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Actividad antifúngica de *Rosmarinus officinalis* Linn. aceite esencial contra *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis* y *Candida krusei*

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ABSTRACT

The antifungal and anti-germ tube formation activity of *Rosmarinus officinalis* Linn. essential oil was tested against four *Candida* strains (*C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. krusei*). Inhibition halo sizes and minimal inhibitory concentrations (MIC) were obtained using radial diffusion and micro dilution tests, respectively. The minimal fungicidal concentration (MFC) was obtained from the MIC assay. Additionally, the effect of the essential oil on germ tube formation in *C. albicans* and *C. dubliniensis* was evaluated. The MIC_{50} ranged from 0.5% to 2%, while the MFC ranged from 1% to 2%. We observed total inhibition of germ tube formation in *C. albicans* and *C. dubliniensis*. The *R. officinalis* Linn. essential oil displayed powerful inhibitory and fungicidal activity against specific *Candida* strains.

Keywords: *Candida* Species; *Rosmarinus officinalis*; Essential Oil.

INTRODUCTION

The uses, effects, and pharmacological properties of medicinal plants have been widely investigated in phytotherapy[1]. Studies have shown that some aromatic plants and spices possess inhibitory activities against bacteria and yeast[2,3]. The specific anti-*Candida* activities of some extracts[4,5,6], essential oils[7,8,9] and their purified components[10,11,12] are well known. Similarly, *Rosmarinus officinalis* Linn. extract has been demonstrated to be active against *Streptococcus sanguinis* (ATCC 10556), *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinus* (ATCC 27609), *Lactobacillus casei* (ATCC 7469)[13] and Herpes Virus type-1[14], and it has also been shown to act as an anti-oxidative stress agent in diabetes[15]. Lima et al.[16] reported the activity of *R. officinalis* Linn. essential oil against *C. albicans* (ATCC-76615). However, few assays have further

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investigated this activity in *C. albicans* and other *Candida* species. Thus, the present study examined the antifungal activity of *R. officinalis* Linn. essential oil against *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. krusei* by determining the effective inhibitory and fungicidal concentrations as well as evaluating the effect of *R. officinalis* Linn. essential oil on germ tube formation in related species.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

This study used *C. albicans* (INCQS 49175), *C. dubliniensis* (CBS 7987), *C. parapsilosis* (ATCC 29019) and *C. krusei* (ATCC 6258). All of the strains were grown on Sabouraud dextrose Agar (Difco Laboratories, Detroit/MI) under aerobic conditions at 37°/C for 24 h before the antifungal assays. The yeast (107 cells/mL) suspensions used in the assays were prepared in sterile phosphate-buffered saline (PBS) at pH 7.2.

OBTAINING *R. officinalis* LINN.

Rosmarinus officinalis Linn. was grown in the botanical garden Jacques Huber, located on the Coordination of Botany, the research campus of the Museu Paraense Emílio Goeldi (Belém, Pará State, Brazil). Plants were grown under 50% shade, potted in black polyethylene with dimensions of 20 × 25 cm, filled with black soil substrate and irrigated as needed to maintain the humidity of the substrate. The specimens were cultured during the rainy season and were not exposed to any type of chemical, such as pesticides or fertilizers. The botanical materials collected (by Gauch, L.M.R 01) were held at the growing site, and the phenophase was adult flower buds. *R. officinalis* Linn. was identified by Ely Simone Cajueiro Gurgel (Museu Paraense Emílio Goeldi, Pará State, Brazil) using MG 204.248.

PREPARATION OF *R. officinalis* LINN. ESSENTIAL OIL

The essential oil was obtained by steam distilling fresh leaves (350 g) for 240 minutes using the Clevenger system. Two milliliters of the essential oil was obtained from this process, and it was stored in a dark, cool place.

ANTIFUNGAL TESTS

The agar diffusion method was conducted as previously described[17]. Briefly, Petri plates containing Sabouraud dextrose agar (Difco Laboratories, Detroit/MI) were inoculated with yeast suspension using a swab. After 15 minutes, 5 mm wells were made, and 100 μL of essential oil was deposited into each well. We tested the susceptibility of the yeasts by radial diffusion using both pure essential oil and an emulsion of essential oil at 8% (8% essential oil, 0.8% Tween 80 in water), as described by Allegrini et al[18]. The plates were incubated at 37°/C for 24 h, and the inhibition halo was measured in millimeters.

The minimum inhibitory concentrations (MIC) were obtained using a microdilution test in 96-well cell culture plates as described by Prabuseenivasan et al[19] using an emulsion of the essential oil (mentioned above) at concentrations ranging from 4% to 0.007% (v/v). The plates were read at A_{630} (TP-Reader, Thermoplate) after a 48-h incubation at 37°/C. MIC_{50} (defined as the minimum concentration that inhibited 50% of the isolate tested such that $DO_{630} \leq 0.05$) was used as the endpoint of inhibition. Next, 10 μL from each well was inoculated on Petri plates containing Sabouraud dextrose agar (Difco Laboratories, Detroit/MI) and incubated at 37°/C for five days to determine the MFC (minimal fungicidal concentration). The MFC (defined as the lowest concentration without visible growth) was used as the endpoint for fungicidal effects.

EFFECT OF *R. officinalis* LINN. ESSENTIAL OIL ON GERM TUBE FORMATION BY *C. ALBICANS* AND *C. DUBLINIENSIS*

The effect of *R. officinalis* Linn. essential oil on germ tubes was evaluated as described by Bernardes et al[20]. Briefly, germ tube formation was rapidly induced in Sabouraud dextrose broth using (10%) fetal bovine serum and a solution of either 4% essential oil from *R. officinalis* Linn. and 0.02% Tween 80 (assay tube) or nothing (control tube), in a final volume of 10 mL (optimization of the concentration of essential oil; unpublished observations). The tube was inoculated with a *C. albicans* or *C. dubliniensis* suspension (100 μL), and the test was conducted at 37°/C for over 3 h. The total number of cells and germ tubes that formed was determined on a Neubauer chamber. The results were described as the percentage of germ tube forming cells out of the total number of cells.

STATISTICAL ANALYSIS

The statistical differences were evaluated using BioEstat version 5.3 software (ANOVA, Tukey test). $P < 0.05$ was considered significant.

RESULTS

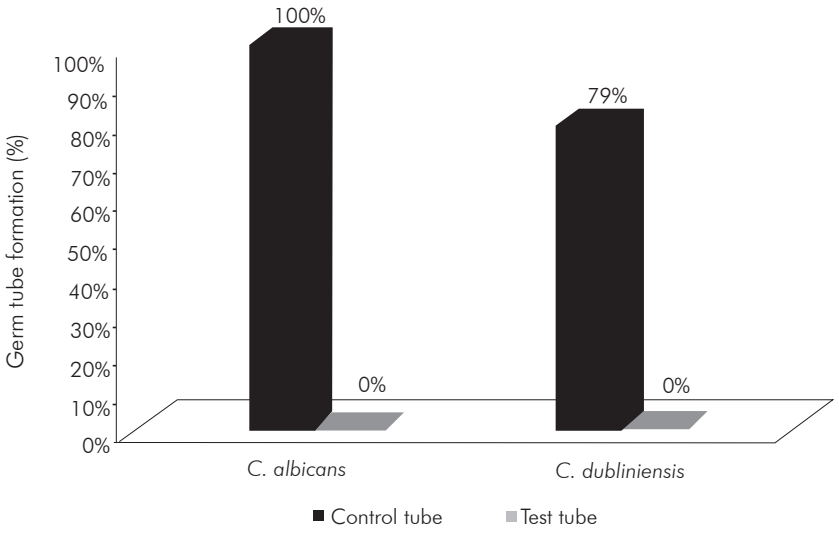
Candida strains were susceptible to the *R. officinalis* Linn. essential oil. Using the agar-diffusion method, we observed inhibition halos ranging from 39 to 47 mm using pure essential oil. Essential oil at a final concentration of 8% had an inhibition halo ranging from 9 to 13 mm. The most susceptible strain was *C. albicans*, for which the MIC_{50} was 0.5%. *C. dubliniensis* and *C. krusei* displayed MIC_{50} values of 1% (75% growth inhibited). We determined that *C. parapsilosis* presented the highest MIC_{50} (2%). Interestingly, while essential oil at a concentration of 2% was able to inhibit greater than 90% of growth for this species, essential oil at a 1% concentration inhibited only 40% of growth. Thus, the MFC was 1% for *C. albicans* and *C. krusei* and 2% for *C. dubliniensis* and *C. parapsilosis*. These results are summarized in table 1.

After incubation with the essential oil, germ tube formation was completely inhibited in both *C. albicans* and *C. dubliniensis* ($p = 0.01$) (Figure 1).

Table 1 – *R. officinalis* Linn. essential oil effect on some species of *Candida*

Strain	Agar-diffusion (mm)		Antifungal test MIC_{50} (% essential oil)	MFC (% essential oil)
	Pure *	8% †		
C. albicans	45	13	0.5	1
C. dubliniensis	39	13	1	2
C. krusei	39	9	1	1
C. parapsilosis	47	11	2	2

*Pure essential oil; † 8% essential oil in an emulsion.



Cell suspensions were pre-incubated in the absence (Control tube) and presence (Test tube) of essential oil (4% v/v). Germ tube formation is expressed as a percentage of cells forming germ tubes relative to the total cell number.

Figure 1 – Effect of *R. officinalis* Linn. essential oil on germ tube formation by *C. albicans* (INCQS 49175) and *C. dubliniensis* (CBS 7987)

DISCUSSION

Some authors have previously studied the antibacterial activity of *R. officinalis* Linn.[13,21,22,23], but few reports have tested the activity of medical plant extracts against strains of *Candida* spp. However, these studies often present conflicting results because there are no standardized techniques for evaluating their antifungal activity[24] (unlike the evaluation of antifungal drugs, which has been standardized by Clinical and Laboratory Standards Institute M27-A3 methodology[25]). Large inhibition halos, ranging from 39 to 47 mm, were obtained from pure essential oil, indicating that it is an effective antifungal. Previous studies using an emulsion of *R. officinalis* Linn. essential oil at a final concentration of 8% indicated that some yeast strains are resistant (*C. albicans* FCF-243 and *C. parapsilosis* ME-2) and some are susceptible (*C. albicans* ATCC-76615, *C. krusei* FCF-281 and *C. parapsilosis* MD-6), with inhibition halos ranging from 10 to 13 mm[16]. If we had used the endpoint of susceptibility described by these authors, only *C. krusei* would have been classified as resistant to an 8% concentration of oil. However, the susceptibility results of these previous studies corroborate our current findings, as the inhibition halos from our 8% essential oil emulsion ranged from 9 to 13 mm, confirming the important anti-*Candida* activity of *R. officinalis* Linn. essential oil. It is difficult to establish the limits of susceptibility and resistance with the agar diffusion test for essential oils because there is no standardized methodology[8,26]. However, it has been suggested that 60% of all essential oils tested possess antifungal activity[5].

Interesting results were obtained for *C. krusei* and *C. parapsilosis*. Using the essential oil emulsion, we observed 50% inhibition of *C. krusei* at 0.5% oil and of *C. parapsilosis* 1% oil. Identical values for the MIC_{50} and MFC were obtained for both species. Nascimento et al[8] reported that assays testing the antifungal activity of essential oils could be inconsistent due to factors such as volatility, water solubility, and viscosity. In the present study, these factors were minimized by including Tween 80 as a surfactant, improving the homogeneity of

the emulsion, and by keeping the plates in the dark to minimize the degradation of the volatile essential oil. We demonstrated that the susceptibility profiles of *C. krusei* and *C. parapsilosis* vary in this study, and we hypothesize that the *R. officinalis* Linn. essential oil tested in the present study contains high concentrations of active compounds such as α-Pinene and 1,8-Cineole[27]. Further studies are underway to determine which fractions of the *R. officinalis* Linn. essential oil were responsible for the observed activity and whether there is any synergy among active compounds.

Aloe vera extract has been previously shown to inhibit germ tube formation in *C. albicans*, and the number of cells forming germ tubes was reduced by approximately 95% using a 10% concentration of extract[20]. Based on concentration optimization studies (data not showed), we used 4% essential oil in the germ tube formation inhibition assay. This concentration was able to inhibit 100% of germ tube formation ($p = 0.01$) for both *C. albicans* and *C. dubliniensis*. These results highlight the potential of *R. officinalis* Linn. essential oil as an antifungal drug candidate. We acknowledge the need to determinate the active compounds that inhibit germ tube formation and their mechanisms of action. Studies to characterize the composition of the oil, with the aim of determining the concentrations of the active components, are being conducted to further reported the antifungal activity of the essential oil of *R. officinalis* used.

CONCLUSION

We identified concentrations of *R. officinalis* Linn. essential oil that are able to inhibit the growth of *C. albicans*, *C. dubliniensis*, *C. krusei* and *C. parapsilosis*. Additionally, 4% essential oil totally inhibited germ tube formation in *C. albicans* and *C. dubliniensis*. These results suggest a need to further evaluate the antifungal performance of *R. officinalis* Linn. essential oil in clinical fungal samples.

Atividade antifúngica de *Rosmarinus officinalis* Linn. óleo essencial contra *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis* e *Candida krusei*

RESUMO

A atividade de formação do tubo antifúngico e antigerme do óleo essencial de *Rosmarinus officinalis* Linn. foi testado contra quatro cepas de *Candida* (*C. albicans, C. dubliniensis, C. parapsilosis* e *C. krusei*). Halos de inibição e concentração inibitória mínima (MIC) foram obtidos utilizando os testes de difusão radial e de microdiluição, respectivamente. A concentração fungicida mínima (MFC) foi obtida por meio de ensaio da MIC. Além disso, o efeito do óleo essencial na formação do tubo germinativo de *C. albicans* e *C. dubliniensi* foi avaliado. A MIC_{50} variou de 0,5% a 2%, enquanto a MFC variou de 1% a 2%. Observou-se a inibição total do crescimento do tubo germinativo em *C. albicans* e *C. dubliniensis*. O óleo essencial de *R. officinalis* Linn. demonstrou potente atividade inibitória e fungicida contra cepas específicas de *Candida*.

Palavras-chave: *Candida*; *Rosmarinus officinalis*; Óleos Voláteis.

Actividad antifúngica de *Rosmarinus officinalis* Linn. aceite esencial contra *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis* y *Candida krusei*

RESUMEN

La actividad de formación del tubo antifúngico y anti-germen del aceite esencial de *Rosmarinus officinalis* Linn. fue verificada en cuatro cepas de *Candida* (*C. albicans, C. dubliniensis, C. parapsilosis* y *C. krusei*). Se obtuvieron halos de inhibición y concentración inhibitoria mínima (MIC) utilizando las pruebas de difusión radial y de microdilución, respectivamente. La concentración fungicida mínima (MFC) se obtuvo a través de ensayo de la MIC. Además, se evaluó el efecto del aceite esencial en la formación del tubo germinativo de *C. albicans* y *C. dubliniensi*. La MIC_{50} varió de 0,5% a 2%, mientras que la MFC varió de 1% a 2%. Se observó la inhibición total del crecimiento del tubo germinativo en *C. albicans* y *C. dubliniensis*. El aceite esencial de *R. officinalis* Linn demostró una potente actividad inhibitoria y fungicida contra cepas específicas de *Candida*.

Palabras clave: *Candida*; *Rosmarinus officinalis*; Aceites Volátiles.

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