Research Paper

Biosynthesis, Characterization and Evaluation of Antimicrobial, Antioxidant and Antiproliferiative Activities of Biogenic Silver Nanoparticles Using *Streptomyces* KBR3

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Facile synthesis of silver nanoparticles was attempted using soil derived *Streptomyces* sp. KBR3 and its antimicrobial, antioxidant and antiproliferiative properties were evaluated *in vitro*. The extracellular synthesis of silver nanoparticles by the biomass of *Streptomyces* sp. KBR3 was confirmed through visual color change and ultraviolet-visible spectral analysis. Results of transmission electron microscopy, selected area electron diffraction and X-ray diffraction analysis showed the crystalline polydispersed spherical shaped nanoparticles with the size of 36 nm. Fourier-transform infrared spectroscopy analysis suggested the role of water soluble polyols present in the *Streptomyces* sp. KBR3 in mediating the synthesis of silver nanoparticles. The synthesized silver nanoparticles showed significant antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. In antioxidant studies, the silver nanoparticles showed maximum scavenging effects of 84 %±1.22 %, 95.8 %±1.32 % and 57.85 %±0.54 % at 1000 µg/ml in 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and metal chelating assay with the half-maximal inhibitory concentration value of 40 µg/ml, 15 µg/ml and 53 µg/ml, respectively. In antiproliferiative study on H357 oral cancer cell line, the silver nanoparticles showed 77.21 %±0.43 % reduction in cell viability at 250 µg/ml with the half-maximal inhibitory concentration values of 9.431 µg/ml. The present study showed that the silver nanoparticles synthesized using the *Streptomyces* sp. KBR3 might be a promising material for different biomedical applications.

Key words: *Streptomyces*, silver nanoparticles, antimicrobial, anti-oxidant, antiproliferiative activity

Threats due to microbial pathogens have been tremendously increasing now-a-days due to the emergence of antibiotic-resistant strains and declined immune compatibility of humans. Hence many pharmaceutical companies have lost their interest in developing new antibiotic compounds due to their narrow profit margin. Despite numerous novel antibiotics and therapeutic agents available in the market, 70 % of them are inactive in treating intracellular infections because of their reduced permeability[1]. In parallel, developing new or better antibiotics and non-antibiotic substances will have a great impact on global public health. To overcome these issues, there is a dire need to develop new class of therapeutic agents with better biocompatibility and efficiency[2].

Nanoparticles are now considered as viable antimicrobial agents and seem to have tremendous potential to solve the challenges of microbial multidrug resistance[3,4]. Nanotechnology provides a platform of excellence to modify and develop the properties of metals by converting them into their nanoform (nanoparticles), that has applications in the treatment of different diseases[5]. Natural biological sources, like *Streptomyces*, may be used to synthesize nanoparticles with various biological activities. Nanotechnology involves the synthesis

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of nanoparticles (i.e., materials not exceeding 100 nm in diameter) through physical, chemical and biological approaches and the integration of the resulting nanostructures into various applications. Nanomaterial's varying by size, distribution and particle morphology. Among them, Silver Nanoparticles (AgNPs) are the most commonly used, given their range of biological activities and good chemical stability[6,7].

Actinobacteria are gram-positive bacteria with high Guanine+Cytosine Deoxyribonucleic Acid (G+C DNA) content in their genome and it remains as one of the largest phylum in the bacterial domain[8,9]. Members of this phylum are capable of producing diffusible pigments which are considered important sources among the microorganisms for the development of secondary metabolites of industrial significance having specific applications[10,11]. Reports claim that more than 75 % of commonly available antibiotics were recovered from the *Actinobacteria* group of microorganisms[12]. In this study, AgNPs were synthesized using the biomass of *Streptomyces* sp. KBR3 and further evaluated for antimicrobial, antioxidant and antiproliferiative properties.

MATERIALS AND METHODS

Description and identification of Actinobacterial strain KBR3:

The Actinobacterial strain KBR3 used in this study was isolated from the rhizosphere soil collected from Udhagamandalam (Lat. 11º 48'N; Long. 76º 77'E), Nilgiris, Tamil Nadu, India by spread plate method using starch casein nitrate agar. Viability of the strain KBR3 was maintained in Yeast Extract Malt Extract (YEME) agar slants as well as in 20 % glycerol broth at 4° and -80°, respectively.

The presence of aerial and substrate mycelium was observed under bright field microscope. Cultural properties such as growth, colony consistency, colour of aerial mycelium, production of reverse side and soluble pigment were studied by growing the strain KBR3 on YEME agar medium for 7-14 d at 28°[13]. Total genomic DNA from the strain KBR3 was isolated using solute ready genomic DNA kit (Himedia- Hi-PurA™) according to the manufacturer's protocol. Further, 16S ribosomal RNA (16S rRNA) gene of the strain was amplified using primers: 27F-5′AGAGTTTGATCMTGGCTCAG3′ (forward)[14] and 1492R- 5′TACGGYTACCTTGTTACGACTT3′ (reverse)[15] and then sequenced at Eurofins Genomics, Bangalore, India. The search program Basic Local Alignment Search Tool (BLAST) was used to screen for close relatives and phylogenetic affiliations. MEGA 7 program was used to align the 16S rRNA gene sequence of strain KBR3 with close relatives and to construct the phylogenetic tree by following neighbour-joining algorithm[16]. The partial 16S rRNA nucleotide sequence of strain KBR3 was deposited to GenBank.

Biosynthesis of AgNPs:

Strain KBR3 was grown in conical flask containing sterile 25 ml YEME broth as seed culture and incubated at 28° for 48 h in a rotary shaker at 200 rpm. Later, 10 % of the inoculum was transferred into 100 ml of sterile YEME broth in 500 ml Erlenmeyer flask at 28° for 72 h in rotary shaker with 200 rpm. The mycelia biomass of the strain KBR3 was collected as pellet after washing twice with distilled water at 10 000 rpm by centrifugation. About 50 ml of Silver nitrate ($AgNO_3$) solution (1 mm) was added to the cell pellet and incubated at 28° in a rotary shaker at 200 rpm for 48 h in dark. The reaction flask was observed intermittently for any color change. Subsequently the reaction solution was centrifuged at 10 000 rpm for 10 min and pellet was dried in an hot air oven at 100°[17].

Characterisation of synthesized AgNPs:

The optical absorption of synthesized AgNPs was studied using Ultraviolet-Visible (UV-Vis) (UV-36000, Shimadzu, Japan) and Fourier-Transform Infrared Spectroscopy (FTIR) (FTIR; IR Affinity-1, Shimadzu Corp, Tokyo, Japan) spectral analysis. UV-Vis spectroscopy analysis was carried out over wavelengths from 200 to 800 nm at a resolution of 1 nm[18]. A FTIR spectrum was recorded using Potassium bromide (KBr) pellets in the range of 500-4000 cm^{-1}[19]. X-Ray Diffraction (XRD) analysis was performed to confirm the crystalline nature of green synthesized AgNPs. The XRD data were obtained using X'Pert Pro X-ray diffractometry (PAN analytical BV) operating at 40 kV voltage and 30 mA current with Cu K alpha radiation[18]. Morphology, size and electron diffraction pattern were examined by Scanning Electron Microscope (SEM) (SEM-JSM-7600F, Japan) and Transmission Electron Microscope ((TEM)-JEM-2100F, Japan) at a voltage of 200 kV, respectively[20]. Selected Area Electron Diffraction (SAED) patterns used graphical method to quantify distances and angles in digitalized patterns by clicking on the two shortest non-collinear vectors

(spots) using user calibration data[18]. Zeta potential analysis was also performed using Malvern Zeta Sizer to evaluate the surface load of nanoparticles[21].

Evaluation of biological activities of synthesized AgNPs:

Antibacterial activity: The antibacterial activity of biosynthesized AgNPs was tested using agar well diffusion bioassay against *Staphylococcus aureus* (*S. aureus*) ATCC 29213 and *Escherichia coli* (*E. coli*) ATCC 25922. 24 h old bacterial culture was spread uniformly over Nutrient Agar (NA) plates using sterile cotton swab. 10 mm diameter wells were made on bacteria swabbed NA plates using sterile cork borer. 200 µl of AgNPs (1 mg/ml) was added to each well. After 24 h of incubation at 37°, the zone of inhibition was measured and expressed as diameter in millimetre[22].

Antioxidant activity of AgNPs:

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity: A solution of DPPH (0.1 mM) was prepared using methanol and covered with aluminium foil. In five different test tubes, about 10, 50, 100, 500 and 1000 µg/ml concentration of AgNPs (2.5 ml) was taken and 500 µl of DPPH solution was added into all the tubes. The reaction mixture was vigorously shaken and permitted to stand for 30 min at room temperature[23]. After incubation, the absorbance was measured at 517 nm using spectrophotometer. Ascorbic acid was used as a standard. The Percent DPPH scavenging effect was calculated using the following equation:

$$\% \text{ inhibition} = (A_0 - A_1/A_0) \times 100 \quad (1)$$

Where, A_0 was the absorbance of control reaction and A_1 was the absorbance in the presence of test or standard sample.

2, 2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) assay: The ABTS assay of AgNPs was examined by the method of Re *et al.*[24]. ABTS solution was prepared with 7 mm ABTS aqueous solution and 2.4 mm of potassium per sulfate and the reaction was kept in dark for 12-16 h at room temperature. This solution was previously diluted in ethanol (around 1:89 v/v) and maintained at 30° to provide a 0.700±0.02 absorption at 734 nm. Briefly, 20 µl of AgNPs with different concentration (10, 50, 100, 500 and 1000 µg/ml) was taken in five different test tubes and 2 ml of ABTS solution was added to all the reaction tubes. The tubes were then incubated for 30 min in dark and the absorbance was taken at 734 nm. The above mentioned equation (1) was used to interpret the results.

Metal chelating assay: AgNPs in different concentrations i.e. 10, 50, 100, 500 and 1000 µg/ml were added to a solution of 0.1 mm Ferrous sulphate ($FeSO_4$) (0.2 ml). The reaction was initiated by the addition of 0.25 mm ferrozine (0.4 ml) and the mixture was vigorously shaken and left for 10 min at room temperature. Absorbance was taken at 562 nm. Ethylenediaminetetraacetic acid was used as standard solution[25]. The percentage of metal chelation was interpreted by the above stated equation (1).

Antiproliferiative activity of AgNPs:

Antiproliferiative activity of the biosynthesised nanoparticles was assessed by 3-(4, 5-Dimethylthiazolyl-2)-2, 5-Diphenyltetrazolium Bromide (MTT) assay on normal Vero cells and oral cancer (H357) cell line. The cells were maintained in minimal essential medium supplemented with 10 % fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in 5 % Carbon dioxide (CO_2) at 37°. The cell lines were seeded at 5000 cells/well in 96-well plate and incubated for 48 h. Various concentrations of AgNPs i.e. 1.56, 3.12, 6.25, 12.50, 25, 50, 100 and 250 µg/ml were added to the 96-well plate and incubated for 24 h at 37°. Later, the medium was removed and washed with phosphate saline solution. The cells were further incubated with 50 µl of MTT solution (5 mg/ml) at 37° for 4 h. The medium with MTT solution was then flicked off and the formed formazan crystals were dissolved in 100 µl of dimethyl sulfoxide, then the absorbance was measured at 570 nm using a micro plate reader and images were taken under fluorescence microscope. The cell inhibition percentage was determined using the formula:

$$\% \text{ inhibition} = 100 - [(T - T_0/(C - T_0)] \times 100 \quad (2)$$

Where, T is the optical density of test sample, T_0 is the optical density at time zero and C is the optical density of control.

RESULTS AND DISCUSSION

The Actinobacterial strain KBR3 produced rough, powdery colonies with creamy yellow colored aerial mycelium and diffusible yellow pigment production on YEME agar plates. Under bright field microscopic observation, strain KBR3 showed presence of both aerial and substrate mycelium without any fragmentation. However, these morphological features alone are inadequate for the generic identification of strain KBR3. The further amplification of 16S rRNA gene yielded a sequence of 1477 bp in size and the same

was deposited in GenBank database with the accession number MT577838.

BLASTN analysis showed that the strain KBR3 shared more than 99 % similarity of 16S rRNA sequence identity with *Streptomyces maritimus* and *Streptomyces rochei*. Phylogenetic tree was constructed using the 16S rRNA gene sequences of these closely related *Streptomyces* sp. (fig. 1). Till now there are no reports on nanoparticle synthesis using *Streptomyces maritimus* or *Streptomyces rochei*. Hence the *Streptomyces* sp. KBR3 reported in this study would be a newly added source for the synthesis of AgNPs.

The brown color appeared on the reaction mixture clearly indicated the formation of AgNPs[26]. This color shift is due to the reduction of Ag^+ ions and the formation of Surface Plasmon Resonance (SPR) in the reaction mixture, whereas no color change was observed in cell pellet without silver nitrate. This observation correlated with the previous reports by Narasimha *et al.*[27] and Zarina *et al.*[28]. The biosynthesis of AgNPs was confirmed by observing the colour change from light blue to dark brown (fig. 2a).

Every element has free electrons, giving rise to the peak of SPR. It is well known that the SPR peak is dependent on the size and shape of the nanoparticles formed. The peak appeared at 420 nm (fig. 2a) was correlated with others findings that indicate the formation of AgNPs[29-31]. Hence, our result from UV spectrophotometer clearly shows that the λ_{max} of biosynthesized AgNPs occurs at 420 nm[7].

The regular XRD pattern of the biosynthesized AgNPs was shown in fig. 2b. The XRD peaks were seen located at 2θ=38.1, 44.6, 64.6 and 77.5 and are assigned to (111), (200), (220), (311) and (222) plane orientation of AgNPs. From the above result, it is clear that the most dominant orientation of *Streptomyces* synthesized silver nano particles must be crystal plane (111). This outcome demonstrates that the crystallinity of AgNPs was upgraded by adequate nucleation vitality from warm treatment. XRD pattern which shows a similar pattern, observed by Skladanowski *et al.*[32].

The size, shape and dispersion of biosynthesized AgNPs were confirmed by SEM. It was clear from SEM analysis that the AgNPs were present in nano form. The white spherical shaped bodies represent the AgNPs which are highly dispersed and small sized (fig. 2d) shows that each element is uniformly distributed throughout the material which correlate with Mohanta *et al.*[33]. Abd-Elnaby *et al.*[34] reported that *Streptomyces* synthesized AgNPs show the size of nanoparticles between 22 and 85 nm. Similarly, others also found that the extracellular biosynthesized AgNPs to be spherical and ranging in the size from 10 to 100 nm[35-37].

TEM images of AgNPs that were scanned up to 10 nm are shown in fig. 2e, which indicates the formation of a uniformly distributed polydispersed spherical structures with 36.5 nm in diameter. The shape and size of synthesized AgNPs were mostly spherical which correlates with Składanowski *et al.*[32] and Zhou *et al.*[38].

The Face-Centered Cubic (FCC) crystalline nature of *Streptomyces* cell pellet associated AgNPs was also confirmed by SAED (fig. 2g). Three well resolved circular rings were indicated by SAED pattern which confirms the (111), (200) and (220) Braggs' reflection planes of XRD pattern. Besides, the circular fringes also indicated the highly crystalline structure of AgNPs and FCC was also confirmed by the report of Zhou *et al.*[38].

The FTIR analysis was used to determine the possible functional groups present in the biosynthesized AgNPs. FTIR spectra show major peaks at 618.52, 1114.11, 1235.15, 1394.18, 1658.16, 2936.22, 3424.43 cm^{-1} which are compared to the bond angles extending of C-S linkage, C-O-C group, alkyl ketone, alkane (C-H) group, aromatics, alkanes (C-H) group, O-H stretching. Similar results were reported by Zarina *et al.*[39], Singh *et al.*[40], Prakasham *et al.*[41] and Lakhsmi *et al.*[42]. The present study showed FTIR spectroscopy peaks at 1658.16, 2936.22 and 3424.43 cm^{-1} which might be the reason in the reduction of silver ions and stabilization of AgNPs (fig. 2c) which also correlates with Sholkamy *et al.*[7].

The stability of biosynthesized AgNPs was determined by Zeta potential analysis. It is mostly used for dispersion stability of nanoparticles. The Zeta potential value of the calculated average was noted as -15.9 mV indicating the stability of synthesized nanoparticles (fig. 2f). This shows a negative value, meaning that nanoparticles are capped with negatively charged constituents that make nanoparticles repulsive and therefore increase stability[43]. This electrostatic repulsion among the negatively charged AgNPs prevents aggregation, which are responsible for their constant stability. Similarly, other researchers also found negative zeta potential values of synthesized nanoparticles of -8.5 mV and -5.08 mV, respectively[44,45]. Our findings more closely correlate with Mohanta *et al.*[46] where the zeta potential of AgNPs synthesized by *Streptomyces* sp. was found

to be -17.7 mV.

In antimicrobial assay, the biosynthesized AgNPs showed 23 mm and 20 mm zone of inhibition against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively. Similarly, other reports also proved that AgNPs synthesized by *Streptomyces* sp. was highly susceptible to Gram-positive bacteria and showed moderate activity against Gram-negative bacteria[47]. Moreover, Vennila *et al.*[48] reported that AgNPs synthesized *Streptomyces* observed highest inhibition against *S. aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Candida albicans*.

In DPPH radical scavenging assay, AgNP showed a maximum of 84 %±1.22 % and 59.52 %±0.45 % scavenging activity at 1000 µg/ml and 500 µg/ml concentrations, respectively, with a Half-Maximal Inhibitory Concentration (IC_{50}) value of 40 µg/ml. Similarly, Kumar *et al.*[49] also reported that the AgNPs exhibited increased radical scavenging activity with respect to increase in concentration. In ABTS assay, the activity of AgNPs is in concentration dependent manner and the maximum radical scavenging activity is 95.8 %±1.32 % inhibition at 1000 µg/ml whereas 58.5 %±0.78 % observed in 500 µg/ml with IC_{50} value of 15 µg/ml. In the metal chelating assay, AgNPs showed a maximum of 57.85 %±0.54 % at 1000 µg/ml and 45.93 %±2.11 % inhibition at 500 µg/ml with an IC_{50} value of 53 µg/ml (fig. 3). Also, Shanmugasundam *et al.*,[17] reported that *Actinobacteria* strain used to synthesize AgNPs showed 68.9 % chelating power at 500 µl (1:1 w/v) concentration.

Antiproliferiative activity of biosynthesized AgNPs against the normal Vero cell line and H357 oral cancer cell line was given in fig. 4. The biosynthesized AgNPs by *Streptomyces* sp. KBR3 was accessed for cytotoxicity (MTT assay) against H357-oral cancer and Vero cell lines (fig. 4). The results revealed that the synthesized AgNPs had maximum inhibition of 77.21 %±0.43 % at 250 µg/ml concentration whereas 54.22 %±1.28 % inhibition was observed in 25 µg/ml with the IC_{50} values of 9.431 µg/ml (fig. 5). The results from this % study indicated that the biosynthesized AgNPs have anticancer activity in desirable concentration and minimal toxicity to non-cancerous cells. Similar to this result, Subbaiya *et al.*[50] reported that AgNPs were highly cytotoxic to the MCF-7 breast cancer cell lines at very low concentrations and IC_{50} dosage differ with time and dose-dependent manner.

Facile synthesis of AgNPs using *Streptomyces* is a simple, inexpensive and efficient method for obtaining nanoparticles in a non-toxic and eco-friendly approach. Interestingly, the biosynthesis of AgNPs by *Streptomyces* sp. KBR3 documented a broad range of promising antimicrobial, antioxidant and antiproliferiative activity. Therefore, the promising *Streptomyces* strain KBR3 could be an ideal model for the development of nano-based medicine for the treatment of various diseases in future.

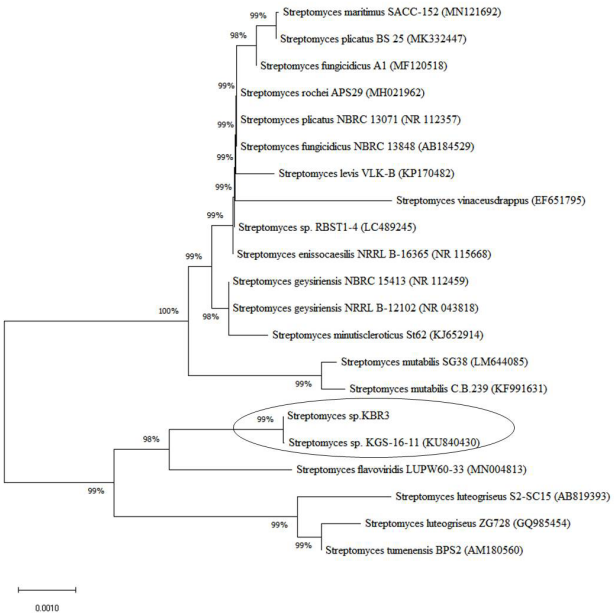

Fig. 1: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences comparing strain KBR3 to *Streptomyces* species. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates; only values >50 % are indicated

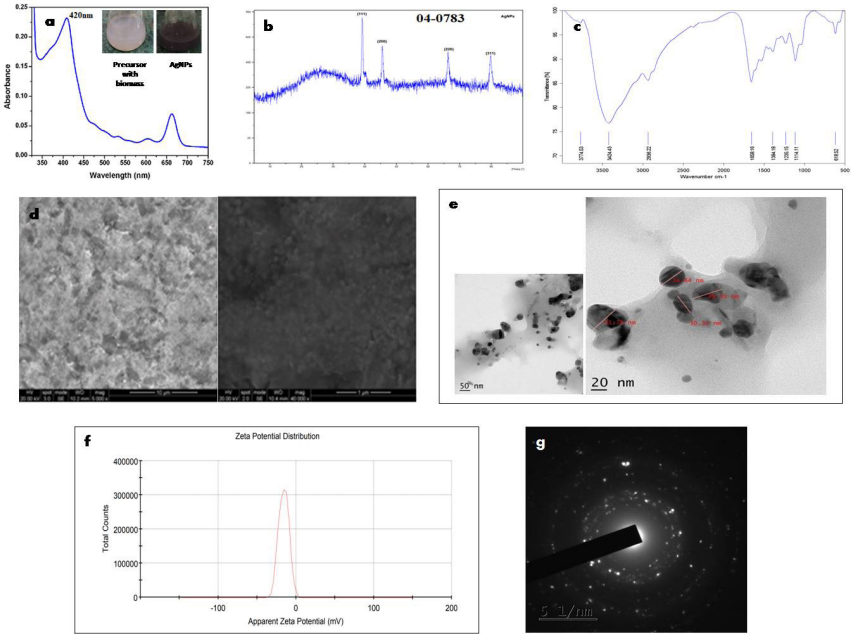

Fig. 2: (a): UV Vis SPECTRUM of AgNPs at 420 nm. Inset shows colloidal AgNPs; (b): XRD pattern of AgNPs; (c): FTIR spectrum of AgNPs; (d): SEM images of AgNPs; (e): TEM images of AgNPs; (f): Zeta potential analysis of AgNPs and (g): SAED diffraction pattern of AgNPs

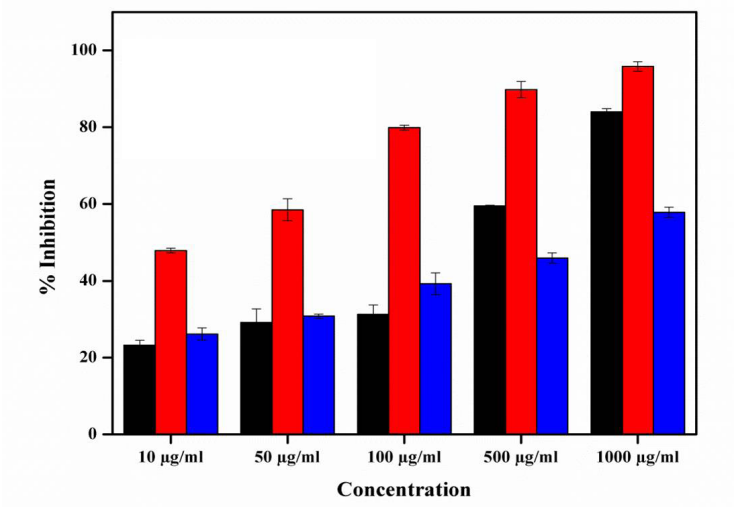

Fig. 3: Different antioxidant assays (DPPH, ABTS and metal chelating) of AgNPs from *Streptomyces* sp. KBR3
Note: ( ): DPPH Assay; ( ): ABTS Assay and ( ): Metal chelation

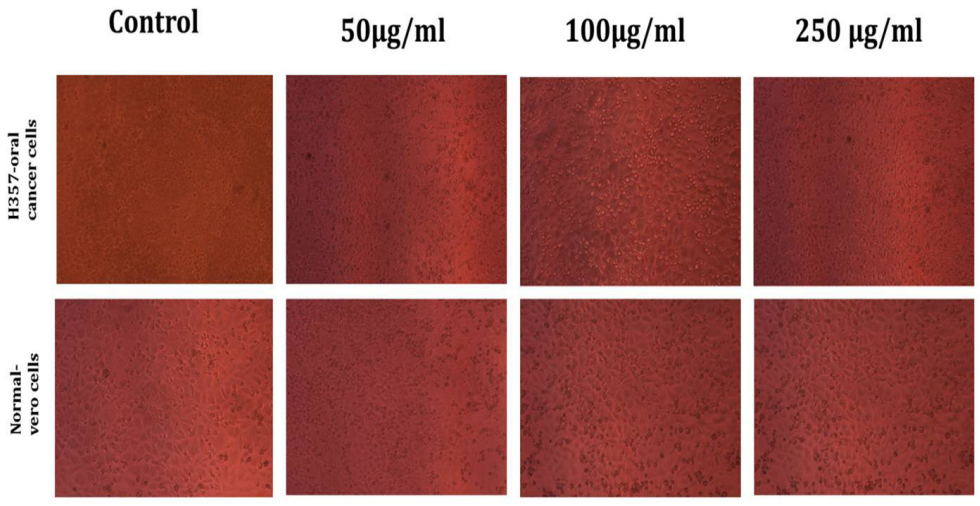

Fig. 4: Microscopic images of AgNPs treated oral cancer cells H357 and Vero cells

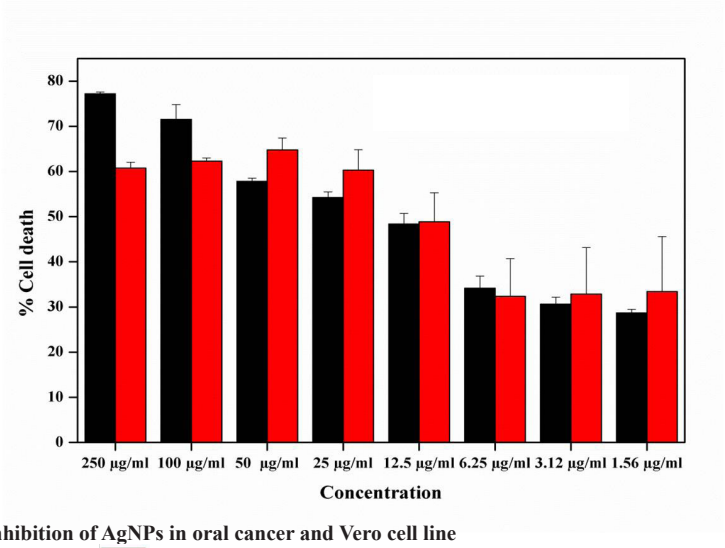

Fig. 5: Graph shows maximum inhibition of AgNPs in oral cancer and Vero cell line
Note: ( ): H357 oral cancer cells and ( ): Normal-Vero cells

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Conflict of interests:

The authors declared no conflict of interests.

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