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Improvement of the Antimicrobial and Antibiofilm Activity of AH Plus Sealer with Chitosan Nanoparticles and Arginine against *Enterococcus faecalis* and *Candida albicans*

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ABSTRACT

The success of endodontic treatments can be significantly impaired by persistent microbial pathogens, especially *Enterococcus faecalis* (*E. faecalis*) and *Candida albicans* (*C. albicans*). The aim of this study was to investigate how the incorporation of chitosan nanoparticles (CsNPs) and arginine (Arg) can enhance the antimicrobial and antibiofilm efficacy of AH Plus, a widely used epoxy resin-based root canal sealer. The CsNPs were synthesized by ionotropic gelation and assessed for their size, morphology, chemical structure, and cytotoxicity. The antimicrobial effectiveness of the modified sealers was evaluated against *E. faecalis* and *C. albicans* by determining the minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration, and agar diffusion method. Additionally, antibiofilm activity was assessed using a microtiter plate assay. Characterization of the CsNPs revealed an average size of 144 ± 12.8 nm by scanning electron microscopy and 182.4 nm by dynamic light scattering with a zeta capacitance of +49.2 mV. CsNPs maintained more than 68% cell viability at 625 µg/mL after 24 and 48 hours. Adding CsNPs and Arg significantly enhanced the impact of AH Plus sealer on antimicrobial and antibiofilm, especially at elevated additive concentrations. This study suggests that AH Plus sealers containing CsNPs and Arg may offer a promising approach to enhance endodontic treatment outcomes by efficiently combating resistant root canal infections.

Keywords: Arginine, *Candida albicans*, chitosan, *Enterococcus faecalis*, nanoparticles

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Introduction

Endodontic therapy treats dental pulp diseases by eliminating the infection and preventing microbial growth. Endodontic fillings consist of gutta-percha and endodontic sealers (1). An ideal root canal sealer should have biocompatibility, antimicrobial properties, efficacious apical sealability, adequate flow, insolubility in biomaterial fluid, and non-staining properties for the teeth (2). Various commercially available root canal sealers contain diverse ingredients, such as glass ionomer cement, zinc oxide-eugenol (ZnO-eugenol), resin, calcium hydroxide $Ca(OH)_2$, silicone, or tricalcium silicate (3). AH Plus, an epoxy resin-based sealer frequently utilized in endodontic therapy, has exceptional physicochemical properties, including long-lasting dimensional stability and good adhesion to dentin. It also has antibacterial properties, possibly due to Bisphenol A diglycidylether's presence or formaldehyde's emission during polymerization (4).

Nanomaterials have enhanced the mechanical integrity of endodontics, as well as antibacterial activity and biomaterial regeneration (5). Chitosan is a unique nanomaterial and a non-toxic polysaccharide originating from the deacetylation of chitin from crustacean shells (6). Chitosan nanoparticles (CsNPs) have been widely explored for drug and gene delivery and exhibit remarkable antibacterial properties. Chitosan is promising for dental applications due to its antiviral, antibacterial, and antifungal properties (7). L-arginine (Arg), an amino acid, inhibits bacterial coalescence, is engaged in cell-to-cell signaling, and alters bacterial metabolism in the oral cavity (8). It also has an antimicrobial impact on monospecies biofilms and stabilizes the hydroxyapatite of tooth enamel at diminished concentrations (9).

The incomplete removal of bacteria from the root canal system during endodontic therapy plays a crucial role in causing periapical inflammation, degradation of biomaterials, periapical lesions, and failure of endodontic treatments (10). Root canal infections can be utilized by various microorganisms, including anaerobes, aerobes, and fungi (11). *Enterococcus faecalis (E. faecalis*), a Gram-positive anaerobic facultative coccus, is frequently found after root canal retreatment, with an incidence ranging from 24% to 77% (12). *E. faecalis* possesses virulence characteristics that allow it to adhere to host cells and the extracellular matrix, facilitating biomaterial invasion. Moreover, it can merge with other bacteria and form biofilms (13). *Candida albicans* (*C. albicans*), a standard component of the natural microbiota, is mainly associated with inefficacious endodontic treatments and can be classified as a dentinophilic microbe (14). It has the extraordinary ability to colonize the tiny canals in the teeth, the so-called dentinal tubules, reaching a depth of about 150 μm (15). Additionally, it can form biofilms even in environments with limited nutrients, including cleaned and filled root canals (16).

Although much research has examined the antimicrobial efficacy of diverse root canal sealers, little research has examined how the incorporation of CsNPs and Arg can enhance the antimicrobial and antibiofilm efficacy of AH Plus by standardized procedures based on microbiological assays. Thus, this study evaluates AH Plus sealer's antimicrobial and antibiofilm capabilities augmented with CsNPs and Arg against *E. faecalis* and *C. albicans*.

Materials and methods

Chemical Compounds and Reagents

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All chemicals and reagents used in this experiment were obtained from reputable suppliers to ensure precision and reproducibility. Chitosan, sodium tripolyphosphate (TPP), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, and glucose were obtained from Sigma (USA). The Institute Pasteur, Tehran, Iran, provided the Vero cell line. Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS), and Roswell Park Memorial Institute (RPMI-1640) medium were acquired from Gibco (USA). Arginine, blood agar, Sabouraud Dextrose Agar (SDA), and Tryptic Soy Broth (TSB) were sourced from Merck (Germany). AH Plus sealer was procured from DENTSPLY DeTrey GmbH (Germany), Muller Hinton Agar (MHA), and Muller Hinton Broth (MHB) from Conda Labs (Spain). These materials played a pivotal role in the assays designed to investigate the antimicrobial and antibiofilm activities of AH Plus Sealer combined with CsNPs and Arg.

Microbial Strains

Specimens of *E. faecalis* ATCC 29212 and *C. albicans* ATCC 10231 were obtained from the microbial bank at Babol University of Medical Sciences. *E. faecalis* was cultured on blood agar under aerobic conditions, while *C. albicans* were cultured on SDA. Both strains were cultured at 37°C for 24–48 h.

Synthesis of CsNPs

CsNPs were produced by ionotropic gelation involving an interaction between chitosan and TPP (17). Initially, chitosan was dissolved in a 1% acetic acid solution. TPP was prepared in a 1% aqueous solution. While the chitosan solution was stirred at room temperature, 1 mL of the TPP solution was incorporated into 25 mL. This mixture was stirred for 20 minutes and then sonicated at 1.5 kW for 10 minutes. The mixture was stirred at 1000 rpm for 25 minutes at ambient temperature until a clear solution was obtained. Subsequently, the solution was frozen at -80°C and freeze-dried at -60 °C for 48 h.

Characterization of CsNPs

The morphology and size of the CsNPs were analyzed employing a scanning electron microscope (SEC model SNE-4500, Korea). Dynamic light scattering (DLS, Microtrac NANOTRAC WAVE II) was used to ascertain the zeta potential and particle size. Fourier transform infrared spectroscopy (FTIR, Agilent, model Cary 630, US) was employed to identify the functional groups of CsNPs within a wavelength range of 400–4000 cm^{-1}.

MIC and MBC/MFC of CsNPs

The microdilution technique, with the concentrations of CsNPs ranging from 2500 to 4.88 µg/mL, was used to determine whether the MIC met the CLSI 2023 guidelines (18). Suspensions of *E. faecalis* ATCC 29212 and *C. albicans* ATCC 10231 were prepared to meet the 0.5 McFarland standard and then diluted 1:20 to achieve a concentration of 5×10^5 CFU/mL in each well. Plates were prepared with media specific to each microorganism, using MHB for *E. faecalis* and RPMI for *C. albicans*. These media were enriched with a gradient of decreasing concentrations of CsNPs. Next, 10 µL of the microbial suspension was added to all wells except the negative control. A positive control contained both the medium and microbial suspension. The microplates were cultured at 37 °C for 16–18 h.

For the MBC and MFC assays, 10 µL samples from each MIC well, 2XMIC, and 4XMIC were cultured for 24 h at 37 °C as described in CLSI 2023.

Assessment Materials Preparation

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AH Plus sealer was modified by adding CsNPs and Arg at 5%, 10%, and 20% concentrations. A control group was utilized without the antimicrobial agents. For the modified samples, CsNPs and Arg powder were mixed proportionally with the base paste of the sealer. The mixture was subsequently blended with the liquid or catalyst paste according to the manufacturer's guidelines; an identical procedure was applied to the control group.

Evaluation of Antimicrobial Activity

The agar well diffusion procedure assessed the antimicrobial efficacy of the AH Plus sealer modified with diverse concentrations of CsNPs and Arg. A 0.5 McFarland microbial suspension was prepared, with *C. albicans* cultured on SDA and *E. faecalis* on MHA. Wells measuring 8 mm in diameter were created in each culture plate. Freshly prepared batches of modified and unmodified AH Plus sealer were added to the wells. The inhibition zone widths were recorded after 24 h of cultivation at 37 °C (19).

Evaluation of Antibiofilm Activity

Following the previous protocol, 96-well microtiter plates were utilized to assess AH Plus sealers with or without diverse concentrations of CsNPs and Arg against *E. faecalis* and *C. albicans* biofilms (20). The biofilms of *E. faecalis* were adjusted to 1×10^8 CFU/mL in TSB with glucose, while *C. albicans* were cultured in RPMI. To prepare, 100 mg of AH Plus sealers containing different concentrations of CsNPs and Arg were soaked in 1 mL of saline and cultured for 48 h at 37°C. After biofilm formation, the wells were washed thrice with 200 µL of PBS, followed by a 24-hour cultivation at 37°C. Subsequently, 200 µL of supernatants were added to each well. After removing the supernatants, the wells were rewashed with PBS. The wells were stained with 200 µL of 0.1% crystal violet solution for 20 minutes at room temperature, and any excess stain was rinsed off with distilled water. After air-drying the plates, 200 µL of 33% acetic acid was used to dissolve the dye bound to the cells. The optical density (OD) at 570 nm was measured using a Bio-Rad microplate reader (USA). Saline was the positive control, while sterile culture medium was the negative control.

Cytotoxicity Analysis of CsNPs

Vero cells, originating from African green monkey kidney biomaterial, were cultured in a complete medium of DMEM containing 10% (v/v) FBS and 10 µL/mL penicillin-streptomycin. Once the cells reached the desired density, they were treated with trypsin-EDTA, and their number was counted using a hemocytometer.

The effect of CsNPs on cell growth inhibition was evaluated using the MTT assay (21). Cells (2×10^4 cells/well) were cultured in 96-well plates overnight at 37°C, 5% CO_2, and 80% humidity. The medium was then replaced with fresh medium containing various concentrations of CsNPs (625 to 78.12 µg/mL) for 24 and 48 h. After adding MTT dye (5 mg/mL), the cells were cultured in the dark at 37°C for 2 to 4 h. OD was measured at 570 nm, and the percentage of viable cells was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD 570 of treated cells}}{\text{Mean OD 570 of control cells}} \times 100$$

Statistical Analysis

Data were analyzed using GraphPad Prism version 9.5, with one-way ANOVA applied to assess group differences, considering $p<0.05$ as the threshold for significance.

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Results

SEM, DLS, and FTIR of CsNPs

The morphology and size of the CsNPs were assessed using SEM, indicating a generally spherical and uniform shape with an average length of 144±12.8 nm, as shown in Figure 1. DLS analysis indicated that the CsNPs had a mean particle size of approximately 182.4 nm and a zeta potential of about +49.2 mV (Figure1).

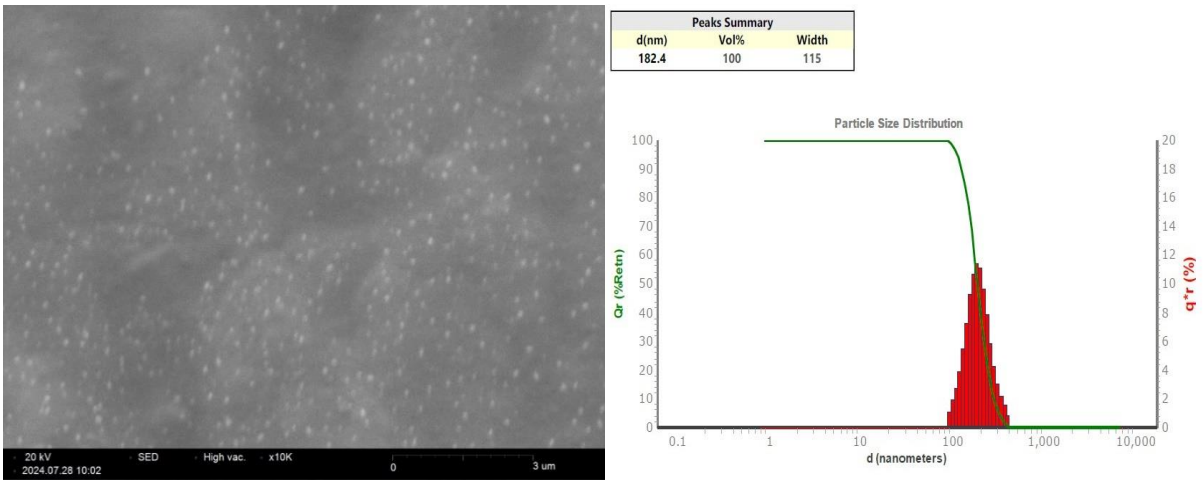

Fig. 1. Morphological and size characterization of CsNPs. SEM images depict CsNPs as generally spherical and uniform, with an average size of 144±12.8 nm. DLS analysis reveals an average particle size of 182.4 nm and a zeta potential of approximately +49.2 mV.

The FTIR spectra of CsNPs revealed several prominent absorption bands: bands at 2920 cm^{-1} and 2870 cm^{-1} corresponding to C-H stretching vibrations; a sharp peak at 1589 cm^{-1} associated with N-H amide bending vibrations; and a broad band between 3600–3100 cm^{-1} attributed to N-H and O-H stretching vibrations. Other notable peaks were 1411 cm^{-1} for CH_2 bending, 1319 cm^{-1} for C-N and N-H amide vibrations, and 1018 cm^{-1} for -C-O-C- stretching. The spectrum also exhibited a peak at 1265 cm^{-1} for P-O stretching, shifted from 1218 cm^{-1} due to interactions with chitosan, and a band at 892 cm^{-1} for P-O-P stretching. These features diverseiate the spectra of pure chitosan from chitosan-tripolyphosphate, confirming phosphate groups (Figure 2).

MIC and MBC/MFC of CsNPs

The MIC and MBC of CsNPs against *E. faecalis* were 312.5 µg/mL and 625 µg/mL, respectively. The MIC and MFC of CsNPs against *C. albicans* were 625 µg/mL. Remarkably, CsNPs had a diminished MIC against *E. faecalis* than against *C. albicans*, indicating a greater susceptibility of *E. faecalis* to CsNPs.

Antimicrobial Activity

Table 1 illustrates the mean inhibition zone diameters of AH Plus sealer utilized with various concentrations of CsNPs and Arg against *E. faecalis* and *C. albicans*, along with the standard deviation (SD) and group differences. AH Plus sealer demonstrated antimicrobial activity against *E. faecalis* and *C. albicans*, increasing efficacy at 5%, 10%, and 20% concentrations of CsNPs and Arg. One-way ANOVA indicated that the addition of CsNPs and Arg at concentrations of 5%, 10%, and 20% substantially augmented

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antimicrobial efficacy against *E. faecalis* compared to no additives ($p<0.05$); however, there were no significant differences among the 5%, 10%, and 20% concentrations ($p>0.05$). Moreover, adding CsNPs at concentrations of 5% and 10% and Arg at 20% substantially enhanced the antimicrobial efficacy against *C. albicans* compared to the sealer without CsNPs or Arg ($p<0.05$). No notable differences were identified among the 5%, 10%, and 20% concentrations ($p>0.05$). Figure 3 shows the growth inhibition zones of *E. faecalis* and *C. albicans* in the presence of the tested materials.

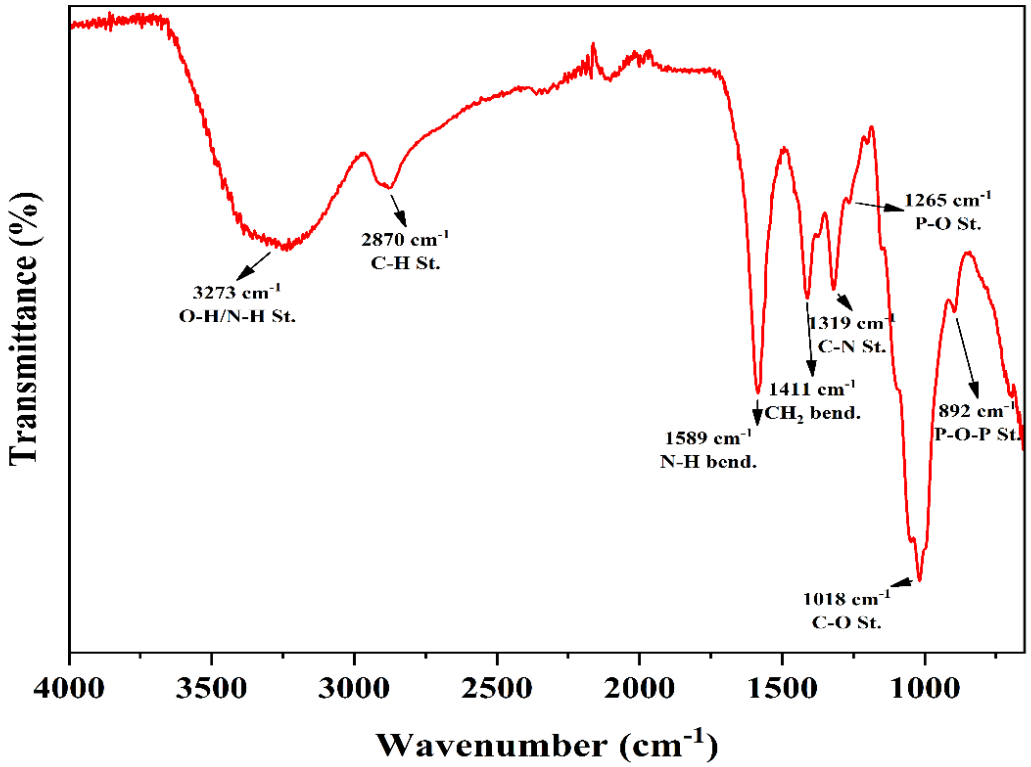

Fig. 2. FTIR spectrum of CsNPs. The FTIR spectrum of CsNPs shows characteristic absorption bands, including N-H and O-H stretching vibrations, C-H stretching vibrations, N-H amide bending, CH_2 bending, C-N and N-H amide vibrations, -C-O-C stretching vibrations, P-O stretching vibrations (shifted due to chitosan interactions), and P-O-P stretching. These features differentiate pure chitosan from chitosan-TPP.

Table 1. Mean and standard deviation (SD) of the inhibition zone (in mm) of AH Plus sealer with different concentrations of CsNPs and Arg against *E. faecalis* and *C. albicans*.

Microorganisms	AH Plus + Various concentrations of CsNPs				AH Plus + Various concentrations of Arg			
	0	5%	10%	20%	0	5%	10%	20%
E. faecalis	12.33 ± 0.4	13.36 ± 0.41	17.73 ± 1.15	24.13 ± 1.16	12.16 ± 0.25	12.73 ± 0.2	14.46 ± 0.35	22.36 ± 0.89
C. albicans	11.96 ± 0.37	12.8 ± 0.36	16.3 ± 0.72	23.43 ± 0.5	11.96 ± 0.5	12.63 ± 0.83	14.03 ± 0.68	21.26 ± 0.41

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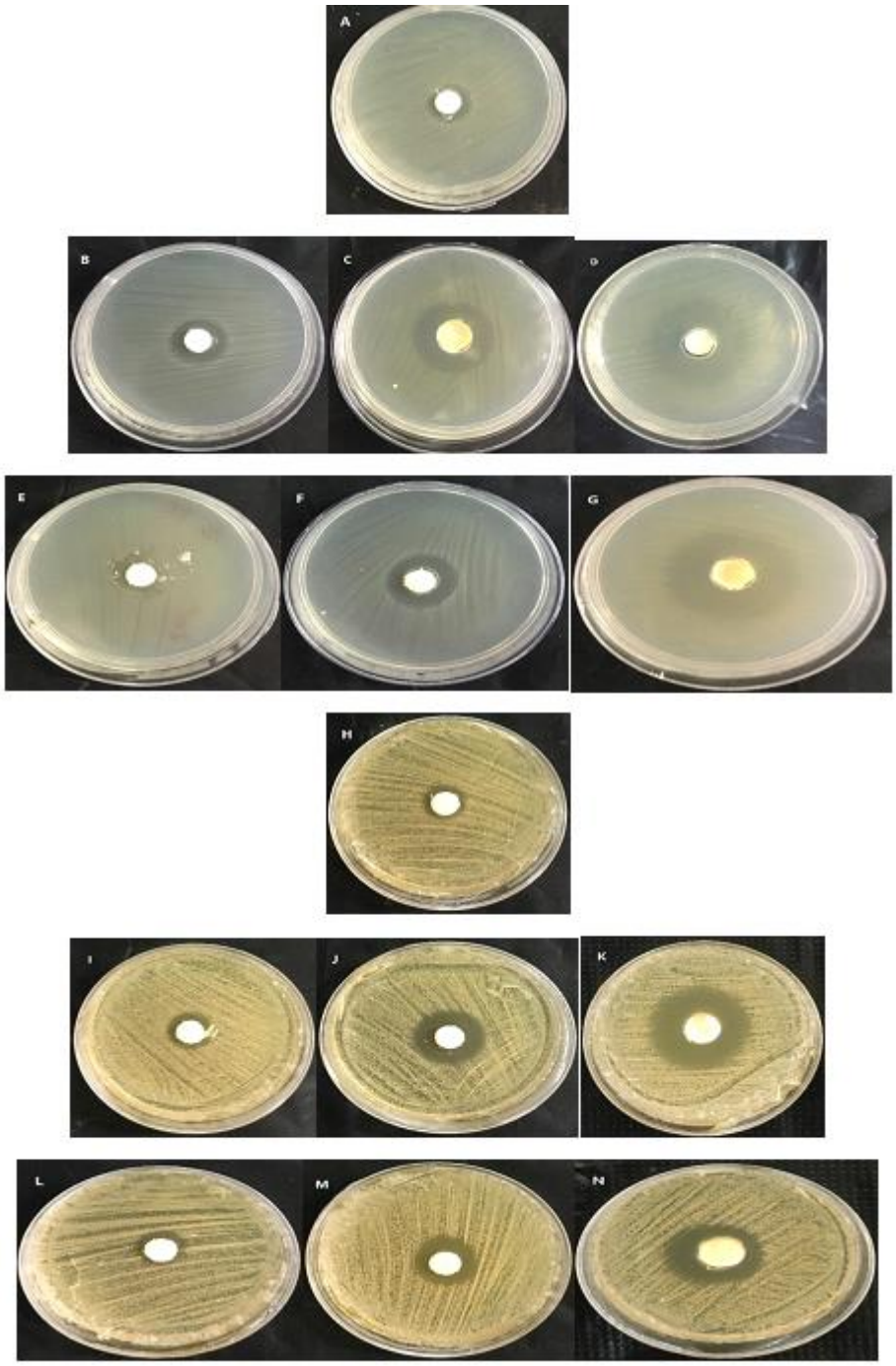

Fig. 3. The growth inhibition zones of *E. faecalis* and *C. albicans* in the presence of the tested materials. **A:** AH Plus against *E. faecalis*. **B:** AH Plus + 5% concentrations of CsNPs against *E. faecalis*. **C:** AH Plus + 10% concentrations of CsNPs against *E. faecalis*. **D:** AH Plus + 20% concentrations of CsNPs against *E. faecalis*. **E:** AH Plus + 5% concentrations of Arg against *E. faecalis*. **F:** AH Plus + 10% concentrations of Arg against *E. faecalis*. **G:** AH Plus + 20% concentrations of Arg against *E. faecalis*. **H**: AH Plus against *C. albicans*. **I**: AH Plus + 5% concentrations of CsNPs against *C. albicans*. **J**: AH Plus + 10% concentrations of CsNPs against *C. albicans*. **K:** AH Plus + 20% concentrations of CsNPs against *C. albicans*. **L**: AH Plus + 5% concentrations of Arg against *C. albicans*. **M**: AH Plus + 10% concentrations of Arg against *C. albicans*. **N**: AH Plus + 20% concentrations of Arg against *C. albicans*.

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Antibiofilm Activity

The formation of biofilms by *E. faecalis* and *C. albicans* in the presence of the AH Plus sealer extract diminished as the concentrations of CsNPs and Arg augmented. One-way ANOVA indicated that the addition of CsNPs and Arg at concentrations of 5%, 10%, and 20% substantially augmented antibiofilm efficacy

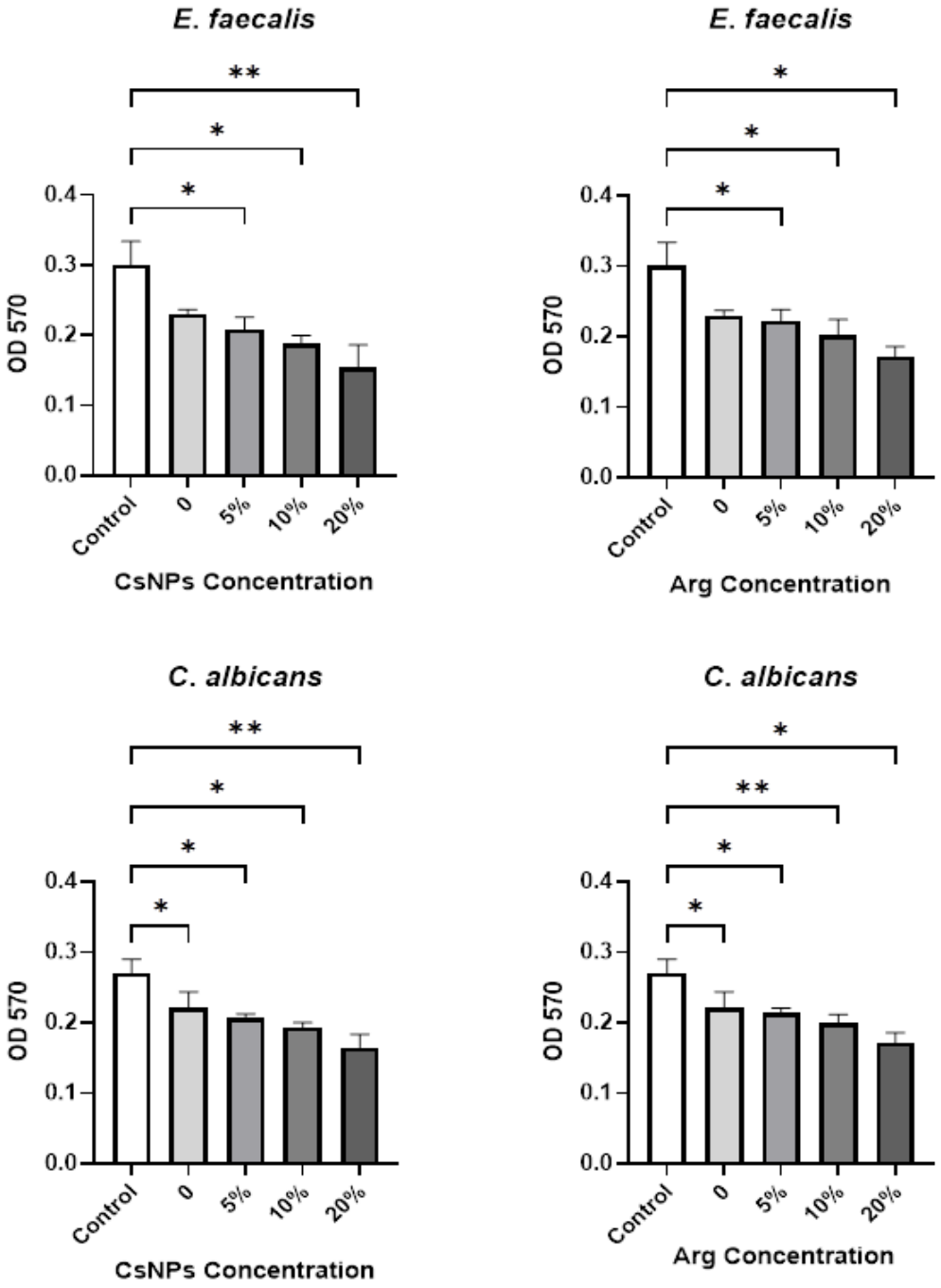

Fig. 4. Effects of AH Plus sealer in combination with different concentrations of CsNPs and Arg on the formation of biofilms by *E. faecalis* and *C. albicans* (* $p < 0.05$, ** $p < 0.01$).

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against *E. faecalis* compared to control (p<0.05); however, there were no significant differences among the sealer without CsNPs or Arg and control (p>0.05). The addition of CsNPs and Arg at concentrations of 5%, 10%, and 20% to sealer and sealer without CsNPs or Arg substantially augmented antibiofilm efficacy against *C. albicans* compared to the control (p<0.05). Figure 4 illustrates the impacts of AH Plus sealer with diverse concentrations of CsNPs and Arg on biofilm formation by E. *faecalis* and *C. albicans*.

Cytotoxic Impact of CsNPs

The cytotoxicity of CsNPs was evaluated using the MTT assay on Vero cells, and the percentage of cell viability was determined at diverse concentrations of CsNPs (Figure 5); as the concentration of CsNPs augmented from 0 to 625 µg/mL, cell viability diminished continuously at both time points. Viability progressively reduced with increasing concentration of CsNPs, reaching approximately 68-71% at 625 µg/mL. Elevated concentrations of CsNPs substantially decrease cell viability, demonstrating a dose-dependent cytotoxic impact on Vero cells (p<0.05).

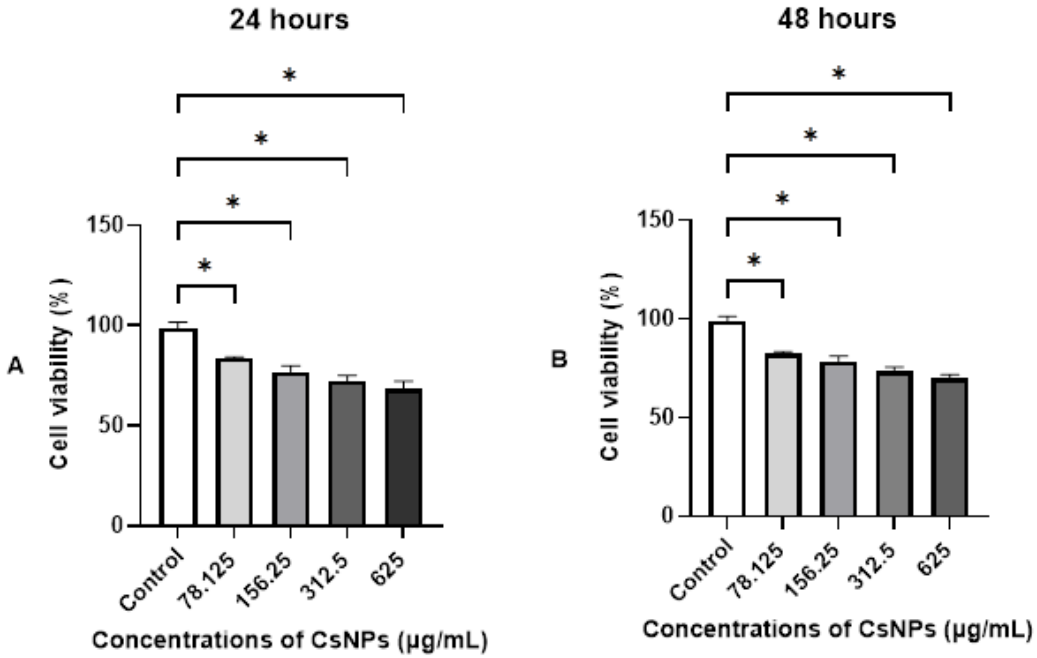

Fig. 5. Cytotoxic effects of different concentrations of CsNPs on Vero cells (* p < 0.05).

Discussion

The current investigation examined the synthesis and characterization of CsNPs, their cytotoxicity, and antimicrobial activity. The antimicrobial and antibiofilm properties of AH Plus sealer were enhanced by incorporating CsNPs and Arg, targeting *E. faecalis* and *C. albicans*, prevalent pathogens in endodontic infections. The outcomes of the current investigation suggested that CsNPs and Arg could substantially enhance AH Plus sealer's efficacy in treating microbial infections in endodontics.

The CsNPs synthesized in the ongoing investigation indicated a small 144 ± 12.8 nm size and a high zeta capacity of +49.2 mV. These characteristics are congruent with previous observations, such as those of Ing *et al*., who observed similar size distributions and positive zeta capacity for CsNPs. This agreement with

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the existing literature exemplifies that the CsNPs synthesized in the current investigation are stable and uniformly formed, highlighting the reliability of the synthesis procedure utilized (22). FTIR analysis confirmed the practical synthesis of CsNPs with absorption bands indicating N-H, O-H, C-H, and phosphate groups. These outcomes are congruent with similar research where critical functional groups of chitosan and crosslinking agents were identified, confirming the NPs' structural integrity and chemical composition (23, 24).

The antimicrobial efficacy of CsNPs against *E. faecalis* and *C. albicans* was assessed using standard microbiological procedures, including MIC, MBC, and MFC assessments. Elshinawy et al. reported MIC and MBC/MFC values of 625 µg/mL and 1250 µg/mL for CsNPs against *E. faecalis* and *C. albicans*, respectively (25). Pandey et al. found MIC and MBC values of 310 µg/mL and 625 µg/mL against *E. faecalis*, which are congruent with the outcomes of the current investigation (26). The antimicrobial properties of CsNPs are attributed to their enhanced ability to interact with and disrupt microbial cell membranes, leading to cell death (27). Notably, the antimicrobial activity of CsNPs was more productive against *E. faecalis* than against *C. albicans*.

Including CsNPs and Arg in AH Plus, the sealer enhanced inhibition zones for *E. faecalis* and *C. albicans*, with the most critical enhancement occurring at a concentration of 20%. Sealers play a crucial role in root canal therapy by sealing the canal and providing antimicrobial protection to prevent reinfection. The antifungal impact of the AH Plus sealer is attributed to its Bisphenol A diglycidylether component, which is also responsible for its antibacterial effect. The enhanced antimicrobial activity of the modified sealer is likely due to the potent bioactive properties of CsNPs and Arg, which further inhibit bacterial growth. This enhancement in antimicrobial efficacy aligns with the findings of Ratih *et al*. (28), who observed that root canal sealers containing CsNPs exhibited increased antimicrobial effectiveness.

Biofilms provide a protective environment for microorganisms and make them resistant to many antimicrobial treatments. In the current investigation, the AH Plus sealer with CsNPs and Arg reduced biofilm formation. The decrease in OD 570 nm values at elevated concentrations of CsNPs and Arg supports the observations of Elshinawy *et al*. (25) and Costa et al. (29), who have declared that CsNPs can interfere with biofilm formation by various pathogens. This phenomenon can be explained by the ability of CsNPs to penetrate and disrupt the biofilm matrix, which is essential for the survival of bacteria within the biofilm. In addition, the sustained release of CsNPs from the sealer may provide a long-lasting antimicrobial impact and prevent biofilm re-formation (30). Furthermore, the unique role of Arg in altering bacterial cell signaling and biofilm dynamics likely plays a vital role in enhancing this anti-biofilm activity (31).

The cytotoxicity data demonstrated a dose-dependent decline in cell viability, a key consideration for clinical applications. The observed cytotoxic impacts at elevated concentrations of CsNPs suggest that although CsNPs are efficacious antimicrobial agents, their concentration needs to be carefully optimized to balance antimicrobial efficacy and biocompatibility. In particular, cytotoxicity assessments demonstrated that CsNPs at a 625 µg/mL concentration maintained a cell viability of over 68% after 24 and 48 h. This contrasts with the observations of Ramar et al., who observed a substantially diminished cell viability of 38% at a comparable concentration of 500 µg/mL (32). Furthermore, Hazarika et al. reported that CsNPs had no adverse impact on the viability of Vero cells, even at concentrations of up to 1000 µg/mL (33). The

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discrepancies in cell viability in this research may be attributed to differences in experimental conditions, nanoparticle characteristics, and the specific cell viability assays utilized.

In conclusion, adding CsNPs and Arg to AH Plus sealer enhances its antimicrobial and antibiofilm properties against *E. faecalis* and *C. albicans*. These observations are consistent with other studies on using CsNPs in antimicrobial applications and support their capacity to enhance the efficacy of endodontic sealers. However, further studies should investigate the long-term stability of these modified sealers and explore the mechanisms by which CsNPs and Arg enhance antimicrobial activity to optimize their clinical application. The current study will improve our understanding of the material's properties and pave the way for developing next-generation endodontic sealers with better therapeutic outcomes.

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