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Antifungal and antiaflatoxigenic activity by vapor contact of three essential oils, and effects of environmental factors on their efficacy

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ABSTRACT

The present investigation reports the antifungal and antiaflatoxigenic properties of essential oils (EOs) from boldo (*Pëumus boldus* Mol.), poleo (*Lippia turbinata* var. *integrifolia* Griseb., clove (*Syzygium aromaticum* L.) and from boldo and poleo mixtures present in the headspace of peanut extract medium at three water activity levels (a_W) (0.98, 0.95 and 0.93). Moreover, the ability of boldo and poleo oils to maintain their antifungal activity was evaluated after subjecting them to environmental variations. Boldo EO at doses $\geq$1500 μL/L showed a highly significant effect on *Aspergillus* section *Flavi* lag phase (>300 h), growth rate (93–100% of inhibition) and aflatoxin B_1 (AFB_1) accumulation (100% of inhibition) at all a_W levels assayed. The antifungal and antiaflatoxigenic effects of poleo and clove EOs were highly dependent on a_W. In general boldo–poleo oil mixtures showed less inhibitory activity on *Aspergillus* strains than the boldo pure oil. The antifungal ability of volatile components released by boldo and poleo EOs was stable against temperature changes; while it was reduced when poleo was stored during six months and when boldo was exposed to sunlight and UV.

Boldo and poleo EO volatile fractions can be used as effective non-toxic biopreservatives in stored peanut industry against AF contamination.

1. Introduction

Among different mycotoxins, aflatoxins (AFs), a difuran-containing polyketide derived *Aspergillus* toxin, are the most significant mycotoxins in peanuts during postharvest processing (Ding, Li, Bai, & Zhou, 2012; Ezekiel et al., 2012; Kamika & Takoy, 2011). Aflatoxins (AFs) in general and specially aflatoxin B_1 (AFB_1) is a genotoxic, immunotoxic and hepatocarcinogenic secondary metabolite (group 1) (IARC, 1993, 2002) produced mainly by *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius* and *Aspergillus pseudotamarii* (Varga, Frisvad, & Samson, 2011). There are reports that hepatic carcinoma and other serious diseases may be induced by consuming food or using raw materials for food processing contaminated with AFs (Li, Yoshizawa, Kawamura, Luo, & Li, 2001; McKean et al., 2006; Turner, Moore, Hall, Prentice, & Wild, 2003). Nearly 5 billion people are exposed to AFs in different developing countries and aflatoxicosis is ranked 6th among the 10 most severe health risks identified by WHO and have ability to accumulate in the organism (Galvano, Ritieni, Piva, & Pietri, 2005). Considering, further the impact on international market is necessary remembering that mycotoxin contamination of agricultural commodities has important economic implications. The losses due to rejection of shipments and lower prices for lower quality can be devastating to developing countries typically grain exporters. For these reasons, AFs levels are highly regulated in peanut and peanut derived products in most countries. Consequently, the European Union and Food and Drug Administration U.S. instituted legislation to protect the health of consumers and set limits for total AFs and AFB_1 in peanuts of 4 and 2 ng/g, respectively (European Commission (EC) Commission Regulation, No 165/2010; FDA U.S. Regulations, CPG Sec. 570.375/09).

The world peanut production obtained during the period 2010/2011 was estimated at 33 million tons, Argentina ranked 14th, contributing with 0.95% of total world production (USDA, 2012). Argentina has established as the world's largest exporter of peanuts and the excellence in this product has given it international prestige to this industry becoming the largest peanut supplier in the European Union (MAGyP, 2012). However, we have studied toxigenic and sclerotial characteristics of *Aspergillus* section *Flavi* populations in the peanut agroecosystem and the 100% of the

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samples were contaminated with potentially toxigenic species (Passone, Rosso, Ciancio, & Etcheverry, 2010).

Although, different synthetic antimicrobials have been successfully commercialized in recent years to minimize such losses, they encounter major problems not only due to their adverse side effects on consumers but also for the development of resistance by microorganisms (Tolouee et al., 2010). Hence, there must be optimization of alternative methods for pest and disease control that produce minimal damage to the environment and human health and with different action mechanisms on the target cell to avoid the development of resistance by microorganisms. Currently, different plant products have been formulated for large scale application in eco-friendly and biorational management of storage pests and are being used as botanical antimicrobials. Amongst plant products the essential oils (EOs) derived from aromatic plants have been well studied during the last two to three decades as a potential candidates against different microbes (Abdollahi, Hassani, Ghosta, Meshkatalsada, & Shabani, 2011; Bullerman, Lieu, & Seiler, 1977; Mallozzi, Correa, Haraguchi, & Brignani, 1996; Marandi et al., 2011; Prakash, Singh, Kedia, Singh, & Dubey, 2012).

The vegetative growth and subsequent aflatoxin by *Aspergillus* section *Flavi* were found to be sensitive to twenty EOs extracted from some medicinal plants (Dubey, Shukla, Kumar, Singh, & Prakash, 2010; Soliman & Badeaa, 2002). Recently, oil extracted from *Hibiscus sabdariffa*, *Nigella sativa*, *Eucalyptus globulus* Labill, *Callistemon lanceolatus* (Sm.) Sweet showed variable fungistatic and fungicidal properties against *Aspergillus* section *Flavi* (El-Nagerabi, Al-Bahry, Elshafie, & AlHilali, 2012; Rocha Vilela et al., 2009; Shukla, Singh, Prakash, & Dubey, 2012). In a contact assay, we demonstrated that the application of high concentrations (2500 μL/L) of *Pëumus boldus* Mol. (boldo) and *Lippia turbinata* var. *integrifolia* (Griseb.) (poleo) oils completely inhibited fungal development. The antiaflatoxigenic property of these EOs (500 μL/L) was more marked with the medium a_W reduction (Passone, Girardi, Ferrand, & Etcheverry, 2012). Besides, oil from the dried flower buds of *Syzygium aromaticum* L. (clove) at the dose of 1500 μL/L completely inhibited the growth of *A. flavus* and *A. parasiticus* strains. Some EOs and their components are bioactive compounds in commercial use as food additives through encapsulation technologies (Ávila-Sosa et al., 2012; Leimann, Gonçalves, Machado, & Bolzan, 2009).

Thus, the objectives of this study were: (a) to examine the efficacy of boldo, poleo and clove oils by vapor contact assay against *A. flavus* and *A. parasiticus* growth and AFB_1 accumulation on peanut meal extract agar under different environmental conditions (0.98, 0.95, 0.93 a_W); (b) to evaluate the antifungal and antiaflatoxigenic properties of boldo and poleo oil mixtures present in the headspace of peanut extract medium at three water activity levels; (c) to determine if boldo and poleo oils have the ability to maintain their antifungal activity after subjecting them to environmental variations that occur in the peanut storage agroecosystem.

2. Materials and methods

2.1. Fungal isolates

Two *A. flavus* Link (RCP08270 and RCP08108) and two *A. parasiticus* Speare (RCP08299 and RCP08300) were used in this study. These strains were originally isolated from stored peanut in Córdoba, Argentina, in August/December 2008 period (Passone et al., 2010), and it was previously demonstrated to be aflatoxin producers in peanut meal extract agar (PMEA; 0.99 a_W; 11 days of incubation at 25 °C; 30.3 ± 4.6 ng/g, 33.0 ± 12.1 ng/g, 953.3 ± 23.1 ng/g and 49.7 ± 39.3 ng/g AFB_1 for RCP08270, RCP08108, RCP08299, RCP08300, respectively). These isolates were deposited in the *Aspergillus* section *Flavi* culture collection, Microbiology and Immunology Department of the National University of Río Cuarto. The fungi were maintained on slants of malt extract agar (MEA) at 4 °C and stored as spore suspensions in 1.5 g/L glycerol at −20 °C.

2.2. Essential oils and chemical characterization

The plant species *P. boldus* Mol. (boldo), *L. turbinata* var. *integrifolia* (Griseb.) (poleo) and *S. aromaticum* L. (clove) used in this study were purchased from a local market. The plant species were stored at −20 °C after harvest (Usai et al., 2011). The plant materials were obtained from dried leaves of *P. boldus*, dried leaves and stems of *L. turbinata* var. *integrifolia* and dried flower buds of *S. aromaticum*. A portion (100 g) of each plant material parts was submitted for 3 h to water-distillation, using an extractor of EOs by steam distillation at laboratory scale (Figmay S.R.L.) (yield 2.0; 1.02 and 10.0 mL/g for boldo, poleo and clove, respectively). The EO was dried over anhydrous sodium sulfate and, after filtration, stored in sterilized vial at 4 °C for up to 1 week until tested. Chemical characterizations of these EOs were previously determined in our laboratory by Bluma and Etcheverry (2008).

2.3. Culture medium

The basic medium used in this study was peanut meal extract agar (PMEA), made by boiling 30 g of dried peanut meal in 1 L water for 60 min. The resulting mixture was filtered through a double layer of muslin and 15 g/L agar was added (Passone, Resnik, & Etcheverry, 2005). The water activity (a_W) of the basic medium (0.99) was adjusted to 0.98, 0.95 and 0.93 by addition of a non-ionic solute, glycerol, according to Dallyn and Fox (1980). PMEA was autoclaved at 121 °C for 20 min, and poured into sterile Petri dishes for volatile assays. The water activity of the medium was checked after autoclaving with an AquaLab Water Activity Meter 4TE (Decagon Devices, Inc.).

2.4. Vapor contact assay

The effect of three EOs (boldo, poleo and clove) on the growth of four aflatoxigenic isolates in PMEA at three a_W levels and 25 °C was studied. Two small Petri dishes (5 cm diameter) containing each one 10 mL PMEA were prepared and placed, without cover, into a big Petri dish (14 cm diameter). The plates were spot inoculated with 2 μL into center of each small Petri dish, with 10^5 spores/mL suspended in 0.2 g/100 mL soft agar (Pitt, 1979). Sterilized cotton was placed in the center of the big Petri dish; that is between the two small PMEA agar plates. Essential oil was added to the cotton, having no direct contact with the PMEA agar plates. In a separate assay, boldo and poleo oil doses were 1000, 1500, 2000, 2500 and 3000 μL per liter of PMEA, while clove EO doses were 1000, 2000, 3000, 4000 and 5000 μL per liter of PMEA. In a mixture assay, boldo and poleo oil doses were 250, 500 and 750 μL/L and 500, 1000 and 1500 μL/L, respectively to obtain the following treatments: B–P mixtures (250 + 500 μL/L), (250 + 1000 μL/L), (250 + 1500 μL/L), (500 + 500 μL/L), (500 + 1000 μL/L), (500 + 1500 μL/L), (750 + 500 μL/L), (750 + 1000 μL/L), (750 + 1500 μL/L). The control plates (without essential oil) were inoculated following the same procedure. The plates were sealed with polyethylene film and incubated at a temperature of 25 °C during 11 days. Those treatments in which no fungal development was observed at 11 days were incubated for a maximum of 35 days. Tests were undertaken in quadruplicate and each colony was daily measured in two directions at right angles to each other to obtain the mean radii. The radii of the colonies were plotted against time, and a linear

regression applied, in order to obtain the growth rate as the slope of the line. Lag phase for growth was calculated mathematically and defined as the time (days) in which each colony reaches a diameter of 5 mm for each treatment, in relation to isolates, EOs and a_W (Marín, Sanchis, & Magan, 1995). In all cases, the experiments were carried out at least in four replicates per treatment. The growth of fungal cultures containing different concentrations of all EOs was compared with that of the control culture with no EOs.

After growth was evaluated, all samples were frozen for later extraction and AFB_1 quantification.

2.5. Aflatoxin B_1 analysis

Extraction of AFs was done following the methodology proposed by Geisen (1996). A piece of PMEA with mycelium (1 cm × 1 cm), which was incubated for 11 days at 25 °C, was taken from inoculated PMEA cultures (aflatoxin producers + different concentrations of EOs), transferred to an Eppendorf tube, into which 500 μL of chloroform was added. The mixture was shaken for 20 min. The piece of agar was then removed and the chloroform extract filtered through a Whatman no. 4 filter paper and allowed evaporation to dryness on N_2 flow. The residue was redissolved in 200 μL acetonitrile/water (9:1) and then derivatized with 700 μL of trifluoroacetic acid/acetic acid/water (20:10:70). Fifty microliters of derivatized solution was analyzed using a reversed-phase HPLC/fluorescence detection system (Trucksses, Stack, Nesheim, Albert, & Romer, 1994). The HPLC system consisted of a Hewlett–Packard 1100 pump (Palo Alto, CA, USA) connected to a Hewlett–Packard 1046 programmable fluorescence detector, interfaced to a Hewlett–Packard Chem Station. Chromatographic separations were performed on a stainless steel Supelcosil LC-ABZ C18 reversed-phase column (150 × 4.6 mm i.d., 5 μL particle size; Supelco, PA, USA). Water/methanol/acetonitrile (4:4:1) was used as the mobile phase, at a flow rate of 1.5 mL/min. Fluorescence of AF derivatives was recorded at excitation and emission wavelengths of 360 and 440 nm respectively. Aflatoxin B_1 standard was obtained from Sigma Chemical (Dorset, UK) and standard curves were constructed with different levels of AFB_1 (5–100 ng/g). This toxin was quantified by correlating peak areas of sample extracts and those of standard curves. The mean recovery of the method used was calculated by spiking peanut seeds at different levels ranging from 5 to 100 ng/g and was estimated at 94.5%. The lowest detection limit was 1 ng/g.

2.6. Effect of environmental factors on the toxicity of boldo and poleo oils

Experiments were performed to determine the thermostable nature of the oils, and the stability during sunlight and UV exposure and storage time. Different glass vials containing 3 mL of each oil were subjected: to different temperature treatments for 1 h in incubators already adjusted at 40, 60 and 80 °C; to sunlight during 30 and 60 min; to UV light (260 nm) during 15 and 30 min; and to storage during 6 months at 4 °C. The fungitoxicity of the treated oils from each set was tested against the test fungi at 2000 μL/L on PMEA by the inoculation technique described above.

2.7. Statistical analyses

All data analyses were performed by analysis of variance (ANOVA). *F*-value was found in ANOVA using SAS program 6.1 SAS Institute, Cary, NC (1998). Posteriori tests were performed using SigmaStat program Version 3.10 (Systat Software, Inc.). The significant differences for lag phase and growth rate were determined using Duncan's New Multiple Range Test at $p < 0.05$ level. Fisher's LSD test ($\alpha = 0.05$) was employed to determine significant differences between treatments and control for AFB_1 accumulation.

3. Results

3.1. Activity of volatile EO treatments on mycelial growth and AFB_1 accumulation

Statistical analyses on growth of *Aspergillus* section *Flavi*; water activity (a_W), isolates (I), essential oils (EOs), concentrations (C) and two-; three-; and four-way interactions indicated that they were statistically significant (Table 1). The major effect was produced by EO, followed by a_W levels and oil C.

Boldo EO was the most effective: at doses of 1000 μL/L and ≥1500 μL/L and at all a_W levels studied, it increased the lag phases to 184.7 h and to more than 300 h, respectively (Table 2). Antifungal effects of poleo and clove EOs were highly dependent of medium a_W. Poleo EO could increase fungal lag phases by 14.7, 16.8 and 137.0 h at 0.98, 0.95 and 0.93 a_W, respectively. The lag phases of four fungal isolates were extended by 1000–5000 μL/L doses of clove oil at the lowest a_W assayed (0.93), whereas it was reduced at 0.95 and 0.98 a_W.

Saturated atmosphere of volatile fraction of boldo at ≥1500 μL/L inhibited between 93.9 and 100% the growth rates of all fungi at all a_W assayed and strongly reduced mycelial development (≥51.3%) at 1000 μL/L (Fig. 1). Treatment with 1000 μL/L of poleo resulted in inhibition growth rates varying between 16.1–75.8% and 3.7–74.7% at 0.98 and 0.95 a_W, respectively; while significant inhibitions ($p < 0.05$) by 39.4–72.8% were observed at 0.93 a_W. Meanwhile, clove EO showed the lowest antifungal activity; inhibitory effects were evident at doses ≥4000 μL/L at 0.98 a_W, while at 2000 μL/L it inhibited fungal growth rate by 37.5–62.7% at 0.93 a_W.

The effect of single factors as well as their two-; three- and four-way interactions on AFB_1 accumulation was also determined by ANOVA (Table 1). AFB_1 accumulation significantly depended on fungal isolate, a_W and their interactions.

The medium water availability affected AFB_1 accumulation by control treatments: the highest levels of this mycotoxin were always detected when *Aspergillus* section *Flavi* isolates grew on PMEA modified at 0.98 a_W (Table 3). These levels decreased by 56.3 and 73.1% as substrate a_W was reduced to 0.95 and 0.93, respectively.

Table 1
ANOVA test. Effects of essential oils (EO) and their concentrations (C) on growth rate and AFB_1 production by *Aspergillus* section *Flavi* isolates (I) grown on PMEA at various a_W levels.

Source of variation	Separate assay				
	DF	Growth rate		Aflatoxin B_1	
		MS	*F* value[a]	MS	*F* value
a_W	2	0.464	789.9*	500,829,949	578.1*
I	3	0.010	17.1*	665,862,002	768.6*
EO	2	0.561	954.5*	102,134,801	117.9*
C	5	0.248	421.0*	160,063,923	184.8*
$a_W \times I$	6	0.017	28.5*	260,948,975	301.2*
$a_W \times EO$	4	0.053	90.5*	52,226,508	60.3*
$a_W \times C$	10	0.007	11.1*	32,918,819	38.0*
$I \times EO$	6	0.006	10.2*	46,403,382	53.6*
$I \times C$	15	0.003	5.9*	137,250,670	158.4*
$EO \times C$	10	0.027	46.4*	10,233,476	11.8*
$a_W \times I \times EO$	12	0.007	11.3*	25,368,775	29.3*
$a_W \times I \times C$	30	0.003	5.6*	26,547,334	30.6*
$a_W \times EO \times C$	20	0.005	8.4*	11,332,221	13.1*
$I \times EO \times C$	30	0.002	3.7*	4,729,428	5.5*
$a_W \times I \times EO \times C$	60	0.002	3.3*	5,056,910	5.8*
Error	648	0.001		866,379	

*Highly significant at $p < 0.001$.
DF: degrees of freedom; MS: mean of squares.
[a] Snedecor's *F* test.

Table 2
Effect of EO volatile fractions on the lag phase of *Aspergillus* section *Flavi* strains on PMEA at different a_W.

Essential oils	Doses (μL/L)	Lag phase (h)[a]			
		A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A. parasiticus* RCP08300
0.98 a_W					
Control	0	43.0 ± 2.4^{d}	19.1 ± 9.5^{b}	32.2 ± 2.8^{cde}	33.2 ± 4.8^{a}
Boldo	1000	94.4 ± 17.4^{d}	72.5 ± 51.4^{b}	250.9 ± 30.5^{a}	236.6 ± 126.7a
	1500	>^{c}	220.5 ± 181.9^{b}	–^{f}	>^{a}
	2000	>^{a}	>^{a}	–^{f}	>^{a}
	2500	>^{b}	>^{a}	–^{f}	>^{a}
Poleo	1000	48.4 ± 2.9^{d}	41.1 ± 1.9^{b}	38.3 ± 8.2^{cde}	34.1 ± 7.8^{a}
	1500	48.9 ± 2.3^{d}	29.0 ± 6.7^{b}	56.3 ± 25.4^{b}	41.1 ± 5.6^{a}
	2000	55.5 ± 7.3^{d}	38.2 ± 8.1^{b}	47.2 ± 3.1^{bcd}	31.1 ± 3.9^{a}
	2500	57.5 ± 5.5^{d}	44.5 ± 4.3^{b}	48.6 ± 3.0^{bc}	62.8 ± 15.9^{a}
	3000	56.9 ± 2.9^{d}	47.3 ± 2.9^{b}	56.4 ± 6.4^{b}	48.5 ± 13.0^{a}
Clove	1000	32.7 ± 0.6^{d}	30.6 ± 1.4^{b}	31.2 ± 2.9^{de}	23.6 ± 10.4^{a}
	2000	32.8 ± 1.1^{d}	28.6 ± 1.6^{b}	30.0 ± 2.0^{e}	29.5 ± 0.5^{a}
	3000	35.2 ± 1.4^{d}	25.3 ± 5.4^{b}	35.8 ± 2.9^{cde}	25.1 ± 3.7a
	4000	16.1 ± 6.5^{d}	25.0 ± 5.5^{b}	7.1 ± 1.5^{f}	23.4 ± 6.4^{a}
	5000	37.0 ± 2.4^{d}	33.8 ± 12.1^{b}	6.2 ± 3.5^{f}	36.7 ± 3.3^{a}
0.95 a_W					
Control	0	61.2 ± 1.4^{c}	72.0 ± 0.1^{d}	66.9 ± 2.4^{b}	55.0 ± 0.3^{bcde}
Boldo	1000	152.1 ± 25.1^{a}	190.0 ± 45.4^{b}	204.9 ± 22.6^{b}	>^{a}
	1500	–^{f}	>^{a}	>^{a}	–^{g}
Poleo	1000	62.6 ± 5.2^{c}	60.0 ± 2.3^{de}	66.7 ± 5.4^{b}	48.5 ± 5.5^{cdef}
	1500	80.1 ± 4.0^{b}	70.3 ± 11.0^{d}	71.0 ± 2.7^{b}	53.1 ± 6.7^{bcdef}
	2000	91.3 ± 9.1^{b}	69.7 ± 4.9^{d}	70.6 ± 3.0^{b}	57.0 ± 3.2^{bcd}
	2500	80.7 ± 4.6^{b}	106.1 ± 15.1^{b}	92.7 ± 4.1^{b}	63.0 ± 2.8^{bc}
	3000	95.4 ± 14.1^{b}	181.0 ± 22.5^{c}	117.2 ± 10.5^{b}	75.6 ± 3.5^{b}
Clove	1000	13.7 ± 8.2^{cd}	38.4 ± 12.8^{ef}	44.1 ± 2.0^{b}	34.3 ± 11.2^{def}
	2000	27.7 ± 17.9^{d}	39.8 ± 13.8^{ef}	29.4 ± 17.3^{b}	40.9 ± 7.4^{cdef}
	3000	44.3 ± 10.0^{d}	33.3 ± 11.9^{ef}	31.5 ± 16.1^{b}	26.8 ± 16.8^{f}
	4000	42.7 ± 12.5^{ef}	32.4 ± 10.5^{f}	30.5 ± 11.8^{b}	33.8 ± 5.7^{def}
	5000	56.0 ± 5.5^{e}	40.2 ± 8.1^{f}	48.0 ± 0.1^{b}	28.7 ± 3.1^{ef}
0.93 a_W					
Control	0	75.8 ± 2.3^{bc}	54.5 ± 9.6^{cde}	65.2 ± 3.9^{bc}	42.2 ± 9.8^{c}
Boldo	1000	–^{c}	194.6 ± 22.6^{a}	–^{c}	–^{c}
Poleo	1000	116.7 ± 40.8^{ab}	68.8 ± 15.7^{cde}	146.8 ± 60.3^{bc}	87.7 ± 17.5^{bc}
	1500	>^{a}	137.5 ± 48.0^{b}	>^{b}	124.8 ± 56.1^{bc}
	2000	149.2 ± 33.8^{bc}	81.8 ± 9.5e	>a	91.9 ± 5.7^{c}
	2500	176.3 ± 14.6^{ab}	108.9 ± 12.1de	163.0 ± 43.3bc	107.8 ± 1.5^{bc}
	3000	–^{c}	177.9 ± 24.9 ab	–c	>^{ab}
Clove	1000	86.6 ± 5.9^{bc}	71.7 ± 3.8cde	118.7 ± 35.1bc	69.1 ± 4.1^{bc}
	2000	90.1 ± 8.1^{bc}	93.2 ± 13.0c	111.0 ± 15.6bc	96.1 ± 27.0^{bc}
	3000	78.8 ± 3.6^{bc}	81.6 ± 6.5cd	118.6 ± 39.8bc	274.0 ± 212.4^{a}
	4000	89.9 ± 13.6^{bc}	69.3 ± 3.4cde	120.8 ± 11.9bc	79.1 ± 7.7^{bc}
	5000	102.3 ± 20.1^{bc}	80.2 ± 9.6cde	89.6 ± 4.8bc	89.1 ± 19.3^{bc}

Key: ≥300 h. (–) Under these conditions the strains were not able to give visible mycelium.
Data with the same letter for each a_W are not significantly different according to Duncan's new multiple range test ($p < 0.05$).
[a] Mean of four replicates.

The presence of boldo EO volatile fraction in the surrounding atmosphere of peanut based medium had a significant effect ($p < 0.001$) on AFB_1. The accumulation of this metabolite was completely inhibited by doses ≥1500 μL/L, regardless of medium a_W. Poleo EO reduced AFB_1 accumulation between 23.1–94.0% and 43.4–100% at 0.98 and 0.95 a_W, respectively. Meanwhile an ambiguous effect was obtained at the lowest a_W level studied (0.93); *A. flavus* strains tended to show AFB_1 stimulation in the presence of poleo EO, but it inhibited (69.0–100%) the synthesis of this metabolite by *A. parasiticus* strains. Clove EO was less effective, the highest concentration assayed (5000 μL/L) was able to reduce the accumulation of AFB_1 by 21.4, 78.7 and 78.2% at 0.98, 0.95 and 0.93 a_W, respectively.

3.2. Activity of EO mixture volatile fractions on Aspergillus growth and AFB_1 accumulation

ANOVA results are shown in Table 4. The single factors substrate water availability (a_W), fungal isolates (I) and boldo and poleo EO mixtures as well as their two- and three-way interactions had a significant impact on the growth rate of *Aspergillus* section *Flavi*. The growth of the four *Aspergillus* isolates was very dependent on substrate a_W followed by EO treatments.

Mean lag phases of the four aflatoxigenic isolates at three a_W levels are shown in Table 5. At all a_W assayed the mixtures with B750–P1000 and B750–P1500 were able to produce a significant increase in lag phase, while the mixture with the lowest dose of boldo and poleo EO (B250–P500) was ineffective.

When *Aspergillus* strains grew on peanut based medium with boldo and poleo EO mixtures in the surrounding atmosphere, it was observed that all isolates has similar responses to the combination of natural preservatives and reduced a_W (Fig. 2). In general, as substrate a_W increased, fungal growth was favored. The increase of poleo concentrations from 500 to 1500 μL/L only showed a significant effect ($p < 0.05$) on *Aspergillus* growth when this oil was combined with the highest dose of boldo assayed (750 μL/L), resulting in growth inhibition increases in the order of 24.2 and 40.3% at 0.98 and 0.95 a_W, respectively. Increases in the growth rate

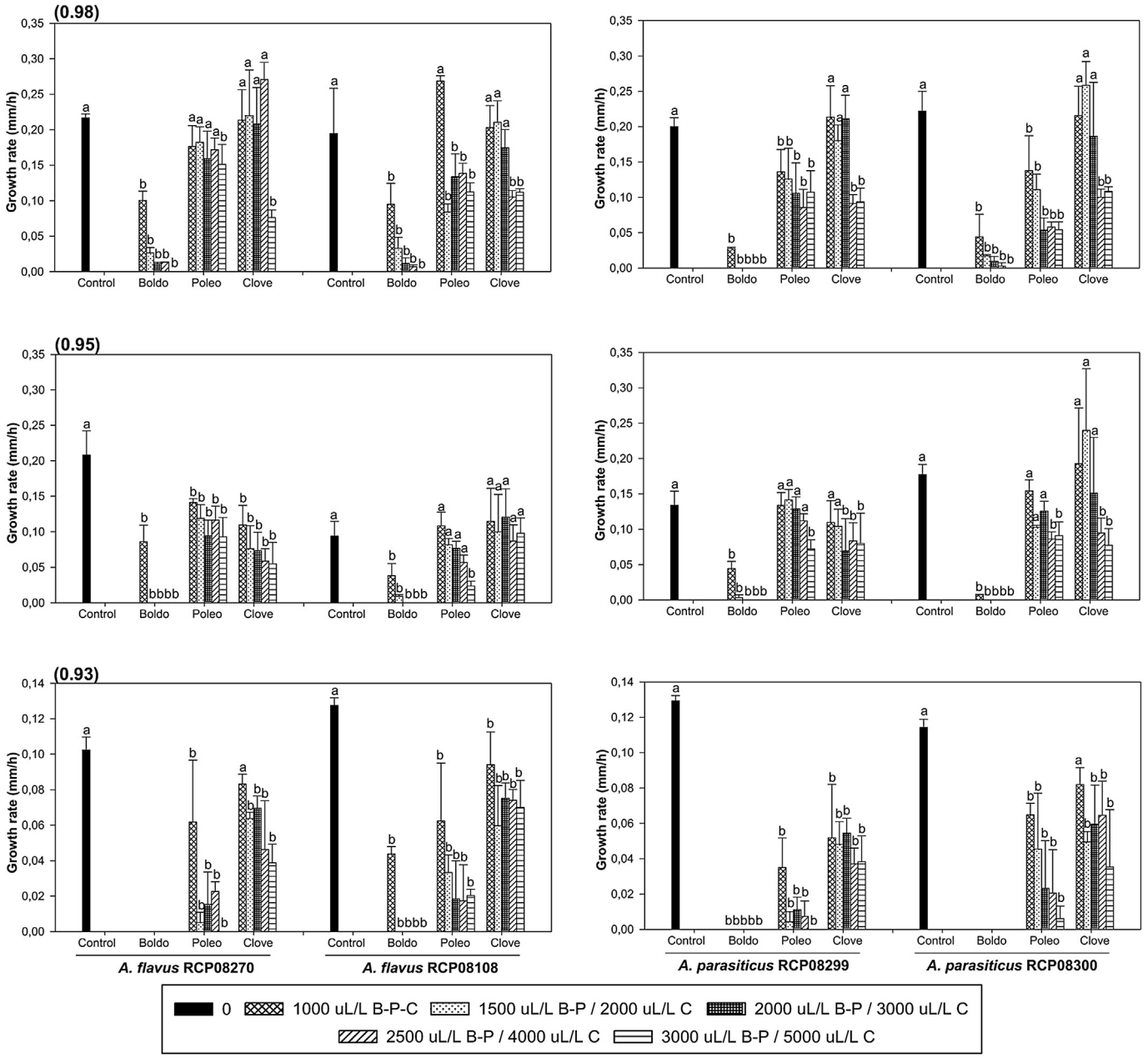

Fig. 1. Effect of EOs and a_W on growth rate of *Aspergillus flavus* and *A. parasiticus* strains on a conducive medium. Data with the same letter for each strain are not significantly different according to Duncan new multiple range's test ($p < 0.05$).

reduction generated by the lowest doses of boldo (250 and 500 μL/L) in combination with increasing doses of poleo (500 and 1500 μL/L) were in the order of 2.1 and 21.3% at 0.98 a_W and 29.4 and 33.3% at 0.95 a_W for B250 and B500, respectively. Meanwhile, under further reduction of medium a_W (0.93) and in the presence of B500–P1500 and mixtures that contained the highest dose of boldo (750 μL/L) the strains were not able to growth.

Statistical analyses showed that medium a_W, EO mixture volatile fractions and interactions between these factors all significantly affected AFB_1 content. The statistical analysis for this dataset is shown in Table 4. The effects of boldo–poleo treatments on AFB_1 accumulation after 11 days over a range of a_W conditions are shown in Table 6. In the *Aspergillus* cultures treated with the highest dose of boldo (750 μL/L), AFB_1 accumulation was reduced by 84.9 and 99.8% at 0.98 and 0.95 a_W, respectively. While the highest AFB_1 inhibition levels were observed at the lowest a_W assayed (0.93); the mixtures that contained 500 and 750 μL/L of boldo EO gave 100% inhibitions. Although, the vapor released by the treatments that contained 500 μL/L of poleo EO significantly reduced (92.9%) ($p < 0.001$) AFB_1 accumulation; the increasing concentrations of this EO (1000–1500 μL/L) did not show major inhibitory effects.

3.3. Impact of storage time, temperature, and sunlight and UV exposition on the antifungal ability of boldo and poleo EOs on Aspergillus section Flavi strains

When boldo EO was exposed to UV light during 30 min, the inhibitory activity on *A. flavus* strains was reduced by 29.1% compared to the EO without treatment, and was not modified on *A. parasiticus* strains (Table 7). Meanwhile, sunlight exposition

Table 3
Effect of EO volatile fractions on AFB_1 accumulation by *Aspergillus* section *Flavi* strains on PMEA at different a_W.

Essential oil	Doses (μL/L)	Aflatoxin B_1 (ng/g)[a]			
		A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A. parasiticus* RCP08300
0.98 a_W					
Control	0	168.4 ± 94.1^{bcd}	3787.5 ± 1939.4^{c}	22,161.3 ± 2187.1^{a}	188.3 ± 9.7^{bc}
Boldo	1000	n.d.^{e}	39.1 ± 33.9^{e}	1375.9 ± 1191.5^{ef}	n.d.^{c}
Poleo	1000	69.6 ± 26.1^{cde}	2911.3 ± 74.2^{c}	10,984.3 ± 982.9^{b}	193.4 ± 27.1^{b}
	1500	337.8 ± 345.1^{a}	1890.1 ± 496.3^{cde}	9412.3 ± 2671.8^{bcd}	41.5 ± 71.9^{bc}
	2000	55.1 ± 95.5^{cde}	208.6 ± 237.9^{de}	3356.9 ± 366.6^{ef}	498.1 ± 431.4^{a}
	2500	37.8 ± 65.4^{de}	36.4 ± 31.5^{e}	4652.7 ± 70.3^{def}	121.9 ± 105.6^{bc}
	3000	11.3 ± 19.5^{de}	267.4 ± 231.5^{de}	1323.3 ± 775.5^{ef}	16.8 ± 14.5^{bc}
Clove	1000	145.9 ± 1.3^{bcde}	2440.7 ± 982.1^{cd}	3513.1 ± 4261.4ef	120.1 ± 11.7^{bc}
	2000	215.9 ± 27.2^{abc}	3570.3 ± 1335.9^{c}	5950.6 ± 3512.6^{cde}	132.6 ± 43.6^{bc}
	3000	234.7 ± 22.4^{ab}	6458.7 ± 2753.2^{ab}	10,099.0 ± 576.9^{bc}	36.4 ± 62.9^{bc}
	4000	49.3 ± 85.4^{de}	5260.1 ± 2561.5^{b}	13,110.1 ± 6022.4^{b}	123.5 ± 28.9^{bc}
	5000	304.2 ± 4.4^{ab}	2224.7 ± 1715.1^{cde}	8716.7 ± 599.9^{bcd}	66.9 ± 57.9^{bc}
0.95 a_W					
Control	0	266.9 ± 136.6^{a}	1225.3 ± 508.5^{b}	10,634.0 ± 703.5^{a}	99.5 ± 21.0^{a}
Boldo	1000	23.6 ± 20.4^{b}	n.d.^{c}	433.3 ± 160.1^{efg}	n.d.^{b}
Poleo	1000	68.7 ± 118.9^{b}	103.2 ± 178.7^{c}	2559.0 ± 518.8^{b}	24.1 ± 41.7^{ab}
	1500	93.9 ± 162.7^{b}	10.3 ± 17.8^{c}	2116.6 ± 845.3^{bc}	56.3 ± 97.5^{a}
	2000	89.2 ± 154.5^{b}	98.8 ± 22.3^{c}	1964.4 ± 125.1^{bc}	24.6 ± 42.7^{ab}
	2500	25.5 ± 44.2^{b}	26.4 ± 45.6^{c}	1642.9 ± 130.9^{cd}	35.7 ± 61.8^{a}
	3000	64.7 ± 112.1^{b}	n.d.^{c}	1056.9 ± 249.3^{de}	13.3 ± 23.1^{ab}
Clove	1000	275.6 ± 49.2^{a}	4421.5 ± 3183.3^{a}	1807.5 ± 208.0^{c}	100.6 ± 174.2^{a}
	2000	279.4 ± 98.8^{a}	1011.9 ± 960.1^{b}	450.9 ± 653.2^{efg}	100.4 ± 5.2^{a}
	3000	278.4 ± 33.4^{a}	3789.4 ± 185.9^{a}	213.2 ± 68.1^{fg}	108.3 ± 26.0^{a}
	4000	140.4 ± 40.9^{ab}	54.3 ± 47.1^{c}	640.4 ± 28.6^{ef}	93.7 ± 162.3^{a}
	5000	66.1 ± 114.4^{b}	105.7 ± 91.6^{c}	242.8 ± 420.5^{fg}	48.8 ± 42.3^{a}
0.93 a_W					
Control	0	n.d.^{d}	20.3 ± 35.2^{d}	2872.6 ± 522.8^{a}	78.2 ± 2.8^{b}
Poleo	1000	101.1 ± 33.5^{bc}	28.3 ± 49.1^{cd}	131.4 ± 24.3^{c}	19.5 ± 33.8^{d}
	1500	24.6 + 21.3^{d}	29.5 ± 51.1^{cd}	665.8 ± 576.6^{b}	n.d.^{d}
	2000	15.4 ± 26.6^{d}	43.5 ± 75.3^{cd}	879.7 ± 662.9^{b}	n.d.^{d}
	2500	n.d.^{d}	29.5 ± 51.0^{cd}	78.9 ± 136.7^{c}	n.d.^{d}
	3000	n.d.^{d}	50.1 ± 86.8^{cd}	n.d.^{c}	n.d.^{d}
Clove	1000	223.9 ± 45.2^{a}	213.4 ± 9.8^{a}	105.6 ± 16.9^{c}	216.7 ± 25.1^{a}
	2000	21.1 ± 36.6^{d}	n.d.^{d}	16.9 ± 29.2^{c}	39.7 ± 34.4^{c}
	3000	35.3 ± 61.1^{cd}	201.5 ± 96.4^{ab}	51.4 ± 40.4^{c}	7.8 ± 13.5^{d}
	4000	149.4 ± 129.4^{b}	116.3 ± 100.7^{bc}	n.d.^{c}	8.1 ± 13.9^{d}
	5000	n.d.^{d}	17.7 ± 82.6^{d}	n.d.^{c}	n.d.^{d}

Data with the same letter for each a_W are not significantly different according to Fisher's LSD test ($p = 0.001$).
[a] Mean of four replicates.

reduced inhibitory ability of this oil by 29.6 and 30.9% at 30 and 60 min, respectively. The thermostability of the oil was tested and it was found that at temperature ranging from 40 to 80 °C, its activity was not altered. Boldo EO showed a stable behavior during the first five storage months; however at six month its inhibitory activity was reduced by 48.4 and 66.5% on *A. flavus* and *A. parasiticus* strains, respectively.

The volatile components activity of poleo EO was not altered when the oil was exposed to heat, sunlight and UV (Table 8). However, a total loss of inhibitory activity was observed at sixth month of storage, even resulting in growth stimulation.

Table 4
ANOVA test. Effects of essential oil (EO) mixtures and their concentrations (C) on growth rate and AFB_1 production by *Aspergillus* section *Flavi* isolates (I) grown on PMEA at various a_W levels.

Source of variation	Mixture assay				
	DF	Growth rate		Aflatoxin B_1	
		MS	*F* value[a]	MS	*F* value
a_W	2	0.7179	2640.57**	178,881,064.3	33.44**
I	3	0.0260	95.67**	4,678,443.9	0.87
EO	9	0.0601	221.12**	105,503,607.8	19.72**
$a_W \times I$	6	0.0083	30.57**	1,655,048.8	0.31
$a_W \times EO$	18	0.0032	11.81**	33,231,844.9	6.21*
$I \times EO$	27	0.0020	7.50**	12,122,130.4	2.27
$a_W \times I \times EO$	54	0.0012	4.56**	4,851,541.3	0.91
Error	360	0.0003		5,349,926.3	

**Highly significant at $p < 0.001$.
*Significant at $p < 0.05$.
DF: degrees of freedom; MS: mean of squares.
[a] Snedecor's *F* test.

4. Discussion

In this article we provided evidences that the volatile fraction of boldo EO has high antifungal activity against spoilage fungi *A. flavus* and *A. parasiticus*. Furthermore, the behavior of the four *Aspergillus* section *Flavi* strains was similar and the inhibition effect of three EOs (boldo, poleo and clove) increased with the a_W substrate reduction. The presence of vapors of boldo EO (≥1500 μL/L) in the surrounding atmosphere of peanut based medium, showed stronger effects on *Aspergillus* section *Flavi* strains at level of lag phase (>300 h) growth rate, (93–100% of inhibition) and AFB_1 accumulation (100% of inhibition), regardless of medium a_W. The application of poleo EO exercised effective control on growth parameters and AFB_1, but was less efficient than boldo. The inhibition produced by the volatile fractions of clove EO was not homogeneous, at doses

Table 5
Effect of EO mixture volatile fractions on lag phase of *Aspergillus* section *Flavi* strains on PMEA at different a_W.

Essential oil mixture doses (μL/L)	Lag phase (h)[a]			
	A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A. parasiticus* RCP08300
0.98 a_W				
Control	43.0 ± 2.4^{d}	19.1 ± 19.5^{e}	32.2 ± 2.7^{b}	33.2 ± 4.8^{f}
B250–P500	23.4 ± 4.2^{e}	45.4 ± 3.1^{cd}	32.5 ± 3.6^{b}	45.9 ± 1.7^{e}
B250–P1000	36.2 ± 3.9^{de}	42.6 ± 4.3^{d}	76.1 ± 4.9^{a}	47.2 ± 1.1^{e}
B250-P1500	62.1 ± 10.3^{abc}	46.0 ± 9.0^{cd}	76.5 ± 1.1^{a}	51.5 ± 1.7^{e}
B500–P500	51.7 ± 2.8^{bcd}	56.0 ± 7.0^{abc}	38.9 ± 6.0^{b}	51.5 ± 2.4^{e}
B500–P1000	41.8 ± 3.8^{d}	53.0 ± 7.9^{bcd}	50.7 ± 2.6^{b}	64.8 ± 3.3^{d}
B500–P1500	46.8 ± 6.2^{cd}	20.1 ± 5.9^{e}	39.1 ± 3.0^{b}	70.7 ± 3.1^{d}
B750–P500	61.5 ± 16.6^{abc}	56.7 ± 2.3^{abc}	51.9 ± 7.2^{b}	82.5 ± 4.6^{c}
B750–P1000	75.6 ± 24.5^{a}	60.3 ± 2.6^{ab}	83.6 ± 2.8^{a}	91.7 ± 0.0^{b}
B750–P1500	66.6 ± 10.2^{ab}	67.4 ± 3.5^{a}	87.3 ± 4.1^{a}	105.9 ± 14.6^{a}
0.95 a_W				
Control	61.2 ± 1.4^{d}	72.0 ± 0.0^{bc}	66.9 ± 2.4^{d}	55.0 ± 0.3^{e}
B250–P500	54.3 ± 1.8^{d}	77.2 ± 2.4^{bc}	55.0 ± 12.3^{d}	67.7 ± 2.3^{d}
B250–P1000	63.1 ± 1.4^{d}	91.6 ± 4.9^{bc}	67.5 ± 2.0^{d}	74.6 ± 4.4^{d}
B250–P1500	67.8 ± 6.6^{d}	94.9 ± 7.3^{bc}	141.7 ± 12.0^{b}	72.2 ± 2.0^{d}
B500–P500	47.9 ± 7.3^{d}	110.5 ± 7.2^{b}	68.1 ± 4.9^{d}	93.2 ± 1.8^{c}
B500–P1000	76.9 ± 15.6^{d}	175.1 ± 24.3^{a}	117.4 ± 26.2^{c}	89.4 ± 11.7^{c}
B500–P1500	97.9 ± 7.0 ^{cd}	59.7 ± 68.9^{c}	146.2 ± 7.4^{b}	97.2 ± 6.6^{c}
B750–P500	168.9 ± 91.1^{b}	111.1 ± 17.1^{b}	103.5 ± 1.4^{c}	124.2 ± 16.5^{b}
B750–P1000	146.2 ± 62.1^{bc}	176.6 ± 25.2^{a}	211.0 ± 19.2^{a}	150.7 ± 0.3^{a}
B750–P1500	>^{a}	–^{d}	–^{e}	159.6 ± 6.9^{a}
0.93 a_W				
Control	75.8 ± 2.3^{b}	54.5 ± 9.6^{b}	65.2 ± 3.8^{c}	42.2 ± 9.8^{cd}
B250–P500	121.3 ± 2.0^{a}	104.1 ± 1.5^{a}	133.3 ± 0.0^{b}	118.4 ± 6.0^{bcd}
B250–P1000	153.6 ± 24.1^{a}	117.2 ± 2.6^{a}	–^{d}	187.4 ± 12.0^{b}
B250–P1500	68.5 ± 79.1^{b}	78.3 ± 90.4^{ab}	–^{d}	231.3 ± 267.1^{ab}
B500–P500	149.2 ± 7.6^{a}	119.1 ± 6.6^{a}	177.9 ± 23.8^{a}	>^{a}
B500–P1000	–^{c}	–^{c}	–^{d}	143.4 ± 19.2^{bc}
B500–P1500	–^{c}	–^{c}	–^{d}	–^{d}
B750–P500	–^{c}	–^{c}	–^{d}	–^{d}
B750–P1000	–^{c}	–^{c}	–^{d}	–^{d}
B750–P1500	–^{c}	–^{c}	–^{d}	–^{d}

Key: ≥300 h. (–) Under these conditions the strains were not able to give visible mycelium.
Data with the same letter for each a_W are not significantly different according to Duncan's new multiple range test ($p < 0.05$).
[a] Mean of four replicates.

≥2000 μL/L and at 0.95 and 0.93 a_W could only significantly ($p < 0.05$) increase lag phase and inhibit fungal development, but some treatments stimulated AFB_1 accumulation. Similar findings were reported by Bluma, Landa, and Etcheverry (2009), who showed that 50 μL of boldo oil per 10 mL of culture medium totally inhibited *Aspergillus* growth at all a_W studied via the volatile diffusion method on maize based medium; while poleo and mountain thyme EOs showed an important antifungal effect which was favored by decreasing a_W.

In a recent work we studied the antifungal and antiaflatoxigenic effects of EOs applied directly on the substrate. In PMEA, boldo, poleo and clove EOs showed the best antifungal effect on growth parameters and AFB_1 accumulation at doses ≥500 μL/L (Passone et al., 2012). Boldo EO was more effective in the vapor assay than in the contact assay, showing growth inhibition percentages in the order of 91.2 and 28.2%, respectively with the application of 1500 μL/L. Several researchers have concurred that the best antifungal activity of volatile compounds is achieved by gaseous contact as opposed to aqueous solution or agar contact (Inuoye et al., 2000; Nielsen & Ríos, 2000; Tullio et al., 2006). Tyagi and Malik (2011a, 2011b) showed that in all fungal strains tested (*Penicillium digitatum*, *A. flavus*, *Aspergillus niger*, *Mucor* spp., *Rhizopus nigricans* and *Fusarium oxysporum*) the zone of inhibition resulting from the exposure to *Mentha piperita* and *Eucalyptus globules* oil vapors in disc volatilization method was significantly larger than that due to the same concentrations of these EOs in liquid phase measured via the well diffusion method. These authors also reported that higher percentage of monoterpene hydrocarbons (54.7%) present in the vapors as compared to the oil (44.5%) could be responsible for the higher antifungal activity. On the contrary, our results showed that aflatoxigenic isolates exhibited greater sensitivity to the treatments with poleo and clove EOs applied in the contact than in the volatile assay. In the first assay doses ≥2500 μL/L completely inhibited the fungal growth, while in the second assay 3000 and 5000 μL/L of poleo and clove EOs reduced fungal growth in the order of 63.6 and 40.2%, respectively. For its part, Matan et al. (2006) confirmed that higher volumes of EOs are required if the EO comes into contact with the contaminating microorganism in the vapor phase. At 3000 μL, the cinnamon and clove volatile oils used in the ratio 1:1 inhibited the growth of *A. flavus* for 19 days and 4000 μL completely inhibited growth of all molds and yeasts for more than 40 days. However, the advantages of using the volatile gas phase of EOs for food products are that it may have less influence on the final taste and aroma of the product and its release may be regulated.

In the present work, a study of the effect of boldo and poleo EO mixtures present in the headspace of peanut medium on growth and AFB_1 accumulation by *Aspergillus* section *Flavi* was performed in order to determine the existence of additive and/or synergistic effects that can increase the effectiveness of oils, to achieve inhibitory effects at lower concentrations. Comparing these results with those obtained in the pure EOs assay, the inhibitions obtained with the application of EO mixtures were similar to or less than those caused by pure boldo and poleo EOs. For example, boldo (1000 μL/L), poleo (1500 μL/L) and the mixture (B750–P1500) reduced growth rate and AFB_1 by 67.8, 39.9 and 47.2% and 98.1, 61.8 and 86.9%, respectively at 0.98 a_W. Bluma (2009) compared the growth rate of *Aspergillus* section *Flavi* strains in corn kernels with the addition of 5 EOs (poleo, anise, clove, thyme and boldo) and 10 mixtures thereof. The mixtures had a lower ability to control aspergilla growth than their respective pure oils or the effects were not statistically different. However, the combination of *Cinnamomum camphora* and *Alpinia galanga* EOs was more effective than the individual EOs, inhibiting fungal growth at 750 μL/L and AFs production at the lowest concentration tested (250 μL/L) (Bhawana, Priyanka, Ravindra, & Dubey, 2007). *Citrus maxima* Burm oil was more effective in comparison with *Citrus sinensis* (L.) Obsbeck oil and the combination of both oils. The inhibition percentages of *A. flavus* growth were 48.1, 46.2 and 44.0% with 500 μL/L of *C. maxima*, *C. sinensis* and their combination, respectively and completely inhibited AFB_1 accumulation (Priyanka et al., 2010). According to Davidson and Parish (1989), the interaction of chemical compounds of different oils can block the action of the main active components with an antagonistic effect. The inherent activity of EO can be explained in relation to the configuration of its components, the proportion in which they are present and their interaction (Delaquis, Stanich, Girard, & Mazza, 2002; Dorman & Deans, 2000; Marino, Bersani, & Comi, 2001). Compositional analyses of the more effective EOs (boldo and poleo) were carried out previously in our laboratory (Bluma & Etcheverry, 2008). The study reveled that α-terpinolene (73.8%) and α-terperpine (15.3%) are the main components present in boldo, while peperitenone oxide (48.6%) and limonene (24.5%) are the main phytochemicals of poleo. To date, there is no bibliography on the direct application of α-terpinolene and peperitenone oxide, the main phytochemicals of boldo and poleo, respectively.

The ultrastructural analysis conducted by Ahmad Khan and Ahmad (2011) has highlighted the multiple sites of action of eight EOs in fungal cells, including damage to the cell walls, cell

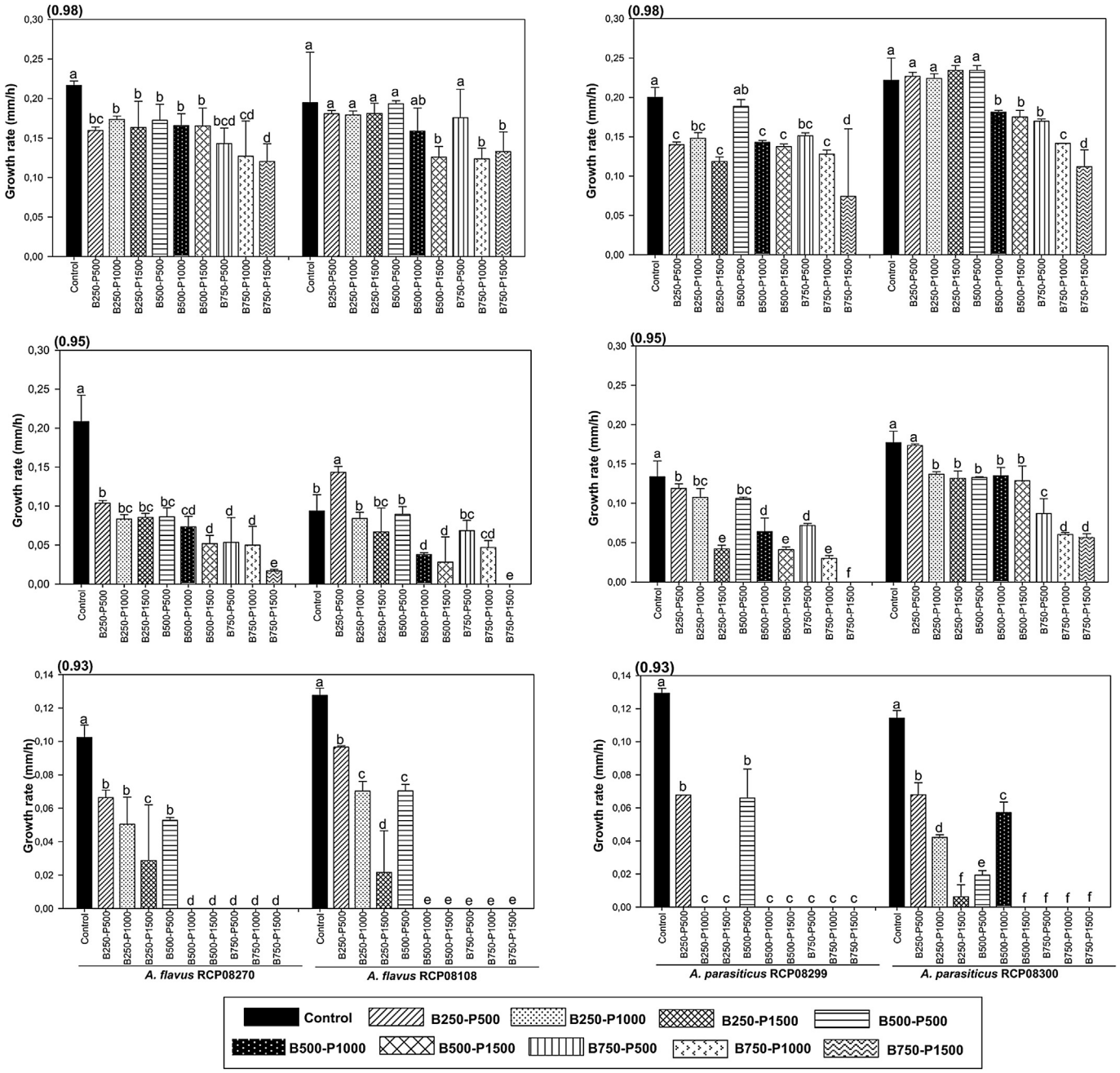

Fig. 2. Effect of EO mixtures and a_W on growth rate of *Aspergillus flavus* and *A. parasiticus* strains on a conducive medium. Data with the same letter for each strain are not significantly different according to Duncan new multiple range's test ($p < 0.05$).

membranes, and cytoplasmic contents of *Aspergillus fumigatus* and *Trichophyton rubrum*. In addition, the authors also demonstrated that test oils inhibited elastase and keratinase activities. It is believed that lipophilic properties of oils may assist in the penetration of cell membranes and in the accumulation of polysaccharides under water stress conditions. This may lead to rupture of plasmalema in fungal cells (Ultee, Bennink, & Moezelaar, 2002).

In this work, we also evaluated the effect of environmental factors on boldo and poleo EOs, and it was shown that the antimicrobial ability of these oils is subject to changes or variations due to many factors related to the type system and storage time. Mishra and Dubey (1994) observed that lemongrass leaves EO (*Cymbopogon citratus*) could inhibit in a 100% *A. flavus* growth at a concentration of 3000 μL/L. These researchers evaluated whether the inhibitory ability of oil is modified when exposed to 5, 10, 40, 80 and 100 °C, ensuring that the EO showed a heat-stable behavior, without modifying its antifungal properties against *A. flavus*. Sharma and Tripathi (2008) conducted experiments to determine the thermostable nature of *C. sinensis* (L.) oil. The authors evaluated the antifungal activity of EO (3.0 mg/mL) in agar medium against *A. niger* after being subjected to temperatures of 40, 60, 80 and 100 °C, and after being autoclaved (121 °C, 15 min). The activity of *C. sinensis* did not change with either treatment. In our study the inhibitory ability of boldo and poleo oil volatile fractions remained stable against temperature changes, while poleo oil completely lost their inhibitory activity at the sixth month of storage. The antifungal activity of boldo EO decreased when exposed to other environmental variables tested. The antimicrobial activity of EOs

Table 6
Effect of EO mixture volatile fractions on AFB_1 accumulation by *Aspergillus* section *Flavi* strains on PMEA at different a_W.

Essential oil mixture doses (μL/L)	Aflatoxin B_1 (ng/g)[a]			
	A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A parasiticus* RCP08300
0.98 a_W				
Control	168.4 ± 94.0^{a}	3787.5 ± 1939.4^{b}	22,161.3 ± 2187.1^{a}	188.3 ± 9.7^{a}
B250–P500	80.3 ± 0.0^{bc}	139.6 ± 14.9^{c}	3857.2 ± 136.2^{c}	38.9 ± 9.8^{bc}
B250–P1000	69.3 ± 20.1^{bc}	75.1 ± 10.3^{c}	906.7 ± 62.6^{e}	25.2 ± 1.6^{bc}
B250–P1500	68.8 ± 59.6^{bc}	44.9 ± 38.9^{c}	2995.0 ± 28.0^{cd}	12.7 ± 11.0^{c}
B500–P500	86.3 ± 30.1^{bc}	217.4 ± 60.2^{c}	3791.8 ± 857.4^{c}	33.3 ± 28.9^{bc}
B500–P1000	126.1 ± 35.1^{ab}	56.1 ± 5.1^{c}	5705.0 ± 812.1^{b}	43.4 ± 12.0^{bc}
B500–P1500	52.5 ± 2.4^{cd}	8138.3 ± 3377.5^{a}	2015.8 ± 247.5^{de}	36.3 ± 1.5^{bc}
B750–P500	n.d.^{d}	236.8 ± 283.6^{c}	3480.2 ± 1128.9^{cd}	62.3 ± 54.0^{b}
B750–P1000	35.7 ± 30.9^{cd}	97.2 ± 84.2^{c}	2646.7 ± 81.3^{cd}	70.2 ± 60.8^{b}
B750–P1500	65.6 ± 0.6^{bcd}	24.4 ± 21.1^{c}	2770.6 ± 284.7^{cd}	n.d.^{c}
0.95 a_W				
Control	266.9 ± 136.6^{ab}	1225.3 ± 508.5^{a}	10,634.0 ± 703.5^{a}	99.4 ± 21.0^{a}
B250–P500	312.4 ± 167.4^{a}	45.7 ± 39.5^{b}	1104.1 ± 46.1^{b}	26.6 ± 8.4^{b}
B250–P100	269.8 ± 79.8^{ab}	53.5 ± 11.6^{b}	716.8 ± 51.9^{bc}	14.3 ± 12.4^{bc}
B250–P1500	221.1 ± 78.5^{abc}	45.5 ± 18.4^{b}	405.2 ± 350.9^{cd}	13.0 ± 11.3^{bc}
B500–P500	99.7 ± 99.3^{cd}	56.0 ± 48.5^{b}	98.4 ± 11.9^{d}	16.1 ± 14.0^{bc}
B500–P1000	129.2 ± 111.9^{bcd}	54.3 ± 47.0^{b}	230.5 ± 199.6^{d}	n.d.^{c}
B500–P1500	n.d.^{d}	n.d.^{b}	57.0 ± 49.4^{d}	n.d.^{c}
B750–P500	n.d.^{d}	n.d.^{b}	168.3 ± 0.0^{d}	n.d.^{c}
B750–P1000	n.d.^{d}	n.d.^{b}	73.3 ± 63.5^{d}	n.d.^{c}
B750–P1500	n.d.^{d}	n.d.^{b}	n.d.^{d}	n.d.^{c}
0.93 a_W				
Control	n.d.^{b}	20.3 ± 35.2^{b}	2872.6 ± 522.8^{a}	78.2 ± 2.8^{a}
B250–P500	29.0 ± 25.1^{a}	n.d.^{b}	16.8 ± 14.5^{b}	n.d.^{b}
B250–P100	25.3 ± 21.9^{a}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B250–P1500	22.8 ± 19.7^{a}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B500–P500	n.d.^{b}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B500–P1000	n.d.^{b}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B500–P1500	n.d.^{b}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B750–P500	n.d.^{b}	n.d.^{b}	n.d.^{b}	n.d.^{b}
B750–P1000	n.d.^{b}	84.8 ± 73.4^{a}	n.d.^{b}	n.d.^{b}
B750–P1500	n.d.^{b}	n.d.^{b}	n.d^{b}.	n.d.^{b}

Data with the same letter for each a_W are not significantly different according to Fisher's LSD Test ($p = 0.001$).
[a] Mean of four replicates.

generally decreases as they age and when exposed to light due to the oxidation suffered by constituents (Burt, 2004).

In conclusion, boldo EO appears to be more effective than the rest of EO evaluated, this suggests that boldo EO volatile fraction at doses ≥1500 μL/L is an effective potential compound for complete inhibition of *Aspergillus* section *Flavi* growth and AFB_1 production over a range of environmental conditions in peanut based medium. However, the results of this work also confirmed that the highest dose of poleo EO presented antifungal and antiaflatoxigenic activity. A further *in situ* study is required to confirm the antifungal activity of these oils in the vapor phase, which may be used for preservation and/or extension of the shelf life of stored peanuts.

Table 7
Impact of storage time, temperature, and sunlight and UV exposition on the antifungal ability of boldo EO on A*spergillus* section *Flavi* strains.

Doses (μL/L)	Treatment		Growth rate (mm/h)[a]			
			A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A. parasiticus* RCP08300
0	Control		0.22 ± 0.00^{a}	0.19 ± 0.06^{a}	0.20 ± 0.01^{a}	0.22 ± 0.03^{a}
2000	Untreated EO		0.01 ± 0.00^{e} (95.4%)	0.01 ± 0.00^{ef} (94.9%)	0.00 ± 0.00^{f} (100%)	0.01 ± 0.00^{e} (95.4%)
	UV	15 min	0.00 ± 0.00^{e} (100%)	0.11 ± 0.02^{bc} (42.1%)	0.01 ± 0.00^{f} (95.0%)	0.00 ± 0.00^{e} (100%)
		30 min	0.08 ± 0.05^{cd} (63.6%)	0.06 ± 0.03^{d} (68.4%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{e} (100%)
	Temperature	40 °C	0.00 ± 0.00^{e} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{e} (100%)
		60 °C	0.00 ± 0.00^{e} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{e} (100%)
		80 °C	0.00 ± 0.00^{e} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{f} (100%)	0.00 ± 0.00^{e} (100%)
	Sunlight	30 min	0.06 ± 0.01^{d} (72.7%)	0.11 ± 0.00^{bc} (42.1%)	0.05 ± 0.01^{e} (75.0%)	0.05 ± 0.01^{d} (77.2%)
		60 min	0.06 ± 0.01^{d} (72.7%)	0.11 ± 0.00^{bc} (42.1%)	0.06 ± 0.00^{d} (70.0%)	0.05 ± 0.00^{d} (77.2%)
	Storage time	T1	0.09 ± 0.00^{c} (59.1%)	0.13 ± 0.01^{bc} (31.5%)	0.07 ± 0.00^{d} (65.0%)	0.09 ± 0.00^{c} (59.1%)
		T2	0.09 ± 0.00^{c} (59.1%)	0.14 ± 0.00^{b} (26.3%)	0.09 ± 0.00^{c} (55.0%)	0.05 ± 0.00^{d} (77.2%)
		T3	0.07 ± 0.00^{cd} (68.2%)	0.03 ± 0.02^{de} (84.2%)	0.05 ± 0.00^{e} (75.0%)	0.08 ± 0.00^{c} (63.6%)
		T4	0.09 ± 0.00^{c} (59.1%)	0.10 ± 0.00^{c} (47.3%)	0.09 ± 0.00^{c} (55.0%)	0.06 ± 0.00^{d} (72.7%)
		T5	0.05 ± 0.00^{d} (77.3%)	0.06 ± 0.01^{d} (68.4%)	0.06 ± 0.00^{d} (70.0%)	0.05 ± 0.00^{d} (77.3%)
		T6	0.13 ± 0.00^{b} (40.9%)	0.09 ± 0.00^{c} (52.6%)	0.13 ± 0.00^{b} (35.0%)	0.16 ± 0.00^{b} (27.3%)

Data with the same letter for each a_W are not significantly different according to Duncan's new multiple range test ($p < 0.05$).
[a] Mean of four replicates.

Table 8
Impact of storage time, temperature, and sunlight and UV exposition on the antifungal ability of poleo EO on Aspergillus section *Flavi* strains.

Doses (μL/L)	Treatment		Growth rate (mm/h)[a]			
			A. flavus RCP08270	*A. flavus* RCP08108	*A. parasiticus* RCP08299	*A. parasiticus* RCP08300
0	Control		0.22 ± 0.00^{b}	0.19 ± 0.06^{b}	0.20 ± 0.01^{b}	0.22 ± 0.03^{b}
2000	Untreated EO		0.16 ± 0.04^{bcd} (27.3%)	0.13 ± 0.03^{cd} (31.6%)	0.10 ± 0.04^{b} (50.0%)	0.05 ± 0.01^{b} (77.3%)
	UV	15 min	0.15 ± 0.07^{bcd} (31.8%)	0.12 ± 0.03^{cde} (36.8%)	0.15 ± 0.08^{b} (25.0%)	0.09 ± 0.01^{b} (59.1%)
		30 min	0.18 ± 0.05^{bc} (18.2%)	0.12 ± 0.04^{cd} (36.8%)	0.04 ± 0.00^{b} (80.0%)	0.11 ± 0.03^{b} (50.0%)
	Temperature	40 °C	0.06 ± 0.04^{gh} (72.7%)	0.07 ± 0.02^{e} (63.1%)	0.20 ± 0.01^{b} (0.0%)	0.12 ± 0.00^{b} (45.4%)
		60 °C	0.03 ± 0.02^{h} (86.4%)	0.08 ± 0.06^{de} (57.8%)	0.21 ± 0.00^{b} (−5%)	0.09 ± 0.01^{b} (59.1%)
		80 °C	0.14 ± 0.00^{cde} (36.4%)	0.15 ± 0.00^{bc} (21.0%)	0.20 ± 0.00^{b} (0.0%)	0.11 ± 0.00^{b} (50.0%)
	Sunlight	30 min	0.11 ± 0.08^{defg} (50.0%)	0.12 ± 0.02^{cde} (36.8%)	0.15 ± 0.06^{b} (25%)	0.10 ± 0.07^{b} (54.5%)
		60 min	0.15 ± 0.05^{cd} (31.8%)	0.12 ± 0.03^{cde} (36.8%)	0.18 ± 0.05^{b} (10%)	0.10 ± 0.03^{b} (54.5%)
	Storage time	T1	0.10 ± 0.01^{defg} (54.5%)	0.12 ± 0.02^{cd} (36.8%)	0.11 ± 0.00^{b} (45.0%)	0.09 ± 0.00^{b} (59.1%)
		T2	0.13 ± 0.00^{cdef} (40.9%)	0.13 ± 0.01^{cd} (31.6%)	0.12 ± 0.00^{b} (40.0%)	0.09 ± 0.00^{b} (59.1%)
		T3	0.11 ± 0.00^{defg} (50.0%)	0.19 ± 0.00^{b} (0.0%)	0.18 ± 0.01^{b} (10.0%)	0.10 ± 0.01^{b} (54.5%)
		T4	0.16 ± 0.03^{bcd} (27.3%)	0.19 ± 0.01^{b} (0.0%)	0.13 ± 0.04^{b} (35.0%)	0.10 ± 0.01^{b} (54.5%)
		T5	0.18 ± 0.02^{bc} (18.2%)	0.16 ± 0.00^{bc} (15.8%)	0.14 ± 0.00^{b} (30.0%)	0.10 ± 0.01^{b} (54.5%)
		T6	0.37 ± 0.00^{a} (−68.2%)	0.27 ± 0.01^{a} (−42.1.%)	0.52 ± 0.55^{a} (−160.0%)	0.54 ± 0.53^{a} (−145%)

Data with the same letter for each a_W are not significantly different according to Duncan's new multiple range test ($p < 0.05$).
[a] Mean of four replicates.

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References

Abdollahi, A., Hassani, A., Ghosta, Y., Meshkatalsada, M. H., & Shabani, R. (2011). Screening of antifungal properties of essential oils extracted from sweet basil, fennel, summer savory and thyme against postharvest phytopathogenic fungi. *Journal of Food Safety, 31*, 350–356.

Ahmad Khan, M. S., & Ahmad, I. (2011). *In vitro* antifungal, anti-elastase and anti-keratinase activity of essential oils of *Cinnamomum*-, *Syzygium*- and *Cymbopogon* species against *Aspergillus fumigatus* and *Trichophyton rubrum*. *Phytomedicine, 19*, 48–55.

Ávila-Sosa, R., Palou, E., Jiménez Munguía, M. T., Nevárez-Moorillón, G. V., Navarro Cruz, A. R., & López-Malo, A. (2012). Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. *International Journal of Food Microbiology, 153*, 66–72.

Bhawana, S., Priyanka, S., Ravindra, S., & Dubey, N. K. (2007). A novel combination of essential oils of *Cinnamomum camphora* and *Alpinia galanga* in checking aflatoxin B1 production by toxigenic strain of *Aspergillus flavus*. *World Journal of Microbiology and Biotechnology, 24*, 693–697.

Bluma, R. (2009). *Sustancias de origen biológico para el control de hongos aflatoxicogénicos en maíz: Eficacia de los extractos bacterianos y vegetales*. PhD Thesis. National University of Río Cuarto.

Bluma, R., & Etcheverry, M. (2008). Application of essential oils in maize grain: impact on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. *Food Microbiology, 25*, 324–334.

Bluma, R., Landa, M. F., & Etcheverry, M. (2009). Impact of volatile compounds generated by EOs on *Aspergillus* section *Flavi* growth parameters and aflatoxin accumulation. *Journal of the Science of Food and Agriculture, 89*, 1473–1480.

Bullerman, L. B., Lieu, E. Y., & Seiler, S. A. (1977). Inhibition of growth and aflatoxin production by cinnamon and clove oils, cinnamic aldehyde and eugenol. *Journal of Food Science, 42*, 1107–1109.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in food, a review. *International Journal of Food Microbiology, 94*, 223–253.

Dallyn, H., & Fox, A. (1980). Spoilage of material of reduced water activity by xerophilic fungi. In G. H. Gould, & E. L. Corry (Eds.), *Society of applied bacteriology technical series* (pp. 129–139). London: Academic Press.

Davidson, P. M., & Parish, M. E. (1989). Methods for testing the efficacy of food antimicrobials. *Food Technology, 43*, 148–155.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology, 74*, 101–109.

Ding, X., Li, P., Bai, Y., & Zhou, H. (2012). Aflatoxin B_1 in post-harvest peanuts and dietary risk in China. *Food Control, 23*, 143–148.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology, 88*, 308–316.

Dubey, N. K., Shukla, R., Kumar, A., Singh, P., & Prakash, B. (2010). Prospectives of botanical pesticides in sustainable agriculture. *Current Science, 98*, 479–480.

El-Nagerabi, S. A. F., Al-Bahry, S. N., Elshafie, A. E., & AlHilali, S. (2012). Effect of *Hibiscus sabdariffa* extract and *Nigella sativa* oil on the growth and aflatoxin B_1 production of *Aspergillus flavus* and *Aspergillus parasiticus* strains. *Food Control, 25*, 59–63.

European Commission. (2010). Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006. Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Official Journal of the European Union, L50*, 8–12.

Ezekiel, C. N., Sulyok, M., Babalola, D. A., Warth, B., Ezekiel, V. C., & Krska, R. (2012). Incidence and consumer awareness of toxigenic *Aspergillus* section *Flavi* and aflatoxin B_1 in peanut cake from Nigeria. *Food Control*, http://dx.doi.org/10.1016/j.foodcont.2012.07.048.

Food and Drug Administration. (2009). *CPG Sec. 570.375 aflatoxin in peanuts and peanut products*. Available at: http://www.fda.gov/ICECI/ComplianceManuals.

Galvano, F., Ritieni, A., Piva, G., & Pietri, A. (2005). Mycotoxins in the human food chain. In D. E. Diaz (Ed.), *The mycotoxin blue book* (pp. 187–225). England: Nottingham University Press.

Geisen, R. (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Applied Microbiology, 19*, 388–392.

International Agency for Research on Cancer (IARC). (1993). *Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins*. In *IARC monographs on the evaluation of carcinogenic risks to humans, aflatoxins*, Vol. 56, (pp. 245–395).

International Agency for Research on Cancer (IARC). (2002). *Some traditional herbal medicines, some mycotoxins, naphthalene and styrene*. In *IARC monographs on the evaluation of carcinogenic risks to humans, aflatoxins*, Vol. 82, (pp. 1–556).

Inuoye, S., Tsuruoka, M., Watanabe, M., Takeo, A., Akao, M., Nishiyama, Y., et al. (2000). Inhibitory effect of essential oils on apical growth of *Aspergillus flavus* by vapor contact. *Mycoses, 43*, 17–23.

Kamika, I., & Takoy, L. L. (2011). Natural occurrence of aflatoxin B_1 in peanut collected from Kinshasa, Democratic Republic of Congo. *Food Control, 22*, 1760–1764.

Leimann, F. V., Gonçalves, O. H., Machado, R. A. F., & Bolzan, A. (2009). Antimicrobial activity of microencapsulated lemongrass essential oil and the effect of experimental parameters on microcapsules size and morphology. *Materials Science and Engineering C, 29*, 430–436.

Li, F., Yoshizawa, T., Kawamura, O., Luo, X., & Li, Y. (2001). Aflatoxins and fumonisins in corn from the high incidence area for human hepatocellular carcinoma in Guangxi, China. *Journal of Agricultural and Food Chemistry, 49*, 4122–4126.

MAGyP, Ministerio de Agricultura, Ganadería y Pesca. (2012). *Informe Mensual de Estimaciones*. Official dates 15/02/2012. Harvest 2010/2011. Available at: www.siia.gov.ar.

Mallozzi, M. A. B., Correa, B., Haraguchi, M., & Brignani, F. N. (1996). Effect of flavonoids on *Aspergillus flavus* growth and aflatoxin production. *Review in Microbiology, 27*, 161–167.

Marandi, R. J., Hassani, A., Ghosta, Y., Abdollahi, A., Pirzad, A., & Sefidkon, F. (2011). Improving postharvest quality of table grape cv. "rish baba" using *Thymus kotschyanus* and *Carum copticum* essential oils. *Journal of Food Safety, 31*, 132–139.

Marín, S., Sanchis, V., & Magan, N. (1995). Water activity, temperature and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Canadian Journal of Microbiology, 41*, 1063–1070.

Marino, M., Bersani, C., & Comi, G. (2001). Impedance measurements to study the antimicrobial activity of essential oils from *Lamiacea* and *Compositate*. *International Journal of Food Microbiology, 67*, 187–195.

Matan, N., Rimkeeree, H., Mawson, A. J., Chompreeda, P., Haruthaithanasan, V., & Parker, M. (2006). Antimicrobial activity of cinnamon and clove oils under modified atmosphere conditions. *International Journal of Food Microbiology, 107*, 180–185.

McKean, C., Tang, L., Tang, M., Billam, M., Wang, Z., Theodorakis, C. W., et al. (2006). Comparative acute and combinative toxicity of aflatoxin B_1 and fumonisin B_1 in animals and human cells. *Food and Chemical Toxicology, 44*, 868–876.

Mishra, A. K., & Dubey, N. K. (1994). Evaluation of some essential oils for their toxicity against fungi causing deterioration of stored food commodities. *Applied Environmental Microbiology, 60*, 1101–1105.

Nielsen, V., & Ríos, R. (2000). Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oils. *International Journal of Food Microbiology, 60*, 219–229.

Passone, M. A., Girardi, N. S., Ferrand, C. A., & Etcheverry, M. (2012). *In vitro* evaluation of five essential oils as botanical fungitoxicants for the protection of stored peanut from *Aspergillus flavus* and *A. parasiticus* contamination. *International Biodeterioration & Biodegradation, 70*, 82–88.

Passone, M. A., Resnik, S. L., & Etcheverry, M. G. (2005). *In vitro* effect of phenolic antioxidants on germination, growth and aflatoxin B_1 accumulation by peanut *Aspergillus* section *Flavi*. *Journal of Applied Microbiology, 99*, 682–691.

Passone, M. A., Rosso, L. C., Ciancio, A., & Etcheverry, M. (2010). Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production. *International Journal of Food Microbiology, 138*, 276–281.

Pitt, J. (1979). *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces*. London: Academic Press.

Prakash, B., Singh, P., Kedia, A., Singh, A., & Dubey, N. K. (2012). Efficacy of essential oil combination of *Curcuma longa* L. and *Zingiber officinale* Rosc. as a postharvest fungitoxicant, aflatoxin inhibitor and antioxidant agent. *Journal of Food Safety*, http://dx.doi.org/10.1111/j.1745-4565.2012.00378.

Priyanka, S., Ravindra, S., Bhanu, P., Ashok, K., Shubhra, S., Prashant, K. M., et al. (2010). Chemical profile, antifungical, antiaflatoxigenic and antioxidant activity of *Citrus maxima* Burm. and *Citrus sinensis* (L.) Osbeck essential oils and their cyclic monoterpene, DL-limonene. *Food and Chemical Toxicology, 48*, 1734–1740.

Rocha Vilela, G., de Almeida, G. S., Regitano D'Arce, M. A. B., Duarte Moraes, M. H., Brito, J. O., da Silva, M. F., et al. (2009). Activity of essential oil and its major compound, 1,8-cineole, from *Eucalyptus globulus* Labill., against the storage fungi *Aspergillus flavus* Link and *Aspergillus parasiticus* Speare. *Journal of Stored Products Research, 45*, 108–111.

Sharma, N., & Tripathi, A. (2008). Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiological Research, 163*, 337–344.

Shukla, R., Singh, P., Prakash, B., & Dubey, N. K. (2012). Antifungal, aflatoxin inhibition and antioxidant activity of *Callistemon lanceolatus* (Sm.) sweet essential oil and its major component 1,8-cineole against fungal isolates from chickpea seeds. *Food Control, 25*, 27–33.

Soliman, K. M., & Badeaa, R. I. (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food and Chemical Toxicology, 40*, 1669–1675.

Tolouee, M., Alinezhad, S., Saberi, R., Eslamifar, A., Zad, S. J., Jaimand, K., et al. (2010). Effect of *Matricaria chamomilla* L. flower essential oil on the growth and ultrastructure of *Aspergillus niger* van Tieghem. *International Journal of Food Microbiology, 139*, 127–133.

Trucksses, M. W., Stack, M. E., Nesheim, S., Albert, R. H., & Romer, T. R. (1994). Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1, G_2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. *Journal of AOAC International, 6*, 1512–1521.

Tullio, V., Nostro, A., Mandras, N., Dugo, P., Banche, G., Cuffini, A. M., et al. (2006). Antifungal activity of essential oils against filamentous fungi determined by broth microdilution and vapour contact methods. *Journal of Applied Microbiology, 102*, 1544–1550.

Turner, P. C., Moore, S. E., Hall, A. J., Prentice, A. M., & Wild, C. P. (2003). Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environmental Health Perspectives, 111*, 217–220.

Tyagi, A. K., & Malik, A. (2011a). Antimicrobial potential and chemical composition of *Eucalyptus globulus* oil in liquid and vapour phase against food spoilage microorganisms. *Food Chemistry, 126*, 228–235.

Tyagi, A. K., & Malik, A. (2011b). Antimicrobial potential and chemical composition of *Mentha piperita* oil in liquid and vapour phase against food spoiling microorganisms. *Food Control, 22*, 1707–1714.

Ultee, A., Bennink, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the foodborne pathogens *Bacillus cereus*. *Applied Environmental Microbiology, 68*, 1561–1568.

Usai, M., Marchetti, M., Foddai, M., Del Caro, A., Desogus, R., Sanna, I., et al. (2011). Influence of different stabilizing operations and storage time on the composition of essential oil of thyme (*Thymus officinalis* L.) and rosemary (*Rosmarinus officinalis* L.). *LWT – Food Science and Technology, 44*, 244–249.

USDA. (2012). *United States Department of Agriculture Oilseeds*. Available at: http://www.fas.usda.gov.

Varga, J., Frisvad, J. C., & Samson, R. A. (2011). Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Studies in Mycology, 69*, 57–80.