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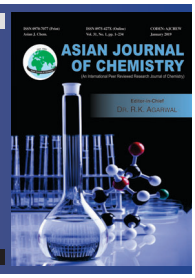

Green Synthesis of Silver Nanoparticles from *Oxynema thaianum* ALU PBC5 and their *in vitro* and *in vivo* Activity Against ESBL Producing MDR *Escherichia coli* and *Klebsiella pneumoniae*

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Antibiotic resistance is a huge problem that stays to challenge the healthcare sector in the world. The spread of multi drug resistant (MDR) bacteria remains a widely an unsolved problem. In the present study, antibacterial efficacy of cyanobacterium, *Oxynema thaianum* mediated silver nanoparticles (AgNPs) against extended-spectrum β-lactamases (ESBL) producing MDR strains were investigated. The UV-visible spectrum of biosynthesized AgNPs exhibited a characteristic SPR peak at 430 nm. The FT-IR, XRD, FESEM, EDAX and HRTEM analysis results confirms that the synthesized AgNPs were crystalline nature and spherical to oval in shape with size of 8-50 nm. The AgNPs synthesized from *O. thaianum* demonstrates high inhibitory activity in disc diffusion, MIC and MBC assays against MDR *Escherichia coli* and *Klebsiella pneumoniae*. Moreover, The LC_{50} value of AgNPs against A549 cells and *Artemia nauplii* were recorded as 7.2 and 53.33 μg mL^{-1}, respectively. Therefore the study concluded that *O. thaianum* mediated AgNPs can be used as an alternative biological agent to control ESBL producing MDR pathogens.

Keywords: Antibiotic resistance, Cyanobacteria, ESBL, AgNPs, Toxicity.

INTRODUCTION

In the past, several repeated infectious diseases have been reported due to extended-spectrum β-lactamases (ESBL) producing multidrug resistant (MDR) enterobacteriaceae [1]. Generally, ESBL producing Gram-negative bacteria are resistant to penicillin and cephalosporin antibiotics. It has ability to hydrolyze broad spectrum antibiotic containing an oximino group and are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. The *E. coli* and *K. pneumoniae* are predominant ESBL producers cause urinary tract infection, diarrhea and life threatening disease like bacteremia in humans. It has been reported that, ESBLs producing MDR Enterobacteriaceae, especially *E. coli* and *K. pneumoniae* are resistance to third-generation cephalosporins antibiotics [2,3]. Furthermore, the infectious antibiotic resistance pathogens are playing an important role in the establishment of unknown diseases in human and animals, which are the major problems in the current scenario.

The applications of nanoparticles are gaining an important in the present state of affairs as they possess well-defined chemical, visual and mechanical attributes. Metal nanoparticles are the most probable agents as they shows outstanding antibacterial activities due to their large surface area-to-volume ratio, hence researchers are focusing in this area for the development of antibacterial agents against multi drug resistance bacteria [4]. Silver has long been known as a valuable antimicrobial agent that reveals low toxicity in humans and comprises various *in vitro* and *in vivo* applications than the other metals. Generally, in ionic or metallic form, silver show high antimicrobial activity through rapidly binding with variety of negatively charged molecules (proteins, DNA and RNA) of the pathogens [5]. The biosynthesized silver nanoparticles (AgNPs) has lot of scope in the biomedical applications like biomolecular detection, catalysis, biosensors and as medicine (antifungal, anti-inflammatory, anticancer and antiangiogenesis activities). Hence, in the recent past, most of the researchers focus on the

synthesis of biocompatible silver nanoparticles due to its non-toxic nature and wide range of biomedical applications including antibacterial activity against drug resistant pathogens [6]. In addition to that, the cytotoxicity studies also comprise prominent significance and numerous studies are underway to elucidate these aspects [7]. The huge development of material science, nanoscience and nanomaterials technology has led to the synthesis and production of several nanostructures are used in various fields such as biomedicine, biotechnology, energy, optics and optoelectronics. Recently, sensors based on different nanosized materials have been developed, achieving a high sensitivity and selectivity [8].

The development of new bioactive agents to combat against multi drug resistant pathogens and cancer is urgently needed. Formulating biocompatible AgNPs using biological organism supports the biomedical field to control the communicable and non-communicable disease without any side effects. Recently, the biosynthesis of nanoparticles using microorganisms received considerable attention because they are non-toxic and eco-friendly [9-11]. The cyanobacteria (blue-green algae) is one of the largest and most primitive ancestral groups of photoautotrophic bacteria on earth. They offer great potential source of fine chemicals, pharmaceuticals, biofuels, pigments/proteins, serum and metabolites. Additionally, the crude extract of cyanobacteria contains a vast array of active biomolecules that may facilitate synthesis and stabilization of the nanoparticles [12]. Phyto-mediated synthesis of AgNPs using marine cyanobacteria is a good sign for a new generation of antimicrobial materials [13]. The EBSL producing MDR *E. coli* and *K. pneumoniae* cause dangerous, life-threatening invasive infections in humans and found to be resistance to broad spectrum antibiotics. Based on the frequent development of resistance from the hospital environment, there is an urgent need for the development of alternative medicines against MDR pathogens. Therefore, the present study aims to synthesis biocompatible AgNPs using *Oxynema thaianum* ALU PBC5 for biomedical applications such as antibacterial against MDR *E. coli* and *K. pneumoniae* respectively.

EXPERIMENTAL

Isolation and cultivation of *O. thaianum*: The cyanobacterial sample of *O. thaianum* was collected from the Palk Strait region *i.e.* Palk Strait and it extends between 9°15' N lat and 78 °50' E Long to 10°20' N lat and 79°55' E Long. The nature of the coast is sandy clay. The cyanobacterium, *O. thaianum* was grown in 250 mL conical flasks containing 100 mL of ASN-III medium adjusted to pH 7.4. The cultured media were incubated at 30 ± 2 °C under illumination of fluorescent lamps with intensity 2500 lux for 14 h light and 8 h dark. The cultures were shaken every day to prevent algal cell clumping and adherence of algal cells to the containers [14].

Preparation of cyanobacterial extract: Log phase culture of cyanobacteria was harvested by centrifugation at 5000 rpm for 10 min (Beckman GPR Centrifuge, Model: SER9D037, USA) at 20 °C and washed several times with sterile distilled water and milli Q water. 1 g of wet weight biomass of cyanobacterial extract of *O. thaianum* culture was homogenized and mixed with 100 mL of sterilized double distilled water and boiled in a water bath at 60 °C for 10 min. After cooling, the mixture was filtered with Whatman No.1 filter paper and the aqueous filtrate was used for further study. The collected filtrate was stored at 4 °C [15].

Biosynthesis of AgNPs: For the biosynthesis of AgNPs, 10 mL of cyanobacterial extract of *O. thaianum* was added to 90 mL of 1 mM aqueous silver nitrate solution The mixture was kept in a magnetic stirrer in the dark condition. The reduction of AgNPs was monitored by observing the colour change of the reaction mixture. The same procedure was followed for the synthesis of AgNPs using cell-free extract of *O. thaianum*. Simultaneously, a control was maintained without *O. thaianum* extract [16].

Microorganisms use: The opportunistic nosocomial infectious bacteria such as Multidrug resistant *E. coli* ALU MDR1 and *K. pneumoniae* ALU MDR2 were obtained from Govt. Hospital, Ariyalur, Tamilnadu, cultured (peptone, 5 g; beef extract, 3 g; sodium chloride, 5 g; pH 7 ± 0.2; distilled water, 1000 mL) and maintained in the laboratory for further study. Prior to antibacterial assays, the bacterial strains were subcultured and incubated at 37 °C for 24 h. The reference strains *E. coli* (MTCC 40) and *K. pneumoniae* (MTCC 432) were obtained from the microbial type culture collection (MTCC), Chandigarh, India and used for the study.

Characterization of AgNPs: The bio-reduction of silver ions to AgNPs was monitored by UV-visible spectra (UV-1800 Shimadzu, Japan) at the wavelength of 300-800 nm. The red solid product was separated by repeated centrifugation at 12,000 rpm for 10 min followed by redispersion of the pellet of silver nanoparticles into deionized water three times. The solid was then dried in an oven at 60 °C. The X-ray diffraction pattern was obtained with a PW 1800 Philips diffractometer using Cu-K_α radiation (k = 0.1541 nm) and the data were collected from 10° to 80° (2 h) with a scan speed of 4 min^{-1}. The possible functional groups responsible for reduction and capping behaviour of biomolecules present in the cyanobacterial extract of *O. thaianum* was examined in the FTIR (Nicolet™ iS™ 5 FT-IR, Thermo Scientific™, Marietta, GA, USA) in the range between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}. A drop of colloidal nanoparticles were coated on a copper plate, dried in a hot air oven and examined using field emission scanning electron microscopy (FESEM) (Quanta 250 FEG, Japan) equipped with energy dispersive X-ray spectroscopy (EDX-JEOL, JSM-5610) analysis. Morphology and size of the nanoparticles were authenticated by the HRTEM image. A drop of AgNPs was coated on a carbon coated copper grid of 200 mesh size and dried for 5min prior to the observation in a high resolution transmission electron microscope (HRTEM) (JEOL JEM 2100HRTEM) operated at an accelerating voltage of 200 kV [17].

Antibacterial activity of AgNPs: The antibacterial effect of AgNPs from cyanobacterial extract of *Oxynema thaianum* was tested against ESBL producing MDR *E. coli* and *K. pneumoniae* by agar disc diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS 2002) [18]. Using a sterile cotton swab, the overnight cultures of *E. coli* and *K. pneumoniae* were uniformly spread over the surface of the agar plates and to absorb the excess moisture, plates were

kept undisrupted for 10 min. In the swabbed plate, discs were punched with a diameter of 10 mm and each disc was loaded with different concentration of AgNPs suspension (25, 50, 75 and 100 μg mL^{-1}). The plates were kept in refrigerator (at 4 °C) for diffusion of AgNPs into agar and then incubated the plates in incubator at 28 °C for 24 h. After incubation, the plates were observed for the zone of inhibition expressed in millimetres.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs: MIC of AgNPs were determined for ESBL producing MDR *E. coli* and *K. pneumoniae* using the susceptibility test was assessed by standard broth dilution method according to National Committee for Clinical Laboratory Standards (NCCLS 2002) [18]. MIC value corresponded to the concentration that inhibited 99 % of bacterial growth and the MBC value corresponded to the concentration where 100 % of the bacterial growth was inhibited, compared to the positive control (*E. coli* and *K. pneumoniae* strains without AgNPs). The MIC and MBC were examined visually, by checking the turbidity of the tubes. The assays were carried out in sterile 96 well microtitre plates (MTP) containing 200 μL broth evenly was added in each well. AgNPs were added at varying concentrations (1 to 400 μg mL^{-1}) and 1 % culture of *E. coli* and *K. pneumoniae* were used as inoculum and the plates were incubated at 37 °C for 24 h. The cell density was measured at 600 nm using multifunctional spectrometer (Spectramax M3, Molecular Devices, USA).

***in vitro* MTT assay:** Human lung cancer cell line (A549) was seeded in 96-well flat bottom microplate and then incubated overnight at 37 °C in 5 % CO_2 for cell attachment. Stock solutions of each compound were prepared in DMSO and diluted with complete medium. An equal volume of DMSO (final concentration < 0.2 %) was added to the controls. The medium was removed and replaced with fresh medium containing various concentrations 25-800 μg of drugs. Cells were incubated at 37 °C in 5 % CO_2 for 48 h. After incubation, 15 μL of MTT (5 mg mL^{-1}) solution was added to each well and then the plates were further incubated for 4 h. After incubation 75 μL of lysis buffer was added to dissolve the formed crystal formazan the optical density was measured using a microplate reader at a wavelength of 570 nm and the percentage of inhibition was calculated using the following formula [19].

$$\text{Cytostasis (\%)} = \left(1 - \frac{A}{B}\right) \times 100$$

where A = the absorbance of treated cells B = the absorbance of the control cells.

in vivo challenge tests using Artemia

***Artemia nauplii* culture:** Brine shrimp *Artemia nauplii* cysts (San Franciso Bay Brand, Sam Franciso CA, USA) were purchased and used for the cytoxicity assay. The Artemia cyst was decapsulated without affecting the viability and hatched *Artemia nauplii* was used for the experiment. *in vivo* Experiments were carried out with five days old larvae of *Artemia nauplii.* 100 mg of cysts were hydrated in 100 mL of seawater for 4 h. Hydrated cysts were well aerated for 24 h to hatch out Artemia larvae [20]. Artemia larvae were cultivated in sets of 20 numbers in glass bowls containing 20 mL of natural seawater with the salinity of 35 ppt. The larvae were fed with suspension of microalgae.

Mortality rate of *Artemia nauplii*: The acute toxicity was determined by measuring the adverse effect of various concentrations of silver nanoparticle on brine shrimp *Artemia nauplii* growth, survival and mortality under intermittent flow-through conditions. The study commenced with 24 h old nauplii in 24-well plate and exposed with different concentration of AgNPs for 24 and 48 h. To conduct this study, 30 healthy *Artemia nauplii* were transferred to the each well that contained 3 mL of 33 ppt and treated with different concentration of AgNPs 25, 50, 75, 100, 125 and 150 μg mL^{-1} along with control (without silver nanoparticles), triplicate was maintained for all the groups. The percentage of mortality was determined after 24 and 48 h exposure of silver nanoparticles with the *Artemia nauplii* [21].

Percentage mortality was calculated by following the formulae:

$$\text{Mortality (\%)} = \frac{\text{Number of } \textit{Artemia nauplii} \text{ dead}}{\text{Initial number of live } \textit{Artemia nauplii}} \times 100$$

Morphological variations of *Artemia nauplii*: The morphological variations in *Artemia nauplii* treated with various concentrations of AgNPs and control were observed using light microscope.

Data collection and statistical analysis: Experiments were conducted in triplicates and significant difference between the means of the parameter was calculated by using Dunnett's ANOVA test ($P < 0.05$) using SPSS Ver. 20.00.

RESULTS AND DISCUSSION

The increase of antimicrobial resistance by ESBL producing MDR strains have become an emerging public health problem due to the clinical failure of empirical treatment protocol. While the mechanism of resistance among ESBL producing *E. coli* and *K. pneumoniae* may be varied; which have been documented that the most common mechanism of resistance to β-lactam antibiotics in *E. coli* and *K. pneumoniae* isolates is β-lactamase production [22].

This is probably the first report in biosynthesis of AgNPs using cyanobacterial extract of *O. thaianum*. After addition of *O. thaianum* extract to the silver nitrate solution, the colourless reaction mixture solution was changed to brown colour within 0.5 h indicates the formation of silver nanoparticles in the solution. Whereas no colour change was observed in the control (Fig. 1). The progress of the reaction, optical density of AgNPs in the colloidal solution, crystalline nature and capping agents was analyzed by UV-visible spectroscopy, XRD and FT-IR analysis respectively.

The mixture of cyanobacterial extract of *O. thaianum* and $AgNO_3$ solution was subjected to ultraviolet-visible (UV-vis) spectroscopy analysis. The UV-visible spectrum of AgNPs is shown in Fig. 1(i). The surface plasmon resonance of the AgNPs was obtained at 430 nm. In present study, the reduction silver ions into silver were confirmed at 430 nm in UV-visible spectrum. The similar findings were reported by Palanisamy *et al.* [23] that bioinspired AgNPs using *Spirulina* extract was obtained at 431 nm. Moreover, *Spirulina platensis* is capable for the formation of AgNPs and showed an absorption peak at

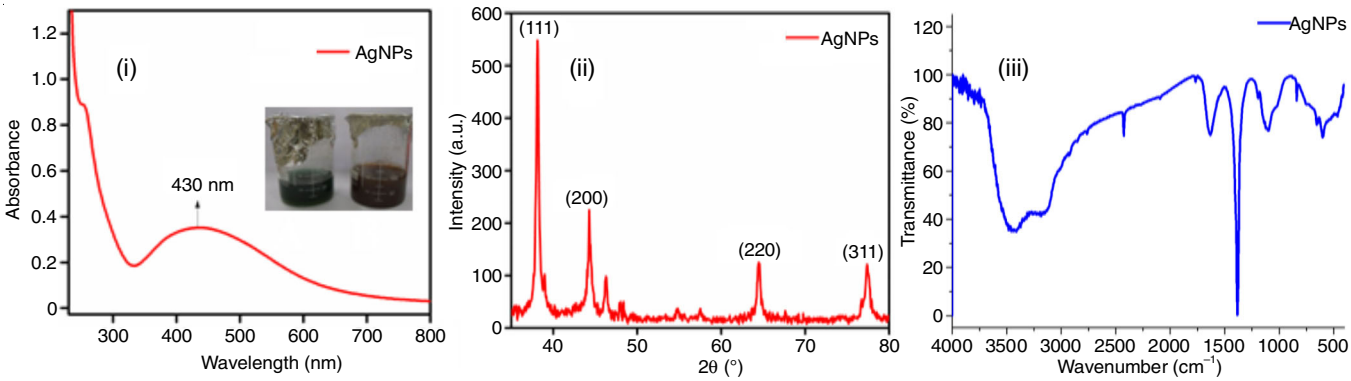

Fig. 1. Characterization of biosynthesised AgNPs: (i) UV-visible spectrum of AgNPs using cyanobacterial extract of *O. thaianum* ALU PBC5; (ii) XRD patter of AgNPs using cyanobacterial extract of *O. thaianum* ALU PBC5. FTIR analysis of *O. thaianum* ALU PBC5 and biosynthesized AgNPs using cyanobacterial extract of *O. thaianum* ALU PBC5

430 nm in UV-visible spectrum [24]. The XRD pattern of biosynthesized AgNPs is shown in Fig. 1(ii). In this diffractogram, four peaks with 2θ values, *viz.*, 38.01°, 44.3°, 64.6° and 77.43° are ascribed by the scattering from (111), (200), (220) and (311) planes, respectively. The above findings coincide with previous studies. An additional peak at 2θ values 37.9° corresponding to (111) lattice plane was observed in AgNPs. These peaks signify face-centered cubic structures of silver and silver oxide (JCPDS 89-3722 and JCPDS 01-1164). The XRD pattern thus clearly confirmed that the biosynthesized AgNPs by *O. thaianum* ALU PBC5 were crystalline in nature [25].

FTIR spectrum of biosynthesized AgNPs showed strong bands at 3411.15, 2425.89, 1423.21, 1383.79, 1098.91 and 601.20 cm^{-1} (Fig. 1 (iii)). The band at 3411.15 cm^{-1} was assigned to carbohydrates and protein of O-H and N-H stretching vibration. The band at 2425.89 cm^{-1} was assigned to C-H stretching vibration of lipids. The band at 1423.21 cm^{-1} was assigned to amide II absorption and N-H stretch vibration of proteins. The two bands were observed at 1383.79 and 1081.73 cm^{-1} assigned to the C-N stretching vibration of of the aromatic and aliphatic amines, respectively. The 600.20 cm^{-1} band at was assigned to the C-CI presence of AgNPs in the cyanobacterial extract of *O. thaianum*. Hence, the FTIR analysis concluded that the proof of protein coat which confirmed the stabilization of AgNPs and it is responsible for stronger affinity to bind with Ag^+ ions and could possibly act as capping and stabilizing agents thereby lowering the aggregation of AgNPs. These results agreed with many reports of cyanobacteria. In addition, the presence of bands at that spectral range suggested that the capping capping agent of biosynthesized nanoparticles possesses an aromatic amines group with specific signatures of amide linkages between amino acid residues in the proteins along with infrared region of the electromagnetic spectrum. The proteins are potential biomolecules responsible for the reduction and capping of the AgNPs synthesized [26,27].

The electron micrograph analysis was accomplished to define the nature and size distribution of the biosynthesized nanoparticles. At room temperature, the addition of $AgNO_3$ to the *O. thaianum* ALU PBC5 which initiated the precipitation of AgNPs at cell surfaces (Fig. 2). The biosynthesized AgNPs seemed to be spherical in shape with different size. The elemental composition of EDAX data revealed the presence of silver in the form of AgNPs in higher amount. Apart from the silver ions, there are other signals of carbon, oxygen, sodium and sulphur which could be the residual presence of cyanobacterial extract of *O. thaianum* which was confirmed the presence of AgNPs in the analysis of EDAX (Fig. 2). The TEM results revealed that biosynthesized AgNPs size ranged from 8 to 50 nm (Fig. 3). Similar results were reported by Morsy *et al.* [28] that the morphology of the AgNPs synthesized by EPS of *N. commune* showed spherical shape with size ranged from 15-45 nm. Similarly Saravanan *et al*. [29] reported that the biosynthesized AgNPs distributed in different shapes, such as spherical and oval shapes with sizes ranging from 34 to 90 nm.

The antibacterial ability of synthesized AgNPs, the cyanobacterial extract of *O. thaianum* was examined against ESBL producing MDR *E. coli* and *K. pneumoniae* using disc diffusion. The maximum zone of inhibition was found at 75 μg mL^{-1}. The synthesized AgNPs from *O. thaianum* ALU PBC5 showed the highest zone of clearance against ESBL producing MDR *E. coli* (14 mm) and *K. pneumoniae* (12 mm) in disc diffusion method. In order to confirm the antibacterial activity of extract, standard antibiotics were used as positive control. Saravanan and Nanda [30] reported that the AgNPs possess significant bactericidal activity against MDR isolates such as *Salmonella typhi* and *Staphylococcus aureus.* The MIC value of *E. coli* and *K. pneumoniae* was 30-50μg mL^{-1} with biosynthesized AgNPs using *O. thaianum* extract. Similarly the MBC values of *E. coli* and *K. pneumoniae* was 60-80 μg mL^{-1} with biosynthesized AgNPs from the extract of *O. thaianum*. The least inhibitory concentration (30 μg mL^{-1}) was recorded against *E. coli* and *K. pneumoniae* whereas the highest bactericidal concentration 80 μg mL^{-1} was registered. Generally, the antibacterial activity of AgNPs against Gram-negative bacteria divided into three steps: (i) nanoparticles mainly in the range of 1-10 nm attach to the surface of the cell membrane and significantly distract its appropriate functions, such as permeability and respiration; (ii) they are able to enter into the bacteria and cause further damage by perhaps interacting with sulfur- and phosphorus-containing compounds such as DNA and (iii) nanoparticles release silver ions, which will have an additional contribution to the bactericidal effect of AgNPs [9].

The effect of biosynthesized AgNPs on cell proliferation and morphology of lung cancer cells. The study revealed that

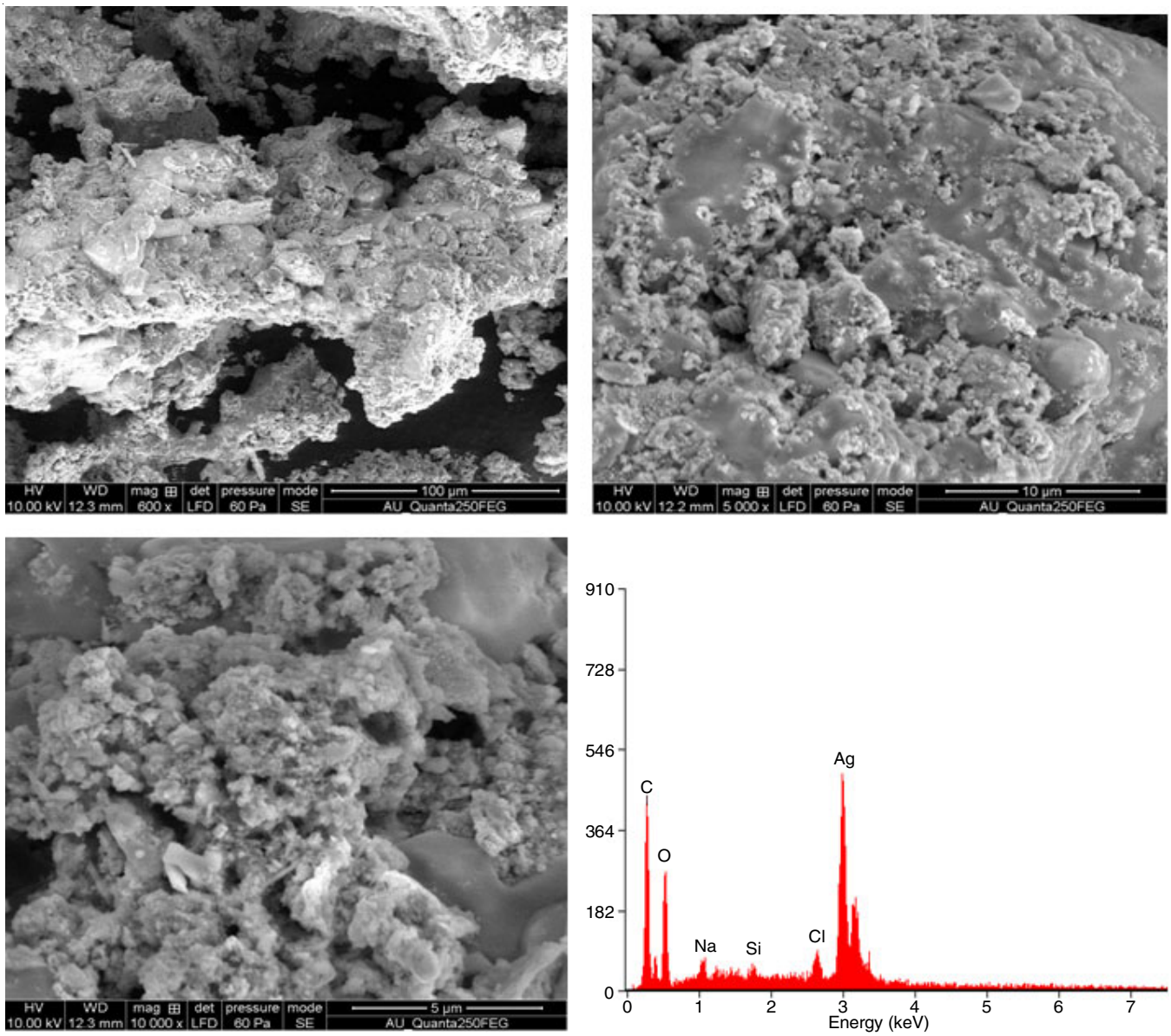

Fig. 2. SEM and EDAX analysis of biosynthesized AgNPs using cyanobacterial extract of *O. thaianum* ALU PBC5

the AgNPs effectively controls the growth of lung cancer (A 549) cells in a dose-dependent manner. The IC_{50} value was 7.2 µg mL^{-1} for AgNPs. To further characterize, images were taken using phase contrast microscopy after 48 h. The AgNPs treated cells become shrunk and loss their morphology at 48 h (Fig. 4). These results demonstrated that silver nanoparticles mediated a dose and time dependent increase in toxicity which concluded that the cyanobacterial extract of *O. thaianum* mediated synthesized silver nanoparticles have great discrimination to cancer cell and can display potential application in cancer chemoprevention and chemotherapy. Silver nanoparticles revealed to have important anti angiogenic properties; thus are attractive for study of their potential antitumor effects. Compounds possessing antiangiogenic properties are known for their potential ability to block the activity of abnormally expressed signaling proteins [31].

The aqueous extract of *O. thaianum* ALU PBC5 and silver nanoparticles showed mortality against *Artemia nauplii* and it was increased proportionally with the increasing concentration of the samples (Fig. 5). *Artemia nauplii* lethality test concluded that the increased nanoparticle aggregates in the guts showed major mortality within 24 h of exposure with different concentrations (25-150 µg mL^{-1}). The LC_{50} value was determined around 53.33 µg mL^{-1} concentration. After 48 h, the mortality rate was twice as compared with the results of the 24 h mortality rate due to the aggregates of AgNPs in the gut. Morphological variation of *Artemia nauplii* treated with AgNPs was clearly observed under the light microscope and the images were photographed. The aggregation of AgNPs inside the gut of *Artemia nauplii* was clearly detected through the light microscope. In general *Artemia* are non-selective filter feeder, it consumes all particles that are below 50 microns in size. The amount of aggregation not only depends on the amount of concentration, but also depends on the amount of consumption of nanoparticles by each individual animal in various concentrations. Our findings were matched with Vijayan *et al.* [32] who reported that *Artemia* cytotoxicity and anticrustacean assays are one of the reliable methods to screen and detect the

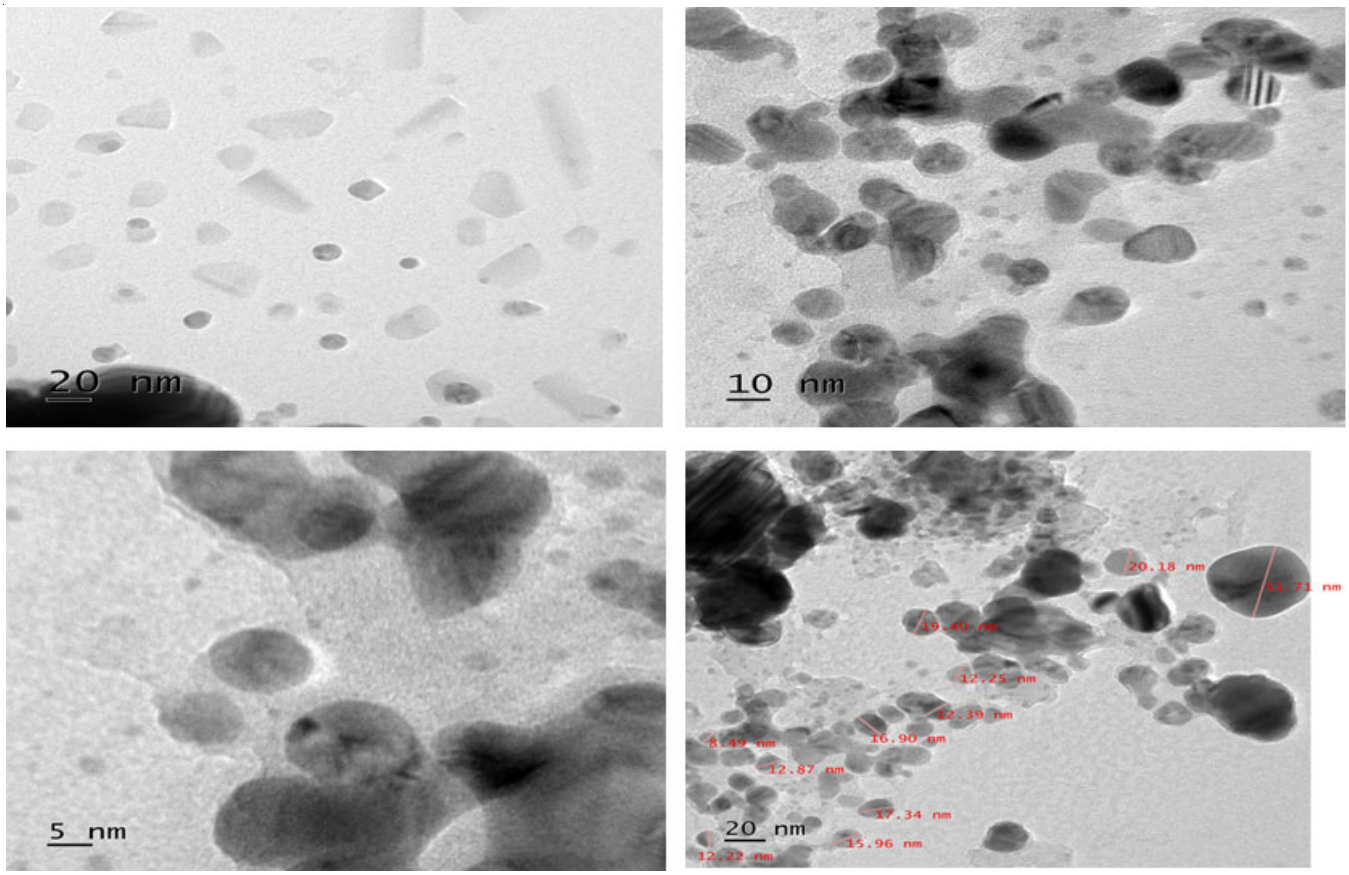

Fig. 3. TEM analysis of biosynthesized AgNPs using cyanobacterial extract of *O. thaianum* ALU PBC5

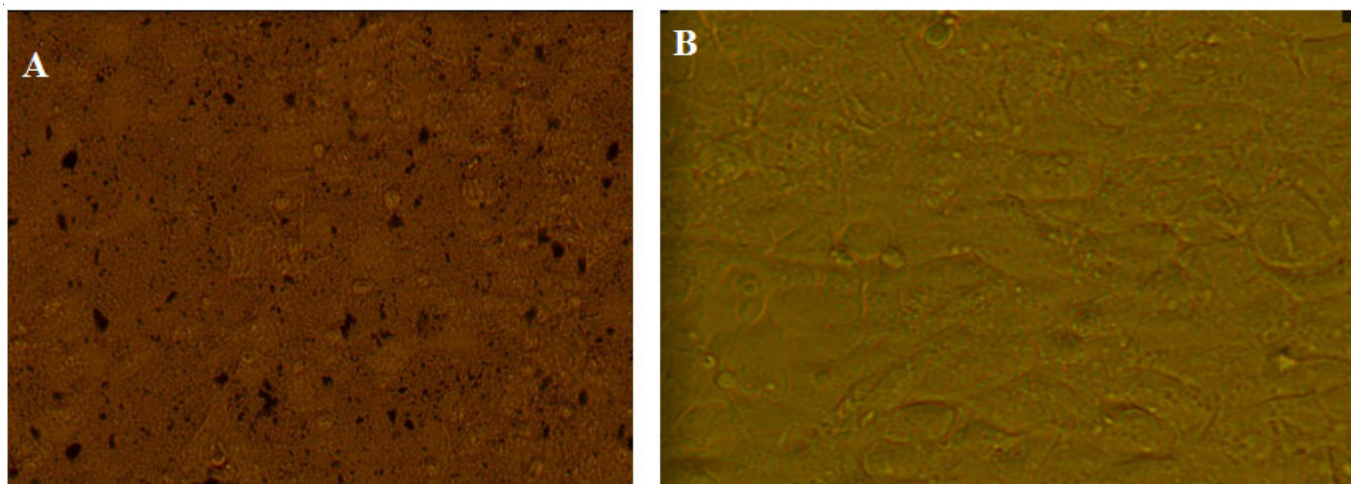

Fig. 4. Drug induces morphological changes in A549 cells. Equal number of cells were plated and allowed to attach for 12 h. Cells were then exposed to vehicle (VEH)-containing media and media containing various doses of 25-800 µg for a period of 48 h. At the end of the experiment, images were taken using phase contrast microscopy. (A) AgNPs from cyanobacterial extract of *O. thaianum* ALU PBC5, (B) control

cytotoxicity of the products. The LC_{50} value on the cytotoxicity of the synthesized colloidal AgNPs was 88.91 ± 5.04 µL mL^{-1} whereas the highest mortality was observed at 160 µL mL^{-1} concentration.

Conclusion

In this present study, it is concluded that AgNPs were successfully synthesized from the cyanobacterial extract of *O. thaianum* ALU PBC5. The synthesized AgNPs are crystalline in nature and exhibit a sharp SPR band width at 430 nm. The TEM result confirms the formation of AgNPs with an average size of 8-50 nm. The biosynthesized nanoparticles effectively controls ESBLs producing MDR *E. coli* and *K. pneumonia*e. The Artemia cytotoxicity assay recorded the LC_{50} value of 53.33 µg mL^{-1} and shows less toxicity. Therefore, the present finding provides a strong foundation for the future

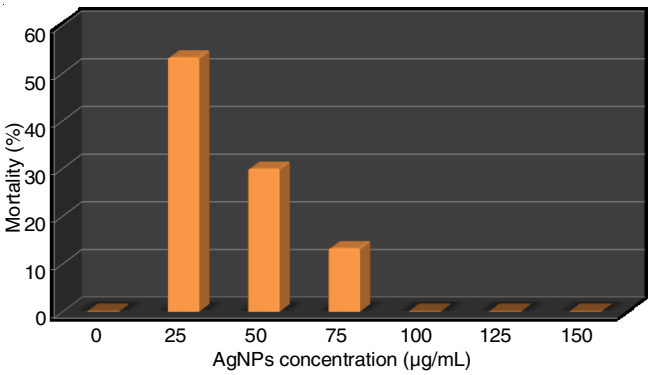

Fig. 5. Artemia cytotoxicity assay from AgNPs of the cyanobacterial extract of *O. thaianum* ALU PBC5 showed different mortality rate

study in the development of nano-biomedicine against for the treatment of MDR human pathogenic infection.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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