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Synthesis and evaluation of silver nanoparticles using *Cymodocea rotundata* against clinical pathogens and human osteosarcoma cell line

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ABSTRACT

Objective: To evaluate the silver nanoparticles (AgNPs) synthesis by aqueous extract of *Cymodocea rotundata* against clinical pathogens and human osteosarcoma cell line. **Methods:** In the present study, AgNPs were synthesized by *Cymodocea rotundata* by reducing 1 mM silver nitrate ($AgNO_3$) solution. The green synthesized AgNPs were characterized by UV-vis spectroscopy, Atomic force microscopy (AFM), Scanning electron microscopy (SEM), Fourier infrared spectroscopy (FTIR) and X-ray diffraction (XRD). **Results:** FTIR spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the AgNPs. Morphologically, the nanoparticles were found to be spherical with an average particle size distribution of 24.2 nm. The green synthesized AgNPs showed powerful antioxidant properties in *in-vitro* antioxidant assays and effective in inhibiting human pathogens. The AgNPs also showed potent cytotoxic effect against MG63 cell lines with an IC_{50} value of 25.31 µg ml^{-1} which was confirmed by MTT assay. **Conclusions:** The results support The advantage of using bio-green method for synthesizing AgNPs with antioxidant, antimicrobial and cytotoxic activities are simple and cost effective.

INTRODUCTION

Over the years the occurrence of the infectious diseases caused by the different pathogenic bacteria has been escalating and the researchers are searching for new antibacterial agents (Rai *et al*., 2009). In the current scenario, nanoscale materials have emerged as novel antimicrobial agents owing to their high surface area to volume ratio and the unique physical and chemical properties. Different types of nanomaterials like copper, titanium, zinc, alginate, gold, magnesium and silver have come up but silver nanoparticles (AgNPs) has been promising and proved to be most effective against bacteria and other eukaryotic microorganisms (Gong *et al*., 2007). The silver nanoparticles have also found diverse applications such as coatings for medical devices, wound dressings, silver nanoparticles impregnated textile fabrics, etc. the advantage of using silver nanoparticles for impregnation is that there is continuous release of silver ions (Ag^+) and the devices can be coated by both inner and outer side hence, enhancing its antimicrobial efficacy. The burn wounds treated with silver nanoparticles shows better cosmetic appearance and scarless healing. In the last decade, biosynthesis of m*et al* nanoparticles is a growing need to develop clean, nontoxic chemicals, environmentally benign solvents and renewable materials and hence the focus turned towards 'green' chemistry and bioprocesses. The synthesis of AgNPs through bacteria, fungi, yeast and plant extracts would benefit from the development of clean, nontoxic and environmentally acceptable 'green chemistry' procedure (Bhattacharya and Rajinder, 2005). Recently, Thirunavoukkarasu *et al*. (2013) reported the potential of leaf extract of *Desmodium gangeticum* in reducing aqueous Ag^+ to Ag^0 ions and the rapid formation of eco-friendly AgNPs with well defined dimensions in the size range of 18-39 nm. Logeswari *et al*. (2013) reported that the AgNPs were synthesized from a silver nitrate solution by commercially available plant powders such as *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica* and *Citrus sinensis*.

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The AgNPs also showed potent cytotoxic effect against MCF-7 breast cancer cell lines with an IC^{50} value of 67 µg ml^{-1} by the MTT assay. In the present research, AgNPs have been synthesized using *Cymodocea rotundata* extract. At the same time *in vitro* antioxidant, antimicrobial and cytotoxic effects of green synthesized silver nanoparticals were evaluated. To our knowledge, this is the first report on the synthesis of AgNPs using *Cymodocea rotundata* extract.

MATERIALS AND METHODS

Sample preparation for synthesis of silver nanoparticles

Fresh *Cymodocea rotundata* (Sea grass) was collected from Thondi coastal area 99^0 44" N and 79 10^0 45" E situated in Palk Strait region of Tamilnadu. The sample was washed thoroughly with distilled water and was uniformly cut it into small pieces. The leaf extract was prepared by boiling 20 g of fresh *Cymodocea rotundata* chopped samples in 100 ml of sterile distilled water for 5 min, cooled and filtered. The extract was stored at 4 °C for further experiments. This filtrate was used as a reducing as well as stabilizing agent for 1 mM of silver nitrate ($AgNO_3$) (Korbekandi *et al*., 2013).

Biosynthesis of silver nanoparticles

The silver nitrate, A.R., used in this study was obtained from Himedia Laboratories Pvt. Ltd., Mumbai, India. 10 ml suspension of the leaf extract was added to 90 ml aqueous solution of $AgNO_3$ (1 mM) solution for the reduction from Ag^+ to Ag^0 nanoparticles by incubation at 35 °C for about 24 h. The primary detection of synthesized AgNPs was carried out in the reaction mixture by observing the colour change of the medium from greenish to dark brown (Thirunavoukkarasu *et al*., 2013).

Characterization of silver nanoparticles

The bioreduction of the Ag in solution was monitored by periodic sampling of aliquots (2 ml). The absorption spectra of the samples were taken 300 to 600 nm using a UV-Vis spectrophotometer (HITACHI, Model U-2800 spectrophotometer). The deionized water was used as the blank. The sample was air dried and was characterized by atomic force microscopy (Model-Nanosurf easyscan 2 AFM, made in Switzerland) for its detailed morphology and size. Scanning electron microscope (SEM) was employed for the analysis of size and shape of AgNPs.

The AgNPs sample was mounted on specimen stubs with double-sided adhesive tape and coated with gold in a sputter coater to avoid charging and was examined under SEM (Zeiss Evo 18). The air dried powder of AgNPs was diluted with potassium bromide in the ratio of 1:100 and the spectrum which was recorded in FTIR in the range of 4000-500 cm^{-1} at a resolution of 4 cm^{-1}. To check phase formation and purity, XRD patterns were recorded using powder X-ray diffractometer (Model-D8 Advance, made in BRUKER Germany) (Chakraborty *et al*., 2016).

In vitro antioxidant assays (DPPH free radical scavenging assay)

DPPH radical scavenging assay for AgNPs was performed at concentration of AgNPs 1 mg ml^{-1} which were separately mixed with 3 ml of 0.1 mM DPPH and incubated in dark for 15 min. After incubation, the absorbance of the samples was measured by using UV-vis spectrophotometer at 517 nm against methanol as blank (Abdel-Aziz *et al*., 2014). Ascorbic acid was used as standard and DPPH methanol reagent without sample was used as control and percentage of inhibition was calculated by the following formula.

% of inhibition = Absorbance of control-Absorbance of test/Absorbance of control

Antimicrobial activity

The AgNPs synthesized from *Cymodocea rotundata* was tested for their antimicrobial activity by well diffusion method against pathogenic organisms which were *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* sp., *Shigella* sp., *Salmonella* sp., *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis* obtained from Microbial Biotechnology laboratory, VIT University. The pure cultures of the organism were sub cultured on nutrient broth at 35 °C on rotary shaker at 120 rpm. Each strain was swabbed uniformly on the individual plates using sterile cotton swab. Wells of size 6 mm have been made on Muller-Hinton agar plates using gel puncture. Using micropipette 25 µl, 50 µl, 75 µl and 100 µl of the sample of nanoparticles solution were poured into wells on all plates. After incubation at 35 °C for 18 h, the different levels of zone of inhibition were measured (Chakraborty *et al*., 2016)

Anticancer effect of AgNPs

The human osteosarcoma cell line (MG63) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% f*et al* bovine serum (FBS). All cells were maintained at 37 °C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

In order to determine the cytotoxic effect of newly synthesized AgNPs, MTT dye reduction assay was performed on human osteosarcoma cell line (MG63) using increasing concentrations (12.5-200 µg ml^{-1}) of AgNPs. The result of the assay depends on the reduction of MTT to a blue colored product by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells. For experimental settings, osteosarcoma cells were harvested and cultured, at a density of 1×10^5 cells ml^{-1} and incubated for 48 h in 5% CO_2 at 37 °C with the increasing concentrations of AgNPs (12.5, 25, 50, 100 and 200 µg ml^{-1}). After the treatment of AgNPs, 15 µl of MTT (5 mg/mL in PBS) was added to each well and the plate was further incubated for 4 h at 37 °C in 5% CO_2. The resulting blue component (reduction of tetrazolium salt of MTT by mitochondrial dehydrogenase) was dissolved in 100 µl dimethyl sulfoxide

(DMSO) and then measured the absorbance at 570 nm using micro plate reader. The percentage of cell inhibition was determined using the following formula (Mosmann *et al*., 1983; Monks *et al*., 1991).

% Cell Inhibition = 100-Abs (sample)/Abs(control)x100.

RESULTS AND DISCUSSION

In this study, the formation of AgNPs by extract of *Cymodocea rotundata* was investigated. The reaction started with in first hour of the incubation with 1 mM $AgNO_3$. This was confirmed by the appearance of brown colour in the reaction mixture. It is well known that silver nanoparticles exhibit a yellowish brown color in aqueous solution due to excitation of surface plasmon vibrations in AgNPs (Jae and Beom, 2009). Biosynthesis of AgNPs from $AgNO_3$ solution was confirmed by UV-vis spectra studies, with the colour change from greenish to brown and recorded maximum absorbance at 420 nm (Figure 1). The observation indicates the release of proteins into solution by *Cymodocea rotundata* by a possible mechanism through reduction of the m*et al* ions present in the solution. Similar observations were also reported in *Solanum tricobatum, Syzygium cumini, Centella asiatica* and *Citrus sinensis*. AFM micrographs of synthesized AgNPs are presented in Figure 2. It is clear that most of the AgNPs were spherical in shape with particle sizes of 24.2 nm. Scanning electron microscopy (SEM) and energy-dispersive X-ray microanalysis (EDX) observation gained further insight into the features of the silver nanoparticles. The synthesized AgNPs were spherical in shape as observed in the SEM image (Fig. 3a) and this confirmed the shape of AgNPs obtained in the AFM image. EDX analysis also showed a peak in the silver region, confirming the formation of silver nanoparticles (Figure 3b) and the optical absorption peak is observed approximately at 2.6 keV. The typical optical absorption between 2-4 keV confirms the metallic nanoparticles due to surface plasmon resonance (Thirunavoukkarasu *et al*., 2013). Figure 4 shows the FTIR spectrum recorded from the air dried powder of AgNPs formed with *Cymodocea rotundata* extract. Peak at 3439 cm^{-1} and 1631 cm^{-1} corresponds to N-H stretching and bending vibrations, respectively in amines from proteins of *Cymodocea rotundata*. The peak at 1087 cm^{-1} is attributed to C–O stretching from ester and ether allowing to functional groups of proteins and metabolites covering the AgNPs. The overall observation confirms the presence of protein in the samples of AgNPs. It is reported earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins (Gole *et al*., 2001) and therefore the stabilization of the AgNPs by protein is a possibility. Further studies were carried out using X-ray diffraction to confirm the crystalline nature of the particle and the XRD pattern obtained has been represented in Figure 5. The XRD pattern showed four intense peaks in the whole spectrum of 2θ value ranging from 30 to 80.

The AgNPs formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 2θ values of 38.25°, 46.37° and 77.62° corresponding to 111, 200 and 311 planes for silver, respectively. The three intense peaks were observed in the spectrum agree to the Braggs's reflection of silver nanocrystals as reported by Lu *et al*. (2003).

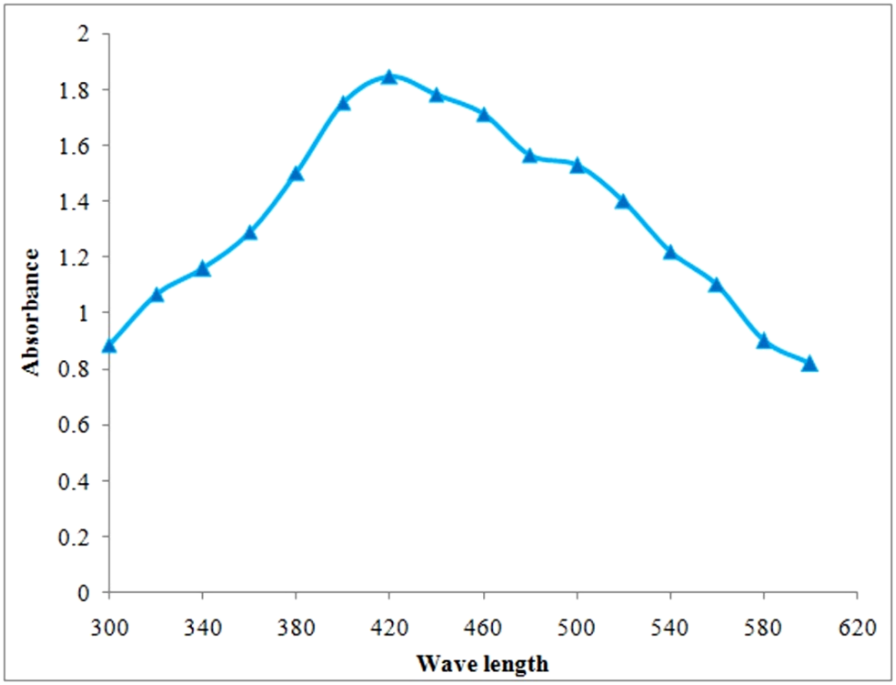

Fig. 1: UV-vis spectra of *Cymodocea rotundata* extract containing AgNPs.

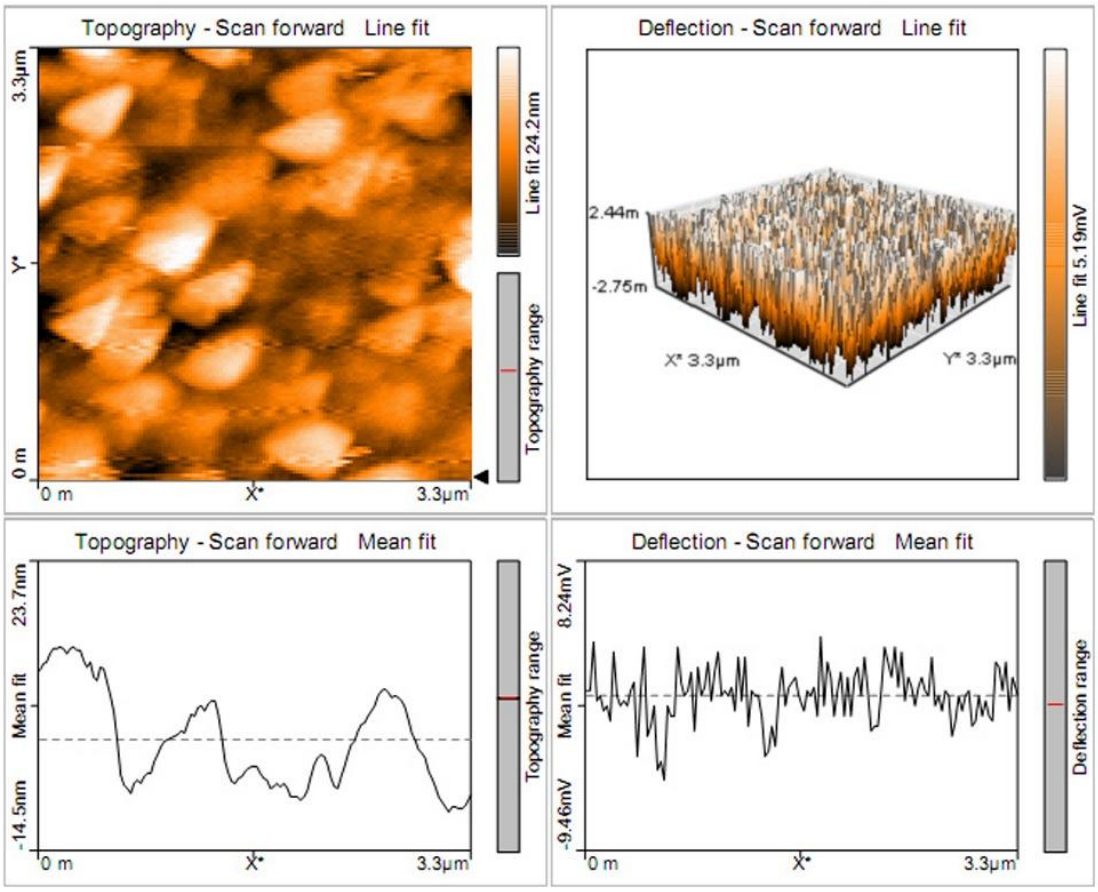

Fig. 2: AFM image of AgNPs.

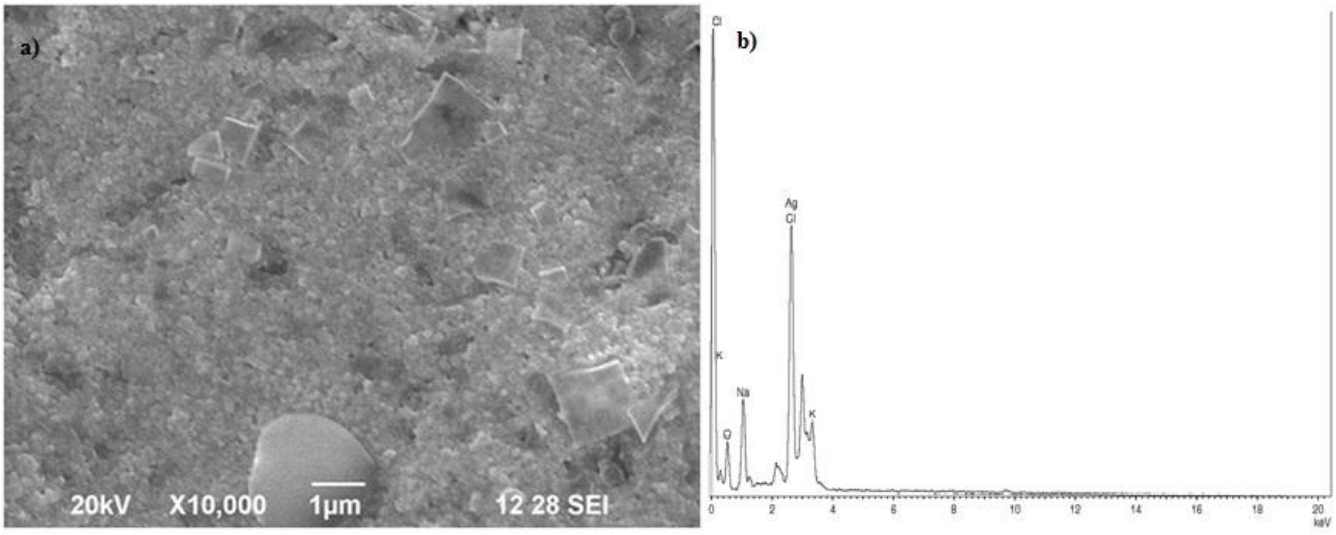

Fig. 3: (a) SEM image of AgNPs and (b) EDX spectrum of AgNPs.

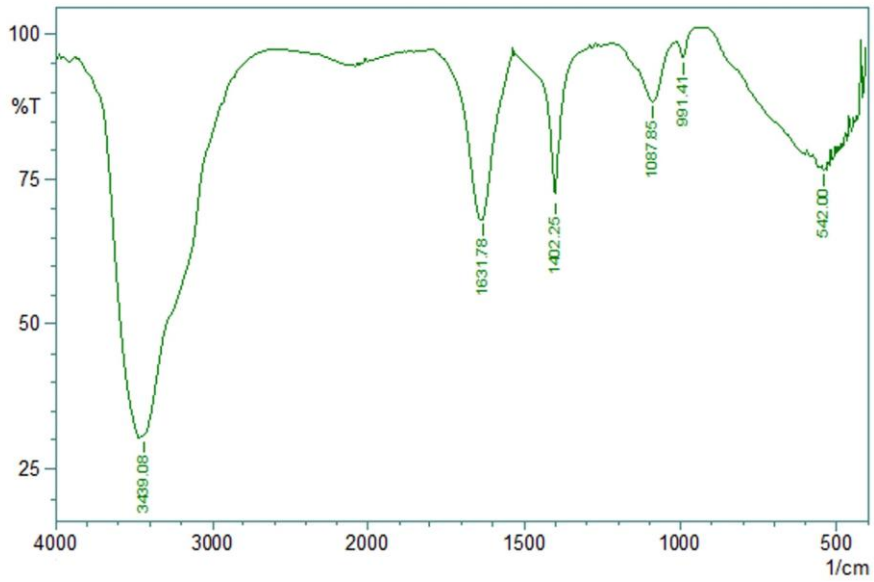

Fig. 4: FTIR spectra of AgNPs.

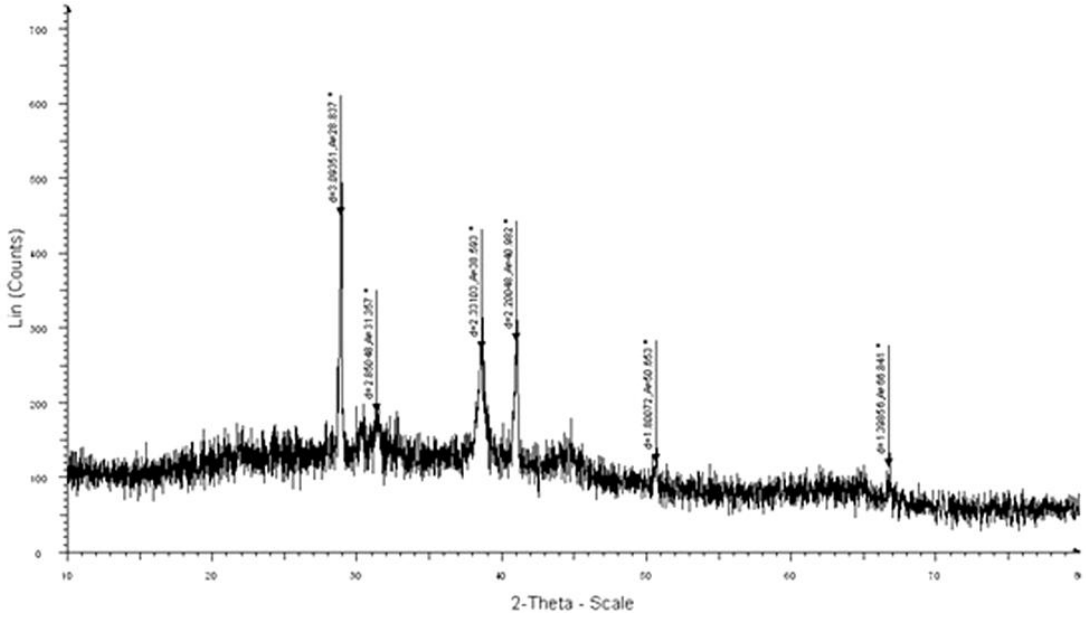

Fig. 5: XRD pattern of AgNPs exhibiting the facets of crystalline silver.

Antioxidant activity of AgNPs was assessed by DPPH scavenging assay by using ascorbic acid as positive control. DPPH is a stable compound and accepts hydrogen or electrons from Ag NPs. The results obtained in the DPPH assay showed effective free radical inhibition by AgNPs. The average percentage inhibition of synthesized AgNPs was found to be 77.3% and the activity was increased with increasing concentrations of AgNPs. Similar observations with enhanced DPPH scavenging activity by selenium, platinum, silver nanoparticles and by torolex and chitosan coated gold nanoparticles have been reported (Ramamurthy *et al*., 2013). The reducing activity of AgNPs was found to be increased with increasing concentrations. Similar observations were made by Dipankar and Murugan (2012) with AgNPs synthesized by *Iresine herbstii*.

Antimicrobial activity of AgNPs was investigated against various pathogenic organisms using the well diffusion method. The highest antimicrobial activity of AgNPs was found against *K. pneumoniae* (13 mm) followed by *Enterococcus* sp. (12 mm) and the lesser antimicrobial activity was found against *S. aureus* with inhibition zone of 11 mm. Recently, Sankar *et al*. (2013) reported the silver nanoparticles showed more than 10 mm zone of inhibition against *E. coli*, *Aeromonas hydrophila*, *Salmonella* sp., and *S. paratyphi*. The highest antimicrobial activity was observed against *Pseudomonas aeruginosa* (16 mm). The lesser

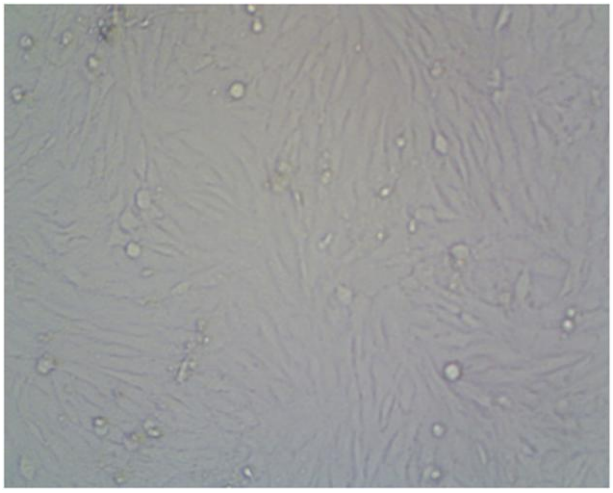

Fig. 6: Image of osteosarcoma cell line MG63 treated with AgNPs.

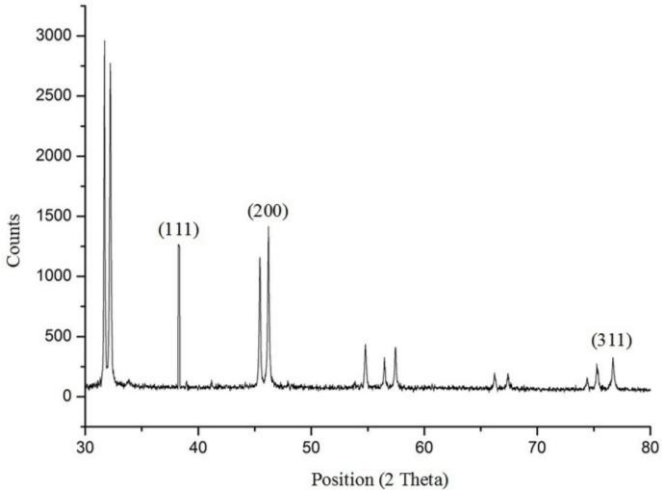

Fig. 7: Smoothen format of XRD pattern showing the presence of different peaks on 2 theta plane indicating the crystalline structure of the synthesized AgNPs.

antimicrobial activity of silver nanoparticles synthesized by both *Citrus sinensis* and *Centella asiatica* against *Staphylococcus aureus* was 8 mm and *Escherichia coli* was 8 mm.

In vitro cytotoxicity of the silver nanoparticles was evaluated against human osteosarcoma cell line MG 63 at different concentrations (12.5-200 µg ml^{-1}) by MTT-assay (Figure 6). In relation to cell death, a minimum of 25 µg ml^{-1} of AgNPs was enough to induce 47.61% of cell mortality. In the present study, the AgNPs were able to inhibit the growth of cell line by 6.76 % at low concentration. In contrast, the presence of 50 µg ml^{-1} of AgNPs significantly inhibited the cell growth by 100%. The IC_{50} value for AgNPs was calculated and it was found to be 25.31 µg ml^{-1}. A few in vitro studies analyzed the translocation of AgNPs in cancer cell line with a LD_{50} value of 300 µg ml^{-1} (Sriram *et al*., 2010).

The AgNPs may induce reactive oxygen species and cause damage to cellular components leading to cell death (Jacob *et al*., 2012). Never the less, this is the first report on cytotoxic effects of green synthesized AgNPs using *Cymodocea rotundata* extract against human osteosarcoma MG63 cells.

CONCLUSIONS

To conclude, we report a simple, speedy and efficient green synthesis of AgNPs from the *Cymodocea rotundata* extract. The characterization with UV-vis spectroscopy, FTIR, AFM, SEM and XRD analysis evidence the formation of nanoparticles. The synthesized AgNPs is substantiated by their potent free radical quenching effect and antimicrobial activity against human pathogenic bacterial strains. In the present study, in vitro cytotoxic activity of green synthesized AgNPs against human osteosarcoma MG63 cell line was remarkable with 47.61% of mortality at 25 µg ml^{-1}.

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Conflict of Interests: There are no conflicts of interest.

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