Chapter 2
Materials and Methods

Chapter 3

Materials and Methods

3.1 Study Site

Six districts situated at the foothills of Bhutan were chosen as the study site namely Baksa, Chirang, Dhemaji, Kokrajhar, Sonitpur and Udalguri. The districts are located in northwest part of the Brahmaputra valley and are considered as the least transformed area of natural vegetation. The landscape of the districts ranges from hills and mountains in the north to plains with mixed vegetations and many small forest interrupted by numbers of small streams and rivers in the middle part and towards the south. Ten villages of which five were near to reserve forests and five close to urban area and inhabited by the Bodos were selected at random for the survey, in each of the districts. The study area encompassed the following villages in each of the six districts.

1. Baksa – Baganpara, Bogulamari, Kumarikata, Merkusi, Naya Basti, Odalbari, Parkijuli, Subankhata, Tupula, Tamulpur.
2. Chirang – Balabari, Bashbari, Bengtol, Bhutiapara, Goibari, Kajalgaon, Khoirabari, Kumguri, Salbari, Sumla bagan,.
3. Dhemaji – Bordoloni naharbari, Dekapam naharani, Dhansiripur nalatol, Kheroni nathgaon, Nalbari, Panbari, Simenmukh, Somkong, Subahi, Ukhamati mingmang.
4. Kokrajhar –Batabari, Boragari, Deborgaon, Gagraguri, Jordonpur, Karigaon, Patgaon, Serfangguri, Simbargaon, Uwabari.
5. Sonitpur – Balabari, Batachipur, Bondaijorani, Doimaluguri, Goroimari, Kathalguri, Num Bagaribari gaon, Pirakata gaon, Nareng guri, Rangapara.
6. Udalguri – Dumuruguri, Bhalukmari, Dwifang, Kalbari, Kundrubil, Naharbari, Swrang hadot, Merbangsuba, Khachibari, Goraimari.

Of the surveyed villages the samples were collected mostly from the four districts namely Baksa, Chirang, Kokrajhar and Udalguri as these districts encompassed the outbreak areas or villages in which edible insects are harvested. Study was focused on wild species of insect. Domesticated edible insects like Eri Silkworm were eliminated from the study. Samples were collected from the districts

during the survey. Permission to work in the area was obtained from the village head of villages (Gaon bura) where the survey was conducted.

3.1.2 Geographical location of the study area

The study site encompassing the six districts of Assam experiences an average temperature that varies from 4°C to 19°C during winter and 26°C to 38°C accompanied by elevated humidity during the summer. This state of Assam predominantly experiences the influence of the southwest tropical monsoon usually from mid April to late October with sporadic and light winter rainfalls. Strong and powerful winds, hailstorms, cyclones, thunder showers and cloudy weather in the month of April and May signify the onset of the monsoons. Flood due to heavy and continuous rainfalls is common during June, July and August. The average rainfall with maximum precipitation recorded for the state for the month of June and July is about 290 cm.

3.2 Data Collection

During 2014 data were collected from the villages and markets of rural areas of the districts through random field studies, questionnaire survey, key informant interviews and group discussion. Group discussions were conducted to gain information on insects utilised and call for volunteers to aid with the surveys. The survey study was scheduled by addressing the local people with questionnaires to gather information on the local identification system, seasonal availability, life cycle and mode of intake of this species. Key informant interviews were held with edible insect dealer from the vicinity to gain information on the harvesting season and costing of the product. Data on wild edible species were collected. Residents referred to edible insects by their vernacular (Bodo) names (ethno-species). Most of the insect species are seasonal in nature and their abundance is dependent on the type of weather and climate.

3.3 Sample Collection

All insect samples used in this study were collected at ideal stages of their lifecycles at which they are consumed by humans. Fresh samples of these species were collected in the months between May to November, 2014 from the chosen study site which was preferentially wild habitats and local markets. Specimens were

collected with the help of local informant, mostly the local residents of the collection site skilled in wild insect harvesting. The insects were collected from various wild habitats, fresh water bodies, paddy fields, vegetable gardens, grasslands and from the local markets. About 100 g of each sample was collected for the nutritional analysis.

3.4 Killing and Preservation

Fresh tissues were transferred to the laboratory and freed from dust and other foreign material by repeated washings. Killing and preservation was followed without delay to circumvent suffer or starve to death by following standard methods (Biosystematics Division, ARC-PPRI, South Africa, 1996). For killing, the species were frozen at -20°C for 48 hours in a deep freezer. After killing the samples were divided into two parts. A few numbers of the whole sample was preserved for identification and the other half was sun dried and pulverized for further nutrient analysis. Samples for identification were directly placed into pample's, fluid a fixative. The fixative was prepared by adding 750 mL of 95% ethanol, 1375 mL of distilled water, 250 mL of 40% formalin and 125 mL of glacial acetic acid. Larvae species were placed to near boiling water to denature their body proteins and prevent decay and directly placed in pample's fluid prior to its transfer to preservative usually 70% alcohol. Samples for nutrient analysis were cleaned by discarding the unwanted portions of the body and kept in open mesh for sun drying. The sun dried sample was milled in an electrical stainless steel grinder using 0.5 mm sieve and stored in glass containers for experimental work.

3.5 Identification

The preserved species for identification were packed in specimen tubes containing 70% alcohol and submitted to Zoological Survey of India (ZSI), Shillong and Kolkata for identification. The specimens were taxonomically identified and classified by entomologists from ZSI, Shillong and Kolkata. Some edible insects could not be properly identified by entomologists at ZSI so some aspects of the analysis were therefore carried out on the ethno-species names, rather than on scientific names.

3.6 Nutritional Analysis

3.6.1 Proximate analysis

3.6.1.1 Determination of moisture content

A sample of 3gm was spread uniformly and dried in a hot air oven for 3 hours at 105°C. Sample was transferred to a dessicator to be cooled and weighed. The differences in weight represented the loss of moisture and were expressed as a percentage of oven dried sample (AOAC 2000).

Calculation:

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

W1 = Weight of sample before drying (g)

W2 = Weight of sample after drying (g)

3.6.1.2 Determination of total solids

Subtraction of the moisture percent from 100 represents the total solid. Total solids were estimated by subtracting the value of moisture from 100 (James 1995).

Calculation:

$$\text{Total solids \%} = 100 - \text{Percent of moisture}$$

3.6.1.3 Determination of protein Content

Total nitrogen and crude protein was estimated using micro kjeldahl method (AOAC 2000). 20 mL of concentrated sulphuric acid was added 1 g of sample in a digestion flask followed by 9.6 g potassium sulphate and 0.4 g copper sulphate and heated gently until frothing ceased. Solution was boiled briskly until clear, allowed to cool and added 60 mL of distilled water. Immediately the flask was connected to digestion bulb on condenser with tip of condenser immersed in standard acid with 5 – 7 drops of mix indicator in receiver. The flask was heated until all ammonia was distilled. The receiver was removed and the tip of the condenser washed. The excess distilled standard acid was titrated against standard sodium hydroxide solution.

Calculation:

$$\text{Nitrogen \%} = \frac{(\text{Sample titre} - \text{Blank titre}) \times \text{Normality HCl} \times 14 \times 100}{\text{Weight of sample} \times 1000}$$

$$\text{Protein \%} = \text{Nitrogen \%} \times 6.25$$

6.25 = the protein to nitrogen conversation factor for fish and its by- products.

3.6.1.4 Determination of ash content

A muffle furnace was used to analyse a 5 g sample for total ash (AOAC 2000). The sample was heated in a silica crucible over low bunsen flame with half covered lid. When fumes were no longer produced, the crucible and lid were placed in muffle furnace and heated at 550°C overnight. After complete heating the lid was placed on the crucible to prevent loss of fluffy ash and cooled in a dessicator. Weight was taken when the sample turned grey.

Calculation:

$$\text{Ash \%} = \frac{\text{Weight of ash (g)}}{\text{Weight of sample (g)}} \text{ x } 100$$

3.6.1.5 Determination of Fat content

Total fat in the sample was estimated in a soxhlet apparatus (AOAC 2000). In a filter paper 3g of sample was weighed and wrapped carefully. The wrapped sample was introduced into the extraction thimble of the soxhlet. 250 mL of petroleum ether was filled into the round bottom flask and placed on the heating mantle. The sample was heated about 14 hours at a heat rate of 150 drop/min. The solvent was evaporated by using vacuum condenser. The bottle was incubated at 90°C until solvent was completely evaporated and the bottle completely dried. The bottle was transferred to a dessicator with partially covered lid and allowed to cool. The bottle and its dried content were reweighed.

Calculation:

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

3.6.1.6 Determination of crude fibres

Fats from sample were extracted with petroleum ether and the sample dried. 2 g of dried sample was boiled with 200 mL sulphuric acid (H_2SO_4) for 30 minutes with bumping chips. It was then filtered through muslin cloth and washed with 25 mL of boiling H_2SO_4, three portions of 50 mL of water and 25 mL of alcohol. The residue was transferred to an ashing dish (W1). After drying the residue was reweighed (W2) and ignited for 30 minutes at 600°C, cooled and reweighed (W3) (Thimmaiah 1999; Maynard 1970; Sadasivam and Manickam 1992)

Calculation:

$$\text{Loss in weight} = (W2 - W1) - (W3 - W1)$$

$$\text{Crude fibre \%} = \frac{\text{On ignition (g)}}{\text{Weight of sample (g)}} \text{ x } 100$$

3.6.1.7 Determination of available carbohydrates

Having estimated all the other fractions by proximate analysis, the available carbohydrate content was obtained by difference (James 1995).

Calculation:

Available carbohydrates (%) = 100 – (Protein + Ash + Moisture + Fat + fibre)

3.6.1.8 Determination of Nutritive Value

The method described by FAO (2003) was pursued to estimate the total energy value in Kcal/100g.

Calculation:

$$\text{Nutritive value} = 4 \text{ x protein \%} + 9 \text{ x fat \%} + 4 \text{ x carbohydrate \%}$$

3.6.1.9 Determination of Starch Content

A sample of 0.5 g was homogenized in hot 80% ethanol to remove sugars. Residue was retained after centrifugation. The residue was washed repeatedly with hot 80% ethanol till the residue did not give colour with anthrone Reagent. The residue was dried in a water bath. To it 5.0 mL of water and 6.5 mL of 52% perchloric acid was added. Extraction was carried out for 20 min at 0ºC, centrifuged and saved the supernatant. The supernatant was centrifuged, pooled and made upto 100 mL. Pipetted out 0.1 mL of the supernatant and adjusted to 1 mL with water. For standard the working standard (glucose) taken as 0.2, 0.4, 0.6, 0.8 and 1 mL and diluted to 1 mL with water. 4 mL of anthrone reagent was added to each and heated for 8 minutes in a boiling water bath. After cooling rapidly the intensity of green to dark green colour was read at 630 nm. The amount of starch content was obtained by multiplying the factor 0.9 to the value of the concentration of glucose (Thimmaiah 1999; Hodge and Hofreiter 1962;Thayumanavan and Sadasivam 1984; Sadasivam and Manickam 1992).

3.6.2 Mineral analysis

3.6.2.1 Sample Preparation for Mineral Analysis

Sample for analysis of minerals were prepared by wet digestion method (Puwastein *et al* 2011; Horwitz 2000; Greenfield and Southgate 1992; Kirk and Sawyer 1991). Dry sample 5.1 g was taken in a Teflon cup with screw cap. 5 mL of concentrated nitric acid (HNO_3) and 1mL concentrated perchloric acid ($HClO_4$) (HNO_3: $HClO_4$ = 5:1) was added. Sample was predigested in tightly closed cup overnight at room temperature. The cup was placed in an oven at 100°C for 5-8 hours, cooled to room temperature in a fume hood, transferred to a 50 mL volumetric flask and diluted to mark with deionised water and mixed well. The solution was filtered through Whatman filter paper No. 541 and transfer to Nalgene plastic bottles for mineral detection.

3.6.2.2 Determination of Calcium and Magnesium

An aliquot of the test solution prepared in section 3.6.2.1 was taken into a volumetric flask. 1% w/v lanthanum chloride $LaCl_3$ solution was added to make a final concentration of 0.1% w/v $LaCl_3$. The solution was diluted to an appropriate volume with deionised water. The solution was then measured for calcium and magnesium by Atomic Absorption Spectrophotometer (AAS). Specific wavelength for each mineral was: Ca = 422.7 nm and Mg = 285.2 nm (Puwastein *et al* 2011; Horwitz 2000; Greenfield and Southgate 1992; Kirk and Sawyer 1991).

Calculation:

$$\text{Ca or Mg (mg/100g)} = \frac{(C_o) \text{ x total volume (mL) x dilution x } 100}{\text{Weight of sample (g) x } 1000}$$

Where,

C = sample concentration (mg/L)

1000 = conversion for mL to L

3.6.2.3 Determination of Potassium and Sodium

An aliquot portion of the sample solution prepared in section 3.6.2.1 was added $CsCl_2$ solution to make a final dilution of 1% w/v $CsCl_2$ prior to the analysis and diluted to an appropriate volume. Determination was done in AAS at their respective element wavelengths of K = 766.5 nm and Na = 589.0 nm (Puwastein *et*

al 2011; Horwitz 2000; Greenfield and Southgate 1992; Kirk and Sawyer 1991).

Calculation:

$$K\ (mg\ /100\ g) = \frac{C_o \times V \times D \times 100}{W \times 1000}$$

Where,

C_o = sample concentration (mg/L)

V = total volume (mL)

D = factor for dilution

W = sample weight g

1000 = conversion for mL to L

3.6.2.4 Determination of Iron, Copper, and Zinc

Organic matter in the sample was destroyed by wet digestion as described in 3.6.2.1 An aliquot portion of the acidified sample was diluted to and read at AAS. Determination was executed at relevant element wavelength (nm)-Fe = 248.3 nm, Zn = 213.9 nm and Cu = 324.7 nm (Puwastein *et al* 2011; Horwitz 2000; Sirichakwal *et al* 1988).

Calculation:

$$\text{Trace element}\ (mg\ /100\ g) = \frac{C_o \times V \times D \times 100}{W \times P \times 1000}$$

Where,

C_0 = sample concentration (mg/L)

V = total volume (mL)

D = dilution factor

W = weight of sample (g)

P = sample solution taken (mL)

1000 = conversion of mL to L

3.6.2.5 Determination of Phosphorus

Phosphorus was determined gravimetric method (Puwastein *et al* 2011; Kolthoff et al 1969; Horwitz 2000; Greenfield and Southgate 1992; Kirk and Sawyer 1991). For dry ash preparation, 2 g of dry sample was taken in a crucible. The dry sample was charred over a hotplate until smoking ceased. Charred sample was

placed in a furnace and incinerate at 525°C for 3-4 h. Ashing was continuous until the sample turned uniformly white or grey. The furnace was turned off and the temperature was allowed to drop to 250°C. The crucible was removed and cooled. The ash was dissolved in 2.5 mL 4N HNO_3 and filtered with Whatman filter paper No. 541 and diluted to 25 mL with DI water. 5 mL test solution was transferred into a 250 mL beaker. The volume was made to 40 mL by adding DI water. The solution was boiled for 3 minutes, added 10 mL concentrated HNO_3, again heated until boiling and swirled for 10 seconds. 50 mL ammonium molybdate solution was added. The solution was covered with watch glass and allowed to stand for 2 hours with timely swirling after every 30 minutes. The solution was left to precipitate at room temperature overnight. The glass filter crucible was cleaned with 95% ethanol and ether and dried in a dessicator under vacuum for 1 h and weighed. The precipitate was transferred from the beaker to the glass filter crucible and filtered using a vacuum pump. The precipitate was rinsed with 95% ethanol and diethyl ether, dried in the dessicator under vacuum for 1hour and weighed.

Calculation:

$$\text{P (mg /100 g)} = \frac{(\text{W2} - \text{W1}) \text{ x Y x } 1559.4557}{\text{Weight of test sample (g)}}$$

where,

W1 = weight of dry glass filter (g)

W2 = weight of glass filter with precipitate (g)

Y = diluting factor

1000 = conversion of mL to L

1559.4557 = calculating factor for P in the precipitate (0.015594557x1000 x100)

A comparision of the mineral contents of the edible insect species with the provided recommended dietary allowance (RDA) per day (DRI 2004) for both male and female human adults of age between 19 - 30 years is provided for each species.

3.6.3 Amino acid analysis

Amino acids were analyzed by High Performance liquid Chromatography (HPLC – Agilent 1100 series, Diode Array Detector) by pre-column dervitization technique with Phenyl Iso Thio Cyanate (PITC) reagent using Agilent TC-C18(2)

analytical Octadecylsilane (ODS) 5.0 µm, 4.6 X 250 mm Column. 32 Amino acids standards from Sigma Aldrich Co. St. Louis MO, USA. in a mixture were separated on the column. The sample was subjected to Gas phase hydrolysis with 6 N HCl under inert conditions followed by incubation at 110ºC for 24 hours. For tryptophan the sample was alkaline hydrolysed with Barium hydroxide and incubated at 120ºC for 15 hours. (Puwastein *et al* 2011). The hydrolyzed sample was abruptly subjected to derivitization. For derivatization mixture 1.0 mol L^{-1} triethylamine in acetonitrile was prepared by adding 417 µL of triethylamine with 2583µL of acetonitrile and 0.1 $molL^{-1}$ PITC in Acetonitrile was prepared by mixing 36 µL of PITC with 2164 µL of acetonitrile. 100 µL of each of the derivatization mixture was added to 200 µL of the sample in a glass vial and kept in the thermomixer for 1 hour at 45°C. Following this 400 µL of hexane was added thoroughly shaken and allowed to stand for 10 min after which the lower level solution was pipetted and defined 20 µl as injection volume was injected into HPLC instrument. The gradient systems used were, 1. Buffer A consisting of 15 g sodium acetate dissolved in 1700 mL Milli Q water adjusted to pH 6.5 with acetic acid. Volume was adjusted to 1850 mL with Milli Q water and finally 140 mL acetonitrile was added and the mixture filtered through 0.45 m membrane. 2. Buffer B consisted of 4:1 V/V acetonitrile-water filtered through 0.45 µm membrane. The flow rate and wavelength were 1 ml/min and 254 nm respectively (Shi *et al* 2013). Quantitative analysis for the amino acid present in the sample was done by comparing with the amino acid standard run simultaneously with the samples. Compound identification and quantification was based on the retention time under definite experimental conditions for each individual amino acid. The pattern of amino acid requirement for preschool children for 2–5 years recommended by FAO/WHO/UNU (1985) was followed to calculate the amino acid score.

Calculation:

$$\text{Amino acid score} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in reference pattern}} \text{ x } 100$$

3.6.4 Determination of Fatty Acids

Lipids from the sample were extracted by the method of Bligh and Dyers (1959). A 20 g sample was homogenized at the speed of 9,500 rpm for 1 min at 4ºC with 16 mL of distilled water, 40 mL of chloroform and 80 mL of methanol. Another 40 mL of chloroform was added and homogenized for 30 seconds followed by addition of 40mL of distilled water and homogenization for other 30 seconds. The homogenate was subjected to centrifugation at 2,000 rpm at 4ºC for 20 min and the supernatant transferred into a separating funnel where the layers were allowed to separate. The chloroform layer was collected and the solvent evaporated to collect the lipid. The fatty acids were analyzed in GC MS QP 2010 Plus – Shimadzu using Helium as carrier gas with a flow rate of 0.75 mL/min. For methylation the extracted fat residue was dissolved in 2.0 mL each of chloroform and diethyl ether. The mixture was evaporated to dryness at 40°C in a water bath under nitrogen stream. 2.0 mL of 14% Boron trifluoride - methanol solution and 1.0 mL of toluene were added and the vials heated at 100°C for 45 minutes in an oven. After every 10 minutes the vials were gently shaken. Vials were cooled at room temperature (24°C). 5.0 mL of Mass spectrometry (MS) grade water, 1.0 mL hexane and 1.0 g sodium sulphate were added and the vials shaken for 1 minute. Layers were allowed to separate. The top layer was carefully transferred to another vial containing 1.0 g sodium sulphate and 2µL of it was injected into GCMS-QP2010 Plus – Shimadzu. The injector and detector ports temperature was maintained at 225ºC and 285ºC. The initial temperature of 100ºC was increased at the rate of 3ºC/min and maintained at 240ºC for 15 min (AOAC 2001). Data file obtained from the GC/MS assay was deconvulated and identified by NIST mass spectral library software, by matching entries in the compound libraries to the measured mass spectra.

3.7 Biochemical evaluation of Proteins

3.7.1 Sample collection

Four wild edible insects belonging to four different orders namely, *Oecophylla smaragdina* (OS) from order (Hymenoptera), *Lethocerus indicus* (LI) (Hemiptera), *Ruspolia baileyi* (RB) (Orthoptera) and *Cybister tripunctatus* (CT)

(Coleoptera) were selected for protein quality evaluation study. The criteria for the selection were:

1. The species are the most commonly consumed insects among the four orders.
2. These species could be harvested abundantly and are available in the market throughout the year.

The edible insects were randomly collected from vendors at different markets of study areas. The unwanted body parts as wings and legs of insects were evacuated and cleaned by repeated washings. The cleaned portions were naturally sun dried following the traditional methods of sun drying till constant weight was achieved. A 500 g sample was pulverized in an electrical stainless steel grinder using 0.5 mm sieve after which the ground samples were stored in air-tight glass containers with screw caps pending use to compound the rat diets.

3.7.2 Animal diets

Following the pattern of Jenkins and Mitchell (1989) and Ekpo (2011) six types of experimental diets were formulated. The proportions of composites of the diet are shown in Table 1 below. The composites were assured proper blending. Prepared diets were stored in labeled polythene bags and stored in the refrigerator at 4^{o} C until needed.

Table 3.1 Test, Reference and Basal Diets compositions (g/kg)

Samples	Weight of samples	Vegetable Oil	Corn Starch	Sucrose	Glucose	Cellulose	Mineral Mix	Vitamin Mix	Protein %
OS	191.90	100	458.1	100	50	50	40	10	10
LI	148.63	100	501.37	100	50	50	40	10	10
RB	331.22	100	318.78	100	50	50	40	10	10
CT	169.5	100	480.5	100	50	50	40	10	10
Casein	100	100	550	100	50	50	40	10	10
Corn Starch(Basal)	-	100	650	100	50	50	40	10	-

OS - *Oecophylla smaragdina*, LI - *Lethocerus indicus*, RB - *Ruspolia baileyi* CT - *Cybister tripunctatus*.

Table 3.2 Composition of mineral mixture (g/kg) and vitamin mix (mg/Kg)

Minerals	g/Kg	Vitamins	mg/kg
Calcium phosphate ($CaHPO_4$)	500	Cholecalciferol	2500
Potassium citrate ($K_3C_6H_5O$)	200	Pyridoxine,-HCL	1600
Sodium chloride (NaCL)	74	Riboflavin	600
Potassium sulphate (K_2SO_4)	52	Thiamin-HCL	600
Magnesium oxide (MgO)	24	Folic acid	200
Zinc carbonate ($ZnCO_3$)	8.0	Menaquinine	50
Ferric citrate (16-17%Fe)	6.0	Amino benzoic acid	6.0
Manganous carbonate, $MnCO_3$(43-48%Mn)	3.5	D-biotin	20
Chromium potassium sulphate [$CrK(SO_4)$, $12H_2O$]	0.55	Nicotinic acid	2.0
Cupric Carbonate, $CuCO_3$ (53-55% Cu)	0.3	Inositol	4.0
Sodium selenite (Na_2SeO_3,$5H_2O$)	0.01	Choline	1.3
Potassium iodate (KIO_3)	0.01	Cyanocobalamin	1.0
Cellulose to make	1000	Ca-pantothenate	0.6
-		Retinyl palmitate, vitamin A activity	320 UI
-		Sucrose to make	1000g

3.7.3 Animal handling

The present study was approved by the Ethical Committee on the use of animals for the research, Department of Biotechnology, Bodoland University, Assam, India. The rats were obtained from the Animal House of Assam Agriculture University, Khanapara, Guwahati, Assam. Male albino rats were maintained at room temperatures of 28 ± 4°C, 50 – 95% of relative humidity on a 12-h light/12-h dark cycle, with access to the diet and distilled water *ad libitum* for 3 days' acclimatization period. Handling of the rats was in accordance with the standard principles of Guide for the care and use of laboratory animals, eighth edition, National Academic press, Washington DC.

3.7.4 Design of animal feed experiment

The feeding regime for the animal feed experiment were designed based on the method devised by Jenkins and Mitchell (1989) and Ekpo (2011). A total of 60 male albino rats (about 23 days old) were divided into two groups. Group A with 30

animals of 5 individuals each in 6 subgroups were on the diet for 14 days and Group B with 30 animals of 5 individuals each in 6 subgroups were on the diet for continuous 28 days. The rat groups were designated on the basis of experimental diets received for 14 and 28 days.

Table 3.4 Design of animal feed experiment for a duration of 14 days

GROUP A	
Group 1a. (OS)	Albino rats received (*O. smaragdina*) diet for 14 days + dH_2O *ad libitum*.
Group 2a (LI)	Albino rats received (*L .indicus*) diet for 14 days + dH_2O *ad libitum.*
Group 3a. (RB)	Albino rats received (*R. baleyi*) diet for 14 days + dH_2O *ad libitum.*
Group 4a. (CT)	Albino rats received (*C.tripunctatus*) for 14 days + dH_2O *ad libitum*.
Group 5a. (Reference)	Albino rats received (Casein) diet for 14 days + dH_2O *ad libitum*
Group 6a. (Basal)	Albino rats received (Corn starch) diet for 14 days + dH_2O *ad libitum.*

Table 3.4 Design of animal feed experiment for a duration of 28 days

GROUP B	
Group 1b. (OS)	Albino rats received (*O. smaragdina*) diet for 28 days + dH_2O *ad libitum*.
Group 2b (LI)	Albino rats received (*L. indicus*) diet for 28 days + dH_2O *ad libitum*.
Group 3b. (RB)	Albino rats received (*R. baleyi*) diet for 28 days + dH_2O *ad libitum*.
Group 4b. (CT)	Albino rats received (*C.tripunctatus*) for 28 days + dH_2O *ad libitum.*
Group 5b. (Reference)	Albino rats received (Casein) diet for 28 days + dH_2O *ad libitum*
Group 6b. (Basal)	Albino rats received (Corn starch) diet for 28 days + dH_2O *ad libitum.*

Every single experimental animal was caged separately for each of the groups to ensure proper metabolic study and monitor. The rats were weighed to obtain their initial weights; after which they were placed on a 3- day adjustment period on the diets. The rats were deprived of food and water for 12 h ahead of commencement of feeding.

All diets contained 10% protein (N x 6.25) by weight with exception to the protein free (Basal) diet. Weighed foods 15 g/rat/day were added about 5 mL of distilled water and mixed to minimize spillage and scattering. The food was served through fixed food dispensers of the metabolic cages. Distilled water ad libitum

were served through a sipper water bottle. Faecal contaminants along with the spilled food were collected daily and dried. The dry spilt food was combined with the dry unconsumed food for the determination of total amount of food actually consumed by the rats in each of the groups. The proximate daily food consumption was estimated by the weight difference between the served food and the unconsumed food plus spilt food. The weight of the rats was examined on a weekly basis. Electronic balance was used to measure the body weight of the test animal and weighing was done in air tight condition.

3.7.5 Collection and Treatment of Urine and Faeces

Urine and faeces were collected daily from each individual animals for the very important period they were on the diets by following the method of Jenkins and Mitchell (1989) and Ekpo (2011). The urine for each cage was collected in urine collection tubes containing 3 ml of (1% v/v) sulphuric acid. Additional precaution against urine losses were taken by washing the floor of the cages with 7 ml of (1% v/v) sulphuric acid and added to that in the sample tube for preventing urine losses. The tubes were stored in the refrigerator at 4°C until analysed for nitrogen. The faecal samples were dried to a constant weight in a hot air oven at 85ºC, grinded to a fine powder and stored in a container in the refrigerator at 4ºC until analysed for nitrogen.

3.7.6 Sacrifice of Animals

After the assigned days of feeding period for each group of animals, the rats were weighed and their physical conditions such as fur, appearance and agility were witnessed and noted prior to sacrificing them. The animals were sacrificed by following the method of Jenkins and Mitchell (1989) and Ekpo (2011). The animals were placed in a sealed container containing diethyl ether to put them to sleep. An incision was made in the abdomen and extended to the thorax. Immediately blood was collected from the heart with a new syringe and needle and shared into 3 different containers containing K3 EDTA, Lithium heparin and the third container with no anticoagulant in it. The kidneys, liver, spleen, heart, testis and brain were dissected out quickly and visually inspected for possible abnormalities such as

colour changes and lesions. The organs were washed with normal saline and their weights determined gravimetrically.

3.7.7 Urine and faecal analysis

A gram of dried faeces and 10 mL of urine were independently digested and analysed for nitrogen using the micro Kjeldal method (AOAC 2000). The values acquired for the protein free group were used to compute endogenous nitrogen losses.

3.7.8 Nutritional Evaluation of the Diets

The protein qualities of the insect/insect larvae, basal and casein diets were biologically evaluated based on their capacity to support growth and nitrogen retention in the albino rats.

3.7.8.1 Protein Efficiency Ratio (PER)

The PER was estimated using method described by Osborne *et al* (1919) by the equation.

$$\text{PER} = \frac{\text{Weight gain (g)}}{\text{Protein consumed (g)}}$$

3.7.8.2 Net Protein Utilization (NPU)

The NPU was determined by the equation of Bender and Miller (1953).

$$\text{NPU} = \frac{\text{Body N of test group} - \text{Body N of non protein group}}{\text{N consumed by test group}} \text{ x } 100$$

3.7.8.3 Biological value (BV)

The nitrogen balance sheet method of Mitchell (1924) was used to estimate the biological value (BV) of the diets. The BV procedure measures the efficiency of utilization of the nitrogen absorbed and is measured by the equation.

$$\text{BV} = \frac{\text{Retained N}}{\text{Absorbed N}} \text{ x } 100$$

$$= \frac{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N}) - (\text{urinary N} - \text{endogeneous urinary N})}{\text{N intake} - (\text{fecal N} - \text{metabolic fecal N})} \text{ x } 100$$

3.7.8.4 Net Protein Ratio (NPR)

Net Protein Ratio was determined using the method described Bender and Doell (1957). NPR was evaluated as-

$$NPR = \frac{\text{Weight gain on test protein} + \text{Weight loss of non protein group}}{\text{Weight of test protein consumed}}$$

3.7.8.5 True protein digestibility

True protein digestibility was determined using the method of (Sarwar and Peace 1986).

$$\text{True digestibility (TD)} = PI - \frac{[FP - MFP]}{PI} x\ 100$$

Where, PI = protein intake, FP = fecal protein, MFP = metabolic fecal protein.

3.7.9 Methods for estimation of biochemical parameters

3.7. 9.1 Haematological Parameters

The relevant hematological parameters measured included:

3.7. 9.1 Determination of total red blood cell (RBC) count

Red blood cells were counted using haemocytometer by the method of Samuel (1986). The method involves an accurate dilution of a measured quantity of blood with a fluid that can prevent its coagulation and is isotonic with blood as well. A dilution volume of 1:200 was used. The diluted blood was placed in a counting chamber and the number of cells in a constrained volume counted under a compound microscope. A neubauer counting chamber with gratings was used. Hayem's fluid was used as a diluting fluid. The composition of this fluid contained:

1. Mercuric chloride - 0.5 g
2. Sodium chloride - 1.0 g
3. Distilled water - 200 mL

Procedure

The RBC pipette was cleaned and blood was drawn upto 0.5 mark. After cleaning the tip of the pipette diluting fluid was drawn upto 101 mark. The blood was mixed for 2 minutes. The fluid was filled in a haemocytometer avoiding air bubbles and examined under a microscope. The RBC's were then counted in the neubauer ruling area. The small squares in the central large 1 mm square were used for counting. The number of cells in the corner groups of 16 squares and one central group were counted. Cells which lie within the area or on the dividing lines to the left or above the section were included in the count.

Calculation:

Total number of cells in 80 small squares = X cells

Area of a small square = 1/400 sq. mm.

Depth of the counting chamber = 1/10 mm.

Volume of a small square = 1/400 x 1/10 = 1/4000 cu. mm.

Dilution volume = 1/200

Therefore, the number of red blood cells (millions/mm^3)

$$= \frac{X \times 4000 \times 200}{80 \times 1 \times 1}$$

3.7. 9.2 Determination of total white blood cell (WBC) count

White blood cells were enumerated by the method of Samuel (1986) using haemocytometer. The method involves an accurate dilution of a measured quantity of blood with a fluid that destroys the red blood corpuscles. A dilution volume of 1:20 is used as WBCs are less in number. The diluted blood was placed in a counting chamber and the number of cells in a constrained volume counted under a compound microscope. A neubauer counting chamber with gratings was used. Composition of White blood cell diluting fluid (Truck's fluid):

1. Glacial acetic acid - 0.5 mL
2. 1% aqueous solution of gentian violet - 1.0 mL
3. Distilled water - 98.0 mL

Procedure

The WBC pipette was cleaned and blood was drawn upto the 0.5 mark. It was adjusted with the WBC diluting fluid upto mark 11. The pipette was held in a horizontal position and rotated between the thumb and fore finger for about a minute and finally shaken. The counting chamber was filled with the fluid holding the pipette at an angle of 45° and lightly touching the tip against the edge of the cover slip. The cells were allowed to settle for 3 minutes and cells in the four corner blocks were counted.

Calculation:

Number of WBC's counted in four corners = X cells.

The volume of a square = 1/10 mm^3.

The blood was diluted to = 1/20

Therefore, Number of white blood cells (thousands/mm^3)

$$= \frac{X \times 10 \times 20}{4}$$

3.7. 9.3 Estimation of Haemoglobin (Hb)

The haemoglobin concentration of blood was estimated by Sahli's haemoglobinometer (Samuel, 1986). Haemoglobin becomes converted to acid haematin when a measured quantity of blood is acidified with hydrochloric acid (0.1 N). Haemoglobin can be estimated by matching the colour of dilute acid haematin solution with a known standard.

Procedure

The graduated glass tube of the haemoglobinometer was filled with 0.1 N HCl upto the level marked 20 and blood was drawn upto 20 µ mark of the haemoglobin pipette. Transferring the blood to the graduated haemoglobin tube containing 0.1 N HCl they were stirred with a glass rod for 3 minutes. While stirring distilled water was added in drops. Stirring was continued till the colour of the diluted blood matched with the colour on the side tubes in the haemoglobinometer. The haemoglobin concentration was read and expressed in gram percentage or g/dl.

3.7. 9.4 Mean Corpuscular Haemoglobin (MCH)

Mean corpuscular haemoglobin was calculated by the method of Merck Veterinary Manual (1979). The absolute amount of haemoglobin per red cell is indicated by the Mean Corpuscular Haemoglobin (MCH). The MCH value was calculated and expressed in pictograms.

Calculation:

$$\text{MCH (pg)} = \frac{\text{Hb (g/dl)} \times 10}{\text{RBC count (millions/mm}^3\text{)}}$$

3.7. 9.5 Estimation of Packed Cell Volume

2 mL of blood was drawn and injected in to the test tube containing the anticoagulant mixture by gently rotating the tube between the palms. 1.5 mL of blood was drawn into a dry and clean Pasteur pipette and the tip of the pipette was inserted into the bottom of haematocrit tube. The blood was slowly expelled into haematocrit tube upto '0' mark. The haematocrit tube filled with blood was

centrifuged at 3,000 rpm for 30 minutes. The relative volume of the height of the RBC's packed at the bottom of the haematocrit tube was recorded as packed cell volume expressed as the percentage of total blood column.

3.7. 9.6 Mean corpuscular haemoglobin concentration (MCHC)

The Mean corpuscular haemoglobin concentration (MCHC) of Merck Veterinary Manual (1979). The average concentration of haemoglobin in red blood cells in a given volume can be reflected by the estimation of MCHC which is expressed in terms of gram percent (gdL^{-1}).

Calculation:

$$\text{MCHC g/dL} = \frac{\text{Haemoglobin (g/dL) x 100}}{\text{Haematocrit (PCV) (\%)}}$$

3.7. 9.7 Estimation of mean corpuscular volume (MCV)

The Mean corpuscular volume (MCV) was calculated by the method of Merck Veterinary Manual (1979). MCV indicates the average size of the red blood cell in a known quantity of blood. MCV is calculated out from haematocrit (%) and RBC count and expressed as femtoliter (fL).

Calculation:

$$\text{MCV(fL)} = \frac{\text{Hematocrit \% x 10}}{\text{RBC count (millions/mm}^3\text{)}}$$

3.7. 9.8 Platelets count

Platelets serve functions in hemostasis, in blood coagulation and in maintaining vascular integrity. Platelets are thin discs shaped colourless blood particles 2-4 μm in diameter and their life span ranges from 7 - 10 days. The methods of Dickinson (1996) and Brecher and Cronkite (1950) were used for estimating the platelets. The standard dilution used in platelet counts was 1:100. The dilution is mixed well and incubated to allow lysis of erythrocytes. After incubation the dilution was mounted on a hemacytometer. The cells were permitted to settle and were counted in a specific area of the hemacytometer chamber under the microscope. Composition of diluents was as follows

Ammonium oxalate - 11.45 g

Sorensen's phosphate buffer - 1.0 g

Thimerosal - 0.1 g

Adjust with distilled water - 1000 mL

Procedure

The capillary pipette was filled with whole blood and the whole blood transferred to a reservoir containing 1.98 mL of the diluent. The pipette was gently inverted several times to thoroughly mix the blood with diluents and permitted to stand for 10 minutes to hemolyze the erythrocytes. Following incubation, the diluted blood was mixed thoroughly by inverting reservoir to resuspend cells and hemacytometer was charged by placing the pipette tip on the edge of the ruled area of the counting chamber. Permitting the cells to settle for 10 minutes, cell counting was done with the low-power (10x) objective in the ruled area and the center large square (1 mm2). The entire center square was inspected for even distribution of platelets, then cautiously switched to the high-dry-power (40x) phase objective for counting platelets. The counts for each center square was recorded.

Calculations

Total number of cells counted x dilution factor x 1/volume factor = cells/mm^3

Cells/mm^3 = cells/µL or cells/µL x 10^3 µL /L = cells x 10^9/L

3.7.10 Toxicological Parameters

Diagnostic Kits from Aspen Laboratories Pvt. Ltd. Delhi, India were used for the estimation of: AST, ALT, ALP, GGT, Triglycerides, Cholesterol, HDL and Urea. The relevant toxicological parameters measured included:

3.7.10.1 Estimation of AST (Aspartate Serum Transaminase)

The reagent kit used was intended for *in-vitro* quantitative determination of AST activity in serum/plasma. The International Federation of Clinical Chemistry (IFCC) - UV kinetic method was followed for the estimation of AST activity (Thomas 1998a; Moss and Henderson 1999; Bergmeyer *et al* 1985). The principle of the reaction was.

$$\text{L-Aspartate} + \text{alpha-Ketoglutarate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L - Malate} + \text{NAD.}$$

Reagent compositions of the kit were

Reagent-1:

R1: TRIS pH 7.8	80mmol/l
L-Aspartate	240mmol/l

MDH (malate dehydrogenase)	≥ 600 U/l
LDH (laclate dehydrogenase)	≥ 600 U/l
R2: α-Ketoglutarate	12 mmol/l
NADH	0.24mmol/l

The working standard was prepared by mixing 4 parts of R1 + 1 part of R2

Procedure

For the estimation of AST activity 100 µL of sample was added 1000 µL of working reagent, mixed well and the absorbance was read at 340 nm after every 1 minute for three times at 37°C. The slope of reaction was decreasing. The kinetic factor for the reaction was 1746. The delay time and delta time were maintained at 60 seconds each. Normal range was upto 37 U/L and linearity upto 340 U/L. The AST activity (U/L) was calculated as.

Calculation:

ΔA/min x Factor = AST activity (U/L)

Factor = 1746

3.7.10.2 Estimation of ALT (Alanine Amino Transferase)

The reagent kit used was intended for *in-vitro* quantitative determination of ALT activity in serum/plasma. International Federation of Clinical Chemistry (IFCC) - UV Kinetic Method was followed for the estimation of ALT activity. (Thomas 1998a; Moss and Henderson 1999; Lorentz 1995).

Principle

$$\alpha\text{-Ketoglutarate} + \text{L-Alanine} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^{+}$$

The rate of NADH consumption is photometrically measured which is directly proportional to the ALT concentration in the sample. Reagent Composition for the estimation was.

R1: TRIS pH 7.5	100 mmol/l
L-Alanine	500 mmol/l
LDH (laclate dehydrogenase)	≥ 1200 U/l
R2: α-Ketoglutarate	16 mmol/l
NADH	0.18 mmol/l

For reagent preparation 4 parts of R1 was mixed with 1 part of R2

Procedure

To estimate the ALT activity 1000 µL of working reagent was added to 100 µL of sample, mixed well and the absorbance was read at 340 nm after every 1 minute for three times at 37°C. The mode of reaction was kinetic with a decreasing slope of reaction and a kinetic factor of 1746. Both the delay time and delta time were maintained at 60 seconds respectively. Normal range was upto 42 U/L and linearity upto 350 U/L. The ALT activity (U/L) was calculated as.

Calculation:

$\Delta A/min \times Factor = ALT\ activity\ (U/L)$

$Factor = 1746$

3.7.10.3 Estimation of ALP (Alkaline Phosphatase)

The reagent kit used was intended for *in-vitro* quantitative determination of (ALP) activity in serum/plasma. The alkaline phosphatase (ALP) estimation was based on the methods recommended by the International Federation of Clinical Chemistry (IFCC) (Thomas 1998b; Moss and Henderson 1999; Tietz *et al* 1983; Burtis and Ashwood 1999a). This method utilises 4-nitrophenylphosphate as the substrate. Under optimised conditions ALP present in the sample catalyses transphosphorylation reaction.

$$4\text{-}NPP + H2O \xrightarrow[Mg\ /Alkaline\ pH]{ALP} 4NPO + Phosphate$$

Reagent composition was as follows

Reagent-1:

R1: 2-Amino-2-Methyl-1-Propanol (pH = 10.4)	0.35 mol/l
Zinc sulphate	1.0 mmol/l
Magnesium sulphate	2.0 mmol/l
EDTA	2.0 mmol/l
R2: p-Nitrophenolphosphate	16.0 mmol/l

Procedure

10 µL of sample was pipetted and added to 400 µL of R1, mixed and incubated for 5 minutes at a constant temperature of 37°C and added 100 µL of R2. The solution was mixed and the change of absorbance (ΔA) was read at 405 nm in a cuvette with 1 cm light path between 60, 120 and 180 seconds at 37°C. The reaction mode was kinetic with increasing slope of reaction and a kinetic factor of 2757. The delay time and read time were maintained at 60 seconds and 180 seconds respectively. Normal range was upto 135 U/L and linearity upto 1700 U/L. The ALP activity (U/L) was calculated as.

Calculation:

ALP activity in U/L = ΔA of test x 2757

Factor = 2757

3.7.10.4 Estimation of GGT (Gamma Glutamyl Transferase) Activity

The reagent kit used was intended for *in-vitro* quantitative determination of GGT activity in serum/plasma. The alkaline phosphatase (ALP) method was based on the Szasz methodology (Szasz 1976; Tietz 1986).

Principle

GLUPA-C + Glycylglycine $\xrightarrow{\text{Gamma GT}}$ L-Gamma-Glutamyl-Glycylglycine + 5-amino-2-nitrobenzoic acid.

GLUPA-C: L-Gamma-Glutamyl-3 Carboxy-p-nitroanilide.

Reagent Composition for the kit were

Reagent	
R1: TRIS pH 8.25	133 mmol/L
Glycylglycine	138 mmol/L
R2: GLUPA-C	23 mmol/L

Reagent was prepared by mixing 4 volume of R1 and 1 volume of R2

Procedure

A 100 µL of sample was added 1000 µL of the working reagent, mixed well and incubated for 1 minute and the absorbance was read at 405 nm in a cuvette with 1 cm light path after every 1 minute for three times at 37°C. Deionized water was used as blank. The reaction mode was kinetic with increasing slope of reaction and a

kinetic factor of 1158. The delay time and delta time were maintained at 60 seconds each. Deionized (DI) water was used as blank Normal range was upto 45 U/L and linearity upto 232 U/L. The GGT activity (U/L) was calculated as.

Calculation:

$\Delta A/min \times Factor = Gamma\ GT\ activity\ (U/L)$

Factor = 1158

3.7.10.5 Cholesterol

The reagent kit used was intended for *in-vitro* quantitative determination of cholesterol in serum/plasma. Cholesterol in blood serum was determined by the CHOD-POD method. (Rifai et al 1999; Artiss and Zak 1997; Deeg and Ziegenhorn 1983; Schaefer and McNamara 1997; Recommendation of the Second Joint Task Force of European and other Societies on Coronary revention 1998).

Principle

The determination of cholesterol is followed after enzymatic hydrolysis and oxidation. Under the catalytic action of peroxidase colorimetric indicator quinoneimine from 4-aminoantipyrine and phenol by hydrogen peroxide is generated (Tinder's reaction).

Cholesterol ester+ H_2O $\xrightarrow{CHE}$ Cholesterol + Fatty acids

Cholesterol + O_2 $\xrightarrow{CHOD}$ Cholesterol – 3 –one + H_2O_2

$2H_2O_2$ +4-Aminoantipyrine + Phenol $\xrightarrow{POD}$ Quinoneimine + 4 H_2O

Reagent composition of the test kit were

Good's buffer	pH 6.7	50 mmol/l
Phenol		5 mmol/l
4- Aminoantipyrine		0.3 mmol/l
Cholesterol esterase	(CHE)	≥ 200 U/l
Cholesterol oxidase	(CHOD)	≥ 50 U/l
Peroxidase	(POD)	≥ 3 kU/l
Standard		200 mg/dl

Procedure

Three test tubes containing 10 µL of sample, 10 µL of standard and a blank were added 1000 µl of reagent, mixed well and incubated for 10 minutes at 37°C. Absorbance was read at 505 nm against blank reagent. The concentration of the standard was 200 mg/dl. The endpoint method was followed and the slope of reaction was increasing. The Normal range was 110 – 200 mg/dl and the linearity was upto 600 mg/dl. Cholesterol was calculated as.

Calculation:

$$\text{Cholesterol} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Concentration of standard (mg/dL)}$$

3.7.10.6 High Density Lipoproteins (HDL) Cholesterol

The reagent kit used was intended for *in-vitro* quantitative determination of HDL cholesterol in serum/plasma. HDL-cholesterol in blood serum was determined by the PEG Precipitation method. (Trinder 1969; Allain *et al* 1974; Flegg 1972; Grillo *et al* 1981; Demacker *et al* 1980).

Principle

Polyethylene Glycol contained in the precipitating reagent, precipitate all the VLDL and LDL present in the serum. The HDL remains in the supernatant and was assayed as a sample for cholesterol using the Cholesterol (CHOD/PAP) reagent.

Procedure

A set of three test tubes were cleaned, dried and labeled as blank (B), Standard (S) and Test (T). 1.0 mL of working reagent was pipetted into each of the test tubes. Into the B test tube 0.05 mL of distilled water was added. The S test tube was added 0.05 mL of HDL standard and into the test tube T 0.05 mL of test sample was added. The contents of the test tubes were mixed well and incubated at 37°C for 5 minutes. The absorbance of the standard (Abs.S) and test Sample (Abs.T) were read against the blank at 505 nm (Hg 546 nm)/ Green, 1 cm light path at 37°C within 60 minutes. The HDL cholesterol was calculated as.

Calculation:

$$\text{HDL Cholesterol in mg/dL} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x 25 x 2}$$

(Where 2 is the dilution factor due to the deprotenization step)

3.7.10.7 Low Density Lipoproteins (LDL) Cholesterol

LDL-Cholesterol (mg/dl) was calculated from the value of HDL Cholesterol by Freidewald's formula (1972).

$$\text{LDL (mg/dL)} = (\text{Total cholesterol}) - \left(\frac{\text{Triglycerides}}{5}\right) - (\text{HDL})$$

3.7.10.8 Triglycerides

The reagent kit used was intended for *in-vitro* quantitative determination of triglycerides in serum/plasma. Triglycerides in blood serum was determined by the GPO-POD Method. (Trinder 1969; Allain *et al* 1974; Flegg 1972; Grillo *et al* 1981; Demacker *et al* 1980).

Principle

Triglycerides were determined after enzymatic splitting with lipoprotein lipase using quinoneimine as indicator, which was generated from 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxide under the catalytic action of peroxidase.

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone-phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{P- Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + \text{HCL} + 4H_2O$$

Reagent Composition was as follows

Good's buffer pH 7.2	50mmol/l
4-Chlorophenol	4mmol/l
ATP	2mmol/l
Mg^{2+}	15mmol/l
Glycerokinase (GK)	$\geq$ 0.4kU/l
Peroxidase (POD)	$\geq$ 2kU/l
Lipoprotein (LPL)	$\geq$ 2kU/l
4-aminoantipyrine	0.5mmol/l
Glycerol-3-phosphate-oxidase	$\geq$ 0.5kU/l
Standard	200mg/dl

Procedure

A set of three test tubes were cleaned, dried and labeled as blank (B), Standard (S) and Test (T). The S test tube was filled with 10 µL of standard and the test tube T was filled with 10 µL of test sample. The test tube B was left blank. Into each test tube were added 1000 µL of the reagent. The contents of the test tubes were mixed well and incubated at 37°C for 10 minutes. The absorbance of the standard and test Sample were read against the blank at 505 nm at 37°C. The slope of reaction was increasing and the endpoint method was followed. The standard concentration was 200 mg/dl. Normal range was upto 150 mg/dl and linearity upto 1000 mg/dl. Triglycerides were calculated as.

Calculation:

$$\text{Triglycerides} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Concentration of standard (mg/dL)}$$

3.7.11. Renal function test

3.7.11.1 Urea

The reagent kit used was intended for *in-vitro* quantitative determination of triglycerides in serum/plasma. Blood serum urea was determined by the Berthelot method. (Thomas 1998; Burtis and Ashwood 1999b).

Principle

Urease hydrolyses urea into ammonia and carbondioxide. In alkaline conditions, ammonia reacts with hypochlorite and salicylate in the presence of nitrupruside to form coloured complex which was read at 578 nm. The intensity of the colour was proportional to the concentration of urea in the sample.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} NH_3 + CO_2$$

$$NH_3 + \text{Salicylate} + \text{Hypochlorite} \xrightarrow{\text{Urease}} \text{Blue Green (Coloured Complex)}$$

Reagent composition was as follows

Reagent 1: Buffer reagent	20 mM/L
Reagent 2: Enzyme reagent	$\geq$ 2U/mL
Reagent 3: Hypochlorite reagent	0.3%
Standaed: Urea	40mg/dL

For reagent preparation 10 mL of Reagent 1 was mixed with 1mL of Reagent 2

Procedure

The enzymatic endpoint method with increasing slope of reaction was followed for the estimation of serum urea. A set of three test tubes were cleaned, dried and labeled as blank (B), Standard (S) and Test (T). The S test tube was filled with 10 µL of standard and the test tube T was filled with 10 µL of test sample. The test tube B was left blank. Into each test tube were added 1000 µL of the reagent. The contents of the test tubes were mixed gently and incubated for 5 minutes at 37°C. The absorbance of the standard and test Sample were read against the blank at 578 nm at 37°C. The standard concentration was 40 mg/dl. Normal range was upto 150 mg/dl and linearity upto 250 mg/dl. Urea was calculated as.

Calculation:

$$\text{Urea (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \text{ x Concentration of standard}$$

3.7.11.2 Blood Urea nitrogen (BUN)

Urea (mg/dl) x 0.467 = BUN (mg/dl)

0.467 = conversion factor

3.7.12 Serum total protein

Total serum protein was determined by the Biuret method (Gornall *et al.*, 1949). The measured reaction product gives a violet colour. Cu^{2+} ions in the Biuret reagent react with biurea a compound produced by heating urea at 180°C at alkaline pH to form a violet complex. The same colour become visible when proteins and peptides are present in samples because of the presence of two peptide (-CONH) bonds. The intensity of colour and the serum proteins concentration are directly proportional.

Reagents were

1. Biuret reagent
2. 0.85% Sodium chloride
3. 2.5 N Sodium hydroxide
4. Standard protein (BSA)

Procedure

A 0.5 mL serum was mixed with 1.5 ml of 0.85% sodium chloride solution in a test tube. 8 mL of biuret reagent was added to the test tubes. A 1 mL of standard protein was mixed with 1 ml of 0.85% sodium chloride solution for standard preparation. To this 8 mL of biuret reagent was added. A blank containing 2 mL of 0.85% sodium chloride solution was mixed with 8 ml of biuret reagent. All the three test tubes were shaken well and allowed to stand for 30 minutes. The test and standard were read against the blank at 520 nm.

3.7.13 Histological Examinations

The methods described by Banchroft (1996) and Lal (2011) were followed for Organ histology.

Reagents were

Composition of alcoholic Bouins (*Gendre's solution*):

95% ethanol saturated with picric acid (5 g per 100 mL)	800 mL
Formaldehyde (37%)	150 mL
Glacial acetic acid	50 mL

Biopsies of samples were taken from tissues of the different animal groups, fixed in alcoholic Bouins *(Gendre's solution)* for 24 h and washed in 70% ethanol, followed by 95% ethanol for several changes. Washing was continued until all picric acid was removed or until the yellow colour disappeared. The tissues were subjected to dehydration through 30%, 50%, 70%, 90% and 100% alcohol serially in a staining tube for 5 minutes in each grade of alcohol. The specimens were cleared in xylene embedded in paraffin in hot air oven at 56 °C for 24 h. The block was trimmed around the embedded material to make a perfect rectangular block for sectioning at 4 µm thicknesses using a rotator microtome. The ribbons were kept over Mayer's albumin coated slides. The sections were flattened over a hot brass plate at nearly 40 – 45 degrees. Subsequent to flattening water was drained off and the slide left overnight at room temperature for drying the ribbons. The ribbon side was marked with a glass marking pencil. The obtained tissue sections on glass slides were dehydrated by immersing in serial dilutions of 100% , 90%, 70%, 50%, 30%

ethyl alcohol-water mixture and cleaned in xylene for 5 – 10 minutes. Next, the specimens were stained with hematoxylin followed by dehydration through ascending series of 30%, 50%, 70% and 90% of alcohols. The slide was immersed in alcoholic eosin for 30 seconds. Eosin was washed in 90% alcohol, in 100% alcohol for 5 minutes and then in xylene for 15 minutes. The slides were then mounted in D.P.X. (Dibutyl Phathalate Xylene) for histopathological examinations. Photomicrographs of the tissue sections were captured using camera under light microscope under magnification power of 10X, 40X and 100X.

3.7.14 Chemicals

Solvents and chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich Co. St. Louis MO, USA.

3.7.15 Statistical analysis

The data from animal experimentation was tested for statistical significance by using IBM SPSS Statistics 19 (Armonk, New York, United States).

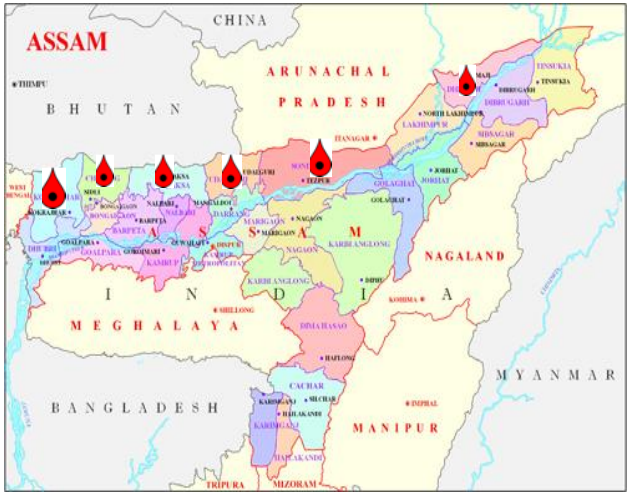

Plate 3.1 Map of the study site