Rosalina, Niaouli and Fir Essential Oils: Strong Antifungal but Weak Antioxidant Activity

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Abstract
Antioxidant and antifungal activities of three essential oils (EOs): *Melaleuca ericifolia* Smith (rosalina; REO), *Melaleuca quinquenervia* (niaouli; NEO) and *Abies alba* (fir; FEO) were determined. The antioxidant capacity was investigated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the disc diffusion method was applied to evaluate their antifungal efficacy (in four concentrations 62.5 µL/L, 125 µL/L, 250 µL/L, 500 µL/L used) against *Penicillium (P.) expansum* microscopic filamentous fungus isolated from bread samples. From the findings it can be clearly evident that antioxidant activities of the EOs were very weak with values ranging from 25.81 ± 7.8 TEAC (6.2 ± 1.4%; FEO) to 162.0 ± 2.1 TEAC (15.9 ± 0.4%; NEO). Regarding antifungal properties, our results revealed that the effects of the EOs on *P. expansum* growth inhibition were dose-dependent, and they were proportionally increased with increasing EOs concentrations. Detected inhibition zones ranged from 0.00 ± 0.00 mm (for all EOs at 62.5 µL/L) to 11.67 ± 1.15 mm (for REO at 500 µL/L). In conclusion, all analyzed EOs possess promising *in vitro* antifungal activity (despite their weak antioxidant capacity) suggesting their use as a promising natural preservative in the food industry.

Keywords: *Melaleuca ericifolia* Smith, *Melaleuca quinquenervia*, *Abies alba*, DPPH assay, disc diffusion method, *Penicillium expansum*

1. Introduction

The ecologically, physiologically, and morphologically very diverse kingdom of Fungi forms one of the largest groups of organisms on Earth, with almost 3 million species [1]. Among the different types, microscopic filamentous fungi have a significant effect on human activities, both in a negative and positive sense. Indeed, they can be used in the production of fermented products, as important producers of enzymes, organic acids, and antibiotics [2], but they can also contaminate various types of food products [3] including cereals, meat, milk, fruits, vegetables, nuts, fats, and fat products [4]. The growth of microscopic filamentous fungi on food products can lead to a plethora of product quality modifications, such as unpleasant taste, acidification, decolorization, and even the product disintegration [5]. *Penicillium* and *Aspergillus* spp. are the most common food spoilage fungi, while *Fusarium* spp. are mostly responsible for significant losses in the yield of small grain cereals and maize [5]. Commonly, many of them are potential producers of mycotoxins (i.e., patulin, and citrinin) having an adverse effect on human and animal health [6]. In

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view of these findings, the identification and early elimination of microscopic filamentous fungi on food products is a pivotal instrument in ensuring food safety and quality [4]. An important challenge for the scientific community is, therefore, to find a suitable way to preserve food products without the use of chemical preservatives, ensuring their shelf-life, quality and safety, as well [3, 7]. In this direction, compounds derived from plants, such as essential oils (EOs), appear to play a fundamental role [8].

Essential oils are characterized as secondary metabolites produced by the synthesis of medicinal and aromatic plants [9]. These substances correspond to a very small amount of plant total structure (less than 5% of their dry matter) [10]. Regarding qualitative properties at ambient temperature, EOs are volatile, mainly liquid, and colorless [11]. From a chemical profile point of view, they are a rich mixture of numerous biologically active substances, such as terpenoids, terpenes, and phenolics [12]. Moreover, EOs have been reported to exhibit a wide range of biological activities including antibacterial, antiviral, insecticidal, and antioxidant ones [13]. Besides them, they have a great potential to reduce spoilage caused by microscopic filamentous fungi [9]. For extraction of EOs, various methods can be applied, i.e., steam distillation, solvent extraction, pressure expression under, supercritical fluid, and subcritical water extractions [13]. In about a total of 3000 EOs identified, only 300 were found to be also commercially used, and a small spectrum of them has been used to manage fungi [9].

Plants of the genus *Melaleuca* (*M.*; Myrtaceae) have long been employed in traditional medicines in many regions of the world [14]. Generally, EOs obtained from *M. alternifolia*, as well as the other species of the genus, have been extensively investigated for their broad-spectrum antimicrobial activity. Nonetheless, EOs obtained from *M. ericifolia* (rosalina; REO) and *M. quinquenervia* (niaouli; NEO) belong to the less known species [15], which have not yet been thoroughly examined in this view. Also, the EO from fir (*Abies alba*, Pinaceae; FEO) is attractive for its refreshing pine-forest aroma. In addition, FEO is known to help the respiratory system, and has an easing and soothing effect on muscles [16]. The proven strong antimicrobial properties of this EO indicate its remarkable phytomedicine potency [17].

In the current study, *P. expansum* (naturally grown on white wheat bread) was isolated and identified, and antioxidant and antifungal properties of three commercial EOs (REO, NEO, and FEO) were tested. Based on the inhibition zones detected by the agar disc diffusion method, the efficacies of EOs as antifungal preservatives were determined in order to assess their practical application as safe and novel preservatives in the food industry.

2. Materials and methods

2.1. Analyzed set of EOs

For all analyses, three commercially available (Hanus Ltd., Nitra, Slovakia) EOs: rosalina EO (REO; *Melaleuca ericifolia* Smith.), niaouli EO (NEO; *Melaleuca quinquenervia*), and fir EO (FEO; *Abies alba*) extracted by steam distillation of fresh needles and leaves were used. The primary volatile substances in the EOs declared by manufacturer were linalool (42.0%; REO), 1,8-cineole (45.0 - 65.0%; NEO), and bornyl acetate (4.0 - 11.0%; FEO).

2.2. Tested fungal strain

Penicillium expansum was applied to carry out the experiment. The strain was firstly isolated from wheat bread, and subsequently it was identified using the macro- and micro-morphological characteristics based on mycological keys [5, 18 19].

2.3. Antioxidant capacity assay

To measure the antioxidant activity (AA) of the EOs, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was applied, as previously described by Valková et al. [20]. 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 EOs samples (TEAC).

2.4. Disc diffusion assay

The antifungal activity of the EOs was evaluated using the agar disc diffusion method according to Valková et al. [20]. To achieve this goal, an aliquot of 0.1 mL of fungal suspension in distilled water was inoculated on Sabouraud dextrose agar (SDA; Oxoid, Basingstoke, UK). Then, the discs of filter paper (6 mm) were impregnated with 10 μL of the EOs samples (in four concentrations: 62.5, 125, 250 and 500 μL/L used), and applied on the SDA surfaces. The fungus was incubated aerobically at 25 °C for 24 h and 5 days. The inhibition zone diameters (in mm) were measured immediately after incubation, and the power of the antifungal activity was expressed as follows: weak antifungal activity (0 - 5 mm) < moderate antifungal activity (5 - 10 mm) < strong antifungal activity (10 - 15 mm).

2.5. Statistical treatment of data

A minimum of three measurements was conducted in the study. One-way analysis of variance (ANOVA) followed by Tukey's test at $P < 0.05$ were performed using Prism 8.0.1 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Antioxidant activity of the EOs

The results from the determination of antioxidant capacity are shown in Table 1. They revealed that all three EOs had low values for AA ($P < 0.05$) ranging from 25.81 ± 7.8 TEAC (FEO) to 162.0 ± 2.1 TEAC (NEO) indicating their weak antioxidant capacity. Considering radical inhibition units, these values were estimated to be 6.2 ± 1.4% in FEO, 15.9 ± 0.4% in NEO, and 17.2 ± 1.4% in REO, with no significant differences between REO and NEO.

The AA of diverse plant EOs has been widely demonstrated, although the mechanism of their action has not been fully elucidated [21]. Several explanations of their machineries have been provided including the sequestration of free radicals, hydrogen donation, metallic ion chelation, or action as a substrate for radicals [22]. In general, the AA of EOs depends not only on their structural characteristics but also on many other factors such as concentration, temperature, light, type of substrate, physical state of the system, as well as on microcomponents acting as prooxidants or synergists [23].

We propose that the weak AA of our EOs may be related to the terpene primary substances creating their conception, i.e., linalool in REO, 1,8-cineole in NEO, and bornyl acetate in FEO which do not have the ability to donate hydrogen atoms and also possess low solubility in the assay medium [24]. These factors may be the pivotal limitation of DPPH radical scavenging activity determination of samples with lipophilic characters including different EOs [25]. Due to the above-mentioned facts, the AA of samples also depends on the type of tests realized [26]. Therefore, the implementation of further methods for AA evaluation (FOMO method), as well as determination of their chemical profile using chromatographic technique will be our next steps.

3.2. *In vitro* antifungal activity of the EOs

The inhibitory effects of the analyzed EOs on the growth of *P. expansum* are demonstrated in Table 2. From it can be seen that the antifungal activity of all EOs significantly ($P < 0.05$) increased with their increasing concentration. In the highest concentration (500 μL/L), REO exhibited the remarkable strongest antifungal activity (with inhibition zone of 11.67 ± 1.15 mm) against the strain among the EOs investigated. Moreover, moderate antifungal efficacy was detected for all EO samples in the concentration of 250 μL/L, and also for 125 μL/L of REO. In the case of 125 μL/L of NEO and FEO, only low values for antifungal efficacy (suggesting their weak activity) were recorded. In the lowest concentration (62.5 μL/L) used, any zones of inhibition (00.00 ± 0.00 mm) were not induced by all EO samples.

Essential oils have long been recognized for their antifungal properties. The results of various experiments confirmed their ability to inhibit the growth of many fungi species [27]. To test this potency of our REO, NEO, and FEO, the *P. expansum* was selected to perform our experiment. In fact, this strain causes blue mold which is the ultimate economically postharvest disease of vegetables and fruit during their storage. Moreover, the fungus produces mycotoxin patulin which is toxic to human health [28]. However, comparison of our results with the data obtained from other research is difficult due to discrepancies in the plant EO chemical profile, methodologies used for

antifungal activity determination, as well as the analyzed microorganisms [27].

Anyway, all EOs analyzed in our study displayed inhibitory effects (in concentrations of ≧ 125 µL/L) on the growth of *P. expansum*; however, the antifungal activities were not equally strong. So, it can be evident that the mycelial growth of *P. expansum* responded differently to the analyzed EOs indicating different modes of the EOs actions. Although several studies have been conducted to understand the mechanism of EOs action, it has still not been fully clarified [29]. One possible explanation is based on the capacity of EOs substances to penetrate the cell of microorganisms, thereby interfering with their cellular metabolism [30].

Finally, our findings allow for the conclusion that REO, NEO, and FEO appear to be promising substances with a proven *in vitro* antifungal effect on the mycelial growth of *P. expansum*. In order to implement them in food practice, it will be necessary to carry out further studies of their antifungal effectiveness on food models, which we plan to realize in the future.

Table 1. Antioxidant activities of analyzed essential oils.

	REO[2]	NEO[3]	FEO[4]
AA[1] (%)	17.2 ± 1.4 [a]	15.9 ± 0.4 [a]	6.2 ± 1.4 [b]
AA (TEAC)	89.3 ± 8.2 [a]	162.0 ± 2.1[b]	25.81 ± 7.8 [c]

Note: Mean ± standard deviation. Values followed by different superscripts within the same row are considerably different ($P < 0.05$).
[1]AA = antioxidant activity.
[2]REO = Rosalina essential oil.
[3]NEO = Niaouli essential oil.
[4]FEO = Fir essential oil.

Table 2. Inhibition zones (mm) of *Penicillium expansum* after exposure to different concentrations of analyzed essential oils.

Concentrations (µL/L)	REO[1]	NEO[2]	FEO[3]
62.5	0.00 ± 0.00 [aA]	0.00 ± 0.00 [aA]	0.00 ± 0.00 [aA]
125	6.33 ± 0.58 [bA]	4.67 ± 0.58 [bB]	3.33 ± 0.58 [bC]
250	9.00 ± 1.00 [cA]	6.67 ± 1.15 [cB]	5.33 ± 0.58 [cB]
500	11.67 ± 1.15 [dA]	9.67 ± 0.58 [dB]	8.67 ± 0.58 [dB]

Note: Mean ± standard deviation. Values in the same column with different small letters, and those in the same row with different upper-case letters are significantly different ($P < 0.05$).
[1]REO = Rosalina essential oil.
[2]NEO = Niaouli essential oil.
[3]FEO = Fir essential oil.
0.00 = Total growth.

4. Conclusions

The present study evaluated the antioxidant potential and *in vitro* antifungal activity of REO, NEO and FEO (in 62.5, 125, 250, and 500 µL/L concentrations) against *P. expansum*. Despite the finding that all tested EOs possessed weak AA, their antifungal effectiveness (in the highest concentrations) was found to be strong. Our results suggest that analyzed EOs have promising potential as innovative antifungal agents for the food industry.

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