Green Synthesis of Silver Nanoparticles Using Cell Extracts of *Anabaena doliolum* and Screening of Its Antibacterial and Antitumor Activity

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In the present work, we describe a simple, cheap, and unexplored method for “green” synthesis of silver nanoparticles using cell extracts of the cyanobacterium *Anabaena doliolum*. An attempt was also made to test the antimicrobial and antitumor activities of the synthesized nanoparticles. Analytical techniques, namely UV-vis spectroscopy, X-ray diffraction, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), and TEM-selected area electron diffraction, were used to elucidate the formation and characterization of silver-cyanobacterial nanoparticles (Ag-CNPs). Results showed that the original color of the cell extract changed from reddish blue to dark brown after addition of silver nitrate solution (1 mM) within 1 h, suggesting the synthesis of Ag-CNPs. That the formation Ag-CNPs indeed occurred was also evident from the spectroscopic analysis of the reaction mixture, wherein a prominent peak at 420 nm was noted. TEM images revealed well-dispersed, spherical Ag-CNPs with a particle size in the range of 10–50 nm. The X-ray diffraction spectrum suggested a crystalline nature of the Ag-CNPs. FTIR analysis indicated the utilization of a hydroxyl (-OH) group in the formation of Ag-CNPs. Ag-CNPs exhibited strong antibacterial activity against three multidrug-resistant bacteria. Additionally, Ag-CNPs strongly affected the survival of Dalton’s lymphoma and human carcinoma colo205 cells at a very low concentration. The Ag-CNPs-induced loss of survival of both cell types may be due to the induction of reactive oxygen species generation and DNA fragmentation, resulting in apoptosis. Properties exhibited by the Ag-CNP suggest that it may be used as a potential antibacterial and antitumor agent.

Keywords: *Anabaena doliolum*, cell extract, silver nanoparticles, antibacterial activity, cytotoxicity.

Introduction

The unique properties and wide application of metal nanoparticles have attracted researchers from all over the world toward study of their synthesis and stabilization [21, 34]. Synthesis of silver nanoparticles (AgNPs) in particular has attracted much attention because of their unique optical, electrical, and chemical properties. AgNPs are used as biolabeling agents, catalysts in chemical reactions, and optical receptors, and in nonlinear optics [10, 21, 34]. AgNPs are also used as a bactericide on burn wounds, fillers in dental cavities to prevent infection, and thin coats on medical devices to prevent bacterial biofilm formation, and in air and water purification systems, wastewater treatment plants, and food processing for controlling microbial contamination [8, 12, 24, 38]. The wide use of AgNPs is also due to the fact that, unlike other metal nanoparticles, they are non-toxic to the human body at
lower concentrations [7, 13, 22, 34].

Several chemical and physical methods for the preparation and stabilization of metal nanoparticles are known; however, more eco-friendly, reliable, energy efficient, and non-toxic methods employing plants and microbes are gaining more popularity [1, 13, 15, 32, 34, 42]. A number of researchers have reported synthesis of silver nanoparticles using bacteria, fungi, and algae, but those reports have focussed on the use of whole cell masses in synthesis [1, 15, 23, 28, 34]. However, very few reports are available on the use of cell extract or culture supernatant of bacteria in synthesis of metal nanoparticles [18, 32]. Cyanobacteria (blue-green algae) form one of the largest and most primitive ancestral groups of photoautotrophic bacteria on Earth [29]. They offer great potential as a source of fine chemicals, pharmaceuticals, and biofuels and are a rich source of pigments/proteins [18]. Additionally, the cell extract of cyanobacteria contains a vast array of active biomolecules that may facilitate synthesis and stabilization of the nanoparticles [18, 38]. The roles of membrane localized oxidoreductases [13], NADH-dependent enzymes [1], and certain other active biomolecules, including phycobiliproteins, have also been suggested in the formation of structures in the nanometer range [18, 27].

To date, synthesis of AgNPs has been achieved by using the whole cells of non-nitrogen-fixing cyanobacteria, namely Plectonema boryanum and the marine Oscillatoria willei [1, 13]. Our interest aroused to test the potential of the cell extract of the N₂-fixing cyanobacterium Anabaena doliiolium in the synthesis process. As the recovery of nanoparticles adhered to the surface of filament or formed inside the cells would require a lengthy/complex extraction process and may not be cost effective, the search for an alternative approach such as the use of cell extract of A. doliiolium was applied in the synthesis. The selection of A. doliiolium was based mainly because of its luxuriant growth in rice fields of North India and its fast growth in simple inorganic medium in the laboratory. To our knowledge, this is the first report wherein the cell extract of the N₂-fixing cyanobacterium has been employed in the synthesis of AgNPs. Furthermore, the role of synthesized silver-cyanobacterial nanoparticles (Ag-CNPs) as biocidal agents against multidrug-resistant bacteria and tumor/cancer cell lines was also a novel finding.

Materials and Methods

Test Organism and Growth Conditions

A. doliiolium, a filamentous heterocystous cyanobacterium, was isolated from a rice field of Banaras Hindu University and is maintained in the laboratory for the last 10 years. Identity of the isolate was confirmed by the sequencing of its 16S rRNA gene, where the sequences showed 99% similarity with A. doliiolium available in the NCBI database (Accession No. JX075257). Axenic culture was routinely grown diazotrophically in BG11 medium [29] in a culture room at 27 ± 2°C and illuminated with Sylvania 40W T12 fluorescent lamps at an intensity of 14.4 ± 1 Wm⁻² for a 14/10 h light/dark cycle. Unless otherwise stated, all the experiments were performed with the log phase cultures, having an initial dry weight of approx. 0.15 mg/ml.

Synthesis of Silver-Cyanobacterial Nanoparticles

Thoroughly washed A. doliiolium pellet (10.0 g wet weight) was suspended in 100 ml of sterile double-distilled water and sonicated in a Branson Sonifier 450 (Branson Ultrasonics Corp., USA) for 5 min at maximum output and duty cycle. The extract was critically examined microscopically to ensure complete breakage of the cells; if the extract showed the presence of filaments/cells, steps of sonication were repeated. If needed, the extract was incubated at ~20°C overnight to attain complete lysis of cells. The resulting cell-free extract was centrifuged at 10,000 × g for 15 min and filtered through Whatman No. 1 filter paper to remove cell debris, if any. For the synthesis of Ag-CNPs, the cell extract (pH 7.0) was distributed equally (50 ml each) into two flasks. To one flask, silver nitrate (AgNO₃) was added to attain a final concentration of 1 mM. The second flask contained only cell extract and served as the positive control. Pure AgNO₃ solution (1 mM) was taken in a flask separately for the negative control. All the flasks were wrapped in aluminum foil to ensure complete darkness and incubated at 25°C in a shaker (150 rpm) for 72 h. The formation of Ag-CNPs was monitored visually at an interval of 2 h by observing the changes in color of the reaction mixture. The original color of the mixture was reddish blue (due to the fluorescence of phycobiliprotein). The bioreduction of silver ions was monitored by the spectroscopic analysis of the reaction mixture in the range of 200–700 nm in a UV-vis spectrophotometer (Shimadzu, Nakagyo-ku, Japan) by withdrawing 1 ml aliquots at known time intervals, lasting up to 60 h. For the quantitative estimation, the nanoparticles formed in the cell extract from known amount of culture (wet weight) were centrifuged at 10,000 ×g at 4°C for 15 min and the weight of the dried pellet was determined.

Characterization of Ag-CNPs

The particle size and morphology of the Ag-CNPs were characterized by transmission electron microscopy (TEM). Briefly, the clear aqueous suspension of Ag-CNPs was dropped onto carbon-coated copper grids and then air dried completely in sterile dust-free conditions. TEM microphotographs were taken in a Transmission Electron Microscope Tecnai 20 FEI (FEI Europe B.V., The Netherlands) operated at an HT of 200 kV. The crystalline nature of the silver nanoparticles was examined with the help of the selected area electron diffraction (SAED) pattern by TEM. The
size distribution and zeta potential value of the nanoparticles (suspended in Milli-Q water) were determined by a Beckman Coulter Delta Nano C Particle Size Analyzer (Beckman Coulter, Inc., USA). For X-ray diffraction (XRD) analysis, the aqueous suspension of the Ag-CNPs was centrifuged twice at 10,000 \( \times \) g for 20 min and the pellet was dissolved in 10 ml of deionized water. After freeze drying of the suspension, spectra were recorded in a Rigaku Miniflex II X-ray Diffractometer (Rigaku Co., Ltd., Japan) operated at 30 kV, 15 mA. The diffraction was recorded at 20 angles from 10 to 80 degrees. XRD was performed for determination of the dimension of biologically synthesized Ag-CNPs with \( h \), \( k \), and \( l \) values. The particle size \( (L) \) of Ag-CNPs was calculated using Debye-Scherrer’s equation: \( L = \frac{0.9λ}{β \cos θ} \), where \( λ \) is the wavelength of the X-ray, \( β \) is full width and half maximum, and \( θ \) is the Bragg’s angle. Fourier transform infrared spectroscopy (FTIR) of synthesized Ag-CNPs was performed after mixing with potassium bromide \((1:100)\) in a Perkin Elmer infrared spectrophotometer (Perkin Elmer Inc., USA) for the analysis of functional groups.

**Antibacterial Assay**

Antibacterial activity of Ag-CNPs was tested on two gram-negative and one gram-positive bacteria; namely, *Klebsiella pneumoniae* DFL25A (HQ114261), *Escherichia coli* DF97TA (HQ163793), and *Staphylococcus aureus* DF8TA (JN642261) respectively, by the disc diffusion technique using the Kirby-Bauer method [5]. All these bacteria were isolated from diabetic foot ulcers of patients admitted to Sir Sundarlal Hospital, Banaras Hindu University, Varanasi, India. Identification of all the three isolates was made by 16S rRNA gene sequencing. Cultures were routinely grown in Luria-Bertani (LB) medium in a bacteriological incubator at 37°C. The antibacterial effect of Ag-CNPs was tested at concentrations of 5–500 \( \mu\)g/disc in Petri plates containing solid LB agar medium. Inoculum \((100 \mu l)\) of each bacterial strain was uniformly spread on solid LB agar medium and then sterilized paper discs \((5 \text{ mm diameter})\) loaded with 5, 50, 100, 250, and 500 \( \mu\)g/disc of Ag-CNPs were carefully placed in each plate. Additionally, 16 antibiotics belonging to eight different classes \((\text{cephalosporin, aminoglycoside, penicillin, lincomamide, tetracycline, carbapenem, dihydrofolate reductase inhibitor, and folate pathway inhibitor})\) were tested for sensitivity against all three strains of bacteria. All the test bacteria showed resistance to as many as 10 antibiotics of the above classes, but only two antibiotics, ampicillin and amikacin, were selected as the positive control in this study. For the negative control, paper discs with sterile water, AgNO\(_3\) \((5 \mu g = 1 \text{ mM/disc})\), or the cell extract of *A. dolichum* were placed over the bacterial lawn in different plates. For loading of Ag-CNPs, antibiotics, or AgNO\(_3\) onto paper discs, a solution of the test materials with desired stock concentration was prepared in sterilized Milli-Q water, and thereafter 20 \( \mu\)l of each was loaded on paper disc by glass micropipette following the steps used in loading of the samples in paper/thin-layer chromatography. The paper discs were dried and sterilized before placing on agar plates. The development of a clear zone around the disc was considered positive for antibacterial activity. The size of zone of inhibition was measured from the periphery of the paper disc. Unless otherwise stated, we have tested the effects of Ag-CNPs in totality; the amount of Ag is approx. 2.6% and accordingly results may be interpreted.

**Test of Antitumor Activity of Ag-CNPs on Dalton’s Lymphoma and Colo205 cells**

Single-cell suspensions of thymocytes were prepared following previously described standard procedures [16, 33], from freshly isolated thymus of mice. Dalton’s lymphoma (DL) cells and colo205 cell line \((\text{colon adenocarcinoma})\) were made available by Prof. S. M. Singh, School of Biotechnology, Banaras Hindu University, Varanasi (India) [11, 33]. Thymocytes, DL cells, and colo205 cells \((1 \times 10^6 \text{ cells/ml})\) were incubated in 96-well culture plates in complete RPMI medium [33] with or without Ag-CNPs and cisplatin (positive control) at 37°C in the humidified atmosphere of a CO\(_2\) \((5\%)\) incubator. Ag-CNPs were dissolved in dimethyl sulfoxide \((\text{DMSO})\) and then diluted in RPMI medium to attain desired concentrations \((0 \text{ to } 50 \mu g/ml)\) before adding to cell cultures. Survival of cells was estimated by the standard MTT \((3-(4,5-\text{dimethylthiazol-2-yl})-2,5-\text{diphenyl tetrazolium bromide})\) assay according to the method of Mosmann [17]. Briefly, MTT was dissolved in phosphate-buffered saline \((\text{PBS})\) at a concentration of 5 mg/ml. Thereafter, 50 \( \mu\)l of the MTT solution was added to each well of the culture plate containing cells \((200 \mu l)\) medium with treatment of varying concentrations of Ag-CNPs. A plate with Ag-CNPs-free PBS was kept as the control. All the plates were incubated at 37°C for 4 h, and thereafter, the medium was carefully removed without disturbing the dark blue formazan crystals. Subsequently, 50 \( \mu\)l of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. Plates were read on a microplate reader \((\text{Labsystems, Finland})\) at a wavelength of 540 nm. Percent cell survival was calculated by normalizing the optical density \((\text{OD})\) values of experimental groups against the control. In vitro survival of the colo205 cell line was compared with normal thymocytes and the value was also compared with a positive control drug \((\text{cisplatin})\).

**Assay of Reactive Oxygen Species (ROS) Production**

Measurement of ROS was done by using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate \((\text{DCFH-DA})\) as described by Kumar et al. [11]. The DCFH-DA assay was employed as it is widely used as a marker of cellular oxidative stress and can detect the ROS \(\text{H}_2\text{O}_2\), hydroxyl, and peroxyl radicals directly in the cell. For the assay, thymocytes, DL cells, and colo205 cells were treated with Ag-CNPs or cisplatin for 3 h and, after washing by PBS, incubated with DCFH-DA at a final concentration 0.1 mM. The cells were further incubated at 37°C for 45 min, followed by washing with PBS. The cells stained with dye were visualized under a fluorescence microscope \((\text{Nikon, Japan})\) at a magnification of 400 \( \times \) and photographed. The amount of staining was quantified by MCID software.
Morphological Evaluation of Apoptotic Cells by Wright-Giemsa Staining

The apoptotic cell population was enumerated by a method described earlier [33]. Cell suspensions, including control cells (without any drug treatment) and cells exposed to Ag-CNPs or cisplatin, were smeared on a slide and air-dried, fixed in methanol, stained with Wright-Giemsa staining solution, mounted in glycerine, and analyzed under a light microscope (Carl Zeiss, Germany) at 400× magnification. Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies; condensed, uniformly circumscribed, and densely stained chromatin; and membrane-bound apoptotic bodies (blebbing) containing one or more nuclear fragments. The percentage of apoptotic cells was determined by counting more than 300 cells in at least three separate microscopic fields.

Estimation of Percent DNA Fragmentation

Ag-CNPs treated and untreated (control) cells of DL and colo205 were lysed in 0.5 ml of Tris-EDTA buffer (pH 7.4) containing 0.2% (v/v) Triton X-100. The fragmented DNA was separated from intact chromatin in a microcentrifuge tube (marked as B) by centrifugation at 10,000 × g at 4°C for 10 min. Supernatant containing the fragmented DNA was transferred to another microcentrifuge tube (marked as T). A volume of 0.5 ml of 25% TCA was added to each marked tube and vortexed vigorously. DNA was precipitated overnight at 4°C and centrifuged at 10,000 × g at 4°C for 10 min. The supernatant was discarded and 80 µl of 5% TCA was added to each pellet. DNA was hydrolyzed by heating at 90°C for 15 min. A blank was also kept, containing 80 µl of 5% TCA. Thereafter, 160 µl of freshly prepared diphenylamine (DPA) reagent (150 mg diphenylamine in 10 ml glacial acetic acid, 150 µl concentrated H₂SO₄, and 50 µl of acetaldehyde solution) was added to each tube and the tubes were allowed to stand overnight at room temperature to develop color. A 100 µl aliquot of this colored solution was transferred to the wells of a 96-well flat bottomed ELISA plate and the absorbance was measured at 600 nm in an ELISA plate reader (Labsystems, Finland). Percent DNA fragmentation was calculated as

\[ \text{DNA fragmentation (\%)} = \frac{\text{T}}{(\text{T+B})} \times 100 \]

where T = absorbance of fragmented DNA, and T + B = absorbance of total DNA.

Statistical Analysis

All the experiments were performed three times under identical conditions. Mean values and standard deviations were determined from three replicates of each treatment. All the experiments were conducted in triplicate. A one-way ANOVA (analysis of variance) was applied to confirm the significance of data according to Duncan’s multiple range test (MRT) at \( p \leq 0.05 \). SPSS-16 software was used for the MRT. Unless otherwise stated, values are the mean ± SD (\( n = 3 \)).

Results

Synthesis of Ag-CNPs

Formation of silver nanoparticles after the addition of the cell extract of A. doliolum to AgNO₃ solution was tested up to 72 h at 25°C (Figs. 1A–1C). Addition of AgNO₃ (1 mM) to the cell extract caused a change in color of the reaction mixture from reddish blue to brown within 1 h. The intensity of color increased with the time of incubation, where maximum change (dark brown) was noted at 60 h (Fig. 1C). On the other hand, the color of the AgNO₃ solution (Fig. 1A) or cell extract of A. doliolum (reddish blue) remained unchanged even after 72 h (Fig. 1B). Changes in the color of the cell extract suggest its reaction with Ag⁺ and possible deposition of silver nanoparticles. That the synthesis of Ag-CNPs indeed occurred became evident from the UV-visible spectroscopic analysis of the reaction mixture. A strong, broad peak appeared at 420 nm and showed increase in absorbance with increasing time of incubation, with maximum absorbance attained at 60 h (Fig. 2). However, the spectra showed increases of ODs in the entire scan range, suggesting the presence of other moieties in the synthesized Ag-CNPs. Work is in progress to obtain pure Ag-CNPs by changing the conditions of synthesis. Quantitative analysis showed that approximately 65 mg of Ag-CNPs is produced from 1.0 g of culture (wet weight) of A. doliolum. The value obtained is the sum of all the constituents (such as the proteins, pigments, and amino acids present in the cell extract) attached to Ag-CNPs, as

![Fig. 1. Change in color of solutions after 60 h of incubation.](image-url)
the content of Ag alone would be very low (approx. 2.6%). It would be worthwhile to analyze the different constituents present in Ag-CNPs.

**Characterization of Ag-CNPs**

Ag-CNPs were characterized in detail by employing various analytical tools. TEM images showed the size of the nanoparticles in the range of 10–50 nm (Figs. 3A and 3B). The TEM-SAED pattern showed prominent silver diffraction rings, suggesting the face cubic centered (fcc) crystalline nature of the Ag-CNPs (Fig. 3C). The size distribution of Ag-CNPs showed a high degree of monodispersed spherical-shaped nanoparticles, as the polydispersity index was noted in the range of 0.1 to 0.2. The average hydrodynamic radii of Ag-CNPs were 35 nm, which is congruent to the diameter of nanoparticles observed in the TEM images (Fig. 4A). Some differences in the size of Ag-CNPs observed in TEM and DLS may be due to the capping of
biomolecules onto nanoparticles. Zeta potential analysis showed the net charge of $-25.52 \text{mV}$ on the Ag-CNPs (Fig. 4B).

The XRD spectra indicated the crystalline nature of Ag-CNPs, as all the reflections were distinctly indexed to a face centered cubic phase of Ag-CNPs. The diffraction peaks were consistent with the standard data files of Joint Committee on Powder Diffraction standards (JCPDX file no -02-1098). Additionally, XRD spectra showed prominent Bragg reflections, which were indexed on the fcc structure of silver. The 2θ values of 111, 200, and 311 confirmed the crystalline nature of Ag-CNPs (Fig. 5). It was also noticed that the diffraction angles of Ag-CNPs were quite close to bulk silver crystal (2θ = 38.2°). The particle size (L) calculated from the XRD data was 20 nm, which matched with the size range (10–50 nm) observed in the TEM images.

**FTIR Analysis**

FTIR analysis of the cell extract and Ag-CNPs showed a number of peaks representing different functional groups of biological origin. The major stretching frequencies at 3,432.19 cm$^{-1}$ (-OH), 2,926.07 cm$^{-1}$ (C-N), 1,640.05 cm$^{-1}$ (C=O), 1,384.35 cm$^{-1}$ (N-O), and 1,072.64 cm$^{-1}$ (C-O) representing different functional groups were observed in the cell extract (Fig. 6A). On the other hand, the stretching frequencies at 3,833.72 cm$^{-1}$ (-OH), 2,930.60 cm$^{-1}$ (C-N), 1,651.13 cm$^{-1}$ (C=O), and 1,076.58 cm$^{-1}$ (C-O) were observed in the Ag-CNPs (Fig. 6B). Comparative analysis of the spectra suggests that the cell extract and Ag-CNPs share certain common functional groups. However, there was significant reduction in the frequency curve from 3,432.19 cm$^{-1}$ to 3,833.72 cm$^{-1}$ noted in the cell extract during the formation of Ag-CNPs. This reduction might be due to the utilization of -OH groups in the reduction of Ag$^+$ to Ag$^0$. Bands observed at 2,926.07 cm$^{-1}$ (cell extract) and 2,930.60 cm$^{-1}$ (Ag-CNPs) correspond to the C-N stretching of amine. Additionally, band of 1,651.13 cm$^{-1}$ in the case of Ag-CNPs could be responsible for the adsorption of biomolecules on their surface. Results of FTIR analysis also point to the release of protein molecules that probably help in the formation and stabilization of Ag-CNPs.

**Antibacterial Assay**

The antibacterial activity of Ag-CNPs was tested on three multidrug-resistant bacteria (K. pneumoniae, E. coli, and S. aureus) isolated from the diabetic foot ulcers of diabetic patients (Figs. 7A–7C). It was evident from the data that Ag-CNPs treatment at a concentration of as low as 5 µg/disc resulted in the formation of a zone of inhibition in all three bacteria. The largest zone of inhibition was formed with 500 µg/disc of Ag-CNPs (Figs. 7A–7C). As expected, the zone of inhibition was not formed in the plates containing discs loaded with the cell extract or Milli-Q water. However, plates containing 5 µg/disc of AgNO$_3$ showed the formation of a zone of inhibition of smaller sizes with all the bacteria.
To test the effectiveness of Ag-CNPs in comparison with antibiotics, discs loaded with the antibiotics ampicillin (Amp) and amikacin (Amk) were used in the assay. Interestingly, neither ampicillin nor amikacin caused zones of inhibition formation in \textit{K. pneumoniae} (Fig. 7A) and \textit{E. coli} (Fig. 7B), even at a concentration of 500 µg/disc. In addition to these two antibiotics, zones of inhibition formation did not appear with a few other antibiotics to which these bacteria were resistant. However, there was a zone of inhibition formation in the gram-positive bacterium \textit{S. aureus} with 100–500 µg/disc of both the antibiotics (Fig. 7C), although the size was smaller than those observed with Ag-CNPs (Table 1). It is evident from the results that the size of zone of inhibition formation did not show much difference above 50 µg/disc (Table 1); however, the largest diameter of zone of inhibition was noted with 500 µg/disc concentration of Ag-CNP in \textit{E. coli}, \textit{K. pneumoniae}, and \textit{S. aureus} (i.e., 33, 36, and 34 mm, respectively). The findings of the disc diffusion assay clearly indicate that there is no correlation between the concentrations of Ag-CNPs used and the size of zone of inhibition formation.

### Table 1. Test of antibacterial activity of Ag-CNPs in selected multidrug-resistant bacteria.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Concentration (µg/ml)</th>
<th>Ampicillin</th>
<th>Amikacin</th>
<th>Ag-CNPs Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Escherichia coli}</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>18 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>23 ± 1.60</td>
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<td>100</td>
<td>-</td>
<td>-</td>
<td>25 ± 2.30</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>27 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>33 ± 1.63</td>
</tr>
<tr>
<td>\textit{Klebsiella pneumoniae}</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>17 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>21 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>29 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>32 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>36 ± 0.82</td>
</tr>
<tr>
<td>\textit{Staphylococcus aureus}</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>11 ± 1.63</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>17 ± 0.81</td>
</tr>
<tr>
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<td>100</td>
<td>16 ± 2.30</td>
<td>19 ± 0.81</td>
<td>20 ± 3.20</td>
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<tr>
<td></td>
<td>250</td>
<td>17 ± 1.60</td>
<td>29 ± 1.60</td>
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</tr>
<tr>
<td></td>
<td>500</td>
<td>17 ± 0.81</td>
<td>29 ± 0.81</td>
<td>34 ± 0.81</td>
</tr>
</tbody>
</table>

All the bacteria were grown under identical conditions and the experiments were performed in triplicate. Plates found with any contamination were immediately discarded. Values represent the mean ± SD. - represents absence of zone of inhibition.

### Effect of Ag-CNP Treatment on Survival of DL and Colo205 Cells

With a view to assess the effects of Ag-CNPs on survival, DL (tumor) and colo205 cells were incubated in medium alone or containing the indicated concentrations of Ag-CNPs for 24 h, followed by estimation of cell survival by MTT assay. Tumor cells (1 × 10^6 cells/ml) exposed to 10 µg/ml of Ag-CNPs showed approx. 47–50% survival, which decreased to 25% with 20 µg/ml (Fig. 8A). However, the increase of concentrations above 20 µg/ml did not show significant changes in percent survival (Fig. 8A), and the complete killing of cells did not occur even at 50 µg/ml of Ag-CNPs. Percent survival of colo205 cells following Ag-CNP treatment was more or less similar to those...
observed with the DL cells. Colo205 cells treated with 10 µg/ml Ag-CNPs showed approx. 62–65% survival, which decreased to 50% with 20 µg/ml (Fig. 9A). That the effects of Ag-CNPs are more pronounced on DL and colo205 cells became evident from the experiments done with thymocyte cells (normal cells). Results showed that the treatment of thymocyte (normal cells) with 10 µg/ml of Ag-CNPs resulted in approx. 10% loss of survival as compared with tumor or colo205 cells where the percentage of killing was more than 40–50% at this particular concentration (Figs. 8B and 9B). Once it became evident that Ag-CNPs indeed induce death of tumor and colo205 cells, our interest aroused to compare its potential with a standard drug, cisplatin. It is evident from the results that cisplatin treatment showed a relatively higher rate of percent survival in both the cell types as compared with Ag-CNPs (Figs. 8A and 9A). However, the percent survival of normal cells (thymocytes) was significantly affected with cisplatin treatment, the value being approx. 55% at 50 µg/ml (Figs. 8B and 9B).

Does Ag-CNP Treatment Induce ROS Generation?

The DCFH-DA method measures intracellular generation of hydrogen peroxide, hydroxyl, and peroxy radicals and therefore could be used as a quantitative method for oxidative stress assessment. In the present study, the intracellular ROS concentration (as evident from fluorescence) was significantly higher in Ag-CNPs-treated tumor and...
Ag-CNP Treatment Induces Apoptosis in DL and Colo205 Cells.

Knowing that survival of DL (tumor) and colo205 cells is severely affected by the treatment of Ag-CNPs, our interest aroused to test the induction of apoptosis and its possible association with cell survival. To achieve this objective, we employed Wright-Giemsa staining and DNA fragmentation test, which are widely and routinely used to assess the events of the apoptotic process. Observations made with Wright-Giemsa staining clearly showed that Ag-CNPs indeed induce apoptosis both in DL (Figs. 8F–8H) and colo205 cells (Figs. 9F–9H). This was evident from the fact that the cells treated with Ag-CNPs exhibited cell shrinkage, nuclear condensation, and cell membrane blebbing (Figs. 8–9G). In fact, cisplatin treatment also elicited the above-mentioned changes in cells, but the percentage was significantly lower than those observed with Ag-CNPs (Figs. 8–9G). DNA fragmentation, another hallmark of apoptosis, was evaluated in Ag-CNP-treated DL (Fig. 8I) and colo205 cells (Fig. 9I) in a dose-dependent manner. The percentage of DNA fragmentation increased significantly with increasing Ag-CNP concentration (Figs. 8–9I).

colo205 cells (10 and 30 µg/ml, respectively) in comparison with the cisplatin (40 µg/ml) treated or untreated cells (Figs. 8C–8E and 9C–9E). ROS measurement was done with a view to test the potential role of oxidative stress, if any, as a possible mechanism of Ag-CNPs-induced toxicity.
Discussion

Several researchers have reported the synthesis of metal nanoparticles through the biological route [23, 28, 34]. However, very few reports are available dealing with the use of cell extracts of bacteria and cyanobacteria in the synthesis process [18, 32, 34]. Our data clearly demonstrate that the cell extract of the cyanobacterium Anabaena doliolum efficiently reacts with AgNO₃ to form Ag-CNPs. This was evident from the change of color of the reaction mixture from reddish-blue to brown and the appearance of a broad absorption peak at 420 nm, which is characteristic of AgNPs [24, 32, 43]. The increase in peak size (absorbance) with increasing time of incubation clearly indicates a gradual increase in production of Ag-CNPs [32]. However, the spectra showed increases of ODs in the entire scan range, implying the purity of synthesized Ag-CNPs is ambiguous and needs further study. Similar to our results, a few other researchers also reported a sharp peak between 420 and 430 nm specific for AgNPs synthesized through a biological route. As such, UV-vis absorption spectra are considered to be quite sensitive for testing the formation of various metal nanoparticles, including AgNPs, and thus could be used as one of the simplest confirmatory tools. As there was marked change in the color of the reaction mixture within 1 h of incubation, we suggest that the process of nanoparticle synthesis is rapid and therefore makes cell extract a potential agent for metal nanoparticle synthesis.

The exact mechanism by which biomolecules present in the cell extract are involved in the synthesis of nanoparticles is poorly understood [18, 25, 32]. Tentatively, the role of biomolecules such as proteins, enzymes, amino acids, carbohydrates, and vitamins present in the cell extract has been implicated in the reduction of Ag⁺ ions [18, 34, 42]. In the case of cyanobacteria (NO₃-grown cultures), the role of nitrate reductase enzyme in the reduction of silver ion has been proposed [1]. However, the role of nitrate reductase in Ag⁺ ions reduction does not apply in this study, as A. doliolum was grown diazotrophically. Most probably, the vast array of metabolites and photosynthetic pigments such as phycobiliproteins, carotenoids, UV-absorbing compound, and mycosporine-like amino acids present in the cell extract of A. doliolum may be involved in Ag⁺ reduction and Ag-CNPs synthesis.

Works done by several researchers suggest that the physicochemical characteristics of the nanoparticles such as shape, size, solubility, and surface charge play key roles in determining their biological responses [20, 23]. Keeping in view the above facts, the size and morphology of Ag-CNPs were characterized by TEM, which revealed the formation of well-dispersed spherical-shaped nanoparticles with size distribution in the range of 10–50 nm. It is pertinent to mention that production of AgNPs of smaller size is desirable as they show pronounced bactericidal effects, possibly due to the availability of a large surface area for interaction [4]. It has been reported that small-sized nanoparticles undergo rapid and efficient urinary excretion and elimination and thus are ideal for intravenous administration with a lower risk of toxicity [4].

The crystalline nature of Ag-CNPs was evident from TEM-SAED and XRD analyses. Available reports suggest that the small size and crystalline structure of the nanoparticles govern their antimicrobial potential and is favored by 111 facets [19, 35]. It has also been reported that the presence of active biomolecules such as amino acids and small secondary metabolites stabilizes the Ag-CNPs for a long duration by avoiding aggregation and growth of nanoparticles [42]. The longer stability of Ag-CNPs could be due to the presence of strong reducing agents in the cell extract of A. doliolum (data not shown). Additionally, the presence of a negative zeta potential value (~25.52 mV) also suggests the role of active biomolecules in augmenting the stability of the Ag-CNPs for a longer period.

Functional group analysis of the Ag-CNPs by FTIR indicated the involvement of hydroxyl, carboxyl, and carbonyl groups of proteins and amino acids in the synthesis and stabilization of nanoparticles. This presumption is based on the fact that the peaks corresponding to the above functional groups were present both in the cell extract and Ag-CNPs, but major changes in stretching frequencies were noted after the synthesis of Ag-CNPs. The role of proteins, especially phycobiliproteins, in the synthesis and stabilization of metal nanoparticles has been reported earlier [18, 38]. As the cell extract of A. doliolum contains a large amount of phycobiliproteins, their role in Ag-CNPs synthesis seems convincing. In the FTIR analysis, a few bands were assigned to C-N stretching of amino acids belonging to the aromatic amino acid group. Most probably, the presence of tyrosine, phenylalanine, and tryptophan moieties in phycobiliproteins induces the formation of Ag-CNPs [18]. Results of FTIR analysis suggest the presence of phycobiliprotein as the major fraction in Ag-CNPs. Additionally, besides providing stability, capping with biomolecules may provide anchoring ability to nanoparticles on bacterial membranes, enabling them to attain antibacterial property.

Ag ions and Ag-based compounds have been used for
decades as antimicrobial agents, as they possess strong growth inhibitory effects against various microorganisms [10, 14, 21, 34]. The antimicrobial activity of biologically synthesized AgNPs has been reported by a few researchers [5, 10, 23, 24, 30] but no report exists on its effect on multidrug-resistant bacteria. As the widespread use of various synthetic antimicrobial agents has resulted in multidrug resistance in a number of bacteria [39], the search for developing an alternative strategy to fight the menace of multidrug resistance is urgently required. To this effect, the findings of the present study seem notable, as the Ag-CNPs synthesized showed strong antibacterial activity against three multidrug-resistant bacteria, E. coli, K. pneumoniae, and S. aureus, at a very low concentration (5 µg/disc). The size of zone of inhibition formation is not critical, as no correlation between increasing concentrations of Ag-CNPs and diameter of zone of inhibition formation was noted. This may be due to the limited diffusion of Ag-CNPs in the solid agar plate. The antibacterial activity of Ag-CNPs is encouraging as the antibiotics ampicillin and amikacin and several others (data not given) were ineffective against E. coli and K. pneumoniae, even at a very high concentration (500 µg/disc). Some differences in sensitivity among test bacteria against Ag-CNPs might be due to the differences in the architecture of the cell wall, as they belong to different genera, including one from the gram-positive group. Gram-positive bacteria have a much thicker outer membrane, the peptidoglycan layer, compared with gram-negative bacteria and this could be the reason for the mild effect of Ag-CNPs on S. aureus. Kim et al. [10] have also reported a poor effect of AgNPs on S. aureus. To our knowledge, this is the first report wherein the inhibitory effect of Ag-CNPs on multidrug-resistant bacteria of diabetic foot ulcers is reported.

The mechanism of action of AgNPs as an antibacterial agent is not fully understood. It has been reported that silver nanoparticles cause formation of perforations/pits in the bacterial cell wall, which change the membrane permeability, leading to the release of vital membrane proteins and lipopolysaccharide molecules [34, 37]. Other reports suggest that the damages to the cell may be caused by the interaction of AgNPs with phosphorus- and sulfur-containing compounds such as DNA and proteins [34, 40]. Additionally, the suppression of respiratory chain enzymes of bacteria by AgNPs has also been reported [30]. In this study, we have demonstrated the antimicrobial property of Ag-CNPs, but the mechanism of its action is not known to us. Most probably, the Ag-CNPs-mediated killing of bacteria is due to the formation of pores/pits in the cell wall or its interaction with vital cellular constituents such as DNA and protein, which in turn lead to the arrest of metabolic activities. Our conclusion is a tentative one, and further study is needed to reveal the exact mechanism(s) of action of Ag-CNPs to confirm its role as an antimicrobial agent. In spite of the poor knowledge of the mode of action, the antibacterial properties of AgNPs have attracted many researchers to exploiting them as antibacterial agents against a wide range of microorganisms [34]. Duran et al. [5] reported the use of Ag-CNPs in hospitals, where these are attached to dressing cloth to minimize infections of multidrug-resistant Staphylococcus aureus. Reports are also available where picomolar concentrations of AgNPs were used as nanoprobes in membrane penetration without producing any significant toxicity to the cells [12, 45]. All these reports suggest that Ag-CNPs synthesized by the cell extract of A. dolichom could be used as a potential antibacterial agent, but it would be necessary to evaluate their effects on a large number of bacteria comprising different groups.

One of the interesting findings other than the antibacterial activity of Ag-CNPs relates to their inhibitory effect on the survival of DL (tumor cells) and human colo205 cancer cells. Similar to our findings, the antitumor activity of AgNPs in Dalton’s lymphoma ascites (DLA) tumor has been reported by Srim et al. [40]. They reported that AgNPs elicit dose-dependent cytotoxic effects in DLA cells through activation of the capsase-3 enzyme and induction of apoptosis. Additionally, biologically synthesized green AgNPs from different sources are known to exhibit anticancer activity [9, 26, 31]. In the present study, a drastic decline in survival of DL (tumor) cells was noted after treatment with Ag-CNPs. About 75% loss of viability was noticed at a concentration of 20 µg/ml in vitro condition in MTT assay. Likewise, survival of human colo205 cells was reduced to 50% with 30 µg/ml of Ag-CNPs. These observations suggest that lower concentrations of Ag-CNPs may be safely used in arresting growth of DL and colo205 cells. It is also interesting to note that the inhibitory effect of Ag-CNPs on the viability of tumor/cancer cells seems specific, as the survival of normal thymocytes was least affected with the concentrations of Ag-CNPs used for testing the viability of tumor/cancer cells. Additionally, findings of the present study suggest that Ag-CNPs have more potential as a biocidal agent, as the survival of DL and colo205 cells with the treatment of standard antitumor drug cisplatin was much higher at similar concentrations of Ag-CNPs used in the assay.

Certain reports suggest that the inhibitory effects of Ag-
CNP s on tumor survival are associated with an augmented induction of apoptosis [26]. The role of reactive oxygen species has also been implicated in Ag-CNPs-induced apoptosis in malignant cells [9]. In this study, several parameters such as generation of ROS, DNA fragmentation, and evaluation of apoptotic cells were included to ascertain the possible mechanism of action of Ag-CNPs on different cell lines. Results showed a marked increase in ROS generation, particularly hydrogen peroxide, hydroxyl, and peroxyl radicals, following Ag-CNPs treatment (30 and 10 µg/ml) in human colo205 and DL cells, respectively, as compared with normal cells (thymocytes). This indicates that Ag-CNPs induce cell death probably through a ROS-mediated apoptotic process, as increased ROS causes extreme cellular oxidative stress. Although we have not detected the generation of ROS radicals, if any, other than those measured by the DCFH-DA method, the role of other ROS radicals in the apoptotic process may not be ruled out. Similar to our results, the preferential ability of biogenically produced nanoparticles to kill HL60 and osteoblast cancer cells as compared with normal peripheral blood mononuclear cells has been reported [31]. AshaRani et al. [2] also observed that the toxicity of AgNPs was more prominent in the cancer cells than in normal human lung fibroblast cells (IMR-90). They suggested that disruption of the mitochondrial respiratory chain by AgNPs leads to the production of ROS and interruption of ATP synthesis, which in turn cause DNA damage [2]. It has been reported that ROS somehow mediates activation of p53, which in turn promotes apoptosis by regulating the expression level of cell survival regulatory molecules.

Findings of this study clearly suggest that Ag-CNP treatment inhibits growth of tumor and cancer cells and induces apoptosis. That the event of apoptosis does take place was noted by the Wright-Giemsa staining, where apoptotic cells were observed in bulk after Ag-CNPs treatment in DL (tumor) and human colo205 cells. In fact, Ag-CNP treatment drastically increased the number of cells undergoing apoptosis, which included contracted cell bodies; condensed, uniformly circumscribed and densely stained chromatin; and membrane-bound apoptotic bodies containing one or more nuclear fragments. It is pertinent to mention that cisplatin treatment also caused apoptosis, but the effect was mild compared with Ag-CNPs. In addition to the morphological features of cells undergoing apoptosis, DNA fragmentation with Ag-CNPs also favors the event of apoptosis. In the present study, approximately 50% DNA fragmentation was observed with Ag-CNPs at 30 and 40 µg/ml in DL (tumor) and colo205 cells, respectively, and the percentage of fragmentation increased with increasing concentrations, due to the increased ROS production. Similar to our results, DNA fragmentation with increasing concentrations of certain other anticancer drugs such as dichloroacetate, cisplatin, and fluorouracil has also been reported [6, 41, 44]. Based on previous reports and results of this study, it may be concluded that DNA fragmentation is most probably due to the activation of intracellular caspase enzyme and oxidative stress caused by Ag-CNPs treatment in the cells [6, 41]. However, further work is needed to decipher the mechanism of DNA fragmentation caused by the Ag-CNPs treatment. This is the shortcoming of this study, but we have provided sufficient evidence showing the biocidal effect of Ag-CNPs more specifically on tumor/cancerous cells. As AgNPs are known to elicit varied effects in humans, the important one being platelet aggregation [36], detailed studies involving several types of cell lines are needed to ascertain the site and exact mechanism(s) of action of Ag-CNPs.

In conclusion, several researchers have reported the biological synthesis of metal nanoparticles, but those studies have focused on the use of cell masses of different microorganisms for the synthesis. This study is the first of its kind that demonstrates the potential of cell extracts of the N2-fixing cyanobacterium A. dolium in the synthesis of Ag-CNPs. Additionally, the strong antibacterial and antitumor activities are novel properties of biologically synthesized Ag-CNPs. Results showing drastic loss of survival of DL (tumor) and human colo205 cancer cells with the treatment of Ag-CNPs suggest its possible application in the management of tumor/cancerous cells. We are currently focusing our studies to understand the exact mechanism(s) of Ag-CNPs synthesis and its mode of action on different cell lines. Attempts are also under way to synthesize pure and well-dispersed Ag-CNPs by manipulating the conditions of synthesis or by using the active ingredient of cell extract alone.

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References