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IN VITRO ANTIFUNGAL EFFECT OF TWELVE ESSENTIAL OILS ON *PENICILLIUM EXPANSUM* GROWTH

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ABSTRACT

The questionable safety of food spoiled by microorganisms is and should be the subject of attention and investigation. Pathogens such as *Penicillium expansum* are more difficult to control due to their resistance and ubiquity. Awareness of plant essential oils (EOs) as alternative natural preservatives is increasing because of their significant antimicrobial activity. The aim of this research work was therefore to contribute to this topic with new results. The antifungal activity of twelve EOs was tested *in vitro* against five strains of *Penicillium expansum*, using the vapour-phase diffusion method. Three EOs, from bergamot, star anise and rosaline, inhibited colony growth during two-week cultivation with a partial efficacy, all other EOs showed complete (100%) inhibitory activity at a concentration of 625 µL.L^{-1}. These (EOs from clove, cinnamon, cinnamon bark, laurel, lemongrass, peppermint, Mitcham mint, spearmint, and lavender) were included in the second part of the trial, the aim of which was to determine their minimum inhibitory doses (MIDs). In the case of peppermint, Mitcham mint, spearmint and lavender EOs, some strains of *P. expansum* grew already at a concentration of 500 µL.L^{-1}. Clove EO stopped the fungal growth of two strains at 250 µL.L^{-1}. Laurel EO was 100% effective at MID 250 µL.L^{-1} throughout the cultivation period. EOs from lemongrass and cinnamon bark had similar efficacy on the strains. Both EOs were able to inhibit one strain at a MID of 125 µL.L^{-1}, cinnamon bark EO inhibited the remaining strains at 250 µL.L^{-1}, lemongrass EO inhibited three strains at 250 µL.L^{-1} and one at 500 µL.L^{-1}. The lowest MID was recorded for cinnamon EO, which effectively inhibited two strains at 125 µL.L^{-1}, two strains at 62.5 µL.L^{-1} and one strain even at 31.25 µL.L^{-1}. The sensitivity of the strains used in this study varied according to the EOs used.

Keywords: EOs, *P. expansum*, antifungal activity, vapour phase

INTRODUCTION

Penicillium expansum is the best known, most studied and at the same time typifying species of the genus *Penicillium*. It occurs both in the air and in the soil, most often in decomposing vegetative matter **(Li *et al*., 2020)**. This species can grow in a more acidic environment (pH 4), represents an exception in the genus in relation to oxygen - growing in weakly anaerobic conditions and it is also considered to be a refrigerator pathogen because of its ability to survives temperatures up to 0 °C **(Tančinová *et al*., 2016; Li *et. al*., 2020)**. Therefore, food commodities are an ideal substrate for this mould. *P. expansum* is a producer of blue rot, one of the main post-harvest diseases of fresh pome fruit. This species can attack fruit during transport, handling and especially storage, causing 15–20% of total harvest losses worldwide **(Gong *et al*., 2019; Lai *et al*., 2021)**. As a necrotrophic pathogen and important source of mycotoxin patulin, *P. expansum* can infect tissues immediately after their contact with spores through superficial injuries **(Wang *et al*., 2019)**, so the need for good preservation is still a current subject of much research. Chemical fungicides and physical methods such as irradiation or heating are methods mainly used to control blue rot **(Luciano-Rosario *et al*., 2020)**, but the demand for natural food treatments is developing the use of natural preservatives more and more. Essential oils (EOs) are metabolites extracted from various parts of plants usually by hydrodistillation. These natural substances contain a complex of bioactive compounds that have proven antibacterial, antioxidant, antiviral or insecticidal effects. The components of EOs affect their aromatic profile, they can contain up to 200 components and the absence of one of them can mean a change in aroma. However, they are mostly characterized by 2-3 main compounds (20-70% of the EO content), namely terpenoids, terpenes and hydrocarbons. Inhibitory effect of different EOs against various types of spoilage microorganisms has already been proven. They are classified as safe, exhibit high degradability and volatility, and have potential as natural antimicrobial agents **(Hashemi *et al*., 2018, Yan *et al*., 2021; Clerck *et al*., 2020)**. Although the exact mechanism of antifungal activity of EOs is not precisely determined, the components of EOs have a broad-spectrum effect and different mechanisms of action on fungal cells **(Mamadalieva *et al*., 2020)**. In general, the inhibitory effect of EOs according to **Nazzaro *et al*. (2017)** and **Sil *et al*. (2020)** categorize as follows: at the cellular level there is disruption or change of the cell membrane, organelles, or cytoplasm, at the molecular level it causes protein leakage, DNA damage, cytotoxicity, dysfunction of fungal mitochondria: inhibition of ATP production or even increased production of reactive oxygen species.

The goal of presented study was to evaluate the antifungal effect of twelve EOs on the growth of five *P. expansum* strains under *in vitro* conditions and therefore to verify the hypothesis that some EOs can and do have strong antifungal effects against known pathogens.

MATERIAL AND METHODS

Essential oils

Twelve plant EOs used in this study were extracted from clove (*Syzygium aromaticum* L.), four types of peppermint (*Mentha x piperita* L., *Mentha spicata* L.- spearmint, *Mentha citrate* Ehrh. – bergamot mint, *Mentha x piperita* L. var. Mitcham), cinnamon (*Cinnamomum zeylanicum* Bloom), lavender (*Lavandula angustifolia* Mill.), lemongrass (*Cymbopogon flexuosus* Nees ex. Steud.), cinnamon bark (*Cinnamomum zeylanicum* Bloom), star anise (*Illicium verum* Hook. f.), laurel (*Litsea cubeba* (Lour.) Pers.) and rosaline (*Melaleuca ericifolia* Sm.) by hydrodistillation as stated by the distributor Sallos.sk. The EOs were hermetically stored at 4 °C and all of them are commercially available.

Chemical composition of EOs

A combination of gas chromatography coupled with mass spectrometry (GC-MS) was used for the quantitative analysis of EOs (Table 1). Specifically, an Agilent 7890B oven coupled to an Agilent 5977A mass detector (Agilent Technologies Inc., Palo Alto, CA, USA) and a CombiPal 120 autosampler (CTC Analytics AG, Zwingen, Switzerland) were used. The essential oil samples were diluted at a ratio of 1:99 in hexane (HPLC ≥97%, Sigma Aldrich GmbH, Germany), followed by injecting one microliter of diluted sample via CombiPal autosampler 120 (CTC Analytics AG, Zwingen, Switzerland) in inlet operated in split mode (1:10; 250 °C). Separation of organic compounds was achieved using a HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Palo Alto, CA, USA). Oven temperature programme was started at

50 °C for the first 5 minutes and increased to 240 °C at a rate of 3 °C min^{-1} and held constant for 2 min. A constant flow of 1.2 $mL.min^{-1}$ of helium carrier gas was used. The ionisation energy of the filament was 70 eV, the temperature of the transfer line was 250 °C, the temperature of the MS source was 230 °C and the quadrupole temperature was 150 °C. The mass spectrometer was programmed under electron impact (EI) in the full scan mode at 40 - 400 m/z. Individual compounds were identified by comparing mass spectra with a commercial database National Institute of Standards and Technology (NIST® 2017, Gaithersburg, MD, USA) and WILEY library (>80% match) and by evaluating retention times of reference standards (linalool, geraniol, α-pinene and β-pinene). By dividing the area of each peak by the total area of all peaks, the relative content (expressed as a percentage) of the determined compounds was calculated. Peaks below 1 % were not counted. Each sample was analysed in triplicate.

Table 1 Semi-quantitative composition of essential oils with percentage representation of the most represented components (above 1%)

Essential oils	Main components	%	RT (min)
Clove	Eugenol	73.3	46.4
	Phenol, 2-methoxy-4-(2-propenyl)-acetate	10.1	47.3
	Caryophyllene	9.23	23.5
	1,4,7, -Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	2.49	26.1
	Caryophyllene oxide	2.19	39.7
Peppermint (mint)	Levomenthol	42.4	25.8
	Menthone	22.5	23.1
	Eucalyptol	7.01	11.1
	Menthol	6.30	23.3
	p-menthone	4.22	24.5
	Levomenthol	3.54	23.9
	Caryophyllene	3.33	23.5
	D-Limonene	2.03	8.47
	Menthofuran	1.62	20.1
Spearmint	(-)-Carvone	72.6	31.8
	D-Limonene	15.2	8.49
	Levomenthol	1.62	25.7
	Cyclohexanone, 2-methyl-5-(1-methylethenyl)-	1.43	28.4
Bergamot	Linalyl acetate	45.0	22.8
	Linalool	33.9	22.2
	Geranyl acetate	5.93	29.3
	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	2.75	28.4
	Levomenthol	2.72	25.6
	Caryophyllene	1.30	23.4
	alpha-Terpineol	1.20	28.0
	D-Limonene	1.07	8.43
	beta-Myrcene	1.00	7.67
Mitcham mint	Levomenthol	41.6	25.8
	p-menthone	19.6	23.1
	Eucalyptol	8.25	11.1
	Menthyl acetate	4.97	23.3
	Methofuran	4.27	20.1
	Cyclohexanone, 5-methyl-2-(1-methylethyl)-, cis-	3.72	24.5
	D-Limonene	3.40	8.44
	Menthol	2.93	23.9
	Caryophyllene	2.02	23.4
	Pulegone	1.86	28.6
	Terpinen-4-ol	1.31	25.1
Lavender	Linalyl acetate	38.1	22.8
	Linalool	33.2	22.2
	(-)-Lavandulol	3.43	24.8
	Caryophyllene	3.32	23.5
	(E)-beta-Famesene	2.58	24.4
	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	2.52	25.1
	alfa-pinene	1.52	9.99
	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	1.18	10.7
	alpha-Terpineol	1.17	28.0
	Eucalyptol	1.01	11.1
Cinnamon	Cinnamaldehyde	73.4	42.6
	o-Metoxicinamaldehyd	13.0	53.2
	Citronellyl propionate	5.46	43.5
	Coumarin	2.68	54.5
Lemongrass	α-Citral	33.6	32.5
	β-Citral	25.9	30.9
	(R)-(+)-Limonene	9.71	8.46
	Geraniol	6.49	31.7
	Geranyl acetate	5.75	29.3
	β-Caryophyllene	2.43	23.4
	6-Methyl-5-heptene-2-one	1.99	17.4
	(-)-β-Pinene	1.39	6.33
	(-)-Linalool	1.13	22.1
Cinnamon bark	Cinnamaldehyde	80.5	42.5
	β-Caryophyllene	5.08	23.4
	o-Metoxicinamaldehyd	3.07	53.1
	(-)-Linalool	2.46	22.1
	Citronellyl propionate	2.46	43.4
Star anise	Anethole	85.5	33.2
	Estragole	5.45	27.3
	E-foeniculin	1.91	44.4
	p-anisaldehyde	1.89	41.3
	(-)-Linalool	1.30	22.1
Laurel	α-Citral	37.8	32.5
	β-Citral	29.7	30.9
	(R)-(+)-Limonene	15.4	8.46
	Sabinene	1.84	6.92
	(+)-α-pinene	1.75	4.41
	Eucalyptol	1.61	11.1
	(-)-Linalool	1.41	22.1
	Geraniol	1.40	31.6
	β-Caryophyllene	1.01	23.4
Rosalina	Linalool	39.9	17.5
	Eucalyptol	22.5	13.5
	Toluene	9.92	8.70
	p-Menth-1-en-4-ol	9.90	21.1
	2,4,6-Octatriene, 2,6-dimethyl-	3.80	14.9
	p-Mentha-1,5,8-triene	3.06	13.2

Legend: RT - retention time

Fungal strains

In this experiment, five *Penicillium expansum* strains were tested, isolated from mouldy types of berries and from tomatoes of various origins, obtained from a retail chain in Slovakia (Table 2). Strains were identified to the species level based on micro- and macro- morphological traits after 7 days of cultivation on identification media for *Penicillium* species, namely MEA (malt extract agar), CYA (Czapek yeast extract agar), YES (agar with yeast extract and sucrose), CREA (creatine sucrose agar). Strains were identified based on the identification keys for the genus *Penicillium* **(Pitt *et* Hocking, 2009; Samson *et* Frisvad., 2004; Samson *et al*., 2002, 2010, 2019; Visagie *et al*., 2014)**. Afterward, 5-day-old cultures cultivated at 25 ± 1 °C on Czapek agar with yeast extract (CYA) were used for testing.

Table 2 Origin and designation of the strains *Penicillium expansum*

Strain	Origin
P. expansum KMi-1020	raspberry, country of origin Morocco
P. expansum KMi-1021	strawberry, country of origin Slovakia
P. expansum KMi-1022	strawberry, country of origin Slovakia
P. expansum KMi-1023	blueberry, country of origin Peru
P. expansum KMi-1024	cherry tomato, country of origin Slovakia

Antimycotic activity of EOs *in vitro*

The vapour-phase diffusion method according to **Císarová *et al.* (2016)** was used to observe the inhibitory abilities of EOs on the growth of *P. expansum* strains. 15 ml of CYA was poured into sterile Petri dishes with a diameter of 9 cm followed by inoculation of 5 µL of spore suspension (10^6 in l mL) of strains (KMi 1020 – 1024). Spore suspension was prepared by purifying and suspending colonies in physiological saline solution supplemented with Tween 80 (0.5%) so that the concentration corresponded to 0.1 McFarland turbidity standards (standard density), using a standardized densitometer (Densilameter II, Erba Lachema s.r.o. Brno), previously verified by a Tom's chamber for each isolate. Filter papers (Whatman No. 1) were placed into the lids of Petri dishes, on the surface of which 50 µL of undiluted EO was applied, which represents concentration of 625 $\mu L.L^{-1}$. The experiment was carried out in three replicates for each strain and using triplicate controls with the same volume of sterilized distilled water instead of the EO concentrate. The Petri dishes prepared in this way were covered around the perimeter with parafilm to prevent EO from leaking into the environment and left to cultivate upside down in the dark at 25 ± 1 °C. Colony growth was measured on the 2nd, 3rd, 4th, 7th, 9th, 11th, and 14th day of cultivation. The diameter of the colonies was measured in two mutually perpendicular planes and the following equation for relative inhibition (RI) was used to express the degree of inhibitory activity of EOs:

$$RI = \left[\frac{(c - t)}{c}\right] \times 100$$

where, c is the diameter of the hyphal extension of the control (mm) and t is the diameter of EO treated colony.

Minimum inhibitory doses

The minimum inhibitory dose (MID) was determined by the vapour-phase diffusion method for EOs with 100% inhibition efficiency against all *P. expansum* strains demonstrated in previous measurements with a concentration of 625 μL.L^{-1}. Petri dishes were pipetted with 40 μL of EOs diluted with dimethyl sulfoxide (DMSO) solution to the desired concentrations, based on the highest concentration of 500 μL.L^{-1}, which was gradually diluted to obtain concentrations: 250; 125; 62.5; 31.25 and 15.625 μL.L^{-1}. Each dose was prepared in six replicates. Fungal growth was evaluated on days 7 and 14, and concentrations of EOs that did not permit any growth compared to controls were evaluated as the minimum inhibitory doses.

Statistical analysis

The growth of each of the 6 replicates was binomial coded (0 or 1) and these data were statistically processed by probit analysis software (Statgraphics Centurion XV (Statgraphics)) for the resulting MID50 and MID90 values, i.e., concentrations at which 50% or 90% of *P. expansum* colonies showed no growth (Tables 5, 6). The averages of the colony diameters were divided into homogeneous groups according to the effectiveness of the essential oils (Table 3) using Statgraphics Centurion XV (analysis of variance –single factor and multifactor ANOVA ($p<0.05$), Tukey HSD test). The results shown in Table 3 (sd – standard deviation, RI – relative inhibition) were calculated using MS Excel program.

RESULTS AND DISCUSSION

This study was focused on the antifungal properties of 12 EOs against the postharvest fruit pathogen *Penicillium expansum*. The antimicrobial activities and potential of EOs as preservatives have been proven in many studies, EOs with the strongest effects include those of thyme, oregano, mint, cinnamon or sage EOs **(Felšöciová *et al.*, 2015)**. Various EOs are currently being tested under different conditions, for example, one of the most recent studies investigated the antifungal effect of the liquid and vapour phases of the EO *Santolina pectinata in vitro* on the growth of *P. expansum* isolated from apples. A significant inhibition ($p < 0.05$) of the pathogen was demonstrated depending on the dose, *P. expansum* showed a strong sensitivity to the investigated EO (**Mounir *et al.*, 2022**).

Evaluation of Antifungal Activity of EOs

The EOs tested in this study can be divided into three groups based on their effectiveness. Three EOs – from star anise, rosaline and bergamot-mint, did not show 100% inhibitory efficiency even at the main concentration 625 μL.L^{-1}. As shown in the Table 3, capability of completely inhibiting the pathogen was observe only for 3 days of cultivation. From these three, EO from bergamot has been showed as the most effective. EO of bergamot caused a delay in the growth of the pathogen during 4 days of cultivation, one strain was inhibited even for a whole week (KMi-1021). The second highest efficiency of the bergamot EO after 14 days of cultivation was with KMi-1022 (83.19%). The least sensitive strain was KMi-1023, with a growth inhibition of 71.71%. According to **Verma *et al.* (2016)**, depending on the different times of extraction, bergamot EO demonstrated antimicrobial activity against the tested bacteria (especially Gram-positive), but the authors also report results of moderate to low activity. Again, it is confirmed that the extraction method influences the composition of the EO, from which the inhibitory efficiency of the EO depends. Next, the effectiveness of star anise EO on KMi-1020 and KMi-1022 dropped to 79.84% and 80.66%, respectively, on the 4th day. The remaining strains were inactivated by this EO up to 7th day of cultivation. On the 14th day of cultivation, four strains (except KMi-1022) filled at least 1/3 of the Petri dish with colonies, while KMi-1020 had the lowest inhibitory activity (48.52%). Contrary to our results according to **Rocha Neto *et al.* (2019)**, the vapour phase of star anise EO completely inhibited *in vitro* germination of *P. expansum* even at low concentrations (0.125 g.L^{-1}). However, the non-significant effect of star anise EO is reported by **Yan *et al.* (2021),** in their research the volatile substances of this EO at 150 μL.L^{-1}inhibited *Rhizopus stolonifer* by less than 30% after 72 hours of incubation. On the fourth day after treatment with EO from rosaline, all strains showed growth, except for KMi-1022, whose growth was inhibited by this EO within 7 days. The inhibitory potency of rosaline EO decreased the most with KMi-1023, on 14th day the growth was not even half inhibited (49.27%). **Sharifi-Rad *et al.* (2017)** reported that the effect of rosaline EO on *B. subtilis*, *E. coli*, *A. niger* and *C. albicans* resulted in inhibition zones between 8 and 18 mm. In their research, methyl eugenol was identified as the main constituent of rosaline EO, which may be the reason for the different effect compared to the effects in our study. Due to the weak effectiveness of these EO, they were eliminated from further trials. The remaining EOs – EOs from clove, cinnamon, lavender, lemongrass, cinnamon bark, peppermint, spearmint, Mitchem mint and laurel, significantly inhibited the growth of the fungal colonies at a concentration of 625 μL.L^{-1} as no measurable growth was observed for the whole period of 14 days and are therefore not listed in the table 3. For those, the minimum inhibitory dose was determined in the following tests.

Table 3 Average colony diameter (in mm, n = 6) and percentage of relation inhibition (RI) of *Penicillium expansum* grown CYA (25 ± 1 °C) in the presence of essential oils applied in the vapour phase (625 μL.L^{-1})

Day	EOs	Strains of *Penicillium expansum*									
		KMi-1020		**KMi-1021**		**KMi-1022**		**KMi-1023**		**KMi-1024**	
		Av ± sd	RI %	Av ± sd	RI %	Av ± sd	RI %	Av ± sd	RI %	Av ± sd	RI %
2nd	**Bergamot mint**	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Rosalina	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Star anise	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Control	13.9 ± 0.19^{b}		13.8 ± 0.24^{b}		14.0 ± 0.36^{b}		12.8 ± 0.36^{b}		12.9 ± 0.19^{b}	
3rd	**Bergamot mint**	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Rosalina	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Star anise	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Control	22.7 ± 0.27^{b}		21.3 ± 0.35^{b}		21.8 ± 0.29^{b}		21.7 ± 0.17^{b}		21.2 ± 0.19^{b}	
4th	**Bergamot mint**	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100	0^{a}	100
	Rosalina	6.11 ± 0.30^{b}	79.9	0^{a}	100	5.49 ± 0.20^{b}	80.3	9.89 ± 0.10^{b}	64.2	6.98 ± 0.16^{b}	75.7
	Star anise	6.12 ± 0.32^{b}	79.8	0^{a}	100	5.39 ± 0.42^{b}	80.7	0^{a}	100	0^{a}	100
	Control	30.4 ± 0.61^{c}		28.1 ± 0.35^{b}		27.9 ± 0.47^{c}		27.6 ± 0.17^{c}		28.8 ± 0.45^{c}	
7th	**Bergamot mint**	7.57 ± 0.48^{a}	83.6	0^{a}	100	0^{a}	100	10.1 ± 0.33^{a}	75.9	7.49 ± 0.13^{a}	83.8
	Rosalina	10.5 ± 0.25^{b}	77.2	0^{a}	100	10.4 ± 0.10^{b}	75.7	16.2 ± 0.51^{c}	61.6	12.9 ± 0.87^{b}	72.0
	Star anise	15.5 ± 0.26^{c}	66.6	5.97 ± 0.26^{b}	86.1	10.5 ± 0.42^{b}	75.4	11.5 ± 0.29^{b}	72.7	12.2 ± 0.66^{b}	73.7
	Control	46.2 ± 0.35^{d}		42.9 ± 1.53^{c}		42.6 ± 0.45^{c}		42.2 ± 0.33^{d}		46.3 ± 0.26^{c}	
9th	**Bergamot mint**	8.45 ± 0.49^{a}	85.4	0^{a}	100	7.71 ± 0.22^{a}	84.9	10.8 ± 0.38^{a}	79.5	10.0 ± 0.09^{a}	81.9
	Rosalina	15.8 ± 0.14^{b}	72.7	4.13 ± 0.25^{b}	92.7	12.7 ± 0.13^{b}	75.2	22.2 ± 0.27^{c}	57.7	15.6 ± 0.20^{b}	71.8
	Star anise	22.0 ± 0.26^{c}	61.8	14.2 ± 0.40^{c}	74.9	13.1 ± 0.32^{c}	74.4	15.7 ± 0.32^{b}	70.2	17.8 ± 0.21^{c}	67.7
	Control	57.7 ± 0.35^{d}		56.4 ± 0.37^{d}		51.2 ± 0.14^{d}		52.6 ± 0.29^{d}		55.2 ± 0.28^{d}	
11th	**Bergamot mint**	9.23 ± 0.15^{a}	84.7	0^{a}	100	9.13 ± 0.29^{a}	85.3	13.6 ± 1.01^{a}	76.1	11.5 ± 0.11^{a}	82.0
	Rosalina	17.3 ± 0.55^{b}	71.2	7.89 ± 0.23^{b}	87.1	17.1 ± 0.10^{c}	72.6	26.7 ± 0.30^{c}	52.8	18.4 ± 0.28^{b}	71.2
	Star anise	24.7 ± 0.37^{c}	59.0	19.2 ± 0.21^{c}	70.1	15.3 ± 0.17^{b}	75.5	21.3 ± 0.26^{b}	62.3	22.9 ± 0.21^{c}	64.0
	Control	60.2 ± 0.24^{d}		60.9 ± 0.11^{d}		62.3 ± 0.31^{d}		56.6 ± 0.45^{d}		63.9 ± 0.20^{d}	
14th	**Bergamot mint**	13.3 ± 0.16^{a}	79.9	0^{a}	100	11.4 ± 0.31^{a}	83.2	17.3 ± 0.32^{a}	71.7	13.1 ± 0.11^{a}	81.9
	Rosalina	24.2 ± 0.17^{b}	63.6	20.7 ± 0.42^{b}	70.5	23.1 ± 0.11^{c}	65.9	31.0 ± 0.64^{b}	49.3	21.6 ± 0.48^{b}	70.1
	Star anise	34.2 ± 0.28^{c}	48.5	33.3 ± 0.20^{c}	52.5	21.6 ± 0.55^{b}	68.1	31.3 ± 0.28^{b}	48.8	30.0 ± 0.38^{c}	58.4
	Control	66.3 ± 0.32^{d}		70.1 ± 0.21^{d}		67.9 ±0.64^{d}		61.1 ± 0.31^{c}		72.1 ±2.34^{d}	

Averages in the column followed by the same letters are not significantly different (ANOVA, Tukey test, $p < 0.05$) for each day.
Legend: n – number of measurements, CYA - Czapek yeast extract agar, av -average, sd – standard deviation, EOs – essential oils

Determination of Minimum Inhibitory Dose

The results of the antifungal activity of the EOs depending on the concentration are shown in Table 4. The next group contains three EOs that completely inactivated growth at the highest concentration (625 μL.L^{-1}) but showed minimal or no activity at diluted concentrations. For peppermint EO, a MID of 500 μL.L^{-1}could be only determined for two strains (KMi-1021, KMi-1022) during the first week of cultivation. The growth of the other strains was not suppressed by this concentration. These results contradict the findings of **Valková *et al.* (2021)**, in their study peppermint EO is proven as the strongest among those investigated, with complete inhibition of *P. expansum* and *P. crustosum* in all concentrations (125, 250 and 500 μL.dm^{-3}), *P. citrinum* was inhibited at the concentration 125 μL.dm^{-3}. The fungicidal activity of peppermint EO against *P. expansum* is also confirmed by **Camele *et al.* (2021)**, EO inhibited mycelial growth at concentrations of 1 and 5 mg.mL^{-1} and at 5 mg. mL^{-1} showed the highest inhibition against *M. fructicola* and *A. niger*. **Felšöciová *et al.* (2020)** found similar results, *P. expansum* was inhibited at a concentration of 0.75 μL.mL^{-1}, the inhibition zone was 9.83 ± 2.56 mm. Other authors indicate that the effectiveness of EO depends not only on the dose but also on the species and strain, as the species *P. citrinum* was not inhibited by these EO at any concentration **(Felšöciová *et al.*, 2020)**, which can possibly be applied to our case as well. Another variety of mint, the Mitcham type and lavender EO as well showed similar lower antifungal effects. During 7 days, both EOs inhibited two strains at 500 uL.L^{-1}, the other strains were inhibited only at 625 uL.L^{-1}.The results of other authors on the effect of lavender EO differ, for example, in the research of **Sumalan *et al.* (2020)** lavender EO completely inhibited the growth of *P. digitatum* mycelium at MID with a fungistatic effect - 200 μL. Nevertheless, lavender EO (125 and 250 μL.L^{-1}) showed no inhibitory effect on the growth of *P. crustosum*, while *P. citrinum* and *P. expansum* were significantly inhibited only at the highest concentrations (250 μL.L^{-1}) **(Valková *et al.*, 2021)**. Lavender EO tested *in vitro* in two doses (200 and 300 μg.mL^{-1}) against phytopathogenic and postharvest fungi did not show any antifungal activity, namely it was ineffective against *Rhizoctonia solani*, *Fusarium equiseti*, *Bipolaris spicifera*, *Fusarium oxysporum lycopersici*, *Fusarium graminearum* and *Alternaria alternata*, the growth of *Penicillium* spp. was even enhanced (20-50%) **(Santamarina *et al.*, 2017)**. Mitcham mint EO was investigated for antimicrobial activity in a study by **Heydari *et al.* (2018)** and they found that EO exhibited strong and dose-dependent antimicrobial activity against *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Differences in the activity of EOs can be largely influenced by their chemical composition. The study **Erland *et al.* (2016)** stated that carvacrol as a minor component of lavender EO was universally effective, while the main component linalool had very weak inhibitory activity, so the antifungal activity in some cases is carried by minor components whose amount in the compound is not sufficient for EO to have inhibitory activity. The peppermint tested by **Valková *et al.* (2021)** had a lower content of methyl acetate, and since this compound was found to cause a decrease in the antifungal properties of EOs, this could have been the reason why their results were positive, unlike ours.

Table 4 Minimum inhibitory doses (μL.L^{-1}) of essential oils on the growth of *P. expansum* colonies (n = 6) after 7 and 14 days of cultivation (CYA, 25 ± 1 °C)

EOs	Day of cultivation	Strains of *Penicillium expansum*				
		KMi-1020	**KMi-1021**	**KMi-1022**	**KMi-1023**	**KMi-1024**
		Minimum inhibitory dose (μL.L^{-1})				
Clove	7th	250	500	250	500	500
	14th	250	500	250	500	500
Peppermint	7th	>500	500	500	>500	>500
	14th	>500	>500	>500	>500	>500
Cinnamon	7th	125	62.5	125	62.5	31.25
	14th	125	62.5	125	62.5	31.25
Spearmint	7th	500	500	>500	500	500
	14th	500	500	>500	500	500
Lavander	7th	500	>500	>500	500	>500
	14th	>500	>500	>500	>500	>500
Lemongrass	7th	250	125	250	250	250
	14th	250	125	250	500	250
Cinnamon bark	7th	250	125	250	250	250
	14th	250	125	250	250	250
Laurel	7th	250	250	250	250	250
	14th	250	250	250	250	250
Mitcham mint	7th	>500	500	>500	>500	500
	14th	>500	>500	>500	>500	>500

Legend: n – number of measurements, CYA - Czapek yeast extract agar, EOs – essential oils

The last group represents the most effective EOs, for which the MID could also be estimated through an experiment and then evaluated statistically. Probit analysis was then used to estimate the concentrations at which the EOs had an inactive effect on 50% (Table 5) or 90% (Table 6) of *P. expansum* growth. The order is from the weakest to the strongest: spearmint, clove, laurel, lemongrass, cinnamon bark, and cinnamon EOs. Spearmint EO was the most effective of the Mentha species. It successfully inhibited four strains of *P. expansum* at a concentration of 500 μL.L^{-1}, the exception being strain KMi-1022 (625 μL.L^{-1}). At half concentration (250 μL.L^{-1}) colonies of all strains grew in at least 1 of 6 replicates and at lower concentrations colonies were present in all (6) replicates. It can be said that the average value of MID50 of spearmint EO for the inhibition of all strains during 7 days of cultivation was 298.06 μL.L^{-1}. The fungitoxic activity of spearmint EO was also confirmed by **Preedy (2016)**, EO inhibited *Aspergillus ochraceus*, *Penicillium digitatum* and *Pyricularia oryzae* within 10-30 minutes and showed maximum effectiveness at a pH level of 4.5-7.5. Clove EO was shown to be 100% antifungal effective when used at a concentration of 250 μL.L^{-1} for 2 strains KMi-1020 and KMi-1022 and at a concentration of 500 μL.L^{-1} to inhibit the remaining three strains. Statistically it was found that the MID90 for strain KMI-1024 was higher than 500 μL.L^{-1}. However, there was no significant difference between the other strains, their reactions to the presence of EO were similar, MID90 148.35 μL.L^{-1} was determined for KMi-1020 and KMi-1022 and MID90 337.49 μL.L^{-1} for KMi-1021 and KMi-1023. In agreement with our results, **Dixit *et al.* (2022)** demonstrated that clove EO showed the best antifungal activity against the tested fungi compared to basil, kananga EO and compared to the synthetic antifungal nystatin. MID against *Aspergillus ochraceus* and *Penicillium verrucosum* was determined to be 1251 ± 42.32 μg.mL^{-1} and 1878 ± 28.47 μg.mL^{-1} and MFC (minimum fungicidal concentration) for fungi in the same order was determined to be 0815 ± 22.69 μg.mL^{-1} and 1146 ± 51.19 μg.mL^{-1}. The MID50 and MID90 values of lemongrass EO remained the same after 7 as well as after 14 days (except for KMi-1023). With lemongrass EO, for the first time in this experiment, a concentration of 125 μL.L^{-1} was sufficient to inhibit at least one strain. In general, the MID90 ranged from 77.03 to 257.33 μL.L^{-1}. Antifungal activity of lemongrass EO is also confirmed by the results of **Mbili *et al.* (2017)**. Their study represents lemongrass EO with complete inhibition of mycelial growth and germination of *B. cinerea*, a common apple pathogen, at concentrations of 0.125-1.0%. Similarly, lemongrass EO at a concentration of 1 g.dm^{-3} inhibited the growth of *F. oxysporum*, with an MID of 0.5 g.dm^{-3} **(Barkaoui *et al.*, 2022)**. From a statistical point of view, laurel, clove, cinnamon, and cinnamon bark EOs maintained more or less the same effectiveness during the entire two weeks of the test, of all the examined EOs, their antifungal effect generally did not decrease linearly with time. MIDs of laurel EO that inhibited three strains of pathogen at the beginning of cultivation were sufficient for mycelium growth inhibition at the end of cultivation as well. Only two strains reacted slightly differently, but even in those cases MID90 increased by a maximum of approximately 22 μL.L^{-1} on day. EOs from laurel were also significantly antifungal in tests against *Fusarium oxysporum* f. sp. fordii, the inhibition rate was over 80% at concentrations of 250 μL.dm^{-3} **(Wang *et al.*, 2022)**. The most promising results in terms of antifungal activity were clearly demonstrated with EOs form cinnamon and cinnamon bark. The connection in the dominant effects of these EOs may be related to their similar composition. Cinnamon EO can be distilled both from the bark, which contains about 4% volatile EO, but also from the leaves and twigs. The main active constituent of cinnamon EO is cinnamaldehyde, while cinnamon bark oil also contains eugenol. Cinnamaldehyde is considered to be a strong antimicrobial compound whose mechanism of action corresponds to the action of EOs: inhibition cell division, disruption of the cell wall, inactivation of synthesizing enzymes or inhibition of ATPase **(Denkova-Kostova *et al.*, 2020; Baker *et* Grant, 2018)**. In this experiment, EO from cinnamon (76 %) had a higher percentage of cinnamaldehyde than EO from cinnamon bark (63.3 %), so this may also result in its slightly better activity. Cinnamon EO was able to completely inhibit the growth of all strains when using a concentration of 125 μL.L^{-1}, three strains were inactivated at half concentration (62.5 μL.L^{-1}), and mycelial growth of one strain was inhibited even at the second lowest concentration so far - 31.25 μL.L^{-1}. Of all the EOs tested, cinnamon EO was the only one active at such a low concentration. Statistically estimated results were significantly lower than before, the maximum effective MID90 during 14 days of cinnamon EO was 66.78 μL.L^{-1}, the lowest value was 16.24 μL. L^{-1}. The growth of *P. expansum* in the presence of EO from cinnamon bark was suppressed at a concentration of 250 μL.L^{-1}, and for one strain at a concentration of 125 μL.L^{-1}. At each lower concentration, *P. expansum* colonies grew sporadically but did not show complete growth (6 out of 6 replicates). On the 7th day, the average MID90 value of cinnamon bark EO on *P. expansum* was 126.75 μL.L^{-1}, and on the 14th day of cultivation it was only slightly higher, 135.72 μL.L^{-1}. Other authors also point to the significant antifungal effect of cinnamon EO. For example, the results of **Lai *et al.* (2021)** demonstrate that the MID of 0.25 mg.dm^{-3} of cinnamon EO shows a stable inhibitory effect against *P. expansum*. After 12 hours, the spore germination rate was below 20%, while it was above 75% in controls not treated with EO. Our results are confirmed by the findings of another study, in which cinnamon EO had the highest antifungal activity among the tested EOs against three strains of *Aspergillus* spp., with the lowest MID inhibition zone in the range from 0.0625 to 0.125 mg.mL^{-1}. Clove EO was the second most effective **(Hu *et al.*, 2019)**. The results of the disc method by

Kačániová *et al.* (2021) showed that cinnamon EO was highly effective against *P. expansum*, with MFC = 0.20 µL.mL^{-1}, *P. citrinum* (MFC = 0.78 µL.mL^{-1}) and *P. crustosum* (MFC = 0.39 µL.mL^{-1}). The vapor phase method was more effective on *Penicillium* spp. as for the bacteria *Stenotrophonomonas maltophilia* and *Bacillus subtilis*. Among the EOs evaluated *in vitro* for antifungal and antimycotoxigenic activity against *Fusarium verticillioides*, cinnamon EO showed the highest fungitoxic activity with an MIC of 250 µg.mL^{-1}, which is probably attributed to its major compound identified in this study, eugenol **(Castro *et al.*, 2020)**. Similar results were obtained in a previous study, cinnamon EO was active against *Alternaria alternata* at MIC = 250 µg.mL^{-1} **(Castro *et al.*, 2017)**.

Table 5 Minimum inhibitory doses (µL.L^{-1}) of essential oils inhibiting 50% growth of *P. expansum* on plates (MID50) determined by probit analyses

EOs	Day of cultivation	Strains of *Penicillium expansum*				
		KMi-1020	**KMi-1021**	**KMi-1022**	**KMi-1023**	**KMi-1024**
Clove	7th	130.5	155.79	98.21	158.37	161.51
	14th	130.5	176.85	130.5	176.85	198.56
Cinnamon	7th	9.63	11.96	17.92	13.24	13.87
	14th	17.92	16.1	28.71	17.67	13.87
Spearmint	7th	260.87	260.87	478.98	260.87	228.72
	14th	260.87	273.29	> 500	352.9	260.87
Lemongrass	7th	119.74	68.38	130.5	136.56	119.74
	14th	119.74	68.38	130.5	228.72	119.74
Cinnamon bark	7th	25.43	27.4	16.77	19.11	16.77
	14th	38.15	27.4	30.81	19.11	16.77
Laurel	7th	130.5	125	119.74	136.56	114.25
	14th	130.5	125	125	136.56	125
Peppermint	7th	478.98	352.9	352.9	478.98	> 500
	14th	> 500	457.06	478.98	> 500	> 500
Lavender	7th	352.9	> 500	> 500	352.9	> 500
	14th	> 500	> 500	> 500	> 500	> 500
Mitcham mint	7th	> 500	352.9	> 500	> 500	352.9
	14th	> 500	> 500	> 500	> 500	478.98

Legend: MID –minimum inhibitory dose, EOs – essential oils

Table 6 Minimum inhibitory doses (µL.L^{-1}) of essential oils inhibiting 90% growth of *P. expansum* on plates (MID90) determined by probit analyses

EOs	Day of cultivation	Strains of *Penicillium expansum*				
		KMi-1020	**KMi-1021**	**KMi-1022**	**KMi-1023**	**KMi-1024**
Clove	7th	148.35	383.54	200.5	270.89	> 500
	14th	148.35	337.49	148.35	337.49	> 500
Cinnamon	7th	53.48	32.86	66.78	43.32	16.24
	14th	66.78	34.53	95.85	44.34	16.24
Spearmint	7th	296.11	296.11	> 500	296.11	257.33
	14th	296.11	307.52	> 500	377.81	296.11
Lemongrass	7th	136.08	77.03	148.35	153.52	136.08
	14th	136.08	77.03	148.35	257.33	136.08
Cinnamon bark	7th	214.06	63.93	108.85	138.05	108.85
	14th	227.07	63.93	140.68	138.05	108.85
Laurel	7th	148.35	142.92	136.08	153.52	128.7
	14th	148.35	142.92	142.92	153.52	149.92
Peppermint	7th	> 500	377.81	377.81	> 500	> 500
	14th	> 500	514.79	> 500	> 500	> 500
Lavender	7th	377.81	> 500	> 500	377.81	> 500
	14th	> 500	> 500	> 500	> 500	> 500
Mitcham mint	7th	> 500	377.81	> 500	> 500	377.81
	14th	> 500	> 500	> 500	> 500	> 500

Legend: MID –minimum inhibitory dose, EOs – essential oils

CONCLUSION

The inhibitory activity of twelve plant EOs: from clove, four types of mint, cinnamon and cinnamon bark, lavender, lemongrass, star anise, laurel, and rosaline, was tested by the vapour-phase diffusion method to inhibit the growth of *P. expansum*. Among them, only three EOs showed partial inhibitory activity, with bergamot mint being the most significant, followed by star anise and rosaline EOs. The remaining 9 plant EOs (clove, peppermint, cinnamon, cinnamon bark, spearmint, lavender, lemongrass, laurel, Mitcham mint) at a concentration of 625 µL.L^{-1} showed complete (100%) antifungal activity against all *P. expansum* strains during the entire two weeks of cultivation. Minimum inhibitory doses were based on an initial concentration of 500 µL.L^{-1}. EOs from Mitcham mint and peppermint showed the weakest activity, failing to inhibit at concentrations lower than 500 µL.L^{-1}. Lavender and spearmint EOs reacted similarly. Clove EO followed, effective on two strains at a concentration of 250 µL.L^{-1} and on three strains at 500 µL.L^{-1}. Laurel EO inhibited the growth of all strains at a concentration of 250 µL.L^{-1}. EOs from lemongrass and cinnamon bark were effective at one strain even with a concentration of 125 µL.L^{-1}. Cinnamon EO proved to be the most effective of the 12 EOs tested, with MID 125 µL.L^{-1} (KMi-1020, KMi-1022), MID 62 µL.L^{-1} (KMi-1021, KMi-1023) and MID 31 µL.L^{-1} (KMi-1024). The strains used in this work were isolated from several mouldy commodities and showed variations in behavior and sensitivity depending on the EO used. Therefore, it is necessary to test multiple strains within a species to obtain more accurate data on the effects of a particular EO on a given species. Our conclusion is that EOs from lemongrass and cinnamon bark, but especially cinnamon EO, have potential for further investigation of their antifungal properties.

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