3. MATERIALS AND METHODS

3.1 Collection and laboratory rearing of lygaeid bugs

Periodical collections of *S. hospes* and *S. pandurus* were made from their common host plant *Calotropis gigantea* by hand picking. The bugs being fairly large in size, could be easily spotted in field and therefore collected with ease. Sometimes, they could also be collected by gently tapping the inflorescence of their host plant into a plastic cover. The bugs were brought to the laboratory within few hours of their collection and maintained on *Calotropis* seeds. Mass cultures of these bugs were maintained using plastic boxes measuring 5 x 4.5 x 4 cm and covered over by a perforated plastic lid to allow proper air flow inside box. Fresh seeds were provided every alternate day and moisture requirements were met by placing cotton wads soaked in water inside the container.

3.2 Studies on nymphal morphology

The eggs laid by field collected adult specimens of the two species were incubated in the laboratory and the nymphs reared on *Calotropis* seeds. On reaching the fifth instar stage they were utilized for morphological studies. This method facilitated correct identification of the nymphs of the two species, since in the field species identification of the nymphs poses problems. Fifth instars nymphs were etherized and immediately taken under the dissection microscopic for morphological studies. Measurements of different body parts of six specimens per species were recorded after calibrating the microscope using the stage and ocular micrometers. Examination of the nymphs revealed variations in shape, colour and markings.
These differences are expressed by production of detailed habitus drawings. The characters illustrated are 1) lateral view of sterna 2) dorsal abdominal gland apertures and evaporative areas, 3) prothoracic femur, 4) Head dorsal view, 5) Dorsal view of pronotum, 6) Dorsal view of abdomen, 7) Pubescence on abdomen, 8) Prothoracic femur, and 9) Mesothoracic wing pads. In order to differentiate the species based on colour, digital photographs of the fifth instar nymphs of the two species were taken using the Wild stereoscopic microscope attached with a digital camera. The magnification and lighting conditions for the two photographs were kept similar. The photos stored in JPEG format was uploaded into the ImageJ software version 1.24. Using the free hand selection tool option, different regions of the photograph was marked. Then under the Plugins option, the analyze button was clicked so as to facilitate the measuring of the RBG characters. The results were then stored as output file in Excel format.

3.3 Allometric growth

Freshly hatched first instar nymphs and subsequent stages of both the species of bugs were fed on the seeds of Calotropis and reared in plastic boxes measuring 5x 4.5 x 4cm separately. Water requirements were met by providing a soaked cotton wad. Measurements of the various body parts were taken immediately after moulting using a calibrated Wild Stereoscopic microscope. Measurements of the following parameters (Fig 1) were recorded (1) total body length, measured from the tip of the head to the abdominal tip, (2) head width, measured as the distance at the eye region and inclusive of the eyes, (3) rostrum length, (4) fore leg length, (5) mid leg length, (6) hind leg length, (7) thorax length, (8) abdomen length (9) head length,
and (10) antenna length. For each parameter, the average of the measurements of six individuals was used for further computation.

The comparative allometry of the two bugs was studied at three levels namely 1) Ontogenic 2) Static and 3) Evolutionary. For ontogenic allometry, the growth trajectory of each of the above ten organ measurements was analyzed relative to the total body size using the data from the first instar nymph to the fifth instar nymph. Static allometry was studied using data of the fifth instar nymph and the adult stage since these two stages represents the maximum growth attained by the bugs at the nymphal and adult stages respectively. The scaling relationship among individuals of a stage was studied between the total body length and measurement of one of the several morphological measurements. In order to visualize the aspect of evolutionary allometry, the data measured for various body parts at the fifth instar stage was compared to similar measurements available in literature of two other species of Lygaeinae namely *Lygaeus equestris* (Linnaeus) and *L. simulans* (Deckert) ; two members of Orsillinae, *Orsillus depressus* Dallas, *Ortholomus punctipennis* (Herrich-Schäffer); two members of Ischnorhynchinae, *Kleedocerys resedae* (Betula) Panzer, *K. truncatulus* (Walker); and two members of Rhyparochrominae, *Rhyparochromus pini* (Linnaeus), *Aphanus rolandri* (Linnaeus).

**Statistical analysis**

All statistical analyses were performed using freeware statistical tools namely 1) PAleontological STatistics (PAST) version 2.16, 2) Standardised Major Axis Test & Routines (SMATR) version 2.0, and 3) Statistical Data Analysis R-2.6.2. The following statistical analysis was carried out- Bivariate analysis, Pearson's
product moment correlation coefficient between pairs of variables, Multivariate analysis, Principal component analysis (PCA), Discriminant analysis (DA), Non-metric multidimensional scaling (MDS), Standard major axis (SMA), major axis (MA), ordinary least square (OLS) regression technique, and cluster analysis.

3.4 Biological studies

3.4.1 Post Embryonic Development

Eggs laid by field collected females were incubated in petridishes under normal environmental conditions. The freshly hatched nymphs were transferred to small plastic vials using a fine Camelin brush and reared by feeding them on fresh C.gigantea seeds. A small cotton wad soaked in water was provided as a source of moisture. The rearing vial was examined on a daily basis and the presence of exuviae indicated that the nymphs have moulted into the next stage. The days spent by the nymphs during each nymphal moult was recorded. After every moult, the exuviae was removed and the vials cleaned. Fresh seeds were provided to the nymphs daily. The total duration taken by the nymphs to reach the adult stage represented the duration of PED (post embryonic development). The data from 6 replications were used for the final analysis.

3.4.2 Fecundity

Fecundity was assessed by confining freshly hatched adult male and female pairs to a plastic cage. Care was taken to see that adult insects used in this study were insects that were reared in the laboratory during their nymphal stages on C. gigantea. Moisture requirements were met by providing a wet cotton wad and
adequate food was also provided. Records were made daily on the number of eggs laid. The eggs laid were immediately transferred to a petridish and kept aside for incubation at room temperature. The total number of eggs laid during the life time of the female represented the fecundity. In the event of the male pair dying before the female, a new male of approximately the same age from the stock culture was introduced into the cage. The data from 6 replications were used for final analysis.

3.4.3 **Food preference test**

The food preference test was carried out using a rectangular nylon netted set up (70 x 50 x 45 cm). Twenty test adult bugs (ten males and ten females) of the almost same age were kept starved for a period of 24 hrs and provided with water to clear off their guts. Fresh *Calotropis* seeds, leaves, flower and cotton wad, were tested as food source and were placed on the four corners of the enclosure. The test bugs were introduced through the top of the net by gently unzipping the enclosure. A time lag of 30 minutes was given for the insects to settle and then the number of bugs feeding in each of the four food source was noted every 30mts for 4 hrs. At the end of every 30 mts, the feeding of the bugs was disturbed. The bugs were collected and the set up was turned through 45°. The bugs were then reintroduced and observations on the choice of food source noted. The whole experiment was repeated two times using fresh insects and food source. This preference test was conducted separately for the two species of *Spilostethus*. 

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3.5 Population Studies

Sampling of population of the two species of lygaeids was carried out on their natural host plant, the milkweed *Calotropis gigantea* R.Br. over a period of two years from January 2011 to December 2012. The vast area of lands at Thiruneermalai (12.98° North latitude, 80.18° East longitude, and 18m above the sea level) was chosen as the site of the study where milkweed plants are common along the bunds of the irrigation fields. The method used for counting these lygaeids were similar to that followed for *Oncopeltus fasciatus* (Sauer and Feir, 1973) since *S. hospes* and *S. pandurus* are fairly large in size with orange and black patterns, which makes them conspicuous and easy to count. The census was carried out by surveying the entire site (approximately 3Km) and recording the number of adults of each species present on fifteen host plants chosen at random. After enumeration, the insects were released into the habitat. Throughout the study period of two years, the census was carried out during the early hours of the day between 09.00 h and 11.00 h, and in the evening between 03.30 h and 05.00h so as to minimize the effect of temperature. During the hotter times of the day, the bugs tend to hide in the crevices of the soil or under fallen leaves or stones and thus may give erroneous observation of the population density. Meteorological data with respect to the abiotic factors of the environment such as the maximum temperature, minimum temperature, relative humidity and rainfall was obtained from the nearest meteorological station. Observations were also made on the parasites, predators and natural enemies of these bugs so as to study the effect of the biotic factors on the population. The effect of these factors on the population dynamics was analyzed statistically using simple correlation and regression analysis. Observations were also recorded on the number of fruiting pods present on twenty randomly selected milkweed plants during each survey day.
3.6 Bacterial association in the two lygaeid species

3.6.1 Isolation of gut bacteria

The digestive system of 5 adult field collected bugs was dissected using the Wild dissection microscope under insect saline and the contents were homogenized in BHI (Brain heart infusion) medium. The contents were then transferred into eppendorf tubes. The eppendorf tubes were mixed thoroughly and an aliquot of 100 μl was transferred to a 50 ml Erlenmeyer containing 20 ml of (BHI) and 100μl glucose (10%). The Erlenmeyer was incubated at 28ºC for 24-48 h, under agitation (80 rpm). After incubation the cultures were serially diluted (10⁻¹ through 10⁻⁷) and an aliquot of 100 μl was transferred to Petri dishes containing Mannitol salt agar, Nutrient Agar and BHI agar. Plates were incubated at 28ºC for 24-48 h.

3.6.2 Characterization and identification of isolated bacteria

Pure cultures were prepared from individual colonies through making simple streak on LB (Lysogeny Broth) agar plates. The predominant pure colonies with individual characters were further analyzed in PGLC based identification system. The identified isolates were tabulated.

3.6.3 Pyrolysis gas-liquid chromatography (PGLC)

Pyrolysis gas-liquid chromatography (PGLC) of bacteria is a technique which produces paralyzed bacterial components with specifically defined patterns. In theory, these patterns when analyzed statistically produce a “fingerprint” for each organism tested. The technique lends itself to automated bacterial identification since systems are available to automatically prepare samples and pyrolyze and chromatograph the resulting volatiles.
3.7 Molecular characterization of the species

3.7.1 DNA Extraction

Adults of *S. hospes* and *S. pandurus* were collected from the common host plant *Calotropis gigantea* (L). About 1 gm of body tissue was ground in liquid nitrogen and incubated overnight at 50°C in 10 ml digestion buffer (0.01M NaCl, 0.1 M Tris-HCl, 0.25M EDTA and 0.5% SDS with 100μg/ml proteinase K). The digested samples were extracted twice with an equal volume of Tris-HCl, saturated phenol (pH 8.0) and then centrifuged at 5000g for 15 min to remove protein contaminants and debris. The supernatant was transferred into a fresh tube and treated with RNAase at 37°C for 30 min, followed by chloroform extraction and centrifugation at 5000g for 15 min. Finally the aqueous phase from each tube was transferred separately to clean centrifuge tubes and mixed with 0.1 volume of 3M sodium acetate, pH 5.2. Genomic DNA was precipitated with two volumes of cold ethanol, spooled, washed twice with 70% ethanol, dried and suspended in 10 mM Tris-CL (pH 8.0).

3.7.2 Qualitative and quantitative determination of DNA by spectrophotometric method

**Principle:** A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purine and pyrimidine. The concentration of nucleic acid in a solution can be
calculated if one knows the value of $A_{260}$ of the solution. A solution of double-stranded DNA at a concentration of 50µg/ml in a 1cm quartz cuvette will give $A_{260}$ reading of 1. A solution of single-stranded DNA/RNA that has $A_{260}$ of 1 in a cuvette with a 1cm path length has a concentration of 40µg/ml. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ratio of absorbance at 260 and 280nm hence provides a clear idea about the extent of contamination in the preparation. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less than 1.8 signify the presence of proteins as impurities. The values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation

**Procedure:**

The UV lamp of the spectrophotometer was switched on. The instrument was set at zero absorbance with T.E buffer or sterile water as blank. 5 or 7µl of the sample was taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution with the sample was read at 260 nm and 280 nm. The concentration of DNA in the sample was calculated using the given formula:

\[
\text{Concentration of dsDNA} = A_{260} \times 50\mu g \times \text{dilution factor}
\]

\[
\text{Purity of the DNA} = A_{260} : A_{280} \text{ ratio} = A_{260} / A_{280}
\]

\[
= 1.8: \text{pure DNA}
\]

\[
= 1.7 – 1.9; \text{fairly pure DNA (acceptable ratio for PCR)}
\]

\[
= \text{less than 1.8; presence of proteins.}
\]

\[
= \text{greater than 1.8; presence of organic solvent}
\]
3.7.3 Agarose Gel Electrophoresis.

**Principle:** For the majority of DNA samples, electrophoretic separation is carried out in Agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size Agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on Agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated: genomic DNA – 0.8% and RNA – 1.5%, mitochondrial DNA – 0.8% and amplified samples at 1.5% was used.

**Procedure:**

1 gm Agarose was weighed and mixed with 100ml 1X TAE buffer. To this a pinch of ethidium bromide was added and this mixture was heated to 100°C. The heated solution was made to cool and was loaded on to the casting plate assembly to form the gel. After loading this gel mixture, a comb was placed to form wells for loading the sample. Once the gel is totally polymerized, the comb was removed and the sample was loaded. The gel assembly was placed in a buffer and was subjected to run under constant electric field (50v). The separated DNA fragments were visualized using UV-transilluminator.
3.7.4 Polymerase Chain Reaction (PCR)

**Principle:** PCR is an invitro method of enzymatic synthesis of specific DNA sequence developed. It is a very simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy. PCR consists of three steps:

1. **Denaturation:** during this step, the two strands melts, open to form single stranded DNA and all enzymatic reaction stoops. This is generally carried out at 92° to 96°C

2. **Annealing:** annealing of primer to each original strand for new strand synthesis is carried out between 40°– 60°C.

3. **Extension:** the polymerase adds dNTPs complementary to the template strand at the 3’end of the primers. Since both the stands are copied in the PCR there is an exponential increase in the number of copies of the required gene.

These 3 steps are repeated for about 20 to 30 times in an automated thermal cycler, which heat and colds the reaction mixture in the tube in a very short time. This result in exponential increase accumulation of the specific DNA fragments.

**Procedure:**

- 100ng of DNA is used for molecular based detection of COI gene.

- The PCR reaction was performed for 20µl.

- PCR reaction was performed for COI gene.

The PCR tubes were placed in thermocycler and the reaction was carried out.
**PCR set up for gene amplification - Standardization**

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>STOCK CONCENTRATION</th>
<th>FINAL CONCENTRATION</th>
<th>VOLUME FOR 20 µl SETUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q water</td>
<td>8.1 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dNTP mix</td>
<td>2mM</td>
<td>0.275mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>25mM</td>
<td>1.5mM</td>
<td>1.2µl</td>
</tr>
<tr>
<td>Taq buffer</td>
<td>10X</td>
<td>1X</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>FORWARD RIMER</td>
<td>3µm</td>
<td>0.375µm</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>REVERSE PRIMER</td>
<td>3µM</td>
<td>0.375µM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>DNA Template</td>
<td>100ng/µl</td>
<td>100ng</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5U/µl</td>
<td>1U</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>

**PCR reaction conditions**

- Initial denaturation : 94°C – 3min
- Denaturation : 94°C – 1min
- Annealing : 46°C – 1min
- Extension : 72°C – 1min 20sec
- Final extension : 72°C – 7min
- Hold : 4°C

Total number of cycles = 40.

This amplified product was further used and sequenced using the ABI sequencer instrument.
3.7.5 Amplification and sequencing of COI gene

The reaction was performed in a final volume of 20 µl, containing 8.1 µl of MilliQ water, 1 µl of 100 ng/µl genomic DNA, 2 µl of dNTPs, 2 µl of Taq buffer, 2 µl of forward (5’-GGT CAA CAA ATC ATA AAG ATA TTG G – 3’) and reverse (5’- TAA ACT TCA GGG TGA CCA AAA AAT CA – 3’) primers each, the Taq DNA polymerase 0.2 µl, 2.5mµ concentration each of dATP, dTTP, dCTP, and dGTP and the remaining glass distilled water. PCR was carried out as per the reaction conditions cited above in a PTC-225 Peltier Thermal Cycler. The amplified product was electrophorered through a 1.5% agarose gel, at 50v for 30 min and the DNA fragments in the gel were visualized using a UV trans- illuminator. I. The PCR products were gel purified and directly sequenced by di deoxy chain termination method using the Big Dye terminator kits in the ABI 3130 Genetic analyser, according to the manufacturer’s protocols. The sequences were then deposited in the (EMBL/DDBI) GenBank data libraries of the NCBI.

3.7.6 Amplification and sequencing of 16S rRNA

The primers used for amplification were forward LR-J-13017: 5'-TTA CGC TGT TAT CCT AA-3’ and reverse LR-N-13398: 5'-CAC CTG TTT AAC AAA AAC AT-3'.

3.7.7 Amplification and sequencing of 28s rRNA:

Primer sequences for the amplification of the 28s rDNA region were: Forward primer 5'-TAC CGT GAG GGA AA G TTG AAA- 3’ and reverse primer 5’ AGA CTC CTT GGT CCG TGT TT-3’.
3.7.8 Sequence analysis and phylogenetic reconstruction

A Blastn similarity search was conducted using the 16S rRNA sequence of *S. hospes* as the query sequence. The nucleotide collection database was searched with the organism key as Lygaeidae (taxid:7533). The programme was optimized for highly similar sequences (megablast). The search with the 16S rRNA sequence of *S.hospes* as query resulted in 22 Blast hits on the query sequence. A similar search was done using the COI sequence of *S.hospes* as query sequence. However the search with the 28S rRNA of *S.hospes* returned with no records in the data bank. Therefore the search parameter was modified to include search of any organism under the organism key and this resulted in 190 blast hits. Of these, top sequences producing significant alignments were selected. These nucleotide sequences were downloaded in FASTA format and aligned using the ClustalW option present in the MEGA5 software (Tamura *et al.*, 2011). After computing the alignment, the data menu was opened and the active data was explored for analysis of various sites such as conserved sites, parsimonious informative sites, variable sites etc., using the highlight section of the sequence data explorer window of the MEGA tool. The statistics of the nucleotide composition was analysed and automatically exported to Microsoft Excel 2007. Further, the aligned sequences were used to find the Best DNA fit model and to compute the pair-wise distance in order to estimate the evolutionary divergence between the sequences. The General Time Reversible (GRT) model with 5 discrete Gamma categories was opted under the rates and patterns option of the MEGA software for the 16S rRNA sequence. For the 28S rRNA sequence, the T92+ G and for the COI sequence, the T92 model was opted for the analysis.
Molecular distinction between *S. hospes* and *S. pandurus* was carried out by analysing the sequences of the three genes for differences in base statistics and the relative synonymous codon usage (RSCU). An attempt was also made to quantify the genetic variation in respect of these three genes in *S. hospes* and *S. pandurus* by calculating the mutation rate and for this the number of nucleotide substitutions per site between the DNA sequences was computed. The rates of transitional and transversional nucleotide substitution (transition-transversion bias) and the deviation of the G+C content (G+C content bias) was calculated. The maximum likelihood estimate of substitution matrix and the maximum composite likelihood estimate of the pattern of nucleotide substitution were also computed.

To construct Phylogenetic trees, the Maximum Likelihood method and the Neighbor-Joining method were employed and the test of phylogeny had 500 bootstrap replications. The evolutionary distance among the species was calculated by computing the distance matrix using the data on the number of base substitutions per site between the sequences. The rate of accumulation of changes in the DNA sequence over evolutionary time was expressed by determining the molecular clock. The observed differences in the evolutionary rates between the two sequences were statistically tested for significance using the Tajima’s Neutrality test and the Tajima’s test for 3 sequences.

The above analyses were conducted separately using the sequences of 16D rRNA, 28S rRNA and COI of two species of *Spilostethus*. Wherever necessary sequences from the GenBank data base was utilized.
3.8 Biochemical Studies

3.8.1 Body cardenolide content

The methodology of Scudder and Duffey (1971) was followed. Fresh samples of adult *S. hospes* and *S. pandurus* were collected from *C. gigantea* host plants and brought to the laboratory. Five individuals of each species were placed in 10 ml of a solvent made of Chloroform and Methanol in the ratio 2:1 for 12 hrs. The intensity of colour developed due to cardenolides present in the body of the insects was quantified using a spectrophotometer.

3.8.2 Protein profile of freshly laid eggs

The protein profile of the eggs of the two species of *Spilostethus* was characterized by running a SD-PAGE gel. 250 μg of eggs was taken and ground with phosphate buffer saline solution. The molecular weight of the proteins separated from the eggs were estimated using standard proteins which were run along with the sample using the formula:

\[ y = mx + b \]

Where,

\[ Y = \log M \]

\[ m = \text{the slope (1.743)} \]

\[ x = \text{Rf (of unknown protein)} \]

\[ b = \text{the y-intercept (2.788)} \]
**SDS-PAGE Principle**

SDS-PAGE is a method widely used for the separation and characterization of proteins. Polyacrylamide gels are formed by polymerizing acrylamide with a cross-linker, bisacrylamide in the presence of detergent SDS, free radical APS and TEMED. The polymerization is initiated when APS undergoes homolytic cleavage in the presence of water, giving rise to persulphate free radicals (unpaired electrons). These persulphate free radicals are short-lived and are transferred to TEMED, which are subsequently transferred to acrylamide monomers. These monomers get activated and polymerize in the presence of bisacrylamide in the form of long chains. SDS-PAGE, works on the basis that proteins are separated based on their charge and mass. Proteins are amphoteric compounds. Depending upon the number and kind of amino acids in proteins, the charges of the proteins vary and hence mobility in an electric field. SDS is an anionic detergent which binds strongly to, and denatures proteins. Thereby, all the proteins get a net, uniform negative charge and hence more towards the anode.

**Reagent preparation.**

**30% Acrylamide mixture:** 29.2g of acrylamide and 0.8g of bis-acrylamide were dissolved in 100ml of double distilled water. The stock so formed was stored in a brown bottle.

**Lower Tris (pH 8.8):** 18.17g of Tris base was dissolved in 50ml of dd.H₂O and the pH adjusted to 8.8. After this, the final volume was made up to 100ml.

**Upper Tris (pH 6.8):** 6.06 g of Tris HCl was dissolved in 50ml of dd.H₂O and the pH adjusted to 6.8. The final volume was made up to 100ml.
10% APS: 1 g of APS was dissolved in 10ml of dd. H₂O care was taken to prepare the solution only before experiment as it should be added fresh.

10% SDS: 1g of SDS was dissolved in 10ml of dd. H₂O

Running buffer: 1.875 g of Tris, 9 g of glycine, 0.625 g of SDS was dissolved in 300ml of dd. H₂O. The pH was set to 8.3 and the final volume made up to 500ml.

Sample buffer: 2.1 ml of 1.5M Tris HCl (pH.6.8), 1ml of 20% SDS, 0.5 ml of 100% glycerol, 0.5 ml of mercaptoethanol and 2.5 mg of bromophenol blue were added to 0.4ml of dd. H₂O.

Staining solution: 2 g of CBB was added to 500 ml of alcohol to which 70 ml of acetic acid was added. The final volume of the solution was adjusted to 1000 ml with dd. H₂O.

Destaining solution: To 200 ml of alcohol, 35 ml of acetic acid was added. The final volume was made up to 500 ml with dd. H₂O

Procedure

The glass plates and spacers were thoroughly wiped with alcohol. White petroleum jelly was then applied to the 2mm spacers.

1. The plates were clamped together with the spaces on two sides and bottom to seal the chamber between the glass plates.

2. 12% of the separating gel was prepared and poured into the gel mold.

3. The top of the gel was layered with double distilled H₂O and allowed to set for 30-60 mins.
4. 5% of the stacking gel was prepared and powered into the gel mold above the separating gel. The comb was inserted without any air bubble.

5. After polymerization, the comb was removed without distorting the shape of the well.

6. The gel plate was fixed in the electrophoretic apparatus and was filled with the electrode buffer without any air bubbles at the bottom of the gel.

7. 50 µl of the samples were loaded to each of the wells along with the standard marker proteins.

8. The tank was then connected to power supply and the gel was run at 100V until the dye reaches the bottom of the gel.

9. When the tracking dye reached the bottom of the gel the power was stopped.

10. The gel was carefully removed from the mold and immersed in staining solution overnight with uniform shaking at 37ºC.

11. The gel was transferred to a suitable container containing the destaining solution and shaken gently and continuously.

12. The process was continued until the background of the gel appeared colourless and the proteins fractionated into bands coloured blue.

13. The gel was then photographed.
**GEL COMPOSITION**

### Stacking gel

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel percentage (%)</td>
<td>5</td>
</tr>
<tr>
<td>30% Polyacrylamide (ml)</td>
<td>0.85</td>
</tr>
<tr>
<td>1M Tris(pH6.8) (ml)</td>
<td>0.625</td>
</tr>
<tr>
<td>10% Ammonium persulfate (ml)</td>
<td>0.05</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.005</td>
</tr>
<tr>
<td>H₂O (ml)</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>Total volume (ml)</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>

### Separating gel

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel percentage (%)</td>
<td>12</td>
</tr>
<tr>
<td>30% Polyacrylamide (ml)</td>
<td>4</td>
</tr>
<tr>
<td>1.5 M Tris(pH8.8) (ml)</td>
<td>2.5</td>
</tr>
<tr>
<td>10% Ammonium persulfate (ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED (ml)</td>
<td>0.004</td>
</tr>
<tr>
<td>H₂O (ml)</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>Total volume (ml)</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>
3.8.3 Assay of Glutathione-S-Transferase Activity

Materials required:

1) 30mM Chlorodinitrobenzene: 0.012g of CDNB was taken and dissolved in 2ml of ethanol.

2) 30mM Reduced Glutathione: 0.018g of Glutathione was dissolved in 2ml of distilled water.

Procedure:

Glutathione-S-Transferase (GST) activity was estimated following the procedure of Habig et al (1974) with slight modification. Insect digestive tissue was ground in phosphate buffer saline solution and the sample was centrifuged at 5000 rpm for 5 min. at 4°C and the resulting supernatant was taken as sample for GST assay. To 100 μl of the homogenate, 0.1ml of 30mM chlorodinitrobenzene (CDNB) was added and the volume adjusted to 2.9 ml with distilled water. After preincubation of the reaction mixture for 5 min at 37°C, 0.1 ml of 30mM reduced glutathione (GSH) was added. The change in the absorbance level was noted at 340 nm for 5 min after every 30 sec in the UV spectrophotometer. Reaction mixture without the enzyme was used as blank.

GST activity was measured using the formula:

\[ \text{Absorbance} \times 1 \times \frac{1000}{9.6} \times 5 \times \text{protein in mg} \]

Absorbance = Final value – Initial value

1 = Total volume of the reaction

1000 = Concentration in μMol of Total reaction

9.6 = CDNB Conjugative Coefficient

5 = Time duration