Original Article

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Dentistry Section

Antimicrobial Efficacy of Calcium Hydroxide with Different Herbal Additives: An In-vitro Study

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ABSTRACT

Introduction: Microorganisms such as *Enterococcus faecalis, Enterobacteriaceae*, and *Candida albicans* are associated with persisting infections and can cause endodontic failure. The addition of herbal additives such as Triphala, Clove, and Activated charcoal to Calcium hydroxide enhances the microbial efficacy within the root canal.

Aim: To evaluate and compare the anti-microbial efficacy of calcium hydroxide with various herbal additives against *E. faecalis* and *C. albicans* using Agar Diffusion Test (ADT) and Direct Contact Test (DCT).

Materials and Methods: The present in-vitro study was conducted in Bangalore, Karnataka, between July 2023 and September 2023. Ethical clearance was obtained from the institution. Sabouraud Dextrose Agar (SDA) and Mueller-Hinton Agar were prepared and used to culture *C. albicans* and *E. faecalis*, respectively. In each plate, four wells were filled with groups (n=10): Group-I: Nanocalcium hydroxide (NCH)+sterile distilled water, Group-II: NCH+Nanoparticle Clove, Group-III: NCH+Nanoparticle Triphala, Group-IV: NCH+nanoparticle activated charcoal. The plates were incubated at 37°C, and zones of inhibition around the wells were measured on the first, third, and seventh day. DCT was evaluated in Colony Forming Units (CFUs)/ml by adding each bacterial suspension to four groups and inoculating on blood agar plates. One-way ANOVA test followed by Tukey's post-hoc analysis was used to compare the mean Zone Of Inhibition (ZOI). Kruskal-Wallis Test followed by Mann-Whitney post-hoc test was used to compare the mean CFUs.

Results: One-way ANOVA test showed that there was a significant difference in the mean ZOI between the four groups at p=0.005. The maximum mean ZOI against *C. albicans* was achieved by the combination of calcium hydroxide and clove with an average mean of 22.20±1.92 mm, 20±1.87 mm, and 17.20±2.28 mm on the 1st, 3rd, and 7th day, respectively. Calcium hydroxide with distilled water combination showed the highest mean ZOI against *E. faecalis* with an average mean of 18.20±0.84 mm on day 1, 17±1 mm on the 3rd and 7th day. The mean CFUs of *E. faecalis* and *C. albicans* showed a significant difference between the four groups at p<0.001.

Conclusion: The addition of herbals to Calcium hydroxide increased anti-microbial activity against *Candida albicans* and *Enterococcus faecalis*.

Keywords: Activated charcoal, *Candida albicans*, *Enterococcus faecalis*, Triphala

INTRODUCTION

Microorganisms play a vital role in the aetiology of pulp and periapical diseases [1]. An important and fundamental goal of root canal treatment is to eliminate bacteria from the root canal to prevent re-infection [2]. Although endodontic infections are reduced by the chemo-mechanical preparation of the root canal, microorganisms can still persist in the complex root canal system [3].

Certain microbial species such as *Enterococcus faecalis (E. faecalis), Enterobacteriaceae,* and *Candida albicans (C. albicans)* are associated with persisting infections and can cause endodontic failure. Researchers have found *C. albicans* in 18% of cases, always associated with other bacteria, and *E. faecalis* in 50% of cases [4]. Their persistence can be explained by their tolerance to anti-microbials and ability to survive in a nutrient-deficient environment [5]. The proportional decrease of facultative bacteria and the concomitant increase of strictly anaerobic bacteria with time are because of oxygen consumption and low oxidation-reduction potential, which collaborate to sustain the growth of these bacteria [6]. Intracanal medicaments are considered mandatory in the management of such cases. Calcium hydroxide is the gold standard material and is widely used as an intracanal medicament for disinfection and to promote periapical healing. The anti-microbial activity is attributed directly to the dissociation and availability of calcium and hydroxyl ions resulting in increased local pH. These hydroxyl ions have the ability to destroy the cytoplasmic membrane, denature bacterial proteins, and damage bacterial DNA [7].

Gum and dental issues have traditionally been treated using herbal treatments. In the recent era, herbal extracts have been used in dentistry as anti-microbials, antiseptics, and anti-bacterials. Clove (*Syzygium aromaticum*), belonging to the family Myrtaceae, is an important culinary plant with immense medicinal use. Clove has been shown to have anti-microbial effects against a variety of bacterial and fungal species. An in-vitro study showed that at a 5% concentration of clove extract, there was no anti-microbial activity, while 10% and 50% concentrations showed anti-microbial activity against E. faecalis. Anti-microbial activity increased as the concentration increased from 5 to 50% [8].

Triphala is an Indian Ayurvedic herbal formulation consisting of dried and powdered fruits. It is an equi-proportional mixture of *Terminalia chebula*, *Terminalia belerica*, and *Emblica officinalis* in a 1:1:1 ratio and is used as a potent anti-inflammatory, antioxidant, and anti-microbial agent against a wide spectrum of microbes [9]. An in-vitro study demonstrated that Triphala extract had anti-microbial properties against *C. albicans* with a maximum ZOI of 20 mm at 9% [10]. Activated charcoal is a black, odourless, flavourless powder with anti-bacterial efficacy that has been used as an emergency anti-poison treatment since the 1800s, as it can bind to a wide variety of drugs, reducing their effects [11].

Nanoparticles are microscopic particles of less than 100 nm in size. The nano form of calcium hydroxide penetrates dentinal tubules and enhances anti-microbial efficacy due to its extended presence in dentinal tubules [12]. An alternative treatment approach has been

developed to treat infections using traditional medicinal herbs, which is clinically safer in combating the negative effects of drug resistance and side effects associated with commonly used antibiotics. Considering the lack of research on the anti-bacterial properties of calcium hydroxide with herbal additives and the advantages of using naturally available herbs, the current in-vitro study was undertaken to evaluate and compare the effect of adding various herbal extracts on the anti-bacterial efficiency of different calcium hydroxide formulations.

MATERIALS AND METHODS

This in-vitro study was conducted at the Department of Microbiology, Rajarajeswari Medical College and Hospital in Bangalore, India from July 2023 to September 2023. The study received approval from the ethical committee of the institution (RRDCH/IEC/2023/88).

Sample size estimation: The sample size was estimated using GPower software v. 3.1.9.4 (Franz Faul, Universität Kiel, Germany). The sample size estimation was performed at a 5% alpha error level (α=0.05), with an effect size of 0.58 (based on the findings from the previous literature by Somayaji SK et al.,) [13], and the study's power set at 80%, indicating that a minimum of 40 samples was needed for the present study. Each group consisted of 10 samples (10 samples×4 groups=40 samples). The sample size in each study group was further subdivided into five samples for ADT and DCT for *E.faecalis* and *C.albicans* (5 samples×2 tests×4 groups×2 organisms=80 samples).

Procedure

The pure strains of *E.faecalis* and *C.albicans* were obtained from the Microbial Type Culture Collection (MTCC) at Rajarajeswari Medical College and Hospital, Bengaluru, Karnataka, India. SDA and MHA media were used to assess the effect of different intracanal medicaments on the growth of *C.albicans* and *E.faecalis*, respectively, using the ADT. Blood agar medium was used to evaluate the impact of various intracanal medicaments on the growth of *C.albicans* and *E.faecalis* using the DCT.

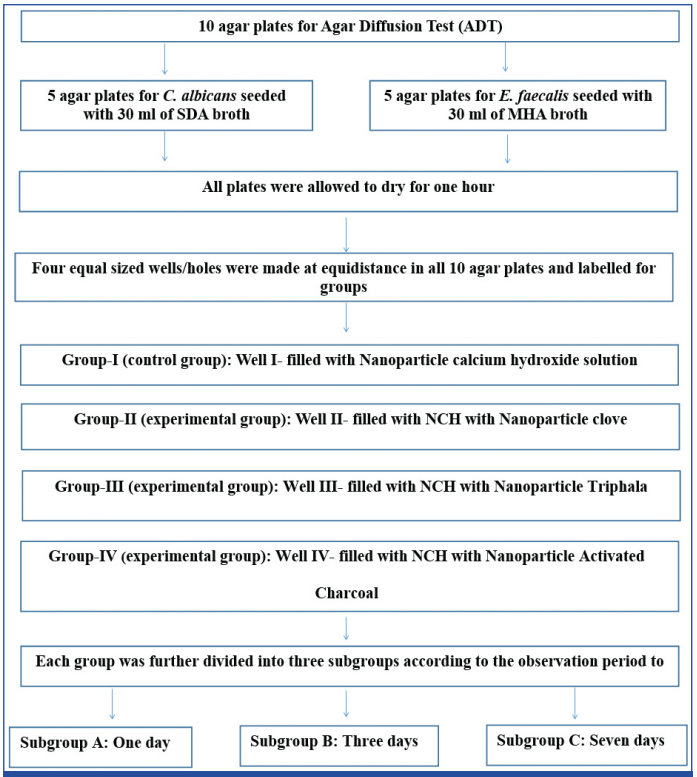
Preparation of Sabouraud Dextrose Agar (SDA) and Mueller-Hinton Agar (MHA): Six grams of SDA and MHA were added to 150 ml of distilled water and then heated to dissolve the agar. Autoclaving was then performed for 15 minutes at 121°C to ensure adequate sterilisation. The mixture was then poured into Petridishes and left to cool and solidify.

Preparation of *E. faecalis* and *C. albicans* Suspension: A sterile swab was used to transfer *C. albicans* and *E. faecalis* growth from the primary culture into the SDA and MHA broth bottles, respectively, and mixed well to form a homogeneous suspension.

Preparation of intracanal medicaments: Two grams of NCH powder (Nanoresearch Laboratory, Jharkhand) were dissolved in 5 mL of distilled water to obtain a liquid consistency, resulting in a 40% concentration solution in a test tube [14]. Additionally, 0.1 gram of nanoparticle Triphala powder (Nanoresearch Laboratory, Jharkhand), 0.5 gram of nanoparticle clove powder (Nanoresearch Laboratory, Jharkhand), and 0.5 gram of nanoparticle activated charcoal powder (Nanoresearch Laboratory, Jharkhand) were added to three different test tubes and dissolved with 1 mL of sterile distilled water. Then, 1 mL of the prepared NCH mixture was added to each test tube to obtain three different formulations [15].

Anti-microbial assay: The anti-microbial activity of different formulations of nanoparticle calcium hydroxide was determined using the agar well diffusion method [Table/Fig-1]. The plates were left at room temperature for one hour and then incubated at 37°C for seven days. Zones of inhibition (in millimeters) were measured at one, three, and seven days.

Direct Contact Test (DCT): Tryptic Soy Broth (TSB) was used to cultivate *E. faecalis* and *C. albicans*, and the cultures were kept for 48 hours at 37°C. The microbial cells were re-suspended in saline to obtain a bacterial solution with 1.5×10^8 cells/mL and a concentration of 0.5 McFarland. Subsequently, the tubes with different formulations were inoculated with 0.01 mL of each bacterial suspension, and then incubated for 24 hours at 37°C [14]. Blood agar plates were used as the medium and divided into groups (Group-I, II, III, and IV). A sterilised inoculating loop was used to inoculate the samples on the blood agar plate using the streak plate method. The anti-microbial activity was evaluated by counting Colony Forming Units (CFUs) after 24 hours.



[Table/Fig-1]: A flowchart of anti-microbial activity test (ADT).

STATISTICAL ANALYSIS

The Statistical Package for Social Sciences (SPSS) for Windows version 22.0, released in 2013 by IBM Corp. in Armonk, NY, was used to perform statistical analyses. A one-way Analysis of Variance (ANOVA) test followed by Tukey's post-hoc analysis was used to compare the mean ZOI (in mm) against *E. faecalis* and *C. albicans* between groups at different time intervals. Repeated measures of ANOVA test followed by Bonferroni's post-hoc test were used to compare the mean ZOI against *E. faecalis* and *C. albicans*. The Kruskal-Wallis test followed by the Mann-Whitney post-hoc test was used to compare the mean CFUs of *E. faecalis* and *C. albicans* between groups. The level of significance (p-value) was set at $p<0.05$.

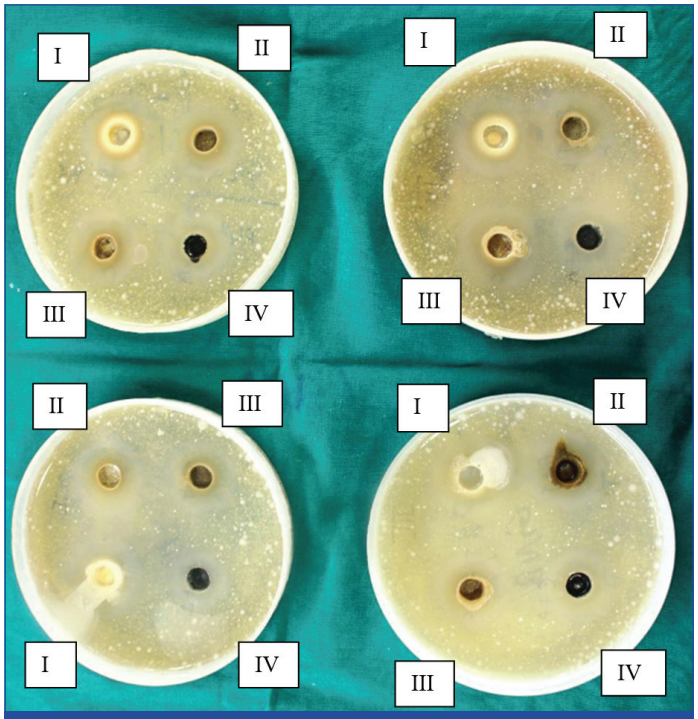
RESULTS

[Table/Fig-2,3] show the mean ZOI against C. albicans between different time intervals in each group. The maximum mean ZOI of 22.20±1.92 mm was achieved by Group-II. On day 3, there was a statistically significant difference in the mean ZOI between groups at p=0.005. On day 1 and day 7, there was no statistically significant difference between groups. [Table/Fig-4] shows the multiple comparison of mean differences between groups, revealing that Group-4 showed a significantly lesser mean ZOI compared to Group-2 and Group-3, which was statistically significant at p=0.004 and p=0.02, respectively. However, no significant difference was observed between the other groups.

[Table/Fig-5,6] show the mean ZOI against *E. faecalis* between different time intervals in each group. The maximum mean ZOI of 18.20±0.84 mm was achieved by Group-1. On Day 1, there was

Time	Groups	N	Mean	SD	Min	Max	p-value
Day 1	Group-I	5	21.60	0.55	21	22	0.27
	Group-II	5	22.20	1.92	20	25	
	Group-III	5	21.60	0.89	21	23	
	Group-IV	5	20.60	1.14	19	22	
Day 3	Group-I	5	18.40	2.07	15	20	**0.005***
	Group-II	5	20.00	1.87	17	22	
	Group-III	5	19.00	1.00	18	20	
	Group-IV	5	15.60	1.52	14	18	
Day 7	Group-I	5	15.60	1.82	14	18	0.13
	Group-II	5	17.20	2.28	14	20	
	Group-III	5	15.80	1.79	13	17	
	Group-IV	5	14.00	2.00	12	17	

[Table/Fig-2]: Comparison of Mean Zone of Inhibition (ZOI) against *Candida albicans* between different time intervals in each group.
*: Statistically Significant



[Table/Fig-3]: Zone Of Inhibition (ZOI) against *Candida albicans* in each group.
• I- Group-I- NCH + sterile distilled water; • II- Group-II- NCH + Nanoparticle clove; • III- Group-III- NCH + Nanoparticle triphala; • IV- Group-IV- NCH + Nanoparticle activated charcoal

Time	Groups	G1 vs G2	G1 vs G3	G1 vs G4	G2 vs G3	G2 vs G4	G3 vs G4
Day 3	Mean Diff	-1.60	-0.60	2.80	1.00	4.40	3.40
	p-value	0.45	0.94	0.07	0.92	**0.004***	**0.02***

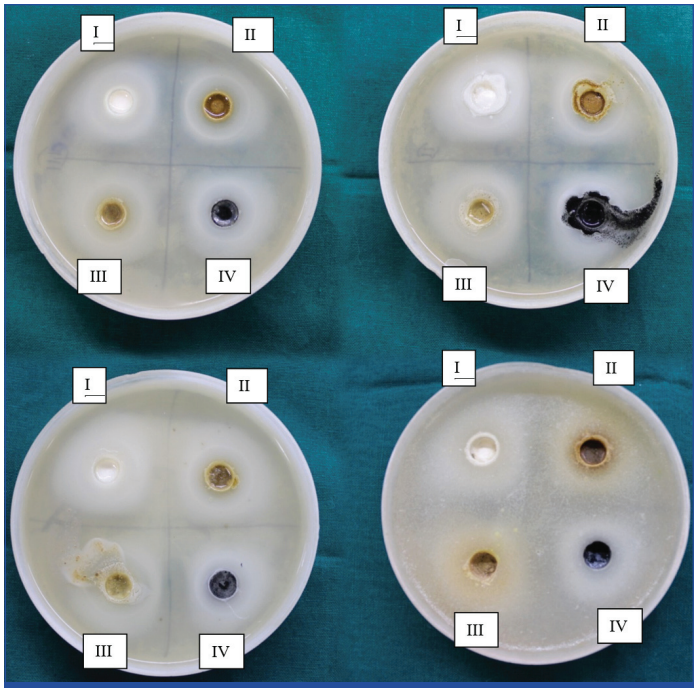
[Table/Fig-4]: Multiple comparison of mean difference in Zone Of Inhibition (ZOI) against *Candida albicans* between 4 groups on Day 3.

no statistically significant difference between the groups, while on Day 3 and Day 7, there was a statistically significant difference in the mean ZOI between the groups at p=0.04 and p<0.001, respectively. [Table/Fig-7] shows the multiple comparison of mean differences between groups on Day 3, revealing that Group-4 showed a significantly lesser mean ZOI compared to Group-1, which was statistically significant at p=0.04. Multiple comparisons on Day 7 revealed that Group-1 showed a significantly higher mean ZOI compared to Group-2, 3, and 4, which was statistically significant at p=0.04, p=0.02, and p<0.001, respectively. However, no significant difference was observed between the other groups.

For mean Colony-Forming Units (CFUs) of *C. albicans* and *E. faecalis*, there was a significant difference between the groups at p<0.001 as shown in [Table/Fig-8]. Multiple comparisons of mean differences

Time	Groups	N	Mean	SD	Min	Max	p-value
Day 1	Group-I	5	18.20	0.84	17	19	0.54
	Group-II	5	17.60	1.14	16	19	
	Group-III	5	17.40	0.89	16	18	
	Group-IV	5	17.20	1.48	15	19	
Day 3	Group-I	5	17.00	1.00	16	18	**0.04***
	Group-II	5	16.20	1.30	15	18	
	Group-III	5	16.40	1.14	15	18	
	Group-IV	5	15.20	1.30	14	17	
Day 7	Group-I	5	17.00	1.00	16	18	**<0.001***
	Group-II	5	15.40	1.14	14	17	
	Group-III	5	15.20	0.45	15	16	
	Group-IV	5	13.80	0.84	13	15	

[Table/Fig-5]: Comparison of Zone Of Inhibition (ZOI) against *E.faecalis* between different time intervals in each group.
*: Statistically significant



[Table/Fig-6]: Zone Of Inhibition (ZOI) against *Enterococcus faecalis* in each group.
• I- Group-I - NCH + sterile distilled water; • II- Group-II - NCH+ Nanoparticle clove; • III Group-III - NCH + Nanoparticle triphala; • IV- Group-IV- NCH + Nanoparticel activated charcoal

Time	Groups	G1 vs G2	G1 vs G3	G1 vs G4	G2 vs G3	G2 vs G4	G3 vs G4
Day 3	Mean diff	0.80	0.60	1.80	-0.20	1.00	1.20
	p-value	0.72	0.86	**0.04***	0.99	0.56	0.21
Day 7	Mean diff	1.60	1.80	3.20	0.20	1.60	1.40
	p-value	**0.04***	**0.02***	**<0.001***	1.00	**0.04***	0.10

[Table/Fig-7]: Multiple comparison of mean difference in Zone Of Inhibition (ZOI) against *E. Faecalis* between 4 groups on day 3 and 7.
*: Statistically significant

between groups revealed that the mean CFUs of *C. albicans* and *E. faecalis* were significantly higher in Group-3 compared to the other groups [Table/Fig-9-11].

DISCUSSION

The present study investigated the efficacy of the combination of nanoparticle calcium hydroxide with various herbal formulations as an intracanal medicament against *Enterococcus faecalis* and *Candida albicans*. The main purpose of endodontic treatment is the elimination of microbiota from the root canal system, a major challenge for all endodontic practitioners. While sporadic species of bacteria affecting the root canal system have a low virulence impact individually, collectively they have a significant virulence impact [16].

	Groups	N	Mean	SD	Min	Max	p-value
Candida albicans	Group-I	5	2.40	0.55	2	3	<0.001*
	Group-II	5	3.40	0.55	3	4	
	Group-III	5	4.40	0.55	4	5	
	Group-IV	5	2.40	0.55	2	3	
Enterococcus faecalis	Group-I	5	0.00	0.00	0	0	<0.001*
	Group-II	5	0.00	0.00	0	0	
	Group-III	5	1200.00	273.86	1000	1500	
	Group-IV	5	0.00	0.00	0	0	

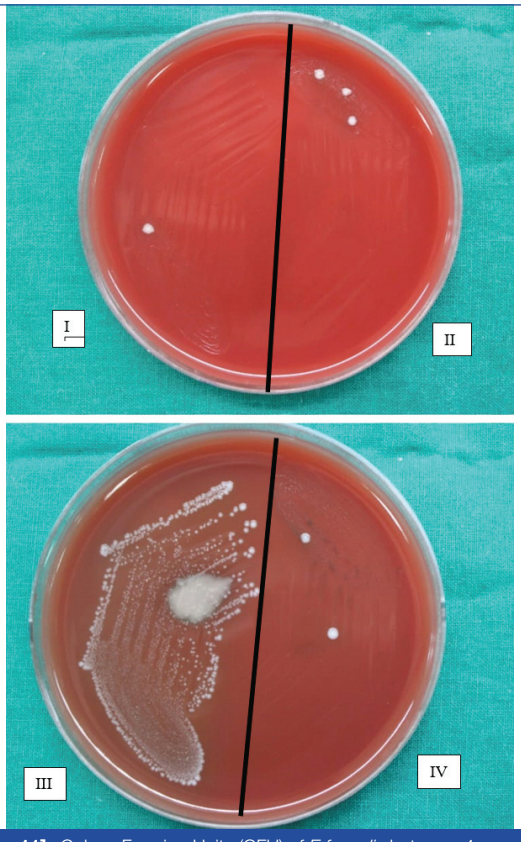
[Table/Fig-8]: Comparison of Mean Colony Forming Units (CFUs) of *C.albicans* and *E.faecalis* between groups.

(I) Groups	(J) Groups	Mean diff. (I-J)	95% CI for the Diff.		p-value
			Lower	Upper	
Group-I	Group-II	-1.00	-1.99	-0.01	**0.04***
	Group-III	-2.00	-2.99	-1.01	**0.002***
	Group-IV	0.00	-0.99	0.99	1.00
Group-II	Group-III	-1.00	-1.99	-0.01	0.16
	Group-IV	1.00	0.01	1.99	**0.04***
Group-III	Group-IV	2.00	1.01	2.99	**0.002***

[Table/Fig-9]: Multiple comparison of mean difference in the Colony Forming Units (CFU) of *C.albicans* between four groups using Dunn's Post-hoc test.

(I) Groups	(J) Groups	Mean diff. (I-J)	95% CI for the Diff.		p-value
			Lower	Upper	
Group-I	Group-II	0.00	-247.77	247.77	1.00
	Group-III	-1200.00	-1447.77	-952.23	<0.001*
	Group-IV	0.00	-247.77	247.77	1.00
Group-II	Group-III	-1200.00	-1447.77	-952.23	<0.001*
	Group-IV	0.00	-247.77	247.77	1.00
Group-III	Group-IV	1200.00	952.23	1447.77	<0.001*

[Table/Fig-10]: Multiple comparison of mean difference in the Colony Forming Units (CFU) of *E.faecalis* between 4 groups using Dunn's Post-hoc Test.



[Table/Fig-11]: Colony Forming Units (CFU) of *E.faecalis* between 4 groups.
• I- Group-I - NCH + sterile distilled water; • II- Group-II- NCH + nanoparticle clove; • III- Group-III - NCH + nanoparticle triphala; • IV- Group-IV- NCH + nanoparticle activated charcoal

Microbial virulence factors include toxins, hydrolytic enzymes, cell surface proteins, and carbohydrates that mediate microbial attachment and protection from the host. Therefore, the goal of root canal treatment is to significantly reduce the number of bacteria and bacterial biofilms by eradicating and killing them, and to modify the conditions in the root canal system to be less favourable for microbial growth [2].

The most commonly used techniques to demonstrate the anti-bacterial activities of materials are the ADT and the DCT. ADT is an accepted method for initially differentiating anti-bacterial activity between materials, and the result depends on the anti-bacterial activity of the material for the particular microorganism and is highly influenced by the diffusibility of the material across the medium [16]. DCT relies on direct and close contact between the test microorganism and the test material, which is virtually independent of the diffusion characteristics of the media and the tested material. These two methodologies were used in the current study [17].

Candida albicans is the most abundant fungus found in the oral cavity and often causes endodontic treatment failure. *Enterococcus faecalis* is one of the most persistent bacteria frequently detected in root canal therapy [4]. The use of intracanal medications may significantly increase the success of root canal treatment by reducing or eliminating bacteria in the root canal system. The material of choice for intracanal medication is calcium hydroxide due to its beneficial properties, such as anti-microbial activity, capacity to limit inflammation, ability to dissolve organic tissues, and inactivation of bacterial endotoxins [2]. Calcium hydroxide has been reported to require a minimum of seven days of contact time to completely eliminate any bacteria that might have survived the chemo-mechanical preparation [18]. To be effective, calcium hydroxide should penetrate dentinal tubules to come in direct contact with microorganisms. Minimising the particle size and producing a nano form of calcium hydroxide may enhance the penetration of the material into the dentinal tubules and improve microbial efficacy due to the longer presence of the drug in the dentinal tubules. The higher surface-to-volume ratio and charge density of these nanoparticles result in their greater interaction with the environment and higher anti-bacterial activity [12]. In the present study, the calcium hydroxide formulation achieved the maximum mean zone of inhibition against *E. faecalis*.

Modern developments in drug research technologies and the quest for unique chemical diversity have boosted efforts to investigate potential therapeutic leads from India's traditional Ayurvedic system [4]. The prevailing notion that "green medicine" is more reliable and safer than expensive synthetic medications, many of which have unfavourable side effects, is the primary reason for the current resurgence of interest in plant-derived pharmaceuticals. The constant increase in antibiotic-resistant strains of microorganisms and the deleterious effects caused by synthetic drugs have led researchers to search for herbal alternatives that are non-toxic or less toxic [9].

The essential oil of cloves is used as a painkiller for dental emergencies in Western herbalism, Chinese medicine, Indian Ayurvedic medicine, and Western medicine. Clove oil is abundantly available, easily accessible, economically feasible, and culturally acceptable, with minimal side effects. It is used in the preparation of toothpastes, laxative pills, and clovacaine solution, a local anaesthetic used in oral ulceration and inflammation [8]. In the present study, the calcium hydroxide with clove formulation showed a maximum mean ZOI of 22.20±1.92 mm and 17.60±1.14 mm against *C. albicans* and *E. faecalis*, respectively. A similar study by Niharika P, showed the anti-bacterial effect of clove and tulsi against *E. faecalis* when compared to calcium hydroxide [19].

Triphala is an herbal mixture used extensively in the Indian system of medicine. The active constituents present in Triphala, such as tannic

Sl. no	Author's name and year	Place of study	Sample size	Materials compared	Parameters assessed	Conclusion
1	Contrast study: Teja KV et al., [20] 2023	Tamil Nadu, India	N=80	Group-I: Calcium hydroxide (CH) Group-II: CH +2%, Group-III: CH +2% chitosan gel Group-IV: CH +0.02% AGNP (silver nanoparticle) gel, Group-V: CH+BAG (Bioactive glass)	Colony-Forming Units (CFU)	Group-V was most the effective ($p<0.001$) against *E. faecalis* followed by Group-IV, Group-II and Group-III. The least effective was Group-I. The current study results have shown CH combinations to be more effective against *E. faecalis* as compared to the CH alone.
2	Similar study: Niharika P, [19], 2023	Tamilnadu, India	-	Group-I: Calcium hydroxide Group-II: Clove and tulsi	Zone Of Inhibition (ZOI) by disk diffusion test	Maximum anti-microbial effect exhibited by 100 µL concentrated solution of clove and tulsi with a mean ZOI of 35 mm. ZOI of 33 mm by 50 µL, 31 mm by 25 µL, and least 30 mm by 10 µL of clove and tulsi herbal extracts against *E. faecalis* when compared against calcium hydroxide.
3	Similar study: Brar R et al., 2019 [15]	Maharashtra, India	N=30	**Study group:** Group-I: 2% CHX - Triphla Group-II 2% CHX - Turmeric Group-III: Triphla - Turmeric **Control Group:** Group-IV: 2% CHX - Saline Group-V: Triphla - Saline Group-VI: Turmeric - Saline	ZOI by Agar Diffusion Test (ADT)	Triphala and turmeric had significant inhibitory effects on *E.faecalis* when compared with the results of the control group
4	Similar study: Elfaramawy M et al., [16] 2023	Egypt	-	Group-I: Conventional Calcium hydroxide Powder Group-II: Conventional Calcium hydroxide Powder + Activated Charcoal Group-III: calcium hydroxide paste with iodoform Group-IV: calcium hydroxide past with iodoform +Activated Charcoal	ZOI by Agar Diffusion Test (ADT)	The addition of activated charcoal did not improve the anti-bacterial effect of conventional calcium hydroxide while it improves the anti-bacterial efficacy of the calcium hydroxide and iodoform (Metapex) against *E. faecalis*.
5	Present study	Bangalore, India	N=80	Group-I: Nanoparticle calcium hydroxide solution Group-II: Nanocalcium Hydroxide (NCH) with Nanoparticle clove Group-III: NCH with Nanoparticle Triphala Group-IV: NCH with Nanoparticle Activated charcoal	ZOI by Agar Diffusion Test (ADT), Colony-Forming Units by DCT	Maximum anti-microbial effect was exhibited by NCH against *E.faecal* and NCH with nanoparticle clove against *C. albicans*.

[Table/Fig-12]: Summary of similar studies and contrast study from the literature [15,16,19,20,Present study].

acid, chebulic acid, and flavonoids, are largely responsible for its therapeutic potential [9]. In the present study, the calcium hydroxide with triphala formulation showed a mean ZOI of 21.60±0.89 mm and 17.40±0.89 mm on day 1 against *C. albicans* and *E. faecalis*, respectively.

Activated charcoal is a natural product used in oral and dental care preparations [11]. In the present study, the calcium hydroxide with activated charcoal formulation showed a significantly smaller mean ZOI compared to the other groups. Similar studies from the literature have been discussed in [Table/Fig-12] [15,16,19,20].

Limitation(s)

All these experiments were conducted under in-vitro conditions; further studies should be carried out to assess the effects of these nanoparticles in clinical settings, as in-vivo conditions vary significantly. The actions and effects of these intracanal medicaments may differ when interacting with periradicular tissues compared to in-vitro controlled conditions. Another limitation of the study could be the small sample size for each group, which could impact the reliability of the findings.

CONCLUSION(S)

The placement of intracanal medicament plays a fundamental role in eliminating endotoxins produced by microbial pathogens. Within the limitations of the study, it can be concluded that the Nanoparticle clove formulation showed the highest mean ZOI against *Candida albicans*, while the Nanoparticle calcium hydroxide formulation showed the highest mean ZOI against *Enterococcus faecalis* when compared to the other three groups. The use of herbal alternatives as intracanal medicament could be safer and more effective, with an additive or synergistic positive effect, as the combination of medications may reduce the development of resistant bacterial strains and ultimately encourage a synergistic action with increased anti-bacterial efficacy.

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