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Antimicrobial activity of *Croton cajucara* Benth linalool-rich essential oil on artificial biofilms and planktonic microorganisms

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We have previously demonstrated that a linalool-rich essential oil from *Croton cajucara* Benth presents leishmanicidal activity. In the present study, we demonstrate that this essential oil inhibits the growth of reference samples of *Candida albicans*, *Lactobacillus casei, Staphylococcus aureus, Streptococcus sobrinus, Porphyromonas gingivalis* and *Streptococcus mutans* cell suspensions, all of them associated with oral cavity disease. The purified linalool fraction was only inhibitory for *C. albicans*. Microbes of saliva specimens from human individuals with fixed orthodontic appliances, as well as the reference strains, were used to construct an artificial biofilm which was exposed to linalool or to the essential oil. As in microbial suspensions, the essential oil was toxic for all the microorganisms, while the purified linalool fraction mainly inhibited the growth of *C. albicans*. The compounds of the essential oil were separated by thin layer chromatography and exposed to the above-cited microorganisms. In this analysis, the proliferation of the bacterial cells was inhibited by still uncharacterized molecules, and linalool was confirmed as the antifungal component of the essential oil. The effects of linalool on the cell biology of *C. albicans* were evaluated by electron microscopy, which showed that linalool induced a reduction in cell size and abnormal germination. Neither the crude essential oil nor the purified linalool fraction is toxic to mammalian cells, which suggests that the essential oil or its purified components may be useful to control the microbial population in patients with fixed orthodontic appliances.

Key words: antimicrobial activity; *Candida albicans*; *Croton cajucara* Benth; essential oil; linalool; oral microorganisms

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Oral diseases are infections caused by microorganisms that colonize the tooth surface at or below the gingival margin (12, 21). Such pathological states, which include dental caries, periodontal diseases and tooth loss, may evolve to more serious manifestations significantly affecting the overall health of the infected individual (20). The occurrence of dental diseases is directly linked with the ability of bacteria to form plaques on the tooth surface. The dental plaque is a complex biofilm accumulated onto the surface of hard tissues (teeth), to which the growing microbial population adheres (16). The adhesion of bacteria to the salivary pellicle is a crucial event in the development of plaque, allowing organisms to remain on tooth surfaces despite the mechanical shearing forces of salivary fluid flow and tongue movement. After the initial bacterial colonization, plaque evolves to contain a variety of microorganisms, including *Candida* species, many of which are potential periodontal pathogens (8). *Candida albicans* is one of many *Candida* species isolated from humans and is responsible for the majority of oral yeast infections (4). It is a pleomorphic, opportunistic pathogen that exhibits a number of different morphological forms under different environmental conditions (18).

The establishment and growth of the dental plaque are considerably favored in individuals using fixed orthodontic appliances (22), which increase the probability of microbial retention and make conditions for oral hygiene more difficult. Patients can develop generalized hyperplasic

gingivitis within 1 or 2 months after the introduction of the appliances (2). Little is known about how to control this microbial population.

Essential oils are odorous, volatile products of plant secondary metabolism, found on many leaves and stems. The use of essential oils as antimicrobial agents has been described qualitatively for many years (10) and, in the specific case of oral microorganisms, essential oil mouthwashes have been demonstrated to be beneficial, safe components of daily oral health routines (1). But little is known about the spectrum of action of essential oils against oral microorganisms.

We have previously demonstrated that a linalool-rich essential oil from *Croton cajucara* presents strong antileishmanial activity (15). In this work, we have investigated the antimicrobial effects of this preparation against fungal and bacterial cells composing an artificial oral biofilm. Our results demonstrate that linalool is the major component involved in the antifungal activity of the essential oil, other unknown components inhibiting the growth of bacterial cells.

Material and methods

Plant material and essential oil extraction

Plant material from *Croton cajucara* Benth was obtained from Embrapa Experimental Farm, Amazonas, Brazil. A voucher specimen was deposited at Embrapa Occidental Amazon Herbarium (registry IAN 165013). Leaves of *C. cajucara* were dried at room temperature and coarsely ground into powder. The oil was obtained by hydrodistillation (5 h) of *C. cajucara* leaves using a modified Clevenger apparatus (5). Taking the dry weight of the original material and the final powdered preparation as a basis, a yield of 0.4% was obtained.

Linalool analysis

Linalool was purified from *C. cajucara* essential oil and identified by gas chromatography coupled with mass spectrometry (GC/MS) as described elsewhere (9, 15). GC-MS was performed using a Hewlett Packard (HP) 6890 gas chromatograph fitted with a BP-5 25 m × 0.33 mm fused silica capillary column, film thickness 0.5 μm, with helium as a carrier gas (1 ml/min). Retention indices were obtained by injection of a series of n-alkanes and compared with literature data. The GC/MS system used was an HP 5973 MSD coupled with an HP 6890 gas chromatograph (9). Results were compared with the Wiley library of spectra.

Microorganisms

Representative microorganisms of the oral microbiota were included in this study. The reference samples representing caries-associated microorganisms were *Lactobacillus casei* (ATCC 4646), *Streptococcus sobrinus* (ATCC 27609) and *Streptococcus mutans* (ATCC 25175). Periodontal disease-related microbes were *Porphyromonas gingivalis* (ATCC 43146) and *Staphylococcus aureus* (ATCC 49456). *C. albicans* (ATCC 51501), a well-characterized oral pathogen (8, 13, 18) was also investigated. Human isolates, obtained from the saliva of patients with fixed orthodontic appliances, included *C. albicans, Lactobacillus* sp., and *Streptococcus* sp. *C. albicans* was isolated and identified by using a selective and indicative medium (CHROMagar Candida, CHROMagar Microbiology, Paris, France), associated with the microbial ability to form germ tubes. *Lactobacillus* sp. was isolated by the pour plate technique in a selective medium (Rogosa agar, Merck, Darmstadt, Germany), associated with the analysis of microscopic aspects of gram-stained cells (14). Streptococci (*S. mutans* and *S. sobrinus*) were isolated in a Mitis Salivarius agar selective medium (Difco Laboratories, Detroit, MI) supplemented with 15% sucrose and Bacitracin (0.02 U/ml), under anaerobic conditions (11).

Antimicrobial assays in cell suspensions

A total of 10^6 cells were incubated into 1 ml of Brain Heart Infusion (BHI) supplemented with varying concentrations (1 pg/ml to 1 mg/ml) of essential oil or purified linalool, diluted in dimethylsulfoxide (DMSO) at a final concentration of 1% (v/v) in the culture media. After 48 h of cultivation at 37°C, the minimal inhibitory concentration (MIC) for each condition was determined visually. MIC was considered the lowest concentration of each substance in which turbidity was not observed. As a control, each system had a final concentration of DMSO equivalent to 1%, even when the antimicrobial compounds were absent.

Antimicrobial assays in artificial biofilms

In vitro biofilms were obtained from a pool of nonstimulated saliva of five individuals with fixed orthodontic appliances. Saliva 20 μl from this pool was spread over cellulose membrane disks (13 mm diameter and 0.22 μm pore diameter) previously placed over plates containing solid BHI. The system was then incubated anaerobically for 72 h at 37°C. After growth of the microbial population, the disks were collected, each placed inside a glass flask containing 1 ml of essential oil or a purified linalool solution (100 μg/ml) and kept in these conditions for 3 min. Control systems were exposed to chlorhexidine (0.12%) or sterile saline for the same periods. To remove nonadherent microbes, the disks were then carefully kept in sterile saline for 27 min and rinsed twice in fresh sterile saline. Microbial cells were extracted from the cellulose matrix by vortexing the disks in 1 ml of sterile saline for 20 s. Vortex-extracted cells were then inoculated into the different solid media described above and cell viability was evaluated by counting the number of colony forming units (CFU). With the exception o of CHROMagar-Candida plates, microorganisms were incubated anaerobically for 72 h at 37°C. The growth of linalool- or essential oil-treated cells was then compared with that of saline- or chlorexidine-treated microbes. The same procedure was performed with the ATCC reference microorganisms listed above. In this case, 20 μl of each microbial suspension (10^4 cells/ml) were spread over cellulose membrane disks to execute the experiment.

Bioautography

Thin layer chromatography (TLC) of the essential oil and purified linalool fraction was performed in Silica gel G60 F_{254} aluminum plates (5 × 8). Plates were developed with n-butanol : acetic acid : water (8 : 1 : 1, by vol.). Bands were visualized under ultraviolet light or after staining with cerric sulfate. Alternatively, plates were placed inside Petri dishes and covered with solid media (10 ml BHI with 1% phenol red). After overnight incubation for diffusion of the separated components, each plate was inoculated with *C. albicans* or the different bacterial reference strains (10^6 cells per plate) and incubated for 48 h at 37°C. Growth inhibition was evidenced after spraying with methylthiazolyltetrazolium chloride (MTT) at 5 mg/ml (17).

Electron microscopy

The effects of the linalool-rich essential oil on *C. albicans* biology were evaluated by scanning and transmission electron microscopy (SEM and TEM, respectively).

For SEM analysis, fungal cells were incubated in the absence or in the presence of 1 μg/ml linalool rich essential oil and purified linalool for 30 min. Control and treated cells were washed twice in phosphate-buffered saline (PBS), pH 7.2, and fixed in 2.5% glutaraldehyde (grade I), 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 5 mM calcium chloride and 3.7% sucrose for 1 h at room temperature. Cells were then rinsed in PBS, pH 7.2, and allowed to adhere to poly L-lysine-coated glass coverslips. Samples were then rinsed in PBS and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.8% potassium ferrocyanide and 5 mM calcium chloride for 30 min at room temperature. Subsequently, samples were dehydrated in graded ethanol, critical point dried in CO_2, coated with gold in a Balzers FL9496 sputter system and imaged in a JEOL-JSM-5310 Scanning Electron Microscope. Images were obtained using secondary electrons.

For TEM analysis, *C. albicans* cells were washed twice in PBS, pH 7.2, and fixed in 2.5% glutaraldehyde (grade I), 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 5 mM calcium chloride and 3.7% sucrose for 1 h at room temperature. Cells were then rinsed in PBS and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer containing 0.8% potassium ferrocyanide and 5 mM calcium chloride for 30 min at room temperature. Cells were rinsed, dehydrated in graded acetone and embedded in Polybed 812. Ultrathin sections obtained with a Reichert Ultracut S ultramicrotome were stained with uranyl acetate and lead citrate and examined in a Zeiss 900 Transmission Electron Microscope operating at 80 kV.

Statistical analysis

All experiments were performed in triplicate and results were expressed as means ± standard deviations. Statistical analysis of the differences between mean values obtained for experimental groups was performed using Student's *t*-test. *P*-values of 0.05 or less were considered significant.

Results

Antimicrobial effects of linalool and essential oil

The MICs of *C. cajucara* essential oil and purified linalool for the growth of *L. casei*, *S. aureus*, *S. sobrinus*, *P. gingivalis, S. mutans*, and *C. albicans* are summarized in Table 1. Although the crude oil preparation inhibited the growth of all the microorganisms tested in different concentrations, the purified linalool fraction only inhibited *C. albicans*. Comparing the MIC of linalool with those detected for the essential oil and the standard drug chlorexidine, it was concluded that the former has a potent antifungal effect.

Susceptibility of microorganisms from the artificial biofilm to crude essential oil and purified linalool

Artificial biofilms were prepared and then exposed to linalool or to the essential oil. Reference strains of oral microorganisms were analyzed, as well as microbes from saliva specimens from human individuals with fixed orthodontic appliances. Confirming the results from Table 1, Fig. 1A shows that the essential oil inhibited all the microorganisms tested; the profile of inhibition was similar to that observed for the standard drug chlorexidine. The purified linalool fraction, however, only inhibited the growth of *C. albicans*. Human isolates were also sensitive to the essential oil in different extents (Fig. 1B); *C. albicans* again was the most sensitive microorganism to purified linalool.

Bioautography

The inhibitory effects of linalool and other components of the essential oil were also

Table 1. MIC (μg/ml) of *C. cajucara* essential oil and purified linalool controlling the growth of *C. albicans* and other microorganisms related to the oral cavity

ATCC strains	Linalool-rich essential oil	Purified linalool	Chlorhexidine*
C. albicans	13.4	0.7*	50
L. casei	22.3	NI	36.5
S. aureus	33.4	NI	40.5
S. sobrinus	13.8	NI	65
P. gingivalis	31.2	NI	48
S. mutans	40.1	NI	55

*Chlorexidine was used as the standard drug.
NI, not inhibitory.

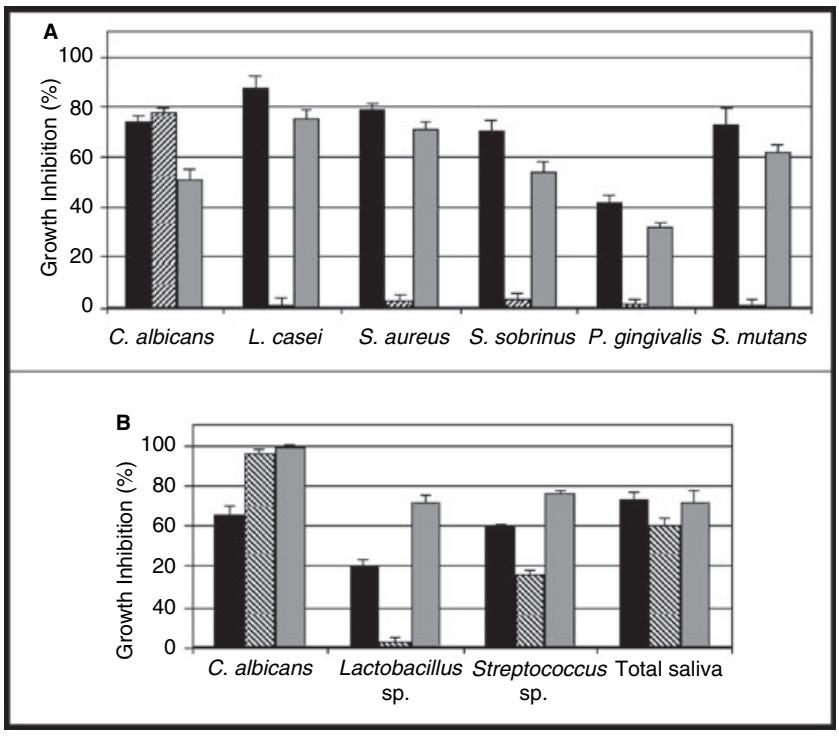

Fig. 1. Growth inhibition of bacterial strains representative of oral microbiota from culture collections (a) or isolated from human saliva from individuals with fixed orthodontic appliances (b). Microbes were exposed during 3 min to the *C. cajucara* essential oil (black column), purified linalool (striped column) or the standard drug chlorexidine (gray column). Results are expressed as means ± standard deviations.

evaluated by the exposition of *C. albicans* and the bacterial strains to different compounds previously separated by TLC. After inoculation of *C. albicans* into the medium recovering the TLC plate followed by incubation for 48 h, regions of growth inhibition were only observed around the zone of migration of linalool, indicating that this was in fact the essential oil component responsible for its antifungal effects (Fig. 2). However, the growth of bacterial cells was inhibited by more polar compounds, suggesting that other compounds in the essential oil, not linalool, present antibacterial activity (data not shown).

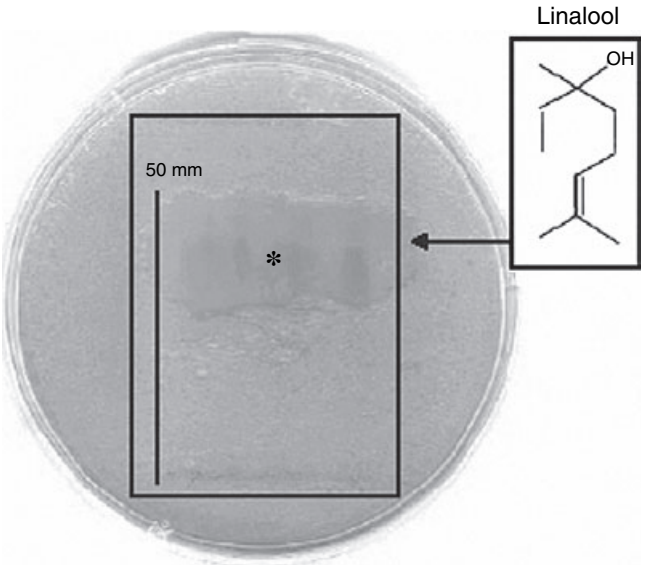

Fig. 2. Evaluation of the antimicrobial effects of TLC-separated essential oil components against *C. albicans*. The zone of growth inhibition is indicated with an asterisk and the migration rate of linalool by its chemical structure. Linalool was apparently the structure responsible for the antifungal activity of the essential oil.

Cellular alterations in *C. albicans* induced by linalool

Scanning and transmission microscopy analyses of *C. albicans* control cells and those treated with the essential oil or purified linalool are shown in Fig. 3. As demonstrated by SEM, no visible changes were seen in the shape or size of the cells after treatment with essential oil. However, when these cells were analyzed by TEM, discrete cytoplasmic alterations were observed. After treatment with linalool, on the other hand, there were significant alterations in the size and shape of cells, as demonstrated by SEM. Comparing these cells with control yeasts, the cells had a wrinkled appearance and were about 25% smaller. The analysis of linalool-treated fungi by TEM revealed that these cells presented an irregular budding pattern associated with several incomplete germ tube emissions.

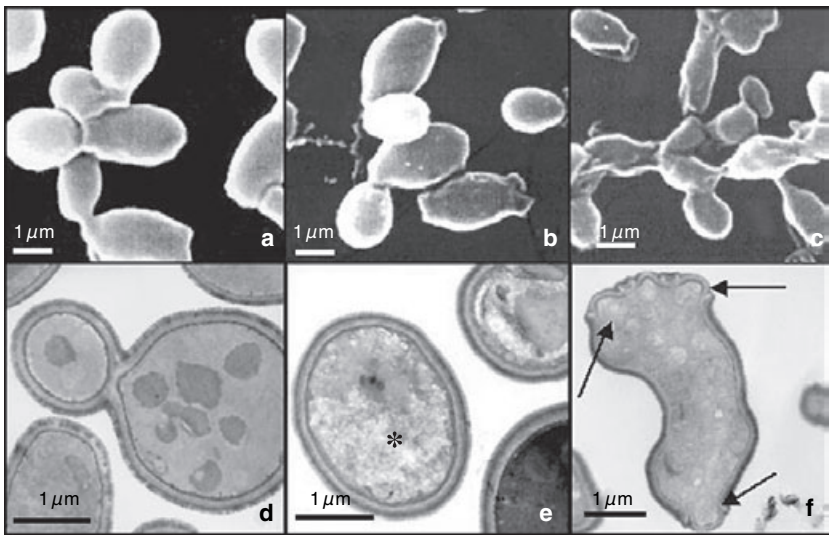

Fig. 3. SEM (a–c) and TEM (d–f) analyses of nonstimulated (a and d), essential oil-treated (b and e), and linalool-stimulated (c and f) *C. albicans*. The asterisk denotes rarefaction of the cytoplasmic matrix in yeast cells exposed to the essential oil. Treatment of *C. albicans* cells with purified linalool showed an irregular aspect and a disordered profile of germination (arrows).

Discussion

The use of fixed orthodontic appliances provides a oral cavity with an increased area for microbial colonization, which is directly associated with an increased rate of food retention and microbial growth (22). Oral hygiene is directly associated with the incidence of caries, and most patients develop generalized hyperplasic gingivitis within 1 or 2 months after placement of the appliances (11, 22). The use of nontoxic antimicrobial preparations is crucial to control the microbial population in these individuals, to prevent the formation of dental plaque and its undesirable effects.

Biofilms are much more resistant to antimicrobial agents than planktonic cells (10, 12). Chlorination of a biofilm is usually unsuccessful because the biocide only kills the bacteria in the outer layers of the biofilm, leaving healthy microorganisms within the internal layers (3). In addition, repeated use of antimicrobial agents on biofilms can select drug-resistant microbes. In this context, new agents that can inhibit the growth of biofilm-associated microorganisms are greatly needed and would enhance the number of effective therapeutic alternatives.

It has been demonstrated that mouthwashes with essential oils can be a beneficial, safe component of daily oral health routines (1), but the antimicrobial activity and selective toxicity of these preparations, as well as their chemical composition, are poorly known. The antiprotozoal activity of an essential oil from *C. cajucara* has been previously demonstrated by our group (15). This preparation was rich in linalool, a therpenic alcohol abundantly present in essential oils with antimicrobial activity (19).

In the present study, we demonstrate that the linalool-rich essential oil from

C. cajucara presents antimicrobial activity against several bacteria associated with the oral cavity and also against the oral opportunistic fungal pathogen *C. albicans*. GC-MS analysis of the essential oil from this plant showed that linalool is a major component. Based on the previously described antimicrobial effects of linalool (15), we have isolated this molecule from the crude extract for further tests of antibacterial and antifungal activity in cell suspensions and in microbes cultivated in an artificial model of biofilm. Interestingly, purified linalool was more effective against *C. albicans*, the antibacterial activity being only observed after using the whole extract. Accordingly, separation of the essential oil components by TLC followed by exposition of the plate to microbial cultures revealed that areas of growth inhibition for fungi and bacteria were clearly distinct. Whereas the only region where fungal cells were unable to grow was the region of linalool migration, the growth of bacteria was inhibited in the area of migration of less polar compounds. This result suggests that, although linalool is a major component of the essential oil from *C. cajucara*, it is not the molecule responsible for its antibacterial effects. Purified linalool, however, strongly inhibited the growth of *C. albicans*, which led us to investigate the effects of this essential oil component on the cell biology of this pathogen.

Treatment of oral candidiasis caused by *C. albicans* is ineffective in a number of cases, which is associated with the resistance to the key anticandidal agent fluconazole (13, 18). The generation of germ tubes in *C. albicans* is crucial for the invasive growth of the fungus *in vivo* (6). Different studies suggest that inhibiting germ tube formation may be an interesting alternative for the treatment of candidiasis. In our experimental conditions, the cellular aspects of yeast cells treated with subinhibitory concentrations of linalool or the original essential oil were dramatically altered. By TEM analysis, it was possible to define cell damage induced by the essential oil through the observation of rarefaction of the cytoplasmic matrix. Linalool-treated yeasts, however, presented an unusual pattern of failing germ tube formation, which was accompanied by significant alterations in cell shape and size, as demonstrated by SEM. Since linalool and the essential oil are not toxic for mammalian cells (15), these results suggest that these compounds may have a clinical application in the treatment of oral candidiasis. In addition, our data indicate that in patients with fixed orthodontic appliances, crude or purified fractions could be used, depending on the predominance of bacterial or fungal populations, respectively. The mechanisms of action of essential oils or their components are unclear, as is the possible existence of resistance mechanisms impairing their antimicrobial activity (7). This condition makes mandatory the development of experimental models evaluating the pharmacological applicability of essential oil components as antimicrobial agents.

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References

1. Claffey N. Essential oil mouthwashes: a key component in oral health management. J Clin Periodontol 2003: **30**: 22–24.
2. Corbett JA, Brown LR, Keene HJ, Horton IM. Comparison of *Streptococcus mutans* concentration in non-banded and banded orthodontic patients. J Dent Res 1981: **60**: 1936–1942.
3. Dunne WM Jr. Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev 2002: **15**: 155–166.
4. Ellepola AN, Samaranayake LP. The effect of limited exposure to antifungal agents on the germ tube formation of oral *Candida albicans*. J Oral Pathol Med 1998: **27**: 213–219.
5. Gottlieb OR, Guimarães MT. Modified distillation trap. Chemist-Analyst 1960: **49**: 114–116.
6. Gow NA. Germ tube growth of *Candida albicans*. Curr Top Med Mycol 1997: **8**: 43–55.
7. Hili P, Evans CS, Veness RG. Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil. Lett Appl Microbiol 1997: **24**: 269–275.
8. Lamfon H, Porter SR, McCullough M, Pratten J. Formation of *Candida albicans* biofilms on non-shedding oral surfaces. Eur J Oral Sci 2003: **111**: 465–471.
9. Lopes D, Bizzo HR, Sobrinho AFS, Pereira MVG. Linalool-rich essential oil from leaves of *Croton cajucara* Benth. J Essent Oil Res 2000: **12**: 705–708.
10. Martindale W. The Chemistry and Manufacture of Cosmetics. Carol Stream, IL: Allured Publishers, 1910: 85–109.
11. Modesto A, Lima KC, Uzeda M. Effects of solutions used in infants' oral hygiene on biofilms and oral microorganisms. ASDC J Dent Child 2000: **67**: 338–344.
12. Parsek M, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 2003: **57**: 677–701.
13. Reichart PA. Oral manifestations in HIV infection: fungal and bacterial infections, Kaposi's sarcoma. Med Microbiol Immunol 2003: **192**: 165–169.
14. Rogosa M, Mitchell JA, Wiseman RF. A selective medium for the isolation and enumeration of oral lactobacilli. J Dent Res 1951: **30**: 682.
15. Rosa MSCS, Mendonça-Filho RR, Bizzo HR, Rodrigues IA, Soares RMA, Souto-Padrón T, et al. Antileishmanial activity of linalool-rich essential oil from *Croton cajucara* Benth. Antimicrob Agents Chemother 2003: **47**: 1895–1901.
16. Rosan B, Lamont RJ. Dental plaque formation. Microb Infect 2002: **2**: 1533–1607.
17. Saxena G, Towers GHN, Farmer S, Handock REW. Use of specific dyes in the detection of antimicrobial compounds from crude plant extracts using a thin layer chromatography agar overlay technique. Phytochem Anal 1995: **6**: 125–129.
18. Shepherd MG. Biology of *Candida* species. In: Samaranayake LP, Macfarlane TW, eds. Oral Candidosis. London: Wright, 1990: 10–20.
19. Sibanda S, Chigwada G, Poole M, Gwebu ET, Noletto JA, Schmidt JM, et al. Composition and bioactivity of the leaf essential oil of *Heteropyxis dehniae* from Zimbabwe. J Ethnopharmacol 2004: **92**: 107–111.
20. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000 2002: **28**: 12–55.
21. Wu CD, Wei GX. Tea as a functional food for oral health. Nutrition 2002: **18**: 443–444.
22. Zachrisson S, Zachrisson BV. Caries incidence and oral hygiene during orthodontic treatment. Scand J Dent Res 1971: **42**: 26–34.