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Ex vivo Antimicrobial Evaluation of Different Drugs Using RT-PCR Test

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Various medicaments are used in conjugation with mechanical cleaning and shaping to render root canals free of microbes. The different medicaments used tend to react among themselves sometimes resulting in deleterious products. For this reason use of alternative medicine, therapy is advocated.
Aim: To evaluate the antimicrobial extent of different homeopathic drugs in comparison to the Ayurvedic and Allopathic medicines.
Materials and Methods: A biofilm model of 8 mm dentine discs was prepared from single-rooted teeth, which were inoculated with E. faecalis for 21 days. 100 samples were divided into six groups Group I: Saline (negative Control), Group II: 2% Chlorhexidine Gluconate, Group III: Propolis, Group IV: RHUS Glabra, Group V: Zincum Oxydatum. The infected biofilm was then treated with the medicaments for seven days followed by testing to obtain threshold cycle (Ct) values using the Real-time polymerase chain reaction technique. DNA isolation was carried out and amplification

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was done using 16S rRNA gene sequence-based nested species-specific primers. DNA denaturation was done using DNA Thermocycler and a threshold cycle was obtained using Real-time Polymerize chain reaction technique.

Results: The threshold cycle (Ct) values obtained were subjected to statistical analysis that revealed a significant difference between the groups and among all 2% Chlorhexidine Gluconate, being the highest (30.3460±0.02505) followed by Propolis (30.1365±0.02621) then RHUS Glabra (30.0865±0.02581) better than Zincum Oxydatum (29.8070±0.02319) and least being Saline (29.6380±0.02505).

Conclusion: All the homeopathic medicines showed antimicrobial properties comparable to the allopathic and ayurvedic drugs. Among RHUS Glabra and Zincum Oxydatum, RHUS Glabra performed significantly better.

Keywords: RHUS glabra; zincum oxydatum; chlorhexidine gluconate; propolis; enterococus faecalis; real-time polymerase chain reaction.

1. INTRODUCTION

Successful endodontic treatment is a result of complete elimination and debridement of the necrotic tissue, microbes that have invaded the root canals through various means; followed by proper and impervious sealing to prevent reinfection [1].However, due to complex anatomy, limitations to access the canal system by irrigants and mechanical instruments complete disinfection is not achieved very often [2].placement of intracanal medicaments helps in reducing the bacterial load that has survived from the chemo-mechanical preparation [3].

Enterococcus faecalis (E. faecalis) is a persistent organism too often found in root canal failure cases due to its ability to survive in starving conditions and high Ph [4] develop stationary phase, ability to form biofilm [5].biofilm formations enhance the survival of the bacteria as the proteins or glycosaminoglycans decreases the effect of drugs through receptor-specific or hydrophobic interactions [6].

Chlorhexidine gluconate(CHX) has been widely accepted as an intracanal medicament over the past decade. The broad-spectrum nature, [7] long-term substantivities, [8]adds to its credibility. But its interaction with sodium hypochlorite results in para- chlorophphenyl isocyanate that degrades slowly to para-chloro-aniline; a carcinogenic product [9].is a major setback for its use as an intracanal medicament.

Ayurvedic or herbal medicines have been used science long due to their excellent biocompatibility, antimicrobial, anti-inflammatory, and antioxidant property.Among them, propolis, a natural resinous compound product made by honey bees (Apis Mellifera) [10].The presence of flavonoids, phenolics, and other aromatic compounds makes it a potent anti-inflammatory agent, capping agents for pulp [11]. It is widely used as an anticaries agent, [12] an avulsed tooth storage medium, [13] capping agents for pulp, and a dentinal hypersensitivity sealant [14]. less toxic than calcium hydroxide [15]. and contains antibacterial, [16] antiviral, antifungal, [17] immunomodulatory, [18] and antioxidant properties [19]. However allergic reactions and their tendency to cause breathing disorder has limited its use [20].

Alternative medicine therapy like, Homeopathy has been accepted by World Health Organization as the second-largest system of medicine; [21] be considered for the ideal endodontic medicaments. Homeopathic medicines work on the concept of “Stimulating Therapy” also known as Hahnemann’s Theory [22]. Zincum Oxydatum a homeopathic medicine contains nanoparticles of zinc oxide [23]. Zinc oxide is antibacterial in nature and is used in composite restorations [24].

Another homeopathic medicine RHUS Glabera is formed from the extract Rhus family native to North America and is widely used in medicine [25]. It is a potent antimicrobial activity against both gram-negative and gram-positive bacteria [26].

Thus this study aimed to compare the antimicrobial efficacy of homeopathic drugs against ayurvedic and allopathic drugs using a biofilm model. Objectives of the study were:

- To evaluate the antimicrobial efficacy of Homeopathic drugs (zincum oxydatum, Rhus glabra), Ayurvedic drug (Propolis),

and allopathic drug (Chlorhexidine Dyclugonate) against E. faecalis

- To compare the antimicrobial efficacy of Homeopathic drugs (zincum oxydatum, Rhus glabra), with allopathic drug (Chlorhexidine Dyclugonate) and Ayurvedic drug (Propolis)
- To find out the feasibility of the best possible Homeopathic drug among Zincum Oxydatum and Rhus Glabra that can be used as an intracanal medicament

2. MATERIALS AND METHODS

The study was conducted in the Department of Oral Pathology and microbiology. 100 single-rooted teeth with fully formed apex were collected; which were extracted for periodontal reasons. Teeth were evaluated under surgical microscope (OPMI pico, ZEISS, Germany) and teeth with open apex and crack lines were excluded from the study.

2.1 Model Preparation

8 mm length discs were prepared from single-rooted teeth after sectioning them vertically in the sagittal plane. For this, sections were made 8 mm from the apex using a diamond disc under water spray. Cleaning and shaping were done till F3 apical size using crown-down technique and rotary instruments (ProTaper, Dentsply Maillefer) 2ml of 3% Sodium hypochlorite and saline was used as intracanal irrigant throughout the instrumentation, final irrigation was carried out by 3ml of 17% EDTA for 1 min and lastly by 5ml of saline. The inner surface of the section was ground to obtain a relatively flat floor. All the samples were autoclaved to prevent any bacterial contamination [27].

2.2 Culture Preparation

E. faecalis (ATCC29212, KwikStik, Himedia®, India) were cultured on Muller Hinton Agar (Himedia®, India) and diluted with broth to match 0.5 McFarland Standards (Himedia®, India). The disc samples along with 2ml broth were then transferred in the culture vials for biofilm growth. The culture medium was replaced every alternate day for 21 days to avoid nutrient depletion. After 21 days samples were divided into groups with 20 samples in each group.The sample was collected from each well using a sterile paper point, and then they were inoculated onto Mueller- Hinton agar plates. Incubation was done at 370C for 24 hours to check for cell viability and purity of culture (Fig. 1).

Group I – Control Group (Normal Saline)
GroupII - 2% Chlorhexidine Gluconate (Dentochlor, Ammdent, India)
Group III - Propolis (Bee Propolis Tincture, Hi-Tech Natural Products Ltd., India)
Group IV - RHUS Glabra (200CH, Dr. Willmer Schwabe India Pvt. Ltd.)
Group V - Zincum Oxydatum (200 CH, Dr. Willmer Schwabe India Pvt. Ltd.)

3ml of the test drugs were added to the respective samples. The culture vials were incubated for 7 days at 370C. The tooth samples were vortexed in sterile saline vials for a few minutes. Then, a serial dilution procedure was carried out for analysis.

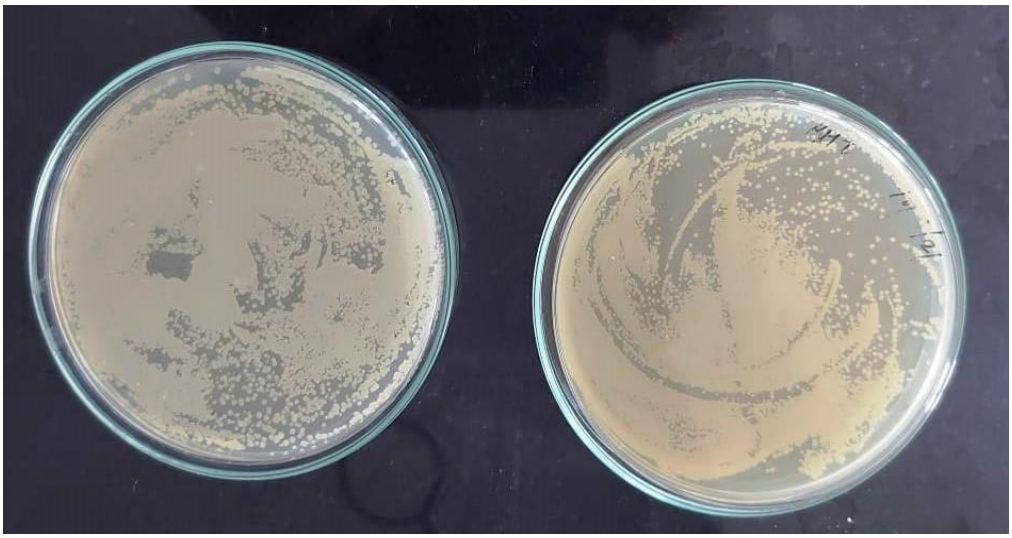

Fig. 1. Culture test for cell viability and purity

2.3 PCR Analysis

Bacterial DNA was isolated [28]using HipurA ®Multisample DNA purification kit (Himdedia laboratories Pvt. Ltd. Mumbai, India). Centrifugation of bacterial culture was carried out at 12000 g for 5 min. pellets obtained were boiled for 10 min. in 200ml lysis buffer (10 mM Tris/HCl buffer, 1 mM EDTA, 1% Triton X-100, pH 80).DNA was collected from the supernatant for PCR analysis that was performed in the CFX-96 Real Time system (BIO RAD). Amplification of PCR products was carried out using 16S rRNA gene sequence-based nested species-specific primers5'-GTT TAT GCC GCA TGG CAT AAGAG-3' (forward primer) and 5'-CCG TCA GGG GAC GTT CAG-3'(reverseprimer)(Eurofins Genomics, India),[29].The samples were subjected to PCR analyses using Hi- SYBr Master Mix (with Taq Polymerase) kit (Himdedia laboratories Pvt. Ltd. Mumbai, India) [30]. DNA thermocycler (C1000 Touch TM Thermal Cycler, BIO RAD) was used for PCR amplification.During amplification Initial denaturation was done at 95.0°Cfor 5:00 min, after that 35 cycles of a final denaturation were performed at 95°C. Finally, Primer annealing was done at 50°Cfor 45 Sec.,later, an extension step at 72°Cfor 30 sec, and a final step of 72°Cfor 2 min [31].

2.4 Statistical Analysis

The statistical analysis was done using SPSS (Statistical Package for the Social Sciences, SPSS Inc., v.16). The descriptive statistics were calculated as mean and standard deviation. The comparison of pre-operative and post-operative values was done using paired t-test. The comparison of values among the study groups was done using Analysis of Variance followed by post-hoc Tukey's test for multiple comparisons. The level of significance for the present study was fixed at a P-value of less than 0.05.

3. RESULTS

The comparison of Ex Vivo values was done using ANOVA (Table 1) which showed that there was a statistically significant difference among the study groups (P<0.001) with mean values of group II being highest (30.3460±0.02505) followed by Group III(30.1365±0.02621) then Group IV(30.0865±0.02581) better than Group V (29.8070±0.02319) and least being Group I(29.6380±0.02505).

Multiple comparisons using Tukey's test (Table 2) showed that there was a statistically significant difference between saline and chlorhexidine (P<0.01), saline and propolis (P<0.01), saline and Glabra (P<0.01), saline and zincum (P<0.01), chlorhexidine and propolis (P<0.01), chlorhexidine and Glabra (P<0.01), chlorhexidine and zincum (P<0.01), propolis and Glabra (P<0.01), propolis and zincum (P<0.01), and Glabra and zincum (P<0.001).

4. DISCUSSION

Intracanal medicaments are used in endodontics for various reasons that encompass elimination of remaining bacteria, reducing inflammation, tissue debris neutralization, inter- appointment barrier [32].Ideal properties of intracanal Medicaments include that it should have a germicidal and fungicidal effect, non-irritating, substantive antibacterial effect, should act in the presence of blood, serum, and protein derivatives of tissue, it should have the least strenuous effect on the tooth structure, it should be easily employed in the root canal, it should prevent coronal microleakage, and should not diffuse through the temporary restoration [33, 34].

The present study was conducted on E. faecalis because of its persistent and protracted presence in root canal failure cases [35].E. faecalis has shown to have high resistance to antimicrobial agents [36] their persistence presence in failed endodontic cases can attribute to form calcified biofilm on root canal dentin [37].The chemical nature of the substrate influences the biofilm-forming capacity and its structural organization. So, its formation on the polycarbonate or glass substrate will not provide true interaction [38].

CHX has been the most widely accepted or as near to the ideal intracanal medicament due to its wide range of antimicrobial activity [7]. Its activity is pH-based, as it dissociates at physiological pH and releases positively charged CH component [39].Its interaction with other irrigants (such as sodium hypochlorite) has led to the formation of para- chlorophphenyl isocyanate that degrades slowly to para-chloro-aniline precipitate (para-chloroanaline)9 and hinders the proper sealing of the obturating material by clogging the dentinal tubules [40].

Use of propolis is advised due to its natural occurrence and does not have any deleterious with other intracanal irrigants [11].Butit has been known to show a certain allergicreaction in some

patients.Compound LB-1 (consisting mainly of three pentenyl esters of caffeic acid) derived from the buds of poplar is a proven contact allergen [41].As per The GC/MS analysis the exact composition of LB-1is 3-methyl-2-butenyl-caffeate (54.2%), 3-methyl-3-butenyl caffeate (28.3%), 2-methyl-2-buthyl-caffeate (4.3%), phenethyl caffeate (7.9%), caffeic acid (1.3%), benzyl caffeate (1.0%)[42].

In the present study based on results obtained and in comparison with the published literature, it can be concluded that both the tested homeopathic medicine showed antimicrobial efficacy against E. faecalis which is well-supported by the various clinical trials and comprehensive reviews that prove homeopathic medicines are effective [43, 44].

Both the tested material showed antimicrobial efficacy. Of the two homeopathic medicines, RHUS Glabra showed statistically better results than Zincum Oxidatum.There is no explanation as to how it happens [45] but scientific body interest hasevidenced Biological effects can be achieved by ultra molecular solutions [46].

Table 1. Comparison of Mean threshold cycle (Ct) among groups, *One- Way analysis of Variance (ANOVA), *P*<0.05

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		P-value
				Lower Bound	Upper Bound	
Saline	20	29.6380	.02505	29.6263	29.6497	
CHX	20	30.3460	.02137	30.3360	30.3560	
Propolis	20	30.1365	.02621	30.1242	30.1488	<0.001*
Glabra	20	30.0865	.02581	30.0744	30.0986	
Zincum	20	29.8070	.02319	29.7961	29.8179	
Total	100	30.0028	.25303	29.9526	30.0530	

Table 2. Intra Group Comparison of threshold cycle (Ct) values; *Statistically significant (*P*<0.05, Post-hoc Tukey's test)

(I) Group	(J) Group	Mean Difference (I-J)	P-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Saline	CHX	-.70800*	<0.001*	-.7295	-.6865
Saline	Propolis	-.49850*	<0.001*	-.5200	-.4770
Saline	Glabra	-.44850*	<0.001*	-.4700	-.4270
Saline	Zincum	-.16900*	<0.001*	-.1905	-.1475
CHX	Propolis	.20950*	<0.001*	.1880	.2310
CHX	Glabra	.25950*	<0.001*	.2380	.2810
CHX	Zincum	.53900*	<0.001*	.5175	.5605
Propolis	Glabra	.05000*	<0.001*	.0285	.0715
Propolis	Zincum	.32950*	<0.001*	.3080	.3510
Glabra	Zincum	.27950*	<0.001*	.2580	.3010

At the ultra molecular level the constituents of the tested material, RHUS Glabra contains antimicrobial agents like methyl ester of 3, 4, 5-trihydroxy benzoic acid (methyl gallate), 4-methoxy-3, 5-dihydroxybenzoic acid, and gallic acid.17 Methyl gallate is a strong antioxidant that that has phytochemical properties [47]. Whereas, zinc oxide nanoparticles show bactericidal properties by interacting with the surface or/and core of the bacteria [48].The bactericidal effect is due to the formation of reactive oxygen species, resulting in elevated membrane lipid peroxidation causing membrane leakage of reducing sugars, DNA, proteins that reduces cell viability [49].

The antimicrobial properties of the tested samples were significantly less than the other tested material. The concentration of the active ingredients in the homeopathic medicine decreases with the increase in the dilution [50].

5. CONCLUSION

In the light of the results, it can be concluded that both homeopathic medicines have shown promising antimicrobial properties in comparison with allopathic and ayurvedic medicines. Out of the two homeopathic medicines tested, RHUS Glabra performed better in ex-vivo conditions than Zincum Oxydatum. But for the use as intracanal medicament, the results of this study should be correlated with the results of the in-vivo test.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

NOTE

The study highlights the efficacy of "RHUS Glabra" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

CONSENT

It is not applicable.

ETHICAL APPROVAL

After taking the approval from the ethical committee the study was conducted.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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