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Antibacterial Efficiency of Chia Seed Oil as an Intra-Canal Medication Against Enterococcus Faecalis Biofilm: An In Vitro Study

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Abstract

Chemo-mechanical endodontic treatment aims to eliminate microbes from the root canal. Applying intra-canal medicaments between visits enhances the disinfection process. Herbal compounds are a new medicinal trend. The study aimed to evaluate the antibacterial efficacy of chia seed oil when used as an intracanal medicament against E. faecalis. The antibacterial efficiency of chia seed oil against E. faecalis was assessed in two sections using different concentrations. The first section was done on the agar, using two methods: agar diffusion and vaporization. The second section was done on the extracted roots contaminated with E. faecalis for 21 days to form biofilms and includes two methods: direct contact and vaporization. Bacterial swabs were taken before and after medication for 3 and 7 days. The canal contents were swabbed using paper points kept for 1 minute in the root canal, and the collected samples were diluted and cultivated on blood agar. Survival fractions were determined by calculating the number of colony-forming units on culture medium after 24 hours. The oil's minimum inhibitory concentration and minimal bactericidal concentration against E. faecalis were determined using the micro-broth dilution method. The tested oil demonstrated antibacterial efficacy in different concentrations and levels. The MBC was 45 µl/ml. Tricresol formalin induced powerful antibacterial action, while calcium hydroxide exhibited less effective antibacterial action as compared to 50% chia seed oil on extracted roots. Within the limits of this study, chia seed oil exhibits strong antibacterial activity against E. faecalis biofilm that has been cultivated in the root canals.

Introduction:

The primary goal of root canal therapy is to thoroughly disinfect the root canal system and completely close it to avoid the infiltration of harmful microorganisms and irritants into the adjacent periapical tissues [1]. Enterococcus faecalis (E. faecalis) remains the gold standard bacteria for antimicrobial research because to its great virulence as the most resistant bacteria identified in root canals [2]. This bacterium causes persistent periradicular lesions after root canal therapy and responsible for 67% to 77% of endodontic failures [3]. 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 [4]. The biofilm concepts in endodontic microbiology advances our understanding of persistent root canal infections.Biofilm-forming microorganisms are one thousand times more resistant than planktonic organisms to antimicrobials and environmental changes [5] . To improve the success rates of endodontic procedures, it is important to address these challenges.

The use of intra-canal medicaments serves as an additional support in achieving full disinfecting of the root canal system[6]. Calcium hydroxide is the most common intra-canal medication, 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 [7]. The majority of intra-canal medications presented are toxic and fail to eliminate all dentinal tubule bacteria.

Biologic medications made from plants are a recent medical trend [8, 9]. Herbal medicines are cheaper, safer, more accessible, longer-lasting, and less microbial-resistant than synthetic ones [10]. Chia is the common name for some species of salvia, among which salvia hispanica L., is an annual herbaceous plant belonging to the lamiaceae family. Today, chia seeds are being used for their medicinal and nutritional properties [11]. Moreover, seeds contain no toxic components and gluten, thus making chia seeds a safe ingredient also gluten free diets [12]. Several studies have been conducted in previous years aimed at the benefits of chia seeds extract for overall human health[13] . They are used as an appetite suppressant for weight loss, blood glucose control, and intestinal regulation due to their high fiber content [14]. The chia seeds are rich in a linolenic acid (C18:3 n-3, ALA), vitamins, and phenolic acids [15]. The presence of fatty acids in chia seed have a prominent role and crucial for health, antimicrobial, and antioxidant activity [11]. It also has a high diversity of secondary metabolic compounds such as terpenes, phenols, and flavonoids, and this enables it to be antimicrobial [16].

Previous studies showed that chia seeds also exhibited antibacterial activity against both gram negative and gram positive strains [17]. There hasn't been any research on chia seed oil's antibacterial effectiveness against E. faecalis in root canal therapy. Therefore, this study aims to evaluate the antibacterial efficacy of different concentrations of natural cold pressed chia seed oil (Sun&Seed/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.

Materials and Methods

The study was approved by the scientific research committee at the College of Dentistry, University of Baghdad (Ref. 904 in 2024).

Patient selection and bacterial isolation

This study includes ten endodontic patients with pulpal necrosis and periapical changes. All of the teeth that have been chosen have a single root. The patients' ages ranged from 25 to 45 years. The teeth were symptomatic, and the diagnosis was verified by radiographic examination. The patients who take antibiotics and teeth with extremely

damaging crowns that limit where the rubber dam can be placed were excluded.

Throughout the aseptic operation, a rubber dam was employed to isolate the specific area, and the tooth and its surrounding area were disinfected using a 10 percent povidone iodine solution. Access cavity preparation involved two processes. First, a sterile carbide fissure was used to remove all carious lesions and coronal restorations, which were then disinfected again. Secondly, a low-speed round carbide bur was used to remove the pulp chamber roof and clean it immediately. Placed a new 20# sterile file inside the root canals to the working length end (verified by x-ray), clockwise twice, and immediately transferred into a tube of sterile transport media (AMIES). E. faecalis is a facultative anaerobe, so half of the samples were transported anaerobically and half aerobically to the lab within two hours for isolation and identification [18].

Morphological characteristic

Blood and Pfizer agar plates were analyzed directly according to Public Health England (2021) recommendations [19].

Gram's stain method

The Gram-positive characteristic of E. faecalis was identified by staining a slide containing the suspected bacteria [19].

Catalase production test

Apply a single droplet of 3% hydrogen peroxide directly over the microorganisms displayed on a sterile slide. A negative catalase test is indicated by the absence of gas bubbles, while a positive test is indicated by the development of gas bubbles [19].

Confirmation by VITEK 2 compact system

The operation was carried out according to the guidelines provided by the manufacturer.

Six levels of confidence are associated with the results produced by the VITEK 2 system: "excellent" (96–99% probability), "very good" (93%–95%), "good" (89–92% probability), "acceptable" (85-88% probability), "low discrimination," and "unidentified result." After completion of the analysis, the identification levels were deemed "excellent" in this study [20].

MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) determinations of the tested oil

The Minimum Inhibitory Concentration (MIC) of tested oil was measured by the broth microdilution method, as outlined by the Clinical and Laboratory Standards Institute (CLSI) , to determine the lowest concentration at which microbial growth is prevented. The Minimum Bactericidal Concentration (MBC) was measured by taking 10 µl from each well and cultured on agar plates to determine the Minimum Bactericidal Concentration (MBC). The MBC was the first higher or equal to the MIC concentration, at which no growth occurs [21].

The agar well diffusion technique

In addition to 100%, four concentrations of chia seed oil (Sun&Seed/UK) were created by diluting it with dimethyl sulfoxide (DMSO): 75%–50%–25%–12.5%. Ratios of 3:1, 1:1, 1:3, and 1:7 were used to combine the experimental oil with 10% DMSO. A 100µl of a 0.5 Mcfarland (turbidity standard) E. faecalis suspension was added to a petri dish with MHA (Himedia/India) media. The inoculum was dispersed throughout using sterile cotton swaps. Every agar plate had wells with a diameter of 6 mm and a depth of 4 mm, which were filled with 100 µl of different test oil concentrations, 10% DMSO solution as a negative control, and $Ca(OH)_2$ as a positive control.

The plates were incubated aerobically at 37 °C for 18–24 hours. After that, an electronic Vernier caliper was used to measure the diameter of the zone of inhibition, which is the area in which there is no bacterial growth. No zone showed complete resistance of the bacteria to the agents.

The vapor diffusion technique

A 5 mm filter paper disc with 15 µl of experimental oil concentrations and

tricresol formalin was placed on the inside lid of each petri dish after sterilization and inversion. Laboratory parafilm covered the petri dish to keep the medication vaporized. The plates were incubated aerobically at 37 °C for 18–24 hours. After that, an electronic Vernier caliper measured the bacteria-free area of inhibition [22].

Preparing tooth samples

A total of 244 extracted teeth from humans with single roots were used. The tooth specimens were selected for examination following a comprehensive evaluation to identify any cracks or faults in the cementum. The teeth were decoronated to obtain root groups of a standardized length of 12 mm (with a range of 10-12 mm). Confirming the existence of apical patency and the formation of a glide path was done using a size 15 K-file [23]. In order to determine the working length, one millimeter was subtracted from the file measurement precisely at the location where it became apparent at the foramen. The root canals were cleansed and shaped in a crown-down manner utilizing the Pro-taper Next file system (X1–X4). One minute was spent using a final irrigants composed of 17% EDTA and 2.5% NaOCl to eliminate all debris involving the smear layer. The canals were then cleansed with sterile distilled water to eliminate any remaining solution. All root samples were sealed at the apex with flowable composite resin following preparation. The specimens were then positioned vertically within the blocks composed of additional silicon impression material. Prior to the inoculation process, the specimens were sterilized through autoclaving at a pressure of 15 psi and a temperature of 121°C for 30 minutes.

Inoculation of a tooth sample

One milliliter of bacterial isolates cultured in BHI-B media for 24 hours was added to five milliliters of new brain heart infusion broth to create the bacterial suspension. Sterilized 1-ml insulin syringes were used to inject the bacterial suspension into each root canal, making sure there was no overflow. With a single inner and outward movement, the sterile K-files #15 were utilized to distribute the bacterial suspension throughout the entire root canal. Next, the roots were kept in a germ-free environment for 21 days by placing them in an incubator set at 37 C°. To maintain the bacteria's viability, new bacterial samples were introduced to the canals every three days.

The SEM (scanning electron microscopy) method

After a 21-day period of bacterial incubation, four roots were randomly selected for inspection using scanning electron microscopy (SEM) to verify the presence of E. faecalis biofilm in the dentinal tubules.

The roots were sectioned longitudinally, and the inner walls of the root canal were investigated under a microscope.

Sample grouping

The root samples were classified based on the type of intra-canal medicament used, its concentration, and the method of application.

Direct contact method

Group I. 20 roots received 5% CHO.
Group II. 20 roots received 10% CHO.
Group III. 20 roots received 25% CHO.
Group IV. 20 roots received 50% CHO.
Group V. 20 roots received $Ca(OH)_2$ calcium hydroxide paste (positive control group).
Group VI. 20 roots received 10% DMSO (Negative control group).

Vaporization method

Group I. 20 roots received 25% CHO.
Group II. 20 roots received 50% CHO.
Group III. 20 roots received 75% CHO.
Group IV. 20 roots received 100% CHO.
Group V. 20 roots received tricresol formalin (positive control group).
Group VI. 20 roots received 10% DMSO (negative control group).

Method of Administration

Direct contact method

A fine-gauge syringe was used for injecting the medications inside the root canals, and cotton pieces were used to remove any extra material. After that, a

temporary filling was used to seal the roots.

Vaporization method

Using a micropipette, 0.025 ml of intra-canal medication was placed inside the canal through the cotton pellet technique. After that, a temporary filling was used to seal the roots.
In order to get baseline microorganism data, root canals were swabbed and cultured prior to the administration of intra-canal medications. Following medications, each group was divided into ten-sample subgroups (n = 10). These subgroups were incubated for 3–7 days. After 3 days, 10 samples per group were collected from the incubator and evaluated. After removing the temporary filling, the medicaments were removed, and the root canals were swabbed with a sterile paper point (size X3) for 1 minute. The samples were diluted and cultured on blood agar plates. After 24 hours, colony-forming units on culture media were used to calculate survival fractions. This allows us to count bacteria in samples. The above method was done at intervals of seven days.

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

Results

The isolated microorganisms' identification
The colonies were gram-positive and catalase-negative, developed well on blood agar, and had no hemolysis. They were sized 1.0–1.5 mm and had a circular, smooth, raised edge. E. faecalis grows effectively on Pfizer agar, resulting in a black colony radius. The VITEK 2 system approved the result with "excellent" confidence and a 99% probability.

MIC and MBC determinations of CHO
Chia seed oil has a MIC of 22.5µl/ml and an MBC of 45µl/ml.

The agar well diffusion technique
The data distribution was normally distributed according to Shapiro-Wilk test. There was a significant difference in inhibition zone between different concentrations of CHO ($p<0.05$) except between 25% and $Ca(OH)_2$ and between 75% and 100%; there was no difference ($p>0.05$). 100% CHO concentration showed a significantly higher inhibition zone than other concentrations except for 75%, as shown in Table 1.

The vapor diffusion technique
The data distribution was normally distributed according to Shapiro-Wilk test. There was a significant difference between each group and the other except between the 75% and negative control groups; there was no significant difference as shown in Table 2.

Direct contact method
The data distribution was normally distributed according to Shapiro-Wilk test. There was a significant difference between each group and the other except between 25% and $Ca(OH)_2$ and between 5% and the negative control at both intervals, and between 25% and 50% and $Ca(OH)_2$ after 7 days there were no significant difference as shown in Table 3.

Vaporization method
The data distribution was normally distributed according to Shapiro-Wilk test. There was a significant difference between each group and the other for each period except between the 25% and 50% and negative control groups, respectively, where there was no significant difference as shown in Table 4.

Discussion
This microbiological investigation evaluated E. faecalis' susceptibility to natural cold-pressed CHO in two sections. The first section covered agar using the agar-well diffusion and the vapor diffusion method. The second section

covers extracted roots and includes direct contact and vaporization method. CHO exhibited a MIC of 22.5 µl/mL and an MBC of 45 µl/mL. This result is different from other studies(24, 25), This could be due to species of bacteria and sources, MIC testing methodology, broth type, or the source of oil [26-28].

In the agar well diffusion technique, CHO has been shown to inhibit E. faecalis growth at different concentrations. As CHO concentration increased from 12.5% to 100%, inhibitory zone sizes increased as shown in Fig. (1). This result agrees with [29] who studied antibacterial activity of chia seeds on periodontal pathogens, including Porphyromonas gingivalis, Fusobacterium nucleatum and Aggregatibacter actinomycetemcomitans and the results supported the hypothesis that higher concentrations of chia seeds oil would improve the inhibition of bacterial.

Statistically, compared to $Ca(OH)_2$, all CHO concentrations (except 25%) showed a highly significant difference, suggesting that they are more efficient against E. faecalis on agar plates. These results are inconsistent with a study conducted by [30], which stated that chia seeds oil did not exhibit inhibitory effects on E. faecalis. The difference may be attributed to variations in procedure, bacterial source, nature, and oil origin.

Calcium hydroxide ($Ca(OH)_2$) had minimal antibacterial effect in agar diffusion, and this result agrees with the findings obtained by [31], which stated that the combination of $Ca(OH)_2$ and distilled water exhibited negligible antimicrobial effects by using agar diffusion.

In the vapor diffusion technique, tricresol (TC) inhibited bacteria growth better than CHO at all concentrations, as demonstrated by the greater inhibition zone as shown in Fig. (2). The findings of this study are verified by the results reported by [18], which indicated that the vapor of TC effectively inhibited E. faecalis in vitro. Chia seed oil's vapor antibacterial action is attributed to the presence of phenolic compounds, which enhance its antioxidant and antimicrobial properties (32).

In the extracted roots section, the selected infection period was sufficient for the growth and development of the bacterial biofilm, as verified using SEM examination as shown in Fig. (3).

To the best of our knowledge, there is currently no published data or research on the application of this oil in the field of endodontics. Specifically, there is no prior documentation about its efficacy in eliminating E. faecalis bacteria in the root canals. Thus, this study is the first attempt to utilize cold-pressed chia seed oil as a medicinal substance within the root canal.

Regarding the duration of medication insertion into the canal, most research confirms that a period of one week is adequate to achieve optimal antibacterial effectiveness[33-35]. This study evaluated antibacterial action across two time periods since certain intra-canal medicaments work better after 48 hours. This study was conducted the antimicrobial evaluations for 3 and 7 days. In the direct contact method, the reduction of Bacterial count increased as the concentration of oil increased from 5% to 50%. After three days, there was a significant difference between 50% CHO concentration and Ca(OH)2. This suggests that 50% concentration of CHO have stronger antibacterial activity as an intra-canal medicament after 3 days as compared to $Ca(OH)_2$. However, there was no significant difference after 7 days between the 25% and 50% CHO and $Ca(OH)_2$. [36] demonstrated that after 1 week, $Ca(OH)_2$ had little effect on eradicating E. faecalis. The causes that could be responsible include dentin's buffering action, the arrangement of bacterial cells that populate the root canal walls, and $Ca(OH)_2$ low efficiency against facultative anaerobes.

In the vaporization method, TC demonstrated a considerably greater reduction in CFUs than CHO at all concentrations tested. The presence of formaldehyde may account for the positive outcomes observed in these compounds . Significant bacterial reduction was accomplished using a compound containing formaldehyde. This finding is in agreement with the study of [37], which found that formaldehyde has strong antibacterial effects that may be had for a long time, enabling its vapor to

reach even the most distant parts of the root canal.

The present investigation carried out on extracted roots demonstrates that the antibacterial effectiveness of CHO declines after a period of three to seven days when used as a root canal medicament. Several factors contribute to this occurrence, such as volatility, evaporation, deterioration, environmental conditions, absorption or binding, and oxidation of oil. These variables have the potential to decrease the concentration or structure of the oil's antibacterial component, thereby reducing its effectiveness against bacteria as time passes [38, 39].

The current study shows that chia seed oil has antibacterial action against E. faecalis biofilm. However, this study focused on the E. faecalis biofilm invasion of root canals. In contrast, clinical endodontic infections typically contain many microorganisms, thereby suggesting more investigation into mixed infections.

Conclusion

Within the limits of this study, chia seed oil, when used as an intra-canal medication, is able to inhibit E. faecalis biofilms from the root canal after 3 days at concentrations of 25% and 50% in the direct contact method and 100% in the vaporization method. This makes chia seed oil a viable alternative to intra-canal medication.

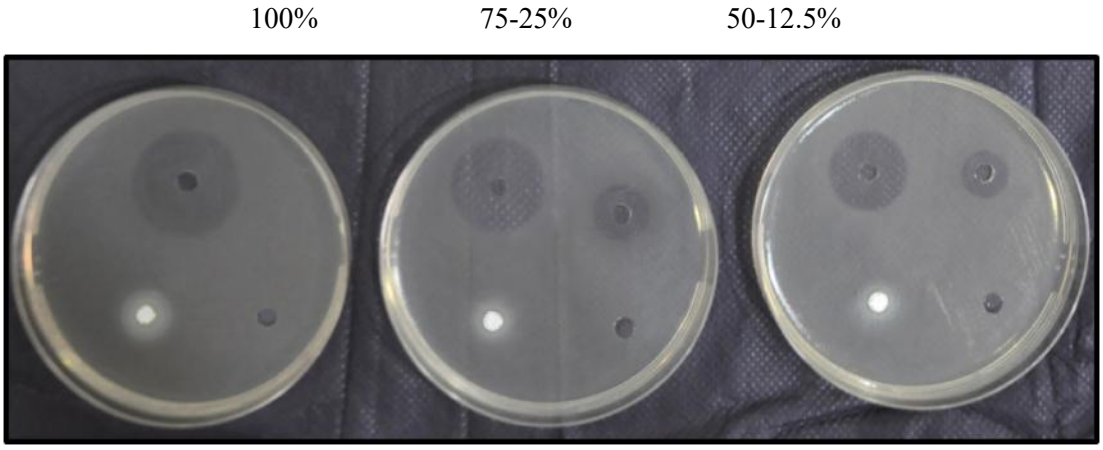

Fig. (1): E. faecalis susceptibility to different CHO concentrations using the agar well diffusion technique.

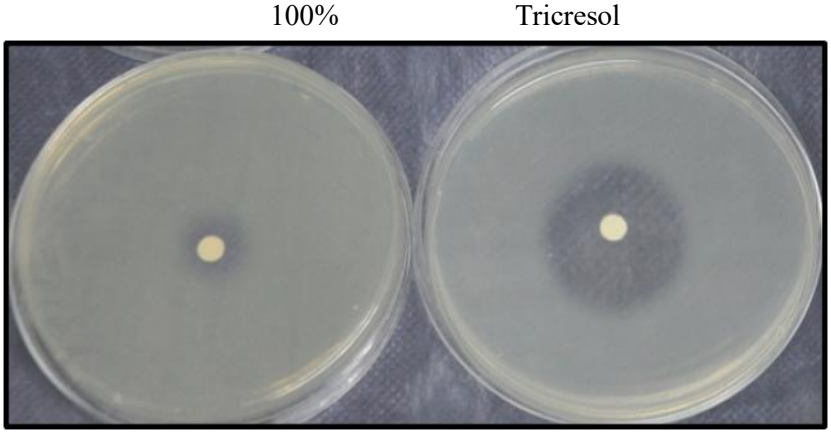

Fig. (2): The sensitivity of E. faecalis to vapor of 100% CHO using vapor diffusion technique.

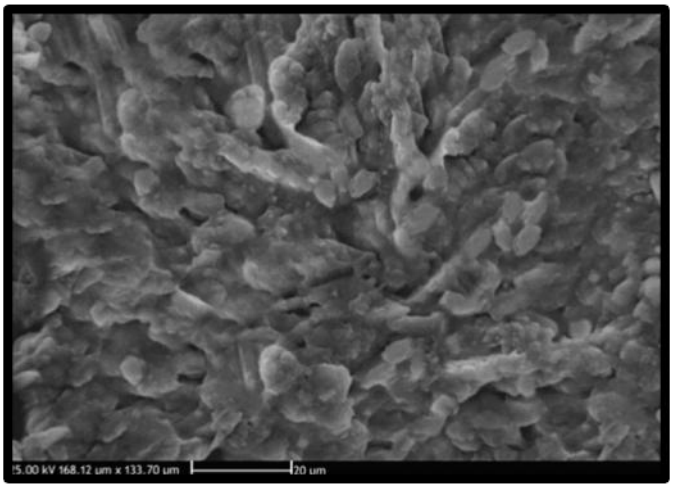

Fig. (3): Well-developed E. faecalis biofilm after 21 days.

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

Agents	N	Mean	Std. Deviation	Minimum	Maximum	p-value*
CHO 12.5%	10	10.07	1.83	7.06	13.08	0.000
25%	10	14.01[a]	1.82	11.01	17.02	
50%	10	23.43	3.43	19.12	27.74	
75%	10	28.56[b]	2.73	24.50	32.62	
100%	10	30.32[b]	3.24	25.21	35.43	
$Ca(OH)_2$ (+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 test.

Table 2: Mean, standard deviation, minimum, maximum of inhibition zone in millimeter of E. faecalis to different concentrations and different agents using the vapor diffusion technique.

Agents	N	Mean	Std. Deviation	Minimum	Maximum	p-value*
CHO 100%	10	11.93	1.86	8.91	14.95	0.000
75%	10	0.00[a]	0.00	0.00	0.00	
Tricresol (+control)	10	41.98	5.18	33.97	49.99	
10%DMSO(-control)	10	0.00[a]	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 3: Mean, standard deviation, minimum, maximum of colony forming unit of E. faecalis to different concentrations and different agents using the direct contact method.

Intervals	Groups	Mean	Std. Deviation	Minimum	Maximum	p-value*
Day 3	CHO 5%	$6.00^{a}\times10^{3}$	3.62	-1.00	11.00	0.000
	10%	27.90×10^{3}	2.64	22.00	32.00	
	25%	$50.40^{b}\times10^{3}$	3.17	46.00	57.00	
	50%	58.30×10^{3}	3.50	52.00	64.00	
	$Ca(OH)_2$ (+control)	$50.10^{b}\times10^{3}$	4.93	42.00	57.00	
	10%DMSO(-control)	$2.40^{a}\times10^{3}$	4.12	-3.00	7.00	
Day 7	CHO 5%	$1.50^{a}\times10^{3}$	4.81	-9.00	6.00	0.000
	10%	21.60×10^{3}	4.86	15.00	29.00	
	25%	$48.90^{b}\times10^{3}$	3.57	43.00	54.00	
	50%	$54.60^{b}\times10^{3}$	4.33	48.00	60.00	
	$Ca(OH)_2$ (+control)	$53.60^{b}\times10^{3}$	4.86	46.00	61.00	
	10%DMSO(-control)	$0.50^{a}\times10^{3}$	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, maximum of colony forming unit of E. faecalis to different concentrations and different agents using the vaporization method.

Intervals	Groups	Mean	Std. Deviation	Minimum	Maximum	p-value*
Day 3	CHO 25%	$0^{a,b}\times10^{3}$	3.46	2.00	8.00	0.000
	50%	$0^{a,c}\times10^{3}$	3.46	4.00	8.00	
	75%	$.40\times10^{3}$	2.72	5.00	23.00	
	100%	$.10\times10^{3}$	5.11	6.00	53.00	
	Tricresol (+control)	$.60\times10^{3}$	4.22	7.00	69.00	
	10%DMSO(-control)	$0^{b,c}\times10^{3}$	1.99	1.00	8.00	
Day 7	CHO 25%	$00^{a}\times10^{3}$	2.91	2.00	8.00	0.000
	50%	$30^{a}\times10^{3}$	4.37	4.00	11.00	
	75%	$.20\times10^{3}$	6.21	5.00	24.00	
	100%	$.00\times10^{3}$	7.69	3.00	39.00	
	Tricresol (+control)	$.90\times10^{3}$	2.02	1.00	67.00	
	10%DMSO (-control)	$0.50^{a}\times10^{3}$	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.

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