Antimicrobial Properties of Selected Essential Oils in Vapour Phase against *Aspergillus flavus*

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Abstract

The aim of this study was to evaluated antifungal and antitoxigenic properties of essential oils (EOs) in vapor phase. *In vitro* antifungal activity of essential oils against three fungal strains (*Aspergillus flavus* (MC1, MC4, and MC33) was evaluated by microatmosphere method and their ability to affect production of mycotoxins by thin-layer chromatography (TLC) method. Each fungus was inoculated in the center on Czapek Yeast Autolysate Agar (CYA) Ø 90 mm dishes. Dishes were incubated for fourteen days at 25 ± 1 °C (three replicates were used for each treatment). The best result were shown by *Origanum vulgare* L. (MFC 6% (6/94; v/v) against all of the strains, followed by *Thymus vulgaris* L. > *Syzygnium aromaticum* L. > *Levadula angustifolia* MILLER > *Mentha piperita* L. > *Salvia officinalis* L. > *Eucalyptus globulus* LABILL. After 14 days of incubation with EOs (100%) with control sets, they were screened for a production of mycotoxins. Result showed, that some of the tested EOs affected mycotoxins production. In conclusion, volatile substances from tested essential oils in this work had a potential antifungal activity against *A. flavus* and their production of mycotoxins, and they should find a practical application in food.

Keywords: *Aspergillus flavus, Essential oils, Mycotoxins, Vapours*

1. Introduction

Fungi are regarded as one of the main concerns in food storage. Fungal contamination with poisonous mycotoxins could lead to major health problems while it obviously deteriorates the nutritive value of food [1, 2]. According to the estimation by FAO, one third of world's crop production and 20% in European Union is annually contaminated by microbes and their mycotoxins [3]. Fungus *Aspergillus flavus* has received a lot of attention due to its severe impact on agriculture and fermented products production caused by aflatoxin secretion [4]. Aflatoxins are carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive, and can inhibit several metabolic systems [5]. Another mycotoxin produce by *A. flavus*, cyclopiazonic acid (CPA), causes necrotic foci in internal organs such as the liver and exerts neurotoxic effects [6]. Isolates of *A. flavus* produce type B (B_1 and B_2) aflatoxins (AFB), or CPA, or both or neither [7–9]. Of great concern is the natural co–occurrence of CPA with aflatoxins since many strains of *Aspergillus flavus* produce both CPA and aflatoxins [10, 11]. In the past decade, due to regarding safety concerns of the synthetic antimicrobial agents, the particular interest has been focused on the potential applications of essential oils as alternative chemical control measure. They have a broad spectrum of antifungal properties [12, 13] and they are environmentally friendly (biodegradable, do not leave toxic residues or by-products to contaminate the environment) [14]. Recently, there has been increasing interest in using naturally occurring compounds, especially EOs, to control food spoilage fungi *in vitro* and *in vivo*

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[15–17]. In previous studies, EOs of *Thymus*, *Eucalyptus*, *Oregano*, *Lavender* and *Cinnamomum* species were tested against *Aspergillus* species [17, 18], including *A. flavus* [19, 20]. The biosynthesis of aflatoxin B_1 (AFB_1) can be inhibited by extracts of certain plants that are toxic to fungi and may be useful in controlling fungal growth and mycotoxin production [21]. Natural compounds, such as flavonoids, stilbene, essential oils (EOs), and others were also active in inhibition of aflatoxin production [22].

This study was undertaken to investigate the *in vitro* inhibitory effects of selected essential oils, differing in chemical composition, by vapours contact, against 3 post-harvest fungi isolated from different food, antifungal activity of EOs *in vitro* as well as their ability to affect production of aflatoxins, concretely AFB_1 and cyclopiazonic acid (CPA).

2. Materials and methods

Fungal isolates. A total of three strains of *Aspergillus flavus* (MC1, MC4 and MC33) isolated from different food products (bread, peanuts, etc.) were used. These isolates belong to the collection of microorganisms at the Department of Microbiology of the SUA in Nitra. They were inoculated on Czapek Yeast Autolysate Agar (CYA) [23] dishes.

Essential plant oils. The essential oils used in this study were extracts of oregano *Origanum vulgare* L (oregano), *Lavandula angustifolia* MILLER. (lavender), *Eucalyptus globulus* LABILL. (eucalyptus), *Salvia officinalis* L. (sage), *Thymus vulgaris* L. (thyme), *Syzygnium armaticum* L. (clove) and *Mentha piperita* L. (mentha). They were all supplied by Calendula company a.s. (Nová Ľubovňa, 238 A, Slovakia). The gas chromatography analysis of main components of each essential oils were determined by Calendula company a.s. (Table 1). Essential oils were extracted by hydro distillation and its quality and stability were certified by suppliers.

Table 1. The major constituents of essential oils analyzed by Calendula company a.s.

Thyme **compound**	**%**	***Lavender*** **compound**	**%**	***Eucalyptus*** **compound**	**%**	***Clove*** **compound**	**%**
ρ –cymen	15–0.28	Limonene cineole	< 1.0	α–pinene β–pinene sabinene	9.0 max. 1.5	ρ–cymene	40±3
linalool	4.0–6.5	3–octanone camphor	< 2.5	α–phellandrene	max. 0.3	thymol	32±2
terpinén–4–ol	0.2–2.5	linalool linalyl	0.1–2.5	limonene 1.8–cineole camphor	max. 1.5 12 min.		
thymol	36–55	acetate terpinen–4–ol lavandulyl acetate	< 1.2 20–45		70		
carvacrol	1.0–4.0	lavandulol α–terpineol	25–46 0.1–6.0		max. 0.1		
β–myrcen	1.0–3.0		> 0.2				
γ–terpinen	5.0–10.0		> 0.1				
			< 2.0				

Sage **compound**	%	***Oregano*** **compound**	**%**	***Mint*** **compound**	**%**
1.8–cineole	min. 5.0	carvacrol	min.50	limonen cineole	1.0–3.5
thujone borneole	min. 15.0			menthon	3.5–8.0
	min. 5.0			menthofuran	14.0–32.0
				izomenthon	1.0–8.0
				menthylacetate	1.5–10.0
				izopulegol	2.8–10.0
				menthol pulegon	max. 0.2
				carvone	30.0–55.0
				cineol/limonene	max. 3.0
					max. 1.0
					min. 2

Microatmosphere method

The antifungal activity of selected EOs was investigated by microatmosphere method. The test was performed in sterile Petri dishes (Ø 90 mm) containing 15 ml of CYA. Evaluation by filter paper was made by the method adapted from [24]. Dishes were kept in an inverted position. A sterilized filter paper (square of 1 x1 cm) was placed in the center of the lid and 50 µl of pure EOs (100/0; v/v; oil/diluent) were added on it. Filter paper disks impregnated with dimethyl sulfoxide (DMSO) (50 µl) were only used as a control to confirm no solvent effect of bioactivity. Each fungus was inoculated in the center on Petri dishes with needle–inoculated. Dishes were tightly sealed with parafilm and incubated for fourteen days at 25 ± 1 °C (three replicates were used for each treatment). Diameters (Ø mm) of the growing colonies were measured at the 3rd, 7th, 11th and 14th day with a ruler. Essential oils able to inhibit each fungus (visible inhibition–non growth of fungus) were used in the following test.

Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration (MFC) of the essential oils with the most significant activity was determined by method of graded concentration of oils. The essential oils dissolved in DMSO (dimethyl sulfoxide) were prepared at concentration of 80/20, 60/40, 50/50, 30/70, 20/80 and 10/90 (v/v; oil/diluent) and 8/92, 6/94, 5/95 and 3/97 (v/v; oil/diluent) for oregano EO. Cultivation was carried out the same way as before. The test were performed in triplicate. The MFC was regarded as the lowest concentration of the oil that did not permit any visible growth in comparison with control sets.

Mycotoxins screening by modified agar plug method

After microatmosphere method of 14 days of cultivation with EOs (antifungal activity of 100 % of each tested essential oil with a control sets), strains of *Aspergillus flavus* were screened for potential production of AFB1 and cyclopiazonic acid (CPA) by TLC method adapted from [23], modified by [25]. Three small pieces (each 5x5 mm) were cut from the colony growing on CYA and placed into 1.5 ml Eppendorf vials. Then 500 µl of extraction solvent (chloroform:methanol, 2:1, v/v) was added to vials containing the agar plugs and shaken on a vortex at least 2 minutes. Extracts (30–50 µl) were applied afterwards as spots to the TLC plate (Silicagel 60, Merck, Germany) 1 cm apart. Consequently, spots were dried and the plates were developed in toluel ene: ethylacetate:formic acid (6:3:1 v/v/v, for AFB1 and 5:4:1 v/v/v for CPA) solvent system that gave an average Rf value of 0.56 for AFB1, and 0.58–0.90 for CPA. Mycotoxin visualization was directly detectable as a colored spots under UV–light (365 nm) for AFB1 (blue spot) and CPA was visualized by spraying with Ehrlich reagent and after drying visualized as a violet tailing–spot in daylight.

Statistical analysis

We calculated the basic variation–statistical values including means and standard deviation from the obtained data using statistical program SAS (one-factorial variance analysis ANOVA) at $P < 0.05$ level of significance.

3. Results and discussion

EOs derived from many different plants has strong antifungal and antibacterial activities. Numerous studies [26, 27] have demonstrated that plant extracts contain diverse bioactive components that can control mould growth. In this study we evaluated antifungal and antitoxic properties of 7 EOs against *A. flavus* strains and their production of aflatoxins. The antifungal test was developed over two weeks. Result of the antifungal activity of pure (100%) tested EOs are summarized in Table 2. All tested EOs were active against *A. flavus* strains (MC1, MC4 and MC33) in the range of tested concentration and cultivation time. All strains were inhibited by oregano, followed by thyme > clove > lavender > mint > sage > eucalyptus EOs in pure (100%) concentration at all days of cultivation. Our results showed that oregano EO was the most effective. Others authors recorded similarly results. Stević, et al. (2014) [18] recorded that EOs from thyme, oregano and savory have antifungal properties against 21 tested fungi isolated from herbal drugs. Kocić–Tanackov et al. (2012) [28] demonstrated the antifungal activity of oregano extract which completely inhibited the growth of *A. wentii* at 2.5 ml/100 ml concentration and growth of

A. carbonarius and *A. niger* was reduced. In our study the second effective EOs were thyme and clove. Also Casella et al. (2002) [29] tested antifungal activity of lavender and tea tree EOs. Results showed that there was a clear antifungal action by both tea tree and lavender essential oils on these organisms grown in culture. In the study of Rasooli and Razzaghi–Abyaneh, (2004) [30] was observed strong fungicidal effect of *Thymus eriocalyx* and *Thymus x–porclock* against *Aspergillus parasiticus*. Moreover, authors Khan and Ahmad (2011) [31] analysed antifungal activity of *Cinnamomum verum*, *Syzygnium aromaticum*, *Cymbopogon citratus* and *Cymbopogon martini* against *Aspergillus fumigatus* and *Trichophyton rubrum*. In this study, clove essential oil belonged into the group of the most effective tested EOs. Inouye et al., (2006) [32] studied antifungal activity of some EOs included oregano, lavender and clove, against *Trichophyton mentagrophytes*. Their results showed that the vapour activity of the six essential oils was ranked in the following order: oregano > clove, perilla > geranium, lavender, tea tree. Where lavender EO did not show strong antifungal effect. In our study lavender seems like to be active, but only from 100% to 60% concentration. Zuzarte et al. (2013) [33] tested lavender and thyme EOs against dermatophyte strains, both EOs showed anti-inflammatory activity. The low antifungal activity of lavender EO in our study was probably due to the activity of minor compounds presented in it (Table 1). EOs of oregano, lavender, thyme and clove, used in our study, were tested for MFC, because of their high antifungal activity after 14 days of cultivation. The mint, sage and eucalyptus EOs were active against *A. flavus* strains, but showed only weak activity. In the study of Felšöciová et al. (2015) [34] was found the lowest antifungal activity by *Pinus mungo* var. *pulmilio*, *Salvia officinalis* L., *Abietis albia etheroleum*, *Chamomila recutita* L. Rauch and *Rosmarinus officinalis*, too. Also Žabka et al. (2014) [27] tested 20 essential oils, include sage, against indoor and outdoor toxigenic and aeroallergenic fungi. They found that sage EOs had only medial antifungal activity. In our study, sage and eucalyptus EO only repressed growth of *A. flavus* strains in comparison with control sets. From these three essential only mint EO were more active. Mint EO inhibited growth of all tested strains after 11 days completely. According to Tyagi and Malik (2011) [35] *Mentha piperita* is more potential inhibitor of food spoiling microbial growth. Freire et al. (2012) [36] also observed a strong inhibitory effect on the growth of various post–harvest pathogenic fungi (*A. flavus, A. niger, A. parasiticus, A. ochraceus, Colletrichum gleosporoides* and *C. musae*) by *Mentha piperita* EO (0.1 and 0.2 ml/100 ml).

Table 2. The antifungal activity of pure of tested essential oils (100 %) to *Aspergillus flavus* strains

Isolates	Day	Essential oils (mean colony diameter in mm ± SD)							
		Thyme	Oregano	Lavender	Eucalyptus	Sage	Clove	Mint	Control
***A. flavus* (MC1)**	**3rd**	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$20.75^{a} \pm 14.32$	$20.88^{a} \pm 14.01$	$0^{a} \pm 0$	$0^{a} \pm 0$	$67.50^{b} \pm 45.00$
	7th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$61.00^{ac} \pm 21.52$	$45.83^{b} \pm 9.65$	$0^{a} \pm 0$	$0^{a} \pm 0$	$90.00^{ac} \pm 0$
	11th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$61.00^{b} \pm 21.52$	$53.88^{a} \pm 20.30$	$0^{a} \pm 0$	$0^{a} \pm 0$	$90.00^{c} \pm 0$
	14st	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$63.33^{b} \pm 25.17$	$52.00^{b} \pm 9.37$	$0^{a} \pm 0$	$6.50^{a} \pm 0.50$	$90.00^{c} \pm 0$
***A. flavus* (MC4)**	**3rd**	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$32.63^{b} \pm 21.98$	$6.50^{a} \pm 7.90$	$0^{a} \pm 0$	$0^{a} \pm 0$	$34.63^{b} \pm 23.25$
	7th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$55.67^{c} \pm 13.61$	$34.07^{b} \pm 17.39$	$0^{a} \pm 0$	$0^{a} \pm 0$	$59.00^{c} \pm 7.94$
	11th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$55.67^{c} \pm 13.61$	$41.33^{b} \pm 17.01$	$0^{a} \pm 0$	$0^{a} \pm 0$	$73.67^{d} \pm 5.13$
	14th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$55.67^{b} \pm 13.61$	$55.33^{b} \pm 31.64$	$0^{a} \pm 0$	$4,67^{a} \pm 2,75$	$90.00^{d} \pm 0$
***A. flavus* (MC33)**	**3rd**	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$40.00^{bd} \pm 34.84$	$11.63^{ac} \pm 8.26$	$0^{a} \pm 0$	$0^{a} \pm 0$	$67.50^{c} \pm 45.00$
	7th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$55.00^{b} \pm 26.00$	$44.83^{ac} \pm 39.18$	$0^{a} \pm 0$	$0^{a} \pm 0$	$90.00^{b} \pm 0$
	11th	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$55.00^{b} \pm 26.00$	$45.17^{b} \pm 38.87$	$0^{a} \pm 0$	$0^{a} \pm 0$	$90.00^{c} \pm 0$
	14st	$0^{a} \pm 0$	$0^{a} \pm 0$	$0^{a} \pm 0$	$56.67^{b} \pm 28.88$	$55.17^{b} \pm 30.25$	$0^{a} \pm 0$	$5,67^{a} \pm 8,14$	$90.00^{c} \pm 0$

Data in the column followed by different letters are significantly different in 95.0% Tukey HSD test, *A.* – *Aspergillus*, SD – standart deviation

Results from the MFC test of thyme, clove and lavender EOs are presented in figure 1–3, for oregano EO in figure 4. The most effective EO was oregano with MFC 6% (6/94; v/v) for all tested strains. The second most active EOs was thyme, which had MFC 30% (30/70; v/v) for strains *A. flavus* (MC1) and *A. flavus* (MC33). Tested strain *A. flavus* (MC4) was more sensitive with MFC 20% (20/80; v/v). Clove EOs it's the third active oil with MFC 30% for all tested strains and EOs which had the least antifungal activity was lavender with MFC 60% (60/40; v/v; oil/diluent) for all tested strains.

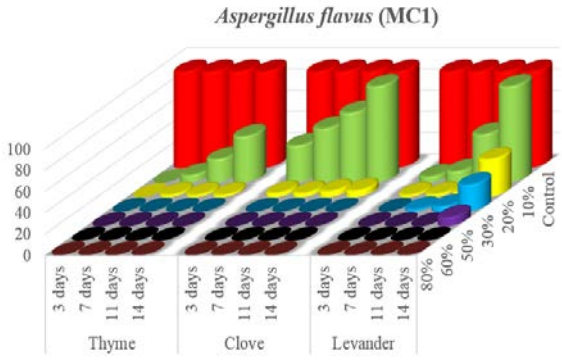

Figure 1. Minimum fungicidal concentration (MFC) of tested essential oils to *Aspergillus flavus* (MC1)

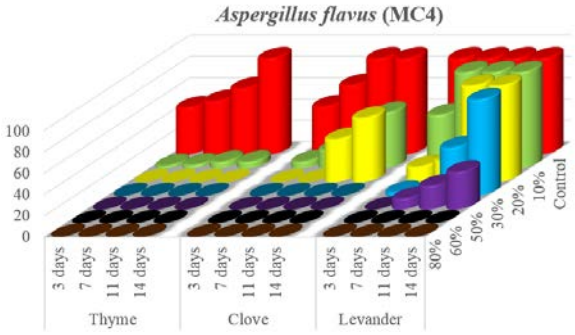

Figure 2. Minimum fungicidal concentration (MFC) of tested essential oils to *Aspergillus flavus* (MC4**)**

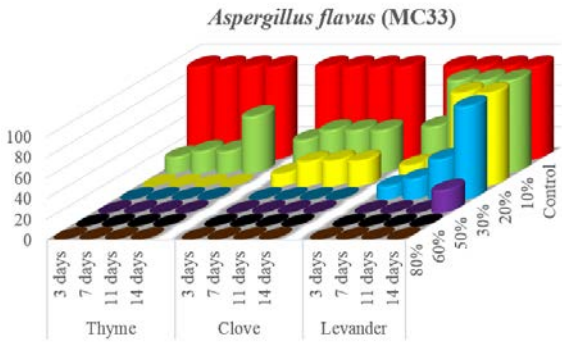

Figure 3. Minimum fungicidal concentration (MFC) of tested essential oils to *Aspergillus flavus* (MC33)

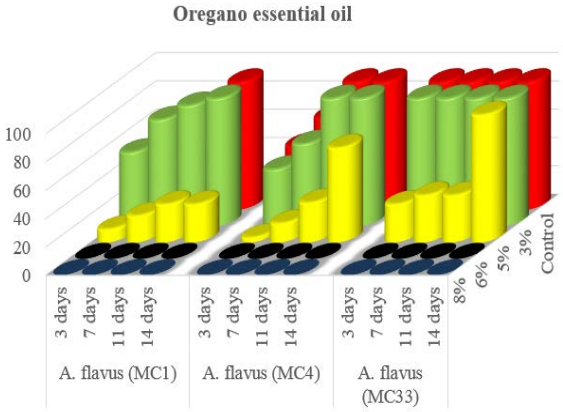

Figure 4. Minimum fungicidal concentration (MFC) of oregano essential oils to *Aspergillus flavus* strains

In the second step of our study we also tested antitoxic properties of EOs. After 14 days of cultivation with EOs (100%) with a control sets, strains *A. flavus* (MC1, MC4 and MC33) were tested for aflatoxin (AFB1) and cyclopiazonic acid (CPA) production by TLC chromatography. Result showed that some of the tested EOs affected mycotoxins production (Table 3). AFB_1 and CPA were not detected only in the samples treated with thyme, clove, oregano and lavender EOs, because they completely inhibited mycelial growth of fungi. In treatments with mint EO, the colonies of strains after 14 days were too small for analysis. Treatments with sage and eucalyptus EOs showed some potential in fungal toxic inhibiting. Production of AFB_1 and CPA by *A. flavus* (MC33) was completely inhibited by sage in all repetitions. Eucalyptus EO inhibited production of AFB_1 in all repetition, but CPA by this strain was produce in one repetition. Strain *A. flavus* (MC1) did not produce AFB_1 only in treatments with sage EO and eucalyptus inhibited their production in two screened repetition. Production of AFB_1 by *A. flavus* (MC4) was inhibited in two repetitions by sage and eucalyptus EO. Production of CPA by *A. flavus* strains (MC1 and MC4) was not affected by sage and eucalyptus EOs.

Vilela et al. (2009) [37] tested antifungal and antitoxic activity of eucalyptus EO and its major compound 1.8–cineole against *A. flavus* and *A. parasiticus* and their production of aflatoxins. They found that AFB_1 production was reduced in headspace volatile assays and partial inhibition was observed at the 200 µl dose of the essential

oil. Bluma and Etcheverry (2008) [38] documented antitoxic activity of thyme, clove, poleo and eucalyptus EOs against *Aspergillus* section *Flavi*. In their study eucalyptus EO was able to inhibit production of mycotoxins only at the highest percentage (85–90%). Aflatoxin inhibition of *A. parasiticus* was also observed by Razzaghi–Abyaneh et al. (2008) [39] at the highest concentration of tested essential oils, than inhibition of its growth.

Table 3. Inhibitory effect of tested EOs (100%) on mycotoxins production by *Aspergillus* sp.

Strains	Screened mycotoxins	Essential oils (100%)					
		Sage		Eucalyptus		Mint	
		Repetition	Control	Repetition	Control	Repetition	Control
***A. flavus* (MC1)**	**AFB_1**	$3^1/0^2$	$3^1/3^2$	$3^1/1^2$	$3^1/3^2$	NS*	$3^1/3^2$
	CPA	$3^1/3^2$		$3^1/3^2$		NS*	
***A. flavus* (MC4)**	**AFB_1**	$3^1/1^2$	$3^1/3^2$	$3^1/1^2$	$3^1/3^2$	NS*	$3^1/3^2$
	CPA	$3^1/3^2$		$3^1/3^2$		NS*	
***A. flavus* (MC33)**	**AFB_1**	$3^1/0^2$	$3^1/3^2$	$3^1/0^2$	$3^1/3^2$	NS*	$3^1/3^2$
	CPA	$3^1/0^2$		$3^1/1^2$		NS*	

NS*–no screened isolates (small diameters of colony), 1–number of screened isolates, 2–number of positive isolates, AFB_1– aflatoxin B_1, CPA–cyclopiazonic acid

4. Conclusions

In conclusion, our findings suggest that oregano, thyme and clove are highly effective in vapor phase and might be a good natural fungicide, because of their MFC values, which were 20% (20/80; v/v) for *A. flavus* (MC4) in treatments with thyme EO and 30% (30/70; v/v) for all tested isolates in treatments with clove. Oregano seems to be the most effective tested EO with MFC 6% (6/94; v/v) against *Aspergillus flavus* strains. Lavender EO showed high antifungal activity only at the highest concentration. In spite of the fact that sage, eucalyptus and mint EOs showed only weak antifungal activity, could be used in food preservation, because of their antitoxic properties. Lavender EO have antifungal activity, but in higher concentration (MFC 60% (60/40; v/v). This fact changes its classification from EOs with higher activity to EOs with lower activity. Because, despite the concentration of EOs is very low, they could still change organoleptic properties of treated foods. Therefore, consumer sensory tests will be needed to evaluate impact of EOs on flavor and aroma properties of particular products.

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