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Evaluation of Antibacterial Efficacy of *Vitex negundo* Linn. extract as Root Canal Irrigant against *Enterococcus faecalis* and its penetration into Root Dentin: An in-vitro study

Research Article

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Abstract

Introduction: The aim of this study was to evaluate the antibacterial efficacy of *Vitex negundo* Linn. extract as root canal irrigant against *Enterococcus faecalis* and its penetration into root dentin. Methods and Materials: Forty single rooted premolars were randomly divided into 4 groups: 3% Sodium hypochlorite (NaOCl), 2% Chlorhexidine (CHX) , 100mg/ml *Vitex negundo Linn.* and saline as control all mixed with Rhodamine B dye. Test samples were analysed for bacterial count before and after irrigation using absorbent paper points and the colony forming units were recorded and measured. Sectioning of the samples was performed at three levels 3mm,6mm,9mm from apex and then these samples were analysed using confocal laser scanning microscopy for penetration depth of the irrigant within the dentinal tubules. Paired t-test and ANOVA test were used to perform statistical analysis with level of significance set at 0.05 Results: The mean CFU/ml count of *Enterococcus facealis* reduced significantly in all the groups post irrigation. All the irrigants showed maximum penetration depth at coronal third level compared to middle and apical third level respectively. The penetration depth of NaOCl group was better when compared to CHX group and *Vitex negundo* Linn. group but the difference was statistically not significant. Conclusion: Although 3% NaOCl was the most effective irrigant, all agents exerted acceptable antimicrobial activity against *Enterococcus faecalis* and penetration depth within tubules of dentin.

Key Words: *Root canal irrigation, Vitex negundo, Enterococcus faecalis, Confocal laser scanning microscope, Herbal irrigants.*

Introduction

Several types of endodontic chemical irrigating solutions have been used for disinfection of root canals. The important properties of root canal irrigants are maximum antibacterial action and pulp tissue dissolving properties with minimal tissue toxicity.(1) Most frequently used intracanal irrigant is sodium hypochlorite because of its high tissue dissolving property. Various concentrations of Sodium hypochlorite (NaOCl) have been used in dentistry but the most effective concentration recommended is 5.25%. Sodium hypochlorite at 2.5%-3% is still the most regularly used concentration of sodium hypochlorite because it causes minimal tissue toxicity. The major drawbacks of this irrigant are its caustic effect on periapical tissues and the potential to weaken dentin and reduce its flexural strength.(2)

Another widely used irrigating solution is Chlorhexidine (CHX) which has the property of substantivity and exceptional action against the canal flora. Now-a-days its use is reduced in endodontics as it causes unwanted discoloration of the tooth structure and its inability to dissolve the pulp.(3)

Inorganic or chemically manufactured irrigants have numerous harmful effects and safety issues. To overcome the harmful effects, herbal substitutes can be recommended (4) Literature has documented various plant extracts having antimicrobial and therapeutic properties which can serve the purpose of endodontic irrigation.(5) *Vitex negundo* Linn, a well-recognized plant in the field of Ayurveda, commonly known as *Nirgundi* which means to protect from all diseases. It has many beneficial actions like antibacterial, anti-inflammatory, analgesic, antifungal, anti-histaminic and antioxidant.(6) Largest components of the *Vitex negundo* Linn. *(Nirgundi)* are glycosides, alkaloids and tannins. Previous studies have shown that the extracts of *Vitex negundo* Linn. *(Nirgundi)* leaf and bark possess

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strong antibacterial and antifungal property. Aqueous and alcoholic extracts of *Vitex negundo* Linn. have antibacterial effect against the bacteria that give positive and negative results in the gram stain test. Literature suggests that the use of the extracts of *Vitex negundo* Linn. may have the potential to be potent against *E.faecalis*.(7)

Keeping the background in mind, present study was planned to evaluate the relative effectiveness of *Vitex negundo* Linn. leaf extract as a root canal irrigant.

Materials and Methods

Study design

This *in vitro* study was conducted at Department of Pediatric and Preventive Dentistry, Sharad Pawar Dental College in collaboration with Vishakha Microbiology Laboratory, Nagpur; Department of Ras-Shastra and Bhaishajya Kalpana, Mahatma Gandhi Ayurveda College, Hospital and Research Centre, Salod (H) and Sophisticated Instrument Centre, Indian Institute of Technology, Indore. The study was reviewed and approved by the Institutional Ethics Committee.

Method

Sample selection and segregation

Forty Premolars were collected and cleaned with 5.25% sodium hypochlorite for 1 hour after ultrasonic scaling. Care was taken that premolars which were included in the study had not been extracted prior to 3 months. Premolars were stored in 0.9% saline at 4 degree celsius until its use. Single rooted premolars extracted for orthodontic purposes were included in the study whereas carious or restored premolars with calcified canals and bifurcated canals were excluded.

After cleaning of the outer surface of premolars, decoronation was done using diamond disk. And the samples were standardized to a length of 14mm. Preparation of the canals was done using hand k-files of no.20 (Dentsply-Maillefer, Ballaigues, Switzerland), along with irrigation with saline. After the working length determination using RVG, the canals were instrumented. The outer surface of the roots of premolars was painted thrice with nail polish to inhibit bacterial invasion.

Cultivation of *Enterococcus faecalis* and contamination of root canals

Enterococcus *faecalis* cultured (ATCC 29212) bacterial strain was stored at 4°C. Streaking of plates containing BHI agar was done and incubated at 37°C for 24 hrs. The obtained colonies

were transported to BHI broth tubes and incubated further for 24 hours at 37°C to obtain a suspension of *Enterococcus faecalis* with McFarland 2 (6x 10^8 CFU/mL). Each root canal was inoculated with 10µl of the *Enterococcus faecalis* suspension using 1ml of sterile insulin syringe under the laminar air flow hood. A sterile 15 no K file was used to uniformly disperse the bacterial suspension till the working length for about 10 seconds**.**

Collection of bacterial samples before instrumentation

After 48 hours of incubation period, contaminated root canals were filled with fresh BHI broth. Samples were obtained by the sterile 20 no. paper points which were kept in the root canal for around 1 min, sterile tweezer was used for collecting the same. Absorbent paper points were removed and were moved into Eppendorf tubes containing 1ml BHI broth. Eppendorf tubes were then vortexed for 30 seconds to uniformly mix the contents of BHI broth. After 10 fold serial dilution, aliquots of 20 µl were plated on sheep blood agar, and incubated at 37°C for 2 days. For every plate, the number of CFU were recorded.

Root canal instrumentation

Premolars used for the study were divided into four groups i.e. Sodium hypochlorite group, Chlorhexidine group, *Vitex negundo Linn.* group and Saline group respectively of 10 teeth each. Canal preparation was done upto size 35 no.K file. Rhodamine B fluorescent dye powder was quantitated to make 0.1m mol/0.1% Rhodamine B solution. Each irrigant (2ml) (NaOCl, CHX, *Vitex .negundo*, saline) was used for irrigation respectively after instrumentation by each file. Irrigation of the root canal was done after instrumentation by each file, using 29 gauge needle and final irrigant volume used was 10ml per canal. Post instrumentation and irrigation again bacterial samples were taken using paper points and were cultured.

Processing of *Vitex negundo* Linn. extract as irrigant (fig 1)

Fresh leaves of Vitex negundo Linn. were collected from herbal garden of Mahatma Gandhi Ayurveda College, Hospital and Research Centre, Salod (H), Wardha.

Twenty gms of *Vitex negundo* Linn. leaves were collected and separated. The leaves were washed with water thoroughly and dried on a filter paper. Then the leaves were cut into thin sections and were grinded in a mortar with a pestle. The entire mixture was then tied in a muslin cloth and placed in thumble and the entire assembly was placed in soxhlet apparatus and mixture was extracted in 100 ml ethanol by soxhlet apparatus at 40-50 degree Celsius. Filtered extract was kept in water bath for elimination of ethanol; 2.29gm extract was collected. The prepared extract was diluted in 10 ml ethanol to obtain mother solution. Serial dilution method was used to obtain 100mg/ml of solution and kept in refrigerator for further use.

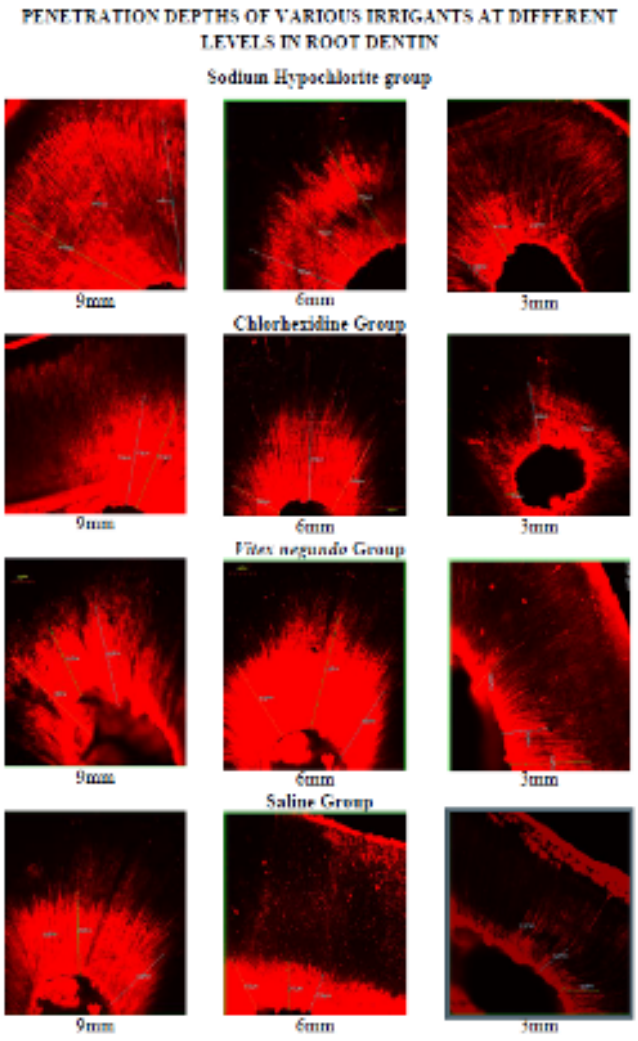
Fig 1. Preparation of *Vitex negundo* Linn. extract as root canal irrigant

Penetration depth of irrigants using CLSM

Two markings were given at the level of the intersection of coronal and middle third and middle and apical thirds and were horizontally segmented using a diamond disc at 3mm, 6mm, 9mm from the apex of the tooth. Each segment was standardized to a size of 1mm and polished.

The root canal slices were visualised under confocal laser scanning microscope (Olympus, fluoview FV 1000 Japan) to evaluate irrigant penetration depth. For accurate visualization of all the images, sections were studied under 10X lens. Wavelength of 540nm was used for absorption of rhodamine dye whereas 590nm was used as emission wavelength. To measure the depth of penetration, the division of the images were done into 3 different regions**.** The final penetration depth was the derived mean out of the 3 readings. OLYMPUS FLUOVIEW Ver 4.2 viewer software was used for measuring the obtained images. Operator was blinded during assessment of all the samples of the groups. (fig 2)

Fig 2- Penetration depths of various irrigants at different levels in root dentin

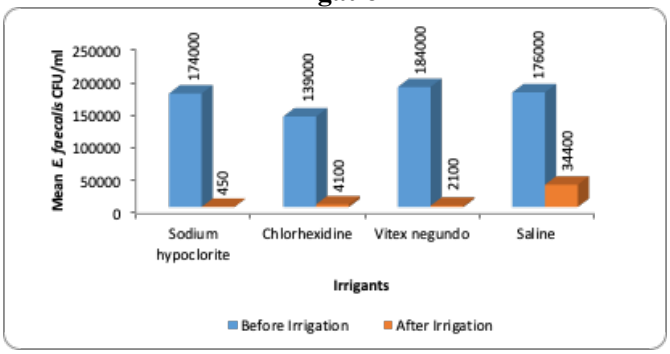


Statistical Analysis

Statistical analysis was carried out by descriptive and inferential statistics using Student's paired t-test, one way ANOVA and Post-hoc Tukey Test. Software used in the analysis was SPSS (Statistical Package for Social Sciences) Version 24.0 (IBM Corporation Chicago, USA) and GraphPad Prism 7.0 version. $p<0.05$ was considered as level of significance.

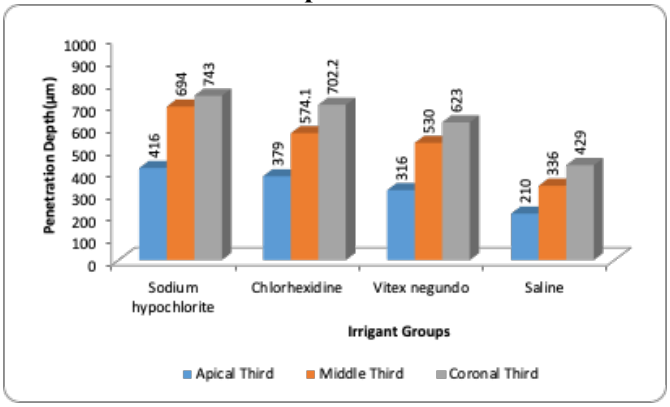
Observations and Results

Graph 1: Comparison of all the groups for the mean CFU/ml of *Enterococcus faecalis* before and after irrigation



The mean reduction comparison of all the groups for the mean CFU/ml of *Enterococcus faecalis* before and after irrigation is shown in graph 1. The mean CFU/ml of *Enterococcus faecalis* before irrigation were higher for *Vitex negundo* Linn. group (184000) followed in decreasing order for saline group (176000), sodium hypochlorite group (174000) and least values for CHX group (139000). After irrigation, CFU/ml values of *Enterococcus faecalis* showed the pattern in decreasing order as: Saline group (34400)> CHX group (4100) >*Vitex negundo* Linn. group (2100)> NaOCl group (450). Sodium hypochlorite group was found to show less CFU/ml of *Enterococcus faecalis* post irrigation and has shown its efficiency to reduce *Enterococcus faecalis* levels significantly in comparison with all other groups of irrigants like CHX group, *Vitex negundo* Linn. group and Saline group.

Graph 2: Summary of Comparison of Penetration Depth in all the groups at Coronal third, Middle third and Apical third levels



Graph 2 shows summary of comparison of penetration depth in all the groups at Coronal third, Middle third and Apical third levels. Penetration depth of irrigants at Coronal third level was highest with sodium hypochlorite group (743µm) followed by CHX group (702.2 µm) followed by *Vitex negundo* Linn. (623 µm) and least depth of penetration with saline group (429 µm). Similarly, for middle third level maximum penetration depth scores obtained in sodium hypochlorite group (694 µm) when compared with CHX group (574.1 µm), *Vitex negundo* Linn. (530 µm) and saline group (336 µm). In the apical third level, values for penetration depth were again higher with sodium hypochlorite group (416 µm) followed by CHX group (379 µm), *Vitex negundo* Linn. group (316 µm). Lesser values were obtained with saline group (210 µm). Sodium hypochlorite group was found to show maximum penetration depth at all levels i.e. Coronal third, middle third and apical third levels, when compared with other group of irrigants like CHX group, *Vitex negundo* Linn. group and Saline group.

Discussion

Endodontic infection consists of a heterogeneous combination of microbial species.(8) Once the bacteria such as *Streptococci, Veillonella parvula, Pepto streptococcus, Propioni bacterium, Lactobacilli, Eubacterium, Actinomyces, Bacteroides, Fusobacterium* invade the pulpal tissue, the root canal becomes a "privileged sanctuary" for clusters of bacteria, their byproducts and degradation products of both pulpal tissue as well as bacterial microorganism.

The failed treatment has been attributed to the flora residing in the lateral canals, tubules of the dentin, ramifications and delta. These viable bacteria constantly act as a source of reinfection or continuous inflammation.(9) The microenvironment of root canal favors the selection of few bacterial species like *Enterococcus faecalis*, *Streptococcus anginosus and Fusobacterium nucleatum*.(10)*Enterococcus faecalis* was chosen for the present study as it is the most common bacteria associated with resistant or recurrent infections leading to endodontic treatment failure. (11,12) *Enterococcus faecalis* is a non-fastidious, gram positive facultative anaerobe that can proficiently invade dentinal tubules, survive during chemo-mechanical instrumentation and intracanal medication, adjust to altered nutrient supply and continue to remain viable inside the dentinal tubules. Prevalence of *Enterococcus faecalis* ranges from 22% to 77% of cases.(13) One of the most potent virulence factors of the *Enteroccoccus* group that enhance the adaptation and also the survival in various environments are collagen binding protein (ace), *Enterococcal* surface proteins (esp) according to a variety of studies.(10,14) They can tolerate an extensive range of growth conditions, including temperatures ranging from10°C to 45°C.(15) It has been suggested in studies that *Enterococcus faecalis* can resist various intracanal treatment procedures.(15,16)

Traditional use of mechanical instrumentation reduces the bacterial load from the canal by approximately 50%. Thus, additional treatment modalities are required to assist in microorganism elimination from the inaccessible areas.(15) Ideally an irrigant should possess qualities such as: powerful antibacterial activity, dissolution of organic tissue remnants, root canal cleansing, expulsion of dentinal debris from canals post instrumentation and the peri-radicular tissue should be free of any cytotoxicity from the contents of the irrigant.(9) A large number of materials have been used for the disinfection of the root canals including normal saline, NaOCl, CHX, EDTA, HEBP, MTAD and newer materials like tetraclean and use of Ozone.(1)

NaOCl acts a potent antimicrobial agent and also has the ability to dissolve organic components of dentin such as the pulpal remnants and collagen. Several concentration of NaOCl ranging from 0.5%-6% have been used for root canal irrigation in various studies.(17-19). In the study conducted by Reyhani et al(20) it was observed that even lowest concentration i.e., 2.5% NaOCl was able to denature endotoxins produced by bacteria and organic tissue dissolution. We used 3% concentration of NaOCl was therefore used as an intracanal irrigant.

The other widely used root canal irrigant in pediatric endodontics is Chlorhexidine (CHX). The antibacterial efficacy of CHX as a root canal irrigant is concentration dependent. Depending on its concentration, CHX can have both bacteriostatic and bactericidal effects. At high concentrations, whereas at low concentrations, CHX is bacteriostatic, It has been demonstrated that 2% CHX has a better antibacterial efficacy than 0.12 % CHX in vitro. Therefore, it was decided to use 2% CHX in the current study.(21) Although NaOCl and CHX have a wide spectrum of useful properties, they have certain disadvantages too.

Undesirable characteristics of NaOCl include its caustic nature, tissue damage risks if expressed under pressure into the periodontal ligament space and also reduction in flexural strength of dentin.(3,21) Similarly, CHX when used as root canal irrigant, causes discoloration of the teeth, tongue and also dryness of the oral cavity.(3, 22-24) Hence, there is always a quest for an effective alternative root canal irrigant that fulfils the requirements of an ideal irrigant, with minimal possible side effects.

Literature has shown that certain natural plant extracts including various herbal extracts like Neem, Triphala, *Aloe vera*, Propolis, Green tea, *Morinda citrifolia*, Chamomile, Garlic extract etc. have antibacterial and medicinal effects, thus implicating their possibility to be used as an endodontic irrigant. (25) *Vitex negundo* Linn., commonly known as *Nirgundi* is a member of the Verbenaceae family, having a wide range of medicinal effects which are attributed to the metabolites like alkaloids, glycosides, tannins, flavonoids, steroids, carbohydrates.(7) *Nirgundi* has been previously used as a mouthwash in treatment of periodontal diseases and in relieving tooth pain.(26) It has also been incorporated in certain commercial products available in the market. Owing to the uses and properties like antibacterial, analgesic, antiviral,

antifungal, anti-helminthic, anti-inflammatory, and immune-enhancing effects. (27)

Study performed by Deogade et al(27) 2016, in which various concentrations of *Vitex negundo* Linn. like 20mg/ml, 40mg/ml, 60mg/ml, 80mg/ml and 100mg/ml had been used. The results of the study stated that 100mg/ml of *Vitex negundo* Linn. plant extract exhibited maximum effectiveness against both Gram positive and Gram negative bacteria respectively. Therefore 100mg/ml concentration of *Vitex negundo* Linn. was used in the present study. Numerous vehicles like ethanol, methanol, ether, ethyl acetate, chloroform and benzene had been used with *Vitex negundo*, but after comparison of solvents it was found that phenol and flavonoid contents were more in ethanolic extract. (28) Hence, in the present study, ethanolic extract of *Vitex negundo* Linn. was used.

Methodology employed in the present study was similar to that used by Nourzadeh et al(29) 2017, in which microbial reduction was assessed after chemo-mechanical debridement. In the same study, microbial samples were taken from the root canals with the use of absorbent paper points before and after chemo-mechanical debridement. Absorbent paper points were used for sample collection in the presentudy so as to obtain optimum growth of microbes.

Microbial count for *Enterococcus faecalis* in the form of colony forming units were recorded in the present study to assess the reduction in microbial growth pre and post irrigation. The mean CFU/ml for *Enterococcus faecalis* before irrigation was 174000±12294 which reduced to 450±263.52 after NaOCl irrigation

After irrigation with CHX, it was found in the present study that the mean CFU/ml for *Enterococcus faecalis* before irrigation were 139000±44833.02 which reduced to 4100±2330.95 post-irrigation. In the present study, mean CFU/ml for *Enterococcus faecalis* in Vitex negundo Linn. group before irrigation were 18400±120296 which reduced to 2100±1173 post irrigation. Sibaram et al(7) (2011)and Kekuda et al(30) (2014) stated that significant antibacterial activity of *Vitex negundo* Linn. against *E. faecalis*. Studies done by Renisheya et al(31) (2011) and Dubey and Padhy(32) (2012) showed that ethanolic extracts of *Vitex negundo* Linn. inhibited microorganisms like *S. aureus* and *Enterococcus faecalis* respectively. Comparable antibacterial activity was found in the present study. Saline group showed significant reduction in bacterial count from 176000±121765 to 34400±22741.05 post irrigation.

All the groups i.e. NaOCl, CHX and *Vitex negundo* Linn. showed significant reduction in bacterial count in intergroup comparision and intragroup comparsion. These results obtained are similar to study carried out by Chandwani et al(5) (2017), where they found that when antimicrobial activity of NaOCl and *Morinda citrifolia* juice as an irrigant was compared, NaOCl was found to show similar results as that of *Morinda citrifolia* juice in terms of reducing mean CFU/ml for *E. faecalis*. Babaji et al(33) (2016), reported that MCJ, Neem and *Aloe vera* extracts when used as root canal irrigants against NaOCl showed lesser antibacterial activity against *Enterococcus faecalis* in terms of inhibition zones in the increasing order of Aloe vera<Neem<MCJ <NaOCl. All the root canal irrigants in the present study were able to show significant bacterial reduction and the effectiveness observed ranged in decreasing order as NaOCl>CHX>*Vitex negundo*> Saline.

Taking into consideration the ideal requirements of the root canal irrigating solutions, it is said that for root canal irrigants, antimicrobial activity is not only important but at the same time it is equally important that it should kill the deeply penetrated bacteria within the dentinal tubules.(9)*Enterococcus faecalis* has been observed to have penetrated up to 1000 µm.(34,35)

For evaluation of penetration depth of root canal irrigants, several methods like Dye bleaching, Radiographic visualization, SEM, Gates Glidden drills and CLSM have been employed by various authors as found in literature.(29,36-38) However, amongst all these methods, CLSM has the advantages like ease in sample processing and preparation which tends to produce smaller number of artefacts.(39,40) Therefore, the method using Confocal Laser Scanning Microscopy was chosen. Rhodamine B dye was used to visualize the penetration using CLSM because of its smaller particle size and increased infusibility in dentinal tubules and easy visualization.(41)

The results of the study revealed that the average penetration depth of 3% NaOCl in coronal third level was 743 µm at 9mm followed by 694µm at 6mm in the mid-level and 416µm at 3mm in the apical third level. The mean penetration depth of 2% CHX in the t study at coronal, middle and apical third levels were 702 µm, 574 µm and 372 µm respectively.. Similar pattern of effectiveness was observed in study done by Vandana et al(40) 2015 in which penetration depth of CHX as irrigant using conventional irrigation method was 138µm, 80µm and 44µm at coronal third, middle third and apical third levels respectively. These results differ in values at all levels when compared to our study. This difference in value could be because of difference in types of teeth used and difference in distance for recording the penetration depth.

Current study shows that the mean penetration depth at 9mm, 6mm and 3mm for *Vitex* group was 623 µm, 530 µm and 316 µm and for saline was 429 µm, 336 µm and 210 µm respectively. Increased penetration was seen in all the groups at the coronal third and middle third portion and least amount was seen in apical one third of root canals which shows similarity in pattern of effectiveness seen in studies done by Llena et al(39) and Vandana et al(40). Their studies showed that the irrigant penetration in the apical most region was the lowest. Reason quoted for the same are: the number of patent dentinal tubules are reduced due to presence of sclerotic dentin in apical region and increased amount of peritubular dentin is also seen.(39,40) Also the anatomy of apical region makes it difficult for irrigants to access and remove the smear layer and debris. These might be some of the causative factors for limited

penetration in the portion near apex. Higher penetration seen in coronal third and middle third levels in the present study could be because of the larger tubular size of the dentin in the coronal part of the canal and the absence of complex anatomical structures.(42) Dentinal tubules hoard the maximum bacteria and thus must be cleaned effectively and efficiently. (43)

In the present study, all the four root canal irrigants, NaOCl, CHX, *Vitex negundo* Linn. and saline showed satisfactory antibacterial activity against *Enterococcus faecalis* as well as penetration depth in dentinal tubules. Comparative order of efficacy of root canal irrigants in decreasing order for both parameters observed was NaOCl> CHX > *Vitex negundo*>Saline.

Owing to the antimicrobial, analgesic, antifungal and anti-inflammatory properties shown by *Vitex negundo,* present study attempted to use *Vitex negundo* Linn. which was found to be successful in showing its effectiveness in terms of its antibacterial effect and penetration ability in dentin.(27) Herbal extracts like *Vitex negundo* Linn. prove to be potential alternatives to commonly available irrigants for root canals of primary teeth and can be used in day to day practice with minimal side effects. Herbal extracts thus must be used in order to reduce potential side effects from occurring and for enhanced antibacterial properties and to overcome resistance to conventional irrigants.(44,45).

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