

Alternative therapies for denture stomatitis treatment: in vivo experimental model in rats

Terapias alternativas para o tratamento da estomatite por prótese total: modelo experimental in vivo em ratos

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ABSTRACT

Background: Denture stomatitis (DS) is a multifactorial condition that commonly affects denture users and is mainly caused by *Candida albicans*. Due to the toxic effects of antifungal therapy, new therapies for DS are claimed. **Objective:** The aim of the study was to evaluate the efficacy of aqueous extract of *Buchenavia tomentosa* and sodium bicarbonate against *C. albicans* in a model of DS in rats. **Material and Methods:** An acrylic resin device simulating a denture base was fixed covering the palate of forty-eight male rats followed by candidiasis induction. Rats were divided into 4 groups (n = 12): Control, sodium bicarbonate, *B. tomentosa* and nystatin (positive control). Each group was subdivided according to the period of treatment; 24 h (n = 6) and 48 h (n = 6). Animals were sacrificed and had their devices removed for *C. albicans* counts and SEM analysis. The palate mucosa was removed and processed for histopathologic analysis. **Results:** After 24 h of treatment, both *B. tomentosa* and nystatin groups reduced significantly *C. albicans* counts when compared to control (nystatin x control, $p < 0.01$; *B. tomentosa* x control, $p = 0.03$). The results were confirmed by the histologic analysis. **Conclusion:** Both the aqueous extract of *B. tomentosa* and sodium bicarbonate was able to significantly decrease *C. albicans* counts in an experimental model of DS.

KEYWORDS

Buchenavia tomentosa; *Candida albicans*; Stomatitis, Denture; Therapies, Alternative.

RESUMO

A estomatite por prótese (EP) é uma condição multifatorial que acomete frequentemente usuários de prótese total e geralmente é relacionada com *Candida albicans*. Devido aos efeitos tóxicos da terapia antifúngica, novas terapias para EP são necessárias. **Objetivos:** O objetivo deste estudo foi avaliar a eficácia do extrato aquoso de *Buchenavia tomentosa* e bicarbonato de sódio frente a *C. albicans* em um modelo de EP em ratos. **Material e Métodos:** Um aparelho de resina acrílica simulando a base da prótese total foi fixado cobrindo o palato de 48 ratos machos seguido por indução da candidose. Os ratos foram divididos em 4 grupos (n=12): controle, bicarbonato de sódio, *B. tomentosa* e nistatina (controle positivo). Cada grupo foi subdividido de acordo com o período de tratamento; 24 horas (n=6) e 48 horas (n=6). Os animais foram sacrificados e os aparelhos foram removidos para contagem de *C. albicans* e análise por microscopia eletrônica de varredura. **Resultados:** Após 24 horas de tratamento, observou-se redução significativa da contagem de *C. albicans* tanto *B. tomentosa* quanto nistatina (nistatina x controle, $p < 0,01$; *B. tomentosa* x controle, $p = 0,03$). Os resultados foram confirmados pela análise histológica. **Conclusão:** Tanto o extrato aquoso de *B. tomentosa* e o bicarbonato de sódio foram capazes de reduzir significativamente as contagens de *C. albicans* em modelo experimental de EP.

PALAVRAS-CHAVE

Buchenavia tomentosa; *Candida Albicans*; Estomatite, Prótese total; Terapias Alternativas.

INTRODUCTION

Denture stomatitis (DS) is a common condition associated to deficient hygiene and maintenance of dentures during sleep, and salivary characteristics among other factors [1,2]. Mostly of the denture wearers are elders, and they show, specially the institutionalized ones, salivary changes that imply on oral function and dentures balance leading to denture stomatitis and oral candidiasis, for example [3].

The amount of biofilm on the denture resin surface, associated to surface roughness, promotes an adequate environment to *Candida* proliferation [4,5]. *C. albicans* is the most common species involved in denture stomatitis cases [6]. It has been shown that the elder denture wearers have a diverse microbiome that changes composition in different sites of the mouth and that teeth presence or absence is very important for this diversity. Though *Candida* spp. provides an acid environment, *Candida*/bacteria interaction is crucial for the development of denture associated injuries [7]. One of the main *Candida* spp. virulence factors is the ability to adhere and colonize abiotic surfaces, including prosthetic dentures [8,9].

Traditional antifungal therapies show some limitations, such as narrow spectrum of action, reduced number of available drugs, occurrence of drug interactions, important side effects, high cost and rising antifungal resistance of some species of *Candida* [10-13]. In addition, the increasing of *Candida* spp. azole resistance and the limited available therapies to oral candidiasis represent a challenge [14,15]. Moreover, despite of being efficient in acute cases traditional therapy shows considerable recurrence rates, especially if combined with deficient hygiene [16].

For these reasons, alternative therapies to denture stomatitis have been proposed with

satisfactory results. Some of the reported therapies are propolis [17], vinegar [5], and topic gel with plant extract [6]. The effect of *Buchenavia* spp. against virus [18] and bacteria [19] was reported in the literature. Previous studies of our group showed a promising in vitro antimicrobial effect of *Buchenavia tomentosa* extracts against cariogenic bacteria [20] and several species of *Candida* [21]. This species, popularly named Tarumarana, is a small-medium tree with comestible leaves, commonly found in Brazilian Pantanal region [22]. Based on our previous findings on *B. tomentosa* anti-candidal activity and the limited results on the literature regarding the benefic effects of sodium bicarbonate, the aim of this study was to evaluate the in vivo effect of *B. tomentosa* extract and sodium bicarbonate to the treatment of denture stomatitis.

MATERIAL AND METHODS

Study groups

The protocol of this study was approved by the Local Research Ethics Committee involving animals (03/11- PA/CEP). All procedures performed in the study were in accordance with the ethical standards of the institution or practice at which the study was conducted. Forty-eight male rats (*Rattus norvegicus* Wistar), weighing about 350 g (approximately 3 months of age) were included in this study. The animals were divided into 4 groups (n = 12): *Buchenavia tomentosa*, sodium bicarbonate, control, and nystatin (positive control). Each group were divided into two subgroups (n = 6) according to the treatment period: 24 h and 48 h.

Preparation of the aqueous extract of *Buchenavia tomentosa*

Leaves of *Buchenavia tomentosa* were collected, identified and the extracts were prepared as previously shown [21]. Shortly, leaves were dried in oven at 38°C, powered and

20 g of the powder was added to 400 ml of water. The aqueous extracts were obtained by decoction in deionized water for 5 min at 100°C.

Preparation of solutions

Solutions of sodium chloride (NaCl) 0.9%, sodium bicarbonate (NaHCO₃) 3% and nystatin 50000 UI were prepared in distilled water and sterilized by filtration in 0.22 µm filter.

Preparation of animals and fixing devices

The animal denture devices were made by acrylic resin and stainless steel wire (0.7 inches). First, the dental arches of some animals were molded with alginate and plaster models were made. Then, using these models, the wires were bent to connect the upper molars both side occlusal surfaces. Prior to molding and fixing of the devices

the animals were anesthetized with a 2% aqueous solution of 2-(2,6-xylydino)-5,6-dihydro-4H-1,3-thiazine hydrochloride (Rompum, Bayer) and Ketamine (Dopalen, AgribRANDS of Brasil Ltda) at a ratio of 1 : 0.5 ml/100 g of body weight. Aztreonam (50 mg/kg subcutaneously) was administered 48 h before the placing the apparatus aiming to reduce local colonization by enteric bacteria (Nett et al., 2010). Subsequently, the wire was anchored on the occlusal surfaces of the right and left side, and fixed with light-cured composite resin (Z350-3M). A small orifice was done on the acrylic resin to facilitate the injection of the solutions at the rat palate/device interface. The process of preparation of the devices is shown in Figure 1.

Infection and treatment of animals

The animals were immunosuppressed with a subcutaneously single dose of cortisone (200

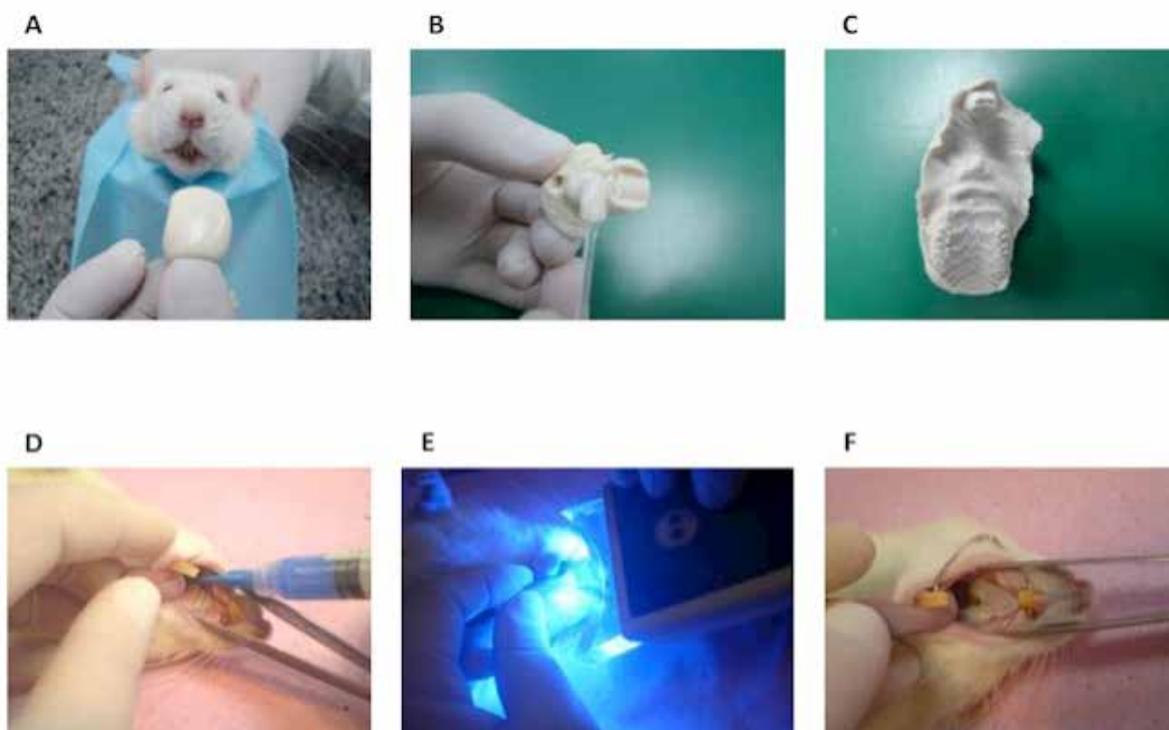


Figure 1 - Process of preparation and fixing of the devices: A) Tray with alginate before palate imprint; B) Alginate imprint of the rat palate; C) Plaster mold used to the resin device confection; D) Teeth conditioning with phosphoric acid before device fixation with resin; E) Polimerization of the resin during the device fixation; F) Palatal device in position after fixation.

mg/kg) according to pre-established protocol [23]. The strain *C. albicans* (ATCC 18804) was used in this study. Standardized inoculum (108 cells/ml) from a 24 h culture growing in Sabouraud dextrose agar (Himedia) was obtained in the NaCl 0.9% solution. The palate of the animals was contaminated by the deposition of 100 μ l of the inoculum under the animal denture devices in order to promote biofilm formation and infection of palatal mucosa. The animals were examined 6 h after placement and contamination of the prosthesis and then every 12 h throughout the study for the detection of visible signs of local inflammation and behavioural alterations of animals.

Treatments

Twenty-four hours after fungal inoculation, treatments were initiated for 24 and 48 hours twice a day (morning and afternoon). An aliquot of 100 μ l of the prepared solutions was inoculated through the device orifice. Treatments were performed according to Table I.

Euthanasia

After 24 and 48 hours of treatment, animals were euthanized with anesthetic overdose. Five animals' palate were removed, fixed in formaldehyde 10% and processed for histological analyses and their correspondent devices were used for UFC/device counting. One device per group was collected for SEM analysis.

Table 1 - Studied groups and correspondent treatments used

Group	Treatment
Control	NaCl 0.9 %
Nystatin (Positive Control)	Nystatin 50000 IU
Sodium bicarbonate	NaHCO ₃ 3% in sterile distilled water
<i>Buchenavia tomentosa</i>	Water extract from 625 mg/ml *

* Obtained according to Teodoro et al.

CFU counting

The removed devices were immersed in 2 ml of sterile 0.9% NaCl solution and vortexed four times for 15 s. Then, the devices were removed and dilutions of 1:10 and 1:100 of the device content were obtained and seeded on Sabouraud dextrose agar with 100 mg/ml chloramphenicol. Plates were incubated at 37°C for 24 h, when the UFC counting per device was done. Samples were duplicated.

Histological analyses

The removed and fixed palatal mucosa were processed and embedded in paraffin and then four 5 μ m semi-serial sections were obtained per slide in a total of 3 slides that were stained with haematoxylin and eosin (HE) and 3 slides stained with Periodic Acid-Schiff (PAS).

On the HE stained slides, the presence or absence of inflammatory infiltrate and the categorization of the infiltrate in mild, moderate or intense infiltrate was done while the PAS staining allowed the identification of *C. albicans* yeast or hyphae in the epithelium keratin layer.

Scanning electron microscope (SEM)

One device per group was analyzed in SEM. The devices were washed with phosphate buffer solution (PBS), fixed in glutaraldehyde 2.5% for 2 h and kept in 70% ethanol overnight. After, they were dehydrated in serial ethanol solutions (75%, 85% and 95%) for 30 min and left at room temperature until ethanol evaporation. For SEM analysis, specimens were bound to an aluminium stub and covered with gold. Images were taken in a magnitude of 150K x.

Statistical analyses

Data were analyzed by Kruskal-Wallis test with Dunn's post-hoc and are expressed as Mean \pm Standard deviation.

RESULTS

During the experiment no visible sign of inflammation could be seen due to the devices fixation in all groups. Immediately after the device removal, the palate was reddish and, in some cases, white spots or areas could be detected.

CFU counting

Data on *Candida* counting were analyzed separated for 24 and 48 hours and each treatment were compared to control group (Table II).

At 24 h, both *B. tomentosa* ($4.02 \times 10^5 \pm 1.75 \times 10^5$; $p = 0.03$) and nystatin ($2.5 \times 10^5 \pm 3.46 \times 10^5$; $p < 0.01$) were able to significantly reduce candidal counts in relation to control ($18.09 \times 10^7 \pm 20.92 \times 10^7$). Nystatin was more effective in reducing the number of UFC/device than sodium bicarbonate ($5.17 \times 10^7 \pm 9.20 \times 10^7$; $p < 0.01$). There was no significant difference between the *B. tomentosa* and nystatin groups ($p = 0.1$), what highlights the efficacy of the phytotherapeutic treatment.

At 48 hours, *B. tomentosa* ($1.33 \times 10^7 \pm 1.04 \times 10^7$) showed lower efficacy than in 24 h and the promoted reduction was not significant compared with the control (NaCl 9%). Nystatin (positive control) ($1.54 \times 10^4 \pm 1.06 \times 10^4$) was the most effective in reducing *Candida* counts when compared to *B. tomentosa* ($p < 0.01$) and control groups ($1.37 \times 10^8 \pm 1.80 \times 10^8$; $p < 0.01$). Counts were also significantly lower

in the sodium bicarbonate group than in control group ($2.69 \times 10^6 \pm 3.18 \times 10^6$; $p = 0.02$).

Scatter plot graph with mean/SD from CFU/device counting data are shown in Figure 2.

Histopathological analysis

The histopathological analyses of the palatal mucosa after HE and PAS stainings are described below. For the analysed HE slides, scores of inflammation (mild, moderate, intense) were attributed. The presence or absence of *C. albicans* could be verified in the PAS stained

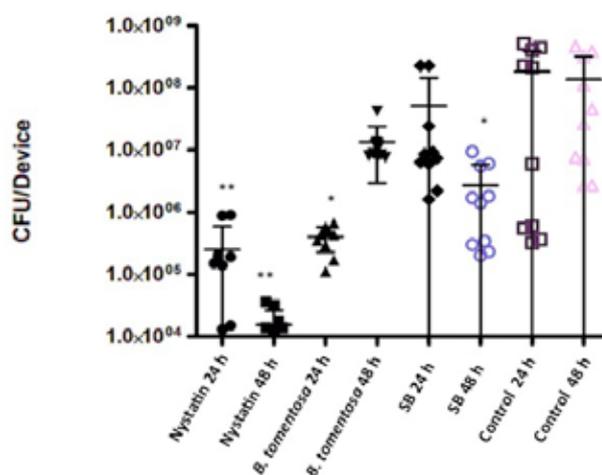


Figure 2 - Scatter plot graph with mean and standard deviation of the fungal counts observed in the studied groups (colony forming units per device, CFU/device).

(*) Mean statistical differences ($p < 0.05$) compared with the control at the same treatment period (24 h or 48 h).

(**) Mean statistical differences ($p < 0.001$) compared with the control at the same treatment period (24 h or 48 h). SB- Sodium bicarbonate.

Table 2 - Means and standard deviations of the colony forming units per device (CFU / device) counting for each studied group at 24 and 48 hours

Group	Counts of Yeasts (Mean + Standard Deviation)	
	24 h	48 h
Control	$18.09 \times 10^7 \pm 20.92 \times 10^7$	$13.7 \times 10^7 \pm 1.80 \times 10^8$
Nystatin (positive control)	$2.5 \times 10^5 \pm 3.46 \times 10^5^*$	$1.54 \times 10^4 \pm 1.06 \times 10^4^{***}$
Sodium bicarbonate	$5.17 \times 10^7 \pm 9.20 \times 10^7$	$2.69 \times 10^6 \pm 3.18 \times 10^6^{****}$
<i>Buchenavia tomentosa</i> extract	$4.02 \times 10^5 \pm 1.75 \times 10^5^{**}$	$1.33 \times 10^7 \pm 1.04 \times 10^7$

* Significantly lower than control ($p < 0.01$) and sodium bicarbonate ($p < 0.01$);

** Significantly lower than control ($p = 0.03$);

*** Significantly lower than control ($p < 0.01$) and *B. tomentosa* ($p < 0.01$);

**** Significantly lower than control ($p = 0.02$).

slides in 24 and 48 hours. None of the groups showed intense inflammatory infiltrate at the studied periods and the presence of PAS positive candidal hyphae could be evidenced in all groups at 48 h. In Figure 3, images illustrate the PAS positive *C. albicans* infection and the inflammation characteristics found in HE staining.

Haematoxylin and Eosin (HE)

The analysed microscopic images showed the presence of a predominantly chronic inflammatory infiltrate both in 24 and 48 hours ranging from mild to moderate. The fragments of palatal mucosa were covered by a stratified pavementous orthokeratinized epithelium presenting hydropic degeneration and exocytosis in the prickle cell layer in all groups. The lamina propria showed a fibrous subepithelial connective tissue turning into soft in the deeper mucosa, well vascularized with several small

vessels spread through the extent of the tissue. The mild to moderate 24 h infiltrate presented macrophages, eosinophils, mast cells, plasma cells and scattered polymorphonuclear cells as well as the moderate 48 h infiltrate.

Periodic Acid-Schiff (PAS)

In the control group, 100% of the animals showed histologic PAS positive hyphae penetration in the epithelium in both studied periods. Besides, in the bicarbonate group, after 24 and 48 hours hyphae penetration scores were 100% and 60%, respectively. PAS positive hyphae was detected both in *B. tomentosa* and nystatin groups only after 48 h of treatment.

Scanning Electron Microscopy (SEM)

The obtained images of the control group 24 h devices showed isolated *C. albicans* cells attached to the acrylic resin that became a better

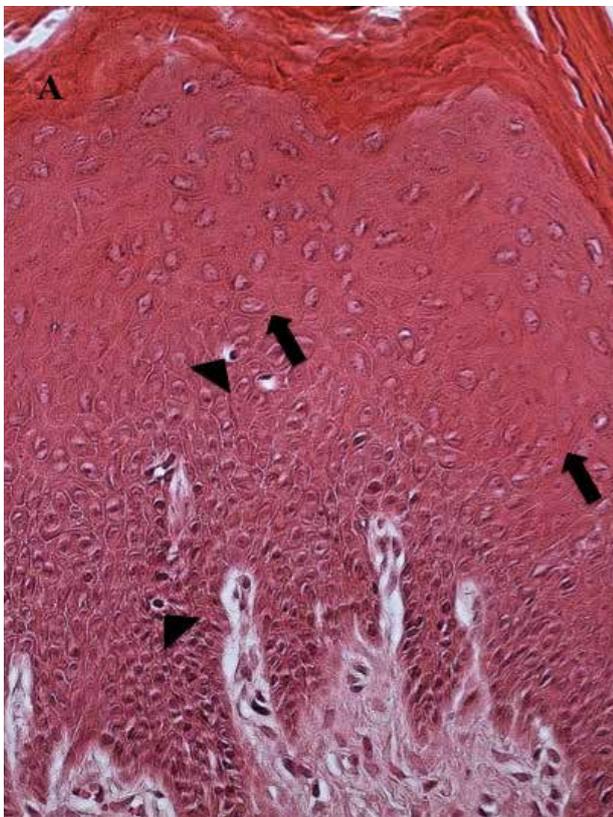


Figure 3 - A) PAS stained slide indicating PAS positive hypha (arrows) characterizing infection with *Candida albicans* – Control group 24 hs after *Candida* infection (400x). B) Illustration of a moderate inflammatory infiltrate in a HE stained slide. Arrow head indicates exocytosis – Sodium bicarbonate group 48 hs after *Candida* infection (200x).

organized biofilm in 48 h with more attached cells immersed in a matrix.

For the sodium bicarbonate, *B. tomentosa* and nystatin groups, SEM images analysis of the devices showed less fungal adhesion to the resin when compared to control group. On the 48 h devices yeasts and some bacteria groups could be seen in addition to the *C. albicans* hyphae.

DISCUSSION

Denture stomatitis (DS) is a multifactorial condition that depends on the *Candida* biofilm formation, host factors, and anatomic location of this biofilm. Reduced salivary flow, especially in denture users, increases candidiasis occurrence. Literature shows that semi-dry like conditions induce filamentation and consequent invasion potential of *C. albicans* [24]. Also, immunosuppression is a risk factor to DS [25], as well as low salivary pH and high sugar ingestion [26].

A study with older individuals, with a majority of institutionalized ones, showed prevalence of 14% for denture stomatitis among the studied population. They associated low saliva pH, never having smoked, regular sugar consumption, and the presence of oral *Candida* with denture stomatitis [26].

Many therapies for DS have been used, for example, denture removal before sleeping, antiseptic and disinfectant methods and antifungal therapy [27]. Due to the not desirable effect related to antifungal therapy, our group has been focusing on natural antifungal alternatives as the *Buchenavia tomentosa* aqueous extract [21], which we decided to test here in an in vivo model of DS.

For experimental DS induction, we used a 24 h biofilm based on previous study [28] that analyzed *C. albicans* biofilm formation in vivo for 6, 24, 48 and 72 hours. Our model of DS induction was proved to be effective by histological evaluation of the rats' palatal mucosa that exhibited chronic inflammatory infiltrate

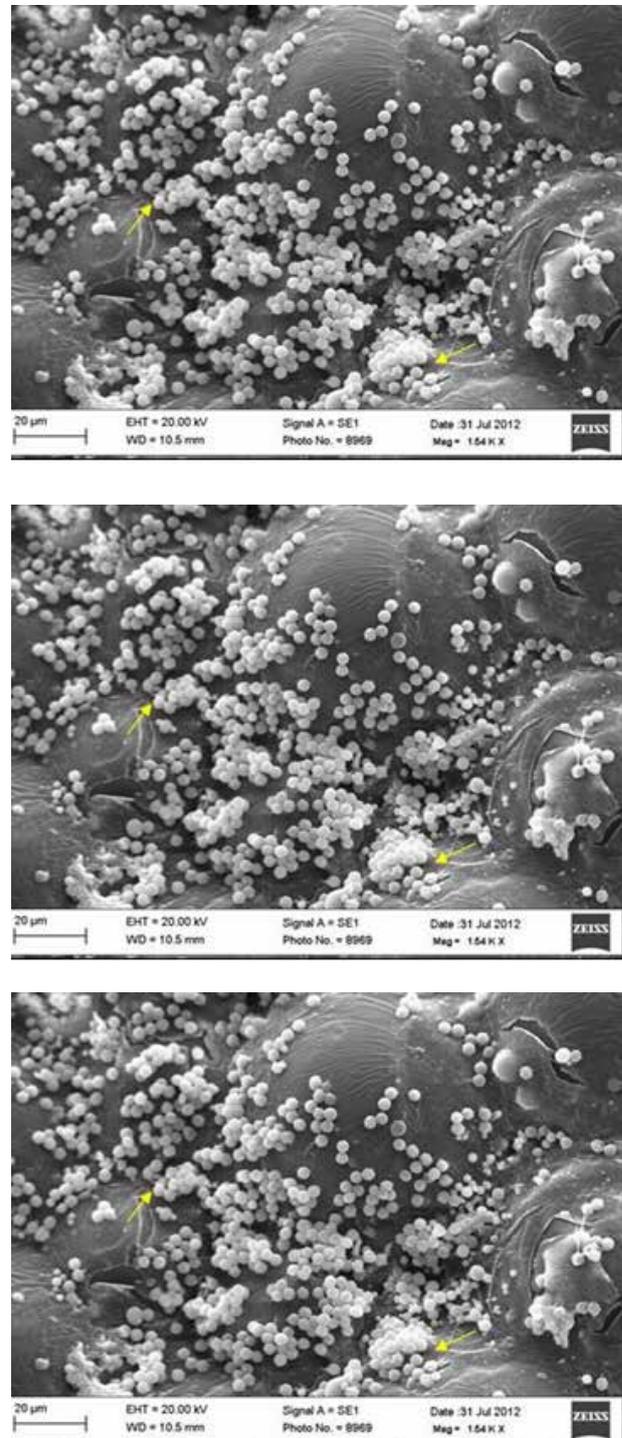


Figure 4 - A) Scanning electron microscopy analysis of the device after 24 hours (control group). Colonization by *Candida albicans* can be seen (arrows). B) Scanning electron microscopy analysis of the device after 48 hours and treatment with *Buchenavia tomentosa*. Sparse yeast cells could be detected (arrows). C) Scanning electron microscopy analysis of the device after 24 hours and treatment with sodium bicarbonate.

and PAS positive hyphae and germinative tubes on the surface of the keratinized epithelium.

Though, the fact that the inflammatory infiltrate ranged from mild to moderate and predominantly chronic but sometimes we could find scattered polymorphonuclear cells in the lamina propria suggests that the time of DS induction used may be a limitation of our study. So the exacerbation of the chronic infiltrate, characteristic of DS, could not happen as well as the induced hyper-keratinization. Nett et al. [28] showed that in 4 days it was possible to point histological signs of acute DS including Munro's abscess among the epithelial cells. In the literature we can find periods of induction ranging from 4 to 8 weeks resulting in clinical local inflammation [28,29] what did not happen in our study even if we have suppressed the rats' immune system. The device/mucosa short period of contact could explain that fact.

The CFU/device counting in 24 hours for both *B. tomentosa* ($4.02 \times 10^5 \pm 1.75 \times 10^5$; $p = 0.03$) and nystatin groups ($2.5 \times 10^5 \pm 3.46 \times 10^5$; $p < 0.01$) were significantly lower than control group ($18.09 \times 10^7 \pm 20.92 \times 10^7$) with no significant correlation between the *B. tomentosa* and nystatin ($p > 0.1$) groups. However, in 48 h the *B. tomentosa* aqueous extract was not as efficient in reducing the CFU/device counting as the nystatin group was when compared with the control and *Buchenavia tomentosa* groups ($p < 0.01$). The possible explanation for this fact is that the extract concentration applied on the animals was correspondent to $\frac{1}{2}$ MIC (minimal inhibitory concentration) for *C. albicans* found in our previous study [21]. Also, the decreased efficiency of the *B. tomentosa* in 48 h could be due the fact that the experimental model applied does not allow us to remove the resin device and clean the area providing a better contact and longer action of the *B. tomentosa* extract with the mucosa as it would in a patient.

Besides the efficiency of nystatin against *C. albicans* reinforced in our study, nystatin known toxicity and DS recurrence rates after

nystatin treatment [11,16] incites the search for DS alternative therapies. Microwave denture disinfection [30], garlic aqueous extract [31], photodynamic therapy [32] and melaleuca and copaiba oil [33] has shown better or similar results compared to nystatin in DS cases.

Our group confirmed the antifungal effect of the *B. tomentosa* aqueous extract not only against *C. albicans*, but also *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei* and *Candida dubliniensis*, even in lower concentrations than the one applied to the *C. albicans* [21].

Plant extracts from the Combrataceae family that includes *B. tomentosa* have shown promising anti-candida effects when applied for denture disinfection [34] as well as the results we found in 24 h for *B. tomentosa*. However, studies with longer periods of application and different concentration should be done to emphasize the fungicidal effect of our extract.

Regarding the sodium bicarbonate results, the in vitro effect in reducing *Candida* biofilm on resin specimens [35] and denture surfaces [34] was previously reported in the literature but there was no in vivo model showing its efficacy against *Candida albicans*. Sodium bicarbonate effect against *C. albicans* induced experimental denture stomatitis was confirmed in our study when applied for 48 h (sodium bicarbonate x control; $p = 0.02$). Also, we found amelioration on the inflammatory infiltrate and decrease in the PAS positive hyphae counting in the same period.

Within the limitations of our study, *B. tomentosa* showed to be a good alternative for DS and the histological profile of *B. tomentosa* and nystatin groups were quite similar during both studied periods and similar results could be found in studies applying other natural therapies [5,6].

In resume, new concentrations of the *B. tomentosa* aqueous extract as well as longer periods of DS induction are needed in future

studies. For instance, we can conclude that the *B. tomentosa* aqueous extract in a concentration of 6.5 mg/ml is an interesting alternative for DS based on our results and has advantages over the traditional therapies as lower cost, easy access to the leaves. Importantly induces less non desirable side effects as it is a natural product.

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