Marine biomolecule mediated synthesis of selenium nanoparticles and their antimicrobial efficiency against fish and crustacean pathogens

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Abstract

Recent techniques of intensive and super intensive aquaculture which involves high rates of stocking and supplementary feedings has substantially enhanced the fish and prawns diseases. Metallic nanoparticles could serve as future substitutes for some conventional antibiotics due to their increase antimicrobial efficiency. The present study reports a simple phytochemical mediated green synthesis of selenium nanoparticles (SeNPs) and evaluates its potential biological application as antimicrobial agent against important fish and crustacean pathogens *Vibrio harveyi* and *Vibrio parahaemolyticus*. The work focused on synthesis and characterization of SeNPs by marine macro algae *Kappaphycus alvarezii(K.alvarezii)* and *Halimeda opuntia (H.opuntia)* .These nanoparticles were characterized by Scanning Electron Microscopy (SEM) ,UV-Visible spectroscopy (UV-Vis), Fourier transform infrared spectroscopy (FT-IR). FTIR spectrum confirms the presence of various functional groups in the plant extract, which may possibly influence the reduction process and stabilization of nanoparticles. Crystal phase composition and average crystalline size of synthesized SeNPs were confirmed with XRD measurements. Characterization of extracted nanoparticles for shape, size, and purity indicated that the particles were spherical with diameters ranging between 30 and 80 nm. Antimicrobial study was done and the results was shown to confirm the activity against marine pathogenic microbes *V. harveyi* and *V. parahaemolyticus.* It was found that SeNPs are able to inhibit the cell growth by dose-dependent manner. The ABTS and DPPH assay results shown that the SeNPs prepared using marine macroalgal extracts possess more antioxidant potential among which *K.alvarezii* shown higher ant oxidative activity when compared to *H. opuntia*.

Introduction

Aquaculture as the world's fastest growing food production sector represents an important impediment to production due to diseases that must be dealt with seriousness. Fish are the most traded food commodity in international trade with an export value of nearly USD150 billion (FAO 2016) and provide numerous ancillary jobs in the secondary sector, such as fish processing, fish feed industry, trade and marketing services (Shruti 2012). This globalization of aquatic products and the rise of aquaculture as a primary supplier of the world's aquatic food supply have been associated with the culture of new aquatic species, the transport of live species to new countries and continents which has simultaneously facilitated the spread of the associated pathogens and diseases (FAO 2014). Disease outbreaks especially caused by bacterial infection, are being increasingly reported hindering the expansion of aquaculture production. *Vibrio spp.* which is considered as the significant pathogen in culture set up caused severe economic losses world wide to the tune of US$ 9 billion per year (Ruwandeepika H.A.D. 2010) affecting large-scale losses of larval and juvenile penaeids and finfishes (Subasinghe et al.,2011; Zorrilla et al.,2003). Marine polysaccharides from several macroalgal species were found to be most active against a number of pathogens like *Vibrio harveyi (V.harveyi)* and *Pseudomonas aeruginosa* (Shaalan et al., 2016) providing protection in aquaculture production. Of which, the red seaweed *Kappaphycus alvarezii (K.alvarezii)* plays a major role in anti-infection and salinity related stress tolerance enhancement in shrimp hatchery culture. *Halimeda opuntia (H.opuntia)*, a well known tropical calcareous algae widely distributed in Indian coast and is known to possess several antibacterial properties.

Nanotechnology has become an extensive field of research due to the unique properties of nanoparticles, which enable novel applications. Nanoparticles have found their way into many applications in the field of medicine, including diagnostics, vaccination, drug and gene delivery (Swain et al. 2014). Nanoparticles have gained much interest as a specific and sensitive tool for diagnosis of several bacterial, fungal and viral diseases in aquaculture (Li et al. 2008). In aquaculture, the metal and metal oxide nanoparticles exhibit effective antimicrobial properties against fish pathogens (Rana 2011) and have been utilized in water decontamination and as antimicrobial agents (Biplab Sarkar et al. 2015; Navarro et al. 2000). Selenium, one of the essential micronutrients, appears as a potent antioxidant with reduced toxicity in its nanoscale form (El-Bayoumy K. 2001). Selenium, a biocatalyst and functional component of numerous

enzymes, is required for the proper functioning of the immune system, and possesses anticarcinogenic effects (Rajasree, 2015; Sassan et al.2010). The preparation of stable selenium nanoparticles as nontoxic biomedical application is still challenge and several work has been undertaken to synthesize by different biological source like microbes (Stoles and Orlando 1999; Dhananjal and Cameotra 2010) and plant sources (Li et al. 2007; Gurunathan et al. 2009; Wang et al. 2010) producing nanoparticles of different size and morphology.

In metabolism, the oxidative stress has generated many reactive oxygen species (ROS), which are highly reactive and harmful to the cells. If it is not eliminated, the ROS is found to be very active and damage important molecules of cells like DNA, proteins and lipids. For the complete scavenging of ROS, the cells possess itself antioxidants enzymes and non enzymatic compounds. Antioxidate enzymes from marine origin can prevent the free radicals .

Herein we report a facile, green synthesis of selenium nanoparticles using marine macroalgae and compared the efficiency of ABTS and DPPH assay to estimate antioxidant activity of macroalgal extracts and selenium nanoparticles synthesized by green and red macroalgae.

Materials And Methods

Sample collection

K. alvarezii thalli were collected from the culture sites located at Muttom, Kanyakumari District, South India and *H. opuntia* were collected from the wild along the coastal waters of Gulf of Mannar, Tamilnadu South east coast of india. Macroalgae were cleaned, washed with distilled water to remove dust and soluble impurities, then shade dried, milled and stored at room temperature in airtight containers.

Preparation of seaweed extracts

The dried powdered algal powders were separately mixed with 100ml distilled water and reflexed for 30mins in the Erlenmeyer flask.The extract obtained was filtered twice with Whatman paper No1 and stored at 4ºC till further use.

Synthesis of selenium nanoparticles

Analytical grade selneious acid was purchased from Merck. About 10ml of macroagal extract was mixed with 90ml of 30 mM selenious acid and 1.8ml of 40mM ascorbic acid.The ascorbic acid is added to induce the synthesis process.These preparations were incubated at room temperature in a shaker incubator at 160–170 rpm for 3 days till it reaches ruby red or brick red color. The solution was then kept in dark for further analysis.

Characterization of biosynthesized selenium nanoparticles

UV–Visible spectroscopy

Noble metal nanoparticles absorb strongly in the visible region due to surface Plasmon resonance. Colour transitions arise due to atomic level structural changes in the substances being tested, leading to related changes in the ability to absorb light in the visible region of the electromagnetic spectrum. Appearance of colour arises from the property of the coloured material to absorb selectively within the visible region of the electromagnetic spectrum. Absorption of energy leads to a transitional change of electron from ground to excited state. Hence the UV–visible absorption spectroscopy is a primary characterization tool to study the metal nanoparticles formation. The nanoparticles were primarily characterized by UV–visible spectroscopy, which proved to be a very useful technique for the analysis of Nanoparticles (Sastry et al. 1998). The reduction of metallic selenium ions was recorded by measuring the UV-Vis spectra of the solution at room temperature with Shimadu UV-vis spectrophotometer UV-2450 at a wave length of 250 -700nm .

Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier Transform Infrared (FT-IR) measurements were carried out to identify the existence of the functional groups in the synthesized selenium nanoparticles. Dry powders of the biomass and SeNPs solutions were centrifuged at 10,000 rpm for 15 min and the resulting suspensions were redispersed in sterile distilled water. The purified pellets were dried and analysed on Shimadzu FT-IR instrument the frequency range of of 4000–400 cm-1 to evaluate the functional groups that might be involved in the absorption process.

X-Ray Diffraction (XRD)

Solids were separated by ultra centrifugation an d12000 RPM for 20 minutes followed by redispersion of SeNPS in to deionized water. The resultant solids were dried in an oven at 55°C. The XRD pattern was obtained with Bruker instrument using Cu- Kα (1.54 °A) source, and the data were collected from 10° to 80° (2θ) with a scan speed of 4 min − 1. XRD analysis was also applied to determine the particle size using Scherrer's formula:

$d = k\lambda\beta(Cos\theta max)$

Where, d is the average crystal size, λ is the X-ray wavelength (0.1541 nm), β is the full-width at half-maximum (FWHM) and θ is the diffraction angle

Scanning Electron Microscopy

Morphology and particle sizes were determined with Scanning Electron Microscopy (SEM) on a JEOL Model 1200 EX instrument operated at an accelerating voltage at 80 kV by focusing nanoparticles following (Rajasree and Gayathri, 2015).

Determination of Minimum inhibitory concentration (MIC) using Resazurin Microtitre Assay

Minimal inhibition concentration (MIC) values of SeNPs and controls were determined based on a microwell dilution method using 96-well sterile microtiter plate for which reasasurin microtitre method (Palomino et al.2002) was employed. The resazurin solution was prepared by dissolving 270 mg in 40 mL of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogenous solution. A sterile 96 well plate was labelled. A volume of 100 µL of the sample was pipetted into the first plate. To all other wells 50 µL of nutrient broth was added and serially diluted it. To each well 10 µL of the resazurin indicator solution was added. 10 µL of bacterial suspension was added to each well. Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. The plate was incubated at 37°C for 18–24 h. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value.

Antioxidant assay

ABTS assay

For ABTS assay, the method of Emad et al.(2013) was followed with some modifications. To 100 µl of sample (different concentration), 0.9 ml of ABTS solution was added. The stock solutions included ,7mM ABTS solution and 2.45 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 4–16 hrs at room temperature in the dark. The resulting solution was then diluted with ethanol by mixing 1 ml of freshly prepared ABTS solution. The extent of decolorization was measured at 734nm to obtain an absorbance of 0.706 ± 0.001 units. Fresh ABTS solution was prepared for each assay. The ABTS

scavenging capacity of the extract was compared with that of BHT(Butylated hydroxytoluene) and percentage inhibition was calculated as;

ABTS radical scavenging activity (%) = [O.D.of control - O.D. of sample/O.D. of control] X 100

DPPH Assay

The DPPH assay was done following the method of (Brand Williams et al.1995) with slight modifications. The radical scavenging and antioxidant potential of the macro algal extracts were determined by the capacity of the extracts to scavenge the stable free radical DPPH and exchange it into Diphenyl picryl hydrazine. The degree of decolourization from purple to yellow color was assessed spectrophotometrically at 517 nm. Different concentrations of *K.alvarezii* and *H. opuntia* extract and the biogenic synthesized SeNPs were separately mixed with 3 ml of 0.1 mmol DPPH and incubated in dark for 15 min. The reaction mixture was mixed well and left in dark for 30 minutes at room temperature. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the extracts was calculated using the following equation:

Antioxidant activity (%) = {(absorbance at blank) – (absorbance at test) / (absorbance at blank)} X 100

Results And Discussion

Metal salts reduction to metal nanoparticles in the presence of biomolecules is always accompanied by color change in the reaction medium. After the addition of macroalgal extracts separately with selenuos acid, the media displayed a time-dependent color change. Biogenic synthesis of selenium nanoparticles was confirmed by the conversion of colourless selenous acid into the brick red colour of selenium nanoparticles in the presence both *K.alvarezii* and *H.opuntia* extracts.

The reduction process took place in 30 minutes when *K.alvarezii* extract was used where as the color change occurred after 2 hrs in case of *H.opuntia.* The occurrence of the red colour indicated the presence of SeNPs and the characteristic red color of the solution was due to excitation of the surface plasmon vibtrations of the SeNPs, a convenient spectroscopic signature of their formation. Earlier work was reported on biosynthesis of SeNPs using green algae *Caulerpa taxifolia* (Men et al. 2009) polysaccharides which was found effective on antiviral bioactivity studies. It is for the first time we are reporting the fast reduction process of biogenic SeNPs by a red and green marine macroalgae.

UV- spectroscopic analysis

The synthesized selenium nanoparticles in the suspensions were primarily confirmed using UV-Vis spectral analysis. The UV-Vis spectra recorded at different time intervals showed increased absorbance with increasing time of incubation and both the samples showed a sharp surface plasmon resonance band at 293nm and 294 nm for *K.alvarezii* and *H.opuntia* respectively (Fig. 1). This clearly indicated that all the selenium nanoparticles had size below 100 nm, as recorded by Chen et al. (2008) who stated that the particle size could be correlated with the nature of the UV-visible spectra and if the particle size below was 100 nm or less, it showed a clear absorption maximum in the UV range. The present results concurred with the findings of Fesharaki et al. (2010) reported the absorption band between 200–300 nm for the nanoselenium synthesized using *Klebsiella pneumonia*. Similar observations was reported by (Zhang et al.2011) by synthesizing nanoselenium using *Pseudomonas alcaliphila* which exhibited absorption band between 200–300 nm .

Fourier Transform Infrared Spectroscopy Analysis

The synthesized selenium nanoparticles were characterized using FTIR in order to investigate the biological compounds responsible for the synthesis and stability of the nanoparticles. The result showed sharp absorption peaks at 1116cm − 1 and 1402 cm − 1. Peak at 1116cm − 1 can be assigned to -OH and the one at 1402 cm − 1 corresponds to the C-H vibration of the aromatic ring (Fig. 2a).

The bands at (Fig. 2b) 1743 cm − 1 and 1670 cm − 1are assigned to the stretching vibrations of C = O and C = C, respectively (Huang et al. 2007; Shankar et al.2004 ; Fayaz et al. 2010). The vibrational bands corresponding to bonds such as C = C and C = O are derived from the compounds such as flavonoids and terpenoids in algal extract. Hence, it may be assumed that these biomolecules are responsible for capping and stabilization. The absorption band at 1517 cm − 1 (Fig. 2c and Fig. 2d) was characteristic of amide II (N = H) bands of proteins. This result suggest that the proteins are interacting with the biosynthesized SeNPs. The other peaks correspond to stretching and vibrational bending of C = C, -NH_2, -COOH, -CH_2 and C = O and thus, indicating the presence of reducing groups responsible for the reduction of SeNPs.

X-Ray Diffraction (XRD)

The crystal structure and the phase composition of selenium nanoparticles were determined using XRD techniques. It is important to know the exact nature of the formed SeNPs and this can be figured out through the XRD spectrum. The pure crystalline natures of the SeNPs have been evidenced by the joint committee on powder diffraction standards (JCPDS) file number 06-0362. The sharpening of the peaks clearly indicated that the particles were in the spherical shape. We observed some noise background in biological method of SeNPs, which may be due to the presence of additional bioactive compounds present. The XRD pattern of powder shows that there is no requirement of post annealing to get desired crystalline phase. The peak (101) at 41 in X-axis also represents selenium as per Kawamura et al.1997.The other peaks (110,021,012) also show the presence of selenium nanoparticles. In the peaks at X-axis (28,31,40) showsthe selenium particles (Fig. 3a&3b).

Surface Morphology Analysis of Synthesised Nanoparticles

SEM is one of the most widely used techniques in characterization of nanomaterials, thanks by its resolution of few nanometers and the possibility to use magnifications from 10 to over 300,000. In a typical SEM, a source of electrons is focused into a beam, with a very fine spot size of *5* nm and an energy ranging from a few hundred eV to 50KeV that is allocated over the surface of the specimen by deflection coils. When electrons strike and penetrate the surface, a number of interactions occurs and result in the emission of electrons and photons from the sample. SEM images are produced by collecting the emitted electrons on a cathode ray tube (CRT). Combining different kind of images, SEM provides information about morphology, microstructures, chemical composition and distribution of bulk and nanomaterials.

In order to characterize SeNPs principally in size, shape and distribution, both chemically and biogenically synthesized SeNPs were analyzed through SEM. The morphology of the prepared nanoparticles was investigated by SEM analysis, which clearly showed that the particle sizes of spherical selenium prepared were in the range of 30–80 nm (Fig. 4a & Fig. 4b).The nanoparticles obtained in the present study were of relatively smaller size than that reported by Eszenyi et al. 2011 based on synthesized nanoselenium using *Lactobacillus spp.* and obtained nanoparticles within the size range of 100–200 nm. The reduction in the size of the nanoselenium obtained could be due to the variation in the plant material used, which differed in the characteristics of polysaccharide present.

Minimum inhibitory concentration (MIC) of SeNPs

Minimal inhibitory concentration of the SeNPs synthesized by different algal extracts against *Vibrio sp*. was analysed, was defined as the lowest concentration at which no increase of the OD_{600} of the pathogens was observed. The synthesized nanoparticles showed reproducible, effective antibacterial activity. Consistently, a comprehensive analysis revealed that neither the presence of algal extracts had a negative effect on the growth kinetics or viability of *Vibrio sp.* In (Table.1) the Sample1 indicates the *V. parahaemolyticus* and the sample 2 was *V. harveyi*. Above result strongly showed that SeNPs has bactericidal properties. Obtained results permit to hypothesize that biogenic SeNPs are able to inhibit bacterial growth because they're able, both using Selenium antimicrobial ability and cap characteristics, to interact with bacteria cells and to inhibit the matrix development.

Antioxidant Capacities of SeNPs In-vitro

The ability of free DPPH radical scavenging consists in measuring the change in absorption of purple DPPH radical solution. The disappearance of the violet color informs about the level of radical neutralization contained in the DPPH solution. In the case of ABTS assay the mechanism is identical, with the only difference that the solution has green colour. The antioxidant capacities *In vitro* of the SeNPs were investigated using the assays reported in literature, most of them can be classified into two types; assays based on electron transfer (ET-based) such as DPPH and ABTS, and assays based on hydrogen atom transfer (HAT-based) reactions such as lipid peroxidation, depending upon the chemical reactions involved. Among them, DPPH was carried out in hydrophobic media, while ABTS was in hydrophilic media. In order to compare the antioxidant activity, the IC_{50} values for all measured parameters were determined. IC_{50} value of parameter defines the weight of the sample material, which causes neutralization of free radical after 2 hours from the start of the test. All prepared materials have the ability to neutralize the DPPH radical. When comparing the IC_{50} values of synthesized SeNPs, nanoparticles synthesized with *K. alvareerzii* (19.4 (µg/ml) had higher value than that with *H. opuntia* 18.7(µg/ml) and has higher ability to scavenge DPPH molecules (Fig. 5b). In ABTS radical scavenging assay, antioxidants respond to ABTS and transform it to blue coloured 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid). Radical scavenging activity is directly proportional to the intensity of ABTS transformation.

When comparing the IC_{50} values of Selenium nanoparticle synthesis of *K alvareerzii* (27.83 (µg/ml)) higher then *H.opuntia* (21.83 (µg/ml) (Fig. 5a). It was observed that the values of RSC% in ABTS were higher than those in DPPH. This feature was due to the effect of the high water solubility of the nanoparticles, which led to the separation of the Se nanoparticle-rich water phase from the free radicalrich lipid phase, and thus reduced the ability of Selenium to capture the free radicals. In general, the total antioxidant activity of SeNPs was reasonably more or less equal to the standard ascorbic acid. Hence, the result of this assay was similar to that of the other two assays. Selenium compounds have higher antioxidant property that delete free radicals *in vitro* and increase seleno enzyme, glutathione peroxidase which plays a key role in preventing free radicals from damaging cells and tissue *in vivo*. Similarly, earlier studies proved that SeNPs exhibited antioxidant activity with lesser toxic effect than selenium Biologically synthesised SeNPs had higher antioxidant action, and was less toxic to normal cell, than selenium dioxide (Forootanfar et al. 2014).

Summary And Conclusion

This green route of biosynthesis of selenium nanospheres is a simple, economically viable and an eco-friendly process resulting in nearly monodispersed highly stable selenium nanoparticles. This method is capable of producing SeNPs in a size range of about 50–150 nm, under ambient conditions. The synthesized nanoparticles can be separated easily from the aqueous sols by a high-speed centrifuge. These selenium nanoparticles were characterized by UV–Vis spectroscopy, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD), FTIR spectrum confirms the presence of various functional groups in the plant extract, which may

possibly influence the reduction process and stabilization of nanoparticles. The antibacterial activityofSeNPs was assayed against *Vibrio* pathogens from aquatics. It was found that SeNPs are able to inhibit the cell growth by dose-dependent manner. The cell wall of *K. alvarezii* is known to be constituted of carrageenan, a sulfated polysaccharide, which may contribute to its antioxidant potential in addition to the presence of ascorbic acid, vitamin A and various phenolics.

Declarations

Ethical Approval and Consent to participate

The data were collected based on seaweed culture collections. Therefore, this study did not require any permits from the authorities. Additionally, the data did not involve any endangered or protected species.

Human and Animal Ethics

Not applicable

Consent for publication

The authors hereby give the consent to publish the work in Thalassas: An International Journal of Marine Sciences.

Availability of supporting data

The data that support the fndings of this study are available from the corresponding author upon reasonable request.

Competing interests

On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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This study did not received funding.

Authors' Contribution Statement

Radhika Rajasree SR conceived and designed the experiments, analyzed the data, prepared fgures and/or tables, authored or reviewed drafts of the paper, approved the final draft. Gayathri S conceived and designed the experiments, analyzed the data, reviewed drafts of the paper, approved the final draft. Gobalakrishnan M performed ,participated in data analysis, provided editorial reviews of the manuscript, and approved the fnal draft.

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Table

Table – 1 Antibacterial activity of SeNPs against pathogenic organisms

S.No	Microorganisms/sample	Growth of inhibition									
		1000 µg	500 µg	250 µg	125 µg	62.5 µg	31.2 µg	15.6 µg	7.8 µg	Positive control 10µg	Culture
	Sample 1										
1	*Vibrio parahaemolyticus*	-	+	+	+	+	+	+	+	-	+
2	*Pseudomonas aeroginosa*	-	+	+	+	+	+	+	+	-	+
	Sample 2										
1	*Vibrio parahaemolyticus*	-	+	+	+	+	+	+	+	-	+
2	*Pseudomonas aeroginosa*	-	-	+	+	+	+	+	+	-	+

Figures

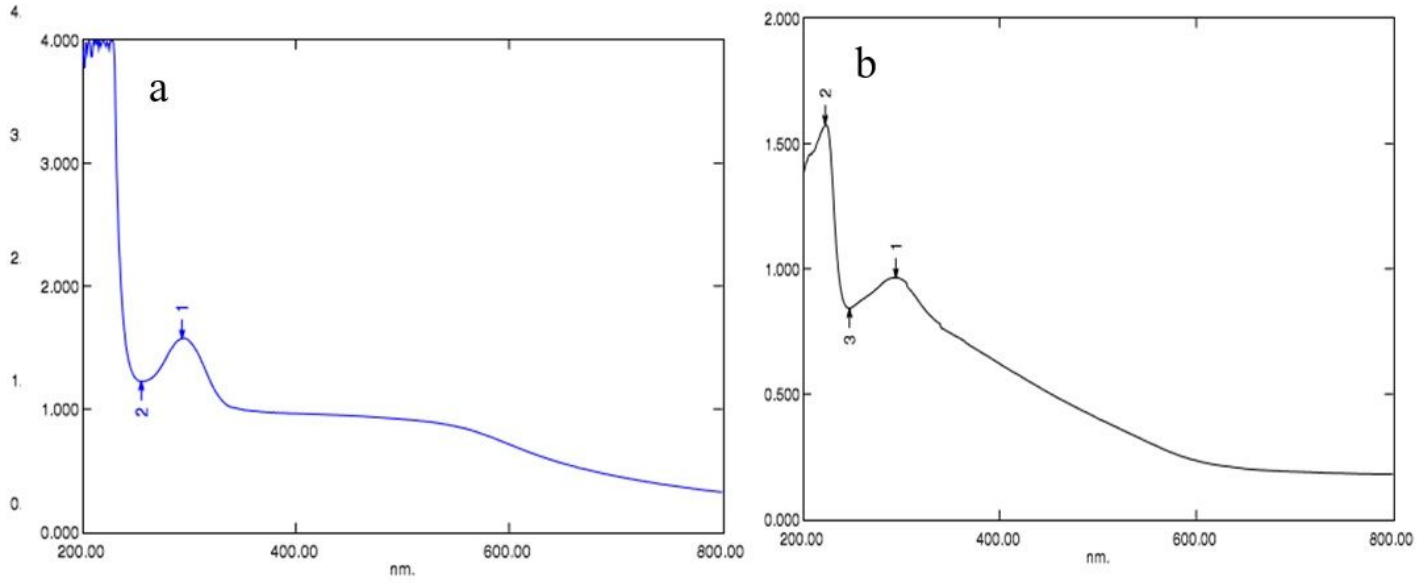

Figure 1

UV- vis spectroscopy images of (a) *Halimeda opuntia (b) Kappaphycus alvarezii*

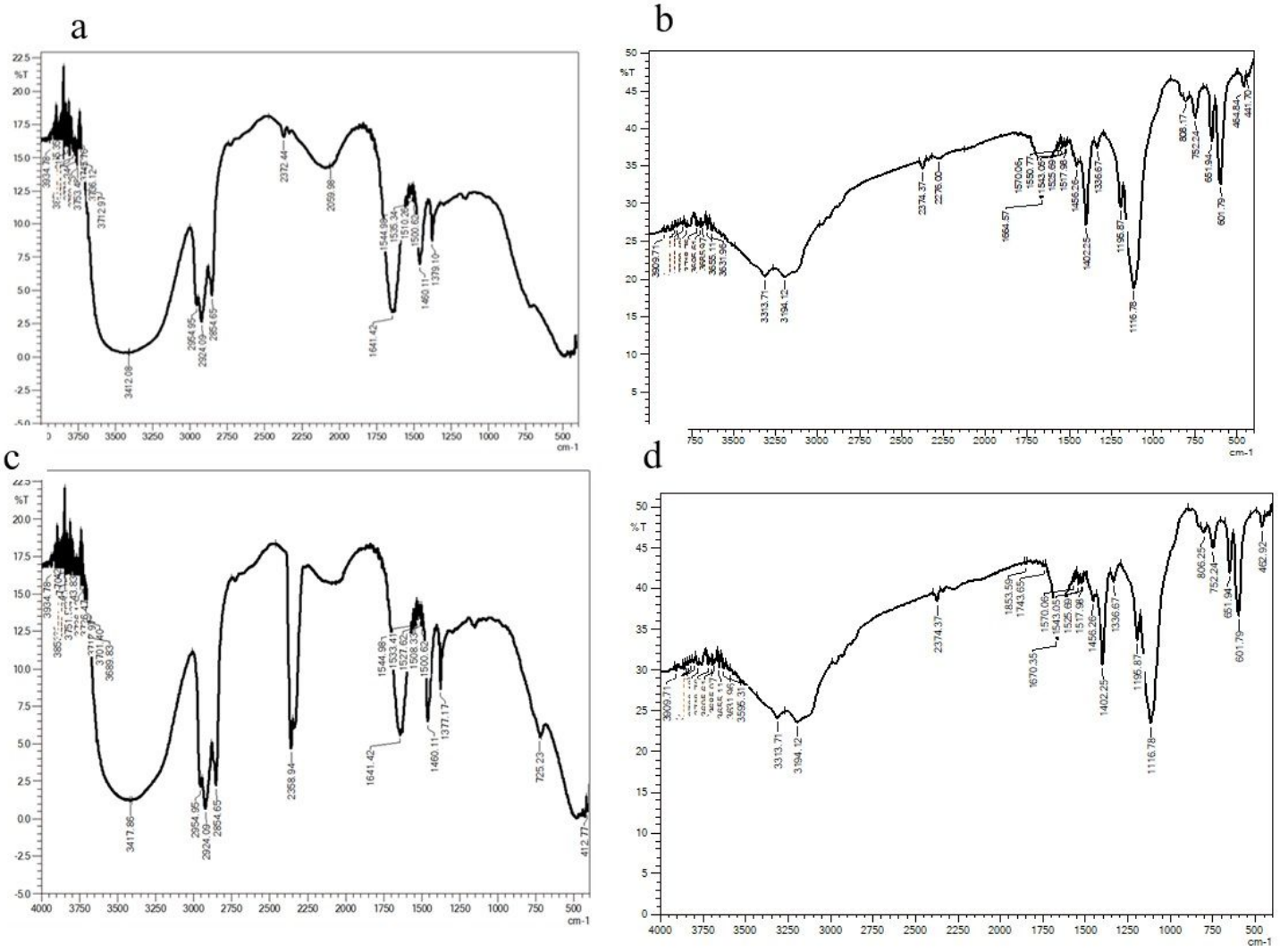

Figure 2

FT-IR Spectra (a) Aqueous extract (*Halimeda opuntia*), (b) Nanoparticles synthesised *(Halimeda opuntia)*,(c) Aqueous extract (*Kappaphycus alvarezii)*,(d) Nanoparticles synthesised (*Kappaphycus alvarezii*

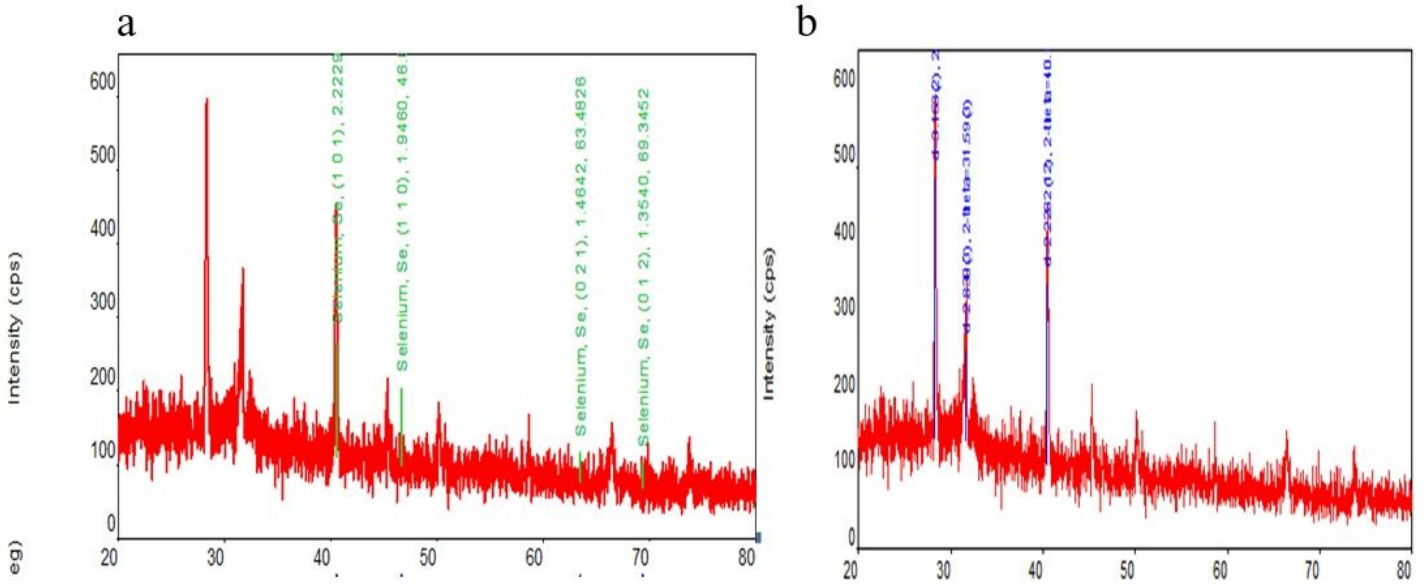

Figure 3

XRD Spectra (A) *Halimeda opuntia (B) Kappaphycus alvarezii*

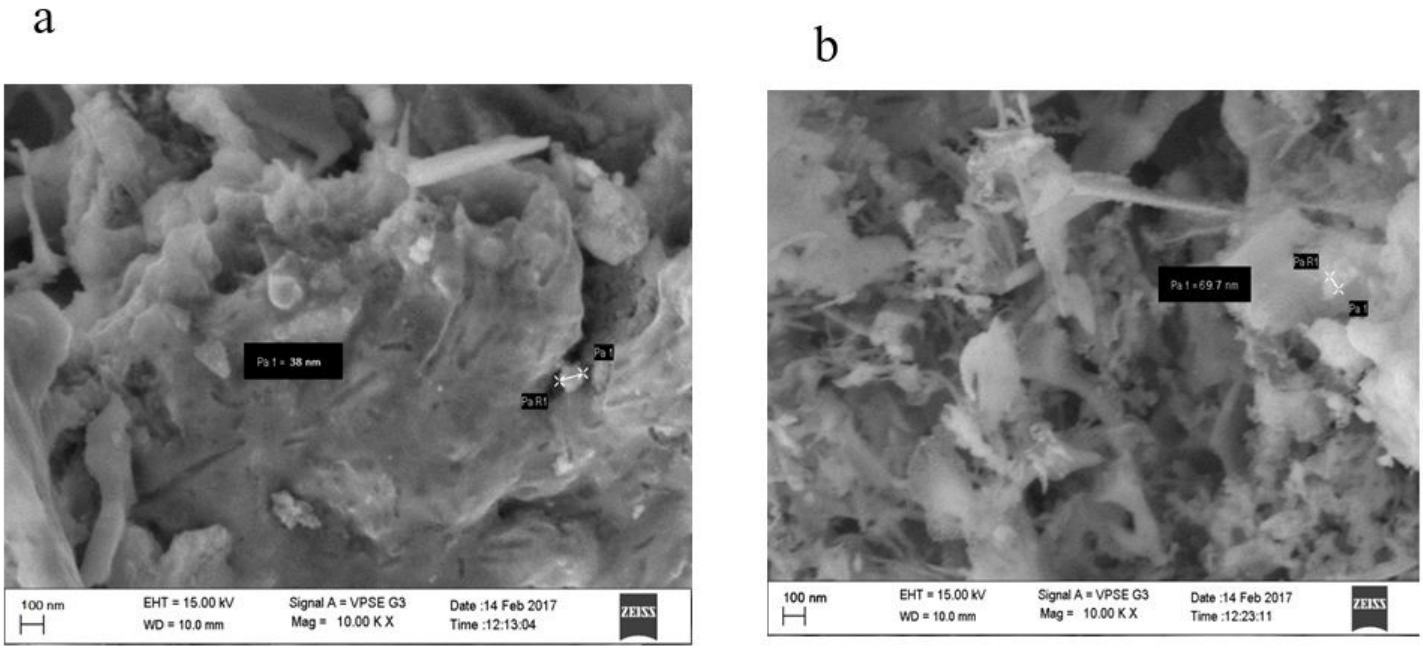

Figure 4

Scanning electron microscope (SEM) images of (a) Nanoparticles synthesised *Halimeda opuntia* ,(b) Nanoparticles synthesised *Kappaphycus alvarezii*

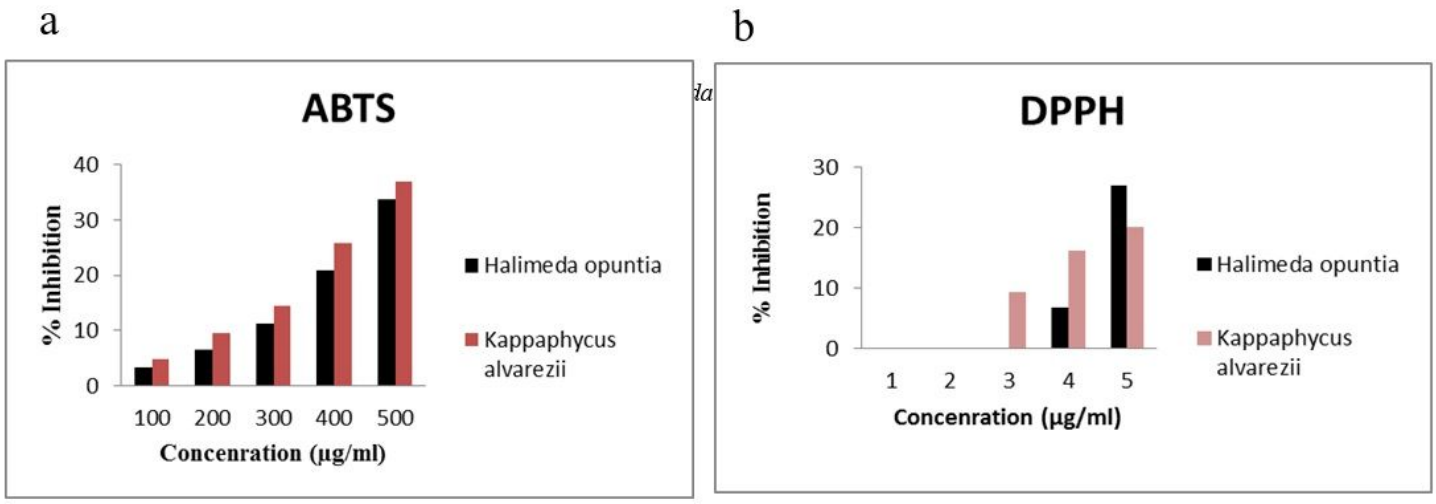

Figure 5

Free radical scavenging ability of SeNPS by using *Halimeda opuntia* and *Kappaphycus alvarezii* ABTS and DPPH method.