Green synthesis of calcium hydroxide-coated silver nanoparticles using *Andrographis paniculata* and *Ocimum sanctum* Linn. leaf extracts: An antimicrobial and cytotoxic activity

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**Abstract**

**Context**: Silver is known for its antibacterial properties since ages. As nanoparticles have smaller size and greater surface area, silver has been utilized in the form of nanoparticles to enhance its antibacterial properties. Calcium hydroxide is a well-known intracanal medicament and serves as a gold standard for root canal disinfection. Using herbal extracts as reducing agents for nanoparticle synthesis appears to be an ecofriendly approach.

**Aim**: The aim of this study was to synthesize calcium hydroxide-based silver nanoparticles using herbs as reducing agents and to test the cytotoxic levels and antimicrobial activity against oral microbes.

**Materials and Methods**: The calcium hydroxide-based silver nanoparticles were synthesized using the leaves of *Andrographis paniculata* and *Ocimum sanctum* Linn. Various properties of the synthesized nanoparticles were also characterized by ultraviolet (UV) spectrophotometer analysis, transmission electron microscopy (TEM), X-ray diffraction (XRD), and Fourier-transform infrared spectroscopy (FTIR) analysis. The cytotoxic effects of these nanoparticles were analyzed using brine shrimp and MTT assay. Antimicrobial activity was assessed by measuring the zone of inhibition. The statistical analysis was done using parametric independent *t*-test. *P* value was set at < 0.05.

**Results**: The calcium hydroxide-based silver nanoparticles were successfully synthesized and were confirmed by UV spectrophotometer analysis, TEM, X-ray diffraction (XRD), and FTIR analysis and showed a minimal cytotoxic effect. They also showed a good antimicrobial activity and a remarkable antifungal activity.

**Conclusions**: The green synthesis of CaOHAgNPs yielded an effective nanoparticle preparation that could be used against common oral pathogens as a potential therapeutic agent in the form of root canal irrigant or intracanal medicament in the field of dentistry.

**Keywords**: Fourier-transform infrared spectroscopy; MTT assay; nanoparticles; ultraviolet-Vis spectrophotometry; X-ray diffraction

**INTRODUCTION**

Nanotechnology has been emerging as a separate entity as it renders benefits to the field of medicine as well as dentistry. The nanomaterial being smaller in size occupies...
a large surface area and can exert better physical, chemical, and biological properties as compared to the conventional materials.\[1\]

The benefits of silver are well known in the field of medicine as it possesses an excellent antimicrobial activity against most of the bacteria as well as fungi. As silver nanoparticles are comparatively smaller in size to their bulk counterparts, they are able to penetrate the cell membranes of bacteria and cause DNA damage and cell death.\[2\] Andrographis paniculata is a well-known medicinal plant belonging to Acanthaceae family and is widely found in all parts of Asia. In the field of medicine, its antibacterial, antiviral, antifungal, and antiparasitic effects have been well documented. The active compound present in A. paniculata is andrographolide (AG), which is thought to be a quorum-sensing inhibitor, and it is also known to inhibit the virulence factor of microbes.\[3\]

It regulates macrophage phenotypic polarization, thereby regulating host immunity and producing Ag-specific antibodies.\[4\] Dedhia et al. used A. paniculata as a root canal irrigant and tested the antimicrobial efficacy against Staphylococcus aureus and Candida albicans and compared with that of sodium hypochlorite and found a significantly higher zone of inhibition against C. albicans and comparable zone of inhibition against S. aureus.\[5\]

Ocimum sanctum Linn. is a well-known basil and has been used in Ayurveda for 1000 of years. It is well known for its excellent healing properties and anti-inflammatory and antioxidant property.

Yamani et al. studied the antimicrobial properties of O. sanctum Linn. and identified that camphor, eucalyptol, eugenol, alpha-bisabolene, beta-bisabolene, and beta-caryophyllene are the main compounds responsible for its antimicrobial action.\[6\] Chandrappa et al. compared the antimicrobial efficacy of tulsi extract with that of 2% chlorhexidine gluconate and found it be effective against Enterococcus faecalis.\[7\]

The root canal system harbors microbes generally in the form of biofilms.\[8\] The microbes are more difficult to eradicate when they are present in the form of biofilms when compared to their planktonic counterparts as they possess resistance to antimicrobial agents. Although mechanical debridement of the root canal and root canal irrigants helps in the reduction of microbial load, the importance of intracanal medicaments in the treatment of apical periodontitis cannot be neglected.

Calcium hydroxide is considered the gold standard, and all the novel intracanal drugs are tested against this. It exerts its antimicrobial effect when it comes in direct contact with the bacteria. It has been well documented in in vitro studies that the bacteria is well eliminated within short time when they come in direct contact with this agent due to its alkaline pH, although in clinical scenarios, this is not always possible.\[9,10\]

The currently available root canal disinfectants have limitations as they are not able to eradicate root canal biofilms; the search for an apt root canal disinfectant continues.

In the present work, we synthesized calcium hydroxide-based silver nanoparticles using the leaves of A. paniculata and O. sanctum Linn. and tested their cytotoxic levels and antimicrobial activity against oral microbes.

**MATERIALS AND METHODS**

**Preparation of plant extract**

Fresh leaves of both the plants, namely A. paniculata and O. sanctum Linn., were collected, dried in shade, and powdered coarsely. 1 g of the powdered leaves was dissolved in 100 ml of distilled water and mixed well and then the solution was boiled for 5 min at 60°C–80°C and filtered and stored in a refrigerator for later use.\[11\]

**Synthesis of Silver nanoparticles**

10 ml of the pure plant extract was added into the 90 ml of 3 mM of silver nitrate solution and was mixed thoroughly, and the solution was kept in a magnetic stirrer for further mixing. The color alteration of the solution indicated the nanoparticle formation. UV Vis spectrophotometer analysis was done to confirm the synthesis of silver nanoparticles. Then, the solution was centrifuged at 8000 rpm for 15 min, filtered, and refrigerated.\[11\]

**Synthesis of Calcium hydroxide-coated silver nanoparticles**

0.5 g of calcium hydroxide powder (Prevest DenPro Limited, India) was dissolved in 50 ml of biosynthesized silver nanoparticle solution and mixed well. To achieve homogenous mix, the solution was boiled for 7–8 h. UV spectrophotometer analysis was done to confirm the synthesis of calcium hydroxide-coated silver nanoparticles. Then, the solution was centrifuged at 8000 rpm for 15 min, filtered, and refrigerated.

**Characterization of CaOH-Ag nanoparticles**

**Ultraviolet-Vis spectrophotometer analysis**

Periodic sampling of the prepared solution was done by subjecting the solution to a spectrophotometer (UV-1800 series).

**Transmission electron microscopy**

Transmission electron microscopy (TEM) was done to analyze the dimensions and morphology of the prepared calcium hydroxide-coated silver nanoparticles.
Fourier-transform infrared spectroscopy analysis and X-ray diffraction assay
The chemical functional groups of calcium hydroxide-coated silver nanoparticles were analyzed by Fourier-transform infrared spectroscopy (FTIR) analysis using a Fourier-transform infrared spectrometer (PerkinElmer, USA), and X-ray diffraction (XRD) assay was performed using X-ray diffractometer (Bruker, Germany) to observe the crystalline structure of newly synthesized nanoparticles.

Antimicrobial activity test
Muller–Hinton agar plates were used to check the antimicrobial activity. The plates were swabbed with Streptococcus mutans, S. aureus, Pseudomonas sp., and E. faecalis. These microbes were isolated from patients. Wells were created, and test suspension as well as calcium hydroxide mixed with distilled water was loaded in the concentration of 25 µL, 50 µL, and 150 µL, respectively. 30 µL concentration of standard antibiotic was used as control. The plates were incubated at 37°C for 48 h, and the zone of inhibition was then measured. Rose bengal was used in a similar manner for C. albicans. The microbiological procedure was done in triplicates.

Assessment of cytotoxicity (brine shrimp lethality assay)
Around 30 g of brine shrimp eggs (Aquatic Remedies Salt Lake Artemia) was added to the tank containing 6 L of distilled water and 50 g of iodine-free salt. Aeration level was maintained according to the manufacturers’ instructions and left undisturbed for 24 h. The hatched nauplii were observed under a stereomicroscope. Test solution was loaded in the test tubes at the concentration of 10 µL, 20 µL, 30 µL, 40 µL, and 50 µL, respectively. To each test tube, nauplii were added. Tube without nauplii served as a control. After 24 h incubation, the live and dead nauplii were counted, and lethality was assessed.

MTT assay
The cytotoxicity was assessed by MTT assay which determines the cell viability and characterizes the cytochemical demonstration of succinic dehydrogenase produced by the cells. Adipose tissue cell line of the mouse (3T3-L1) was utilized. The cells were seeded onto 96-well microplates at a density of 1 × 10⁴ cells/100 µL per well and were incubated with nanoparticles and calcium hydroxide mixed with distilled water for 48 h at the concentrations of 10–50 µg/mL. 100 µL of MTT solution (0.5 mg/mL MTT in PBS) was added to it. The cells were incubated for 4 h in a CO2 incubator. Purple color formation indicated the formation of formazan. The MTT-purple formazan was dissolved in 0.1 N isopropanol/hydrochloric acid, and optical densities of the solutions were measured by absorbance at 570 nm in an enzyme-linked immunosorbent assay plate reader.

% Inhibition = Absorbance of control − absorbance of sample/absorbance of control × 100

Statistical analysis
The data were analyzed using SPSS 23.0 statistical package (IBM SPSS Statistics, Version 23.0. Armonk, NY: IBM Corp). As the data were continuous, parametric independent t-test was used. The mean and standard deviation were calculated. P value was set at < 0.05.

RESULTS
Ultraviolet spectrophotometer analysis
The calcium hydroxide-coated silver nanoparticles were successfully formed as noticed by visual color change and were confirmed by spectrophotometer analysis (UV-1800 series).

The absorption peak was recorded at 288 nm. The absorption peak of silver is around 420 nm, which is not visible in the graph suggestive of complete absorption of silver nanoparticles into crystalline structure of calcium hydroxide [Figure 1a].

Transmission electron microscopy
There was a uniform distribution of silver nanoparticles of size around 15–20 nm and spherical in shape. Some nanoparticles are very small in size around 10 nm. Agglomerations of silver nanoparticles were also observed. Calcium hydroxide particles coated the surface of silver nanoparticles [Figure 1b]. Similar results were reported by Kodeh et al.

Fourier-transform infrared spectroscopy analysis
The FTIR spectra of CaOH Ag nanoparticles showed absorption peaks at 418 cm⁻¹ corresponding to (O-H) and (Ca-O) vibration; the band at 873 cm⁻¹ and 1409 cm⁻¹ is due to (C-O) bond related to carbonation of CaO nanoparticles. Sharp peak at 3639 cm⁻¹ corresponds to hydroxyl group (O-H) bonds due to the presence of CaOH [Figure 1c].

X-ray diffraction analysis
The initial diffraction peaks were observed at 20 = 12.48°, 18.27°. This may correspond to crystalline and amorphous organic phases of the plant extract. The intense peak was observed at 20 = 29.60°, 34.28°, 50.97°, and 62.69° corresponding to (111), (200), (311), and (222) orientation planes, respectively, which is a usual 20 value calcium oxide nanoparticles. The peaks observed at 71.89° and 84.88° correspond to (311) and (222) crystalline planes of silver nanoparticles, respectively. These are the usual diffraction peaks for silver.

It can be inferred that at the initial phase of the reaction, there was a reduction of calcium hydroxide
by plant extract. At the later phase of the reaction, silver nanoparticles were formed. Hence, there was a successful formation of calcium hydroxide-coated silver nanoparticles [Figure 1d].

**Antimicrobial activity**

There was no statistically significant difference in antimicrobial activity of CaOH and CaOH AgNP against S. mutans, E. faecalis, and Pseudomonas at all tested concentrations. The mean zone of inhibition was greater for CaOH AgNP as compared to CaOH at all tested concentrations against S. aureus although it was not statistically significant. The mean zone of inhibition was greater for CaOH AgNP as compared to CaOH at 25 µL and 50 µL with statistically significant difference at 150 µL concentration against C. albicans [Table 1].

**MTT assay**

At all the concentrations, more than 70% of cells were viable. There was no statistically significant difference in cell viability between CaOH AgNP and CaOH although the mean cell viability percentage was higher for CaOH AgNP as compared to CaOH at all the concentrations [Table 2].

This may be due to reason that for the synthesis of nanoparticles, the plant extract was used as reducing and capping agents, which would have reduced the cytotoxicity levels of these nanoparticles.

**Brine shrimp lethality assay**

No cytotoxic effects were observed at concentration of 10 µL. Minimum cytotoxicity was observed at higher concentrations. It is noteworthy that even at the highest concentration, around 70% of nauplii were alive.

**DISCUSSION**

In the present study, we synthesized calcium hydroxide-based silver nanoparticles using the leaves of A. paniculata and O. sanctum Linn. plant and check their cytotoxicity and antimicrobial activity against oral microbes.

To the best of our knowledge, there are no studies which evaluated the antimicrobial effect of the green synthesized (using plant extract of A. paniculata and O. sanctum Linn.) silver nanoparticles and calcium hydroxide.

The nanoparticles were synthesized and characterized and proved to have a good antimicrobial activity with minimum cytotoxic effects.

To achieve complete success for any therapy, the disinfection plays an important role. Eradication of microbes requires adequate cleaning and shaping of the root canals along with proper disinfection protocol. Calcium hydroxide serves as the gold standard for root canal disinfection, but there are various conflicting studies, which reported the inability of calcium hydroxide to eradicate E. faecalis.[10,16]

The concerns have been raised by various authors regarding buffering effect of dentin, hydroxyapatite, and pupal remnants on calcium hydroxide.[17]

Nanoparticles can be synthesized by physical, chemical, and biological means. The nanoparticles prepared using plant extract have the advantage of being a cost-effective, quick process. It avoids the usage of high temperature, pressure, energy, and toxic chemicals and hence can be considered an environment-friendly approach. The synthesis of nanoparticles using plant extracts occurs due to the presence of nanoparticles.
Table 1: Mean and standard deviation of antimicrobial activity of AgCaOH nanoparticles and CaOH

| Microorganisms          | Concentration (µL) | Groups         | Mean zone of inhibition (mm) | SD  | P   |
|-------------------------|--------------------|----------------|------------------------------|-----|-----|
|                         |                    |                | Calcium hydroxide            |     |     |
| *Streptococcus mutans*  | 25                 | AgCaOH NP      | 10.6667 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 16.3333 0.57735             |     |     |
|                         | 50                 | AgCaOH NP      | 13.6667 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 18.3333 0.57735             |     |     |
|                         | 150                | AgCaOH NP      | 16.6667 0.57735             | 0.561|0.673 |
|                         |                    | Calcium hydroxide | 20.0000 1.00000            |     |     |
| *Staphylococcus aureus* | 25                 | AgCaOH NP      | 12.0000 1.00000             | 0.561|0.66091|
|                         |                    | Calcium hydroxide | 10.6667 0.57735             |     |     |
|                         | 50                 | AgCaOH NP      | 13.3333 0.57735             | 0.561|0.561 |
|                         |                    | Calcium hydroxide | 11.0000 1.00000            |     |     |
|                         | 150                | AgCaOH NP      | 15.3333 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 13.6667 0.57735             |     |     |
| *Enterococcus faecalis* | 25                 | AgCaOH NP      | 11.6667 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 11.6667 0.57735             |     |     |
|                         | 50                 | AgCaOH NP      | 14.3333 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 12.3333 0.57735             |     |     |
|                         | 150                | AgCaOH NP      | 15.6667 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 14.3333 0.57735             |     |     |
| *Pseudomonas*           | 25                 | AgCaOH NP      | 11.6667 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 0.6667 0.57735              |     |     |
|                         | 50                 | AgCaOH NP      | 12.3333 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 11.6667 0.57735             |     |     |
|                         | 150                | AgCaOH NP      | 15.3333 0.57735             | 1.00|     |
|                         |                    | Calcium hydroxide | 13.6667 0.57735             |     |     |
| *Candida albicans*      | 25                 | AgCaOH NP      | 26.0000 1.00000             | 0.205|0.99047|
|                         |                    | Calcium hydroxide | 10.6667 2.08167            |     |     |
|                         | 50                 | AgCaOH NP      | 26.3333 0.57735             | 0.145|0.81733|
|                         |                    | Calcium hydroxide | 11.3333 2.51661           |     |     |
|                         | 150                | AgCaOH NP      | 30.3333 0.57735             | 0.044*|0.891 |
|                         |                    | Calcium hydroxide | 12.3333 2.30940           |     |     |

*Indicates significant difference (Independent t-test, P<0.05). SD: Standard deviation

Table 2: Mean and standard deviation of percentage cell viability (MTT assay) of AgCaOH nanoparticles and CaOH

| Concentration (µg) | Groups | Mean cell viability (%) | SD  | P   |
|--------------------|--------|-------------------------|-----|-----|
| 10                 | AgCaOH | 81.7833                 | 1.00167|0.305|
|                    | CaOH   | 78.5733                 | 0.40415|     |
| 20                 | AgCaOH | 80.5833                 | 0.81733|0.891|
|                    | CaOH   | 74.4567                 | 0.76376|     |
| 30                 | AgCaOH | 80.6700                 | 0.66091|0.384|
|                    | CaOH   | 74.6467                 | 1.26231|     |
| 40                 | AgCaOH | 81.1167                 | 1.45029|0.673|
|                    | CaOH   | 72.5467                 | 0.99047|     |
| 50                 | AgCaOH | 78.6100                 | 0.54836|0.470|
|                    | CaOH   | 71.4467                 | 0.85874|     |

Independent t-test, P<0.05. SD: Standard deviation

Various noble metals have been utilized to formulate nanoparticles. Among them, silver has gained special attention owing to its distinct properties, such as favorable electrical conductivity, chemical stability, catalytic, and antibacterial activity. The bacterial cells are negatively charged; silver has high affinity to negatively-charged molecules, and it inactivates functions of bacterial cells and inhibits bacterial growth and biofilm formation,[19] and when combined with calcium hydroxide, antimicrobial action seems to be enhanced as evident from the results of the present study.

There is a strong evidence supporting the enhancement of antimicrobial efficacy against *E. faecalis* biofilm when calcium hydroxide was combined with silver nanoparticles as compared to calcium hydroxide used alone. Afkhami *et al.* compared the CaOH + AgNP with CaOH + CHX and CaOH used alone against *E. faecalis* and found the CaOH + AgNP to be most effective.[20] Zhang *et al.* evaluated the efficacy of CaOH + AgNP against *E. faecalis* in the starvation phase and compared it with AgNP and CaOH used alone and found the combination to be most effective.[21] Javidi *et al.* observed that the colony-forming units were significantly lesser when CaOH + AgNP was used when compared to CaOH used solely.[22] Balto *et al.* confirmed that there was no significant difference between the CaOH + AgNP and triple antibiotic paste against *E. faecalis* biofilm.[23] Tülü *et al.* compared the antimicrobial efficacy of CaOH, CaOH + AgNP, 2%CHX gel, and 2% CHX gel + AgNP and found that CaOH + AgNP was most effective against multispecies (*E. faecalis, S. mutans, Lactobacillus acidophilus*, and *Actinomyces naeslundii*) biofilm.[24]

**CONCLUSION**

Calcium hydroxide coated silver nanoparticles were effectively synthesised using leaf extracts of *Andrographis paniculata* and *Ocimum sanctum* Linn. They were proved to have similar antimicrobial property as calcium hydroxide and found to have better antifungal properties when compared to calcium hydroxide. In terms of cytotoxicity, they were proven to be less cytotoxic when compared to CaOH used alone. Afkhami *et al.* compared the CaOH + AgNP with CaOH + CHX and CaOH used alone against *E. faecalis* and found the CaOH + AgNP to be most effective. Afkhami *et al.* compared the antimicrobial efficacy of CaOH, CaOH + AgNP, 2%CHX gel, and 2% CHX gel + AgNP and found that CaOH + AgNP was most effective against multispecies (*E. faecalis, S. mutans, Lactobacillus acidophilus*, and *Actinomyces naeslundii*) biofilm. A strong evidence supporting the enhancement of antimicrobial efficacy against *E. faecalis* biofilm when calcium hydroxide was combined with silver nanoparticles as compared to calcium hydroxide used alone. Afkhami *et al.* compared the CaOH + AgNP with CaOH + CHX and CaOH used alone against *E. faecalis* and found the CaOH + AgNP to be most effective. There is a strong evidence supporting the enhancement of antimicrobial efficacy against *E. faecalis* biofilm when calcium hydroxide was combined with silver nanoparticles as compared to calcium hydroxide used alone. Afkhami *et al.* compared the CaOH + AgNP with CaOH + CHX and CaOH used alone against *E. faecalis* and found the CaOH + AgNP to be most effective. Zhang *et al.* evaluated the efficacy of CaOH + AgNP against *E. faecalis* in the starvation phase and compared it with AgNP and CaOH used alone and found the combination to be most effective. Javidi *et al.* observed that the colony-forming units were significantly lesser when CaOH + AgNP was used when compared to CaOH used solely. Balto *et al.* confirmed that there was no significant difference between the CaOH + AgNP and triple antibiotic paste against *E. faecalis* biofilm. Tülü *et al.* compared the antimicrobial efficacy of CaOH, CaOH + AgNP, 2%CHX gel, and 2% CHX gel + AgNP and found that CaOH + AgNP was most effective against multispecies (*E. faecalis, S. mutans, Lactobacillus acidophilus*, and *Actinomyces naeslundii*) biofilm.

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**Conflicts of interest**
There are no conflicts of interest.

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