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Original Article

Dentistry Section

Evaluation of Antibacterial Properties, Cytotoxicity and Setting Time of an Epoxy Resin-based Root Canal Sealer After Incorporation of Chitosan Nanoparticles at Different Concentrations: An In-vitro Study

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ABSTRACT

Introduction: Persistence of microorganisms within the root canal system is a major cause of endodontic failure, with *Enterococcus faecalis* commonly implicated in post-treatment disease owing to its ability to survive under adverse conditions and penetrate dentinal tubules. Although Epoxy Resin-based (ERB) root canal sealers possess favourable sealing properties, their antibacterial efficacy is limited and diminishes over time. Incorporation of bioactive nanoparticles such as chitosan may enhance antimicrobial activity while maintaining biocompatibility.

Aim: To evaluate the setting time, antibacterial properties and cytotoxicity of chitosan nanoparticles in combination with a root canal sealer across various concentrations (0%, 10%, 20%, 30%) of chitosan nanoparticles.

Materials and Methods: An in-vitro experimental study was conducted at Rajiv Gandhi Institute of Information Technology and Biotechnology and Katraj Metallurgical Laboratory, Pune, Maharashtra, India from July 2025 to December 2025. A total of four groups were included based on chitosan nanoparticle concentrations (0%, 10%, 20%, and 30%) incorporated into an epoxy resin–based sealer. The inclusion criteria comprised standardised sample preparation and the use of *Enterococcus faecalis* {American Type Culture Collection (ATCC) 29212}. Antibacterial activity was evaluated using the agar diffusion test, cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on Vero cell lines, and setting time using the Gilmore needle test. Data were analysed using the Kolmogorov–Smirnov test and One-way Analysis of Variance (ANOVA) followed by post-hoc Tukey test, with significance set at $p<0.05$.

Results: Chitosan incorporation demonstrated a concentration-dependent antibacterial effect against *Enterococcus faecalis*. The control group (0%) and the 10% chitosan group exhibited no zone of inhibition (0 mm), indicating absence of antibacterial activity. In contrast, the 20% and 30% chitosan groups showed measurable zones of inhibition, which were significantly greater than both the control group and the 10% chitosan group ($p<0.05$). Among all groups, the 30% chitosan group demonstrated the highest antibacterial efficacy ($p<0.05$). Cytotoxicity analysis revealed significantly higher cell viability in chitosan-modified groups compared to the control ($p<0.05$). The 30% group demonstrated the longest setting time, whereas the 10% group showed the shortest setting time ($p<0.05$). All groups exhibited setting times within International Organisation for Standardisation (ISO) recommended limits.

Conclusion: Chitosan nanoparticle incorporation significantly enhanced the antibacterial activity and cytocompatibility of the ERB sealer, without adversely affecting setting time at specific concentrations. This modification shows potential for improving the biological performance of endodontic sealers.

Keywords: Biocompatible materials, Cell survival, Dental materials, Drug delivery systems, Root canal filling materials

INTRODUCTION

A successful root canal obturation procedure depends on achieving a three-dimensional seal of the canal system to entomb residual microorganisms and prevent coronal and apical leakage. Root canal sealers are used in combination with gutta-percha to fill canal irregularities and create an interface between the core material and the dentinal walls [1,2]. The ideal root canal sealer should be biocompatible, possess antimicrobial properties, demonstrate adequate flow and sealing ability, exhibit low solubility in tissue fluids, and prevent tooth discolouration [3].

The ERB sealers are commonly used in endodontic therapy because of their favourable physicochemical properties, including efficient apical sealing, low solubility, and good adhesion to root canal dentin [4]. However, a study has reported that ERB sealers exhibit limited inherent antibacterial activity, which may compromise their effectiveness against persistent endodontic microorganisms [5]. *Enterococcus faecalis* is frequently isolated from cases of post-treatment apical periodontitis and is known for its resistance to intracanal disinfection procedures and its ability to survive in nutrient-deprived conditions [6].

The incorporation of nanoparticles into dental materials has emerged as a promising strategy to enhance antimicrobial properties and improve dentin interaction [7]. Chitosan, a biocompatible and biodegradable polysaccharide, has demonstrated significant antibacterial activity against endodontic pathogens. Previous studies have shown that chitosan nanoparticles can effectively inhibit *E. faecalis* while maintaining favourable biological properties [8,9].

The incorporation of chitosan nanoparticles into ERB sealers may enhance antibacterial efficacy; however, any modification should not adversely affect critical physical properties such as setting time and biocompatibility, particularly when the material is extruded into periapical tissues. Root canal obturation materials should comply with the standards established by the International Organisation for Standardisation (ISO 6876) to ensure adequate physicochemical

and biological performance for clinical use [10]. These standards specify essential properties such as setting time, flow, solubility, and biocompatibility, which are critical for the long-term success of endodontic treatment. Any modification to root canal sealers must therefore be carefully evaluated to ensure that these properties are not adversely affected.

The selection of nanoparticle concentrations (10%, 20%, and 30%) in the present study was based on previously reported ranges demonstrating a concentration-dependent antibacterial effect while maintaining acceptable physicochemical properties. Lower concentrations may be insufficient to produce significant antimicrobial activity, whereas higher concentrations may adversely affect the material properties [8].

Therefore, the aim of the present study was to evaluate the effect of incorporating chitosan nanoparticles at different concentrations (0%, 10%, 20%, and 30%) into an epoxy resin-based root canal sealer on antibacterial activity, cytotoxicity, and setting time.

Specifically, the study assessed the antibacterial efficacy of the modified sealer against *Enterococcus faecalis*, evaluated its cytotoxicity using Vero cell lines, and determined the influence of nanoparticle incorporation on the setting time of the sealer. It was hypothesised that the incorporation of chitosan nanoparticles would have no significant effect on these properties (null hypothesis), whereas the alternative hypothesis proposed that the incorporation would result in a significant effect on antibacterial activity, cytotoxicity, and setting time.

MATERIALS AND METHODS

This in-vitro experimental study was conducted from July 2025 to December 2025. The antibacterial and cytotoxicity tests were carried out at Rajiv Gandhi Institute of Information Technology and Biotechnology, while the setting time evaluation was performed at Katraj Metallurgical Laboratory, Pune, Maharashtra, India Approval was obtained from the Institutional Research Committee (IRC No: BVDU/DCH/292/2024-25) prior to commencement of the study.

Inclusion and Exclusion criteria: The inclusion criteria comprised standardised preparation of samples using Teflon moulds and evaluation against *Enterococcus faecalis* {American Type Culture Collection (ATCC) 29212}. Samples with visible defects, improper setting, or contamination were excluded.

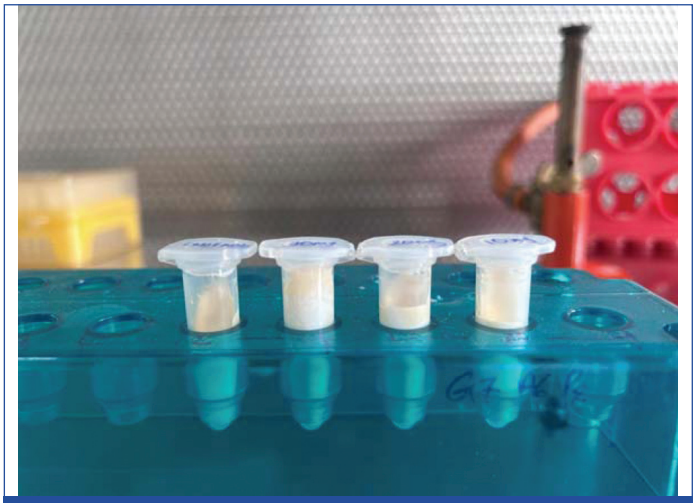
The sample size was determined based on previously published in-vitro studies evaluating similar parameters, which utilised comparable group sizes for adequate statistical analysis. A total of 132 samples were included, with 44 samples allocated to each experimental assessment (antibacterial activity, cytotoxicity, and setting time), and further subdivided into four groups (n=11 per group). A convenience sampling technique was employed, and the study was time-bound, wherein all samples prepared during the study period were included.

The chitosan nanoparticles used in present study were procured from Nano Research Lab (International Organisation for Standardisation (ISO), World Health Organisation (WHO)-GMP (Good Manufacturing Practices), Food and Drug Administration (FDA), and Conformité Européenne (CE) certified. As per manufacturer specifications, the nanoparticles had a particle size ranging between 50-100 nm, with a polydispersity index <0.3 and a degree of deacetylation >85%. A total of 132 samples were fabricated using Teflon moulds (5x2 mm), in accordance with previously published methodologies and ISO recommendations to ensure standardisation.

Sample size calculation: The sample size was estimated based on data from a previous study by Ratih DN et al., which evaluated the antibacterial activity of a root canal sealer modified with chitosan nanoparticles using a similar experimental model [8]. Considering the expected Standard Deviations (SD) of the two groups to be 1.07 and 1.43, a mean difference of 2.34, an alpha error of 0.05, and a study power of 99%, the minimum sample size was calculated to be 11 samples per group.

Study Procedure

The samples were equally distributed into three experimental assessments (44 samples each), and each assessment was further divided into four groups (n=11): Group I (control) received epoxy resin–based sealer (AH Plus, Dentsply DeTrey, Germany) without chitosan nanoparticles; Group II received sealer with 10% chitosan nanoparticles; Group III received sealer with 20% chitosan nanoparticles; and Group IV received sealer with 30% chitosan nanoparticles. A simple random sampling technique was used to allocate the samples into the respective groups.The concentrations were prepared on a weight/weight (w/w) basis by incorporating 10 mg, 20 mg, and 30 mg of chitosan nanoparticles into 100 mg of sealer to obtain 10%, 20%, and 30% formulations, respectively. The mixtures were prepared aseptically using a standardised spatulation technique, and homogeneity was ensured by continuous mixing for 60 minutes until a uniform paste was obtained. The uniformity of the final mixtures was verified by visual and tactile assessment, confirming consistent colour, smooth texture, and absence of visible agglomerates or particulate clustering within the sealer matrix. Group I received unmodified sealer [Table/Fig-1].



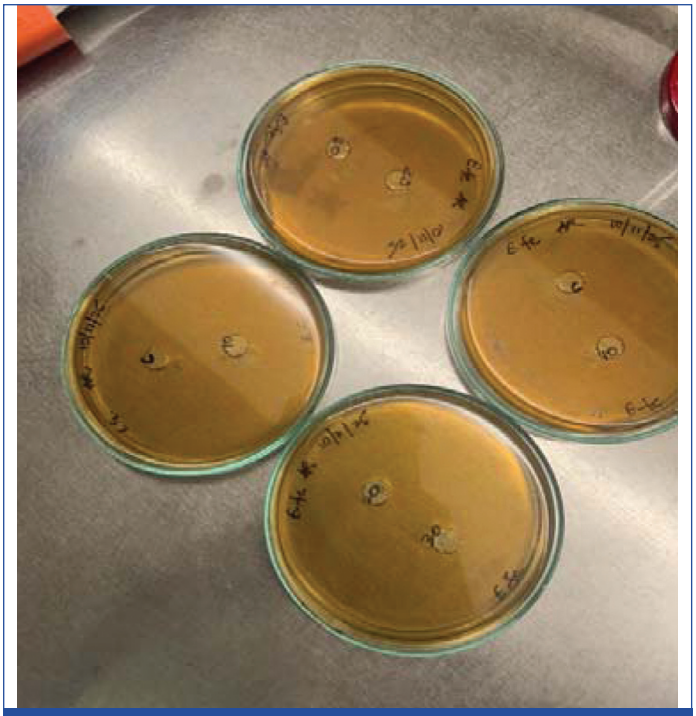
[Table/Fig-1]: Sample preparation illustrating the four experimental groups.

Antibacterial Assessment: For antibacterial evaluation, 44 samples (n=11 per group) were tested using the agar well diffusion method against *Enterococcus faecalis* {American Type Culture Collection (ATCC) 29212}. A bacterial suspension equivalent to 0.5 McFarland standard {($1x10^8$ Colony Forming Unit (CFU)} was prepared from a 24-hour culture grown on Brain Heart Infusion agar and adjusted using a spectrophotometer. Mueller-Hinton agar plates were uniformly inoculated using sterile swabs and allowed to stand for 10-15 minutes at room temperature to facilitate uniform bacterial adherence.

Subsequently, four wells (6 mm in diameter and 4 mm in depth) were created in each plate using a sterile glass tube, and 50 µL of the experimental sealer was immediately introduced into each well [Table/Fig-2]. The plates were then incubated at 37°C for 24 hours, after which the zones of inhibition were measured using a digital sliding caliper with a precision of 0.02 mm. The agar diffusion method was performed in accordance with previously established protocols for antimicrobial testing [11].

Cytotoxicity Assessment: For cytotoxicity analysis, 44 samples (n=11 per group) were prepared using Teflon moulds and allowed to set for 24 hours at 37°C. The set samples were used for sealer extract preparation without pulverisation to maintain standardised surface area and avoid exaggerated cytotoxic effects.

The extracts were prepared in accordance with ISO guidelines by immersing the samples in Dulbecco's Modified Eagle Medium



[Table/Fig-2]: Mueller-Hinton agar plates with wells prepared for the agar well diffusion assay to evaluate antibacterial activity.

at a standardised surface area-to-volume ratio of 3 cm^2/mL and incubated at 37°C for 24 hours.

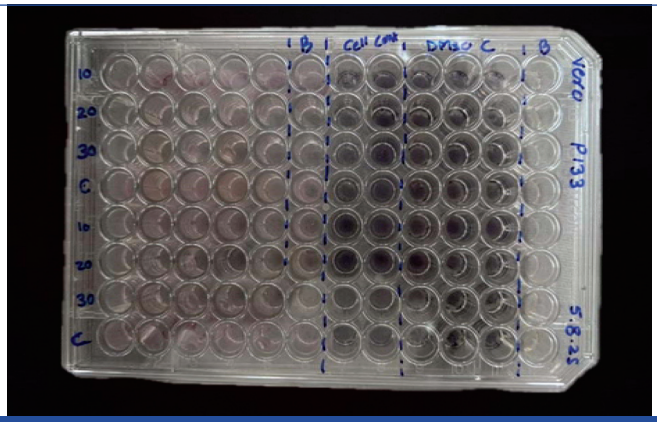
Vero cells (procured from National Centre for Cell Science (NCCS), Pune, India) were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced every three days. Cells were harvested using Trypsin-Ethylenediamine Tetracetic Acid (EDTA), and viable cell count was determined using Trypan Blue dye exclusion method with a Neubauer chamber.

Cells were seeded at a density of $1x10^4$ cells per well in 96-well plates and incubated for 24 hours to allow adequate cell attachment and formation of a subconfluent monolayer [Table/Fig-3]. All procedures were performed under aseptic conditions in a laminar airflow cabinet.

All procedures were performed by a single calibrated operator under aseptic conditions in a laminar airflow cabinet. Sterility was maintained using standard aseptic protocols; ultraviolet irradiation was not employed to avoid potential alteration of material properties and culture conditions.

Sealer extracts were prepared in accordance with International Organisation for Standardisation (ISO) 10993-12 guidelines, maintaining a standardised surface area-to-volume ratio of 3 cm^2/mL, and incubated at 37°C for 24 hours.

The cell culture medium was then discarded and replaced with 100 µL of the respective sealer extract for each group, while fresh culture medium served as the negative control. The plates were further incubated for 24 hours prior to cytotoxicity evaluation.



[Table/Fig-3]: Total 96-well microtiter plates used for cytotoxicity assessment.

MTT Assay: Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay, which reflects mitochondrial metabolic activity of viable cells. The sealer extracts were incubated with the cells for 24 hours. Following incubation, 10 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated at 37°C for three hours to allow formation of formazan crystals. The culture medium was then carefully removed, and 100 µL of Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the crystals.

The Optical Density (OD) was measured using an Enzyme-linked Immunosorbent Assay (ELISA) reader at a wavelength of 570 nm. The percentage of cell viability was calculated using the following formula:

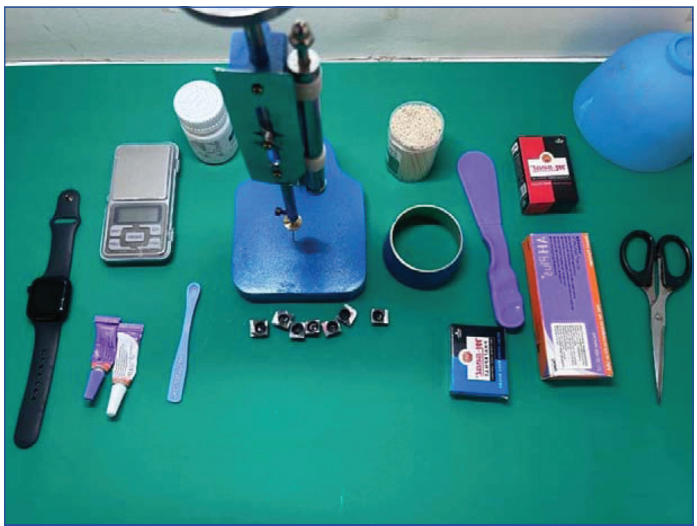
Cell viability (%) = (OD of experimental group/OD of control group) × 100

The MTT assay procedure and calculation method were performed in accordance with established protocols [12].

Setting Time Evaluation: For the evaluation of setting time, forty-four samples (n=11 per group) were prepared using an epoxy resin-based sealer (AH Plus, Dentsply DeTrey, Germany). The sealer was dispensed in equal proportions of base paste and catalyst paste (1:1 ratio) onto a clean glass slab and mixed using a stainless steel spatula according to the manufacturer's instructions. Chitosan nanoparticles were incorporated into the sealer at concentrations of 10%, 20%, and 30% (w/w), and the mixture was blended using a standardised spatulation technique for 60 minutes to ensure uniform distribution and homogeneity. The mixed material was immediately transferred into metallic moulds of dimensions 5x2 mm.

The samples were maintained at 37°C and 95% relative humidity. The initial and final setting times were determined using a Gilmore needle apparatus in accordance with ISO standards. The initial setting time was assessed using a Gilmore needle of 100 g weight with a tip diameter of 2 mm, while the final setting time was determined using a needle of 456 g weight with a tip diameter of 1 mm.The needles were applied perpendicular to the sample surface at regular intervals (approximately every 5 minutes) until no indentation was observed [Table/Fig-4] [13].

The initial setting time was defined as the time elapsed from the start of mixing until the material exhibited sufficient resistance to indentation by the 100 g needle, indicating the onset of setting. The final setting time was recorded when the 456 g needle failed to produce any visible indentation on the surface of the material, indicating complete setting. The procedure was carried out in



[Table/Fig-4]: Gilmore needle apparatus employed for the determination of initial and final setting times of the experimental sealers.

accordance with ISO 6876 specifications for root canal sealing materials [13].

STATISTICAL ANALYSIS

Data were entered and tabulated using Microsoft Excel (Version 2016) and analysed using Statistical Package for Social Sciences (SPSS) software (IBM Corp., version 26.0). Descriptive statistics were computed for all variables and expressed as mean±SD, along with minimum and maximum values. Normality of data distribution was assessed using the Kolmogorov-Smirnov test. Cell viability values were obtained as proportional data (range: 0-10) and used in this form for inferential analysis. For ease of interpretation, these values were converted into percentages (×100) and presented in descriptive tables. Intergroup comparisons among the four groups were performed using ANOVA. When a statistically significant difference was observed, post hoc pair-wise comparisons were carried out using Tukey's Honestly Significant Difference (HSD) test. All statistical tests were two-tailed, with a confidence interval of 95%, and a p-value<0.05 was considered statistically significant.

RESULTS

The descriptive statistics of the antibacterial property measured as the zone of inhibition (in mm) across four groups with varying concentrations of chitosan nanoparticles has been depicted in [Table/Fig-5]. The control group (0%) and 10% chitosan nanoparticle group showed no antibacterial activity, with a mean zone of inhibition of 0.00 mm. In contrast, the 20% chitosan nanoparticle group demonstrated a mean zone of inhibition of 17.36±1.21 mm, while the 30% group exhibited the highest antibacterial activity with a mean of 27.45±0.82 mm. This indicates a concentration-dependent increase in antibacterial efficacy with higher percentages of chitosan nanoparticles.

Groups	n	Minimum	Maximum	Mean±SD
Group 1: 0% chitosan nanoparticle (control group)	11	0	0	0
Group 2: 10% chitosan nanoparticles	11	0	0	0
Group 3: 20% chitosan nanoparticles	11	16.000	20.000	17.363±1.206
Group 4: 30% chitosan nanoparticles	11	26.000	29.000	27.454±0.820

[Table/Fig-5]: Descriptive statistics of antibacterial property (zone of inhibition in mm) in different groups.
SD: Standard Deviation; mm: millimeter; values expressed as mean±standard deviation.

The overall intergroup comparison using One-way ANOVA has been depicted in [Table/Fig-6]. The results revealed a statistically highly significant difference among the groups (F=3813.262, p=0.001), indicating that at least one group differs significantly from the others in terms of antibacterial activity.

Comparison groups	Sum of squares	df	Mean square	F-value	p-value
Group 1: 0% chitosan nanoparticle (control group) vs Group 2: 10% chitosan nanoparticles vs Group 3: 20% chitosan nanoparticles vs Group 4: 30% chitosan nanoparticles	6083.886	3	2027.962	3813.262	0.001

[Table/Fig-6]: Intergroup comparison of antibacterial property (zone of inhibition in mm) between different groups.
ANOVA: Analysis of Variance; F: F-statistic value; p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant

The pair-wise multiple comparisons between groups has been depicted in [Table/Fig-7]. There was no significant difference between the control group (0%) and the 10% chitosan nanoparticle group (p=1.000), confirming the absence of antibacterial activity in both groups. However, both 20% and 30% chitosan nanoparticle groups showed statistically significant differences when compared to the control and 10% groups (p<0.001), indicating markedly higher antibacterial activity. Additionally, a statistically significant difference was observed between the 20% and 30% groups (p <0.001), with the 30% group demonstrating superior antibacterial efficacy. Overall, the findings suggest that chitosan nanoparticles exhibit significant antibacterial properties at higher concentrations, with 30% concentration showing the greatest zone of inhibition. This suggests a dose-dependent increase in antibacterial activity with increasing concentration of chitosan nanoparticles.

(I) Groups	(J) Groups	Mean difference (I-J)	p-value
Group 1: 0% chitosan nanoparticle (control group)	Group 2: 10% chitosan nanoparticles	0.000000	1.000
	Group 3: 20% chitosan nanoparticles	-17.363636*	0.001*
	Group 4: 30% chitosan nanoparticles	-27.454545*	p<0.001
Group 2: 10% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	0.000000	1.000
	Group 3: 20% chitosan nanoparticles	-17.363636*	p<0.001
	Group 4: 30% chitosan nanoparticles	-27.454545*	0.0008*
Group 3: 20% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	17.363636*	0.001*
	Group 2: 10% chitosan nanoparticles	17.363636*	p<0.001
	Group 4: 30% chitosan nanoparticles	-10.090909*	p<0.001
Group 4: 30% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	27.454545*	p<0.001
	Group 2: 10% chitosan nanoparticles	27.454545*	p<0.001
	Group 3: 20% chitosan nanoparticles	10.090909*	p<0.001

[Table/Fig-7]: Pair-wise multiple intergroup comparison of antibacterial property (zone of inhibition in mm) between different groups.
Tukey test: Post-hoc multiple comparison test; mm: millimeter
*p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant

The descriptive statistics of cell viability expressed as relative metabolic activity (%) across the four groups, with the control group standardised to 100% has been depicted in [Table/Fig-8]. The control group showed a mean value of 100%, with a range of 90.1% to 118.9%.

Groups	n	Mean viability (%)	Standard Deviation	Minimum (%)	Maximum (%)
Group 1: 0% chitosan nanoparticle (control group)	11	100	10.8	90.1	118.9
Group 2: 10% chitosan nanoparticles	11	245	53.2	189.2	351.4
Group 3: 20% chitosan nanoparticles	11	316	57.6	218.0	378.4
Group 4: 30% chitosan nanoparticles	11	685	121.5	441.4	801.8

[Table/Fig-8]: Descriptive statistics of cell viability (%) in different groups.
SD: Standard deviation
Note: Cell viability values were analysed as proportions (0-10 scale) for statistical testing and are presented here as percentages (×100) for ease of interpretation.

The 10% group exhibited a mean value of 245%, with a wider range (189.2%-351.4%), indicating enhanced metabolic activity compared to the control. Similarly, the 20% group demonstrated a mean value of 316% (218.0%-378.4%).

The highest values were observed in the 30% group, with a mean of 685% and a range of 441.4% to 801.8%, along with the greatest variability (SD=121.5). These elevated values reflect increased mitochondrial metabolic activity as measured by the MTT assay and should be interpreted as relative changes compared to the control rather than absolute cell viability.

The present intergroup comparison of cell viability (%) among the four groups using one-way ANOVA. The analysis revealed a statistically highly significant difference among the groups (F=152.6, p<0.001), indicating that varying concentrations of chitosan nanoparticles significantly influence cell viability has been depicted in [Table/Fig-9,10].

Comparison groups	Sum of squares	df	Mean square	F-value	p-value
Group 1: 0% chitosan nanoparticle (control group) vs Group 2: 10% chitosan nanoparticles vs Group 3: 20% chitosan nanoparticles vs Group 4: 30% chitosan nanoparticles	2.934	3	0.978	152.6	<0.001*

[Table/Fig-9]: Intergroup comparison of cell viability (%) between different groups.
ANOVA: Analysis of Variance; F: F-statistic value; p<0.05 considered statistically significant.
Note - Analysis performed on proportional (0-10) scale values.

(I) Group	(J) Group	Mean difference (I-J)	p-value
Group 1: 0% chitosan nanoparticle (control group)	Group 2: 10% chitosan nanoparticles	-0.161	<0.001*
	Group 3: 20% chitosan nanoparticles	-0.240	<0.001*
	Group 4: 30% chitosan nanoparticles	-0.649	<0.001*
Group 2: 10% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	0.161	<0.001*
	Group 3: 20% chitosan nanoparticles	-0.079	0.002*
	Group 4: 30% chitosan nanoparticles	-0.488	<0.001*
Group 3: 20% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	0.240	<0.001*
	Group 2: 10% chitosan nanoparticles	0.079	0.002*
	Group 4: 30% chitosan nanoparticles	-0.409	<0.001*
Group 4: 30% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	0.649	<0.001*
	Group 2: 10% chitosan nanoparticles	0.488	<0.001*
	Group 3: 20% chitosan nanoparticles	0.409	<0.001*

[Table/Fig-10]: Pair-wise multiple Intergroup comparison of cell viability (%) between different groups.
*p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant.
Note: Mean differences are expressed on proportional (0-10) scale.

The descriptive statistics of initial and final setting times across the four groups has been depicted in [Table/Fig-11]. The 10% chitosan nanoparticle group exhibited the lowest mean initial setting time (531.45±32.25 minutes), indicating a faster setting reaction compared to the other groups. The control group (0%) showed a moderate initial setting time (598.00±43.15 minutes), while the 20% (626.90±24.14 minutes) and 30% (682.36±19.32 minutes) groups demonstrated progressively higher initial setting times.

A similar trend was observed for final setting time, where the 10% group showed the shortest time (947.18±38.66 minutes), followed by the control group (1056.81±131.14 minutes), while the 20% (1252.45±26.78 minutes) and 30% (1385.36±16.31 minutes) groups showed substantially prolonged setting times. These findings suggest that lower concentrations may accelerate the setting reaction, whereas higher concentrations tend to delay it.

The intergroup comparison using One-way ANOVA has been depicted in [Table/Fig-12]. A statistically significant difference was observed among the groups for both initial setting time (F=44.991, p=0.001) and final setting time (F=85.931, p=0.002), indicating that

Groups	Setting time	n	Minimum (minutes)	Maximum (minutes)	Mean±SD
Group 1: 0% chitosan nanoparticle (control group)	Initial	11	550.00	679.00	598.00±43.148
	Final	11	901.00	1232.0	1056.81±131.137
Group 2: 10% chitosan nanoparticles	Initial	11	500.00	586.00	531.45±32.250
	Final	11	898.00	1002.0	947.18±38.659
Group 3: 20% chitosan nanoparticles	Initial	11	581.00	655.00	626.90±24.143
	Final	11	1217.0	1298.0	1252.45±26.778
Group 4: 30% chitosan nanoparticles	Initial	11	650.00	701.00	682.36±19.324
	Final	11	1345.0	1400.0	1385.36±16.311

[Table/Fig-11]: Descriptive statistics of initial and final setting time in different groups.
SD: Standard deviation; min: minutes; values expressed as mean±standard deviation

Comparison groups	Setting time	Sum of squares	df	Mean square	F value	p-value
Group 1: 0% chitosan nanoparticle (control group) vs Group 2: 10% chitosan nanoparticles vs Group 3: 20% chitosan nanoparticles vs Group 4: 30% chitosan nanoparticles	Initial	130189.364	3	43396.455	44.991	0.001*
	Final	1268012.364	3	422670.788	85.931	0.002*

[Table/Fig-12]: Intergroup comparison of setting time between different groups.
*p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant

the variation in chitosan nanoparticle concentration significantly influences setting characteristics.

The pair-wise comparisons for initial setting time has been depicted in [Table/Fig-13]. The 10% group showed a significantly shorter setting time compared to all other groups (p<0.001). The 30% group demonstrated a significantly longer setting time compared to all groups (p<0.001). The difference between the control group and 20% group was also statistically significant (p=0.045), indicating a gradual increase in setting time with increasing concentration beyond 10%.

The pair-wise comparisons for final setting time. All intergroup comparisons were statistically significant (p<0.01 or p<0.001) has been depicted in [Table/Fig-14]. The 10% group consistently showed significantly reduced final setting time compared to all

(I) Groups	(J) Groups	Mean difference (I-J)	p-value
Group 1: 0% chitosan nanoparticle (control group)	Group 2: 10% chitosan nanoparticles	66.54545*	p<0.001
	Group 3: 20% chitosan nanoparticles	-28.90909	0.045*
	Group 4: 30% chitosan nanoparticles	-84.36364*	p<0.001
Group 2: 10% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	-66.54545*	p<0.001
	Group 3: 20% chitosan nanoparticles	-95.45455*	p<0.001
	Group 4: 30% chitosan nanoparticles	-150.90909*	p<0.001
Group 3: 20% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	28.90909	0.045*
	Group 2: 10% chitosan nanoparticles	95.45455*	p<0.001
	Group 4: 30% chitosan nanoparticles	-55.45455*	0.001*
Group 4: 30% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	84.36364*	p<0.001
	Group 2: 10% chitosan nanoparticles	150.90909*	p<0.001
	Group 3: 20% chitosan nanoparticles	55.45455*	0.001*

[Table/Fig-13]: Pair-wise multiple intergroup comparison of initial setting time between different groups.
*p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant

(I) Groups	(J) Groups	Mean difference (I-J)	p-value
Group 1: 0% chitosan nanoparticle (control group)	Group 2: 10% chitosan nanoparticles	109.63636*	0.004*
	Group 3: 20% chitosan nanoparticles	-195.63636*	p<0.001
	Group 4: 30% chitosan nanoparticles	-328.54545*	p<0.001
Group 2: 10% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	-109.63636*	0.004*
	Group 3: 20% chitosan nanoparticles	-305.27273*	p<0.001
	Group 4: 30% chitosan nanoparticles	-438.18182*	p<0.001
Group 3: 20% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	195.63636*	p<0.001
	Group 2: 10% chitosan nanoparticles	305.27273*	p<0.001
	Group 4: 30% chitosan nanoparticles	-132.90909*	p<0.001
Group 4: 30% chitosan nanoparticles	Group 1: 0% chitosan nanoparticle (control group)	328.54545*	p<0.001
	Group 2: 10% chitosan nanoparticles	438.18182*	p<0.001
	Group 3: 20% chitosan nanoparticles	132.90909*	p<0.001

[Table/Fig-14]: Pair-wise multiple intergroup comparison of final setting time between different groups.
*p-value <0.05 statistically significant, <0.01 highly significant, <0.001 very highly significant

other groups, while the 30% group exhibited the highest setting time, significantly differing from all other groups. The 20% group showed intermediate values but was still significantly different from both lower and higher concentration groups. Overall, the results demonstrate a concentration-dependent effect of chitosan nanoparticles on setting time. Lower concentration (10%) accelerates both initial and final setting times, whereas higher concentrations (20% and 30%) significantly prolong the setting process.

DISCUSSION

The present study was designed to test the null hypothesis that the incorporation of chitosan nanoparticles at varying concentrations would not significantly affect the antibacterial activity, cytotoxicity, or setting time of the epoxy resin-based root canal sealer. However, the results demonstrated a clear concentration-dependent effect on all evaluated parameters. Therefore, the null hypothesis was rejected, as significant differences were observed in antibacterial efficacy, cell viability, and setting time among the experimental groups.

The present in-vitro study evaluated the effect of incorporating chitosan nanoparticles at different concentrations (0%, 10%, 20%, and 30%) into an epoxy resin-based root canal sealer on its antibacterial activity against *Enterococcus faecalis*, cytotoxicity, and setting time. The findings demonstrated a concentration-dependent influence on both the biological and physicochemical properties of the sealer.

With respect to antibacterial activity, a statistically significant increase in the zone of inhibition was observed with increasing concentrations of chitosan nanoparticles, with the 20% and 30% groups showing significantly greater efficacy compared to the control and 10% groups (p<0.001). The absence of antibacterial activity in the control and 10% groups suggests that lower concentrations of chitosan may be insufficient to exert a measurable antimicrobial effect under agar diffusion conditions. The enhanced antibacterial activity at higher concentrations can be attributed to the polycationic nature of chitosan, which facilitates interaction with negatively charged bacterial cell membranes, resulting in increased membrane permeability, leakage of intracellular components, and subsequent cell death [14].

Enterococcus faecalis is a well-recognised pathogen associated with persistent endodontic infections due to its resistance to adverse environmental conditions and its ability to form biofilms [15]. The observed dose-dependent antibacterial activity is therefore of considerable clinical relevance, indicating improved microbial control within the root canal system. These findings are consistent with previous studies on nanoparticle-modified endodontic materials, where incorporation of antibacterial nanoparticles significantly enhanced antimicrobial efficacy [16]. Furthermore, Ratih DN et al., reported improved antibacterial properties of epoxy resin–based sealers modified with chitosan nanoparticles, supporting the results of the present study [8].

In terms of cytotoxicity, a significant increase in cell viability was observed with increasing concentrations of chitosan nanoparticles, indicating reduced cytotoxicity. The control group demonstrated comparatively lower cell viability, whereas the 30% group exhibited the highest values. Values exceeding 100% indicate enhanced cellular metabolic activity relative to the control, reflecting increased cell proliferation. The improved cytocompatibility of chitosan-modified sealers may be attributed to its inherent biocompatibility, biodegradability, and bioadhesive properties [17].

Additionally, chitosan may form a protective polymeric coating over the sealer surface, thereby limiting the diffusion of unreacted epoxy resin components and reducing cytotoxic effects. Its buffering capacity and interaction with cellular membranes may further promote favourable cell–material interactions and cellular proliferation [18,19]. These findings are in agreement with previous studies demonstrating improved biocompatibility of chitosan-containing biomaterials and nanoparticle-modified sealers [8,16,17].

Regarding setting time, a concentration-dependent increase in both initial and final setting times was observed, with the 10% group exhibiting the shortest setting time and the 30% group the longest. This prolongation may be attributed to the interference of chitosan nanoparticles with the polymerisation kinetics of the epoxy resin matrix. The presence of nanoparticles may hinder the mobility of reactive components and reduce cross-linking efficiency, thereby delaying the setting reaction [20,21]. Similar observations have been reported in studies evaluating nanoparticle incorporation in dental materials, where modifications altered setting characteristics without compromising clinical acceptability [16].

Importantly, all groups demonstrated setting times within the limits prescribed by ISO 6876, indicating that the modifications did not adversely affect clinical usability. From a clinical perspective, a moderate increase in setting time may be advantageous by providing extended working time during obturation procedures.

The incorporation of chitosan nanoparticles into epoxy resin-based sealers may therefore enhance antimicrobial efficacy while simultaneously improving biocompatibility. This is particularly relevant in cases of persistent endodontic infections and in situations involving sealer extrusion into periapical tissues, where reduced cytotoxicity and improved cellular response may contribute to favourable healing outcomes [22].

In-vivo studies and randomised clinical trials are required to validate the clinical applicability and long-term success of these modified materials. Future studies should focus on assessing long-term performance, including sealing ability, durability, and behaviour under simulated clinical conditions.

Limitation(s)

The present study has certain limitations that should be considered while interpreting the results. The present study was conducted under in-vitro conditions, which may not fully replicate the complex biological environment of the root canal system. Factors such as long-term solubility, dimensional stability, interaction with dentinal

fluids and mechanical stresses were not evaluated. The antibacterial activity was evaluated using the agar diffusion method, which is dependent on the diffusibility of the material and may not accurately represent antimicrobial effectiveness within the root canal space.

CONCLUSION(S)

Within the limitations of present in-vitro study, the addition of chitosan nanoparticles to an ERB root canal sealer resulted in a substantial enhancement of antibacterial properties against *Enterococcus faecalis* and cytocompatibility. Among the concentrations tested, the 20% chitosan nanoparticle group showed an acceptable setting time with improved biological properties. Although the higher concentrations showed longer setting times, they were all within the acceptable limits. The results of present study suggest that chitosan nanoparticle modified ERB sealers have the potential to enhance biological properties without compromising handling characteristics. In-vivo and long-term studies are recommended to validate their clinical utility.

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