EXPERIMENTAL PROCEDURE

The methodology followed in the present study entitled “Nutritional and Safety Evaluation of Underexploited Seaweeds and Nutraceutical Potentials of Ulva fasciata” consisted of the following phases;

A. Phase I - Nutrient and nutraceutical analysis of underexploited seaweeds

B. Phase III - Development and acceptability trial of selected seaweeds in traditional recipes

C. Phase IV - Evaluation of antidiabetic activity of Ulva fasciata

D. Phase VI - Evaluation of anticancer activity of Ulva fasciata and its nanoparticle extract

E. Phase VII - Statistical analysis and interpretation

A. PHASE I NUTRIENT AND NUTRACEUTICAL ANALYSIS OF UNDEREXPLOITED SEAWEEDS

1. Selection of area

Ramanathapuram District in Tamil Nadu was selected as the locale for the study. Gulf of Mannar with all 21 islands along the 140 Km stretch between Tuticorin and Rameswaram (Lat. 8° 55” – 9° 15”N and Long. 78° 0” – 79° 16” E) has been rightly considered for a Marine Biosphere Reserve. Gulf of Mannar coast covers Tuticorin, Tirunelveli, and a portion of Kanyakumari and Ramanathapuram Districts stretching from Kanyakumari in the southern end of the Indian Peninsula to Pamban in the north.

The Indian coasts have a rich biodiversity of marine algae with about 850 recorded species. The sea coast of Gulf of Mannar and Palk Bay of Ramanathapuram district-Tamil Nadu contains a rich vegetation
Experimental procedure

of marine algae. Figure 7 shows the map of Ramanathapuram district of Tamil Nadu depicting the coastal regions.

MAP OF RAMANATHAPURAM DISTRICT IN INDIA
FIGURE 7

2. Selection of Underexploited Seaweeds

As the sea coast of Gulf of Mannar and Palk Bay of Ramanathapuram District contains a rich vegetation of marine algae and the use of seaweeds as food is not quite popular in India, the present investigation has been chosen on seaweeds. Based on the results of consumption pattern of seaweeds in our earlier study (Kowsalya and Abirami, 2011), five underexploited seaweeds were selected for the study. The selected seaweeds include red seaweeds namely Acanthophora spicifera and Gracilaria edulis, brown seaweeds namely Padina gymnospora and green seaweeds namely Ulva fasciata and Enteromorpha flexuosa which are grown in abundance as dominant community. The selected seaweeds were authenticated by scientists at Central Salt and Marine Chemicals Research Institute (CSMCR), Mandam
Experimental procedure

area of Ramanathapuram District. Plate 4 shows the underexploited seaweeds selected for the study.

3. Evaluation of Nutrient Content of Underexploited Seaweeds

a. Macronutrient content

The selected seaweeds were washed thoroughly in seawater and then in tap water. The seaweeds were again washed in distilled water, the remaining water was drained and the fresh seaweeds were used for nutrient and nutraceutical analysis. All the marine algae are rich in nutrients and novel components when compared to higher plants (Fleurence, 1993). Macronutrient and micro nutrient components were analysed for selected seaweeds.

Macronutrients were analysed for both seaweeds by the following methods. Carbohydrate was analysed by Anthrone method (Hedge and Hofreiter, 1962), protein was analysed by Lowrey’s method (Lowery et al., 1951) and fat was analysed by Soxhlet method (Sadasivam and Manikam, 1996).

b. Micronutrient content

Micronutrients were analysed for the selected seaweeds. The seaweeds contain a wealth of mineral elements. The micronutrients namely iron, phosphorus, calcium, magnesium and niacin were analysed by standard AOAC methods (Raghuramulu et al., 2003).

c. Heavy metal and mineral analysis

The dried and ground seaweed samples were placed in a crucible overnight in an electric muffle furnace maintained at a temperature between
Experimental procedure

400°C and 420°C, because loss of zinc might occur at >450°C and for potassium, loss might occur if the temperature is too high at >480°C. Ashing will destroy all the organic materials present in the samples. The crucible containing pure ash was kept in desiccators.
Experimental procedure

(Red seaweed)

*Acanthophora spicifera*  
*Gracilaria edulis*

(Brown seaweed)

*Padina gymnospora*

(Green seaweed)

*Ulva fasciata*  
*Enteromorpha flezuosa*

UNDEREXPLOITED SEAWEEDS SELECTED FOR THE STUDY

PLATE 4
Experimental procedure

Then, the ash was digested with the triple acid mixture of nitric acid: sulphuric acid: perchloric acid (11:6:3), a clear solution was obtained when dissolved in HCl. This solution was made up to 25 ml with water. The yield of the ash of each sample was determined by Atomic Absorption Spectrophotometer (AAS).

4. Evaluation of Nutraceutical Potentials of Underexploited Seaweeds

Evaluation of nutraceutical potentials of underexploited seaweeds was carried out. Marine algae are among the richest source of known and novel bioactive compounds with valuable antioxidant potentials and application as a therapeutic agent (Strayo et al., 2005).

a. Non enzymatic antioxidant analysis

Antioxidants like total carotenoid and beta carotene was analysed by HPLC, Vitamin C was analysed by dichlorophenol indophenol method (Sadasivam and Manikam, 1995), chlorophyll a, b and total chlorophyll were analysed by spectrophotometric method (Witham et al., 1971).

b. Phytochemical screening

The methanolic extracts obtained from underexploited seaweeds were used for phytochemical studies. Phytochemicals like alkaloids, glycosides, carbohydrates, proteins and amino acids, flavonoid, sterols, saponins, tannins, gums and mucilage, terpenoids, phenols, starch and quinones were analysed by qualitative chemical method.

5. Microbial Analysis of Underexploited Seaweeds

The selected five species of seaweeds were analyzed for the microbial load for coliforms, Escherichia coli, Salmonella typhii, Staphylococcus aureus, faecal streptococci and standard plate count by standard techniques (James et al., 2008).
6. Antibacterial Activity of Underexploited Seaweeds

Seaweeds are known to contain bioactive compounds that display antibacterial, antiviral and antifungal properties. The determination of antimicrobial activity of the methanol extracts of *Acanthophora spicifera*, *Gracilaria edulis*, *Padina gymnospora*, *Ulva fasciata* and *Enteromorpha flexuosa* were evaluated against bacterial strains like Gram positive *Staphylococcus aureus*, *Streptococci*, *Bacillus cereus* and Gram negative *Klebsiella aerogenes* and *E.coli* respectively. Microbes were grown in Mueller Hinton Agar medium by using agar well diffusion method. The bacterial strains were inoculated on nutrient broth and incubated for 24 hours at 30±1°C. Adequate amounts of autoclaved Mueller Hinton Agar medium were dispensed into sterile plates and allowed to solidify under aseptic conditions. Then bacterial strains were placed over plates containing Mueller Hinton Agar medium using cotton swabs. Wells were made by using well cutters, 40 and 60 µg concentration of extracts were used in the medium (39g/1000 ml). Briefly, the selected seaweed extracts were dissolved in dimethyl sulfoxide (DMSO). Then wells were filled with extracts of different concentration under sterilized condition in laminar airflow chamber, and then petridishes were covered with thin films and kept the plate under incubation overnight at 37°C. Also amikacin was used as positive control against bacterial strains (10 and 20 µg/disk). After incubation, all the plates were observed for zones of growth inhibition and the diameters of these zones were measured. Inhibitory activity of DMSO was also tested. All tests were carried out under sterile conditions in triplicate (NCCLS, 2001).

7. Antifungal Activity of the Selected Seaweeds

Antifungal activity of methanolic extracts of *Acanthophora spicifera*, *Gracilaria edulis*, *Padina gymnospora*, *Ulva fasciata* and *Enteromorpha flexuosa* were tested against *Aspergillus niger* and *Candida albicans* using the diffusion plate method. In this, 0.1 ml of fungal spore suspension (growth for
three days on 10 ml of nutrient dextrose agar) was thoroughly mixed with 25 ml of melted potato dextrose agar and was poured into sterilized petri plates. When the agar solidified, 8mm diameter wells were made on each of the seeded plates. These cups were filled with test samples of various concentrations (40 µg and 60 µg) and standard fluconazole (100 µg/ ml) was kept as control. The petri plates were incubated at 280°C for 2-4 days. All the culture plates were examined from 24hours onwards till 48hours and the results were tabulated. The inhibition zones produced by the test samples were compared with the inhibition zone produced by fluconazole which acted as the standard (Latha and Latha, 2011).

8. In Vitro Antioxidant Activity of Underexploited Seaweeds

Dietary antioxidants, including polyphenolic compounds, vitamin E and C and carotenoids are believed to be the effective nutrients in the prevention of oxidative stress related diseases. Antioxidants have thus become a topic of increasing interest recently. The methods followed to determine the antioxidant activity of methanolic extract of the selected seaweeds were done by in vitro are given below. Plate 5 shows the extract preparation by using soxhlet extractor.
Experimental procedure

PREPARATION OF EXTRACT IN SOXHLET EXTRACTOR

PLATE 5

a. Determination of total phenol content

The strong association between fruit and vegetable intake and degenerative disease prevention has been explained by the aspect of antioxidant phytonutrients. In recent years, particular attention has been given to a specific class of antioxidant phytochemicals, the polyphenols. These polyphenols comprise basically of phenolic acids including benzoate and hydroxycinnamate derivatives and flavonoids. Polyphenolic substances are naturally present in essentially all lower and higher plants.
Experimental procedure

Phenols react with phosphomolybdic acid in Folin Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue) that can be estimated colorimetrically at 650nm.

The content of total phenol was determined by Folin Ciocalteau method. An aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin Ciocalteau reagent was added. After three minutes, 2 ml of 20% sodium carbonate was added and mixed thoroughly. The results were expressed as Tannic acid equivalent (mg TAE/g).

c. DPPH radical scavenging assay

A simple method that has been developed and extensively applied to determine the antioxidant activity of foods utilizes the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant are shown in Figure 8. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolourization is stoichiometric with respect to the number of electrons captured.

![DPPH Radical Scavenging Activity](image)
Experimental procedure

FIGURE 8

Since the seaweeds have shown the presence of polyphenolic compounds, their oxygen radical scavenging capacity has been attempted. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging was assayed as described by Hwang et al. (2001). One ml of DPPH solution (0.1 mM/L in 95% ethanol (v/v) was incubated with different concentrations of the extract, the reaction mixture was shaken and incubated for 20 minutes at room temperature and the absorbance was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equations,

\[
\% \text{ Radical Scavenging activity} = \left[ \frac{1(Abs \text{ Control} - Abs \text{ sample})}{Abs \text{ control OD}} \right] \times 100
\]

BHA was taken as reference standard. The percentage inhibition versus concentration was plotted.

d. Nitric oxide radical inhibition assay

Nitric oxide, a free radical gas, is formed in the atmosphere during lightening storms. Less dramatically, but with far-reaching biological consequences, it is also formed in an enzyme-catalysed reaction between molecular oxygen and L-arginine. The convergence of several lines of research led to the realisation that NO is a key signaling molecule in the cardiovascular and nervous systems, and that it has a role in post defence.

A physiological function of NO was discovered in the vasculature when it was shown that the endothelium-derived relaxing factor is accounted for by the formation of NO by endothelial cells. NO is the endogenous activator of soluble guanylate cyclase, leading to the formation of cyclic GMP, which functions as a second messenger in many cells including nerves, smooth muscles, monocytes and platelets. Nitrogen and oxygen are neighbours in the
periodic table, and NO shares several properties with O₂, in particular a high affinity for haem and other iron-sulphur groups. This is important for activation of guanylate cyclase, which contains a heam group and for the inactivation of NO by haemoglobin.

Nitric oxide radical inhibition was assayed by incubating 2 ml sodium nitroprusside (10mM), 0.5 ml phosphate buffer saline and 0.5 ml (0.25 mg) of extract solution at 25°C for 150min. Then one ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) was added to 0.5 ml of reaction mixture for 55min to complete diazotization, subsequently 1 ml naphthyl ethylene diamine dihydrochloride (NEDD) was added and allowed to stand for 30 min at 25°C. The absorbance of these solutions were measured at 540nm (Lekameera et al., 2008).
C. PHASE III DEVELOPMENT AND ACCEPTABILITY TRIAL OF SELECTED SEAWEEDS IN TRADITIONAL RECIPES

1. Standardization of recipes

The United States Department of Agriculture (USDA) defines a standardized recipe as one that has been tried, adapted and retried several times for use by a given food service operation and has been found to produce the same good results and yield every time when the exact procedures are used with same type of equipment and the same quantity and quality of ingredients. The use of standardized recipes ensure that menu items will be consistent in quality each time they are prepared and served. It will also ensure that nutritional values per serving are valid and consistent.

The recipes were standardized in terms of the ingredients used, the quantity of each ingredient and the time taken for cooking.

2. Sensory evaluation of the selected recipes

Sensory properties, among many other factors influence considerably the quality of food products. A product could have an excellent composition and satisfy the highest nutrition criteria, but if it is unsatisfactory in appearance, taste or odour it will not find the way to consumers.

Sensory evaluation has been defined as a scientific method used to evoke measure, analyze and interpret those responses to products as perceived through the senses of sight, smell, touch, taste and hearing. The developed recipes were organoleptically evaluated by 25 panel members. The panel members were selected on the basis of their age, health, cooperation, willingness and knowledge of sensory analysis and also the ability to discriminate the various criteria’s for sensory evaluation.
Experimental procedure

The sensory evaluation was carried out in a suitable time around 11 am to 12 pm before lunch time. The result were recorded and analyzed appropriately.

Traditional recipes were prepared in the foods laboratory in the Food Science and Nutrition Department at Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore. Water was provided to sensory evaluator before and after tasting the recipe. Three trials were conducted for all the standardized 10 recipes, vegetarian items namely seaweed soup, rasam, chappathi, dosa, poriyal, pickle and non vegetarian items namely chicken biriyani, scrambled egg, fish curry, and chicken gravy.

Score card is a tool which help in evaluation through direction and degree of judgement using suitable defined scores. The panel members did the organoleptic evaluation of these recipes based on five point scale developed by Peryam and Pilgrim (1957). A five point Henodic Scale was developed for the purpose of evaluation of the appearance, flavour, texture and overall acceptability. The mean score of the organoleptic evaluation was calculated and on the basis of the total obtained scores.

In general, the acceptability trial revealed that Ulva fasciata incorporated recipes were most acceptable compared to other seaweeds. Hence all the seaweeds may be promoted among the masses, Ulva fasciata can incorporated in their daily diet to improve the nutritional status.

D. PHASE IV EVALUATION OF ANTIDIABETIC ACTIVITY OF ULVA FASCIATA

1. Selection and grouping of animals:

Wistar strains of male albino rats weighing between 180-200g were used for this study. The animals were housed in large spacious cages and
they were fed with commercial pellets and access to water *ad libitum*. The animals were well acclimatized to the standard environmental condition of temperature (22°C ± 5°C) and humidity (55 ±5%) and 12 hour light dark cycles throughout the experimental period.

2. **Induction of Diabetes mellitus**

Diabetes mellitus was induced in wistar rats by a single intraperitoneal injection of a freshly prepared solution of alloxan monohydrate (150 mg/kg BW) in physiological saline after overnight fasting for 12 hrs. The development of hyperglycemia in rats was confirmed by plasma glucose estimation 72 hrs post alloxan injection. The rats with a fasting plasma glucose level of 200-260 mg/dl were used for this experiment.

3. **Experimental procedure**

In this experiment, a total of 30 rats (24 diabetic surviving rats and six normal rats) were used. Diabetes was induced in rats three days before starting the experiment. The rats were divided into five groups after the induction of alloxan diabetes. In the experiment, six rats were used in each group. Group-I consisted of normal rats given 10 ml/Kg of normal saline. Group-II consisted of diabetic control which received 150 mg/Kg of alloxan monohydrate through i.p. Group-III consisted of positive control i.e diabetic rat which received glipizide (10 mg/Kg i.p) for 28 days. Group-IV was treatment group which consisted of diabetic rats which received low dose (200 mg/Kg) of aqueous extract of *Ulva fasciata* daily for 28 days. Group-V consisted of diabetic rat received high dose (400 mg/Kg) of aqueous extract of *Ulva fasciata* daily for 28 days.

4. **Sample collection**

After 28 days of treatment blood was collected retro-orbitally from the eye under light ether anesthesia using capillary tubes. Blood was collected in
Experimental procedure

Fresh vials containing EDTA as anticoagulant agent and plasma was separated in a T8 electric centrifuge at 2000rpm for two minutes. Then the animal was sacrificed by decapitation. Liver and pancreas were immediately dissected out, washed in ice-cold saline to remove the blood and the liver was used for estimation of enzyme activity.

5. Estimation of blood glucose

Fasting blood glucose levels was determined in all experimental rats initially to determine the diabetic status and thereafter every week during the 28 day study period. Blood was obtained by snipping tail of rat with the help of sharp razor and blood glucose levels were determined using glucometer (Ultra Touch Two, Johnson and Johnson). Each time the tail of the rat was sterilized with spirit.

6. Hepatic glucokinase and hexokinase activity

The liver was perfused with ice cold 0.15M KCl and 1mM EDTA solution and homogenized with twice its weight of ice cold buffer (0.01 cysteine and 1mM EDTA in 0.1 ml Tris-HCL., pH 7.4) and centrifuged for 20 minutes at 40°C. Glucose phosphorylation was assayed by means of the glucose 6 phosphate dependent spectrophotometric method at 340nm (Chou and Wilson, 1975).

7. Glucose-6-phosphatase activity

The liver glucose-6-phosphatase activity was measured according to standard protocol (Swanson, 1955). Tissue was homogenized in ice cold 0.1M phosphate buffer saline (pH=7.4) at the tissue concentration of 50 mg/ml. In a calibrated centrifuge tube, 0.1 ml of 0.1 M glucose-6-phosphate solution and 0.3 ml of 0.5 M maleic acid buffer (pH=6.5) were taken and brought to 37°C in water bath for 15 min. The reaction was stopped with 1 ml
of 10% trichloroacetic acid (TCA) followed by chilling in ice and centrifuged at 3000 × g for 10 min. The optical density was noted at 340 nm.

8. Glycogen content

The tissue sample was digested by hot concentrated 30% KOH and treated with anthrone reagent. Glycogen content was determined colorimetrically as glucose (Swanston et al., 1990).

9. Estimation of glycosylated haemoglobin

After 28 days of experimental period, the 12 hour fasted rats were sacrificed by anaesthesia, blood was withdrawn by retro orbital puncture under light ether anesthesia and the glycosylated hemoglobin was estimated (Sadasivam and Manickam, 1996).

10. Histopathological examination

Pancreas tissue was separated from all the groups of albino rats, a cross section was fixed in 4g/L formaldehyde and embedded in paraffin. Paraffin section was then stained with Hematoxylin-eosin. Each sample was observed at 400X magnification and scored according to the injuries (Li, 2001).

F.PHASE VI EVALUATION OF ANTICANCER ACTIVITY OF ULVA FASCIATA

1. Preparation of Extract

The dried seaweeds were coarsely powdered and 250 g of this seaweed powder was packed in soxhlet extractor of one litre capacity. The solvent methanol was added into the flask and heated. The temperature was maintained at 60°C to 70°C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. The methanolic extract was
then cooled and concentrated by using a rotary evaporator at 30–45°C. The extract was stored in labeled sterile screw-capped bottles at 4°C until it was used (Ganesan et al., 2011). As mentioned in the earlier section, aqueous extract was prepared and used as a dosing regimen.

2. Selection of Animals

Healthy adult male Swiss albino mice of commonly used laboratory strains were employed, weighing 20-25 g. Each animal at the commencement of its dosing was between 8 and 12 weeks old and its weight was in an interval within ± 20% of the mean weight of any previously dosed animals. They were allowed to acclimatize in the departmental animal facility for one week before the start of the experiment. They were fed a standard diet and water *ad libitum*. The study room was maintained at approximately 25 ± 2°C in a 12-hours light/dark cycle and were housed in individual standard isolation cages (45×35×25 cm). After sufficient period of acclimatization, they were used to evaluate anticancer activity.

3. Induction of Cancer using DAL Cells

Dalton’s Ascites Lymphoma (DAL) cells were supplied by Amala Cancer Research Centre, Trissur - Kerala, India. The cells were maintained *in vivo* in Swiss albino mice by intraperitonial transplantation. While transforming the cancer cells to the grouped animal, the DAL cells were aspirated from peritoneal cavity of the mice using saline. The cell count was done and further dilutions were made so that total cell should be $1 \times 10^6$, this dilution was given intraperitoneally (i.p) and the cancer cells were allowed to grow in the mice for a minimum of seven days before starting the study. Ascitic fluid was drawn from cancer-bearing mice at the log phase (days 7-8 of cancer bearing) of the
cancer cells. Each animal received 0.2 ml of cancer cell suspension containing \(1 \times 10^6\) cancer cells (i.p).

4. Grouping of animals

Swiss albino mice were divided in to six groups of six each. All the animals in four groups were injected with DAL cells \((1 \times 10^6\) cells/mouse) intraperitonally, and the remaining one group from the normal control group. Group I served as the normal control. Group II served as the cancer control. Group I and II received normal diet and water. Group III served as the positive control, was treated with injection flurouracil at 20 mg/kg body weight, Group IV served as the treatment control, which was treated with methanolic extract of *Ulva fasciata* (MEUF) at 200 mg/kg body weight, in i.p (as per LD\(_{50}\) Value). Group V served as treatment control which was treated with aqueous extract of *Ulva fasciata* (AEUF) at 200 mg/kg of body weight, in i.p. Group VI was treated with seaweed nanoparticle which was given about 100 mg/kg of body weight. The extract was dissolved in distilled water and seaweed nanoparticle was dissolved in DMSO which was used as a dosage regimen. In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days. Twenty-four hours of last dose and 18 hour of fasting, all mice from each group were sacrificed; the blood was withdrawn from each mice by retro orbital plexus method and the following parameters were analysed.

a. Hematological parameters- WBC count, RBC count, Hb content, Platelet count, Packed Cell Volume. (pentra-120 Automated Hematology Analyzer)

b. Lipid profile- Cholesterol, Triglycerides-CHOD PAP method

c. Liver function test- AST, ALP, ALT- Modified IFCC/UV kinetic method

d. Derived parameter- Body weight, Life span (%), Cancer Cell Count.
4. Percentage increase in life span (ILS)

The mortality was recorded by monitoring the effect of MEUF, AEUF and *Ulva fasciata* nanoparticle on cancer growth and percentage increase in life span (ILS %) was calculated.

\[ \% \text{ILS} = \frac{\text{Lifespan of treated group}}{\text{Lifespan of control group}} - 1 \times 100 \]

5. Assessment of Cancer Cell Count

The fluid (0.1 ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold Normal saline or sterile Phosphate Buffer Solution (PBS) and 0.1 ml of trypan blue (0.1 mg/ml) and total number of the living cells were counted using haemocytometer (improved Neubauer chamber).

Total number of cells per ml = average no of cells × dilution factor $2 \times 10^4$

G. PHASE VI STATISTICAL ANALYSIS AND INTERPRETATION OF DATA

The data was consolidated and tabulated and subjected to statistical analysis with appropriate tools using the Graphpad Prism 5 version. The following statistical tests were applied in the different phases.

Phase I

Descriptive statistics (mean and standard deviation) was used to represent the basic distribution of nutrients.

Phase II

Dunnett’s multiple comparison test was used to determine One way ANOVA to interpret intra group variations; Data were accepted as statistically
significant difference at \( p<0.05 \), to compare the significant differences in the mean of haematological and biochemical parameters of the control and experimental groups.

**Phase III**

Newman Keul’s multiple range tests was used to determine One way ANOVA to compare mean of the haematological and biochemical parameters of the control and experimental groups to determine the significant difference between the groups. Data were accepted as statistically significant difference at \( p<0.01 \) and \( p<0.05 \). Diabetic control was compared with the normal, experimental groups were compared with diabetic control and non diabetic control.

**Phase VI**

Newman Keul’s multiple range tests was used to determine One way ANOVA to compare mean of methanolic and aqueous extract of *Ulva fasciata* extract and its nanoparticle. Cancer cell count, life span, hematological and biochemical parameters of the control and experimental groups to determine the significant difference between the groups. Data were accepted as statistically significant difference at \( p<0.01 \) and \( p<0.05 \). Cancer control was compared with the normal, experimental groups were compared with cancer control.

**Validation of Data**

The procedures involved in the biochemical analysis in blood were standardized in normal blood samples. The analysis of biochemical parameters in the test samples of the present investigation was carried out after this. Proper quality control was maintained in the protocol throughout the study period. The investigator herself carried out the entire analytical procedures. She underwent prior training in handling of animals and the
Experimental procedure

procedures involved in animal experimentation. Further, she also underwent training in phytochemical analysis and animal tissue culture to conduct the anticancer study. Hence, there is no probability of bias or error in obtaining the analytical data in the present study.