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Green synthesis of silver nanoparticles using red seaweed *Portieria hornemannii* (Lyngbye) P.C. silva and its antifungal activity against silkworm (*Bombyx mori* L.) Muscardine pathogens

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Abstract
Silver nanoparticles play an important role in the field of sericulture. This study aims at evaluating the antifungal properties of green synthesised silver nanoparticles from *Portieria hornemannii* extract against two silkworm larval pathogenic fungus *Beauveria bassiana* and *Metarhizium anisopliae*. Recently by adopting greener and eco-friendly method of synthesis of silver nanoparticles developed dynamically. The present work is investigated on the green synthesis of silver nanoparticles by using marine red seaweed *P. hornemannii*. The synthesized nanoparticles were scrutinized by UV-visible spectrophotometer shows the crest at 430 nm. X-ray crystallography confirmed the silver nanoparticles crystalline personality. The structure and size distribution of the silver nanoparticles were examined by Transmission Electron Microscope (TEM) and the mean average sizes ranges from 9 to 80 nm was clearly shown and most of the nanoparticles were in spherical in shape. Silver nanoparticle of *P. hornemannii* concentration of 100 µl showed maximum zone of inhibition against *B. bassiana* (22.6 mm) and *M. anisopliae* (21.0 mm). The present study elucidates *P. hornemannii* derived silver nanoparticles can play a vital role in nano-based therapy in sericulture industry.

Keywords: Portieria, silver nanoparticle, beauveria, *Metarhizium*, antifungal activity

Introduction
In India the success of sericulture industry comes from after the successful harvesting of cocoon crops. Perhaps the major problem for sericulture in a tropical country like India is the high incidence causes of diseases. The major diseases affecting mulberry silkworm are muscardine (fungal disease), flacherie (bacterial disease), grasserie (viral disease) and pebrine (protozoan disease) (Govindhan *et al.*, 1998) [9]. *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae* (Mets.) Sorokin both are most destructive fungal pathogens to the larvae of silkworm, *Bombyx mori* L. causing muscardine diseases and their impact to reduce considerable level of cocoon loss of 30-40% (Nataraju *et al.*, 2005) [18].
Nanotechnology applications are highly suitable for biological molecules, because of their unique properties which will further undergo building of assembly by assembly for controlled and efficient delivery of many forms of metal based nanoparticle synthesis which was found to be reliable and eco-friendly (Harekrishna *et al.*, 2009) [10]. The field of nanotechnology advancing much more in different field is gaining and playing it as a one of the promising area of research in the modern field of material science, engineering, biology and agricultural sciences. Nanoparticles which are typically in the dimensions of 1-100nm, shows electrical, optical properties, and physical properties *viz.*, size distribution and surface morphology of the particles. Nanoparticles and nanomaterials are paving rapidly in interdisciplinary fields (Kaviya *et al.*, 2011) [13].
Nanoparticles is free of metals by showing extensively because of their uniqueness of physical properties, chemical reactivity and, among all special attention is given to many applications *viz.*, catalysis, biological labelling, biosensing, drug delivery, antifungal, antibacterial, antiviral activities detection of genetic disorders, gene therapy and DNA sequencing (Thirumurugan *et al.*, 2010) [34]. To date, metallic oxide based nanoparticles are synthesized and prepared from noble metals, *i.e.* silver (Ag), platinum (Pt), gold (Au) and palladium (Pd) (Norziah *et al.*, 2002) [19]. Among the noble metals, silver (Ag) is the metal of choice and showing interest in the research field of biological systems, living organisms and medicine (Parashar *et al.*, 2009) [20]. The main advantages of silver nanoparticles over with other metal nanoparticles (*e.g.* gold and copper) is because of the surface plasmon resonance energy of silver is located far from the interband transition energy.

Most of the chemical and physical synthesis protocols are still in the infancy stage and problems are much experienced with the stability of the nanoparticles preparations control of the crystal growth and aggregation of the particles (Mandal *et al*., 2005) [16]. Recent advancements of green chemistry approaches ensure that nanotechnology will play a pivotal role in enhancing the crucial role in physical, biological, pharmaceutical and biomedical applications. This will boost the growing need of developing an environmentally friendly synthesis of nanoparticles through greener approach. In some cases the synthesis of nanoparticles by using various plant based extracts can be advantageous over other biological synthesis processes which involves the complex procedures for maintaining the microbial cultures (Sastry *et al*., 2003) [29]. Seaweeds or benthic are the two most types of marine algae and are classified under the category of plants that live either in marine or brackish water. The synthesis route of nanoparticles using algae as source has been found least significance and underexploited. According to scientific literature report the algae is being widely used as a biofactory for synthesis of metallic nanoparticles. Studies have indicated that biomolecules like protein, phenols and flavonoids not only play a role is reducing the ions but also reduces to nano dimension, it also work as the capping agent of the nanoparticles (Shankar *et al*., 2004) [31]. Nanoparticles can also be stabilized by addition of protein derived molecules during the process (Duran *et al*., 2005) [4]. Biomolecules with as reducing agents and protecting agents are found to have a significant advantage over their counterparts (Huang *et al*., 2007) [11].

In the recent days, marine algal mediated silver nanoparticles have been synthesized by green/biological methods *viz*., *Sargassum wightii* (Govindaraju *et al*., 2009; Sunitha *et al*., 2015) [7, 32], *Ulva lactuca* (Bharathi Raja *et al*., 2012) [2], *Sargassum plagiophyllum*, *Ulva reticulate*, *Enteromorpha compressa* (Dhanalakshmi *et al*., 2012) [3], *Turbinaria conoides* (Rajeshkumar *et al*., 2012a) [22], *Padina tetrastromatica* (Rajeshkumar *et al*., 2012b) [23], *Padina pavonica* (Sahayaraj *et al*., 2012) [26], *Urospora* sp. (Suriya *et al*., 2012) [33], *Sargassum longifolium* (Saraniya Devi *et al*., 2013) [27], *Cystophora moniliformis* (Prasad *et al*., 2013) [21], *Sargassum longifolium* (Rajeshkumar *et al*., 2014) [25], *Hypnea muciformis* (Saraniya Devi and Valentin Bhimba, 2014) [28]. Through an elaborate screening involving number of seaweeds it is observed that *Porteria hornemannii* was a potential source for green synthesis of silver nanoparticles.

Materials and Methods

Source of seaweed

Red seaweed, *Portieria hornemannii* were collected by hand picking method in Mandapam coastal regions (Lat.09˚17.417 N; Long.079˚ 08.558 E) of Gulf of Mannar, Tamil Nadu and these were identified by Botanical Survey of India, Southern Regional Centre, TNAU Campus, Coimbatore, Tamil Nadu, India.

Preparation of seaweed extract

Seaweed was firstly surface sterilized with tap water to remove extraneous substances followed by distilled water. The seaweed was identified, shade dried for 15 days and powdered using mixer grinder. The dried algae of *P. hornemannii* were washed with distilled water to remove the waste materials. Algal extract used for the synthesis was prepared from 5 g of thoroughly washed seaweed in a 500-mL Erlenmeyer flask and boiled in 100 mL double-distilled water for 15 min. Filtered algae extract was stored at -15°C for further use, being usable for several weeks (Rajeshkumar *et al*., 2013) [24].

Green synthesis of *Portieria hornemannii* silver nanoparticle

In this typically synthesis process of silver nanoparticles, 10 mL of pure algal extract was added into the 90mL of 1mM of silver nitrate solution in 250 mL conical flask. The reaction mixture was kept at room temperature. The colour change was noted and nanoparticles formation was monitored using UV-vis Spectrophotometer periodically (Rajeshkumar *et al*., 2012).

Characterization of silver nanoparticles (AgNPs)

Characterization of silver nanoparticles was performed in sequence using UV-Visible spectrophotometer based upon colour change, X-ray diffraction (XRD), Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) measurements were done the particle characterization like crystallinity, size and morphology. To perform FT-IR spectrum analysis, the silver nanoparticles which were synthesized by using were centrifuged at 12,000 rpm for 15 min to remove free constituents or components present in the solution. The centrifuged and vacuum dried powder sample was placed in attenuated total reflection (ATR) sample holder of PerkinElmer Spectrum-One FT-IR instrument for measurement.

Antifungal activity

Antifungal activity of *P. hornemannii* assisted silver nanoparticles was carried out by well diffusion method against silkworm white muscardine (*Beauveria bassiana*) and green muscardine (*Metarhizium anisopliae*) pathogens. Silkworm fungal pathogen cultures were maintained at Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore, India.

Results

Synthesis and Characterization of silver nanoparticles

Pure seaweed extract was added into silver nitrate solution. Within few minutes the appearance of light red colour was observed and it indicates the formation of silver nanoparticles. After the 1 h incubation the colour was changed into dark brown due to the excitation of free electrons in the reaction mixture. The reduction of silver ions into silver nanoparticles by using *P. hornemannii* seaweed was analyzed by UV-vis Spectrophotometer. Figure 1 shows the UV absorption spectra of the synthesized silver nanoparticles using the extract of red seaweed, *P. hornemanni* recorded as the function of reaction time. Absorption spectrum showed that the peak positioned at 430 nm indicated the formation of silver nanoparticles.

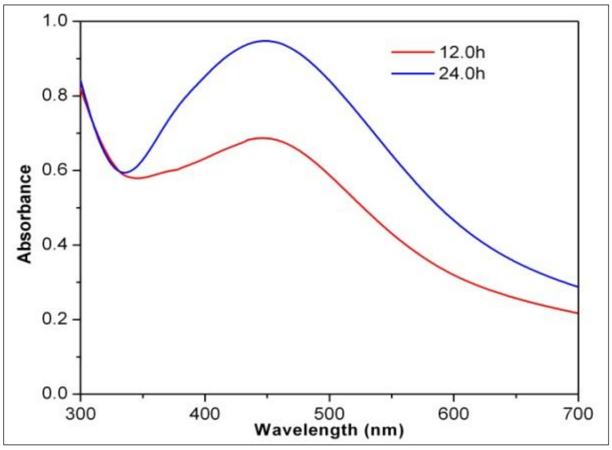


Fig 1: UV-Vis spectra recorded as a function of time of reaction of aqueous solution of silver nitrate with *P. hornemannii* extract

Results of the XRD patterns (Fig.2) with peaks assigned to the corresponding diffraction signals of 2θ value of 38.18(111), 44.42 (200), 64.4 (220) and 77.36 (311) facts of silver. The result reveals that the Ag^+ reduced to Ag^0 by *P. hornemannii* which are crystalline in nature.

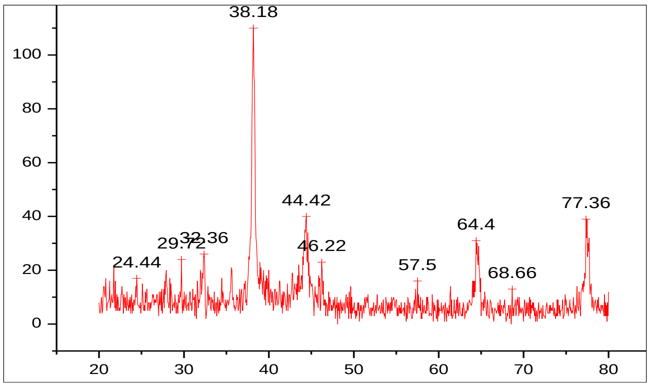


Fig 2: XRD patterns of silver nanoparticles synthesized by treating *P. hornemannii* extract with silver nitrate aqueous solution

Both SEM and TEM measurements were recorded from drop coated films of the 'Ag' nanoparticles synthesized by using *P. hornemannii* for 12 h (Fig.3 and Fig.4). The SEM image showed nanoparticles with variable shapes but mostly in spherical with the particle size range 9 to 80 nm.

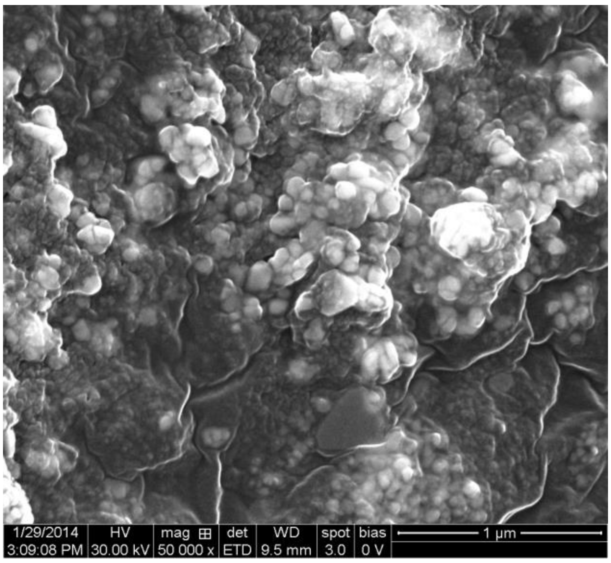


Fig 3: SEM image of *P. hornemannii* mediated silver nanoparticles

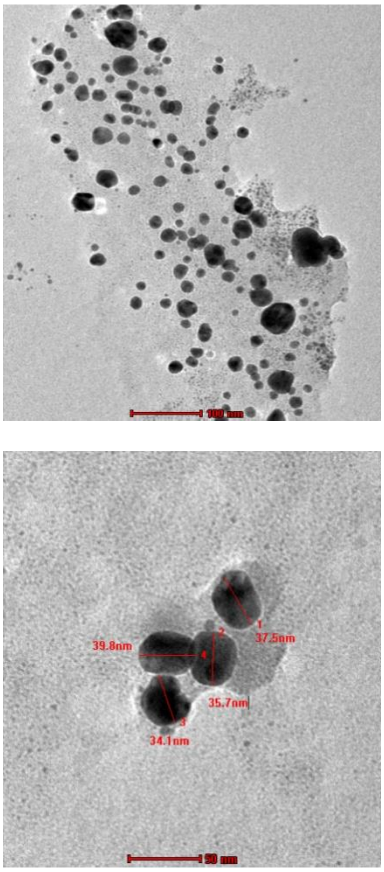
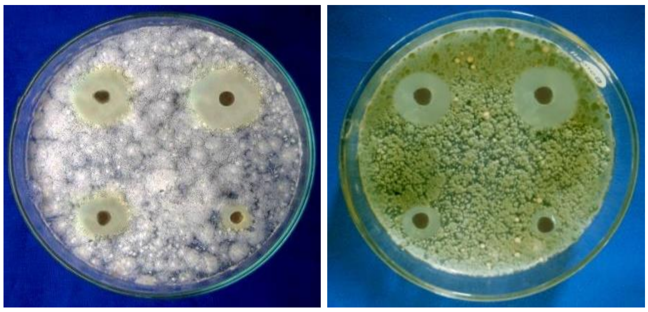


Fig 4: TEM image of silver nanoparticles formed Ag^+ ions using *P. hornemannii* extract

The biologically synthesized silver nanoparticles of the present finding exhibited excellent antifungal activity against the silkworm (*B. mori*) larval fungal green muscardine *M. anisopliae* (21.0 mm) and white muscardine pathogen *B. bassiana* (22.6 mm) in Figure (5) showed the zone of inhibition of the subjected fungal growth (Table 1).

Table 1: Antifungal activity of red seaweed mediated silver nanoparticles

Concentrations	Zone of inhibition (mm)	
	Beauveria bassiana	***Metarhizium anisopliae***
50 µl	9.2	8.5
75 µl	16.5	15.2
100 µl	22.6	21.0
Control	5.8	5.2



Beauveria bassiana *Metarhizium anisopliae*

Fig 5: Antifungal activity of silver nanoparticles against silkworm white and green muscardine pathogens

Discussion

Several reports have been employed for the synthesis of silver nanoparticles for its beneficial applications. Silver nanoparticle were synthesized and characterized in ambient conditions with an average size of 16 nm. Silver nanoparticles were formed by the reduction of Ag^+ into Ag^0 with the addition of seaweed extraction to the solution of 1 mM $AgNo_3$. The solution of $AgNo_3$ is in colorless stage initially and subsequently turned into dark brownish yellow color indicating the formation of silver nanoparticles. The formation of silver nanoparticles was monitored by UV-vis absorption spectra at 200 to 600 nm where an intense band was clearly detected at 430 nm (Surya *et al*., 2012) [33]. The shape of the band was symmetrical, suggesting uniform dispersal of cubic to spherical shape nanoparticles (Travan *et al.*, 2009) [35].

The similar XRD results of four distinct diffraction peaks of the 2θ values of 38.27^0, 44.16^0 and 65.54^0 could be assigned to the plane of (1 1 1), (2 0 0), (2 2 0) and (3 1 1) respectively, indicating that the silver nanoparticles are partially cubic to spherical crystalline in nature (Govindaraju *et al*., 2009; Rajeshkumar *et al*., 2014) [7, 25]. The synthesized silver nanoparticles are in comparison with the standard silver nitrate and pure silver particles which are published by Joint Committee on Powder Diffraction Standards (File nos. 04-0783 and 84-0713). The size of the *P. hornemanni* extract mediated nanoparticle was around 9 to 28 nm. A similar result was recorded by Jain *et al*. (2009) [12] revealed that papaya extract act as reducing as well as capping agent. Cubical to spherical shaped nanoparticles were observed through SEM and TEM investigations (Govindaraju *et al*., 2009) [7].

Performance of silver nanoparticles was depends upon both dosage and particle size. Metal nanoparticles show large surface to volume ratio and exhibit antimicrobial properties due to their ability to interact with cellular membranes through disruption of cell wall structure (Trop *et al*. 2006; Ahmad *et al.* 2013) [36, 1]. Especially silver is known for its strong toxicity against a wide range of microbes including bacteria and fungi (Narayanan and Park, 2014) [17].

According to Kim *et al.* (2009a) [15], silver nanoparticle affect fungus cells by attacking their membranes, thus disrupting the membrane potential. The biologically synthesised *P. hornemanni* mediated silver nanoparticles prepared by direct reduction method showed antifungal activity against silkworm muscardine pathogens of *B. bassiana* and *M. anisopliae* using agar well method. The zone of inhibition produced by various antibiotics were compared with the inhibitory zone produced by the silver nanoparticles, were also demonstrated (Geoprincy *et al*., 2011) [5]. However, the anti-fungal activity of AgNPs depends on the nature and type of fungus along with size of AgNPs and also closely associated with the formation of pits in the cell wall of microorganism (Shafaghat, 2015) [30].

The antifungal activity of silver nanoparticles is attributed to its effects on the mycelia. The mode of action of nano-Ag on fungi targeting the yeast cell membranes and disrupting membrane potential (Kim *et al*., 2008) [14]. The antimicrobial activity of *Solanum torvum* mediated silver nanoparticles was performed against bacteria pathogens (*Staphylococcus aureus*, *Bacillus rhizoids*, *Escherisia coli* and *Pseudomonas aeruginosa*) of silkworm *B. mori* and *Spirulina platensis* mediated AgNPs exhibit strong antifungal activity against mulberry fungal pathogens (*Cerotelium fici*, *Cercospora moricola* and *Phyllactinia corylea*) were demonstrated by Govindaraju *et al.* (2010; 2008) [8, 6].

Conclusion

Results support the hypothesis that the silver nanoparticles can be prepared in a simple, eco-friendly and cost effective manner and are suitable for formulation of new technique of fungal control. The biosynthesized silver nanoparticles suggested low concentration that greater significance in the prevention and management of silkworm muscardine pathogens during rearing environment in future

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