4. MATERIALS AND METHODS

4.1. Study Area

The Seaweed samples were collected from the Coastal region of Manapad, South India, 60 Kms from Tuticorin and 18 Kms South of Tiruchendur. Coordinates for the collection site is 8°22′28″N 78°3′34″E.

Figure 3: Map showing the study area of Algal Collection

4.2. Collection and Processing of Algal Sample

The three different types of seaweeds viz., Sargassum wightii, Caulerpa scalpelliformis and Cheilosporum spectabile belonging to the family Phaeophyceae, Chlorophyceae and Rhodophyceae respectively were collected from the Manapad Coastal region from the Tuticorin district of Tamil Nadu during the month of December 2010 (Plate 5). The algal samples were handpicked and washed thoroughly with seawater to remove debris, sand particles and epiphytes. It was kept in an ice box containing slush ice,
transported to the laboratory and washed thoroughly with tap water to remove the salts from
the surface of the samples. The water was drained off and the algal material was spread on
blotting paper to remove excess water. The algal samples were identified by Dr. E. Santosh
Kumar, Scientist-D, Tropical Botanical Garden and Research Institute, Palode,
Thiruvanthapuram.

Plate 5: Sample Collection sites from Manapad Coast

A                                                                              B
A, B, C, D: Different sites of Manapad Coast

C                                                                              D
Plate 6: Air dried Seaweeds

A: *C. scalpelliformis*, B: *C. spectabile*, C: *S. wightii*.

Plate 7: Preparation of Seaweed Liquid Fertilizer (SLF)

Flow chart for the Preparation of Seaweed Liquid Fertilizer

1. Fresh Seaweeds
2. Washed thoroughly to remove all epiphytes and sand particles with tap water
3. Shade dried for seven days
Hand crushed

Algal sample was ground using mixer-grinder

Coarse powder collected

Coarse powder was mixed with distilled water 1:20 (W/V)

Boiled at 60°C for 45 minutes

Filtered through muslin cloth

Filtrate was collected and is known as Seaweed Liquid Fertilizer and stored at 4°C for future studies

Different concentrations of SLF were prepared from seaweed extract

The three different seaweeds were shade dried (Plate 6) and made into powder form and they were boiled separately with one litre of distilled water for an hour and filtered. The filtrate was taken as 100% concentration of seaweed extract (Plate 7) and from this it was made into different concentrations viz., 10%, 20%, 30%, 40% and 50% respectively using distilled water. The prepared Seaweed Liquid Fertilizers were refrigerated and used for further studies. The Chemical liquid fertilizer (CLF) was bought from the local fertilizer shop located in Kadayam.

4.3. Elemental analysis in Seaweeds

The physical nature of the seaweeds such as colour, pH and the chemical characteristics such as Sodium, Potassium, Magnesium, Calcium, Silicon, Chloride, Sulphur, Phosphorus, Nitrate, Iron, Zinc and Copper contents were analyzed by the method described
by the American Public Health Association (APHA, 1995). The macro and microelements present in the seaweeds were analyzed by Atomic Absorption Spectrophotometer.

4.4. Field Trial

The vegetable plant, *Solanum lycopersicum* was selected as the experimental plant for the present study. The seeds of the plant were collected from Agriculture Union, Kadayam, Tirunelveli district, Tamil Nadu, India. Healthy seeds which were free from visible infection with uniform size and colour were segregated and then stored in metal tin containers as suggested by Rao (1976) and used for the experimental purpose.

Seed Pre-treatment

The seaweed liquid fertilizer (SLF) and chemical liquid fertilizer (CLF) was prepared in different concentrations viz., 10%, 20%, 30%, 40% and 50%. The seeds were later soaked in particular concentration of SLF and CLF for 24 hrs (Plate 9). One batch of seaweed was kept as control and treated with water. The seeds were sowed and observed for their germination and early growth. In the field experiment, ten seeds were sowed per set of experiment. The weeds were removed regularly and watering was done once in every 2 days for the test plant. The experiment was carried out in triplicates.

Experimental Analysis

Plants from each concentration were drawn for various analysis. Plants from the field were uprooted carefully, washed in tap water. They were then processed for different analysis. The growth parameters and biochemical parameters were analyzed at the end of 60 days after sowing. Triplicate samples were used for all the parameters and the mean values were represented.
Plate 9: Seeds soaked in different concentrations of SLF and CLF

A: C. scalpelliformis, B: S. wightii, C: C. spectabile

Plate 8: Field trial of SLF and CLF treated S. lycopersicum
4.5. Growth Parameters

4.5.1. Germination Percentage of Seeds

A single sheet of paper was placed in the bottom of a flat tray of about 30 cm square and 5 cm deep to cover the drainage holes. It was then filled with clean garden soil. Laboratory germination tests were conducted at room temperature. Three rows of 10 seeds each were sowed for the five different concentrations of CLF and SLF. Seeds were sowed at normal seeding depth of 2-3 cm. Seedlings were counted after 7 to 10 days when the majority of seedlings were up.

4.5.2. Measurement of Root and Shoot Length

The shoot length was measured from the collar region to the tip of the shoot and the root length was calculated to the tip of the primary root of the plants grown in different concentrations of SLF and CLF and recorded. The mean values were measured and expressed in centimetres (cms).

4.5.3. Number of Branches

The total number of branches was counted manually after 90 days from the control and the test plants.

4.6. Reproductive Parameters

4.6.1. Number of Flowers

The total number of flowers in the plants treated with different concentrations of chemical liquid fertilizers and three different seaweed liquid fertilizers were manually counted and it was recorded on 90 days.
4.6.2. Number of Fruits

The total number of fruits were counted manually after 120 days from the treated plants of different concentrations of chemical liquid fertilizer and three different seaweed liquid fertilizers and expressed in grams.

4.7. Bio-Chemical Parameters

The uprooted plants were washed and separated into root, shoot and leaf. They were blotted in blotting paper and weighed with the help of an electronic weighing balance. Samples were analysed and the parameters were represented in mean values.

4.7.1. Estimation of Chlorophyll

Leaf samples of 0.5 grams were collected from Solanum lycopersicum test plant, grounded with motor and pestle by adding 80% acetone till they became a smooth paste. The homogenate was transferred to a centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The clear supernatant was transferred to a measuring cylinder and the volume was made up to 20ml by using 80% acetone. The presence of non-green pellet indicates the complete extraction of the pigment. If the pellet is green, it was again subjected to grinding and centrifugation using 80% acetone to extract the chlorophyll completely. The solution was measured in spectrophotometer at 645nm using 80% acetone as blank. The chlorophyll was estimated spectrophotometrically according to the method of Arnon (1949).

4.7.2. Estimation of Carotenoids

Leaves of Solanum lycopersicum weighing about 0.5 gram were collected from the different concentrations of SLF and CLF test plants and was grinded with motor and pestle by adding 80% acetone till they became a smooth paste. The homogenate was transferred to a centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The clear supernatant was
transferred to a measuring cylinder and the volume was made up to 20ml by using 80% acetone. The presence of non-green pellet indicated the complete extraction of the pigment. The extract was measured at 480nm in spectrophotometer to estimate the carotenoid content (Krick and Allen, 1965).

4.7.3. Estimation of α- Amylase

α-Amylase activity was determined by the method of Hall et al., (1970) with an assay based on the hydrolysis of amylose azure (20 mg ml⁻¹). The assay mixture was prepared in 20 mM sodium-phosphate buffer (pH 6.0) containing 2 mM CaCl₂ and 5 mM β - mercapto-ethanol. Assays were conducted at 40°C for 10 min and the reaction was stopped with 18% acetic acid (v/v). One unit (U) of activity is defined as the amount of enzyme liberating coloured soluble material from amylose azure corresponding to 2.5 absorbance units/min at 595 nm under described assay conditions.

4.7.4. Estimation of β-Amylase

β-amylase was measured by the method of Bernfeld (1955). The leaves were homogenized with 4 ml ice-cold 16 mM sodium acetate buffer in pH 4.8. The homogenate was centrifuged at 12,000 g for 15 min and the supernatant was used for determining β-amylase activity. To 0.5 ml of 1% potato starch, 16mM sodium acetate buffer was equilibrated at 37°C for 2 min, 0.5 ml enzyme extract was added, vortexed and incubated with shaking for 5 min at 37°C. To the reaction mixture, 0.5 ml of 3, 5-dinitrosalicylic acid (DNSA) reagent was added and then boiled for 5min. Absorbance at 540 nm was read after adding 4.5 ml of distilled water. DNSA reagent consisted of 1% 3, 5- dinitrosalicylic acid, 0.4 M NaOH and 1 M potassium sodium tartarate. A standard curve using maltose solution was prepared. β-amylase activity was expressed in units g⁻¹ F.w. One unit is defined as the amount of enzyme liberating 1 mg maltose from starch in 5 min at 37°C and pH 4.8.
4.7.5. **Estimation of Total Soluble Sugars**

Fresh leaves from different concentrations of CLF and SLF were hydrolyzed in 5 ml of 2.5 N HCl for 3 hrs. The hydrolyzed extract was cooled and neutralized with sodium carbonate until effervescence was stopped. Volume was made to 100 ml with deionized water and centrifuged at 4,472xg for 10 min. The supernatant (0.5 ml) was collected and mixed with equal volume of distilled water to which 4 ml of Anthrone reagent was added. Test tubes were placed in boiling water for 8 min and allowed to cool at room temperature. Optical density was measured at 490 nm. Amount of total soluble sugars was estimated from the standard curve prepared with D-glucose (Yemm and Willis, 1954).

4.7.6. **Reducing Sugars**

About 1 gm of fresh leaf samples after 90 days were homogenized with 10 ml hot 80% ethanol. The extract was filtered and the filtrate was evaporated in boiling water. To dissolve the sugars in samples, 10 ml of distilled water was added to the extract. To 0.5 ml of the above prepared extract, 2.5 ml of distilled water and 3 ml of dinitrosalicylic acid reagent were added. Test tubes were placed in boiling water for 5 min upon which the colour developed. Following this, 1 ml of 40% Rochelle salt solution (sodium potassium tartarate solution) was added. Test tubes were cooled and the absorbance was read at 510 nm. Amount of reducing sugars was estimated from the standard curve prepared with D-glucose (Miller, 1959).

4.7.7. **Estimation of Total Free Amino acids**

One ml of ethanol extract was taken in 25 ml test tube and neutralized with 0.1N sodium hydroxide using methyl red indicator. One ml of Ninhydrin reagent was added (800 mg Stannous chloride in 500 ml litre buffer, pH-5, 20 g Ninhydrin in 500ml, both solutions were mixed). The contents were boiled in a water bath for 20 min and n-propanol was mixed
in equal volume added, cooled and diluted to 25 ml with distilled water. The absorbance was measured at 570 nm in a spectrophotometer. The standard graph was prepared, total amino acid content was determined according to the method of Moore and Stein (1948).

4.7.8. Estimation of Protein

Fresh leaves of 0.5 g at 90 days of growth were homogenized in 5 ml of phosphate buffer (pH 7.0). The homogenate was filtered through four muslin cloth layers and the filtrate was centrifuged at 6,440xg for 10 min at 4°C. To the aliquot, 5 ml of Bradford reagent was added and vortexed. Absorbance was read at 595 nm (Bradford, 1976). Concentration of soluble proteins was calculated and quantified against a standard curve prepared using bovine serum albumin.

4.7.9. Lycopene Analysis

The extraction of lycopene pigment was based on modified method of Fish et al., (2002). Three or four freshly harvested tomato fruits (140 days) were taken and pulped well to a smooth consistency in a mixer grinder. Five gm of tomato pulp was weighed and extracted repeatedly with acetone in a mortar and pestle until the residue was colourless. The acetone extract was pooled and transferred to a separating funnel containing 20 ml of petroleum ether and the contents were mixed gently. To this, 5% sodium sulphate solution was added. To reduce the loss due to evaporation, 20 ml of petroleum ether was added. The two solvent phases were separated and the lower aqueous phase was re-extracted with additional petroleum ether until it was colourless. The above extracts containing carotenoids were pooled into a brown bottle containing anhydrous sodium sulphate and allowed to stand for 30 min. The extracts were transferred to a volumetric flask through a funnel containing cotton/glass wool. The sodium sulphate slurry was washed with petroleum ether until it was colourless, transferred to a volumetric flask and the volume was made to 100 ml with
deionized water. Using petroleum ether as a blank, absorbance of supernatant containing lycopene was read at 503 nm. One mole of lycopene when dissolved in 1 L petroleum ether (40-60°C) and measured in a spectrophotometer at 503 nm in 1 cm light path gives an absorbance of $17.2 \times 10^4$. Therefore, a concentration of 3.1206 μg lycopene ml gives unit absorbance. Amount of lycopene in tissues was estimated by the formula:

$$\text{Lycopene (mg/g)} = \frac{X}{Y} \times A_{503} \times 3.12$$

Where $X$: amount of petroleum ether (millilitres), $Y$: weight of fruit tissue (grams), $A_{503}$: Absorbance at 503 nm, 3.12: Extinction coefficient.

4.7.10. Estimation of Vitamin C (Ascorbic acid)

To prepare a standard solution (ascorbic acid - 100 μg/ml), 4% oxalic acid was added and titrated against 2, 6- dichlorophenolindophenol dye (V1). End point was detected by the appearance of a pink colour which persisted for 5 min. Amount of dye consumed is equivalent to the amount of ascorbic acid. Tomato pulp (0.5 gm) was taken from 140 day old plant and extracted in 10 ml of 4% oxalic acid. The volume was made up to 100 ml with deionized water and centrifuged at 5,411xg for 10 min. To the above prepared extract, 4% oxalic acid was added and titrated against the dye (V2) (Thimmaiah, 1999). The ascorbic acid concentration was calculated by:

$$\text{Ascorbic acid (mg /100gm)} = \frac{0.5 \text{mg} \times V2 \times 100 \text{ ml}}{V1 \times 15 \text{ ml} \times W}$$

Where, $W =$ weight of the sample (grams).

4.7.11. Estimation of Starch

Estimation of starch was done in 90-day old leaves by the method described by Thimmaiah (1999). Fresh leaf tissue (0.1 g) was homogenized in 10 ml hot 80% ethanol and centrifuged at 4,472xg for 10 min. The resulting pellet was washed repeatedly with ethanol
and dried in a water bath. The dried pellet was redissolved by adding 5.0 ml H$_2$O and 6.5 ml perchloric acid and centrifuged at 0°C for 20 min at 4,472xg. The pellet formed was repeatedly washed with perchloric acid. The supernatants obtained from repeated washings were then pooled and the volume was made up to 100 ml using distilled water. To 0.1 ml of the above prepared solution, 0.9 ml of water and 4 ml of anthrone reagent were added. The contents were heated for 20 min in boiling water and cooled. The absorbance was read at 630 nm. Amount of glucose content was estimated from the standard curve prepared with D-glucose. The concentration obtained was multiplied by a factor 0.9 to calculate the starch content (Hassid and Neufeld, 1964).

4.8. Earthworm Study

4.8.1. Collection of Earthworms

The African variety of earthworm, *Eudrilus eugeniae* were obtained from the Vermiary Laboratory of the Department of Biotechnology, Manonmaniam Sundaranar University, Alwarkurichi.

Earthworms have been studied as a readily available easily maintainable and cheap test species for assessing the chemical pollution. The current investigation aims to study the effect of Chemical Liquid Fertilizer and also Seaweed Liquid Fertilizer in earthworms.

4.8.2. Mass culture of *Eudrilus eugeniae*

*Eudrilus eugeniae* was mass cultured in cement tanks with 1x1x0.3m dimensions. The feed medium was prepared to a height of 0.8m with leaf litters, cow dung and garden soil into which the adult clitellate worms were introduced with water sprinkling on everyday to maintain the moisture content. The worms started feeding vigorously by gathering around the surface layer as the worms are of surface feeders in nature.
Plate 10: Diluted concentrations of SLF and CLF

Diluted *C. spectabile* in 100 ml of water

Diluted *C. scalpelliformis* in 100 ml of water

Diluted *S. wightii* in 100 ml of water

Diluted CLF in 100 ml of water

Plate 11: Experimental Setup of SLF and CLF for earthworm studies
4.8.3. Collection and Preparation of Medium

The main constituent in the medium were garden soil and cowdung. The cow dung was used in the preparation of the standard bedding material which was collected from the nearby locality. Cow dung was shade dried for 3 to 4 days. Later it was grinded and powdered. The plastic tubs used for the present study were washed well in ground water. About 2 kg of cowdung was weighed and used for vermibed preparation in each troughs. They were maintained in triplicates. Pre-weighed breeders of earthworms, *Eudrilus eugeniae* each numbering twenty was introduced manually in each tubs.

4.8.4. Exposure of Seaweed Liquid Fertilizer and Chemical Fertilizer to earthworms

The seaweed liquid fertilizer and chemical liquid fertilizer was taken in two concentrations viz., 0.50 % and 1.00% respectively which was diluted in 100 ml of tap water (Plate 10) and sprinkled in the experimental tubs. The experiments were carried out in triplicates. A control tub was maintained during the study period without incorporation of fertilizer but with earthworms alone. (Plate 11). The worms slowly worked their way down through the mass to the bottom of the tube. The experimental tubs were placed indoor in the laboratory to avoid direct sunlight, rain and to protect the worms from the predators. Water was sprinkled on alternate days to maintain the optimal moisture of the beds. The moisture content of the vermibed was tested by grabbing a fistful and squeezing it such that no water arrives out.

4.9. Growth Studies of Earthworms

4.9.1. Biomass

Biomass of *Eudrilus eugeniae* in different concentrations of SLF and CLF were determined by removing the worms from the substrate which was washed with tap water and dried on paper towels. They were then weighed fortnightly in water filled pre-weighed boats.
This was done to prevent the worm from drying out and dying. The biomass was weighed with the help of electronic weighing balance. The obtained biomass was recorded.

The development of the clitellum was used as one of the parameter to determine the influence of fertilizers on the sexual development of worms. Worms were observed closely every ten days starting from four weeks after they hatched out. Worms were classified as juveniles, pre-clitellates and clitellates.

4.10. Physical Parameters of earthworm amended medium

The physical parameters (pH, Electrical conductivity, Porosity, Moisture) were determined (Chandrabose et al., 1988).

4.10.1. Determination of pH

The pH is defined as the negative logarithm to the base 10 of the $H^+$ ion concentrations. The pH of the bedding material was determined by potentiometric method using a digital pH meter.

Fifteen gram of the air dried sample of each concentration of CLF and SLF was passed through a 2 mm sieve and transferred to a clean 100 ml beaker to which 30 ml of distilled water was added. The contents were stirred intermittently and the suspension was again stirred just before taking the reading. The electrodes were immersed into the beaker containing the sample and water suspension. The metre reading both in the supernatant and the suspension were recorded.

4.10.2. Determination of Electrical Conductivity (EC)

An electrical conductivity meter (EC meter) measures the electrical conductivity in a solution. It is generally used in hydroponics, aquaculture and freshwater system to monitor the amount of nutrients, salts or impurities of water. It is the measurement of the total amount of soluble salts present in the sample and it is expressed in millisimens/cm (ms/cm). To the 5gm of each concentrations of CLF and SLF treated compost sample, 50 ml of distilled water
was added, stirred well and the suspension was allowed to settle down for 8 hrs. The electrode of the conductivity cell was immersed into the sample solution and readings were noted.

4.10.3. Determination of Porosity

Twenty gram of different concentrations of the three different SLF and CLF of the sample was weighed and transferred in small quantities to a 100 ml measuring cylinder with glass stopper by gently tapping the cylinder. The volume of the sample was noted. A known volume of 50 ml of water was added to the sample for complete soaking of the sample. The cylinder was kept undisturbed for proper filling of the pore space with water. The volume of the sample and water was recorded at the end of the experiment and the percentage of the pore space was calculated:

\[
\text{Volume of the sample taken} : W \text{ gm}
\]
\[
\text{Volume of the sample} : P \text{ ml}
\]
\[
\text{Volume of the water added} : Q \text{ ml}
\]
\[
\text{Volume of the sample and water} : P+Q \text{ ml}
\]
\[
\text{Volume of the sample and water at the end of the experiment} : r \text{ ml}
\]
\[
\text{Pore space} (P+Q)-r : t \text{ ml}
\]
\[
\text{Percentage of pore space} : (t/p \times 100)
\]

4.10.4. Determination of Moisture Content

A weighing bottle with its stopper was placed in an electric oven separately at 105ºC for about 15 min. The stopper was replaced and the moisture bottle was removed from the oven, cooled in a dessicator and weighed. The loss in weight was calculated and expressed in percentage using the following calculation:

\[
\text{Weight of moisture bottle} : A \text{ gm}
\]
\[
\text{Weight of moisture bottle + sample} : B \text{ gm}
\]
Weight of sample taken : (A-B) gm
Weight of moisture bottle + sample after drying in the oven : C gm
Weight of moisture in the sample : (B-C) gm
Percentage of moisture in the sample : \( \frac{(B-C) \times 100}{(B-A)} \)

4.11. Histological Study of earthworms

Histological techniques provide a visual means for the examination and analysis of cell or tissue physiologically and morphologically at the microscopic level. The earthworms from the different concentrations of SLF and CLF were taken with distilled water after which 50 ml jars were filled with 30 ml of 1.5% agar prepared with distilled water. After getting cooled and solidified in the jars, the agar was taken out and cut into small pieces. The earthworms were transferred separately into the jars and kept for 96 hrs to remove all soil from their gut (Pokarzhevskii et al., 2000). Fixation preserves the structure and morphology of the specimen throughout the harsh conditions of dehydration, clearing, embedding, sectioning and staining.

4.11.1. Preparation of Histological Specimens

The earthworms were taken out from the beaker and then placed in the dissection board. The surgical blade was set in the holder. The dissection was carried out with a new sterile blade. The earthworms were amputated at the middle of the gut region.

Preparation of histological specimens undergoes the following steps:

1. Fixation
2. Dehydration
3. Clearing
4. Embedding
5. Sectioning
6. Mounting and
7. Staining

4.11.2. Fixation

Fixation is a chemical process used to preserve the tissue from decay either through autolysis or putrefaction. It is to preserve the cell structure of the material in a life-like condition and it was done by a compound fixative called Zenkar’s fixative. The chemical composition of Zenkar’s fixative is

\[
\begin{align*}
\text{HgCl}_2 & \quad : 5 \text{ gm} \\
\text{K}_2\text{Cr}_2\text{O}_7 & \quad : 2.5 \text{ gm} \\
\text{Na}_2\text{SO}_4 \cdot \text{H}_2\text{O} & \quad : 1 \text{ gm} \\
\text{Distilled water} & \quad : 100 \text{ ml}
\end{align*}
\]

Fixation should be carried out immediately after removal of the tissues as it involves the chemical cross linking of proteins. The live earthworms were cut into pieces and transferred to Zenkar’s fixative for 12 hrs. After fixation was accomplished, the excess fixative was washed in running tap water to prevent interference with subsequent process (Pantin, 1969).

4.11.3. Dehydration

During this procedure, various cellular components were dissolved by dehydrating fluids. For example, certain lipids were extracted by anhydrous alcohols and water soluble proteins get dissolved in the lower aqueous alcohols. Dehydration is a process of gradual or stepwise removal and replacement of water from the tissues. Ethyl alcohol was used as a dehydrant. The dehydration was achieved by immersing the tissue in a series of solution of ethyl alcohol in water with gradually increasing the percentages of alcohol. Changing through solutions of 25%, 50%, 75% and 100% reduced some of the shrinkage occurring in the tissue. The time required for each step was about 5 hrs.
4.11.4. Clearing

The process of dehydration led to the saturation of tissues with alcohol. After the dehydration process, the tissues were impregnated with paraffin wax to make it ideal for the purpose of section cutting. This means that the paraffin had to remove the alcohol from the tissues to take its place. However, the diffusion of paraffin into the tissue to replace the alcohol was not possible, as the paraffin was immiscible with alcohol. Therefore, after dehydration, the tissue had to pass through an intermediate step into which it was placed in a fluid which being miscible with alcohol and paraffin made the paraffin to infiltrate into the tissues. This is called clearing.

The clearing of the tissues was done by xylene. This clearing agent not only brought the infiltration of paraffin into the tissues but also made them transparent by removing their opacity. The tissue was placed in xylene at 55-60ºC for 3 hrs. Prolonged treatment with xylene tends to make the tissues brittle. Xylene brought about the quick removal of alcohol from the tissues and speeded up the infiltration of paraffin into them. The tissues were transferred directly from xylene to paraffin (melting point 58º-60ºC). The oven temperature was kept high enough to maintain the paraffin in a molten state. This lessened the overheating of tissues. The paraffin must be in molten condition to infiltrate the tissues effectively.

Properties of paraffin wax are as follows:

Paraffin wax is a polycrystalline mixture of solid hydrocarbons produced during the refining of coal and mineral oils. It is about two thirds the density and slightly more elastic than dried protein. The properties of paraffin wax are improved for histological purposes by the inclusion of substances added alone or in combination to the wax.

- Improve ribboning
- Increased hardness
• Decreased melting point

• Improve the adhesion between specimen and wax

4.11.5. Embedding

Embedding is the process by which tissues are surrounded by a medium such as wax which when solidified will provide sufficient external support during sectioning. As soon as the tissues were thoroughly infiltrated with paraffin they were ready to be embedded. The L-shaped iron blocks were used for block making. The blocks were kept close and adjusted in such a way to accommodate the object. The molten paraffin wax was poured into this mould. The impregnated tissue from the vial was placed in the mould and oriented according to the plane of sections needed. The blocks were allowed to cool and stored until for further use.

4.11.6. Sectioning

The section was prepared from the embedded block using the microtome. It gives the clear appearance of cell structures when visualized under microscope. Microtome is an instrument which is used to cut the tissues or section of uniform thickness. Commonly used microtome is a rotatory microtome. The blade was fixed in a blade holder and adjusted at an angle of 50°. The embedded blocks was fixed in the block holder and fitted with microtome using block adjustment clamp. Using operating handle, the microtome was rotated at a required micron (5-6µ). The ribbon like structure was obtained during each rotation and they were placed in a clean slide.

4.11.7. Mounting

One drop of egg albumin was placed over one end of the slide and eventually spread over the slide to fix the section. The slide was placed in a slide warmer. The ribboned section was placed over the albumin slide. The folds on the section were removed by pouring few drops of water over it and were kept in a warmer at 55-60°C to remove the wax. The melted
section was suddenly placed in xylene to remove the wax for 5 minutes after which the slide was placed in isopropyl alcohol for 5 minutes.

4.11.8. Staining

Staining is the process by which it gives colour to the section. Haematoxylin and Eosin are the two most commonly used routine stains in histology. Haematoxylin, a basic dye stains acidic structures purplish blue. Nuclei (DNA), ribosomes and rough endoplasmic reticulum (with their RNA) are therefore stained blue. Eosin, in contrast is an acidic dye which stains basic structures red or pink. Most cytoplasmic proteins are basic and therefore stained pink or pinkish red. The hydrated section was then kept in basic stain haematoxylin for 5 minutes. Later the excess stain was washed with water and then dipped in 10% of acid alcohol. It was washed in running tap water for 10 minutes. The section was stained with eosin, an acidic dye for 30 seconds. An excess stain, eosin was washed in running tap water for 10 minutes. The sectioned tissue was dehydrated by isopropyl alcohol for 2 minutes and allowed for air drying. After this, the dehydrated section was cleared in xylene for being more transparent.

4.11.9. Visualization

After completion of staining, the slide was ready for visualization. Sections were examined and photographed under phase contrast microscope.

4.12. Preparation of Solvent Extracts of Seaweeds

For the extraction of bioactives, the shade dried seaweeds (30g) were finely powdered and the algal materials were soaked in 50 ml of different solvents. The extraction was carried out separately with different organic solvents in the order of increasing polarity: Chloroform, Benzene, Acetone, Diethyl ether and Methanol. The powdered samples were percolated in different solvents for 30 days. The extracts were filtered using muslin cloth and again filtered
by filter paper. The organic extracts were concentrated to be solvent free by evaporation at 30°C. The residues obtained were finally dried and dissolved in the respective solvents.

4.13. Collection of Test Microbial Cultures

Seven bacterial cultures viz; *Serratia* sp., *Erwinia caratovora*, *Citrobacter* sp., *Xanthomonas vesicatoria*, *Bacillus subtilis* and *Bacillus thuringiensis* and four fungal cultures viz; *Aspergillus niger*, *Aspergillus terreus*, *Trichoderma viridae* and *Aspergillus flavus* were used as the test cultures. These plant pathogenic cultures were collected from Tamil Nadu Agricultural University, Coimbatore.

4.14. Antibacterial Activity Test

The solvent extract of the three different test seaweeds were subjected to antimicrobial assay by agar well diffusion method. The sterilized Muller Hinton Agar (20ml) was poured into sterile petriplates and allowed for solidification. The inoculum was swabbed in each petriplate with cotton swab. After swabbing, five uniform wells were made in each petriplate by using sterile 6 mm tips. A drop of molten nutrient agar was used to seal the base of each well. The wells were filled with 60 µl of the test seaweed solvent extract and allowed for diffusion for 45 min. The solvents were used for the control. The plates were incubated at 37°C for 24 hours for bacteria. The zones of inhibition were measured with antibiotic zone scale millimetre.

4.15. Antifungal Activity Test

The solvent extract of the three different test seaweeds were subjected to antifungal assay by agar well diffusion method. About 20 ml of sterilized Potato Dextrose Agar was poured into sterile petriplates and were allowed to solidify. The inoculum was swabbed in each petriplate with cotton swab. After swabbing, five uniform wells were made in each
petriplate by using sterile 6mm tips. A drop of molten nutrient agar was used to seal the base of each well. The wells were filled with 60 µl of the seaweed solvent extract and allowed for diffusion for 45 min. The solvents were used for the control. The plates were incubated at 28°C for 48 hours for fungi. The zones of inhibition were measured with antibiotic zone scale millimetre.

4.16. Ash Content

Total ash and acid insoluble ash have been analyzed as per the protocols in Ayurvedic Pharmacopoeia (1999).

4.16.1. Determination of Total Ash

About 2-4 gm of powdered *C. scalpelliformis* was accurately weighed in previously ignited weighed crucibles. The samples were ignited by gradually increasing the heat not exceeding to dull red hot of silica crucible (450°C) until they became white. The crucibles were cooled in dessicators and weighed. The percentage of ash was calculated with reference to the air dried powder. The procedure was repeated to get the constant weight.

4.16.2. Determination of Acid insoluble Ash

The acid insoluble ash was determined from the total ash obtained. About 25 ml of 10% w/v diluted hydrochloric acid was added to the crucible containing total ash and was covered with watch glass and boiled for five minutes. Watch glass was rinsed with 5 ml of hot distilled water and added to the crucible. It was then filtered through an ashless filter paper (Whatman No. 41) and the insoluble matter was collected. It was washed with hot distilled water until the filtrate became neutral. The filter paper was then transferred to previous crucible and dried on hot plate. It was ignited to constant weight and the residue was cooled in a dessicator for 30 min and weighed. The total acid insoluble ash was calculated as mg per gm of air dried material.
4.16.3. Determination of Sulphated Ash

About 1 gm of the *C. scalpelliformis* powder was weighed in a silica crucible. The powder was moistened with 1 ml of concentrated sulphuric acid to which a few drops of concentrated Sulphuric acid was added, ignited and cooled until the difference of weight was not more than 0.5 gm and calculated the percentage of sulphated ash with reference to air dried material.

4.16.4. Determination of Water Soluble Ash

The total ash content of *C. scalpelliformis* was boiled with 25 ml of water and filtered through an ashless filter paper (Whatman No.41). It was washed with hot water and the filter paper was ignited in the silica crucible, cooled and the water soluble matter was weighed. The water insoluble ash value was calculated by subtracting the water soluble matter from the total ash.

4.17. Phytochemical Analysis

4.17.1. Qualitative Phytochemical Analysis of Seaweeds

Phytochemical screening determines the biologically active compounds present in the different solvent extracts and aqueous extracts of *Caulerpa scalpelliformis*. All the extracts were tested for the presence of different phytochemicals like alkaloids, steroids, tannin, flavonoids, glycosides and phenolics.

4.17.1.1. Test for Alkaloids

About 1 ml of each solvent extract was boiled in 2 ml of 1 % Hydrochloric acid in a water bath for 5 minutes. The mixture was allowed to cool and filtered. The filtrate was shared in equal proportion into 5 test tubes and named as A, B, C, D and E. 1 ml portion of the filtrate was treated with drops of the following reagents. With Dragendroff’s reagent, a
red precipitate was shown. With Mayer’s reagent, a creamy white coloured precipitate indicates the presence of alkaloids (Harborne, 1973).

4.17.1.2. Test for Steroids

To 0.5 ml of the ethanolic extract of each sample, 2 ml of acetic anhydride was added. Two ml of sulphuric acid was mixed to the test tube and the colour change was observed from violet to blue or green which indicated the presence of steroids.

4.17.1.3. Test for Tannins

About 2 ml of the solvent extract was boiled with 5 ml of 45 % ethanol for 5 minutes. The mixture was cooled and filtered. 1 ml of the filtrate was added to 3 drops of lead subacetate solution. A gelatinous precipitate was observed which indicates the presence of Tannins. Another 1 ml of the filtrate was diluted with distilled water and 2 drops of ferric chloride was added. A transient green colour indicates the presence of Tannins (Trease and Evans, 1996).

4.17.1.4. Test for Flavonoids

About 2 ml of the solvent extract was introduced into 10 ml of ethyl acetate and heated in a boiling water for 3 minutes. The mixture was then filtered and the filtrate was used for the following tests. About 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution (1%). The layers were allowed to separate. A yellow colouration observed at the ammonia layer is an indicative of the presence of flavonoids (Harborne, 1973).

4.17.1.5. Test for Glycosides

About 2 ml of the solvent extract was added to 10 ml of distilled water and it was heated for 5 minutes in a water bath, filtered using Whatman No. 1 filter paper. Two ml of
the filtrate was added to 0.2 ml of Fehling’s solution B until it turns alkaline and heated in a water bath for 5 minutes. Development of a light blue colouration is an indicative of the absence of glycosides (Oloyede, 2005).

4.17.1.6. Test for Phenolic Compounds

To the solvent extracts, two ml of ethanol was added to which a few drops of ferric chloride were incorporated. Formation of blue colour represented the presence of phenolic compounds.

4.17.1.7. Test for Saponins

Small amount of the test extract was boiled with 5 ml of distilled water for 5 minutes. Mixture was filtered while still hot and the filtrate was used for the following tests. To 1 ml of the filtrate, 2 drops of olive oil was added. The mixture was shaken and observed for the formation of emulsion. 1 ml of the filtrate was diluted with 5 ml of distilled water. The mixture was vigorously shaken and observed for frothing (Trease and Evans, 1996).

4.17.1.8. Test for Phlorotannins

To the test extract, 1% aqueous Hydrochloric acid was added and boiled. Development of red precipitate confirms the presence of Phlorotannins in the sample.

4.17.1.9. Test for Terpenoids

To 5 ml of test extract of *C. scalpelliformis*, 2 ml of chloroform and 3 ml of concentrated sulphuric acid was added. Reddish brown colour formation indicated the presence of terpenoids in the sample.
4.17.2. Quantitative Phytochemical Analysis

4.17.2.1. Total Protein Content

The total protein content was calculated by the method followed by Lowry et al., (1951). To 1 ml of the *C. scalpelliformis* extract or standard, 5 ml of alkaline copper sulphate reagent was added, mixed well and allowed to stand for 10 min. Later 0.5 ml of Folins-Ciocalteau’s reagent was added and mixed well. The mixture was allowed to stand under dark for 30 min. The blue colour developed was read at 660 nm using UV visible spectrophotometer (Systronics, 119, India). The protein content of the extract was calculated from the standard graph of Bovine Serum Albumin and the results were expressed as % w/w.

4.17.2.2. Total Carbohydrate Content

The total carbohydrate content was evaluated by following the method of Hedge et al., (1962). To 0.5 ml of *C. scalpelliformis* extract or standard, 0.5 ml of water was added and the volume was made to 1 ml to which a volume of 4 ml of anthrone reagent was added. The mixture was heated for 8 min in boiling water bath and cooled. The green colour developed was read at 630 nm using UV visible spectrophotometer (Systronics, 119, India). The carbohydrate content of the extract was calculated from the standard graph of glucose and the results were expressed as % w/w.

4.17.2.3. Total Lipid Content

The total lipid content was estimated by the method Zlatkis et al., (1953) with minor modifications. About 0.1 ml of the *C. scalpelliformis* extract, supernatant or standard was made up to 5 ml with working ferric chloride acetic acid reagent and the tubes were kept at room temperature for 10 min. Three millilitres of 85% concentrated sulphuric acid was added. The mixture was kept in an ice cold condition for 20 min. The pink colour formed was read at 540 nm using UV visible spectrophotometer (Systronics, 119, India). The lipid
content of the extract was calculated from the standard graph of cholesterol and the results were expressed as % w/w.

4.18. Separation of active components in \textit{C. scalpelliformis} by TLC

4.18.1. Preparation of TLC plates

The slurry was prepared by mixing silica gel with distilled water in the ratio of 3:2. The slurry was coated onto the glass plate at a thickness of about 0.25 mm and the plates were allowed to dry at room temperature for 15-30 min. Then the plates were kept in a hot air oven at 100-120°C for 1-2 hrs to remove the moisture and activate the absorbent on the plate.

4.18.2. Sample Application

A line was drawn on the plate to about 1.5-2.0 cm from the bottom. The samples were spotted on the plates using micropipette. The spots were allowed to dry and spotting can be done repeatedly for a more concentrated sample spot.

4.18.3. Development of TLC Chromatogram

To obtain the best separation of \textit{C. scalpelliformis} extracts the following solvent systems were tested. Pure solvents of petroleum ether and acetone were used as mobile phase with different ratios as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. The solvent (mobile phase) was poured into the tank. The tank was covered with a glass plate and it was allowed to stand for an hour to ensure that the atmosphere within the tank becomes saturated with solvent vapours. This process is called equilibration. After equilibration, the plate was placed vertically in the tank so that it stands in the solvent with the spotted end dipping in the solvent. The solvent moves upwards due to capillary action and the compounds get separated. As soon as the solvent reached the top of the plates, it was removed from the tank.
4.18.4. Partial purification of active compounds from the TLC plates

Using a sterile surgical blade, the solid adsorbent coat (silica gel) of the bands and blank areas were scrapped separately from the plates and collected carefully into 15ml centrifuge tubes. While collecting the sample, extreme care has been taken to avoid mixing of the compounds. The collected samples were extracted twice with the respective solvents. The silica gel was discarded and the organic phases were combined and brought to dryness by evaporation. The residue was redissolved in 20 μl of the same solvents or DMSO and kept at 4°C for further studies.

4.19. GC-MS analysis of *C. scalpelliformis* extract

GC-MS analysis of the test extracts were performed using Thermo GC-Trace Ultra VER: 5.0 (Bremen, Germany). For MS detection, a Thermo MS DSQ II electron ionization mode with ionization energy of 70 eV and a mass range at m/z 50-650 was employed. A DB 5-MS capillary standard non-polar column (30 m × 0.25 mm, film thickness 0.25 μm) was used for the GC-MS. The column temperature was programmed from 70-260°C (rate = 6°C/min) with the lower and upper temperatures being held for 3 and 10 min respectively. Total GC running time was 43.2 min. The GC injector and MS transfer line temperatures were set at 280 and 290°C respectively. All analysis was done in the split-less mode. Helium (99.9%) was used as a carrier gas (flow rate = 1.0 ml/min) and an injection volume of one μl was used for the analysis. Major and essential compounds were identified by their retention times and mass fragmentation patterns.

4.20. Sporicidal Activity and Preparation of Conidial Suspension

Conidia of *Aspergillus niger* was taken from 12 days culture and conidial suspension (103 conidia/ml) were treated by the aqueous extract of *C. scalpelliformis*. Conidial suspensions were taken in small Petri plates and were kept at 28°C for 24 hrs. A drop of the
treated conidial suspension was taken on concave slides in different times of incubation in moisture chambers. A drop of lactophenol cotton blue was added to the conidial suspensions on the slides. The slides were examined under microscope (10X, 40X) for observing the inhibition percentage of conidial germination using the following formula:

\[
\text{Inhibition (\%)} \text{ of conidial germination} = \frac{\text{Total no. of conidia} - \text{No. of germinated conidia}}{\text{Total no. of conidia}} \times 100
\]

4.21. *InVitro Nematicidal Activity of Seaweeds*

The crude methanol extracts of *C. scalpelliformis*, *S. wightii*, *C. spectabile* seaweeds were tested for the invitro nematicidal activity against *Meloidogyne javanica*, a root-knot nematode. The assay was carried out in triplicates, as presented in the flowchart.

Crude methanol extract of seaweed in 6000 μg / 6 ml of MeOH

Two ml of each placed in cavity block

Kept over night to evaporate the solvent

Dispensed 4 ml of medium in each test tube

Add 2 ml nematode larval suspension in each cavity block

Observe at 24 hours interval

Calculation of % mortality by the following formula:

\[
\% \text{ Mortality} = \frac{\text{No. of dead nematode}}{\text{Total no. of nematode}} \times 100
\]
The methanol extract of the three different seaweeds and Carbofuran (nematicide) was taken in a separate watch glass. Ten juveniles of *M. javanica* were transferred to the watch glass containing seaweeds and Carbofuran. The watch glass without any crude extract served as the control. The number of dead juveniles was recorded after 24, 48 and 72 hrs using stereomicroscope. Mortality was confirmed by touching the larvae with a fine needle (Atta-ur-Rahman *et al.*, 1997).

### 4.22. Bioformulation of *C. scalpelliformis* Extract

The benzene extract of *C. scalpelliformis* was used to make formulation with different natural binding agents such as Maida flour, Coconut oil and Wood ash. The benzene free extract (evaporated by room temperature) was used to prepare the powdery formulation with Maida flour and Wood ash. These extracts were suspended in coconut oil for liquid formulation. Disease was caused in the test plant by spraying *Xanthomonas vesicatoria* culture and polythene cover was used to tie around the plant. The plant respired through holes made in the polythene cover. After one week, the above bioformulation was tested against the disease affected tomato plants. Normal tomato plant served as the control.