COMPARATIVE EVALUATION OF ANTIMICROBIAL EFFICACY OF VARIOUS PHYTOCHEMICAL IRRIGANTS AND 3% SODIUM HYPOCHLORITE AGAINST AN ENDODONTIC BIOFILM MODEL AND SCANNING ELECTRON MICROSCOPIC OBSERVATION OF SURFACE MORPHOLOGY OF ROOT CANAL DENTIN AFTER TREATMENT WITH DIFFERENT IRRIGANTS - AN EX VIVO STUDY.

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CERTIFICATE

This is to certify that this dissertation titled **"Comparative Evaluation Of Antimicrobial Efficacy Of Various Phytochemical Irrigants And 3% Sodium Hypochlorite Against An Endodontic Biofilm Model And Scanning Electron Microscopic Observation Of Surface Morphology Of Root Canal Dentin After Treatment With Different Irrigants - An Ex Vivo Study"** is a bonafide record of work done by **Dr. S. JAYALAKSHMI** under my guidance and to my satisfaction during her postgraduate study period between 2012-2015. This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the award of the degree of Master of Dental Surgery in Conservative Dentistry and Endodontics, Branch IV. It has not been submitted (partial or full) for the award of any other degree or diploma.

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INTRODUCTION

The success of endodontic treatment requires effective debridement and disinfection of the root canal system [37]. Currently, the eradication of a microbial infection is accomplished mainly through mechanical instrumentation and chemical irrigation [41]. Although mechanical preparation of the infected root canal has been shown to be most effective in reducing the number of bacteria, it alone is unreliable in achieving adequate disinfection [10, 50]. Irrigation allows for cleaning beyond what might be achievable through instrumentation because it enhances further bacterial elimination and facilitates necrotic tissue removal from the anatomic complexities of the root canal system, and prevents the packing of infected debris apically [28].

A broad antimicrobial spectrum against anaerobic and facultative microorganisms, biofilms and the ability to dissolve or remove the smear layer formed during instrumentation are amongst the major requirements of root canal irrigants. They should also be nontoxic and noncaustic to the periapical and periradicular tissues [77].

Sodium Hypochlorite (NaOCl) has been the most widely used root canal irrigant for several decades. Its excellent properties of tissue dissolution and antimicrobial activity make it the irrigating solution of choice for endodontic treatment [67]. However it has several undesirable characteristics such as tissue toxicity, risk of emphysema, allergic potential, and disagreeable smell and taste [45, 53,54].

In the last few decades, the medical fraternity has evinced keen interest in using natural preparations for treating various ailments, which has lead to various researches in

phytotherapeutics. To overcome problems associated with currently used irrigants, use of natural plant extracts as endodontic irrigants might be of interest to professionals as part of a growing trend to seek natural remedies in dental treatment. The use of natural derivatives may have a greater level of tolerance by the body with exhibition of fewer side effects [22]. According to the W.H.O medicinal plants would be the best source to obtain a variety of drugs [22]. Plant derived natural products represent a rich source of antimicrobial compounds, the beneficial medicinal effects of which typically result from the combinations of secondary products present, such as *tannins, saponins, phenolic compounds, essential oils and flavonoids,* which disrupt the permeability barrier of cell membrane structures and thus inhibit the bacterial growth**.** It has been proposed that the mechanism of antimicrobial effects involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally, ion leakage from the cells. Due to their beneficial effects, certain of these plant derivatives have been tried in endodontics over conventional irrigants.

The majority of endodontic biofilm studies have been conducted using models with monospecies bacterial cultures grown on membranes, glass or plastic, either under continuous or frequent supply of nutrients [8, 11, 12, 19, 20, 76]. Recently, mixed-species dentin infection models have been developed to study factors affecting biofilm pathogenicity and the effects of different disinfecting solutions [38, 74]. However, most biofilm models used thus far do not adequately reflect the complexity of the root canal anatomy, and they do not simulate the clinical situation. Therefore, it is of importance to develop

multispecies biofilm models resembling in vivo endodontic biofilms for studying root canal disinfection [39].

Thus the purpose of this study was

(1) To find out the best yield and to determine the optimum concentration of herbal extracts namely ***Acacia nilotica*** **(Babool),** ***Azadirachta indica*** **(Neem),** ***Cinnamomum zeylanicum*** **(Cinnamon),** and ***Syzygium aromaticum*** **(Clove),** for their antimicrobial activity.

(2) To introduce a novel multispecies biofilm model in extracted single-rooted teeth.

(3) To use the model to test the efficacy of herbal irrigation together with instrumentation in the removal of endodontic biofilms.

(4) To analyze the surface morphology of root canal dentin after treatment with different irrigants (Sodium Hypochlorite and different Herbal extracts) using Scanning Electron Microscope.

AIM AND OBJECTIVE

The aim of this study was to determine the optimum concentration of herbal extracts namely ***Acacia nilotica*** **(Babool),** ***Azadirachta indica*** **(Neem),** ***Cinnamomum zeylanicum*** **(Cinnamon),** and ***Syzygium aromaticum*** **(Clove),** for their antimicrobial activity, and to test these herbal irrigants against Sodium Hypochlorite for their antimicrobial efficacy in multispecies endodontic biofilm model.

REVIEW OF LITERATURE

Saini *et al*., (2008) [61] examined the comparative antimicrobial studies of *Acacia* species namely *Acacia nilotica*, *Acacia tortilis*, *Acacia senegal*, *Acacia catechu*, *Acacia jacquemontii* which were tested for preliminary ethnomedicinal and antimicrobial screening using the disc diffusion method against three bacterial (*Escherichia coli, Staphylococcus aureus* and *Salmonella typhi*) and two fungal strains (*Candida albicans* and *Aspergillus niger*). Subsequently, the two most active species: *A. catechu* and *A. nilotica* were further considered for detailed pharmacognostical studies. The authors found out that *A. catechu* and *A. nilotica* exhibited the highest antibacterial activity among the species against the tested microorganisms.

Vijayashanthi *et al*., (2011) [73] evaluated the antibacterial potential of various solvent extracts of *Acacia nilotica* leaves. Amongst six microorganisms investigated, two Gram-positive bacteria were *Staphylococcus aureus*, *Bacillus subtilis* while four Gram-negative bacteria were *Escherichia coli* MTCC 2961, *Pseuodomonas aeruginosa* MTCC 4676, *Klebsiella pneumoniae* MTCC 432 and *Salmonella typhi* MTCC 733. Antimicrobial activity was carried out by the disc diffusion method with Dimethylsulfoxide as negative control and Chloramphenicol as positive control. The authors concluded that crude alkaloids of *A.nilotica* leaves had higher inhibitory potential against tested bacterial pathogens.

Nagumanthri *et al*., (2012) [47] screened the antimicrobial activity of *Acacia nilotica, Ziziphus mauritiana, Bauhinia variegate* and *Lantana camara* against some selected clinical isolated bacterial strains. The fresh parts (leaves, barks & pods) of the

test medicinal plants were collected and methanol, ethanol and ethyl acetate extracts were prepared. Antibacterial susceptibility test was done by using Agar diffusion assay method. The authors found out that *Lantana camara* showed the highest antimicrobial activity followed by *Acacia nilotica* against the microorganisms tested and yielded the most potent antimicrobial extracts respectively.

Deshpande (2013) [16] performed a preliminary phytochemical analysis and in vitro investigation of antibacterial activity of *Acacia nilotica* against clinical isolates. Tests revealed the presence of alkaloids, carbohydrates, saponins, tannins, flavanoids, cardiac glycosides and anthraquinone in both ethanol and petroleum ether extracts while fixed oils, fats, proteins and amino acids were absent. Antimicrobial activity of the extracts against clinical isolates was performed by agar diffusion method. The author found that the extracts exhibited potent activity against all clinical isolates. The minimum inhibitory concentration for ethanol extract was 5 mg/ml while it was 10 mg/ml for petroleum ether extract.

Siswomihardjo *et al.*, (2007) [68] determined the antibacterial effect of ethanolic neem leaves and stick extract in inhibiting the growth of *Streptococcus mutans*. Ethanol extracts of neem leaves and stick were prepared at 10% and 20% concentration respectively and the antibacterial effect was determined by agar well diffusion method in which the Muller Hinton agar had been inoculated with *Streptococcus mutans*. The inhibition diameters were measured after 24 hrs of incubation. The results showed that

neem leaves and stick ethanolic extracts had good antibacterial effect on *Streptococcus mutans* and neem leaves extract had higher antibacterial properties than the stick extract.

Irshad *et al.,* (2011) [31] evaluated the antibacterial activity of Neem (*Azadirachta indica*) and Peppermint (*Mentha piperita*) by using agar diffusion assay and gel filtration chromatography against different bacterial strains. The authors found that, by agar diffusion assay the acetone extract of Neem and Peppermint Oil showed the maximum antibacterial activity as compared to other solvent extracts. Then, distilled water macerated form of Neem and Peppermint was used for gel filtration chromatography technique in order to determine the fraction containing the active components. Fraction 8 of both Neem and Peppermint showed maximum antibacterial activity against all above mentioned bacterial strains.

Sarmiento *et al*., (2011) [62] performed a study to determine if Neem leaf extract (*Azadirachta indica*) has antibacterial properties against Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* and to compare the antistaphylococcal properties of Neem leaf extract with Oxacillin, Vancomycin, Mupirocin, and Povidone iodine. Ethanol extract of Neem leaf was diluted to produce 25%, 50%, 75%, and 100% concentrations. Antimicrobial activity was seen by agar diffusion method and zones of inhibition were checked. The authors noted a trend of increasing antibacterial activity with increasing concentration of the extract. Zones of inhibition started to appear at 50% concentration for *S. aureus* and 75% for MRSA. They concluded that ethanol extract

from Neem leaves exhibits in vitro antibacterial activity against both *Staphylococcus aureus* and MRSA with greatest zones of inhibition noted at 100% concentration.

Maragathavalli *et al*., (2012) [42] determined the antimicrobial activity of neem (*Azadirachta indica*) leaf alcoholic extract against *E.coli, Staphylococcus aureus, Pseudomonas aeruginosa , Salmonella typhimurium,* and *Bacillus pumilus.* Varying concentration of the extract 200mg/ml, 150 mg/ml, 100mg/ml, 50mg/ml, 25mg/ml was prepared and antimicrobial activity was determined by using disc diffusion method. When compared with the control Gentamycin, the results revealed that methanol and ethanol extract showed maximum inhibition on *Bacillus pumillus, Pseudomonas aeruginosa* and *Staphylococcus aureus* in an ascending order.

Reddy *et al*., (2013) [58] compared the antimicrobial efficacy of aqueous extracts of leaf, bark and seeds of *A. Indica* against human pathogenic bacteria (S*taphylococcus aureus, Enterococcus feacalis, Proteus mirabilis* and *Pseudomonas aeuroginosa*) and fungi (*Aspergillus fumigatus* and *Candida albicans*). Agar well diffusion method and micro-broth dilution methods were used to determine the minimum inhibitory concentration (MIC). Results showed that leaf extract exhibited strong antimicrobial activity against bacteria and fungi at all the concentrations tested (500, 1000 and 2000µg/ml). Antimicrobial activity of bark extract was found to be moderate on bacteria and fungi (effective at 1000 and 2000µg/ml), whereas seed extract exhibited least antimicrobial activity.

Panchal *et al*., (2013) [51] evaluated the antibacterial activity of methanolic Neem leaf extract against *E.coli* and *Salmonella* using the Zone of Inhibition (ZOI) method. Methanol extracts of varying concentrations 0.5, 1.0, 1.5, and 2.0% was prepared and tested against test organisms using agar diffusion method. Gentamycin of same varying concentrations was used to compare the effect of antimicrobial activity of methanol leaf extract. The authors found that methanol extract of Neem showed the highest antimicrobial activity as compared to other extracts.

Mishra *et al*., (2013) [44] determined the antibacterial effects of *Azadirachta indica* against *Escherichia coli* and *Staphylococcus aureus.* Methanol extracts of varying concentrations 0.5, 1.0, 1.5, and 2.0% was prepared and tested against test organisms using agar diffusion method with Gentamycin as control. The results showed that *A.indica* leaves possessed good antibacterial activity and the authors concluded that the extract of *A.indica* when used as medicinal plant, could be useful for the growth inhibition of the carcinogenic bacterium, *S. sobrinus*.

Gende *et al*., (2008) [23] studied the physicochemical properties, composition and antimicrobial activity of cinnamon essential oil (*Cinnamomum zeylanicum*). The bioactivity of this essential oil against *Paenibacillus larvae* was analyzed by means of a combination of *in vitro* techniques, such as the tube dilution method and bioautography, a method employed to localize antibacterial activity on a chromatogram. Results revealed that Cinnamaldehyde and eugenol possessed antibacterial effects against *P. larvae.* The

authors concluded that Essential oil and especially, two of its main components presented inhibitory capacity against strains of *P. larvae*.

Ramya and Ganesh (2012) [55] performed phytochemical analysis and compared the effect of *Cinnamomum zeylanicum, Piper nigrum* and *Pimpinella anisum* with selected antibiotics and its antibacterial activity using the disc diffusion method against *Enterobacteriaceae* family. The spices were tested against the organisms such as *Escherichia coli*, *Salmonella* species, *Shigella* sp., *Klebsiella* sp., and *Proteus* sp. Results showed that phytochemical screening and qualitative estimation of the crude yield of *Cinnamonmum zeylanicum, Piper nigrum, and Pimpinella anisum* were rich in alkaloids, flavanoids, terpenoids, and saponins. The authors concluded that the presence of phytochemicals in spices has bacteriostatic and bactericidal activity and thus the spices extracts could serve as a source of drugs useful in chemotherapy.

Nimje *et al*., (2013) [49] compared the antibacterial activity of the essential oil from bark of two cinnamon species, *Cinnamomum zeylanicum* and *Cinnamomum cassia* and their chemical constituents against *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Efficacy of the essential oil of Cinnamomum species was compared using Disc Diffusion method and Minimum Inhibitory Concentration was calculated. Gentamycin, an anitibiotic, was used as positive control. The authors found that all three bacteria were found to be sensitive towards the essential oil of Cinnamomum species. However, the essential oil of *Cinnamomum cassia* was found to have more effective antimicrobial activity showing its maximum efficacy for *E.coli*.

Sofia *et al*., (2007) [69] tested the antimicrobial activity of different Indian spice plants such as mint, cinnamon *(Cinnamomum zeylanicum)*, mustard, ginger, garlic and clove *(Syzygium aromaticum)* against *Escherichia coli (E. coli), Staphylococcus aureus* and *Bacillus cereus* by the disc diffusion method. The only sample that showed complete bactericidal effect against all the food-borne pathogens tested was the aqueous extract of clove (*Syzygium aromaticum*) at 3%. At 1% concentration, clove extract showed good inhibitory action.

Aneja and Joshi (2010) [1] investigated the antimicrobial activity of clove *(Syzygium aromaticum)* and clove bud oil by agar well diffusion method against five dental caries causing microorganisms namely *Streptococcus mutans, Staphylococcus aureus, Lactobacillus acidophilus, Candida albicans* and *Saccharomyces cerevisiae*. The results indicated that clove and clove oil have potent antimicrobial activity against the tested dental caries causing microorganisms. The highest antimicrobial activity of clove was found against *Saccharomyces cerevisiae* in methanolic extract and that of clove oil was found against *Streptococcus mutans*. The authors concluded that clove and clove bud oil can be used as an antimicrobial agent to cure dental caries.

Khan *et al*., (2009) [33] compared the antimicrobial activities of the crude ethanolic extracts of five plants namely *Acacia nilotica*, *Syzygium aromaticum, Cinnamum zeylanicum*, *Terminalia arjuna,* and *Eucalyptus globules* against multidrug resistant (MDR) strains of *Streptococcus mutans, Staphylococcus aureus, Enterococcus faecalis, Streptococcus bovis, Pseudimonas aeruginosa, Salmonella typhimurium, Escherichia coli, Klebsiella pneumoniae* and *Candida albicans.* Results showed that the MDR strains

were sensitive to the antimicrobial activity of *Acacia nilotica*, *Syzygium aromaticum* and *Cinnamum zeylanicum*, whereas they exhibited strong resistance to the extracts of *Terminalia arjuna* and *Eucalyptus globulus*. This study concludes that *A. nilotica*, *C. zeylanicum* and *S. aromaticum* can be used against multidrug resistant microbes causing nosocomial and community acquired infections.

Prabhakar *et al*., (2010) [54] evaluated the antimicrobial efficacy of Triphala, green tea polyphenols (GTP), MTAD, and 5% sodium hypochlorite against *E. faecalis* biofilm by disc diffusion method. The results showed complete inhibition of bacterial growth with Triphala, MTAD and NaOCl, except GTP and saline, which showed presence of bacterial growth. The authors concluded that 5% sodium hypochlorite showed maximum antibacterial activity against *E. Faecalis* biofilm formed on tooth substrate. Triphala, green tea polyphenols and MTAD showed statistically significant antibacterial activity. They suggested that the use of herbal alternatives as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of NaOCl.

Badr *et al.,* (2010) [2] evaluated the antibacterial and cytotoxic effects of Liquorice as a root canal medicament compared with calcium hydroxide $Ca(OH)_2$ against *Enterococcus faecalis*. Agar-well diffusion methods, broth microdilution tests and biofilm susceptibility assays were used to determine the antibacterial activity. Human PDL fibroblast tissue culture was used to assess the cytotoxicity. The authors found that Liquorice extract either by itself or in combination with $Ca(OH)_2$ had a significant

inhibitory effect against *Enterococcus faecalis* compared with that of $Ca(OH)_2$ alone and it also retained significantly more viable PDL cells than $Ca(OH)_2$, which had a strong lethal effect on the cells. The authors concluded that Liquorice extract either separately or as Liquorice/$Ca(OH)_2$ mixture had potent bactericidal effect against *Enterococcus faecalis* and retained compatibility with fibroblasts in tissue culture compared to the commonly used root canal medicament $Ca(OH)_2$.

Bohora *et al*., (2010) [7] compared the antibacterial efficiency of neem leaf extract and 2% sodium hypochlorite against *E. faecalis, C. albicans* and mixed culture in vitro. Ethanolic extract of neem leaf was prepared and its antimicrobial efficacy was checked by agar well diffusion method with 2% NaOCl as control. The authors found that 2% NaOCl showed comparatively less antimicrobial effect than neem, and they concluded that neem leaf extract has a significant antimicrobial effect against *E. faecalis* and *C.albicans* and mixed state.

Singhal *et al*., (2011) [65] compared the antimicrobial efficacy of conventional endodontic irrigants and herbal products, like neem and green tea, alone and with calcium hydroxide (intracanal medicament) against *Enterococcus faecalis*. The herbal extracts were prepared and the samples were divided into five groups:

GROUP I – Conventional irrigants (Sodium Hypochlorite and Chlorhexidine Gluconate)

GROUP II – Herbal irrigants (Green tea and Neem)

GROUP III - Conventional irrigants + Calcium hydroxide

GROUP IV - Herbal irrigants + Calcium hydroxide

GROUP V – Control group (distilled water)

Antibacterial activity of the materials was evaluated by disc diffusion method and zones of inhibition were calculated. The authors concluded that neem had significant antimicrobial action against *E.faecalis* alone and with calcium hydroxide.

Kumar and Sidhu (2011) [36] evaluated the antimicrobial activity of *Azardirachta indica, Glycyrrhiza glabra, Cinnamum zeylanicum, Syzygium aromaticum, Acacia nilotica* on *S.mutans* and *E.faecalis* in vitro. Antibacterial activity of ethanol extracts of Neem, Liquorice, Cinnamon, Clove and Babool was tested against *Streptococcus mutans* and *Enterococcus faecalis* by disc diffusion method at concentrations of 10% and 50% at varying volumes for 24 hours. Results showed that Babool *(Accacia nilotica)* extract at 50% concentration showed the maximum zone of inhibition against *S.mutans* and *E.faecalis* followed by Liquorice *(Glycyrrhiza glabra).* The authors concluded that Babool and Liquorice extracts were effective against cariogenic pathogens like *S.mutans* and Babool and Clove extracts were effective against *Enterococcus faecalis* and can be used to reduce root canal microflora and root canal failures.

Chandra and Kumar (2011) [13] evaluated the antibacterial efficacy of aloe vera *(Aloe barbadensis Miller)* extract on resistant antimicrobial strains in endodontics. Chloroform, methanol and water extract of aloe vera pulp was obtained and its antibacterial efficacy was tested against *E.faecalis* and *Candida albicans* through the agar diffusion method with Calcium hydroxide as control. Zones of inhibition were greater for ethanol extract than chloroform extract for both *E.faecalis* and *Candida albicans*. The

authors concluded that the results achieved with alcohol and chloroform extracts suggest that the components of aloe vera are more soluble in those liquid extract media and possessed good antibacterial properties.

Singh and Das (2012) [64] evaluated the effectiveness of different concentrations of passion fruit pulp extract against *Streptococcus mutans*. Effectiveness was assessed by agar diffusion method and bacterial inhibition zones were measured for each concentration and compared. The authors found that the most effective concentration of passion fruit pulp extract was 40% to 45% against *Streptococcus mutans*. They suggested that passion fruit extract can be used as an alternative, inexpensive, good in taste, simple and effective method for sanitization of tooth cavity as well as root canal system.

Balakrishnan *et al*., (2012) [3] compared the antimicrobial efficacy of *Triphala, Morinda citrifolia, Aloe-vera* and *Vitex negundo* herbal extracts with the standard irrigant 5.25% Sodium hypochlorite against *Enterococcus faecalis* using agar disc diffusion method. The results showed that 5.25%NaOCl had the maximum zone of inhibition. Among the herbal irrigants, *Triphala* showed maximum zone of inhibition followed by *Morinda citrifolia, Aloe-vera* and *Vitex negundo*. The authors concluded that the in vitro observations of herbal products appear to be promising but more preclinical and clinical trials are needed to evaluate the biocompatibility and safety factor before they could be used as intra canal irrigating solutions and medicaments.

Hedge *et al*., (2012) [30] evaluated the antimicrobial activity of aqueous and hydro-alcoholic *Curcuma longa* (turmeric) extracts against endodontic pathogens (*Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans*) by agar well diffusion method. Aqueous and hydro-alcoholic extracts of the roots of *Curcuma longa* rhizome were prepared and solutions of different concentrations of the extracts were made. The Minimum inhibitory concentration (MIC) and the Minimum bactericidal concentration (MBC) were calculated. 2.3% sodium hypochlorite solution was used as the positive control. The authors found out that both extracts showed good antimicrobial properties against the endodontic pathogens and concluded that its future use as an endodontic irrigant or medicament should be considered.

Gupta *et al*., (2013) [26] evaluated the antimicrobial efficacy of *Ocimum sanctum, Cinnamomum zeylanicum, Syzygium aromaticum* and 3% sodium hypochlorite (NaOCl) against *Enterococcus faecalis* in planktonic suspension and biofilm phenotypes. The antibacterial efficacy of different concentrations of aqueous ethanolic extracts of *O.sanctum, C.zeylanicum* and *S.aromaticum* against *E.faecalis* at various time intervals was assessed using the agar well diffusion test, microdilution test and biofilm susceptibility assay (BSA) on cellulose nitrate membrane as well as in a tooth model with NaOCl as the positive control. The authors concluded that *C.zeylanicum, S.aromaticum* and *O.sanctum* demonstrated antimicrobial activity against planktonic and biofilm forms of *E. faecalis* with *C.zeylanicum* and *S.aromaticum* having better antimicrobial efficacy than *O.sanctum*. NaOCl had superior antimicrobial efficacy amongst all the groups.

Rosaline *et al.,* (2013) [59] assessed the antibacterial efficacy of three different herbal irrigants namely *Morinda citrifolia, Azadiracta indica* and green tea as a final rinse on the adherence of *Enterococcus faecalis*. Teeth inoculated with *E. faecalis* were randomly divided into three experimental and two control groups. Group 1 specimens were treated with 5.2% NaOCl for 30 min followed by 5 mmol/L EDTA for 5 min and saline as final irrigant. Group 2 specimens were treated with and 5.2% NaOCl for 30 min as final irrigant. Groups 3, 4 and 5 were treated with *Morinda citrifolia, Azadiracta indica* and green tea respectively for 30 min as final irrigant. The dentin specimens were examined in a confocal laser scanning microscope. The authors found that significantly fewer bacteria were found adhering to the samples treated with Neem followed by NaOCl, green tea, *Morinda citrifolia and* Saline. They concluded that Neem is effective in preventing adhesion of *E. faecalis* to dentin.

Lin *et al.,* (2013) [39] present a standardized biofilm model in extracted teeth with an artificial apical groove to quantify the efficacy of hand, rotary nickel-titanium, and self-adjusting file (SAF) instrumentation in biofilm bacteria removal. Thirty-six extracted single-rooted teeth were selected, split longitudinally, 0.2-mm-wide groove was placed in the apical 2 to 5 mm of the canal and mixed bacteria biofilm was grown inside the canal under an anaerobic condition. The split halves were reassembled in a custom block, and the teeth were randomly divided into 3 treatment groups using the K-file, ProFile and the SAF respectively. Irrigation consisted of 10 mL 3% NaOCl and 4 mL 17% EDTA. The authors found out that even though all techniques equally removed bacteria outside the groove, the SAF reduced significantly more bacteria within the apical groove and no

technique was able to remove all bacteria. They suggested that the biofilm model represents a potentially useful tool for the future study of root canal disinfection.

Valgas *et al*., (2007) [72] evaluated the technical variants used in screening methods to determine antibacterial activity of natural products. A varied range of natural products of plant, fungi and lichen origin were tested against two bacterial species, *Staphylococcus* and *Escherichia coli* by two variants of the agar diffusion method (well and disc), two variants of the bioautographic method (direct and indirect) and by microdilution assay. The authors concluded that the well-variant of the diffusion method was more sensitive than the disc-variant, whilst the direct-variant of the bioautographic method exhibited a greater sensitivity if compared to indirect variant. Bioautographic and diffusion techniques were found to have similar sensitivity; however the latter technique provided more suitable conditions for microbial growth.

MATERIALS AND METHODS

Materials used

- Extracted human teeth – single canal premolars
- Herbal powders - *Acacia nilotica, Azadirachta indica, Cinnamomum zeylanicum, Syzygium aromaticum* (Agricultural College and Research Institute, Coimbatore)
- Ethanol (Merck)
- Saline (0.9% w/v sodium chloride injection, NS, Baxter, India)
- Sodium hypochlorite (Prime dental, India)
- Ethylene Diamine Tetra Acetic acid (Dentsply Maillefer, USA)
- Muller Hinton Agar 173 (Himedia, India)
- Antibiotic discs (Himedia, India)
- Sticky wax (Coltene Whaledent)

Armamentarium

- Electronic weighing balance
- Spatula
- Conical flasks
- Measuring jars
- Beaker
- Test tubes (Borosil 27 ml, Riviera 15 ml)
- Petri dish
- Funnel
- Tripod stand
- Hot air oven
- Aluminium foil
- Electronic Shaker
- Incubator (NSW, India)

- Refrigerator
- Autoclave (Unique clave C-79, Confident)
- Scanning Electron Microscope (Carl Zeiss)
- Filter paper (Whatman's paper No 1)
- Paper points (Dentsply)
- Sterile swab
- Micropipette (Eppendorf)
- Microcentifuge tube (1.5ml)
- Absorbent paper
- Diamond saw
- Endomotor (X-Smart, Dentsply Maillefer, Japan)
- K files (Mani, Japan)
- Gates glidden drills (Mani, Japan)
- Disposable syringe (Dispovan)

Phase I – obtaining the maximum yield and optimum concentration of the herbal extracts

Obtaining the herbal powders

Leaves of ***Acacia nilotica*** **(Gum Arabic tree, Babool)** (Fig 1) and ***Azadirachta indica*** **(Neem)** (Fig 2) were collected from the gardens of Agricultural College and Research Institute, Coimbatore (Tamilnadu Agricultural University). Dried bark of ***Cinnamomum zeylanicum*** **(Cinnamon)** (Fig 3) and dried buds of ***Syzygium aromaticum*** **(Clove)** (Fig 4) were also collected. The taxonomic identity of these plants was confirmed at Department of Botany, Agricultural College and Research Institute, Coimbatore. All the herbs were dried in shade and pulverized grounded to coarse powder (Fig 5).

Herbal extracts - Obtaining the maximum yield

All the herbal powders were weighed electronically – each portion consisting of 5 grams and then suspended in varying volumes of 100% ethanol in conical flasks (Fig 6).

1: 2 (5 gms in 10 ml of ethanol – wt/vol)

1: 5 (5 gms in 25 ml of ethanol – wt/vol)

1: 10 (5 gms in 50 ml of ethanol – wt/vol)

Fig 1 : Acacia nilotica (Babool)

Fig 2 : Azadirachta indica (Neem)

Fig 3 :Cinnamomum zeylanicum (Cinnamon)

Fig 4 : Syzygium aromaticum (Clove)

Fig 5 : Herbal powders

Fig 6 : Herbal powders dissolved in ethanol

All conical flasks were covered with double thickness aluminum foil to prevent evaporation of ethanol. After a week's time the flasks were activated in a shaker (Fig 7) for about 18 hrs. Using Whatman's filter paper No 1 (Fig 8) the herbal solutions were filtered (Fig 9) and the beakers containing the filtrate were left to facilitate evaporation of ethanol. Ten days time was taken for complete evaporation of ethanol, at the end of which the extract was obtained (Fig 10). The ratio of 1:10 weight by volume of herbal powders to ethanol gave the maximum yield of the extract.

Determining the optimum concentration for antimicrobial activity

The extracts were redissolved in Dimethyl Sulfoxide (DMSO) in varying concentrations – 1mg/ml, 2mg/ml 3mg/ml, 4mg/ml and 5mg/ml.

Sub gingival plaque samples (Fig 11) 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.

Antibacterial activities of the extracts of varying concentrations were determined by the agar well diffusion assay. The zones of inhibition obtained with the herbal extracts were compared with the zones formed with the standard antibiotic discs (Fig 12 & 13) - Amoxicillin 10mcg, Ciprofloxacin 10mcg and Metronidazole 4mcg.

Fig 7 : Ethanol dissolution in electronic shaker

Fig 8 & 9 : Filtration of Extracts with Whatman's filter paper

Fig 10 : Herbal extracts

Fig 11 : Subgingival plaque sample

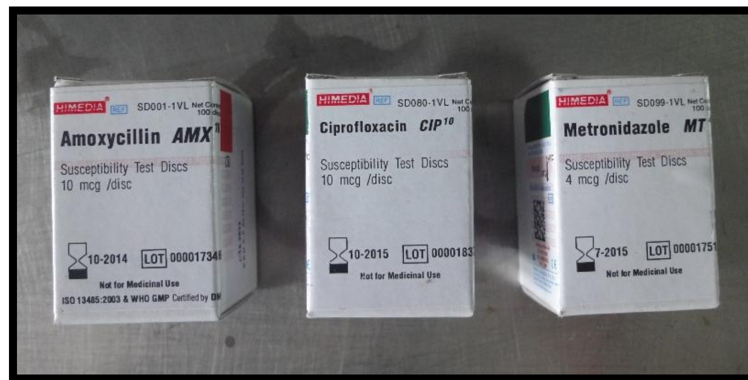


Fig 12 : Antibiotic discs

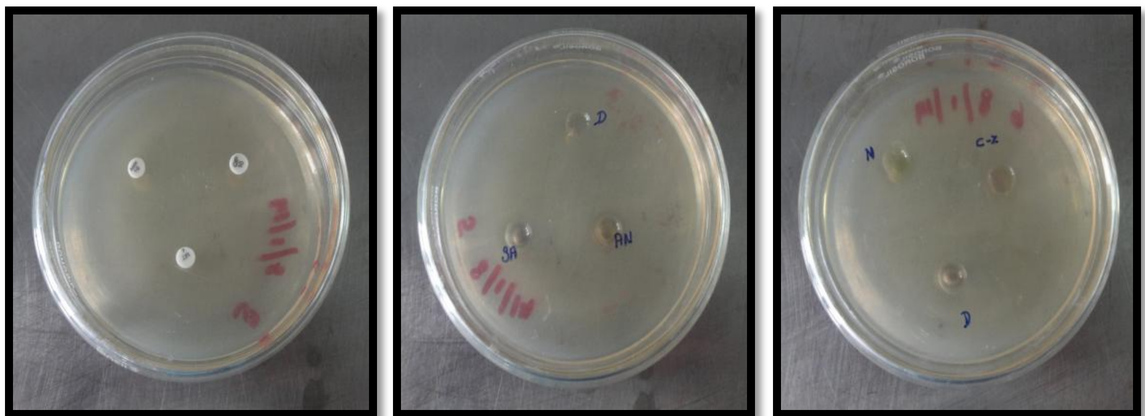
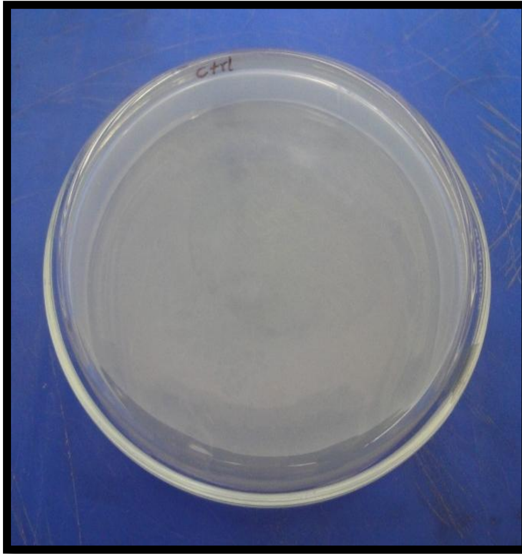


Fig 13 a : Antimicrobial activity - Antibiotic discs placed in culture plate

Fig 13 b & c : Herbal extracts placed in wells created in culture plates



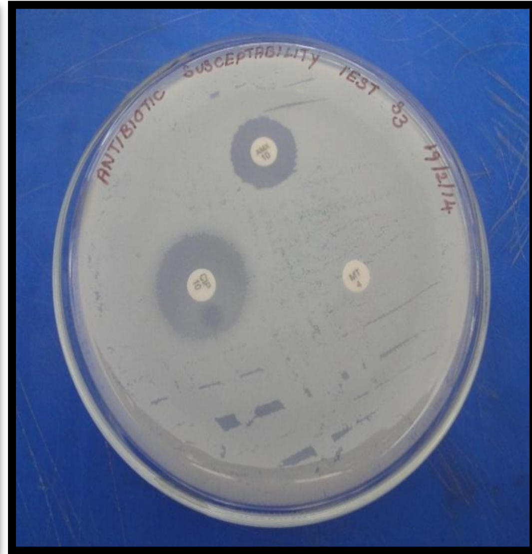


Fig 14 : Control

Fig 15 : Antibiotic sensitivity

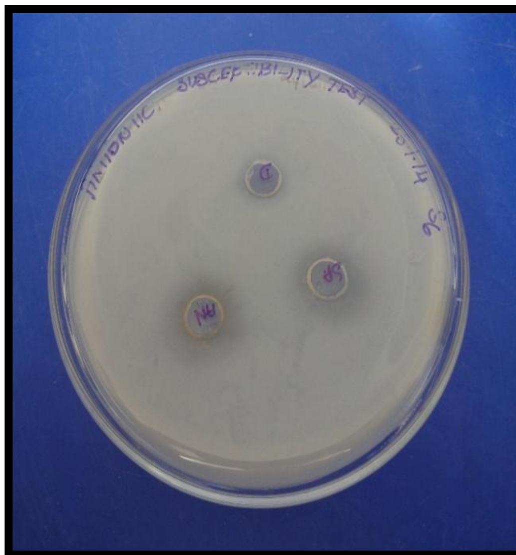


Fig 16 & 17 : Zone of inhibition in herbal groups

Zones obtained with the herbal extracts at 1 mg/ml concentration were very minimal when compared to the antibiotic control, therefore the same procedure was repeated with increasing concentration of the herbal extracts – with 2 mg/ml 3 mg/ml, 4 mg/ml and 5 mg/ml. Since the zones of inhibition obtained with a concentration of 5 mg/ml was equivalent or comparable to that of the control antibiotic discs, 5 mg/ml concentration of the herbal extract was taken as the optimum concentration for use in endodontic therapy of teeth in vitro (Fig 14 - 17).

Phase II - Antibacterial activity in teeth

Standardized Biofilm Tooth Model

Teeth selection and Standardization of Working Length

Sixty 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. The tooth specimens were sectioned below the cementoenamel junction with a diamond disc to obtain a standardized tooth length of 13 mm (Fig 18). The canals were accessed, and initially a size #10 Stainless Steel (SS) K was file 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.

Fig 18 : Decoronated teeth

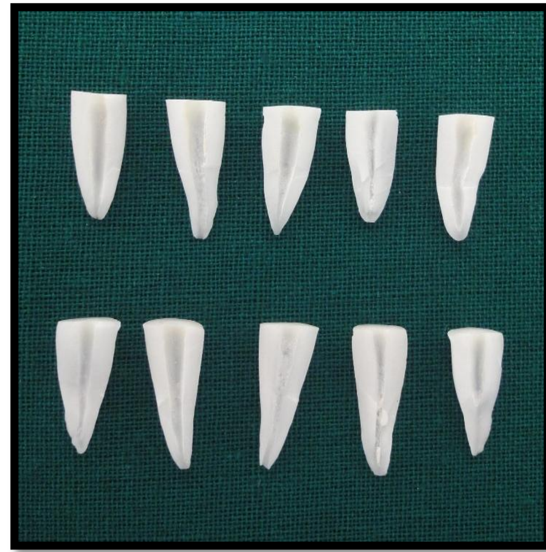


Fig 19 : Vertical sectioning for SEM analysis

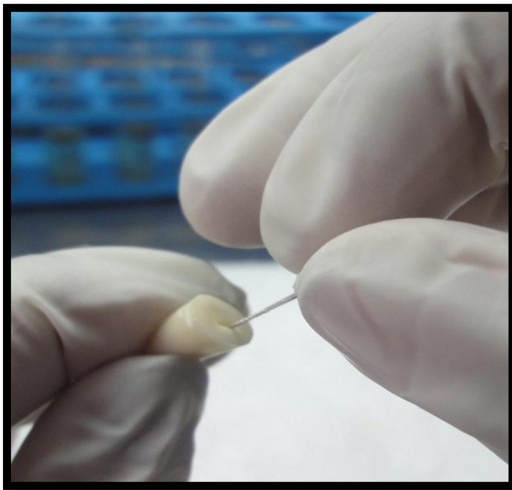


Fig 20 : Cleaning & Shaping of teeth

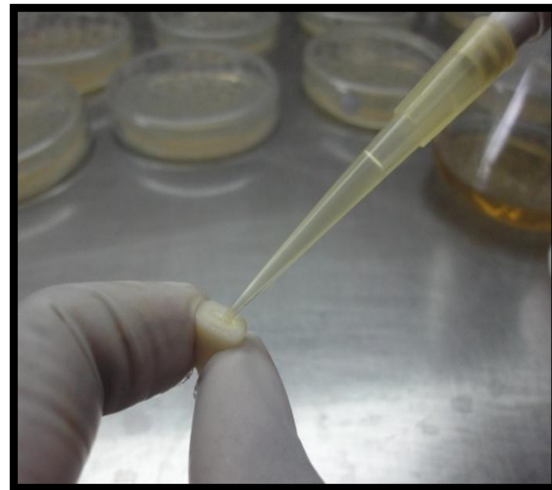


Fig 21 : Inoculation of bacterial sample into teeth

Standardization of Apical Canal Dimension

Coronal enlargement of the canal was done using Gates Glidden drills – Sizes #1, #2 and #3 respectively. To facilitate the standardization of the apical canal geometry, the canal was hand instrumented to the WL using SS K-files until the apex was enlarged upto size #35 K file (Fig 20). Further apical shaping was accomplished by using the balanced-force technique with SS K file #40 as the master apical file to the WL and then stepping back 1mm shorter for each subsequent file size (ie, for #45, #50 respectively). A #15 file was used for recapitulation to the WL in between each file. Using a syringe attached to a 30-gauge side-vented needle (Max-i-Probe; Dentsply Rinn, Elgin, IL), the canal was filled with 3% NaOCl solution during instrumentation. Approximately 1ml irrigant was exchanged after each recapitulation. Irrigation was accomplished using the manual dynamic agitation technique with in-and-out movements of the needle during irrigant delivery. A further 10 ml 3% NaOCl rinse with the needle tip inserted without binding to within 3 mm of the apical foramen was performed after the last instrument. A 2-minute rinse with 4 ml 17% EDTA was used as the final irrigant.

Teeth sectioning

10 teeth (2 in each group) were selected for viewing under Scanning Electron Microscope (SEM) after the irrigation protocol. These teeth were prepared by sectioning them longitudinally (Fig 19) and reapproximated prior to the procedure. Grooves were made on the buccal and lingual surface of the tooth with a low-speed abrasive diamond

disc (Brasseler, Savannah, GA), and the tooth was sectioned longitudinally through the center of the canal in the buccolingual dimension. The sectioned halves were examined to confirm that they can be reapproximated predictably.

Sterilization of teeth

All the prepared teeth and the sectioned teeth were packed in suitable autoclave pouches and autoclaved at 121°C. As a sterility check, each tooth was placed in a 1.5ml Eppendorf tube, immersed in sterile nutrient broth, sealed and incubated for 1 week at 37°C (inspected daily) to ensure that the nutrient broth showed no signs of turbidity. From this step forward, all specimens were processed using strictly aseptic protocols.

Reapproximation of the split tooth

The split halves of the tooth were reapproximated using 0.2 g utility wax (Coltene-Whaledent, Cuyahoga Falls, OH) and the patency of the canals were checked with size #15 K file.

Growing Bacterial Biofilm in Root Canal

The root canals were rinsed with 3 ml 17% EDTA for 3 minutes to remove the smear layer followed by a 10 ml wash using physiologic saline for 10 minutes. Each canal was inoculated with mixed human subgingival plaque bacteria (Fig 21) from an

adult volunteer. The plaque sample in nutrient broth was homogenized by pipetting for 30 seconds, evenly divided to each specimen, and incubated at 37°C for 24 hours. After 24 hours bacterial growth (biofilm) could be seen as confirmed at high magnifications with the SEM.

Irrigation protocol in standardized biofilm tooth model

The teeth specimens were randomly divided into 5 groups consisting of 10 teeth each and 2 of sectioned teeth as mentioned previously and treated with the various irrigants to be tested. Irrigation was accomplished using the manual dynamic agitation technique (Fig 22).

Group I – 10 ml of 3% Sodium hypochlorite for 5 minutes

Group II – 10 ml of *Acacia nilotica* extract (5mg/ml wt/vol) for 5 minutes

Group III – 10 ml of *Azadirachta indica* extract (5mg/ml wt/vol) for 5 minutes

Group IV – 10 ml of *Cinnamomum zeylanicum* extract (5mg/ml wt/vol) for 5 minutes

Group V – 10 ml of *Syzygium aromaticum* extract (5mg/ml wt/vol) for 5 minutes

Antibacterial efficacy

The antibiotic efficacy of the herbal extracts compared to NaOCl was evaluated by Turbidity Testing (Optical Density at 600nm) and Culture Study (Colony Counting).

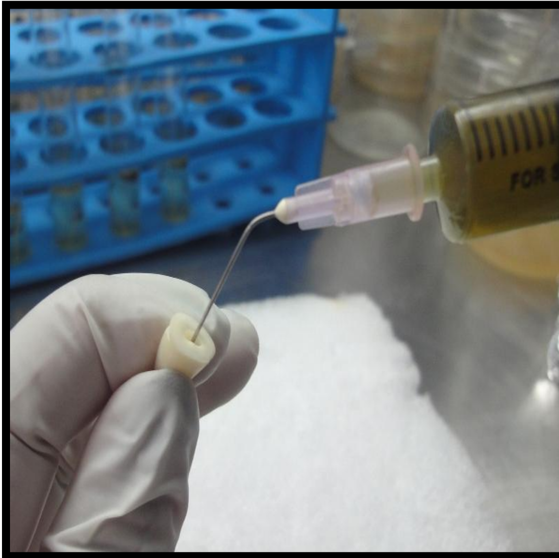
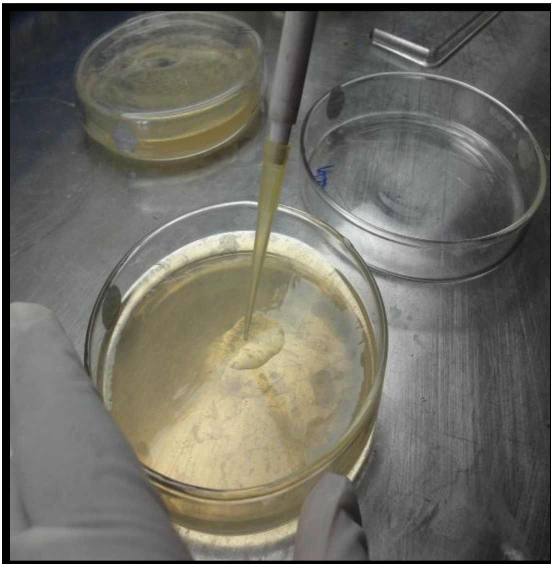


Fig 22 : Irrigation with different irrigants

Fig 23 : Samples collected for Turbidity Testing



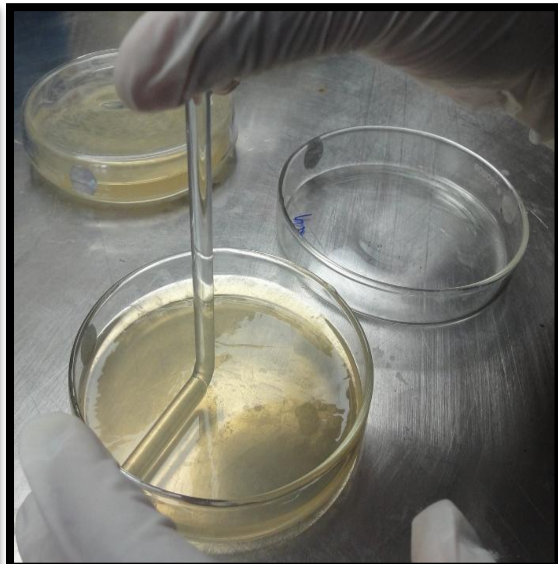


Fig 24 & 25 : Samples collected and cultured in MH agar for Colony Counting

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 23). 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 irrigation (Fig 26 - 30).

Culture study - After the irrigation protocol, sterile size #30 absorbent points were used to take sample from the root canals. These absorbent points were introduced into a test tube containing sterile nutrient broth and incubated at 37°C for 24 hours, after which lawn culture of the sample was done in Muller Hinton agar and incubated for another 24 hours (Fig 24 & 25). 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 irrigation (Fig 31 - 35).

SEM analysis – 2 specimens from each group which were split longitudinally were viewed under the Scanning Electron Microscope to analyze the surface morphology of root canal dentin after irrigation with different irrigants (Fig 36 & 37).

<u>Statistical analysis</u>

The statistical analysis was processed with the SPSS 17 software system (Chicago, USA). Descriptive statistics was performed. For analyzing Colony Counting and Optical Density One Way Anova followed by Tukey HSD (Post Hoc) was done at $P < 0.05$.

RESULTS

Table 1. Mean optical density across the experimental groups

Group	Minimum	Maximum	Mean	Std. Deviation
Sodium hypochlorite	.224	.471	.348	.086
Acacia Nilotica	.252	.652	.412	.141
Azadirachta indica	.359	.682	.484	.114
Cinnamomum zeylanicum	.359	.491	.445	.039
Syzygium Aromaticum	.293	.550	.443	.088

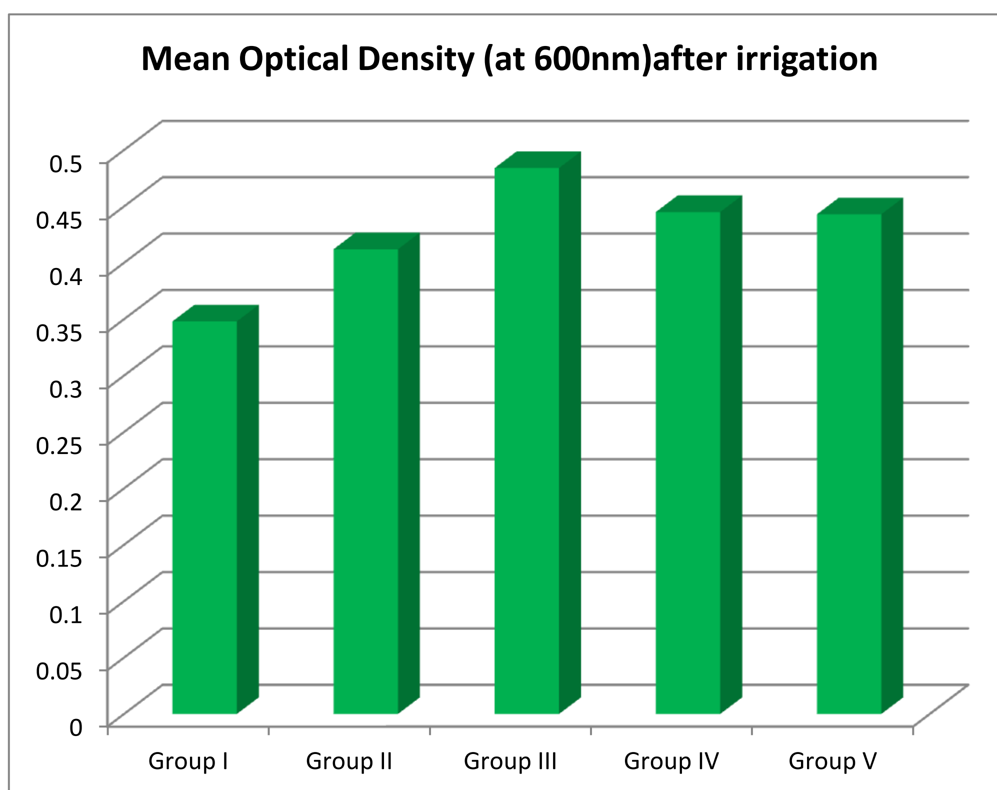
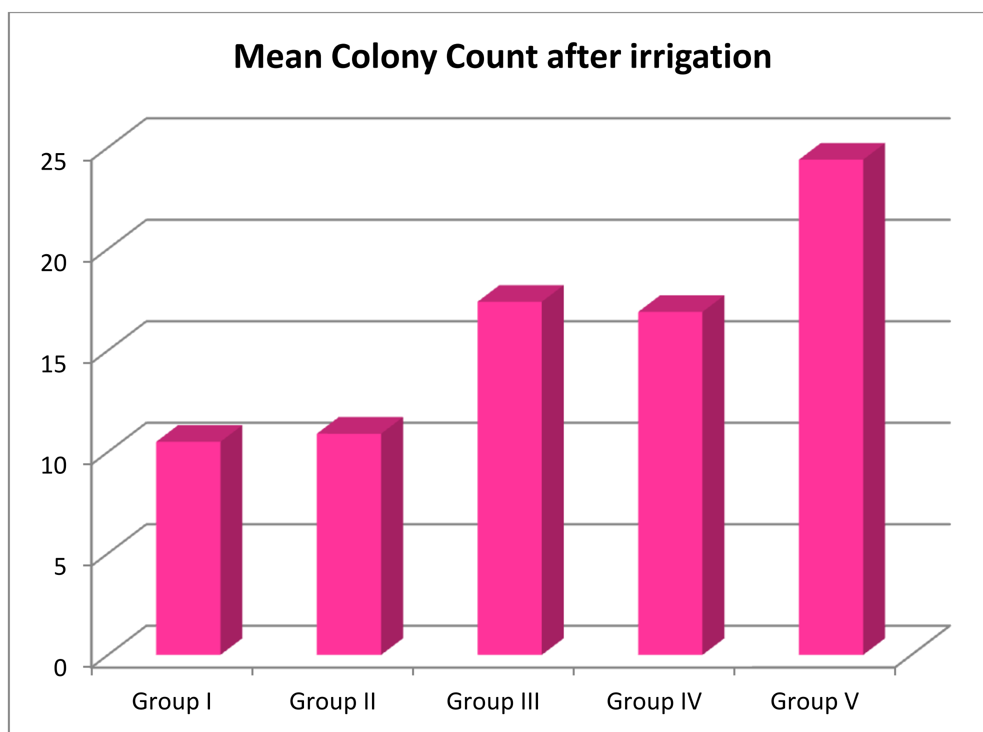


Table 2. Mean colony counts across the experimental groups

Group	Minimum	Maximum	Mean	Std. Deviation
Sodium hypochlorite	6.0	16.0	10.5	3.2
Acacia Nilotica	6.0	17.0	10.9	3.7
Azadirachta indica	8.0	29.0	17.4	6.1
Cinnamomum zeylanicum	7.0	26.0	16.9	6.4
Syzygium Aromaticum	11.0	44.0	24.4	9.9



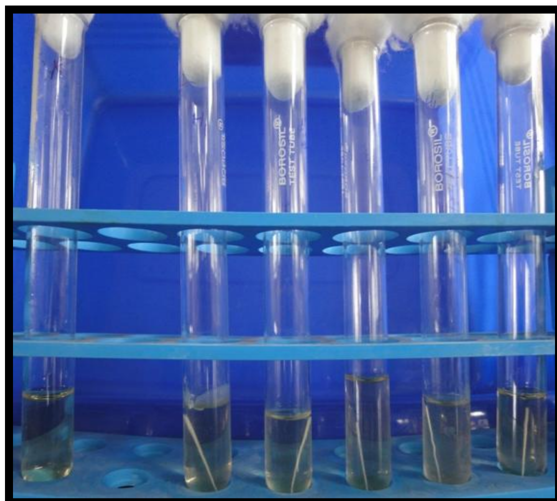


Fig 26 : Group I samples (Sodium Hypochlorite) Turbidity Testing after incubation

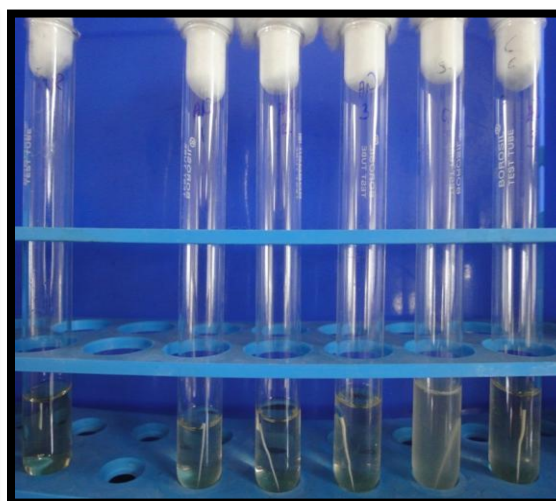


Fig 27 : Group II samples *(Acacia nilotica)* for for Turbidity Testing after incubation

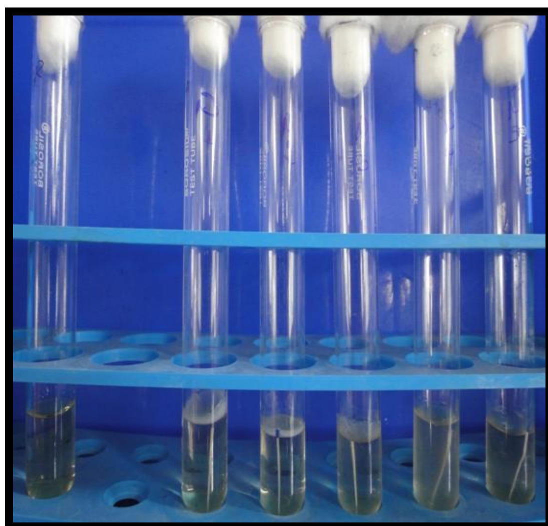


Fig 28 : Group III samples *(Azadirachta indica)* for Turbidity Testing after incubation

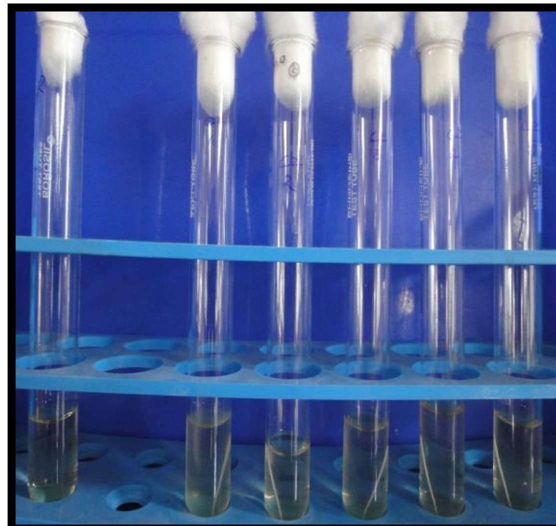
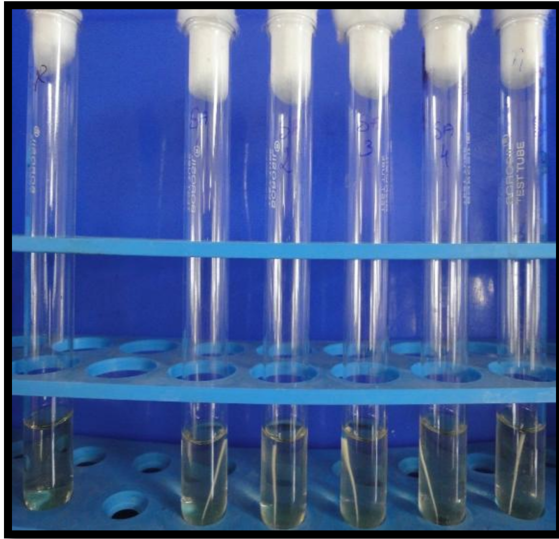


Fig 29 : Group IV samples *(Cinnamomum zeylanicum)* for Turbidity Testing after incubation



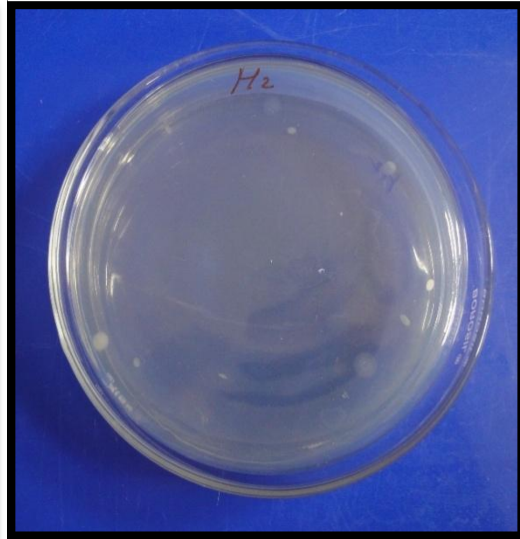


Fig 30 : Group V samples *(Syzygium aromaticum)* for Turbidity Testing after incubation

Fig 31 : Group I Colony counting

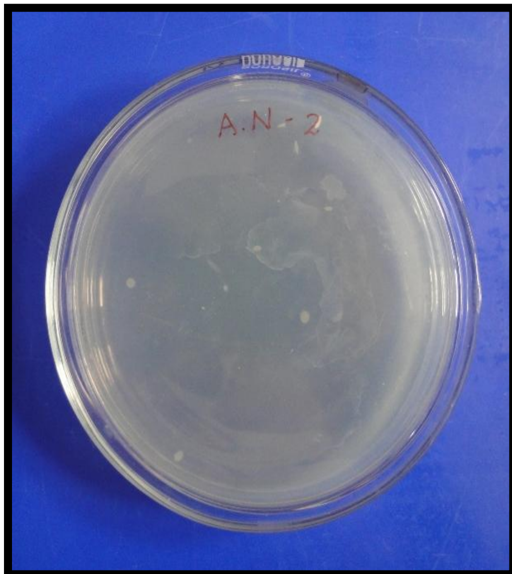


Fig 32 : Group II Colony Counting

Fig 33 : Group III Colony Counting

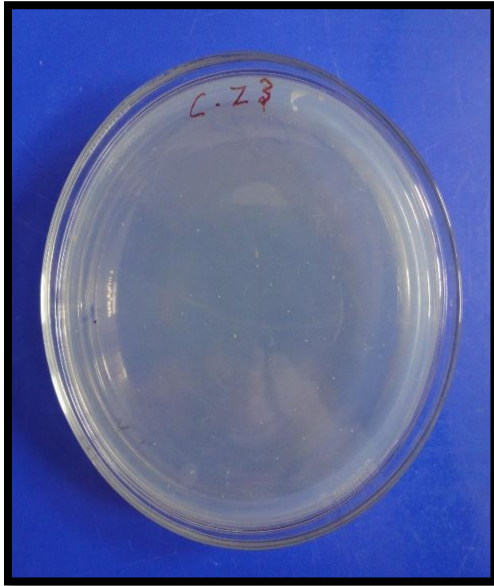


Fig 34 : Group IV Colony Counting

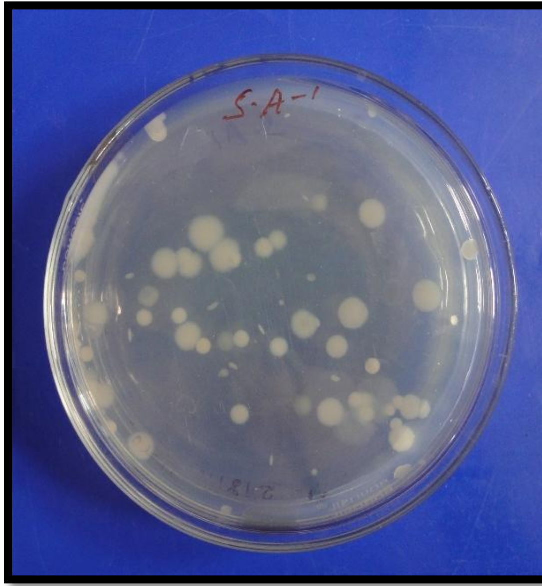


Fig 35 : Group V Colony Counting

Fig 36 : Scanning Electron Microscope

Fig 37 : Gold sputter coating of teeth specimens

Table 3. Comparison of optical density among five groups

Group	Mean	Std. Deviation	F value	P value
Sodium hypochlorite	.348	.086	2.601*	**0.048***
Acacia Nilotica	.412	.141		
Azadirachta indica	.484	.114		
Cinnamomum zeylanicum	.445	.039		
Syzygium Aromaticum	.443	.088		

Table 4. Post hoc analysis for optical density

Group	Group	Mean difference	p value
Sodium hypochlorite	*Acacia Nilotica*	-.06	.606
	Azadirachta indica	-.14	**.029**
	Cinnamomum zeylanicum	-.10	.207
	Syzygium Aromaticum	-.09	.228
Acacia Nilotica	*Azadirachta indica*	-.07	.492
	Cinnamomum zeylanicum	-.03	.947
	Syzygium Aromaticum	-.03	.959
Azadirachta indica	*Cinnamomum zeylanicum*	.04	.901
	Syzygium Aromaticum	.04	.881
Cinnamomum zeylanicum	*Syzygium Aromaticum*	.00	1.00

* Tukey HSD

Table 5. Comparison of colony counts among five groups

S. No	Group	Mean	Std Deviation	F value	P value
1	Sodium hypochlorite	10.5	3.2	8.085*	**<0.001***
2	*Acacia Nilotica*	10.9	3.7		
3	*Azadirachta indica*	17.4	6.1		
4	*Cinnamomum zeylanicum*	16.9	6.4		
5	*Syzygium Aromaticum*	24.4	9.9		

* One way ANOVA

Table 6. Post hoc analysis for colony counts

Group	Group	Mean difference	p value
Sodium hypochlorite	*Acacia Nilotica*	-.40	1.00
	Azadirachta indica	-6.90	.124
	Cinnamomum zeylanicum	-6.40	.177
	Syzygium Aromaticum	-13.90	**<.001**
Acacia Nilotica	*Azadirachta indica*	-6.50	.165
	Cinnamomum zeylanicum	-6.00	.230
	Syzygium Aromaticum	-13.50	**<.001**
Azadirachta indica	*Cinnamomum zeylanicum*	.50	1.00
	Syzygium Aromaticum	-7.00	.115
Cinnamomum zeylanicum	*Syzygium Aromaticum*	-7.50	.078

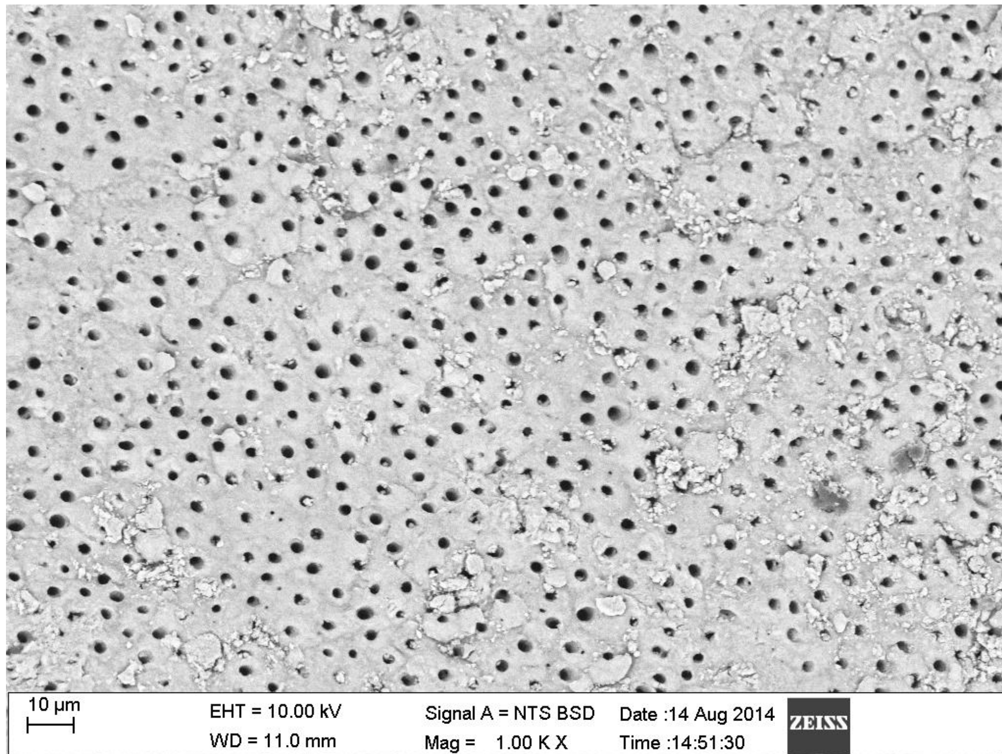
* Tukey HSD

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

Table 1 depicts the mean Optical Density (OD) at 600 nm for all the groups. The lowest mean OD was shown by Group I (0.348 ± 0.086) and the highest mean OD was shown by Group III (0.484 ± 0.114). Table 3 illustrates the comparison of OD among the 5 groups. Through One Way Anova test, the F value was found to be 2.601, which was found to be statistically significant at $P <0.05$. On Post Hoc analysis (Table 4) by Tukey HSD, a statistically significant difference was found between Group I and Group III with a mean difference of -0.14 and a P value of <0.05. There was no difference between the other groups. The best antibacterial activity through Optical Density was shown by Group I (Sodium Hypochlorite), followed by Group II *(Acacia nilotica)* and the least antibacterial activity among the groups was shown by Group III *(Azadirachta indica).*

Table 2 depicts the mean colony count for all the groups. The lowest mean colony count was shown by Group I (10.5 ± 3.2) and the highest mean colony count was shown by Group V (24.4 ± 9.9). Table 5 illustrates the comparison of Colony Count among the 5 groups. Through One Way Anova test, the F value was found to be 8.085 which was found to be statistically very significant at $P < 0.001$. Post Hoc analysis (Table 6) by Tukey HSD revealed statistically very significant results at $P < 0.001$ for Group I and Group V, Group II and Group V, with a mean difference of -13.9 and -13.5 respectively. The best antibacterial activity through Colony Counting was shown by Group I (Sodium Hypochlorite), followed by Group II *(Acacia nilotica)* and the least antibacterial activity among the groups was shown by Group V *(Syzygium aromaticum).*

Scanning Electron Microscopy analysis of the experimental specimens at various magnifications was seen after the irrigation protocol to analyze the surface morphology of the dentin after treatment with different irrigants (sodium hypochlorite and different herbal extracts). SEM images revealed that among the experimental groups, root canal dentin surface after treatment with Sodium Hypochlorite (Group I) showed relatively greater number of clear, open dentinal tubules with the least amount of debris present on the dentin surface (Fig 38 & 39). The dentinal tubule orifices were patent and the orifice boundaries were clearly demarcated. Root canal dentin treated with *Cinnamomum zeylanicum* (Group IV) (Fig 44 & 45) and *Syzygium aromaticum* (Group V) (Fig 46 & 47) were better than *Acacia nilotica* (Group II) (Fig 40 & 41) and *Azadirachta indica* (Group III) (Fig 42 & 43) but were inferior to the Sodium Hypochlorite group (Group I). Group IV and Group V showed moss like depositions on the dentin surface and on higher magnifications, it was seen that the dentinal tubules were patent, but the boundaries of the dentinal tubule orifices were not clearly demarcated. Group II also showed more deposition on the dentin surface with florid debris present. On higher magnifications, the number of patent dentinal tubules seen was lesser than that of Group IV and V, but the boundaries of the dentinal tubule orifices were clearer than in Group IV and V. Group III showed mat like depositions on dentin surface. On higher magnifications, it was seen that the dentinal tubules were not patent due to the deposition, and slit like appearance was seen in the areas where the dentinal tubules were present.



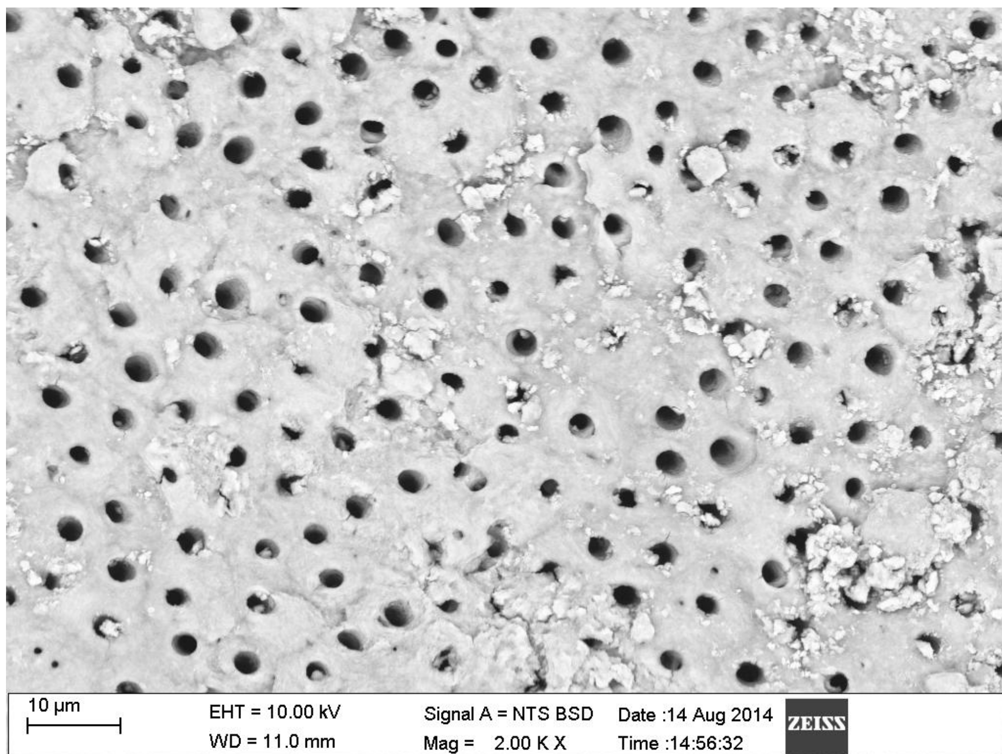
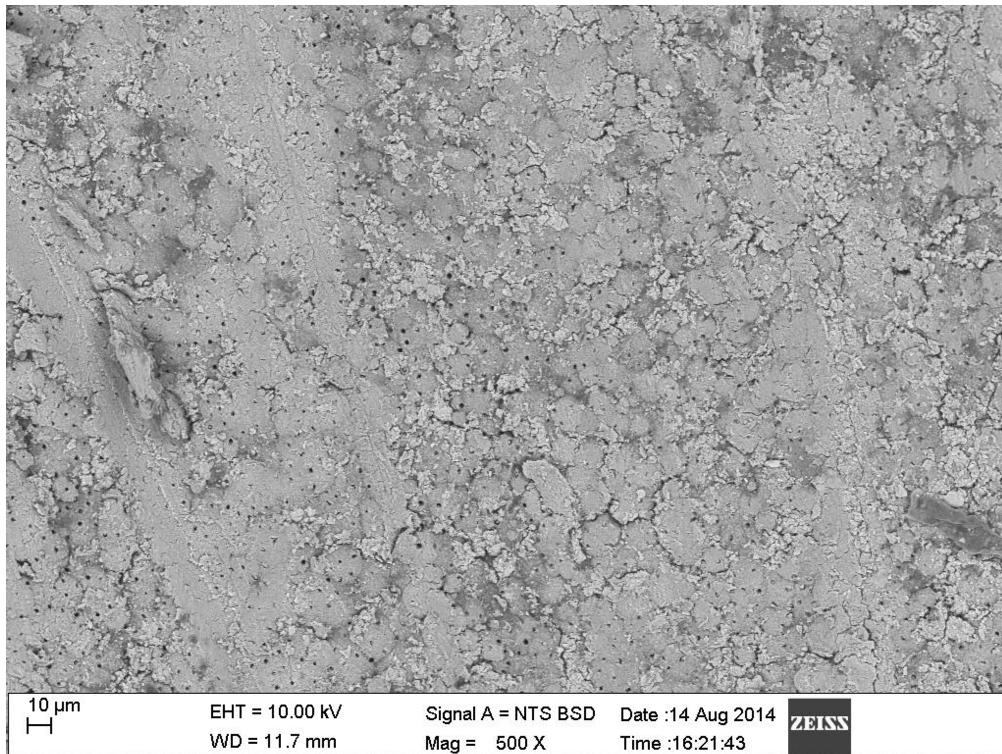


Fig 38 & 39 : SEM images of Group I (Sodium Hypochlorite)sample at 1000x and 2000x magnification



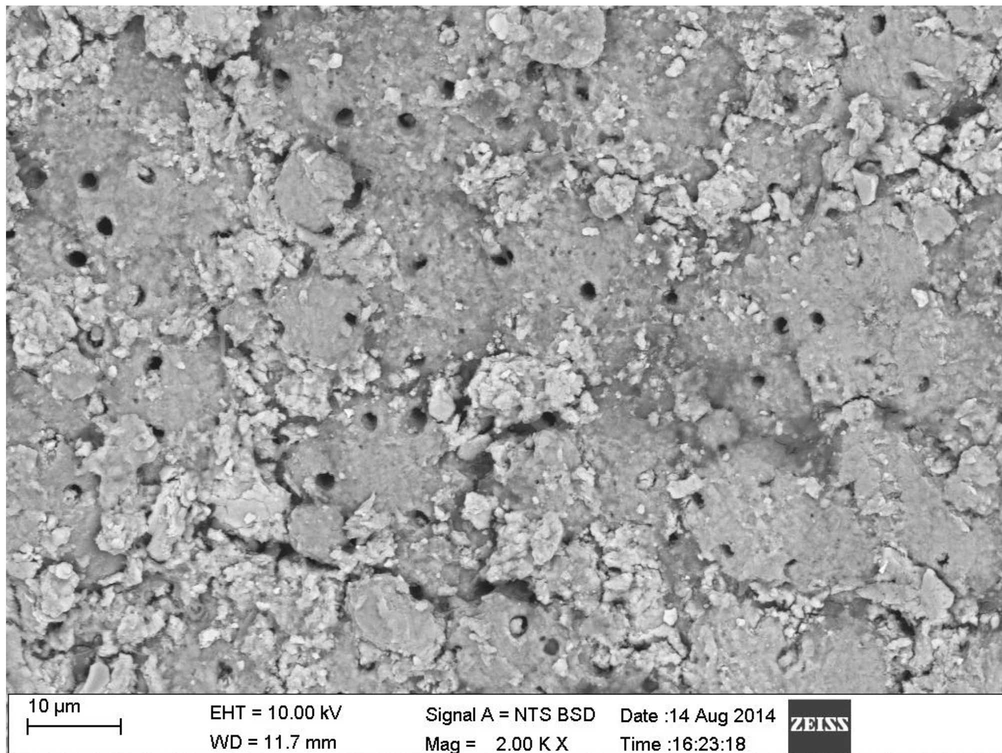
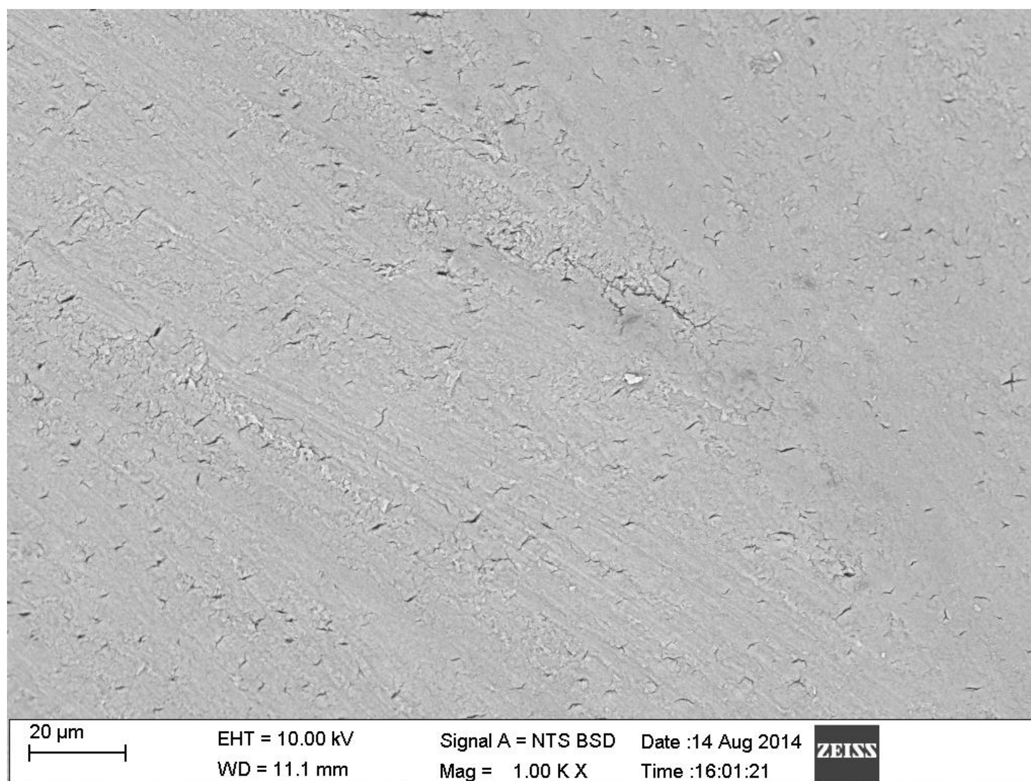


Fig 40 & 41 : SEM images of Group II *(Acacia nilotica)* sample at 500x and 2000x magnification



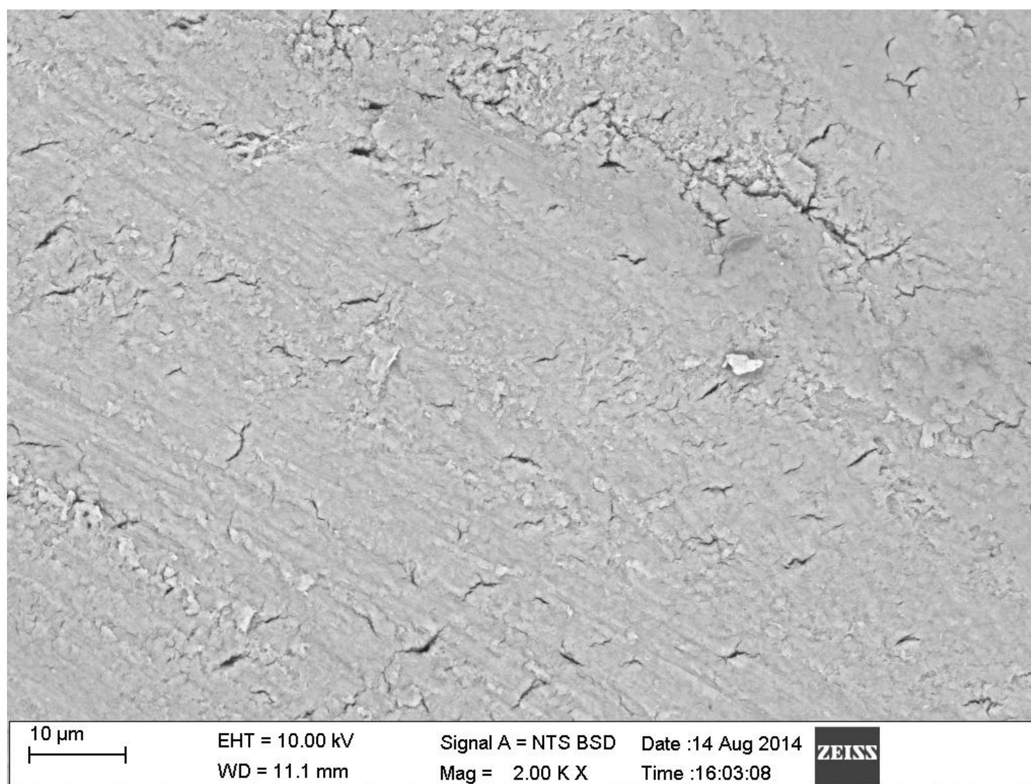
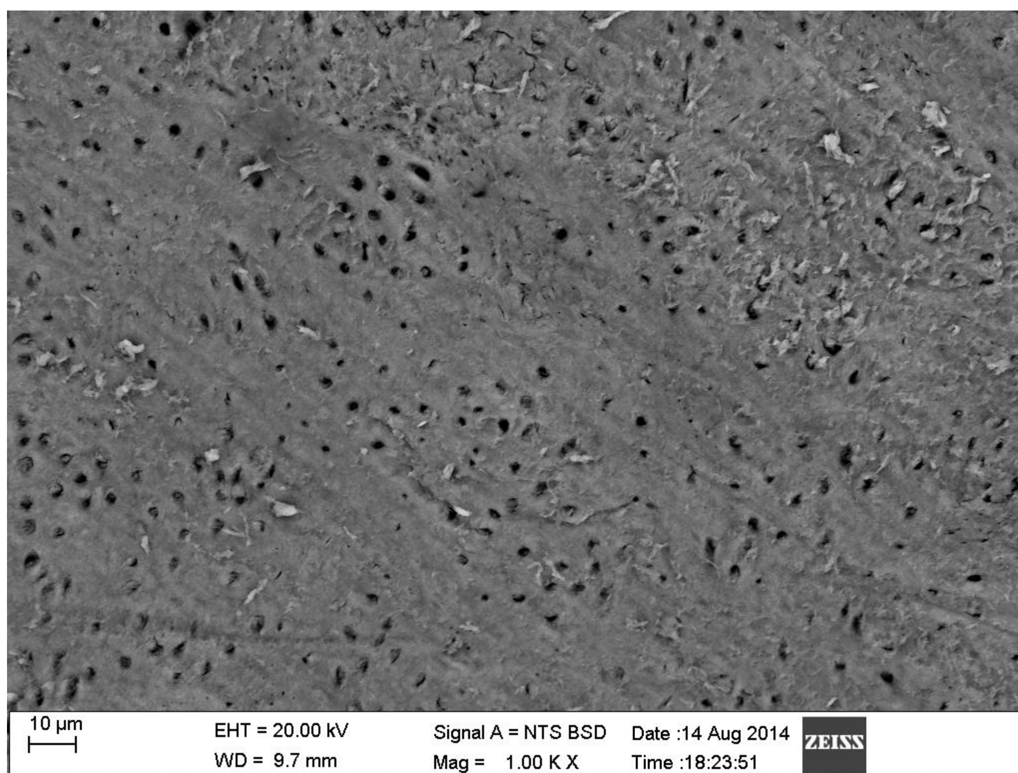


Fig 42 & 43 : SEM images of Group III *(Azadirachta indica)* sample at 1000x and 2000x magnification



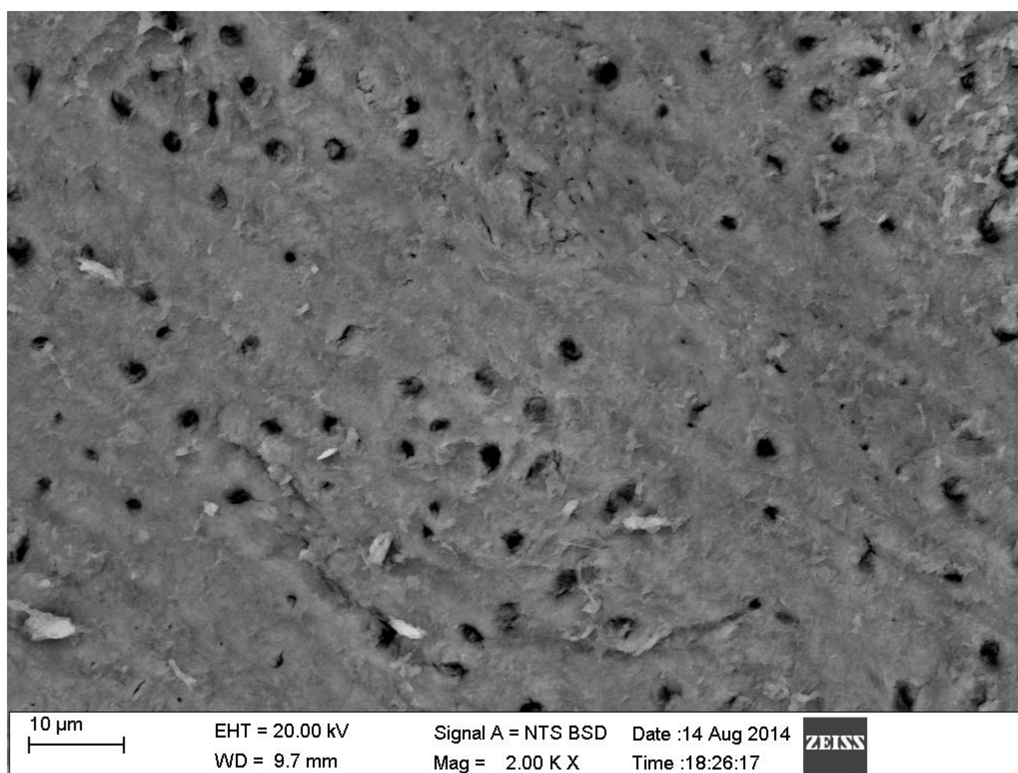
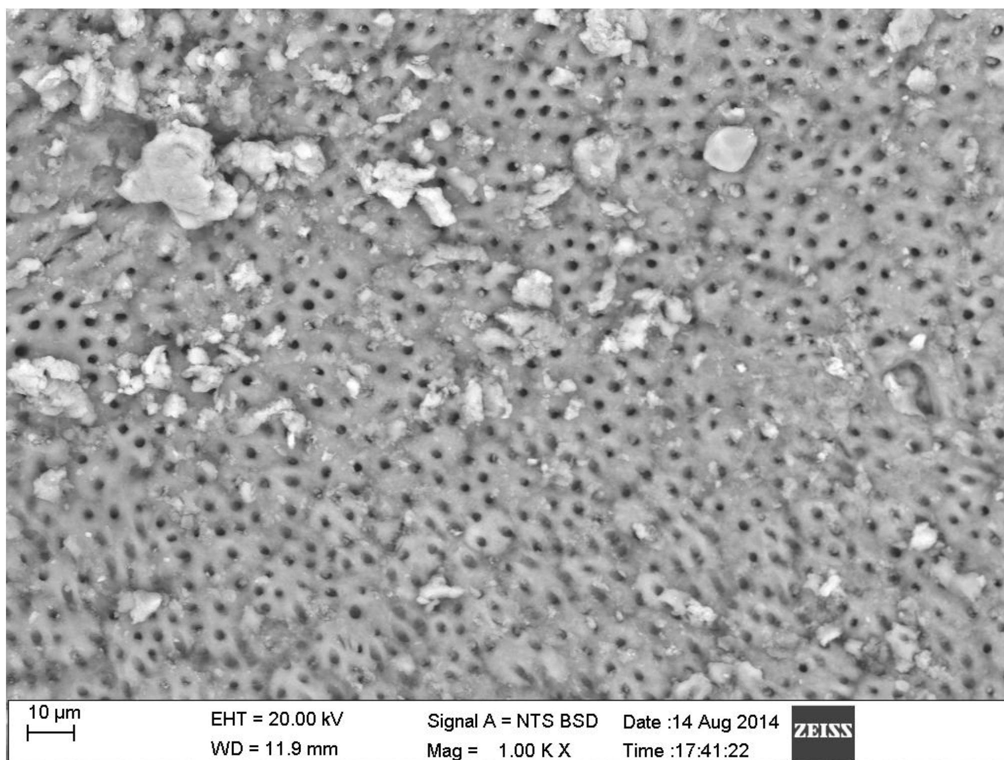


Fig 44 & 45 : SEM images of Group IV *(Cinnamomum zeylanicum)* sample at 1000x and 2000x magnification



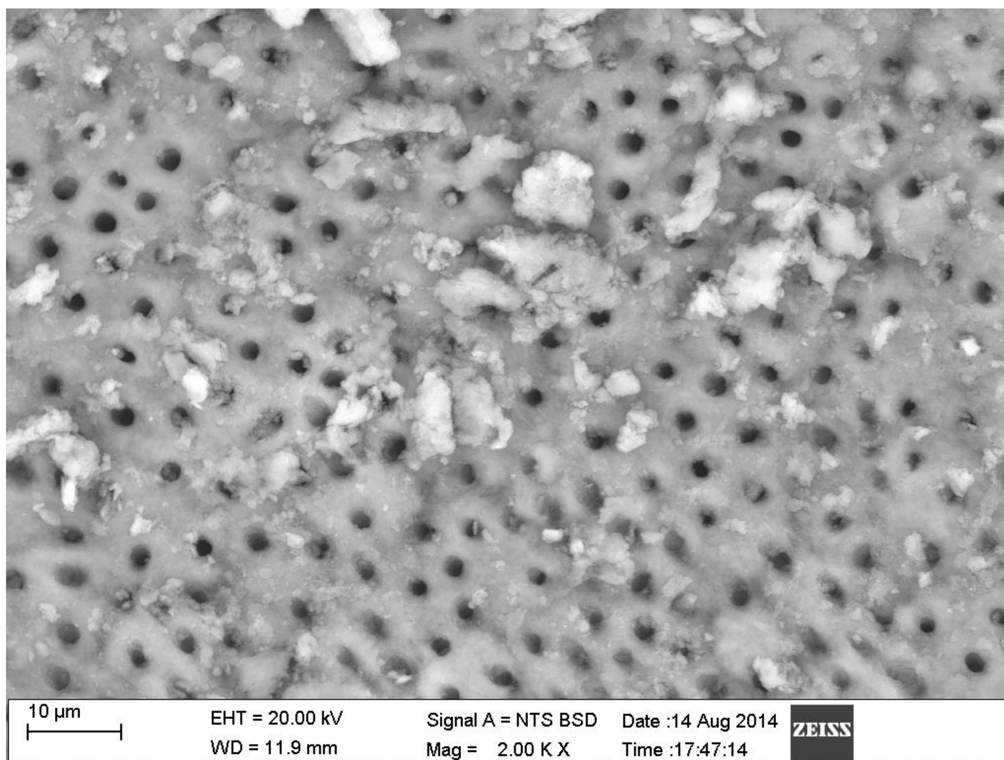


Fig 46 & 47 : SEM images of Group V *(Syzygium aromaticum)* sample at 1000x and 2000x magnification

DISCUSSION

The prime objective of root canal treatment is to clean the root canal system thoroughly, free of microbiota and debris, so that it can be sealed with a 3 dimensional hermetic filling. This procedure mainly revolves around the process of **“cleaning and shaping”**, wherein chemically active solutions (irrigants) are used along with mechanical instrumentation of the root canal space [66].

Irrigation allows for cleaning beyond what might be achievable through instrumentation because it enhances further bacterial elimination and facilitates necrotic tissue removal from the anatomic complexities of the root canal system, and prevents the packing of infected debris apically [28]. A broad antimicrobial spectrum against anaerobic and facultative microorganisms & biofilms is a major requirement of root canal irrigants. They should also be nontoxic and noncaustic to the periapical and periradicular tissues [77].

The most commonly used irrigant in endodontics is Sodium Hypochlorite [NaOCl] in concentrations ranging from 1-6%. The preference for this chemical over other irrigants stems from its unique ability to dissolve pulp tissue, and its excellent antimicrobial potency [67]. However the use of NaOCl holds certain disadvantages such as tissue toxicity, risk of emphysema, allergic potential, and disagreeable smell and taste [45, 53, 54]. Recent studies have shown that long-term exposure of dentin to high concentrations of sodium hypochlorite can have a detrimental effect on dentin elasticity and flexural strength, thereby predisposing the tooth to vertical fracture, which has a hopeless

prognosis [25, 77]. The constant increase in antibiotic resistant strains and side effects caused by synthetic drugs has prompted researchers to look for natural alternatives.

To overcome problems associated with currently used irrigants, use of natural plant extracts as endodontic irrigants might be of interest to professionals as part of a growing trend to seek natural remedies in dental treatment [40]. Screening of medicinal plants for bioactive compounds leads to the development of less expensive new antimicrobial agents with improved safety and efficacy. The use of natural derivatives may have a greater level of tolerance by the body with exhibition of fewer side effects. Plant derived natural products represent a rich source of antimicrobial compounds and certain of these derivatives have been tried in endodontics as irrigants.

The beneficial medicinal effects of plant derivatives typically result from the combinations of secondary products present in the plant, such as *tannins, saponins, phenolic compounds, essential oils and flavonoids,* which possess the following mechanisms of action:

- Disrupt the permeability barrier of cell membrane thus inhibit the bacterial growth.
- Antimicrobial effect involves the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally, ion leakage from the cells.

Since plant derived natural products represent a rich source of antimicrobial compounds, certain of these derivatives have been incorporated into oral hygiene products. Natural extracts of *Arctium lappa, Morinda citrofolia juice*, *Green tea*

polyphenols, *Berberine, Propolis, Triphala* etc have been tested for their antimicrobial efficacy against *E.faecalis* in endodontics [53]. The efficacy of *Morinda citrofolia* was found to be similar to that of NaOCl when used as an intracanal irrigant. *Morinda citrofolia* juice appears to be the first possible alternative to NaOCl as an intracanal irrigant [29]. *Berberine* when combined with Chlorhexidine was comparable with NaOCl in its antibacterial property [6]. *Propolis*, a compound derived from bees wax was found to be effective against *E.faecalis*, however its activity did not exceed that of Chlorhexidine [5].

Other plant derived natural extracts such as ***Acacia nilotica, Azadirachta indica, Cinnamomum zeylanicum, Syzygium aromaticum*** have been reported to possess inherent antibacterial properties. However studies pertaining to the antimicrobial efficacy of these extracts against endodontic pathogens are lacking in literature and their effects in a biofilm tooth model have not been studied earlier.

Acacia is a genus of shrubs and trees belonging to the subfamily *Mimosoideae* [48, 57], of the family *Fabaceae* or *Leguminosae* [21, 46], first described by the Swedish botanist **Carl Linnaeus (1773).** *Acacia nilotica* commonly called as Gum Arabic tree, Babool is a tropical species widely spread in subtropical and tropical countries. Almost all its parts are used in medication including root, bark, leaves, flower, gum, and pods etc [60]. It is a multipurpose plant, used for treatment of various diseases as the plant contains a variety of bioactive components. It contains secondary metabolites including amines and alkaloids, flavanoids, saponins, polysaccharides, proanthocyanidins which possesses good antibacterial activity [63], and this has been proved by several other studies [4, 16, 61].

Azadirachta indica belongs to the family ***Meliaceae***, commonly known as neem. It is an indigenous plant widely distributed in India. It is a multipurpose tree with multiple health benefits and has been used in traditional medicine as a source of many therapeutic agents. Different parts of the plant are shown to exhibit antimicrobial effects against a wide variety of microorganisms. Its leaf, bark and seed are known to contain antibacterial, antifungal & antiviral activities against different pathogenic microorganisms [71]**.** Presence of high concentration of *azadirachtins, quercetin and β-sitosterol* in *A. Indica* leaves might be responsible for strong antibacterial and antifungal activity [70]**.** *Azadirachta indica* leaves possesses good anti bacterial activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care [35]**.**

Cinnamomum zeylanicum is a tree of the laurel family ***(Lauraceae).*** Cinnamon comes from the inner bark of the shoots of a tree that grows predominantly in India, China, and Ceylon. The inner bark is a pungent, sweet, hot herb that treats many illnesses. The active constituents of *C.zeylanicum* are cinnamaldehyde (65–80%) and volatile oils (90%) [14]. Terpenes and eugenol constitute the major part of volatile oil. Several possible mechanisms have been proposed to describe the antimicrobial activity of cinnamaldehyde. It may destroy the bacterial cell surface [34], inhibit amino acid decarboxylase activity [75], and decrease cellular glutathione levels. Terpenes acts mainly through disruption of cell membranes by lipophilic compounds [14]**. Gupta *et al.,* (2008)** [27]**,** found that ethanol extract of bark of *Cinnamomum zelyanicum* possessed good antimicrobial properties against *Bacillus subtilis, B.cereus*. **Matan *et al*., (2006)** [43] also

confirmed the antibacterial properties of cinnamomum. **Ranasinghe *et al*., in 2013** [56] reviewed various studies pertaining to the antimicrobial properties of Cinnamomum and confirmed that it possessed good antibacterial properties against a wide range of bacteria.

Syzygium aromaticum (clove) is an aromatic herb and belongs to the family ***Myrtaceae.*** One of the main constituents of clove oil **(eugenol)** exhibits broad antimicrobial activities against both Gram-positive, Gram-negative and acid-fact bacteria, as well as fungi. The major component of *S. aromaticum* is an essential oil (up to 20%), which is characterized by the presence of eugenol (60–95%), eugenol acetate (2–27%) and a- and b-caryophyllene (5–10%). The probable antimicrobial activity of eugenol is through its action on cytoplasmic membrane**.** The volatile oil of cloves (about 85-92% eugenol) was highly active against a range of test microorganisms, being classified as bactericidal in nature. **Aneja and Joshi (2010)** [1] investigated the antimicrobial activity of clove *(Syzygium aromaticum)* and clove bud oil by agar well diffusion method against five dental caries causing microorganisms and found that the highest antimicrobial activity of clove was found against *Saccharomyces cerevisiae* in methanolic extract and that of clove oil was found against *Streptococcus mutans*. The antibacterial potential of *Syzygium aromaticum* has been proved by various studies [9, 17, 18, 69].

Khan *et al*., (2009) [33] compared the antimicrobial activities of the crude ethanolic extracts of five plants namely *Acacia nilotica*, *Syzygium aromaticum, Cinnamum zeylanicum*, *Terminalia arjuna,* and *Eucalyptus globules* against multidrug resistant microorganisms, and found that extracts of *A. nilotica*, *C. zeylanicum* and *S. aromaticum*

possessed the best antimicrobial activity and can be used against multidrug resistant microbes causing nosocomial and community acquired infections. In this study we selected *Acacia nilotica*, *Syzygium aromaticum, Cinnamum zeylanicum* along with *Azadirachta indica* as our experimental groups.

Dimethyl Sulfoxide (DMSO) was used as a solvent for the herbal extracts in Phase I study, although they were readily soluble in water. DMSO is a clean, safe, highly polar, aprotic solvent that helps in bringing out the pure properties of all the components of the herb being dissolved [15,32]. Antibacterial inertness of 10% DMSO was confirmed with the disc diffusion test.

The concentration of the herbal extract was kept constant for all the experimental groups namely 5mg/ml wt/vol in ethanol in order to standardize the concentration and to find the herb with most potent antimicrobial activity at that concentration.

The majority of endodontic biofilm studies have been conducted using models with monospecies bacterial cultures grown on various mediums [8, 11, 12, 19, 20, 76]. Recently, mixed-species dentin infection models have been developed to study factors affecting biofilm pathogenicity and the effects of different disinfecting solutions [38, 74]**.** However, most biofilm models used thus far do not adequately reflect the complexity of the root canal anatomy, and they do not simulate the clinical situation. Therefore, it is important to develop multispecies biofilm models resembling in vivo endodontic biofilms for studying root canal disinfection.

Endodontic diseases are polymicrobial infections in which the interactions between microorganisms play a significant role in determining the ecologic environment and the establishment of an endodontic habitat-specific multispecies microbiota. Intracanal microbial biofilms formed on the root canal dentin exhibit morphologically distinct types of bacteria. The model described here provides a method for studying multispecies biofilms that have the following important similarities with those found in vivo [39]**:**

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

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.

Colony Counting and Turbidity Testing (Optical Density at 600nm) were chosen to evaluate the antibacterial efficacy of the herbal extracts as they would signify the quantity of live residual bacteria present in the root canals after the irrigation protocol.

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**.** Sterile nutrient broth incubated at $37^{O}C$ for 24 hours was used as a control. 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 irrigation. In this study, the least turbidity was shown by Sodium Hypochlorite which showed significant difference with *Azadirachta indica* but there was no significant difference between the other groups.

Microbiological root canal culturing is commonly used to assess the effectiveness of endodontic treatment measures. **Valgas *et al*., in 2007** [72] evaluated the technical variants used in screening methods to determine antibacterial activity of natural products and concluded that the well-variant of the diffusion method was more sensitive than the disc-variant method due to which we used the well variant method in our study.

In this present study the best antibacterial activity through Colony Counting was exhibited by 3% Sodium Hypochlorite and *Acacia nilotica* which showed almost similar results followed by *Cinnamomum zeylanicum* and *Azadirachta indica* and last by *Syzygium aromaticum*.

These findings are similar to the study by **Gupta *et al*., (2013)** [26] in which the antibacterial efficacy of 3% Sodium Hypochlorite was compared with *Ocimum sanctum, Cinnamomum zeylanicum, Syzygium aromaticum*. In their study 3% Sodium Hypochlorite showed the best antibacterial properties, followed by *Cinnamomum zeylanicum* and *Syzygium aromaticum* and the least by *Ocimum sanctum*, which is similar

to the results obtained from this study. In another study by **Kumar and Sidhu (2011)** [36] who evaluated the antimicrobial activity of *Azardirachta indica, Glycyrrhiza glabra, Cinnamum zeylanicum, Syzygium aromaticum, and Acacia nilotica* on *S.mutans* and *E.faecalis* in vitro, *Acacia nilotica* exhibited the best antibacterial property, which is also similar to the results obtained in this study. The results were also in accordance with the study by **Khan *et al*., (2009)** [33] in which *A. nilotica*, *C. zeylanicum* and *S. aromaticum* possessed the best antibacterial effect respectively among the tested herbs.

However, in this study, the antibacterial efficacy of *Azadirachta indica* is inferior to 3% Sodium Hypochlorite, unlike the results of the study by **Bohora *et al*., in 2008** [7], in which the authors compared the antibacterial efficiency of ethanolic neem leaf extract and 2% sodium hypochlorite against *E. faecalis, C. albicans* and mixed culture in vitro. This may be because of the lower concentration of Sodium Hypochlorite (2%) used in that study, or due to the fact that the irrigants were tested against monospecies culture (E.fecalis) unlike the multispecies mixed biofilm used in this study.

Scanning Electron Microscopy analysis was done after the irrigation protocol which showed moss like deposits on the dentin surface in Group IV and V and mat like depositions in Group I and II, whereas dentin surface in Group I showed relatively clear dentinal surface with patent dentinal tubules. These deposits (in the herbal extract groups) could be due to the deposition of the extracts after the evaporation of ethanol solvent. The deposition might have been higher in *Acacia nilotica and Azadirachta indica* due to the higher viscosity of the extracts when compared to *Syzygium aromaticum* and

Cinnamomum zeylanicum. A criticism of the SEM may be that only topographic assessment of the observed structures is possible and it is not possible to differentiate between live and dead bacterial load.

Each herb is unique and has got specific properties. Herbal extracts exhibit maximum antibacterial efficacy at different concentrations. In this study, the concentration of all the herbal extracts was fixed to compare their potency at a standardized concentration. A limitation of all sampling techniques is that inaccessible areas within the root canal system such as fins, accessory canals, and isthmuses cannot be adequately evaluated. Furthermore, bacteria that exist in a biofilm may assume a state of low metabolic activity for the majority of time, similar to that of a stationary-phase planktonic state. These bacteria in the low metabolic activity state may be undetectable by regular culture techniques.

The concentration of each herbal extract could be customized to obtain the maximum antibacterial activity. Alternative solvents could be tried to dissolve the extracts which could prevent evaporation of the solvent, thereby preventing the possible deposition of the extract on the dentin surface. Another alternative option which could be tried is to use EDTA as the final irrigant after the use of herbal extracts to flush the root canals, so that it removes the smear layer as well as the extract deposition, thereby providing a clear dentin surface. Further studies should be conducted about the reaction between the herbal extracts and EDTA.

SUMMARY AND CONCLUSION

The current study aimed to determine the optimum concentration of herbal extracts namely ***Acacia nilotica*** **(Babool),** ***Azadirachta indica*** **(Neem),** ***Cinnamomum zeylanicum*** **(Cinnamon), and** ***Syzygium aromaticum*** **(Clove)** for their antimicrobial activity and to test the efficacy of herbal irrigation against 3% Sodium Hypochlorite, together with instrumentation in the removal of multispecies endodontic biofilm.

Leaves of *A.nilotica* and *A.indica*, dried bark of *C.zeylanicum* and dried buds of *S.aromaticum* were collected. All the herbs were dried in shade and pulverized or grounded to coarse powder, then suspended in ethanol in 1:2, 1:5 and 1:10 weight / volume concentration for 7 days to identify the suspension which gives the maximum yield. After filtration and evaporation of ethanol, the extracts were dried and redissolved in dimethyl sulfoxide (DMSO) in 3 various concentrations – 1mg/ml, 2mg/ml, and 5mg/ml to check their antibacterial activity. The susceptibility of the mixed microbial strain to different antibiotics vs herbal extracts were tested using well diffusion method. Best yield and optimum concentration of the herbal extracts were found out.

Sixty single-rooted human mandibular premolars were decoronated, debrided and following cleaning and shaping, they were randomly grouped into five groups consisting of 12 teeth each.

Group I – conventional irrigation consisting 3% Sodium Hypochlorite (NaOCl)

Group II – irrigation with *Acacia nilotica* extract

Group III – irrigation with *Azadirachta indica* extract

Group IV – irrigation with *Cinnamomum zeylanicum* extract

Group V – irrigation with *Syzygium aromaticum* extract

Two teeth in each group were selected for viewing under Scanning Electron Microscope (SEM) after the irrigation protocol. These teeth were prepared by sectioning them longitudinally and reapproximated prior to the procedure. All the prepared teeth and the sectioned teeth were sterilized and the canals were inoculated with mixed human subgingival plaque bacteria and incubated at 37°C for 24 hours to allow the biofilm to form.

The teeth specimens were treated with the various irrigants to be tested and the antibacterial efficacy of the herbal extracts compared to NaOCl was evaluated by Culture Study (Colony Counting) and Turbidity Testing (Optical Density at 600nm).

The results showed that complete elimination of bacteria was not achieved in any of the experimental groups. The best antibacterial activity through Optical Density was shown by Group I (Sodium Hypochlorite), followed by Group II *(Acacia nilotica)* and the least antibacterial activity among the groups was shown by Group III *(Azadirachta indica).* The best antibacterial activity through Colony Counting was shown by Group I (Sodium Hypochlorite), followed by Group II *(Acacia nilotica)* and the least antibacterial activity among the groups was shown by Group V *(Syzygium aromaticum).* Scanning Electron Microscopy analysis was done after the irrigation protocol which showed moss like depositions on the dentin surface in Group IV and V and mat like depositions in

Group I and II, whereas dentin surface in Group I showed relatively clear dentinal surface with patent dentinal tubules.

Within the limitations of this study, 3% sodium hypochlorite showed maximum antibacterial activity, followed by *Acacia nilotica* which showed almost similar results. The use of herbal alternatives as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of Sodium Hypochlorite. Further research is needed to overcome the deposition of extracts on the dentin surface and conclusively recommend herbal solutions as root canal irrigants.

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