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INHIBITORY EFFECT OF ESSENTIAL OILS ON THE GROWTH OF *GEOTRICHUM CANDIDUM*

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ABSTRACT

The aim of this study was focused on the determination of the inhibitory effect of selected essential oils on the growth of five isolates of *Geotrichum candidum*. The isolates were obtained from the dairy products of domestic origin (sour cream, raw cow's milk, base for production of the brand, unsalted cheese, and cheese made from cows' milk). We studied the impact of five essential oils [pure essential oil EOs (100 %)] - clove, basil, rosemary, fennel and thyme at concentration of 0.625 $\mu L.cm^{-3}$ of air. Gas chromatography-mass spectrometry (GC-MS) analysis was used for the identification of the main components of EOs. We tested the effect of the essential oils by the gaseous diffusion method. The isolates were cultivated on PDA (Potato dextrose agar), in the dark, at 25 ±1 °C, 14 days. The diameter growing of colonies we continuously measured on the 3rd, 7th, 11th, and 14th day of cultivation. The results of the paper suggest that clove and thyme essential oil had 100 % inhibitory effect on the growth of all tested isolates. Fennel, basil and rosemary essential oil had not significant inhibitory effects on tested isolates *Geotrichum candidum*. However, these oils affected the growth of colonies throughout the cultivation. Fennel EO had 100 % inhibition only on the growth of one isolates of *Geotrichum candidum* to the 3rd day of cultivation. Basil EO had the stimultating effect on the growth of two isolates of *G. candidum* (KMi 322 and KMi 189). Our results showed that clove and thyme essential oil could be used as a natural preservative useful in the food industry.

Keywords: essential oils, inhibitory effect, *Geotrichum candidum*

INTRODUCTION

The genus *Geotrichum* is classified as microscopic filamentous fungi (**Pitt and Hocking, 2009, Samson *et al.*, 2010**), but also as yeast (**Boutrou and Guéguen, 2004**) and some characterize it as a filamentous fungus that resembles yeast (**Pottier *et al.*, 2008**). *G. candidum* is generally associated with milk and milk products. Its occurrence in food has no clear meaning. Its presence in certain dairy products such as butter, a cream, fresh cheese is considered undesirable (**Laurenčík *et al.*, 2008**).Moulds are common contaminants of milk and milk products and contribute to their spoilage. Today, it is very important to find out the protection of products of natural origin as an alternative to synthetic fungicides. The promising alternative is the use of the essential oils. Essential oils from plants have great potential as a new source of fungicide to control the pathogenic fungi (**Císarová *et al.*, 2016a**). Essential oils have deserved much attention in the past decades for their antimicrobial activity, since many of them have demonstrated efficacy against food-borne pathogenic and spoilage microorganisms (**Bassanetti *et al.*, 2017**). Essential oils have been identified as natural food additives which can find useful application in food preservation (**Davidson *et al.*, 2013**). Nowadays, natural biological preservatives are mainly extracted from edible spices, and the essential oils from these spices have been found to be effective preservatives (**Burt, 2004**). Natural flavours, this is primarily the essential oils, as well as individual aromatic components that are derived from essential oils by physical methods. Gas chromatogra-phy coupled with mass spectrometry (GC–MS) and flame ionizationdetector (GC-FID) are used for chemical composition analysis ofisolated oils (**Matos, 2012; Smelcerovic *et al.*, 2013; Frolova *et al.*, 2019**). Both the essential oil and some of its components, e.g. carvacrol, thymol, gamma-terpinene, alpha-terpinene, or para-cymene, have well-documented biological activity. Carvacrol exerts antimicrobial (**Kotan *et al.*, 2012**), antifungal, antioxidant, analgesic, and antiinflammatory effects (**Moghadam, 2015**). Another phenolic compound present in the essential oil, i.e. thymol, has antimicrobial, expectorant and disinfectant effect (**Moghtader, 2012**). Limited information is available on the antifungal activity of plant extracts against *Geotrichum candidum*. The aim of the present research was to determine the inhibitory effect of vapor phase of five essential oils on the growth of different strains of *Geotrichum candidum* isolated from moldy milk products.

Scientific hypothesis

The aim of the present research was to determine the inhibitory effect of five essential oils on the growth of different *Geotrichum candidum* isolates.

MATERIAL AND METHODS

Fungal isolation and identification

Five strains (Table 1) from different moldy milk products of domestic origin were used. These strains belong to the Collection of Fungi of Department of Microbiology; Faculty of Biotechnology and Food Sciences SUA in Nitra, Slovakia. Isolates of the genus *Geotrichum* were identified to the species level according to morphological characteristics based on microscopy. To determine particular species, diagnostic literature was used as follows: **Pitt and Hocking** (**2009**), **Samson *et al.*,** (**2010**). Strains were inoculated in three points on the identification media MEA (malt extract agar). Inoculated media were incubated at 25 ± 1 °C, 7 days in the dark.

Table 1 Origin of the strains *Geotrichum candidum*

Strains	Origin
G. *candidum* KMi 329	sour cream
G. *candidum* KMi 120	raw cow's milk
G. *candidum* KMi 189	base for production of the brand
G. *candidum* KMi 322	unsalted cheese
G. *candidum* KMi 172	cheese made from cows' milk

Essential oils

The essential oils used in this study were obtained from clove (*Syzygnium aromaticum* L.), basil (*Oscimum basilicum* L.), rosemary (*Rosmarinus officinalis* L.), fennel (*Foeniculum vulgare* L.), thyme (*Thymus vulgaris* L.). The used oils were supplied by Calendula company a.s. (Nová Ľubovňa, Slovakia) and Hanus (Nitra, Slovakia). All essential oils were extracted by hydrodistillation and stored in air-tight sealed glass bottles at 4°C.

Antifungal activity of essential oils

The antifungal activity of selected essential oils 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 **Guynot *et al.*, (2003)**. Essential oils were tested in concentration 0.625 μL.cm^{-3} of air. A round sterile filter paper (Ø 9 cm) was placed in the lid of Petri dish and 50 μL of essential oil was pipetted by micropipette to the paper. Dishes were kept in inverted position. Filter paper discs impregnated with sterilized distilled water were used as a control to confirm no solvent effect of bioactivity. Each isolate was inoculated in the center of Petri dishes with needle. Dishes were tightly sealed with parafilm and incubated for seven 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. The antifungal activity was expressed in terms of percentage of mycelial growth inhibition and calculated according to the following formula:

Mycelial growth Inhibition: MGI % = $[(d_c-d_t)/d_c]$ *100
Where d_c=average (mm) increase in mycelial growth in control, d_t=average (mm) increase in mycelial growth in treatment (**Marandi *et al.*, 2011**).

GC-MS analysis of essential oils

Essential oils constituent were identified and the relatively composition of the oil was determined by gas chromatography followed by mass spectrometry (GC-MS). Prior to the analysis, essential oils were diluted in hexane to a concentration of l μL/mL. Analyses were carried out using an Agilent 7890A GC coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS column (30 m × 0.25 mm, 0.25 mm film thickness). One microliter of the sample was injected in split mode 1:12, at an injector temperature of 250°C and at electron ionization energy of 70 eV. Analysis were measured in SCAN mode, mass range was 40-400m/z. Starting at 60°C, the oven temperature was increased at a rate of 3°C/min to a maximum of 231°C, where it was kept constant for 10 min. The identification of constituents was based on a comparison of their mass spectra and relative retention indices (RI) against the National Institute of Standards and Technology Library (NIST, USA), as well as authentic analytical standards and data from the literature. Relative proportions were calculated by dividing individual peak area by total area of all peaks. The response factor was not taken into account. Only compounds over 1% were included. Peaks under 1% were not counted. The following standards were used: α-pinene, Camphene, β-Pinene, p-Cymene, (R)-(+)-Limonene, 1,8-cineole, terpinolene, linalool, geraniol, (−)-Bornyl acetate, Trans-anetole, Eugenol, β-caryophyllene, α-caryophyllene and Caryophyllene oxide.

Statistical analysis

All analyses were performed in triplicate and the results were expressed as the mean of the data obtained in each replicate. Statistical analyses were performed with descriptive statistics.

RESULTS AND DISCUSSION

In our study were isolated the genus *Geotrichum* from different moldy milk products of domestic origin. Based on phylogenetic and morphologilcal studies were identified to the species of *Geotrichum candidum*. The colonies of all isolates had relatively rapidly growth on MEA with diameters between 50–65 mm, softer, yeast-like texture (Fig. 1A). Conidiophores were undifferentiated hyphae, fragmented almost to form arthroconidia, which were hyaline and cylindrical (Fig. 1B).

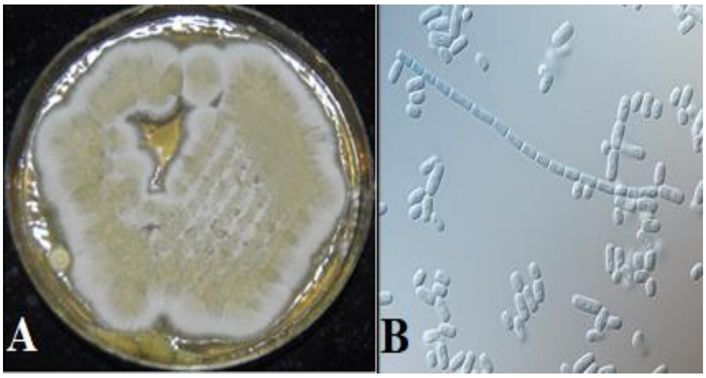

Figure 1 *Geotrichum candidum* - 7 days of cultivation at 25 ± 1 °C in the dark, colonies on MEA (A), conidia (B)

Currently, there is a growing interest in using new methods in food industry. These methods include the use of essential oils as preservatives to prolong food durability (**Adelakun *et al.*, 2016**). Many species effectively inhibit the growth of some pathogens such as e.g. *Escherichia, Shigella, Enterococcus, Bacillus, Pseudomonas, Streptococcus*, because of their excellent antimicrobial properties (**Burt, 2004**). However, essential oils are also characterized by their antifungal activity. This activity can be maintained even in the gas phase (**Reyes-Jurado *et al.*, 2019**). The aim of our paper was to determine the activity of volatile components of selected essential oils - clove (*Syzygnium aromaticum* L.), basil (*Oscimum basilicum* L.), rosemary (*Rosmarinus officinalis* L.), fennel (*Foeniculum vulgare* L.) and thyme (*Thymus vulgaris* L.) on the growth of five *Geotrichum candidum* isolates. *Geotrichum candidum* isolates reacted differently to the presence of the EOs. Among the essential oils, the ones that totally inhibited growth of all tested isolates were clove and thyme regardless of time of the days of cultivation. Basil, rosemary and fennel were also able to inhibit the growth of mould, but depending of the isolates and time of incubation. The results are presented in Table 2.

Table 2 Mycelial growth inhibition (%) of *Geotrichum candidum* by tested essential oils after 14th days of cultivation

Essential oils	Tested isolates				
	Mycelial growth inhibition (%)				
	KMi 329	KMi 120	KMi 189	KMi 322	KMi 172
Clove	100	100	100	100	100
Basil	0	1.51	**-7.59**	**-2.62**	7.81
Thyme	100	100	100	100	100
Rosemary	2.81	**-9.56**	19.62	12.67	6.94
Fennel	8.43	**-1.09**	10.97	13.68	28.19

The rosemary and fennel essential oil showed only partial inhibitory effects on the growth of *G. candidum* KMi 329 (Fig. 2). Basil EO had no effect on the growth of this isolate after 14 days of cultivation. The most resistant isolate was *G. candidum* KMi 120. Its growth was stimulating by two EOs - rosemary and fennel after 14 days of cultivation. In the presence of basil EO its growth was slightly inhibited (1.51%). Also the stimulating effect was detected in the presence of basil essential oil on the last day of cultivation (14th) for both, *G. candidum* Kmi 189 (-7.59%) and KMi 322 (-2.62%). The isolates *G. candidum* KMi 322 and KMi 189 have also similar responses to the presence of fennel essential oil. The most sensitive isolate was the *G. candidum* KMi 172. The fennel essential oil showed the most potency after thyme and clove EOs (28.19%) Basil and rosemary had only a partial inhibitory effect on the growth of the strain throughout the cultivation period. Essential oils that have the strongest antifungal activity include clove, oregano, cinnamon and thyme oils (**Taylor, 2015**). This allegation has also been confirmed when the thyme and clove essential oils showed 100% antifungal activity regardless of the culture day. **Kalemba and Kunicka (2003)** report that the antibacterial activity of thyme, cinnamon and rosemary has been demonstrated against undesirable pathogens of *Agrobacterium tumefaciens* and *Erwinia carotovora*. **Božik *et al.*, (2016)** investigated the antifungal action of thyme (but also other essential oils) on the growth of three species of the genus *Aspergillus* (*A. parasiticus, A. flavus and A. clavatus*). This study confirmed the 90% antimicrobial action of thyme essential oil at a concentration of 500 μl/l air for the growth of *Aspergillus* microscopic filamentous fungi. Very strong inhibitory effect of thyme is results based oil (**Císarová *et al.*, 2016a**) was confirmed against *A. niger* and *A. tubingensis*. This essential oil was able to completely inhibit the growth of all tested isolates using a minimum inhibitory dose of 125 μl.l^{-1}. The 100 % inhibition effect of thyme, red thyme, mint, and savory essential oils (625 μL.L^{-1} of air) against *Rhizopus* spp. has shown **Tančinová *et al.* (2018)**. Based on our results, basil essential oil showed partial inhibitory effects on the growth of all tested strains. In the case of the two tested isolates (KMi 189 and KMi 322), there were also stimulatory effects. However, **Kocić-Tnackov *et al.*,** (**2012**) report that basil essential oil is effective against the *Penicillium* species, in particular *P. aurantiogriseum*, *P. glabrum*, *P. chrysogenum* and *P. brevicompactum*.

Based on the growth of tested isolates during the cultivation days under treatments with EOs, we constructed growth curves for each isolate. The results showed that EOs inhibit the tested isolates of *G. candidum* differently in depend of the isolates and time of cultivation. From day 7 of cultivation, the diameter of the colonies increased gradually. When using rosemary essential oil, the growth of colonies has increased since the beginning of the cultivation, and on the last day of the cultivation we also noticed the stimulating effects on mycelium growth.

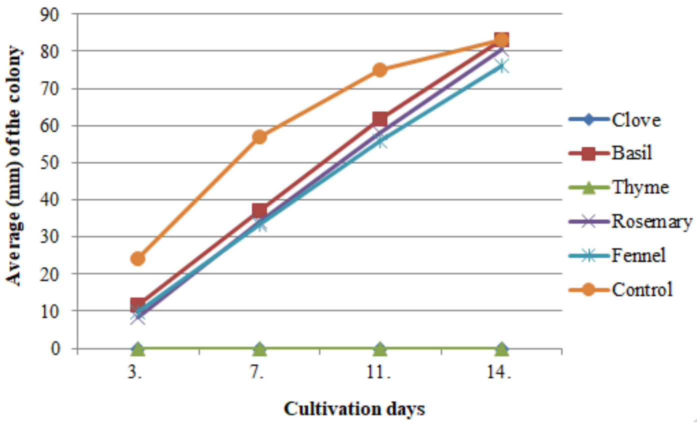

Figure 2 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *G. candidum* KMi 329

Fig. 3 shows the growth curves of *G. candidum* KMi 120. In the presence of fennel essential oil on day 3 of cultivation, the colony size was low. From day 7 of cultivation, the diameter of the colonies increased gradually. When using rosemary essential oil, the growth of colonies has increased since the beginning of the cultivation, and on the last day of the cultivation we also noticed the stimulating effects on mycelium growth.

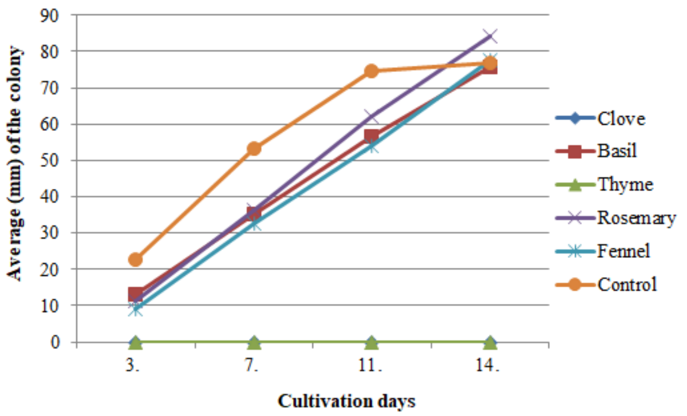

Figure 3 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *G. candidum* KMi 120

Fig. 4 shows the average efficiency of essential oils for the growth of *G. candidum* KMi 189. The stimulating effect was detected in the presence of basil essential oil on the last day of cultivation, with the lowest growth inhibitory effect of this isolate. **Císarová *et al.***, (**2016b**) observed the ability of basil to inactivate the growth of representatives of the genus *Aspergillus*. In this study, the antifungal effect of this oil showed up only for a limited period of time, because from the 7th day the growth of the isolates did not suppress.

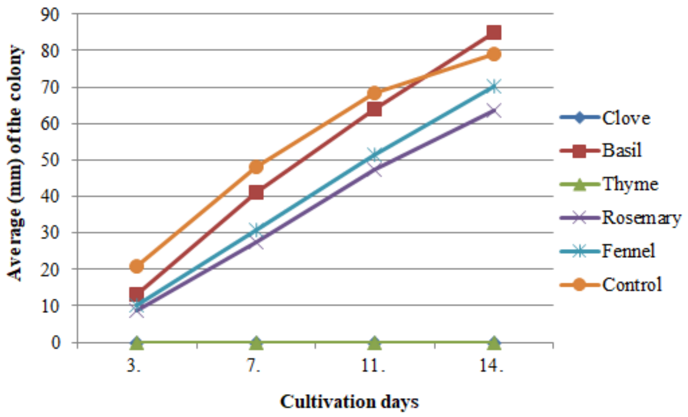

Figure 4 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *G. candidum* KMi 189

Fennel had a potent inhibitory effect by day 3 of cultivation on the growth of isolate KMi 322. The basil EO had the lowest antimicrobial effect on the growth of this isolate during the cultivation period, with stimulatory effect on the last day of cultivation (Fig. 5). The fennel EO showed 100 % inhibition effect on 3rd day of cultivation on the growth of isolate KMi 172, but the inhibitory effect gradually decreased over the next days of cultivation (Fig. 6).

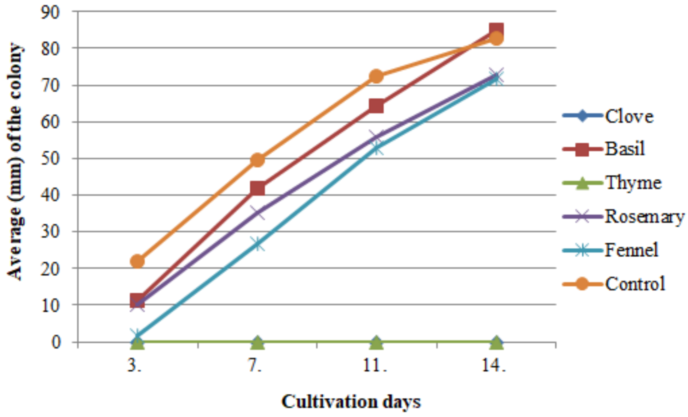

Figure 5 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *G. candidum* KMi 322

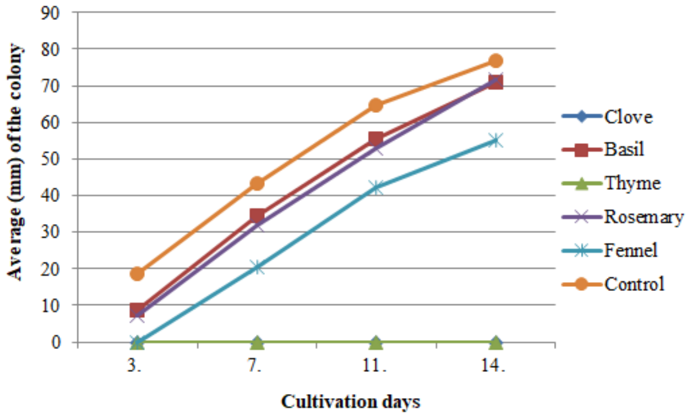

Figure 6 Antifungal activity of tested EOs (0.625 µL.cm^{-3}) to *G. candidum* KMi 172

The antifungal activity of essential oils depends largely on their chemical composition **(Coban *et al.*, 2018)**. Therefore, in our study, we subjected tested essential oils to qualitative and quantitative analysis by gas chromatography. Qualitative and quantitative analysis of the tested EOs is listed in Table 3.

Table 3 Qualitative and quantitative analysis (%) of the used essential oils by GC-MS

RI[b]	Component	Clove	Basil	Thyme	Rosemary	Fennel
938	**α-pinene[a]**			2.5	10.3	2.1
953	**Camphene[a]**				4.8	
981	**β-Pinene[a]**				7.2	
1029	**p-Cymene[a]**			39.1	3.0	
1033	**(R)-(+)-Limonene[a]**				2.6	5.1
1034	**1,8-cineole[a]**		3.5		42.9	
1090	**terpinolene[a]**					4.5
1101	**linalol[a]**		1.2	5.1		
1148	(−)-Isopulegol				13.1	
1192	α-terpineol			1.1		
1200	Estragol		88.6			4.4
1257	**geraniol[a]**					1.1
1287	**(−)-Bornyl acetate[a]**				1.3	
1289	**Trans-anetole[a]**					79.9
1298	(+)-Menthofuran			43.1		
1363	**Eugenol[a]**	82.3				
1419	**β-caryophyllene[a]**	6.0			3.6	
1454	**α-caryophyllene[a]**	1.6				
1531	acetyleugenol	7.0				
1574	**Caryophyllene oxide[a]**	1.3				
total		**98.90**	**97.80**	**97.70**	**98.00**	**99.90**

Legend: [a] – Identification confirmed by co-injection of authentic standard; [b] – RI: identification based on Kovatś retention indices (HP-5MS capillary column) and mass spectra

The main component of the clove EO was eugenol (82.3%) and the main component of the thyme EO was (+)-Menthofuran (43.1%). These oils were the most effective against tested isolates of *G. candidum*. Our results agree with other authors. **Císarová *et al.*,** (**2014**) tested the inhibitory activity of volatile components of five essential oils. Specifically, they monitored the impact of clove, jasmine, thyme, basil and rosemary against *Eurotium* sp., *E. chevalieri* and *E. rubrum*, and found that these oils had 100% antimicrobial activity included clove and thyme essential oil. **Cortés-Rojas *et al.*,** (**2014**) reports that clove essential oil compared to other essential oils such as e.g. oregano, mint and cinnamon oil have much stronger antioxidant and antimicrobial effects. Antimicrobial properties are due to clove oil's presence of eugenol. Eugenol is a phenolic compound that participates in protein denaturation and affects phospholipid membranes, changing the permeability of these membranes and thereby inhibiting microorganisms (**Shahavia *et al.*, 2015**). **Wenqiang *et al.*, (2007)** reported that clove essential oil also exhibits strong inhibitory activity against species such as *Aspergillus flavus*, *Penicillium citrinum* and *Rhizopus nigricans*. Estragole was identified as the major component of basil EO (88.6%). Rosemary EO contained mainly 1,8-cineole (42.9%) and in fennel as the main component was Trans-anetole determined (79.9%). Also **Kalleli *et al.* (2019)** investigated chemical composition of five *Foeniculum vulgare* cultivars and their found that trans-anethole was the main component of each cultivars. But in the study of **Coban *et al*. (2018)** was the major compound in the fennel fruit oil determined Anethole (90.71–91.62%), followed by estragole (3.60–4.02%), limonene (2.19–3.24%), and fenchone (0.96–1.55%), respectively. Cheese is one of the most popular dairy products which is a rich source of essential nutrients but it also can be attacked by both bacteria and fungi. However, the type of the spoilage varies depending on the type of cheese. Yeasts frequently found in spoiled cheese include *Candida* spp., *Yarrowia lipolytica*, *Pichia* spp., but most frequently is *Geotrichum candidum*. Natural preservatives, such as essential oils have proven popularity such that interest continues in substituting artificial additives with natural. For example in the study of **Perdones *et al.*,** (**2016**), was thyme oil added to contaminated milk. Its presence was able to control bacterial growth, protect milk and prolong its shelf life. So the use of essential oils into the food could be very profitable, because their can improve the flavour, odour and extend the shelf-life of dairy products.

CONCLUSION

In this study, we evaluated the inhibitory effect of selected essential oils clove (*Syzygnium aromaticum* L.), basil (*Oscimum basilicum* L.), rosemary (*Rosmarinus officinalis* L.), fennel (*Foeniculum vulgare* L.) (*Thymus vulgaris* L.) on the growth of five *Geotrichum candidum* isolates. Our results showed that clove and thyme essential oil had a 100% inhibitory effect on the growth of all tested isolates of *Geotrichum candidum* throughout the cultivation days. The conclusions indicate that volatile phase of combinations of clove oil and thyme oil showed good potential to inhibit growth of *Geotreichum candidum.* Other essential oils demonstrated different effects on the growth of isolates. Even though that essential oils such as basil, rosemary and fennel had not antifungal activity like thyme and clove essential oils, they should find a practical application in the inhibition of the fungal mycelial growth. According to all, essential oils could be used as a natural preservative in the food industry.

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