



Comparison of the Antimicrobial Efficiency of Neem Leaf Extract and 17% Edta with 3% Sodium Hypochlorite against *E. faecalis*, *C. albicans* – An *in vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TS and SDPA design of the study, collected the data, analysis of data, wrote the results and prepared the manuscript. Author TS has made the tabulation. Author NPM supervised the laboratory process. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: *Candida albicans* and *Enterococcus faecalis* are the most predominant microorganisms found in the canals of failed root canal treated teeth. Thorough debridement of an infected root canal and complete elimination of microorganisms are objectives of effective endodontic therapy. For thousands of years, humans have used herbs as the primary means to sort out health issues and illnesses. Not all herbs have shown to provide scientific evidence as medicine to illness. Neem is a Botanical herb that is truly remarkable with its scientific value, a

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tropical evergreen native to India. Although different agents have been suggested as root canal irrigants, sodium hypochlorite and EDTA are the most popularly used.

Aim: To compare the antimicrobial efficacy of commercially available irrigants, 17% EDTA and a herbal product, neem leaf extract compared with 3% sodium hypochlorite as a control against *Enterococcus faecalis* and *Candida albicans*.

Materials and Methods: The antimicrobial activity was determined using the agar well diffusion method. Freshly prepared neem leaf extracts, 17% EDTA, 3% Sodium hypochlorite Standard strain of *E. Faecalis* and *C. Albicans* were used in this study. These isolates were subcultured on to Brain heart infusion agar (BHI agar) and HI-chrome candidal differential media respectively. The agar well diffusion test was performed in brain heart infusion media and broth. The plates were incubated at 37°C for 24 hours and the zone of inhibition was recorded and analyzed statistically.

Results: Zones of inhibition were present with Neem leaf extract suggestive of antimicrobial properties. Zones of inhibition were greater with Neem extract than 17% EDTA. Hence, further research should be directed towards the use of this herbal extract as an irrigant clinically with endodontic therapy.

Conclusion: Within the limitation of this study, it was found that neem leaf extract had a significant antimicrobial effect against *E. Faecalis* and *C. Albicans* compared to EDTA and Sodium Hypochlorite. Since the study is a qualitative analysis, further testing needs to be done to final quantitative analysis of the antimicrobial activity of neem leaf extract.

Keywords: Antimicrobial efficacy; herbal irrigant; microbial infection; neem leaf; root canal irrigation.

1. INTRODUCTION

Successful endodontic treatment can be determined through the complete removal of infective microflora from the infected root canal treated teeth. The primary etiology of periapical bone lesions and the failure of endodontic treatment is well known as the microorganisms which have also been recognized in the development of necrotic pulp chambers[1].

Moreover, viable bacteria harboring the less accessible areas of the root canal system, and dentinal tubules caused reinfection and continued periapical inflammation to occur. However, many studies have shown root canal systems with anatomic variations are difficult to debride and achieve complete disinfection.

Enterococcus faecalis is an infectious microorganism that is predominantly found 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. found in failed or unhealed teeth. This microorganism has the ability to accumulate and adhere to the walls of the canal forming communities or colonies organized into biofilm, which enables the bacteria to become highly resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm-producing organisms [2]. *E.Faecalis* may also survive chemo-mechanical anatomical niches of the

root canal system and reinfect the filled root canal [3].

Fungi constitute a small part of the oral microbiota. Fungal microbiota mainly consist of *Candida* species. The incidence of *Candida albicans* in the oral cavity has been reported to be 30-45% in healthy adults and 95% in patients infected with human immunodeficiency virus. The presence of *C. Albicans* is prevalent in both initial infections and persistent infections of root canal systems [4].

Cleaning and shaping of the canals followed by adequate irrigation protocol plays an important role in the disinfection of the root canal system and is an integral part of root canal biomechanical preparation procedures. The most frequently used irrigants are sodium hypochlorite (NaOCl), EDTA, and chlorhexidine gluconate [5]. 17% EDTA interferes with the bond strength between the dentinal wall and calcium silicate based cements and also inhibits the setting of MTA [6].

Azadirachta indica is a commonly seen medicinal tree in India, which is considered holy. They are most popularly known as "Indian neem / Margosa tree" or "Indian lilac", in India and its neighboring countries. For centuries it has been believed that neem tree leaves have biological values that are widely used [7]. In dentistry, *Azadirachta indica* has been investigated, due to its antimicrobial potential against oral microorganisms [8].

Furthermore, it also has an anti-adherence activity by altering bacterial adhesion and the ability of the organism to colonize. Its nature to be biocompatible to human periodontal ligament fibroblasts is a key factor favoring its clinical use. Neem extract as an endodontic irrigant is advantageous compared to other irrigants due to its antioxidant behavior and biocompatibility, and very little to no chances of incidents like hypochlorite accidents. *Azadirachta indica* has complex of various constituents including nimbin, nimbidin, nimbolide, and limonoids and such types of ingredients play role in diseases management through modulation of various genetic pathways and other activities [9].

Candida albicans and *Enterococcus faecalis* are present predominantly in failed root canal treated teeth. Elimination of microorganisms and Complete debridement of an infected root canal is the objective of successful endodontic treatment. For thousands of years, humans have used herbs as the primary means to sort out health issues and illnesses. Not all herbs have shown to provide scientific evidence as medicine to illness. Neem is a Botanical herb that is truly remarkable with its scientific value, a tropical evergreen native to India [10]. Although different agents have been suggested as root canal irrigants, sodium hypochlorite and EDTA are the most popularly used.

We have numerous highly cited publications on well designed clinical trials and lab studies [11–26]. The present study was aimed to compare the in vitro effectiveness of neem leaf extracts and 17% EDTA with 3% sodium hypochlorite (commonly used concentration) against *E. Faecalis* and *C. Albicans* by agar diffusion testing [27].

2. METHODS AND MATERIALS

2.1 Study Design

The study was carried out in the microbiology laboratory of the institution under controlled environment.

2.2 Microorganisms Used

Standard strain of *E.Faecalis* and *C.Albicans* were used in this study. These isolates were subcultured on to Brain heart infusion agar (BHI agar) and HI-chrome candidal differential media respectively. They were further confirmed by

standard biochemical tests and stored at 4 degree C for further use.

2.3 Preparation of the Test Plates

Six wells were punched in the agar. The wells were estimated to be at a safe distance from the edge and from each other to avoid overlapping the zones of inhibition around the wells.

2.4 Preparation of NEEM Leaf Extract

Fresh neem leaves were used for the study, which was first washed and cleaned using distilled water and allowed to dry. It was then weighed about 25gms and mixed with 50ml of absolute alcohol. For 1- 2 mins the mixture was macerated and then the contents were filtered using a muslin cloth filter. The process was repeated with the course residue using alcohol. These two extracts were mixed together and filtered again and about 25ml was taken after separation of alcohol from the mixture. It was stored in a dark or amber colored container [28].

2.5 Preparation of Medium

Freshly prepared culture plates of Mueller Hinton agar (HI media) were prepared. Mc Farland standard 0.5 turbidity adjusted suspensions of *E.Faecalis* and *C.Albicans* were lawn cultured onto the media plates respectively. After a brief drying, wells were made using a sterile well cutter and 10 microliters 3% sodium hypochlorite (Prime Dental), 17% EDTA (Prevest DenPro) and neem leaf extract were added in each one of the wells respectively. Group I - 3% sodium hypochlorite, Group II -17% EDTA and Group III -neem leaf extract . 3 groups of plates were prepared as each group consists of all 3 of manipulated variables. Here, 3% of sodium hypochlorite acts as the control for this study. 2 Muller-Hilton agar plates were prepared and inoculated (one for each microorganism) to serve as a positive control group and tested for their susceptibility to the 3 irrigants used. These plates were incubated at 37 degrees celsius for 24hrs. Results were recorded by measuring the zone of inhibition of each irrigant against *E.Faecalis* and *C.Albicans* after 24 hrs.

2.6 Statistical Analysis

To compare the mean values between groups one way ANOVA is applied. SPSS version 22.0 was used to analyze the data. Significance level was fixed at 5% ($\alpha = 0.05$).

3. RESULTS AND DISCUSSION

The mean values of growth inhibition (Figs. 3 and 4) produced by different irrigant groups against the test microorganisms are given. Table 1 shows the mean zone of inhibition of 3% sodium hypochlorite, 17% EDTA and neem leaf extract and there was a statistically significant difference between the groups. 3% sodium hypochlorite performed better than all the test groups, followed by neem leaf extract and, 17% EDTA against all the tested microorganisms after 24 hrs incubation. The diameter of the inhibition zone was measured in (mm) by a transparent scale. Measurement was done on the backside of the plate. Two readings were taken for each sample (well) and averaged as one reading. A second examiner repeated these measurements. The results from the first and second examiners were compared. There were no differences.

Several studies on the antimicrobial activity of irrigation solutions in endodontics, such as 1%, 3%, and 5% sodium hypochlorite, EDTA, 2% chlorhexidine gluconate are found in the literature. Also, *E. Faecalis* was taken in the present study because it has been identified as the main microorganism that causes pulpal disease. The use of natural extracts in endodontics is gaining fame because of their reduced side effects, the most common microorganism in the root canal. Some studies presented that herbal extracts are moderately effective as irrigants [29]. Different laboratory methods are available to evaluate the in vitro antimicrobial activity of an extract. The most known and basic methods are the disc-diffusion method. It is commonly used because of providing a direct estimation of its antimicrobial activity against a specific microorganism and added advantages of simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease of results interpretation [30]. Though there are new technologies in the field of microbiology, disc diffusion is still one of the preliminary tests to assess the antimicrobial activity of newer material. To get precise data, further studies are required to evaluate the antimicrobial effect of material in-depth, time-kill test and flow cytofluorometric methods are recommended, which provide information on the nature of the inhibitory effect [31].

Interest in this substance is based on its properties like antibacterial, antifungal, antiviral, anti-inflammatory. The inclusion of *E. Faecalis* in

this study was based on the literature that relates these microorganisms to pulp infection, mainly in recalcitrant infection after endodontic treatment [32]. The study design followed the standard established for agar dilution tests. Ideal irrigants should combine antimicrobial action and the capacity to dissolve organic and inorganic remnants. NaOCl at its strongest concentration is well known for its bactericidal action and cytotoxicity. Moreover, its anti-adherence activity by altering bacterial adhesion and the ability of organisms to colonize also stimulated the study of this substance [33]. Apical periodontitis is an inflammation of the periodontium that occurs due to trauma, irritation, or infection through the root canal, It represents the main indication for root canal treatment [34].

Mainstream 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. The role of antimicrobial plants has interested microbiologists for two reasons. The first reason being the presence of phytochemicals in the arsenal of antimicrobial drugs prescribed by physicians. The second reason being the scientists have pieces of evidence stating that the effective span of synthetic antibiotics is shortened due to drug resistance. Herbal extracts (neem leaf) were used in this study, which is commonly used in gastrointestinal disorders. *Enterococcus faecalis* are the reason for human enterococcal infections of about 80–90% [35], which is most commonly found in failed root canal walls or obturate material. This verity indicates that in failed endodontic treatment, *E. Faecalis* has a pathogenic role. The Enterococci are capable of transmitting genetic information by both plasmid and transposon exchange [36].

The genetic material included in enterococcal plasmids and transposons may be drug resistance determinants of virulence genes. The most cited virulence factors are surface adhesions [37], sex pheromones, lipoteichoic acid, extracellular superoxide production [38], 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 [39]. *E. Faecalis* are capable of resisting the high pH of intracanal medicament due to its functioning Proton - pump mechanism. Due to

which in the obturated root canals with chronic apical periodontitis, the infected organisms are enclosed or resist the defense mechanisms of the body. In most environmental niches [40], these bacteria thrive and multiply not as planktonic cells suspended in liquids, but as

surface-attached biofilms and form complex communities of microorganisms [41]. They activate sets of genes that are inactive with renewed genetic characteristics, to produce complicated colonies/communities that can be 1500 times more resistant [42].

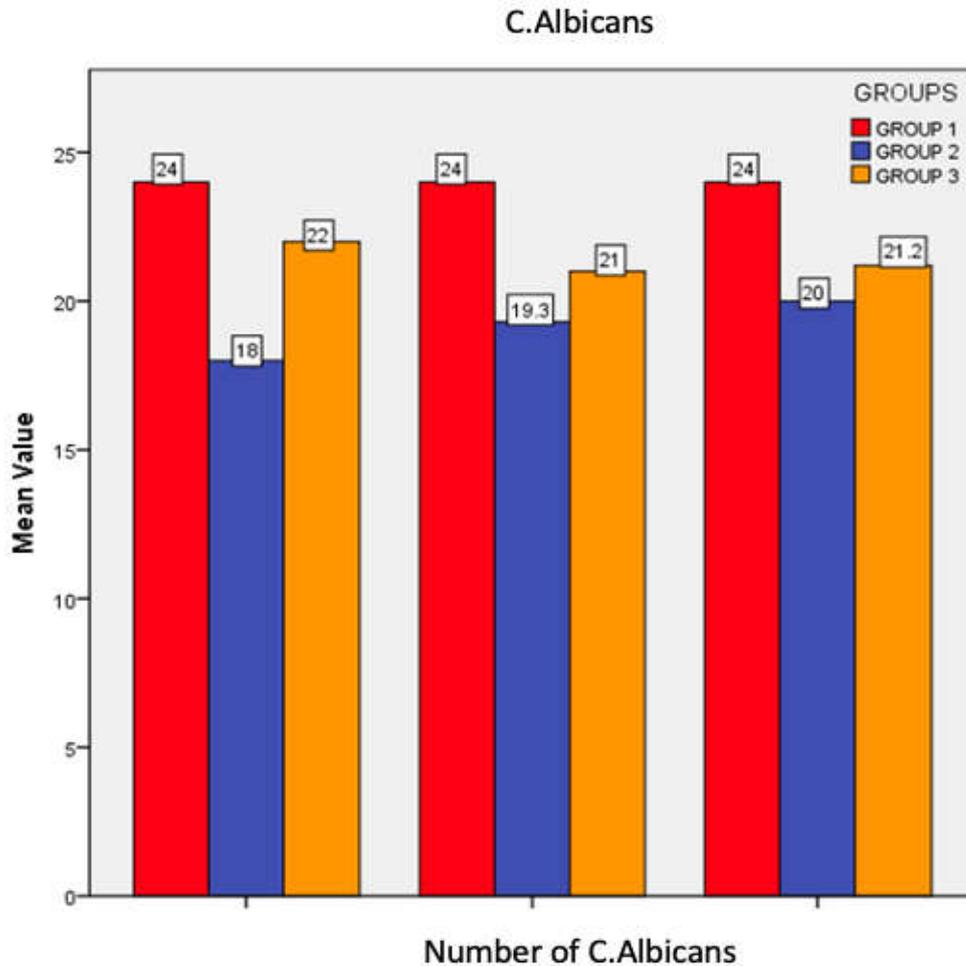


Fig. 1. Bar chart shows the mean zone of inhibition for *C. albicans* for 3% hypochlorite, 17% EDTA and neem leaf extract
 (X-axis represents the three groups of the extract; Y-axis represents the mean value). This graph inferred that 3% sodium hypochlorite (Red color) shows the highest mean zone of inhibition followed by herbal extract (Orange color) and 17% EDTA (Blue-green color)

Table 1. Mean zone of inhibition of 3% hypochlorite, 17% EDTA and Neem Extract against *C. albicans* and *E. faecalis*

Variables	Group	Mean	Std. Dev	F-Value	P-Value
<i>Candida albicans</i>	Group -I	24.00	1.225	276.652	<0.05
	Group -II	18.00	.985		
	Group -III	22.00	1.105		
<i>E. Faecalis</i>	Group -I	35.00	2.000	412.732	<0.05
	Group -II	20.00	1.124		
	Group -III	24.00	1.332		

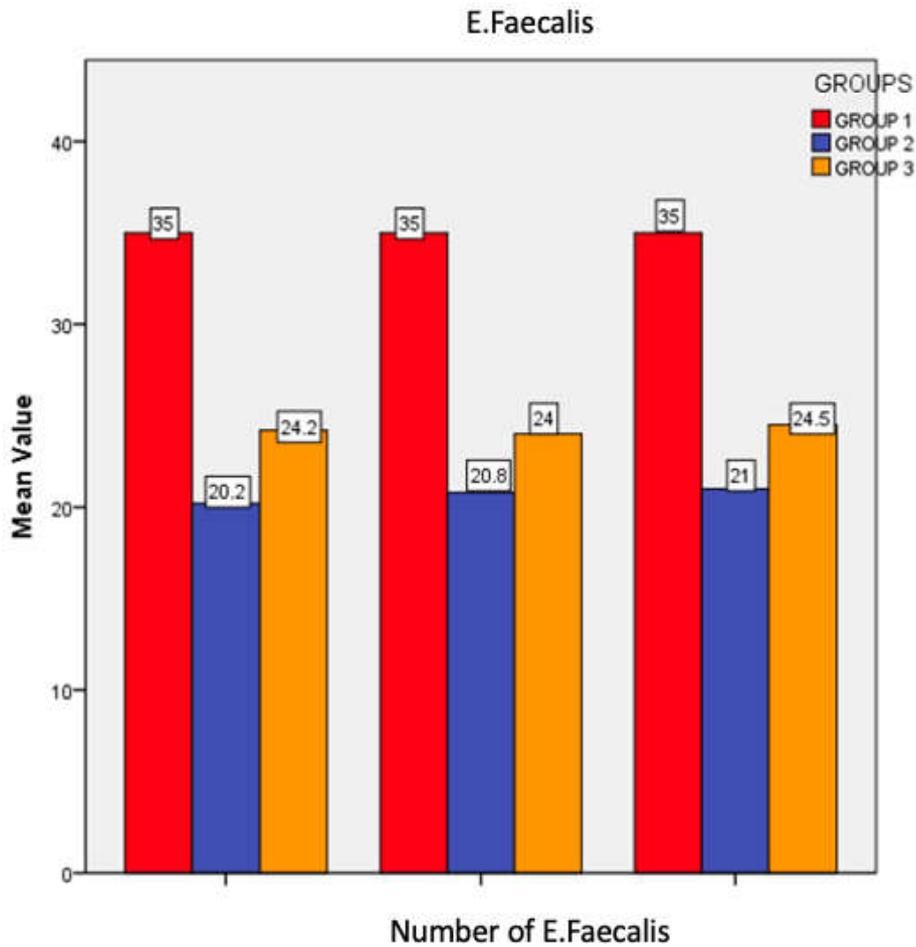


Fig. 2. Bar chart shows the mean zone of inhibition for *E. faecalis* for 3% hypochlorite, 14% EDTA and neem leaf extract

(X-axis represents the three groups of the extract; Y-axis represents the mean value). It is inferred that the 3% sodium hypochlorite (Red color) shows the highest mean zone of inhibition followed by herbal extract (Orange color) and 17% EDTA (Blue color)

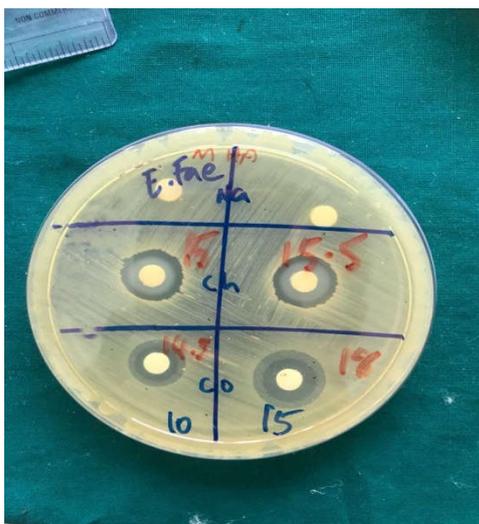


Fig. 3. Zones of Bacterial growth inhibition for *E. Faecalis* using the three irrigants



Fig. 4. Zones of Bacterial growth inhibition for *C. Albicans* using the three irrigants

A biofilm is a group of bacteria or other microbes organized with an extensive exopolymer that is considered to be highly structured habitats with spatial and physiological heterogeneity [43]. Cell to cell signaling are done within the biofilm with a quorum-sensing system along with deposition of enzymes by the organism within the matrix. In a biofilm, the microbes get adsorbed onto a solid non-shedding surface and are embedded in a common self-produced extracellular matrix [44]. The characteristic features of biofilm allow efficient transfer of nutrients, removal of waste materials, and circulation of secondary metabolites and pheromones. The alterations in their genetic and microbial process combined with extracellular matrix resist the actions of antimicrobials. The surface attributes of the substratum play an important role in biofilm-forming capacity [45].

In this study, the use of neem extract as an irrigant might be advantageous because it is biocompatible, antioxidant, and thus not likely to cause severe injuries to patients that might occur with NaOCl accidents. In comparison with NaOCl, the irrigant is having less toxicity and foul taste. EDTA is proposed to be an alternative to NaOCl in NaOCl allergic patients. Neem extract has moderate activity against *E. Faecalis*. However, the present study shows the least activity against *C. Albicans*. The antimicrobial activity of neem extract is similar to 17% EDTA against *Enterococcus faecalis*.

4. CONCLUSION

Within the limitations of the study, it can be concluded that neem extract demonstrated antimicrobial activity against *E.Faecalis*, and *C.Albicans*, Since the study is a quantitative analysis further testing needs to be done to final quantitative analysis of the antimicrobial activity of neem leaf extract. Henceforth, further dilution studies need to be carried out to find out the better activity of the extract. This study indicates the use of herbal-based non-irritant, non-toxic irrigant in place of chemical ones.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the research ethics committee to Saveetha Dental College and Hospitals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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