CHAPTER 3
MATERIALS AND METHODS

3.1 Study area

3.1.1 Location

Kokrajhar district (26.401436 latitude and 90.266701 longitude) is one of the districts of Assam and head quarter of Bodoland Territorial Region, an autonomous territory in Assam. It is situated on the western part of Assam and occupies an area of 3129 km^2. The Kokrajhar district is located on the north bank of river Brahmaputra. Kokrajhar shares its boundary with Chirang, West Bengal, Barpeta, Bhutan and Dhubri.

3.1.2 Topography

The district is situated in a humid sub-tropical climate, a characteristic of lower Brahmaputra valley of Assam. The soil throughout the district is fertile, composing of sand and clay in varying proportion making it suitable for paddy cultivation (Figure 2).

3.1.3 Meteorological data

The meteorological data were recorded during the entire study period and the maximum and minimum temperature, relative humidity, and rainfall were recorded.

3.1.4 Study period

The study period was for five years (2018-2023) for evaluation of host plants, rearing of silkworms and biochemical analysis.

3.2 Evaluation of host plants

The available literature regarding the host plants of eri silkworm were reviewed. The information on the effect of rearing eri silkworms on different food plants based on previous studies was compiled. The selection of the host plants was done based on the available literature and the availability and distribution of those host plants in the study area. The proper taxonomic identification of the selected host plants was done in Dept. of Botany, Bodoland University, Kokrajhar.

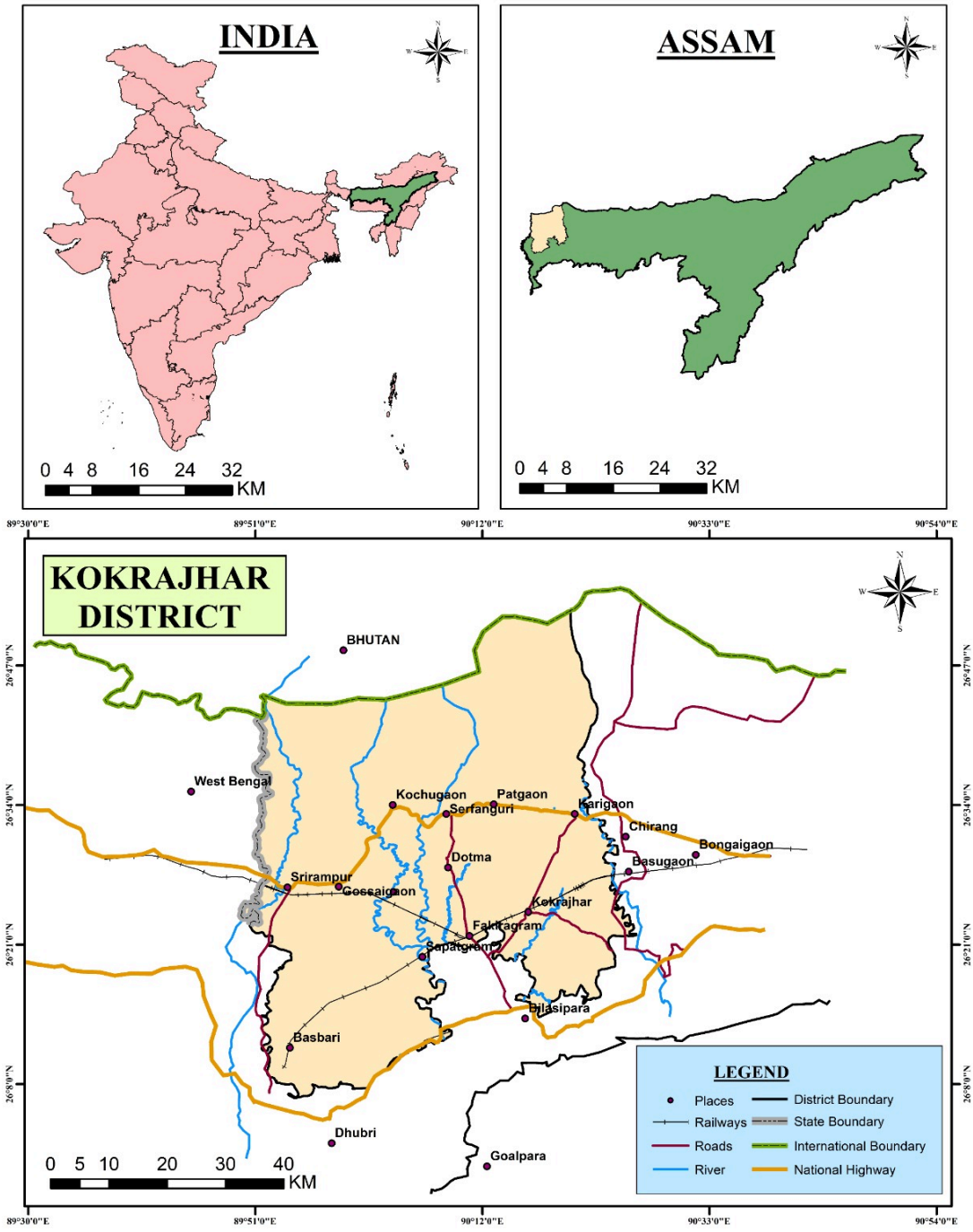

Figure 2: Map of Kokrajhar district, Assam.

3.3 Collection of eri seed

Eggs that were confirmed to be free of any infectious diseases were obtained from the Directorate of Sericulture, Kokrajhar, and then the eggs were reared using the methods given by Sarkar (1980). The eggs were placed in a piece of fabric, which was then tied off, and the incubation process was carried out at normal room temperature, at around 26.92ºC during

Season 1 (February-April), 30.23ºC during Season 2 (May-July), 31.43ºC during Season 3 (August-October) and 22.82ºC during Season 4 (November-January).

3.4 Rearing of *S. ricini*

Rearing of the larvae was done by following the standard procedures as recommended by Sarkar (1980). The room used for rearing, montage trays, and other materials used for the rearing were cleaned before starting the experiment. Then, the eggs were divided into five groups names as Sample C, Sample T, Sample G, Sample K and Sample P consisting of equal number of eggs in each (n=100). The larvae hatched out of the eggs were transferred to rearing trays and four experimental host plants (Plate 1) were used for feeding the silkworms in addition to the control group. The silkworm samples were designated as Sample 'C', 'T', 'G', 'K' and 'P' based on the first letter of the common name of the plant used for rearing the silkworms as mentioned below

Sample C: Reared on host plant *R. communis* from I to V instar.

Sample T: Reared on host plant *R. communis* from I to II instar and *Manihot esculenta* from III to V instar.

Sample G: Reared on host plant *R. communis* from I to II instar and *Gmelina arborea* from III to V instar.

Sample K: Reared on host plant *R. communis* from I to II instar and *Heteropanax fragrans* from III to V instar.

Sample P: Reared on host plant *R. communis* from I to II instar and *Carica papaya* from III to V instar.

3.4.1 Rearing techniques

The rearing of the eri silkworm was divided into two phases based on the nutritional requirement needed during the rearing, *viz.,*

(i) Young age silkworm: First and second instars larvae.

(ii) Late age silkworm: Third to fifth instars larvae stage.

I. Rearing of young age eri silkworm

The young larvae of first and second instar stages were reared on rearing tray. The hygiene of the rearing room was maintained since the larvae are very sensitive. Tender and good quality leaves were used to feed the larvae.

II. Rearing of late age silkworm

The late age larvae from third to fifth instar stages were fed with semi-matured and mature leaves. They are reared on a rearing tray and transferred during the late stage for formation of pupa.

III. Feeding frequency

Healthy sampled leaves were collected for feeding the silkworms. The collected leaves were washed properly in water and stored in a cool dry place for 24 hours during which dried leaves were discarded and only the fresh leaves were used for feeding.The young age silkworms were fed 2-3 times per day and the late age silkworms were fed 4-5 times per day.

IV. Bed cleaning

The regular bed cleaning is necessary for hygiene maintenance of the silkworms. Cleaning was done before and after each moulting stage from first to fourth larval stages. During the fifth larval stage, daily bed cleaning was done. The excreta were and dried leaves were removed and fresh leaves were given after cleaning the bed of the silkworms.

V. Mounting and harvesting cocoons

Matured larvae of fifth instar stage were collected and transferred to montage tray for spinning of cocoons. The cocoons thus produced were harvested after 5-6 days during summer and 7-8 days during winter.

3.5 To study total and differential haemocyte count of the haemolymph of *S. ricini* reared on different host plants

1.5.1 Extraction of haemolymph

Haemolymph were collected from the matured fifth instar larvae from each sampled group (in triplicate). Firstly, the larvae were kept in a jar with ethyl ether mixed cotton for 2 mins for anaesthesia. The larvae were then placed in warm water (40-50°C) for 5 mins to fix the haemocytes and the excess water was removed using filter paper. The haemolymph was

then extracted by puncturing the proleg on the seventh abdominal segment using sterilized dissecting scissors and the haemolymph thus bled was collected (Zhou et al., 2015).

1.5.2 Total haemocyte count

Total haemocyte count was estimated using a haemocytometer following the standard protocol as given by Jalali and Saheli (2008). The collected haemolymph was drawn to the pipette up to 0.5 marking and diluted using physiological saline up to 11 marking on the pipette. The pipette was then shaken to mix the haemolymph with the saline. The first drop was discarded and the haemocytometer was filled with remaining haemolymph. The total haemocyte was counted in the four corners of the Neubauer's chamber and triplicate results were obtained. Total haemocyte counts (THC) were determined mL^{-1} of haemolymph and THC per mm^3 was estimated.

$$THC = \frac{\text{Haemocytes in five } 1mm^2 \text{ squares} \times \text{Dilution} \times \text{depth factor of the chamber}}{\text{Number of squares counted}}$$

Where,

Dilution = 20 times

Depth factor of the chamber = 10 (constant)

Number of squares counted = 5

1.5.3 Differential haemocyte count

Differential haemocyte count was done using smear preparation (Bhagawati and Mahanta, 2012). Few drops of haemolymph obtained from the silkworm were taken on a clean glass slide and a thin smear was prepared which was allowed to dry overnight at 32ºC. The dried slide was dipped in absolute alcohol for 3-4 mins and stained using 6% Giemsa stain for 8-10 mins. The slide was then dipped in distilled water to remove the excess stain and the slide was air-dried and mounted in DPX. For differential haemocyte count, 1000 cells were counted randomly in each slide using a microscope and the cell type was categorized on the basis of morphological description as given by Al-Robai et al. (2002).

3.6 Study of biological parameters of eri *S. ricini* reared on different food plants

From each sampled group, the rearing and growth parameters like effective rate of rearing, fecundity, hatchability, the number of larvae that survived to maturity, and the survival rate of the larvae were observed during the experiment. Cocoon traits such as weight of cocoon,

shell and ratio of the silk were also noted. Every observation of the parameters was carried during four different seasons such as Season 1 (February-April); Season 2 (May-July); Season 3 (August-October) and Season 4 (November-January) for five years (2018-2023). The different parameters observed were as follows

3.6.1 Larval duration (in days)

It is the total time taken by the larva to complete its life cycle from an egg stage to the spinning stage.

3.6.2 Pupal duration (in days)

It is the total time taken by the pupa during metamorphosis transitioning from the larval to an adult moth form.

3.6.3 Fecundity (in numbers)

Fecundity is the total number of eggs laid by a female moth. A random moth from each group was examined and the number of eggs laid by each moth was recorded and the mean fecundity was calculated.

3.6.4 Hatchability (in %)

The hatchability was calculated by examining 100 disease-free laying eggs which were kept in Petri dish and allowed to hatch at room temperature.

$$\text{Hatchability} = \frac{\text{Total number of eggs hatched} \times 100}{\text{Total number of eggs examined}}$$

3.6.5 Effective rate of rearing (ERR %)

The effective rate of rearing (ERR%) is a measure of the success in rearing the silkworm that reflects the percentage of silkworms that has reached the spinning stage of development. The effective rate of rearing was calculated by using the formula

$$\text{ERR} = \frac{\text{Total number of larvae spinning}}{\text{Total number of larvae hatched}} \times 100$$

3.6.6 Emergence rate (%)

The rate of emergence is the total number of moths that have emerged from the total seed cocoon preserved.

$$\text{Emergence rate} = \frac{\text{Total moths emerged}}{} \times 100$$

Total number of cocoons

3.6.7 Survival ratio (%)

The survival rate of the eri silkworms reared on different host plants was recorded. It was calculated by the differences in the total number of fifth instar larvae and the total number of larvae hatched from the total number of eggs.

3.6.8 Cocoon and shell weight (g)

The weight of the cocoon was measured together with the pupa as well as after removing the pupa. All the weights were measured using a digital weighing balance.

3.6.9 Shell ratio (%)

The shell ratio refers to the percentage of cocoon weight that is contributed by the silk fibre. It is one of the crucial factors in sericulture that helps in assessing the quality of silk production. A higher shell ratio indicates the greater yield of silk. Shell ratio was calculated using the formula

$$\text{Silk Ratio} = \frac{\text{Weight of single shell}}{\text{Weight of single cocoon}} \times 100$$

3.7 Study on biochemical parameters of *S. ricini* reared on different host plants

The biochemical analysis of *S. ricini* fed on five selected host plants was analysed using standard methodologies for each parameter. Experiments were done in triplicates and results were analysed.

Extraction of haemolymph: Haemolymph was collected from the matured fifth instar larvae. The larvae were first placed in warm water for few minutes to fix the haemolymph and the excess water was removed using filter paper. The haemolymph was extracted by puncturing the proleg on the seventh abdominal segment using sterilized dissecting scissors and the haemolymph thus bled was collected in a pre-cooled tube containing few crystals of Phenylthiourea. The sample was then centrifuged at 4°C for 10mins and the resultant supernatant was stored at -20°C until further use. The supernatant was used for the estimation of biochemical parameters (Zhou et al., 2015).

3.7.1 Total protein assay of haemolymph

The total protein assay was done by following the standard method of Lowry et al. (1951) by taking Bovine albumin serum (BSA) as standard. In brief, 1mL of supernatant was mixed with 1.5mL of protein reagent and incubated for 10mins after which 0.5mL of Folin ciocalteau reagent (FCR) was added and the reaction mixture was incubated for 30mins in dark at room temperature. Absorbance of the sample was measured at 660nm spectrophotometrically against the blank. The standard graph was plotted with absorbance on Y-axis and concentration on X-axis. The concentration of unknown was determined using the stand graph plotted and results were expressed in mg of protein per ml of haemolymph.

3.7.2 Total carbohydrate assay of haemolymph

The total carbohydrate assay was done by following the standard Anthrone method as given by Sadasivam and Manickam (2008). Anthrone reagent (2mL) was added to 1mL of supernatant and heated in a boiling water bath for 10mins. Once cooled, the absorbance was recorded at 630nm. Standard graph was plotted with absorbance on Y-axis and concentration on X-axis. Concentration of unknown was determined using the stand graph plotted and result was expressed in mg of protein per mL of hemolymph.

3.7.3 Total free amino acid estimation of haemolymph of *S. ricini*

The total amino acid content of the haemolymph was determined using ninhydrin method outlined by Moore (1968). The standard solution was prepared by taking different concentration of working standard of leucine. In brief, 1mL of ninhydrin reagent was added to the test tubes containing the supernatant (0.1mL haemolymph) and the standard solution (leucine) and diluted with distilled water up to 2mL. The test tubes containing the mixture were then heated in a boiling water bath for 20mins. 5mL of the diluent solution (n-propanol and distilled water in 1:1 ratio) was added to it and heated again for 15mins in a boiling water bath. After cooling, the absorbance of the purple was measured at 570nm (green filter) against the blank containing (80% ethanol). The amount of total free amino acid of the haemolymph was calculated by using standard curve was plotted against the standard. The experiment was done in triplicates and the result indicates the mean of three observation (n=3) and expressed as mg/mL of sample.

3.7.4 Mineral content estimation of *S. ricini*

Sample preparation: The sample preparation for mineral analysis was done using the wet digestion method (Puwastein et al., 2011). The dried samples of *S. ricini* fed on different host plants were taken in Teflon cups and Nitric acid (HNO_3) and Perchloric acid ($HClO_4$) at 4:1 ratio was added to the sample. Sample was then pre-digested overnight in tightly closed cups at room temperature. The sample was then heated in an oven for 4-8 hours at 100°C until the sample is fully digested. The sample was allowed to cool and transferred to a conical flask which was diluted to a final volume of 100mL using deionized water. The solution was mixed well and filtered through Whatman filter paper and transferred to Nalgene tubes for mineral estimation. The leaves of the host plants were also taken, and the sample was prepared in a similar manner.

3.7.4.1 Determination of Calcium (Ca) and Magnesium (Mg)

An aliquot of test solution was taken into a volumetric flask and lanthanum chloride ($LaCl_3$) solution (1 %, w/v) added to make the final concentration of 0.1%.The solution was diluted by adding distilled water to get the desired volume. The Ca and Mg content were determined using an Atomic Absorption Spectrophotometer (AAS). The Ca and Mg were measured at particular wavelengths of 422.7 nm and 285.2 nm, respectively, according to Puwastein et al. (2011). The concentration of Ca and Mg was estimated using the following formula

$$\text{Concentration of Ca/Mg (mg/100 g)} = \frac{(C) \times total\ volume\ (mL) \times dilution \times 100}{weight\ of\ sample\ (g) \times 1000}$$

Where, C= concentration of sample (mg/L)

1000= conversion for mL to L.

3.7.4.2 Determination of Potassium (K) and Sodium (Na)

Aliquot of the test solution was transferred into a volumetric flask and then diluted with an aqueous solution of caesium chloride (CsCl) before analysis. The determination of potassium (K) and sodium (Na) levels was performed using Atomic Absorption Spectroscopy (AAS) at the respective wavelengths of 766.5nm and 589.0nm, as reported by Puwastein et al. (2011). The concentration of potassium (K) and sodium (Na) was determined using the following formula

Concentration of K/Na (mg/100 g) = $\frac{(C) \times total\ volume\ (mL) \times dilution \times 100}{weight\ of\ sample\ (g) \times 1000}$

Where, C= concentration of sample (mg/L)

1000= conversion for mL to L.

3.7.4.3 Determination of Iron (Fe), Copper (Cu) and Zinc (Zn)

An aliquot of the test solution was diluted to the required volume. The Fe, Cu, and Zn content was then determined by Atomic Absorption Spectroscopy (AAS) at their respective wavelengths of 248.3 nm, 213.9 nm, and 324.7 nm. These methods were described by Puwastein et al. (2011). The concentrations of iron (Fe), copper (Cu), and zinc (Zn) were determined using the following formula

Concentration of Fe/Cu/Zn (mg/100 g) = $\frac{(C) \times total\ volume\ (mL) \times dilution \times 100}{weight\ of\ sample\ (g) \times 1000}$

Where, C= concentration of sample (mg/L); 1000= conversion for mL to L.

3.7.4.4 Determination of Phosphorus (P)

An aliquot of the test solution was dissolved with water and then further series of dilutions was done with a standard phosphate solution. The acid molybdate reagent and 1, 2, 4-Aminonapthol sulphonic acid reagent are introduced into each dilution. After duration of 10 mins, the measurement of absorbance is recorded at a wavelength of 660nm. These methods were described by Puwastein et al. (2011). The phosphorus concentration was determined using the following formula

Concentration of P (mg/100 g) = $\frac{(C) \times total\ volume\ (mL) \times dilution \times 100}{weight\ of\ sample\ (g) \times 1000}$

Where, C= concentration of sample (mg/L)

1000= conversion for mL to L.

3.8 To study nutritional content of the larvae of *S. ricini* reared on different host plants

3.8.1 Sample preparation

The sample preparation for the study of nutritional content of *S. ricini* was done following the method of Longvah et al. (2011). Matured fifth instar larvae were taken for the experiment. The silkworms reared on selected five host plants were washed separately using warm water and the excess water was removed using filter paper. The cleaned silkworms were then transferred to a petri dish which was kept in an oven overnight at 60°C. The dried silkworms were then homogenized and used for the biochemical analysis.

3.8.2 Proximate analysis of the larvae of *S. ricini*

3.8.2.1 Determination of moisture content

Moisture content of the silkworm was determined using oven dry method (Association of Official Analytical Chemists, 2000). About 3g of homogenized sample was spread uniformly on a petri dish and dried in a hot air oven for 3hours at 105°C. The dried sample was then transferred to a desiccator and allowed to cool. The weight of the cooled sample was observed. The difference in the weight represents the loss of moisture and expressed as percentage of dried sample.

$$\text{Moisture (\%)} = \frac{W2-W1}{W1} \times 100$$

Where, W1= Weight of sample before drying (g)

W2= Weight of sample after drying (g)

3.8.2.2 Determination of ash content

A muffle furnace was used to determine the ash content. About 5g of sample was heated in a silica crucible on a Bunsen flame with lid half covered. The crucible was placed in a muffle furnace when the fumes were no longer produced and heated overnight at 550°C. The crucible was covered with lid to prevent loss of ash and allowed to cool in a desiccator. The weight of the burned sample was taken and the percentage of the ask content was calculated (Ismial, 2017)

$$\text{Ash (\%)} = \frac{W2\text{-}W1 \times 100}{W}$$

Where, W1= weight of empty crucible (g)

W2= weight of empty crucible (g)

W= weight of test sample (g)

3.8.2.3 Determination of crude fibre

The crude fibre content was determined by using the method of A.O.A.C (2000). Fat and moisture free silkworm sample (4g) was digested with 200 mL of 1.25% sulphuric acid (H_2SO_4) for 30 mins. The sample was removed from the acid solution by decanting the acid solution and washing the sample with hot water. The acid free sample was then treated with 200 mL of 1.25 % sodium hydroxide (W/V) solution for 30 mins. The top layer was discarded and the solid sample was filtered through weighed filter paper. The solid sample was repeatedly washed with hot water and then with alcohol and ether in order to make it free from alkali. The material was then dried in an oven at 100°C for five hours and weighed (W1). The material was then transferred to a crucible and heated in a muffle furnace at 60°C for three hours after which it was cooled and weighed again (W2). The difference in weight (W1-W2) represents the weight of crude fibre.

$$\text{Crude fibre content (\%)} = \frac{W2-W1}{Weight\ of\ sample}$$

3.8.2.4 Determination of fat content

The fat content of the silkworm sample was estimated by using the method of A.O.A.C. (2000) in a Soxhlet apparatus. A total of 3 g sample was weighed in a pre-weighed bottle and wrapped carefully in a filter paper which was then introduced into the extraction thimble of the Soxhlet. A round bottom was filled with 250 mL petroleum ether and kept on a heating mantle. The heating mantle was switched on after turning on the water to cool them and connecting the Soxhlet apparatus. The sample was heated for about 13-14 hrs at the rate of 150 drop per min. The bottle was incubated at 80-90°C in a vacuum condenser until the solvent is completely evaporated. When the bottle was dried, it was transferred to a desiccator to cool and the weight of the dried sample was measured which represents the weight of fat.

$$\text{Fat (\%)} = \frac{\text{Weight of fat(g)}}{\text{Weight of sample (g)}} \times 100$$

3.8.2.5 Determination of crude protein

The crude protein content of the silkworm was estimated using Kjeldahl method (1983) (A.O.A.C 1970). It was calculated by multiplying the total nitrogen content with a conversion factor of 6.25. About 50 mg of sample was taken in a digesting tube along with 1 g of Kjeldahl digesting tablet mixture (catalyst) which contains (K_2SO_4, HgO and $CuSO_4$) and 2 mL of concentrated sulphuric acid (H_2SO_4). Digestion was done at 370°C until the solution turned colourless. The digest was allowed to cool and transferred to the distillation apparatus and

diluted with 60 mL of distilled water. The flask was connected to the digesting bulb on condenser with the tip of condenser dipped in standard acid containing Boric acid (4% w/v) solution along with 3-4 drops of indicator solution containing one part of 0.2 % methyl red in ethanol and five parts of 0.2 % bromocresol green in ethanol. The flask was then heated until distillation was complete and the ammonia on the standard acid was collected. The condenser was removed and the solution was titrated against the standard sulphuric acid solution (0.02N). A blank sample containing equal volume of distilled water was taken for titration.

$$\text{Nitrogen content (\%)} = \frac{(sample\ titrated - blank\ titrated) \times Normality\ of\ HCl \times 14 \times 100}{Weight\ of\ sample \times 1000}$$

$$\text{Crude Protein (\%)} = Nitrogen\ content\ (\%) \times 6.25.$$

Where,

6.25= Protein to nitrogen conversion factor.

3.8.3 Fatty acid profiling using Gas Chromatography-Mass Spectrometry (GC-MS)

Lipid Extraction

Lipid extraction was done using the method described by Folch et al. (1957). In brief, 2.25g of sample was taken and homogenized with Chloroform: Methanol (2:1 v/v) mixture using a magnetic stirrer for 30mins. The mixture was filtered and kept in a separating funnel, 20mL of distilled water was added and the mixture was swirled gently. The mixture was then let to stand overnight until two layers were formed. The upper methanol layer was discarded and the lower layer was collected and evaporated in a rotary evaporator until the solution was nearly dry.

Preparation of Fatty acid methyl ester (FAME): The lipid extract was mixed with 10% Boron Trifluoride (BF_3) in methanol (1:9 v/v) in a conical flask and kept in an oven for 6mins at 83°C after which the solution was transferred to separating funnel with addition of 4mL Hexane. Then 4mL of saturated NaCl brine solution was added and shaken vigorously for 5mins. The lower salt was discarded, and the upper FAME was collected which was used for the analysis.

Fatty Acid analysis: The GC-MS analysis of the FAME extract of the silkworm samples were carried out with Perkin Elmer (USA) make GCMS instrument, Clarus 680 GC & amp; Clarus 600C MS which comprises of a liquid auto-sampler. The software used in the system is TurboMass Ver. 6.4.2. The capillary column used is 'Elite-5MS' having dimension length-60m, ID- 0.2 mm and film thickness was 0.25 µm, the stationary column was 5% diphenyl 95% dimethyl polysiloxane.

GC Protocol: Helium gas (99.99%) was used as mobile phase at the flow rate of 1 mL/min. Injection volume of 1 µl was employed, temperature of injector was 280°C while the ion-source was 180°C. The temperature of the oven was programmed at 60°C (for 1 min) with and increase at 7°C per min to 200°C (for 3 min) which was again increased at 10°C per min to 300°C (for 5 mins). The total run time was around 39 mins. Solvent delay was kept for 8 mins. MS Protocol Mass Spectra was taken in Electron Impact positive (EI+) mode at 70 eV. Mass range i.e., m/z (mass/charge) range was 50-600amu.

Identification of Peaks: The interpretation of the peaks appeared in GC chromatogram was done by library search of the mass spectrum of corresponding peaks using the database software of National Institute Standard and Technology- 2014 (NIST-2014). The mass spectra of the unknown component were compared with the known component spectrum of NIST library, and the compounds of the chromatogram were identified based on the name, molecular weight, empirical formula etc.

3.8.4 Amino acid profiling using Ultra Performance Liquid Chromatography (UPLC)

About 5mg of sample was taken and dissolved in 1mL of MQ-water and incubated at 45℃ in a thermomixer for 30mins. After incubation, the protein was precipitated by adding 4mL of methanol and incubated overnight at -20℃ after which the sample was centrifuged at 4000rpm for 30 mins, and supernatant was transferred to a clean tube and completely evaporated under Nitrogen atmosphere at 60℃. Derivatization was done by adding 80µl of borate buffer and 20µL Accq Tag ultra-reagent to the supernatant which was then incubated at 55℃ for 10mins. After incubation, 2µL sample was loaded into UPLC (Waters Acquity UPLC, Column temperature: 55℃, PDA detector 260nm at the flow rate of 0.5mL/min). Mobile phase A (Accq. Tag Ultra eluent A1) and mobile phase B (Accq. Tag Ultra eluent B) were used. The standard amino acids obtained from Sigma Aldrich were also allowed to run at the same condition. The sample and standard were allowed to run at the flow rate of 0.5mL/min at 55℃

column temperature, PDA detectors of 260nm. The quantification and amino acid profiling was done by comparing the retention time and the peak obtained with that of the standard amino acids.

3.9 Study on scavenging and antioxidant activity of *S. ricini* fed on different host plants

Preparation of sample: The larvae of eri silkworm reared using different host plants were taken from all the five groups for sample preparation to determine the antioxidant activity. The larvae of all the five samples were dried in hot air oven overnight at 30°C. The dried larvae were then powdered using mortar and pestle. About 10g of dried silkworm larvae was taken and dissolved in 100mL methanol for 48 hours which was then filtered to prepare the methanolic extract of larvae. The filtrate was then evaporated using rotary evaporator at low temperature below 35°C and the methanolic extract of the larvae thus prepared was kept at 4°C to be used in studying the antioxidant properties through DPPH, ABTS and FRAP assay.

3.9.1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay was determined by following the method given by Benzie et al. (2018). The FRAP reagent consisting of three different solution was prepared by adding 25mL acetate buffer (300 mM, pH=3.6), 2.5mL TPTZ (in 40mM HCl) and 2.5mL of 20mM $FeCl_3.6H_2O$ solution. Standard solutions of Gallic acid was prepared in different concentrations (10, 20, 50, 100 and 200 µg/mL) to obtain the calibration curve. FRAP reagent (4mL) was added to the Gallic acid solutions. The mixture was then incubated in room temperature for 30mins at dark and the absorbance of the sample was measured at 593nm using UV-VIS spectrophotometer (Systronics, Double Beam Spectrophotometer 2203). Then, 40 µL of sample extract was taken and added to 4mL of FRAP solution and mixed which was then incubated for 30mins at dark and the absorbance was measured at 593nm. The FRAP value was obtained from the regression equation obtained from the standard graph plotted. The data was expressed in µm Gallic acid equivalent (GAE)/g of dry sample extract.

3.9.2 DPPH free radical scavenging assay

The free radical scavenging activity of Eri silkworm, *S. ricini* was studied by using standard DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay given by Brand-Williams et al. (1995). Firstly, a solution of DPPH in methanol with a concentration of (6×10^{-5} M) was prepared. The samples were prepared in different concentrations (10, 20, 50, 100 and 200µg/mL in test tubes to which DPPH solution in a 1:1 ratio was added and firmly mixed using a vortex mixer for

15-20mins. The combination was allowed to incubate for 60mins in a dark environment at room temperature. Standard solutions of ascorbic acid were also prepared using the same procedure in the same concentrations (10, 20, 50, 100 and 200µg/mL) as sample. Blank solution consists of methanol and the control consists of 1mL methanol and 3mL of methanol DPPH solution. The UV-Vis Spectrophotometer (Systronics, Double Beam Spectrophotometer 2203) was used to measure the absorbance at 517nm at various time intervals. The concentration (µg/mL) of the sample was plotted against the percentage inhibition and the IC_{50} value was calculated from the regression equation calculated from the graph. The transition from purple to yellow in the reaction mixture, accompanied by a decrease in absorbance, was regarded as an indication of increased scavenging activity. The inhibition percentage of radical scavenging activity was determined using the following formula

$$\text{Inhibition (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

3.9.3 ABTS radical scavenging activity

The radical cation scavenging activity of ABTS (2, 2 azobis, 3-ethyl benzothiozoline-6- sulphonic acid) was determined by the method of Shah et al. (2015). ABTS cation radical was produced by the reaction between 5 mL of ABTS dissolved in water and 5mL of potassium persulfate (2.45 mM) which was stored in dark at room temperature for 12-16hours. The ABTS solution was then diluted with methanol until the absorbance was recorded to be 0.70 at 734nm. The sample and standard (Gallic acid) were taken in different concentrations (20, 40, 80, 100, 200µg/mL) and 2mL of ABTS solution was added to the test tubes and mixed. The absorbance of solution was measured after 6mins at 734nm using UV-VIS spectrophotometer (Systronics, Double Beam Spectrophotometer 2203). Methanol (1mL) and 2mL of ABTS solution was taken as control and methanol was taken as blank solution. The concentration (µg/mL) of the sample was plotted against the percentage inhibition and the IC_{50} value was calculated from the regression equation calculated from the graph.

$$\text{ABTS scavenging activity (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

3.10 Study on the immunological responses of *S. ricini* using enzyme assay

3.10.1 Glutathione-S-transferase (GSTs)

Glutathione-S-transferase (GSTs) is a diverse group of enzymes belonging to the class transferase. These enzymes are found in all organisms and play important role detoxification xenobiotic and endogenous compounds by catalysing their conjugation with glutathione (GSH) (Sivori et al., 1997). GSTs neutralize the thiol group of electrophilic centres of various substances by forming a conjugate which facilitates the water solubility of the substance.

The GST enzyme assay of *S. ricini* was studied using the method described by Habig et al. (1974). This assay was based on the reaction between GST and 1-chloro-2, 4-dinitrobenzene (CDNB), which served as a substrate that is conjugated with glutathione (GSH) in the presence of the enzyme. The GST catalyses the conjugation of L-glutathione to CDNB through the thiol group of glutathione.

$$\text{GSH+CDNB} \longrightarrow \text{GS-DNB conjugate + HCl.}$$

The reaction product GS-DNB conjugate absorbs light at 340nm which was measured spectrophotometrically. The rate of increase in absorption was proportional to the GST activity in the sample.

$$\text{GST activity} = \frac{((\Delta A340) \times V\,(ml) \times dil)}{\varepsilon mM \times Venz(ml)} = \mu mol / mL/ min$$

Where,

$(\Delta A_{340})/min = A_{340}(final\ read) - A_{340}(initial\ read) \div$ reaction time (min.)

V(mL)= reaction volume of sample

dil= dilution of the sample

ε_{mM}=extinction coefficient for CDNB conjugate at 340nm

V_{enz}= Volume of enzyme sample tested.

3.10.2 Catalase enzyme assay

Catalase is an oxido-reductase enzyme present in the cells of various organisms. It is an important enzyme responsible for degradation of the reactive oxygen species, hydrogen peroxide. It promotes the conversion of Hydrogen Peroxide (H_2O_2) to water and oxygen

molecule. Haemolymph of the fifth instar larvae of *S. ricini* was taken for the analysis of Catalase enzyme activity using the method given by Sinha et al. (2008).

The catalase activity was determined by measuring the decrease in H_2O_2 concentration observed after incubating the enzyme sample with H_2O_2 (65mM) standard solution. The decomposition of peroxide depending on the catalytic activity of the protein sample was measured spectrophotometrically at 240 nm against blank sample containing PBS buffer. One unit of catalase activity was defined as 1µmol conversion of H_2O_2 per min / mL protein sample.

$$\text{Catalase Units/mL} = \frac{((\Delta A240 \text{ of sample} - A240 \text{ of blank}) \times V\,(\text{ml}) \times \text{dil})}{\varepsilon 240 \times b} \times \frac{1}{\text{mg}}\, protein$$

Where,

(ΔA_{240})/min = A_{240}(final read) – A_{240}(initial read) ÷ reaction time (min.)

V= volume of enzyme sample

dil= dilution factor

ε_{240}= extinction coefficient of H_2O_2 (65mM) at 240 nm.

3.11 Statistical Analysis

The data obtained from the studies were determined by using statistical tools such as mean, standard deviation (SD), ANOVA and Kruskal Wallis test by using the software PAST Version 4.14.