ORIGINAL RESEARCH

Effect of Herbal Extract of *Spilanthes acmella* and Cinnamon Oil on *Enterococcus faecalis* Biofilm Eradication: An *In Vitro* Study

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Abstract

Introduction: *Enterococcus faecalis* has a pathogenic role in failed endodontic treatments. The study aimed to assess the efficiency of *Spilanthes acmella* (SA) and cinnamon oil (CO) extract on *E. faecalis* biofilm eradication.

Materials and methods: The antibacterial efficacy of SA and CO against *E. faecalis* was assessed by the tests of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), and further, the interaction with agents was evaluated at different time intervals by a time-kill assay. The inhibition efficacy of both agents was determined by biofilm adhesion reduction crystal violet assay.

Results: The MIC of SA was 25 µg/mL, and for CO, it was 12.5 µg/mL. The time-kill assay revealed that antibacterial efficacy was identified till 36 hours by both the test materials. The mean biofilm reduction at 25 µg/mL of calcium hydroxide [$Ca(OH)_2$], SA, and CO was 1.53 ± 0.05, 1.83 ± 1.57, and 2.06 ± 0.05, respectively.

Conclusion: SA and CO demonstrated promising antibacterial efficacy against *E. faecalis* and CO presented significant eradication of biofilms compared to SA.

Keywords: Antibacterial, Antimicrobial efficacy, Biofilm, Calcium hydroxide, Cinnamon oil, Root canal therapy, Virulence factor.

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Introduction

Microorganisms present in the oral cavity and root canals cause periapical lesions and infections. Endodontic treatment aims to control and prevent these periapical lesions.[1] Several measures are being advocated to reduce microorganisms present in the endodontic spaces, including various biomechanical preparation techniques, irrigation methods, and intracanal medicaments.[2] Most common gram-positive bacteria present in periradicular infections are streptococci, lactobacilli, *E. faecalis*, staphylococci, and *Actinomyces* spp. There are gram-negative anaerobes like *Prevotella* spp and *Fusobacterium nucleatum*.[1] There are various virulence factors associated with such bacteria that can be sustained intraorally. *E. faecalis*, the dominant *enterococcus* species, is responsible for most human enterococcal diseases. It is the only bacterial species isolated from endodontically treated teeth; it has been documented to be associated with chronic pulpal infections. The virulence of *E. faecalis* might be because it resists intracanal medicaments and its survival as a sole microorganism without the support of any other bacteria.[2]

The gel or liquid forms of sodium hypochlorite (NaOCl) or chlorhexidine (CHX) have been used efficiently for eradicating *E. faecalis* during endodontic therapies. NaOCl possesses certain disadvantages, such as having an unpleasant taste, caustic actions, high toxicity, and instrument staining, whereas CHX demonstrates a few disadvantages like the inability to dissolve in necrotic material, dentin staining, and the inability to remove the smear layer.[3] Using ancient medicines and the development of medicinal plants has various benefits, like cost-effectiveness when treating numerous diseases.[4] Nowadays, in many developed realms, the use of medicinal herbs and their derivatives as medical drugs is over 25%. *E. faecalis* has shown resistance to the universally utilized intracanal medicament, that is, calcium hydroxide [$Ca(OH)_2$], which prompted researchers and clinicians to seek an alternative safe and reliable medicinal agent to counteract the deleterious effect of bacteria. Few evidence from ancient paramedical sciences have gained attention toward herbal products and extracts available freely and abundantly in nature for usage owing to their advantages.[5]

Spilanthes acmella (SA) has a major contribution to the medicinal compositions for short-term or long-lasting treatment of microbial infections, specifically, oral pathogens, dental caries, gingival and periodontal diseases, and reduction of plaque. It demonstrated vasorelaxant and antioxidant actions, as mentioned by Murr et al.[6] Some studies revealed that extracts of SA ethyl alcohol leave exerted noticeable analgesic properties. This might be because of the flavonoids present in the leaf extract that reduce prostaglandin E2 (PGE2), prostaglandin F2 alpha (PGF2), and prostaglandins that are involved in the pain perception mechanism.[7] Cinnamon is a very well-known traditional culinary herb with various medicinal uses. It

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has demonstrated antibacterial, antifungal, and anti-inflammatory properties, and it is also used as a vasorelaxant in the medical field. Hence, essential oils, ethanolic extracts, and pure compounds of cinnamon have been used in toothpaste, mouthrinse, and root canal irrigation solutions.[8]

The antimicrobial properties of extracts of cinnamon or cinnamaldehyde are responsible for the alterations in the arrangement of cell envelopes. Because of glucose inhibition followed by inhibition of energy generation by a microorganism, the membrane permeability of the cells might be affected.[9] Previous research has already proved the bacteriostatic effect of SA extract on *Porphyromonas gingivalis* and the bactericidal effect on *Streptococcus mutants* to prevent the progress in caries and periodontal disease.[10]

Considering the scientific and therapeutic benefits of these materials in other medicinal fraternity and dentistry, the abovementioned herbal extracts can be used in root canal therapies as an intracanal medicine. This study aimed to evaluate the efficiency of SA and CO extract on *E. faecalis* biofilm eradication.

Materials and Methods

The present experimental study was performed in the Department of Pedodontics and Preventive Dentistry, as well as the Department of Microbiology (Maharashtra Institute of Dental Sciences and Research (MIDSR), Latur, Maharashtra, India) after receiving approval from the Institutional Ethics Committee (IEC, reference number MUHS/Acad/EO/3065/2019 Date: 01/08/2019) (Table 1).

Bacterial Strain and Culture Conditions

E. faecalis—NCIM 5367-NCIM, NCL, Pune, India (corresponding to ATCC 29212) was used in this study. The lyophilized microorganisms were revived in sterile brain heart infusion (BHI) broth at 37°C. The broth was cultured on BHI agar plates at 37°C. Fresh BHI broth was used to dilute the isolated colonies. The turbidity of inoculi was fixed to 0.5 McFarland standards on the opacity scale (corresponding to 1.5 × 108 CFU/mL).[11]

Preparation of Ethanolic Extract of *Spilanthes acmella*

Spilanthes acmella extract (SIGMU Group, Astrology Bhavan, Bhubaneswar, Odisha, India) was extracted using the reflux method and concentrated using a rotary evaporator. A total of 10 gm of SA powder was weighed and placed into the flask. Around 100 mL of ethanol solvent with a concentration of 99% (Sigma-Aldrich) was added into the flask, and this flask was placed on a rotary shaker overnight. The extract obtained thus was filtered by using Waterman filter paper. The ethanolic extract was further evaporated using a rotary evaporator and sterilized in the filter for use in the study.[12]

Preparation of Stock Solution

The standardized extracts were converted into a concentration of 100 µg/mL as stock solution. The pure extract of 100% cinnamon bark oil (*Cinnamomum zeylanicum*) (100% pure bark essential oil, Mesmara™ Hyderabad, India) was purchased and used in the study after the stock solution was prepared. Preparation of stock solution was carried out by dissolving it in 10% dimethyl sulfoxide (DMSO) and vortexed (M. C. Dalal and Co. Chennai, India) and sterilized by filtration through 0.45 µm Millipore filters.

A recently prepared ethanolic extract of SA was separately mixed into 10 mL DMSO and vortexed. The homogeneous solution was obtained by these two test materials having a concentration of 100 µg/mL. To prevent dehydration and oxidation of the components till their further usage, the stock solutions were kept in light-proof containers.[13]

Calcium hydroxide (Neelkanth Health Care (P) Ltd. Jodhpur, India) is used as the positive control, and normal saline is used as the negative control in the study.

Table 1: Test materials used in the study

Group	*Test materials*
Group I: Positive control	$Ca(OH)_2$
Group II: Negative control	Normal saline
Group III: Test material—I	SA —pure extract (SA)
Group IV: Test material—II	CO—pure extract (CO)

Quantification of Minimum Inhibitory Concentration of Test Materials

Minimum inhibitory concentration (MIC) was accomplished using the serial dilution method as suggested by Clinical and Laboratory Standard Institute (CLSI) guidelines. Using DMSO, the stock solution of SA and CO was prepared. Twofold serial dilutions of SA and CO using double dilution method in BHI broth from 100 µg/mL to 0.4 µg/mL respectively. Bacterial inoculum was added to a 96-well flat-bottom microtiter plate at a concentration of 10^6 cells/mL. The microtiter plate was incubated at 37°C for 48 hours in an anaerobic environment. Following the incubation, the growth of the bacteria was evaluated visually and confirmed through optical densitometry (OD_{600}: 0.6–0.7). The MIC values for both test materials were noted.[14]

Quantification of Minimum Bactericidal Concentration of Test Materials

The minimum bactericidal concentration (MBC) was evaluated through the well diffusion method. The aliquot of 5 µL showing no growth was spread on the BHI agar plate, then incubated for 48 hours at 37°C anaerobically. After this period, the growth of bacterial colonies was observed, and the extracts were classified as bacteriostatic or bactericidal. DMSO was considered a negative control in the study at 3% (v/v).[14]

Time-kill Assay

Considering the CLSI guidelines, the broth microdilution method was used to conduct the time-kill assay. Test tubes containing the bacterial culture along with BHI broth were incubated with MIC and MBC values of the SA extract and CO for 0–48 hours at 37°C in an anaerobic environment. The plates were cultured by plating 10-fold dilutions of the extracts on the BHI agar plate. After incubation at 37°C for 48 hours, the CFU relating to set time intervals were obtained. Following the incubation at 37°C for 24 hours, colonies were reckoned, and the number of sustainable cells was considered as CFU/mL.[11]

Bacterial Adhesion Assay

A bacterial adhesion assay was carried out to evaluate the efficacy of bacteria in forming biofilms. Bacteria were grown overnight anaerobically in BHI broth.

Around 200 µL of bacterial broth were mixed with MIC and MBC concentrations of SA extract and CO in a 96-well flat-bottom microtiter plate. The growth medium without any test material was taken as a negative control. These plates were later incubated in an anaerobic environment (menadione, thioglycolate agar with hemin and vitamin K, and tryptic soy agar were incubated at 37°C along with

blood agar in an anaerobic incubator. The incubation was carried out for the duration of 48–72 hours in an environment with 80% N_2 and 10% CO_2) to facilitate *E. faecalis* growth at 37°C for 12 hours. The unbound bacteria were removed with phosphate-buffered saline (Dulbecco's PBS) by washing the decanted culture supernatant, which had a pH of 7.2. For the fixation of the bound bacteria, methanol was used for 15 minutes and then air-dried. Crystal violet (0.1% w/v) was used for 5 minutes to stain the bounded cells. Unbound dye was washed away with PBS. The bound dye was dissolved in 95% ethanol (v/v) following air-drying the microtiter plate. Abs_{570} (absorbance at 570 nm) was used to record the results of the test.[15–18]

Biofilm Adhesion Reduction–Crystal Violet Assay

E. faecalis bacteria developed in BHI broth was poured into the wells of a microtiter plate (200 µL/well). Then, the patient was kept for incubation at 37°C for 72 hours in an anaerobic condition. Every single well was then added with MIC and MBC concentrations of SA and CO and kept for incubation for 24 hours. The bounded bacteria were fixed with methanol. Viable cells were stained using crystal violet (0.1% w/v) for 5 minutes, followed by washing with PBS. Quenching of the reaction mixture was done using 95% ethanol. The results were recorded at 572 nm using a microplate reader. Here, only broth was kept as a negative control and blank.

Statistical Analysis

The data obtained was entered into Microsoft Excel 2013. Statistical Package for the Social Sciences (SPSS) version 24 (IBM, United States of America) was used to analyze all the statistical analyses. Descriptive numbers were obtained as mean, standard deviation (SD), and standard error. For the comparison of all the test groups, analysis of variance (ANOVA) was used for intergroup comparison, and an unpaired *t*-test was used for intragroup comparison. The *p*-value ≤ 0.05 was stated as statistically significant.

Results

Quantification of Minimum Inhibitory Concentration of Test Materials

The antibiotic activity of SA and CO for *E. faecalis* was evaluated using the standard broth dilution method. SA and CO were observed to have significant inhibition properties, as mentioned in Table 2.

Table 2: Intergroup and intragroup comparison in MIC between calcium hydroxide, CO, and SA at different concentrations

Concentration	*Groups*	*Groups*	*Mean difference*	*p-value*	*F-value*	*p-value*
100 µg/mL	$Ca(OH)_2$	SA	0.13	0.000 (HS)	155.27	<0.001 (HS)
		CO	–0.02	0.147 (NS)		
	SA	$Ca(OH)_2$	–0.13	0.000 (HS)		
		CO	–0.15	0.000 (HS)		
	CO	$Ca(OH)_2$	0.02	0.147 (NS)		
		SA	0.015	0.000 (HS)		
50 µg/mL	$Ca(OH)_2$	SA	–0.010	<0.001 (HS)	385.85	<0.001 (HS)
		CO	–0.20	<0.001 (HS)		
	SA	$Ca(OH)_2$	0.09	<0.001 (HS)		
		CO	–0.10	<0.001 (HS)		
	CO	$Ca(OH)_2$	0.20	<0.001 (HS)		
		SA	0.10	<0.001 (HS)		
25 µg/mL	$Ca(OH)_2$	SA	–0.21	<0.001 (HS)	603.00	<0.001 (HS)
		CO	–0.27	<0.001 (HS)		
	SA	$Ca(OH)_2$	0.21	<0.001 (HS)		
		CO	–0.06	0.001 (HS)		
	CO	$Ca(OH)_2$	0.27	<0.001 (HS)		
		SA	0.06	0.001 (HS)		
12.5 µg/mL	$Ca(OH)_2$	SA	0.036	0.023 (S)	512.35	<0.001 (HS)
		CO	–0.043	0.011 (S)		
	SA	$Ca(OH)_2$	–0.036	0.023 (S)		
		CO	–0.080	<0.001 (HS)		
	CO	$Ca(OH)_2$	0.043	0.011 (S)		
		SA	0.08	<0.001 (HS)		
6.25 µg/mL	$Ca(OH)_2$	SA	0.01	0.551 (NS)	0.800	0.492 (NS)
		CO	0.01	0.551 (NS)		
	SA	$Ca(OH)_2$	–0.01	0.551 (NS)		
		CO	0.00	1.000 (NS)		
	CO	$Ca(OH)_2$	–0.07	0.551 (NS)		
		SA	0.00	1.000 (NS)		

Contd…

Contd…

Concentration	*Groups*	*Groups*	*Mean difference*	*p-value*	*F-value*	*p-value*
3.125 µg/mL	$Ca(OH)_2$	SA	0.01	0.551 (NS)	0.800	0.492 (NS)
		CO	0.01	0.551 (NS)		
	SA	$Ca(OH)_2$	–0.01	0.551 (NS)		
		CO	0.00	1.000 (NS)		
	CO	$Ca(OH)_2$	–0.01	0.551 (NS)		
		SA	0.00	1.000 (NS)		
1.60 µg/mL	$Ca(OH)_2$	SA	–0.01	0.835 (NS)	0.296	0.754 (NS)
		CO	0.00	0.988 (NS)		
	SA	$Ca(OH)_2$	0.00	0.835 (NS)		
		CO	0.00	0.758 (NS)		
	CO	$Ca(OH)_2$	–0.00	0.988 (NS)		
		SA	–0.00	0.758 (NS)		
0.80 µg/mL	$Ca(OH)_2$	SA	–0.00	0.768 (NS)	0.333	0.729 (NS)
		CO	0.00	1.000 (NS)		
	SA	$Ca(OH)_2$	0.00	0.768 (NS)		
		CO	0.00	0.768 (NS)		
	CO	$Ca(OH)_2$	0.00	1.000 (NS)		
		SA	–0.00	0.768 (NS)		
0.40 µg/mL	$Ca(OH)_2$	SA	–0.00	0.768 (NS)	0.333	0.729 (NS)
		CO	0.00	1.000 (NS)		
	SA	$Ca(OH)_2$	0.00	0.768 (NS)		
		CO	0.00	0.768 (NS)		
	CO	$Ca(OH)_2$	0.00	1.000 (NS)		
		SA	–0.00	0.768 (NS)		

$p \leq 0.001$ (HS, highly significant); $p \leq 0.05$ (S, significant); $p \geq 0.05$ (NS, nonsignificant)

At the concentration of 25 µg/mL of $Ca(OH)_2$, SA, and CO, the mean optical density was found to be 0.13 ± 0.01, 0.34 ± 0.01, and 0.40 ± 0.01, respectively. $Ca(OH)_2$ showed less bacterial growth than SA and CO. These differences were found to be statistically highly significant ($p \leq 0.001$). The intragroup comparison showed a statistically highly significant difference in the mean bacterial growth at the concentration of 25 µg/mL between all three test materials ($p \leq 0.001$).

At the concentration of 12.5 µg/mL of $Ca(OH)_2$, SA, and CO, the mean optical density was found to be 0.05 ± 0.01, 0.02 ± 0.01, and 0.10 ± 0.01, respectively. It was observed that SA showed the least bacterial growth. $Ca(OH)_2$ showed more growth of bacteria than SA but was lower than CO. These differences were highly significant ($p \leq 0.001$). The intragroup comparisons revealed a significant difference in the growth of *E. faecalis* at the concentration of 12.5 µg/mL between all three test groups ($p \leq 0.001$).

Spilanthes acmella demonstrated antibacterial activity against *E. faecalis* at the concentration of 25 µg/mL (OD_{600} = 0.34 ± 0.01), which will be considered its MIC value. Similarly, the MIC value for CO was detected at the concentration of 12.5 µg/mL (OD_{600} = 0.10 ± 0.01). Figure 1 represents the MIC of SA, and the MIC of CO is presented in Figure 2. Table 2 represents a comparison in MIC between $Ca(OH)_2$, CO, and SA at different concentrations.

Quantification of MBC of Test Materials Using Well Diffusion Method

The MBC of the test material was evaluated in the form of inhibition zones appearing on the culture media as a clear halo. The mean value obtained for the zone of inhibition in $Ca(OH)_2$ and SA was 19.50 ± 6.36 and 19.50 ± 9.19, respectively, and in CO, it was observed to be 16.00 ± 8.48. The zone of inhibition of *E. faecalis* was found to be more at 25 than 12.5 µg/mL. One sample *t*-test was applied to evaluate the significant difference in the $Ca(OH)_2$, SA, and CO in the inhibition zone against *E. faecalis*. It was detected that there was no statistically significant difference in the zone of inhibition against *E. faecalis* between $Ca(OH)_2$, SA, and CO, respectively ($p \geq 0.05$). Table 3 and Figure 3 represent the MBC of all test materials against *E. faecalis*.

Time-kill Assay

The time-kill assay was used to evaluate the antibacterial activity of SA and CO against *E. faecalis*. For conducting the test, bacteria were exposed to MIC and MBC concentrations of SA and CO at different time intervals. At MIC values (25 and 12.5 µg/mL), bacterial count was reduced gradually till 36 hours for the test materials, but after 36 hours, it was observed that there was an increase in the CFU count, which means their antibacterial properties were compromised after 36 hours. There was a rapid increase in the CFU count at 48 hours (10^7 CFU/mL for SA and 10^6 CFU/mL for CO), which makes it evident that there is a reduction in the antibacterial properties. Tables 4 and 5 represent the viable cell count at different time intervals at the MIC concentration of test materials.

Biofilm Adhesion Reduction–Crystal Violet Assay

The antiadhesion effect of SA and CO against *E. faecalis* was evaluated using microtiter plate bacterial adhesion reduction—crystal violet assay. This assay revealed that MIC and MBC concentrations of SA and CO reduced a significant number of attached *E. faecalis* cells. The mean biofilm reduction at 25 µg/mL of $Ca(OH)_2$, SA, and CO were observed to be 1.53 ± 0.05, 1.83 ± 1.57, and 2.06 ± 0.05, respectively. Biofilm reduction was observed to be greater in CO

than in $Ca(OH)_2$ and SA. A high statistically significant difference was observed in biofilm reduction at 25 µg/mL between$Ca(OH)_2$, SA, and CO ($p \leq 0.001$).

At 12.5 µg/mL $Ca(OH)_2$, SA, and CO were observed to show biofilm reduction at 1.93 ± 0.057, 1.93 ± 0.057, and 2.36 ± 0.057, respectively. It was observed to be similar in $Ca(OH)_2$ and SA. CO showed a higher assay than SA and $Ca(OH)_2$. A high statistically significant difference was observed in biofilm reduction at 12.5 µg/mL between $Ca(OH)_2$, SA, and CO ($p \leq 0.001$). Table 6 and Figures 4 and 5 represent biofilm adhesion-reduction crystal violet assay for all the test materials.

Discussion

On investigation, microbiota associated with endodontic infections has many bacteria such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Streptococcus* species, *Staphylococcus* species *E. faecalis*. However, it was

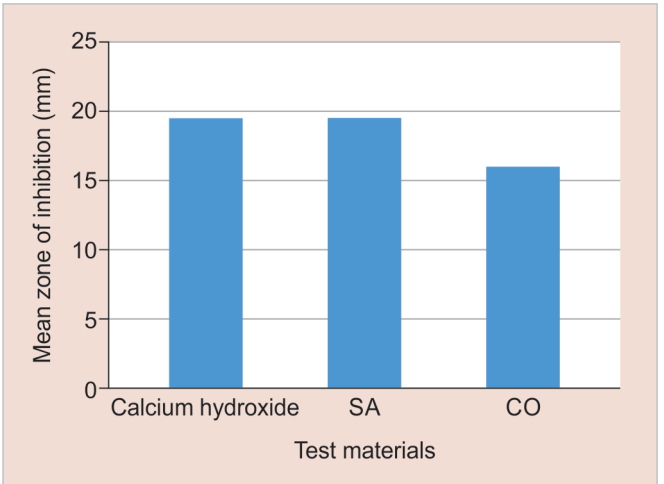

Fig. 3: Zone of inhibition of the test materials

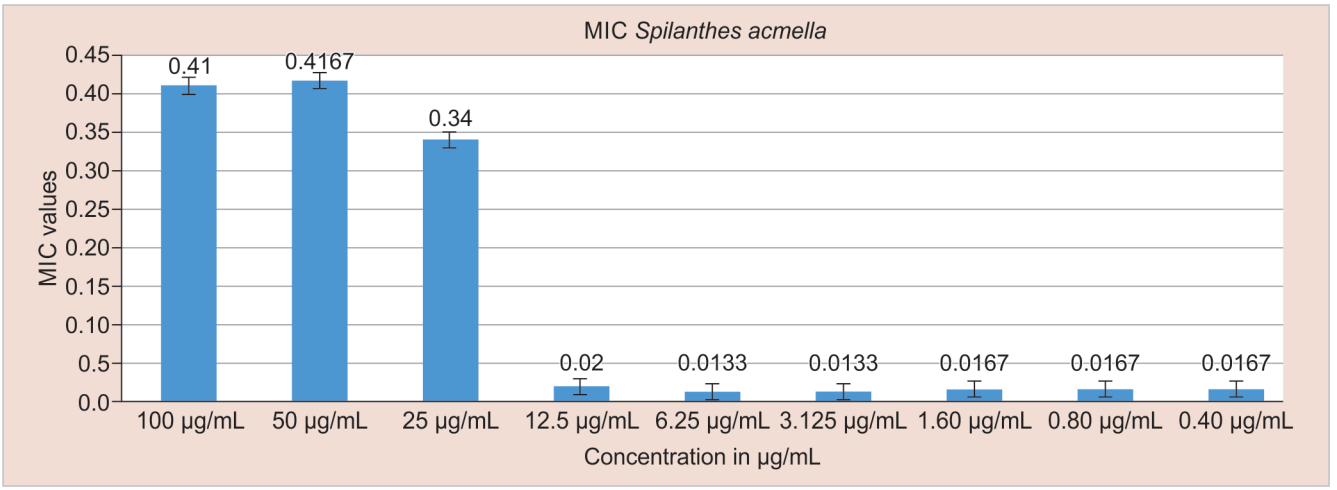

Fig. 1: *E. faecalis* inhibition by SA

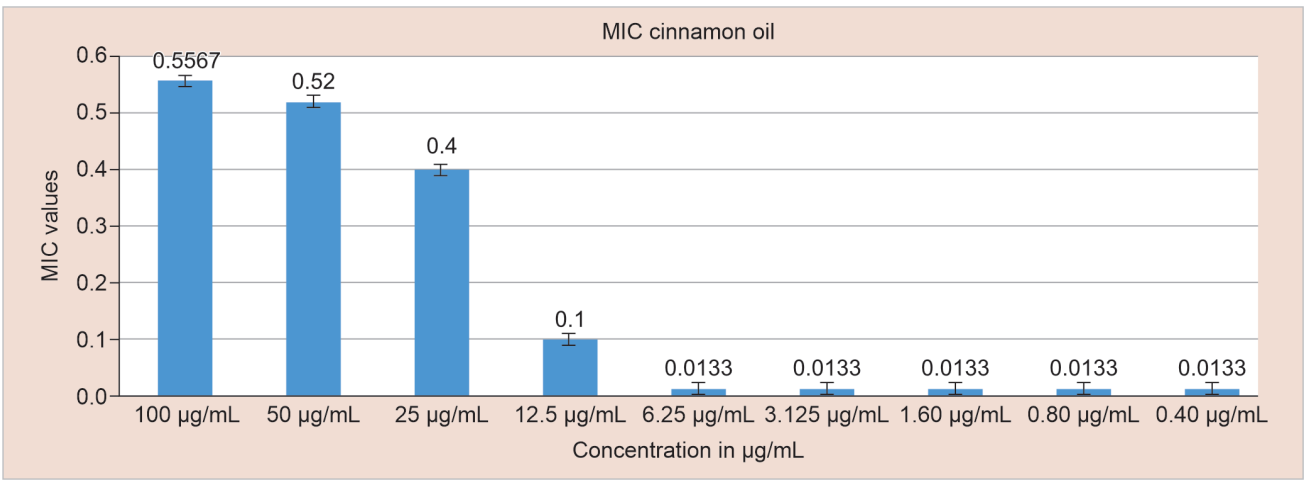

Fig. 2: *E. faecalis* inhibition by CO

Table 3: One sample "*t*-test" comparing zone of inhibition between 25 and 12.5 µg/mL

Zone of inhibition against E. faecalis	*Mean*	*SD*	*t-value*	*p-value*
$Ca(OH)_2$	19.5000	6.36396	4.333	0.144 (NS)
SA	19.5000	9.19239	3.000	0.205 (NS)
CO	16.0000	8.48528	2.667	0.228 (NS)

$p \leq 0.001$ (HS, highly significant); $p \leq 0.05$ (S, significant); $p \geq 0.05$ (NS, nonsignificant); SD, standard deviation; *t*-value, the ratio of the difference between the mean of sample sets

Table 4: CFU/mL at the concentration of 25 µg/mL

	Time interval in hours MIC					*Time interval in hours MBC*				
Test materials	0	12	24	36	48	0	12	24	36	48
$Ca(OH)_2$	00	00	10^5	–	–	–	–	10^5	–	–
SA	00	10^6	50,003	70,385	10^7	00	10^4	72,450	86,310	10^5
CO	00	10^5	50,203	73,564	10^8	00	10^4	71,402	82,031	10^6

Table 5: CFU/mL at the concentration of 12.5 µg/mL

	Time interval in hours MIC					*Time interval in hours MBC*				
Test materials	0	12	24	36	48	0	12	24	36	48
$Ca(OH)_2$	00	00	10^5	–	–	–	–	10^6	–	–
SA	00	10^4	59,343	75,385	10^4	00	10^4	70,252	80,310	10^5
CO	00	10^5	65,203	82,564	10^4	00	10^4	71,452	85,031	10^6

Table 6: Intergroup and intragroup comparison of biofilm reduction between calcium hydroxide, CO, and SA at different concentrations

Concentration	*Group-wise comparisons of the test materials*		*Mean difference*	*p-value*	*F ANOVA*	*p-value*
100 µg/mL	$Ca(OH)_2$	SA	−0.13	0.006 (S)	13.00	0.007 (S)
		CO	−0.03	0.483 (NS)		
	SA	$Ca(OH)_2$	−0.13	0.006 (S)		
		CO	0.10	0.024 (S)		
	CO	$Ca(OH)_2$	0.03	0.483 (NS)		
		SA	−0.10	0.024 (S)		
50 µg/mL	$Ca(OH)_2$	SA	−0.13	0.006 (S)	76.000	≤0.001 (HS)
		CO	−0.33	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.13	0.006 (S)		
		CO	−0.20	≤0.001 (HS)		
	CO	$Ca(OH)_2$	0.33	≤0.001 (HS)		
		SA	0.20	≤0.001 (HS)		
25 µg/mL	$Ca(OH)_2$	SA	−0.30	0.002 (S)	64.333	≤0.001 (HS)
		CO	−0.53	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.30	0.00 (HS)		
		CO	−0.23	0.006 (S)		
	CO	$Ca(OH)_2$	0.53	≤0.001 (HS)		
		SA	0.23	0.006 (S)		
12.5 µg/mL	$Ca(OH)_2$	SA	0.00	1.000 (NS)	56.333	≤0.001 (HS)
		CO	−0.43	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.00	1.000 (NS)		
		CO	−0.43	≤0.001 (HS)		
	CO	$Ca(OH)_2$	0.43	≤0.001 (HS)		
		SA	0.43	≤0.001 (HS)		
6.25 µg/mL	$Ca(OH)_2$	SA	−0.43	≤0.001 (HS)	122.33	≤0.001 (HS)
		CO	−0.73	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.43	<0.001 (HS)		
		CO	−0.30	0.002 (S)		
	CO	$Ca(OH)_2$	0.73	≤0.001 (HS)		
		SA	0.30	0.002 (HS)		
3.125 µg/mL	$Ca(OH)_2$	SA	−0.30	0.002 (S)	174.33	≤0.001 (HS)
		CO	−0.87	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.30	0.002 (S)		
		CO	−0.57	≤0.001 (HS)		
	CO	$Ca(OH)_2$	0.87	≤0.001 (HS)		
		SA	0.57	≤0.001 (HS)		

Contd…

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Concentration	*Group-wise comparisons of the test materials*		*Mean difference*	*p-value*	*F ANOVA*	*p-value*
1.60 µg/mL	$Ca(OH)_2$	SA	−0.54	≤0.001 (HS)	405.33	≤0.001 (HS)
		CO	−1.33	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.54	≤0.001 (HS)		
		CO	−0.80	≤0.001 (HS)		
	CO	$Ca(OH)_2$	1.33	≤0.001 (HS)		
		SA	0.80	≤0.001 (HS)		
0.80 µg/mL	$Ca(OH)_2$	SA	−0.30	0.002 (S)	160.33	≤0.001 (HS)
		CO	−0.83	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.30	0.002 (S)		
		CO	−0.53	≤0.001 (HS)		
	CO	$Ca(OH)_2$	0.83	≤0.001 (HS)		
		SA	0.53	≤0.001 (HS)		
0.40 µg/mL	$Ca(OH)_2$	SA	−0.46	≤0.001 (HS)	114.33	≤0.001 (HS)
		CO	−0.70	≤0.001 (HS)		
	SA	$Ca(OH)_2$	0.47	≤0.001 (HS)		
		CO	−0.23	0.006 (S)		
	CO	$Ca(OH)_2$	0.70	≤0.001 (HS)		
		SA	0.23	0.006 (S)		

$p \leq 0.001$ (HS, highly significant); $p \leq 0.05$ (S, significant); $p \geq 0.05$ (NS, nonsignificant)

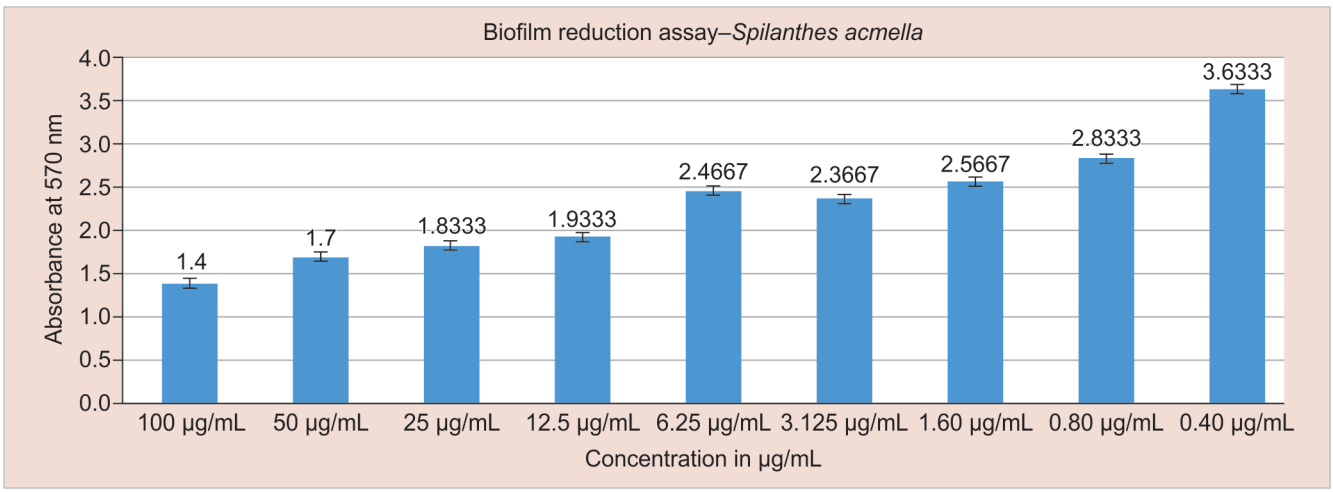

Fig. 4: Biofilm eradication by SA

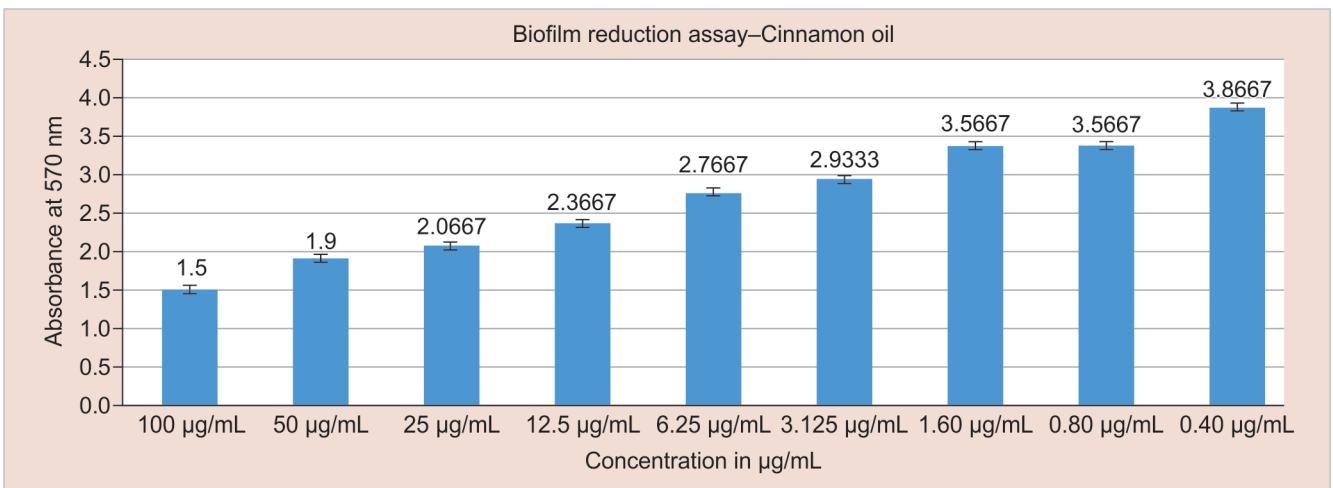

Fig. 5: Biofilm eradication by CO

stated that *E. faecalis* is a more commonly found bacteria in endodontic canals,[19] and hence it is used in this study. *E. faecalis* is a saprophytic organism of enteric flora. It is present either as a single bacterium or as a major bacterium in the oral microflora.[3] The virulence feature of *E. faecalis* consists of cytolysin, aggregation substance, and lipoteichoic acid. Lymphocytic actions that potentially contribute to root canal treatment failure are supported by these.[20] In the mutilated tooth, various bacteria are responsible for the infection. These bacteria form colonies inside the root canals as well as in the dentin tubules. These colonies are present either as surface aggregates (in nutritious surroundings) or adherent colonies that contain nonliving materials and islands of viable microorganisms that damage the dentin.[21] Though many studies are following the pathogenicity of *E. faecalis* in pulpal diseases, some experimental researchers oppose its importance as the most dominant bacteria in the etiology of pulpal diseases, as enterococci are not only common colonizers present in the oral microflora.[21,22]

Calcium hydroxide was selected as a positive control in this experiment since it is the gold standard for intracanal medicament.[23,24] The discharge of hydroxyl ions is the primary reason for the antimicrobial properties of $Ca(OH)_2$. Various experimental researches have revealed that after the $Ca(OH)_2$ application inside the root canal, the hydroxyl ions are released through the dentin on the exterior side of the root.[23,25] Outcomes of the research are in agreement with the previous research,[26–29] that reported the least antibacterial efficacy of $Ca(OH)_2$ contrary to *E. faecalis*.[23] However, a study conducted by Baik et al.[30] stated that $Ca(OH)_2$ can be used as a detoxifying agent for lipoteichoic acid, an important virulence factor of many bacteria. These results demonstrate a decrease in the inflammatory response to *E. faecalis*.[23] This variance might be because $Ca(OH)_2$ was used against planktonic bacteria in their study, whereas the authors used it against the biofilm structure in this study.[23]

Since herbal products have various antibacterial agents, they are integrated into commercial products used for maintaining oral hygiene. SA has been considered to have a great contribution to traditional treatment for tooth, gingiva, and throat infectious diseases. Pungent isobutylamide "spilanthol" is the major constituent of SA. It is also known for its antibacterial efficacy and alkamide (immune stimulator). Previous research has discussed its antibacterial efficacy against common root canal bacteria *viz E. faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus mutans*, *Candida albicans*, *Klebsiella pneumoniae*, *Staphylococcus albus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.[31] Thus, in the pursuit of discovering an alternative to frequently used intracanal medicament, a flower head extract of SA was selected to determine its antibacterial efficacy against the commonly found pathogen *E. faecalis*.

The mechanism of action for essential oils is very complex. It relies on the composition of bioactive materials.[21] Essential oil tends to reach extreme concentrations in the biofilms of bacteria and thus can proficiently eliminate *E. faecalis*, despite being organized in biofilms.[21,32,33] Cinnamon, a common domestic spice, contributed traditionally as remedies for many intraoral diseases like pain in the decayed teeth and gingival inflammation because of its antimicrobial properties. Extracts for this study were obtained from the bark of the cinnamon.

In the current study, the antibacterial efficacy of SA and CO was assessed and compared against *E. faecalis* in terms of MIC and MBC. Further, it was confirmed by evaluating the antibacterial efficacy at different time intervals at MIC and MBC concentrations using a time-kill assay. Their potential to inhibit bacterial growth and biofilm reduction was evaluated by biofilm adhesion reduction–crystal violet assay. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested.[34] MBC is the lowest concentration of antibacterial agent required to kill a particular bacterium.[35] MBC values were determined by the agar well diffusion method by measuring the width of the inhibition zone on BHI Agar and ranked arbitrarily depending on the proportional distribution of the dataset.[36–38] The result obtained as inhibition and assassination of bacteria by CO was probably due to the major compound in the CO, which is cinnamaldehyde, followed by α-Pinene and cis-beta Ocimene.[39] Cinnamaldehyde develops high antimicrobial properties because of interference caused by an electronegative molecule in the cellular biological process, mostly proteins and nucleic acid. El Atki et al., in their study, mentioned that CO inhibits bacterial growth by inhibiting cell division, ATPase, and altering the lipid profile[40] The antibacterial activity of different concentrations of SA extract might be due to the presence of amide spilanthol and alkamides. Krishnaswami et al. also reported the presence of nonvolatile sesquiterpenoids and saponins, which may be responsible for the antibacterial and antifungal activity of SA.[12,41]

Adhesion is an essential property for saving the energy of bacteria and protecting their environmental niche.[11] This property of the bacterium plays a substantial role in its development and colonization, which leads to inflammation of the tissues. The current experimental research revealed that thin filaments of $Ca(OH)_2$ are not very efficient in eradicating *E. faecalis*. These results were in the consensus with those suggested by other researchers. Estrela et al. stated that $Ca(OH)_2$ had not presented any antibacterial activity against *E. faecalis*.[42] Behnen et al. suggested that thin $Ca(OH)_2$ medicament was least efficient in the eradication of *E. faecalis* from root canals.[43] The probable reason that *E. faecalis* might survive intracanal treatment of $Ca(OH)_2$ could be either because *E. faecalis* unreceptively maintains pH homeostasis by cytoplasm's buffering capacity and by the proton pump or due to the buffering ability of dentin, pH of calcium hydroxide cannot be maintained constantly at 11.5.[12] Sathyaprasad et al. observed the similar results, where they observed that calcium hydroxide eliminated *E. faecalis* but at higher concentration and SA has got bactericidal properties and is effective in eliminating the *E. faecalis*.[12]

Microbial-related factors in *E. faecalis* infection, like proton pump activity, adherence to collagen, or inter- and intraspecies gene transfer, increase the challenges of failed endodontic therapy. Hence, the purpose of selecting this study is to search for ecofriendly and biocompatible intracanal medicament. Considering the facts, as the root canal procedure is persistently progressing, it can be asserted that natural products can be considered as an efficient alternative medicament against *E. faecalis* to be used in endodontic procedures.[1] Considering that the colonization of the bacteria present in the pulp takes place by various species of bacteria, more research is required to examine the efficacy of the essential oils and herbal medicaments as this is an *in vitro* study.

Limitations of the Study

This experimental research was an *in vitro* study evaluating the antibacterial efficacy of the test materials against *E. faecalis*. Though

the test materials have shown better antimicrobial effects than conventional ones, the study has a few limitations.

- It is an *in vitro* study; it can't be replicated clinically.
- Only one bacterial strain was selected for the study.
- Antibacterial property of the test material was compromised after 36 hours.

Conclusion

Considering the abovementioned limitations of this microbial study, based on the employed methodology and the results obtained in the study, it was concluded that:

- Both SA and CO have demonstrated promising antibacterial efficacy against *E. faecalis*.
- Cinnamon oil has presented significant biofilm eradication against the bacteria in comparison with SA and $Ca(OH)_2$.

Ethical Approval and Consent to Participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. The study was started after gaining approval from the Institutional Ethics Committee. This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of Data and Material

The data which supports the conclusion of the article are included in this article.

Author Contributions

- Study conception and design: PTD, SAB.
- Data collection: SAB, PTD.
- Conducting the experiment: SAB.
- Analysis and interpretation of results: PTD, SAB, YJK, MVD.
- Draft manuscript preparation: SAB, YJK, SBK.

All the authors reviewed the results and approved the final version of the manuscript. The authors confirm sole responsibility for the following—study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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