Original Article

Comparative Evaluation of Antimicrobial Efficacy of Silver Nanoparticles and 2% Chlorhexidine Gluconate When Used Alone and in Combination Assessed Using Agar Diffusion Method: An *In vitro* Study

Abstract

Context: Silver nanoparticle (AgNP) is a potent antimicrobial that is widely used in several fields of medicine. Chlorhexidine (CHX) gluconate is a well-known agent used in dentistry to eliminate oral microbial flora. **Aims:** The aim of this study is to evaluate the efficacy of AgNPs, 2% CHX gluconate, and the combination of two solutions against endodontic pathogens such as *Enterococcus faecalis*, *Klebsiella pneumoniae*, and *Candida albicans*. These organisms are frequently found in the root canal space and their persistence may lead to endodontic failure. The synergistic effect of the two solutions has been evaluated in this study. The antibiotic gentamycin was taken as the control group. **Settings and Design:** Agar well diffusion method was used and minimum inhibitory concentration of AgNP was found to be 15 µg/mL. AgNPs were synthesized from the aqueous plant extract of *Cassia roxburghii*. The combination of CHX-AgNP solution was stirred together by a glass rod. The values were tabulated and subjected to statistical analysis using the SPSS software version 20. One-way ANOVA test was used to compare within the groups and between groups. The level of significance was set at 5%. **Results:** CHX-AgNP combined solution exhibited the highest efficacy in comparison to these solutions used alone. They showed the highest efficacy against *C. albicans* among the three organisms tested. **Conclusion:** The present study demonstrates the antimicrobial efficacy of a novel mixture of CHX-AgNP solution, and it may be developed as a promising antimicrobial agent against endodontic flora.

Keywords: *Candida albicans, Enterococcus faecalis, Klebsiella pneumoniae, silver nanoparticle*

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Introduction

Silver is one of the most versatile metals in use from the primordial age. It is well known for its antimicrobial property and is being incorporated in several fields of medicine.[1] Silver interferes with an enzyme present in the microbe that is liable for oxygen uptake and inhibits it.[2] Nanoparticles are diminutive in array ranging from 1 to 100 nm. Owing to their minuscule mass, surface area is augmented leading to amplified effect against microbes.[3] The variations in unambiguous characteristics such as size, distribution, and morphology of particles attribute to the unique properties of nanoparticles.[4] The field of bionanotechnology has developed and introduced nanoparticles from metallic silver. Silver nanoparticles (AgNPs) have received many accolades due to its relatively less toxic effects on human beings at a lower concentration and also its far-ranging activity against innumerable microorganisms.[5] They do not usually cause microbial resistance since the viable cells are not adversely affected.[6]

Intracanal medicaments are used against endodontic microbial flora. They are proven to be effective against microbes that survive instrumentation and irrigation.[7] It is imperative to exterminate the intracanal microbes for the success of a root canal treatment therefore interappointment medicaments are applied. It should provide disinfection, should be efficacious throughout the given period, and pervade through the dentinal tubules annihilating bacteria that maybe present, with little toxicity to the periradicular tissues.[8] *Enterococcus faecalis* is the most persistent organism in the periradicular area. It is an unrelenting microbe that resists many intracanal drugs and endures

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harsh environment in the root canal.[5] This indomitable property is due to its high alkali tolerance and its tubular invasion potentiality.[9] Secondary intraradicular infections are caused during treatment or between appointments or posttreatment.[10] *Candida albicans* are found in such infection. They can infiltrate the pulp space even after the completion of root filling.[10] It is a fungus which invades deep into the dentinal tubules due to its thigmotropic properties, proteinase secretion, phenotypic switching phenomenon, and hyphal growth. It is a pleomorphic organism exhibiting variable forms, giving rise to blastospores and chlamydospores[11,12] *Klebsiella pnuemoniae* is a facultative, Gram-negative, nonsporulating bacteria that persist in the oral cavity during high prevalence of dental caries, poor maintenance of oral hygiene and in teeth with multiple canals having limited access during endodontic therapy.[13,14]

Chlorhexidine (CHX) is an extensively used potent intracanal medicament against Gram-positive and negative organisms. It is also very effective against *Candida albicans*. The antimicrobial properly is essentially attributed to its substantivity and protein-binding properties which thence increases the duration of the antimicrobial effects. It also has low toxicity.[8] Numerous studies have been carried out comparing the antimicrobial efficacy of CHX and AgNPs as an intracanal medicament independently.[5,6,8,9,11] However, the synergistic effect of CHX and AgNP concocted together as a solution has not been assessed yet. AgNPs undoubtedly have excellent antimicrobial properties as substantiated by the literature. Similarly, CHX independently is good antimicrobial agents that are being widely used in clinical dentistry. Combining both the compounds can result in a potentially superior medicament. Thus, the aim of the present study is to compare the antimicrobial efficacy of AgNPs, 2% CHX gluconate, and combination of 2% CHX and AgNPs solution.

Materials and Methods

This study was designed as an *in vitro* evaluation to compare the antimicrobial efficacy of three intracanal medicaments against *E. faecalis*, *K. pneumonia*, and *C. albicans*. All the analytical grade chemicals were procured from Sigma-Aldrich Co. (St Louis, MO, USA). Silver nitrate was procured from HiMedia Laboratories (Mumbai, India). The bacteria were obtained from the Culture Collection Facility at the Centre for Advanced Studies in Botany, University of Madras, Chennai, and the fungus *C. albicans* was obtained from Fungal Culture Collection Facility at the Centre for Advanced Studies in Botany, University of Madras, Chennai.

Synthesis of silver nanoparticles

The AgNPs were synthesized from the aqueous extracts of *C. roxburghii*. The healthy leaves of the plant were amassed, methodically washed with distilled water, and later air-dried at room temperature for 3 days.

Preparation of plant extract

Using a blender, the air-dried leaves were kibbled to coarse powder. About 4 g of this powder was then mixed with 100 ml distilled water. This mixture was kept at 55°C for 15 min. It was then cooled down to room temperature and was filtered using Whatman No. 1 filter paper.

Synthesis of silver nanoparticles

One milliliter of *C. roxburghii* leaf aqueous extract was added to 9 mL of 1 mM solution of silver nitrate ($AgNO_3$) in a 15 mL test tube. The reaction was carried out in the dark at room temperature and was left overnight to curtail photoactivation of $AgNO_3$. The aqueous leaf extracts of *C. roxburghii* and $AgNO_3$ solution were used as control. After the solicited reaction period, the solution containing the AgNPs was centrifuged at 15,000 rpm for 10 min. The obtained pellet was then congregated and redispersed in glass-distilled water to remove interactive biological molecules if present. The above step was repeated thrice to asseverate better separation of the AgNPs. AgNPs synthesized by this green method has a face-centered crystalline structure. The amino groups of *C. roxburghii* are used for the encapsulation and stabilization of the AgNPs. Flavonoids act as a reducing agent thereby reducing Ag+ to Ag0. The synthesized AgNPs were then subjected to characterization.

Characterization of silver nanoparticles

Ultraviolet visible spectroscopy

The formation of AgNPs was confirmed by the development of dark brown color. The ultraviolet (UV)-visible spectroscopy using Hitachi U-2900 UV-visible spectrophotometer recorded the reduction of pure AgNPs between 300 nm and 700 nm. The UV-visible spectra of the plant leaf extract and $AgNO_3$ solution were also recorded. The UV-spectroscopy shows strong absorbance peak at 425 nm. Figure 1a shows the graph generated by UV-visible spectroscopy.

High-resolution scanning electron microscopy and energy dispersive X-ray spectroscopy (EDX)

The AgNPs were placed on a carbon-coated copper grid. The high-resolution scanning electron microscopy (HR-SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis were done using the Quanta 200 FEG scanning electron microscope at the Indian Institute of Technology, Madras. HR-SEM in the magnification of ×200,000 reveals polydispersed spherical crystalline particles of size ranging from 9.8 nm to 13.8 nm. EDX revealed the chemical purity and composition of the AgNPs. Figure 1b shows HR-SEM images of the synthesized AgNPs and Figure 1c shows the EDX analysis.

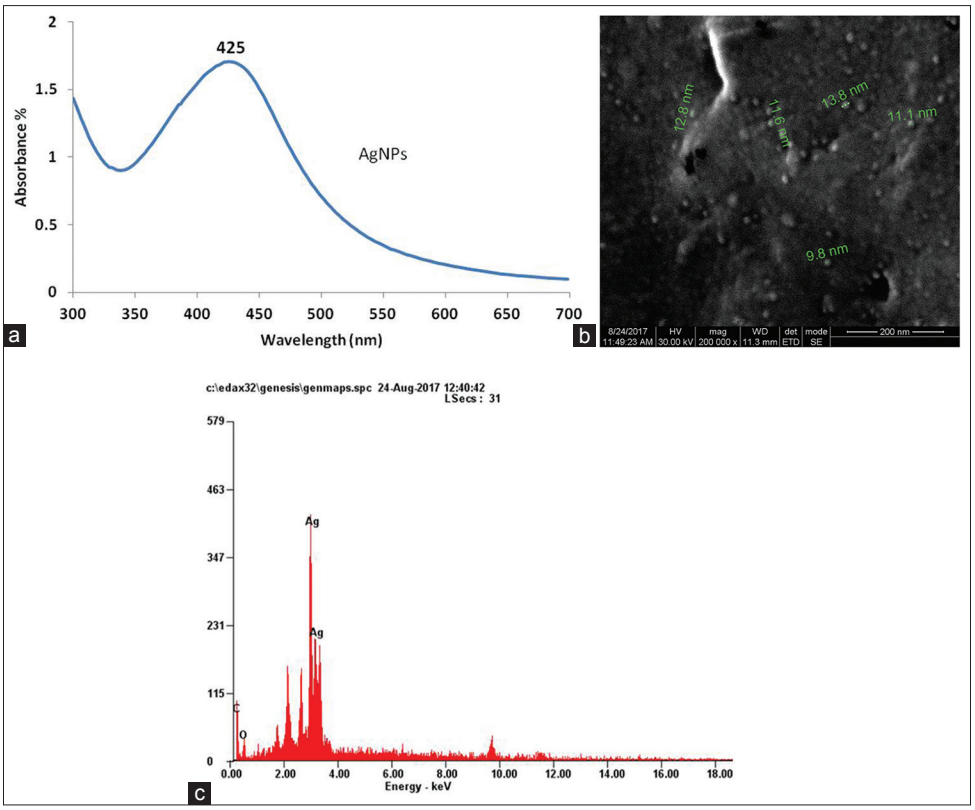

Figure 1: (a) Ultraviolet spectroscopy of silver nanoparticles. (b) High-resolution scanning electron microscopy image of silver nanoparticles. (c) Energy dispersive X-ray spectroscopy analysis of silver nanoparticles

Preparation of microorganisms

The test organisms were inoculated individually into a tube containing 5 ml of 85% saline. Adjustments of the suspension were made in such a way that they were equivalent to 0.5 Mcfarland scale – 1.5×10^8 colony-forming units. Mueller-Hinton agar medium and potato dextrose agar medium were used for the culture of the bacteria and fungus, respectively. Freshly prepared bacterial and fungal suspensions were swabbed on the suitable growth mediums. Ten samples of each test organism were chosen. The three organisms – *E. faecalis*, *C. albicans*, and *K. pneumoniae* were categorized into Group 1, 2, and 3, respectively.

Antimicrobial activity

Two tests were carried out to assess the antibacterial activity of AgNPs. The first test was to identify the minimum inhibitory concentration (MIC) of the synthesized AgNPs. The second test consisted of agar well diffusion method where the zone of inhibition determines the extent of antibacterial activity for all the three solutions.

Minimum inhibitory concentration

The MIC of AgNPs was identified to determine the lowest concentration that inhibits the visible growth of the test organisms. Suspensions of the test organism were swabbed on the culture medium. Wells were then carved on the plates. Different concentrations of the AgNPs (5, 10, 15, and 20 μg/mL) were added to the wells. All the plates were incubated at 37°C for 24 h in order to determine the inhibitory growth of the AgNPs on particular pathogen. The procedure was repeated three times and the mean value was taken into consideration.

Preparation of chlorhexidine and silver nanoparticle solution

Equal amounts of 2% CHX and 15 μg/mL of AgNPs were stirred together using a glass rod to obtain a uniformly mixed solution.

Assessing antibacterial and antifungal activity by agar well diffusion method

The antibacterial and antifungal activities of the sample were determined by following the agar plate well diffusion method. Ten agar plates were sterilized and then seeded with freshly prepared samples of the pathogens. With the help of a sterile stainless steel cork borer, four agar wells of 6 mm diameter each were contrived in all the agar plates used. The wells were labeled as Group A, B, C, and D. The wells A and B were loaded with 20 μL of the CHX and AgNPs (15 μg/mL), respectively. The well C was loaded with 40 μg/mL of the (AgNPs + ChX) combination. The well D was loaded with 20 μg/mL of gentamicin which was used as the positive control. The plates were incubated at 37°C for 24 h and the zone of inhibition (ZOI; mm) that appeared around the wells was recorded.

The values were tabulated using Microsoft Office Excel 2007 and subjected to statistical analysis using the SPSS software version 20 (IBM, Armonk, NY, USA). One-way ANOVA test was used to compare within the groups and between groups. The level of significance was set at 5%.

Results

MIC was calculated for AgNPs. [Table 1] shows the MIC. It was found to be 15 µg/mL for the synthesized AgNPs. [Figure 2a-c] shows the MIC of AgNPs for *E. faecalis*, *K. pneumonia* and *C. albicans* respectively.

[Table 2] shows the Inter-group comparisons of all the four intra-canal medicaments against the test organisms. All the four medicaments had good antibacterial and antifungal activity. [Figure 3a-c] shows the antibacterial and antifungal activity for the three intra-canal medicaments. ANOVA analysis showed differences within the group. It was found to be statistically significant ($P = 0.001$).

It was seen that Group C containing the CHX-AgNPs combination solution had the highest efficacy against all the three microorganisms when compared to the test groups. Intergroup comparison showed that Group 2A containing CHX as the test drug had the highest efficacy against *K. pneumoniae* among the three test organisms. Group 3C containing AgNPs as the test drug showed the highest efficacy against *C. albicans*. Group 3C containing the test drug CHX-AgNP mixture showed the highest efficacy against *C. albicans*. The control group 2D containing the antibiotic gentamycin showed the highest efficacy against *K. pneumoniae*. The diameter of zone of inhibition was highest for CHX and AgNP combined solution against *C. albicans* followed by *K. pnuemoniae* and *E. faeclis.*

Discussion

Intracanal medicaments are necessary to eliminate the endodontic microbial flora during root canal treatment, the failure of which may lead to chronic periapical infection and subsequent retreatment.[15] Studies have shown that *E. faecalis* is usually the most persistent primary organism causing refractory endodontic infection.[16] It is found that *E. faecalis* can tolerate harsh environment due to its high alkali tolerance, possession of various virulence factors which are also shared among other organisms further aggravating the condition. It is shown to exhibit genetic polymorphism, ability to sustain long periods of starvation

Table 1: Antibacterial activity of the biologically synthesized silver nanoparticles and minimum inhibitory concentration

Name of the organism	Synthesized AgNPs			
	Zone of inhibition (mm in diameter)			
	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL
Enterococcus faecalis	9	11	13	14
Klebsiella pneumoniae	8	9	10	12
Candida albicans	9	12	15	16

AgNPs: Silver nanoparticles

Table 2: Comparison of zone of inhibition among different antibiotic regimens and for different organisms

Groups	*Enterococcus faecalis* (1)	*Klebsiella pneumoniae* (2)	*Candida albicans* (3)	*P*
CHX (A)	7.93±0.2	12.01±0.2	8.02±0.2	0.001*
AgNPs (B)	13.98±0.2	13.91±0.2	14.99±0.2	0.001*
CHX + AgNPs (C)	15.98±0.2	16.96±0.2	19.02±0.2	0.001*
Gentamycin (D)	18.08±0.2	22.10±0.2	19.00±0.1	0.001*
P	0.001*	0.001*	0.001*	

*Significant at 1% interval. CHX: Chlorhexidine; AgNPs: Silver nanoparticles

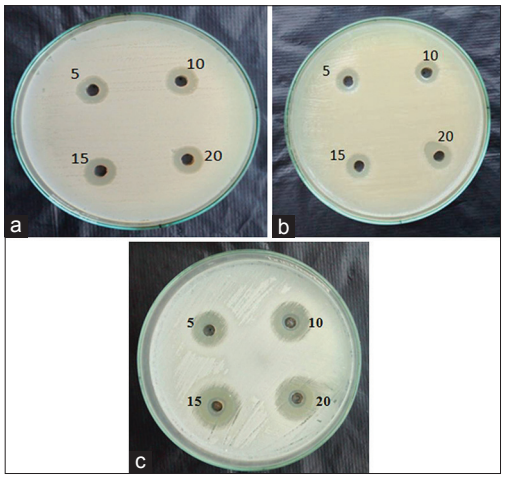

Figure 2: (a) Minimum inhibitory concentration of silver nanoparticles against *Enterococcus faecalis*. (b) Minimum inhibitory concentration of silver nanoparticles against *Klebsiella pneumoniae*. (c) Minimum inhibitory concentration of silver nanoparticles against *Candida albicans*

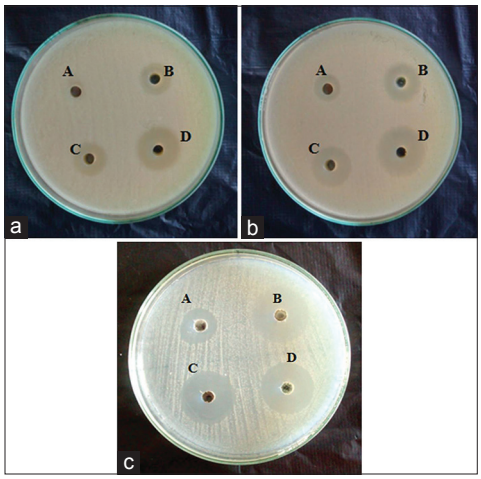

Figure 3: (a) Antimicrobial activity of chlorhexidine gluconate against the test organisms. (b) Antimicrobial activity of silver nanoparticles against the test organisms. (c) Antimicrobial activity of chlorhexidine + silver nanoparticles against the test organisms

and has protein binding properties that make it adherent with dentin.[17-21]

The fungal species *C. albicans* is also a common inhabitant of root canal space that may cause pulpal and periapical infection. Waltimo *et al*.[22] has shown that *C. albicans* is prominent in 7%–18% cases of persistent apical periodontitis. Studies have shown that *Candida* can tolerate even higher alkalinity compared to *E. faecalis*. It is reported to be resistant against calcium hydroxide.[23,24] Tronstad *et al*.[14] has reported that *K. pneumoniae* are found in the case of compromised endodontic therapy due to the presence of inaccessible canals, inaccurate surgical procedure, and multiple canal opening. These microorganisms are frequently found pathogens of root canal space which are often associated with endodontic failures.[5,10] Hence, the antimicrobial action against these three organisms was assessed in the present study.

AgNPs possess bactericidal activities since they have the ability to bind with proteins and enzymes, thereby interfering with the integrity of the bacterial cell wall.[25,26] Roe *et al*.[27] has shown that coating of AgNPs as an anti-biofilm agent over catheters increased its potency against bacteria *E. coli*, *Staphylococcus aureus*, and the fungi *C. albicans* and 50% inhibition in the case of *Enterococcus* sp. and *Pseudomonas aeruginosa*. In the current study, AgNPs of size 11 nm were used since smaller particles yield greater surface area and heightened antimicrobial properties.[28,29] CHX is effective against both Gram-positive and Gram-negative organisms. It most often does not lead to the development of antimicrobial resistance.[30-32] Studies have shown that CHX is more effective against 40 oral microorganisms when compared to other antibacterial agents.[33] It has been found that 2% CHX is effective against *E. faecalis* even after 3 weeks of root dentin treatment.[34]

Multiple studies have evaluated and compared CHX combined with other intracanal medicaments; however, the coaction of CHX and AgNP as an intracanal medicament has not been assessed yet. In one of the studies by Seneviratne *et al*.,[35] the antimicrobial efficacy of CHX encapsulated in silica nanoparticles against oral biofilm has been evaluated. The present study aims to compare the antimicrobial effectiveness of CHX, AgNP solution, and CHX-AgNP mixed together against *E. faecalis, K. pneumoniae*, and *C. albicans*. The current study was initiated as a simplified pilot study and hence agar well diffusion method was employed for the evaluation of antibacterial and antifungal potency. In the former method, effectiveness of the intracanal medicament was noted against organisms in a grown culture. The latter method uses serial dilution to determine the lowest concentration of the drug that will be effective against the microbes. For CHX as an intracanal medicament, 2% concentration is considered the standard value in literature. Hence, in the present study, MIC was calculated only for the newly synthesized AgNPs.

The results of the current study are in harmony with the above findings. The zone of inhibition formed around *C. albicans* was marginally more than that of the other two organisms when AgNps was used alone. CHX solution developed zone of inhibition against the microorganisms employed in this study which was also found to be statistically significant. It revealed that the antimicrobial activity of AgNP-CHX solution was found to be higher in comparison with nanosilver solution and 2% CHX used individually. However Mozayeni *et al*.[36] in his study combining nanosilver-CHX gel against *C. albicans* and *E. faecalis* found that the synergistic effect was not as high as CHX or nanosilver alone. His study further concluded that the effect of nanosilver gel individually against *E. faecalis* was reduced when compared to CHX and calcium hydroxide. He stated that the use of methyl cellulose as a gelling agent could have hindered the antimicrobial action, whereas in the present study, the solutions were mixed physically and no gelling agents were incorporated. Souza-Filho *et al*.[37] in his study concluded that CHX when combined with other medicaments such as calcium hydroxide, reduced the antimicrobial efficacy of CHX.

The results of the current study are contradictory to the above findings. AgNPs when combined with CHX proved to enhance the antimicrobial effectiveness. However, this method is done under *in vitro* conditions, and hence the presence of blood, temperature changes, or variations in the oxidation and reduction at different areas of the oral cavity may affect the results.[38] In contrary, the *in vivo* condition of the root canal space is unique and relatively different. Further studies should be conducted to determine the penetration of the irrigants or the intracanal medicaments in the dentinal tubules and the efficacy of the medicaments in the presence of dentin against wide range of root canal pathogens.

Conclusion

Within the limitations of the present study, it can be concluded that the synergistic effect of CHX-AgNP solution has better antimicrobial effect against *E. faecalis*, *K. pneumonia*, and *C. albicans* when compared to each of those solutions used individually. Intracanal medicaments are indispensable for successful endodontic treatments. The diverse potential of NPs should be judiciously exploited to develop novel medicaments which can combat problems of antimicrobial resistance.

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Conflicts of interest

There are no conflicts of interest.

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