

**THE EFFECT OF BRAZILIAN PROPOLIS ON THE GERM TUBE FORMATION  
AND CELL WALL OF *CANDIDA ALBICANS***

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**Summary**

Candidal adherence has been implicated as the first step in the pathogenesis of oral candidosis, and germ tube formation by *Candida albicans* has been attributed as co-factor that promotes adherence. Propolis, a natural product produced by the honeybee, has been shown to possess antifungal activity although the mechanism of its action remains unclear. The aim of this study was to investigate the germ tube formation capacity of *C.albicans* (ATCC18804) following its exposure to Brazilian Green Propolis (BGP) at different concentrations. The ultrastructural topographic features of the yeast cells exposed to propolis using transmission electron microscopy (TEM) and light microscopy (LM) were performed to investigate the morphology of the yeast. Yeast cell suspensions were added to tubes containing foetal calf serum medium (2 h-37°C). Nystatin and *C. glabrata* (ATCC 2001) were used as control. Absence of germ tube formation (LM) occurred at 0,33 µg/mL. The ultrastructural findings (TEM) showed hyperplasia and changes in the cell surface at 0,43 µg/mL . It is suggested that the antifungal activity of propolis is due to changes in the cell wall leading to an increase of volume and membrane rupture. The positive results suggest that propolis should be further tested as an alternative therapy for infectious conditions of the oral cavity, such candidiasis and denture stomatitis.

**Key words:** *Candida albicans*; germ tubes; Brazilian Green Propolis; MIC; Electronic Microscopy

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### Introduction

Development of effective strategies for treatment of candidosis and other oral fungal diseases has been a challenge, considering the increase of opportunistic fungal infections in immunocompromised patients<sup>1</sup>. Some of the drugs used for the treatment of candidosis, such as amphotericin B, are very toxic, and others, such as fluconazole, are limited because the high rate of cell spontaneous mutation for fungal resistance<sup>2</sup>. Thus, searching for alternative antifungal compounds has been a concern in recent years.

Propolis has been widely employed in popular medicine, mainly in communities with inadequate conditions of public health. Propolis and its compounds have been studied in order to be effective against bacteria and fungi<sup>3,4,5,6,7,8</sup>. Brazilian Green Propolis (BGP) is collected by bees *Apis mellifera* of the *Baccharis dracunculifolia*<sup>9,10,11</sup> and exhibit *in vitro*<sup>12,13</sup> and *in vivo*<sup>14</sup> antimicrobial activity against *Candida albicans* and other gram positive and gram negative oral microorganisms.

The aim of this study was to evaluate the germ tube formation of *C.albicans* following its exposure to propolis at different concentrations focusing on the ultrastructural topographic features of the yeast cells.

### Material and Methods

The Brazilian Green Propolis (BGP), origin from *Baccharis dracunculifolia*, was collected from the honey bee *Apis mellifera* in Minas Gerais State, Brazil. The 20% ethanolic propolis extract used in this study was extracted by PharmaNéctar® (Belo Horizonte, Brazil). Crude propolis samples were further dehydrated with a low-vacuum pump, and the extracts of the dried propolis were prepared as described by Park et al.<sup>15</sup>. The dried propolis samples were ground into fine powder, and 2.0g of propolis was mixed with 25 ml of 80% aqueous ethanol in a test tube and shaken at 70°C for 30 min. After extraction, the mixture was centrifuged at 8.000 rpm to obtain the supernatants, which were named as BGP.

The MIC of BGP Extract was determined against the yeast in accordance to National Committee for Clinical Laboratory Standards-NCCLS<sup>16</sup>. MIC was determined in RPMI 1640 (Gibco, Invitrogen Co., New York, USA) with MOPS, pH 7.0. *C. albicans* (ATCC 18804) was grown in Sabouraud dextrose agar (Difco, Detroit, Michigan, USA) plates at 37°C for 24-48h. Samples of *C. albicans* (ATCC 18804) were displayed in different concentrations of BGP Extract. The starting inoculum was 1.0 x 10<sup>6</sup> CFU/ml. Microtitre trays were incubated at 37°C in dark chamber, and MIC were recorded after 48 h of incubation. MIC was determined as the lowest concentration of the propolis extract, which inhibited the growth of the tested microorganisms. Nystatin (Sigma, USA) was used as positive control.

The germ tube tests were made with human serum (Sigma, USA). The yeast was displayed in different concentrations of BGP and Nystatin and incubated at 36°C in water bath during 2 hours. *C. glabrata* was used as the negative control for germ tube formation. After 48 hours at 37°C aerobic incubation, in foetal calf serum, aliquots of the microorganisms cultures suspensions, containing different concentrations of propolis, were observed through Light Microscopy (LM). For conventional electron microscopy<sup>1</sup>, the yeast treated with BGP was fixed for 2 hours at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After that, it was carried out in 1% osmium tetroxide in cacodylate buffer containing 0.8% potassium ferrocyanide and 5 mM CaCl<sub>2</sub>. The cells were dehydrated in acetone and embedded in Epon. Ultrafin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss CEM-900 scanning electron microscope.

### Results

The MIC values for *C. albicans* and *C. glabrata*, the germ tube formation and Colony Forming Units (CFU) are reported in Table 1. These results showed that BGP has an influence on the cellular morphology of *C. albicans* and act on the germ tube formation. The propolis antifungal activity seems to be associated to the microorganism cellular wall, as showed on Table 2.

Table 1: Minimum Inhibitory Concentration (MIC) of Brazilian Green Propolis Extract (BGP) and Nystatin (NYS) against *C. albicans* and *C. glabrata*, CFU inhibition and germ tubes formation.

Microorganism	MIC ( $\mu\text{g/mL}$ )			
	BGP	NYS	Germ tube inhibition	CFU inhibition
<i>C. albicans</i>	0.00	0.00	no	no
	0.33	0.49	yes	no
	0.90	1.38	yes	yes
<i>C. glabrata</i>	0.00	0.00	-	no
	0.85	0.38	-	yes

Table 2: *C. albicans* cell morphology alterations in different Brazilian Green Propolis concentrations.

Propolis - $\mu\text{g/ml}$	<i>Candida albicans</i> / Cell morphology alterations			
	Cell agglomeration	Cell edema	Cell rupture	Cell division
0.350	Yes	Yes	Yes	No
0.175	Yes	Yes	Yes	No
0.87	Yes	Yes	Yes	No
0.43	Yes	Yes	Yes	No
0.21	No	No	No	No
0.10	No	No	No	Yes
0.05	No	No	No	Yes
0.025	No	No	No	Yes
0.012	No	No	No	Yes

Scanning electronic microscopy analysis of the ultrastructure of the yeast cells revealed changes in the cell wall. Scanning images showed control yeasts with a normal budding profile. Changes in the outer layer of the cell wall, showing irregular budding sites, were observed in treated *C. albicans* (Fig.1).

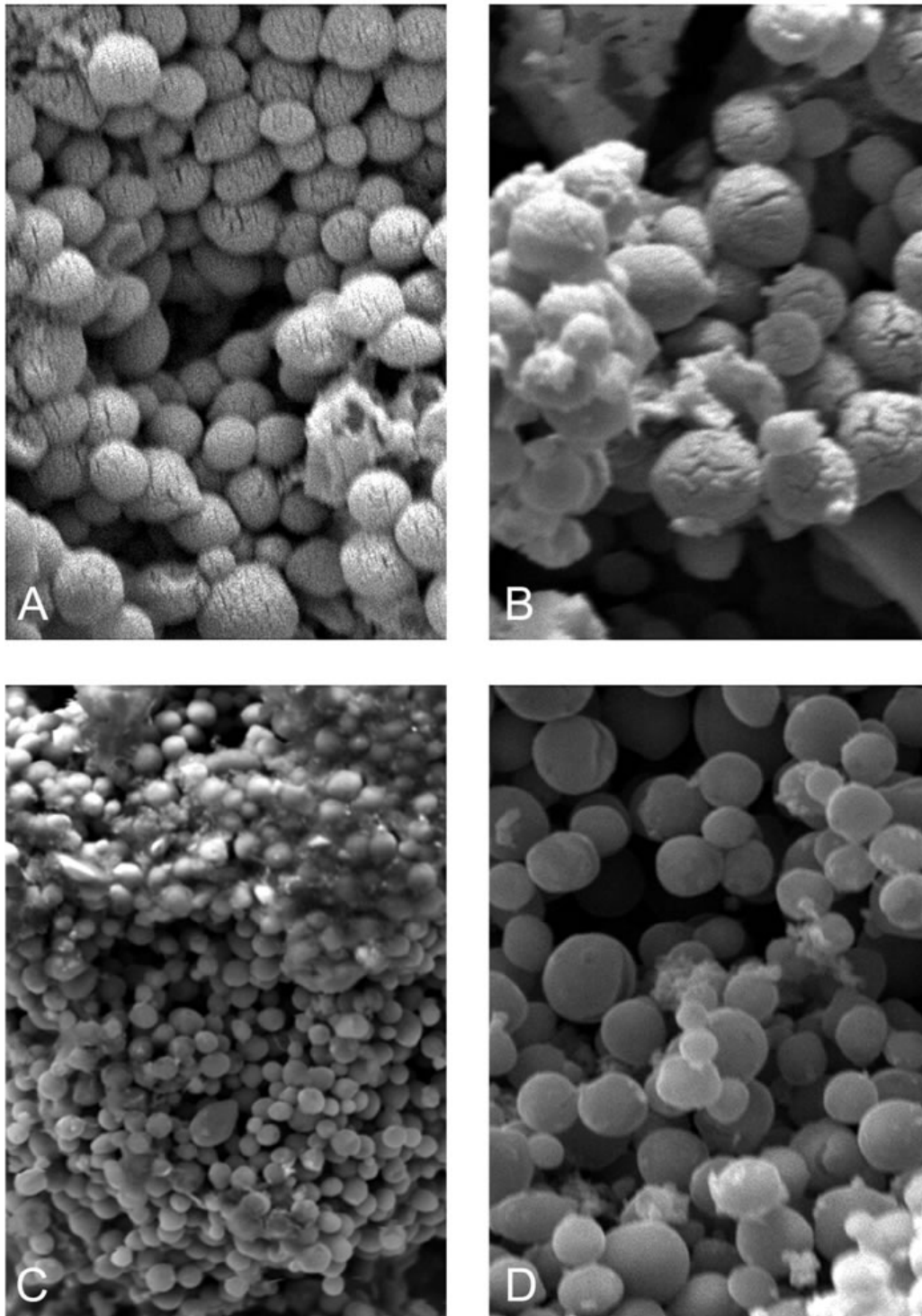


Figure 1: Micrographs showing *C. albicans* treated for 24h with subinhibitory concentrations of Brazilian Green Propolis extract (BGP). Scanning electron micrographs: Treated (panels A, B, and C) and untreated (panel D). A and B: cell wall detachment. C: cell agglomeration.

### Discussion

*Candida* species are the most frequent causes of oral and systemic mycosis in our era<sup>20</sup>. Resistant species of *Candida* to usual antifungal drugs in hospital environment and oral lesions have been reported<sup>21</sup>.

Although propolis is not widely used in conventional healthcare, propolis have been recommended for use as home remedies for the treatment of oral candidiasis, denture stomatitis and skin lesions by numerous books and articles in the popular press<sup>14,20</sup>. Although many studies have focused on showing the antifungal activity of propolis extract, few have demonstrated their effects on the morphology and structure of *Candida albicans*<sup>23,24</sup>.

This study reports the ultrastructural alterations of *C. albicans* seen in electron microscopy when treated with propolis extract. The morphological changes included detachment of the fungal cell wall, and disturbance of division resulting in defects in the texture of the daughter-cell wall. Furthermore, BGP were able to reduce the appearance of germ tubes after 2 h of exposure without affecting cell growth. This inhibition of germ tube formation is probably due to the different chemical content of propolis. Although the mechanism is not clear, we can speculate that one possible mechanism for propolis inhibition of yeast growth and germ tube formation in *C. albicans* is due to an interaction with cellular sulphhydryl compounds. Comparison of these results with those induced by imidazole derivatives in *Candida* reveals some similarities<sup>25</sup>. Invagination of the plasmalemma was observed in yeasts treated with saperconazole and low doses of miconazole and clotrimazole and vesicles were also observed with saperconazole, causing disruption of the dynamic relationship between ergosterol and chitin biosynthesis<sup>26</sup>. Several studies have demonstrated the antimycotic effect of Brazilian propolis<sup>14,27</sup>.

Although the antifungal activity of propolis is well established, we have demonstrated in this study the effect of Brazilian Green propolis on the *in vitro* germ tube formation of *C. albicans*. The deleterious effect of the BGP on the cell wall of the *C. albicans* may be the main reason for the decrease in the rate of yeast budding, because the integrity of the cell wall is necessary for cell division<sup>27</sup>. In addition, a general change in the morphology of the yeasts was frequently observed, which could also be due to the loss of integrity of the cell wall. Consequently, we suggest that the probable mode of action of propolis against fungi could be attributed to an alteration in cell permeability, which explain the changes in the morphology and size of the internal organelles such as mitochondria and vacuoles<sup>1</sup>.

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