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Synthesis, Characterization and Remedial Action of Biogenic Silver Nanoparticles and Chitosan-Silver Nanoparticles against Bacterial Pathogens

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ABSTRACT

Custard apple is a dry land fruit. Its leaves exhibit different pharmacological activities. In the present study, both silver (Ag) nanoparticles and chitosan-coated Ag (Chi-Ag) nanoparticles were fabricated using the aqueous leaf extract of the custard apple plant. During preliminary phytochemical analysis, various types of phytocompounds were found in the aqueous leaf extract of the same plant. Next, both nanoparticles were physiochemically characterized. FTIR analysis exhibited the fingerprint vibrational peaks of active bioactive compounds in plant extract, Ag nanoparticles, and Chi-Ag nanoparticles. UV/Visible spectral analysis revealed the highest absorbance peak at 419 nm, indicating the presence of Ag nanoparticles. XRD analysis presented the face-centered cubic (FCC) structure of both prepared nanomaterials. Further, the average crystalline size of both Ag nanoparticles and Chi-Ag nanoparticles was calculated to be 23 and 74 nm, respectively. FESEM analysis showed the spherical and cubical shapes of Ag nanoparticles and Chi-Ag nanoparticles, respectively. EDS analysis indicated a peak around 3.29 keV, conforming to the binding energies of Ag ions. The biogenic nanomaterial also showed strong antibacterial activity against all tested bacterial pathogens.

KEYWORDS

Custard apple; chitosan; Ag nanoparticles; Chi-Ag nanoparticles; antibacterial

1 Introduction

In recent years, the field of nanoscience has grown, and we have explored several nanomaterials in numerous biomedical, agricultural, and sensing applications [1]. In general, the synthesis of inorganic and organic nanoparticles with uniform size distribution and controlled monodispersity is a significant area of research in

nanotechnology. Among inorganic nanoparticles, metal and metal-oxide nanoparticles play a significant role in having larger surface areas and more fractionally surface atoms because of their exceptional physicochemical properties [2–6]. Due to this, they become excellent materials for biomedical applications.

Polymers form an important class of materials and are classified into three types: natural polymer, synthetic polymer, and semi-synthetic polymer. Among these polymers, Chitosan is a natural amino polysaccharide having a unique structure with multidimensional properties. It has a wide range of applications in the biomedical field due to its excellent biocompatibility, biodegradability, low immunogenicity, and good antimicrobial activity [7–11]. The physicochemical properties of chitosan polymer were further improved by fabricating their composites. The polymer composites are hybrid nanomaterials in which Polymers coated nanoparticles and a core-shell structure can be created [12–14]. Therefore, the metal nanoparticles coated with chitosan polymer into a single "hybrid" nanosystem give good physicochemical and biological properties [15–18].

Over the past decades, various metals (e.g., Au, Ce, Cu, Pt, Zn, Ce, Cd, Fe, Al, Ti, Ni, Co, Br, etc.) have been brought to nm size with various dimensions and morphologies using multiple experimental techniques for diverse applications. Among these nanoparticles, silver is one of the most preferred metal choices due to its elegant applications in the biomedical field [19]. Many methods are implemented for the preparation of nanoparticles, including chemical, physical, and biological processes. The physical and chemical methods are not generally environmentally friendly in nature. They have some disadvantages such as toxic chemicals, involvement of expensive instruments (PVD, CVD, Laser, high beam, etc.), which lead to high cost, the requirement of high energy and pressure. Biological mediated nanoparticle synthesis is gaining importance since this process is non-toxic, biocompatible, and eco-friendly [20–22].

The use of plants for nanoparticle biosynthesis is an exciting possibility that needs to be explored more. The foremost benefit of the plant (leaf, root, stem, seed, peel, flower, etc.) extract mediated synthesis of nanoparticles is that they are safe and non-toxic in most cases due to the presence of different medicinally essential phytochemicals that can aid the stabilize and reduction of metal ions to metallic nanoparticles, also readily available and low cost (Scheme 1) [23]. In 2020, Renuka et al., used the fruit extract of the *Phyllanthus emblica* plant to synthesize silver nanoparticles, which exhibited strong antibacterial properties against *Klebsiella pneumoniae* and *Staphylococcus aureus* [24].
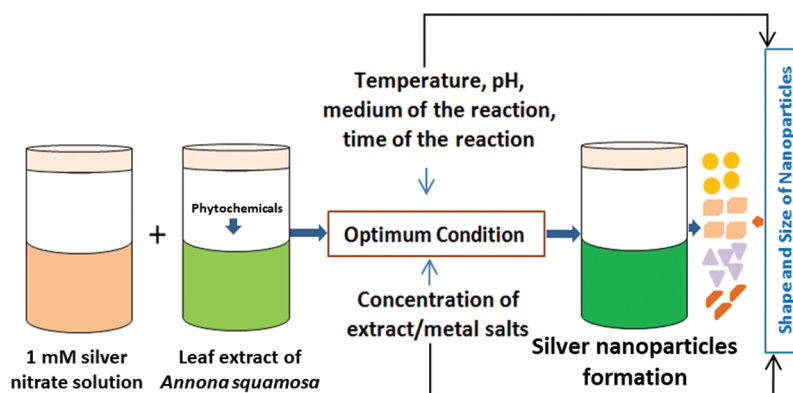
Scheme 1: The diagram shows the formation of biogenic Ag nanoparticles using the custard apple plant leaf extract (*Annona squamosa* L.)

Annona squamosa L. (Family-Annonaceae), also known as "custard apple" is a tropical endemic species. The leaf, root, fruit, peel, stem, and seed extracts of this plant have been utilized in traditional pharmacological applications [25–27]. This study demonstrated the biosynthesis of custard apple's leaf extract mediated silver,

and chitosan-coated silver nanoparticles. Further, we have given in detail the physicochemical properties of these nanoparticles and showed their antibacterial properties against human bacterial pathogens.

2 Materials and Methods

2.1 Materials

The silver nitrate and chitosan were obtained from Himedia, India. The organic solvents were procured from Merck, USA, and all the glasswares were obtained from Borosil, India.

2.2 Preparation of the Plant Extract and Preliminary Phytochemical Screening

The leaves of the custard apple plant were rinsed with distilled water to remove the attached dust particles and dried. The preparation of aqueous leaf extract was initiated by taking 10 g of powdered leaf in 100 mL distilled water and boiling for the next 20 min. The crude solution was left to cool at room temperature. Next, the prepared leaf extract was filtered using Whatman filter paper no. 1, and the collected leaf extract was stored at 4°C for further preliminary phytochemical screening. The presence of saponins, alkaloids, phenols, flavonoids, tannins, and glycosides in the aqueous leaf extract was determined through standard protocols [28,29].

2.3 Fabrication of Ag Nanoparticles and Chi-Ag Nanoparticles

Initially, a 1 mM aqueous solution of silver nitrate was prepared. Then, 20 mL of prepared aqueous leaf extract was added to the reaction mixture. The pH of the resulting solution was calculated to be 7.0. Then, the solution was kept for the subsequent 24 h incubation at room temperature. After incubation, the brown colour of the reaction mixture was obtained, indicating the fabrication of Ag nanoparticles via one-pot green synthesis method. The prepared solution was centrifuged for 20 min at 10,000 rpm and the obtained pellet was dried at 60°C to get the final product. Also, the reduction of Ag ions in aqueous leaf extract solution was monitored through UV/Visible spectroscopy (Data not shown).

Next, 0.2 g chitosan was dissolved in a 10 mL solution of 2% glacial acetic acid. Then, Ag nanoparticles solution was added dropwise until 2 h and left for 24 h in a dark room. The reaction mixture was centrifuged, and the obtained pellet was dried at 40°C for 3 h and finally stored at room temperature.

2.4 Physicochemical Characterizations of Ag Nanoparticles and Chi-Ag Nanoparticles

Different physicochemical techniques characterized both nanoparticles. FTIR spectroscopy was used to investigate the bonding linkage of fabricated nanoparticles with the functional groups of various bioactive compounds present in the custard apple leaf extract through bridging linkage. The vibrational peaks were recorded by Shimadzu FTIR spectrometer in the wavenumber region of 450–4000 cm^{-1}. Next, an X-ray diffractometer (PAN analytical BV) was used to measure the diffraction pattern of both nanoparticles in the diffraction angle range of 10°–80°. The surface morphology and particle size of Ag nanoparticles and Chi-Ag nanoparticles were analyzed by Field-Emission Scanning Electron Microscopy (HITACHI SU6000 FESEM). Furthermore, the chemical composition of the prepared nanomaterial was studied through HITACHI SU6000 SEM equipped with an EDX attachment.

2.5 Antibacterial Activity

The antibacterial properties of Ag nanoparticles and Chi-Ag nanoparticles were examined against human pathogenic bacterial by standard well diffusion technique. The inoculum of tested bacteria was prepared by growing pure isolates in nutrient broth at 37°C for overnight. The agar plates were prepared by the pour plate method using MHA medium. 1 mL of bacterial culture was poured and swabbed into the sterile Petri-dishes and left the bacteria to grow. About 30 μL of prepared 1 mg/mL concentrations of plant extract, $AgNO_3$, Ag nanoparticles, and Chi-Ag nanoparticles was added to each well to find their antibacterial properties. The assay plates were further incubated at 37°C for 24 h, and the observed zone of inhibition was measured.

3 Results and Discussion

3.1 Phytochemicals Screening

The preliminary phytochemical screening of the aqueous leaf extract of the custard apple plant is represented in Table 1. We observed the presence of alkaloids, flavonoids, saponins, carbohydrates, glycosides, phenols, proteins, and tannins in the leaf extract. Such secondary metabolites in the plant are responsible for different pharmacological actions [30–33].

Table 1: Preliminary phytochemical screening of aqueous leaf extract of custard apple plant

S. No.	Phytochemicals test	Aqueous extract of custard apple leaf
1	Alkaloids	Positive
2	Flavonoids	Positive
3	Saponins	Positive
4	Carbohydrates	Positive
5	Glycosides	Positive
6	Phenols	Positive
7	Proteins	Positive
8	Tannins	Positive

3.2 Biogenic Synthesis of Ag Nanoparticles and Chi-Ag Nanoparticles

In this study, we carried out the biogenic synthesis of Ag nanoparticles using custard apple aqueous leaf extract. We added 1 mM $AgNO_3$ in leaf extract solution then the colour of the reaction mixture was changed from yellow to brown which indicated the reduction of Ag^+ ions and formation of Ag nanoparticles. The reduction of Ag^+ ions was analyzed using a UV-visible spectrophotometer. We observed a strong peak at 419 nm (Fig. 1), and this peak provides a convenient surface plasmon resonance signature for the formation of Ag nanoparticles [34,35]. Further, the chitosan was coated on the surface of Ag nanoparticles, forming Chi-Ag nanoparticles.
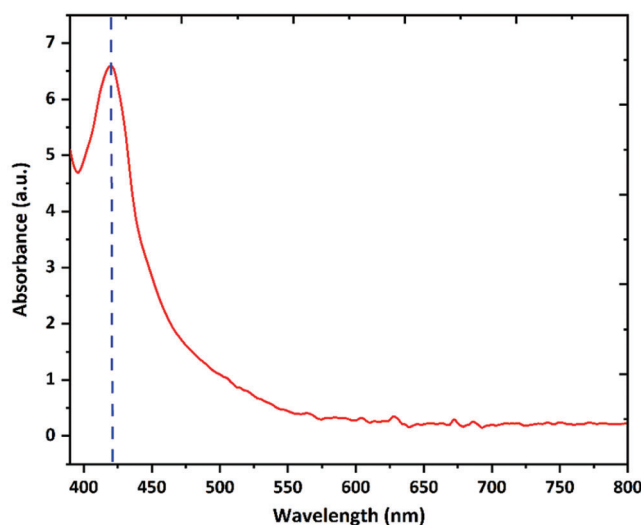

Figure 1: UV-Visible spectrum of biogenic Ag nanoparticles in the wavelength range of 390–800 nm

3.3 FTIR Analysis

Different techniques physicochemically characterized the fabricated Ag nanoparticles and Chi-Ag nanoparticles. FTIR spectrum of aqueous leaf extract of custard apple plant, Ag nanoparticles, and Chi-Ag nanoparticles was displayed in Fig. 2. The plant extract presented several peaks indicating their complex compositions. The bands appearing at 3365.14 cm^{-1}, 1814.09 cm^{-1}, 1634.64 cm^{-1}, 1327.03 cm^{-1}, 1026.13 cm^{-1}, and 678.94 cm^{-1} were assigned to O-H, C=O, C=C, and C-N functional groups of alcohol, phenol, hydroxyl, amine, carboxylic acids, and halo compounds. However, the biogenic Ag nanoparticles showed a shift in the peaks at 3672.47 cm^{-1}, 3340.71 cm^{-1}, 2885.51 cm^{-1}, 1728.22 cm^{-1}, 1519.91 cm^{-1}, 1327.03 cm^{-1}, and 817.82 cm$^{-1,}$ which were assigned to N-H, O-H, C-H, C=C, C=O, and N-O functional groups of alcohol, secondary amine, alkaline, aldehydes, and alkene. In addition, the Chi-Ag nanoparticles displayed the absorbance peaks at 3039.81 cm^{-1}, 2059.98 cm^{-1}, 1743.65 cm^{-1}, 1597.06 cm^{-1}, 1512.19 cm^{-1}, 1334.74 cm^{-1}, 1211.30 cm^{-1}, 887.26 cm^{-1}, and 810.10 cm^{-1} that were given to O-H, N=C=N, C=O, N=H, N-O, C-O, and C-C functional groups of phenol, alcohol, amine, nitro compound, and alkene.
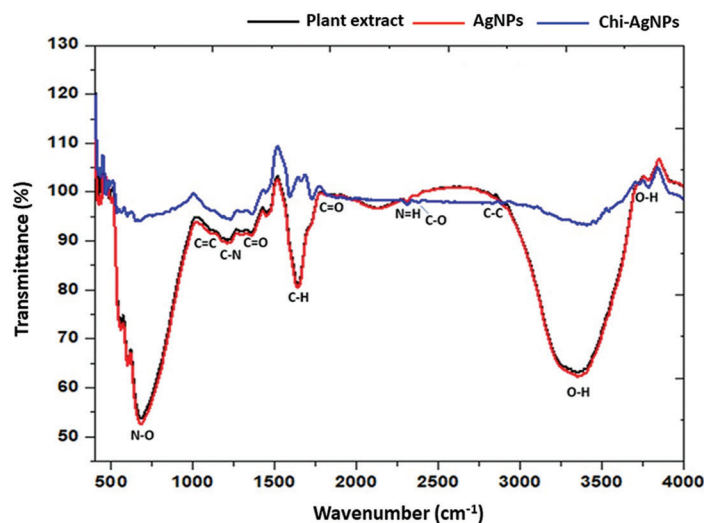

Figure 2: FTIR spectrum of aqueous leaf extract of custard apple plant, Ag nanoparticles, and Chi-Ag nanoparticles in the wavenumber range of 450–4000 cm^{-1}

These results confirmed the presence of phenols, hydroxyl, and carboxylic acid groups derived from the aqueous leaf extract of the custard apple plant. Hence, the FTIR spectrum indicated the presence of different functional groups such as phenols, hydroxyl, and carboxylic acids that are being capped on the surface of the prepared Ag nanoparticles. The bonding vibration of C-N and C-O indicates the presence of amine and carboxylic groups in prepared Chi-Ag nanoparticles, which may be due to chitosan polymer ($C_{56}H_{103}N_9O_{39}$), which clearly indicated the prepared Ag nanoparticles are conjugated with the chitosan. The plant-derived phytocompounds and chitosan lead to the reduction, formation, and surface coating of Ag nanoparticles and Chi-Ag nanoparticles where various functional groups lead to the different applications of prepared bionanomaterials [36–38].

3.4 X-Ray Diffraction Analysis

The XRD patterns of Ag nanoparticles and Chi-Ag nanoparticles were exposed in Fig. 3. We observed the major and minor diffraction peaks conforming to the Miller indices [hkl] of silver and silver oxide (100, 110, 111, 200, 211, 211, 220, 310, and 311). This confirmed the FCC configuration of Ag nanoparticles [39]. The XRD spectrum of both samples showed two different phases of silver and silver oxide. The peaks obtained at 2Θ = 27.81°, 32.20°, 54.85°, 57.49°, and 76.77° that presented to the Miller indices [hkl] of silver oxide (100, 110, 211, 211, and 310) matching with JCPDS file no. 00-076-1393 [40,41] while the peaks at 2Θ = 38.44°, 46.54°, 64.73°, 76.93°, and 77.66° representing the Miller indices [hkl] of silver (111, 200, 220 and 311) matching with JCPDS file no. 04-0783 [42,43]. The prepared samples showed two peaks around 44.51° and 74.76° in the XRD spectrum due to the crystallization phase of phytochemicals [44,45]. Furthermore, the Scherrer equation calculated the average crystallite size of Ag nanoparticles, which was 23 and 74 nm from the breadth of (110 and 111) reflection [46]. In earlier reported studies, the silver oxide nanoparticles are further reduced to silver nanoparticles [47,48].
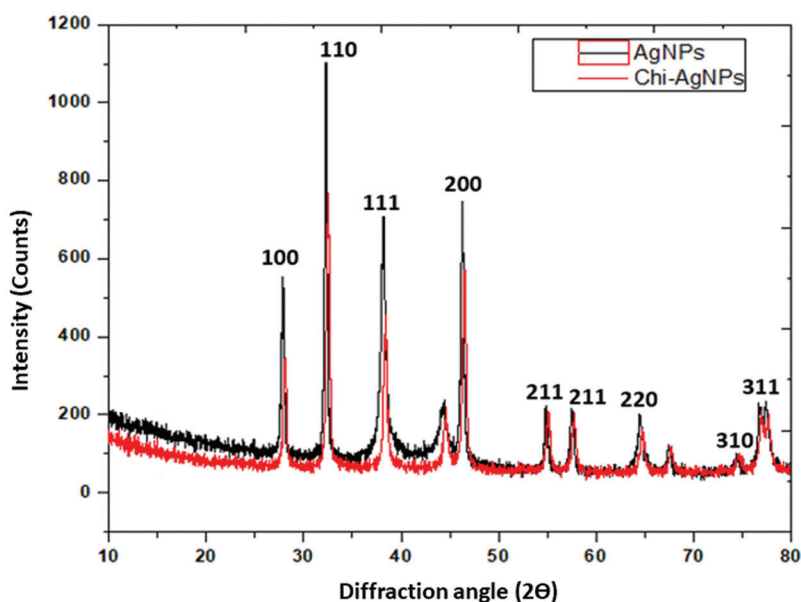

Figure 3: XRD patterns of Ag nanoparticles and Chi-Ag nanoparticles in the diffraction range of 10°–80°

3.5 FESEM-EDS Analysis

FE-SEM determined the surface morphologies of Ag nanoparticles and Chi-Ag nanoparticles. We showed the uniform spherical shape of Ag nanoparticles. Next, the average particle size of Ag nanoparticles was calculated to be 38.99 nm using their particle size distribution curve (data not shown). These nanoparticles were also showing high agglomeration and clustering of Ag nanoparticles that may be attributed to the interaction between phytocompounds present in the custard apple leaf extract and nanoparticles (Fig. 4). However, the Chi-Ag nanoparticles presented their uniform cubical shape with an average particle size of 241.82 nm and the obtained results indicated that the surface of the nanoparticles was successfully capped by the polymeric structure of chitosan (Fig. 4). The rationale behind the high agglomeration of Ag nanoparticles can be due to different types of capping agents present in the aqueous leaf extract of the custard apple plant.
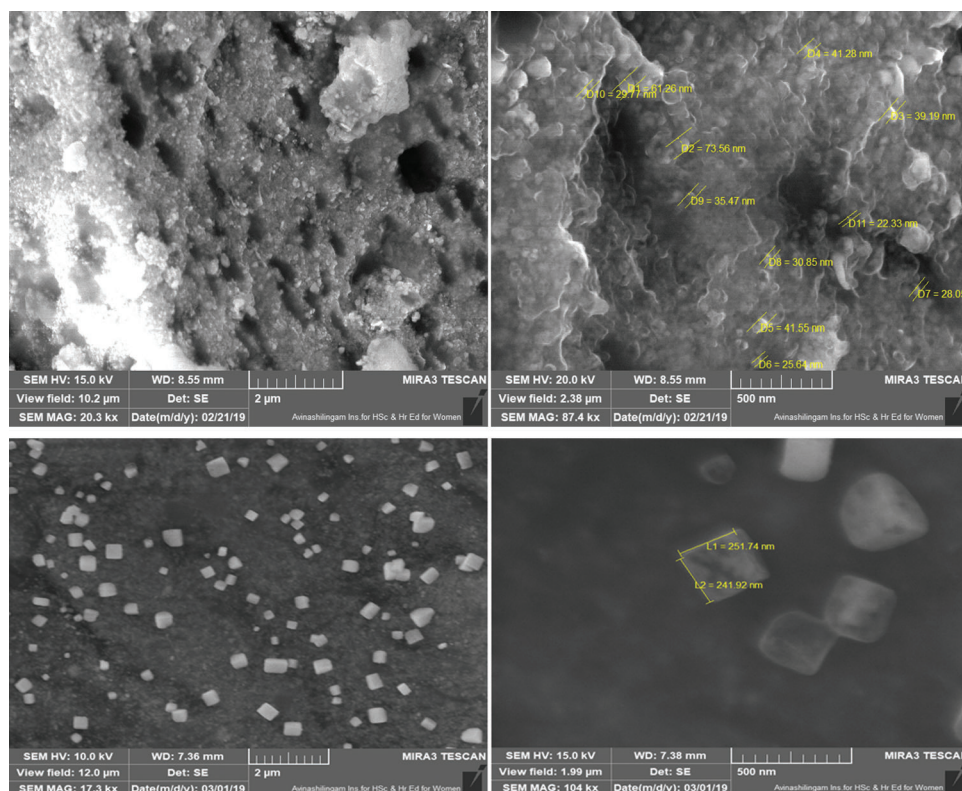
Figure 4: FESEM images of Ag nanoparticles (Upper panel) and Chi-Ag nanoparticles (Lower panel)

In our study, the elemental analysis of prepared Ag nanoparticles and Chi-Ag nanoparticles was performed using their corresponding EDS spectrum. We showed the determined peaks around 3.29 keV conforming to the binding energies of Ag ions (Fig. 5). Furthermore, the Chi-Ag nanoparticles showed an absorbance peak of nitrogen (N) in its EDS spectrum; it may have come from the amine (-NH$_2$) group of chitosan (C$_{56}$H$_{103}$N$_9$O$_{39}$), which indicates the excitation of X-ray from polymer. Further, other peaks in the EDS spectrum suggest mixed precipitates present in the prepared plant extract [49].

3.6 Antibacterial Activity

The antibacterial properties of both Ag nanoparticles and Chi-Ag nanoparticles were investigated against different human bacterial pathogens. We observed that both nanoparticles presented significant antibacterial activity against chosen human bacterial pathogens (Fig. 6).

In this test, Chi-Ag nanoparticles showed the maximum zone of inhibition (ZoI) against *E. coli* (21 mm) followed by *Klebsiella pneumoniae* (18 mm), *Staphylococcus aureus* (17 mm), and *Pseudomonas aeruginosa* (16 mm). On the other hand, biogenic synthesis Ag nanoparticles showed significant antibacterial activity against *E. coli* (17 mm) and *Klebsiella pneumoniae* (13 mm). These Ag nanoparticles also exhibited moderate antibacterial action against *Staphylococcus aureus* (9 mm) and *Pseudomonas aeruginosa* (8 mm). However, the aqueous leaf extract of custard apple and the positive control solution 1 mM AgNO$_3$ displayed the minimum antibacterial activity around 0.8 to 1 mm against all chosen bacterial pathogens. Thereby, the prepared hybrid Chi-Ag nanoparticles showed higher antibacterial properties against all tested bacterial pathogens compared to crude leaf extract and Ag nanoparticles. In a study, Klueh et al., reported that the positively charged Ag ions play a vital role in the antibacterial properties of Ag nanoparticles. In addition, the electrostatic attraction between the positively

charged nanoparticles and negatively charged bacterial cells also leads to the antibacterial activity of nanoparticles [50,51]. In some studies, it was reported that the antibacterial property of nanoparticles mostly depends upon the shape, size, ionic strength of the medium, and type of capping agent present on the surface of nanoparticles. Nevertheless, the explicit antibacterial mechanisms of the nanoparticles have not been completely explained. Further, based on the other reports, it was suggested that the antibacterial and anticancer effects of the nanomaterials and nanocomposites can involve many possible mechanisms (Fig. 7), for example—cell-surface membrane interaction, formation of free radicals inside the bacterial or cancer cell, inhibition of enzyme activities, inhibition of cell wall synthesis, ribosome disassembly followed by the inactivation of protein synthesis and structure modification of essential proteins, interface with cell signaling and incorporation into the DNA bases to DNA damage [52–56]. Based on this literature, our present study clearly indicates that the Chi-Ag nanoparticles showed the potential antibacterial property, and it will be useful for the treatment and management of various microbial infections.
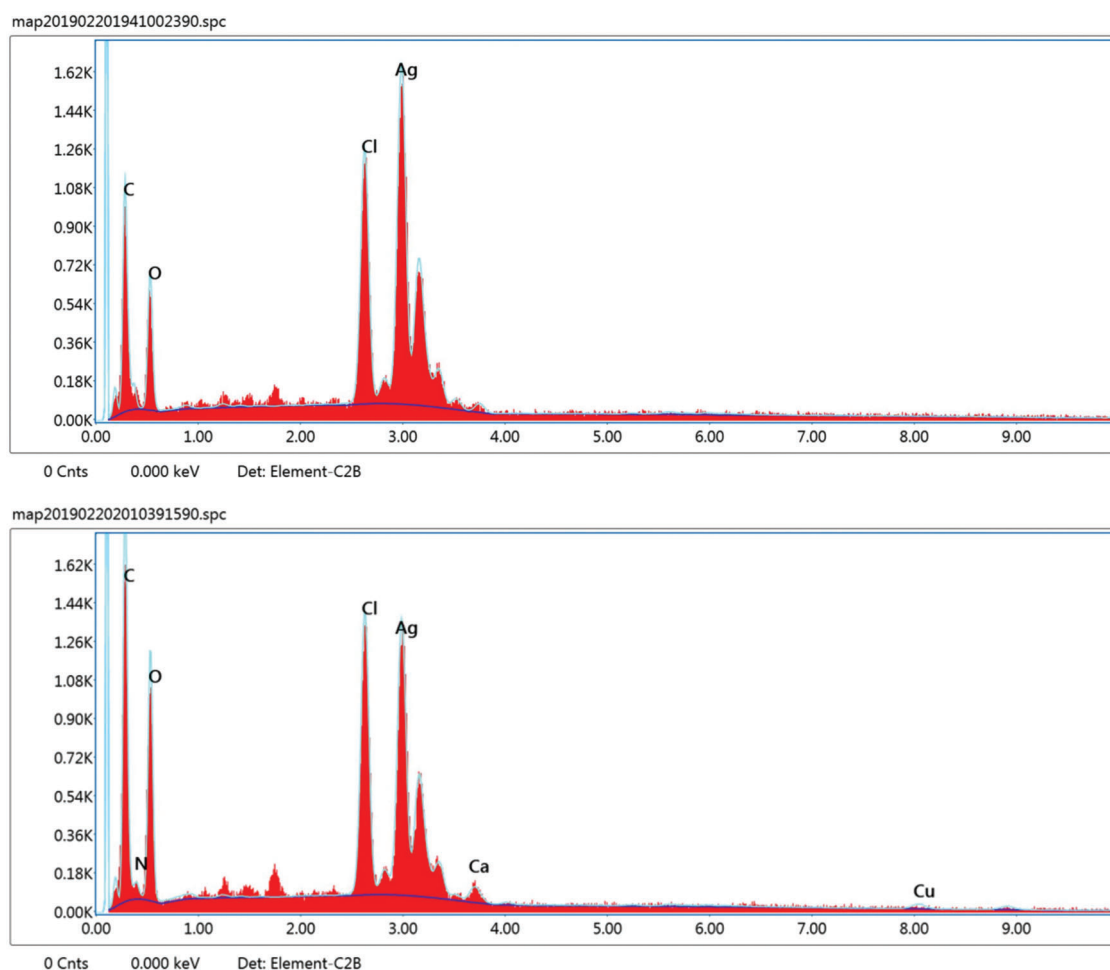

Figure 5: EDS spectrum of Ag nanoparticles (Upper panel) and Chi-Ag nanoparticles (Lower panel)
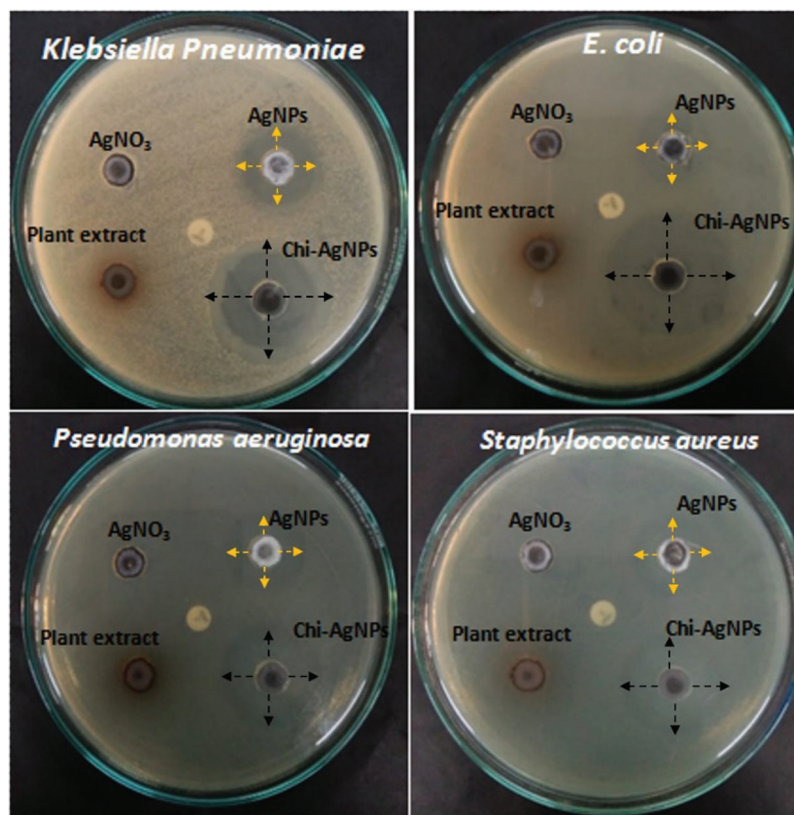
Figure 6: Antibacterial activity of custard apple plant's leaf extract, Ag nanoparticles, and Chi-Ag nanoparticles against different bacterial pathogens
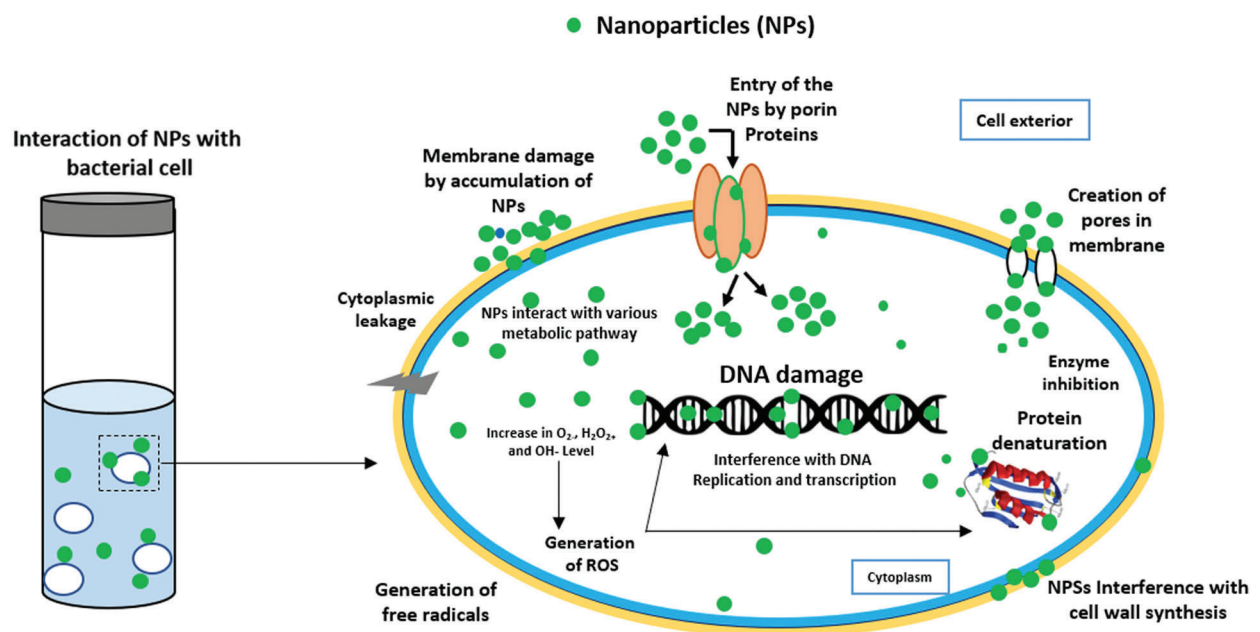

Figure 7: Possible mechanisms of the antibacterial activity of nanoparticles [56]

4 Conclusion

In this present study, we used the aqueous leaf extract of the custard apple plant as a reducing agent to prepare Ag nanoparticles. In preliminary phytochemical screening, the crude aqueous leaf extract showed the occurrence of flavonoids, alkaloids, saponins, carbohydrates, glycosides, phenols, proteins, and tannins that could reduce Ag^+. In the UV/Visible spectrum, an absorbance peak was observed at 419 nm, indicating the formation of Ag nanoparticles. In general, chitosan and its derivatives have been widely used in different biomedical applications. We further prepared Chi-Ag nanoparticles to enhance the biomedical properties of Ag nanoparticles. The crystal size, surface morphology, elemental composition, and functional molecular analysis of prepared Ag nanoparticles and Chi-Ag nanoparticles were investigated with XRD, FESEM, EDS, and FTIR spectroscopy. XRD spectrum indicated the presence of organic constituents and Ag nanoparticles, which was again confirmed by FTIR analysis. The calculated crystallite size of Ag nanoparticles and Chi-Ag nanoparticles was calculated to be 23 and 74 nm, respectively. FESEM analysis showed the spherical shape of Ag nanoparticles and the cubical shape of Chi-Ag nanoparticles. Furthermore, the Chi-Ag nanoparticles showed strong antibacterial activity against tested bacterial pathogens like *S. aureus, E. coli, K. pneumoniae*, and *P. aeruginosa*. Next, the Ag nanoparticles showed a moderate antibacterial effect followed by custard apple leaf extract. Hence, the present study concludes that the biopolymer-coated metal nanoparticles can open new avenues in various biomedical applications.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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