CHITOSAN-CAPPED SILVER NANOPARTICLES AGAINST OPPORTUNISTIC AND HUMAN PATHOGENS: A PROMISING WEAPON IN THE FIGHT AGAINST INFECTIONS

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Abstract

This pioneering study synthesises silver nanoparticles (AgNPs) using Carica papaya fruit extract and chitosan capping, yielding homogeneous, spherical nanoparticles prepared at an average size of 20 nm and characterised by SEM-EDAX and TEM. Antimicrobial activity studies showed that the opportunistic and human pathogenic ones, S. aureus*, S. typhimurium and E. coli, were susceptible to the chitosan-capped AgNPs. This study signifies the novel synthesis approach, commensurate particle size, universal antimicrobial efficacy, and compilation of a comparative antibiotic resistance index. Chitosan-capped AgNPs acted effectively towards multidrug-resistant bacterial strains, which gives the opportunity to consider this material as a potential weapon in the fight against microbial infections, being eco-friendly.*

Keywords: *silver nanoparticles, chitosan-capping, papaya fruit, antibacterial potential, eco-friendly*

Received: 28.10.2024
Accepted: 07.07.2025

1. Introduction

A clear demarcation exists between the opportunistic pathogens and the human pathogens that target humans in various body regions, so it is essential for one to be very careful when contracting these pathogens. As compared to the proto-pathogens, the opportunistic pathogens are uncommon to be pathogenic to healthy people, but they are very dangerous for the immunocompromised individuals, particularly due to their multiple antibiotic resistances and simplified modes of transmission in the healthcare centres. On the other hand, human pathogens are directly oriented towards human beings. These pathogens release toxin, which destroys human tissue components and disrupts normal cellular processes, or tissue penetration to reproduce, inflame or hurt tissues, cells and organs in the human body. The two mentioned types of pathogens require one to exercise a lot of caution so that they do not spread and can be easily treated [1].

Nanoparticles (NPs) have been of great significance in changing several fields of medicine, drug analysis, engineering, and tribology, considering their size, surface area, adsorption capacity, and enhanced bioavailability [2, 3]. Silver nanoparticles (AgNPs) have been widely studied for their properties, which include being an antimicrobial, antifungal and antiviral compound, making them effective in combating drug-resistant microbes, fungi, and viruses. Also, AgNPs are endowed with antioxidant features, therefore improving their application possibilities. The most common method for synthesising AgNPs is by chemical reduction, which utilises organic and inorganic reducing agents [4]. However, various methods of synthesis have drawbacks, such as the use of dangerous reagents, such as borohydride, 2-mercaptoethanol, citrate, and thioglycerol, which decreases their biomedical and pharmaceutical uses [5].To counter this challenge, green NP synthesis technology is developed using plant extracts, microorganisms (such as bacteria, fungi and yeast), and biowaste [6]. This method is less environmentally toxic than conventional methods, thus being eco-friendly [7, 8].

Silver nanoparticles, AgNPs, have recently gained a reputation as effective antimicrobial agents that can contribute to fighting antibiotic resistance. However, their aggregation in solution confines them to their practical uses. Chitosan, an abundant biopolymer present in nature, can also be used in a stabilising mode for disaggregation and to improve the activities of AgNP [9–11]. Chitosan is a biodegradable polysaccharide and is therefore suggested as a safe capping agent in the synthesis of AgNP. In addition, using chitosan in AgNPs production has proved to be more efficient than other polymers because of its high antimicrobial activity, low cytotoxicity, stability and biocompatibility [12, 13].

This paper aims to investigate a cost-effective, eco-friendly synthesis of chitosan-capped AgNPs utilising aqueous extract of *Carica papaya* fruit. Characterisation of the synthesised nanoparticles and chitosan-capped AgNPs was conducted using different techniques, e.g., SEM-EDAX (Scanning Electron Microscopy- Energy Dispersive X-ray Analysis), TEM (Transmission Electron Microscopy). The antimicrobial activity of chitosan-capped AgNPs was tested against selected opportunistic and human pathogenic organisms: *Pseudomonas* sp., *Salmonella* sp., *Shigella* sp., *Staphylococcus* sp., *Klebsiella* sp., *Proteus* sp., *Escherichia coli* sp. and *Bacillus* sp. with the cross-agar diffusion technique. This work aims to create a novel antimicrobial material with long-lasting performance and possible uses in healthcare sectors.

2. Materials and Methods

2.1. Materials

Materials used included chitosan (moisture 5.2%, viscosity 212 cps, purchased from ISF Chitin and Marine Products LLP, Ernakulam, Kerala), silver nitrate, sodium borohydride, sodium hydroxide, and glacial acetic acid of AR (Analytical Reagent) grades procured from Merck. Fresh fruits of *Carica papaya* were collected from Kalady, Ernakulam, Kerala. The cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 14153), *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022), *Bacillus subtilis* (ATCC 6051a) and *Escherichia coli* (ATCC 25922) (American Type Culture Collection) were obtained from the Centre for Water Resources Development and Management (CWRDM), Kozhikode, Kerala.

2.2. Synthesis of AgNPs from Fruit Extract of *C. papaya*

Fresh fruits of *C. papaya* were collected and cleaned using distilled water. A portion of fruits without skin and seeds was taken, dried in the shade, and then finely chopped into small pieces. After drying, the samples were blended and ground into a fine powder. The powder (25 g) was weighed in a beaker and boiled with 100 ml of water for about 30 minutes at 60°C; then it was filtered to obtain the fruit extract. 45 ml of 0.012 M $AgNO_3$ solution was added to 5 ml of this extract, mixed and kept in a dark place for 24 hours. Colour change was noted. The formation of silver nanoparticles from the fruit extract of *C. papaya* was confirmed by TEM analysis.

2.3. Synthesis of Chitosan-Capped AgNPs From Fruit Extract of *C. papaya*

C. papaya fruit extract (5 ml) and 3 ml 1% chitosan solution were mixed for 30 minutes. Then 1 ml of 1 mM $AgNO_3$ solution was added. The mixture was stirred at 70°C and then incubated for 2 hours. Colour changes were noted. Then, the solution containing *C. papaya* fruit extract, chitosan and $AgNO_3$ was dripped into 20 wt.% NaOH solution to obtain silver nanoparticle-chitosan spheres [14]. Here, silver nanoparticles are produced from $AgNO_3$ solution, where *C. papaya* fruit extract acts as the reducing agent, while chitosan acts as a stabilising agent. Chitosan spheres without AgNPs were also synthesised by treating 1% chitosan solution (in acetic acid) with NaOH solution.

2.4. Characterisation of AgNPs and Chitosan-Capped AgNP spheres

2.4.1. Transmission Electron Microscopy of AgNPs

To determine the size of the synthesised AgNPs, TEM analysis using a JEOL JEM 2100 Transmission Electron Microscope was performed. A drop of the dispersion of the sample in deionised water was placed on a staining mat. A copper grid with carbon coating was inserted into the drop. After 10 minutes, the grid was removed, dried and screened using the instrument. Magnification was obtained from the ratio of the distance between the objective lens and the specimen and the distance between the objective lens and its image plane.

2.4.2. Scanning Electron Microscopy-Energy Dispersive X-ray Analysis of Chitosan-Capped AgNPs Spheres

To obtain the morphology and size of chitosan-capped AgNPs spheres, scanning electron microscopy-energy dispersive X-ray analysis (SEM EDAX) using JEOL 6390 LA (with 7000× magnification and energy range 0.5 - 30 kV) was done.

2.5. Antibacterial Potential of Chitosan-Capped AgNPs

2.5.1. Antibacterial Properties of Common Antibiotics

The bacterial cultures were maintained in the laboratory as pure cultures. Further, 10 µl of each culture was inoculated into sterilised nutrient broth and kept for overnight incubation. The susceptibility of the antibiotics towards the cultured bacteria was analysed according to the Clinical and Laboratory Standards Institute (CLSI, 2010) guidelines. The inocula were prepared by adjusting the turbidity to 0.5 McFarland's standard and spreading them on MHA plates. Antibiotic discs containing ciprofloxacin (30 µg) (CIP 30), colistin (10 µg) (CL10), erythromycin (10 µg) (E10), gentamicin (10 µg) (GEN10), amoxycillin (10 µg) (AMX10) and linezolid (30 µg) (LZ30) (Himedia Laboratories, Mumbai, India) were used according to the manufacturer's instructions. The plates with the antibiotic disc were incubated at 37°C for 24 hours. The zones of incubation were measured.

2.5.2. Antibacterial Properties of Chitosan-Capped AgNPs

The susceptibility of synthesised chitosan-capped silver nanoparticles was tested using the agar diffusion method [15] against the panel of bacterial strains given in section 2.5.1. A standing stock of the chitosan-capped AgNPs was made from the stock solution in sterile distilled water at a 1000 ppm concentration to get the working solutions of 500 ppm. Sterile nutrient agar (NA) plates were then streaked with each bacterial strain using a sterile cotton swab [16]. The stock chitosan-capped AgNP solution (50 µL) was pipetted onto NA plates and allowed to diffuse into the agar at room temperature for 30 minutes. The plates were then incubated in a CO_2 incubator at 37°C for 18 - 24 hours. Upon incubation, the diameter of zones formed around each concentration of chitosan-capped AgNPs and the control was measured [17, 18]. Collected data were used to determine the chitosan-capped AgNPs' ability to eradicate bacterial strains [19]. All procedures were done under sterile conditions, and all safety measures were strictly followed.

3. Results and Discussion

3.1. Green Synthesis of AgNPs from Fruit Extracts of *C. papaya*

Silver nitrate solution, when reacted with the fruit extract of *C. papaya*, produced silver nanoparticles. The change in colour during the reaction indicated the formation of AgNPs (Figure 1).

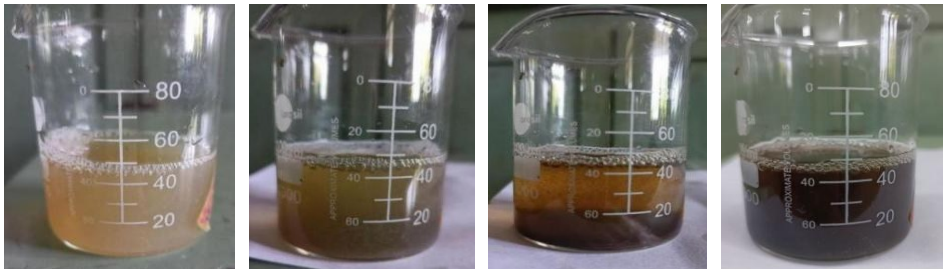

Figure 1. Colour changes during the AgNPs formation.

The *C. papaya* fruit extract acted as a reducing agent. The flavonone and terpenoid constituents stabilise the formation of AgNPs, and the reduction of silver ions occurs due to the action of simple sugars, polyols and other constituents. The plant extract also contains enzymes, polysaccharides, tannin, and phenol, which also favour the AgNP

formation. The bioactive compounds present in the *C. papaya* fruit extract contain hydroxyl and carbonyl groups, which donate electrons to silver ions, reducing them to metallic silver. This reduction is often indicated by a colour change in the solution, confirming the formation of AgNPs. Also, adsorption of these bioactive compounds onto the surface of the newly formed nanoparticles can act as capping agents and prevent aggregation, which leads to improved stability [20].

3.2. Green Synthesis of Chitosan-Capped AgNPs from Fruit Extracts of *C. papaya*

Fruit extract *of C. papaya* was then utilised to synthesise chitosan-capped silver nanoparticles. The extract was treated with an acidic solution of chitosan, followed by the addition of silver nitrate solution. The chitosan-capped silver nanoparticles formed were visually characterised by the colour change from light yellow to dark brown, an effect of the surface plasmon resonance at a wavelength of 430 nm. AgNPs formed by applying the green synthesis method were stabilised by chitosan. In this reaction, the Ag^+ ions react with both *C. papaya* extract components (see section 3.1) and chitosan amino groups fixed on polymer chains, as amine groups have strong complex formation potential with the metal ions. During the reduction of Ag^+ to Ag^0, glycosidic bonds present in chitosan can participate in the electron transfer process, facilitating the formation of AgNPs. Chitosan, a polysaccharide composed of glucosamine and *N*-acetylglucosamine units, contains glycosidic linkages. As these linkages are susceptible to oxidation under certain conditions, they can participate in Ag^+ reduction, leading to the formation of ketones or esters in the glycosidic ring [21]. Thus, chitosan can play a significant capping agent role and a minor role as a weak reducing agent. Such prepared chitosan-capped nanoparticles were used in the formation of chitosan-capped AgNP spheres, as given in section 2.3.

3.3. Formation of Chitosan-Capped AgNP Spheres

Previous studies have reported and demonstrated that chitosan-capped silver nanoparticles show a low cytotoxicity compared to silver nanoparticles alone [21].

Both neat chitosan and chitosan-capped AgNP composite spheres formed are indicated in Figures 2 and 3.

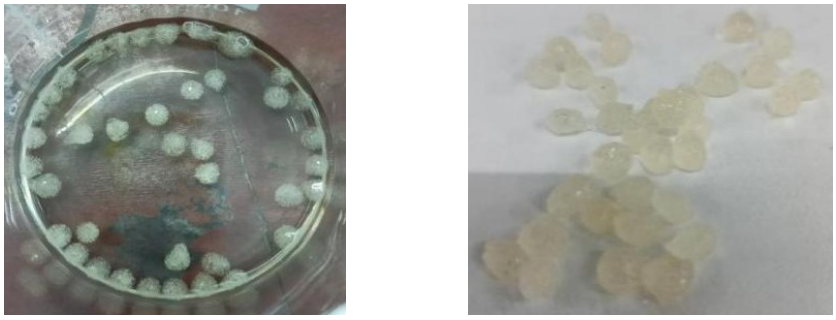

Figure 2. Chitosan beads in NaOH solution and neat chitosan beads.

The chitosan spheres without entrapped silver nanoparticles have a white colour. To obtain chitosan-capped AgNP spheres, a solution containing chitosan and AgNPs was dripped into 20% NaOH. The obtained chitosan-capped AgNP composite spheres were almost uniform in size, having a diameter of about 5 mm and being soft. Silver nanoparticles were produced using $AgNO_3$ solution, while NaOH acts as the precipitating agent and chitosan as the AgNPs stabilising agent.

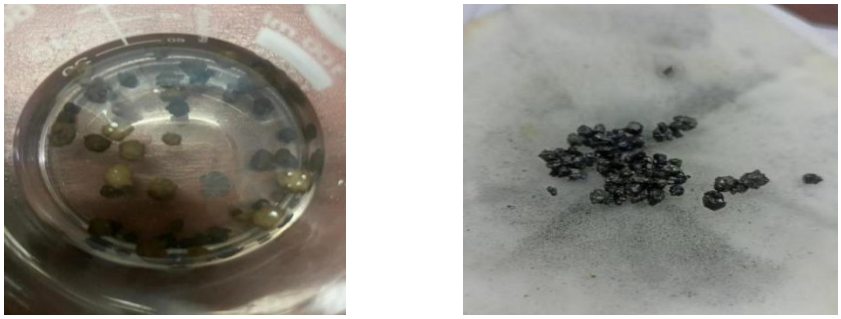

Figure 3. Chitosan-capped AgNP spheres in NaOH solution (left) and removed from the solution (right).

3.4. Characterisation of AgNPs and Chitosan-Capped AgNP Spheres

3.4.1. TEM Analysis of AgNPs

TEM analysis is a primary tool to determine the physical characteristics of AgNPs. The TEM image (Figure 4) showed that silver nanoparticles are predominantly spherical in shape, and many particles fell within the range of ~ 20nm.

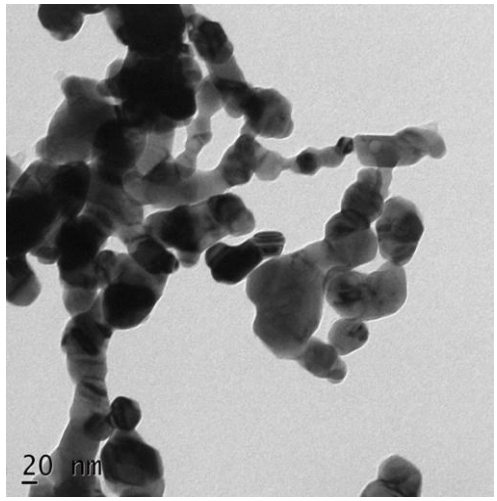

Figure 4. TEM image of AgNPs.

3.4.2. SEM EDAX of Chitosan-Capped AgNP Spheres

SEM-EDAX analysis was performed to understand the morphology and chemical composition of the developed chitosan-capped AgNP spheres. The SEM-EDAX results are presented in Figure 5 and in Table 1. The EDAX spectrum of chitosan-capped AgNP spheres (Figure 5) confirmed the presence of silver in chitosan-capped AgNP spheres. It was found that these Ag content reaches were 2.26 wt.%. The presence of other elements was also noted, most of them resulting from chitosan's chemical structure, i.e., carbon, oxygen, and nitrogen. The presence of Na resulting from the NaOH solution applied as a precipitating agent was also confirmed.

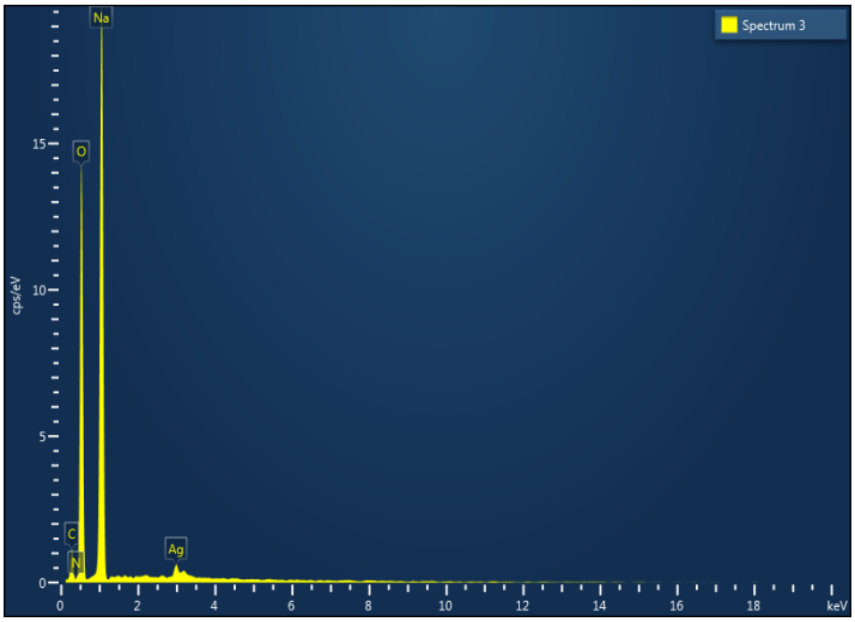

Figure 5. EDAX spectrum of chitosan-capped AgNP spheres.

Table 1. Elemental composition of chitosan-capped AgNP spheres.

Element	Line Type	Element content	
		[wt.%]	[atomic %]
C	K series	9.06	13.28
N	K series	6.21	7.81
O	K series	46.01	50.62
Na	K series	36.46	27.92
Ag	L series	2.26	0.37
Total		100.00	100.00

3.5. Antibacterial Potential of Chitosan-Capped AgNP spheres

3.5.1. Antibacterial Activity of Selected Antibiotics

To compare and understand the antibiotic susceptibility of the selected bacterial strains, the Kirby-Bauer disc diffusion test on MHA agar was conducted, and the results are depicted in Table 2. All bacterial isolates studied were sensitive to colistin (CL10), as seen by small incubation zones, which, for each species, were above the breakpoints provided by The Clinical & Laboratory Standards Institute. It is a last-line antibiotic employed to manage infections due to MDR/OxyHSP (Multi Drug Resistant) organisms [22]. This observation indicates that the spread of multidrug resistance in the bacterial population under study is not very high. This observation is particularly so as the threat of multidrug-resistant bacteria has become increasingly apparent on the international scene [23]. Large amounts of antibiotics can be used as a driving force in developing selectors for resistance mechanisms and achieve even worse results in the treatment of microbial infections [24].

Table 2. Antibiotic susceptibility of the bacterial isolates expressed by the incubation zones.

Organism	Incubation zones [mm]					
	AMX10	CIP30	E10	GEN10	LZ30	CL10
***Pseudomonas* sp.**	45	35	34	30	30	12
***Salmonella* sp.**	45	36	40	35	31	14
***Shigella* sp.**	50	37	40	33	33	12
***Staphylococcus* sp.**	46	33	34	32	35	12
***Klebsiella* sp.**	46	32	40	30	30	12
***Proteus* sp.**	40	33	40	33	34	12
***E.coli* sp.**	36	32	34	30	36	15
***Bacillus* sp.**	46	39	38	30	36	15

3.5.2. Antibacterial Activity of Chitosan-Capped AgNPs

By applying the agar diffusion assay, it was possible to find that chitosan-capped AgNPs have a moderate ability to inhibit the growth of the selected bacterial strains, which comprise both opportunistic and human pathogens. The experimental results are depicted in Figures 6 and 7.

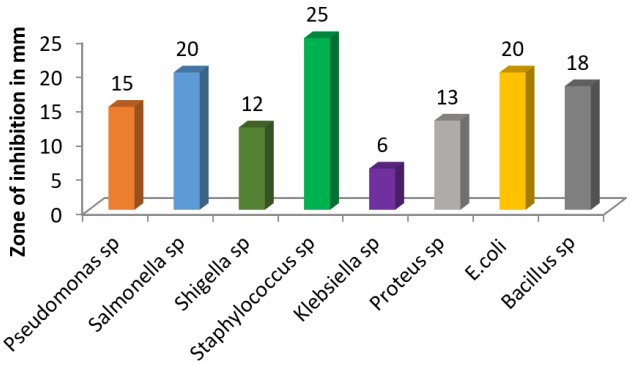

Figure 6. Zone of inhibition for different bacterial strains using chitosan-capped AgNPs.

According to these data, *Staphylococcus aureus*, a typical human pathogen, was the most sensitive to the chitosan-capped AgNPs, having the largest zone of lysis (25 mm) (Figure 7H). Similar susceptibility was exhibited by *Salmonella typhimurium* (Figure 7F), a human pathogen that causes food poisoning, and *Escherichia coli* (Figure 7B), an opportunistic pathogen, with zones of lysis of 20 mm each. *Bacillus cereus*, another opportunistic pathogen, had a moderate susceptibility of 18 mm (Figure 7A), along with *Pseudomonas aeruginosa* (Figure 7E), an opportunistic pathogen that causes severe infections, and *Proteus mirabilis* (Figure 7C) associated with urinary infections (15 mm and 13 mm, respectively).

The zones of inhibition produced by the other tested organisms are as follows: *Shigella flexneri*, a human pathogen that causes dysentery, exhibited a susceptibility zone of 12 mm (Figure 7G); and *Klebsiella pneumoniae*, an opportunistic pathogen responsible for

pneumonia, also showed a zone of 12 mm (Figure 7D). From the above results, it can be proposed that chitosan-capped AgNPs possess a broad spectrum of antibacterial activity against opportunistic and human pathogenic bacteria. The observed variation in susceptibility of bacteria to chitosan-capped AgNPs might be due to differences in cell wall thickness and membrane fluidity [25].

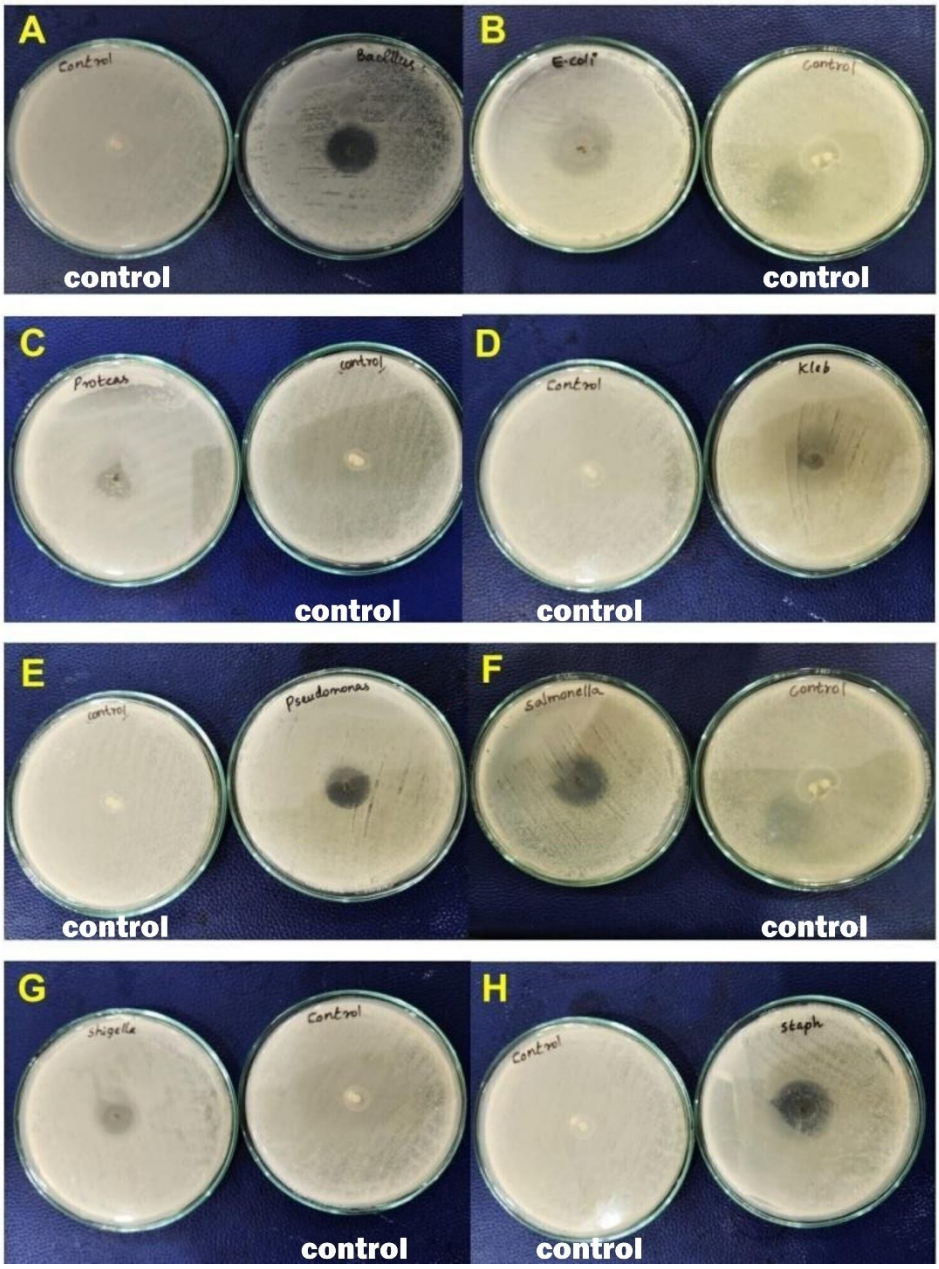

Figure 7. Lysis zone of (A) *Bacillus* sp., (B) *E. coli* sp., (C) *Proteus* sp., (D) *Klebsiella* sp., (E) *Pseudomonas* sp., (F) *Salmonella* sp., (G) *Shigella* sp., and (H) *Staphylococcus* sp. using chitosan-capped AgNPs.

It is argued that the antimicrobial activity of AgNPs is rather synergistic and depends on a variety of factors. One of the main ways is the process of bacterial cell membrane damage. The nanoparticles AgNPs penetrate the area of bacterial membranes and affect their stability, causing leakage and release of vital components [26]. This is even more so the case with Gram-negative bacteria, mainly as a result of the relative thinness of the layer of peptidoglycan compared with Gram-positive bacteria [27]. Moreover, silver ions are capable of chelating with sulphur-containing groups of proteins and enzymes and derailing their activities and hence crucial metabolism of the cell, leading to cell death [28].

The other active process is ROS (Reactive Oxygen Species) production. It involves the production of reactive oxygen species by AgNPs. ROS cause oxidative stress and DNA and protein damage, leading to cell death [29]. Moreover, the antimicrobial activity of AgNPs can be further influenced by surface modifications, such as chitosan capping, which may enhance their stability, biocompatibility, and interaction with bacterial cells. Chitosan capping could potentially modulate the release of silver ions, affect the nanoparticles' affinity for bacterial membranes, or alter the production of reactive oxygen species, thereby impacting the overall efficacy of AgNPs as antimicrobial agents.

It was also found that chitosan coating can improve the antimicrobial activity of the AgNPs through preventing nanoparticle aggregation [30]. Chitosan, a natural polysaccharide, enhances the AgNPs' biocompatibility and eliminates the AgNPs' toxicity effects on the human cells [31]. From the potential therapeutic point of view, this improved biocompatibility is very important. Moreover, the bacterial cell walls can also be penetrated by chitosan, enhancing the interaction of AgNPs with intracellular targets [32]. The antimicrobial activity of chitosan-stabilised AgNPs observed in this study needs further research to conclusively compare their efficacy with AgNPs without chitosan stabilisation [33].

Additional elaborate investigations should be done to research carefully the specifics of the interaction of chitosan-capped AgNPs with opportunistic and human pathogenic bacteria species and their suppression mechanisms [34]. It is essential to identify the minimum inhibitory concentration (MIC) of the AgNPs for each bacterial species to properly evaluate them for their therapeutic potential and further use [35].

4. Conclusions

In conclusion, this study successfully synthesised chitosan-capped silver nanoparticles (AgNPs) using a green synthesis method, employing *C. papaya* fruit extract. The characterisation results confirmed the formation of homogeneous, spherical AgNPs with an average size of 20 nm. The synthesised chitosan-capped AgNPs exhibited significant broad-spectrum antimicrobial activity against various opportunistic and human pathogens, including multidrug-resistant bacteria. These findings highlight the potential of green synthesis methods in the production of effective antimicrobial nanoparticles.

Simultaneously, the study did not directly compare the stability, biocompatibility, or antimicrobial activity of AgNPs with and without chitosan as a capping agent. Further research is needed to evaluate these aspects. This study contributes to the development of sustainable and efficient methods for producing antimicrobial nanoparticles, offering a promising alternative to traditional antibiotics.

5. References

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