

Antifungal Activity of the Essential Oil of *Cymbopogon citratus* (DC) Stapf. An *in vitro* study.

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Abstract: Aim. To evaluate the antifungal potential of the essential oil of *Cymbopogon citratus* by determining the Minimum Inhibitory Concentration (MIC) and the Minimum Fungicidal Concentration (MFC) for *Candida albicans* (ATCC 90029), *Candida albicans* (CBS 562), *Candida tropicalis* (ATCC 705) and *Candida tropicalis* strains (CBS 94), as well as to analyze the possible mechanism of action of the oil through the addition of sorbitol to the culture medium. Methods. For the MIC determination, inocula were previously adjusted through spectrophotometry and 100µL were added to the wells of plates already containing the culture medium and 100µL of the serial dilutions of the oil, incubating them in aerobiosis for 24 hours, with subsequent staining by 1% TCT. For the MFC, 50µL of the supernatant from the MIC assay wells were dripped onto Petri dishes and incubated in aerobiosis for 24 hours. Tests were performed in triplicate and data analysed by descriptive statistics. Results. It was determined that the MIC for *C. albicans* was 125 µg/mL while MIC for *C. tropicalis* was 250 µg/mL, with the essential oil presenting fungicidal effect for both analyzed yeasts. Conclusion. The essential oil of *Cymbopogon citratus* does not act at the cellular wall level and demonstrated an antimicrobial effect on *Candida albicans* and *Candida tropicalis*, therefore acting as a fungicide.

Keywords: phytotherapy, microbial sensitivity test, candidiasis.

INTRODUCTION.

A biofilm is a complex three-dimensional structure generally adhered to a solid surface, known as substrate, with time representing a favorable factor for biofilm maturation, resulting in the adsorption of a greater number of microbial species and in a greater degree of interspecies interaction, thus making it difficult to remove or disorganize.^{1,2}

In medicine, fungi are responsible for infections, mainly of the skin, gastrointestinal tract and respiratory system, and the biofilm structure is responsible for antimicrobial resistance and for the difficulty in treating these diseases.¹ Yeasts of the genus *Candida spp.* are strongly resistant to antifungal therapies, since they can organize themselves into biofilms,³ causing serious infections with high morbidity and mortality rates, even in the hospital environment.⁴⁻⁶ In dentistry, *Candida albicans* biofilms, besides causing diseases such as candidiasis and other opportunistic infections, can cause changes in the mechanical properties of glass ionomer cement, such as reduction of surface hardness after exposure to the biofilm formed by these microorganisms.⁷

Regular and adequate biofilm control is the best way to prevent diseases,

and the use of auxiliary substances with antimicrobial effect^{2,4} is recommended. Plant products may present antimicrobial activity and contribute to the chemical control of the biofilm.³ This makes the possibility of innovation of therapeutic proposals more effective with less potential to promote undesirable effects,⁸⁻¹⁰ as in the case of *Cymbopogon citratus*, known by several popular names: *capim-santo*, *capim-limão*, *capim-cidreira*, among others.¹¹⁻¹⁴ Antimicrobial, anti-inflammatory, antiproliferative of tumor-cells and wound-healing properties make *Cymbopogon citratus* a potentially beneficial product for use in the health care area.¹⁵⁻¹⁷ In this context, the aim of this study was to evaluate the antifungal effect of the essential oil of *Cymbopogon citratus*, as well as to verify the possible mechanism of action of the vegetal active pharmaceutical ingredient under testing.

MATERIALS AND METHODS.

Chemical Characterization

The essential oil of *Cymbopogon citratus* was obtained from a reference company in the marketing of essential oils and essences (Quinari Fragrâncias e Cosméticos Ltda., Ponta Grossa, PR, Brazil). It was chemically analyzed in order to compare the results to the information in the leaflet provided by the producer. The essential oil was prepared for Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) evaluations.

Minimum Inhibitory Concentration Determination

Standard *Candida albicans* ATCC 90029, *Candida albicans* CBS 562, *Candida tropicalis* ATCC 705 and *Candida tropicalis* CBS 94 strains were reactivated in Sabouraud agar medium (BD, Heidelberg, Germany) and inocula were prepared the day before the test in aerobiosis and adjusted by spectrophotometry to 5×10^6 CFU/ml (at 530nm), corresponding to 0.5 in the McFarland scale. Then, the inoculum was diluted, and the final concentration in the plate wells was 2.5×10^3 CFU/ml.

For the MIC determination, the Serial Microdilution technique methodology was used,¹⁸ and 100µL of Sabouraud broth medium (BD, Heidelberg, Germany) was added to the wells of plates, into which 100µL of the essential oil were added and serially transferred from well to well to achieve the test dilutions, ranging from 2000µg/mL to 15.6µg/mL. Finally, 100µL of the previously adjusted

inocula were added. Assays were run in triplicate and the plates incubated in aerobiosis at 37°C for 24 hours.

Controls of the viability of the test strains were carried out, using medium with inoculum only, and positive control, using medium with inoculum and nystatin solution starting at 100µg/mL. After the 24 hour period, 50µl of 1% TCT solution (2,4,6-Trichloro-1,3,5-triazine) was placed in the wells of plates, to colorimetrically determine the MIC, by confirming the viability of the microorganisms as reflected by the activity of dehydrogenase enzymes involved in the process of the cellular respiration,¹⁶ and which were then incubated for a further 24 hours at 37°C.

Minimum Fungicide Concentration Determination

To determine the Minimum Fungicide Concentration, 50µL from the wells corresponding to MIC, MICx2 and MICx4 were dripped onto Petri dishes containing Sabouraud Dextrose agar medium (BD - Germany), and incubated in aerobiosis for 24 hours at 37°C.¹⁷

Mechanism of Action: Analysis with Addition of Sorbitol

To determine the mechanism of antifungal action against *Candida albicans* ATCC 90029, Sorbitol, a component present in the cell wall, was added to the test culture medium at a concentration of 0.8 M, acting as an osmotic protector.⁸

The test followed the standard protocol for MIC determination using antifungal Caspofugin at concentration 2.5µg/ml as a positive control, since this drug has a mechanism of action known to inhibit fungal cell wall synthesis.

The MIC values with the addition of Sorbitol were determined. Considering that the test product, by inhibiting cell wall synthesis, will stimulate the yeast to use the Sorbitol richly available in the culture medium, it will lead to increased fungal growth.²⁰ Therefore, in the presence of Sorbitol the MIC should be observed at higher values than those presented for MIC determined in plates containing culture medium only.¹⁹

Chemical Characterization

The test substance was characterized by chromatography using gas chromatograph coupled to mass spectrometer (GCMS Shimadzu model QP2010, Japan).

Statistical analysis

The results were tabulated and analyzed using descriptive statistics.

Table 1. MIC and MFC of *Cymbopogon citratus* and positive control on *Candida spp.*

STRAIN	<i>C. citratus</i>		Nystatin	
	MIC µg/ml	MFC µg/ml	MIC µg/ml	MFC µg/ml
<i>C. albicans</i> ATCC 90029	125	125	0.4	0.8
<i>C. albicans</i> CBS 562	125	125	0.4	0.8
<i>C. tropicalis</i> ATCC 705	250	250	0.4	0.4
<i>C. tropicalis</i> CBS 94	250	250	0.4	0.4

Table 2. MIC obtained with and without the osmotic protector sorbitol, against *Candida albicans*.

STRAIN	<i>C. citratus</i>		Caspofungin	
	MIC µg/ml	MIC Sorbitol µg/ml	MIC µg/ml	MIC Sorbitol µg/ml
<i>C. albicans</i> ATCC 90029	125	125	0.078	0.15

RESULTS.

The chemical characterization showed that the major component of the product is citral, corresponding to approximately 84% of its constituents, a little more than the 80% informed in the leaflet from the producer. Other constituents were identified as myrcene and geraniol, but at very low concentrations.

The data obtained in the antimicrobial evaluation allow defining the vegetal active principle as antifungal, with fungicidal effect, without alterations by the addition of Sorbitol to the culture medium (Table 1 and Table 2). The fungicidal effect was observed on the MFC test, which did not detect fungal growth.

DISCUSSION.

The use of phytotherapeutic agents in dentistry has been an alternative therapeutic option²¹ and the most important group of active compounds in dental formulations of natural origin include essential oils and monoterpenoids.²²

It is suggested that terpenes are phyto-constituents responsible for the antimicrobial activity of this essential oil, with the main focus on citral,^{23,24} which is the major component of the test product, and which may be responsible for the antifungal effect observed. However, differences in constituents may occur according to variations on the location and time of collection of

the botanical samples of *Cymbopogon citratus*.²⁵ Such variations make it necessary to verify the composition of plant active principles used in studies, justifying the CG/MS analysis applied here, in order to be able to make comparisons with other studies.

The antifungal effect observed in this study corroborates the literature findings, showing action on *Candida spp.* species at low concentrations.^{7,15,26} However, there is disagreement about the fungicide potential, since some authors observed a fungistatic effect.¹⁵ A previous study has shown that even though antifungal activity on clinical *Candida albicans* and *Candida tropicalis* isolates was found, the essential oil of *Cymbopogon citratus* was effective only on 70% of *Candida albicans* isolates and 50% of *Candida tropicalis* isolates.¹⁵

When using the agar diffusion methodology with the essential oil in the liquid and vapor phase, some authors reported a potent inhibitory effect of the oil,¹⁶ and the results obtained for the vapor phase test were superior to those of the liquid phase, suggesting that this finding may be due to volatile compounds present in the oil. However, another study demonstrated that cellular defects were more evident for the vapor phase.²⁶

The results of the present study for the MIC of *Candida albicans* (125µg/mL) are lower than those described by other authors, who found MIC of 288µg/mL.²⁶ This result can be justified by climate variations and the manner in

which the material used to produce the oil was collected, resulting in the vegetal active principle being used in this research having a higher amount of citral.

There is no consensus in the literature regarding the concentration of the substance that can determine its microbicidal potential, and it may be possible to attribute different classifications to the essential oil studied here. The results obtained for MIC in this study can be classified as very good by some authors, since they are below 1mg/mL.²⁷

However, for other researchers, the classification would be of potent substance, since the MIC was lower than 500µg/mL.³¹ Finally, differing from previous studies,^{27,28} the effect of the essential oil on yeast in this study can be considered moderate, as the MIC is between 100 and 500µg/mL.²⁹

Reinforcing the importance of studies using the essential oil of *Cymbopogon citratus* as study material, the literature also highlights this vegetal active principle as promising to prepare mouthwashes, dental creams and to be part of the therapeutic arsenal used in Dentistry. Considering that many biofilm infections due to *Candida spp.* may begin in the oral cavity³ the control of the dental biofilm through the use of this substance may be a prophylactic alternative.³⁰

The literature also reports the anti-inflammatory potential as an advantage of its topical use, which at concentrations of 10mg/kg or 10µL/area was able to reduce atrial edema in animal models.¹⁷ These findings

instigate new investigations and place the essential oil of *Cymbopogon citratus* in a favorable condition to be applied in mouthwash solutions, which is the proposal of this research.

Antifungal Caspofungin acetate inhibits the synthesis of essential constituents of the cell wall³¹ and was chosen as a positive control in the present research. When compared to findings for the essential oil, the fungal strains studied did not respond to the addition of Sorbitol to the culture medium when treated with *Cymbopogon citratus*, indicating that the cell wall is not the site of action of the essential oil in these microorganisms and instigates the performance of further studies in order to elucidate the still not clarified mechanism of action of this vegetal material.

Since essential oils have many associated phyto-components that can act synergistically at various sites, it is difficult to identify a specific and unique mechanism of action. However, when the mechanism of antimicrobial action is due to interference with the cell wall of the microorganism, there is an advantage for its medicinal use because of cell selectivity, being less toxic to human cells.¹⁹

CONCLUSION.

The essential oil of *Cymbopogon citratus* does not act at the cellular wall level and has demonstrated antimicrobial effects on *Candida albicans* and *Candida tropicalis*, being therefore a fungicide.

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