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Research Article

Comparison of Antibacterial Activity of Calcium Hydroxide, Azadirachta Indica (Neem), Ocimum Tenuiflorum (Tulsi) and Punica Granatum (Pomegranate) Gels as Intracanal Medicaments Against Enterococcus Faecalis: An *In-vitro* Study

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ABSTRACT

Aim: This study aimed to evaluate the antibacterial activity of Calcium hydroxide, Azadirachta indica (Neem), Ocimum tenuiflorum (Tulsi) and Punica granatum (Pomegranate) gels as intracanal medicaments against Enterococcus faecalis. Methodology: Crude extracts of Pomegranate peel, Neem leaves and Tulsi leaves were used to determine MIC following which gels of 5% neem, 10% tulsi and 10% pomegranate were prepared. Forty-eight single rooted human premolars were procured and inoculated with E.faecalis for 7 days. Specimens were then randomly distributed into 4 groups.
Group I- Calcium hydroxide
Group II- 5% A. indica (Neem) gel
Group III- 10% O. tenuiflorum (Tulsi) gel
Group IV-10% P. granatum (Pomegranate) gel
The experimental gels were then introduced into the samples and were sealed at both ends. The antimicrobial activity of medicaments was assessed by measuring CFU/ml at the end of 1, 3 and 5 days. Results: Calcium hydroxide showed the maximum antibacterial activity ($5.3X10^4$ CFU/ml) followed by Pomegranate gel ($5.4\ X10^4$ CFU/ml) with no statistically significant difference between them. Similarly, no statistically significant difference was observed between the mean CFU/ml values of the neem ($10.2\ X10^4$ CFU/ml) and tulsi gel ($10.2\ X10^4$ CFU/ml). However, pomegranate gel showed statistically significant antibacterial activity when compared to Neem and Tulsi. (Table 1, Table 2, Table 3, table 4) (Figure 1). Conclusion: Calcium hydroxide showed the best antibacterial activity against E.faecalis. Among herbal gels, pomegranate showed the maximum antibacterial activity, however, further in-vivo research is required for it to be used as a sole intracanal medicament clinically.

Key words: Intracanal medicaments, Calcium hydroxide, Herbal, Enterococcus faecalis, Root canal therapy, Microbial sensitivity tests.

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INTRODUCTION

It is well established that bacteria play a crucial role in the development of apical periodontitis.[1] Invariably, elimination of all bacteria from the root canal is one of the main objectives of endodontic treatment as their persistence results in endodontic infection.[2] This goal is achieved by the process of mechanical instrumentation along with the usage of various irrigants and intracanal medicaments between appointments.

E. faecalis is a gram-positive cocci and facultative anaerobe which can survive extreme pH (9.6), salt concentration and a temperature of 60°C for 30 min.[3-4] It has a key role in the formation of peri-radicular lesions post endodontic treatment. This persistent pathogen can survive within the root canal system as an isolated organism making up the major part of the flora and is known to be found in 22 – 77% of endodontic failure cases.[3] Because of its ability to form biofilm, E. Faecalis becomes more impervious to phagocytic activity, antibodies and antibacterial agents making it survivable in harshest conditions.[5] Both forms of peri-radicular diseases, primary (4–44%) and persistent endodontic infections (24–74%) have been known to be associated with E. faecalis. The resistance of the E. faecalis increases from 1000-fold to 10,000-fold when it is devoid of nutrition.[6]

Since the 1920s, calcium hydroxide [$Ca(OH)_2$] is the most frequently placed endodontic medicament.[7] Calcium hydroxide has many favorable properties such as high pH (12.5–12.8 approximately), insolubility in alcohol & low solubility in water. Its low water solubility is particularly advantageous as it provides us with a longer period before it dissolves in tissue fluids.[8] However, Calcium hydroxide has been reported inefficient against E.faecalis.

E. faecalis has the ability to colonize inside of dentinal tubules and thus can elude the hydroxyl ions. Also, ramifications, irregularities & isthmuses contain necrotic tissue that can shield bacteria from effect of calcium hydroxide.[9]

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Table 1: Descriptive statistics and ANOVA of different groups across different time intervals (All mean values are in 10^4CFU/ml).

DAYS	DAY 1				DAY 3				DAY 5			
GROUPS	I	II	III	IV	I	II	III	IV	I	II	III	IV
N	4	4	4	4	4	4	4	4	4	4	4	4
Mean	8.60	10.60	11.10	8.73	7.20	10.30	10.87	7.60	5.30	10.27	10.20	5.47
S.D.	0.72	0.92	0.20	0.42	0.26	0.17	0.21	0.26	0.10	0.12	0.10	0.15
S.E.	0.42	0.53	0.12	0.24	0.15	0.10	0.12	0.15	0.06	0.07	0.06	0.09
95% C.I.	6.81-10.39	8.32-12.88	10.06-11.59	7.69-9.76	6.54-7.86	9.87-10.73	10.35-11.38	6.94-8.26	5.05-5.55	9.98-10.56	9.95-10.45	5.08-5.85
ANOVA												
df	3				3				3			
F	12.46				194.52				1661.55			
Significance	0.002				0.001				0.001			

Table 2: Groupwise comparison of Mean CFU/ml at Day 1.

Groups	Groups	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-2.00	0.51	0.02	-3.64	-0.36
	3	-2.50	0.51	0.01	-4.14	-0.86
	4	-0.13	0.51	0.99	-1.77	1.51
2	1	2.00*	0.51	0.02	0.36	3.64
	3	-0.50	0.51	0.77	-2.14	1.14
	4	1.87*	0.51	0.03	0.23	3.51
3	1	2.50*	0.51	0.01	0.86	4.14
	2	0.50	0.51	0.77	-1.14	2.14
	4	2.37*	0.51	0.01	0.73	4.01

*. The mean difference is significant at the 0.05 level.

Table 3: Groupwise comparison of Mean CFU/ml at Day 3.

Groups	Groups	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-3.10	0.19	0.00	-3.70	-2.50
	3	-3.67	0.19	0.00	-4.27	-3.06
	4	-0.40	0.19	0.23	-1.00	0.20
2	1	3.10*	0.19	0.00	2.50	3.70
	3	-0.57	0.19	0.07	-1.17	0.04
	4	2.70*	0.19	0.00	2.10	3.30
3	1	3.67*	0.19	0.00	3.06	4.27
	2	0.57	0.19	0.07	-0.04	1.17
	4	3.27*	0.19	0.00	2.66	3.87

The mean difference is significant at the 0.05 level.

Table 4: Groupwise comparison of Mean CFU/ml at Day 5.

Groups	Groups	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-4.97	0.10	0.00	-5.28	-4.66
	3	-4.90	0.10	0.00	-5.21	-4.59
	4	-0.17	0.10	0.38	-0.48	0.15
2	1	4.97*	0.10	0.00	4.66	5.28
	3	0.07	0.10	0.90	-0.25	0.38
	4	4.80*	0.10	0.00	4.49	5.11
3	1	4.90*	0.10	0.00	4.59	5.21
	2	-0.07	0.10	0.90	-0.38	0.25
	4	4.73*	0.10	0.00	4.42	5.05

The mean difference is significant at the 0.05 level.

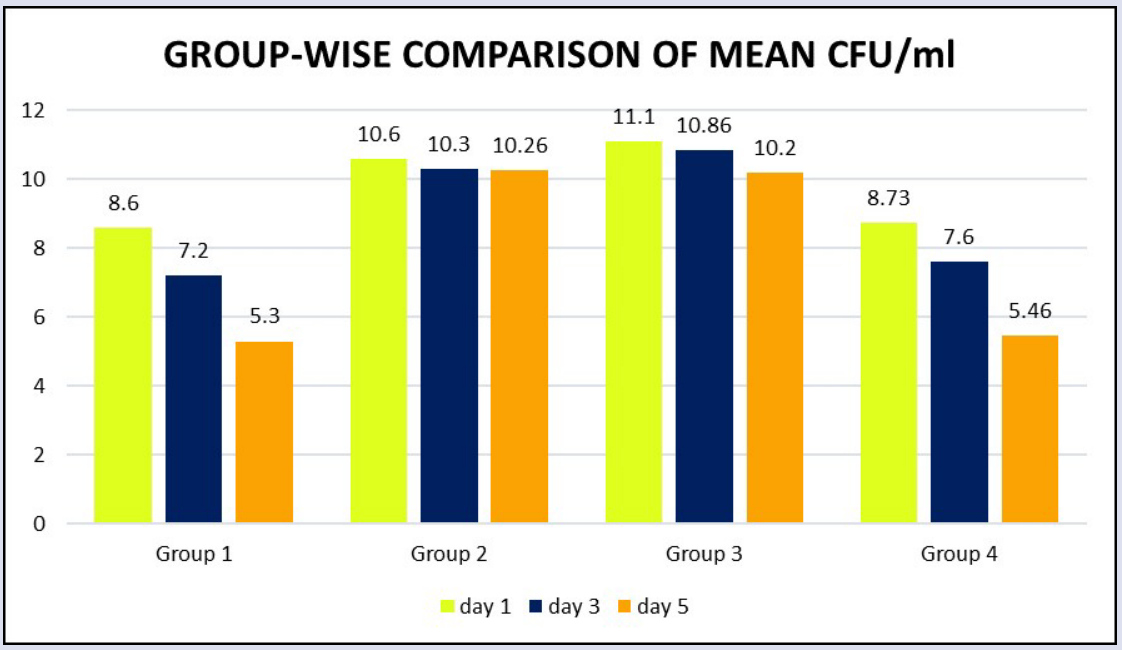

Figure 1: Group-wise comparison of Mean CFU/ml over different time intervals.

Presently, most of the commercial products used in Endodontics as intracanal medicaments are cytotoxic and are unable to eradicate bacteria from the dentinal tubules, leading to rapid inclination towards the usage of biologic medication derived from natural plants. The main advantages of utilizing herbal alternatives are their cost-effectiveness, low toxicity, easy availability, increased shelf life, and decreased microbial resistance.[10]

Azadirachta Indica (Neem) contains immense amount of chemically diverse biologically active compounds that inhibits the vitality of the bacteria by hampering the respiratory chain.[11]

These active compounds include azadirachtin, nimbidin, margolone, nimbin, nimbolide, gedunin, cyclictrisulphide, and mahmoodin. They inhibit the "mitochondrial oxidative phosphorylation" thereby inhibiting the "respiratory chain".[12]

In India, Tulsi has been famously considered as "The Queen of the herbs" for its restorative and spiritual properties.[13] Tulsi (Ocimum sanctum) contains active components such as Eugenol, ursolic acid and carvacrol, which are responsible for its antimicrobial activity.[14]

Pomegranate (Punica granatum) belonging to family punicaceae, is a small tree or shrub native to Asia. It contains phenolic compounds, including flavonoids (catechins, anthocyanins, and other complex flavonoids) and hydrolysable tannins (pedunculagin, punicalagin, punicalin, gallic and ellagic acid). Punica granatum peel has free radical-scavenging properties.[15] Besides accounting for majority of its antioxidant activity, these compounds also exhibit antifungal, antibacterial and antiproliferative properties.[16]

It is essential to explore new antimicrobial agents that are productive in eradicating the tenacious bacteria in the root canal systems. Therefore, in this study, three herbal agents were chosen to test their antibacterial efficacy against E.faecalis.

METHODOLOGY

Punica granatum (Pomegranate) peel powder, Azadirachta indica (Neem) and Ocimum tenuifloram (Tulsi) leaves powder was procured commercially. The aqueous extract solutions were prepared of 80% concentration which were used for MIC determination using Broth micro dilution method.

Preparation of specimens

A total of 48 freshly extracted, intact single rooted, permanent mandibular premolars extracted due to orthodontic reasons, were collected. The teeth were decoronated at CEJ (Cementoenamel junction) using a sterile diamond disc to obtain 14mm of root structure.

The patency of the apical foramen was established. Canals were then prepared with Protaper-gold files (PTG, Dentsply Sirona) up to an apical size of F3. The specimens were then kept in an ultra-sonic bath of 17% EDTA for 5 minutes followed by 3% NaOCl for 5 minutes to remove inorganic and organic debris. They were then immersed in ultrasonic bath containing distilled water for removal of remaining chemical traces for 5 minutes and were then dried using a size 25 sterile paper point for one minute. Two cycles of autoclaving were done for all the specimens. Cycle one was carried out at 121°C and cycle two with the specimens immersed in 1mL of Brain Heart Infusion (BHI) broth in individual microcentrifuge tubes.

Contamination of the specimens

Blood agar plates were used for cultivation of E. faecalis (ATCC 29212) over 24 hours following which a suspension of this culture was prepared in 5ml of BHI broth. Incubation at 37°C was carried out for four hours and turbidity of this suspension was adjusted to 0.5 McFarland. Each of the prepared tooth samples was put in the sterile microcentrifuge tubes with 1 mL of sterile BHI Broth. Fifty microlitres of the initially prepared E. faecalis inoculum was added to each microcentrifuge tube. Purity of the culture was confirmed by subculturing 5ml of the broth taken from the incubated samples on blood agar plates. Contamination of the samples was conducted for a period of 7 days.

Antimicrobial assessment

After completion of 7 days, 5 mL of sterile saline was used to remove the incubation broth from each sample. They were then randomly distributed into 4 groups (n= 12 teeth).

Group I- Calcium hydroxide mixed with saline till a paste like consistency was obtained

Group II- 5% Neem gel prepared using carboxy-methyl cellulose as the thickening agent

Group III- 10% Tulsi gel prepared using carboxy-methyl cellulose as the thickening agent

Group IV- 10% Pomegranate gel prepared using carboxy-methyl cellulose as the thickening agent

The medicaments were then introduced within the canals and secured with paraffin wax at both ends. Then incubation was carried out in aerobic environment at 37°C. A microbial cells assessment was carried out with 4 specimens at the end of 1,3 and 5 days.

The H-file was placed to the working length, and with filing motion, the dentinal shavings were scraped from apical third to a coronal third of a canal. The obtained dentinal shavings were then placed into 1 mL of sterile BHI broth and incubated in aerobic environment at 37°C for 24 hours. After 24 hours, fifty µL of the dilution was plated on BHI agar plates and again incubation was carried out for 24 hours. Colonies were counted and readings recorded.

STATISTICAL ANALYSIS

The data obtained was tabulated and analysed using SPSS software version 23.0. The results in the study were subjected to One way – ANOVA test and Post- hoc Tukey test.

RESULTS

All the experimental groups in the study showed antibacterial activity. Table 1 displays the antibacterial activity, at 3 selected time intervals (1, 3 and 5 days). Calcium hydroxide showed the maximum antibacterial activity followed by Pomegranate gel with no statistically significant difference between them. Similarly, there was no statistically significant difference observed between the mean CFU/ml values of the neem and tulsi gel, at different time intervals. However, pomegranate gel showed statistically significant antibacterial activity when compared to Neem and Tulsi. On the basis of above analysis, the order of microbial efficacy of different medicaments derived was-

$Ca(OH)_2$ > Pomegranate > Neem > Tulsi.

DISCUSSION

In this study, mandibular first premolars were used rather than bovine teeth as they simulate the clinical scenario better in assessment of endodontic medicaments efficacy in disinfection of dentinal tubules.[17] First and third day were chosen to evaluate the antimicrobial action as it has been previously reported that antibacterial efficacy of intra-canal medicaments reduces after 48 hours.[18] An additional evaluation was done on the 5th day based on a study by Beltes *et al* which reported that sufficient hydroxyl ions are released by $Ca(OH)_2$ over a period of 5 days.[19]

In this study, gel form of medicament was chosen as it renders ease of placement inside the root canal and better stay of the material within the canal walls. Also, probability of extrusion of material beyond the apex is minimized with a gel-based consistency.[20] For antibacterial assessment, Colony Forming Units were counted as it has been previously reported as the gold standard method. One of its advantages is that it does not include dead bacteria or debris and only the viable bacteria are counted.[21]

For the antimicrobial assessment, mean CFU/ml values obtained were subjected to statistical analysis which revealed that among the four experimental groups, $Ca(OH)_2$ demonstrated the maximum antibacterial efficacy which was comparable to the pomegranate gel group. However, the mean CFU/ml values of pomegranate and calcium hydroxide was significantly lower as compared to the neem and tulsi group. Our study demonstrated that out of the three herbal gels, only 10% pomegranate gel was efficient enough to reduce the E faecalis count to a level comparable to the gold standard calcium hydroxide over a period of five days. Our results were in accordance with study published by Ahmet D. Duman *et al* in 2009 in which P.granatum showed antibacterial efficacy against E.faecalis at 40µg/ml.[22] Visveswaraiah *et al* in 2015 also reported inhibitory effect of P.granatum on E.faecalis at MIC of 100 µg/ml.[15] Another study conducted by S. Aravindraj *et al* in 2017 reported prominent antibacterial efficacy of P. Granatum peels against E. faecalis.[23]

The reason for higher minimum inhibitory concentrations in the present study might be due to the use of distilled water-based extracts and not any other solvent based, as solvents (like benzene and alcohol based) enhance the antimicrobial potential of the actual extracts and also have toxic effects on periapical tissues.[24] For eg., if exposed in larger amounts & for longer duration, benzene exposure has been shown to cause an inflammatory response, hematotoxicity and leukemia. Likewise, alcohol based extracts are said to disrupt the physical structure of cell membranes, if not used judiciously.[25]

Not many articles are reported in previous literature regarding antibacterial efficacy of P. granatum against E.faecalis, however, Abdollahzadeh *et al* and Saad Sabbar Dahham *et al* in 2010 reported excellent antibacterial activity of P.granatum against S.aureus.[26-27] In our study, significant antibacterial activity of P.granatum peel powder can be attributed to the presence of substantial amounts of phenolic compounds, including flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolysable tannins (punicalagin, punicalin, pedunculagin, gallic and ellagic acid), gallic acid and ellagic acid, which have free radical-scavenging properties, and account for 92% of the antioxidant activity.[28]

5% Neem was selected as another experimental group because of its antibacterial activity attributed to the presence of astringents and salts like calcium, chloride, sulphur and fluoride. Neem contains sterols, oils, resins, silica, flavonoids, gum and alkaloids. Calcium present in neem, acts as an abrasive agent and is responsible for tooth polishing. Analgesic property is due to the Tannins present as astringent. In the present study, though Neem gel showed antibacterial activity against E.faecalis, it did not prove to be as effective as calcium hydroxide or pomegranate gel. Many previous studies like Rosaline *et al* (2013), Mistry *et al* (2014), Mustafa M (2016), Bharadwaj *et al* (2017) reported significant antibacterial activity of neem against E.faecalis.[11,29,30] However, none of these studies compared antibacterial activity of neem with calcium hydroxide or pomegranate. Also, another study Hugar *et al* in 2017 reported ineffectiveness of neem in oil form against E.faecalis.[31]

10% Tulsi gel in our study reduced the colony count of E.faecalis. This is in accordance with previous literature like Mistry *et al* (2014), Mukka *et al* (2017), Kalita C *et al* (2019), all of which demonstrated antibacterial activity of tulsi against E.faecalis.[29,32] It contains Eugenol, ursolic acid and carvacrol as the active component which are responsible for its antimicrobial activity. However, Jain *et al* (2015), Bharadwaj *et al* (2017), Kalita *et al* (2019) compared the antibacterial efficacy of neem and tulsi extracts against E.faecalis and concluded that neem was more efficacious than tulsi, as observed in this study.[33]

Calcium hydroxide proved to be the most efficacious medicament against E.faecalis. This is in accordance with several studies Varshini *et al* (2019), Samiei *et al* (2018), Louwakul *et al* (2016).[34-35] The antibacterial efficacy of calcium hydroxide can be attributed to the release and diffusion of hydroxyl ions (OH-) that results in a highly

alkaline environment which is not favorable for the survival of micro-organisms. Its fatal effects are due to various mechanisms such as A) direct action of hydroxyl ions cause damage to the cytoplasmic membrane of bacteria B) suppression of enzyme activity and cellular metabolism disruption C) Splitting DNA thereby inhibiting DNA replication.[36]

However, there are many studies such as DiFiore *et al.* (1983), Haapasalo & Ørstavik (1987), Siqueira & Uzeda (1996), Weiger *et al.* (2002), Ballal *et al.* (2007), Krithikadatta *et al.* (2007) which reported the inefficacy of $Ca(OH)_2$ against E.faecalis. This may be due to dense bacterial biofilms and escape of E. faecalis from hydroxyl ions as it penetrates into the canal isthmus and irregularities.[37] Also, due to the proton pump present in E.faecalis that acidifies the cytoplasm, enables it to resist the calcium hydroxide action at or below pH= 11.1.[38]

Sjögren *et al.*[39] demonstrated that using calcium hydroxide powder with different vehicles such as saline, distilled water and anaesthetics help in maintaining the high pH for at least 7 days. Polyethylene glycol, glycerine and other viscous mediums lead to very slow release of calcium and hydroxyl ions for a longer period. In the situations, that calcium hydroxide has to be placed in canal for a long time for it to be beneficial.[40] Behnan *et al* in 2001 demonstrated that less viscous preparations were more efficacious in removal of E.faecalis as compared to viscous preparations upto 24 hours. Therefore, in this study saline was selected as a vehicle for $Ca(OH)_2$ as the experimental time was 5 days.

This study provided evidence that over the five-day period pomegranate was successful as an intracanal medicament and this has not been previously reported in scientific literature. Therefore, there is a scope for further research with a longer observation period.

Further long-term clinical trial must be conducted to know the effectiveness of the extract solutions as intracanal medicaments. The extracts should also be tried in-vivo, to evaluate their toxicity and allergic potential.

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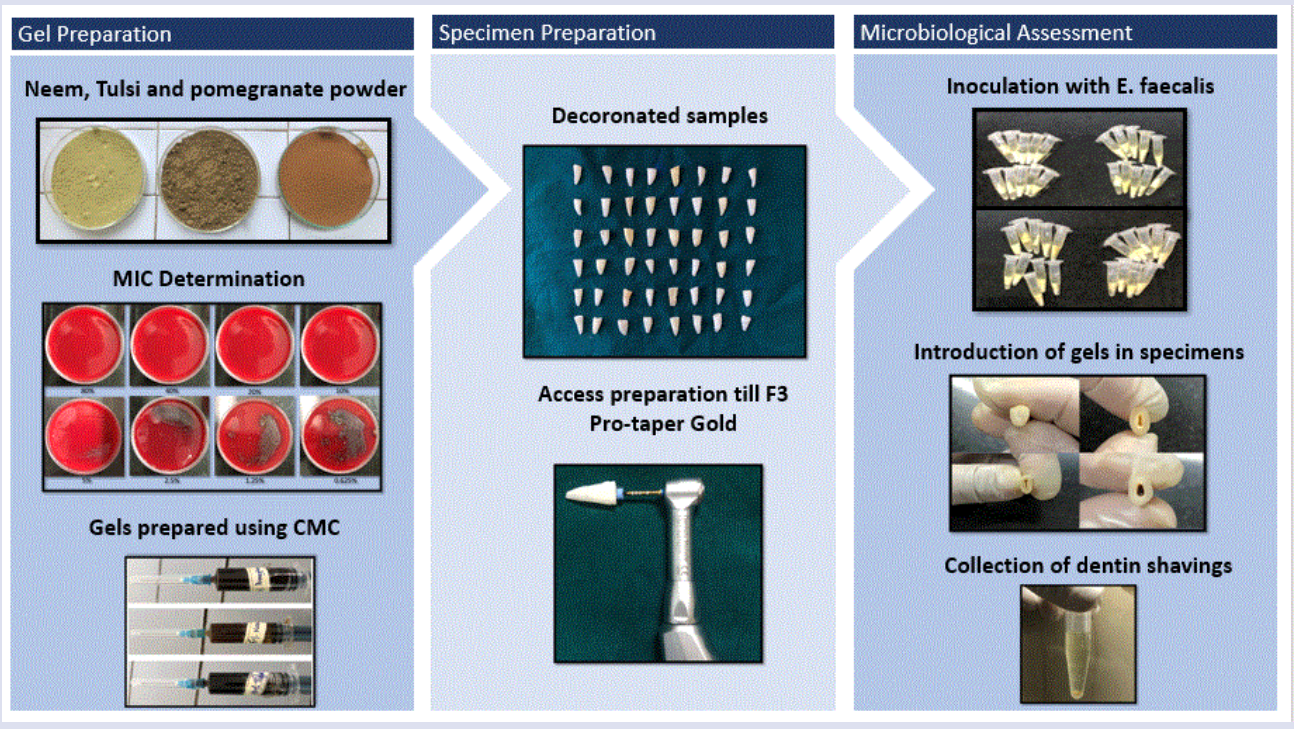
GRAPHICAL ABSTRACT



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