Food Control 110 (2020) 107007

Contents lists available at ScienceDirect

Food Control

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

# The *in vitro* and *in situ* effect of selected essential oils in vapour phase against bread spoilage toxicogenic aspergilli

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ARTICLE INFO

*Keywords:*
*Aspergillus* spp.
Aflatoxins
Ochratoxins
Bread
Essential oils
Antifungal activity
Vapour contact

ABSTRACT

The major objective of this study was to determine the antifungal, anti-toxicogenic and anti-sporulation effect of selected essential oils in the control growth of *Aspergillus* spp. by contact vapour method in *in vitro* and *in situ* condition. In continue, the effect of the essential oils treatment on the sensory profile of the bread was evaluated. In this study, ten essential oils (EOs), namely: thyme, clove, laveder, cumin, oregano, cinnamon, basil, rosemary, sage and lemongrass were tested against four strains of *Aspergillus* [*Aspergillus flavus* (SLO-B-201), *Aspergillus parasiticus* (SLO-B-219), *Aspergillus ochraceus* (SLO-B-245) and *Aspergillus westerdijkiae* (SLO-B-249)] isolated from bread samples. The *Aspergillus* strains were tested for potential production of mycotoxins by thin layer chromatography (TLC). The chemical composition of all tested EOs was identified by GC-MS and by gas chromatography with flame ionization detector (GC-FID). The antifungal activity, the minimum inhibitory doses (MIDs), anti-toxicogenic and antisporulation effect of the selected EOs were investigated by gas diffusion method in both, *in vitro* and *in situ* analyses. The results indicated that the thyme, clove, oregano, cinnamon and lemongrass were highly effective against tested toxigenic *Aspergillus* species in vapour phase. A treated bread sample with EOs does not have negative effect on the sensory properties of the breads, with exception of oregano and lemongrass. In this context, application EOs in vapour phase seems to be an effective, sustainable and safe method and promising alternative to the use of chemical inhibitors for bread preservation.

## 1. Introduction

Bread is an essential and primary food resource in many cultures. It contains essential constituents such as proteins, fiber, lipids, vitamins, salts, that collectively make bread an important product in the traditional diet and resource of nutrients and energy (Herras-Mozos et al., 2019; Shewry, Halford, Belton, & Tatham, 2002). Because of its water activity and storage conditions bread is a highly perishable product (Legan, 1993) and is prone to mould spoilage (Magan, Arroyo, & Aldred, 2003). Spoilage caused by filamentous fungi is serious economic concern due to incurred losses (1–5%), but another concern is the possibility of mycotoxin production (Saranraj & Sivasakthivelan, 2016). Only a small number of mycotoxins are important for bread and bakery products, in terms of economic effect, toxicity and frequency of occurrence. One of them are aflatoxins and ochratoxin A (de Koe & Juodeikiene, 2012). Aflatoxins (AFs) are highly toxic secondary metabolites produced by some species of *Aspergillus*, especially *A. flavus* and *A. parasiticus* (Khayoon et al., 2010). Moreover, *A. flavus* produces as well cyclopiazonic acid (Lalitha Rao & Husain, 1985; Sacchi et al., 2009) which occurs as a co–contaminant with aflatoxins (Pitt, Taniwaki, & Cole, 2013). Aflatoxin $B_1$ ($AFB_1$) is the most potent mammal hepatocarcinogen known and the International Agency of Research on Cancer (IARC, 1993) classifies it as a Group 1 carcinogen. The majority of OTA-producing species are included in the genus *Aspergillus* section *Circumdati*. Although, *A. ochraceus* used to be considered as the most important OTA producer, the new species are also able to produce OTA, in particular *A. steynii* and *A. westerdijkiae*. OTA has been considered as an important contaminant in different dietary

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https://doi.org/10.1016/j.foodcont.2019.107007
Received 27 August 2019; Received in revised form 13 November 2019; Accepted 13 November 2019
Available online 14 November 2019
0956-7135/ 

products commonly consumed by humans (Frisvad, Frank, Houbraken, Kuijpers, & Samson, 2004; Varga, Baranyi, Chandrasekaran, & Vágvölgyi, 2015). The International Agency for Research on Cancer (IARC, 1993) classified this toxin as a possible human carcinogen (group 2B). EU regulation (No. 1881/2006) defines maximum level for the mycotoxins, aflatoxin (2 μg/kg) and ochratoxin A (OTA) (3 μg/kg) in all the cereal products (European Commission, 2010). One of the traditional ways of managing the moulds spoilage of food is through the addition of synthetic preservatives. But their continuous application has led to the development of fungal resistance (Gemeda, Woldeamanuel, Asrat, & Debella, 2014) and some of these chemicals exhibit side effects on human health and environment (Damalas & Eleftherohorinos, 2011; Prakash, Kedia, Mishra, & Dubey, 2015). However, consumers do not like artificial preservatives in their food, especially not in their everyday bread (Krisch, Rentskenhand, Horváth, & Vágvölgy, 2013). In this context, plant essential oils (EOs) which have been used in traditional medicine and pharmaceutical preparations are gaining interest by the food industries for the development of eco-friendly food preservatives with functional properties (Cherrat, Espina, Bakkali, Pagán, & Laglaoui, 2014; Corthouts & Michiels, 2016; Prakash et al., 2015). EOs are a good source of several bioactive compounds which possess antioxidative and antimicrobial properties. Several studies with EOs have reported their antifungal activity (Rajkovic, Pekmezovic, Barac, Nikodinovic-Runic, & ArsićArsenijević, 2015; Tyagi, Malik, Gottardi, & Guerzoni, 2012), inhibition of spore formation and germination in *Aspergillus* species (Gemeda et al., 2014; Paranagama, Abeysekera, Abeywickrama, & Nugaliyadde, 2003), or the effect of EOs on aflatoxins biosynthesis (Ferreira et al., 2013; Quiles, Manyes, Luciano, Mañes, & Meca, 2015). On the other hand, the unique odour associated with the volatiles may limit the use of EOs in some foods since it may alter the typical smell/flavour of foods (Tongnuanchan & Benjakul, 2014). Therefore, one advantage of EOs is their biological activity in the vapour phase (Bernardos et al., 2015; Tyagi et al., 2012) and in this case, they could be used at lower concentrations (Laird & Phillips, 2012).

Thus, the major objective of this study was to determine the efficacy of selected essential oils in the control growth of *Aspergillus* spp. by contact vapour method. Further objectives were to evaluate minimum inhibitory doses (MIDs) of selected essential oils, to investigate any potential influence on the sporulation and production of mycotoxins, as well as to evaluate the effect of essential oil treatments on the sensory acceptability of bread.

## 2. Material and methods

### 2.1. Essential oils samples

The original essential oils (EOs) of thyme (*Thymus vulgaris* L.), clove (*Syzygnium aromaticum* [L.]), lavender (*Lavandula angustifolia* Mill.), cumin (*Carum carvi* L.), oregano (*Origanum vulgare* L), cinnamon (*Cinnamomum zeylanicum* Nees. [*C. verum* J.S.Presl.]), basil (*Ocinum basilicum* L.), rosemary (*Rosmarinus officinalis* L.), sage (*Salvia officinalis* L.) and lemongrass (*Cymbopogon citrati* [DC] Stapf.) were obtained from commercial supplier Calendula a.s. (Nová Ľubovna, Slovakia). All samples were obtained by hydrodistillation and stored in hermetically sealed flasks at 4 °C in the dark until *in vitro* and *in situ* analysis. The chemical composition of all tested EOs was identified by GC-MS and relative proportions of EOs constituents were assessed by gas chromatography with flame ionization detector (GC-FID) as described by Božik et al. (2017). The relative proportion of the EOs constituents were confirmed by comparison their spectra with the authentic standards (Sigma-Aldrich, CZ) (Table 1).

### 2.2. Fungal strains

Four strains of *Aspergillus*, namely *Aspergillus flavus* (SLO-B-201), *Aspergillus parasiticus* (SLO-B-219), *Aspergillus ochraceus* (SLO-B-245) and *Aspergillus westerdijkiae* (SLO-B-249) were used. All tested strains were previously isolated from bread samples collected from different commercial markets in Skovakia. The fungal isolates were identified by ITS rDNA sequencing (Císarová et al., 2016). Fungal strains were cultivated on Sabouraud dextrose agar (Oxoid, Czech rep.) at 25 °C for four days before the *in vitro* and *in situ* analysis. Sequences of used isolates were deposited in genbank database under following accession numbers: *A. flavus* isolate (SLO-B-201), ITS: MH517574. *A. parasiticus* isolate (SLO-B-219), ITS: MH517575. *A. ochraceus* isolate (SLO-B-245), ITS: MH517572. *A. westerdijkiae* isolate (SLO-B-249), ITS: MH517573.

### 2.3. In vitro antifungal analysis

The antifungal activity of selected EOs was investigated by gas diffusion method following the method of Guynot et al. (Guynot et al., 2003) with some modifications. Czapek yeast extract agar (CYA, Sigma-Aldrich, Germany) was used for fungal strains cultivation, in this study. In the first step, all EOs were tested at the highest concentration (500 μL/L of air). Ethyl acetate was used for dilution of EOs to give the final volume of 100 μL. This solution was evenly distributed on a sterile filter paper. Then the paper was left to dry for 1 min to evaporate ethyl acetate by previous study Klouček et al., (2012) and Božik et al. (2017). Blank filter papers with ethyl acetate served as positive control. Diameters of the growth colonies were measured on the 3rd, 7th, 11th and 14th day of incubation in two perpendicular directions. The percentage of mycelial growth inhibition was calculated according to the following formula:

$$\text{Mycelial growth inhibition (MGI \%)} = [(d_c - d_t)/d_c] \times 100$$

Where $d_c$ = Average (mm) increases in mycelial growth in control, $d_t$ = Average (mm) increases in mycelial growth in treatment (Marandi et al., 2011).

After incubation, the minimum inhibitory doses (MIDs) of EOs with the most significant activity were recorded, using the method by Klouček et al. (Klouček et al., 2012). EOs were tested at concentration range between 15.625 and 250 μL/L$^{-1}$ of air. The MID was defined as the lowest concentration of the oil that cause any visible growth after all days of cultivation (14) in comparison with control.

### 2.4. In situ antifungal analysis on bread

#### 2.4.1. Breads inoculation and treatment with essential oils

The bread samples (obtained from different markets in Slovakia) without damage on the surface were cut into slices (height of the bread slices was 150 mm). The bread slices were placed into 0.5 L sterile glass jars (Bromioli Rocco, Italy). Fungal spore suspension of each strain (final concentration of spores $1 \times 10^6$ spores/mL) was prepared (Božik et al., 2017). This suspensions were diluted in 20 mL sterile phosphate–buffered saline with 0.5% Tween 80 by adjusting the density to 1–1.2 McFarland, depending on the strain (Espinel-Ingroff & Kerkering, 1991; Petrikkou et al., 2001). Then, 5 μL of inoculum was added on top of the bread at four different places.

Thyme, clove, oregano, lavender, cumin, lemongrass and cinnamon at concentration of 125, 250 and 500 μL/L of air were used for the treatment. EOs were selected according to their best activity in evaluation of MIDs. Next, 100 μl of solution (EOs + ethyl acetate) was evenly distributed on a sterile paper–filter disc (6 cm). The paper–filter disc with EOs was inserted into the cover of the jar. The control group did not receive any EOs treatment. Jars were hermetically closed and kept at room temperature (22 °C ± 1 °C) for 14 days in the dark. After 14 days of storage, the colonies with visible mycelial growth and visible sporulation were counted.

#### 2.4.2. Production of mycotoxins and anti-toxicogenic effect of essential oils

*Aspergillus* strains were tested for potential production of

**Table 1**
Essential oils composition determined using by GC–MS and quantified by GC–FID techniques.

| RI[b] | | Component | Thyme[c] | Clove | Lavender | Cumin | Oregano | Cinnamon | Basil | Rosemary | Sage | Lemongrass |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 938 | [a] | α-Pinene | 1.31 | 0.12 | 1.13 | 0.10 | 0.27 | 1.17 | 0.26 | 10.57 | 5.52 | 0.37 |
| 953 | [a] | Camphene | 1.52 | | 0.69 | 0.10 | 0.29 | 0.41 | 0.11 | 5.01 | 6.09 | 2.13 |
| 964 | [a] | Benzaldehyde | | | | | | 0.18 | 0.05 | | | |
| 980 | [a] | β-Pinene | 0.12 | | 0.83 | | | 0.31 | 0.30 | 7.39 | 2.27 | |
| 982 | | 1-Octen-3-ol | 0.73 | | 0.19 | | | | | | | 1.86 |
| 993 | | β-Myrcene | 1.36 | | 0.89 | 0.20 | 0.47 | | 0.17 | 0.91 | 0.52 | 0.11 |
| 997 | | 3-Octanol | | | | | 0.21 | | | | | |
| 1006 | [a] | α-Phellandrene | 0.14 | | | | | 0.36 | | 0.16 | | |
| 1019 | [a] | α-Terpinene | 1.12 | | 0.15 | | 0.91 | | | | | |
| 1029 | [a] | p-Cymene | 17.88 | | 0.58 | | 7.43 | 1.79 | 0.80 | 2.57 | 2.59 | |
| 1031 | [a] | D-Limonene | 0.36 | | 1.34 | 41.15 | | 0.77 | 0.25 | 2.13 | 1.98 | 0.42 |
| 1032 | | β-Phellandrene | | | | | | | 0.15 | | | |
| 1034 | [a] | Eucalyptol | 1.47 | 0.26 | 12.01 | | 1.19 | 0.17 | 3.78 | 43.11 | 10.87 | |
| 1062 | [a] | γ-Terpinen | 5.27 | | 0.14 | | 3.49 | | | 0.77 | 0.48 | |
| 1074 | | Linalool oxide | | | 0.51 | | | | | | | 1.23 |
| 1090 | [a] | Terpinolene | 0.13 | | | | 0.12 | | | 0.18 | 0.30 | |
| 1091 | | Nonanone | | | | | | | | | | 0.11 |
| 1101 | | Linalool | 4.81 | | 40.59 | 0.86 | 3.19 | 2.48 | 1.53 | 0.60 | 0.39 | 1.50 |
| 1108 | | α-Thujone | | | | | | | | | 21.19 | |
| 1119 | | β- Thujone | | | | | | | | | 6.46 | |
| 1147 | [a] | Camphor | 1.98 | | 4.62 | | 0.89 | | 0.33 | 12.89 | 19.18 | |
| 1158 | [a] | (+/-)-citronellal | | | | | | | | | | 0.39 |
| 1160 | | Isoborneol | | | | | | | | | | 0.24 |
| 1168 | [a] | Borneol | 1.85 | | 7.43 | | 0.87 | | | 3.89 | 4.05 | 0.81 |
| 1171 | | Lavandulol | | | 3.36 | | | | | | | |
| 1179 | [a] | 4-Terpineol | 1.93 | | 6.93 | | 0.77 | 0.11 | | 0.46 | 0.48 | 0.45 |
| 1192 | | α-Terpineol | 0.18 | | 0.73 | 0.18 | 0.41 | 0.28 | | 2.27 | | 0.78 |
| 1198 | | γ-Terpineol | | | | 0.78 | | | | | | 0.27 |
| 1202 | | Methyl chavicol | | | | | | | 86.58 | 0.33 | | 0.21 |
| 1222 | | (+)-cis-Carveol | | | | 0.52 | | | | | | |
| 1234 | | b-citronellol | | | | | | | | | | 0.19 |
| 1238 | | Thymol methyl ether | 0.70 | | | | | | | | | |
| 1244 | [a] | Neral | | | | | | | | | | 31.65 |
| 1247 | [a] | (−)-carvone | 1.01 | | | 54.93 | 0.10 | | | | | |
| 1259 | [a] | Geraniol | | | | | | | | | | 4.76 |
| 1260 | | Linalyl anthranilate | | | 10.15 | | | | | | | |
| 1272 | [a] | Cinnamaldehyde | | | | | | 1.12 | | | | |
| 1273 | [a] | Geranial | | | | | | | | | | 40.06 |
| 1287 | | Bornyl acetate | | | 0.12 | | | | 0.16 | 1.28 | 2.37 | 0.17 |
| 1289 | | Safrole | | | | | | 1.40 | | | | |
| 1296 | [a] | Thymol | 44.34 | | | | 4.06 | | | | | |
| 1297 | | Menth-1-en-9-ol | 2.88 | | | | | | | | | 0.12 |
| 1302 | | Cinnamyl alcohol | | | | | | 0.16 | | | | |
| 1306 | [a] | Carvacrol | | | | | 69.78 | | | | | |
| 1360 | [a] | Eugenol | 0.22 | 82.3 | | | 0.15 | 72.53 | | | | 0.14 |
| 1370 | | m-Eugenol | | | | | | | | | | 0.21 |
| 1373 | | Benzenepropyl acetate | | | | | | 0.22 | | | | 0.15 |
| 1376 | | Copaene | 0.16 | | | | 0.14 | 0.81 | | 0.16 | | 0.14 |
| 1386 | [a] | Geranyl acetate | | | 0.26 | | | | | | | 4.08 |
| 1399 | | Vanillin | | | | | | 0.18 | | | | |
| 1412 | | Isocaryophillene | | 0.18 | | | | | | | | |
| 1420 | [a] | Caryophyllene | 6.94 | 7.40 | 3.79 | | 1.84 | 2.69 | 0.14 | 3.76 | 5.38 | 1.99 |
| 1435 | | α-Bergamotene | | | | | | | 2.28 | | | |
| 1445 | | Cinnamyl acetate | | | | | | 2.01 | | | | |
| 1448 | | Citronellyl propionate | | | | | | | | | | 0.37 |
| 1452 | [a] | α-Caryophyllene | | 2.18 | 0.12 | | 0.45 | 0.52 | | 0.33 | 6.35 | 0.24 |
| 1478 | | Germacrene D | | | | | | | 0.73 | | | |
| 1481 | | α-Curcumene | | | | | | | 0.14 | | | |
| 1491 | | Varidiflorene | | | | | | | | | | 0.11 |
| 1493 | | α-Zingiberene | | | | | | | | | | |
| 1505 | | α-Bisabolene | | | | | 0.11 | | | | | |
| 1510 | | γ-Cadinene | | | 0.12 | | | | 0.45 | | | 1.95 |
| 1521 | | β-Sesquiphellandrene | | | | | | | 0.94 | | | |
| 1524 | | δ-Cadinene | 0.32 | 0.46 | | | 0.44 | 0.19 | | | | 0.46 |
| 1525 | | Aceteugenol | | | | | | 2.67 | | | | 0.16 |
| 1531 | | Eugenol acetate | | 6.10 | | | | | | | | |
| 1570 | | Caryophyllenyl alcohol | | | | | | | | | | |
| 1574 | [a] | Caryophyllene oxide | 0.19 | 0.94 | 0.26 | | 0.44 | 1.35 | | 0.25 | 0.92 | 0.52 |
| 1627 | | tau.-cadinol | | | | | | | 0.58 | | | |
| 1725 | | Benzyl Benzoate | | | | | | 4.13 | | | | |
| | | **total** | **98.92** | **99.94** | **96.90** | **98.81** | **98.02** | **98.01** | **99.71** | **99.01** | **97.39** | **98.35** |

[a] Identification confirmed by co-injection of authentic standard.
[b] RI: identification based on Kovat's retention indices (HP-5MS capillary column) and mass spectra.
[c] Relative proportions were calculated in % by dividing individual peak area by total area of all peaks.

mycotoxins by thin layer chromatography (TLC) (Labuda & Tancinová, 2006). The method is described by Císarová et al. (Císarová, Tančinová, & Brodová, 2015). *A. flavus* (SLO-B-201) was tested for production of aflatoxin $B_1$ and cyclopiazonic acid, *A. parasiticus* (SLO-B-219) was tested for production of $AFB_1$ and $AFG_1$. *A. ochraceus* (SLO-B-245) and *A. westerdijkiae* (SLO-B-249) were tested for production of ochratoxin A (OA). After developing the plates, aflatoxins spots $AFB_1$ ($R_f = 0.65$), $AFG_1$ ($R_f = 0.39$) and OA ($R_f = 0.45$) were observed directly under UV light (366 nm) as blue (AFB1), blue-green ($AFG_1$) and bluish-green (OA) spots respectively. CPA ($R_f = 0.65$) was observed by spraying Ehrlich reagent (Sigma-Aldrich, Germany), which revealed as a violet tailing-spot visible at daylight. The mycotoxins were determined by visual comparison with prepared standards (Sigma-Aldrich, Germany). The same method was used to detect production of mycotoxins after treatments with EOs in both, *in vitro* (after treatment of fungal strain with the concentration of 500 μL/L of air) and *in situ* (after treatment of fungal strain on bread samples with the concentration range from 500 to 125 μL/L of air) analysis. The inoculated growing colonies on the surface of the bread samples were used for *in situ* analysis of anti-toxicogenic effect of EOs by tested fungi after 14 days. The inhibition of mycotoxin production was expressed in percentage (%) over control. Anti-toxicogenic effect was determined only with essential oils treatments, which did not inhibit the growth of tested strains completely in *in vitro* and *in situ* assays.

### 2.5. Sensory evaluation of treated bread

Non-inoculated bread slices treated as described in section 2.4.1 with three concentrations (125, 250 and 500 μL/L of air) of the most effective EOs were used for sensory evaluation. Triangle tests (two-tail) were used to evaluate any differences between sensory characteristic of treated samples and their respective control samples. The tests were performed according to ISO Standards 4120 (2004) with a sensory panel consisting of 15 untrained assessors. Each panelist received three coded samples each time, two from the same treatment and one without treatment. The subjects were asked to select samples that differ from the other two to assess the acceptability of the odour and taste of the samples. Water was provided to assessor as neutralizer of the taste and coffee beans as neutralizer of the odour between the samples. Sensory analysis was accomplished on the 3rd, 7th, 11th and 14th day of storage at 22 ± 1 °C in the dark.

### 2.6. Statistical evaluation

All experiments were carried out in triplicate in this study, independently. Analysis of variance (ANOVA) and Tukey HSD 95% multiple range test were performed at a significance level of $p < 0.05$ using SAS (one-factorial variance analysis and multifactorial variance analysis ANOVA 16.1 statistical program). The results shown in Table 4 of the $MFC_{50}$ and $MFC_{90}$ value (concentration causing 50% and 90% reduction of mycelial growth) were estimated by probity analysis. Suitable regression model was selected by Statgraphic Centurion XV software and calculated by log-log linear regression with following equation Radial growth = 10^(slope*log(Days of cultivation) + Yintercept). Regression curves and values were obtained where using GraphPad Prism 6 software. The results shown in Table 5 were expressed by percent of spore germination inhibition in comparison with the control assay.

Triangle tests, concretely two-tail test were analysed by consulting tables from ISO Standards 4120:2004. When the number of correct replies was greater than or equal to the value in the table, it can be stated that there is a difference between samples. The significant differences between treated and control samples were evaluated by the chi-square binomial distribution with value of α-risk = 0.05 by program R Core Team 2016.

## 3. Results and discussion

### 3.1. Essential oils analyses

Qualitative and quantitative analysis of the essential oils are listed in Table 1. The main essential oils (EO) and their components which inhibited growth of Aspergilli strains completely were followed. Clove (*Syzygium aromaticum* L.) with content of eugenol (82.3%) and cinnamon (*Cinnamomum zeylanicum* Nees. [*C. verum* J.S.Presl.]) (72.53%). Authors (Pinto, Vale-Silva, Cavaleiro, & Salgueiro, 2009) studied antimicrobial effect of eugenol as main compounds of clove essential oils against fungi (*Aspergillus* and *Candida*) and they confirmed mechanism of action as lesion on cytoplasmic membrane which leads to inherent loss of cell viability. Further, authors determined changes in metabolic activity by FUN 1 membrane-permeant fluorescent probe, where detected that increasing of eugenol proportion in essential oils is directly dose-depended. Also these authors (Pinto et al., 2006) confirmed that thyme (*Thymus vulgaris* L.) essential oil inhibit cell wall growth by disruption of normal sterol biosynthetic pathway which leads to reduction in ergosterol biosynthesis. The main compounds in thyme essential oils were thymol (44.34%) and p-Cymene (17.88%). Cumin (*Carum carvi* L.) as the only one contained carvone (54.93%) whose mechanisms of action is not clear and completely understood, but authors Oosterhaven et al., 1995 demonstrated that it acts by disrupting the metabolic energy status of the cell, whereas Helander et al. (1998) found that it did not affect the membrane system or the adenosine triphosphate pool. Carvacrol as main compounds in oregano (*Origanum vulgare* L.) and it's using as antimicrobial agent may be leads to inactivation of essential enzymes, react with cell membrane or disrupt genetic material functionality (Davidson, 2001). Neral and geranial as two isomeric acyclic monoterpene aldehydes are a natural mixture of compounds known as citral founded in lemongrass (*Cymbopogon citrati* [DC] Stapf.) (Silva, Guterres, Weisheimer, & Schapoval, 2008). Neral as cis-citral (citral B) (31.65%) and genarial as trans-citral (citral A) (40.06%). Both these compounds neral and geranial can inhibit spore germination in *Aspergillus* species. It was found that citral injected the wall and the membrane of *A. flavus* spore, resulting in a decrease in it's elasticity. After entering the cell, citral not only affected by the genetic expression of mitochondrion reduplication and it's morphology, but also changed the aggregation of protein-like macromolecules. As results, cell, organelles and macromolecules lost normal structures and functions, eventually leading to the loss of germination ability of *Aspergillus flavus* spores (Luo et al., 2004; Xie, Fang, & Xu, 2004; Zhang, Wei, Shen, & Jiang, 2011). In the context of lavender (*Lavandula angustifolia* Mill.) essential oil was found to be more effective in the inhibition of germ tube than of hyphal growth. It was confirmed that gaseous contact suppressed growth of filamentous fingi but not solution contact (Inouye et al., 1998). This was reported to be caused by the direct binding of gaseous oil on the arial mycelia that formed the spore-forming organ. When the activity of two major constituents was examined, linalyl acetate was found to be capable of suppressing spore formation while linalool was not inhibitory for sporulation but was effective for the inhibition of germination and fungal growth. The inhibition of sporulation appeared to arise from respiratory suppression of arial mycelia (Cavanagh & Wilkinson, 2002). Mechanisms of action of essential oils are different and it is depended on contained compounds in specific essential oils as describe collective of authors (Tian et al., 2012) in their study where they tested dill (*Anethum graveolens* L.) essential oils against *Aspergillus flavus*. Dill essential oils contain mainly compounds as D-limonene, carvone and dihydrocarbone (Delaquis, Stanich, Girard, & Mazza, 2002). Authors Tian et al., 2012 detected more mechanisms of action in antimicorbial activity of mentioned dill essential oil. They detected the following mechanisms of action: lesion of cytoplasmic membrane which can leads to loss cell viability, decreasing of ergosterole in plasmic membrane, reduction of mitochondrial membrane potential (MMP) in mitochondria, acidification of

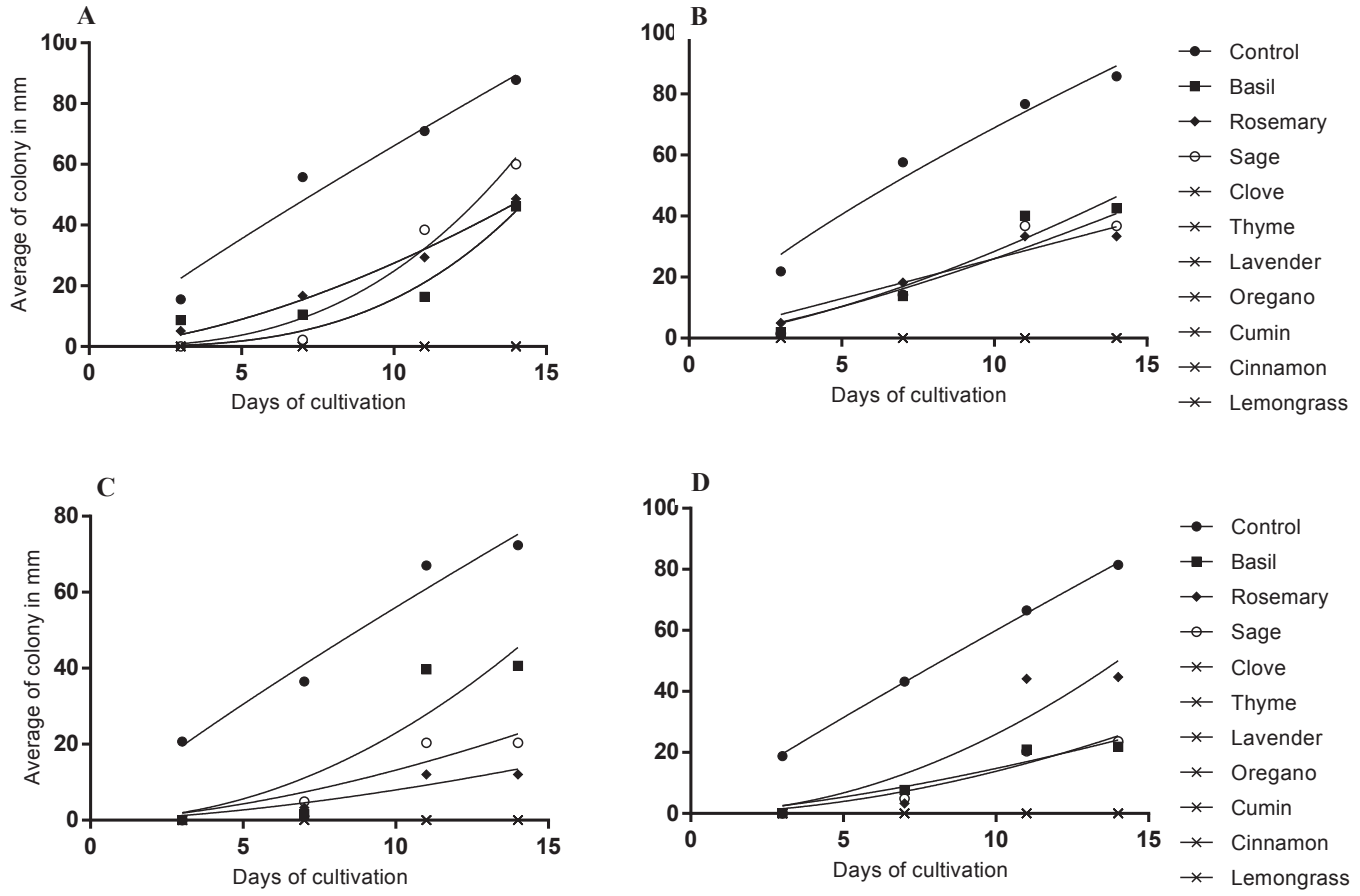


**Fig. 1.** Regression growth lines (mm/d) of fungi on CYA in treatment with essential oils (500 μL/L of air). (A) *A. flavus* (SLO-B-201), (B) *A. parasiticus* (SLO-B-219), (C) *A. ochraceus* (SLO-B-243), (D) *A. westerdijkiae* (SLO-B-249).

external medium, decreasing of ATPase and dehydrogenase activities in A. flavus were also observed in a dose-dependent manner.

### 3.2. In vitro fungicidal effect of essential oils

Foods preserved with natural additives have become popular due to greater consumer awareness and concern regarding to synthetic chemical additives. This has led researchers and food processors to look for a natural food additives with a broad spectrum of antimicrobial activity (Marino, Bersani, & Comi, 2001). Among promising alternative methods to control food spoilage belong use of essential oils (EOs) and their activity in vapour phase (Kloucek et al., 2012). The fungicidal effect of the 10 essential oils was evaluated by using the gas diffusion method in this study. At first, the concentration of EOs (500 μL/L of air) was used for all tested strains: *Aspergillus flavus* (SLO-B-201), *Aspergillus parasiticus* (SLO-B-219), *Aspergillus ochraceus* (SLO-B-243) and *Aspergillus westerdijkiae* (SLO-B-249). The results showed a different activity against all tested strains of *Aspergillus*. The EOs caused significant differences ($P < 0.05$) in the mycelial growth of all tested strains during the 14 days of cultivation presented in Fig. 1 as timeline regression curves with log-log linear regression values showed in Table 2.

Seven essential oils, namely thyme, clove, cinnamon, lemongrass, cumin, oregano and lavender totally inhibited the growth of all tested fungi (100%) during the all days (14 days) of cultivation. Similar results for these EOs (thyme, clove, cinnamon, lemongrass, cumin, oregano and lavender) have been reported by numerous studies (Bluma & Etcheverry, 2008; Carmo, Lima, De Souza, & De Sousa, 2008; Santamarina, Roselló, Giménez, & Amparo Blázquez, 2016; Tarazona et al., 2018; Tian et al., 2012).

Only three essential oils, namely basil, rosemary and sage, partially inhibited the growth of all *Aspergillus* strains tested. In model conditions, at the higher doses assayed, the most resistant fungi against the sage essential oil were *A. flavus* (SLO-B-201) and *A. parasiticus* (SLO-B-219), with a lowest differences in mycelial growth inhibition compared

**Table 2**
Regression values in mycelial growth of *Aspergillus* strains after essential oils treatment.

| Species | Reg. value | Essential oils | | | | |
|---|---|---|---|---|---|---|
| | | T/CL/LA/CU/O/CI/LE | B | R | S | Control |
| ***AF*-SLO-B-201**[a] | YIntercept | ND | −1.909 | −0.1769 | −1.330 | 0.9234 |
| | Slope | | 3.105 | 1.616 | 2.726 | 0.8966 |
| | $R^2$ | | 0.8340 | 0.9451 | 0.9261 | 0.9568 |
| ***AP*-SLO-B-219**[a] | YIntercept | ND | 0.0001762 | 0.4083 | 0.2638 | 1.072 |
| | Slope | | 1.453 | 1.007 | 0.2431 | 0.7662 |
| | $R^2$ | | 0.9214 | 0.9154 | 0.8898 | 0.9313 |
| ***AO*-SLO-B-243**[a] | YIntercept | ND | −0.6735 | −0.6588 | −0.4989 | 0.8719 |
| | Slope | | 2.034 | 1.560 | 1.619 | 0.8764 |
| | $R^2$ | | 0.8323 | 0.8684 | 0.8529 | 0.9353 |
| ***AW*-SLO-B-249**[a] | YIntercept | ND | −0.2869 | −0.5321 | −0.6721 | 0.8447 |
| | Slope | | 1.456 | 1.947 | 1.813 | 0.9335 |
| | $R^2$ | | 0.9069 | 0.8280 | 0.9206 | 0.9948 |

Regression model was calculated by log-log linear regression. Regression equation is following Radial growth = 10^(slope[a]log (Days of cultivation) + Yintercept).

T-Thyme, CL-Clove, LA-Lavender, CU-Cumin, O-Oregano, Ci-Cinnamon, LE-Lemongrass, B-Basil, R-Rosemary, S-Sage.

[a] -strain ID, AF- A. flavus, AP- A. parasiticus, AO- A. ochraceus, AW-A. westerdijkiae.

to the control, where sage essential oil inhibited growth of *A. flavus* about 31.56% and A. parasiticus about 57.05%, respectively. Despite the fact that *A. parasiticus* (SLO-B-219) is phenotypically similar to *A. flavus*, the most resistant were found against basil (specifically 50.45%), further to sage (57.05%) and the greater inhibitory effect on this species showed rosemary essential (61.09%) oil after 14 days of cultivation (Fig. 1B). Also Atanda et al. (Atanda, Akpan, & Oluwafemi, 2007) recorded that sweet basil oil at optimal protective dosage of 5% (v/v) was fungistatic on *A. parasiticus* CFR 223. The sage and basil essential oils also inhibited the growth of *A. flavus* (SLO-B-201) until the 3rd day of cultivation only. On the other hand, basil essential oil in comparison to the control and *A. parastitcus* (SLO-B-219) species had better inhibitory effect on the growth of *A. flavus* (SLO-B-201) until the 11th day of cultivation (Fig. 1A). The most effective antifungal activity against *A. ochraceus* (SLO-B-243) was shown after 14 days of cultivation by rosemary oil, where 83.34% inhibition compared to control were found. Further, continued inhibitions by sage oil (71.91%) and basil oil (43.86%) with significant differences ($P < 0.05$) between each other. Basil essential oil was the least effective during the whole cultivation period. Similar results were observed against *A. westerdijkiae* (SLO-B-249), however this strain was more inhibited by sage (75.65%), followed by basil (73.12%) and the least by rosemary essential oil (45.13%) after 14 days of cultivation and during the whole cultivation period. Sage essential oil inhibited the growth of *A. ochraceus* and *A. westerdijkiae* until the 7th day as well as rosemary essential oil (Fig. 1C and D). Stupar et al. (Stupar et al., 2014) tested oregano, rosemary and lavender essential oils against some fungi species, included *A. ochraceus*. Their results showed that rosemary oil had a strong inhibition effect on this species but with lower inhibition potential.

Therefore, the lower concentrations (15.625–250 μL/L of air) of these EOs were used to determine the minimum inhibitory doses (MIDs) on tested fungi. The obtained results of MIDs determination are summarized in Table 3.

The highest MIDs value against the tested aspergilli was found in lavender (MIDs 250 μL/L of air) for *A. westerdijkiae* (SLO-B-249) strain and this essential oil had no inhibition effect at lower concentration on all other tested strains. Stupar et al. (Stupar et al., 2014) also m obtained highly values of MIC and MFC for *L. angustifolia* (from 10.0 to 100.0 μL/L) oil tested by gas diffusion method. Therefore, due to the low ability to inhibit strains in evaluating of MIDs, lavender oil was not included in *in situ* experiments. The best MIDs values obtained against the tested fungal strains were lemongrass (15.625 μL/L of air), followed by clove oil (31.25 μL/L of air), both for all tested strains. The most sensitive strain was *A. flavus* (AF-SLO-B-201) with the best values of MIDs after 14 days of cultivation for lemongrass + cinnamon (15.625 μL/L of air) > thyme + oregano + clove (31.25 μL/L of air) EOs. Cinnamon and lemongrass EOs has been used in study of Mahilrajan et al. (Mahilrajan, Nandakumar, Kailayalingam, & Manoharan, 2014) against *A. niger, A. flavus* and *Penicillium* spp. They recorded a strong inhibition activity for cinnamon oil with MIC value of 10, 15 and 25 mL/dL and for lemongrass oil were obtained MIC value of 25 mL/dL. The strong inhibitory activity of lemongrass oil was also confirmed by Tyagi and Malik (Tyagi & Malik, 2010). They found that the vapour phase of essential oils is more effective (MID 32.7 mg/L) than adding the oil to cultivated medium (288 mg/L). In our study, cumin essential oil were effective against all tested strains at lower concentration of 62.5 (μL/L of air), except AF-SLO-B-201 strain. Here was obtained the MID value of 125 (μL/L of air).

The better results for cumin essential oil was obtained by Zhaveh et al. (Zhaveh et al., 2015). They found the minimum inhibitory concentration of free and encapsulated *C. cyminum* EOs against *A. flavus* under sealed condition at 650 and 350 ppm, respectively. Different results obtained could be explained due to various method used for evaluating of the antifungal activity.

**Table 3**
Minimum inhibitory doses (μL/L of air) of essential oils in vapour phase effective against the tested aspergilli strains on CYA at 25 ± 1 °C after 14 days.

| Essential oils | MIDs (μL/L of air) of essential oils | | | |
|---|---|---|---|---|
| | AF-SLO-B-201 | AP-SLO-B-219 | AO-SLO-B-243 | AW-SLO-B-249 |
| Thyme | 31.25 | 31.25 | 15.625 | 15.625 |
| Clove | 31.25 | 31.25 | 31.25 | 31.25 |
| Cumin | 125 | 62.5 | 62.5 | 62.5 |
| Lavender | NA | NA | NA | NA |
| Cinnamon | 15.625 | 31.25 | 31.25 | 31.25 |
| Oregano | 31.25 | 62.5 | 62.5 | 62.5 |
| Lemongrass | 15.625 | 15.625 | 15.625 | 15.625 |

NA-non-active, CYA-Czapek yeast extract agar.

**Table 4**
Minimum inhibitory doses ($MID_{50}$ and $MID_{90}$) for used essential oils able to inhibit growth of *Aspergillus* species on bread samples at 25 ± 1 °C after 14 days.

| Species | | Essential oils | | | | | |
|---|---|---|---|---|---|---|---|
| | | CI | CL | T | LE | O | CU |
| *AF*-SLO-B-201[a] | $MID_{50}$ | 131.62 | 445.90 | 296.01 | 96.91 | 156.22 | 367.17 |
| | **$MID_{90}$** | 558.44 | 679.49 | 474.20 | 134.12 | 319.85 | 636.47 |
| *AP*-SLO-B-219 | **$MID_{50}$** | 357.44 | 488.90 | 267.55 | 70.26 | < 125 | 453.58 |
| | **$MID_{90}$** | 575.41 | 866.27 | 564.41 | 235.06 | 711.00 | 802.28 |
| *AO*-SLO-B-243 | **$MID_{50}$** | 226.74 | 343.40 | 70.81 | 106.65 | 167.91 | 364.06 |
| | **$MID_{90}$** | 445.90 | 788.08 | 587.27 | 141.52 | 351.77 | 643.65 |
| *AW*-SLO-B-249 | **$MID_{50}$** | 227.83 | 205.02 | < 125 | 96.91 | 126.65 | 304.58 |
| | **$MID_{90}$** | 417.64 | 505.69 | 430.52 | 134.12 | 285.37 | 586.97 |

$MID_{50}$-concentration causing 50% reduction in mycelial growth, $MID_{90}$-concentration causing 90% reduction in mycelial growth.

[a] -strain ID, T-Thyme, CL-Clove, CU-Cumin, O-Oregano, CI-Cinnamon, LE-Lemongrass, *AF- A. flavus*, *AP- A. parasiticus*, *AO- A. ochraceus*, *AW- A. westerdijkiae*.

**Table 5**
The effect of essential oils on spore germination of *Aspergillus* species at 22 ± 1 °C after 14 days of cultivation in in situ condition (results expressed in percent of spore germination inhibition in comparison with the control assay).

| Essential oils | conc. (μL/L of air) | Species | | | |
|---|---|---|---|---|---|
| | | *AF*-SLO-B-201[a] | *AP*-SLO-B-219 | *AO*-SLO-B-243 | *AW*-SLO-B-249 |
| **Cinnamon** | 125 | 41.67% | 58.33% | 66.67% | 75% |
| | 250 | 83.33% | 66.67% | 75% | 83.33% |
| | 500 | 91.67% | 91.67% | 100% | 100% |
| **Clove** | 125 | 66.67% | 75% | 58.33% | 50% |
| | 250 | 83.33% | 83.33% | 83.33% | 66.67% |
| | 500 | 91.67% | 91.67% | 91.67% | 91.67% |
| **Thyme** | 125 | 75% | 66.67% | 50% | 83.33% |
| | 250 | 91.67% | 91.67% | 75% | 91.67% |
| | 500 | 100% | 100% | 100% | 100% |
| **Lemongrass** | 125 | 100% | 100% | 100% | 100% |
| | 250 | 100% | 100% | 100% | 100% |
| | 500 | 100% | 100% | 100% | 100% |
| **Oregano** | 125 | 83.33% | 83.33% | 75% | 58.33% |
| | 250 | 91.67% | 91.67% | 91.67% | 83.33% |
| | 500 | 100% | 100% | 100% | 100% |
| **Cumin** | 125 | 25% | 33.33% | 0% | 25% |
| | 250 | 50% | 50% | 33.33% | 50% |
| | 500 | 66.67% | 66.67% | 50% | 75% |
| **Control** | | 0%[NT] | 0% [NT] | 0% [NT] | 0% [NT] |

[a] -strain ID, conc.-concentration, [NT] – Not treated with essential oils, *AF-A. flavus, AP-A. parasiticus, AO-A. ochraceus, AW-A. westerdijkiae*.

### 3.3. In situ antifungal assays on the bread samples

The determination of $MID_{50}$ and $MID_{90}$ values of tested essential

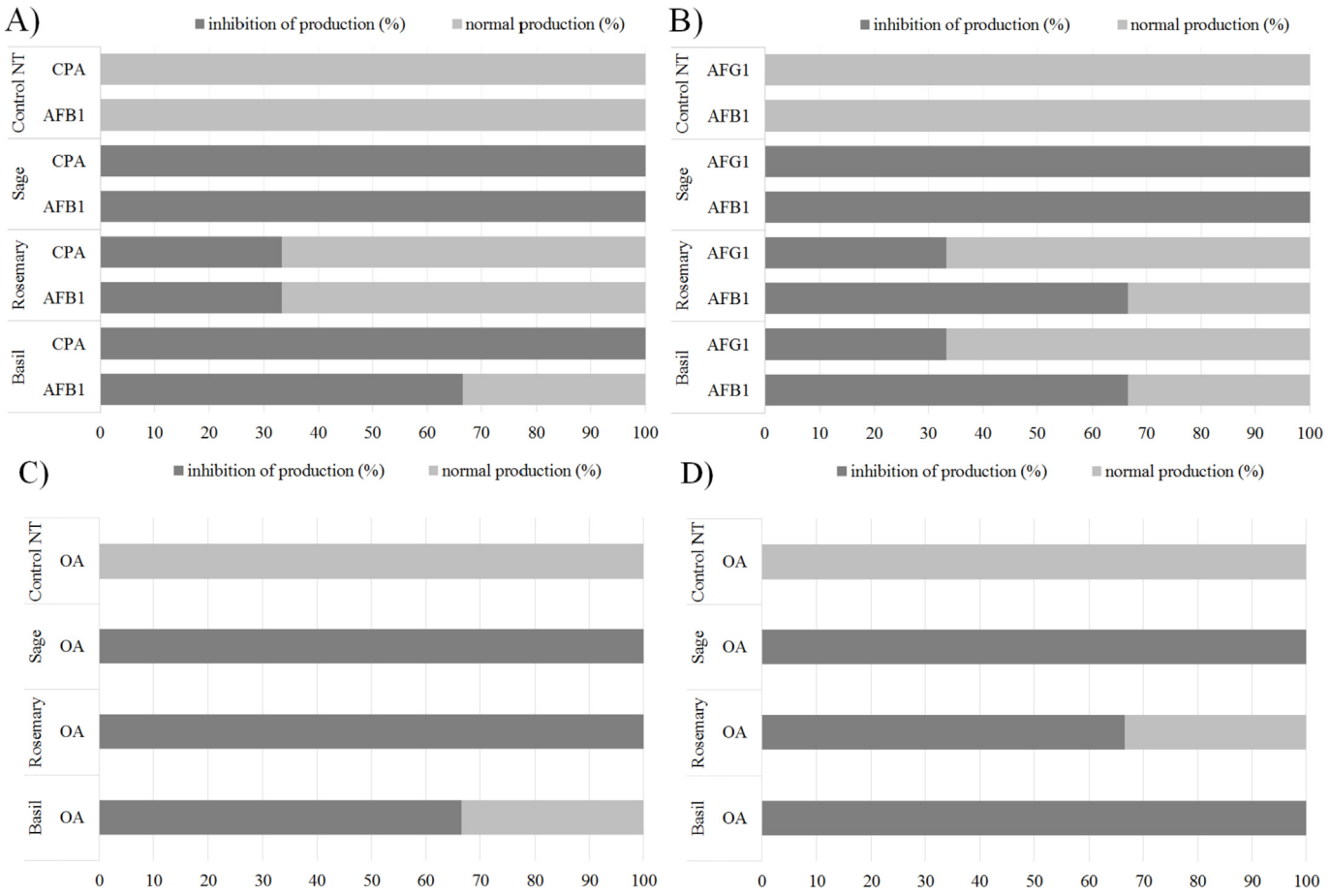


**Fig. 2.** Inhibitory effect of essential oils (500 µL/L of air) on production mycotoxins by *Aspergillus* species after 14 days of cultivation. (A) *A. flavus* (SLO-B-201), (B) *A. parasiticus* (SLO-B-219), (C) *A. ochraceus* (SLO-B-243), (D) *A. westerdijkiae* (SLO-B-249), AFG1-aflatoxin $G_1$, AFB1-aflatoxin $B_1$, CPA-cyclopiazonic acid, OA-ochratoxin A, Control NT-control non treated.

oils, namely thyme, clove, cumin, oregano, cinnamon and lemongrass, was evaluated in hermetically closed sterile glass jars for 14 days at 25 ± 1 °C in the dark after treatment using concentration of 125, 250 and 500 µL/L of jars volume. The results were estimated by probity analysis and are summarized in Table 4.

The lowest value of $MID_{50}$ was determined for lemongrass (70.26 µL/L of air), followed by thyme (70.81 µL/L of air) > oregano (< 125 µL/L of air) > cinnamon (131.62 µL/L of air) > clove (205.02 µL/L of air) and finally for cumin essential oil (304.58 µL/L of air). Our results showed that lemongrass essential oil strongly inhibited the growth of three tested strains at relatively low concentrations: *A. parasiticus* (AP-SLO-B-219) with $MID_{50}$ 70.26 (µL/L of air), *A. flavus* (AF-SLO-B −201) and *A. westerdijkiae* (AW-SLO-B-249) with $MID_{50}$ of 96.91 (µL/L of air), respectively. According the probity analysis, the best $MID_{90}$ values were found at concentration 134.12 (µL/L of air), which inhibited the radial mycelial growth of *A. flavus* and *A. westerdijkiae* during 14 days. Krisch et al. (Krisch et al., 2013) tested the antifungal activity of marjoram and clary sage essential oils against bread spoilage moulds *A. niger, Penicillium chrysogenum* and *Rhizopus* spp. by the reversed Petri dish method on wheat, wheat-rye mixed, and rye bread slices by modelling an active packaging. Their results showed that the essential oils are able to inhibit and significantly reduce the growth of all investigated moulds by vapour treated bread slices. However, they found that *A. niger* was the most resistant fungi.

Our results showed that essential oils were effective but at higher concentration in comparison with the *in vitro* evaluation. For example, thyme essential oil vapour completely eliminated *Alternaria alternata* growth in *in vitro* but only reduced growth on cherry tomatoes (Feng, Chen, Zheng, & Liu, 2011). The reason of different antifungal activity is the food products, due to high concentrations needed to achieve sufficient antimicrobial activity. In many food products, the hydrophobic essential oil constituents are impaired by interactions with food matrix components, such as starch, fat and proteins (Rattanachaikunsopon & Phumkhachorn, 2010; Kyung, 2012). The antimicrobial effect of EOs and their constituents also depends on used extraction methods of EOs, temperature and pH (Rattanachaikunsopon & Phumkhachorn, 2010), and the contamination level of food products (Hyldgaard, Mygind, & Meyer, 2012).

The effect of EOs on the sporulation of tested strains is shown in Table 5. The essential oils of lemongrass (all tested concentration), oregano and thyme (concentration of 500 µL/L of air) showed strongest inhibitory effect (100%) on the sporulation of all tested *Aspergillus* species.

Table 5: The effect of essential oils on spore germination of Aspergillus species at 22 ± 1 °C after 14 days of cultivation in in situ condition (results expressed in percent of spore germination inhibition in comparison with the control assay)

At the same concentration, clove and cinnamon oil inhibited more than 90% of sporulation. Moderate inhibitory effect was observed for all essential oils at concentration 250 (µL/L of air), except cumin essential oil which showed the very poor inhibitory effect on sporulation at the highest concentration (less than 80%). Our results agree with authors Paranagama et al. (Paranagama et al., 2003), Tzortzakis and Economakis (Tzortzakis & Economakis, 2007) or Aguiar et al. (Aguiar et al., 2014), who clearly demonstrated that lemongrass oil at different concentrations completely retarded and inhibited fungal sporulation.

*A. ochraceus* was the most resistant species from all tested strains in our study. Thyme oil was able to inhibited sporulation of this strain only at concentration of 500 μL/L of air completely. The lower concentration (125 μL/L of air) inhibited only 50% of sporulation. Cumin essential oil at the same concentration had no or stimulating effect on sporulation of *A. ochraceus*. Numerous studies (Kadoglidou et al., 2011; Kuate et al., 2006) demonstrated that essential oils could have the stimulating effect on sporulation of fungi. The same authors suppose that sporulation *per se* is part of a defence mechanism enabling fungi to overcome the chemical stress caused by the monoterpenoids in their growth medium. Moreover, the differentiating part of the aerial hyphae seems to be more sensitive to the respiratory inhibition than other aerial and vegetative hyphae. Interestingly enough, the antisporulating effect was observed only by gaseous contact, but not by solution contact. Gaseous oils can directly bind to reproductive hyphae, but the oils in the medium cannot bind to hyphae standing in air (Inouye, 2003).

Anti-toxicogenic effect of essential oils in *in vitro* and *in situ* condition.

The production of mycotoxins was confirmed by TLC method for all tested strains. The presence of aflatoxin $B_1$ and aflatoxin $G_1$ was confirmed in *Aspergillus parasiticus* (SLO-B-219). *Aspergillus ochraceus* (SLO-B-245) and *A. westerdijkiae* (SLO-B-249) produced ochratoxin A. Cyclopiazonic acid and aflatoxin $B_1$ were confirmed for *Aspergillus flavus* (SLO-B-201). The potential inhibitory effect on mycotoxins production by *Aspergillus* species was tested after treatment with essential oils (500 μL/L of air, 14 days of cultivation). For each assay was selected only essential oils which did not inhibit the growth of the tested strains completely in both, *in vitro* and *in situ* assay. Fig. 2 show *in vitro* anti-toxicogenic activity of basil, rosemary and sage essential oils.

Results showed a various spectrum of fungal toxicity inhibition by EOs in *in vitro* screening. The best results were obtained from treatments with sage essential oil, which totally (100%) inhibited the mycotoxin production of all tested strains after 14 days. The production of $AFB_1$ and CPA by *A. flavus* (SLO-B-201) (Fig. 2A), $AFB_1$ and $AFG_1$ by *A. parasiticus* (SLO-B-219) (Fig. 2B) and OA synthesis by *A. ochraceus* (SLO-B-245) (Fig. 2C) and *A. westerdijkiae* (SLO-B-249) (Fig. 2D) was inhibited, respectively. The complete anti-toxicogenic effects of sage essential oil have also been confirmed in previous studies (Císarová et al., 2015; Císarová, 2015; Císarová et al., 2016). Basil oil was able to completely (100%) inhibited production of CPA by *A. flavus* (SLO-B-201) and OA production by *A. westerdijkiae* (SLO-B-249). The least effective essential oil was rosemary, in this study. This oil completely (100%) inhibited only production of OA by *A. ochraceus* (SLO-B-245). The different effects of individual oils on mycotoxins production or growth of fungi could be explained by using different species of microscopic fungi. For example, Santos et al. (Santos et al., 2010) in their study demonstrated that OA production was the most inhibited in *A. tubingensis* and the least in *A. westerdijkiae* species. Inhibitory effects of essential oils on mycotoxins accumulation are not always associated (Bluma, Amaiden, & Etcheverry, 2008). Prakash et al. (Prakash et al., 2015) suggested that lack of sporulation in fungal mycelia treated with essential oils may be a strong reason for their anti-mycotoxigenic activity as such correlation between secondary metabolite production and sporulation. This suggestion correlated with our results from *in situ* assay on bread samples (Table 6).

Lemongrass, oregano, thyme, cinnamon and clove belonged to the most effective essential oils, with the best inhibition effect on sporulation of all tested *Aspergillus* strains. Lemongrass completely inhibited the growth and sporulation of each fungus, so the anti-toxicogenic effect of this essential oil could not be demonstrated by TLC method. Very good anti-toxicogenic activity of lemongrass EO recorded Paranagama et al. (Paranagama et al., 2003) against aflatoxin $B_1$ produced by *A. flavus* on rice. Cinnamon and clove essential oils completely inhibited all mycotoxin production only at a high concentration (500 μl/L of air), cinnamon in *A. parasiticus* (SLO-B-219) and *A. flavus* (SLO-B-201) and clove in all tested species. Only oregano oil was able to inhibit the aflatoxin production in both, *A. parasiticus* (SLO-B-219) and *A. flavus* (SLO-B-201), without concentration dependent manner, and inhibition of OA production by this oil was very good in a comparison with control sets. Thyme oil inhibited completely the production of OA and aflatoxin at concentration up to 250 μl/L of air in all tested strains. Cumin essential oils showed very poor or no effect on mycotoxins production by tested *Aspergillus* strains. Types of the EOs, the concentration used and time of incubation markedly influenced the anti-toxicogenic activity of the tested oil. Similar results were obtained by Azaiez et al. (Azaiez, Meca, Manyes, & Fernández-Franzón, 2013). They investigated the gaseous phase of allyl (AITC), phenyl (PITC) and benzyl isothiocyanates (BITC) on the bread inoculated with some toxigenic fungi. They also found that the fumonisins $B_2$ production was reduced by73–100%, but this reduction was depended on the dose and time of exposure. In addition, the TLC method used in this study was a qualitative method; therefore, if there is any difference, it will not be detectable. However, it is rapid and low-cost method in a comparison with other methods (UHPLC, HPLC, LC-MS) used for detection of mycotoxins.

**Table 6**
In situ inhibitory effects of essential oils at 22 ± 1 °C after 14 days of cultivation on the mycotoxin production by *Aspergillus* spp. [three replications in treatments with each essential oil were screened (four colonies in three repetition, n = 12)].

| Essential oils | conc. (μL/L of air) | AF-SLO-B-201[a] | AP-SLO-B-219 | AO-SLO-B-243 | AW-SLO-B-249 |
|---|---|---|---|---|---|
| | | number of tested strains/number of positive strains | | | |
| Cinnamon | 125 | 7/2 | 12/2 | 7/2 | 8/2 |
| | 250 | 3/1 | 7/1 | 8/1 | 7/1 |
| | 500 | 2/0 | 3/0 | NA | NA |
| Clove | 125 | 8/2 | 11/5 | 9/4 | 7/3 |
| | 250 | 4/1 | 9/2 | 7/4 | 6/2 |
| | 500 | 3/0 | 6/0 | 4/0 | 1/0 |
| Thyme | 125 | 11/6 | 5/2 | 6/3 | 4/2 |
| | 250 | 3/0 | 1/0 | 3/0 | 2/0 |
| | 500 | NA | NA | 2/0 | 1/0 |
| Lemongrass | 125 | NA | NA | NA | NA |
| | 250 | NA | NA | NA | NA |
| | 500 | NA | NA | NA | NA |
| Oregano | 125 | 2/0 | 4/0 | 7/3 | 2/2 |
| | 250 | 1/0 | 3/0 | 4/2 | 6/2 |
| | 500 | NA | 2/0 | NA | NA |
| Cumin | 125 | 12/12 | 12/12 | 10/9 | 9/9 |
| | 250 | 6/6 | 7/6 | 9/8 | 8/8 |
| | 500 | 4/4 | 6/6 | 3/1 | 2/2 |
| Control | | 12 | 12 | 12 | 12 |

[a] -ID of tested strain, conc.-concentration, NA-not analysed (no visible growth of colony), *AF-A. flavus*, *AP-A. parasiticus*, *AO-A. ochraceus*, *AW-A. westerdijkiae*, CPA-cyclopiazonic acid, OA-ochratoxin A, AFB1/AFG1-aflatoxins.

### 3.4. Sensory properties

The use of antimicrobials as preservatives in food systems can negatively influence the organoleptic properties, especially the use of essential oils. So the one objective of this study considered their impact on organoleptic properties of the used bread samples. In our study, the triangle test was chosen to distinguish between samples without necessity to specify the differing sensory characteristics (Lawless & Heymann, 2010). The results and the number of correct responses identified the treated samples in the triangle test and are summarized in Table 7. The treatment of bread samples with EOs did not have impact on the odour and taste, with the exception of oregano and lemongrass EOs. Nearly half of the panellists detected differences between breads treated with oregano and lemongrass at all days of treatment (3rd, 7th, 11th and 14th days). The most significant difference ($p < 0.001$) was observed at the concentration of 250 and 500 μl/L of air for both, oregano and lemongrass oil. The breads treated with the lowest

**Table 7**
Sensory evaluation of the sliced bread treated by different essential oils 3rd, 7th, 11th and 14th days after storage at 22 ± 1 °C in the dark.

| Essential oils | conc. μL/L | 3rd day | | 7th day | | 11th day | | 14th day | |
|---|---|---|---|---|---|---|---|---|---|
| | | Correct replies (%) | *p-value* | Correct replies (%) | *p-value* | Correct replies (%) | *p-value* | Correct replies (%) | *p-value* |
| Thyme | **125** | 10 (22.22%) $^{ns}$ | *0.153* | 12 (26.66%) $^{ns}$ | *0.429* | 15 (33.33%) $^{ns}$ | *1* | 19 (42.22%) $^{ns}$ | *1* |
| | **250** | 12 (26.66%) $^{ns}$ | *0.430* | 12 (26.66%) $^{ns}$ | *0.429* | 13 (28.88%) $^{ns}$ | *0.635* | 14 (31.12%) $^{ns}$ | *0.874* |
| | **500** | 15 (33.33%) $^{ns}$ | *1* | 15 (33.33%) $^{ns}$ | *1* | 16 (35.56%) $^{ns}$ | *0.753* | 16 (35.56%) $^{ns}$ | *0.753* |
| Clove | **125** | 9 (20.00%) $^{ns}$ | *0.058* | 11 (24.44%) $^{ns}$ | *0.267* | 11 (24.44%) $^{ns}$ | *0.267* | 12 (26.67%) $^{ns}$ | *0.268* |
| | **250** | 10 (22.22%) $^{ns}$ | *0.153* | 10 (22.22%) $^{ns}$ | *0.153* | 10 (22.22%) $^{ns}$ | *0.153* | 11 (24.45%) $^{ns}$ | *0.268* |
| | **500** | 12 (26.26%) $^{ns}$ | *0.429* | 13 (28.88%) $^{ns}$ | *0.636* | 13 (28.88%) $^{ns}$ | *0.635* | 14 (31.12%) $^{ns}$ | *0.874* |
| Cinnamon | **125** | 8 (17.77%) $^{ns}$ | *0.940* | 8 (17.77%) $^{ns}$ | *0.940* | 8 (17.77%) $^{ns}$ | *0.940* | 9 (20.00%) $^{ns}$ | *0.268* |
| | **250** | 9 (20.00%) $^{ns}$ | *0.058* | 9 (20.00%) $^{ns}$ | *0.058* | 10 (22.22%) $^{ns}$ | *0.153* | 11 24.45%) $^{ns}$ | *0.268* |
| | **500** | 20 (44.44%) $^{ns}$ | *0.116* | 20 (44.44%) $^{ns}$ | *0.116* | 22 (48.89%) $^{ns}$ | *0.208* | 22 (48.89%) $^{ns}$ | *0.209* |
| Lemongrass | **125** | 21 (46.66%) $^{ns}$ | *0.001* | 21 (46.66%) $^{ns}$ | *0.006* | 22 (48.89%) $^{ns}$ | *0.001* | 24 (53.34%) | *0.001* |
| | **250** | 27 (60.00%) | *0.001* | 27 (60.00%) | *0.000* | 28 (62.22%) | *0.001* | 30 (66.67%) | *0.001* |
| | **500** | 30 (71.11%) | *0.001* | 30 (71.11%) | *0.000* | 33 (73.33%) | *0.001* | 33 (73.33%) | *0.001* |
| Oregano | **125** | 19 (42.22%) $^{ns}$ | *0.001* | 19 (42.22%) $^{ns}$ | *0.000* | 20 (44.44%) $^{ns}$ | *0.001* | 24 (53.34%) | *0.001* |
| | **250** | 23 (51.11%) $^{ns}$ | *0.001* | 23 (51.11%) $^{ns}$ | *0.002* | 23 (51.11%) $^{ns}$ | *0.001* | 24 (53.34%) | *0.001* |
| | **500** | 29 (64.44%) | *0.001* | 29 (64.44%) | *0.000* | 30 (66.67%) | *0.001* | 30 (66.67%) | *0.001* |
| Cumin | **125** | 5 (11.11%) $^{ns}$ | *0.888* | 5 (11.11%) $^{ns}$ | *0.888* | 6 (13.34%) $^{ns}$ | *0.822* | 6 (13.34%) $^{ns}$ | *0.822* |
| | **250** | 6 (13.33%) $^{ns}$ | *0.058* | 6 (13.33%) $^{ns}$ | *0.153* | 7 (15.55%) $^{ns}$ | *0.153* | 8 (17.78%) $^{ns}$ | *0.153* |
| | **500** | 10 (22.22%) $^{ns}$ | *0.153* | 12 (26.66%) $^{ns}$ | *0.429* | 12 (26.66%) $^{ns}$ | *0.429* | 13 (28.88%) $^{ns}$ | *0.636* |

conc.- concentration, ns-Not significantly different. For $n = 45$ the difference between samples would be significant if the number of correct answer was 24 or more ($p \leq 0.01$) ISO Standards 4120:2004, *p-value*-calculated according the correct and incorrect answers by the chi-square binomial distribution with value of α-risk = 0.05 (R Core Team, 2016).

concentration (125 μL/L of air) of oregano and lemongrass essential oil were acceptable until 11th day of treatment. However, oregano EO caused the spicy flavour in treated bread and lemongrass EO caused an unacceptable citrus flavour. Thus, these EOs cannot be used at the most effective concentration against the growth of pathogenic fungi on bread. The treatment with other EOs did not have any significant influence on the sensory evaluation. The best result was obtained in treatment by thyme and cumin EOs at all used concentrations (125–500 μL/L of air) throughout the all storage period. Also none of the triangle test attained the 24 correct replies needed (except with treatment by oregano and lemongrass EOs) to establish significant differences. According to these results, the use of EOs such as thyme, clove, cinnamon and cumin as sprout inhibitors in vapour phase does not imply any effect on the sensory properties of bread.

## 4. Conclusions

In this study, we have demonstrated that the antifungal, anti-toxicogenic and antisporulation effect of EOs from thyme, clove, oregano, cinnamon and lemongrass are highly effective against tested toxigenic *Aspergillus* species in vapour phase in both, *in vitro* and *in situ* condition. Lemongrass showed the strongest activity, followed by thyme, cinnamon, clove and oregano. Other tested EOs, such as basil, rosemary and sage had not any significant antifungal activity, but they should find a practical application in the inhibition of the mycotoxin production of fungi. Moreover, a treated bread sample with EOs does not have negative effect on the sensory properties of the breads, with exception of oregano and lemongrass. In conclusion, it is possible to test a combination of different essential oils in order to reduce the growth, sporulation and mycotoxins, mainly aflatoxin and ochratoxin production of aspergilli. The use of essential oils promising alternative to the use of chemical inhibitors to avoid the growth and mycotoxins production without affecting the overall bread quality.

## Declaration of competing interestCOI

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work was supported by project no. FPPV-09-2018 and by Slovak Research and Development Agency under Contract no. APVV-16-0289.f

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