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Effect of commercial ethanol propolis extract on the *in vitro* growth of *Candida albicans* collected from HIV-seropositive and HIV-seronegative Brazilian patients with oral candidiasis

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Abstract: The present study assessed the susceptibility of *Candida albicans* strains, collected from HIV-positive patients with oral candidiasis, to a commercial 20% ethanol propolis extract (EPE) and compare it to the inhibitory action of the standardized antifungal agents nystatin (NYS), clotrimazole (CL), econazole (EC), and fluconazole (FL). Twelve *C. albicans* strains collected from HIV-positive patients with oral candidiasis were tested. The inhibition zones were measured with a pachimeter and the results are reported as means and standard deviation ($M \pm SD$). Data were analyzed statistically by the non-parametric Kruskal-Wallis test. EPE inhibited all the *C. albicans* strains tested. No significant difference was observed between the results obtained with NYS and EPE, while significant differences were observed between EPE and other antifungals. The *C. albicans* strains tested showed resistance to the remaining antifungal agents. The propolis extract used in this study inhibited the *in vitro* growth of *C. albicans* collected from HIV-seropositive Brazilian patients, creating/forming

inhibition zones like those ones formed by NYS. This fact suggests that commercial EPE could be an alternative medicine in the treatment of candidiasis from HIV-positive patients. However, *in vivo* studies of the effect of EPE are needed to determine its possible effects on the oral mucosa. (J. Oral Sci. 44, 41-48, 2002)

Key words: oral candidiasis; *Candida albicans*; HIV patients; Brazilian propolis.

Introduction

Opportunistic infections caused by *Candida* species are particularly common in immunocompromised patients (1). It is known that more than 90% of Human Immunodeficiency Virus- infected patients (HIV- positive patients) suffer from oral candidiasis at some point during their disease (2,3).

Many antifungal agents are available for the treatment of candidiasis, such as polyenes, azoles and the DNA-analogue 5-fluorocytosine. Despite the availability of such an array of agents, failure of therapy is not uncommon (4,5). Different studies have suggested the resistance of some *C. albicans* strains from HIV-positive patients to antifungal agents (6-9). Propolis is a resinous hive product collected by bee from tree buds and mixed with secreted beeswax. The propolis is used by bees as a glue to seal the opening of the hives (10), and thus being considered a natural

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antibiotic (11). Propolis has been used as a therapeutic agent by the world population since the time of Hippocrates (12). It is known that the ethanolic extract of propolis (EPE) exhibits some pharmacological activities, such as antibacterial, antiviral, antifungal, anti-inflammatory, anesthetic and cytostatic properties (13-15). A number of investigations have shown the antimicrobial activity of propolis against various pathogenic microorganisms (10,16,17). Furthermore, earlier reports demonstrated that the EPE inhibited the *in vitro* growth of *Candida albicans* (18-20).

Therefore, pharmacological applications of propolis preparations have led to an increased interest in its chemical composition. So far, polyphenolic compounds, e.g. flavonoid aglycones, phenolic acids and their esters, and phenolic aldehydes, have been mainly identified in propolis collected by bees in different regions (21). The flavonoid aglycones have been considered the main active substance in propolis. However, the composition of propolis is quantitatively and qualitatively variable, depending on area and ecology (10,20,22,23).

The composition of propolis can affect their biological activity. Considering that in Brazil the EPE is not recognized as a medicine, the access of population is made easy by the low price of the product in market and this same population uses it discriminated, the aim of the present paper was to evaluate the susceptibility of *Candida albicans* from HIV-positive patients with oral candidiasis to a commercial 20% (EPE) and compare it to the inhibitory action of some popular antifungal agents.

Materials and Methods

Microorganism strains

Candida albicans strains were collected from oral lesions of pseudomembranous and erythematous candidiasis of 12 HIV-positive patients seen at the Reference Center Treatment for Sexually Transmitted Diseases of Belo Horizonte, Minas Gerais State (PR1, PR2, PR3, PR4, PR5, PR6, PR7, PR8, PR9, PR10, PR11 and PR12). Twelve patients, eight male and four female, with ages ranging from 22 to 46 years (mean = 31.1), were selected. The strains were isolated in Sabouraud broth (Difco, USA) containing chloramphenicol and incubated at 37°C for 48 hours and identified by colony morphology, germinative tubes formation, and biochemically (Candifast, Microbio, France). *Candida albicans* was collected from 12 normal soronegative patients (PN1, PN2, PN3, PN4, PN5, PN6, PN7, PN8, PN9, PN10, PN11 and PN12), six male and six female, with ages ranging from 26 to 48 years (mean = 34.7) with erythematous atrophic candidiasis (Denture stomatitis).

Propolis solutions

Ethanol extract containing 20% propolis (EPE) (Nectar Farmacêutica, Belo Horizonte, MG, Brazil) was purchased at random in stores specialized in apicultural products in Belo Horizonte (Minas Gerais State, South-Eastern Brazil) and tested for their inhibitory ability for the microorganism growth *in vitro*. The original formula of the propolis solution obtained commercially was used in the experiments.

Test of antimicrobial susceptibility (agar diffusion test)

The susceptibility test of *C. albicans* to the propolis solution was performed by agar diffusion according to the method of Collins et al. (24). A 0.1 ml aliquot of 48 hour cultures of each 12 *Candida* strains incubated at 37°C in dextrose Sabouraud, corresponding to 0.5 turbidity on the McFarland scale (10^8 UFC/ml), was plated onto 30 ml agar Sabouraud (Difco, USA) previously melted and distributed on Petri dishes measuring 150 mm in diameter.

Sterile blank disks (CECON, São Paulo, Brazil) were soaked in 20 μ l of the ethanol propolis solution (20%) and applied to the agar surface previously seeded with the microorganism. Econazole (EC, 25 mg), clotrimazole (CL, 50 mg), and fluconazole (FL, 25 mg) (CECON, São Paulo, Brazil) discs were used to compare the effect of the EPE. Positive and negative controls of the discs containing 100 IU nystatin (NYS) (CECON, São Paulo, Brazil) and 20 μ l sterile distilled water (DW) were used, respectively. The effect of 93.2° alcohol (AL) was also determined, because it is the solvent in the EPE.

After 48 hours of incubation at 37°C, the diameters of the inhibition zones were measured and compared according to the method of Hindler and Jorgensen (25). For each *Candida* sample, three experiments were made in three different times.

Statistical analysis

The results of the diameters of the inhibition zones are reported as means $\pm$ standard deviation ($M \pm SD$) and were analyzed statistically by the non-parametric Kruskal-Wallis test. Differences at the 1% level of variation ($P < 0.05$) were considered to be significant.

Results

EPE was effective in inhibiting the *in vitro* growth of all *C. albicans* strains tested (Fig. 1). The mean values of the inhibition zones of *C. albicans* isolates from 12 HIV-positive and 12 HIV-negative patients exposed to different substance are shown in Table 1 and Table 2, respectively.

There were no significant differences between the data obtained for EPE and NYS ($P < 0.05$). However, there was significant difference between NYS and other antifungal agents. Strains PR9 and PR11 of *C. albicans* were significantly more susceptible to EPE than NYS. The mean diameters of the inhibition zones caused by EPE and NYS were similar for strains PR3, PR6, PR7 and PR8.

EC showed significantly lower inhibition zones for 10 of the 12 *C. albicans* strains tested when compared to EPE and NYS. EC was ineffective against *in vitro* growth of strains PR2, PR8 and PR11. CL and FL did not inhibit the *in vitro* growth of the 12 *C. albicans* strains tested. Double inhibition zone areas were observed around the discs, showing microorganism resistance to these antifungal

agents (Fig. 1).

However, all the strains of *C. albicans* collected from HIV-seronegative patients with Denture stomatitis showed significant susceptibility to all antifungals and EPE tested. When compared to EC, CL and FL, the inhibition zones were bigger and were significantly different from those seen with NYS and EPE. The inhibition zones included by AL were significantly smaller than those observed for all antifungal agents tested, included EPE.

Discussion

This paper studied the *in vitro* susceptibility of *C. albicans* to one 20% commercial brand ethanol extract of Brazilian propolis. The sample was used as it is presented

Table 1 Mean* and Standard Deviation ($M \pm SD$) inhibition zones values of *in vitro* susceptibility of *Candida albicans* from HIV-seropositive patients exposed at commercial EPE, antifungal agents and 93.2° alcohol

Isolate	EPE 20%	EC	NYS	AL	CL	FL
PR1	17.6 $\pm$ 1.52	3.3 $\pm$ 5.77	17.0 $\pm$ 1.00	10.3 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR2	21.3 $\pm$ 4.10	0.0 $\pm$ 0.00	15.3 $\pm$ 1.15	11.2 $\pm$ 2.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR3	16.6 $\pm$ 0.50	6.6 $\pm$ 5.77	17.0 $\pm$ 1.00	6.0 $\pm$ 5.10	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR4	16.3 $\pm$ 2.30	10.6 $\pm$ 1.15	17.6 $\pm$ 1.15	5.30 $\pm$ 4.60	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR5	20.3 $\pm$ 2.51	3.3 $\pm$ 5.77	16.6 $\pm$ 0.57	10.3 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR6	18.6 $\pm$ 1.50	6.6 $\pm$ 5.77	17.0 $\pm$ 1.77	9.5 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR7	17.3 $\pm$ 0.50	7.3 $\pm$ 6.83	18.0 $\pm$ 1.00	9.5 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR8	16.6 $\pm$ 3.50	0.0 $\pm$ 0.00	15.3 $\pm$ 0.50	9.5 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR9	29.6 $\pm$ 0.50	7.3 $\pm$ 6.40	20.0 $\pm$ 0.00	9.0 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR10	23.0 $\pm$ 2.80	3.3 $\pm$ 5.77	18.0 $\pm$ 1.77	11.0 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR11	25.6 $\pm$ 1.15	0.0 $\pm$ 0.00	17.0 $\pm$ 0.00	11.6 $\pm$ 1.15	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
PR12	20.0 $\pm$ 0.00	3.3 $\pm$ 5.77	20.0 $\pm$ 0.00	9.0 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00

Legend: EPE = Ethanol Propolis Extract; EC = econazole; NYS = nystatin; AL = alcohol;

CL = clotrimazole; FL = fluconazole

*Mean of three measurements

Table 2 Mean* and Standard Deviation ($M \pm SD$) inhibition zones values of *in vitro* susceptibility of *Candida albicans* from HIV-seronegative patients exposed at commercial EPE, antifungal agents and 93.2° alcohol

Isolate	EPE 20%	EC	NYS	AL	CL	FL
PN1	19.0 $\pm$ 1.73	27.1 $\pm$ 2.60	18.0 $\pm$ 0.00	10.3 $\pm$ 1.15	25.6 $\pm$ 2.08	22.6 $\pm$ 1.15
PN2	17.3 $\pm$ 2.80	26.0 $\pm$ 1.00	17.0 $\pm$ 1.73	11.2 $\pm$ 2.00	24.6 $\pm$ 0.50	23.3 $\pm$ 1.15
PN3	21.0 $\pm$ 1.73	27.6 $\pm$ 2.08	18.0 $\pm$ 0.00	6.0 $\pm$ 5.10	25.6 $\pm$ 0.57	24.00 $\pm$ 0.00
PN4	21.0 $\pm$ 1.00	25.6 $\pm$ 0.50	18.0 $\pm$ 0.00	5.30 $\pm$ 4.60	25.3 $\pm$ 1.50	22.6 $\pm$ 1.15
PN5	20.3 $\pm$ 1.15	25.0 $\pm$ 0.00	18.0 $\pm$ 0.00	10.3 $\pm$ 1.15	25.3 $\pm$ 0.50	22.3 $\pm$ 0.50
PN6	18.3 $\pm$ 0.50	25.6 $\pm$ 0.50	17.3 $\pm$ 1.15	9.5 $\pm$ 1.15	25.3 $\pm$ 0.50	22.3 $\pm$ 0.50
PN7	19.6 $\pm$ 1.50	26.3 $\pm$ 0.50	17.6 $\pm$ 0.57	9.5 $\pm$ 1.15	25.3 $\pm$ 0.50	24.0 $\pm$ 0.00
PN8	20.6 $\pm$ 0.50	25.6 $\pm$ 1.15	18.0 $\pm$ 0.00	9.5 $\pm$ 1.15	25.3 $\pm$ 1.15	22.3 $\pm$ 0.50
PN9	19.0 $\pm$ 1.70	26.0 $\pm$ 0.00	18.0 $\pm$ 1.00	9.0 $\pm$ 0.00	25.0 $\pm$ 0.00	22.6 $\pm$ 1.15
PN10	20.3 $\pm$ 1.15	25.6 $\pm$ 0.50	18.0 $\pm$ 0.00	11.0 $\pm$ 0.00	25.0 $\pm$ 1.00	22.0 $\pm$ 0.00
PN11	19.6 $\pm$ 1.50	25.6 $\pm$ 0.50	18.0 $\pm$ 0.00	11.6 $\pm$ 1.15	25.0 $\pm$ 0.00	23.0 $\pm$ 1.00
PN12	17.0 $\pm$ 1.15	25.6 $\pm$ 0.50	18.6 $\pm$ 1.15	9.0 $\pm$ 0.00	25.6 $\pm$ 0.50	23.3 $\pm$ 1.15

Legend: EPE = Ethanol Propolis Extract; EC = econazole; NYS = nystatin; AL = alcohol;

CL = clotrimazole; FL = fluconazole

*Mean of three measurements

to the population, without modifications.

The chemical compounds of propolis have been isolated and analyzed in different countries, with caffeic acid, coumaric acid and flavonoid aglycones possibly accounting for the antibiotic power of this resin (26). The chemical constitution of propolis varies according to the type of bee, the geographic area of origin and the extraction and quality control methods used by each manufacturer (7). Chemometrics has been shown quite efficient in uncovering relationships between chemical composition of a sample and its geographical origin. Forty propolis samples originating from the South and South East of Brazil were analyzed by high performance liquid chromatography (HPLC) and 18 compounds of interest were studied which included: caffeic, p- coumaric and ferulic acids, and some of their derivatives, pinobanksin, a derivative of kaempferol and five phenolic compounds (assigned as 3-prenyl-4-hydroxycinnamic acid (PHCA); 2,2-dimethyl-6-carboxyethyl-2H-1-benzopyran (DCBEN); 3,5-diprenyl-4-hydroxycinnamic acid (DHCA); compound E (still unknown) and 6-propenoic-2,2-dimethyl-8-prenyl-2H-1-benzopyran acid (DPB). Principal Component Analysis (PCA) indicated three different groups of propolis samples, having the same typical chromatogram, evaluated by HPLC. Samples from the South East group were rich in derivatives of kaempferol. Samples from the South group had a high content of DPB compound, but a low concentration of kaempferol derivatives and of DCBE compound. Samples from the South group II were

characterized by a high concentration of DCBEN, DHCA, p-coumaric and DPB compounds.

Therefore, the identification of new compounds in Brazilian propolis can give useful information about the plant sources of a given geographic area (27).

Several studies have demonstrated the antimycotic effect of propolis (11,28). Velikova et al. (29) tested twenty-one propolis samples produced by 12 different Meliponinae species which were analyzed by GC-MS. Several chemical types of stingless bees' propolis could be grouped, according to the prevailing type of compounds like: "gallic acid," "diterpenic" and "triterpenic" types. The results confirm that neither the bee species nor the geographical location determine the chemical composition of Meliponinae propolis and the choice of its plant source, respectively. This could be explained by the fact that Meliponinae forage over short distances (maximum 500 m) and thus use as propolis source the first plant exudate they found during their flights. The antibacterial, antifungal and cytotoxic activities of the samples were also investigated. Most samples had weak or no activity against *E. coli*, and weak action against *Candida albicans*. Some of them showed significant activity against *S. aureus*, presumably connected to the high concentration of diterpenic acids.

Several investigators have demonstrated the antifungal action of propolis against *C. albicans* (12,30), but few workers have documented the effect of the antifungal activity of Brazilian propolis on *in vitro* growth of *C. albicans* of HIV-seropositive Brazilian patients. The mean inhibition zones caused by the positive control (NYS) in all *Candida species* tested corresponded to the pattern previously defined in the literature (4,31). The *C. albicans* resistance to EC, CL and FL observed in this work is explained by the fact that the HIV- positive patients have already been treated with these antifungal agents. The zones of inhibition induced by EPE were similar to those observed for NYS, with no significant difference in HIV-positive *C. albicans* inhibition. However, the EPE mechanism of action on *C. albicans* needs more studies. The mechanism of the antimicrobial action of propolis seems to be complex and is not completely understood. According to Amoros et al. (32) and Bonhevi et al. (17), its activity against microorganisms is more related to the synergistic effect of flavonoids (and other phenolics) than to the individual compounds. These findings are in agreement with those of Takaisikikuni and Schilcher (33), who observed that the antibacterial action against *Strep. agalactiae* was complex, involving several mechanisms such as the formation of pseudomulticellular streptococci; disorganization of the cytoplasm, the cytoplasmatic

Fig. 1 Inhibition zones of *in vitro* culture of *Candida albicans* collected from HIV-positive patients exposed to Ethanol Extract Propolis (EPE), and antifungal agents.

P = Ethanol Propolis Extract, EC = econazole, NYS = nystatin, AL = alcohol, CL = clotrimazole, FL = fluconazole, DW = distilled water

membrane, and the cell wall, partial bacteriolysis; and inhibition of protein synthesis. They concluded that a simple analogy cannot be made with the mode of action of any classic antibiotics.

Marcucci et al. (34) isolated four compounds from Brazilian propolis. The authors identified them as: 1) 3-prenyl-4-hydroxycinnamic acid (PHCA), 2) 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyrane (DCBEN), 3) 3,5-diprenyl-4-hydroxycinnamic acid (DHCA), and 4) 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzopyran (DPB). The structures of the compounds were determined by MS and NMR techniques. All compounds were assayed against *Trypanosoma cruzi* and the bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus faecalis*. Compounds (1) to (4) were active against *T. cruzi*. With the exception of (1), all compounds presented activity against the bacteria tested. However, the mechanism of action of propolis was not known until now.

De Paula et al. (35) demonstrated that 7 of the 14 Brazilian brands of ethanol extract solutions of propolis tested presented a pattern of inhibition similar to NYS against the “*in vitro*” growth of *C. albicans*. The author previously demonstrated that EPE induced zones of inhibition varied according to different *C. albicans* strains. The variations observed in the diameter of inhibition zones around the discs containing propolis were probably related to the type and concentration of chemical components, and also to bee species and to the geographic region where they were produced (10,12,27,29,36-38).

Propolis is a resinous hive product collected by honeybees from various plant sources. The composition of the propolis depends upon the time, vegetation and the area of collection. Thus, quality evaluation of the propolis is important, before use in food and beverages. For this propose, methanol and water extracts of six different propolis from Brazil were studied by chromatography (39). The authors (39) demonstrated 39 compounds with high intensity variety between B1-M/ B1-W and B6-M/ B6-W propolis. In this study, the B5-M propolis sample represent the EPE of our experiment.

All experiments present samples of microorganism resistance to 93.2° alcohol as shown in figure 1, a finding not observed with EPE. In our experiments, we used 20% EPE as a pharmaceutical solution that is sold in market. The southwestern Brazilian region is the source of the product, specifically the Minas Gerais State. According to Koo et al. (10), there are differences in quality and quantity in chemical compounds of propolis solution collected from different areas. However, this fact does not alter the antifungal activity of the samples.

Flavonoids are well known for their antibacterial, antifungal and antiviral action and are thought to be responsible for the beneficial properties of propolis (13,15). Esters of phenolic acids and specially caffeates and ferulates have also been identified as antibacterial, antifungal and antiviral principles of propolis (18,26,40). This is in good correlation with the action observed from propolis from many countries (20). In the latter, however, the decrease of the phenolic compounds content is not accompanied by a decrease of activity. Obviously, there are other compounds that contribute to the biological activity in this case. The triterpenes (lanosterol, cycloartenol, -amyrene and one of its isomers), might play a role, but there could be other active principles, still unidentified. Brazilian propolis samples, including those from indigenous bees, contained little or no phenolic acid esters, and most of them contained only traces of flavonoid aglycones. Their activities, however, were similar to those of European samples (20). The large amounts of aromatic (prenylated derivatives of coumaric acid) and diterpenic acids might contribute to the antibacterial activity of these samples (27,39,41).

Some flavonoids are considered antimicrobial, such as pinocembrin, galangin, and sakuranetin (13,42,43). Osawa et al. (44) reported that kaempferol had antimicrobial activity against *Strep. mutans* and *Act. viscosus* and Cai and Wu (45) showed that kaempferol also inhibited the growth of *Porphy. gingivalis* and *Prev. intermedia*. Aga et al. (41) showed that some hydroxycinnamic acid, isolated and identified in Brazilian propolis, showed antimicrobial activity against *Bacillus cereus*, *Enterobacter aerogenes*, and *Arthroderma benhamiae*.

When the intra-pattern variations of the 12 *C. albicans* strains from HIV patients were compared, EPE and NYS invariably suppressed the *in vitro* growth of *Candida* species. This suggest that NYS and EPE potentiate a fundamental change by inhibiting metabolic precursors involved in *C. albicans* metabolism, even after exposure to the drugs, whereas the other drugs seem to be less potent.

The antifungal activity of CL, EC, FL and NYS observed in the strains collected from HIV-seronegative patients (Table 2) was expected and showed the efficacy of these drugs, even now confirmed in literature (9). However, when these results and those from HIV- seropositive patients are compared, the profile of inhibition was significantly different. However, azoles are usually used in complicated cases of candidiasis when the possibility of using NYS is over (46).

Propolis has been used as a medicine all over the world in a random, indiscriminate manner without medical indication, and is considered to belong to the cosmetic

group. Thus, there is no systematization or control of its use by the population. The ethanol solution is the form most frequently available in grocery stores, pharmacies and stores specializing in apicultural products. Considering the free access of the population to propolis solutions and the fact that they are natural medicines with no therapeutic contraindication, our study suggest that EPE can be used topically in HIV patients infected by oral candidiasis.

Brazilian propolis has been studied all over the world from different viewpoints (4,10,47,48), nevertheless *in vitro* results cannot be directly extrapolated to *in vivo* biological conditions. *In vivo* studies should be carried out for a better evaluation of the activity of propolis against microorganisms of the *Candida* species, considering the dynamics of human beings as a whole, as well as the interference of systemic parameters and oral ecology.

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