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Assessment of Disinfectants on Adherence of Candida Albicans to Soft Denture Liner

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ABSTRACT

Aim: The aim of the study is to assess and compare the efficacy of four different disinfectants on adherence of Candida albicans to soft denture liner.

Materials and Methods: A total of 55 samples of soft lined acrylic blocks are fabricated and subjected to adherence testing for Candida albicans. The samples were then immersed in disinfectant solutions such as Distilled water, 100% Tea tree oil, 100% Neem extract, 2 % Chlorhexidine and 0.5% Sodium hypochlorite. Using the digital colony counter the number of cfu/ml of Candida albicans were determined on each sample before and after disinfection.

Statistical Analysis Used: Kruskal Wallis ANOVA and Post hoc tests were performed to analyze pairwise differences between the study groups at each of the dilutions used in this study.

Results: Tea tree oil demonstrate-d highest inhibitory effect on the growth of Candida albicans at each of the three dilutions tested. At dilutions 10 and 100, neem extract was observed to be having the next highest inhibitory effect on Candida albicans after tea tree oil; however, the efficacy of neem extract reduced with an increase in dilution with chlorhexidine demonstrating the second highest inhibitory effect on Candida albicans adherence at 10^3 dilution. Post hoc tests were performed to analyze pairwise differences between thestudy groups at each of the dilutions used in this study. At a dilution of 10, tea tree oil demonstrated significant difference with all other disinfectants except neem extract. At a dilution of 100, similar observations were made with no significant difference in the Candida adherence between tea tree oil and neem extract. However, at a dilution of 1000, tea tree oil showed significant difference only with sodium hypochlorite.

Conclusion: It was observed that there exists a significant difference between the disinfectants used in this study on adherence of Candida albicans to the soft denture liner.

Keywords: Candida Albicans, Soft denture liner, Acrylic blocks, Tea tree oil, Neem extract, Chlorhexidine, Sodium hypochlorite, Colony Counter

Introduction

Denture soft liners distribute the functional load evenly on the denture-bearing area to avoid local stress concentration and improve denture retention by engaging undercuts.

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They are primarily used for patients with atrophic ridge, bony undercuts, bruxism, congenital or acquired oral defects, xerostomia, dentures opposing natural teeth, traumatic ulceration.

Soft liners made from silicone or acrylic-based material, both of which may be heat cure or self-cure.[1,2,3] The favourable properties of them include high bond strength to the denture base, dimensional stability, colour stability, easy application, biocompatibility, and low cost and their disadvantages are loss of softness[2,3]

Candida albicans is an opportunistic pathogenic yeast. It is usually a commensal organism but can become pathogenic due to local andsystemic predisposition caused by chronic irritation or immunocompromised patients. Some natural alternatives have received increased popularity in recent decades.[4] Efforts to validate their use as therapeutics have comefor increasing scrutiny in-vitro and in-vivo. Tea tree oil (TTO) is a volatile essential oil derived mainly from the Australian native plant, Melaleuca alternifolia. Tea tree oil is the most active ingredient in many topical formulations used to treat cutaneous infections as it has antimicrobial activity.

The literature documents several reports conducted to evaluate and compare the effectiveness of Chlorhexidine, Sodium hypochlorite, Tea tree oil and Neem extract on adherence of Candida albicans to the soft liners. However, till recent day no studies have been conducted to evaluate and compare the effectiveness of Chlorhexidine, Sodium hypochlorite, Tea tree oil and Neem extract on adherence of Candida albicans to the soft liners. The present in-vitro study was undertaken to evaluate and compare the antifungal effectiveness of 2%Chlorhexidine, 0.5%Sodium hypochlorite, 100%Tea tree oil and 100%Neem extract on the adherence of Candida albicans to soft denture liner.

Materials and methods

An in-vitro study was done in the Department of Prosthodontics and Crown & Bridge, Sibar Institute of Dental Sciences, Takellapadu, Guntur. Metal dies and molds fabricated at Solidworks, Patancheru, Telangana. Adherence testing and colony counting were done in the Department of Microbiology, Vignan University, Guntur.

Standardized aluminium mold preparation: (Figure -1)

Two aluminium blocks of 100 × 70 x12 mm taken and marked for cutting a mold space of 4×4×2mm and 4x4x4mm. After the markings are done on the block, it is cut using a computerized milling machine into the predetermined dimensions.

Preparation Of Samples:

Total of 55 samples prepared following the below steps.

- Preparation of wax patterns
- Preparation of Poly Methyl Meth Acrylate (PMMA) resin samples
- Packing of the soft liner on resin samples

Preparation of wax patterns:

Fifty-five wax patterns were fabricated by pouring molten modelling wax into the customized mold space of dimensions 4×4×2mm (Figure-2), coated with wax separator for easy retrieval of the wax patterns. The mold with the wax patterns was allowed to solidify in cold water andlater retrieved for investment.

Preparation of PMMA resin samples:

Dental plaster was mixed according to the manufacturer instructions and poured into the

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base of the dental flask. The wax patterns were invested into the dental plaster to half of their height(Figure-3). After the dental stone set, cold mold seal was applied and is allowed to dry. Dental stone is then mixed and poured into the flask until it is filled to form the second pour. Aftercompleting the stone's setting, de-waxing did by immersing the flask in the de-waxing unit at 100ºC for 4 minutes. The flask removed from the de-waxing unit, and residual wax was flushed outusing hot water (Figure-4).
After the mould is dried, a cold mold seal applied. The heat polymerized resin was mixed in a porcelain jar according to the manufacturer's instructions. The heat-cured acrylic resin packed into the stone mold in the dough stage in both the compartments, and the flask is placed under hydraulic pressure up to 1000 psi. Trail closure also was done, and the flash was removed.
The flasks are again placed back in the hydraulic press, and the resin was allowed to bench cure for 30 minutes. Flask was removed from the hydraulic press and then it was attached to the clamp and placed in a temperature-controlled acrylizer for polymerization. Short curing cycle was followed by putting flask at 74ºC for 2 hours, and the temperature was raised to 100 ºC and processed for 1 hour. Flask was allowed to cool down to room temperature and then removed from the water bath and deflasked. The heat-cured acrylic specimens were then retrieved carefully, trimmed by using the acrylic trimmer and sandpapered using sandpaper 80 µm,120 µm,220µm grit. Finishing and polishing are avoided to simulate the complete denture's intaglio surface.

Packing of the soft liner:

GC SOFT (auto-polymerized acrylic -based-long term) denture liner consists of the two-component system supplied as powder and liquid. The standard powder/liquid ratio is 2.2 g/1.8 g. It was measured into the glass jar and mixed for 30-60 seconds. The mixture was blended to avoid bubbles using a plastic spatula until all of the powder particles were moistened entirely.
The thickness of a soft liner to be packed over the surface of the heat-cured acrylic component was 2 mm (Figure-5). The heat-cured acrylic component was fabricated earlier with similar dimensions of 4mm × 4mm × 2mm (Figure-6) using a standardized aluminium mold.These heat-cured blocks were placed into the aluminium mold of size 4x 4 x 4mm. The bonding surface of the heat-cured acrylic component was dried thoroughly. Then the mixture is packed into mold space above the heat-cured acrylic resin block. The custom-made mould was then covered from the top by a cellophane sheet, and glass slab was pressedfirmly against the mold to remove excess material and shape the specimens. Once the material was set, the sample was removed fromthe mold, placed in water and extra material was trimmed at the periphery using a heated BP blade. In total, 55 GC SOFT liner samples were prepared in the same manner as mentioned above. The samples were stored in a glass container with a lid that contained physiological saline.

Culture of Candida albicans:
Pure Lyophilized culture of Candida albicans MTCC 1637 was purchased and inoculated on Yeast Extract Peptone Dextrose growth medium (yeast extract, peptone, dextrose, agar) and incubated at 37^{o}c for 24 hours. Subculture was done on Sabouraud's dextrose agar medium (dextrose, peptone, agar) (Figure-7) by taking 5µl of previous incubated culture growth on YEPD medium using a micropipette. It was then incubated for 48 hours at 37^{o}c and checkedfor purity. Serial dilution is used in microbiology to estimate the concentration or number of cells/organisms in an incubated plate for a simple counting number of colonies. The culture was taken in a test tube and 9 test tubes, each with 9ml of

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sterile diluents, and then at a rate of 0.9% saline.
Serial dilution of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, dilutions were done by taking 1ml of Candida albicans subculture and mixed with 9ml of physiological saline solution. 0.1ml of this solution from each serial dilution was spread on Sabouraud's dextrose agarmedium with L shape glass rod to enable candidal growth. These Petri plates incubated at 37 ºC for 48 hrs (Figure-8).
After this incubation period, colony counting was done to know the number of candidal colonies before disinfection (Figure-9).

Adherence testing:
A suspension containing 10^6 viable cells per millimetre was prepared in a saline solution using UV-Spectrophotometer at a wavelength of 600 nm, and the absorbance of -0.003 is obtained (Figure-10). Adherence testing was performed in an aseptic environment in a laminar airflow chamber using 24- wells plates for cell culture. For adherence testing, the Sabouraud's dextrose broth 350 µl, 50 µl of Candida albicans standardized suspension and one soft-lined acrylic block (sample) added to each well (Figure-11). And the plates were sealed with parafilm and incubated at 37^o c for 24 hours.

After the incubation period:
The specimens were washed with 1ml of sterile distilled water and randomly divided equally into five groups according to the disinfecting solution tested.

Group A	Distilled water (control group)
Group B	100 % Tea Tree Oil
Group C	100 % Neem extract
Group D	2 % Chlorhexidine
Group E	0.5 % Sodium Hypochlorite

Disinfection of specimens:
According to the group, the specimens immersed for 10 minutes in 10 ml of the corresponding solution. The samples were then removed, washed with 2ml of sterile distilled water and transferred to test tubes containing 1ml of sterile physiological saline solution. The test tubes were agitated in a vortex stirrer for 60 seconds, and the adhered cells dispersed. (Figure-12)

Colony counting:
For proper colony count, the initial suspension diluted 10, 100, and1000 times (Figure- 13) in physiological saline solution in the same manner as serial dilution which explained above and 0.1 ml of each suspension plated on growth medium. After 48 hours of incubation at 37^oC, the number of colony-forming units per specimen determined using a colony counter (Figure-14). All the media and the armamentarium used for culture autoclaved at temperature 121 ^{o} C for 30 minutes and 15 psi of pressure.

Results
It was observed that there exists a considerable difference between the disinfectants used in this study on adherence of Candida albicans to the soft denture liner. Tea tree oil demonstrated the highest inhibitory effect on the growth of C. albicans at each of the three

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dilutions tested (Table 1). At dilutions 10 and 100, neem extract was observed to be having the next highest inhibitory effect on C. albicans after Tea tree oil; however, the efficacy of neem extract reduced with an increase in dilution with chlorhexidine demonstrating the second-highest inhibitory effect on C. albicans adherence at 10^3 dilutions. Post hoc tests were performed to analyze pairwise differences between the study groups at each of the dilutions used in this study. At a dilution of 10, tea tree oil demonstrated significant difference with all other disinfectants except neem extract. At a dilution of 100, similar observations were made with no significant difference in the candida adherence between Tea tree oil and neem extract. However, at a dilution of 1000, tea tree oil showed a significant difference only with sodium hypochlorite.

Table 1: - Comparison of efficacy of the disinfectants on adherence ofCandida albicans to soft denture liner at various dilutions

Dilution	Disinfectant	Mean±SD	Mean rank	P-value
10	Distilled water	47.64 ±58.74	38.23	0.001*
	Tea tree oil	1.91 ± 3.08	13	
	Neem extract	19.73 ±38.82	22.23	
	Chlorhexidine	35.91 ±63.31	32.14	
	Sodium hypochlorite	12.73 ± 8.86	34.41	
100	Distilled water	7.18 ± 11.07	31.95	0.001*
	Tea tree oil	0.27 ± 0.64	13.32	
	Neem extract	2.36 ± 4.41	22.18	
	Chlorhexidine	5.91 ± 9.64	29.32	
	Sodium hypochlorite	12.27 ± 8.34	43.23	
1000	Distilled water	2.73 ± 3.63	27.86	0 .016*
	Tea tree oil	0.91 ± 1.57	17.23	
	Neem extract	2.09 ± 1.92	29.23	
	Chlorhexidine	1.82 ± 1.83	25.5	
	Sodium hypochlorite	8.18 ± 10.4	40.18	

Except with Tea tree oil at a dilution of 100, no significant differences were found for distilled water with any other disinfectant at dilutions of 100 and 1000. At a dilution of 10, distilled water showed a significant difference in candidal adherence with Tea tree oil and neem extract.

In intra-group comparisons at various dilutions, only chlorhexidine showed a significant difference in an inhibitory effect on C.albicans with a change in dilution. All the other disinfectants did not show significant difference in their inhibition of C. albicans with increasingdilution.

Discussion

Pain and difficulty are experienced by many patients using dentures constructed with hard denture bases. On this basis, the resilient materials used to increase resiliency during function and under pressure. Soft lining materials can be defined as soft, resilient, elastic materials which form a cushioned layer between the hard denture base and the oral mucosa.[5] Denture soft lining materials are broadly divided into two groups of materials. The first group includes the tissue conditioners and temporary soft lining materials typically based on polyethyl methacrylate, an aromatic ester, and ethyl alcohol. The second group comprises permanent soft lining materials based on silicone rubber or acrylic resin. Both of

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the groups may be of self-cure (chemically activated) or heat-cure (heat activated).[6,7,8]
Tissue conditioners or short-term soft liners are uncross-linked, which are formed by polymer chain entanglements. The polymer powder generally consists of poly ethyl methacrylate (PEMA) of molecular weights ranging between 1.79×10^5 and 3.25×10^5 with no initiator[9,10,11].The liquid comprises an ester-based plasticizer and 4–50 wt% ethyl alcohol (EtOH) and contains no monomer.[9,12] Short-term soft liners are tissue conditioners which remain soft for a limited period. It should be replaced with a fresh mix every 2 to 3 days for adequate cushioning.[13]
This study aims to assess the efficacy of various disinfectant solutions on adherence of Candida albicans to soft denture liner. According to disinfecting solution tested, samples are divided into group A (distilled water) control group, group B (Tea Tree Oil), group C (Neem Extract), group D (2% Chlorhexidine), group E(0.5%hypochlorite). Each specimen was immersed in 10 ml the corresponding solution for 10 minutes. After immersion, the specimens were removed, washed with 2 ml of sterile distilled water. Then each sample was transferred to test tubes containing 1 ml of clean physiological solution. The tubes were agitated in a vortex shaker for 60 seconds to disperse adhered cells. To get proper colony count the initial suspension was diluted 10, 100 and 1,000 times in physiological solution and 0.1 ml of each suspension was taken and plated on Sabouraud dextrose agar. After 48 hrs. of incubation at 37° C, the numbers of colony-forming units per specimen (CFU/specimen) were determined using a colony counter.
From the results obtained, Table 1 depicts that there exists a significant difference between the disinfectants used in this study on adherence of Candida albicans to the soft denture liner. Multiple pairwise comparisons on the effectiveness of the disinfectants on adherence of Candida albicans to soft denture liner at multiple dilutions and Intra-group comparison of the efficacy of disinfectants on Candida adherence different dilutions. Tea tree oil demonstrated the highest inhibitory effect on the growth of C. Albicans at each of the three dilutions tested (Table1). At dilutions 10 and 100, neem extract was observed to be having the following highest inhibitory effect on C. Albicans after Tea tree oil; however, the efficacy of neem extract reduced with an increase in dilution with Chlorhexidine demonstrating the second-highest inhibitory effect on C. Albicans adherence at 1000 dilution. At a dilution of 10, tea tree oil showed significant difference with all other disinfectants except neem extract. At a dilution of 100, similar observations were made with no significant difference in the candida adherence between Tea tree oil and neem extract. However, at a dilution of 1000, tea tree oil showed a significant difference only with sodium hypochlorite.
The present study's result was congruent with the study conducted by **Pachava K R et al. (2015)[14]**. They showed that 15% of Tea Tree Oil had significant antifungal activity against Candida albicans on heat- cured acrylic denture base material. **Barua et al. (2017)[15]** reported that the significant benefits of using neem are its easy availability, cost-effectiveness, good shelf life, low toxicity, and no micro-organisms resistance. **Dalwai S et al. (2014)[16]** suggested that Tea tree oil exert a significant antifungal effect (similar to chlorhexidine gluconate 2%). Tea Tree Oil was effective in inhibiting C. Albicans.[17]. **Iqbal et al. (2016)[18]** stated that Tea tree oil-modified tissue conditioners showed inhibitory and fungicidal activity on C. Albicans and effective in treating the denture stomatitis. Sodium hypochlorite was an effective agent in reducing adherent Candida albicans both in vitro and in vivo which justifies its application as a cleaning protocol.[19] **R. Sushma et al. (2017)[20]** stated that Chlorhexidine has a broad spectrum of activity against various organisms, including C. Albicans, the susceptibility of C. Albicans biofilms to Chlorhexidine was significantly reduced compared to its action against suspended organisms.

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Conclusion

It was thus concluded that there exists a significant difference between the disinfectants used in this study on adherence of Candida albicans to the soft denture liner. Tea tree oil demonstrated the highest inhibitory effect on the growth of C. Albicans. At a dilution of 10, tea tree oil showed significant difference with all other disinfectants except neem extract. At a dilution of 100, similar observations were made with no significant difference in the candida adherence between Tea tree oil and neem extract. However, at a dilution of 1000, tea tree oil showed a significant difference only with sodium hypochlorite.

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Nil.

Conflicts of interest

There are no conflicts of interest

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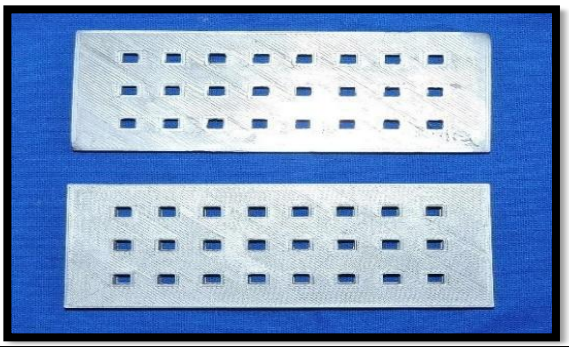

Figure- 1 Two Standard Aluminium blocks with mold size of 4mm x 4mm x 2mm & 4mm x 4mm x 4mm

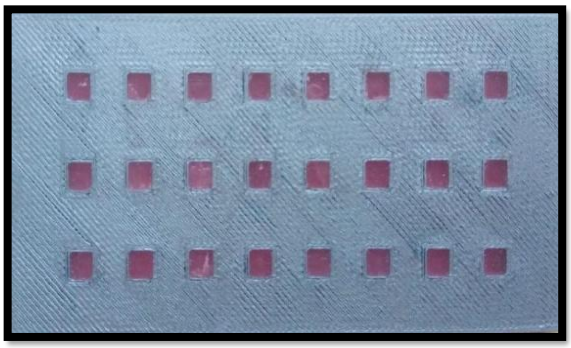

Figure- 2 Preparation of wax patterns

Figure- 3 Wax patterns invested into the dental plaster

Figure- 4 Dewaxing of wax patterns

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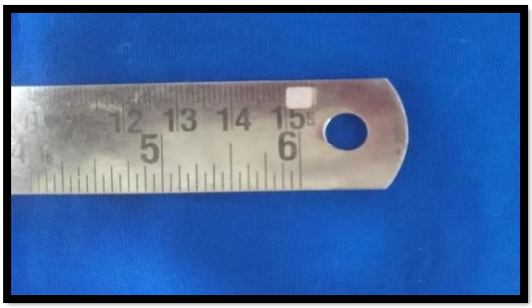

Figure- 5 Thickness of a soft liner – 2mm

Figure- 6 Heat-cured acrylic specimens-4 x 4 x2mm

Figure- 7 Subculture of Candida albicans

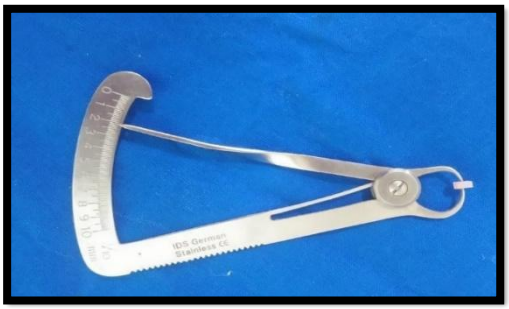

Figure- 8 Candida albicans growth after incubation

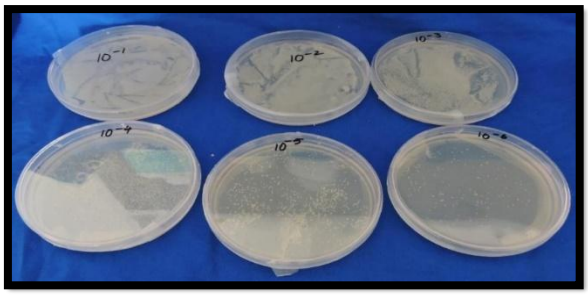

Figure- 9 Colony counting before disinfection

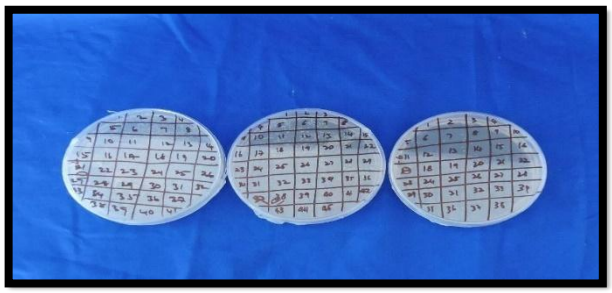

Figure- 10 UV-Spectrophotometer absorbance and wavelength reading

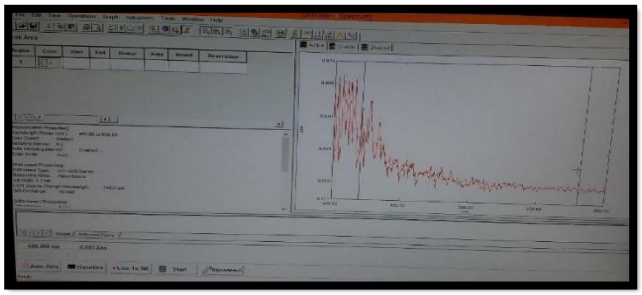

Figure- 11 Adherence testing by adding specimen with candida broth.