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THE *CITRUS RETICULATA* ESSENTIAL OIL: EVALUATION OF ANTIFUNGAL ACTIVITY AGAINST PENICILLIUM SPECIES RELATED TO BAKERY PRODUCTS SPOILAGE

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ABSTRACT

Fungal food spoilage plays a key role in the deterioration of food products, and finding a suitable natural preservative can solve this problem. Therefore, antifungal activity of green mandarin (*Citrus reticulata*) essential oil (GMEO) in the vapor phase against the growth of *Penicillium* (*P.*) *expansum* and *P. chrysogenum* inoculated on wheat bread (*in situ* experiment) was investigated in the current research. The volatile compounds of the GMEO were analyzed by a gas chromatograph coupled to a mass spectrometer (GC–MS), and its antioxidant activity was determined by testing free radical-scavenging capacity (DPPH assay). Moreover, the disc diffusion method was used to analyze the antifungal activity of GMEO in *in vitro* conditions. The results demonstrate that the *Citrus reticulata* EO consisted of α-limonene as the most abundant component (71.5%), followed by γ-terpinene (13.9%), and β-pinene (3.5%), and it displayed the weak antioxidant activity with the value of inhibition 5.6 ±0.7%, which corresponds to 103.0 ±6.4 μg TEAC.mL^{-1}. The findings from the GMEO antifungal activity determination revealed that values for the inhibition zone with disc diffusion method ranged from 0.00 ±0.00 (no antifungal effectiveness) to 5.67 ±0.58 mm (moderate antifungal activity). Finally, exposure of *Penicillium* strains growing on bread to GMEO in vapor phase led to the finding that 250 μL.L^{-1} of GMEO exhibited the lowest value for mycelial growth inhibition (MGI) of *P. expansum* (-51.37 ±3.01%) whose negative value reflects even supportive effect of the EO on the microscopic fungus growth. On the other hand, GMEO at this concentration (250 μL.L^{-1}) resulted in the strongest inhibitory action (MGI: 54.15 ±1.15%) against growth of *P. chrysogenum*. Based on the findings it can be concluded that GMEO in the vapor phase is not an effective antifungal agent against the growth of *P. expansum* inoculated on bread; however, its antifungal potential manifested against *P. chrysogenum* suggests GMEO to be an appropriate alternative to the use of chemical inhibitors for bread preservation.

Keywords: *Citrus reticulata*; volatile compound; DPPH assay; antifungal properties; bread

INTRODUCTION

Essential oils (EOs) are complex mixtures of water-vapor aromatic substances (mainly terpenoids, less frequently aromatic and aliphatic compounds) derived from diverse parts of plants in which they determined their pleasant aroma **(Denkova-Kostova et al., 2021)**. They possess a broad range of various biological activities such as antimicrobial, antioxidant, anti-inflammatory, and anticancer activities **(Sharifi-Rad et al., 2017)** which were documented in many preclinical studies. In addition, their antifungal properties have been screened on a global scale as potential sources of novel antimicrobial compounds, promoting food preservation **(Chouhan, Sharma and Guleria, 2017)**.

Citrus essential oils (EOs) have a volatile fraction usually >90% in which monoterpenes and sesquiterpenes are found with limonene being the major compound **(Raspo et al., 2020)**. From them, mandarin EO is extracted from *Citrus reticulata* of the *Rutaceae* family and has some great properties to help relieve stress and digestive problems. Also, it is used to increase circulation to the skin, reducing fluid retention, and to help prevent stretch marks **(Fayed, 2009)**. In general, there are three kinds of mandarin EO, i.e., green, yellow, and red, all derived from the same fruit, but at different stages of maturity. Green mandarin essential oil (GMEO) is generally sharper and with more of a "peel" note compared to red mandarin **(Boughendjioua, Mezedjeri and Idjouadiene, 2020)**. From a chemical profile point of view, the mandarin EO contains α-pinene, β-pinene,

camphene, citral, citronellal, γ-terpinolen, geraniol, citric, linalool, methyl myrcene, sabine, and terpineol **(Denkova-Kostova et al., 2021)**. It is well-known for its antibacterial and antifungal actions in a wide spectrum. Indeed, its strong activity against some microorganisms related to food spoilage and food safety including *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Aspergillus flavus*, and *P. digitatum* was reported **(Gao et al., 2010; Tao et al., 2009; Wang et al., 2012)**.

The major purpose of our study was to evaluate the antifungal potential of GMEO against selected *Penicillium* (*P.*) species (*P. expansum* and *P. chrysogenum*) using the contact vapor method. Moreover, the chemical composition of the GMEO, as well as its antioxidant properties and antifungal effect in *in vitro* conditions were established. The obtained findings of the studied GMEO would give a reason for their inclusion in the development of biopreservation strategies for the food industry.

Scientific hypothesis

Since *Citrus reticulata* essential oil represents a rich source of bioactive monoterpenes, its antifungal potential against *Penicillium* spp. could be expected.

MATERIAL AND METHODOLOGY

Samples

Green mandarin essential oil (GMEO; *Citrus reticulata*) was purchased from Hanus Company (Ltd, Hrochoť, Slovakia).

Wheat bread was obtained from Laboratory of Cereal Technologies (AgroBioTech Research Centre, Slovak University of Agriculture in Nitra).

Chemicals

All chemicals were analytical grade, and were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (Schnelldorf, Germany).

Sabouraud Dextrose Agar (SDA; Schnelldorf, Germany).

Animals and Biological Material

The fungi *P. expansum* and *P. chrysogenum* were isolated from grape and bread samples, respectively. Then, they were identified with the MALDI-TOF MS Biotyper and 16S rRNA sequencing.

Instruments

Mass spectrophotometer (MALDI-TOF MS Biotyper, Bruker, USA).

Spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, USA).

Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA).

Quadrupole mass spectrometer 5975B (Agilent Technologies, Santa Clara, CA, USA).

Spiral mixer (Diosna SP 12 D, Diosna, Germany).

Fermentation cabinet (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

Steamy oven (MIWE cube, Pekass s.r.o., Plzeň, Czech Republic).

Laboratory Methods

Determination of volatile compounds

The chemical profile of GMEO was performed using an Agilent 6890N gas chromatograph coupled to quadrupole mass spectrometer 5975B as reported by **Valková et al. (2021)**. The individual volatile constituents of the injected EO samples were identified based on their retention indices **(Adams, 2007)** and compared with reference spectra (Wiley and NIST databases). The retention indices were experimentally determined using the standard method **(Van Den Dool and Kratz, 1963)** which included retention times of n-alkanes (C6-C34), injected under the same chromatographic conditions. The percentages of the identified compounds (amounts higher than 0.1%) were derived from their GC peak areas.

Determination of radical scavenging activity

The radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to measure the antioxidant activity (AA) of GMEO described previously by **Galovičová et al. (2021)**. The AA was expressed as the percentage of DPPH inhibition, and was calculated according to the formula: (A0 – A1)/A0 × 100; where A0 was the absorbance of DPPH and A1 was the absorbance of the sample. The AA values increased in the following manner: weak (0 – 29%) < medium-strong (30 – 59%) < strong (60 and more %). Moreover, the value of total AA was expressed according to the calibration curve as 1 μg of standard reference Trolox to 1 mL of the GMEO sample (TEAC).

Evaluation of antifungal activity

The evaluation of the antifungal activity of the GMEO was performed using the agar disc diffusion method according to the **Valková et al. (2021)** with minor modifications. For this purpose, an aliquot of 0.1 mL of fungal in distilled water was inoculated on SDA (60 mm). Subsequently, the discs of filter paper (6 mm) were impregnated with 10 μL of GMEO samples (in four concentrations: 62.5, 125, 250, and 500 $\mu L.L^{-1}$), then applied on the SDA surface, and incubated at 25 °C for 5 days. The disks impregnated with ethanol served as negative controls. After incubation, the diameters of the inhibition zones in mm were measured. Each test was repeated three times (one repeat reflected one separate plate). The values of inhibitory activity increased in the following manner: weak antifungal activity (5 – 10 mm) < moderate antifungal activity (5 – 10 mm) < very strong antifungal activity (zone > 15 mm).

Bread making procedure

Wheat bread, as a substrate for fungal growth, was made according to the procedure by **Kačániová et al. (2020a)** in the Laboratory of Cereal Technologies (AgroBioTech Research Centre, the Slovak University of Agriculture in Nitra).

Antifungal analyses on bread loaves model

First, the bread samples were cut into slices with 150 mm height and placed into 0.5 L sterile glass jars (Bormioli Rocco, Fidenza, Italy). The fungal spores were used for bread inoculation. The GMEO in concentrations of 62.5, 125, 250, and 500 $\mu L.L^{-1}$ (diluted in ethyl acetate) were evenly distributed in a volume of 100 μL on a sterile paper–filter disc (6 cm), which was inserted into the cover of the jar, except for the control group. The jars were hermetically closed and kept at 25 ±1 °C for 14 days in the dark **(Kačániová et al., 2020a)**. The size of the microfungal colonies with visible mycelial growth and visible sporulation was evaluated using stereological methods. In this concept, the volume density of the colonies was firstly assessed using ImageJ software (National Institutes of Health, Bethesda, MD, USA), counting the points of the stereological grid hitting the colonies and those falling to the reference space (growth substrate used, i.e., bread). The

antifungal activities of the EOs were expressed as the percentage of mycelial growth inhibition (MGI), which was calculated using the formula: MGI = [(C − T)/C] × 100, where C = volume density of the fungal colony in the control group and T = volume density of that in the treatment group **(Sempere-Ferre et al., 2021)**.

Description of the Experiment

Sample preparation: 42
Number of samples analyzed: 42
Number of repeated analyses: 4
Number of experiment replication: 3

Statistical Analysis

The obtained data were statistically evaluated using Prism 8.0.1 (GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) followed by Tukey's test was used to evaluate the significance of differences between analyzed groups of samples. The level of significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of GMEO

Although the mode of action of EOs has not been fully elucidated it is well-known that their primary effects are related to their chemical composition **(Burt, 2004; Cox and Markham, 2007)**. Indeed, these compounds, typically monoterpenes or phenylpropanoids, often exhibit antioxidant capacity and also antimicrobial activity against a wide range of bacterial and fungal species **(Sikkema, de Bont and Poolman, 1995; Behbani et al., 2017)**. Moreover, the interaction between the various compounds of EOs can lead to antagonistic, additive, or synergistic effects **(Burt, 2004)**. Therefore, the identification of individual volatile substances may be a useful tool for the characterization of EOs, and may help to understand the key points in their antioxidant and antimicrobial activity, thus being possible to employ either alone or in combination with other additives in food preservation techniques **(Viuda-Martos et al., 2007; Miladinovic et al., 2015)**.

GC-MS analysis was also used in our study to determine the chemical composition of GMEO. It revealed that a total of 28 compounds, accounting for 99.7% of the whole constituents, were identified in the EO chemical profile. The major compound was shown to be α-limonene (71.5%), and other major ones included γ-terpinene (13.9%), β-pinene (3.5%), p-cymene (3.1%), α-pinene (2.6%), and β-myrcene (2.2%), as presented Table 1.

Our results are in agreement with the previous findings reported by **Viuda-Martos et al. (2009)**, who consider α-limonene (74.7%) and γ-terpinene (15.7%) as the major oil compounds of the mandarin EO. The same observation was also demonstrated by **Espina et al. (2011)** and **Denkova-Kostova et al. (2021)**, who found that the main compound of *C. reticulata* EO was limonene which comprised 74.4% and 84.88% of the EO, respectively. In contrast to our findings, **Yabalak, Eliuz and Nazlı (2021)** detected eucalyptol (7.2%), methyl palmitate (3.8%), and α-terpineol (3.7%) as the most represented substances in their *C. reticulata* EO, whereby α-limonene (71.5%) or γ-terpinene were absent in its chemical profile. However, we assume that the differences in the EO chemical composition between these studies might be due to various factors such as genetic factors (genotype or variety), geographical locations, environmental conditions, season, cultivation practices, fertilizer application, stress during growth or maturity, harvesting time, stage of maturity, as well as processing methods which strongly influenced it **(Burt, 2004; Sandeep, Sanghamitra and Sujata, 2015; Srinivasan et al., 2016)**.

Table 1 Main volatile compounds of green mandarin essential oil.

Compounds	(%)
α-limonene	71.5
γ-terpinene	13.9
β-pinene	3.5
p-cymene	3.1
α-pinene	2.6
β-myrcene	2.2
α-terpineol	1.0
α-terpinolene	0.7
α-thujene	0.5
n-decanal	0.4
sabinene	0.3
(*E*)-β-ocimene	0.1
trans-limonene oxide	tr
4-terpinenol	tr
α-copaene	tr
(*E*)-caryophyllene	tr
(*E*,*E*)-α-farnesene	tr
δ-cadinene	tr
TOTAL	99.7

Note: tr – compounds identified in amounts less than 0.1%.

Summary, based on the findings we can propose that the high proportion of monoterpenes (mainly α-limonene, γ-terpinene, and β-pinene) in our analyzed GMEO can predict its promising use as a preservative in the food industry as it was stated in the study by **Badawy, Lotfy and Shawir (2020)**.

Antioxidant activity of GMEO

It is generally known that EOs have antioxidant properties, which are subjected to analyses performed in many scientific papers **(Diniz do Nascimento et al., 2020; Kačániová et al., 2020b; Boudiba et al., 2021)**.

The DPPH method, also used in our study, is considered a simple and sensitive technique applicable to most plant extracts including EOs **(Noipa et al., 2011)**. DPPH is a stable free radical with deep violet color, which accepts an electron or hydrogen radical to create a stable diamagnetic molecule with discoloration. The degree of discoloration indicates the free radical-scavenging potential of the sample **(Schaich, Tian and Xie, 2015)**. Interestingly, employing this assay we have found that values for AA of the EO of *C. reticulata* were 103.0 ±6.4 μg TEAC.mL^{-1}, with 5.6 ±0.7% free radical-scavenging inhibition linked to a weak AA. Dissimilar to our results, **Boudries et al. (2017)** reported a higher value of % of inhibition associated with a stronger AA of mandarin EO which was even the highest between all analysed citrus fruit EOs (mandarin, clementin, wilking).

Also, the highest DPPH activities (78.0%; 73.32%) of EO from *Citrus reticulata* were found in the studies by **Denkova-Kostova et al. (2021)** and **Ishfaq et al. (2021)**, respectively. Most probably, different chemical compositions and amounts of individual constituents (depending on various aforementioned factors) of the mandarin EOs used in our and all mentioned studies could be responsible for the discrepancies in AA of the EOs demonstrated by their results.

In vitro antifungal properties of GMEO

Data from the inhibitory effects of GMEO against two tested *Penicillium* spp. fungi (*P. expansum*, and *P. chrysogenum*) assessed by the disc diffusion method are shown in Table 2. Our results revealed that the growth inhibition of fungi strains depends on the concentration of the GMEO applied. In effect, moderate antifungal activity (inhibition zone: 5.67 ±0.58 mm) was observed at the highest concentration (500 μL.L^{-1}) of GMEO against the growth of *P. chrysogenum*. This effect was statistically different (p <0.05) from those exhibiting at the lowest concentration (62.5 μL.L^{-1}) of the GMEO which showed no inhibitory activity (inhibition zone: 0.00 ±0.00 mm) against this fungus strain. On the other hand, the growth of *P. expansum* was only weakly inhibited (inhibition zone: 1.67 ±0.58 mm) by GMEO in the concentration of 500 μL.L^{-1}, and this inhibition even significantly differ from those (inhibition zone: 0.00 ±0.00 mm) displayed by remaining concentrations (62.5, 125, 250 μL.L^{-1}) of GMEO.

Several scientific studies have demonstrated that citrus EOs have been shown to reduce or completely inhibit the growth of microscopic fungi depending on their concentration **(Sharma and Tripathi, 2006; Sharma and Tripathi, 2008)**. **Droby et al. (2008)** observed that citrus EOs (mandarin, lemon, grapefruit, and orange) in concentration of 20.0 μL.mL^{-1} stimulated the growth of *P. italicum* and *P. digitatum*. On the other hand, grapefruit EOs (in concentration of 40.0 μL.mL^{-1}) had moderate antifungal activity against *P. digitatum*. Moreover, it was found that some microscopic fungi can catalyze the conversion of antifungal compounds in various plant extracts (including those obtained in EOs). Such conversion has also been reported for instance in *P. digitatum* which was able to convert limonene to α-terpineol, cis- and trans-p-menth-2-en-1-ol, neodihydrocarveol and limonene oxide **(Tan, Day and Cadwallader, 1998; Demyttenaere, Van Belleghem and De Kimpe, 2001; Badee, Helmy and Morsy, 2011)**, i.e., to less effective substances related to antifungal activity. Therefore, we assume that a similar type of conversion may explain why GMEO was more powerful against *P. chrysogenum* as compared to *P. expansum*, as was reported in our study.

In situ antifungal vapor contact assay of GMEO

In general, antifungal agents are used in the food industry for the preservation of food (control natural spoilage processes) or their safety (prevent or control the growth of microorganisms; **Shaaban, 2020)**. As spoilage of bakery products is often caused by microscopic fungi including *Penicillium* spp. **(Salas et al., 2017)** the antifungal properties of GMEO in the vapor phase on bread as a substrate for the growth of these species were evaluated in our study. The relative volatilities of the EOs compounds determine the characteristics of their vapors which have impacts on antimicrobial potential **(Tullio et al., 2007)**. Several studies have shown that the vapor phases of EOs are more effective than liquid ones **(Soylu, Soylu and Kurt, 2006; Mondello et al., 2009; Tyagi and Malik, 2010)**. This fact can be attributed to the free adhesion of EOs to the substrate (vapor phase), while in the aqueous phase the lipophilic molecules combine to form micelles suppressing the adhesion of EOs to the substrate **(Inouye et al., 2003)**.

The inhibitory effects of our GMEO on the growth of the *Penicillium* spp. inoculated on wheat bread are demonstrated in Table 3 and Figure 1. From the results, it

Table 2 Antifungal activity of EOs (*in vitro*).

Fungal strains	The inhibition zones (mm)			
	GMEO (μL.L^{-1})			
	62.5	125	250	500
P. expansum	0.00 ±0.00 ^{a}	0.00 ±0.00 ^{a}	0.00 ±0.00 ^{a}	1.67 ±0.58 ^{b}
P. chrysogenum	0.00 ±0.00 ^{a}	2.67 ±0.58 ^{b}	4.00 ±1.00 ^{b}	5.67 ±0.58 ^{b}

Note: Means ± standard deviation. Values followed by different superscript within the same row are significantly different (p <0.05). GMEO – Green mandarin essential oil; 00.00 – total growth.

Table 3 Mycelial growth inhibition of the GMEO.

Fungal strains	MGI (%)			
	GMEO (μL.L^{-1})			
	62.5	125	250	500
P. expansum	3.49 ±1.23 a	-11.77 ±0.40 b	-51.37 ±3.01 c	-15.35 ±0.99 d
P. chrysogenum	14.17 ±0.53 a	20.02 ±2.13 b	54.15 ±1.15 c	45.61 ±0.88 d

Note: Means ± standard deviation. Values followed by different superscript within the same row are significantly different (p <0.05). MGI – Mycelial growth inhibition; GMEO – Green mandarin essential oil; 00.00 – total growth. The negative values indicate a profungal activity against *Penicillium* strains.

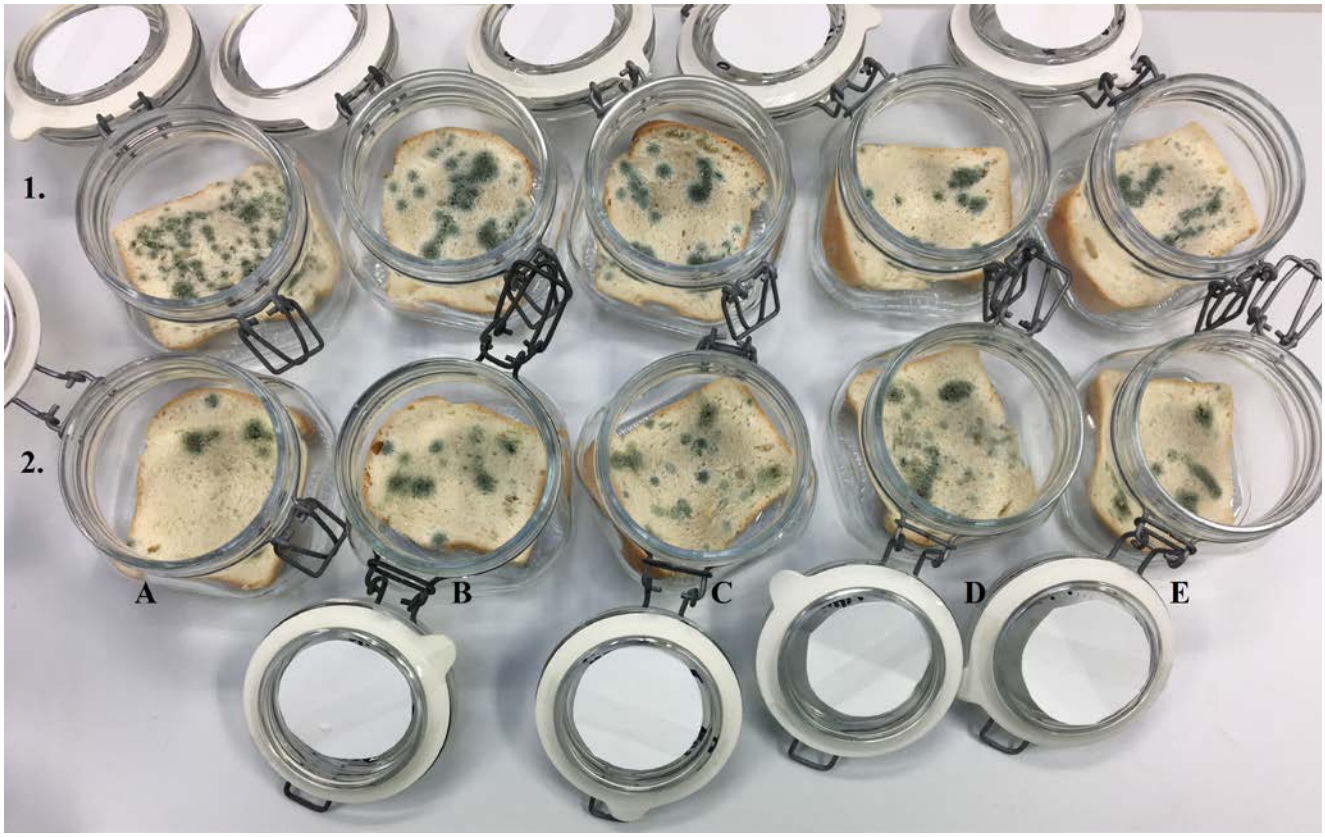

Figure 1 *In situ* antifungal analyses of bread with *Penicillum expansum* and *Penicillium chrysogenum* in vapor phase. Note: 1 – *P. chrysogenum*; 2 – *P. expansum* and after their treatment with different GMEO concentrations (A – control; B – 62.5 µL.L^{-1}; C - 125 µL.L^{-1}; D – 250 µL.L^{-1} and E – 500 µL.L^{-1}).

can be evident that all GMEO concentrations analyzed (62.5, 125, 250, and 500 µL.L^{-1}) exhibited antifungal potential against *P. chrysogenum*, whereby the concentration of 250 µL.L^{-1} showed the best efficiency (54.15 ±1.15%). Moreover, between all concentrations used, statistically significant differences (p <0.05) were noted. Interestingly, the growth of *P. expansum* was only very weak (3.49 ±1.23%) inhibited by the lowest concentration (62.5 µL.L^{-1}) of GMEO. On the other hand, the remaining concentrations used (125, 250, and 500 µL.L^{-1}) acted proactively on the growth of this fungus which was indicated by the negative values of -11.77 ±0.40, - 51.37 ±3.01, and -15.35 ±0.99%, respectively.

We hypothesize that the weak antifungal activity of GMEO against *P. expansum* may be due to the high resistance of this strain **(Adams, 2007)**. Therefore, we assume that GMEO used can be more effective against other species of microscopic fungi as was shown in the case of *P. chrysogenum*. Interestingly, *P. chrysogenum* was the most sensitive to the concentration of 250 µL.L^{-1} of the EO. For this reason, in our further research activities, we will deal with the optimization of GMEO concentration to obtain its highest possible efficiency. Moreover, the results are following our previous researches, in which the antifungal properties of other EOs, such as *Citrus aurantium* EO or **(Kačániová et al., 2020a)** coriander EO **(Kačániová et al., 2020b)**, against the fungi of *Penicillium* spp. analysed were confirmed. These findings are of particular relevance for food production because by introducing GMEO, the contamination with pathogens could be avoided, and the growth of the microscopic fungi could be inhibited, which is primarily important for the food industry **(Denkova-Kostova et al., 2021)**.

CONCLUSION

The aim of the present research was to assess the antifungal activity of GMEO in the vapor phase on the growth of selected *Penicillium* species inoculated on wheat bread. The chromatography analysis has shown that the major chemical compounds of the EO were α-limonene (71.5%), γ-terpinene (13.9%) and β-pinene (3.5%). The mandarin EO exhibited only weak AA comparable with Trolox as a standard with a value of 103.0 ±6.4 µg.mL^{-1} for DPPH, reflecting 5.6 ±0.7% of free radical-scavenging inhibition. The antifungal activity of GMEO against the tested fungi was shown to be not effective or moderate *in vitro*. Similar trend was also found for *in situ* analysis in which a maximum value for MGI (54.15 ±1.15%) was observed against *P. chrysogenum* using 250 µL.L^{-1} of GMEO. Thus, our results pointed to the fact that GMEO has weak both AA and inhibitory action against the growth of *P. expansum*. However, against *P. chrysogenum* it can be considered an appropriate alternative to use synthetic inhibitors for the preservation of bakery products such as wheat bread.

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