A COMPARATIVE EVALUATION OF ANTIBACTERIAL EFFICACY OF NEEM AND TURMERIC WITH AND WITHOUT ADDITION OF CALCIUM HYDROXIDE AGAINST *ENTEROCOCCUS FAECALIS* -AN IN VITRO STUDY

A Dissertation submitted
in partial fulfilment of the requirements
for the degree of

MASTER OF DENTAL SURGERY

BRANCH – IV
CONSERVATIVE DENTISTRY AND ENDODONTICS

THE TAMILNADU Dr.M.G.R. MEDICAL UNIVERSITY
CHENNAI- 600032
2015 – 2018

ADHIPARASAKTHI DENTAL COLLEGE AND HOSPITAL

MELMARUVATHUR – 603319.

DEPARTMENT OF CONSERVATIVE DENTISTRY AND ENDODONTICS

CERTIFICATE

This is to certify that **Dr.A.KARTHIKEYAN,** Post Graduate student (2015-2018) in the Department of Conservative Dentistry and Endodontics, Adhiparasakthi Dental College and Hospital, Melmaruvathur -603319, has done this dissertation titled **"Comparative Evaluation Of Antibacterial Efficacy Of Neem And Turmeric With And Without Addition Of Calcium Hydroxide Against *Enterococcus faecalis* –An Invitro Study**under our direct guidance and supervision in partial fulfilment of the regulations laid down by the Tamilnadu Dr.M.G.R Medical University, Chennai – 600032 for MDS., (Branch-IV) **CONSERVATIVE DENTISTRY AND ENDODONTICS** degree examination.

Guide
Dr.S.Thillainayagam M.D.S.,
Professor
Department of Conservative
Dentistry and Endodontics

Head of the Department
Dr.Prabhakar Joseph M.D.S.,
Professor
Department of Conservative
Dentistry and Endodontics

Principal
Dr.S. Thillainayagam M.D.S.,
Department of Conservative
Dentistry and Endodontics

ACKNOWLEDGEMENT

I thank **ALMIGHTY GOD** for answering my prayers and making me what I am today.

I avail this opportunity to express my gratitude and reverence to my beloved Teacher **Dr.S.Thillainayagam M.D.S**., Professor Department of conservative dentistry and endodontics, Adhiparasakthi Dental College and Hospital Melmaruvathur. His pursuit for perfection and immense support were a source of constant inspiration to me. Words cannot express my gratitude for his quiet confidence in my ability to do the study, his willingness to help and clear the stumbling blocks along the way and his tremendous patience till the completion of this study.

A special mention of thanks to **Dr.Prabhakar Joseph M.D.S.,** professor and HOD, department of conservative dentistry and endodontics for valuable suggestions and encouragements throughout my completion of my Main dissertation.

It is my duty to express my thanks to my Co- guide **Dr.Bharath M.D.S.,** Reader for his valuable suggestions and encouragements.

I am thankful and express my gratitude to my previous teacher **Dr. S. Sathyakumar M.D.S.,** Professor **Dr. D.S Dinesh M.D.S.,** Professor **Dr. P. Prashanth M.D.S.,** Reader**,** for their excellent guidance, immense help and support for the initiation of this study.

I thank our Managing Director **Dr. T. Ramesh MD**., for his vital support and allowing us to utilize all the facilities provided.

I am thankful to **Dr. S. Thillainayagam M.D.S.,** our beloved Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur for providing us the opportunity to utilize the facilities of the college.

I am thankful to express my gratitude to my teachers **Dr.A.Jayasenthil M.D.S.,** Reader, **Dr.S.Karthikeyan M.D.S.**, Reader, **Dr.N.Ragunathan. M.D.S.,** Senior lecturer, **Dr.M.Purushotham M.D.S.,** Senior lecturer **Dr. E. Premkumar M.D.S.,** Senior lecturer, and **Dr.P.Kaushalya M.D.S.,** Senior lecturer.

I would like to thank **Dr.K.Thirugnanasambantham**,.Research officer, Pondicherry centre for biological sciences for their support in granting me permission to conduct the study in their department and helping me to bring out my study.

I would like to thank Statistician **Mr.K.Bhoopathi M.Sc., MBA.** Department of Biostatistics, Chennai (ICMR) for helping me in statistical works.

I also wish to thank my Co-Pg **Dr.S.Saravanan**, **Dr.Yalamanchianusha** and my senior **Dr.C.Ravivarman**, **Dr.S.Srikanth** and I warmly acknowledge my juniors **Dr.Y.Reeja, Dr.K.Santhoshkumar, Dr.N.Mohankumar, Dr. A. Yogahapadhma, Dr.T.Vaibhavi, and Dr.V.Kanagapriyaa.**

I owe my gratitude to my father Late **R**.**Arumugam MA.BEd,.** and my mother **A.Gandhimathi,.** who stood beside me during my hard time and sacrificed so much to make me what I am today. A special thanks to my loving wife **Dr.S.Sangavi** and my cute son master **K.Mughilan** for their sacrifice and encouragement throughout my career.

My acknowledgement wouldn't be complete without mentioning my teaching and non-teaching staffs, Office staffs, and Librarian **Mr.P.Maveeran**, **and Mr.K.Selvakumar** for being my strength, by giving me continual support all the time.

Dr.A.Karthikeyan

Post Graduate Student

DECLARATION

TITLE OF THE DISSERTATION	"Comparative Evaluation Of Antibacterial Efficacy Of Neem And Turmeric With And Without Addition Of Calcium hydroxide Against *E.faecalis*-An In Vito Study"
PLACE OF THE STUDY	Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319
DURATION OF THE COURSE	3 years
NAME OF THE GUIDE	Dr.S.ThillainayagamMDS.,
NAME OF THE CO- GUIDE	Dr. N. Bharath MDS.,

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Guide **Head of the department**

Signature of the candidate

ABSTRACT

Complete disinfection of the root canal system is essential for the success of root canal therapy. This requires the use of an intra-canal medicament. *Enterococcus faecalis* is the most frequently found species in persistent/secondary intracanal infection associated endodontic treatment failure. This study evaluates the antibacterial property of neem and turmeric as a intracanal medicament in combination with calcium hydroxide. The activity of neem, turmeric and calcium hydroxide against E.*faecalis* planktonic cells was measured on well diffusion assay. Broth dilution method was done to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the medicament used. Seven days *E.faecalis* biofilm was inoculated on root canal surface to check herbal activity with and without addition of calcium hydroxide. Dentin samples were collected on root canal to quantitatively analysing the colony forming units by pour plate's method.

AIM:

The aim of the invitro study is to compare the antimicrobial activity of neem (*Azadirachta Indica)*and turmeric (*Curcuma Longa*) with and with out Addition of Calcium hydroxide Against *E.faecalis* Biofilm.

MATERIALS AND METHODS:

Sixty single rooted human mandibular premolars extracted for orthodontic purpose has been selected. The external surface of the

teeth and the cementum were cleaned using periodontal curettes. Samples were decoronated at the level of CEJ to obtain the length of 13mm. Cleaning and shaping were done on rotary protaper universal file up to F3 size. The samples were divided in to 6 Groups(n=10).Group I (neem), Group II(turmeric), Group III (calcium hydroxide), Group IV (neem+calcium hydroxide),Group V (turmeric+calcium hydroxide), Group VI(saline).The tooth samples were inoculated for 7 days in *E.faecalis* broth. At the end of 7 days tooth samples were rinsed with saline superficially and placed in a medicament for 24 hrs. Dentin samples were collected from the root canal using H files size 30 placed in broth solution. Colony forming units were counted using pour plate's method.

RESULTS:

The result of the present study showed that neem+calcium hydroxide combination is a maximum inhibition of bacterial count. Neem alone showed good antibacterial activity when it combined with calcium hydroxide it is showed even improved antibacterial activity compared to all other Groups. Saline (control) showed more colony forming units when compared with other Group.

CONCLUSION:

Within the limitation of this study it was found that neem+calcium hydroxide showed maximum antibacterial activity against 7 days *E.faecalis* biofilm formed on the tooth substrate followed by neem, calcium hydroxide, turmeric + calcium hydroxide.

But none were able to completely eradicate *E. faecalis* biofilm in dentinal tubules. Further research is needed to conclusively recommend herbal as a root canal medicament.

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LIST OF ABBREVIATIONS

ANOVA	-	One way analysis of varience
BSA	-	Bovine serum albumin
CHX	-	Chlorhexidine
CEJ	-	Cemento Enamel junction
CFU	-	Colony forming units
CLSI	-	Clinical and laboratory standards institute
DMSO	-	Dimethyl sulfoxide
EDTA	-	Ethylene diamine tetra acetic acid
GE	-	Garlic extract
hPDLSCS	-	Human periodontal ligament stem cells
MHA	-	Mueller-hinton agar
MIC	-	Minimum inhibitory concentration
MBC	-	Minimum bactericidal concentration
MTCC	-	Microbial type cell culture
NIH	-	National institute of science
NaOCl	-	Sodium hypocholorite
MHB	-	Mueller-hinton broth
PMN	-	polymorph nuclear
rRNA	-	Ribosomal ribonucleic acid

INTRODUCTION

The successful endodontic treatment depends on elimination of infective microflora from the necrotic root canal system[1]. Microorganisms have long been recognized as the primary etiology in the development of periapical bone lesions and the failure of endodontic treatment[2].

Reinfection and continued periapical inflammation may occur from viable bacteria residing in the complex root canal system and dentinal tubules. However, studies have shown that complete disinfection of the root canal space is difficult to achieve because of the anatomic complexities of the root canal system[3.]

Enterococcus faecalis has frequently been isolated as the sole infectious microorganism in infected root canals although endodontic infections are typical of a polymicrobial etiologic. Being a facultative anaerobic gram-positive coccus, it is the most common *Enterococcus sp*. cultured from non-healing cases. It can adhere to the root canal walls, accumulate and form communities organized into biofilm, which enables the bacteria to become highly resistant to phagocytosis, antibodies and antimicrobials than non–biofilm-producing organisms. *E.Faecalis* may also survive chemo mechanical anatomical niches of the root canal system and reinfect the filled root canal[4].

One of the principle roles of root canal medicament is to assist in the killing of microorganism and the removal of the bacterial

infection from uninstrumented root canal surface. Intracanal medicament and other locally used disinfecting agents plays a key role in the eradication of microbes[5].

An ideal root canal medicament should have a broad antimicrobial spectrum and high efficacy against microorganisms while being systemically nontoxic and non-irritating to periodontal tissues. Plant-derived natural products that represent a rich source of antimicrobial compounds have been incorporated into oral hygiene products. However, their application in endodontics is not much documented.

Turmeric (*Curcuma longa*) belongs to the ginger (Zingiberaceae) family. It is extensively used as a spice, food preservative and colouring material in countries like India, China and South East Asia. From the time of Ayurveda (1900 BC) numerous therapeutic activities have been assigned to turmeric for a wide variety of diseases and conditions, including skin, pulmonary and gastrointestinal systems, and also aches, pains, wounds, sprains and liver disorders. Components of turmeric are named as curcuminoids, which predominately includes curcumin (diferuloyl methane), demethoxycurcumin and bisdemethoxycurcumin[6].

Neem (*Azadirachta indica*)is a well known traditional medicine in India and its neighbouring countries. It is the most versatile medicinal plant with wide spectrum of biological activity and most commonly used medicine for household remedies. Nimbidin, a major

crude extract from the seed *A. Indica* demonstrates several biological activities. Few tetranortriterpenes including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated. Antimicrobial effects of neem leaf extract have been demonstrated against *Streptococcus mutants* and *E.faecalis* which are also used to treat dental plaque and gingivitis. Neem leaf extract has significant antimicrobial effect against *E.faecalis* derived from infected root canal samples[7].Its antioxidant and antimicrobial properties makes it a potential agent for root canal medicament as an alternative to calcium hydroxide.

Calcium hydroxide plays a major role as an inter-visit dressing in the disinfection of the root canal system. Though it cannot be categorized as a conventional antiseptic, it kills bacteria in root canal space. Direct contact experiments in vitro require a 24 hour contact period for complete destruction of *Enterococci*. It not only kills bacteria, but it also reduces the effect of lipo-polysaccharide, the remaining cell wall material. Calcium hydroxide has a wide range of antibacterial activity against common endodontic pathogens, but is less effective against *Enterococcus faecalis* and *Candida albicans*. Calcium hydroxide is also an effective anti-endotoxin agent. However, its effect on microbial biofilms is controversial[8].

Various studies have reported that short comings of calcium hydroxide has been rectified by using chlorhexidine and herbals but no studies have reported the effects of calcium hydroxide mixed with

herbal extract on biofilm. This study is to evaluate the antimicrobial property of neem and turmeric with and without calcium hydroxide against the *E.faecalis* planktonic cells and biofilm.

AIM AND OBJECTIVES

Aim:

The aim of the invitro study is to compare the antibacterial activity of neem (*Azadirachta Indicia)* and turmeric (*Curcuma Longa*) with and with out addition of calcium hydroxide against *E.faecalis* biofilm.

Objectives:

To evaluate the antibacterial activity of two herbal extract as intracanal medicament against *E.faecalis* biofilm.

To evaluate the antibacterial activity of two herbal extract in combination with and without calcium hydroxide as intracanal medicament against *E.faecalis* biofilm.

REVIEW OF LITERATURE

Costerton JW., 1999 [12]stated that in a biofilm, the microbes get adsorbed onto a solid nonshedding surface, and are embedded in a common self-produced extra-cellular matrix. The structural features of biofilm allow efficient transfer of nutrients, removal of waste materials, and circulation of secondary metabolites and pheromones. When bacteria grow as biofilm, the altered genetic and metabolic processes of bacteria along with its complex matrix prevent the entry and action of antimicrobial agents. Subsequently the colonizing organism gains protection against unfavorable, environmental and nutritional conditions. The antibiotic resistance has been found to increase up to 1500 times when bacteria is grown as biofilm, compared to planktonic cells. Furthermore, there is constant detachment of cells from a fully matured biofilm, and the detached cells serve as a steady source for chronic infection. Thus, the concept of bacterial biofilm is associated with a wide range of persistent infections.

Love R M .,2001[16] identified a possible mechanism that would explain how *E.faecalis* could survive and grow within dentinal tubules and reinfect an obturated root canal. He postulated that a virulence factor of *E.faecalis* in failed endodontically treated teeth may be related to the ability of *E.faecalis* cells to maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum.

Leonardo MR., 2002[13] suggested the presence of bacterial biofilm at the apical portion of the root canal and at the extraradicular region on clinical examination of root tips of teeth associated with refractory periapical periodontitis. *Enterococcus faecalis* is the most predominant bacteria in teeth with failed root canal therapy and is found to survive harsh conditions prevailing in the root canals of endodontically treated teeth.

Evans *et al.*,2002[10] **conducted a** study to clarify the mechanisms that enable *E.faecalis* to survive the high pH of calcium hydroxide.In his study he used *E.faecalis* strain JH2-2 was exposed tosublethal concentrations of calcium hydroxide, with and without various pretreatments. Blocking agents were added to determine the role of stress-induced protein synthesisand the cell wall-associated proton pump and concluded that Survival of *E.faecalis* in calcium hydroxide appears to be unrelated to stress induced protein synthesis, but a functioning proton pump is critical for survival of *E.faecalis* at high pH.

Evans M *et al.*, 2002[17] studied the mechanisms that enable *E.faecalis* to survive the high pH of calcium hydroxide. They concluded that survival of *E.faecalis* in calcium hydroxide appears to be unrelated to stress induced protein synthesis, but a functioning proton pump is critical for survival of *E.faecalis* at high pH.

Tronstad*et al.*, 2003[14] demonstrated that the biofilm mode of growth is a survival strategy and harsh environmental conditions existing in the root canal favors the growth of bacteria as a biofilm. This aspect is supported by the fact that clinically isolated *E.faecalis* possess increased adhering capacity, increased virulence factors and increased resistance to antimicrobials that are all characteristics of biofilm style of growth.

Chávez de Paz LE et al., 2003[19] determined whether there is a pattern for certain bacteria to remain after chemo-mechanical treatment of root canals in teeth with apical periodontitis. In the present study, Gram-positive bacteria were recovered from root canals of teeth receiving root-canal treatment for either symptomatic or non symptomatic apical periodontitis. In several cases the organisms remained or new ones appeared in subsequent samples, despite chemo-mechanical treatment and antimicrobial intracanal dressings, suggesting that conditions for their survival and growth prevailed in these root canals. Gram-negative anaerobes were conspicuous by their relatively rare occurrence in contrast to primary infections of teeth with necrotic pulps and apical periodontitis. Their findings suggested that the treatment procedures carried out, especially in teeth with necrotic pulps, were more effective against Gram-negative bacteria but less so towards Gram-positive organisms. Hence, these results seem to provide support for the hypothesis that endodontic procedures may select for the more resilient organisms, while the susceptible Gram-negative anaerobes are more easily eliminated. The supposedly higher resistance of Gram positive

bacteria may be related to different factors, as for example cell-wall structure, metabolic products secreted, and resistance towards medicaments. Another commonly recovered bacterial group was Enterococcus spp. In comparison with all other organisms, Enterococcus spp. more than doubled their percentage share of the total number of strains over the treatment period, while other organisms either had a slight increase or decrease or had disappeared totally. They concluded that findings of the current study justify the view that nonmutans group *streptococci*, *enterococci* and *lactobacilli* commonly survive antimicrobial endodontic treatment.

Gomes BPFA *et al.*,2003[25] conducted in vitro study to evaluate the effectiveness of 2% chlorhexidine gel and calcium hydroxide against *E.faecalis*. They concluded that 2% chlorhexidine gel alone was more effective against *E.faecalis* than calcium hydroxide.

Chavez de Paz LE *et al.*,2004[20] identified Gram-positive rods from root canals of teeth with apical periodontitis and examined their associations with other species. The most frequent interactions in the present study were given by Lactobacillus spp. and Grampositive cocci, e.g. *Lactobacillus casei* with Enterococcus spp. Such associations have not been reported previously. Other non-significant associations were also determined possibly due to the low number of strains recovered, thus, it can be speculated that with a larger number of samples such associations may have been found to be significant. They concluded that the results of this study contribute to the study of bacterial flora

remaining after root canal treatment. In addition to Enterococcus spp. other bacteria seem to remain after root canal treatment. Further research is required to determine whether these prevalent organisms have any pathogenic implications in root canal treatment failures.

Nageswarrao*et al.*,2004[26] conducted a study to evaluate the efficacy of an intra canal medicament comprising of calcium hydroxide and 2% chlorhexidine against *E.faecalis*. The results showed that the paste made from calcium hydroxide and 2% chlorhexidine was significantly more effective than that made from alone calcium hydroxide and 2% chlorhexidine.

Reynaud afGeijersstam A *et al.*,2005[21] measured the release of hydrolytic enzymes [elastase, cathepsin G and collagenase-2 (MMP-8)] from human polymorph nuclear leukocytes (PMNs) during interaction with strains of *Enterococcus faecalis* isolated from endodontic infections. The periapical bone resorption during the acute phase of the infection may be followed by a quiet phase with an infective flora dominated by *E.faecalis*. These results also indicate that a microbiological diagnosis to species level may not be a sufficient indicator of the virulence of the isolated strains, as for *E.faecalis* certain strains are clearly more virulent than others. The results of the present study indicate a variable potential for different endodontic *E.faecalis* strains to induce proteinase release from PMNs. Only one of the six strains of *E.faecalis* used in the study induced considerable

PMN degranulation. This possibly reflects the clinical observation that endodontic retreatment cases seldom show acute inflammatory reactions. These findings emphasize the need for well characterized *E.faecalis* strains when studying interactions between host defense protagonists and these bacteria.

Sedgley CM *et al.*,2005[22] tested the hypotheses in this study were that: (i) *Enterococcus faecalis* can survive long-term entombment in root filled teeth without additional nutrients, (ii) initial cell density influences the survival of *E.faecalis* in instrumented root canals and (iii) gelatinase-production capacity influences the survival of *E.faecalis* in root canals. Viable *E.faecalis* was recovered from all root filled teeth and from 95–100% of unfilled inoculated teeth. Initial cell density and gelatinase production did not influence the recovery of viable *E.faecalis*. *Enterococcus faecalis* 16S rRNA gene products were present in all inoculated teeth and absent in all non inoculated controls. Dentinal tubule infection was evident under light microscopy in sections from inoculated teeth after 48-h, 6- and 12-month incubation. *Enterococcus faecalis* inoculated into root canals maintained viability for 12-months ex vivo. The clinical implications are that viable *E.faecalis* entombed at the time of root filling could provide a long-term nidus for subsequent infection.

Kayaoglu G *et al.*,2005[23] evaluated the effect of growth at pH levels from 7.1 to 9.5 on the adherence of *Enterococcus faecalis* to bovine serum albumin (BSA) and collagen type I.. The adhesion of *E.faecalis*

to BSA-coated surfaces decreased inversely with alkalinity of the growth medium. The pH 7.1-grown bacteria bound to BSA significantly more than the other BSA groups. On the contrary, the adhesion to collagen type I-coated surfaces of bacteria grown at pH 8.0 and 8.5 was significantly greater than for those grown at pH 7.1. A minor increase in pH up to 8.5, which may be a consequence of insufficient treatment with alkaline medicaments such as calcium hydroxide, increases the collagen-binding ability of *E.faecalis*, in vitro. This can be a critical mechanism by which *E.faecalis* predominates in persistent endodontic infections.

Ferrari PHP *et al.*,2005[24] detected Enterococcus, enteric bacteria and yeast species from the canals of teeth with primary endodontic infections before and after canal preparation and to test the antibiotic susceptibility of *Enterococcal* strains isolated from infected root canals. Amongst the total target microorganisms isolated in this study, three yeast strains were identified as *Candida albicans* (one), *Candida glabrata (one) and Candida magnoliae (one).* Three enteric bacteria strains were identified, one being *Klebsiella pneumoniae* and two *Enterobacter sakazaki*. Of the total isolated enterococci, two strains were *E. casseliflavus*, four *E. faecium* and six were *E.faecalis*. *Enterococci*, enteric bacteria and yeasts were present in primary endodontic infections; enterococci were the most frequently isolated. Enterococci, particularly *E.faecalis* and *E. faecium*, were more resistant to root canal preparation and intracanal dressing.

Krithikadatta *et al.*,2007[11] conducted a study *on* Disinfection of Dentinal Tubules with 2% Chlorhexidine, 2% Metronidazole, Bioactive Glass Compared with Calcium Hydroxide as Intracanal Medicaments antibacterial efficacy of the four medicaments against *Enterococcus faecalis* was assessed in vitro using extracted premolar teeth at the depths of 200µm and 400µm and concluded that concluded from the present study that 2% chlorhexidine gel alone was most effective against *E.faecalis* when compared to other medicaments.

Valera MC *et al.*, 2010[27] evaluated the antimicrobial activity of 2% chlorhexidine gel associated with various intracanal medicaments against *Candida albicans* and *Enterococcus faecalis* inoculated in root canals. They concluded that the use of 2% chlorhexidine gel reduces the number of microorganisms significantly, only the calcium hydroxide and calcium hydroxide associated with chlorhexidine are able to eliminate these microorganisms completely.

Ehsani M *et al.*, 2013 [28] compare the antibacterial activity of Chlorhexidine with two natural drugs. The antibacterial activities of three different propolis extracts (alcohol concentrations: 0, 15, 40%) and Aloevera gel on *E.faecalis* were compared. The results of the study showed the hydro alcoholic extracts of propolis and Aloe vera gel had antibacterial effects on *E.faecalis*, however, propolis is more potent than Aloe vera. They concluded that appropriate concentrations of alcoholic extracts of propolis and some fractions of Aloe vera gel

might be good choices for disinfecting the root canal in endodontic treatments.

Valera MC *et al.*, 2013[29] evaluated the antimicrobial activity of auxiliary chemical substances and natural extracts on *Candida albicans* and *Enterococcus faecalis* inoculated in root canals. They concluded that 2.5% sodium hypochlorite and 2% chlorhexidine gel were more effective in eliminating *C. albicans* and *E.faecalis*, followed by the castor oil and glycolic ginger extract. The Aloevera extract showed no antimicrobial activity.

Maekawa LE *et al.*, 2013[30] evaluated the effectiveness of glycolic propolis and ginger extracts, calcium hydroxide, chlorhexidine gel and their combinations as ICMs against *Candida albicans*, *Enterococcus faecalis, Escherichia coli* and endotoxins in root canals. They concluded that all ICMs were able to eliminate the microorganisms in the root canals and reduce their amount of endotoxins; however, calcium hydroxide was more effective in neutralizing endotoxins and less effective against *C. albicans* and *E.faecalis*, requiring the use of medication combinations to obtain higher success.

Kumar H *et al.*, 2013[31] valuated the antimicrobial efficacy of *Curcuma longa*, Tachyspermumammi, chlorhexidine gluconate gel and calcium hydroxide as intracanal medicaments against *Enterococcus faecalis*. Author concluded that *Curcumalonga* can be used as intracanal medicament in endodontic failure cases.

Castilho AL *et al.*, 2013[32]evaluated 25 plant extracts obtained from Brazilian forests against planktonic *E.faecalis* and were subjected to two traditional antibacterial assays, the microdilution broth assay and the disk diffusion assay, using chlorhexidine as a control. The results of the study discoved six active extracts against planktonic *E.faecalis* and support further testing via assays involving biofilm formation, as well as the determination of the compounds' chemical profiles, as their activity was significantly better than that observed for chlorhexidine.

Prasanna neelakantan et al., 2013[9] compared the antimicrobial efficacy of curcumin against *Enterococcus faecalis* biofilm formed on tooth substrate using sodium hypocholorite and chlorhexidine (CHX) served as standards for comparison and concluded that Sodium hypochlorite (3%) showed maximum antibacterial activity against *E.faecalis* biofilm formed on the tooth substrate, followed by curcumin and CHX.

Mistry KS *et al.*, 2014[33]checked the antimicrobial activity of *Azadirachta indica* (Neem), *Ocimum sanctum* (Tulsi), *Mimusopselelngi* (Bakul), *Tinosporacardifolia* (Giloy) and Chlorhexidine Gluconate (CHX) on common endodontic pathogens like *Streptococcus mutans*, *Enterococcus faecalis* and *staphylococcus aureus*. They concluded that Methanolic extract of *A.Indica, O.sanctum, M. Elengi, T.cardifolia* and Chlorhexidine Gluconate has considerable antimicrobial activity against *S. mutans, E.faecalis and S. aureus*.

Sponchiado EC *et al.*, 2014[34] done a study was to assess the antimicrobial activity of an intracanal medication containing the ethyl-acetate fraction of Pothomorpheumbellata against *Enterococcus faecalis*. They concluded that Ethyl-acetate fraction of P. umbellate was efficient against *E.faecalis*, making this phytotherapy a viable option for endodontic treatment.

Birring OJ *et al* ., 2015[35] done a study to assess the anti-microbial efficacy of garlic extract (GE) against *Enterococcus faecalis* biofilm and its ability to penetrate into root dentin. The results indicate that GE has a potential to serve as an alternative herbal root canal irrigant being an effective and biocompatible anti-microbial agent with good dentinal penetration property.

Karkare SR *et al.*,2015[36] compare the antimicrobial activity of saturated and diluted (1:1) hydro alcoholic extract of Aloe vera, garlic, and 5% NaOCl against *E.faecalis* using the commonly used agar diffusion method. The results of the study showed saturated hydro alcoholic extract of A. vera showed the highest zone of inhibition against *E.faecalis*. NaOCl, which is considered as gold standard, also showed higher zones of inhibition.

Chandrappa PM *et al.*, 2015[37]assessed the antimicrobial activity of herbal medicines tulasi extract and neem extract and chlorhexidine against *E.faecalis*.they concluded that both 2 herbal extracts showed significant inhibitory effect against *E.faecalis* compared to 2%

chlorhexidine. Thus these can be used as alternatively as endodontic irrigants or medication.

Chan LK *et al* .,2015[38] evaluated the cytotoxic effect of a novel nano-silver particle (25.2±6.5 nm) endodontic irrigant and compared it with 3% sodium hypochlorite. They used two cell types, mouse fibroblast National Institutes of Health 3T3 (NIH 3T3) and primary human periodontal ligament stem cell (hPDLSCs) in a test for the effect of direct and indirect exposure to the two solutions and in the direct exposure experiment, ten groups of cell cultures were exposed to one dilution (3:1, 2:1, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6 or 1:7) of a nano-silver irrigant for 48 hours. They found that toxicity of the test and control group on both mouse fibroblasts and primary human periodontal ligament stem cell was not statistically different and concluded that the nano-silver irrigant was non-cytotoxic to both NIH 3T3 and hPDLSCs.

Zhang R *et al*.,2015[39] compared the antibacterial and residual antimicrobial activities of five root canal irrigants (17% EDTA,2% chlorhexidine,0.2% cetrimide, MTAD, and QMix) in a model of *Enterococcus faecalis* biofilm formation. They observed that no bacteria were seen in the blank control group whereas the number of viable *E.faecalis* was significantly fewer in the irrigant-treated groups compared with the untreated control. Their results also showed that among the five irrigants, QMix had the strongest antibacterial activity and the residual antimicrobial activities of CHX were significantly

higher at 12 h, 24 h and 36 h compared to untreated control and all five root canal irrigants were effective to some extent against *E.faecalis*, but QMix and CHX had the strongest, and CHX the longest (up to 36 h), antimicrobial activity.

MATERIALS AND METHODS

STUDY ON THE *ENTEROCOCCUS FAECALIS* (MTCC 2527) PLANKTONIC CELLS

Two standardized testing methods recommended by Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS, to determine the antibacterial activity of neem, turmeric, calcium hydroxide, on *Enterococcus faecalis* (MTCC 2527) culture.

1. Well Diffusion Test
2. Broth Dilution

PREPARATION OF NEEM AND TURMERIC EXTRACT

CRUDE NEEM LEAF EXTRACT[40, 48]

250grms of mature, fresh neem leaves were collected and washed in distilled water(fig-3).The leaves were dried using autoclave .Then the leaves were ground into fine powder and packed in muslin cloth bag for the process of extraction.100ml of 99.9% of ethanol were used as a solvent. The extraction was done using soxhlet apparatus (fig-4). The samples were macerated in ethanol for 3 to 4 hrs at temp of 50^{o} matching the boiling point of solvent. The extraction was followed by re-flux method. Water was supplied continuously to the condenser to cool the solvent in order to prevent the evaporation and facilitate the process of extraction. The procedure was continued till the crude neem extract was obtained. The extract was distilled to remove the solvent in

order to get a concentrated extract. Solvent thus extracted was evaporated by hot water bath and the extract was stored in refrigerator.

ETHANOLIC TURMERIC EXTRACT[40,41].

500grms of dry rhizomes (fig-5) (*curcuma longa*) were collected and thoroughly washed with water to remove the soil particles and chopped in to small pieces, powdered using a grinder. The samples were weighed and transferred to a cylinder which is attached to the soxhlet apparatus. 100ml of 99.99% of ethanol were used as a solvent. The samples were soaked in ethanol for 3to4hrs at temp of 50^{o} matching the boiling point of solvent. The extraction was followed by re-flux method. Water was supplied continuously to the condenser to cool the solvent in order to prevent the evaporation and facilitate the process of extraction (fig-6). The procedure was continued till the crude turmeric extract was obtained. The extract was distilled to remove the solvent in order to get a concentrated extract. Solvent thus extracted was evaporated by hot water bath and the extract was stored in refrigerator.

PREPARATION OF TEST SOLUTIONS

Neem

1gm of neem extract (Rohini pharmaceuticals Ltd., Chennai, India) was dissolved in 10ml of 10% Dimethyl Sulfoxide (S.D. Fine Chem Pvt Ltd., India)

Turmeric

1gm of Turmeric (Rohini pharmaceuticals Ltd., Chennai, India.) was dissolved in 10ml of 10% Dimethyl Sulfoxide (S.D. Fine Chem Pvt Ltd., India).

Calcium hydroxide

1gm of Calcium hydroxide (Prime Dental , India) was dissolved in 10ml of saline (Aculife, Gujarat, India).

WELL DIFFUSION TEST[41, 42, 48]

Well diffusion test is a more practical and convenient method for testing multiple antibacterial agents against bacterial strain. Using the well diffusion susceptibility test, antibacterial resistance can be detected by challenging bacterial isolates with antibiotic test solution that are placed on the surface of an agar plate that has been seeded with a lawn of bacteria.

Enterococcus faecalis MTCC 2527 was obtained from the Pondicherry center for biological science, Pondicherry, India. A loop full of culture grown on Mueller-Hinton agar (MHA, Himedia, India) was inoculated in 100ml of Mueller-Hinton broth (MHB, Himedia, India) and incubated at 37^{o}C for overnight and diluted with sterile MHB to obtain optical density 1.0 at 600nm in spectrophotometer (JASCO UV-VIS spectrophotometer., Japan)

Mueller-Hinton agar

Ingredients

- Beef Extract - 2.0gm
- Acicase peptone - 7.5gm
- Starch - 1.5gm
- Agar - 17gm
- Distilled water - 1000ml
- pH - 7.4+/- 0.2

Mueller-Hinton broth

Ingredients

- Beef Extract - 2.0gm
- Acicase peptone - 7.5gm
- Starch - 1.5gm
- Distilled water - 1000ml
- pH - 7.4+/- 0.2

Well of 6 mm diameter, were made on the agar plates and adding 10µl of test solutions (neem extract, turmeric extract, calcium hydroxide). The broth culture of *E.faecalis* was swabbed on sterile Mueller Hinton agar plates using sterile swabs. With the help of micro pipette the test solutions were placed on the medium and the plates were incubated at 37^0C for overnight. Standard ciprofloxin (µ mcg),and was included for comparison. 10% DMSO were also included to see if it shows any significant zone of inhibition.

BROTH DILUTION[44]

Broth dilution testing involves challenging the organism of interest with antimicrobial agents in a broth environment. In this procedure a series of doubling dilution of an antibacterial agent was prepared in the broth medium, the lowest antimicrobial concentration that completely inhibits visible bacterial growth is recorded as the Minimal Inhibitory Concentration (MIC) (fig-10) and complete eradication of growth was recorded as a minimum bactericidal concentration (MBC)

From the prepared test solutions doubling dilutions were done from higher dilution 100mg/ml to lower dilution in a series of test tubes (50,25,12.5,6.25,3.125,. 0.406,0.203mg/ml).The first test tube was test solution control (neem, turmeric, calcium hydroxide). To all the other test tubes containing doubling dilution, 100 µl of E.*faecalis* suspension was added and incubated at 37^0C for overnight. The MIC was regarded as the lowest concentration in the series of dilution which did not permit the growth (turbidity) of *E.faecalis*

TIME KILL STUDIES[43,47]

Another approach to examine bactericidal activity involves exposing a bacterial isolate to a concentration of antibiotic in a broth medium and measuring the rate of killing over a specified period. This was done by the addition of 2ml of test solution (neem, turmeric, calcium hydroxide) in broth containing inoculum. At regular time intervals (2, 4, 6…) a loop full of sample was plated in Mueller-Hinton

agar and incubated at 37º C for 24 hours. The number of viable bacteria from each sample is plotted over time so that the rate of killing can be calculated.

Test Solution	Time
Neem	6 mins
Turmeric	6 mins
Calcium hydroxide	12hrs

STUDY ON *ENTEROCOCCUS FAECALIS* (MTCC 2527) BIOFILM

Since the herbal test solutions showed antibacterial effect on planktonic cells, We expanded our study on the *Enterococcus faecalis* (MTCC 2527) Biofilm formed on the tooth substrate.

TOOTH SAMPLE PREPARATION[45]

Freshly extracted, intact, non-carious, 60 single rooted human mandibular premolars with fully formed apices were chosen for the study. The teeth were cleaned of superficial debris, calculus, tissue tags and stored in normal saline. The teeth were then radiographed to confirm the presence of a single canal.

The tooth specimens were sectioned below the cementoenamel junction (CEJ) with a diamond disc to obtain a standard tooth length of 13mm. This also enabled us avoiding cervical interference, easy exploration and to achieve better cleaning and shaping of the canal.

A size 10k-file was used for scouting and establishing patency till the tip of the file was seen at the apical foramen, from which 1mm was subtracted in order to establish the working length. All the samples were instrumented using Protaper universal rotary system in a crown down technique using NSK endomotor. The apical third of each canal was enlarged to F3 size to allow adequate flushing and penetration of the irrigating solution. During instrumentation 2ml of 2.5% NaOCl solution was used as a working solution, delivered by a 30 gauze needle placed as far apically as possible into the canal without binding[45].

STERILIZATION OF TOOTH SAMPLES

The prepared tooth samples were placed in the sterilization pouches and sterilized twice in autoclave at 120^{o} for 30 min .To insure the sterility and to avoid contamination a swab was taken from the tooth samples cultured before it proceed to quantitative assay.[41]

BIOFILM FORMATION

The wells containing the tooth samples were inoculated with 2ml of *Enterococcus faecalis* MTCC 2527 (Pondicherry Centre For Biological Science, Pondicherry, India) cultured overnight (37ºC) in Mueller-Hinton broth (MHB, Himedia, India), adjusted to an optical density of 1 at 600nm having 10^{8}Cells/ml using Spectrophotometer (JASCO UV-VIS spectrophotometer., Japan). To avoid nutrient depletion and accumulation of toxic end products the culture medium (Mueller-Hinton broth) was replaced every alternate day. The culture

purity was checked by inoculating a loop full of culture media onto Mueller-Hinton agar (MHA, Himedia, India) and Gram Staining.[46,31.]

At the end of 7th day, the incubated samples were randomly assigned to all the following 6 Groups of test solutions.

The test Groups are as follows:

GROUPS	TEST SOLUTION	QUANTITATIVE
GROUP I	NEEM	n=10
GROUP II	TURMERIC	n=10
GROUP III	CALCUM HYDROXIDE	n=10
GROUP IV	NEEM+CALCIUM HYDROXIDE	n=10
GROUP V	TURMERIC+CALCIUM HYDROXIDE	n=10
GROUP V1	SALINE	n=10

PREPARATION OF TEST SOLUTIONS

NEEM

Sufficient quantity of solution was prepared with concentration of 40mg/ml by dissolving 40mg of neem extract (Rohini pharmaceuticals Ltd., Chennai, India) 1ml of 10% Dimethyl Sulfoxide (S.D. Fine Chem Pvt Ltd., India).

TURMERIC

Sufficient quantity of solution prepared with concentration of 40mg/ml by dissolving turmeric extract (Rohini pharmaceuticals Ltd., Chennai, India) in 1ml of 10% Dimethyl Sulfoxide (S.D. Fine Chem Pvt Ltd., India).

CALCIUM HYDROXIDE

Sufficient quantity of solution was prepared with concentration of 1mg/ml by dissolving calcium hydroxide (Prime Dental, India) in saline (Aculife, Gujarat, India).

NEEM +CALCIUM HYDROXIDE

Sufficient quantity of solution was prepared with concentration Of 40mg/ml by dissolving 40mg Of Neem extract in 1 ml of 10%DMSO. 100ml of neem solution was mixed 1mg of calcium hydroxide.

TURMERIC +CALCIUM HYDROXIDE

Sufficient Quantity Of Solution Was Prepared With Concentration Of 40mg/ml. By dissolving 40mg Of Turmeric Extract In 1ml of 10%DMSO.100ml of turmeric solution was with 1mg of calcium hydroxide.

SALINE

0.9%saline was freshly dispensed before each use according to manufacturer instruction (Aculife, Gujarat, India)

QUANTITATIVE ASSAY

Seven day Biofilm tooth samples (n=10) was rinsed with saline twice for few minutes. The tooth samples were then treated with test Groups in a sterile Tissue Culture Wells. The medicaments were applied for 24 hrs. In Group I, Group II, Group III. For Group IV and Group V the medicament applied for 10 min. Dentin samples were retrieved from the canal using H files size 30, added to 10ml of

saline(Master dilution).The number of colonies formed were counted by Pour plate method. Pour plate method is used to count the number of living bacteria or Groups of bacteria in a liquid culture or suspension.

SERIAL DILUTION

Nine sterile test tubes, each containing 9ml of sterile saline were taken in a test tube rack.1 ml of Master dilution (saline vortexed with tooth sample) is added to 9 ml of sterile saline in a sterile test tube and mixed thoroughly. From the first test tube ,1ml of the solution was added to the second test tube and the same procedure was repeated till the ninth test tube and 1ml is finally discarded. The dilutions obtained are from 10^{-1} to 10^{-10}.1ml of suspension from the above dilutions were transferred in to nine sterile Petri plates using sterile pipette.9ml of Mueller-HintonAgar (melted ad cooled to 45º C) was added to each plate, mixed by rotating gently and incubated at 37ºC for 24 hours [43].

FLOW CHART OF METHODOLOGY

QUANTITATIVE ASSAY

60 Tooth samples

↓

E.faecalis inoculation and incubation

↓

Group I n=10	Group II=10	Group III=10	Group IV n=10	Group V n=10	Group VI n=10
Neem	Turmeric	Calcium hydroxide	Neem+ Calcium hydroxide	Turmeric+ Calcium hydroxide	Saline

↓

At 7^{th} day

↓

All tooth samples washed with saline twice

↓

Medicaments were placed 24hrs for all Groups

↓

After 24 hrs all the samples were washed once with saline

↓

Dentin chips were retrieved from each Group using 30 size H files

↓

Serial dilution was made and the number of cells was calculated by

Pour plate method

ARMAMENTARIUM AND MATERIALS USED

1. 60 freshly extracted single rooted mandibular premolars
2. Airotor hand piece(NSK, Japan.)
3. Straight hand piece(NSKEX, Japan)
4. Diamond disk
5. K files size – 10 and 15 (Mani, inc, Japan)
6. Prtotaper universal– S1-F3 (dentsply maillefe, rballaigues)
7. Syringes (Dispovan, Hindustan syringes & medical device, Faridabad india)
8. Endomate (NSK, Japan)
9. Neem Ethonolic Extract (Rohini pharmaceuticals pvt ltd)
10. Turmeric Ethonolic Extract (Rohini pharmaceuticals pvt ltd)
11. 99.9%Ethonol
12. Soxhlet Apparatus
13. *Enterococcus faecalis* (MTCC2527)
14. Calcium Hydroxide (prime dent, india)
15. Autoclave (confident pvtltd, india)
16. Incubator
17. Laminar flow chamber
18. Eppendorf tubes
19. Absorbent paper points – 25size
20. 2.5% Sodium hypochlorite
21. Saline(Aculife, Gujarat, india)
22. Tweezer
23. Mueller-Hinton broth (MHB, Himedia, India)

24.10%DMSO(S.D. Fine Chem Pvt Ltd., India)

25.Petri Dish

INCLUSION CRITERIA

Single rooted mandibular premolars that has been extracted for orthodontic purpose

EXCLUSION CRITERIA

Teeth with dental caries, cervical abrasions, previous restorations, cracks, fractures and dilacerations are excluded

Figure 1: Extracted Human Mandibular Premolar Decoronated

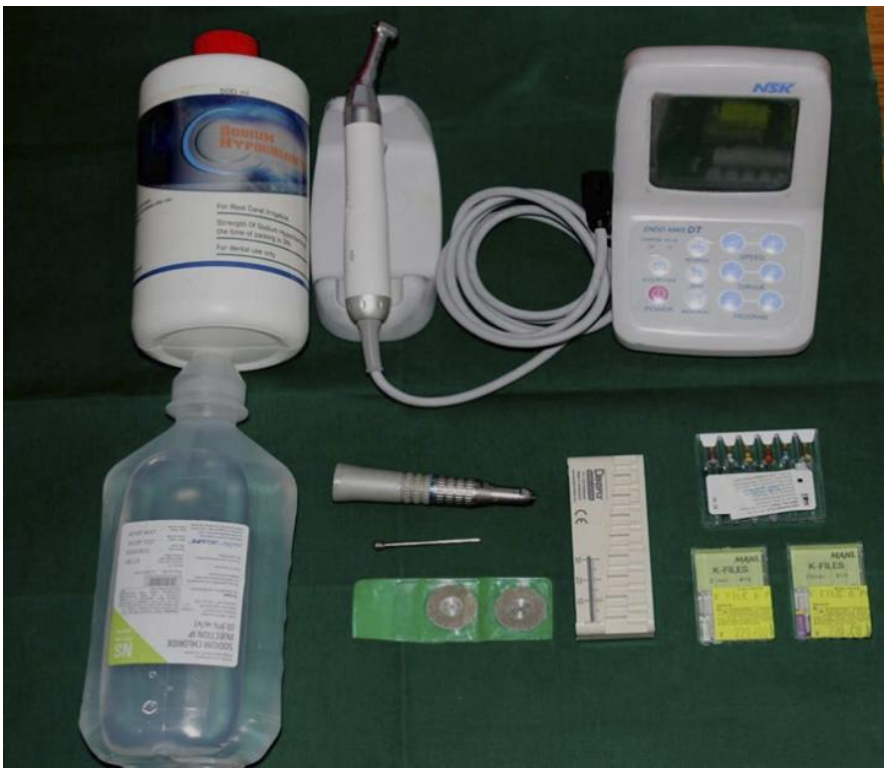


Figure 2: Armamentarium For Root Canal Preparation

Figure 3: Mature Dry Neem Leaves

Figure 4: Preparation Of Ethanolic Extract Of Neem

Figure 5: Dry Turmeric Rhizomes

Figure 6: Preparation Of Ethanolic Extract Of Turmeric

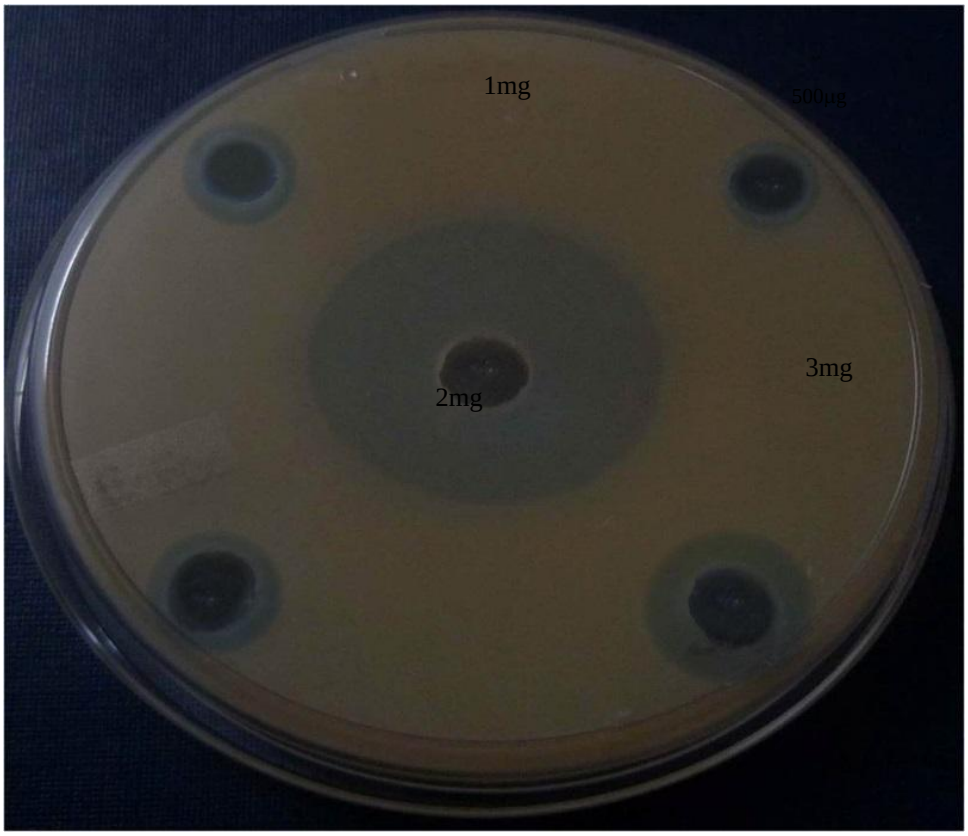


Figure 7: Zone Formation In Neem

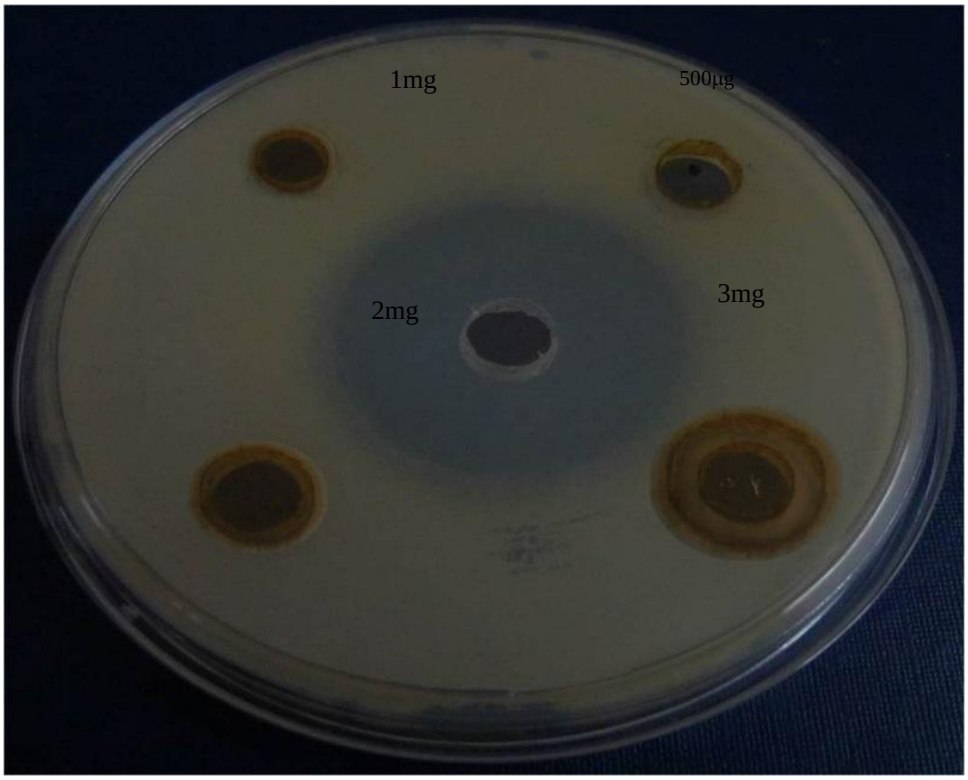


Figure 8: Zone Formation In Turmeric

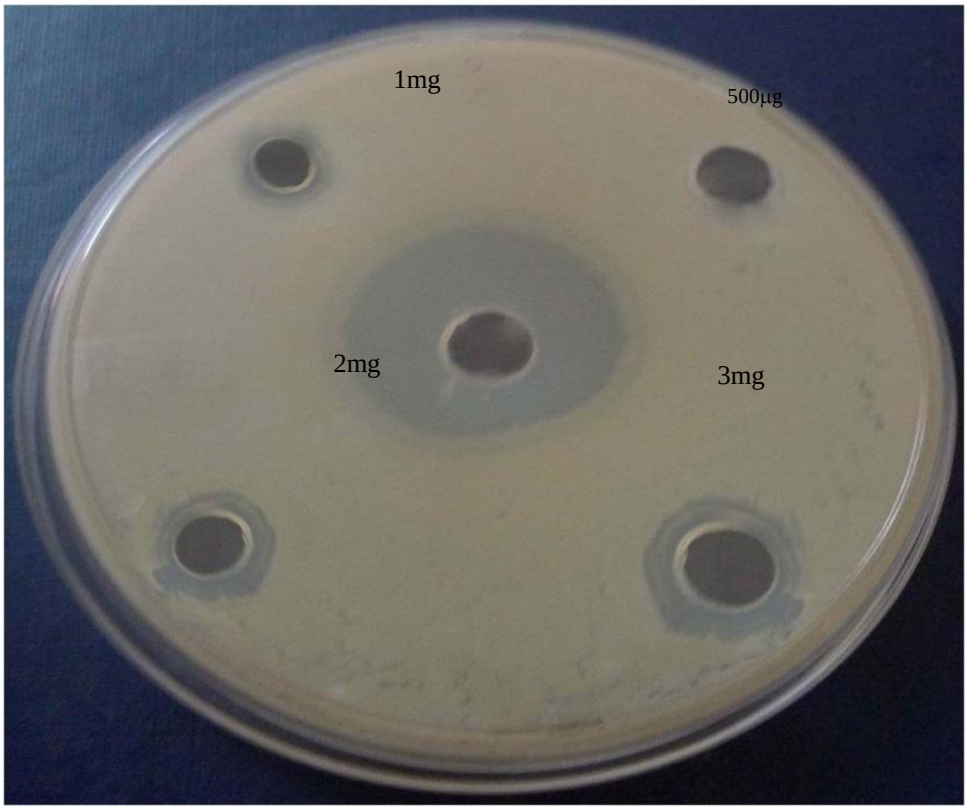


Figure 9: Zone formation In Calcium Hydroxide

Figure 10: Minimum Inhibitory Concentration Of Test Solutions

Figure 11: Laminar Flow Chamber

Figure 12: Retrieval of Dentin From Root Canal

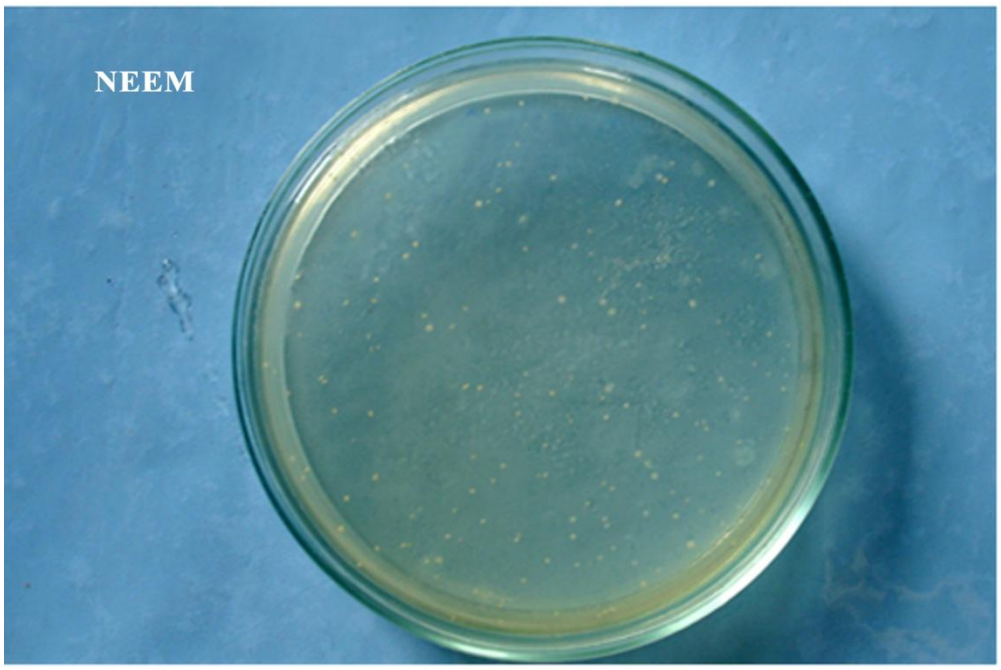


Figure 13: Colony Forming Units Of Neem

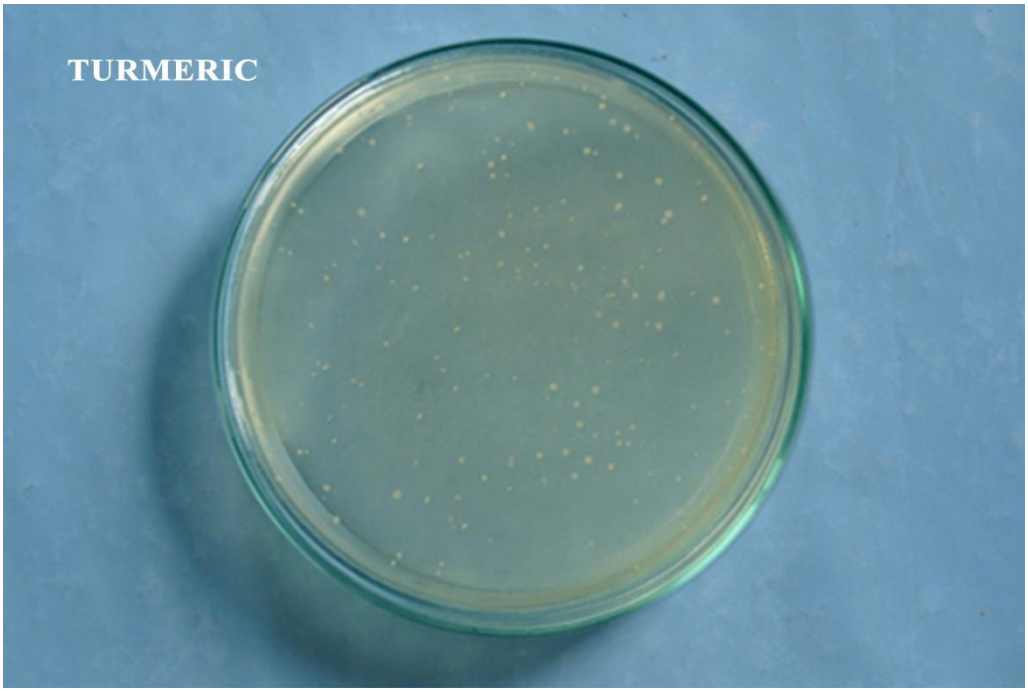


Figure 14: Colony Forming Units Of Turmeric

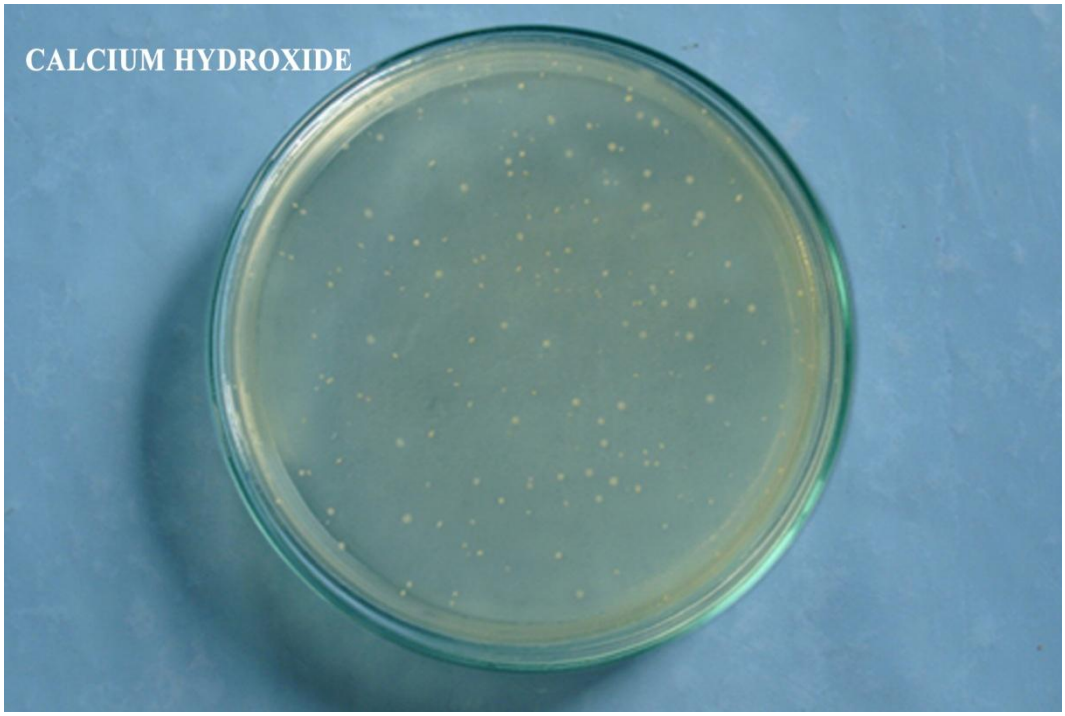


Figure 15: Colony Forming Unit Of Calcium Hydroxide

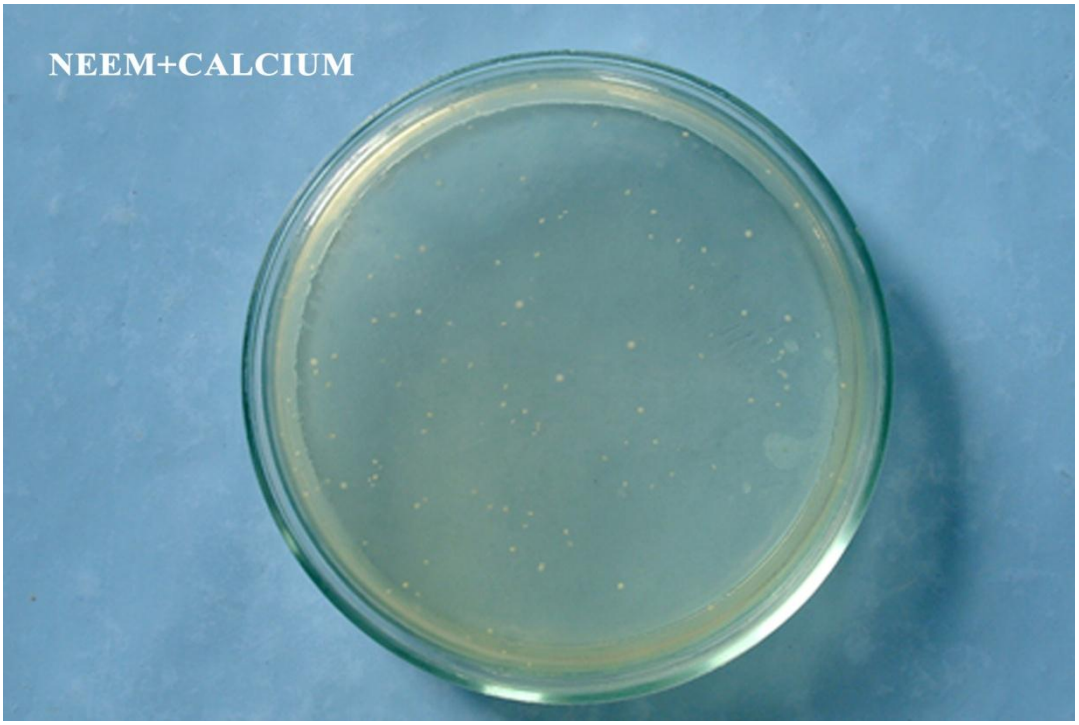


Figure 16: Colony Forming Unit of Neem + Calcium Hydroxide

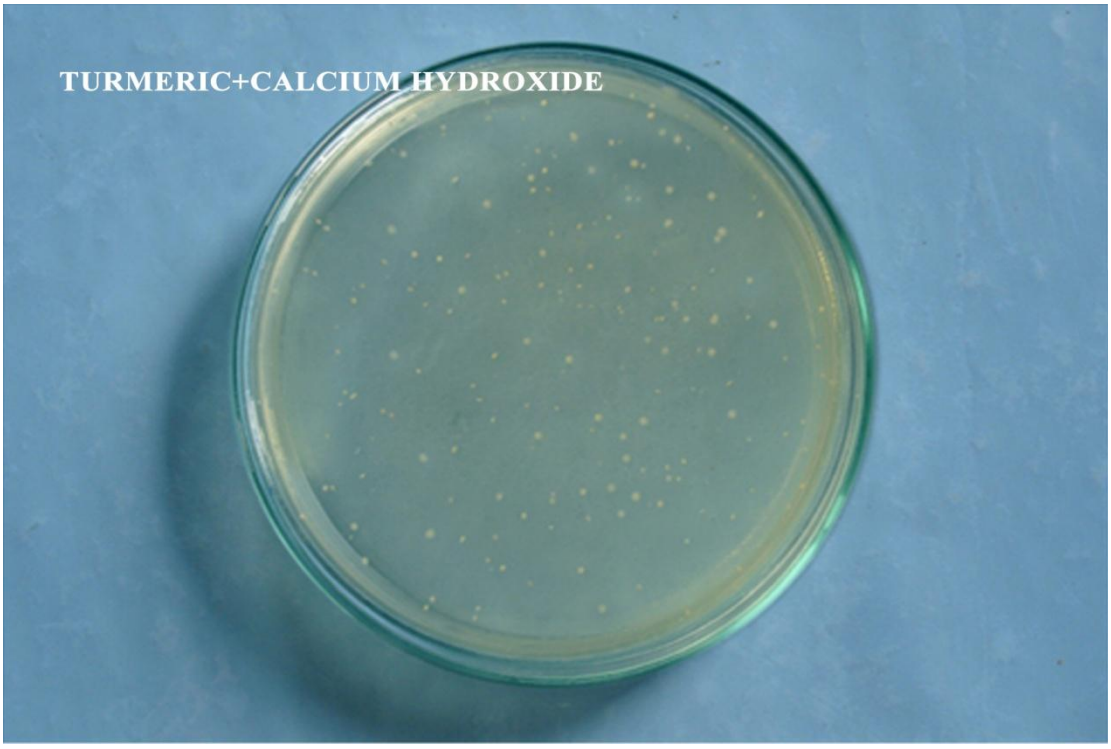


Figure 17: Colony Forming Unit of Turmeric + Calcium

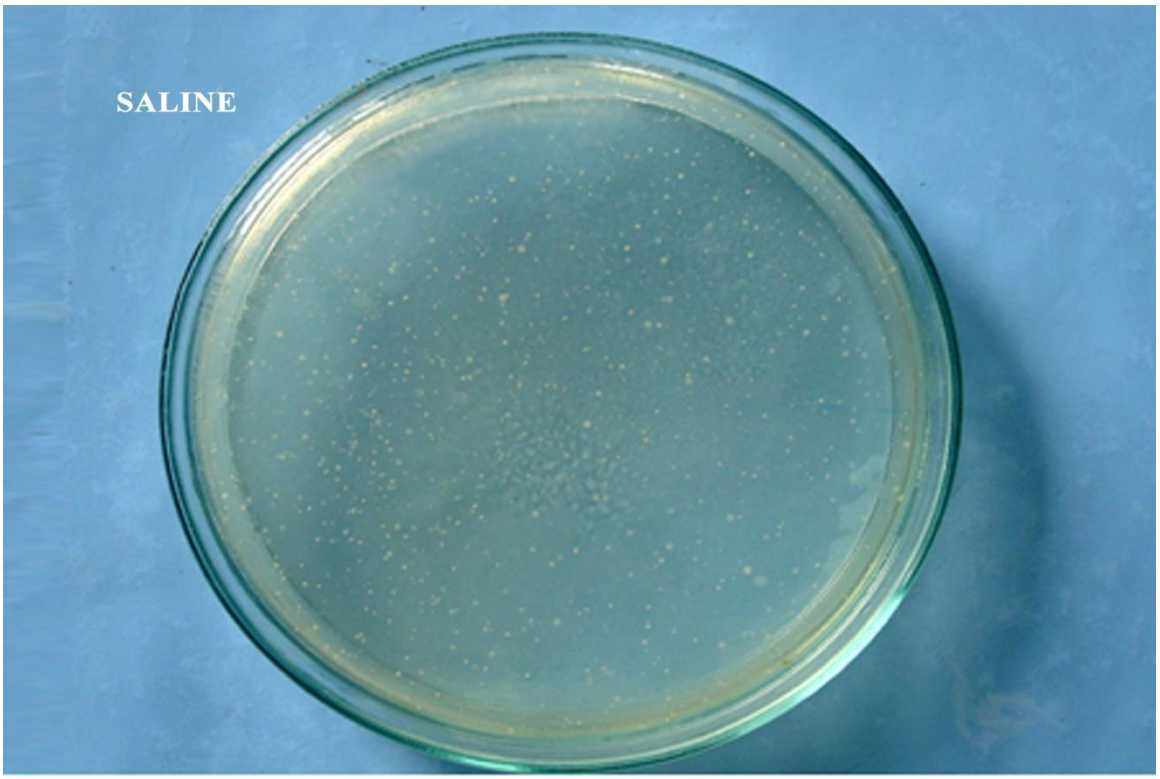


Figure 18: Colony Forming Unit Of Saline

RESULTS

STATISTICAL ANALYSIS

Data entry and data base management was done in SPSS (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp. Released 2013) is used. Significance level is fixed as 5% ($\alpha = 0.05$).

Descriptive statistics was used to calculate mean and standard deviation for all the groups

The results of the present study were subjected to statistical analysis to interpret the significant differences in *E.faecalis* count within each group and also between the groups using one way anova and Tamhane's Post hoc tukey tests.

One way analysis of variance (ANOVA) is used to study the overall variance within groups. It is the extension of the between groups t-test to the situation in which more than two groups are compared simultaneously. However, it is not possible to identify the difference between the various subgroups with the help of the P values obtained from ANOVA. Therefore a specific statistical test was used for intra group comparison. Hence, Tamhane's Post hoc tukey tests is done in order to determine which groups differ from each other. The Post hoc Tukey Test is a post hoc test designed to perform a pair wise comparison of the means to identify the specific groups in which significant difference expression occurs.

TABLE-1SUSCEPTIBILITY OF *E.FAECALIS* MTCC 2527 AGAINST THE TEST SOLUTIONS

Test Solution	Zone of Inhibition At 3mg	Minimum Inhibitory Concentration	Minimum Bactericidal Concentration
Neem	18mm	1mg/ml	2mg/ml
Turmeric	8mm	3mg/ml	6mg/ml
Calcium Hydroxide	19mm	315µg/ml	1.25mg/ml
Ciprofloxin 5µg	23mm	1 µg/ml	2 µg/ml

Table 1 shows the zone of inhibition, MIC, and MBC of test solutions for *E.faecalis* (MTCC 2527). All test solutions have shown a significant zone of inhibition in the well diffusion method when compared with ciprofloxin. No zone of inhibition was shown by 10% DMSO. Maximum inhibition was observed by in neem followed by calcium hydroxide and turmeric.

TABLE 2: ONE -WAY ANOVA ANALYSIS FOR MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT SAMPLES

Groups	N	Mean CFU (10^3cfu/ml)	Std. Dev	p-Value
Group 1(Neem)	10	1.65	0.649	
Group 2(Turmeric)	10	3.49	0.423	<0.001
Group 3(Calciumhydroxide)	10	1.65	0.649	
Group 4 (Neem + Calciumhydroxide)	10	0.64	0.109	
Group 5(Turmeric + Calciumhydroxide)	10	2.58	0.429	
Group 6(Saline)	10	643000	53758.7	

Table 2 shows the mean values of colony forming units(CFU) using one way ANOVA in root samples. Statistical result denotes that there is a significant differences among all the groups. Group I mean was 1.65±0.65, Group II mean was 3.49±0.42, Group III mean was 1.65±0.65 and Group mean IV 0.64±0.11,Group mean V was 2.58±0.43 and Group mean VI 643000±537587.7 for which the p value was < 0.001 which was statistically significant 1% level.

TABLE 3: PAIRWISE COMPARISONS OF MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT CANAL SAMPLES GROUP I WITH ALL OTHER GROUPS USING TAMHANE'SPOST HOC ANALYSIS

Groups	P Value
Neem × Turmeric	0.002
Neem × Calcium Hydroxide	0.026
Neem × Neem+Calcium Hydroxide	<0.001
Neem × Turmeric+Calcium Hydroxide	0.026
Neem × Saline	<0.001

Table 3 denotes pairwise comparisons of mean colony counts of *E.faecalis* in root canal samples using tamhane's post hoc analysis in which group I is statistically significant to group IV and group VI p<0.001.group II, group III and group V is not statistically significant.

TABLE-4 PAIRWISE COMPARISONS OF MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT CANAL SAMPLES GROUP II WITH ALL OTHER GROUPS USING TAMHANE'S POST HOC ANALYSIS

Groups	P Value
Turmeric × Neem	<0.001
Turmeric × Calcium Hydroxide	0.002
Trumeric × Neem+ Calcium Hydroxide	<0.001
Turmeric × Turmeric+ Calcium Hydroxide	<0.001
Turmeric × Saline	<0.001

Table 4 denotes pairwise comparisons of mean colony counts of *E.faecalis* in root canal samples using tamhane's post hoc analysis in which group II is statistically significant to group I, group IV, group V, group VI p<0.001. group III is not statistically significant.

TABLE-5: PAIRWISE COMPARISONS OF MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT CANAL SAMPLES GROUP III WITH ALL OTHER GROUPS USING TAMHANE'S POST HOC ANALYSIS

Groups	P Value
Calcium Hydroxide × Neem	<0.001
Calcium Hydroxide × Turmeric	<0.001
Calcium Hydroxide × Neem+ Calcium Hydroxide	0.012
Calcium Hydroxide × Turmeric+ Calcium Hydroxide	1.000
Calcium Hydroxide × Saline	<0.001

Table 5 denotes pairwise comparisons of mean colony counts of *E.faecalis* in root canal samples using tamhane's post hoc analysis in which group III is statistically significant to group I, group II, group VI $p<0.001$. group IV and group Vis not statistically significant.

TABLE -6: PAIRWISE COMPARISONS OF MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT CANAL SAMPLES GROUP IV WITH ALL OTHER GROUPS USING TAMHANE'S POST HOC ANALYSIS

Groups	P Value
Neem+ Calcium Hydroxide × Neem	<0.001
Neem+ Calcium Hydroxide × Turmeric	<0.001
Neem+ Calcium Hydroxide × Calcium Hydroxide	0.012
Neem+ Calcium Hydroxide × Turmeric+ Calcium Hydroxide	1.000
Neem+ Calcium Hydroxide × Saline	<0.001

Table 6 denotes pairwise comparisons of mean colony counts of *E.faecalis* in root canal samples using tamhane's post hoc analysis in which group IV is statistically significant to group I, group II, group VI $p<0.001$. group III and group Vis not statistically significant.

TABLE-7: PAIRWISE COMPARISONS OF MEAN COLONY COUNTS OF *E.FAECALIS* IN ROOT CANAL SAMPLES GROUP V WITH ALL OTHER GROUPS USING TAMHANE'S POST HOC ANALYSIS

Groups	P Value
Turmeric+ Calcium Hydroxide × Neem	0.026
Turmeric+ Calcium Hydroxide × Turmeric	<0.001
Turmeric+ Calcium Hydroxide × Calcium Hydroxide	1.000
Turmeric+ Calcium Hydroxide × Neem+ Calcium Hydroxide	0.012
Turmeric+ Calcium Hydroxide × Saline	<0.001

Table 7 denotes pairwise comparisons of mean colony counts of *E.faecalis* in root canal samples using tamhane's post hoc analysis in which group V is statistically significant to group II, group VI $p<0.001$. group II, group III, group IV is not statistically significant.

Chart 1:Comparison of mean change in colony forming unit control group with study group.

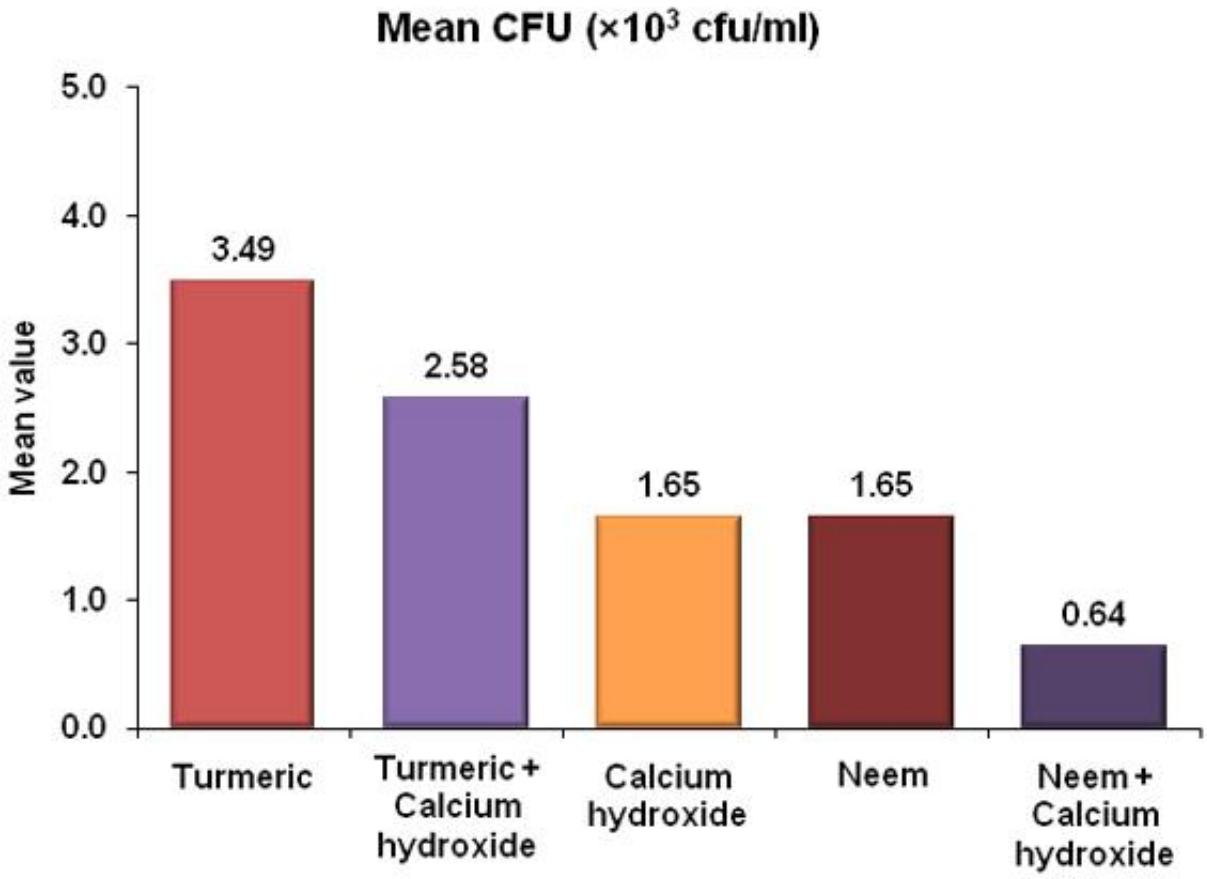
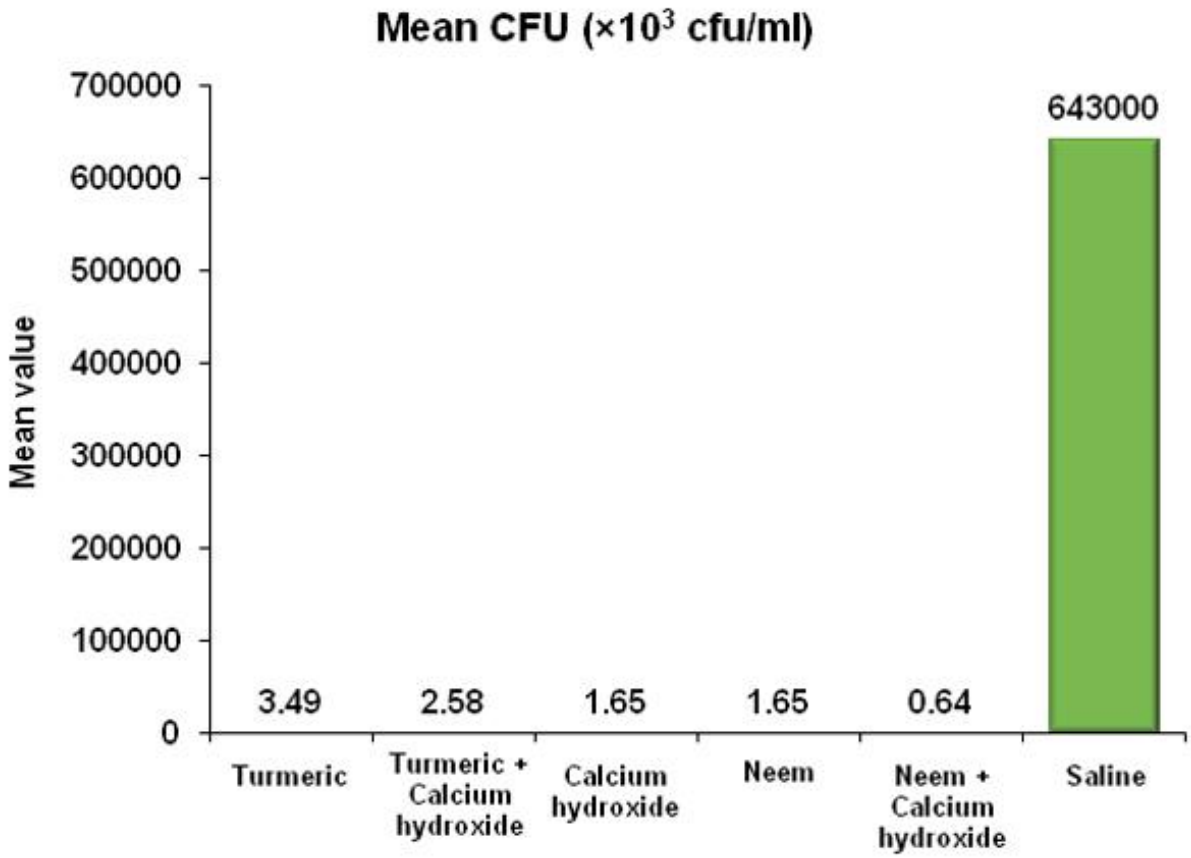


Chart 2: Comparison of mean change in colony forming unit control group with study group.



All treated groups have shown a significant reduction of bacterial population compared with the control group, In treated groups, group II has shown maximum bacterial count (3.49×10^3cfu/ml), group IV shows maximum inhibition in bacterial count (0.64×10^3cfu/ml).

DISCUSSION

Main stream medicines are either synthetic or semi-synthetic and their toxic nature has started to raise concern amongst the public. The WHO estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care or their active components. Clinical microbiologists have three specific reasons to be interested in the role of antimicrobial plant extracts. First reason being the phytochemicals have found their way in to the arsenal of antimicrobial drugs prescribed by physicians. Second reason being the scientists have realized that drug resistance has shortened the effective span of synthetic antibiotics. Thirdly, public awareness of limited span of antibiotics, its abuse and ill effects. Two herbal extracts were used in this study, which are commonly used in gastrointestinal disorders, as *E.faecalis* is one of the microorganisms of concern in these conditions.

Enterococcus faecalis, which is responsible for 80–90% of human enterococcal infections[49], is the dominant *Enterococcus species*[50,62] and commonly the only species recovered from the obturated root canal[51,62] . These facts indicate that *E.faecalis* has a pathogenic role in chronic endodontic treatment failure. The Enterococci are capable of transmitting genetic information by both plasmid and transposon exchange. The genetic material included on enterococcal plasmids and transposons may be drug resistance determinants or virulence gene[52] .

The most-cited virulence factors are surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin. Each of them may be associated with various stages of an endodontic infection as well as with periapical inflammation. Functioning Proton-pump mechanism enables *E.faecalis* to resist high pH of intracanal medicaments[55].

In the obturated root canals with chronic apical periodontitis, the infected organisms are partly shielded from the defence mechanisms of the body. In most environmental niches, these bacteria survive and multiply not as planktonic cells suspended in liquids, but as surface attached biofilms and form complex communities of microorganisms. They activate sets of genes that are dormant with renewed genetic instruction, to form complicated slimy communities that can be 1500 times more antibiotic resistant than free floating bacteria[54,63].

A biofilm is a consortium of bacteria or other microbes organized with an extensive exopolymer[55,64], biofilms are considered to be highly structural habitats with spatial and physiological heterogenecity[56] .Organisms can deposit enzymes within the matrix and cell to cell signalling is an important feature in biofilm regulation, with the quorum sensing system. In a biofilm, the microbes get adsorbed onto a solid nonshedding surface, and are embedded in a common self-produced extra-cellular matrix[57] .The structural features of biofilm allow efficient transfer of nutrients, removal of waste

materials, and circulation of secondary metabolites and pheromones. The altered microbial genetic and metabolic processes, along with the extra-cellular matrix, are thought to resist the actions of antimicrobials[58,65.] It is crucial to realize that the biofilm forming capacity of microorganisms depends upon the surface attributes of the substratum.

In this study biofilm was grown on the root canal surface to provide the tooth substrate for the organism to form the biofilm, which will be in sequence with the clinical condition. Haapasalo reported that dentin has the ability to inactivate intracanal medicament when tested in vitro[59]. It is established that the biofilm forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria–substrate interaction and therefore tooth substrate was chosen.

The tooth specimens were sectioned below CEJ to obtain a standardized tooth length of 13 mm for uniform specimen. The 13 mm tooth samples with average weight of 0.12 gram to 0.13 gram were chosen for standardization which will influence the quantitative assay (CFU)[60,66].

The standard chart established with measurement of zone diameter for ciprofloxin against *E.faecalis* MTCC 2527 strain was used as international bench mark to compare sensitivity of our test solutions. Oral isolates were not preferred over MTCC strain because

these are resistant traits of particular clinical relevance as they confer resistance to agents used in the treatment of serious infections and can abolish the activity of the therapeutic regimes with proven bactericidal activity.

7 days old biofilm were chosen to evaluate the antibacterial efficacy because the incubation period was sufficient for the formation of clumps of bacteria bounded by carbohydrate matrix, which was feature normally found in biofilm. In fact the starvation condition of the *E.faecalis* biofilm could develop as early as after one day of incubation period[47.]

The concentration of the herbal solutions was increased, as the fact that sessile bacteria on surfaces or present within biofilm are much less readily inactivated than planktonic cells. Several reasons have been put forward for this difference. Diffusion of biocides into biofilm and interaction with biofilm are both important aspects,[61, 67] but are not the only factor responsible. A Biocide gradient is produced throughout the biofilm, so that in thick biofilm there will be an 'in-use' concentration as the biocide penetrates into the community. The concentration of 40mg/ml used in this study was found fruitful as an antibacterial against *E.faecalis* and further reduction in concentration, when used in vivo is still feasible and effective as the bacterial count expected to be much less than what we have used in this study.

Neem has been considered as a potential source of many therapeutic agents[72]. Earlier research on Neem showed that it contains various active constituents with diverse medicinal properties. The aqueous extract of Neem leaves had shown a good therapeutic potential as anti-hyperglycaemic agent. Neem leaf extract is also used to treat dental plaque and gingivitis[73]. Neem has been extensively used in Ayurveda, unani, homeopathy and siddha. Neem *(Azadirachta Indica)* leaf extract has significant antimicrobial effect against *E.faecalis* derived from infected root canal samples. Its antioxidant and antimicrobial properties makes it a potential agent for root canal medicament as an alternative to calcium hydroxide[68]. In this study alcoholic neem extract where used to see antimicrobial efficacy on *E.faecalis* biofilm.

Curcuma longa, a member of a ginger family has been widely reported to possess anti-inflammatory, antioxidant, antimicrobial and anti-cancer activity[75]. Components of turmeric are named curcuminoids, which mainly include curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. Curcumin, a yellow coloured pigment which is phenolic, is the most important fraction which is responsible for the biological activities of turmeric. Curcumin possesses antibacterial property against a number of Gram positive and Gram negative bacteria. Curcumin being a highly pleiotropic molecule is capable of interacting with numerous molecular targets involved in inflammation. Curcumin has been shown to regulate numerous

transcription factors, cytokines, protein kinases, adhesion molecules, redox status and enzymes that have been linked to inflammation[71].

Calcium hydroxide, discovered by Hermann in 1920, has been advocated and used as an intra-canal medicament . Its anti-microbial properties have been attributed to its High pH (11-12.5). Its dissociation into the highly interactive and lethal hydroxyl ions which kill bacterial cells by damaging the cytoplasmic membrane, protein denaturation and damaging the DNA. It has ability to absorb carbon dioxide, which deprives capnophillic bacteria, which mainly rely on it for their nutrition for thriving. Its physical presence, prevents the ingress of bacteria either coronally or apically. It enhances the tissue dissolution action of sodium hypochlorite .Even with all these anti-microbial actions calcium hydroxide has been shown to be incapable in eliminating *E.faecalis* and certain other organisms, which are present deep within the dentinal tubules as it needs direct contact with the bacteria for acting[45.]

In this present study, The antimicrobial sensitivity test was observed on neem, turmeric, and calcium hydroxide by well diffusion and broth dilution method on *E.faecalis* planktonic cells. Neem performed better zone formation than the calcium hydroxide and turmeric, but all the test solution were confirmed to be a sensitive.

In time kill study shows neem and turmeric took 6min to kill *E.faecalis* planktonic cells. Whereas calcium hydroxide took 12hrs to

kill viable bacteria it could be because of calcium hydroxide acts by changing the pH to alkaline and it takes time to act.

In Quantitative assay with 7days biofilm, CFU (colony forming unit) count was observed with all test solutions.

The CFU (colony forming unit) were observed and counted in all the test solution. Neem performed better in 7 days biofilm compared to the Turmeric and calcium hydroxide. Turmeric and calcium hydroxide performance was reduced in 7days biofilm. Neem was 5 log reduction in the microbial population when compared with control group saline. Turmeric and Calcium hydroxide showed only less than 4 log. Based on the microbial count calcium hydroxide and turmeric is not as much effective against *E.faecalis* when compared with neem.

When neem is combined with calcium hydroxide the antimicrobial efficacy was increased further which may be due to the synergistic effect of calcium hydroxide and neem. It shows 6 log reduction in microbial colonies when comparison with other groups. Whereas combination of turmeric and calcium hydroxide showed only 5 log reduction in microbial colonies.

According to National Committee for Clinical Laboratory Standards 2005, the definition of bactericidal activity usually requires ≥ 99.9% killing of the initial inoculum and is determined by noting the presence or absence of a 3-log 10-unit decrease in the CFU per milliliter. Generally any antibacterial agent showing four log reduction

from the original count is considered to be highly sensitive against particular organism that is been tested.

It was known that the antibacterial activity of Ca(OH)2 depends on its high pH. The alkalinity of this agent destroys bacterial cell membranes and protein structures. However, the initial high pH of Ca(OH)2 at 12.3 will be reduced to a pH of 10.3 when it is placed into the root canals. This pH reduction is due to the buffering effect of root canaldentine . It has been known that the *E.faecalis* can survive at a pH as high as 11.5, hence, with the lower pH value of Ca(OH)2, the *E.faecalis* in the dentinal tubules could not be removed effectively[74].

Turmeric has a less effect on *E.faecalis* 7 days biofilm due to the reason of inability to penetrate in the dentinal tubules, inadequate exposure, inadequate concentration used. Thus the optimal exposure and concentrations are needed for further investigation.

Among the two herbal alternatives, neem showed more potency to eliminate the *E.faecalis*. It may be either due to the presence of nimbidin, and nimbolide in neem which possess antibacterial, antioxidant and antifungal property causing bacterial cell lysis. All the test solutions in this study showed statistically significant antibacterial efficacy against *E.faecalis* when compared with the control.

A combination of neem and calcium hydroxide is proved to be the best among the test solutions which exhibited excellent antibacterial activity in 7 days biofilm. Antimicrobial and antibioflim of neem and calcium hydroxide are therefore promising in future control of biofilm formation.

SUMMARY

Antibacterial effect of neem (*Azadirachta Indica*) and turmeric (*Curcuma longa*) and calcium hydroxide were estimated on planktonic *E.faecalis* by well diffusion and Broth dilution method as per NCCLS recommendations. Since the herbal test solutions showed antibacterial effect on planktonic cells, we expanded our study on the *Enterococcus faecalis* (MTCC 2527) Biofilm in combination with and without addition calcium hydroxide.

Quantitative assay were done on 60 teeth samples which were freshly extracted, inoculated with *E.faecalis* (MTCC 2527) and were incubated for 7days. The samples were divided into 6 groups namely neem, turmeric, calcium hydroxide, neem+ calcium hydroxide, turmeric + calcium hydroxide, saline (control). In quantitative assay, CFU (Colony forming unit) was estimated using Pour plate method and the results were statistically analyzed using One - Way Anova and Tamhane's Post hoc tukey tests.

CONCLUSION

Within the limitation of this study it was found that neem + calcium hydroxide showed maximum antibacterial activity against 7 days *E.faecalis* biofilm formed on the tooth substrate followed by neem, calcium hydroxide, turmeric + calcium hydroxide. Hence, it is good notion to try herbal alternatives, out of which neem combine with calcium hydroxide seems to be the most effective, but none were able to completely eradicate *E.faecalis* biofilm in dentinal tubules. Further research is needed to conclusively recommend herbal as a root canal medicament.

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INSTITUTIONAL ETHICS COMMITTEE AND REVIEW BOARD

ADHIPARASAKTHI DENTAL COLLEGE AND HOSPITAL

Melmaruvathur, Tamilnadu-603019

An ISO 9001:2008 certified institution. Accredited by NAAC with "B" grade.

Recognised by DCI, New Delhi. Affiliated to: The Tamil Nadu Dr. M.G.R. Medical University, Chennai.

This ethical committee has undergone the research protocol submitted by **Dr. A. KARTHIKEYAN** Post Graduate Student, Department of Conservative Dentistry and Endodontics under the title **"A COMPARATIVE EVALUATION OF ANTIBACTERIAL EFFICACY OF NEEM AND TURMERIC WITH AND WITHOUT ADDITION AF CALCIUM HYDROXIDE AGAINST *ENTEROCOCCUS FAECALIS* - AN IN VITRO STUDY"** Reference No: 2015- MD-BrIV-SAT-09/APDCH under the guidance of **Dr.S.THILLAINAYAGAM MDS.,** for consideration of approval to proceed with the study.

This committee has discussed about the material being involved with the study, the qualification of the investigator, the present norms and recommendation from the Clinical Research scientific body and comes to a conclusion that this research protocol fulfils the specific requirements and the committee authorizes the proposal.

Date: **Member secretary**