EFFECT OF *CINNAMOMUM ZEYLANICUM* BLUME ESSENTIAL OIL ON THE GROWTH AND MORPHOGENESIS OF SOME POTENTIALLY PATHOGENIC *ASPERGILLUS* SPECIES

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**ABSTRACT**

*Cinnamomum zeylanicum* Blume is known for a wide range of medicinal properties. This study aimed to assess the interference of *C. zeylanicum* essential oil on the growth and morphogenesis of some potentially pathogenic *Aspergillus* species. The essential oil presented strong antifungal effect causing the growth inhibition of the assayed strains and development of large growth inhibition zones. MIC₅₀ and MIC₉₀ values were 40 and 80 µL/mL, respectively. 80, 40 and 20 µL/mL of the oil strongly inhibited the radial mycelial growth of *A. niger*, *A. flavus* and *A. fumigatus* along 14 days. 80 and 40 µL/mL of the oil caused a 100% inhibition of the fungal spore germination. Main morphological changes observed under light microscopy provided by the essential oil in the fungal strains were decreased conidiation, leakage of cytoplasm, loss of pigmentation and disrupted cell structure indicating fungal wall degeneration. It is concluded that *C. zeylanicum* essential oil could be known as potential antifungal compound, particularly, to protect against the growth of *Aspergillus* species.

**Key words:** *Aspergillus*, *Cinnamomum zeylanicum* Blume, essential oil, antifungal activity.

**INTRODUCTION**

Aspergilli are ubiquitous fungi found in air, soil, plants and decayed organic materials. Human contamination by *Aspergillus* has been related to the inhalation of conidia, colonization of wounds and/or penetration in human tissues through surgical interventions (13,30). Aspergillosis is an opportunistic infection which can attack the lungs, ears, eyes, digestive system, kidneys and brain (12,19).

Aspergillosis generally rises as a respiratory disease characterized for the presence of granulomatous lesions in lungs or bronchi, followed by dissemination to other organs throughout the bloodstream (3,14). Cutaneous aspergillosis, aspergillar otomycosis, aspergillar onychomycosis, invasive lung aspergillosis, aspergillar sinusitis, immunoallergic aspergillosis, aspergilloma and mycotoxicosis are some clinical forms of aspergillosis (19,30).

The growth of Aspergilli in foodstuffs is toxicologically significant since some species are known to produce mycotoxins. Aflatoxins-B₁, B₂, G₁ and G₂ (produced by *A. flavus* and *A. parasiticus*), aspergillic acid (produced by *A. flavus*), hydroxyaspergillic acid (produced by *A. flavus*), ochratoxins (produced by *A. ochraceus*), oxalic acid (produced by *A. flavus* and *A. glaucus*), terreic acid (produced by *A. terreus*) are some mycotoxins produced by *Aspergillus* species (6,37). The consumption of mouldy products can cause human or animal mycotoxicoses, and more importantly some mycotoxins are potent carcinogens (23,26).

Resistance of Aspergilli to some clinically used antifungals brings a worrying clinical prognostic in people attacked by...
aspergillosis (11,14). For over fifty years antibiotics have been applied for treating or inhibiting infections. The wide use and sometimes misuse of chemo-antimicrobials in both human and animal medicine has been responsible for the selection of resistant strains (17,21).

Regarding the increasing clinical importance given to fungi causing infections and the development of drug resistance many scientific studies focusing the antifungal properties of plant products have been carried out (4,41). Antifungal effect of medicinal plants and derivatives has been scientifically proven in assays with essential oils, extracts and isolated phytochemicals (20,26,42). Essential oils as antimicrobial agents present two main characters: their natural origin generally means more safety to people and environment; and they can be considered at low risk for development of microbial resistance since they are mixtures of compounds which may present different mechanisms of antimicrobial activity (15,29).

*Cinnamomum zeylanicum* Blume, Lauraceae, has many biological properties as analgesic, antiseptic, antispasmodic, aphrodisiac, astringent, carminative, haemostatic, insecticidal and parasiticide. Barks from branches, without the epidermis and suberous layer, is marketed as the commercial cinnamon which has long use in perfumery, culinary and native medicine fields (2,20). Previous research has revealed interesting antimicrobial effect in *C. zeylanicum* essential oil (10,27,28).

Camphene, linalool, α-phellandrene, α-terpinene, limonene, β-cymene, α-carophyllene, cinnamaldheyde and eugenol are some of the compounds found in *C. zeylanicum* essential oil (24,44).

The present study aimed to evaluate the effect of *C. zeylanicum* Blume essential oil on the growth and morphogenesis of some *Aspergillus* species known as potential etiological agent of fungal infections.

**MATERIAL AND METHODS**

**Essential oil**

*C. zeylanicum* Blume essential oil was supplied by Ferquima Ind. Com. Ltda. (Vargem Grande Paulista, São Paulo, Brazil) and its quality parameters (appearance, color, purity, odor, density -20°C, refraction index -20°C) were described in an accompanying technical report. The essential oil was assayed at concentrations ranging from 320 to 5 µL/mL and the solutions were prepared according to Souza et al. (42).

**Fungal strains**

*Aspergillus fumigatus* (ATCC-16913 and ATCC-40640), *A. niger* (P-03 and LM-257), *A. flavus* (ATCC-16013 and LM-247), *A. parasiticus* (ATCC-15517 and NRRL-2999), *A. terreus* (UP-03 and ATCC-7860) and *A. ochraceus* (ATCC-7860 and LM06) strains were used as test microorganisms. These strains were obtained from the Microorganisms Collection, Laboratory of Clinical Mycology, Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. Stock cultures were maintained on Sabouraud agar (SA) slants and stored in a refrigerator (7°C, ±1°C).

**Minimum Inhibitory Concentration - MIC**

The MIC of the essential oil was determined by a qualitative method using the solid medium diffusion procedure (22). For this, 1 mL of the fungal homogenous suspension (approximately 10³ spores/mL) prepared according to Rana et al. (32) was uniformly spread on sterile SA Petri dishes. After inoculum absorption by SA, wells were made using sterile glass stems (diameter 6 mm) which were filled with 50 µL of the essential oil solution. The incubation period was 7-10 days at 25-28°C. At the end of the incubation period, the MIC was the lowest essential oil concentration showing growth inhibition zones with diameter equal to or greater than 10 mm (41,42).

Amphotericin B (100 µg/mL) and ketoconazole (50 µg/mL) were used as control using the solid medium diffusion procedure using filter paper discs (Cecon, diameter 6 mm) (7). The control of viability of the assayed fungal strains was carried out by observing their capability of growing on SA without adding the essential oil or standard antifungals.

**Mycelial growth inhibition**

Inhibition of the fungal mycelial growth was determined using the poisoned substrate technique by the daily measure of the radial mycelial growth on SA added of 80, 40 and 20 µL/mL of the essential oil (1,15). For this, a 2 mm plug taken from a 10-days-old fungal colony cultivated on SA slants was placed on the center of the sterile SA Petri dishes containing the essential oil and incubated at 25-28°C. At different time intervals (1, 2, 4, 6, 9 and 14 days) of incubation, the radial mycelial growth was measured (mm) using calipers. The control was the observation of the radial mycelial growth on SA added of ketoconazole (50 µg/mL) and without adding the essential oil.

**Spore germination assay**

An aliquot of 0.2 mL of each concentration of the essential oil (80, 40 and 20 µL/mL) was mixed with 0.2 mL of the fungal spore suspension (approximately 10⁶ spores/mL). The mixture was placed on separated glass slides which were incubated in a moisture chamber at 25-28°C for 24 hours. At the end of the incubation period, each slide was fixed with lacto-phenol-cotton blue stain and observed under the microscope for spore germination. Control without essential oil was tested in the same way. About 200 spores were counted and the per cent of spore germination was calculated in comparison with the control assay (32,38).

**Fungal morphogenesis study**

For evaluating morphological alterations caused by the essential oil in *A. niger* P-03 a sample of mycelium was taken
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from the periphery of a 10-days-old fungal colony grown on SA at 25-28°C containing the essential oil (80 µL/mL). The samples were fixed in lacto-phenol–cotton blue stain and observed under the microscope at 400 x to examine morphological abnormalities. Control assay without essential oil was tested in the same way (38).

All antifungal assays were carried out in duplicate and the results were expressed as an average of the two parallel assays.

Statistical analysis
Statistical analysis was performed to determine significant differences (P < 0.05) by the Tukey test in the mycelial radial growth assays. For this the Sigma stat 2.03 computer program was used.

RESULTS

Results of the inhibitory effect of C. zeylanicum essential oil in a solid medium on some potentially pathogenic Aspergillus species are shown in Table 1. The oil at 320 - 80 µL/mL strongly inhibited the growth of all assayed strains. 40 µL/mL was the MIC 50 (lowest concentration causing a growth inhibition of 50 percent or more of the assayed strains), while 80 µL/mL was the MIC 90 (lowest concentration causing a growth inhibition of 90 percent or more of the assayed strains) found for C. zeylanicum essential oil. The essential oil at 320 to 40 µL/mL provided growth inhibition zones with diameter equal to or higher than the ones caused by standard antifungals (amphotericim B and ketoconazole).

Fig. 1, 2 and 3 show the effect of C. zeylanicum essential oil (80, 40 and 20 µL/mL) and ketoconazole on the radial mycelial growth of A. flavus LM-247, A. fumigatus ATCC-40640 and A. niger P-03 using the poisoned substrate technique. The essential oil provided a fungicidal effect noted by total inhibition of the mycelial growth along 14 days of exposure. The oil provided significant (P < 0.05) inhibitory effect on the mycelial growth when compared with the control assay and ketoconazole. Only A. niger showed small mycelial growth up to 8 days of exposure when exposed to 20 µL/mL of the oil.

Ketoconazole showed no significant (P < 0.05) reduction in the mycelial growth after 14 days of exposure in comparison with the control assay. Ketoconazole was inserted in the radial mycelial growth assay because no tested strain showed resistance to it in the MIC assay.

Results obtained from the effect of C. zeylanicum essential oil (80, 40 and 20 µL/mL) on spore germination of A. flavus LM-247, A. fumigatus ATCC-40640 and A. niger P-03 are showed in Table 2. The oil at different concentrations caused an interesting inhibition of the spore germination. A 100% inhibition was found at 80 and 40 µL/mL of the oil, while it was over 90 and 80% at 20 µL/mL for A. flavus and A. fumigatus, respectively. A. niger presented a 25% of spore germination inhibition at 20 µL/mL.

Figure 1. Effect of C. zeylanicum essential oil and ketoconazole on the radial mycelial growth kinetic of A. flavus LM-247.

Figure 2. Effect of C. zeylanicum essential oil and ketoconazole on the radial mycelial growth kinetic of A. fumigatus ATCC-40640.
Spores which germinated when exposed to 20 µL/mL of essential oil produced smaller germ tubes (early growing hyphae) in comparison with the control assay (data not showed).

Observations of *A. niger* examined under the light microscope at 400 x magnification after exposure to 80 µL/mL of *C. zeylanicum* essential oil showed some morphological abnormalities (Fig. 4). Microscopic examination of the control mycelium (untreated cell) showed a regular cell structure with homogenous cytoplasm, clearly visible sterigmata bearing conidia and profuse conidiation on a large and radiated conidial head. The mycelial growth of *A. niger* in the medium containing the essential oil appeared to present morphological changes with a heterogeneous mycelial structure. In addition, the alterations observed included decreased conidiation (lack of sporulation), visible loss of cytoplasm content, loss of pigmentation, aberrant development of hyphae and fragmentation. The essential oil clearly caused reduction in conidial heads, with distorted presence of conidiophores.
The capability of essential oils to inhibit fungal spore germination has been noted in other studies using different testing methods (31,34,38). Earlier reports showed that some essential oils are able to inhibit the mycelial growth of fungi in laboratorial media and foodstuffs (4,33,43). However, the antifungal property of different essential oils ranges from a narrow to wide spectrum depending on the assayed essential oil, its concentration and the target fungal species (8,15,18,36,46).

The antifungal property of phytochemicals found in C. zeylanicum essential oil (e.g. mono- and sesqui-terpenes, phenols, cinnamaldehyde) could involve inhibition of extracellular enzymes synthesis and the disruption of the cell wall structure resulting in lack of cytoplasm, damage of integrity and ultimately the mycelial death. Cytoplasm granulation, cytoplasm membrane rupturing, cytoplasm hyperacidity, break down of the electron transport chain, H⁺-ATPase and channel inhibition are some structural and metabolic events possibly related to the antifungal property of essential oils (9,10,25). It is also reported that essential oils are able to interfere into the mitochondrial membrane system by a membrane-disruptive activity closely associated with the enzymatic reactions, such as respiratory electron transport, protein transport and coupled phosphorylation (4,35).

Velluti et al. (11) report that eugenol (a phenolic compound known as major component of C. zeylanicum essential oil) presents its antimicrobial activity attributed to the presence of an aromatic nucleus and a phenolic OH group known to be reactive and to form hydrogen bonds with active sites of target enzymes. Although, the antimicrobial activity of an essential oil is attributed mainly to its major components, the synergistic or antagonistic effect of compounds in minor percentage in the mixture has to be considered (15,42).

The observations of light microscopy showed that the main morphological changes caused by C. zeylanicum essential oil on A. niger were associated with the degeneration of fungal hyphae causing leakage of cytoplasm content and loss of conidiation. These modifications in the cytological structure may be related to the interference of the essential oil with the enzymes responsible for wall synthesis as previously cited by other researchers (39,45). De Billerbeck et al. (16) noted that Cymbopogon nardus (L.) W. Watson essential oil was able to cause morphological changes in A. niger including heterogeneous mycelium structure, granular/vesicular aspect of the cytoplasm content and complete degeneration of fungal hyphae resulting in an empty hyphae tip and cytoplasm retraction. Ultrastructural changes were smooth cell walls, depressions (like craters) on the cell surface. Such changes could be related to the interference of its components on enzymatic reactions of wall synthesis, which affects fungal morphogenesis and growth.

Sharma and Tripathi (38) noted that Citrus sinensis (L.) Osberk essential oil caused reduction in the conidial heads, poorly developed sterigmata and distorted (squashed and...
flattened) conidiophores in *A. niger*. Anomalous empty, budded and flattened hyphae and cell wall destruction was also found. These effects results in the death of hyphae suggesting that the essential oil antifungal property is a result of its attack on the cell wall and retraction of cytoplasm. Rasooli and Owlia (34) observed that thyme oils provided irreversible damage to cell wall (degenerative changes), cytoplasm membrane (irregular, dissociated from cell wall, invaginated) and nuclear membrane (folding) of *Aspergillus parasiticus*.

In conclusion, our results indicate that essential oils could find a practical and rational use in the inhibition of mould growth. Particularly, *C. zeylanicum* essential oil possesses strong anti-Aspergillus activity inhibiting the growth, spore germination and causing deleterious cellular morphological changes of different *Aspergillus* species. The broad inhibition of fungal growth and sporulation by *C. zeylanicum* essential oil, in addition to its availability as natural volatile product, justifies its possible rational use as an alternative antifungal compound to control the growth and dissemination of pathogen *Aspergillus* species.

**RESUMO**

**Efeito do óleo essencial de Cinnamomum zeylanicum Blume sobre o crescimento e morfogênese de algumas espécies de Aspergillus potencialmente patogênicas**

*Cinnamomum zeylanicum* Blume é uma planta conhecida por apresentar ampla variedade de propriedades medicinais. Portanto, este estudo teve por objetivo avaliar a interferência do óleo essencial *C. zeylanicum* sobre o crescimento e morfogênese de algumas espécies de *Aspergillus* potencialmente patogênicas. O óleo essencial testado apresentou potente efeito antifúngico demostrado pela visualização de grandes zonas de inibição de crescimento de todas as linhagens testadas. Os valores de CIM<sub>50</sub> e de CIM<sub>90</sub> foram 40 e 80 µg/mL, respectivamente. Nas concentrações de 80, 40 e 20 µg/mL o óleo demonstrou um potente efeito fumigante, inibindo o crescimento micelial radial de *A. niger*, *A. flavus* e *A. fumigatus* ao longo de 14 dias de exposição. A 80 e 40 µg/mL o óleo promoveu inibição de 100% da germinação de esporos, das três espécies de *Aspergillus* citadas anteriormente. Além disso, alterações morfológicas no crescimento fúngico foram observadas sob microscopia óptica após exposição ao óleo essencial, como diminuição da conidiogênese, perda citoplasmática, perda de pigmentação e rompimento da estrutura fúngica (hifa) indicando degeneração da parede celular. Diante do exposto, conclui-se que o óleo essencial de *C. zeylanicum* poderia ser empregado como potente composto antifúngico, particularmente, prevenindo crescimento de espécies de *Aspergillus*.

**Palavras-chave:** *Aspergillus, Cinnamomum zeylanicum, Blume, óleo essencial, atividade antifúngica.*

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