ANTIMICROBIAL EFFICACY OF DIFFERENT INTRACANAL MEDICAMENTS ON ENTEROCOCCUS FAECALIS AND CANDIDA ALBICANS – AN IN VITRO STUDY

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfilment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

APRIL 2017

CERTIFICATE

This is to certify that this dissertation titled **"Antimicrobial efficacy of different intracanal medicaments on Enterococcus faecalis and Candida albicans- An In Vitro Study"** is a bonafide record of work done by **Dr. DEVINA DINAKAR** under my guidance and to my satisfaction during her postgraduate study period, 2014 – 2017. This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfilment for the award of the degree of Master of Dental Surgery in Conservative Dentistry and Endodontics, Branch IV. It has not been submitted (partially or fully) for the award of any other degree or diploma.

_____ _____

Dr. Subha Anirudhan, MDS,
Guide, Reader,
Department of ConservativeDentistry and Endodontics,
Sri Ramakrishna Dental College and Hospital.
Coimbatore.

Dr. V. Prabhakar, MDS,
Principal, Professor and HOD
Sri Ramakrishna Dental College and Hospital
Coimbatore

Date:

Place: Coimbatore

ACKNOWLEDGEMENT

This thesis is the result of work done with immense support from many people and it is with immense pleasure that I express my heartfelt gratitude to all of them.

I devote my heartfelt thanks to **Dr. V. Prabhakar, MDS**, **Principal & Head of Department**, whose discipline and skills that run deep under his authoritative yet natural care during my post graduate period which enabled me to successfully conclude my thesis.

I would like to thank and acknowledge **Dr. Subha Anirudhan, MDS**, **Reader,** my Guide who has always been a source of support and encouragement at any moment, in and out of the department. I am grateful to her for her innovative ideas, constructive suggestions, valuable criticism and constant encouragement.

I take this opportunity to express my sincere gratitude to **Dr. Minu Koshy, MDS, Professor**, **Dr. S. Sudhakar, MDS, Reader and Dr. Sriman Narayanan, MDS, Senior Lecturer, Dr. Gayathri V, MDS, Senior Lecturer and Dr.Mohan Kumar S** for their valuable guidance that enabled me to comprehend this dissertation and reach its successful culmination.

This study wouldn't have come to existence without the effort and time of the faculties and residents at the **Department of Microbiology, PSG College of Arts and Science, Coimbatore**. I sincerely acknowledge **Dr.A. Michael, PhD, Associate Professor and Head, Department of Microbiology,** PSG College of Arts and Science, **Dr.Sentila Rajan, Ms. Divya. P** for their valuable guidance, encouragement and support for the successful completion of this study.

I am thankful to **Ms.Aditi Rao**, for her guidance in the statistical works of this study and for her valuable guidance that enabled me to comprehend this dissertation and reach its successful culmination. I am grateful to her for sparing her valuable time in guiding me through this thesis.

I am thankful to my seniors, my colleagues and my juniors,who have been together as friends and of great support throughout my period of study here. I am thankful to all other department staff members, my fellow colleagues in other departments, all UG staff members and non-clinical staffs of my department for their great support and encouragement.

I express my dearest gratitude to **parents, my brother**, and the special people in my life who contributed in various ways towards my study and this dissertation.

Last but not the least, I am greatly indebted to **God the Almighty**, for blessing me with all the good things in my life and guiding me throughout.

Dr. DEVINA DINAKAR

CONTENTS

INTRODUCTION

Elimination of microorganisms, debris and complete removal of pulp tissues from root canal system during endodontic therapy is an important determinant of its success. Hence, endodontic treatment requires effective debridement and disinfection of the root canal system[1]. Microorganisms are the primary etiological factor in the development of pulp and periapical diseases[2]. Chemo-mechanical preparations of the root canal reduce endodontic infection, but microorganisms are able to survive within the complex anatomy of the root canal system[3]. Nearly 30- 40% of the canal remain untouched despite proper instrumentation. Thus the use intracanal medicaments between appointments can enhance bacterial elimination before canal filling[4].

Root canal infections have a polymicrobialnature[4], hence anaerobic and facultative anaerobic microorganisms are usually found together in endodontic flare-ups and cases with post treatment diseases[5]. To ensure complete elimination of bacteria, an effective antimicrobial agent in the root canal is required for a predetermined period of time to ensure its efficacy and complete eradication of any remaining bacteria[7]. Therefore, antimicrobial agents used as inter-appointment medicaments must be able to penetrate through the dental tissues in the presence of microbes to reach a sufficiently high concentration in order to eliminate the disease-causing bacteria in a predictable manner [8].

Traditionally calcium hydroxide has been the choice as an intracanal medicament, because of its wide spectrum of action against many endodontic pathogens, which is thought to be mainly due to its alkalinity causing destruction of bacterial cell membrane[9]. However, calcium hydroxide is not equally effective against all bacterial species found in the root canals[10].

Nisin, a naturally occurring antimicrobial peptide, has antimicrobial activity against a wide rage of gram-positive bacteria and their spores[11], even against drug resistant E. faecalis isolates[12]. To increase the intracanal medicament stability and insolubility, chitosan can be used as a drug where it has added advantage of slow and controlled release of intracanal medicament[13, 14]. Till date only a few studies have shown the antimicrobial effect of intracanal medicaments using chitosan as a carrier on *Candida albicans* and *Enterococcus faecalis*[1].

The question of the role of intracanal medicaments becomes more complex in treatment of apical periodontitis with variable types of microorganisms. Most models used so far do not adequately reflect the complexity of the canal anatomy, and they do not simulate the clinical condition. Therefore, it is of importance to develop multispecies biofilm models resembling in vivo endodontic biofilm for studying root canal disinfection[15].

AIM AND OBJECTIVE

Thus purpose of this study was

(i) Isolation of *Enterococcus faecalis* and *Candida albicans* from plaque specimen and formation of biofilm model in teeth.

(ii) In vitro testing of antimicrobial effects of test medicaments (Nisin and Chitosan) with Calcium hydroxide used in root canal treatment in tooth biofilm model.

REVIEW OF LITERATURE

Shaik et al. (2014)[1]analyzed the sustained release of Triple antibiotic paste (TAP) and calcium hydroxide as intracanal medicaments in root canals using chitosan as a carrier and testing their antimicrobial efficacy against *C.albicans* and *E.faecalis* over a period of 2, 7, 21 days. Theyconcluded that combining TAP and calcium hydroxide with chitosan had a good antimicrobial effect against *C. albicans* and *E. faecalis.*

Gomes et al. (2003)[5]assessed the effectiveness of 2% chlorhexidine gluconate gel and $Ca(OH)_2$, separately and combined, as intracanal medicaments, in cylindrical specimens of bovine root dentine against E. faecalis. They concluded that chlorhexidine gel has a greater antibacterial activity against E. faecalis than $Ca(OH)_2$, but it loses this property if used for longer periods.

Gomes et al. (2003)[10]evaluated the effectiveness of 2% chlorhexidine gluconate gel and calcium hydroxide ($Ca(OH)_2$) as intracanal medicaments, in cylindrical specimen of bovine root dentine against *Enterococcus faecalis* for a period of 7 days. They concluded that 2% chlorhexidine gel alone was more effective against E. faecalis than calcium hydroxide. However, its antibacterial activity depended on how long it remained inside the root canal.

Ballal et al. (2009)[13]analysed the sustain release of Chlorhexidine with Chitosan and to investigate the antimicrobial activity of 2% Chlorhexidine gel, 2% Chitosan gel and their combination against *Candida albicans* and *Enterococcus faecalis.* They were examined at 24 and 72 hours interval. They concluded that combining Chlorhexidine gluconate gel with Chitosan gel may improve the antimicrobial activity of Chlorhexidine gel against *C. albicans* and *E. faecalis in vivo* rather than using 2% Chlorhexidine gel or 2% Chitosan gel alone.

Atila-Pekas et al (2013)[16] compared the disinfection capacities of calcium hydroxide, 1% chlorhexidine gluconate gel, bioactive glass, calcium hydroxide plus point(medicated gutta-percha with calcium hydroxide) and Activ Point (medicated gutta-percha with chlorhexidine diacetate) against *Enterococcus faecalis and Streptococcus mutans* for a period of 1 week. They concluded that chlorhexidine-impregated medicaments were more efficient than alkaline-pH-acting medicaments.

Lima et al. (2012)[17]evaluated the antimicrobial efficacy of calcium hydroxide-based intracanal medications used for different time periods against *E.faecalis.* They concluded that all calcium hydroxide-based medicaments were able to significantly reduce the presence of *E.faecalis* in the root canal system. The associations of Calen/CMCP (14 days) and Calen/CHX (7 or 14 days) were more effective in eliminating E.faecalis.

Madhubala et al. (2011)[18] evaluated and compared the antimicrobial activity of calcium hydroxide, triantibiotic mixture (TAM), and an ethanol extract of propolis as intracanal medicaments on *Enterococcus faecalis*–infected root canals and concluded that Propolis was more effective than TAM against E. faecalis at a 2-day time period, and both were equally effective at 7 days.

Lee et al. (2013)[19]evaluated the antibacterial efficacy of human b-defensin-3 (HBD3) compared with calcium hydroxide and chlorhexidine after 24 hours of treatment using commercial biofilm/ viability assay kit. The authors found out that HBD3 peptide exhibited more antibacterial activity against mature multispecies biofilms in vitro than either Calcium hydroxide or Chlorxidine.

Wu et al. (2014)[20]evaluated the antibacterial efficacy of silver nanoparticles (AgNPs) as an irrigant (irrigation for 2 minutes) or medicament (for a period of 7) days against *Enterococcus faecalis* biofilms formed on root dentin. They results showed that the antibiofilm efficacy of AgNPs depends on the mode of application. AgNPs as a medicament and not as an irrigant showed potential to eliminate residual bacterial biofilms during root canal disinfection.

D.A. Attia et al. (2015)[21]compared the antimicrobial effect of Calcium hydroxide paste (CaOH), Chlorhexidine gluconate (CHX) gel and Antibiotic- Corticosteroid paste against *Streptococcus mutans, Enterococcus faecalis* and *Candida albicans* in root canal

lumen and radicular dentin for a period of 7 days. They concluded that CHX was the best medication used to eliminate the three different tested organisms at the two experimental sites, root canal lumen and radicular dentin. *S. mutans* was the most sensitive tested microorganism to the whole tested medications, whereas *C.albicans* was the most resistant one. *E. faecalis* was more sussibtable to CHX than the other medications.

Chai et al. (2013)[22]evaluated the antimicrobial effectiveness of local application of two antibiotics- erythromycin, oxytetracycline and $Ca(OH)_2$ against *E. faecalis* biofilm in dentin for a period of 21 days. They concluded that the antimicrobial efficacy of both the antibiotics was shown to be more effective than the $Ca(OH)_2$, but none were able to completely eradicate *E. faecalis* biofilm in dentinal tubules.

Hemadri et al. (2011)[23]evaluated the antimicrobial efficacy of Nisin against *E.faecalis* in solution and also evaluated and compared the effect of Nisin and Calcium hydroxide against *E.faecalis* within the root canal system for a period of 14 days. They concluded that Nisin was effective at eradicating E.faecalis in pure culture and was more effective when compared to Calcium hydroxide in the elimination of this species with the root canal system.

Estrela et al. (2009)[24]discussed the antibacterial efficacy of intracanal medicament on bacterial biofilm. They reported that endodontic infections and the high clinical success required adequate disinfection assisted by the intracanal medicaments.

This reduces the bacterial population and favors the prognosis. The antimicrobial efficacy of intracanal medicaments on bacterial biofilm still needs to be confirmed.

Abbaszadegan et al. (2014)[25]synthesized and characterized silver nanoparticles (Ag NPs) with different surface charges in order to evaluate their cytotoxicity and antibacterial activity in the absence and presence of dentine compared with Sodium hypochlorite and chlorhexidine. The authors concluded that Ag NP surface charge was important in bactericidal efficacy against E. faecalis. The positively charged imidazolium-based ionic liquid- protected Ag NPs showed promising antibacterial results against E. faecalis and exhibited a high level of cytocompatibility to L929 fibroblasts.

Awawdeh et al. (2009)[26] investigated the antimicrobial activity of propolis-based intracanal medicament against *Enterococcus faecalis,* to find minimum time needed to achieve its optimal antibacterial effect using infected dentine models, and compared its antimicrobial efficacy with that of the non-setting calcium hydroxide paste when used as a short-term medication for 1 and 2 days. They concluded that propolis is very effective *ex vivo* in eliminating *E. faecalis* within 1 day and its effectiveness is not weakened by dentine. However, using calcium hydroxide alone in resistant endodontic cases where *E. faecalis* play a major role seems to be questionable.

Chau et al.(2015)[27]determined the relationships between the antibacterial activity of NaOCl and treatment time and biofilm age in early Enterococcus faecalis biofilms using a linear fitting procedure. They concluded that anti-bacterial activity of NaOCl

against early E. faecalis biofilms in root canals might follow a linear pattern depending on biofilm age or treatment time.

Nara et al. (2010)[28]compared the antimicrobial efficacy of 3 % NaOCl , MTAD and propolis against *E.* faecalis for a period of 48 hours. They concluded that MTAD was more effective than 3% NaOCl and propolis against *E. faecalis.*

Javidi et al. (2014)[29] evaluated the efficacy of $Ca(OH)_2$ with or without a silver nanoparticle suspension to eliminate *Enterococcus faecalis* from root canals. The samples were obtained at 1 and 7 days after root canal preparation. They concluded that the combination of $Ca(OH)_2$ and nanosilver as an intracanal medication significantly reduced the number of intracanal*E. faecalis* microorganisms. This study highlighted the efficacy of nanosilver in conjunction with calcium hydroxide to reduce colonies of *E. faecalis*, indicating its potential use as a root canal interappointment medicament.

Liu et al. (2012)[30]tested a casein peptide in its glycosylated form (kappa-casein glycopeptide, KCGP) and its non-glycosylated form (kappa-casein peptide, KCP) for antibacterial efficacy against Enterococcus faecalis in planktonic and biofilm cultures. They concluded that the casein-derived antimicrobial peptides KCGP and KCP inhibited growth of *E. faecalis* in the form of planktonic cells and also inhibited biofilm formation by the bacterium. These peptides, together with other antimicrobial agents, may have potential in the control of bacterial infection.

De Lucena et al. (2013)[31] evaluated the viability of E. faecalis in root canal dentine after placement of different root canal medications based either on CHX or on octenidine in vitro. They concluded that in contrast to calcium hydroxide, both CHX and octenidine-based intracanal medicaments were effective in decreasing the viability of E. faecalis. OCT showed the most favourable results and may have potential as an endodontic medicament.

Mejia. (2014)[32]evaluated the efficacy of Ca(OH)2, 2% CHX, and propolis against both *E. faecalis* and *C. albicans* using infected dentine models at two different depths (100 and 200 lm) after 14 days of application. He concluded that both CHX and propolis were the most effective against E. faecalis, whereas only CHX had the highest antifungal activity on C. albicans in dentine of extracted teeth.

Menezes et al. (2004)[33] evaluated the in vitro antimicrobial effectiveness of sodium hypochlorite (NaOCl), chlorhexidine (CHX) and five intracanal medicaments on C.albicans and E.faecalis. They concluded that 2.0% CHX solution was a more effective irrigant solution than 2.5% NaOCl against E. faecalis and $Ca(OH)_2$paste mixed with CPMC (Calen) was a more effective intracanal medicament than $Ca(OH)_2$ alone against E. faecalis and C. albicans inoculated in root canals.

Sathorn et al. (2007)[34] determined the extent to which calcium hydroxide intracanal medication eliminate bacteria from human root canals, compared with the same canals before medication, as measured by the number of positive cultures, in patients undergoing root canal treatment for apical periodontitis. They concluded that calcium hydroxide has limited effectiveness in eliminating bacteria from human root canal when assessed by culture techniques.

Siren et al. (2004)[35] measured the antibacterial effect of combinations of calcium hydroxide with iodine potassium iodide or Chlorhexidine against E. faecalis in a dentine infected model and evaluated the cytotoxicity of the combinations as compared to their components alone. They concluded that The antibacterial effect of IKI or CHX in combination with calcium hydroxide may prove to be of benefit in the treatment of certain types of persistent infections in primary and particularly in retreatment cases where E. faecalis is the most common isolate.

Turner et al. (2004)[36]determined whether nisin, a bacteriocin, would be effective at killing *Enterococcus faecalis* and *Streptococcus gordonii* cells in solution and within the root canal system. They concluded that nisin was effective at eradicating E. faecalis and S. gordonii cells in pure culture and was comparable with $Ca(OH)_2$ in the elimination of these species from within the root canal system.

Ambikathanaya. (2014)[37]Disinfection of pulp space is an important step during and after cleaning and shaping. Intracanal medicaments are used for root canal disinfection. It plays a vital role in the success of root canal treatment from the past multivisit to todays single visit technique in various forms. Recent advances in various fields led to the development of introducing newer medications as well as modifying the existing ones and their mode of applications.

Chinni et al. (2016)[40] determined the efficacy of Nisin against *E. faecalis* and its efficiency is compared with other intracanal medicaments like Calcium hydroxide, Chlorhexidine in human radicular dentin. They concluded that Nisin was effective at eradicating *E. faecalis* cells in pure culture and was comparable with chlorhexidine, positive control Vancomycin in elimination of *E. faecalis* from within the root canal system.

Somanath et al. (2015)[42]compared the efficacy of 2% CHX, Linezoid, and Nisin in reducing the colony forming unit (CFU) of E.faecalis and to compare the rapidity with which these medications act at intervals of 24 h, 72 h, and 1 week. They concluded that Nisin was found to be the most effective in reducing the bacterial count of Enterococcus faecalis in one week. It's action was found to be comparable with Chlorhexidine. Linezolid was found to be short acting with gradual decrease in antimicrobial action after 72 hours.

Tong et al. (2011)[43] determined whether MTAD in combination with nisin could exert a stronger inhibitory effect against E. faecalis, and also compared the antibacterial activities of MTAD, MTAN (substitution of doxycycline with nisin) and MTADN (doxycycline in conjunction with nisin) and investigated the synergetic effect of doxycycline and nisin on E. faecalis. They concluded that the combination of MTAD and nisin has significant activity against E. faecalis in vitro. We hope that these findings will lead to new treatment strategies for the eradication of E. faecalis, which is closely associated with persistent endodontic infection.

Rahman et al. (2013)[48] evaluate the antimicrobial activity of Matricariachamomilla, Chlorhexidine gel, Chitosan gel and their combination against Candida albicans and Enterococcus faecalis. They concluded that combining Chlorhexidinegluconate gel with Chitosan gel may improve the antimicrobial activity of Chlorhexidine gel against C.albicans and E.faecalis rather than using 1% Chlorhexidine gel or 1% Chitosan gel alone. Matricariachamomilla as 15% aq. base is not effective against E.faecalis and C.albicans.

Taneja et al. (2015)[58]compared the antimicrobial efficacy of an oxazolidinone (linezolid [LZ]), lantibiotic (nisin) and calcium hydroxide against *Enterococcus faecalis* biofilm formed on tooth substrate after 2 and 7 days. They concluded that LZ showed maximum antimicrobial potential against *E. faecalis* biofilm followed by nisin after 2 and 7 days. Calcium hydroxide showed the least antimicrobial potential against *E. faecalis* biofilm after 2 and 7 days. The antimicrobial effect of LZ and nisin was not

affected with lapse of time, but that of calcium hydroxide decreased significantly with increasing time period.

Grover et al. (2014)[60]investigated the release of calcium ions and measured the pH change in the surrounding environment when calcium hydroxide was combined with different vehicles at different time intervals. They concluded that all vehicles used except gutta- percha points containing calcium hydroxide maintained an alkaline pH for over 7 days Propylene glycol and chitosan as vehicles maintain an alkaline pH for a period of 1 month, in comparison with distilled water and calcium hydroxide points.

Elsaka et al. (2012)[61]evaluated the antibacterial activity of $Ca(OH)_2$ combined with Chitosan as an intracanal medicament and the effect of this new intracanal medicament on the bond strength of RealSeal sealer to radicular dentin. They concluded that $Ca(OH)_2$ intracanal medicament incorporating Chitosan solution as a vehicle exhibited an inhibitory effect on the growth of E. faecalis in the radicular dentin, compared to $Ca(OH)_2$ mixed with saline.

Peters et al. (2002)[62]**e**valuated the fate of microorganisms in root canals of teeth with infected pulps and periapical bone lesions with and without the use of calcium hydroxide medication. They concluded that although a calcium hydroxide paste was placed in the prepared canals, the number of positive canals had increased in the period between visits. However, the number of microorganisms had only increased to 0.93% of

the original number of CFU. Hence calcium hydroxide and sterile saline slurry, limits but does not totally prevent regrowth of endodontic bacteria.

Mohammadi & Abbott.(2009)[63] reviewed that disinfection of root canal systems is the primary aim of root canal treatment. This can be achieved by using various antimicrobial agents in the form of irrigants and medicaments. These agents are only used for relatively short periods of time ranging from minutes (for irrigants) up to days or several weeks (for medicaments) and therefore their long-term antimicrobial effects rely on whether or not the particular agent has any properties of substantivity. The short-term substantivity of commonly used antimicrobial agents show that substantivity of chlorhexidine lasts for up to 12 weeks and tetracycline for up to 4 weeks. However, it is not known whether the substantivity of these agents will last for longer periods of time as this has not been investigated.

Kawashima et al. (2009)[64] said that intracanal medicaments have been thought an essential step in killing the bacteria in root canals. Formocresol and its relatives were frequently used as intracanal medicaments, but it was pointed out that such bactericidal chemicals dressed in the canal distributed to the whole body from the root apex and so might induce various harmful effects including allergies. In modern endodontics, biocompatibility and stability are essential properties for intracanal medicaments. The more modern meaning of intracanal dressing is for a blockade against coronal leakage from the gap between filling materials and cavity wall. Calcium hydroxide has been determined as suitable for use as an intracanal medicament as it is stable for long periods,

harmless to the body, and bactericidal in a limited area. It also induces hard tissue formation and is effective for stopping inflammatory exudates.

MATERIALS AND METHODS

Materials used

- Extracted human teeth – single canal premolar
- Intracanal medicaments – Calcium hydroxide, Nisin, Chitosan
- Saline (0.9% w/v sodium chloride injection, NS, Baxter, India)
- Sodium Hypochlorite (Prime dental, India)
- Ethylene Diamine Tetra Acetic acid (Dentsply Maillefer,USA)
- Mitis Salivarius Agar (MSA)
- Sabouraud Dextrose Agar (SDA)

Armamentarium

- Test tubes (Borosil 27 ml, Riviera 15 ml)
- Petri dish
- Hot air oven
- Incubator (NSW, India)
- Autoclave (Unique clave C-79, Confident)
- Paper points (Dentsply)

- Sterile swab
- Micropipette (Eppendorf)
- Absorbent paper
- Diamond saw
- Endomotor (NSK, Endomate DT)
- K files (Mani, Japan)
- Disposable syringe (Dispovan)

Phase I- obtaining plaque sample and identification of microorganism

Plaque sample collection and Processing

Sub gingival plaque samples were collected from healthy human volunteers with a sterile periodontal curette from the subgingival area of upper first molar. Plaque samples were carried in 1.5ml eppendorf tubes filled with nutrient broth(Fig 1). Plaque samples are then inoculated onto 2ml of sterile Brain Heart Infusion Broth (BHIB) and Sabouraud Dextrose Broth (SDB).

Identification of microorganism

The overnight grown inoculated SDB and BHIB tubes were further streaked onto MitisSalivarius Agar (MSA) Sabouraud and Dextrose Agar (SDA) for the isolation of root canal pathogens (*Enterococcus faecalis*(fig 2)*& Candida albicans*(fig 3)).

Phase II- Antibacterial activity in teeth

Teeth selection and Standardization of Working Length

Ninety single-rooted human mandibular premolars with closed apices, extracted for orthodontic reasons were used in this study. The teeth were cleaned of superficial debris, calculus, and tissue tags and stored in normal saline to prevent dehydration before use. Each tooth was radiographed to confirm the presence of a single patent canal. Teeth with curved roots and those with more than one canal were excluded from the study. The tooth specimens were sectioned below the cementoenamel junction with a diamond disc to obtain a standardized tooth length of 13 mm (Fig 4). The canals were accessed, and initially a size #10 Stainless Steel (SS) K file was inserted into the canal until the file tip was just visible at the apical foramen. The working length (WL) was kept 1mm short of the apical foramen.

Standardization of Apical Canal Dimension

To facilitate the standardization of the apical canal geometry, the root canals were instrumented using Protaper rotary system in conjuction with 2.5% Sodium hypochlorite

(NaOCl) irrigation. Canals were prepared with ProTaper instruments were used according to manufacture's instructions upto F3.

Sterilization of tooth

The roots were rinsed in water for 30 min, then rinsed with EDTA for 5 min in an ultrasonic bath to remove smear layer and rinsed in water for a further 30 mins. The teeth were then stored in sterile water until used. Each root was dried, coated externally with clear nail varnish and autoclaved for further study.

Experimental Inoculation of Root Canal:

Each root canal was inoculated with cultured bacterial solution *of Enterococcus faecalis* and *Candida albicans* upto the canal entrance using a sterile pipette. The specimens were divided into three groups with 30 teeth in three experimental group (Fig 6).

Group I – *Enterocccus faecalis*

Group II - *Candida albicans*

Group III – Blank (uninfected + untreated).

All samples were incubated in a closed eppendorf at 37^{o}C for 14 days. The canals are re-inoculated with fresh bacterial samples at every 3 days interval. The inoculum establishment of *Enterococcus faecalis* and *Candida albicans* inside the root canal was

confirmed by streak plate using toothpicks onto sterile BHI agar and SD Agar plates.

Placement of Intracanal Medicaments:

After 14days, the canal contents were rinsed with 5 ml saline and dried. The specimens were then subdivided into five subgroups (fig 7-10) with six teeth under each medicament (fig 11)

Subgroup A – Saline (Negative control)

Subgroup B – Calcium hydroxide

Subgroup C - Nisin

Subgroup D – Chitosan

Subgroup E – Chlorhexidine

The canals were then sealed with dental wax and all samples were incubated at 37^{o}C. Readings were then taken at 1, 7, 14 and 21 days. The wax seal was then removed from each of the canals. Sterile paper points were inserted into root canals. After adsorption of the canal contents for 1min, the points were dipped into sterile broth medium, incubated overnight under appropriate condition. After incubation, they were plated onto sterile BHI agar and SD Agar for count of *E.faecalis* and *C.albicans* in terms of CFU/ml (fig 12).

Antibacterial efficacy

The antibacterial efficacy of the intracanal medicaments against various microorganisms was evaluated by Turbidity Testing (Optical Density at 600nm) and Culture Study (Colony Counting).

Turbidity Testing – once the sampling from the root canals were done with absorbent paper points, it was introduced into another test tube containing sterile nutrient broth and incubated for 24 hours to check for turbidity (fig 13). The intensity of turbidity was as checked by the optical density in spectrophotometer which corresponded to the amount of residual bacteria present in the root canals after placement of medicament (fig 16-25).

Culture Study - After the placement of medicaments, absorbent points were used to take sample from the root canals. These absorbent points were introduced into a test tube containing sterile broth and incubated at 37^{o}C for 24 hours. After incubation the samples were plated onto sterile BHI agar and SD agar for count of *E.faecalis* and *C.albicans* in terms of CFU/ml (fig 14,15)). Colony counting was done to determine the antibacterial efficacy. The number of colonies is directly proportional to the amount of residual bacteria present in the root canals after placement of medicament.

Statistical Analysis

The statistical analysis was processed with the SPSS 17 software system (Chicago, USA). As the data does not follow normality, non parametric test was used to analyse the data . For analyzing Colony Counting and Optical Density Friedman test and paired t test was done at $P < 0.05$.

Fig 1:Subgingival plaque sample

Fig 2: *E. fecalis*

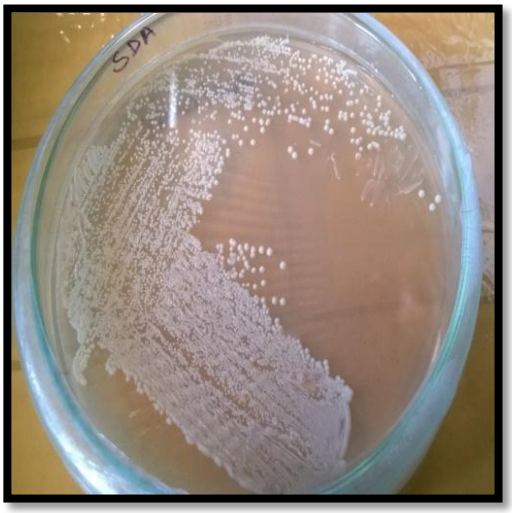


Fig 3: *Candida albicans*

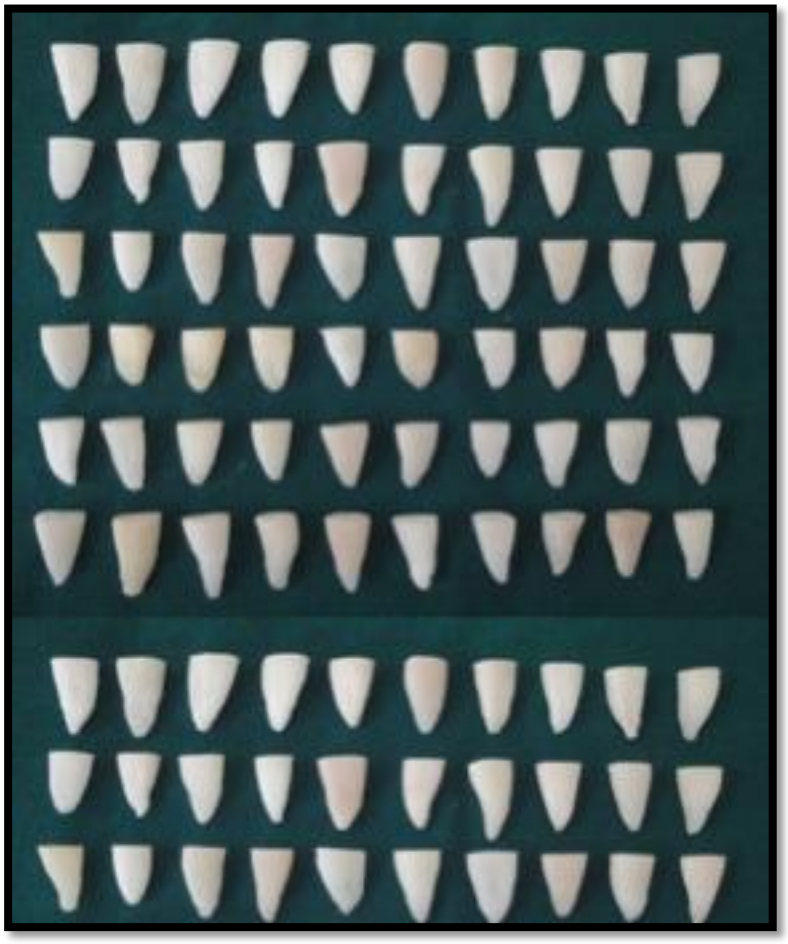


Fig 4: Decoronated Teeth

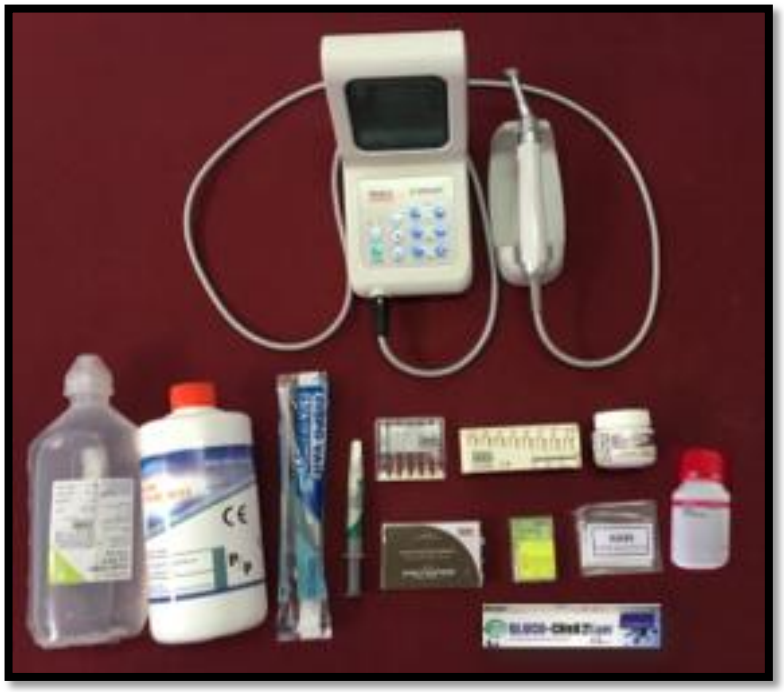


Fig 5: Materials used for the sudy

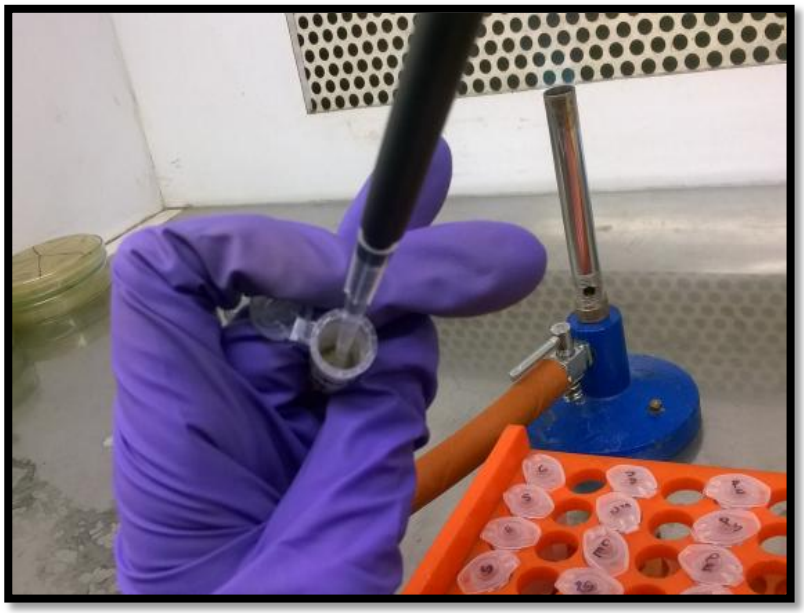


Fig 6: Inoculation of bacterial sample into teeth

Fig 7: Calcium hydroxide

Fig 8: Nisin

Fig 9: Chitosan

Fig 10: Chlorhexidine

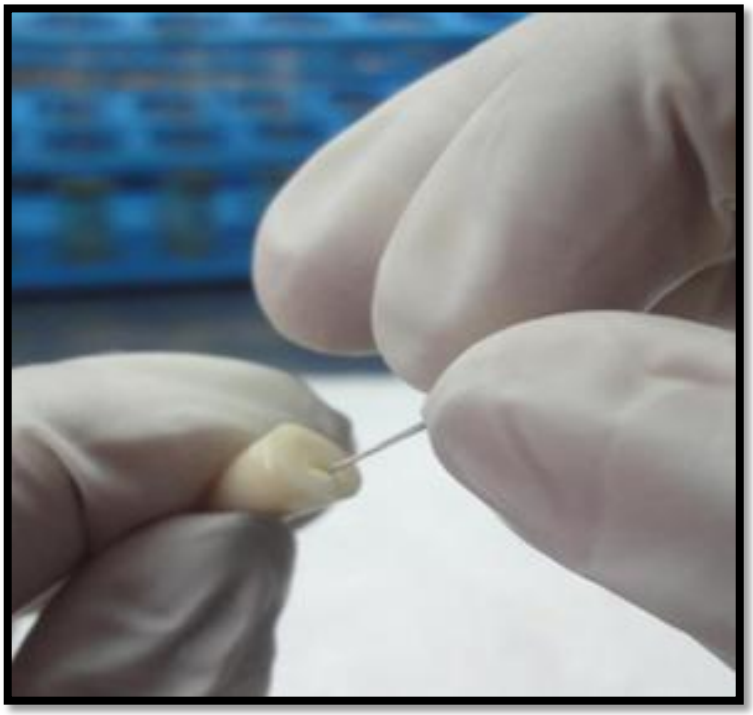


Fig 11: Placement of medicament

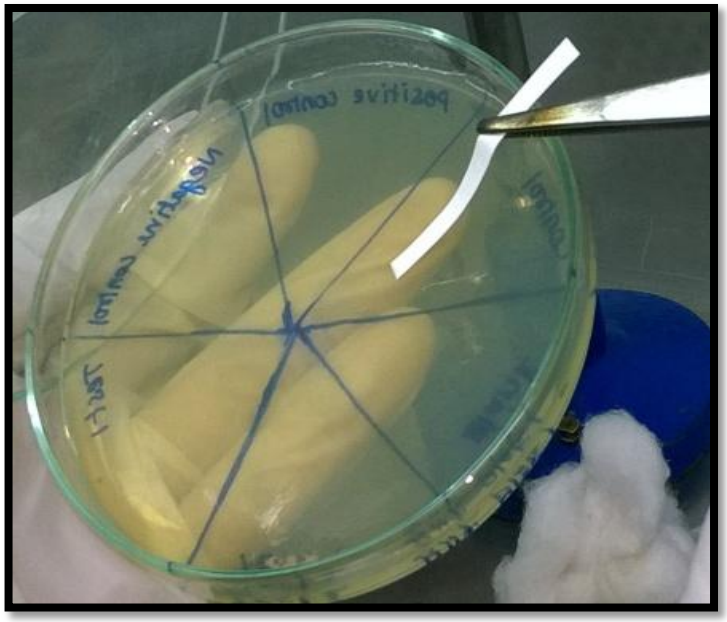


Fig 12: Plating

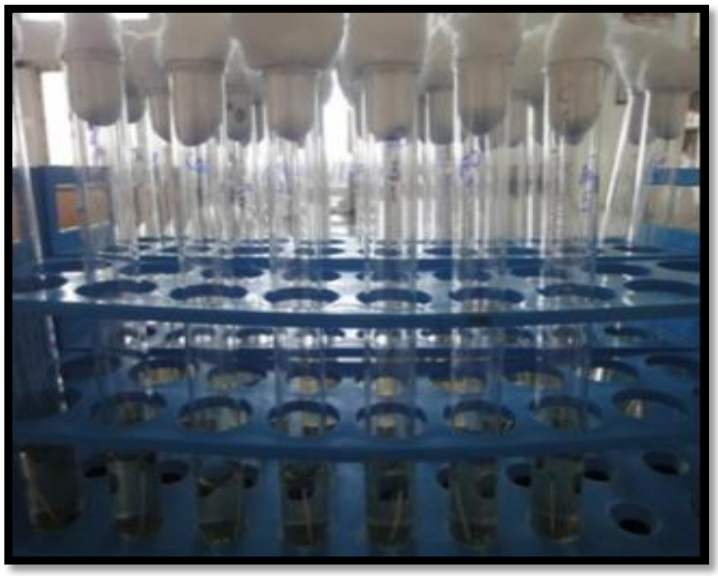
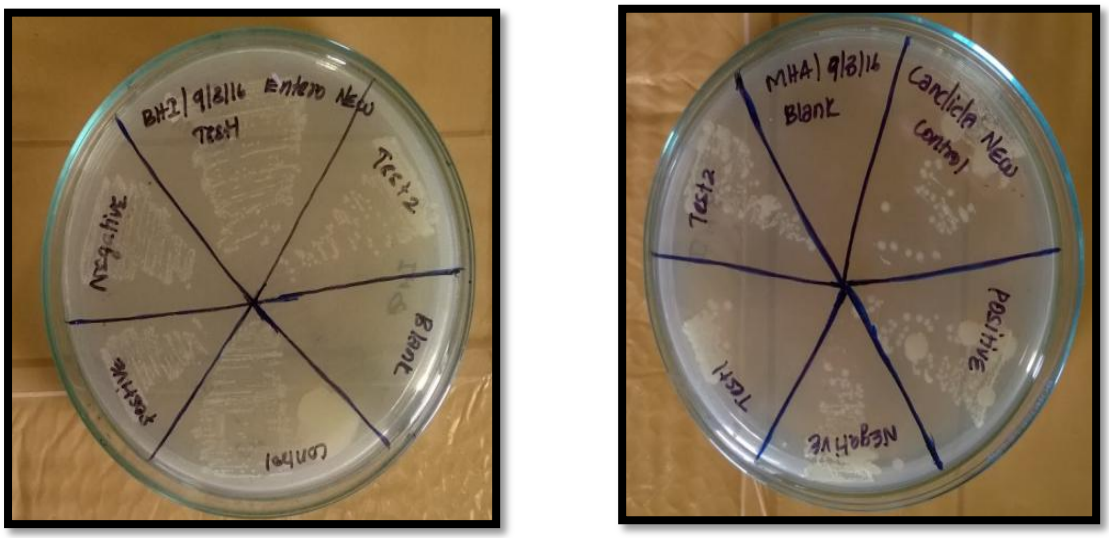


Fig 13: Samples collected for turbidity testing

RESULTS

The results obtained from Turbidity Testing and Colony Counting revealed that complete elimination of bacteria was not achieved in any of the experimental groups.

The colony counts for different medicaments were obtained on Day 1, Day 7, Day 14 and Day 21. As the same measure was obtained across different time points the Friedman Test was used to estimate the significance of use of various medicaments. Results of the Friedman test showed that Chitosan was most effective in causing reduction in colony counts and was highly significant (χ^2 (3) = 0.029, $p<0.05$) followed by Nisin (χ^2 (3) = 0.032, $p<0.05$) and Chlorhexidine (χ^2 (3) = 0.042, $p<0.05$). Calcium hydroxide and Saline did not show statistically significant difference.



Enterococcus faecalis **Candida albicans**

Fig 14, 15 : Confirmation of inoculum in root canal by streak plating on BHI and SDA agar

To better study the variation is colony counts the %RCC (percentage of reduction in colony count) was estimated using the formula:

$$\% \text{ RCC} = \frac{\text{Initial colony count} - \text{Final colony count}}{\text{Initial colony count}} \times 100$$

The results are provided in the Tables:

Graph1: Showsthe percentage of reduction in colony count between days 1-7

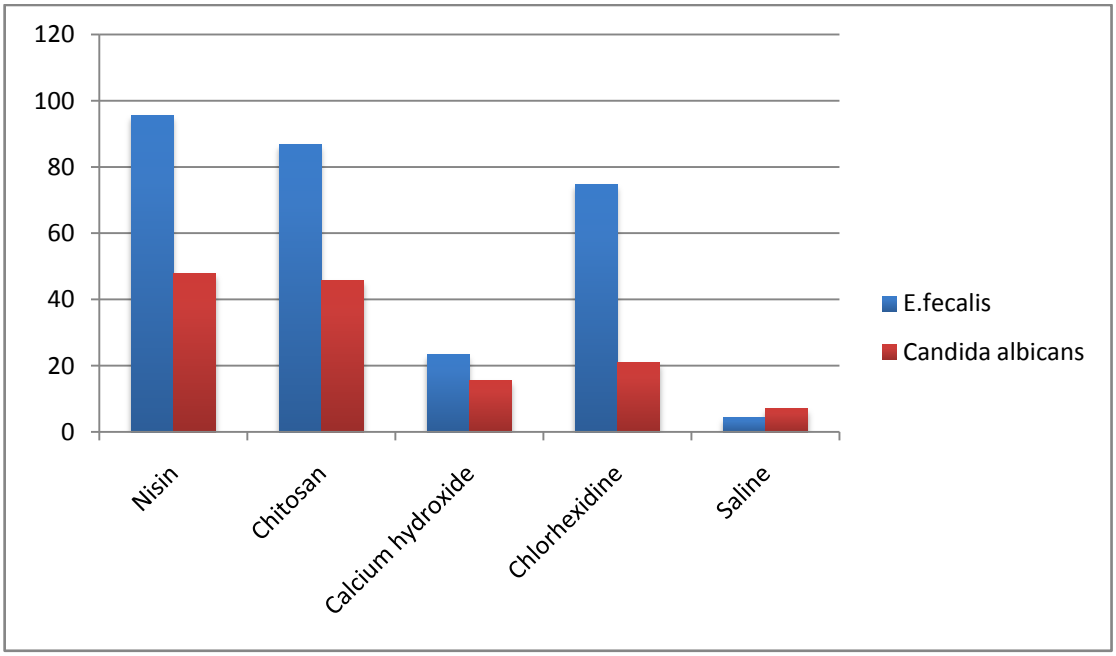


Table 1: Shows the percentage of reduction in colony count between days 1-7

	Nisin	Chitosan	Calcium Hydroxide	Chlorhexidine	Saline
E.faecalis	95.6	86.77	23.49	74.76	4.28
Candida albicans	48	45.83	15.55	21.05	7.14

Graph2: Shows the percentage of reduction in colony count between days 7- 14

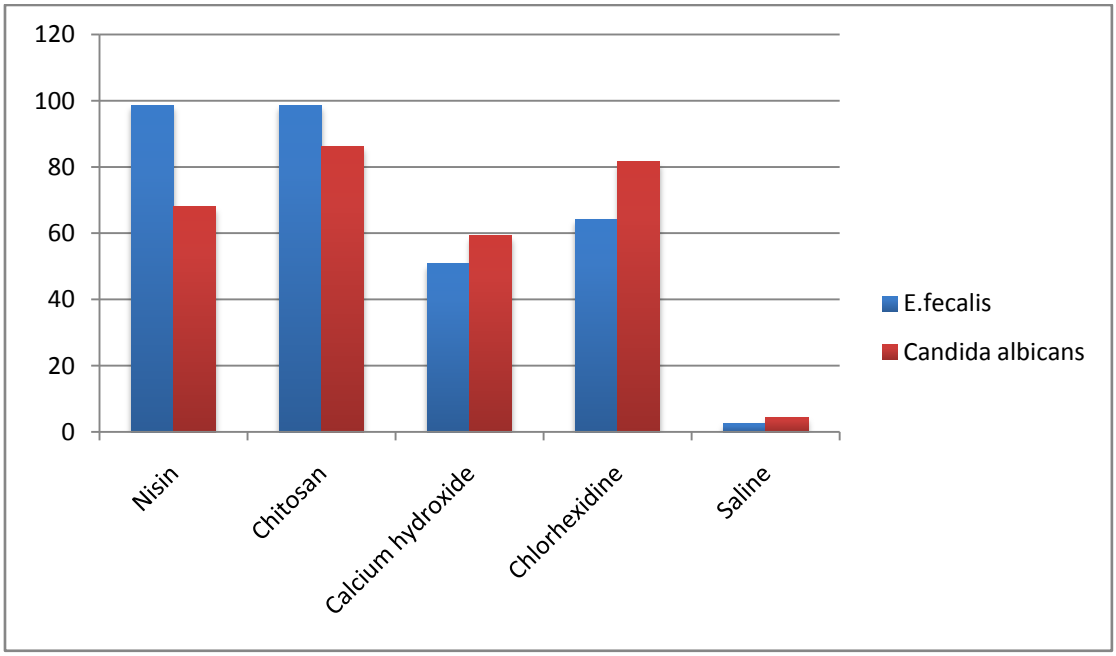


Table 2: Shows the percentage of reduction in colony count between days 7- 14

	Nisin	Chitosan	Calcium Hydroxide	Chlorhexidine	Saline
E.faecalis	98.6	98.70	50.79	64.28	2.5
Candida albicans	68	86.25	59.25	81.57	4.47

Graph3: Shows the percentage of reduction in colony count between days 14- 21

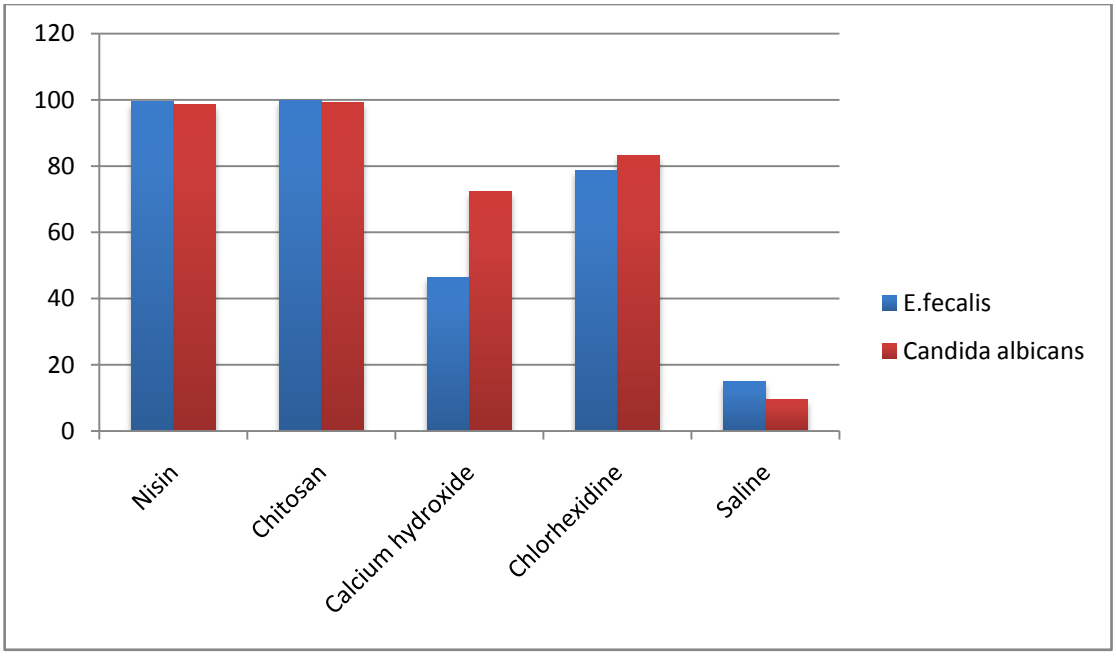


Table 3: Shows the percentage of reduction in colony count between days 14-21

	Nisin	Chitosan	Calcium Hydroxide	Chlorhexidine	Saline
E.faecalis	99.36	99.93	46.5	78.57	15
Candida albicans	98.5	99.16	72.22	83.15	9.52

The inhibition growth of *E.fecalis*at the end of 1, 7, 14 and 21 days was shown to be maximum for Nisin (Table 1,2,3, Graph 1,2,3). Nisin and Chitosan showed the maximum %RCC compared with Chlorhexidine, Calcium hydroxide and Saline. Table 3, Graph 3 shows that Chitosan exhibited maximum reduction for *Candida albicans* at days 14-21. Nisin requires 14 days to exert its antibacterial efficacy (99.75%) whereas Chitosan requires 21 days to exert its maximum antibacterial efficacy (99.93%).

To analyse the variation in colony counts using Nisin and Chitosan, difference in colony counts between Day 1 and Day 21 were obtained. The difference in counts obtained was then statistically analysed using Wilcoxon Sign Rank Test which showed statistically significant difference between the two groups (p=0.028).

Optical density across the experimental groups

Fig 16 Turbidity testing for control with E.faecalis

Fig 17 Turbidity testing for control with Candida albicans

Fig 18 Turbidity testing for Nisn withE.faecalis

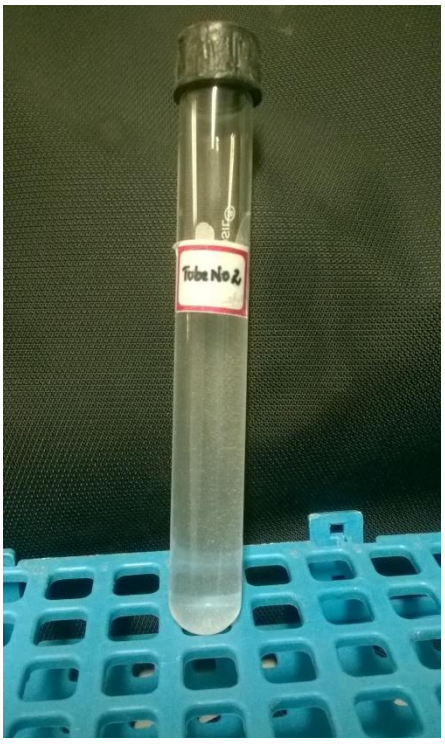


Fig 19 Turbidity testing for Nisn with Candida albicans

Fig 20 Turbidity testing for Chitosan with E.faecalis

Fig 21 Turbidity testing for Chitosan with Candida albicans

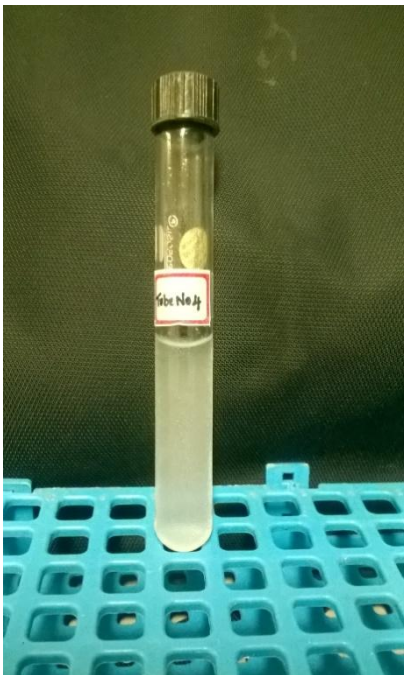


Fig 22 Turbidity testing for Calcium hydroxide with E.faecalis

Fig 23 Turbidity testing for Calcium hydroxide with Candida albicans

Fig 24 Turbidity testing for Chlorexidine with E.faecalis

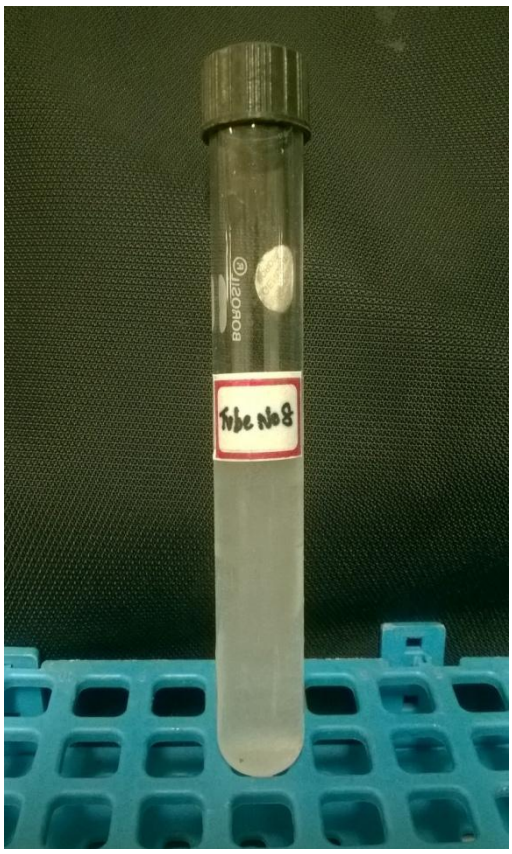


Fig 25 Turbidity testing for Chlorexidine with Candida albicans

To statistically analyse the difference in optical density between Day 1 and Day 21, paired t test was used (Table 4). On analysis it was seen that Chitosan showed highly significant difference $t(2)= 0.019$ ($p<0.05$), and Nisin showed significance as well, $t(2)= 0.042$ ($p<0.05$). However Chlorhexidine and Calcium hydroxide did not show statistically significant difference ($p>0.05$).

Table 4: OPTICAL DENSITY using Paired Samples Test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Nisin	.40000	.16371	.09452	.00667	.80667	4.232	2	.042
Chitosan	.34667	.08386	.04842	.13833	.55500	7.160	2	.019
Calcium hydroxide	-.01667	.56695	.32733	-1.42505	1.39172	-.051	2	.964
Chlorhexidine	-.18333	.39171	.22615	-1.15638	.78972	-.811	2	.503

This shows that each medicament has varied levels of action on the bacteria. However seeing the mean values of difference in colony counts it can be said that more reduction was seen using Chitosan >Nisin>Chlorhexidine> Calcium hydroxide (as Chitosan showed most reduction in colony counts and Calcium hydroxide showed the least).

DISCUSSION

Disinfection of pulp space is an important step during and after cleaning and shaping. Intracanal medicaments are used for root canal disinfection[37]. **Walton** wrote, "Intracanal medicaments have traditionally gone hand-in-glove with endodontics. They are generally considered to be an integral part of treatment and important to the success of root canal therapy"[38]. Intracanal medicaments have been thought an essential step in killing the bacteria in root canals; however, in modern endodontics, cleaning and shaping may be assuming greater importance than intracanal medicaments as a means of disinfecting root canals. The more modern meaning of intracanal dressing is for a blockade against coronal leakage from the gap between filling materials and cavity wall[39].

Calcium hydroxide has been suitable for use as an intracanal medicament for a long period of time. It plays an important role in endodontics through its versatile action like inducing tissue formation, exerting antibacterial action, and interrupting the nutrient supply to remaining bacteria[40]. The antimicrobial ability of calcium hydroxide is dependent upon direct contact with bacteria. Moreover, calcium hydroxide is not very effective in eliminating bacteria from the dentinal tubules. *Enterococcus faecalis* is small enough to proficiently invade and live within dentinal tubules[13]. **Orstavik et al** reported that *Enterococcus faecalis* present in the dentinal tubules are resistant to calcium hydroxide intracanal dressing over 10 days[41].

Nisin (NI) is a naturally occurring antimicrobial peptide produced by strains of *Lactococcus lactis* and was discovered in 1928[42]. It has been found to inhibit the growth and spore germination of many gram-positive bacteria and has an antibacterial efficacy against multidrug-resistant bacteria including *Enterococcus faecalis*[43]. It also disrupts the cellular membrane inducing leakage of small intracellular contents from the cell[37]. Nisin acts by inserting into the bacterial plasma membrane and triggering the activity of bacterial murein hydrolases which results in damage or degradation of the peptidoglycans and lysis of cells. This induces the leakage of small intracellular contents from the cell[36].It has been found to have a broad spectrum of activity against gram-positive organisms and weak antibacterial activity against gram-negative bacteria[44].

Chitosan is a cationic polymer derived from the exoskeleton of crustaceans (such as crabs)[45]. It is composed of copolymers of glucosamine and N-acetyl glucosamine[46]. An important property of chitosan for study as an excipient is its ability to become hydrated and form gels in acidic aqueous environments and is thus used to prepare slow release drug delivery systems[47]. **Rahman et al** conducted a study by using a Combination of CHX and Chitosan. Even though Chitosan has antimicrobial effect against C. albicans and E. faecalis, it proved to be less effective than Chlorhexidine gluconate when it was used alone[48].

Chlorhexidine (CHX) is an antiseptic with a broad antimicrobial spectrum and high substantivity[21]. Chlorhexidine gluconate gel is an alternative for root canal

medication because of its broad-spectrum antimicrobial effect[49]. It acts by adsorbing onto the cell wall of microorganisms causing intracellular component leakage[16]. Its efficacy is because of the interaction of the positive charge of the molecule and the negatively charged phosphate groups on the microbial cell walls there by altering the cells osmotic equilibrium. This increases the permeability of cell wall which allows the CHX molecule to penetrate into the bacteria[50].

The use of intracanal medications possessing antimicrobial properties between appointments may reduce or eliminate bacteria in the root canal system and significantly increase the success of root canal treatment [51]. This study was conducted to compare and evaluate the antimicrobial effect of different intracanal medications including Calcium hydroxide, Chlorhexidine gluconate gel, Nisin and Chitosan against two different microorganisms *E. faecalis* and *C. albicans.*

The biofilm model used in the current study attempts to replicate the heterogeneous nature of an in vivo biofilm and the versatility and high reproducibility of this model makes it a potentially useful vehicle to study the effects of treatment on biofilm removal. The model described here provides a method of studying the biofilm that have the following similarities with those found in vivo[15]:

1. They are grown from subgingival bacteria, which are the source of bacteria in endodontic infections.
2. They are grown on root dentin

Colony Counting and Turbidity Testing (Optical Density at 600nm) were chosen to evaluate the antibacterial efficacy of intracanal medicaments as they would signify the quantity of live residual bacteria present in the root canals. Microbial root culturing is commonly used to assess the effectiveness of endodontic treatment measures. In a study by **Gomes et al. 1995,** evaluated themoleculartechnique and culture technique and concluded that molecular techniques cannot differentiate between viable and dead cultures. On the other hand, using culture techniques a minimum concentration of microorganism is necessary for their isolation, and hence for their recognition in the clinical situation.

Optical density measured in a spectrophotometer at 600nm, can be used as a measure of the concentration of bacteria in a suspension. As visible light passes through the bacterial suspension, the light is scattered. The intensity of turbidity as checked by the optical density in spectrophotometer corresponds to the amount of residual bacteria present in the root canals after placement of medicament. In this study, Chitosan and Nisin showed significant difference between day 1 and day 21 but there was no significant difference between the other groups.

Enterococcus faecalis has been found to be one of the most persistent microorganisms, with a prevalence of 24 to 77% in root filled teeth with periradicular lesions[52]. Studies have shown *E.faecalis* to be viable inside the root canal dentin up to a period of 12 months[53]. Therefore, it is important to consider treatment regimens aimed at

eliminating or preventing the infection of E.faecalis during all the phases of root canal treatment.

Candida albicans is the most common species of fungi cultured from root canals of teeth with failed endodontic treatment[54]. The virulence mechanism of *C. albicans* that would promote colonisation in the root canal may be due to its collagenolytic activity that may make it possible to use dentin as a nutrient source[55]. Studies have reported that both *C. albicans* and *E. faecalis* are resistant to the antimicrobial action of calcium hydroxide, a commonly used intracanal medicament, but are sensitive to antimicrobial action of Chlorhexidine gluconate[56,57].

In the present study, the antibacterial activity through colony counting was obtained on day 1, day 7, day 14 and day 21 they showed that chitosan was most effective followed by Nisin, Chlorhexidine, Calcium hydroxide.

To better study the variation is colony counts the %RCC (percentage of reduction in colony count) was also estimated.

The inhibition growth of *E.fecalis* at the end of 1, 7 14 and 21 days was shown to be maximum for Nisin. These findings are similar to the study by **Taneja et al[58], Hemadri et al (2011)[23]** and **Turner et al(2004)[36]** in which the antibacterial efficacy of Nisin against *E.fecalis* was compared with calcium hydroxide. The studies showed that Nisin was able to effectively reduce the bacterial count of *E.fecalis* when compared to

calcium hydroxide. This might be due to the different mode of action of Nisin than that of calcium hydroxide

Studies by **Rahman et al (2013)[48]**, **Elaka et al (2012)[59]**, **Grover et al (2014)**[60] showed that the antibacterial efficacy of Chitosan gel, Chlorhexidine gel and their combination against *C.albicans* and *E.fecalis* and was found that combination of chlorhexidine with Chitosan was better than using plain Chlorhexidine or Chitosan. In another study by **Shaik et al (2014)**[1], **Elsaka et al (2012)[61]** , $Ca(OH)_2$ + chitosan combination was more effective in inhibiting the growth of *E. faecalis* and *C. albicans* when compared with $Ca(OH)_2$ + saline combination. In the present study, Chitosan exhibited maximum reduction for *Candida albicans* than *E.fecalis.*

In this study, the antibacterial efficacy of Chlorhexidine was inferior to Chitosan, unlike the results of the study by **Ballal et al. (2009)[13]**,in which the author compared the antibacterial efficiency of Chitosan, Chlorhexidine and their combination. The antimicrobial effect of Chitosan against *C. albicans*and *E. faecalis*, proved to be less effective than Chlorhexidine gluconate when it was used alone. This could be because strains of E.fecalis and C.albicans were used in that study, unlike a biofilm model that was used in our study.

The findings of these study demonstrated that Nisin and Chitosan have a good anti- microbial effect against *C. albicans* and *E. faecalis*. However, the medicament that is effective against single microbe *in vitro* may not necessarily be effective against the

same microbe *in vivo* because root canal system contains multiple microorganisms. Further studies using the same medicaments in failed root canal cases *in vivo* have to be conducted. The duration of action and the depth of penetration into the dentinal tubules of these medicaments also need to be investigated and compared.

The endodontic biofilm used in the present study was monospecies bacterial cultures. The biofilm models used does not adequately reflect the complexity of the root canal environment, and they do not replicate the clinical situation. Therefore, it is important to develop multispecies biofilm models resembling in vivo endodontic biofilms for studying root canal disinfection.

As Chitosan has mucoadhesive property, whether it has got similar adhesive property to the root canal dentin also needs to be investigated because this property might help in prolonged action of the medicament within the root canal system[13].

The ability of Nisin to effectively kill *E.faecalis* by a mechanism that is not reliant on achieving a high pH may provide a means to eliminate this species by a method to which it has no defense mechanism. Nisin is reported to be active against a broad range of Gram-positive bacteria. However, infection of radicular dentinal tubules is dominated by Gram-positive species namely, *Streptococci* and *Enterococci*. Given the association of *E. faecalis* in cases of chronic failure in endodontically treated teeth, a medication aimed specifically at this species may be of value. This may be especially pertinent in cases of conventional endodontic retreatment where *E. faealis* is the most commonly recovered species[40].

In this in vitro study, Chitosan and Nisin showed better results when compared to chlorhexidine and calcium hydroxide. Investigations regarding the efficacy of these intracanal medicament in vivo and multispecies biofilm model needs to be further studied for better understanding.

SUMMARY AND CONCLUSION

The current study aimed to isolate *Enterococcus faecalis and Candida albicans* from the plaque sample and determine their antimicrobial efficacy of the test medicaments- Nisin and Chitosan against theses microorganisms.

Plaque samples were collected from healthy human volunteers and incubated overnight. They were further streaked onto Mitis salivarius agar and Sabouraud Dextrose Agar for the isolation of *Enterococcus faecalis* and *Candida albicans.*

Ninety single-rooted human mandibular premolars were decoronated, debrided and following cleaning and shaping, they were randomly grouped into three groups consisting of 30 teeth each.

Group I – *Enterocccus faecalis*

Group II - *Candida albicans*

Group III – Blank (uninfected + untreated).

All the samples were incubated at 37°C for 14 days for the growth of the microorganisms. After 14 days, the contents were rinsed with 5ml saline and were further subdivided into five subgroups with six teeth under each medicament.

Subgroup A – Saline (Negative control)

Subgroup B – Calcium hydroxide

Subgroup C - Nisin

Subgroup D – Chitosan

Subgroup E – Chlorhexidine

The canals were then sealed and all the samples were incubated at 37°C and the readings were taken on days 1,7, 14, and 21.

The antibacterial efficacy of the intracanal medicaments against various microorganisms was evaluated by Turbidity Testing (Optical Density at 600nm) and Culture Study (Colony Counting).

The results obtained from Turbidity Testing and Colony Counting revealed that complete elimination of bacteria was not achieved in any of the experimental groups. Chitosan was most effective in causing reduction in colony counts followed by Nisin and Chlorhexidine. The inhibition growth of *E.fecalis* at the end of 1, 7, 14 and 21 days were shown to be maximum for Nisin. Nisin and Chitosan showed the maximum %RCC compared with chlorhexidine, calcium hydroxide and saline. Chitosan exhibited maximum reduction for *Candida albicans* at days 14-21. Nisin requires 14 days to exert its antibacterial efficacy (99.75%) whereas Chitosan requires 21 days to exert its maximum antibacterial efficacy (99.93%). This shows that each medicament has varied levels of action on the bacteria. However seeing the mean values of difference in colony

counts it can be said that more reduction was seen using Chitosan >Nisin>Chlorhexidine> Calcium hydroxide (as Chitosan showed most reduction in colony counts and Calcium hydroxide showed the least).

Nisin and Chitosan have a good antimicrobial effect against *C. albicans* and *E. faecalis*. However, the medicament that is effective against single microbe *in vitro* may not necessarily be effective against the same microbe *in vivo* because root canal system contains multiple microorganisms. Further studies using the same medicaments in failed root canal cases *in vivo* have to be conducted.

BIBLIOGRAPHY

1- Shaik J, Garlapati R, Nagesh B, Sujana V, Jayaprakash T, and Naidu S. Comparative evaluation of antimicrobial efficacy of triple antibiotic paste and calcium hydroxide using chitosan as carrier against Candida albicans and Enterococcus faecalis: An *in vitro* study. J Conserv Dent.2014 Jul-Aug; 17(4): 335–339.

2- Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J Endodontic Supplement 2007;52:(1 Suppl):S64-S82

3- Lana PEP , Scelza MFZ , Silva LE , Mattos-Guaraldi AL , Hirata- Ju´nior R . Antimicrobial activity of calcium hydroxide pastes on Enterococcus faecalis cultivated in root canal systems. Braz Dent J 2009;20:32-6.

4- Bystrom A, Claesson R, Sundqvist G. The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. Dent trautamol 1985 October;1(5):170-175.

5- Gomes BP, Pinheiro ET, Gade-Neto CR, Sousa EL, Ferraz CC, Zaia AA, Teixeira FB, Souza-Filho FJ. Microbiological examination of infected dental root canals.Oral Microbiol Immunol. 2004 Apr;19(2):71-6.

6- Siqueira JF, Rocas IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009 Nov;88(11):969-81

7- Turk BT, Sen BH, Ozturk T. *In vitro* antimicrobial activity of calcium hydroxide mixed with different vehicles against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod.2009;108:297–301.

8- Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32:361- 369.

9- Tronsted L, Andreasen JO, Hasselgren G, Kristerson L, Riis I. pH changes in dental tissues after root canal filling with calcium hydroxide. J Endod 1980; 7:17-21.

10- Gomes BP, Ferraz CC, Vianna ME et al. (2002) In vitro antimicrobial activity of calcium hydroxide pastes and their vehicles against selected microorganisms. Braz. Dent. J.2002; 13(3):155–61.

11- Kuipers OP, Rollema HS, Yap WMGJ, Boot HJ, Siezen RJ, de Vos WM. Engineering dehydrated amino acid residues in the antimicrobial peptide Nisin. J. Biol. Chem. 1992; 267, 24: 340-346.

12- Severina E, Severin A, Tomasz A. Antibacterial efficacy of Nisin against multidrug-resistant Gram positive pathogens. J Antimicrob Chemoth 1998; 41: 341-347.

13- Ballal N, Kundabala M, Bhat KS, Acharya S, Ballal M, Kumar R, et al. Susceptibility of Candida albicans and Enterococcus faecalis to Chitosan, Chlorhexidine gluconate and their combination *in vitro*. Aust Endod J.2009;35:29–33.

14- Wang JJ, Zeng ZW, Xiao RZ, Xie T, Zhou GL, Zhan XR, et al. Recent advances of chitosan nanoparticles as drug carriers. Int J Nanomedicine.2011;6:765–74.

15- Lin j, Shen y, Haapasalo m. A comparative study of biofilm removal with hand, rotary nickel-titanium, and self-adjusting file instrumentation using a novel in vitro biofilm model. J Endod. 2013 May;39(5):658-63

16- B. Atila-Pektas, P. Yurdakul, D. Gulmez, O. Gorduysu. Antimicrobial effects of root canal medicaments against Enterococcus faecalis and Streptococcus mutans. Int Endontic J 2013; 46: 413-418.

17- Lima RKP, Guerreiro-Tanomaru JM, Faria-Junior JB & Tanomaru-Filho M. Effectiveness of calcium hydroxide-based intracanal medicaments against Enterococcus faecalis. Int Endontic J 2012; 45:311-316.

18- Madhubala MM, Srinivasan N,and Ahamed S. Comparative Evaluation of Propolis and Triantibiotic Mixture as an Intracanal Medicament against Enterococcus faecalis. J Endod 2011 Sept; 37:1287-1289.

19- Lee J, Chang SW, Perinpanayagam H,Lim S, Park Y,Han SH, Baek, Qiang Zhu SH, Bae K and Kum KY. Antibacterial Efficacy of a Human b-Defensin-3 Peptide on Multispecies Biofilms. J Endod 2013; 39:1625-1629.

20- Wu D,Fan W,Kishen A, Gutmann JL,and Fan B. Evaluation of the Antibacterial Efficacy of Silver Nanoparticles against Enterococcus faecalis Biofilm. J Endod 2014; 40:285-290.

21- Attia DA , Farag AM , Afifi IK, AM. Darrag. Antimicrobial effect of different intracanal medications on various microorganisms. Tanta Dent J 2015; 12: 41-47.

22- Chai WL, Hamimah H & Abdullah M. Evaluation of Antimicrobial Ef cacy of Antibiotics and Calcium Hydroxide against *Enterococcus faecalis* Biofilm in Dentine. Sains Malaysian 2013; 42(1): 73-80.

23- Hemadri M ,Thakur s,Sajjan G. Nisin Vs Calcium Hydroxide – Antimicrobial Efficacy On Enterococcus Feacalis – An In-vitro Study. Int J contemp dent June 2011; 2(3): 55- 61.

24- Estrela C, Blitzkow G, Poli J.A, Rodrigues C. Antibacterial efficacy of intracanal medicaments on bacterial biofilm: a critical review.J Appl Oral Sci. *2009;17(1):1-7.*

25- Abbaszadegan A, Nabavizadeh M, Gholami A, Aleyasin Z. S, Dorostkar S, Saliminasab M, Ghasemi Y, Hemmateenejad B& Sharghi H. Positively charged Imidazolium-based ionic liquid-protected silver nanoparticles: a promising disinfectant in root canal treatment. Int Endontic J 2015; 48: 790-800.

26- Awawdeh L, Beitawi M , and Hammad M. Effectiveness of propolis and calcium hydroxide as a short-term intracanal medicament against *Enterococcus faecalis* :A laboratory study. Aust Endod J 2009; 35: 52–58.

27- Chau NPT, Chung NH & Jeon JG. Relationships between the antibacterial activity of sodium hypochlorite and treatment time and biofilm age in early Enterococcus faecalis biofilms. Int Endontic J 2015; 48: 782-789.

28- Nara A, Dhanu, ChandraP, Anandakrishna L. Comparitive Evaluation of Antimicrbial Efficacy of MTAD, 3% NaOCl and Propolis Against *E.faecalis.* J Clin Pediatr Dent January-April 2010;3(1):21-25.

29- Javidi M, Afkhami F, Zarei M, Ghazvini K, and Rajabi O. Efficacy of a combined nanoparticulate/calcium hydroxide root canal medication on elimination of *Enterococcus faecalis.* Aust Endod J 2014; 40: 61–65.

30- Liu V, Dashper S, Parashos P, Liu S. W, D Stanton, P Shen, P Chivatxaranukul, EC Reynolds. Antibacterial efficacy of casein-derived peptides against *Enterococcus faecalis*. Australian Dental Journal 2012; 57: 339–343.

31- Lucena JMVM, Decker EM, Walter C, Boeira LS, Lost C& Weiger R. Antimicrobial effectiveness of intracanal medicaments on Enterococcus faecalis: chlorhexidine versus octenidine. Int Endontic J 2013; 46:53-61.

32- Jeison B. Carbajal M. Antimicrobial effects of calcium hydroxide, chlorhexidine, and propolis on Enterococcus faecalis and Candida albicans . J Investig and Clin Dent 2014; 5:194–200.

33- Menezes MM, Valera MC, Jorge AOC, Koga-Ito CY, Camargo CHR&Mancini MNG. In vitro evaluation of the effectiveness of irrigants and intracanal medicaments on microorganisms within root canals. Int Endontic J 2004 ;37: 311-319.

34- Sathorn C, Parashos P & Messer H. Antibacterial efficacy of calcium hydroxide intracanal dressing: a systematic review and meta-analysis. Int Endontic J 2007; 40: 2–10.

35- Eva K. Siren, Markus PP. Haapasalo, Tuomas M. T. Waltimo, Ørstavik D. In vitro antibacterial effect of calcium hydroxide combined with chlorhexidine or iodine potassium iodideon Enterococcus faecalis. Eur J Oral Sci 2004; 112: 326–331.

36- Turner SR, Love RM& Lyons KM. An in-vitro investigation of the antibacterial effect of Nisin in root canals and canal wall radicular dentine. Int Endontic J 2004; 37: 664–671.

37- Ambikathanaya. Intracanal antiseptic medications; a review. Ujmds 2014, 02 (03): page 136-142.

38- Peters O A, Peters C I. Cleaning and shaping of the root canal system. *In*: Cohen S, Hargreaves KM, eds. *Pathways of the pulp*. 9th ed 290-357. St. Louis: Mosby Elsevier, 2006.

39- Kawashima N, Wadachi R and Suda H. Root canal medicaments. Int Dent J 2009; 59: 5-11.

40- Chinni S K, Veni A B, Manali R. Srinivasan, Rajamani I. An in vitro investigation of a newer intracanal medicament Nisin on Enterococcus faecalis in comparison with chlorhexidine and calcium hydroxide. J Int Dent Res Organ 2011; 3(1):21-24.

41- Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endod Dent Traumatol 1990;6:142-149.

42- Somanath G, Samant PS, Gautam V, Singh Birring OJ. To comparatively evaluate the antimicrobial ef cacy of chlorhexidine, nisin and linezolid as an intracanal medicament on *Enterococcus faecalis*: An *in vitro* study. Indian J Dent Res 2015;26:613-8.

43- Tong Z, Zhou L, Kuang R, Lv H, Qu T, Ni L. *In vitro* evaluation of MTAD and nisin in combination against common pathogens associated with root canal infection. J Endod 2012;38:490-4.

44- Breukink E, de Kruijff B. Lipid II as a target for antibiotics. Nat Rev Drug Discov 2006;5:321–32.

45- Gagné N, Simpson BK. Use of proteolytic enzymes to facilitate the recovery of chitin from shrimp wastes. Food Biotechnol 1993;7:253-63.

46- Petri DF, Donega J, Benassi AM, Bocangel JA. Prelimi- nary study on chitosan modified glass ionomer restora- tives. Dent Mater 2007; 23: 1004-10.

47- Knapczyk J. Chitosan hydrogel as a base for semisolid drug forms. Int J Pharm 1993;93:233-7.

48- Rahman H, Chandra R, Singh S, Chandra A. A combination approach to fight against E.faecalis and C.albicans. Int J Contemp Dent 2013; 4(2):1-5.

49- Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza-Filho FJ. Comparative study of the antimicrobial efficacy of chlorhexidine gel, chlorhexidine solution and sodium hypochlorite as endodontic irrigants. Braz Dent J 2007;18: 294–8.

50- Seltzer S Pulp Irritants Microbial The Dental Pulp All India Distributors ltd 173-193.

51- Sathorn C, Parashos P, Messer H. Australian endodontists' perceptions of single and multiple visit root canal treatment. Int Endod J 2009;42:811-8.

52- Rocas IN, Siqueira JF, Santos KRN. Association of Enterococcus faecalis with different forms of periradicular diseases. J Endod. 2004; 30(5):315-20.

53- Sedgley CM, Lennan SL & Appelbe OK. Survival of Enterococcus faecalis in root canals ex vivo. Int Endod J. 2005; 38(10):735-742.

54- Waltimo TMT, Siren EK, Torkko HLK, Olsen I, Happasalo MPP. Fungi in therapy-resistant apical periodontitis. Int Endod J 1997; 30: 96–101.

55- Hagihara Y, Kaminishi H, Cho T, Tanaka M, Kaita H. Degradation of human dentin collagen by an enzyme produced by the yeasts *Candida albicans*. Arch Oral Biol 1988; 33: 617–19.

56- White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine.J Endod 1997; 23: 29–31.

57- Heling I, Sommer M, Steinberg D, Friedman, Sela MN. Microbiological evaluation of the efficacy of chlorhexidine in a sustained release device for dentin sterilization. Int Endod J 1992; 25: 15–19.

58- Taneja S, Pragya Kumar P, Malhotra K, and Dhillon J. Antimicrobial effect of an oxazolidinone, lantibiotic and calcium hydroxide against *Enterococcus faecalis* biofilm: An *in vitro* study. Indian J Dent. 2015 Oct-Dec; 6(4): 190–194.

59- White RR, Hays GL, Janer LR. Residual antimicrobial activity after canal irrigation with chlorhexidine.J Endod 1997; 23: 29–31.

60- Grover C, Shetty N. Evaluation of calcium ion release and change in pH on combining calcium hydroxide with different vehicles. Contemp Clinical Dentistry Oct-Dec 2014; 5(4):434-439.

61- Elsaka SE, Elnaghy. Antibacterial activity of calcium hydroxide combined with chitosan solutions and the outcomes on the bond strength of RealSeal sealer to radicular dentin. J Biomed Res 2012; 26(3): 193-199.

62- PetersLB, vanWinkelhoffA-J, BuijsJF, WesselinkPR. Effects of instrumentation, irrigation and dressing with calcium hydroxide on infection in pulpless teeth with periapical bone lesions. Int Endontic J 2002; 35: 13–21.