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Biogenic synthesis of silver nanoparticles using *Paenibacillus* sp. *in-vitro* and their antibacterial, anticancer activity assessment against human colon tumour cell line

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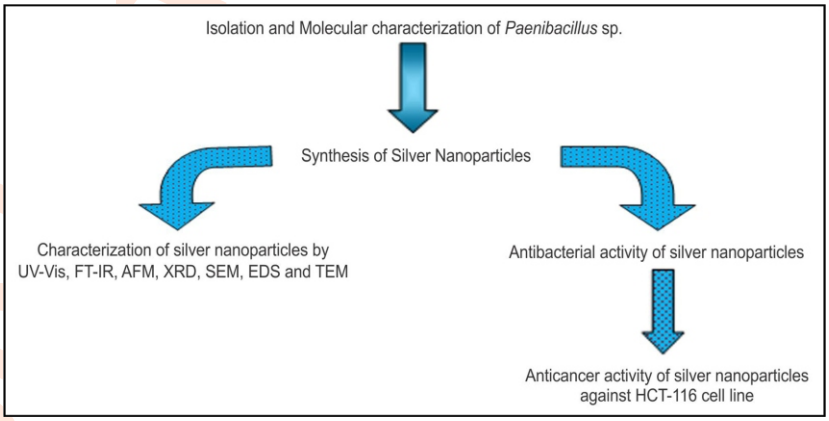
Abstract

Aim: To evaluate the antibacterial and anticancer activities of silver nanoparticles (AgNPs) synthesized from aqueous extract of *Paenibacillus* sp. strain NS-36.

Methodology: The green synthesized AgNPs were characterized by UV-Vis. spectroscopy, Fourier Transform Infrared Spectroscopy, Atomic Force Microscopy, X-Ray Diffraction, Scanning Electron Microscopy, Energy Dispersive X-ray Spectroscopy, and Transmission Electron Microscopy. Antibacterial activity was assessed against pathogenic bacteria by using agar well diffusion method and the anticancer activity was evaluated against Human Colon Tumour-116 cell line using 96 well plate cell proliferation assay.



Results: The synthesized AgNPs showed UV-Vis absorbance peak at 416 nm. The characterization analyses revealed the shape as spherical and size ranging from 17.49 to 52.85 nm and the presence of different functional groups and elements that involved in the stabilization and capping. The antibacterial activity revealed that AgNPs have good inhibitory action on pathogens, whereas the results of anticancerous assessment indicated that AgNPs have a dose-dependent activity on the Human Colon Tumour-116 cancer cells and the IC_{50} value was found to be 81.45 µg ml^{-1}.

Interpretation: The AgNPs exhibited considerable antibacterial activity against bacterial pathogens by rupturing and damaging the cell membrane. The AgNPs release silver ions into the cell once they attached to the cell membrane and disrupt the bacterial DNA replication. The AgNPs are toxic to tumour cells and induce intracellular reactive oxygen species which damage cells. Along with ROS, the rounding and shrinkage of tumour cells caused by AgNPs reduced the % viability of cancer cells.

Key words: Antimicrobial activity, Cytotoxicity, *Paenibacillus* sp, Silver nanoparticles, Tumour cells

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Introduction

Nanoparticles exhibit completely new or improved properties due to their small size and large surface to volume ratio. Nanoparticles can be synthesized from various metals, such as gold, silver, copper, magnesium, zinc, titanium, chromium, etc. (Nayak *et al.,* 2020). However, silver nanoparticles (AgNPs) are of particular interest due to their specific properties and wide applications in different fields, such as bio-sensing, imaging, drug delivery, medicine, etc. (Ahmad *et al.,* 2007; Ali *et al.,* 2018). There are different methods for synthesizing nanoparticles, like chemical, physical and biological methods. The physical and chemical methods used for the synthesis of AgNPs are marked as harmful to the environment as they release a large amount of toxic and hazardous by-products (Singh *et al.,* 2018). Among them, the biological or green synthesis method of nanotechnology is considered to be the easier, comparatively inexpensive and environmentally friendly, however, the synthesis of AgNPs using biological entities, for instance, plant parts, bacteria, fungi, actinomycetes, cyanobacteria and algae are gaining interest (Mouxing *et al.,* 2006; Ahmed and Mustafa, 2019).

Microorganisms produce various enzymes that are industrially important and the culture supernatant contains reductase enzyme responsible for the synthesis of nanoparticles. Among the microbial synthesis methodologies; bacteria have received great attention because of rapid growth, easy handling and genetic modification (Akter and Huq, 2020). Some inorganic materials are produced extracellular or intracellular by bacteria and in some silver resistant bacteria, ionic silver can accumulate on the cell wall as much as 25% in case of dry weight biomass. The extracellular method of nanoparticle synthesis using various microorganisms is considered as an easy method, although large-scale production of AgNPs requires optimum temperature, pH, incubation period and exact amount of oxidant and reductant (Deepika *et al.,* 2013; Ameen *et al.,* 2019).

Some studies have indicated that the AgNPs can be synthesized by using the culture supernatants of bacteria, especially the mesophiles and there is a need to extend the search for microbes, such as thermophiles and psychrophiles from different environments. In the external environment of a cell, the precipitation of nanoparticles occurs due to metabolic activities of microorganisms (Sadowski *et al.,* 2008; Shivaji *et al.,* 2011; Ameen *et al.,* 2019). Soil is a good source of microbial diversity and these microbes can be used for human welfare. Soil bacteria also need inexpensive and less nutrient media for growth that makes them a perfect candidate for utilization (Amdadul, 2020). Green nanotechnology-based AgNPs are characterized by low toxicity to humans and high bactericidal potential.

The AgNPs have a high surface area to volume ratio that makes them an important antimicrobial agent against a wide range of microorganisms, including multidrug-resistant bacterial pathogens (Hamouda *et al.,* 2019). The unique physico-chemical properties and biofunctional features, such as antibacterial, antifungal, antiviral, antiplatelet, anti-angiogenesis, and anti-inflammatory activities of AgNPs play an important role in development and implementation of novel biomedical strategies (Roy *et al.,* 2019). Recently, the AgNPs have gained much importance as promising anticancer drugs by triggering apoptotic mechanisms against various human cancer cell lines (Burdusel *et al.,* 2018; Ahmed *et al.,* 2019). Cancer is considered as one of the leading causes of mortality due to the fact that it causes one out of six deaths worldwide annually (Bray *et al.,* 2018). In recent years, nanotechnology-based cancer diagnosis and therapy methods are gaining attraction in the medical field and has unfolded a newer horizon of interdisciplinary areas like, chemistry, medicine, engineering and biology. AgNPs can induce cytotoxicity in tumour cells by altering the cell morphology, inducing oxidative stress, and reducing cell viability in various kinds of cancers (Bhatnagar *et al.,* 2019; Gnanakani *et al.,* 2019; Ratan *et al.,* 2020). Nanomedicine is a promising area that can induce changes affecting cancer treatment protocols, and the green synthesized AgNPs are effective anticancer mediators used for detection, prevention and bioremediation of tumour cells. AgNPs are intimately investigated for their promising anticancer property exhibited against different human cancer cell lines, like IMR-90, MCF-7, U251, MDA-MB-231, HT-29, HeLa, A-549 and HepG2 tumour cells (Burdusel *et al.,* 2018; Burlacu *et al.,* 2019; Nayaka *et al.,* 2020; Rajoka *et al.,* 2020).

Paenibacillus sp. strain NS-36 is a Gram-positive, aerobic, endospore-forming, rod-shaped and motile bacterium with 51% Guanine+Cytosine content. It belongs to family *Paenibacillaceae* and class *Bacilli.* The bacterium is a mesophile and can grow in the temperature range of 10 to 37 °C with pH ranging from 5.0 to 9.0. The members of genus *Paenibacillus* can be detected in rhizosphere soil and vegetable matters (Piuri *et al.,* 1998; Girardin *et al.,* 2002; Von-der *et al.,* 2003). The antimicrobial activities of some *Paenibacillus* sp. have been reported and the results have proved that the species of genus *Paenibacillus* possess good antimicrobial activities against many pathogenic Gram-positive and Gram-negative bacteria, including some fungi (Aw *et al.,* 2016; Huang *et al.,* 2017; Pajor *et al.,* 2020; Zhang *et al.,* 2020). Keeping in view the in-vitro antimicrobial studies of different species of *Paenibacillus*, the present investigation aimed to study the antimicrobial activity and potential anticancer activity of synthesized AgNPs from the isolated bacterium *Paenibacillus* sp. strain NS-36.

Materials and Methods

Isolation of bacterial strains: In the present study, the soil samples were collected from in and around Karnatak University Campus, Dharwad, Karnataka, India, during February 2019. The samples were serial diluted up to 10^{-5} dilution and inoculated on Nutrient agar medium. The culture plates were incubated at 35 °C for 48 hr and a total of 38 aerobic bacterial strains were isolated (Holt *et al.,* 1994). From these 38 isolated strains, a single bacterium was selected based on its unique morphological

growth features, like colonial pattern forming and the selected bacterium was designated as strain NS-36.

Genotypic characterization of isolate: The genomic DNA was extracted from fresh culture of NS-36 strain using Hi-PurA bacterial DNA purification kit according to manufacturer's instructions. The 16S rRNA gene was PCR amplified using two universal primers, bacterial-domain-specific primer 8F and prokaryote-specific primer 1490R (Sekiguchi *et al.,* 1998). Purified PCR amplicons were sequenced (Sanger Sequencing 3500 Series, Genetic Analyzer) and deposited to the NCBI database via nucleotide BLAST web portal. The DNA sequences were selected based on similarity to construct a phylogenetic tree as per the standard procedure by using the MEGA 7.0 software (Tamura *et al.,* 2015).

Preparation of cell-free extract: The NS-36 strain was inoculated into sterile nutrient broth in a 250 ml Erlenmeyer flask. The culture flasks were incubated on a rotating shaker set at a speed of 200 rpm for 48 hr at room temperature. After incubation, the broth containing strain NS-36 culture was centrifuged at 12,000 rpm (REMI R-24) for 10 min to obtain cell-free supernatant.

Biosynthesis of AgNPs: The cell-free supernatant of NS-36 strain (80 ml) was mixed with an aqueous solution of 0.1 mM silver nitrate. The pH was adjusted to 8.5 and the mixture was incubated in dark for 5 days. Reduction of silver ion was examined by change in colour of the solution at different time intervals.

Characterizations of AgNPs

UV-visible spectroscopy: The test solution was taken in a cuvette (quartz) and analyzed for optical density from 300 to 700 nm wavelength using an UV-Vis. spectrophotometer (Double beam spectrophotometer, METASH UV-9000A).

Fourier Transform Infrared Spectroscopy: FT-IR spectroscopy was performed using FT-IR spectrophotometer (Nicolet 6700, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The pellets were prepared by mixing AgNPs dry powder with KBr and scanned in the range of 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} in transmittance mode.

X-ray Diffraction: The particle size and crystal structure of synthesized nanoparticles was studied by XRD (Rigaku, Smartlab SE) instrument (CuKα radiation, λ = 0.15418 nm) running at 40 kV and 30 mA. The sample was prepared by making a smooth surface of AgNPs powder on the sample holder and diffracted intensities were recorded from 30.010 to 79.990 of 2θ angles. The crystalline size was calculated from FWHM of diffraction peaks of XRD pattern with the help of Debye-Scherrer equation.

Atomic Force Microscopy: Morphology and size of AgNPs were ascertained by atomic force microscopy (Nano Surf Flex AFM). The sample was prepared by spin coating the AgNPs solution onto the glass slide and the slide was dried at room temperature and subjected to AFM analysis.

Scanning Electron Microscopy and Energy Dispersive X-ray analysis: The surface images and concentration of metal ions were obtained using Energy dispersive X-ray coupled with scanning electron microscope (JSM-IT 500). The chemical state and composition of the elements present in the AgNPs were examined using gold-coated AgNPs onto the sample holder.

Transmission Electron Microscopy: The surface morphology and size of AgNPs were analysed by TEM (Hitachi, Model: S-3400N) at an accelerating voltage of 80 kV. The particle size and surface morphology of nanoparticles were evaluated using Image J 1.45s software.

Antibacterial assay: The antagonistic activity of biosynthesized AgNPs was tested against four pathogenic bacteria, namely *Staphylococcus aureus* (MTCC6908), *Enterococcus faecalis* (MTCC6845), *Streptococcus pneumoniae* (MTCC2672) and *Escherichia coli* (MTCC40) by agar well diffusion technique, in which 6 mm wells were cut in nutrient agar medium by swabbing the pathogens uniformly in each plate using cotton swabs. The working solution was prepared by making a suspension of 1 mg AgNPs in 1 ml distilled water. Later, 25 µl, 50 µl, 75 µl and 100 µl of AgNPs suspension was pipetted into each well and incubated at 37 °C overnight. After incubation period, the diameter of inhibition zone around each well was measured and expressed in millimeters.

Anticancer activity: MTT assay is used for the determination of cytotoxicity and cell proliferation, based on the reduction of yellow-coloured water-soluble tetrazolium dye MTT to formazan crystals. Human Colon Tumor (HCT-116) cells were procured from NCCS, Pune and subcultured and incubated at 37 °C in 5% CO_2 incubator before analysis. The cells were plated in 96 well plates (Thermoscientific Nunc, Cat.No.167008) at a required cell density of 20,000 cells per well without the test agent. Appropriate concentrations (12.5, 25, 50, 100 and 200 µg ml^{-1}) of AgNPs suspension in DMSO was added in each well, excluding the wells with 15 µM Camptothecin drug as positive control and the well with only medium and cells as negative control. The cells were incubated for 24 hr, the MTT reagent to a final concentration of 0.5 mg ml^{-1} of total volume was also added. The plates were incubated for 24 hr at 37 °C in 5% CO_2 atmosphere and then the MTT reagent was removed and 100 µl of solubilisation solution (DMSO) was added. The number of viable cells was determined by recording the absorbance at 570 nm on ELISA reader at 630 nm wavelength.

Results and Discussion

Among 38 bacterial isolates, the strain NS-36 was selected and further used for the synthesis of AgNPs. The Erlenmeyer flask with bacterial cell-free supernatant was pale

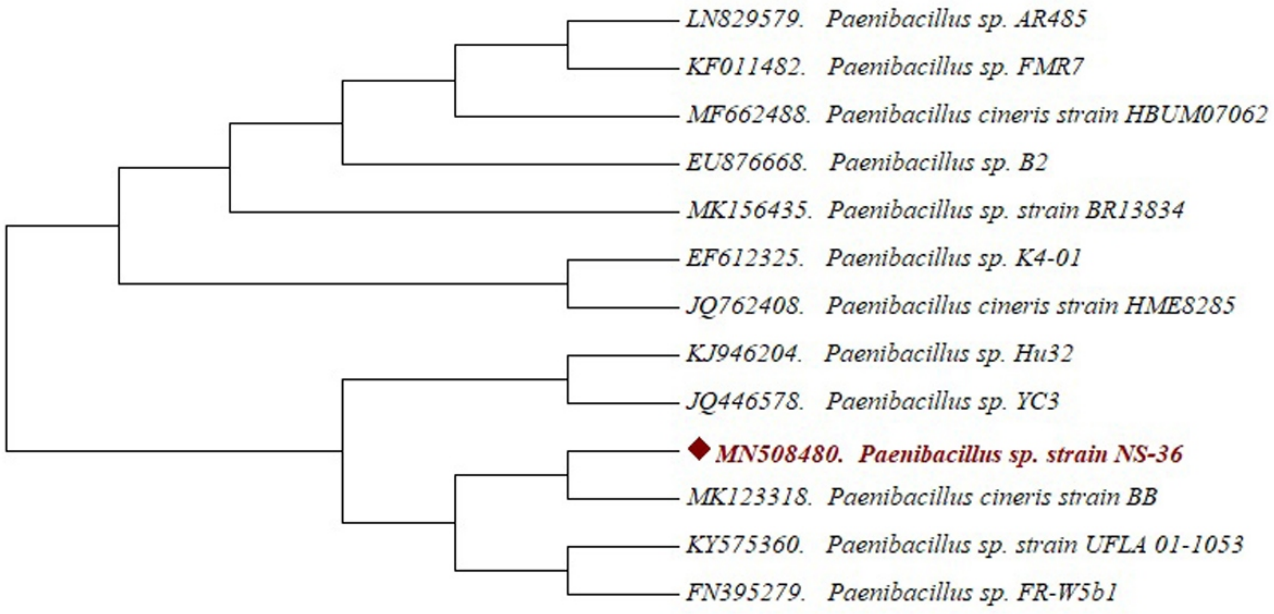

Fig. 1: Phylogenetic tree of the isolate *Paenibacillus* sp. NS-36.

yellow prior to silver nitrate addition, however, the colour changed from pale yellow to brown at the end of incubation period. The appearance of brown colour was a clear indication of the formation of AgNPs due to reduction of Ag^+ ions to Ag^0 (elemental state) by the reducing agents present in the cultural supernatant. Similar results about the colour change of the reaction mixture after addition of $AgNO_3$ followed by incubation in dark condition have been reported and also suggests that microorganism extract containing various secondary metabolites may act both as reducing and capping agent in AgNPs synthesis (Kalishwarlal *et al.,* 2010; Gnanakani *et al.,* 2019; Priya *et al.,* 2020).

The genomic DNA was extracted and 1554 base pairs of 16S rRNA gene sequence were submitted to the NCBI database (Accession Number: MN508480). The sequence was initially characterized by nucleotide BLAST analysis, which hit 100 homologous 16S rRNA genes from various species. Most homologies of sequence were found with 16S rRNA gene sequence of the strain NS-36 and it shared 98.36% similarity with *Paenibacillus cineris* strain BB (Accession Number: MK123318). A phylogenetic tree was constructed with a complete gene sequence and manifested in Fig. 1. Similar reports have been documented by Meenal *et al.* (2012) and Aw *et al.* (2016), where a marine and a soil bacteria were identified as *Idiomarina* sp. PR58-8 and *Paenibacillus tyrfis* MSt1T, based on nucleotide homology and phylogenetic tree analysis. The UV-Vis. absorption spectrum of AgNPs synthesized by the strain NS-36 (Fig. 2A) indicated a strong surface plasmon resonance (SPR) maximum at 416 nm as a characteristic peak for AgNPs. The increase in the intensity of SPR peak could be due to the increasing number of nanoparticles formed as a result of reduction of Ag^+ ions present in the aqueous extract. The surface plasmon band in the AgNPs aqueous solution remain between 410 to 430 nm throughout the reaction period, indicating the particles are dispersed with no evidence for aggregation (Ahmad *et al.,* 2007; Karthik *et al.,* 2012; Allam *et al.,* 2019). FT-IR spectrum showed bio-fabrication of AgNPs mediated by cell-free supernatant of NS-36 strain, when scanned from 4000 to 400 cm^{-1} (Fig. 2B). The intense bands at 3360, 2926 cm^{-1} were a characteristic group of C-H stretching alkenes, 2369 and 2342 cm^{-1} corresponded to P-H stretching phosphine and 1651 cm^{-1} was identified as C=C alkenes. The peak at 1568 cm^{-1} was identified as carboxylic acid and at 1416 cm^{-1} corresponded to C-C aromatic compounds.

The peak of 1304 cm^{-1} assigned to N-O aliphatic nitro compounds, whereas the peak of 1270 cm^{-1} and 1149 cm^{-1} were assigned to the stretching of C-O alkyl ether and P-H bending phosphine. The peak of 1108 cm^{-1} and 1033 cm^{-1} corresponded to C-N amines and S=O sulphoxide or carboxylic acid. The peak of 668 cm^{-1} and 650 cm^{-1} attributed to the stretching of C-Br and C-H bending alkenes. Secondary metabolites, such as terpenoids, flavonoids, alkaloids, phenolics, proteins, carbohydrates, lipids and peptides present in the supernatant are also involved in capping and stabilization of AgNPs (Srivastava *et al.,* 2012; Yurtluk *et al.,* 2018; Gnanakani *et al.,* 2019). The phase purity and composition of AgNPs and the crystalline nature were examined by XRD. The XRD spectrum (Fig. 2C) showed four distinct diffraction peaks at 31.87^0, 45.58^0, $64.62^0$and 77.51^0 corresponding lattice plane value was indexed at (111), (200), (220) and (311) planes of face-centred cubic silver with a lattice

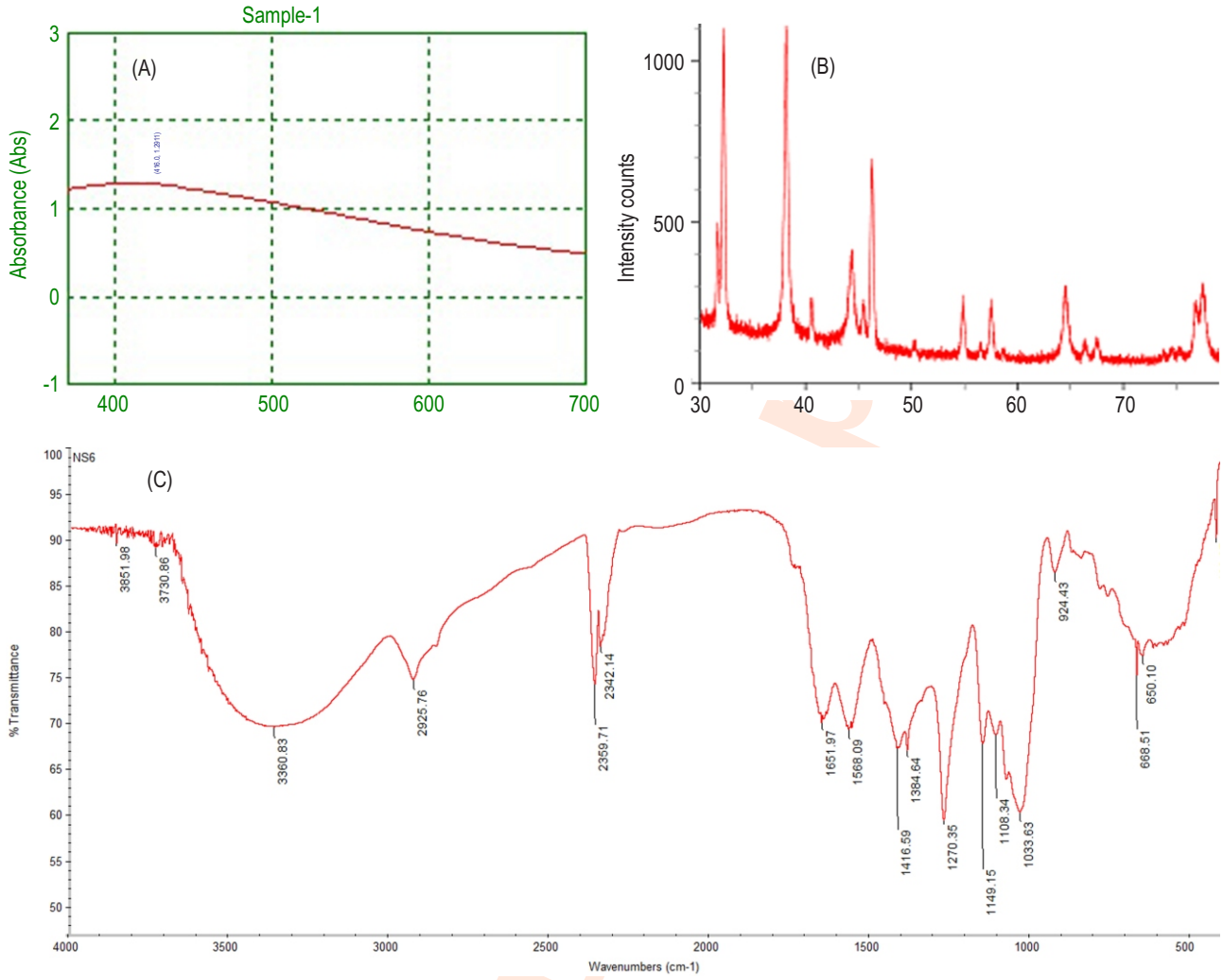

Fig. 2: Characterization of *Panibacillus* sp. NS-36 mediated AgNPs (A) UV-Vis spectra of NS-36 AgNPs; (B) XRD pattern of NS-36 AgNPs, and (C) FTIR spectrum of NS-36 AgNPs.

parameter of a=4.08 A^0. The average particle size estimated was approximately 27.48 nm. Similarly, Gurunathan *et al.* (2009) and Meenal *et al.* (2012) reported the size of AgNPs synthesized from bacterial extracts between 20 to 50 nm. In another report, the characteristic Bragg reflection peaks of Ag crystallites were observed at (111), (200), (220) and (311) with an average size of 49.5 nm (Hossain *et al.,* 2019).

A two-dimensional horizontal cross-section of AgNPs indicated that the nanoparticles were well defined, spherical and poly-dispersed. The three-dimensional image showed the surface morphology and homogeneity in the distribution of nanoparticles. The AFM data revealed that the size of AgNPs ranged from 17.49 nm to 52.85 nm (Fig. 3A and 3B) with a distance of 39.06 nm from each other (Fig. 3C). These observations can be correlated with previous studies, where synthesized AgNPs were spherical and their size ranged between 13 to 75 nm, poly-dispersed, and some of them were agglomerated (Saravanan *et al.,* 2018; Sowmya *et al.,* 2019). SEM images showed that most of the AgNPs were predominantly spherical having a smooth surface and well dispersed with a close compact arrangement (Fig. 4A). The EDX spectrum of synthesized nanoparticles suggested the presence of silver as the major ingredient element. AgNPs gave a strong signal peak at 3 keV and the quantitative information of biosynthesized AgNPs with the presence of various elements was reported (Fig. 4B). This corroborates the study of Nadhe *et al.* (2019), who identified the presence of AgNPs on the EDX spectrum, while their morphology was studied by SEM. In other reports, the SEM images showed polydispersive nature of AgNPs and the EDX spectrum showed the peak in the silver region at 3 keV, which is typical for metallic silver nanocrystals due to surface plasmon resonance (Nayaka *et al.,* 2020; Priya *et al.,* 2020). Transmission electron microscopic images of AgNPs synthesized from the NS-

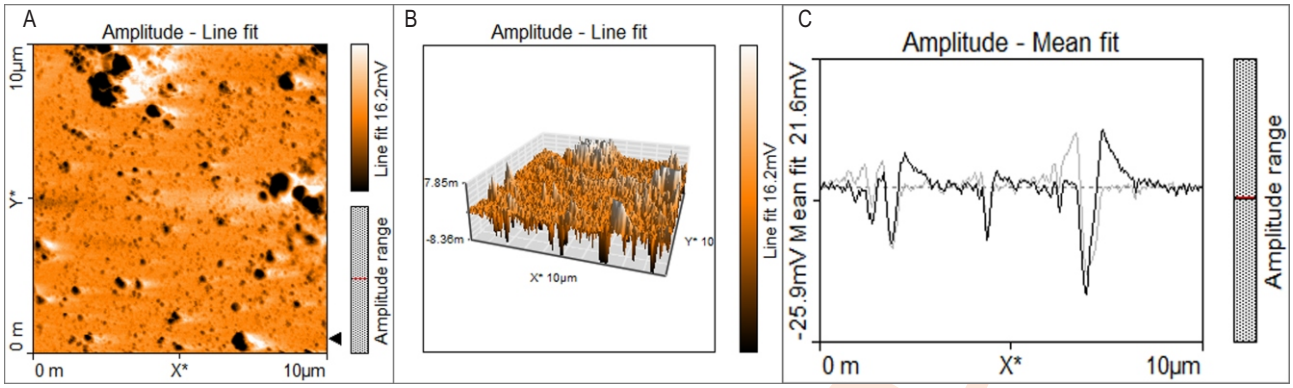

Fig. 3: AFM analysis of *Panibacillus* sp. NS-36 mediated AgNPs: (A) 2D structure of NS-36 AgNPs; (B) 3D structure of NS-36 AgNPs and (C) Particle size distribution of NS-36 AgNPs.

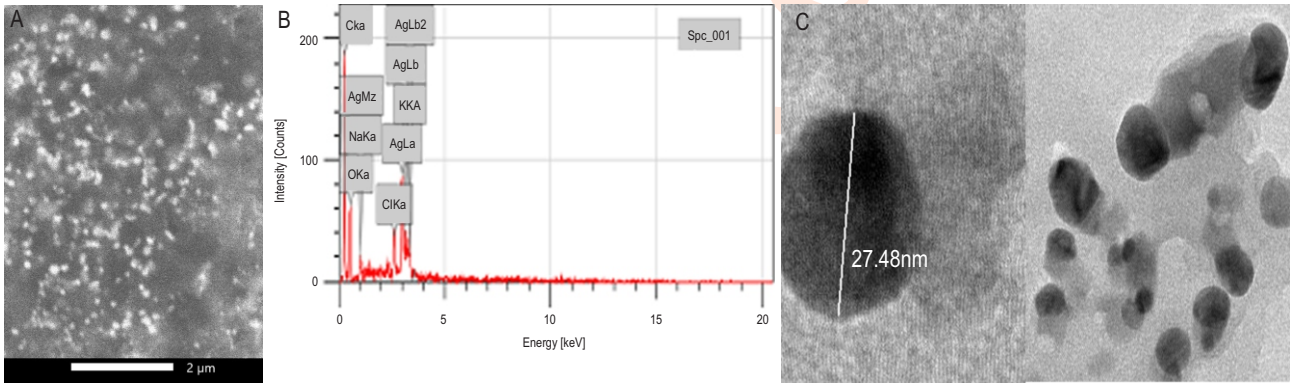

Fig. 4: Characterization of *Panibacillus* sp. NS-36 mediated AgNPs: (A) SEM analysis of NS-36 AgNPs; (B) EDX analysis of NS-36 AgNPs and (C) TEM micrograph of NS-36 AgNPs.

36 strain at various magnifications confirmed the shape of AgNPs as spherical, size ranging between 17.49 to 52.85 nm, with average size of 27.48 nm (Fig. 4C). In a similar study, Kalishwarlal *et al.* (2010) reported that the synthesized AgNPs were spherical, well dispersed and the size averaged at 50 nm with a uniform diameter. Recently, Dhanaraj *et al.* (2020) and Rajoka *et al.* (2020) reported that the synthesized AgNPs were spherical with size ranging from 10 to 100 nm with average size around 45 nm.

The synthesized AgNPs showed good antibacterial activity against the selected pathogenic bacteria (Fig. 5A-D). Among the bacterial pathogens, *E. faecalis* was found to be more sensitive to AgNPs synthesized from the isolate *Paenibacillus* sp. strain NS-36, while *S. pneumoniae* was found to be least sensitive and Fig. 5E representing histogram of inhibition zone. The AgNPs showed formation of moderate inhibition zones on *S. aureus* and *E. coli.* The size and high surface area of AgNPs play an important role by enabling them to reach easily the nuclear content of bacteria to exhibit relatively high antibacterial activity (Yurtluk *et al.,* 2018). Similar reports have been documented by Elsayed *et al.* (2018) and Sowmya *et al.* (2019) suggested that AgNPs exhibit antibacterial activity by releasing Ag^{+} ions, which gets attached to the surface of bacterial cell membrane and may disrupt membrane permeability, DNA replication, induce ROS and subsequently leading to bacterial cell death.

The morphological changes of Human colon adenocarcinoma HCT 116 cell line on adding different concentrations of AgNPs were recorded using an inverted phase-contrast tissue culture microscope (Fig. 6A-G). The results of negative and positive controls are depicted in Fig. 6A, B respectively. The changes in the morphology of cells, such as rounding or shrinking of cells at varying degree were visible in the images and it was a clear indication of cytotoxicity. In this study, the cytotoxicity was evaluated on HCT 116 cells *in-vitro* by MTT assay. About 50% of HCT 116 cells died when treated with AgNPs at concentrations between 12.5 to 200 µg ml^{-1} (Fig. 6C-G). The observations of cell cytotoxicity study by ELISA reader suggested

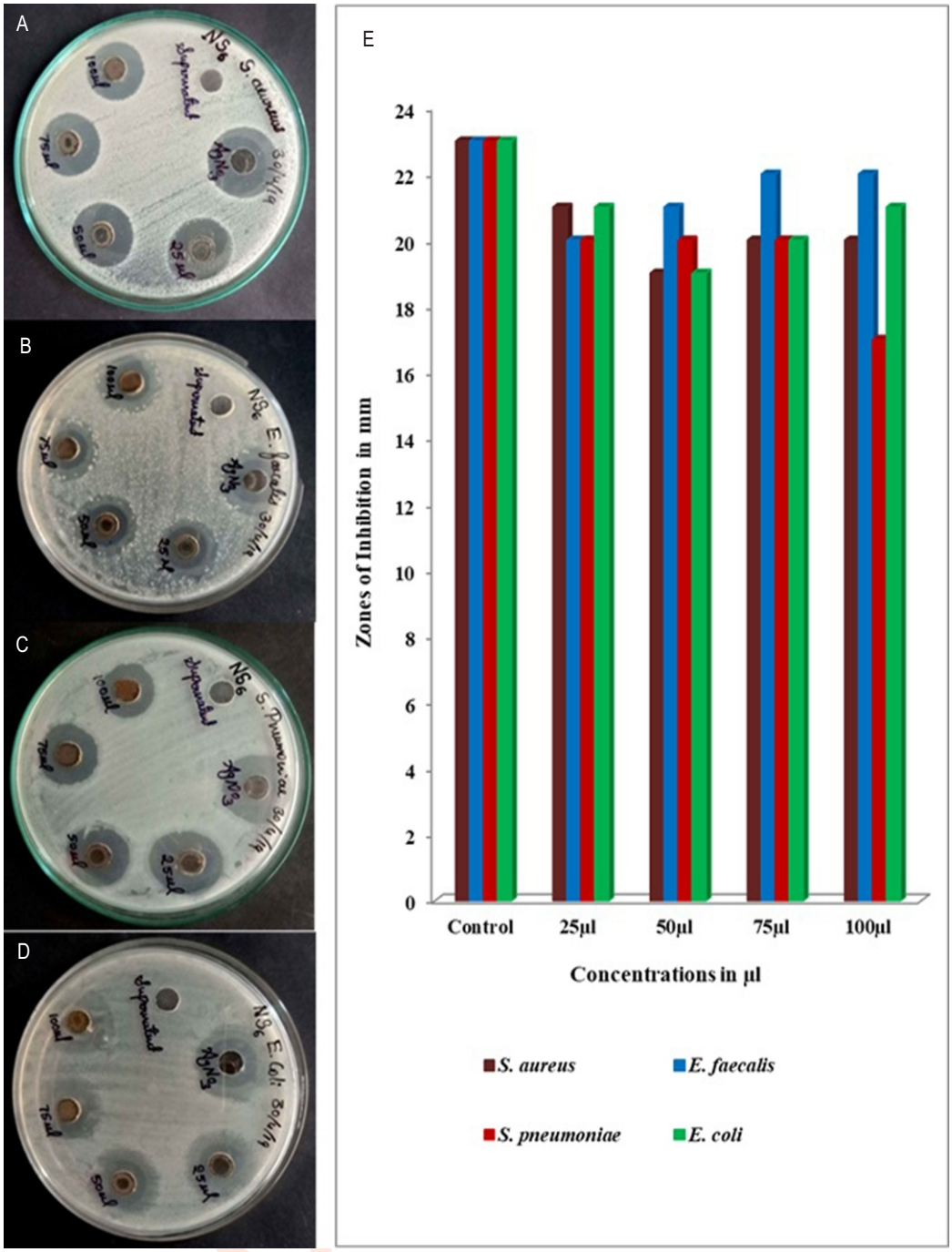

Fig. 5: Antibacterial activity of Panibacillus sp. NS-36 mediated AgNPs against bacterial pathogens: (A) *S. aureus*; (B) *E. faecalis*; (C) *S. pneumoniae*; (D) *E. coli* and (E) Histogram of zone of inhibition.

that AgNPs from *Paenibacillus* sp. strain NS-36 showed IC_{50} concentration at 81.45 µg ml^{-1} against HCT-116 cell line. The results suggests that the test compound may have good cytotoxic potential against HCT-116 cells, and the effect of AgNPs on the viability percentage of HCT-116 cell line showed a dose-dependent activity. Ramalingam *et al.* (2014) reported that very low concentration of AgNPs showed high activity *i.e.,* 80% inhibition on MCF-7 cells. At higher concentration (10-100 µg ml^{-1}), no significant difference in the prevention of cancer cell growth was observed with AgNPs and IC_{50} value for AgNPs > 10

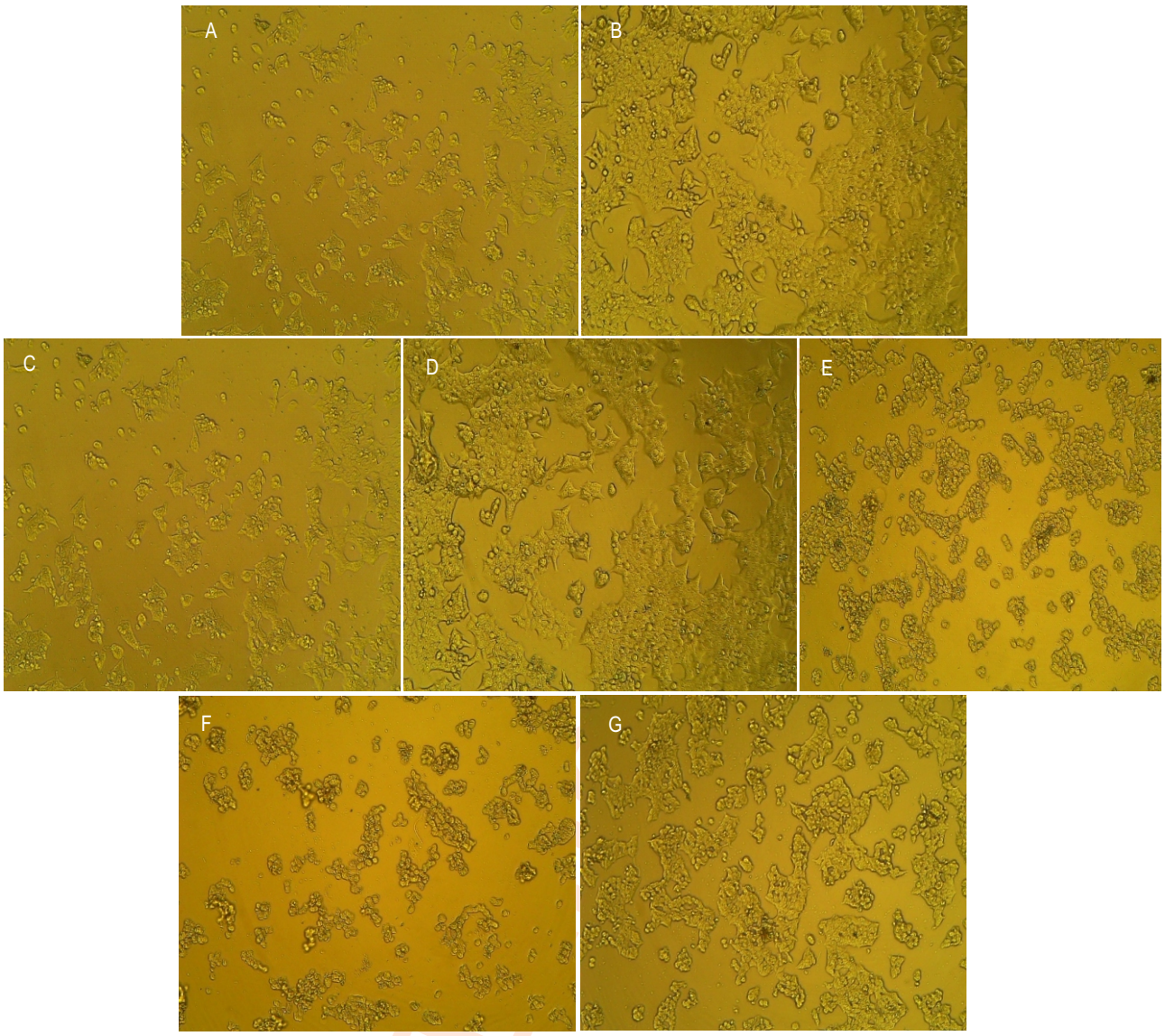

Fig. 6: Anticancer activity of *Panibacillus* sp. NS-36 mediated AgNPs at different concentrations against HCT-116 cell line: (A) Negative control (medium with cells); (B) Positive control (15μM Camptothecin); (C) 12.5 μg ml^{-1}, (D) 25 μg ml^{-1}, (E) 50 μg ml^{-1}, (F) 100 μg ml^{-1} and (G) 200 μg ml^{-1}.

μg ml^{-1}. Also, Buttacavoli *et al.* (2018) and Siddiqi *et al.* (2018) found that HT-3, HT-29, HCT-116, SKBR3, 8701-BC and Caco-2 cells were the most sensitive cancer cell lines when studied with the cytotoxic activity of AgNPs. Based on these findings, AgNPs may lead to valuable applications as antimicrobial agents in the medicinal field and it was also observed that anticancer activity of AgNPs induced intracellular ROS generation which caused cell damage. AgNPs are involved in the selective interruption of mitochondrial respiratory chain resulting in the production of ROS. These ROS species induce the expression of genes associated with DNA disruption and produces apoptosis of tumour cells (Bhatnagar *et al.,* 2019; Priya *et al.,* 2020; Ratan *et al.,* 2020). Overall, the present work demonstrates an eco-friendly and low-cost synthesis procedure for AgNPs using the isolate *Paenibacillus* sp. strain NS-36. The results of this study provide useful information for designing a much better antibacterial and anticancer compound in the near future using bacterial mediated AgNPs.

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Add-on Information

Authors' contribution: N. Sreenivasa: Designed the concept, supervised and wrote the manuscript; **B.P. Meghashyama:** Wrote the manuscript and carried out experimental work; **S.S. Pallavi, C. Bidhayak:** Carried out experiments and edited the manuscript; **A. Dattatraya, H. Halaswamy and S. B. Dhanyakumara:** Edited manuscript and validated the data; **R. Muthuraj and K.N. Shashiraj:** Edited manuscript and prepared figures/graphs; **M.D. Vaishnavi:** Carried out the laboratory work

Research content: The research contents is original and has not been published elsewhere

Ethical approval: Not Applicable.

Conflict of interest: The author declares that there is no conflict of interest.

Data from other sources: Not Applicable

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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