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ANTIBACTERIAL ACTIVITY AND IN-VITRO CYTOTOXICITY ASSAY AGAINST BRINE SHRIMP USING SILVER NANOPARTICLES SYNTHESIZED FROM *SARGASSUM ILICIFOLIUM*

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The increasing commercial applications of nanoparticles have wide pertinent in the field of medicine. Biological synthesis of Silver nanoparticles (AgNPs) are effective and environment friendly than chemical methods. In the present study, we demonstrated extracellular synthesis of AgNPs from *Sargassum ilicifolium* rapidly. The UV-Vis spectra of aqueous medium containing Ag^+ ions showed peak at 414 nm corresponding to the plasmon resonance of AgNPs. SEM and TEM analysis showed formation of well-dispersed AgNPs in the range of 33-40 nm. Antibacterial activity against five clinical pathogens at various concentrations shows impediment in growth at various nanomolar concentrations. Further, the toxicity of biosynthesized AgNPs was tested against *Artemia salina* to evaluate the cytotoxic effect that displayed LD_{50} value of 10nM/ml.

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1. Introduction

Rapid development in the synthesis of nano-sized materials has become important in the field of nanotechnology and its wide applications in biomedical science. Assessment based on toxicity of nanoparticles (NPs) dimension below 100 nm exhibit improved properties for the welfare of living systems and their efficacy inside their system is not well understood [1]. At present, a number of approaches are being used for the synthesis of silver nanoparticles (AgNPs) but still the need for economic, commercially viable, environmental safe synthesis of AgNPs is an issue [2-11]. Biological synthesis of AgNPs has an outstanding numerous benefits without the use of toxic chemicals and posses advancement over both physical and chemical methods [12-15].

Mono-valent silver ions are used extensively for antimicrobial treatment over decades that have impediment over microorganisms even before antibiotics were introduced. Moreover, use of silver played a vital role in water purification, wound dressing, dental hygiene and eye related problems. After the discovery of penicillin [16, 17] which minimized the use of silver against bacterial infection in this decade. Now, due to the development of antibiotic-resistant strains that possess a threat to clinical disease have been a research of interest. Silver have been established for the second time against bacteria in the form of complex or colloids, which possess antibacterial activity. The use of metal particles that are nano-sized exhibit special properties with increased catalytic activity than its normal state or bulk material.

Sargassum ilicifolium are widespread along the coast of India and little information is conceded about its chemical constituents and biological activity. Several species of brown algae

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are found to have immune-stimulant, antitumoral and antiviral activity [18]. This provides valuable ideas for the development of new drugs from commercially important marine renewable resources such as seaweed. In the present study, we reported the synthesis of AgNPs from *S. ilicifolium* and examined its antibacterial potency. Further, brine shrimp cytotoxicity assay was conducted, because it is considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and can be extrapolated for cell-line toxicity and anti-tumor activity [19, 20].

Naturally, occurring seaweed poses antitumour compounds and are being exploited for their beneficiary purpose. Brine shrimp assay provides better prescreening of nanoparticles having antitumour activity [21]. To our knowledge, the present study demonstrated on biological synthesis of AgNPs in *S. ilicifolium* has not yet been reported earlier and it is the first report to examine AgNPs bactericidal efficacy and evaluate cytotoxicity using brine shrimp, *Artemia salina* lethality assay.

2. Experimental details

2.1. Sample collection and extract preparation

Sargassum ilicifolium were collected along the coast of Puthumadam, Gulf Of Mannar Marine Biosphere Reserve Trust (GOMBRT), brought to the laboratory; shade dried and grounded using mortar and pestle. About 100 mg of mixture was dissolved in 100 ml of deionized water, boiled to 80°C and the extract was filtered using whatman no.1 filter paper followed by centrifugation at 8000 rpm for 10 minutes to remove residual debris. The algal extract (AE) was stored in refrigerator until further analysis.

2.3 Synthesis of silver nanoparticles (AgNPs)

To 100 ml of prepared AE, 100 µl of 1M silver nitrate solution was added to form 1mM $AgNO_3$ solution. The aqueous medium was heated up to 60 °C for 20 min and the change in color to dark brown was visualized. The bioreduction was characterized by using UV-Vis spectroscopy, Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM)

2.4 Characterization

2.4.1 UV-Vis Spectrophotometer

The bioreduction of Ag^+ ions was monitored by UV-Visible spectrophotometer (model-UV-2450 Shimadzu) immediately after the synthesis of AgNPs by diluting a small aliquot of samples into distilled water.

2.4.2 Scanning Electron Microscope

Scanning Electron Microscope (Hitachi-S-4500) analyses was done by preparing thin films of samples in carbon copper grid and were allowed to dry by placing them in mercury lamp for 5 min.

2.4.3 Transmission Electron Microscope

Samples for Transmission Electron Microscopic (TEM) analysis were prepared by drop coating Ag nanoparticles solutions onto carbon coated copper TEM grids. The films on the TEM grids were allowed to dry and remaining solution was removed using a blotting paper. TEM measurements were performed on a TECNAI instrument operated at an accelerating voltage of 80 keV.

2.5 Preparation of nanomolar concentration for AgNPs

X. Liu *et al* [22] and Kalishwaralal *et al* [23] have previously reported the preparation of nanomolar concentration (nM) in gold and silver respectively and the same procedure was adopted throughout the experiment for both antibacterial activity and brine shrimp lethality assay.

2.6 Antibacterial activity

Antibacterial activity of AgNPs were performed against bacterial cultures brought from Microbial Type Culture Collection Centre (MTCC), Chandigarh, India such as *Escherichia coli* (MTCC 1687), *Klebsiella pneumoniae* (MTCC 530), *Salmonella typhii* (MTCC 531), *Staphylococcus aureus* (MTCC 96), and *Vibrio cholerae* (MTCC 3906). Disk diffusion method was adopted to evaluate bactericidal activity against test strains on LB agar (Luria Bertani agar). The plates were incubated at 37 °C for 24 h and the zone of inhibition (ZoI) was measured using vernier caliper. The experiments were performed in quadrates to calculate mean.

2.7 Brine shrimp lethal assay

Brine shrimp *Artemia salina* cysts were purchased and maintained in the laboratory conditions and were used for cytoxicity assay [21]. Briefly, *Artemia salina* cysts of 1 gm were aerated in 1 L capacity of glass jar containing 3.2 % of saline water (3.2 gm NaCl in 100 ml of distilled water). The jar was aerated constantly for 48 h at room temperature (25-29 °C). After hatching, active free-floating nauplii was collected from bright illumination and were used for the bioassay. Experiment was performed in 2 ml sterile eppendorf with 3.2 % of saline water. Parallel vehicle control (with AE) and negative control (without AE and AgNPs) was also included for the experimental setup. Each nauplii were transferred with the addition of desired concentration of AgNPs (from 10 nm to 100 nm) respectively. The experimental setup was allowed to remain 24 h in darkness and nauplii were counted after incubation time. Percentages of mortality, LD_{50} for tested concentration of AgNPs were determined using probit analysis [24].

3. Result and discussion

3.1. Synthesis and characterization of silver nanoparticles:

The colloidal AgNPs suspended in the aqueous medium pose dark brown color (Fig.1) after the process of bioreduction formed due to Surface Plasmon Resonance (SPR) [25]. Absorption spectra of AgNPs have been recorded at 414 nm after 20 min of reaction time, indicates the change in reaction when compared to the AE without silver nitrate. Due to transition of electrons, AgNPs strongly absorb radiation near wavelengths of 400 nm and 300 nm (Fig. 2). Scanning Electron Microscope (SEM) results reveal spherical shaped particles with 40 nm in size (Fig. 3). Further, the size of AgNPs was also determined by using Transmission Electron Microscope (TEM) with an average size of 33 - 44 nm with spherical morphology (Fig. 4). The particles were distributed evenly in the aqueous medium due to some repulsive forces exerted by these particles.

Fig 1. Color change of Algal Extract (AE) after the synthesis of AgNPs

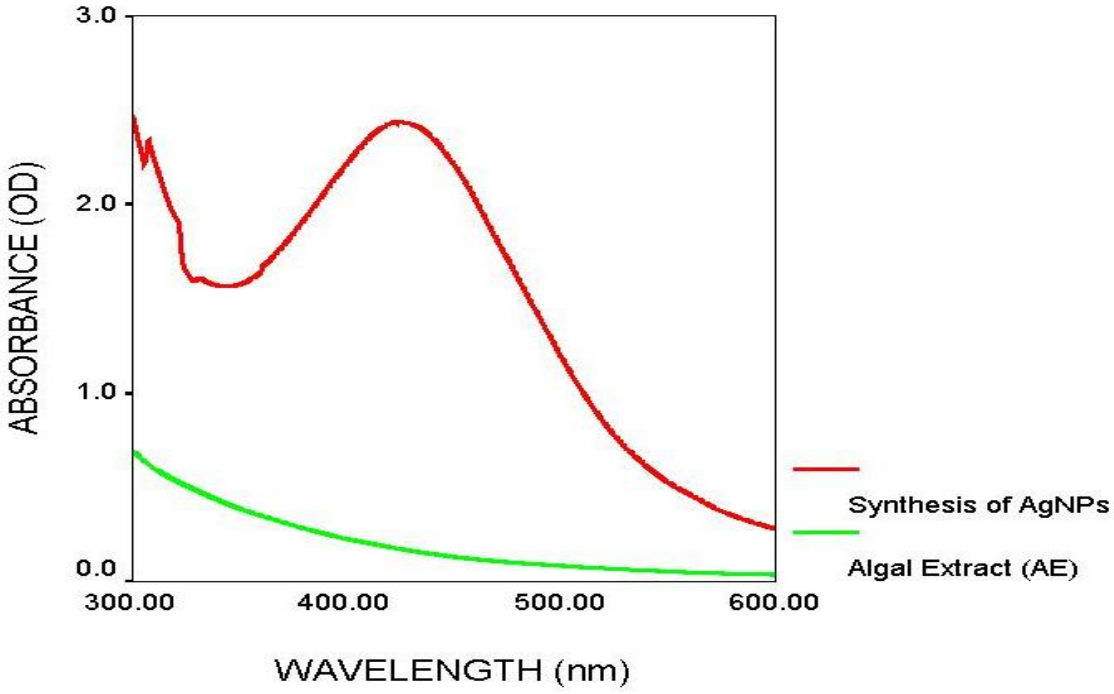

Fig 2. UV-Vis absorption spectra of AgNPs synthesized at 414 nm

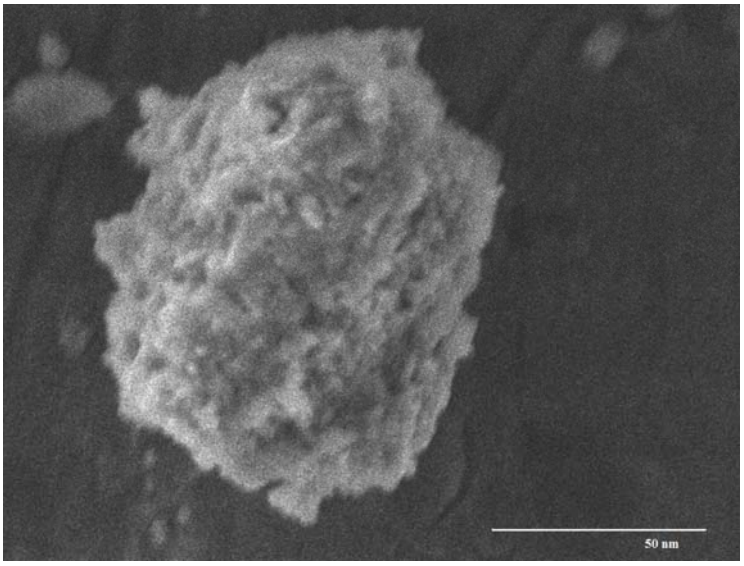

Fig 3. SEM image of AgNPs formed by S. ilicifolium

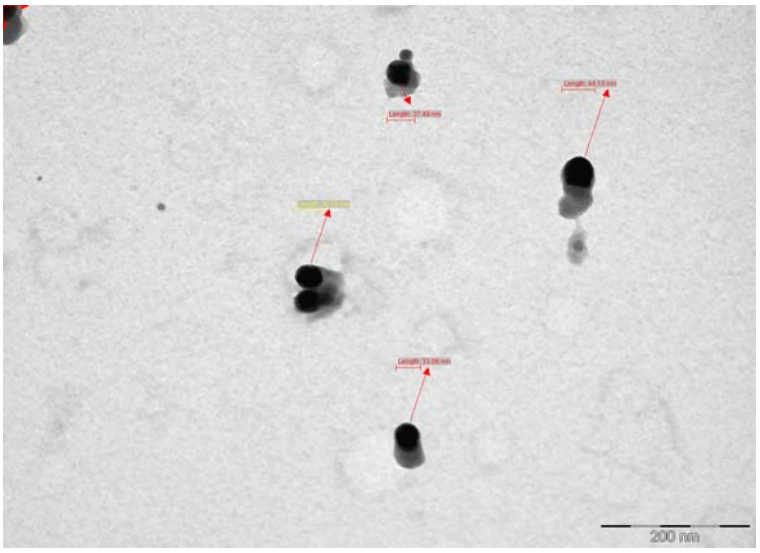

Fig. 4. TEM image of AgNPs recorded from a small region of drop-coated film

3.2. Antibacterial assay

The bactericidal potency of silver nanoparticles was tested against five prominent pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhii*, *Staphylococcus aureus*, and *Vibrio cholerae* (Fig. 5). Nanomolar concentrations of AgNPs were highly effective against all pathogens. The maximum zone of inhibition was found at 100 nM compared to 10nM and 50 nM (Table. 1). In contrast, AE possess no zone of inhibition against any of the pathogenic strains used. The synthesized AgNPs acts against cell wall by disrupting the bacterial cells by inducing toxicity have reported earlier [26, 27]. Normally AgNPs increases the permeability of cell wall and finally cause cell death [28].

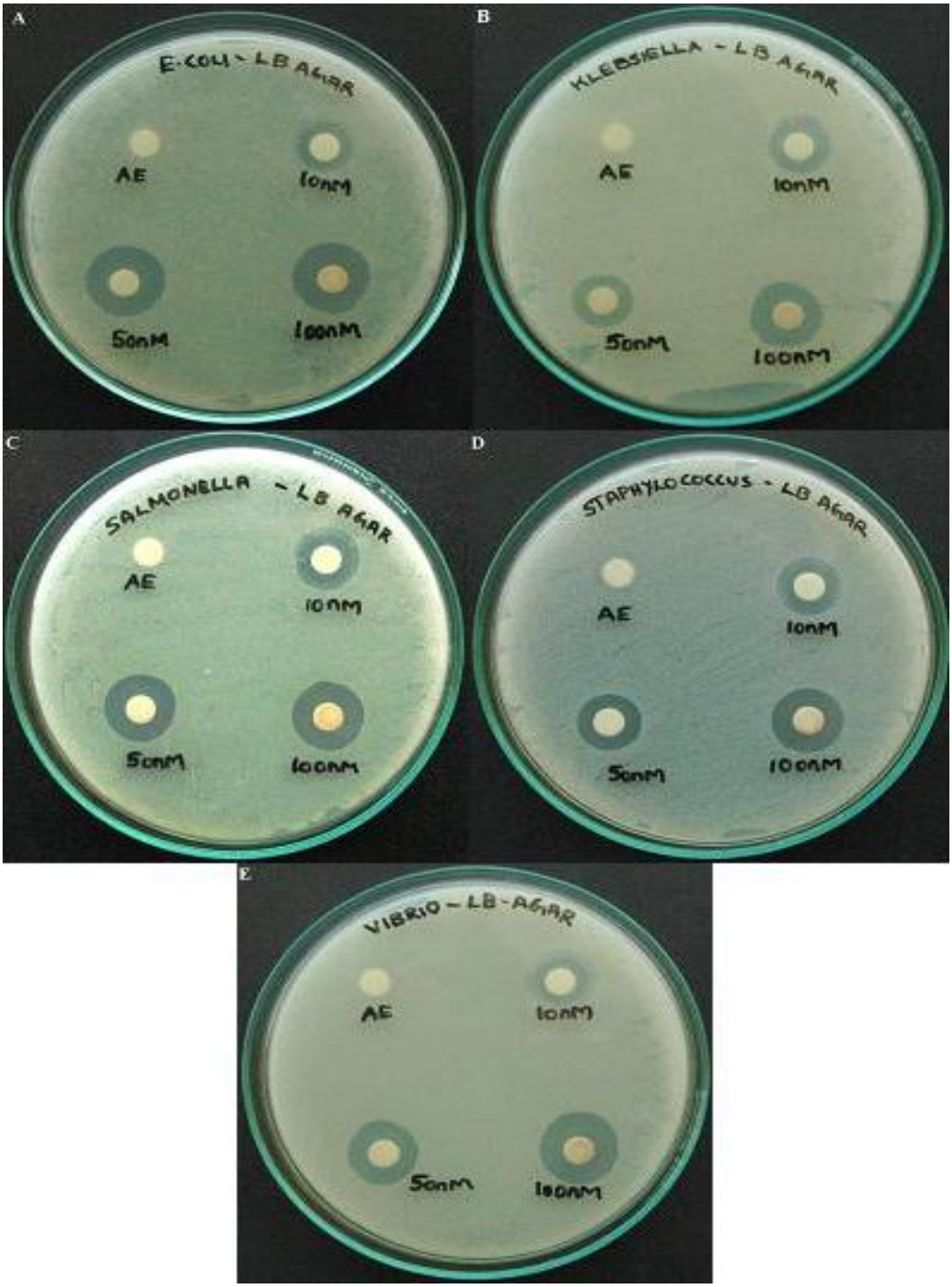

Fig. 5. Antibacterial effect of AgNPs against prominent pathogenic strains.

Table. 1. Antibacterial activity by measuring Zone of Inhibition (mm).

Strains used	Zone of Inhibition in mm			
	AE	**10 nM**	**50 nM**	**100 nM**
E. coli	0	12.6	16.8	18.2
K. pneumoniae	0	13.2	13.6	16.2
S. typhii	0	14.2	16.5	17.1
S. aureus	0	13.5	13.9	16.8
V. cholerae	0	12	15.3	17.3

3.3. In-vitro cytotoxicity assay

It has been demonstrated that, early developmental stages of *Artemia* is highly vulnerable to toxins [20, 29]. In cytoxicity test, *Artemia salina* tested with AgNPs showed outstanding results when compared to AE (Fig. 6). Literature related to cytotoxic effect of *S. ilicifolium* against *A. salina* is insufficient. The lethality was found to be directly proportional to the concentration of extract. Maximum mortality rate was observed at 100 nM concentration while 50 % mortality was observed at 10 nM concentration. Aseer Manilal [31], have reported 100 % of inhibition of hatching after 24 h at lower concentration of about 100µg/ml in *Laurencia brandenii* algal fraction. Similarly Kladiet and Zubia *et al* [32, 33], have reported that the extract of *Laurencia obtuse* and *Asparagopsis Armata* have potent cytotoxic effect against cancer cell lines. El-Baroty *et al* [34], have studied, the cytotoxic effect of powdered *Asparagopsis taxiformis* against *Daphna magna*. Similarly, various cytotoxic effects against cancer cells have been reported using algal extract when compared to AgNPs [35, 36, 37]. Seaweeds possess cytotoxic compound such as fucoidans, laminarians and terpeniods, which have anticancer, antitumour and antiproliferative properties [18]. Identification of these compounds can lead to discovery of novel products from seaweeds to prevent cancer [39]. Brine shrimp lethality test, to detect antitumoural compounds is simple and it has been reported in many terrestrial plants extracts. In the present study, AgNPs have showed better cytotoxic effect against *Artemia salina*.

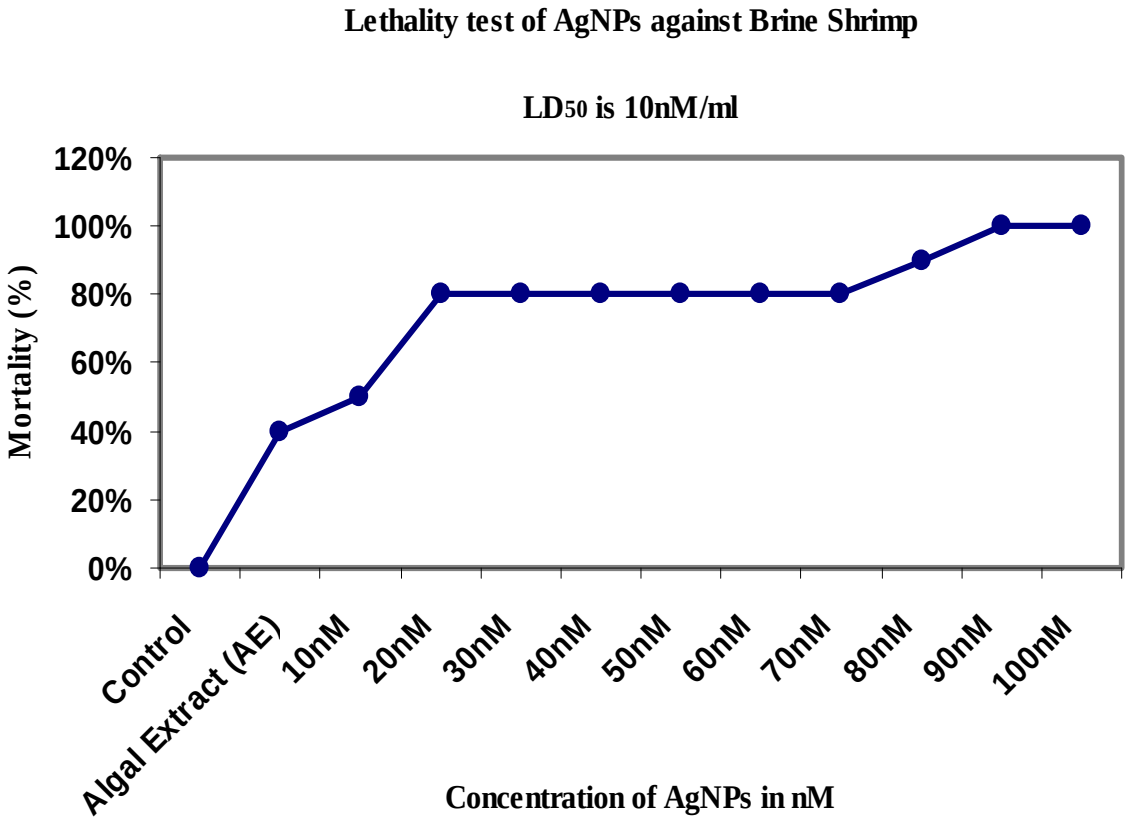

Fig. 6. Effect of synthesized AgNPs on Brine shrimp

4. Conclusion

To conclude, this method is simple and rapid for synthesis of colloidal silver nanoparticles, which have been accomplished by bio-reduction of seaweed *Sargassum ilicifolium*. An important potential benefit of the described method is that they are quite stable in solution. Antibacterial activity of the synthesized AgNPs is prominently effective among various bacterial isolates. While brine shrimp lethality assay elucidates their importance in pharmacological industry. Further, the study of active compounds present in seaweed after the synthesis of AgNPs can develop novel drugs for human welfare in near future.

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