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# ANTIFUNGAL ACTIVITY OF *CYMBOPOGON WINTERIANUS* JOWITT EX BOR AGAINST *CANDIDA ALBICANS*

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## ABSTRACT

*Candida albicans* is an opportunistic yeast and a member of the normal human flora that commonly causes infections in patients with any type of deficiency of the immune system. The essential oils have been tested for antimycotic activity and pose much potential as antifungal agents. This work investigated the activity of the essential oil of *Cymbopogon winterianus* against *C. albicans* by MIC, MFC and time-kill methods. The essential oil (EO) was obtained by hydrodistillation using a Clevenger-type apparatus. It was tested fifteen strains of *C. albicans*. The MIC was determined by the microdilution method and the MFC was determined when an aliquot of the broth microdilution was cultivated in SDA medium. The phytochemical analysis of EO showed presence of citronellal (23,59%), geraniol (18,81%) and citronellol (11,74%). The EO showed antifungal activity, and the concentrations 625 µg/mL and 1250 µg/mL inhibited the growth of all strains tested and it was fungicidal, respectively. The antimicrobial activity of various concentrations of EO was analyzed over time, it was found concentration-dependent antifungal activity, whose behavior was similar to amphotericin B and nystatin.

**Key words:** *Candida albicans*, antifungal, *Cymbopogon winterianus.*

## INTRODUCTION

*Candida albicans* is an opportunistic yeast and a member of the normal human flora. The individual is colonized at birth or near birth and transmission is primarily through physical contact. This yeast normally colonizes the skin and mucosal epithelium in healthy individuals (25). In patients hospitalized with cancer, immunocompromised or who use immunosuppressive drugs colonization may progress to invasion, resulting in severe systemic disease (26, 38).

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Systemic infection by *C. albicans* is often associated with catheters, surgery, parenteral nutrition and damage the skin, mucous membranes and the digestive tract (39). Invasive fungal infections are associated with high mortality rates and over 40% in bloodstream infections caused by *C. albicans* (8). The common sites for superficial candidiasis include under the breasts, in the groin, in skin folds of obese persons and the perineal area (18). The major antifungal agents used in the therapy of these infections are azoles such as fluconazole and itraconazole, and the polyene compounds such as amphotericin B and nystatin (6, 30).

The increasing resistance of fungi to azole derivatives as a result of long-term therapies may limit the use of these drugs in the future (10, 17, 30). Due to limitations of current antifungal therapy and the emergence of resistant strains, it is necessary the search for new antifungal drugs through the testing of substances from plants (3, 34). Among the candidates, the essential oils are known to possess several biological activities, such as antibacterial and antifungal action (2, 7).

*Cymbopogon winterianus* is a plant belonging to the family Poaceae, popularly known as citronella, which is cultivated in India and Brazil. This plant demonstrated depressant effect on the central nervous system, anticonvulsant effect (33), larvicidal effect against *Aedes aegypti* (27), antibacterial (14,31) and antifungal activity (18), including anti-*Candida* action (13).

This study aimed to evaluate the antimicrobial activity of the essential oil of *Cymbopogon winterianus* against *Candida albicans* and to characterize the relationship between the concentration this essential oil with the rate and extent of its antifungal activity.

## MATERIAL AND METHODS

### Essential oil

*Cymbopogon winterianus* Jowitt ex Bor was grown, collected and had the essential oil extracted by hydrodistillation using a Clevenger-type apparatus at the Center for Technology Training at the Federal University of Paraíba, Bananeiras city (Paraiba, Brazil) by Prof. Dr. Paulo Alves Wanderley.

The plant was identified by Prof. Dr. Rita Baltazar de Lima in Laboratory of Botany, Department of Systematics and Ecology of the Center of Exact and Nature Sciences of the Federal University of Paraíba. The voucher specimen was deposited in the Herbarium Prof. Lauro Pires Xavier of the Department of Systematics and Ecology, Federal University of Paraíba under the code JPB 41387.

### Essential oil analysis

The essential oil was analyzed using a gas chromatograph (GC) fitted to a mass spectrometer (MS) (GC-MS-Schimadzu QP-5050A) instrument equipped with a GC Schimadzu 17A. Fused silica capillary column was 30 m x 0.25 mm i.d., with film thickness 0.25 µm. Helium was used as the carrier gas at 0,9 mL/min, with inlet pressure 48,9 psi. Injector and MS transfer line temperatures were at 280 and 170°C, respectively. The initial column temperature was 60°C, and then gradually increased to 240°C at the rate of 3°C/min. It was kept at 240°C for 10 minutes. For GC-MS detection an electron ionization system was used with ionization energy of 70eV. Samples were diluted 1/1000 (v/v) in hexane and 1.0 µL were injected in the splitless mode (1). The compounds were identified by comparing their fragmentation patterns reported in the mass spectra with those present in the library of mass spectrometers NIST 98 (National Institute of Standards and Technology, USA) and with reports from the literature. The components quantification was based on the area percentage of the peak of each component in relation to the total area of all standardized peaks in the chromatogram.

Fungal samples

The strains of *C. albicans* tested belong to the collection of the Mycology Laboratory, Federal University of Paraíba and include ATCC 76485, ATCC 76615, ATCC 13803, LMV 42,

ICB 12, M101, LM 968, LM 68, LM 16, LM 018, LM 023, LM 601, LM 290, LM 052, LM 087.

**Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

The MIC was determined by the microdilution method. Cultures of *Candida albicans* were placed on Sabouraud Dextrose Agar (SDA) and incubated for 24-72 hours at temperature 37ºC. Colonies of this culture were suspended in sterile 0.85% NaCl and the inoculum was standardized according to the scale of 0.5 McFarland (1-5 x $10^6$ CFU/mL). In a 96-well plate was added Sabouraud broth and essential oil of *C. winterianus* concentrations of 10.0000 to 39µg/mL. The MIC determination was conducted with approximately 1-5 x$10^5$ CFU/mL of the microorganism in each well. The plates were incubated at 37°C for 24-48 hours. In 24-48 hours there was a visual observation of fungal growth. To determine the MFC, 10µL of each of the wells without fungal growth was seeded on a plate containing SDA, the SDA plating were incubated at 37°C for 24-48 hours. The MFC was considered as the lowest concentration cultivated in plate with SDA in which growth was less than 3 CFU. Afterwards, 20 µL of 0.5% triphenyl tetrazolium chloride (TTC) was added to each of 96 wells for MIC determination, and the plate incubated for 24 hours. The MIC was determined as the lowest oil concentration that inhibited visible growth of the microorganism, as also indicated by the TTC by reading the plates of 96 wells. There were three independent experiments on different occasions (11, 15, 21, 40).

**Time-kill**

The time-kill of *C. albicans* in presence of the essential oil was performed according to Klepser *et al*. (22), with some modifications. Before testing, the microrgamism was cultivated in SDA. Colonies derived from culture were suspended in 0.85% NaCl and turbidity adjusted to the range of 0.5 McFarland (1-5 x $10^6$ CFU/mL). One milliliter of fungal suspension was added to 9 mL of broth Sabouraud with or without the essential oil in various appropriate concentrations. The initial inoculum contains 1-5 x $10^5$ CFU/mL. Concentrations of essential oil of *C. winterianus* tested were 0.5, 1, 2, 4 and 8 times the MIC. These cultures were incubated at 37°C and at various time periods (0, 1, 2, 4, 6, 8, 12 and 24 hours), an aliquot of 100 µL was removed from each solution and diluted 1:10. An aliquot of 10µL of each dilution was removed and plated on SDA. The plates were incubated at 37°C for 24-48 hours and the number of colony forming units (CFU) was counted. When less than 1000 CFU/mL was expected, 10 µL sample was plated directly into SDA without dilution. The experiment was performed in duplicate. The minimum detection limit of this method is 100 CFU/mL (23).

The $\log_{10}$ CFU/mL was plotted on a graph as function of time and used to compare the rate and extent of antifungal activity in various concentrations of essential oil. It was considered fungicidal activity when there was a decrease greater than or equal to 3 $\log_{10}$ CFU/mL of the initial inoculum, resulting in reduction of 99.9% or more CFU/mL in 24 hours compared with the initial inoculum. Activity lower than that described was considered fungistatic (16, 24, 35).

## RESULTS

**Chemical characterization of oil constituents**

Essential oil of *C. winterianus* was subjected to GC and GC-MS analysis. The phytochemicals and their respective percentage in the essential oil composition and retention times are shown in Table 1. The majority constituents were citronellal (23,59%), geraniol (18,81%) and citronellol (11,74%).

**Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)**

By the broth microdilution technique was determined the MIC and the MFC, which are shown in Table 2.

As seen in Table 2, the MIC of the essential oil tested ranged between 78 and 625 µg/mL. The concentration of 625 µg/mL inhibited the growth of all strains, while 312 µg/mL was able to inhibit 60% of the strains tested. MFC of microorganisms ranged between 312 and 1250 µg/mL, being the latter fungicidal for all strains tested.

**Table 1.** Chemical composition of essential oil from *C. winterianus.*

| Peak No. | Compounds | Composition (%) | Retention time (min) |
|---|---|---|---|
| 1 | 2-methyl-2-hepten-6-one | 0.13 | 6,99 |
| 2 | β-myrcene | 0.07 | 7.18 |
| 3 | Limonene | 3.39 | 8.58 |
| 4 | Linalool | 1.34 | 11.13 |
| 5 | Citronellal | 23.59 | 13.81 |
| 6 | Citronellol | 11.74 | 17.00 |
| 7 | Geraniol | 18.81 | 18.65 |
| 8 | Citronellyl acetate | 5.29 | 22.30 |
| 9 | β-elemene | 6.40 | 22.50 |
| 10 | Eugenol | 10.34 | 23.96 |
| 11 | Germacrene | 2.63 | 27.69 |
| 12 | Δ-cadinene | 2.27 | 29.44 |
| 13 | Elemol | 6.73 | 30.67 |
| 14 | Endo-1-bourbonanol | 1.01 | 31.52 |
| 15 | Farnesol | 0.60 | 33.01 |
| 16 | γ-eudesmol | 1.00 | 33.66 |
| 17 | Torreyol | 1.65 | 34.08 |
| 18 | Trans-farnesol | 3.01 | 34.69 |

**Table 2.** Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of essential oil of *C. winterianus* against strains of *C. albicans.*

| ***C. albicans* samples** | **MIC (µg/mL)** | **MFC (µg/mL)** |
|---|---|---|
| 1- ATCC 76485 | 78 | 312 |
| 2- ATCC 76615 | 78 | 312 |
| 3- ATCC 13803 | 625 | 1250 |
| 4- LMV 42 | 312 | 625 |
| 5- ICB 12 | 625 | 1250 |
| 6- M101 | 312 | 625 |
| 7- LM 968 | 156 | 312 |
| 8- LM 68 | 625 | 1250 |
| 9- LM 16 | 625 | 1250 |
| 10- LM 018 | 312 | 625 |
| 11- LM 023 | 312 | 625 |
| 12- LM 601 | 312 | 625 |
| 13- LM 290 | 625 | 1250 |
| 14- LM 052 | 312 | 625 |
| 15- LM 087 | 625 | 1250 |

**Time-kill**

The microorganism growth was analyzed over time when it was subjected to various concentrations of essential oil of *C. winterianus*. Two strains of *C. albicans* (ICB 12 and ATCC 13803) were submitted to the experiment of microbial time-kill (Figures 1 and 2).

The graphs show the $\log_{10}$ of CFU/mL versus time for several multiples of the MIC (625 µg/mL) and the control without essential oil. The analysis of the graph to point out that at concentrations less than or equal to 1xMIC, the essential oil has fungistatic activity (reduction of less than 99.9% or $3\log_{10}$ the number of CFU/ml of initial inoculum), and at concentrations greater than or equal to 2xMIC it has fungicidal activity (reduction greater than or equal to 99.9% or $3\log_{10}$ the number of CFU/mL of initial inoculum), therefore, the essential oil of *C. winterianus* has concentration-dependent fungicidal activity.

Although the rate and extent of antifungal activity has varied slightly between the two strains tested, their behavior in front of the essential oil was similar. The antifungal activity improved with increasing concentration of essential oil, like this, when the higher concentration of essential oil, the lower time is required for fungicidal activity.

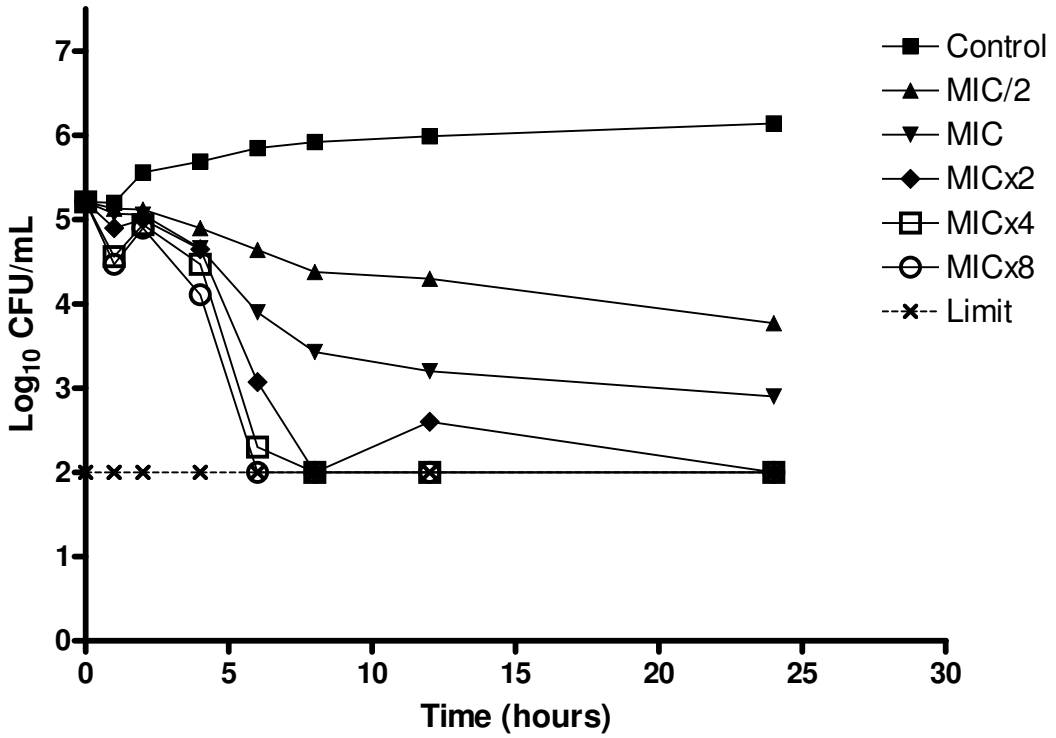


**Figure 1.** Time-kill of *C. albicans* ATCC 13803 when exposed to various concentrations of essential oil of *C. winterianus*.

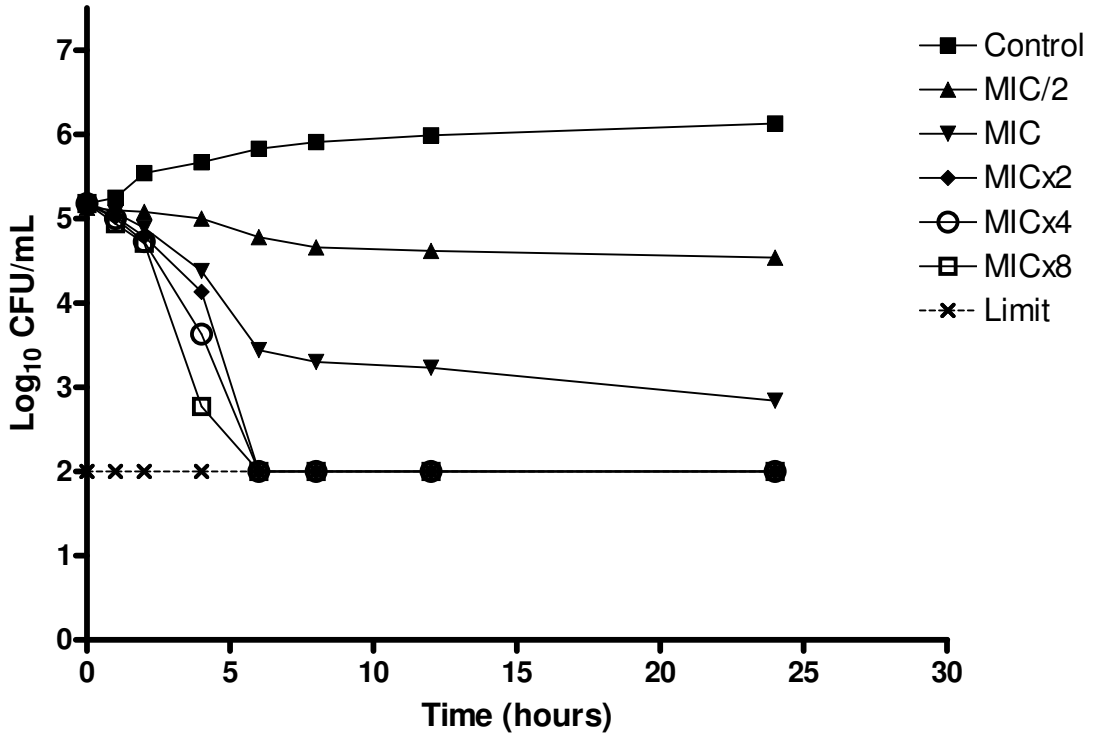


**Figure 2.** Time-kill of *C. albicans* ICB 12 when exposed to various concentrations of essential oil of *C. winterianus*.

## DISCUSSION

Substances from plants are used in the treatment of various diseases, but the potential of plants as a source of new drugs is still poorly explored. The estimated number of plant species, only a small percentage had their pharmacological properties studied (34), like this, there is need to search for new drugs from plants that have therapeutic potential. Therefore, the essential oils are important because their several pharmacological activities including anti-fungal, antibacterial, antiparasitic (2,4,37).

The GC-MS analysis resulted in the identification of 18 components. Among the phytochemicals, citronellal, geraniol and citronellol are the majority constituents. Other studies recorded that citronellal, citronellol and geraniol are the main constituents of essential oil of *C. winterianus* (5, 9, 13, 33).

Among the identified compounds, some were previously reported to have antibacterial activity, including geraniol against *E. coli* (14), *Listeria monocytogenes, Salmonella enterica* and *Salmonella typhimurium* (36). Mesa-Arango *et al*. (28) showed that citronellal and geraniol were active against *Candida parapsilosis, C. krusei, Aspergillus flavus and A. fumigatus.*

The MIC and the MFC in all strains were 625 µg/mL and 1250 µg/mL, respectively. It was reported that the essential oil of this plant had antibacterial activity against *Escherichia coli* O157: H7 (MIC > 0,8% v/v), *Salmonella typhimurium* (MIC 0,4% v/v), *Staphylococcus aureus* (MIC 0,05% v/v), *Listeria monocytogenes* (MIC 0,4% v/v) (31); against enterotoxigenic, enteroinvasive and enteropathogenic serotypes of *E. coli*, with MICs ranging between 200 and 800 µg/mL (14) and antifungal activity against *Trichophyton* species (18) and against *Candida albicans* (MIC 600µg/mL) (13). MIC found in this study is according to values obtained in the other studies.

Due to its pronounced anti-*C. albicans* activity, the essential oil of *C. winterianus* had its action studied in more detail through the time-kill method. When analyzing the graphic $\log_{10}$ CFU/mL versus time, it can identify the transition between the levels of fungistatic and fungicidal activity of a substance, the essential oil of *C. winterianus* showed concentration-dependent fungicidal activity. Another type of antifungal activity occurs for example with fluconazole, whose activity is independent of concentration, because there is no significant increase in activity with increasing concentration (6). Among the drugs commonly used to treat fungal infections, amphotericin B and nystatin are fungicidal concentration-dependent (22, 29).

It was reported that the essential oil of *Ocimum gratissimum* L. had antifungal activity against *C. albicans*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* with MICs ranging between 750 and 1500 µg/mL and in the test of time-kill this oil showed concentration-dependent antifungal activity (29).

The MFC found when an aliquot of the broth microdilution was cultivated in SDA medium was 1250µg/mL (2xMIC). This value is related to the concentration able to inhibit the growth of 99.9% or more CFU/mL in 24 hours compared with the initial inoculum, information that can be observed analyzing the time-kill graphs.

Clinically, differences in fungal dynamics can influence the selection of optimal regimes of doses of an antifungal. Agents in which the rate and extent of antifungal activity improve with increasing concentration (e.g. amphotericin B) can be optimized by the administration of large doses. In contrast, the activity of antifungal agents like fluconazole does not improve with increase in the concentration higher than the MIC. Therefore, the administration of large doses of fluconazole cannot change the rate and extent of fungal eradication (16).

Pauli and Schilcher (32) suggest that oral administration of essential oil compounds is not suitable to cure severe infections in children. Topical application or inhalation of selected compounds for the treatment or additional treatment of mild infections is reasonable. Dorman and Deans (12) reported that administered orally, volatile oils may be able to control a wide range of microbes, but there is also the possibility that they may cause an imbalance in the gut microflora, allowing opportunistic pathogens coliforms to become established in the gastrointestinal tract with resultant deleterious effects. Other possible side effects of essential oils are irritation, allergic contact dermatitis and spasmolytic or spasmogenic properties (7). The essential can to be used for treatment some diseases like dermatomycoses and superficial candidiasis mainly in topical applications (18). The essential oils can be used alone or in combination with other drugs which can exhibit synergism (19, 20).

The increased resistance of *C. albicans* and the nature of the fungistatic azoles have valued the search for new antifungal agents, once the activity against this type of pathogen is essential (16). The antifungal activity demonstrated by the essential oil of *C. winterianus* makes it a potential candidate as a major agent in controlling fungi growth that cause infections or are present in the environment.

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