

EVALUATION OF VAPOR-PHASE ANTIFUNGAL ACTIVITIES OF SELECTED PLANT ESSENTIAL OILS AGAINST FUNGAL STRAINS GROWING ON BREAD FOOD MODEL

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ABSTRACT

The current study aimed to investigate antifungal activities of two commercially available essential oils (EOs), specifically Tea tree oil (*Melaleuca alternifolia*; TTEO) and St. John's wort oil (*Hypericum perforatum*; HPEO) against three *Penicillium* (*P.*) species: *P. citrinum*, *P. expansum*, and *P. crustosum* in *in situ* conditions. For this purpose, EOs were applied in the vapor phase to determine the growth inhibition of fungi artificially inoculated on sliced bread. Changes in colony growth rate were evaluated as markers for the mycelial growth inhibition (MGI) effect of the EOs. The antioxidant activities of the EOs were evaluated using the DPPH method. The moisture content (MC) and water activity (a_w) of bread as a substrate for fungal growth were also measured. From the DPPH assay, we have found that both EOs (TTEO, HPEO) exhibited strong antioxidant activity ($64.94 \pm 7.34\%$; $70.36 \pm 1.57\%$, respectively). The values for bread MC and a_w were $43.01 \pm 0.341\%$ and 0.947 ± 0.006 , respectively. Our results suggest that HPEO is the only weak inhibitor of *P. citrinum* and *P. crustosum* colony growths. Also, the highest concentrations of TTEO display only the weak capability of mycelial growth inhibition of *P. citrinum* and *P. crustosum*. By contrast, the colony growth of *P. expansum* was enhanced by both EOs at all levels used. In conclusion, the application of both EOs in the vapor phase against selected *Penicillium* species seems not to be a promising alternative to chemical inhibitors used for bread preservation.

Keywords: essential oil; *Penicillium* sp.; antioxidant activity; antifungal activity; bakery product

INTRODUCTION

Bread is a staple cereal-based product consumed worldwide. The shelf life of bread is reduced due to the action of many factors such as its moisture content (MC), water activity (a_w), and storage conditions (Legan, 1993). Bread spoilage is often caused by microscopic fungi, among which the most common species are *Penicillium* (*P.*), *Aspergillus*, *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium*, and *Rhizopus* (Passarinho et al., 2014). Although *P. expansum* is mainly associated with apple spoilage, many studies focused on the antimicrobial properties of bread have selected *P. expansum* as the target form because of its high resistance as compared to other microscopic fungi and mycotoxin production, as well (Balaguer et al., 2013; Belz et al., 2012; Luz et al., 2018).

To solve the problem with the shelf life of bakery products, diverse preservatives (such as propionic acid or sorbic acid) are added to the dough. Currently, however, most consumers avoid chemical preservatives present in daily foods in the context of a healthy lifestyle. Therefore, the use of EOs in packaging materials vaporized in the gas

phase can be a solution for this concern (Krisch, Tserennadmid and Vágvölgyi, 2011).

Essential oils (EOs) are defined as odoriferous and volatile products of plant secondary metabolism which are applicable in almost every field of life including folk medicine, food flavorings, and preservation (Kalemba and Kunicka, 2003). These liquid, volatile, pure, and colored mixtures of several aromatic compounds are obtained from the different parts of plants, mainly from herbs and spices. However, various new sources of EOs such as food or secondary waste are currently being explored (Ravindran and Jaiswal, 2016; Wu et al., 2017). EOs are extracted from different techniques and the most preferable method of extraction is hydrodistillation which is cheap and easy to use (Irshad et al., 2020). About 3,000 different types of EOs are known to date, of which about 300 are in commercial use (Burt and Reinders, 2003). Due to chemical composition, many EOs have strong antimicrobial activities, pharmacological and neuroprotective properties and some of them have been also shown to affect behavioral characteristics (Lis-Balchin, Deans and Eaglesham, 1998).

Tea tree essential oil (TTEO) is obtained by distillation from the leaves and terminal branches of the narrow-leaf tea tree *Melaleuca alternifolia*. The EO is known mainly for its use in the treatment of many skin diseases. Also, it can be present in topical pharmaceuticals, cosmetics, and household products (de Groot and Schmidt, 2016). The antifungal activity of TTEO is attributed to the presence of compounds such as terpinen-4-ol, α -terpineol, linalool, α -pinene, β -pinene, β -myrcene, and 1,8-cineole (Puvača et al., 2018).

Hypericum perforatum (common St. John's wort) is one of the best-known and most commonly used herbs in recent years often associated with the treatment of anxiety and depression (Lyles et al., 2017). In the plant, a wide range of biologically active substances such as naphthodianthrones (Kitanov, 2001), acylphloroglucinol (Verotta et al., 2000), xanthenes (Sparenberg, 1993), flavonoids (Jürgenliemk and Nahrstedt, 2002), tannins (Barnes, Anderson and Phillipson, 2001), and lipids (Omarova and Artamonova, 1999) has been identified. *Hypericum perforatum* essential oil (HPEO) is composed mainly of 2-methyl octane, α -pinene, and caryophyllene (Mathis and Ourisson, 1964; Schwob, Bessière and Viano, 2002). This EO is a popular home remedy for the recovery of cuts, burns as well as peptic ulcers (Yeşilada et al., 1995).

In the current study, the antifungal activities of TTEO and HPEO against three *Penicillium* (*P.*) species (*P. expansum*, *P. crustosum*, and *P. citrinum*) inoculated on sliced bread were evaluated. To date, such research has not been done before our experiment. The efficacy of the selected EOs as antifungal preservatives in wheat bread will allow us to assess the possibility of their practical application as novel and safe preservatives.

Scientific hypothesis

There is growing evidence that various types of essential oils (EOs) obtained from plants show effective antimicrobial and antifungal properties. Our study aimed to evaluate the antifungal properties of EOs (Tea tree oil and St. John's wort oil) against selected *Penicillium* species, providing the possibility of used these EOs for long self-life products in the food industry.

MATERIAL AND METHODOLOGY

Samples

Tea tree essential oil (TTEO) and St. John's wort (*Hypericum perforatum*) essential oil (HPEO) were purchased from Gratis Company (Ltd, Istanbul, Turkey).

Chemicals

All chemicals were analytical grade and were purchased from Merck (Germany) and Sigma Aldrich (USA).

Animals and Biological Material

The fungi *P. expansum*, *P. crustosum*, and *P. citrinum* were isolated from grape samples and identified with the MALDI-TOF MS Biotyper and 16S rRNA sequencing.

Sample preparation:

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

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

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).

Water activity analyzer (Lab Master aw Standard, Novasina, Switzerland).

Moisture analyzer (Kern DBS 60-3, Kern & Sohn GmbH, Germany).

Laboratory Methods

Radical Scavenging Activity—DPPH Method

The radical scavenging activities of the EOs were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method according to Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) with minor modifications. The 0.1 mL of each EO oil sample was mixed with 3.9 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). The absorbance of the reaction mixture was determined with The Agilent Cary 60 UV-Vis spectrophotometer at 515 nm. The final scavenging activity was calculated as a percentage (AA%) and determined according to the following formula: $AA\% = [(A_0 - A_1)/A_0 \times 100]$; where A_0 is the absorbance of the control reaction (DPPH radical); A_1 is the absorbance of the tested sample.

Bread making process

The baking formula consisted of wheat flour T650 (250 g), water (150 mL), sucrose (2.5 g), salt (5 g), and yeast (5 g). All the ingredients were mixed in a spiral mixer for 6 min. The prepared dough was placed into an aluminum vessel and transferred to a fermentation cabinet at 32 °C and 85% relative humidity for 40 min. The loaves were baked in two stages: (i) at 180 °C for 17 min with the addition of 160 mL water and (ii) at 210 °C for 10 min in a steamy oven. Following the baking, the bread was left to stand at the laboratory temperature for 2 h and then analyzed.

Water activity (a_w) and moisture content (MC) of bread

Bread a_w was measured using the Lab Master a_w Standard. For this purpose, 2.0 g of crumbs was cut into a cube and placed into a sample pan. The value of a_w was measured automatically at 25 °C for 15 – 20 min.

Bread MC was determined using a moisture analyzer Kern DBS 60-3. In this case, 1.0 g of crumbs was weighed on the sample plate and measurement was done at 120 °C for 10 – 15 min.

Vapor-phase of the antifungal assay with bread

The bread samples were cut into slices with a height of 150 mm and placed into 0.5 L sterile glass jars (Bormioli Rocco, Fidenza, Italy). A fungal spore of each strain was used for bread inoculation. Essential oil concentrations of 125, 250, and 500 $\mu\text{L}\cdot\text{L}^{-1}$ (EOs + ethyl acetate) were evenly distributed on a sterile paper-filter disc (6 cm), which was inserted into the cover of the jar, except for the treatment of the control group. The jars were hermetically closed and kept at 25 °C for 14 days in the laboratory incubator. The colonies with visible mycelial growth and visible sporulation were evaluated. The antifungal activity was expressed as a percentage of mycelial growth inhibition (MGI) which was calculated using the formula: $MGI = [(C - T) / C] \times 100$, where C = fungal growth in the control and T = fungal growth in the treatment group, as was

previously described (Aman and Rai, 2015; Talibi et al., 2012).

Description of the Experiment

Sample preparation: 1
 Number of samples analyzed: 3
 Number of repeated analyses: 3
 Number of experiment replication: 3

Statistical analysis

MBIC50 and MBIC90 values (concentration causing 50% and 90% reduction of fungal growth) were estimated by the logit analysis for evaluation antifungal activity of EOs. In addition to MBIC50 and MBIC90 values, statistical evaluation of the obtained data was performed using the GraphPad Prism 8.0.1 (GraphPad Software Incorporated, San Diego, California, USA). One-way analysis of variance (ANOVA) followed by the Tukey test was used for statistical analysis.

RESULTS AND DISCUSSION

Antioxidant activity of selected EOs

The scavenging ability of DPPH free radical is commonly used to evaluate the antioxidant potential of diverse plant extracts including EOs (Fadel et al., 2020; Kačániová et al., 2020a; Kačániová et al., 2020b). Following the assay, the abilities of TTEO and HPEO to act as donors of hydrogen atoms or electrons in the transformation of DPPH radical into its reduced form DPPH-H were in our research investigated. As shown in Table 1, both EOs exhibited high free-radical scavenging activity through the used assay. Also, no significant difference ($p = 0.25$) was observed between analyzed EOs.

In accordance with our study, the evident antioxidant capacity of TTEO (even stronger than vitamin E and α -tocopherol) was reported by Zhang et al. (2018). In addition to this, Kim et al. (2004) have found that although terpinen-4-ol is the major bioactive chemical compound of TTEO, the majority of antioxidant activity in TTEO was attributed to the three terpenic compounds, i.e., α -terpinene, α -terpinolene, and γ -terpinene. The higher antioxidant activity of HPEO than TTEO determined in our research can be attributed to flavonoids and phenolic acids presented in HPEO molecular compositions. From them, hyperoside, rutin, quercitrin, and quercetin are the most abundant in *Hypericum perforatum* (Orčić et al., 2011; Orhan and Kartal, 2015).

Generally, antioxidant properties of various bioactive substances including medicinal and aromatic plants play a key role in counteracting the deleterious impact of free radicals in biological systems (Raj Kapoor, Burkan and Senthilkumar, 2010; Pirbalouti et al., 2014). In foods (such as bread), poly-unsaturated fatty acids are particularly susceptible to oxidation by free radicals during the storage (Donnelly and Robinson, 1995), manufacturing, distribution, and final preparation of foods. The oxidative changes can cause rancidity such as off-flavors, loss of color, altered nutrient value, and may produce toxic compounds with an adverse effect on the health of consumers (Ahmed et al., 2016). We propose that the antioxidant capacity of TTEO and HPEO demonstrated in our study could have a positive effect on bread shelf life

which could be attributed to their hydrogen donating ability consequently reducing the lipid oxidation in bread. However, to confirm the assumption, further analyses are needed.

Moisture content and water activity of bread loaves

Generally, microscopic fungi can grow in conditions related to sufficient moisture and nutrition. Wheat bread is classified as intermediate moisture food (IMF). The product usually contains 20 – 50% of MC (Vermeulen et al., 2015). In agreement with the findings, our bread as a substrate for the growth of *Penicillium* species had MC 43.01 ± 0.341 .

Water in food is a very important factor in the quality and safety of food products (Al-Muhtaseb, McMinn and Magee, 2002). It is commonly observed that foods showing rapid degeneration due to chemical and biological changes are typically those with high MC (Abdullah, Nawawi and Othman, 2000). Moreover, it has been recognized that the fungal growth of products is also significantly affected by a_w (Davey, 1989). Water activity in food is a term that expresses the availability of water to participate in physical, chemical, and microbial reactions (Al-Muhtaseb, McMinn and Magee, 2002). According to Labuza et al. (1972), a_w above 0.7 is known to provide suitable conditions for fungal growth. Since an approximate value for a_w in wheat bread is within the range of 0.94 to 0.97 (Roos, Jouppila and Söderholm, 1999), which is in line with our finding ($a_w 0.947 \pm 0.006$), the bread is susceptible to microbial spoilage with the main effect coming from the growth of various molds. Therefore, the shelf life of white bread is relatively short. Considering these facts, bread was chosen as a substrate for fungal growth in our experiment.

In situ antifungal vapor contact assay

Essential oils and closely related components are well-known for their antimicrobial properties (Chouhan, Sharma and Guleria, 2017; Wińska et al., 2019). As antimicrobial systems, EOs in the vapor phase be effective for many applications including the food industry (Laird and Phillips, 2012). In our study, the antifungal activities of TTEO and HPEO against 3 *Penicillium* species artificially inoculated on bread were evaluated (Figure 1). The activities are presented as MGI in Table 2.

Our results revealed that the growing colony of *P. citrinum* was weakly reduced by all used concentrations of HPEO, and only slightly inhibited by high levels ($\geq 250 \mu\text{L.L}^{-1}$) of TTEO. The highest concentrations of both EOs are the only weak inhibitors of *P. crustosum* growth. On the other hand, the mycelial growth of *P. expansum* was stimulated by all concentrations of both EOs. In previous studies, we evaluated the antifungal effect of Coriander essential oil and Citrus aurantium essential oil against the same species of fungi (*P. citrinum*, *P. expansum*, *P. crustosum*). In contrast to current results, these essential oils have been found to have a strong inhibitory effect on fungal growth. These differences may be due to other types of essential oils used, which have different chemical compositions (Kačániová et al., 2020a, Kačániová et al., 2020b).

Table 1 Antioxidant activity of chosen EOs used in experimental design.

| EOs | Antioxidant activity (%) |
|------|--------------------------|
| TTEO | 64.94 ± 7.34 |
| HPEO | 70.36 ± 1.57 |

Note: The values are expressed mean ± standard deviation. Values in the same column are not significantly different.

Table 2 Percentage of mycelial growth inhibition (MGI) of *Penicillium* strains expressing the antifungal potential of investigated EOs against the artificially inoculated fungi on bread.

| Fungi strains | MGI (%) | | | | | |
|---------------------|----------------------------|-------|-------|----------------------------|--------|--------|
| | TTEO (µL.L ⁻¹) | | | HPEO (µL.L ⁻¹) | | |
| | 125 | 250 | 500 | 125 | 250 | 500 |
| <i>P. crustosum</i> | -6.72 | -6.11 | 3.05 | -28.11 | 0.00 | 4.68 |
| <i>P. citrinum</i> | -11.36 | 8.61 | 11.36 | 16.87 | 22.38 | 23.24 |
| <i>P. expansum</i> | -9.72 | -9.29 | -6.70 | -36.29 | -33.26 | -32.40 |

Note: The negative values indicate a profungal activity against *Penicillium* strains.

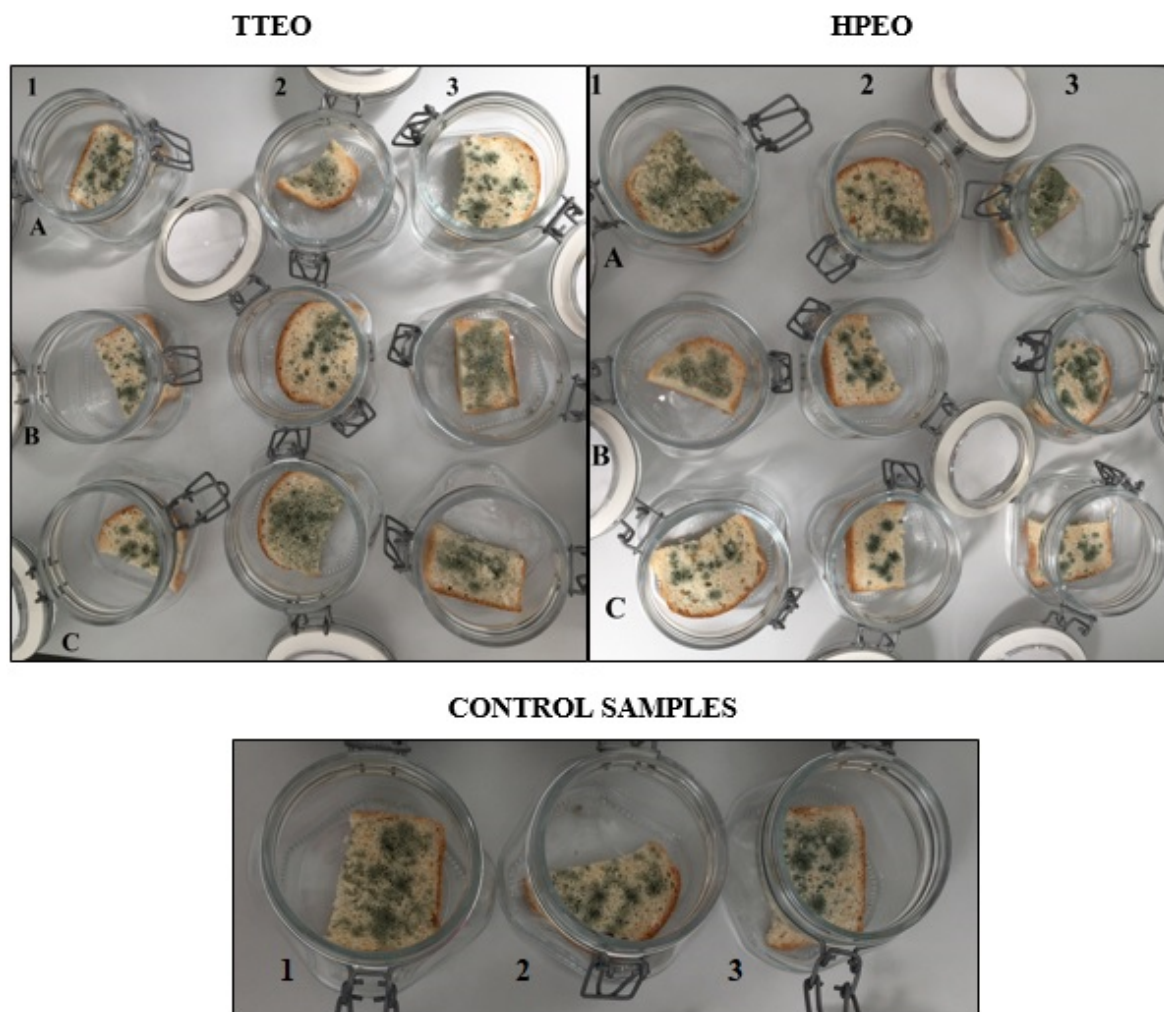


Figure 1 *In situ* antifungal analyses of bread inoculated with *Penicillium* spp. (1 – *P. citrinum*; 2 – *P. expansum* and 3 – *P. crustosum*) after their treatment with different TTEO and HPEO concentrations (A – 125 µL.L⁻¹; B – 250 µL.L⁻¹ and C – 500 µL.L⁻¹).

Generally, EOs exert various mechanisms to inhibit the growth of pathogens. For instance, due to the hydrophobicity of their compounds (such as terpenoid derivatives), they can penetrate the lipid bilayer membrane of the cells leading to higher cell permeability and leakage of vital cell contents (Burt, 2004). As a result, disruption of cellular functions such as inhibition of ionic transport and cellular respiration is observed in treated microorganisms (Cox et al., 2001). Also, their entrance to the cell membrane may be associated with swelling and reducing membrane function inducing cell death (Patel et al., 2005). The study by Cox et al. (2000) has demonstrated the ability of TTEO to disrupt the permeability barrier of microbial membrane structures as a mechanism of its action. The main compound responsible for the antifungal activity of TTEO was shown to be terpinen-4-ol (Yu et al., 2015). In contrast to our study, the antifungal activity of *Melaleuca alternifolia* extract was displayed against *P. chrysogenum* (Rogawansamy et al., 2015), *P. italicum* Wehmer, *P. digitatum* Sacc. (Zhang et al., 2018), *P. verrucosum* and *P. funiculosum* (Chidi, Bouhoudan and Khaddor, 2020). Taking into account all the aspects we propose that the slight degree of antifungal activity of TTEO against *P. citrinum* and *P. crustosum* investigated in our study could be connected with the weak capacity of the EO compounds to penetrate the cellular membranes and to inhibit the growth of the fungi. Moreover, the findings by Li et al. (2017) showed dose-dependent strong inhibition of mycelial growth of *P. expansum* (inoculated on cherry fruits) by TTEO which strongly contradicts our study. However, this discrepancy with our results can be linked to different chemical composition, used TTEO concentrations, as well as food substrates.

Against *P. citrinum*, higher antifungal activity of HPEO than TTEO (at all used levels) was found in our research. *Hypericum perforatum* is an antifungal inhibitor that was also demonstrated against *P. canescens* (Milosevic, Solujic and Sukdolak, 2007) and *P. funiculosum* (Rančić et al., 2005). Besides, the antifungal activity of *Hypericum* species was reported also against *P. verrucosum* (Grafakou et al., 2020). The antifungal potential of EOs from many *Hypericum* species is attributed to their dominant components, such as α - and β -pinene, as well as β -caryophyllene (Crockett, 2010). The compounds could also contribute to slightly mycelial growth inhibition of *P. citrinum* in our experiment.

CONCLUSION

Our study suggests that although EOs from Tea tree and St. John's wort exhibit strong antioxidant activity, they are in vapor phase-only slight inhibitors of *P. citrinum* growth in *in situ* conditions. Against other tested fungal strains (*P. crustosum* and *P. expansum*), their very low or even no growth inhibitory potentials were reported. Thus, our results allow for the conclusion that TTEO and HPEO in investigated concentrations are not suitable alternatives to the chemical inhibitors of fungal growth on bread substrate.

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