Effect of aqueous extract from Neem (Azadirachta indica A. Juss) on hydrophobicity, biofilm formation and adhesion in composite resin by Candida albicans

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Summary

Objective: Azadirachta indica, a Meliaceae family tree, has been used in India for many years in the treatment of several diseases in medicine and dentistry. Current research analyses the effects of the leaf aqueous extract from Azadirachta indica (Neem) on the adhesion, cell surface hydrophobicity and biofilm formation, which may affect the colonisation by Candida albicans.

Methods: Azadirachta indica extract was tested in vitro on strains of Candida albicans 12A and 156B. Changes in hydrophobicity were reported in assays of yeast adhesion to hydrocarbons, in biofilm formation with glucose and in the adhesion of the microorganisms on light cured composite resin. Assays involved enumeration of candidal colony-forming units together with scintillation counting of radiolabelled Candida and compared to a solution of chlorhexidine digluconate 0.125% widely used in dentistry. Results: Yeast growth in Neem extract was not inhibited in concentrations ranging from 0.1 mg/ml. A statistically significant increase ($p < 0.05$) in cell surface hydrophobicity was evident for the two strain tested and there was also an associated
Introduction

Candidiasis is the most common mouth fungus infection in humans, primarily caused by *C. albicans*.\(^1,2\) *Candida* are important opportunistic pathogens owing to the high frequency with which they cause infections in immunodeficient individuals such as cancer patients, those receiving broad spectrum antibiotics, and HIV-infected and AIDS patients.\(^2-4\)

Since adhesion is an essential prerequisite in colonisation and infection, its role in the pathogenesis of several diseases by fungus is widely acknowledged. Several researches have suggested that the initial stage of various microbial diseases involves the adhesion of microorganisms to the target tissue.\(^1-5\) So that the buccal mucosa might be colonised and infected, *C. albicans* must actually survive antimicrobial activities and saliva cleansing.\(^1,2,4,6,7\) Several specific interactions between the genus *Candida* and other organisms, medical devices and host tissues\(^3,8\) have been described and may facilitate survival in mixed communities. Dentures and orthodontic appliances, are liable to colonisation and pathogenesis by yeasts in the mouth cavity. Liability increase for stomatitis may occur when other characteristics of host are present.\(^2,8-10\)

Several researchers suggest that the level of cell-surface hydrophobicity is closely related to pathogenic potential.\(^11,12\) When hydrophobic and hydrophilic cells are compared, the former adhere more to the surface and to inanimate objects, they are more resistant to phagocytosis and have greater capacity to form the germ tubes.\(^12\) Cell surface hydrophobicity in the genus *Candida* varies with growth conditions, cell morphology, growth phase conditions and even among different species of the genus.\(^12-15\)

Since several strategies may control biofilm accumulation in the mouth cavity, daily oral hygiene has an important role in maintaining teeth and gums healthy.\(^16\) Research has been undertaken with various substances in clinical and laboratorial assays to inhibit the growth of microorganisms that cause diseases of the mouth cavity. These substances include topically applied iodine solution,\(^17\) chlorhexidine\(^18,19\) and others sold on the market, and natural compounds such as a propolis solution,\(^20\) oolong tea\(^21\) and the cocoa extract.\(^22\) Chlorhexidine is perhaps the most effective compound and frequently used as a mouth antiseptic. Chlorhexidine has become a paradigm among mouth antiseptics due to its clinical efficiency on a wide range of microorganisms in the mouth cavity.\(^18,19,23-25\)

For thousands of years parts of a tree, called Neem, have been used in India and South Asia in teeth cleansing to maintain teeth and gum health. Teeth brushing with Neem sticks and the chewing of its leaves and seeds after meals became a habit in mouth hygiene.\(^16\) The literature shows that the extract from *Azadirachta indica* is a powerful inhibiting agent against the increase and the establishment of microorganisms that cause infectious diseases in the mouth cavity.\(^26\) Clinical studies have shown that the extract decreases the dental plaque index,\(^16\) whereas in vitro studies have demonstrated that the formation of the bacterial plaque has been positively affected.\(^27\) Certain anti-plaque traits may be due to the fibrous nature of these sticks, which may mechanically cause plaque removal. The plant, however, may contain anti-plaque chemotherapeutical agents.\(^27\) Neem is neither toxic nor does it have any mutagenic properties. Besides containing long-known bactericidal traits, it also seems to have anti-inflammatory, astringent, antiseptic, anti-ulcer, antiviral, antihyperglycaemia and immunostimulant properties.\(^16,26,28\)

The deployment of natural substances in dentistry is just starting and research in the field endeavours to analyse the chemical properties and the workings of these compounds.\(^20\) Since the use of antibacterial agents may be restricted by side effects,\(^19,24\) great importance is given to natural alternatives for the prevention or decrease of microorganism adhesion. The aim of current research is the evaluation of Neem extract as a potential agent in the inhibition of colonisation. Tests have been undertaken to evaluate the effects of the aqueous extract in the adhesion, hydrophobicity and biofilm formation by *C. albicans*.

Materials and methods

Strains and culture conditions

*Candida albicans* serotypes 12A and 156B were obtained from the Fungal Culture Collection of the Microbiology Department of the Instituto de
Ciências Biomédicas (ICB)-São Paulo University. Strains were maintained in Sabouraud dextrose agar (SDA) at room temperature and cultured (18–24 h) by growth in SDA at 37 °C, prior to each experiment.

**Preparation of yeast suspension**

Isolated colonies of 18–24 h cultures of strains 12A and 156B of *C. albicans* were suspended in a 0.85% saline solution until the required concentration for each experiment (determined by transmittance with Bausch & Lomb spectrophotometer at 530 nm). Final cell concentration in each suspension was monitored microscopically in a Neubauer chamber.

**Preparation of Azadirachta indica extract**

Extract was prepared from the dry leaves of the *Azadirachta indica* A. Juss (Meliaceae) obtained from the Paraná Agronomic Institute (IAPAR). The leaves were ground and 100 g obtained. Material was extracted in distilled water (100 g/1000 ml) during 5 h, by shaking (120 rpm), in the shade. After shaking the material was filtered in common filter paper and in Whatman n.1 (Whatman, U.K.), lyophilised and stored in amber flasks. One gram of lyophilised material was dissolved in 100 ml distilled water (0.01 g/ml) for the preparation of the aqueous extract.

**Minimum inhibitory concentration (MIC) determination**

Minimum inhibitory concentration was determined by medium dilution technique according to standard protocol NCCLS (1997). Serial dilutions were undertaken with a 5 × 10^5 cells/ml inoculum from a 0.1 g/ml concentration of aqueous Neem extract. Chlorhexidine solution (chlorhexidine digluconate 2%, FGM, Brazil), employed for comparison, was diluted as from 2%. Assays were done in quadruplicate.

**Adhesion assays**

Adhesions assays were done according to counting technique of colony-forming units, proposed by Samaranayake and MacFarlane, with slight changes. Test-bodies were made of light cured composite resin (Fill Magic, Vigodent, Brazil) in 8 mm by 4 mm by 1 mm bands, polished with polish disks and diamonded paste. Tests were done for each strain in triplicate. Sterile composite resin bands (auto-claved in distilled water) were placed aseptically in 5 mm diameter wells on a sterile serum plate. Each test well contained inoculum (150 μl; 2 × 10^5 yeast) and either aqueous Neem extract (150 μl; final concentration of 0.01 g/ml) or chlorhexidine (0.125% final concentration). Controls used sterile distilled in place of the antimicrobial. Plates were incubated for 1 h at 37 °C under continuous reciprocate shaking (Shaker NT712). Bands were then removed aseptically from wells and smoothly cleansed three times with a 0.85% sterile saline solution to remove non-adherent cells. Test-bodies were transferred to 10 cm × 7 cm tubes with phosphate buffered saline solution (PBS) and 1.5 g of glass beads (1–2 mm diameter from Roni Alzi Scientific Glass). After 1 min vortex shaking of the washed bands, 20 μl of inoculum was inoculated on SDA medium. Plates were incubated at 37 °C for 24 h and the colony-forming units (cfu) counted.

In the radiolabelled adhesion assay, inocula of the two strains were incubated in Erlenmeyer flasks containing 4 ml of Sabouraud dextrose broth (SDB) and 28 μl of methyl-3H thymidine (Amersham) at 37 °C, for 18 h, under continuous shaking (Shaker NT712), according to Gasparetto. Cultures were then centrifuged at 3000 × g and supernatants removed. Cleansing was done three times with distilled water. Cell suspensions adjusted to (1–2) × 10^5 cells/ml final concentration and deployed to verify adhesion of strains in test-bodies [test-bodies were 10 mm diameter and 1 mm thick disks of light cured composite resin (Fill Magic, Vigodent, Brazil)]. Test-bodies were immersed in water for total hydration, autoclaved and fixed in polyethylene plates. Disks were covered by the cell suspension as described above, and completed by a similar quantity of distilled water as control. In the case of treatments, test-bodies disks were covered with cell suspension completed by a similar quantity of aqueous extract of Neem 0.02 g/ml or by a solution of chlorhexidine 0.25% to obtain 0.01 g/ml and 0.125%, respectively, in final concentrations. Tubes were then incubated for 1 h at 37 °C under continuous shaking. After incubation, the test-bodies were washed three times with sterile water and then transferred to a 1 ml scintillation solution. A 50 μl cell suspension aliquot from each strain was transferred to an Eppendorf tube and completed with scintillating solution for adhesion control by radiation emission. Readings were undertaken in scintillation analyser (TRI-CARB 2100 TR Liquid Scintillation Analyzer, Canberra Packard Company, USA). Results were given in number of adhered cells per square millimetre of test-bodies disks. The number of cells was determined by calculating the test-bodies area and comparing the radiation emitted by the controls (the Eppendorf containing only the inoculum, which had a known cellular
concentration) with the radiation emitted by the each test body.

**Cell surface hydrophobicity (CSH) assay**

Hydrophobicity was assessed according to Rosen-berg et al., modified by Anil et al. After a 48 h culture in Sabouraud dextrose broth (SDB), cells of *C. albicans* were centrifuged at 3000 × g for 5 min, cleansed in a saline-phosphate buffer solution (pH 7.2) and re-suspended in the same buffer (10⁶ cells/ml) and deployed as inoculum in tubes with buffer only (control), 1.5 ml buffer solution completed by 1.5 ml Neem extract 0.02 g/ml, and 1.5 ml buffer solution completed by 1.5 ml of chlorhexidine solution 0.25% for comparison. Tubes were incubated for 48 h at 37 °C by shaker (Shaker NT712), cleansed three times, and re-suspended in a phosphate—urea—magnesium buffer. Three millilitres of aliquots of suspensions were transferred to 13 mm × 100 mm tubes; 400 μl n-hexadecane (Sigma Chemical Co., USA) were then added. Mixtures were incubated in a warm bath (Quimis) for 10 min, at 30 °C, and then homogenised in tube shakers (Vortex AP56, Phoenix, Brazil) for two 30 s intervals, with a 5 s pause in between. Absorbances were determined by spectrophotometer (Bausch & Lomb) at 530 nm after the separation of the two phases. Results were given as percentages of adsorbed cells by n-hexadecane and contrasted to control. Tests were done in triplicates and strains compared to their capacity of being adsorbed in n-hexadecane. A control absorbance tube for yeast suspension and another for Neem extraction were prepared.

**Biofilm formation**

Inocula containing 3 ml saline with 2 × 10⁷ cells/ml concentration were prepared in tubes from each yeast cultivated in SDA for 24 h at 25 °C. So that the effect of Neem extract and chlorhexidine solution in biofilm formation might be verified, 50 μl inoculum were added in 3 ml dextrose SDB (6%) (control) and in solutions of Neem extract (0.01 g/ml) and of chlorhexidine (0.125%) final concentrations, also in 3 ml dextrose SDB (6%). After tubes were incubated for 48 h at 37 °C under continuous shaking (Shaker NT712), they were carefully removed from shaker. Reading was done by subjective quantification, according to suggestion by Solano et al.

**Scanning electron microscopy (SEM)**

Composite resin strips (Fill Magic, Vigodent, Brazil), 0.8 cm long by 0.4 cm wide by 0.1 cm thick were incubated with a 2 × 10⁷ cells/ml suspension for 6 h with distilled water (control group) and with the same volume of or Neem aqueous extract (treatment groups). Test-bodies were fixed in glutaraldehyde solution 10% and fixed on an aluminium support adapted to the microscope, and then metalised in Shimadzu IC-50 Ion Coater (Shimadzu Biotech., Japan) apparatus. Scanning electron microscopy assays were undertaken in non-quantitatively form by electron scanning microscope Shimadzu SS-550 Superscan (Shimadzu Biotech., Japan).

**Statistic analysis**

Results were analysed by ANOVA and Tukey tests to compare strains and treatments. Dunnet test compared control and treatment of the same strain. Significance level was 5% and tests were done by Graph Pad Prism (Graph Pad Software Inc.).

**Results**

**Minimum inhibitory concentration**

Reading of minimum inhibitory concentration assays was done visually, according to protocol NCCLS (1997). Although Neem aqueous extract did not inhibit the growth of any strain in concentrations as from 0.1 g/ml, growth of yeast colonies occurred in all tested dilutions. Chlorhexidine solution inhibited the growth of two strains as from 0.03% concentration. Test was done in quadruplicate.

**Cell surface hydrophobicity assay**

The two strains showed hydrophilic behaviour. Fig. 1 shows that a high and statistically significant increase \( p < 0.05 \) occurred in percentages of cells partitioned to n-hexadecane after Neem extract had been added at concentration 0.01 g/ml. Chlorhexidine solution, with 0.125% concentration, triggered a significant increase too \( p < 0.05 \) in the samples’ hydrophobicity. Strain 156B had higher hydrophobicity than that of strain 12A in control and in treatments. Microorganism self-aggregation has been reported visually in current and in other experiments in which strains had been incubated with Neem extract.

**Adhesion assays**

Colony-forming units (cfu) showed a significant statistical decrease \( p < 0.05 \) (Fig. 2) when Neem extract was added and cfu compared to controls of both strains. When incubated with a chlorhex-
idine solution, cfu decrease, reaching zero in most assays, with statistically significant differences ($p < 0.001$) (Fig. 2). Adhesion experiments radiolabelling (Fig. 3) showed an adhesion decrease in the two strains after contact with Neem extract. On the other hand, strain 12A presented only a slight adhesion decrease after contact with chlorhexidine solution, while strain 156B increased the number of cells adherent to the test-bodies disks posterior to contact with chlorhexidine solution (Fig. 3).

**Biofilm formation**

Incubation of microorganisms in SDB tubes plus 6% glucose showed the capacity of the two strains in forming the biofilm even in tubes with an addition of chlorhexidine solution (Table 1). Strain 156B produces more biofilm than 12A (Table 1). Fig. 4 shows an increase in biofilm formation in strain 156B when Neem extract was added. The same occurred in strain 12A (data not shown).

**Scanning electron microscopy**

Since the two strains showed a high increase in hydrophobicity and a decrease in adhesion capacity after contact with Neem aqueous extract, only strain 12A was analysed by electron microscopy. Fig. 5 shows adhesion pattern of strain 12A when incubated with composite resin test-body without Neem extract. Cells and hyphae adhered throughout the test-body. Fig. 6 presents adhesion pattern of the same strain to the test-body in the presence of 0.01 g/ml concentration of Neem extract. Fig. 5 reports a more pronounced adhesion cells without Neem extract than with Neem extract (Fig. 6).
However, adhesion of cells in presence of Neem did not occur throughout the test-body surface, but only in higher retention areas, either where polishing failed to be effective or on the rim of composite resin.

Discussion

Anti-fungal agents may enter the oral cavity in concentrations, which are equal to or over minimum inhibitory concentrations (MIC). When present in in vitro-determined concentrations, they may inhibit microorganism development for a short period of time due to their natural dilution and inactivation by saliva, expectoration or deglutition.1,33 Longer effects depend on the subsistence of these agents, or rather, on the duration of their retention to oral surfaces.19,33 In this way, the capacity of a bactericidal agent to reduce the population of a certain microorganism may, in practical terms, result in a similar effect to that produced by another agent that promotes bacterial desorption or impairs its adhesion to the mouth surface.24,34 Minimum inhibitory concentration assays showed that Neem aqueous extract does not seem to have a fungicidal effect on the strains of *C. albicans* tested. This result is similar to that obtained by Wolinski et al.,27 who verified behaviour changes in different species of *Streptococcus* when in contact with Neem extract, while no decrease in the growth of microorganisms was found. Several concentrations of the extract were initially tested in the assays and the experiments showed better results at lower extract concentrations. The use of more concentrated extracts is made difficult by the components’ low solubility in water. In fact, extracts become highly viscous and difficult to handle at high concentrations. The lack of fungicidal effect of the Neem extracts in the assays may be due to low concentrations of the bioactive compound, which may be increased when alcoholic extracts are involved. However, it seems that the aqueous extract is more appropriate for use in the mouth cavity.27 The fact that chlorhexidine solution inhibits the growth of the strains at the concentration of 0.03% endorses its use in mouth cleansing at the concentration of 0.12%.19

The adhesion process of *Candida* is a complex issue and involves biological and non-biological factors. The adhesion capacity of *Candida* seems to influence its infection and colonisation potential. Adhesion is not only affected by yeast conditions; variations in the epithelial surface also have a critical role in the adhesion phenomenon.7 The significance of adhesion as an ecological determinant is suggested by the

Figure 4 Subjective quantification of biofilm with dextrose, strain 156B from left to right: (−) absence of biofilm; (+) biofilm done by adding chlorhexidine solution; (+++) biofilm done by strain 156B without any treatment; (+++) biofilm done by adding Neem extract.

Figure 5 Adhesion of *C. albicans*, strain 12A, in test-body of light cured composite resin after 6 h incubation. Enlargement 2000×.

Figure 6 Adhesion of *C. albicans*, strain 12A, in test-body of light cured composite resin after 6 h incubation with Neem 0.01 g/ml extract. Enlargement 2000×.
relationship between the in vivo adhesion of oral bacteria to different surfaces and their proportional distribution in the mouth. The effects of restorative materials on microorganisms in the mouth should also be taken into account, as they are incorporated in the mouth and the adhesion of microorganisms to them is of paramount importance in the pathogenesis of the mouth cavity.

Cell surface hydrophobicity, which contributes to the interaction between the cells and the surfaces, seems to be an important factor in the adhesion of C. albicans. There are statistically significant correlations between cell hydrophobicity and the adhesion of C. albicans to the epithelial cells of the mouth and acrylic resin surfaces. Hydrophobicity assays revealed initial hydrophilic behaviour in the two strains, with a low percentage of cells adhering to \( n \)-hexadecane. However, there was a statistically significant increase \((p < 0.05)\) in the hydrophobicity of the cell surfaces of the two strains after contact with Neem extract (Fig. 1). Adhesion assays based on the hydrophobicity of the cell surface essentially test the conjunction of all the structural and physicochemical factors involved in microbial adhesion and not only one isolated factor, and there are no strains or species that can always be classified hydrophobic or hydrophilic. Therefore, when analysing CSH, it should be kept in mind that it is a dynamic process which may be modified by various factors. Although previous literature shows that hydrophobic yeasts are more virulent than non-hydrophobic ones, research by Cai et al. failed to find a correlation between hydrophobicity and adherions in S. mutans cells incubated with different bactericidal and/or bacteriostatic agents, demonstrating that the hydrophobicity of a cell alone is not sufficient for the activity of adhesion. Also, in a study by Gasparetto, the hydrophobicity of C. albicans 12A and 156B evaluated by the n-hexadecane technique did not show results compatible with any of the assays; either those of adherence or those of hydrophobicity through contact angle determination. When incubated with Neem extract, the strains showed a visible aggregation, which also occurred with bacteria in experiments by Wollinski et al. The self-aggregation of yeasts observed after the addition of extract may be explained by the pronounced increase in hydrophobicity.

In the same manner as in the studies by Cai et al. and Gasparetto, although the results obtained in the hydrophobicity assays show an increase in the CSH of the two strains after contact with the extract, the adhesion assays did not show the same pattern. A statistically significant fall in the numbers of cfu in both strains was observed after contact with Neem extract (Figs. 2 and 3). The fact that the increase in hydrophobicity was not directly related to an increase in adhesion supports research by van der Mei et al., who believe that the technique of determining hydrophobicity through adhesion to hydrocarbons is inaccurate. Assays by scanning electron microscopy confirm the fall observed in yeast adhesion when incubated with Neem extract (Figs. 5 and 6).

Regarding the differences found between the two samples in values for hydrophobicity, greater capacities of adhesion and biofilm formation were observed in sample 156B (Table 1 and Fig. 2), as well as greater hydrophobicity both before and after the addition of Neem extract. The difference in behaviour between the two strains has also been verified in studies by Gasparetto, where, taking into account the techniques employed, strain 12A revealed homogeneous behaviour, contrary to strain 156B. The studies suggest that such differences may not be attributed exclusively to the various methodologies used but, possibly, to the different constituents of the cell wall of the yeasts. Similar results were also reported by Pires et al. with regard to the adhesion of serotypes A and B in cell lineages (He La and Vero cells). The results obtained in the study showed further that the strains of the two serotypes showed a clear difference between themselves: while both demonstrated reactions to Neem extract, the values found, in actual numbers such as the quantity of cfu, hydrophobicity and rates of adhesion, differed between the strains \((p < 0.05\) for the values for cfu) (Fig 1).

The radiolabelled adhesion and cfu counting assays suggest Neem extract has an anti-adhesive effect (Figs. 2 and 3), even though incubation of the strains with the extract demonstrates a greater capacity of biofilm formation (Fig. 4, Table 1). The effects of Neem extract and chlorhexidine solution on the capacity of biofilm formation correspond to the findings of increased hydrophobicity obtained in the assays. However, no correlation was found with the observed reduction in adhesion, with the exception of the radiolabelled assays with chlorhexidine for strain 156B, which demonstrated a greater number of adhered cells in relation to the control (Fig. 3). The difference in values found for chlorhexidine in the assays of adhesion by counting cfu and by radiolabelling are probably due to the quantity of cells, which remain viable after contact with the solution. In their adhesion assays, Kimura and Pearsall verified that even when cells of C. albicans were dead, due to heat or to the action of formaldehyde, the amount of adhesion of yeasts treated with saliva remained the same, which suggests that the viable yeasts incubated in saliva...
undergo changes that increase their capacity of adhesion to epithelial cells. Similar results have been found in the comparison of radiolabelled adhesion experiments with tests of adhesion by counting cfu, where almost no growth of yeasts incubated with chlorhexidine was observed, and when the rate of adhesion found in radiolabelled cells was verified, high numbers of cells adhered to the test-bodies were found in strain 156B.

Cell death caused by contact with chlorhexidine may be the explanation for this, as the concentration of the solution used was higher than the MIC, which impedes its growth in the colony growth counting tests. However, the alterations that occurred in the cell surface do not prevent cells adhering to the test-body, or even contributing to biofilm formation (Figs. 1—3). As for the increase in hydrophobicity observed after contact with chlorhexidine solution, it may simply be related to the increase in adhesion found in the radiolabelling assays.

In spite of the great benefit of the reach of bactericides, the application of antiseptic agents may be accompanied by side effects. Daily mouth rinsing with chlorhexidine stains the tongue and teeth, produces changes in tastes and buccal mucosa irritation. Several studies indicate that chlorhexidine has toxic effects on human cells and on granulation tissues. Concentrations of chlorhexidine well below those used in clinical dentistry have been reported as the cause of injuries, cell death and inhibition of protein synthesis in human fibroblast culture and HeLa cells. Antiseptic agents effectively kill microorganisms but fail to remove the biofilm. Consequently, growth of microorganisms may still occur from the pre-existing biofilm. In fact, the remaining biofilm may mediate the link between viable microorganisms, as observed in the values for radiolabelled adhesion, and biofilm formation after contact of the strains with the 0.125% chlorhexidine solution, a concentration above the MIC. Therefore, antimicrobial strategies in conjunction with agents that are non-lethal to the microorganisms, with different mechanisms of action, are of greater interest for the control of biofilms, such as the use of anti-adhesive substances that inhibit the initial adhesion of the microorganisms.

The results suggest that Neem leaves have a potential anti-adhesive effect on the samples studied in vitro. Generally, the beneficial effects attributed to the plants may be due to one or more photochemicals, including antioxidants, flavins, and other substances present in the extract. While the effects of the extract cannot be attributed to a specific component, this study supports the belief in the anti-adhesive nature of the plant extract. The results of this study reinforce the possible effect of the extract in the prevention of diseases of the buccal cavity, although further clinical studies are needed prior to its use.

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