Short research article

Antifungal effectiveness of *Citrus sinensis* against *Candida albicans* in the dental field. *In vitro* study

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SUMMARY

Introduction: This study focuses on the antifungal effectiveness of *Citrus sinensis* essential oil against *Candida albicans*, a common fungal infection in the oral cavity. **Objective:** To analyze the efficacy of *Citrus sinensis* essential oil against *C. albicans* at different concentrations. **Methodology:** A microbiological analysis using different concentrations of *Citrus sinensis* essential oil and the evaluation of its effect by measuring inhibition halos were included. Non- parametric statistical methods, such as the Kruskal-Wallis test, were applied to analyze the relationship between the concentration of the oil and its antifungal efficacy. **Results:** It was shown that, although *Citrus sinensis* oil did not present statistically significant differences in its antifungal activity at the different concentrations, an increasing trend in efficacy was observed as the concentration increased. This suggests that its use at higher concentrations could be beneficial in complementary treatments. **Conclusion:** *Citrus sinensis* essential oil could be considered as a complementary option in the treatment of fungal infections, but more research is needed to understand its synergy with other oils and its application in dentistry.

Keywords*: Candida albicans*; oral candidiasis; *Citrus sinensis*; microbial sensitivity.

RESUMEN

Eficacia antifúngica de *Citrus sinensis* contra *Candida albicans* en la cavidad bucal. Estudio *in vitro*

Introducción: Este estudio se centra en la eficacia antifúngica del aceite esencial de *Citrus sinensis* contra *Candida albicans*, una infección fúngica común en la cavidad bucal. **Objetivo:** Analizar la eficacia del aceite esencial de *Citrus sinensis* contra *C. albicans* en diferentes concentraciones. **Metodología:** Se realizó un análisis microbiológico con diferentes concentraciones de aceite esencial de *Citrus sinensis* y se evaluó su efecto mediante la medición de halos de inhibición. Se aplicaron métodos estadísticos no paramétricos, como la prueba de Kruskal-Wallis, para analizar la relación entre la concentración del aceite y su eficacia antifúngica. **Resultados:** Se demostró que, si bien el aceite de *Citrus sinensis* no presentó diferencias estadísticamente significativas en su actividad antifúngica a las diferentes concentraciones, se observó una tendencia creciente en la eficacia a medida que aumentaba la concentración. Esto sugiere

que su uso a concentraciones más altas podría ser beneficioso en tratamientos complementarios. **Conclusión:** El aceite esencial de *Citrus sinensis* podría considerarse una opción complementaria en el tratamiento de infecciones fúngicas, pero se necesita más investigación para comprender su sinergia con otros aceites y su aplicación en odontología.

Palabras clave: *Candida albicans*; candidiasis oral; *Citrus sinensis*; sensibilidad microbiana.

RESUMO

Eficácia antifúngica do óleo essencial de *Citrus sinensis* contra *Candida albicans* no ambiente odontológico. Estudo *in vitro*

Introdução: Este estudo concentra-se na eficácia antifúngica do óleo essencial de *Citrus sinensis* contra *Candida albicans*, uma infecção fúngica comum na cavidade oral. **Objetivo:** Analisar a eficácia do óleo essencial de *Citrus sinensis* contra *C. albicans* em diferentes concentrações. **Metodologia:** Foi realizada uma análise microbiológica utilizando diferentes concentrações do óleo essencial de *Citrus sinensis* e a avaliação do seu efeito pela medição dos halos de inibição. Métodos estatísticos não paramétricos, como o teste de Kruskal-Wallis, foram aplicados para analisar a relação entre a concentração do óleo e sua eficácia antifúngica. **Resultados:** Demonstrou-se que, embora o óleo de *Citrus sinensis* não tenha apresentado diferenças estatisticamente significativas em sua atividade antifúngica nas diferentes concentrações, observou-se uma tendência crescente de eficácia com o aumento da concentração. Isso sugere que seu uso em concentrações mais elevadas pode ser benéfico em tratamentos complementares. **Conclusão:** O óleo essencial de *Citrus sinensis* pode ser considerado uma opção complementar no tratamento de infecções fúngicas, mas são necessárias mais pesquisas para compreender sua sinergia com outros óleos e sua aplicação na odontologia.

Palavras-chave: *Candida albicans*; candidíase oral; *Citrus sinensis*; sensibilidade microbiana.

1. INTRODUCTION

Oral diseases, according to the World Health Organization, dental caries, periodontal disease and candidiasis are part of a public health problem [1]. The etiologic agent of oral candidiasis, *C. albicans*, an opportunistic dimorphic yeast, is part of the normal oral microbiota in 30-60% of healthy adults [2, 3]. However, in circumstances such as immunosuppression or the use of dental prostheses may provide its pathogenic transformation, causing recurrent fungal infections [4–6]. Oral candidiasis, which presents as white plaques on the oral mucosa, is clinically diagnosed and treated with topical or systemic antifungals in severe cases [6, 7].

C. albicans has the ability to form biofilms, which increases resistance to conventional treatments, particularly with polyenes and azoles, due to mechanisms such as efflux pumps, gene regulation and enzyme mutations [1, 8, 9]. This phenomenon highlights the need to explore new therapeutic alternatives to combat antifungal resistance and avoid the toxicity generated by these conventional therapies [10, 11].

Phytotherapy, based on natural compounds derived from plants, has gained attention for its antimicrobial potential [12]. Essential oils present antifungal, anti-inflammatory and antioxidant properties acting through mechanisms such as changes in cell membranes and formation of reactive oxygen species [13–16]. These mechanisms act as cell membrane variation and the reproduction of oxygen in reactive species. In this research we will focus on the essential oil of *Citrus sinensis*, where previous in vitro studies have reported its efficacy against various Candida species [16–18]. In view of the above, the present investigation was aimed at evaluating the antifungal effectiveness of *Citrus sinensis* essential oil against *C. albicans* at different concentrations, applying it in dentistry.

2. METHODOLOGY

This is a longitudinal laboratory study, carried out in the laboratories of the Center for Research, Innovation and Technology Transfer of the Catholic University of Cuenca (CIITT), under the approval of CEISH-UCACUE code 108-2024, with exempt category, this project responds to one of the objectives of a research project approved in the call for Sustainable Development Goals (ODS-UCAUCE) whose coding corresponds to PICODS21-21.

2.1. Inclusion criteria:

Candida albicans ATCC 60193 (resistant strain), pure and certified oils (*Citrus sinensis*).

2.2. Exclusion criteria:

Oils with other compounds, sensitive *C. albicans ATCC* strain, clinical isolation of *C. albicans* of human origin and contaminated samples.

2.3. Microbiological analysis

2.3.1. Activation and seeding of Candida albicans: The strain was activated and cultured MuellerHinton (MH) agar medium, prepared according to the manufacturer's technical specifications, ensuring the quality and reproducibility of the culture medium. Before inoculation, dilution of the strain was performed by adjusting it to a density of 0.5 on the McFarland scale, which is approximately equivalent to 1.5×10^8 colony forming units per milliliter (CFU/mL), ensuring a standard concentration of microorganisms for microbiological tests.

Inoculation was carried out using the mass seeding technique inside a laminar flow cabinet (BIOAIR-TopSafe), which allowed uniform distribution of the microorganism over the surface of the culture medium, minimizing contamination and ensuring consistent results. Subsequently, the inoculated plates were incubated in a laboratory oven (Memmert model UF110) at a controlled temperature of 25 °C, for a period of 24 to 48 hours, sufficient time to observe the growth and inhibitory effects of the substances under study.

2.4. Preparation of concentrations and controls:

Solutions of the oil to be evaluated were prepared in concentrations of 25%, 50%, 75% and 100%, using fluconazole (positive control) and distilled water (negative control). All concentrations were worked in triplicate to guarantee the reproduction and precision of the results. The technique used for the inoculation of *Candida albicans* was by mass seeding in the triplicate boxes. Blank sensitivity discs were placed on the medium and the different concentrations of the oil and the controls were deposited on the corresponding discs.

2.5. Observation, measurement and analysis of results:

After the incubation time, the formation of inhibition halos was evaluated in each of the boxes. The measurements of the halos were recorded and analyzed to determine the antifungal activity of the different concentrations of the oil, comparing the results with the positive controls. This procedure allowed an analysis and control of the antifungal activity, ensuring optimal conditions for reproducibility and reliability of the results (Figure 1).

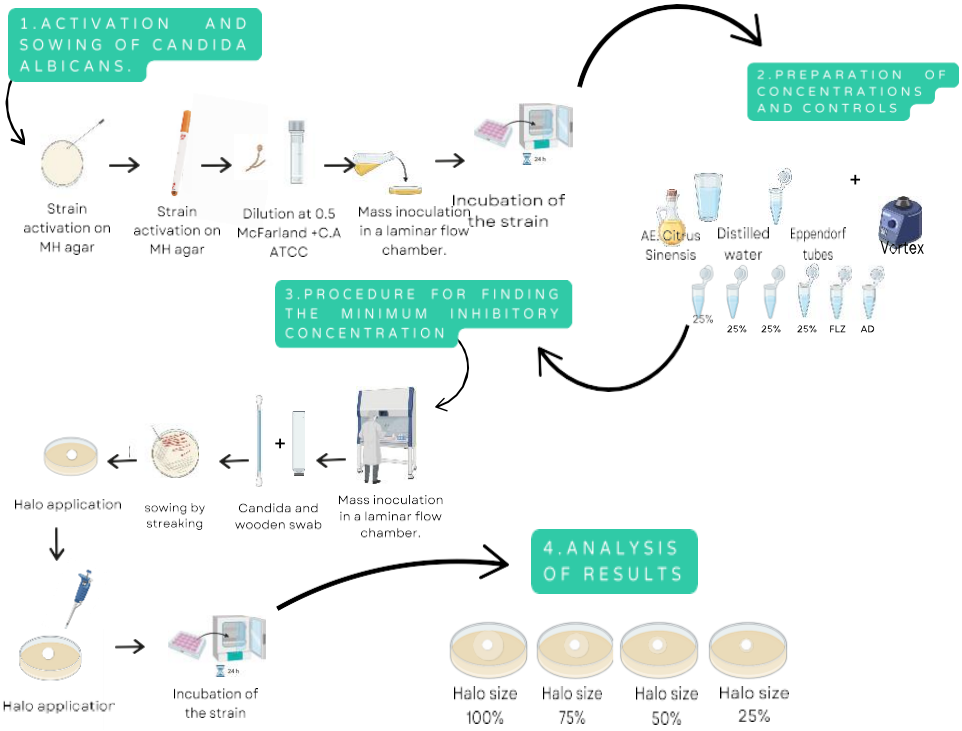

Figure 1. Flow chart of the microbiological analysis and antifungal effectiveness of the essential oil *Citrus Sinensis*. Authors' elaboration.

3. RESULTS

A statistical analysis was performed using non-parametric methods to evaluate the relationship between the variables, concentration and halo. For this purpose, the Kruskal-Wallis test was implemented and the data were analyzed by means of a box plot. All the analysis was performed using SPSS version 26 software, guaranteeing accuracy and reliability in the statistical calculations. The data did not have normality, so nonparametric analysis of the independent variable was performed. The Null Hypothesis states that the halo distribution is the same among the different *Citrus sinensis* concentration categories. To evaluate this hypothesis, a Kruskal-Wallis test for independent samples was carried out. The significance value (Sig.) obtained was 0.069. Based on this result, it was decided to retain the null hypothesis. The data did not have normality, so the nonparametric analysis of the independent variable was performed. The significance value of 0.069 shows that there is not enough statistical evidence to reject the null hypothesis at the 0.05 significance level. Under the conditions of the study, *Citrus sinensis* essential oil showed no significant difference in its antifungal activity against *Candida albicans* among the different concentrations evaluated.

Table 1. Inhibition halo measurements according to *Citrus sinensis* concentrations and controls.

Plant species	Concentrations (%)	Measuring ranges (mm)
Citrus sinensis	25	5
	25	5
	25	6
	50	9
	50	9
	50	6
	75	13
	75	11
	75	6
	100	13
	100	13
	100	9
Positive control (Flz)		19
Negative control (Distilled water)		0

The measurements and concentrations presented show that for the 5 mm value the concentrations are 25% on two occasions; for the 6 mm value, there are concentrations of 25% on one occasion and 50% on two occasions; then, for the 9 mm measurement, there are concentrations of 50% on two occasions and 100% on one occasion. The 11mm measurement has a concentration of 75%, while for the 13 mm value, there are concentrations of 75% on one occasion and 100% on two occasions. In the 19 mm measurement, a fluconazole value is mentioned (positive control) and finally, the 0 mm measurement represents distilled water (negative control), indicating no concentration.

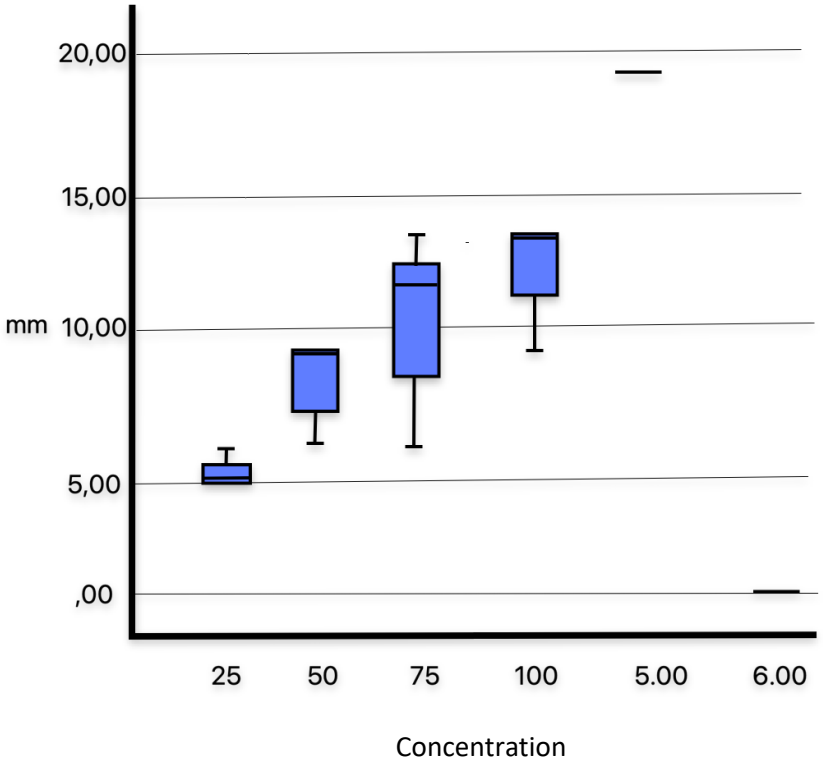

Figure 2. This graph shows the results with respect to the inhibition halos found.

Concentrations of 25%, 50%, 75% and 100% inhibition halo were evaluated by the KirbyBauer method to analyze the antifungal effect. Controls were established, where the positive control (5) showed a high performance, indicating a significant effect against *Candida albicans,* and the negative control (6) showed a null effect, showing a lack of antifungal activity. As for the results by concentration, at 25% there was a minor inhibition halo, indicating limited antifungal activity. At 50%, a modest increase in activity, while at 75% a significant increase was achieved compared to the lower concentrations. Finally, at 100%, the highest antifungal activity was reflected, approaching the results of the positive control. The results show a clear increasing trend in antifungal activity with increasing concentration of *Citrus sinensis* essential oil, suggesting a cumulative effect. This analysis was carried out using the latest generation SPSS software, which allowed a more accurate interpretation of the data.

3.1. Analysis of results

At the 25% concentration, the median is 5 mm, indicating low antifungal activity. The dispersion of the data is very narrow, with a small interquartile range (IQR), suggesting that the measurements are homogeneous and show little variability. Furthermore, no outliers are identified, reinforcing the consistency of the results. When increasing to 50%, the median reaches approximately 7 mm, suggesting an improvement in antifungal activity, and although the scatter increases, showing longer whiskers on the graph, no visible outliers are observed, maintaining the relative consistency of the data [19]. At the 75% concentration, the median is noticeably higher, close to 10 mm, reflecting evident antifungal activity. However, the variability is greater than at the 50% concentration, with a wide IQR and longer whiskers, suggesting considerable scatter in the data; here an outlier is detected near 20 mm, indicating the need for further analysis to understand its origin. Finally, at the 100% concentration, the median resembles that of 75%, albeit with a slightly lower range, evidencing a high level of antifungal activity. The variability is lower compared to the previous concentration, with a smaller IQR and shorter whiskers, indicating more concentrated data in its distribution, and no outliers are observed, suggesting greater stability in the comparative results [20].

4. DISCUSSION

This research focuses on the antifungal activity of *Citrus sinensis ATCC* essential oil, with the objective of analyzing the efficacy of the essential oil against *C. albicans* at different concentrations.

The results obtained for *Citrus sinensis* essential oil indicate that, although no statistically significant differences were found in its antifungal activity at different concentrations, an increasing trend in efficacy was observed as the concentration increased. This suggests that, although *Citrus sinensis* essential oil may not be the most potent, its use at higher concentrations could be beneficial, especially in a context of complementary treatments. However, it was noted that at concentrations of 50% and 75% the effect was minimal.

In comparison with the study of Ortiz-Timbi, the essential oil of *Cinnamomum verum* showed remarkable antifungal activity, with a minimum inhibitory concentration (MIC) of 39 ppm, indicating a high sensitivity of *Candida albicans* [21]. Similarly, in the study by Velasteguí-Pazos, *Melaleuca alternifolia* oil proved to be effective at concentrations of 75% and 100%, presenting significant inhibition halos [22]. These findings position cinnamon oil and tea tree

oil as more potent options in the fight against fungal infections. According to research conducted by Guazanda-Orrala, the essential oil of *Piper imperiale* also showed considerable potential, with a MIC of 6894.286 μg/mL, suggesting that it may be a viable alternative to traditional antifungal treatments [23]. The ability of this oil to inhibit the growth of *Candida spp*. highlights the importance of exploring essential oils as therapeutic options.

On the other hand, citing the work of Vasquez-Gavidia, the essential oil of *Matricaria chamomilla* (chamomile) was shown to have a significant antifungal effect, especially at higher concentrations, suggesting its potential in the treatment of fungal infections [24]. Likewise, according to Valverde-Quinaluisa, oregano oil has been recognized for its antimicrobial properties, making it an interesting candidate for inclusion in antifungal treatments [25].

According to Lalangui-Pazmiño and E.W. Palacios-Paredes alike to Echeverría-Erazo, *Schinus molle* and *Eucalyptus globulus* oils, known for their antimicrobial properties, also deserves attention, although its specific efficacy against *Candida spp.* requires further research [26, 27]. Moreover, analysis reported by Alberca-Torres and Dilas-Castillo regarding ginger oil (*Zingiber officinale*), its antifungal potential has been documented, suggesting that it could be useful in the formulation of combination treatments [28].

The research by Paucar-Rodríguez *et al.* indicates that the essential oil of *Minthostachys mollis* showed remarkable inhibitory activity against several pathogens, including *Candida albicans*, suggesting its potential in the treatment of fungal infections [29]. Finally, Churata-Oroya *et al.* indicated that *Citrus paradisi* (grapefruit) oil presented the most effective antifungal activity against clinical strains of *Candida albicans* at a concentration of 25% [30].

Analysis of *Citrus sinensis* essential oil showed moderate antifungal activity that improved with concentration, with inhibition halos of 5 mm at 25% and 10 mm at 75%. At 100%, the activity remained high, indicating a positive cumulative effect. In comparison, *Cinnamomum verum* stands out with an MIC of 39 ppm, demonstrating high sensitivity against *Candida albicans*, consolidating itself as one of the most effective oils. Similarly, *Melaleuca alternifolia* showed significant efficacy at concentrations of 75% and 100%, positioning itself next to cinnamon oil in effectiveness. On the other hand, *Piper imperiale* has a MIC of 6894.286 μg/mL, suggesting lower effectiveness compared to the previous ones, although, its potential as an alternative is recognized, so *Citrus sinensis* oil offers moderate antifungal activity, while *Cinnamomum verum* oil and *Melaleuca alternifolia* oil are more potent options for the treatment of fungal infections.

5. CONCLUSION

Research on the antifungal effectiveness of *Citrus sinensis* essential oil against *Candida albicans ATCC* 60193 has revealed important findings. Although *Citrus sinensis* oil shows moderate antifungal activity compared to other essential oils and no significant differences in efficacy were detected among the concentrations studied, its safety profile and availability make it an interesting candidate for use in combination with more effective oils.

It is essential to continue exploring natural alternatives such as essential oils, not only to diversify therapeutic options, but also to address the increasing resistance to conventional antifungals. Future research could focus on the synergy between *Citrus sinensis* essential oil and other oils, as well as its application in complementary treatments for fungal infections, especially in patients with compromised immune systems. Although the current results do not support the oil as a conclusive solution against candidiasis, they open the door to further research that could expand our knowledge and offer new hope in the treatment of these infections.

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CONFLICT OF INTEREST

It is explicitly stated that there is no conflict of interest in conducting this research.

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