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INHIBITORY EFFECTS OF ESSENTIAL OILS WITH POTENTIAL TO BE USED IN FOOD INDUSTRY

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Abstract

Lately, essential oils are trying to be used in food industry because of their biological activity. Essential oils (EOs) are natural compounds with a complex composition which have the potential to be used as antimicrobial agents in food system due to the fact that contain active principles present in plants, as many research have been already demonstrated.
The objective of this study is to show that EOs have the potential to be used in food system like antifungal agents by inhibiting the growth of pathogenic moulds.
The method used to evaluate the in vitro inhibition effect of essential oils is Agar diffusion disk method. A known amount of limonene, nonane, pinene and nerol was applied on 6 mm diameter disks and placed onto the agar surface where an inoculum with filamentous fungi from the genus Aspergillus was spread. After this, the plates were incubated for 9 days at 25^{0}C under aerobic conditions. By evaluating and measuring the colony diameter of the fungus colonised daily and comparing it with the diameter of the control it could be told that nerol and pinene have antifungal activity.
Essential oils can be used in consumer goods as a natural alternative to conventional food preservatives because it can prevent the spoilage of the products and can increase the shelf life, this way ensuring the microbiological safety of food products.

Keywords*: antimicrobial, essential oils, food processing, microorganism inactivation*

INTRODUCTION

The contamination of spoilage microorganisms during storage is a major problem for the food industry and consumers. Fungi, especially some *Aspergillus* species, are responsable for spoilage and poisoning of food, having the ability to grow in various climates (Garcia et al., 2011). It is established that the microorganisms from the *Aspergillus* genus represent very serious risks for the health of consumer because they produce dangerous mycotoxines, which are a chemical risk in food products. *Aspegillus niger* and *Aspergillus ochraceus* are known to produce ochratoxine (OTA). The mycotoxin OTA derives its name from *Aspergillus ochraceus*, the first mould from which it was isolated. It is the main toxic component in cultures of this mould, but it is also produced by other ubiquitous moulds such as various strains of *Aspergillus* and *Penicillium* (Meca and Ritieni, 2009). *Aspergillus flavus* is known to produce aflatoxins which are a group of common, extremely hazardous, and carcinogenic metabolites (Passone et al., 2012).

Curently, the demand of consumers to reduce or eliminate these food-related microorganisms during the shelf life of food products is growing. This fact has led to the research and development of alternative treatments. There is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes, as well residual toxicity. For these reasons, there is an increasing interest in the use of natural compounds, antimicrobial agents from herbs and plants. Antimicrobial properties of herbs and spices have been recognized and used since ancient times for food preservation and in medicine (Omidbeygi et al., 2007). EOs may be an alternative to common chemical control agents because they constitute a rich source of bioactive compounds (Burt, 2004) which can reduce the environmental risk, increase the shelf life and safety of food products and satisfy the consumer's request.
According to the 8th Edition of the French Pharmacopeia (1965), EOs are products of complex general composition that contain volatile principles present in plants, more or less modified during their preparation.

The composition of EOs includes a complex mixture of several compounds. In general, most chemical components of essential oils are terpenoids, including monterpenes, sesquiterpenes, and their oxygenated derivates, all characterized by low molecular weight. Terpenes are among the most active antimicrobial compounds of essential oils (Bakkali et al., 2008, Tian et al., 2012).
Volatile oils contain two or three major components at high concentrations (20-70%) compared to the other components which are found in trace amounts. Mono- and sesquiterpenoids are the major components of essential oils, which are known as phenolic compounds in nature. Aromatic compounds occur with less frequency than the terpenes, but the antimicrobial effect of essential oils depends on the content of phenolic components (Cakir et al., 2004).
Several authors have demonstrated the antifungal activity of plant extracts and their ability to inhibit mycotoxin production (Rasooli et al., 2006, Tzortzakis et al., 2007, Tian et al., 2011, Phillips et al., 2012, Tian et al., 2012, Ferreira et al., 2013). In addition, they have attempted to elucidate the effect of bioactive chemicals on growth and morphological features and on primary and secondary fungal metabolism (Ferreira et al., 2013).
The present study aimed to determine the antifungal efficacy of 4 essential oils (nonane, pinene, nerol and limonene) against three common food spoilage-related fungi from the genus *Aspergillus* (*A. ochraceus*, *A. niger*, *A. flavus*) with emphasis for the future use of the essential oils as alternative antimould compounds.

MATERIALS AND METHODS

1. Essential oils
The commercial EOs used in this study were purchased from MERCK and Sigma-Aldrich. S-(-)-Limonene for synthesis, n-Nonane, 1 S-(-) -α-Pinene is from Merck. Nerol 97% and Malt Extract Broth is from Sigma Aldrich Fluka.

2. Microbial strains and growth conditions
Three food spoilage-related microorganisms were used to assess the antifungal properties of essential oils used in this study. All strains of *Aspergillus* (aflatoxin producer *Aspergillus ochraceus* mi 152, *Aspergillus flavus* 3-88 and *Aspergillus niger* 3-200) were provided from the collection of Faculty of Biotechnology, University of Agronomic Sciences and Veterinary Medicine Bucharest and maintained on Malt Extract Broth at 25^0C for 15 days. Spore suspensions were prepared and diluted in sterile water to a concentration of approximately 10^6 spores/ml. Spore population was counted using a haemocytometer.

3. Agar disk diffusion assay
The EOs were screened for antimicrobial activity using the agar disk diffusion method and 3 target microorganims. Malt Extract Broth was sterilized in an autoclave and cooled to 45-50^0C before being poured into 90 mm Petri dishes. After solidifying, under aseptic conditions, in the middle of each Petri dish was seeded 2 µl inoculum suspension of each microorganism. Sterile filter diskes Whatman (6 mm diameters) containing 0.5/1/2/5/7,5/10µl of pinene, nonane and limonene, respectively 0.25/0.5/1/2 µl of nerol were applied to the surface of agar plates. Control plates (without essential oils) were inoculated using the same procedure. All inoculated plates were incubated at 25^0C for 9 days. Fungal growth was visually appreciated by measuring the colony diameter of the fungus daily based. The diameter of the growing fungal colonies was measured with a rule, in two directions at right angles to each other to obtain the mean diameter for each colony. The values were expressed in mm diameter/day. The growth of fungal cultures containing different concentrations of all EOs was compared with that of the control culture that was grown with no EOs. Samples were examine in duplicate.

4. Statistical analysis
Data were analyzed statistically using analysis of variance (ANOVA) and differences among the means were determined for significance at $P<0.05$ by SPSS software. All experiments were repeated twice.

RESULTS AND DISCUSSIONS

The activity of the four essential oils used in this study was evaluated against the strains *A.*

ochraceus, *A. niger* and *A. flavus*. The best result were obtained for nerol and pinene.

Mycelia growth of the three fungi species treated with nerol during the nine days incubation at 25^0C are shown in figure 1. The results indicated that mycelia growth was significantly ($P<0.05$) influenced by incubation time and essential oil concentration. Mycelia growth was considerably reduced with increasing concentration of essential oil while their growth increased with incubation time. It is evident that nerol exhibited both fungistatic and fungicidal activities on the test molds, depending on the concentration used. From figure 1 it can be noticed that nerol completely inhibited the growth of all the food spoilage fungi tested when it was used in a higher concentration (1 and 2 µl). In the case of *A. flavus*, nerol exhibited fungicidal activity starting with the concentration of 0.5 µl. The mycelia growth was retarded by 4 days for *A. flavus* when it was used the smallest concentration of nerol (0.25 µl nerol). The mycelia growth inhibition percentage was determine at day 9. When nerol oil was used in a concentration of 0.25 µl it significantly reduced mycelium growth of *A. ochraceus, A. niger* and *A. flavus* (61.82%, 19.43%, and 63.14% reduction respectively). When used in a higher concentration at 0.5 µl, the percentage reduction was 73.96% for *A. ochraceus*, 44.53% for *A. niger*, respectively 100% for *A. flavus*. The fungal inhibition observed when nerol was used may be caused by the hydroxyl group present in this compound that can form hydrogen bonds with active enzymes resulting the deactivation (Tian et al., 2011).

In the case of nonane and limonene, these essential oils didn't inhibit the mycelial growth of the tested microorganisms. Limonene is a terpene that can be found in citrus oil. It has been reported that his antifungal activity is weakly inhibitory against *A. niger* (Moleyar et al., 1986). Caccioni (1998) showed in his study that the antimicrobial effect of citrus oil was produced by the synergy between monoterpenes other than limonene and sesquiterpene content. In a study made by Matan N. and Matan N. (2008) it was shown that lime oil and tangerine oil, which contain limonene as their main constituents can inhibit the growth of *A. niger* but the concentration needed is higher than that used to inhibit *Penicillium sp.*

In a study realized by Phillips et al. (2012) it was tested the effect of the citrus EO vapour on mycelial growth and spore germination of the *A. niger* in culture and also determined the growth of this mould on grain. The citrus EO vapour under test (Citri-V™®) contains limonene which is being present in the highest quantities and linalool, citral and β-pinene also present and the later three components having been previously identified as the active antimicrobial compounds. A 15 min treatment with the citrus EO vapour used in this study reduced growth of *A. niger* in culture by 66.9%, although the citrus EO vapour was an effective treatment to reduce the growth of *A. niger* by 50% on grain over 10 days, suggesting its possible use in reducing spoilage in grain by this specie, especially as this treatment has previously been shown not to affect the organoleptic properties of raw vegetables. Similar results were obtained by M. Viuda-Martos et al. (2008) who showed that essential oils of lemon, mandarin, grapefruit and orange inhibited completely the growth of *A. niger* when a concentration of 0.94% of any of these EOs was used. Orange EO is the most effective against *A. niger*, while mandarin is the best inhibitor of *A. flavus*.

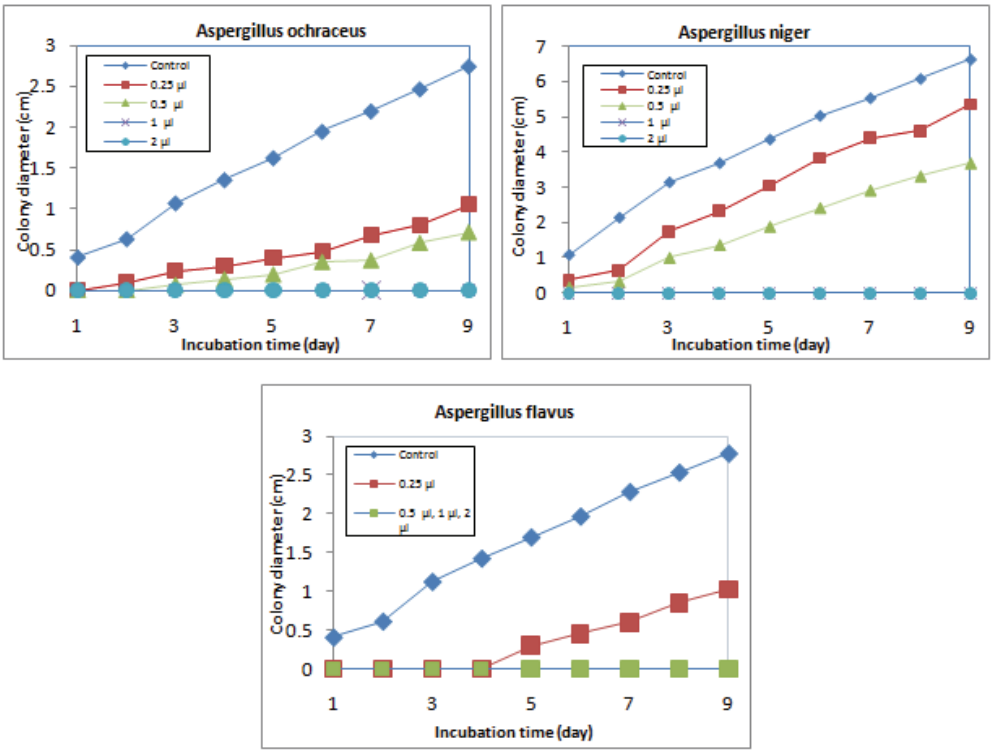

Figure 1. Effect of different concentrations of nerol on colony growth of *A. ochraceus*, *A. niger* and *A. flavus* raised in MEA. Plates were incubated at a temperature of 25^{0}C for 9 days.

When pinene was used against the tested moulds, it was noticed that this monoterpene has antifungal activity only against *A. flavus*. From figure 2 it can be seen that when the concentration of pinene was higher (7.5 and 10 µl), this monoterpene had fungicidal activity. The antifungal activity of pinene was in direct ratio to the amount of pinene added to the tested samples. The mycelia growth of *A. flavus* was reduced with a percentage reduction of 17.30% for 2 µl pinene, respectively 26.28% for 5 µl. Similar results were obtained by Lopez-Malo et al. (2007) who showed that cinnamon extracts have fungistatic effect when the concentration was smaller and fungicidal effect at higher concentrations. Colony diameter decreases with the increasing antimicrobial concentration.

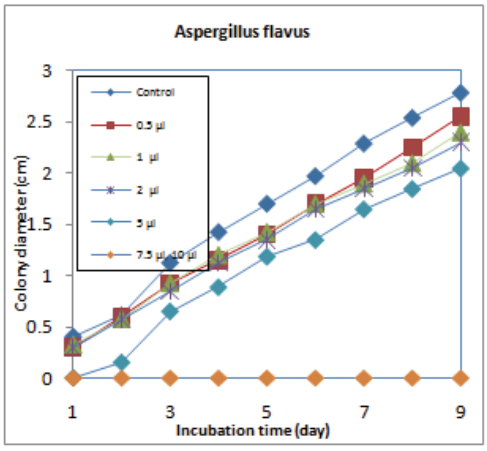

Figure 2. Effect of different concentration of pinene on colony growth of *A. flavus* raised in MEA. Plates were incubated at a temperature of 25^{0}C for 9 days.

Several authors study the mechanism of inhibition of *Aspegillus* growth at ultra-structural level. Rassoli et al. (2006) showed with a transmission electron microscopy (TEM) that *A. niger* exposed to MIC levels of *Thymus eriocalyx* and *Thymus x-porlock* presented irreversible damage to cell wall, cell membrane and cellular organelles. The mycelium exposed to the thyme oils showed morphological changes in the hyphae, plasma membrane

disruption and mitochondrial destruction. In accordance with this study, Tolouee (2010) reported the same result when it was used *Matricaria Chamomilla* L. flower essential oil against *A. niger.* When it was used a scanning electron microscopy, the major changing observed were swelling and deformation of hyphal tips, formation of short branches, and collapse of entire hyphae. A change in cell permeability might result in an imbalance in intracellular osmotic pressure, subsequent disruption of intracellular organelles, leakage of cytoplasmic contents and finally cell death.

CONCLUSIONS

In the present study, nerol and pinene were reported as potential inhibitors of the in vitro *Aspergillus* tested strains.

The results of this study showed that only nerol and pinene have antifungal activity, the highest effect on the growth of the three tested species of *Aspergillus* being demonstrated by nerol. Nerol exhibited 100% inhibition of the growth of *A. ochraceus*, *A. niger* and *A. flavus* when it was used in a higher concentration (1, 2 µl). It can be concluded that nerol can inhibit ochratoxigenic and aflatoxigenic moulds. Due to the antifungal activity of nerol, it can be exploited as a suitable alternative to chemical additives for use in food industry, attending to the needs for safety and satisfying the demand of consumers for natural components.

Pinene also can be an alternative antifungal agent, our study showing that the highest action of this essential oil was against *A. flavus.*

These oils could be used as food preservatives in some food products in which *A. flavus*, *A. niger* and *A. ochraceus* growth and potential production of mycotoxines are considered healthy hazards. For the practical use of these oils as novel fungal-control agents, further research is needed on safety issues for human health. It is also necessary to establish the level of essential oil needed to inhibit the fungal growth in food matrix, knowing that in food products the amount of essential oil is higher than *in vitro*, most probably due to interactions between phenolic compounds and the food matrix.

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