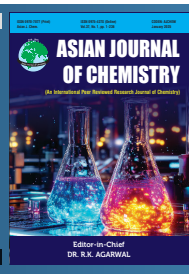
Asian Journal of Chemistry; Vol. 37, No. 5 (2025), 1197-1203

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2025.33680




# Antimicrobial, Antioxidant and Anticancer Activities of Green Synthesized Silver Nanoparticles using Sea Weed *Spongomorpha indica*

Saisha Vinjamuri[1], Lakshmi Pethakamsetty[2,*], Sharad S. Achar[3] and Narasimha Reddy Parine[4]

[1]Department of Biotechnology, B.M.S. College of Engineering, P.O. Box No. 1908, Basavanagudi, Bengaluru-560019, India
[2]Department of Microbiology, Andhra University College of Science and Technology, Andhra University, Visakhapatnam-530003, India
[3]Department of Biotechnology, Padmashree Institute of Management and Sciences, Kommaghatta, Bengaluru-560060, India
[4]Department of Biochemistry, Genome Research Chair, College of Science, King Saud University, Riyadh, Kindgom of Saudi Arabia

*Corresponding author: E-mail: lmkandregula@gmail.com

*Received*: 14 March 2025; *Accepted*: 16 April 2025; *Published online*: 30 April 2025; AJC-21988

With the increase in antimicrobial resistance, there is always a dire need for the bioactive molecules that can serve as novel therapeutic regiments. The aim of this study is to develop a straight forward scheme of silver nanoparticle synthesis using algal extracts as reducing agent and also to explore optimal parameters for controlling particle morphology. The current research explores the use of biological methods to synthesize silver nanoparticles (AgNPs) using the aqueous extracts of *Spongomorpha indica*, a Chlorophycean member. The effect of metal ion concentration from 5 mM to 12.5 mM and algal extract 10 mL of 20% (w/v) has been studied for a time interval of 2 h. The biosynthesized AgNPs were characterized by UV-VIS spectroscopy, X-ray diffractometer and field emission gun-scanning electron microscope technique. This confirmed the formation of metallic silver in a face-centered cubic (FCC) crystalline form, with an average crystallite size of approximately 18 nm. Furthermore, the peak patterns of HPTLC chromatograms of aqueous extracts of *Spongomorpha indica* showed the presence of phenolic compounds especially gallic acid as major phytoconstituent, which might primarily be responsible for the production of AgNPs. The green synthesized nanoparticles of different concentrations were initially tested for antimicrobial properties and MICs were worked out for two susceptible bacterial strains.

**Keywords: *Spongomorpha indica*, Silver nanoparticles, Antimicrobial activity, Anticancer activity, Antioxidant activity, HPTLC.**

## INTRODUCTION

The growing threat of antimicrobial resistance is making the treatment of microbial infections progressively more difficult. The phenomenon of drug resistance commonly represents an evolutionary adaptation, occurring *via* horizontal gene transfer or selective pressure exerted by antibiotic usage. This process culminates in the re-emergence of diseases that were previously effectively managed [1,2]. Increased antimicrobial resistance necessitates higher antibiotic doses, leading to severe side effects and toxicity. The present medical calamity of microbial resistance and drug susceptibility has incited the scientific research for exploring other options to treat microbial infections and combat diseases [3,4].

The interdisciplinary scope of nanotechnology has resulted in its global prominence with navigating into nano industrial revolution and applications in catalysis, cosmetics, drug delivery systems, molecular diagnostics, *etc.* [5-7]. The huge variety of the nanoparticles which display new and enhanced size-dependent properties when assessed to their bulk material is being utilized as antimicrobials for doctoring infectious diseases [8]. Several nanodevices *viz.* quantum dots, carbon nanotubes and the polymeric micelles are reported to possess antibacterial properties [9,10] are manufactured as conventional productive nano systems. The surface properties, particle size and porosity can be controlled and tailored to create an equivalent physico-chemical profile of guest components tailored to designated applications [11]. Moreover, by attaching functional groups, stimuli-sensitive molecules and targeting molecules to the silica pore surfaces, both inside and outside, improvement of disperity, delivery of carriers to targeted sites is achieved [12].

Seaweeds are a fascinating and diverse group of marine algal forms living in the seas and oceans that occupy more than 71% of the earth surfaces. Nearly/more than 1, 50,000 algal species are found in the oceans of the globe but only a few of them were identified, under water forests and/or floating on the ocean's surface [13]. *Spongomorpha* is a genus of thalloid, Chlorophycean algae comprising of approximately 14 species occurring in tide pools, mid to low littoral waters of South Asian countries. They were commonly referred as motsure-gusa or hoso-kagimotsure. The thallus takes a crustose form; branching is irregular, reaching around 2 cm in size. Plants are unisexual and reproduce by asexual and sexual reproduction [14]. Optimal culture condition for growth and reproduction of the algae was 5 ºC and long day length. The gametophyte shows abnormal development at 15 ºC. The sporophyte developed normally at 15 ºC, but do not produce zoospores [15].

The present study explains an easy and rapid biosynthesis of AgNPs using silver nitrate solution and aqueous extract of *Spongomorpha indica*, green algae (Chlorophyceae) as reducing agent. It emphasizes an economical and eco-friendly approach with the most effective parameters, like reaction time, concentration of the metal ions and concentration of algal extracts for the synthesis of AgNPs with controlled particle morphology. The green technology of AgNPs synthesis from $AgNO_3$ using aqueous extracts of *S. indica* is the first attempt to the best of our knowledge.

## EXPERIMENTAL

**Collection of algal material:** During low tide, the seaweed species of *Spongomorpha indica* which was exposed on rocks along the coastal areas of Visakhapatnam, India were collected in bulk quantity. The algal material was washed thoroughly under running water to remove attached debris, epiphytes, epizoons, animal castings, sand particles, *etc.* and finally washed with distilled water and air dried.

**Synthesis of AgNPs using aqueous extracts of *S. indica*:** The cleaned *S. indica* was dried in dark and crushed into powder. The extract was prepared by dissolving 1 g of *S. indica* powder in 20 mL deionized water. The crude extract thus developed was further filtered with Whatman No. 1. filter paper for the subsequent reactions. In this typical experiment, 10 mL of 0.005 M, 0.075 M, 0.010 M, 0.0125 M aqueous silver nitrate solutions was mixed with 20 mL of algal extract in different vessels and stirred continuously to obtain reaction mixture. During the reaction, aliquots of the reaction mixture was taken out at regular time span and was measured by UV-vis spectroscopy. The remaining reaction mixture was centrifuged at 12000 rpm, washed in double distilled water followed by acetone and ethanol and finally kept in oven at 70 ºC to remove the moisture content.

**UV-vis analysis:** The UV-vis spectra were measured for different metal ion concentration with aqueous extract of *S. indica* at various specific time periods. The absorption peak readings were taken on aliquots and analyzed at 0 min and then at 2 h intervals up to 16 h, followed by 24 h and 2 days.

**HP-TLC analysis:** Sample was applied on to the plate with programmed CAMAG Linomat 5 Linomat 5_211497 (Camag HPTLC system with Linomat 5 sample applicator) and TLC scanner (CAMAG TLC, Scanner_220256) with CATS software for quantitative evaluation. A 20 cm × 20 cm twin trough glass chamber were used for developing the plates. TLC plates 60 $F_{254}$ 10 × 10 cm with 0.2 mm thickness with silica aluminum gel coating (Merck, Germany) was used for all trials. Five different sample extracts (ethanol, methanol, acetone, chloroform, ethylacetate) along with two standards namely gallic acid (phenol) and quercitin (flavonoid) were applied on TLC for the development of calibration curve. A steady application of 0.2 µL was exercised with a band width of 6 mm. The dimension of slits was kept at 4.00 mm × 0.30 mm and the speed of scanning was of 20 mm/s. The bandwidth between the tracks was set at 11.6 mm. The mobile phase contained ethylacetate:acetic acid: formic acid:water at 68:7.5:7.5:17. Each plate consumed 15mL of mobile phase. The chamber saturation time of mobile phase was optimized to 20 min at relative humidity of 60% at room temperature (25 ºC). The chromatogram was run for 7.0 cm. The chromatographic plates were air dried for 10 min. After drying, the plates were placed in for 15 min at 110 ºC in a preheated oven and the plates were scanned immediately using densiometric scanner in remission mode at 254 nm. The spots or peaks detected were calculated for their $R_f$ values and area under the peak.

**Antibacterial activity:** Antibacterial activity was carried out for biosynthesized AgNPs against pathogenic Gram-positive *S. aureus*, *S. pyogenes*, *B. pumilis*, *B. subtilis* and Gram-negative *K. pneumoniae*, *P. aeuriginosa*, *P. vulgaris*, *E. coli* bacterial strains. All the cultures of bacteria were obtained from National Collection of Industrial Microorganisms (NCIM) laboratory, Pune, India. The antibacterial activity testing was performed by cup-plate agar diffusion method as described earlier [16]. Different concentrations were obtained by serial dilution from the original solution of 10 mM concentration of $AgNO_3$ with 8 mL of aqueous algal extract for a reaction time of 2 h. The tests were carried out in triplicates and the average inhibition zone diameter was measured.

**Determination of MIC:** Minimum inhibitory concentrations (MIC) was reported for two bacterial strains namely *Bacillus subtilis* and *Escherichia coli*, which displayed maximum susceptibility by macrodilution broth method [17] using Muller-Hinton broth following the standards. A 1 mL of reaction mixture sample of various concentrations ranging from 3.125 to 100 mg/mL in a two-fold serial dilution were added (spread plate technique) and placed in an incubator at 30 ºC for 8 h, respectively. The plates were checked for viability by observing the colonies. Chloramphenicol (100 µg/mL) was used as standard and DMSO as control.

**Antifungal activity:** The fungal test organisms *Aspergillus niger* (ATCC 430), *Aspergillus fumigatus* (ATCC 439), *Aspergillus flavus* (ATCC 433), *Saccharomyces cerevisiae* (ATCC 656) and *Candida albicans* (ATCC 523) were studied in this work. The cup and plate agar diffusion method as described by Lakshmi *et al.* [16] was followed to assess the antifungal activity. Different concentrations were prepared by serial dilution from stock solution of 10 mM concentration of $AgNO_3$ with 8 mL of aqueous algal extract for a reaction time of 2 h. PDA petriplates

were incubated at 37 ºC for 48 h. Experiments were performed in triplicate and the average diameter of inhibition zones was measured.

**Antioxidant assay:** The AgNPs were also tested for antioxidant property by DPPH assay following the standard procedure as described by Brand-William *et al.* [18]. Various concentrations of 3.125 µg/mL to 100 µg/mL of AgNPs was considered. The standard solution was ascorbic acid, whereas the positive control was gallic acid solution. The solvents without DPPH were regarded as blank, while the solvent with DPPH were utilized as negative control. Triplicate measurements were performed for all experiments and absorbance values were recorded at 517 nm. The percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

A graph of percentage inhibition against concentration was also plotted to determine the $IC_{50}$ value.

**Cyotoxicity assay:** The anticancer activity was assayed by performing the cytotoxicity assay with HCT 116 cell lines. The McCoy's 5a modified medium containing 10% fetal bovine serum was used to culture the cell lines in corning T-75 flasks incubated in a humidified atmosphere consisting of 5% $CO_2$ at 37 ºC. The assay was carried out at 70 to 80% confluence by seeding the cells to 96 well plates. After 24 h of incubation, the medium in the cell culture was replaced with 250 µL and 500 µL of AgNPs. Cells without any treatment were considered as control. All the tests were carried out in triplicate and cultured for 24 and 72 h. Upon completion of the treatment period, each well received MTT with a concentration of 5 mg/mL, with PBS. This was again incubated at 5% $CO_2$ for 3 h. Subsequently, 150 µL of DMSO was added to all wells to dissolve the formazan crystals and absorbance was read at 570 nm using a microplate reader [19].

## RESULTS AND DISCUSSION

The biosynthesis of AgNPs from *Spongomorpha indica* sea weed is confirmed with UV-vis spectroscopy, with variations in parameters including the quantity of algal extract and metal ion concentration, allowing the process to occur for 2 h at ambient temperature. The influ-ence of metal ion concentration on the synthesis of AgNPs were studied at different concentrations of $AgNO_3$ (ranging from 5-12.5 mM) by fixing the concentration and amount of algal extract (10 mL of 20%). The strong surface plasmon resonance peak at approximately 434 nm confirms AgNP formation, with its intensity steadily increasing without a shift as the metal ion concentration ranged from 5-10 mM. Further by increasing the metal ion concentration to 12.5 mM, the shifting of peak position to greater wavelength with higher width represents the increase in the size of AgNPs. Further optimization was carried out by the varying the algal extract concentration ranging from 20-100%, setting metal ion concentration at 10 mM. The UV-vis spectra analysis revealed that as algal extract concentration increased from 20% to 80%, peak intensity increased sharply. At 100%, peak broadening indicated rapid AgNP formation, possibly causing agglomeration or larger particle sizes. The SPR peak in spectra of AgNPs occurs at 434 nm acting as a specific indication of the AgNP formation. The spectra recorded for every 2 h time interval and its absorption maxima peak progressively increased. However, UV-Vis spectra indicated that the colour intensity did not increase beyond 16 h. Subsequent spectra recorded at 24 h showed no further increase in absorption, confirming reaction completion within 16 h. AgNPs showed no change in peak wavelength or intensity even after 2 days (Fig. 1).

**Morphological studies:** The biosynthesized AgNPs were subjected for SEM analysis at different resolutions (FEG-SEM: JSM-7600F), which displayed a mixture of spherical and cuboidal structures with an average particle size of 18 nm. The SEM images also showed larger particles which might be the aggregates of some smaller particles (Fig. 2).

**XRD studies:** Face-centered cubic (FCC) patterns have been identified for biosynthesized AgNPs by X-ray diffractometer (PAN analytical: XPERT-PRO). The results are presented as peak positions at 2θ and intensity of X-ray counts in the form of X-Y plot. The peaks obtained in XRD at 2θ of 30.409º, 44.652º, 64.757º and 77.661º could be correspond to the (111), (200), (220) and (311) crystallographic planes (Fig. 3), which indicate the crystalline nature of AgNPs formed (JCPDS file no. 01-087-0717). The unknown phases were determined by comparing of their *d*-spacings with the d-spacings of known material. The Debye-Scherrer formula was used to calculate average nanocrystalline size, particle diameter size $D = k\,\lambda/\beta \cos\theta$, where k is a constant equals 1; λ is wavelength of X-ray source (0.1541 nm); β is the FWHM (full width at half maximum) and θ is the diffraction angle corresponding to the lattice plane (111). The crystallite particle size (average dimensions) calculated from XRD analysis was found to be 18 nm, which is fairly in accordance with Debye-Scherrer equation.

**HPTLC analysis:** Among the different solvent extracts of *S. indica*, ethanol extract exhibited maximum level of phytocompounds followed by acetone, while the remaining extracts showed lower level. HPTLC chromatogram data (peaks, $R_f$ values and area) for solvent extracts, obtained at UV 254 nm are shown in Fig. 4, which displayed the clear separation of all the sample constituents without any tailing and diffusion. It is evident that six peaks were observed for ethanol extract containing a minimum of six different components. Among the six components, the ones exhibiting $R_f$ values 0.24, 0.29, 0.33 occupied major area and the intensity of area was found to be 11748.4, 14666.5 and 12966, respectively. Apart from these, methanol extract exhibits 3 peaks, acetone extract exhibits 5 peaks followed by 2 peaks with chloroform and ethyl actetate extracts. The peak pattern of HPTLC chromatograms of extracts (Fig. 4a-e) is found similar to the standard gallic acid (phenol) (Fig. 4f), rather than quercitin, which is a flavonoid (Fig. 4g). This shows the presence of phenolic compounds especially gallic acid as major phytocompounds which are primarily responsible in AgNP synthesis. Similar findings stood concluded by Li *et al.* [20]. According to their report, gallic acid mediated synthesis of AgNPs with antimicrobial action and low cytotoxicity.

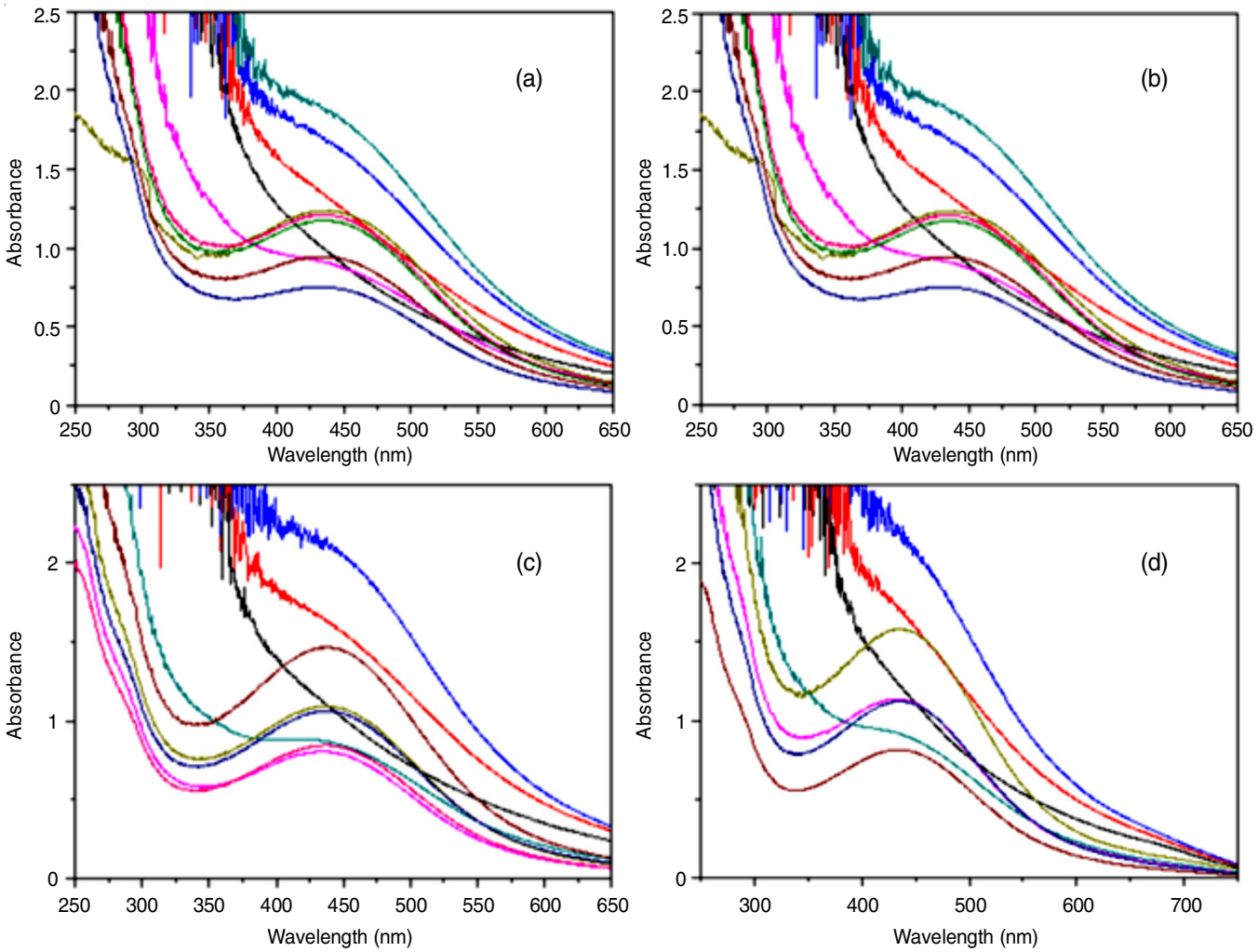



Fig. 1. UV-vis absorption spectra of various concentrations of $AgNO_3$ in aqueous algal extract; (a) 5 mM, (b) 7.5 mM, (c) 10 mM, (d) 12.5 mM

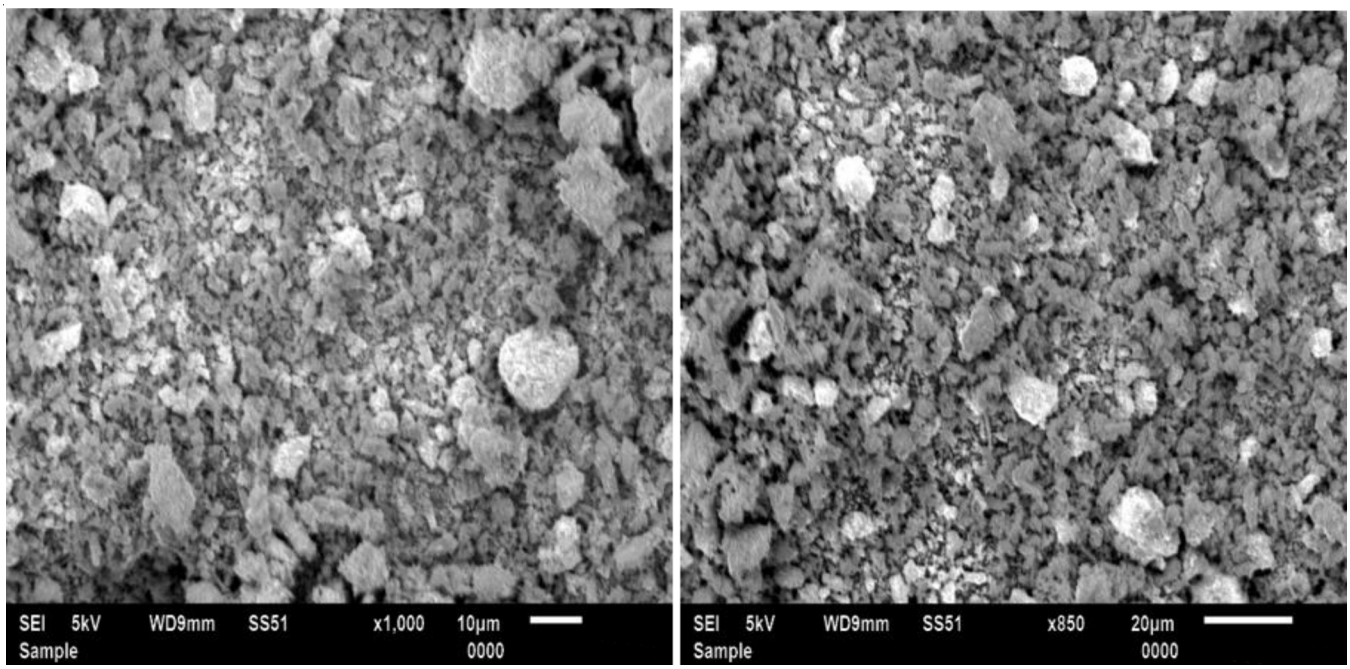



Fig. 2. SEM images displaying the spherical and cuboidal structures of AgNPs

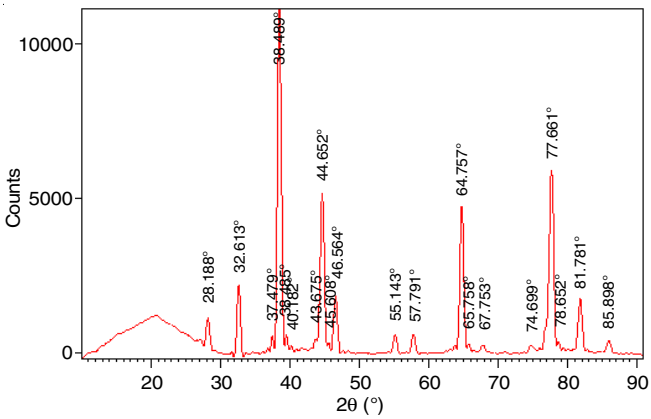



Fig. 3. Face-centered cubic (FCC) patterns identified for biosynthesized AgNPs by X-ray diffractometer (PAN alytical: XPERT-PRO)

**Antibacterial activity:** The bacterial test organisms exhibited maximum susceptibility in the presence of biosynthesized AgNPs extracted using algal extracts of *S. indica.* The activity is more pronounced against Gram-positive bacteria compared to Gram-negative bacteria and were improved with increased dosage levels. They exhibited slight activity towards *Bacillus pumilis* and *Proteus vulgaris* demonstrated appreciable activity against *K. pneumonia*, *S. pyogenes*, *S. aureus* and the maximum activity against *B. subtilis*, *E. coli* and *P. aeuriginosa* (Table-1).

**MIC:** Bacterial growth was inhibited in a concentration-dependent manner was observed with increased AgNPs concentrations. The inoculated plates containing *B. subtilis* and *E. coli* exhibited MIC values ranging from 6.25 to 12.5 mg/mL and 12.5 to 25 mg/mL, respectively. The plates above 25 µg/mL exhibited no bacterial growth, which was evident as identified from lacking any colony formation in the plates compared to the control plates. Fig. 5 displays the growth inhibition of *B. subtilis* exposed to different concentrations of AgNPs.

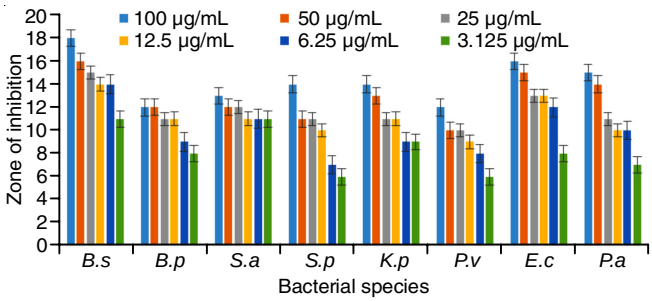



Fig. 5. Graph of antibacterial assay with minimum inhibitory concentration

**Antifungal activity:** The algal synthesized AgNPs exhibited reasonable activity against *A. niger* and *A. fumigatus,* exhibited slight activity towards *A. flavus* and almost no activity

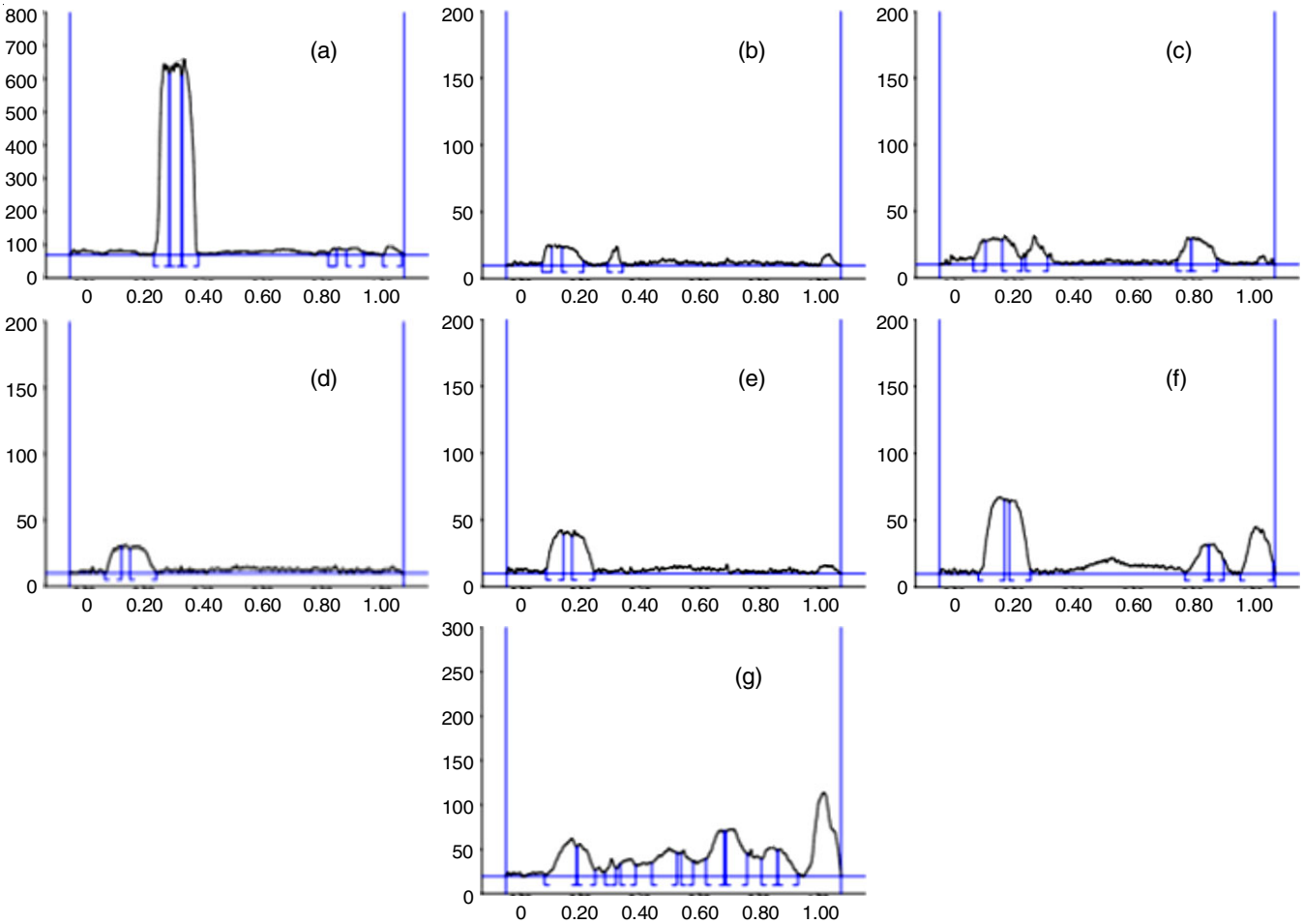



Fig. 4. HP-TLC chromatogram of various extracts of *Spongomorpha indica*; (a) ethanol extract, (b) methanol extract, (c) acetone extract, (d) chloroform extract, (e) ethyl acetate extract, (f) standard gallic acid (phenol), (g) standard quercetin (flavonoid)

TABLE-1
THE ANTIBACTERIAL ACTIVITY OF AgNP MADE FROM AQUEOUS EXTRACTS OF *Spongomorpha indica*

| Test sample (µg/mL) | Gram-positive bacteria | | | | Gram-negative bacteria | | | |
|---|---|---|---|---|---|---|---|---|
| | *S.a* | *B.p* | *B.s* | *S.p* | *K.p.* | *E.c* | *P.v* | *P.a* |
| 3.125 | 11 | 8 | 11 | 6 | 9 | 8 | 6 | 7 |
| 6.25 | 11 | 9 | 14 | 7 | 9 | 12 | 8 | 10 |
| 12.5 | 11 | 11 | 14 | 10 | 11 | 13 | 9 | 10 |
| 25 | 12 | 11 | 15 | 11 | 11 | 13 | 10 | 11 |
| 50 | 12 | 12 | 16 | 11 | 13 | 15 | 10 | 14 |
| 100 | 13 | 12 | 18 | 14 | 14 | 16 | 12 | 15 |
| Control (DMSO) | 10 | 08 | 7 | 09 | 08 | 07 | 08 | 10 |
| Antibiotic (CP) | 25 | 26 | 23 | 29 | 21 | 27 | 23 | 26 |

Cup diameter = 6 mm, *S.a = Staphylococcus aureus*, *B.p = Bacillus pumilis, B.s = Bacillus subtilis S.p = Streptococcus pyogenes, K.p = Klebsiella pneumoniae, E.c = Escherichia coli, P.v = Proteus vulgaris P.a = Pseudomonas aeuriginosa*, Antibiotic CP: Chloramphenicol, Zone of inhibition (6 mm)

TABLE-2
ANTIFUNGAL PROPERTY OF AgNP SYNTHESIZED FROM AQUEOUS EXTRACTS OF *Spongomorpha indica*

| Test sample (µg/mL) | *Aspergillus fumigatus* | *Aspergillus flavus* | *Aspergillus niger* | *Candida albicans* | *Sacharomyces cerevisiae* |
|---|---|---|---|---|---|
| 3.125 | 7 | – | – | – | – |
| 6.25 | 8 | 7 | – | – | – |
| 12.5 | 10 | 8 | 8 | – | 7 |
| 25 | 11 | 9 | 12 | 7 | 7 |
| 50 | 11 | 10 | 12 | 7 | 8 |
| 100 | 13 | 12 | 13 | 8 | 8 |
| Control | 08 | 09 | 09 | 08 | 08 |
| Antibiotic (Standard) | 23 | 19 | 23 | 21 | 20 |

Cup diameter: 6 mm

towards *S. ceriviseae* and *C. albicans* (Table-2). AgNPs causes inhibition of mycotic cell cycles and membrane disruption owing to the presence of cationic silver that causes membrane depolarization and pits in the fungal cell wall [21,22]. In present study, the mild antifungal activity might be due to the lack of poor penetration of nanoparticles into the fungal components.

**Antioxidant assay:** The inhibition percentage of biosynthesized AgNPs at 12.5 µg/mL was found to be maximum (62.34%), however, the maximum inhibition percentage (83.21%) of ascorbic acid was found to be at 6.25 µg/mL. The $IC_{50}$ values of ascorbic acid (standard) and AgNPs appeared to be 0.41 µg/mL and 0.62 µg/mL, respectively (Fig. 6).

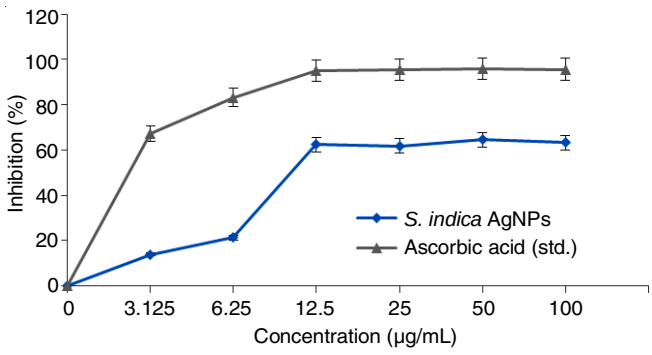



Fig. 6. Graph of antioxidant assay

**Cytoxicity assay:** The AgNP demonstrated exceptional cytotoxic properties. The cell death was more severe in 72 h culture than in 24 h culture when compared to the control cells. After 24 h of treatment, the percentage of cell death was higher in 500 mL sample. After 72 h of treatment, the higher concentration exhibited significantly increased cell toxicity, while the lower concentration of 250 mL demonstrated reduced cytotoxic effects.

## Conclusion

This study demonstrates that eco-friendly and simple green synthesis of AgNPs using *Spongomorpha indica*, halloid Chlorophycean alga, could be a competitive alternative to conventional chemical methods. The phenolic and alkaloid contents and seasonal change of this seaweed have been studied. The biosynthesized AgNPs were spherical and crystalline with controlled morphological size of 18 nm. Biosynthesized AgNPs showed dose-dependent antibacterial activity. The antioxidant activity was effective, with an $IC_{50}$ value of 0.62 µg/mL for DPPH. They also strongly promoted apoptosis, which inhibited human colorectal cancer cells. Thus, the controlled nanoparticle shape by biological reduction using algal extracts, which could be used in biosensing, microelectronics, biodiagnostics, bioimaging and drug delivery systems for the cancer treatment.

## ACKNOWLEDGEMENTS

The authors are thankful to Dr. Y Sarojini, Women Scientist, College of Science and Technology, Andhra University, Vizag, India for providing the authenticated material sea weed *S. indica.*

## CONFLICT OF INTEREST

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

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