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Original Article

Dentistry Section

Antimicrobial Effectiveness of Different Root Canal Irrigants on Viablility of Root Canal Flora

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ABSTRACT

Introduction: Cleaning and shaping of sophisticated root canal system plays an important role in the success of endodontic treatment. In addition to root canal instrumentation, irrigation also becomes a crucial aspect in the effective disinfection of root canal system. The chemical substances like acids, chelating agents, alkaline solutions, Sodium Hypochlorite (NaOCl), oxidative agents, and normal saline are used as root canal irrigants. But each irrigant has its own limitations. With the advent of novel materials and techniques, the search for the optimal root canal irrigant still continues.

Aim: To evaluate and compare the antimicrobial effectiveness of various root canal irrigant solutions such as combination of 5.25% NaOCl and 2% Chlorhexidine gluconate, 2% Chlorhexidine gluconate, Miswak extract and normal saline for root canal irrigation.

Materials and Methods: In this prospective observational study was conducted from February 2021 to April 2021, on 10 patients with persistent endodontic infection on single rooted teeth. After obtaining access to the root canals, paper points were inserted and the soaked paper points were placed immediately in a microtube with two mL of Reduced Transport Fluid (RTF) to obtain the bacterial suspension. The sensitivity of Gram negative anerobic bacteria and *E.faecalis* to different irrigants was assessed with disc diffusion test and bacterial load was evaluated with Colony Forming Unit (CFU) assay. The study was analysed statistically using Kruskal-Wallis test and Dunn test (post-hoc test).

Results: The results of disk diffusion method showed that the zone of inhibition for 2% chlorhexidine gluconate was 29 mm for Gram negative anerobic bacteria and 22 mm for *E.faecalis*, and the zone of inhibition for Miswak extract was 8 mm for Gram negative anerobic bacteria and 10 mm for *E.faecalis*. Gram negative anerobic bacteria on the culture plates treated with Miswak extract had 6.41 CFU/mL, and *E.faecalis* had 5.21 CFU/mL. Gram negative anerobic bacteria on the culture plates treated with normal saline had a CFU/mL of 6.77, and *E.faecalis* had a CFU/mL of 5.77. The antibacterial activity of miswak extract was lower than that of other irrigants. The normal saline lacked any antimicrobial qualities.

Conclusion: The antibacterial effectiveness of the combination of 5.25% NaOCl and 2% Chlorhexidine gluconate was found to be higher than, 2% Chlorhexidine gluconate, Miswak extract and normal saline for root canal irrigation. A combination of 5.25% NaOCl and 2% Chlorhexidine gluconate can be used as a better choice over the other three irrigants.

Keywords: Chlorhexidine, Colony forming units assay, Disk-diffusion method, *Enterococcus faecalis*, Sodium hypochlorite

INTRODUCTION

A successful root canal treatment depends on the removal of organic matter and microorganism from the root canals. After endodontic therapy, residual bacteria in the pulpal spaces and dentinal tubules may lead to a persistent infection. Irrigating solutions are used during mechanical instrumentation to clean the root canal system. Along with root canal preparation, a range of antibacterial irrigation treatments can be utilised in different concentrations to irrigate and disinfect root canals. Endodontic infections are initiated by bacteria. A diverse genera of Gram negative anerobic bacteria colonise in the root canals. *E.faecalis* which colonise in the root canals are the most frequently seen bacteria in persistent endodontic infection [1]. The chemical substances like acids, chelating agents, proteolytic agents, alkaline solutions, NaOCl, oxidative agents, and normal saline are used as root canal irrigants. Certain natural extracts like Neem leaf extract, Propolis extract, Miswak extract are also being studied for their antibacterial effect [2].

An irrigant that could incorporate all the ideal requirements is yet to be discovered. Sodium hypochlorite is the most frequently used root canal irrigant. It has proteolytic, anti-bacterial and tissue dissolving properties. Chorhexidine gluconate is a broad spectrum antimicrobial agent which is active against Gram negative and Gram positive bacteria. It is bactericidal in high concentrations. Substantivity of this irrigant causes antimicrobial activity for 72 hours when used as a root canal irrigant [3]. Miswak is short twig that is obtained from an Arak tree (Salvadora persica). It is active against Gram negative and Gram positive organism [4].

Mechanical instrumentation alone cannot be an effective tool for root canal disinfection. It should be always followed by irrigation. Here various irrigants has its significance. Gutierrez and Goldman in the study by Davis SR et al., proved that there are areas in a root canal that harbours microorganism even after biomechanical preparation [5].

Natural products have been used in cleaning and disinfecting root canals either as irrigants or as intracanal medicaments. Potent anti-bacterial properties against *Enterococcus faecalis (E.faecalis)*, *Streptococcus mutans, Actinomyces viscosus*, and *Streptococcus sanguis* were observed when liquorice ethanolic extract (*Glycyrrhiza glabra*) [6], Miswak extract [7], essential oil of *L.sidoides,* methanolic extract of Azadirachta indica (*Neem*), Ocimum sanctum (*Tulsi*), *Mimusops elelngi* (Bakul), and *Tinospora cardifolia* (*Giloy*) [8], *Morinda citrifolia juice* 'Triphala' [9], T*erminalia bellerica*, *Terminalia chebula*, and *Emblica officinalis*, propolis extract [10].

The synthetic chemicals used as irrigation solutions generally do not have the ideal properties of an irrigation material [11]. Studies have indicated that natural alternatives for endodontic practice are highly promising [6-10]. Researchers have been looking for cures using herbal and natural ingredients. The reasons for this include the unfavourable and inadequate characteristics of the available

irrigants, the steadily increasing number of strains that are resistant to solutions, and the negative effects of synthetic medications. Several synthetic irrigation agents have been studied thus far, and others are being looked into. Similar to in medicine, there is a movement to revert to natural therapies in the fields of dentistry and endodontics. In this context, herbal irrigation techniques also seem promising. This study analyses the antimicrobial effectiveness of a natural irrigant with four chemical irrigants. The adequate usage of root canal irrigants with root canal instrumentation is the key to successful root canal treatment. The in-vitro study mentioned in this article evaluates the antimicrobial effectiveness of different root canal irrigants on the viability of root canal flora.

MATERIALS AND METHODS

This prospective observational study was performed in Teerthanker Mahaveer Dental College and Research Center Moradabad from February to April 2021. Ethical clearance was obtained from Institutional Ethical Committee (ref. No-: TMDCRC/IEC/SS/22-23/CDE 01).

Inclusion criteria: Those patients with persistent endodontic infection on single rooted teeth were selected.

Exclusion criteria: The patients who were on antibiotics therapy for two weeks prior to the treatment and those cases where impossibility to reach the full length of the canal was evident were excluded from the study.

Procedure

After obtaining proper access through the definite restoration, the existing root filling material was removed. The gutta percha points were removed with the help of a No.20 H-File. Root canal humidification was done with the sterile saline. Paper points ISO 25 or 30 were inserted into the root canals for sampling [3]. Radiographic length was estimated. Care was taken to insert the paper points 1 mm short of this length. Paper point was placed inside the root canal for 60 seconds with pumping movements in order create a suspension inside the canal. Collection of the soaked paper points were done without any external contaminators. Paper points were placed immediately in a microtube with two mL of Reduced Transport Fluid (RTF) [12]. This procedure was repeated for each paper point to obtain the bacterial suspension.

A sterile cotton swab was taken and dipped in the bacterial suspension. The culture plates were opened and the swab was wiped inside the plate in order to obtain unform layer of bacteria. Brain Heart Infusion media (BHI) was selected as the culture media. BHI was used for the propagation of pathogenic cocci and other fastidious organisms associated with infected root canals. The samples were cultured and incubated for 48 hours in 37ºC. Turbidity during the incubation period was indicative of positive growth. Gram negative anerobic bacteria which are most frequently seen in infected root canals were cultured and incubated in a candle jar. A 24-hour pure culture of *E.faecalis* (ATCC 19433) verified by polymerase chain reaction was grown in a different BHI broth culture plate. *E.faecalis* is the bacteria frequently associated with persistent endodontic infection. Microbiological evaluation was done in Goel Diagnostics Kashipur, Uttarakhand.

Disk Diffusion Test: The culture of Gram negative anerobic bacteria and *E.faecalis was* picked with the help of a sterilised wire loop. This wire loop was dipped in the RTF to make a bacterial suspension. A sterile cotton swab was taken and dipped in the bacterial suspension both for Gram negative bacteria and *E.faecalis.* Mueller Hinton Agar (MHA) was selected for the disk diffusion test to evaluate the sensitivity of different irrigants. Two MHA plates were selected for Gram negative anerobes and *E.faecalis,* respectively. Then the cotton swab was wiped all over the MHI plates. Preformed disks of following irrigants such as a combination of 5.25% NaOCl and 2% Chlorhexidine gluconate, 2% Chlorhexidine gluconate, *Salvadora persica* (Miswak) extract and normal saline were obtained. Miswak extract was made available from Shiv Sales Corporation, New Delhi, India. These disks were picked with the help of a sterile forceps and placed on the MHA plates. The disks were pressed gently. There was be a gap of atleast 25 mm between the disks. The plate lid was closed. The plates were inverted and incubated overnight. Following incubation, the plates were taken out and examined for the zone of inhibition. The diameter of the zone of inhibition was measured with the help of a ruler. The measurements were noted and interpreted.

Colony Forming Unit (CFU) assay: Ten culture plates of nutrient agar were made. Combination of 5.25% NaOCl and 2% Chlorhexidine gluconate, 2% Chlorhexidine gluconate, Miswak Extract and normal saline were added to the nutrient agar culture plates. Each irrigant was added to two nutrient agar plates. These plates were autoclaved. Once the nutrient agar was cooled to 55ºC, blood was added to it to make it blood agar. Two culture plates were not incorporated with any irrigants. These plates were converted to blood agar in the same way. Four nutrient agar plates incorporated with the four different irrigants were inoculated with Gram negative bacteria which were cultured before. The rest four nutrient agar plates incorporated with these four different irrigants were inoculated with *E.faecalis* which was cultured before. Two plates which were not incorporated with any irrigants were also inoculated with Gram negative anerobic bacteria and *E.faecalis,* respectively. All five culture plates inoculated with Gram negative anerobic bacteria was cultured at 37ºC overnight in a candle jar. The other five culture plates inoculated with *E.faecalis* with was cultured overnight at 37ºC. The turbidity on the plates indicated positive bacterial growth. These plates were then microscopically evaluated for the microorganism and bacterial load. The CFU were carefully examined and evaluated.

STATISTICAL ANALYSIS

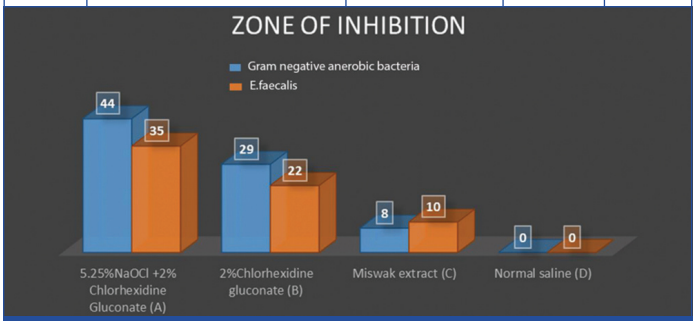
Data was analysed using the statistical package Statistical Package for Social Sciences (SPSS) version 26.0 (SPSS Inc., Chicago, IL) and level of significance was set at $p<0.05$. Descriptive statistics was performed to assess the mean and standard deviation of the respective groups. The study was analysed statistically using Kruskal-Wallis test and Dunn test (post-hoc test) Normality of the data was assessed using Shapiro Wilkinson test. Since the data was following normal distribution and parametric test were used for the data analysis. Inferential statistics to find out the difference between the groups was done using One-way Analysis of Variance (ANOVA) by Tukey's Honest Significant Difference (HSD) post-hoc analysis to find out the difference between any two groups.

RESULTS

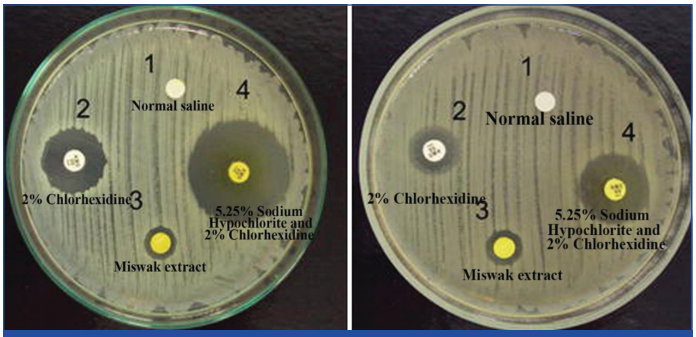
The disk diffusion method was used as a means for examining the sensitivity of root canal irrigants. It was seen that combination of 5.25% NaOCl and 2% Chlorhexidine gluconate had a maximum zone of inhibition of 44 mm for Gram negative anerobic bacteria and 35 mm for *E.faecalis*, while the zone of inhibition for 2% Chlorhexidine gluconate, was 29 mm for Gram negative anerobic bacteria and 22 mm for *E.faecalis*, and the zone of inhibition for Miswak extract, was 8 mm for Gram negative anerobic bacteria and 10 mm for *E.faecalis*. Normal saline didn't show any zone of inhibition [Table/Fig-1-3]. As there was no zone of inhibition for normal saline, bacteria didn't show any sensitivity to this irrigant. The irrigant that represented with largest zone of inhibition showed greatest anti-bacterial properties.

Presence of turbidity was seen on all the plates suggestive of bacterial growth. A tremendous distinction ($p<0.05$) among the whole range of CFUs of Gram negative anerobic bacteria

	Irrigant	Gram negative anerobic bacteria	Zone of inhibition (mm)	p-value
Groups	5.25% NaOCl+2% Chlorhexidine Gluconate (A)	44±1.84	35±1.93	**0.0001***
	2% Chlorhexidine gluconate (B)	29±1.02	22±1.32	**0.0001***
	Miswak extract (C)	8±0.85	10±0.56	**0.03***
	Normal saline (D)	0	0	0.99
Tests		**p-value**		
Kruskal Wallis		**0.0001***	**0.0001***	
Post-hoc	A vs B	**0.0001***	**0.0001***	
	A vs C	**0.0001***	**0.0001***	
	A vs D	**0.0001***	**0.0001***	
	B vs C	**0.0001***	**0.0001***	
	B vs D	**0.0001***	**0.0001***	
	C vs D	**0.0001***	**0.0001***	



[Table/Fig-1]: Comparison of zone of inhibition (mm).
*p-value <0.05 is statistically significant



[Table/Fig-2]: Antimicrobial activity of the irrigants in-vitro against normal root canal flora. The zone of inhibitiojn is in millimeter.
[Table/Fig-3]: Antimicrobial activity of the irrigants in vitro against *E.faecalis*. The zone of inhibitiojn is in millimeter. (Images from left to right)

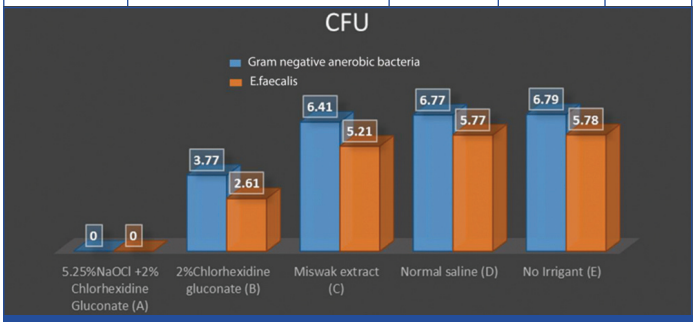
and *E.faecalis* was seen inside the experimental groups. The culture plates treated with combination of 5.25% NaOCl and 2% Chlorhexidine gluconate showed no growth, while those treated with 2% chlorhexidine gluconate produced 3.77 CFU/mL for Gram negative anerobic bacteria CFU/mL and 2.61 CFU/mL for *E.faecalis*. Miswak extract showed higher antimicrobial effect than normal saline, but its effect was considerably lower than the other chemical irrigants [Table/Fig-4].

Significant differences in the ability of 2% chlorhexidine gluconate, Miswak extract, combination of 5.25% NaOCl and 2% Chlorhexidine and normal saline to disinfect the canals was found using Kruskal-Wallis test and Dunn test (post-hoc) [Table/Fig-1,4].

DISCUSSION

The primary goal of endodontic treatment must be proper disinfection of root canal and to prevent reinfection [13]. This study aimed to evaluate and compare the antimicrobial effectiveness of three chemical irrigants and a natural irrigant on the root canal flora. Al-Sabawi NAK et al., concluded that 15% alcoholic extract of *Salvadora persica* (Miswak) had significant anti-microbial effect which was not significantly different from sodium hypochlorite and chlorhexidine, and significantly different from normal saline [14].

CFU comparative analysis	Irrigant	Gram negative anerobic bacteria	*E.faecalis*	p-value
Groups	5.25% NaOCl+2% Chlorhexidine Gluconate (A)	0	0	-
	2% Chlorhexidine gluconate (B)	3.77±0.23	2.61±0.23	**0.0001***
	Miswak extract (C)	6.41±0.87	5.21±0.87	**0.0001***
	Normal saline (D)	6.77±0.79	5.77±0.79	**0.0001***
	No Irrigant (E)	6.79±0.85	5.78±0.85	**0.0001***
Tests		**p-value**		
Kruskal Wallis		**0.0001***	**0.0001***	
Post-hoc	A vs B	**0.0001***	**0.0001***	
	A vs C	**0.0001***	**0.0001***	
	A vs D	**0.0001***	**0.0001***	
	A vs E	**0.0001***	**0.0001***	
	B vs C	**0.0001***	**0.0001***	
	B vs D	**0.0001***	**0.0001***	
	B vs E	**0.0001***	**0.0001***	
	C vs D	0.67	0.77	
	C vs E	0.85	0.67	
	D vs E	0.79	0.56	



[Table/Fig-4]: Comparison of Colony Forming Unit (CFU).
*p-value <0.05 is statistically significant

The present study implies that in addition to the chemical irrigants used, the natural irrigants also showed mild but significant anti-bacterial properties. Many natural products with anti-bacterial properties have to be tested to see their suitability as a root canal irrigant. By modifying the natural irrigants like Miswak extract, the authors may be able to enhance the anti-bacterial properties of these irrigants that could compete with the chemical irrigants used in this study. According to Shingare P and Chaugule V Miswak could be a good natural substitute to sodium hypochlorite when tested on chronically exposed primary teeth [2]. Thabet MS concluded that 10% water extracted Miswak showed antibacterial property which is comparable with the antibacterial property of Sodium hypochlorite and Chlorhexidine [15].

All the three studies mentioned above compared the anti-bacterial properties of Miswak extract with chemical irrignats like NaOCl and CHX. These studies showed comparable anti-bacterial effectiveness of Miswak extract with the chemical irrigants which is contrasting to the present study where this study results showed mild anti-bacterial property for Miswak extract. Gram negative anerobic bacteria and *E.faecalis* is usually associated with a root canal with persistent infection. *E.faecalis* is reported to cause continual apical infection in scientific conditions and secondary endodontic infections. Chlorhexidine is a good final irrigating solution for *E.faecalis* eradication [16,17]. The combination of 5.25% NaOCl and 2% Chlorhexidine gluconate showed greater antibacterial properties. When these irrigants are mixed together, they form an orange precipitate. The large amount of precipitate seems to more quickly eliminate *E.faecalis*. One possible explanation would be that larger amounts of parachloronanaline produce strong changes in the liquid media, leading to a more rapid microbial death.

Several microorganisms are unable to grow on parachloroanaline environments. Only a few species are able to metabolise it, and even then, further degradation of chlorocatechols, the most likely intermediate product of chloroaniline deamination, often becomes a rate-limiting step for microbial growth. So even though the orange precipitate forms, the precipitate medium helps in the degradation of microbes [18]. According to Kuruvilla JR and Kamath MP this could be due to formation of a byproduct called "chlorhexidine chloride," which increases the ionising capacity of the chlorhexidine molecule [19].

When the microbial samples treated with these irrigants were compared, the usage of 2% Chlorhexidine, Miswak, and normal saline, the results of this study demonstrated that combination of 5.25% NaOCl and 2% Chlorhexidine gluconate dramatically reduced intracanal bacteria levels [18-20]. Vianna ME and Gomes BP investigated in-vitro efficacy of the combination of sodium hypochlorite (NaOCl) and chlorhexidine (CHX) in different concentrations against *E.faecalis*. They concluded that the combination of NaOCl and CHX improved the antibacterial property of the irrigating solution [18]. Kuruvilla JR and Kamath MP concluded that the use of sodium hypochlorite and chlorhexidine gluconate combined within the root canal resulted in the greatest percentage reduction of post-irrigant positive cultures [19]. Basrani BR et al., found that the combination of CHX and NaOCl forms a precipitate called Parachloroanaline. It reduces microbial development and subsequent degradation of chlorocatechols, the most likely intermediate result of chloroaniline deamination [20]. The nascent chlorine that is present in sodium hypochlorite is responsible for its anti-bacterial activity. Chlorhexidine is bactericidal in high concentrations. It alters the intergrity of bacterial cell membrane, thereby leading to cell death. A combination of these two irrigants could give a predictable antimicrobial activity [21]. Results from the studies of Haque MM and Alsareii SA, and Jaiswal N et al., contradicts the results obtained from this study [22,23]. The study's microbiological sampling procedure techniques may be responsible to account for the discrepancies in the results.

Miswak is reported to have many pharmacological benefits such as anti-plaque, anti-caries, anti-periopathic, anti-ucerogenic, anti-inflammatory, anti-mycotic, anti-diabetic and anti-viral properties [24-26]. Such anti-microbial effect of Miswak extract is believed to be due to its high chemical contents of chlorides, tannins, trimethylamine, salvadorine, nitrate, thiocynate and sulpher [24,25,27]. This could be the reason for the anti-microbial effect of Miswak as a root canal irrigant. Normal saline has got good flushing action, but it lacks anti-microbial action.

The approach utilised in this study was designed to mimic an in-vitro clinical scenario in order to assess the efficacy of combination of 5.25% NaOCl and 2% Chlorhexidine gluconate, 2% Chlorhexidine Gluconate, Miswak extract, and normal saline on *E.faecalis* and Gram negative anerobic bacterial flora [28,29].

Limitation(s)

Since it was an observational study done on microbiological laboratory conditions, it was not possible to replicate the conditions in an organism. The authors in this study didn't evaluate the antimicrobial properties of NaOCl alone. Chemical agents like 5.25% NaOCl and 2% Chlorhexidine gluconate have already proved to be satisfying irrigants with their antimicrobial action. In order to confirm the antimicrobial effectiveness of miswak extract, further research and in-vitro tests are required.

CONCLUSION(S)

Root canal irrigants play an important role in eradicating microbes from the root canal system. The antibacterial effectiveness of combination of 5.25% NaOCl and 2% Chlorhexidine gluconate was found to be higher than 2% Chlorhexidine gluconate, Miswak extract and normal saline for root canal irrigation. A combination of 5.25% NaOCl and 2% Chlorhexidine can be used as a better choice over the other three irrigants for root canal disinfection. In near future more natural irrigants must be introduced in dentistry which could substantially reduce cytotoxicity and increase the rate of root canal disinfection. Judicious use of root canal irrigants and proper mechanical instrumentation can reduce the bacterial load and thereby enhance the success of root canal treatment.

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