ORIGINAL ARTICLE

Use of Flaxseed Oil as a Root Canal Medicament Against Enterococcus Faecalis Biofilm: An In-Vitro Study

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Abstract

Purpose: To evaluate the antibacterial efficacy of different concentrations of natural cold-pressed flaxseed oil when used as an intra-canal medicament against Enterococcus faecalis.

Materials and Methods: The antibacterial efficiency of flaxseed oil against Enterococcus faecalis was assessed in two sections using different concentrations. Both sections were compared to calcium hydroxide and tricresol formalin. The first section was on the agar, using two methods: agar diffusion and vaporization. The second section is on the extracted roots contaminated with E. faecalis for 21 days to form biofilms, confirmed by SEM examination, and includes two different methods: direct contact and vaporization. Bacterial swabs were collected before and after medication throughout two time periods (3 and 7 days). The canal contents were swabbed using paper points and kept for 1 minute in the root canal, and the collected samples were diluted and cultivated on plates containing blood agar. Survival fractions were determined by calculating the number of colony-forming units on the culture medium after 24 hours.

The oil's Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) against E. faecalis were determined using the micro-broth dilution method.

The active components in flaxseed oil were evaluated using GC-MS and HPLC analysis.

Results: The tested oil demonstrated antibacterial efficacy against E. faecalis in different concentrations and levels. The MBC was 22.5 µl/ml. Tricresol formalin-induced powerful antibacterial action, while calcium hydroxide exhibited less effective antibacterial action than flaxseed oil. Flaxseed oil contains numerous biologically active components.

Conclusion: Flaxseed oil exhibits strong antibacterial activity when evaluated against E. faecalis biofilm cultivated in root canals.

Keywords: Flaxseed Oil; Enterococcus Faecalis; Intra-Canal Medicaments; Calcium Hydroxide; Antibacterial.

DOI:

1. Introduction

Endodontic treatment primarily involves the chemo-mechanical preparation of the root canal system, which involves mechanical cleaning, shaping, and microbiological disinfection [1]. Despite canal cleaning methods, washing solutions, and antimicrobial medications, resistant microbes like Enterococcus faecalis still challenge root canal therapy [2]. Enterococcus faecalis (E. faecalis) remains the gold standard bacteria for antimicrobial research because of its great virulence as the most resistant bacteria identified in root canals [3]. This bacterium causes persistent peri radicular lesions after root canal therapy and is responsible for 67% to 77% of endodontic failures [4]. The ability to form biofilms is an important virulence feature for this Gram-positive facultative anaerobic bacteria, and it also has a high tolerance for high pH and concentrations of saline solution [5]. To improve the success rates of endodontic procedures, it is important to address these challenges.

Biomechanical preparation and irrigation are key to root canal disinfection. However, biomechanical preparation alone cannot completely cleanse the permanent root canal system due to its anatomical complexity [6]. Therefore, the intra-canal medicaments is served as an additional support in achieving full disinfecting of the root canal system [7]. The enhanced antibacterial efficacy of intra-canal medicament is attributed to its disinfection by penetration into the dentinal tubules [8]. Calcium hydroxide is the most frequently used intra-canal medicament, however, it is inactivated by dentin's buffering action of hydroxyapatite, reduces compressive strength, is difficult to remove, and has limited effectiveness against Enterococcus faecalis [9]. Due to synthetic drug side effects, herbal medicine use has been considered [10]. Linum usitatissimum, commonly referred to as common flax seeds, is an annual herb belonging to the Linum genus in the Linaceae family. The seeds yield a stable oil commonly referred to as flax seeds oil.

Flax seed is currently becoming recognized as a beneficial food component for human nutrition [11]. Nutritionists and medical researchers are becoming interested in flax seed due to its biologically active components ALA, lignan-Secoisiciresinol Diglycoside (SDG), and dietary fiber [12]. Flax seeds rank highest in terms of polyphenol content among all plant species, and phenolic compounds are excellent natural antioxidants [13, 14]. Consumption of flax seeds reduces tumor growth, serum cholesterol, and breast, prostate, and colon cancers [15-18]. The positive health effects of flax seeds come from their richness of biologically active components [19-21].

Previous studies have demonstrated that flaxseed oil exhibits antibacterial properties against multiple types of microbes [22, 23].

No research has focused on the antibacterial efficacy of flaxseed oil in root canal therapy against E. faecalis. Therefore, this study aimed to evaluate the antibacterial efficacy of different concentrations of Cold pressed flax seeds oil (Oils4life/UK) when used as an intra-canal treatment against enterococcus faecalis, which was obtained from infected root canals. Its effect is compared to that of $Ca(OH)_2$ (Metapaste, Meta Biomed. Korea) and Tricresol (PD/Swiss), which are frequently used for this objective.

2. Materials and Methods

2.1. Selection of Patients and Bacterial Isolation

Patients who obtained a diagnosis of pulpal necrosis and periapical alterations and are requiring endodontic therapy are subjects of this study. The diagnosis was verified through a radiographic assessment.

- Inclusion criteria:

  1- Single rooted teeth.

  2- Patients' ages ranged from 25-45 years.

  3- Symptomatic teeth

- Exclusion criteria:

  1- Patients who are receiving antibiotics.

  2- The teeth with highly destructive crowns that restrict the rubber dam's placement

  3- Cracked teeth

A rubber dam was used to isolate the location throughout the aseptic procedure, and the tooth and its surroundings were disinfected with a ten percent povidone-iodine solution. Two steps were taken to prepare the access cavity. First, a sterile carbide fissure was used to remove all carious lesions and coronal restorations, after that, disinfect the location again.

The second step was to remove the pulp chamber's roof and clean it immediately with a low-speed round carbide bur. A new 20# sterile file was placed inside the root canals to the working length end (confirmed by x-ray), clockwise twice, and then transferred immediately into a tube of sterile transport media (AMIES) [24]. E. faecalis is a facultative anaerobe, therefore, half of the samples were transferred to the laboratory using anaerobic conditions, while the other half was transported aerobically within two hours for isolation and identification [25].

2.2. Microorganism Isolation and Identification

1- Morphological characteristic: The analysis followed the guidelines described by Public Health England (2021) and was performed directly on the blood and Pfizer agar plates [26].

2- Gram stain: E. faecalis' Gram-positive feature was determined by staining a slide of suspected bacteria [26].

3-Catalase Production Test: Immediately apply a single droplet of 3 percent hydrogen peroxide over the microorganisms exhibited on a sterile slide. No reaction (absence of gas bubbles) indicates a negative catalase test; the formation of gas bubbles indicates a positive test [26].

4- Confirmation by Vitek 2 compact system:

The operation was performed in accordance with the manufacturer's instructions.

The VITEK 2 system provides results with six levels of confidence: "Excellent" with a probability of 96-99%, "Very Good" with a probability of 93-95%, "Good" with a probability of 89-92%, "Acceptable" with a probability of 85-88%, "low discrimination", and "unidentified result". After completion of the results, this study considered the identification levels to be "Excellent" [27].

2.3. Determination of MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of the Tested Oils

2.3.1. MIC Determination of the Tested Oil

The Minimum Inhibitory Concentration (MIC) was determined using the broth microdilution method, as described by the clinical and laboratory standards institute [28], to identify the lowest concentration at which microbial growth is inhibited.

2.3.2. MBC Determination of the Tested Oil

The ultimate subculture was obtained by acquiring 10 µl from every well, which had been well combined, and cultured it on agar plates to identify the Minimal Bactericidal Concentration (MBC). The MBC refers to the lowest concentration, equivalent to or greater than the Minimum Inhibitory Concentration (MIC), at which no growth is observed [29].

1- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil Against E. Faecalis on Agar

A- Agar Diffusion Method

Flaxseed oil (oils4life/UK) was diluted with dimethyl sulfoxide (DMSO) to make five concentrations (75%-50%-25%-12.5%) in addition to 100%. Experimental oil was mixed with 10% DMSO in 3:1, 1:1, 1:3, and 1:7 ratios. A petri dish with MHA (Himedia/India) media was inoculated with 100µl of 0.5 McFarland (turbidity standard) E. faecalis suspension. Using a sterilized cotton swap, the inoculum was spread all around. Each agar plate contained 6 mm diameter and 4 mm depth wells filled with 100µl of various test oil concentrations. As a negative control, another well was filled with 10%DMSO solution, and the last with $Ca(OH)_2$.

The plates were incubated aerobically at 37 °C for 18-24 hours. The diameter of the zone of inhibition, which refers to the area where no bacteria growth occurs, was measured using an electronic Vernier caliper. No zone indicated total bacterium resistance to the agents [28].

B- Vaporization Antibacterial Method

After sterilizing and inverting the Petri dishes, a 5 mm filter paper disc was impregnated with 15µl of experimental oil concentrations and tricresol formalin and placed on the inside lid of each plate. To keep the medicament vapor, the petri dish was coated in laboratory para-film.

Plates were aerobically incubated at 37℃ for 18-24 hours. A digital Vernier caliper evaluated the bacteria-free zone of inhibition.

2.3.3. Preparation of Tooth Specimens

A total of 244 Single-rooted extracted teeth from humans were utilized. The tooth specimens were chosen for investigation after undergoing a thorough assessment for the presence of cracks or defects on the cementum. The teeth were decoronated to obtain root segments of a standardized length of 12 mm (10^{-12} mm). The presence of apical patency and the establishment of a glide path were verified using a size 15 K-file. The working length was established by decreasing one millimeter from the file measurement at the exact point where it became visible at the foramen. The root canals were cleaned and shaped using the Pro-taper Next system (X1-X4) in a crown-down manner. A final irrigate made of 2.5% NaOCl and 17% EDTA was used for one minute to get rid of all the waste, including the smear layer. Then, sterile distilled water rinsed the canals and removed any remaining solution. After the preparation, all root specimens were sealed at the apex using flowable composite resin. Before the inoculation, the specimens underwent sterilization through autoclaving at a temperature of 121°C for 30 minutes under a pressure of 15 psi. Subsequently, the specimens were vertically placed in the blocks made of additional silicon impression material [30].

2.3.4. Tooth Specimen Inoculation

The suspension of bacteria was produced by inoculating 1 ml of bacterial isolates grown in BHI-B media for a period of 24 hours into five milliliters of newly prepared Brain Heart Infusion broth. The bacterial suspension was carefully injected into each root canal using sterile 1-ml insulin syringes, ensuring that there was no overflow. The sterile K-files #15 were used to transfer the bacterial suspension throughout the length of the root canal with a single inward and outward movement. Subsequently, the roots were placed in an incubator at a temperature of 37°C for 21 days, ensuring a sterile and germ-free environment. Every three days, fresh bacterial samples were added to the canals to keep the bacteria viable.

2.3.5. Scanning Electron Microscopy (SEM) Technique

Following 21 days of bacterial incubation, four roots were chosen at random for examination using Scanning Electron Microscopy (SEM) in order to confirm the presence of E. faecalis biofilm.

The teeth were sectioned longitudinally and the inner walls of the root canal were examined under magnification.

2- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil Against E. Faecalis on Extracted Roots

- **Categorizing Samples**

The root specimens were categorized according to the type of intra-canal medicament, its concentration, and the mode of application.

A- Direct Contact Method:

Group I. 20 roots were treated with 5% flaxseed oil; by flooding it in the root canal.

Group II. 20 roots were treated with 10% flaxseed oil; by flooding it in the root canal.

Group III. 20 roots were treated with 25% flaxseed oil; by flooding it in the root canal.

Group IV. 20 roots were treated with 50% flaxseed oil; by flooding it in the root canal.

Group V. 20 roots were treated with $Ca(OH)_2$ calcium hydroxide paste (Metapaste, Meta Biomed. Korea); by compacting it into the root canal (Positive control group).

Group VI. 20 roots were medicated by 10% DMSO; by flooding it in the root canal. (Negative control group).

B- Vaporization Method:

Group I. 20 roots were treated with 25% flaxseed oil; by the cotton pellet approach.

Group II. 20 roots were treated with 50% flaxseed oil; the cotton pellet approach.

Group III. 20 roots were treated with 75% flaxseed oil; the cotton pellet approach.

Group IV. 20 roots were treated with 100% flaxseed oil; the cotton pellet approach.

Group V. 20 roots were treated with tricresol formalin; the cotton pellet approach (Positive control group).

Group VI. 20 roots were treated with 10% DMSO by cotton pellet technique (Negative control group).

- **The Procedure of Medication**

A-Direct Contact Method

The intra-canal medicaments were applied using a fine-gauge syringe, followed by the removal of any excess material using cotton pieces. Finally, the roots were sealed with a temporary filling.

B-Vaporization Method

An amount of 0.025 ml of intra-canal medicaments was placed in the canal using the cotton pellet approach with a micropipette. Afterward, the teeth were sealed with a temporary filling.

2.4. Bacterial Sampling

1- Before administering intra-canal medications, root canals were swabbed and cultured to get baseline microorganism data.

2- Following the application of the medicaments, each group was subdivided into subgroups consisting of ten samples (n=10). These subgroups were then incubated for a duration of three and seven days. Following a period of 3 days, a total of 10 samples per group were taken from the incubator and subjected to evaluation. Following the removal of the temporary filling, the medicaments were removed and the contents of the root canals were swabbed using a sterile paper point (size X3) and kept for 1 minute in the root canal. Afterward, the collected samples were diluted and cultivated on plates containing blood agar. Survival fractions were determined by calculating the number of colony-forming units on the culture medium after 24 hours. This enables us to determine how many bacteria are contained in the samples. The above procedure was repeated at a time interval of 7 days.

2.5. HPLC Analysis of FO

Samples from FO made in the United Kingdom were analyzed by High-Performance Liquid Chromatographic (HPLC). The separation occurred on HPLC-UV: LC800-0101-column: ODS C18 column (4.6 × 250 mm, 5 µm). The flow rate was 1.0 mL/min, and fractions were detected at 210 nm. Injection volume: 1µl; mobile phase: Eluent A was acetonitrile (0.2% formic acid), and eluent B was water (0.2% formic acid). An isocratic elution program was used, which was 80% A.

2.6. GC-MS analysis of FO

Samples from FO made in the United Kingdom were analyzed by GC-MS under the following conditions:

Gas Chromatograph: Agilent Technologies (7820A), GC Mass Spectrometer (5977E) USA.

Analytical Column: Agilent HP-5ms Ultra unit (30 m length x 250 µm inner diameter x 0.25 µm film thickness), Injection Volume: 11, Pressure: 11.933 psi, GC Inlet Line Temperature: 250 ˚C, Aux Heaters Temperature: 300 ˚C, Carrier Gas: 99.99%, Injector Temperature: 250 ˚C, Scan Range: m/z 25-1000 and Injection Type: Splitless

2.7. Statistical Analysis

Data were analyzed using SPSS version 26. Normality was assessed using the Shapiro-Wilks test. One-way ANOVA, multiple comparisons using Tukey, and Independent sample t-tests were used. The significance level was set at $p<0.05$.

3. Results

3.1. The Isolated Microorganisms' Identification

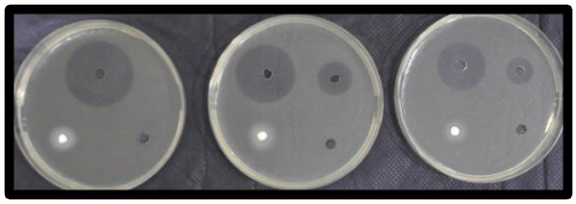
The colonies under investigation exhibited Gram-positive characteristics, tested negative for catalase, demonstrated robust growth on blood agar, and did not have any hemolysis. They also had a round, smooth, raised edge and measured between 1.0 and 1.5 mm. On Pfizer agar, E. faecalis grows well, and the colony's surrounding area appears black. The result was confirmed VITEK 2 system and the level of confidence was "Excellent" with a 99% probability.

3.2. MIC and MBC Determination of Flaxseed Oil

Flaxseed oil has a MIC of 11.25µl/ml and an MBC of 22.5µl/ml.

1- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil Against E. Faecalis on Agar

A- Agar Diffusion Method



100 % 75%-25% 50%-12.5%

Figure 1. Susceptibility of E.faecalis to different concentrations of flaxseed oil

- **Normality**

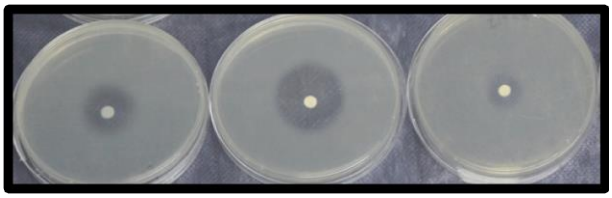
The data of the Agar well diffusion was normally distributed according to the Kolmogorov-Smirnov (KS) and Shapiro-Wilk test.

- **Comparison of Inhibition Zone**

The diameter of the inhibition zone (clear zone of no growth of E.faecalis around each well) was found to increase as the concentrations of the oil increased. Ten percent of DMSO showed no zone of inhibition while $Ca(OH)_2$ showed small to moderate zones of inhibition compared to the tested oil concentrations except in concentration 12.5%.

There was a significant difference in the inhibition zone between different concentrations of flax ($p<0.05$) except between 12.5% and positive control there was no difference ($p>0.05$). 100% concentration showed a significantly higher inhibition zone than other concentrations as shown below (Table 1).

B- Vaporization Antibacterial Method



100 % Tricresol 75%

Figure 2. Susceptibility of E. faecalis to vapor of different concentrations of flaxseed oil

- **Normality**

The data were normally distributed according to the Kolmogorov-Smirnov (KS) and Shapiro-Wilk test.

- **Comparison of Inhibition Zone**

There was a significant difference in inhibition zones between different groups ($p<0.05$). +control showed significantly higher IZ compared to other groups followed by 100% and 75% concentrations of flax with significant differences between them all ($p<0.05$), as shown below (Table 2).

Table 1. Mean, standard deviation, minimum, and maximum of inhibition zone in millimeters of E. faecalis to different concentrations and different agents using the agar well diffusion method

Concentration	N	Mean (mm)	Std. Deviation	Minimum	Maximum	p-value*
12.5%	10	13.01[a]	1.76	10.01	16.20	0.000
25%	10	18.50	2.03	15.10	21.89	
50%	10	27.21	2.43	23.01	31.40	
75%	10	30.10	2.68	26.01	34.20	
100%	10	36.41	2.79	32.27	40.54	
Ca(OH)2 (+control)	10	15.01[a]	1.83	12.01	18.02	
10%DMSO(-control)	10	0.00	0.00	0.00	0.00	

*One-way ANOVA. Identical superscript small letters represent non-significant differences between relevant groups according to Tukey HSD

Table 2. Mean, standard deviation, minimum, and maximum of inhibition zone in millimeters of E. faecalis to different concentrations and different agents using the vaporization antibacterial method

Groups	N	Mean (mm)	Std. Deviation	Minimum	Maximum	p-value*
75%	10	10.07	1.86	7.05	13.10	**0.000**
100%	10	17.42	3.47	12.03	22.81	
Tricresol (+control)	10	41.98	5.18	33.97	49.99	
10%DMSO(-control)	10	0.00	0.00	0.00	0.00	

*One-way ANOVA. Each group showed a significant difference from all other groups according to Tukey HSD

- **Scanning Electron Microscope Images of Roots before Placement of Medication**

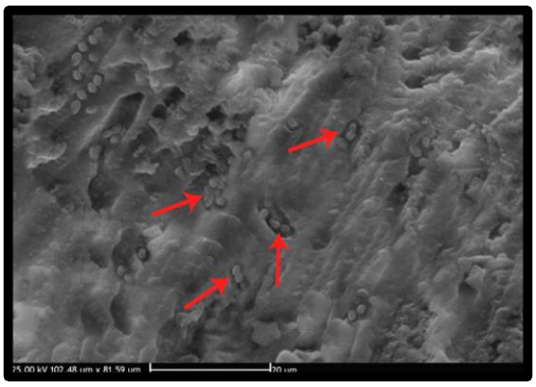


Figure 3. E. faecalis biofilm (shown by red arrows) has deeply penetrating dentinal tubules

2- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil as an Intra-Canal Medicament Against E. Faecalis Biofilm on Extracted Roots

A- Direct Contact Method

- **Normality**

Data of difference between before and after counts were normally distributed according to the Kolmogorov-Smirnov (KS) and Shapiro-Wilk test.

- **Comparison of Differences between Groups**

There was a significant difference between groups as shown below. There was a significant difference between each group and the other except between 25 and 50% of groups there was no significant difference as shown below (Table 3).

B- Vaporization Method

- **Normality**

Data were normally distributed according to the Kolmogorov-Smirnov (KS) and Shapiro-Wilk test.

- **Comparison of Differences between Groups**

There was a significant difference between each group and the other except between 25% and negative control groups there was no significant difference as shown below (Table 4).

Table 3. Mean, standard deviation, minimum, maximum of colony forming unit of E. faecalis to different concentrations and different agents using the direct contact method

Group	Mean	Std. Deviation	Minimum	Maximum	p-value*
After 3 days					
5%	21.70×10^3	4.45	16.00	32.00	0.000
10%	35.70×10^3	4.60	29.00	42.00	
25%	$61.70^{a}\times10^3$	4.00	54.00	68.00	
50%	$60.50^{a}\times10^3$	3.10	55.00	64.00	
$Ca(OH)_2$ (+control)	50.10×10^3	4.93	42.00	57.00	
10%DMSO(-control)	2.40×10^3	4.12	-3.00	7.00	
After 7 days					
5%	14.50×10^3	4.09	6.00	20.00	0.000
10%	31.90×10^3	3.84	27.00	38.00	
25%	$58.30^{a,b}\times10^3$	3.37	55.00	65.00	
50%	$60.20^{a}\times10^3$	2.53	56.00	64.00	
$Ca(OH)_2$ (+control)	$53.60^{b}\times10^3$	4.86	46.00	61.00	
DMSO(-control)	0.50×10^3	3.66	-4.00	7.00	

*One-way ANOVA. Identical superscript small letters represent NON-significant differences between relevant groups for each period, according to Tukey HSD.

Table 4. Mean, standard deviation, minimum, and maximum of colony forming unit of E. faecalis to different concentrations and different agents using the vaporization method

Group	Mean	Std. Deviation	Minimum	Maximum	p-value
After 3 days					
25%	$5.60^{a}\times10^{3}$	3.53	-1.00	10.00	0.000
50%	10.90×10^{3}	2.81	6.00	16.00	
75%	33.10×10^{3}	3.48	29.00	38.00	
100%	52.70×10^{3}	3.09	47.00	57.00	
Tricresol (+control)	62.60×10^{3}	4.22	57.00	69.00	
DMSO(-control)	$4.20^{a}\times10^{3}$	1.99	1.00	8.00	
After 7 days					
25%	$2.50^{a}\times10^{3}$	3.10	-3.00	7.00	0.000
50%	$5.40^{a,b}\times10^{3}$	4.30	1.00	14.00	
75%	27.90×10^{3}	4.95	23.00	39.00	
100%	46.60×10^{3}	4.74	40.00	58.00	
Tricresol (+control)	63.90×10^{3}	2.02	61.00	67.00	
10%DMSO (-control)	$0.50^{a,b}\times10^{3}$	3.24	-5.00	7.00	

*One-way ANOVA. Identical superscript small letters represent NON-significant differences between relevant groups for each period, according to Tukey HSD

- **HPLC Analysis of FO**

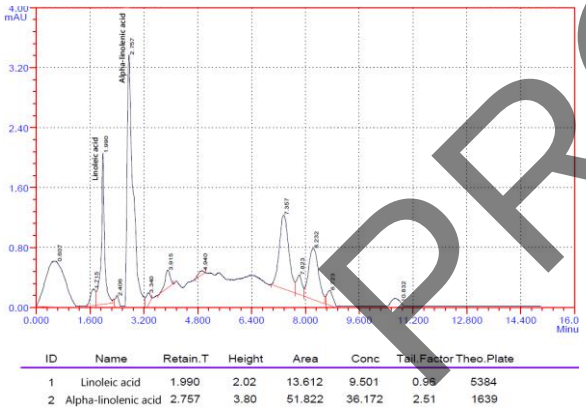


Figure 4. HPLC chromatogram of linoleic and alpha-linolenic acid formulations in FO

- **GC-MS Analysis of FO**

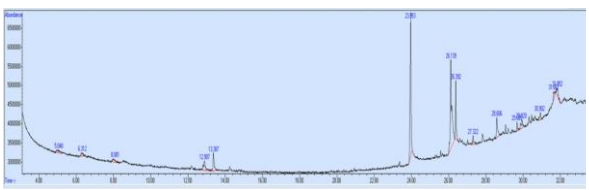


Figure 5. GC-MS chromatogram of FO

4. Discussion

Modern endodontic intra-canal medications have a different rationale [31]. Using an intra-canal medication can help prevent the regrowth of bacteria, enhance bacterial suppression, and prevent pathogen entry via leaky restorations between appointments [32].

Currently, majority of commercially available intra-canal medications are cytotoxic and fail to eradicate bacteria from the dentinal tubules. A modern medicinal trend refers to the use of biological medications derived from natural plants [33]. Herbal medications give several advantages over synthetic alternatives, including cost-effectiveness, minimal toxicity, availability, extended shelf life, and reduced microbial resistance [34].

In this microbiological study, the susceptibility of E. faecalis to natural cold pressed of FO was evaluated in two sections. The first section was on the agar, using two methods: the agar-well diffusion method and the disk vaporization method. The second section is on the extracted roots and includes two different methods: the direct contact method and the vaporization method.

Table 5. Compounds of FO

No. of peaks	Name of compound	Retention time	Area %	Molecular formula
1	Propionic acid	5.052	1.66	$C_3H_6O_2$
2	cis-10-Nonadecenoic acid.	6.315	1.66	$C_{19}H_{36}O_2$
3	Hexadecanoic acid, methyl ester	8.081	2.41	$C_{17}H_{34}O_2$
4	tetradecanoic acid	12.911	4.84	$C_{14}H_{28}O_2$
5	(9Z)-octadec-9-enoic acid	13.387	4.53	$C_{18}H_{34}O_2$
6	cis,cis-9,12-Octadecadienoic acid. C18:2	23.955	27.61	$C_{18}H_{32}O_2$
7	cis,cis-9,12-Octadecadienoic acid. C18:3	26.136	28.55	$C_{18}H_{30}O_2$
8	icosanoic acid	26.396	9.48	$C_{20}H_{40}O_2$
9	8-Methyl-6-nonenamide	27.322	2.24	$C_{10}H_{19}NO$
10	hexadecanoic acid	28.603	5.01	$C_{16}H_{32}O_2$
11	(Z)-hexadec-9-enoic acid	29.685	1.60	$C_{16}H_{30}O_2$
12	Octadecanoic acid	29.918	2.99	$C_{18}H_{36}O_2$
13	Pentadecanoic acid	30.905	1.86	$C_{15}H_{30}O_2$
14	Octatriacontyl pentafluoropropionate	31.658	2.65	$C_{41}H_{77}F_5O_2$
15	(Z)-docos-13-enoic acid	31.848	2.92	$C_{22}H_{42}O_2$

4.1. Determination of MIC and MBC of FO

FO has been found to have a MIC of 11.25µl/ml and an MBC of 22.5µl/ml. This result is different from other studies [35, 36], which could be attributed to various bacterial species and sources, the MIC study methodology, the type of broth utilized, or the source of the oil [37, 38].

1- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil Against E. Faecalis on Agar

A- Agar Diffusion Method

It has been demonstrated that FO inhibits E. faecalis growth at various concentrations and levels. The dimensions of the inhibition zones exhibited an increase with the elevation of the FO concentration from 12.5% to 100%. This result agrees with [39] who studied the antibacterial activity of flaxseed extracts on Streptococcus mutans and the results confirmed the hypothesis that higher concentrations of flaxseed oil would enhance the suppression of bacterial growth. A higher flaxseed oil concentration can release more antibacterial or inhibitory substances.

Statistically, all concentrations of FO except 12.5% exhibited a highly significant difference, indicating that they possess greater antimicrobial activity against E. faecalis compared to $Ca(OH)_2$. This result is inconsistent with the results reported in a study conducted by [40], who stated that FO did not exhibit any inhibitory effects on E. faecalis. The differences in methods, bacterial source, nature, and origin of the oil used could be responsible for this difference.

Calcium hydroxide $Ca(OH)_2$ demonstrated limited antibacterial effects in the agar diffusion method, and this result is consistent with the findings of [41, 42] who reported that $Ca(OH)_2$ along with distilled water had minimal antimicrobial properties in agar diffusion method.

B- Vaporization Antibacterial Method:

In this study, TC demonstrated a greater capacity to inhibit the growth of bacteria compared to FO at all tested concentrations, demonstrated by the larger inhibition zone observed. The results of this study are consistent with [25], who observed that the vapor of TC efficiently suppressed E. faecalis in vitro. Flaxseed oil's antibacterial action is attributed to the presence of volatile phenolic compounds, which enhance its antioxidant and antimicrobial properties [43].

2- Antibacterial Efficiency of Different Concentrations of Flaxseed Oil Against E. Faecalis Biofilm on Extracted Roots

The current study used natural cold-pressed Flaxseed Oil (FO) as an intra-canal medicament. As far as we know, there is no data available or research on the utilization of this oil in the field of endodontics. More precisely, there is no previous documentation of its effectiveness in eradicating E. faecalis bacteria in root canals. Therefore, this study represents the first attempt to use cold-pressed flaxseed oil as an intra-canal medicament.

The introduction of the biofilm concept to the field of endodontic microbiology represents an important step in the understanding of persistent root canal infections. Microorganisms producing biofilms are 1,000 times more resistant to antimicrobials and environmental changes than planktonic organisms [44].

In this section, the chosen infection period was enough for the growth and penetration of the bacterial biofilm inside dentinal tubules, as confirmed by SEM examination. Regarding the duration of medicament insertion within the canal, many studies agree that a one-week time is sufficient to achieve maximal antibacterial efficacy [45-47]. Two time periods were used in this study to assess the disinfection antibacterial action, as several intra-canal medicaments exhibit increased effectiveness after 48 hours. Hence, the time intervals selected for the antibacterial evaluation comprised 3 and 7 days.

A. Direct Contact Method

FO reduces Colony-Forming Units of bacteria (CFUs) at different levels. As FO concentration increased from 5% to 50%, CFUs decreased. Increased concentration of FO might improve its antibacterial action by increasing its active component contents. After 3 days, 25% and 50% FO exhibited significantly higher bacterial reduction compared to $Ca(OH)_2$, whereas there was no significant difference between 25% FO and $Ca(OH)_2$ after 7 days.

In the current study, 50% FO exhibits the highest level of reduction in CFUs after 3 days while $Ca(OH)_2$ showed maximum reduction in CFUs after 7 days against E. faecalis. [48] showed that $Ca(OH)_2$ was not effective in eliminating E. faecalis after 1 week. This could be attributed to the limited effectiveness of $Ca(OH)_2$ against facultative anaerobes, the buffering action of dentin, and the arrangement of bacterial cells that colonize the walls of the root canal.

B. Vaporization Method

T.C exhibited significantly more CFUs reduction compared to FO at all tested concentrations. The presence of formaldehyde in these compounds may explain this good result [41]. A chemical containing formaldehyde achieved a significant bacterial reduction. This conclusion coincides with the findings of [49] who stated that formaldehyde exhibits potent and long-range antibacterial properties, allowing its vapor to effectively penetrate even the furthest regions of the root canal.

Current study on extracted roots reveals that the antibacterial efficacy of FO decreases after three to seven days of use as a root canal medicament in extracted roots. Many factors contribute to this phenomenon including volatility and evaporation, degradation, environmental factors, absorption or binding, and oxidation of oil. All these variables may reduce the oil's antibacterial component concentration or structure, decreasing its potency against bacteria over time [50, 51].

4.2. HPLC Analysis of Cold-Pressed FO

The α-linolenic and Linoleic acids peaks were identified and their amounts were also calculated as well. Findings obtained from HPLC analysis revealed that α-linolenic and Linoleic acids were the predominant unsaturated fatty acids in the oil. The Linoleic acid and α-linolenic acid demonstrated strong antibacterial activities against several types of gram-positive and gram-negative bacterial biofilm formation [52, 53].

4.3. GC-MS Analysis of Cold-Pressed FO

The GC-MS analysis provides different peaks that helps to enable the prediction of the formula and structure of 15 biomolecules fatty acids.

FO was found to be rich in fatty acids, especially unsaturated fatty acids. Unsaturated fatty acids were found to suppress bacterial growth better than saturated fatty acids of the same carbon chain length [54]. Furthermore, recent research has shown that unsaturated fatty acids can prevent or eliminate biofilms created by several types of bacteria [55-57]. These results are comparable to those reported in the current study. Therefore, unsaturated fatty acids may be the next generation of antimicrobial agents for the treatment of biofilm-associated infection [58].

Flaxseed was also reported rich in Secoisolariciresinol Di Glucoside (SDG), a lignan precursor with several health benefits. The SDG is antioxidant, anti-viral, antibacterial, and anti-fungal, and enhances the immune system [59].

Mazur *et al.* in 1996 stated the flax plant possesses a lignan content that is up to 800 times higher than that of other plant-based foods [60]. It was found that the lignans in FO hulls inhibited the growth of a number of bacteria. The antibacterial activity of FO can be attributed to all these components.

5. Conclusion

The current study demonstrates the superior antibacterial efficacy of flaxseed oil against E. faecalis biofilm as compared to calcium hydroxide agents. However, this study focused on E. faecalis biofilm infection of root canals, whereas clinical endodontic infections often involve many microorganisms, therefore, mixed infection requires more investigation.

Under the limitations of this study, it can be concluded that flaxseed oil at concentrations of 25% and 50% in the direct contact method and 100% in the vaporization method can be successfully used for the inhibition of E. faecalis biofilms from the root canal when used as intra-canal medicament and can be considered an alternative intracanal medicament.

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