"COMPARATIVE EVALUATION OF LEAF EXTRACTS OF *MANGIFERA INDICA*, *ANACARDIUM OCCIDENTALE* AND 0.2% CHLORHEXIDINE GLUCONATE ON DISINFECTION OF MAXILLOFACIAL SILICONE MATERIAL SURFACE CONTAMINATED WITH MICROORGANISMS - AN INVITRO STUDY"

BY

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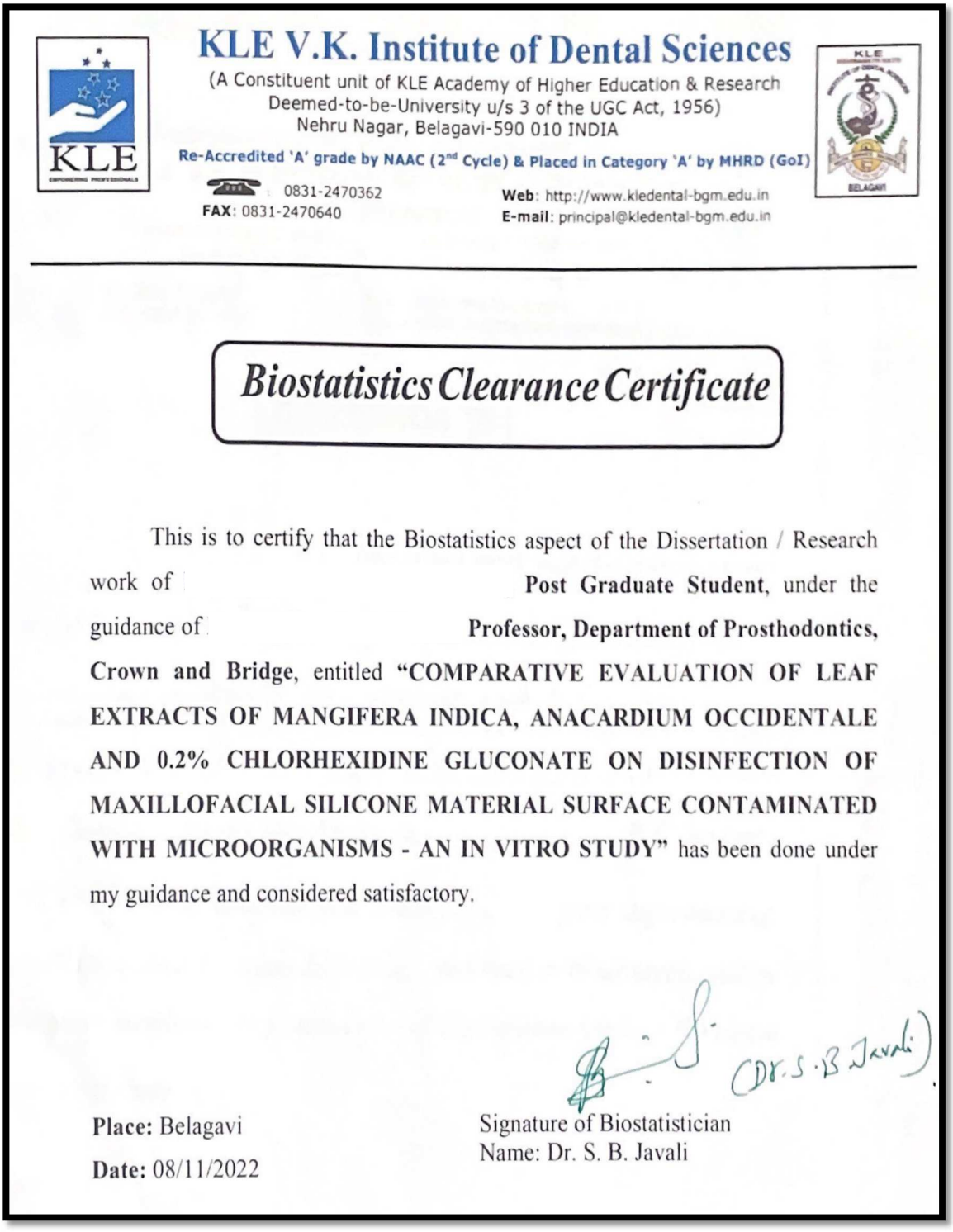

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LIST OF ABBREVIATIONS USED IN THE STUDY

CHX	Chlorhexidine
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
BHI	Brain Heart Infusion
CFU	Colony Forming Units
SD	Standard Deviation
ANOVA	Analysis of Variance
%	Percentage
^{0}C	Degree Celsius
S.aures	*Staphylococcus aures*
C.albicans	*Candida albicans*
M.indica	*Mangifera indica*
A.occidentale	*Anacardium occidentale*

ABSTRACT

STATEMENT OF PROBLEM

Maxillofacial prostheses gives a pleasing appearance and allow patients to resume full participation in society. Thermally activated acrylic resin and silicones are currently the most frequent materials utilised to produce facial prostheses and silicone is the preferred material due to its flexibility and skin-like texture.

Maintaining the quality and hygiene of maxillofacial prosthesis and results in maintaining the health of the residual tissues, therefore necessitates cleaning. Sampling the maxillofacial prostheses, has relieved presence of *Staphylococcus aureus* and *Candida albicans* colonisation on silicone surfaces. Cleaning procedures of maxillofacial silicones that have been used in the past include: Biofilm can be removed by mechanical means, such as manual brushing or washing using water with neutral soap as adjunctive or using chemical means like cleansing tablets, sodium hypochlorite, and chlorhexidine gluconate.

More research is needed to develop alternate disinfection methods which does not modify the silicone surface and are safe and non-toxic. Due to rising microorganism resistance and fewer adverse effects, phytoextracts appear to be a viable.

The leaves of *Mangifera indica* and *Anacardium occidentale* are reported to have antimicrobial properties, and leaf extracts from these plants were utilised to test their effectiveness against the growth of *Candida albicans* and *Staphylococcus aureus*, two microorganisms commonly found in maxillofacial prosthesis. Furthermore, it will be compared to 0.2% chlorhexidine gluconate.

PURPOSE

To evaluate leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms (*Staphylococcus aureus and Candida albicans*).

METHODS

Maxillofacial silicone elastomer (Silastic MDX4-4210) was used to fabricate 150 discs using metal mould of diameter- 5 mm, thickness- 2 mm**.** 75 silicone samples were contaminated with *S. aureus* and 75 were contaminated with *C.albicans*. The disc was rolled on blood agar in a 1cmx1cm area. Pre-disinfection CFUs were evaluated and the discs were subjected to disinfection protocols. The contaminated discs with *S. aureus* and *C.albicans* were disinfected using *M.indica* leaf extracts, *A.occidentale* leaf extracts and 0.2% chlorhexidine. Post disinfection CFUs were evaluated. The results were tabulated and analysed using dependent t-test , one-way ANOVA and Tukeys multiple posthoc procedure.

RESULTS:

Pair-wise comparison of 0.2% chlorhexidine, *M. indica* and *A. occidentale* with pre and post-disinfection log CFU counts of *S. aureus* was done by Tukeys multiple posthoc procedure, it was found that there is statistical significance between 0.2% chlorhexidine and *M. indica* (p=0.001); *M. indica and A. occidentale* (p=0.001). No statistically significant results were found between 0.2% chlorhexidine and *A. occidentale* (p=0.1988).

Pair wise comparison of 0.2% chlorhexidine, *M.indica and A. occidentale* of the difference of the log CFU from pre-disinfection to post-disinfection of *C. albicans* was done by Tukeys multiple posthoc procedure, found that statistically significant between all the three groups; 0.2% chlorhexidine v/s *M. indica* (p=0.001); *M. indica* v/s *A. occidentale* (p=0.001) and 0.2% chlorhexidine v/s *A. occidentale* (p=0.001).

CONCLUSION

Given the limitations of the current research, it was found that 0.2% chlorhexidine gluconate showed the most CFU reduction post disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* followed by *A. occidentale* leaf extract and *M. indica* leaf extract with statistically significance results were seen between 0.2% chlorhexidine and *M. indica; M. indica and Anacardium occidentale*. 0.2% chlorhexidine gluconate showed the most CFU reduction post disinfection of maxillofacial silicone material surface contaminated with *Candida albicans* followed by *A. occidentale* leaf extract and *M. indica* leaf extract. Statistically significant results were seen between all three groups.

KEYWORDS

Anacardium Occidentale, Antimicrobial Solution, Chlorhexidine, Disinfection, Facial Prosthesis, *Mangifera Indica*, Microbial Biofilms, Silicone Elastomers

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INTRODUCTION

Maxillofacial prosthetics is the science and art of reconstructing anatomically flawed or missing parts of the head and neck to improve their function and appearance.[1] Carcinomas, infections and developmental anomalies can all result in the loss of parts of the head and neck region.[1,2.] Patients with such defects have psychological and social challenges, which have a significant impact on the overall quality of life. The use of maxillofacial prostheses provides a pleasing appearance and allows patients to resume daily routine in society.[3]

Maxillofacial prostheses offer a non-invasive, low-cost, and pleasant appearance, allowing patients to continue their normal lives.[5] Currently, silicones and heat-activated acrylic resin are used more extensively in fabrication of facial prostheses. For fabricating maxillofacial prostheses, the introduction of room-temperature vulcanizing polymers (e.g., MDX-4-4210; VST-50) has been an improvement over polymethyl methacrylate, polyvinyl chloride, and polyurethane[3]. Due to its adaptability, patient comfort, skin-like smoothness, and capacity for both intrinsic and extrinsic colour matching, silicone is the material of choice. Polymethylmethacrylate, polyurethanes, latex, and silicone elastomers are among the materials used to make external prosthesis. Silicone elastomers are chemically inert and have sufficient tear strength, durability, and ease of manipulation. [2-6]

A delicate synergy prevails between the skin's bacterial ecology and the host. This balance may be altered when a silicone elastomeric prosthesis is placed on the skin. Biofilm can develop on prostheses due to mucosa, humidity, and skin secretions (such as perspiration, sebum, and other compounds released by the skin).[4] The compression, heating, and contact from the prosthesis could induce dermatitis.

Problems associated with the microflora of these prostheses include endophthalmitis, bacterial dermatitis, disagreeable odours, and black patches on the prosthesis.[4-7]

The skin and surfaces of the prosthesis have been routinely found to be colonized with *Staphylococcus aureus*. It is also a pathogenic bacterium that has been used to examine the effectiveness of antibiotics. Colonization of *Candida albicans* on silicone surfaces has been associated with the staining of maxillofacial prostheses. The presence of bacteria and yeast is observed after sampling the maxillofacial prosthetics' surfaces. The most common bacterial species found were *Staphylococcus epidermidis, Staphylococcus schleiferi, Staphylococcus xylosus,* and *Staphylococcus capitis*, whereas the most common yeast species were *Candida albicans, Candida parapsilosis,* and *Candida famata*.[5] On prosthetic surfaces, microbial adherence and biofilm growth are significant contributors to material deterioration and skin irritation, which can contribute to facial prosthesis failure. It is critical to disinfect and conserve maxillofacial prostheses to preserve both their quality and the well-being of the tissues around them.[5]

Under all of the current cleaning techniques, patients are obliged to clean their prostheses. Some cleaning methods include wiping down with a cotton ball immersed in a moderate soapy fluid, using a brush with soap, washing in water, patting dry with a napkin, and keeping in a container out of direct sunlight. Some of the most often used cleansing agents for the facial silicone elastomer include neutral soap, peroxides, acid enzymes, sodium hypochlorite, cleansing tablets, and chlorhexidine.[1,2,7,8]

However, these may cause surface abrasion, which is undesirable and harmful to the prosthesis. When it comes to disinfecting maxillofacial elastomer, chemical soaking is the method of choice. Cleansing with a 2-4% chlorhexidine gluconate by

spraying or dipping in solution for 1 minute, followed by washing under running water, can adequately condition to minimize the quantity of bacterial contamination without jeopardizing the prosthesis. The physical and mechanical characteristics of maxillofacial silicone elastomers could be altered by frequent application of chlorhexidine gluconate, leading to loss of smooth surface and a rise in microhardness[8]

As a result, more study is required to seek different disinfection methods that do not affect the silicone surface and are both safe and non-toxic.[1] One of the key strategies for overcoming these challenges is the use of phytoextracts, which appear to be a viable disinfectant due to increased microorganism resistance and fewer side effects. The recent resurgence of natural health has contributed to an increase in interest in available naturopathic therapies. Medicinal plant extracts and essential oils like *Melaleuca alternifolia* oil (Tea tree oil), *Origanum* oil, Lemongrass essential oil, etc have been used in developing countries as alternative treatments to health problems.

Mangifera indica (mango) leaves have been shown to have antimicrobial properties. Phytochemical analysis of *M.indica* leaf extracts indicated that there was active pharmacological components which include tannins, saponins, flavonoids, alkaloids, and mangiferin. Leaf extracts are used as astringents and antiseptics, and the ash of burned leaves is used to treat burns and scalds.[9-11,28,48]

Because of its antioxidant, antibacterial, and anticancer properties, *Anacardium occidentale* (cashew) plants are used medicinally in many nations. Plant metabolites such as phenols, flavonoids, and tannins are found in leaf extracts and are

responsible for their antibacterial properties. Toothache and sore gums are treated with an infusion of leaves and stem bark.[11-14,39,49]

There has been no research on the disinfectant properties of *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) for polymers used in maxillofacial prostheses. The leaves of *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) are reported to have antibacterial and antifungal properties.

Thus, this research is performed with an aim is to evaluate leaf extracts from *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) and to test their effectiveness against the growth of *Candida albicans* and *Staphylococcus aureus*, two microorganisms commonly found in the maxillofacial prosthesis, and it will be compared with 0.2% chlorhexidine gluconate disinfectant.

NEED FOR THE STUDY

Maxillofacial prostheses gives a pleasing appearance and allow patients to resume full participation in society. Thermally activated acrylic resin and silicones are currently the most frequent materials utilised to produce facial prostheses and silicone is the preferred material due to its flexibility and skin-like texture.[2]

When tissue is in contact with the surgical defect, human fluids contaminate maxillofacial prostheses. There have been reports of problems with maxillofacial prosthetics, such as microbial colonisation. The presence of these microorganisms has been linked to cases of bacterial dermatitis and endophthalmitis. [8] . Biofilm growth on prosthetic surfaces are well-known causes of material breakdown and skin irritation, ultimately leading to facial prosthesis malfunction. [3]

Maintaining the quality and hygiene of maxillofacial prosthesis and results in maintaining the health of the residual tissues, therefore necessitates cleaning. Sampling of the maxillofacial prostheses has reported the existence of *Staphylococcus aureus* and *Candida albicans* colonisation on silicone surfaces. Cleaning procedures of maxillofacial silicones that have been used in the past include: Biofilm can be removed by mechanical means, such as manual brushing or washing using water with neutral soap as adjunctive or using chemical means like cleansing tablets, sodium hypochlorite, and chlorhexidine gluconate.

However, repeated exposure to these solutions may cause the surface of the prosthesis to become uneven. The physical and mechanical characteristics of maxillofacial silicone elastomers could be altered by frequent application of chlorhexidine gluconate, leading to loss of smooth surface and a rise in

microhardness. [6]As a result, more research is needed to develop alternate disinfection methods which does not modify the silicone surface and are safe and non-toxic[1]. Due to rising microorganism resistance and fewer adverse effects, phytoextracts appear to be a viable disinfection choice.

Mangifera indica (mango) leaves have been shown to have antimicrobial properties.. Leaf extracts are used as astringents and antiseptics as they contain active pharmacological components which include tannins, saponins, flavonoids, alkaloids, and mangiferin [8-11] Because antioxidant, antibacterial, and anticancer properties, *Anacardium occidentale* (cashew) plants are used medicinally in many nations. Plant metabolites such as phenols, flavonoids, and tannins are found in leaf extracts and are responsible for their antibacterial properties.[12-14]

There have been no research on the disinfectant properties of *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) for polymers used in maxillofacial prostheses. The leaves of *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) are reported to have antibacterial and antifungal properties, and leaf extracts from these plants will be utilised to test their effectiveness against the growth of *Candida albicans* and *Staphylococcus aureus*, two microorganisms commonly found in maxillofacial prosthesis. Furthermore, it will be compared to 0.2% chlorhexidine gluconate.

HYPOTHESIS

NULL HYPOTHESIS

There is no significant difference of leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms.

ALTERNATIVE HYPOTHESIS

There is significant difference of leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms.

AIM AND OBJECTIVES

AIM OF THE STUDY

To evaluate leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms (*Staphylococcus aureus and Candida albicans*).

OBJECTIVES

1. To evaluate leaf extracts of *Mangifera indica, Anacardium occidentale* on disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans*
2. To evaluate 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans.*
3. To compare leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans*

REVIEW OF LITERATURE

1. **Steven P Haug (1992)** examined how four maxillofacial elastomers' physical qualities were affected by weather, sunshine, cleaning products, cosmetics, adhesives, and time. The samples were immersed in the cleaning solution, sealed, and left in the same dark environment at room temperature for 30 hours. A-2186, while being one of the softer materials tested, demonstrated significant strength ratings. Unfortunately, the majority of the studied environmental variables weakened and made this new material stiffer, causing it to lose these beneficial properties.[22]

2. **H. Nikawa et al. (2001)** performed a study on the growth *Candida albicans* on commercial maxillofacial materials that were protein-coated and thermo-cycled and used for facial prosthesis, revealing that the deterioration of the material and the host's body fluid aided fungal growth on maxillofacial materials.[20]

3. **G. S. Bbosa et al (2007)** *Mangifera indica* (L.) leaf extracts were tested for their ability to combat *Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa.* When compared to the positive control, the extracts' antibacterial activity against the study organisms using the agar-well diffusion and gradient serial dilution procedures was low. Chemical analyses revealed that the ethanolic portion of the plant extract contained anthracenocides, flavanones, and reducing sugars, while the aqueous portion contained alkaloids, coumarins, flavonoids, reducing sugars, catechol and gallic tannins, saponins, steroids, and triterpenoids. These findings indicate that *M. indica* leaf extracts have some antibacterial activity, which may be the basis for their potential medical use.[37]

4. **Y. Shi et al (2008)** The study's goal was to see how effective human beta-defensin-3 (HBD3) was at eliminating *Staphylococcus aureus* and *Candida albicans* on the surface of a maxillofacial silicone elastomer. In 30-minute immersion, the active recombinant HBD3 eradicated *S. aureus* and *C. albicans* microorganisms from the surface of the maxillofacial elastomer. The difference between the rHBD3 group and the sodium hypochlorite 5.25 % group was not statistically significant.[21]

5. **Marcelo Coelho Goiato et al (2009)** described the maintenance of the prosthesis' quality requires both cleaning and care. This demonstrated the proper techniques for cleaning and caring for the patient's prosthesis and surrounding tissue, beginning with expert teaching and training. It has been confirmed that silicone, when used to make maxillofacial prostheses, is the material that holds microorganisms on its surface. Therefore, it is advised to clean prosthetics with water, neutral soap, and chlorhexidine. When it comes to taking care of the surrounding tissues, it is advised to remove the prosthesis before going to bed and to wash the tissues that are in contact with the prosthesis with water and neutral soap or with a solution of hydrogen peroxide and water. While cleaning the mucosal surfaces of the ocular cavity at least three times per day with physiological solution or filtered, boiling water is advised.[3]

6. **O.O. Ayepola and RO Ishola (2009)** investigated *Anacardium occidentale* leaves and stem bark extracts phytochemically for secondary metabolite content and in vitro antibacterial activity. The screening for phytochemicals found alkaloids and tannins. In comparison to the aqueous extracts, the leaf methanol

extracts showed significant activity. The antibacterial activity was most effective against Candida albicans, Bacillus anthracis, and K. pneumoniae.[12]

7. **Ariani N et al. (2012)** studied the microorganism present on healthy skin without prosthesis to the microorganism on silicone prostheses. Mixed bacterial as well as yeast biofilms were discovered under scanning electron microscopy. In all samples, bacterial diversity outnumbered yeast diversity. Occlusion of the epidermis by prostheses produced a favourable environment for opportunistic microorganisms like *Candida spp. and Staphylococcus aureus.*[5]

8. **A Atay et al. (2013)** determined the lowest surface contact angle and fungus adhesion on popular maxillofacial silicone materials. The maxillofacial silicone materials VST-50, A-2006, and A-2186F were used. On the sample made of A-2006 silicone material, the lowest surface contact angle values and the maximum Candida adhesion amount were measured. The three maxillofacial silicone materials varied from one another statistically and substantially.[23]

9. **Salah Khalaf Al-Askari et al. (2014)** examined the differences in the adhesion of *Staphylococcus aureus, Streptococcus mutans, and Candida albicans t*o surfaces made of silicone elastomers used in maxillofacial prostheses. Microscopy showed that bacteria had adhered to the silicone elastomers' surface. In addition to causing undesired infections and microorganism proliferation in dental offices, the presence of these germs on external maxillofacial prostheses may also lead to re-infection, resulting in cross-contamination. [24]

10. **Chabi Sika K. et al. (2014)** studied the antibacterial properties of extracts from leaf and bark two different cashew tree components in aqueous, ethanol, ethyl acetate, and dichloromethane. *S. aureus and C. albicans* displayed the lowest MBC for the acetyl acetate leaf extract. The outcomes demonstrate that acetyl acetate leaf extract and ethanol were both more effective than ethanol bark extract.[13]

11. **Ariani N et al. (2015)** investigated the effectiveness of several cleaning agents in eliminating bacteria and yeast in a mixed species biofilm on silicone facial prosthesis. The most promising cleansing agent was found to be 0.2 percent chlorhexidine digluconate. The mouthwash containing essential oils was shown to be as effective as ethanol. The least effective agents were antibacterial soap and buttermilk.[18]

12. **Anand G et al (2015)** investigated the antimicrobial activity of ethanol extracts of cashew and mango leaves. In comparision to povidone-iodine rinse, cashew and mango leaf extracts provided a considerably greater zone of inhibition against *E.faecalis, S.aureus, S.mutans, E.coli, and C.albicans.*[11]

13. **Aimée Maria Guiotti et al (2016)** compared the antimicrobial activity of standard disinfectant solutions (water, neutral soap, and 4% chlorhexidine) and extracts of *Cymbopogon nardus and Hydrastis canadensis* on maxillofacial silicone samples contaminated with *Candida albicans* and *Staphylococcus aureus* biofilms. They concluded that all disinfection treatments resulted in a statistically significant decrease in biofilm viability for *C. albicans* and *S. aureus*. Lowering biofilm viability via the use of water and neutral soap was considerably more

effective than soaking in disinfectants. Photomicrographs show that 4% chlorhexidine transformed the polymer's interface.[2]

14. **Smriti Balaji et al (2016)** analyzed the effectiveness of 4 different disinfectants in disinfecting acrylic resins. Chlorhexidine digluconate is found to show good disinfecting ability when compared to vinegar. Percentage reduction in CFU seen with vinegar is less than 15% against *C.albicans* as well as *S.mutans.*[54]

15. **Juliana Barchelli Pinheiro et al (2017)** evaluated the effectiveness of 0.12% chlorhexidine gluconate, 10% Ricinus communis solutions for reducing CFU in *C.glabrata, S. aureus,* on MDX 4-4210 and Bio-Skin, respectively. The most effective antimicrobial treatment, was immersion in 0.12% chlorhexidine gluconate, proceeded by mechanical brushing techniques.[19]

16. **Disegha, G. C. & Akani, N.P.(2017)** studied antifungal activity of different concentrations of *Mangifera indica* (mango) crude leaf extracts fresh cold aqueous (FCAE), fresh hot aqueous (FHAE) and fresh ethanol extract (FEE) while cold distilled water, hot distilled water and 95% ethanol were used as controls on some selected fungal species, namely, *Candida albians, Aspergillus flavus, Aspergillus fumigatus, and Aspergillus niger.* The study has revealed that *M. indica* extracts can be used as an antifungal agent in the treatment of infectious diseases implicating the test organisms.[26]

17. **A M Guiotti et al. (2018**) identified and examined the microbes in conjunctival secretions in anophthalmic cavities of users of ocular prostheses as well as on the prostheses used by them, associating them with the microbiota of the

contralateral eye. Irrespective of where the sample was from, it was proven that *S. aureus* and *S.epidermidis* were the most prevalent microorganisms.[36]

18. **Sophia Tetteh et al (2018)** used tea tree oil and manuka oil were used to disinfect test specimens from staphylococcus epidermidis. The hardness and elongation at the break of the test samples varied significantly over time, although the tear and tensile strength characteristics of the test samples did not.[7]

19. **Anna Serene Babu et al (2018)** in their research concluded regardless of the disinfectant utilised, chemical disinfection caused considerable changes in colour and surface roughness but did not influence Shore a hardness.[27]

20. **Martin Kalumbi et al. (2018)** investigated the effect of *Mangifera indica* leaf extract concentration and processing technique on their antibacterial activity against *S. aureus.* The processing method (mortar and pestle, blender, laboratory mill, and stone). The highest inhibitory zones were found at 50 mg/ml concentration and laboratory mill (14.5mm) (16.7mm).[35]

21. **Olasehinde G. I. et al (2018)** investigated the aqueous and ethanolic extracts of *Mangifera indica* leaf extract"s for their phytochemical make-up and antimicrobial properties. The water extract with MIC were more sensitive to *S. aureus.* Phytochemical screening showed the presence of active pharmacological components such as tannins, saponins, cardiac glycoside, flavonoid and alkaloids.[28]

22. **Ruth Bright Chirayath et al (2019**) tested the antibacterial efficacy of the methanol extract of mango leaf against *S. aureus* and it was found to be an effective anti-staphylococcal agent, non-mutagenic, and contains phytochemicals such as tannins, saponins, flavonoids, phenols, and coumarin.[29]

23. **Nayane de Lanes Gonçalves et al (2020)** examined the antibacterial efficacy of photodynamic treatment (PDT) utilising methylene blue (MB) and laser to eradicate *Staphylococcus aureus* biofilm that had formed on the surface of scleral acrylic resin. PDT presents itself as a viable option for cleaning ocular prosthetics.[15]

24. **Sahal G et al (2020)** stated that *Cymbopogon citratus* oil can be used as a antifungal and antibiofilm agent on silicone where *C. tropicalis* pose a serious risk for skin problems and could reduce the lifespan of the prosthesis..[17]

25. **Abdul-Ameer FM (2020)** concluded that after disinfection, the tear strength of all groups decreased, with the experimental group having the highest values. The tear strength and hardness of the HTV control and the hardness of the RTV experimental specimen were not affected by 10% *Salvadora persical*.[30]

26. **Bahare Salehi et al (2020)** reviewed the antioxidant, antibacterial, and anticancer activity are among the various bioactive effects of *Anacardium* species that have drawn the most attention. Hydroethanolic extracts of *A. occidentale* demonstrated positive results against *S.aureus*[14].

27. **Victoria Cardenas et al. (2020)** assessed the antibacterial activity of four dilutions of *Mangifera indica* L. hydroalcoholic extract (MHE) and ethanol extract (MEE) on cultures *of S. aureus* ATCC 6538TM in comparison to the

positive control (chlorhexidine 0.12%) and the negative control (alcohol 96°). Mango leaf extracts have strong antibacterial properties, demonstrating that 100% MHE is more efficient and demonstrating the existence of active components in medicinal plants.[31]

28. **Amalia Moreno et al (2020)** assessed how disinfectants affected the biofilm of *Staphylococcus aureus and Staphylococcus epidermidis* that had developed on the acrylic surface of ocular prostheses. When compared to their control groups, 0.5% CHX10, 2% CHX10, 4% CHX10, and Efeeredent15 significantly reduced the number of CFU/mL for both bacteria.[33]

29. **Ashok Kumar et al (2021)** stated that it has been established that both silicone and acrylic resin may harbour bacteria when used to construct maxillofacial replacements, but silicone is more porous and so more prone to microbial adherence.[16]

30. **Mariana Neves de Azevedo et al (2021)** concluded that *S. aureus* biofilm was successfully removed from samples of N1 acrylic resin specifically designed for ocular prosthesis and maxillofacial elastomer by immersion in a 10% green propolis alcohol solution for 5 minutes. [8]

31. **Shamsiahwati Mat-Rani (2021)** et al assesses the effectiveness of lemongrass essential oil in eliminating *Candida albicans* biofilm that has already been developed on the maxillofacial silicone specimens. A fungicidal impact was seen in the *C. albicans* biofilm that had already been developed on silicone discs made by various silicone manufacturers after exposure to lemongrass essential oil. The

oil's fungicidal activity against the developed fungal biofilm was also dose-dependent.[34]

32. **Malateaux G et al (2021)** used a variety of treatments, including distilled water, 0.12% chlorhexidine, UV-C LED light, and dimethyl sulfoxide (DMSO) as the adverse control and came to a conclusion that medical silicone used in facial prosthetics had a lower in vitro microbial cell survival after being exposed to UV-C LED light, confirming the original colour stability.[42]

33. **Karl M Lyons et al (2022)** noted that it is crucial to reduce the risk of systemic and local infections in immunocompromised cancer patients who have maxillary abnormalities. The function of saliva in microbial attachment to obturator materials and to create materials with longer lifespans and surface properties that encourage less microbial adhesion than other materials is required.[32]

MATERIAL AND METHODOLOGY

SOURCE OF DATA:

This in-vitro study was conducted in-

KAHER's

- Central research facility, KLE Shri BMK Ayurveda Mahavidyalaya, Belagavi. (authentication, preparation of extract and preparation of disinfecting solution)
- Department of Prosthodontics and Crown and Bridge, KLE Vishwanath Katti Institute of Dental Science, Belagavi. (fabrication of maxillofacial silicone material disc specimens)
- Department of Microbiology, Jawaharlal Nehru Medical College, Belagavi. (microbial analysis)

PERMISSIONS TAKEN:

Permissions were taken from –

- Ethical committee (Annexure-I)

KAHER's

- Central research facility, KLE Shri BMK Ayurveda Mahavidyalaya, Belagavi.
- Department of Prosthodontics and Crown and Bridge, KLE Vishwanath Katti Institute of Dental Science, Belagavi.
- Department of Microbiology, Jawaharlal Nehru Medical College, Belagavi.

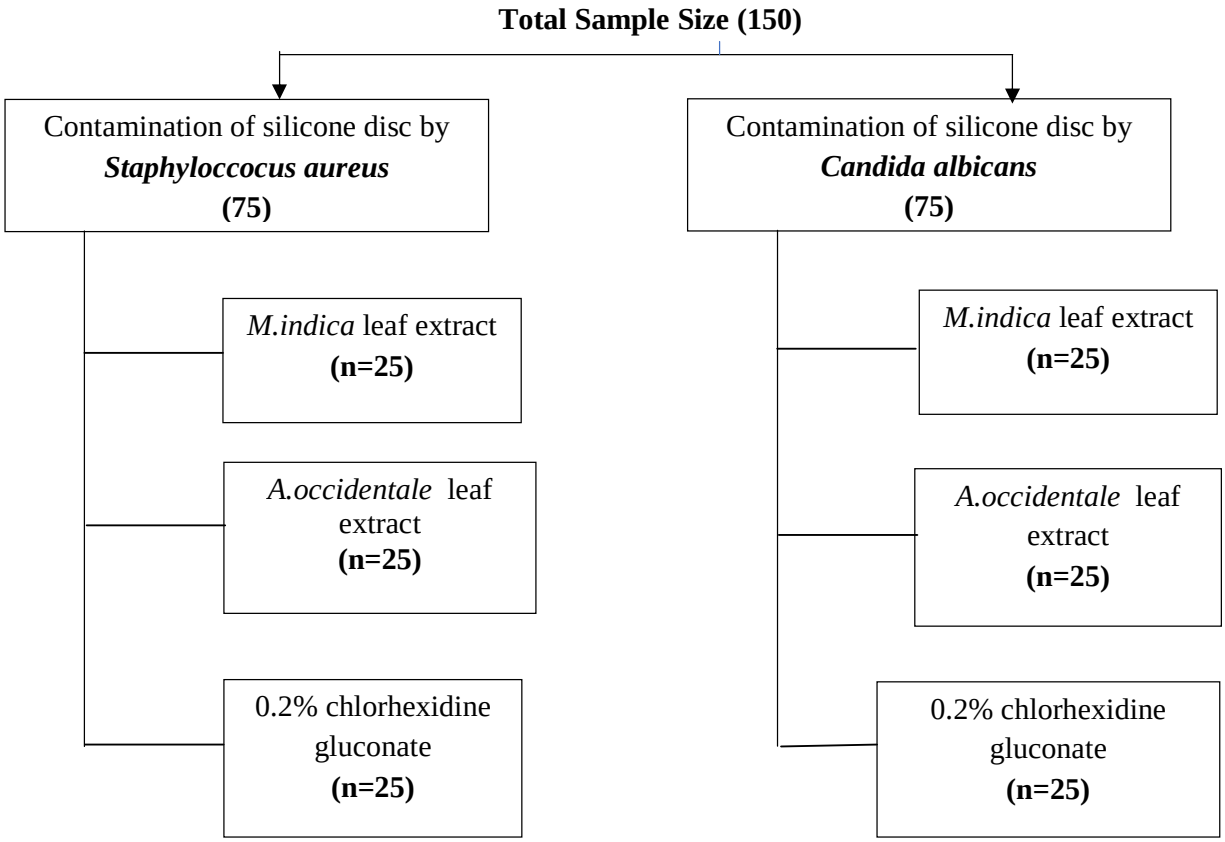
SAMPLE SIZE ESTIMATION:

$$n = \frac{2S^2(Z_{1-\alpha/2}+Z_{1-\beta})^2}{d^2}$$

n=25 samples in each group

Total groups=6; Total sample size=150

- Where, standard deviation S_1= 0.010 ; S_2=0.030
- $Z_{1-\alpha/2}$ = α-error at 5% = 1.960
- $Z_{1-\beta}$ = power of test at 85% = 1.037
- d = mean difference = 0.019



INCLUSION CRITERIA:

- Disc-shaped specimens of the size-diameter-5mm;^{2}-thickness - 2mm^2
- Completely cured specimens.
- Specimens without visual surface defects, deformities and gross irregularities.

EXCLUSION CRITERIA:

- Specimens with visual surface defects, deformities and gross irregularities.
- Specimens with inaccurate dimensions.

STUDY DESIGN

- An In Vitro Comparative Study

MATERIALS AND ARMAMENTARIUM

MATERIALS

- Dried *Mangifera indica* leaves (mango)
- Dried *Anacardium occidentale* leaves (cashew)
- Brain heart infusion broth
- Blood agar
- Whatman filter paper
- Distilled water

Table 1: Materials used in the study.

MATERIALS	DESCRIPTION	MANUFACTURER
Silastic MDX4-4210	BioMedical Grade Elastomer	Dow Corning Corp,USA
Hexidine	0.2% Chlorhexidine gluconate	ICPA Health Products LTD
Ethyl alcohol	Ethanol , absolute	Changshu Hongsheng Fine Chemical Co., Ltd, Jiangsu Province
Candida albicans fungal strain	ATCC 10231	
Staphylococcus aureus bacterial strain	ATCC 25923	

ARMAMENTARIUM

- Vacuum mixing machine
- Mortar and pestle
- Soxhlet extraction apparatus
- Weighing machine
- Incubator
- Metal mould (5mmx2mm)
- BP blade and handle
- Scissors
- Spatula
- Beakers
- Dappen dish
- Petri dishes
- Test tubes
- Sterile containers
- Inoculation loop

Table 2: Armamentarium used in the study

MATERIALS	DESCRIPTION	MANUFACTURER
Incubator	Sr no. ZBCT-08444	Remi elektrotechnik ltd India
Vacuum mixing machine	Model no. 26090	Easymix Bego Wilhelm – Herbst -str
Soxhlet extraction apparatus	431/2 29/32 24/29 100	Jain scientific works product

METHODOLOGY IN FLOWCHART:

A) Preparation of *Mangifera indica* and *Anacardium occidentale* leaf extracts.

↓

B) Determination of Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration of *Mangifera indica* and *Anacardium occidentale* leaf extracts against *Staphylococcus aureus* and *Candida albicans.*

↓

C) Preparation of disinfecting solutions.

↓

D) Fabrication of maxillofacial silicone material discs.

↓

E) Contamination of maxillofacial silicone material discs with *Staphylococcus aureus and Candida albicans.*

↓

F) Disinfection of maxillofacial silicone material discs with leaf extracts of *Mangifera indica, Anacardium occidentale* and 0.2% chlorhexidine gluconate.

↓

Pre and post disinfection **Colony Forming Units** were evaluated.

METHODOLOGY IN DETAIL

A) Preparation of *Mangifera indica* and *Anacardium occidentale* leaf extracts.

Fresh leaves were collected from an infestation free *Mangifera indica* and *Anacardium occidentale* tree in the region of Thivim, Goa, India. The leaves authenticated by the Central research facility of Shri BMK Ayurveda Mahavidyalaya, Belagavi (Annexure II). Leaves were washed two to three times under running water and made free from contaminant. The leaves were shade dried for a period of 15 days and then made into a medium-coarse powder by using a mortar and pestle and stored in an airtight container until extract is made. A weighed quantity of the leaf powders was extracted with 99% ethyl alcohol as solvent by the Soxhlet extraction method. (Fig 5) The extracts were filtered and concentrated using a water bath till all the solvent evaporates. The extracts were stored in a refrigerator until further use. (Fig 6)

B) Determination of Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration of *Mangifera indica* and *Anacardium occidentale* leaf extracts against *Staphylococcus aureus* and *Candida albicans*.

By using the broth dilution procedure, the Minimum Inhibitory Concentration (MIC) of the leaf extracts was determined Suspension of *S. aureus* and *C. albicans* were added to BHI broth to which graded amount of freshly prepared leaf extracts were added. Series of dilution were prepared containing same volume of media inoculated.

One test tube was left without extract, to serve as positive control and one without organism to serve negative control. Final volume per tube was 1ml which includes 10 microliters of organism in each tube and different concentration of extract and the BHI broth.

The solutions will be incubated at 37^0C for 24 hours. MIC was taken as the least concentration of extracts that showed no observable growth (no turbidity). Samples from the tube that showed no visible bacterial growth during MIC determination was inoculated on separate agar plates and incubated at 37^0C for 24 hours. The least concentration of the extracts that showed no colonies on the medium after incubation period was regarded as Minimum Bactericidal/Fungicidal concentration (MBC/MFC).The value of MBC of *Mangifera indica* was 75 microliters against *Staphylococcus aureus* and *Candida albicans* and the value of MBC of *Anacardium occidentale* against *Staphylococcus aureus* was 25 microliters and *Candida albicans* was 100 microliters. (Annexure III)

C) Preparation of Disinfecting Solutions.

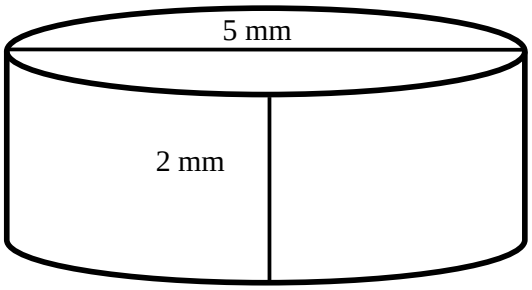
To prepare the disinfecting solution, the highest effective concentration was chosen of the Minimal Bactericidal/Fungicidal Concentration values of freshly prepared extracts.

The solutions were prepared by mixing the effective concentration values of the extracts in non-ionized distilled water until a homogenous solution is obtained and were stored in sterile conditions.

D) Fabrication of Maxillofacial Silicone Material Discs.[2]

Processing of the maxillofacial silicone elastomer (Silastic MDX4-4210) discs was done using a metal mould of diameter- 5 mm, thickness- 2 mm.[2] (Fig 9) In the Vacuum mixing machine **(**Easymix Bego Wilhelm – Herbst –str) silicone catalyst was combined with base paste in accordance with manufacturer recommendations. Following its mixing, the silicone was inserted into the mould matrix and its surface was flattened with a spatula at the matrix's edge to a thickness of 2 mm. To finish the

polymerization process, the matrix containing the silicone samples was left in the mould for three days with the exterior surface exposed to the room environment. After this time, the specimens were taken out of the mould using a fine-pointed tool, and extra material and imperfections were trimmed out using thin, curved scissors.[2] A uniform surface smoothness will be maintained for cell attachment, but the surface won't be finished or polished, simulating what is typically done in processing these prostheses, in which frequently only the edges are trimmed.[2]



E) Contamination of maxillofacial silicone material discs with *Staphylococcus aureus and Candida albicans.*

The sterilised silicone disc was placed in a test tube (Fig 9)

↓

Contamination with *Staphylococcus aureus/Candida albicans*
1 mL of BHI broth +2 µL of standardized inoculum ($1x10^6$cells) and incubate for 24 hours at 37^0C aerobically

↓

Disc was rolled on blood agar in a 1cmx1cm area (Fig 12)

↓ ↓

The disc was placed back in the above solution incubated for 2 hours at 37^0C aerobically

↓

Blood agar was incubated for 24 hours at 37^0C aerobically and CFU was counted for baseline microbial study

Pre disinfection CFUs were evaluated. Discs were subjected to disinfection protocols

F) Disinfection of maxillofacial silicone material discs.

The contaminated discs with *Staphylococcus aureus* and *Candida albicans* were disinfected for 10 minutes by the following protocols. (Fig 14)

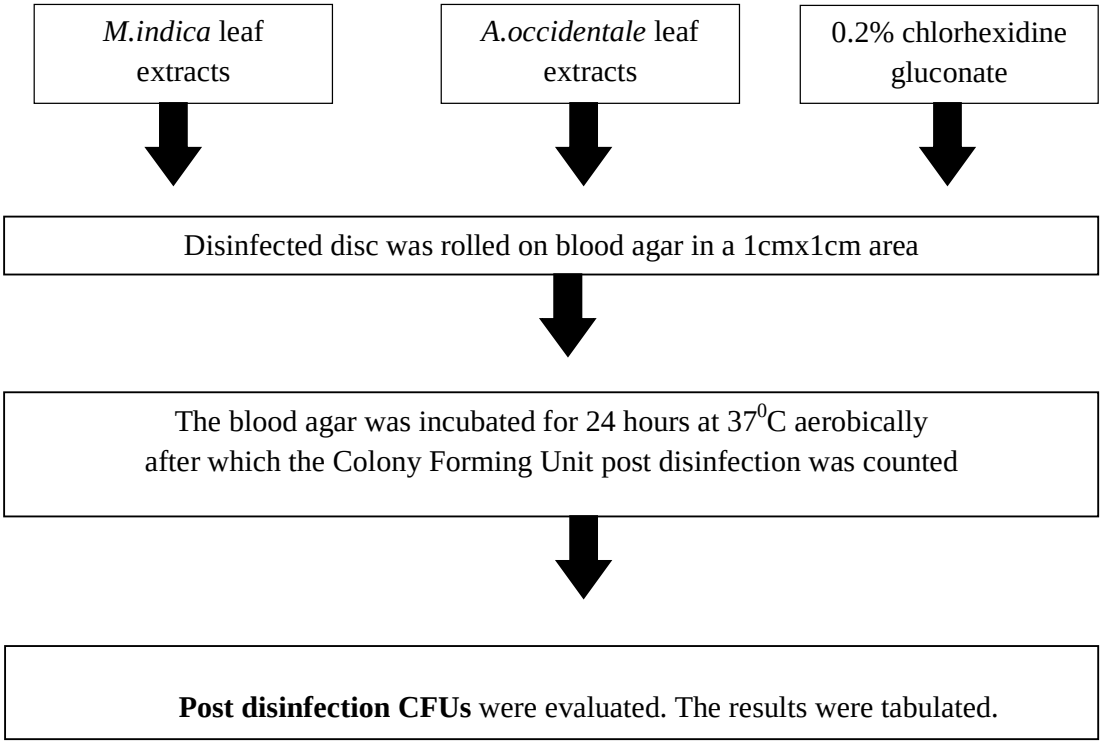


Figure 1- Dried leaves of *Mangifera indica*

Figure 2 - Dried leaves of *Anacardium occidentale*

Figure 3 - Mortar and pestle

Figure 4 - *Mangifera indica* and *Anacardium occidentale* leaves powder

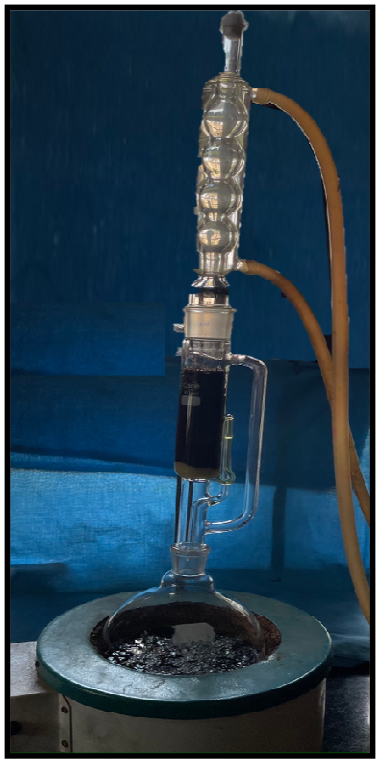


Figure 5 - Soxhlet extraction apparatus

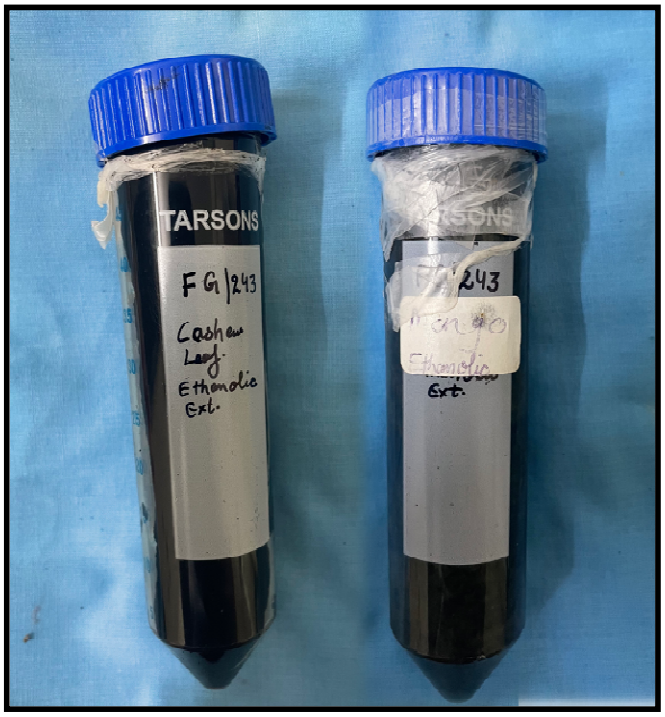


Figure 6 - Ethanolic extracts of *Anacardium occidentale* leaf extracts and *Mangifera indica* leaf extract

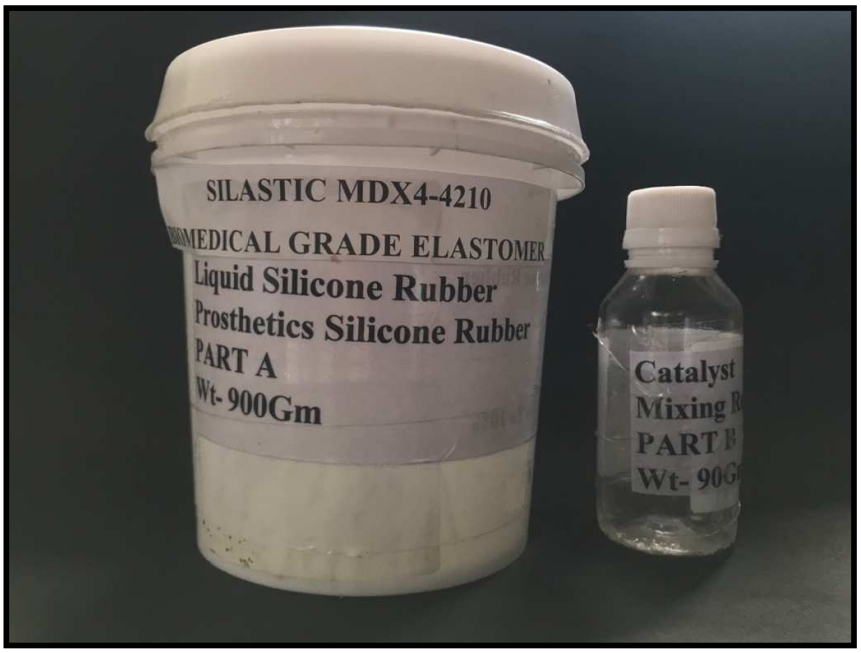


Figure 7 - Silastic MDX 4-4210

Figure 8 - Vacuum mixing machine

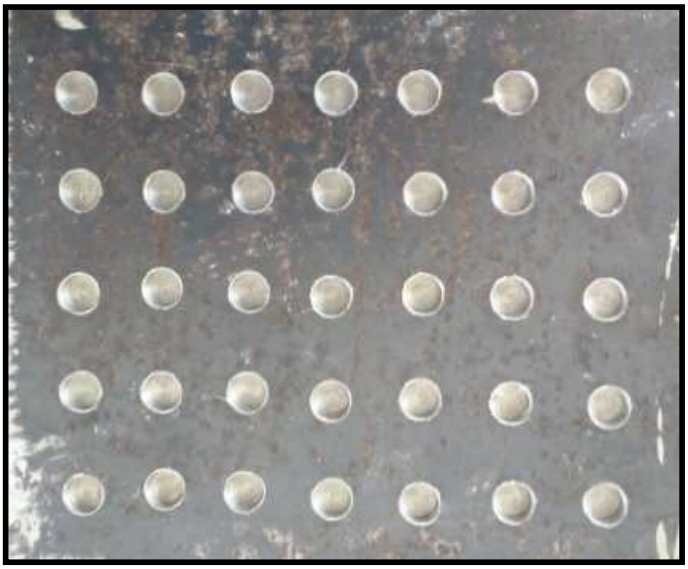


Figure 9 - Metal mould

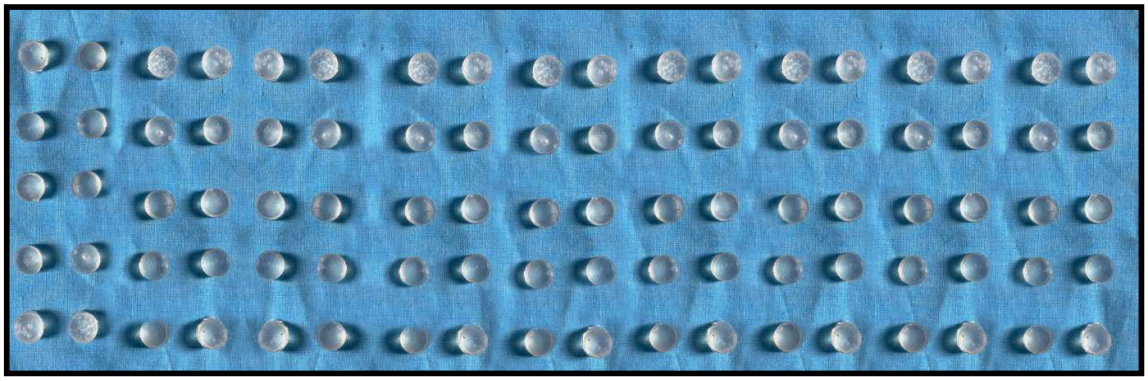


Figure 10 - Silicone samples

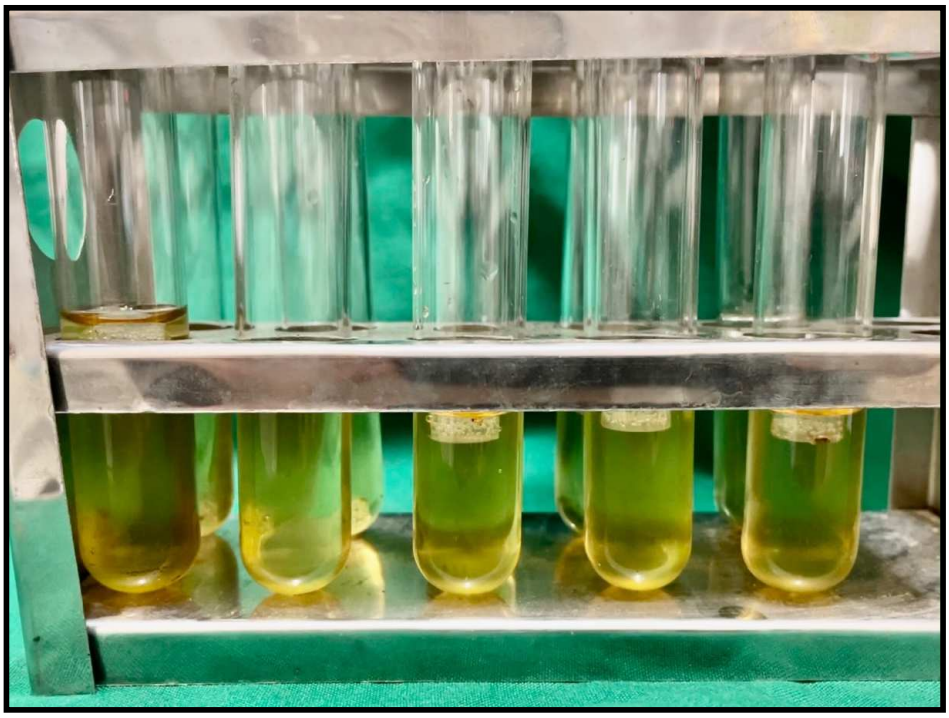


Figure 11 - Silicone disc placed in test tube containing BHI broth and organism for contamination of discs

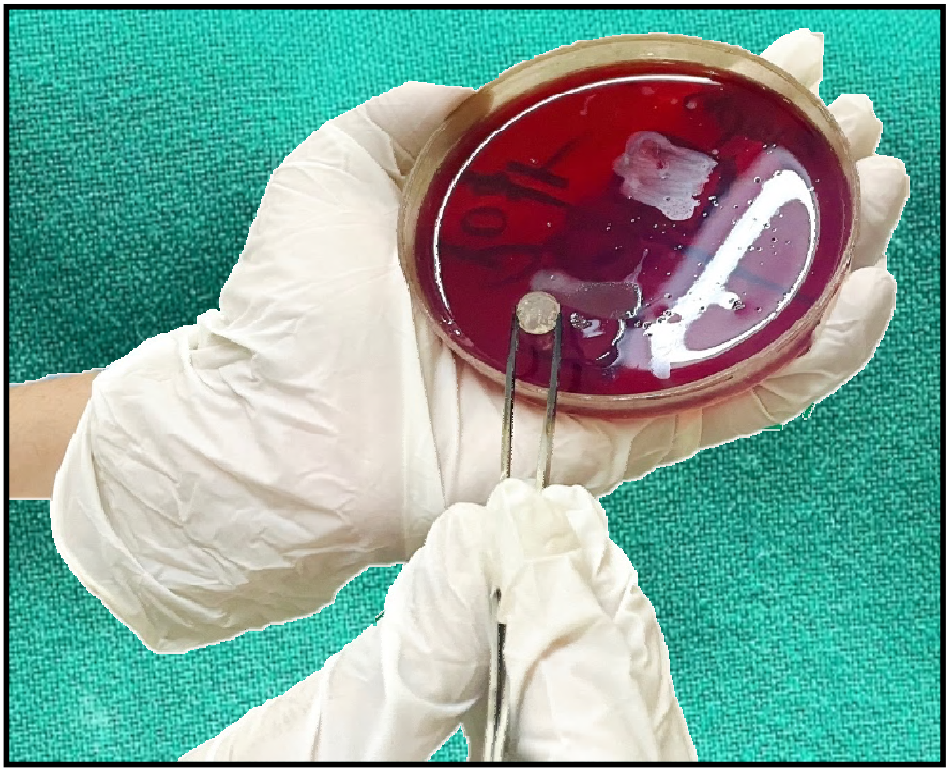


Figure 12 - Silicone disc rolled in a 1cmx1cm square area onto the blood agar.

Figure 13 - Hexidine – 0.2% CHX

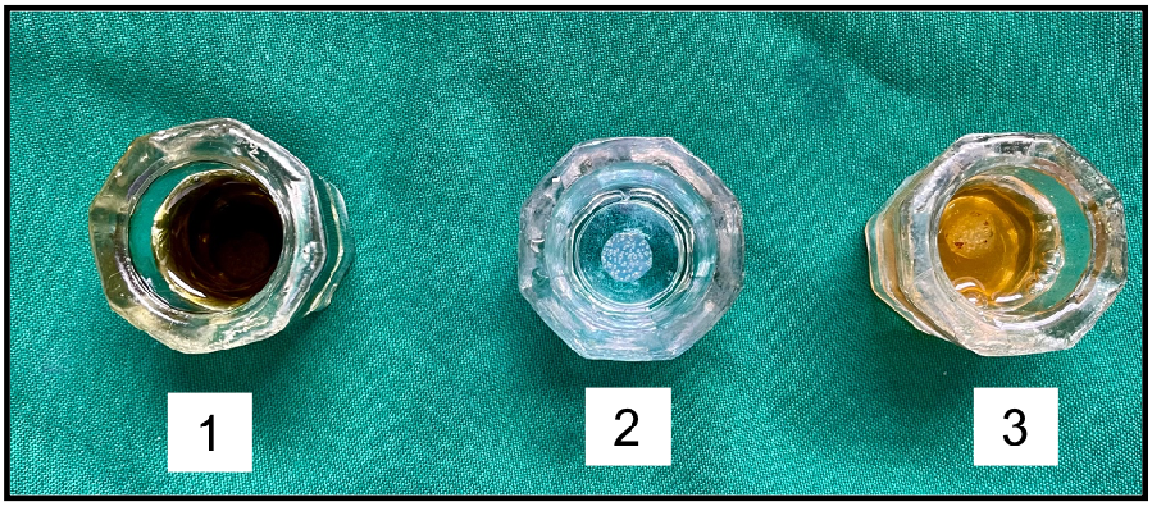


Figure 14 - Decontamination of silicone disc 1- *A. occidentale* leaf extract, 2- CHX, 3- *M. indica* leaf extract,

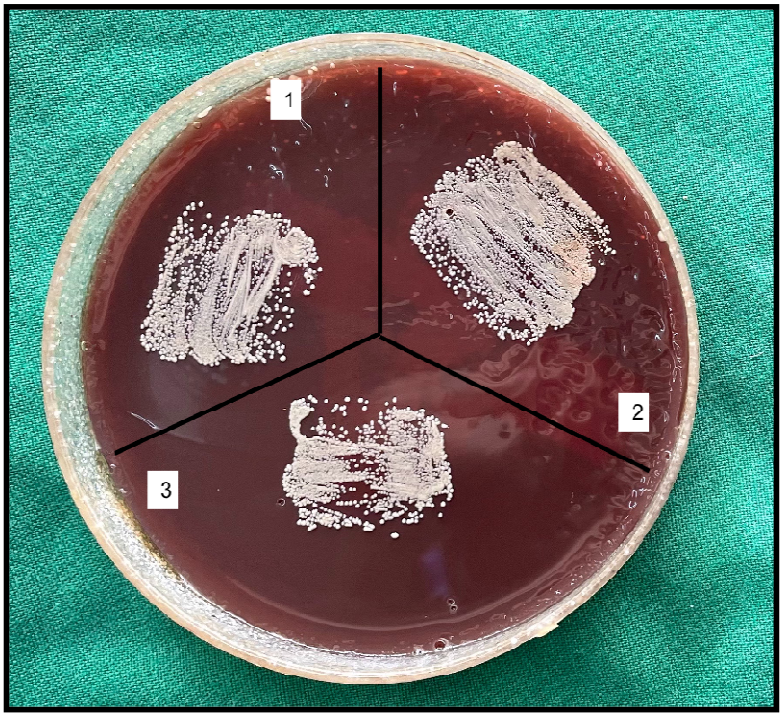


Figure 15 - *Staphylococcus aureus* colonization on blood agar pre-disinfection

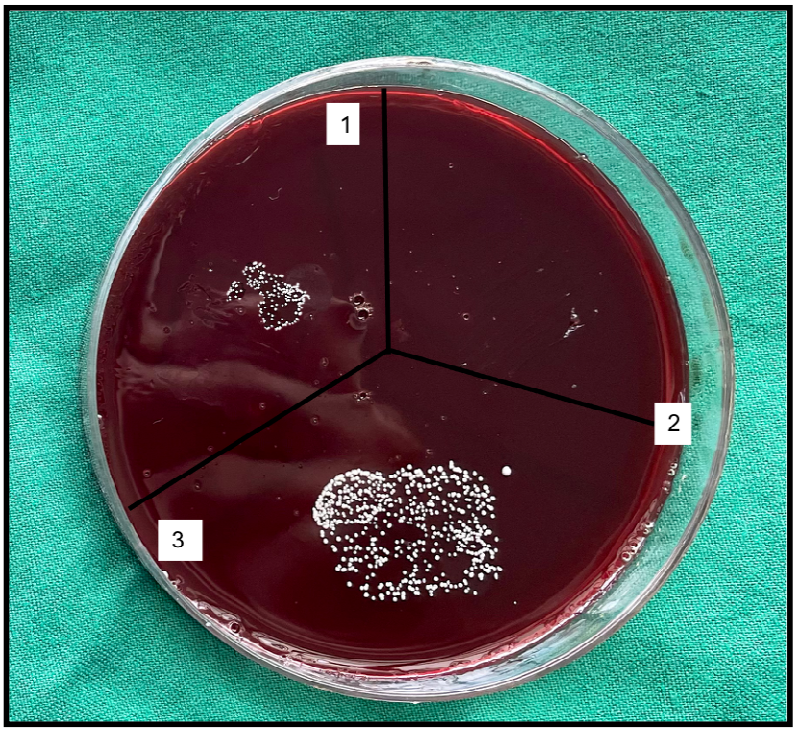


Figure 16 - *Staphylocoocus aureus* colonization on blood agar post-disinfection. 1- *A. occidentale* leaf extract, 2- CHX, 3- *M. indica* leaf extract

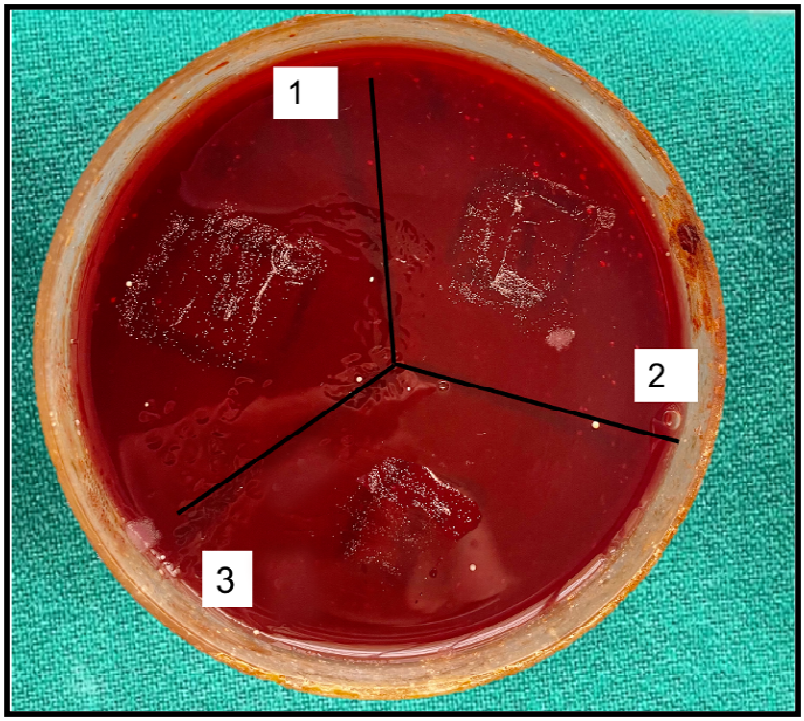


Figure 17 - *Candida albicans* colonization on blood agar pre-disinfection

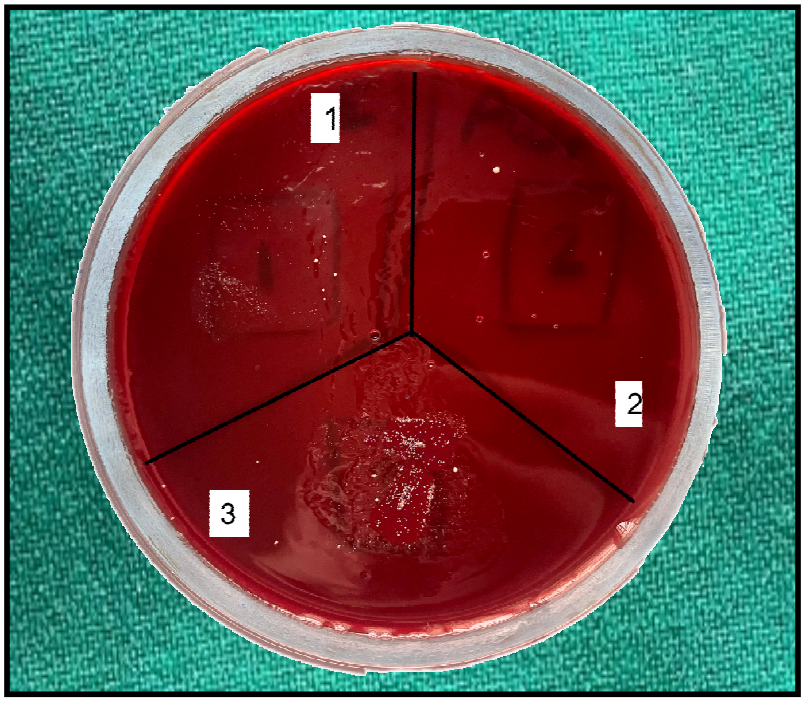


Figure 18 - *Candida albicans* colonization on blood agar post-disinfection. 1- *A. occidentale* leaf extract, 2- CHX, 3- *M. indica* leaf extract

RESULTS

The colony forming units/ml of the respective microbial count was converted to log CFU and tabulated to evaluate and compare the leaf extracts of *Mangifera indica*, *Anacardium occidentale* and 0.2% chlorhexidine on disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans.*

The results were tabulated and analysed and subjected to statistical analysis. Descriptive statistical measures such as Mean, Standard deviation were calculated for all the study groups pre-disinfection and post-disinfection for maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans.*(Table no. 3 and 4)

The mean and standard deviation of the pre-disinfection of *S. aureus* was 8.12(0.008), 8.13(0.008), 8.13(0.008) in the 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* groups respectively. Mean and standard deviation of the post-disinfection was 0.48(0.59), 1.95(0.73), 0.87(1.03) in the 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* groups respectively. The dependent t-test was applied to evaluate pre disinfection v/s post-disinfection of *Staphylococcus aureus in* each group, which showed statistically significant (p=0.0001) in all the groups. (Table no 5, graph no 1).

A one-way ANOVA was performed to compare the three groups i.e 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* on pre- and post-disinfection of *Staphylococcus aureus.* The test revealed that there was no

statistically significant (p=0.9401) in the pre disinfection, whereas in the post disinfection it was statistically significant between the groups (p=0.0001) (Table 6.)

Pair-wise comparison of 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidenta*le with pre and post-disinfection log CFU counts of *Staphylococcus aureus was done* by Tukeys multiple posthoc procedure, it was found that there is statistically significance between 0.2% chlorhexidine and *Mangifera indica* (p=0.001)*; Mangifera indica and Anacardium occidenta*le (p=0.001). No statistically significant were found between significant 0.2% chlorhexidine and Anacardium occidentale (p=0.1988). (Table no 7)

The difference of the log CFU from pre-disinfection to post disinfection of *Staphylococcus aureus* was tabulated where the mean difference for chlorhexidine was 7.64, *Mangifera indica was 6.18* and 7.26 for *Anacardium occidenta*le. (Table no 8)

A one way ANOVA was performed to compare the difference of the log CFU from pre-disinfection to post disinfection of *Staphylococcus aureus in* the three groups, which showed that there was statistical significance (p= 0.0001) between the groups. (Table no 9)

Pair wise comparison of 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidental* of the difference of the log CFU from pre-disinfection to post disinfection of *Staphylococcus aureus was done* by Tukeys multiple posthoc procedure, found statistically significant result between 0.2% chlorhexidine and *Mangifera indica (p=0.001); Mangifera indica and Anacardium occidenta*le

(p=0.001). No statistically significant results were found between significant 0.2% chlorhexidine and *Anacardium occidenta*le (p=0.2121). (Table no 10)

The mean and standard deviation of the pre-disinfection of *C.albicans* was 6.02(0.06), 6.04(0.07), 6.03(0.07) in the 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* groups respectively. Mean and standard deviation of the post-disinfection was 0.22(0.55), 1.91(0.53), 1.06(0.77) in the 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* groups respectively. The dependent t-test was applied to evaluate pre disinfection v/s post-disinfection of *Candida albicans* in each group which showed statistically significant result (p=0.0001) in all the groups. (Table no 11)

A one-way ANOVA was performed to compare the three groups i.e. 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* on pre- and post-disinfection of *Candida albicans.* The test revealed that there was no statistically significant (p=0.7506) in the pre disinfection, whereas in the post disinfection it was *statistically significant* between the groups (p=0.0001) (Table no 12)

Pair wise comparison of 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidental* of the difference of the log CFU from pre-disinfection to post disinfection of *Candida albicans was done* by Tukeys multiple posthoc procedure, found that there was statistical significance between all the three groups; 0.2% chlorhexidine v/s *Mangifera indica (p=0.001); Mangifera indica v/s Anacardium occidenta*l*e (p=0.001) and* 0.2% chlorhexidine v/s *Anacardium occidenta*l*e* (p=0.001). (Table no 13)

The difference of the log CFU from pre-disinfection to post disinfection of *Candida albicans* was tabulated where the mean difference for chlorhexidine was 5.80, *Mangifera indica was 4.12* and 4.96 for *Anacardium occidenta*le. (Table no 14)

A one way ANOVA was performed to compare the difference of the log CFU from pre-disinfection to post-disinfection of *Candida albicans in* the three groups, which showed that there was statistical significance (p= 0.0001) between the groups. (Table no 15)

Pair wise comparison of 0.2% chlorhexidine, *Mangifera indica* and *Anacardium occidentale* of the difference of the log CFU from pre-disinfection to post-disinfection of *Candida albicans was done* by Tukeys multiple posthoc procedure, found statistically significant result between all the three groups; 0.2% chlorhexidine v/s *Mangifera indica (p=0.001); Mangifera indica v/s Anacardium occidenta*l*e (p=0.001) and* 0.2% chlorhexidine v/s *Anacardium occidenta*l*e* (p=0.001). (Table no 16)

Table no. 3: Descriptive statistics of pre-disinfection and post-disinfection log CFU counts of *S. aureus in* three groups

Groups	Pre-disinfection				Post-disinfection			
	Mean	SD	95% CI		Mean	SD	95% CI	
			Lower	Upper			Lower	Upper
CHX	8.12	0.08	8.09	8.15	0.48	0.59	0.24	0.72
Mangifera indica	8.13	0.08	8.09	8.16	1.95	0.73	1.65	2.25
Anacardium occidentale	8.13	0.08	8.09	8.16	0.87	1.03	0.45	1.29
Total	8.12	0.08	8.11	8.14	1.10	1.01	0.87	1.33

Table no. 4: Descriptive statistics of pre-disinfection and post-disinfection log CFU counts of *C .albicans in* three groups.

Groups	Pre-disinfection				Post-disinfection			
	Mean	SD	95% CI		Mean	SD	95% CI	
			Lower	Upper			Lower	Upper
CHX	6.02	0.06	6.00	6.05	0.22	0.55	-0.01	0.44
Mangifera indica	6.04	0.07	6.01	6.06	1.91	0.53	1.69	2.13
Anacardium occidentale	6.03	0.07	6.00	6.06	1.06	0.77	0.75	1.38
Total	6.03	0.06	6.01	6.04	1.07	0.93	0.85	1.28

Table no. 5: Comparison of pre- disinfection and post-disinfection log CFU counts of *S .aureus in* Chlorhexidine, *Mangifera indica and Anacardium occidentale*) by dependent t test

Groups	CHX		*Mangifera indica*		*Anacardium occidentale*	
	Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection
Mean	8.12	0.48	8.13	1.95	8.13	0.87
SD	0.08	0.59	0.08	0.73	0.08	1.03
Mean Diff	7.64		6.18		7.26	
SD diff	0.57		0.75		1.03	
% of change	94.13		76.01		89.29	
t value	67.34		41.3099		35.2106	
p value	**0.001***		**0.001***		**0.001***	

Table no 6: Comparison of in three groups Chlorhexidine, *Mangifera indica and Anacardium occidentale*) with pre- disinfection and post- disinfection log CFU counts of *S. aureus* by one way ANOVA

Times	Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Pre-disinfection	Between extracts	2	0.0008	0.0004	0.0619	0.9401
	Within extracts	72	0.4812	0.0067		
	Total	74	0.4821			
Post-disinfection	Between extracts	2	29.0852	14.5426	22.5894	**0.0001***
	Within extracts	72	46.3521	0.6438		
	Total	74	75.4373			

*$p<0.05$

Table no 7 Pair wise comparison three groups Chlorhexidine, *Mangifera indica and Anacardium occidentale*) with pre-disinfection and post-disinfection log CFU counts of *S. aureus* by Tukeys multiple posthoc procedures

Time points	Extracts	CHX	*Mangifera indica*	*Anacardium occidentale*
Pre-disinfection	Mean	8.12	8.13	8.13
	SD	0.08	0.08	0.08
	Mangifera indica	P=0.9503	-	
	Anacardium occidentale	P=0.9503	P=1.0000	-
Post-disinfection	Mean	0.48	1.95	0.87
	SD	0.59	0.73	1.03
	Mangifera indica	**P=0.0001***	-	
	Anacardium occidentale	P=0.1988	**P=0.0001***	-

*p<0.05

Table no 8: Descriptive statistics of changes in log CFU counts of *S. aureus in* three groups

Groups	Mean	SD	SE	95% CI	
				Lower	Upper
CHX	7.64	0.57	0.11	7.41	7.88
Mangifera indica	6.18	0.75	0.15	5.87	6.49
Anacardium occidentale	7.26	1.03	0.21	6.83	7.68
Total	7.03	1.01	0.12	6.79	7.26

Table no 9: Comparison of changes in log CFU counts of *S. aureus in* three groups from pre-disinfection to post-disinfection by one way ANOVA

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between extracts	2	28.8668	14.4334	22.2883	**0.0001***
Within extracts	72	46.6255	0.6476		
Total	74	75.4923			

*p<0.05

Table no 10: Pair wise comparison of three groups with changes in log CFU counts of *S. aureus* from pre-disinfection to post-disinfection by Tukeys multiple posthoc procedures

Groups	CHX	*Mangifera indica*	*Anacardium occidentale*
Mean	7.64	6.18	7.26
SD	0.57	0.75	1.03
Mangifera indica	**P=0.0001***	**-**	
Anacardium occidentale	P=0.2121	**P=0.0001***	-

*p<0.05

Table no 11. Comparison of pre- disinfection and post-disinfection log CFU counts of *C. albicans* in three groups Chlorhexidine, *Mangifera indica and Anacardium occidentale*) by dependent t test

Groups	CHX		*Mangifera indica*		*Anacardium occidentale*	
	pre	post	pre	post	pre	post
Mean	6.0	0.22	6.04	1.91	6.003	1.0
SD	0.06	0.55	0.07	0.53	0.07	0.77
Mean Diff	5.80		4.12		4.96	
SD diff	0.56		0.51		0.76	
% of change	96.38		68.31		82.33	
t value	52.0056		40.2926		32.7426	
p value	**0.001***		**0.001***		**0.001***	

*p<0.05

Table no 12: Comparison of in three groups Chlorhexidine, *Mangifera indica and Anacardium occidentale*) with pre- disinfection and post- disinfection log CFU counts *C. albicans* by one way ANOVA

Times	Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Pre-disinfection	Between extracts	2	0.0025	0.0012	0.2880	0.7506
	Within extracts	72	0.3101	0.0043		
	Total	74	0.3126			
Post-disinfection	Between extracts	2	35.9053	17.9527	45.7674	**0.0001***
	Within extracts	72	28.2426	0.3923		
	Total	74	64.1480			

*$p<0.05$

Table no 13: Pair wise comparison three groups Chlorhexidine, Mangifera *indica and Anacardium occidentale*) with pre-disinfection and post-disinfection log CFU counts of *C. albicans* by Tukeys multiple posthoc procedures

Time points	Groups	CHX	*Mangifera indica*	*Anacardium occidentale*
Pre-disinfection	Mean	6.02	6.04	6.03
	SD	0.06	0.07	0.07
	Mangifera indica	P=0.7293	-	
	Anacardium occidentale	P=0.9239	P=0.9239	-
Post-disinfection	Mean	0.22	1.91	1.06
	SD	0.55	0.53	0.77
	Mangifera indica	**P=0.0001***	-	
	Anacardium occidentale	**P=0.0001***	**P=0.0001***	-

*p<0.05

Table no 14: Descriptive statistics of changes in log CFU counts of *C albicans from disinfection in* three groups

Groups	Mean	SD	SE	95% CI	
				Lower	Upper
CHX	5.80	0.56	0.11	5.57	6.03
Mangifera indica	4.12	0.51	0.10	3.91	4.33
Anacardium occidentale	4.96	0.76	0.15	4.65	5.28
Total	4.96	0.92	0.11	4.75	5.18

Table no 15: Comparison of changes in log CFU counts of *C. albicans in* three groups from pre-disinfection to post-disinfection by one-way ANOVA

Sources of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F-value	p-value
Between extracts	2	35.3109	17.6555	46.1608	**0.0001***
Within extracts	72	27.5384	0.3825		
Total	74	62.8493			

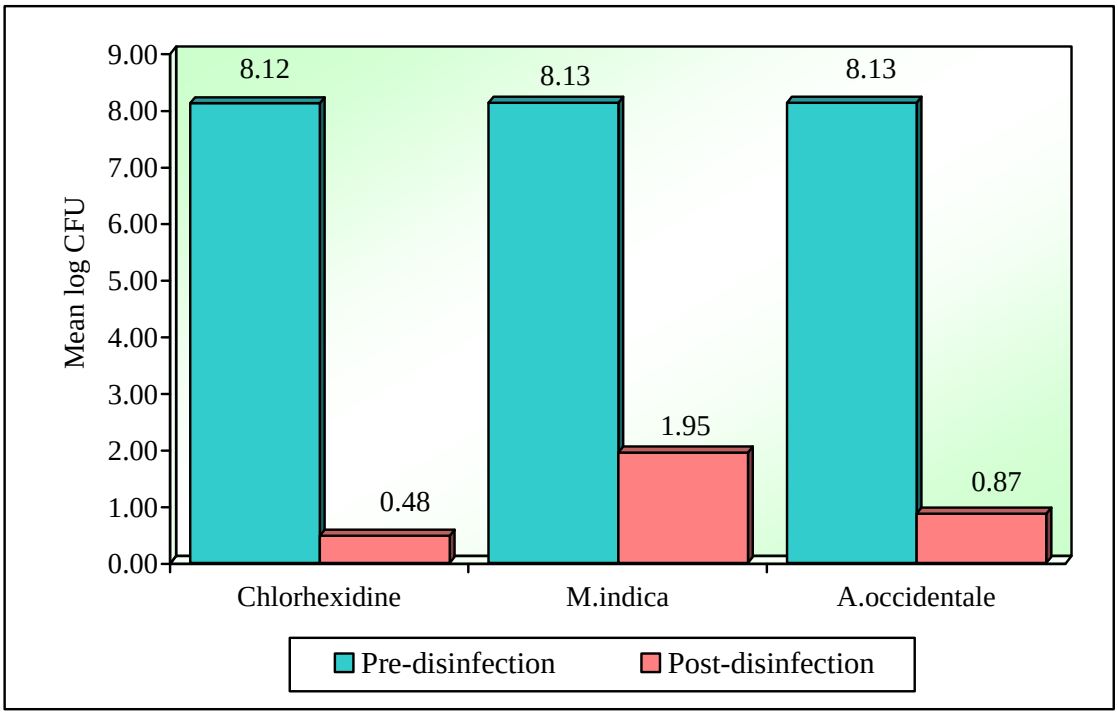
*p<0.05

Table no 16: Pair wise comparison of three groups with changes in log CFU counts of *C. albicans* from pre-disinfection to post-disinfection by Tukeys multiple posthoc procedures

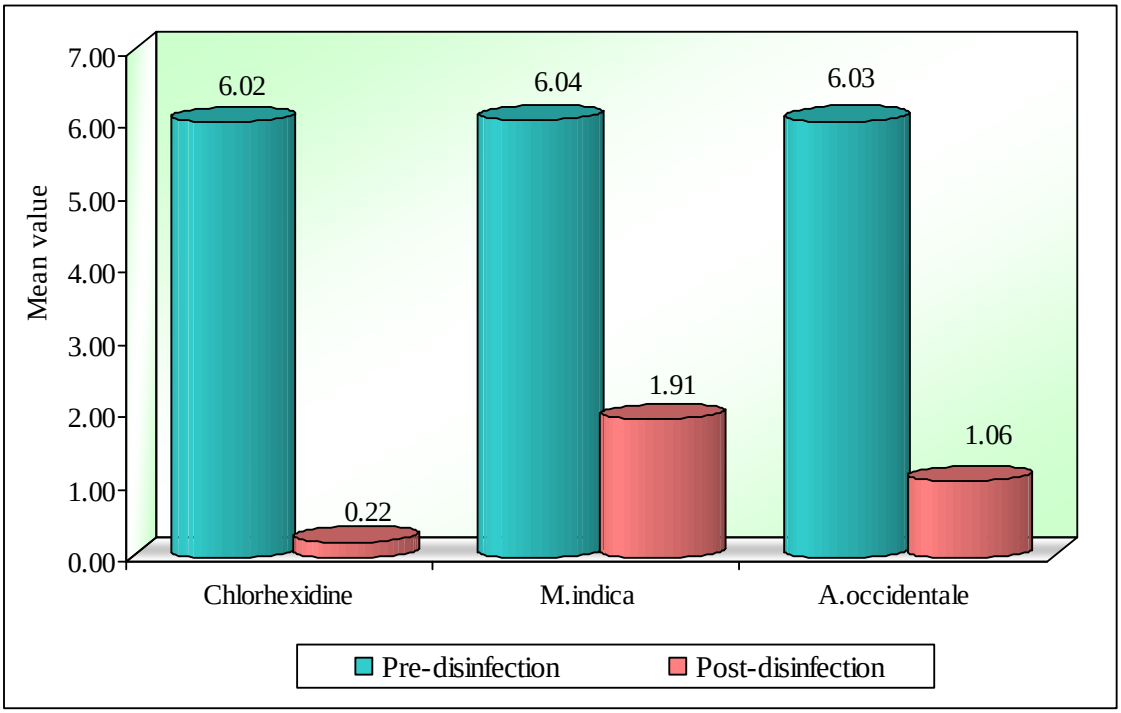
Groups	CHX	*Mangifera indica*	*Anacardium occidentale*
Mean	5.80	4.12	4.96
SD	0.56	0.51	0.76
Mangifera indica	**P=0.0001***	-	
Anacardium occidentale	**P=0.0001***	**P=0.0001***	-

*p<0.05

Graph no 1: Comparison of pre- disinfection and post-disinfection log CFU counts of *S. aureus* in Chlorhexidine, *Mangifera indica* and *Anacardium occidentale*.



Graph no 2 : Comparison of pre- disinfection and post-disinfection log CFU counts of *C. albicans* in three groups Chlorhexidine, Mangifera *indica and* *Anacardium occidentale*.



DISCUSSION

Maxillofacial prostheses offer a non-invasive, low-cost, and pleasant appearance, allowing patients to continue their normal lives.[5] Due to its adaptability, patient comfort, skin-like smoothness, and capacity for both intrinsic and extrinsic colour matching, silicone is the material of choice. Polymethylmethacrylate, polyurethanes, latex, and silicone elastomers are among the materials used to make external prosthesis.[2-6] Biofilm can develop on prostheses due to mucosa, humidity, and skin secretions (such as perspiration, sebum, and other compounds released by the skin).[4] The compression, heating, and contact from the prosthesis could induce dermatitis. The presence of bacteria and yeast is observed after sampling the maxillofacial prosthetics' surface. The most common bacterial species found on maxillofacial prosthesis were *Staphylococcus epidermidis*, *Staphylococcus schleiferi*, *Staphylococcus xylosus*, and *Staphylococcus capitis*, whereas the most common yeast species were *Candida albicans, Candida parapsilosis,* and *Candida famata*.[5] On prosthetic surfaces, microbial adherence and biofilm growth are significant contributors to material deterioration and skin irritation, which can contribute to facial prosthesis failure It is critical to disinfect and conserve maxillofacial prostheses to preserve both their quality and the well-being of the tissues around them.[5]

Under all of the current cleaning techniques, patients are obliged to clean their prostheses. Some cleaning methods include wiping down with a cotton ball immersed in a moderate soapy fluid, using a brush with soap, washing in water, patting dry with a napkin, and keeping in a container out of direct sunlight. Some of the most often used cleansing agents for the facial silicone elastomer include neutral soap; peroxides, acid enzymes, sodium hypochlorite, cleansing tablets, and chlorhexidine.[1,2,7,8] These

mechanical methods of cleaning maxillofacial facial prosthesis can cause increased surface roughness, loss of color stability leading to degradation of prosthesis which will decrease the life of the prosthesis.

When it comes to disinfecting maxillofacial elastomer, chemical soaking is the method of choice. Cleansing with a 2-4% chlorhexidine gluconate spraying or dipping in solution for 1 minute, followed by washing under running water, can adequately condition to minimize the quantity of bacterial contamination without jeopardizing the prosthesis. The physical and mechanical characteristics of maxillofacial silicone elastomers could be altered by frequent application of chlorhexidine gluconate, leading to loss of smooth surface and a rise in microhardness.[8]

The focus of oral hygiene has switched to plant-based solutions as a result of the increasing number of strains that are multi-drug resistant and possible negative effects from conventional mouthwash. The use of phytoextracts appears to be a viable disinfectant due to increased microorganism resistance and fewer side effects. The recent resurgence of natural health has contributed to an increase in interest in available naturopathic therapies.

In the current research, taking this into account in the comparison was done between 0.2% chlorhexidine gluconate, *Mangifera indica* (mango), *Anacardium occidentale* (cashew) leave extracts disinfectant solution. The disinfectant solution was formulated and prepared for the purpose of this study and mango and cashew were chosen as the plant extracts for their established antifungal and antimicrobial properties in previous studies.[9-14] Also these plants are indigenous and locally available and thereby acceptable for clinical use and have an added economic

advantage. 0.2% chlorhexidine gluconate was selected for the comparison and control purpose as it is the most common, economical and easily available in Indian markets.

The study was carried out using 75 silicone samples contaminated with *Staphylococcus aureus* and 75 with *Candida albicans*. The evaluation followed by comparison was carried out by counting the pre and post disinfection colony-forming units of *Staphylococcus aureus and Candida albicans* on blood agar. According to the findings of the current study, 0.2% chlorhexidine gluconate, *Mangifera indica* (mango), *Anacardium occidentale* (cashew) leave extracts significantly decreased the number of microorganisms, namely *C. albicans and S. aureus.* However, statistically considering, 0.2% chlorhexidine gluconate was superior to all other groups in terms of both organisms.

The findings regarding the cleaning procedures using submersion in 0.12% and 2% chlorhexidine gluconate is shown to be successful in lowering the CFU of the microorganisms in previous studies conducted by Ariani et al [5], Pinheiro JB et al [19] and de Azevedo MN [8]. In contrast, a study conducted by Guiotti et al[2], the presence of 50% viable *C. albicans* after 10 minutes of 4% chlorhexidine gluconate was observed and concluded that the most effective regimen for maintaining silicone prosthesis against *S. aureus* and *C. albicans* was hand washing with water and neutral soap. [2]

Even if chlorhexidine gluconate is showing effective anti-fungal and anti-microbial properties, 4% chlorhexidine gluconate is said to present a modified surface leading to unevenness and eventually microbial adhesion. [1,2,19]

Mangifera indica (mango) leaves have been shown to have antimicrobial properties. Initial research is being done on a substance called beta-carotene, lutein, and alpha-carotene, as well as polyphenols like quercetin, kaempferol, gallic acid, caffeic acid, catechins, tannins, and the special mango xanthonoid called mangiferin, which may be able to prevent various disease processes. The mango leaves and stems' aqueous and ethanol extracts, have been demonstrated to have considerable action against microorganisms like *Staphylococcus aureus,* S*treptococcus pneumoniae, Pseudomonas aeruginosa, Candida albicans.* Gallotannin and mangiferin are assumed to be responsible for the antibacterial effects of mango extract.[9-11] Mangiferin has strong antioxidant and antilipid peroxidation properties. Therefore, the rupture of the microbial biofilm may have been brought on by the polyphenolic component found in plant extracts.[11]

Significant antibacterial and antifungal activity were discovered in an ethanol extract of *A occidentale* leaves.[11,38] *Anacardium occidentale* (cashew) plants are used medicinally in many nations. Plant metabolites such as phenols, flavonoids, and tannins are found in leaf extracts and are responsible for its antibacterial properties. High levels of tannins in cashews interact and precipitate proteins thereby inhibiting the growth of pathogens. [11-14] This prompts further probing and research into various therapeutic and pharmacologic applications of *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) leave extracts to utilize as disinfecting solutions. In comparison to the water-based extracts, the leaf alcoholic extracts showed more anti-bacterial and anti-fungal activity; this might be because alcoholic extract can better extract the bioactive agents and producing more anti-microbial properties[12]. Hence ethanolic extracts *of M. indica and A. occidentale* were used.

In the present study, *Anacardium occidentale* leaf extract disinfectant performed statistically better than *Mangifera indica* leaf extracts among the two extracts for both *S aureus* and *C. albicans.*

In a study conducted by Anand G et al [11] there was no difference in the antimicrobial action of cashew leaf extract against *S. aureus, C. albicans* compared to CHX-based mouth rinse.[11]

Other studies have stated that used plant-based extract solutions, such as *C nardus* (citronella)[2], alcoholic solution of 10% green propolis[8], 10% *Ricinus communis*[19] solution were effective in eliminating *S. aureus* biofilms from maxillofacial elastomers.

0.2% chlorhexidine gluconate had a superior disinfecting action on *S aureus* and *C. albicans* followed by A. *occidentale and M. indica.* The action of phyto-ingredients of *M.indica and A. occidentale* gave statistically significant and overall acceptable action against biofilm formation of S. *aureus and C. albicans.* Hence the use of these natural disinfectants can be a reliable alternative for maintenance of not only maxillofacial prosthesis but even denture hygiene.

SCOPE OF THE STUDY

- Additional studies can be conducted to gauge the impact of the disinfectant solutions on the colour stability, shore A hardness, tear strength, and surface roughness of silicone materials.
- Assessment of the effect of different time duration for disinfection on the micro-organisms.
- Further research is suggested to assess the combined effect of *Mangifera indica* leaf and *Anacardium occidentale* leaf extracts on the micro-organisms commonly seen in the oral cavity.
- A combination of mechanical methods of brushing and immersion in disinfectant solutions can be studied.
- Since this is an in-vitro study, further in-vivo parameters should be considered with variable clinical conditions and outcomes.

LIMITATIONS OF THE STUDY

- Since this is an in-vitro study, the application of the results in clinical conditions might yield a different result.
- Room temperature vulcanizing was evaluated in this study. Heat temperature vulcanizing silicone might yield different results owing to the different surface properties.
- Leaves from different geographical areas, in different seasons, can yield different results.
- The time period considered for disinfection may be different from that determined for individuals who have maxillofacial prostheses of various types and sizes.
- Only one species of biofilm was used to test the efficacy of the hygiene regimens.

CLINICAL IMPLICATIONS

- In this study, disinfection of maxillofacial silicone samples using 0.2% chlorhexidine gluconate, *Mangifera indica* (mango) and *Anacardium occidentale* (cashew) leaf extracts showed reduction in the colony forming units of *S. aureus* and *C. albicans.* 0.2% chlorhexidine gluconate was superior to all other groups in terms of both organisms.
- Shifting the focus to plant-based solutions because of the increasing number of strains that are multi-drug resistant and possible negative effects from conventional disinfectant is the need of the hour. The use of phytoextracts appears to be a viable disinfectant due to increased microorganism resistance and fewer side effects. *Anacardium occidentale* leaf extract disinfectant performed better than *Mangifera indica* leaf extracts among the two extracts for both *S aureus* and *C. albicans.*
- It is important to give proper prosthesis hygiene instructions to patients and educate them about the importance of maintaining proper oral and prosthesis hygiene along with regular recall and follow-up of the patient.

CONCLUSION

Given the limitations of the current research, the following conclusions can be made.

1. *Anacardium occidentale* leaf extract and *Mangifera indica* leaf extract when used for the disinfection of maxillofacial silicone material surface *contaminated with Staphylococcus aureus* and *Candida albicans* resulted in a significant reduction in post-disinfection CFU.

2. 0.2% chlorhexidine gluconate when used for the disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* and *Candida albicans* resulted in a significant reduction of the post-disinfection CFU.

3. 0.2% chlorhexidine gluconate showed the most CFU reduction post disinfection of maxillofacial silicone material surface contaminated with *Staphylococcus aureus* followed by *Anacardium occidentale* leaf extract and *Mangifera indica* leaf extract. No statistically significant were found between 0.2% chlorhexidine *and Anacardium occidentale.* And statistically significance results were seen between 0.2% chlorhexidine *and Mangifera indica; Mangifera indica* and *Anacardium occidentale.*

4. 0.2% chlorhexidine gluconate showed the most CFU reduction post disinfection of maxillofacial silicone material surface contaminated with *Candida albicans* followed by *Anacardium occidentale* leaf extract and *Mangifera indica* leaf extract. Statistically significant results were seen between all the three groups.

SUMMARY

The present study was conducted with the aim to evaluate leaf extracts of *M. indica, A. occidentale* and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms *Staphylococcus aureus and Candida albicans.*

Maxillofacial silicone elastomer (Silastic MDX4-4210) was used to fabricate 150 discs using metal mould of diameter- 5 mm, thickness- 2 mm**.** 75 silicone samples were contaminated with *Staphylococcus aureus* and 75 were contaminated with *Candida albicans*. The disc was rolled on blood agar in a 1cmx1cm area. Pre-disinfection CFU's were evaluated and the discs were subjected to disinfection protocols. The contaminated discs with *S. aureus* and *C.albicans* were disinfected using *M.indica* leaf extracts, *A.occidentale* leaf extracts and 0.2% chlorhexidine. Post disinfection CFU's were evaluated. The results were tabulated and analysed and subjected to statistical analysis using the dependent t-test, one-way ANOVA and Tukeys multiple posthoc procedure.

Given the limitations of the current research, it was found that here 0.2% chlorhexidine gluconate showed the most CFU reduction post disinfection of maxillofacial silicone material surface contaminated *Staphylococcus aureus* followed by *A. occidentale* leaf extract and *M. indica* leaf extract. No statistically significant result were found between 0.2% chlorhexidine and *A. occidentale*. Statistically significant results were seen between 0.2% chlorhexidine *and M. indica; M. indica and A. occidentale.* 0.2% chlorhexidine gluconate showed the most CFU reduction post-disinfection of maxillofacial silicone material surface contaminated *Candida albicans* followed by A. *occidentale* leaf extract and *M. indica* leaf extract. Statistically significant results were seen between all the three groups.

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ANNEXURE – I

ETHICAL CLEARANCE

Research and Ethics Committee
KLE V K INSTITUTE OF DENTAL SCIENCES
KLE University
Accredited 'A' Grade by NAAC Placed in Category 'A' by MHRD (GoI)
Nehru Nagar, Belagavi - 590 010, Karnataka State
☎: 0831-2470362
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E-mail:principal@kledental-bgm.edu.in

Sl. No. : 1456

CERTIFICATE

This is to Certify that the synopsis titled

Comparative evaluation of leaf extracts of mangifera indica, anacardium occidentale and 0.2% chlorhexidine gluconate on disinfection of maxillofacial silicone material surface contaminated with microorganisms - Invitro study *Submitted by*

Dr. _____ *P. G. Student /*

Staff, Guided by _____ *from Department of*

Prosthodontics & Crown & Bridge *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

MEMBER SECRETARY
Research & Ethical Committee
KLEVK Institute of Dental Sciences
BELAGAVI.

Research and Ethical Committee
Belgaum

ANNEXURE – II

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA
A constituent unit KLE Academy of Higher Education & Research
Deemed–to–be–University
Central Research Facility
DRUG AUTHENTICATION REPORT

Submitted By:

Submitted Date: 28/10/2020

Date of Issue: 31/10/2020

SN	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as			
						Ayurvedic Name	Scientific Name	Family	Part Authenticated
1	Kajutaka	*Anacardium occidentale* L.	Anacardiaceae	Leaf	CRF/Auth/ 2020/Oct/56	Kajutaka	*Anacardium occidentale* L.	Anacardiaceae	Leaf

Signature:
Authentication Expert Name:
Date: 31/10/2020

(MSc.Botany)

Signature of Coordinator
ASU Drug Testing Laboratory

SHRI B.M.K. AYURVEDA MAHAVIDYALAYA

A constituent unit KLE Academy of Higher Education & Research
Deemed–to-be-University
Central Research Facility
DRUG AUTHENTICATION REPORT

Submitted By:

Submitted Date: 28/10/2020

Date of Issue: 31/10/2020

S N	Sample Name	Scientific Name	Family	Part submitted	CRF Code	Authenticated as			
						Ayurvedic Name	Scientific Name	Family	Part Authenticated
1	Amra	*Mangifera indica* L.	Anacardiaceae	Leaf	CRF/Auth/ 2020/Oct/55	Amra	*Mangifera indica* L.	Anacardiaceae	Leaf

Signature:
Authentication Expert Name:
Date: 31/10/2020

Sc.Botany)

CRF SHAHAPUR, BELGAUM

Signature of Coordinator
ASU Drug Testing Laboratory

K.L.E University's
(Accredited 'A' Grade BY NAAC (2nd Cycle) & Placed in Category 'A' by MHRD, GoI)

Shri B.M.Kankanawadi Ayurveda Mahavidyalaya

Shahapur,Belgaum-590003 Karnataka-India

Central Research Facility

Form-50 [See Rule 160-D (f)]

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No.: CRF/FG/242/20-21
Researcher:

Date of Receipt: 28/10/2020
Report Date : 29/12/2020

Name of the extract: Mango leaf Ethanolic extract

Activity: Antifungal activity- MIC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
C. albicans	NT	NT	NT	NT	NT	T	T	T	T	T	T	NT

*NT- No turbidity, T- Turbidity

Name of the extract: Mango leaf Ethanolic extract

Activity: Antifungal activity - MBC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	G	NG
C. albicans	NG	NG	NG	NG	NG	G	G	G	G	G	G	NG

*NG- No growth, G- Growth

Microbiologist

CRF SHAHAPUR, BELGAUM

Authorized signatory

K.L.E University's
(Accredited 'A' Grade BY NAAC (2nd Cycle) & Placed in Category 'A' by MHRD, GoI)

Shri B.M.Kankanawadi Ayurveda Mahavidyalaya

Shahapur,Belgaum-590003 Karnataka-India

Central Research Facility

Form-50 [See Rule 160-D (f)]

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No.: CRF/FG/242/20-21
Researcher:

Date of Receipt:29/10/2020
Report Date : 29/12/2020

Name of the extract:Mango Leaf Ethanolic extract

Activity: Antibacterial activity- MIC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
S.aureus	NT	NT	NT	NT	NT	T	T	T	T	T	T	NT

*NT- No turbidity, T- Turbidity

Activity: Antibacterial activity- MBC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
S.aureus	NG	NG	NG	NG	NG	G	G	G	G	G	G	NG

*NG- No growth, G- Growth

Microbiologist

K.L.E University's Shri B. M. Kankanwadi Ayurveda Mahavidyalaya
CRF
SHAHAPUR, BELGAUM

Authorized signatory

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Shahapur,Belgaum-590003 Karnataka-India
Central Research Facility
Form-50 [See Rule 160-D (f)]
(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No.: CRF/FG/243/20-21
Researcher

Date of Receipt:29/10/2020
Report Date : 29/12/2020

Name of the extract: Cashew Leaf Ethanolic extract

Activity: Antibacterial activity- MIC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
S.aureus	NT	NT	NT	NT	NT	NT	NT	T	T	T	T	NT

*NT- No turbidity, T- Turbidity

Activity: Antibacterial activity- MBC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
S.aureus	NG	NG	NG	NG	NG	NG	NG	G	G	G	G	NG

*NG- No growth, G- Growth

Microbiologist

K.L.E University's Shri B. M. Kankanwadi Ayurveda Mahavidyalaya CRF SHAHAPUR, BELGAUM

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Central Research Facility

Form-50 [See Rule 160-D (f)]

(AYUSH Approved ASU Drug Testing Laboratory Lic. No.TL-8/2011)

Reference No.: CRF/FG/243/20-21
Researcher:

Date of Receipt: 28/10/2020
Report Date : 29/12/2020

Name of the extract: Cashew leaf Ethanolic extract

Activity: Antifungal activity- MIC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	No drug	No organism
C. albicans	NT	NT	NT	NT	T	T	T	T	T	T	T	NT

***NT- No turbidity, T- Turbidity**

Activity: Antifungal activity - MBC

	1	2	3	4	5	6	7	8	9	10	PC	NC
	200microL	150	125	100	75	50	25	12	10	5	G	NG
C. albicans	NG	NG	NG	NG	G	G	G	G	G	G	G	NG

***NG- No growth, G- Growth**

Microbiologist

K.L.E University's Shri B. M. Kankanawadi Ayurveda Mahavidyalaya
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SHAHAPUR, BELGAUM

Authorized signatory

ANNEXURE – IV

Silicone samples contaminated with *S. aureus* (Log CFU)					
CHX		*A. occidentale*		*M. indica*	
Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection
8.18	0.85	8.18	1.85	8.18	1.95
8.18	0.00	8.18	1.70	8.18	0.00
8.18	0.00	8.18	1.78	8.18	0.00
8.18	0.00	8.18	1.18	8.18	0.00
8.00	0.00	8.00	0.60	8.00	0.00
8.18	1.00	8.18	0.00	8.18	0.00
8.18	0.70	8.18	0.00	8.18	0.00
8.18	1.28	8.18	2.00	8.00	0.00
8.18	1.60	8.18	2.04	8.18	2.06
8.18	1.18	8.00	2.30	8.00	2.11
8.18	1.11	8.00	2.30	8.18	2.00
8.00	1.18	8.18	2.26	8.00	2.04
8.18	1.54	8.18	2.40	8.18	2.00
8.18	0.00	8.00	2.40	8.18	2.23
8.18	0.00	8.18	2.41	8.18	2.32
8.00	0.00	8.00	2.41	8.18	2.00
8.00	0.00	8.18	2.41	8.18	0.00
8.18	0.00	8.18	2.40	8.18	1.00
8.00	0.78	8.18	2.31	8.18	0.00
8.18	0.70	8.18	2.02	8.18	0.00
8.18	0.00	8.18	2.41	8.00	0.00
8.00	0.00	8.00	2.41	8.18	0.00
8.18	0.00	8.18	2.40	8.18	0.00
8.00	0.00	8.00	2.39	8.00	0.00
8.00	0.00	8.18	2.38	8.00	2.04

Silicone samples contaminated with *C.albicans*					
CHX		*A. occidentale*		*M. indica*	
Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection	Pre-disinfection	Post-disinfection
6.00	1.70	6.00	2.00	6.00	2.00
6.00	0.00	6.00	2.05	6.00	2.00
6.00	0.00	6.00	1.18	6.00	0.00
6.00	0.00	6.00	1.04	6.00	0.00
6.00	0.00	6.00	0.00	6.00	0.00
6.00	0.60	6.00	1.30	6.00	1.78
6.00	1.30	6.00	1.95	6.00	1.00
6.00	0.00	6.18	2.41	6.00	1.18
6.18	0.00	6.18	2.26	6.18	1.78
6.00	0.00	6.00	2.30	6.00	1.85
6.00	0.00	6.00	2.30	6.18	2.00
6.00	0.00	6.18	2.32	6.00	2.08
6.00	0.00	6.00	2.32	6.00	0.00
6.00	0.00	6.00	1.90	6.00	0.00
6.00	0.00	6.00	1.70	6.00	0.00
6.18	0.00	6.18	2.04	6.18	1.48
6.00	0.00	6.00	2.00	6.00	1.11
6.00	0.00	6.00	2.08	6.00	0.70
6.18	0.00	6.18	2.11	6.18	0.95
6.00	0.00	6.00	2.19	6.00	1.00
6.00	0.00	6.00	2.05	6.00	1.00
6.00	0.00	6.00	2.10	6.00	1.70
6.00	0.00	6.00	2.13	6.00	1.54
6.00	0.00	6.00	2.04	6.00	0.00
6.00	1.85	6.00	2.04	6.00	1.48