**Full Article**

**TITLE OF THE MANUSCRIPT:**

**Evaluation Of The Efficacy Of Fennel Seed Extract (*Foeniculum Vulgare*) In Inhibition Of Growth Of *Enterococcus Faecalis* Biofilm Formed On Tooth Substrate: An *Ex Vivo* Study.**

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**Abstract**

The aim was to determine the Minimal Inhibitory Concentration (MIC) of the fennel seed extract against Enterococcus faecalis and to evaluate and compare the efficacy of fennel seed extract and chlorhexidine in inhibiting the growth of Enterococcus faecalis biofilm formed on tooth substrate. Ethanolic extract of Fennel was prepared and (MIC) values were determined against Enterococcus faecalis by Broth Microdilution. Dentine block of 5mm long was prepared and contaminated with E. faecalis (ATCC 29212) for 21 days. Then samples were divided into three groups (n=15), 2% Chlorhexidine, fennel seed extract, Saline. The respective medicaments were placed in root canal and incubated. At the end of 5 days dentin harvesting was carried out at 200 and 400 μm depth, then plated and colonies were counted.The MIC was assessed to be 2.5 mg/mL(0.25%). The evaluation of antimicrobial efficacy showed the mean CFU (x105) at 200µm for saline, fennel seed extract, 2% chlorhexidine, groups along with the standard deviation values were 5.3 ± 0.61, 3.5 ± 0.37, 1.9 ± 0.30 and at 400µm were 5.5 ± 0.44, 3.9 ± 0.55, 2.7 ± 0.43 respectively. Study concludes that Fennel seed extract has proved to be a potent antimicrobial agent against E faecalis.

**Keywords**: E faecalis, rootcanal medicaments,Foeniculum Vulgare, rootcanal treatment

**Introduction:**

Proper debridement and disinfection of root canals results in long-term and predictable success of root canal treatment. This involves chemicomechanical preparation of the canals(1). The preparation of root canal removes infected and necrosed pulp tissue, and majority of the infecting bacteria. Although cleaning and shaping by instrumentation and by use of intracanal medicament is an effective way of removing majority of bacterial debris, some part of the flora always survives(2). Concern exists with the survival and fate of the microorganisms left behind in the root canal, as it is practically not possible to achieve a sterile root canal space in a single-visit even after effective cleaning and shaping with instruments and antimicrobial irrigants. Hence, multiple-visit root canal treatment is preferred over one-visit treatment.The remaining microorganisms in the root canal can replicate in-between patient visits, if inter-appointment antimicrobial intracanal dressing is not placed(3).

The most common pathogenic bacteria cultured from persistent, non-healing endodontic infectious cases is Enterococcus faecalis(4). It’s belongs to the group of gram-positive cocci, and also is a facultative anaerobic bacterium (5).

The ability of this bacterial to reinfect the canals can be attributed to two factors. Firstly, this organism has the ability to survive harsh environment with scant nutrient availability and where the presence of commensal bacteria is minimum. Secondly, the unique ability of the bacteria to grow through the formation of a biofilm. This enables E. *faecalis* to survive in harsh environments such as obturated root canal system(4).

Hence, there is a need for an effective intra canal medication. Ideally an intra canal medicament should be active throughout its period of application between appointments, it should have the capacity to penetrate dentinal tubules and to eliminate the bacteria that may be present in them with least possible toxicity to the periradicular tissues(1).

Extracts of herbal and natural products are being described with anti-bacteria, anti-fungal and anti-viral activity. In this stage of emerging antibiotic resistance this calls for an effort to be made in this field of research for identification and in the studying of the biologically active ingredients, to test their potency for scientific validation for their practical and significant beneficial application in multiple fields (6).

*Foeniculum vulgare* (Fennel seed) belongs to the Apoideae subfamily. Fennel seed extract that is, the drug and as an essential oil is well known for its hepatoprotective and antispasmodic effect(7). *Foeniculum vulgare* also has anti-inflammatiry, antimicrobial, analgesic and antioxidant properties.

The aim of the present study was to evaluate the efficacy of *Foeniculum vulgare* (Fennel seed) extract as an intracanal medication against *E.faecalis* in the dentinal tubules of human mandibular premolars.

**Aim and objective:**

1. To determine the minimal concentration of the fennel seed extract that substantially inhibits the growth of*Enterococcus faecalis*.
2. To evaluate and compare the efficacy of fennel seed extract and chlorhexidine in Inhibiting the growth of a single species of*Enterococcus faecalis* biofilm formed on Tooth Substrate

**Materials and Methods:**

Ethanolic extract of Fennel was prepared according to the Soxhlation method described by Dr. F. Soxhlet.(8) The MIC value was determined against*Enterococcus faecalis* in accordance with the National Committee for Clinical Laboratory Standards (NCCLS Guidelines. An overnight culture of bacterial inoculum in Brain heart infusion broth was standardized to 0.5 McFarland standards prior to use. Different concentrations of Fennel extract were prepared to get final concentrations of 5, 2.5,1.25, 0.625, 0.312mg/mL. 100 μL of the test were added to 100 μL of bacterial inoculum in each of the wells. Chlorhexidine was taken as known drug control, broth with bacterial inoculum as positive control and 1% Dimethyl sulfoxide(DMSO) solution as negative control. For each concentration of Fennel, individual blanks containing all the constituents except the bacterial inoculum were taken in separate wells. The microtitre plate was incubated anaerobically at 370C for 24 hours at the end of which a microplate reader was used to calculate the final optical density. The first well i.e , the lowest concentration of Fennel extract to show a sharp decline in absorbance at 600 nm or the first well to show an absorbance value of less than or equal to 0.05, was considered as the Minimal Inhibitory Concentration(MIC) of the fennel extract against *Enterococcus faecalis.*

***Preparation of samples for evaluation***

A modification of model proposed by Haapasalo M and Orstavik D was used in the study.(9) A total of 45 human mandibular single rooted premolar teeth freshly extracted for orthodontic reasons were selected. So as to standardize the root canal only teeth with single root canal system were used. Using the help of digital radiograph the type I canal configuration was selected. The collected teeth were decoronated below the Cemento-Enamel Junction and sectioning of the apical portion was done using a rotary diamond disc so as to get middle third of root which was 5 mm long. Standardization of the root canal was done after mounting the teeth on a specialized jig using No. 3 Gates Glidden drill (Mani Inc, Tochigi, Japan) in a slow speed contra- angled hand piece. The removal of organic and inorganic debris was done by placing the teeth in an ultrasonic bath of 17% EDTA for a period of 5 minutes followed by 5% NaOCl for 5 minutes. Further dentine samples were immersed in ultrasonic bath with distilled water for five minutes so as to remove the traces of the chemicals and then autoclaved.

***Contamination of the Specimens***

*E. faecalis*(ATCC 29212)was used as test organism for the present study. The turbidity of *E. faecalis* bacteria culture in BHI was adjusted at 0.5 McFarland standards. Pre-sterilized tubes containing 1 mL of the BHI broth were taken and each dentine blocks were placed in it. A 50 μL of the inoculums containing *E.faecalis* was transferred into each of the tubes. All procedures were carried out under laminar flow (Thermo Fisher Scientific Inc, Waltham, MA USA).Every second day the canals were reinoculated with fresh bacteria sample. Contamination of the dentin samples was done for a period of 21 days.

***Antimicrobial Assessment***

All the samples were irrigated with 5 mL of sterile distilled water so as to remove the incubation broth at the end of 21 days. They were randomly stratified into three groups (n=15). Group 1: 2% Chlorhexidine (RC-Chlor, Azure Lab, Kochi, India), Group 2: fennel seed extract, Group 3:Saline (Nice Chemicals, Kochi, India). The respective medicaments were placed in root canal and sealed; and incubated in an anaerobic environment for 37°C. Microbial cells assessment was carried out at the end of 5 days of incubation, “Harvesting of dentin was carried out at two depths (200 μm and 400 μm) by preparing the root canal circumferentially using sterile Gates Glidden drills no.4 and no.5 respectively “(Mani Inc, Tochigi, Japan)” in slow speed handpiece with the help of customized Jig. The fine dentin shavings were collected in a test tube containing 1 mL of sterile BHI broth and each sample were mixed for 1 minute and serially diluted and then platted on BHI agar plates (Himedia Laboratories, Mumbai, India) and incubated for 24 hour at 37°C in CO2 incubator. Colonies were counted using a digital colony counter and the readings were tabulated.

**Results:**

The dry weight of the Fennel seeds used to prepare the extract was 100 grams and upon Soxhlation using 99.9% Ethanol as solvent, it yielded 5.457 grams of extract. Thus, the yield was calculated to 54.57 mg / gram of fennel seed.

The lowest concentration of Fennel extract to show a sharp decline in the absorbance was 2.5 mg/ml (0.25%) which showed an inhibition of 69.5 % of bacterial growth. 2 % Chlorhexidine showed a 82.2 % inhibition of growth of the bacteria in the same study. Further, at higher concentrations 5 mg/ml (0.5%), 79.8% inhibition of bacterial growth was seen which was comparable to the effect of 2% Chlorhexidine. Thus, the Minimal Inhibitory Concentration (MIC) was assessed to be 2.5 mg/mL(0.25%) and the Chlorhexidine equivalent was 5mg/ml(0.5%).Lower concentrations of Fennel extract, 1.25 mg/ml, 0.625 mg/ml 0.312 mg/ml gave 37%, 19% and 11% respectively.(Tab.1)

The mean CFU (x105) at 200µm for saline, fennel seed extract, 2% chlorhexidine, groups along with the standard deviation values were 5.3 ± 0.61, 3.5 ± 0.37, 1.9 ± 0.30 respectively.(Fig.1)

 The mean CFU (x105) at 400µm for saline, fennel seed extract, 2% chlorhexidine, groups along with the standard deviation values were 5.5 ± 0.44, 3.9 ± 0.55, 2.7 ± 0.43 respectively.(Fig.2)

**Discussion:**

Fennel is a rich sources of potassium, sodium , phosphorus and calcium(10). The total flavonoid content of hydroalcoholic extract of fennel is estimated to contain about 12.3 ± 0.18 mg/g. Among the flavonoids, most common were Quercetin-3-glucoronide and Kaempferol-3-glucoronide. Its reported that Alcoholic extract of *Foeniculum vulgare* contain more polyphenolic content than flavonoid content. The most abundantly found phenol being Gallic acid , Rosmarinic and chlorogenic acids.These flavonoids and polyphenols contribute to the antioxidant and antimicrobial properties of Fennel.

The antimicrobial activity of Fennel extract against E *faecalis* was tested by Broth microdilution assay. Several authors have stated the challenge when it comes to natural plant extracts that are unable to diffuse well on the agar, thus, in such cases, the broth micro dilution method offers a much more reliable assay for determination of MIC as the extract is directly added to the bacterial inoculums.(11–13). The MIC determined was 2.5mg/mL (0.25 %) fennel seed extract which showed a 69.5% inhibition of bacterial growth and antimicrobial efficacy against e faecalis was done using this concentration of fennel. A study done by K.A. Hammer et al to evaluate the antimicrobial activity of essential oil and plant extracts also found similar results.

Haapasalo and Orstavik proposed a dentin block model used to assess the efficacy of endodontic armamentarium in the disinfection of dentinal tubules (9). The study included human premolars instead of bovine teeth. The cementum was not removed as suggested by Haapasalo and Orstavik. The standardization of canal was done using a customized jig in which the position of micro motor hand piece was stabilized and it can be moved up and down only in one plane. The specimen can be mounted into the jig. This set up aided in standardization of the angles of the teeth by avoiding any untoward changes in the angulations of hand piece attribute to human error such as in advertent shaking of the hand and change in the orientation of the tooth held.

Inter group comparison of CFU count between the groups were done with one way ANOVA. A significant difference was found between mean CFU counts of 2% Chlorhexidine and saline in both 200µm and 400µm (p< 0.001)(Tab.2). 2% Chlorhexidine and Fennel seed extract, and also in fennel seed extract and saline were compared. A high significant difference was found between mean CFU counts of Chlorhexidine and Fennel seed extract, and also in fennel seed extract and saline in both 200µm and 400µm (p< 0.001)(Tab.3,4). To compare the efficacy of the medicaments on CFU counts at different depths (200µm and 400µm) paired t test was used. There was no statistical significant difference in CFU count at different depth.

There is no study in literature that evaluated the ex vivo model of evaluation of efficacy of fennel seed extract against E faecalis. The present study shows the activity of fennel seed extract in inhibiting the growth of E faecalis but it is not close enough to the efficacy of commercially available 2% chlorhexidine solution.

The current model used in this study simulates the condition of a longstanding endodontic infection such as non vital tooth, chronic abscess and lesions with sinus drainage. Antibacterial effect of different disinfecting solutions on young and established E faecalis biofilms in dentine canals were evaluated by Wang et al. They concluded that that inside the dentin canals, endodontic medicaments have reduced efficacy on bacteria in established biofilm than bacteria in young biofilm. This can be a reason why in the present study the fennel seed extract was not showing a very high activity.(14) . Hence single intracanal dressing for the period of 5 days may not be able to achieve complete eradication of bacteria from root canal system. However multiple intracanal dressings or dressing with higher concentration using this may be required for attaining complete asepsis. Its concluded that Fennel seed extract has proved to be a antimicrobial agent and can be developed further to purify and isolate the active ingredients to form an effective and safe oral care product.

**Disclosure statement:** No potential conflict of interest was reported by the authors

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**Table.1:The percentage inhibition of bacterial growth by each concentration of Fennel extract and 2% Chlorhexidine for determining Minimal inhibitory concentration.**

|  |  |  |
| --- | --- | --- |
| **Groups** | **Bacterial Growth(Abs at OD600)** | **Percentage Inhibition of bacterial growth (%)** |
| **Positive control (Untreated)** | 0.1899 | 0 |
| **Known Drug control (CHX)** | 0.0349 | 82.2 |
| **Fennel 0.312** **mg/mL** | 0.1702 | 11 |
| **Fennel 0.625** **mg/mL** | 0.1557 | 19 |
| **Fennel 1.25** **mg/mL** | 0.1203 | 37 |
| **Fennel 2.5 mg/mL** | 0.058 | 69.5 |
| **Fennel 5mg/mL** | 0.0384 | 79.8 |

**Table.2 Inter group comparison of CFU count for 2% chlorhexidine and saline with one way ANOVA.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Depth | Groups | Mean diff | S.E | ‘p’ |
| 200µm | **2% chlorhexidine****saline** | -3.4 | 0.2 | 0.000\*\*\* |
| 400µm | **2% chlorhexidine****saline** | -2.6 | 0.2 | 0.000\*\*\* |

**Table.3: Inter group comparison of CFU count for fennel seed extract and saline with one way ANOVA.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Depth | Groups | Mean diff | S.E | ‘p’ |
| 200µm | **Fennel seed extract****saline** | -1.8 | 0.2 | 0.000\*\*\* |
| 400µm | **Fennel seed extract****saline** | -1.5 | 0.2 | 0.000\*\*\* |

**Table.4: Inter group comparison of CFU count for 2% Chlorhexidine and fennel seed extract with one way ANOVA.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Depth | Groups | Mean diff | S.E | ‘p’ |
| 200µm | **2% Chlorhexidine Fennel seed extract** | -1.6 | 0.2 | 0.000\*\*\* |
| 400µm | **2% Chlorhexidine Fennel seed extract** | -1.1 | 0.2 | 0.000\*\*\* |

**Fig.1 CFU count for various Groups at 200µm dep**

**Fig.2 CFU count for various Groups at 400µm depth.**