Anti-adherent activity of *Rosmarinus officinalis* essential oil on *Candida albicans*: an SEM analysis

Atividade antiaderente do óleo essencial de *Rosmarinus officinalis* em *Candida albicans*: uma análise por MEV

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Abstract

Purpose: To evaluate the anti-adherent activity of *Rosmarinus officinalis* (Rosemary) essential oil on *Candida albicans* (ATCC289065).

Methods: The effect of the essential oil of *R. officinalis* at concentrations of 0.56 mg/mL, 1.12 mg/mL and 2.25 mg/mL on *C. albicans* adherence was examined using Scanning Electron Microscopy (SEM) analysis. A test piece of acrylic resin, 2 mL of Sabouraud Dextrose broth, 0.2 mL of fungal inoculum and 2 mL of *R. officinalis* essential oil in the various test concentrations were combined in sterile glass tubes. Sterile distilled water and nystatin (100.000 IU/mL) were used as controls. The essential oil test products were combined with the fungal samples under two different conditions: at the same time as the other components of the adhesion device (t=0 h) or 24 h after the combination of the other components (t=24 h). The tubes were incubated at 37 °C for 48 h. The specimens were fixed and prepared for SEM analysis.

Results: At 0.56 mg/mL, the effect of *R. officinalis* essential oil was similar to that of nystatin (t=0h and t=24 h). At 2.25 mg/mL, the essential oil caused significant cell disruption and inhibition of adhesion. An intermediate effect was observed at 1.12 mg/mL. Greater inhibition of adhesion was observed at t = 24 h.

Conclusion: The essential oil of *R. officinalis* had an anti-adherent effect on *C. albicans*. Greater inhibition of adhesion was observed in cultures undergoing cellular aggregation (t=24 h) and at higher concentrations of the natural product.

Key words: Essential oils; natural products; oral candidiasis; antimicrobial activity

Resumo

Objetivo: Avaliar a atividade antiaderente do óleo essencial de *Rosmarinus officinalis* (Alecrim) sobre *Candida albicans* (ATCC289065).

Metodologia: O óleo essencial de *R. officinalis* foi avaliado nas concentrações 0,56 mg/mL; 1,12 mg/mL e 2,25 mg/mL, através de Microscopia Eletrônica de Varredura (MEV). Adicionou-se, em tubos de vidro estéreis: 1 corpo de prova de resina acrílica; 2 mL de caldo Sabouraud-Dextrose; 0,2 mL do inoculo fúngico e 2 mL do óleo essencial de *R. officinalis* nas concentrações testadas. Água destilada estéril e nistatina (100.000 UI/mL) funcionaram como controle. Os produtos testados foram adicionados em duas condições: junto aos demais componentes do dispositivo de aderência (t=0h); e após 24 h da inserção desses componentes (t=24 h). Os tubos foram incubados a 37 °C durante 48 h. Os espécimes foram fixados e preparados para análise em MEV.

Resultados: Na concentração 0,56 mg/mL o óleo essencial de *R. officinalis*, foi semelhante a nistatina (t=0 h e t=24 h). Na concentração 2,25 mg/mL, o produto natural provocou inibição significativa da aderência e rompimento celular. Efeito intermediário foi observado em 1,12 mg/mL. Maior inibição da aderência foi observada em t=24 h.

Conclusão: O óleo essencial de *R. officinalis* apresentou atividade antiaderente sobre *C. albicans*. Maior inibição da aderência foi observada para o estado de agregação celular (t=24 h) e maior concentração do produto natural.

Palavras-chave: Óleos essenciais; produtos naturais; candidíase bucal; produtos com ação antimicrobiana

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Introduction

Oral candidiasis is one of the most common fungal infections among humans. It is described as an opportunistic infection, often involved in the alteration of oral microflora, systemic diseases and reduced immunity of the host (1,2). Among the strains involved in the development of oral candidiasis, *Candida albicans* is the most prevalent and pathogenic species (1,3).

According to Rex et al. (4) and Khan et al. (5), *Candida* species have proven to be resistant to a number of synthetic antifungal drugs. To identify substances that might be alternatives to traditional medicines, studies were conducted on the antimicrobial activity essential oils (6-9).

Natural products that provide effective antifungal activity against resistant microorganisms are a necessary alternative for the control of oral candidiasis (6,9). The fungistatic activity of *Rosmarinus officinalis* (Rosemary) essential oil was reported by several studies (6-8). According to Fontenelle et al. (7), the essential oil of *R. officinalis* has antifungal activity against *Candida* at low concentrations. However, there were no studies on the anti-adherent activity of this natural product. Thus, there is a need to further investigate the antifungal activity of these products to justify and validate the clinical use of essential oils.

The mechanism of action by which essential oils might be effective has not been fully described. However, Hammer et al. (10) suggest that essential oils are able to interact with lipid structures and cause changes in cell membranes. Phongpaichit et al. (11) identified changes in the cell walls of filamentous fungi after exposure to natural products. Thus, this research is justified by the need to examine the microscopic, morphological effect of essential oils on *Candida* species.

The aim of this study was to evaluate the anti-adherent activity of *R. officinalis* (Rosemary) essential oil against *C. albicans*.

Methods

We conducted a study with an inductive approach, a descriptive and comparative procedure and direct documentation in the laboratory (12).

To generate the fungi adhesion devices, colorless acrylic resin (that can be chemically polymerized) test substrates were prepared (Vipi Flash VIPI Produtos Odontológicos Ltda., Pirassununga, SP, Brazil), with dimensions of 10x10x5 mm. The substrates were prepared according to the manufacturer's instructions (25 °C, in equal proportions of powder and liquid, without incorporation of air bubbles) and then immersed in water for 24 h at 37 ºC to release the residual monomer.

The surfaces of the substrates were subjected to finishing and polishing with water and 200-, 400- and 600-grit sandpaper (3M-ESPE, Campinas, SP, Brazil) and then cleaned with ultrasonic equipment. All substrates were stored in a glass tube with distilled water and were sterilized by autoclaving (121 ºC for 20 min) for later use.

The reference strain used in this study was *C. albicans* (ATCC 289065) from the *American Type Culture Collection*. The strain was reactivated in Sabouraud Dextrose broth (DIFCO®, Detroit, Michigan, USA) at 37 °C and stored in Sabouraud Dextrose Agar 4% (DIFCO®, Detroit, Michigan, USA). To conduct the study, fungal suspensions were prepared in saline at a concentration of 1.5 x 10^6 microorganisms/mL, equivalent to the 10^6 tube of the MacFarland scale.

For the *in vitro* evaluation of anti-adherence activity, we used *R. officinalis* (rosemary) essential oil obtained from Ferquima® (Ind. e Com. Ltda.), which provided a technical report with specifications as shown in Table 1.

Preliminary study of the antifungal activity of *R. officinalis* essential oil against *C. albicans* (ATCC 289065) identified the MIC and MFC to be 0.56 mg/mL. In the present study, the anti-adherent activity of the *R. officinalis* essential oil was evaluated at concentrations 0.56 mg/mL, 1.12 mg/mL and 2.25 mg/mL.

The *R. officinalis* essential oil used in this study was initially diluted in sterile distilled water and emulsifier (Tween 80) to obtain an initial concentration of 0.25% (2.25 mg/mL). To dilute the essential oil, we considered the density to be 0.9 g/mL, according to vendor specifications. We used the dilution method described by Lima et al. (6) and Souza et al. (13). Briefly, 0.05 mL of *R. officinalis* essential oil, 0.05 mL of Tween 80 and 20 mL of sterile distilled water were combined in sterile Falcon tubes. The mixture was stirred for 5 minutes in a Vortex solution agitator (Model AP56, Phoenix), and the concentration obtained was 0.25% (2.25 mg/mL). To obtain the subsequent dilutions, serial dilutions were performed with the addition of 10 mL sterile distilled water to 10 mL of the previous dilution.

Table 1. Technical specifications of the essential oils used in this study, according to the technical report issued by Ferquima® (Ind. e Com. Ltda).

Essential oils	Batch	Impurities	Density (g/mL, 20°C)	Origin	Main components
Rosmarinus officinalis (Rosemary)	141	Free	0.912	Tunisia	1.8 Cineole + limonene + para-cymene α-pinene Camphor

To examine anti-adhesion activity, one sterile acrylic resin substrate, 0.2 mL of the fungal suspension, 2 mL of sterile Sabouraud-Dextrose broth and 2 mL of diluted *R. officinalis* essential oil, distilled water (growth control) or nystatin 100.000 IU/mL (positive control) (14) were combined in sterile glass tubes. The tubes were covered and incubated in a bacteriological incubator at 37 °C for 48 h. The sterility of the culture medium and the substrates was verified through the absence of turbidity in the medium in the absence of an inoculum or the test products.

The anti-adherence activity of *R. officinalis* essential oil and of the positive control was assessed under two conditions: the product was combined with the substrate at the same time as the other components (t=0 h); and the product was added to the substrate 24 hours after the combination of the other components (t=24 h). Under the first condition, the planktonic cells composed the majority of the culture. Under the second condition, cellular aggregates were observed as a result of the secretion of extracellular matrix and subsequent adhesion (15). Under this condition, the *R. officinalis* essential oil or nystatin 100.000 UI/mL (positive control) were added only after the incubation process had begun.

Subsequently, the substrates were removed, transferred to sterile glass tubes and subjected to the process of fixation for viewing by scanning electron microscopy (SEM). Buffer and fixative solutions were prepared as follows. The buffer solution was made using 39 mL of monobasic sodium phosphate (NaH_2PO_4) and 61 mL of dibasic sodium phosphate (Na_2HPO_4). The fixative solution was prepared by adding 68 mL of buffer solution to 70 mL of 2% glutaraldehyde. The substrates were immersed in fixative solution for 3 hours and subsequently placed in buffer solution for 24 hours. The substrates were removed and dried in a bacteriological incubator at 37 °C for 48 hours (16).

The samples were prepared for descriptive analysis by SEM, in which the aim was to identify the morphology of fixed cells on the surface of the substrate. We used a magnification of 500x at 10 kV. The results obtained using the *R. officinalis* essential oil were compared with those obtained with the positive and growth controls.

Results

The photomicrographs depicting the anti-adhesion activity of the products tested against *C. albicans* on the surface of the substrates at t=0 h are shown in Fig. 1. The photomicrographs depicting the anti-adhesion activity of the products tested against *C. albicans* on the surface of the substrates at t=24 h are shown in Fig. 2.

The SEM analysis revealed that the tested products inhibited the adhesion of *C. albicans* by denaturing cellular structures. At 0.56 mg/mL, the cells suffered disruption of the cell wall when in the planktonic phase (t=0 h) and the alteration of cell permeability when in the aggregation phase (t=24 h). Concentrations above 0.56 mg/mL of the natural product caused the loss of cellular integrity and resulted in the significant inhibition of adhesion. Essential oil at a concentration of 1.12 mg/mL produced an intermediate effect to that of 0.56 mg/mL and 2.25 mg/mL. There was greater inhibition of cell adhesion and extracellular matrix at t=24 h and when essential oil at 2.25 mg/mL was used.

Discussion

According to Fontenelle et al. (7), *R. officinalis* essential oil presented MICs between 0.62 mg/mL and 2.50 mg/mL and MFCs from 1.25 mg/mL to 2.50 mg/mL when was used on *C. albicans* and *C. tropicalis* as determined using the micro-dilution technique. Likewise, the antifungal activity of *R. officinalis* essential oil was demonstrated by Lima et al. (6) and Packer and Luz (17), who observed fungistatic activity at concentrations above 8% using the agar diffusion technique. According to Scorzoni et al. (18), the micro-dilution technique is more sensitive for the determination of MIC and MFC in the assessment the antifungal activity of natural products. We found no reports in the literature regarding the anti-adherent activity of *R. officinalis* essential oil. Thus, to determine the most appropriate concentration of *R. officinalis* essential oil to use for the assessment of anti-adherent activity, we elected to use the methodology of Fontenelle et al. (7) because of its technical standardization and increased data reliability. As observed in this study, the essential oil of *R. officinalis* caused changes in both the cellular morphology and the adherence of *C. albicans* at the tested concentrations.

To validate the technique used in this study and for comparison with the effect of essential oil, a positive control (nystatin – *commercially available suspension* – 100,000 IU/mL), a growth control and sterility controls were employed. The sterility control confirmed the absence of extrinsic contamination from the culture medium and on the substrates. The growth control confirmed the viability of the strains and allowed the demonstration of cell morphology in the absence of antifungal treatment. The absence of fungal growth in the presence of nystatin (100,000 IU/mL) demonstrated the susceptibility of the samples to a synthetic antifungal agent. As observed in this study, the effects produced by the positive control and by the *R. officinalis* essential oil at a concentration of 0.56 mg/mL were similar (Fig. 1 and 2).

The informed components of the products tested in this investigation are consistent with the components reported to be active in the literature. According to Souza et al. (13) and Mondello et al. (19), the biological activity of essential oils has been shown to be dependent on their main chemical components, including cineole, limonene and cymene. These components are responsible for the antiseptic and antimicrobial properties of the *R. officinalis* essential oil, and their physico-chemical and biological properties should be studied (13,19).

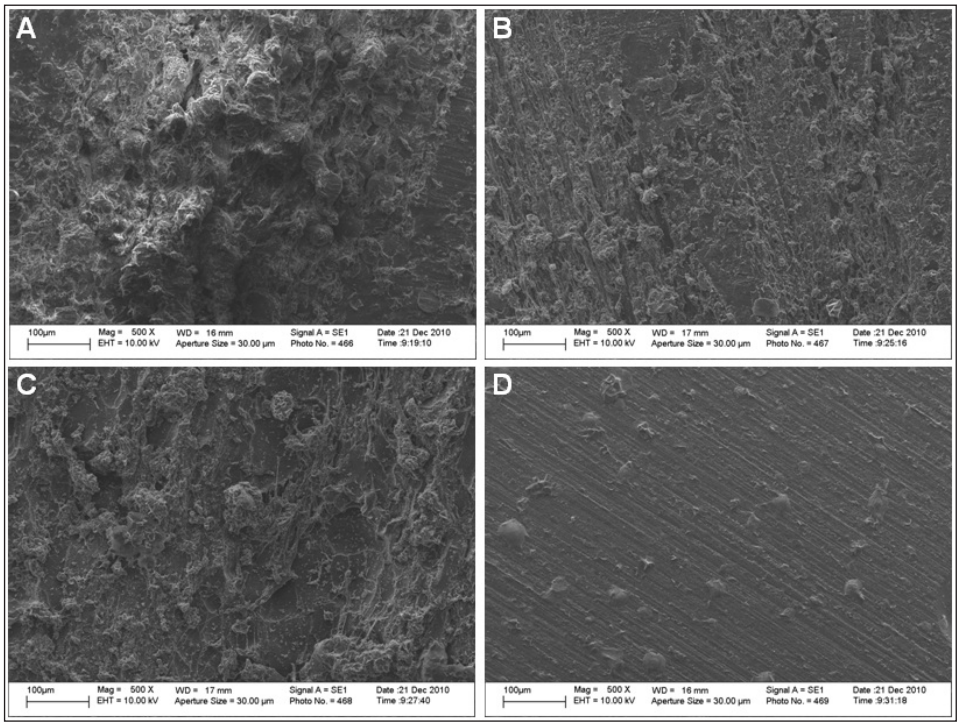

Fig. 1. Photomicrographs demonstrating the anti-adherent activity of the tested products on *C. albicans* on the surface of the substrates at t=0 h. **(A)** Growth Control. **(B)** Substrate exposed to the Positive Control (Nystatin 100.000IU/mL). **(C)** Substrate exposed to the essential oil of *R. officinalis* at 0.56 mg/mL. **(D)** Substrate exposed to the essential oil of *R. officinalis* at 2.25 mg/mL.

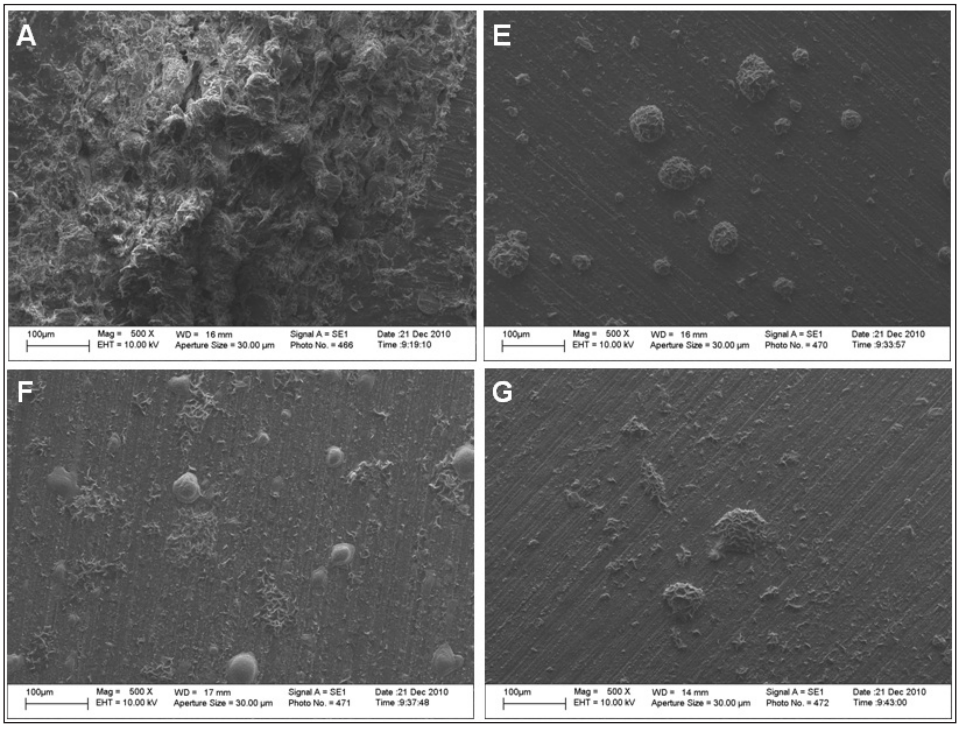

Fig. 2. Photomicrographs demonstrating the anti-adherent activity of the tested products on *C. albicans* on the surface of the substrates at t=24 h. **(A)** Growth Control. **(B)** Area exposed to the Positive Control (Nystatin 100.000 IU/mL). **(C)** Surface exposed to the essential oil of *R. officinalis* at 0.56 mg/mL. **(D)** Surface exposed to the essential oil of *R. officinalis* at 2.25 mg/mL.

Studies in the literature have indicated that the mechanism of action of essential oils is related to changes in cell membrane permeability. The liposoluble nature of essential oils and their constituents facilitates their interaction with cellular structures that have lipid components, resulting in increased membrane permeability, which in turn can cause electrolyte imbalance and cell death (9,10,20). When Hammer et al. (10) evaluated the antifungal action of the *M. alternifolia* essential oil against *C. albicans*, *C. glabrata* and *Saccharomyces cerevisiae*, they observed that *M. alternifolia* essential oil altered the permeability and membrane fluidity of these fungi between concentrations of 0.25% and 1.0%. This suggests that the antifungal activity of this essential oil has its effect by compromising the function and altering the physical properties of membranes. In this study, SEM analysis revealed morphological changes caused by the essential oil, which confirms the hypothesis that these products alter cell permeability.

When Phongpaichit et al. (11) and Ibrahim Osman (21) analyzed cell morphology by SEM, they showed that fungal cultures suffered cellular collapse and denaturation after exposure to the extracts of natural products. Phongpaichit et al. (11) found that structures such as hyphae and blastoconidia had shrunk and were altered after exposure to the natural product. Consistent with findings in the literature, we report that the denaturation of cellular structures was observed when the fungal strain was exposed to the essential oil of *R. officinalis* or to the positive control. According to Ibrahim and Osman (21), this phenomenon may be associated with the leakage of the cytosol through the cell wall or with alterations in membrane permeability caused by the tested products.

According to Chandra et al. (15), cells of *C. albicans* have the ability to perform co-aggregation and excrete an extracellular matrix that contributes to cellular adherence to acrylic and polymeric surfaces. Chandra et al. (15) demonstrated that, while in planktonic state, cells of *C. albicans* are more susceptible to antifungal action. The authors considered that the secretion of the extracellular matrix and the aggregation state contributed to higher antifungal resistance (15). Here, we observed differences between the activity of compounds tested in the planktonic phase (t=0 h) and in the aggregation phase (t=24 h), differences that were supported by the lower adhesion of cells and the extracellular matrix at t=24 h. These results are in contrast to the findings of Chandra et al. (15) because they indicate a greater inhibition of adhesion at t=24 h.

The action of *R. officinalis* essential oil at 0.56 mg/mL, t=0 h, was observed to produce a disorganized matrix, disruption of the cell wall and denatured structures. At t=24 h, shrunken cells were identified, without the loss of cell wall integrity and in the absence of extracellular substance, although there was a greater degree of inhibition of adhesion. At the 2.25 mg/mL concentration, a significant inhibition of adhesion and cell disruption at both t=0 h and t=24 h were observed.

In the conditions presented in this study, the essential oil of *R. officinalis* showed anti-adherent activity against *C. albicans,* as observed by cellular denaturing, fragmentation of structures and a small quantity of adhered components. The interaction of *C. albicans* with other microorganisms in the constitution of oral biofilms may contribute to the increased susceptibility of this strain to the action of antifungal agents (16). Further studies should consider the action of this product on clinical strains and other standardized strains using techniques that take into consideration the influence of human saliva and its interaction with other oral microorganisms.

Conclusions

We conclude that *R. officinalis* essential oil, at the tested concentrations, has anti-adherent activity against *C. albicans* and can additionally affect changes in the cellular morphology of these strains. Greater inhibition of adhesion was observed during the cellular aggregation phase and with higher concentrations of the natural product.

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