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SILVER NANOPARTICLES BIOSYNTHESIS USING MARINE ALGA *PADINA PAVONICA* (LINN.) AND ITS MICROBICIDAL ACTIVITY

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There have been impressive developments in the field of nanotechnology in the recent past, with numerous methodologies formulated to synthesize bionanoparticles using plants. Currently, there is a growing need to develop environmentally benign nanoparticles synthesis processes that do not use toxic chemicals in the synthesis protocol. We report the use of marine algae *Padina pavonica* (Linn.) thallus broth in the extra cellular synthesis for bio-silver nanoparticles. The biosynthesized silver nanoparticles were characterized with UV-vis Spectroscopy, FTIR, XRD, SEM and TEM. The thallus extract as well as silver-based nanoparticles of marine alga, *P. pavonica* (Linn.) were tested against two important pathogens of cotton. Fusarium wilts (*Fusarium oxysporum* f.sp. *vasinfectum*) and bacterial leaf blight (*Xanthomonas campestris* pv *malvacearum*) are responsible for significant yield losses in cotton worldwide. The *P. pavonica* based silver nanoparticles inhibited the growth of the test pathogens (12.33±0.33 mm and 10.33±0.33 mm for *F. oxysporum* and *X. campestris* respectively). This novel highly stable spherical polydispersed bionanoparticle was synthesized using a simple environmental friendly green method, and can be used for the management of cotton phytopathogens.

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Keywords: Silver-based bionanoparticles; Marine algae; Reduction capping method; Cotton pathogen; Antimicrobial activity; Agar well diffusion technique

1. Introduction

Metal nanoparticles have received considerable attention in recent years because of their unique properties and potential applications in catalysis, photonics, optoelectronics, biological tagging, agriculture and pharmaceuticals. Their performance depends critically on their size, shape, reducing agents and composition [1]. Biosynthesis of nanoparticles as an emerging field, highlights the intersection of nanotechnology and biotechnology and has attracted increased attention due to the growing need to develop environmentally benign technologies in material synthesis. The use of plant extract for the synthesis of nanoparticles could be advantageous over other environmentally benign biological processes by eliminating the elaborate process of maintaining cell cultures. Gardea-Torresday et al. [2] demonstrated that silver nanoparticles were synthesized within live alfalfa plant from solid media. Extracellular nanoparticles synthesis using plant leaf extracts rather than whole plants would be more economical owing to easier downstream processing.

Shankar et al. [3] reported that pure metallic silver and gold nanoparticles were synthesized by the reduction of Ag^+ and Au^+ ions using neem (*Azadirachta indica*) leaf broth. There have been recent reports on phytosynthesis of silver and gold nanoparticles by employing lemon grass extract [3, 4], *Arbutus unedo* leaf extract [5], sun dried *Artemesia nilagirica* [6],

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Sesbania drammandii [7], green tea (*Camellia sinensis*) [8], *Dioscorea bulbifera* tuber extract [9], Phyllanthin extract [10], purified apilin of henna leaves [11], *Acalypha indica* [12] and *Hibiscus rosa sinensis* [13] as reducing agents.

Similar to plant, there are reports of marine algae being used as a "bio-factory" for the synthesis of metallic nanoparticles. Recently, Singaravelu et al. [14] adopted a systematic approach to study the synthesis of metallic nanoparticles by *Sargassum wightii* (Greville). This is the first report in which a marine alga has been used to synthesize highly stable extracellular gold nanoparticles in a short period of time, compared to that of other biological procedures [12]. Silver is known for its antimicrobial properties and has been used for years in the medical field for antimicrobial applications. The mechanism of the bactericidal effect of silver and silver nanoparticles remains to be exploited and understood. Several studies propose that silver nanoparticles (AgNP's) may attach to the surface of the cell membrane, disturbing the cell permeability and respiration [15]. Marine algae are widely spread throughout the coastal areas around many continents. Almost all investigations were carried out on these materials focused on the different aspects concerned with their nature and growth. However, very little information is available about its antimicrobial properties. Marine algae have rich source of structurally important, novel and biologically active metabolites [16], with antifungal, antibacterial and antiviral activities [17], and pharmaceutical importance [18].

With the prevalence and increase of microorganisms resistance to multiple microbicides and the continuing emphasis on crop protection, many researchers have tried to develop new, effective antimicrobial agents, free of resistance and cost-effective. Such problems and needs have led to the resurgence in the use of marine algae–based microbicides that may be linked to broad-spectrum impact and far lower propensity to induce microbial resistance than synthetic fungicides and microbicides. Cotton is primarily the world's major fibre used in almost half of all textiles, apart from the seed being used as a source of food. Cotton is highly prone to diseases in the rain-fed areas where opportunities for growing alternative crops are limited [19, 20]. Bacterial leaf blight (BLB), caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (= *Xanthomonas axonopodis* pv *malvacearum* (Smith) Dye) [21, 22] has become an increasing problem to cotton production world-wide. *Fusarium oxysporum* f.sp. *vasinfectum* (Atk.) Snyder and Hansen (Ascomycota) is a filamentous fungus, widely distributed in the soil, causing Fusarium wilt (FW) in cotton, an economically important disease inflicting plant mortality resulting in severe yield loss [19]. In this paper, we report on the synthesis of pure and stable metallic nanoparticles of silver by the reduction of aqueous Ag^+ ions with the thallus broth of marine alga, *P. pavonica* (Pahaeophyceae) and we further investigated the impact of the synthesized nanoparticles against *F. oxysporum* and *X. campestris* using agar well diffusion method.

2. Experimental

2.1. Preparation of alga thallus broth

Padina pavonica was collected by hand picking method from the submerged marine rocks from Tuticorin district, Tamil Nadu during low tide at 6 AM. Collected algae were washed thoroughly with tap water to remove both epiphytes and necrotic plants and then, once rinsed with sterile distilled water to remove any associated debris if any. These clean, fresh materials were shade-dried for two weeks, and powdered using domestic blender. For the alga thallus broth preparation, ten gram of the dried alga powder was boiled with 100 mL of deionised distilled water. The resulted infusion was filtered thoroughly until no insoluble material appeared in the alga leaf broth. The qualitative phytochemical analyses of the alga thallus broth was performed as described by Brinda *et al.* [23] method and confirmed by Harborne [24].

2.2. Synthesis and characterization of silver nanoparticles

The materials used for the synthesis of silver nanoparticles are $AgNO_3$ and algal thallus extract. Exactly 17 mg of $AgNO_3$ was dissolved in 100 mL distilled water (10^{-3}M). Ten mL of algal thallus extract was added to 90 mL of 10^{-3}M $AgNo_3$ solution for reduction of Ag^+ ions. The reduction of pure Ag^+ ions was monitored by measuring the UV-vis spectra of the solution at

regular intervals after diluting a small aliquot (0.2 mL) of the sample 20 times. UV-vis spectra were recorded as a function of reaction time on a UV- 1601Shimadzu spectrophotometer with samples in Quartz cuvette operated at a resolution of 1 nm. X–ray diffraction (XRD) pattern of the alga thallus broth reduced Ag nanoparticles were obtained using Siemens D5005 XRD (X- ray diffractometer) with Cu Kα radiation (λ = 0.1542). XRD patterns were analyzed to determine peak intensity, position and width. The particle size was calculated using the Scherrer formula:

$$d = 0.9\lambda / \beta\cos\theta$$

where, d is the mean diameter of the nanoparticles, λ, the wavelength of X-ray radiation source and β, the angular FWHM of the XRD peak at the diffraction angle θ [25].

The alga thallus broth reduced Ag nanoparticles solution was centrifuged at 13,000 rpm for 15 minutes, redispersed in sterile distilled water to get rid of any uncoordinated biological molecules for Fourier transform infrared (FTIR) spectroscopy measurements. Centrifugation and the redispersion were repeated thrice in order to ensure better separation. The purified KBr pellets were then air dried at room temperature and powdered subjected to FTIR spectroscopy measurement (Shimadzu FTIR - 8300S). The morphology of the alga thallus broth reduced Ag nanoparticles was recorded using the JSM-6390 Scanning electron microscope (SEM). Samples for SEM were prepared by drop coating the Ag nanoparticles solutions onto carbon copper grid. The films on the grids were allowed to dry prior to SEM measurement. To record the size and shape of alga thallus broth reduced Ag nanoparticle, samples for Transmission Electron Microscopy (TEM) were prepared by drop-coating the Ag nanoparticle solution onto carbon-coated copper grids. The films on the TEM grids were allowed to stand for two minutes, following which the extra solution was removed using a blotting paper and the grid allow drying prior to measurement. TEM measurements were performed on a JEOL model 3010 instrument operated at an accelerating voltage at 120 kv.

2.3. Agar well diffusion bioassay to evaluate microbicidal activity

Fusarium oxysporum f.sp. *vasinfectum* and *Xanthomonas campestris* pv *malvacearum* were isolated from infected cotton plants and were used for the experiment. These pathogens were isolated, sub-cultured on Sabouraud Dextrose Agar (SDA) for fungi and Nutrient Agar (NA) for bacteria and identified using standard protocol [26]. Antimicrobial activity was carried out using agar well diffusion method. Petri plates were prepared with 20 mL each of sterile Mueller Hinton Agar (MHA) and SDA for bacteria and fungi respectively. Wells were made using sterile cork borer under aseptic condition. The alga thallus broth-based nanoparticles with various concentrations (25 µL, 50 µL, 75 µL, 100 µL) were added to the wells. Carbendazim (Bavistin) (0.03%) (BASF, Mumbai, India) and Chloramphenicol (0.1%) (HiMedia, Mumbai, India) were used as positive control for fungus and bacteria respectively and the distilled water was maintained as negative control for both microorganisms. The zone of inhibition was measured using a ruler and expressed in mm [27].

3. Result

A detailed study on the extra cellular synthesis of silver nanoparticles by *P. pavonica* was carried out, and the microbicidal effect of AgNP's against the fungi and bacteria of cotton was reported from this work. Terpenoids, phenolic compounds and saponins were observed in the alga thallus broth. Figure 1 shows Erlenmeyer flasks containing the filtrate of *P. pavonica* biomass with Ag^+ ions at the initial time point and after 24 h of the reaction end point, respectively. The change in color of the filtrate of *P. pavonica* was noted by visual observation. The excitation spectra of the AgNP's sample were characterized by UV-vis spectroscopy. The technique outlined above has proved to be very useful for the analysis of nanoparticles [28]. The UV-vis spectra recorded from the *P. pavonica* reaction vessel at different times of reaction are plotted in figure 2. The strong surface plasmon resonance centred at 422 nm clearly indicated an increase in intensity with time and stability after 24 h of reaction. The metal particles were observed to be stable in solution even two years after synthesis.

Fig. 1. Erlenmeyer flasks containing bio-synthesized silver nanoparticles by Padina pavonica, before reaction (left) after reaction (right)

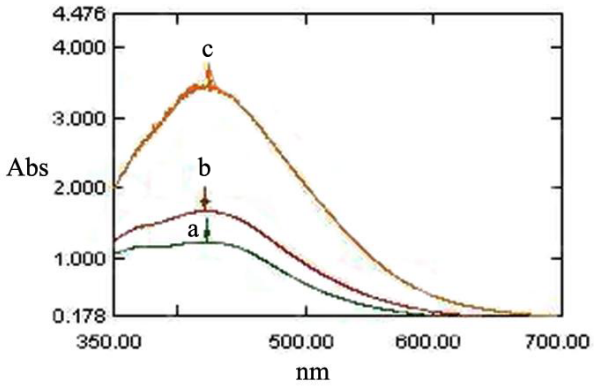

Fig. 2. UV-Vis spectra of silver nanoparticles synthesized by treating Padina pavonica extract with $10^{-3}M$ $AgNo_3$ solution a) 16 hours; b) 24 hours c) 16 weeks

Fig. 3 shows the XRD patterns of silver nanoparticles synthesized using marine brown alga, *P. pavonica.* A number of Bragg reflections with 2θ values of 38.03°, 46.18°, 63.43° and 77.18° sets of lattice planes are observed which may be indexed to the 111, 200, 220 and 311 facets of silver respectively. X–Ray diffraction pattern thus clearly illustrates that the silver nanoparticles formed in this present synthesis are crystalline in nature and the size was found to be ~54nm. The metallic silver nano-crystals showed typically optical absorption peak approximately at 3 KeV due to surface plasmon resonance [29].

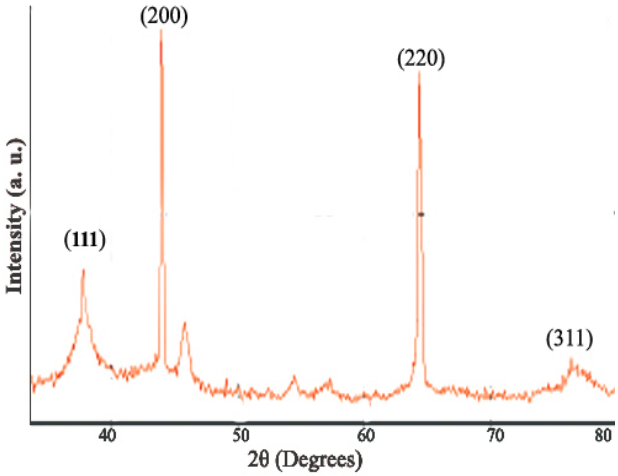

Fig. 3. XRD pattern of silver nanoparticles synthesized by treating Padina pavonica extract with $10^{-3}M$ $AgNo_3$ solution

Fourier Transform Infrared spectroscopy (FTIR) measurements are carried out to identify the possible bio-molecules responsible for the reduction of the Ag^+ ions and capping of the bio-reduced AgNP's synthesized by *P. pavonica*. The FTIR spectrum is showed in fig. 4a and 4b. The observed peaks were more characteristic of terpenoids that are very abundant in algal thallus broth [30]. The presence of terpenoids in algal thallus broth was also confirmed by phytochemical analysis. The peaks observed in crude algal thallus broth at 1415.65 cm^{-1} (C-C groups or from aromatic rings) (Table 1), suggest the presence of terpenoids whereas in the biosynthesized silver nanoparticles the peak has been shifted. It is also possible that the terpenoids play a role in the reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acids [3].

Figure 5 shows the SEM image of the biosynthesized silver nanoparticles by *P. pavonica.* The alga thallus broth synthesized nanoparticles were spherical with sizes ranged from 45 to 64 nm. TEM images recorded from drop-coated films of the silver nanoparticles synthesized by treating silver nitrate solution with alga thallus broth for 24 h. the silver nanoparticles formed were predominantly spherical and polydispersed with diameters in the range 10 to 72 nm (mean value = 46.8 nm) (Figure 6). Under careful observation, it is noted that the silver nanoparticles are surrounded by a faint thin layer of other material, which we suppose is the capping organic material from alga thallus broth.

The AgNP's synthesized by *P. pavonica* possessed antibacterial and antifungal activities against the test pathogens (Table 2). The AgNP's inhibited the growth of *F. oxysporum* and *X. campestris*. However, aqueous crude extract of *P. pavonica* showed no activity against these pathogens, hence it can be concluded that the antimicrobial activity is due to the presence of AgNP's.

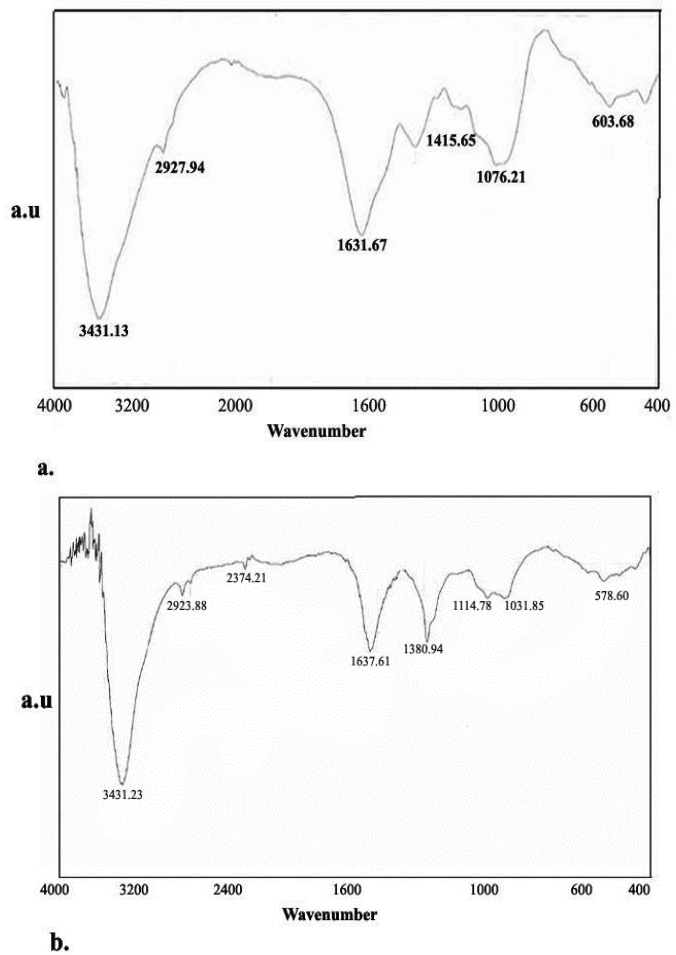

Fig. 4. FTIR spectra of Padina pavonica crude extract (a) and silver nanoparticles synthesized using P. pavonica extract with 10^{-3}M $AgNo_3$ solution (b)

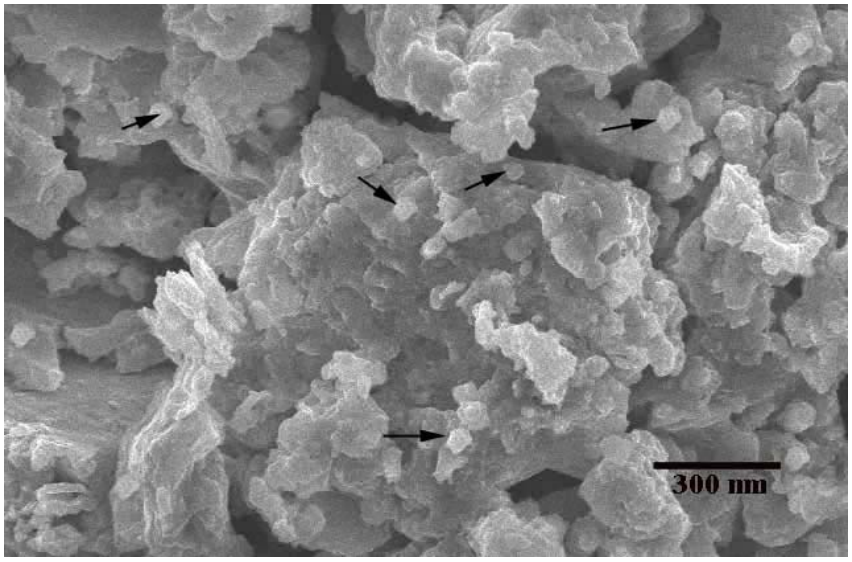

Fig. 5. SEM images of silver nanoparticles synthesized by treating Padina pavonica extract with 10^{-3}M $AgNO_3$ solution

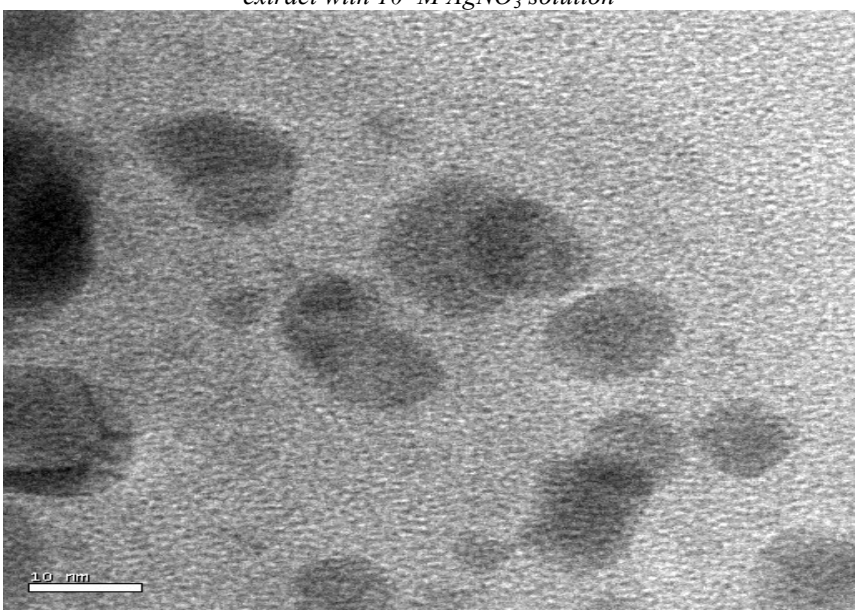

Fig. 6. TEM images of silver nanoparticles synthesized by treating Padina pavonica extract with 10^{-3}M $AgNO_3$ solution

Table 1. FTIR analysis of Padina pavonica crude extract and silver nanoparticles synthesized using with 10^{-3}M $AgNo_3$ solution

***Padina pavonica* crude thallus extract**			***Padina pavonica*-based AgNP**		
Frequency (cm^{-1})	**Bond/stretching**	**Functional group**	**Frequency (cm^{-1})**	**Bond/stretching**	**Functional group**
3431.13	O-H stretch, H-bonded	Alcohols, Phenols	3431.13	O-H stretch, H-bonded	Alcohols, Phenols
2927.74	C-H stretch	Alkanes	2923.88	C-H stretch	Alkanes
1631.67	N-H bend	Primary amines	2854.45	C-H stretch	Alkanes
1415.65	C-C stretch (in-ring)	Aromatics	1631.67	N-H bend	Primary amines
1251.72	C-H wag (-CHX)	Alkyl halides	1114.78	C-N stretch	Aliphatic amines
1076.21	C-N stretch	Aliphatic amines	1031.85	C-N stretch	Aliphatic amines
603.68	C-Br stretch	Alkyl halides	578.60	C-Br stretch	Alkyl halides

Table 2. Mean Zone of inhibition (mm) of biologically synthesized silver nanoparticles by Padina pavonica against two cotton pathogens (Mean±SE).

Amount (in µl)	***F. oxysporum* f.sp. *vasinfectum***	***X. campestris* pv *malvacearum***
Silver Nanoparticles		
25	5.66±0.33	-
50	8.33±0.33	8.66±0.33
75	11.66±0.33	10.33±0.33
100	12.33±0.33	10.33±0.33
Thallus broth		
25	0.00	0.00
50	0.00	0.00
75	0.00	0.00
100	0.00	0.00
Positive control	18.66±0.33 (Carbendazim 0.03%)	23.66±0.33 (Chloramphenicol (0.1%)
Negative control (Distilled water)	0.00	0.00

4. Discussion

The study of nanomaterials biosynthesis offers a valuable contribution to nano-biotechnology. The biosynthetic methods have been investigated as an alternative to chemical and physical ones. In this regard alga, *P. pavonica* proves to be an important biological component for extra-cellular biosynthesis of stable AgNP's. It was observed that the reduction of the Ag^+ ions during the exposure to *P. pavonica* thallus broth could easily be followed by visual observation and UV-Vis spectroscopy. It is well known that AgNP's exhibit a brown colour in aqueous solution; this colour results from the excitation of surface plasmon vibrations in the metal nanoparticles [31]. It is observed from the spectra that the surface plasmon resonance band of AgNP's occurs at 422 nm a characteristic peak of AgNP's as reported by Petit *et al.* [32]; Ahmad *et al.* [33]; Kong and Jong [28]; Singaravelu *et al.* [14], and this absorption steadily increases in intensity as a function of time of reaction, indicating the presence of AgNP's in the solution. Due to the excitation of plasma resonances on inter-band transitions, some metallic nanoparticles dispersions exhibit unique bands/peaks [34]. The broadness of the peak is a good indicator of the size of the nanoparticle. As the particle size increases, the peak becomes narrower with a decreased bandwidth and increased band intensity [32, 28]. Results showed that the biosynthesized nanoparticle are about 10 to 72 nm in size similarly neem-based [3] and black tea leaf extract [1] synthesized silver nanoparticles are spherical and poly-dispersed as observed for *P. pavonica*. Using FTIR analysis, Shankar *et al*. [3] postulated that terpenoids present in the neem plant extract contribute to reduction of Ag^+ ions. Our FTIR result shows the involvement of terpenoids (1415.65 cm^{-1} C-C groups or from aromatic rings) in the reduction of Ag^+ ions. Terpenoids could be adsorbed on the surface of metal nanoparticles, possibly by interaction through carbonyl groups or π-electrons in the absence of other strong ligating agents in sufficient concentration. It is also possible that the terpenoids play a role in reduction of metal ions by oxidation of aldehydic groups

in the molecules to carboxylic acids [3]. Paula and co-workers [35] identified xeniane a terpenoid from this plant.

Nanoparticles have substantially different physiochemical properties from those of bulk materials of the same composition, possible resulting in different toxicity mechanisms to biological systems [36]. The mode of action by the AgNP's could be the inhibition of the microbial processes on the cell surface and in the cell. Previous research demonstrated that AgNP's attach to the surface of cell membrane, affecting membrane permeability, dissipation of the ATP pool and Proton Motive Force (PMF) and finally caused cell death [37, 38, 39]. In the cell, silver ions may deactivate cellular enzymes and DNA by reacting with electron-donating groups such as thiol (-SH) groups and generate Reactive Oxygen Species [40, 41]. Thus, it is reasonable to infer that the bio-synthesized AgNP's can be used to control the disease caused by *F. oxysporum* and *X. campestris* on cotton plant, and there is a high possibility of generating a new microbicidal agent. The mechanism of silver action is linked with its interaction with thiol group compounds found in the respiratory enzyme of bacterial cells. Silver binds to the bacterial cell wall and cell membrane and inhibits the respiration process [42]. Studies show that Ag slightly binds with *X. campestris* as a result it showed only 39% activity in 75 and 100µl of metal nanoparticles. This might be probably due to larger size (~54 nm) of the synthesized metal nanoparticles. However, it was suggested that nanoparticles release silver ions into the bacterial cell, resulting in bactericidal activity [38, 43]. The surface plasmon resonance plays a major role in the determination of optical absorption spectra of metal nanoparticles, which shifts to a longer wavelength with increase in particle size. The size of the nanoparticle implies that it has a large surface area to come in contact with the bacterial cells and hence, it will have a higher percentage of interaction than bigger particles [38, 44-48]. The mechanism of the antimicrobial action of silver ions is not properly understood, however the effect of silver ion on bacteria can be observed by the structural and morphological changes [49]. Further they suggested that when the silver ions penetrated inside the bacterial cell the DNA molecules turn into condensed form and lose their replication ability leading to cell death.

5. Conclusions

Present green synthesis clearly brought out that the environmentally benign and renewable algal extracts can be used as an effective capping as well as reducing agent for the synthesis of silver nanoparticles. Silver nanoparticles synthesized by the above method are quite stable and no visible changes are observed even after four months or so if only the nanoparticle solutions are kept in light proof condition. Synthesis of metallic nanoparticles using green resources like seaweed extract is a challenging alternative to chemical synthesis, since this novel green synthesis is pollutant free and eco-friendly synthetic route for synthesis of silver nanoparticles. Our SEM, XRD and UV-vis spectroscopic studies showed that there is a major distribution of particle size. The biosynthesized nano particle is spherical and poly-disperse shaped with the size ranged from 10 to 72 nm (46.8 nm). It is interesting to note that the biosynthesized AgNP's showed higher antimicrobial activity compared to the crude extracts. Further research is required to formulate AgNP's and for field application.

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