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Antibacterial Activity of The Vapor Phase of *Thymus mastichina* Essential Oil

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Abstract: Essential oils have a diverse spectrum of biological activities, they are also low-toxic, and easily degradable in the human body. These properties make them suitable candidates for the protection and shelf-life extension of agricultural products. The aim of our study was to evaluate the efficacy of the vapor phase of *Thymus mastichina* essential oil against microorganisms on model fruit and vegetable crops. We focused on comparing the efficacy of the essential oil in the vapor phase and contact application using the disk diffusion method against the tested microorganisms. Based on the methods disc diffusion method for contact application and antimicrobial activity of vapor phase on model crops for vapor phase we used, we concluded that *Thymus mastichina* essential has higher efficacy in a vapor application. For most of the tested microorganisms and on all the tested crops, the most significant inhibition was detected at the lowest tested concentration of 62.5 µL/L. Only moderate antimicrobial activity was detected in contact application and lower efficacy compared to antibiotics. These findings suggest that in the future *Thymus mastichina* essential oil could find application in crop storage to prevent crop deterioration due to microbial pathogens. Due to the need for low concentrations, it is assumed that the sensory properties of the crop for the consumer will not be affected. The replacement of synthetic fungicides and bactericides with natural alternatives could have a positive impact on the environment.

Keywords: essential oil, Thymus mastichina, vapor phase.

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1. INTRODUCTION

Thymus mastichina is also known as "Spanish Thyme" is native to the Iberian Peninsula. Thymus mastichina belongs to the Lamiaceae family and is a semi-woody shrub growing to a height of about 0.5 meters (Arantes et al. 2019). This plant is very resistant to frost, diseases, and pests. Thymus mastichina is mainly used for its antimicrobial, antioxidant, antirheumatic, and antitussive properties (Méndez-Tovar et al. 2015). For centuries, Thymus mastichina has been used as a spice to flavor food. From this mother plant, the essential oil of Thymus mastichina (TMEO) is extracted which is used in the pharmaceutical, food, cosmetic, and fragrance industries (Taghouti et al. 2019).

Essential oils (EOs) are volatile, aromatic compounds extracted from different parts of plants that possess diverse biological properties (Ishaq et al. 2017). EOs, due to their pronounced vapor-phase biological activities, low toxicity to humans, easy degradability, and antimicrobial effects, could find application in protecting and extending the shelf life of agricultural products during storage (Gutiérrez et al. 2009).

Vegetables and fruits are among the perishable products with a short shelf life after harvesting. These commodities are at risk of mechanical damage, variation in physical parameters, and contamination during handling by various microbial pathogens (Ding and Lee, 2019). Control of postharvest microbial pathogens is largely provided by synthetic fungicides and bactericides (Palou 2018). The use of these synthetic products can lead to the emergence of

resistance of pathogens to the products used as well as high residues on agricultural products posing a high risk to consumer health and the environment (Hosseini et al. 2020).

Our study aimed to evaluate the efficacy of the vapor phase of Thymus mastichina essential oil against microorganisms on fruit and vegetable crops model. To compare the efficacy of vapor phase essential oil with contact application against the microorganisms tested.

2. MATERIAL AND METHOD

2.1. Essential oil

Thymus mastichina essential oil was purchased from the Slovak company Hanus S.R.O. The essential oil was stored in the dark at 4 °C throughout the experiment. The composition of the essential oil were stated by the manufacturer is cineol 45-65 %, linalool 8-30 %, 4-terpineol, β-pinene, α-pinene, limonene, α-terpineol.

2.2. Tested microorganisms

The tested microorganisms were obtained from the Czech collection of microorganisms Brno. Gram-negative bacteria *Yersinia enterocolitica* CCM 7204 and *Haemophilus influenzae* CCM 4454 were used. Gram-positive bacterias were *Listeria monocytogenes* CCM 4699 and *Enterococcus faecalis* CCM 4224.. Candida tropicalis CCM 8264 was used as yeast. The biofilm-producing bacterium *Pseudomonas fluorescens* was obtained from a fish sample.

2.3. Antimicrobial activity of TMEO contact application

The antimicrobial activity of the contact application of TMEO was determined by the disk diffusion method. The bacterial inoculum was cultured for 24 hours in Tryptone soy agar (TSA, Oxoid, Basingstoke, UK) at 37 °C. The yeast inoculum was cultured for 24 h in Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK) at 25 °C. The inoculum was adjusted to an optical density of 0.5 McFarland's standard (1.5 x 10^8 CFU/mL). The disc diffusion method was analyzed on Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK). 100 µL of modified inoculum were applied to Petri dishes (PD) containing MHA, to bacteria and yeast, respectively Sterile paper discs (Oxoid, Basingstoke, UK) with a diameter of 6 mm were placed on the PDs. The discs were saturated with 10 µL of TMEO. Samples were incubated for 24 h at 37 °C for bacteria and 25 °C for yeast. Two antibiotics (cefoxitin for gram-positive bacteria, gentamicin for gram-negative bacteria; Oxoid, Basingstoke, UK) and one antifungal (fluconazole; Oxoid, Basingstoke, UK) were used as positive controls for gram-negative, gram-positive bacteria and yeasts. An inhibition zone above 10 mm was determined as very strong antimicrobial activity, an inhibition zone of 5-10 mm was determined as moderate activity, and an inhibition zone below 5 mm was determined as weak activity. Antimicrobial activity was measured in triplicate (Kačániová et al. 2020).

2.4. Antimicrobial activity of vapor phase TMEO on model crops

The antimicrobial activity of vapor phase TMEO on model crops (apple, pear, carrot, white radish) was tested on gram-negative, gram-positive bacteria and yeast. SDA for yeast and MHA for bacteria was poured into 60 mm PD and capped. Sliced model crops (0.5 mm) were placed on agar. Using a microbial needle, three injections of inoculum were applied to the sliced model foods. TMEO was diluted in ethyl acetate to concentrations of 500, 250, 125, and 62.5 µL/L. A sterile filter paper was placed in the lid onto which 100 µL of the appropriate concentration was injected using a micropipette. The filter paper was dried for 1 min to evaporate the remaining ethyl acetate, then the plates were sealed and incubated at 37 °C for bacteria and 25 °C for yeast for 7 days.

Growth inhibition was assessed by stereological methods. Volume density (Vv) was estimated using ImageJ software. Colony (P) and substrate (p) stereological grid points were calculated. Growth density was calculated as % by the formula $Vv = P/p \times 100$. The antimicrobial activity of EO was expressed as growth inhibition $BGI = [(C - T)/C] \times 100$, where C was the growth density in the control group and T was the growth density in the treated group (Aman 2015; Talibi et al. 2012). Negative results represented growth stimulation.

2.5. Statistical data processing

One-way analysis of variance (ANOVA) followed by Tukey's test at $p < 0.05$ was used to statistically process the data using Prism 8.0.1 (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1. Antimicrobial activity of TMEO contact application

Based on the disc diffusion method, we found that TMEO achieved moderate inhibitory activity on how many inhibition zones in the range of 5-10 mm were observed for all tested microorganisms (Table 1). The highest zone of inhibition was detected against the yeast *C. tropicalis* (8.66 mm) and the gram-negative bacteria *H. infuenzae* (8.33 mm). Compared to the antibiotic control, the effect of TMEO was lower.

Table 1. Antimicrobial activity of TMEO contact application.

Microorganisms	Inhibition zones (mm)	ATB (mm)
Yersinia enterocolitica	5.66±0.58	29.66±0,58
Haemophylus influenzae	8.33±1.15	30.54±1,53
Lysteria monocytogenes	6.33±1.66	30.67±1.15
Enterococcus faecalis	7.54±0.58	28.88±0.33
Pseudomonas fluorescens-biofilm	5.33±1.15	25.66±0.58
Candida tropicalis	8.66±0.58	31.66±1.15

The inhibition zone above 10 mm was determined as very strong antimicrobial activity, the inhibition zone 5-10 mm was determined as moderate activity, and the inhibition zone below 5 mm was determined as weak activity. Antimicrobial activity was measured in triplicate.

3.2. Antimicrobial activity of vapor phase TMEO on model crops

In the analysis of the antimicrobial activity of vapor phase TMEO on the model apple crop (Table 2.), we detected inhibition in all tested microorganisms and at all tested concentrations, except for *E. faecalis* with a concentration of 250 µL/L and for *P. fluorescens* biofilm with a concentration of 125 µL/L where we observed stimulation of growth. The most pronounced inhibition was observed at the lowest tested concentration, 62.5 µL/L, for all tested microorganisms.

Table 2. Antimicrobial activity of TMEO vapor application on model food apple.

Apple	**BGI (%)**			
	Con. (µL/L)			
Microorganisms	**62.5**	**125**	**250**	**500**
Y. enterocolitica	54.40±0.08 [a]	44.23±0.99 [b]	36.39±2.12 [c]	26.02±2.09 [d]
H. influenzae	35.25±1.07 [a]	22.65±0.89 [b]	15.83±0.83 [c]	12.68±0.67 [d]
L. monocytogenes	44.74±0.95 [a]	34.35±0.98 [b]	13.32±1.05 [c]	8.32±0.52 [d]
E. faecalis	34.71±0.95 [a]	6.73±1.20 [b]	-9.28±0.55 [c]	2.76±0.42 [d]
C. tropicalis	53.40±1.15 [a]	-24.46±2.06 [b]	24.68±0.42 [c]	8.74±0.57 [d]
P. fluerescens-biofilm	36.55±2.03 [a]	21.06±0.63 [b]	12.03±1.60 [c]	2.99±0.59 [d]

Mean ± standard deviation. Values followed by different superscripts within the same line are significantly different ($P < 0.05$). Con.—concentration. BGI – bacterial growth inhibition.

In the analysis of the antimicrobial activity of vapor phase TMEO on the model crop pear (Table 3.), we detected inhibition in all tested microorganisms and at all concentrations. The most pronounced inhibition was observed at the lowest tested concentration, 62.5 µL/L, for all microorganisms.

Table 3. Antimicrobial activity of steam application of TMEO on model crop pear.

Pear	**BGI (%)**			
	Con. (µL/L)			
Microorganisms	**62.5**	**125**	**250**	**500**
Y. enterocolitica	36.63±2.04 [a]	33.72±5.34 [a]	12.36±0.84 [b]	6.84±0.95 [c]
H. influenzae	36.55±2.03 [a]	25.36±0.11 [b]	15.17±0.61 [c]	0.81±0.56 [d]
L. monocytogenes	44.09±2.28 [a]	23.85±2.03 [b]	12.56±0.48 [c]	5.37±0.44 [d]
E. faecalis	54.78±1.46 [a]	36.39±2.12 [b]	26.52±2.39 [c]	8.48±0.59 [d]
C. tropicalis	36.39±2.12 [a]	23.32±0.95 [b]	14.45±0.86 [c]	5.37±0.40 [d]
P. fluerescens-biofilm	64.89±1.00 [a]	36.46±2.72 [b]	17.43±0.06 [c]	6.98±0.97 [d]

Mean ± standard deviation. Values followed by different superscripts within the same line are significantly different ($P < 0.05$). Con.—concentration. BGI – bacterial growth inhibition.

In the analysis of the antimicrobial activity of vapor phase TMEO on a model carrot crop (Table 4.), we detected inhibition in all tested microorganisms and at all concentrations. The most pronounced inhibition concentration for all microorganisms except L. monocytogenes was observed 62.5 µL/L, and for L. monocytogenes 125 µL/L

Table 4. Antimicrobial activity of steam application of TMEO on carrot model crop.

Carrot	BGI (%)			
	Con. (µL/L)			
Microorganisms	**62.5**	**125**	**250**	**500**
Y. enterocolitica	19.32±0.95 [a]	14.05±1.50 [b]	8.28±1.47 [c]	2.16±0.86 [d]
H. influenzae	56.18±1.17 [a]	43.66±1.02 [b]	27.47±2.13 [c]	8.27±0.52 [d]
L. monocytogenes	16.34±1.85 [a]	25.54±0.39 [b]	7.16±1.63 [c]	4.44±0.46 [d]
E. faecalis	44.21±0.48 [a]	28.17±5.90 [b]	13.24±0.55 [c]	3.43±0.83 [d]
C. tropicalis	56.05±0.95 [a]	33.72±1.06 [b]	12.47±0.45 [c]	4.96±0.34 [d]
P. fluerescens-biofilm	47.87±1.35 [a]	27.46±1.06 [b]	14.00±1.16 [c]	5.21±0.30 [d]

Mean ± standard deviation. Values followed by different superscripts within the same line are significantly different ($P < 0.05$). Con.—concentration. BGI – bacterial growth inhibition.

In the analysis of the antimicrobial activity of vapor phase TMEO on a model crop of white radish (Table 5.), we detected inhibition in all tested microorganisms and at all tested concentrations. The most pronounced inhibition was observed for *Y. enterocolitica, E. faecalis* and *C. tropicalis* at the lowest concentration tested, 62.5 µL/L. Against other microorganisms, efficacy was observed at higher concentrations for *H. influenzae* and *L. monocytogenes* at 250 µL/L and biofilm-producing *P. fluorescens* at 125 µL/L.

Table 5. Antimicrobial activity of steam application of TMEO on a model crop of white radish.

White radish	BGI (%)			
	Con. (µL/L)			
Microorganisms	**62.5**	**125**	**250**	**500**
Y. enterocolitica	43.62±0.56 [a]	23.65±1.53 [a]	17.67±0.48 [b]	8.87±0.59 [c]
H. influenzae	15.68±0.57 [a]	5.20±3.68 [b]	35.30±1.06 [c]	35.21±0.76 [c]
L. monocytogenes	25.93±1.80 [a]	23.49±18.65 [ac]	87.67±1.48 [b]	14.26±0.57 [c]
E. faecalis	86.97±1.34 [a]	4.26±1.08 [b]	16.52±2.02 [c]	37.10±1.85 [d]
C. tropicalis	55.85±1.14 [a]	32.80±1.00 [b]	23.65±1.53 [c]	16.37±0.79 [d]
P. fluerescens-biofilm	35.28±0.95 [a]	75.47±1.33 [b]	24.45±2.41 [c]	8.43±0.71 [d]

Mean ± standard deviation. Values followed by different superscripts within the same line are significantly different ($P < 0.05$). Con.—concentration. BGI – bacterial growth inhibition.

4. DISCUSSION AND CONCLUSIONS

Rodrigues et al. (2020) in their work found inhibition zones of TMEO against *L. monocytogenes* of 9.7-12.3 mm and for *P. fluorescens* of 9-10 mm, depending on the origin of the EO and the plant part used. The findings for *L. monocytogenes* are in agreement with our results for *P. fluorescens* we detected a lower zone of inhibition which could be because it is a biofilm-producing strain that is more resistant to inhibition. Faleiro et al. (2003) detected in their work the inhibitory activity of TMEO against *L. monocytogenes* with a zone of inhibition of 9.7 mm and also report that TMEO is most effective against the *Candida* genus with a zone of inhibition above 10 mm. These findings confirm our results. Ballester-Costa et al. (2013) in their work found a zone of inhibition against *P. fluorescens* of 9.0 mm. In our work, we detected a lower zone of inhibition which could be because it is a biofilm-producing strain which is more resistant to inhibition.

To our knowledge, TMEO has not yet been tested in the vapor phase on food models. In a previous study, we analyzed the vapor phase effect of *T. vulgaris* detecting a very good antifungal as well as antibacterial effect (Galovičová et al. 2021). Paris et al. (2020) in their work analyzed the antifungal and antimicrobial effect of EOs in washed and contact applications against fruit spoilage pathogens and concluded that the vapor phase is more effective than the contact phase as EOs are rich in volatile compounds which confirms our findings. In contrast, Ács et al. (2018) in their study reported that their EOs were stronger inhibitors in liquid form, which is probably due to direct contact with the pathogen. This finding contradicts our results which may be due to the different chemical compositions especially the content of volatile compounds which are more effective in the vapor phase.

The results of our study show that TMEO has higher efficacy in vapor application where we detected the most significant inhibition at the lowest tested concentration of 62.5 µL/L for most of the tested microorganisms and on all crops. In contact application, only moderate antimicrobial activity was detected, and lower efficacy compared to antibiotics. These findings suggest that in the future TMEO could find application in the storage of horticultural crops to prevent their deterioration due to microbial pathogens. In the future, it would be advisable to test even lower concentrations of TMEO as the trend of antimicrobial effect showed that in most cases the lowest concentration was the most effective. Due to the need to use very low concentrations, it is assumed that the sensory properties of the crop for the consumer will not be affected. The replacement of synthetic fungicides and bactericides with

natural alternatives could have a positive impact on the environment.

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Conflict of Interest
The authors have no conflicts of interest to declare.

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