and a reactive phenolic OH group may form

Table 3. Effect of EO from eucalyptus, pirul and thymus on radial growth rate of *A. parasiticus* and *F. moniliforme* in Czapek agar.

Essential oil (μL L^{-1})	*A. parasiticus*			*F. moniliforme*		
	Eucalyptus	Pirul	Thymus	Eucalyptus	Pirul	Thymus
CONt	0.19 ± 0.005	0.19 ± 0.005	0.19 ± 0.005	0.20 ± 0.004	0.22 ± 0.01	0.21 ± 0.006
CONc	0.20 ± 0.005	0.20 ± 0.005	0.20 ± 0.005	0.19 ± 0.005	0.24 ± 0.01	0.22 ± 0.009
CONv	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0.20 ± 0.004	0.19 ± 0.005	0.20 ± 0.007	0.19 ± 0.005	0.20 ± 0.009	0.20 ± 0.005
10	0.19 ± 0.003	0.19 ± 0.005	0.18 ± 0.009	0.19 ± 0.005	0.21 ± 0.006	0.20 ± 0.005
50	0.19 ± 0.003	0.19 ± 0.005	0.17 ± 0.012	0.19 ± 0.004	0.21 ± 0.005	0.20 ± 0.006
100	0.20 ± 0.004	0.19 ± 0.006	0.15 ± 0.008	0.19 ± 0.004	0.21 ± 0.006	0.19 ± 0.004
250	0.19 ± 0.004	0.19 ± 0.006	0.15 ± 0.008	0.19 ± 0.004	0.21 ± 0.005	0.19 ± 0.004
500	0.19 ± 0.005	0.19 ± 0.006	0.07 ± 0.005	0.19 ± 0.003	0.20 ± 0.005	0 ± 0
1000	0.19 ± 0.005	0.18 ± 0.006	0 ± 0	0.19 ± 0.003	0.17 ± 0.006	0 ± 0
2500	0.18 ± 0.005	0.13 ± 0.006	0 ± 0	0.19 ± 0.004	0.08 ± 0.006	0 ± 0
5000	0.17 ± 0.004	0.09 ± 0.004	0 ± 0	0.17 ± 0.008	0.07 ± 0.006	0 ± 0
10000	0.15 ± 0.01	0.08 ± 0.004	0 ± 0	0 ± 0	0.06 ± 0.004	0 ± 0

Values represent means of measurements made on four repetitions. CONc (control Czapek), CONt (control Tween), CONv (control Terravax).

Table 4. Inhibition of spore germination in Czapek liquid with EO from eucalyptus, thymus and pirul after 24 h.

Treatment	IC_{50} (μL L^{-1})	*A. parasiticus*	IC_{50} (μL L^{-1})	*F. moniliforme*
CONt	NA*	0 ± 0^{d}	NA*	0 ± 0^{c}
CONc	NA*	0 ± 0^{d}	NA*	0 ± 0^{c}
CONv	NA*	98.5 ± 0.8^{a}	NA*	100 ± 0^{a}
Eucalyptus	4209	99.3 ± 1.0^{a}	1105	84.6 ± 9.2^{a}
Thymus	110	92.5 ± 2.4^{b}	68	30.6 ± 1.2^{b}
Pirul	3377	53.8 ± 10.6^{c}	418	34 ± 9.6^{b}

Values represent means made from three repetitions. Values within a column followed by the same letter do not significantly differ at $P = 0.05$ according to Tukey's test. CONc (control Czapek); CONt (control Tween); CONv (control Terravax). NA* = Not applicable.

Table 5. Effect of EO from eucalyptus, thymus and pirul in spore germination rate of *A. parasiticus* and *F. moniliforme*.

Treatment	IC_{50} (μL L^{-1})	*A. parasiticus*	IC_{50} (μL L^{-1})	*F. moniliforme*
		k		***k***
CONc	NA*	0.83 ± 0.1	NA*	0.46 ± 0.03
CONt	NA*	0.27 ± 0.1	NA*	0.50 ± 0.03
CONv	NA*	0.08 ± 0.1	NA*	0 ± 0
Eucalyptus	4209	2.37 ± 24.1	1105	0.36 ± 0.30
Thymus	110	1.63 ± 1.5	68	0.50 ± 0.18
Pirul	3377	0.41 ± 0.1	418	0.14 ± 0.05

Values represent means of measurements made on three repetitions. CONc (control Czapek); CONt (control Tween); CONv (control Terravax). NA* = Not applicable.

hydrogen bonds with active sites of target enzymes. Aldehydes such as formaldehyde and glutaraldehyde are known to possess antimicrobial activity. The conjugation of an aldehyde group to a carbon- carbon double bond has been proposed as a way of producing a highly electronegative arrangement, which may explain their activity (Moleyar & Narasimham, 1986). These electronegative compounds may interfere in biological processes such as electron transfer, react with proteins and nucleic acids and, therefore, inhibit the growth of microorganisms.

3.4 Effect of essential oils in spore viability

Results obtained from cell viability determination in *A. parasiticus* are presented in Figure 1. Significant differences among tested EO ($P \leq 0.05$) were observed. Thymus EO caused the highest effect at all concentrations; spore proliferation was lower than 20%. The effect of eucalyptus EO increased when the oil concentration increased, and the highest effects were observed when 5000 and 10,000 μL L^{-1} were used. Pirul EO was significantly ($P \leq 0.05$) different only at the highest concentration. For *F. moniliforme* eucalyptus EO also produced high effects on spore viability, followed by thymus EO (Figure 2). Pirul EO had an effect at the highest concentrations (> 2500 μL L^{-1}). Thymus EO was the most effective for both fungi.

EO may cause a depolarization of mitochondrial membranes in eukaryotic cells. This may be produced by decreasing the membrane potential, affecting ionic Ca^{++} cycling and other ionic channels, reducing the pH gradient, and affecting the proton pump and the ATP pool (Richter & Schlegel, 1993). Such oils change membranes fluidity, which become abnormally permeable, resulting in leakage of radicals, cytochrome C, calcium ions, and proteins, as in the case of oxidative stress and energy failure (Vercesi et al., 1997). Permeabilization of

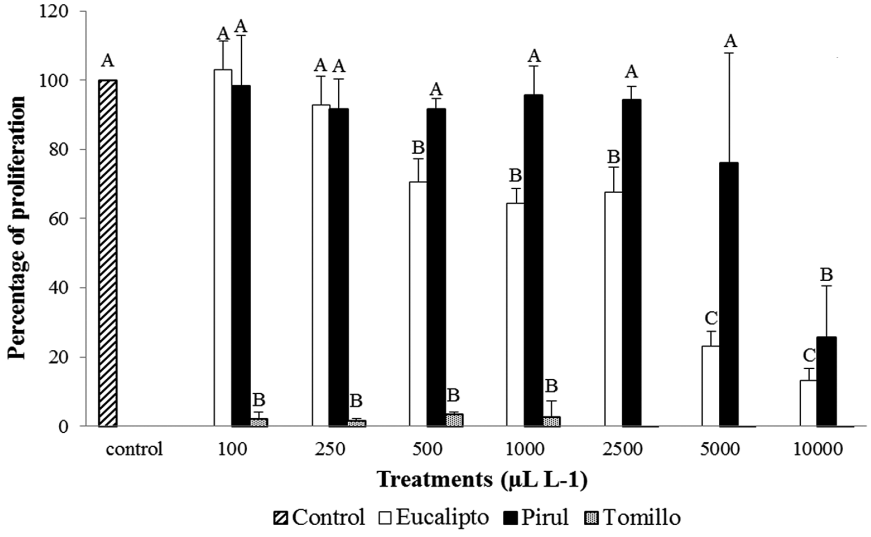

Figure 1. Effect of EO from eucalyptus, pirul and thymus on *A. parasiticus* spore viability.

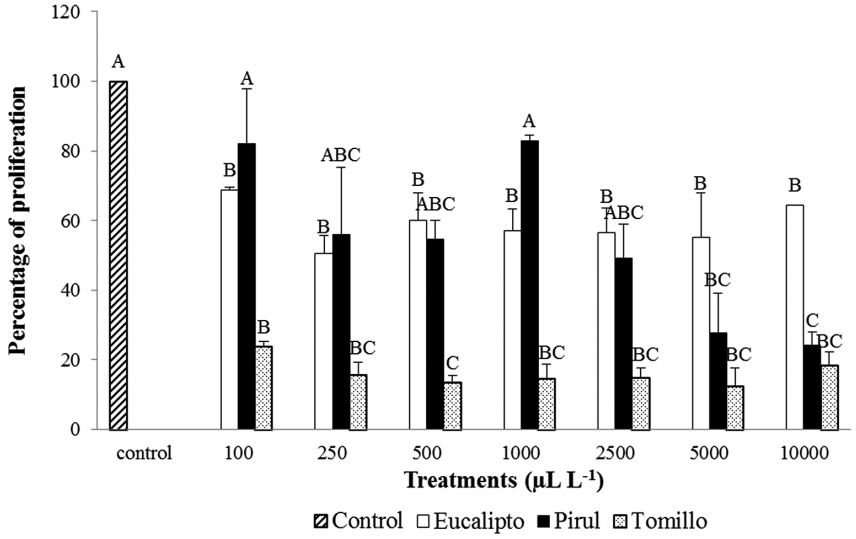

Figure 2. Effect of EO from eucalyptus, pirul and thymus on spore viability of *F. moniliforme*.

Table 6. Effect of EO from eucalyptus, pirul, and thymus on total aflatoxins and fumonisins production.

Treatment	IC_{50} (μL L^{-1})	AFBs (ng g^{-1})	IC_{50} (μL L^{-1})	FBs (μg kg^{-1})
CONa	NA*	800 ± 57.7^{ab}	NA*	5.0 ± 0.4^{b}
CONt	NA*	1000 ± 57.7^{ab}	NA*	4.9 ± 1.1^{b}
CONv	NA*	867 ± 33.3^{ab}	NA*	5.2 ± 1.2^{b}
Eucalyptus	4208	1233 ± 185.6^{a}	1105	1.5 ± 1.0^{c}
Thymus	110	767 ± 66.7^{a}	68	7.5 ± 3^{a}
Pirul	3377	833 ± 33.3^{ab}	418	7.5 ± 2.3^{a}

Values represent means made from three repetitions. Values within a column followed by the same letter do not significantly differ at $P = 0.05$ according to Tukey's test. CONa (control water); CONt (control Tween); CONv (control Terravax). NA* = Not applicable.

outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Armstrong, 2006). The presence of thymol and carvacrol has been reported mainly in thymus EO, and due to their hydrophobicity, they could affect cell membrane permeability leading to leakage of cell contents and formation of channels (Chavan & Tupe, 2014), reducing proliferation capacity. The use of this assay has not been reported for EO and, for this reason, some problems came up that caused the test to be inappropriate to evaluate cell viability. The major problem was volatilization of EO that inhibited fungi growth in the microplates. In addition, MTT reacted with thymus EO producing a dark purple color that interferes with the readings.

3.5 Mycotoxins production

Results for total aflatoxins and fumonisins production by studied fungi, in the presence of the tested EO at their IC_{50}, are presented in Table 6. Significant effects ($P < 0.05$) were observed for aflatoxins production by *A. parasiticus* in the presence of thymus EO, which was reduced in 4.0%. *A. parasiticus* in the presence of EO from the other plants, produced equal or more mycotoxins than controls. These results differ from those from other authors. Vilela et al. (2009) reported that 500 μL of eucalyptus EO totally inhibited the aflatoxin B_1 production by *A. flavus*. Also, Rasooli & Abyaneh (2004) reported similar findings when applying *Thymus eriocalyx* EO. Fumonisin production in control plates was statistically ($P < 0.05$) lower than those amended with pirul and thymus EO (Table 6). Only eucalyptus EO was capable of causing a reduction in fumonisin production.

Fandohan et al. (2004) found that EO of *Cymbopogon citratus, Ocimum gratissimum,* and *O. basilicum* ranging from 4.8 to 8.0 μL g^{-1}, reduced fumonisin production and kernel germination. Dambolena et al. (2008) studied the effects of terpenes on fumonisin B_1 production and found that thymol and limonene (75 μg L^{-1}) were the most active compounds to inhibit mycotoxin production. However, this was not observed in the present study, even when Hayouni et al. (2008) indicated that EO from *S. molle* is high in monoterpenes, such as limonene, α-phellandrene, and sesquiterpenes.

4 Conclusions

A 1.7% yield of pirul EO can be achieved from pirul plant by hydrodistillation. EO from eucalyptus, thymus and pirul affect the normal growth of *A. parasiticus* and *F. verticillioides* by delaying the spore germination. *F. moniliforme* is more susceptible to tested EO than *A. parasiticus*. The EO interfered with spore viability determination by MTT assay. A small but significant reduction in aflatoxin production can be achieved only when *A. parasiticus* is exposed to thymus EO; however, eucalyptus and pirul EO caused the opposite in *F. moniliforme* fumonisin production.

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