Essential oil from *Cymbopogon citratus* (DC) Stapf: a promising natural product against *Malassezia* spp.

Óleo essencial de *Cymbopogon citratus* (DC) Stapf: um produto natural promissor contra *Malassezia* spp.

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ABSTRACT

This study evaluated the sensitivity of *Malassezia* spp. strains, the etiologic agent of pityriasis versicolor, to essential oil from *Cymbopogon citratus*. The chemical composition of the essential oil was analysed by GC-MS**,** and the major constituents were: geraniol (52.80%), neral (36.65%) and myrcene (3.73%). The minimum inhibitory concentration (MIC) of *C. citratus* essential oil on to 22 strains of *Malassezia* spp. were determined by agar dilution technique in the presence and absence of ergosterol (50-250 μg/mL) and sorbitol (0.8 M). Increased MIC values in the presence of ergosterol indicate an effect on fungal membrane, and the higher MIC values in the presence of sorbitol indicate a mechanism of action on the cell wall. The MIC ranged from 0.31 to 1.25 μL/mL and it increased fourfold in the presence of ergosterol, regardless of concentration tested**;** and this fact was most likely related to the occurrence of the oil and exogenous ergosterol complexes. No change in MIC values in the presence of sorbitol was found. These data infer that *C. citratus* essential oil causes an effect on *Malassezia* spp. plasma membrane synthesis by binding to ergosterol. This study contributes to the development of new antifungal drugs, especially against *Malassezia spp*.

Keywords. *Cymbopogon citratus*, *Malassezia*, pityriasis versicolor, fungal infection.

RESUMO

Este estudo avaliou a sensibilidade de cepas de *Malassezia* spp., agente etiológico da pitiríase versicolor, ao óleo essencial de *Cymbopogon citratus*. A composição química do óleo essencial, obtido por hidrodestilação, foi analisada por CG-EM. Os constituintes majoritários do óleo foram: geraniol (52,80%), neral (36,65%) e mirceno (3,73%). A concentração inibitória mínima (CIM) do óleo essencial de *C. citratus* foi determinada para 22 cepas de *Malassezia* spp. pela técnica de diluição em ágar, na presença e ausência de ergosterol (50-250 μg/mL) e sorbitol (0,8 M). Aumento nos valores de CIM na presença de ergosterol indica interferência na membrana fúngica; e valores mais elevados de CIM na presença de sorbitol indicam mecanismo de ação sobre a parede celular. A CIM variou de 0,31 a 1,25 μL/mL e aumentou quatro vezes na presença de ergosterol, independentemente da concentração testada, fato provavelmente relacionado à formação de complexo do óleo com ergosterol exógeno. Não houve alteração nos valores de CIM na presença de sorbitol. Em conclusão, sugere-se que o óleo essencial de *C. citratus* atue sobre a síntese da membrana plasmática de *Malassezia* spp. ligando-se ao ergosterol**.** Este estudo contribui no desenvolvimento de novos antifúngicos, especialmente contra *Malassezia* spp.

Palavras-chave. *Cymbopogon citratus*, *Malassezia*, pitiríase versicolor, infecção fúngica.

INTRODUCTION

In recent years, there was a significant increase in fungal infections worldwide, especially in immunocompromised patients. Among fungal infections that frequently appear are highlighted those produced by *Malassezia* spp[1,2].

Malassezia spp. is a lipophilic yeast, which inhabits the human skin saprophytes, but may cause fungal infections in susceptible individuals, when using corticosteroids, transplant recipients, patients with cancer, diabetes and generally immunocompromised. As most common pathologies associated are cited pityriasis versicolor, dandruff, seborrheic dermatitis, atopic dermatitis, folliculitis and, less frequently may be involved in systemic infections, usually by colonization of intravascular catheters, especially in pediatric patient when using parental lipid-rich nutrition[2-5].

The treatment of infections caused by *Malassezia* spp. varies with the seriousness they generally use imidazole derivatives such as fluconazole and itraconazole[6]. These skin diseases have been of concern to researchers and pharmaceutical industry worldwide. Besides the great problem of the many adverse reactions to medicines available in the market can cause, there are still two other problems: the emergence of resistant microorganisms arising from the indiscriminate use of antifungal agents and the issue of common relapses, for example, in people with pityriasis versicolor[2,7,8]. Thus, it is evident that the development of new antifungal agents is a necessary strategy to overcome problems encountered in treating these diseases[9].

In this regard, several studies aim new sources of substances with antimicrobial activity, with fewer side effects, low cost, greater safety and efficacy for the population. In part, this search is oriented to the use of medicinal plants and their respective isolate secondary metabolites[10,11]. Among plants used for medicinal purposes herbs are a prominent group of plants, mainly for its essential oils[12].

Cymbopogon citratus (DC) Stapf is a perennial herb, popularly known in Brazil as "holy grass" and worldwide as "Lemongrass". It is a plant native to India and cultivated in the tropics and subtropics. Several studies have reported the antimicrobial activities of its oil against different Gram positive and Gram negative pathogenic bacteria, yeasts and filamentary fungi[10,13-16]. Some components of the essential oil of *C. citratus* have been reported, such as myrcene and citral. Some authors attributed the oil's antimicrobial properties to the presence of citral in its composition[17]. Based on the information about antimicrobial properties of essential oil of *C. citratus*, this study evaluated its antifungal activity against *Malassezia* yeasts.

MATERIAL AND METHODS

Botanical Material

Essential oil of *C. citratus* acquired from Tekton Essential Oils LTDA, whose chemical analysis certificate was issued by the University of Caxias do Sul (Brazil), under no. 019/08.

Essential Oil analysis

Analysis of the oil was performed on Shimadzu GC-17A / MS QP5050A (GC/MS system): OV-5 capillary column (30 m × 0.25 mm id, 0.25 μm film thickness); carrier gas: helium 1,7 mL/min; column inlet pressure 48,7 kPa; linear velocity = 36.0 cm/sec; total flow 50 mL/min; carrier flow 24 mL/min; injector temperature 250 °C; detector temperature 280 °C; column temperature 40 (2 min) – 180 °C (1 min) at 4 °C /min, then 180-280 °C at 10 °C /min (10 min). Mass spectrometer operating conditions 70 eV ionization energy. Identification of Individual components was based on their mass spectral fragmentation using two computer library MS searches (wiley 229), retention indices and comparison with literature data[18].

Synthetic antifungal agent

In the study on antifungal activity of essential oils the ketoconazole disc was used as the standard at 50 μg/mL concentration, obtained from the Center for Disease Control and Diagnostics Products – CECON/Sao Paulo.

Fungal species

Twenty two strains of *Malassezia* were isolated and identified from clinical samples from patients attending the dermatology clinic of University Hospital Lauro Wanderley, Federal University of Paraíba (ethics committee no. 0037), as described by Erchiga et al[19]. Identified species were maintained in Mycosel medium supplemented with olive oil and ox bile. Cultures were incubated and maintained at 32 °C[20].

Inoculum

From the recent crops and kept in the Mycosel medium with olive oil and ox bile for seven days at 32 °C, the inoculum was prepared and standardized in sterile saline solution. Initially a comparative suspension with tube 0.5 Scale of McFarland was prepared and cell count in a Neubauer chamber. It was adjusted in the spectrophotometer (Leitz-Fhotometer 340-800 nm) to contain about 10^6 CFU/mL[21].

Minimum Inhibitory Concentration (MIC)

The MIC determination of essential oil of *C. citratus* was performed by the agar dilution technique proposed by Hadacek and Greger[22] with some modifications. For the technique, originally Petri dishes were prepared (Petri-Dispo®) with 21 mL of culture medium (Mycosel, ox bile and olive oil), plus the essential oil of *C. citratus* at 20-0.16 µL/mL concentrations, according to Allegrini et al[23]. Subsequently, 10 µL of fungal inoculums were inoculated on the surface of the solidified culture medium. The system was incubated at 32 °C for seven days. After the appropriate incubation time, the reading was carried out. MIC was defined as the lowest oil concentration able to inhibit fungal growth visually. Tests were performed in duplicate and the geometric mean of results was calculated. MIC_{90} values were interpreted as the MIC at which there was 90% inhibition growth of strains tested[24]. Feasibility controls were also performed, only with the microorganisms in the medium and positive, in which the organism was challenged with the antifungal drug ketoconazole at 50 µg/mL concentration.

Sorbitol Bioassay

The test to determine the MIC of essential oil of *C. citratus* was performed as previously described, but 0.8 M sorbitol was added to the medium as osmotic support. Plates were incubated for seven days at 32 °C[25]. It is considered as mechanism of action of the compounds on fungal cell wall if the compounds MIC in the presence of sorbitol are higher than in its absence. Tests were performed in duplicate and the geometric mean of results was calculated[26].

Ergosterol Bioassay

Parallel to the determination of MIC, it is also determined the MIC in the presence of different ergosterol concentrations (50-250 µg/mL) from Sigma-Aldrich®. It is considered as mechanism of action of the compounds on fungal plasma membrane when the compound's MIC in the presence of ergosterol is greater than in its absence. Tests were performed in duplicate and the geometric mean of results was calculated[27,28].

RESULTS AND DISCUSSION

The GC-MS analysis of *C. citratus* essential oil resulted in the identification of 8 components (Table 1). Geranial, neral and myrcene are the major components, representing over 90% of phytochemicals. These results confirm previous studies performed by Kasali et al.[29] and Andrade et al[30].

Table 1. Chemical Composition of the Essential Oil of *C.* citratus

Constituents	Rt*(min)	(%)
6-methyl-5-hepten-2-one	9.72	1.97
myrcene	10.00	3.73
α-terpinolene	13.94	0.80
Neral	19.12	36.65
trans-geraniol	19.63	2.15
Geranial	20.20	52.80
2-octanone	20.97	1.10
2-undecanone	27.75	0.79

*Retention time

Due to some difficulties related to the growth of *Malassezia* spp. using conventional media such as Sabouraud dextrose agar and RPMI – 1640, because of the lipophilic character of this yeast, the technique of agar dilution proposed by Hadacek and Greger[22] was modified using the Mycosel medium plus oil 1% olive oil. In Table 2 are recorded the MIC results of the essential oil of *C. citratus* on strains of the genus *Malassezia* determined by the agar dilution technique. There was MIC_{90} values ranging from 0.31 to 1.25 µL/mL, depending on the *Malassezia s*pecies tested.

Results found for the oil of *C. citratus* corroborate previous studies on the antimicrobial potential of this plant product. Antimicrobial activities of the essential oil of *C. citratus* were attributed to citral in a study conducted by Guerra et al[31]. The myrcene did not show antimicrobial activity, but when combined with citral it has potentiated its effect[32].

In 2003, Belém et al.[33] performed a chemical and antifungal evaluation of natural and synthetic products against *Malassezia furfur*. In this study, the oil of *C. citratus* inhibited strains of *M. furfur* in all concentrations.

Table 2. Antifungal activity of *C. citratus* essential oil on strains of *Malassezia* spp.

Microorganisms	MIC range (μL/mL)*	MIC_{90} (μL/mL)*	Control	
			Viability	Ket (50 μg/mL)
M. sympodialis (N=12)	1.25 – 0.15	1.25	+	-
M. furfur (N=6)	0.62 – 0.15	0.62	+	-
M. globosa (N=2)	0.62 – 0.31	0.62	+	-
M. obtusa (N=1)	0.62	0.62	+	-
M. slooffiae (N=1)	0.31	0.31	+	-

*Results expressed as geometric mean of two experiments; (+) presence of fungal growth; (-): absence of fungal growth; ket: ketoconazole.

Table 3. MIC values of *C. citratus* essential oil on strains of *Malassezia* spp in the presence and absence of sorbitol (0.8M).

Microorganisms	Essential oil (μL/mL)*	Essential oil + sorbitol*	Control	
			Sorbitol	Viability#
M. sympodialis LM-02	1.25	1.25	+	+
M. furfur LM-06	0.62	0.62	+	+
M. obtusa LM-01	0.62	0.62	+	+
M. globosa LM-02	0.31	0.31	+	+
M. slooffiae LM-01	0.31	0.31	+	+

*Results expressed as geometric mean of two experiments; (+) presence of fungal growth; #Viability without sorbitol.

The MIC was achieved at a 0.25% concentration with halos on average 10 mm in diameter and at this same concentration there was 80% inhibition of its strains.

The activity of the essential oil *C. citratus* was investigated against other yeasts, such as those of the genus *Candida* isolated from nosocomial infections. To conduct the study were analyzed 24 isolates of *Candida albicans* and 15 isolates of *Candida tropicalis* originated from patients with suspected nosocomial infection and a standard strain of *C. albicans* ATCC 10231, by agar diffusion technique. The essential oil of *C. citratus* showed antifungal activity against 100% of isolates at 25% concentrations[14].

Confirming the antifungal potential of essential oil of *C. citratus,* we carried out a research to investigate possible mechanisms of action. Considering a possible interference of the essential oil in fungal cell wall, the essential oil was tested by the sorbitol bioassay. In Table 3, are exposed the MIC values of essential oil of *C. citratus* in the presence of 0.8 M sorbitol osmotic protector and as can be seen, sorbitol did not protect cells from the inhibitory effects of the essential oil, suggesting another mechanism.

This research the mechanism of antifungal action does not involved interaction with the cell wall and similar results using the same methodology were observed by Pereira[34] where it was ruled out the essential oil of *Cymbopogon winterianus* Jowitt ex Bor as acting on the cell wall, whereas unchanged MIC values for oil in the presence and absence of sorbitol versus dermatophytes of the genus *Trichophyton*.

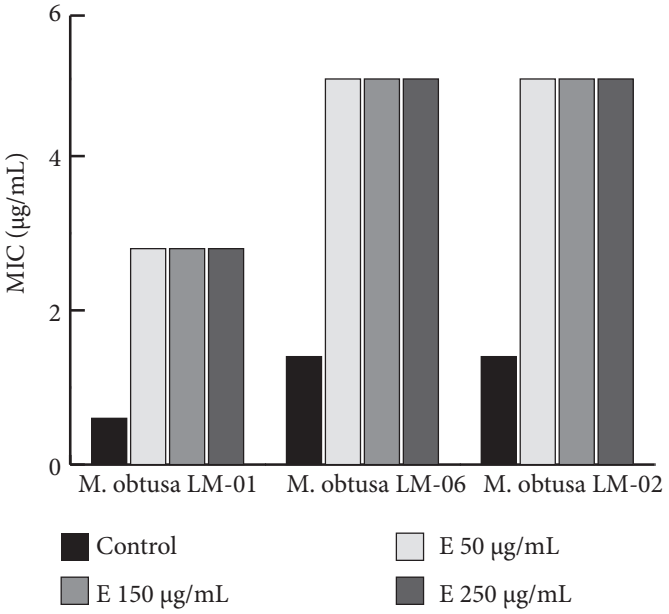

Figure 1. MIC values of *C. citratus* essential oil on strains of *Malassezia* spp. in the presence and absence of several concentrations of ergosterol (E). The results are expressed as geometric mean of two experiments

Moreover, Frost et al.[25] evaluating the activity of inhibitors of cell wall as papulacandin B (inhibitor of

β-1, 3 glucan biosynthesis) against strains of *Candida albicans* in the absence and presence of sorbitol, found respectively, increased MIC from 1.0 µg/mL to 250 µg/mL. Similar phenomenon occurred with a chitin synthesis inhibitor, the Congo red, which had its MIC increased by one hundred fold in the presence of sorbitol.

Additionally the test of ergosterol was conducted to verify the effects of essential oil of *C. citratus* on cell membrane of *Malassezia* strains. The MIC_{90} before 0.31-1.25 µL/mL, increased four times its value in the presence of ergosterol (see Figure 1), regardless of concentration tested. This fact can be associated with a complexion of the oil with the exogenous ergosterol, suggesting a possible mechanism of action on the ergosterol of fungal membrane.

Similar studies involving the test of ergosterol were conducted by other researchers, but with different results. For example, in a study by Escalante et al.[27] with B Phytolaccoside isolated from *Phytolacca tetramer* versus *Saccharomyces cerevisiae* it was observed that the MIC was unchanged in the presence of different concentrations of exogenous ergosterol (50-250 µg/mL), suggesting that the B Phytolaccoside would not act by binding to membrane's ergosterol, but by binding to other components of the fungal membrane whereas the amphipathic nature of the molecule tested.

Noting the possibility of ellagic acid has activity on fungal membrane ergosterol of *Saccharomyces cerevisiae*, Silva Junior et al.[28] observed MIC values in the presence of increased concentrations of ergosterol and, due to no changes in these values; it was suggested different mechanism of action. Then, by testing with sorbitol, it was found a MIC increase from 62 to 250 µg/mL in the presence of 0.8 M sorbitol, concluding a possible action of the acid on the synthesis of fungal cell wall.

Currently, there are drugs available for clinical use that directly interact with ergosterol, causing irreversible damage to the fungal cell membrane, like that of B amphotericin[35]. The association of essential oil of *C. citratus* with ergosterol, the main sterol present in fungal membrane, can cause a disruption of their functions up to cause the release of their cell constituents[9]. The structure and function of plasma membrane in fungal cells is essential for fungus survival, whereas the occurrence of changes in the synthesis or maintenance of cell membranes usually results in letality[36]. Therefore, data found in this study prove to be quite promising.

It is observed that the essential oil of *C. citratus* may be a promising antifungal agent to improve the treatment available, especially against *Malassezia* yeasts, whereas in addition to the antimicrobial activity observed, a possible mechanism of action on fungal plasma membrane ergosterol was observed.

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