

The effect of *Rosmarinus officinalis* essential oil on germ tube formation in *Candida dubliniensis* recovered from denture users

O efeito do óleo essencial de *Rosmarinus officinalis* sobre a formação do tubo germinativo em *Candida dubliniensis* isolada de usuários de dentadura

El efecto del aceite esencial de *Rosmarinus officinalis* sobre la formación del tubo germinativo en *Candida dubliniensis* aislada de usuarios de dentadura

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ABSTRACT

Candida dubliniensis is an emerging pathogenic yeast that is closely related to *C. albicans* and colonizes or infects the oral cavities of denture users. Similar to *C. albicans*, *C. dubliniensis* produces germ tubes as a major virulence factor. Due to the scarcity of data demonstrating that natural products have anti-*C. dubliniensis* activity, the objective of this study was to evaluate the effect of an essential oil from *Rosmarinus officinalis* on *C. dubliniensis* germ tube formation. Six isolates of *C. dubliniensis* were recovered from the oral cavities ($n = 5$ /denture base and $n = 1$ /palatal mucosa) of six denture users either with ($n = 4$) or without ($n = 2$) stomatitis. Germ tube formation was induced in Sabouraud dextrose broth containing 10% fetal bovine serum in the presence (test) or absence (control) of 4% *R. officinalis* essential oil. The complete inhibition of germ tube formation for all of the tested isolates of *C. dubliniensis* was observed ($p = 0.004$). Our results demonstrate the potent anti-*C. dubliniensis* effect of *R. officinalis* essential oil, indicating the need to further investigate the pharmaceutical use of *R. officinalis* essential oil, particularly as it impacts the colonization of denture resin and stomatitis.

Keywords: *Candida dubliniensis*; *Rosmarinus officinalis*; Stomatitis, Dentures.

INTRODUCTION

Candida dubliniensis has been described by Sullivan et al¹ as a group of atypical isolates of *Candida* due to the significant genomic differences from *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. kefyr*, and *C.*

krusei. *C. dubliniensis* is closely related to *C. albicans*; both are chlamyospore and germ-tube producing species that are differentiated by the use of a variety of techniques for yeast identification, including the VITEK 2 ID-YST system and differential media², such as Tobacco agar³. Reports on the isolation of *C. dubliniensis* show a link to oral infections in HIV-negative and HIV-positive individuals⁴. *C. dubliniensis* can cause oral infections in denture users (with or without stomatitis), either in the presence⁵ or absence⁶ of *C. albicans*. The pathogenesis of oral candidiasis due to *C. dubliniensis* begins with the initial adherence of the organism to mucosal surfaces via a mechanism that directly involves cell surface hydrophobicity and then

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switches to pseudohyphae or true hyphae⁷, considered the major virulence factor⁸. Therefore, this species is considered an emerging yeast associated with oral candidiasis⁹, despite being the smallest constituent of the oral microflora in humans¹⁰. Within this context, studies have demonstrated the effectiveness of compounds (synthetic or natural) for inhibiting the pathogenicity of *C. dubliniensis* recovered from the oral cavity¹¹. Indeed, previous reports have indicated the antifungal activity of the essential oils of medicinal plants¹² against *Candida* spp., including compounds from *Rosmarinus officinalis*^{13,14}. Therefore, the aim of the present study was to evaluate the inhibition of germ tube formation by *C. dubliniensis* recovered from denture users using *R. officinalis* essential oil.

MATERIALS AND METHODS

FUNGAL ISOLATES AND GROWTH CONDITIONS

A total of six isolates of *C. dubliniensis* were recovered from the oral cavities of six denture users (Table 1). The yeasts were recovered from the denture base or palatal mucosa using a sterile swab. Sampling was conducted from March 2012 to October 2012 at the dental school clinic of the Universidade Federal do Pará, Brazil. The study was approved by the Research Ethics Committee of the Instituto Evandro Chagas (CEP/IEC 032/10; January 13, 2011), and all denture users gave informed consent. The isolates were identified from a carbohydrate assimilation profile using the VITEK 2 system (bioMérieux, Marcy l'Etoile, France). After 3 hours of incubation in fetal bovine serum at 37° C¹⁵, germ tube formation and the production of wrinkled colonies with the presence of hyphae and chlamydozoospores in Tobacco agar³ were used to confirm the presence of *C. dubliniensis*. The *C. dubliniensis* isolates were then cultivated in Sabouraud dextrose agar (Difco, Laboratories, Detroit, MI, USA) under aerobic conditions at 37° C for 24 hours. A suspension of 10⁶ cells/mL in sterile (pH 7.2) phosphate-buffered saline (PBS) was produced for each isolate and used to assay the inhibition of germ tube formation.

EXTRACTION OF *R. OFFICINALIS* ESSENTIAL OIL

R. officinalis was collected (collector: Gauch, L.M.R 01) and identified by Ely Simone Cajueiro Gurgel (Museu Paraense Emílio Goeldi, Pará State, Brazil) under registration code MG 204.248. The essential oil was obtained from fresh leaves (350 g) by steam distillation for 240 minutes using the Clevenger system. In this process, 2 mL of the essential oil was obtained and subsequently stored under cool, dark conditions. The experiments were conducted using the pure essential oil without fractionation.

GERM TUBE INHIBITION TEST

The effect of *R. officinalis* essential oil on germ tube formation was evaluated as described by Bernardes et al¹⁷. Briefly, germ tube formation was induced in Sabouraud dextrose broth containing fetal bovine serum (10%) and the essential oil from *R. officinalis* (4%) (assay tube) or broth alone (control tube). The yeast suspension (100 µL) was inoculated, and the assay was performed at 37° C for 3 hours. The total cell number was determined using a Neubauer chamber, and germ tube formation was expressed as the percentage of germ tube-forming cells relative to the total number of cells.

STATISTICAL ANALYSIS

The data were analyzed using BioEstat version 5.3 (Mann-Whitney, Kruskal-Wallis, and Fisher exact tests), with $p \leq 0.05$ considered to be statistically significant.

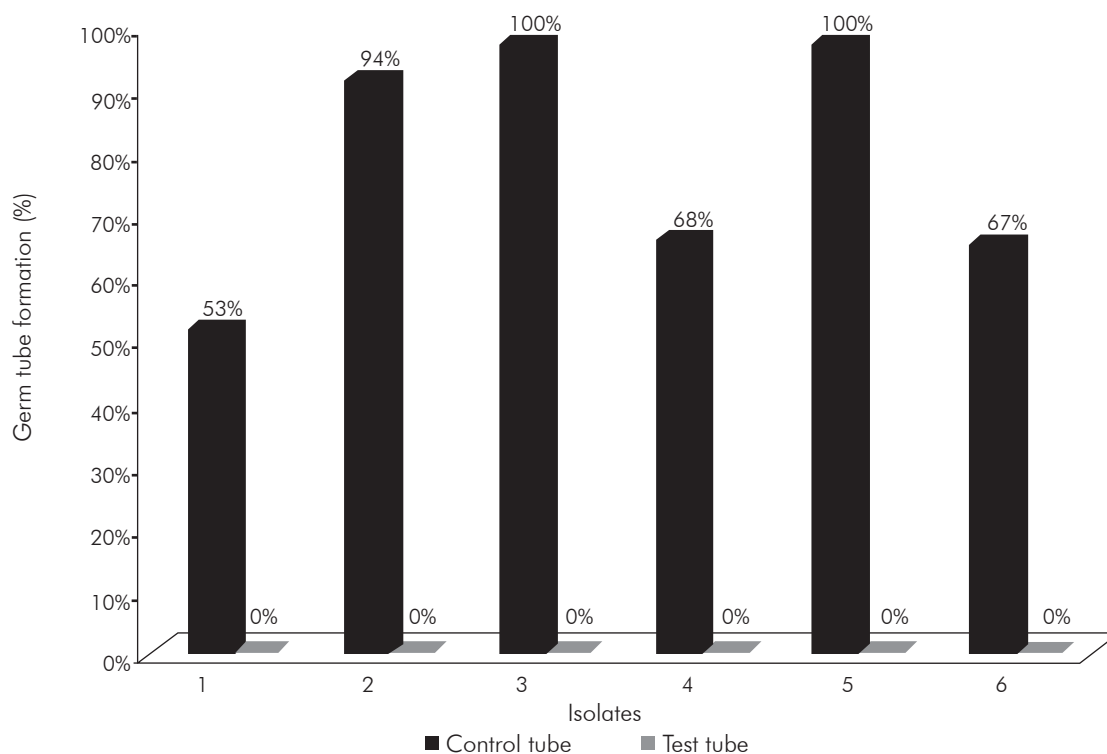
RESULTS

The percentage of cells from each isolate tested, forming germ tube ranged from 53% to 100% (Figure 1), with no significant difference indicated by either the variation in germ tube formation ($p = 0.06$) or the presence or absence of stomatitis ($p = 0.47$) (Table 1, Figure 1). Following the exposure to 4% *R. officinalis* essential oil, a complete inhibition of germ tube formation was observed for all isolates of *C. dubliniensis* tested ($p = 0.004$) (Figure 1). Figure 2 illustrates the morphology observed during the germ tube inhibition assay.

Table 1 – Summary data of denture users from whom *C. dubliniensis* were recovered

Number	Gender	Age	Specimen from	Stomatitis*
1	F	67	Denture	Without stomatitis
2	F	52	Denture	Without stomatitis
3	F	46	Denture	Type II
4	M	63	Denture	Type III
5	M	58	Palatal Mucosa	Type I
6	M	58	Denture	Type I

* Stomatitis type according to Newton's classification¹⁶; M: Male; F: Female.



Tests were performed in duplicate.

Figure 1 – The effect of *R. officinalis* essential oil on germ tube formation in six *C. dubliniensis* isolates recovered from denture users ($p = 0.004$)

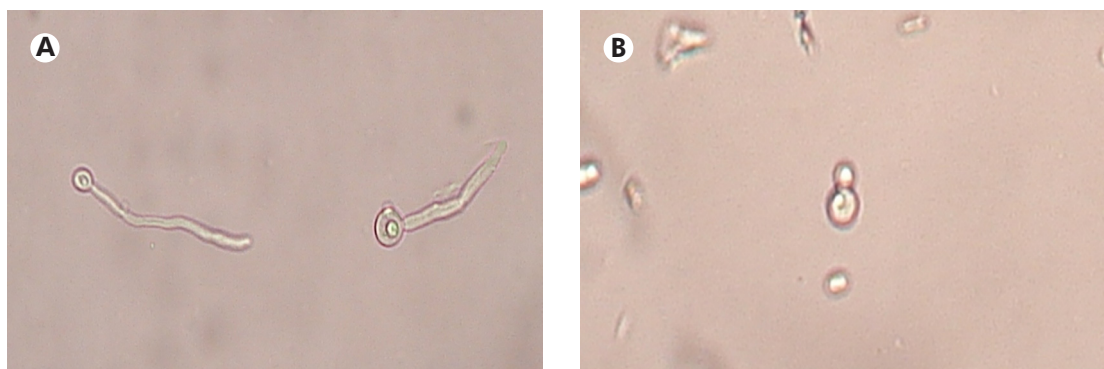


Figure 2 – Morphologies of the *C. dubliniensis* yeasts used herein to assess the effect of the *R. officinalis* essential oil on germ tube formation

DISCUSSION

In the present study, six isolates of *C. dubliniensis* recovered from the oral cavities of denture users were tested, reflecting the low frequency of this species in the microbiota of the individuals included in the study. This finding is corroborated by studies of *C. dubliniensis* isolated from the oral cavity¹⁵ and from denture users presenting with stomatitis^{5,6}. Although the pathogenesis of oral infection due to *C. dubliniensis* is poorly understood, germ tube formation is the primary virulence factor that triggers yeast proliferation during the infection process^{7,18}. Depending on the isolate, different percentages of cells forming germ tubes were observed (Figure 1, $p = 0.06$), variation that might be related to variability in the adaptation of each isolate to the incubation conditions in the test. Indeed, the

development of hyphae by *C. dubliniensis* depends on the association between the activation level of transcription factor Crz1 and the composition of the medium that induces filamentation¹⁹. The results from the inhibition of germ tube formation assay observed in this study agree with those described by Pozzatti et al²⁰, reinforcing the need for the evaluation of pharmaceuticals based on the essential oil of *R. officinalis*.

Additionally, the reduction in *C. dubliniensis* colonization of denture resins has been described in an assay using sub-therapeutic concentrations of chlorhexidine gluconate (0.005% and 0.0025%) that are capable of reducing the hydrophobicity of the yeast cell surface¹¹ and inhibiting the formation of germ tubes²⁰, thus contributing to the reduction of *in vivo* pathogenicity. Such reduced pathogenicity is due

to reduced host cell adherence and the prevention of morphology switching. In comparison, gels containing 1% *Pelargonium graveolens*²² or *Satureja hortensis*²³ essential oil have been described, and a significant reduction in stomatitis caused by *C. albicans* was observed. These data strongly encourage further studies aimed at evaluating the effects of natural products or synthetic compounds on oral infections caused by yeasts of the *Candida* genus, such as *C. dubliniensis*, particularly with regard to the targeted inhibition of its filamentous form.

Using 4% *R. officinalis* essential oil, the present study showed a complete inhibition of germ tube formation for all isolates of *C. dubliniensis* tested. Our data demonstrate the ability of this oil to potently inhibit *C. dubliniensis*, which is important considering the lack of data showing the activity of natural products against this species. Given that four of the six *C. dubliniensis* isolates tested in this study were recovered from cases of stomatitis (Table 1) and that all were susceptible to the

inhibition of germ tube formation in the presence of this essential oil (Figure 1), it is imperative to study this oil more thoroughly. It will be necessary to investigate the applicability of pharmaceutical preparations containing *R. officinalis* essential oil as inhibitors of denture resin colonization and its effectiveness in treating stomatitis caused by this species of *Candida*.

CONCLUSION

The present study describes the complete inhibition of germ tube formation in six isolates of *C. dubliniensis* by exposure to 4% *R. officinalis* essential oil. The development of pharmaceutical preparations containing this essential oil and the assessment of their potential for the treatment of stomatitis associated with *C. dubliniensis* are suggested.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.



O efeito do óleo essencial de *Rosmarinus officinalis* sobre a formação do tubo germinativo em *Candida dubliniensis* isolada de usuários de dentadura

RESUMO

Candida dubliniensis é uma levedura patogênica emergente, muito semelhante à *C. albicans*, que coloniza ou infecta as cavidades bucais de usuários de dentaduras. Similar à *C. albicans*, *C. dubliniensis* produz tubos germinativos com fator de virulência maior. Devido à escassez de dados, demonstrando que produtos naturais têm atividade anti-*C. dubliniensis*, o objetivo deste estudo foi avaliar o efeito de um óleo essencial de *Rosmarinus officinalis* sobre a formação de tubo germinativo em *C. dubliniensis*. Seis isolados de *C. dubliniensis* foram coletados de cavidades orais (n = 5/base da dentadura e n = 1/mucosa palatal) de seis usuários de dentaduras com (n = 4) ou sem (n = 2) estomatite. A formação do tubo germinativo foi induzida em caldo Sabouraud dextrose, contendo soro fetal bovino a 10% na presença (teste) ou ausência (controle) de óleo essencial de *R. officinalis* a 4%. Observou-se a completa inibição da formação do tubo germinativo de todos os isolados de *C. dubliniensis* (p = 0,004). Nossos resultados demonstraram o potente efeito anti-*C. dubliniensis* do óleo essencial *R. officinalis*, indicando a necessidade maiores investigações sobre o uso farmacêutico do óleo essencial *R. officinalis*, particularmente o seu impacto sobre a colonização da resina para dentaduras e a estomatite.

Palavras-chave: *Candida dubliniensis*; *Rosmarinus officinalis*; Estomatite; Dentaduras.

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RESUMEN

Candida dubliniensis es una levadura patógena emergente, muy parecida a la *C. albicans*, que coloniza o infecta las cavidades bucales de usuarios de dentaduras. Similar a *C. albicans*, *C. dubliniensis* produce tubos germinativos con un mayor factor de virulencia. Debido a la escasez de datos, demostrando que productos naturales tienen una actividad anti-*C. dubliniensis*, el objetivo de este estudio fue el de evaluar el efecto de un aceite esencial de *Rosmarinus officinalis* sobre la formación de tubo germinativo en *C. dubliniensis*. Se recolectaron seis aislados de *C. dubliniensis* de cavidades orales (n = 5/base de la dentadura y n = 1/mucosa palatal) de seis usuarios de dentaduras con (n = 4) o sin (n = 2) estomatitis. La formación del tubo germinativo fue inducida en caldo Sabouraud dextrosa, conteniendo suero fetal bovino a 10% en presencia (prueba) o ausencia (control) de aceite esencial de *R. officinalis* a 4%. Se observó la completa inhibición de la formación del tubo germinativo de todos los aislados de *C. dubliniensis* (p = 0,004). Nuestros resultados demostraron el potente efecto anti-*C. Dubliniensis* del aceite esencial *R. officinalis*, indicando la necesidad de más investigaciones sobre el uso farmacéutico del aceite esencial *R. officinalis*, particularmente su impacto sobre la colonización de la resina para dentaduras y la estomatitis.

Palabras clave: *Candida dubliniensis*; *Rosmarinus officinalis*; Estomatitis; Dentaduras.



REFERENCES

- 1 Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE, Coleman DC. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiol.* 1995 Jul;141(7):1507-21.
- 2 Moran GP, Coleman DC, Sullivan DJ. *Candida albicans* versus *Candida dubliniensis*: why is *C. albicans* more pathogenic? *Int J Microbiol.* 2012 Sep;2012:205921.
- 3 Khan ZU, Ahmad S, Mokaddas E, Chandy R. Tobacco agar, a new medium for differentiating *Candida dubliniensis* from *Candida albicans*. *J Clin Microbiol.* 2004 Oct;42(10):4796-8.
- 4 Sullivan DJ, Moran GP, Coleman DC. *Candida dubliniensis*: ten years on. *FEMS Microbiol Lett.* 2005 Dec;253(1):9-17.
- 5 Gasparoto TH, Dionísio TJ, Oliveira CE, Porto VC, Gelani V, Santos CF, et al. Isolation of *Candida dubliniensis* from denture wearers. *J Med Microbiol.* 2009 Jul;58:959-62.
- 6 Marcos-Arias C, Vicente JL, Sahand IH, Eguia A, De-Juan A, Madariaga L, et al. Isolation of *Candida dubliniensis* in denture stomatitis. *Arch Oral Biol.* 2009 Feb;54(52):127-31.
- 7 Whiteway M, Bachewich C. Morphogenesis in *Candida albicans*. *Annu Rev Microbiol.* 2007 Jun;61:529-53.
- 8 Gutiérrez J, Morales P, González MA, Quindós G. *Candida dubliniensis*, a new fungal pathogen. *J Basic Microbiol.* 2002;42(3):207-27.
- 9 Nonaka CFW, Nascimento GJF, Goulart Filho JAV, Lima KC, Milan EP. *Candida dubliniensis*: emergent yeast associated with oral candidosis. *Rev Odontol UNESP.* 2008;37(2):125-31.
- 10 Loreto ES, Scheid LA, Nogueira CW, Zeni G, Santurio JM, Alves SH. *Candida dubliniensis*: epidemiology and phenotypic methods for identification. *Mycopathologia.* 2010 Jun;169(6):431-43.
- 11 Ellepola ANB, Joseph BK, Khan ZU. Cell surface hydrophobicity of oral *Candida dubliniensis* isolates following limited exposure to sub-therapeutic concentrations of chlorhexidine gluconate. *Mycoses.* 2013 Jan;56(1):82-8.
- 12 Castro RD, Lima EO. Atividade antifúngica in vitro do óleo essencial de *Eucalyptus globulus* L. sobre *Candida* spp. *Rev Odontol UNESP.* 2010 mai-jun;39(3):179-84.
- 13 Lima IO, Oliveira RAG, Lima EO, Farias NMP, Souza EL. Antifungal activity from essential oils on *Candida* species. *Braz J Pharmacogn.* 2006 Apr-Jun;16(2):197-201.
- 14 Castro RD, Lima EO. Atividade antifúngica dos óleos essenciais de sassafrás (*Ocotea odorifera* Vell.) e alecrim (*Rosmarinus officinalis* L.) sobre o gênero *Candida*. *Rev Bras Pl Med.* 2011;13(2):203-8.
- 15 Arikan S, Darka O, Hascelik G, Gunalp A. Identification of *Candida dubliniensis* strains using heat tolerance tests, morphological characteristics and molecular methods. *Mikrobiyol Bul.* 2003 Jan;37(1):49-57.
- 16 Newton AV. Denture sore mouth: a possible etiology. *Br Dent J.* 1962;112:357-60.
- 17 Bernardes I, Rodrigues MPF, Bacelli GK, Munin E, Alves LP, Costa MS. Aloe vera extract reduces both growth and germ tube formation by *Candida albicans*. *Mycoses.* 2012 May;55(3):257-61.
- 18 Ge YP, He GX, Lin T, Lu GX, Shen YN, Liu WD. First isolation of *Candida dubliniensis* from oral cavities of dermatological patients in Nanjing, China. *Mycopathologia.* 2011 Dec;172(6):465-71.
- 19 Chen YL, Brand A, Morrison EL, Silao FGS, Bigol UG, Malbas Junior FF, et al. Calcineurin controls drug tolerance, hyphal growth, and virulence in *Candida dubliniensis*. *Eukaryot Cell.* 2011 Jun;10(6):803-19.
- 20 Pozzatti P, Loreto ES, Nunes Mario DA, Rossato L, Santurio JM, Alves SH. Activities of essential oils in the inhibition of *Candida albicans* and *Candida dubliniensis* germ tube formation. *J Mycol Med.* 2010 Sep;20(3):185-9.
- 21 Ellepola ANB. The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine gluconate on germ tube formation of oral *Candida dubliniensis*. *Mycoses.* 2010 Sep;54(5):e330-5.
- 22 Sabzghabae AM, Shirdare Z, Ebadian B, Aslani A, Ghannadi A. Clinical evaluation of the essential oil of *Pelargonium graveolens* for the treatment of denture stomatitis. *Dent Res J (Isfahan).* 2011 Dec;8(Suppl 1):S105-8.
- 23 Sabzghabae AM, Davoodi N, Ebadian B, Aslani A, Ghannadi A. Clinical evaluation of the essential oil of "*Satureja Hortensis*" for the treatment of denture stomatitis. *Dent Res J (Isfahan).* 2012 Mar-Apr;9(2):198-202.