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Antifungal Efficacy of Mint Essential Oil Against *Penicillium* spp. Inoculated on Carrots

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Abstract: Current research aims to investigate the chemical composition, antioxidant, and *in vitro* and *in situ* antifungal activities of commercially available *Mentha arvensis* essential oil (mint, MEO). The identification of the volatile substances was done using Gas chromatography–Mass spectrometry (GC-MS) analysis. A total of 42 components representing 99.5% of the total oil were identified. The main compounds in the oil were menthol (37.3%), menthone (17.4%), neo-menthol (14.1%), and 1,8-cineole (4.9%). Antioxidant assays (1,1-diphenyl-2-picrylhydrazyl radical) demonstrate only weak activity for the MEO in values 195.00 ± 5.30 µg TEAC.mL^{-1}, with 22.8 ± 1.2% free radical-scavenging inhibition. Evaluation of *in vitro* and *in situ* antifungal activities of MEO (in four concentrations: 62.5 µL/L, 125 µL/L, 250 µL/L, and 500 µL/L) against three strains of *Penicillium* (*P.*) spp. fungi strains (*P. expansum*, *P. citrinum*, *P. crustosum*) were assessed by disc diffusion method and vapor contact method on the carrot as model food, respectively. The suitability of carrots as a substrate for analyzes was verified by determining moisture content (MC) and water activity (a_w), which showed values of 82.80 ± 2.33% and 0.959 ± 0.001, respectively. MEO exhibited promising antifungal activity against analyzed strains of test fungi as a diameter of zones of inhibition (from 2.88 ± 0.55 to 12.33 ± 1.14 mm), as well as the effectiveness of this oil was detected on the carrot model (from -5.41 ± 7.35 to 100.00 ± 0.00%). Moreover, it can be concluded that the growth inhibition of fungi strains significantly depends ($P < 0.05$) on the concentration of the MEO used in both procedures. Our results suggest that MEO, as a promising natural antifungal agent, can be applied in the innovative packaging of food products including carrots.

Keywords: *Mentha arvensis*, DPPH assay, volatile compounds, antifungal activity, model food.

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1. INTRODUCTION

Currently, the cultivation of herbs and aromatic plants to derive essential oils (EOs) is greatly increasing primarily due to the expanding demand generated by the food, pharmaceutical, and cosmetics industries (Lubbe and Verpoorte, 2011). Among medicinal plants, the production of mint (*Mentha* species) is very agriculturally profitable because of a large number of practical applications (Souza et al., 2014).

Mentha (M; Lamiaceae) is a well-known genus including approximately 30 reported species that grow across the world in temperate areas (Trucker and Naczi, 2007). The most common species of *Mentha* include *M. aquatica*, *M. arvensis*, *M. citrata*, *M. longifolia*, *M. piperita*, *M. pulegium*, *M. rotundifolia*, and *M. spicata* (Anwar et al., 2019) which are widely used in savory dishes, food, beverages, and confectionary products (Tafrihi et al., 2021). These plants exhibit a great chemical diversity with respect to their EOs and important biological activities (Trucker and Naczi, 2007) which are used in management of plant pathogens and insect pests, in traditional medicine, as well as in culinary and cosmetics (Singh and Pandey, 2018). In general, EOs are a highly volatile lipophilic mixture produced in plant secondary metabolism responsible for plant protection and communication (Saeed et al., 2022). At the laboratory scale, steam distillation and hydrodistillation are the most commonly used methods for their extraction (Ribeiro-Santos et al., 2018).

Mentha arvensis, popularly known as corn mint, wild mint or field mint (Nazim et al., 2020), has unique importance among the mint family due to its high concentration of menthol (Thawkar, 2016) ranging up to 71.40% (Pandey et al., 2003). In effect, menthol has antiseptic, carminative, refrigerant, stimulative, and diuretic properties (Thawkar et al., 2016). Other primary compounds of *M. arvensis*, responsible for its typical aroma, are menthone and its isomers, menthyl esters, and piperitone (Trucker and Zarowin, 2006). Generally, essential oil obtained from *Mentha arvensis* (MEO) is yellow in color with a very strong and persistent odor of mint (Makkar et al., 2018). Its chemical profile is affected by many factors, such as environmental and cultivar conditions, soil nutrients, humidity, temperature, and biotic and abiotic stress (de Sousa Barros et al., 2015). Regarding its biological properties, MEO was found to be a potential candidate for antimicrobial (Bokhari et al., 2016; Bibi et al., 2021), antioxidant (Benabdallah et al., 2018), and fungicidal activities (Makkar et al., 2018).

In this report, the antifungal activity of MEO and its possible application as a bio-preserver of carrots were evaluated. For a detailed description of the EO, its chemical profile and antioxidant properties were also taken into consideration.

2. MATERIAL AND METHOD

2.1. Essential Oil

Mint EO (MEO; *Mentha arvensis*) was extracted by steam distillation of flowering stems. This EO was obtained by a commercial producer Hanus Ltd. (Nitra, Slovakia), and was preserved at 4 °C in the laboratory refrigerator until their next application.

2.2. Chemical Analysis

The chemical composition of the MEO was analyzed using gas chromatography with mass spectrometry (GC-MS), as it was described by Valková et al. (2022a). In brief, the analysis was carried out by Agilent Technology 6890N (Agilent Technologies, Santa Clara, CA, USA) coupled to quadrupole mass spectrometer 5975B (Agilent Technologies, Santa Clara, CA, USA). Separation of compounds was carried out using HP-5MS capillary column (30 m × 0.25 mm × 0.25 m). The temperature program was as follows: 60 °C to 150 °C (increasing rate 3 °C/min) and 150 °C to 280 °C (increasing rate 5 °C/min), using helium 5.0 as the carrier gas with a flow rate of 1 mL/min. Samples of essential oils were dissolved in pentane, and injection volume was 1µL. The split/splitless injector temperature was set at 280 °C. The investigated samples were injected in the split mode with a split ratio at 40.8:1. Electron-impact mass spectrometric data (EI-MS; 70 eV) were acquired in scan mode over the m/z range 35–550. The mass spectrometry ion source temperature was 230 °C, while the temperature of MS quadrupole was set at 150 °C. Solvent delay time of 3 min. After the separation, the components were identified based on the comparison of their relative retention index and compared with the library mass spectral database (Wiley and NIST databases). The percentage composition of compounds (relative quantity; amounts higher than 0.1%) was measured based on the peak area. The retention indices were experimentally determined by injection of standard n-alkanes (C6–C34) under the same chromatographic conditions.

2.3. Determination of MEO Antioxidant Activity

To measure the antioxidant activity (AA) of MEO, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used, as previously described by Galovičová et al. (2021). The AA was expressed as the percentage of DPPH inhibition, which was calculated using the following equation: $(A0 - A1)/A0 \times 100$; where A0 was the absorbance of DPPH and A1 was the absorbance of the sample. The power of AA was recognized as follows: weak (0–29%) < medium–strong (30–59%) < strong (60 and more %). Moreover, the value for total AA was expressed according to the calibration curve as 1 µg of the standard reference Trolox to 1 mL of the MEO sample (TEAC).

2.4. Evaluation of MEO Antifungal Potential

2.4.1. Fungal Strains and Culture Media

In the current study, three strains of genus *Penicillium* (*P. expansum*, *P. crustosum*, *P. citrinum*), isolated from berry samples of *Vitis vinifera* were employed. Consequently, the microscopic filamentous fungi were classified using a reference-based MALDI-TOF MS Biotyper, and validated by comparison with the taxonomic identification using 16S ribosomal RNA (16S rRNA) gene sequences analysis.

2.4.2. *In Vitro* Antifungal Activity of MEO

Evaluation of the *in vitro* antifungal activity of the EOs was performed using the agar disc diffusion method, according to Valková et al. (2022a). For this purpose, an aliquot of 100 µL of culture media was inoculated on SDA. To prepare culture media, the strains were inoculated in Sabouraud Dextrose Agar (SDA; Oxoid, Basingstoke, UK) and incubated for 5 days at 25 °C. Subsequently, small aliquots of the fungi were transferred to test tubes, each containing 3 mL of distilled water. The inoculum concentration was

standardized by comparison with the 0.5 McFarland scale (1.5 × 108 CFU/mL). After that, the discs of filter paper (6 mm) were impregnated with 10 µL of MEO sample (in four concentrations: 62.5, 125, 250, and 500 µL/L), and applied on the SDA surfaces. Fungi were incubated aerobically at 25 ± 1 °C for 5 days. After the incubation, diameters of the inhibition zones in mm were measured. The values for inhibitory activity increased in the following manner: weak antifungal activity (5 - 10 mm) < moderate antifungal activity (10 - 15 mm) < very strong antifungal activity (zone > 15 mm).

2.4.3. *In Situ* Antifungal Activity of MEO

All three fungal strains (*P. expansum*, *P. crustosum*, and *P. citrinum*) were used to evaluate the antifungal activity of the EOs *in situ*.

2.4.4. Food Model

Carrot was applied as substrates for the growth of the fungi. This vegetable was purchased at the local market (Nitra, Slovakia).

2.4.5. Moisture Content and Water Activity of Food Model

To predict the suitability of substrates for fungal growth, moisture content (MC) and water activity (a_w) were determined, as reported by Valková et al. (2022a).

2.4.6. Vapor Contact Method

The experiment itself was performed as reported by Valková et al. (2022a). Firstly, sliced carrot (5 mm) was placed on the bottom of Petri dishes (PDs), and the inoculum was applied by stabbing one time with an injection pin on the vegetable surface. Further, 10 µL of the MEO (in the same four concentrations) was applied on the sterile filter paper disc (60 mm), then, it was placed at the top of PD. Subsequently, PDs were hermetically closed using parafilm and cultivated at 25 °C for 14 days.

2.4.7. Determination of Fungal Growth Inhibition

In situ fungal growth was determined using stereological methods. In this concept, the volume density (Vv) of visible fungal colonies was firstly established using ImageJ software counting the points of the stereological grid hitting the colonies (P) and those (p) falling to the reference space (growth substrate used: bread, carrot, and potato). The volume density of strain colonies was consequently calculated as follows: Vv (%) = P/p. Finally, the antifungal potential of the EOs was expressed as the percentage of fungal growth inhibition (FGI) according to the formula FGI = [(C − T)/C] × 100, where C and T is the growth of fungal strains (expressed as Vv) in the control and treatment group, respectively (Valková et al., 2022a).

2.5. Statistical Analysis

The data were submitted to one-way analysis of variance (ANOVA) and the means were compared by the Tukey test at 5% of probability using statistical software Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). All analyses were performed in triplicate.

3. RESULTS

3.1. Chemical Profile of MEO

GC-MS analysis revealed that a total of 42 substances, accounting for 99.5% of the whole constituents, were identified in the MEO chemical compostition. The major compounds were shown to be menthol (37.3%), menthone (17.4%), neo-menthol (14.1%), and 1,8-cineole (4.9%), as presented in Table 1.

Table 1. Chemical composition of MEO

NO	RI [a]	Compound [b]	% [c]
1	926	α-thujene	0.1
2	938	α-pinene	2.3
3	948	camphene	0.3
4	977	sabinene	0.5
5	980	β-pinene	1.5
6	992	β-myrcene	0.4
7	993	3-octanol	0.6
8	1004	α-phellandrene	tr
9	1016	α-terpinene	0.1
10	1023	p-cymene	1.0
11	1028	α-limonene	3.6
12	1033	1,8-cineole	4.9
13	1047	(*E*)-β-ocimene	tr
14	1060	ɣ-terpinene	0.5
15	1088	α-terpinolene	0.4
16	1148	isopulegol	1.7
17	1151	menthone	17.4
18	1160	pinocarvone	1.2
19	1162	iso-menthone	1.0
20	1164	neo-menthol	14.1
21	1170	borneol	0.2
22	1173	menthol	37.3
23	1189	α-terpineol	0.6
24	1217	trans-carveol	0.1
25	1229	cis-carveol	tr
26	1239	pulegone	0.9
27	1241	carvone	0.2
28	1253	3-carvomenthenone	1.0
29	1254	(*Z*)-anethole	0.4
30	1276	*p*-penth-1-en-7-al	5.2
31	1289	(*2E*)-hexenyl valerate	0.3
32	1297	menthyl acetate	tr
33	1298	*p*-menth-1-en-9-ol	tr
34	1378	α-ylangene	tr
35	1379	α-copaene	tr
36	1385	β-bourbonene	0.2
37	1388	β-elemene	tr
38	1422	(*E*)-caryophyllene	0.8
39	1443	aromadendrene	tr
40	1483	germacrene D	0.2

Table 1. Chemical composition of MEO (continue)

41	1525	δ-cadinene	0.4
42	1583	caryophyllene oxide	0.1
Total			99.5

Note: [a] Values of retention indices on on HP-5MS column; [b] Identified compounds; [c] Percentage of identified compounds; tr - compounds identified in amounts less than 0.1%.

3.2. Antioxidant Activity of MEO

It was found that values for AA of the MEO were 195.00 ± 5.30 μg TEAC.mL^{-1}, with 22.8 ± 1.2% free radical-scavenging inhibition linked to a weak AA.

3.3. *In vitro* Antifungal Activity of MEO

Results from the antifungal effects of MEO against *P. expansum*, *P. crustosum* and *P. citrinu*m are shown in Table 2. It can be concluded that the growth inhibition of fungi strains significantly depends ($P < 0.05$) on the concentration of the MEO used. Concretely, moderate antifungal activities (12.13 ± 0.48 mm, 11.56 ± 0.86 mm, and 12.33 ± 1.14 mm) were observed at the highest concentration (500 μL/L) of MEO against the growth of *P. expansum*, *P. crustosum* and *P. citrinu*m, respectively. On the other hand, 125 μL/L and 250 μL/L concentrations of MEO showed weak antifungal effects against all evaluated strains; whereas the lowest concentration of MEO resulted in only a very low inhibitory efficiency.

Table 2. *In vitro* antifungal activity of MEO in analyzed concentrations (inhibition zones in mm)

Fungi	MEO (μL/L)			
	62.5	125	250	500
P. expansum	2.88 ± 0.55 [a]	5.92 ± 0.39 [b]	8.13 ± 1.25 [c]	12.13 ± 0.48 [d]
P. crustosum	4.36 ± 0.78 [a]	6.23 ± 0.44 [b]	7.89 ± 0.96 [c]	11.56 ± 0.86 [d]
P. citrinum	3.71 ± 0.61 [a]	5.84 ± 1.06 [b]	8.56 ± 1.01 [c]	12.33 ± 1.14 [d]

Note: Mean ± standard deviation. MEO - Mint essential oil. Values in the same line with different small letters are significantly different ($P < 0.05$).

3.4. Moisture content and water activity of carrot

The results from the moisture content (MC) and water activity (a_w) measurements showed that the parameters of carrot in our study had values of 82.80 ± 2.33% and 0.959 ± 0.001, respectively.

3.5. *In situ* antifungal activity of MEO

The antifungal effectiveness of MEO on the growth of the *Penicillium* spp. inoculated on carrots are demonstrated in Table 3. From the findings it is clearly evident that with an increasing concentration, the MEO exhibited an enhancing antifungal effects against all analyzed strains, with the strongest one in the highest concentrations (500 μL/L).

Table 3. *In situ* antifungal activity of MEO on carrot.

Fungi	MGI (%)			
	MEO (μL/L)			
	62.5	125	250	500
P. expansum	28.57 ± 4.31 [a]	88.89 ± 5.12 [b]	89.09 ± 6.13 [b]	98.08 ± 4.78 [c]
P. crustosum	-5.41 ± 7.35 [a]	3.68 ± 2.93 [a]	72.04 ± 6.71 [b]	98.43 ± 4.88 [c]
P. citrinum	-23.33 ± 6.09 [a]	71.81 ± 8.12 [b]	96.92 ± 5.13 [c]	100.00 ± 0.00 [c]

Note: Mean ± standard deviation. MEO - Mint essential oil. Values in the same line with different small letters are significantly different ($P < 0.05$). The negative values indicate a profungal activity against Penicillium strains.

4. DISCUSSION AND CONCLUSIONS

Generally, the biological potencies of plant EOs were attributable to their chemical composition and especially to their major substances (Kasrati et al., 2015). Therefore, the detection of individual volatile components, which we implemented in our study, is an important tool for knowing the effect of EOs. In line with our findings Pandey et al. (2008), Chagas et al. (2020), and Mahn and Tuyet (2020) detected the major substance in EO obtained from *M. arvensis* menthol (71.4%, 86.1%, 66.04%, respectively). However, in our analyzed MEO was presented in lower concentration (37.3%). A similar lower concentration of this substance (21.35%) was also confirmed by Khan et al. 2019. The authors also find high content of menthone (29.42%) in its conception which also creates a high concentration of our oil sample (17.4%). We assume that differences in the percentage of the chemical components in MEO between mentioned studies may be related to varying cultivars of mint or different growing stages of plants (Verma et al., 2010).

DPPH assay is a widely employed procedure to estimate the free radical scavenging ability of materials due to its simplicity and rapidity (Gudimella et al., 2021). This method is based on the reduction of the commercially available radical (DPPH) and shows a color change from deep purple to pale yellow upon reaction (Higgins et al., 2021). Due to its properties, this technique is often used to analyze the antioxidant characteristics of EOs (Valková et al., 2022a,b,c). In our study, we found that despite the diverse chemical profile, MEO showed weak values for AA (195.00 ± 5.30 μg TEAC.mL^{-1}, with 22.8 ± 1.2%). Accordingly, in previous research works, we also noted weak antioxidant activity in green mandarin EO (Valková et al., 2021a), rosalina EO, fir EO and niaouli EO (Valková et al., 2022b). We propose that the weak AA of our MEO may be related to the high concentration of monoterpene and sesquiterpene hydrocarbons in its conception, which has low solubility in the assay medium and also does not have the ability to donate hydrogen atoms (Mata et al., 2007). These properties can be an essential limitation for the determination of the DPPH radical scavenging activity of some types of samples, including various EOs (Viuda-Martos et al, 2010).

Therefore, the choice of methodology largely affects the antioxidant activity of the samples. In the future, we plan to carry out the determination of antioxidant activity using several methods, including the FOMO and the ABTS methods.

Essential oils from *Mentha* spp. were screened for their antifungal activities (Saba and Anwar, 2018). Confirming our findings, Hussain et al. (2010) also detected the antifungal effects of *M. arvensis* EO against seven fungi strains including *Aspergillus* (*A.*) *flavus*, *Alternaria* (*A.*) *solani*, *Fusarium* (*F.*) *solani*, *Rhizopus* (*R.*) *solani*, *A. alternata*, *A. niger* and *Rhizopus* spp. Their results from the disc diffusion method indicated that MEO showed maximum antifungal activity with large inhibition zones varied from 16 to 30 mm against fungi strains. The antifungal efficacy of MEO may be related to its high menthol content, which we also detected in our study (37.3%). It is known that menthol exhibits antifungal effects against various fungi strains including *Candida (C.) albicans* (Piran et al., 2017), *Aspergillus* (*A.*) *niger*, *A. fumigatus*, *A. flavus*, *A. ochraceus*, *A. alternata*, *Botrytis* (*B.*) *cirenea*, *Cladosporium* spp., *P. citrinum*, *P. chrysogenum*, *F. oxysporum* and *Rhizopus oryzae* (Abbaszadeh et al., 2014). Although the exact mechanism of menthol action is not fully understood, its antifungal effect can be resulted from a perturbation of the lipid fraction of fungi plasma membrane, resulting in alterations of membrane permeability and in leakage of intracellular materials (Trombetta et al., 2005). Moreover, Samber et al. (2015) demonstrated the efficacy of menthol due to its integration with PM-H+ ATPase enzyme which possibility an electrochemical proton gradient across the cell membrane necessary for nutrient uptake. However, we assume that individual compounds of MEO and their interaction are crucial for their final inhibitory effects on mycelial growth. Therefore, the antifungal efficacy of our EO may be related to other volatile components present in its conception, as well.

MC and a_w largely influence the ability of microorganisms to grow on food products (Qiu et al., 2019). The presence of water in the form of MC is the major factor aiding the growth and activities of the microorganisms because it increases their metabolic activities. Without water or in the presence of a limited volume of water, agricultural products will become inhospitable to the microorganism and inhibit their growth (Rajeev et al., 2012). In this context, many fresh foods including vegetables are perishable due to their high MC (> 40%; Akdogan, 1999; Dagnas et al., 2017). Further a_w is defined as the ratio of the vapor pressure of water over a substrate compared to that over pure water at the same temperature and pressure (Cazier and Gekas, 2001).Concerning a substantial impact on the growth of microorganisms it was found out that a_w above 0.7 supporting the microbial spoilage (Syamaladevi et al., 2016). In line with our findings, similar values for MC (82.80 ± 2.33%) and a_w (0.959 ± 0.001) of carrot we detected in our last research (86.83 ± 0.42%; 0.945 ± 0.002; Valková et al., 2022c). Our results indicate the suitability of carrot as food model for *in situ* antifungal analysis of the MEO investigated.

Some studies have reported that vapor generated by EOs has a greater antifungal effect compared with EOs in liquid form applied by direct contact (Tullio et al. 2007; Fisher and Phillips 2008). Moreover, the vapor phase allows free attachment of EOs to microorganisms, unlike lipophilic molecules in the liquid phase associated to form micelles which restrain the attachment of EOs to microorganisms (Boukhatem et al., 2014). In this way, the vapor phase of EOs has a specific impact on fungi due to their superficial growth reflecting more susceptibility to EO volatile compounds (Edris and Farrag 2003). Furthermore, the composition of the food system impacts the antifungal effectiveness of EOs, and this activity is typically decreased in in situ conditions compared to in vitro ones. However, the low-fat content of vegetables can participate in the successful antifungal effects also on the food model (Burt, 2004). Therefore, in the current study, this effect has been investigated for carrots as food substrates. In accordance with our results, in our previous study we noted the antifungal effect of MEO obtained from *M. piperita* against the same fungi strains (*P. expansum*, *P. citrinum*, and *P. crustosum*) inoculated on bread (Valková et al., 2021b). In both cases, the effect of essential oils on food models was dependent on their concentration, with the highest efficiency recorded at the highest concentrations (500 µL/L), with the exception of *P. citrinum* inoculated on bread (Valková et al., 2021b), when the highest efficiency was recorded at concentrations 125 µL/L. Moreover, our findings are in accordance with our previous studies, in which the antifungal efficacies of various types of EOs, such as mandarin EO (Valková et al., 2021a), coriander EO (Kačániová et al., 2020), fir EO, rosalina EO, and niaouli EO (Valková et al., 2022c), against the same fungi species analysed were confirmed.

From the results of all our analyses, it can be concluded that MEO may be a promising agent with potential use for extending the shelf-life of vegetables including carrots on the commercial scale of the food industry.

Ethics Committee Approval

N/A

Peer-review

Externally peer-reviewed.

Author Contributions

Conceptualization: V.V., H.Ď., M.K.; Investigation: V.V., H.Ď., L.G., N.L.V., M.V., M.K.; Material and Methodology: V.V., H.Ď., L.G., N.L.V., M.V., M.K.; Supervision: M.K.; Visualization: V.V., H.Ď.; Writing-Original Draft: V.V., H.Ď.; Writing-review & Editing: V.V., H.Ď., M.K.; Other: All authors have read and agreed to the published version of manuscript.

Conflict of Interest

The authors have no conflicts of interest to declare.

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