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Original Article

Preparation and Characterization of Silver Nanoparticle Cross-linked Polymeric Cages by Freshwater Cyanobacteria and their Bactericidal Evaluation against Multi-Drug Resistant Bacteria

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ABSTRACT

Introduction: Over the past 60 years, numerous modern types of antibiotics have been developed. Antibiotic overuse has been largely responsible for spreading resistant strains that endangering the public's health at an alarming rate. It prompted the pacing advert of new resistance mechanisms and a lessening in the productivity of treating common infectious. **Objectives:** The present study emphasizes using cyanobacteria silver nanoparticle conjugates (Ag-NPCs) as a drug delivery system to predominantly control Multi-Drug Resistant bacteria. **Materials and Methods:** The Ag-NPCs were prepared using a sodium alginate calcium chloride cross-linking process. The Ag-NPCs properties have been characterized by UV-vis-Spectroscopy, FTIR (Fourier Transform Infrared Spectroscopy), Transmission Electron Microscopy (TEM), EDAX (Energy Dispersive Analysis X-Ray), and Scanning Electron Microscopic analysis (SEM) analysis. **Results:** The SEM and TEM analysis showed that Ag-NPCs have been bio-reduced to the size of 20-22nm that are trapped on the surface. FTIR analysis and EDAX analysis are used to confirm the bio-reduction of Ag-NPCs. The antibacterial activity of Ag-NPCs showed prominent results against four MDR bacteria by exhibiting a zone of inhibition with a range of 2-10 mm.

Keywords: Cyanobacteria, Multi-Drug Resistant bacteria, Cyanobacteria Silver nanoparticle conjugates, Drug delivery system, Antibacterial activity.

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INTRODUCTION

At an Estimated scale, about 100,000 tons of antibiotics are being manufactured worldwide annually; this is leading to a profound impact on the bacteria procuring resistance towards many antibiotics and chemotherapeutic agents, the phenomenon of multidrug resistance.[1] The frightening situation of pathogen-resistant microbiological infections has emerged as the most pressing issue confronting physicians today, as well as a serious global public health concern. Drug-resistant bacteria could emerge as a result of several circumstances, including the broad and inappropriate use of antibiotics, as well as their widespread usage as growth promoters in animal feeds.[2] Worldwide, the concern has risen towards the threat of antibiotic resistance, which has promoted the development of new approaches to improve effective therapies for serious infections.[3] Nanobiotechnology, a developing field of nanoscience, uses nano-based systems for different biomedical applications. Based on their size and shape, nanoscale particles and molecules show a viable biological impact on the treatment of disease that contrasts with customary small-molecule drugs.[4]

On the other hand, since classical times the major disinfectant in use is silver. It has a variety of properties, including exceptional antibacterial activity, poor solubility, and high stability, making it difficult for bacteria to acquire resistance and being harmless to human cells.[5-7] Among the most extensively used nanoparticles in science are silver nanoparticles with several characteristics exclusively one of the high characteristics is an antibacterial property.[8] The antimicrobial property of nanoparticles is intensified when combined with silver nanoparticles collected in an aggregate of atoms in the range of 1-100nm that show unique physicochemical along with optoelectronic properties. There are a variety of physical and chemical approaches for producing nanoparticles of various sizes, shapes, and compositions, but the biological method is believed to be more environmentally friendly and safer. Green nanoparticle synthesis is preferable to chemical synthesis because the biomolecules used in green nanoparticle synthesis are less toxic and act as fictionalizing ligands. Among

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all biological systems, cyanobacteria synthesize a wide range of polysaccharides, lipids, vitamins, proteins, and minerals.[9,10] Solvent extracts of microalgae produce a large number of bioactive chemicals with a wide range of therapeutic potential, which has piqued the pharmaceutical industry's interest. To date, important molecules with antibacterial, antiviral, and antitumor action have been discovered in microalgae.[11,12] Because of the presence of activity like capping and reducing agents with functional groups like hydroxyl, carboxyl, and amino, the coating of metallic nanoparticles to these groups is ensured by microalgae extracts.[13,14]

Uniquely the usage of Nanomedicine is simultaneously allowed in drug delivery, wound healing, and tumor cell targeting.[15] Whereas alginate one of the most generously available biosynthesized biomaterial alginate is an anionic hydrophilic polysaccharide.[16,17] On account of its wide extent of uses as a biomaterial, it centers specific interest, particularly as the supporting matrix or delivery system, biocompatibility, biodegradability, non-antigenicity, and chelating capacity thus alginate is generally utilized in different biomedical applications, including drug delivery.[18] Typically, Ca^{2+} is one of the most much of the time utilized divalent cations used to ionically cross-link alginate and calcium carbonate. Given some organic or inorganic nano-carriers, some nano-drug delivery systems (nano-DDSs) have been intended to get prevail over the MRD sickness which likewise upgrades the drug efficacies against both drug-sensitive and drug resistance by improving medication bioaccessibility.[19,20] Our recent research shows how calcium alginate is used as a crosslinker to attach silver nanoparticles to freshwater cyanobacteria, acting as a stable carrier for silver nanoparticle immobilization and their bio evaluation against Multi-Drug Resistance Bacteria obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

MATERIALS AND METHODS

Isolation and development of cultures of cyanobacterial from freshwater

To synthesize silver nanoparticles, two cyanobacterial strains were collected from freshwater habitats in Warangal. Cyanobacteria are recuperated from their native habitat during isolation utilizing various enrichment strategies. Based on the presence of cyanobacterial mat and growth, water samples were taken from the surface scum and water column, respectively. The samples were collected, infected using the pour plate method on BGA and Allen Arnon media, and simultaneously grown in a broth of similar media. The samples were then incubated at 30°C for 20 days while being continuously exposed to white fluorescent light sustained for 16x 8 L/D cycles. The colonies were maintained in BG11 culture media under laboratory settings mimicking isolation using a conventional subculturing procedure. By microscopic filament identification, species colony mat creation, and heterocyst development, cyanobacteria species were traditionally identified. Microalgae are identified using molecular methods such as PCR paired with RFLP, direct rRNA gene sequencing, and isozyme analysis. The 18sRNA gene is the most significant and popular molecular marker for the identification of Eukaryotes; consequently, new cyanobacterial taxa are proposed using the same 18sRNA gene. The isolates were identified as *Characium typicum, Mariniradius saccharolyticus* based on their morphological and physiological characteristics.[20]

Synthesis of Silver nanoparticles

Silver nanoparticles were chemically synthesized by adding 5 mL of 1% trisodium citrate to 1mm silver Nitrate solution dropwise at 85°C on Persistent Stirring at 50-70 rpm.[21] The formation of AgNPs was examined visually. Generally, nanoparticle formation is visually considerable after 4 min of the starting of the reaction from a colorless solution to pale yellowish color.

Preparation of Cyanobacterial cells for Immobilization

0.2 g of 15 days old harvested cyanobacterial cultures of *Mariniradius saccharolyticus* and *Characium typicum* were dissolved in 10mL of PBS buffer separately.[22]

Cyanobacteria linker solution preparation

By dissolving 70mg of sodium alginate in 20mL of distilled water, sodium alginate with 3mM concentration was prepared. By continuous stirring, to the sodium alginate mixture, calcium chloride solution (0.03M) was added dropwise. After 10 min, cyanobacterial samples dissolved in PBS were added drop by drop to the aforesaid mixture. These solutions were used in 5:1:4 proportions.[22]

Linker solution for silver nanoparticles and cyanobacterial conjugation

To immobilize silver nanoparticles, 5mL of cyanobacterial–linker sample is mixed with 5mL of silver nanoparticle solution on constant stirring for 3 to 4 hr. Which was additionally measured and portrayed by UV-vis-spectrophotometer (Shimadzu, Model: UV-2450) with a precision of 1nm in the range of up to 800nm, Scanning Electron Microscopic analysis (SEM), Transmission Electron Microscopy (TEM), and FTIR (Fourier Transform Infrared Spectroscopy) which was carried out in the range of 400-4000 cm^{-1} at a resolution of 4 cm^{-1}, and followed by EDAX (Energy Dispersive Analysis X-Ray) analysis.

Test organisms

Methicillin-resistant *Staphylococcus aureus* (CI153), ampicillin-resistant *Escherichia coli* (CI18), *Klebsiella pneumoniae* (BAA-2785), erythromycin-resistant *Streptococcus pyogenes*, and multidrug-resistant *Pseudomonas aeruginosa* (CI3) was provided by the Microbial Type Culture Collection Centre in Chandigarh

(MTCC), India. The bacteria were subcultured and stored at 35°C on Mueller-Hinton and Blood agar media.

Antibacterial assay

The disc diffusion method was used to investigate the antibacterial susceptibility of silver nanoparticles (Ag-NPCs). Bacterial broth cultures were prepared and used for the antimicrobial assay before the experimental setting. To disseminate the bacterial culture, a sterile L-shaped glass rod was placed across the surface of the agar plates to disseminate bacterial lawns (40 µL). Using a sterile borer (6 mm), wells were punched in the agar plates. 40l µL, 60l µL, and 80l µL of conjugated silver nanoparticles with linker solution of cyanobacterial were filled the wells utilizing a micropipette, individually. The sample plates were maintained by incubating at 30°C for 24 hr. The diameter of the inhibitory zone was manually measured on a millimeter-scale after the incubation period.[23]

RESULTS AND DISCUSSION

Stable cross-linked polymer cages by cyanobacterial (conjugates) secondary metabolites were prepared where the divalent Ca^{+} ionically linked the alginate with calcium carbonate forming a perfect carrier for nanoparticles embedded with cyanobacterial secondary metabolites that dissolved into PBS buffer. The synthesis of Ag-NPCs by cyanobacterial cells was determined using the UV absorbance peak, which was created due to considerable absorption of visible light due to nanoparticle excite coupled with Surface Plasmon Resonance, a prominent property of silver nanoparticles.[24-27] UV absorbance of *Characium typicum* showed a large peak of 0.663abs at 434nm presented in Figure 1. *Mariniradius saccharolyticus* has an absorbance UV peak of 2.243abs at 442nm presented in Figure 2. A little peak at 425nm and a large peak at 490nm, as per Pal *et al.*, 2007, correspond to silver nanoparticles of 29 and 89nm, respectively. Silver particle reduction could occur as a result of the capping of microalgal protein metabolites, bringing about the generation of silver nanoparticles.[28] Silver nanoparticle retention spectra show a maximum extreme peak height of somewhere between 420nm and 450nm, with either a blue or red shift as molecule size increases.[29,30]

The silver particles were thought to require the NADH-dependent nitrate reductase chemical for their reduction.[31-33] Cell extract, which contains enzymes, proteins, lipids, and carbohydrates (sugars), is thought to act as reducing agents mostly in the biological formation of nanoparticles. Active functional groups including hydroxyl groups of tyrosine residues, glutamic acid residues, and carbonyl groups in aspartic acid have been used to reduce silver and synthesize silver nanoparticles.[34,35]

SEM and TEM (Scanning Electron Microscopy and Transmission Electron Microscopy)

The morphological characterization of biosynthesized *Characium typicum* and *Mariniradius saccharolyticus* silver nanoparticles was revealed by SEM studies. The reduced silver nanoparticles were installed arbitrarily into the cell surface, as indicated by light microscopy. The silver nanoparticles appear to be trapped in aggregates or scattered on the cellular structures. The particle size generated by biomass by two strains was between 12nm and 20nm, according to the SEM analysis as shown in Figure 3.

Transmission electron microscopy was used to determine the morphology and size of the silver nanoparticles (TEM). The form and size of Ag-NPCs varied significantly across the species studied, as seen by TEM pictures. Within the organic matrix, probably polysaccharide, the particles have a predisposition to agglomerate, in the case of *Mariniradius saccharolyticus* and ovoid in the case of *Characium typicum*, and the particles have a spherical shape in the case of *Mariniradius saccharolyticus* and ovoid in the case of *Characium typicum*. A considerable number of nanoparticles are generated or confined within the matrix, as previously described by Morones *et al.*, 2005. According to the TEM investigation, polydisperse Ag-NPs with an average size of 22.73nm in *Mariniradius saccharolyticus* and 20.67nm in *Characium typicum* were generated in the 20-23nm range as shown in Figure 4. The selected area electron diffraction pattern confirmed the particles' crystalline form (SAED).

FTIR analysis

FTIR examination of cyanobacterial silver nanoparticles linked conjugates (Ag-NPCs) and cyanobacterial cell biomass revealed a variety of peaks matching several biological functional groups. *Mariniradius saccharolyticus* Ag-NPCs showed a peak at 3493cm^{-1} as shown in Figure 5, which is attributed to O-H stretching in corresponds to alcohol and phenol compounds. C-N and C-H

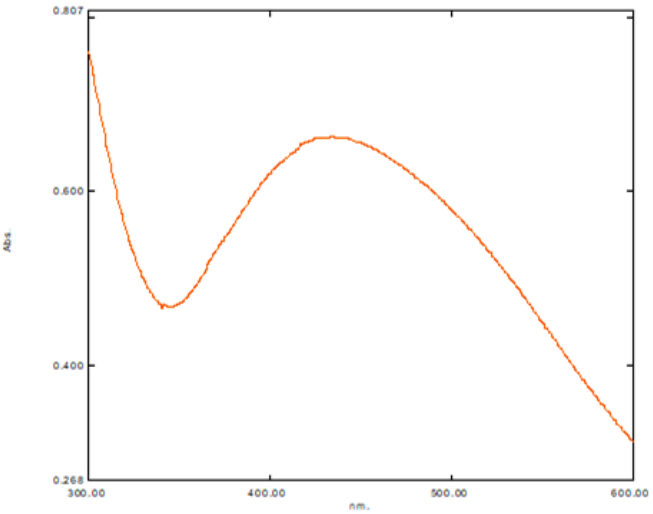

Figure 1: UV Absorance spectra *of Characium typicum* Ag-NPCs.

alkanes are ascribed to the peaks at 2924cm^{-1} and 2854cm^{-1}. At 1315cm^{-1}, stretching was also seen. The stretching at 1651cm^{-1} and 1546 cm^{-1} is attributed to primary and secondary amides, namely the amide I and amide II bonds combining carbonyl and proteins that stretch in the N-H direction. After bio-reduction biomass revealed a variation in the position of a few peaks, as well as the emergence of new peaks at 3493cm^{-1}, 1400cm^{-1}, and 995cm^{-1}, which could indicate biomolecule adsorption on their surface. A thorough analysis of the spectrum bands indicated that the cell density and Ag-NPCs indeed have the same basic functional groups. Furthermore, during the reduction of Ag+ to Ago in the cell extract during the production of Ag-NPCs, –OH groups are used, the frequency curve was drastically lowered from 2926cm^{-1} to 2924cm^{-1}, 1661cm^{-1} to 1651cm^{-1}, and 1547cm^{-1} to 1546cm^{-1}.

Characium typicum's stretching and bending frequencies had large peaks as shown in Figure 6. The bending frequencies of amide I as well as amide II are represented by the FTIR peaks of 1658cm^{-1} and 1547cm^{-1} of Ag-NPCs, respectively. The reduction of Ag^{+} to Ag^{o} causes the peaks to shrink from 2926cm^{-1} to 2924cm^{-1}, and from 1658cm^{-1} to 1654cm^{-1}.

The presence of carboxyl, carbonyl, hydroxyl, protein groups, and amino acids in the manufacturing and stability of nanoparticles is reflected in the functional group analysis of Ag-NPCs as shown in Figure 7. As indicated by these perceptions, silver ions were reduced in the presence of nitrate reductase.[36] Ascension to comprehend peaks in the infrared portion of the electromagnetic spectrum is provided by amino acid residues bound by an amide bond. Apart from providing nanoparticles with a harbor on bacterial membranes, which allows them to acquire antibacterial properties[37] Silver nanoparticles can also be stabilized through protein nanoparticle interactions, which can occur whether it be through free amino groups or cysteine residues in the protein or by means of the electrostatic attraction of negatively charged carboxylate groups in enzymes found in the cell wall.[23,38]

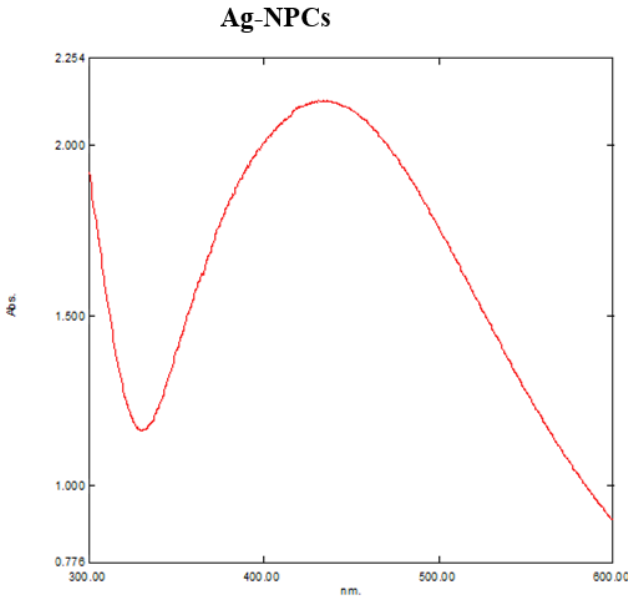

Figure 2: UV Absorance spectra of *Mariniradius saccharolyticus* Ag-NPCs.

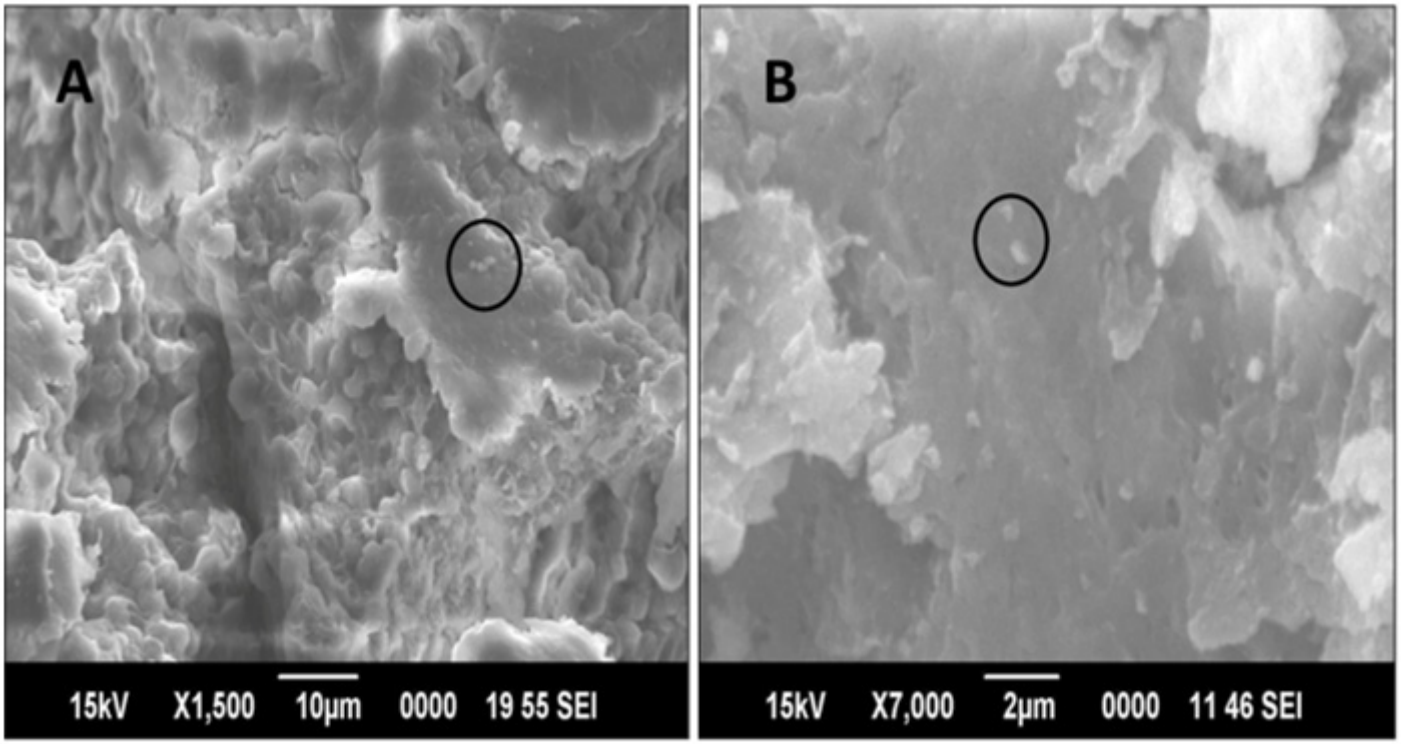

Figure 3: SEM images of A: *Characium typicum*; B: *Mariniradius saccharolyticus* with spherical shape nanoparticles.

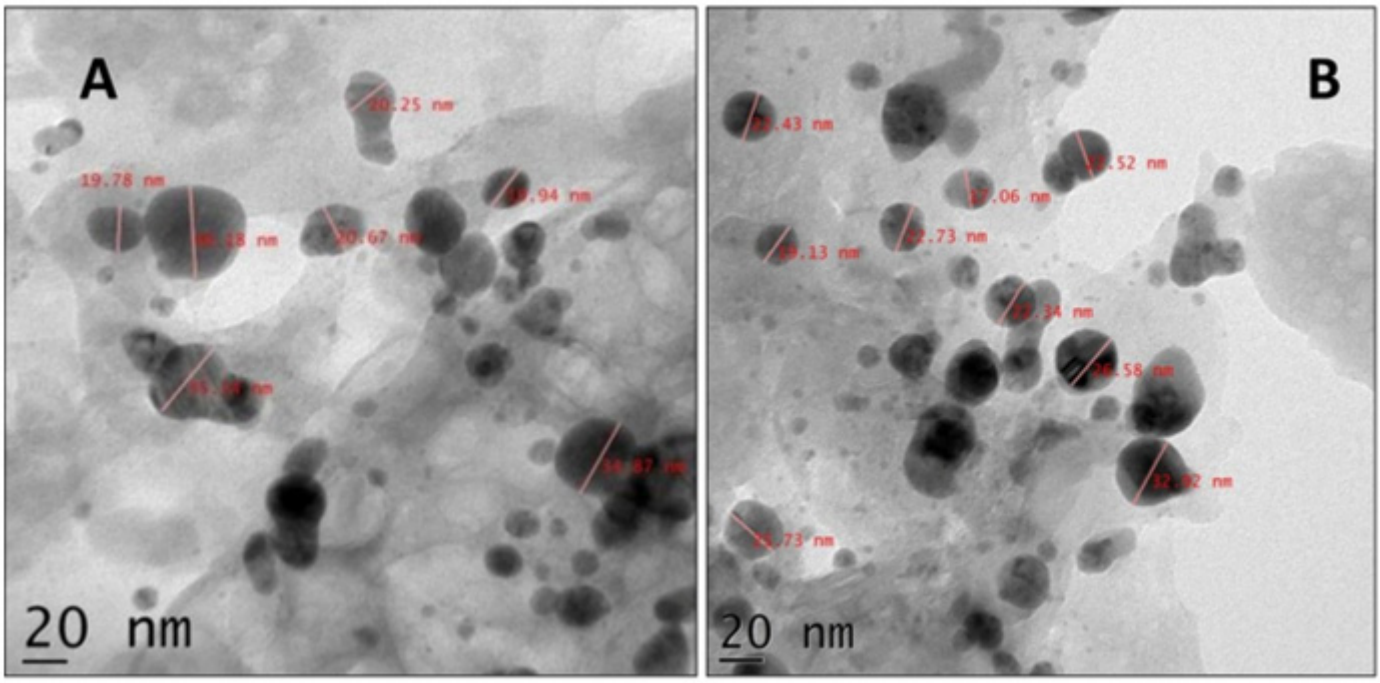

Figure 4: TEM images of A: *Characium typicum*; B: *Mariniradius saccharolyticus*.

Table 1: Antibacterial assay MDR bacteria where $AgNO_3$ as positive control and PBS buffer as a negative control; MDR-Multi Drug Resistance.

Test Organisms	Zone of against Inhibition in mm											
	Silver nitrate (positive control)			PBS Buffer (Negative control)			*Mariniradius saccharolyticus* conjugated linked silver nanoparticles			*Characium typicum* conjugated linked silver nanoparticles		
	40µL	60µL	80µL	40µL	60µL	80µL	40µL	60µL	80µL	40µL	60µL	80µL
	0	1	3	0	0	1	2	3.5	5	3	5	6
Ampicillin resistant *Escherichia coli*	0	1	3	0	0	1	2.6	5	7	3	7	10
Ampicillin resistant *Klebsiella pneumonia*	0	0	4	0	0	1	0	2	4	1	3	2.5
Erythromycin resistant *Streptococcus pyogenes*	0	0	3	0	0	1	3	7	7.3	3	3.1	5
Multidrug resistant *Pseudomonas aeruginosa*	0	1	3.2	0	0	1	2	5	9	3	7	10

Antibacterial Assay

The ability of antibacterial activity by cyanobacteria-linked silver nanoparticle conjugates was performed against Multidrug-Resistant bacteria by the Disc diffusion method and the results were recorded by measuring the zone of inhibition in mm. Silver nitrate serves as positive control and PBS buffer as a negative control. The Ag-NPCs showed considerable activity against all test organisms at the volume of 80 µL, 60 µL, and 40 µL tabulated in Table 1. Results explain that both the *Characium typicum* Ag-NPCs and *Mariniradius saccharolyticus* Ag-NPCs showed the zone of inhibition against methicillin-resistant *Staphylococcus aureus*, ampicillin-resistant *Escherichia coli*, and *Klebsiella pneumonia*, erythromycin-resistant *Streptococcus pyogenes*, and multidrug-resistant *Pseudomonas aeruginosa* ranging between 5-10mm volume of 80 µL. 60 µL and 40 µL volumes of the test sample showed inhibition activity at a range of 3-9mm.

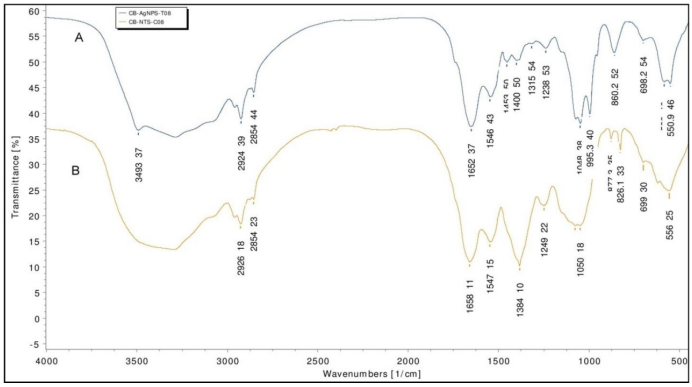

Figure 5: FTIR spectra of (A) *Mariniradius saccharolyticus* Ag-CNPS (B) Extract without silver nitrate (control).

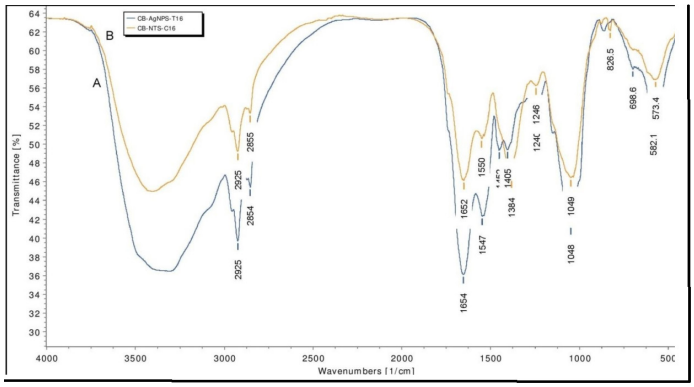

Figure 6: FTIR spectra of (A) *Characium typicum* Ag-CNPS (B) Extract without silver nitrate (control).

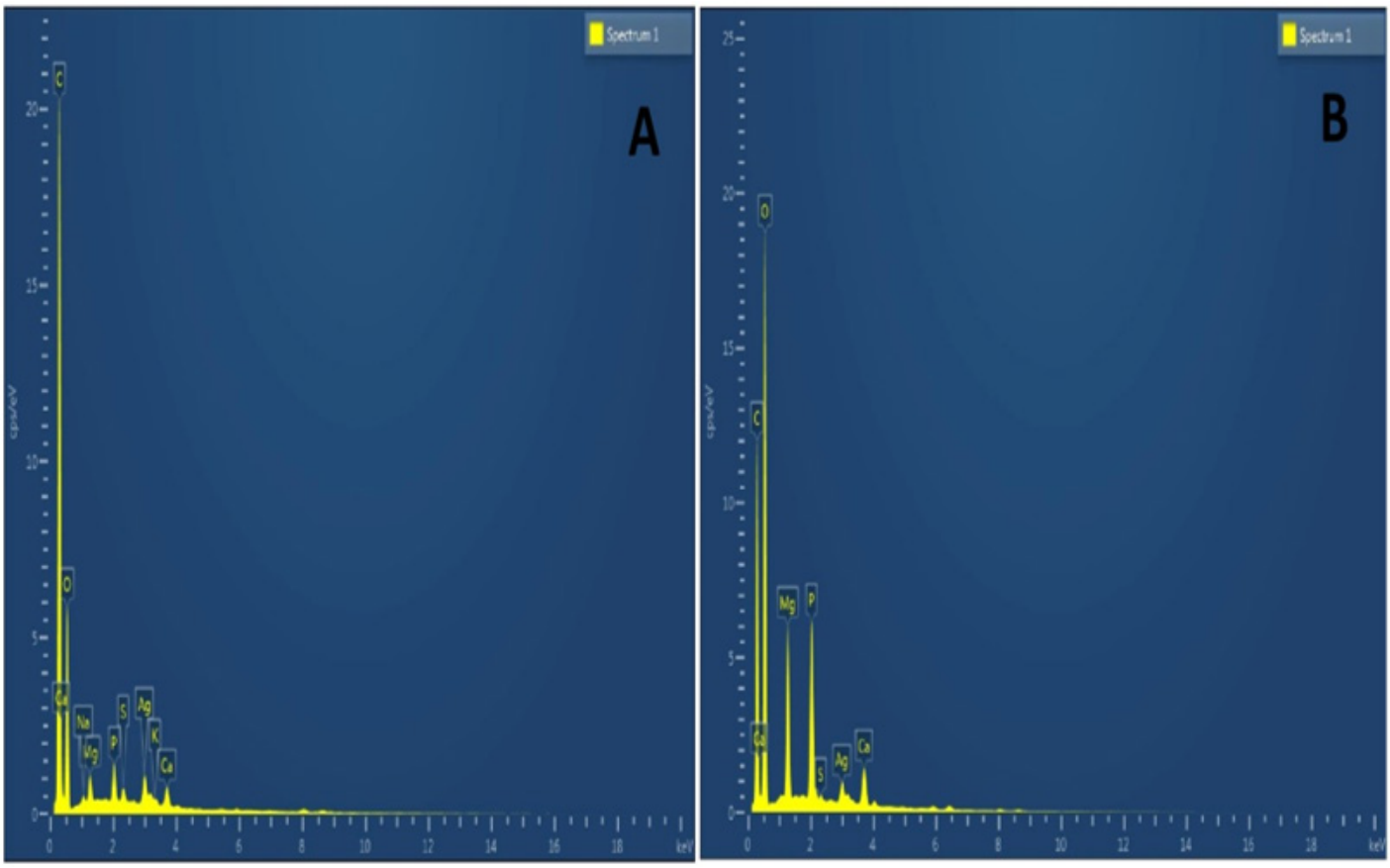

Figure 7: EDAX Spectra of A: *Characium typicum* B: *Mariniradius saccharolyticus* Ag-NPCs displaying major emission energies for silver (Ag) that showed over all weight percentage of silver.

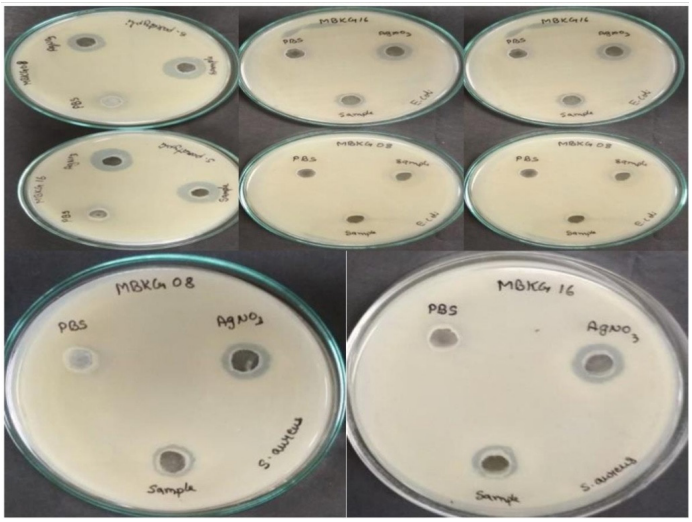

Figure 8: Antibacterial activity by cyanobacterial conjugated linked silver nanoparticles against Multidrug-Resistant bacteria that show a zone of inhibition.

Gram-negative bacteria and Gram-positive have distinct antibacterial actions due to differences in cell wall composition. Silver nanoparticles are known to be hazardous to microorganisms due to their bacteriostatic and antibacterial properties. As per past exploration, silver nanoparticles can stick to the outer layer of cell films, hindering cell vulnerability and respiration. The silver nanoparticles may potentially be able to penetrate the bacteria.[39] As per our examination, because of the slenderer peptidoglycan layer and the prevalence of porins, silver nanoparticles have superior antibacterial activity against gram-negative bacteria than gram-positive bacteria.[36] When attempted against pathogenic organisms, the antibacterial action of the test i.e., When compared to conjugate-linked silver nanoparticles, cyanobacteria-linked silver nanoparticles, and silver nanoparticles alone as a positive control was shown to be lower, which could be a direct outcome. This showed that nanoparticles linked to cyanobacteria could coagulate and stay constant over a period of time due to cyanobacteria protein discharge, which acted as a capping agent to prevent certain conjugates from losing their bioactive therapeutic efficacy, despite the fact that non-formed silver nanoparticles had a smaller zone of restraint and lower bioactivity as shown in Figure 8.

CONCLUSION

A sodium alginate linker solution was used to make silver nanoparticles with a mean diameter of 20nm in our research. UV-vis-spectroscopy was used to identify the nanoparticles. For the silver nanoparticles, UV/Vis-spectra revealed a Plasmon absorption peak extending between 420nm and 470nm. The decrease of Ag^{+} to Ag^{o} was confirmed by FTIR analysis. The findings demonstrate that cyanobacterial-conjugated silver nanoparticles suppressed the bacteria's turn of events and growth, including drug-resistant *S. paratyphi, S. typhi, S. aureus*, and strains. Silver nanoparticles generated by cyanobacteria work on the stability of silver nanoparticles by secreting proteins (capping agents). The Bio-Nano-formulation of cyanobacteria and silver nanoparticle conjugates was more effective than silver nanoparticles alone against harmful bacteria. As a result, these conjugates could be used in biological, food, pharmacological, health, and agriculture applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

Ag-NPs: Silver Nanoparticles; **Ag-NPCs:** Silver Nanoparticle Conjugates; **UV-vis:** Ultraviolet-Visible; **SEM:** Scanning Electron Microscopy; **TEM:** Transmission Electron Microscopy; **FTIR:** Fourier Transform Infrared Spectroscopy; **EDAX:** Energy Dispersive Analysis X-Ray; **MDR:** Mutli Drug-Resistant; **PBS Buffer:** Phosphate buffered saline buffer; **Rpm:** Rotation per minute; **SAED:** Selected Area Electron Diffraction.

SUMMARY

Nanoscience, which makes use of nanoparticles, is a new way to combat MDR infections. Nanomedicine is permitted in the mucoadhesive drug delivery system at the same time. In order to improve the drug efficiency against both drug sensitivity and drug resistance by boosting drug bioaccessibility, the bio nanoformulation of cyanobacteria as a nano-drug delivery system has been emphasised in this case study. The evaluation of cyanobacteria nanoconjugates revealed that the electromagnetic radiance, size, and shape of nanoparticles are suitable for drug administration in this particular study. The antibacterial assay of these nanoparticles confirmed the cyanobacterial conjugates' static nature.

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