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Synthesis of silver nanoparticles using red algae, characterization and effect on beneficial soil microbes

V. Murugammal[1], G. Flora[2]*

Department of Botany, St. Mary's College (Autonomous), Thoothukudi-628 001, Tamil Nadu, India.

E-mail : florachandran@gmail.com Contact No. : +91 - 9976940601

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Abstract

The synthesis and characterization and application of biologically synthesised nanomaterials are important aspect in nanotechnology**.** The present study focused on the synthesis of silver nanoparticles using red algae characterization and effect on beneficial soil microbes. The formation of AgNPs were performed by UV-Visible spectrophotometer revealed surface plasmon resonance at 420nm .The structure and size distribution of the silver nanoparticles were examined by Transmission Electron Microscopy (TEM) which showed that the particles are spherical in shape with size ranging from 50nm. Finally the compounds responsible for the silver nanoparticles biosynthesis were studied using Fourier Transmission-Infrared (FT-IR) spectroscopy analysis showed that the synthesised nano-Ag was capped with bimolecular compounds which are responsible for reduction of silver ions. Moreover the microbes isolated from garden soil of synthesised silver nanoparticles exhibited potential inhibitory activity against ten tested microbes showed that (*Bacillus spp, Bacillus substilis, Staphyllococcus epidermidis, Serratia spp*, *Pseudomonas fluorescens*, *Pseudomonas spp, Aspergillus flavus*, *Aspergillus fumigates*, *Alternaria altermata*, *Cladosporium spp)*.

Key words : Antioxidant activity, FT-IR, Marine macroalgae -*Rhodymenia palmata*, Silver nanoparticles, Soil microbes, TEM and UV- Vis.

INTRODUCTION

Nanoparticles (NPs) of different size and physicochemical properties have been introduced to many fields of life and biomedical sciences over the last decade [1]. In this respect, NPs opened a new era in biomedical sciences and have been used specifically as gene or agent carriers, and in drug design, modification of therapeutics, labeling of fluorescents, and tissue engineering [2-6].Among the NPs, silver nanoparticles (AgNPs) have received attention for their antimicrobial activities [7,8] and have been used for different purposes including the manufacture of disinfectants, shampoos, deodorants, humidifiers, wound dressings, and various textile products [9-11], they have also been used as a coating for various implantable devices such as catheters, heart valves, and implants [12,13]. Despite their benefits, there has been serious concern about the possible side effects of AgNPs.

MATERIALS AND METHODS

COLLECTION AND PREPERATION OF FOR ANALYSIS

The red algae *Rhodymenia palmata* Grev. **(**Rhodophyceae) was collected from punnakayal, Thoothukudi district. The collected sample were washed with sea water and immediately transported to a laboratory in polythene bags containing natural sea water to prevent evaporation. Algal material was washed with distilled water to remove the dust and soil. After cleaning, the fresh algae were shade dried at room temperature for a week. Collected seaweed was identified on the basis of pigmentation, morphology and authenticated by Dr.P.Anantharaman, Associate Professor, CAS in Marine Biology, Annamalai University in Parangipettai, India. Dried seaweeds were powdered with the help of mixer grinder. Seaweed was collected during low tide in the forenoon during January 2016.

PREPERATION OF SEAWEED EXTRACT

The seaweed powder (5g) was soaked for 24h in 1L of sterile water. Then the crude extract was blended thoroughly and filtered using a Whatman No.1 filter (24µm) twice. The filtrate was used for further analysis.

SYNTHESIS OF SILVER NANOPARTICLES

In the seaweed extract, 1mM silver nitrate solution was added. The reduction of silver nitrate occurred within 10min which resulted in colour change (dark brown), as noted by visual observations indicating the formation of AgNPs. As per the absorption spectrum, this medium remained stable for more than 3 months. The absorbance of aliquots of the reaction solution was measured using a UV-2371 spectrophotometer operated at a resolution of 1nm [14].

ANTIOXIDANT ACTIVITY

Seaweeds contain many phytochemicals including compounds with antioxidant activity, which are mostly phenolic compound [15, 16]. Compounds with antioxidant activity are mainly phenolic acids, flavonoids and polyphenols, so content of total phenol [17], flavonoid [18], tannin [19], terpenoid [20, 21] and tocopherol [22], were investigated in *Rhodymenia palmata* and *Rhp*-AgNPs (aqueous extract).

CHARACTERIZATION OF Ag-NPs

The different techniques were used to characterized the *Rhp*-AgNPs such as UV-vis (2371) used to know the band at nanometer, FT-IR (Fourier Transform Infrared spectroscopy) from (Systronics 166) type FTIR spectrometer which was used in the range 400 to 4000 cm^{-1} by KBr pellet method for extract powder and silver nanoparticles TEM (JEM 2100) analysis were carried out to confirm the image of specimen by magnified focus on imaging device.

ISOLATION OF SOIL MICROBES [23, 24]

The isolation of microorganisms was carried out using a serial dilution technique. Aliquots of 100µL of different dilutions of garden soil were spread onto plates of nutrient agar medium for bacteria and potato dextrose agar for fungi. The plates were incubated at 28˚C for 5 days under aerobic conditions. Developed colonies were picked and isolated following morphological

criteria. Purified isolates were obtained by repeatedly streaking colonies on a TSA (Trypticase soy agar medium) and observing them using light microscopy. The identification and classification of the colony morphotypes were achieved using five parameters: colony size, form, colour, texture and margin. The isolated bacterial and fungal colonies were identified by Dr. D. Arvind Prasanth, Assistant Professor of Microbiology, Periyar University, and Salem. The effect of synthesized nanoparticles on soil microbes were tested using these isolated microbial colonies.

ANTIBACTERIAL ASSAYS

CALORIMETRIC BROTH ASSAY [25].

Overnight cultures of microbial isolates were subcultured in the nutrient broth. Samples of 3ml of microbial culture were placed into test tubes and 1, 1.5 and 2µl of appropriate dilutions of *Rhp*-AgNPs were added. After 24 h incubation at 37 °C, the optical density (OD_{520}) was measured using the spectrophotometer. The MIC (Minimum Inhibition Concentration) for growth was defined as the lowest concentration of NPs, which inhibited bacterial and fungal growth.

AGAR WELL DIFFUSION ASSAY FOR BACTERIA

The antibacterial assays were done on the garden soil bacterial isolates by standard well method. Nutrient broth/agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculums of each culture were spread on to nutrient agar plates using sterile cotton swabs. The well made in the agar plate using cork borer. The silver nanoparticles along with the sample (2 µg) were poured over the well of inoculated plates followed by incubation overnight at 37º C. The antibacterial activity was assigned by measuring the diameter of the zone of inhibition around the well.

AGAR WELL DIFFUSION ASSAY FOR FUNGI

Antifungal activity *Rhp*-AgNPs against garden soil fungal isolates were determined by using well agar diffusion method. Stock cultures were prepared and maintained in Potato dextrose agars were also done parallel. The plates were examined for evidence of zone inhibition, which appear as a clear around the well. The diameter of such zone of inhibition was measured using a meter ruler. Mean value was calculated by performing the experiments in triplicates.

RESULTS

SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES

The UV-vis spectra of AgNPs synthesised by *R. palmata* are shown in Plate 1. UV-visible spectrum of reaction mixture at different wavelength ranging from 416-591nm. Fig.1 showed strong absorbance peak at 338-490nm indicates the formation of AgNPs Fig.1 confirming the formation of silver nanoparticle [26, 27]. The peak indicated a surface plasmon resonance (SPR), which has already been recorded for various metal nanoparticles which ranged from 2 to 100nm in size [28, 29].

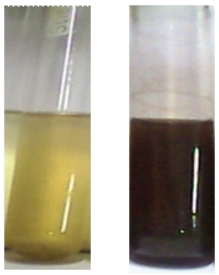


Fig. 1:
Plate 1: Digital photograph showing in the colour change of AgNO3 on addition of aqueous extract of seaweeds.

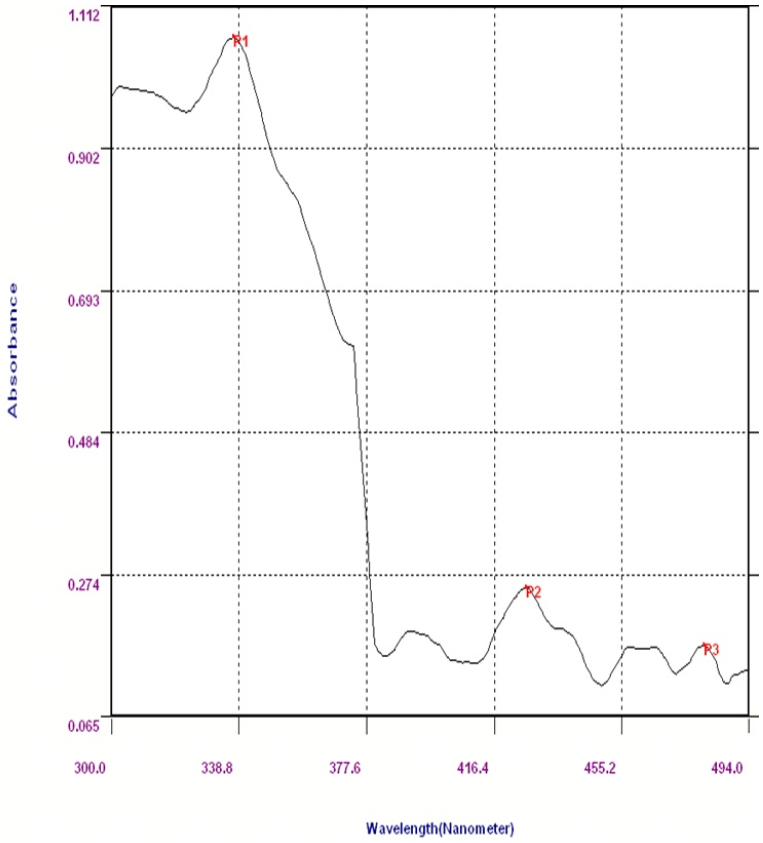


Fig. 1:
UV-Visible spectra of the synthesised silver nanoparticles from the aqueous extracts of seaweeds Rhp-AgNPs.

ANTIOXIDANT ACTIVITY

The antioxidant capacity observed on the total phenols, tannins, flavonoids, tocopherol and terpenoids content as shown in Fig. 2. The substantial amount of antioxidants in aqueous extracts of *Rhodymenia palmata* and *Rhp*-AgNPs presented in Fig.2, indicated these chemical content were predominantly higher in aqueous extract *Rhodymenia palmata* compared to the seaweed reduced *Rhp*-AgNPs. But tocopherol content higher in *Rhp*-AgNPs was noted. Polyphenolic compounds are natural antioxidants which are found mostly in seaweeds[30].

FT-IR ANALYSIS OF SILVER NANOPARTICLES

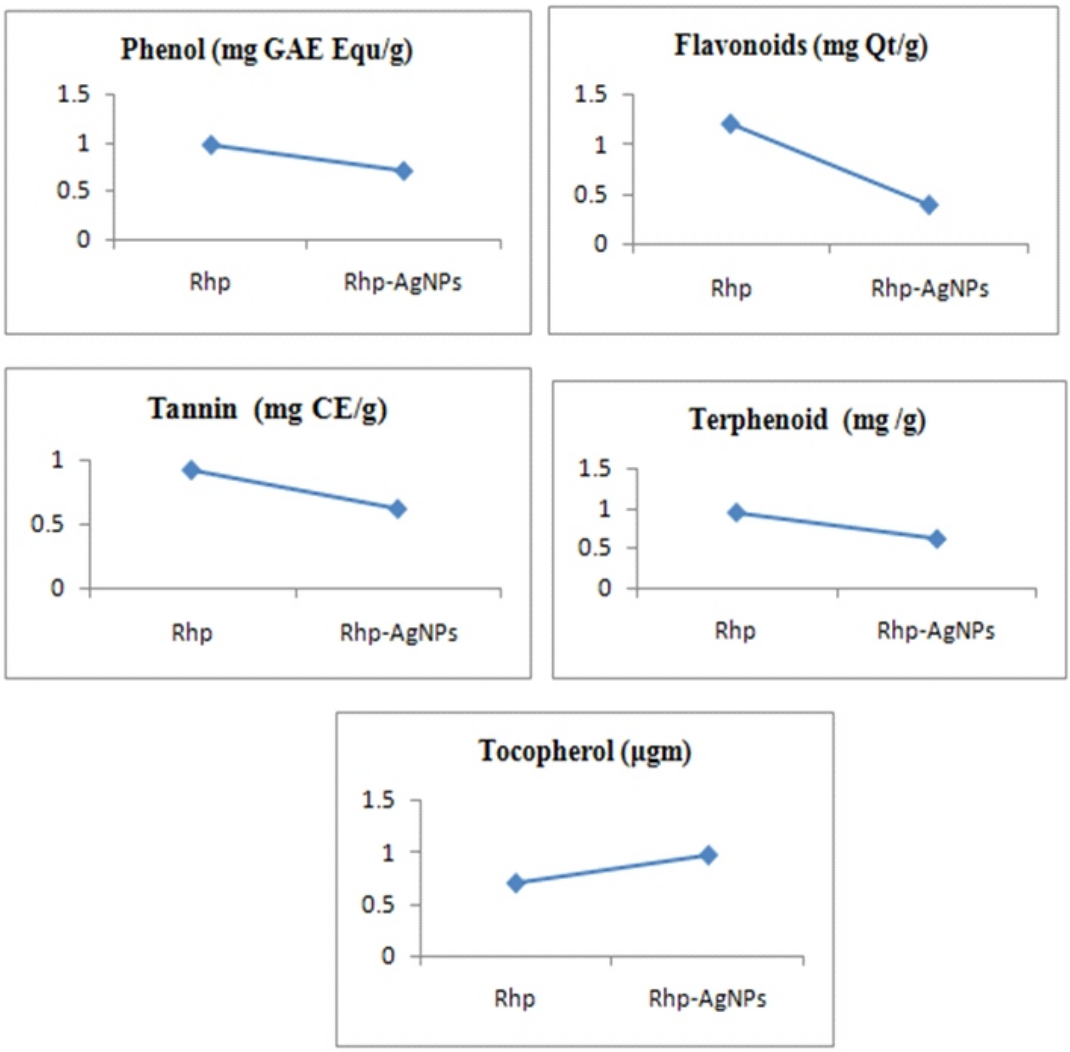
The presences of some functional groups are revealed by FTIR spectral analysis is shown in Fig. 3. FTIR spectrum of *R. palmata* before and after reaction with silver nitrate were represented in Fig.3 (a) control shows different major peaks positioned at 1437.82, 1560.13, 1637.14cm^{-1} indication the presence of alkene (C=C) bond and C-H of alkanes. 3447.30cm^{-1} it can be assigned of N-H stretching vibration of amine, respectively [31]. After reaction with $AgNO_3$ there was a shift in the following peaks 1639.89cm^{-1}, which gives indication of presence of (C=C) alkene,1384 indicating that C-H of alkanes and 3424.23 cm^{-1} were assingned to N-H stretching vibration of amines. Were as C=O vibration broad and strong peak appears at 1762.88cm^{-1} for carboxylic acid and derivatives on the surface of *R. palmata* may be participating in the process of nanoparticle synthesis Fig.3 (b)[32].

TEM ANALYSIS

The Ag-NPs synthesised by the help of *R. palmata* extract were scanned using TEM from which the average mean size of the AgNPs was 50nm and seems to be spherical in morphology as shown in Plate 2[33].

EFFECT OF GREEN SYNTHESIZED NANOPARTICLES *RHP*-AgNPs ON SOIL MICROBIAL ISOLATES

The effect of *Rhp*-AgNPs on the microbial isolated from garden soil were studied. The isolation of microorganism was carried out using serial dilution technique. Aliquots of 100 µl of different dilution of garden soil were spread onto plates of nutrient agar medium for bacteria and potato dextrose agar for fungi. The plates were incubated at 28º C for 5 days under aerobic



Rhp - Rhodymenia palmate; Rhp-AgNPs -Rhodymenia palmate

Fig. 2: Comparison of amount of antioxidant contents in seaweed and seaweed synthesis silver nanoparticles

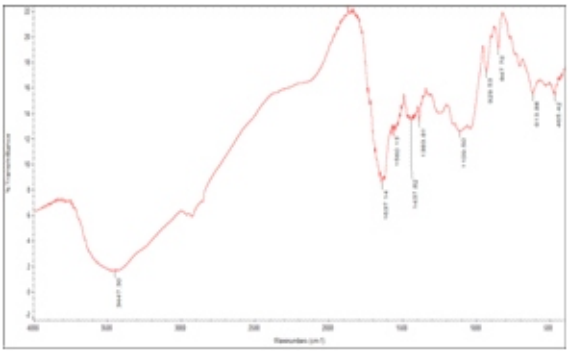


Fig. 3 (a) FT-IR spectra of R. palmata

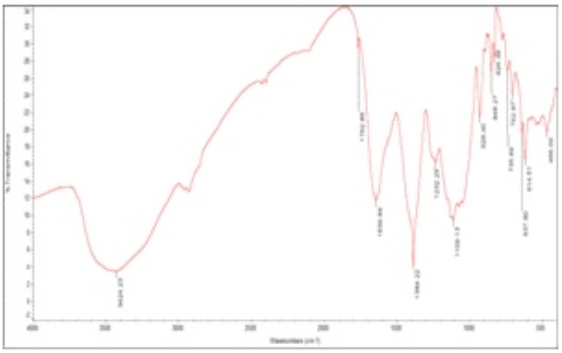


Fig. 3 (b) FT-IR spectra of Rhp-AgNPs

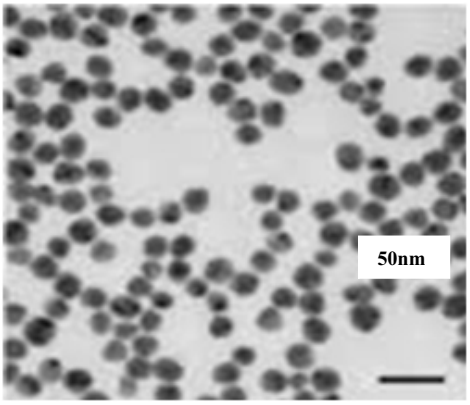


Plate 2: Transmission Electron Microscopy (TEM) micrographs of synthesised seaweeds silver nanoparticles Rhp-AgNP

conditions. Developed colonies were picked and isolated based on morphological criteria and the isolated bacteria were sub-cultured as pure culture. Pure cultured microbes were identified as *Bacillus spp, Bcillus subtilis, Staphylococcus epidermidis, Serratia spp, Pseudomonas spp, Pseudomonas fluorescens* and isolated fungi were *Aspergillus fumigates, Aspergillus flavus, Alternaria alternata, Cladosporium spp***.** The bacteria isolated from the garden soils are soil N-cycle, nitrifying bacteria. To study the effect of silver nanoparticles on soil microbes 2 type of *in vitro* assay were carried out they are calorimetric broth assay and agar well diffusion assay [34].

Overnight cultures of these microbes were subcultured in a nutrient broth. Sample of 3ml of microbial culture were placed into test tubes and 1, 1.5 and 2µl of appropriate dilutions of *Rhp*-AgNPs were added. After 24 hours incubation, absorbance reading at 520nm wavelength for seaweed was measured post incubation at 37º C for 12 hours. Bacterial cell viability and minimum inhibitory concentration (MIC) values were determined by observing the turbidity and the absorbance reading of the suspension post incubation. The lowest-concentration of synthesized nanoparticles with clear suspensions was considered as the MIC values. The suspensions of isolated microbial inoculums with all different concentrations (1µl, 1.5µl, 2µl) of *Rhp*-AgNPs in broth assay method were very cloudy Fig. 4-5 and that remained throughout the incubation period. This observed the visual suspension for determining the MIC as the turbidity due to bacterial and fungal growth.

The calorimetric broth microbial growth assay gave MIC value is 1 µl (Fig.4-5) for all the tested 6 bacteria and 4 fungi. 1µml of *Rhp* -AgNPs showed maximum growth inhibitory activity on *Pseudomonas fluorescens* and *Cladosporium spp*. The results of our study showed significant antimicrobial activities in calorimetric broth assay

The result of the agar well diffusion assay tests of *Rhp*-AgNPs were tested against soil microbial isolates using agar well diffusion technique. The synthesized AgNPs were, were found to

Table 1: Bactericidal activity of silver nanoparticles synthesised by *R.Palmata* against garden soil bacterial isolates (Zone of inhibition (mm).

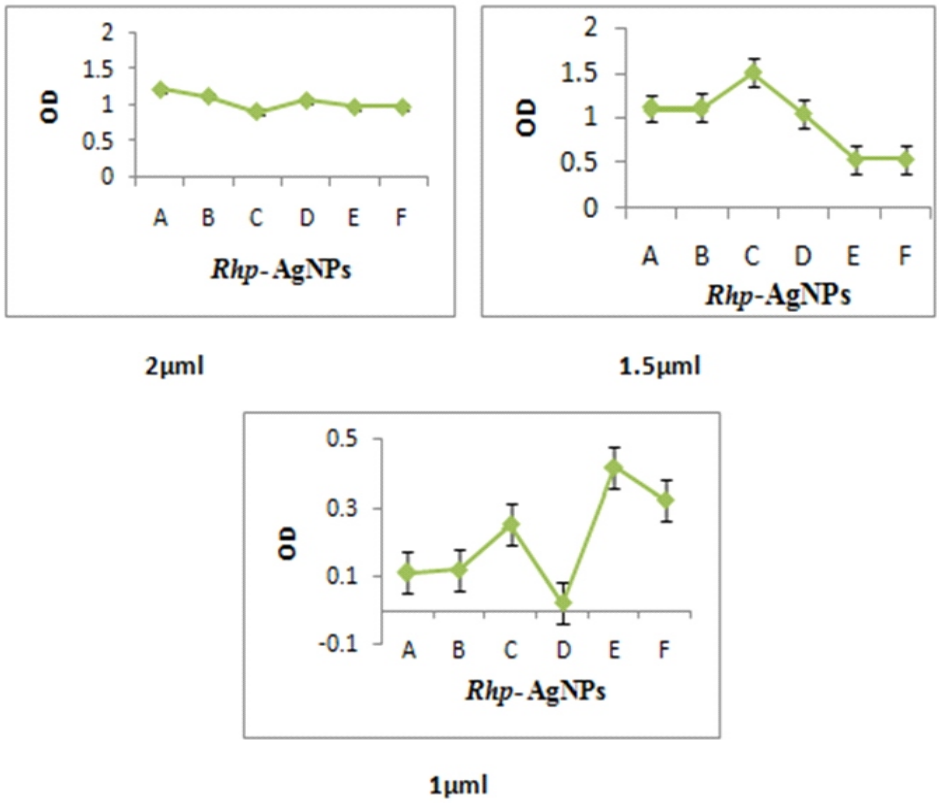
Bacteria	Control			*Rhp*-AgNPs
	W	AgNPs	Str	
Bacillus subtilis	NI	7	1 ± 0.076	NI
Bacillus spp	NI	NI	1 ±0.26	5 ± 0.008
Serratia spp	NI	NI	8 ±0.097	8 ± 0.004
Staphylococcus epidermidis	NI	NI	NI	NI
Pseudomonas fluorescens	NI	NI	1 ± 0.021	NI
Pseudomonas spp	NI	NI	1 ± 0.120	1 ± 0.027

Values are mean of 3 replicate ± SD
W- Water; AgNPs - Nanoparticles synthesis; Str Streptomycin; NI- No inhibition.

Table 2: Fungicidal activity of silver nanoparticles synthesised by *R.Palmata* against garden soil fungal isolates (Zone of inhibition (mm).

Fungi	Control			*Rhp*-AgNPs
	W	AgNPs	Str	
Aspergillus flavus	3 ± 0.76	3 ±0.76	2 ±0.022	9 ± 0.0.92
Aspergillus fumigatus	NI	5 ± 0.06	2 ± 0.004	7 ± 0.034
Alternaria alternata	NI	NI	2 ± 0.702	1 ± 0.024
Cladosporium spp	NI	NI	2 ± 0.032	NI

Values are mean of 3 replicate ± SD
W- Water; AgNPs - Nanoparticles synthesis; Str Streptomycin; NI- No inhibition



Values are mean of 3 replicate ± SD
A- *Bacillus spp* B- *Bacillus subtilis* C- *Staphyllococcus epidermidis* D- *Serratia spp*
E- *Pseudomonas fluorescens* F- *Pseudomonas spp*

Fig. 4: Bactericidal activities of 2µml, 1.5µml and 1µml of .silver nanoparticles on bacterial isolates.

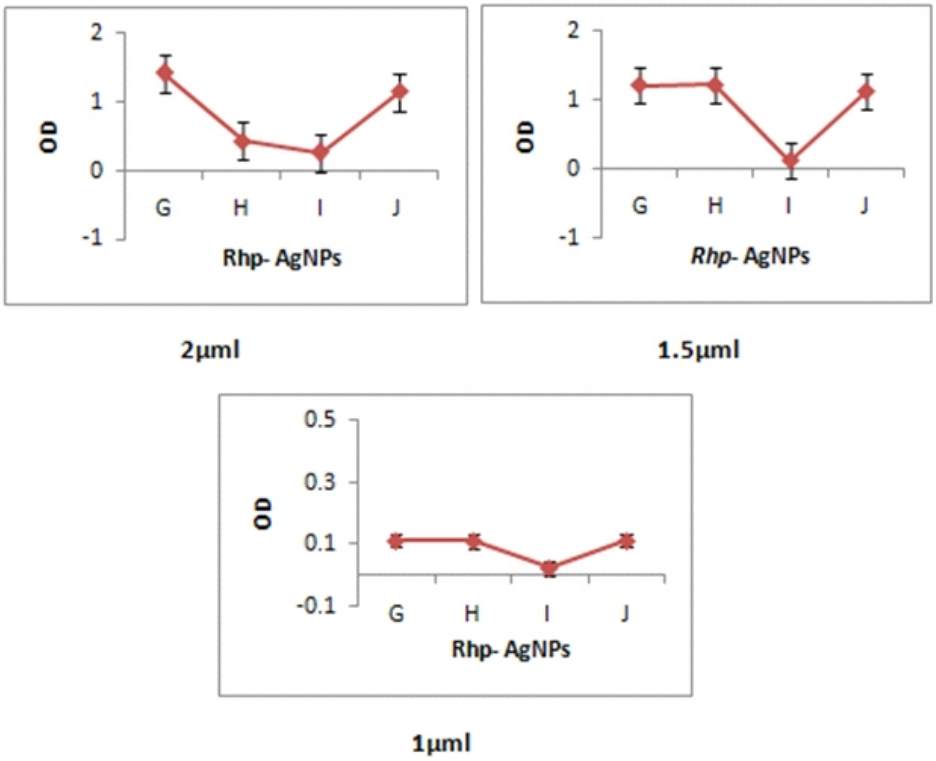
be ineffective or showed poor inhibition on *Bacillus spp, Bcillus substilis, Pseudomonas fluorescens, Pseudomonas spp, Staphylococcus epidermidis, Aspergillus flavus*, *Alternaria alternata*, and *Cladosporium spp* bacterial and fungal growth Table 1-2. The larger zone of inhibition was observed on *Serratia spp* (8mm) and *Aspergillus fumigates* (7mm). Though minimum inhibition on soil microbes by synthesized silver nano particles have been observed in our study.

DISCUSSION

In this study, UV-visible spectrum of AgNPs synthesised by *R. palmata* at different wavelength ranging from 416-591nm and strong absorbance peak at 338-490nm confirming the formation of AgNPs [26, 27]. The peak indicated a surface plasmonresonance (SPR), which has already been recorded for various metal nanoparticles which ranged from 2 to 100nm in size [28, 29].

The antioxidant capacity observed on the total phenols, tannins, flavonoids, tocopherol and terpenoids content. The substantial amount of antioxidants in aqueous extracts of *R.palmata* and *Rhp*-AgNPs. Polyphenolic compounds are natural antioxidants which are found mostly in seaweeds [30].

The FTIR studies revealed the presence of some functional groups, inferring that presence of phenolic compounds in the



Values are mean of 3 replicate ± SD
G - *Aspergillus flavus* H- *Aspergillus fumigatus* I- *Alternaria altermata*
J - *Cladosporium spp*

Fig. 4: Fungicidal activities of 2µml, 1.5µml and 1µml of silver nanoparticles on fungal isolates.

seaweed extract responsible for the reduction of the metallic salt silver nanoparticles [31, 32]. The AgNPs synthesised by the help of *R. palmata* extract were scanned using TEM from which the average mean size of the AgNPs was 50nm and seems to be spherical in morphology reported by [33].

The effect of *Rhp*-AgNPs on the microbial isolated from garden soil were studied. Pure cultured microbes were identified as *Bacillus spp, Bcillus subtilis, Staphylococcus epidermidis, Serratia spp, Pseudomonas spp, Pseudomonas fluorescens* and isolated fungi were *Aspergillus fumigates, Aspergillus flavus, Alternaria alternata, Cladosporium spp***.** The bacteria isolated from the garden soils are soil N-cycle, nitrifying bacteria [34]. To study the effect of silver nanoparticles on soil microbes 2 type of *in vitro* assay were carried out they are calorimetric broth assay and agar well diffusion assay . The maximum growth inhibitory activity appeared on (1µl) *Pseudomonas fluorescens* and *Cladosporium spp*. The results of our study showed significant antimicrobial activities in calorimetric broth assay [36].

As a result of synthesized AgNPs were, found to be ineffective or showed poor inhibition on *Bacillus spp, Bcillus substilis, Pseudomonas fluorescens, Pseudomonas spp, Staphylococcus epidermidis, Aspergillus flavus*, *Alternaria alternata*, and *Cladosporium spp* bacterial and fungal growth. The larger zone of inhibition was observed on *Serratia spp* (8mm) and *Aspergillus fumigates* (7mm). Though minimum inhibition on soil microbes by synthesized silver nano particles have been observed in our study [37, 38]. Findings suggest that bacteria with a tolerance for a toxic agent may appear with time [39] and the antimicrobial activity of synthesised NPs could possibly be reduced by bacterial self protection mechanism for instance. The idea that microorganisms are resistant, resilent, and functionally redundant is pervasion in ecology [40]. The AgNP-toxicity to nitrification bacteria has been reported to be highly dependent on their size, where AgNPs with less than 5nm diameter were reported to significantly inhibit the nitrification bacteria [41-43]. Our result showed that the average particle size in 25 to 50nm. AgNPs interactions with bacteria have been found to be dependent on the size and shapes of the NPs. AgNPs have spherical (7and29nm) and Pseudospherical shape (89nm) with a narrow size distribution. Among these, [44], found that the 7nm AgNPs presented best activity against *E.coli* and *S.aureus*. Because of their size, 7nm AgNPs can easily reach the nuclear contact of bacteria and they present the greatest surface area; therefore the contact with bacteria is the greatest [45]. Basically, the smaller size they are, the greater their surface area to volume ratio and higher their microbial contacting efficiency [46].

CONCLUSION

Using of marine red algae for the bio synthesis of silver nanoparticles is an ecofriendly and low cost effective.The interaction of the algae with the nanoparticles are expected to have remarkable applications in pharmaceutical and biomedical fields. Furthermore they are widely used in a range of materials and consumer products, including plastics, textiles, surface coatings on buildings and cosmetics. Their use is increasing and they are therefore more likely to be released to the environment where they may have damaging effects on ecologically-important bacteria and other living organism. So some guidance is needed as

to which precautionary measures are warranted in order to encourage the development of " green nanotechnologies" and their further innovative technologies, while at the same time minimizing the potential for adverse effects on human health and/or the environment. Thus there is urgent need for a systematic evaluation of the potential adverse effect of nanotechnology. It is therefore recommended that the ecotoxicological effect of nanoparticle be clarified before their application.

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