3. MATERIALS AND METHODS

3.1. Seaweeds collection site

Seaweeds are occurring on the rocky substratum or shallow muddy waters along the inter-tidal or sub-tidal regions of coastal waters. Thondi is located (Lat: $9^\circ 44' 10"$ N and Long: $79^\circ 00' 45"$ E Palk Bay) in the heart of Palk strait (Palk Bay) in Ramanathapuram District of Tamil Nadu, India(Map 1) known for historical minor port right of early Pandiya’s kings. This coastal shore naturally of shallow waters contain loose mud and sand which habour quite number of diversified seaweeds belonging to \textit{Rhodophyceae, Phaeophyceae} and \textit{Chlorophyceae}. The ropes permanently immersed in the seawater used for tying the mechanized and country boats operated for fishing in this coastal regions also showed luxuriant growth of different types of seaweeds.

3.2 Diversity study

Four different localities such as Nambutalai (Station-I), Thondi (Station-II), Muthuramalingapattanam or M R Pattanam (Station-III) and Theerthandathanam (Station-IV) with luxuriant growth of seaweeds were selected for this study.

Occurrence and distribution of seaweeds at four Stations were studied in four seasons namely post-monsoon (January, February, March), summer (April, May, June), pre-monsoon (July, August, September) and monsoon (October, November, December) seasons for one year during 2007. The seaweeds were collected during spring tides and their occurrence was recorded as most abundant (++++)), abundant (+++), less abundant (++), sparse (+) and absent (-), based on the visible observation made at the collection localities. Herbarium prepared from each alga and the portion
of same alga preserved in 5% formalin in seawater as voucher specimens were used for identification (Umamaheswara Rao, 1970, 1987; Krishnamurthy and Joshi, 1970; Oza and Zaidi, 2001). The herbaria and voucher specimens were stored in the plant museum, Department of Botany, Alagappa Government Arts College, Karaikudi-630 003, Tamilnadu, India.

Seaweeds recorded along the Thondi coastal regions are listed as follows:

**Rhodophyta (Red algae):**

1. *Amphiroa fragilissima* (Linnaeus) Lamouroux
2. *Jania rubens* (Linnaeus) Lamouroux
3. *Grateloupia filicina* (Lamouroux) C. Agardh
4. *Gracilaria corticata* var. *corticata* (J. Agardh) J. Agardh
5. *G. corticata* var. *cylindrica* (J. Agardh) Umamaheswara Rao
6. *G. edulis* (S. Gemelin) P. Silva (Plate I)
7. *G. canaliculata* (Sonder) (= *G. crassa* )
8. *G. verrucosa* (Huds.) Papenfuss
9. *G. foliifera* (Forsskal) Borgesen
10. *Hypnea flagelliformis* (Harvey) Homersley
11. *H.musciformis* (Hulgen) Lamouroux
12. *H. valentiae* (Turner) Montagne (Plate II)
14. *Centroceras clavulatum* (C. Agardh) Montagne
15. *Spiridia hypnoides* (Bory de Saunt-Vincent) Papenfuss
16. *Acanthophora spicifera* (Vahl) Borgesen

**Phaeophyta (Brown algae):**
1. *Dictyota dichotoma* (Huds.) Lamouroux

2. *Chnoospora implexa* (Herring) J. Agardh (Plate III)

Chlorophyta (*Green algae*):

1. *Enteromorpha flexuosa* (Hudgen) J. Agardh
2. *E. intestinalis* (Linn.) Nees
3. *Ulva lactuca* Linn.
4. *Ulva reticulata* Forssk. (Plate IV)
5. *Chaetomorpha linum* (O. F. Muller) Kuetzing
6. *Caulerpa scalpeliformis* (R. Braun ex Turner) C. Agardh
7. *Cladophora fascicularis* Kutzing (Plate V)

### 3.3 Biochemical studies—Proximate studies in seaweeds

Chemicals and solvents used in the present investigation were Analytical grade (AR) and solvents were distilled before use.

#### 3.3.1 Collection of seaweeds

Fresh, matured, disease free and healthy sample weighing approximately 1 kg of each seaweeds (14 red, 2 brown and 5 green) collected along the coast of Thondi during monsoon season (November) in the year 2007 during spring tide was washed thoroughly in seawater followed by tap water to remove the epiphytes and other extraneous materials. Then they were brought to laboratory and stored at -0°C till conducting further studies.

Dry weight, ash content, total chlorophyll, accessory pigments (phycocyanin, allophycocyanin and phycoerythrin) and total lipids were estimated from the frozen samples of the seaweeds whereas proximate compositions such as total carbohydrate, total protein, total amino acids, total phenol, water retention capacity (WRC) and sulphate were recorded from the crude carbohydrate extracts obtained from the frozen sample of seaweeds. Apart from these parameters, agar yield was recorded in
agarophytes (Gracilaria corticata var. corticata, G. corticata var. cylindrica, G. edulis, Gracilaria foliifera, G. canaliculata (Sonder) (=G. crassa) and G. verrucosa) and carrageenan yield was estimated from carrageenophytes (Grateloupia filicina, Hypnea flgelliformis, H.musiformis, H. valentiae and Grateloupia filicina). Sodium alginate was recorded from the brown seaweed Dictyota dichotoma.

3.3.2 Extraction of crude carbohydrate for proximate analyses

Each seaweed weighing 100 grams of frozen samples were soaked in 500 ml of distilled water and heated up to 80 °C for 30 minutes under agitated condition. Then the mixture was filtered through muslin cloth under warm condition. The procedure was repeated for three times. The extracts were combined and kept at –10°C under freeze-drying by high vacuum dehydration.

3.3.2.1 Dry weight analysis

Ten grams of frozen sample (W1) of each seaweed weighed after blotting excess water was kept in an oven at 60° C till achieving constant weight (W2). Dry weight of seaweed was calculated as W2/W1 x 100.

3.3.2.2 Estimation of total ash content (Triebold,1946)

Each alga weighing 1 gram of dry sample obtained as described above (W1) was kept at 550 °C for 4 h in muffle furnace in silica crucibles. After charring, the content of crucibles was weighed (W2) for ash content. Percentage of total ash of the sample was calculated as W2/W1 x 100.

3.3.2.3 Estimation of total chlorophyll (Jeffrey and Hymphrey, 1975)

One gram of frozen sample each seaweed after thawing after blotted with excess water was ground with glass mortar and pestle by adding 10 ml of ice-cold 80 % acetone. The homogenate was centrifuged at 1400g for 2 minutes and collect the supernatant. Repeat the procedure at least thrice until the pellet was
colourless. Samples values were adjusted to 10ml with the help of 80% acetone and stored in the dark at 4°C until absorbence read. Absorbency was read at 645 and 663 nm against 80% acetone blank. The following formula was applied for the calculation of total chlorophyll.

\[
\text{Total chlorophyll (a and b) (mg/L)} = 20.2(A_{645}) + 8.02(A_{663}).
\]

3.3.2.4 Extraction of Phycobiliprotein (Bennet and Bogard, 1973)

One gram of frozen sample of each seaweed was homogenated in ice cold mortar and pestle with 10 ml of 0.05 M phosphate buffer solution (pH 6.7). 0.05 M phosphate buffer was made by mixing equal volume of 0.1 M KH₂PO₄ and 0.1 M K₂HPO₄ solutions and stored at 4°C. Approximately 20 mg of acid washed sand was added to facilitate grinding. Extraction was repeated at least thrice. The extracts were combined and kept at 4°C over night under dark condition. Then the extracts were centrifuged at 8000 rpm for 15 minutes. The supernatant was collected and made up to 30 ml.

3.3.2.5 Estimation of accessory pigments- phycocyanin, phycoerythrin and allophycocyanin

Absorbance of the supernatant was measured at 615 nm and 652 nm against 0.05 M phosphate buffer blank using spectrophotometer. The concentration of phycocyanin, phycoerythrin and allophycocyanin in the extracts were calculated (in milligrams / milliliter) using the following equations.

\[
\text{Phycocyanin (PC)} = \frac{(OD_{615}) - 0.474(OD_{652})}{5.34}
\]

\[
\text{Phycoerythrin (PE)} = \frac{(OD_{652}) - 2.41(OD_{615}) (PC) - 0.849 (APC)}{9.62}
\]
3.3.2.6 **Total lipid content** (Folch et al., 1957)

Ten grams of frozen seaweed sample, sample after thawing was homogenated using glass mortar and pestle. Then the homogenate was transferred to separating funnel containing 50 ml of chloroform: methanol (2:1 v/v) and added with 5 ml of distilled water. The setup was kept undisturbed for 30 minutes. The resulting mixture was separated into 2 phases; the lower phase was the total pure lipid was collected in a pre-weighed beaker. The extraction was repeated for three times until the extract become colourless. The solvents were evaporated to dryness and the total lipids were measured gravimetrically.

3.3.2.7 **Total carbohydrate** (Dubois et al., 1956)

**Reagents**

(i) Phenol 5% solution

Five grams of phenol was dissolved 100 ml and made up to distilled H₂O and stored at room temperature.

(ii) Conc.H₂SO₄.

**Procedure**

One gram of frozen sample of crude carbohydrate was ground as fine powder using mortar and pestles with 10 ml of distilled water and kept the volume for 30 minutes with intermittent grinding for obtaining homogenate solution. To an aliquot of 1.0 ml of sample in a test tube, 1 ml of 5% phenol and of 5 ml of conc. H₂SO₄ were added. Rapid addition of conc. H₂SO₄ promoted the mixing and heat development that was necessary for the assay. The sample was allowed to stand for 30 minutes at room temperature and the absorbance was read at 485 nm against blank without sample.
The amount of total carbohydrate was determined from a standard graph prepared with different concentrations of D-galactose (10-100 μg ml⁻¹).

3.3.2.8 Estimation of protein (Lowry et al.,1951)

Principle: The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex with maximum absorption in the region of 660 nm with Folin – Ciocalteau reagent. Thus the intensity of the colour depends on the amount of these aromatic amino acids present and will thus vary for different proteins.

Reagents:

a. Alkaline copper reagents:

Solution A: 2% sodium carbonate in 0.1N sodium hydroxide solution.

Solution B: 0.5% copper sulphate solution

Solution C: 1 % sodium potassium tartarate solution

50ml of solution A was mixed with 0.5ml of solution B and 1ml of solution C just before use.

b. Folin-Ciocalteau reagent solution: Hundred grams of sodium tungstate, 25 grams of sodium molybdate, 700ml of water, 50ml of 85% orthophosphoric acid and 100ml of concentrated hydrochloric acid were taken in a 1500ml round bottom flask. The mixture was refluxed gently for 10 hours. To this 150g of lithium sulphate, 50ml of water and a few drops of bromine were added and the mixture was boiled for 15 minutes to remove excess bromine. This was diluted 1:2 with distilled H₂O just before use.

Extraction of crude protein: One gram of frozen crude carbohydrate sample was taken and homogenized in a prechilled mortar and pestle with 10 ml of ice cold Tris buffer (0.1M pH-7). The extract was centrifuged at 8000 rpm for 10 minutes and
the supernatant was collected. It was added with 10% TCA (twice the volume) and kept overnight at 40°C. The sample was centrifuged at 8000 rpm for 10 minutes and the pallet was collected, dissolved in Tris buffer and was used for the estimation of protein. The final protein extract was made into 30 ml using 10 % TCA solution.

**Procedure**: An aliquot of 0.2ml of sample was diluted to 5ml with distilled water and then added 2ml of alkaline copper sulphate reagent. Mix the solutions well. This solution was incubated at room temperature for 10 minutes. Then 0.2ml of Folin-Ciocalteau reagent solution was added and incubated for 30 minutes. Optical density was read against the blank at 660nm and protein was calculated from the standard curve prepared by using BSA.

3.3.2.9 **Estimation of total amino acids** (Dave and Chauhan, 1993).

**Reagents**

80% ethanol : 80 ml absolute alcohol was made up to 100 ml with glass distilled water

0.1N Na OH : 0.4 g of NaOH pellets was dissolved and made upto 100 ml using glass distilled water.

1% Ninhydrin : 1 g of Ninhydrin was dissolved and made up to 100 ml with acetone

Methyl red indicator

n-Propanol

**Extraction**

One gram of frozen crude carbohydrate sample was taken and homogenized with 4 ml of 80 % ethanol and pooled in a test tube. The homogenization was
repeated at least thrice. The pooled extracts were centrifuged at 8000 rpm for 10 minutes and the supernatant was made up to 15 ml.

**Estimation**

To 1 ml of the above sample in a test tube, 1 drop of methyl red indicator was added and neutralized with 0.1 N NaOH. To this, 1 ml of Ninhydrin reagent was added and mixed thoroughly. The mouth of the test tubes were covered with glass beads and heated in a boiling water bath for 20 minutes. Then 5 ml of diluents (n-propanol: distilled water 1:1 v/v) was added to the mixture while the samples were kept in the water bath. The test tubes were cooled under running tap water. The purple colour of the solution was read at 560 nm using spectrophotometer against a blank prepared without sample solution. Amino acid was calculated by using a standard graph prepared by varying concentration of glycine ranging from 10–100 µg ml⁻¹.

**3.3.2.10. Total phenol** (Kuda et al., 2005)

**Reagents**

a. **Folin-Ciocalteau reagent solution**: Hundred grams of sodium tungstate, 25 grams of sodium molybdate, 700 ml of water, 50 ml of 85% orthophosphoric acid and 100 ml of concentrated hydrochloric acid were taken in a 1500 ml round bottom flask. The mixture was refluxed gently for 10 hours. To this 150 g of lithium sulphate, 50 ml of water and a few drops of bromine were added and the mixture was boiled for 15 minutes to remove excess bromine. This was diluted 1:2 with distilled H₂O just before use.

b. 20% sodium carbonate solution
Extraction

One gram of frozen crude carbohydrate sample was taken and homogenised with 5 ml of 80 % ethanol and pooled in a test tube. The homogenisation was repeated at least thrice. The pooled extracts were centrifuged at 8000 rpm for 10 minutes and the supernatant was made up to 15 ml.

Estimation

To 1 ml of the above sample in a test tube, 1 ml of the 10% Folin-Ciocalteu-phenol reagent was added. After 3 min, 2 ml of the 20% sodium carbonate solution was added. The contents were mixed thoroughly using glass rod and heat at boiling water bath exactly 1 minute and left to stand at room temperature for 1h. Optical density was read at 650 nm using a spectrophotometer against blank containing all reagents except sample. Catechin was used for the preparation of the standard curve and the results were expressed as mg of catechin g⁻¹ dry weight of alga.

3.3.2.11 Estimation of sulphate by turbidometric method
(Verma et al., 1977)

Reagents

a. 6 M HCl
b. 1 N HCl
c. 70% Sorbitol
d. Barium chloride

Procedure

Ten milligrams of crude carbohydrate extract of each seaweed sample was homogenized with 6 ml of 1 N HCl and transferred to a hydrolyzing
tube with proper sealing and subjected to hydrolysis in a boiling water bath for a period of 4 h. The sample was then cooled to room temperature. The solution was filtered through Whatman No.1 filter paper and volume was made up to 25 ml with double distilled water.

Ten ml of solution was taken for the estimation. To this, 1 ml of 6 M HCl and 5 ml of 70% sorbitol were added. The sample was then stirred with a magnetic stirrer while stirring 1 gram of barium chloride crystals was added and read at 470 nm within one minute. The amount of sulphate in the sample was calculated from a standard graph prepared in the same manner as that of the sample with different concentrations of potassium sulphate ranging from 10 to 100 μg ml\(^{-1}\).

3.3.2.12 Extraction of agar (Visweswara Rao et al., 1965)

Hundred grams of the frozen sample of seaweed after thawing was kept overnight in freshwater and then chopped into small pieces. Water was added to the samples in the ratio of 1:20 w/v, pH was adjusted to 6.5 and autoclaved at 120\(^0\)C at 15 lb per square inch for 2 h.

The hot extract was recovered on a tray by filtering through a muslin cloth and the residue was again filtered by adding 100 ml of hot water. The tray was left at room temperature allowing the filtrate to gel. Then the sample was kept at 0\(^0\)C for 24 h and later thawed at room temperature and after decanting the water, the sample was kept at 60\(^0\)C for 48 h for drying. The yield of agar was expressed as percentage dry weight.

3.3.2.13 Extraction of Carrageenan (Craigie and Leigh, 1978)

Hundred grams of the frozen samples of each seaweed was treated with 400 ml of 0.5 M sodium bicarbonate solution (1: 40 w/v) and autoclaved
at 120°C at 15 lb per square inch for 2 h. The hot extract was recovered on a tray by filtering through a muslin cloth; the residue was again filtered by adding 100 ml of hot water. Then 1% KCl was added and left at room temperature allowing the filtrate to gel. Then the sample was kept at 0°C for 24 h and then thawed at room temperature. After decanting the water, the sample was kept at 60°C for 48 h for drying. The yield of caragenan was expressed as percentage dry weight.

3.3.2.14 Extraction of sodium alginate (Mc Hugh, 1987)

Hundred grams frozen sample of each seaweed after thawing was kept overnight in freshwater and then chopped into small pieces. Then the sample was digested with 25 ml of 3% Na₂CO₃ solution for 24 h. To this 100 ml of distilled H₂O was added, filtered and the crude sodium alginate was collected. To this, 30 ml of 1N HCl was added, and filtered, and after filtration the insoluble alginic acid was collected, mixed with 3% Na₂CO₃ solution until the pH was 7.0 and 10 ml of ethanol was added. The sodium alginate was collected and dried at 70°C for 8 h. The yield of sodium alginate was expressed as g⁻¹ dry weight of alga.

3.4 Monthly biochemical analysis in seaweeds based on meteorological parameters

Based on the occurrence, distribution, dry weight, total carbohydrate and agar yield in red seaweeds Gracilaria corticata var. corticata, G. corticata var. cylindrica, G. edulis and G. verrucosa recorded monthly basis from October 2006 to September 2007 were statistically correlated with atmospheric meteorological parameters such as mean maximum temperature, mean minimum temperature, mean relative humidity,
total rainfall and mean wind speed obtained from the Thondi meteorological station, Indian Meteorological Department, Government of India in order to find a suitable atmospheric condition for the growth of seaweeds along the coast of Thondi climatic regions.

3.5 **Amino acid analysis on HPLC (High Performance Liquid Chromatography)**

Based on the previous investigations, red seaweeds *Gracilaria edulis* and *G. verrucosa*; and green seaweeds *Ulva lactuca* and *Chaetomorpha linum* collected during spring tide November 2008 along the coast of Thondi were taken up for the following study.

3.5.1 **Extraction of crude amino acids** (Moss, 1952)

Healthy, matured and disease free sample of seaweeds weighing approximately 1 kg fresh weight of *Gracilaria edulis*, *G. verrucosa*, *Ulva lactuca* and *Chaetomorpha linum* collected along the coast of Thondi were washed thoroughly in seawater followed by fresh water to remove the epiphytes and other extraneous materials. Then they were brought to the laboratory and air dried under shade in dark condition for 3 days. The shade dried samples were milled using mixie grinder machine as fine powder. Then the powdered samples of each specimen weighing 100 grams were transferred into 250 ml of Erlenmeyer conical flasks containing 150 ml of 75 % ethanol for extraction for 5 days under dark condition in room temperature. The extraction was repeated at least thrice until the extracts were colourless. The extracts were combined and kept each extraction type separately were centrifuged at 12000 rpm for 10 minutes. The supernatants were concentrated
using flash evaporator at 45°C under reduced pressure. The concentrated extracts were stored at 0°C till further study.

3.5.2 Preparation of samples for detecting amino acids profile using HPLC (Sánchez-Machado et al., 2003)

**Reduction of disulphide compounds and deproteinisation:**
In a test tube, 1 mg crude amino acid extracts prepared from seaweeds and 1 ml of β-mercaptoethanol were added and mixed thoroughly, allowed to stand for 5 minutes at room temperature. Then 0.5 ml of ice cold methanol was added and mixed thoroughly using vortex mixture for 5 minutes and allowed the tubes to stand for 15 minutes in an ice bucket. Then the mixture was centrifuged at 5000 rpm for 15 minutes and the supernatant was collected and immediately processed by HPLC analysis.

**Alkylation of –SH compounds:** An equimolar concentration of 0.1 mM of amino acids mixture or 0.1 ml of deproteinated amino acid methanol crude were mixed with 50 μl of sodium borate buffer (0.5 M, pH 10.5) containing iodoacetate (0.2 M) followed by 25 μl of OPA reagent (Ortho-pthalaldehyde (OPA) reagent: Prepared, just before use, by dissolving 10 mg of OPA (Sigma Chem. Co., USA) in 1 ml of methanol-sodium borate buffer (1:9 v/v) mixture containing 0.01% (v/v) of mercaptoethanol). This derivatised solution was made up to 1 ml by adding 825 μl of start eluent (mixture of acetate buffer (0.2 M iodoacetate (Sigma Chem. Co, USA) in 0.5 M borate buffer, pH 10.5), 0.05 M, pH 6.8: methanol 4:2 v/v). OPA derivatised amino acids were injected to HPLC system (Shimadzu, Japan) equipped with 20 μl (100 μl for seaweed extract) injection loop and C18 column (25 cm) fitted with a guard column (1 cm) housed in an incubator oven set at 40°C constant temperature. Individual amino acids were separated by reverse phase (gradient
of 0.05 M sodium acetate buffer, pH 6.8 (solvent A) and methanol (solvent B) with a flow rate of 1.0 ml per minute. Resolution of amino acid derivatives were monitored through fluorescence detector with excitation and emission set at 330 nm and 450 nm respectively.

**Peak identification and quantification:** Amino acids were detected based on the retention time established for the individual amino acid under defined experimental conditions. Linearity of the peak areas for different concentrations, ranging from 20 - 200 picomoles, of individual amino acids was determined. Calculation was based on the area under peak established for a given amino acid of known concentration.

### 3.6 Evaluation of Immunostimulation potential of seaweeds under *in vivo*

Generally Murine albino rats are used for numerous experiments in the field of pharmacology to anticipate similar results in the human system as both are mammals having same metabolic functions at some extend. This basic concept led to pharmacologist to choose the rat models for *in vivo* experiments.

#### 3.6.1 Selection of experimental animals

This present investigation was conducted in the animal house laboratory, Jay Nine Biotechnology centre, Cauvery Nagar East, Thanjavur, Tamil Nadu, India. Adult Swiss male albino mice each weighing 20-25 grams procured from Animal Experimental Laboratory of Thanjavur Medical College, Thanjavur, and Tamilnadu were used throughout the study. The mice were housed in surface sterilized(wiped with rectified sprit using absorbent cotton) polypropylene cages(Plate VI) containing sterilized paddy husk as bedding material with a maximum of 10 animals in each cage. Animals were acclimatized to the
experimental room at 25±2°C with controlled humidity, and 12: 12 hour light and dark condition. The animals were fed on autoclaved standard mice food pellets (Rat Feeds, Government Poultry Form, Tamilnadu Veterinary College, Chennai, Tamilnadu, India) and water ad libitum. The animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC). As per the standard practice, healthy mice were segregated 15 days before the commencement of the experiment. Each treatment group containing 10 animals were housed in a cage kept in the randomized block design and exposed to the following treatments for 21 days along with regular feed (Plates VI).

Rats were subjected to immunosuppressive state by injecting Azathioprine as 100mg kg⁻¹ animal weight that is approximately 15 mg rat⁻¹. Azathioprine is an immunosuppressive agent, generally used in combination with a corticosteroid to prevent rejection following allergenic (from a genetically different donor) kidney transplants and to manage severe cases of rheumatoid arthritis in adults when other treatments have failed. In the present study, Azathioprine as a tablet form weighing 50 mg manufactured and marketed by Ranbaxy Pharmaceuticals, Bombay, India was used.

3.6.2 Preparation of seaweed powder and crude amino acids for rat feeds

Powder samples and concentrated crude amino acids extracts of *Gracilaria edulis*, *G. verrucosa*, *Ulva lactuca* and *Chaetomorpha linum* obtained for HPLC study as per the method of Moss(1952) described earlier were used as feeds to rats for immunostimulation investigation. Powdered samples were reconstituted in distilled H₂O and dried as pellets and given to animals along the regular diets
whereas concentrated amino acids extracts were reconstituted in 0.9 % saline H2O and they were given orally to rats using syringe in the following ways.

3.5.3 Experimental Design

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Description of each treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control -1</strong> (Normal)</td>
<td>Rats administrated with 0.9% normal saline H2O day⁻¹ for 21 days.</td>
</tr>
<tr>
<td><strong>Control-2</strong> (Immunosuppressive control)</td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily for 21 days.</td>
</tr>
<tr>
<td><strong>Treatment -1</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th day followed by 30 mg of crude amino acid extracts of <em>Gracilaria edulis</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment -2</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th day followed by 30 mg of crude amino acid extracts of <em>Gracilaria verrucosa</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-3</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th day followed by 2.5 grams powder of <em>Ulva lactuca</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-4</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th followed by 2.5 grams powder of <em>Chaetomorpha linum</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-5</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th followed by 2.5 grams powder of <em>Gracilaria edulis</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-6</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th followed by 2.5 grams powder of <em>Gracilaria verrucosa</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-7</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th followed by 2.5 grams powder of <em>Ulva lactuca</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
<tr>
<td><strong>Treatment-8</strong></td>
<td>Rats administrated with 15 mg of Azathioprine day⁻¹ daily from 1st-7th followed by 2.5 grams powder of <em>Chaetomorpha linum</em> day⁻¹ daily from 8th-21st day.</td>
</tr>
</tbody>
</table>

After 21 days, blood samples were collected through syringe aseptically from six individuals randomly from each treatment group and the immunostimulation properties of seaweeds were evaluated through estimating the following parameters from the blood samples of treated animals.
3.6.4 Method of blood sample collection

Rats were sacrificed using chloroform anaesthesia and blood samples of 10 ml were obtained from each animal through cardiac puncture into EDTA capped bottles using a 10 ml syringe. Samples were mixed gently and put on a roller mixer until analysis made.

1. Determination of hematological parameters
   a. Red blood cell (RBC) count
   b. White blood cell (WBC) count
   c. Differential cell count

2. Differential leucocytes count (DLC)
   a. The Polymorphonuclear neutrophils
   b. The Polymorphonuclear eosinophils

3. Total leucocyte count

4. Estimation of neutrophil adhesive

5. Immunopotential study

3.6.5.1. Hematological parameters

   a. Red blood cells (RBC) count (Dacie and Lewis, 2001)

   The blood was drawn up to 0.5 marks. Then the pipette was filled to the 11 mark with Hayem’s red cell dilution fluid (0.5 gram mercuric chloride, 5 gram sodium sulphate and 1 gram sodium chloride were dissolved in 200 ml distilled water) and mixed thoroughly. Blood was diluted to 1:200 with Hayem’s fluid which preserved the corpuscles. The first few drops of diluted blood were blown out and a few drops were allowed to place in the counting chamber of Haemocytometer. A cover slip was placed over the chamber so that the fluid runs under the slip, filling the chamber. To
count red blood cells, the counting chamber was placed under the microscope at low power. The cells were counted in the four fields. The average number of cells in each small square was counted. The total number of cells $^{-1} \text{mm}^3$ was calculated.

**b. White blood cells (WBC) count** (Brown, 1974)

The blood was drawn up to 0.5 mark, diluted with white cell diluting fluid - Turk’s fluid (1ml of 1 % glacial acetic acids + 99 ml distilled H$_2$O) up to the mark 11 in a ratio of 1:20 v/v and mix thoroughly, first few drops were discarded and the fluid was placed in the counting chamber of haemocytometer. One cover slip was placed over the chamber and counting was taken under the microscope at low power. Then the total count of white blood cells was calculated from number of cells $^{-1} \text{mm}^3$ of the areas of counting chamber.

**c. Differential count of blood cells (Leishman’s method)**

**Preparation of Leishman’s stain**

A Romanowsky type stain was designed to differentiate Leucocytes. It was prepared by

Leishman’s powder (Methylene blue, Eosin) – 0.15gram

Methanol – 100ml

Dissolving 0.15 gram of Leishman’s stain powder in few ml of Methanol and made the volume up to 100ml.

**Preparation of Buffer**

1 N KH$_2$PO$_4$ - 50.4ml

1N Na$_2$ HPO$_4$ - 49.6ml

Both solutions were mixed thoroughly and adjusted the pH to 7.2.
The blood film was prepared by spreading a large drop of blood in a clean grease-free slide. Then the blood film was dried in air. The Leishman’s stain was added on the dried blood film and evenly distributed over the entire slide. After one minute, double the quantity of buffer (pH 6.8) was carefully added and mixed with the stain by means of a clean pipette. The film was allowed to stain for 7-8 minutes and the excess stain was removed by washing with the distilled H₂O for 2 minutes. The water was then washed off with fresh distilled H₂O. The film was dried in air and cleaned with xylol mounted with DPX.

3.6.5.2 Differential leucocytes count (DLC):

After the general examination of the blood film, the differential leucocytes were observed. Counted about 200 to 500 cells and taken an average percent of the cells counted. The following leucocytes count was observed.

a. The Polymorphonuclear neutrophils

Showed a faintly pinkish tinged cytoplasm filled with nearly uniform, fine granules, which take a pink colour. The nucleus was divided irregularly into two to five lobes which were connected by fine bands. It was a round cell with a distinct nuclear membrane.

b. The Polymorphonuclear eosinophils

Compact coarse granules with eosin colour distinguish it with circular in shape, blobbed nucleus looked like spectacles.

3.6.5.3 Total leucocytes count (TLC)

This method used for estimating the total number of white cells in a cubic millimeter of blood. It is important in the diagnosis of diseases when accompanied by a differential white cell count.
**Diluting Fluid**

White blood cell diluting fluid contains a weak acid that lyses the red blood cells and a stain for staining the nucleus of white blood cells e.g. Turke’s fluid.

- Glacial acetic acid: 1.5 ml
- 1% aqueous solution of Gentian violet: 1.0 ml
- Distilled H$_2$O: 98.0 ml

(A pinch of thymol was added to the diluting fluid to prevent growth of molds)

**Counting Chamber**

The chamber normally used for cell counts is the improved Neubauer chamber which has an area of 9sq mm$^{-1}$ and a depth of 0.1mm.

**Methods**

Using a WBC pipette of a haemocytometer, drew well mixed venous blood or capillary blood up to 0.5 mark level. Clean the tip of the tube. Then drew WBC diluting fluid till the 11 mark (or to 0.38 ml of diluting fluid and add 0.02 ml of blood with a Hb pipette). Mixed the fluid and blood mixture gently, avoiding bubbling. Placed the cover slip on the counting chamber at the right place. Shook the fluid-blood mixture and transferred the mixture using a fine bore Pasteur pipette on to the counting chamber (called charging the chamber). Care must be taken that the mixture does not over flow, wash and dry the chamber to be recharged again. Allowed the cells to settle to the bottom of the chamber for 2 minutes. See that fluid does not get dried up. (For preventing this, Petri dishes were placed with a wet filter at its bottom and then placed under charged chamber gently and close off the dishes for about 2 minutes.

For counting, clean the under part of the chamber and if it was left in the Petridish. Placed it on the stage microscope. Using 10 x or low power objective
WBCs were counted uniformly present in the four larger squares. Cells found on the outermost lines should be counted on one side and those found on the line opposite should not be counted.

Number of cells per cubic millimeter of blood was calculated as follows:

\[
\text{Cells counted} \times \text{blood dilution} \times \text{Chamber depth} / \text{Area of chamber counted} = \frac{\text{number of cells counted} \times 20 \times 10 \text{ (depth factor)} / 4}{50}
\]

Calculation:

\[
\frac{\text{Number of nucleated RBCs}}{100 + \text{number of nucleated RBCs}} \times \text{TLC}
\]

\[
= \text{Nucleated RBC/cu mm}
\]

Corrected count = TLC - Nucleated RBC count.

3.6.5.4 Estimation of neutrophil adhesive

In the differential count, the neutrophil cells were identified through the Leishman’s staining techniques. They appeared as pinkish tinged cytoplasm filled with nearly uniform, fine granules which took a pink colour. The neutrophil index was estimated through the reference neutrophil chart. Generally the total number of neutrophil index were subtract from neutrophil cells and then calculated in percentage level which denotes the values of neutrophil number, index and neutrophil adhesive factors.

3.7 Immunopotential study

Antibodies are specific immunoglobulin proteins that act against the foreign substances called antigens. The antibody attaches to the invader creating an antibody-antigen complex, after which it stimulates the immune system to recognize the invader. Antibody response can be either primary, occurring at the first exposure to an
antigen or secondary after exposure to the same or similar antigen at a later date. This is called sensitization. It is the reason why certain pollens, for example, only affect people who have become sensitized. The most common antibodies (Immunoglobulin type) are IgG, IgA, and IgE and high levels in the blood indicate strength of immunosystem. Among the antibodies, IgG is the most abundant antibody in the blood serum, comprising about 60-70% of the total. In the early stages of infection, increasing IgG can improve the speed of the immune response. In many cases of chronic inflammation and allergy it is important to modulate IgG response downward to reduce the inflammation.

3.7.1 Collection of serum from blood

Blood collected from the rats as described earlier was kept 37°C for 12 h and at 5°C for 12 h. Then the samples were centrifuged at 2,500×g for 10 minutes and serum supernatant was collected. Sera of six rats selected randomly from each group of treatment were pooled and stored at -20°C till further study.

3.7.2 Estimation of Total protein in the blood serum (Bradford, 1976)

Reagents

1. 0.1 M phosphate buffer (pH 7.0): 61ml of 0.2M Na2HPO4 was added to 39ml of 0.2 M NaH2PO4 and diluted to 200 ml with glass distilled water.
2. 10% trichloro acetic acid (ice cold): 10g of trichloro acetic acid was dissolved and made upto 100ml using glass distilled water.
3. 1N NaOH: 4 g of NaOH pellets was dissolved and made upto 100ml using glass distilled water.
4. Preparation of protein reagents: 100mg of Coomassie Brilliant Blue G – 250 was dissolved in 50ml of 93% ethanol. To this, 100 ml of 85 % phosphoric acid was added and diluted to 1000ml with glass distilled water.
Extraction of protein

Serum sample of 10 ml (1:10 serum:10% sucrose v/v) was added with 10 ml of phosphate buffer. The extract was centrifuged at 10,000 x g for 15 minutes and the supernatant was collected. Protein was precipitated by adding equal volume of 10% ice cold trichloro acetic acid. The pellet collected after centrifugation at 12000 x g for 10 minutes was redissolved in 2 ml of 1N Na OH.

Estimation

To 0.1ml of the above sample, 5 ml of protein reagent was added and mixed thoroughly. The absorbance was read at 595 nm against a reagent blank. The amount of protein was calculated by using a standard graph prepared with varying concentrations of Bovine Serum Albumin( BSA) from 10µg to 100µg⁻¹ ml. The data were expressed in terms of µg protein ml⁻¹ serum.

3.7.3 Separation of blood serum protein through polyacrylamide gel electrophoresis (Davis ,1964; Brewer and Ashworth ,1969)

Electrophoresis was run in native state in a vertical slab gel electrophoresis unit

Procedure

Two thoroughly cleaned slides (8.5×0.3×8.5 cm) were placed on a leveled table. Longer glass plate was laid down first and placed two spaces and two margins of the plates. Then equal size of other glass plate was kept over rectangular plate laid down. Put the clip and stand it up. Polyacrylamide gel was prepared (0.38 M Tris and 0.06 M HCl, pH 8.9; 7.5% acrylamide, 0.18% Bis, 0.03% TEMED and 0.07% ammonium persulphate) and poured in between the space of slap of glass plates and allowed for 15-20 minutes to form a gel. The gel appeared as translucent. On top of the gel 10 wells were made using the comb. The spacer combs were removed. The gel was placed in the buffer chamber and running gel buffer (Tris-glycine buffer,pH 8.3)
was added into the chamber. Serum samples of 5 µl (1:10 serum:10% sucrose v/v) were placed on each well. Careful observation was made that the buffer in the upper chamber did not cause any leakage. Power was connected and gel was allowed to run with suitable power pack at a constant current of 30 mA. Electrophoresis was stopped when the sample moved approximately 1 cm above the end of the glass plates. After running, gel was removed from the buffer chamber and all four screws of the clamp assembly and glass plate sandwich from it were dismantled. Gel was transferred to staining solution (16 ml ortho-phosphoric acid in 768 ml of distilled H₂O + 80 gram of ammonium sulfate+ 16 ml of 5% Coomassie Brilliant Blue R 250 in distilled H₂O. Before use, 200 ml of methanol was added to the solution) for 1 h. Then the gel was destained (in several changes of 7% acetic acid). Then the gel was transferred to cellophane paper and dehydrated in a gel dryer(Model 583-Bio-Red) at 80°C for 24 h. The gel was scanned at 605 nm using a densitometer (Model GS-700 - Bio-Rad) programmed with the "Molecular Analyst" software (Bio-Rad).

Analyses were made from six samples for each experiments and data were statistically analyzed using SPSS 14.