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ORIGINAL ARTICLE

Inhibition of adherence of *C. albicans* to dental implants and cover screws by *Cymbopogon nardus* essential oil and citronellal

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Abstract
Objective This study investigated the biological activity of the essential oil from *Cymbopogon nardus* and of the phytoconstituent citronellal on *Candida* strains as to the inhibition of adherence to dental implants and cover screws.
Material and methods The essential oil was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and had its MIC and MFC determined against 12 strains of *Candida.* Then, tests of inhibition of adherence to the dental implants and cover screws were carried out using the MIC of the substances, followed by scanning electron microscopy analysis. Nystatin and chlorhexidine were used as positive controls, and experiments were performed in triplicate.
Results The analysis by GC-MS of the essential oil identified citronellal as the major compound. The MICs of the essential oil, citronellal, chlorhexidine, and nystatin—able to inhibit 100 % of the strains—were found to be 64, 512, 64, and 32 μg/ml, respectively. The essential oil significantly inhibited the adherence of *Candida albicans* to the dental implants and cover screws ($p<0.001$). Citronellal inhibited yeast adherence only to the dental implants ($p<0.001$), and no significant results were found for the cover screws ($p>0.05$) compared to the growth control.
Conclusion The essential oil and citronellal have proven antifungal activity and are able to inhibit the in vitro adherence of *C. albicans*.

Clinical relevance: There has been a search for alternative natural product-containing formulations that should be effective in inhibiting adherence of yeasts to the surfaces of materials and also able to treat oral fungal infections. Further trials could make these products an alternative to chemical removal of peri-implant biofilm.

Keywords *Cymbopogon* · Citronellal · *Candida* · Dental implant · Cover screw

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Introduction

The use of dental implants has become an alternative in the rehabilitation of edentulous patients, with a success rate of approximately 96 %. In spite of that, a considerable number of implant failures occurs throughout the years [1]. The causes of the failures may be related to endogenous and exogenous factors. Endogenous factors include those affecting the patient's general health and oral hygiene habits, which can trigger peri-implant infections known as peri-implantitis. Exogenous factors, in turn, are related to the type of material used in the manufacture of implants, biocompatibility, surface features, and implant design [2, 3].

Peri-implantitis is defined as an inflammation of the mucosa with the presence of suppuration, bleeding, and continuous bone loss around the implants [4]. Risk factors for peri-implantitis include early exposure of implants and poor oral hygiene, resulting in accumulation of biofilm [5] that can be initiated on the surface of the implant as soon as it is exposed in the oral cavity [6, 7]. The microbiota found in the peri-implant disease is similar to that found insites with gingivitis and advanced periodontitis [8]. In addition to periodontopathogenic bacteria, peri-implant disease includes microorganisms of the resident microbiota of the oral cavity,

such as *Candida* spp., which are capable of damaging the tissue due to proteinase production, enhance formation of diverse and potentially pathogenic biofilms, besides modulating the host immune response, which in the case of peri-implant infection, promotes tissue destruction, particularly the activation of osteoclasts [9–11].

In general, bacterial and/or fungal biofilms have greater resistance to host defenses and to conventional antimicrobial therapy, resulting in serious and persistent infections [12, 13]. The adherence of microorganisms to titanium surfaces has already been described in vivo and in vitro; however, there is little information in the literature about the inhibition of yeast adherence to the surfaces of dental implants and cover screws, even considering that these devices provide the substrate necessary for formation of fungal biofilms and thus can serve as a reservoir for infection/reinfection by *Candida albicans* [14].

C. albicans is a commensal and opportunistic pathogen that causes infections usually as a result of a change in the host immune response. Its virulence is attributed to morphological plasticity and ability to form biofilms [15]. The early and essential step in the pathogenesis of oral candidiasis involves the attachment of *C. albicans* to a receiving surface or implanted device, such as prostheses and dental implants [12, 13]. In general, yeast cells have a high potential for adherence to artificial materials, in much the same way as to the oral tissues [16].

Certain factors are essential to make the mechanical control of biofilm successful, for instance, frequency, technique, and brushing time, in addition to patient's motivation and performance. Some studies [7–9, 17] point out that the use of antimicrobials (systemic, local, or combined) is beneficial in the treatment of peri-implant infections; however, there is not a specific protocol so far, but only proposals addressing the antimicrobial treatment of peri-implantitis.

Chlorhexidine is proven to act upon biofilm formation with a broad antimicrobial spectrum against both bacteria and fungi [18]. As such, it is indicated as adjunctive chemical method for the treatment of peri-implantitis [19–21]. However, adverse effects of chlorhexidine, such as unpleasant taste, taste changes, pigmentation of teeth, and oral desquamation, are factors limiting its prolonged use [22, 23].

New agents have been proposed in order to minimize unwanted reactions presented by users with the use of antimicrobials, as well as increased resistance by microorganisms. Microbial resistance levels have been increasing due to the indiscriminate use of antimicrobials, which is driving researchers to study new antimicrobial substances from various sources, including medicinal plants [24]. Medicinal plants play an important role in global health and continue to be used despite the great advances observed in modern medicine. It is estimated that 25–30 % of all drugs considered as therapeutic agents are derived from natural products [25].

Among the substances obtained from plants, the essential oils stand out as natural, complex compounds characterized by strong odor and volatility. They are produced by aromatic plants as secondary metabolites and have about two or three major compounds at relatively high concentrations (20–70 %) [26]. Thus, the present study aimed to evaluate the inhibition of adherence of *C. albicans* to dental implants and cover screws by *Cymbopogon nardus* essential oil and citronellal.

Materials and methods

Microbiological tests were performed in the Mycology Laboratory of the Department of Pharmaceutical Sciences, Center for Health Sciences, and scanning electron microscopy analysis was performed at the Technology Center, Federal University of Paraíba (UFPB).

The dental implants DSP BIOMEDICAL® OSTEOFIT HE 4.1×11.5 mm SLA ZIRCON® and the cover screws DSP BIOMEDICAL® were used in this study, as well as *C. nardus* essential oil (QUINARI®) and the phytoconstituent citronellal (SIGMA-ALDRICHT®). In order to investigate the biological activity of the products, the following clinical and collection strains were used: *C. albicans* (ATCC 76485); *C. albicans* (ATCC 76645); *C. albicans* (LM P20); *C. albicans* (LM 62); *C. albicans* (LM 122); *C. albicans* (LM 108); *C. albicans* (LM 86); *C. albicans* (LM 111); *C. tropicalis* (LM 45); *C. tropicalis* (ATCC 13803); *C. tropicalis* (LM 14); and *C. tropicalis* (LM 20).

Nystatin (SIGMA-ALDRICHT®) and chlorhexidine (RIOQUÍMICA®) were used as positive controls in the tests of antimicrobial susceptibility. The culture media used were RPMI-1640 (HIMEDIA®), Sabouraud Dextrose Broth (SDB), and Sabouraud Dextrose Agar (SDA) (DIFCO®), which were prepared according to the manufacturers'recommendation.

For the preparation of the inoculum, the selected strain was maintained in SDA for 24–48 h at 35 °C and subsequently standardized in sterile saline solution using a suspension of barium sulfate n. 0.5 of the McFarland scale. After being stirred with the aid of a Vortex device (FANEM) for 2 min, the suspension was adjusted to 90 % transmittance on a spectrophotometer (LEITZ-PHOTOMETER 340-800) to contain approximately 10^6 CFU/ml [27–29].

Chromatography and mass spectrometry

The compounds were identified using a gas chromatograph coupled to a mass spectrometer (SHIMADZU GC-MS-QP5050A) with capillary column (J & W SCIENTIFIC®) and a stationary phase of 5 % phenyl and 95 % dimethylpolysiloxane measuring 30-m length, 0.25-mm inner diameter, and 0.25-mm film thick. The initial temperature program was 60 to 240 °C (3 °C/min). The programming time of the run was 60 min, and the injector oven temperature was 250 °C. Helium was used as carrier gas (mobile phase) at a flow rate of 1.0 ml/min with a 1:20 split ratio and injection volume of 1 μl.

The ionization of the components was performed by electron impact at 70 eV, using a 1.25-kV detector. The spectrometer was operated in SCAN mode, scanning a range of masses from 40 to 500 atomic mass unit (a.m.u). The ion source temperature was 250 °C, and the compounds were identified by comparing their mass spectra with those existing in the equipment database (MIST library, 2008).

The essential oil sample was injected at a concentration of 2 ppm, and hexane was used as solvent. Analyses of the chromatogram and mass spectra were performed using the equipment library, and the integration parameters used were width, 3, and slope, 2000.

Minimum inhibitory concentration (MIC)

The determination of MIC of the essential oil, phytoconstituent, nystatin, and chlorhexidine was performed by the microdilution technique [27, 28]. Initially, 100 μl of RPMI-1640 culture medium was added to the wells of the plate. Then, 100 μl of the experimental substances was also added to the wells at the concentration of 1024 μg/ml, and both the medium and the sample were homogenized. Subsequently, the sample was serially diluted by transferring an aliquot of 100 μl from the first well to the following ones. The concentrations ranged from 1024 to 8 μg/ml. Aliquots of 10 μl of the inoculum were dispensed into the wells of each column. In parallel, controls of strain viability and susceptibility to nystatin and chlorhexidine were also carried out using the same technique. Tests were performed in triplicate, and plates were incubated at 35 °C for 24–48 h. The reading for MIC determination was performed by the visual method, taking into consideration whether or not there was formation of cell clusters at the bottom of the wells. As such, MIC was considered as the lowest concentration of the test product capable of producing visible growth inhibition of yeast strains used in microbiological assays [30].

Minimum fungicidal concentration (MFC)

After determining the MIC, the content of the wells corresponding to the inhibitory and two higher concentrations (MIC, MICx2, and MICx4) as well as that of the positive controls was subcultured on SDA plates. The reading of MFC was based on the growth of the controls after 24–48 h of incubation at 35 °C. MFC was considered as the lowest concentration of the test product that hampered visible growth in the subculture. Tests were performed in triplicate [31].

Inhibition of fungal adherence to dental implants and cover screws

The strain of *C. albicans* (ATCC 76645) was used in the test of inhibition of adherence, performed in triplicate. This fungal species is known for its virulence associated with morphological plasticity and ability to form biofilms on different substrates. Furthermore, the use of an ATCC reference strain

Table 1 Characterization of *C. nardus* essential oil by GC-MS

Peak#	R. Time	I. Time	F. Time	Area	Area%	Name	Base m/z
1	8.892	8.830	8.990	349264	3.17	Limonene	68.10
2	9.226	9.160	9.395	856389	7.78	Piperidine<1-methyl->	99.10
3	11.581	11.515	11.640	52458	0.48	Linalool	71.10
4	13.554	13.490	13.655	95429	0.87	Isoisopulegol	41.10
5	13.889	13.765	13.995	4157403	37.75	Citronellal	41.10
6	17.183	17.085	17.285	1572018	14.27	Citronellol	41.10
7	18.380	18.250	18.495	2075063	18.84	Geraniol	69.10
8	19.082	19.015	19.135	45593	0.41	Geranial	69.10
9	22.698	22.615	22.815	365114	3.31	Citronellylacetate	81.10
10	22.973	22.900	23.070	70762	0.64	Eugenol	164.10
11	24.047	23.960	24.125	270668	2.46	Nerylacetate	69.10
12	24.468	24.385	24.545	212422	1.93	Elemene<beta->	93.10
13	28.311	28.220	28.390	114515	1.04	Cadinene<gamma->	161.15
14	29.111	29.030	29.190	52993	0.48	Muurolene<alpha->	105.10
15	29.690	29.610	29.765	59400	0.54	Cadinene<gamma->	161.20
16	30.065	29.975	30.165	188826	1.71	Cadinene<delta->	161.20
17	31.107	31.020	31.210	285007	2.59	Elemol<alpha->	93.10
18	32.194	32.115	32.305	121511	1.10	Muurolene<gamma->	161.20
19	35.328	35.245	35.405	69435	0.63	Cadin-4-em-10-ol	161.20
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enables the reproducibility of the experimental conditions and comparison with other studies. The strain was maintained in SDA, incubated at 35 °C for 24–48 h. The implants and cover screws were placed individually into glass vials containing 1.9 ml of SDB plus the product to be tested in a sufficient amount capable of providing a final concentration corresponding to the MIC. A total of 100 μl of yeast suspension (10^6 CFU/ml) were also added, and the tubes were incubated at 35 °C for 48 h. Both the implants (n=3) and cover screws (n=3) were randomly allocated into five groups: (A) growth control; (B) *C. nardus*; (C) citronellal; (D) nystatin; and (E) chlorhexidine, totalizing 15 dental implants and 15 cover screws.

After the incubation period (48 h), the materials were washed with 1 ml of sterile distilled water and placed in sterile vials containing 5 ml of saline solution (0.85 % NaCl). Then, the vials were stirred in a Vortex apparatus for 60 s. After this procedure, 10 μl of the solution was seeded on Petri dishes containing SDA, which were incubated at 35 °C for 48 h. Ultimately, the counting of CFU/ml was made [32].

Scanning electron microscopy (SEM) analysis

In order to conduct the SEM analysis, baseline micrographs of the surfaces of one dental implant and one cover screw were taken prior to incubation of the microorganisms. To allow the adherence, five dental implants and five cover screws were individually placed in sterile vials containing 1 ml of SDB, 0.1 ml of yeast suspension (10^6 CFU/ml), and 1.0 ml of the test products at MIC (groups B, C, D, and E). Then, the vials were incubated at 35 °C for 24–48 h. In group (A), the vials contained only 1.0 ml of SDB and 0.1 ml of yeast suspension (10^6 CFU/ml).

After the incubation period, the materials were removed from the vials and underwent metallization with gold plasma (Emitech K550x®). Posteriorly, microbial adherence was visualized under a scanning electron microscope (LEO 1430®).

Statistical analysis

The data were analyzed on the software GraphPad Prism 4. Analysis of variance (ANOVA) with Tukey's posttest was carried out, with a confidence interval of 95 %.

Results

Chromatographic profile and identification of compounds

The analysis by gas chromatography couple to mass spectrometry (GC-MS) allowed the characterization of *C. nardus* essential oil and identification of compounds based on information of the retention time and molecular weight. Additionally, the compounds were quantified according to the peak areas (Table 1). The data were compared to the library available in the chromatographer.

The terpenoid citronellal was found to be the major compound (37.75 %), followed by geraniol (18.84 %), and citronellol (14.27 %).

Table 2 MIC and MFC of the products tested against strains of *C. albicans* and *C. tropicalis*

Strains	*C. nardus*		Citronellal		Chlorhexidine		Nystatin	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 76485	64	128	256	512	64	128	32	128
C. albicans ATCC 76645	32	32	16	16	32	32	8	8
C. albicans LM P20	32	32	256	256	32	32	8	8
C. albicans LM 122	32	32	512	1024	64	64	8	8
C. albicans LM 108	64	64	512	512	64	64	8	8
C. albicans LM 86	64	64	512	1024	64	128	8	8
C. albicans LM 111	32	32	256	256	64	128	8	8
C. albicans LM 62	32	128	16	64	64	256	8	32
C. tropicalis ATCC 13803	32	32	128	128	8	8	8	8
C. tropicalis LM 45	32	32	256	256	64	64	8	16
C. tropicalis LM 14	32	32	256	256	64	128	16	64
C. tropicalis LM 20	32	32	256	512	64	64	16	16

Values are expressed as μg/ml

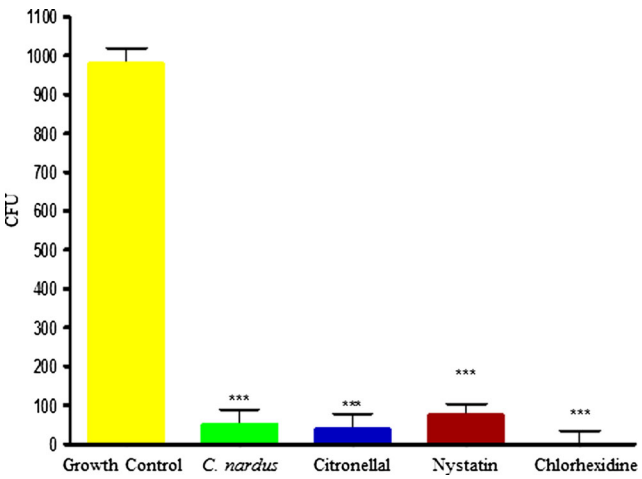

Fig. 1 In vitro evaluation of the inhibition of adherence to dental implants (***p<0.001)

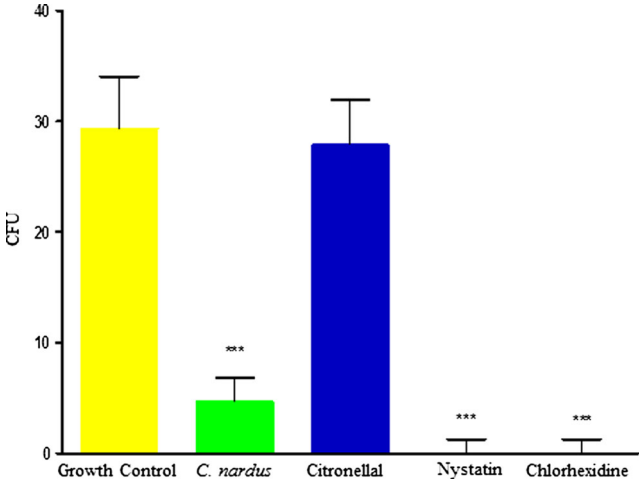

Fig. 2 In vitro evaluation of the inhibition of adherence to cover screws (***$p<0.001$)

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The products evaluated were able to inhibit the growth of all *Candida* strains tested herein. *C. nardus* essential oil inhibited 75 % ($MIC_{75\%}$=32 μg/ml) of the strains at a concentration of 32 μg/ml, although *C. albicans* ATCC 76485, *C. albicans* LM 108, and *C. albicans* LM 86 were only susceptible at the concentration of 64 μg/ml (Table 2). This essential oil had $MFC_{66.6\%}$ of 32 μg/ml, and the strains of *C. albicans* ATCC 76485 and *C. albicans* LM 62 were found to be more resistant (MFC of 128 μg/ml).

Citronellal presented $MIC_{75\%}$ and $MFC_{58.3\%}$ of 256 μg/ml, although MIC and MFC values ranged between 16 and 512 μg/ml and 16 and 1024 μg/ml, respectively. *C. albicans* LM 122 and *C. albicans* LM 86 were found to be among the most resistant strains. Chlorhexidine at the concentrations between 8 and 64 μg/ml was able to inhibit all strains, with $MFC_{58.3\%}$ of 64 μg/ml.

Lower values of MIC and MFC were observed for nystatin, which had $MIC_{75\%}$ of 8 μg/ml and $MFC_{75\%}$ of 16 μg/ml. *C. albicans* ATCC 76485 and *C. tropicalis* LM 14 showed higher resistance to nystatin, with MFC of 128 and 64 μg/ml, respectively.

Inhibition of fungal adherence to the dental implants and cover screws

The test of inhibition of adherence to the dental implants (Fig. 1) indicated that the experimental substances evaluated, as well as nystatin and chlorhexidine, were able to inhibit fungal growth, with a reduction in the number of colony-forming units (CFU). All substances showed statistically significant results compared to the growth control ($p<0.001$), but with no statistical difference when compared between each other ($p>0.05$). As to the inhibition of adherence to the cover screws (Fig. 2), *C. nardus* essential oil, nystatin, and chlorhexidine also showed statistically significant results when compared to the growth control ($p<0.001$), with no significant difference when compared between each other ($p>0.05$). Citronellal was not effective in inhibiting adherence to the cover screws, with results similar to those of the growth control.

Scanning electron microscopy (SEM) analysis

The SEM analysis was performed in order to obtain images of the surfaces of the dental implants and cover screws after treatment with the experimental substances at MIC. Firstly, a micrograph of the surfaces of the implant (Fig. 3a) and cover screw (Fig. 4a) was taken to allow the visualization of the surfaces with no microorganisms. Then, micrographs of the dental implants (Fig. 3b) and cover screws (Fig. 4b) were taken to show *C. albicans* adherence to the surfaces. The figures 5 and 6 reveal absence of yeast adherence to the surfaces after treatment with the antifungal agents tested.

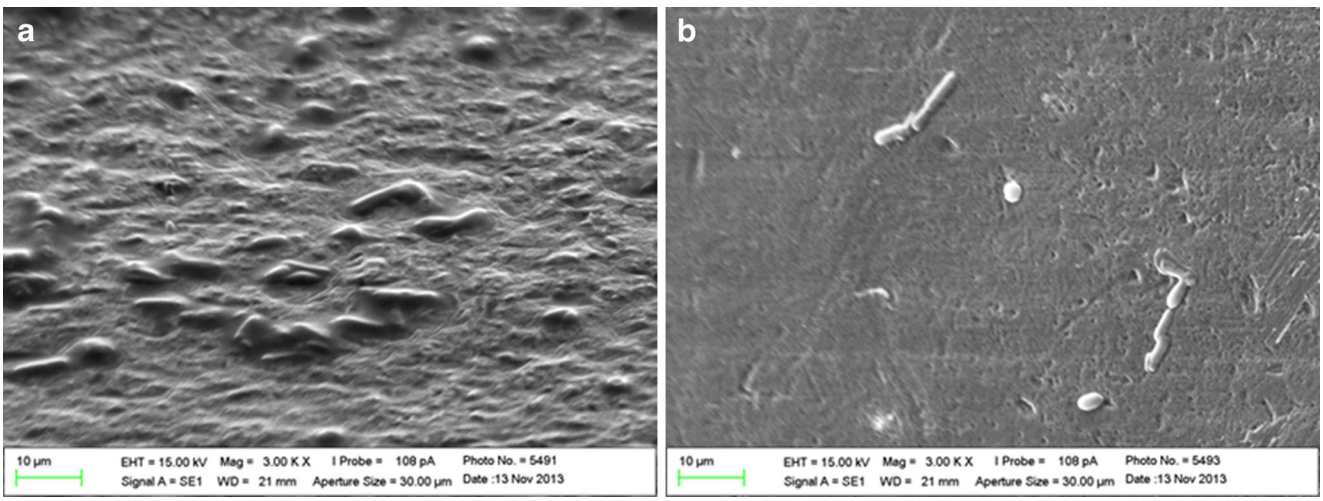

Fig. 3 **a** Surface of the dental implant—SLA Zircon®; **b** *C. albicans* adhered to the surface of the cover screw (×3000)

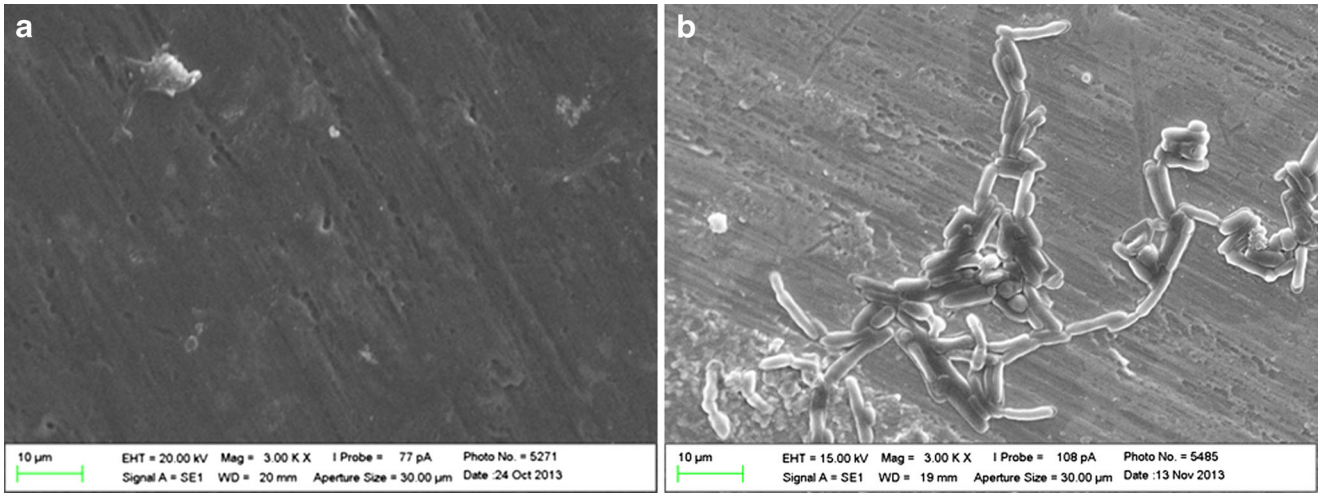

Fig. 4 **a** Surface of the cover screw; **b** growth control—*C. albicans* adhered to the surface (×3000)

Discussion

The interest in studying the anti-*Candida* activity of *C. nardus* essential oil and citronellal is based on their proven activity against fungal species [33, 34] and on their notable low toxicity on human epithelial cells. A previous study [35] showed that these products allow 75 % cell viability at the concentration of 75 µg/ml, which is much higher than that proposed in our study.

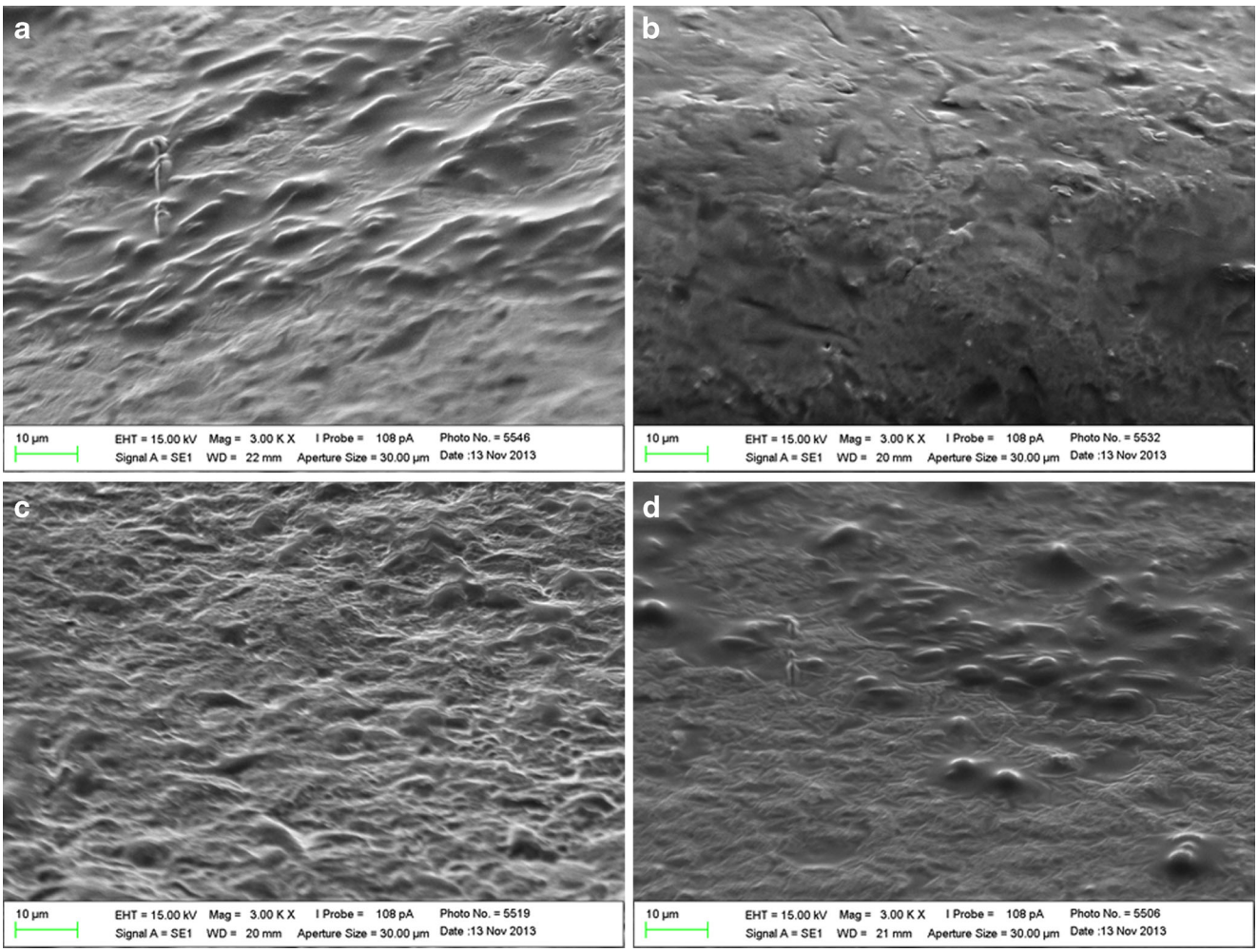

Fig. 5 Surfaces of the dental implants treated with the following substances at MIC: **a** *Cymbopogon nardus*; **b** citronellal; **c** nystatin; **d** chlorhexidine. Note the lack of adherence of the yeasts to the surface of the implants (×3000)

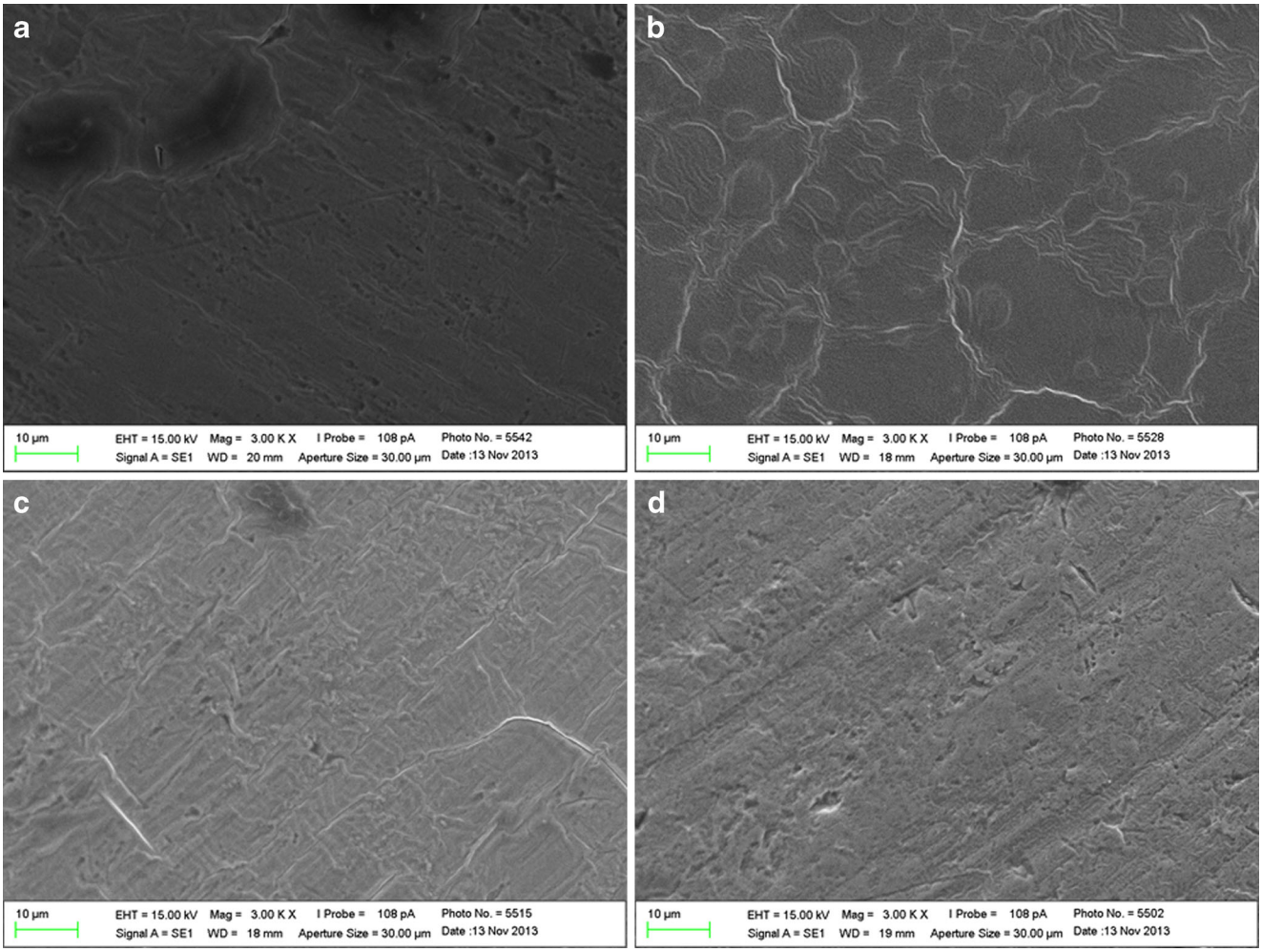

Fig. 6 Surfaces of the cover screws treated with the following substances at MIC: **a** *Cymbopogon nardus*; **b** citronellal; **c** nystatin; **d** chlorhexidine. Note the lack of adherence of the yeasts to the surface of the cover screws (×3000)

The analysis of *C. nardus* essential oil by GC-MS identified the terpenoid citronellal as the major compound (37.75 %). This finding is in agreement with other studies in the literature [35–38], in which citronellal was quantified ranging from 29.6 to 35.9 %. A number of factors may influence the chemical composition of the essential oil. The most important ones include the following: the plant part used for obtaining the oil, its origin, development stage, and climatic conditions [39].

The results of this study showed that *C. nardus* essential oil had lower values of MIC and MFC in 83.3 % of the tested strains when compared to citronellal. This fact may be due to the existing synergism between the compounds of the essential oil, which makes it to display a better biological activity [40].

Even though some studies have shown that *C. nardus* essential oil as well as its major constituent citronellal have antifungal properties [34, 41], their biological activity against *Candida* species remains poorly studied. Furthermore, little information is known about their antibacterial activity, particularly against oral pathogens, even though the essential (whose major compound is citronellal) was found to be active against periodontopathogenic bacteria [42].

The present study demonstrated that the essential oil and the phytoconstituent citronellal were able to inhibit 100 % of the strains at the concentrations of 64 and 512 µg/ml, respectively. Investigating the antifungal activity of *C. nardus* essential oil on *C. albicans*, another study [43] found MIC of 450 µg/ml. These differences in the MIC values can be attributed to the chemical composition of the essential oil and to the different strains of microorganisms, although belonging to the same species.

The essential oil also had lower MIC and MFC values in relation to chlorhexidine against most of the strains studied. Chlorhexidine has proven antimicrobial action upon biofilm formation. However, adverse effects such as unpleasant taste, taste changes, pigmentation of teeth, and oral desquamation are limiting factors for its prolonged use [22, 23].

Although nystatin had the lowest MIC and MFC values against most of the strains tested, some authors [44, 45] showed that *Candida* species may exhibit resistance to this

standard antifungal. This resistance can be caused by the indiscriminate use of antimicrobials, which makes it interesting to search for new substances, mainly of natural origin. Therefore, further in vitro and in vivo studies of *C. nardus* essential oil and citronellal should be carried out, in view of the utmost importance of elucidating issues like safety, tolerability, and possible adverse effects related to the use of these chemical agents.

In general, essential oils act by breaking or disrupting membranes due to their lipophilic compounds, which leads to loss of several enzymes and nutrients through the cell membrane [46, 47]. In the test of inhibition of adherence to the dental implants, *C. nardus* essential oil and the phytoconstituent citronellal were able to inhibit fungal growth and showed statistically significant results compared to the growth control ($p<0.001$), with no statistical difference when compared to nystatin and chlorhexidine ($p>0.05$) though. As to the inhibition of adherence to the cover screws, *C. nardus* essential oil, nystatin, and chlorhexidine inhibited the growth of *C. albicans* with statistically significant results compared to the growth control ($p<0.001$). However, no significant difference was found when these groups were compared between each other ($p>0.05$). Citronellal was not effective in inhibiting fungal adherence, displaying results similar to those of the growth control. *C. nardus* essential oil showed better results compared to citronellal likely due to the synergism between the compounds present in the oil, leading to a better biological activity.

In the qualitative analysis by SEM, *C. nardus* essential oil and citronellal were able to inhibit the adherence of the tested strains to the surface of the dental implants and cover screws. This study demonstrated by SEM analysis that *C. albicans* was able to adhere to the surfaces of dental implants and cover screws, corroborating other reports [10, 12, 13, 48, 49] on the ability of this species in adhering to surfaces and forming biofilms thereon. The dental implants used in this study had undergone surface treatment, which favors cell adherence and accelerates the process of osseointegration in the rehabilitation of edentulous patients [50]. It is worth noting that the SEM analyses were based on a qualitative methodological approach complementary to the findings of the microbiological assays.

In general, dental implants have roughness that are, on average, greater than 2 μm [51, 52], which provides an effective adherence of *C. albicans* [53, 54] and leads the biofilm to be more resistant to chemical agents [13].

Conclusion

The essential oil from *C. nardus* showed antifungal activity and was able to inhibit the adherence of *C. albicans* to the surfaces of the materials assayed, similarly to nystatin and chlorhexidine (standard drugs). Nevertheless, further in vitro and in vivo research should be undertaken to investigate the possible mechanisms of action and toxicological effects, with the purpose of developing an effective natural product-containing formulation able to inhibit fungal adherence and infections.

Conflict of interest The authors declare that they have no conflict of interest.

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