4.1. COLLECTION AND STORAGE:

4.1.1. Materials:

1) Sharp scrapper/knife
2) Insulated basket
3) Ice
4) 75% alcohol
5) Lyophillizer

4.1.2. Principle:

The samples of zoanthids are soft coral marine invertebrates. They are in the form of mats and incorporate sand and detritus in them. As they adhere on to the rocky shore strongly, they are required to be removed by using sharp scrapper/knife type of objects. As the samples are required for molecular analysis further, they should be stored in alcohol. 75% grade of alcohol is considered best for storage of samples for longer durations as 25% of water present in it would provide enough hydration effect and prevent the samples from total dehydration upon long standing. Storage of samples at -20°C and lyophillization are two processes which can preserve them for long. Lyophillization is the process of removing all the moisture from the samples under vacuum. This process leads to complete drying of the samples with little damage to the tissues and their biochemical composition. The tissues so obtained can be crushes and powdered easily and still the chemistry of the organism will not be affected[83].
4.1.3. **Procedure:**

1) The samples of zoanthids are collected from the rocky intertidal region of Anjuna beach (Goa) (Fig.4.1. & Fig.4.2.).
2) During collection, sharp scrapper/knife type of objects are used, but care was taken as to not to damage the mats as far as possible.

3) Immediately after collecting the samples, they were transferred into 75% alcohol and kept in the ice.

4) After transporting back to the lab, some samples are removed and kept at -20°C in the freezer and some are left as such in alcohol.

5) The samples left in alcohol were further used for histological processing.

6) Some of the samples from the freezer were then lyophilized (Fig. 4.3.) and powdered, which can be further utilized for molecular and protein analyses.

Fig. 4.3. Lyophilizer
4.2. HISTOLOGICAL ANALYSIS:

4.2.1. Materials:

4.2.1.1. Decalcification & Desilification treatment:

1) Zoanthid samples
2) Citric acid (20%)
3) Formic acid (50%)
4) Hydrofluoric acid (HF) (15%)

4.2.1.2. Histology:

1) Ethanol (70%, 90%, 100%)
2) Xylene
3) Delafield’s Hematoxylin
4) Eosin
5) Paraffin wax (58°-60°C)
6) Microtome

4.2.2. Principle:

4.2.2.1. Decalcification and desilification treatment:

The sample of zoanthids are in the form of mats which embed a lot of sand and detritus in them. Hence the samples are treated with decalcification agents like the combination of citric and formic acids. These react with the calcium present usually in the form of carbonate and liberate carbon dioxide, which can be noticed by the release of bubbles. The cessation of bubble release is the indication of decalcification limit. Similarly, the sand present in the mats is also
removed by desilification treatment with agents like HF. Mild concentrations of HF are used to slowly dissolve the sand granules which can be checked by their absence[68].

4.2.2.2. **Histology:**

Histology is the microscopical study of an organism. The basic steps included in histology are:

1. **Tissue fixation:** Fixation is a complex series of chemical events that lead to the formation of a bond between the protein in a tissue and the fixative so as to keep the *in vivo* conditions as such. Ethanol is a good fixative for samples which have to utilized especially for molecular analysis.

2. **Tissue processing:** It aims to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable their sections to be cut, and yet soft enough not to damage the knife or tissue. It is further divided into three stages – dehydration (to remove fixative and water from the tissue and replace them with dehydrating fluid like absolute alcohol), clearing (replacing the dehydrating fluid with a fluid that is totally miscible with both the dehydrating fluid and the embedding medium like xylene) and embedding (is the process by which tissues are surrounded by a medium – in a mould – which when solidified will provide sufficient external support during sectioning.
3. Sectioning: It is the process of cutting biological specimens into very thin segments for microscopic examinations using instruments like a microtome.

4. Staining: The sections of the tissues obtained are then stained with staining reagents so as to clearly visualize different tissue components. Usually Hematoxlin and Eosin stains are used which are charge-based stains[84].

4.2.3. Procedure:

4.2.3.1. A. Decalcification & desilification treatment:

1) The samples from 75% ethanol were cut into smaller portions and transferred into 10% formalin seawater for 24 hrs.

2) Then these portions were decalcified by chelation with a 1:1 mixture of 20% citric acid and 50% formic acid subsequently diluted 50% with distilled water.

3) The decalcification procedure was stopped when bubbles stopped emitting from the solution.

4) Then the sample portions were rinsed with distilled water overnight, with distilled water being changed periodically.

5) Polyps were then treated with 15% HF with pH<1 for 48hrs (during desilification treatment all safety protocols were followed – triple gloves, safety goggles, conducted in fume hood etc.).

6) After desilification treatment, the polyps were rinsed overnight with multiple changes of distilled water until the pH was approximately 7.0.
7) After the completion of decalcification and desilification treatments, the sample of zoanthid polyps were stored in 70% ethanol till further processing[68].

4.2.3.2 Histology:

1) The small portions of mat of zoanthids after the treatment were then cut into individual polyps.

2) The polyps were then dehydrated in 70% alcohol for 2hrs followed by 90% alcohol for 3hrs twice.

3) Then they were transferred into absolute (100%) alcohol for 1hr twice and were kept along with the alcohol in vacuum for approximately 30min to remove air bubbles in the coelenteron.

4) The dehydrated specimen polyps were then clarified with xylene for 1hr to remove the dehydrating agent.

5) The specimen polyps were then transferred into a beaker containing molten paraffin wax kept at its lower end of melting point range for about half an hour.

6) The previous step ensured a thorough coating of the sample zoanthis with the paraffin wax, so as to avoid any air bubbles around it during moulding.

7) Traditional paper boat system was followed to prepare the moulds and molten paraffin wax was poured into these.

8) The specimen polyps from the molten wax container were then transferred into the paper boat moulds containing the molten paraffin wax. (Care was taken during placement of the
specimens as proper orientation of the samples (Fig.4.4.) is required for correct sectioning).

9) The moulds were allowed to cool and then sectioned using mechanical rotary microtome (Fig.4.5.) into 5-10µm sections.

10) The sections so obtained were collected on slides and then stained with Delafiel’s Hematoxylin and Eosin.

11) The sections so stained were then observed under light microscope (Olympus 1X51 Inverted Microscope)[84].
4.3. MOLECULAR ANALYSIS:

4.3.1. Materials:

4.3.1.1. DNA Isolation:

1) Lyophilized and powdered Zoanthid sample

2) Lysis buffer:
   - Tris HCl (pH 8) – 100mM
   - Na₂EDTA (pH 8) – 100mM
   - NaCl – 1.5M
   - CTAB – 1%

3) Phenol : Chloroform : Isoamyl alcohol

4) Chloroform : Isoamyl alcohol

5) Ethanol

6) Sterile distilled water

4.3.1.2. B. PCR Amplification:

1) DNA extract of zoanthids – 1µl

2) Forward primer (16Sant0a) – 1µl

3) Reverse primer (16SbmoH) – 1µl

4) dNTP’s (20mM) – 2µl

5) PCR buffer (10x) – 2.5µl

6) Taq. Polymerase – 0.2µl

7) Sterile distilled water – 17.3µl

Note: 16Sant0a 5’-GAAGTAGGCTTTGAGCCAGCCA-3’
4.3.1.3. **Agarose Gel Electrophoresis:**

1) Agarose (molecular biology grade).

2) Running buffer: 50x TAE buffer pH 8.0:
   a. Tris - 242 g
   b. glacial acetic acid - 57.1 mL
   c. EDTA (0.5M) - 100 mL
   d. sterile distilled water - 1 L.

3) Sterile distilled water

4) Microwave oven

5) Loading buffer 6X:
   a. Glycerol - 50% (v/v)
   b. EDTA, pH 8.0, 50 mM
   c. bromophenol blue - 0.125% (w/v)
   d. xylene cyanol - 1.125% (w/v)

6) DNA ladder 100bp

7) Ethidium bromide: 10 mg/mL dissolved in H$_2$O. Store at 4°C in a container wrapped in tin foil.

8) UV transilluminator (300 nm).

9) Polaroid camera or gel documentation system.
4.3.2. Principle:

4.3.2.1. DNA Isolation:

DNA isolation is a procedure to collect DNA for subsequent molecular or forensic analysis. There are four basic steps in DNA extraction.

1. Breaking the cells open, commonly referred to as cell disruption or cell lysis, to expose the DNA within. This is commonly achieved by grinding or sonicating the sample.

2. Removing membrane lipids by adding a detergent.

3. Removing proteins by adding a protease or by precipitating with sodium or ammonium acetate or extracting with phenol-chloroform mixture.

4. Precipitating the DNA with an alcohol – usually ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation. This step also removes alcohol-soluble salts.

Other than these, chelating agents are also added to sequester divalent cations such as Mg$^{2+}$ and Ca$^{2+}$, which prevent enzymes like DNAase from degrading the DNA. CTAB is a unique detergent which has the ability to separate polysaccharides from nucleic acids[85].
4.3.2.2. **PCR Amplification:**

The polymerase chain reaction (PCR) is a method of amplifying a target sequence of DNA. PCR provides a sensitive, selective, and extremely rapid means of amplifying a desired sequence of DNA. Specificity is based on the use of two oligonucleotide primers that hybridize to complementary sequences on opposite strands of DNA and flank the target sequence (Fig. 4.6.). The DNA sample is first heated to separate the two strands; the primers are allowed to bind to the DNA; and each strand is copied by a DNA polymerase, starting at the primer site. The two DNA strands each serve as a template for the synthesis of new DNA from the two primers. Repeated cycles of heat denaturation, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase, result in the exponential amplification of DNA segments of defined length. A heat-stable DNA polymerase from *Thermus aquaticus* (or the corresponding DNA polymerase from other thermophilic bacteria), an organism that lives and replicates at 70–80 °C, has made possible automation of the reaction, since the polymerase reactions can be run at 70 °C without denaturation of the enzyme. This has also improved the specificity and the yield of DNA[86].
4.3.2.3 Agarose Gel Electrophoresis:

Agarose gel electrophoresis is a widely used method that separates molecules based upon charge, size and shape. It is particularly useful in separating charged biomolecules such as DNA, RNA and proteins. The gel is made by dissolving agarose powder in boiling buffer solution. The solution is then cooled to approximately 55°C and poured into a mould containing a comb that serves as a template. Samples are prepared for electrophoresis by mixing them with components that will give the mixture density, such as glycerol or sucrose. This makes the samples denser than the electrophoresis buffer. These samples can then be loaded with a micropipette or transfer pipet into wells that were created in the gel by a template during casting. The dense samples sink through the buffer and remain in the wells. A direct current power supply is
connected to the electrophoresis apparatus and current is applied. Charge molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). Within a range, the higher the applied voltage, the faster the samples migrate. The buffer serves as a conductor of electricity and to control pH. The pH is important to the charge and stability of biological molecules[87].

4.3.3. Procedure:

4.3.3.1. DNA extraction:

1) 1ml of lysis buffer was in a 1.5ml tube containing 200mg of lyophilized zoanthid powder.
2) It was stirred well with the help of the tooth picks.
3) It was left for 1hr incubation at 60°C in water bath.
4) Then it was centrifuged (Fig.4.7.) for 15min at max RPM of 12,000

Fig.4.7. Cooling Centrifuge
5) The supernatant was collected into a fresh 1.5ml tube and to it 500 µl of Phenol : Chloroform : Isoamyl alcohol was added in the proportion of 25:24:1.

6) Then the tube was subjected to rotor mixing (Fig.4.8.) for 30 min

7) After mixing thoroughly, it was centrifuged at 12,000 RPM for 10 min

8) With the supernatant obtained in the above step, step 5 to 7 were repeated

9) To the supernatant obtained, 500µl of Chloroform : Isoamyl alcohol was added in the proportion of 24:1, mixed well and centrifuged at 12,000 RPM for 10 min

10) The above supernatant is collected in a fresh 1.5ml tube and to it equal volume of 100% ice cold ethanol was added and centrifuged.
11) To the pellet obtained in the above step, 70% ethanol wash was given, incubated for 5-10 min on ice and again centrifuged.

12) After discarding the ethanol, the pellet was allowed to dry free of any residual solvent and then 50µl of sterile distilled water was added to solubilize the DNA.

13) The DNA so extracted was verified on 0.8% agarose gel[88,89].

4.3.3.2. PCR Amplification of 16S mt rDNA and Sequencing:

1) 25µl of PCR mix, of the DNA extract along with the 16S mt rDNA zoanthid specific primers (16S ant0a and 16SbmoH), was prepared.

2) PCR (Fig4.9.) was performed using the following thermal cycle conditions: Initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 1 minute denaturation at 95°C, 30 seconds annealing at 52°C and 90 seconds extension at 72°C, followed by 7 minutes extension at 72°C.

![PCR Thermal Cycler](image)
3) The amplified PCR products were checked by agarose (1.2%) gel electrophoresis.

4) Cycle sequencing (Fig.4.10.) was accomplished in both directions using the forward and reverse primers separately. (Reagents and reaction conditions were as specified in the ABI Prism Big Dye v.3.1 Terminator cycle sequencing ready reaction kit, PE Applied Biosystems, Foster City, CA, USA.)

5) The sequences were analysed by Sequencing Analysing Software v5.3.1., ABI Software Division of Perkin Elmer, Foster City, CA, USA)[90].

4.3.3.3. **Agarose Gel Electrophoresis:**

1) The casting tray is thoroughly cleaned and the open ends are closed by using rubber dams.
2) Appropriate quantities of agarose (0.24g for 0.8% and 0.4g for 1.2%) are weighed and taken in a conical flask.

3) 30ml of 1X TAE buffer was added to the conical flask and mixed thoroughly by swirling.

4) The above gel mix was then heated in a microwave oven for a minute for the agarose to completely dissolve in the buffer.

5) The flask was taken out and cooled till the temperature is around 50°C or just bearable to hold.

6) To this luke warm gel mix, 4µl of Ethidium bromide solution was added and poured into the casting tray.

7) The coomb was then properly placed, without touching the bottom of the tray, so that wells are formed correctly.

8) The gel was allowed to set for 20 to 25 minutes.

9) After the gel is set, the coomb and the rubber dams are carefully removed and the tray with the gel is placed in the electrophoresis unit (Fig.4.11.).

10) Enough running buffer was added to the unit so as to completely submerge the electrodes and the gel.

11) The samples of DNA were appropriately mixed with loading dye and loaded into the wells in one smooth pipette motion.

12) DNA ladder was added to the end wells of the gel.

13) The gel was run at 90V, till the bromophenol blue dye has travelled two-third of the way down the gel.
After electrophoresis, the gel was removed from the apparatus and viewed on UV transilluminator (Fig. 4.12.).

Photograph of the gel was taken using a gel documentation system [91].
4.4. PROTEIN EXTRACTION:

4.4.1. Materials:

1) Sample of zoanthids (lyophilized and powdered)

2) Lysis buffer (LB)I: (with EDTA & PMSF; pH 7.8 at 37°C)

   Tris HCl – 20mM
   NaCl – 150mM
   EDTA – 5mM
   Triton X-100 – 1%
   PMSF – 10mM
   D.H₂O – upto 50ml

3) Lysis buffer (LB)II: (with chitinase; pH 6.0 at 37°C)

   Tris HCl – 20mM
   NaCl – 150 mM
   Triton X-100 – 1%
   Chitinase – 0.1U/ml
   Pot.Ortho.Phos – 50mM
D.H2O – upto 50ml

4) Lysis buffer (LB)III: (with chitinase & PMSF ; pH 6.0 at 37°C)

   Tris HCl – 20mM
   NaCl – 150mM
   Triton X-100 – 1%
   Chitinase – 0.1U/ml
   PMSF – 10mM
   Pot.Ortho.Phos – 50mM
   D.H2O – upto 50ml

4.4.2. Principle:

A lysis buffer is used to break the cells and bring protein into solution, which is usually composed of the following:

1) Salts - Tris-HCl, NaCl – to maintain the pH between 7.0 to 9.2, that coincides with the typical physiological pH of most living organisms.

2) Chelating agents – EDTA – to sequester any metal ions such as Ca2+ and Fe2+ and to disrupt the integrity of the cell wall.

3) Surfactants – Triton X-100 – to permeabilize unfixed eukaryotic cell membranes and solubilize their membrane proteins in the native state.

4) Protease inhibitors – PMSF – to inhibit the proteases present in the cell to deactivate the proteins during their solubilisation, inhibitors like PMSF are used, which form an irreversible
complex with the serine residue present in the majority of the proteins and thus inhibit them.

5) Digestive enzyme - Chitinase – that break down glycosidic bonds in chitin. As zoanthid cell wall is composed of some chitin element, a small amount of chitinase is also used. Chitinase is active at pH 6.0 and requires Pot.Ortho.Phosphate for dissolution.

6) Time duration – Marine invertebrate – time duration spent for the lysis is an important factor, as certain tough tissues like marine invertebrates require long incubation hours with the lysis buffers for extraction to be complete[92].

4.4.3. Procedure:

1) Three conical flasks of 100ml capacity were taken and labelled as Lysis I, Lysis II and Lysis III respectively.

2) 1g each of the lyophilized samples of zoanthids were taken in the flasks and 48ml of each of the lysis buffers were added respectively.

3) 2ml of each of the buffer solutions were kept aside to use as blank in further experiments.

4) The flasks were then plugged with cotton wool.

5) They were then placed in a shaker incubator (Fig.4.14.) at 37°C for 24hrs.
After 24hrs, the flasks were removed and the crude extract was separated by filtration[93].

4.5. PROTEIN PURIFICATION:

The total proteins from the curde extract are separated by precipitation method followed by their purification using dialysis.

4.5.1. Materials:

4.5.1.1. Precipitation:

1) Crude extract
2) Ammonium sulphate
3) PBS buffer

4.5.1.2. Dialysis:

1) Dialysis tubing
2) PBS buffer
3) Sterile distilled water
4.5.2. Principle:

4.5.2.1. Precipitation:

Ammonium sulphate precipitation method is used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out.

The solubility of proteins varies according to ionic strength of the solution, and hence according to the salt concentrations. Two distinct effects are observed: at low concentration, the solubility of proteins increases with increase salt concentration (i.e. increase in ionic strength) an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the proteins begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). The precipitated proteins are recovered by centrifugation and dissolved in fresh buffer for the next stage of purification. Usually 75% of saturation recovers the maximum amount of reactive proteins[94].

4.5.2.2. Dialysis:

Dialysis tubing or visking tubing is a type of semi or partially permeable membrane tubing made from regenerated cellulose or cellophane. It can be used for diffusion with solutes or osmosis with water only. It allows only those molecules to pass through it which
are small enough to fit through the membrane or a membrane’s pore[95].

4.5.2.3. **PBS Buffer:**

PBS buffer is a buffer solution commonly used in biological research. It is a water-based salt solution containing NaCl, Na3PO4, KCl, and K3PO4. It is isotonic and non-toxic to cells. It is used as a diluent in methods to dry biomolecules like proteins, as water molecules within it will be structured around the substance and immobilized to a solid surface. The thin film water that binds to the substance prevents denaturation or other conformational changes.

**4.5.3. Procedure:**

4.5.3.1. **Precipitation:**

1) 20ml of the crude filtrate was taken in a beaker, placed on magnetic stirrer, in a cold room and ammonium sulphate was gradually added till 75% saturation (9.52g).

2) The set up was over-night till the precipitation was completed.

3) The precipitate was then separated by centrifugation at 10,000rpm for 15 min at 4°C.

4) The supernatant was discarded and the pellet of protein precipitate so obtained was dissolved in 2ml of PBS buffer[93].

4.5.3.2. **Preparation of Dialysis Tubing:**

1) The tubing was cut into pieces of convenient length (10-20cm)
2) Then it was boiled for 10min in a large volume of 2% (w/v) sodium bicarbonate and 1mM EDTA (pH 8.0)
3) The tubing was then rinsed thoroughly in distilled H2O
4) Again it was boiled for 10min in 1mM EDTA (pH 8.0)
5) It was then allowed to cool and stored at 4°C (Care was taken to ensure that the tubing was completely submerged)
6) From then onwards, the tubing was always handled with gloves on.
7) Before the use, the tubing was washed inside and out with distilled water[94].

4.5.3.3. Purification of the Sample:

1) Three tubings were selected for three different samples.
2) One end of the tubing was clamped tightly, the sample was added and then the other end of the tubing was clamped tightly.
3) The clamps were accordingly numbered
4) The tubings with the samples were kept in 1L PBS (1X) buffer; in a cold room and dislysed while stirring gently.
5) The dialysis was carried on for 16hrs.
6) Then the tubings with the samples were transferred into 1L sterile distilled water and again carried on for 8hrs, followed by 16hrs in PBS (1X) buffer.
7) Finally the concentrated and purified total protein solution obtained was carefully transferred into sterile eppendorf tube (1.5ml) and stored at -20°C till further analysis[95].
4.6. PROTEIN ASSAY:

To determine the amount of total proteins present in the crude as well as the purified proteins were assayed using Bradford’s assay method.

4.6.1. Materials:

1) Crude protein extract
2) Purified protein extract
3) Bovine Serum Albumin (BSA)
4) Bradford’s reagent (CBB G-250)

4.6.2. Principle:

The Bradford’s assay, a calorimetric assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue (CBB) G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. During the formation of this complex, two types of bond interaction take place: the red form of Coomassie dye first donates its free electron to the ionisable groups on the protein, which causes a disruption of the proteins native state, consequently exposing its hydrophobic pockets. These pockets on the proteins tertiary structure bind non-covalently to the non-polar region of the dye via van der waals forces, positioning the positive amino groups in proximity with the negative charge of the dye. The bond is further strengthened by the ionic interaction
between the two. The binding of the protein stabilized the blue form of the Coomassie dye; thus the amount of the complex present in the solution is a measure of the protein concentration, and can be estimated by use of an absorbance reading. The bound form of the dye has an absorption spectrum maximum historically held to be at 595nm. The cationic (unbound) forms are green or red. The binding of the dye to the protein stabilizes the blue anionic form. The increase of absorbance at 595nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample[96].

4.6.3. Procedure:

1) Stock of BSA standard (1mg/ml) was prepared by dissolving 10mg of BSA in 10ml of buffer solution.

2) Serial dilutions of the protein standard (BSA) were prepared ranging from 0,10,20,30,40,50,60,70,80,90 and 100 µg/ml concentrations using distilled water to a total volume of 100µl.

3) Each of the above concentrations were taken in 11 test tubes respectively and labelled.

4) To each of the test tube 3ml of Bradford’s reagent was added and mixed well.

5) Absorbance of the above prepared standards was noted at 595 nm using Jasco UV-Visible spectrophotometer (Fig.4.15.).
6) The standard graph was plotted using concentrations on the x-axis and the absorbance on the y-axis

7) Two different dilutions (1:4 & 3:2) of the three crude and three dislyzed/purified extracts of the zoanthid samples were made using distilled water to a volume of 100µl

8) To a total of 12 of the above samples, 3ml of Bradford’s reagent was added and individual absorbance noted at 595nm[97].

4.7. PROTEIN ANALYSIS:

The proteins present in the crude as well as the purified extracts were analysed for their approximate molecular weights using SDS-PAGE and spectral (UV and fluorescent) properties using respective spectroscopy instruments.
4.7.1. Materials:

4.7.1.1. SDS-PAGE:

1) Crude extracts of zoanthids
2) Purified extract of total proteins
3) Protein Molecular Weight Marker: 14.3 – 97.4 KDa
   - Phosphorylase b – 97.4 KDa
   - Bovine Serum Albumin – 66.0 KDa
   - Ovalbumin – 43.0 KDa
   - Carbonic Anhydrase – 29.0 KDa
   - Soyabean Trypsin Inhibitor – 20.1 KDa
   - Lysozyme – 14.3 KDa
4) Separating/Resolving gel (12%): 10ml
   - Acrylamide (30%) – 4ml
   - Tris buffer (1.5M) pH 8.8 – 2.5ml
   - SDS (10%) – 0.1ml
   - APS (10%) – 0.1ml
   - TEMED – 0.010ml
   - S.D.Water – 3.3ml
5) Stacking/Loading gel (5%): 3ml
   - Acrylamide (30%) – 0.51ml
   - Tris buffer (0.5M) pH 6.8 – 0.75ml
   - SDS (10%) – 0.03ml
   - APS (10%) – 0.03ml
   - TEMED – 0.003ml
   - S.D.Water – 1.7ml
6) Loading buffer/dye (2x): 10ml
   - Tris HCl (100mM) pH 6.8 – 0.121g
   - SDS (4%) – 0.04g
   - DTT (200mM) – 1.55g
Bromophenol blue (0.2%) – 0.02g
Glycerol (20%) – 2ml
7) Electrode Buffer (10x) pH 8.8: 1000ml
   Glycine (1.92M) – 144g
   Tris base (0.25M) – 30g
   SDS (10%) – 50ml
   D. Water – upto 1000ml

4.7.1.2. Silver Staining:

1) Run SDS-PAGE gels
2) Fixation Solution:
   Ethanol – 50%
   Acetic acid – 12%
   Formalin – 0.05%
   D. Water – upto 50ml
3) Washing Solution:
   Ethanol – 20%
   D. Water – upto 50ml
4) Sensitizing Solution:
   Sod. Thiosulphate – 0.02%
   D. Water – upto 50ml
5) Staining Solution:
   Silver nitrate – 0.2%
   Formalin (37%) – 0.076%
   D. water – upto 50ml
6) Developing Solution:
   Sod. Carbonate – 6%
   Sod. Thiosulphate – 0.0004%
Formalin – 0.05%
D. Water – upto 50ml

7) Terminating Solution:

Acetic acid – 12%
D. Water – upto 50ml

4.7.1.3. **UV Spectroscopy:**

1) Crude extracts of Zoanthids
2) Purified extract of total proteins
3) Blank solutions - Three lysis buffers (I,II,III)

4.7.1.4. **Fluorescence Spectroscopy:**

1) Crude extracts of Zoanthids
2) Purified extract of total proteins

**4.7.2. Principle:**

4.7.2.1. **SDS-PAGE:**

Polyacrylamide gels (PAG) are employed in electrophoresis (PAGE) as they possess several electrophoretically desired features that make them a versatile media for the application. PAGE separates protein molecules according to both size and charge. It is a synthetic gel, thermo-stable, transparent, strong, relatively chemically inert, can be prepared with a wide range of average pore sizes.

The gels usually consist of acrylamide, bisacrylamide, SDS and a Tris-HCl buffer with adjusted pH. The solution is degassed under vacuum to prevent air bubbles during polymerization. Ammonium
persulphate and TEMED are added when the gel is ready to be polymerized. The separating or resolving gel is usually more basic and has a higher polyacrylamide content than the loading or stacking gel.

An electric field is applied across the gel towards the anode. Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel while larger ones will have more difficulty, as they encounter more resistance. After a set amount of time, the proteins will have differentially migrated based on their size; smaller proteins will have travelled farther down the gel, while larger ones will have remained closer to the point of origin. Therefore, proteins may be separated roughly according to size and therefore, molecular weight[98].

4.7.2.2. Silver Staining:

Silver staining is used as a sensitive procedure to detect trace amounts of proteins in gels. Classical Coomassie Brilliant Blue staining can usually detect a 50ng protein band. Silver staining increases the sensitivity typically 50times. Many variables can influence the colour intensity and every protein has its own staining characteristics; clean glassware, pure reagents and water of highest purity are the key points to successful staining[99].
4.7.2.3. **UV Spectroscopy:**

The absorption spectrum of a molecule is obtained by measuring the decrease in light intensity passing through the sample (compared to a suitable “blank”) as a function of wavelength (Fig.4.16): 

\[
\text{\textbf{ABSORPTION}}
\]

\[I_0 \quad \text{(vary } \lambda \text{)} \quad \text{(Sample)} \rightarrow I \quad \text{(transmitted light, measured as a function of wavelength)}
\]

\[\text{Fig.4.16. Principle of absorption}\]

According to the Beer-Lambert law, the intensity of the transmitted light is related to the initial intensity as \(I = I_0 \times 10^{-A}\), where \(A\) is called absorbance. For sufficiently diluted samples, absorbance is directly proportional to concentration \((c)\) of the analyte and pathlength \((l, \text{ in cm})\) of the sample holder, \(A = \varepsilon cl\). The proportionality constant, \(\varepsilon\), is called molar absorptivity, and it is usually determined from the slope of a plot of absorbance against concentration at a known path length. Each molecule is characterized by a unique dependence of \(\varepsilon\) on the absorption wavelength[100].

4.7.2.4. **Fluorescence Spectroscopy:**

The fluorescence spectrum is measured passing a light beam through the sample, and measuring the light emitted from the sample as a function of wavelength (Fig.4.17):
That is, one particular wavelength is chosen to excite the sample and the various fluorescence wavelengths emitted are followed. The light emitted is usually measured perpendicular to the direction of the excitation beam. The excitation spectrum is similar to an absorption spectrum. The excitation wavelength is varied while the fluorescence intensity at one wavelength is followed [101] (Fig. 4.18.):
4.7.3. Procedure:

4.7.3.1. SDS-PAGE:

1) The gel (stacking gel upon resolving gel) was cast in a gel casting apparatus and the whole assembly of electrophoresis was set up (Fig.4.19.).

2) The conductivity of gel was checked after adding the electrode buffer at 80V using Biopac.

3) All the samples (3 crude extracts, 3 purified protein extracts and the protein molecular weight marker) were denatured by adding equal volume of the loading dye and heating at 100°C for 2 min.

4) Out of the 9 wells of the gel, 1st, 5th and 9th well were utilized to load the protein molecular weight marker (3µl each).

5) The 2nd, 3rd, and 4th well were utilized for loading the crude extracts of zoanthids (15µl each)

6) The 6th, 7th and 8th well were utilized for loading the purified protein extracts (3µl each of the sample 1 and 2 and only 1µl of the sample 3)

7) Electrophoresis was performed on the gel was run at 80V for 2hrs
8) After the 2hrs, the gel was carefully removed and proceeded for silver staining.

4.7.3.2. Silver Staining:

1) The gel after electrophoresis, was transferred to a box containing fixation solution and incubate it for 2hrs.

2) The fixing solution was then discarded and the washing solution was then added and kept for 20 min. This procedure was repeated for 2 more times.

3) The washing solution was discarded and sensitizing solution was added and the gel was incubated for 2 min.

4) The sensitizing solution was then discarded and the gel was washed twice with distilled water for 1 min each.

5) The freshly prepared staining solution (in cold state) was then added and shook for 20 min.
6) The staining solution was then discarded and the gel was washed with large volume of distilled water for 1 min and the same was repeated one more time.

7) The gel was then rinsed with developing solution for ~10 to 30 sec.

8) The developing solution was discarded and again fresh developing solution was added and kept for 2 to 5 min.

9) As soon as the bands start appearing, the reaction was stopped by adding terminating solution and gently agitating the gel for 10 min[99].

4.7.3.3. UV Spectroscopy:

1) The 6 samples (3 crude extracts and 3 purified protein extracts) were diluted with distilled water in the ratio of 1:9 and were analysed for their spectral behaviour in the ultra-violet and visible region.

2) The lysis buffers I, II and III, which were kept aside initially, were used as the blanks for the respective samples.

3) The obtained spectra of all the samples were recorded with an emphasis on the red-shifted absorbance peak.

4) The values of the red-shifted absorbance peaks were then considered for fluorescence spectroscopy.
4.7.3.4. **Fluorescence Spectroscopy:**

1) The 6 samples (3 crude extracts and 3 purified extracts) were then observed (Fig. 4.20.) for their emission and excitation peaks individually at their respective red-shifted absorbance peaks.

2) First the emission peak was scanned at the absorption maximum of the red region (670nm).

3) Then the excitation peak was identified by using the obtained emission peak.

![Spectrofluorophotometer](image-url)