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MICROATMOSPHER AND MICRODILUTION METHODS TO EVALUATE ANTIFUNGAL ACTIVITY OF SIX ESSENTIAL OILS ON *ASPERGILLUS NIGER* AND *PENICILLIUM SP* ISOLATED FROM THE UNIVERSITY OF YAOUNDE I LIBRARY

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ABSTRACT

A. niger and *Penicillium sp* are the most frequent molds in libraries. Their presence can cause allergy-like reactions as well as infections in library users. They can also attack collections and library buildings due to their cellulolytic abilities. The aim of this study was to investigate the antifungal effect of six essential oils (EOs) against fungi isolated from a university library. This antifungal activity was assessed using broth dilution, and micro-atmosphere methods. Micro-atmosphere methods were carried out in terms of minimal inhibitory concentration, minimal fungicidal concentration (MIC, MFC in µl/mL of air) and percentage of mycelia growth inhibition of the tested fungus, while broth microdilution method was used to determined MIC and MFC in mg/mL. The whole EOs samples were found to be efficient in inhibiting the mycelia growth with complete inhibition on *Penicillium sp* and *Aspergillus niger* at the MIC value of 0.5 µL/mL of air. The MICs for radial mold growth ranged from 0.125 µL/mL to 1 µL/mL and CMFs from 0.125 µL/mL to 1.25 µL/mL. In broth microdilution method, MICs ranged from 0.19 to 6.25 mg/mL for the EOs and from 0.03 mg/mL for Nystatin ® on the two fungal isolates. Overall, the EO of *Eugenia caryophylla* showed better activity with a fungicidal effect on the fungi isolates tested. All of these results allow to conclude on a potential use of EOs in library disinfection.

KEYWORDS: Essential oils, Antifungal activity, Micro atmosphere, Broth dilution.

1. INTRODUCTION

Recently, research into indoor air quality has increased because of increasing awareness of the variety of health problems potentially caused by airborne microorganisms.

Microbial pollution, one of the key constituents of indoor air pollution, comprises hundreds of bacterial and fungal species, and particularly filamentous fungi (molds), which grow indoors when sufficient moisture is available.[1]

Molds are microscopic filamentous fungi, capable of colonizing different substrates such as food products, textiles, paper and wood. They can be useful in certain industries such as the food, cosmetic or pharmaceutical industries, but they can also be harmful by altering the physical and chemical properties of the substrate that they colonize like paper. When humidity and temperature conditions are right, these molds can produce secondary metabolites.[2,3,4] Among these secondary metabolites, mycotoxins produced by *Aspergillus niger* are likely to represent a danger to human and animal health.

Different mold genera (*Alternaria* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp.), could be found around indoor habitats,[5,6] Thus, it has been shown that inhalation of fungal spores is associated with allergic symptoms manifested clinically by asthma, rhinitis, and even hypersensitivity pneumonitis.[7,8] Filamentous fungi have also been associated to unhealthy building syndrome (SBS), by some authors, which manifests itself by breathing difficulties, headache, conjunctivitis, and even nausea [9]. Mycotoxins can be transported by dust and thus be aerosolized and inhaled causing them to deposit in lungs being dangerous for human health.

Given the severity of the precarious situation, microbiological purification is imperative for public health preservation and that of buildings. Antimicrobial agents described as disinfectants are often alternatively used as sterilizing agents and hygienic products. They are mostly strong and toxic biocidal chemical compounds or antimicrobials that are applied on contaminated surfaces.[10]

However, there is increasing consumer concern regarding the safety of synthetic chemicals used to

disinfect and their side effects. Much attention is being given worldwide to minimize the use of synthetic antibacterial agents for disinfection. Therefore, there has been great interest in identifying natural and safe antibacterial compounds from various natural sources. Substitution of the usual products by essential oils (EOs) will be an interesting approach by virtue of the fact that some studies have proven that EOs exhibit good antibacterial, antifungal, insecticidal, antioxidant and anti-inflammatory properties.[11,12] At physical level, essential oils stimulate and modify our physiological states by acting directly on the central nervous system as well as a well-targeted olfactory atmosphere can bring a lot of benefits and have a lot of positive repercussions. Essential oils help to maintain a healthy atmosphere, fight against diseases and microbes, and maintain good concentration.[13]

The choice of essential oils in this scientific approach is justified not only by their low toxicity but also by their antimicrobial, deodorizing and relaxing virtues. Indeed, many of these natural products have an adequate chemical composition and have the advantage of being significantly less toxic than other synthetic disinfectants for a comparable result.[13]

Therefore, in this study, we evaluated the antifungal potential of six essential oils against two dominant fungal species, isolated from the central library of the University of Yaoundé I.

2. MATERIAL AND METHODS

2.1 Microbial strains

Aspergillus niger and *Penicillium sp* used in this study were isolated from the Central Library of the University of Yaoundé I. The isolates were identified on the basis of macroscopic and microscopic observation using Dufresne identification keys.[14,15]

2.2 Plant material

For essential oil extraction, six aromatic plants including *Citrus sinensis* (L.) Osbeck (Rutaceae), *Cymbopogon citratus* DC Stapf (Poaceae), *Eugenia caryophylla* (Spreng.) Bullock & S.G. Harrison (Myrtaceae), *Mentha* sp. cf. *piperita* L. (Lamiaceae), *Cananga odorata* Hook & Thomson (Annonaceae) and *Eucalyptus globulus* Labill (Myrtaceae) were harvested or purchased and identified at the National Herbarium of Cameroon. Essential oil extractions were carried out by hydrodistillation using a Clevenger apparatus.[16]

2.3 Antifungal assay

2.3.1. Microatmosphere method

The microatmosphere method were used to determine the Minimum Inhibitory and Fungicidal Concentrations (in air) (MIC and CMF expressed in µL / mL of air) as well as the inhibition percentages.[17]

Petri dishes of diameter 90 mm are prepared immediately by filling with 20 mL of Sabouraud medium (20 mL of Sabouraud medium provides 80 mL of air in each dish). Inoculation is carried out on the surface, in the form of deposits from the mycelial disc (6 mm) in the center of the box. 80 mm diameter filter papers are placed at the bottom of the lid of each petri dish and impregnated with different quantities of EOs : 0 (control), 10, 20, 40, 80 and 100 µL / disc (equivalent to concentrations of 0; 0.125; 0.25; 0.5; 1 and 1.25 µL / mL of air, respectively). The dishes are immediately sealed with parafilm to prevent evaporation of EO, then incubated at 25 ± 2 ° C for 5 days. For each fungal specie at each concentration, three repetitions were carried out and three boxes are used per test. Mycelial growth was followed by measuring the average of two perpendicular diameters passing through the center of each box. The fungitoxicity, expressed in terms of percentage inhibition of mycelium growth was calculated according to the following formula.[18]

$$\text{Mycelial growth inhibition (\%)} = \frac{g_c - g_i}{g_t} \times 100$$

Where,

gc = growth of mycelial colony in control set after incubation period subtracting the diameter of inoculums disc.

gt = growth of mycelial colony in treatment set after incubation period subtracting the diameter of inoculums disc.

These measurements were used to determine the minimum concentrations inhibiting the radial growth of molds (MIC expressed in µL / mL of air).

The Minimum Fungicidal Concentrations (MFC) were determined by transfer of the mycelial discs from the petri dishes, where the inhibition by EOs was complete, in a new Sabouraud medium free of EOs. EO is fungistatic if the growth of the fungus resumes again and fungicidal or lethal if there is no growth.

2.3.2. Broth dilution method: Determination of the Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration (MFC)

The MIC determination was conducted in triplicate in a liquid medium by direct exposure of fungal spores to increasing concentrations of the tested antifungal agents according to the method described by CLSI.[19] and incubated at 25° C for 6 days. The MIC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth compared to that produced by the growth control tube. In order to evaluate the MFC, fractions of 50 µl from the tubes showing no growth were aseptically transferred into new tubes containing 150 µl of sterile sabouraud broth. After an incubation period of 6 days at 25 °C, the tubes were examined. The MFC was defined as the lowest antifungal agent concentration at which there was a visual absence of growth.

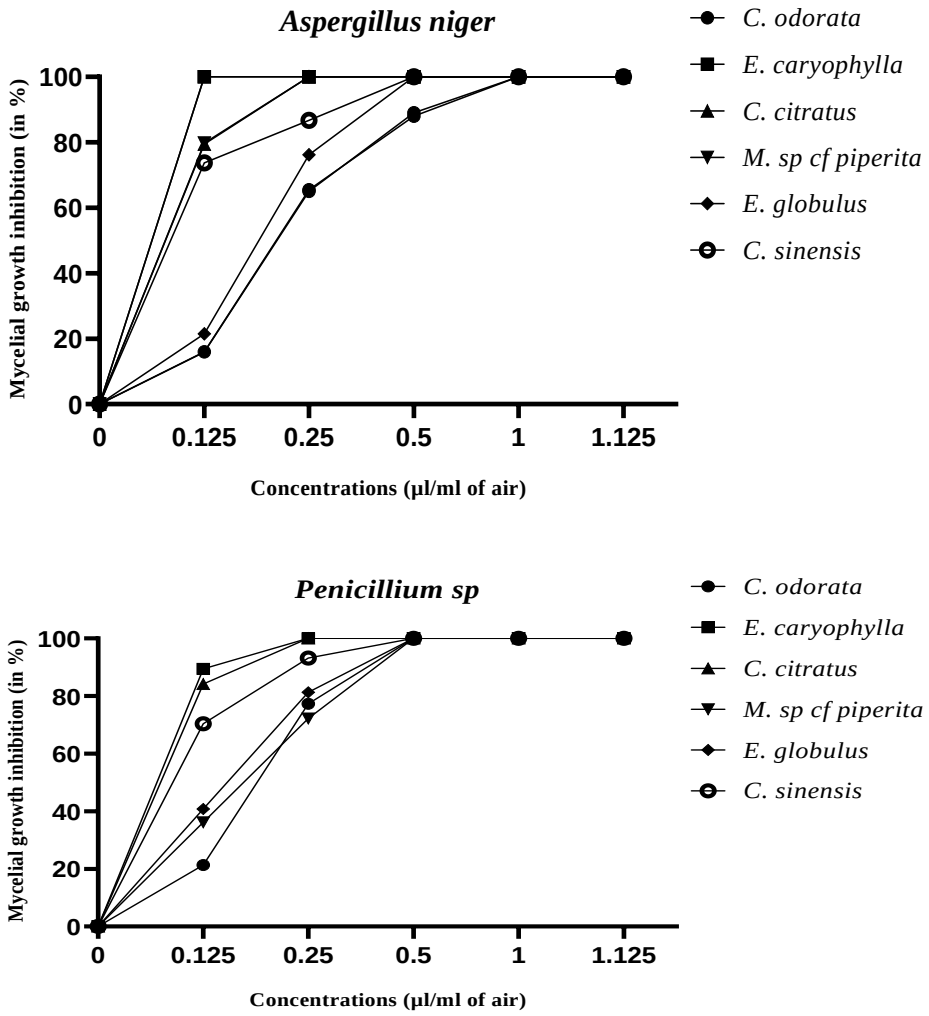
3. RESULTS

3.1. Micro atmosphere method

3.2. Percentage of mycelia growth inhibition

Figure 1 shows the Inhibition Percentages of mycelial growth of *A. niger* and *Penicillium sp* as a function of the concentration of EOs.

The results reported in FIG. 1 show that all of the EOs tested completely inhibited the mycelial growth of *Penicillium sp* and *A. niger* with an MIC equal to 0.5 µL / mL of air, apart from the EO of C. odorata which showed a percentage inhibition of 88.40 ± 0.24 on *A. niger* at this concentration.



mycelia growth inhibition of *Aspergillus niger* and *Penicillium sp*

3.3. Radial mold growth inhibition parameters by microatmospher method (MIC, MFC)

Results of minimum inhibitory concentration (MIC) and of minimum fungicidal concentration have been collated in Table I below.

The MICs for radial mold growth ranged from 0.125 µL / mL to 1 µL / mL and CMFs from 0.125 µL / mL to 1.25 µL / mL.

The nature of fungitoxicity (CMF) of essential oils has been studied by transferring the mycelial discs of the strains tested, whose growth inhibition is complete, into a new SDA medium without essential oil. The EO of *Eugenia caryophylla* showed the best activity with a fungicidal effect at a concentration of 0.125 µL / mL of air on *A. niger* and 0.25 µL / mL of air on *penicillium sp*.

Tableau I: Inhibition parameters (MIC, MFC in µL / mL of air) by micro atmosphere.

Essential oils	Isolates	MIC (in µL/mL of air)	MFC (in µL/mL of air)
Cananga odorata	*Aspergillus niger*	1	ND
	Penicillium sp.	0.5	ND
Eugenia caryophylla	*Aspergillus niger*	0.125	0.125
	Penicillium sp.	0.25	0.25
Cymbopogon citratus	*Aspergillus niger*	0.25	0.5
	Penicillium sp.	0.25	0.25
Mentha sp cf piperita	*Aspergillus niger*	0.5	1
	Penicillium sp.	0.5	1
Eucalyptus globulus	*Aspergillus niger*	0.5	1
	Penicillium sp.	0.5	ND
Citrus sinensis	*Aspergillus niger*	0.5	1.25
	Penicillium sp.	0.5	1.25

Figure 2 below illustrates the results observed when determining the inhibition parameters in micro-atmosphere of the EO of *Mentha sp cf piperita* on *A. niger* and of the EO of *Cymbopogon citratus* on *Penicillium sp.* This image shows a complete inhibition of the growth of *A. niger* at a concentration of 0.5 µL / mL of air (MIC). This concentration is 0.25 µL / mL of air on *Penicillium sp.*

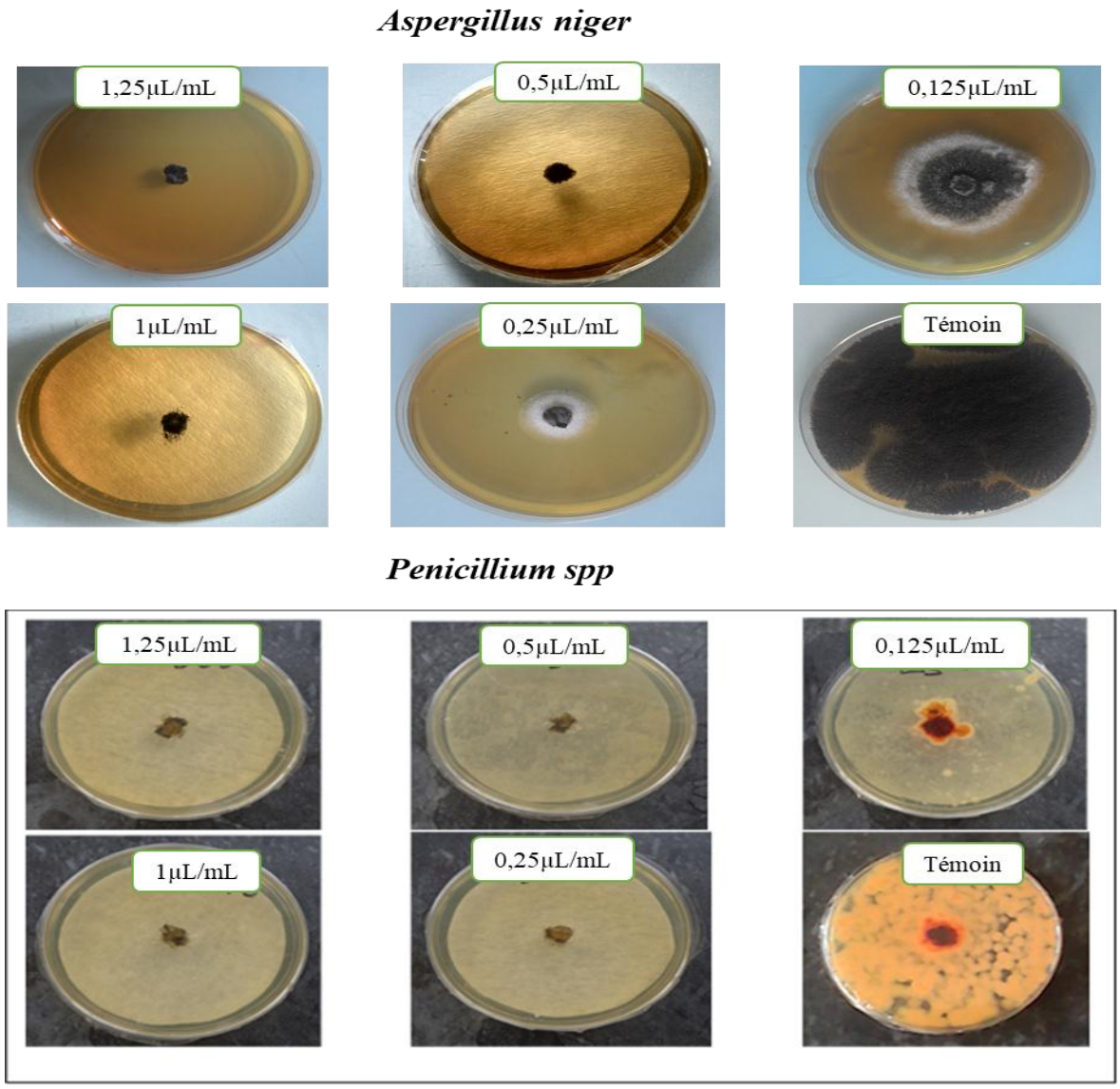


Figure 2: Photograph of inhibitory concentrations of radial growth of *Aspergillus niger* and *Penicillium sp.*

3.4. Inhibition parameters (MIC and MFC) of essential oils by microdilution

Regarding the inhibition parameters of EOs on molds, results are shown in Table II below. It appears from Table II that all the tested EOs showed activity on *A. niger* and on *Penicillium sp* with MICs ranging from 0.19 to 6.25 mg / ml for the EOs and from 0.03 mg / ml for Nystatin ® on the two fungal isolates. Overall, the EO of *E. caryophylla* showed better activity, with a fungicidal effect on the fungal isolates tested.

Tableau II: Inhibition parameters of essential oils.

Isolats microbiens	Paramètres d'inhibition (mg/mL)	Nystatine®	*Cananga odorata*	*Eugenia caryophylla*	*Cymbopogon citratus*	*Mentha sp cf piperita*	*Eucalyptus globulus*	*Citrus sinensis*
Aspergillus niger	**CMI**	0.03	6.25	0.19	0.39	3.125	1.56	0.39
	CMF	0.03	ND	0.39	0.39	6.25	12.5	1.56
	CMF/CMI	1	ND	2	1	2	8	4
Penicillium sp	**CMI**	0.03	6.25	0.19	0.19	3.125	0.78	0.39
	CMF	0.03	ND	0,39	1,56	ND	12.5	6.25
	CMF/CMI	1	ND	2	8	ND	16	16

Legend: MIC: Minimal Inhibitory Concentration; MFC : Minimal Fungicidal Concentration; ND= Not Determined

4. DISCUSSION

After carring out the tests in micro-atmosphere on Aspergillus *niger* and *Penicillium sp*, in order to show the activity of the volatile fraction of the EOs, we noted an inhibitory effect of the volatile constituents of the EOs on the growth of these microorganisms. This could be explained by the composition of these EOs rich in volatile antibacterial compounds such as monoterpenes which constitutes more than 50% of their chemical composition.[16] The antimicrobial activity of volatile compounds has already been shown to result from the combined effect of the direct absorption of vapor on microorganisms and the indirect effect via the vapor absorbing medium.[20] A significant contribution of volatile compounds by the absorption of agar has been reported for *E. coli*.[20]

Several studies have shown the lethal effect of volatile constituents of EOs on various microorganisms. Laghchimi in 2014 demonstrated that essential oils tested in the gaseous phase had a lethal effect on molds responsible for apple rot.[17]

The results obtained from the effect of the volatile fraction of the six essential oils used in our study on mycelial growth of *A. niger* and *Penicillium* sp report complete inhibition of radial mold growth by all EOs at MICs ranging from 0.125 to 1 µl / ml air.

Dilution tests are reliable, widely accepted and promising tests for determining an organism's sensitivity to inhibitors. The micro dilution method is considered to be the "gold standard".[21,22] It is a quantitative method which makes it possible to calculate the MIC and makes it possible to understand the power of the essential oils.[23,22] One of the most problematic characteristics of essential oils being their volatility, the micro-dilution technique helps to bypass this problem because it allows less evaporation by the mixture of the essential oil in the broth.[24]

The EOs tested in this study showed good activities in a liquid medium with MICs ranging from 0.19 to 6.25 mg / mL. Given the large number of groups of chemical compounds present in EOs, it is likely that their antifungal activity is not attributable to a specific mechanism, but that there are several targets in the cell. An important characteristic of EO and their components is their hydrophobic character, which allows them to distribute themselves in lipids of the fungal cell membrane and mitochondria, to disturb the structures and to make them more permeable. Leakage of ions and other cellular contents can then occur and cause the death of a fungal cell.[25]

The best activity observed with *E caryophylla* essential oil is attributed to its chemical composition rich in eugenol (70 %) which is a phenolic compound.

Indeed, the antimicrobial activity of the family of phenolic compounds is due to their structure (aromatic nucleus linked to the hydroxyl group in different positions). This structure allows them to form hydrogen bonds with the SH groups in the active sites of target enzymes, which leads to the deactivation of these enzymes in fungi.[26]

It is reported that the antimicrobial mechanism of cyclic hydrocarbons, such as eugenol, is related to its lipophilic character in that they increase the fluidity and permeability of the cell membrane of microorganisms. In fact, these compounds interfere with ion transport, unbalancing osmotic conditions in the membrane and making its associated proteins inefficient. In any case, this can lead to inhibition of microbial growth, and death or cell lysis.[27]

However, the value of an essential oil lies in its "totum", that is to say in all of its components and not only in its majority compounds.

5. CONCLUSION

Given the results described above, selected Eos were found to have a potential inhibitory effect on *Aspergillus* niger and *Penicillium sp.* EOs possess good antifungal activity. In addition, these EOs are highly effective in both gaseous and liquid phase and might be considered as promising candidates for the development of natural antimicrobials for the control of indoor microbial contaminants.

ACKNOWLEDGEMENTS

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