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Comparative Antimicrobiotic Efficacy Test of Triple Antibiotic Paste, Double Antibiotic Paste with Fungicide and Calcium Hydroxide with Chitosan as Vehicle against *Enterococcus faecalis*: An *In vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author SN designed the study, performed the statistical analysis, wrote the protocol, and wrote the manuscript's first draft. Author MN analysed the study. Author GDD managed the literature searches. Authors TS and SP drafted the final manuscript. Author MBB performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: This present research aimed to assess the in vitro antimicrobial efficacy of triple antibiotic paste and calcium hydroxide with two different vehicles against *Enterococcus faecalis (E. faecalis).*

Materials and Methodology: An agar well diffusion assay was used to determine the experimental medicaments' efficacy against *E. faecalis.* Medicaments were divided into six groups, which includes Triple antibiotic powder (TAP) with saline or chitosan, Double antibiotic powder with fungicide (DAP 1) with saline or Chitosan, and calcium hydroxide with saline or Chitosan. These medicines were tested in an agar well diffusion test for three days, i.e., 1,4,7 days. The diameters

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of growth inhibition zones were recorded and compared for each group were tested in an agar well diffusion test for three days, i.e., 1,4,7 days. The diameters of growth inhibition zones were recorded and compared for each group. These medicaments were evaluated for three days an agar well diffusion test, i.e., on 1,4,7 days. The inhibition zones diameters for each group were recorded and compared — the differences between groups analyzed by Kruskal-Wallis and Mann-Whitney U tests.

Results: The largest inhibition zones were observed for the triple antibiotic powder with chitosan and the smallest for $Ca(OH)_2$ with saline. As days progress, they produced lesser antibacterial effects in all groups.

Conclusion: Triple antibiotic paste and Chitosan were more effective in eliminating microorganisms than calcium hydroxide and DAP 1.

Keywords: Antimicrobial efficacy; calcium hydroxide, chitosan; double antibiotic paste; E. faecalis; triple antibiotic paste; intracanal medicaments; root canal medicaments.

1. INTRODUCTION

Pulpal and periapical diseases are primarily related to microorganisms and their byproducts in the root canal system. Endodontic treatment's primary objective is to eliminate microorganisms from the root canal system and prevent their reinfection [1]. However, due to its complicated nature of the root canal system and the presence of many inaccessible areas, chemomechanical preparation alone cannot eliminate all the microorganisms, so interappointment medicaments are recommended [2].

Polymicrobial endodontic infections are mainly composed of anaerobic species: *E. faecalis*, the most crucial bacterium in resistant infections. One of the commonly found organisms in cases of failed endodontic infections and endodontic flare-ups is *E. faecalis*. As a single individual, it can thrive in the root canal system without other bacteria's assistance and is small enough to penetrate and reside within the dentinal tubules proficiently [3]. It has been stated by several other studies that *E. faecalis* can enter at a variable depth into the dentinal tubules. So, it was challenging to eliminate the root canal bacteria, an active antimicrobial agent in the root canal required for a predetermined period for complete eradication of any remaining bacteria.

Calcium hydroxide [$Ca(OH)_2$] has widely used as an intracanal medicament due to its antimicrobial properties and its high alkalinity. The high pH level of CH alters the lipopolysaccharide portion of gram-negative bacteria in the cell wall, which inactivates membranes' transport and ultimately leads to cell death [4]. Nonetheless*, E. faecalis*, due to its ability to infiltrate further into the dentinal tubules, where the buffering action of dentin neutralizes the pH of the drug [5,6]. Besides, dentin's flexural strength is decreased by CH, and its effectiveness in removing bacteria has been doubted, even after sustained interaction with root canal walls [7]. However, it has limited action against *E. faecalis* [8].

The triple antibiotic paste was recently used as an intracanal medicament for disinfecting the root canal system during regenerative procedures [9]. Triple antibiotic paste (TAP) is a combination of metronidazole, ciprofloxacin, and minocycline. The purpose of using TAP when an infected root canal is inaccessible to the local immune system and the drug concentration that reaches the canal space after administration of systemic antibiotics is minimal, and it was unable to eliminate bacterial growth. Therefore, the local application of intracanal medicaments within the root canal system may be a more effective mode of delivering the drug [10].

Chitosan is used as a drug carrier where it has an added advantage of slow and controlled release of intracanal medicament and increases the medicament's constancy. The partial deacetylation of chitin produces Chitosan. The second most abundant natural polysaccharide is chitin. It is composed of β (1→4) linked N-acetyl glucosamine units. Chitosan has many critical pharmacological applications. It has a broad diversity of drug delivery uses, has an absorption enhancer, colon targeting, and gene delivery [11].

Till now, there is no adequate data on the antimicrobial efficacy of TAP, DAP 1, and CH against the effect of intracanal medicaments using Chitosan as a carrier on*E. faecalis.*This study comparedto evaluate the antimicrobial

efficacy of Triple antibiotic paste, Double antibiotic paste with fungicide and Calcium hydroxide, and two different vehicles such as Saline and Chitosan as root canal medicaments used in endodontic therapy against *E. faecalis*.

2. MATERIALS AND METHODS

With ethical Approval from the Institution's Ethics Committee,the present in vitro study was done at the Department of Conservative and Endodontics in Collaboration with the Department of Microbiology at Sree Sai Dental College and Research Institution, Srikakulam, Andhra Pradesh, India.

The materials tested were:

- Triple antibiotic paste (ciprofloxacin, metronidazole, doxycycline).
- Double antibiotic paste + fungicide (DAP 1) (ciprofloxacin, metronidazole, fluconazole)
- Calcium hydroxide
- Saline
- Chitosan

The methodology used in this study was done by an agar diffusion method.

2.1 Sample Preparation - Agar Diffusion Method

Standard resistant strains of *E. faecalis* (American Type Culture Collection [ATCC] 1827) obtained for this study were cultured on Brain heart infusion (BHI) agarmedium, respectively. The agar plates were prepared in sterile glass Petri dishes and kept overnight for sterility at 37°C. After ensuring sterility, inoculate the strains within the sterile saline, and the turbidity was compared using McFarland's turbidity standard tube No 0.5. These inoculations were used to make the organism's lawn culture using sterile cotton swabs on BHI agar. According to Kirby Bauer's punch well method, the holes were punched in the cultivated agar plates (4mm in-depth, 6mm in diameter). A total of 120 wells in 60 BHI agar plates for *E. faecalis* were prepared (1 plate = 1 well). The medicaments tested were in powdered form. They were as follows:

The details of medicaments tested against *E. faecalis.*

Groups	Description
1	Triple antibiotic paste + saline
2	Double antibiotic paste (with fungicide) 1 + Saline
3	Calcium hydroxide + saline
4	Triple antibiotic paste + Chitosan
5	Double antibiotic paste (with fungicide) 1 + Chitosan
6	Calcium hydroxide + Chitosan

In each well, introduced these paste with the help of a sterile spatula. The plates were kept under appropriate atmospheric conditions (80% N_2, 10% CO_2, 10% H_2) in an incubator at 37°C under anaerobic conditions for 24 hrs in a CO_2 incubator (KGH, Dept of Microbiology Lab, Visakhapatnam). The microbial zones of inhibition around the wells containing the test substances were recorded after the period of incubation. The inhibitory zone was observed in millimeters by measuring the shortest distance between the outer margin of the well and initial microbial growth. The inhibition zones were recorded on the first day, the fourth day, and the seventh day against *E. faecalis.*

3. RESULTS

All the intracanal medicaments used in this study exerted antimicrobial activity against *E. faecalis*. TAP + Chitosan had the highest antimicrobial activity compared with TAP + saline, DAP 1+ Chitosan, saline, $Ca(OH)_2$ + saline Chitosan [Table 1]. TAP + chitosan has shown better antimicrobial efficacy against *E. faecalis* compared with DAP 1+Chitosan, $Ca(OH)_2$ + Chitosan [Tables 2 and 3]. For all the six groups, bacterial inhibition was significantly more on the first day than the fourth and seventh day. It indicates that Chitosan with TAP has decreased the microbial load after the placement of intracanal medicament.Mean, and the standard deviation was estimated for all the six different groups. Tables 1,2,3 shows the mean counts and standard deviation of different diameters of microbial inhibition zones between 6 different groups. All the intracanal medicaments tested reduced the bacterial load in the root canal system, but TAP + Chitosan ($P > 0.001$) showed the most antibacterial efficacy while $Ca(OH)_2$ + saline showed the least antibacterial efficacy. The statistical package SPSS software (Statistical package for social science) is used for statistical analysis. The data were analyzed with Mann-Whitney U tests to check the microbial inhibition zones of differences between the groups ($P < 0.05$).

Table 1. Antimicrobial activity against *E. faecalis* (TAP with CHITOSAN)

Enterococcus faecalis						
Day	TAP with saline		TAP with chitosan		Difference	P value
	MEAN	SD	MEAN	SD	MEAN±SD	
1	47.00	2.67	49.40	3.92	2.40±1.25	0.127 NS
4	43.20	1.23	47.80	4.08	4.60±2.85	0.003 S
7	34.40	10.12	47.00	4.85	12.60±5.27	0.002 S

Statistical Analysis: Independent sample t-test. Statistically significant if P<0.05

Table 2. Antimicrobial activity against *E. faecalis* (DAP1 with CHITOSAN)

Enterococcus faecalis						
Day	DAP 1 with saline		DAP1 with chitosan		Difference	P value
	MEAN	SD	MEAN	SD	MEAN±SD	
1	39.60	1.58	44.60	2.72	5.00±1.14	0.000 S
4	38.80	2.25	44.80	3.91	6.00±1.66	0.001 S
7	36.00	3.20	43.60	3.75	7.60±0.55	0.000 S

Statistical Analysis: Independent sample t-test. Statistically significant if P<0.05

Table 3. Antimicrobial activity against *E. faecalis* ($Ca(OH)_2$ with CHITOSAN)

Enterococcus faecalis						
Day	$Ca(OH)_2$ with saline		$Ca(OH)_2$ with chitosan		Difference	P value
	MEAN	SD	MEAN	SD	MEAN±SD	
1	7.60	4.09	39.80	6.51	32.20±2.42	0.000 S
4	0.00	0.00	39.00	6.70	39.00±6.70	0.000 S
7	0.00	0.00	34.80	4.54	34.80±4.54	0.000 S

Statistical Analysis: Independent sample t-test. Statistically significant if P<0.05

4. DISCUSSION

The golden rule of successful endodontic therapy is the complete elimination of infection and three-dimensional obturation of the root canal system to prevent recurrent infections [12]. However, nowadays, current debridement techniques leave some parts of the root canals untouched due to limitations, which include the anatomical variation, constrictions, and curved roots. The fulfillment of root canal treatment depends on microbial control and the complete filling of the root canal system. Chemo mechanical preparation alone could not eliminate bacteria because of the complexity of the root canal system and limitation of access by instruments and irrigants [13], so the use of different intracanal medicaments proposed to eliminate the bacteria.

*E. faecalis*is one such bacterium that resists elimination and is persistent in apical periodontitis [14]. These bacteria can proliferate into the deeper layer of the dentin [15]. Hence, the penetration of the medicament and the antibacterial efficiency of medicaments were evaluated in the study. As this bacterium is suggested to be mostly associated with endodontic failures, it has been one of the most frequently investigated bacterial species [16]. Hence, the medicament's penetration and the antibacterial efficiency of medicaments evaluated in the study by the agar diffusion test used to evaluate the antimicrobial activities of medicaments as this method in many studies for evaluations of antibacterial effects of various endodontic materials. The development of bacterial strain resistance was reduced by using the combination of various irrigants and medicaments and produced a synergistic effect whose antimicrobial action lasts longer and also sustains the release of medicaments occurs [17].

Chitosan is a β-1,4-linked polymer of glucosamine (2-amino-2-deoxy-β-D-glucose) natural polysaccharide comprising copolymers glucosamine and N-acetyl glucosamine. Due to its biodegradable and nontoxic properties, this helped prepare nanoparticles for various applications. It is insoluble in acidic conditions, and free amino groups on its polymeric chain protonate and donates to its positive changes. Its cationically charged amino group might combine with N-acetyl muramic acid, sialic acid, and

neuramic acid, an anionic component on the cell surface, suppressing bacterial growth by compromising the exchanges with medium, chelating transition metal ions, and inhibiting enzymes [18]. There have been many studies on the ability of Chitosan to act as a delivery vehicle for antimicrobial drugs [19]. Therefore, incorporating Chitosan to TAP, $Ca(OH)_2$ in a trial against *E. faecalis* for the potential additive or synergistic effect on their viability.

The ideal or optimum vehicle for delivery of antibiotics in root canals should have the ability to facilitate better diffusion of medicament through dentinal tubules and anatomical aberrations like fins, isthmuses, and blocked canals [20]. In this study, Chitosan and saline were used as vehicles for the antimicrobials tested. In the present study, TAP + Chitosan has shown better antibacterial efficacy against *E. faecalis* [Figs. 1,3] compared with TAP + saline, DAP 1 + saline, and Chitosan, $Ca(OH)_2$+ saline and Chitosan, [Fig. 2] which is statistically significant. $Ca(OH)_2$ has an insignificant ability to eliminate bacterial cells inside dentinal tubules because of its low solubility, diffusibility, and dentin-buffering ability, suppressing its antibacterial effect. It could explain that $Ca(OH)_2$ alone had a limited antimicrobial effect when differentiate with $Ca(OH)_2$ with Chitosan against *E. faecalis* [21].

In our current study, results showed that TAP+Chitosan was the most effective intra-canal medicament when compare to the other medicaments tested and followed by TAP + Saline, DAP 1 + Saline, and Chitosan, $Ca(OH)_2$ + Saline and Chitosan in descending order.Calcium hydroxide has been used in dentistry as an intracanal medicament for almost a century (Siqueira & Lopes 1999). Bystrom & Sundqvist documented the antibacterial efficacy of calcium hydroxide in human root canals [22]. Calcium hydroxide was the standard against which other medicaments compared [23]. To enhance the antimicrobial activity of $Ca(OH)_2$, different substances have used as vehicles [24]. The primary mechanism of action of $Ca(OH)_2$ + Chitosan might be due to the reason of Chitosan has positively charged NH3, and glucosamine group that interacts with the negatively charged unit of bacteria, resulting in extensive cell surface attraction, that led to the leakage of intracellular substances, and cause damage to vital bacterial functions [25]. It had a possibility that $Ca(OH)_2$ combined with Chitosan inhibits the growth of*E. faecalis*and affects their re-entry and recolonization. A synergistic/additive effect was found in $Ca(OH)_2$ combined with Chitosan against *E. faecalis* when measured with $Ca(OH)_2$ combined with saline.

TAP, including ciprofloxacin, metronidazole, and minocycline, had an appropriate effect against *E. faecalis,* and this is in agreement with the study done by Adl et al. [26]. A study done by Ballal et al. in 2010 concluded that the addition of Chitosan to $Ca(OH)_2$ paste as an intracanal medication has shown to promote prolonged calcium ion release [27]. Recently, triple antibiotic paste (a mixture of metronidazole, ciprofloxacin, and minocycline) has been used as an intracanal medicament for disinfecting the root canal regenerative procedures. Sato et al. showed that triple antibiotic paste could destroy the bacteria in the root canal system's deep areas [28].

Here in this study, two formulations of triple antibiotic paste were used.

- Triple antibiotic paste: (ciprofloxacin, metronidazole, doxycycline).
- Double antibiotic paste 1 (with fungicide): (ciprofloxacin, metronidazole, flucanazole)

In the present study, TAP + Chitosan showed better antimicrobial efficacy against *E. faecalis*. The probable reason might be that Chitosan, as a drug carrier, has the mechanism of slow and controlled drug release, which improves drug solubility, stability, enhancing efficacy, and reduced toxicity [29]. Jadhav et al. did a study, in their case series, when equal proportions of the components of TAP mixed with distilled water to form a thick paste-like consistency resulted in successful revascularization of immature maxillary anterior teeth [30]. In the present study, the TAP + chitosan combination has shown better antibacterial activity against *E. faecalis* when compared with the TAP + saline combination.

A study done by Hoshino et al. stated that the antibacterial effect of TAP with or without the inclusion of rifampicin on bacteria from root canal infected tooth and stated that none of the drugs alone resulted in a complete defeat of the bacteria. So, therefore, in combination, these drugs were able to sterilize all samples consistently. *In vitro*, TAP was effective in removing endodontic pathogens from infected root canals [31]. Many *in vitro* studies have also stated that TAP was an effective disinfectant [32].

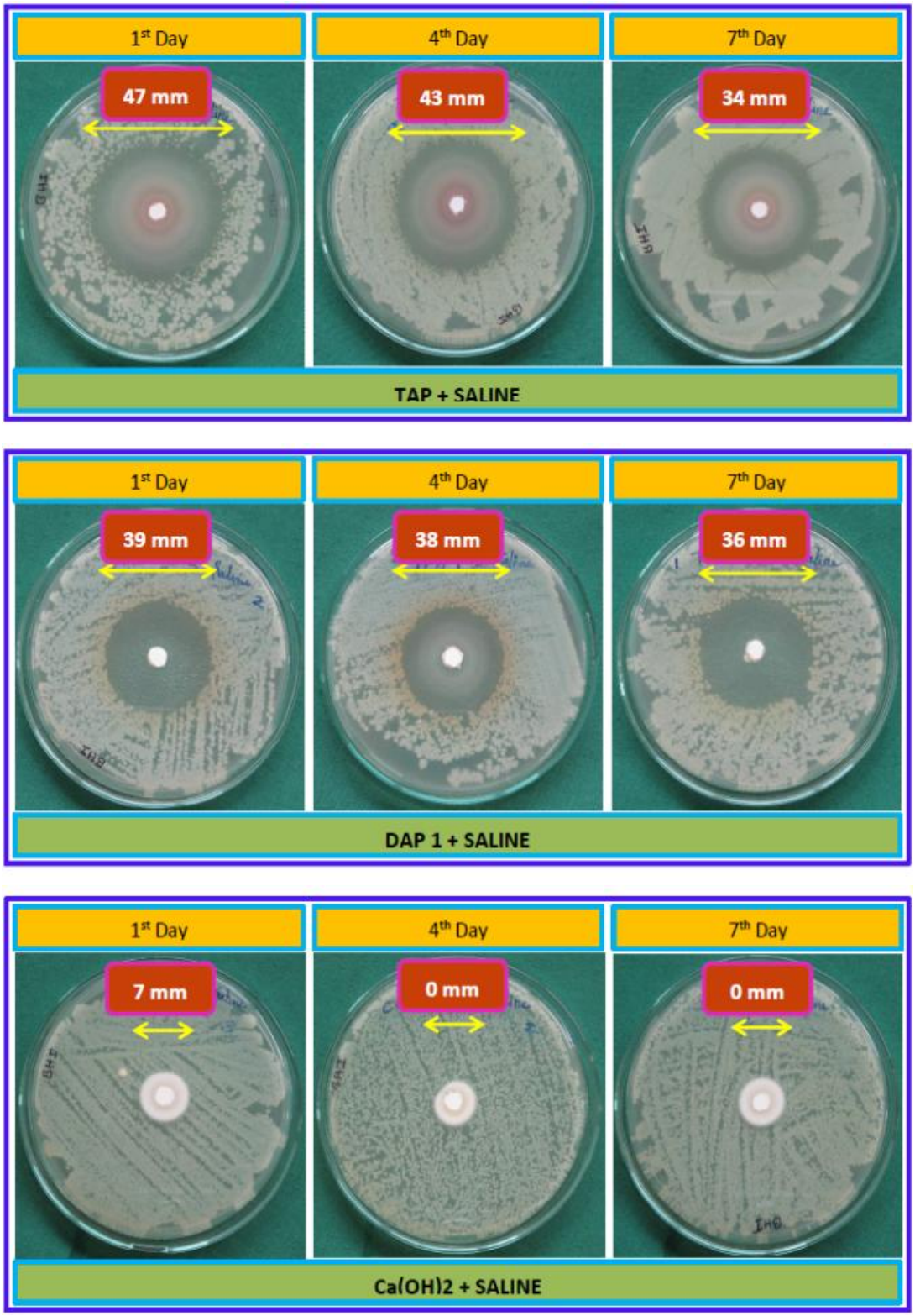

Fig. 1. Clinical representation of all mean zones of diameter in "mm" of various intracanal medicaments on the first, fourth & seventh day by saline treatment

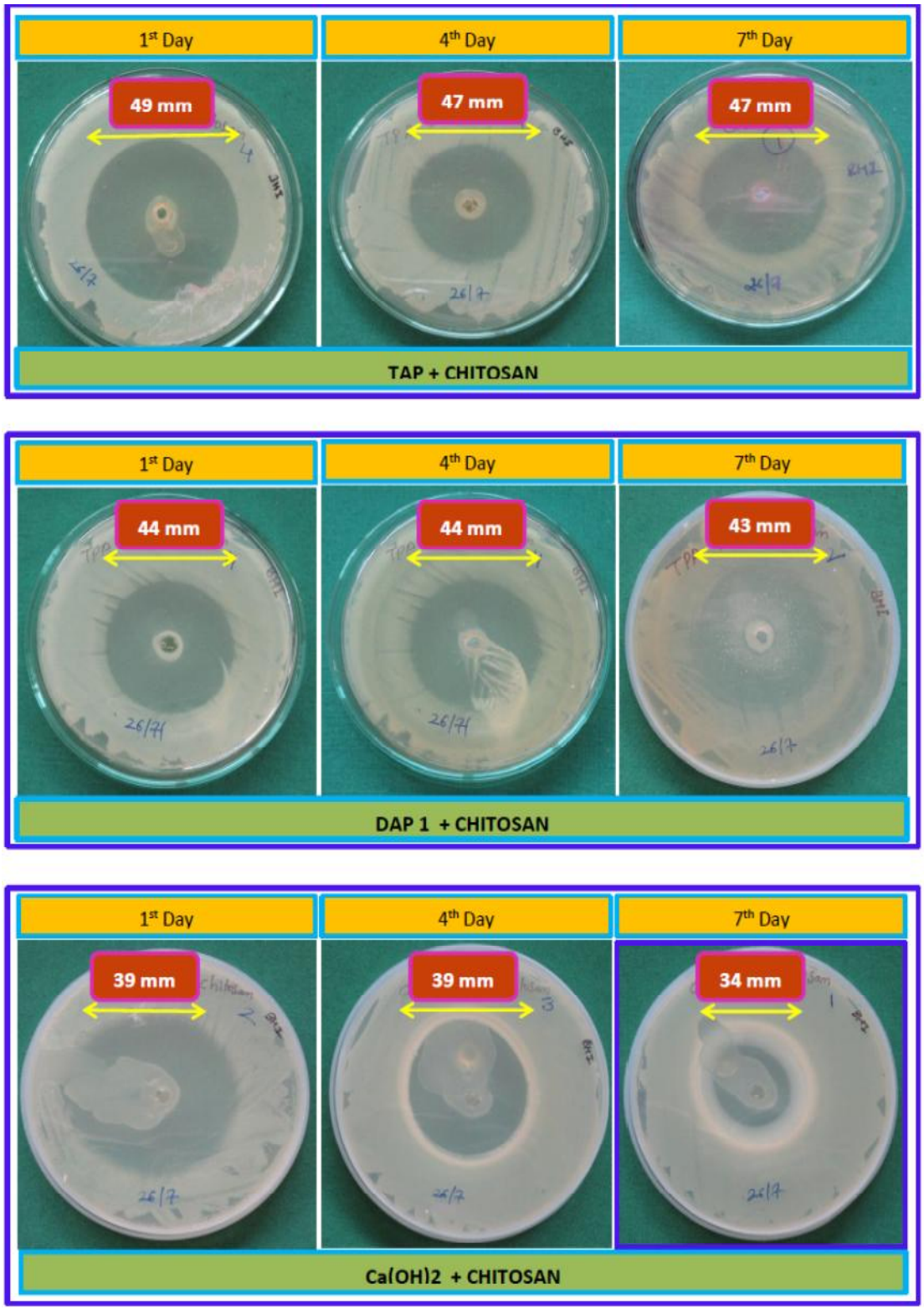

Fig. 2. Clinical representation of all mean zones of diameter in "mm" of various intracanal medicaments on the first, fourth & seventh day by chitosan treatment

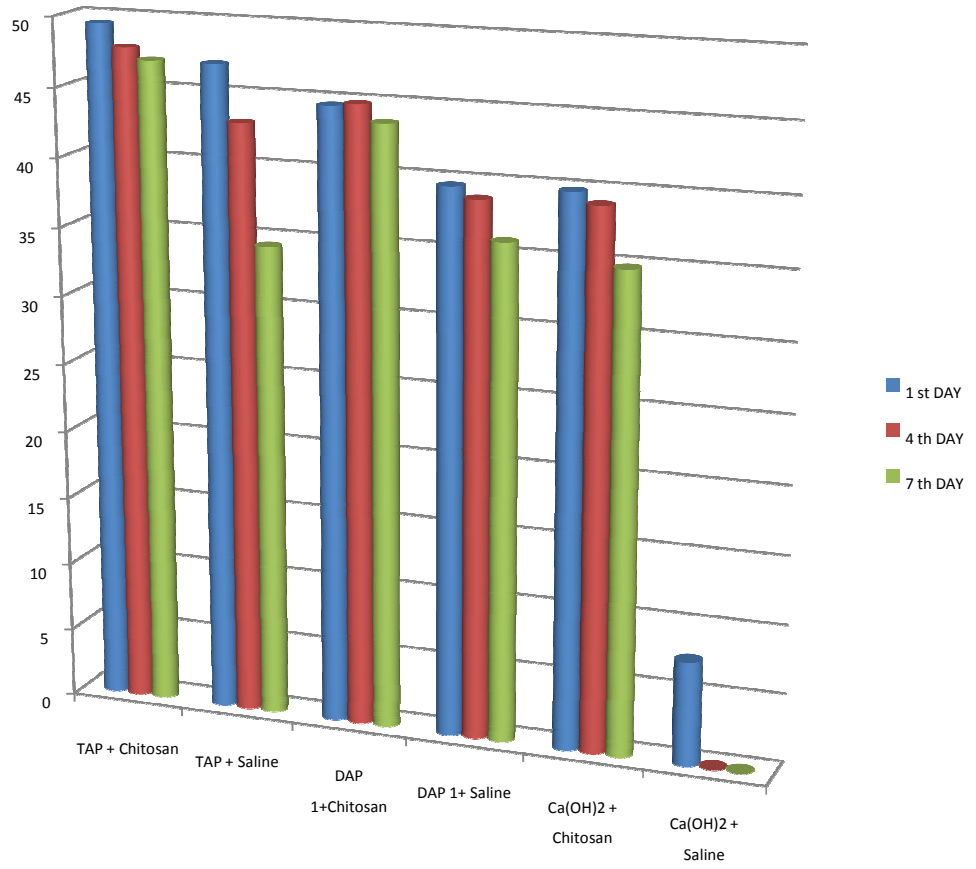

Fig. 3. Graphical representation of mean zone of diameter in "mm" of various intracanal medicaments on the first day, fourth day & seventh day

Bose et al. stated that when $Ca(OH)_2$ and TAP were used as intracanal medicaments in immature necrotic teeth, they showed the pulp dentin complex development [33]. Sato et al. stated that TAP destroys bacteria in deep areas of root canals [34]. Another study done by Sonali et al. stated that nonsurgical healing of large periapical lesions when TAP is used as an intracanal medicament [35]. This present study's findings demonstrated that TAP + chitosan combination had shown higher antibacterial activity against *E. faecalis.*

This study showed triple antibiotic paste mixed with Chitosan showed maximum inhibitory zone whereas minimum with DAP 1 + Saline and Chitosan, calcium hydroxide + saline, and Chitosan. This finding confirms the study conducted by Madhubala et al., in which propolis and TAP showed higher antibacterial effects than Calcium hydroxide on *E. faecalis* [19]. It also shows that Chitosan used as a versatile drug delivery agent that can enhance antimicrobial effects — so coinciding with studies conducted by Jayakumar, dai, et al. [36].

In this study, triple antibiotic paste with Chitosan showed the largest inhibitory zone for *E. faecalis.* It is due to the presence of different constituents in these paste. Hence, furtherstudies on the microbial species to be done to determine the antibacterial effect of intracanal medicaments and gel form for better prognosis.

5. CONCLUSION

Complete eradication of microbes in the root canal system is an impossible task, while reducing the microbes to the maximum helps in the endodontic therapy's long-lasting success. Under the limitations of this *in vitro* study, calcium hydroxide and Chitosan were not as

effective as TAP. A combination of TAP + Chitosan produced better results than the combination of medicaments with TAP 1 + Saline and Chitosan, $Ca(OH)_2$+Chitosan, and saline. The findings of this present in vitro study demonstrated that combining TAP with Chitosan as a carrier had an excellent antimicrobial effect against *E. faecalis.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

Before carrying out the present in vitrostudy, institutional ethics committee approval was obtained from the college.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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