



An in Vitro Study Comparing the Anti-Candidal Efficiency of Neem Solution with Various Cleaning Techniques on the Orthodontic Acrylic Base Material.

Dr. Prerna S. Badgujar¹, Dr. Nitin Gulve², Dr. Amit Nehete³, Dr. Shivpriya Aher⁴, Dr. Vedant Gambhire¹, Dr. Ruchita Tayade¹.

1. Student, Department of Orthodontics and Dentofacial Orthopedics, M.G.V.'s K.B.H Dental College and Hospital, Nashik
2. Head of the Department, Professor, Department of Orthodontics and Dentofacial Orthopedics, M.G.V.'s K.B.H Dental College and Hospital, Nashik
3. Professor, Department of Orthodontics and Dentofacial Orthopedics, M.G.V.'s K.B.H Dental College and Hospital, Nashik
4. Assistant Professor, Department of Orthodontics and Dentofacial Orthopedics, M.G.V.'s K.B.H Dental College and Hospital, Nashik

Running title

Anticandidal efficacy of Neem solution on orthodontic acrylic base material

Corresponding Author

1. Dr. Prerna Shiram Badgujar, Student, Department of Orthodontics and Dentofacial Orthopedics, M.G.V.'s K.B.H Dental College and Hospital, Nashik
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ABSTRACT:

Introduction: Individuals pursuing orthodontic treatment aim to rectify malocclusions and improve various aspects of health and self-esteem. Self-polymerizing acrylic resin, prevalent in orthodontic applications, is prone to microbial biofilm formation due to its intrinsic properties and poor oral hygiene. *Candida albicans*, a prevalent oral microorganism, asymptotically colonizes over 60% of healthy individuals, facilitated by the hydrophobic nature of acrylic materials, potentially leading to enamel demineralization through cariogenic biofilms. The neem tree, *Azadirachta indica*, native to India, is recognized for its therapeutic properties, with its seeds containing valuable oil and bioactive compounds. Due to its antibacterial and anti-inflammatory characteristics, neem oil is extensively employed in both traditional practices and contemporary medicine.

Objectives: The present study aimed to assess the effectiveness of different techniques in eliminating *Candida albicans* from the self-polymerizing acrylic base material utilized to create removable orthodontic appliances.

Methods: The orthodontic acrylic base material bars were prepared and incubated in a *Candida albicans* suspension for 2 hours at 37 °C. The samples were divided into three groups (with fifteen bars in each) based on the cleaning method used: (1) soaking in distilled water, (2) soaking in a 0.2% Chlorhexidine commercial mouthwash solution, and (3) soaking in Neem solution. After cleaning, any yeast that remained attached to the bars was dislodged by vortexing in a growth medium and plated on Sabouraud dextrose agar. After cleaning, the reduction in yeast colony count was calculated and expressed as the number of colony-forming units per acrylic bar (CFU/bar).

Results: Results showed significant differences in the anti-candida effectiveness of the three cleaning techniques assessed at the 24-hour mark, as evaluated by the number of colony-forming



units (colony-forming unit $\times 10^3$). Distilled water had the highest average colony-forming unit count (15.06 ± 3.51), indicating the least effectiveness. In contrast, Chlorhexidine mouthwash inhibited *Candida* growth (0 ± 0 colony-forming unit), demonstrating the greatest effectiveness. Neem solution exhibited moderate effectiveness with an average colony-forming unit count of 4.06 ± 1.79 . The comparison between groups indicated a statistically significant F value of 531.168 ($p < 0.001^{**}$), emphasizing notable differences.

Conclusions: All three cleaning methods examined in this study were effective to a certain degree in eliminating *C. albicans* from the orthodontic acrylic samples. The method with the highest effectiveness and accessibility is the 0.2% Chlorhexidine mouthwash.

1. Introduction

Individuals seeking orthodontic treatment have grown not only to correct malaligned teeth but also to enhance chewing, speech, appearance, overall well-being, comfort, and confidence. Self-polymerizing acrylic resin is a commonly used material for orthodontic devices, and it is susceptible to microbial colonization and the development of biofilms in the oral environment. This susceptibility is attributed to various factors such as the material's porosity and roughness, inadequate oral hygiene maintenance.¹

Candida albicans is a common microorganism found in the oral cavity of over 60% of healthy individuals without causing any symptoms. The acrylic material of the appliance also provides a hydrophobic surface that allows *Candida albicans* to adhere through hydrophobic interactions and van der Waals forces. These factors may increase the chances of *Candida* colonization and proliferation, as well as contribute to enamel demineralization by enhancing the cariogenic potential of *Streptococcus mutans*-containing biofilms.²⁻⁷

Chlorhexidine is considered the gold standard in antimicrobial agents. By adhering to negatively charged surfaces, this cationic chlorophenyl biguanide has broad-spectrum antibacterial action, including against *Candida albicans*. It is commonly used to treat periodontal disorders and as a topical antiseptic in medical settings. Chlorhexidine works well against suspended *Candida albicans*, but it has a much reduced capacity to break up yeast biofilms, which makes it less efficient against the organism's biofilm form.^{6,7}

The neem tree, *Azadirachta indica*, is indigenous to the Indian subcontinent and well-known for its therapeutic qualities. Its seeds are rich in odorous and bitter chemicals and contain around 20% oil. Because of its antibacterial, anti-inflammatory, and insecticidal properties, neem oil is widely utilized in both traditional and modern medicine.^{2,6,7}

Many traditional cleaning techniques are combined with mechanical aids, and examining their combined effects on reducing fungal presence can help improve cleaning methods and establish better practices for preventing fungal infections, particularly among high-risk individuals. With increasing interest in natural healthcare solutions, investigating the potential role of neem in oral care could offer alternative or complementary approaches for treating fungal infections, potentially reducing the reliance on synthetic antimicrobial agents, which may have undesirable side effects.^{2,5,6}

However, a notable absence of research has been undertaken to evaluate the comparative effectiveness of Neem solution as an antifungal agent on self-polymerizing acrylic resin base material. Therefore, the study aims to evaluate the anticandidal activity of a neem solution and compare it with other standard cleaning methods, such as distilled water, and chlorhexidine mouthwash.

2. Objectives

I) To evaluate the colony-forming unit of *Candida albicans* on an orthodontic acrylic base after soaking it in distilled water.

II) To evaluate the colony-forming unit of *Candida albicans* on an orthodontic acrylic base after soaking it in Neem solution.



III) To evaluate the colony-forming unit of *Candida albicans* on an orthodontic acrylic base after soaking it in 0.2% chlorhexidine mouthwash.

IV) To compare the colony-forming unit of *Candida albicans* on an orthodontic acrylic base after soaking it in denture cleansing solution, neem solution, and 0.2% chlorhexidine mouthwash.

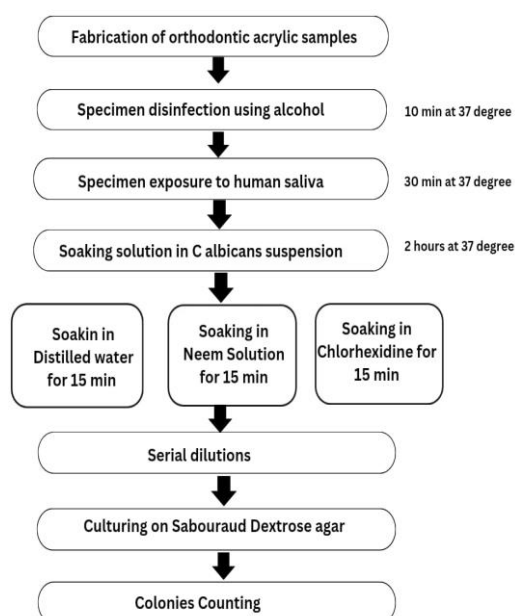


Fig 1. Flow chart of method employed in the study.

3. Methods

The study was conducted in the Department of Orthodontics and Dentofacial Orthopedics and Microbiology laboratory. The steps followed were as follows:

Fabrication of orthodontic acrylic samples

Forty-five rectangular bars with dimensions 12x25x2 mm were fabricated using orthodontic acrylic base material. The self-polymerization process was used for all the samples according to the manufacturer's instructions. The acrylic bars were polished and finished on only one side to simulate the creation of removable orthodontic appliances.

Isolation and identification of *Candida albicans*

Using a sterile swab to sample the palatal surface of the patient's removable orthodontic device, fresh *Candida*

albicans clinical isolates were obtained. The samples were then plated on Sabouraud Dextrose Agar with Chloramphenicol and incubated for 48 hours at 37°C.

Coating acrylic bars with human saliva

The acrylic bars were coated with human saliva to simulate in vivo conditions, and the saliva supernatant was kept for subsequent use at room temperature.

Ethical approval and consent forms

Following approval of the study by the Ethical Committee, and providing verbal explanation of the intended research.

Preparation of *Candida albicans* to coat acrylic

The clinical isolate of *Candida albicans* was cultured for 48 hours at 37 °C using Sabouraud dextrose agar and chloramphenicol.

Attachment of *Candida albicans* to acrylic bars

After immersing in 70% v/v ethanol for 10 minutes, the bars were rinsed with distilled water for one minute.

After being exposed to 2 ml of the previously prepared human saliva in test tubes at room temperature for half an hour, each bar was rinsed with water for a minute. After that, each acrylic bar was put in a test tube with 8 ml of the *Candida albicans* suspension, and it was kept in an incubator set at 37° C. Each sample was taken out using sterile tongs after being incubated for 2 hours.

Cleaning of specimens

A total of 45 samples were randomly divided into three groups (15 samples per group). Each group underwent a different cleaning regimen: Group 1 soaked in distilled water for 15 minutes, Group 2 soaked in the Neem solution (water: neem; 4:1) for 15 minutes, and Group 3 soaked in Chlorhexidine (0.2%) for 15 minutes.

Assessment of efficacy of the cleaning methods

After cleaning, samples were immersed into a broth containing 1 mL of Sabouraud Dextrose agar with Chloramphenicol and vortexed for 2 mins, and subjected to tenfold serial dilutions (ranging from 10² to 10³). Diluted samples were then plated on Sabouraud dextrose



agar and incubated at 37°C for 48 hours. The *Candida albicans* colonies were colony-forming units manually counted, and the colony-forming units per milliliter were determined. The effectiveness of the different cleaning methods was evaluated by comparing the counts before and after cleaning, and the percentage reduction in *Candida albicans* was calculated.

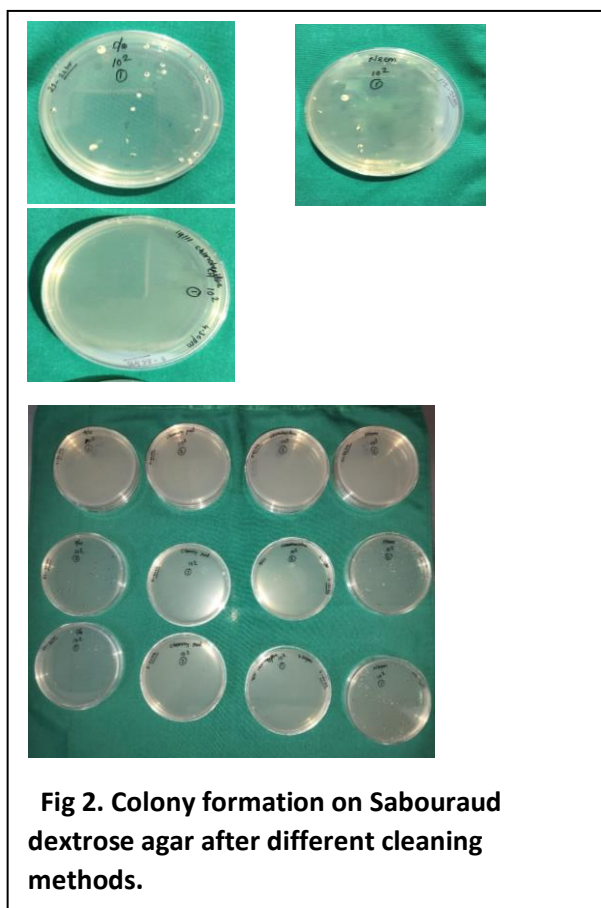


Fig 2. Colony formation on Sabouraud dextrose agar after different cleaning methods.

The *Candida albicans* colonies on the agar plates were counted manually (the total number of viable *Candida albicans* cells = the number of colonies \times the dilution factor \times 10) and expressed in several colony-forming units per milliliter (colony-forming unit /mL) which is equivalent to the colony-forming unit /bar. The percentage reduction due to the cleaning was calculated using the following formula: percentage reduction = (colony-forming unit /barc – colony-forming unit /bart) \div colony-forming unit /barc \times 100 where colony-forming

unit /barc = colony-forming unit /bar with no treatment (control) and colony-forming unit /bart = colony-forming unit /bar after treatment.

Statistical Analysis

The Statistical analysis of ordinal data was obtained and performed using Statistical Package for Social Science (SPSS) version 21 for Windows (SPSS Inc, Chicago, IL). Quantitative continuous data will be expressed in mean and standard deviation respectively. Post hoc analysis was used to compare the anticandidal efficacy between the groups.

4. Results

According to Table 1, and Graph 1, the results of the study demonstrate significant differences in the anti-candida efficacy among the three cleaning methods tested at 24 hours, as measured by colony-forming units (colony-forming unit \times 10³). Distilled water showed the highest mean colony-forming unit (15.06 \pm 3.51), indicating the least efficacy. In contrast, Chlorhexidine mouthwash achieved almost complete inhibition of candida growth (0 \pm 0 colony-forming unit), demonstrating the highest efficacy. Neem solution exhibited intermediate effectiveness with a mean colony-forming unit of 4.06 \pm 1.79.

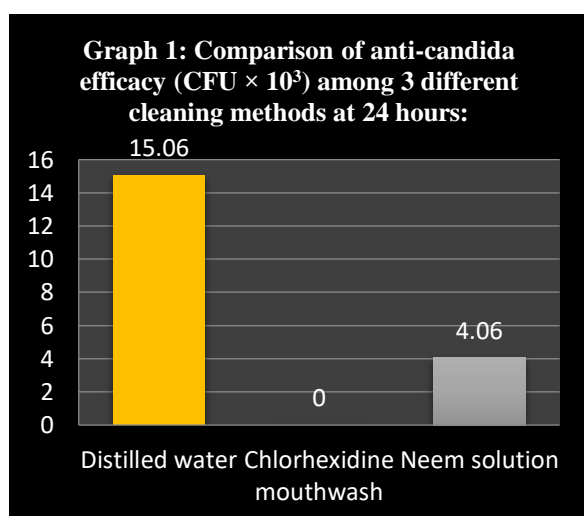
The intergroup comparison yielded a statistically significant F value of 531.168 (p<0.001**), indicating substantial differences among the groups. Post-hoc analysis revealed significant differences in all pairwise comparisons: distilled water vs. Chlorhexidine mouthwash (p<0.001**), distilled water vs. Neem solution (p<0.001**), and Chlorhexidine mouthwash vs. Neem solution (p<0.001**). These findings highlight the superior anti-candida efficacy of Chlorhexidine mouthwash, with Neem solution also showing considerable effectiveness compared to distilled water.

Table 1: Comparison of anti-candida efficacy (colony-forming unit \times 10³) among 3 different cleaning methods at 24 hours:

Cleaning method	Mean \pm SD	Intergroup comparison F value, p value
Distilled water	15.06 \pm 3.51	F=531.168, p<0.001**



Chlorhexidine mouthwash	0 ± 1	
Neem solution	4.06 ± 1.79	
Post-hoc comparison		
Distilled water vs Chlorhexidine mouthwash		p<0.001**
Distilled water vs Neem solution		p<0.001**
Chlorhexidine mouthwash vs Neem solution		p<0.001**



5. Discussion

Removable orthodontic appliances serve as an effective tool for orthodontists when addressing various malocclusions. Orthodontic appliances have been noted to elevate the rate of oral *Candida* colonization and modify *Candida* levels during treatment. Recent findings indicate that any objects placed in the mouth, whether fixed or removable, seem to change the microbial environment by offering surfaces for *Candida* to adhere to within the oral cavity.^{6,7,8}

A more careful strategy is advisable for orthodontic patients with compromised immune systems to prevent candidal infections. Understanding how orthodontic appliances influence bacterial and fungal colonization inside the mouth is vital, particularly since acrylic resin plates are commonly utilized as removable orthodontic

devices. Research has shown that using an orthodontic appliance can lead to an increase in stimulated salivary flow rate, buffer capacity, and salivary pH, which improve the anticaries characteristics of saliva.^{6,8,9,10}

The result of our study showed that soaking acrylic samples in different cleaning agents can reduce viable colony count. Chlorhexidine (0.2 %) was almost completely effective in reducing the viable colony count, followed by Neem solution, almost 96%, and the least reduction was seen with distilled water.

Ojah P et al. examine the anticandidal properties of Triphala, aloe vera, neem, and denture cleansers on heat-polymerized acrylic resin, which is used for fabrication of dentures. They discovered that denture cleansing tablets were the most effective at reducing *Candida* colonies, but neem, among natural products, exhibited the strongest antifungal effects, followed by Triphala and aloe vera. The accessibility and affordability of neem make it a promising alternative to commercial denture cleaning tablets.⁷

A study by Charmaine A.C. and Lloyd et al. examined the antifungal effects of ten different neem seed kernel extracts against *Candida* species. Although its antibacterial properties have not been extensively researched, three solvent extracts showed strong anticandidal effects. Future ethnopharmacological and antimicrobial research may uncover new compounds with better antibacterial properties.⁶

The main limitation of this study is that it was undertaken in a laboratory. Since it was an in vitro study, other oral parameters like temperature and stress were not considered. It was performed on an acrylic base plate, not an actual appliance.

Further studies can be done with a higher concentration of neem solution and on actual removable orthodontic appliances.

6. Conclusion

This study has shown that chlorhexidine gluconate (0.2%) and Neem solution can remove *Candida albicans* from the surface of the orthodontic acrylic base material. Chlorhexidine mouthwash was proven to be the most effective method followed by the Neem solution and the least with distilled water.



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