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Antimicrobial Activity Evaluation of Cetylpyridinium Chloride - Chlorhexidine Combination Versus Different Types of Endodontic Irrigants

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Abstract

Background: Endodontic irrigants play a crucial role in disinfecting the root canal system. However, none of the currently available irrigants can be considered perfect. Nevertheless, most irrigants containing surfactants in their composition are believed to enhance their antimicrobial activity.

Aim: This study aimed to compare the antimicrobial activity of different active ingredients of root canal irrigants; cetylpyridinium chloride- chlorhexidine combination, chlorhexidine, and MTAD irrigants on different microorganisms (*E. faecalis, S. aureus*, and *Candida albicans).*

Method: The direct contact test was used to evaluate the antimicrobial activity of three irrigants (2% chlorhexidine, 2% chlorhexidine + 0.2% cetylpyridinium chloride surfactant, and 100% Biopure MTAD) against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*. The antimicrobial activity was assessed after a 5-minute contact time between the microbial suspension and different irrigant solutions. Subsequently, 10 µl of the treated suspension was cultured and incubated at 37°C for 24 hours. The results were expressed as the kill percentage of viable microbe. Statistical analysis was performed using the Kruskal-Wallis test and the Multiple Wilcoxon sum rank test.

Result: No statistically significant differences were observed in the killing percentages of chlorhexidine, chlorhexidine + cetylpyridinium, and MTAD on *Enterococcus faecalis* and *Staphylococcus aureus* ($p > 0.05$). Against *Candida albicans* subgroups, both chlorhexidine and chlorhexidine + cetylpyridinium were more effective and statistically significant than MTAD, while chlorhexidine and chlorhexidine + cetylpyridinium have comparable effective.

Conclusion: The addition of cetylpyridinium chloride surfactant to chlorhexidine irrigant did not cause modification or diminishing the in the killing percentages or the antimicrobial activity of chlorhexidine. So, cetylpyridinium chloride can be used in combination with chlorhexidine to overcome some of its drawbacks without changing its antimicrobial activity.

Key Words: Cetylpyridinium chloride, surfactant, chlorhexidine, MTAD, direct contact, antimicrobial

Introduction

Disinfection of the root canal system is one of the main aims of endodontic treatment, and accomplished by removing the microorganisms and their by-products by chemomechanical preparation (Wong et al., 2021). Although mechanical preparation is the primary mechanism to reduce the microbial load in the canals, certain portions of the root canal walls appear to be unprepared during mechanical instrumentation (Mohammadi & Abbott, 2009; Xu et al., 2020). Therefore, it was crucial to use antimicrobial irrigants with mechanical instrumentation to get rid of microorganisms from the canal and achieve successful root canal treatment (Yeung et al., 2014; De Andrade et al., 2015). However, certain microorganisms can still be inside the canals after the chemomechanical preparation such as *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*, and responsible of the failure of the endodontic treatment (Nirupama et al., 2014; Ghivari et al., 2017).

The irrigants used in endodontic treatment should possess specific characteristics to function efficiently. One of these characteristics is broad-spectrum antimicrobial activity. Other important features include the ability to lubricate the canals, flush the debrise and dissolve both organic and inorganic material. Furthermore, it must not harm the surrounding tissue or compromise the strength of the teeth (Haapasalo et al., 2010).

One of the most commonly used endodontic irrigants is chlorhexidine (CHX) (Dutner et al., 2012). CHX is a bisbiguanide antiseptic agent with broad-spectrum antimicrobial activity. CHX is active against Gram-positive and Gram-negative bacteria, yeasts, and viruses (Karpiński and Szkaradkiewicz, 2015). It is available in different concentrations (0.12%–2%) solutions; however, endodontic irrigation uses the 2% solution. CHX produces positively charged ions that attach to the dentin and prevent further microbial colonization for an extended period, and CHX is well known for this substantivity (Thirunarayanan and Hegde, 2022).

Studies found that adding surfactant reduces the surface tension of some irrigants up to 50%, thereby increasing wettability and antimicrobial activity. In addition, certain commercial irrigants, like biopure MTAD, are marketed as having higher antimicrobial activity than their conventional counterparts because they contain modifiers or surfactants in their composition (Lopes et al., 2015; Ravinanthanan et al., 2022). Biopure MTAD comprises 3% doxycycline, 4.25% citric acid, and 0.5% Tween 80 (surfactant) (Srikumar et al., 2013). The surfactant in MTAD helps reduce the surface tension of

the irrigant, there by enhancing the flow and penetration of irrigating solutions more profoundly into the dentinal tubules and thus wholly disinfecting the canal spaces.

The combining of cetylpyridinium chloride (CPC) surfactant with CHX irrigant to enhance the penetration depth of CHX by reducing its surface tension was first reported by Thirunarayanan and Hegde in 2022. Cetylpyridinium chloride is a quaternary ammonium salt antiseptic. It is commonly in aqueous solutions with a broad-spectrum antimicrobial activity used in mouthwash solutions or lozenges to treat minor mouth and throat infections (Hoang et al., 2021). The antimicrobial activity of using CPC and CHX together as irrigant solution was not assessed in previous studies, so it must be investigated.

The purpose of this study was to assess the antimicrobial activity of this new combination(CHX+CPC) against a variety of microorganisms while comparing it with that of CHX and MTAD. The null hypothesis is that there are no differences in the killing percentages of CHX, CHX+CPC, and MTAD against *E. faecalis, S. aureus,* and *Candida albicans*.

Material And Methods

This study received ethical approval (No. The MUOPR 30) from the Ethical-Scientific Committee of a local institution (College of Dentistry, Mustansiriyah University). The present in vitro comparative study was conducted at the College of Health and Medical Technologies - Baghdad's educational laboratories. The following endodontic irrigants were employed in the present study: 2% CHX, a combination of 2% CHX and 0.2% CPC made in the lab by dissolving CPC powder (0.2 g) (Sigma-Aldrich, Schnelldorf, Germany) in 100 ml of CHX irrigant (Thirunarayanan and Hegde, 2022), and Biopure MTAD (Tulsa, Dentsply) prepared in accordance with manufacturer's guidelines. The irrigants were placed in sterile test tubes after being filtered with a syringe filter with pore size 0.22 µm to standardized the solutions and ensure sterilization. This step was taken to prevent contamination by other microorganisms during the preparation process or through the repeated opening and closing of bottles during the experiment (Figure 1).

The Ministry of Health's Educational Labs provided pure cultures of the microorganisms used, which were cultivated on brain heart infusion plates. The colonies of these microorganisms were subject to microscopical examination to identify their morphology and reaction to gram stain. The VITEK 2 compact system was used to conduct biochemical tests for further identification.

Overnight planktonic cultures of the selected microorganisms were prepared at 37°C by inoculating isolated colonies from the pure cultures in brain-heart infusion (BHI) broth to produce a cell suspension. The cell suspension was then adjusted with sterile saline (0.9% NaCl) to match the optical density of 0.5 McFarland scale (1.5×10^8 cfu/ml) by using the spectrophotometer at 600 nm. (Nirupama et al., 2014; Ravinanthanan et al., 2018; Ravinanthanan et al., 2022)

Under sterilized conditions, 1ml of the prepared cell suspension of each microorganism was obtained and added up to a tube holding one milliliter of the used irrigant solutions (CHX, CPC+CHX, MTAD) for 5 minutes contact time (Figure 3) (Ravinanthanan et al., 2018;

Ravinanthanan et al., 2022). After the specified duration of exposure, 100 µL of samples were taken, mixed with 900 µL of NaCl in a serial of five Eppendorf tubes for serial dilution (Figure 4). A vortex mixer (Gemmy Industrial Crop) consistently mixes the tubes contents. On Mueller-Hinton agar plates, 10 µL droplet from each Eppendorf tubes and undiluted suspension were cultivated and incubated for 24 hours at 37°C C to count the number of colonies by a colony-counting device (Suntex, Taiwan). This process was repeated for ten times per subgroup. The sample number was calculated using G power 3.0.10 (program written by Franz-Faul, University of Kiel, Germany) with the power of study =80%, alpha error of probability =0.05 two-sided, assuming the effect size of F is 0.6 (Small =0.1, medium =0.25, large=0.4.) (Ravinanthanan et al., 2018; Goud et al., 2018; Ravinanthanan et al., 2022).

The killing effect of each test agent on microbes was determined by calculating the kill percentage of surviving bacteria from the initial inoculum. The formula calculated the percentage of kills for each test irrigant:

1-(Colony forming unit (CFU) [test agent]/CFU [initial inoculum]) × 100

Statistical Analysis

The statistical package for social science (SPSS version 22, Chicago, Illinois, USA) was used for data analysis and presentation. The data were found to be non-normally distributed by the Shapiro-Wilk test, which led to the use of the Kruskal-Wallis test to ascertain whether there was a significant difference among the groups. Multiple comparisons between groups were done by the Wilcoxon sum rank test that was corrected by the Dunn-Bonferroni method, with a probability value (*P* - value) of 0.05.

Results

The mean ranks of the killing percentage for each subgroup were shown in (Figure 6). The result of Kruskal-Wallis test showed that statistically no significant differences was seen in the killing percentages of the used irrigants against *E. faecalis* and *S. aureus* (p = 0.325, 0.066) respectively. While against *C. albicans*, the killing percentages of the irrigant solutions varied significantly (p = 0.008) (see Table 1).

From Table 1 and Figure 6, CHX+CPC have the highest mean rank of eradication effect on *E. faecalis* (17.50) with a killing percentage of (100%). At the same time, the lowest mean rank of eradication effect on *E. Faecalis* was seen in the MTAD group (14.30), with a killing percentage of (99.990%). The results of the Kruskal-Wallis test showed that there was no significant difference between the various irrigants (*p* = 0.325).

Regarding *S.aureus* subgroups, the highest mean rank value was in the CHX group (18.00) with a killing percentage of (100%). The lowest mean rank of eradication effect on *S. aureus* was in the MTAD group (12.20), with a killing percentage of (99.990%). The results of the Kruskal-Wallis test showed that there was no significant difference between the various irrigants (*p* = 0.066), but by measuring the effect size found that there was medium practical significance (effect size = 0.13).

Regarding *C. albicans* subgroups, the highest mean rank value was in the CHX+ CPC group (19.50) with a killing percentage of (100%). The lowest mean rank of eradication effect on *C. albicans* was in the MTAD group (9.50) with a killing percentage of (99.993%). The Kruskal-Wallis test showed a significant difference

was present when comparing the mean rank of different irrigants ($p = 0.008$).

Multiple pairwise comparisons of *C. albicans* subgroups using the Multiple Wilcoxon sum rank test adjusted by the Dunn Bonferroni method revealed a statistically significant difference between the effect of CHX irrigant and MTAD irrigant on *C. albicans* ($p = 0.019$) and between CHX+CPC and MTAD ($p = 0.003$) (Table 2).

Discussion

Root canal infections are polymicrobial infections in nature (Wong et al., 2021). Numerous microorganisms can be detected in endodontic infections, particularly in persistent root canal infections, including *E. faecalis, S. aureus*, and *C. albicans*, which were used in the present study (Ghivari et al., 2017).

The mechanical instrumentation, irrigation, and irrigating solutions' antibacterial properties all work together to eradicate microorganisms from the root canal (Prada et al., 2019). In previous years, antibacterial compounds and surfactants have been incorporated into the primary endodontic irrigants to increase their antibacterial activity, boost their therapeutic efficacy, and overcome some drawbacks of these irrigants (Rossi-Fedele et al., 2013; Ravinanthanan et al., 2017). One of these surfactants used is CPC, which was used in a previous study in combination with CHX to reduce the surface tension of the CHX and improve its permeability (Thirunarayanan and Hegde, 2022). This combination's antimicrobial activity was not evaluated. The addition of the surfactant to CHX might have modified its antimicrobial activity. This study additionally included MTAD because there was debate regarding its antimicrobial activity in relation to CHX. Although the majority of researchers have stated that MTAD is more efficient than CHX as it demonstrated the highest wettability and substantivity compared to 2% CHX (Kamberi et al., 2012; Agrawal et al., 2013; Sharaf and Alshareef, 2019), others have reported that there is no such distinction or they are equally effective (Kho et al., 2006; Portenier et al., 2006). So, this study assessed the impact of adding CPC surfactant to CHX antimicrobial activity and contrasted it with CHX alone and MTAD on different microorganisms.

The VITEK 2 system was used to identify the selected microorganisms because it provides rapid, highly accurate, reproducible results (Kim et al., 2022). A spectrophotometer at a wavelength of 600 nm was used to standardize the microbial suspension to be equal to 0.5 McFarland since this wavelength is known to minimize cellular damage and proliferation and is not naturally destructive (Nirupama et al., 2014).

The direct contact test was used in this study because of its quantitative nature and reproducibility, which are unaffected by the solubility and diffusibility of an antimicrobial agent (Nirupama et al., 2014; Ji et al., 2022). In this test, a specific amount of the microbial suspension came in close contact with specific amount of the antimicrobial agent. Then the effect of this direct, close contact was evaluated at a given time (Luddin & Ahmed, 2013). Following the manufacturer's guidelines, MTAD required a 5-minute contact period. So, the antimicrobial activity of the irrigants employed in the present study was evaluated after 5 minutes (Ravinanthanan et al., 2018; Ravinanthanan et al., 2022).

The results of the this study showed that all the used irrigants had effcient antimicrobial activity against *E. faecalis, S. aureus*, and *C. albicans*. All the used irrigants have comparable effect against *E. faecalis* and *S. aureus*, so the null hypothesis was accepted in this part, this may be related to the extended duration of contact for 5 minutes. Luddin & Ahmed showed that, the microorganisms in planktonic form can be eliminated in a short time using direct contact test (Luddin and Ahmed, 2013). So, after 5 minutes all the irrigants can produce negative cultures and have comparable effects. In *C. albicans* subgroups, a significant differences was evaluated in the killing percentage of the different irrigants. Thus, the null hypothesis was rejected in this part. CHX and the combined irrigant demonstrated significantly higher killing percentages than MTAD. This difference may be attributed to the varying mechanisms of action of the different irrigants.

The antimicrobial activities of CHX and CPC are related to their cationic molecules that promote binding to negatively charged components found on the microbial cell wall. When chlorhexidine is used at a concentration of 2%, it causes cytolysis, which results in the death of cells by precipitating or coagulating the cell's cytoplasmic protein and releasing crucial intracellular components like nucleotides. (Karpiński and Szkaradkiewicz, 2015). CPC interacts with the microbial cell wall, it modifies the cytoplasmic membrane's integrity, inhibits cellular activity, and causes cell death (Paunovska et al., 2019). MTAD's antimicrobial activity is related to it's doxycycline component, which is a bacteriostatic antibiotic with a wide range of activity against a variety of bacteria and limited activity against fungi. Doxycycline binds reversibly to the 30s ribosomal subunits in the susceptible microorganisms and inhibits protein synthesis (Sharaf and Alshareef, 2019).

The findings of the present study were consistent with those of Mohammadi, who concluded that MTAD and CHX had comparable effects against the planktonic form of *E. faecalis* (Mohammadi, 2015). These results also align with those of Ozkan et al., who demonstrated that CHX and MTAD irrigants exhibited similar antibacterial activities against *E. faecalis* (Ozkan et al., 2020).

On the contrary, studies done by Mattigatti et al. and Misuriya et al. assessed the antimicrobial activities of CHX and MTAD on various microorganisms. The antimicrobial activity of MTAD and CHX was shown to differ from one another. MTAD was found to be more efficient on *E.faecalis* than CHX in both investigations, and less efficient against S. aureus. These results disagreed with the present study findings, and this might have to do with the various approaches taken to evaluate the irrigants' antimicrobial activities; in their investigations, the agar diffusion test was used, which is influenced by the diffusibility of the irrigant solution, that impacted by irrigant surface tension. Because, MTAD contains a surfactant, which lowers surface tension compared to 2% CHX, it exhibited larger zones of inhibition (Mattigatti et al., 2012; Misuriya et al., 2014). While the direct contact test used in this study is independent of the solubility and diffusibility of the studied agent, which gives more accurate results (Nirupama et al., 2014). These results also disagree with Ravinanthanan et al. findings; they report that CHX is more effective than MTAD, and this may be related to the fact that they

depend on the colony-forming unit in the statistic, not the killing percentage (Ravinanthanan et al., 2022)

Regarding *C. albicans*, both Misuriya et al. and Mattigatti et al. conclude that MTAD was less effective than CHX against *C. albicans,* and these findings agreed with the present results (Mattigatti et al., 2012; Misuriya et al., 2014).

Few studies have investigated the antimicrobial activity of CPC as an endodontic irrigant. In their experiment to assess the antibacterial effect of CPC against *E. faecalis* in contaminated root canals, Estrela et al. found equal antimicrobial activity of 0.2% CPC and 2% CHX. In the agar diffusion method, CPC produced significant inhibition zones of microbial growth comparable to that of 2% CHX produced (Estrela et al., 2012). The antimicrobial capability of CHX combined with CPC surfactant as an endodontic irrigant has not been evaluated in previous studies. Becker et al. Evaluated the antibacterial activity of CHX and CHX + CPC mouthwashes against the oral biofilms and showed that both groups have comparable efficacy, and no variations were observed between them (Becker et al., 2021). However, García-Gargallo et al. found that combining CHX with CPC at varying concentrations had a synergistic effect that increased the mouthwash's overall antimicrobial efficacy (García-Gargallo et al., 2017). The differences in these findings may be attributed to differences in methodologies and the microorganisms used to evaluate the antimicrobial effects of these solutions.

This study has limitations as it was carried out in vitro, no tooth modules were used, and planktonic cultures were used instead of biofilm mode of microbial growth.

Conclusion

Within the limitations of our study, it can be concluded that, after five minutes of contact time, the antibacterial activity of all the irrigants utilized, is equivalent against both *S. aureus* and *E. faecalis*. More efficient antimicrobial activity against *Candida albicans* is shown by the combined irrigant (2% CHX + 0.2% CPC) and 2% CHX compared to MTAD. Certain CHX drawbacks can be addressed by combining CHX and CPC without compromising the antimicrobial activity of the former.

Conflect of interest:….

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Tables

Table (1): Analysis of the mean ranks of the kill percentage for each group using the Kruskal-Wallis test.

Microorganisms		CHX	CHX+CPC	MTAD	Kruskal-Wallis	*P* value
E. faecalis	Killing %	100.000	100.000	99.990		
	Mean rank	14.70	17.50	14.30	2.248	0.325
S. aureus	Killing %	100.000	99.990	99.990		
	Mean rank	18.00	16.30	12.20	5.442	0.066
C. albicans	Killing %	99.990	100.000	99.993		
	Mean rank	17.50	19.50	9.50	9.704	**0.008**

Note: The Kruskal-Willis Test showed that the C. albicans subgroups differed significantly from one another as $P \leq 0.05$.

Table (2): Multiple pairwise comparisons of the killing % among C. albicans group.

Microorganism	Groups		Z test	*P* value
C. albicans	CHX	CHX+CPC	0.589	0.556
		MTAD	2.355	**0.019**
	CHX+CPC	MTAD	2.944	**0.003**

Note. Bold values indicate significant differences between the tested subgroups.

$p > 0.05$: Non- significant, $p \leq 0.05$: Significant

FIGURES

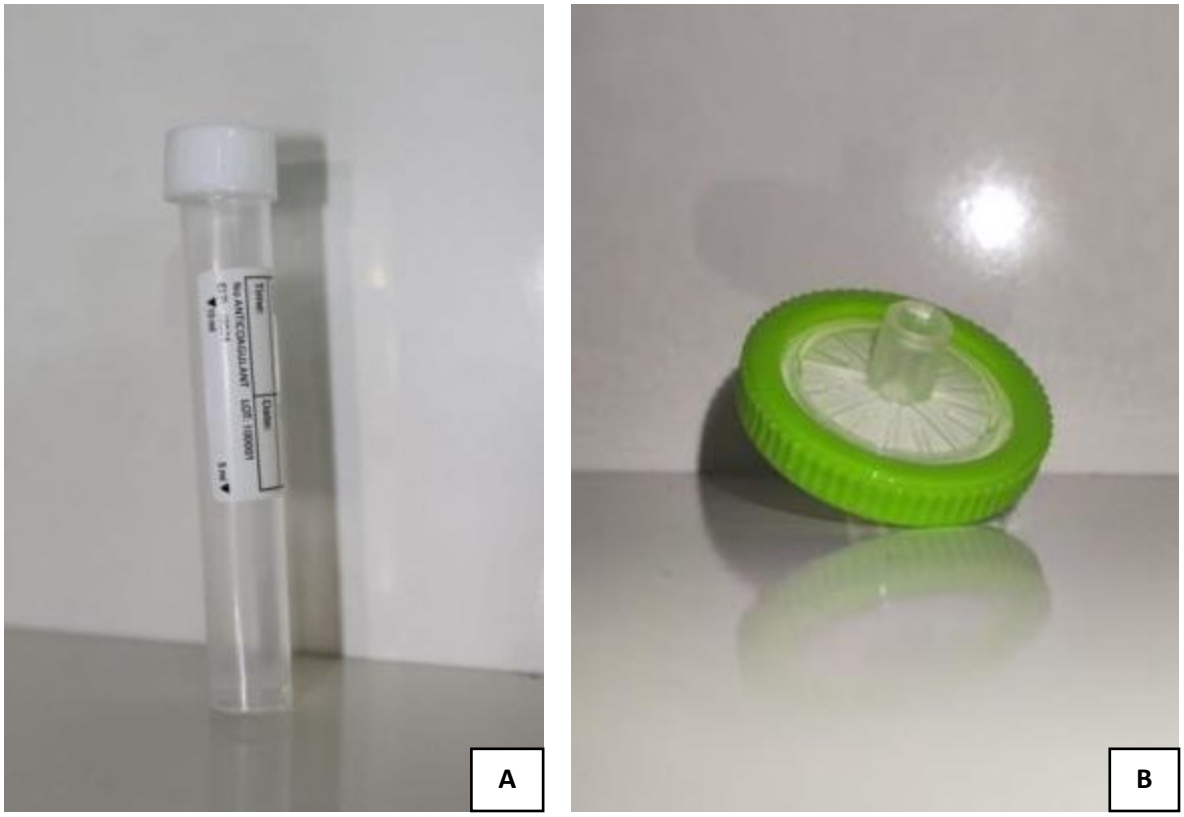

Figure 1: A- Autoclavable test tube B- 0.22 µm Millipore syringe filter

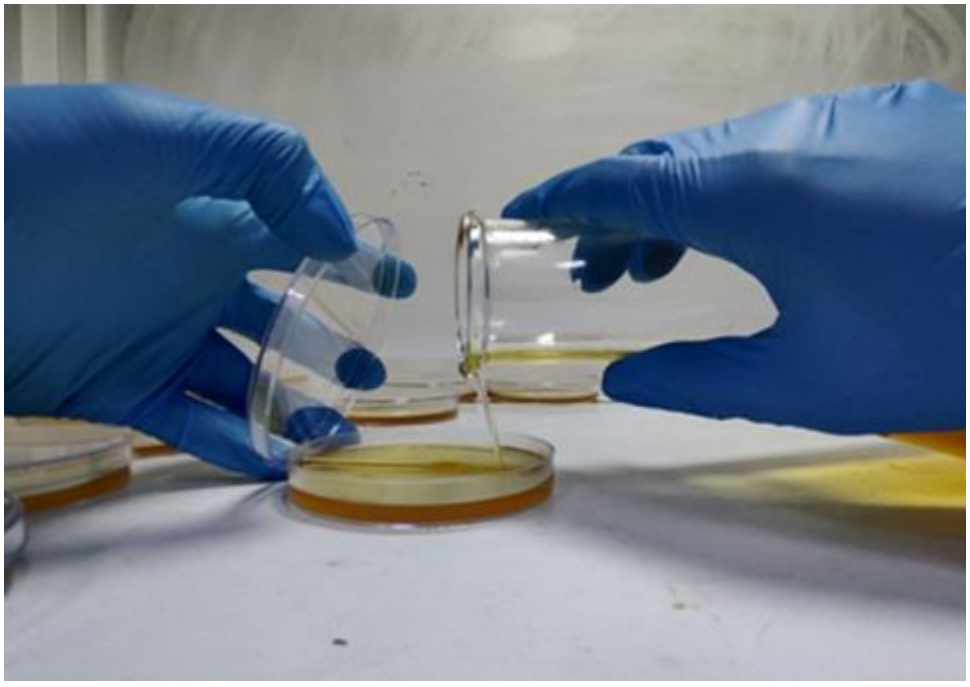

Figure 2: Pouring of Mueller-Hinton agar plates

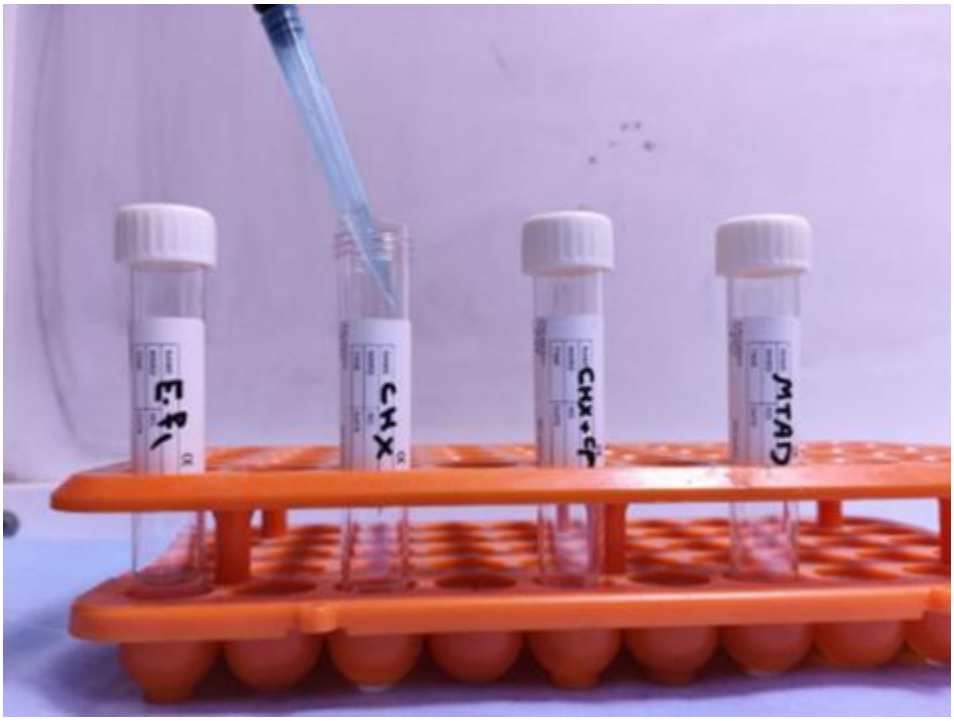

Figure 3: Treating the irrigant solutions with the microbial suspension

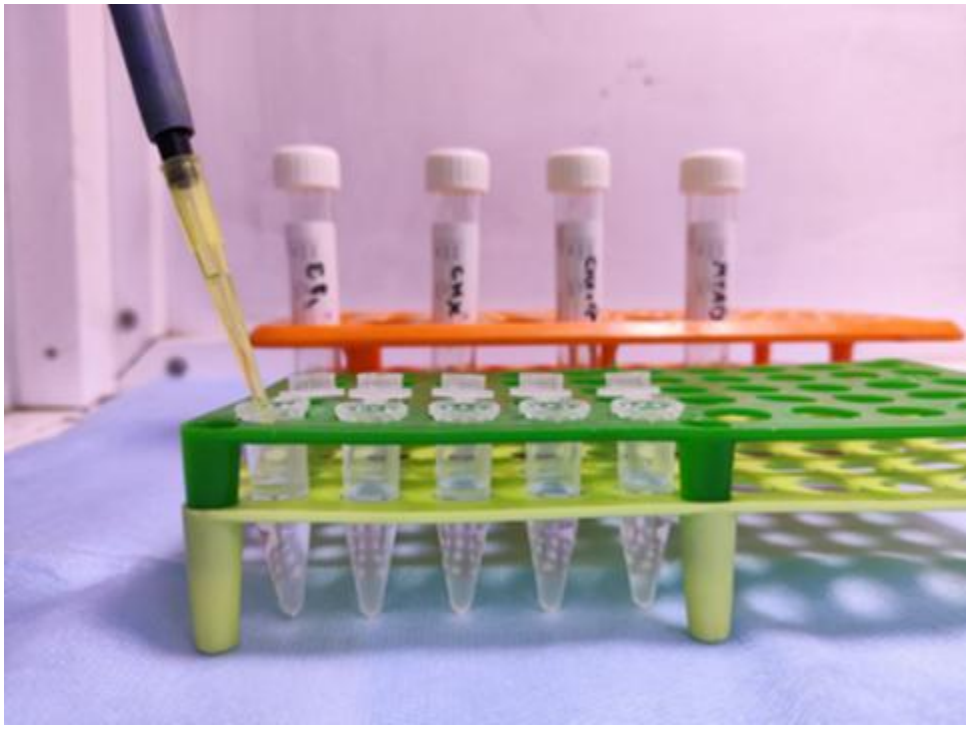

Figure 4: Serial dilution process

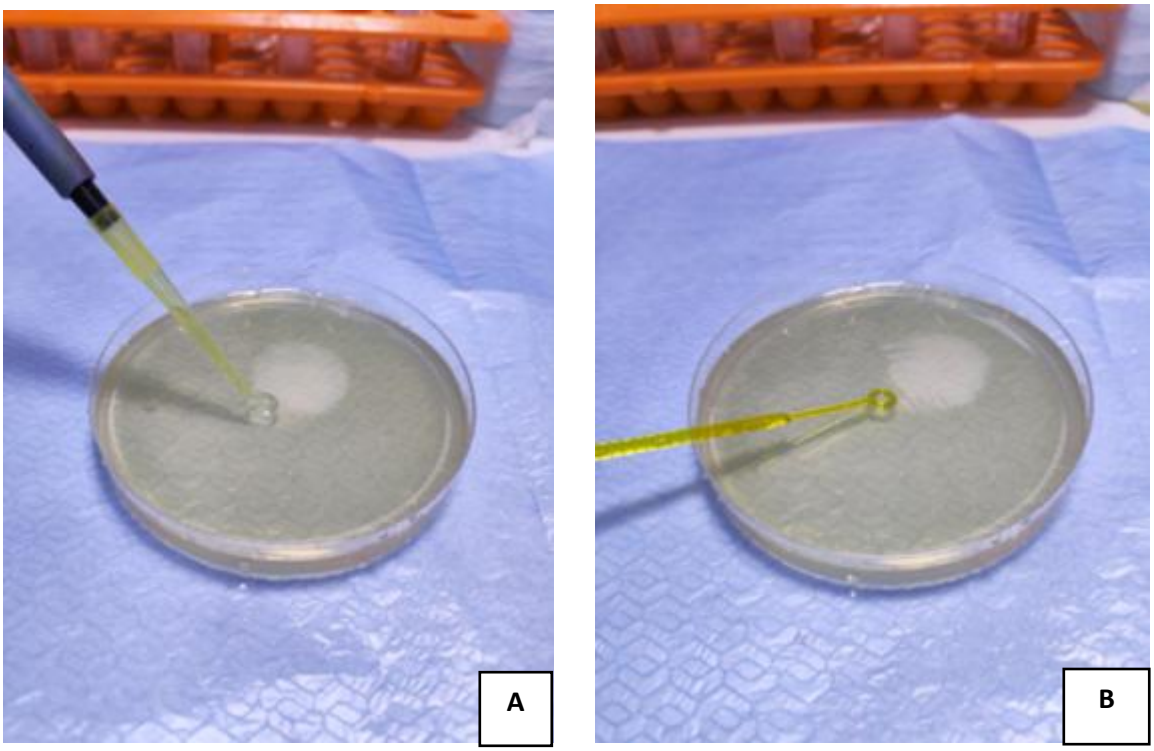

Figure 5: Culturing of sample A: Placing of 10 µl drop of final suspension on MHA plates B: Spreading of 10 µl of treated microbial suspension on MHA plates

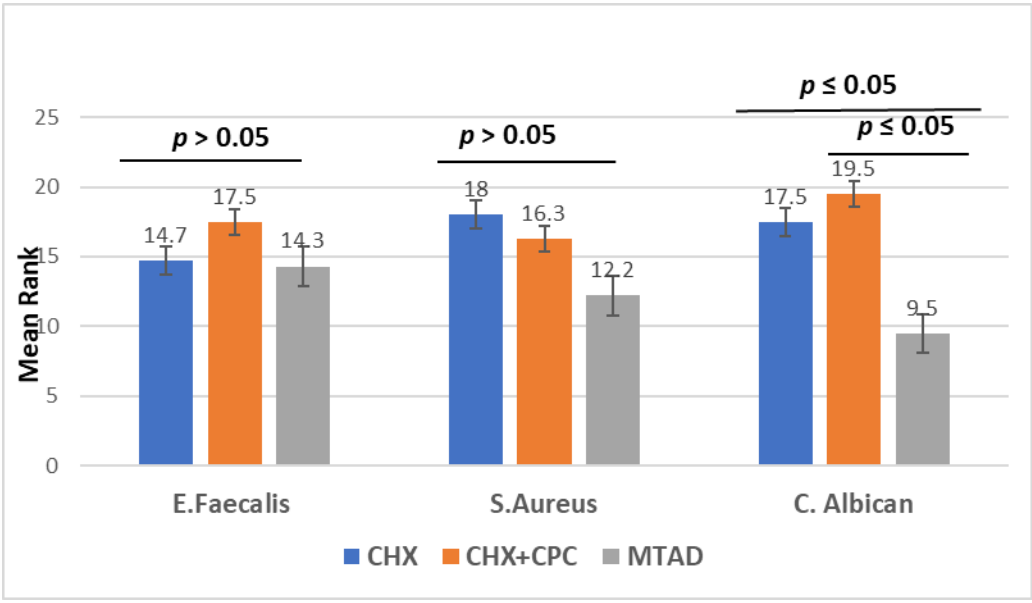

Figure 6: Bar chart of Killing percentage mean rank among the groups. The antibacterial activity of CHX, CHX + CPC, and MTAD against *E. faecalis* and *S. aureus* was not statistically different (p = 0.325 and 0.066, respectively). The antibacterial activity of different irrigant solutions on Candida albicans differed significantly (p = 0.008).

$p > 0.05$: Non- significant, $p \leq 0.05$: Significant