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RESEARCH ARTICLE

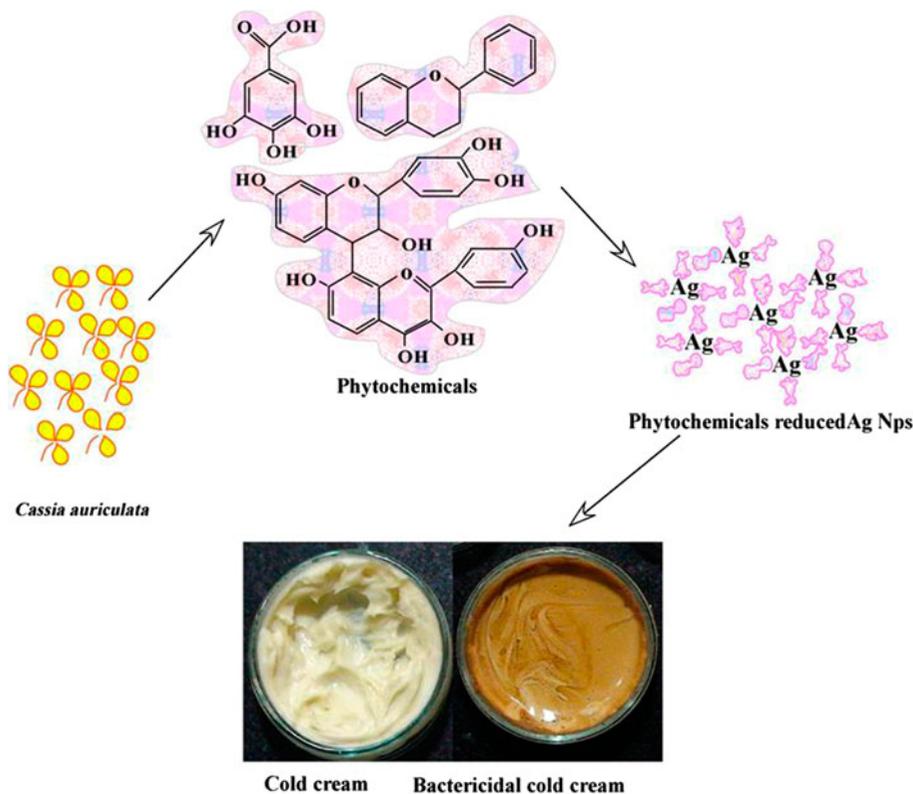
Formulation of bactericidal cold cream against clinical pathogens using *Cassia auriculata* flower extract-synthesized Ag nanoparticles

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A simple formulation of bactericidal cold cream using the biosynthesized silver nanoparticles (AgNPs) from *Cassia auriculata* flower extract and their antibacterial activity was tested against various clinical pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*. An eco-friendly method was followed for the biosynthesis of AgNPs using *C. auriculata* flower extract as a reducing agent at room temperature. The effect of different concentrations of flower extract and the various pH conditions of the reaction medium toward the formation of NPs were studied. Surface plasmon resonance peaks were obtained from 403 nm to 428 nm. Further, the synthesized NPs were characterized by dynamic light scattering particle size analysis, Zeta potential analysis, atomic force microscope, and high-resolution transmission electron microscopic analysis.



Keywords: biosynthesis; silver nanoparticles; *Cassia auriculata*; bactericidal cold cream; antibacterial activity

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R. Sahana and S.C.G. Kiruba Daniel contributed equally.

1. Introduction

In recent trends, nanobiotechnology has emerged as a promising field in nanomedicine which involves development in biological, non-polluting, eco-friendly method for the synthesis of nanoparticles (NPs) (1–3). Size distribution and morphology-dependent properties of NPs make them as an indistinguishable source of material for various applications in the field of electronic, magnetic, information storage, drug delivery, biomolecular detection, diagnostics, and antimicrobial therapeutics (4, 5). Among the noble metal NPs, silver has attained much interest due to their higher surface area in correspondence to the decrease in the size of particles (6–8). This unique property of silver provides them better contact with the micro-organism in comparison with other salts. The possible antibacterial mechanism of silver nanoparticles (AgNPs) is that they get attached and penetrate into the bacterial cell wall followed by the gradual release of silver ions (9), interacting with phosphorous- and sulfur-containing compounds like deoxyribonucleic acid (DNA) and proteins which result in damages to DNA replication, ATP production, cell division, cellular respiration, and cell wall permeability. One of the major advantages of AgNPs is that at low concentration they are safe to human cells but lethal to microbes (10–12). Thus due to their enhanced antimicrobial activity, AgNPs-embedded creams are formulated and commercially used for treating burns and wounds. These NPs' dressings are found to exhibit fast and broad spectrum of antibacterial activity against Gram-positive and Gram-negative organisms. Previous studies reported on the antibacterial activity of ZnO NPs emulsified in cold cream against *Propionibacterium acnes* (13, 14).

Most of the methods adopted for the synthesis of NPs involve the usage of toxic chemicals, low material conversions, high energy requirements, and difficult purification process. Therefore, naturally occurring biological sources are best alternative for the synthesis of NPs. The usage of plant extract for the synthesis of NPs is that they are easily available, safe to handle, has a broad variable of metabolites, and, moreover, it eliminates the maintenance of microbial cell culture (15–16), among others. *Cassia auriculata* is a herb locally known as avaram belongs to a family of Caesalpiniaceae profoundly used in ayurvedic and siddha medicines due to its antipyretic, hepatoprotective, antidiabetic, antiperoxidative, and antihyperglycemic activity (17, 18).

Silver sulfonamide NP-based cream was formulated and analyzed for burn wound healing applications (19) but chemically synthesized NPs were used, for

which we propose the use of biosynthesized AgNPs with *C. auriculata* flower extract which itself has medicinal properties. The purpose of this study is to examine the antibacterial activity of bactericidal cream formulated using the biosynthesized AgNPs from *C. auriculata* flower extract against prominent clinical isolates.

2. Materials and methods

2.1. Materials

Silver nitrate (AgNO_3), liquid paraffin, borax, and bees wax were purchased from Sigma Aldrich. *C. auriculata* flowers were collected from Udayanpatti village, Tiruchirappalli. Clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* were obtained from local hospitals.

2.2. Preparation of flower extract

C. auriculata flowers were washed thoroughly with Milli-Q water. The flower extract was prepared by taking 20 gm of *C. auriculata* flowers in 100 ml of Milli-Q water ($18.2\ \Omega$ cm resistivity at 25°C), boiled for 1 hour at 70°C , was filtered through Whatman No. 1 filter paper, was stored at 4°C , and used for the biosynthesis of AgNPs as reported earlier (20). Figure 1 shows the schematic representation for the biosynthesis of AgNPs using *C. auriculata* flower extract.

2.3. Biosynthesis of AgNPs

The aqueous silver nitrate solution was prepared by adding 0.016 gm (1 mM) of silver nitrate solution into

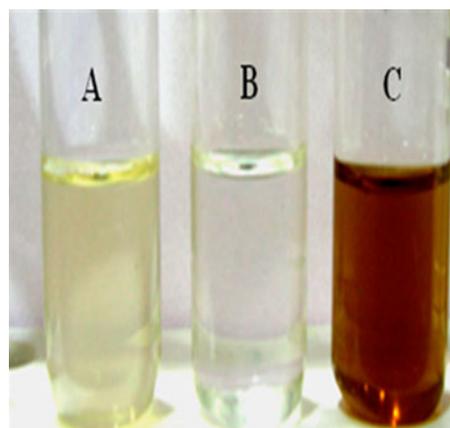


Figure 1. AgNPs formed after the addition of 50 μl flower extract to aqueous 5 ml of 1 mM silver nitrate solution. (A) Flower extract, (B) aqueous silver nitrate, and (C) AgNPs.

100 ml of Millipore water. Then 500 μl of *C. auriculata* flower extract was added into the prepared aqueous AgNO_3 solution for the reduction of AgNO_3 into AgNPs (20) and kept in a magnetic stirrer for 1 hr at room temperature. Additionally, the effect of concentration of flower extract on the formation of NPs was investigated by varying the flower extract concentration as 50 μl , 100 μl , 250 μl , 500 μl , and 1000 μl . The effect of pH of the reaction medium on the formation of AgNPs was also studied by adjusting the solutions to different pH levels as pH 3, 5, 7, 9, and 11.

2.4. Formulation of antimicrobial cream

The nature of cream is water in oil emulsion and three different creams were prepared, one with biosynthesized AgNPs, one with plant extract alone, and another as control. Two-gram bees wax and 6-ml liquid paraffin were taken in a porcelain cup and heated in a water bath at 70°C. The aqueous phase was prepared by heating 0.1 gm of borax in 2 ml of Milli-Q water in another porcelain cup at 50°C. The aqueous phase was transferred to a mixture of bees wax and liquid paraffin along with 2 ml of biosynthesized AgNPs or flower extract with continuous stirring at room temperature and kept at an airtight container for further studies (21).

2.5. Characterization of NPs

Characterization of the biosynthesized AgNPs was carried out by the following standard techniques (20). The absorption spectra analysis for the biosynthesized AgNPs was carried out using JASCO V-650 UV spectrophotometer. Dynamic light scattering (DLS) analysis was performed (Malvern V 6.20 version) to measure the average particle size of the NPs. Before analyzing, the sample was sonicated for 5 min to obtain a uniform dispersion of NPs. To investigate the morphology and monodispersity of AgNPs, atomic force microscope (AFM) studies have been performed (Park systems). Images were obtained by scanning in non-contact mode. Both two-dimensional and three-dimensional images were obtained. Morphology and size of the biosynthesized AgNPs were investigated by high-resolution transmission electron microscopic (HRTEM) and selected area electron diffraction (SAED) images (Phillips, TECHNAI FE 12). Thin film of the sample was prepared on a carbon-coated copper grid and dried under lamp for analysis.

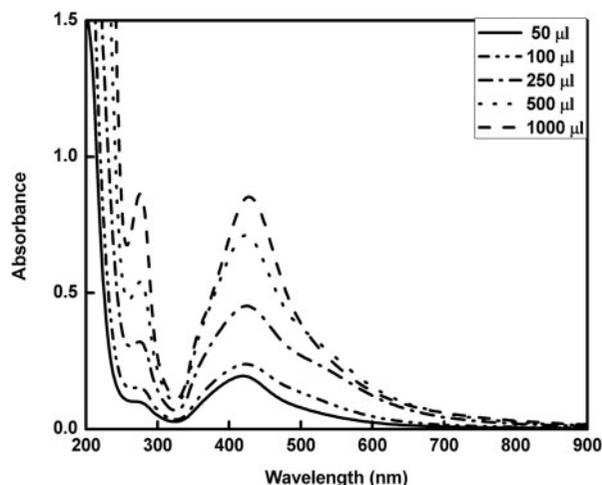


Figure 2. UV-visible spectra for different concentration of flower extract to 1 mM aqueous silver nitrate solution increase in absorbance value were observed.

2.6. Antibacterial assay

Antibacterial assays were performed for bactericidal cold cream containing biosynthesized AgNPs, the another cream containing flower extract and control cream against the clinical isolates such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis* through standard disk diffusion method as done previously (20). Briefly, lysogenic broth (LB) medium was used to cultivate the bacteria. Fresh, overnight bacterial cultures were used for the experiment. A 15-ml LB agar was poured in well-rinsed, autoclaved petri plates, bacterial cultures were spread uniformly on the surface of LB plates, the disks of diameter 0.5 mm

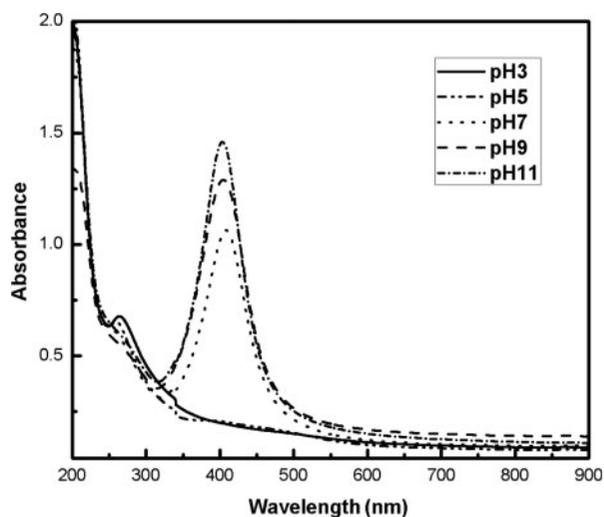


Figure 3. UV-visible spectra of AgNPs formed at different pH 3, 5, 7, 9, and 11 and as the pH value is increased blue shift was observed.

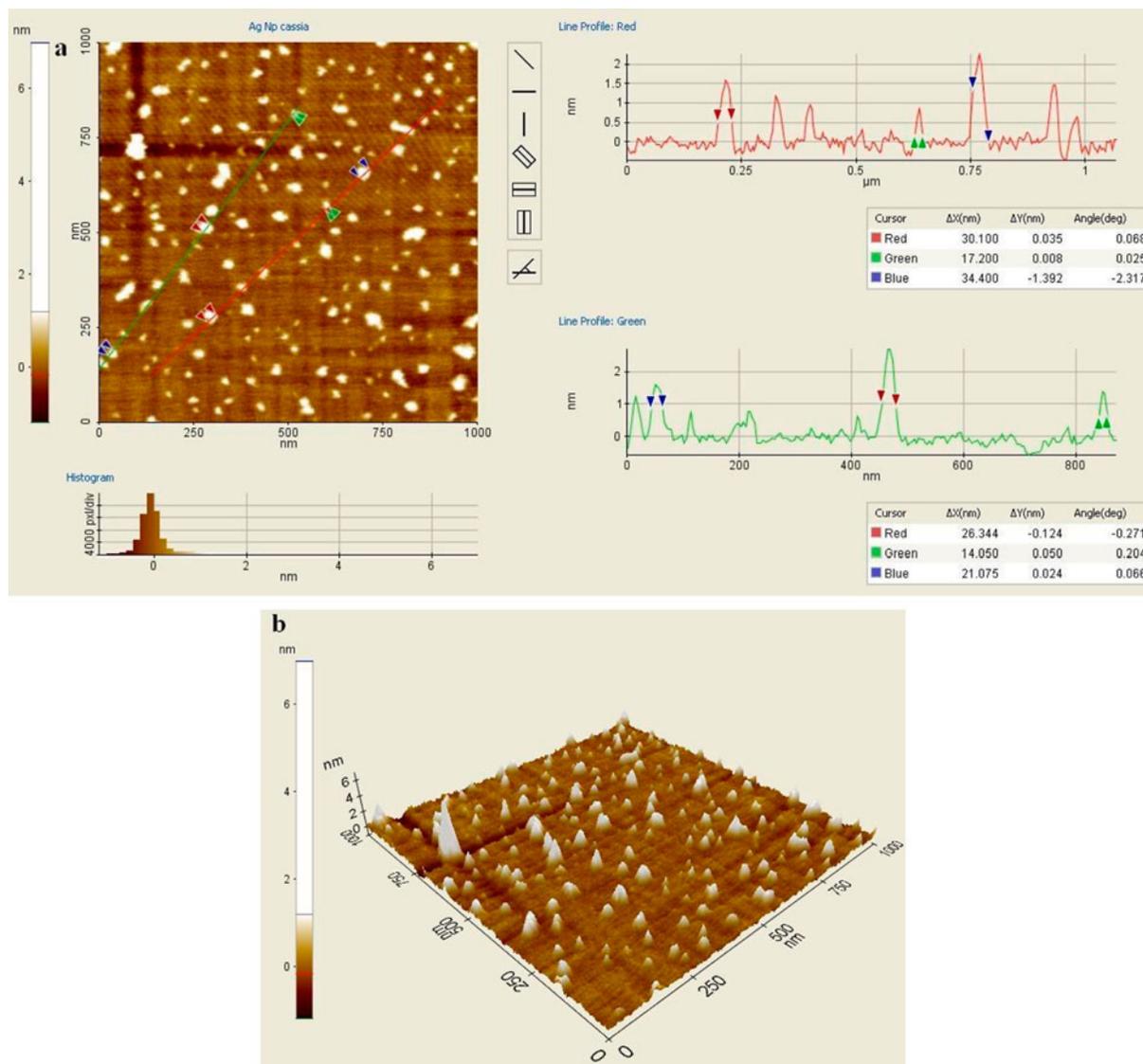


Figure 4. AFM (2D and 3D) image of *C. auriculata* flower extract-biosynthesized AgNPs exhibiting a size range of 20–70 nm.

were autoclaved, dipped in the cream for 6 hours, and placed over the bacteria-inoculated medium at 37°C for 24 hours. The zone of inhibition was determined by measuring the diameter of the inhibition zone.

3. Results and discussion

3.1. Synthesis of AgNPs

In recent years, many physical and chemical methods have been adopted for the synthesis of NPs which involve in the usage of organic surfactants and strong reducing agents like cetyl trimethylammonium bromide (CTAB), sodium borohydride etc., thus the NPs synthesized using toxic chemicals are not applicable for the biological applications. This is because the surfactants cannot be completely removed from the

metallic NPs (22). Dhas et al. reported the synthesis of AgNPs from *C. auriculata* flower extract and evaluated their antibacterial activities against bacterial strains such as *Bacillus licheniformis*, *Klebsiella planticola*. The synthesized NPs showed a broad absorption spectrum which may be due to the presence of NPs in a wide size range (23). We investigated the role of concentration of flower extract and pH of the reaction medium toward the formation of AgNPs. We have also formulated a bactericidal cold cream and tested its efficiency against various clinical pathogens.

The reaction mixture containing the aqueous solution of AgNO_3 and flower extract starts to change the color from yellowish brown to reddish brown as shown in Figure 1, indicating the characteristic optical properties of the AgNPs. Thus the plant

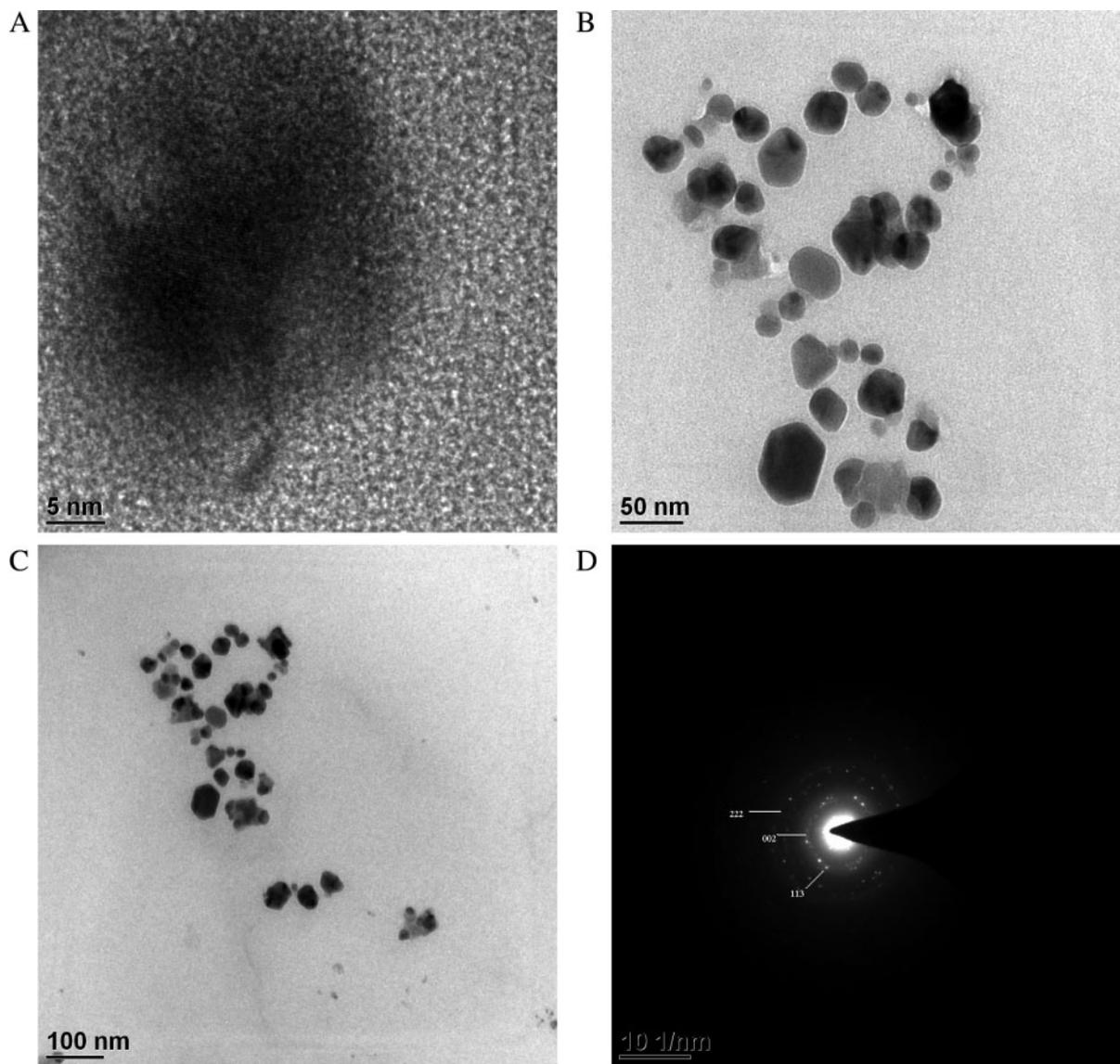


Figure 5. HRTEM images of AgNPs formed after the addition of *C. auriculata* flower extract shown at different scales of (a) 5 nm, (b) 50 nm, (c) 100 nm, and (d) SAED pattern.

phytochemicals such as terpenoids, tannin, flavonoids, proteins, phenols, and saponin present in the flower extract (24) play an important role in the formation of NPs. So that once these phytochemicals enter into the body along with the NPs, they do not cause any harm. The possible mechanism involving in the reduction of AgNO_3 ions is due to the formation of intermediate complexes with OH groups of phenolic compounds in the hydrolysable tannins, which undergoes oxidation to quinone forms resulting in the reduction of Ag^+ to AgNPs (25, 26).

3.2. UV-visible spectral analysis

AgNPs exhibit unique, tunable optical properties on account of their surface plasmon resonance (SPR),

depending on shape, size, and distribution of the NPs (27). Further, the AgNPs have free electrons which give rise to SPR band, this is due to the combined vibrations of electrons of the metal NPs. Some of the important parameters such as temperature pH, dielectric properties, shape, and size of the NPs influence the absorbance and position of the SPR band (28, 29). Thus the formation of AgNPs was confirmed using UV-spectral analysis. Figure 2 shows the UV-visible spectra for AgNPs formed at different concentrations of flower extract. From the graph, it was noticed that the lowest amount of flower extract concentration was effective for the synthesis of AgNPs. The absorbance peak was centered between 422 nm and 427 nm which is the characteristic peak of AgNPs. Also the

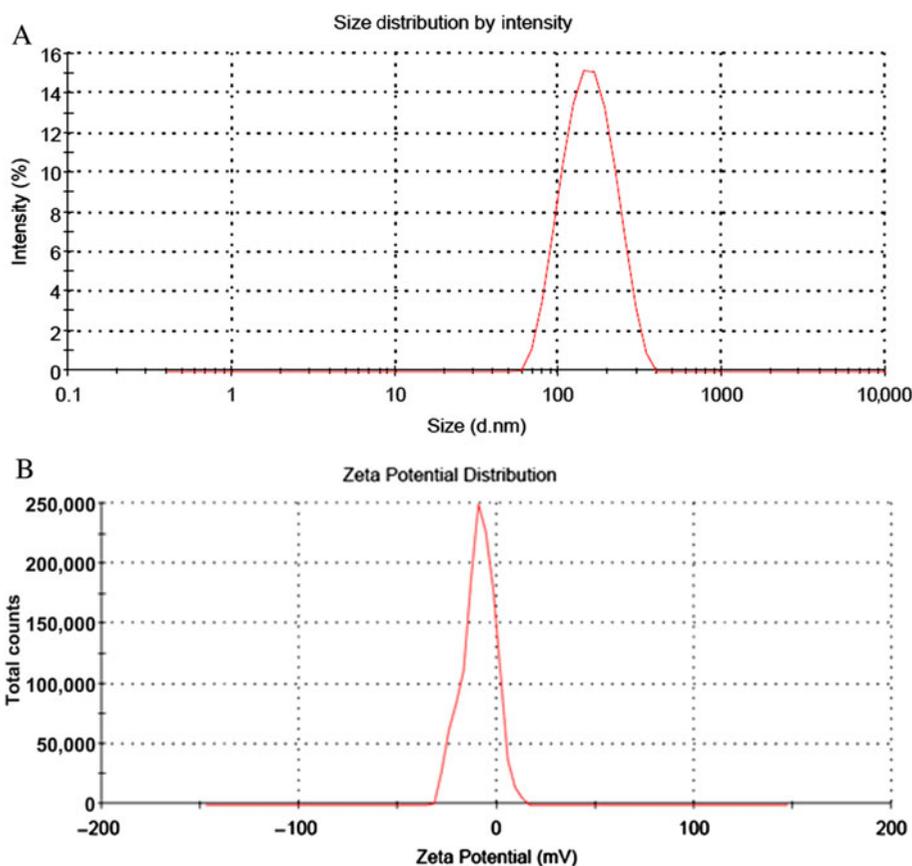


Figure 6. (a) DLS (particle size analysis) of biosynthesized AgNPs. (b) Zeta potential analysis of biosynthesized AgNPs.

sharpness of the absorption peak is dependent on the concentration of flower extract, which gets further sharpened at increase in the concentration of flower extract – this might be due to the narrow size distribution of the NPs. Thus the amount of flower extract used for the synthesis of AgNPs was found to be less when compared with the previous reports (30).

The effect of pH on the formation of AgNPs was analyzed using UV–visible spectrophotometric studies. From the results, it was found that the formation of AgNPs was observed once the pH of the medium reached pH 7 and the absorption peak was obtained at 408.5 nm. At pH 9 and 11, the color intensity increases from orange to reddish brown and the



Figure 7. Antimicrobial cream formulated, (A) control cream without the addition of AgNPs and (B) antimicrobial cream formulated by adding 2 ml as synthesized bioAgNPs.

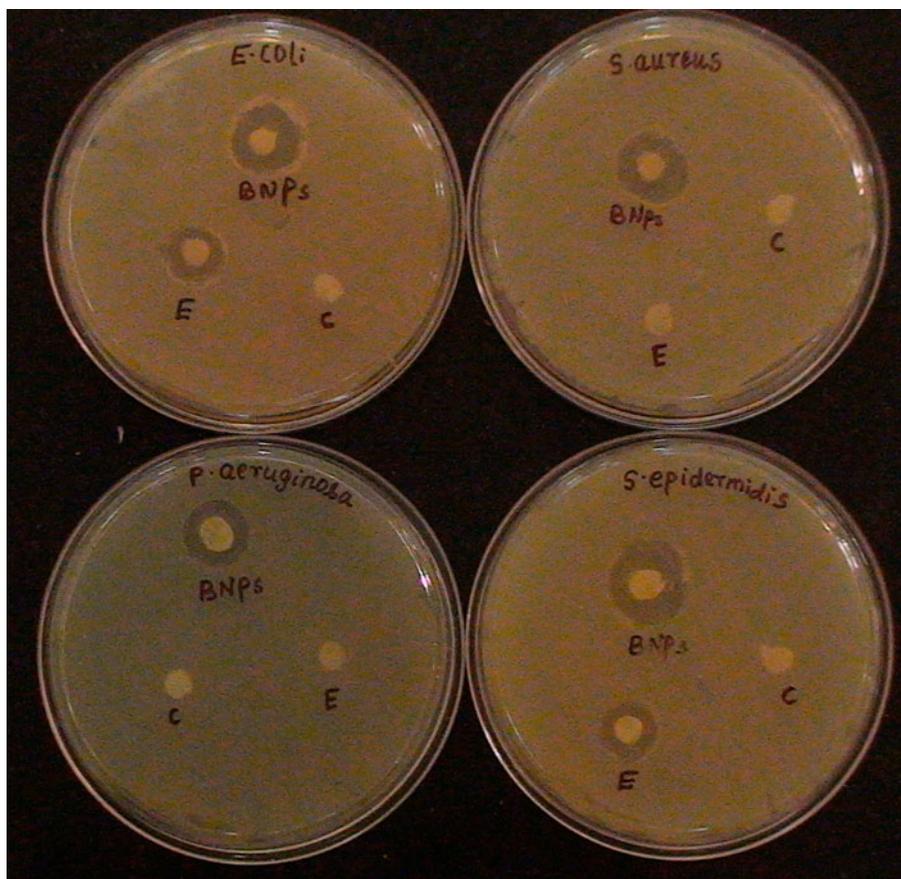


Figure 8. Antibacterial assays: zone of inhibition (mm) observed around the disks containing creams C – control cream, E – extract containing cream, and BNPs – bactericidal cream having AgNPs tested against (A) *E. coli*, (B) *S. aureus*, (C) *P. aeruginosa*, and (D) *S. epidermidis*.

absorption peak was obtained at 404 nm and 403.5 nm, respectively (Figure 3). Thus the formation of AgNPs occurs rapidly at the neutral pH and this might be due to the ionization of phenolic groups present in the *C. auriculata* flower extract. At acidic condition of the reaction medium (pH3–pH5), there were no development of brown color or characteristic peak of AgNPs. So that from the results it was understood that the variety of biomolecules involved in the formation of AgNPs are inactive under acidic pH condition (31). Further, the slow rate of formation and aggregation of NPs at the acidic pH might be due to the electrostatic repulsion of anions present in the

reaction medium. The variation of color at different pH condition is related to the difference in the dissociation constant (pKa) of various functional groups present in the flower extract that play an important role in the reduction process (32).

3.3. Analysis of particle size and topography

AFM studies showed that the AgNPs were similar in shape and size, ranges between 20 nm and 70 nm. The two-dimensional and three-dimensional view of the AgNPs was shown in Figure 4. HRTEM analysis was performed to analyse the size and shape of the AgNPs.

Table 1. Zone of inhibition (mm) observed around the disks containing creams.

S. No	Bacteria	Zone of inhibition (AgNPs)	Zone of inhibition (Flower extract)
A	<i>E. coli</i>	14 mm	7 mm
B	<i>S. aureus</i>	12 mm	–
C	<i>P. aeruginosa</i>	11 mm	–
D	<i>S. epidermidis</i>	16 mm	9 mm

From the HRTEM images, it was found that the biosynthesized AgNPs were mostly hexagonal and irregular in shape with narrow size distribution as shown in Figure 5. Most of the AgNPs are sized between 30 nm and 70 nm. A similar result was reported by Kumar et al. on the synthesis of AgNPs from *Annona squamosa* peel extract. But, in their studies synthesized NPs were polycrystalline and irregular narrow size distribution was observed. The average particles size was found to be ± 35 nm (33). SAED pattern of the biosynthesized AgNPs reveals that they are in phases of 222, 113, and 002 (Figure 5f). DLS results show that the average particle size of the biosynthesized AgNPs was around 152.4 d.nm which is shown in Figure 6a. The DLS measures the hydrodynamic radii of the NPs and hence the particle size value was found to be high. The surface charges of AgNPs were determined using Zeta potential measurement and it exhibited a value of -9 mV (Figure 6b), which suggests that the AgNPs are highly stable and the large negative Zeta potential value might be due to the capping of polyphenolic constituents present in the flower extract of *C. auriculata* (34).

3.4. Antibacterial analysis of cold cream

A bactericidal cold cream containing biosynthesized AgNPs using *C. auriculata* flower extract, and control cream containing flower extract was formulated and it is shown in Figure 7. Also their antibacterial efficiency was tested against the clinical isolates such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis* through standard disk diffusion method. It was found that the bactericidal cold cream exhibited an excellent antibacterial activity against both Gram-positive and Gram-negative organisms such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*. The maximum zone of inhibition for bactericidal cream was found to be 14 mm, 11 mm, 12 mm, and 16 mm which is shown in Figure 8. The cold cream containing the flower extract alone showed a minimum inhibitory effect on the pathogens. But the bactericidal cold cream containing the NPs synthesized from the flower extract showed an excellent antibacterial activity shown in Table 1. This bactericidal activity is mainly because of the release of silver cations from AgNPs which act as the reservoir for the Ag^+ bactericidal agent. The interaction of the silver cations with the cell membrane of bacteria leads to the increased membrane permeability of the bacteria (35, 36). Kaviya et al. reported on the antibacterial activity of biosynthesized AgNPs using *Citrus sinensis* peel extract against *E. coli*, *P. aeruginosa*, and *S. aureus* (37).

4. Conclusion

Thus an eco-friendly, nontoxic method and cost-effective method have been developed for the biosynthesis of AgNPs using *C. auriculata* flower extract. The phytochemicals such as terpenoids, tannin, flavonoids, proteins, phenols, and saponin present in the *C. auriculata* flower extract play an important role as reducing as well as capping agent at room temperature. So that this method is found to be a best alternative when compared with the chemical synthesis of NPs and it can be also adopted for the biosynthesis of NPs at a large scale. A bactericidal cold cream formulated using biosynthesized AgNPs showed an excellent antibacterial activity against various clinical pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. epidermidis*, and further studies are being going on to investigate it on animal models and to bring the formulated bactericidal cold cream as a cost-effective, patient-affordable cream in the market against bacterial infections.

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