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Green synthesis of silver nanoparticles using two seaweeds and their potential towards environment

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ABSTRACT

Nano-biotechnology is recognized as offering revolutionary changes in various fields of medicine. Biologically synthesized silver nanoparticles have a wide range of applications. The biosynthesis of silver nanoparticles is an eco-friendly method in the field of nanotechnology. Seaweed extracts of *Chondrococcus hornemannii*(Lyngbye) F. Schmitz and *Gracilaria corticata* J. Agardhwas used as a reducing agent in the eco-friendly extracellular synthesis of silver nanoparticles from an aqueous solution of silver nitrate ($AgNO_3$). High conversion of silver ions to silver nanoparticles was achieved with *Chondrococcus hornemannii* at reaction temperature of 100°C and a seaweed extract concentration of 10% with a residential time of 1h using reflux extractor when compared with the other methods. Formation of silver nanoparticles was characterized by spectrophotometry and the electron microscopic technique. The average particles size was ranging from 35 to 75 nm. Antimicrobial activities indicate the minimum inhibitory concentration of biologically synthesized nanoparticles tested against the pathogen *Staphylococcus aureus*(1 mg/ml). High inhibitions over the growth of *Pseudomonas aeruginosa*, *Vibrio cholerae*and *Escherichia coli* were witnessed against the concentrations of 1 mg/ml. Enzyme assay of the collected seaweeds performed using standard protocol to assess the potency level. Furtherseed germination test proved that synthesized nanoparticles were environmentally safe.

Introduction

Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. In the era of growing awareness about increasing pollution and global warming, developing new methods of synthesis of nanomaterial with green technologies is a challenge [1]. The involvement of biological entities in the nanoparticle growth is gaining tremendous advantages such as production of anisotropic nanoparticles with size and shape control and intensive energy [2, 3].

Among the metal nanoparticles, silver has been consumed largely due to their antimicrobial and pharmaceutical applications. Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process. Characterization of nanoparticles is very important for optimization of the various parameters involved during the synthesis of nanoparticles [5, 6].

Seaweed-mediated synthesis has been suggested as possibly eco-friendly, cost-effective, and less time-consuming process and do not involve any toxic chemicals [9, 10 and 11]. The advantage of using seaweeds for the synthesis of nanoparticles is that they are easily available, safe to handle, and possess a broad variability of metabolites that may aid in reduction [17, 19, and 20]. Recently, there is a study on the extracellular biosynthesis of monodisperse gold nanoparticles using the marine alga, *Sargassumwightii*(31). From the reports of Mehrdad F *et al* (2010) the green synthesis of nanoparticle avoids the toxicrisk and reduces the cost over physico-chemical method.

Thus an environment friendly method is necessary for synthesis of nanoparticle [28, 29, 30]. From the studies made by Ingle A(2008), Seaweed has various phytochemicals including carbohydrates, alkaloids, steroids, phenols, saponins and flavonoids. New research by Mansuya P (2010) has proved that the reduction of silver nanoparticle from seaweed is a green chemical method that exhibit good antibacterial activity. These bioinorganic materials can exhibit exquisite hierarchical ordering from the nanometer to macroscopic length scales [12, 13 and 15]. No information is available on the uptake and toxicity of AgNPs to algae. Hence our current research focuses on phytochemical analyses, enzyme assays, antioxidant potency, nanoparticle synthesis, antimicrobial activity and phytotoxicity of selective seaweeds *Chondrococcus hornemannii* (Lyngbye) F. Schmitz and *Gracilaria corticata* J. Agardh.

Experimental section

Methods

Collection and processing of seaweeds

From the coastal areas of Rameshwaram, *Chondrococcus hornemannii*(Lyngbye) F. Schmitz and *Gracilaria corticata* J. Agardh were collected. Some of the collected samples were kept frozen and few were shade dried, powdered for further analyses.

Seaweed extracts preparation and phytochemical estimation

5g sample were dissolved in sterile water, methanol, ethyl acetate, hexane and kept under repeated freeze thawing followedby sonication (1 min on, 10 sec off condition) for three days as per standard protocol. After incubation the samples were dried under room temperature, weighed and stored at -4°C until use. Phytochemical estimation was performed in triplicates to

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avoiderror as follows, steroids, alkaloids, triterpenoids, phenolics, flavonoids, catechins, saponins, tannin, carbohydrates, protein, carboxylic acids, coumarines, xanthoproteins and anthroquinones [14].

Enzyme assays

Enzyme assay was performed by preparing the extract from fresh seaweeds using extraction buffer maintained at 4°C. 0.5g fresh seaweed was grinded with sodium phosphate buffer/extraction buffer according to the assay and centrifuged to collect the supernatant. This solution was used for further determination of assay. Determination of catalase (CAT, EC 1.11.1.6) [4], ascorbate peroxidase (APX, EC 1. 11. 1. 11) [8], peroxidase (POX, EC 1.11.1.7) [18] and polyphenol oxidase activity assay (PPO, EC 1. 14. 18. 1) [7] was carried out.

Antioxidant activity of seaweed extracts

Some of the antioxidant assays were performed by following standard protocols such as, total phenol [32], total condensed tannin [16],hydrogen peroxide radical scavenging activity [22], DPPH assay [21], total reducing power assay and total antioxidant capacity [23, 24].

Nanoparticle synthesis

Silver nitrate stock solution (10^{-3} concentration) was prepared using sterile deionized water.

By boiling sample extract

5g of each sample was weighed and dissolved in sterile deionized water and boiled for 1hour. After cooling, extract was filtered and 5ml of the same was added to 95ml of $AgNo_3$ stock solution and kept for incubation under dark until colour change occurs.

By boiling extract with $AgNo_3$ solution

5g of each sample was weighed, dissolved in sterile deionized water and incubated for 48hours at room temperature. After incubation extract was filtered and 5ml of the extract was added to 95ml of $AgNo_3$ stock solution. This mixture was kept in boiling water bath for 30mins.

By using reflux extractor

5g of each sample was weighed, dissolved in sterile deionized water and kept for refluxing at 70°C for 1hr. 95ml of $AgNo_3$ stock solution was added to 5ml of the prepared extract and incubated under dark until colour change.

Characterization of synthesized nanoparticles

UV-Visible Spectroscopy

UV-Vis spectrophotometer experiment was carried out on a Shimadzu UV-8500 PC scanning spectrometer using $AgNO_3$ as the reference. 2-3 drops of the sample was pipetted into quartz UV Cell (2ml) and diluted with deionized water, followed by immediate spectral measurements. Deionized water was used as blank for all measurements.

Fourier Transform Infrared Spectroscopy

FT-IR (Spectrum RX-1 instrument) was used for the analysis of the water extract of sample and sample synthesized nanoparticles. The spectrum was focus in the range of 400-4000 cm^{-1} by the KBr pellet technique.

X-Ray Diffraction (XRD) analysis

Crystallographic information of the bioreduced Silver Nitrate solution was dried. XRD patterns were recorded by a SEIFERT X-ray diffractometer with cu Kα radiation. The samples were scanned in the 2θ range of 10°-70°.

High Resolution - Transmission Electron Microscopy (TEM)

Examination of the nanoparticle morphology by transmission electron microscopy was performed on a CM12 Philips model in SAIF, IIT Madras with an electron kinetic energy of 120 KV. For sample preparation a drop of silver solution was dispensed onto a carbon coated copper grid.

Antibacterial activity of the extracts and nanoparticles synthesized

Escherichia coli, *Staphylococcus aureus, Vibrio cholerae, Pseudomonas aeruginosa* were maintained in Muller Hinton broth for determining the activity of the reflux extract and synthesized nanoparticles [25, 26]. The agar well diffusion method was employed to determine the antimicrobial activities of the respected extracts. Well diffusion assay was found to be a simple, cheap and reproducible practical method [27]. A suspension of each sample tested micro-organism diluted prior to 10^{-1}, 10^{-2} and 10^{-3} - (1 ml of 10^8cells/ml) was spread on a solid agar medium in petri dishes (Mueller-Hinton agar). Finally the well were pierced using cork-borer and samples along with positive and negative controls were loaded onto the wells after labeling the plates, then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimeter.

Phytotoxicity by seed germination test (Raquel Barrena*et al.*, 2009)

The phytotoxicity of NPs was evaluated by the seed germination technique. The germination index has been extensively used as an indicator of phytotoxicity in soils [34, 35]. Cucumber (*Cucumissativus*) seeds were used for this test. The seed germination percentage and root length of cucumber seeds were determined after 7 days of incubation at 25°C. The seed germination percentage and root elongation of both seeds in distilled water were also measured and used as a control. Experiments were done in triplicate. The percentages of relative root elongation (E) and germination index (GI) were calculated according to standard methods [34].

Relative root elongation (E) = (Mean root length with NPs) / (Mean root length with control) X 100

Germination index (GI) = (Relative seed germination) X (Relative root elongation) / 100

where,

Relative seed germination = (Seeds germinated with NPs) / (Seeds germinated with control) X 100

It is important to note that the germination index combines germination and root growth and consequently and therefore reflects the toxicity more completely. The root elongation is the percentage of root length compared to control and it can be an indication of the presence of stress effects or other non-acute toxicological effects in the plant evolution. Hence, the root elongation can be more sensitive than the germination index when the toxicity directly affects the root development.

Results and Discussion

The main objective of the work was to evaluate and compare the ability of macroalgal species from southwest coast of India to synthesis nanoparticles which are environmentally safe. The study also focuses on the antimicrobial potential of the extracts and synthesized sample for biomedical applications.

Phytochemical screening

Theethyl acetate, methanol and hexane extracts of seaweed samples were obtained for phytochemical analyses (Table 1). Triplicates were performed for this assay. From the results, the maximum no. of phytochemicals (steroids, alkaloids, triterpenoids, phenolics, saponins, carbohydrates, protein and carboxylic acids) were present in ethyl acetate extracts of *Chondrococcus hornemanni*iwhereas, hexane extracts shows least no. of phytochemicals.

Enzyme assays

Enzyme assays was not carried out as frequently from the analyzed data's, thus this study focuses on some of the enzyme activity of fresh seaweeds. From the enzyme assay data evaluated, *Chondrococcus hornemannii* shows maximum 187%

of catalase activity and 160% in *Gracilaria corticata*. Activity of ascorbate peroxidase was 152% in *Chondrococcus hornemannii* whereas 128% only in *Gracilaria corticata*. Maximum of peroxidase activity was showed 145% in *Chondrococcus hornemannii* and 128% in *Gracilaria corticata*. Polyphenol oxidase activity showed maximum of 170% in *Chondrococcus hornemannii*and 128% in *Gracilaria corticata*(Table 2).

In vitro antioxidant activity

Maximum IC50 value of total phenolwas showed in ethyl acetate extract of *Gracilaria corticata*(62.43±0.07) and minimum in the same hexane extract (55.32±1.05). Total condensed tannin was showed maximum in methanolic extract of (59.12±0.04)*Chondrococcus hornemannii* and minimum in ethyl acetate extract of (49.05±3.42) *Gracilaria corticata*.

The total antioxidant activity was maximum in ethyl acetate extract (67.31±1.02) of *Chondrococcus hornemannii* whereas hexane extract (51.01±3.06 and 58.71±1.23) was minimum in both the samples. Considering, total reducing power assay *Chondrococcus hornemannii* showed better reducing power (77.24±0.04) than *Gracilaria corticata*(73.31±0.04). Many species of seaweed possess scavenging ability of hydrogen peroxide. The maximum scavenging activity was shown by the ethyl acetate extract of *Gracilaria corticata*(73.31±0.4) and minimum in *Chondrococcus hornemannii* (66.24±0.34). The antioxidant activity of α-tocopherol was high in ethyl acetate extracts of *Chondrococcus hornemannii*followed by methanol, aqueous and hexane extracts. Least activity was shown in hexane extracts of both the sample extracts (Table 3).

Nanoparticle synthesis and characterization

Among the three methods verified, extract obtained from reflux extractor shows more synthesizing capacity. Color change from pale brown to deep red indicates the formation of silver nanoparticles.

Characterization of synthesized particles

UV-Spectroscopic data revealed the absorbance peak at 430nm for *Chondrococcus hornemannii* and 420nm for *Gracilaria corticata*.Stability was maintained for *Chondrococcus hornemannii* synthesizing nanoparticles (Figure 1 & 2).

FT-IR spectroscopic data predicts the molecular configuration of different functional group present in the seaweed extract. Considerable absorption peak were found at 3475.33 cm^{-1}, 2071 cm^{-1}, 2073 cm^{-1}, 1638 cm^{-1}, 698 cm^{-1}, 1069 cm^{-1}, 1637 cm^{-1}, 689 cm^{-1}, 3432 cm^{-1}, 1121 cm^{-1}, 1637 cm^{-1}, 2676 cm^{-1}, 2777 $cm^{-1}$and 686 cm^{-1}respectively.The peak corresponding to 3432 cm-1 indicates the presence of intermolecular hydrogen bonding with hydroxyl group with polymeric association.The peak formed at 2777 cm^{-1}, 2676 cm^{-1} is because of C-H stretching and symmetric stretching of methoxy groups present.A small peak at 2071 cm-1 is an attribute to S-H stretching vibration mode. The stretch between 2155 cm^{-1} – 2161 cm^{-1} typically formed by triple C ≡ C bond in alkynes or because of cumulative double bond in ketones.

A minute peak with less intensity at 1069 cm^{-1} indicates the presence of asymmetric CH_3 bending modes of the methyl groups of proteins. The absorption peak formed at 1069 cm^{-1} is due stretching C-N, deformation N-H and deformation of C-H. Peak at 1121 cm^{-1} and 1069 cm^{-1} is responsible for polysaccharides and C-O stretch associated with glycogen respectively (Figure 3).

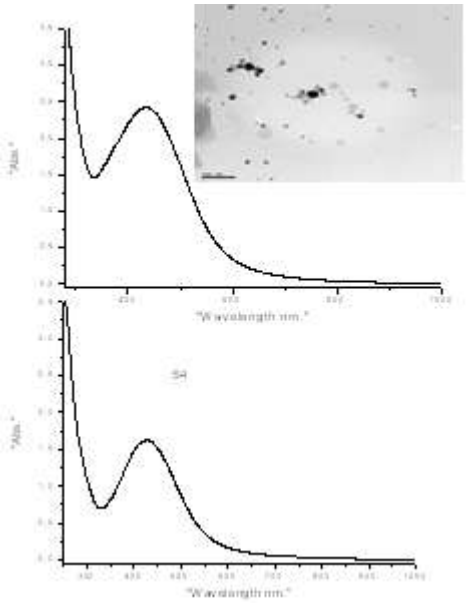

Figure 1 (above).UV-Visible spectral imageof*C.hornemannii* with inlet showing HR-TEM image.
Figure 2 (below). UV-Visible spectral image of *G. corticata*

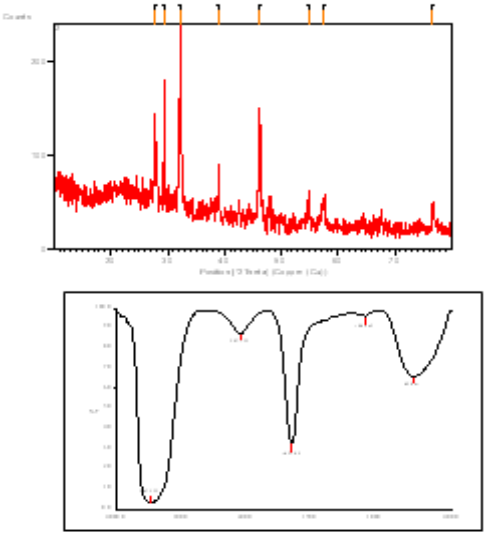

Figure 3. XRD and FT-IR spectral images of *C.hornemannii* synthesized nanoparticles

From the spectroscopic studies, *Chondrococcus hornemannii* was preceded for HR-TEM, XRD, photocatalytic degradation and seed germination studies. HR-TEM image of *Chondrococcus hornemannii*(Figure 1 inlet) showed similar dispersed spherical shape when compared with others showing aggregates. From the microscopical analyses and particle size analyser data, particle size ranging from 35nm – 75nm was shown in *Chondrococcus hornemannii*.To analyse the structure and composition of the nanoparticles synthesized using seaweeds, X-ray diffraction analysis was performed with Cu Kα radiation in a θ - 2 θ configuration. This data confirmed that metallic silver nanoparticles of size 2-5nm were being formed. The crystallite domain size was calculated from the width of the XRD peaks, assuming that they are free from non-uniform strains, using the Scherrer's formula (Figure 3).

Table 1.Phytochemical screening of seaweed extracts

Solvents	Seaweeds	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aq	*C. hornemannii*	-	-	+	+	-	-	+	-	-	+	+	-	-	-
	G. corticata	-	-	+	+	-	-	+	-	-	+	+	-	-	-
EtAc	*C. hornemannii*	+	+	+	+	-	-	+	-	+	+	+	-	-	-
	G. corticata	+	-	+	+	-	-	+	-	+	+	+	-	-	-
Hx	*C. hornemannii*	+	-	+	+	-	-	+	-	+	+	+	-	-	-
	G. corticata	-	-	+	+	-	-	+	-	+	-	+	+	-	-
Me	*C. hornemannii*	+	+	+	+	-	-	+	-	-	+	+	-	+	-
	G. corticata	+	+	+	+	-	-	+	-	+	+	+	-	-	-

Present: +, Absent: -

Aq- Aqueous, **EtAc**- Ethyl acetate, **Hx**- Hexane, **Me**- Methanol

Table 2.Enzyme assays of seaweeds

Sample	Amount in µm/g	APX activity (nmol min^{-1}) at 290nm	Amount of POD in U/mg	PPO (A575/min/g Fresh weight)
Control	0.15±0.05 (100)	7.26±0.01 (100)	2.26±0.01 (100)	-
C. hornemannii	0.36±0.03 (162)	9.09±0.05 (119)	3.09±0.05 (109)	0.125
G. corticata	0.4±0.05 (180)	13.4±0.02 (173)	4.4±0.02 (173)	0.205

Table 3.Antioxidant assays

Assays	Samples	Methanol	Ethyl acetate	Hexane	Aqueous
Total condensed tannin	***C. hornemannii***	59.12±0.04	56.03±1.04	54.51±2.04	49.21±0.03
	G. corticata	55.21±0.01	49.05±3.42	50.01±0.07	45.87±3.04
Total phenol	***C. hornemannii***	59.43±0.01	54.76±5.04	46.32±0.05	43.47±0.07
	G. corticata	59.21±0.01	62.43±0.07	55.32±1.05	56.53±7.06
Total Antioxidant Capacity assay (IC50 value)	***C. hornemannii***	67.31±1.02	59.23±1.42	51.01±3.06	59.31±0.64
	G. corticata	66.45±0.47	59.36±0.84	58.71±1.23	60.61±0.01
Total Reducing Power assay (IC50 value)	***C. hornemannii***	77.24±0.04	68.16±0.14	56.01±1.03	64.51±0.02
	G. corticata	73.31±0.04	65.08±0.02	53.21±0.51	72.09±0.41
DPPH assay (IC50 value)	***C. hornemannii***	66.24±0.34	58.26±0.54	36.21±0.03	64.21±1.32
	G. corticata	73.31±0.4	64.28±0.62	43.33±1.54	72.09±1.45
Hydrogen Peroxide Radical Scavenging assay (IC50 value)	***C. hornemannii***	68.01±0.02	60.31±1.05	50.51±0.09	59.76±0.01
	G. corticata	67.05±1.07	56.23±0.04	55.01±0.03	59.01±6.01

Extracellular synthesis of nanoparticles could be highly advantageous from the point of view of synthesis in large quantities and easier downstream processing as compared to the intracellular synthesis.

Figure 5.Morphological changes of Water, Reflux extract and *C.hornemannii* nanoparticles by Seed germination test

Antibacterial activity of the extract and synthesized particles

Considering the reflux extracts, maximum zone of inhibition was obtained in *Gracilaria corticata* (11mm) against *Pseudomonasaeruginosa* and minimum of 7mm against *Staphylococcus aureus* at 1mg/ml concentration. Whereas, *Chondrococcus hornemannii* at 1mg/ml concentration against all the four pathogens showed least maximum zone of inhibition of only 9mm diameter.

Comparatively, while assaying the synthesized particles of both samples, maximum zone of inhibition of 18mm was shown by *Chondrococcus hornemannii* against *Staphylococcus aureus* at 1mg/ml concentration, whereas *Gracilaria corticata* against *Pseudomonasaeruginosa* (12mm at 1mg/ml), *Escherichiacoli* and *Vibrio cholera* (11mm at 1mg/ml) and no activity even at 1mg/ml concentration against *Staphylococcus aureus*. From the results, *Chondrococcus hornemannii* was highly active to all the tested microorganisms. The production of antimicrobial activities was considered to be an effective indicator of the capability of the seaweeds to synthesize bioactive secondary metabolites (Table 4 & 5).

Seed germination test

Seed germination test was done for determination of phytotoxicity of nanoparticles synthesized using seaweed extracts by measuring the relative root elongation, germination index and relative seed germination of the seeds for various concentrations after 7 days of incubation. The concentrations ranging from 0.5 µg/ml to 5 µg/ml concentrations was tested, from which 5 µg/ml concentration showed maximum growth in seaweed reflux extract, water and nanoparticle synthesized. Maximum length of both root and shoot was obtained on 5 µg/ml concentration of *Chondrococcus hornemannii*synthesizednanoparticles, whereas minimum growth was seen on reflux extracts of the sample. Root and shoot growth was very least in water (Figure 5). From the formula stated in materials and methods, germination index, root elongation and shoot length were calculated accordingly by standard deviation and results were tabulated. These calculated results show that *Chondrococcus hornemannii* nanoparticles have shown maximum growth and germination index at 5 µg/ml concentration.

Table 4. Antibacterial activity of seaweed extracts

Sample	Concentration (µg/ml)	Antibacterial activity of seaweed reflux extract (well diffusion method) in mm			
		E. coli	*P.aeruginosa*	*V. cholerae*	*S.aureus*
C. hornemannii	250	7	6	-	-
	500	8	7	-	-
	750	9	8	8	-
	1000	9	9	9	9
G. corticata	250	-	9	7	-
	500	-	10	8	-
	750	8	10	8	-
	1000	9	11	9	7

Table 5. Antibacterial activity of synthesized nanoparticles

Sample	Concentration (µg/ml)	Antibacterial activity of synthesized nanoparticles (well diffusion method) in mm			
		E. coli	*P. aeruginosa*	*V.cholerae*	*S.aureus*
C. hornemannii	250	10	11	10	16
	500	10	11	10	16
	750	11	12	11	17
	1000	11	13	13	18
G. corticata	250	9	10	9	-
	500	10	11	10	-
	750	10	11	10	-
	1000	11	12	11	-

Conclusion

The green approach to synthesis of nanoparticles using plant materials, such as reducing and capping agents, could be considered attractive in nanobiotechnology. In recent years, studies on the improvement of biological techniques for synthesis of nanoparticles have been extensively increased due to the harmful effects of chemical preparation methods, in this present work, a nontoxic, green and eco-friendly protocol for the synthesis of silver nanoparticles using seaweeds has been adopted.

The remarkable difference between our results and the results obtained in previous studies may be due to several factors. The main reason can be due to the difference in the seasonal variation of the seaweeds which has been well established from our results. Another important reason can be due to difference in the extraction procedure to recover the active metabolites and differences in assay methods that would result in different susceptibilities of the target strains. The prevalence of antimicrobial activities observed in the extracts from the southwest coast of India provides credible evidence that seaweeds maintain effective antimicrobial chemical defences. From the present study, it can be concluded that the

macroalgae are potential sources of bioactive compounds. Further studies are necessary to identify the anticancer activity and catalytic degradation of the synthesized particles.

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