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Original Article

Free and nanoemulsified Cymbopogon flexuosus essential oil: antifungal effect on Candida spp. in vitro

Óleo essencial de Cymbopogon flexuosus livre e nanoemulsionado: efeito antifúngico sobre Candida spp. in vitro
Aceite esencial de Cymbopogon flexuosus libre y nanoemulsionado: efecto antifúngico sobre Candida spp. in vitro

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ABSTRACT

Background and Objectives: The *Cymbopogon* genus has attracted interest due to its bioactive compounds with antifungal and antimicrobial potential. This study investigated the *in vitro* antifungal activity of free and nanoemulsified essential oil of *Cymbopogon flexuosus* against ATCC standard strains of *Candida* spp. **Methods:** The strains used in the assay were *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata*, tested alongside a positive control (Fluconazole and Amphotericin B) and a negative control (distilled water + Tween 80 at 2%). The agar diffusion technique was employed. After treatment, the assays were incubated at 36 °C for 48 hours and performed in six replicates. **Results:** Antifungal activity was observed against all ATCC strains tested. The free oil at concentrations of 10%, 15%, and 70% showed activity similar to the positive control for *Candida tropicalis*. The nanoemulsified oil at a concentration of 5% presented results comparable to the positive control for *Candida glabrata*. **Conclusion:** Among the species tested, *Candida albicans* proved to be the most sensitive at all concentrations, whereas *Candida krusei* was the most resistant to essential oil treatment. Further toxicological and clinical studies are needed to complement our findings.
Keywords: *Cymbopogon. Nanoparticles. Phytochemicals.*

RESUMO

Justificativa e Objetivos: O gênero *Cymbopogon* sp. tem despertado interesse devido aos seus compostos bioativos com potencial antifúngico e antimicrobiano. Este estudo investigou a atividade antifúngica *in vitro* do óleo essencial de *Cymbopogon flexuosus* livre e nanoemulsionado contra cepas padrão ATCC de *Candida* spp. **Métodos:** Foram utilizadas no ensaio as cepas de: *Candida albicans*, *Candida tropicalis*, *Candida Krusei* e *Candida glabrata*, comparadas a um controle positivo (Fluconazol e Anfotericina B) e controle negativo (água destilada+Tween 80 a 2%). A técnica utilizada foi de difusão em ágar. Após o tratamento, os ensaios foram incubados a 36°C, por 48 horas, sendo realizados com seis repetições. **Resultados:** No ensaio foi observada atividade antifúngica para todas as cepas ATCC testadas. O óleo livre nas concentrações de 10, 15 e 70% foi semelhante ao controle positivo para *Candida tropicalis*. A concentração de 5% do óleo nanoemulsionado apresentou resultado semelhante ao controle positivo para *Candida glabrata*. **Conclusão:** Entre as espécies testadas, a *Candida albicans* mostrou ser mais sensível em todas as concentrações; a *Candida krusei* foi a cepa mais resistente para o uso de óleo essencial. Novos estudos, de cunho toxicológico e clínico, são necessários para complementar nossos achados.
Descritores: *Cymbopogon. Nanopartículas. Compostos Fitoquímicos.*

RESUMEN

Justificación y Objetivos: El género *Cymbopogon* ha despertado interés debido a sus compuestos bioactivos con potencial antifúngico y antimicrobiano. Este estudio evaluó la actividad antifúngica *in vitro* del aceite esencial de *Cymbopogon flexuosus* libre y nanoemulsionado contra cepas estándar ATCC de *Candida* spp. **Método:** En el ensayo se utilizaron las cepas *Candida albicans*, *Candida tropicalis*, *Candida krusei* y *Candida glabrata* comparadas con un control positivo (Fluconazol y Anfotericina B) y un control negativo (agua destilada + Tween 80 al 2%). Se empleó la técnica de difusión en agar. Tras el tratamiento, los ensayos fueron incubados a 36°C durante 48 horas y realizados con seis repeticiones. **Resultados:** En el ensayo se observó actividad antifúngica para todas las cepas ATCC evaluadas. El aceite libre en concentraciones del 10%, 15% y 70% mostró actividad similar al control positivo para *Candida tropicalis*. La concentración del 5% del aceite nanoemulsionado presentó resultados comparables al control positivo para *Candida glabrata*. **Conclusión:** Entre las especies analizadas, *Candida albicans* demostró ser la más sensible en todas las concentraciones, mientras que *Candida krusei* fue la cepa más resistente al uso del aceite esencial. Se requieren nuevos estudios, de carácter toxicológico y clínico, para complementar nuestros hallazgos.
Palabras Clave: *Cymbopogon. Nanopartículas. Fitoquímicos.*

INTRODUCTION

Yeasts can colonize humans and loss of the parasite-host balance cause localized or disseminated infectious, conditions that have shown increasing incidence. Among yeasts, the genus *Candida* sp. is greatly relevant due to its high colonization and infection in human hosts.[1] *Candida albicans* is the species most frequently isolated from superficial infections, followed by *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida lusitaniae*.[2]

The treatment for *Candida* sp. pathogenicity involves antifungals, drugs that are often ineffective due to fungal resistance.[3] *Candida* sp. resistance to antifungal treatment has been attributed to recurrent infections and intermittent and continuous exposure to antifungals.[4] Thus, the decrease in yeast sensitivity to conventional antifungals has increased the interest in natural antifungal products to find new alternatives to treatment with good efficacy, spectrum of action, and tolerability.[3]

Essential oils (EOs) stand out among the novel alternatives for their phytochemical pharmacological properties in in vitro models and antibacterial and antifungal action.[5] Studies with EOs show that these oils have *in vitro* antifungal activity in *Candida* spp. strains.[6]

Of the plants producing OEs, the genus *Cymbopogon* sp. and the species *Cymbopogon flexuosus* (Nees ex Steud) Will Watson (popularly known as lemongrass) stands out. The main constituent in its EO is (3,7-dimethyl-2,6-octadienal), followed by geraniol, citronellol, and citral.[5,7] The encapsulation of bioactive compounds such as EOs aims to protect them against degradation and improve their stability to increase their durability up to fungal death.[8] Nanoemulsions (nanometric emulsions of green synthesis mediated by plants) are among the most advantageous alternatives for encapsulating EOs compared with other chemical and physical methods due to their relatively small droplet sizes, with average radii of up to 200nm.[9]

Thus, natural products have a promising potential in the development of new antifungal options. Thus, this study aims to evaluate the antifungal activity of free and nanoemulsified EOs of *C. flexuosus* against *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*.

METHODS

The EO was extracted from fresh *C. flexuosus* leaves at the Unijuí Oleochemical Complex in Três Passos/RS (27°26’02.4” latitude S and 53°57’06.7” longitude W). A modified D20 Clevenger, manufactured by LINAX, was used for 1 h 30 min for hydrodistillation. The plant was identified in the Botany Laboratory of the Universidade Regional do Noroeste do Estado do Rio Grande do Sul. The species was cataloged and registered in the Rogério Bueno University Herbarium under number 8113 (*C. flexuosus*) according to Flora do Brasil.[10]

The nanoemulsions were developed at the Nanotechnology Laboratory of Universidade Francisca in Santa Maria/RS by a high-energy method without organic solvents and with temperature control.[11] The formulations (n=3) were obtained after an injection of an oily phase (5% oil) and 2% sorbitan monooleate (Span 80®) in the aqueous phase (2% polysorbate 80 - Tween 80® - and ultrapure water) under high agitation in an T18 Ultra-Turrax® (IKA®, Germany at 10,000 rpm). After mixing, the mixture was agitated at 17,000 rpm for 45 minutes. During the obtention of the nanoemulsions, a temperature control (ice bath) was carried out to prevent volatilization and/or the degradation of the oil constituents. For comparisons, negative control formulations were prepared (n=3) using capric/caprylic triglyceride mixture. All formulations were prepared in triplicates and stored in the dark. The final concentration of the nanoemulsified EO totaled 5%.

Antifungal activity was evaluated by agar diffusion with ATCC strains of *C. albicans* (CAMT05), *C. tropicalis* (CTMT16), *C. krusei* (ATCC6258), and *C. glabrata* (CGMT01) strains that were donated by the Applied Mycology Research Laboratory at Universidade Federal do Rio Grande do Sul. The strains were seeded in sterile plates with a Sabouraud agar medium by loop sowing. Cell density was adjusted with a spectrophotometer, adding enough saline solution up to a standard solution equivalent to the 0.5 McFarland scale at a 625-nm wavelength. After being seeded with the fungus, the surface of the plates was drilled at different points, forming holes with about 5 mm in diameter.

For the antimicrobial susceptibility tests, the concentrations were prepared from free and nanoemulsified EO, which were diluted in water and emulsified in 2% polysorbate 80 (Tween™ 80) (Table 1).

Table 1. Concentrations (%) of free and nanoemulsified essential oils prepared for testing.

	Tested concentrations (µL mL⁻¹)	Concentration OE in %	Description
Treatment 1 - Free EO	25; 50; 100; 300; 700; 1000µL	2.5, 5, 10, 15, 30, 70, 100%	Free *Cymbopogon flexuosus* + sterile distilled water
Treatment 2 – Nanoemulsified EO b*	15; 30; 60; 125; 1000µL	0.3125, 0.625, 1.25, 2.5, 5%	Nanoemulsified *Cymbopogon flexuosus* + sterile distilled water
Positive control	-	-	Fluconazole and Amphotericin B
Negative Control	-	-	Sterile distilled water with Tween 80 at 2%

Abbreviation: b* At the end of the emulsification, the nanoemulsified essential oil had a 5% concentration.

The concentrations were prepared in sterile Eppendorf microtubes in sufficient quantities for 1mL. After adding the components, the microtubes were subjected to a mechanical vortex agitator for two minutes; an operation that was immediately repeated before each experiment.

In the plates, 20-µL (microliter) aliquots of essential oil from each solution concentration were applied to each orifice. After preparing the plates with the treatments, the plates were incubated at 36°C for 48 hours. Then, the diameters of the inhibition halos were measured in mm. In total, two positive controls were used: a) The results were evaluated by measuring the diameters of the growth inhibition halos (mm). The assay was performed in six replicates.

The results were tabulated on Microsoft Office Excel® and analyzed on the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), version 23.0. The data are shown as means and standard deviations. For each tested fungus, all concentrations were compared by the one-way analysis of variance and the Tukey's post-hoc tests.

Based on the project description, this research required no consideration by the Ethics Committee for Research with Human Beings or by the Ethics Committee for the Use of Animals since no human beings or vertebrate animals were used in the experimental stages of this research.

RESULTS

This study obtained positive results (Table 2). The 24-hour results for *C. glabrata* and *C. tropicalis* strains and the 10, 15, 30, and 70% of free EO concentrations did not statistically differ from the positive control. For *Candida albicans*, the 10 and 70% doses showed efficacy but with no differences from the positive control. *C. krusei* showed no inhibition halo in any free EO concentration. At the 100% free EO concentration, the plates showed no fungal growth for any tested species 24 hours after incubation since such EO concentration inhibited any growth on the entire plate in the test, making it impossible to read the halo.

The mean free EO 10, 15, 30, and 70% concentrations show no mean differences for *C. albicans*, *C. glabrata*, and *C. tropicalis* 48 hours after incubation when compared with the positive control, which showed a 29.17-mm inhibition halo, indicating good antifungal activity.

Table 2. Inhibition halos (mm) formed by the antifungal action of free *C. flexuosus* essential oil on C. spp. strains after 24 and 48 hours.

	Candida albicans CAMT05						*Candida glabrata CGMT01*					
	24h			48h			24h			48h		
Concentration in %	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2
2.5	13.17	1.47	B	21.50	4.37	B	14.83	1.83	B	18.83	0.98	B
5	19.00	0.89	B	20.50	2.17	B	18.67	1.21	B	20.33	1.86	B
10	27.17	1.17	A	29.67	1.51	A	27.83	1.47	A	26.83	3.13	A
15	22.67	1.75	B	28.67	3.01	A	24	3.01	A	23.83	1.94	A
30	19.67	1.51	B	28.33	2.66	A	25.83	3.92	A	25.83	3.92	A
70	25.83	2.99	A	26.83	2.40	A	25.33	4.18	A	27.17	3.13	A
100	0	0		0	0		0	0		0	0	
C+	28.33	1.21	A	29.17	1	A	26.67	4.32	A	28.17	3.43	A
C-	0	0	B	0	0	B	0	0	B	0	0	B
p1	<0.001			<0.001			<0.001			<0.001		

	Candida krusei ATCC6258						*Candida tropicalis CTMT16*					
	24h			48h			24h			48h		
Concentration In %	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2
2.5	0	0	B	0	0	B	18.67	2.73	B	16.83	2.23	B
5	0	0	B	0	0	B	20.33	1.97	B	16.67	2.07	B
10	0	0	B	0	0	B	32.67	2.42	A	24.69	1.79	A
15	0	0	B	0	0	B	31.33	1.63	A	29.83	2.71	A
30	0	0	B	0	0	B	27.84	2.34	A	28.67	3.27	A
70	0	0	B	0	0	B	28.50	2.81	A	23.17	3.13	A
100	0	0	B	0	0	B	0	0	B	0	0	B
C+	21.67	2.34	A	25.33	3.01	A	29.00	3.52	A	29.33	3.50	A
C-	0	0	B	0	0	B	0	0	B	4.67	11.43	B
p1	<0.001			<0.001			<0.001			<0.001		

Abbreviation: c+ = positive control; c−= negative control; SD=standard deviation; p1=1-way analysis of variance. P2=post hoc test to compare each concentration and c+; A (no significant difference between concentrations and c+; b (value significantly lower than c+).

The nanoemulsified EO showed inhibition halos at 1.2–-5% concentrations for all strains. For *C. tropicalis*, 24h after the incubation, only the 5% concentration had an effect equal to that of the positive control (Table 3); the 2.5 and 5% doses stood out at 48 hours. For *C. krusei*, at 48h, the 1.25, 2.5, and 5% concentrations showed inhibition halos equal to the control group, as in *C. albicans* at 24h and 48h. The 0.3% concentration showed no inhibition halos in the tested strains, except in *C. glabrata*, which averaged 12±14mm. *C. krusei* and *C. tropicalis* better resisted the 0.325 and 0.625% concentrations of the nanoemulsified oil.

Table 3. Nanoemulsified EO data. Inhibition halos (mm) formed by the antifungal action of nanoemulsified *C. flexuosus* essential oil on strains *Candida* spp. after 24 and 48 hours.

	Candida albicans *CAMT05*						*Candida glabrata* *CGMT01*					
	24h			48h			24h			48h		
Concentration in %	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2
0.31	0	0	B	0	0	B	12.333	1.5055	B	14.667	3.7238	B
0.63	15.33	3.93	B	19.67	1.97	B	13.67	2.66	B	16.33	4.46	B
1.25	21.67	4.63	A	25.33	4.84	A	25.17	3.82	A	25.50	3.78	A
2.5	19.50	2.66	B	24	3.88	A	17.33	3.72	A	21.67	3.67	A
5	20.33	5.28	A	20.33	7.20	B	25.67	3.44	A	29.67	3.20	A
C+	26.00	3.10	A	28.00	4.56	A	23.17	4.92	A	27.00	4.52	A
C-	0	0	B	0	0	B	0	0	b	0	0	B
p1	<0.001			<0.001			<0.001			<0.001		
	Candida krusei *ATCC6258*						*Candida tropicalis* *CTMT16*					
	24h			48h			24h			48h		
Concentration in %	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2	Mean	SD	p2
0.31	0	0	B	0	0	B	0	0	B	0	0	B
0.63	0	0	B	7.33	5.75	B	0	0	B	0	0	B
1.25	21.67	2.34	B	23.17	3.92	A	20.33	1.97	B	21.67	2.34	B
2.5	22.67	1.63	A	22.33	1.51	A	25.67	3.44	B	28.67	2.73	A
5	27.33	6.15	A	23.33	7.45	A	29.33	3.01	A	26.00	3.10	A
C-	27.00	3.29	A	29.00	2.76	A	30.33	3.44	A	32.00	1.79	A
C-	0	0	B	0	0	B	0	0	B	0	0	B
p1	<0.001			<0.001			<0.001			<0.001		

Abbreviation: c+ = positive control; c-= negative control; SD= standard deviation; p1=1-way analysis of variance. P2=post hoc test to compare each concentration and C+; A (no significant difference between concentrations and c+; b (value significantly lower than c+). Source: developed by the author, 2024.

DISCUSSION

The antifungal biological activity of the free and nanoemulsified EOs in this study inhibited fungal growth. This effect is related to their complex phytochemical composition, which includes citral, pinene, cineole, caryophyllene, elemene, furanodiene, limonene, eugenol, eucalyptol, carvacrol, and others. Such constituents have antiseptic, antibacterial, antifungal, and antiparasitic properties.[12]

Similar research has evaluated the antifungal activity of the *Cymbopogon winterianus* and *Cymbopogon martinii* OEs in *C. albicans* strains. It observed that at a 15% concentration, both oils showed growth inhibition halos that averaged above 32mm, corroborating this study, which found that free *C. flexuosus* at 10 and 15% concentrations showed similar results for *C. albicans*, averaging 29mm. In our assays, the *C.* spp. strains, except *C. krusei*, showed inhibition due to low *C. flexuosus* concentrations, just as in a study on *Cymbopogon citratus* EO in isolated strains of *C. albicans* and *C. tropicalis* in 10, 15, 25, 35, 50, and 60% concentrations.[13] It observed that, at the 15% concentration, the EO showed halos averaging 21mm in *C. albicans*, similar to the results in our study, which, at this concentration, showed a 22-mm mean halo.

A study evaluated the fungicidal effect of *Rosmarinus officinalis* Linn EO in an agar diffusion in *C. dubliniensis*, *C. albicans*, *C. parapsilosis*, and *C. krusei* strains.[14] Its results showed inhibition halos ranging from 39 to 47mm due to its free essential oil. Those authors also observed an inhibition halo for *C. krusei*, unlike our study. *R. officinalis* EO has α-Pinene and 1,8-Cineole (cineole) in its phytochemical composition, which may be responsible for the antifungal effect against this strain. Note the divergent methodologies between that study and ours, which may contribute to such findings due to the absence of standardized techniques to evaluate antifungal activity (unlike the evaluation of antifungal drugs, which was standardized by the Clinical and Laboratory Standards Institute M27-A3 methodology).

C. krusei, responsible for invasive infections, differs from other species due to its intrinsic resistance to the antifungal fluconazole stemming from its morphological and metabolic characteristics. Such information may influence the therapeutic approach and pathogenicity of this species when compared to others, which may explain our findings, in which *C. krusei* showed no fungal growth for free EO, only halo formation at 1.25, 2.5, and 5% concentrations of the nanoemulsified EO.[15] Free EOs are volatile, have a low molecular weight, and enter a gaseous state when exposed to the environment. On the other hand, nanoemulsions protect EOs against degradation in harsh environments, providing sufficient stability, persistence, and permeability over time.[16] This may justify the results of this study, indicating a potential use of nanoemulsified *C. flexuosus* EO on *C.* spp.

Researchers have conducted a similar investigation on the effect of free and nanoemulsified *Origanum vulgari* L., (oregano) EO in a 5% concentration by oil mass.[17] They found that free and nanoemulsified OEs showed effective activity against the tested strains, especially for *C. albicans*. These results corroborate this research, in which the free and nanoemulsified *C. flexuosus* EOs also showed antifungal effects against *C. albicans*. Although these above used different EOs, both have terpenes, which may be responsible for the observed effect.

Researchers have also evaluated the effect of the antifungal activity of EOs, including of *Laurus nobilis*, *Thymus vulgaris*, *Mentha piperita*, *Cymbopogon citratus*, and *Lippia junellian* on clinical *C. krusei*, *C. albicans*, *C. glabrata*, and *C. parapsilosis* strains.[18] Among the tested oils, the one with the greatest activity on these strains was that of *C. citratus* and *L. nobilis*. Of the tested strains, *Candida albicans* showed the greatest sensitivity to these EOs. Both EOs showed efficacy against the tested strains, obtaining antifungal effects at low concentrations. The phenolic compounds responsible for the antifungal activity of EOs can be evalulated at the macromorphological and cellular levels.[8] Macromorphological changes include lack of sporulation or pigmentation, change in the number of conidia, increase in the branching of the hyphae, or change in their size.[19] EOs can inhibit the synthesis of DNA, RNA, proteins, and polysaccharides in fungi and bacterial cells, which can cause similar changes to the mechanism of antibiotic activity.[19,20]

The findings evince that the tested free and nanoemulsified EO showed significant fungal activity in the tested species and that the 10, 15, and 70% concentrations obtained similar results to the positive control. The free EO 100% concentration failed to inhibit the halos of all tested strains. The nanoemulsified EO at 1.25, 2.5, and 5% showed inhibition halos for all tested strains; its 5% concentration showed a result similar to the positive control. *C. albicans* showed greater inhibition halos. Comparing the means of the free and nanoemulsified EO inhibition halos obtained positive results.

Despite its promising results, this study has some limitations that require consideration. It only carried out *in vitro* tests, which limits the extrapolation of its data to clinical applications as it ignored issues such as toxicity, safety, and efficacy in more complex biological models. It also neither investigated the mechanisms of action of the active compounds in the EOs nor the stability of the nanoemulsions over time.

Free and nanoemulsified *C. flexuosus* EOs showed antifungal activity against the tested strains. However, it is crucial to carry out more studies to better understand the mechanisms of action of EOs and to consider safety and toxicity issues to develop formulations that guarantee stable compounds. Ultimately, research on EOs offers promising prospects for the development of new therapeutic strategies to combat fungal infections. We emphasize the need for clinical studies to evaluate its effectiveness.

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AUTHORS' CONTRIBUTIONS

Dara Monize Pazze and **Christiane de Fátima Colet** contributed to the bibliographic research, writing of the abstract, introduction, methodology, and discussion, interpretation and description of the results, preparation of tables, conclusions, review, and statistics. **Kétlin Luiza Strada** and **Karine Raquel Uhdich Kleibert** contributed to project management, bibliographic research, writing of the abstract, introduction, methodology, and discussion, interpretation and description of results, conclusions, review, and statistics. **Gabriela Matte Bertoldi**, **Ivan Ricardo Carvalho** and **José Antônio Gonzalez da Silva** contributed to the writing of the abstract, and methodology, interpretation of results, conclusions, review, and statistics. **Fernanda Wagner Boz** contributed to the writing of the abstract, review, and statistics. **Patrícia Gomes** and **Giane Engel Montagner** contributed to project administration, fund acquisition, literature research, proofreading, and statistics. **Dara Monize Pazze** and **Christiane de Fátima Colet** contributed to project management, bibliographic research, writing of the abstract, introduction, methodology, and discussion, interpretation and description of results, conclusions, review, and statistics.

All authors approved the final version to be published and are accountable for all aspects of this study, including ensuring its accuracy and integrity.

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