CHAPTER- IV

MATERIALS AND METHODS

Collection of seaweeds

Seaweeds, a group of non-flowering plants endowed with broad spectrum of
neutraceutical and pharmaceutical components with significant species diversity are
found inhabited in Hare island of Gulf of Mannar. Hare Island is situated 4.5 Km
away from Tuticorin Port beach with shallow region consisted of sand, rocks, mud
and coral reefs supporting luxuriant growth of seaweeds. A field survey was
undertaken in the selected sampling stations of Hare Island of Tuticorin coast over a
period of twelve months from January to December 2011. Seaweed samples were
periodically collected in pre-monsoon (June -September), monsoon (October -
January) and post – monsoon seasons (February - May) during low tide by random
sampling method. After collection, seaweeds were washed with seawater to remove
all extraneous matters such as epiphytes, shells, associated fauna and adhering sand
particles. Later, seaweeds were thoroughly cleaned with tap water to remove salt on
the surface and preserved by wet (5% formalin in seawater) and dry preservation
(herbaria) methods. Seaweed species were identified by referring to authentic floras
and books (Srinivasan 1969; Misra, 1996; Fritsch, 1977). Diversity and seasonal
availability status of seaweeds were recorded as most abundant (+++), abundant
(++), less abundant (+) and sparse (+). Seaweeds encountered in Hare Island were
also photographed.

Dominated species of seaweeds *Ulva lactuca* Linn., *Ulva reticulata* Forsk.,
*Enteromorpha intestinalis* (L.) Link f., *Enteromorpha compressa* (L.) Grev, *Codium
tomentosum* (Hudson) stack, *Codium bursa* C. Agardh, *Caulerpa racemosa* (Forsk)
Weber-van-Bosse, *Caulerpa sertularioides* (Gmelin) Howe, *Caulerpa scalpelliformis*

**Preparation of seaweed powder:**

Selected seaweeds were air-dried under shade at 30 to 35°C and 65-70% relative humidity and were powdered in an electrical miller, sieved and stored. These powdered samples were used to estimate the nutritional constituents such as total carbohydrate, fibre, protein, protein digestibility, free amino acids, amino acids profile, lipids, fatty acids profile, phenols, flavonoids, tannin, vitamin-C, vitamin-E, free radical scavenging activity, superoxide radical assay, reducing power and total antioxidant activity.

**4.1 Determination of nutritive components in seaweeds**

**4.1.1 Total carbohydrate** (Dubois, 1956)

Reagents

5% Phenol
96% Sulphuric acid

100 mg of leaf tissue was homogenized with 10 ml of distilled water and filtered through a muslin cloth. To 0.1 ml of the filtrate, 0.9 ml of distilled water, 1 ml of 5% phenol and 5 ml of 96% sulphuric acid were added. The contents were shaken well and after 30 minutes, absorbance was read at 490 nm. Glucose was used as standard.

4.1.2 Dietary fibre (AOAC, 2000)

Reagents

1.25% Sulphuric acid

1.25% NaOH

Crude fibre was determined by sequential extraction of seaweed samples with 1.25% H2SO4 and 1.25% NaOH using the fibre-bag as a container. For drying and ashing, the crucible with sample was dried in an oven for 5 hours at 105°C and ashed in the muffle furnace (Carbolite, United Kingdom) at 525°C overnight. The weight of crucible with sample after drying and ashing was recorded and the crude fibre content was calculated.

4.1.3 Total soluble protein (Lowry et al., 1951)

Reagents

Alkaline copper reagent

Solution A – 2% Sodium carbonate in 0.1N sodium hydroxide

Solution B – 1% Sodium potassium tartrate

Solution C – 0.5% Copper sulphate

To prepare 100 ml alkaline copper reagent, 98 ml of solution A, 1 ml of B and 1 ml of C were mixed together freshly.
Folin-ciocalteau reagent (commercial reagent was diluted in the ratio of 1:1 distilled water at the time of use).

100 mg of sample was homogenized in 10 ml of distilled water and filtered through a muslin cloth and centrifuged at 3000 rpm for 10 minutes. To the supernatant 10% trichloro acetic acid (TCA) was added in 1:1 ratio and left in an ice bath for 30 minutes to precipitate protein. Then, centrifuged at 3000 rpm for 5 minutes and discarded the supernatant. The precipitate was dissolved in 0.1 N sodium hydroxide and diluted to a known volume.

To 0.5 ml of protein extract, 5 ml of alkaline copper reagent was added. After thorough mixing 0.5 ml of folin ciocalteau reagent was added and allowed to stand for 30 minutes; the blue colour appeared and absorbance was measured at 650 nm using UV visible spectrophotometer (Model No: UV 2371). Amount of protein was calculated and expressed as mg/g DW. Bovine serum albumin (BSA) was used as standard.

4.1.4 In vitro protein digestibility (IVPD) Hsu et al., (1977)

Protein digestibility was assayed by in vitro method described by Hsu et al., (1977). The enzymes used for IVPD were purchased from Sigma Chemical Co., St. Louis, MO, USA. Calculated amount of control (casein) and sample were weighed out, hydrated in 10ml of distilled water and refrigerated at 5°C for 1 hour. The samples containing protein and enzymes were maintained at pH 8.0 and 37°C. In vitro protein digestibility was determined by sequential digestion of the samples containing protein with a multi-enzyme mixture (trypsin, α- chymotrypsin and peptidase) at 37°C followed by protease at 55°C. pH drop of the samples from pH 8.0 was recorded after
20 min of incubation. The IVPD was calculated according to the regression equation
\[ Y = 234.84 - 22.56X \], where \( Y \) is the % digestibility and \( X \) the pH drop.

### 4.1.5 Free amino acids - Ninhydrin method (Moore and Stein, 1948)

**Reagent**

Ninhydrin (200 mg ninhydrin was dissolved in 100 ml of acetone)

**Procedure:**

100 mg of the sample was homogenized in 10 ml of 80% ethanol and filtered. To 2 ml of the filtrate, 2 ml of ninhydrin reagent was added. The mixture was kept in boiling water bath for about 20 minutes. After cooling 3 ml of 50% ethanol was added and absorbance was read at 570 nm. Leucine was used as standard and the results were expressed as mg/g DW.

### 4.1.6 Analysis of amino acids by High performance liquid chromatography (HPLC)

Free and protein amino acids were estimated by O-pthaldialdehyde method described by Rajendra, (1987). Concentrated 80% ethanolic extract was directly used for qualitative and quantitative estimation of free amino acids. For protein amino acids, protein in the extract was precipitated by adding equal volume of 10% TCA and dried in vacuum. To known quantities of dried protein (usually 75 mg), 2.0 ml of 6.0 N HCl was added and hydrolyzed at 110°C for 18 hrs. After hydrolysis, the hydrolysates were allowed to evaporate to dryness and the dried material was used for HPLC analysis (LACHROM L-700 and D-70000 HPLC).

**Reagents**

Borate buffer (0.4M): Boric acid (2.47g) was dissolved in 100 ml of water and pH was adjusted to 9.5 with 4.0N NaOH
Methanol tetrahydrofuron was prepared by the addition of 30 ml of tetrahydrofuron to 970 ml of methanol.

Ortho-phtaldialdehyde reagent: Anhydrous ortho-phtaldialdehyde (50 mg) was dissolved in a mixture of 2.0 ml of methanol, 8.0 ml of borate buffer and 5.0 ml of β-mercaptoethanol. The reagent was prepared fresh for every estimation.

**Procedure:**

Ortho-phtaldialdehyde reagent (1 ml) was added to 200 µl of the amino acid sample, mixed thoroughly and kept undisturbed for 2 minutes for derivatization. The sample was then filtered and 20 µl was injected for analysis.

Operational conditions: Column: C 18’ 4.6 x 250 mm, 5 µm packing was loaded and run with mobile phase A: 0.1 M acetate buffer (pH 7.2) and mobile phase B: 3% tetrahydrofuron in methanol at flow rate 1.5 ml/min. The column gradient was maintained as 10 - 42% B for 15 min., 42% B for 10 min., 42% - 50% B for 3 min., 50% - 70% B for 7 min., 70% - 90% B for 4 min., 90% - 100 % B for 1 min., 100% B for 2 min., 100% - 10% B for 1 min., 10% B for 2 min. Standard amino acid mixture (5 µl) was also run separately, and the chromatograms were compared with standard and quantified.

**4.1.7 Lipid** (Bligh and Dyer, 1959)

One Gram of seaweed sample was ground in a pestle and mortar with about 10 ml of distilled water. The pulp was transferred to a conical flask and 30 ml of chloroform-methanol mixture (2:1 v/v) was added and mixed well. For complete extraction, it was kept for overnight at room temperature under darkness. At the end of this period, 20 ml chloroform and 20 ml water was added. The resulting solution
was subjected to centrifugation, and 3 layers were formed. A clear lower layer of chloroform containing all the lipids, a coloured aqueous layer of methanol with water soluble material and a thick pasty interface were seen. The methanol layer was discarded and the lower layer was carefully collected free of interface by sucking out with fine capillary tube and transferred to a pre-weighed beaker and evaporated. When the solution is free of organic solvents, the weight was determined again. The difference in the weight was recorded as weight of lipid. The total amount of lipid was expressed in mg g⁻¹ DW.

4.1.8 Estimation of fatty acids (Miller and Berger, 1985)

Fatty acids in seaweed samples were identified and quantified as methyl esters. One Gram of each seaweed powder was extracted with chloroform-methanol mixture (2:1 v/v) using a glass pestle and mortar with a pinch of glass powder. The homogenate was taken in a container, sealed air tight and incubated overnight in an oven at 50°C. The sample was once again extracted as described above and filtered. The filtrate was added with enough quantity of anhydrous sodium sulphate to dehydrate. The resulting concentrate was taken in a Pasteur pipette, dried at room temperature by passing nitrogen gas and stored in a sealed airtight container. Fatty acids in lipid samples thus prepared were then converted to methyl esters in the following manner.

Reagents for preparation of fatty acid methyl esters (FAMES)

a) Saponification reagent: 45 g of NaOH in 300 ml of methanol: water mixture (1:1v/v).

b) Methylation reagent: 325 ml of 6.0 N HCl mixed with 275 ml of CH₃OH
c) Extraction solvent: 200 ml of hexane mixed with 200 ml of anhydrous
diethyl ether.

d) Base wash: 10.8 g of NaOH dissolved in 900 ml of distilled water.

100 mg of each sample was taken in teflon-lined screw cap tube and 1.0 ml of
saponification reagent was added. The tube was closed and boiled for 30 minutes with
intermittent shaking. Then 2 ml of methylation reagent was added and boiled in a
water bath at 80°C for 20 minutes, with intermittent shaking. Again 2 ml methylation
reagent was added and boiled in a water bath at 80°C for 20 minutes. Following
cooling of the tube to room temperature, 1.25 ml of extraction solvent was added and
the tube was rotated end to end for 10 minutes. After discarding the lower aqueous
phase, 3 ml of base wash was added and the tube was shaken thoroughly for 10
minutes. The upper layer, an organic phase containing the fatty acid methyl ester was
used for analysis by gas chromatography (ASHMACO, Japan; Model No: ABD20A). Two
microliters of each sample was injected into the GC column and the fatty acids were
identified and quantified by comparing the peaks with that of standard fatty acid
methyl esters. The column contained 10% diethylene glycol succinate. The oven
temperature was 180°C isothermal and injection port temperature was 200°C.
Nitrogen was used as carrier gas and the flow rate was 30 µl per minute.

4.1.9 Potassium, Calcium, Magnesium and Sodium (AOAC, 1970)

1g of seaweed sample was mixed with 10 ml of concentrated nitric acid, 4 ml
of 60% (v/v) perchloric acid and 1 ml of concentrated sulphuric acid. The mixture
was left undisturbed overnight. After that it was heated on a hot plate containing
concentrated sulphuric acid in a beaker until brown fumes ceased to come out and
then it was allowed to cool. After cooling, it was filtered through Whatmann No. 42
filter paper. The filtrate was made up to 100 ml with deionised distilled water and was used for the estimation of K, Ca, Mg and Na using flame photometer Model 125 with KCl, CaCl₂ and MgCl₂ and NaCl as standards for calibration.

4.1.10 Iron, Zinc, Copper, Mercury and Cadmium (Issac and Johnson, 1975)

1g of seaweed sample was digested with a mixture of 10 ml concentrated nitric acid, 4 ml of 60% perchloric acid and 1ml of concentrated sulphuric acid. After cooling, the digest was diluted with 50 ml of deionised distilled water, filtered through Whatman No. 42 filter paper and the filtrate was made up to 100 ml in a glass volumetric flask with deionised distilled water. The filtrate was analysed by atomic absorption spectrophotometer (AAS 6300).

4.2. Determination of antioxidant chemicals

4.2.1 Total phenol (Duan et al., 2006)

100 mg of seaweed sample was homogenized with 10 ml of distilled water and filtered through a muslin cloth. 1 ml of the filtrate 1.5 ml of deionized water and 0.5 ml of 50% Folin ciocalteau reagent were added and the content was mixed thoroughly. After one minute, 1 ml of 20% sodium carbonate solution was added, and mixed. The control contained all the reagents except sample. After 30 min of incubation at 37°C, absorbance was measured at 750 nm (UV-visible spectrophotometer - Model No: UV 2371). Total phenolics were expressed as gallic acid equivalent (GAE) per gram dry weight.

4.2.2 Total flavonoid (Zhinshen et al., 1999)

100 mg of seaweed sample was homogenized with 10 ml of distilled water and filtered through muslin cloth. 250 µl aliquot of the extract was mixed with 1.25 ml of distilled water and 75 µl of 5% NaNO₂ solution. After 6 minutes, 150 µl of 10%
AlCl₃·H₂O solution was added. After 5 minutes, 0.5 ml of 1M NaOH solution was added and then the total volume was made up to 2.5 ml with distilled H₂O. Following thorough mixing of the solution, absorbance against blank was determined at 510 nm. Quercetin was used as standard and the results were expressed as mg quercetin equivalents (QE)/g dry weight.

4.2.3 Tannin (Julkunen - Titto, 1985)

100 mg of the sample was homogenized with 10 ml of distilled water and filtered through a muslin cloth. 0.1 ml aliquot of aqueous extract was mixed with 3 ml of 4% vanillin (prepared with methanol) and 1.5 ml of concentrated HCl. The solution was shaken vigorously and left to stand at room temperature for 20 minutes in darkness. Absorbance against blank was read at 500 nm using UV visible spectrophotometer. Results were expressed as mg catechin equivalent (CE)/g dry weight.

4.2.4 Estimation of thiamine (B₁), riboflavin (B₂), niacin or niacinamide (B₃) and pyridoxine hydrochloride (B₆)

The B vitamins were analysed with HPLC method equipped with UV detector and auto- injector using the modified methods described by Aslam et al., (2008).

Preparation of extraction solution

It was made by mixing 5 ml of acetonitrile with 10 ml of glacial acetic acid and the volume was finally made up to 1000 ml with double distillled water.

Preparation of buffer and mobile phase

To prepare buffer, 1.08 g of hexane sulphonic acid sodium salt and 1.36 g of potassium dihydrogen were dissolved in 940 ml distilled water and 5 ml of tri-ethylamine was added to it. pH was adjusted to 3.0 with orthophosphoric acid. For the
preparation of mobile phase, buffer and methanol were mixed in a ratio of 96:4 and filtered through 0.22 µm membrane filter and sonicated for degassing in an ultrasonic bath.

Standard stock solution - Dissolved accurately weighed quantities of niacin (41.5 mg) in 25 ml of double distilled water, pyridoxine hydrochloride (20.8 mg) in 25 ml of double distilled water, riboflavin (6.9 mg) in 100 ml of extraction solution and Thiamine (2.24 mg) in 25 ml of double distilled water.

**Preparation of samples**

10 g of each sample was ground with 25 ml of extraction solution, kept on shaking in water bath at 70°C for 40 minutes and finally the volume was made up to 50 ml with extraction solution. Then the extract was filtered through filter tips and aliquots of 20 µl from this solution were injected into HPLC by using auto- sampler.

Chromatographic conditions: Analytical reversed phase C-18 column (ODS column, 250 x 4.6 mm, 5 µm, phenomenex, Inc.) was used for separation. Mobile phase containing a mixture of buffer and methanol in the ratio of 96:4 was delivered at a flow rate of about 1 ml / min with UV detection at 210 nm. 20 µl aliquots of the standard solutions and sample solutions were injected.

**4.2.5 Vitamin-C (Ascorbic acid) – Baker and Frank, (1968)**

Indophenol reagent (20 mg of dichlorophenol indophenol was dissolved in 10 ml of warm distilled water).

DT reagent (2 g of 2, 4 dinitrophenyl hydrazine and 1 g of thiourea were dissolved in 100 ml of 9 N sulphuric acid).

100 mg of each sample was homogenized with 10 ml of 5% trichloro acetic acid (TCA). The homogenate was centrifuged at 3000 rpm. To 2 ml of protein free
supernatant, 1 drop of indophenol reagent and 0.5 ml of DT reagent were added and incubated at 10°C for 1 hour. Then cooled in ice bath and 2.5 ml of 85% sulphuric acid was added. After intermittent shaking for 30 minutes (until red colour appeared), absorbance was measured at 540 nm. L-ascorbic acid was used as standard and the results were expressed as mg/g DW.

4.2.6 Tocopherol (Rosenberg, 1992)

The seaweed sample (2.5 g) was homogenized in 50 ml of 0.1 N sulphuric acid and allowed to stand overnight. The content in the flask was shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate was used for estimation.

Into stoppered centrifuge tubes, 3 ml of extract and 3 ml of water were pipetted out separately. To both the tubes, 3 ml of ethanol and 3 ml of xylene were added, mixed well and centrifuged. Xylene (2.0 ml) layer was transferred into another stoppered tube. To each tube, 2.0 ml of dipyridyl reagent was added and mixed well. The mixture (3 ml) was pipetted out into a cuvette and the extinction was read at 460 nm. Ferric chloride solution (0.66 ml) was added to all the tubes and mixed well. The red colour developed was read exactly after 15 minutes at 520 nm. Tocopherol was used as standard.

Amount of tocopherol in the sample was calculated using the formula,

\[
\text{Tocopherol (µg)} = \frac{\text{Sample } A_{520} - A_{460} \times 0.29 \times 0.15}{\text{Standard } A_{520}}
\]

4.2.7 Antioxidant activity

Crude extracts of twelve seaweeds were prepared by pouring 100 ml of distilled water in a conical flask containing 10 g of each seaweed powder separately in
the ratio of 10:1 (v/w). After 24 hours, the mixture was filtered through Whatman No.1 filter paper and the filtrate was evaporated to dryness. Crude (aqueous) extracts of all the samples (1 mg / ml) were used for the determination of free radical scavenging activity, superoxide anion radical scavenging, ferric ion reducing power and total antioxidant activity.

4.2.8 Free radical scavenging assay (Hatano et al., 1988)

1 ml aliquot of test sample was added to 3 ml of 0.004% DPPH (2, 2-diphenyl-1-picrylhydrazine) solution prepared in methanol. The mixture was vortexed for 1 minute and kept at room temperature for 30 minutes in darkness. The absorbance was read at 517 nm. A low absorbance of the reaction mixture indicated a high free radical scavenging activity.

\[
\text{DPPH scavenging activity (\%) = } \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

Where, \(A_{\text{control}}\) is the absorbance of the DPPH solution without test solution, \(A_{\text{test}}\) is the absorbance of DPPH with test solution. Methanol was used as blank. Ascorbic acid was used as control.

4.2.9 Superoxide anion radical scavenging assay (Dasgupta and De, 2004).

To 3 ml of reaction mixture (50 mM of phosphate buffer pH 7.8, 13 mM methionine, 2 \(\mu\)M riboflavin, 100 M EDTA, 1 ml of 75 \(\mu\)M nitroblue tetrazolium (NBT) and 1 ml of the test solution were added. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 minutes of illumination from a fluorescence lamp. The percentage inhibition was calculated by following the formula,

\[
\text{Inhibition (\%) = } \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]
Where \( A_{\text{control}} \) is the absorbance of the sample without test solution, \( A_{\text{test}} \) is the absorbance of the test solution. Sample without NBT and test solution was used as blank. Ascorbic acid was used as control.

4.2.10 Ferric ion reducing antioxidant assay (Oyaizu, 1986).

1 ml of the test solution was mixed with 1 ml of phosphate buffer (0.2 M, pH 6.6) and incubated at 50°C in a water bath for 20 min. The reaction was stopped by adding 1 ml of 10% TCA solution and then centrifuged at 5000 rpm for 10 min. The supernatant (1.5 ml) was mixed with 1.5 ml of distilled water 0.1 ml of 0.1% ferric chloride solution and allowed to stand for 10 min. The absorbance was measured at 700 nm and higher absorbance indicated greater reducing power. Ascorbic acid was used as control.

4.2.11 Total antioxidant activity (Prieto et al., 1999).

Preparation of reaction mixture:

7.45 ml sulphuric acid (0.6 M), 0.9942 g sodium phosphate and 1.2359 g ammonium molybdate were mixed together in 250 ml with distilled water.

1 ml of each sample was mixed with 3 ml of reaction mixture and was incubated at 95°C for 90 min. Absorbance was measured at 695 nm. Total antioxidant activity is expressed as equivalence of ascorbic acid in milligram per gram of extract.

4.3 Antimicrobial activity

The powdered seaweed samples (20 gms) were extracted with hexane, benzene, chloroform, methanol, petroleum ether and water using soxhlet apparatus. Solvent was evaporated under vacuum and the concentrates were used for antifungal and antibacterial assay.
4.3.1 Antifungal assay- Paper disc diffusion method (Perez et al., 1990)

Antifungal activity of each seaweed extract was analysed using human pathogens., *Candida albicans* (MTCC 227), *Trichophyton simii* (110/02) and *T. rubrum* (MTCC 296) and plant pathogens *Aspergillus niger* (MTCC1344) and *Curvularia lunata* (46/01) obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, and Chandigarh, India. Each fungal pathogen was sub cultured in sabouraud’s dextrose agar (SDA) medium and maintained. The inocula of the respective fungi were seeded on sabouraud’s dextrose agar plates. Sterile filter paper (Whatman No.1) discs (8 mm) were impregnated with different seaweed concentrations: 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml and 3 mg/ml and dried aseptically. The impregnated discs were placed on pre inoculated sabouraud’s dextrose agar medium and incubated at 37°C for three days. Fluconazole (100 μg/ml) was used as positive control. Hexane, benzene, chloroform, methanol, petroleum ether and water (100%) were also used as negative controls. The diameter (mm) of the growth inhibition halo caused by each extract was measured. All the assays were carried out in triplicates. The seaweed extract concentration which has effected minimum inhibition (MIC = 1 mg/ml) was used for further studies.

4.3.2 Antibacterial activity – Disc diffusion Assay (Bauer et al., 1966).

Antibacterial activity of each seaweed extract was analysed using human pathogens., Gram positive bacteria, *Bacillus subtilis* and Gram negative bacteria *Escherichia coli*, (MTCC 50), *Salmonella typhi* (MTCC 1357), *Pseudomonas aeruginosa* (MTCC 424) and *Klebsiella pneumoniae* (MTCC 3384) obtained from the Department of Microbiology; St. Mary’s College (Autonomous), Thoothukudi. Each bacterial pathogen was subcultured in agar medium and maintained. What man No. 1 sterile
filter paper discs (6mm) were impregnated with different seaweed extract concentrations 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, and 3 mg/ml and dried aseptically at room temperature. The spread plates were prepared by proper concentration of inocula. Each sample loaded disc was placed in the seeded agar plate. After 24-48 hours of ±37°C incubation, the diameter of the inhibition zone was measured. For positive control, streptomycin disc (100 µg/ml) was used, whereas for negative control, respective solvents were loaded on sterile discs. All the assays were carried out in triplicates. The seaweed extract concentration which has effected minimum inhibition (MIC = 2.5 mg/ml) was used for further studies.

4.4 Biosynthesis and characterization of silver nanoparticles

1 g of each seaweed powder was suspended in 100 ml deionised water and was incubated for 24 hours at ambient temperature. Then, the suspension was filtered to obtain clear aqueous seaweed extract. To synthesis silver nanoparticles 5 ml of each aqueous seaweed extract was taken in 250 ml flask separately and 95 ml of AgNO₃ (1 mM) solution was added. The colour change of the solution from pale yellow to dark brown was checked periodically, upto 24 hours. The colour of the solution changing into dark brown indicated the formation of silver nanoparticles.

4.4.1 UV-visible spectroscopy:

The seaweeds mediated synthesized silver nanoparticles (AgNPs) were monitored by ultra violet – visible (UV-visible) absorption measurement using UV-visible spectrophotometer model No: UV2371. Formation of silver nanoparticles observed at multiple time intervals from 0-24 hours were analysed for their absorption between 200 and 800 nm ranges and the absorption spectra were drawn.
4.4.2 SEM (Scanning electron microscope) analysis

Seaweeds mediated synthesized nanoparticles were subjected to lyophilization using Vir Tis bench top machine. After lyophilization the purified silver nanoparticle were coated. Coating of the sample was done by placing the purified silver nanoparticles in a vacuum evaporator, pumping the chamber down to a vacuum level equivalent to that in the SEM specimen chamber and heated the conductive metal (gold palladium) upto vapourization. A small part of the cloud of metallic vapour hit and stuck to the surface of the specimen, and formed a thin metallic vapour covered all the sides. The coated sample was mounted on SEM stub, in such a way that it could be tilted in any angle for easy viewing. Observations were made with different magnifications and size, shape and arrangement pattern of AgNPs were analysed by SEM image (JEOL JSM 35 CF SEM).

4.4.3 TEM (Transmission electron microscope) analysis (Cressey and Schofield, 1996)

Transmission electron microscope (TEM) analysis was employed for detecting size, shape and morphology of silver nanoparticles. TEM gives high-resolution images of the sample under study. Transmission electron microscope works as same principle as an optical microscope, but it measures the electrons scattered from the sample. Because electrons can be accelerated by greater electric potential, the TEM is capable of magnifying images up to 200,000 times. The sample of silver nanoparticles synthesized using sea weed extract was prepared by placing a drop of the reaction mixture over carbon coated copper grids and allowing the acetone to evaporate. The sample was dried in order to take electron micrograph using Joel, JEM 2100
4.4.4 Antibacterial activity

Silver nanoparticles were also tested for antibacterial activity by disc diffusion method. (Bauer et al., 1966).

4.4.5 FT–IR (Fourier transforms infra-red spectroscopy) spectroscopic analysis

(Vijayabaskar and Shiyamala, 2012)

One milligram seaweed powder was mixed with 100 mg of dry potassium bromide (FT-IR grade) and then compressed into a pellet using hydraulic press (5000 - 10000 psi). The pellet was immediately put into the sample holder and FT-IR (Systronics 166) spectra were recorded in the range of 400 to 4000 cm\(^{-1}\).

Statistical analysis:

Statistical analysis was worked out by using data analysis tool package of Microsoft Excel 2007 (Gurumani, 2005). Data collected during this study were analysed by analysis of variance (one way ANOVA) at 1 % and 5% levels of significance (Steele and Torrie, 1980) and Karl Pearson’s correlation (Neil Salkind, 2003).